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**Nonthermal Processing Technologies for the Mitigation of
Soy Protein Isolates Immunoreactivity – with Focus on
their Sensory and Physicochemical Properties**

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**The important thing is not to stop questioning. Curiosity
has its own reason for existing.**

Albert Einstein (1879 - 1955)

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PRELIMINARY REMARKS

The work presented in this thesis is a selection of papers published in international peer reviewed journals, which are listed below.

Full Papers

1. MEINLSCHMIDT, P., SUSSMANN, D., SCHWEIGGERT-WEISZ, U., EISNER, P. (2016). Enzymatic treatment of soy protein isolates: effects on the potential allergenicity, technofunctionality, and sensory properties. *Food Science & Nutrition*. 4(1), 11-23.
2. MEINLSCHMIDT, P., SCHWEIGGERT-WEISZ, U., BRODE, V., EISNER, P. (2016). Enzyme assisted degradation of potential soy protein allergens with special emphasis on the technofunctionality and the avoidance of a bitter taste formation. *LWT - Food Science and Technology*. 68, 707-716.
3. MEINLSCHMIDT, P., SCHWEIGGERT-WEISZ, U., EISNER, P. (2016). Soy protein hydrolysates fermentation: effect of debittering and degradation of major soy allergens. *LWT - Food Science and Technology*. 71, 202-212.
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Further scientific contributions resulted from the same period.

Review

1. MEINLSCHMIDT, P. AND SCHWEIGGERT-WEISZ, U. (2016). Mitigation of Soybean Immunoreactivity by Processing Technologies and Their Effect on the Sensory and Technofunctional Properties - A Comprehensive Review. *in preparation*

Oral Presentations

1. MEINLSCHMIDT, P. AND SCHWEIGGERT-WEISZ, U. (2016) Food Allergy - Promising Mitigation Methods are on the Way! 4th International ISEKI_Food Conference, 6-8 July 2016, Vienna, Austria
2. MEINLSCHMIDT, P. (2016). Low-Allergen Food Ingredients - An insight into Nonthermal Processing Technologies to Mitigate Soybean Allergy! 10th European PhD Workshop on Food Engineering and Technology, 28-29 April 2016, Uzwil, Switzerland
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4. MEINLSCHMIDT, P., UEBERHAM, E., LEHMANN, J., SCHWEIGGERT-WEISZ, U., EISNER, P. (2015). Fermentation - a powerful method to overcome soybean allergenicity! 29th EFFoST International Conference *Food Science Research and Innovation: Delivering sustainable solutions to the global economy and society, Bioprocessing and fermentation technology Session*, 10-12 November 2015, Athens, Greece

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3. MEINLSCHMIDT, P., SCHWEIGGERT-WEISZ, U., UEBERHAM, E., LEHMANN, J., REINEKE, K., SCHLÜTER, O., EISNER, P. (2015). Mitigation of soybean allergy by pulsed ultraviolet light, nonthermal atmospheric plasma, and γ -irradiation. In: Proceedings of the 2015 International Nonthermal Processing Workshop. Taoukis P., Stoforos N., Gogou E. (Eds.). ISBN: 978-618-82196-2-5. Athens, Greece.

Poster Presentations

1. MEINLSCHMIDT, P., SCHWEIGGERT-WEISZ, U., UEBERHAM, E., LEHMANN, J., REINEKE, K., SCHLÜTER, O., EISNER, P. (2015). Mitigation of soybean allergy by pulsed ultraviolet light, nonthermal atmospheric plasma, and γ -irradiation. 2015 International Nonthermal Processing Workshop *Sustainable Innovation Based on Science and Applied Research of Nonthermal Technologies* 12-13 November 2015, Athens, Greece
2. MEINLSCHMIDT, P., SCHWEIGGERT-WEISZ, U., EISNER, P. (2015). Mitigation of soybean allergy - effects of processing on the immunoreactivity and techno-functional as well as sensory properties of soy protein. Innovations in Food Packaging, Shelf Life and Food Safety Conference, 15-17 September 2015, Erding (Munich), Germany
3. MEINLSCHMIDT, P., SCHWEIGGERT-WEISZ, U., EISNER, P. (2015). Enzymatic hydrolysis as an effective approach for the reduction of soybean allergenicity: effects on the degree of hydrolysis and sensory properties. IFT15 Annual Meeting & Food Expo McCormick Place South, 11-14 July 2015, Chicago, IL USA
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1. Winner of the Julius Maggi Research Award 2016 at the 10th European PhD Workshop on Food Engineering and Technology, 28-29 April 2016, Uzwil, Switzerland
2. Winner of the "Best Student Poster Award" at the 2015 International Non-thermal Processing Workshop, 12-13 November 2015, Athens, Greece

3. Food Engineering Division Graduate Student Research Paper Poster Competition Finalist at the IFT15 Annual Meeting & Food Expo McCormick Place South, 11-14 July 2015, Chicago, IL USA

Patents

1. Meinschmidt, P., Eisner, P., Schweiggert-Weisz, U., Sussmann, D., Pickardt, C., Muranyi, I., Keitzel, A. (2016) Techno-funktionelle Fraktion aus Pflanzenproteinen. DPMA Application No. *116381PDE* (submitted 08.04.2016)

For all publications listed above that constitute the basis for the thesis presented here the author of this thesis is the main author. This means that Dipl.-Ing. **Pia Meinschmidt** has basically been at the design and execution of all scientific experiments. The intellectual processing of experimental information gathered and the setup of the scientific context as well as the actual writing of the papers are practically exclusive her product.

The co-authors' contributions to the papers presented in CHAPTER 1 to 6 are specified as follows:

The work presented in this thesis was carried out under the supervision of Professor **Horst-Christian Langowski**, Fraunhofer Institute for Process Engineering and Packaging (IVV), and Professor **Dietrich Knorr**, Institute of Food Biotechnology and Food Chemistry, Berlin University of Technology.

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GENERAL INTRODUCTION

1 Food allergy

Since the beginning of medicine (Hippocrates, 460-377 BC), there has been acknowledgment that food can cause health problems in some individuals. Currently, the incidence and prevalence of food allergies is on the rise and a significant amount of research is dedicated to find the cause, treatment, and a remedy for food allergies.

1.1 General aspects of food allergy

All *individualistic* adverse reactions to foods or food ingredients are collectively defined as food sensitivity (TAYLOR, 1987). These adverse reactions are divided into primary and secondary sensitivities (see Figure 1).

Primary sensitivities include immunologically mediated food allergies and non-immunological mediated food intolerances. Food intolerances are further classified into anaphylactic reactions, metabolic disorders, and idiosyncratic reactions. Secondary sensitivities can occur with or as a result of other conditions and can result in the same ailments as primary sensitivities due to the effects provoked by numerous gastrointestinal conditions or drug treatments. Secondary conditions are usually temporary but can enhance the chances of developing permanent food allergies, lactose intolerance, or celiac disease (TAYLOR & HEFLE, 2001).

A true food allergy, also called food hypersensitivity, is defined as adverse reactions of the immune system arising from a specific immune-mediated response towards food components, typically proteins (TAYLOR & HEFLE, 2001). COOMBS & GELL (1963) separated hypersensitivities into four classes: Type I hypersensitivity is responsible for most food allergic reactions and is characterized by the presence of food-specific serum immunoglobulin (Ig) E antibodies. Type II hypersensitivity is

an IgG-mediated reaction. Both allergic reactions (Type I and II) are induced by soluble antigens. The third Type is an IgG-mediated reaction, which is induced by cell-surface antigens (Type III). The symptoms of Type I-III commonly arise within a few minutes to 1 h after ingestion of the allergenic food. In contrast, the Type IV hypersensitivity takes often two or three days to develop and is not antibody-mediated, but is a T cell-mediated response. The probability of occurrence is relatively low and the underlying mechanism is not precisely known (TAYLOR & HEFLE, 2001).

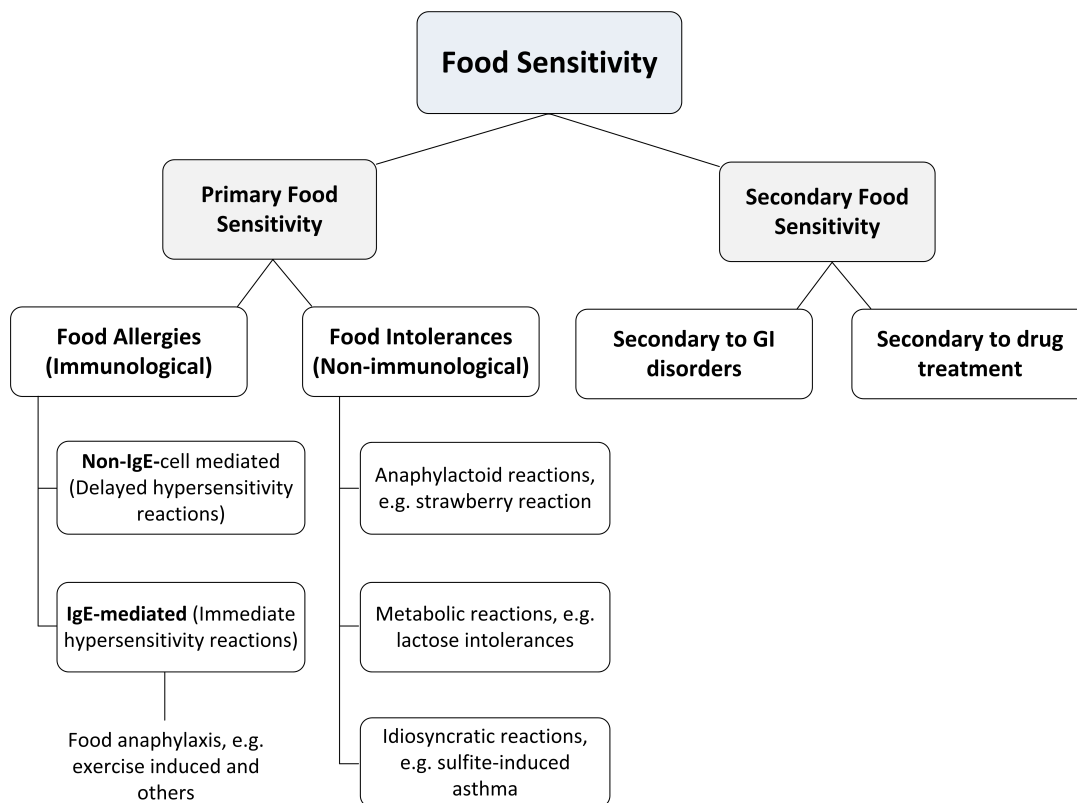


Figure 1. Classification of different types of food sensitivities (modified according to TAYLOR & HEFLE, 2001).

Type I hypersensitivity regulated by IgE antibodies could result in rapid-onset of severe reactions and may manifest with a variety of symptoms. The symptoms range from mild dermatological (hives, local swelling, dermatitis, and eczema), gastrointestinal (nausea, vomiting, diarrhea, and abdominal pain), respiratory (runny nose, asthma, and tightening of the throat) to systemic responses such as anaphylactic shocks, organ failure, cardiac arrhythmia, and death (CORDLE, 2004; TAYLOR & HEFLE, 2001). IgE-mediated food allergies have a low tolerance to specific foods, and trace amounts are sufficient to cause an allergic reaction in some individuals.

The World Allergy Organization (WAO) estimated approximately 220 to 250 million people worldwide suffering from some kind of food allergy. It is supposed that about 0.1 - 3.2 % of the adults and about 0.1 - 5.7 % of infants and young children (< 3 years) are affected by food allergies in Europe. However, uncertainties about the exact prevalence of food allergies still remain (VERHOECKX et al., 2015).

1.2 Food allergens

A specific food component, which is able to induce the immune system to produce IgE antibodies, bind to those IgE antibodies and elicit as a consequence an allergic response, is called food allergen (AALBERSE, 2000).

Food allergens are primarily proteins or glycoproteins (BREITENEDER & RADAUER, 2004), which act as storage or defense proteins within plant cells. Traditional allergens are heat-, enzyme-, low pH-resistant water- and /salt-soluble glycoproteins with molecular sizes of between 10 and 70 kDa. Representatives of this called class I allergens are soybean and peanut, which can induce systemic reactions primarily via the gastrointestinal tract (HAN et al., 2012). In contrast, class II allergens such as apple and celery proteins are heat-labile and susceptible to digestion. Their high homology with pollen proteins cause reactions limited to oral allergy symptoms as a consequence of respiratory sensitization to cross-reactive pollen (HAN et al., 2012; LORENZ, 2015). Furthermore, food allergens can be classified into "major" and "minor" allergens. Food allergens which bind IgE antibodies from more than 50 % of sera from at least ten allergic individuals are termed "major", whereas a "minor" allergen react with less than 50 % of these sera (CANTANI et al., 2008).

It becomes obvious, that various classification systems of allergens exist. To standardize the classification, the International Union of Immunological Societies (IUIS) Allergen Nomenclature (<http://www.allergen.org>) established by the World Health Organization (WHO) categorized allergens according to the taxonomy of their source: the first three letters of the genus name and the first letter of the species name, followed by a number, assigned to the allergen in the chronological order of purification. Hence, soybean glycinin became *Glycine max* allergen 6 or according to the taxonomy Gly m 6.

According to the Allergome database (<http://www.allergome.org>) more than 200 out of 700 allergens are derived from foods. The most comprehensively characterized allergens are plant-derived and can be classified into families and superfamilies on the basis of their structural properties (BREITENEDER & EBNER, 2000; MATSUO

et al., 2015; PUNTA et al., 2012). The most widespread groups of plant proteins that contain allergens are the prolamine, cupin, and plant defense system families. Detailed information about this classification system that is emerging from the synopsis of allergology and protein evolution is described in BREITENEDER & RADAUER (2004).

The potential of any protein to be a food allergen is difficult to predict. Although several characteristics have been proposed to be shared by food allergens, the molecular basis of them has not yet been fully understood. Therefore, a decision-tree approach has been developed by the Food and Agriculture Organization (FAO) and WHO in 2001. Questions are included whether a protein exhibits characteristics that might increase its probability of being an allergen such as sequence-relationship to other known allergens, its membership of a certain protein family, its abundance, and stability to processing and digestion. Such a decision tree, however, is only a guide and not a good rigorous predictor of allergenic potential or potency. Accordingly, the Codex Alimentarius and the European Food Safety Authority (EFSA) recommended the use of a weight-of-evidence approach.

1.2.1 The most common allergenic foods

Any food protein has the potential to cause an allergic reaction. Until now, more than 160 foods have been documented as causing food allergies. However, most confirmed food allergies are limited to only few allergen sources (TAYLOR & HEFLE, 2001). It was found that nearly 90 % of all documented allergic reactions to food in the U.S. are triggered by eight main protein sources, which are referred to the so-called "big 8" according to the guidelines of the FAO and WHO. These include cow's milk, eggs, fish (all fish species), crustacean (shrimp, crab, crayfish, and lobster), peanuts, tree nuts (almonds, Brazil nuts, pecans, walnuts, cashews, pistachios, hazelnuts, pine nuts, macadamia nuts, chestnuts, and hickory nuts), wheat and soybeans (FAO, 1995; FARRP, 2015; HEFLE et al., 1996).

In addition, labeling of the "big 8" is also mandatory according to the European Union (EU) regulations, which follows the Codex Alimentarius recommendations. In a recent amendment, decided by the Nutrition and Allergies panel (Annex IIIa of 2003/89/EC) of the European labeling directive (2000/13/EC and Commission-Directive 2006/142/EC), this list has been expanded in the EU in number to 14, including lupins, celery, mustard, sesame, molluscs, and sulphur dioxide at levels above 10 mg/kg or 10 mg/litre. On 13th December 2014, the European legislation

(EU Food Information for Consumers Regulation No.1169/2011) came fully into force and prescribed that all of the 14 food allergens have to be emphasized on the label, if they are used as ingredients in a pre-packaged food.

1.3 Basic mechanisms of an IgE-mediated allergic reaction

The mechanisms of an IgE-mediated food allergy are basically composed of two phases as depicted in Figure 2.

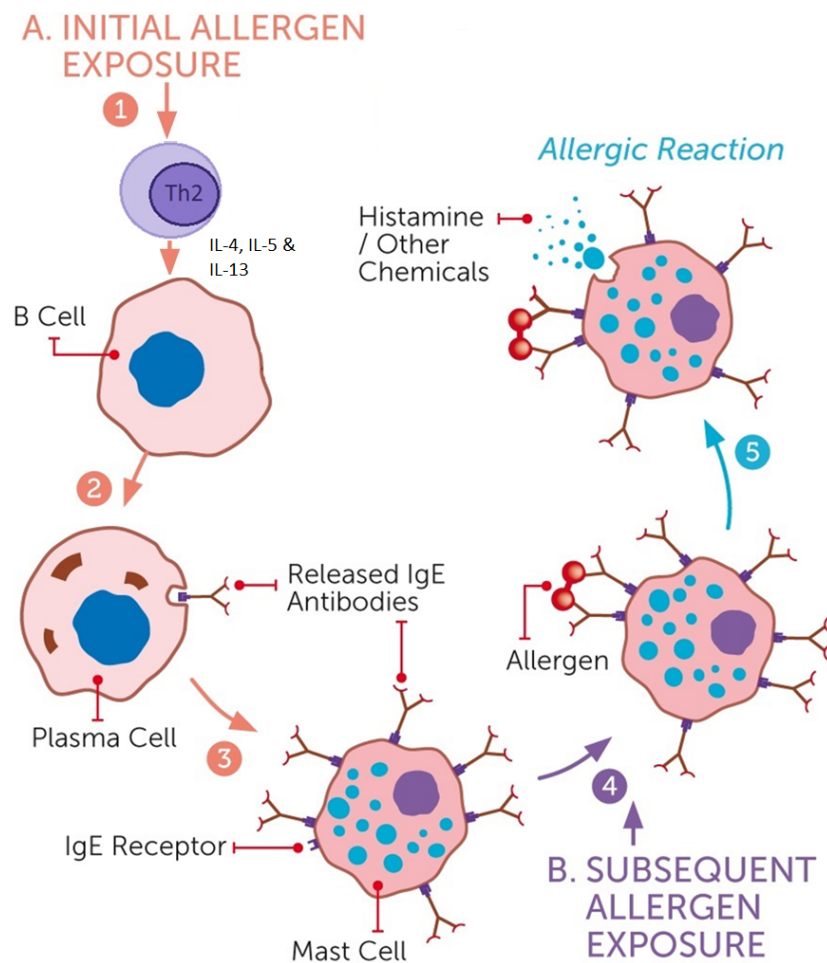


Figure 2. Mechanisms of an IgE-mediated allergic reaction (modified according to STARK et al., 2015). **1.** Initial allergen exposure; **2.** IgE production; **3.** IgE-binding on mast cell; **4.** Subsequent allergen exposure; **5.** Release of mediators.

(1) The first phase (= sensitization phase) involves the initial contact with the intact allergen, which enters the body through the gastrointestinal tract, skin, or mucosa. A special set of helper T cells (Th2) are stimulated by the antigen, which causes

the release of specific signal molecules called interleukins (IL-4, IL-5, and IL-13), which in turn activate and convert B cells into IgE-producing plasma cells. IgEs are quickly bound by high-affinity FcεRI receptors on the mast cell, which contain large amount of mediators, in particular histamine. (2) The second phase (= induction phase) results from the subsequent oral intake of the same antigen as in the first phase. IgE antibodies bind the allergen, which results in cross-linking of the allergen with at least two receptor-bound IgE antibodies on the mast cells. As a result, the mast cell releases pre-formed mediators, which results in an break-out of allergic symptoms (MATSUO et al., 2015; TAYLOR & HEFLE, 2001).

1.3.1 The allergenic determinant - Epitopes

The antigenic determinant of a protein, which makes a harmless protein to a harmful allergen, is called epitope. The word epitope was first defined by JERNE (1960) as an antigenic determinant or region of a molecule recognized by an antibody. Currently, epitopes describe the binding site of a molecule at the amino acid level for both B and T cells. In order to enter IgE cross-linking, more than one epitope or IgE-binding site is required per fragment of an allergen (SATHE et al., 2005).

Allergenic epitopes can be classified into linear (= sequential, continuous) and conformational (= discontinuous) types (CORDLE, 2004; TAYLOR & HEFLE, 2001) (see Figure 3).

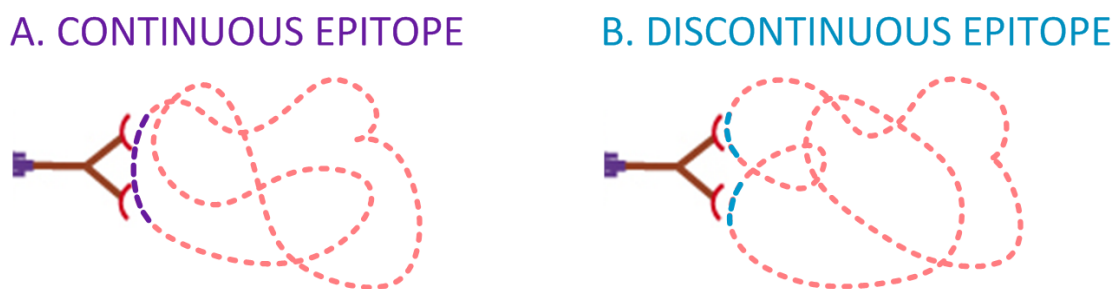


Figure 3. Peptide antigen design for (A) continuous (= linear, sequential) and (B) discontinuous (= conformational) epitopes.

The linear epitopes comprise continuous amino acid sequences, while the conformational epitopes are formed from a number of segments of the polypeptide chain. These segments might be distant in the amino acid sequence of a protein, but are

brought together spatially by the protein's three-dimensional structure (VAN REGENMORTEL et al., 1996).

Even though epitopes are suggested to consist of at least 8 - 16 amino acid residues, energy calculations have indicated that 5 - 7 amino acids or 3 - 4 sugar residues are the actual contributors to the binding between the epitope and antibodies (BANNON & OGAWA et al., 2006; LAVER et al., 1990; VAN REGENMORTEL et al., 1996). An allergenic protein may contain a single epitope that is repeating or may have several different epitopes. Most epitopes are thought to be conformational in nature and are particularly difficult to define in relation to food allergens where processing can have such a disruptive effect on native protein structure (MILLS et al., 2009). Until now, epitopes are not fully characterized by their primary protein structure, but also by their tertiary conformation (DALL'ANTONIA et al., 2014; LEHRER et al., 1996). Most IgE epitopes are conformational, whereas some antibodies can bind linear epitopes, but with lower affinity (POMÈS, 2010).

2 Soybean (*Glycine max* (L.) MERR.)

Soybean (*Glycine max* (L.) MERR.) belongs to the Leguminosae family, and is cultivated worldwide for food and animal feed (FRIEDMAN & BRANDON, 2001). Soybean has become an important agriculture crop with a steady increase in worldwide annual production, which rised from 82.8 million metric tons to 108 million metric tons between 2012 and 2014 (FAOSTAT, 2014, www.soystats.com). Soybean is now an essential and dominant source of protein and oil. The USA is the world's leading soybean producer representing 34 % of the world's production, followed by Brazil with 30 %, Argentina with 18 %, China with 4 %, India with 3 %, Paraguay with 3 %, and Canada with 1 % (FAOSTAT, 2014).

Due to its high protein and oil contents, soybean is one of the most important vegetable protein and oil source for food industry. The expansion in soybean production and the increasing importance of soybean as a world crop could be *inter alia* predominantly attributed to the increasing consumer awareness of soybean-based food as natural health-promoting products (MESSINA & MESSINA, 1991).

Native to China and Southeast Asia, soybeans are still important staples in those countries. Soybeans are processed into various fermented and non-fermented traditional dishes such as soy sauce, miso, natto, yogurts, kinako, protein crisp, desserts, baby food, and soy milk, which is further processed into tofu and yuba. The pro-

duction and consumption of soy products in Europe has started in the beginning of the 18th century, and has increased enormously in the last decade. In contrast to the large variety of products in the Asian countries, soybean products in Europe are primarily milk, tofu, soy sprouts and edamame. A new category of texturized soybean products such as meat-like products but also yoghurt and soy ice cream have emerged on the market in the last years (HAMMOND & JEZ, 2011).

Furthermore, soy-based ingredients have been developed for a great variety of mainstream food uses and include soybean oil, texturized soy protein, soy flour (50 % protein), flakes, grits, soy protein concentrate (65 - 85 % protein), soy protein isolate (> 85 % protein), soybean lecithin, and soybean fiber (ALVAREZ & BOYE, 2012; TAYLOR et al., 2015). Soy proteins are additionally introduced into the diet early in life particularly for infants with intolerance to cow's milk (BUSINCO et al., 1992). Soybean-based products are also used as the primary protein source for those with several other disorders such as lactose intolerance and severe gastroenteritis in infants (BUSINCO et al., 1992).

2.1 Composition and nutritional value of soybeans

Mature soybean seeds are composed of approximately 35 - 45 % proteins, 17 - 20 % oil, 31 - 35 % carbohydrates, 5 % ash/minerals and 12 % moisture (L'HOCINE & BOYE, 2007; USDA, 2013).

Several health benefits have been reported associated with the consumption of soybeans. Since the FDA approved the soy protein health claim, in which they proposed that 6.25 g of soy protein per serving size must be contained in the respective food product, several studies have been conducted to support this claim. These studies have demonstrated the link between soy product consumption and the prevention of coronary heart disease and cancer, improvement of bone mineral density as well as lowering cholesterol levels and low-density lipoprotein cholesterol level (ANDERSON et al., 1995; TAYLOR & HEFLE, 2001; TAYLOR et al., 2015). Health-promoting bioactive components such as lecithin, isoflavones, and saponins were also found (HE & CHEN, 2013). However, anti-nutritive factors have also been reported in soy. Approximately 6 % of soy proteins are classified as inhibitors of trypsin and chymotrypsin such as Kunitz trypsin and Bowman-Burk inhibitor, and approximately 0.5 % are sugar-binding lectins (TAYLOR et al., 2015).

2.2 Soybean proteins

Soy proteins account for up to 35 - 45 % of the whole soybean seed (L'HOCINE & BOYE, 2007; USDA, 2013). They belong to the storage proteins and consist of 90% salt-soluble globulins and 10% water-soluble albumins (BELITZ, 2009). The globulins can be classified on the basis of their Svedberg (S) coefficient into 2S, 7S, 11S, and 15S fraction (Table 1) (KINSELLA, 1979; SVEDBERG, 1933).

Table 1. Distribution of soy storage proteins (KINSELLA, 1979; SVEDBERG, 1933)

Protein fraction	MW (kDa)	Percent of total protein (%)	Principal components
2S	8 - 50	8 - 22	Trypsin inhibitor, cytochrome C
7S (β -conglycinin)	150 - 200	35 - 37	Lipoxygenase, β -amylase, lectin, globulins, vicilin-like
11S (glycinin)	300 - 400	31 - 52	Globulins, legumin-like
15S	600	5 - 11	Polymers

MW = molecular weight in kilo Dalton (kDa); S = Svedberg coefficient

The 2S fraction comprises several low molecular mass components like the trypsin inhibitors Bowman-Birk inhibitor, cytochrome C, and Kunitz trypsin inhibitor (KINSELLA, 1979, L'HOCINE & BOYE; 2007). The legumin-like protein (glycinin, 11S globulin) and the vicilin-like protein (β -conglycinin, 7S globulin) account for 65 - 80 % of the total soluble proteins. The characterization of the 15S fraction is not yet completed; however, it is believed that it is composed of protein polymers (L'HOCINE & BOYE, 2007).

Soybean glycinin is a hexameric protein of approximately 350 kDa with a compact quaternary structure stabilized via disulfide, electrostatic and hydrophobic interactions. The subunit composition of glycinin varies according to cultivar (MORI et al., 1981). It is composed of five A-SS-B pairs of non-identical subunits, G1 - G5 (UTSUMI et al., 1997). Each subunit has an acidic (A, \sim 34 - 39 kDa, pI = 4.2 - 4.8, A1 - A5) and a basic polypeptide chain (B, \sim 20 kDa, pI = 8.0 - 8.5, B1 - B4) interlinked via a single disulfide bond, except for the acidic chain A4, present in G4 subunit (RENKEMA et al., 2001).

Soybean β -conglycinin is a densely packed trimeric glycoprotein and composed of four subunits: three major subunits α (\sim 68 kDa), α' (\sim 72 kDa) and β (\sim 53 kDa),

which are associated via hydrophobic interactions and a minor subunit γ . All of the monomers include a single asparagine-linked glycosylation site, which are glycosylated by addition of a polymannose or complex glycan chains (MARUYAMA et al., 2003).

2.3 Soybean allergy

The first report describing food allergy and asthma to soy in humans was described in 1934 (Duke, 1934). For many years, soy was considered a weak sensitization protein on the basis of animal studies (JEDRYCHOWSKI & WICHERS, 2009). Since the 1960s, there is no doubt that soy is an important food allergen. Therefore, many attempts have been made to investigate the detailed mechanisms of soy allergy, including the molecular structure of the epitopes. As a consequence, the Federal Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004 includes soybean in its definition to the "big 8" most significant food allergens as already described in paragraph 1.2. Furthermore, the FALCPA obliges that all soy-containing foods must list the word "soy" on the label in the U.S. and EU among others (GENDEL, 2012).

Although the prevalence of soy allergy is not precisely known and contradictory results exist, it is expected to escalate due to the increasing consumption of soy-containing food products (FAO, 1995). A recent EFSA supporting publication reviews the prevalence of soy allergy being between 0% and 2.7% (EFSA, 2007; VERHOECKX et al., 2015). Soy allergy is more common among infants and young children than adults due to the exposure to soybean-based infant formula (CORDLE, 2004). Soy is one of the major allergens to which children under three years of age generate a food hypersensitivity reaction. However, many children outgrow their allergy, wherefore the prevalence in adults is lower (SAMPSON & SCANLON, 1989). Symptoms range from mild skin reactions to even severe systemic reactions including anaphylaxis. There are three main types of soybean allergic reactions. The first type consists of IgE-mediated reactions that can produce respiratory, cutaneous, and gastrointestinal symptoms. The second type is a non-IgE-mediated reaction, and includes soy-induced enterocolitis. The third and least common reactions are anaphylactic reactions, which are rarely known (WILSON & BAHNA, 2005).

Up to now, standards for acceptable minimum allergen doses have not been set. The estimated soy allergy threshold is low and small amounts of allergens, ranging from

1.3 μg to 500 mg, may be enough to trigger an allergy (FDA, 2004; L'HOCINE & BOYE, 2007). For comparative purposes, CORDLE (2004) set the "safe" soy protein dose for soy-sensitive individuals to approximately 400 mg of soybean proteins.

2.4 Soybean allergens

Up to now, at least 21 allergenic soy proteins with molecular masses ranging from 7.0 to 76.2 kDa have been identified (FARRP, 2015). However, only eight of them have been characterized and registered by the WHO-IUIS Allergen Nomenclature Sub-Committee (WILSON & BAHNA, 2005). Gly m1 and Gly m2 are soybean hull proteins. In addition, Gly m1 consists of the two hull-proteins Gly m1A and Gly m1B, which are very similar in their chemical structure and therefore called isoallergens. Gly m3 indicated profilin, which is related to the birch-pollen-related allergen. The fourth allergen (Gly m4) is a stress-induced pathogenesis-related starvation associated message protein. The two storage proteins β -conglycinin (Gly m5) and glycinin (Gly m6) belong to the allergenic proteins as well. At least, Gly m7 and Gly m8 are dedicated to the seed biotinylated protein and the 2S albumin, respectively (<http://www.allergen.org>). In addition to the official IUIS allergens, there are various other proteins that have been shown to bind to IgE from soy-sensitive individuals. All potential soybean allergens currently available in literature have been summarized in Table 2.

OGAWA et al. (1993; 2000) and TSUJI et al. (1997) identified three major soybean allergens: Gly m Bd 60K, Gly m Bd 30K and Gly m Bd 28K. Gly m Bd 30K, also known as P34, a monomeric glycoprotein with thiol protease activity of the papain family, is the strongest immunodominant allergenic protein, which is recognized by 65 % of soy-sensitive individuals with atopic dermatitis. Gly m Bd 60K is the α subunit of β -conglycinin and Gly m Bd 28K, minor glycoprotein has been recognized by soybean sensitive patients with atopic dermatitis with about 25 % of incidence (OGAWA et al., 2000). Recently, Gly m IFR, an isoflavone reductase, and five IgE-binding proteins have been identified in soy lecithin (P7, P12, P57, P39, and STI) (GU et al., 2001; XIANG et al., 2008). Furthermore, a protein, called P 22-25, has been found to induce strong antibody response in calves (HESSING et al., 1996).

As the focus of this work was on the reduction of Gly m6 and primarily Gly m5, these two soy allergens will be described in more detail. For a further characterization of the other allergens, Table 2 and the respective literature could be used as comprehensive information material.

Sensitization to major soybean proteins Gly m5 and Gly m6 has been shown to be potential indicator for severe allergic reactions to soy (HOLZHAUSER et al., 2009). Recent studies have shown the sensitivity and IgE reactivity to all three major subunits of Gly m5 (HOLZHAUSER et al., 2009; KRISHNAN et al., 2009). An IgE-binding to Gly m5 and Gly m6 was found in 86 % of persons showing symptoms of anaphylaxis after soy consumptions and 55 % with moderate reactions had IgE to Gly m5 or Gly m6 (BALLMER-WEBER et al., 2007). European and Japanese IgE-binding studies respectively found up to 67 % recognition of Gly m5 by soy allergic sera (HOLZHAUSER et al., 2009; ITO et al., 2011). Gly m5 has also been identified as the soybean allergen responsible for food induced anaphylaxis after the consumption of tofu (ADACHI et al., 2009). The acidic polypeptides of G1 and G2 of glycinin are mainly reported to be allergenic, whereas also all five subunits are known to react with IgE (BEARDSLEE et al., 2000; HELM & BURKS, 2000; HOLZHAUSER et al., 2009). In a recent study, IgE binding studies in Europe and Japan respectively found up to 56 % recognition of Gly m6 by soy allergic sera (HOLZHAUSER et al., 2009; ITO et al., 2011). Both Gly m5 and Gly m6 can bind IgE through linear and conformational epitopes (HELM & BURKS, 2000; OGAWA et al., 1995). Eleven linear epitopes have been identified in glycinin by B-cell epitope mapping among which, four are immuno-dominant (HELM & BURKS, 2000). Gly m5 and Gly m6 are stable proteins and potent allergens due to the combination of linear and conformational epitopes and their structural resistance to denaturation from dense packing and disulfide bonds.

Table 2. Soybean (*Glycine max* (L.) MERR.) allergens

MW (kDa)	Allergen Nomenclature	Biochemical Name & Protein Family	References
7.0 - 8.0	Gly m1*:	Non-specific lipid transfer protein (Prolamin)	GONZÁLEZ et al. (1991)
7.0	Gly m1B (1.0102)*	Hydrophobic seed protein (S1), Lipid transfer protein; aeroallergen	KLEIN-TEBBE et al. (2002); GONZÁLEZ et al. (1991)
7.5	Gly m1A (1.0101)*	Hydrophobic seed protein (S2), Lipid transfer protein, aeroallergen	KLEIN-TEBBE et al. (2002); HELM et al. (1998)
8.0	Gly m2*	Defensin (hull protein), aeroallergen	CODINA et al. (1997)
7.0 - 9.0	Gly m LTP	Lipid transfer protein (Prolamin)	WILSON et al. (2005)
10	Gly m CPI	Cysteine protease inhibitor	NATARAJAN et al. (2006)
12	Gly m8*, Gly m 2S	2S albumin	EBISAWA et al. (2013); Gu et al. (2001); MITTAG et al. (2004)
14	Gly m3*	Profilin, 2S-Globulin, panallergen	AMNUAYCHEEWA & DE MEJIA (2010); OGAWA et al. (2000); RIHS et al. (1999)
15	Gly m EAP	Embryonic abundant protein	NATARAJAN et al. (2006)
17	Gly m4*	Pathogenesis-related (PR)-10, 2S-Globulin, Bet v1-like homologue, starvation associated message 22 (SAM22)	OGAWA et al. (1991); MITTAG et al. (2004); KLEINE-TEBBE et al. (2002)
20 - 21	Gly mTI	Kunitz-Trypsin inhibitor (KTI)	OGAWA et al. (1991); BURKS et al. (1991)
18 - 22	Gly m6.0201 (G2)*	Glycinin: basic subunit, whey fraction (11S-legumin)	HELM et al. (2000)
23	23-kDa	Chitinase	HIEMORI et al. (2004); XIANG et al. (2004)
23 - 28	Gly m Bd 28K	Vicilin (P28), 7S-Globulin	OGAWA et al. (2000); TSUJI et al. (1997); GONZÁLEZ et al. (1991)
29 - 31	Gly m6*	A1, A2, A4 polypeptides, soy whey	OGAWA et al. (1991)
32	Gly m lectin	Soybean lectin, soybean agglutinin	MITTAG et al. (2004); BREITENDER & EBNER (2000); AMNUAYCHEEWA & DE MEJIA (2010)
34	Gly m Bd 30K	Thiol-protease (P34), vacuolar protein oil-body glycoprotein	OGAWA et al. (1993); HELM et al. (1998); GONZÁLEZ et al. (1991)
39	Gly m 39kD	39 kDa protein (P39-1)	XIANG et al. (2008)
35 - 40	Gly m6.0101 (G1)*	Glycinin: acidic subunit (11S-legumin)	BAERDSLEE et al. (2000)
50	Gly m Bd 50K	50 kDa protein, hull protein homology to chlorophyll A-B binding protein	CODINA et al. (2002)
47 - 52	Gly m5.0301* Gly m5.0302*	Beta-subunit of beta-conglycinin (Cupin, 7S-vicilin)	KRISHNAN et al. (2009)
53	Gly m oleosin	Oleosin	LIN et al. (2006); PONS et al. (2002)
61.2	Gly m6 (G4)*	Glycinin (11S-legumin)	NATARAJAN et al. (2006)
63 - 67	Gly m5.0101*, Gly m Bd 60K, Gly m Bd 68K, Gly m Bd 70K	Alpha-subunit of beta-conglycinin (Cupin, 7S-vicilin)	OGAWA et al. (2000); TSUJI et al. (1997); OGAWA et al. (1995); HOLZHAUSER et al. (2009); RIHS et al. (1999)
71 - 72	Gly m5.0201*	Alpha'-subunit of beta-conglycinin (Cupin, 7S-vicilin)	KRISHNAN et al. (2009); RIHS et al. (1999)
76.2	Gly m7*	Seed biotinylated protein	NATARAJAN et al. (2006)
94	Gly m LOX	Lipoxygenase, enzyme	BAUR et al. 1996

* Soy allergens registered by the International Union of Immunological Societies (IUIS)

3 Soybean allergy mitigation methods

Currently, total avoidance of soy-containing foods and prompt treatment of allergic shocks with medicine i.e. epinephrine are still likely to be the only way to avoid severe outcome.

Due to the various health benefits and economic importance of soy products and concomitantly the considerable increase of food allergies, investigations on the mitigation of soy allergy are indispensable. As a consequence, science and industry were searching for thermal and nonthermal technologies - or combinations thereof - to control food allergy, in particular soybean allergy (for review see SHRIVER & YANG, 2011; VERHOECKX et al., 2015). A variety of processing technologies have been studied with varying success in proof-of-concept investigations into the design of low-allergen foods. An overview of those technologies is shown in Figure 4.

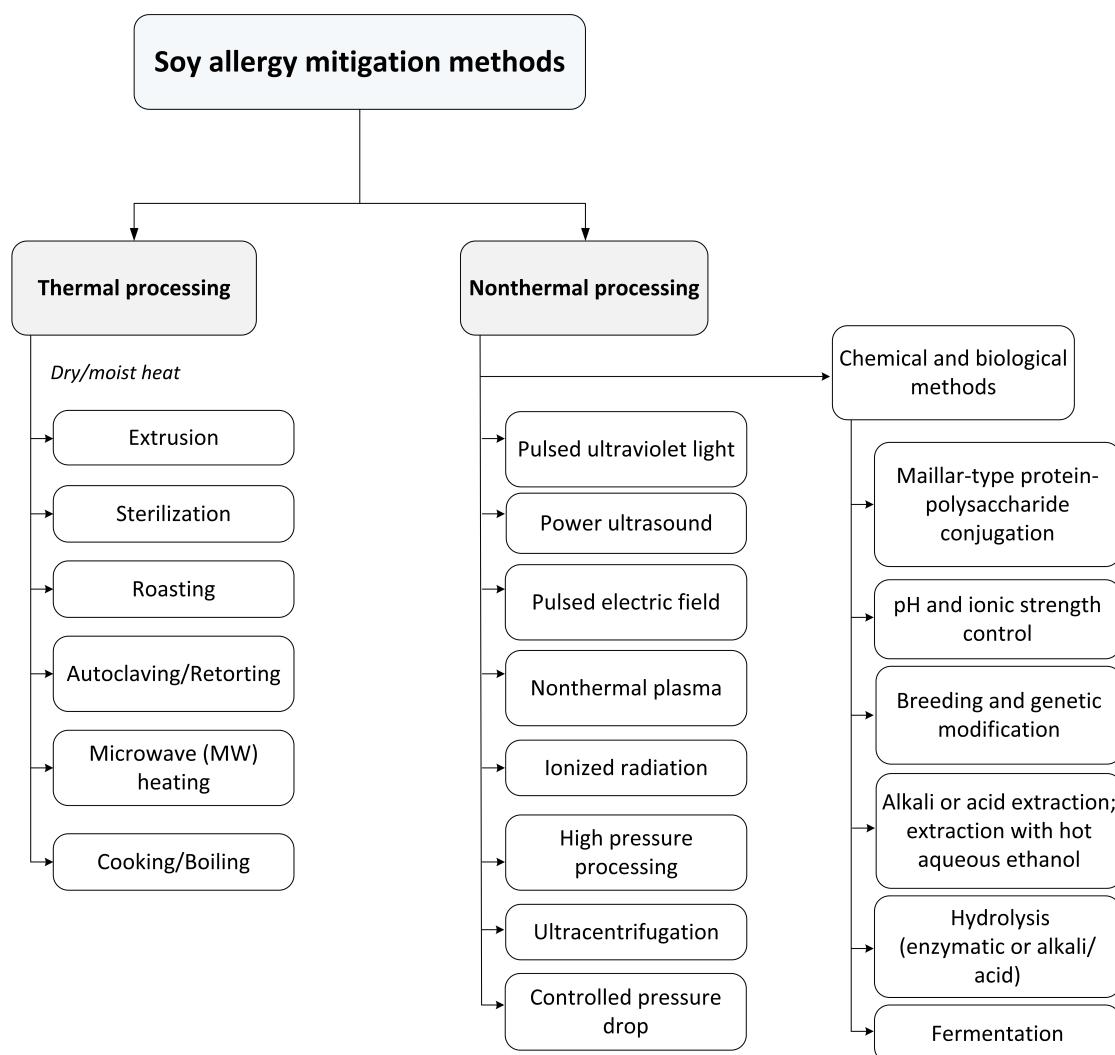


Figure 4. Thermal and nonthermal soy immunoreactivity mitigation methods.

Thermal treatments include processes applying dry heat, including roasting, or moist heat such as retorting, cooking, extrusion, and boiling. Nonthermal treatments comprise biological and chemical methods such as fermentation and enzymatic hydrolysis as well as emerging technologies, including ionized radiation, high pressure processing, pulsed ultraviolet light and nonthermal plasma.

Multiple mechanisms for the reduction of the allergenicity exist, but the underlying concept of all of them is always the modification of the protein structure in order to affect the epitopes. Therefore, an effective treatment method must be able to interfere with the amino acid sequence at a specific position (= linear epitope) or to alter the protein conformation (= conformational epitope) of the allergen (SATHE et al., 2005). This modification could result in the destruction, modification, masking and unmasking of previously hidden epitopes. In addition, immunoreactive epitopes concealed inside the tertiary structure might be revealed, which leads to the formation of new epitopes, called "neoallergens/neoantigens/neoepitopes" (PASCHKE & BESLER, 2002). In general, conformational epitopes are expected to be more susceptible to processing induced destruction than linear epitopes.

Thermal and nonthermal technologies offer great opportunities to modify the nature of allergenic epitopes by commonly four basic mechanisms: protein fragmentation, denaturation, aggregation and covalent modification including crosslinking and covalent attachment of chemical compounds through i.e. Maillard-type conjugation, (FRIAS et al., 2008; WILSON & BAHNA, 2005). Since each type of process might differ in its effectiveness on epitope modifications (Figure 5), individual treatments must be considered carefully.

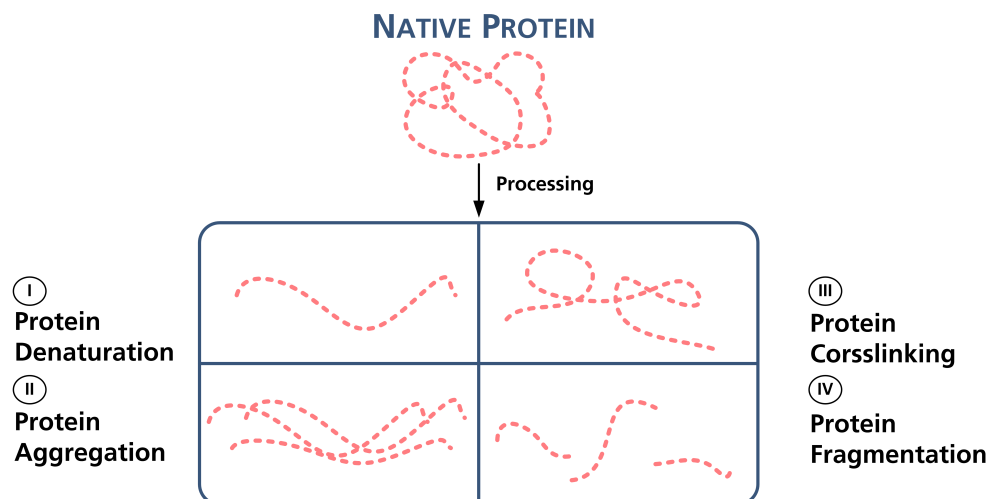


Figure 5. Putative inactivation mechanisms caused by food processing on food allergens (modified according to SHRIVER, 2011). Allergen in native state might undergo (I) protein denaturation, (II) aggregation, (III) crosslinking, or (IV) fragmentation following food processing.

For conformational epitope modification, heat treatment or high pressure processing are suggested, since heat or pressure cause partial unfolding of soy proteins (HUANG et al., 2014). As linear epitopes are not as easily accessible to food processing as conformational epitopes, they might be altered through acid or enzymatic hydrolysis, which is able to destroy the primary protein sequence (THOMAS, 1998). Consequently, any modification, deletion, or substitution of linear epitopes may result in a loss of IgE-binding and may in turn potentially result in the reduction of soybean allergenicity.

The objective of this chapter was to provide an overview of the major aspects of soybean allergy mitigation methods. The examples of thermal and nonthermal processes described in the following sections illustrate the diversity and complexity of issues to mitigate soy allergy. Hereby, emphasis was put on high pressure processing, pulsed ultraviolet light, cold atmospheric pressure plasma, ionized radiation as well as fermentation and enzymatic hydrolysis as the work performed within this thesis focused on nonthermal technologies. These technologies are able to preserve the original characteristics, organoleptic properties and nutritional benefits of food products compared to thermal processing. Although all methods might reduce the allergenicity of foods, certain limitations remain in their practical application.

3.1 Thermal processing

Thermal processing was traditionally applied for improving food safety, modifying food texture and for the modification of the flavor profile as a consequence of the Maillard-reaction during baking for example (DAVIS et al., 2001). In addition, heat treatment might reduce the allergenicity of proteins by inducing denaturation (i.e. tertiary and secondary conformational), cross-linking (= masking linear epitopes) as well as aggregation or rearrangement of disulfide bonds (BOYE et al., 2010).

The effect of thermal processing on soybean allergenicity has been extensively investigated in the last decades. Several researchers investigated the effect of heating (SHIBASAKI et al., 1980), boiling (MÜLLER et al., 1998), microwave heating (VIETHS et al., 1995) and twin-screw extrusion of raw soybeans (FRANCK et al., 2002; OHISHI et al., 1994; SAITOH et al., 2000) on allergen reduction. All of these methods revealed a considerable reduction in allergenicity. However, other researchers showed contrary findings. An elevated allergenicity has been found after heating/autoclaving of soy proteins primarily at temperatures higher than 120 °C (SHIBASAKI et al., 1980; YA-

MANISHI et al., 1995). The increase of allergenicity was explained by the formation of so-called "neoantigens" due to heat-induced conformational changes. It could be assumed that buried epitopes will be uncovered or new epitopes will be generated due to thermal treatment (DAVIS et al., 2001; CODINA et al., 1998; HERIAN et al., 1990).

Although heat treatment might have the ability to reduce soybean allergy at certain processing conditions, the major obstacle associated with high-temperature treatment is the risk to alter the nutritional value as well as the organoleptic properties and the unique functional of soybean proteins.

3.2 Nonthermal processing

The change in consumer demand and food safety issues coupled with the awareness that traditional thermal technologies result in nutritional losses and deteriorated functional and organoleptic properties led to the emergence of nonthermal technologies. More recently, novel nonthermal technologies i.e. irradiation and plasma treatment have emerged in the food industry due to their negligible effects on food properties (for comprehensive review see HUANG, et al. 2014; SHRIVER & YANG, 2011; VERHOECKX et al., 2015).

Different nonthermal processing technologies have been investigated with respect to their effects on soy allergy, including physical treatments such as ultracentrifugation (SAMOTO et al., 1994) and instant controlled pressure drop (CUADRADO et al., 2011; TAKÁCS et al., 2014) as well as chemical modifications such as Maillard-type carbohydrate conjugation or transglutaminase treatment (ARITA et al., 2001; BABIKER et al., 1998; USUI et al., 2004; VAN DE LANGEMAAT et al., 2007). However, all of these technologies were limited due to their restricted effectiveness on soy allergen removal. As a biological method, genetic modification has gained much attention despite its controversial nature. HERMAN (2003) used transgene-induced gene silencing to prevent the accumulation of Gly m Bd 30K in soybean seeds. Similar studies have been conducted by OGAWA et al. (2000) and SAMOTO et al. (1997), who eliminated the α -subunit of Gly m5 and Gly m Bd 28K through breeding. However, genetic modification has still a poor reputation in society as the fear of allergic reactions related with genetically modified crops remained.

The production of soy protein isolate uses an acid and alkali extraction of defatted soybeans, or an extraction of soy flour with hot ethanol. Within the studies conducted by HERIAN et al. (1990; 1993), they demonstrated that commercial soy

protein isolates have diminished IgE-binding compared to whole soybeans, whereas soy products such as soy flour and soy protein concentrate retained most of the allergenicity. Immunoreactivity of soy can also be controlled by pH and ionic strength (L'HOCINE & BOYE, 2007). Pulsed-electrical fields and high intensity ultrasound treatment for soy allergen reduction have so far not been reported in literature. However, high intensity ultrasound was already successfully applied for the reduction of shrimp allergenicity (LI et al., 2006). TOSHIKO et al. (2004) investigated the effect of pulsed-electrical fields on various allergen-antibody interactions and found that only ovalbumin showed a drastically reduced binding-ability in ELISA.

As the focus of this thesis was put on high pressure assisted enzymatic hydrolysis, enzymatic hydrolysis, fermentation, pulsed ultraviolet light as well as cold atmospheric pressure plasma and gamma-irradiation, these methods will be reviewed in the following sections in detail.

3.2.1 High pressure processing

High pressure processing (HPP) has attracted much attention in the field of food science in the last decade as excellent nonthermal technology for food preservation, addressing most of the recent challenges faced by the food industry (KNORR et al., 1992; RASTOGI et al., 2007). The effects of HPP are investigated at different levels, ranging from proteins and enzymes to microorganisms. The main application of HPP is the decontamination of foods, thereby extending shelf life, with little effects on flavor and nutritional value (KNORR et al., 1992).

HPP causes reversible or irreversible structural modifications in proteins, which in turn leads to protein denaturation, aggregation, or gelatinization (MILLS et al., 2009). Furthermore, HPP is known to unfold proteins, in particular to rupture the noncovalent interactions within the protein molecule. As a consequence, the tertiary and quaternary structure of proteins will be destroyed (HUANG et al., 2014). As the tertiary structure of a food allergen is the key to conformational epitopes, HPP has a great potential in reducing food allergenicity. However, only a few studies exist investigating the effect of HPP on soybean allergenicity. PEÑAS et al. (2011) compared the effects of high pressure on the protein pattern, amino acid composition, and immunoreactivity of soybean, tofu, and bean sprouts. HPP did not affect the protein profile of all products, but a lower intensity of some protein bands could be detected. The results confirmed an important reduction in immunoreactivity of

pressurized soybean sprouts, which was assessed by ELISA. LI et al. (2012) found that HPP could be applied to reduce the allergenicity of soy protein isolate for infant formula, which was assessed by a commercial sandwich ELISA. The IgG-binding was reduced by 48.6 % after pressurization at 300 MPa for 15 min.

Another promising approach for HPP application is its combination with enzymatic hydrolysis to destroy food allergens. It has been shown, that HPP could promote/facilitate the diffusion of the enzyme preparations into the food or protein matrix enhancing the enzymatic hydrolysis reactions due to an advanced enzyme accessibility. Only very few researchers ascertained this combination process so far, but all of them have shown similar findings - an improved enzymatic hydrolysis due to HPP application (CHICON et al., 2008; GARCIA-MORA et al., 2015; PEÑAS et al., 2004; PEÑAS et al., 2006a; PEÑAS et al., 2006b). In particular Peñas and colleagues confirmed a positive impact of HPP on hydrolysis of soybean whey proteins, where HPP was performed prior to or during hydrolysis. Compared to enzymatic treatment at 0.1 MPa, HPP at 100 MPa enhanced the proteolytic activity of certain proteases (PEÑAS et al., 2004). At a pressure of 300 MPa and a subsequent enzymatic hydrolysis, a marked decrease in Gly m1 immunoreactivity could be detected using ELISA with anti-Gly m1 monoclonal specific antibodies and no antigenicity could be detected using ELISA with sera from three children allergic to soy, when combining enzymatic hydrolysis with Alcalase, Neutrase or Corolase PN-L with HPP at 300 MPa.

Most researchers investigated the effect of HPP alone or combined with enzymatic hydrolysis on residual Gly m1 or Gly m Bd 30K allergenicity. However, to the best of our knowledge, comprehensive research about the combined effect of HPP and enzymatic hydrolysis on the residual immunoreactivity of major soy allergens Gly m5 and 6 as well as the resulting sensory perception and physicochemical properties are not available in literature so far.

3.2.2 Pulsed ultraviolet light

More recently, the use of pulsed ultraviolet (PUV) light treatment has attracted considerable attention as an alternative food preservation method. In the PUV technology, the electrical energy is stored in a capacitor and released as short-period, intermitted high-peak power pulses. An inert gas within the PUV lamp is ionized by electrical energy and produces broad spectra of light containing wavelength from near-infrared to ultraviolet (KOUTCHMA et al., 2009). The efficiency of

PUV has been attributed to photochemical, photothermal, and photophysical reactions (KRISHNAMURTHY et al., 2009; YANG et al., 2010), which might contribute to changes in protein structure, thereby reduction in IgE-binding ability.

Previous studies have shown that PUV has the ability to reduce the level of allergenicity in peanut products as well as soybean, shrimp, almond, and wheat extracts (CHUNG et al., 2008; LI et al., 2013; NOOJI, 2011; SHRIVER, 2011; YANG et al., 2010; YANG et al., 2012). YANG et al. (2010) treated soybean extracts with PUV light at a distance of 13.2 cm for 2, 4, and 6 min, which resulted in a residual IgE-binding ability of 20 %, 44 % and 50 %, respectively. However, PUV light only destroy food allergens on the food surface as the penetration depth is restricted, and therefore has limited ability to have a complete effect on food allergens.

3.2.3 Cold atmospheric pressure plasma

Cold atmospheric pressure plasma (CAPP) is electrically energized and highly energetic gaseous matter, which can be generated by electrical discharge across an electrical field. Plasma is generated when a significant number of gas atoms are energized by heat or another energy source which ionizes the atoms and release electrons, which is the fourth state of matter (LAROUCSI, 2005). Application of CAPP has gained much attention as an alternative microbial inactivation technology due to its germicidal effects (EHLBECK et al., 2011).

The effect of CAPP on protein structure and the mechanisms being involved in the reduction of immunoreactivity remain still uncertain. Conformational epitopes can be altered by plasma-induced aggregation into insoluble aggregates or crosslinking of proteins as a consequence of a loss of protein solubility, whereas linear epitopes can be affected by fragmentation. Plasma-immanent species present as hydroxyl radicals can cleave peptide bonds and oxidize amino acid side chains, which might lead to fragmentation. Oxygen radicals are involved in etching processes and the oxidation of proteins (SUROWSKY et al., 2013). Furthermore, the cleavage of disulfide bonds within a peptide due to dissociative addition of a hydroxyl radical to form RSH and RSO \cdot at the cleavage site are conceivable. Reactive oxygen radicals (ROS), as atomic oxygen or hydroxyl radicals, may attack amino acids, which are sensitive to oxidation.

Only a small number of studies showed that CAPP might be an effective method to reduce immunoreactivity of whey, α -casein, wheat and shrimp proteins (TAMMINEEDI et al., 2013; NOOJI, 2011; SHRIVER, 2011). SHRIVER (2011) observed a drastically

reduced allergenicity of the allergenic shrimp protein tropomyosin up to 76 % applying direct CAPP with a voltage of 30 kV and a frequency of 60 Hz for 5 min. NOOJI (2011) was able to reduce wheat allergenicity up to 37 % with direct CAPP in his studies. In contrast, TAMMINEEDI et al. (2013) could not find changes in antibody-binding ability of α -casein and whey solutions after samples were exposed to plasma afterglow. However, to the best of our knowledge, reports on the effect of CAPP on soy immunoreactivity are not available in the literature up to now, thus further research is urgently needed.

3.2.4 Ionized radiation

As a nonthermal method, ionized radiation (irradiation), in particular high-energy, short-wavelength electromagnetic gamma-irradiation turned out to be an effective preservation method, extending the shelf-life of perishable foods (KASERA et al., 2012). Ionizing radiation with low dose rates up to 1 kGy is usually applied to control food-borne pathogens as well as to reduce the microbial load and insect infestation, thereby extending the shelf-life of perishable products for commercial purposes (FDA, 1997).

As side effect, this technology may change antigenicity of food proteins by the destruction or modification of epitopes. Although the utilization of gamma-irradiation is limited to a few food applications depending on the individual national legislation, the ability of irradiation to reduce the allergenicity of quite different allergenic food products, including almond, cashew nut, walnut proteins, ovalbumin, bovine serum albumin, milk proteins (beta-lactoglobulin) as well as shrimp tropomyosin and legume proteins has been reported (BYUN et al., 2002; KASERA et al., 2012; SEO et al., 2007; SU et al., 2004). It has been assumed that irradiation structurally alters IgE-binding epitopes by generating primary free radicals, reacting with proteins, which results in protein fragmentation, polymerization/dimerization, and aggregation (KUAN et al., 2013). In contrast, MORIYAMA et al. (2013) found that gamma-irradiation of soybeans between 2.5 and 30 kGy resulted in apparent band profiles of major soy allergens Gly m5, Gly m Bd 30K, Gly mTI, and Gly m4 and band intensities were not significantly changed by irradiation. ELISA analyses suggested no significant changes in the allergen contents, except for a decrease in Gly mTI. Soybean treatment with gamma-irradiation conducted by MANJAYA et al. (2007) resulted in soybean mutants lacking subunits of major soy allergens glycinin and β -conglycinin. Further investigations into the impact of food irradiation on allergen structure and allergenic potential are still warranted.

3.2.5 Fermentation

A regulation of immunogenicity and possibly reduction of allergenicity by fermentation has been hypothesized recently (GRANATO et al., 2010), but has rarely been investigated.

A few studies have confirmed the degradation of soybean allergens during fermentation by microbial proteolytic enzymes in fermented soybean foods such as soy sauce, natto, miso, tempeh, soybean ingredients and feed-grade soybean meals (FRIAS et al., 2008; KOBAYASHI, 2005; SONG et al., 2008; SONG et al., 2010; TSUJI et al., 1995). The effect of the fermentation of whole soybeans with *Bacillus natto* on the allergenicity was also investigated by YAMANISHI et al. (1995). It has also been shown that various lactic acid bacteria (LAB) are able to hydrolyze α' - and α -subunits of soybean β -conglycinin (AGUIRRE et al., 2008). The studies of FRIAS et al. (2008) and SONG et al. (2008) demonstrated that both solid- and liquid-state fermentation of cracked soybean seeds, flour, or meals by various mold strains and bacteria effectively reduced IgE-immunoreactivity by 65 to 99 % as evaluated by an indirect ELISA with human sera. A fermentation process of steamed soybeans using a combination of three microorganisms (*Lactococcus lactis* subsp., *Aspergillus oryzae* and *Bacillus subtilis*) resulted in a soybean product with amino acids and polypeptides of less than 10 kDa (SON et al., 2004). These peptides were tested against sera from soy-sensitive individuals, and antigenicity could not be detected. However, some researchers found that soy could retain its allergenicity through fermentation. In a study conducted by HERIAN et al. (1993), commercial- and acid-hydrolyzed soy proteins and consequently fermented soy products such as miso, tempeh, and tofu were found to retain their allergenicity. Further, HEFLE et al. (2005) reported that a residual allergenicity of 10 - 30 % could be found in soy sauce.

3.3 Enzymatic hydrolysis

3.3.1 Enzymatic hydrolysis of proteins

Since the 1940's, protein hydrolysates have been used for nutritional and clinical management due to their various physiological and physical functions (ADLER-NISSEN, 1984). Hydrolysis is a widely used technique to modify the techno-functional and sensory properties of proteins (PANYAM & KILARA, 1996). The hydrolysis

reaction depends on protease specificity and activity, extent of protein denaturation, enzyme to substrate ratio (E/S), hydrolysis conditions, including temperature, time, pH, ionic strength as well as the presence or absence of inhibitory substances (KILARA, 1985).

Hydrolysis of proteins involves the cleavage of protein peptide bonds, which results in a breakdown of the native protein structure into peptides and amino acids (see Figure 6).

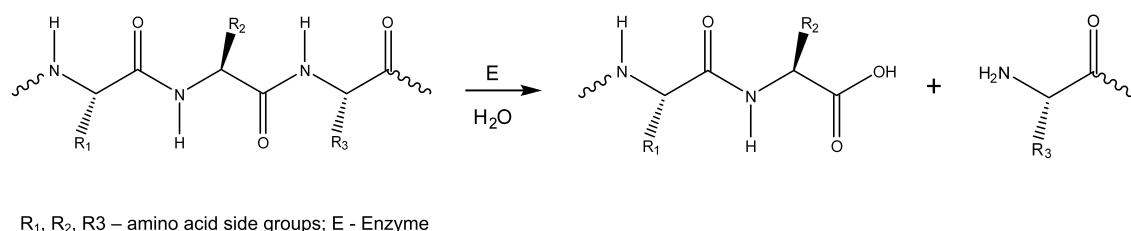


Figure 6. Enzymatic hydrolysis of a peptide bond (SUJITH & HYMAVATHI, 2011).

In protein hydrolysates, the degree of hydrolysis (DH), average peptide chain length, and average molecular weight are the most often used parameters to describe the extent of hydrolysis. The DH value is defined as the fraction of the peptide bonds cleaved and can be calculated as follows:

$$\text{DH (\%)} = \frac{h}{h_{\text{tot}}} \cdot 100$$

, where h_{tot} is the total number of moles of peptide bonds in one mole of protein and h is the number of moles of peptide bonds cleaved per mole of protein.

A wide variety of proteases of different origin has been used for hydrolysis of soy proteins. Proteases can be classified into exopeptidases (= peptidases) and endopeptidases (= proteinases). Endopeptidases hydrolyze amino acids of the interior of the polypeptide chain, while exopeptidases hydrolyze from either the N-terminal (aminopeptidases) or the C-terminal (carboxypeptidases) end of the protein. Based on the mechanism of catalysis, proteinases are divided into four subgroups according to the EC nomenclature, which are the serine (EC 3.4.21), cysteine (EC 3.4.22), aspartic (EC 3.4.23), and metallo-proteinases (EC 3.4.24) (SUJITH & HYMAVATHI, 2011). Proteases can also be mixtures of endo- and exopeptidases e.g. Flavourzyme[®] from Novozymes A/S (Bagsvaerd, Denmark), although most commercial proteases are endopeptidases (HAMADA, 2000).

3.3.2 Effects of enzymatic hydrolysis on soybean allergy

Hypoallergenic foods currently available on the market are mainly produced using enzymatic hydrolysis, which has proven to be an efficient process for disrupting linear and conformational epitopes. A considerable quantity of research was directed on enzymatic hydrolysis using different proteases of microbial or plant origin to mitigate soy allergy (WILSON et al., 2005). The prerequisites for removal of allergenicity are sufficient contact between the allergenic epitope and the enzyme and sufficient control of undesirable side effects that may affect the sensory quality or functionality (FUKUSHIMA, 2004). With the progress of hydrolysis, proteins are broken down to a greater degree, which in turn could result in a loss of the allergenicity (NAGODAWITHANA et al., 2010).

FRANCK et al. (2002) compared the allergenicity of commercial infant formula, which were prepared using enzymatic hydrolysis. These formula obtained proteins with molecular masses below 28 kDa and showed a lack of allergenicity, which was measured by immunoblotting. Hydrolysis with trypsin, pepsin and chymotrypsin is frequently used to prepare hypoallergenic formulas, but other enzymes of bacterial and fungal origin are also investigated. BURKS et al. (1991) artificially hydrolyzed soy proteins with trypsin, chymotrypsin, pepsin, and intestinal mucosal peptidases, which resulted in a 10-fold decrease of IgE-binding capacity.

The combination of different food technologies may also affect the susceptibility of allergens to digestion. For example heat treatment of proteins prior to digestion may affect the digestibility, and has therefore been investigated (TSUMURA et al., 1999; TSUMURA et al., 2004; TSUMURA et al., 2009; VAN BOXTEL et al., 2009). In a study from VAN BOXTEL et al. (2008) it could be shown that pre-heating of soy before digestion by pepsin significantly accelerated the digestibility. As a consequence, IgE-binding capacity of glycinin was reduced to non-detectable. Several researcher groups found a drastically reduced or non-detectable allergenicity after enzymatic hydrolysis of raw or autoclaved soybeans using various proteases from *Bacillus subtilis*, whereas Proleather and Protease N were most effective (ASTWOOD et al., 1996; TSUMURA et al., 1999; TSUMURA et al., 2004; TSUMURA et al., 2009; YAMANISHI et al., 1995; YAMANISHI et al., 1996). The use of sequential enzymatic hydrolysis with pepsin and chymotrypsin has been investigated by LEE et al. (2007). Although a reduction of IgE binding for overall Gly m6 was observed, a 20 kDa peptide was still immunoreactive.

However, a major obstacle associated with enzymatic hydrolysis was the deteriorated sensory and techno-functional properties of the resulting hydrolysates. The production of better tasting hydrolysates with improved functional properties would be a benefit for both industry and consumer.

4 Sensory perceptions of soy hydrolysates

A serious quality concern facing the manufacturer of hydrolysates is the formation of a bitter taste, which often restricts the commercial exploitation as food ingredients. Sensory attributes of soy products, in particular bitterness, continues to be a concern in food industry. Therefore, soy flavor was recognized as an "off-flavor" by the U.S. consumers and foods labeled "containing soy" were perceived negatively for flavor impressions (WANSINK & PARK, 2002).

4.1 Soy protein hydrolysates bitterness

Bitterness of soy protein hydrolysates is often caused by the occurrence low molecular weight peptides, which mainly contain hydrophobic amino acid residues, in particular leucine, proline, phenylalanine, tryptophan, isoleucine, and tyrosine (ISHIBASHI et al., 1988). However, the exact cause is not fully understood and conflicting data exists.

The hydrophobicity, primary sequence, spatial structure, molecular weight, and bulkiness of peptide have been suggested as possible influences for elucidating bitter taste sensation (Figure 7) (KIM et al., 2008). Furthermore, bitterness development depends on the DH value, protein source as well as the protease type and specificity (ADLER-NISSEN & OLSEN, 1979; AUBES & COMBES, 1997; KODERA, et al. 2006). In addition, hydrolysate manufacturing conditions such as pH, time of hydrolysis, enzyme-to-substrate (E/S)-ratio and also the solid content might play an important role in bitter taste formation (FITZGERALD & O'CUINN, 2006; SPELLMAN et al., 2005; SUJITH & HYMAVATHI, 2011).

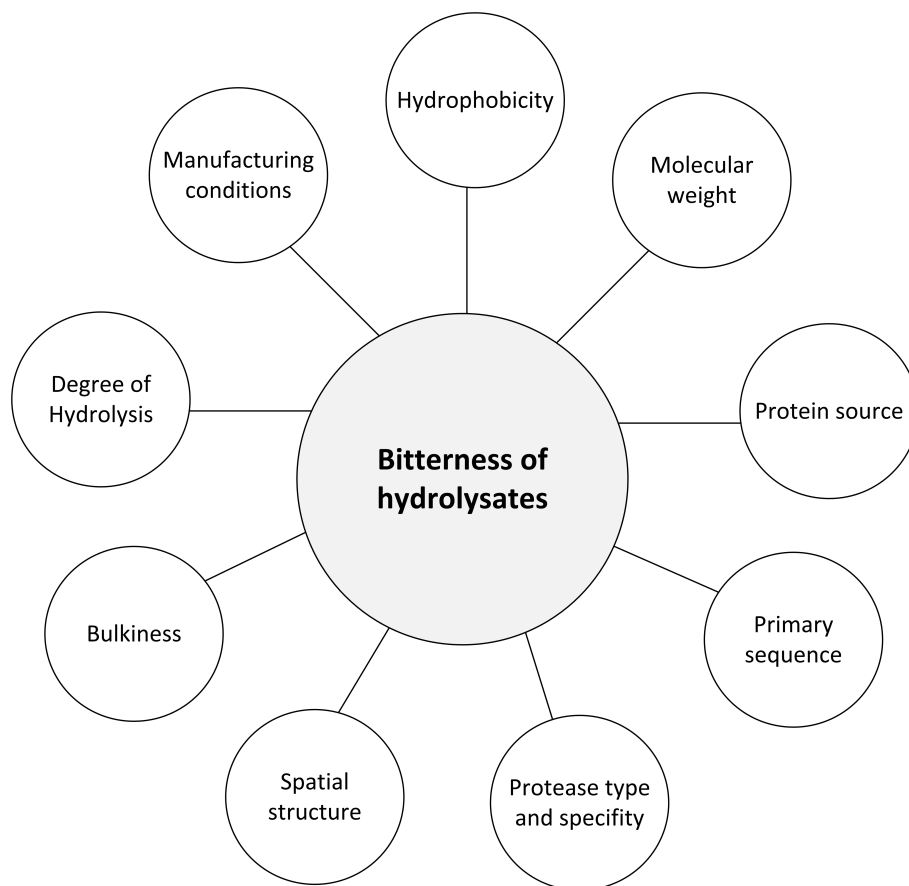


Figure 7. Factors affecting the formation of bitter taste in soy protein hydrolysates.

Traditionally, bitterness is strongly related to the average hydrophobicity of peptides. The "Q-rule" devised by NEY (1971), established a quantitative relationship between amino acid composition of a peptide and its bitterness (for comprehensive review, see FITZGERALD & O'CUINN, 2006; SUJITH & HYMAVATHI, 2011). Recently, however, the general accuracy of the "Q-rule" has come into question. Steric parameters and spatial structure showed also great impact on bitter taste formation, but are not reflected by the average hydrophobicity (KIM et al., 2008). Furthermore, one basic and one or two hydrophobic groups, separated by 4.1 Å (0.41 nm), and a pocket size of 15 Å (1.5 nm) are essential requirements for a bitter taste (ISHIBASHI et al., 1988; TAMURA et al., 1990). Molecular weight limits between 10 and 6 kDa were reported to bitter taste, whereas hydrolysates with a relative molecular weight below 6 kDa were considered not bitter.

In general, it is believed that intact food proteins do not display bitterness as hydrophobic side-chains are oriented towards the interior of the molecule, which are unable to interact with taste receptors (MATOBA & HATA, 1972; SUJITH & HYMAVATHI, 2011). Consequently, proteins are not bitter themselves, but hydrolysis lead

to the exposure of hydrophobic regions, which results in a bitter taste formation (MAEHASHI et al., 2008). A complete hydrolysis into free amino acids decreases bitterness again, because hydrophobic peptides are considerably more bitter than the corresponding mixture of free amino acids (WIESER & BEELITZ, 1976). ADLER-NISSEN & OLSEN (1979) proposed a qualitative relationship between the DH value and bitter taste formation. Consequently, the control of the proteolytic reaction and its termination at a given DH value is in general a preferable way of securing a low level of hydrolysate bitterness.

4.2 Soy protein hydrolysate debittering

The practical use of protein hydrolysates for food purpose requires the reduction of bitterness by removal or masking of bitter peptides. Therefore, many attempts have been made to prevent, reduce, eliminate or even mask the bitterness of food hydrolysates. These methods include selective separation or adsorption of bitter peptides such as treatment with activated carbon, extraction with alcohol, isoelectric precipitation, chromatography on silica gel, hydrophobic interaction chromatography (SAHA & HAYASHI, 2001). The bitterness in hydrolysates was also masked via the addition of various agents such as polyphosphates, asparagine and glutamine as well as α -cyclodextrin and through the admixture with intact proteins to mask the bitter taste (FITZGERALD & O'CUINN, 2006; SUJITH & HYMAVATHI, 2011; SUN, 2011). Debittering via transpeptidation, T-plastein reaction in addition to cross-linking using transglutaminase as well as the modification of taste signaling (blocking bitter taste perception) and bitter blocking (transduction pathways) have also been investigated (SUJITH & HYMAVATHI, 2011; SUN, 2011). YEOM et al. (1994) demonstrated that chemical modification of lysine by acetylation decreased the bitterness of lysine-acetylated soy protein. Bio-based methods include a further enzymatic hydrolysis of bitter peptides with exopeptidases, which is part of this thesis and, therefore, will be explained in detail.

However, all of these methods have their own limitations. Most of them are commonly cost-effective, need specific equipment, may decrease solubility of the product, result in a low recovery yield, and can cause a considerable loss of essential function of amino acids, thus reducing the nutritional value (SUN, 2011). Furthermore, the functional and biological activity of peptides could be destroyed as well as a soapy and brothy off-flavor often develops and a certain intensity of bitterness still remains (SUJITH & HYMAVATHI, 2011).

Considerable attention has been paid to peptidase-mediated debittering of protein hydrolysates. This means an enzymatic hydrolysis of bitter peptides with, particularly exopeptidases from different sources, destroying terminal bitter peptides, condensation of bitter peptides using proteases and use of *Lactobacillus* as a debittering starter adjunct (CHAE et al., 1998; FITZGERALD & O'CUINN, 2006; ISHIBASHI et al., 1988; SAHA & HAYASHI, 2001). KODERA et al. (2006) purified a novel soy protease D3 from germinated soy cotyledon, which preferred hydrophobic amino acid residues. This new protease generated a less bitter taste than other proteases. Special attention has been given to proline-specific exopeptidases as proline gives a unique contribution to hydrolysate bitterness. In general, aminopeptidases are not able to hydrolyze amino bonds, thus certain bitterness still remains (FITZGERALD & O'CUINN, 2006). Enzymes hydrolyzing single proline or pairs of proline residues in bitter peptides are of particular interest due to its unusual structure, which is commonly resistant to enzymatic hydrolysis as it oftentimes limits the depth of hydrolysis. BOUCHIER et al. (2001) successfully combined a proline-specific exopeptidase together with an aminopeptidase of broad specificity to drastically lower bitterness of a tryptic hydrolysate of β -casein.

Microorganisms are also an important source of exopeptidases, which are expressed in the media to hydrolysate proteins as nutrient source. Microbial strains such as the genus *Lactobacillus* are extensively used as a debittering starter adjunct for the production of protein hydrolysates, in particular milk proteins, lacking bitter taste (SAHA & HAYASHI, 2001). Similar effects have been observed during hydrolysis with fungal protease preparations and microbial aminopeptidases from different sources for example *Aeromonas caviae* T-64 and *Streptococcus thermophilus* (PepS) hydrolyzing bitter peptides at the N-terminus (IZAWA et al., 1997). L-leucine aminopeptidases are commonly used in food industry for debittering as they are effective in removing single or pairs of hydrophobic amino acids. Some of the commercial aminopeptidases that are used are from lactic acid bacterial and fungal enzyme preparations such as *Rhizopus oryzae*, *Aspergillus oryzae*, and *Aspergillus sojae* (NAMPOOTHIRI et al., 2005). In addition, the carboxypeptidases from i.e. *Actinomucor elegans* are well known to reduce bitterness by releasing bitter peptides at the C-terminus (FU et al., 2011; LI et al., 2008; UMETSU & ICHIMISHIMA, 1988).

5 Functionality and application of soy hydrolysates

Protein hydrolysates have a wide range of application in both food and allied health-care sector (FITZGERALD & O'CUINN, 2006). Food protein hydrolysates can be used as nitrogen fortification agents in beverages, as pre-digested ingredients for enteral/parenteral nutrition and as enriched/isolated peptide preparations for beneficial physiological functions. The functionality of soy proteins is an important attribute for food ingredients and relevant properties for food application comprise protein solubility, water- and oil-binding and emulsifying capacity as well as foamability, and gelation behaviour (KINSELLA, 1979). These properties are mainly governed by the two storage proteins β -conglycinin and glycinin.

Enzymatic hydrolysis significantly impacts the functional properties of the resultant hydrolysate. The hydrolysis of soybean proteins with proteases can increase the protein solubility (MOLINA ORTIZ & WAGNER, 2002), thereby providing functional properties that depends on protein solubility such as foaming and emulsifying properties (JUNG et al., 2005; MOLINA ORTIZ & WAGNER, 2002; TSUMURA et al., 2009). Several researchers have used various protease enzymes to improve their solubility and to achieve the desirable functionality (DE LA BARCA et al., 2000; KIM et al., 1990; MOLINA ORTIZ & CRISTINA AN, 2000; MOLINA ORTIZ & WAGNER, 2002). To obtain desired functional properties of soy protein hydrolysates, hydrolysis must be carried out under strictly controlled conditions to a specified degree of hydrolysis. A limited degree of hydrolysis usually improves solubility, emulsifying and foaming capacities (DE LA BARCA et al., 2000; ORTIZ & WAGNER, 2002). Molecular size of the peptides decreases as a result of hydrolysis, which has a major effect on many functional properties. Larger peptides of 2 - 5 kDa are ideal for functional ingredients in food, medium sized peptides of 1 - 2 kDa are ideal for sports nutrition (FRØKJAER, 1994; SIEMENSMA & KUNST, 1999) and clinical nutrition (SCHMIDL et al., 1994), while smaller peptides of < 1 kDa are ideal for hypoallergenic infant formulas (SIEMENSMA et al., 1993). With a high nutritional value, soy protein is widely used in the food industry, and the production of infant formula is one of its important applications. Soybean is a suitable alternative food source for babies who are allergic to cow milk (EL-AGAMY, 2007). Besides molecular weight of the hydrolysate, surface activity, hydrophobicity, carbohydrate interaction, and mineral interaction influence various functional properties.

However, in some cases, excessive enzymatic hydrolysis may impair some functional properties of food proteins i.e. emulsifying capacity as well as foaming stability and density (TSUMURA et al., 2005).

6 Aims of the study

The primary aim of this study was the development of an effective nonthermal processing technology for the mitigation of soybean (*Glycine max* (L.) MERR.) immunoreactivity. Emphasis was put on nonthermal processing technologies, which have so far only partially or not been considered appropriately in literature. For this purpose, interdisciplinary and innovative approaches have been investigated. Furthermore, the physicochemical and sensory properties of soy protein isolate should not be negatively affected by this mitigation technology, and have therefore been examined as well.

Hypoallergenic foods currently available on the market are mainly produced by means of enzymatic hydrolysis, which has proven to be efficient to attenuate soy immunoreactivity (WILSON et al., 2005; YAMANISHI et al., 1996). Therefore, food-grade enzyme preparations were screened in order to find an appropriate protease to sufficiently degrade major soy allergens Gly m5 and Gly m6. In addition, functional characteristics required for consumer acceptance such as good sensory and physicochemical properties in terms of protein solubility, emulsifying capacity, water- and oil-binding capacity as well as foaming properties (activity, stability, and density) are required for consumer acceptance were evaluated as well (CHAPTER 1).

Since soy protein is a nutritious while cheap source of high quality proteins, development of better tasting soy-based foods and food ingredients are indispensable to make the products more interesting for food industry. Effective debittering methods, however, have not been successfully developed yet. Therefore, the efficiency of enzyme combination systems applying endo- and exoproteases simultaneously or concomitantly for the avoidance or reduction of bitter taste was investigated. Furthermore, the physicochemical properties of the resulting hydrolysates were investigated in this context (CHAPTER 2).

Despite the improved sensory characteristics, a complete removal of bitterness of soy could not be achieved, wherefore a certain bitter taste remained. Therefore, the efficiency of subsequent fermentation of soy protein isolate hydrolysates was examined. For the fermentation of the soy hydrolysates, various bacterial and mold strains have been investigated to study their potential ability to further reduce bitter taste. The physicochemical properties have been analyzed as well (CHAPTER 3).

In continuation of the investigation on the debittering effect of combined enzymatic hydrolysis and fermentation, it has to be proven whether fermentation alone is effective in the reduction of soy immunoreactivity. Up to now, data about the effect of different food processing technologies on the reduction of major soy allergen Gly m5 do not exist. Gly m5 is one of the most abundant storage proteins of soy and sensitization against this protein is highly indicative for severe allergic reactions (HOLZHAUSER et al., 2009). Therefore, the residual Gly m5 immunoreactivity of fermented soy protein isolate was investigated by *in vitro* methods such as sandwich enzyme-linked immunosorbent assay (ELISA) and western blot using newly developed epitope-specific mouse monoclonal anti-Gly m5 antibodies (mAbs) and sera from soy-sensitive patients. In addition, the effects on the sensory profile and physicochemical properties have been considered (CHAPTER 4).

The processes comprising enzymatic hydrolysis and fermentation used so far might be limited by incomplete allergen destruction due to the compact structure and folding of proteins, thus, epitopes are difficult to access or not susceptible for the proteolytic enzymes. In addition, enzymatic hydrolysis can have an adverse impact on food structure and organoleptic properties, liberating a strong bitter taste, which impedes the utilization as food ingredient (SHRIVER & YANG, 2011). Recently, novel nonthermal food processing technologies have emerged in the food industry due to their negligible effects on food properties (HUANG, et al. 2014; SHRIVER & YANG, 2011).

Consequently, the final objective of this work was to study the efficiency of various emerging technologies to attenuate soy immunoreactivity and their effect on the sensory and physicochemical properties. Therefore, high pressure processing assisted enzymatic hydrolysis will be analyzed (CHAPTER 5).

The study described in CHAPTER 6 was designed to evaluate the effectiveness of other emerging nonthermal technologies such as gamma-irradiation, pulsed ultraviolet light, and cold atmospheric pressure plasma.

Collectively, the processing technologies presented in this thesis provide comprehensive and feasible technological approaches to mitigate soybean immunoreactivity, while sensory and physicochemical properties could be maintained or even improved. The results from this study will be helpful for the production of low-allergen food ingredients with good taste and enhanced functional properties, thereby addressing the growing society concern about food allergies. In addition, these food processing technologies are feasible to other plant-derived proteins and will find application in the food industry.

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CHAPTER 1: Enzymatic treatment of soy protein isolates: effects on the potential allergenicity, technofunctionality, and sensory properties¹

Abstract

Soybean allergy is of great concern and continues to challenge both consumer and food industry. The present study investigates the enzyme-assisted reduction of major soybean allergens in soy protein isolate using different food-grade proteases, while maintaining or improving the sensory attributes and techno-functional properties. SDS-PAGE analyses showed that hydrolysis with Alcalase, Pepsin, and Papain was most effective in the degradation of the major soybean allergens with proteolytic activities of 100 %, 100 % and 95.9 %, respectively. In the course of hydrolysis, the degree of hydrolysis increased, and Alcalase showed the highest degree of hydrolysis (13 %) among the proteases tested. DSC analysis confirmed the degradation of major soybean allergens. The sensory experiments conducted by a panel of 10 panelists considered the overall improved sensory properties as well as the bitterness of the individual hydrolysates. In particular, Flavourzyme and Papain were attractive due to a less pronounced bitter taste and improved sensory profile (smell, taste, mouthfeeling). Techno-functional properties showed a good solubility at pH 7.0 and 4.0, emulsifying capacity up to 760 mL g⁻¹ (Flavourzyme) as well as improved oil-binding capacities, while the water-binding properties were generally decreased. Increased foaming activity for all proteases up to 3582 % (Pepsin) was observed, whereas lower foaming stability and density were found. The hydrolysates could potentially be used as hypoallergenic ingredients in a variety of food products due to their improved techno-functional properties and a pleasant taste.

Keywords: Soybean allergens, enzymatic hydrolysis, proteolytic enzymes, Bitterness, SDS-PAGE

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Introduction

Due to its considerable amounts of high-quality proteins, soy has found wide usage in processed foods during many years. It is applied in numerous food products such as baked, cereal, and meat-based products as well as hypoallergenic infant formula and vegetarian foods to provide specific functional properties such as improved texture, moisture, fat retention, emulsifying and protein fortification (Sun 2011).

However, one of the major drawbacks of soy-containing food products is the allergenic potential of soy. Soybean is listed among the "big 8" most allergenic foods comprising those foods that cause 90% of all immunoglobulin E (IgE)-mediated food allergenic reactions (FDA 2004). Soy allergies can provoke mild symptoms but can also be the cause of life-threatening reactions, ranging from severe enterocolitis atopic eczema to immediate IgE-mediated systematic multisystem reactions (Shriver and Yang 2011). Small regions of allergenic proteins, known as epitopes, are responsible for the allergenic reaction by acting with a corresponding antigen (FDA 2004). Even though 42 reactive proteins allergenic proteins have been identified as related to soybean allergy, just the two storage proteins glycinin and β -conglycinin are considered as major soybean allergens (Holzhauser et al. 2009; Amnuaycheewa and de Mejia 2010).

Numerous investigations in the elimination or hypoallergenization of soy ingredients and products have been conducted in recent years. Various thermal and nonthermal processing steps have been applied to combat soybean allergy, including microwave, ultrafiltration, high pressure processing, pulsed ultraviolet light, pulsed electrical fields, irradiation, high intensity ultrasound, genetic or chemical modifications (Shriver and Yang 2011; Verhoeckx et al. 2015). However, most of these methods could not destroy the responsible allergenic epitopes sufficiently or the methods have not yet been investigated in detail.

A more effective approach to reduce the allergenicity of soy proteins is their enzymatic hydrolysis, which has been successfully proven in different studies (Yamanishi et al. 1996; Wilson et al. 2005). Besides the reduction or elimination of the allergenic potential, the destruction of soy proteins due to enzymatic hydrolysis is also accompanied by a loss or change in their functional properties such as solubility as well as foaming, emulsifying, and gelation properties (De la Barca et al. 2000; Ortiz and Wagner 2002; Jung et al. 2004; Tsumura et al. 2005; Yin et al. 2008). In addition, enzymatic hydrolysis could lead to the formation of bitter-tasting peptides, which also impedes the utilization of hydrolysates in food (Ishibashi et al. 1988; Saha and Hayashi 2001).

Up to now, a feasible technology to reduce soy allergenicity is not implemented in the food industry. As a consequence, total avoidance of soy-containing products is mandatory to prevent allergenic reactions. However, this is difficult due to the ubiquitous presence of soy proteins in food products. As enzymatic hydrolysis is one of the most effective approaches, it should be investigated in more detail. Former studies have described the effects of proteases either on the level of allergenicity and organoleptic properties or technofunctionality. Literature data about the simultaneous determination of the reduction in the allergenic potential and the alteration of the functional as well as organoleptic properties are not available. This knowledge is a prerequisite for the development of a high- quality soy- based food ingredient.

The present study was conducted to (1) assess the effectiveness of different proteases on the degradation of the major soybean allergens (glycinin, β -conglycinin), (2) investigate the effects on the sensory perception with a specific emphasis on the bitter taste, and (3) determine the denaturation profile (DSC) and technofunctional properties of the resulting hydrolysates. The degree of allergenic protein degradation was evaluated and quantified by SDS-PAGE and by the analysis of the degree of hydrolysis. The organoleptic characteristics with a specific emphasis on the bitter taste were identified. The technofunctional characteristics (protein solubility, emulsifying, foaming, water- and oil- binding capacity) of the obtained hydrolysates have been investigated and their correlation with the observed degradation of the major soybean allergens was examined.

Material and Methods

Raw materials and chemicals

Soybeans (*Glycine max* (L.) MERR.) were purchased from Naturkost Ernst Weber (Munich, Germany). Enzymes used in this study including Alcalase[®] 2.4 L FG (endoprotease from *Bacillus licheniformis*), Flavourzyme[®] 1000 L (endoprotease and exopeptidase from *Aspergillus oryzae*), Protamex[®] (endopeptidase from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*), Neutrase[®] 0.8 L (endoprotease from *Bacillus amyloliquefaciens*), and Pancreatic Trypsin Novo[®] 6.0 S (endopeptidase from Porcine pancreatic glands) were kindly provided by Novozymes A/S (Bagsvaerd, Denmark). Papain (cysteineprotease from papaya latex) (E.C. 3.4.22.2, Sigma no P4762) and Pepsin (endoprotease from Porcine gastric mucosa) (E.C.

3.4.23.1, Sigma no P6887) were purchased from Sigma- Aldrich Inc. (St. Louis, MO) and Corolase[®] 7089 (endopeptidase from *Bacillus subtilis*), Corolase[®] 2TS (endopeptidase from *Bacillus stearothermophilus*) as well as Protease N- 01 (endoprotease from *Bacillus subtilis*) were kindly provided by AB Enzymes GmbH (Darmstadt, Germany) and ASA Spezialenzyme GmbH (Wolfenbüttel, Germany), respectively.

All chemicals used in this study were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany).

Preparation of soy protein isolates (SPI)

Soybeans were dehulled with an underflow peeler (Streckel & Schrader KG, Hamburg, Germany), classified in an air-lift system (Alpine Hosakawa AG, Augsburg, Germany) and flaked using a roller mill (Streckel & Schrader KG). Soybean flakes were defatted with *n*-hexane in a percolator (volume 1.5 m³, e&e Verfahrenstechnik GmbH, Warendorf, Germany) and flash desolventized with *n*-hexane (400-500 mbar) prior to steam desolventation. For the preparation of SPI, soy flakes were mixed with acidic water (pH 4.5; 1:8 w/v flakes to water ratio). The suspension was stirred for 1 h at room temperature and separated with a decanter (4400 U min⁻¹) for 60 min at 4 °C. For protein extraction, the solid phase was stirred in alkaline water (1:8 w/v), which was adjusted to pH 8.0 with 3 mol L⁻¹ NaOH. After 60 min of extraction, the suspension was separated (4400 U min⁻¹, 60 min) to obtain a clear protein extract, which was adjusted to pH 4.5 with 3 mol L⁻¹ HCl (room temperature) to precipitate the proteins. After separation by centrifugation at 5600 *g* for 130 min, the isoelectric precipitated protein was neutralized with 3 mol L⁻¹ NaOH, pasteurized (70 °C, 10 min) and spray dried.

Enzymatic hydrolysis of SPI

Enzymatic hydrolysis of SPI was performed with different proteases (Table 1) in thermostatically controlled reaction vessels. Therefore, SPI was dispersed in deionized water (5% w/w) utilizing an Ultraturrax for 1 min at 5000 U min⁻¹. The obtained slurry was adjusted to enzyme-specific temperature and pH value (Table 1). After adding the enzyme (E/S-ratio, see Table 1), the mixture was stirred, maintaining enzymes' optimum temperature and pH value. Aliquots of 100 mL were

taken at time intervals of 10, 30, 60, and 120 min to obtain SPI hydrolysates with different degrees of hydrolysis. Reaction conditions for Papain were chosen according to the method of Tsumura et al. (2004). Enzymes were inactivated at 90 °C for 20 min in a water bath. Control SPI dispersions were prepared under the same incubation conditions and inactivation treatment, but without enzyme addition. The samples were frozen at - 50 °C and lyophilized. All experiments were performed in duplicate.

Table 1. Degree of hydrolysis (%) of SPI hydrolysates obtained by different protease treatments.

Protease	E/S (%)	Temperature (°C)	pH value (-)	Degree of hydrolysis (%)				
				Time of hydrolysis (min)				
				0	10	30	60	120
Alcalase® 2.4 L	0.5	50	8.0	2.1 ± 0.0 ^a	7.5 ± 0.6 ^b	9.0 ± 0.5 ^c	10.1 ± 0.8 ^c	13.0 ± 0.8 ^d
Corolase® 7089	0.5	55	7.0	2.1 ± 0.0 ^a	5.2 ± 0.3 ^b	5.9 ± 0.3 ^{b,c}	5.9 ± 0.5 ^{b,c}	6.8 ± 0.4 ^c
Corolase® 2TS	0.5	70	7.0	2.1 ± 0.0 ^a	6.8 ± 0.3 ^b	7.1 ± 0.2 ^b	7.2 ± 0.2 ^b	7.8 ± 0.9 ^b
Flavourzyme® 1000 L	0.5	50	6.0	2.1 ± 0.0 ^a	5.0 ± 0.7 ^b	6.1 ± 0.3 ^{b,c}	6.8 ± 0.9 ^{b,c}	8.5 ± 1.1 ^c
Neutrase® 0.8 L	0.5	50	6.5	2.1 ± 0.0 ^a	3.7 ± 0.1 ^b	4.9 ± 1.0 ^{b,c}	5.8 ± 0.7 ^c	6.3 ± 0.8 ^c
PTN® 6.0 S	0.5	50	9.0	2.1 ± 0.0 ^a	2.7 ± 0.1 ^b	2.7 ± 0.2 ^b	2.7 ± 0.2 ^b	2.8 ± 0.1 ^b
Papain	0.2	80	7.0	2.1 ± 0.0 ^a	4.9 ± 0.0 ^b	4.8 ± 0.1 ^b	4.7 ± 0.1 ^b	4.6 ± 0.4 ^b
Papain	0.05	80	7.0	2.1 ± 0.0 ^a	3.5 ± 0.2 ^b	3.6 ± 0.1 ^b	3.8 ± 0.1 ^b	3.8 ± 0.3 ^b
Pepsin	0.5	50	2.0	2.1 ± 0.0 ^a	7.6 ± 0.7 ^{b,c}	7.9 ± 0.4 ^b	9.3 ± 0.3 ^c	10.6 ± 0.1 ^d
Protamex®	0.5	60	8.0	2.1 ± 0.0 ^a	3.3 ± 0.3 ^b	3.9 ± 0.3 ^{b,c}	4.7 ± 0.8 ^{b,c}	5.4 ± 0.8 ^c
Protease N-01	0.5	55	7.2	2.1 ± 0.0 ^a	4.3 ± 0.3 ^{b,c}	4.4 ± 0.1 ^b	4.5 ± 0.2 ^{b,c}	4.8 ± 0.1 ^c

Results are expressed as means ± standard deviation (n = 2). Means with different letters within one row indicate significant differences ($P < 0.05$) relating to one protease. Each protease was statistical analyzed separately due to different hydrolysis conditions following ANOVA (Bonferroni).

Determination of protein degradation due to enzymatic hydrolysis

Degree of hydrolysis using the *o*-phthaldialdehyde (OPA) method

The degree of hydrolysis (DH) was calculated by determining the free α -amino groups with *o*-phthaldialdehyde (OPA) using serine as standard (Nielsen et al. 2001).

The percentage of DH was calculated as follows: $DH = h / h_{tot} * 100\%$; where h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds. The h_{tot} factor was 7.8 (based on soy) according to Adler-Nissen (1986). Six measurements were performed for each sample.

Molecular weight distribution applying sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight distribution of all samples was determined according to Laemmli (1970) using SDS-PAGE under reducing conditions. The samples were suspended with 1 x Tris- HCl treatment buffer (0.125 mol L⁻¹ Tris-HCl, 4% SDS, 20% v/v Glycerol, 0.2 mol L⁻¹ DTT, 0.02% bromophenol blue, pH 6.8), boiled for 3 min to cleave noncovalent bonds and centrifuged at 12,100 *g* for 4 min (Mini Spin, Eppendorf AG, Hamburg, Germany). The electrophoresis was performed on 4-20% midi Criterion™ TGX Stain-Free™ precast gels and the proteins were separated using the Midi Criterio™ Cell from Bio- Rad (Ismaning, Germany). A molecular weight marker (10-250 kDa, Precision Plus Protein™ Unstained Standard, Bio-Rad Laboratories Inc., Hercules, CA, USA) was additionally loaded onto the gel. Electrophoresis conditions were 200 V, 60 mA, 100 W at room temperature and protein visualization was performed by Criterion Stain-Free Gel Doc™ EZ Imager (Bio-Rad).

Denaturation profile of the hydrolysates using differential scanning calorimetry (DSC)

The denaturation profiles of all samples were investigated using the differential scanning calorimetry (DSC) method according to Ahmed et al. (2006) with slight modifications using the DSC Q 2000 system from TA Instruments (New Castle, DE). Briefly, the samples were diluted in distilled water to obtain a protein content of 20% (w/w). About 15 mg of the dispersions was weighed into DSC pan. An empty DSC pan was taken as reference. Samples were heated with a heating rate of 2 K min⁻¹ in two cycles from 40 to 105 °C. All samples were immediately rescanned, after cooling down to 40 °C, to investigate reversibility. Peak denaturation temperatures (T_d), onset temperatures (T_{onset}), and relating enthalpies (ΔH) were calculated by the TA Universal Analysis software. Triplicate determinations were done throughout.

Chemical composition and technofunctional properties of soy hydrolysates

Chemical composition

The chemical composition (protein, ash, and dry matter) was determined as described by AOAC methods (AOAC 2005a,b). The protein contents were calculated based on the nitrogen content ($N \times 6.25$) according to the Dumas combustion method (AOAC 2005b). Dry matter and ash content were analyzed in a thermogravimetric system (TGA 601, Leco Corporation, St. Joseph, MI) at 105 and 950 °C, respectively.

Technofunctional properties

Emulsifying capacity

The emulsifying capacity (EC) was determined in duplicate as suggested by Wang and Johnson (2001). Protein solution samples of 1% (w/w) were prepared utilizing an Ultraturax[®] (IKA- Werke GmbH & Co. KG, Staufen, Germany) at 18 °C. Rapeseed oil was added by a titration system (Titrino 702 SM, Metrohm GmbH & Co. KG, Hertisau, Switzerland) at a constant rate of 10 mL min⁻¹ until phase inversion of the emulsion was observed, accomplished by continuous determination of the emulsion's conductivity (conductivity meter LF 521 with electrode KLE 1/T, Wissenschaftlich-technische Werkstätten GmbH, Weilheim, Germany). The volume of oil needed for phase inversion was used to calculate the EC (mL oil per g sample).

Foaming activity, density, and stability

Foaming activity was determined according to Phillips et al. (1987). Protein solution samples (5% w/w) were whipped using the Hobart 50-N whipping machine (Hobart GmbH, Offenburg, Germany) for 8 min. The relation of the foam volume before and after whipping was utilized for the calculation of the foaming activity. The foaming density was measured by weighing a specified quantity of foam volume. The ratio of foam volume to foam weight was defined as foaming density in g L⁻¹. The foaming stability was estimated as the percent loss of foam volume after 60 min.

Water- and oil-binding-capacity

Water-binding capacity (WBC) was analyzed according to the AACC 56- 20 official method (AACC 2000). Oil-binding capacity (OBC) was determined using the method described by Ludwig et al. (1989).

Protein solubility

Protein solubility was analyzed at pH 4.0 and 7.0 following the method of Morr et al. (1985). For each pH, 1 g of the sample was suspended in 50 mL 0.1 mol L⁻¹ sodium chloride solution. The pH was adjusted using 0.1 mol L⁻¹ NaOH or 0.1 mol L⁻¹ HCl, while the suspension was stirred at ambient temperature for 1 h. Nondissolved fractions of the samples were separated by centrifugation at 20,000 *g* for 15 min. Afterward, the protein content of the supernatant was determined according to AOAC (2005b).

Sensory analysis of the protein hydrolysates**Training of the panelists**

A sensory panel consisted of 10 panelists had been trained for bitterness evaluation over 2 month (1 h per session, twice a week) using the DIN 10959 threshold tests with caffeine solutions at concentrations of 0, 0.025, 0.05, 0.075, 0.1, 0.125, 0.15, 0.175, 0.2, and 0.225 g L⁻¹, respectively. Since the bitter profile of caffeine, which was included to select bitter-taster, is slightly different from a protein hydrolysate solution, an Alcalase hydrolysate was additionally added to the training session. The Alcalase hydrolysate was prepared by incubation of 5% SPI dispersion with 0.5% Alcalase at pH 8.0, 60 °C for 3 h without pH adjustment. The hydrolysate was then diluted to obtain solutions of 0.05, 0.1, 0.25, 0.5, 1.0, 1.5, and 2.5 g L⁻¹, respectively.

Bitter taste evaluation

A 10-cm line scale anchored from 0 (not detectable) to 10 (intense) was used. For scale calibration, Alcalase hydrolysates with a concentration of 1.0 and 2.5 g L⁻¹ were evaluated by the panel to correspond to a bitter intensity of 5 and 10, respectively.

Profile analysis

In addition to the determination of the bitter intensity, a profile analysis of the samples was obtained. A broad list of attributes characteristic for the individual samples was developed within the panel. The attributes in terms of smell ("fresh", "fruity", "beany"), taste ("sour", "salty", "bitter", "fresh", "beany"), and mouthfeeling ("mouthcoating", "astringent") were also rated on the 10-cm line scale. The attributes "fresh" and "fruity" are associated with the smell and taste of a lemon, whereas "beany" describes the soybean-like aroma. "Sour", "salty", and "bitter" are associated with fundamental taste sensations elicited by acids, salt, and caffeine, respectively. "Mouthcoating" describes the degree of coating inside the mouth after swallowing, while "astringent" is the trigeminal sensation elicited by grapefruit juice.

Sample preparation

Samples were mixed and stirred with tap water to prepare 2.5% (w/w) solutions. This sample concentration was found to be most appropriate for identifying and evaluating the attributes precisely. The pH was adjusted to pH 7.0 with 1 mol L⁻¹ NaOH. Each panelist was presented with eight samples (10 mL) per session, which were served to the panel in a random order at room temperature in plastic cups, which were coded by arbitrary numbers (three digits).

Sample evaluation

Each sensory evaluation was conducted by the trained panel (performed in 10 sessions, 1 h each). Water and plain crackers were provided for palate cleansing in between. Sensory analyses were carried out in a sensory panel room at 21 ± 1 °C. Solutions containing 2.5% SPI, 1.0% and 2.5% Alcalase hydrolysate were prepared as standard for each session, respectively. The assessors were instructed to evaluate bitterness and the attributes mentioned above in relation to the bitterness and attributes of the standard solutions using the standard 10-cm line scale. Each panelist did a monadic evaluation of the samples at individual speed. Two replicated measurements were made for each sample and replicates were randomized within the same session in order to avoid replicate effects.

Statistical analysis

All data are expressed as means \pm standard deviation of at least two independent measurements ($n = 2$). All chemical data were statistically analyzed by one-way Analysis of variances (ANOVA) and means were generated and adjusted with Bonferroni post hoc test using SPSS 20.0 (SPSS for windows, SPSS Inc. Chicago, IL). Sensory data ($n = 10$) were also subjected to ANOVA with the use of the Tukey's HSD average post hoc test. Statistically significant differences were considered at $P < 0.05$.

Results and Discussion

The enzymatic hydrolysis of SPI, containing a dry matter of 94.4%, a protein content of 94.6%, and an ash content of 4.6%, was conducted in two parts. First, a screening of 10 proteases was carried out. The DH, molecular weight distribution (SDS-PAGE), and the bitter taste were analyzed to estimate the degradation of the molecules as an indication for the reduction in the allergenic potential. Based on these results, selected proteases were investigated in more detail by determining the denaturation profile (DSC) as well as the technofunctional and sensory (profile analysis) properties.

Screening of different enzyme preparations

Effect of the enzymatic treatment on the protein degradation

Degree of Hydrolysis (DH)

The DH gives an initial indication for the change in the molecular integrity and thus for the reduction in allergenic compounds as presented in several studies (Kong et al. 2008; Tavano 2013). During protein hydrolysis, the large complex structured protein molecules are broken down into smaller sized peptides and specific amino acids. The DH was continuously monitored during enzymatic treatment of SPI. As shown in Table 1, the unhydrolyzed SPI showed an average DH value of 2.1%. In the course of enzymatic hydrolysis, the DH increased significantly ($P < 0.05$). The highest DH value of 13% was achieved after treatment of SPI for 2 h with Alcalase followed by

DH values of 10.6%, 8.5%, 7.8%, and 6.8% by using Pepsin, Flavourzyme, Corolase 2TS, and Corolase 7089, respectively. The lowest DH of 2.8% after a 2 h hydrolysis was achieved by Pancreatic Trypsin. This is probably attributed to the presence of the Kunitz Trypsin Inhibitor, inhibiting the proteolytic action of trypsin. The hydrolysis of the proteins was only caused by the enzyme activities as an increase in the DH values could not be observed in the reference experiments (no enzyme addition).

Electrophoretic analysis (SDS-PAGE)

A further initial indication for a reduced allergenicity of the hydrolysates was achieved by SDS-PAGE analyses (Fig. 1A-E). Specific emphasis has been given to the two major soybean allergens (glycinin, β -conglycinin) (Holzhauser et al. 2009; Amnuaycheewa and de Mejia 2010). In Figure 1, selected SDS-PAGE profiles are shown exemplarily. The unhydrolyzed SPI and reference (no enzyme addition) presented typical electrophoretic patterns for soy proteins (Fig. 1 A). The first three bands are α' (\sim 67-72 kDa), α (\sim 63 kDa), and β subunits (\sim 47 kDa) of β -conglycinin. Glycinin is composed of two subunits, the acidic subunit "A" (\sim 29-33 kDa) and the basic subunit ("B") at about 22 kDa (Amnuaycheewa and de Mejia 2010). Already after a 10 min hydrolysis with Alcalase, β -conglycinin was completely decomposed, while small amounts glycinin remained still within 30 min of hydrolysis. The acid subunit was eliminated after 60 and 120 min of hydrolysis, respectively, while the basic subunit was not completely destroyed. Similar observations could be obtained by the Pepsin preparation (Fig. 1 B). The decreased intensity of the acidic subunit of glycinin was more substantial for proteases such as Alcalase, Pepsin, and Papain than for the other proteases examined. In addition, an increasing reaction time led to a progressive disappearance of the basic subunit. This might be due to the fact that the basic group is located inside the glycinin complex and was therefore less exposed to hydrolysis. In contrast the acidic subunit, which is at the exterior of the complex, was degraded by almost all proteases (Yin et al. 2008).

Pepsin and Papain turned out to be the most effective enzyme preparations (Fig. 1 B and C). Already after 10 min of hydrolysis, β -conglycinin and glycinin were completely decomposed. A Papain concentration of 0.05% (data not shown) was also examined, which led to similar result as observed for the 0.2% treatment, indicating the high efficiency of Papain (Tsumura et al. 2005). These results were not expected taking the findings of the DH experiments into account as the DH values of the 0.2% and 0.05% Papain hydrolysates were relative low with 4.6% and 3.8% after 2 h, respectively. These differences might be caused by a weak reaction of the OPA reagent with the cysteine residues released during hydrolysis with Papain

(cysteine-protease) (Chen et al. 1979). The SDS-PAGE profiles of the hydrolysates obtained by the other enzymes showed a considerable deviating pattern in comparison to Alcalase, Papain, and Pepsin. The SDS-PAGE profiles of Corolase 2TS and Flavourzyme are shown as an example (Fig. 1 D-E) as the SDS-PAGE profiles obtained by the other enzyme preparations are quite similar (data not shown). It could be shown that these enzymes could slightly deteriorate β -conglycinin, but the glycinin subunits remained unchanged. Although Flavourzyme showed only slight changes in the SDS-PAGE patterns, the DH of 8.5% was contrary high. This might be attributed to the fact that Flavourzyme contains exoproteases, which cleave small peptides at the end of proteins, liberating groups for acting with the OPA reagent.

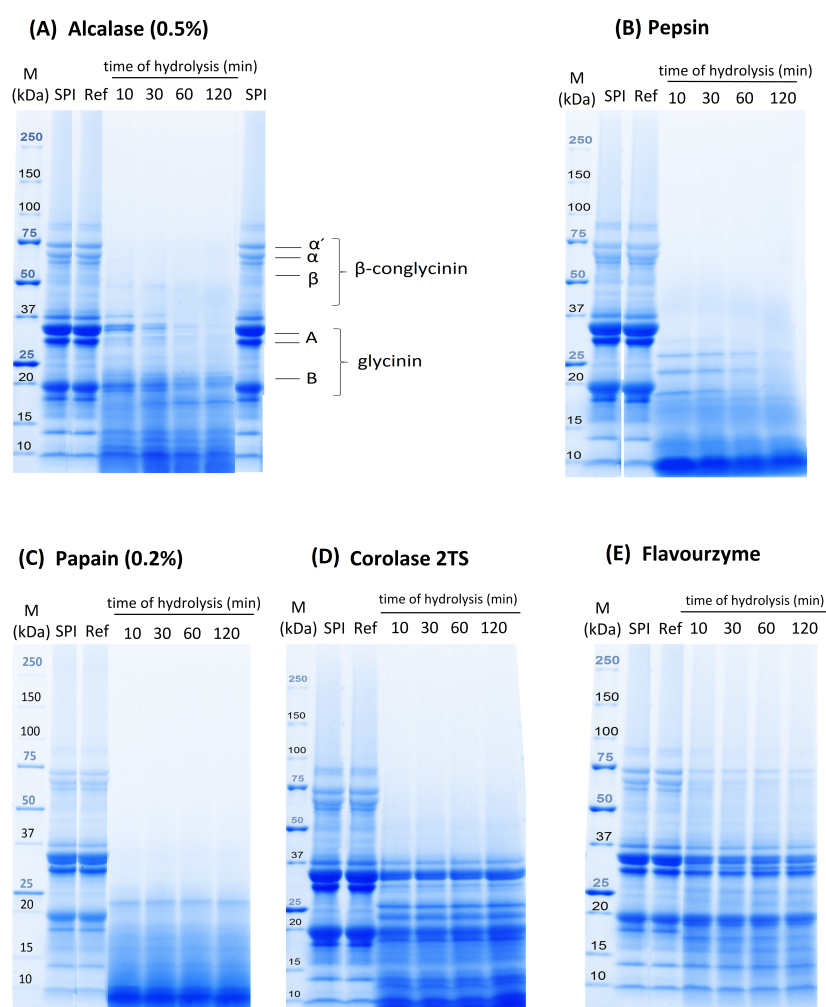


Figure 1. SDS-PAGE patterns of SPI hydrolysates obtained by different protease treatments. *M* molecular weight standard indicated in kilo Dalton (kDa); *SPI* soy protein isolate; Ref reference of each protease (no enzyme addition) after 120 min; Electrophoresis was carried out with 4-20% polyacrylamide gradient gels. For each protein, 50 μ g were loaded per well and visualized by UV activation. α' -, α -, and β -subunits of β -conglycinin; A and B: acidic and basic subunit of glycinin.

Effects of the enzymatic treatment on the bitterness of SPI

Due to the presence of strongly hydrophobic bitter peptides arising as natural degradation products of proteolytic reactions, enzymatic hydrolysates are often associated with a strong bitter taste (Adler- Nissen 1986; Ishibashi et al. 1988; Saha and Hayashi 2001; Sun 2011).

Native SPI showed a bitter intensity of 2.8. The bitterness of all hydrolysates increased with increasing reaction time with an exception of the hydrolysate prepared by Flavourzyme, (Table 2). The bitter intensity of the Flavourzyme hydrolysate, increased within the first hour of hydrolysis from initially 2.8 to 4.3, but decreased after 2 h to an intensity of 2.1, which is even lower than the bitterness of native SPI. Flavourzyme contains both endoprotease and exopeptidase activities. The latter can selectively release hydrophobic amino acid residues from the protein molecules, having a debittering effect (Saha and Hayashi 2001).

The highest bitter intensity of 9.2 was achieved using Alcalase followed by Corolase 2TS, Corolase 7089 and Neutrase with bitter intensities of 7.7, 7.6, and 7.1, respectively. The high bitter intensity of the hydrolysates produced by Alcalase is probably caused by the tendency of this enzyme to hydrolyze hydrophobic amino acid residues. Thereby, nonpolar amino acid residues at the C-terminus of the resulting peptides remain and cause a relatively high bitterness (Adler- Nissen 1986; Ishibashi et al. 1988; Saha and Hayashi 2001; Sun 2011).

The hydrolysis with 0.2% and 0.05% Papain for 120 min results in low bitterness intensities of 3.1 and 3.0, respectively. Hydrolysis applying the other enzyme preparations resulted in samples with bitter intensities in the range of 5.5 and 6.4 (Table 2).

Among the proteases investigated, Alcalase, Pepsin, and Papain turned out to be most efficient in the degradation of proteins into small-sized peptides as evidenced by the DH (except Papain) and SDS-PAGE analysis (Table 1 and Fig. 1), while hydrolysis with Flavourzyme and Papain resulted in hydrolysates with the lowest bitter intensities (Table 2).

Table 2. Sensory perception (bitterness) of hydrolyzed SPI obtained by different protease treatments.

Protease	Intensity of bitterness				
	Time of hydrolysis (min)				
	0	10	30	60	120
Alcalase [®] 2.4 L	2.8 ± 0.9 ^a	8.7 ± 1.4 ^b	8.6 ± 1.5 ^b	9.2 ± 0.8 ^b	9.2 ± 1.1 ^b
Corolase [®] 7089	2.8 ± 0.9 ^a	4.5 ± 1.2 ^a	4.7 ± 1.5 ^{a,b}	6.9 ± 1.2 ^{b,c}	7.6 ± 1.7 ^c
Corolase [®] 2TS	2.8 ± 0.9 ^a	6.8 ± 1.7 ^b	6.3 ± 1.7 ^b	7.0 ± 1.5 ^b	7.7 ± 1.8 ^b
Flavourzyme [®] 1000 L	2.8 ± 0.9 ^a	4.1 ± 1.2 ^{a,b}	4.5 ± 1.4 ^b	4.3 ± 1.8 ^{a,b}	2.1 ± 1.7 ^{a,b}
Neutrase [®] 0.8 L	2.8 ± 0.9 ^a	4.4 ± 1.5 ^{a,b}	4.5 ± 1.4 ^{a,c}	7.0 ± 1.2 ^{b,c}	7.1 ± 0.9 ^c
PTN [®] 6.0 S	2.8 ± 0.9 ^a	3.5 ± 1.3 ^a	4.0 ± 1.5 ^a	3.8 ± 1.8 ^a	3.9 ± 1.5 ^a
Papain	2.8 ± 0.9 ^a	4.3 ± 1.4 ^b	4.8 ± 1.1 ^b	3.1 ± 1.1 ^{a,b}	3.1 ± 1.5 ^{a,b}
Papain	2.8 ± 0.9 ^a	3.3 ± 1.5 ^a	3.3 ± 1.9 ^a	3.0 ± 1.1 ^a	3.0 ± 1.2 ^a
Pepsin	2.8 ± 0.9 ^a	6.4 ± 1.1 ^{a,b}	4.4 ± 1.6 ^{a,b}	5.4 ± 1.6 ^{a,b}	6.4 ± 1.7 ^b
Protamex [®]	2.8 ± 0.9 ^a	2.7 ± 1.9 ^{a,b}	4.5 ± 1.6 ^{a,b}	4.6 ± 1.8 ^{a,b}	5.5 ± 1.3 ^b
Protease N-01	2.8 ± 0.9 ^a	3.3 ± 1.0 ^{a,b}	6.2 ± 1.1 ^{b,c}	4.4 ± 1.3 ^{a,c}	6.1 ± 1.3 ^c

Results are expressed as means ± standard deviation (n = 2). Means with different letters within one row indicate significant differences ($P < 0.05$) relating to one protease. Each protease was statistical analyzed separately due to different hydrolysis conditions following ANOVA (Bonferroni).

The effect of enzymatic hydrolysis of SPI on its potential allergenicity, technofunctionality and sensory properties

The most promising enzymes Alcalase, Flavourzyme, Pepsin, Papain, Corolase 7089 as well as Corolase 2TS with respect to a less bitter taste and an effective degradation of molecular weight distribution were analyzed in more detail. The enzymatic hydrolysis was repeated under the same reaction conditions as described in the screening experiments, but the incubation time was changed. Enzymatic hydrolysis with Alcalase, Flavourzyme, Pepsin was performed for 120 min, the treatment with Corolase 7089 and Papain was conducted for 30 min and with Corolase 2TS for 10 min. For Papain, a lower enzyme concentration of 0.05% was applied due to the high reactivity of this enzyme preparation.

Effect on the protein degradation

Degree of Hydrolysis (DH)

In accordance with the screening trials, Alcalase, Pepsin, and Flavourzyme showed the highest DH values of about 13.6%, 10.0%, and 9.4%, respectively. Lower DH values of about 5.8%, 5.8%, and 3.9% were obtained after hydrolysis with Corolase 7089, Corolase 2TS, and Papain, respectively.

Electrophoretic analysis (SDS-PAGE)

The individual bands of glycinin and β -conglycinin units were quantified by Image Lab™ Software (Bio-Rad, Hercules, CA, USA). The relative hydrolyzation in relation to the unhydrolyzed fractions was calculated (Table 3). Alcalase, Pepsin, and Papain were the most efficient proteases for the overall degradation of the major allergens with a proteolytic activity of about 100%, 100%, and 95.9%, respectively (Table 3). Alcalase, Corolase 2TS, Pepsin, and Papain hydrolyzed the basic subunit of glycinin with varying degree (Fig. 1 and Table 3). In general, glycinin was least degraded due to its molecular structure and location of the basic subunit, which is covered in the interior of the glycinin complex (Yin et al. 2008).

Hydrolysates prepared with Corolase 7089 and Flavourzyme showed smaller changes in the molecular weight distribution. A complete degradation of the α and β -subunits was observed (Table 3), while the α' -subunit was reduced by 70.5% and 61.0%, respectively. However, the acid and basic subunits of glycinin were only slightly affected.

Table 3. Degradation of the main allergen fractions of SPI obtained by different protease treatments. Mean value in each column having different letters are significantly ($P < 0.05$).

	Soy protein isolate (% hydrolyzed) ¹					
	β -conglycinin			Glycinin		Total average
	α'	α	β	A	B	
Alcalase® 2.4 L	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a
Flavourzyme® 1000 L	61.0 ± 1.0 ^b	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	27.0 ± 1.0 ^b	27.0 ± 2.0 ^b	63 ± 0.7 ^b
Pepsin	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a
Corolase® 7089	70.5 ± 2.5 ^c	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	45.4 ± 1.6 ^c	35.2 ± 1.4 ^b	70.2 ± 0.7 ^c
Corolase® 2TS	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	85.3 ± 1.3 ^d	64.5 ± 1.5 ^c	90.0 ± 0.5 ^d
Papain (0.05%)	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	100.0 ± 0.0 ^a	79.5 ± 0.5 ^d	95.9 ± 0.2 ^e

α , α' , β , subunits of β -conglycinin; A, acidic fraction of glycinin; B, basic fraction of glycinin.

¹ of hydrolysis of each main fraction of soy protein isolate treated with different proteases with respect to those of SPI without treatment.

Differential scanning calorimetry (DSC)

DSC analysis was applied to examine the secondary and tertiary structural changes of SPI due to enzymatic hydrolysis, which can give an additional evidence for the destruction of allergenic proteins. Figure 2 depicts characteristic DSC curves corresponding to unhydrolyzed SPI and three hydrolysates prepared with Flavourzyme, Corolase 7089, and Corolase 2TS while all other hydrolysates exhibit no peaks, indicating complete denaturation of the proteins (data not shown).

SPI showed two endothermic thermal transitions, the major peak denaturation temperatures (T_d) were at approximately 71.7 °C ($T_{\text{onset}} = 68.8$ °C) and 91.7 °C ($T_{\text{onset}} = 87.0$ °C) along with denaturation enthalpies of 0.03 and 0.32 J g⁻¹, respectively. These results are consistent with previous reports where the onset denaturation temperature of glycinin is around 80-90 and 60-70 °C for β -conglycinin (Renkema et al. 2002; Ahmed et al. 2006). Slight variations can be due to genotypic differences in the raw material or varied processing conditions, that is, temperature (Riblett et al. 2001).

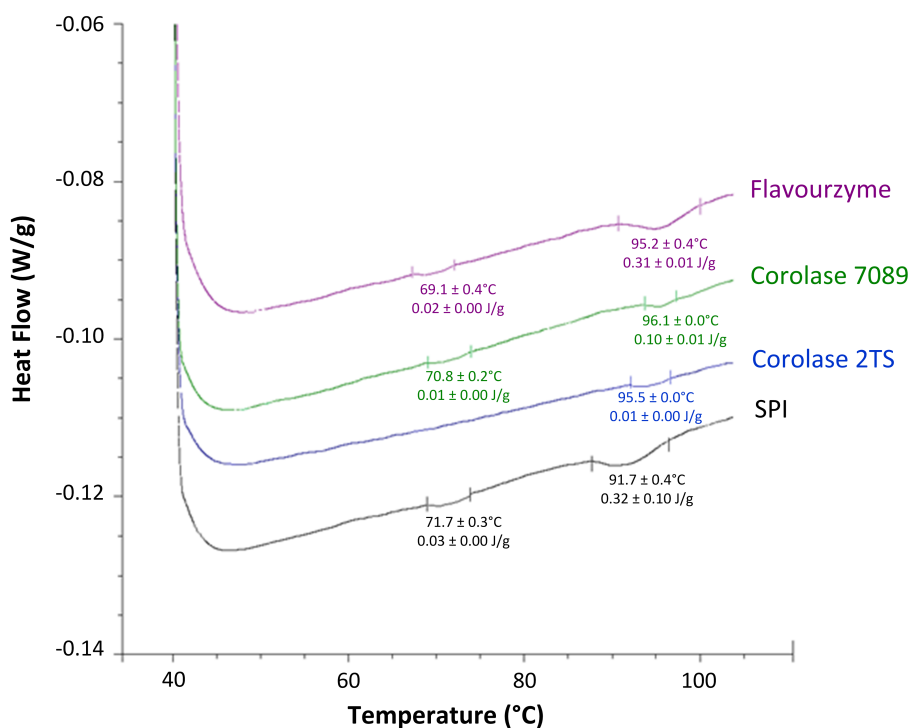


Figure 2. Differential scanning (DSC) thermogram of a 20% unhydrolyzed SPI, Flavourzyme, Corolase 7089, and Corolase 2 TS hydrolysates dispersions.

The Flavourzyme and Corolase 7089 hydrolysates were likely to be partially denatured or rather partially degraded since the first denaturation point (°C) decreased to 69.1 and 70.8 °C, respectively with an enthalpy of 0.02 to 0.01 J g⁻¹.

The enthalpy of the second denaturation point of the Flavourzyme hydrolysates of about 95.2 °C was not significantly ($P < 0.05$) lower compared to native SPI being 0.31 J g⁻¹, while a shift of the second denaturation temperature toward higher temperatures was detected. In contrast, the Corolase 7089 hydrolysate exhibited a denaturation point of about 96.1 °C with a lower denaturation enthalpy of 0.10 J g⁻¹. The Corolase 2TS hydrolysate showed one denaturation temperature at 93.5 °C and the enthalpy of denaturation being 0.01 J g⁻¹ was significantly ($P < 0.05$) lower than that for SPI, Flavourzyme, and Corolase 7089 hydrolysates. The β -conglycinin fraction was completely denatured, whereas the glycinin complex was only slightly affected as evidenced by a decreased denaturation enthalpy. These findings are in great accordance with the SDS-PAGE analyses (Table 3).

Effects on the sensory profile of SPI

Evaluation of the SPI (Fig. 3) by trained panelists resulted in the following smell-scaling: "fresh" (5.0), "fruity" (2.7), and "beany" (4.8); taste-scaling: "sour" (1.5), "salty" (0.9), "bitter" (3.2), "fresh" (4.7), and "beany" (3.8); mouthfeeling-scaling: "mouthcoating" (3.9) and "astringent" (3.2). Compared to SPI, all hydrolysates exhibited a significantly ($P < 0.05$) lower "beany" smell as well as a "fresh" and "beany" taste.

The Alcalase hydrolysate (Fig. 3) showed the highest bitter intensity of 8.2, and therefore, the application of the Alcalase hydrolysate in food systems might be limited. In contrast, the Pepsin hydrolysate (Fig. 3) showed a predominantly "fresh" and "fruity" smell, but the "sour" taste and "astringent" mouthfeeling were significantly ($P < 0.05$) higher than for the other hydrolysates tested. The application of the pepsin hydrolysate as food ingredient might be limited due to its extreme "sour" taste as well as "astringent" mouthfeeling.

The hydrolysate prepared with Papain showed improved sensory properties in comparison to hydrolysates prepared with other proteases tested in terms of "bitter" (2.8) and "beany" taste (3.5), "mouthcoating" (3.2) and "astringency" mouthfeeling (1.8). This sensory profile is even better than the sensory properties of the unhydrolyzed SPI. The sensory properties of the Flavourzyme hydrolysate were comparable to those of native SPI, having the following smell-scaling: "fresh" (3.4), "fruity" (2.6), and "beany" (2.0); taste: "sour" (1.7), "salty" (1.7), "bitter" (3.7), "fresh" (2.9), and "beany" (3.7); mouthfeeling: "mouthcoating" (3.6) and "astringent" (3.4). The taste profiles of Corolase 2TS and Corolase 7089 were also similar to those of native

SPI except of a higher bitter intensity of about 4.9 and 5.3, respectively, and increased "mouthcoating" (4.8 and 5.1) and "astringency" mouthfeeling (4.1 and 3.9) (data not shown).

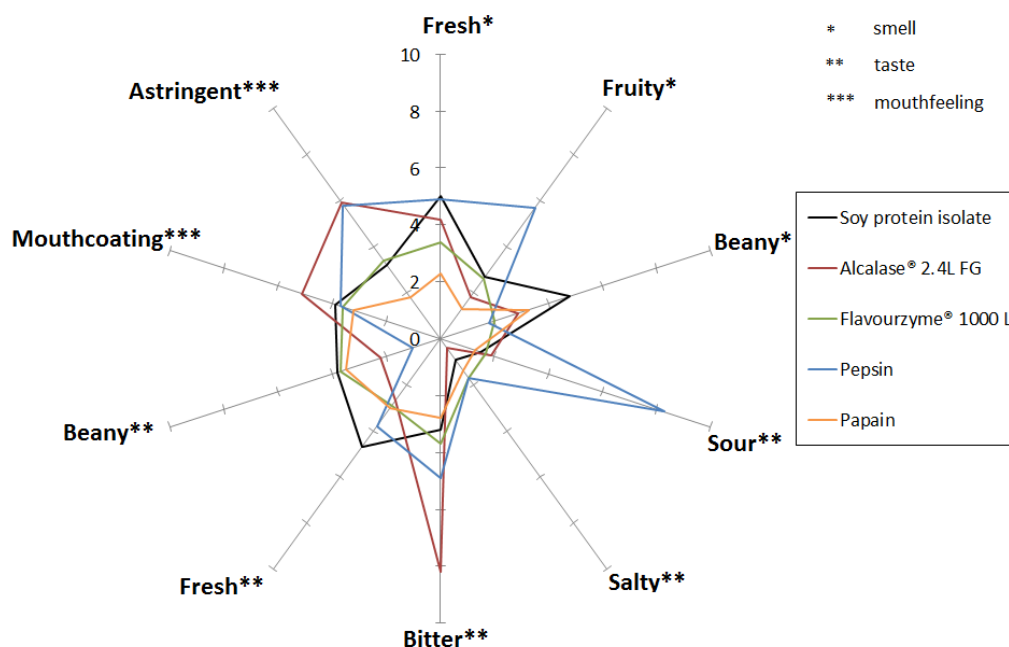


Figure 3. Taste profile (descriptive analysis) of unhydrolyzed SPI , Alcalase, Pepsin, Papain, and Flavourzyme hydrolysates. Each value is expressed as mean \pm standard deviation scored on a 10-cm line scale, ranging from 0 (not detectable) to 10 (intense), by 10 panelists ($n = 10$, 2 x replicates), $P < 0.05$ (ANOVA, Tukey's HSD).

Effects on the technofunctionality of SPI

Technofunctional properties including solubility, gelation, emulsifying, and foaming of proteins connote the physicochemical properties which govern the behavior of protein in the food matrix. Applying enzymatic hydrolysis, functional properties of proteins are modified (Were et al. 1997; De la Barca et al. 2000; Ortiz and Wagner 2002). Enzymatic hydrolysis decreases the molecular weight and increases the number of ionizable groups in proteins and expose hydrophobic groups which change the physical and chemical interactions (Creusot et al. 2006). Soybean proteins glycinin and β -conglycinin mainly reflect the functional properties of SPI and show considerable differences in functional properties such as emulsifying due to their diverse molecular structure (Utsumi and Kinsella 1985).

Protein solubility

Solubility is the most important technofunctional property due to its considerable effect on other technofunctional characteristics, particularly gelation, foaming, and emulsifying, which depend on an adequate initial solubility of proteins (Vojdan 1996).

The solubility of all samples is shown as a function of pH 4.0 and 7.0 in Figure 4. The minimum solubility of 5.0% of the unhydrolyzed SPI was detected at pH 4.0, at the isoelectric point of soybean protein, but was significantly increased after hydrolysis by all proteases. At pH 4.0, the hydrolysates prepared with Alcalase and Pepsin exhibit the highest solubility of 77.4% and 84.3%, respectively. The highest solubility of 91.3% was achieved at pH 7.0 using Corolase 7089 followed by the solubility of 90.5%, 84.5%, and 82.9% by using Pepsin, Corolase 2TS, and Alcalase, respectively. It has been proposed that the reduction in the secondary structure of proteins and the release of smaller peptides, and the corresponding increase in ionizable amino and carboxyl groups are responsible for increased solubility of hydrolysates, increasing the interactions with water molecules (Adler-Nissen 1986; Ortiz and Wagner 2002). At pH 4.0 the solubility of all other hydrolysates was significantly ($P < 0.05$) lower, ranging from 30.3% to 42.1% and at pH 7.0 between 56.2 and 58.3%.

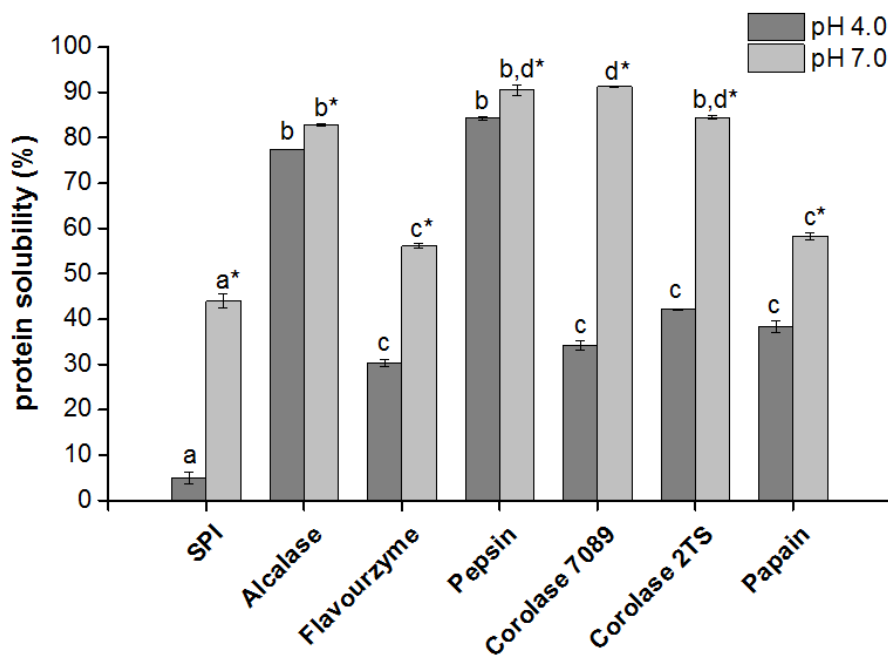


Figure 4. Solubility of SPI and SPI hydrolysates at pH 4.0 and pH 7.0. Means with different letters within one figure indicate significant differences ($P < 0.05$) following ANOVA (Bonferroni). *indicates the solubility at pH 7.0. Results are expressed as means \pm standard deviation ($n = 2$).

Emulsifying properties

The EC of the unhydrolyzed SPI and hydrolysates was determined. SPI had an EC of 660 mL g⁻¹ while all SPI hydrolysates - except hydrolysates generated by Alcalase and Pepsin - showed significantly increased ($P < 0.05$) EC. The Flavourzyme, Corolase 7089, Corolase 2TS, and Papain hydrolysates had EC's of about 760, 730, 670, and 705 mL g⁻¹, respectively. Enzymatic hydrolysis has already been used in scientific approaches to improve the emulsifying properties (Wu et al. 1998; Jung et al. 2004). De la Barca et al. (2000) demonstrated an increased emulsification activity after enzymatic hydrolysis of soy protein, which is comparable to the present results. The increased emulsifying properties may be due to the degradation of large protein molecules, exposure of hydrophobic groups and enhanced protein solubility implicating an improved protein surface activity and therefore a better emulsifying activity (Wu et al. 1998). However, the EC of the Alcalase and Pepsin hydrolysates decreased to 438 and 220 mL g⁻¹, respectively, indicating significant ($P < 0.05$) differences compared to unhydrolyzed SPI. The reason for this might be due to the excessive protein hydrolysis; thus, a sharp degradation to smaller peptides as evidenced by DH (Table 1) and SDS- PAGE results (Table 3). The molecular structure of the protein might be altered, particularly with respect to its interfacial adsorptivity and reduction in continuous phase viscosity, which is essential for the ability to form emulsions (Kinsella et al. 1985).

It has been reported that the EC of hydrolysates is closely related to the degree of hydrolysis, with a low DH (3-5%) increasing and a high DH (~8%) decreasing EC (Achouri et al. 1998). The obtained results in this study cannot entirely confirm these statements. A high DH does not always results in a reduced EC as evidenced by the increased EC of the Flavourzyme hydrolysate, which had a high DH of about 9.4%, but also the highest EC of 760 mL g⁻¹.

Water- and oil-binding capacity

The WBC of almost all hydrolysates was significantly ($P < 0.05$) lower compared to the unhydrolyzed SPI. The hydrolytic action of proteases causes disruption of the protein network, which is responsible for the inhibition of water- holding properties. The WBC decreased from an initial WBC of 2.6 - 1.8 mL g⁻¹, 0.9, and 0.2 mL g⁻¹ after hydrolysis with Flavourzyme, Pepsin, and Alcalase, respectively, while no WBC for the Corolase 7089 and Corolase 2TS hydrolysates was observed. However, the Papain hydrolysate showed a significantly ($P < 0.05$) higher WBC with values of 3.9 mL g⁻¹.

In contrast, all SPI hydrolysates enhanced the OBC from initially 0.0 - 3.3 mL g⁻¹, 3.3, 3.3, 2.9, 2.8, and 2.1 mL g⁻¹ after hydrolysis with Corolase 2TS, Corolase 7089, Papain, Flavourzyme, Pepsin, and Alcalase, respectively. The presence of OBC might be attributed to the exposure of hydrophobic groups after enzymatic hydrolysis allowing the physical entrapment of oil.

Foaming properties

The foaming properties are usually characterized in terms of foaming density, activity and stability. Proteins in dispersions cause a lower surface tension at the air-water interface, thus creating a foam (Surowka and Fik 1992). As shown in Table 4, all hydrolysates presented an improved foaming activity. Enzymatic hydrolysis results in smaller peptides with improved foaming activity by rapid diffusion to the air-water interface (Tsumura et al. 2005). Furthermore, native SPI has limited foaming due to its quaternary and tertiary structure, whereas hydrolyzed SPI lost the tertiary structure, which leads to improved foam activity (Yu and Damodaran 1991). Among the proteases studied, the highest foaming activity of 3582% was achieved after hydrolysis with Pepsin, while the hydrolysate prepared with Flavourzyme showed the lowest foaming capacity of 1201% among the hydrolysates. There is an evidence of a trend toward increased foaming activity when the *beta*-conglycinin fraction faded and the glycinin fraction becomes dominant, which is supported by SDS-PAGE profiles (Fig. 1 and Table 3), where only the hydrolysates generated with Flavourzyme and Corolase 7089 showed a slight degradation of the β -conglycinin fraction.

Table 4. Foaming properties (foaming activity, density, and stability) of SPI and SPI hydrolysates.

	Foaming activity (%)	Foaming stability (%)	Foaming density (g L ⁻¹)
SPI	552 ± 5 ^a	90 ± 0 ^a	215 ± 5 ^a
Alcalase [®] 2.4 L	2766 ± 10 ^b	0 ± 0 ^b	32 ± 0 ^b
Flavourzyme [®] 1000 L	1201 ± 31 ^a	86 ± 0 ^c	88 ± 3 ^c
Pepsin	3582 ± 236 ^{a,b,c}	66 ± 2 ^d	27 ± 1 ^b
Corolase [®] 7089	2095 ± 47 ^{b,c}	74 ± 2 ^{a,c,d}	48 ± 1 ^d
Corolase [®] 2TS	2315 ± 111 ^{a,b,c}	68 ± 2 ^{a,c,d}	38 ± 1 ^{b,d}
Papain (0.05%)	2583 ± 0 ^c	78 ± 0 ^{a,c,d}	37 ± 0 ^{b,d}

Results are expressed as means ± standard deviation (n = 2). Means with different letters within one column indicate significant differences ($P < 0.05$) following ANOVA (Bonferroni).

Although the foaming activities of the hydrolysates were higher compared to SPI, their stability and density decreased (Table 4). The most stable foam was obtained after hydrolysis with Flavourzyme with a stability of 86%, which is near to native SPI with a stability of 90%. For all other hydrolysates, the foaming stability was markedly decreased (Table 4). For foam stabilization some larger protein components are needed, but only few large peptides were found in the hydrolysates, which led to weak foaming stability. The trend of increased foaming activity coupled with decreased foaming stability has been reported in previous studies (Were et al. 1997; De la Barca et al. 2000; Tsumura et al. 2005).

Conclusions

The aim of this study was to investigate the effect of enzymatic hydrolysis with various proteases on the potential allergenicity, technofunctionality, and sensory properties of SPI. The results clearly demonstrate that enzymatic hydrolysis is an effective approach to reduce the level of allergenicity, while sensory and technofunctional properties can be improved depending on the proteases used. According to the findings, Papain turned out to be the most appropriate proteases for improving the technofunctionality and sensory characteristics, while effectively decreasing the molecular weight of SPI. SDS-PAGE and the DH were used to examine the degradation the soybean allergens to enable a first evaluation of the level of allergenicity. As this is an indirect method, further research is required to get detailed knowledge of the allergen structure as well as specific and reliable detection methods.

Although the sensory analysis showed promising results, the bitter taste of the produced hydrolysates is still a challenge. Further investigation needs to be carried out focusing on debittering hydrolysates to expand the use in food systems. Studies on enzymatic hydrolysis through various combinations of exo- and endopeptidases and other methods for reducing the level of bitterness and allergenicity are ongoing in our laboratory, which might lead to the development of hypoallergenic SPI with pleasant taste and good technofunctionality.

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Conflict of Interest

The authors have declared no conflicts of interest.

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CHAPTER 2: Enzyme assisted degradation of potential soy protein allergens with special emphasis on the techno-functionality and the avoidance of a bitter taste formation²

Abstract

Soy protein hydrolysates are often rejected by consumers due to their unpleasant bitter taste. In the present study, soy protein isolate (SPI) was hydrolyzed using five proteases and combinations thereof to prevent bitterness. Hydrolysis was performed as one- and two-step process and the extent of hydrolysis was evaluated by SDS-PAGE and degree of hydrolysis analyses. Enzymatic hydrolysis performed as both, one- and two-step process, increased the degree of hydrolysis from initially 2.1 % up to 30 %. Most peptides generated were < 20 kDa and major soybean allergens (glycinin, β -conglycinin) were effectively degraded as shown by SDS-PAGE and liquid chromatography mass spectrometry (LC-MS/MS) analyses. The bitterness of SPI hydrolysates was evaluated by a sensory panel on a 10-cm continuous scale, ranging from 0 (no perception) to 10 (strong perception). Bitterness of the resulting hydrolysates was remarkably reduced to a minimum of 1.3 compared to unhydrolyzed SPI with a bitterness intensity of 2.8. Hydrolysates showed increased protein solubility at both pH 4.0 and 7.0, emulsifying capacity up to 810 mL g⁻¹ and foaming activity up to 2706 %. The study shows that enzyme combinations are an effective approach to produce hypoallergenic soy hydrolysates that combine low bitterness and superior techno-functional properties.

Keywords: Soy allergy; Enzymatic hydrolysis; Enzyme combination; Debittering; Techno-functional properties

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1. Introduction

Soybean (*Glycine max* (L.) MERR.) is the most important valuable crop that provides the largest source of vegetable protein to human diet. Particularly, soy protein isolates are often favoured as functional and nutritional ingredients due to the high protein content (ca. 90 %).

However, consumption of soy-containing food products can cause severe and even fatal allergic reactions such as anaphylactic shock. The Food and Agriculture Organization of the United Nations (FAO) listed soybean as one of the eight priority allergens ("big 8") which comprises those foods that cause 90 % of all Immunoglobulin E (IgE)-mediated allergies (FDA, 2004). At least 16 IgE-binding soy proteins have been characterized, but only the two storage proteins Gly m5 (β -conglycinin) and Gly m6 (glycinin) have been suggested to be the major fractions containing allergens (Amnuaycheewa & de Mejia, 2010; FARRP, 2015; Holzhauser et al., 2009).

In recent years, the need to control soybean allergy by methods other than avoidance has spurred the development of new technologies, including genetic modifications as well as thermal and non-thermal treatments (Shriver & Yang, 2011; Verhoeckx et al., 2015). An effective approach to reduce the allergenicity of soybean is the enzymatic hydrolysis, which has already been proven in numerous studies using selective proteases from animal, plant or microbial origin (Wilson, Blaschek, & de Mejia, 2005; Yamanishi et al., 1996). Generally, enzymatic hydrolysis is widely applied to upgrade the functional features, including solubility, foaming and emulsifying properties (Ortiz & Wagner, 2002; Tsumura et al., 2005).

However, protein hydrolysates are accompanied by the formation of bitter peptides, which impedes their utilization as food ingredients. The bitterness of hydrolysates is due to the release of low molecular weight peptides composed of mainly hydrophobic amino acid residues (Ishibashi et al., 1988; Ney, 1979; Seo, Lee, & Baek, 2008). Thus, the prevention, reduction, or elimination of bitter taste is essential to make the hydrolysates acceptable to consumers.

Effective debittering methods, however, have not yet been successfully developed. Numerous options have been investigated in the debittering of food protein hydrolysates including selective separation or adsorption of bitter peptides such as treatment with activated carbon, extraction with alcohol, isoelectric precipitation, and chromatography (Saha & Hayashi, 2001). The bitterness in hydrolysates was also masked via the addition of various agents such as polyphosphates, specific amino acids and through the admixture with intact proteins (FitzGerald & O'Cuinn, 2006; Sujith & Hymavathi, 2010; Sun, 2011). Debittering via transpeptidation of specific

amino acids by T-plastein reaction in addition to cross-linking using microbial transglutaminase as well as the modification of taste signalling have also been conducted (Sun, 2011).

Much effort has been made in peptidase-mediated debittering of protein hydrolysates that means an enzymatic hydrolysis of bitter peptides with, particularly exo-peptidases such as aminopeptidases, alkaline/neutral proteases and carboxypeptidases. A reduction in bitterness has been observed in proteinase hydrolysis of food proteins during concomitant or subsequent incubation with exopeptidase-rich enzyme preparations which cleave adjacent to hydrophobic amino acid residues (Chae, In, & Kim, 1998; FitzGerald & O’Cuinn, 2006).

Although many studies have been applied in either the reduction of the allergenicity or the debittering of hydrolysates, literature on an effective enzyme combination for sufficiently reducing both allergenicity and bitterness, while improving the techno-functional properties, is not available.

The present study was conducted to investigate (1) the effectiveness of different enzyme combinations on the degradation of the major soybean allergens (glycinin, β -conglycinin), (2) their effects on the bitter taste, and (3) the techno-functional properties of the resulting hydrolysates. Protein degradation was evaluated by determining the degree of hydrolysis as well as electrophoresis (SDS-PAGE) and mass spectrometry (LC-MS/MS) analyses. A specific emphasis has been given to the debittering effect of enzyme combinations. The effects on the techno-functional properties (protein solubility, emulsifying capacity, foaming activity, density, and stability) were also studied.

2. Material and Methods

2.1. Raw materials and chemicals

Soybeans (*G. max* (L.) MERR.) were purchased from Naturkost Ernst Weber (Munich, Germany). Alcalase[®] 2.4 L FG (2.4 AU-A/g, endoprotease from *Bacillus licheniformis*), Flavourzyme[®] 1000 L (1000 LAPU/g, endo- and exoprotease from *Aspergillus oryzae*), and Neutrase[®] 0.8 L (0.8 AU-N/g, endoprotease from *Bacillus amyloliquefaciens*) were kindly provided by Novozymes A/S (Bagsvaerd, Denmark). Papain (cysteine-protease from papaya latex) (≥ 10 units/mg, E.C. 3.4.22.2, Sigma no P4762) was purchased from Sigma-Aldrich Inc. (St. Louis, U.S.A) and Corolase[®] 7089 (850 UHb/g, endopeptidase from *Bacillus subtilis*) was kindly provided by AB

Enzymes GmbH (Darmstadt, Germany).

All chemicals used were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany) if not stated separately.

2.2. Preparation of soy protein isolates (SPI)

Soy protein isolate (SPI) was prepared from soybean seeds (*G. max* (L.) MERR.) as previously described by Meinschmidt, Sussmann, Schweiggert-Weisz, and Eisner (2016).

2.3. Enzymatic hydrolysis of SPI

For the experiments, five different commercially available food-grade enzymes were used. These protease preparations are commonly used in the food industry for the production of protein hydrolysates. Reaction conditions (50°C and pH 8.0) were chosen according to producers' application sheet. The dosage of 0.5%, 0.05% and 1% for endo- and exoproteases has been applied according to unpublished preliminary experiments.

Enzymatic hydrolysis was performed with various enzyme combinations (Table 1) in thermostatically controlled reactors. Therefore, SPI was dispersed in deionized water at a protein concentration of 5% (w/w) and adjusted to 50°C and pH 8.0 with 1 M NaOH prior to enzyme addition. A total volume of 1000 mL was used for the proteolysis reaction. Hydrolysis was performed either as one-step or two-step process and 10 enzyme combinations were carried out using various endo- and exoproteases (Table 1). The enzyme concentrations used were 500 LAP-U/g for Flavourzyme, 0.6 AU-A/g for Alcalase, 212.5 UHb/g for Corolase 7089, 0.2 AU-N/g for Neutrase and ≥ 250 units/g for papain, which correspond to an enzyme to substrate ratio (E/S) of 1% (v/w), 0.5% (v/w), 0.5% (v/w), 0.5% (v/w) and 0.05% (w/w) of the total protein, respectively.

For the one-step process, enzymes were simultaneously added to the vessel and SPI was digested for 4 h, while aliquots were taken after 30, 120, and 240 min. During the two-step process, an endoprotease was applied in the first hydrolysis stage for pre-digestion (see Table 1). After 1 h incubation, Flavourzyme (1% v/w) was added for subsequent debittering by additional hydrolysis for another 2 h and aliquots were taken after 30, 120, and 240 min. During hydrolysis, the mixture was

stirred, while the pH-value and temperature were maintained constantly.

Hydrolysis was stopped by heat treatment at 90°C for 20 min. Control dispersions (no enzyme addition) were prepared under the same incubation conditions and heat inactivation treatment. All samples were frozen at -50°C and lyophilized (BETA 1-8, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). Experiments were performed in duplicate.

Table 1

Combination of protease preparations for SPI hydrolysis

Process	System	Endoprotease (E/S %)				Exoprotease (E/S %)
		Alcalase	Corolase 7089	Neutrase	Papain	Flavourzyme
one-step	S1	0.5	-	-	-	1
	S2	-	0.5	-	-	1
	S3	0.5	0.5	-	-	1
	S4	0.5	0.5	0.5	-	1
	S5	0.5	-	0.5	-	1
	S6	-	0.5	0.5	-	1
	S7	-	-	-	0.5	1
two-step	S8*	0.5	-	-	-	1
	S9*	-	0.5	-	-	1
	S10*	-	-	-	0.5	1

* SPI was hydrolyzed for 1h with the endoprotease and after 1h the exoprotease Flavourzyme was added and hydrolysis was continued

2.4. Determination of protein degradation due to enzymatic hydrolysis

2.4.1. Degree of hydrolysis (DH) using the *o*-phthaldialdehyde (OPA) method

The DH of all samples was calculated in triplicate ($n = 3$) by determining the free α -amino groups with *o*-phthaldialdehyde (OPA) using serine as standard according to Nielsen, Petersen, and Dambmann (2001).

2.4.2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight distribution of the samples was determined according to Laemmli (1970) using SDS-PAGE under reducing conditions. The sample preparation and gel running conditions were chosen as described elsewhere (Meinlschmidt et al., 2016). Protein visualization was performed by Criterion Stain-Free Gel DocTM EZ Imager (Bio-Rad).

2.5. Protein identification by liquid chromatography tandem mass spectrometry (LC-MS/MS)

2.5.1. Sample preparation for LC-MS/MS analysis

Protein separation was performed using SDS polyacrylamide gel electrophoresis (SDS-PAGE). The soy protein isolate was mixed with 1 x Laemmli Buffer and incubated for 5 min at 95°C and proteins were alkylated by adding acrylamide and incubation at room temperature (RT) for 30 min. An aliquot of 10 mL (\approx 50 mg protein) was loaded onto a 4-20% precast gel (Mini-PROTEAN[®] TGX[™] Gels 4-20%, 10-well comb, Bio-Rad Laboratories) and the separation was performed at 200 V for 35 min using a Mini-PROTEAN[®] Tetra Cell (Bio-Rad Laboratories). After electrophoresis the proteins were visualized by staining with Coomassie Brilliant Blue (PageBlue[™] Protein Staining Solution, Thermo Fisher Scientific). The gel lane was manually cut into 20 slices and each slice was subsequently cut into 1 mm³ gel pieces. Gel pieces were destained twice with 100 mL 50% (v/v) acetonitrile (ACN, Merck KGaA), 20 mM ammonium bicarbonate (ABC, SigmaAldrich Chemie GmbH) at 37°C for 30 min and dehydrated with 100 mL 100% ACN at RT for 10 min. Excess solvent was removed using a vacuum centrifuge (SAVANT DNA120 SpeedVac Concentrator, Thermo Fisher Scientific) for 30 min. The dried gel pieces were rehydrated in 20 mL 10 ng/mL trypsin solution (Trypsin NB Sequencing Grade, SERVA Electrophoresis GmbH) for 1 h on ice and 50 mL 10% (v/v) ACN, 20 mM ABC were added. The digestion was performed over night at 37°C by gently mixing (350 rpm) and stopped by addition of 5 mL 10% (v/v) ACN, 0.5% (v/v) trifluoroacetic acid (TFA, J.T. Baker) for 30 min at RT. The supernatants were transferred into fresh reaction tubes (Safe-Lock Tubes, Eppendorf AG) and peptides were extracted twice

using 20 mL 10% (v/v) ACN, 0.2% (v/v) TFA for 5 min at RT. The extracts were combined with the supernatants and dried in a vacuum centrifuge. The dried peptide extracts were redissolved in 30 mL 2% (v/v) ACN, 1% (v/v) TFA by shaking at 600 rpm for 30 min. After centrifugation at 13,000 $\times g$ for 10 min (Centrifuge 5417 C, Eppendorf AG) 20 mL of each sample were transferred into HPLC-vials prior to MS-analysis.

2.5.2. LC-MS/MS analysis

Nano-LC-ESI-MS/MS analysis was performed as described recently (Schröder, Rohrbeck, Just, & Pich, 2015; Zeiser, Gerhard, Just, & Pich, 2013) using a nano-flow ultra-high pressure liquid chromatography system (RSLC, Thermo Fisher Scientific) coupled online to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). An adequate amount of the proteolytic digests of the protein samples were injected and preconcentrated on a reversed phase trapping column (Acclaim PepMap[®], C18 particle 3 mm size, 2 cm length, 75 μ m i.d., pore size 100 Å, Thermo Fisher Scientific) at a flow rate of 6 mL/min with 0.1% (v/v) TFA in 5 min, followed by a separation on a 50 cm reversed phase separating column (Acclaim PepMap[®] RSLC nanoViper, C18 particle 2.4 mm size, 50 cm length, 75 μ m i.d., pore size 100 Å, Thermo Fisher Scientific). The peptides were eluted with a multi-step binary gradient (solvent A: 0.1% (v/v) formic acid (FA, Merck KGaA), solvent B: 0.1% (v/v) FA 80% (v/v) ACN) at a flow rate of 250 nL/min.

MS survey scans were acquired in a mass range of m/z 300-1600 at a resolution of 60,000 at 400 m/z in the orbitrap analyzer. The 10 most intense ions were selected for CID fragmentation (MS/MS) with a normalized collision energy of 38 in the ion trap, taking into account a minimum intensity of 2000 counts and a dynamic exclusion of 10 s.

Raw MS data were processed using the software PEAKS Studio (Version 7.0, Ma et al. 2003) and searched against *G. max* UniProtKB/Swiss-Prot database. Proteins were stated identified by a false discovery rate of 0.01 on protein and peptide level and a minimum number of five unique peptides.

2.6. Determination of the chemical composition and techno-functional properties

2.6.1. Chemical composition

The protein and dry matter content was determined as described by AOAC methods using a protein calculation factor of $N \times 6.25$ (AOAC, 2005a, 2005b).

2.6.2. Techno-functional properties

2.6.2.1. *Protein solubility.* Protein solubility of the samples was determined at pH 4.0 and 7.0 following the method of Morr et al. (1985).

2.6.2.2. *Emulsifying capacity.* The emulsifying capacity (EC) was determined in duplicate as suggested by Wang and Johnson (2001).

2.6.2.3. *Foaming properties.* Foaming activity was determined according to Phillips, Haque, and Kinsella (1987). The foaming density was measured by weighing a specified quantity of foam volume. The ratio of foam volume to foam weight was defined as foaming density in g L^{-1} . The foaming stability was estimated as the percent loss of foam volume after 60 min.

2.7. Bitterness evaluation of SPI hydrolysates

Bitterness of all samples was quantified as previously described in Meinschmidt et al. (2016). A ten-member sensory panel (7 female, 3 male) had been screened and trained with caffeine solutions over two months (1h per session, twice a week) according to DIN 10959. An Alcalase hydrolysate (E/S 0.5 % v/w, production conditions: pH 8.0, 60 °C, 180 min without pH adjustment) at different solution concentrations was chosen to assess bitterness (Meinschmidt et al., 2016). A 10-

cm continuous scale anchored from 0 (no perception) to 10 (strong perception) was taken for bitterness evaluation. For scale-calibration, Alcalase hydrolysates with concentrations of 1.0 and 2.5 g L⁻¹ (w/w) were estimated by the panel to correspond to bitter intensities of 5 and 10, respectively.

For sample evaluation, SPI hydrolysates were mixed with tap water to a protein equivalent of 2.5 g L⁻¹ (w/w) and the pH was adjusted to pH 7.0 with 1 M NaOH. The samples (10 mL) were randomly presented to the panel in small plastic cups, which were coded by arbitrary numbers (three-digits). At each session (performed in 10 sessions, 1h each) the panel was firstly supplied with the reference solutions containing 1.0 and 2.5 % Alcalase hydrolysate. Panellists were instructed to assign bitterness scores to the samples (eight samples per session) on the 10-cm continuous scale. Water and plain crackers were served for palate cleansing in between. Each panellist did a monadic evaluation of the samples at individual speed. Sensory evaluation was at least performed in duplicate.

2.8. Statistical analysis

All data are expressed as means \pm standard deviation from at least two independent experiments. Chemical data were analyzed using one-way Analysis of variances (ANOVA) and means were generated and adjusted with Bonferroni post hoc test. Sensory data were also subjected to ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test. Statistical analysis was performed with SPSS 20.0 (SPSS for windows: SPSS Inc. Chicago, IL, USA) and probability $p < 0.05$ indicated statistically significant differences.

3. Results and Discussion

The experiments were divided into two parts, first various enzyme combination systems S1-S10 (Table 1) were screened to find a proper combination for the hydrolysis of SPI (dry matter 94.4 %; protein content 94.6 %), while producing a low bitter taste. Based on these results, individual hydrolysates were analyzed in more detail, including the techno-functional properties.

3.1. Screening of different enzyme combination systems

3.1.1. Effect on protein degradation

3.1.1.1. *Degree of Hydrolysis*. The DH can give an initial indication for the reduction of allergenic compounds as enzymatic hydrolysis involves the cleavage of peptide bonds, resulting in a breakdown of the intact protein into peptides (Sujith & Hymavathi, 2010).

The DH was monitored during hydrolysis of SPI and the results are shown in Table 2 and Figure 1. Untreated SPI had a DH value of 2.1 %, whereas the DH significantly ($p < 0.05$) increased in the course of hydrolysis.

Among the one-step process hydrolysates, S5 reached the highest DH value of about 29.9 % after 4h, followed by S3, S4, and S1 with DH values of 28.9 %, 27.3 %, and 23.6 %, respectively. These hydrolysates were prepared with enzyme combination systems containing Alcalase. Meinschmidt et al. (2015) hydrolyzed SPI with single enzymes, but the same reaction conditions and a DH value of about 13 % was reached after 2h of hydrolysis with Alcalase. In contrast, hydrolysis for 2h with a combination containing Alcalase increased the DH up to 26.9 % (S6). The lowest DH values of 16.8 %, 16.0 %, and 14.0 % after digestion for 4h were found for S2, S6, and S7, respectively.

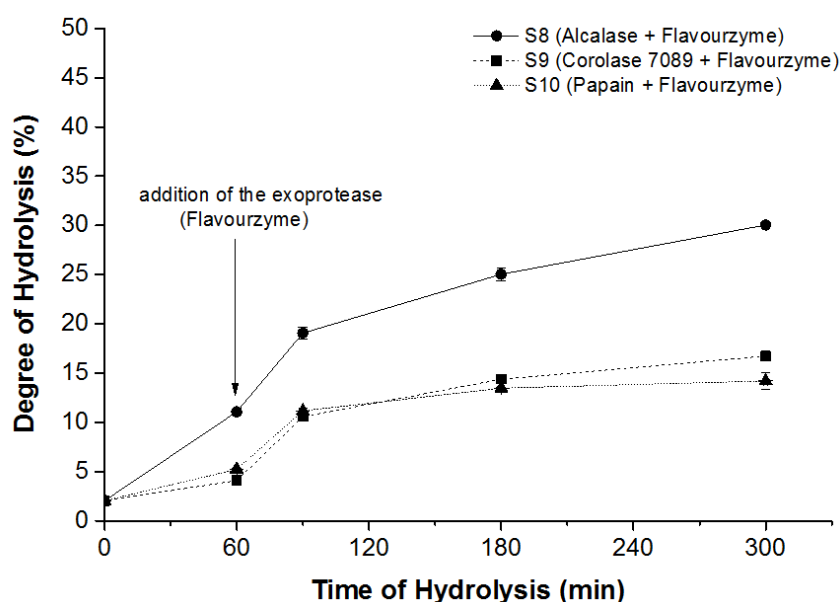


Fig. 1. Hydrolysis curves of the two-step process enzyme combination systems S8 (Alcalase + Flavourzyme), S9 (Corolase 7089 + Flavourzyme), and S10 (papain + Flavourzyme). Results are expressed as means \pm standard deviation.

The two-step enzyme combinations S8 - S10 resulted in a low initial hydrolyzation rate during the first hour of pre-digestion as evidenced by low DH values (Table 2 and Figure 1). After the addition of Flavourzyme, the hydrolyzation rate increased rapidly during the initial stage. The cleavage of peptide bonds by the endopeptidases increases the number of end peptide sites for the action of the exoprotease Flavourzyme. S8, a combination of Alcalase and Flavourzyme, results in the highest DH value of 30 % after 5h, while S9 and S10 had lower DH values of about 16.7 % and 14.2 %, respectively.

Table 2

Degree of Hydrolysis (DH) (%) of hydrolyzed SPI by different protease combinations obtained by the screening trials

Process	System	Degree of Hydrolysis (%)							
		Time of Hydrolysis (min)							
		0	30	60	90	120	180	240	300
one-step	S1	2.1 ± 0.0 ^a	11.6 ± 0.3 ^b	-	-	21.5 ± 1.7 ^c	-	23.6 ± 0.2 ^c	-
	S2	2.1 ± 0.0 ^a	10.1 ± 1.6 ^b	-	-	13.5 ± 1.6 ^{b,c}	-	16.8 ± 1.6 ^{c,d}	-
	S3	2.1 ± 0.0 ^a	15.5 ± 1.3 ^b	-	-	24.7 ± 1.3 ^c	-	28.9 ± 0.8 ^d	-
	S4	2.1 ± 0.0 ^a	15.2 ± 0.4 ^b	-	-	22.3 ± 0.8 ^c	-	27.3 ± 0.8 ^d	-
	S5	2.1 ± 0.0 ^a	22.2 ± 1.1 ^b	-	-	26.9 ± 1.6 ^c	-	29.9 ± 0.2 ^c	-
	S6	2.1 ± 0.0 ^a	10.0 ± 0.5 ^b	-	-	14.1 ± 0.5 ^c	-	16.0 ± 0.5 ^d	-
	S7	2.1 ± 0.0 ^a	9.0 ± 0.3 ^b	-	-	12.0 ± 0.1 ^c	-	14.0 ± 0.3 ^d	-
two-step	S8	2.1 ± 0.0 ^a	-	11.1 ± 0.3 ^b	19.1 ± 0.6 ^c	-	25.0 ± 0.7 ^d	-	30.0 ± 0.2 ^e
	S9	2.1 ± 0.0 ^a	-	4.1 ± 0.1 ^b	10.6 ± 0.4 ^c	-	14.4 ± 0.2 ^d	-	16.7 ± 0.1 ^e
	S10	2.1 ± 0.0 ^a	-	5.2 ± 0.1 ^b	11.2 ± 0.2 ^c	-	13.5 ± 0.1 ^d	-	14.2 ± 0.9 ^d

Results are expressed as means ± standard deviation. Means followed with different superscript letters within each column are significantly different ($p < 0.05$) relating to one enzyme combination system (one-way ANOVA, Bonferroni).

3.1.1.2. *Electrophoretic analysis (SDS-PAGE) and mass spectrometry (LC-MS/MS) analyses.* SDS-PAGE profiles of native SPI, references and selected SPI hydrolysates are exemplarily shown in Fig. 2. As shown in Fig. 2A, native SPI and the reference presented characteristic electrophoretic patterns for soy proteins: The first three bands are α' (~ 72 kDa), α (~ 68 kDa), and β (~ 53 kDa) subunits of β -conglycinin (Gly m5), followed by glycinin (Gly m6), which is composed of the acidic ("A", ~ 29-33 kDa) and basic subunit ("B", 18-22 kDa) (Amnuaycheewa & de Mejia, 2010).

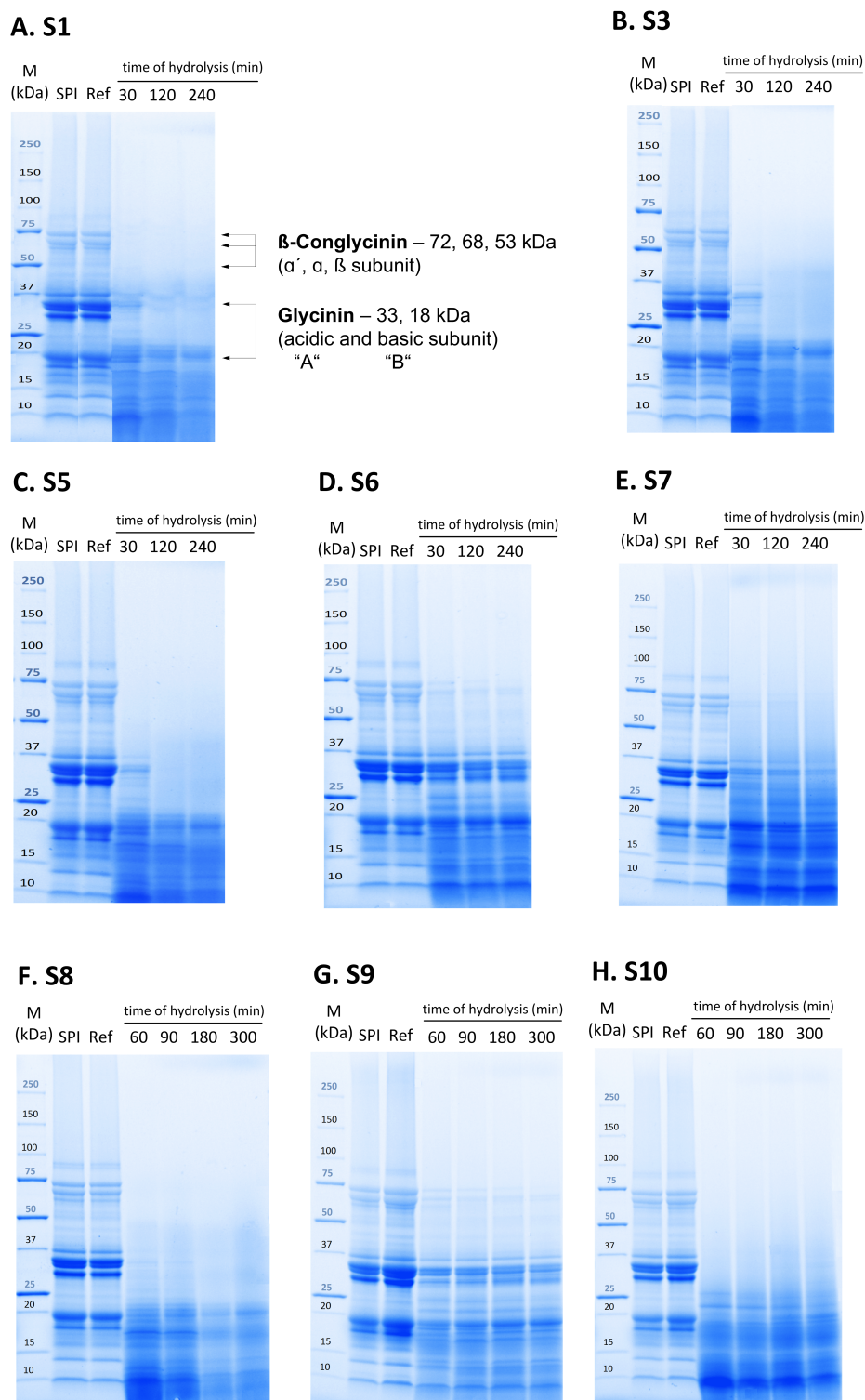


Fig. 2. SDS-PAGE patterns of SPI hydrolysates prepared with commercial available proteases in combination (screening). M- Molecular weight standard indicated in kilo Dalton (kDa); SPI- soy protein isolate; Ref- reference of each protease (no enzyme addition); Electrophoresis was carried out with 4-20 % polyacrylamide gradient gels. α'-, α- and β-subunits of β-conglycinin; "A" and "B": acidic and basic subunit of glycinin.

Gel-based LC-MS/MS analyses of native SPI were additionally performed to further verify the identity and presence of the two major soy allergens β -conglycinin and glycinin, including their subunits (Fig. 3 and Table 3). Therefore, a total of 20 protein slices, consisting of both abundant and less abundant proteins were selected, digested with trypsin and analyzed. Data listed in Table 3 include selected slices and the corresponding subunits of major soy allergens, the accession number of the proteins as in *G. max* UniProtKB/Swiss-Prot database, protein confidence score ($-10 \lg P$), number of high-confidence supporting peptides, number of high confidence supporting peptides that are uniquely mapped to the protein as well as the sequence coverage. Although a few additional proteins have been identified, the subunits of β -conglycinin and glycinin represent the proteins with the highest score and number of supporting peptides within the assigned gel slices (see Fig. 3, slices 6, 7, 9, 11, 12, 15).

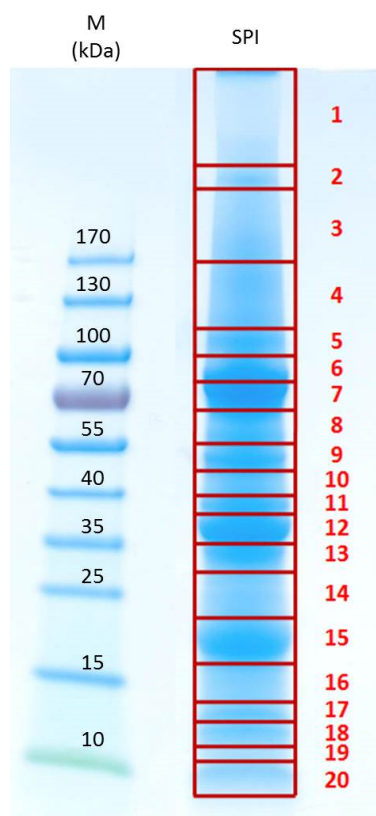


Fig. 3. SDS-PAGE pattern of untreated SPI. Excised protein bands used for LC-MS/MS identification of the subunits of β -conglycinin and glycinin. M - Molecular weight standard indicated in kilo Dalton (kDa); SPI - soy protein isolate; the gel lane was cut into 20 slices as indicated by red rectangles. Each slice was analyzed separately in order to determine the location of the proteins of interest.

LC-MS/MS analysis of trypsin-digested 72, 68, and 53 kDa proteins indicated that these proteins were the α' (alphaprime)-, α (alpha)-, and β (beta)-subunits of β -conglycinin, having a high sequence coverage of 74, 62, and 84%, respectively. Furthermore, the high score values ranging from 402.64 to 463.36 gives an extremely high level of confidence that this identification is correct. The acidic and basic subunits of glycinin are represented as full-length proteins within the database. Therefore, differentiation of the location of the acidic and basic polypeptide chains in the SDS-PAGE was performed based on the respective sequence coverage and number of supporting peptides and confirmed by the high score - 10 lgP values (Table 3). Hence, the acidic polypeptide chains (A1-A5) were identified with higher sequence coverage (see Table 3, sequence coverage 71-91%) and a larger number of high confident peptides in slices 11 and 12, whereas the basic polypeptide chains (G1-G5) were identified with high confidence in slice 15, corresponding to a molecular mass of 29-33 and 18-22 kDa, respectively.

Already after 30 min of hydrolysis with S1, β -conglycinin was completely decomposed and small amounts of glycinin remained within 4 h. Glycinin "A" was partially hydrolyzed, whereas glycinin "B" was more resistant. S3 (Fig. 2B), S4 (data not shown), and S5 (Fig. 2C) showed similar profiles than S1, but were more effective as glycinin "A" was instantly hydrolyzed after 2 h. Profiles of S2 (data not shown), S6 (Fig. 2D), and S9 (Fig. 2G) were merely the same and exhibit a low activity against Gly m6. Gly m5 diminished within the first 30 min, and almost completely vanished after 3 h (S9) and 4 h (S2 and S6).

The most effective combinations were the two-step systems S8 and S10, hydrolyzing all allergens to a greater degree and most of the peptides obtained after 60 min of pre-digestion were < 20 kDa. After the addition of Flavourzyme, the SDS-PAGE only slightly changed. Peptides released after pre-hydrolysis were further hydrolyzed by the exo-proteases of Flavourzyme, which cleave small peptides at the end of proteins, leading to no visible effect on the electrophoretic profile.

SDS-PAGE profile of S7 (Fig. 2E) showed great changes in the molecular weight distribution, but being less effective than S10 (Fig. 2H). It is noteworthy that SDS-PAGE results of S7 (one-step) and S10 (two-step), both combinations of papain and Flavourzyme, showed visible differences in the electrophoretic profiles. These findings suggest that enzymes might compete and negatively affect each other, when hydrolyzing concomitantly. The same trend can be found for S1 and S8, where the two-step process seems to be more effective in hydrolyzing soybean allergens.

Table 3

Identification of the subunits of β -conglycinin and glycinin in native SPI using LC-MS/MS analysis

Slice	Protein (Subunit)	Accession #	Score - 10 lgP	# Peptides	# Unique Peptides	Sequence Coverage (%)
6	α' subunit β -conglycinin	P11827	463.36	188	169	74
7	α subunit β -conglycinin	P13916	402.64	56	47	62
9	β' subunit β -conglycinin	P25974	412.70	123	109	84
11	acidic subunit (A3) glycinin G5	P04347	368.98	84	72	79
12	acidic subunit (A1) glycinin G1	P04776	423.36	125	90	91
	acidic subunit (A2) glycinin G2	P04405	400.02	95	51	92
	acidic subunit (A) glycinin G3	P11828	360.06	69	34	90
	acidic subunit (A4,5) glycinin G4	P02858	395.39	66	58	71
15	basic subunit glycinin G1	P04776	508.83	129	87	85
	basic subunit glycinin G2	P04405	471.94	113	78	88
	basic subunit glycinin G3	P11828	431.35	67	35	71
	basic subunit glycinin G4	P02858	522.81	116	85	73
	basic subunit glycinin G5	P04347	436.32	71	40	73

Accession: The accession number of the protein as in database.

Score (-10lgP): The protein confidence score; p: probability.

Peptides: The number of high-confidence supporting peptides.

Unique peptides: The number of high-confidence supporting peptides that are uniquely mapped to the protein. Unique peptides with the same sequence but different modifications are only counted once in this number.

Sequence coverage: The percentage of the protein sequence covered by supporting peptides.

→ Explanations given by the Software PEAKS (User Manual).

3.1.1.2. Effects on the bitterness

One of the attributes that reduces the consumer acceptance of protein hydrolysates is the presence of a strong bitter taste due to the presence of low molecular weight peptides composed of mainly hydrophobic amino acids (Ney, 1979; Saha & Hayashi, 2001). Bitterness was evaluated by a sensory panel on the basis of the 10- cm continuous scale (Table 4).

Native SPI showed a bitterness score of 2.6. With an exception of the hydrolysates prepared with enzyme combinations containing Alcalase (S1, S3, S4, S5, and S8), the bitterness of all hydrolysates remained widely unchanged within the first hour of hydrolysis compared to native SPI, but clearly tend to decrease at the end of hydrolysis.

All hydrolysates generated with combination systems containing Alcalase (S1, S3, S4, S5, and S8) reached higher bitterness scores up to 7.9 (S8). These results may be expected when the DH values of Alcalase containing hydrolysates were considered. It has previously been shown that hydrolysate bitterness increases with increasing

DH (Barry, O’Cuinn, Harrington, O’Callaghan, & FitzGerald, 2000). According to the findings, this hypothesis cannot be entirely confirmed as the bitterness score of hydrolysate S8 decreased in the course of hydrolysis to a minimum of 3.4 (5 h), while the DH increased from 11.1% to 30%. However, the DH values were the highest among all combinations tested, which means that bitterness can be expected to be high at high DH values.

Hydrophobicity, primary sequence, spatial structure, molecular weight, and bulkiness of peptides have been studied as possible influences in bitter taste of hydrolysates (Kim, Kawamura, Kim, & Lee, 2008). Several research groups found that Alcalase has the tendency to hydrolyze at hydrophobic amino acid residues, which cause a high bitterness (Adler-Nissen, 1986; Ishibashi et al., 1988). Reduction in bitterness might be due to the fact that Flavourzyme is a mixture of endo- and in particular exo-peptidase activities, which selectively release N-terminal amino acid residues, degrading bitter peptides.

Table 4

Sensory perception (bitterness) of hydrolyzed SPI by different proteases of the screening trials

Process	System	Bitterness Intensity								
		Time of Hydrolysis (min)								
		0	30	60	90	120	180	240	300	
one-step	S1	2.6 ± 0.9 ^a	4.1 ± 1.3 ^{a,b}	-	-	4.6 ± 1.5 ^b	-	4.5 ± 1.5 ^b	-	
	S2	2.6 ± 0.9 ^a	3.0 ± 1.6 ^a	-	-	3.7 ± 1.5 ^a	-	2.6 ± 2.2 ^a	-	
	S3	2.6 ± 0.9 ^a	3.8 ± 1.3 ^{a,b}	-	-	5.7 ± 1.0 ^c	-	4.9 ± 1.6 ^{b,c}	-	
	S4	2.6 ± 0.9 ^a	4.6 ± 1.8 ^b	-	-	3.8 ± 1.4 ^{a,b}	-	6.4 ± 1.6 ^c	-	
	S5	2.6 ± 0.9 ^a	5.4 ± 1.4 ^b	-	-	4.6 ± 1.4 ^b	-	4.0 ± 1.4 ^{a,b}	-	
	S6	2.6 ± 0.9 ^a	2.1 ± 1.6 ^a	-	-	2.5 ± 1.6 ^a	-	2.5 ± 1.7 ^a	-	
	S7	2.6 ± 0.9 ^a	3.4 ± 1.6 ^a	-	-	2.4 ± 1.2 ^a	-	2.3 ± 1.5 ^a	-	
two-step	S8	2.6 ± 0.9 ^a	-	7.9 ± 1.8 ^b	6.3 ± 1.2 ^{b,c}	-	4.9 ± 1.5 ^{c,d}	-	3.4 ± 1.5 ^{a,d}	
	S9	2.6 ± 0.9 ^a	-	4.4 ± 1.6 ^a	2.0 ± 1.5 ^a	-	3.2 ± 1.5 ^a	-	1.9 ± 1.6 ^a	
	S10	2.6 ± 0.9 ^a	-	2.4 ± 1.5 ^a	2.6 ± 1.5 ^a	-	1.9 ± 1.5 ^a	-	2.1 ± 0.8 ^a	

Results are expressed as means ± standard deviation. Means followed with different superscript letters within each column are significantly different ($p < 0.05$) relating to one enzyme combination system (one-way ANOVA, Tukey’s HSD).

3.2. Effects of selected enzyme combinations on the potential allergenicity, technofunctionality and sensory properties

Enzymatic hydrolysis with enzyme combination systems S3, S4, S5, S6, S7, S9, and S10 were repeated with respect to a less bitter taste and an effective degradation of the major soy allergens. The time of hydrolysis for each combination was

chosen individually to find a proper reaction condition and enzyme combination for hydrolyzing SPI (Table 5). In addition, the techno-functional properties in terms of protein solubility, emulsifying capacity, foaming activity as well as foaming density, and stability were examined.

3.2.1. Effect on the protein degradation

3.2.1.1. *Degree of Hydrolysis*. The results of the DH values were in accordance with the screening trials (see 3.1). The highest DH values of about 31.0 %, 22.0 %, and 21.6 %, respectively, could be obtained by S3, S4, and S5. Lower DH values of 15.8 %, 14.7 %, 13.0 %, and 11.9 % were achieved after incubation with S9, S7, S6, and S10, respectively.

3.2.1.2. *Electrophoretic analysis (SDS-PAGE)*. In addition to the SDS-PAGE profiles (Fig. 2), individual bands of glycinin and β -conglycinin were quantified by Image Lab™ software. SPI hydrolysis was expressed as percentage of disappearance of the protein fraction bands with respect to native SPI fractions (Table 5).

Table 5

Degradation of SPI main allergen fractions by different proteases. Results are expressed as means \pm standard deviation. Means followed with different superscript letters within each column are significantly different ($p < 0.05$) following one-way ANOVA (Bonferroni)

Process	System	Time of Hydrolysis (min)	Soy protein isolate (% hydrolyzed) ^a					
			β -conglycinin			Glycinin		Total average
			α'	α	β	A	B	
one-step	S3	30	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	79.2 \pm 0.8 ^a	95.8 \pm 0.4 ^a
	S4	120	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	90.5 \pm 1.0 ^b	98.1 \pm 0.4 ^b
	S5	240	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	88.0 \pm 1.3 ^b	97.6 \pm 0.6 ^b
	S6	120	79.6 \pm 1.0 ^b	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	39.3 \pm 0.9 ^c	83.8 \pm 0.4 ^c
	S7	240	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	89.7 \pm 0.9 ^b	97.9 \pm 0.4 ^b
two-step	S9	300	67.6 \pm 0.7 ^c	100.0 \pm 0.0 ^a	27.9 \pm 0.1 ^b	60.7 \pm 0.3 ^b	25.8 \pm 0.2 ^d	56.4 \pm 0.1 ^d
	S10	90	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	100.0 \pm 0.0 ^a	97.6 \pm 0.4 ^e	99.5 \pm 0.2 ^b

α , α' , β , subunits of β -conglycinin; A, acidic fraction of glycinin; B, basic fraction of glycinin.

^a % of hydrolysis of each main fraction of soy protein isolate treated with different enzyme combination systems with respect to those of SPI without treatment

Enzyme combinations S3, S4, S5, S7, and S10 were most effective in the overall degradation with proteolytic activities of 95.8%, 98.1%, 97.6%, 97.9%, and 99.5%,

respectively. These combinations completely hydrolyzed β -conglycinin subunits and glycinin "A", whereas glycinin "B" was affected with varying degrees (Fig. 2 and Table 5). The hydrolysate generated with S6 showed a less effective overall proteolytic activity of about 83.8%, evidenced by SDS-PAGE analysis (see Fig. 2G). The a and b subunit of β -conglycinin and glycinin "A" were completely degraded, whereas the α' subunit and glycinin "B" were only reduced by 79.6% and 39.3%, respectively. S9 showed the smallest changes as the overall proteolytic activity was 56.4%.

3.2.2. Effects on the bitterness

The panel was asked again to evaluate the bitter taste of the hydrolysates on the basis of the 10-cm continuous scale and the results are presented in Fig. 4.

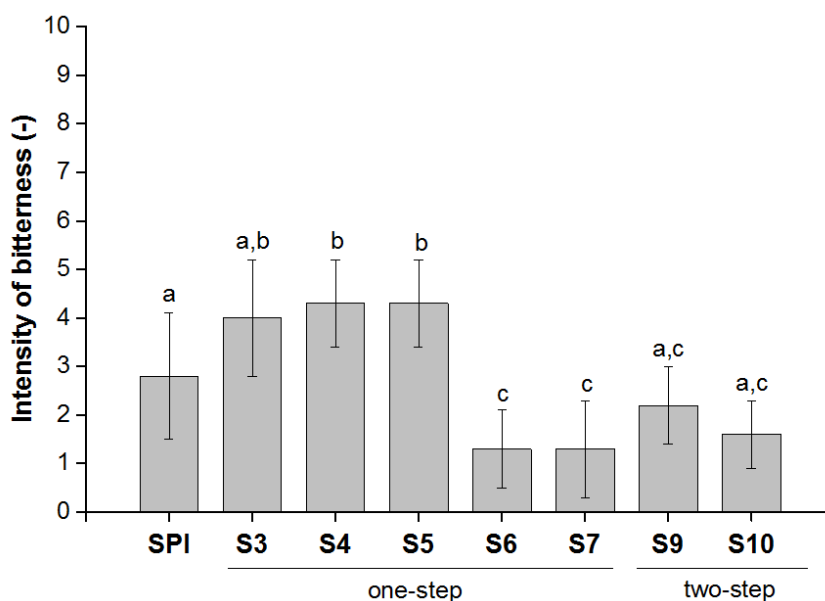


Fig. 4. Bitterness intensities of SPI and SPI hydrolysates. Each value is expressed as means \pm standard deviation scored on a 10-cm line scale, ranging from 0 (no perception) to 10 (strong perception), by ten panellists. Means followed with different superscript letters (a,b,c) are significantly different ($p < 0.05$, 10 panellists) following one-way ANOVA (Tukey's HSD).

Native SPI was judged with a bitterness intensity of 2.8. The bitter taste, bitterness score 4.0, of S3 was not significantly ($p < 0.05$) higher than that of native SPI, whereas the hydrolysates generated with S4 and S5 resulted in significantly (p

< 0.05) higher bitterness of 4.3 and 4.3, respectively. Instead, enzyme combinations containing no Alcalase preparation led to significantly lower bitterness intensities of 1.3 (S6 and S7), 1.6 (S10) and 2.2 (S9). As previously outlined, incubation of SPI with an enzyme combination containing Alcalase resulted in the highest bitterness. This fact was expected, because Alcalase was known to produce a strong bitter taste due to the release of bitter peptides, which are mainly composed of hydrophobic amino acid residues (Seo et al., 2008). Meinschmidt et al. (2016) found a bitterness score of 8.2 for a hydrolysate obtained only by Alcalase, whereas an enzyme combination containing Alcalase facilitated a bitter intensity of 4.0 (S3).

In addition, the panel judged all protein hydrolysates as having good organoleptic properties in terms of less intense *beany* and *green* smell and taste, which are commonly negative attributes associated with soybean products (profile analysis, data not shown).

3.2.3. Effects on the technofunctionality

Soy protein hydrolysates are used in foods as functional and nutritional ingredients, and enzymatic hydrolysis is one of the most efficient methods for modifying the functional properties (Ortiz & Wagner, 2002).

3.2.3.1. *Protein solubility.* Protein solubility is considered a key functional property since good solubility is a prerequisite for the formation of foams and emulsions. The results are represented graphically as a function of pH 4.0 and 7.0 in Fig. 5.

The minimum solubility of 5.0% of native SPI was detected at pH 4.0, near the isoelectric point of soybean proteins, but was significantly ($p < 0.05$) higher after hydrolysis with all enzyme combination systems. Enzyme combinations containing Alcalase (S3, S4 and S5) had the highest solubility at pH 4.0 of 70.3%, 79.9% and 87.0%, respectively, whereas the other systems had a solubility between 36.0% and 41.9%. At pH 7.0 a generally higher solubility could be measured. Native SPI had a solubility of 44.0%. Applying S3, S4, S5 and S6 caused a solubility of 80.5%, 85.7%, 91.6% and 86.0%, respectively. These enzyme combinations were most effective in the reduction of the molecular weight thereby creating smaller peptides. Adler-Nissen (1986) and Ortiz and Wagner (2002) introduced that the release of smaller peptides and the corresponding increase in ionizable groups such as amino and carboxyl groups, which can interact with water molecules, are responsible for enhanced solubility.

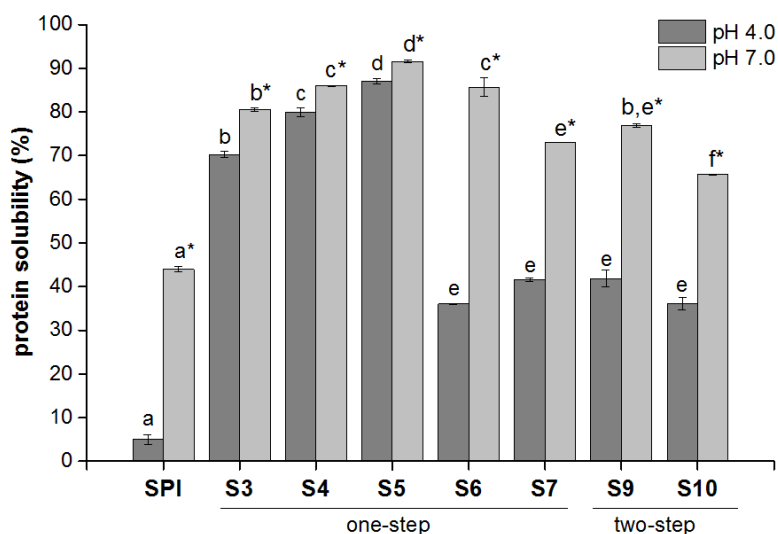


Fig. 5. Solubility of SPI and SPI hydrolysates at pH 4.0 and pH 7.0. Results are expressed as means \pm standard deviation. Means followed with different superscript letters (a,b,c,d,e,f,) are significantly different ($p < 0.05$) following ANOVA (Bonferroni). *indicates the solubility at pH 7.0.

3.2.3.2. *Emulsifying capacity.* Native SPI had an EC of 660 mL g^{-1} , while all hydrolysates, except hydrolysates generated with S3, S4 and S5, showed a significantly ($p < 0.05$) enhanced EC. The EC increased to 810 mL g^{-1} , 750 mL g^{-1} , 700 mL g^{-1} , and 693 mL g^{-1} after hydrolysis with S10, S6, S7, and S9, respectively. Enzymatic hydrolysis has already been shown by several studies to improve the emulsifying properties (Wu, Hettiarachchy, & Qi, 1998). The increased EC may be due to the degradation of large protein molecules, exposure of hydrophobic groups and enhanced protein solubility, wherefore protein surface activity was improved, resulting in a better emulsifying activity (Wu et al., 1998). S3 showed an EC of about 645 mL g^{-1} , which is comparable to native SPI. However, the EC of hydrolysates prepared with S4 and S5 decreased to 560 mL g^{-1} and 480 mL g^{-1} , respectively, which might be due to a high content of small peptides, negatively affecting protein film formation since a minimum peptide length is desirable for an effective protein-protein interaction.

3.2.3.3. *Foaming properties.* Table 6 summarizes the foaming properties. Foaming activity of all hydrolysates was remarkably improved up to 2706% (S3), which is nearly 4-times higher than that of native SPI (552%). Due to hydrolysis, more flexible and low molecular weight aggregates were formed. As a consequence, pep-

tides could transfer to the air-water interface more rapidly and the protein-protein interaction improved the strength of the viscoelastic cohesive film, dropping the surface tension efficiently (Tsumura et al., 2005). The compact structure of native SPI molecules led to a lower transferring-speed of proteins to the air-water interface, lowering the foaming activity.

S6 and S9 had the lowest foaming activity of about 1522% and 1576%, respectively. There is an evidence of a trend towards increased foamability when β -conglycinin faded and glycinin became dominant (Tsumura et al., 2005). This might explain the lower foamability of S6 and S9, which were less effective in degrading β -conglycinin as evidenced by SDS-PAGE analysis (Fig. 2 and Table 5).

The results of the foaming stability and density indicated no improvement compared to native SPI (Table 6). The most stable foam was achieved with S10 (88%), which is near to that of native SPI (90%), followed by S6, S7, S9, and S3 with 80%, 80%, 66%, and 16%, respectively. The hydrolysates prepared with S4 and S5 exhibited no foaming stability (foam decomposes directly after whipping), which might be due to excessive hydrolysis of proteins, leading to a reduced ability of polypeptides to interact at the air-water interface so that less viscoelastic films will cause decreased foaming stability (Martinez, Sanchez, Ruiz-Henestrosa, Patino, & Pilosof, 2007). Some larger protein components were essential for stable foams, which are indeed not present in S4 and S5 due to excessive hydrolysis (SDS-PAGE, Fig. 2 and Table 5).

Table 6

Foaming properties (activity, density, and stability) of SPI and SPI hydrolysates

Process	System	Foaming activity (%)	Foaming stability (%)	Foaming density (g L ⁻¹)
one-step	-	552 ± 5 ^a	90 ± 0 ^a	215 ± 5 ^a
	S3	2706 ± 0 ^b	16 ± 0 ^b	34 ± 0 ^b
	S4	2395 ± 0 ^c	0 ± 0 ^c	30 ± 0 ^c
	S5	2173 ± 0 ^d	0 ± 0 ^c	29 ± 0 ^d
	S6	1522 ± 2 ^e	80 ± 0 ^d	65 ± 0 ^e
	S7	2016 ± 0 ^f	80 ± 0 ^d	45 ± 0 ^f
two-step	S9	1576 ± 0 ^g	66 ± 0 ^e	59 ± 0 ^g
	S10	2173 ± 5 ^d	88 ± 0 ^f	41 ± 0 ^h

Results are expressed as means ± standard deviation. Means with different letters within one column indicate significant differences ($p < 0.05$) following ANOVA (Bonferroni).

4. Conclusions

The study was conducted to investigate the effects of enzymatic hydrolysis with various enzyme combinations on the potential allergenicity, technofunctionality, and sensory properties of SPI. The results revealed that hydrolysis with enzyme combinations turned out to be a great approach for enhancing the DH, thereby degrading the major soybean allergens, while improving the techno-functional properties. However, SDS-PAGE and the DH are indirect methods to investigate the reduction of the allergic potential. Therefore, further research should be conducted to get detailed knowledge of the allergen structure. Epitope-specific antibodies against soy allergens should be developed to assess the residual immunoreactivity using enzyme-linked immunosorbent assays (ELISA). In addition, sera from soy allergy suffering people could be useful in different immunoblots to get a deep insight in soy protein allergy. A specific emphasis has been given to the utility of enzyme combinations for debittering SPI hydrolysates, which was indeed successfully achieved, decreasing the bitter taste to a minimum of 1.3 (S6 and S7, see Figure 4). According to the results, the two-step combination system S10 (papain + Flavourzyme) turned out to be the most appropriate enzyme combination to address a low level of intact allergenic proteins, low bitter taste, and superior techno-functional properties. Besides the organoleptic tests, the analysis of bitter peptides - their characterization and quantification - might be of particular importance to get a deeper insight in the enzyme assisted hydrolytic reactions.

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Conflict of Interest

The authors have declared no conflicts of interest.

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CHAPTER 3: Soy protein hydrolysates fermentation – tation: effect of debittering and degradation of major soy allergens³

Abstract

The debittering effect of induced liquid state fermentation (*Lactobacillus perolens*, *Rhizopus oryzae*, and *Actinomucor elegans*) on different soy protein hydrolysates has been investigated. The hydrolytic action was monitored by SDS-PAGE and degree of hydrolysis analyses. Sensory perception using Quantitative Descriptive Analysis (QDA), employing multivariate statistical Principal Component Analysis (PCA), techno-functional properties, and the growth behavior of microorganisms (MALDI-TOF MS) have been evaluated. SDS-PAGE profiles evidenced that the enzyme preparations degraded most of major soy allergens (β -conglycinin, glycinin), while subsequent fermentation did not further change the profiles. All strains investigated effectively reduced bitterness to a minimum of 0.7 on a 10-cm continuous scale (0 = no perception; 10 = strong perception) compared to non-fermented hydrolysates (2.8 - 8.0) and native soy protein isolate (2.8). Protein solubility, emulsifying and oil-binding capacity as well as foaming activity and gelation behaviour were enhanced depending on the protease used; subsequent fermentation further improved foaming stability and gelation concentration. PCA of descriptive sensory data revealed that fermentation apparently upgrade the organoleptic perception by effectively decreasing the bitter taste, simultaneously reducing the beany off-flavour of soy. Consequently, enzymatic hydrolysis combined with subsequent fermentation represents a promising method for producing hypoallergenic soy hydrolysates with pleasant taste and great technofunctionality.

Keywords: Soybean allergens; Fermentation; Bitter taste; Techno-functional properties; Principal Component Analysis (PCA)

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1. Introduction

Food allergy is a common disease and affects approximately 220-250 million people worldwide (WAO, 2013). Both the number of incriminated foods and the frequency of severe reactions appear to be rising dramatically. Food allergens are naturally occurring proteins of the allergenic foods, and small regions, called epitopes, are responsible for the immunoglobulin E (IgE)-mediated allergic response by acting as an antigen (Taylor & Hefle, 2001).

Soybean (*Glycine max* (L.) MERR.) is one of the most important valuable crops in the world due to its considerable amount of high quality proteins and excellent functional properties. However, soy ranks among the "big 8" allergens which comprises those foods that causes over 90 % of all documented food allergies in the U.S. (FDA, 2004; Taylor & Hefle, 2001). Although 16 IgE-reactive allergenic proteins have been identified, just the two storage proteins glycinin (Gly m6) and β -conglycinin (Gly m5) are considered as major soybean allergens (Amnuaycheewa & de Mejia, 2010; FARRP, 2015; Holzhauser et al., 2009).

The only method currently available to avoid any allergic reaction is complete dietary exclusion of the offending foods. As food intolerances emerged considerably in the last years, science and food industry are searching for new technologies, including thermal and non-thermal technologies to control soybean allergy other than avoidance (Shriver & Yang, 2011; Verhoeckx et al., 2015). The technological approach hitherto to decrease allergenicity has largely been empirical due to the lack of detailed knowledge on integral epitope structure. There are very few practical applications of food processing that effectively reduce allergenicity, including enzymatic hydrolysis, which has proven to be efficient in destroying epitopes (Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016; Wilson, Blaschek, & de Mejia, 2005; Yamanishi et al., 1996). However, a serious quality concern facing the manufacturer of hydrolysates is the formation of bitter taste, often restricting the commercial exploitation as food ingredient. Bitterness is often attributed to the release of low molecular weight peptides containing hydrophobic amino acid residues, particularly leucine, proline, phenylalanine, and tyrosine (Ishibashi et al., 1988), although the exact cause is not fully understood and conflicting research exists in the literature. The hydrophobicity, primary sequence, spatial structure, molecular weight, and bulkiness of peptide have been studied as possible influences for bitterness (Kim, Kawamura, Kim, & Lee, 2008).

Many attempts have been made to prevent, reduce, eliminate or even mask the bitterness of food hydrolysates. These include selective separation or adsorption

of bitter peptides such as treatment with activated carbon, extraction with alcohol, isoelectric precipitation, chromatography on silica gel, hydrophobic interaction chromatography (Saha & Hayashi 2001). The bitterness in hydrolysates was also masked via the addition of various agents, and through the admixture with intact proteins (FitzGerald & O’Cuinn, 2006; Sujith & Hymavathi, 2010; Sun, 2011). Debittering via transpeptidation, cross-linking as well as the modification of taste signalling have also been conducted (Sun 2011). These methods, however, are commonly cost-effective and can cause a significant loss of essential amino acids (Pedersen, 1994).

Considerable attention has been paid to peptidase-mediated debittering of protein hydrolysates. That means an enzymatic hydrolysis of bitter peptides with, particularly exo-peptidases from different sources (FitzGerald & O’Cuinn, 2006; Ishibashi et al., 1988; Meinlschmidt, Schweiggert-Weisz, Brode, & Eisner, 2016). Exopeptidases cleave adjacent to hydrophobic amino acid residue at the C- or N-terminus of the proteins/peptides. Based on specificity for hydrolysis of synthetic substrates, they are classified as aminopeptidases, alkaline/neutral proteases and carboxypeptidases (Ishibashi et al., 1988). Microorganisms are an important source of exopeptidases, which are expressed in the media to hydrolysate proteins as nutrient source. Microbial strains such as the genus *Lactobacillus* are extensively used as a debittering starter adjunct for the production of protein hydrolysates lacking bitter taste (Raksakulthai & Haard, 2003; Saha & Hayashi, 2001). Similar effects have been observed during hydrolysis with fungal protease preparations and microbial aminopeptidases hydrolyzing bitter peptides and liberating aromatic amino acids, which are important precursors of aroma compounds (Izawa, Tokuyasu, & Hayashi, 1997). Commonly, L-leucine aminopeptidases (LAP) are commonly used for debittering as they are efficient in removing single or pairs of hydrophobic amino acids. Some of the commercial aminopeptidases that are used to reduce bitterness in foods are also from lactic acid bacteria (LAB) and fungal enzyme preparations such as *Rhizopus oryzae*, *Aspergillus oryzae* and *Aspergillus sojae* (Nampoothiri, Nagy, Kovacs, Szakacs, & Pandey, 2005). But also the carboxypeptidases from *Actinomucor elegans* are well known to reduce bitterness (Fu, Li, & Yang, 2011; Li et al., 2008).

The objective of this study was to investigate the degradation of major allergens (glycinin and β -conglycinin) of soy protein isolates (SPI) using different microorganisms as well as their ability to diminish the bitter taste. SPI hydrolysates prepared with Alcalase, papain, or an enzyme combination (papain + Flavourzyme, two-step process) were inoculated with the LAB *Lactobacillus (L.) perolens* as well as the two mold strains *Actinomucor (A.) elegans* and *Rhizopus (R.) oryzae*. Their effect on major soybean allergens (glycinin, β -conglycinin) by means of degree of hydrolysis

and SDS-PAGE has been investigated. Sensory characteristics (smell, taste, and mouthfeeling) were analyzed using principal component analysis (PCA), along with quantitative descriptive analysis (QDA). In addition, the techno-functional properties (protein solubility, emulsifying, foaming, water- and oil-binding, gelation) were also regarded as the final protein preparation should be applied as food ingredient

2. Material and Methods

2.1. Raw materials and chemicals

Soybeans (*G. max* (L.) MERR.) were purchased from Naturkost Ernst Weber (Munich, Germany). Alcalase[®] 2.4 L FG (2.4 AU-A/g, endoprotease from *Bacillus licheniformis*) and Flavourzyme[®] 1000 L (1000 LAPU/g, endo- and exoprotease from *A. oryzae*) were kindly provided by Novozymes A/S (Bagsvaerd, Denmark). Papain cysteine-protease from papaya latex (≥ 10 units/mg, E.C. 3.4.22.2, Sigma no P4762) was purchased from Sigma-Aldrich Inc. (St. Louis, U.S.A). *Lactobacillus perolens* DSM 12744, *A. elegans* DSM 1174, and *R. oryzae* DSM 2200 were purchased from the Germany Resource Center (DSMZ, Braunschweig, Germany).

All chemicals used in this study were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany) if not stated separately.

2.2. Preparation of soy protein isolates (SPI)

SPI was prepared from soybean seeds using the technique as previously described by Meinschmidt, Schweiggert-Weisz, et al. (2016). Briefly, soybeans were de-hulled, flaked, and defatted with *n*-hexane. SPI was prepared by acidic pre-extraction (pH 4.5, 1:8 w/v flakes to water ratio, 1 h) of soybean flakes. After stirring for 1 h at room temperature, the suspension was separated using a decanter (3250 x *g*, 60 min). Subsequently, alkaline protein-extraction (pH 8.0, 1:8 w/v, 1 h) of flakes residue was performed and the suspension was separated (3250 x *g*, 60 min). The supernatant was adjusted to pH 4.5 for protein precipitation, followed by centrifugation (5 600 x *g*, 130 min). The obtained SPI was neutralized, pasteurized (70 °C, 10 min) and spray-dried.

2.3. Enzymatic hydrolysis of SPI

The enzymatic hydrolysis using Alcalase, papain, and the enzyme combination prepared with papain and Flavourzyme were performed as previously described Meinschmidt, Schweiggert-Weisz, et al. (2016) and Meinschmidt, Sussmann, et al. (2016). Briefly, for each hydrolysis, dispersions of SPI in sterile deionized water (5 g/100g) were prepared and transferred into thermostatically-controlled reactors.

The hydrolysis using Alcalase (E/S: 0.5 mL/100g protein) was conducted at 50 °C and pH 8.0 for 2 h, while the papain (E/S: 0.05 g/100g protein) hydrolysate was prepared at 80 °C and pH 7.0 for 30 min (Meinschmidt, Sussmann, et al., 2016). The enzyme combination was performed as two-step process, where SPI was pre-digested with papain (E/S: 0.05 g/100g protein) for 1 h at 50 °C and pH 8.0 and subsequently incubated with Flavourzyme (E/S: 1 mL/100g protein) for 30 min (Meinschmidt, Schweiggert-Weisz, et al., 2016). During hydrolysis, the mixture was stirred, while pH and temperature were kept constant. To terminate the hydrolysis, samples were boiled at 90 °C for 20 min. After cooling, the hydrolysates were kept at 4 °C prior to fermentation (Figure 1). Each hydrolysis was performed in duplicate.

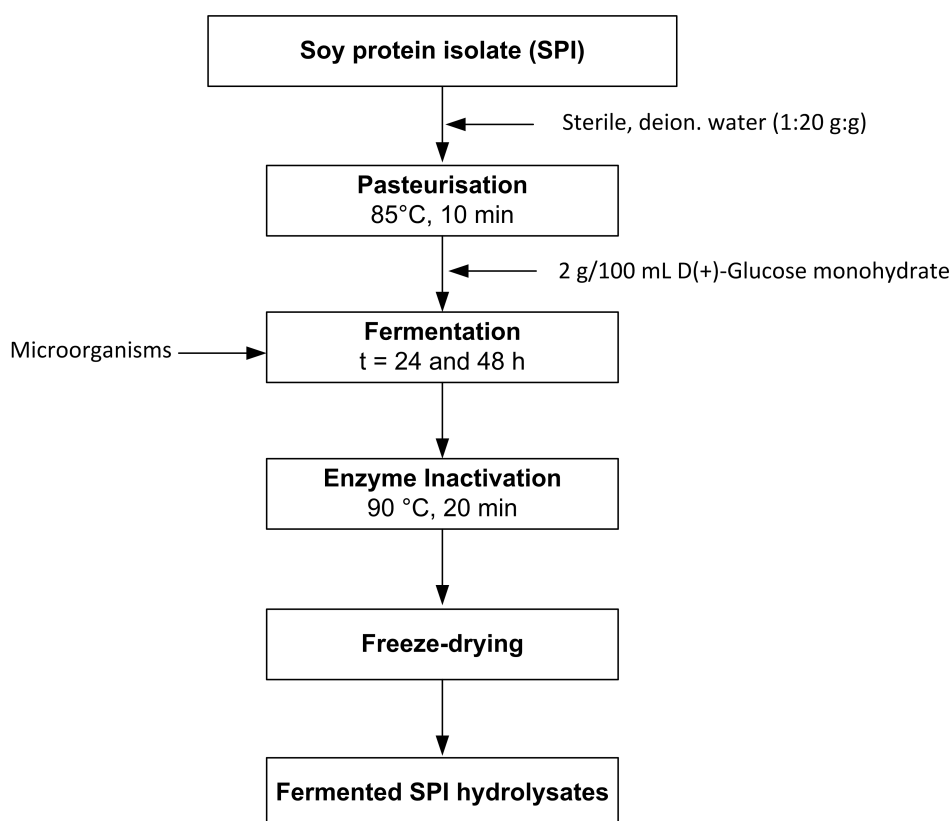


Fig. 1. Process scheme for the enzymatic hydrolysis and subsequent fermentation of SPI.

2.4. Fermentation of SPI hydrolysates

2.4.1. Strains, media, growth conditions, and preparation of inocula

L. perolens, *A. elegans*, and *R. oryzae* were cultivated overnight under their respective optimal growth temperature in deMan Rogosa Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA), yeast phosphate soluble starch (YPSS) medium (15 g L⁻¹ starch, 4.0 g L⁻¹ yeast extract, 1.0 g L⁻¹ K₂HPO₄, and 0.5 g L⁻¹ MgSO₄·7H₂O) and potato dextrose (PD) broth (Carlo-Roth GmbH, Karlsruhe, Germany), respectively (see Table 1).

For solid media, 15 g L⁻¹ agar was added to the respective broth. After cell enumeration, a calculated aliquot of the preculture was centrifuged for 10 min at 9000 x *g*, the pelleted cells were washed twice with Ringer solution (Merck KGaA, Darmstadt, Germany) and were resuspended in 1 mL sterile distilled water, which was used as inocula.

Table 1

List of employed strains including abbreviations, and growth/culture conditions.

Strain	No.	Abbreviation	Type	Growth/Culture conditions
<i>Actinomucor elegans</i>	DSM 1174	<i>A. elegans</i>	mold	24 °C, aerob, YPSS
<i>Lactobacillus perolens</i>	DSM 12744	<i>L. perolens</i>	Lactic acid bacteria	28 °C, aerob, MRS
<i>Rhizopus oryzae</i>	DSM 2200	<i>R. oryzae</i>	mold	30 °C, aerob, potato dextrose

DSM = Deutsche Sammlung von Mikroorganismen; MRS = DeMan, Rogosa and Sharpe;
PD = potato dextrose; YPSS = yeast phosphate soluble starch

2.4.2. Induced liquid-state fermentation of SPI

Prior to induced liquid-state fermentation, 2 g/100mL of glucose (Sigma-Aldrich Inc., St. Louis, USA) was dispensed into the hydrolysates to favour the growth of the inoculated strains. Three liquid-state fermentations for each SPI hydrolysate were performed, including induced fermentations with *L. perolens*, *A. elegans*, and *R. oryzae* (Figure 1). The hydrolysates were inoculated with a start inoculation concentration of either 1 x 10⁸ colony forming units (CFU) per milliliter of *L. perolens* or 10 mL/100mL of *A. elegans* or 20 mL/100mL of *R. oryzae* from precultures

(based on preliminary studies, data not shown).

The fermentation process was carried out aerobically at 30 °C for 6 h in conical flasks without agitation. Samples were collected after 6 h for pH value and microbial measurement and fermentation was terminated by heat treatment at 90 °C for 20 min. The pH value of the samples was measured initially and after fermentation using a disinfected pH electrode (WTW, Weilheim, Germany) calibrated at pH 7.0 and 4.0. After inactivation, the fermented samples were frozen at -50 °C and lyophilized utilizing a lyophilisator (BETA 1-8, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). All fermentations were performed in two independent replicates.

2.5. Microbiological analyses

2.5.1. Determination of the total viable counts by measuring the colony forming units (CFU)

At time intervals of 0 h (directly after inoculation) and 6 h, the fermentation process was monitored by determining the total viable counts by measuring the number of colony forming units. Aliquots (100 $\frac{1}{4}$ L) of fermented SPI hydrolysates were in triplicate serially diluted with Ringer solution and 100 μ L of certain dilutions was spread homogenously with a Drigalski spatula in duplicate on the surface of pre-dried plate count agar-plates (Merck KGaA, Darmstadt, Germany) for measuring the total viable counts. The hydrolysates, which were fermented with *L. perolens*, were also plated and counted on MRS agar to quantify and favour the growth of this microorganism. All dishes were incubated aerobically at 30 °C for 48 ± 2 h. The colonies were counted and expressed as colony forming units per milliliter (CFU mL^{-1}).

2.5.2. Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS was performed to verify that the inoculated strains were dominant in the samples during fermentation. Fresh colony material (a single colony) was smeared on a polished steel MSP 96 target (Bruker Daltonik), spotted with 1 μ L of a saturated α -cyano-4-hydroxy-cinnamic acid matrix solution in 50 mL/dL

acetonitrile-2.5 % trifluoroacetic acid (Bruker Daltonik) and air dried at room temperature. The analysis ($n = 3$) of the mass spectra was performed by a Microflex LT mass spectrometer (Bruker Daltonik) using the MALDI Biotyper software package (version 3.0). For external mass calibration, a bacterial standard (Bruker Daltonics) was used.

2.6. Determination of protein degradation due to enzymatic hydrolysis and fermentation

2.6.1. Degree of hydrolysis (DH) using the *o*-phthaldialdehyde (OPA) method

The DH of all samples was calculated in triplicate by the determination of free α -amino groups with *o*-phthaldialdehyde (OPA) using serine as a standard according to Nielsen, Petersen, and Dambmann (2001) and as described in Meinschmidt, Sussmann, et al. (2016).

2.6.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight distribution of the samples was determined according to Laemmli (1970) using SDS-PAGE under reducing conditions. Sample preparation and running conditions were chosen according to Meinschmidt, Sussmann, et al. (2016) using 4-20 % midi CriterionTM TGX Stain-FreeTM precast gels. Protein visualization was performed by Criterion Stain-Free Gel DocTM EZ Imager (Bio-Rad).

2.7. Determination of the chemical composition and techno-functional properties

2.7.1. Chemical composition

The protein and dry matter content was determined as described by AOAC methods using a protein calculation factor of $N \times 6.25$ (AOAC, 2005a; AOAC, 2005b).

2.7.2. Techno-functional properties

2.7.2.1. *Protein solubility.* Protein solubility of the samples was determined at pH 4.0 and 7.0 following the method of Morr et al. (1985). Briefly, 1 g of the sample was suspended in 50 mL 0.1 mol L⁻¹ sodium chloride solution, which was adjusted to pH 7.0 and 4.0 using 0.1 mol/L NaOH or 0.1 mol/L HCl, respectively. Non-dissolved fractions of the samples were separated by centrifugation at 20,000 x g for 15 min and the protein content of the supernatant was determined according to AOAC (2005b).

2.7.2.2. *Emulsifying capacity.* The emulsifying capacity was determined in duplicate as suggested by Wang and Johnson (2001). 2.7.2.3. *Water and oil binding capacity.* Water binding capacity was analyzed according to the AACC 56-20 official method (AACC 2000). Oil binding capacity was determined using the method described by Ludwig, Ludwig, and Pingel (1989). Briefly, protein solutions of 1 g/100g were prepared using an UltraturraxTM (IKA-Werke GmbH & Co. KG, Staufen, Germany). Rapeseed oil was added at a constant rate of 10 mL min⁻¹ until phase inversion of the emulsion was observed, accomplished by continuous determination of the emulsion's conductivity (conductivity meter LF 521 with electrode KLE 1/T, Wissenschaftlich-technische Werkstätten GmbH, Weilheim, Germany). The volume of oil needed for phase inversion was used to calculate the EC (mL oil per g sample).

2.7.2.4. *Foaming properties.* Foaming activity was determined according to Phillips, Haque, and Kinsella (1987) using a whipping machine (Hobart 50-N, Hobart GmbH, Offenburg, Germany). Briefly, a 5 g/100g protein solution was whipped for 8 min. The relation of the foam volume before and after whipping of the sample was utilized for the calculation of the foaming activity. The foaming density and stability were analyzed as described elsewhere (Meinlschmidt, Sussmann, et al., 2016). In brief, the foaming density was measured by weighing a specified quantity of foam volume, where the ratio of foam volume to foam weight was defined as foaming density in g L⁻¹. The foaming stability was estimated as the percent loss of foam volume after 60 min.

2.7.2.5. *Gelation*. The least gelation concentration of all samples was determined according to Sathe, Deshpande, and Salunkhe (1982). Briefly, protein samples were dispersed in 5 mL distilled water in concentrations between 2 and 20 g/100mL. The test tubes containing the dispersions were boiled for 1 h followed by cooling in an ice bath (4 °C) for 2 h. The least gelation concentration was determined by inversion of the test tubes.

2.8. Sensory evaluation

2.8.1. Quantitative descriptive analysis (QDA)

QDA is based on the ability of panellists to reproducibly measure specific attributes of a sample to yield a comprehensive quantitative product description amenable to statistical analysis (Stone & Sidel, 1985). A ten-member sensory panel (7 female, 3 male) was selected and trained over two months (1 h per session, twice a week) according to the principles of QDA (Stone & Sidel, 1985). The panel generated a broad list of attributes characteristic for the samples in terms of smell, taste, and mouthfeeling during several sessions (1 month, 2-times per week). The attributes and definitions are listed in Table 2.

Table 2

Sensory attributes and definitions used in the profile analysis of SPI, SPI hydrolysates, and the fermented SPI hydrolysates.

Attribute group	Attribute	Description
Smell	<i>buttery</i>	Smell associated with diacetyl and cream cheese
	<i>beany</i>	Smell of raw soybeans/legumes; dusty/musty
Taste	<i>buttery</i>	Taste associated with diacetyl and cream cheese
	<i>bitter</i>	Fundamental taste sensation elicited by caffeine, quinine
	<i>beany</i>	Legume- or soybean-like, green; dusty/musty; aroma, flavour and aftertaste associated with raw soybeans
Mouthfeeling	<i>astringent</i>	Degree of coating inside the mouth after swallowing
	<i>mouthcoating</i>	Trigeminal sensation and aftertaste elicited by pure cranberry juice

A specific emphasis has been given to the evaluation of the bitter taste and the bitterness was quantified as previously described (Meinlschmidt, Sussmann, et al., 2016). The sensory panel had been screened and trained with caffeine solutions according to DIN 10959 to evaluate bitterness Alcalase hydrolysates (E/S 0.5 mL/100g, production conditions: pH 8.0, 60 °C, 180 min without pH adjustment) with concentrations of 1.0 and 2.5 g L⁻¹ were estimated by the panel to correspond to bitter intensities of 5 and 10, respectively. Each panellist did a monadic evaluation of the samples at individual speed. Sensory evaluation was at least performed in duplicate.

2.8.2. Sample preparation and sensory evaluation

For sample evaluation (performed in eight sessions, 1 h each), samples were mixed with tap water to a protein equivalent of 2.5 g/100mL and neutralized (pH 7.0) with 1 mol/L NaOH. The samples (10 mL) were randomly presented to the panel at room temperature in plastic cups, which were coded by arbitrary numbers (three-digits). To every session, the reference solutions containing 1.0 g/100mL and 2.5 g/100mL Alcalase hydrolysate were given to the panellists. Water and plain crackers were provided for palate cleansing in between. The panel was instructed to evaluate the attributes (Table 2) of four samples at each session on a 10-cm continuous scale, ranging from 0 (= no perception) to 10 (= strong perception).

2.8.3. Multivariate statistical data analysis - Principal component analysis (PCA)

PCA is a widely used multivariate analytical statistical technique that can be applied to QDA data to reduce the set of dependent variables to a smaller set of underlying variables based on patterns of correlation among the original variables. PCA attempts to identify underlying variables or factors that explain the pattern of correlations within a set of observed variables. It is a powerful method to identify factors that do have impact on the sensory profile. Therefore, the results of QDA were processed by means of PCA using the R-based PanelCheck software (Nofima, Ås, Norway; version 1.4.0.).

2.9. Statistical analysis

Statistical analysis of chemical data was performed using SPSS 20.0 (SPSS v 20.0 SPSS Inc. Chicago, IL, USA). All data were analyzed using one-way analysis of variances (ANOVA) and means were generated and adjusted with the Bonferroni post hoc test. Descriptive sensory data were also subjected to one-way ANOVA with the use of Tukey's honestly significant difference (HSD) post hoc test. Probability $p < 0.05$ indicated statistically significant differences.

3. Results and Discussion

3.1. Microbial growth during fermentation

In a preliminary study (data not shown), 25 different microorganisms from lactobacilli, molds and yeasts were screened in different inoculation concentrations. Beyond the LAB group, *L. perolens* showed the most promising results for bitterness reduction of SPI. This microorganism was therefore included in this study, although it is known to produce off-flavours in soft drinks (Back et al., 1999) and is therefore commonly not tolerated in foods. The yeast *Saccharomyces cerevisiae* was not included in this study as the sensory characteristics of the SPI hydrolysate after fermentation with the yeast was not acceptable. Beyond the mold group, *A. elegans* and *R. oryzae* - their debittering potential was already described in Li et al. (2008) and Chien et al. (2002) showed also the most promising results in the preliminary study.

The microbial growth was monitored by the pH changes during fermentation, the total viable counts as well as MALDI-TOF-MS analysis. Untreated SPI, containing a dry matter content of 94.4 g/100g, a protein content of 94.6 g/100g, and an ash content of 4.6 g/100g, had an initial pH value of 6.7. As a representative of LAB, *L. perolens* convert glucose into mainly L(+)-lactic acid. Therefore, the pH of all SPI hydrolysates decreased within the first 6 h from 8.0 (hydrolysate from Alcalase and the enzyme combination) and 7.0 (hydrolysate from papain) to 7.5 and 6.1, respectively. This acidification - characteristic for LAB - led to an inhibition of the growth of other competing or pathogen microorganisms (Song et al. 2008). This ability of *L. perolens* to dominate fermentation could be monitored by the determination of the total viable counts and MALDI-TOF-MS analysis of fermented

samples. A slight growth of *L. perolens* was determined with 1.5×10^8 CFU mL⁻¹. MALDI-TOF-MS analyses reported a reliable identification of the inoculated strain with log(score) values of ≥ 2.3 (highly probable species identification) and confirmed the absence of other competitive strains and the dominance of *L. perolens* in the fermented samples.

Similar results have been obtained for *A. elegans* and *R. oryzae*. The pH value decreased from 8.0 (Alcalase and the enzyme combination) and 7.0 (hydrolysate from papain) to approximately 7.6 and 6.9, respectively. MALDI-TOF-MS analysis could not be conducted as the characteristic wooly texture of the mycelia made a MALDI-TOF-MS analysis impossible. Nevertheless, the visual colonies on the surface of strain-specific plates confirmed the ability of these strains to dominate the fermentation, which was also observed by Song et al. (2008).

3.2. Degradation of soy proteins due to enzymatic hydrolysis and subsequent fermentation

3.2.1. Degree of hydrolysis (DH)

The DH value gives a first indication for the reduction of allergenic compounds as the enzymatic digestion of proteins results in a breakdown of the intact protein structure into smaller peptides and amino acids due to the cleavage of peptide bonds (Nagodawithana, Nelles, & Trivedi, 2010; Sujith & Hymavathi, 2010). Untreated SPI had an average DH value of 2.1 %, whereas the DH value significantly ($p < 0.05$) increased in the course of enzymatic hydrolysis. The highest DH value of 12.2 % was achieved after hydrolysis with Alcalase followed by DH values of 9.7 % and 4.0 % by applying the enzyme combination and papain, respectively. These findings are in great accordance with previous results (Meinlschmidt, Schweiggert-Weisz, et al., 2016; Meinlschmidt, Sussmann, et al., 2016). However, fermentation did not lead to a change in the DH value (data not shown), which means that the proteins were not further hydrolyzed by microbial strains.

3.2.2. Electrophoretic analysis (SDS-PAGE)

Figure 2 displays the densitograms corresponding to the bands obtained by SDS-PAGE analysis of the standard marker, untreated SPI and the SPI hydrolysates. In addition, individual bands glycinin and β -conglycinin units were quantified by Image Lab[®] Software and their relative hydrolyzation in relation to untreated SPI fractions was calculated.

Untreated SPI (Figure 2 B) presented typical electrophoretic patterns for native soy proteins, showing high intensity protein bands corresponding to β -conglycinin subunits α' (\sim 72 kDa), α (\sim 68 kDa), and β (\sim 53 kDa), followed by the two subunits of glycinin, the acidic subunit ("A") at approximately 29 - 33 kDa and the basic subunit ("B") at around 18 - 22 kDa (Amnuaycheewa & de Mejia 2010). These bands have previously been identified and characterized using LC-MS/MS analysis (Meinlschmidt, Schweiggert-Weisz, et al., 2016).

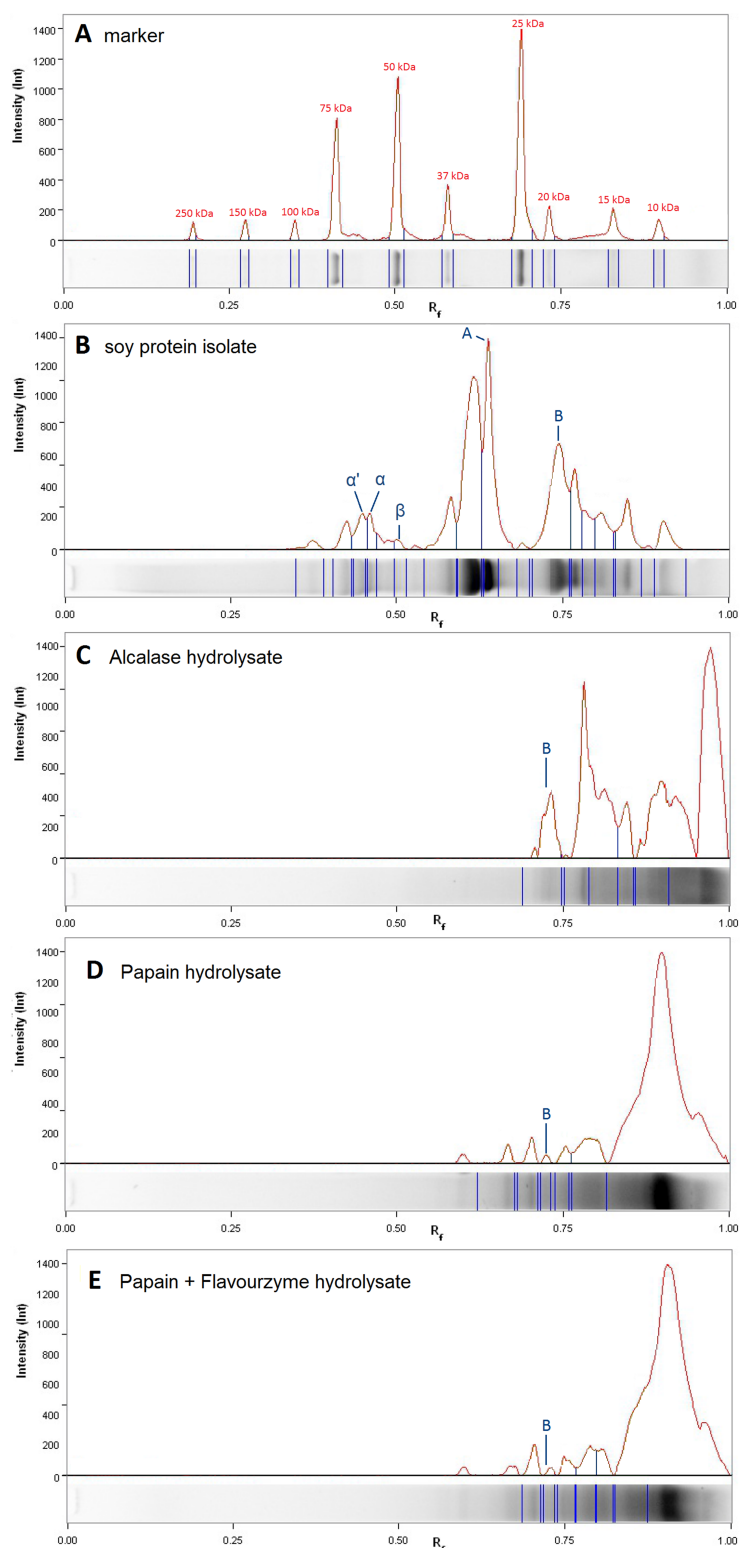


Fig. 2. Electropherograms and the corresponding electrophoretic mobility maps obtained by SDS-PAGE of (A) molecular weight standard (marker), (B) SPI, (C) Alcalase hydrolysate, (D) papain hydrolysate, and (E) the enzyme combination (papain and Flavourzyme, two-step process). R_f is the relative front/mobility; α' , α , β are the subunits of β -conglycinin; "A" and "B" are the acidic and basic subunit of glycinin.

Figure 2 C-E showed that the β -conglycinin and the acidic subunit of glycinin completely disappeared after enzymatic hydrolysis applying Alcalase, papain, or the enzyme combination. However, the basic subunit of glycinin was more resistant to enzymatic hydrolysis as this subunit was not completely hydrolyzed. The subunit "B" was reduced by 97.7 %, 97.6 %, and 90.8 % after enzymatic treatment with papain, the enzyme combination, and Alcalase, respectively. In addition, most of the peptides were < 20 kDa (Figure 2 C-E), considering the resulting hydrolysates as less allergenic or even hypoallergenic (Nagodawithana et al., 2010).

However, electrophoretic profiles of the fermented hydrolysates did not show visually changes (data not shown).

3.3. Effects of enzymatic hydrolysis and subsequent fermentation on the sensory perception of SPI

3.3.1. Bitterness intensities

Figure 3 shows the bitterness scores of all samples. Untreated SPI had a bitter intensity of 2.8 and the hydrolysates generated with papain and the enzyme combination resulted in comparable bitterness scores of 3.0 and 2.8, respectively. The bitterness of the Alcalase hydrolysate showed a significantly ($p < 0.05$) higher value of 8.0. Subsequent fermentation with all strains led to significantly ($p < 0.05$) reduced bitterness scores. After fermentation, the bitterness values of the papain hydrolysate (3.0) and the hydrolysates prepared with the enzyme combination (2.8) were 4-times lower (0.7 - 1.3) compared to non-fermented samples. The Alcalase hydrolysate with an initial bitterness value of 8.0 was nearly halved by fermentation with the strains to scores of 3.9, 5.1, and 5.4, respectively. Alcalase is a serine endoprotease and has the tendency to hydrolyze at hydrophobic amino acid residues, thereby, nonpolar amino acid residues at the C-terminus of the resulting peptides remain and cause a relatively high bitterness (Ishibashi et al., 1988; Saha & Hayashi, 2001; Sun, 2011). Fu et al., (2011) and Li et al. (2008) reported that the bitterness of soybean hydrolysates produced with Alcalase decreased when the hydrolysate was treated concomitant or subsequent with an *A. elegans* extract. They concluded that the exopeptidases, particular the dominant carboxypeptidases, of *A. elegans* were responsible for the debittering effect by selectively releasing hydrophobic amino acids from the C-terminus of proteins or peptides. The exoproteolytic LAP of *R. oryzae*

is able to mainly remove leucine from the N-terminus (Rahulan, Dhar, Nampoothiri, & Pandey, 2011). However, the LAP activity of *R. oryzae* required a significantly higher dose rate (20 mL/100mL) to generate a similar reduction in bitterness than the *A. elegans* carboxypeptidases (10 mL/100mL). This might be explained by the fact that bitterness increases with the number of hydrophobic amino acids such as proline, leucine, tyrosine, and the carboxypeptidases of *A. elegans* is able to hydrolyze most of these residues, whereas the LAP of *R. oryzae* mainly cleaves L-leucine residues from the N-terminus. Fermentation with *L. perolens* was equally effective in debittering SPI hydrolysates than the two mold strains.

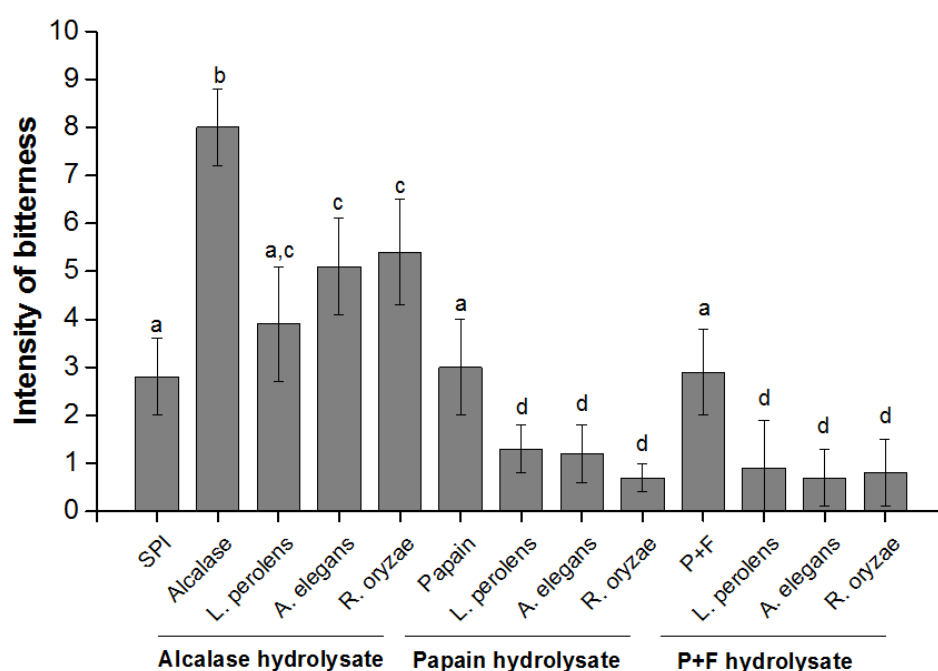


Fig. 3. Bitterness intensities of SPI, SPI hydrolysates, and fermented SPI hydrolysates. Each value is expressed as means \pm standard deviation scored on the 10-cm continuous scale (0 = no perception; 10 = strong perception) by ten panelists. Means followed with different superscript alphabet are significantly different ($p < 0.05$) following one-way ANOVA (Tukey's HSD).

3.3.2. Descriptive sensory analysis (QDA): sensory profile

The effect of fermentation on the sensory profile (sensory attributes, see Table 2) using QDA by rating on the 10-cm continuous scale (0 = no perception; 10 = strong perception) has been determined and the results are shown in Table 3.

Table 3

Sensory evaluation (QDA) results of each sensory attribute (smell, taste, mouthfeeling) for SPI, SPI hydrolysates, and the fermented SPI hydrolysates.

	Smell		Taste			Mouthfeeling	
	<i>buttery</i>	<i>beany</i>	<i>buttery</i>	<i>bitter</i>	<i>beany</i>	<i>astringent</i>	<i>mouthcoating</i>
SPI	2.9 ± 0.8 ^a	4.6 ± 1.1 ^a	1.3 ± 1.2 ^a	2.8 ± 0.8 ^a	6.4 ± 0.6 ^a	4.6 ± 1.3 ^a	3.3 ± 1.8 ^a
Alcalase hydrolysate	3.3 ± 0.9 ^a	4.5 ± 0.9 ^{a,b}	0.7 ± 0.9 ^a	8.0 ± 0.8 ^b	2.7 ± 1.4 ^b	3.3 ± 1.5 ^{a,b}	5.1 ± 1.0 ^a
<i>L. perolens</i>	6.1 ± 1.2 ^b	1.3 ± 1.1 ^c	5.3 ± 1.1 ^b	3.9 ± 1.2 ^{a,c}	1.6 ± 1.0 ^c	2.4 ± 1.2 ^b	3.4 ± 1.9 ^a
<i>A. elegans</i>	1.2 ± 0.8 ^c	3.1 ± 1.2 ^{a,c}	0.8 ± 0.8 ^a	5.1 ± 1.0 ^c	3.3 ± 1.4 ^{b,c}	2.3 ± 1.6 ^b	2.4 ± 1.0 ^{a,b}
<i>R. oryzae</i>	2.3 ± 1.6 ^{a,c}	3.3 ± 1.3 ^{a,c}	2.0 ± 1.3 ^a	5.4 ± 1.1 ^c	3.3 ± 0.9 ^{b,c}	2.4 ± 1.2 ^b	2.4 ± 1.1 ^{a,b}
Papain hydrolysate	3.8 ± 1.3 ^a	3.6 ± 1.9 ^a	0.8 ± 1.0 ^a	3.0 ± 1.0 ^a	3.8 ± 1.8 ^{b,c}	4.4 ± 1.7 ^a	3.3 ± 1.0 ^a
<i>L. perolens</i>	6.4 ± 1.4 ^b	1.1 ± 1.2 ^c	6.2 ± 1.5 ^b	1.3 ± 0.5 ^d	1.5 ± 0.9 ^{b,c}	3.1 ± 1.0 ^a	1.6 ± 1.4 ^{a,b}
<i>A. elegans</i>	2.2 ± 1.0 ^{a,c}	3.6 ± 1.8 ^a	1.1 ± 0.7 ^a	1.2 ± 0.6 ^d	3.2 ± 1.4 ^{b,c}	2.7 ± 1.2 ^b	2.7 ± 1.2 ^{a,b}
<i>R. oryzae</i>	4.2 ± 1.4 ^a	2.9 ± 1.4 ^{a,c}	1.8 ± 1.2 ^a	0.7 ± 0.3 ^d	2.9 ± 1.6 ^{b,c}	2.7 ± 1.5 ^b	2.2 ± 1.4 ^{a,b}
P + F hydrolysate	3.1 ± 1.5 ^a	2.5 ± 1.6 ^{a,c}	2.3 ± 1.4 ^a	2.9 ± 0.9 ^a	2.9 ± 1.2 ^{b,c}	3.3 ± 1.3 ^{a,b}	4.1 ± 1.1 ^a
<i>L. perolens</i>	5.2 ± 1.1 ^b	1.8 ± 1.3 ^c	5.3 ± 1.2 ^b	0.9 ± 1.0 ^d	2.1 ± 1.1 ^{b,c}	2.1 ± 1.1 ^b	1.6 ± 1.1 ^{a,b}
<i>A. elegans</i>	3.1 ± 1.5 ^a	3.3 ± 1.5 ^{a,c}	2.4 ± 1.5 ^a	0.7 ± 0.6 ^d	2.4 ± 1.1 ^{b,c}	1.9 ± 1.3 ^b	1.2 ± 1.0 ^b
<i>R. oryzae</i>	3.7 ± 1.5 ^a	2.4 ± 1.1 ^b	2.5 ± 1.5 ^a	0.8 ± 0.7 ^d	2.8 ± 1.4 ^{b,c}	2.6 ± 1.2 ^b	1.7 ± 1.5 ^{a,b}

Results are expressed as means ± standard deviation. Means followed with different superscript letters within each column are significantly different ($p < 0.05$, 10 panelists) following one-way ANOVA (Tukey's HSD). For the sensory evaluation the 10-cm continuous scale was used (0 = no perception; 10 = strong perception).

Evaluation of untreated SPI resulted in the following smell-scaling: *buttery* (2.9) and *beany* (4.6); taste-scaling: *buttery* (1.3) and *beany* (6.4); mouthfeeling-scaling: *mouthcoating* (4.6) and *astringent* (3.3). Soy proteins are often not recognized due to their unpleasant beany off-flavour, which is due to lipid oxidation, polar lipids, and lipoxygenase catalyzed oxidation of soybean oil to volatile compounds such as isopentanol, *n*-hexanal and hexanol (Shogren, Mohamed, & Carriere, 2003). After enzymatic hydrolysis and fermentation, the beany taste was significantly ($p < 0.05$) reduced from initially 6.4 to a minimum of 1.5 (Table 3). This was also observed by Pinthong, Macrae, and Rothwell (1980), who found that fermented soy milk contained less *n*-hexanal, which was converted into *n*-hexanoic acid, reducing the beany off-flavour. After fermentation with *L. perolens*, the fresh buttery smell and taste of all hydrolysates were significantly ($p < 0.05$) higher compared to untreated SPI and SPI hydrolysates, increasing from initially 2.9 and 1.3 to 6.4 and 6.2, respectively, as observed for the papain hydrolysate. *L. perolens* belongs to the heterofermentative LAB family, converting the available sugars into lactic acid, acetic acid, ethanol and CO₂ via pyruvate to produce energy (Leroy & De Vuyst, 2004). Pyruvate can be converted to diacetyl, which is well known to produce a buttery flavour, might also be responsible for a lower bitterness, acting as masking agent.

3.3.3. Multivariate statistical analysis (PCA)

PCA was applied on QDA data to analyze the interrelationships among the samples with respect to the sensory attributes. The resulting bi-plots of the uncorrelated principal components (PCs) 1 and 2 of each hydrolysate, including untreated SPI and fermented hydrolysates, and the scaled loadings are represented in Figure 4 A-C and Table 4.

PCA on sensory data of SPI, the Alcalase hydrolysate and the fermented Alcalase hydrolysates (Figure 4 A) showed that PC1 and PC2 explained 45.1 % and 36.1 %, respectively, of the observed variation (81.2 % in total). The major components contributing to the PC1 dimension (Table 4, factor loadings > 0.550) were *buttery* (-0.795/-1.000) as well as *beany* (0.796/0.764) smell and taste, representing a strong contrast between these two attributes. In contrast, PC2 is mainly described by the attributes *bitter* (-1.000) and *beany* (0.625) taste. The bitter taste is loaded on the Alcalase hydrolysate, whereas the hydrolysate fermented with *L. perolens* was well characterized by the attribute *buttery*. Untreated SPI and the hydrolysates fermented with the two mold strains were correlated with the attributes *beany*, *mouthcoating*, and *astringent*. According to the scaled loadings in Table 4, the attributes *beany* smell and taste highly loaded on PC1, dominating SPI and hydrolysates fermented with *A. elegans* and *R. oryzae*.

Figure 4 B presented the PCA results of untreated SPI, the papain hydrolysate and the fermented samples. PC1 and PC2 accounted for 93.9 % of the total data variance (PC1 = 78.1 %; PC2 = 15.8 %). The *beany* smell and taste (scaled loadings: 0.611/0.742) positively correlate with the PC1 dimension, where untreated SPI and the papain hydrolysate are loaded. PC2 was negatively correlated to all sensory attributes; consequently, the samples fermented with the two mold strains *A. elegans* and *R. oryzae*, which are located in proximity with high intensity in the positive side of PC2, were nearly "neutral" tasting. Here, again, samples following fermentation with *L. perolens* are highly explained by *buttery* smell and taste (-0.678/-1.000).

The PCA results of untreated SPI and the samples prepared with the enzyme combination (papain + Flavourzyme, two-step), including the fermented hydrolysates, are visualized in Figure 4 C and Table 4, where PC1 (16.0 %) and PC2 (77.2 %) explained 93.2 % of the total variance. PC1, where untreated SPI is located, is highly correlated to the *beany* smell and taste (0.572/1.000) and *mouthcoating* mouthfeeling (0.615), but negatively linked to *buttery* taste (-0.773). The hydrolysate is located on the PC2 dimension, which is not described by the attribute *bitter* (-1.000). Untreated SPI and the hydrolysate prepared with the enzyme combination

are described by the terms beany, bitter, mouthcoating and astringent, whereas fermentation led to a loss of these sensory attributes often negative associated with native soybeans. Fermentation with *L. perolens*, again, led to the formation of a *buttery* taste (-0.773), whereas samples fermented with the two mold strains were less explained by all sensory attributes, suggesting nearly "neutral" tasting products.

Table 4

Principal Component (PC) scaled loadings for SPI, SPI hydrolysates, and the fermented SPI hydrolysates obtained by Principal Component Analysis (PCA).

Scaled Loadings	Attributes	PC1	PC2
Alcalase hydrolysate	<i>Buttery</i> *	-0.795	0.077
	<i>Beany</i> *	0.796	-0.103
	<i>Buttery</i> **	-1.000	0.235
	<i>Bitter</i> **	0.195	-1.000
	<i>Beany</i> **	0.764	0.625
	<i>Astringent</i> ***	0.429	0.286
	<i>Mouthcoating</i> ***	0.063	-0.408
Papain hydrolysate	<i>Buttery</i> *	-0.678	-0.759
	<i>Beany</i> *	0.611	-0.073
	<i>Buttery</i> **	-1.000	-0.853
	<i>Bitter</i> **	0.273	-0.977
	<i>Beany</i> **	0.742	-1.000
	<i>Astringent</i> ***	0.250	-0.981
	<i>Mouthcoating</i> ***	0.338	-0.205
P + F hydrolysate	<i>Buttery</i> *	-0.453	-0.527
	<i>Beany</i> *	0.572	0.361
	<i>Buttery</i> **	-0.773	-1.000
	<i>Bitter</i> **	0.538	-0.554
	<i>Beany</i> **	1.000	-0.303
	<i>Astringent</i> ***	0.615	-0.435
	<i>Mouthcoating</i> ***	0.508	-0.688

Loadings with an absolute letter greater 0.550 are shown in bold type. * smell; ** taste; *** mouthfeeling

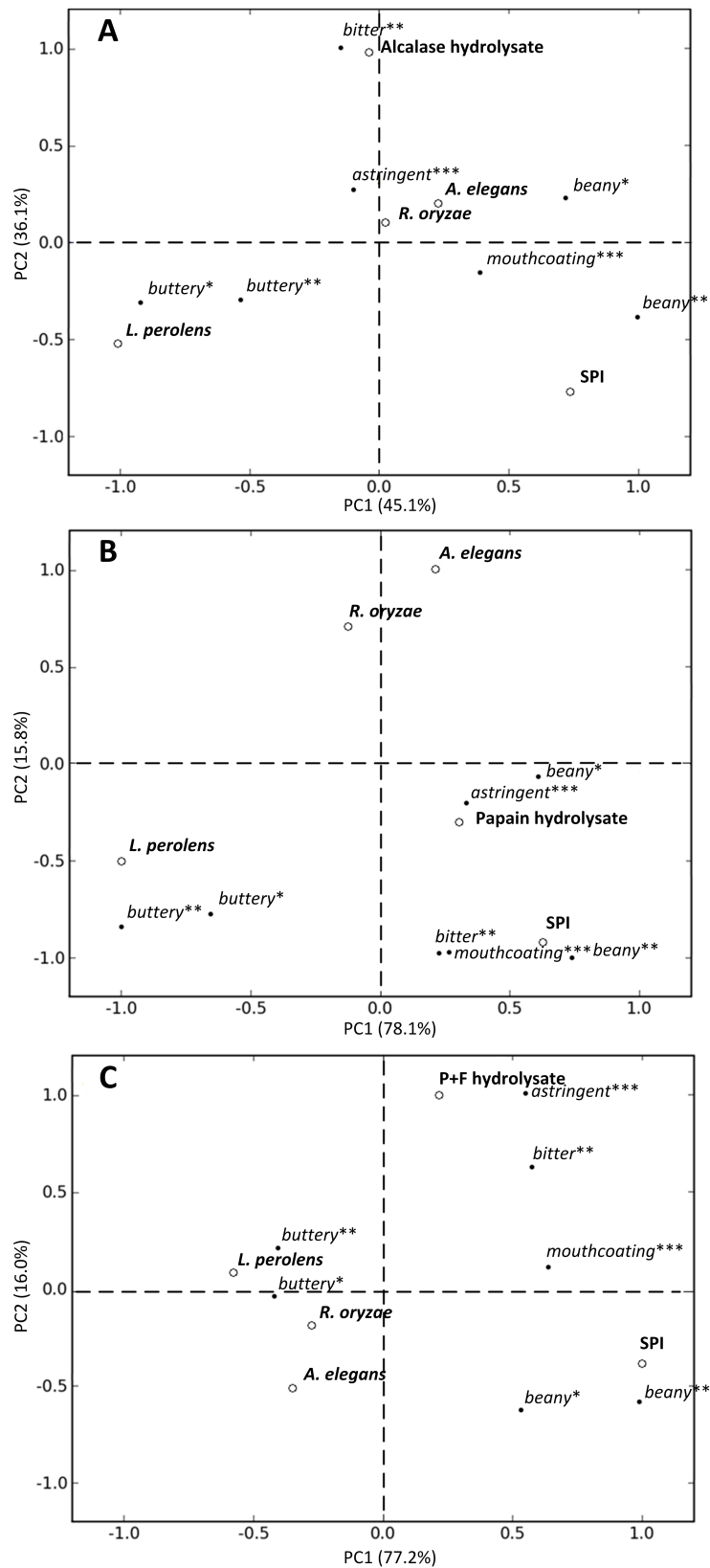


Fig. 4. Bi-plots obtained from PCA of sensory attributes (smell, taste, and mouthfeeling) for SPI, SPI hydrolyzed with Alcalase (A), papain (B), and the enzyme combination (B), and the fermented SPI hydrolyzates. * smell; ** taste; ***mouthfeeling.

3.4. Effects of enzymatic hydrolysis and subsequent fermentation of SPI on the techno-functional properties

Functional properties of proteins connote the physicochemical characteristics, which govern the behaviour of proteins in the food matrix. Enzymatic hydrolysis is a powerful tool in the modification of technofunctionality (Ortiz & Wagner, 2002).

Untreated SPI exhibited a protein solubility at pH 4.0 and 7.0 of 5.0 % and 44.0 %, respectively. Enzymatic hydrolysis significantly ($p < 0.05$) increased the protein solubility at both pH 4.0 and 7.0 to a maximum of 77.4 % and 82.9 % (Alcalase), respectively, as previously described in Meinschmidt, Schweiggert-Weisz, et al. (2016) and Meinschmidt, Sussmann, et al. (2016). Irrespective of the applied strain, subsequent fermentation did not lead to further changes. The same trend can be found for the EC (Table 5), WBC and OBC, where subsequent fermentation did not lead to further changes compared to SPI hydrolysates (Meinschmidt, Schweiggert-Weisz, et al., 2016; Meinschmidt, Sussmann, et al., 2016).

Enzymatic hydrolysis applying Alcalase, papain, or the enzyme combination greatly enhanced the foaming activity from initially 552 % up to 2766 %, 2583 %, and 2173 %, respectively, whereas fermentation did not further changed the foaming activity (Table 5). However, fermentation of the hydrolysate prepared with the enzyme combination resulted in a significantly ($p < 0.05$) reduced foaming stability from initially 90 %-14 % (*A. elegans*) and 4 % (*R. oryzae*), whereas fermentation with *L. perolens* increased the stability to 94 %. As might be expected, the foaming density of all hydrolyzed and fermented samples significantly ($p < 0.05$) decreased from initially 215 g L⁻¹ to a minimum of 40 g L⁻¹. The least gelation concentration was determined as well. Untreated SPI was able to form a gel at a protein concentration of 10 g/100mL. Hydrolysis of SPI with Alcalase yielded in a deteriorated gelation concentrations of 12 g/100mL, which was further negatively affected by fermentation (gelation concentration 16 - 18 g/100mL). In contrast, enzymatic treatment using papain or the enzyme combination increased the gelation concentration to 8 g/100mL, while further fermentation - except fermentation with *L. perolens* - did not further change the gelation concentration. The improved gelation concentration of 4 g/100mL following fermentation with *L. perolens* of the hydrolysate prepared with the enzyme combination might be due to synthesized exopolysaccharides (EPS). These EPS can form an adherent cohesive layer and interact with proteins to form a gel network, which might also be the reason for increased foaming stability (94 %).

Table 5

Techno-functional properties, including emulsifying capacity and foaming properties (activity, stability, and density), of SPI, SPI hydrolysates, and fermented SPI hydrolysates.

Sample	Emulsifying capacity (mL g ⁻¹)	Water-binding capacity (mL g ⁻¹)	Oil-binding capacity (mL g ⁻¹)	Foaming activity (%)	Foaming stability (%)	Foaming density (g L ⁻¹)
SPI	660 ± 5 ^a	2.6 ± 0.1 ^a	0.0 ± 0.0 ^a	552 ± 5 ^a	90 ± 0 ^a	215 ± 5 ^a
Alcalase hydrolysate	438 ± 8 ^b	0.2 ± 0.2 ^b	2.1 ± 0.1 ^b	2766 ± 0 ^b	0 ± 0 ^b	32 ± 0 ^{b,c}
<i>L. perolens</i>	508 ± 18 ^b	0.2 ± 0.1 ^b	1.7 ± 0.1 ^c	2442 ± 16 ^{c,d}	0 ± 0 ^b	37 ± 1 ^{b,c}
<i>A. elegans</i>	512.5 ± 4 ^b	0.3 ± 0.1 ^b	2.3 ± 0.2 ^b	2316 ± 16 ^{d,e,f}	4 ± 0 ^{b,c}	37 ± 4 ^{b,c}
<i>R. oryzae</i>	513 ± 4 ^b	0.0 ± 0.0 ^c	2.3 ± 0.0 ^b	2363 ± 32 ^e	2 ± 0 ^b	39 ± 2 ^{b,c}
Papain hydrolysate	705 ± 0 ^{a,c}	3.9 ± 0.2 ^d	3.3 ± 0.0 ^d	2583 ± 0 ^c	78 ± 0 ^{d,e,f}	37 ± 0 ^b
<i>L. perolens</i>	795 ± 10 ^{d,e}	3.8 ± 0.1 ^d	2.1 ± 0.0 ^b	2551 ± 63 ^c	70 ± 6 ^{d,e,f}	36 ± 1 ^{b,c}
<i>A. elegans</i>	795 ± 0 ^{d,e}	0.0 ± 0.0 ^c	2.1 ± 0.0 ^b	2268 ± 0 ^{d,e,f}	78 ± 2 ^{d,e,f}	41 ± 2 ^{b,c}
<i>R. oryzae</i>	798 ± 3 ^{d,e}	0.0 ± 0.0 ^c	2.1 ± 0.0 ^b	2300 ± 32 ^{d,e,f}	70 ± 2 ^{e,f}	45 ± 2 ^{b,c}
P + F hydrolysate	810 ± 5 ^{d,e}	0.0 ± 0.0 ^c	1.8 ± 0.1 ^c	2173 ± 0 ^f	88 ± 0 ^{a,d}	41 ± 0 ^{b,c}
<i>L. perolens</i>	818 ± 8 ^{d,e}	0.0 ± 0.0 ^c	2.2 ± 0.1 ^b	1171 ± 40 ^g	94 ± 0 ^a	88 ± 2 ^d
<i>A. elegans</i>	848 ± 53 ^d	0.0 ± 0.0 ^c	2.0 ± 0.1 ^b	1924 ± 0 ^h	14 ± 0 ^c	41 ± 3 ^{b,c}
<i>R. oryzae</i>	763 ± 4 ^{c,e}	0.0 ± 0.0 ^c	2.3 ± 0.0 ^b	1970 ± 16 ^h	4 ± 0 ^{b,c}	47 ± 1 ^c

Results are expressed as means ± standard deviation. Means followed with different superscript letters within each column are significantly different ($p < 0.05$) following one-way ANOVA (Bonferroni).

4. Conclusions

The debittering of different soy hydrolysates could be achieved to a large extent by fermentation using *L. perolens*, *A. elegans* and *R. oryzae*. However, a complete debittering of the hydrolysates was not reached, because neither carboxy- nor aminopeptidases are able to hydrolyze proline residues, which are responsible for a strong bitter taste. In order to provide non-bitter protein base as food ingredient, other microorganisms containing prolyl dipeptidyl aminopeptidase activities such as *Lactococcus lactis* might provide a powerful method to completely debitter protein hydrolysates as they can hydrolyze dipeptides with the sequence X-proline, releasing proline residues (Zevaco, Monnet, & Gripon, 1990). In addition, elucidation of the mechanisms for bitter taste perception of peptides would aid in evaluating the sensory quality of the fermented hydrolysates and for further research on general food acceptability. Identification of the resulting bitter peptides and free amino acids might also be worth further research activities in order to gain deeper insight into

peptide structure and correlation between bitter peptides and taste perception.

Further research on the evaluation of the residual immunoreactivity and allergenicity using *in vivo* and *in vitro* test methods such as immunoassays including sandwich ELISA and western blot as well as clinical trials comprising oral challenge tests are indispensable future research activities. Those studies are in particular essential prior to the utilization of these fermented hydrolysates as low-allergen or hypoallergenic food ingredient with pleasant taste and superior techno-functional properties as we have shown in the current study.

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Conflict of Interest

The authors have declared no conflicts of interest.

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CHAPTER 4: Immunoreactivity, sensory and physicochemical properties of fermented soy protein isolate⁴

Abstract

The effect of induced liquid state fermentation (*Bacillus subtilis*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *Lactobacillus helveticus*) on immunoreactivity, physicochemical and sensory properties of soy protein isolate (SPI) was studied. *L. helveticus* revealed the most abundant reduction in terms of immunoreactivity within soluble protein fractions up to 100 %, which could be measured by *in vitro* sandwich ELISA using mouse monoclonal anti-Gly m5 antibodies (mAbs). Almost no binding in western blot using mouse monoclonal mAbs and sera from soy sensitive individuals was found. Fermentation increased water- and oil-binding capacity as well as protein solubility at pH 4.0. Foaming activity was nearly doubled compared to non-fermented SPI. A decreased emulsifying capacity, foaming density, and quantity of soluble proteins at pH 7.0 were observed. Principal component analysis (PCA) confirmed decreased *bitter* and *beany* off-flavors of fermented samples compared to non-fermented SPI. Consequently, fermentation might be a promising method to produce tasty low-allergen food ingredients with good physicochemical properties.

Keywords: Soybean allergy; Fermentation, Sandwich-ELISA and western blot; Principal Component Analysis (PCA); Physicochemical properties

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1. Introduction

Food allergy is an abnormal immune response to certain food components. The prevalence of food allergies is rising dramatically, and approximately 220 to 250 million people worldwide suffer from some kind of food allergy (WAO, 2013). Food allergens are typically naturally occurring proteins, and small regions, called epitopes, are responsible for the immunoglobulin E (IgE)-mediated allergic response (Taylor & Hefle, 2001).

Soybean (*Glycine max* (L.) MERR.) is an important vegetable protein source for the food industry due to its considerable amount of high quality proteins and nutritional value. However, soybean is among the so-called "big 8" food allergens, which together account for over 90 % of all documented food allergies in the U.S. (FDA, 2004; Taylor & Hefle, 2001). The prevalence of soy allergy is not precisely known and it is expected to escalate due to the increasing consumption of soybean products. Currently, eight allergenic proteins (Gly m1 - Gly m8) with molecular masses ranging from 7.5 to 97 kDa have been registered by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (www.allergen.org). However, the two storage proteins β -conglycinin (Gly m5) and glycinin (Gly m6) have been related to severe reactions in European allergic population (Amnuaycheewa & de Mejia, 2010; Holzhauser et al., 2009; Verhoeckx et al., 2015). The estimated allergy threshold is low and small amounts of soy protein, ranging from 0.0013 to 500 mg, may be enough to trigger an immune response (FDA, 2004).

So far, the only way to prevent unintended exposure to a food allergen is complete avoidance of the offending food. Recently, the need to control soy allergy by processing treatments rather than avoidance has pushed the development of new technologies, including genetic modification, thermal and non-thermal treatments (Verhoeckx et al., 2015). Currently, enzymatic hydrolysis using proteases of microbial or plant origin is the most efficient process for disrupting sequential and conformational epitopes, thereby reducing the allergenicity (Wilson, Blaschek, & de Mejia, 2005; Meinschmidt, Scheiggert-Weisz, Brode, & Eisner, 2016; Meinschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016).

Fermentation is a traditional processing technology in food industry. Besides the functions of improving the bioavailability of nutrients and reducing pathogenic bacteria in the gastrointestinal tract, a regulation of immunogenicity and possibly reduction of allergenicity by fermentation has been hypothesized (Granato, Branco, Cruz, Faria, & Shah, 2010), but has rarely been investigated. Few studies (Frias, Song, Martinez-Villaluenga, Gonzalez de Mejia, & Vidal-Valverde, 2008; Kobayashi,

2005; Song, Frias, Martinez-Villaluenga, Vidal-Valdeverde, & de Mejia, 2008; Song, Pérez, Pettigrew, Martinez-Villaluenga, & de Mejia, 2010; Yamanishi, Huang, Tsuji, Bando, & Ogawa, 1995) have confirmed the degradation of soybean allergens, particularly Gly m Bd 30K, also known as P34, during fermentation by microbial proteolytic enzymes in fermented soybean foods such as soy sauce, miso, tempeh, soybean ingredients and feed-grade soybean meals. It has also been shown that various lactic acid bacteria (LAB) are able to hydrolyse α' - and α -subunits of soybean β -conglycinin (Aguirre, Garro, & de Gioria, 2008). The studies of Frias et al. (2008) and Song et al. (2008) demonstrated that both solid- and liquid state fermentation of cracked soybean seeds, flour, or meals by various mold strains and bacteria effectively reduced IgE-immunoreactivity by 65 - 99 % as evaluated by an indirect ELISA with human sera. However, these research groups did not consider the effect of fermentation on sensory and physicochemical properties of the respective soy product as fermentation can often lead to off-flavors or a loss of technofunctionality (Quinn & Beuchat, 1975; Shrestha, Dahal, & Ndungutse, 2013). As sensory and physicochemical properties are equally important parameters for the food industry, the simultaneous investigations are of considerable importance, but not examined to our knowledge up to now. Consequently, the evaluation of residual immunoreactivity of these two proteins is indispensable for the assessment of potential allergenicity of the processed food. However, reports are not available in the literature so far.

The objective of this research was to evaluate the degradation of major soybean allergens Gly m5 and Gly m6 (SDS-PAGE) and the reduction of Gly m5 immunoreactivity (sandwich ELISA and western blot) by induced liquid state fermentation of SPI using *Bacillus (B.) subtilis*, *Rhizopus (R.) oryzae*, *Saccharomyces (S.) cerevisiae*, and *Lactobacillus (L.) helveticus*. The sensory (smell, taste, and mouthfeeling) and physicochemical properties (protein solubility, emulsifying, foaming, oil- and water-binding capacity) have been analyzed as well.

2. Material and Methods

2.1. Raw materials and chemicals

Untoasted soybeans (*G. max* (L.) MERR.) were purchased from Naturkost Ernst Weber (Munich, Germany). *L. helveticus* DSM 20075, *B. subtilis* DSM 10, and *R. oryzae* DSM 2200 were purchased from the Germany Resource Center (DSMZ,

Braunschweig, Germany), while *S. cerevisiae* TMW 3.210 was obtained from the collection of the chair ‘Lehrstuhl für Technische Mikrobiologie’ in Weihenstephan (TMW, Freising, Germany).

All chemicals used in this study were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany) if not stated separately.

2.2. Preparation of Soy Protein Isolate (SPI)

SPI was prepared from untoasted soybean seeds using the technique as previously described in Meinschmidt et al. (2016b).

2.3. Fermentation of SPI

2.3.1. Strains, media, growth conditions, and preparation of inocula

All strains were cultivated overnight under their respective optimal growth conditions (see Table 1).

Table 1

List of employed strains including abbreviations, and growth/culture conditions.

Strain	No.	Abbreviation	Type	Growth/Culture conditions
<i>Bacillus subtilis</i>	DSM 10	<i>B. subtilis</i>	bacteria	30 °C, aerob, nutrient
<i>Lactobacillus helveticus</i>	DSM 20075	<i>L. helveticus</i>	Lactic acid bacteria	37 °C, anaerob, MRS
<i>Rhizopus oryzae</i>	DSM 2200	<i>R. oryzae</i>	mold	30 °C, aerob, potato dextrose
<i>Saccharomyces cerevisiae</i>	TMW 3.210	<i>S. cerevisiae</i>	yeast	30 °C, anaerob, potato dextrose

DSM = Deutsche Sammlung von Mikroorganismen; TMW = Technische Universität Weihenstephan;
MRS = DeMan, Rogosa and Sharpe

For solid media, 15 g L⁻¹ agar was added to the respective broth. After cell enumeration, a calculated aliquot of the preculture was centrifuged for 10 min at 9000 x *g*, the pelleted cells were washed twice with Ringer solution (Merck KGaA, Darmstadt, Germany) and were resuspended in 1 mL sterile distilled water, which was used as inocula.

2.3.2. Experimental design of induced liquid state SPI fermentation

Liquid state fermentations were performed in SPI that was suspended in sterile deionized water (5 % w/w, pH 6.7). Prior to inoculation, the SPI dispersion was pasteurized at 85 °C for 10 min in order to reduce the endogenous microbiota of the raw materials. After cooling down, the dispersions were supplemented with 2 % (w/v) glucose (Sigma-Aldrich Inc., St. Louis, USA) to favour the growth of the inoculated strains. Two independent replicates of the SPI dispersion were inoculated with strains from precultures to obtain a concentration of either 1×10^8 colony forming units (CFU) per milliliter of bacteria/yeast or 10 % (v/v) mold.

Fermentations were carried out under strain-specific growth conditions (Table 1) for 24 and 48 h in conical flasks without agitation. Fermentation was stopped by heat treatment at 90 °C for 20 min to terminate residual proteolytic activity. All samples were neutralized (pH 7.0) with 1 M NaOH, kept at -50 °C and lyophilized (BETA 1-8, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The pH value of the samples was measured initially and after fermentation using a disinfected pH electrode (WTW, Weilheim, Germany) calibrated at pH 7.0 and 4.0. All fermentations were performed at least in duplicate.

2.4. Microbiological analysis

2.4.1. Determination of the total viable counts by measuring the colony forming units (CFU)

Directly after inoculation and at time intervals of 24 h and 48 h, the fermentation process was monitored by determining the total viable counts. Aliquots (100 μ L) of fermented SPI were in triplicate serially diluted 1:10 (v/v) with Ringer solution (Merck, Germany) and 100 μ L of certain dilutions was spread homogenously with a Drigalski spatula in duplicate on the surface of pre-dried plate count agar-plates (Merck KGaA, Darmstadt, Germany). In addition, fermented samples were plated on strain specific agar to quantify and favor the growth of the respective strain. Plate-count agar plates were incubated aerobically at 30 °C for 48 ± 2 h, while all other plates were incubated under strain specific growth conditions (see Table 1). The colonies were counted and expressed as colony forming units per milliliter (CFU mL⁻¹).

2.4.2. Matrix Assisted Laser Desorption Ionization-Time of flight-mass spectrometry (MALDI-TOF MS)

MALDI-TOF-MS was performed to verify that the inoculated strains were dominant in the samples during fermentation. Fresh colony material (a single colony) was smeared on a polished steel MSP 96 target (Bruker Daltonik), spotted with 1 μ L of a saturated α -cyano-4-hydroxy-cinnamic acid matrix solution in 50 % acetonitrile-2.5 % trifluoroacetic acid (Bruker Daltonik) and air dried at room temperature. The analysis ($n = 3$) of the mass spectra was performed by a Microflex LT mass spectrometer (Bruker Daltonik) using the MALDI Biotyper software package (version 3.0). For external mass calibration, a bacterial standard (Bruker Daltonics) was used.

2.5. Molecular weight distribution applying sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight distribution was determined using SDS-PAGE according to Laemmli (1970) under reducing conditions. Sample preparation and running conditions were chosen according to Meinschmidt, Sussmann, et al. (2016) using 4 - 20 % midi CriterionTM TGX Stain-FreeTM precast gels. Protein visualization was performed by Criterion Stain-Free Gel DocTM EZ Imager (Bio-Rad).

2.6. Sandwich enzyme-linked immunosorbent assay (ELISA)

Mouse monoclonal anti- β -conglycinin antibodies (mAbs) were generated by immunizing female BALB/c mice (Janvier Labs, Le-genest saint-Isle, France) with β -conglycinin (SIGMA, Deisenhoven, Germany). Mice with maximum antibody titers were used as donors of splenic cells, which were fused to X63.Ag8.653 myeloma cells (ACC 43, DSMZ, Braunschweig, Germany). Three positive clones (Izimab-Glym5-3, Izimab-Glym5-4 and Izimab-Glym5-5) were selected according to standard protocols (Davis, Pennington, Kubler, & Conscience, 1982) and were used in three sandwich variants to avoid detection losses due to insufficient capture efficiency. Three positive clones were selected to assemble sandwich ELISAs for determining β -conglycinin. For Sandwich 1, Izimab-Glym5-4 and Izimab-Glym5-5 were used

as capture and detection antibody, respectively, whereas Izimab-Glym5-3 (capture) and Izimab-Glym5-5 (detection) were applied in Sandwich 2. In contrast, Izimab-Glym5-5 (capture) and Izimab-Glym5-4 (detection) were chosen for Sandwich 3. Capture antibodies were immobilized onto 96-well plates (maxisorp; Nunc, Germany) at 4 °C overnight. Plates were washed three times with phosphate buffered saline, 154 mM NaCl, pH 8.0, 0.05 % Tween (3xPBS-T) and blocked with Superblock blocking buffer (Life Technology GmbH, Germany). SPI and all other samples were centrifuged at 15,000 x *g*, 10 min and supernatants (soluble protein) were incubated 1 h at RT in duplicate. After a further wash-step (3xPBS-T) the POD-conjugated detection-antibody was bound 1 h at RT. POD activity was determined following a third wash step (3xPBS-T) by incubating the plate with TMB-E substrate (DUNN Labortechnik, Asbach, Germany) and stopping reaction after 5 min with 0.3 M H₂SO₄. Color intensity represents a signal for the amount of captured antigen and was measured at 450 nm. Signal intensities were calculated by extrapolation to a calibration curve from standard-SPI consisting of seven known concentrations. Each sample was analyzed in duplicate.

2.7. Western blot

Monoclonal antibodies specific for β -conglycinin were used for detecting β -conglycinin by western blot. SPI and fermented samples were separated by SDS-PAGE using precast gradient (4 - 15 %) tris-glycine gels (BioRad, M¹ünchen, Germany) according to standard protocol (Laemmli, 1970). After electrophoresis, proteins were blotted onto nitrocellulose membranes using semi-dry blotter Perfect blue (Peqlab, VWR, Erlangen, Germany) according to manufacturer's instructions using Towbin buffer (25 mM Tris, 192 mM glycine with 10 % MetOH, 0.02 % SDS) as transfer buffer. Membranes were blocked with 5 % skim milk powder in PBS-T (phosphate-buffered saline, 154 mM NaCl, 0.05 % Tween 20, pH 8.0) and incubated overnight with mAbs at 4 °C. After washing in PBS-T (phosphate-buffered saline, 154 mM NaCl, 0.05 % Tween 20, pH 8.0), membranes were incubated with detection antibody Horse-radish-peroxidase (HRP)-conjugated goat anti-mouse IgG (Fc Fragment specific) (Dianova, Hamburg, Germany) for 1 h at room temperature.

Serum samples of allergy patients were collected in Dermatology, Venereology and Allergology Clinic, University of Leipzig (Leipzig, Germany ethics proposal 428-12-17122012). Three serum samples out of 50 different patients suffering from soy allergy, showed *in vitro* Gly m5-reactivity (CAP class 2, f432, measured with Pha-

dia 100, Phadia, Thermo Scientific, Freiburg Germany). Two of these serum samples were used to verify results, obtained with mouse monoclonal anti- β -conglycinin Abs in western blot. Conditions for electrophoresis and blotting were the same as used for analysis with mAbs. Human serum was diluted two-fold and detection of bound Abs was carried out with mouse-anti-human-IgE (ϵ -chain specific)-HRP (Biozol, Eching, Germany). HRP activity was visualized by using chemiluminescence substrate supersignal west femto (Thermo Fisher, Pierce, Bonn, Germany) with subsequent exposure of CL-XPosure films.

2.8. Chemical composition and physicochemical properties

2.8.1. Chemical composition

The protein and dry matter content was determined as described by AOAC methods using a protein calculation factor of $N \times 6.25$ (AOAC, 2005a, 2005b).

2.8.2. Physicochemical properties

2.8.2.1. *Protein solubility.* Protein solubility of the samples was determined at pH 4.0 and 7.0 following the method of Morr et al. (1985).

2.8.2.2. *Emulsifying capacity.* The emulsifying capacity (EC) was determined in duplicate as suggested by Wang and Johnson (2001).

2.8.2.3. *Foaming properties (activity, density, and stability).* Foaming activity was determined according to Phillips, Haque, and Kinsella (1987), whereas the foaming density and stability were analyzed as described elsewhere (Meinlschmidt, Sussmann, et al., 2016).

2.8.2.4. *Water- and oil-binding capacity.* Water-binding capacity (WBC) was analyzed according to the AACC 56-20 official method (AACC, 2000). Oil-binding capacity (OBC) was determined as described by Ludwig, Ludwig, and Pingel (1989).

2.9. Sensory evaluation

2.9.1. Quantitative Descriptive Analysis (QDA)

Sensory profiling of the samples was performed using descriptive sensory analysis as described in ISO 6564:1985. The sensory panel consists of ten panellists (7 female, 3 male), selected and trained according to guidelines in ISO 8586-1:1993. A broad list of attributes characteristic for the samples in terms of smell (*fermented*, *beany*, *yeasty*), taste (*fermented*, *bitter*, *beany*), and mouthfeeling (*mouthcoating*, *astringent*) have been developed by the panel (1 month, 2-times per week). The attributes were rated on a 10-cm continuous scale (0 = no perception; 10 = strong perception).

2.9.2. Samples preparation and sensory evaluation

For sensory evaluation, samples were mixed with tap water to a protein concentration of 2.5 % (w/w) and adjusted to pH 7.0 with 1 M NaOH. The panel was presented with five samples (10 mL) per session (performed in two sessions, 1 h each), which were served to the panel in randomized order at room temperature in small plastic cups, coded by arbitrary numbers (three-digits). Water and plain crackers were provided for palate cleansing in between. The panel was instructed to evaluate the sensory attributes for smell, taste, and mouthfeeling as mentioned in 2.9.1 using the 10-cm scale. Each panellist did a monadic evaluation of the samples at individual speed. Sensory evaluation was performed in duplicate.

2.10. Statistical analysis

Statistical analysis was performed using SPSS (SPSS v 20.0 SPSS Inc. Chicago, IL, USA). All chemical data were analyzed using the one-way analysis of variances (ANOVA) and means were generated and adjusted with the Bonferroni post hoc test. Statistically significant differences were considered at $p < 0.05$.

QDA data were also analyzed using the one-way ANOVA, followed by Tukey's honest significance difference (HSD) test. In order to find similarities and differences

between the samples and relations between the attributes, the results of the descriptive sensory analysis were processed by means of multivariate statistical PCA using the R-based PanelCheck software (Nofima, Ås, Norway; version 1.4.0.).

3. Results and Discussion

For the experiments, a SPI, containing a dry matter content of 94.4 % and a protein content of 94.6 % was prepared (see 2.2).

All fermentations were monitored for 24 and 48 h, but all samples fermented for 48 h - except *L. helveticus* - were not suitable as food ingredients as the sensory properties were negatively affected. Therefore, fermentations with all strains were performed for 24 h, while fermentation with *L. helveticus* was additionally carried out for 48 h due to the pleasant taste.

3.1. pH value and microbial growth during fermentation

Fermentation with *L. helveticus* showed a progressive reduction in pH value after 24 and 48 h with an initial pH value of 6.7 lowering to pH 4.5 and 4.2, respectively. This sharp decrease ($\Delta\text{pH} = 2.2 - 2.5$) is characteristic for LAB strains. During fermentation, *L. helveticus* grew rapidly, converting the supplemented glucose into organic acids, mainly lactic-acid, thereby lowering the pH and inhibiting the growth of other competing microorganisms (Doblado, Frias, Munoz & Vidal-Valverde, 2003). SPI was inoculated with *L. helveticus* at a cell density of 1×10^8 CFU mL⁻¹, and in the course of fermentation a growth up to 7.5×10^9 CFU mL⁻¹ after 48 h was detected. After 24 h, *B. subtilis*, *S. cerevisiae*, and *R. oryzae* lowered the pH value to pH 5.1, 5.9, and 6.5, respectively. These strains produce only minor organic acids from fermentable sugars (hexoses) and, therefore, the lowering of the pH was rather minor compared to *L. helveticus*. This phenomenon was also observed by Song et al. (2008). An increase in viable cells was detected for both *B. subtilis* and *S. cerevisiae*, rising from initially 1×10^8 CFU mL⁻¹ up to 5×10^8 CFU mL⁻¹ or 7.5×10^8 CFU mL⁻¹, respectively. Following fermentation with *R. oryzae*, the visual colonies displayed the wooly texture on the surface on the plates, thereby the growth of this mold and the absence of other bacteria on plate-count agar.

MALDI-TOF-MS analyses reported a reliable identification of the inoculated strains with log(score) values of ≥ 2.3 (highly probable species identification) and

confirmed the absence of competitive strains and the ability of the inoculated strains to dominate fermentation.

3.2. Molecular weight distribution and immunoreactivity

Figure 1A shows the electrophoretic patterns of all samples. The electrophoretic profile of non-fermented SPI (lane 2) presents high intensity bands of β -conglycinin subunits α' (~ 72 kDa), α (~ 68 kDa), β (~ 53 kDa), and glycinin, which is mainly composed of the acidic subunit "A" (29 - 33 kDa) and the basic subunit ("B", 18 - 22 kDa) (Amnuaycheewa & de Mejia, 2010). The protein identification by means of LC-MS/MS was described previously Meinschmidt, Schweiggert-Weisz, et al. (2016).

Among the fermented samples, electrophoretic patterns of samples fermented with *B. subtilis* (Figure 1 A, lane 3) and *L. helveticus* (Figure 1A, lanes 6 and 7) showed the greatest reduction in protein band intensity corresponding to β -conglycinin subunits. In contrast, both acidic and basic subunits of glycinin were less affected. This might be due to the complex structure and disulfide bridges of glycinin, which impedes enzymatic hydrolysis of proteins. Aguirre et al. (2008) reported that β -conglycinin subunits were the preferred substrate for different LAB strains. Various different proteolytic enzymes have been identified in LAB (Savijoki, Ingmer & Varmanen, 2006). It is well known that exploitation of milk proteins in the course of fermentation is initiated by cell-envelope proteinases (CEP), a subtilisin-like serine protease, that degrades proteins into oligopeptides, shorter peptides and amino acids (Christensen, Dudley, Pederson & Steele, 1999). In addition, a number of distinct intercellular peptidases, including endo-, amino-, tri- and dipeptidases are involved, which might hydrolyze soy proteins (Christensen et al., 1999). The degradation of major soybean allergens following fermentation with *B. subtilis* was also reported by Yamanishi et al. (1995) and Frias et al. (2008). *S. cerevisiae* (Figure 1 A, lane 5) and *R. oryzae* (Figure 1A, lane 4) were less effective in the degradation of soy proteins as the electrophoretic pattern did not changed compared to non-fermented SPI.

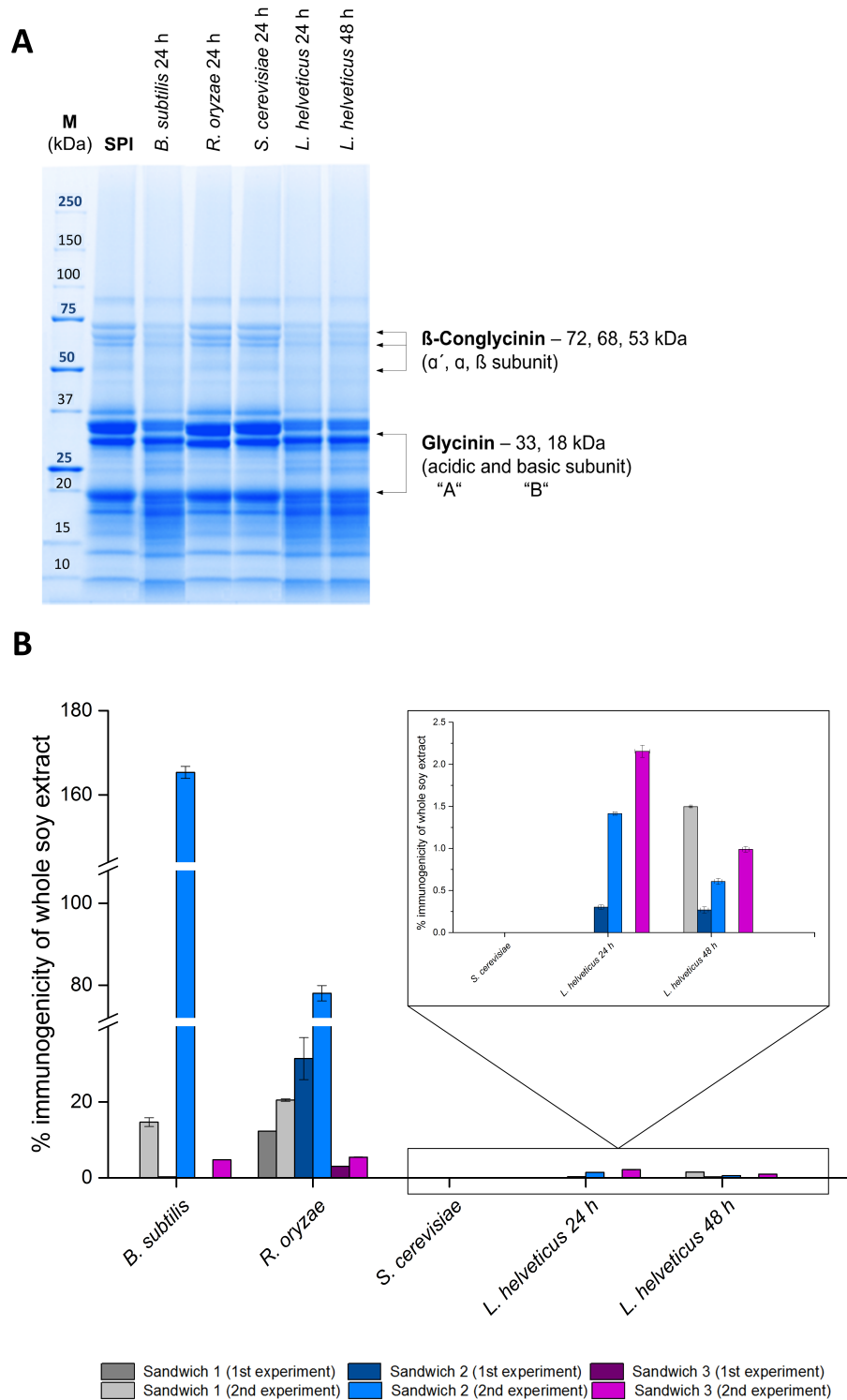


Fig. 1. SDS-PAGE patterns (A) and sandwich ELISA (B) using mouse monoclonal anti- β -conglycinin (Gly m5) Abs (Izimab-Glym5-3, Izimab-Glym5-4, Izimab-Glym5-5). M- Molecular weight standard indicated in kilo Dalton (kDa); SPI- Soy protein isolate; Electrophoresis was carried out with 4-20 % polyacrylamide gradient gels. α' -, α - and β - subunits of β -conglycinin; "A" and "B": acidic and basic subunit of glycinin. Results of three different sandwich ELISAs are shown. Each ELISA was performed in duplicate (1st and 2nd experiment).

The residual immunoreactivity of the soluble proteins of all samples is presented in duplicate (1st and 2nd experiment) in Figure 1B (sandwich ELISA) and Figure 2 (western blots). Each sandwich ELISA (Sandwich 1-3) in Figure 1B is composed of two different antibodies as described in the Material and Method section (see 2.6), recognizing different epitopes of β -conglycinin. Therefore, the result of ELISA analysis represents the concerted immunoreactivity of both epitopes. The immunoreactivity was calculated in relation to non-fermented SPI, which was set to 100 %. Reduced immunoreactivity is measured if either of both epitopes failed in binding. Hence separate tests for each antibody were performed in western blot, and both capture and detection Abs revealed reduced immunoreactivity in the fermented samples (Figure 2 A and B). These results verified the ELISA-data (Figure 1B). All fermented samples showed a reduction in immunoreactivity in the first experiment of fermentation in western blot using any antibody applied previously in ELISA regardless of the strain. However, differences among both experiments (1st and 2nd experiment) and the strains can be found.

For all strains assayed - except for *S. cerevisiae* and *R. oryzae* - SDS-PAGE results greatly coincide with the ELISA and western blot results. Although SDS-PAGE analysis of the sample prepared with *S. cerevisiae* and *R. oryzae* suggests that this strain did not alter the allergic potential of SPI, ELISA and western blot analyses disprove this assumption. A reason could be that both strains changed the conformational epitopes on the surface of soy proteins, which did not lead to changes in electrophoretic patterns (Figure 1A, lane 4 and 5), but lead to great changes in IgE-binding ability to human serum.

The greatest reduction of up to nearly 100 % in immunoreactivity to mAbs against β -conglycinin was achieved by fermentation with bacteria (*B. subtilis* and *L. helveticus*) and yeast (*S. cerevisiae*). Molds like *R. oryzae* grow at slower growth rate compared to bacteria, generating lower β -conglycinin alteration, thereby giving higher immune response with anti- β -conglycinin mAbs (Figure 1B and 2). Among the strains assayed, almost no binding to soy sensitive serum (Figure 2D and E) was found for samples fermented with *L. helveticus*, presumably as a result of a combined effect of proteolysis and acid-induced protein denaturation. The enormous reduction in immunoreactivity due to fermentation of soybean meals and flour following fermentation with a LAB (*Lactobacillus plantarum*) and yeast (*S. cerevisiae*) was also observed by Song et al. (2008) and Frias et al. (2008). They attributed the reduction in immunoreactivity to the decreased protein solubility due to fermentation. Reasons for decreased protein solubility (see 3.3.2, protein solubility) at neutral pH could be due to the fact that microorganisms and enzymes involved in the ferment-

tation process can easily hydrolyze soluble proteins, modifying epitopes with lower affinity to IgE-Abs. Further, new peptides were formed, and the conditions used to extract original proteins were not adequately extracting the new peptides. In addition, during fermentation, proteases and peptidases produced by LAB might cleave proteins into peptides and amino acids; hence, the structure of the epitopes might be altered or even destroyed (El-Ghaish et al., 2011).

In the present paper only β -conglycinin was monitored with specific Abs in the different fermented SPIs. Other allergenic components of soy e.g. glycinin (Gly m6), Kunitz inhibitor (Gly mTI) or proflin (Gly m3) could contribute to the allergenicity as well. However β -conglycinin (Gly m5) and glycinin (Gly m6) are the most abundant storage proteins of soybeans and sensitization against Gly m5 and Gly m6 is highly indicative for severe allergic reactions (Holzhauser et al., 2009). We could show by SDS-PAGE analysis that exactly proteins with molecular weights matching that of the subunits of Gly m5 and Gly m6 disappear by fermentation using *B. subtilis* and *L. helveticus* in soluble extracts. Additionally, two human serum samples from soy sensitive individuals show an overall decrease in immunoreactivity in the fermented protein extracts and not solely in immunoreactive bands corresponding to β -conglycinin.

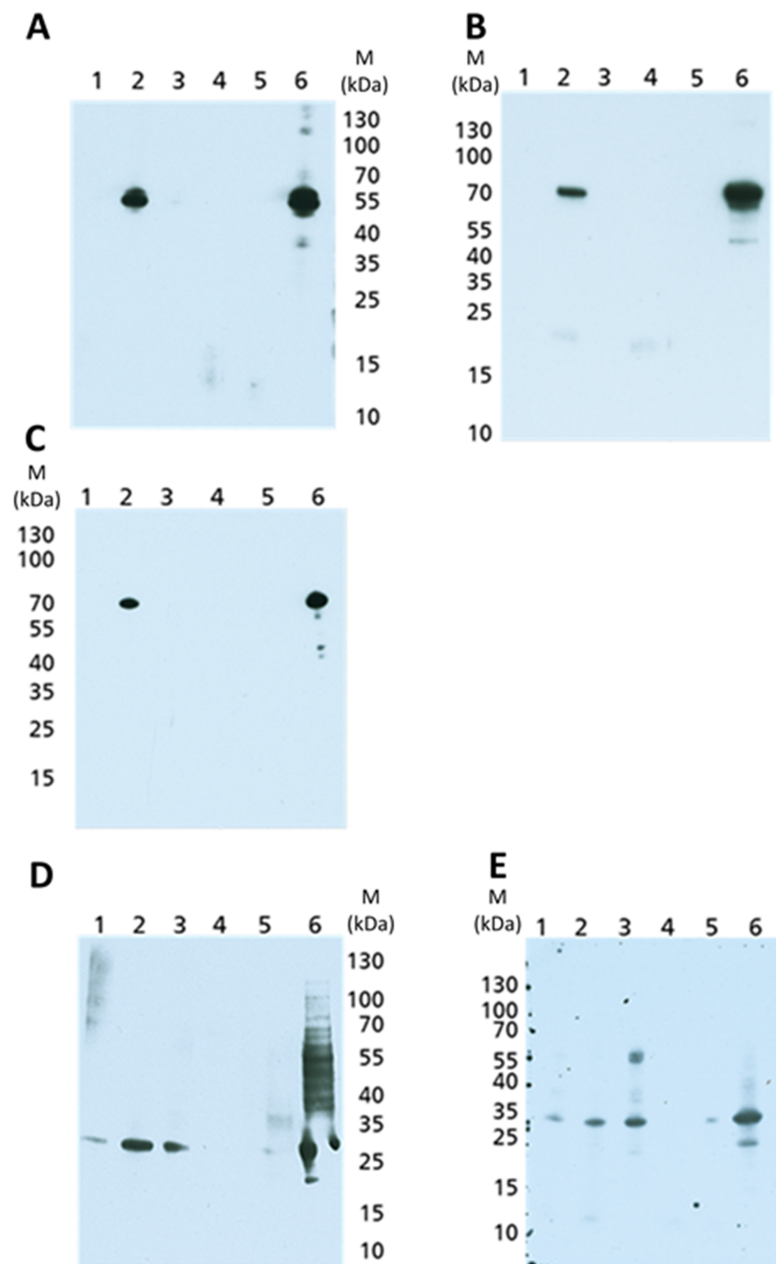


Fig. 2. Western blot analysis of all samples applying three different mouse monoclonal antibodies (Izimab-Glym5-3, Izimab-Glym5-4, Izimab-Glym5-5) used in the ELISA experiments above as coating-Abs (A, B, C) and two sera from soy sensitive individuals (D and E). Representative samples containing equal protein contents (determined by Bradford assay) of SPI, fermented by microorganisms or non-fermented (1- *B. subtilis* 24 h, 2- *S. cerevisiae* 24 h, 3- *R. oryzae* 24 h, 4- *L. helveticus* 48 h, 5- *L. helveticus* 24 h and 6- non-fermented SPI), were blotted onto nitrocellulose membranes. M- Molecular weight standard indicated in kilo Dalton (kDa). Electrophoresis was carried out with 4 - 15 % polyacrylamide gradient gels. Membranes were incubated with mouse monoclonal anti- β -conglycinin antibodies (A, B, C) and serum samples from soy sensitive individuals (D and E).

3.3 Effects of fermentation using *L. helveticus* on the sensory profile and physicochemical properties of SPI

According to the results given, *L. helveticus* was most effective in the reduction in immunoreactivity and almost no binding to monoclonal mouse Abs and human serum in western blot could be found. Therefore, samples fermented with *L. helveticus* for 24 and 48 h were analyzed in more detail, including the physicochemical and sensory properties.

3.3.1. Sensory profile

3.3.1.1. *Quantitative Descriptive Analysis (QDA)*. The results of QDA are shown in Figure 3A. Non-fermented SPI evaluation resulted in the following smell-scaling: *fermented* (0.0), *beany* (4.6), *yeasty* (0.0); taste-scaling: *sour* (0.4), *bitter* (2.8) and *beany* (6.4); mouthfeeling-scaling: *mouthcoating* (4.6) and *astringent* (3.3). During fermentation, the samples exhibited a *fermented* (2.6 - 3.3) and *yeasty* (1 - 1.3) smell as well as a slight *sour* (0.7 - 1.1) taste. In general, panellists agreed that all negative attributes associated with soybeans like *beany*, *bitter*, *mouthcoating* and *astringent* were significantly ($p < 0.05$) lower following fermentation compared to non-fermented SPI. Soy is often rejected due to a *beany* off-flavor, which results from lipid oxidation and lipoxygenase catalyzed oxidation of soybean oil to volatile compounds such as isopentanol, *n*-hexanal and hexanol (Shogren, Mohamed & Carriere, 2003). Pinthong, Macrae, and Rothwell (1980) observed that fermented soy milk contained less *n*-hexanal, which was converted into *n*-hexanoic acid, thereby reducing the *beany* off-flavor. The fermented and sour taste might be attributed to the production of lactic acid, which results in a tangy lactic acid taste characteristic for fermented soy products. During fermentation, bitterness was significantly ($p < 0.05$) reduced from initially 2.8 to 0.6 and 0.2 after 24 and 48 h, respectively. Bitterness, which is believed to result from the accumulation of hydrophobic peptides, i.e. peptides rich in proline and leucine, is a serious quality concern facing the food industry. Specific enzymatic debittering strategies have focused on the application of proline specific exo- and endopeptidases given the contribution of proline residues to peptide/hydrolysate bitterness. Other exopeptidase activities, including carboxypeptidases, have been detected in a number of lactic acid bacteria, but their role has mainly been described with respect to the dairy processes (Savijoki et al., 2006). In addition, the debittering effect of fermentation on SPI hydrolysates have

previously described by Meinschmidt et al. (2016b). *L. helveticus* is an important industrial starter culture that is known to express endopeptidases with post-proline specificity such as PepO2 and PepO3, which sufficiently degrade bitter peptides in cheese during ripening, which might explain the reduced bitterness of the fermented samples (Soervanranata, Powers & Unlu, 2007).

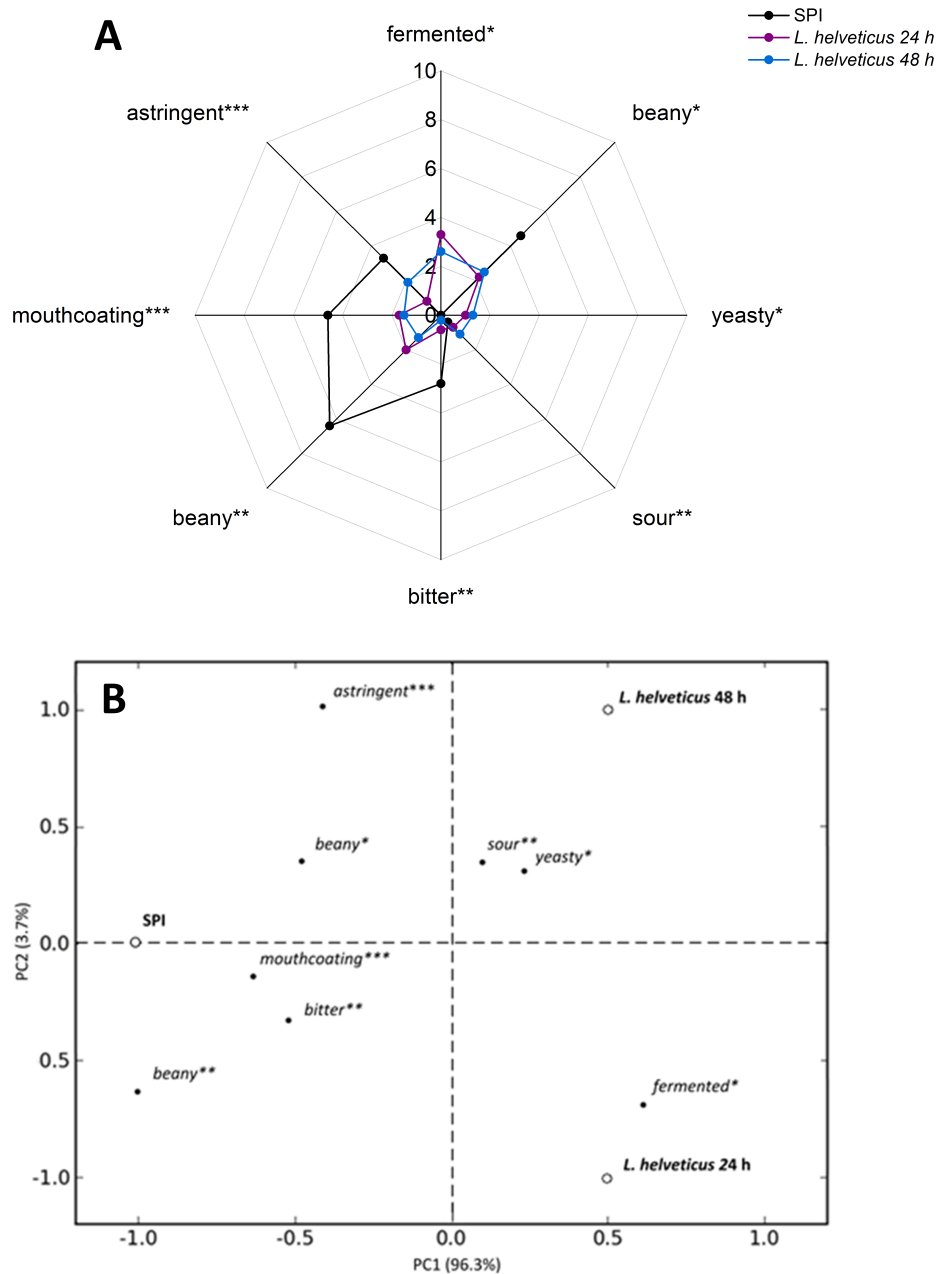


Fig. 3. Taste profile (A) and bi-plot (B) showing non-fermented SPI and SPI fermented with *L. helveticus* for 24 and 48 h in relation to descriptive attributes based on the descriptive sensory data. Each value is expressed as mean scored on the 10-cm continuous scale from 0 (no perception) to 10 (strong perception) by a 10-member panel. * smell; ** taste; *** mouthfeeling.

3.3.1.2. *Principal Component Analysis (PCA)*. PCA was employed on QDA data to identify the most important sensory attributes that do have impact on samples characteristics. The results are shown in Figure 3B (bi-plot) and Table 2 (scaled scores and loadings).

The extracted and uncorrelated principal components (PCs) PC1 and PC2 accounted for 96.3 % and 3.7 % (100 % in total) of the variation across the samples, respectively. The bi-plot (Figure 3B) reveals great differences between non-fermented SPI and fermented samples. The PC1 dimension (factor loadings > 0.550) was mainly described by the attributes *fermented* smell (0.613), *beany* taste (-1.000), and *mouthcoating* mouthfeeling (-0.632). In contrast, the PC2 dimension is mainly explained by the attributes *fermented* smell (-0.693), *beany* taste (-0.630), and *astringent* mouthfeeling (1.000). According to the scaled loadings and the location (scaled scores) in Table 2, PC1 mainly explain a contrast between fermented samples and non-fermented SPI (*beany*), while PC2 presents a contrast between *fermented* and *astringent*.

Table 2

Principal Component (PC) scaled scores and scaled loadings for SPI and SPI samples fermented with *L. helveticus* for 24 and 48 h obtained by Principal Component Analysis (PCA).

Scaled Scores			Scaled Loadings		
Samples	PC1	PC2	Attributes	PC1	PC2
SPI	-1.000	0.005	<i>fermented</i> *	0.613	-0.693
<i>L. helveticus</i> 24 h	0.496	-1.000	<i>beany</i> *	-0.475	0.350
<i>L. helveticus</i> 48 h	-1.000	0.995	<i>yeasty</i> *	0.234	0.307
			<i>sour</i> **	0.099	0.338
			<i>bitter</i> **	-0.514	-0.340
			<i>beany</i> **	-1.000	-0.630
			<i>mouthcoating</i> ***	-0.632	-0.145
			<i>astringent</i> ***	-0.411	1.000

Loadings with an absolute letter greater 0.550 are shown in bold type. * smell; ** taste; *** mouthfeeling

Non-fermented SPI loaded on the negative side of PC1 and is explained by the attributes *beany* (smell and taste), *bitter*, and *mouthcoating* (Figure 3B). In contrast and in great accordance with the QDA results, the fermented samples were less explained by these attributes, but *fermented*, *sour* and *yeasty* became dominant. The attribute fermented was loaded on the sample following fermentation for 24 h, while attributes such as *sour* and *yeasty* became dominant after 48 h of fermentation. This might presumably be due to further production of lactic acid and aroma compounds characteristic for fermented products.

3.3.2. Physicochemical properties

The results of the physicochemical properties (protein solubility, emulsifying, water- and oil-binding capacity, foaming activity, density, and stability) are summarized in Table 3.

3.3.2.1. *Protein solubility.* Non-fermented SPI had a protein solubility of 5.0 and 44.0 % at pH 4.0 and 7.0, respectively. Data presented in Table 3 indicated that fermentation drastically lowered protein solubility at pH 7.0, while the solubility at acidic conditions was increased. Protein solubility at pH 7.0 was nearly halved by fermentation compared to non-fermented SPI. *L. helveticus* produced a large amount of acid, which might result in irreversibly coagulation of proteins, and thereby decreasing protein solubility. Further, enzyme/microorganisms inactivation (90 °C, 20 min) might have promoted aggregation and cross-linking of partially hydrolyzed soy proteins. In contrast, protein solubility at pH 4.0 was almost doubled after fermentation. Sadowska, Fornal, Vidal-Valverde, and Frias (1999) and Elkhailifa, Schiffler and Bernhardt (2005) also reported the enhancing effect of fermentation on protein solubility of lentils and sorghum flour in acidic pH range, whereas it was decreased at neutral pH value.

3.3.2.2. *Emulsifying capacity.* Non-fermented SPI exhibited the highest EC of 660 mL g⁻¹, while fermentation resulted in a significantly ($p < 0.05$) decreased EC (475 - 483 mL g⁻¹). As mentioned before, the exposure of buried hydrophobic regions and high-temperature enzyme/microorganism inactivation promoted aggregation and cross-linking of partially hydrolyzed proteins. Soluble proteins are surface active and known to promote oil-in-water emulsions. At pH 7.0, the solubility of the fermented samples was reduced, thus the ability to form an emulsion was nega-

tively affected. This was in confirmation with observations reported by Quinn and Beuchat (1975), who also found a reduced EC of defatted peanut flour following fermentation with *R. microsporus var. oligosporus*.

Table 3

Physicochemical properties in terms of protein solubility, emulsifying capacity, water- and oil-binding capacity, and foaming properties of SPI and the SPI samples fermented with *L. helveticus* for 24 and 48 h.

	Protein solubility		Emulsifying capacity (mL g ⁻¹)	Water-binding capacity (mL g ⁻¹)	Oil-binding capacity (mL g ⁻¹)	Foaming activity (%)	Foaming stability (%)	Foaming density (g L ⁻¹)
	pH 4.0	pH 7.0						
	(%)	(%)						
SPI	5.0 ± 1.1 ^a	44.0 ± 0.6 ^a	660 ± 5 ^a	2.6 ± 0.1 ^a	0.0 ± 0.0 ^a	552 ± 5 ^a	90 ± 0 ^a	215 ± 5 ^a
<i>L. helveticus</i> 24 h	11.3 ± 0.8 ^b	18.2 ± 0.4 ^b	483 ± 3 ^b	3.4 ± 0.0 ^b	2.7 ± 0.1 ^b	996 ± 42 ^b	90 ± 0 ^a	114 ± 0 ^b
<i>L. helveticus</i> 48 h	12.4 ± 2.8 ^b	16.5 ± 0.0 ^c	475 ± 10 ^b	3.5 ± 0.1 ^b	2.9 ± 0.1 ^b	1085 ± 13 ^c	90 ± 0 ^a	91 ± 2 ^c

Results are expressed as means ± standard deviation. Means followed with different superscript letters within each column are significantly different ($p < 0.05$) relating to one enzyme combination system (one-way ANOVA, Bonferroni).

3.3.2.3. Water- and oil-binding capacity. Fermented SPI samples exhibited an increased WBC up to 3.5 mL g⁻¹ (48 h), while non-fermented SPI had a WBC of about 2.6 mL g⁻¹. An enhanced WBC might be due to the decrease in solubility, which means that the higher content of insoluble proteins were able to bind more water. Non-fermented SPI did not show any OBC, whereas fermentation increased OBC to 2.9 mL⁻¹. This might be due to a higher content of hydrophobic or lipophilic groups, which enables oil retention (Dixon & Larry, 1981). Ahmed, Ramaswamy, and Alli (2006) and Elkhailifa et al. (2005) also found an enhanced OBC and WBC after fermentation of peanut flour and sorghum meal and flour with fungal and LAB strains.

3.3.2.4. Foaming activity, density and stability. Non-fermented SPI had a foaming activity of 552 %, which was nearly doubled (996-1085 %) after fermentation. Due to the partial hydrolysis of soy proteins, more flexible and low-molecular weight peptides were formed. Thereby, peptides could transfer to the air-water interface more rapidly and the protein-protein interaction improved the strength of the viscoelastic cohesive film, dropping the surface tension (Tsumura et al., 2005). As might be expected, the foaming density of the fermented samples significantly ($p < 0.05$) decreased compared to non-fermented SPI. The foaming density was reduced from initially 215 g L⁻¹ to 114 and 91 g L⁻¹ after fermentation for 24 and 48 h, respectively. However, a foaming stability of about 90 % could be found for all samples.

4. Conclusions

In conclusion, fermentation of SPI significantly reduced the immunoreactivity of soluble soy proteins tested in this study as demonstrated by sandwich ELISA and western blot. The highest performance among the strains assayed was obtained by fermentation with *L. helveticus*. Furthermore, *L. helveticus* exhibit great sensory properties with significantly ($p < 0.05$) decreased bitter taste and good physico-chemical properties in terms of increased foaming activity and maintained foaming stability. *L. helveticus* is a "generally recognized as safe" (GRAS) LAB, which can be applied as "natural" food preservative and starter culture for the production of hypoallergenic food ingredients that combine pleasant taste and good physicochemical properties. In addition, *L. helveticus* is known to possess many common probiotic properties such as prevention of gastrointestinal infections, making the fermented food products healthier (Taverniti & Guglielmetti, 2012).

Although Gly m5 immunoreactivity was almost completely inhibited, other allergenic components of soy e.g. glycinin (Gly m6), Kunitz Trypsin Inhibitor (Gly mTI) or profilin (Gly m3) could contribute to the allergenicity as well. Therefore, the immunoreactivity of these protein fractions has to be analyzed by further ELISAs and western blots. The level of immunoreactivity reduction in this study might be important for a lot of allergic individuals depending upon their sensitivity threshold; however, the clinical relevance of these findings still needs to be confirmed by i.e. controlled human challenge studies. However, it is worth note that aggregation and loss of protein solubility, rather than the epitope destruction, may be responsible for the observed decrease in immunoreactivity of the tested soy epitope. Future studies on immunoreactivity of variety of soluble and insoluble protein fractions are necessary to understand mechanism of inactivation of soy allergenic proteins (epitopes) by fermentation.

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Conflict of Interest

The authors have declared no conflicts of interest.

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CHAPTER 5: High pressure processing assisted enzymatic hydrolysis – An innovative approach for the reduction of soy immunoreactivity⁵

Abstract

Soybean (*Glycine max* (L.) MERR.) is recognized as a potent food allergen causing one of the most frequent food allergies worldwide. The effect of high pressure processing (HPP) prior to and during enzymatic hydrolysis using the enzyme preparation Flavourzyme[®] on the degree of hydrolysis (DH), molecular weight distribution (SDS-PAGE) and β -conglycinin (Gly m5) immunoreactivity of soy protein isolate (SPI) was studied. Enzymatic hydrolysis was carried out at atmospheric pressure (0.1 MPa) and HPP (100-600 MPa) at 50 °C for 15 min. Pressures higher than 300 MPa enhanced the degradation of Gly m5, which was confirmed by SDS-PAGE and LC-MS/MS analyses. The immunoreactivity of the samples was assessed by in vitro sandwich ELISA using mouse monoclonal anti-Gly m5 antibodies. Depending on the antibody tested, the residual immunoreactivity was completely inhibited or significantly impaired up to 99.5 % applying HPP during hydrolysis at 400 and 500 MPa. By means of principal component analysis, the beany and green off-flavors characteristic for unprocessed SPI could be reduced by pressure enhanced hydrolysis at 400-500 MPa. The resulting hydrolysates possessed improved protein solubility, foaming activities and oil-binding capacities, which were improved by 45 %, 66 %, and 210 %, respectively. HPP prior to and during enzymatic hydrolysis at 400-500 MPa constitutes an innovative approach for the production of low-allergen food ingredients that combine good taste and enhanced functional properties.

Keywords: Soybean allergy; Sandwich ELISA; High pressure processing (HPP); Mass spectrometry (LC-MS/MS); Principal component analysis (PCA); Physicochemical properties

⁵MEINLSCHMIDT, P., BRODE, V., SEVENICH, R., UEBERHAM, E., SCHWEIGGERT-WEISZ, U., LEHMANN, J., RAUH, C., KNORR, D., EISNER, P. (2016). High pressure processing assisted enzymatic hydrolysis – An innovative approach for the reduction of soy immunoreactivity. *Innovative Food Science & Emerging Technologies*. Doi: 10.1016/j.ifset.2016.06.022. Reproduced with permission.

1. Introduction

Due to the rising prevalence and incidence of food allergies, public awareness and concern have initiated intensified efforts to treat and mitigate allergic reactions. The World Allergy Organization (WAO) estimated that 250 million people worldwide suffer from some kind of food allergy, where infants are more affected (5 - 8 %) than adults (1 - 2 %) (Fiocchi et al. 2003). Food allergy is an immunoglobulin E (IgE)-mediated adverse immunological response of the immune system towards specific food components, which are triggered by antigenic proteins with molecular masses ranging from 7 to 71 kDa (Wilson et al. 2005). The antigenic determinants of the proteins, which can be recognized by IgE antibodies (= allergenic epitopes) can be categorized into linear (continuous) and conformational (discontinuous) epitopes (Shriver and Yang 2011).

Soybean (*Glycine max*) is an attractive ingredient for the production of a variety of foods due to its high functionality as well as nutritional value. However, soy belongs to the eight priority food allergens ("big 8") that are believed to be responsible for 90 % of all IgE-mediated allergic reactions in the U.S. (Taylor and Hefle 2001). The European Food Safety Authority (EFSA) reviews reported that soy allergy prevails among 2.7 % of the population. Up to now, eight allergenic soy proteins (Gly m1 - Gly m8) have been registered by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (www.allergen.org), but only the two storage proteins β -conglycinin (Gly m5) and glycinin (Gly m6) have been related to severe allergic reactions (Holzhauser et al. 2009).

Although symptoms of food allergies can be treated with medicine, including antihistamines and epinephrine, the only prophylactic approach hitherto to avoid an allergic response is the complete dietary exclusion of the offending food. So far, as food allergies emerged considerably in the last years, science and industry were searching for new thermal and nonthermal technologies to control food allergy, in particular soybean allergy (Shriver and Yang 2011; Verhoeckx et al. 2015). Hypoallergenic foods currently available on the market are mainly produced using enzymatic hydrolysis, which has proven to be efficient to attenuate soy immunoreactivity (Wilson et al. 2005). Nevertheless, only complete hydrolysis up to amino acid level was shown to reduce the allergenicity to a level of safe consumption. The reason for mostly incomplete allergen destruction is the compact structure and folding of proteins, thus, epitopes are difficult to access or not susceptible for the proteolytic enzymes. In addition, enzymatic hydrolysis can have an adverse impact on food structure and organoleptic properties, liberating a strong bitter taste, which

impedes the utilization as food ingredient (Seo et al. 2008; Shriver and Yang 2011).

Nonthermal processing technologies, in particular high pressure processing (HPP), have emerged in the field of food science in the last decade as effective food preservation alternatives to conventional heat treatment. The main application of HPP is the decontamination of foods, thereby extending shelf life, with little effects on flavor and nutritional value (Knorr et al. 1992; Oey et al. 2008; Tao et al. 2014; Guerrero-Beltran et al. 2004). HPP is known to unfold proteins due to its substantial impact on tertiary and quaternary structure of proteins, mainly maintained by noncovalent (hydrogen, ionic and hydrophobic) interactions between amino acid side chains. While most applications of HPP are the inactivation of enzymes, i.e. lipoxygenase (LOX) (van der Ven et al. 2005), there is evidence that HPP can reduce immunoreactivity of proteins and might stabilize or even activate enzymes (Dhakal et al. 2014; Eisenmenger and Reyes-De-Corcuera 2009). Recent research focused on the effect of HPP on immunoreactivity, which is explained by different mechanisms, i.e. protein-denaturation and thus the induction of protein conformational changes, leading to unfolding of proteins into monomers, and allergen removal by extraction (Kato et al. 2000; Houska et al. 2009; Li et al. 2012).

HPP combined with enzymatic hydrolysis may have a practical relevance as an attractive technology for modifying allergy-relevant epitopes by promoting hydrolysis due to enhanced accessibility to enzymes. However, few researchers have ascertained this process (Peñas et al. 2004; Garcia-Mora et al. 2015; Chicon et al. 2008). In particular Peñas and colleagues confirmed a positive impact of HPP on hydrolysis of soybean whey proteins as they observed a reduced Gly m1 immunoreactivity combining HPP and hydrolysis using various food-grade enzymes (Peñas et al. 2004; Peñas et al. 2006b; Peñas et al. 2006a).

However, data about the effect of HPP combined with hydrolysis on the reduction of other potent soy allergens (Gly m5 and Gly m6) remain unstudied. As these two proteins are the most abundant storage proteins of soy and sensitization against these proteins is highly indicative for severe allergic reactions (Holzhauser et al. 2009), the evaluation of their residual immunoreactivity is indispensable for the assessment of the potential allergenicity of the modified food.

Therefore, the aim of this study was to determine the effect of HPP (100 - 600 MPa) prior to and during enzymatic hydrolysis on (i) the degradation of major soy allergen Gly m5 (SDS-PAGE and LC-MS/MS), and (ii) the residual immunoreactivity (sandwich ELISA) using mouse monoclonal anti-Gly m5 antibodies (mAbs). (iii) As the sensory and physicochemical properties of the resulting hydrolysates are of overriding importance for food industry, these characteristics have been analyzed.

The food-grade Flavourzyme preparation was applied for enzymatic hydrolysis as this enzyme is - outstandingly effective by reducing or eradicating the bitter taste (Meinlschmidt et al. 2016a). A bitter taste is often perceived in protein hydrolysates due to the emergence of bitter peptides and might impair their application as food ingredients,. However, Flavourzyme is not able to sufficiently degrade soy proteins at atmospheric pressure (Meinlschmidt et al. (2016b) and the enzyme hydrolysis might be promoted by HPP treatment.

2. Material and Methods

2.1. Raw materials and chemicals

Untoasted soybeans (*Glycine max* (L.) MERR.) were purchased from Naturkost Ernst Weber (Munich, Germany). The enzyme preparation Flavourzyme® 1000 L (1000 LAPU/g, Leucine Amino Peptidase from *Aspergillus (A.) oryzae*) was kindly provided by Novozymes A/S (Bagsvaerd, Denmark).

All chemicals used in this study were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany) unless stated otherwise.

2.2. Preparation of soy protein isolates (SPI)

SPI was prepared from soybean seeds using the technique as described elsewhere (Meinlschmidt et al. 2016b). The SPI obtained had a protein content of 94.6 % and a dry matter content of 94.4 %.

2.3. High pressure (HP) Equipment and high pressure processing (HPP) assisted enzymatic hydrolysis of SPI

2.3.1. HP Equipment

Pressurization was conducted in a laboratory system with indirect pressure generation (High Pressure Single Vessel Apparatus U4000, Institute of High Pressure Physics, Warsaw, Poland), a maximum operating pressure of 800 MPa, a volume of approximately 750 mL and a theoretically operable temperature range of 25 to 80 °C. This unit is composed of a biphasic pressure build-up. With the initial pump (piston) a pressure with 50 MPa less of the desired pressure is achieved up to 600 MPa. The fine tuning up to the desired pressure level is done in a second phase by another intensifier. Both intensifiers have a transformation ratio of 1:16. The pressure build-up up to 600 MPa took 5 s per 100 MPa (30 s from 0.1 to 600 MPa). A mixture of propane-1,2-diol (Sigma-Aldrich Corporation, St. Louis, Missouri) and deion. water (1:1) was used as pressure transferring liquid (PTL) for the experiments.

2.3.2. HPP assisted enzymatic hydrolysis

For all experiments described below, SPI dispersions containing a protein concentration of 5 g per 100 mL deion. water were prepared and adjusted from initially pH 6.7 to pH 8.0 with 1 M NaOH prior to treatment. Two sets of experiments were performed and are visualized in Figure 1 (A: HPP prior to enzymatic hydrolysis; B: HPP during enzymatic hydrolysis).

HPP prior to enzymatic hydrolysis. SPI dispersions were packed into in bags made of pressure-stable foil (Whirl Pak, Nasco, Fort Atkinson, Wisconsin, USA) and vacuum sealed (PlusVac 23, Komet Maschinenfabrik GmbH, Plochingen, Germany). The vessel was tempered with a connected water bath (DC10-K20, Thermo Haake GmbH, Karlsruhe, Germany) to a specific temperature between 38 and 48 °C to reach a final temperature of 50 °C due to adiabatic compression (temperature increase: 2 °C per 100 MPa). Samples were given into the high pressure unit prior to treatment and subjected to pressures of 100, 200, 300, 400, 500 and 600 MPa for dwell times of 15 min. The temperature was continuously controlled in the PTL

by a circulating thermostatic bath. All samples were treated under isothermal and isobaric conditions during pressure dwell time. Directly after pressure release, the pre-treated SPI dispersions were enzymatically hydrolyzed at atmospheric pressure (0.1 MPa) at 50 °C for 15 min in thermostatically-controlled reaction vessels using the enzyme preparation Flavourzyme at an enzyme to substrate ratio (E/S) of 0.5 % (w/v) without pH adjustment during hydrolysis.

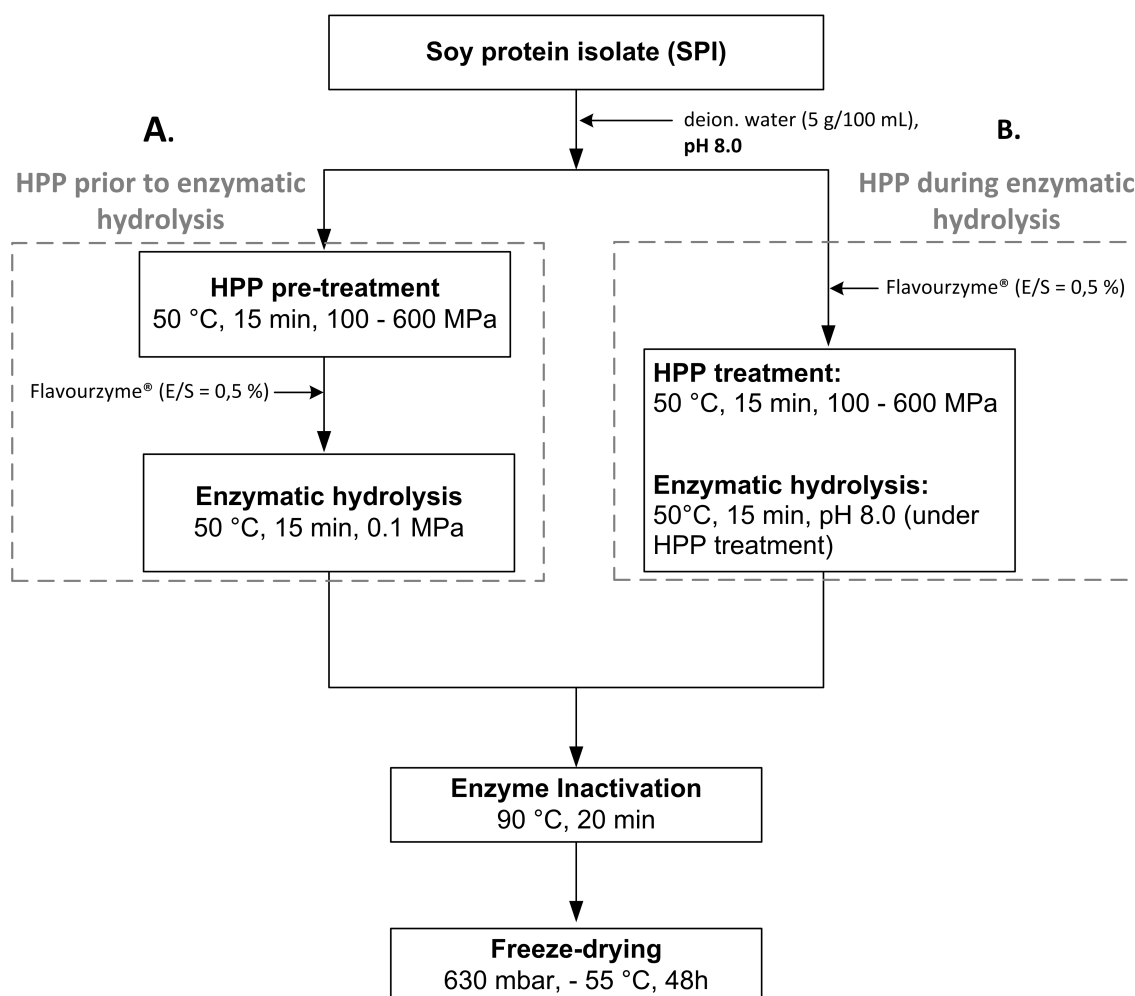


Fig. 1. Experimental set-up of high pressure processing (A.) prior to and (B.) during enzymatic hydrolysis using the enzyme preparation Flavourzyme®.

HPP during enzymatic hydrolysis. The enzyme preparation Flavourzyme was added to the SPI dispersions at an E/S of 0.5 % (w/v). The samples were vacuumed into pressure-stable bags, heat sealed and pressurized at 100, 200, 300, 400, 500 and 600 MPa for 15 min at 50 °C. All samples were treated under isothermal and isobaric conditions during pressure dwell time.

Preparation of control samples. Two different control samples were prepared. First, SPI dispersions were also pressurized (200, 400, and 600 MPa, 50 °C) for 15 min without enzyme addition. Secondly, enzymatic hydrolysis was carried out at atmospheric pressure (0.1 MPa) at 50 °C for 15 min without pressure treatment.

Enzyme activity was terminated by heat treatment at 90 °C for 20 min in a water bath. All samples were freeze-dried utilizing a lyophilisator BETA 1-8 (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). Therefore the samples were frozen to -50 °C prior to freeze-drying. Samples were lyophilised at a pressure of 630 mbar with an increasing shelf temperature continuously from 5 to 40 °C for 48 h. Temperature of the ice condenser was -55 °C.

2.4. Determination of the extent of hydrolysis

2.4.1. Degree of hydrolysis (DH) using the *o*-phthaldialdehyde (OPA) method

The DH was measured in triplicate by quantification of cleaved peptide bonds by the OPA spectrophotometric assay using serine as a standard according to Nielsen et al. (2001).

2.4.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight distribution of the samples were determined according to Laemmli (1970) using SDS-PAGE analysis under reducing conditions. Sample preparation and running conditions were chosen as described in (Meinlschmidt et al. 2016b). Protein visualization was performed by Criterion Stain-Free Gel Doc™ EZ Imager (Bio- Rad).

2.5. Protein identification by liquid chromatography tandem mass spectrometry (LC-MS/MS)

2.5.1. Sample preparation for LC-MS/MS analysis

The sample preparation prior to LC-MS/MS analysis was performed similar to Meinschmidt et al. (2016a) . As described there, each gel lane was cut into slices. Six slices were excised from the lanes corresponding to the samples, which were HPP treated prior to enzymatic hydrolysis. Seven slices were excised from the lanes corresponding to the samples, which were treated with HPP during enzymatic hydrolysis. Protein digestion and dissolution of the peptide extracts were performed as described recently (Schröder et al. 2015; Meinschmidt et al. 2016a).

2.5.2. LC-MS/MS analysis

LC-MS/MS analysis was performed using a nano-flow ultra-high pressure liquid chromatography system (RSLC, Thermo Fisher Scientific) coupled online to a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). The peptide samples were analyzed as described recently (Schröder et al. 2015; Meinschmidt et al. 2016a) using a 50 cm reversed phase separation column (ReproSil-Pur 120 C18-AQ, C18 particle 2.4 μm size, 50 cm length, 75 μm i.d., pore size 120 Å, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany).

2.5.3. Database query and analysis of proteomic data

Raw MS data were processed using MaxQuant software (Version 1.5.3.8, Cox and Mann, 2008) and searched against Glycine max UniProtKB/Swiss-Prot database with a false discovery rate of 0.01 on protein and peptide level.

2.6. Determination of the immunoreactivity

2.6.1. Sandwich enzyme-linked immunosorbent assay (ELISA)

Quantification of β -conglycinin (Gly m5) was performed with sandwich ELISA using two different capture Gly m5-specific mAbs (Izimab-Glym5-3, Izimab-Glym5-4) as previously described in Meinschmidt et al. (2016c).

Capture antibodies were immobilized onto 96-well plates (maxisorp; Nunc, Germany) at 4 °C overnight. Plates were washed three times with phosphate buffered saline, 154 mM NaCl, 0.05 % Tween (3xPBS-T) and blocked with Superblock blocking buffer (Life Technology GmbH, Germany). Suspensions of SPI and all other samples were centrifuged at 15, 000 x *g* for 5 min, and soluble supernatants were incubated for 1 h at RT in duplicate. After a further wash-step (3xPBS-T), the POD-conjugated detection-antibody (Izimab-Glym5-4, Izimab-Glym5-5) was incubated 1 h at RT. POD activity was determined following a third wash-step (3xPBS-T) by incubating the plate with TMB-E substrate (3,3',5,5'-tetramethylbenzidine) (DUNN Labortechnik, Asbach, Germany) and yielding a yellow color after acidification. Color intensity represents a signal for bound detection antibodies and was measured at 450 nm. Signal intensities were calculated by extrapolation to a calibration curve from standard-SPI consisting of seven known concentrations (2000, 500, 125, 31.3, 7.8, 2.0, and 0.5 ng mL⁻¹). Each sample was analyzed at least by duplicate.

2.7. Chemical composition and physicochemical properties

2.7.1. Chemical composition

The protein and dry matter content was determined as described by AOAC methods using a protein calculation factor of N x 6.25 (AOAC 2005a, b).

2.7.2. Physicochemical properties

Protein solubility. Protein solubility (pH 4.0 and 7.0) was determined in duplicate following the method of Morr et al. (1985). Protein solubility was calculated according to the formula:

$$\text{Protein solubility (\%)} = \frac{\text{Total volume extract (mL)} \times \text{Protein content supernatant (mg/mL)}}{\text{Initial weight of residue (mg)} \times \text{Protein content of residue (\%)}} \cdot 100$$

Equation 1

Emulsifying capacity. The emulsifying capacity was analyzed in duplicate as suggested by Wang and Johnson (2001):

$$\text{Emulsifying capacity (mg/mL)} = \frac{\text{Oil added (mL)}}{\text{Initial weight of protein product (g)}}$$

Equation 2

Water- and oil-binding capacity. Water-binding capacity (WBC) was analyzed according to the AACC 56-20 official method (AACC 2000). Oil-binding capacity (OBC) was determined as described by Ludwig et al. (1989).

Foaming properties (activity, density, and stability). Foaming activity was determined according to Phillips et al. (1987). The relation of foam volume before and after whipping was utilized for the calculation of foaming activity:

$$\text{Foaming activity (\%)} = \frac{\text{Foam volume after whipping (mL)}}{\text{Volume before whipping (mL)}} \cdot 100$$

Equation 3

Foaming density and stability were analyzed as previously described in (Meinlschmidt et al. 2016b).

2.8. Sensory analysis

Sensory evaluation was performed using quantitative descriptive analysis (QDA) according to ISO 6564:1985 and as previously described in Meinlschmidt et al. (2016b). A 10-member sensory panel (7 female, 3 male) was selected and carefully trained according to the guidelines in ISO 8586-1:1993. A broad list of attributes in terms of smell (beany, rancid, green), taste (beany, bitter, green), and mouthfeeling (astringent, mouthcoating) have been elaborated by the panel and the attributes were rated on the basis of a 10-cm continuous scale, ranging from 0 (= no perception) to 10 (= strong perception).

For sensory evaluation, samples were mixed with tap water to a protein concentration of 2.5 % (w/w). The panel was presented with four samples (10 mL) per session (performed in four sessions, 1 h each), served in randomized order at RT (20 ± 3 °C) in small plastic cups, coded by arbitrary numbers (three digits). Water and plain crackers were provided for palate cleansing in between. The panel was instructed to assess the attributes (smell, taste, and mouthfeeling) on the basis of the 10-cm continuous scale. Each panellist did a monadic evaluation in duplicate.

2.9. Statistical analysis

All data are expressed as the mean \pm standard deviation (SD) of at least duplicate measurements from two independent experiments ($n=4$). Data were analyzed using SPSS version 21 computer program (SPSS Statistical Software, Inc., Chicago, IL, USA). The data obtained by the degree of hydrolysis and sandwich ELISA analysis were analyzed following one-way analysis of variance (ANOVA), where mean comparison of data were generated and adjusted using Games-Howell post hoc test. Statistical significance was established at $p < 0.05$ to describe means with 95 % confidence.

In order to identify similarities and differences among the samples and relations between the sensory attributes, mean QDA data from sensory analysis were processed by multivariate statistical principal component analysis (PCA) using R-based PanelCheck software (Nofima, Ås, Norway; version 1.4.0.).

3. Results and Discussion

Reaction conditions for enzymatic hydrolysis (50 °C, pH 8.0) were chosen according to the Flavourzyme specification. Reaction time was set to 15 min for a better comparison with literature data (Peñas et al. 2004; Peñas et al. 2006a; Peñas et al. 2006b). Due to the high cost of pressure vessels, pumps, intensifiers, and sealing systems, the economic holding times for industrial application of 5 min or less are desired and are limited to holding times no longer than 10 min (FDA 2015). However, preliminary experiments showed that a holding time of 10 min was less effective as 15 min in order to ensure sufficient destruction of major soy allergens, which was assessed by an in-house sandwich ELISA applying polyclonal rabbit Abs.

3.1. Effects of HPP on the extent of hydrolysis of SPI

3.1.1. Degree of hydrolysis (DH)

Untreated SPI showed a mean DH value of 2.1 %, whereas all treated samples exhibited a significantly ($p < 0.05$) higher DH value (Figure 2). The DH value of pressurized samples without enzyme addition (control sample) showed no changes compared to untreated SPI (data not shown).

The highest DH value of 9.6 % was achieved when hydrolysis was performed during HPP at 100 MPa, but a considerable diminution was detected with rising pressure. These results might suggest an activation of proteolytic enzyme activity at moderate pressures of 100 and 200 MPa, while a further rise of the pressure from 300 MPa to 600 MPa presumably caused a loss of enzyme activity or efficiency. It has been reported that pressures above 300 MPa might have adverse effects on the substrate-enzyme interaction (Stapelfeldt et al. 1996). Changes in the three-dimensional structure of the enzyme could also affect its active site, resulting in a modified activity or a change in substrate specificity (Claeys et al. 2003). Although the enzyme activity was negatively affected by HPP at pressures above 200 MPa, the enzyme was still able to hydrolyze the unfolded protein, which resulted in a higher or even comparable DH value than that of the control sample.

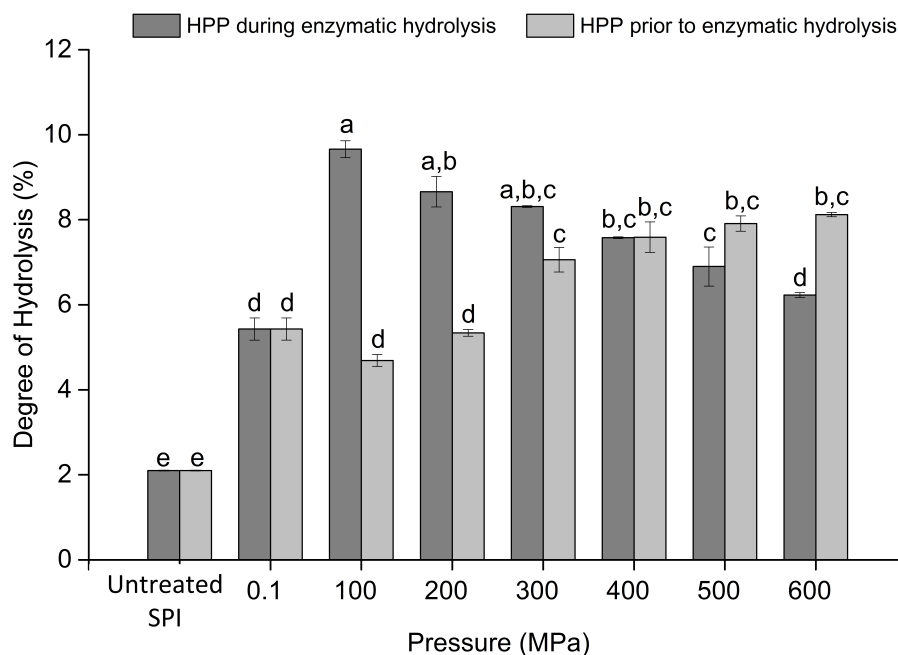


Fig. 2. Degree of hydrolysis of untreated SPI, SPI hydrolyzed at atmospheric pressure (0.1 MPa) and HPP (100-600 MPa) by Flavourzyme: HPP during and prior to enzymatic hydrolysis at 50 °C for 15 min (pH 8.0). Each value is expressed as means \pm SD of duplicate measurements from two independent experiments ($n=4$). *a,b,c,d,e*: different subscripts indicate significant differences ($p < 0.05$; one-way ANOVA, Games-Howell).

An opposite trend was observed for HPP applied prior to hydrolysis, where the DH continuously increased up to 8.1 % with higher pressures (600 MPa). Interestingly, applying 100 MPa and 200 MPa, a significant ($p < 0.05$) increase of the DH could not be found in contrast to the samples obtained after HPP treatment during enzymatic hydrolysis. HPP treatment at moderate pressure levels (100-300 MPa) prior to enzymatic degradation seems to be not strong enough for protein unfolding, making the proteins more susceptible for enzyme degradation. This corroborates the assumption made above that higher DH values obtained by HPP treatment during hydrolysis could mainly be attributed to the enzyme activation. At HPP higher than 200 MPa, DH values were significantly ($p < 0.05$) higher than the control sample. The major storage proteins of soy have a globular structure that makes them more resistant to digestive enzyme hydrolysis. The application of HPP changes the conformation of the respective proteins and makes them more susceptible for enzymatic hydrolysis. Probably the exposure of new cleavage sites on the substrate through

several pressure-induced phenomena such as protein unfolding (denaturation) and enhancement of structural flexibility enhance enzyme-assisted protein degradation as the accessibility for enzymes was increased (Kajiyama et al. 1995). Puppo et al. (2004) found that HPP higher than 200 MPa produced important changes of proteins i.e. partial unfolding of 7S and 11S globulins. Although the mechanism behind HPP-induced protein unfolding is not yet fully understood, it has been suggested that HPP causes changes in protein structure through cleavage of noncovalent bonds (Tedford et al. 1999).

3.1.2. Molecular weight distribution (SDS-PAGE)

As shown in Figure 3, untreated SPI (lane 2) presents a typical electrophoretic profile characteristic for soy proteins with high intensity bands corresponding to Gly m5 (β -conglycinin) (Amnuaycheewa and de Mejia 2010; Meinlschmidt et al. 2016a). The first bands at approximately 72, 68, and 53 kDa are the α , α' and β subunits of β -conglycinin, respectively. The protein identification by means of LC-MS/MS was already described in Meinlschmidt et al. (2016a).

SDS-PAGE profiles of both HPP prior to and during hydrolysis showed the same molecular weight distribution, thus, only the electrophoretic profile of HPP during hydrolysis is shown in Figure 3. There is a controversy regarding SDS-PAGE and DH value results at 100 and 200 MPa, where both treatments showed an opposing trend for the DH with rising pressure. This could likely be ascribed to the modification of the substrate or binding of substrate to enzyme induced by HPP, which might affect the size of peptides produced during hydrolysis. Pressurization alone without enzyme addition did not visually alter the electrophoretic profile of SPI (Figure 3, lane 3-5). SDS-PAGE profile confirmed that hydrolysis at 0.1 MPa (Figure 3, lane 6) was less effective than combined with HPP as only Gly m5 subunits were slightly affected, which was previously reported by Meinlschmidt et al. (2016b). Digestion concomitantly or subsequent to HPP - regardless of the pressure level applied - led to qualitative differences in the molecular weight distribution (Figure 3, lane 7-12). Protein bands corresponding to Gly m5 were absent or drastically reduced, consequently a greater intensity was observed at low molecular weight bands at approximately 15-20 and 23-30 kDa as indicated in Figure 2 (lane 9-12). These bands might be derived from the proteolytic action of Flavourzyme on different soy proteins into smaller peptides, suggesting these peptides are fragments of Gly m5.

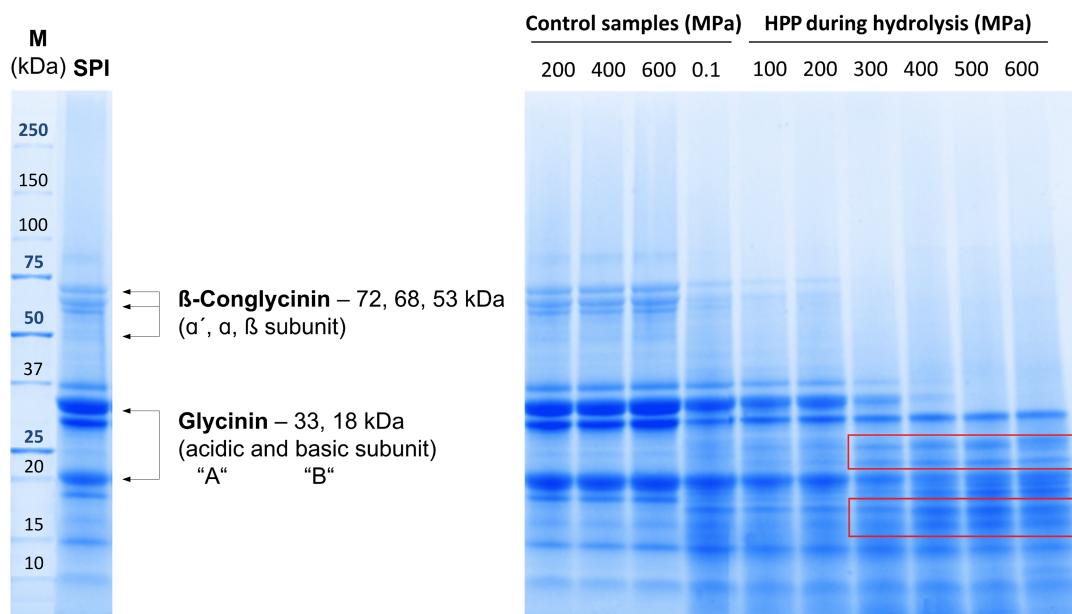


Fig. 3. SDS-PAGE profiles of SPI, control samples and SPI hydrolysates prepared with Flavourzyme: HPP during enzymatic hydrolysis at 100 to 600 MPa (50 °C, 15 min, and pH 8.0). M- Molecular weight standard indicated in kilo Dalton (kDa); SPI- soy protein isolate. Electrophoresis was carried out with 4-20 % polyacrylamide gradient gels. α (α), α' (α') and β (β) subunits of β -conglycinin.

3.1.3 Identification of degraded proteins by liquid chromatography tandem mass spectrometry (LC-MS/MS)

Considerable effects on the electrophoretic profile due to HPP treatment (prior to/during) combined with enzymatic hydrolysis were evident at pressures above 300 MPa. LC-MS/MS analysis was conducted using the samples prepared with HPP at 400, 500 and 600 MPa (prior to/during enzymatic hydrolysis), to corroborate the enzymatic degradation of Gly m5 and to tentatively identify the emerging bands (Figure 2) to be derived from the proteolytic action.

The MS results are presented exemplarily for the three subunits (α , α' and β) of β -conglycinin (Gly m5) in Figure 4. Untreated SPI showed a typical electrophoretic profile characteristic for soy proteins like Gly m5, which was confirmed by the LC-MS/MS determined abundance of the individual subunits with respect to their molecular weight distribution (Figure 3, SPI). Furthermore, LC-MS/MS data (Figure 4, 400, 500 and 600 MPa) confirmed a degradation of all

subunits of β -conglycinin due to the treatment of SPI with HPP combined with enzymatic hydrolysis, which could result in the observed reduced band intensities at approximately 70-75 ($\langle\alpha>'$), 65-70 ($\langle\alpha>$) and ≈ 50 ($\langle\beta>$) kDa.

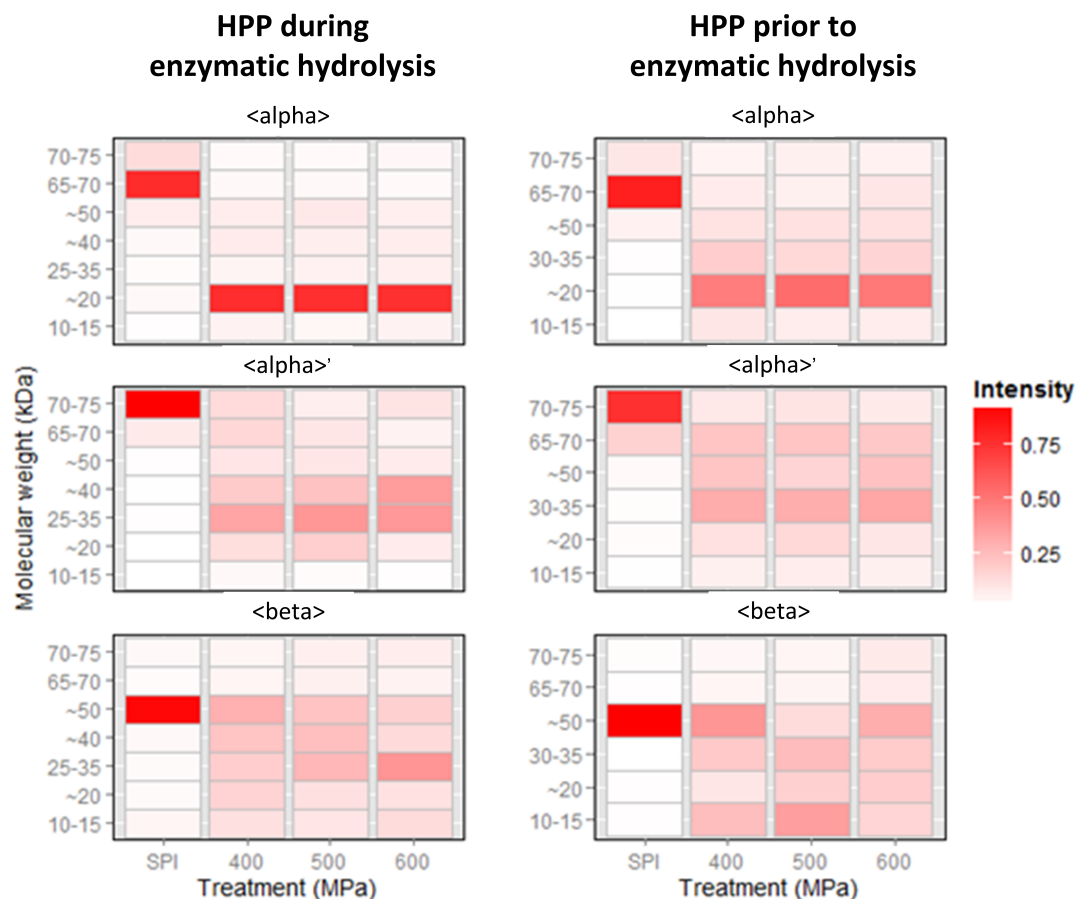


Fig. 4. Relative abundance of the three subunits ($\langle\alpha>$, $\langle\alpha>'$ and $\langle\beta>$) of β -conglycinin (Gly m5) in the analyzed sections of the SDS-PAGEs determined by LC-MS/MS. The abundance of the individual subunits is presented as the percentage of the intensity of the respective subunit added up for all excised bands within one treatment. The results are visualized in dependence on the molecular weight determined according to the position of the analyzed SDS-gel bands.

The results also indicate a proteolytic cleavage into fragments of about 15 to 35 kDa (Figure 3 and 4). LC-MS/MS analysis indicated that these fragments derived from Gly m5 contribute to the observed low molecular weight bands (3.1.2, Figure 3). Especially the alpha subunit (65-70 kDa) seemed to be hydrolyzed in equal fragments with a molecular size of about 20 kDa, as indicated by the deter-

mined sequence coverage (Table 1). In addition, Figure 5 visualizes the sequence coverage of the identified tryptic peptides of the purified full length alpha subunit of β -conglycinin (66.9 %) and degraded protein at approximately 20 kDa (57.7 %, HPP during enzymatic hydrolysis at 400 MPa). Consequently, the remaining immunoreactivity measured by sandwich ELISA (Figure 6) could be explained by the existence of low-molecular weight fragments, which may still contain the required epitope for antibody binding, thus contributing to the residual allergenicity. A similar molecular weight distribution of the degraded proteins regardless of the HPP treatment was also observed. A significant difference in the abundance of the proteins with respect to the application of different pressure levels cannot be stated based on the LC-MS/MS results, because no biological replicates have been measured.

Sequence coverage <alpha> subunit of β -conglycinin (P13916)

SPI (65 - 70 kDa)

Signalpeptide Propeptide Protein Sequence →

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MMRARFPLLGLLVFLASVSVSGIAYWEKENPKHNKCLQSCNSERDSYRNQACHARC�LLKVEKEECEEGEIPRFRPQHPEREPQQPGEKEEDEDEQ
PRPIPFPRPQPRQEEHEQREEQEMPRKEEKRGEKGSSEEEDEDEDEEQDERQFFFRPPHQQEERNEEEDDEEQRESESESESELRRHKCNKPNPFLFGS
NRFETLQKQYGRIRVLQRFNQRSPLQNLRDYRILEFNKPNNTLLLPNHADADYLIVILNGTAILSLVNNDDRDSYRLQSGDALRVPSGTTYVVPDN
NENLRLITLAIIPVNKPRFESFFLSSTEAAQSYLQGFNRNILEASYDTKFEEINKVLFVSREEGQQQGEQRLOESVIVEISKEQIRALSKRAKSSSRKTIS
SEDKFFNLRSDPIYSNKLGFKEITPEKNPQLRDLDIFLSIVDMNEGALLLPHFNSKAIIVILVINEGDANIELVGLKEQQQEQQEQEOPLEVRKYRAEL
SEQDIFVIFAGYFVVVFNATSNLNFFAIGINAENNRNFLAGSQDNVISQIPSQVQELAFPGSAQAVEKLLKNQRESYFVDAQPKKKEEGNKGRKGPLSSI
LRAFY
    
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400 (≈ 20 kDa)

Signalpeptide Propeptide Protein Sequence →

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MMRARFPLLGLLVFLASVSVSGIAYWEKENPKHNKCLQSCNSERDSYRNQACHARC�LLKVEKEECEEGEIPRFRPQHPEREPQQPGEKEEDEDEQ
PRPIPFPRPQPRQEEHEQREEQEMPRKEEKRGEKGSSEEEDEDEDEEQDERQFFFRPPHQQEERNEEEDDEEQRESESESESELRRHKCNKPNPFLFGS
NRFETLQKQYGRIRVLQRFNQRSPLQNLRDYRILEFNKPNNTLLLPNHADADYLIVILNGTAILSLVNNDDRDSYRLQSGDALRVPSGTTYVVPDN
NENLRLITLAIIPVNKPRFESFFLSSTEAAQSYLQGFNRNILEASYDTKFEEINKVLFVSREEGQQQGEQRLOESVIVEISKEQIRALSKRAKSSSRKTIS
SEDKFFNLRSDPIYSNKLGFKEITPEKNPQLRDLDIFLSIVDMNEGALLLPHFNSKAIIVILVINEGDANIELVGLKEQQQEQQEQEOPLEVRKYRAEL
SEQDIFVIFAGYFVVVFNATSNLNFFAIGINAENNRNFLAGSQDNVISQIPSQVQELAFPGSAQAVEKLLKNQRESYFVDAQPKKKEEGNKGRKGPLSSI
LRAFY
    
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Fig. 5. Sequence alignment of the amino acid sequences of untreated SPI and the samples prepared with HPP during enzymatic hydrolysis at 400 MPa, and sequence coverage of the identified tryptic peptides of the purified <alpha> subunit of β -conglycinin (Gly m5) and degraded protein at approximately 20 kDa.

Table 1

Comparison of the sequence coverage of the α subunit of $\beta</math>-conglycinin (Gly m5) at 65-70 kDa and 20 kDa in dependence on the sample treatment.$

	Sequence coverage alpha subunit Gly m5 (%)*			
	SPI	400 MPa	500 MPa	600 MPa
	65 - 70 kDa	20 kDa	20 kDa	20 kDa
HPP during enzymatic hydrolysis	66.9	57.7	57.7	59.2
HPP prior to enzymatic hydrolysis	68.3	59.7	58.3	63.0

*Sequence coverage: The percentage of the protein sequence covered by supporting peptides.

3.2. Effects of HPP assisted enzymatic hydrolysis on the residual immunoreactivity

The effect of HPP (200, 400, and 600 MPa, 50 °C, 15 min) without enzyme addition on soy immunoreactivity was investigated in preliminary trials using an in-house inhibition assay with polyclonal rabbit anti-soy antibodies. Application of HPP alone did not alter the immunoreactivity of untreated SPI (data not shown).

Three newly developed mouse monoclonal anti-Glym5 mAbs were used as capture or detection antibodies to establish a sandwich ELISA. These antibodies are comparable with the corresponding antibodies in the human system as recently shown by comparing western blots using human sera or alternatively Izimab-Glym5-3, Izimab-Glym5-4 or Izimab-Glym5-5 (Meinlschmidt et al. 2016c). The ELISA results confirm the reduction in residual immunoreactivity as visualized in Figure 6A and B using the two different sets of antibody pairs Izimab-Glym5-3/Izimab-Glym5-4 and Izimab-Glym5-4/Izimab-Glym5-5, respectively, recognizing different epitopes of $\beta</math>-conglycinin. Therefore, the result of ELISA analysis represents the concerted immunoreactivity of both epitopes. The immunoreactivity was calculated in relation to untreated SPI, which was set to 100 %. A remarkable difference between these two antibody variants could be found. Although the residual immunoreactivity measured by Izimab-Glym5-3/Izimab-Glym5-4 pair (capture/detection mAb) shown in Figure 6A was considerably higher, a similar tendency compared to Izimab-Glym5-4/Izimab-Glym5-5 pair (Figure 6B) could be detected. The results imply that the specific epitope, which can be recognized by the Izimab-Glym5-4/Izimab-Glym5-5 pair was sufficiently destroyed in the course of hydrolysis as the immunoreactivity was drastically reduced (Figure 6B). In contrast, the epitope, which is recognized$

by the Izimab-Glym5-3/Izimab-Glym5-4 pair, was less affected as the immunoreactivity was only slightly reduced (Figure 6A). Hydrolysis at 0.1 MPa already reduced soy immunoreactivity of about 90 % applying the Izimab-Glym5-4/Izimab-Glym5-5 pair, whereas an approximately 30 % reduction could be measured, using the Izimab-Glym5-3/Izimab-Glym5-4 mAb pair (Figure 6A and B).

With focus on the Izimab-Glym5-4/Izimab-Glym5-5 mAb pair (Figure 6B), the application of a pressure of 400 MPa prior to enzymatic hydrolysis reduced the immunoreactivity from approximately 10.% at atmospheric pressure to 0.5 %. A pressure increase to 500 MPa further diminished the residual immunoreactivity to nearly non-detectable (100 %). However, a higher pressure of 600 MPa seems to inhibit the activity of Flavourzyme as the destruction of antigenic binding-sites for antibodies was less effective and a higher residual immunoreactivity of approximately 7.5 % could be measured. The samples obtained after enzymatic hydrolysis during HPP with pressures of 300, 400, 500, and 600 MPa showed an almost complete loss of immunoreactivity taking the Izimab-Glym5-4/Izimab-Glym5-5 pair mAb into consideration (Figure 6B).

HPP probably caused structural changes by disrupting hydrophobic and electrostatic interactions, which might led to unfolding of proteins. Consequently, this HPP-induced protein unfolding makes epitopes more susceptible for proteases. Peñas et al. (2004; 2006a; 2006b) also found a marked reduction in soy Gly m1 and bovine whey β -lactoglobulin immunoreactivity after combining HPP and enzymatic hydrolysis applying different protease preparations, which was evaluate by an indirect ELISA test with test polyclonal rat antibodies.

In conclusion, the ELISA results coincide with the results obtained by SDS-PAGE (Figure 3) and LC-MS/MS (Figure 4 and 5) analyses, where Gly m5 was sufficiently degraded at 400 and 500 MPa, combining HPP prior to and in particular during enzymatic hydrolysis.

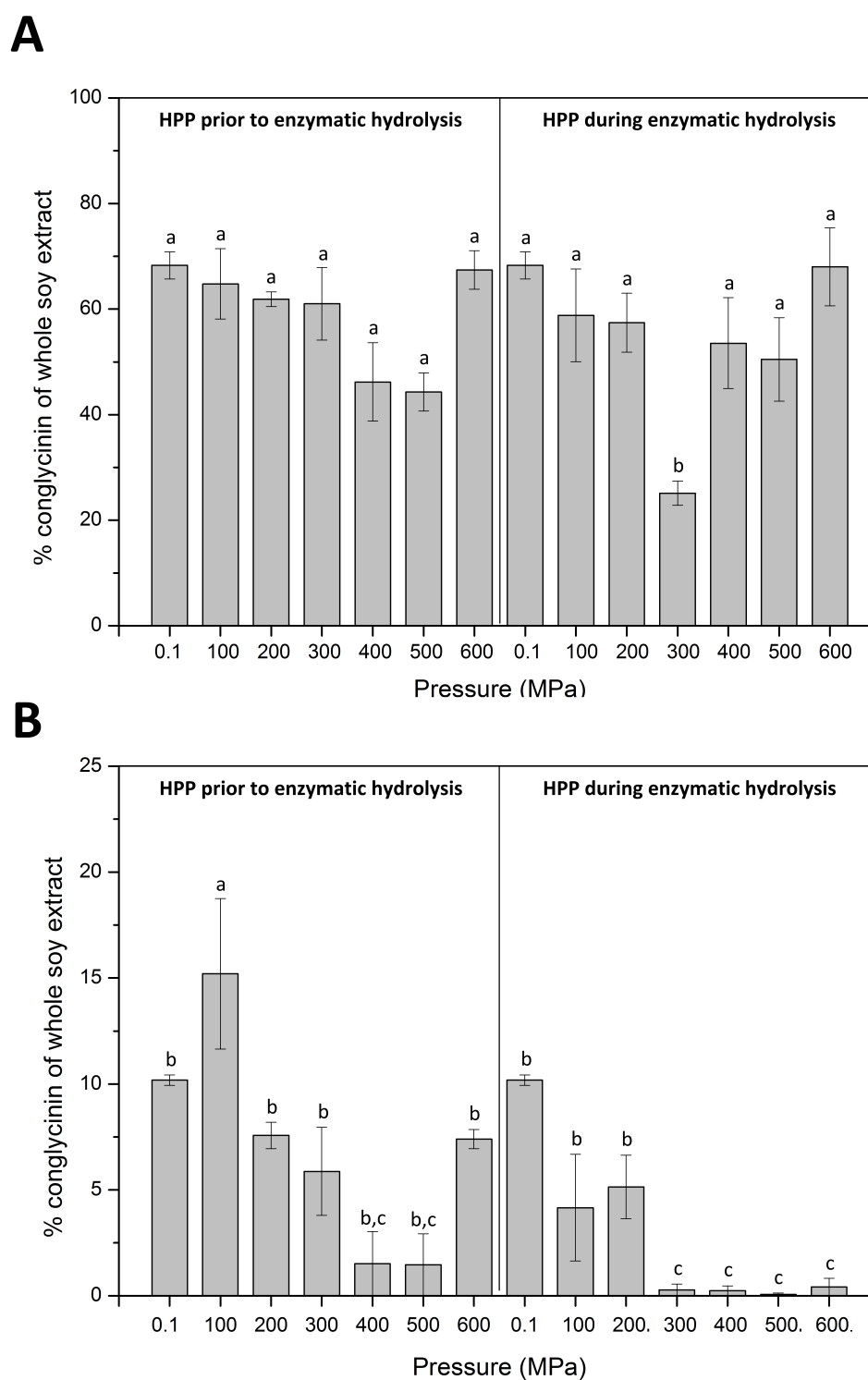


Fig. 6. Sandwich ELISA using different mouse monoclonal anti- β -conglycinin (Gly m5) Abs: (A) Izimab-Glym5-3 and (B) Izimab-Glym5-4. Results of two different sandwich ELISAs are shown. All ELISA values are means \pm SD of duplicate measurements from two independent experiments ($n=4$).^{a,b,c}: different subscripts indicate significant differences ($p < 0.05$; one-way ANOVA, Games-Howell).

3.3. Effects of HPP assisted enzymatic hydrolysis of selected hydrolysates on the sensory and physicochemical properties

The sensory and physicochemical properties of the aforementioned samples in comparison with the untreated SPI were analyzed in detail.

3.3.1. Sensory evaluation

Principal Component Analysis (PCA). PCA was applied on QDA data to analyze the interrelationships among the samples with respect to the sensory attributes. The resulting bi-plot is shown in Figure 7.

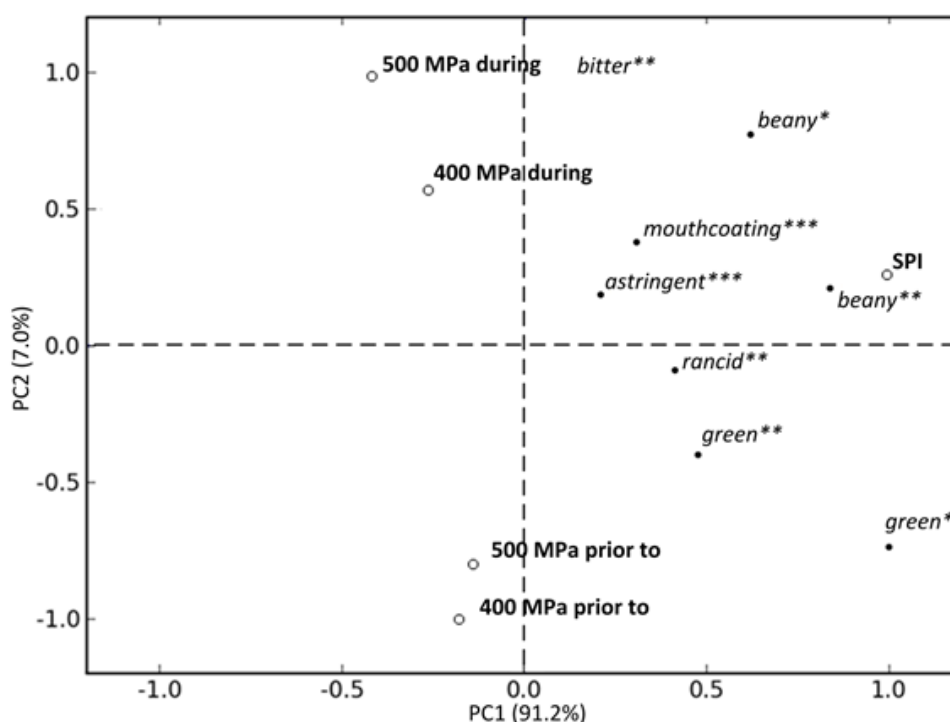


Fig. 7. Bi-plots obtained from PCA of sensory attributes (smell, taste, and mouthfeeling) for untreated SPI and SPI hydrolysates prepared with HPP prior to and during enzymatic hydrolysis at 400 and 500 MPa. *smell; **taste; ***mouthfeeling.

The two principal components (PCs) 1 and 2 describe 91.2 % and 7.0 %, respectively, of the total variance in the data set. The major attributes positively

contributing to the PC1 dimension (Table 2, factor loadings > 0.550) were a *beany* smell/taste (0.621/0.837) and *green* smell (1.000). In contrast, PC2 is mainly described by the attributes *bitter* taste (1.000) as well as *beany* (0.771) and *green* smell (-0.739). Untreated SPI is loaded on the positive PC1 dimension, which means that this sample is mainly described by sensory attributes characteristic for raw soybeans like *rancid*, *beany* and *green*. These undesirable flavors could likely be ascribed to lipid oxidation, polar lipids, and lipoxygenase catalyzed oxidation of soybean oil to volatile compounds such as isopentanol, *n*-hexanal and hexanol (Shogren et al. 2003). *n*-Hexanal is known to contribute a disagreeable green/beany and grass-like aroma to soy proteins (Ames et al., 1984).

Combining HPP with enzymatic hydrolysis, the *green* and *beany* off-flavors of untreated SPI were significantly ($p < 0.05$) reduced in the samples (Figure 6 and Table 2).

Samples treated with HPP prior to and during hydrolysis are located on opposite sides along the PC2 axis: Samples prepared with HPP during enzymatic hydrolysis at 400 and 500 MPa are highly loaded on the positive dimension of PC2, which is described by a *bitter* taste (1.000). This observation might corroborate the assumption that HPP induced a change of enzyme specificity and activity, which might have resulted in the formation of bitter peptides. In contrast, samples prepared with HPP prior to enzymatic hydrolysis at 400 and 500 MPa are loaded on the negative PC2 dimension. These samples are not described by the attribute *bitter*, which can be ascribed to the dominant exoproteolytic activity of Flavourzyme at 0.1 MPa. The key enzyme activity of Flavourzyme at 0.1 MPa is provided by exopeptidases that cleave adjacent to hydrophobic amino acids, which may have led to a lower bitter taste sensation.

3.3.2. Physicochemical properties

The results of the physicochemical properties in terms of protein solubility, emulsifying capacity, foaming properties (activity, stability, and density) as well as water- and oil-binding capacities (WBC/OBC) are summarized in Table 2.

Table 3

Physicochemical properties (protein solubility, emulsifying capacity, water- and oil-binding capacity, foaming activity, density, and stability) of untreated SPI and SPI hydrolysates prepared with HPP (400 and 500 MPa) prior to/during enzymatic hydrolysis.

	Protein solubility		Emulsifying capacity	Water binding capacity	Oil binding capacity	Foaming activity	Foaming stability	Foaming density
	pH 4.0	pH 7.0						
	(%)	(%)	(mL g ⁻¹)	(mL g ⁻¹)	(mL g ⁻¹)	(%)	(%)	(g L ⁻¹)
SPI	5.0 ± 1.1 ^a	44.0 ± 0.6 ^a	660 ± 5 ^a	2.6 ± 0.1 ^a	0.0 ± 0.0 ^a	552 ± 5 ^a	90 ± 0 ^a	215 ± 5 ^a
HPP during enzymatic hydrolysis								
400 MPa	25.3 ± 0.1 ^b	56.9 ± 0.8 ^b	640 ± 10 ^a	0.0 ± 0.0 ^b	1.9 ± 0.1 ^{bc}	1604 ± 28 ^b	56 ± 2 ^b	56 ± 5 ^b
500 MPa	21.4 ± 0.2 ^c	52.7 ± 0.2 ^b	635 ± 5 ^a	0.0 ± 0.0 ^b	1.9 ± 0.0 ^{bc}	1470 ± 0 ^c	58 ± 2 ^b	59 ± 5 ^b
HPP prior to enzymatic hydrolysis								
400 MPa	23.9 ± 0.1 ^d	79.4 ± 0.1 ^c	690 ± 15 ^b	0.0 ± 0.0 ^b	1.7 ± 0.0 ^b	1419 ± 0 ^c	54 ± 2 ^b	61 ± 5 ^c
500 MPa	21.9 ± 0.0 ^d	75.4 ± 0.0 ^c	705 ± 5 ^b	0.0 ± 0.0 ^b	2.1 ± 0.1 ^c	1419 ± 25 ^c	24 ± 4 ^c	62 ± 5 ^c

Results are expressed as means ± standard deviation. The mean difference is significant at a level of $p < 0.05$ as indicated by different superscript letters (a,b,c,d) within one column following one-way ANOVA (Bonferroni).

Untreated SPI exhibit a protein solubility of 44.0 % and 5.0 % at pH 7.0 and pH 4.0, respectively. Solubility at both pH 7.0 and pH 4.0 for all hydrolyzed and HPP treated samples was significantly ($p < 0.05$) enhanced up to 79.4 % and 25.3 %, respectively.

HPP prior to hydrolysis resulted in a solubility (pH 7.0) between 75.4 % and 79.4 % (400 and 500 MPa), followed by 56.9 % and 52.7 % performing the hydrolysis during HPP at 400 and 500 MPa, respectively. The discrepancy between protein solubility at pH 7.0 observed for both HPP prior to and during digestion might be related to the modification of the substrate or binding of substrate to enzyme induced by HPP, which could affect the size and characteristic of peptides produced during hydrolysis.

SPI showed an emulsifying capacity of 660 mL g⁻¹, which was significantly ($p < 0.05$) enhanced to 690 mL g⁻¹ and 705 mL g⁻¹, when HPP was applied prior to hydrolysis at 400 and 500 MPa, respectively. Soluble proteins are surface active and known to promote oil-in-water emulsions. As solubility of proteins is required to form an emulsion, the emulsifying capacity of samples prepared with HPP prior to enzymatic hydrolysis was greatly enhanced as the protein solubility was significantly ($p < 0.05$) increased by approximately 33 % at pH 7.0. The emulsifying capacity of samples prepared with HPP during hydrolysis did not changed compared to untreated SPI, although the protein solubility was also slightly increased. However, the solubility enhancement of about 11 % seems to be not enough for a significant improvement of the emulsifying capacity. Nevertheless, the great emulsifying capacity of untreated SPI could be maintained after HPP treatment and enzymatic hydrolysis.

Enzymatic hydrolysis combined with HPP (prior to/during) led to a complete loss of the WBC of untreated SPI of 2.6 mL g^{-1} . A decreased WBC might be due to the increase in protein solubility as well as the size of protein particles and surface topography, which influences protein-water interactions. In contrast, untreated SPI did not possess an OBC, whereas enzymatic hydrolysis combined with HPP (prior to/during) increased the OBC up to 2.1 mL g^{-1} (HPP prior to enzymatic hydrolysis at 500 MPa).

The initial foaming activity of untreated SPI of about 552 % was significantly ($p < 0.05$) enhanced applying HPP prior to and during enzymatic hydrolysis. The highest foaming activity of 1604 % was achieved after a HPP treatment at 400 MPa during hydrolysis. More soluble proteins/peptides obtained after enzymatic hydrolysis combined with HPP (during/prior to) could diffuse more rapidly to the air-water interface, concentrate and reduce the surface tension efficiently (Tsumura et al. 2005). In contrast, foaming stability and density were reduced. This was expected as smaller peptides as a result of enzymatic hydrolysis and presumably the presence of hydrophobic groups impede the formation of viscoelastic film for foam stabilization.

4. Conclusions

In conclusion, HPP (prior to/during) combined with enzymatic hydrolysis is capable of reducing Gly m5 immunoreactivity and may thus represent a powerful tool in allergenicity-reducing food-processing technology. HPP at pressures higher than 300 MPa for both HPP prior to and during hydrolysis enhanced the proteolytic activity of Flavourzyme. According to the SDS-PAGE and LC-MS/MS results, a sufficient degradation of major soy allergen Gly m5 could be observed. In addition, the sensory and physicochemical properties were maintained or even improved compared to untreated SPI.

As only the immunoreactivity of Gly m5 was measured, immunoreactivity of Gly m6 and other soy allergy related allergenic proteins of soy e.g. Kunitz Trypsin Inhibitor (Gly mTI) or profilin (Gly m3) should be analyzed as well. Another important aspect is the investigation of reactivity with other allergenic components such as Gly m4 (starvation-associated message 22), which is a Bet v 1-related pathogenesis-related protein 10 (PR-10) from soy. Further, the level of immunoreactivity reduction in this study might be important for a lot of allergic individuals depending upon their sensitivity threshold. However, the clinical relevance of these findings

still needs to be verified by in vivo methods i.e. controlled human challenge studies. In addition, a basophil degranulation test (BDT) might constitute a reliable fast test method before running prick test or CAP test.

The microbial counts, in particular pathogens, will be analyzed and intermediate pressure levels between 400 and 500 MPa will be studied to find a proper pressure level for the reduction of allergenic potential of soy. More research is needed to understand the impact of pressure treatment on structural changes of soy proteins. The analysis of i.e. the surface hydrophobicity and conformational characterization using for example circular dichroism spectra (UV-CD), multi-angle laser light scattering (MALLS) as well as quasi-elastic light scattering (QELS) or fourier transform infrared (FTIR) spectroscopy might be powerful tools to corroborate the present results.

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Conflict of Interest

The authors have declared no conflicts of interest.

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CHAPTER 6: The effects of pulsed ultraviolet light, cold atmospheric pressure plasma, and gamma-irradiation on the immunoreactivity of soy protein isolate⁶

Abstract

This study investigates the effect of nonthermal processing technologies on soy immunoreactivity. Soy protein isolate was treated with pulsed ultraviolet (PUV) light, direct and remote cold atmospheric pressure plasma (CAPP), and gamma-irradiation (3 - 100 kGy). Sample weight, surface temperature, hydrogen peroxide content, and pH value have been measured. SDS-PAGE analysis revealed reduced protein intensity bands corresponding to major soy allergens β -conglycinin (Gly m5) and glycinin (Gly m6). Sandwich ELISA using specific mouse monoclonal anti-Gly m5 antibodies (mAbs) confirmed a loss of soy immunoreactivity following PUV light, direct NTAP, and gamma-irradiation with increasing dose and time. The maximum reduction in immunoreactivity (91 - 100 %) in the soluble protein fraction was achieved by direct CAPP as well as PUV light and gamma-irradiation treatment. A decreased immunoreactivity up to 89 % was observed for samples treated with remote CAPP. These innovative technologies might have great potential for industrial application due to their effectiveness in reduction of soy immunoreactivity.

Keywords: Soy immunoreactivity; Sandwich ELISA; cold atmospheric pressure plasma; gamma-irradiation; pulsed ultraviolet light; SDS-PAGE

⁶MEINLSCHMIDT, P., UEBERHAM, E., LEHMANN, J., REINEKE, K., SCHLÜTER, O., SCHWEIGERT-WEISZ, U., EISNER, P. (2016). The effects of pulsed ultraviolet light, cold atmospheric pressure plasma, and gamma-irradiation on the immunoreactivity of soy protein isolate. *Innovative Food Science & Emerging Technologies*. Doi: 10.1016/j.ifset.2016.06.007. Reproduced with permission.

1. Introduction

The ubiquitous presence of allergens in human food supply coupled with an increased awareness of food allergies has intensified increasing effort for developing allergy mitigation methods. Food allergy results from an adverse immunoglobulin E (IgE)-mediated reaction of the immune system towards dietary antigens, commonly proteins. The antigenic determinant of allergenic proteins is called epitope, which can be classified into linear and conformational epitopes (Taylor and Hefle 2001). Although nearly any food is capable of causing an allergic reaction, it was found that nearly 90 % of all allergic reactions in the U.S. are triggered by eight main protein sources, which compromise milk, eggs, fish, crustacean/shellfish, tree nuts, peanuts, wheat, and soy. These foods are called the "big 8", which were defined as "major food allergens" by the Food Allergen Labelling and consumer Protection Act of 2004 (FALCPA).

Allergy to soy is one of the most common food allergies, especially among infants. Up to now, eight allergenic soy proteins (Gly m1 - Gly m8) have been registered by the International Union of Immunological (IUIS) Societies Allergen Nomenclature Sub-Committee (www.allergen.org), but only the two storage proteins β -conglycinin (Gly m5) and glycinin (Gly m6) have been identified as related to severe allergic reactions (Holzhauser et al. 2009). Although the prevalence of soy allergy is not precisely known, it is expected to escalate due to the increasing consumption of soy-containing food products (FAO 1995).

Total avoidance of soy-containing foods and prompt treatment to allergic shocks with medicine i.e. epinephrine are still likely to be the only way to avoid severe outcome. Consequently, a great demand for methods, which reduce food allergens without affecting the nutritional value, is becoming a popular topic of various research activities. Science and industry are searching for thermal and nonthermal technologies to control soy allergy by modifying epitopes (Shriver and Yang 2011). The technological approach hitherto has mainly been focused on thermal technologies, which commonly retain the ability of soy to elicit an immune response. More recently, nonthermal food processing technologies have emerged in the food industry due to their negligible effects on food properties (Huang et al. 2014; Shriver and Yang 2011). Different studies have shown that nonthermal technologies such as high pressure processing (HPP) hold a great promise for the development of food ingredients with reduced allergenicity (Meinlschmidt et al. 2016a; Tammineedi et al. 2013; Yang et al. 2010).

Besides HPP, high-energy, short-wavelength electromagnetic gamma(γ)-irradiation

turned out to be an effective preservation method, extending the shelf-life of perishable foods (Kasera et al. 2012). Although the utilization of gamma irradiation is limited to a few food applications depending on the individual national legislation, the ability of irradiation to reduce the allergenicity of quite different allergenic food products, including almond, cashew nut, walnut proteins, ovalbumin, bovine serum albumin, milk proteins (beta-lactoglobulin) as well as shrimp tropomyosin and legume proteins (kidney bean, peanut, black gram) has been reported (Byun et al. 2002; Kasera et al. 2012; Seo et al. 2007; Su et al. 2004). It has been assumed that irradiation structurally alters IgE-binding epitopes by generating primary free radicals, reacting with proteins, which results in protein fragmentation, polymerization (dimerization), and aggregation (Kuan et al. 2013). In contrast, Moriyama et al. (2013) found that gamma-irradiation of soybeans applying a dose rate between 2.5 and 30 kGy resulted in apparent band profiles of major soy allergens Gly m5, Gly m Bd 30K, Gly mTI, and Gly m4, while protein band intensities were not significantly changed by irradiation. ELISA analyses using allergen-specific antibodies (Gly m5, Gly m Bd 30K, Gly mTI, and Gly m4) suggested no significant changes in the allergen contents, except for a decrease in Gly mTI. Chemical changes of proteins that are caused by gamma-irradiation are commonly fragmentation (depolymerization), inter-protein cross-linking (aggregation), including the formation of disulfide bonds, hydrophobic interactions that could lead to protein aggregation, and oxidation by oxygen radicals that are generated in the radiolysis of water (Davies and Delsignore 1987; Lee and Song 2002). Irradiation emits electrons and generates radicals from the breakdown of the cobalt-60 isotope. Proteins can be converted into higher molecular weight aggregates due to the generation of inter-protein cross-linking reactions, hydrophobic and electrostatic interactions as well as formation of disulfide bonds due to the water-radiolysis (Davis and Delsignore 1987). Gamma-irradiation as a physical mean of decontamination by photon-induced changes at the molecular level. Ionizing radiation with low dose rates up to 1 kGy is usually applied to control food-borne pathogens as well as to reduce the microbial load and insect infestation, thereby extending the shelf-life of perishable products for commercial purposes (FDA 1997). As side effect, this technology may change antigenicity of food proteins by the destruction or modification of epitopes. According to Vaz et al. (2013) the types of modification that food proteins might undergo during irradiation, including protein unfolding and aggregation, could be not observed at a low-dose range. Therefore, the potential of gamma-irradiation to affect soy Gly m5 immunoreactivity after a low dose compared to a high dose of radiation was investigated in this study.

More recently, the use of pulsed ultraviolet (PUV) light treatment, a high-peak power technology, has attracted considerable attention as an alternative food preservation method that consists of intense flashes of broad-spectrum of light containing wavelength from near-infrared to ultraviolet (Koutchma et al. 2009). Previous studies have shown that PUV has the ability to reduce the level of allergenicity in peanut products (Chung et al. 2008; Yang et al. 2012) as well as soybean (Yang et al. 2010), shrimp (Shriver 2011), almond (Li et al. 2013), and wheat extracts (Nooji 2011). The efficiency to reduce immunoreactivity has been attributed to photothermal, photochemical, and photophysical reactions, contributing to a change in protein structure and reduction in IgE-binding ability (Krishnamurthy et al. 2009; Yang et al. 2010). PUV is commonly regarded as nonthermal if the time of exposure is limited to some seconds where the temperature rise is insignificant (Krishnamurthy et al. 2009). However, recent studies confirmed that prolonged exposure with PUV light can induce significant photothermal effects by the infrared portion of PUV light spectra (Krishnamurthy et al. 2009; Yang et al. 2010). Consequently, a considerable temperature rise, moisture loss, and simultaneously sample weight loss could occur due to increased energy absorbance (Chung et al. 2008; Li et al. 2013; Nooji 2011; Yang et al. 2010).

Application of cold atmospheric pressure plasma (CAPP) has gained considerable attention as an alternative microbial inactivation technology due to its germicidal effects (Ehlbeck et al. 2011). Although the effect of CAPP on protein structure is not well studied so far, it has been assumed that CAPP might promote reactions in liquids by injecting reactive oxygen radicals, altering the epitope structure. Recent studies showed that CAPP might be an effective method to reduce immunoreactivity of wheat and shrimp proteins (Nooji 2011; Shriver 2011). However, to the best of our knowledge, reports on the effect of CAPP on the residual soy immunoreactivity are not available in the literature up to now.

Currently, little is known about how gamma-irradiation, PUV light, and CAPP treatment may alter food allergens, and hence there is a need to investigate the relationship between food protein allergenicity and the effect of food irradiation. Further, the effect of these technologies on soy Gly m5 immunoreactivity has not been investigated so far. As Gly m5 is one of the most abundant proteins in soy and sensitization against this protein is highly indicative for severe allergic reactions (Holzhauser et al. 2009), the evaluation of its residual immunoreactivity is indispensable for the assessment of potential allergenicity of modified foods. Therefore, this study aimed to investigate the effect of PUV light, gamma-irradiation as well as direct and remote CAPP on soy immunoreactivity. The degradation of major soy

allergens Gly m5 and Gly m6 and residual immunoreactivity have been evaluated by SDS-PAGE analysis and sandwich ELISA using mouse monoclonal anti-Gly m5 antibodies (mAbs). In addition, sample weight, surface temperature, hydrogen peroxide content and pH value have been measured.

2. Material and Methods

2.1. Raw materials and chemicals

Untoasted soybeans (*Glycine max* (L.) MERR.) were purchased from Naturkost Ernst Weber (Munich, Germany).

All chemicals used in this study were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany) if not stated separately.

2.2. Preparation of soy protein isolates (SPI)

SPI was prepared from soybeans using the technique as previously described in Meinschmidt et al. (2016c). Briefly, soybeans were de-hulled, flaked, and defatted with *n*-hexane. SPI was prepared by acidic pre-extraction (pH 4.5, 1:8 w/v flakes to water ratio, 1 h) of soybean flakes. After stirring for 1 h at room temperature, the suspension was separated using a decanter (3250 x *g*, 60 min). Subsequently, alkaline protein-extraction (pH 8.0, 1:8 w/v, 1 h) of flakes residue was performed and the suspension was separated (3250 x *g*, 60 min). The supernatant was adjusted to pH 4.5 for protein precipitation, followed by centrifugation (5 600 x *g*, 130 min). The obtained SPI was neutralized, pasteurized (70 °C, 10 min) and spray-dried.

2.3. Nonthermal food processing technologies

2.3.1. Pulsed ultraviolet (PUV) light treatment

SPI dispersions (5 mg mL⁻¹, 10 mL each in an aluminum dish with a diameter of 7 cm) were treated in a pulsed light chamber (Claranor, Avignon, France),

which was equipped with a three Xenon tubes reflector. The lamp is connected to a capacitor and emits a broad spectrum intense light flash of 200 to 1100 nm. The applied voltages ranged between 1.5 to 2.8 kV, which corresponded to a total available energy of 0.27 and 0.98 J cm⁻² s⁻¹ at a distance of 10 and 8 cm from the central axis of the pulsed UV lamp system, respectively. The total energy input was determined with a Solo2 Power and Energy Meter (Gentec, Quebec City, Canada). Three pulses per second with a width of 300 μs were produced. Treatment duration was set to 1, 2, 4 and 6 min (three replicates each). Sample weights, pH value using pH-indicator strips (pH 2.0 - 9.0; Merck, Darmstadt, Germany), and surface temperature using a noncontact infrared thermometer (MiniTemp MT6, Raytek GmbH, Berlin, Germany) of all samples were recorded before and after PUV treatment. All samples were stored at -20 °C prior to chemical analyses. For chemical analyses, including molecular weight distribution and residual immunoreactivity, as described in the following sections, samples were conditioned to RT.

2.3.2. Gamma-irradiation treatment

SPI dispersions (50 mg mL⁻¹, 15 mL) were sealed in polyethylene bags and kept cold (4 °C) prior to and during irradiation. Samples were irradiated at Synergy Health Allershausen GmbH (Allershausen, Germany) using a (radioisotope) cobalt-60 source (1.17 and 1.33 MeV) irradiator with an activity of 1.48 x 10¹⁷ Bq (MDS Nordion International Inc., Ontario, Canada). Target doses were 3, 5, 10, 25, 50, and 100 kGy. The radiation dose was computed by taking into account the source strength and the time of exposure. After treatment, the pH value using pH-indicator strips (pH 2.0 - 9.0; Merck, Darmstadt, Germany) was measured immediately. All samples were stored at -20 °C prior to chemical analyses and experiments were done in triplicate.

2.3.3. Cold atmospheric pressure plasma (CAPP) treatment

Two types of plasma devices were used. A direct plasma treatment using a surface dielectric-barrier air-discharge (SDBD) system similar to that described by Oehmigen et al. (2010) and a remote (indirect) plasma treatment with a microwave generated plasma (Schnabel et al. 2012).

Direct CAPP treatment. The SDBD plasma source consists of an array of concentric ring-shaped electrodes (85 mm outer diameter) embedded in a 1.5 mm thick epoxy-glass bulk material mounted into the upper shell of a Petri dish (90 mm diameter) as described in Bußler et al. (2015a).

Aliquots (15 mL) of SPI dispersions (5 mg mL^{-1}) were put in sterile Petri dishes (30 mm diameter), which were fixed on a holder under the plasma source at a distance of 12 mm. In order to prevent interactions between plasma-immanent species and ambient atmosphere, the airtight treatment chamber was filled with ambient air and was hermetically sealed during plasma treatment. CAPP was generated at the surface of the dielectric epoxy glass by applying sinusoidal peak-to-peak (pp) voltage of 9, 10, and 11 kV_{pp} at a frequency of 3.0 kHz delivered by a commercial arbitrary waveform generator (max. 20 MHz, DG1022, Rigol, Puchheim, Germany). The current voltage was controlled through a built-in two channel digital storage oscilloscope (max. 500 MHz, TDS 2001C, Tektranx, Beaverton, USA). CAPP exposure was set to 1, 2.5, 5, 7.5 and 10 min without stirring. The pH value and hydrogen peroxide content using pH-indicator strips (pH 2.0 - 9.0; Merck, Darmstadt, Germany) and QUANTOFIX[®] test stripes (MACHEREY-NAGEL Inc., PA, USA), respectively, were measured directly after treatment. Samples were stored at $-20 \text{ }^{\circ}\text{C}$ prior to chemical analyses and trials were performed at least in triplicate.

Remote (indirect) CAPP treatment. Plasma processed air (PPA) was used for the remote plasma treatment, which was generated by a microwave-driven plasma torch (Plexc[®], INP, Greifswald, Germany). The microwave had a frequency of 2.45 GHz and a power of 12 kW. The process gas was air with a gas flow of 18 slm and the microwave plasma had a peak temperature of about 3700 K. An optical emission spectrum of the used microwave plasma torch is described in Pipa et al. (2012) and a detailed description of the experimental set-up is given in Hertwig et al. (2015).

Aliquots (15 mL) of SPI dispersion was transferred into the reaction chamber, which was afterwards filled with PPA. Bottles were shaken to obtain homogenous treatment and treatment time was set to 15, 30, 60, and 90 min. The treatment temperature was kept below $30 \text{ }^{\circ}\text{C}$. After treatment, remaining PPA was removed. The pH value and hydrogen peroxide content using pH-indicator strips (pH 2.0 - 9.0; Merck, Darmstadt, Germany) and QUANTOFIX[®] test stripes (MACHEREY-NAGEL Inc., PA, USA), respectively, were measured. Samples were measured directly after treatment. Samples were stored at $-20 \text{ }^{\circ}\text{C}$ prior to chemical analyses and trials were performed in triplicate.

2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight distribution was determined according to Laemmli (1970) using SDS-PAGE under reducing conditions. Sample preparation and running conditions were performed as suggested by Meinlschmidt et al. (2016c). Electrophoresis was performed on 4 - 20 % midi Criterion™ TGX Stain-Free™ precast gels and proteins were separated using the midi Criterion™ Cell from Bio-Rad (Ismaning, Germany). Protein visualization was performed by Criterion Stain-Free Gel Doc™ EZ Imager (Bio- Rad).

2.5. Sandwich enzyme-linked immunosorbent assay (ELISA)

Three newly generated mouse monoclonal anti- β -conglycinin (Gly m5) antibodies (mAbs) called Izimab-Glym5-3, Izimab-Glym5-4, and Izimab-Glym5-5 were applied in the sandwich ELISA experiments as described elsewhere in detail (Meinlschmidt et al. 2016c). To avoid detection losses due to insufficient capture efficiency, three sandwich variants were performed. The antibodies as well as the sandwich ELISA procedure have been described in detail in a previous study (Meinlschmidt et al. 2016c). In brief, capture antibodies were immobilized onto 96-well plates (maxisorp; Nunc, Germany) at 4 °C overnight. Plates were washed three times with phosphate buffered saline, 154 mM NaCl, pH 8.0, 0.05 % Tween (3xPBS-T) and blocked with Superblock blocking buffer (Life Technology GmbH, Germany). SPI solutions and all other samples were centrifuged at 15,000 x *g* for 10 min prior to ELISA analysis. The supernatants were incubated 1 h at RT in duplicate. After a further wash step (3xPBS-T), the POD-conjugated detection-antibody was incubated for 1 h at RT. The POD activity was determined following a third wash step (3xPBS-T) by incubating the plate with TMB-E substrate (DUNN Labortechnik, Asbach, Germany) and stopping the reaction after 5 min with 0.3 M H₂SO₄. The color intensity of the samples represents a signal for the amount of captured antigen, which was measured at a wavelength of 450 nm. Signal intensities were calculated by extrapolation to a calibration curve from standard-SPI consisting of seven known concentrations (2000, 500, 125, 31.25, 7.8125, 1.953125, and 0.48828125 ng mL⁻¹). The immunoreactivity was calculated in relation to untreated SPI, which was set to 100 %. Reduced immunoreactivity is measured if either of both epitopes failed in binding. Each sample was determined in duplicate.

2.6. Statistical analysis

All data are expressed as mean \pm standard deviation (SD) of at least two or three independent measurements. A statistical analysis was performed using the SPSS version 21 computer program (SPSS Statistical Software, Inc., Chicago, IL, USA). Pulsed UV light data were subjected to two-way analysis of variances (ANOVA) to compare the effects of distance and time on the sample weight loss as well as final temperature and temperature increases, and their interaction. Direct CAPP data were also analyzed using the two-way ANOVA approach to determine if voltage and time and the interaction of both are significant. Fisher's least significant difference (LSD) test was used to describe means with 99 % confidence ($p < 0.01$).

One-way ANOVA was carried out for analyzing the residual immunoreactivity after PUV, CAPP (direct and remote) and gamma-irradiation treatment. Whenever differences were significant, a 95 % confidence level (statistical significance at $p < 0.05$) was used in a within-subjects design with pairwise Bonferroni post hoc test.

3. Results and Discussion

3.1. Pulsed ultraviolet (PUV) light treatment

For PUV light experiments, distances of 8 and 10 cm between the light source and the sample material as well as treatment times of 1, 2, 4, and 6 min have been investigated in order to sufficiently reduce soy Gly m5 immunoreactivity.

3.1.1. Surface temperature, mass loss and pH value after PUV light treatment

The results of sample surface temperature and sample weight loss are summarized in Table 1, where a significant ($p < 0.05$) increase in temperature was attenuated by a concurrent decrease in sample weight with prolonged treatment time. In general, a higher temperature rise and sample weight loss was observed for samples exposed to PUV light at a distance of 8 cm, which is due to the higher energy dose applied. Results from the two-way ANOVA demonstrated that all independent variables in-

cluding time, distance and the interaction of distance and time (DxT) do have a significant effect on sample weight loss ($R^2 = 0.986$; $p < 0.01$), final temperature ($R^2 = 0.962$; $p < 0.01$), and temperature increase ($R^2 = 0.967$; $p < 0.01$). Consequently, for both 8 and 10 cm distance, a longer treatment time resulted in a higher temperature increase and simultaneous sample weight loss up to 81 °C and 58 %, respectively. Already after 4 min at a distance of 8 cm, a sample weight loss of 58 % could be detected, wherefore the maximum treatment time was set to 4 min. The present results are in consistency with the results obtained by Yang et al. (2010), who also observed a loss of water up to 50 % after 6 min of treatment time as a result of water evaporation. In addition, the color of SPI dispersions changed from transparent-light-yellow to opaque yellow and aggregates became visible, which implicates the formation of heat-induced aggregates.

The pH value of PUV-treated soy proteins decreased significantly ($p < 0.01$) from initially pH 6.7 to a minimum of pH 5.5 for all samples, indicating acidification of samples as a consequence of PUV light treatment. The reduction in pH value was, however, not significantly ($R^2 = 0.996$; $p > 0.01$) affected by the treatment time, distance and the interaction of distance and treatment time.

Table 1

Energy input, sample weight loss as well as final temperature and temperature increase due to pulsed ultraviolet light treatment at a distance of 8 and 10 cm.

Distance	Treatment time	Energy input	Weight loss*	Final temperature*	Temperature increase (ΔT)*	pH value
(cm)	(min)	(J cm ⁻²)	(%)	(°C)	(K)	(-)
0	-	-	-	-	-	6.7 ± 0.0 ^a
8	1	58.8	7.6 ± 0.6 ^d	70.1 ± 1.9 ^b	47.6 ± 1.6 ^b	5.5 ± 0.0 ^b
	2	117.6	24.0 ± 2.5 ^b	74.9 ± 1.7 ^{a,b}	53.6 ± 1.7 ^{a,b}	5.5 ± 0.0 ^b
	4	235.2	58.3 ± 5.9 ^a	80.8 ± 6.3 ^a	60.4 ± 5.6 ^a	5.5 ± 0.0 ^b
10	1	16.2	1.8 ± 0.6 ^d	43.1 ± 1.3 ^d	22.5 ± 1.2 ^d	5.5 ± 0.0 ^b
	2	32.4	4.9 ± 0.3 ^d	58.3 ± 0.5 ^c	38.1 ± 1.2 ^c	5.5 ± 0.0 ^b
	4	64.8	15.9 ± 0.9 ^c	68.0 ± 0.0 ^b	48.0 ± 0.4 ^b	5.5 ± 0.0 ^b
	6	97.2	30.9 ± 0.6 ^b	73.3 ± 1.3 ^{a,b}	52.5 ± 1.7 ^{a,b}	5.5 ± 0.0 ^b
Two-way ANOVA		Distance	< 0.01	< 0.01	< 0.01	n.s.
		Time	< 0.01	< 0.01	< 0.01	n.s.
		DxT**	< 0.01	< 0.01	< 0.01	n.s.
		R ²	0.986	0.962	0.967	0.996
		R ² (adj)	0.982	0.951	0.958	0.992

*All values are means ± SD of triplicate measurements from two independent experiments (n=6). ^{a,b,c,d}: different subscripts within one column indicate significant differences ($p < 0.01$; two-way ANOVA, LSD).

**DxT = Distance x Time interaction effect. n.s. = not significant.

3.1.2. Molecular weight distribution and residual immunoreactivity of PUV-treated samples

Figure 1 A and B show the electrophoretic profile of untreated SPI and SPI samples treated with PUV light at a distance of 10 and 8 cm, respectively. The electrophoretic profile of untreated SPI (Figure 1 A and B, lane 2) presents high intensity protein bands characteristic for native soybean proteins. The first three bands are the subunits of β -conglycinin (Gly m5: α' = \sim 72 kDa, α = \sim 68 kDa, and β = \sim 53 kDa) as well as glycinin (Gly m6: acidic "A" = \sim 29-33 kDa and basic "B" = \sim 18-22 kDa), which have previously been identified as being Gly m5 and Gly m6 using LC-MS/MS analysis (Meinlschmidt et al. 2016b).

SDS-PAGE profiles of samples treated at a distance of 10 cm (Figure 1 A) revealed almost completely invisible protein bands corresponding to major soybean allergens Gly m5 and Gly 6 after 6 min treatment time (lane 6). Already after 2 min of exposure, Gly m5 subunits could not be detected, while Gly m6 was more resistant as it disappeared after 6 min. These results are coherent with the results described by Yang et al. (2010), who found a notable reduction of soy protein bands, applying PUV light at a distance of 13.2 cm to soybean extracts. The same trend could be found for samples treated at a distance of 8 cm (Figure 1 B), but already after 1 min most of Gly m5 and Gly m6 diminished and completely vanished after 2 min treatment time. The bands were presumably not visible on the SDS-PAGE due to PUV treatment, damaging proteins, but also as a consequence of protein precipitation, decreasing the band intensity and leading to the appearance of smeared bands following PUV-induced intramolecular crosslinking of proteins (Figure 1 A and B). Smearing might indicate a cross-linking between soy proteins, thus the presence of aggregates, which were not able to run through the gel smoothly. Proteins that become aggregated due to treatment conditions may become too large to travel through the polyacrylamide gel and consequently get stuck in SDS-PAGE wells (see Figure 1 A and B). The increased thermal load of the samples, which were heated up to 81 °C at a distance of 8 cm for 4 min, might be a further factor contributing to the differences in protein stability. According to Herrero et al. (2009) heat has been shown to unfold proteins, which lead to the aggregation and consequently to the disappearance in the electrophoretic patterns. This phenomenon was also previously observed by Yang et al. (2010) and Chung et al. (2008). In addition, the UV portion of PUV can form hydroxyl and hydrogen radicals (OH^- and H^+), which can result in the formation of ozone and protein cross-linking and protein-fragmentation as they are known to be powerful protein-modifying agents (Krishnamurthy et al.

2009).

Sandwich ELISA using three mouse anti-Gly m5 mAbs (Izimab-Glym5-3, Izimab-Glym5-4, and Izimab-Glym5-5) was performed to determine the residual immunoreactivity of PUV-treated samples and the results are shown in Figure 1 C (8 and 10 cm). In general, a great change in immunoreactivity was observed for all samples, using the antibodies Izimab-Glym5-4 and Izimab-Glym5-5, whereas almost no changes were found using Izimab-Glym5-3. The samples positioned at a distance of 8 cm from the xenon lamp showed a higher reduction in immunoreactivity than samples placed at 10 cm (Figure 1 C). This can be explained by the fact that samples at a distance of 8 cm received a higher energy dose along with an increase in temperature, which consequently modified mainly conformational epitopes more effectively due to heat-induced conformational changes. Only the samples treated at 8 cm distance for 4 min showed a reduced immunoreactivity up to 91 % for all three antibodies tested. As mentioned before, it is believed that the photothermal, photophysical and photochemical effects caused by PUV treatment seems to alter allergen conformation or cause protein crosslinking and aggregation, which results in reduced antibody-binding ability as a results of modified epitopes (Chung et al., 2008; Krishnamurthy et al., 2009; Yang et al., 2010). Further, radicals might have been induced due to molecular ionization under high-energy burst, leading to modification of epitopes, thereby reducing the immunoreactivity (Krishnamurthy et al., 2009). Yang et al. (2010) noticed a pronounced reduction of Gly m6 subunits and the β -subunit of Gly m5 along with a reduction in allergenicity up to 50 % as measured by indirect ELISA with pooled plasma from soy-sensitive individuals after PUV treatment of soy extracts for up to 6 min.

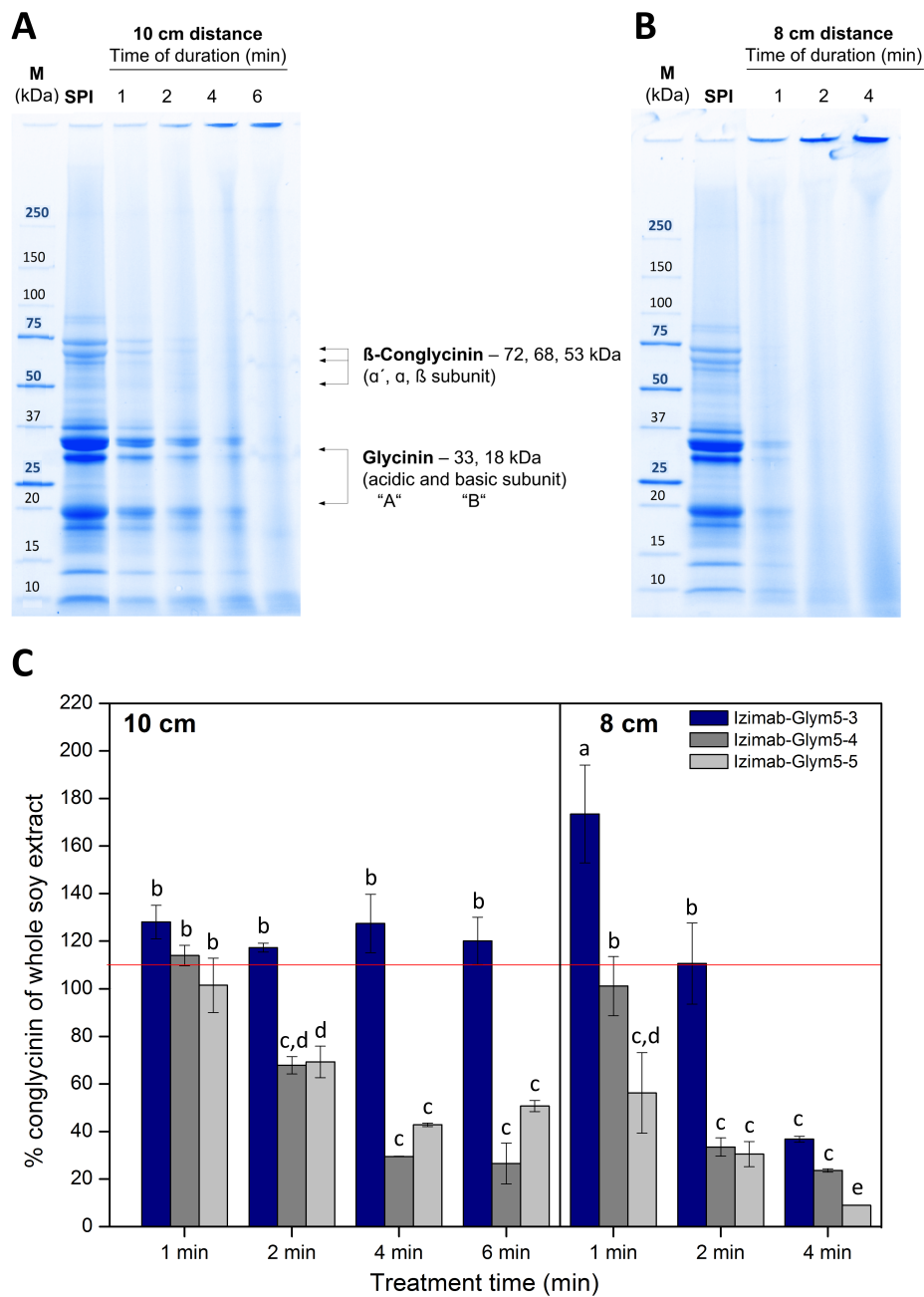


Fig. 1. SDS-PAGE profiles (A and B) and sandwich ELISA results (C) of SPI and SPI dispersions treated with PUV light at a distance of 10 cm (A) and 8 cm (B). M- Molecular weight standard indicated in kilo Dalton (kDa); SPI- soy protein isolate. Electrophoresis was carried out with 4-20 % polyacrylamide gradient gels. α' -, α - and β - subunits of β -conglycinin; "A" and "B": acidic and basic subunit of glycinin. Results of three different sandwich ELISAs are shown. Immunoreactivity of SPI dispersions treated with PUV light (8 and 10 cm) was measured by sandwich ELISA with three sets of monoclonal mouse anti-Gly m5 Abs (Izimab-Glym5-3, Izimab-Glym5-4, and Izimab-Glym5-5). All ELISA values are means \pm SD of duplicate measurements from two independent experiments (n=4). *a,b,c,d,e*: different subscripts indicate significant differences ($p < 0.05$; one-way ANOVA, Bonferroni).

3.2. Gamma-irradiation treatment

3.2.1. Molecular weight distribution and residual immunoreactivity of irradiated samples

Figure 2 A presents the SDS-PAGE profiles of non-irradiated SPI and SPI samples irradiated at 3, 5, 10, 25, 50, and 100 kGy. In general, the electrophoretic profile corresponding to major soy allergens Gly m5 and Gly m6 could be significantly changed following gamma-irradiation depending upon the dose.

The band profile of irradiated samples showed that irradiation at dose rates between 5 and 25 kGy caused a slight break down of Gly m5 subunits. At low dose rates up to 25 kGy, even under the denaturing conditions of SDS-PAGE, fragmentation and molecular aggregation were not observed (see Figure 2 A, lanes 3-6). However, at dose levels above 25 kGy, Gly m6 additionally diminished, and completely vanished at 100 kGy. SDS-PAGE analysis indicated that a high dose of gamma-radiation of 50 and 100 kGy induces peptide bond cleavages which later undergo aggregation as aggregates become visible at the top of the SDS-PAGE gels (Figure 2 A, lanes 7-8). It is believed that proteins were cross-linked and aggregated, following irradiation treatment of proteins in an aqueous system, which causes disappearance on SDS-PAGE due to the formation of higher molecular weight polymers that probably could not pass through the gradient gel (Byun et al. 2002). It has been speculated that gamma-irradiation structurally alters the IgE-binding epitopes of egg (Seo et al. 2007), milk (Lee et al. 2001), and shrimp allergens (Byun et al. 2000; Zhenxing et al. 2007) by creating free radicals, which might have caused protein fragmentation and aggregation. Chemical changes of proteins that are caused by gamma-irradiation are commonly fragmentation (depolymerization), inter-protein cross-linking (aggregation), including the formation of disulfide bonds, hydrophobic interactions that could lead to protein aggregation, and oxidation by oxygen radicals that are generated in the radiolysis of water (Davies and Delsignore 1987; Lee and Song 2002). Irradiation emits electrons and generates radicals from the breakdown of the cobalt-60 isotope. Proteins can be converted into higher molecular weight aggregates due to the generation of inter-protein cross-linking reactions, hydrophobic and electrostatic interactions as well as formation of disulfide bonds due to the water-radiolysis (Davis and Delsignore 1987). The formation of high molecular aggregates was negligible at low-dose range between 3 and 25 kGy, but increased at higher doses above 25 kGy.

In addition, the pH value, measured by pH-indicator strips on the surface of the sample, remained at 6.7 after irradiation, which means that no statistical significantly ($p > 0.01$) changes in the pH value due to gamma-irradiation treatment could be measured.

The residual immunoreactivity of irradiated samples is shown in Figure 2 B. At dose rates of 3 and 5 kGy, the allergenicity is slightly exacerbated using Izimab-Glym5-3 and Izimab-Glym5-4. This phenomenon comply with the hypothesis and results described by Vaz et al. (2013), who found an elevated antigenicity of Con-A after irradiation at low-dose between 1 and 25 kGy. However, irradiation with a dose higher than 25 kGy, the immunoreactivity of irradiated samples continuously decreased with rising dose level up to 100 kGy for all three antibody-pairs tested. However, a complete inhibition of antibody-binding ability was not achieved. The effect of an increased allergenicity at a low dose rate may be due to increased exposure of conformational and linear epitopes resulting from the formation of partially unfolded and aggregated species after irradiation. The highest reduction up to 91 % was detected for samples irradiated with a dose rate of 100 kGy (Figure 2 B). However, a dose of a minimum of 25 kGy is rather high for food applications, while a maximum dose of approximately 10 kGy has been shown to be safe for human consumption (FDA 2004).

As a nonthermal method, gamma-irradiation is believed to change the epitope-structure through protein fragmentation and aggregation. At a high dose range, more free radicals were generated and led to other reactions such as deamination, decarboxylation, reduction of disulfide linkages, which might have resulted in the destruction and modification of antibody binding epitopes (Zhenxing et al. 2007). Kasera et al. (2012), Byun et al. (2002) and Seo et al. (2007) found a reduced allergenicity of various food proteins, including legume proteins (kidney bean, black gram, peanut), shrimp tropomyosin as well as β -lactoglobulin and egg albumin (ovalbumin) after gamma-irradiation at 25, 10, and 100 kGy, respectively. By means of spectrometric measures, Byun et al. (2002) monitored that the conformational epitopes of shrimp heat-labile proteins were destroyed. Radiation damages allergens directly by rupturing covalent bonds as a result of transfer of photon energy, or indirectly, by producing reactive oxygen species which can reduce allergenicity by compromising structure-function relationships (Vaz et al. 2012). By the use of Far-UV CD spectra and DSC analyses, Vaz et al. (2013) demonstrated that irradiated Con-A showed a shape characteristic of proteins with a high degree of aggregation, which led to a decreased antigenicity at 25 kGy. Additionally, the results revealed that high dose rates of 25 kGy led to conformational changes, lacking of β -sheet

structures, which indicated the accumulation of completely unfolded, fragmented peptides after high dose irradiation. All of these structural changes suggest that conformational changes might have led to a certain loss of conformational epitopes, which consequently resulted in a reduced antibody-binding as evidenced by sandwich ELISA (Figure 2 B).

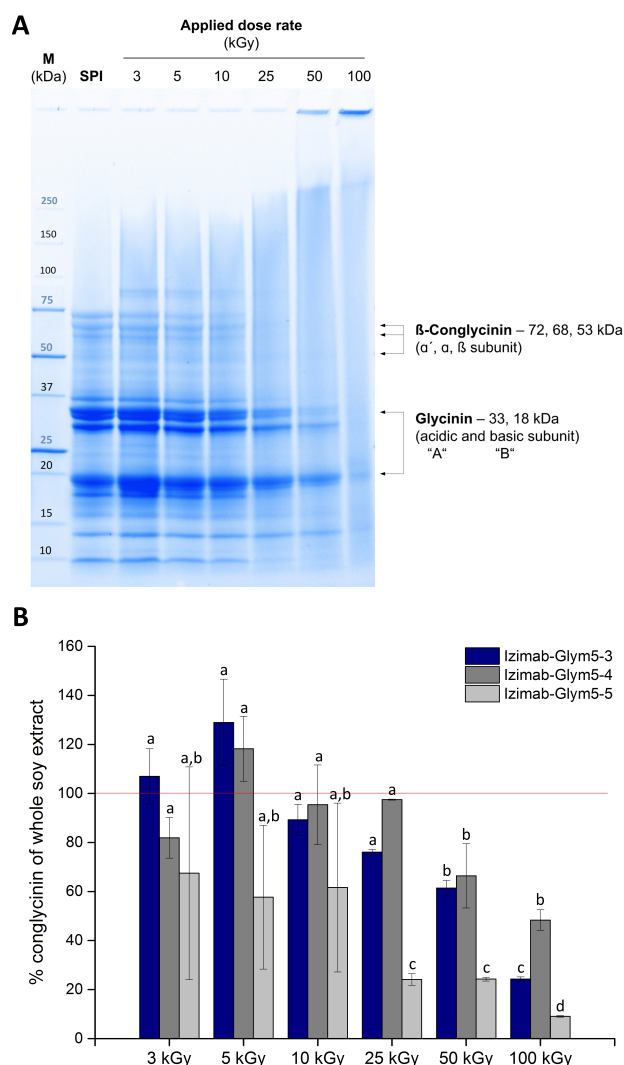


Fig. 2. SDS-PAGE profiles (A) and sandwich ELISA results (B) of SPI and irradiated SPI dispersions. M- Molecular weight standard indicated in kilo Dalton (kDa); SPI- soy protein isolate. Electrophoresis was carried out with 4-20 % polyacrylamide gradient gels. α' -, α - and β - subunits of β -conglycinin; "A" and "B": acidic and basic subunit of glycinin. Results of three different sandwich ELISAs using are shown. Immunoreactivity of irradiated SPI dispersions was measured by sandwich ELISA with three sets of monoclonal mouse anti-Gly m5 Abs (Izimab-Glym5-3, Izimab-Glym5-4, and Izimab-Glym5-5). All ELISA values are means \pm SD of duplicate measurements from two independent experiments (n=4). *a,b,c,d*: different subscripts indicate significant differences ($p < 0.05$; one-way ANOVA, Bonferroni).

3.3. Cold atmospheric pressure plasma (CAPP) treatment

3.3.1. Hydrogen peroxide content and pH value after direct and remote CAPP treatment

The pH value and hydrogen peroxide content of samples treated with remote and direct CAPP have been measured. Remote CAPP treatment of samples resulted in no statistical significantly ($p > 0.01$) changes in pH value (pH 6.7) and hydrogen peroxide content (0 mg mL^{-1}), wherefore only the results of direct CAPP treatment are summarized in Table 2.

Applying direct CAPP, a significant acidification and concomitantly decrease of the pH value already after 1 min could be observed regardless of the voltage (kV_{pp}) applied (see Table 2). The pH value was reduced from initially 6.7 (untreated SPI) to a minimum of 2.5 after 10 min treatment time, irrespective of the voltage applied. This change presumably derived from an interaction between water ions and molecules, which can lead to the production of hydronium ions (H_3O^+).

With increasing treatment time, the hydrogen peroxide content gradually increased to at least 100 mg mL^{-1} . However, a lower hydrogen peroxide production at 10 and 11 kV_{pp} than at 9 kV_{pp} could be found. Hydrogen peroxide production could be a result of dismutation (O_2^-). The reactive species probably included ozone (O_3), hydroxyl radical ($\text{OH}\cdot$), superoxide radical ($\text{O}_2^{\cdot-}$) and when introduced to a liquid environment, hydrogen peroxide (H_2O_2) will be produced (Oehmigen et al. 2010; Surowsky et al. 2015). A reason for a lower hydrogen peroxide concentration at higher voltage of 10 and 11 kV_{pp} and prolonged treatment time of 7.5 and 10 min might be explained by the fact that the further reaction between the produced hydrogen peroxide and OH radicals might have been promoted by higher energy input, which consequently resulted in a lower H_2O_2 concentration.

According to the two-way ANOVA, only the treatment time had a significant effect on the pH value ($R^2 = 0.736$; $p < 0.01$) and hydrogen peroxide content ($R^2 = 0.907$; $p < 0.01$), whereas the voltage and the interaction of time and voltage ($V \times T$) showed no statistical significantly ($p > 0.01$) effects (Table 2).

Table 2

Hydrogen peroxide content and pH value of samples treated with direct CAPP.

Peak-to-peak-voltage	Time time (min)	pH value* (-)	Hydrogen peroxide content* (mg mL ⁻¹)	
0	0	6.7 ± 0.0 ^a	0 ± 0 ^f	
	9	1	3.6 ± 0.1 ^b	5 ± 0 ^e
		2.5	3.5 ± 0.3 ^b	10 ± 0 ^d
		5	2.5 ± 0.4 ^c	30 ± 0 ^c
		7.5	2.5 ± 0.6 ^c	50 ± 0 ^b
10	2.5 ± 0.1 ^c	> 100 ± 0 ^a		
10	1	4.2 ± 0.8 ^b	5 ± 0 ^e	
	2.5	3.3 ± 0.5 ^b	30 ± 0 ^c	
	5	3.3 ± 0.5 ^b	30 ± 0 ^c	
	7.5	3.0 ± 0.2 ^c	50 ± 0 ^b	
	10	2.5 ± 0.4 ^c	50 ± 0 ^b	
11	1	3.3 ± 0.2 ^b	10 ± 0 ^d	
	2.5	3.3 ± 0.1 ^b	10 ± 0 ^d	
	5	3.0 ± 0.2 ^c	30 ± 0 ^c	
	7.5	2.5 ± 0.4 ^c	30 ± 0 ^c	
	10	2.5 ± 0.7 ^c	50 ± 0 ^b	
Two-way ANOVA	Voltage	n.s.	n.s.	
	Time	< 0.01	< 0.01	
	VxT**	n.s.	n.s.	
	R ²	0.736	0.907	
	R ² (adj)	0.489	0.820	

*All values are means ± SD of triplicate measurements from two independent experiments (n=6). *a,b,c,d,e,f*: different subscripts within one column indicate significant differences ($p < 0.01$; two-way ANOVA, LSD). **VxT = Voltage x Time interaction effect. n.s. = not significant.

3.3.2. Molecular weight distribution and residual immunoreactivity of CAPP-treated samples

Electrophoretic profiles of untreated SPI and SPI samples treated with direct CAPP (9 and 11 kV_{pp}) and remote CAPP are shown in Figure 3 A and B, respectively. SDS-PAGEs of samples treated with direct CAPP at 10 kV_{pp} are not included as no visual differences compared to 9 kV_{pp} could be observed.

As shown in Figure 3 A, the electrophoretic profile of the samples treated at a voltage of 9 kV_{pp} already changed after 2.5 min of exposure. In particular, the protein bands corresponding to Gly m5 became less intensive. After 5 and 7.5 min, a newly generated band emerged at approximately 50 kDa (Figure 3 A, lanes 5, 6, 10, and 11). It is well known that atmospheric plasma offers a source of reactive oxygen (ROS; e.g., atomic oxygen (O), ozone (O₃), hydroxyl radical (OH·)) and nitrogen species (RNS; e.g., N₂, NO, NO₂, nitric oxide radical (NO·)) as well as UV-A and UV-B radiation (Bufler et al. 2015a; Laroussi and Leipold 2004), which might have an adverse effect on protein structure. Lacroix et al. (1998) and Zhenxing et al. (2007) reported that this phenomenon might be due to the formation of new proteins by cross-linkage of free amino acids to the protein and protein-to-protein aggregation, resulting in insoluble aggregates. However, the protein bands almost diminished after a treatment time of 10 min. As soluble aggregates could not be observed in the wells at the top of the gel, it could be excluded that direct CAPP resulted in soluble protein aggregation. Presumably, a decreased protein solubility, and concomitantly the formation of insoluble aggregates, due to direct and remote CAPP treatment led to disappearance of protein bands in SDS-PAGE as only the soluble proteins can be detected in the electrophoretic profile. A reduced protein solubility due to direct and remote CAPP was indeed observed by Bufler et al. (2015b), who investigated the impact of thermal treatment versus direct CAPP on the techno-functional protein properties from *Pisum sativum* 'Salamanca'. In contrast, at a voltage of 11 kV_{pp}, protein disappearance could be detected already after 5 min of plasma treatment.

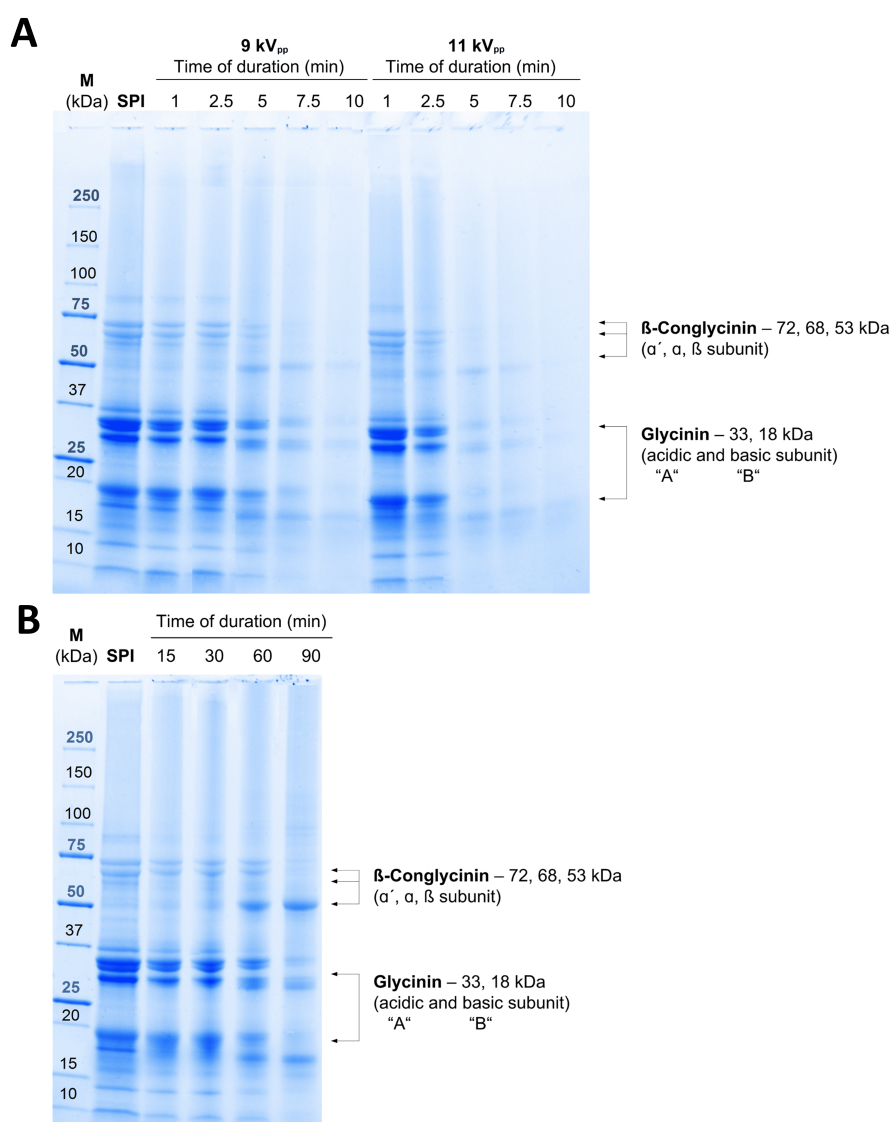


Fig. 3. SDS-PAGE profiles of SPI, direct plasma-treated (A) and SPI dispersions treated with remote plasma (B). M- Molecular weight standard indicated in kilo Dalton (kDa); SPI- soy protein isolate. Electrophoresis was carried out with 4-20 % polyacrylamide gradient gels. α' -, α - and β - subunits of β -conglycinin; "A" and "B": acidic and basic subunit of glycinin.

Figure 3 B presents the electrophoretic pattern of samples treated with remote (plasma afterglow) CAPP for up to 90 min, where a new protein band at 50 kDa could be found after 60 and 90 min treatment time (Figure 3 B, lanes 5 and 6). After 15 min of exposure, protein bands became already smeared, indicating protein cross-linking. A noticeable reduction in protein bands intensity corresponding to Gly m5, Gly m6 and low-molecular weight bands at approximately 10 and 15 kDa was found.

Sandwich ELISA using specific mouse monoclonal anti-Gly m5 antibodies was carried out to assess the retained immunoreactivity of plasma-treated SPI samples, and the results are shown in Figure 4 A, B, C (direct CAPP), and D (remote CAPP). Sandwich ELISA analysis revealed a profound change in soy immunoreactivity up to 100 % applying direct CAPP as indicated in Figure 4 A, B, and C. Remote CAPP was also effective as the immunoreactivity was reduced up to 89 % after 90 min treatment time, when using Izimab-Glym5-4 (Figure 4 D).

Shriver (2011) also observed a drastically reduced allergenicity in shrimp tropomyosin up to 76 % using direct CAPP with a voltage of 30 kV and a frequency of 60 Hz for 5 min at ambient temperature. Direct CAPP was generated by dielectric discharge between two electrodes fitted with a dielectric barrier using air as working medium. Nooji (2011) used the same plasma equipment for his experiments and was able to reduce wheat allergenicity up to 37 % with direct CAPP for 5 min. However, Tammineedi et al. (2013) found no changes in antibody-binding ability of α -casein and whey solutions after samples were exposed to plasma afterglow (remote CAPP). According to our findings, it can be concluded that both direct and remote CAPP are effective in the attenuation of soy Gly m5 immunoreactivity.

However, the major mechanisms being involved in the reduction of immunoreactivity remains still uncertain. There are multiple mechanisms, which can affect food allergen reactivity, but the underlying concept is always the modification of the conformational as well as linear epitopes. For example, conformational epitopes can be altered by aggregation into insoluble aggregates or crosslinking of proteins as a consequence of a loss of protein solubility, whereas linear epitopes can be affected by fragmentation. Plasma-immanent species present as hydroxyl radicals can cleave peptide bonds and oxidize amino acid side chains, which might lead to fragmentation. Oxygen radicals are involved in etching processes and the oxidation of proteins (Surowsky et al. 2013). Further, the cleavage of disulfide bonds within a peptide due to dissociative addition of a hydroxyl radical to form RSH and RSO \cdot at the cleavage site are conceivable. Reactive oxygen radicals (ROS), as atomic oxygen or hydroxyl radicals as well as reactive nitrogen species (NOS), may attack amino acids, which are sensitive to oxidation, presumably resulting in destruction of binding sites for the antibodies investigated in this study.

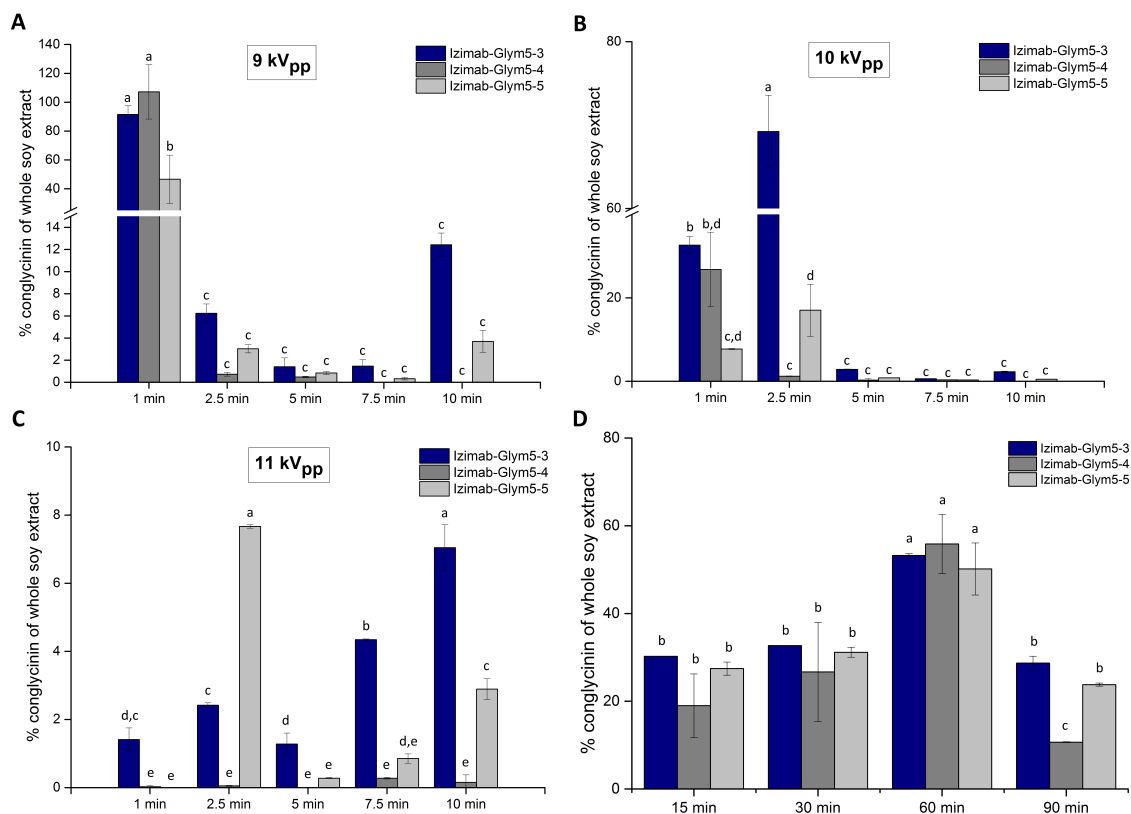


Fig. 4. Sandwich ELISA results of SPI dispersions treated with direct plasma at 9 kV_{pp} (A), 10 kV_{pp} (B), and 11 kV_{pp} (C) and remote plasma (D) using mouse monoclonal anti-Gly m5 Abs. Results of three different sandwich ELISAs are shown. Immunoreactivity of SPI dispersions treated with direct and remote CAPP was measured by sandwich ELISA with three sets of monoclonal mouse anti-Gly m5 Abs (Izimab-Glym5-3, Izimab-Glym5-4, and Izimab-Glym5-5). All ELISA values are means \pm SD of duplicate measurements from two independent experiments ($n=4$). *a,b,c,d,e*: different subscripts indicate significant differences ($p < 0.05$; one-way ANOVA, Bonferroni).

4. Conclusions

This study investigated the effect of nonthermal processing technologies, including PUV light, gamma-irradiation as well as direct and remote CAPP treatment, on soy immunoreactivity. The results clearly showed that all technologies investigated seem to be efficient approaches to reduce Gly m5 immunoreactivity, while direct CAPP treatment turned out to be most effective in sufficient reduction of immunoreactivity up to nearly 100 % as assessed by sandwich ELISA using specific

mouse monoclonal anti-Gly m5 antibodies. However, other allergenic components of soy e.g. glycinin (Gly m6), Kunitz Trypsin inhibitor (Gly mTI), or profilin (Gly m3) could contribute to the potential allergenicity as well. Consequently, further investigations using in vivo clinical tests i.e. skin prick test (SPT) and oral food challenge tests (OFC) need to be conducted to verify the present sandwich ELISA results.

The change in consumer demand and food safety issues coupled with the awareness that traditional thermal technologies result in nutritional losses and adverse effects on organoleptic quality of foods led to the emergence of nonthermal technologies. Among these nonthermal technologies, irradiation could be effective in eliminating allergens applying a high dose above 25 kGy. However, the legislation allows a dose rate up to 10 kGy for specific commercial food items up to now (FDA 2004), which is not sufficient to degrade soy proteins according to the present results. Further investigations into the impact of food irradiation on allergen structure and allergenic potential are still warranted. Although decades of research worldwide have shown that irradiation of food is an effective way to kill food-borne bacteria and extend shelf-life, irradiated foods are not widely available yet. CAPP treatment (direct and remote) and PUV light, which are currently *inter alia* applied for surface disinfection, show limitations in practice due to limited penetration depth, shadow effects, and the considerable increase in temperature accompanied by samples weight loss.

Yet, the exact mechanisms, including conformational and linear epitopes modifications, remains to be further investigated for all of these technologies. The effects on protein characteristics as well as toxicological safety have to be elucidated thoroughly. Furthermore, the recovery of protein structure during storage prior to analysis has to be evaluated by further experiments. Depending on the structural changes induced by the processing technology applied, some proteins possess the ability to recover their native ordered structure or even to form a new structure (Guo et al. 2015).

Although various reservations about the industrial application of these technologies exist, our study showed that they have great potential for sufficient reduction of soy immunoreactivity, which could help to produce low-allergen food ingredients in future. However, crucial aspects associated with these processes have to be clarified before reconsidering the application of these technologies for the production of low-allergen food ingredients.

Conflict of Interest

The authors have declared no conflicts of interest.

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CONCLUDING REMARKS

Food allergies have emerged as a growing health concern in recent years. Therefore, food industry has taken numerous initiatives to meet the requirements of allergic consumers, but a remedy for food allergy does not exist and the mainstay of dietary management of food allergies is the complete dietary exclusion of the offending foods (BOYCE et al., 2011). An avoidance of diet is successful, but causes stress and a diminution of the consumers' quality of life (DUNNGALVIN et al., 2008).

As soy is recognized as a potent food allergen producing one of the most frequent food allergies worldwide, the production of hypoallergenic soy products would be a desirable commodity given the presence of soy in a large fraction of processed foods. Hence, any critical step leading to an allergy has to be blocked. One important step might be the modification of harmful epitopes through processing. Therefore, various processing technologies have been investigated. However, these investigations are still in their infancy and the technological approach hitherto has largely been empirical due to the lack of detailed knowledge on integral allergen and epitope structure. As a consequence, an allergy mitigation method is still not implemented in food industry. Most research activities have focused on thermal processes, which commonly retain the ability of soy to elicit an immune response and causes a loss of the sensory and functional properties (JIMENEZ-SAIZ et al., 2015). Recently, non-thermal processing technologies have emerged in food industry due to their negligible effects on these important food characteristics. Yet, the impact of these technologies on soy allergy has scarcely been investigated. Thus, it is of crucial importance to give prominence to nonthermal technologies as potential soy allergy mitigation methods.

1 Soy allergy assessment

The physicochemical modification of proteins by processing technologies does not consequently allow the abolition of all epitopes of native proteins (MOORE et al.,

1985). This is due to the fact that each food contains a number of allergenic proteins, each with multiple epitopes, which could still elicit an allergic reaction in sensitive individuals. As only the immunoreactivity of Gly m5 was measured in this study, the residual immunoreactivity of glycinin (Gly m6) and other soy allergy related allergenic proteins e.g. Kunitz Trypsin Inhibitor (Gly mTI), profilin (Gly m3) or PR-10 (Gly m4) should be analyzed as well.

In general, the degree to which the immunoreactivity of the samples was reduced in this study might be important for a lot of soy-sensitive patients, depending upon their sensitivity threshold. Within this thesis, the evaluation of the residual immunoreactivity of intact proteins and peptide fragments has been accomplished by the use of SDS-PAGE, ELISA, and western blot. For the measurement of the degradation of major soy allergens Gly m5 and 6, SDS-PAGE and DH value analysis were used in the first three studies (CHAPTER 1 - 3), giving an initial evaluation of the level of allergenicity. However, as degraded proteins might still be able to interact with IgE antibodies, which could not be determined by these indirect test methods, more appropriate *in vitro* methods such as immuno-assays including sandwich enzyme-linked immunosorbent assay (ELISA) and western blot as well as *in vivo* test methods such as skin prick tests have to be performed in order to investigate the clinical relevance of these findings. Although these *in vitro* immunological methods are currently preferred, confirmatory alternatives are still needed as they do not determine whether the allergen can cause cross-linking of FcεRI bound IgE antibodies, which causes allergic symptoms. It is well known that cross-linking of FcεRI bound IgE antibodies is a requisite for degranulation response of the mast cells and basophils (STONE, 2010). So far, not enough emphasis has been given to understand the way, the insidious proteins interact with the epitopes. This knowledge is of particular importance for the development of a proper modification method of these allergenic protein sites.

As the intensity of allergic reactions and the tolerance among patients could be extreme different, a predication that the allergic reaction has been suppressed is not possible, unless it is certain that no clinical symptom will arise after ingestion. Hence, the allergenic potential of the modified sample to elicit an immune response has to be evidenced by means of sophisticated cellular assays such as human basophil degranulation and basophil activation tests (BENVENISTE, 1981; MCGOWAN et al., 2013). Consequently, all samples prepared within this study require careful *in vivo* controlled human challenge studies including skin prick and oral challenge tests to verify the *in vitro* results (Sathe & Sharma, 2009). Due to ethical reasons, sensitization studies cannot be performed in humans without permission by the ethics

commission. However, these investigations are indispensable for the complete assessment of the residual allergenic potential. Animal models, despite their individual limitations, are regarded as good alternatives to study the allergic response *in vivo* (FRITSCH, 2009; HELM, 2002).

It is worth note that aggregation and loss of protein solubility, rather than the epitope destruction, may be responsible for the observed decrease in immunoreactivity of the tested soy epitopes in CHAPTER 4. Future studies on the immunoreactivity of a variety of soluble and insoluble protein fractions are necessary to understand the mechanisms involved in the inactivation of allergenic epitopes by fermentation. In order to optimize allergy mitigation methods, it is essential to understand the impact of processing on the allergenic potential at the molecular level. Hence, further research must be conducted to get deeper and detailed knowledge of the allergen structure and the impact of food processing on epitope destruction/alteration. The key factors that contribute to an allergen's allergenicity and whether the structure of the specific epitope can be destroyed by processing are important issues for future research activities. Understanding the impact of all processing technologies on the sensitization potential of food allergens and the thresholds for elicitation of allergic reactions in sensitized individuals is crucial for the management of food allergy (MILLS et al., 2009). The regulations of the European Union for labeling infant formulas as having reduced allergenicity are based arbitrarily on a content of immunoreactive protein of lower 1 % of total nitrogen containing substances (COMMISSION OF THE EUROPEAN COMMUNITIES, 1996), but there is no evidence that such a threshold of immunogenic proteins would ensure a reduced clinical allergenicity, which has to be examined in future. "Hypoallergenic" foods for the consumption by food-allergic individuals should have a significantly lower *in vivo* allergenicity compared to naturally occurring foods (MAHLER, 2015). Up to now, only pure amino acid mixtures are considered to be "non-allergenic" (HØST & HALKEN, 2004). The exact threshold of allergenic soy proteins necessary to elicit an immune response is still uncertain. However, it is important to note that the production of "zero- or non-allergenic" food ingredients is almost impossible when considering the sensory and functional properties.

Although the increasing number of studies and the present study demonstrated that soy allergy can be increased or decreased to a certain degree by processing, it is not reliable predictable which effect processing have on the residual allergenicity. This is complicated by the fact that besides the processing technology applied, the matrix components such as polyphenols, lipids, or carbohydrates can strongly influence the allergen modification. Furthermore, it is also acknowledged that food processing can

affect the responsiveness of the immunoassay methods used to monitor allergens in foods and equipment clean-down (BARBOSA-CANOVAS et al., 2009; POMS et al., 2004). Thus, processing could lead to impaired analytical sensitivity for allergens that are not significantly reduced in their allergenicity, which would cause distorted results. To be able to predict the allergenicity of a certain protein would be particularly useful as a part of process designing of a mitigation method. However, models that would permit accurate assessment of allergenic potential of proteins unrelated to known allergen do not exist up to now. A better understanding of how processing affects allergen structure and allergen screening assays, would support the interpretation of immunoassay results especially when used to monitor highly processed ingredients.

2 Soy allergy mitigation methods

Within this work, novel and optimized nonthermal soy allergy mitigation technologies, including enzymatic hydrolysis, fermentation, high pressure processing as well as emerging technologies such as pulsed ultraviolet light, cold atmospheric pressure plasma and gamma-irradiation have been successfully developed.

2.1 Enzymatic hydrolysis

"Hypoallergenic" foods currently available on the market are mainly produced by means of enzymatic hydrolysis, which has proven to be efficient to attenuate soy immunoreactivity (WILSON et al., 2005; YAMANISHI et al., 1996).

In the first study, various food-grade proteases with respect to their effects on the potential allergenicity of soy protein isolate (SPI) were screened (CHAPTER 1). The results clearly demonstrate that enzymatic hydrolysis is quite effective in the alteration of the molecular weight distribution of soy proteins, depending on the protease applied. The extent of degradation was assessed by DSC, SDS-PAGE, and degree of hydrolysis (DH) analyses. This study was important for the subsequent developments, investigating debittering methods presented in CHAPTER 2 and 3.

In the following study (CHAPTER 2), the combination of various enzyme preparations, applied as one- or two-step process, was successfully conducted for enhancing the extent of hydrolysis of major soy allergens Gly m5 and 6. The results revealed

that enzymatic hydrolysis turned out to be a great approach for increasing the DH value up to 30 % and generating proteins with low molecular weight sizes. As evidenced by SDS-PAGE and LC-MS/MS analysis, enzyme combinations support the degradation of major soy allergens more effectively compared to the utilization of single enzyme preparations as described in CHAPTER 1. In particular, the initial application of an endoprotease and subsequently with an exoprotease was most effective. This could be ascribed to the fact that enzymes might compete and negatively affect each other, when hydrolyzing SPI concomitantly.

According to the results given, the exact inactivation mechanisms of allergenic epitopes by enzymes would be worth further and detailed studies. For this purpose, the exact epitope structure at the amino level is necessary in order to model the exact destruction of the allergenic epitopes. Furthermore, if the exact structure of allergenic epitopes is known, specific enzymes could be designed, which can exactly cleave amino acids at the position known to be recognized by IgE-antibodies.

Commercial available "hypoallergenic" formulas are processed by means of enzymatic hydrolysis of different protein sources such as bovine casein/whey and soy followed by further processing such as thermal treatment and commonly ultrafiltration, or they are based on the admixture of amino acids, which are considered to be "non-allergenic" (HØST & HALKEN, 2004). As some extensively hydrolyzed and amino-acid-based products have met these criteria, it would be interesting to study further combination processes including enzymatic hydrolysis with i.e. subsequent filtration or physical processes such as high pressure processing or high pressure homogenization.

2.2 Fermentation

In CHAPTER 3, the combination of enzymatic hydrolysis and subsequent fermentation was performed. With respect to the degradation efficiency of this combination process, the SDS-PAGE and DH value results evidenced that fermentation does not show further impact on the molecular weight distribution of soy hydrolysates. However, this combination process was initially conducted in order to subsequently degrade the bitter taste perception of SPI hydrolysates, and these results are discussed in section 3 in detail.

Although the enzyme assisted fermentation process was not as successful as expected, the fermentation of the native SPI was investigated and showed interesting results (CHAPTER 4). The data obtained by ELISA and western blot confirmed

a significantly reduced Gly m5 immunoreactivity of soy proteins by induced liquid state fermentation of SPI using *Lactobacillus helveticus*, *Rhizopus oryzae*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*.

Among the strains assayed, *L. helveticus* revealed the most abundant reduction in terms of immunoreactivity within the soluble protein fractions up to 100 %. Furthermore, almost no binding to anti-Gly m5 mAbs and sera from soy sensitive patients was found in western blots. In addition, it could be verified by different analytical methods comprising the colony forming unit, pH value, and MALDI-TOF-MS analysis that the inoculated *L. helveticus* was able to dominate the fermentation process. Consequently, every change in molecular weight distribution and immunoreactivity can be ascribed to this strain.

L. helveticus is a "generally recognized as safe" (GRAS) lactic acid bacteria, which can be applied as "natural" food preservative and starter culture for the production of hypoallergenic food ingredients. Furthermore, *L. helveticus* is known to possess many common probiotic properties such as prevention of gastrointestinal infections, making the fermented food products healthier (TAVERNITI & GUGLIELMETTI, 2012). Another worthwhile topic to investigate is the simultaneously application of different microbial and mold strains, which might be complementary, making it possible to further reduce the immunoreactivity.

2.3 Emerging nonthermal technologies

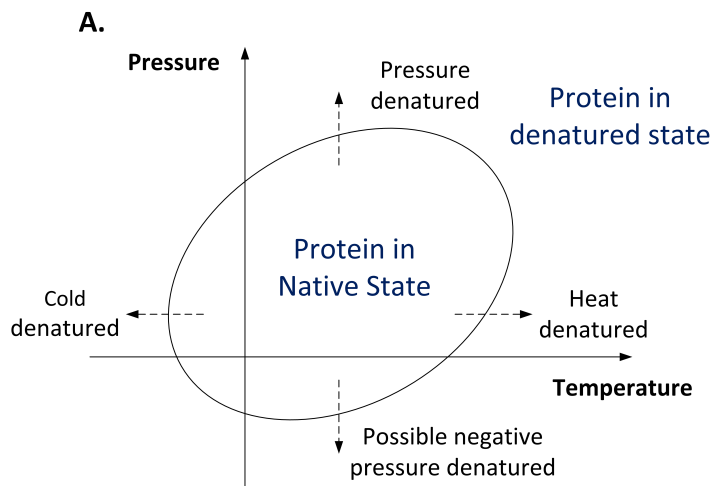
Due to the ability of high pressure processing to unfold proteins (see Figure 1 A) and to alter enzyme activity/specificity, the effect of high pressure assisted hydrolysis on residual soy immunoreactivity has been presented in CHAPTER 5. For the experiments, Flavourzyme® was used due to its ability to remain or even reduce the *bitter* taste of soy. However, it was also shown in CHAPTER 1 that Flavourzyme® is not effective in the degradation of soy allergens at atmospheric pressure. The DH value, SDS-PAGE, and LC-MS/MS results evidenced that this combination process - high pressure processing prior to and during enzymatic hydrolysis - increased the proteolytic activity and protein accessibility of Flavourzyme®. This study confirmed a drastically or completely inhibited immunoreactivity of Gly m5 at pressure levels between 400 and 500 MPa.

From the perspective of food safety, this technique presents a "hurdle concept", in which high pressure support enzymatic hydrolysis, when applied concomitantly to enzymatic hydrolysis to attain high level of soy allergen elimination. Simultaneously,

this technique allows the removal of pathogenic microorganisms from raw materials and food, which reduce concerns about food safety; thus, this aspect of microbial safety would worth further study, investigating the residual microbial load, including pathogens and spores, of the prepared samples. These advantages are not offered by other food processing techniques up to now, thus high pressure is a highly beneficial technique (HUANG et al., 2014).

Furthermore, more research is needed to understand the impact of pressure on structural changes of proteins. The analysis of i.e. the surface hydrophobicity and conformational characterization using i.e. circular dichroism spectra (UV-CD), multi-angle laser light scattering (MALLS) as well as quasi-elastic light scattering (QELS) or Fourier transform infrared spectroscopy (FTIR) might be powerful tools to corroborate the present results.

The functional associations of pressure, temperature and exposure time should be provided by means of pressure-temperature diagrams (pT-diagrams) as exemplarily presented in Figure 1 B and C, which show pressure-temperature combinations yielding to a desired reaction (e.g. allergen destruction) rate. In preliminary trials (unpublished data) temperatures of 20 °C, 50 °C, and 70 °C as well as pressures between 100 and 600 MPa with respect to Gly m5 destruction and residual immunoreactivity using specific anti-Gly m5 antibodies were investigated. Based on these experiments, isokinetic lines for Gly m5 destruction as a results of high pressure processing prior to enzymatic hydrolysis on the basis of SDS-PAGE results were exemplarily modeled (see Figure 1 B). For the calculation of the isokinetic lines the Weibullian approach as described in SEVENICH et al. (2015) with slight modifications was applied using the analytical software "Geeraerd and Van Impe Inactivation model Fitting Tool" (GinaFit Version 1.6 March 2012, Katholieke Universiteit Leuven). Consequently, a deeper insight into the effect of high pressure processing on enzyme activity and specificity as well as on epitope destruction and consequently reduction in allergenicity as a function of pressure and temperature on the basis of immunoassay data would be intriguing topics of further research studies. Therefore, the development of a predictive and quantitative model for epitope degradation along with allergenicity reduction by high pressure assisted hydrolysis would be of vital importance for process modeling. As the processing time was set to 15 min in this study, variation of processing and corresponding holding time - less or more than 15 min - would be worth continued investigations.



B. High pressure processing prior to enzymatic hydrolysis

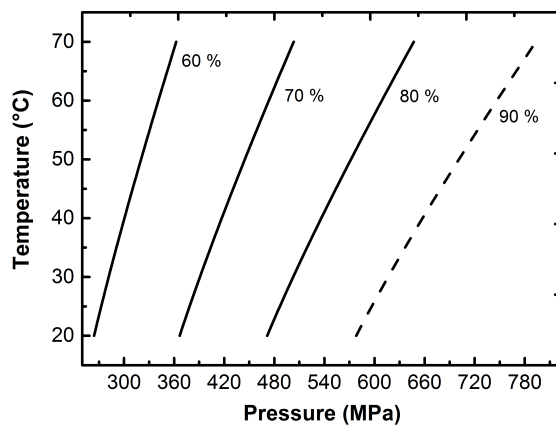


Figure 1. (A) Schematic elliptic phase diagrams of proteins (SMELLER, 2002) and (B) schematic isokinetic lines of reduction (%) of major soy allergens Gly m5 in the p,T-domain for high pressure processing prior to enzymatic hydrolysis (Flavourzyme®) for 15 min at 50°C based on SDS-PAGE analysis results.

The last study was designed, to evaluate the effects of other emerging technologies, including gamma-irradiation, pulsed light, and cold atmospheric pressure plasma on residual soy immunoreactivity (CHAPTER 6). The results revealed that all technologies investigated seem to be efficient approaches to reduce Gly m5 immunoreactivity. The maximum reduction up to 100 % was achieved by direct plasma treatment as well as pulsed light and gamma-irradiation treatment. To the best of our knowledge, reports on the effect of plasma treatment on soy immunoreactivity are not available in the literature up to now, and the results obtained by direct and remote plasma treatment revealed a first insight into their potential to attenuate soy immunore-

activity for the first time. In contrast to MORIYAMA et al. (2013), who could not find changes in Gly m5 immunoreactivity after gamma-irradiation treatment of soy, it was found that gamma-irradiation is effective in eliminating allergens applying a dose rate above 25 kGy. However, the legislation allows a dose rate up to 10 kGy for specific commercial food items up to now (FDA, 2004), which is not sufficient to degrade soy proteins according to the present results. Although decades of research worldwide have shown that irradiation of food is an effective way to kill food-borne bacteria and extend shelf-life, irradiated foods are not widely available yet. Cold atmospheric pressure plasma and pulsed light treatment, which are currently *inter alia* applied for surface disinfection, show limitations in practice due to limited penetration depth, shadow effects, and the considerable increase in temperature accompanied by samples weight loss. The effects on protein characteristics as well as toxicological safety have to be elucidated and clarified before reconsidering the application of these technologies for the production of food ingredients. Although various reservations about the industrial application of these technologies exist, the present study showed that they have great potential for sufficient reduction of soy immunoreactivity, which could help to produce low-allergen food ingredients and food products in future.

3 Sensory perceptions of soy hydrolysates

Besides a reduced immunoreactivity, another aim of this thesis was the production of soy samples with a pleasant taste and good technofunctionality. Although certain flavor compounds with *beany* and *bitter* taste and smell pre-exist in maturing soybeans, food processing commonly lead to an amplified *bitter* taste, which impedes their utilization as food ingredient (FITZGERALD & O'CUINN, 2006; SHRIVER & YANG, 2011). As good taste is required for commercial utilization, better tasting ingredients are desired. Considering this drawback associated with enzymatic hydrolysis, special attention has been given to the prevention or even reduction of bitter taste throughout this thesis.

As shown in CHAPTER 1, enzymatic hydrolysis applying single enzyme preparations revealed a maintained or unfortunately significantly increased bitterness of SPI, depending on the proteases used. Applying exoproteolytic enzyme preparations such as papain and Flavourzyme, bitterness was effectively in maintaining or even decreasing bitterness. In contrast endoproteolytic enzyme activities, including Alcalase and Corolase preparations, were not suitable due to the formation of a strong bitterness.

Many attempts have been made to prevent, reduce, eliminate or even mask the bitterness of food hydrolysates. However, an effective debittering method is still not implemented in food industry (SAHA & HAYASHI, 2001). Therefore, further studies on enzymatic hydrolysis through various combinations of exo- and endopeptidases for the prevention of *bitter* taste formation and even reduction of the initial bitterness were conducted, which was indeed successfully achieved (CHAPTER 2). Bitterness of the resulting hydrolysates was remarkably reduced to a minimum of 1.3 compared to unhydrolyzed SPI with a bitterness intensity of 2.6.

As microorganisms are an important source of exopeptidases, which are known to cleave adjacent to hydrophobic amino acid residue at the C- or N-terminus of peptides, the effects of fermentation using *Lactobacillus perolens*, *Aspergillus elegans*, and *Rhizopus oryzae* on the debittering of SPI hydrolysates have been investigated (CHAPTER 3). Principal component analysis revealed that fermentation of soy hydrolysates ensured nearly complete reduction of *bitter* taste perception. Concomitantly, other off-flavors associated with raw soy like *beany* and *grassy* were drastically reduced. Hence, these samples were nearly "neutral" tasting. All strains used in this study have high potential for effectively debittering SPI hydrolysates, which could be successfully applied in food products. Furthermore, fermentation with i.e. *Lactobacillus perolens* is capable for the formation of emerged aroma compounds such as *buttery*, which could be beneficial in certain food products for flavor-improvement. However, complete debittering was not achieved, because neither carboxy- nor aminopeptidases are able to hydrolyze imino bonds and proline residues, which are responsible for a strong *bitter* taste (FITZGERALD & O'CUINN, 2006; SAHA & HAYASHI, 2001). Special attention should be given to proline-specific exopeptidases as proline gives a unique contribution to hydrolysate bitterness. Thus, in order to provide non-bitter protein-based food ingredients, the prolyl dipeptidyl aminopeptidase in preparations of *Lactococcus lactis* might provide a powerful method for a completely debittering of protein hydrolysates as they can hydrolyze dipeptides with the sequence X-Pro, releasing proline residues (ZEVACO et al. 1990). Besides organoleptic tests, the analysis of bitter peptides - their characterization and quantification - might be of particular importance to get a deeper insight in the enzyme assisted hydrolytic reactions. Identification of the resulting *bitter* peptides and free amino acids might also be an interesting research area to gain deeper insight in peptide structure and correlation between bitter peptides and taste perception. Although enzymatic debittering of soy protein isolates is of superior technological and nutritional value, limitations regarding their economic viability and the availability of specific enzymes exist. The cost of enzymes in conventional, batch-type hydrolytic

system is relatively high. Enzymatic hydrolysis unfortunately involves an increase in amino acids, which is accompanied by an additional taste, often referred to as off-flavours, derived from free amino acids (KODERA et al., 2006). Thus, controlled enzymatic debittering using exopeptidases and subsequent removal of free amino acids might be an interesting and powerful way to produce non-bitter hydrolysates. The experiments presented in CHAPTER 4 unexpectedly showed that SPI samples obtained after fermentation with *L. helveticus* exhibited great sensory properties with almost non-detectable *bitter* and *beany* off-flavors compared to non-fermented SPI. Hence, these samples showed upgraded sensory properties, wherefore the resulting samples are well-suited as food ingredient. *L. helveticus* is an important industrial starter culture that is known to express endopeptidases with *post*-proline specificity such as PepO2 and PepO3, which sufficiently degrade bitter peptides in cheese during ripening, which might explain the nearly non-detectable bitterness of the fermented samples (SOERYAPRANATA et al., 2007).

Up to now, comprehensive research on high pressure assisted enzymatic hydrolysis on the sensory perception is not available in literature so far. It could be shown in CHAPTER 5 by principal component analysis, that the *beany* and *green* off-flavors, characteristic for untreated SPI, could be reduced by the combination of high pressure processing and enzymatic hydrolysis. The bitterness of the samples could not be reduced applying high pressure processing during enzymatic hydrolysis. In contrast, the samples obtained after a high pressure treatment prior to enzymatic hydrolysis were not described by a *bitter* taste. This can be ascribed to the dominant exoproteolytic activity of Flavourzyme® at 0.1 MPa. This observation might corroborate the assumption that high pressure induced a change of enzyme specificity and activity. Finally, the application of the hydrolyzed soy protein isolates as food ingredients in several products such as mayonnaise, soft drinks, alternative dairy products i.e. soy yoghurt or bakery products and their impact of sensory product characteristics has to be investigated in further studies.

4 Techno-functional properties of soy hydrolysates

Their excellent techno-functional properties make soy proteins a popular functional ingredient for food industry. However, proteins undergo several changes during processing, which might improve or impair their functionality. As the allergenic epitopes integrity is not a *conditio sine qua non* for proper biological functioning of a given property, specific epitope-destruction in order to reduce the allergenic potential of

soy proteins must not consequently negatively affect the functionality of proteins; thus the chance to produce hypoallergenic foods with good functionality is enhanced. For the evaluation of the hydrolyzed SPI as food ingredients, their techno-functional properties should be investigated since they play an important role in the physical behavior of foods or ingredients during processing and storage. These properties include the protein solubility, emulsifying capacity, oil- and water-binding capacity as well as the foamability (activity, stability, and density) and gelation behavior have been analyzed extensively within this thesis (CHAPTER 1 - 5).

In general, all processing technologies applied showed great potential to produce low-allergen food ingredients with outstanding functional properties, which is highly dependent on the technology applied. As good solubility is required for various other functional properties such as emulsification and foamability, the protein solubility was initially determined. All technologies investigated - except fermentation (CHAPTER 4) - led to an increased protein solubility at both pH 4.0 and 7.0 compared to native SPI. It has been proposed that the reduction of the secondary structure of proteins, the release of smaller peptides and in turn the increase of ionizable amino and carboxyl groups are responsible for the improved solubility (ADLER-NISSEN, 1986; ORTIZ & WAGNER, 2002). However, fermentation resulted only in an increased solubility at pH 4.0. In contrast, solubility at pH 7.0 was nearly halved by fermentation compared to non-fermented SPI. Lactic acid bacteria such as *L. helveticus* produces a large amount of acid, which might result in irreversibly coagulation of proteins, and thereby decreasing protein solubility at neutral pH, which support the findings of ELKHALIFA et al. (2005) and SADOWSKA et al. (1999).

Depending on the process applied, the emulsifying capacity of native SPI could be improved or reduced. In order to upgrade this functional property, enzymatic hydrolysis applying single enzymes and enzyme combinations alone or in combination with subsequent fermentation would be the method of choice. However, all samples prepared with Alcalase or fermentation alone showed a decreased emulsifying capacity. This can be ascribed to the fact that fermentation lowered the protein solubility at pH 7.0; consequently a reduced emulsifying capacity at pH 7.0 could be observed. High pressure treatment or homogenization could be taken into account in order to subsequently increase the emulsifying capacity. The water-binding capacity decreased due to the increase in protein solubility as well as the size of protein particles and surface topography, which influences protein-water interactions. Only fermentation revealed an increased water-binding capacity. Also an increased oil-binding capacity could be found for all samples prepared in this study.

According to the results, it could be concluded that all mitigation methods investigated must be conducted under strict controlled reaction conditions in order to

maintain or even improve the techno-functional characteristics. Depending on the desired technofunctionality, each process has the opportunity to raise these characteristics. Furthermore, it is indispensable to analyze if these enhanced techno-functional properties provided by the samples are still present in a final food product. Consequently, application experiments with the samples as ingredient have to be performed.

5 Final Conclusions

Finally, the objective of further studies might be the investigation of the anti-nutritive components in soy before and after processing. Although soy is a major source of both high quality vegetable protein and edible oil, it contains several anti-nutrients that limit their utilization. These components include *inter alia* phytic acid, protease inhibitors, tannins as well as lectins and flatulence-causing oligosaccharides such as raffinose, stachyose, and verbascose (HEFNAWY, 2011). It is of importance to remove these anti-nutrients to improve the nutritional quality of soy. As these factors are heat- and processing-labile, they probably could have been destroyed due to the technologies investigated in this thesis, contributing to the health benefits of the resulting samples.

In conclusion, even though the processing technologies developed within this study for soy allergy mitigation require some advances, they still have great potential and provide a comprehensive and feasible technological basis for the production of low-allergen soybean-based food ingredients with pleasant taste and enhanced techno-functional properties (see section 3 and 4). Further combinations of physical, chemical, and biological technologies might be effective to maximize the alleviation of soybean allergy and to minimize the change in protein characteristics such as sensory and functional properties. More specifically, application of a "hurdle-concept" in allergen mitigation, in which one technology is combined with another, can be quite effective to prepare soy products with a low level of residual immunoreactivity as already shown in CHAPTER 5. Furthermore, for soy-sensitive patients, in particular young children, food products with reduced allergenicity, if developed, could be beneficial and may raise the threshold of the amount of allergenic proteins required to trigger an allergic response. Managing allergens in a manner that minimizes the risk to allergic consumers, while maximizing their choices as well as ensuring the overall sustainability of food production processes remains a huge subject of considerable work (CREVEL, 2015).

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SUMMARY

The increasing prevalence of food allergies is currently a serious health concern. Up to 250 million people worldwide suffer from some kind of food allergy. A food allergy is an immunoglobulin E (IgE)-mediated adverse reaction of the immune system towards food components, commonly proteins. Although almost any food is capable of causing an allergy, it was found that nearly 90 % of all allergic reactions are triggered by eight main protein sources, including soy (*Glycine max*).

The ubiquitous presence of soy allergens in human food supply coupled with an increased awareness of food allergies has intensified much effort for developing food allergy mitigation methods. The technological approach hitherto has mainly been focused on thermal processing technologies, which commonly retain the ability of soy to elicit an allergy and result in deteriorated functional and sensory properties. Recently, novel nonthermal food processing technologies have emerged in the food industry due to their negligible effects on food properties. However, these technologies have so far only partially or not been considered appropriately in literature with respect to their potential utility to reduce food allergy. Consequently, the present thesis aimed to develop nonthermal food processing technologies for the production of low-allergen soy products that combine a pleasant taste and good techno-functional properties.

The initial part of this thesis (CHAPTER 1) consisted of a comprehensive screening of various microbial- and plant-derived proteases to find appropriate enzymes for the sufficient degradation of major soy protein isolate (SPI) allergens β -conglycinin (Gly m5) and glycinin (Gly m6). As the sensory perception and technofunctionality in terms of protein solubility, emulsifying capacity, water- and oil-binding capacity, and foamability (activity, density, stability) of SPI are indispensable for commercial utilization as food ingredient, these characteristics have been analyzed as well. The degree of hydrolysis (DH), differential scanning calorimetry (DSC), and SDS-PAGE analyses revealed that enzymatic hydrolysis is effective in the destruction of Gly m5 and 6 and the initial DH value of SPI (2.1 %) was enhanced up to 13 %. The sensory perception, in particular bitterness, and the functional properties could

be maintained, depending on the protease applied. Papain was the most appropriate protease preparation, which effectively decreased the molecular weight of SPI and concomitantly improved the technofunctionality and sensory characteristics; however, a certain bitterness still remained. In contrast, the enzyme preparation Flavourzyme® was very attractive due to its ability to prevent or even reduce the bitter taste of native SPI, whereas it was not effective in the degradation of major soy allergens at atmospheric pressure. Therefore, following studies for both further degradation of soy allergens and reduction of bitter taste were performed (CHAPTER 2 and 3).

An effective debittering method has not successfully been developed up to now. Therefore, the efficiency of enzyme combinations applying five proteases and combinations thereof for the prevention of bitter taste formation or even reduction of the initial bitterness of non-hydrolyzed SPI was investigated. Hydrolysis was performed as one- and two-step process. Furthermore, the technofunctionality of the resulting hydrolysates was also examined. The results obtained by SDS-PAGE, DH, and LC-MS/MS analyses confirmed that major soy allergens treated with enzyme combinations were degraded remarkably or totally, thus they were more effectively compared to single enzymes. A DH value up to 30 % could be achieved and proteins with molecular masses lower 20 kDa were generated. More interestingly, the resulting hydrolysates possessed a considerable lower bitterness compared to non-hydrolyzed SPI. Furthermore, the emulsifying capacity, solubility, and foaming ability of untreated SPI were greatly improved.

Despite the improved sensory perception, a complete removal of bitterness of SPI could not be achieved. As a consequence, a subsequent fermentation step of SPI hydrolysates (*Lactobacillus perolens*, *Rhizopus oryzae*, *Actinomucor elegans*) after the enzymatic hydrolysis was investigated in order to eliminate the bitter taste. The microbial growth and functional properties have been analyzed as well. The electrophoretic profiles evidenced that subsequent fermentation did not further alter the electrophoretic profiles of SPI hydrolysates. All strains investigated drastically reduced the bitterness to almost non-bitter compared to non-fermented hydrolysates and untreated SPI. The techno-functional properties of the hydrolyzed and fermented isolates showed improved foaming stabilities (90 %) and gelation formation concentrations (10 %) of SPI up to 94 % and 4 %, respectively. Principal component analysis (PCA) revealed that fermentation resulted in well and nearly "neutral" tasting products as the bitter taste and beany off-flavors characteristic for soy could be considerably reduced.

In continuation of the above mentioned work, the effect of liquid state fermentation (*Bacillus subtilis*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *Lactobacillus helveticus*) without enzymatic hydrolysis on the immunoreactivity, functional and sensory

properties of SPI was investigated (CHAPTER 4). *Lactobacillus helveticus* revealed the most abundant reduction in terms of immunoreactivity up to 100 %, which could be measured by *in vitro* sandwich ELISA using specific mouse monoclonal anti-Gly m5 antibodies (mAbs). The immunoreactivity was calculated in relation to non-fermented SPI, which was set to 100 %. Almost no binding in western blot using anti-Gly m5 mAbs and sera from soy-sensitive patients was found. Fermentation increased water- and oil-binding capacity as well as solubility at pH 4.0 and the foaming activity of SPI was nearly doubled. However, a decreased emulsification, foaming density, and protein solubility at pH 7.0 were observed. PCA analysis confirmed that these samples are suitable as food ingredient due to their indiscernible bitterness.

The final objective of this work was to investigate the effect of various nonthermal technologies including high pressure assisted hydrolysis, pulsed UV light, gamma-irradiation as well as direct and remote cold atmospheric pressure plasma to attenuate soy immunoreactivity (CHAPTER 5 and 6). The efficiency of high pressure processing (prior to/during) assisted enzymatic hydrolysis using the enzyme preparation Flavourzyme[®] on the residual immunoreactivity of SPI was examined. Within the tested conditions of the study, the results revealed that this combination process is strongly capable of almost completely inhibiting immunoreactivity at 400 and 500 MPa. According to the DH, SDS-PAGE, and LC-MS/MS results, pressures above 300 MPa - prior to and during hydrolysis - enhanced the proteolytic activity of Flavourzyme[®], thus a sufficient degradation of Gly m5 could be observed. Furthermore, the beany, bitter and green off-flavors were almost completely removed. The resulting hydrolysates possessed improved protein solubility, foaming activities, oil-binding and emulsifying capacities. In the last study, the potential of other emerging technologies such as pulsed UV light, gamma-irradiation as well as direct and remote cold atmospheric pressure plasma for the mitigation of SPI immunoreactivity was studied. This study provided preliminary evidence that all technologies investigated are efficient approaches to reduce Gly m5 immunoreactivity. The maximum reduction in immunoreactivity (91 - 100 %) was achieved by direct plasma, pulsed UV light, and gamma-irradiation treatment. Remote plasma treatment resulted also in a loss of the immunoreactivity up to 89 %.

In summary, the findings of this thesis demonstrate that all processes applied provide a comprehensive and feasible technological approach to mitigate soy immunoreactivity. Consequently, these results will be helpful for the preparation of low-allergen food ingredients with pleasant taste and enhanced functional properties, which in turn will make an important contribution to food safety.

ZUSAMMENFASSUNG

Aufgrund der steigenden Prävalenz allergischer Reaktionen stellen Nahrungsmittelallergien derzeit ein ernstzunehmendes Gesundheitsproblem dar. Nahezu 250 Millionen Menschen weltweit leiden bereits unter einer Nahrungsmittelallergie. Darunter ist eine Immunoglobulin E (IgE)-vermittelte Reaktion des Immunsystems gegenüber Lebensmittelbestandteilen, regelmäßig Proteine betreffend, zu verstehen. Obgleich fast jedes Lebensmittel eine Allergie hervorrufen kann, wurde festgestellt, dass fast 90 % aller Allergien von acht Proteinen, einschließlich Soja (*Glycine max*), ausgelöst werden.

Die allgegenwärtige Präsenz von Sojaproteinen in der menschlichen Nahrung und das damit verbundene erhöhte Bewusstsein für Allergien haben die Bemühungen, Prozesse zur Reduktion der Allergenität zu entwickeln, verstärkt. Der bisherige technologische Ansatz konzentrierte sich dabei vornehmlich auf thermische Verfahren, welche jedoch das allergene Potential von Soja beibehalten und zu verschlechterten funktionellen und sensorischen Eigenschaften führen. Zwischenzeitlich sind in der Lebensmittelindustrie nicht-thermische Verfahren aufgrund ihres geringen Einflusses auf die zuvor genannten Eigenschaften in der Lebensmittelindustrie auf ein breites Interesse gestoßen. Gleichwohl wurden diese Technologien im Hinblick auf ihren potentiellen Einsatz zur Reduzierung der Allergenität bisher kaum in der Literatur betrachtet. Die vorliegende Arbeit beschäftigt sich daher mit nicht-thermischen Technologien zur Herstellung niedrig-allergener Produkte auf Basis von Soja unter Berücksichtigung der sensorischen und techno-funktionellen Eigenschaften.

Der erste Teil der Arbeit (KAPITEL 1) bestand aus einem umfassenden Screening von Enzymen mikrobiellen und pflanzlichen Ursprungs, um geeignete Enzyme für den Abbau der Hauptallergene von Sojaproteinisolat (SPI), β -Conglycinin (Gly m5) und Glycinin (Gly m6), zu finden. Da die sensorischen und techno-funktionellen Eigenschaften insbesondere die Proteinlöslichkeit, Emulgierkapazität, Wasser- und Fettbindekapazität und die Schaumeigenschaften (Aktivität, Stabilität, Dichte) von SPI unverzichtbar für den kommerziellen Einsatz als Lebensmittelzutat sind, wurden diese ebenfalls untersucht. Die Bestimmung des Hydrolysegrades (DH), die

dynamische Differenzkalorimetrie (DSC) und SDS-PAGE Analyse ergaben, dass die Hydrolyse effektiv Gly m5 und 6 zerstört und der DH vom nativen SPI (2,1 %) auf bis zu 13 % erhöht werden konnte. Je nach eingesetztem Enzympräparat, konnten sowohl die sensorische Wahrnehmung, insbesondere die Bitterkeit, als auch die techno-funktionellen Eigenschaften beibehalten werden. Papain bewies sich als besonders geeignet, da neben einer Verbesserung der funktionellen und sensorischen Eigenschaften auch der stärkste Abbau der Hauptallergene erreicht werden konnte. Allerdings wiesen die Proben eine deutlich wahrnehmbare Bitterkeit auf. Dagegen war das Enzympräparat Flavourzyme[®] wegen seiner Fähigkeit die Entstehung eines bitteren Geschmack zu verhindern oder sogar die Bitterkeit vom nativen SPI zu reduzieren interessant; jedoch zeigte sich dieses Enzym nicht wirksam beim Abbau der Allergene unter atmosphärischen Bedingungen. Daher war sowohl der weitere Abbau der Allergene als auch die Reduzierung des bitteren Geschmacks Gegenstand fortführender Untersuchungen (KAPITEL 2 und 3).

Eine wirksame Methode zur Verringerung der Bitterkeit konnte bislang nicht erfolgreich entwickelt werden. Folglich wurde der Einsatz von Enzymkombinationen, bestehend aus fünf Proteasen, zur Prävention eines bitteren Geschmackes und Verringerung der ursprünglichen Bitterkeit des SPI untersucht. Die Hydrolyse wurde als ein- und zweistufiges Verfahren durchgeführt und die techno-funktionellen Eigenschaften wurden ebenfalls analysiert. Die Ergebnisse vom DH, der SDS-PAGE und LC-MS/MS Analyse konnten bestätigen, dass die Hauptallergene durch Enzymkombinationen im Vergleich zu einzelnen Enzymen effektiver abgebaut wurden. Ein DH von bis zu 30 % und eine Molekulargewichtsverteilung kleiner 20 kDa wurde erreicht. Interessanterweise wiesen die Hydrolysate eine deutlich geringere Bitterkeit als das native SPI auf. Zudem wurden die Emulgierfähigkeit, Proteinlöslichkeit und das Schaumvermögen deutlich verbessert.

Trotz der verbesserten Sensorik, war eine gewisse Bitterkeit nach wie vor vorhanden, weshalb die Wirksamkeit einer anschließenden Fermentation (*Lactobacillus perolens*, *Rhizopus oryzae*, *Actinomyces elegans*) der SPI Hydrolysate zum weiteren Abbau der Bitterkeit untersucht wurde. Das mikrobielle Wachstum und die funktionellen Eigenschaften wurden ebenfalls analysiert. Mittels SDS-PAGE konnte gezeigt werden, dass die Fermentation der Hydrolysate keine Veränderung in der SDS-PAGE bewirkt, jedoch die Bitterkeit vom nativen SPI und den SPI Hydrolysaten jedoch drastisch reduziert wurde. Die techno-funktionellen Eigenschaften der hydrolysierten und fermentierten Isolate zeigten verbesserte Schaumstabilitäten (90 %) und Gelbildungskonzentrationen (10 %) vom nativen SPI bis zu 94 % bzw. 4 %. Die Hauptkomponentenanalyse (PCA) ergab, dass durch die Fermentation nahezu "neutral" schmeckende Proben mit deutlich reduzierten Fehlgerüchen vom SPI wie Bitterkeit und bohniiger Geschmack hergestellt werden konnten.

In Weiterführung der vorangegangenen Arbeit, wurde der Einfluss der Fermentation (*Bacillus subtilis*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *Lactobacillus helveticus*) ohne vorherige Hydrolyse auf der Immunreaktivität, funktionellen und sensorischen Eigenschaften des SPI untersucht (KAPITEL 4). Hierbei zeigte *Lactobacillus helveticus* die größte Reduktion der Immunreaktivität bis zu 100 %, welche mittels *in vitro* Sandwich ELISA und monoklonalen Maus anti-Gly m5 Antikörpern (mAk) bestimmt wurde. Die Proben wiesen nahezu keine Bindung zu den mAk und Humanseren von Soja-Allergikern im Western Blot auf. Die Fermentation erhöhte das Wasser- und Fettbindevermögen sowie die Proteinlöslichkeit bei pH 4,0; zudem wurde die Schaumaktivität von SPI nahezu verdoppelt. Die Emulgierkapazität, Schaumdichte und Proteinlöslichkeit bei pH 7,0 verschlechterten sich. Die Eignung dieser Proben als Nahrungsmittelzutat aufgrund ihrer kaum wahrnehmbaren Bitterkeit wurde durch die PCA zweifelsfrei bestätigt.

Im letzten Abschnitt der Arbeit wurde der Einfluss nicht-thermischer Technologien wie die hochdruck-unterstützte enzymatische Hydrolyse, gepulstes UV-Licht, Gamma-Bestrahlung sowie Niedertemperatur-Plasma im Hinblick auf die Minderung der Immunreaktivität von Soja untersucht (KAPITEL 5 und 6). Die Effizienz der Hochdruckbehandlung vor und während der enzymatischen Hydrolyse, unter Einsatz des Enzympräparats Flavourzyme[®], wurde auf die verbleibende Immunreaktivität analysiert. Die Ergebnisse bewiesen, dass dieses Kombinationsverfahren bei Drücken von 400 und 500 MPa eine fast vollständige Hemmung der Immunreaktion bewirkt. Die DH, SDS-PAGE und LC-MS/MS Analytik zeigte, dass Drücke höher als 300 MPa - vor und während der Hydrolyse - die proteolytische Aktivität von Flavourzyme[®] verbesserten; ausserden konnte ein erhöhter Abbau von Gly m5 beobachtet werden. Ferner wurden die bohningen, bitteren und grünen Fehlgerüche von SPI fast vollständig entfernt. Die Hydrolysate besaßen eine verbesserte Proteinlöslichkeit, Schaumaktivität sowie Fettbinde- und Emulgierkapazität. In der letzten Studie wurde das Potenzial anderer Technologien wie gepulstes UV-Licht, Gamma-Bestrahlung sowie direktes und indirektes Niedertemperatur-Plasma zur Abschwächung der Immunreaktivität von Soja untersucht. Die Ergebnisse lieferten erste Hinweise, dass alle Verfahren effektiv die Immunreaktivität senken. Die maximale Reduktion der Immunreaktivität (91 - 100 %) wurde durch direktes Plasma, gepulstes UV Licht und Gamma-Bestrahlung erreicht. Indirektes Plasma führte ebenfalls zu einem Verlust der Immunreaktivität von bis zu 89 %.

Insgesamt ist festzuhalten, dass die entwickelten Verfahren einen umfassenden und übertragbaren Ansatz zur Herstellung niedrig-allergener Lebensmittelzutaten mit angenehmen Geschmack und guten funktionellen Eigenschaften bieten und damit einen wertvollen Beitrag zur Lebensmittelsicherheit leisten.