TECHNISCHE UNIVERSITÄT MÜNCHEN

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Effects of Enzymatic Hydrolysis and Fermentation of Pea Protein Isolate on its Functional, Sensory and Immunogenic Properties

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“The secret to finding your passion is to bring it to everything you do.”

--Marie Forleo

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Summary

Peas (*Pisum sativum* L.) were one of the first cultivated crops. Currently, the global pea protein ingredient market is growing fast, as plant-based diets gain popularity. Peas have high protein content including all essential amino acids. They can replace soybeans as their proteins are not considered main allergens and their production is more sustainable. However, pea proteins exhibit poor functional properties and an unpleasant flavor compared to soybean proteins. Furthermore, recent studies have shown an increase in pea allergy prevalence, which might hinder their application in food products. Pea proteins can be used as functional ingredients or added to food products for protein enrichment. Several technological treatments are known to modify protein functionality, aroma and allergenicity of legumes; nevertheless, there is a lack of studies on modification of pea protein considering all three aspects altogether. Therefore, the focus of the present dissertation was to investigate the effect of enzymatic hydrolysis, fermentation and cultivar selection on protein functionality, sensory profile, changes in the molecular weight distribution and its effect on the degradation of allergen fractions and immunogenicity of pea protein isolates.

The first study (Chapter 1) investigated the effect of eleven proteolytic enzymes on functionality, sensory profile and main allergen fractions of a pea protein isolate. Different hydrolysis times were correlated to the degree of hydrolysis and further functional properties. The electrophoretic results showed changes in the molecular weight distribution and suggested degradation of main allergens with specific enzymes, especially for longer hydrolysis times. Furthermore, higher degrees of hydrolysis were correlated with higher protein solubility and improved foaming properties; however, there was a correlation between a high degree of hydrolysis and an increased bitter taste of the hydrolysates. The increase in bitterness is known to lower the consumer acceptance of hydrolysates and requires the usage of appropriate methods to mask or reduce the bitter taste.

Microbial fermentation is a well-known process to modify the organoleptic characteristics of food. Thus, the second study (Chapter 2) aimed to disclose the effects of lactic acid fermentation on the properties of pea protein isolate from the same cultivar. This study was mainly focused on changes of the sensory profile but also of crucial functional properties and a potential degradation of allergen fractions. Six strains were investigated and proven successful in reducing characteristic *bitter* taste and *beany* off-flavor after 24 h; however, with longer times of fermentation (48 h) undesirable flavors increased, which impaired the acceptance of the fermented samples. Moreover, all fermented samples showed reduced functional properties regardless of pH and no significant changes on the allergen fractions. These results highlight the potential of controlled 24-h fermentation for sensory improvement of pea protein isolates. However, the limited functionality

needed to be addressed in order to maintain the versatile application range of pea protein isolates.

From the first study, three proteolytic enzymes (papain, Esperase® and trypsin) were identified as promising for improved functionality and reduced immunogenicity of pea proteins. In the second study, the sample fermented with *Lactobacillus plantarum* for 24 h showed the most neutral taste, a reduction in *bitter* taste and highest overall acceptance. Thus, the third study (Chapter 3) dealt with a favorable combination of the three enzymes with subsequent or preceding microbial fermentation using *L. plantarum* to obtain a highly functional pea protein with neutral flavor and reduced immunogenicity. The order of treatment affected the results significantly, in which fermentation followed by enzymatic hydrolysis showed the most promising results regarding all evaluated aspects. Electrophoretic and gel filtration results revealed a reduction in the molecular weight distributions of the protein fractions and a significant degradation of the allergen fractions. An indirect Enzyme-linked Immunosorbent Assay (ELISA) was performed to confirm changes in the allergen fractions *in vitro*. The ELISA confirmed a reduced immunogenicity of treated samples, especially when hydrolyzed with trypsin, which suggested a reduction in immunogenicity of the total protein. For all treatment combinations, the protein solubility and foaming capacity were improved and the characteristic pea off-flavors were reduced. The results show the advantages of combining non-thermal treatments to produce highly functional pea protein ingredients with neutral taste. Furthermore, the reduced antibody-binding suggests that the treated pea proteins might promote allergic reactions to a lower extent.

The pea protein composition is affected by genotype, environmental conditions, harvest and storage. Thus, pea cultivars might show differences on the chemical composition, molecular weight distribution, protein functionality and sensory profile of their protein isolates. The final study (Chapter 4) screened different pea cultivars for their suitability regarding the production of sensory appealing and functional protein isolates. The goal was to understand the impact of the cultivar characteristics on protein isolate properties. The standardized production of the pea protein preparations included milling the cotyledons, solubilizing the proteins using alkaline extraction prior to isoelectric precipitation and spray-drying. Although the molecular weight distribution did not show significant differences, principal component analyses of protein functionalities and sensory profiles showed different clusters with significant differences between *pea-like* and *bitter* attributes. These results suggest that the proper selection of a pea cultivar or a combination of cultivars is important depending on the final application and that proteolysis or fermentation might not have the same effect among cultivars.

In summary, the findings of this thesis demonstrate that enzymatic hydrolysis, fermentation and a combination of both might be a promising approach to obtain valuable pea protein ingredients suitable for versatile food applications. The results demonstrate the importance of raw material

selection and process parameters in order to produce food ingredients with a high sensory acceptance and functional properties. The results suggest that the modification of pea proteins by enzymatic hydrolysis alone or in combination with microbial fermentation might result in reduced allergenic reactions after consumption.

Zusammenfassung

Erbsen (*Pisum sativum* L.) gehören zu den ältesten Kulturpflanzen. Derzeit verzeichnet der Weltmarkt für Erbsen-Proteinzutaten ein starkes Wachstum, da die pflanzliche Ernährung global einen immer wichtigeren Stellenwert einnimmt. Erbsen weisen einen hohen Proteingehalt mit allen essentiellen Aminosäuren auf und stellen aktuell kein Hauptallergen dar. Die Erbsenproduktion ist nachhaltiger, als die von anderen Hülsenfrüchten und so stellen Erbsen-Proteinzutaten, wie Erbsenproteinisolate, einen potenziellen Sojaersatz dar. Allerdings weisen Erbsenproteinisolate im Vergleich zu Sojaproteinisolaten eine verminderte Funktionalität und einen unangenehmen Geschmack auf. Darüber hinaus zeigen jüngste Studien eine Zunahme der Prävalenz von Erbsenallergien, was ihre Anwendung in Lebensmittelprodukten zukünftig beeinträchtigen könnte. Erbsenproteinisolate können als funktionelle Zutat verwendet, oder Lebensmitteln zur Proteinanreicherung zugesetzt werden. Es ist bekannt, dass technologische Verfahren sowohl die Funktionalität, das Aroma als auch die Allergenität von Zutaten aus Leguminosen verändern können; jedoch fehlen Studien zur gezielten Modifikation von Erbsenproteinen, die alle drei Aspekte simultan berücksichtigen.

Das Ziel der vorliegenden Dissertation war es daher den Einfluss einer enzymatischen Hydrolyse und Fermentation, sowie der Auswahl des Cultivars auf die Proteinfunktionalität, das sensorische Profil, Änderungen der Molekulargewichtsverteilung, den Abbau von Allergenfraktionen und die *in vitro* Immunogenität von Erbsenproteinisolaten zu ermitteln.

Die erste Studie (Kapitel 1) untersuchte die Wirkung von elf proteolytischen Enzymen auf die Funktionalität, das sensorische Profil und den Abbau der Hauptallergenfraktionen eines Erbsenproteinisolats. Die Hydrolysezeit wurde mit dem Hydrolysegrad und ausgewählten funktionellen Eigenschaften korreliert. Die elektrophoretischen Ergebnisse zeigten signifikante Veränderungen in der Molekulargewichtsverteilung und deuteten auf einen Abbau der Hauptallergene durch spezifische Enzyme hin, insbesondere bei längeren Hydrolysezeiten. Darüber hinaus wurden höhere Hydrolysegrade sowohl mit einer höheren Proteinlöslichkeit als auch mit steigenden Schaumeigenschaften korreliert; jedoch korrelierte ein steigender Hydrolysegrad auch mit einer Zunahme des *bitteren* Geschmacks der Hydrolysate. Da eine Bitterkeit die Verbraucherakzeptanz von Proteinzutaten verringert, sind geeignete Verfahren zur Maskierung oder Reduzierung des *bitteren* Geschmacks erforderlich.

Die mikrobielle Fermentation ist bekannt dafür, die organoleptischen Eigenschaften von Lebensmitteln zu verändern. Daher zielte die zweite Studie (Kapitel 2) darauf ab, die Auswirkungen der Milchsäuregärung auf die Eigenschaften des Erbsenproteinisolats, welches aus derselben Sorte hergestellt wurde, aufzudecken. Diese Studie konzentrierte sich

hauptsächlich auf Veränderungen des sensorischen Profils, aber auch auf entscheidende funktionelle Eigenschaften und einen möglichen Abbau der Hauptallergene. Sechs Mikroorganismenstämme wurden untersucht und erwiesen sich als erfolgreich zur Reduzierung des charakteristischen *bitteren* und *bohnigen* Geschmacks, insbesondere nach 24 h. Nach 48 h Fermentation nahmen jedoch unerwünschte Geschmacksnoten zu, welche die Akzeptanz der fermentierten Proben beeinträchtigten. Darüber hinaus zeigten alle fermentierten Proben unabhängig vom pH-Wert verminderte funktionelle Eigenschaften und keine signifikanten Veränderungen hinsichtlich der Allergenfraktionen. Diese Ergebnisse unterstreichen das Potenzial einer kontrollierten 24-Stunden-Fermentation zur sensorischen Optimierung von Erbsenproteinisolaten. Allerdings ist eine Refunktionalisierung erforderlich, um das vielseitige Anwendungsspektrum von Erbsenproteinisolaten zu erhalten.

Aus der ersten Studie wurden drei proteolytische Enzyme (Papain, Esperase® und Trypsin) als vielversprechend für eine verbesserte Funktionalität und reduzierte Immunogenität von Erbsenproteinen identifiziert. In der zweiten Studie zeigten die über 24 h mit *Lactobacillus (L.) plantarum* fermentierten Proben den neutralsten Geschmack, eine Verringerung des *bitteren* Geschmacks und die höchste Gesamtakzeptanz. So befasste sich die dritte Studie (Kapitel 3) mit einer günstigen Kombination der drei Enzyme mit anschließender oder vorgeschalteter mikrobieller Fermentation unter Verwendung von *L. plantarum*. Ziel war es, ein hochfunktionelles Erbsenproteinisolat mit neutralem Geschmack und reduzierter Immunogenität zu erhalten. Die Reihenfolge der Behandlung beeinflusste die Ergebnisse erheblich, wobei die Fermentation gefolgt von der enzymatischen Hydrolyse die vielversprechendsten Ergebnisse hinsichtlich aller drei Aspekte zeigte. Elektrophorese- und Gelfiltrationsergebnisse zeigten eine Verringerung der Molekulargewichtsverteilung der Proteine im Bereich der Allergenfraktionen, was auf eine verminderte Immunreaktivität hindeutete. Das indirekte antikörperbasierte Nachweisverfahren Enzyme-linked Immunosorbent Assay (ELISA) wurde herangezogen, um den Abbau der Allergenfraktionen *in vitro* zu bestätigen. Der ELISA zeigte eine verringerte Antikörperbindung, insbesondere für die mit Trypsin behandelten Proben und bekräftigte damit die reduzierte Immunreaktivität des Gesamtproteins. Bei allen Behandlungskombinationen wurden Proteinlöslichkeit und Schaumbildevermögen erhöht und das charakteristische Erbsenaroma reduziert. Die Ergebnisse zeigen die Vorteile der Kombination von nicht-thermischen Behandlungsmethoden zur Herstellung hochfunktioneller Erbsenproteinisolate mit neutralem Geschmack. Darüber hinaus deutet die reduzierte Antikörperbindung darauf hin, dass die behandelten Erbsenproteine allergische Reaktionen in geringerem Maße fördern könnten.

Die Zusammensetzung der Erbsenproteine wird durch Genotyp, Umweltbedingungen, Ernte und Lagerungsbedingungen beeinflusst. Daher können Proteinisolate verschiedener

Erbsensorten Unterschiede in der chemischen Zusammensetzung, Molekulargewichtsverteilung, Proteinfunktionalität und dem sensorischen Profil aufweisen. In der abschließenden Studie (Kapitel 4) wurden deshalb verschiedene Erbsensorten auf ihre Eignung zur Herstellung von sensorisch ansprechenden und funktionellen Proteinisolaten untersucht. Ziel war es, den Einfluss der Sorteneigenschaften auf die Eigenschaften von Proteinisolaten zu beleuchten. Die standardisierte Herstellung der Erbsenproteinisolate umfasste das Mahlen der Kotyledonen, das Solubilisieren der Proteine durch alkalische Extraktion, die isoelektrische Präzipitation und die Sprühtrocknung. Die vergleichbare Molekulargewichtsverteilung der Proteinisolate deutete auf eine ähnliche Proteinverteilung innerhalb der Erbsensorten hin. Die Hauptkomponentenanalysen von Proteinfunktionalitäten und sensorischen Profilen unterschiedliche Cluster mit signifikanten Unterschieden zwischen Erbsengeschmack und Bitterkeit. Diese Ergebnisse legen nahe, dass die Auswahl der Erbsensorte oder eine Kombination von Sorten insbesondere Einfluss hat auf die Funktionalität und das sensorische Profil der daraus hergestellten Proteinisolate. Die passende Sortenauswahl ist von der späteren Anwendung abhängig.

Zusammenfassend wurde in der vorliegenden Arbeit gezeigt, dass die enzymatische Hydrolyse, Fermentation und eine Kombination aus beiden Verfahren ein vielversprechender Ansatz sein kann, um hochwertige Zutaten aus Erbsen für vielseitige Lebensmittelanwendungen zu erhalten. Für die Herstellung von Lebensmittelzutaten mit hoher sensorischer Akzeptanz und funktionellen Eigenschaften waren insbesondere die Auswahl von Rohstoff und Prozessparameter relevant. Die Ergebnisse deuten darauf hin, dass die proteolytische Modifikation von Erbsenproteinen durch enzymatische Hydrolyse allein oder in Kombination mit einer mikrobiellen Fermentation zu einer Verringerung der allergischen Reaktion nach dem Verzehr führen könnte.

Contents

Preliminary Remarks

The work presented in this thesis is a selection of papers published in international peer-reviewed journals and presented as oral presentations and scientific posters in international conferences, all of which are listed below. Further scientific contribution resulted from the same period are marked with an asterisk (*).

Full papers

1. García Arteaga, V., Apéstegui Guardia, M., Muranyi, I., Eisner, P., & Schweiggert-Weisz, U. (2020). Effect of enzymatic hydrolysis on molecular weight distribution, techno-functional properties and sensory perception of pea protein isolates. *Innovative Food Science & Emerging Technologies*, 102449. doi:10.1016/j.ifset.2020.102449
2. García Arteaga, V., Leffler, S., Muranyi, I., Eisner, P., & Schweiggert-Weisz, U. (2021). Sensory profile, functional properties and molecular weight distribution of fermented pea protein isolate. *Current Research in Food Science, 4*, 1-10. doi:10.1016/j.crfs.2020.12.001
3. García Arteaga, V., Kraus, S., Schott, M., Muranyi, I., Schweiggert-Weisz, U., & Eisner, P. (2021). Screening of Twelve Pea (*Pisum sativum* L.) Cultivars and Their Isolates Focusing on the Protein Characterization, Functionality, and Sensory Profiles. *Foods, 10* (4), 758. doi:10.3390/foods10040758
4. García Arteaga, V., Demand, V., Kern, K., Strube, A., Szardenings, M., Muranyi, I., Eisner P. & Schweiggert-Weisz, U. (2022). Enzymatic Hydrolysis and Fermentation of Pea Protein Isolate and Its Effects on Antigenic Proteins, Functional Properties, and Sensory Profile. *Foods, 11*(1), 118. doi:10.3390/foods11010118
5. * Lidzba, N., García Arteaga, V., Schiermeyer, A., Havenith, H., Muranyi, I., Schillberg, S., Lehmann, J., Ueberham, E. (2021). Development of Monoclonal Antibodies against Pea Globulins for Multiplex Assays Targeting Legume Proteins. *Journal of Agricultural and Food Chemistry, 69*(9), 2864-2874. doi:10.1021/acs.jafc.0c07177

Oral presentations

1. García Arteaga, V., Apéstegui Guardia, M., Muranyi, I., Eisner, P., & Schweiggert-Weisz, U. (2019). Breaking peas-full proteins –Enzymatic hydrolysis for production of valuable and allergen-reduced food ingredients. 33rd EFFoST International Conference, Sustainable Food Systems - Performing by Connecting. 12-14 November 2019 Rotterdam, The Netherlands
2. García Arteaga, V., M., Muranyi, I., Eisner, P., & Schweiggert-Weisz, U. (2021). Effect of enzymatic hydrolysis and fermentation on the functional properties, sensory perception and allergen fractions of pea protein isolates. Online-Conference on Food Reformulation – Regulation and Marketing. 17-18 June, 2021.

Poster presentations

1. García Arteaga, V., Apéstegui Guardia, M., Muranyi, I., Eisner, P., & Schweiggert-Weisz, U. (2019). Breaking peas-full proteins –Enzymatic hydrolysis for production of valuable and allergen-reduced food ingredients. 33rd EFFoST International Conference, Sustainable Food Systems - Performing by Connecting. 12-14 November 2019 Rotterdam, The Netherlands

General Introduction

In the last few years, there has been an increase in the awareness of chronic diseases, climate change and the relationship of each with dietary patterns (An, Ji, & Zhang, 2018; Ritchie, Reay, & Higgins, 2018; Springmann, Godfray, Rayner, & Scarborough, 2016). The transition from animal-based to plant-based diets helps improve overall health (Catsburg et al., 2015; Kahleova, Levin, & Barnard, 2017; Kahleova, Tura, Hill, Holubkov, & Barnard, 2018; McMacken & Shah, 2017; Satija et al., 2016; Satija et al., 2017; Zoltan et al., 2016) and reduce the environmental impact through the cutback of fresh water consumption, greenhouse gas emissions and land use (Clune, Crossin, & Verghese, 2017; Hallstrom, Carlsson-Kanyama, & Borjesson, 2015; Hess, Chatterton, Daccache, & Williams, 2016; Ranganathan et al., 2016; van de Kamp, Seves, & Temme, 2018; Westhoek et al., 2014). These benefits have motivated a considerably large population to follow vegetarian or vegan diets. To fulfill the demands arising from this new trend, the food industry is looking for innovative ways to introduce new plant-based products that welcome consumers to healthier and tasteful options (Nettle, 2020).

Legumes play an important role in the plant-based transition because of their high protein content and their contents of fiber, vitamins and minerals. All these legume nutrients have shown positive effects on health, acting as metabolism regulators, anti-inflammatory, and anti-carcinogenic agents, and modulating intestinal microbiota, bowel mobility and glucose homeostasis (Clemente & Olias, 2017). A wide variety of food products based on legumes is now on the market, such as milk-, yogurt, -cheese-, and meat-alternatives. However, the development of the latter products requires profound research focusing on functional and sensory properties to optimize their flavor and appearance.

Moreover, the improvement of current food systems is necessary to increase sustainability; Niavis, Kleisiari, Kyrgiakos, and Vlontzos (2021) assessed the effects of soybean monoculture on land productivity and urged the need to replace soybeans with other leguminous crops. Peas have been increasingly used as ingredients for the production of plant-based foods in order to substitute soybeans (Boukid, Rosell, & Castellari, 2021). Depending on the end product or on the aimed functionality, pea ingredients can be used as flours or as protein concentrates or isolates.

Pea

Agricultural and economical importance of peas

Peas (*Pisum sativum*) are adapted to different conditions but are mostly grown in mild temperatures, at high altitudes, and in well-drained and light textured soils (Pavek, 2012; Schatz et al., 2016). The cultivation of peas offers several benefits when used in rotation and intercropping with cereals (Powers & Thavarajah, 2019). Such benefits are the protection for cereals against diseases and weed as well as the restoration of minerals, such as nitrogen, phosphorus and organic carbon levels in the soil. Peas have a lower carbon footprint compared to other protein-rich food products (Foyer et al., 2016; Poore & Nemecek, 2018; Stagnari, Maggio, Galieni, & Pisante, 2017). The latter benefits, together with the increase in plant-based diets, have rendered peas a popular crop for cultivation. According to the Food and Agriculture Organization database (FAOSTAT, 2021), the world production of dry pea seeds increased around 37% in the last decade with Canada, Russia, China, USA and India as major producers with a total of 9,890,127 tons in 2019 (Figure 1). During the same year in Europe, France and Germany were the biggest dry pea seed producers with the production of 709,380 tons and 228,200 tons, respectively. According to a market report (Mordor Intelligence), the European pea market is expected to grow up to 4.8% CAGR until 2025.

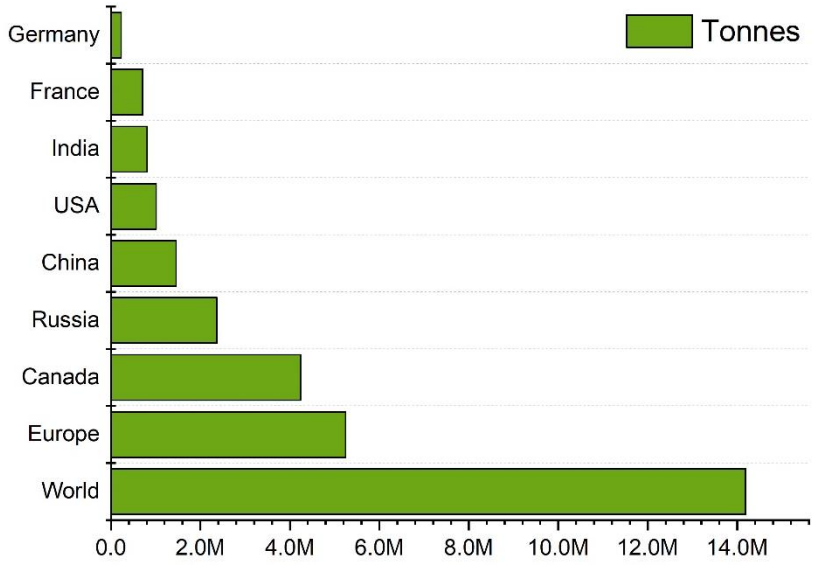


Figure 1 Production of dried peas in 2019 according to the Food and Agriculture Organization database. USA: united states of America; M: million

Pea classification and morphology

Peas belong to the legume family (*Fabaceae*) and to the category of pulses comprising all cool season legumes. Peas are one of the oldest cultivated seeds; they were first cultivated around 10,000 years ago (Jing et al., 2010; Zohary & Hopf, 1973). The *Pisum* genus comprises around 1200 *Pisum sativum* (garden pea) cultivars, which display a large number of polymorphisms (Jing et al., 2010; N. F. Weeden, 2001). The fast evolution and breeding of *Pisum sativum* genera keeps the number of cultivars growing (Kreplak et al., 2019). Several studies attempted to classify and disclose relationships among different subspecies of peas, wild and landrace genotypes (Jain, Kumar, Mamidi, & McPhee, 2014; Kreplak et al., 2019; N. Weeden, 2018; Zaytseva, Bogdanova, & Kosterin, 2012). Kreplak et al. (2019) found that 52% of the alleles are shared among wild (*Pisum sativum ssp. elatius)*, landrace and cultivar (*Pisum sativum ssp. sativum)* accessions; however, they found clustered accessions according to their cultivated status and their geographical origin and usage type (dry or fresh seeds). *Pisum sativum ssp. sativum* comprises different varieties; however, the most known and most cultivated is *Pisum sativum ssp. sativum var. arvense* L. also known as field pea (Kalloo, 1993).

Peas are grown in vines with weak stems reaching up to 60 cm height in the wild or up to 150 cm when cultivated. The vine consists of one stem and its leaves consist of two leaflets and one tendril (Schatz et al., 2016). The flowers can be white, purple or pink depending on the variety; each flower produces a closed-pod fruit ranging from 2.5 to 10.0 cm length (Pavek, 2012).

Ripening of the pods depends on environmental factors, where extreme temperature, salinity and acidity of the soil could hinder its proper development. The pea kernel is formed by a seed coat (hull), mainly constituted by cellulose, a storage cotyledon, mainly containing protein and starch, and the embryonic axis, mainly containing protein and lipids (Kosson, Czuchajowska, & Pomeranz, 1994b). Field peas can also be classified according to their seed morphology, such as the hull and cotyledon color, shape and surface. The latter is described as smooth or wrinkled, whereas, the shape might be described as elliptical, cylindrical, rhomboid and irregular (Santos et al., 2019). The hull color varies from light cream yellow to green, dark green, orange brown and brown depending on the tannin content (Kalloo, 1993); on the other hand, the cotyledons are yellow, orange or green and they differ mainly in their lutein and chlorophyll content (Holasová, Dostálová, Fiedlerová, & Horácek, 2009). For selecting the appropriate field pea cultivar, one should consider the market class (food or feed), yield, ease to harvest, seed size, disease tolerance and pea nutrient composition (Schatz et al., 2016).

Pea composition and nutritional value

Peas are rich in carbohydrates, proteins, and minerals and they include some vitamins (B vitamins and folate) and polyphenols, while being low in fat and sodium. Their composition depends on the genetic and environmental factors such as location, precipitation and heat (Al-Karaki & Ereifej, 2001; Nikolopoulou, Grigorakis, Stasini, Alexis, & Iliadis, 2007; N. Wang, Hatcher, Warkentin, & Toews, 2010). Several studies have focused on the chemical composition of peas (Table 1).

The pea hulls contain polyphenols, such as condensed tannins (Troszynska & Ciska, 2002), insoluble fiber, and some anti-nutritional factors (lectins, phytates, alkaloids, saponins and enzyme inhibitors); whereas, the cotyledon complies the highest number of nutrients and mostly soluble fibers (Singh, Singh, Shevkani, Singh, & Kaur, 2017). Several reviews have compiled the benefits of legume and pea consumption on health (Clemente & Olias, 2017; Dahl, Foster, & Tyler, 2012; Roy, Boye, & Simpson, 2010; Singh et al., 2017). These benefits include a risk reduction of cardiovascular diseases, diabetes, cancer and obesity. Furthermore, Robinson and Domoney (2021) summarized the genetic nutritional traits which might be used to improve pea cultivars while taking a pleiotropic effect under consideration.

Table 1 Field pea nutrient composition

Nutrient	Amount	Nutrient	Amount
Composition (mg/100 g dry matter)		*Minerals (mg/100 g dry matter)*	
Protein	23.3[1]	Calcium	77.7[1]
Carbohydrate	60.1[2]	Copper	0.7[1]
Starch	44.9[1]	Iron	5.9[1]
Total dietary fiber	21.2[2]	Potassium	1152.5[1]
Sugar	6.5[2]	Magnesium	152.8[1]
Fat	1.4[1]	Manganese	1.3[1]
Ash	2.9[1]	Phosphorus	489.5[1]
		Zinc	3.2[1]
Vitamins (mg/kg)			
Thiamin (B1)	5.30[3]		
Riboflavin (B2)	0.70[3]		
Folate (B9)	0.54[3]		

[1] N. Wang and Daun (2004); [2] Tulbek, Lam, Wang, Asavajaru, and Lam (2017);[3] Robinson, Balk, and Domoney (2019)

Oligosaccharides present in field peas are responsible for producing flatulence (Fleming, 1981). Different processing methods of legumes reduce the concentration of these antinutrients (Khattab & Arntfield, 2009; Ma, Boye, & Hu, 2018). However, the Bowman-Birk trypsin inhibitors, phytic acid and lectins have shown some positive effects on health such as anti-inflammatory and anti-carcinogenic properties (Clemente, Sonnante, & Domoney, 2011; Duranti, 2006; Gautam, Sharma, Sharma, & Saini, 2020; Konietzny & Greiner, 2003). Although the bioavailability of

micronutrients is affected by the aforementioned antinutrients, iron, zinc, calcium, potassium, magnesium and copper constitute the main minerals from pea seeds (Boye & Ma, 2015; De Angelis et al., 2021; N. Wang & Daun, 2004). Field peas are also a good source of folate and carotenoids (Ashokkumar et al., 2015; Marles, Warkentin, & Bett, 2013; Sen Gupta et al., 2013)

The 1-4% fat content in peas (Kosson et al., 1994b) is relatively low compared to the 18-22% in soybeans (Zaaboul, Zhao, Xu, & Liu, 2022). Lipids are mainly found on the seed embryo (Kosson, Czuchajowska, & Pomeranz, 1994a). The main fatty acid found in peas is linoleic acid (C18:2) and moderate amounts of linolenic acid (C18:3) and oleic acid (C18:1) have been found (Kosson et al., 1994a; Villalobos Solis, Patel, Orsat, Singh, & Lefsrud, 2013).

Starch and fibers are the main carbohydrate components in peas. Depending on the phenotype surface, the starch in field peas varies from 30-50% (Kringel, El Halal, Zavareze, & Dias, 2020). Their amylose to amylopectin ratio affects the digestibility as well as their duration or resistance to digestion. The decrease in starch digestion in the small intestine or the resistant starch promotes low glycemic index effects, which are favorable for managing type 2 diabetes. Similarly, pea fibers help regulate blood sugar levels and promote a reduction in blood cholesterol. In the colon, the resistant starch and fibers are fermented by bacteria promoting the production of short chain fatty acids resulting in several health benefits (Silva, Bernardi, & Frozza, 2020; Topping & Clifton, 2001).

Pea proteins

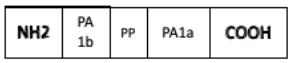
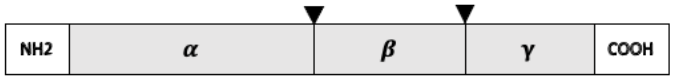
Field peas contain around 15-30% of protein and, similarly to all legumes, globulins represent the most abundant class of storage proteins. According to the Osborne (1924) classification, vegetable proteins can be classified regarding their solubility properties. Globulins, albumins, and prolamins are classified as salt-, water-, and ethanol soluble, respectively. Glutelins are another group of proteins, mostly soluble in weak acids, alkalis or SDS solutions. Proteins are also classified according to their sedimentation coefficient (*s*); the latter is defined as the speed at which particles sediment when a centrifugal force is applied. The *s* is expressed in Svedbergs unit (S), which corresponds to 10^{-13} seconds. The molecular mass, shape and density of the proteins determine the *s*, where heavier and larger particles have higher *s* values as they sediment faster (Halsall & Wermeling, 1982). Proteins can also be classified according to their function and biochemical properties in storage proteins, structural, metabolic and protective proteins (Shewry & Casey, 1999); however, some proteins differ from these functions. Protein bodies are organelles that contain storage proteins to separate them from metabolic ones (Chéreau et al., 2016; Shewry & Casey, 1999). Storage proteins act as nitrogen, carbon and sulfur reserve while non-storage

proteins play a role in stress responses, energy, metabolism and storage of non-protein compounds (Bourgeois et al., 2009; Shewry & Casey, 1999). Albumins and globulins are the main proteins in legumes representing 10-35% and 55-90%, respectively; the albumin/globulin ratio depends on different factors such as pea seed cultivar, environmental factors and harvesting stages (Sell, Steinhart, & Paschke, 2005).

The overall molecular weight of albumins ranges from 5 kDa to 110 kDa (Dziuba, Szerszunowicz, Nalecz, & Dziuba, 2014). Pea storage proteins include 2S albumins, known as PA1 (10-11 kDa) and PA2 (25-28 kDa). The PA1 is encoded by at least four different genes and consists of two subunits, namely, PA1a (~6 kDa) and PA1b (~4 kDa) (Shewry & Pandya, 1999). Although pea albumin fractions are rich in cysteine, PA1 subunits are not linked via disulfide bonds (Shewry & Pandya, 1999). PA2 albumin also consists of two fractions, namely PA2a (~25 kDa) and PA2b (~24 kDa) (Bourgeois et al., 2009; Croy, Hoque, Gatehouse, & Boulter, 1984; Higgins et al., 1987). Albumins have different biological activities and may act as trypsin inhibitors, as antifungal proteins and as allergens (Reinkensmeier, Bußler, Schlüter, Rohn, & Rawel, 2015; Shewry & Pandya, 1999).

Globulins are divided into the 11S and 7S fractions corresponding to the legumin and vicilin-convicilin protein fractions, respectively. Pea globulins represent 50-80% of the total protein content of pea seeds (Tzitzikas, Vincken, de Groot, Gruppen, & Visser, 2006). Pea globulins are deficient in sulfur-containing amino acids, cysteine and methionine; however, the Cys/Met content might vary among cultivars and among the globulin classes (Casey & Domoney, 1999; Kluth, Mantei, Elwert, & Rodehutscord, 2005; N. Wang & Daun, 2004). Legumins are hexamers of acid (40 kDa) and basic (20 kDa) subunits bound by disulfide bonds; however, legumin also includes some minor polypeptides ranging from 18-75 kDa (Bourgeois et al., 2009; Casey & Domoney, 1999). Vicilin is formed by three subunits of 50 kDa forming a heterotrimer; due to their lack of cysteine residues, no sulfide bridges are formed; however, they can go through post-translational modifications resulting in different protein fractions ranging from 11-140 kDa (Bourgeois et al., 2009; Casey & Domoney, 1999). The 47-50 kDa fraction is known as mature vicilin, which has two cleavage sites resulting in peptide fractions of different sizes (Table 2).

Table 2 Pea globular protein fractions and subunits

Fraction	Abundance of protein (%)	MW (kDa)	Fraction structure
Albumin	14-42		
PA1		10-11[1]	
PA2		25-28[2,3]	
Legumin	7-25	60-65[4]	
Acid subunit		35-43	
Basic subunit		21-23	
Vicilin	26-52		
$\alpha\beta\gamma$		47-50[4,5]	
α		18-19	
β		13-13.5	
γ		12-14	
$\alpha\beta$		30-33	
$\beta\gamma$		25-30	
Convicilin	4-8	68-70[4]	

PP: two propeptides; (▼) cleavage site; [1] Croy et al.(1984); [2] Eyraud et al. (2013); [3] Higgings et al. (1987); [4] Tzitzikas et al. (2006); [5] Bourgeois et al. (2009).

Convicilin (290 kDa) polypeptides range from 60-83 kDa and they do not suffer any translational modification, except for the removal of one signal peptide (Tzitzikas et al., 2006). However, two other smaller fractions (22 and 33 kDa) were identified as convicilin as they have the same accession numbers as the other convicilin fractions (Bourgeois et al., 2009; Croy, Gatehouse, Tyler, & Boulter, 1980). Convicilin is closely related to vicilin although it has one cysteine- and one methionine residue per subunit and it differs from vicilin by the N-terminal (Bown, Ellis, & Gatehouse, 1988; Croy et al., 1980; Tzitzikas et al., 2006). Vicilins are the main globulins found in peas with up to 8-fold and 11-fold higher concentrations than legumin and convicilin, respectively (Tzitzikas et al., 2006); however, these ratios might differ depending on the protein extraction method, pea seed cultivar and environmental conditions (Barac et al., 2010; Gueguen & Barbot, 1988).

The complete amino acid profile of peas includes high values of glutamic acid and aspartic acid and low values of tryptophan, methionine, and cysteine (Table 3). Field peas have shown a higher content of essential amino acids compared to the pattern suggested by the FAO/WHO, especially

isoleucine, histidine, valine and lysine (N. Wang & Daun, 2004). Moreover, the main and non-essential amino acids present in peas are aspartic- and glutamic acids.

Table 3 Amino acid composition of total pea proteins, protein fractions and individual globulin fractions of pea seeds from *Pisum sativum* L. [g/100 g]

				Globulin fractions		
	Pea protein [1,2,3,4]	**Albumin** [3,5]	**Globulin** [3]	**Legumin** [5]	**Vicilin** [5]	**Convicilin** [6]
Essential amino acids						
Methionine	0.8 ± 0.3	1.4 ± 0.6	0.6	1.0	0.7	0.1
Cysteine	0.9 ± 0.5	1.6 ± 2.2	1.2	0.0	0.0	0.1
Valine	4.3 ± 1.0	4.4 ± 1.1	4.9	3.0	3.9	4.5
Leucine	7.2 ± 1.1	4.3 ± 1.2	8.9	4.9	8.7	8.7
Isoleucine	3.8 ± 1.0	3.6 ± 1.1	4.7	2.5	4.2	3.9
Lysine	6.7 ± 1.1	10.2 ± 0.1	7.0	5.0	11.1	8.2
Threonine	3.4 ± 0.5	4.5 ± 0.1	3.2	5.0	6.3	2.6
Histidine	2.1 ± 0.4	3.2 ± 0.5	2.2	2.4	3.2	2.2
Arginine	7.3 ± 1.4	6.3 ± 3.1	8.1	3.0	4.1	8.2
Tyrosine	3.1 ± 0.4	4.1 ± 0.3	3.4	2.6	3.5	2.6
Phenylalanine	4.5 ± 0.7	3.8 ± 1.1	5.5	3.0	5.5	3.3
Tryptophan	0.9 ± 0.1	0.6 ± 0.9	1.0	0.0	0.0	-
Non-essential amino acids						
Aspartic acid[a]	11.4 ± 0.5	12.2 ± 1.1	12.0	8.7	15.1	11.6
Glutamic acid[b]	15.8 ± 1.7	16.0 ± 0.9	16.9	12.6	20.5	22.1
Serine	4.5 ± 0.8	4.3 ± 0.2	4.9	3.5	5.5	6.4
Glycine	4.0 ± 0.7	6.0 ± 0.5	3.8	2.7	3.3	5.9
Alanine	4.1 ± 0.5	5.2 ± 0.0	4.0	2.6	3.1	4.2
Proline	4.0 ± 0.9	4.9 ± 0.6	4.5	3.9	4.9	5.5

[a]aspartic acid + asparagine, [b]glutamic acid + glutamine. [1] Reichert and MacKenzie (1982); [2] Gorissen et al. (2018); [3] Leterme, Monmart, and Baudart (1990); [4] Pownall, Udenigwe, and Aluko (2010); [5] Rubio et al. (2014); [6] Bown et al. (1988)

Different pea cultivars and protein extraction methods have shown a slight difference in their amino acid composition (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015). The pea protein fractions show differences in their amino acid profiles, where albumins have particular higher levels of lysine, while the globulins have higher levels of leucine and arginine. Few studies have investigated the amino acid profile of the globulin fraction; however, a homology of vicilin and convicilin in the amino acid profile has been shown and both fractions have higher levels of amino acids than the legumin fraction (Table 3).

Pea protein extraction

Pea protein ingredients can be found as concentrates (40-85%) or isolates (>85%). Different extraction methods exist for the enrichment or isolation of legume protein ingredients. These methods are classified as dry or wet extractions and influence the protein structure, functional properties, sensory profile and nutritive value. Recent reviews have studied in detail the effects of

the different extraction methods on protein yield and functionality (Assatory, Vitelli, Rajabzadeh, & Legge, 2019; Momen, Alavi, & Aider, 2021; Schutyser & van der Goot, 2011; Vogelsang-O'Dwyer, Zannini, & Arendt, 2021). J. Yang, Zamani, Liang, and Chen (2021) investigated the effects of different extraction methods on pea protein composition, structure and functionality.

Dry processing methods include air classification, which allows the separation of particles by the dispersion of flour into an air stream based on the size of their components, and electrostatic separation, which separate charged particles using electrical forces created by high voltage electrodes (Vitelli et al., 2020). These methods deliver protein fractions with lower denaturation levels compared to wet methods although the protein content is lower (Assatory et al., 2019; Jafari, Rajabzadeh, Tabtabaei, Marsolais, & Legge, 2016; Pelgrom, Boom, & Schutyser, 2015; Schutyser & van der Goot, 2011).

Wet processes are more complex than dry ones as they use more energy, resources and thus, they represent higher costs. Depending on the raw material, a de-fatting step might be necessary. Protein fractions extracted using wet processing normally contain more than 90% protein of dry matter and can be classified as protein isolates; however, depending on the scale and individual extraction methods, the protein content might be lower. The end-products resulting from these wet methods will be referred as protein isolates. The common wet methods include salt extraction (SE), ultrafiltration (UF) and isoelectric precipitation.

Isoelectric precipitation

Protein recovery by isoelectric precipitation (IEP) is the most common and efficient method to obtain protein isolates. Alkaline extraction (AE) is necessary prior the isoelectric precipitation. Further, the pH of the alkaline extract is decreased to the isoelectric point (*pI)* of the raw material. Proteins have no net charge at their *pI* and thus, the solubility of the protein is significantly reduced due to hydrophobic interactions and van der Waals forces (Singhal, Karaca, Tyler , & Nickerson, 2016). Factors such as precipitation pH, temperature, and washing steps affect protein yields, which vary among studies and scale sizes (Fuhrmeister & Meuser, 2003; McCurdy & Knippel, 1990). The precipitated protein is further centrifuged or filtered and can be washed to increase the quality of the proteins. The precipitated protein is resuspended for neutralization followed by lyophilization or spray-drying. The obtained protein isolate has a higher globulin content as well as higher concentration of ash and salts due to pH adjustment steps (Alonso-Miravalles et al., 2019; Boye et al., 2010). Several studies have investigated the effect of IEP on pea protein yield and have compared it to other methods (Boye et al., 2010; Fuhrmeister & Meuser, 2003; Stone et al., 2015). The protein yield of IEP has been lower compared to UF (Boye et al., 2010) and SE (Stone et al., 2015), however, Karaca, Low, and Nickerson (2011) found higher protein yields of

pea protein using IEP compared to SE. Fuhrmeister and Meuser (2003) found that IEP of pea proteins resulted in higher protein yields (~60%) when the precipitation was performed at higher temperatures (85 ºC) rather than at room temperature (~35%). Furthermore, the protein contents of the IEP pea extracts were lower compared to UF (Boye et al., 2010; Fuhrmeister & Meuser, 2003) but higher compared to SE (Stone et al., 2015). Gao et al. (2020) found that the protein yield was significantly influenced by the pH of the alkaline extraction, where a high pH of 9.5 yields the highest protein recovery with around 58%; however, the chemical composition and molecular weight distribution were similar regardless of the pH of extraction.

The combination of dry-, wet- and novel methods, such as air classification or electrostatic separation followed by ultrafiltration, might promote more economic and sustainable protein extractions while increasing protein yield and proteins with highly functional, sensory and nutritional properties (Avila Ruiz, Arts, Minor, & Schutyser, 2016; Kumar et al., 2021; Pojić, Mišan, & Tiwari, 2018).

Functionality of pea proteins

The functionality of a food or food ingredient is defined by any of their properties that affect its usage (Pour-El, 1981). The functionality of proteins depends on different factors such as protein fraction composition, protein structure, pH, ionic strength, temperature, and extraction and drying methods (Casey & Domoney, 1999; Gao et al., 2020; Karaca et al., 2011; Shevkani, Singh, Kaur, & Rana, 2015; Stone et al., 2015), which challenges the comparison among studies.

Protein solubility

Protein solubility is the most investigated functional property, as it might be relevant for other functional characteristics. The protein solubility of plant proteins is also important for different food applications, especially for dairy alternatives. The solubility of proteins is usually investigated in a range of pH values. Pea proteins have shown a solubility behavior similar to those from soybean and other legumes; a reduction in protein solubility of pea protein isolates (PPI) has been found closer to and at the net zero charge at the isoelectric point (p*I*) around pH 4.5, promoting hydrophobic interactions and protein aggregation. Adebiyi and Aluko (2011) showed that albumins are highly soluble over a wide pH range, whereas globulin proteins showed the typical bell-shape curve behavior with the lowest solubility at the p*I*. However, even at pH values away from the p*I*, pea globulins' solubility was significantly lower than those from albumins. Thus, protein fractions play an important role in the solubility of PPI. Pea albumins consist of hydrophilic and hydrophobic groups, where low MW albumins correspond to more hydrophilic groups (Lu, Quillien, & Popineau,

2000); pea vicilins have shown lower hydrophobicity than legumin, which could result in higher protein solubility (Barac et al., 2010; Barac, Pesic, Stanojevic, Kostic, & Bivolarevic, 2015). Stone et al. (2015) and Karaca et al. (2011) investigated the effects of IEP and SE on the functionality of PPI using the same protocol. However, the former found that SE produced PPI with higher protein solubility, whereas Karaca et al. (2011) found significantly higher protein solubility from the PPI extracted by AE-IEP. The difference between these studies might be related to the pea cultivar and their protein fractions (Barac et al., 2010; Tzitzikas et al., 2006). Moreover, the pH of extraction using AE-IEP have also shown effects on the solubility of PPI. Gao et al. (2020) showed that higher alkaline conditions during the protein extraction reduced the overall solubility of the PPI. They attributed this effect to a higher protein aggregation promoted by changes in the protein structure (Bogahawaththa, Bao Chau, Trivedi, Dissanayake, & Vasiljevic, 2019; Sternberg & Thornton, 1977). Taherian et al. (2011) found similar U-shape behavior of PPI extracted using UF/diafiltration; they also investigated different extraction conditions and found that the phytic acid concentration changed and that PPI diafiltrated with pH 6 showed lower phytic acid concentration and higher solubility. Furthermore, the adjustment of the ionic strength of PPI solutions increases solubility by promoting electrostatic repulsion and hydration of the charged residues; this hydration promotes more protein-solvent interactions (Bogahawaththa et al., 2019; Burger & Zhang, 2019).

Emulsifying properties

Emulsions are thermodynamically unstable dispersions of immiscible liquids which can be separated over time into their components by physicochemical characteristics (Fligner & Mangino, 1991; McClements, Bai, & Chung, 2017). Emulsifiers promote the stabilization of emulsions by reducing the interfacial tension at the interface (Kim, Wang, & Selomulya, 2020). The interface could correspond to an oil-in-water emulsion, which is formed when oil is dispersed in water forming small droplets, such as milk and milk alternatives, ice creams, mayonnaise; or to water-in-oil emulsions such as in margarine and butter. Some of these products used their naturally occurring proteins as emulsifiers and recently, plant proteins are widely investigated as alternatives to synthetic emulsifiers (Arancibia, Riquelme, Zúñiga, & Matiacevich, 2017; Burger & Zhang, 2019; Kim et al., 2020). Figure 2 represents the behavior of pea proteins at the oil-water interface. The amphiphilic character of proteins promotes their adsorption to the interface where partial denaturation is necessary to expose hydrophobic amino acids; this exposure promotes the reorientation of the protein into their hydrophobic and hydrophilic fractions facing the oil or water phase, respectively. The formation of a viscoelastic film around the oil droplets promotes the reduction of interfacial tension and the stabilization of the interface (Burger & Zhang, 2019; Karaca

et al., 2011; Lam & Nickerson, 2013; Shao & Tang, 2014). Protein characteristics such as low molecular weight, balanced hydrophobic- and hydrophilic fractions, flexibility and their diffusion into the interface are important players in their suitability as emulsifiers for oil-water emulsions (Barac et al., 2015; Burger & Zhang, 2019; Shevkani et al., 2015).

In the literature, different parameters are used to study the suitability of proteins as emulsifiers. The emulsifying capacity (EC) is the amount of oil that can be held per grams of protein before the inversion of the emulsion; the emulsifying activity (EA) refers to the total surface area of the emulsion; turbidity measurements allow to determine the emulsification activity index (EAI) as the area of the interface stabilized per unit weight of protein; and the emulsion stability (ES), which measures the maintenance of the structure and texture of the emulsion over a period (Fligner & Mangino, 1991).

Pea protein and their fractions have been studied for their ability to form emulsions. From the protein fractions, albumins have shown better emulsion formation and better stability at pH 4 (Adebiyi & Aluko, 2011; Lu et al., 2000). Lu et al. (2000) found that the PA2 was important for the stabilization of emulsions probably due to its flexible structure, which promotes rearranging of the hydrophilic and hydrophobic residues at the interface. On the other hand, globulins have shown higher EC and ES at pH 7, which might result from a cohesiveness increase in the interfacial proteins (Adebiyi & Aluko, 2011). Vicilins have formed better emulsions compared to legumins (Dagorn-Scaviner, Gueguen, & Lefebvre, 1987; Koyoro & Powers, 1987; Shevkani et al., 2015); however, the ratio of vicilin/legumin in PPI did not show significant effects on the emulsion formation. The EC might be attributed to the presence of polar lipids in PPI, especially phosphatidylcholine, as well as to partial denaturation (Dagorn-Scaviner et al., 1987; McClements et al., 2017). Barac et al. (2015) found significant differences in the emulsion formation and stability among different pea cultivars, where the lowest EC was at pH 5 probably due to reduced protein solubility. Other authors have found also positive correlation between protein solubility and EC (Barac et al., 2010; Fuhrmeister & Meuser, 2003). However, other studies have found no correlation or negative correlation between protein solubility and EC (Shevkani et al., 2015).

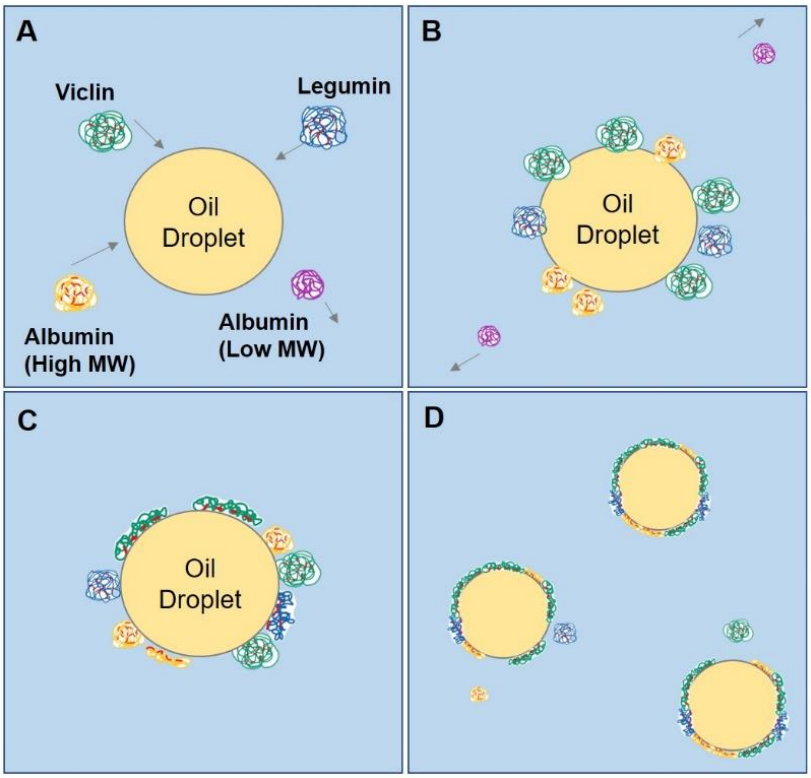


Figure 2 Pea protein behavior at the oil-water interface in a sequence of (A) migration, (B) adhesion, (C) partial denaturation and reorientation, and (D) formation of viscoelastic film at the interface. Adapted from Burger and Zhang (2019) and Kim et al. (2020)

Some studies have compared the functionality of PPI and other legumes; Aluko, Mofolasayo, and Watts (2009) found that the PPI at pH 7 formed better emulsions compared to soy protein isolates, whereas other studies have found that the PPI showed lower EC and higher ES than soy protein isolates and kidney bean protein isolates (Barac et al., 2010; Shevkani et al., 2015). However, the two latter studies attributed this effect to different protein fractions; Barac et al. (2010) attributed the higher EC to a lower ratio of vicilin/legumin, whereas Shevkani et al. (2015) attributed the higher EC to higher vicilin content. The different results among studies might be influenced by the legume genotypes used, the processing methods of the protein isolates, protein fractions in the isolates and emulsion methods.

Foaming properties

Foaming properties are essential for the food industry for products such as ice creams, meringue, and whipped creams. Similar to the emulsifying properties, the formation of foam occurs when non-polar air is dispersed in the water, which increases the number of small air cells, and thus, the surface tension (Fligner & Mangino, 1991). Proteins decrease the surface tension by

interaction of their hydrophobic and hydrophilic fractions with air and water, respectively, which are exposed during whipping. The formation of foams using proteins as foaming agents has been divided into three steps: first, soluble proteins are diffused into the air-water interface reducing the surface tension; second, proteins unfold and reorient their hydrophobic and hydrophilic fractions facing the air or water phase, respectively; third; adsorbed at the interface, proteins form a film around air cells promoting foam formation and stabilization (Zayas, 1997). Suitable foaming proteins are characterized by good protein solubility, rapid unfolding at the air-water interface, high surface hydrophobicity, protein flexibility, small net charge and readily protein denaturation (Adebiyi & Aluko, 2011; Fligner & Mangino, 1991; Shevkani et al., 2015); the foaming capacity (FC) is affected by the protein source, processing parameters such as isolation, temperature, pH, protein concentration, and the foaming method (Zayas, 1997).

The foaming properties of pea albumins are influenced by the pH, where they showed higher FC at acid pH (Lu et al., 2000). The same authors found that the PA2 fraction is essential for better foaming properties. Similarly, Adebiyi and Aluko (2011) found that albumins showed significantly higher foam formation than globulins or the PPI at pH 4 and pH 7, which was attributed to a higher solubility. In the globulin fraction, a higher vicilin/legumin ratio could promote a longer foam stability as vicilin contains lower sulfur amino acids, which reduces the ability to form disulfide bridges and therefore, form more flexible films (Zayas, 1997). Some studies have found higher FC away from the isoelectric point probably due to increased solubility and to an increase in net charge promoting protein unfolding and flexibility (Barac et al., 2010; Chao & Aluko, 2018; Stone et al., 2015); however, higher FC near the isoelectric point has been attributed to a higher surface hydrophobicity of the soluble proteins, to a more active protein surface and a reduction in electrostatic charge leading to an increased adsorption (Gharsallaoui, Cases, Chambin, & Saurel, 2009; Koyoro & Powers, 1987; Zayas, 1997). Moreover, Taherian et al. (2011) showed that an increase in protein solubility by the addition of salt (0.25%) increased FS as the NaCl might promote the diffusion of proteins into the interface. Another factor affecting the FC of protein solutions might be related to the fat content in the protein isolates. The mechanism of fats as antifoam agents depends on protein hydrophobic surfaces that allow the fat droplets to enter into the air-water interface (Denkov, Marinova, Denkov, & Marinova, 2006). Moreover, Stone et al. (2015) found that the extraction method and pea cultivar were significant factors for the formation of foam and its stability; SE pea proteins showed higher FC than those from AE-IEP but they showed lower FS. Sumner, Nielsen, and Youngs (1981) found that PPI from SE showed higher FC after spray- and drum drying, whereas the PPI from AE-IEP showed higher FC after freeze drying; they also found that the SPI both from SE and AE-IEP resulted in less FC than the PPI. On the other hand, Barac et al. (2015) found that the SPI showed higher FC and FS than the PPI

at different pH values. Furthermore, the extraction and drying methods not only affect the functionality of the protein but also might strongly impact the sensory characteristics of PPI and their products.

Sensory profile of pea proteins

Although the popularity of PPI in the food industry is increasing, its usage is still limited due to its characteristic flavor and taste. The flavor is mainly composed of volatile and non-volatile compounds, corresponding to the aroma perceived by nasal receptors and the taste (sweet, bitter, sour, salty, and umami) perceived by the tongue and mouth receptors (Roland, Pouvreau, Curran, van de Velde, & de Kok, 2017). Astringency is another influencing perception in the mouth caused by the interaction of non-volatile compounds with salivary proteins and mucins (Roland et al., 2017). Talking about the characteristic flavors of peas, they are generally referred as off-flavor; this term is used to describe unpleasant flavors innate or developed in the raw material (Roland et al., 2017). The off-flavors are influenced by genetic, environmental, harvesting and processing conditions (Heng, Vincken, Hoppe, et al., 2006; Heng, Vincken, van Koningsveld, et al., 2006; Roland et al., 2017). These off-flavors are formed through enzymatic, non-enzymatic, and thermal treatments by oxidation of fatty acids (linoleic and linolenic acids), Maillard reactions, and degradation of phenolic compounds, carotenoids and vitamins (thiamine) (Roland et al., 2017). Compared to soybeans, peas can develop stronger off-flavors due to their higher content of leucine, serine and threonine, which are involved in off-flavor development (Zhang, Hua, Li, Kong, & Chen, 2020).

Several studies have investigated the flavor compounds of peas and their products and have found alcohols, aldehydes and ketones as main ones (Table 4). Four main compounds have been found in peas through the different processing steps including hexanal, 1-pentanol, 1-octanol, 1-Octen-3-ol and 2,3-diethyl-5-methylpyrazine. The oxidation of lipids, mainly linoleic and linolenic acids, catalyzed via lipoxidase activities promotes the formation of aldehydes and aliphatic and unsaturated alcohols; however, these compounds can also be developed from autoxidative decomposition and from physical damage of seeds (Azarnia et al., 2011). Further oxidation of aldehydes promotes the formation of ketones though different pathways (Grebenteuch, Kanzler, Klaußnitzer, Kroh, & Rohn, 2021). Methylpyrazines are formed by the reaction of monosaccharides with amino acids (Maillard reaction) and they represent a potent aroma compound of peas and other foods due to their low thresholds (Grosch, 1994; Mortzfeld, Hashem, Vranková, Winkler, & Rudroff, 2020).

Table 4 Flavor compounds found in different pea protein products

Compounds	PF	PE	PPI	Flavor, off-flavor, taste
Aldehydes/Ketones				
Hexanal*	[1,3]	[4,5]	[2,4,6]	fatty, green, grassy, fruity odor and taste
2-Methyl-pentanal	[1]	[4]	[4]	acrid, pungent odor, warm, slightly fruity and nut-like taste
(E)-2-Nonenal		[4]	[4]	fatty, cucumber
Benzaldehyde	[2]	[5]	[2,4]	almond, marchpane, vanilla
Octanal	[3]		[4]	lemon, fruity
Nonanal	[2]		[4]	plastic, citrus, fruity
Trans-2-octenal	[1]			green-leafy odor
Trans-2-heptenal	[1]	[5]		pungent green fatty odor
(E,E)-2,4-Nonadienal	[3]			cucumber, green
(E,E)-2,4-Decadienal	[2,3]		[2]	fatty, nut, meat
3-Methylbutanal		[5]		pungent, cheese
n-Pentanal		[5]		green, milky
Hexenal		[5]		tea like
2-Pentenal		[5]		fruity, banana
Heptanal			[2]	fresh, green
3-Methylthiopropanal			[2]	potato, French Fries
(E)-2-Octenal			[2]	green, cucumber
Ketones				
3,5-Octadien-2-one			[2]	mushroom
2-Butanone	[1]	[5]	[4]	sweet apricot-like odor
2-Pentanone	[1]			acetone-like
Octan-2-one		[5]		soapy, fruity
1-Octen-3-one		[4]	[6]	metallic, mushroom
2-Methyl-3-heptanone	[2]		[2]	
2-Undecanone	[2]		[2]	fruity
Alcohols				
1-Hexanol	[2,3]	[5]		lemon, grass, green
3-Methyl-1-butanol		[5]	[6]	
1-Pentanol*	[1,2]	[4,5]	[4,6]	fermented, pungent, bread
2-Ethyl-1-hexanol			[2]	citrus, fresh, floral
1-Octanol*	[1,2,3]		[4]	fresh, orange-rose odor and an oily, sweet taste
1-Octen-3-ol*	[1,2,3]	[4,5]	[4,6]	mushroom
2-Methyl-propanol	[1]		[4]	alcoholic odor, ripe and fruity flavor
2-Heptanol,3-methyl	[1]			aromatic and fatty odor and a pungent spicy taste
1-Nonanol	[2,3]			fatty, green, waxy, citrus
1-Penten-3-ol	[2]	[5]	[4]	bread-like
n-Heptan-1-ol		[5]		mushroom, earthy, burnt
Esters				
Ethyl benzoate	[1]			fruity odor
Propanoic acid ethyl ester			[2]	
Pyrazines				
2,3-Diethyl-5-methylpyrazine*		[4,5]	[6]	green, vegetable
2-Methoxy-3-isopropyl-methyl pyrazine	[2,3]		[2]	earthy, spicy, plastic, hay
2-Ethyl-6-methylpyrazine		[5]		
2,5-Dimethylpyrazine		[4,5]		nutty, moldy, cereal-like
Saponins				
Saponin B	[7]			bitter
DDMP	[7]			bitter
Soyasaponin I			[8]	bitter

The main pea aroma compounds are marked with an asterisk (*). PF: pea flour; PE: pea protein extract; PPI: pea protein isolate; DDMP: 2,3-dihydro-2,5-dihydroxy-6- methyl-4H-pyran-4-one. [1] Azarnia et al. (2011), [2] Murat et al. (2013), [3] Zhang et al. (2020), [4] Cui et al. (2020), [5] Schindler et al. (2012) , [6] Gao et al. (2020), [7] Heng et al. (2004), [8] Gläser et al. (2020)

Proteins can interact with flavor compounds affecting the product perception (Heng et al., 2004). The protein fractions might also be an important factor in the sensory perception of PPI and factors such as chain length, concentration, pH and heat affect the binding of aroma compounds to these protein fractions (Heng et al., 2004). Volatile compounds found in protein ingredients change depending on the extraction method and each step of it, namely, alkaline extraction (step 1), isoelectric precipitation (step 2) and neutralization of the precipitated protein (step 3) (Cui, Kimmel, Zhou, Rao, & Chen, 2020). Larger differences have been found between step 1 and step 3 among cultivars (Cui et al., 2020). Drying methods can also affect the flavor-protein binding, where spray-drying could promote Maillard reactions and, thus, the formation of volatile compounds, whereas lyophilization might not have much influence (Cui et al., 2020; Gao et al., 2020). Gao et al. (2020) found that 1-octen-3-ol and hexanal levels significantly increased with increasing pH of the alkaline extraction (from pH 8.5 to 9.5). On the other hand, 1-pentanol, 1-octen-3-one and the pyrazine were not significantly different at different pH values of extraction. Using PPI alkaline extraction at pH 6.5 and spray-drying, more than 65 and 71 volatile molecules have been identified in the pea flour and PPI, respectively (Murat, Bard, Dhalleine, & Cayot, 2013). Among studies and steps of extraction, hexanal, 1-pentanol, 1-octanol, 1-Octen-3-ol and 2-methoxy-3-isopropyl-(5or6)-methylpyrazine seem to be the main aroma compounds found in pea ingredients (Azarnia et al., 2011; Cui et al., 2020; Gao et al., 2020; Murat et al., 2013; Schindler et al., 2012; Zhang et al., 2020). The above-mentioned studies suggest that the aroma compounds of PPI depend not only in the extraction method, but also the cultivar, drying method, extraction of volatile compounds and the mass spectrometry method.

Moreover, the bitterness of peas might depend on the interaction of proteins with saponins, which the latter might also affect interactions with volatile compounds. Saponins are non-volatile, amphiphilic, surface-active glycosides found naturally in many plants including peas (Heng et al., 2004). Saponins are known to interact with proteins (Morton & Murray, 2001; Potter, Jimenez-Flores, Pollack, Lone, & Berber-Jimenez, 1993), which was believed to increase bitterness perception of the PPI (Heng, 2005); however, Gläser et al. (2020) found that although the bitter taste perception threshold induced by the pea protein isolate saponin (soyasaponin I) was high (1.62 mmol/L), the saponin showed a low dose-overthreshold factor (0.7) which indicates a minor influence on the bitter taste. A major influence on bitterness was found coming from 1-linoleoyl glycerol and from products resulting from the oxidation of α-linolenic acid, linoleic acid, and trihydroxyoctadecenoic acids of pea protein isolates (Gläser, Mittermeier-Kleßinger, Spaccasassi, Hofmann, & Dawid, 2021).

Pea allergy

Another factor hindering the usage of ingredients in food products is their ability to trigger allergic reactions. The reactions after food consumption can be classified in two groups, the non-immunologically or immunologically mediated such as food intolerance or food allergy, respectively. The immunological reactions are also divided in the immunoglobulin E (IgE) mediated, like food allergy, and the non-IgE mediated, like enterocolitis or proctitis. In the last decades, the prevalence and increase of food allergies has been recognized and studied by different authors (Valenta, Hochwallner, Linhart, & Pahr, 2015; Verma, Kumar, Das, & Dwivedi, 2013). Recent studies have found that food allergies affect up to 2-8% of children and 3-6% of adults (Lyons et al., 2019; Lyons et al., 2020; Rachid & Keet, 2018). Although there is no comprehensive understanding of the mechanisms behind this increase, it has been stated that a relationship between allergic diseases and epigenetic modifications might be involved in such conditions (Tezza, Mazzei, & Boner, 2013). In other words, factors such as environment pollution, lifestyle and food habits might have an effect in gene expression resulting in the increase of food allergies and other diseases (Blanchard, 2017; Bunning, DeKruyff, & Nadeau, 2016; Scott H. Sicherer & Sampson, 2018).

In western countries, more than 170 foods have been considered as the cause of allergic IgE reactions promoting food allergy as well as other conditions such as allergic rhinitis, asthma, and atopic dermatitis (Moore, Stewart, & deShazo, 2017; Sears, 2014; S. H. Sicherer & Sampson, 2010). Allergens can be classified as complete allergens when the sensitization has been induced by specific IgE or as incomplete allergens when they trigger a reaction through a cross-reaction with an IgE from a similar allergen (Masilamani, Commins, & Shreffler, 2012). An antigen do not bind completely to IgE antibodies, but just the antigens recognition sites or epitopes, which present a characteristic sequence of adjacent amino acids (linear epitope) or amino acids that have been brought together due to chain folding (conformational) (Andjelkovic, 2021). In food products, only 8 foods are considered as global allergens according to the Food Allergen Labelling and Consumer Protection Act of 2004 (FALCPA). These allergens belong to milk, eggs, nuts, fish, shellfish, peanuts, wheat and soybeans, which account for about 90 % of all food allergies (Andjelkovic, 2021). Besides peanuts and soybeans, other legumes such as lupins, lentils, chickpeas and peas, have allergenic fractions which could trigger allergic reactions through own specific IgE or through cross-reactivity (Barre, Borges, & Rouge, 2005; Lima-Cabello, Robles-Bolivar, Alché, & Jimenez-Lopez, 2016; López-Torrejón et al., 2003; Peeters et al., 2007; Popp et al., 2020; Sanchez-Monge et al., 2004).

In Europe, peas are used in food products as an alternative to known allergen protein sources such as soybean and lupin. However, recent studies have investigated the prevalence of pea

allergy and the potential allergic reactions after ingestion of food products containing peas (Codreanu-Morel, Morisset, Cordebar, Larré, & Denery-Papini, 2019; Dreyer et al., 2014; Lavine & Ben-Shoshan, 2019; Popp et al., 2020; Smits et al., 2021). The results from these studies suggest that peas have become an important potential allergenic food causing severe reactions and their addition to the allergen list might be necessary. The International Committee of Allergen Nomenclature recognize three pea allergens, namely Pis s1, Pis s2 and Pis s3. Sanchez-Monge et al. (2004) used anti-Len c1 serum and patient serum pools for IgE immunodetection of crude pea extracts. They found the mature vicilin (~47 kDa) and one of its proteolytic fragments (~32 kDa) as major pea allergens corresponding both to Pis s1; they also identified a convicilin fraction (~63 kDa) as the second type of allergen (Pis s2). They highlighted the degree of sequence identity between Pis s1 and lentil (Len c1) and peanut (Ara h1) allergens corresponding to 90% and 52%, respectively. Bogdanov et al. (2016) isolated a novel lipid transfer protein (Ps-LTP1) of 9.4 kDa which shared degree of sequence identity with allergens from lentil (Len c3, 81%), peanut (Ara h9, 69%), green bean (Pha v3, 68%) and peach (Pru p 3, 68%). They identified the Ps-LTP1 as a third pea allergen (Pis s3) due to its specific IgE binding to Pru p3 sera and its high identity with the previously mentioned allergens according to different guidelines (Ladics, 2008; Thomas et al., 2009). Other studies have investigated pea 2S albumins as potential allergens (Malley, Baecher, Mackler, & Perlman, 1975; Sell et al., 2005; Vioque et al., 1998); however, the evidence for IgE binding is scarce which hampers their recognition as allergens. A recent study investigated the IgE binding of sera from 19 pea-sensitized children against pea total protein extract, natural Pis s3 and recombinant (r) rPis s1, rPA1, and rPA2 using IgE immunoblot/inhibition (Popp et al., 2020). They found that 63% of the children were sensitized to Pis s1 and that Pis s1 inhibited 58% of the IgE binding capacity of pea total protein extract. They assumed that IgE binding capacity with un-inhibited high molecular weight proteins in two children's sera could correspond to Pis s2 although as a minor allergen. On the other hand, they found low evidence for the Pis s3 and 2S albumins, although they attributed it to possible changes in the protein structure due to the reducing conditions. This study shows that Pis s1 was a major allergen in pea-allergic children.

The increasing prevalence of food allergy and the identification of pea allergens urge to find methods for developing pea ingredients with reduced allergenic potential. Dong, Wang, and Raghavan (2020) reviewed studies using different novel processing techniques, which are promising for the modification food allergens and a possible reduction of food immunoreactivity.

Modification of pea proteins

Proteins are inherent in some food products and food ingredients and they are frequently modified (e. g. heated, sheared) prior to consumption. These modifications can be induced by thermal or non-thermal treatments. On the one hand, thermal processing (cooking, boiling, roasting) are commonly used methods which can alter functionality, flavor and allergenicity of food products; on the other hand, non-thermal treatments are gaining more attention. The latter treatments include enzymatic hydrolysis, fermentation, high-pressure processing, pulse light, pulse electric field, cold plasma, and ultrasound or a combination of these. In particular, enzymatic hydrolysis and fermentation have been widely studied as they use milder conditions than thermal treatments and produce different bioactive peptides (Daliri, Oh, & Lee, 2017).

Enzymatic hydrolysis of pea proteins

Enzymatic hydrolysis of proteins is an extensive studied process within the food industry. Proteolytic enzymes break peptide bonds leading to an increase of smaller peptides, where the latter promote different effects depending on the raw material, the cleavage site and peptide size. Protein hydrolysis catalyzed by enzymes is a preferred method due to its short reaction time, repeatability and adaptability to larger production (Daliri et al., 2017). The proteolytic enzymes are classified according to their cleavage site in endo- or exoproteases. Endopeptidase activities catalyze the breakage of bonds within the peptide chain, while exopeptidases catalyze it at the amino- or carboxy-terminal of the protein chain releasing single amino acids (Figure 3). The mechanism of action of these enzymes depends on the raw material, protein structure, enzyme to substrate (E/S) ratio, enzyme specificity, pH, and temperature (Deng, 2018).

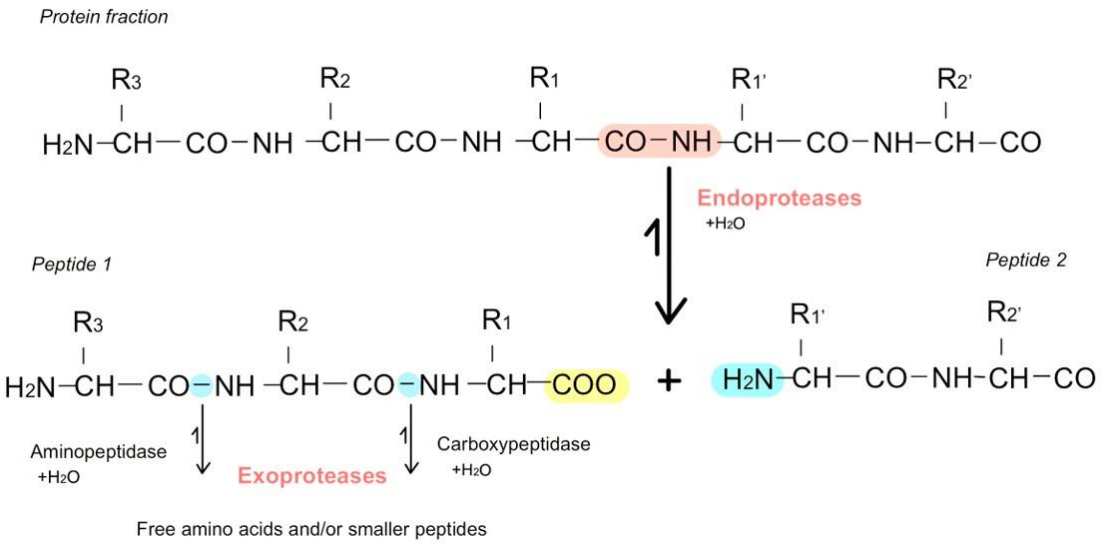


Figure 3 Enzymatic proteolysis

The efficiency of the hydrolysis can be measured as the degree of hydrolysis (DH), which is defined as the percentage of peptide bonds cleaved compared to the total number of peptide bonds. However, the analysis methods for the DH varies among research groups which might hamper the comparison of results; these methods include the formol titration, the trinitrobenzenesulphonic acid (TNBS), the o-phthaldialdehyde (OPA), the trichloroacetic acid soluble nitrogen (SN-TCA), and pH-stat method (Rutherfurd, 2010). The first three methods measure the amino groups released; the SN-TCA, as the name implies, measures the soluble nitrogen and the pH-stat method measures the number of protons released (Rutherfurd, 2010). Moreover, the extent of hydrolysis has been also analyzed through electrophoretic methods to observe changes in the molecular weight (MW) distribution of protein fractions. It has been found that the fastest increase in DH and changes in the MW distribution occurs in the first 15 min of hydrolysis (Barac et al., 2012; Sijtsma, Tezera, Hustinx, & Vereijken, 1998).

Although enzymatic hydrolysis has been extensively studied for its effects on different functional properties of plant proteins (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016), less research has been done regarding the effects of hydrolysis on the functionality, sensory profile and allergenicity of pea proteins. Table 5 shows different enzyme activities investigated for the hydrolysis of PPI and their effects on protein functionality, sensory profile and allergenicity.

Effect of hydrolysis on protein functionality

Protein hydrolysis promotes the exposure of hydrophobic residues increasing interfacial tension and possible changes in the p*I* of the proteins due to the available acid or basic amino acids in the solution (Klost & Drusch, 2019; Tamm, Herbst, Brodkorb, & Drusch, 2016). Through limited DH, these changes had shown to improve protein solubility (Barac et al., 2011; Klost & Drusch, 2019; Sijtsma et al., 1998; Tamm et al., 2016); however, further increase in the DH has opposite effects, which is attributed to an increased hydrophobicity and basic amino acids in solution.

Furthermore, correlations between improved protein solubility and emulsifying properties have not been consistent among different studies. However, limited DH has also shown to improve emulsifying capacity. Trypsin has been effectively used to improve emulsifying properties of pea proteins and has shown better results than other enzymes such as Alcalase®, Flavourzyme®, papain, and chymotrypsin (Humiski & Aluko, 2007; Klost & Drusch, 2019; Sijtsma et al., 1998; Tamm et al., 2016). The difference among these hydrolysates depends on the enzyme specificity and affinity to pea proteins, the final peptide charge, as well as the particle and peptide size (Burger & Zhang, 2019; Humiski & Aluko, 2007; Robins, 2000).

Table 5 Effects of pea protein hydrolysis using different enzymes and conditions

Raw material	Enzyme	Enzyme Activity	E/S ratio	DH	Effects		Reference
AE-IEP PPI	Trypsin	Serine-Endopeptidase	0.06% and 0.12%	2% and 4%	F	Improved solubility specially near the p*I*. Decreased solubility at pH 3 and 7. Emulsions were formed but creaming occurred specially at higher DH.	Klost & Drusch, 2019
Comm. PPI (Cosucra)	Trypsin	Serine-Endopeptidase	0.15% - 0.5%	2% - 8%	F	Up to 4% DH improved emulsion, upon further hydrolysis EAI decreased.	Tamm et al., 2016
	Alcalase®	Serine-Endopeptidase	0.14% - 1.0%	2% - 8%	F	Poor interfacial properties compared to trypsin hydrolysates	
AE-IEP PPI	Papain	Cystein-Endopeptidase	0.50%	12-17%	F	Improved solubility at pH 5 but impaired at pH 7. Improved EAI (depended on pea cultivar, pH and time of hydrolysis). Improved FC and FS.	Barac et al., 2012
	Streptomyces griseus protease	Combination of Exo- and Endoproteases	0.50%	46-57%	F	Improved solubility at pH 5 but significantly impaired at pH 7. Improved EAI (depended on pea cultivar, pH and time of hydrolysis). Improved FC at low pH values.	
AE-IEP PPI	Chymosin	Aspartic-Endopeptidase	0.50%	3.9% - 4.7%	F	Improved solubility at pH 5 but significantly impaired at pH 7. Improved EAI at low pH. Improved FC but impared FS at pH 7	Barac et al., 2011
Comm. PPI (Propulse)	Alcalase®	Serine-Endopeptidase	4.00%	NM	S	Most bitter +++++	Humiski & Aluko, 2007
	Flavourzyme	Exo- and Endoprotease	4.00%	NM	S	Bitter ++++	
	Papain	Cysteine-Endopeptidase	4.00%	NM	S	Least bitter +	
	Trypsin	Serine-Endopeptidase	4.00%	NM	F, S	Emulsion with the smaller particle size. Bitter +++	
	Chymo-trypsin	Serine-Endopeptidase	4.00%	NM	S	Bitter ++	
Comm. PPI (Propulse)	Protamex	Endoprotease	0.50%	1.8%-5.1%	F	Improved solubility. Up to 3.7% DH improved EAI, upon further hydrolysis EAI decreased.	Sijtsma et al., 1998
SE PPI	Alcalase®	Serine-Endopeptidase	12.50 %	0%-16.5%	S	Increased DH resulted in increased aldehyde and disulfide retention and decreased ketone and ester binding.	Wang and Arntfield 2016
SE PPI	Trypsin	Serine-Endopeptidase	0.60%	2% - 6%	A	Reduction of immunogenic potential	Fraczek 2007 and Fraczek 2008
Pea protein fractions	Alcalase®	Serine-Endopeptidase	30 mAU/g protein	NM	A	Reduction in immunoreactivity of pea legumin and albumin (ELISA). The antigenicity remained high (Serum test)	Szymkiewicz 2008
AE-IEP PPI	Flavourzyme®	Exo- and Endoprotease	0.50%	NM	A	Significant reduction of immunoreactivity, especially Pis s1 and to a lesser extent Pis s2	Lidzba et al., 2021
	Papain	Cysteine-Endopeptidase	0.50%	NM			
	Pepsin	Aspartic-Endopeptidase	2.00%	NM			

Comm. PPI: commercial pea protein isolate; AE-IEP: alkaline extraction-isoelectric precipitation; SE: salt extracted; E/S: enzyme to substrate; NM: not mentioned; F: functionality; S: sensory; A: allergenicity; DH: degree of hydrolysis; EAI: emulsifying activity index; FC: foaming capacity; FS: foaming stability; '+' represent bitterness intensity;

Foaming properties of PPI hydrolysates have been investigated in a lesser extent. Barac et al. (2011) and Barac et al. (2012) showed that hydrolysis of PPI, regardless of the enzyme, improved foaming capacity; however, the results of foaming stability were less clear.

In general, the ability of enzymes to improve functionality of protein hydrolysates has been demonstrated to depend on the enzyme used, the conditions, E/S ratio and the DH.

Effect of hydrolysis on the sensory profile

Changes in the sensory profile of proteins after enzymatic hydrolysis are attributed to the increase in smaller peptides with mainly hydrophobic residues, to the length of the peptides, to the free amino acid content, to the peptide-flavor interactions and to the accumulation of salty off-flavor (Adler-Nissen, 1976; Leksrisompong, Miracle, & Drake, 2010; Maehashi & Arai, 2002). The effects of protein hydrolysis on the sensory profile have been mainly investigated on animal and soybean proteins (Leksrisompong et al., 2010; Maehashi & Arai, 2002).

The development of bitter taste is one of the sensory attributes most studied in protein hydrolysates. The increased bitterness has been attributed to hydrophobic peptides (Figure 4) and their interaction with taste bud receptors and to the DH (Adler-Nissen & Olsen, 1979); however, at extreme DH, the bitterness decreases due to the increase in free amino acids which are known to be less bitter (Adler-Nissen & Olsen, 1979; Matoba & Hata, 1972).

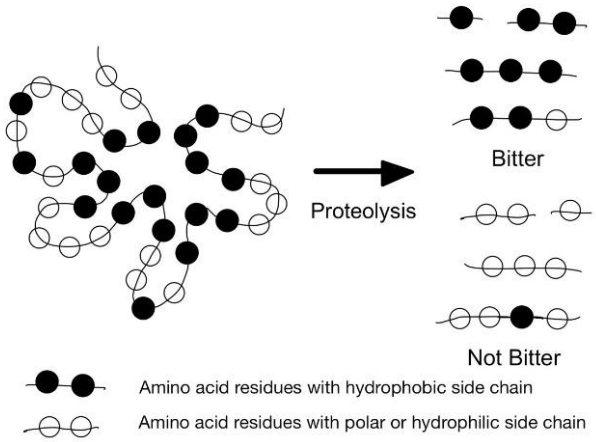


Figure 4 Release of bitter or non-bitter peptides after enzymatic hydrolysis (adapted from Matoba and Hata (1972))

Proteolysis of plant proteins results in hydrolysates with higher bitterness perceptions. Hydrolysis of soy and lupin proteins with Alcalase® had resulted in hydrolysates with an increased

bitter taste compared to other enzymes (Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016; Schlegel, Sontheimer, et al., 2019), whereas Flavourzyme® hydrolysates had shown less bitter. However, in another study both Alcalase® and Flavourzyme® hydrolysates showed the highest bitterness after hydrolysis of PPI (Humiski & Aluko, 2007), probably attributed to their broad specificity resulting in smaller peptide fractions. Furthermore, hydrolysis of PPI with papain produced the largest peptide fractions and the least bitter hydrolysates probably due to its specificity for His-Asn bonds (Cstorer & Ménard, 1994; Humiski & Aluko, 2007). Differences in bitterness between different legume hydrolysates are probably attributed to the specific amino acid profile, E/S ratio, conditions and methods used for the measurement of the bitterness.

The sensory perception of the solutions is also affected by the interaction of aroma compounds and proteins, which depend on changes in the protein structure, enzyme used and hydrolysis conditions (Tromelin, Andriot, & Guichard, 2006). It seems that only one study has investigated the effects of enzymatic hydrolysis of PPI on aroma binding. K. Wang and Arntfield (2016) found that higher DH increased retention of aldehyde and disulfide in PPI Alcalase®-hydrolysates but reduced ketone and ester binding. They suggested that changes in the exposure of functional groups and a decrease in hydrophobic regions were responsible for the increased binding of off-flavors like octanal and for the release of 2-octanone, respectively.

To increase the acceptance of the hydrolyzed proteins several studies have attempted to reduce or mask the bitter taste or off-flavors of protein hydrolysates by selective separation of bitter peptides, the usage of different enzyme activities (aminopeptidases, carboxypeptidases, alkaline/neutral peptidases) and lactic acid fermentation (Saha & Hayashi, 2001).

Effect of hydrolysis on allergenicity

The hydrolysis of proteins can affect the structure of the epitopes, hindering the binding to IgE antibodies (Besler, Steinhut, & Paschke, 2001). Therefore, extensively hydrolyzed proteins have been used to produce hypoallergenic infant formulas (Kiewiet, Faas, & De Vos, 2018). However, the structural modification and unfolding can also promote the formation of neoallergens (Vanga, Singh, & Raghavan, 2017; Verma et al., 2013). The allergenicity of different legume hydrolysates has been investigated (Aluko, 2008; Kasera, Singh, Lavasa, Prasad, & Arora, 2015; Meinlschmidt, Sussmann, et al., 2016); however, only few studies have looked into the effects of hydrolysis on the allergenicity of pea proteins.

Frączek, Kostyra, Kostyra, and Krawczuk (2007) investigated the immunogenic properties of tryptic hydrolysis of salt-extracted pea proteins by direct and competitive ELISA methods. Proteolysis of pea protein using trypsin significantly reduced the immunogenicity of the extracts, which was even further decreased with higher DH. A recent studied investigated the effects of PPI

hydrolysis with Flavourzyme®, papain and pepsin by means of direct, indirect and peptide-specific ELISA (Lidzba et al., 2021). All hydrolysates showed a significant reduction in immunoreactivity, especially for Pis s1. Szymkiewicz and Jędrychowski (2008) hydrolyzed pea extracts with Alcalase® and found a reduction in immunoreactivity of pea legumin and albumin by means of ELISA; however, when they used patient's serum, the antigenicity remained high especially for the vicilin fraction.

The results from these studies and studies from other legumes suggest that enzymatic hydrolysis could be an effective treatment to reduce the allergenicity of legume proteins but additional studies are required to confirm the panallergens and neoallergens in the legume family (Hauser, Roulias, Ferreira, & Egger, 2010).

Fermentation of pea proteins

Since ancient times, fermentation has been used as a food preserving and flavor enhancing method. The popularity of fermented foods has increased due to their potential health benefits (Frias, Peñas, & Martinez-Villaluenga, 2017; Şanlier, Gökcen, & Sezgin, 2019). For this reason, different studies have investigated which microorganisms can be used in plant-based products to enhance product functionality, flavor and health benefits (Wuyts, Van Beeck, Allonsius, van den Broek, & Lebeer, 2020). Lactic acid bacteria (LAB) are the most studied microorganisms, as they are responsible for the fermentation of milk and vegetables. LAB require nitrogen sources for growth; this nitrogen is provided by oligopeptides and free amino acids resulting from the hydrolysis of proteins initiated by a cell-envelope proteinase (Ibrahim, 2016; Y. Wang et al., 2021). The extent of this indirect enzymatic hydrolysis of proteins depends on the microorganism strain, substrate, and fermentation conditions (Daliri et al., 2017; Pessione & Cirrincione, 2016); this proteolysis might also affect the functionality, flavor profile and allergenicity of the food products.

Effect of fermentation on functionality

Fermentation is expected to affect functionality of protein solutions as microbial cells are amphipathic with electrical charges and surface hydrophobic character (Daeschel & McGuire, 1998). The interaction of microbial cells with proteins could alter the properties of the interface as they might attract each other reducing the ability to solubilize and interact with oil and air in emulsions and foams, respectively.

Few studies have investigated the effects of fermentation on functional properties of pea proteins. Fermentation of pea protein enriched flour and PPI with *L. plantarum* for around 10 h showed an increase in the DH and changes in the surface hydrophobicity affecting protein

functionality such as protein solubility, emulsifying and foaming capacities, and water and oil holding capacities (Cabuk, Stone, Korber, Tanaka, & Nickerson, 2018; Shi, Singh, Kitts, & Pratap-Singh, 2021). However, LAB are not the only microorganisms affecting functionality, fermentation of PPF with *Aspergillus oryzae* and *Aspergillus niger* up to a 10% DH showed improved water and oil binding properties, attributed to changes in the protein conformation and exposure of hydrophilic and hydrophobic residues, respectively (Kumitch, Stone, Nickerson, Korber, & Tanaka, 2020). Fermentation of other legume flours and proteins have shown similar and contradictory results regarding functional properties (Lampart-Szczapa et al., 2006; Sadowska, Fornal, Vidal-Valverde, & Frias, 1999; Schlegel, Leidigkeit, Eisner, & Schweiggert-Weisz, 2019).

Effect of fermentation on the sensory profile

The effects of fermentation on the aroma and taste of legumes and their different products have been extensively studied as a way to improve acceptability by the consumer (Boyaci Gunduz, Gaglio, Franciosi, Settanni, & Erten, 2020; Kaczmarska, Chandra-Hioe, Frank, & Arcot, 2018).

Lactobacillus plantarum has been widely used for fermentation of different foodstuff such as meat, dairy, fruits, vegetables, cereals, and legumes to modify flavor. A recent study investigated the effects of PPI fermentation with *L. plantarum* for 10 h and found a decreased aldehyde and ketone concentrations changing the aroma profile of the fermented samples (Shi et al., 2021). Similarly, Schindler et al. (2012) found a reduction in n-hexanal after fermentation of pea protein extracts which contributed to the reduction or masking of off-flavors. Other LAB has been studied alone or in combination for the fermentation of pea protein extracts, PPI and PPI emulsions. They found that the beany (leguminous) and green flavors were significantly reduced and that most of the aldehyde, furan and ketone molecules were degraded, while other volatile compounds were generated masking pea off-flavors (Ben-Harb et al., 2019; El Youssef et al., 2020; Schindler et al., 2012).

Fermentation has been studied for mitigation or debittering of proteins and its hydrolysate (Meinlschmidt, Schweiggert-Weisz, & Eisner, 2016; Schlegel, Leidigkeit, et al., 2019); this debittering effect depend on the strain used and its release of aminopeptidases, which might cleave hydrophobic amino acid residues and particularly Pro residues (El Abboudi et al., 1992; Song et al., 2020; Tchorbanov, Marinova, & Grozeva, 2011). Although few studies have investigated the effects of fermentation of pea proteins on sensory profile, to my knowledge, there are no studies focusing on the debittering of pea proteins and its hydrolysates by fermentation.

Effect of fermentation on allergenicity

As the awareness and increased incidence of pea allergy is relatively recent, there are few

studies focusing in the reduction of allergens or mitigation of allergenicity (Shiferaw Terefe & Augustin, 2020). Fermentation of lupin protein isolates showed no effect on lupin major allergens, however, a combination of enzymatic hydrolysis and further fermentation were able to reduce Lup an 1 to a residual immunogenicity level of <0.5% (Schlegel, Lidzba, Ueberham, Eisner, & Schweiggert-Weisz, 2021). Similarly, soybean meal treated by combination of fermentation and enzymatic hydrolysis showed a decreased antigenicity of β-conglycinin and glycinin by means of SDS-PAGE and ELISA (H. Yang et al., 2020). However, there is only one study focusing on the allergenicity of fermented pea proteins. Pea flour was individually fermented with three LAB, a *Rhizopus microspores var. oligosporus* and a *G. candidum* was analyzed using SDS-PAGE, immunoblotting and sandwich ELISA, where samples fermented with LAB and *R. oligosporus* showed reduced antigenicity to 10% of the unfermented pea flour (Barkholt et al., 1998).

Aims of study

Legumes have played a significant role in the consumption of plant-based diets. Not only for their high protein content but also for their contents of fiber, vitamins and minerals and other essential nutrients such as folate and lysine. The usage of pea proteins has substituted the one from soybeans in different products due to economic and environmental reasons. Unfortunately, pea proteins exhibit lower functionality and higher off-flavors limiting its use in different products. Furthermore, peas are not considered main allergens; however, an increase in pea allergy incidence and the cross-reactivity of its allergens with specific antibodies from other legumes has encouraged more studies to target this topic. Therefore, the main objective of this work was to investigate the effects of different non-thermal treatments in order to increase functionality, optimize sensory profile and reduce the immunogenicity of pea protein isolates. To fulfill this goal, different hypotheses were established and investigated through different approaches. These hypotheses were formulated considering previous published works from other authors.

Enzymatic hydrolysis is commonly used to modify different features of proteins. Hence, the hypothesis for this chapter was that the proteolysis of pea protein isolates would significantly affect the functional properties of the treated isolates and would affect or degrade allergen fractions. To investigate this hypothesis, different proteases were used to find appropriate enzyme activities that can not only improve functional properties such as protein solubility, emulsifying capacity and foaming properties, but also could change the molecular weight profile reducing main allergen fractions. In addition, a sensory evaluation was carried out to observe changes in main pea off-flavors and bitter taste (CHAPTER 1).

One of the main reason consumers do not fully accept products with a higher content of pea proteins is the off-flavors such as *beany* and *green*. Fermentation has successfully shown the reduction of these off-flavors in peas and other legumes. Therefore, the hypothesis for the second chapter was that lactic acid fermentation of pea protein isolates can change the sensory profile and might increase acceptance among consumers. To target this, six commonly used lactic acid bacteria were applied to ferment pea protein isolates for 24 h and 48 h. A trained panel evaluated the fermented samples regarding off-flavor intensities and taste providing also an indication for preference. Moreover, the effects on functionality and on main allergens were also assessed (CHAPTER 2).

Previous studies have shown that protein hydrolysates have an increased bitterness, whereas fermentation has shown no effect or negative effects on protein functionality. The third hypothesis was that a combination of enzymatic treatment and fermentation can create pea protein ingredients with improved functionalities and reduced bitterness and off-flavors. Hence, three enzymes, selected from chapter one, were chosen according to their improvements on

functionality and their effects on allergen fractions; one microorganism from chapter two was selected regarding its aroma and taste profile. The experiments were carried out in two different ways, first enzymatic hydrolysis followed by fermentation or the other way around. The functional properties, the effects on allergen fractions and immunogenicity and the sensory profile were analyzed (CHAPTER 3).

Pea cultivars vary among countries and regions, which has made its genomic study more challenging. Pea protein and its fractions can significantly change depending on seed cultivar, environmental factors, harvesting and storage conditions. There are few studies regarding the differences of protein fractions and protein functionality from different pea cultivars from Germany; however, their differences regarding main allergens and sensory profile have not been investigated. The objective of this chapter was to screen different pea cultivars commonly used in Germany and France regarding their functional properties, pea off-flavors and presence of main allergens (CHAPTER 4).

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CHAPTER 1

Effect of enzymatic hydrolysis on molecular weight distribution, techno-functional properties and sensory perception of pea protein isolates[1]

Abstract

The increasing incidence of food allergies and the awareness of pea allergy urge finding new methods to reduce allergens and diminish potential allergic reactions. Pea protein isolates from the cultivar “Navarro” were hydrolyzed using different proteolytic enzymes to investigate their effects on functional and sensory properties of the pea proteins. Electrophoresis was used as indication of the effects of proteolysis on the main pea allergen fractions. High degrees of hydrolysis were correlated with changes in the molecular weight distribution and the improvement of protein solubility and foaming properties. At first sight, some enzymes were able to degrade one or two of the known pea allergens. The study is of high interest as it investigates the simultaneous effects on these three categories, which might help to select the best treatment for the production of highly functional ingredients with high consumer acceptance and a reduced allergenic potential.

1 García Arteaga, V., Apéstegui Guardia, M., Muranyi, I., Eisner, P., & Schweiggert-Weisz, U. (2020). Effect of enzymatic hydrolysis on molecular weight distribution, techno-functional properties and sensory perception of pea protein isolates. *Innovative Food Science & Emerging Technologies*, 102449. doi:10.1016/j.ifset.2020.102449

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Effect of enzymatic hydrolysis on molecular weight distribution, techno-functional properties and sensory perception of pea protein isolates

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ABSTRACT

Pea protein isolate (*Pisum sativum* "Navarro") was hydrolyzed with 11 proteolytic enzymes at different hydrolysis times (15, 30, 60, and 120 min) to improve techno-functional and sensory properties. The degree of hydrolysis and changes within the molecular weight distribution were used as indicators for a reduced allergenic potential. The highest degree of hydrolysis was reached by Esperase hydrolysates (9.77%) after 120 min of hydrolysis, whereas Chymotrypsin hydrolysates showed the lowest (1.81%). Hydrolysis with Papain, Trypsin, Bromelain, Esperase, Savinase, and Alcalase suggested an effective degradation of the 72 kDa-convicilin fraction. Papain and Trypsin hydrolysates showed a degradation of the 50 kDa-mature vicilin after 15 min of hydrolysis. Most hydrolysates showed a significant increase in protein solubility at pH 4.5 at all times of hydrolysis. Trypsin hydrolysates showed the highest foaming (2271%) and emulsifying (719 mL/g) capacities. The bitterness of the hydrolysates was strongly correlated ($P < 0.05$) with the degree of hydrolysis. In general, enzymatic hydrolysis improved techno-functional properties indicating their potential usage as food ingredients.

Industrial relevance: Due to their high protein content, peas are becoming an attractive ingredient for the food industry. However, pea protein isolates are often characterized by poor techno-functional and sensory properties. Enzymatic hydrolysis is known to change the molecular weight distribution of proteins. Consequently, the techno-functional and immunogenic properties might be altered selectively. In this study, enzymatic hydrolysis was applied, resulting in highly functional pea protein hydrolysates with a hypothesized reduction of main allergens. The lower bitter perception highlights their high potential as valuable functional food ingredients.

1. Introduction

The use of protein-rich raw materials for food applications has become increasingly important in recent years. Within the legume family, peas (*Pisum sativum* L.) are an auspicious raw material due to the high amounts of proteins as well as to their absence in the allergen list of *Official Journal of the European Union* (O.J.E.U., 2011). The pea protein content ranges between 20 and 30% (Koyoro and Powers, 1987), and the proteins are mainly composed of salt-soluble globulins (55–80% of the total protein) and water-soluble albumins (18–25% of the total protein). The ratio of these storage proteins depends on genetic and environmental characteristics such as maturation, fertilizers, soil nutrients and cultivation temperature (Barac et al., 2015.; Gueguen and Barbot, 1988; Nikolopoulou et al., 2007).

Depending on the production conditions, pea protein isolates (PPI) are characterized by deficient techno-functional properties, in particular, their low foaming and emulsifying capacities, and by unpleasant sensory properties. Several approaches are described in the literature for the alteration of protein structures in order to improve the techno-functional as well as the sensory properties (Adler-Nissen and Olsen, 1979; Angioloni and Collar, 2013; Buchert et al., 2010; Raksakulthai and Haard, 2003). Among them, enzymatic hydrolysis has shown to be one of the most promising methods for the modification of tailor-made protein preparations (Lqari et al., 2005; Meinlschmidt et al., 2016; Polanco-Lugo et al., 2014; Schlegel et al., 2019). Proteolytic active enzymes cleave peptide bonds, resulting in a mixture of peptides of different sizes and free amino acids (Wouters et al., 2016). Proteases are classified as endopeptidases or exopeptidases depending on their mechanism of action and catalytic site. The efficiency of the enzymatic hydrolysis mainly depends on the enzymes applied and hydrolysis

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conditions (time, temperature, pH) used, where the resulting cleavage products have a decisive influence on the hydrophobicity of the peptides and thereby the techno-functional properties (Singhal, Karaca, Tyler, & Nickerson, 2016). Barac et al. (2011, 2012) studied the influence of enzymatic hydrolysis (chymosin, Papain, and *Streptomyces griseus protease*) on pea protein isolates. All hydrolysates showed an improvement in protein solubility (pH 5) and better emulsifying and foaming capacities. However, they focused only on the functional properties for food application, and no attention was given regarding the sensory perception of the hydrolysates.

Enzymatic hydrolysis also affects the peculiar sensory properties of plant proteins such as the green, bitter, or astringent attributes (Adler-Nissen, 1986a; Saha and Hayashi, 2001). The extent of changes in sensory properties is attributed to the degree of hydrolysis and, in particular, to the release of low molecular weight peptides constituted of hydrophobic amino acids. This release depends on the enzyme and the substrate used (Raksakulthai and Haard, 2003; Saha and Hayashi, 2001). Humiski and Aluko (2007) demonstrated that Papain and α-Chymotrypsin hydrolysates from pea proteins were less bitter, while those hydrolyzed with Flavourzyme and Alcalase preparations resulted in an increased bitterness. On the other hand, hydrolysis of soy protein isolates with Flavourzyme showed similar bitterness to the untreated isolate (Meinlschmidt et al., 2016).

Although pea proteins are not included in the list of main allergens, there is some evidence in the literature that also pea proteins, in particular, Pis s 1 (vicilin) and Pis s 2 (convicilin), exhibit an allergenic potential (Codreanu-Morel et al., 2019; Dreyer et al., 2014; Sanchez-Monge et al., 2004). Sanchez-Monge et al. (2004) identified three major pea allergens by immunodetection, immunoblot inhibition assays and cDNAs encoding of pea vicilin. These fractions are a 63 kDa convicilin (Pis s 2), a 47 kDa mature vicilin (Pis s 1), and its 32 kDa proteolytic fragment, which are recognized by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee. The results for pea albumin (2S) potential allergens such as PA1 (6.5 kDa) and PA2 (26 kDa) are ambiguous, and the proteins are yet not recognized as allergens (Malley et al., 1976; Mierzeiewska, Mitrowska, Rudnicka, Kubicka, & Kostyra, 2008). Among the approaches to reduce this allergenic potential, enzymatic hydrolysis has been investigated in detail for different legume proteins such as peanut, soy, and lupin (Kasera et al., 2015; Meinlschmidt et al., 2016; Schlegel et al., 2019). but few data are available for pea proteins (Frączek et al., 2008; Szymkiewicz and Jędrychowski, 2008).

As mentioned above, some studies have focused on the effect of enzymatic hydrolysis on techno-functional and sensory properties of pea protein isolates and, to a lesser extent, on the mitigation of pea allergenicity. However, only the simultaneous study of all effects of proteolysis will enable the production of highly functional and appealing food ingredients where changes in the molecular weight distribution might have an effect on the reduction of allergens.

Therefore, the present study aimed to investigate the influence of enzymatic hydrolysis on the techno-functional properties such as protein solubility, emulsifying capacity, foaming capacity, and foam stability as well as the sensory profile of PPI and its hydrolysates. An indication of the degradation of the main pea allergens Pis s 1 and Pis s 2 was reached by determination of molecular weight distribution.

2. Materials and methods

2.1. Materials

Peas (*Pisum sativum* L. cultivar "Navarro") were provided by Norddeutsche Pflanzenzucht Hans-Georg-Lembke KG (Germany). Enzymes Alcalase® 2.4 L FG 1, Flavourzyme®, Neutrase®, Protamex®, and Savinase® 16 L were from Novozymes (Denmark); Trypsin, Bromelain, and Esperase® 8.0 L were obtained from Sigma-Aldrich (Germany); Chymotrypsin, Corolase® 7089 and Papain were from Merck KGaA (Germany), AB Enzymes (Germany) and Carl Roth GmbH (Germany), respectively. Broad Range™ Unstained Standard, 4–20% Criterion™ TGX Stain-Free™ Precast Gels and Coomassie Brilliant Blue R-250 were purchased from Bio-Rad Laboratories GmbH (Germany). Sodium dihydrogen phosphate, sodium dodecyl sulfate, sodium tetraborate decahydrate, o-phthaldialdehyde, and sodium monohydrogen phosphate were from Sigma-Aldrich (Germany). All chemicals used in this study were of analytical grade.

2.2. Production of pea protein isolate

Peas were dehulled and split using an underflow peeler (Streckel & Schrader KG, Germany) and separated using an airlift system (Alpine Hosakawa AG, Germany). Subsequently, the split pea seeds were milled using a pilot-plant impact mill (Alpine Hosakawa AG, Germany) with 0.5-mm-sieve insertion. The isolation of pea protein was performed according to Tian, Kyle, & Small, (1999) with few changes. An aqueous alkaline extract of the pea flour was prepared in deionized water at a ratio of 1:8 (w/v) at pH 8.0 ± 0.1 using 3.0 mol/L NaOH under constant stirring for 60 min. The protein extract was removed by means of a decanter (3,300 rpm). For isoelectric precipitation, the protein extract was adjusted to pH 4.5 using 3.0 mol/L HCl. After 60 min, the precipitated proteins were separated from the clear supernatant in an SC 20-disc separator (GEA Westfalia Separator Group GmbH, Germany) at 12,000 rpm. The isolate was neutralized with 3.0 mol/L NaOH, pasteurized (70 ± 2 °C) for 2 min and spray-dried.

2.3. Enzymatic hydrolysis of PPI

For enzymatic hydrolysis, a 9% (w/w) PPI dispersion was prepared in deionized water in a thermostatically controlled reactor with temperature and pH adjusted to the optimum conditions of each enzyme (Table 1) according to product data sheet. The enzyme to substrate ratio (E/S) was chosen according to literature. After enzyme addition, the

Table 1
Enzymes preparations used for the hydrolysis of pea protein isolate and the respective hydrolysis conditions applied.

Enzyme	E/S (%)	T (°C)	pH value (−)	Activity	Origin
Alcalase® 2.4 L FG	0.5	65	8	Serine Endoprotease	*Bacillus licheniformis*
Bromelain	0.1	50	7	Cysteine Endoprotease	Pineapple stem
Chymotrypsin	0.1	50	8	Serine Endoprotease	Bovine pancreas
Corolase® 7089	0.5	50	7	Endoprotease	*Bacillus subtilis*
Esperase® 8.0 L	0.5	65	8	Serine Endoprotease	*Bacillus sp.*
Flavourzyme®	0.5	50	7	Endo- and *exo*-protease	*Aspergillus oryzae*
Neutrase® 0.8 L	0.5	50	7	Metallo Endoprotease	*Bacillus amyloliquefaciens*
Papain	0.1	65	7	Cysteine Endoprotease	Papaya latex
Protamex®	0.5	65	7	Endoprotease	*Bacillus licheniformis* and *amyloliquefaciens*
Savinase® 16 L	0.5	50	8	Serine Endoprotease	*Bacillus*
Trypsin	0.1	50	8	Serine Endoprotease	Bovine pancreas

E/S: enzyme to substrate ratio, T: temperature.

suspension was continuously stirred and the temperature and pH were maintained constant. Aliquots of approximately 900 mL were transferred to smaller reactor vessels after 15, 30, 60 and 120 min for enzyme inactivation at 90 °C for 10 min. The hydrolysates were cooled to room temperature and neutralized to pH 7.0 ± 0.1. Aliquots of 5 mL were stored at −20 °C for a minimum of 24 h until electrophoretic analysis. The rest of the samples was lyophilized and ground for 10 s at 7,500 rpm (Grindomix GM200, Retsch GmbH, Germany). The control samples were treated with the same conditions but without the addition of the enzymes. The hydrolysis and controls were performed in duplicate.

2.4. Chemical composition

The dry matter and ash content of the samples were determined by means of a thermogravimetric method (TGA 701, Leco Instruments, Germany). The protein content was determined according to the Dumas combustion method (TruMac N, Leco Instruments, Germany) using the average nitrogen-to-protein conversion factor of N x 6.25. All analyses were performed in duplicate and according to AOAC Official Methods (AOACa, 2003; AOACb, 2003).

2.5. Determination of protein degradation

2.5.1. Molecular weight distribution

The molecular weight distribution was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to Laemmli (1970) with slight modifications. Briefly, depending on protein content and dry matter, aliquots between 5.8 and 7.6 µL of the liquid hydrolyzed samples were suspended in 60% (v/v) 2× Tris-HCl treatment buffer, 30% (v/v) phosphate buffer (pH 7) and 10% (v/v) HPLC water to reach a protein concentration of 5 µg/µL. The samples were heated at 95 °C for 5 min (300 rpm) prior to centrifugation at 13,400 rpm for 3 min (Mini Spin Centrifuge, Eppendorf AG, Germany). An aliquot of 3 µL was added into the gel pocket of the Bio-Rad 4–20% Criterion™ TGX Stain-Free™ Precast Gels. The Broad Range™ Unstained Standard (Bio-Rad Laboratories, Germany) was used as a standard molecular weight marker. Gels were run for 30 min at 200 V, 60 mA, and 100 W at room temperature. Staining of the gel was performed using 0.02% Coomassie Brilliant Blue R-250 solution. Finally, gel images were obtained with the Coomassie Blue Gel Doc™ EZ Imager (Bio-Rad Laboratories, Germany). The SDS-PAGE was performed in duplicate, with each sample being prepared two times independently.

2.5.2. Degree of hydrolysis

The degree of hydrolysis (DH) was analyzed according to Nielsen et al. (2001). The DH was calculated based on the total number of peptide bonds per protein equivalent (h_{tot}), and the number of hydrolyzed bonds (h) using the following equation:

$$DH = h/h_{tot} \cdot 100\%$$

The constant values used for α, β and h_{tot} factor were 1.0, 4.0, and 8.0, respectively, according to theoretical general values for unexamined raw material (Nielsen et al., 2001). The sample preparation was performed in duplicate with each preparation measured in triplicate.

2.6. Techno-functional properties

2.6.1. Protein solubility

The protein solubility was performed according to Morr et al. (1985). A 3% (w/v) sample solution was prepared in 50 mL of 0.1 mol/L NaCl solution and adjusted to pH 4.5 and 7.0 using 0.1–1 mol/L NaOH or 0.1–1 mol/L HCl. After constant stirring for 1 h at room temperature, the non-dissolved fraction was centrifuged at 13,650 rpm for 15 min at 15 °C (3 K30 Sigma Laborzentrifugen GmbH, Germany). The supernatant was then filtrated in Whatman No.1 filter paper and frozen until analysis (−20 °C). The protein content was determined using the Dumas combustion method (AOACb, 2003). The protein solubility was analyzed for all four times of hydrolysis of each sample.

2.6.2. Foaming properties

The foaming capacity and foam stability were analyzed according to Phillips et al. (1987) using a whipping machine (Hobart N50, Hobart GmbH, Germany). A 5% sample solution (w/w) was gently stirred for 15 min before whipping (580 rpm) for 8 min until the formation of stable foam. Based on the relation of the foam volume before and after whipping the foaming capacity was calculated. The foam stability was assessed as the percent loss of foam volume after 60 min. The foaming properties of each sample were analyzed after 15 min and 120 min of hydrolysis.

2.6.3. Emulsifying capacity

The emulsifying capacity was determined according to Wang and Johnson (2001). A 1% sample solution (w/w) and 125 mL of Mazola corn oil were placed in a reactor system (IKA®-Werke GmbH & Co. KG, Germany). After 1 min homogenization at 11,000 rpm using an Ultra-Turrax (IKA-Werke GmbH & Co. KG, Germany), 10 mL/min oil were added using a dispenser (IKA®- Werke GmbH & Co. KG, Germany), while measuring constantly the emulsion conductivity using a conductivity meter (LF 521 with electrode KLE 1/T, Wissenschaftliche-Technische Werkstätten GmbH, Germany). The measurement was stopped as a phase inversion was reached (< 10 µS/cm) and the volume of added oil was used to calculate the emulsifying capacity (mL oil/g sample). The emulsifying capacity was analyzed in the samples hydrolyzed for 15 min and 120 min, respectively.

2.7. Sensory analysis

2.7.1. Sample preparation

For sensory analysis, 2% solutions (w/w) of PPI and hydrolysates inactivated after 15 min and 120 min were prepared with tap water. The samples were adjusted to pH 7.0 with 1 mol/L NaOH and coded using three-digit random numbers. Water and plain crackers were provided for palate cleansing in between.

2.7.2. Sample evaluation

The sensory evaluation was conducted according to DIN 10967-1-1999. For selection of the main attributes, a ten-member trained panel evaluated attributes regarding retronasal aroma, taste and trigeminal sensation of the PPI and its hydrolysates. Attributes selected by more than five assessors were chosen for further sensory analysis such as pea-like (3-s-butyl-2-methoxypyrazine), green (hexanal), earthy (geosmin), roasted (furaneol/acetylpyridine), cooked potato (3-(methylthio-) propanal), salty, astringent, and bitter.

For sensory analysis, 20 mL of each sample were presented at room temperature, in glass cups and random order. Six samples were presented per session. The panelists assessed the sample intensities of the attributes on a 0 (not noticeable) to 10 (strongly noticeable) ranging scale. Furthermore, overall intensity (0 = not perceivable, 10 = very strong perception) and hedonic scale (0 = dislike, 5 = neutral, 10 = like) were assessed. The results are presented as the mean values among all panelists.

2.8. Statistical analysis

All results, expressed as mean values ± standard deviation of at least two measurements (n = 2), were analyzed by one-way analysis of variance (ANOVA). Additionally, a two-way ANOVA was used to analyze the influence of pH and time of hydrolysis on the protein solubility. The mean values were compared using Tukey's post-hoc test. The

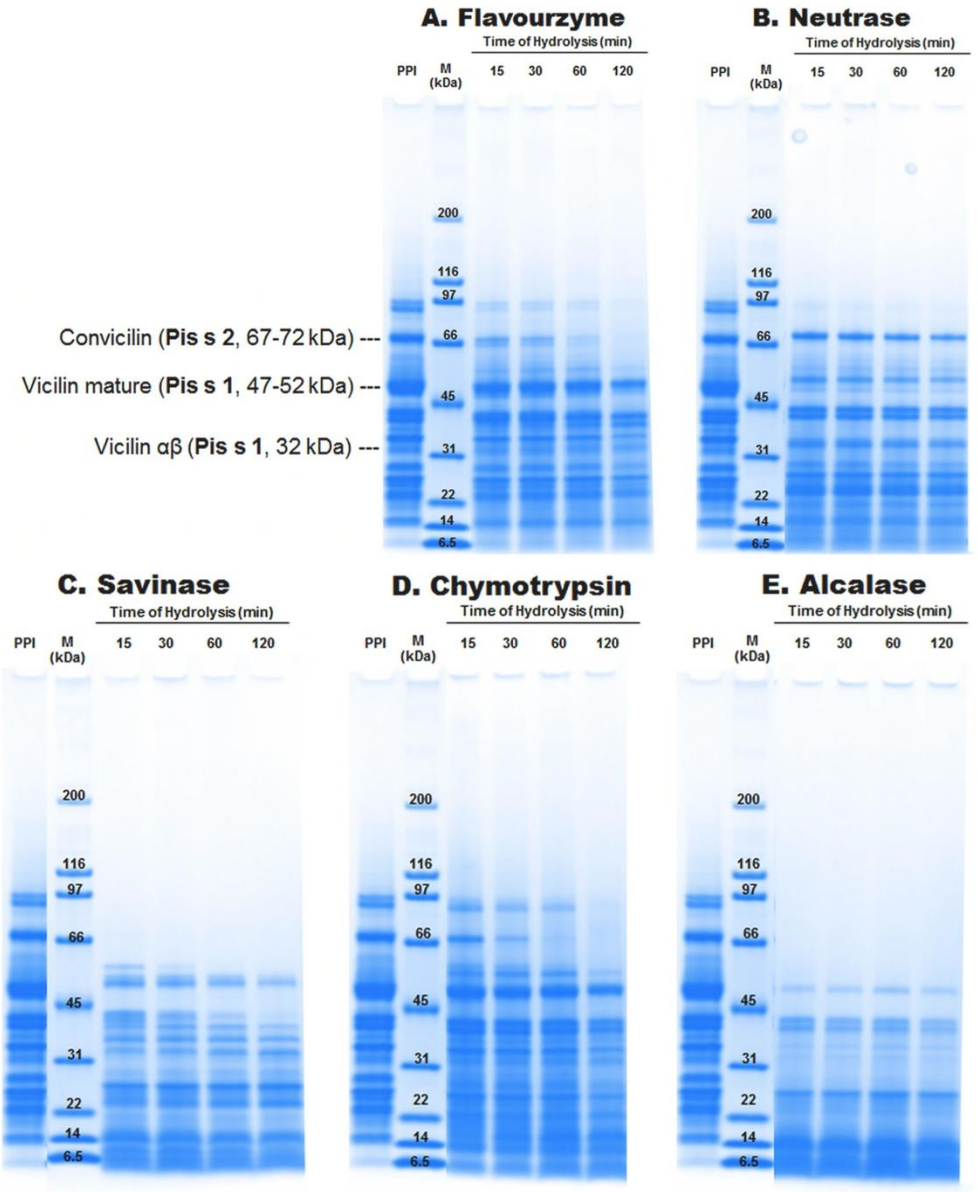

Fig. 1. Molecular weight distribution of the untreated pea protein isolate (PPI) and PPI hydrolysates (Flavourzyme, Neutrase, Savinase, Chymotrypsin, and Alcalase) obtained at different times of hydrolysis as determined by SDS-PAGE under reducing conditions. M = molecular weight standard, indicated in kilo Dalton (kDa).

relationship among DH, protein solubility, and bitterness was analyzed using the Pearson correlation coefficient. All statistical analyses were performed using OriginPro 2018b and were considered statistically significant at $P < 0.05$.

3. Results and discussion

The reference PPI showed 83%of protein, 92% of dry matter, and 5% ash content. The hydrolyzed PPI solutions showed an average protein content of 83%, dry matter of 95%, and ash content of 6%. Complete data can be found in Table A-1 in the Mendeley dataset (García Arteaga et al., 2020).

3.1. Effects of enzymatic hydrolysis on protein degradation

3.1.1. Molecular weight distribution

The molecular weight distribution was analyzed in order to investigate the effect of enzymatic hydrolysis on the pea proteins and on

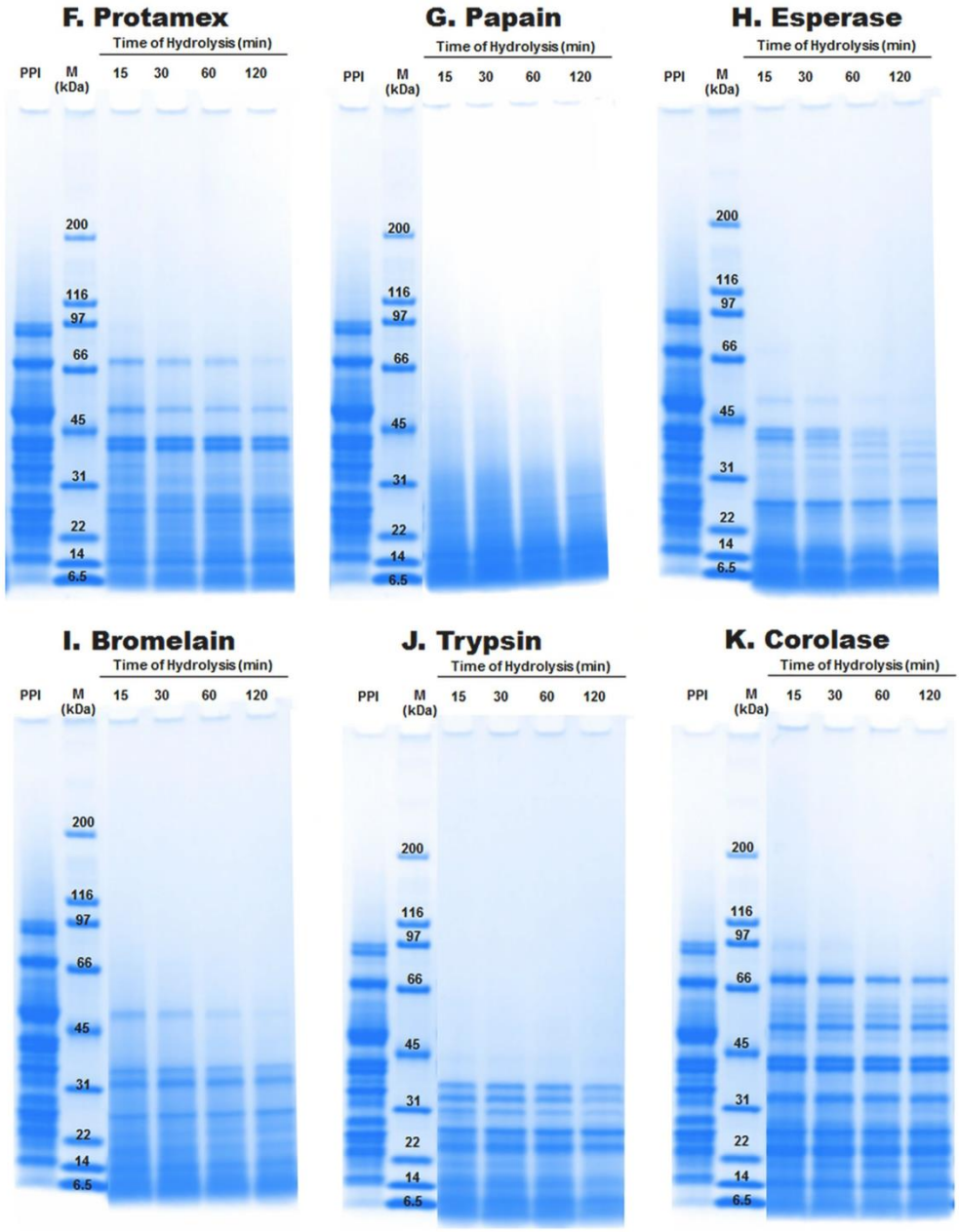

Fig. 2. Molecular weight distribution of the untreated pea protein isolate (PPI) and PPI hydrolysates (Protamex, Papain, Esperase, Bromelain, Trypsin, and Corolase) obtained at different times of hydrolysis as determined by SDS-PAGE under reducing conditions. M = molecular weight standard, indicated in kilo Dalton (kDa).

the main allergens. Protein fractions of the PPI ranged from 97 to 7 kDa (Figs. 1 and 2), which is in accordance to pea SDS-PAGE profiles available in literature (Barac et al., 2012). Enzymatic hydrolysis showed an influence on molecular weight distribution, especially regarding the high molecular weight fractions (Figs. 1 and 2).

3.1.1.1. Pis s 2 degradation. Pis s 2 (~72 kDa) was degraded almost completely after 15 min of hydrolysis with Savinase, Alcalase, Papain, Esperase, Bromelain, and Trypsin. Hydrolysis with Neutrase and Corolase showed no effect on this protein fraction; whereas hydrolysis with Flavourzyme, Chymotrypsin, and Protamex showed a

reduction of this fraction with longer times of hydrolysis. Furthermore, Flavourzyme, Neutrase, Chymotrypsin, and Corolase hydrolysates showed one neo-band at around 55 kDa, which has been previously described by Le Gall et al. (2005) as a possible cleaved-peptide of convicilin.

3.1.1.2. Pis s 1 degradation. Pis s 1 (~50 kDa) was completely hydrolyzed by Papain and Trypsin within the first 15 min of hydrolysis, whereas Esperase and Bromelain cleaved this fraction only after 60 and 120 min, respectively. Alcalase, Protamex, and Savinase reduced the Pis s 1-intensity by approximate 71%, 38%, and 20%, respectively, after 120 min of hydrolysis. The mature Pis s 1 vicilin is composed by different polypeptides such as vicilin αβ (30–36 kDa) and vicilin-γ (12–16 kDa), and the breakdown of mature Pis s 1 could result in an increase of these fractions. The vicilin αβ has been also described as one major pea allergen (Sanchez-Monge et al., 2004). Except for all Papain hydrolysates and Esperase 120-min hydrolysate, all other enzymes were unable to hydrolyze the vicilin αβ fraction, which might indicate a preservation of the allergenic potential of this protein fraction.

Different results within the same protease family might be due to substrate specificity. From the cysteine endopeptidases, Papain shows a preference for bulky hydrophobic residues, whereas Bromelain shows a preference for polar amino acids in both P1 and P1′ position (Cstorer and Ménard, 1994; Rowan, 2013). The electrophoretic results from this study suggest that the PPI probably had more of the hydrophobic residues such as leucine or glycine, which enabled Papain to cleave peptide bonds within the protein efficiently. Similarly, hydrolysates from serine proteases showed different degradation patterns suggesting different substrate specificities. Furthermore, as pea protein composition depends on the botanical variety, time of harvest, and environmental conditions, further studies of the PPI "Navarro," such as amino acid profile and protein fractioning, are necessary to understand the mechanism of action of these enzymes. A comparison of electrophoretic results in this study with those from literature is difficult as different pea varieties and enzyme conditions have been used.

Comparable to the results of Le Gall et al. (2005), the PA2 albumin fraction (26 kDa) showed resistance to proteolysis in all hydrolysates except for Papain hydrolysate. The complete and partial degradation of Pis s 2 and Pis s 1, respectively, indicates that enzymatic hydrolysis might represent an effective method to destroy the main allergens of pea proteins.

3.1.2. Degree of hydrolysis

The DH was analyzed with the OPA reagent, which forms a complex with free primary α- and ε-amino groups, which is then photometrically detected. The DH increased significantly with longer times of hydrolysis (Table 2). Among the serine proteases, Esperase hydrolysate showed the highest DH after 120 min (9.77%) followed by Alcalase (9.24%) and Savinase (8.62%) hydrolysates after 120 min of hydrolysis. Trypsin hydrolysates showed lower DH with 7.59% after 120 min of proteolysis, while Chymotrypsin hydrolysates showed the lowest DH with 1.81% after 120 min. As mentioned in the previous section, different results within the same protease family might be due to substrate specificity, however, the presence of a *Pisum sativum* Trypsin inhibitor (PSTI II) could have an influence on the hydrolysis with Trypsin and Chymotrypsin (Pouvreau et al., 1998), thus reducing their proteolytic mechanism of action. Another explanation for the low DH of Chymotrypsin hydrolysates might be the low amounts of methionine and tryptophan in pea proteins reducing the enzyme-substrate interactions (Hedstrom et al., 1992).

Although Papain and Bromelain showed noticeable changes in the molecular weight distribution, the DH values of 5.04% and 3.57% were unexpectedly low after 120 min. A reason could be an unstable and weak reaction of the OPA reagent with cysteine, as postulated by Chen et al. (1979). Hydrolysates from Protamex, Corolase, Flavourzyme, and Neutrase showed a lower increase in the DH with 4.15%, 4.65%, 4.70%, and 5.16% after 120 min of hydrolysis, respectively.

3.2. Effects on techno-functional properties

According to the molecular weight distribution and DH, the hydrolysates with the most changes in the electrophoretic profile (Papain, Trypsin, Esperase, Bromelain, and Alcalase hydrolysates) and hydrolysates with the least changes (Chymotrypsin hydrolysates) are shown in tables and figures of further sections. Complete data can be found in Tables B-1 and B-2 in the Mendeley dataset (García Arteaga et al., 2020).

3.2.1. Protein solubility

Enzymatic hydrolysis promotes the interaction of hydrophilic groups with water molecules by decreasing peptide size, hence increasing protein solubility (Wouters et al., 2016). Consequently, an increase in protein solubility could be attributed to changes in the protein structures, the release of smaller peptides and hydrophilic amino acids as well as changes in the electrostatic forces (Lam et al., 2016).

Protein solubility was analyzed at pH 4.5 (general isoelectric point of pea proteins) and pH 7.0 as well as after the four different hydrolysis times. The PPI showed a low protein solubility of 2% at pH 4.5, while the protein solubility at pH 7.0 was 51%. Fig. 3 shows the protein solubility of the different hydrolysates. Except for Chymotrypsin at 15 min and 30 min, all the hydrolysates improved protein solubility significantly at pH 4.5. Esperase hydrolysates showed the highest protein solubility at pH 4.5 and pH 7.0, with 71% and 78%, respectively, after 120 min. Trypsin, Savinase, and Alcalase hydrolysates followed

Table 2
Degree of hydrolysis of pea protein isolate (PPI, 0 min) and PPI hydrolysates after 15, 30, 60 and 120 min of hydrolysis.

	Degree of hydrolysis (%)				
	0 min	15 min	30 min	60 min	120 min
Flavourzyme	2.36 ± 0.16^{a}	2.96 ± 0.46^{a,b}	3.41 ± 0.21^{b,c}	3.88 ± 0.05^{c}	4.70 ± 0.24^{d}
Neutrase	2.36 ± 0.16^{a}	4.12 ± 0.15^{b}	4.14 ± 0.34^{b}	4.73 ± 0.15^{c,d}	5.16 ± 0.14^{d}
Savinase	2.36 ± 0.16^{a}	5.40 ± 0.57^{b}	6.45 ± 0.81^{b,c}	7.44 ± 1.04^{c,d}	8.62 ± 1.07^{d}
Chymotrypsin	2.36 ± 0.16^{a}	1.43 ± 0.70^{a}	1.50 ± 0.80^{a}	1.74 ± 0.75^{a}	1.81 ± 0.70^{a}
Alcalase	2.36 ± 0.16^{a}	7.15 ± 0.59^{b}	7.77 ± 0.69^{b}	8.40 ± 0.99^{b,c}	9.24 ± 0.28^{e}
Protamex	2.36 ± 0.16^{a}	2.79 ± 0.69^{a}	2.92 ± 0.71^{a}	3.21 ± 0.80^{a}	4.15 ± 0.67^{a}
Papain	2.36 ± 0.16^{a}	4.41 ± 0.44^{b}	4.67 ± 0.35^{b}	4.81 ± 0.33^{b}	5.04 ± 0.37^{b}
Esperase	2.36 ± 0.16^{a}	5.96 ± 0.19^{b}	7.05 ± 0.76^{b,c}	8.15 ± 0.75^{c}	9.77 ± 0.51^{d}
Bromelain	2.36 ± 0.16^{a}	2.29 ± 1.28^{a}	2.71 ± 1.05^{a}	2.48 ± 0.43^{a}	3.57 ± 0.87^{a}
Trypsin	2.36 ± 0.16^{a}	3.29 ± 0.48^{a}	4.72 ± 0.80^{a,b}	6.08 ± 0.88^{b,c}	7.59 ± 1.67^{c}
Corolase	2.36 ± 0.16^{a}	3.38 ± 0.41^{a,b}	3.94 ± 0.19^{b,c}	4.26 ± 0.67^{b,c}	4.65 ± 0.45^{c}

Results are expressed as means ± standard deviation (n = 4). Means with different letters within one row indicate significant differences (Tukey, $P < 0.05$).

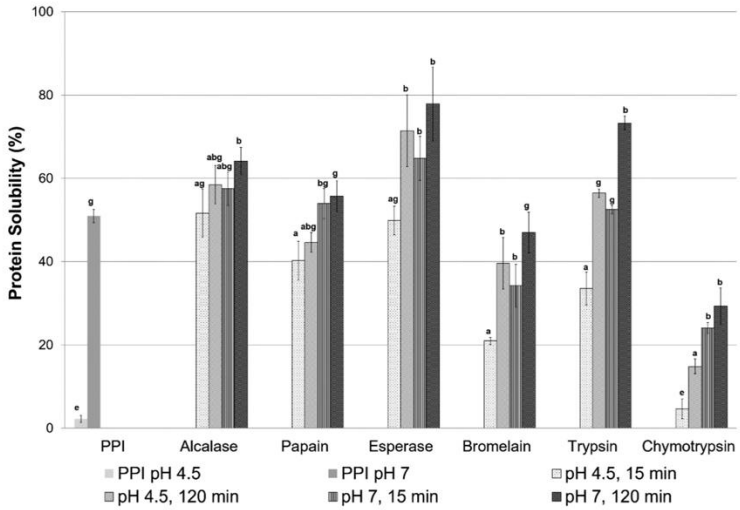

Fig. 3. Protein solubility of the untreated pea protein isolate (PPI) and PPI hydrolysates at different pH and hydrolysis times. Results are expressed as means ± standard deviation (PPI $n = 2$, Hydrolysates $n = 4$). Means with different letters within each enzyme indicate significant differences with the untreated PPI (Tukey, $P < 0.05$).

with 56%, 65%, and 59%, respectively, at pH 4.5 and 73%, 68%, and 64% at pH 7.0, respectively. Serine endopeptidases such as Alcalase, Esperase, Savinase hydrolyze peptide bonds with tyrosine, phenylalanine or leucine at the carboxyl side (Mahajan and Badgujar, 2010) which might have a positive effect on protein solubility (Adler-Nissen, 1986b; Molina Ortiz and Wagner, 2002). However, Chymotrypsin showed the lowest protein solubility at both pH 4.5 and pH 7.0, which might be attributed to previously discussed reasons in Section 3.1.2.

Moreover, Flavourzyme hydrolysates showed the second lowest protein solubility at pH 7.0, followed by Protamex, Neutrase, and Corolase. Papain (45%) and Bromelain (40%) improved significantly the solubility at pH 4.5, especially after 120 min, while at pH 7.0 their hydrolysates showed no significant difference to the PPI. Protein solubility correlated strongly with the DH after 15 min and 120 min.

3.2.2. Foaming properties

Proteins are known as good foaming agents that distribute homogeneously fine air cells, especially if they have a low molecular weight, a highly hydrophobic surface, and a low electrostatic repulsion as well as a low surface tension (Barac et al., 2015; Lam et al., 2016; Zayas, 1997).

The method used in this study considers that a sample is able to form a foam, only when no liquid remains visible in the whipping bowl directly after whipping. According to this method, the untreated PPI was unable to properly form foam, probably due to its higher molecular weight and unfolded structure. On the other hand, all hydrolysates showed a significant improvement of the foaming capacity and foam stability (Table 3). This might have been caused by changes in the molecular peptide size and surface hydrophobicity. The improvement of protein solubility is known to impact the surface hydrophobicity (Molina Ortiz and Wagner, 2002), and although the solubility was correlated with the degree of hydrolysis, there were no significant correlations between the foaming capacity and the degree of hydrolysis. Thus, to some extent, the average hydrophobicity of the released peptides might have played an essential role in foaming capacity (Lam et al., 2016). Trypsin hydrolysates showed the highest foaming capacity of 2271% after 120 min of hydrolysis, followed by the samples obtained after 15 min of Esperase hydrolysis (2237%). However, the foaming capacity decreased significantly after a 120 min treatment with Esperase (1859%). A similar tendency was observed for the foaming capacity of Savinase hydrolysates (15 min: 2013%, 120 min: 1798%),

Table 3
Foaming properties of the pea protein isolate (PPI) and PPI hydrolysates after 15 min and 120 min of hydrolysis.

	Time (min)	Foaming capacity (%)	Foam stability (%)
Alcalase	15	1940 ± 35^{a}	81 ± 6^{a}
	120	1806 ± 60^{b}	80 ± 6^{a}
Papain	15	2119 ± 72^{a}	97 ± 1^{a}
	120	2101 ± 167^{a}	97 ± 2^{a}
Esperase	15	2237 ± 124^{a}	90 ± 5^{a}
	120	1859 ± 78^{b}	74 ± 12^{b}
Bromelain	15	1710 ± 19^{a}	87 ± 5^{a}
	120	1830 ± 80^{b}	81 ± 16^{a}
Trypsin	15	2065 ± 122^{a}	93 ± 2^{a}
	120	2271 ± 19^{b}	95 ± 3^{a}
Chymotrypsin	15	1619 ± 11^{a}	88 ± 4^{a}
	120	1831 ± 18^{b}	79 ± 3^{a}

Results are expressed as means ± standard deviation (n = 4). Means with different letters within each enzyme indicate significant differences in each experiment (Tukey, $P < 0.05$).

Alcalase hydrolysates (15 min: 1939%, 120 min: 1806%), and Papain hydrolysates (15 min: 2119%, 120 min: 2101%). Flavourzyme hydrolysates showed the lowest foaming capacity of 1614% and 1611% at 15 min and 120 min of hydrolysis, respectively, followed by Corolase, Neutrase, Protamex, and Bromelain hydrolysates.

Horiuchi et al. (1978) suggested that the foam stability of enzymatic hydrolysates improves with an increase in the hydrophobic surface of the protein molecules rather than with the release of hydrophobic amino acids. In our study, Papain hydrolysates showed the highest foam stability (97%) after 15 min and 120 min of hydrolysis. On the other hand, Neutrase hydrolysates showed the lowest foam stability after 15 min (19%) and 120 min (12%) of hydrolysis, followed by Flavourzyme hydrolysate (22%) after 120 min and Protamex hydrolysate (34%) after 15 min of hydrolysis. These results suggest that the higher hydrolyzed isolates might have formed peptides with larger hydrophobic surfaces resulting in higher stabilities.

3.2.3. Emulsifying capacity

Emulsions are dispersions of two immiscible liquid phases, which are generally unstable due to high interfacial tension. Proteins have the

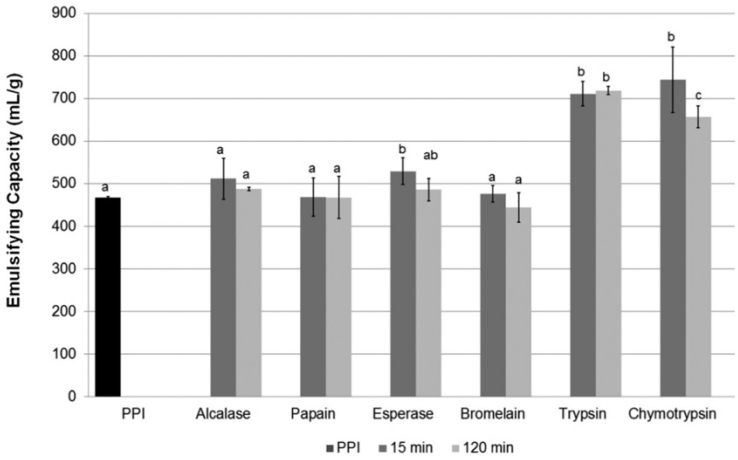

Fig. 4. Emulsifying capacity of the untreated pea protein isolate (PPI) and PPI hydrolysates after 15 min and 120 min of hydrolysis. Results are expressed as means ± standard deviation (PPI $n = 2$, Hydrolysates $n = 4$). Means with different letters within each enzyme indicate significant differences with the untreated PPI (Tukey, $P < 0.05$).

ability to reduce the tension between the two phases by redirecting their amphiphilic residues towards the water and oil phase resulting in smaller droplets.

The PPI showed an emulsifying capacity of 467 mL/g (Fig. 4). After 15 min of hydrolysis, Chymotrypsin hydrolysate showed the highest emulsifying capacity with 727 mL/g, followed by Flavourzyme (715 mL/g) and Trypsin (711 mL/g) hydrolysates. Savinase (499 mL/g) and Esperase (529 mL/g) hydrolysates showed a slight increase in the emulsifying capacity after 15 min of hydrolysis but it decreased with longer hydrolysis times (120 min). Papain, Bromelain, and Alcalase hydrolysates were not significantly different from the PPI. Hydrolysates from Neutrase and Corolase improved the emulsifying capacity significantly after both times of hydrolysis, ranging from 592 mL/g to 641 mL/g. Protamex hydrolysates showed only a significant increase in emulsifying capacity after 15 min of hydrolysis with 645 mL/g.

Negative correlations between the degree of hydrolysis and emulsifying capacity have been reported in the literature (Achouri et al., 1998; Adler-Nissen and Olsen, 1979; Klost and Drusch, 2019). Thus, the molecular protein size might influence protein-protein and protein-oil interactions. Peng et al. (2016) suggested that higher molecular weight and bigger hydrodynamic diameter of the proteins might improve emulsifying capacity. They also suggested that heat treatment and larger peptide sizes increase surface hydrophobicity, promoting hydrophobic interactions between protein-oil droplets, which result in higher emulsifying capacity. However, Barac et al. (2012) suggested that high molecular weight aggregates decreased emulsifying capacity but formed more stable emulsions.

The present study showed a weak negative correlation between the degree of hydrolysis and emulsifying capacity. The weakness of this correlation was mainly due to trypsin hydrolysates as they showed higher emulsifying capacity compared to other highly hydrolyzed samples. One explanation might be that the trypsin hydrolysates maintained the protein fractions between 35 kDa and 22 kDa. These two protein fractions might provide an amphiphilic character to the trypsin hydrolysates since the hydrolysates without these fractions presented lower emulsifying capacity. However, the emulsifying capacity of trypsin hydrolysates was also higher compared to some of the less hydrolyzed samples. Therefore, trypsin might have facilitated the unfolding of hydrophobic side chains of the pea proteins, promoting optimal interaction with the oil.

3.3. Effects on sensory properties

The retronasal aroma of the PPI resulted in attributes such as pea-like (4.2), green (2.9), earthy (1.6), roasted (2.3), and cooked potato (3.8), whereas the main taste attributes were salty (2.1), astringent (1.9), and bitter (3.0) with an overall intensity of 4.9 and a preference indication (hedonic) of 4.3. Compared to the PPI aroma profile, bitterness was the only attribute with a significant change after 15 min and 120 min (Fig. 5). Complete data can be found in Tables C-1 and C-2 in the Mendeley dataset (García Arteaga et al., 2020).

The bitter intensity of the Savinase and Alcalase hydrolysates (15 min of hydrolysis) increased significantly to 6.7 and 6.5, respectively, compared to the untreated PPI (3.0); however, with longer hydrolysis times (120 min), the bitterness of those samples was reduced to 6.5 and 5.4, respectively. On the other hand, the bitterness of Esperase hydrolysates increased significantly to a score of 6.4 only after 120 min of hydrolysis. After 15 min of hydrolysis, Bromelain (2.4), Protamex (2.5), Trypsin (2.6), and Papain (2.7) hydrolysates showed lower bitter intensities compared to the PPI followed by Chymotrypsin (3.5), Corolase (3.5) and Neutrase (3.7) hydrolysates. The lowest bitterness in the samples (2.2) was obtained by hydrolysis with Chymotrypsin and Protamex after 120 min of hydrolysis followed by Neutrase (2.4) and Corolase (2.4). These results suggested a strong correlation between the bitterness and the DH. The correlation between the DH and the formation of bitter peptides has been extensively studied (Adler-Nissen and Olsen, 1979; Meinlschmidt et al., 2016; Saha and Hayashi, 2001; Sun, 2011), where the cleavage of peptide bonds and release of small peptides with hydrophobic amino acid residues leads to an increase in bitterness.

The highest overall intensity after 15 min of hydrolysis was observed in Alcalase (5.9) and Savinase (5.7) hydrolysates; whereas Papain (4.1) and Trypsin (4.1) hydrolysates showed the lowest overall intensity. However, after 120 min of hydrolysis, Esperase hydrolysate showed the highest overall intensity of 6.9 followed by Savinase (5.9), Alcalase (5.4), and Trypsin (5.0) hydrolysates. The high overall intensity results suggest that the panelist perceived this intensity as an increase in bitterness. Accordingly, Esperase (2.9, 1.9), Savinase (1.7, 1.8), and Alcalase (1.6, 2.0) hydrolysates were the least favorite among the panelist after 15 min and 120 min of hydrolysis, respectively. After 15 min of hydrolysis, Protamex hydrolysate (5.5) was the favorite

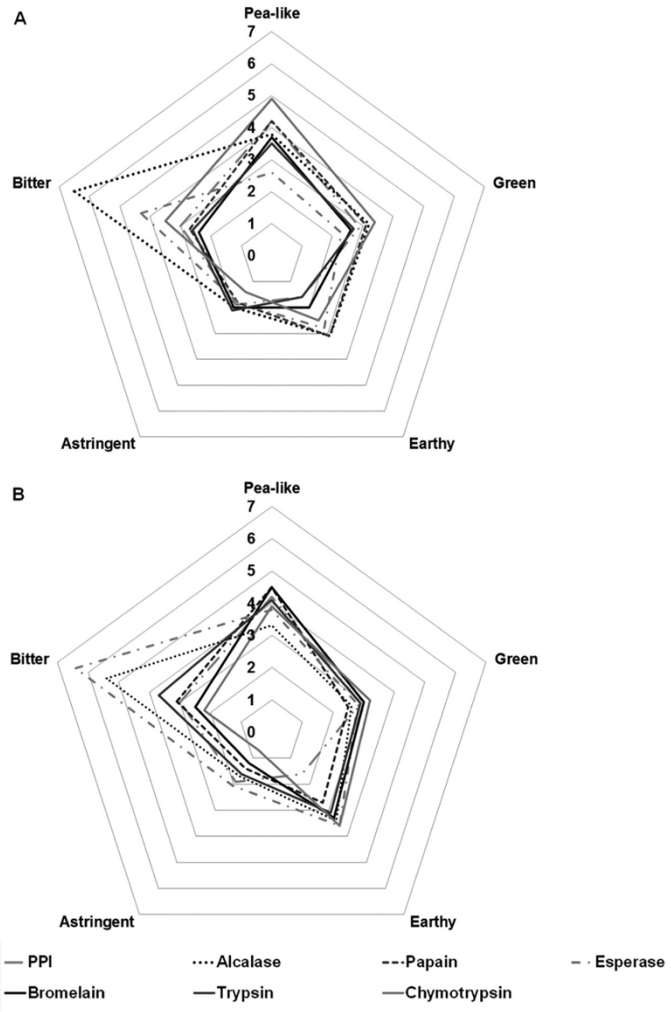

Fig. 5. Retronasal aroma and taste profile of the untreated pea protein isolate (PPI) and PPI hydrolysates after 15 min (A) and 120 min (B) of hydrolysis. Results are expressed as means (n = 10).

sample among the panelists, followed by Chymotrypsin hydrolysate (4.8), Bromelain hydrolysate (4.6), and Trypsin hydrolysate (4.2). These results suggest that bitterness is an important factor influencing the acceptance by the panelist (Fig. 6).

4. Conclusions

The present study aimed to investigate the effect of hydrolysis of PPI with different enzyme preparations on the techno-functional and sensory properties as well as on the degradation of potential allergens through changes in the molecular weight distribution. Of the 11 enzyme preparations investigated by SDS-PAGE, only Papain, Trypsin, Esperase, Bromelain, and Alcalase hydrolysates showed major changes in the molecular weight distribution with a degradation of high molecular weight peptides and an increase in low molecular weight peptides. This was particularly evident in the Papain and Trypsin hydrolysates. Although these electrophoretic results might indicate a degradation of the main pea allergens, the SDS-PAGE gives only an indication of molecular changes, and further immunological studies are necessary to evaluate a possible reduction in the allergenic potential. Most enzymes improved the techno-functional properties of the PPI, especially protein solubility at pH 4.5 and foaming capacity. Regarding sensory properties, only bitterness changed significantly after enzymatic hydrolysis. This increase in bitterness might affect their usage as a food ingredient; therefore, ongoing studies such as the combination of enzymes and fermentation of hydrolysates are being considered to reduce bitterness while maintaining improved techno-functional properties.

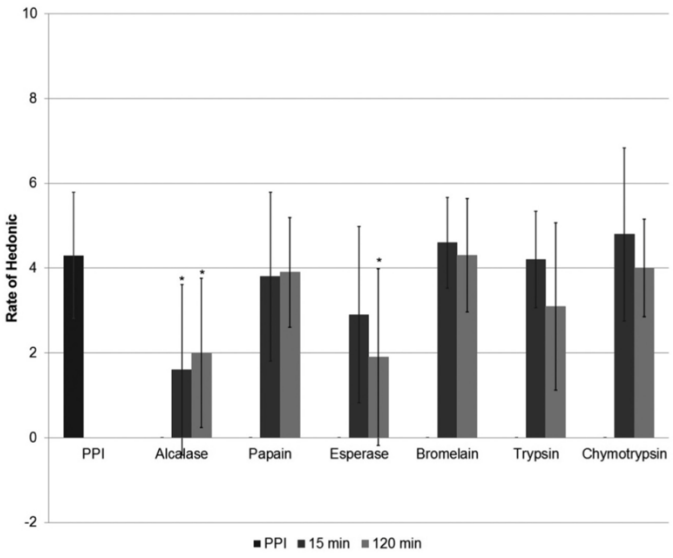

Fig. 6. Hedonic evaluation of the untreated pea protein isolate (PPI) and PPI hydrolysates after 15 min and 120 min of hydrolysis. Results are expressed as means ± standard deviation (n = 10). Means marked with an asterisk (*) indicate significant differences between the individual sample and the untreated PPI (Tukey, $P < 0.05$).

Author agreement statement

The authors hereby declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 2

Sensory profile, functional properties and molecular weight distribution of fermented pea protein isolate[2]

Abstract

Fermentation is known to change the organoleptic characteristics of different raw material and food products. In the present study, different lactic acid bacteria were used for the fermentation of the pea protein isolate prepared from the cultivar “Navarro” to investigate their effects on the sensory profile and on functional properties of the pea proteins. Electrophoresis was used as indication of the effects of proteolysis on the main pea allergen fractions. Most microorganisms were able to grow in the PPI solution and successfully reduced pea off-flavors depending on the strain and fermentation times used. Shorter times of hydrolysis were related to a higher acceptance by the consumer. The protein solubility and emulsifying capacity of the fermented samples were decreased. The electrophoretic results showed a slight reduction in the intensity of pea allergens. The study is of high interest as it highlights the effects of lactic acid fermentation on these three categories simultaneously, giving an overview of the fermentation’s optimal parameters for the production of higher quality pea protein ingredients.

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Research Paper

Sensory profile, functional properties and molecular weight distribution of fermented pea protein isolate

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ABSTRACT

Pea protein isolate (PPI, from *Pisum sativum* L.) was fermented with six different lactic acid bacteria strains for 24 h and 48 h. The fermented samples were analyzed regarding their retronasal aroma and taste, their protein solubility, emulsifying and foaming capacity. Changes in the molecular weight distribution were analyzed to monitor potential effects of fermentation on the main allergenic protein fractions of PPI. After 24-h fermentation, PPI's characteristic aroma attributes and bitter taste decreased for all fermented PPI. However, after 48-h fermentation, cheesy aroma, and acid and salty tastes were increased. The PPI fermented with *L. plantarum* showed the most neutral taste and the panel's highest preference; instead, fermentation with *L. fermentum* led to a fecal aroma and was the least preferred. The protein solubility and emulsifying capacity decreased after PPI fermentation, while foaming capacity remained constant in comparison to the untreated PPI. The electrophoretic results showed a reduction in the intensity of the allergenic protein fractions; however, these changes might be attributed to the reduced protein solubility rather than to a high proteolytic effect of the strains. Fermentation of PPI for 24 h and 48 h might not be a suitable method for the production of highly functional pea proteins. Further modification methods have to be investigated in the future.

1. Introduction

The food industry is looking for functional and appealing plant-based ingredients to meet the growing demand for alternative protein sources. Peas (*Pisum sativum* L.) are an attractive raw material for vegetable food products due to their extensive plantation and good availability (Cernay et al., 2016). Furthermore, peas are rich in proteins featuring all essential amino acids. However, the use of pea proteins in the food industry is limited due to present green and grassy sensory attributes resulting from compounds such as aldehydes, ketones, and alcohols (Heng, 2005). A large part of the components responsible for the characteristic off-flavors of peas can be traced back to oxidation and enzymatic degradation products of unsaturated fatty acids during harvest, storage (Roland, Pouvreau, Curran, van de Velde and de Kok, 2017) and further processing (Azarnia et al., 2011; Heng et al., 2004; Lan et al., 2019). Fermentation has been widely used to improve sensory properties of different cereal and legume products (Ferri et al., 2016; Kaczmarska et al., 2018; Meinlschmidt et al., 2016a; Schlegel et al., 2019). During fermentation, biochemical changes occur, such as degradation and formation of organic substances developing a more intense aroma profile (Adewumi, 2019; Cabuk et al., 2018). To the best of our knowledge, only one study has investigated a 48-h lactic acid fermentation of pea protein extracts to improve the aroma profile while reducing present off-flavors (Schindler et al., 2012).

Metabolic enzymes and metabolites released during fermentation could affect the protein functionality. Few studies have investigated the functional properties of fermented pea proteins. Cabuk et al. (2018) and Kumitch et al. (2020) fermented protein-enriched pea flours with lactic acid bacteria (LAB) and fungi; they found a negative influence of fermentation on protein solubility, emulsifying and foaming capacity. These properties are relevant for several food products such as vegetable milk alternatives, ice cream, and mayonnaise. Therefore, the control and selection of appropriate microorganisms are essential for the later application potential of the fermented pea protein products.

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Additionally, there are indications for a reduction in the allergic potential of fermented food products and ingredients from legumes such as soy protein isolates (Chen et al., 2020; Meinlschmidt et al., 2016b; Zhou et al., 2013). Peas are known for their low allergenic potential; however, Sanchez-Monge et al. (2004) identified two main pea allergens, increasing pea allergy awareness. Other studies investigated the incidence of pea allergies and demonstrated cross-reactivity with different nuts and legumes (Codreanu-Morel et al., 2019; Dreyer et al., 2014; Lavine and Ben-Shoshan, 2019; Richard et al., 2015). To our knowledge, only one study has focused on reducing the allergenic potential of pea flour by fermentation (Barkholt et al., 1998). They showed that 48-h fermentation with LAB could reduce the antigenicity to 10% compared to the unfermented pea flour. Thus, the fermentation of pea protein isolate (PPI) could present an important approach to reduce its allergenic potential.

The main objective of the present study was to investigate the impact of lactic acid fermentation on the sensory profile of PPI. In addition, the effects on the functional properties and on the degradation of allergenic proteins were investigated to consider the value of fermented PPI as food ingredient with lower allergenic potential.

2. Materials and methods

2.1. Materials

Pea seeds (*Pisum sativum* L., cultivar Navarro) were provided by Norddeutsche Pflanzenzucht Hans-Georg-Lembke KG (Germany). Broad Range™ Unstained Protein Standard, 4–20% Criterion™ TGX stain-free™ precast polyacrylamide gels, Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories GmbH (Germany). Sodium dihydrogen phosphate, sodium dodecyl sulfate, sodium tetraborate decahydrate, o-phthaldialdehyde, and sodium monohydrogen phosphate were purchased from Sigma-Aldrich (Germany). All chemicals used in this study were of analytical grade unless otherwise indicated.

2.2. Production of pea protein isolate

Peas were dehulled and split using an underflow peeler (Streckel & Schrader KG, Germany) and separated using an airlift system (Alpine Hosakawa AG, Germany). The split pea seeds were milled by an impact mill (Gebrüder Jehmlich GmbH using a REKORD A) at maximum peripheral speed of 135 m/s with a 0.5 mm sieve. The isolation of pea protein was performed according to Arteaga, Apéstegui Guardia, Muranyi, Eisner, and Schweiggert-Weisz (2020a). In brief, an aqueous alkaline extract (pH 8.0) of the pea flour was prepared in DI water while stirring constantly for 60 min. The protein extract was adjusted to pH 4.5 for isoelectric precipitation of the proteins. The precipitated proteins were separated, neutralized, pasteurized (70 ± 2 °C) for 2 min and spray-dried.

2.3. Fermentation

2.3.1. Strains, media, growth conditions and preparation

Six microorganisms were selected according to literature regarding their ability to improve the sensory profile of legumes and their proteolytic activity (Barkholt et al., 1998; Ben-Harb et al., 2019; Schindler et al., 2012). All microorganisms were cultivated for 48 h in 150 mL MRS-broth at their individual conditions (Table 1). The liquid preculture (1 mL) was serially diluted in Ringer solution (1:10 v/v) and incubated in MRS-Agar plates for 48 h at the optimal conditions of each microorganism to determine the number of colony-forming units (CFU). The CFU enabled the calculation of the aliquots required for fermentation (8 Log CFU/mL). The required aliquot was centrifuged for 10 min at 9000 rpm. The pellets were used for inoculation.

Table 1
Microorganisms and growth conditions.

Microorganism	Abbreviation	Specie No.	Growth/Culture conditions		
			T (°C)	Type	Medium
Lactobacillus plantarum	*L. plantarum*	DSM-20174	30	Anaerobe	MRS
Lactobacillus perolens	*L. perolens*	DSM-12744	30	Aerobe	MRS
Lactobacillus fermentum	*L. fermentum*	DSM-20391	37	Aerobe	MRS
Lactobacillus casei	*L. casei*	DSM-20011	30	Aerobe	MRS
Leuconostoc mesenteroides subsp. cremoris	*Lc. cremoris*	DSM-20200	30	Aerobe	MRS
Pediococcus pentosaceus	*P. pentosaceus*	DSM-20336	30	Anaerobe	MRS

DSM: Deutsche Sammlung von Mikroorganismen (German Collection of Microorganisms); T: temperature; MRS: De Man, Rogosa and Sharpe.

2.3.2. Fermentation of PPI dispersions

A 9% (w/v) PPI dispersion was prepared in sterile DI water and homogenized for 7 min using an Ultraturrax (IKA® Werke GmbH & Co KG, Germany). The dispersion was pasteurized at 80 °C for 30 min in a thermostatically controlled reactor. Before inoculation, the dispersion was cooled down to the respective temperature (Table 1) and 0.5% (w/v) glucose was added. Aliquots of 990 mL were transferred to sterile 2 L Schott-Duran bottles, where the fermentation took place. The dispersions were inoculated and the fermentations were carried out for 24 h and 48 h under strain-specific conditions (Table 1) without stirring. The anaerobe fermentation was performed by closing the bottle lid completely, whereas the aerobe fermentation was done with semi-opened lid. Aliquots of each sample were taken to determine changes in viable cell count prior to inactivation at 90 °C for 10 min, neutralization, and lyophilization. All fermentations were performed in duplicate. The fermentation times were selected based on previous studies, in which 48-h fermentation improved aroma profile and reduced antigenicity (Barkholt et al., 1998; Schindler et al., 2012).

2.3.3. Growth determination

Liquid aliquots were taken after 5 min (0 h), 24 h and 48 h of inoculation of the PPI. The viable cell counts were determined on MRS-Agar plates by serial dilutions as described in section 2.3.1.

2.3.4. Determination of pH

The pH was measured every 30 min during 48 h of fermentation using a disinfected WTW ProfiLine pH 3310 pH electrode (Xylem Analytics Germany GmbH, Germany). The pH measurements were performed on an additional bottle with the same conditions for each microorganism.

2.3.5. D-Glucose and D-/L-lactic acid

The determinations of D-glucose and D-/L-lactic acid were performed using Enzymatic BioAnalysis test kits from R- BIOPHARM AG (Germany). The samples were prepared according to the manufacturer's instructions.

2.4. Chemical composition

The dry matter content (105 °C), ash content (950 °C) and protein content (N x 6.25) were performed in duplicate and according to AOAC Official Methods (AOACa, 2003; AOACb, 2003) by means of a thermogravimetric method (TGA 701, Leco Instruments, Germany) and Dumas combustion method (TruMac N, Leco Instruments, Germany).

2.5. Sensory analysis

2.5.1. Sample preparation

Dispersions of the PPI and dispersions of the 24 h and 48 h fermented samples (2%, w/w) were prepared with tap water. The respective samples were adjusted to pH 7.0 with 1 mol/L NaOH. The samples were coded using three-digit random numbers.

2.5.2. Sample evaluation

The sensory evaluation was conducted according to DIN 10967-1-1999. First, for the selection of the main attributes, an eight-member trained panel evaluated attributes regarding retronasal aroma and taste of the PPI and 48-h fermented samples. The panel was trained to identify legume aroma profile attributes; the aroma attributes were compared to specific aroma compounds provided in aroma pens. Each sample (20 mL) was presented at room temperature in glass cups and random order. Attributes selected by more than five assessors were chosen for further sensory analysis.

Second, for sensory analysis of the PPI and all fermented samples, 20 mL of each sample were presented at room temperature in glass cups and random order. The sensory analysis was divided into two sessions, where six fermented samples and the unfermented isolate were presented per session. Water and plain crackers were provided for palate cleansing in between. The panelists assessed the intensities of the attributes on a 0 (attribute not perceivable) to 10 (very strong perception of the attribute) ranging scales. The overall intensity (0 = not perceivable) to 10 (10 = very strong perception) and the indication of preference using a hedonic scale (0 = dislike, 5 = neutral, 10 = like) were assessed.

2.5.3. Principal component analysis (PCA)

The results of sensory evaluation were assessed using PCA covariance matrix to analyze the aroma attributes. The PCA was performed using OriginPro 2018b.

2.6. Functional properties

All functional experiments were performed in duplicate.

2.6.1. Protein solubility

The protein solubility was performed according to Morr et al. (1985) at different pH (pH 3.0 – pH 8.0). The protein content was determined using the Biuret method (550 nm) from the Approved Methods of Analysis (AACC, 2000) with bovine serum albumin (BSA) as calibration standard.

2.6.2. Foaming properties

The foaming capacity and foam stability were analyzed according to Phillips et al. (1987) using a whipping machine (Hobart N50, Hobart GmbH, Germany).

2.6.3. Emulsifying capacity

The emulsifying capacity was determined according to Wang and Johnson (2001) using an 1L-reactor equipped with a stirrer and an Ultraturrax (IKA-Werke GmbH & Co. KG, Germany). The oil was added gradually (10 mL/min) until a phase inversion occurred (<10 μS/cm). The volume of added oil was used to calculate the emulsifying capacity (mL oil/g sample).

2.7. Determination of protein degradation

2.7.1. Molecular weight distribution

The molecular weight distribution was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing and reducing conditions according to Laemmli (1970) with slight modifications and described in detail in García Arteaga, Apéstegui Guardia et al. (2020). Briefly, protein solutions of 5 μg/μL based on the dry matter content were prepared in treatment buffer. For reducing conditions, the samples were heated prior to centrifugation. The supernatants were mixed with treatment buffer. For the electrophoresis, an aliquot of 5 μL of the sample mixture was added into the gel pocket of the Bio-Rad 4–20% Criterion™ TGX Stain-Free™ Precast Gels. The Broad Range™ Unstained Protein Standard (Bio-Rad Laboratories, Germany) was used as molecular weight marker. Gels were run for 30 min and stained using Coomassie Brilliant Blue R-250 as described by Garcia et al. (2020b). Finally, gel images were obtained using an EZ Imager (Bio-Rad Laboratories, Germany). Protein band intensities were calculated using the Image Lab Software (Bio-Rad Laboratories, Germany).

2.7.2. Degree of hydrolysis

The degree of hydrolysis (DH) was performed according to the o-phthaldialdehyde (OPA) method (Nielsen et al., 2001). The DH value was calculated based on the total number of peptide bonds per protein equivalent (h_{tot}), and the number of hydrolyzed bonds (h) using the following equation:

$$DH = h / h_{tot} \cdot 100\%$$

The constant values used for α (degree of dissociation of the α-amino group), β (slope of calibration through linear regression) and h_{tot} factor were 1.0, 4.0, and 8.0, respectively, according to theoretical general values for unexamined raw material (Nielsen et al., 2001). The sample preparation was performed in duplicate with each preparation measured in triplicate.

2.8. Statistical analysis

PPI fermentation was performed in duplicate for each microorganism. All other experiments were performed in duplicate unless otherwise stated. Complete raw data can be found in Mendeley Data files (García Arteaga, Leffler, Muranyi, Eisner and Schweiggert-Weisz, 2020b). The results, expressed as mean values ± standard deviations, were analyzed by one-way analysis of variance (ANOVA). Kruskal-Wallis was used when the ANOVA assumptions were not satisfied. The mean values were compared using Tukey's post-hoc test. The relationship among functional properties, bitterness, protein band intensities and DH was analyzed using the Pearson correlation coefficient. All statistical analyses were performed using OriginPro 2018b and were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Chemical composition

The unfermented PPI contained 84.9% ± 1.4 protein, 95.5% ± 0.3 dry matter, and 5.0% ± 0.2 ash content. The average content of protein, dry matter, and ash in the fermented samples was 80.1% ± 1.8, 96.8% ± 0.6, and 6.8% ± 0.4, respectively. Only the PPI fermented with *Lc. cremoris* for 24 h showed a significant lower protein content (75.7% ± 3.7) compared to the unfermented PPI. This might suggest that PPI was a good source of nitrogen for *Lc. cremoris* which would increase the conversion to lactic acid and further by-products (Coelho et al., 2011). The significant increase in the ash content from all samples might be attributed to the increase in salts resulting from the neutralization of the samples.

3.2. Microbial growth

The growth of the selected microorganisms was evaluated through the total viable cell counts (Log CFU/mL, Table 2A), changes in the pH, the consumption of glucose and the production of D-/L-lactic acid (Table 2B).

Log CFU/mL. With an exception of *Lc. cremoris*, which remained constant with 8.29 Log CFU/mL during the 48-h fermentation, all other

Table 2
(A) Viable cell count (Log CFU/mL) and (B) D- and L-Lactic acid concentrations after 0 h, 24 h and 48 h fermentation of pea protein isolate (PPI).
A

	Log CFU/mL		
	0 h	24 h	48 h
L. plantarum	8.87 ± 0.05^{a}	9.02 ± 0.01^{a}	9.13 ± 0.25^{a}
L. perolens	8.17 ± 0.07^{b}	8.34 ± 0.26^{bc}	8.89 ± 0.11^{bc*}
L. fermentum	7.38 ± 0.14^{c}	8.29 ± 0.04^{c}	8.35 ± 0.01^{bd*}
L. casei	8.50 ± 0.02^{ab}	8.89 ± 0.01^{ab}	8.90 ± 0.02^{ac}
Lc. cremoris	8.29 ± 0.00^{b}	8.29 ± 0.04^{c}	8.29 ± 0.19^{d}
P. pentosaceus	8.08 ± 0.12^{b}	8.37 ± 0.00^{bc}	8.55 ± 0.06^{bcd}

	D-Lactic acid (g/L)			L-Lactic acid(g/L)		
	0 h	24 h	48 h	0 h	24 h	48 h
L. plantarum	0.00 ± 0.00^{a}	1.85 ± 0.23^{a}	2.75 ± 0.15^{a}	0.03 ± 0.02^{a}	1.48 ± 0.13^{a}	2.20 ± 0.19^{a}
L. perolens	0.00 ± 0.00^{a}	0.01 ± 0.02^{b}	0.02 ± 0.01^{b}	0.18 ± 0.11^{a}	4.38 ± 1.41^{b}	5.26 ± 0.62^{b}
L. fermentum	0.00 ± 0.00^{a}	0.79 ± 0.19^{c}	0.51 ± 0.25^{c}	0.06 ± 0.03^{a}	1.47 ± 0.20^{a}	0.99 ± 0.27^{c}
L. casei	0.00 ± 0.00^{a}	0.29 ± 0.08^{bd}	0.60 ± 0.34^{c}	0.36 ± 0.15^{a}	4.52 ± 0.14^{b}	4.73 ± 0.20^{b}
Lc. cremoris	0.02 ± 0.03^{a}	0.60 ± 0.22^{cd}	0.66 ± 0.02^{c}	0.01 ± 0.01^{a}	2.75 ± 0.22^{c}	2.39 ± 0.05^{a}
P. pentosaceus	0.00 ± 0.00^{a}	0.62 ± 0.10^{cd}	0.78 ± 0.06^{c}	0.08 ± 0.05^{a}	1.78 ± 0.27^{ac}	1.89 ± 0.15^{ac}

Results are expressed as means ± standard deviation (CFU n = 2, Glucose and Lactic acid n = 4). Means marked with different letters indicate significant differences between the fermented samples within fermentation times in the Log CFU/mL and within microorganisms in the lactic acid values (Tukey, $P < 0.05$). Means marked with an asterisk (*) indicate significant differences between the initial (0 h) and end (48 h) times of fermentation within one microorganism.

microorganisms were able to grow in the PPI, although they showed a low growth rate. In contrast, the fermented sample with *L. fermentum* showed the highest increase after 24 h of fermentation from 7.38 Log CFU/mL to 8.29 Log CFU/mL and continued to increase after 48 h (8.35 Log CFU/mL). The samples fermented with *L. perolens* and *L. casei* showed an increase to 8.34 Log CFU/mL and 8.89 Log CFU/mL after 24 h, respectively, and to 8.89 Log CFU/mL and 8.90 Log CFU/mL after 48 h, respectively. After 24-h fermentation with *P. pentosaceus*, the sample showed an increase to 8.37 Log CFU/mL and to 8.55 Log CFU/mL after 48 h. Lastly, the sample fermented with *L. plantarum* showed an increase to 9.02 Log CFU/mL and 9.13 Log CFU/mL after 24 h and 48 h, respectively. The ability of LAB to grow in substrates depend on the nutrients present (Ciani et al., 2013). Pea proteins are known to contain low amounts of methionine and tryptophan; the latter is an important nutrient for the growth of *L. plantarum, Lc. cremoris, P. pentosaceus, and L. fermentum* (Corsetti et al., 2016; Holzapfel et al., 2006; Liu, 2016; Verce et al., 2020). This might explain the reduced growth of *Lc. cremoris, L. plantarum,* and *P. pentosaceus*; the growth of *L. fermentum* might be explained by its ability to adapt to non-optimal growth conditions by means of the arginine deiminase pathway (Vrancken et al., 2009).

Although the growth rates of the individual microorganisms were rather low, the microorganisms continued to metabolize, as shown below by the decrease in pH, the decrease in glucose and the increase in lactic acid described below as well as by the changes in the molecular weight distribution and degree of hydrolysis (described in section 3.5).

pH value. The PPI solutions showed an average initial pH of 6.5. Fermentation with *L. casei* lowered the pH of the sample to 4.6 after 12 h of fermentation, while all other strains were below pH 5.0 after 24 h. After 24-h fermentation, the pH of the samples fermented with *L. perolens* and *L. casei* remained constant at pH 4.7 and pH 4.5, respectively. Fermentation with *L. plantarum* and *L. pentosaceus* for 48 h reduced the pH down to pH 4.6 and to pH 4.8, respectively. Samples fermented with *Lc. cremoris* and *L. fermentum* after 24 h showed a pH of 4.7 and 4.8, respectively; which increased after 48 h to pH 4.8 and 5.8, respectively. This increase might suggest an alkalization due to the decarboxylation and/or deamination of the released amino acids into alcohols, ammonia or aldehydes (Ben-Harb et al., 2019; Liu, 2016).

Glucose content. Glucose was used by all microorganisms as a fast energy source. In particular, *L. perolens*, *L fermentum*, and *L. casei* metabolized the entire amount of added glucose after 24 h of fermentation. In the samples fermented with *L. plantarum*, *Lc. cremoris* and *P. pentosaceus*, residual amounts of glucose were detected after 24 h; however, after 48 h of fermentation, all fermented samples showed a complete depletion of the glucose.

Lactic acid content. The results obtained after fermentation with *L. plantarum, L. fermentum, L. casei, Lc. cremoris* and *P. pentosaceus* for the production of D- and L-lactic acid are consistent with the literature, as these LAB are known to produce both D- and L-lactic acid (Chun et al., 2017; Corsetti et al., 2016; González-Vara et al., 1996; Raccach, 1987; Verce et al., 2020).

The fermentation of PPI with *L. perolens* showed the highest production of L-lactic acid after 24 h and 48 h with 4.38 g/L and 5.26 g/L, respectively, followed by the fermentation with *L. casei* (4.52 g/L and 4.73 g/L, respectively). The high production of lactic acid by these two strains might suggests an adequate ability to grow in PPI solutions. Fermentation with *Lc. cremoris* showed a production of 2.39 g/L of L-lactic acid and 0.66 g/L of D-lactic acid after 48 h.

Fermentation of PPI with *L. plantarum* showed a production of D- and L-lactic acid of 2.75 g/L and 2.20 g/L, respectively, after 48 h, whereas fermentation with *P. pentosaceus* increased D- and L-lactic acid concentrations up to 0.78 g/L and 1.89 g/L, respectively. These LAB are known to produce larger concentrations of L-lactic acid under anaerobic conditions (Corsetti et al., 2016; Raccach, 1987). However, as oxygen was not removed prior to fermentation, the residual oxygen content could have contributed to microaerobic fermentation, which slowed down the production of lactic acid, and promoted D-lactic acid and acetate production by *L. plantarum* (Raccach, 1987).

Fermentation with *L. fermentum* showed the lowest L-lactic acid concentration after 24-h (1.47 g/L) and after 48-h (0.99 g/L) fermentation. These low concentrations could indicate that this specific strain produces mainly other by-products as soon as the carbohydrate substrates are depleted. A comparative genomic analysis of 28 strains of *L. fermentum* by Verce et al. (2020) revealed the production of acetate, ethanol, glycerol, diacetyl (2,3-butanedione), and 2,3-butanediol besides lactic acid production. With a pKa of 14.9, 2,3-butanediol is considered a strong base and it is also known to hinder the production of acid compounds (Ciani et al., 2013; Ji et al., 2011), which could be related to both, the low amounts of lactic acid and the increase of pH after 48 h fermentation.

3.3. Sensory analysis

Throughout fermentation, microorganisms metabolize the substrate resulting in the production of different volatile and non-volatile compounds characteristic for the fermented products. The identified attributes (and specific compounds compared to aroma pens) were: pea-like (isopropyl-methoxypyrazine), green (hexanal), earthy (geosmin), roasted (furaneol/acetylpyridine), buttery (2,3-butanedione), cheesy (3-methylbutanoic acid), greasy (2-nonenal), spicy (sotolone), oatmeal, fermented, floury and fecal. A principal component analysis was applied to analyze relationships between samples and sensory attributes. Fig. 1 shows the biplot of the principal components 1 and 2 using the standardized scores of the PPI and fermented samples.

Aroma. For the retronasal aroma attributes (Fig. 1A), the first two components of the PCA explained 73.2%. The sensory attribute with the strongest influence on PC1 was cheesy (0.68), whereas green showed the strongest influence on PC2 (0.62). The unfermented PPI scored the highest in the PC2 (2.58) and was in the nearest proximity to the green attribute, which is known to be one primary off-flavor of peas (Roland et al., 2017; Schindler et al., 2012). Aroma profiles of samples fermented

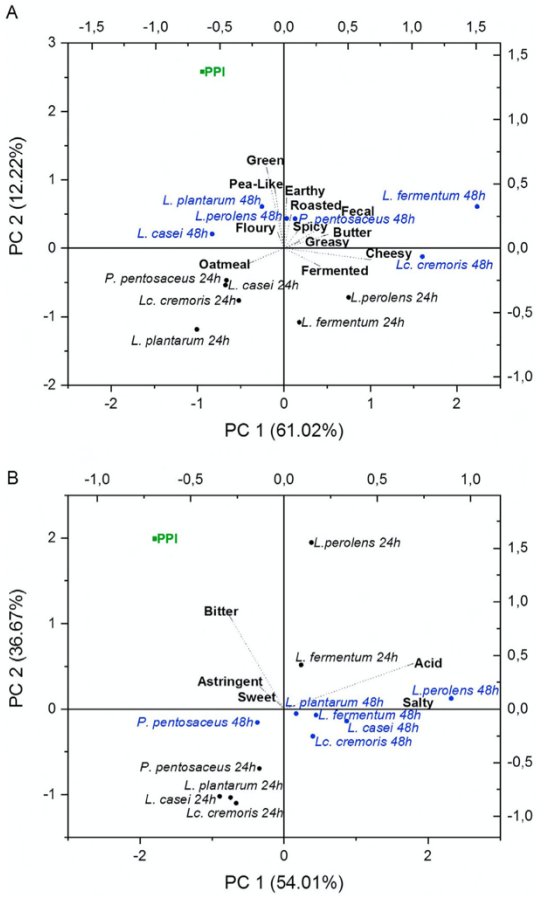

Fig. 1. Biplot of retronasal aroma (A) and taste (B) of the unfermented pea protein isolate (PPI) and PPI fermented for 24 h (black) and 48 h (blue) with different microorganism strains. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

for 24 h and 48 h were distinct from the unfermented PPI and each other.

The PPI solutions fermented for 24 h were found in the negative quadrants of the PC2 independently of the microorganism. The attributes of these samples were farther away from the unfermented PPI, which suggests greater differences in aroma. The lowest pea-like aroma was achieved after 24 h fermentation with *L. perolens, L. casei, L. plantarum, and L. fermentum*. Fermentation with *L. plantarum* for 24 h also masked other aroma attributes of the unfermented PPI (−1.00/-1.18) such as green and earthy. The PPI fermented with *L. perolens* for 24 h showed the highest buttery aroma, which could be attributed to the metabolism of *L. perolens*. Back et al. (1999) reported that *L. perolens* produced notably high concentrations of diacetyl, which might explain the pronounced buttery aroma in this study. Fermentation with *P. pentosaceus* reduced characteristic aromas from peas such as pea-like, green, and earthy, and showed the highest production of the floury attribute.

The 48-h fermentation of PPI resulted in less variation in PC2, whereas in PC1, differences were more pronounced, especially in samples fermented with *L. fermentum* (2.23) and *Lc. cremoris* (1.60). The fermentation of PPI with *L. fermentum* for 48 h was characterized by a fecal aroma usually produced by the catabolism of aromatic amino acids and the generation of undesirable compounds such as p-cresol, indole, and skatole (Ganesan and Weimer, 2017; Ibrahim, 2016). PPI fermented with *Lc. cremoris* for 48 h showed the most intense cheesy aroma, for which this microorganism is used in cheese production (Liu, 2016). The characteristic pea off-flavors were probably masked after fermentation due to the production of other aroma attributes such as buttery and cheesy (Schindler et al., 2012).

Taste. Regarding the taste attributes, PC1 and PC2 represented 90.7% of the total variance (Fig. 1B). The bitter taste showed the strongest influence on PC2 (0.87), whereas acid accounted for the strongest influence on PC1 (0.69). Fig. 1B shows clusters of the fermented samples after 24 h and 48 h, with an exception for the samples fermented with *L. perolens* and *L. fermentum* for 24 h. The unfermented PPI scored the highest in the PC2 (1.99), which can be attributed to the characteristic bitter taste from peas. In contrast, samples fermented with *L. plantarum* and *Lc. cremoris* for 24 h showed lower bitter intensities as well as low intensities of other taste attributes. The fermentation of PPI for 24 h with *L. perolens* led to strong bitter and acid tastes. After 48-h fermentation, *L. casei* showed the lowest bitter taste intensity, which might be attributed to its strong activity peptidase against bitter peptides (Arora and Lee, 1990; El Abboudi et al., 1992). The proteolytic effects during fermentation depend on the LAB species, the specific strains, the individual proteins and their cleavage sites. As a result, smaller peptides, responsible for the bitter taste in unfermented samples, might have been degraded, leading to changes in the taste profile (Saha and Hayashi, 2001).

Overall intensity. The highest overall intensity was perceived in the PPI solution fermented with *L. fermentum* for 48 h, followed by the 48-h fermented PPI with *Lc. cremoris,* and *L. perolens* (Fig. 2A). In contrast,

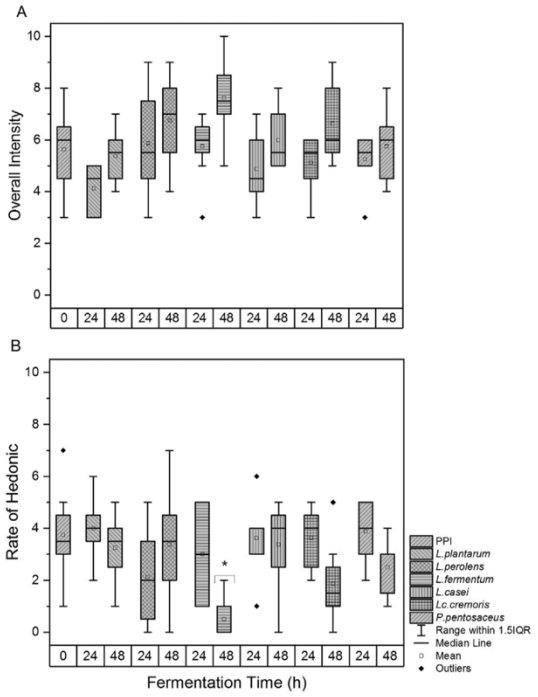

Fig. 2. Overall intensity (A) and rate of hedonic (B) of the unfermented pea protein isolate (PPI) and PPI fermented for 24 h and 48 h with different microorganism strains. Bars marked with an asterisk (*) indicate significant differences between the individual sample and the unfermented PPI (Tukey, $P < 0.05$).

L. plantarum produced the lowest overall intensity after 24 h fermentation.

Preference. Fig. 2B shows the trend for the preference of each sample. All microorganisms reduced the bitter and astringent attributes; however, the sample preference was not significantly improved. The sample fermented with *L. plantarum* for 24 h was rated slightly higher than the unfermented PPI, whereas the preference for the PPI fermented with *L. fermentum* for 48 h was significantly reduced. As previously mentioned, the low preference for the latter was most likely related to the production of fecal aroma attributes. The preference for samples fermented for 24 h was higher than the ones fermented for 48 h. The lower acceptance after longer times of fermentation might be attributed to the possible production of acetate and 2–3 butanediol and other undesired aroma compounds such as p-cresol, indole, and skatole. There was a negative correlation (−0.79, $P < 0.05$) between the overall intensity and the preference among fermented samples. This suggests that the samples were less preferred by the panelists when they had higher overall intensities.

3.4. Functional properties

3.4.1. Protein solubility

Table 3 shows the pH-dependent protein solubility profiles of the unfermented and fermented PPI samples for both fermentation times.

Table 3
Protein solubility (%) of unfermented pea protein isolate (PPI) and PPI fermented for 24 h and 48 h with different microorganism strains.

		Protein Solubility (%)					
	Time	pH					
	(h)	3.0	4.5	5.0	6.0	7.0	8.0
PPI	0	36.3 ± 3.3	7.9 ± 1.6	7.1 ± 0.3	12.3 ± 1.0	43.4 ± 4.0	48.3 ± 2.8
L. plantarum	24	12.7 ± 1.6*	10.6 ± 1.2	11.9 ± 0.4*	14.7 ± 1.8	17.8 ± 1.5*	17.0 ± 2.4*
L. perolens		10.8 ± 2.6*	10.1 ± 1.2	10.6 ± 0.7*	14.4 ± 1.8	14.8 ± 2.5*	13.5 ± 2.4*
L. fermentum		12.4 ± 1.0*	10.4 ± 2.0	11.4 ± 1.2*	15.3 ± 2.1	16.8 ± 3.3*	16.1 ± 1.1*
L. casei		12.8 ± 1.9*	8.8 ± 1.1	11.6 ± 1.9*	12.8 ± 1.9	14.0 ± 2.0*	15.6 ± 1.2*
Lc. cremoris		13.4 ± 1.0*	11.5 ± 1.6*	12.7 ± 2.0*	15.3 ± 2.1	16.9 ± 1.9*	18.4 ± 1.7*
P. pentosaceus		11.2 ± 0.7*	11.3 ± 2.0*	11.5 ± 1.3*	13.6 ± 1.6	15.1 ± 1.4*	15.7 ± 1.5*
L. plantarum	48	12.6 ± 1.4*	10.8 ± 1.0	11.3 ± 1.4*	14.9 ± 0.8	15.0 ± 1.5*	16.8 ± 0.9*
L. perolens		11.4 ± 1.1*	9.8 ± 0.7	12.7 ± 0.9*	14.1 ± 2.0	15.4 ± 2.6*	14.8 ± 2.1*
L. fermentum		13.7 ± 0.6*	11.9 ± 0.7*	13.4 ± 1.2*	17.4 ± 3.3*	17.2 ± 2.4*	16.0 ± 1.0*
L. casei		11.4 ± 1.5*	11.8 ± 1.7*	11.9 ± 1.6*	13.0 ± 1.7	14.9 ± 1.8*	14.1 ± 1.7*
Lc. cremoris		12.3 ± 0.5*	10.9 ± 0.9	11.2 ± 1.2*	13.5 ± 1.3	14.9 ± 0.5*	16.7 ± 2.2*
P. pentosaceus		12.5 ± 0.2*	10.5 ± 0.7	12.0 ± 0.5*	11.3 ± 2.7	16.8 ± 2.2*	17.5 ± 2.6*

Results are expressed as means ± standard deviation (n = 4). Means marked with an asterisk (*) indicate significant differences between the individual sample and the unfermented pea protein isolate (PPI) (Tukey, $P < 0.05$).

The maximum protein solubility of unfermented PPI was shown at pH 8.0 (48.3), whereas the minimum protein solubility at pH 5.0 (7.1%). After 24-h and 48-h fermentation, all samples showed a significant improvement in protein solubility at pH 5.0 but a significant decrease at pH 3.0, 7.0, and 8.0. At pH 4.5, the protein solubility increased significantly after 24-h fermentation with *Lc. cremoris* and *P. pentosaceus* and after 48 h with *L. fermentum* and *L. casei*. Fermentation with *L. fermentum* for 48 h improved the protein solubility significantly at pH 6.0. The PPI fermented with *L. plantarum* for 24 h showed the highest protein solubility at pH 7.0 (17.8%), whereas fermentation with *Lc. cremoris* reached the highest protein solubility at pH 8.0 (18.4%). In contrast, after 48- h fermentation, the sample fermented with *L. fermentum* showed the highest protein solubility at pH 7.0 (17.2%), while the one with *P. pentosaceus* did at pH 8.0 (17.5%).

Fermentation of pea flour, soy and lupin protein isolates has shown similar effects on the protein solubility in other studies (Cabuk et al., 2018; Kumitch et al., 2020; Meinlschmidt et al., 2016b; Schlegel et al., 2019). The decline of protein solubility by fermentation might be related to different factors such as 1) changes in the protein surface, leading to exposure of hydrophobic groups and protein-protein interactions, 2) changes in the surface charge of the samples, and 3) increase in biomass due to microorganisms' growth. These factors might induce interactions and aggregation between proteins, microbial cells, lactic acid, and other compounds produced during fermentation and neutralization of the samples. In particular, the hydrophobicity of the LAB cell surfaces might play a role in the interaction with hydrophobic proteins and by-products leading to the precipitation of these agglomerates (Daeschel and McGuire, 1998; Marín et al., 1997).

3.4.2. Foaming capacity

The foaming capacity of proteins depends on different physicochemical characteristics such as surface tension and hydrophobicity, electrostatic repulsion, and molecular weight (Zayas, 1997). The unfermented and fermented PPI were unable to form foams. The lack of foam formation by the unfermented PPI might be attributed to the alkaline extraction method (Stone et al., 2015). In addition, the possible agglomeration between LAB cells, proteins, and by-products during fermentation could have reduced protein-air-water interactions preventing the formation of foams. To our knowledge, there are no studies regarding the functional properties of fermented PPI; however, studies on pea protein enriched-flour reported no effect or even a decrease in foaming capacity after fermentation (Cabuk et al., 2018; Kumitch et al., 2020).

3.4.3. Emulsifying capacity

The fermentation of the PPI significantly decreased the emulsifying capacity of the pea proteins. Unfermented PPI showed an emulsifying capacity of 548 mL/g ± 33. The fermented samples with the highest emulsifying capacity were those fermented with *L. plantarum* for 24 h and 48 h with 370 mL/g ± 62 and 385 mL/g ± 24, respectively. The PPI fermented with *L. perolens* showed the lowest emulsifying capacity with 204 mL/g ± 27 and 180 mL/g ± 4 after 24 h and 48 h, respectively. Samples fermented with *L. fermentum* and *P. pentosaceus* showed emulsifying capacities of 320 mL/g ± 17 and 348 mL/g ± 11 after 24 h, respectively, and 275 mL/g ± 19 and 322 mL/g ± 18 after 48-h fermentation. Fermentation with *L. casei* for 48 h increased the emulsifying capacity significantly (300 mL/g ± 4) compared to the 24-h sample (219 mL/g ± 19). *Lc. cremoris* fermented samples showed emulsifying capacities of 290 mL/g ± 21 and 310 mL/g ± 11 after 24-h and 48-h fermentation, respectively. Other authors reported a reduction in emulsifying capacity with longer fermentation times of different legume preparations (Cabuk et al., 2018; Kumitch et al., 2020; Meinlschmidt et al., 2016b; Schlegel et al., 2019). A positive correlation (0.78, $P < 0.05$) was found between the protein solubility (pH 7.0) and the emulsifying capacity, thus, low emulsifying capacities might be attributed to the agglomeration of the proteins and the interaction of by-products. These

agglomerates could prevent the hydrophobic interactions between protein and oil molecules and reduce the amphiphilic character of the proteins.

3.5. Proteolysis of PPI

Fig. 3 shows the electrophoretic results of the unfermented and fermented PPI. The protein fractions of the unfermented PPI ranged from 91 to 6.5 kDa for both conditions (reduced and non-reduced). Sanchez-Monge et al. (2004) identified fractions of 67 kDa (convicilin, Pis s 2), and 47 kDa (mature vicilin, Pis s 1) as the main allergens; in addition, they found that the 32 kDa proteolytic fraction (αβ) from the mature vicilin was also a major allergen. In the present study, these fractions were found in both the unfermented and fermented samples.

Protein fractions of the fermented samples ranged from 70 to 6.5 kDa and 90–6.5 kDa under non-reducing and reducing conditions, respectively. Vicilin and convicilin fractions lack disulfide bonds; thus, allergen fractions were expected to remain in the PPI solutions under both conditions. However, mature vicilin can undergo post-translational cleavage resulting in different fragments, one of them being the major allergen at 32 kDa, which can be further cleaved (Tzitzikas et al., 2006). Protein volume intensities of each allergenic protein fraction as detected by the Image Lab Software are shown in Table 4. The unfermented PPI under non-reducing conditions showed protein volume intensities of 303, 320, and 142 for Pis s 2, Pis s 1, and Pis s 1 αβ, respectively. Under reducing conditions, the unfermented PPI showed intensities of 272, 313, and 98 for Pis s 2, Pis s 1, and Pis s 1 αβ, respectively.

Effect of fermentation on Pis s 2 protein fraction. Under non-reducing conditions, fermentation significantly reduced the protein band intensity of Pis s 2 after 24 h and 48 h compared to the PPI. After 24-h fermentation, isolates fermented with *Lc. cremoris* showed the lowest intensity under non-reducing conditions, whereas the ones with *L. plantarum* showed the highest. However, under reducing conditions, only PPI fermented with *L. fermentum* for 48 h showed a significant reduction in intensity. Under reducing conditions, fermentation for 24 h with *L. perolens* showed the lowest protein band intensity, whereas *P. pentosaceus* showed the highest. Longer fermentation (48 h) with *L. fermentum* showed a further reduction of this protein fraction intensity in both conditions. In contrast, fermentation for 48 h with *L. plantarum*, *L. perolens,* and *L. casei* showed an increase in intensity of this fraction under non-reducing conditions.

Effect of fermentation on Pis s 1 protein fraction. Pis s 1 mature vicilin and its proteolytic fraction showed a reduced intensity after fermentation with the different LAB. Under non-reducing conditions, all fermented samples showed a significant reduction in protein band intensities at both fermentation times. After 24 h fermentation, the highest reduction in mature vicilin was achieved by *L. perolens* under both conditions. However, after fermentation for 48 h, PPI fermented with *L. fermentum* showed the lowest protein band intensities under both conditions. Regarding the proteolytic fraction (αβ) of Pis s 1, the lowest

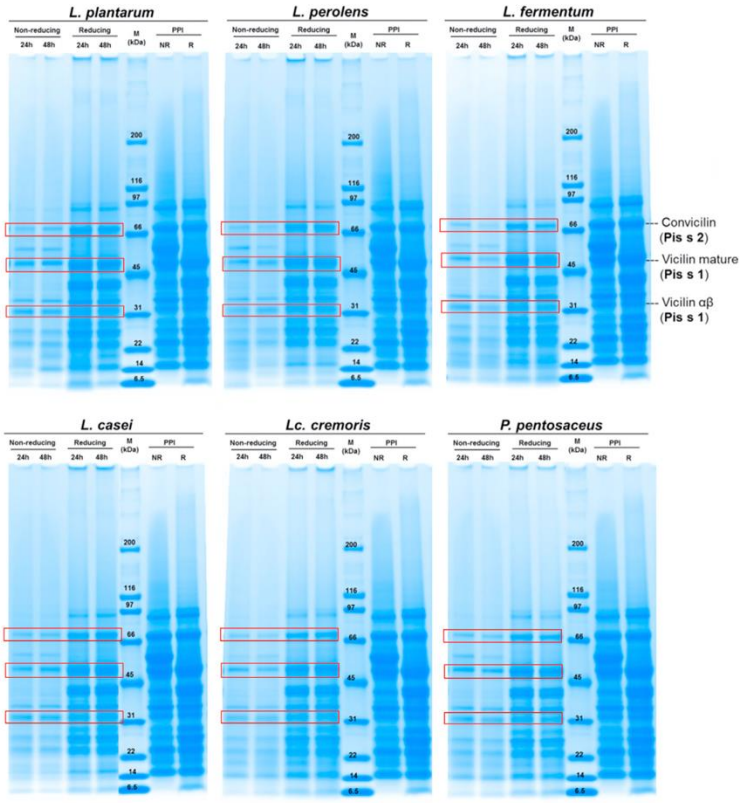

Fig. 3. Molecular weight distribution of the unfermented pea protein isolate (PPI) and PPI fermented for 24 h and 48 h with different microorganism strains by SDS-PAGE under non-reducing (NR) and reducing (R) conditions. M = molecular weight standard indicated in kilo Dalton (kDa).

Table 4
Protein band volume intensities of the main pea allergens of the unfermented pea protein isolate (PPI) and PPI fermented for 24 h and 48 h analyzed by SDS-PAGE under non-reducing and reducing conditions.

		Volume (Int)		
	Time	Pis s2	Pis s1	Pis s1 (αβ)
A) NON-REDUCING	(h)	(~70 kDa)	(~50 kDa)	(~32 kDa)
PPI	0	303 ± 70	320 ± 72	142 ± 27
L. plantarum	24	76 ± 45*	158 ± 40*	115 ± 26
L. perolens		40 ± 14*	96 ± 2*	68 ± 8
L. fermentum		47 ± 14*	108 ± 32*	86 ± 42
L. casei		61 ± 10*	142 ± 29*	134 ± 10
Lc. cremoris		39 ± 6*	99 ± 7*	84 ± 17
P. pentosaceus		55 ± 12*	139 ± 13*	79 ± 36
L. plantarum	48	90 ± 32*	151 ± 11*	105 ± 40
L. perolens		64 ± 10*	127 ± 4*	85 ± 28
L. fermentum		35 ± 22*	75 ± 16*	62 ± 28
L. casei		71 ± 7*	146 ± 10*	143 ± 13
Lc. cremoris		39 ± 6*	97 ± 6*	74 ± 22
P. pentosaceus		52 ± 3*	123 ± 14*	81 ± 24
		Volume (Int)		
	Time	Pis s2	Pis s1	Pis s1 (αβ)
B) REDUCING	(h)	(~70 kDa)	(~50 kDa)	(~32 kDa)
PPI	0	272 ± 35	313 ± 79	98 ± 9
L. plantarum	24	159 ± 15	228 ± 14	55 ± 14
L. perolens		148 ± 5	224 ± 26	56 ± 6
L. fermentum		161 ± 36	279 ± 24	60 ± 1
L. casei		158 ± 29	259 ± 2	73 ± 4
Lc. cremoris		160 ± 31	276 ± 18	80 ± 3
P. pentosaceus		178 ± 39	275 ± 20	76 ± 1
L. plantarum	48	146 ± 9	233 ± 40	60 ± 24
L. perolens		129 ± 13	223 ± 14	66 ± 14
L. fermentum		131 ± 58*	221 ± 24	32 ± 1
L. casei		163 ± 5	260 ± 13	73 ± 11
Lc. cremoris		158 ± 40	273 ± 15	61 ± 1
P. pentosaceus		165 ± 43	267 ± 2	70 ± 2

Results are expressed as means ± standard deviation (n = 2). Means marked with an asterisk (*) indicate significant differences between the individual fermented sample and the unfermented pea protein isolate (PPI) (Tukey, $P < 0.05$).

protein band intensities were shown by PPI fermented with *L. perolens* under non-reducing and with *L. plantarum* under reducing conditions after 24 h. On the other hand, *L. fermentum* showed the lowest intensity after 48 h fermentation under both conditions.

Under both conditions, the allergen fractions of fermented samples were less intense than of the unfermented PPI, especially under non-reducing conditions. However, changes in the intensity of the protein fractions might be attributed to low protein solubility of the fermented sample and not to a high proteolytic effect during fermentation. Pearson correlations were calculated to support the latter assumption, where strong correlations (>0.80, $P < 0.05$) were found between the protein solubility at pH 7.0 and the protein band intensities of Pis s 2 (both conditions) and Pis s 1 (non-reducing). The difference in protein band intensities might be attributed to each microorganism and their 1) release of proteolytic enzymes, 2) production of biomass, and 3) specificity for the substrate. Some authors have investigated the effect of fermentation on allergens from different plant substrates, and they have found a reduction in immunogenicity with different microorganisms (Barkholt et al., 1998; Licandro et al., 2020; Meinlschmidt et al., 2016b). Further immunological analyses such as Western-Blot or ELISA are necessary to understand the effect of the lactic acid fermentation on main pea allergen fractions.

Effect of fermentation on the degree of hydrolysis. The proteolytic activity was also measured by means of the total amount of hydrolyzed peptide bonds. The degree of hydrolysis (DH) started with 1.73% for the unfermented PPI (Table 5). The DH of the fermented samples ranged between 1.70 -3.02% after 24 h fermentation and between 2.19-3.75% after 48 h fermentation. A significant increase was observed after 24-h fermentation with *L. perolens*, *L. fermentum*, *L. casei*, and *P. pentosaceus*; furthermore, after fermentation for 48 h, all microorganisms showed a significant increase in the DH. The lowest DH was shown after fermentation with *Lc. cremoris* (1.70%, 2.68%) and *L. plantarum* (1.74%, 2.19%) after 24 h and 48 h fermentation. Fermentation with *L. perolens* showed the highest DH and increased significantly after 24 h (3.02%) and 48 h (3.75%). Although fermented samples with the lowest and highest DH are consistent with the growth of viable total cell counts, statistical correlations were not found. Compared to the functional properties and SDS-PAGE results, higher DH were expected. However, the overall low DH might be attributed to the raw material, the strains of each microorganism, and the determination method. The agglomeration of the proteins or interactions of the by-products and the OPA reagent might have concealed the primary amino groups affecting the measurement.

Table 5
Degree of hydrolysis of unfermented pea protein isolate (PPI) and PPI fermented for 24 h and 48 h.

Sample	Degree of Hydrolysis (%)		
	Fermentation Time (h)		
	0	24	48
L. plantarum	1.73 ± 0.05^{a}	1.74 ± 0.22^{a}	2.19 ± 0.16^{b}
L. perolens	1.73 ± 0.05^{a}	3.02 ± 0.18^{b}	3.75 ± 0.18^{c}
L. fermentum	1.73 ± 0.05^{a}	2.43 ± 0.27^{b}	3.21 ± 0.15^{c}
L. casei	1.73 ± 0.05^{a}	2.61 ± 0.25^{b}	2.78 ± 0.10^{b}
Lc. cremoris	1.73 ± 0.05^{a}	1.70 ± 0.06^{a}	2.68 ± 0.12^{b}
P. pentosaceus	1.73 ± 0.05^{a}	2.02 ± 0.13^{b}	2.80 ± 0.08^{c}

Results are expressed as means ± standard deviation (n = 4). Means marked with different letters within one row indicate significant differences (Tukey, $P < 0.05$).

3.6. General remarks

Despite the low growth rate and low degree of hydrolysis, significant changes were found in the production of lactic acid, the functional properties and the electrophoretic results. On the one hand, temperature treatments used during processing, such as pasteurization, during fermentation and inactivation might have affected the protein structure of the proteins. The decrease of pH during fermentation could also have contributed to a partial denaturation of the proteins. Either by temperature or by pH, the unfolding of proteins exposes hydrophobic regions, which causes an increase in protein-protein interactions and the formation of aggregates. On the other hand, neutralizing the fermented samples with NaOH might have resulted in the formation of sodium lactate. This compound is high-soluble (Yen et al., 2010), and it might have competed for interaction with water molecules before potential soluble proteins increased their net charge. Moreover, these interactions might lead to agglomeration and thus, reduced protein solubility and in consequence reduced emulsifying and foaming capacities. Certain degrees of aggregation are known to improve emulsion and foaming capacities (Peng et al., 2016). However, a higher number of aggregates might conceal the hydrophobic moieties from interaction with oil and air, respectively, hence hindering an optimal orientation of the proteins towards oil- and air-interfaces and reducing emulsifying and foam capacities. Limited hydrolysis through reduced fermentation times might improve functional properties by a lower degree of denaturation and fewer interactions between proteins, metabolites and LAB cell surfaces, which would allow the smaller peptides to interact with the solvent and oil-water and air-water interfaces.

4. Conclusion

The present study investigated the effects of fermentation with six different LAB on the sensory profile, functional properties, and changes in the molecular weight distribution as well as in the allergenic protein fractions of PPI. Overall, fermentation of PPI reduced aroma attributes

that characterize PPI, such as pea-like, green, and earthy. The aroma properties of the fermented samples depended mainly on the LAB used, their specific metabolism and the associated release of acids and other metabolites. Similarly, changes in the bitterness of the samples depended on the microorganisms, suggesting that some LAB might have higher activity against bitter peptides. PPI fermentation for 24 h resulted in higher acceptance compared to the 48-h fermented samples, which suggest that longer times of fermentation might induce the production of further compounds that are no longer attractive for consumers. Regarding the aroma profile, fermentation of PPI with *L. plantarum* for 24 h achieved the most neutral retronasal aroma, low bitter taste, lowest overall intensity, and highest preference among all fermented samples.

The fermentation of PPI significantly decreased the functional properties. These results might be attributed to an agglomeration of the proteins and their interaction with by-products released during fermentation. Regarding the effects on the allergenic protein fractions and molecular weight distribution, the samples need to be further investigated by immunological in vitro and in vivo assays to be able to draw a more precise conclusion about the reduction of the allergenic potential of the modified pea protein isolates.

This study aimed to investigate whether fermentation is a suitable method to improve the sensory profile and functional properties of pea protein isolates to be used as food ingredients. Unfortunately, the selected microorganisms and fermentation times were not suitable for producing good-tasting and highly functional ingredients. Shorter fermentation times and other microorganisms should be additionally investigated. Furthermore, other methods, such as enzymatic hydrolysis before or after fermentation, might be worth investigating.

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CRediT authorship contribution statement

Verónica García Arteaga: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Sophia Leffler:** Investigation. **Isabel Muranyi:** Methodology, Writing - review & editing, Supervision. **Peter Eisner:** Resources, Writing - review & editing, Supervision. **Ute Schweiggert-Weisz:** Resources, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 3

Enzymatic Hydrolysis and Fermentation of Pea Protein Isolate and Its Effects on Antigenic Proteins, Functional Properties, and Sensory Profile[3]

Abstract

To include protein ingredients in food products, it is necessary to consider not only their functionality but also their sensory profile. According to the result of previous chapters, three enzymes (papain, Esperase®, trypsin) were selected for further research in combination with one lactic acid bacteria (*Lactobacillus plantarum*). The combination of treatments was performed in two different order to assess changes in the enzyme activity or LAB metabolism affecting functionality, sensory profile and immunogenicity. Fermentation followed by enzymatic hydrolysis showed the most promising results regarding all evaluated aspects. Lower molecular weight fractions were found through SDS-PAGE and gel filtration and indirect ELISA showed a reduction of immunogenicity. All treated samples showed a significant improvement in protein solubility and foaming capacity, whereas emulsifying capacity was either not affected or slightly impaired. The combined methods resulted in proteins with lower pea off-flavors. These results suggest that the combination of methods to treat pea proteins might be an effective approach to produce pea protein ingredients with improved functionality and more neutral taste. Furthermore, the results indicate a decrease in allergenic reactions after consumption.

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Enzymatic Hydrolysis and Fermentation of Pea Protein Isolate and Its Effects on Antigenic Proteins, Functional Properties, and Sensory Profile

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Abstract: Combinations of enzymatic hydrolysis using different proteolytic enzymes (papain, Esperase®, trypsin) and lactic fermentation with *Lactobacillus plantarum* were used to alter potential pea allergens, the functional properties and sensory profile of pea protein isolate (PPI). The order in which the treatments were performed had a major impact on the changes in the properties of the pea protein isolate; the highest changes were seen with the combination of fermentation followed by enzymatic hydrolysis. SDS-PAGE, gel filtration, and ELISA results showed changes in the protein molecular weight and a reduced immunogenicity of treated samples. Treated samples showed significantly increased protein solubility at pH 4.5 (31.19–66.55%) and at pH 7.0 (47.37–74.95%), compared to the untreated PPI (6.98% and 40.26%, respectively). The foaming capacity was significantly increased (1190–2575%) compared to the untreated PPI (840%). The treated PPI showed reduced pea characteristic off-flavors, where only the treatment with Esperase® significantly increased the bitterness. The results from this study suggest that the combination of enzymatic hydrolysis and lactic fermentation is a promising method to be used in the food industry to produce pea protein ingredients with higher functionality and a highly neutral taste. A reduced detection signal of polyclonal rabbit anti-pea-antibodies against the processed protein preparations in ELISA furthermore might indicate a decreased immunological reaction after consumption.

Keywords: pea protein isolate; lactic fermentation; *Lactobacillus plantarum*; enzymatic hydrolysis; functional properties; protein solubility; pea allergens; sensory properties; bitterness

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1. Introduction

Peas (*Pisum sativum* L.) are increasingly used due to their sustainable production [1], economic benefits [2], high protein content (15–30%), and alleged low allergenicity. They belong to the legume family (Fabaceae) and their proteins are classified as salt-, water-, and ethanol soluble, corresponding to globulins, albumins, and prolamins, respectively [3].

Peas are not on the list of main allergens and do not need to be declared as allergenic in food products; however, two allergenic protein fractions from the storage proteins have been identified [4] and are recognized by the International Committee of Allergen Nomenclature as main pea globulin allergens. The allergen Pis s1 correspond to the mature vicilin (47–50 kDa) as well as to one of vicilin's proteolytic fractions (32 kDa). The Pis s2 correspond to convicilin (67–70 kDa). The ability of allergen proteins (antigen) to cause

an immune response (immunogenicity) depends on different factors, such as the antigen dose, exposure, and host genetic background [5], and thus, their ability to cause allergic reactions [6]. Moreover, pea allergens have shown homology between epitopes (recognition sites) from other legume allergens [7] and serological cross-reactivity has been proved [4,8,9]. Different methods to modify food allergens and their impact on food allergenicity have been reviewed [10].

Enzymatic hydrolysis is one of the most common methods used for this purpose and has been proven effective in allergen degradation of different legumes [11–13]. Modification of pea allergens by enzymatic treatment has been studied to a lesser extent. Pea protein isolate (PPI) treated with trypsin [14], Alcalase [15], flavourzyme, papain, and pepsin [16] have shown a reduced immunogenicity by means of ELISA methods. Frączek, Kostyra [14] found that a higher degree of hydrolysis resulted in a higher reduction in immunogenic potential. Moreover, changes in the molecular weight distribution of proteins are also known to affect functional and sensory properties. Partial hydrolysis was shown to increase protein solubility and emulsifying capacity; however, further hydrolysis reduced both [17–19]. Depending on the composition, the low molecular weight peptides formed during enzymatic hydrolysis can promote a bitter taste. The mechanism is not yet fully understood but mainly hydrophobic amino acid residues appear responsible [20].

For debittering of protein hydrolysates, fermentation has been widely studied [21–25]. Lactic acid bacteria reduced the bitterness of hydrolysates by releasing aminopeptidases cleaving hydrophobic amino acid residues [23]. There are several studies focusing on changes in the aroma profile of fermented pea, pea proteins, and pea products [26–28]; however, to our knowledge, there are no studies focusing on the debittering of pea protein hydrolysates by lactic fermentation.

The effects of fermentation on the functional properties have been studied for different legumes [21,29,30], and, to a lesser extent, for peas [31–33]. Moreover and to our knowledge, only one study has investigated the effects of fermentation on the antigenicity of pea flour [34].

The combination of enzymatic hydrolysis and microbial fermentation seems very promising for the production of low-allergenic and tasty functional food ingredients. A combination has been investigated for soy [22] and lupin protein isolate [35,36], but not yet for pea. For this reason, this study aimed to investigate the effects of combining enzymatic hydrolysis and fermentation on allergenic proteins (Pis s1 and Pis s2), as measured by SDS-PAGE and the ability of polyclonal sera to recognize antigens, functional properties and on the debittering and characteristic off-flavors of pea proteins. According to previous findings, papain, Esperase®, trypsin and *Lactobacillus plantarum* were selected for enzymatic hydrolysis [18] and fermentation [33], respectively. The specificity of an enzyme is determined by the arrangement of amino acids within the active site and the structure of the substrates. The acidification during fermentation could cause protein aggregation hiding protein parts from binding with the enzyme active site. Therefore, two sequences of the reactions, enzymatic treatment and fermentation, were investigated as the order of the method combination might be relevant for changes in the molecular weight distribution of the hydrolysates, functional properties and taste. Moreover, the treatments and the order of the method combination might also change the epitope binding sites and thus, the immunogenicity of pea allergenic proteins.

2. Materials and Methods

2.1. Materials

Pea seeds (*Pisum sativum* L., cultivar Navarro) were provided by Norddeutsche Pflanzenzucht Hans-Georg-Lembke KG (Holtsee, Germany). Trypsin and Esperase® 8.0 L were obtained from Sigma-Aldrich (Munich, Germany). Papain was from Carl Roth GmbH (Karlsruhe, Germany). *L. plantarum* (DSM 20174) was purchased from the German collection of microorganisms and cell cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). Broad Range™ Unstained Protein Standard, 4–20% Criterion™

TGX stain-free™ precast polyacrylamide gels, Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories GmbH (Feldkirchen, Germany). Sodium dihydrogen phosphate, sodium dodecyl sulfate, sodium tetraborate decahydrate, o-phthaldialdehyde, and sodium monohydrogen phosphate were purchased from Sigma-Aldrich (Munich, Germany). All chemicals used in this study were of analytical grade unless otherwise indicated.

2.2. Production of Pea Protein Isolate

Pea flour was prepared by dehulling, splitting and impact-milling pea seeds as described by García Arteaga, Leffler [33]. The pea protein isolation was performed according to García Arteaga, Apéstegui Guardia [18]. Briefly, an alkaline protein extract (pH 8.0) was adjusted to pH 4.5 for protein isoelectric precipitation. The precipitated proteins were neutralized, pasteurized (70 ± 2 °C) for 2 min and spray-dried.

2.3. Pea Protein Isolate Modification

The PPI was treated by enzymatic treatment, microbial fermentation or a combination of both. Table 1 shows the specific conditions for the enzyme preparations and microbial strain. The combination experiments were carried out as follows: enzymatic hydrolysis with the individual enzyme preparations followed by fermentation (HyF), and fermentation followed by hydrolysis (FdH), and are presented in Table 2. A 9% (*w*/*w*) PPI dispersion in DI water was homogenized using an Ultra-Turrax (IKA® Werke GmbH & Co KG, Staufen, Germany) for 90 s at 11,000 rpm and pasteurized at 80 °C for 10 min. The pH and temperature were adjusted to the optimal conditions (Table 1) prior to the addition of enzymes or of *L. plantarum* in each treatment. The pH was adjusted using 3.0 mol/L hydrochloric acid or 3.0 mol/L sodium hydroxide. Inactivation of enzymes or microorganisms was performed at 90 °C for 10 min before proceeding to the next treatment or finalizing the experiment. The denatured enzyme and the inactivated microbial cells were not removed from the samples. The final samples were neutralized (pH 7.0) at room temperature, lyophilized and grinded for 10 s at 7500 rpm (Grindomix GM200, Retsch GmbH, Haan, Germany). An untreated PPI dispersion was used as reference. Samples of each treatment were prepared in duplicate.

Table 1. Optimal conditions of commercial enzyme preparations and microorganism.

Enzyme/Microorganism	Amount	Temp. (°C)	pH Value (-)	Activity	Origin
Papain	0.1% E/S	65	7	Cysteine Endoprotease	Papaya latex
Esperase® 8.0 L	0.5% E/S	65	8	Serine Endoprotease	*Bacillus* sp.
Trypsin	0.1% E/S	50	8	Serine Endoprotease	Bovine pancreas
Lactobacillus plantarum	7 Log CFU/mL	30	6.5	Anaerobe	Pickled cabbage

E/S: enzyme/substrate ratio; Temp: temperature.

Table 2. Treatment sample code.

Sample Code	Treatment 1	Treatment 2
Untreated PPI	-	-
Fermented PPI	*L. plantarum*	-
P_Hy	Papain	-
P_HyF	Papain	*L. plantarum*
P_FdH	*L. plantarum*	Papain
E_Hy	Esperase®	-
E_HyF	Esperase®	*L. plantarum*
E_FdH	*L. plantarum*	Esperase®
T_Hy	Trypsin	-
T_HyF	Trypsin	*L. plantarum*
T_FdH	*L. plantarum*	Trypsin

2.3.1. Fermentation

Growth and Culture Conditions

To optimally cultivate *L. plantarum* strains, a late exponential growth phase was chosen. Briefly, a 200-µL aliquot of *L. plantarum* in MRS (De Man, Rogosa, and Sharpe) covered with 50 µL sterile paraffin oil was incubated using a microplate reader (Synergy HTX, BioTek Instruments GmbH, Waldbronn, Germany). The OD was measured every 15 min at a wavelength of 600 nm. The exponential phase lasted approximately from the 11 h until the 24 h since beginning of fermentation; thus, a late exponential phase was selected at 18 h to obtain inocula of *L. plantarum*.

Determination of Viable Cell Counts for Inoculum and after Fermentation

The *L. plantarum* was incubated in MRS-broth for 18 h at 30 °C under anaerobic conditions. Serial dilutions were used for the determination of viable bacteria cell and OD measurements to select the OD corresponding to a viable cell count of a 7-log colony forming units per milliliter per sample (CFU/mL). The OD 0.1 was used as reference for liquid cultured aliquots before each fermentation. The log CFU/mL of fermented samples were determined at the beginning and the end of the fermentation on MRS agar from 100 µL of diluted sample.

Fermentation of PPI Dispersions

The pasteurized PPI dispersions or inactivated PPI hydrolysates were transferred into sterile 2-L Schott flasks. Prior to inoculation, the solutions were adjusted to pH 6.5 and cooled down to 30 °C before 0.5% (*w*/*v*) glucose was added. The aliquot taken for CFU determination represented the initial viable cell number t = 0 h after 10 min inoculation. The flasks were flushed with nitrogen to achieve anaerobic conditions and the fermentation was carried out for 24 h without stirring. The pH was assessed after 24 h. After inactivation, the HyF samples were cooled to room temperature, neutralized, and lyophilized. For the FdH samples, the inactivated fermented solutions were adjusted to the optimal conditions of each enzyme.

2.3.2. Enzymatic Hydrolysis

The pasteurized PPI dispersions or inactivated fermented PPI were transferred to thermostatically controlled stainless-steel reactors and the optimal conditions for each enzyme were set. The enzyme to substrate ratio was calculated based on the protein content. The hydrolysis was carried out for 2 h with constant stirring (80 rpm) using an agitator (R50-20D, Phoenix Instruments GmbH, Garbsen, Germany) and maintaining optimal conditions. After inactivation, the HyF samples were cooled and adjusted to the optimal conditions for fermentation. The FdH samples were cooled to room temperature, neutralized, and lyophilized. The sample codes are shown in Table 2.

2.4. Chemical Composition

The dry matter content (105 °C), ash content (950 °C) and protein content (N × 6.25) were analyzed according to AOAC Official Methods [37,38] by means of a thermogravimetric method (TGA 701, Leco Instruments, Germany) and the Dumas combustion method (TruMac N, Leco Instruments, Mönchengladbach, Germany), respectively.

2.5. Determination of Protein Degradation

2.5.1. Molecular Weight Distribution

The molecular weight distribution was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [39] with slight modifications and as described in detail in García Arteaga, Apéstegui Guardia [18]. Briefly, 5 µg/µL protein solution (based on dry matter) was prepared in 1× reducing buffer (50% (*v*/*v*) 2× Tris-HCl reducing buffer, 50% (*v*/*v*) phosphate buffer (pH 7)). The samples were heated (95 °C, 5 min) prior to centrifugation at 12,045× *g* for 3 min (MiniSpin, Eppendorf AG,

Hamburg, Germany). An aliquot of 3 µL of the supernatants was added into the gel pocket of the Bio-Rad 4–20% Criterion™ TGX Stain-Free™ Precast Gels. The Broad Range™ Unstained Protein Standard was used as the molecular weight marker. The running time was 30 min, followed by staining using Coomassie Brilliant Blue R-250. Finally, gel images were obtained using an EZ Imager (Gel Doc™ EZ Imager, Bio-Rad Laboratories, Feldkirchen, Germany). SDS-PAGE was performed in duplicate, with each sample being prepared two times independently.

2.5.2. Degree of Hydrolysis

The degree of hydrolysis (DH) was determined according to Nielsen, Petersen [40] using o-phthaldialdehyde (OPA). The DH was calculated based on the total number of peptide bonds per protein equivalent (h_{tot}). The constant values used for α (degree of dissociation of the α-amino group), β (slope of calibration through linear regression) and htot factor were 1.0, 4.0, and 8.0, respectively, according to theoretical general values for unexamined raw material [40]. The DH was calculated according to the following equations:

$$Serine\text{-}NH_2 = \frac{Abs_{sample} - Abs_{blank}}{Abs_{standard} - Abs_{blank}} \times 0.951 \frac{meqv}{L} \times \frac{V_{sample} \times 100}{m_{sample} \times PC_{sample}}$$

$Serine\text{-}NH_2$ = meqv $serine\text{-}NH_2$/g protein;
Abs_{sample} = sample absorbance value;
Abs_{blank} = blank absorbance value;
$Abs_{standard}$ = standard absorbance value;
V_{sample} = volume of sample solution (L);
m_{sample} = weight of sample (g);
PC_{sample} = protein content of sample (%);

$$h = \frac{Serine\text{-}NH_2 - \beta}{\alpha}$$

$Serine\text{-}NH_2$ = meqv $serine\text{-}NH_2$/g protein;
h = number of hydrolyzed peptide bonds;
β = slope of calibration through linear regression;
α = degree of dissociation of the α-amino group;

$$DH = \frac{h}{h_{tot}} \times 100$$

DH = degree of hydrolysis (%)
h = number of hydrolyzed peptide bonds;
h_{tot} = total number of peptide bonds per protein equivalent.

The sample preparation was performed in duplicate and each prepared sample was measured in triplicate.

2.5.3. Gel Filtration Chromatography

Two grams of untreated and treated samples were solubilized in 2 mL of 50 mM Tris-HCl and 100 mM KCl, pH 7.5. Samples were centrifuged in an Eppendorf centrifuge 5424 R at 20,000× g. Supernatant (1.6 mL) was applied to Superdex 200 gel filtration column (26/600, GE Healthcare; 60 cm × 26 mm) using ÄKTA avant System. The sample was processed at a flow rate of 2 mL/min in 50 mM Tris-HCl and 100 mM KCl, pH 7.5. Peak eluate fractioning was used to collect the eluate in 2.5-mL fractions. Elution was monitored at 280 nm. On average 74 fractions were collected.

2.5.4. Generation of Polyclonal Rabbit Sera

The immunization of three rabbits ("Continental Giant") with a suspension of the untreated PPI powder was performed by a certified external supplier (Seramun Diagnostica GmbH, Heidesee, Germany). Three rabbits are required to obtain a complete coverage of all proteins. A basic immunization with 1 mg and Complete Freund's Adjuvant was followed by one booster injection on day 21 using 0.5 mg in combination with Incomplete Freund's Adjuvant. The serum was recovered 7 days after the booster injection. Final sera showed at >1,000,000 dilution >5× binding to PPI compared to the pre-immune serum.

2.5.5. Immunogenicity Measured by Enzyme-Linked Immunosorbent Assay (ELISA)

Purified sample fractions were measured in duplicate by indirect ELISA. MaxiSorp 96-well immuno plates (Life Technologies) were coated by adding 100 µL of gel filtration fractions to each well. The plates were incubated at 4 °C for 20 h. The wells were emptied and 100 µL 5% NFDM (blocking buffer) in PBS was added to each well. The plates were incubated for 1 h at 4 °C. After 3× washing with 0.1% Tween/PBS, the plates were incubated with the rabbit sera immunized with PPI (1:2000 in blocking buffer) at 4 °C for 1 h. Another washing step with 0.1% Tween/PBS was performed. Moreover, 100 µL/well the detection antibody (Goat-anti-rabbit IgG, Dianova 111-035-003, 1:5000 in blocking buffer) was added and incubated at 4 °C for 1 h. The plate was washed twice with 0.1% Tween/PBS and once with PBS. The color reaction was developed by the addition of 100 mL of TMB Microwell Substrate System (BioLegend) to each well and incubation at room temperature for 5 min. The reaction was stopped by the addition of 50 µL of 20% H_2SO_4 to each well. The color developed was measured at optical density (OD) 450 nm using a TECAN Infinite® M1000 microtiter plate reader. Background for the intensity calculation were wells coated with blocking buffer only.

2.6. Functional Properties

All functional experiments were performed in duplicate.

2.6.1. Protein Solubility

The protein solubility was performed according to Morr, German [41] at pH 4.5 and 7.0. The soluble protein was determined using the Biuret method (550 nm), according to the AACC Approved Methods of Analysis [42], using bovine serum albumin (BSA) as standard.

2.6.2. Emulsifying Capacity

The emulsifying capacity was determined according to Wang and Johnson [43] using an 1 L-reactor equipped with a stirrer and an Ultra-Turrax (IKA-Werke GmbH and Co. KG, Staufen, Germany). Mazola corn oil was added gradually (10 mL/min) to 1% (w/w) neutralized sample dispersions until a phase inversion occurred (<10 µS/cm). The volume of added oil was used to calculate the emulsifying capacity (mL oil/g sample).

$$EC = \frac{V_{oil}}{m_{sample}}$$

EC = emulsifying capacity (mL/g);
V_{oil} = volume of oil used until phase inversion (mL);
m_{sample} = weight of sample (g).

2.6.3. Foaming Properties

The foaming capacity and foam stability were analyzed according to Phillips, Haque [44] using a whipping machine (Hobart N50, Hobart GmbH, Offenburg, Germany). Briefly, 5% (w/v) dispersions were adjusted to pH 7.0 and stirred for 15 min. The dispersions

were whipped (580 rpm) for 8 min and the foaming capacities determined as the relation between the initial and final volume.

$$FC = \frac{V_2}{V_1} \times 100$$

FC = foaming capacity (%);
V_1 = volume of sample solution before whipping (mL);
V_2 = volume of foam after whipping (mL).

2.7. Sensory Analysis

2.7.1. Sample Preparation

The sensory analysis was performed using the combined treated samples (HyF and FdH) and the PPI. Sample solutions (2%, w/w) were prepared with tap water and coded using three-digit random numbers.

2.7.2. Sample Evaluation

The sensory evaluation was conducted according to the ISO 8587:2006 Sensory analysis—Methodology—Ranking, which compares different products according to the intensity of a given characteristic or property. First, a ten-member panel ranked attributes regarding bitterness and plant-like (pea-like/green/beany) flavor. These attributes were evaluated on a 1 (attribute not perceivable) to 7 (very strong perception) ranging scales.

2.8. Statistical Analysis

Complete raw data of untreated PPI, treated PPI and controls (temperature treatment) can be found in Mendeley Data files [45]. All results are expressed as mean values ± standard deviations. The microbial growth results were analyzed using the two-sample t-test. Further results were analyzed by one-way analysis of variance (ANOVA). The mean values were compared using Tukey's post-hoc test. All statistical analyses, except those from the sensory analysis, were performed using OriginPro 2018b and were considered statistically significant at $p < 0.05$. A Friedman Test and Duncan Test as post-hoc test were used to analyze the results from the sensory analysis ($p < 0.10$). Ranking recording and statistical analyses of sensory data were carried out using RedJade software (RedJade Sensory Solutions, LLC, Martinez, CA, USA).

3. Results and Discussion

3.1. Microbial Growth

L. plantarum requires tryptophan, arginine, glutamate and branched-chain amino acids (isoleucine, leucine, valine) for growth [46]. Besides of tryptophan, PPI is a good source of all the required amino acids; thus, *L. plantarum* was able to grow both, in the PPI dispersion and hydrolyzed PPI (Table 3). However, fermentation of PPI hydrolysates resulted in significantly higher viable cell counts compared to the fermented PPI. This could be due to some release of amino acids and peptides during hydrolysis, which provide a readily available source of nutrients for *L. plantarum* growth. The hydrolysates showed slight differences in CFU after fermentation, with P_HyF showing the highest value of 9.53 Log CFU/mL followed by E_HyF and T_HyF with 9.30 Log CFU/mL and 9.17 Log CFU/mL, respectively. The pH was measured after 24 h of fermentation and was similar for all fermented samples (pH 4.5 ± 0.2). A recent study showed that lactic fermentation of hydrolyzed lupin protein isolate resulted in similar pH values regardless of the enzyme used [35].

Table 3. Colony forming units (CFU) after inoculation and 24 h of fermentation.

	Log CFU/mL	
	0 h	**24 h**
Fermented PPI	7.40 ± 0.10 [a]	8.89 ± 0.09 [b*]
P_HyF	7.41 ± 0.03 [a]	9.53 ± 0.45 [b]
E_HyF	7.37 ± 0.15 [a]	9.30 ± 0.01 [b]
T_HyF	7.39 ± 0.01 [a]	9.17 ± 0.03 [b]

Results are expressed as means ± standard deviation (n = 2). Means marked with different letters indicate significant differences between 0 h and 24 h within same row (two-sample *t*-test, $p < 0.05$). Means marked with an asterisk (*) indicate significant differences between fermented pea protein isolate (PPI) and fermented hydrolysates (HyF) within the same column (One-way ANOVA, Tukey, $p < 0.05$). P: papain; E: Esperase®; T: trypsin.

3.2. Chemical Composition

The untreated PPI showed a protein content of 84.7 ± 0.1% (Table 4). The average protein content of PPI hydrolysates (83.4 ± 1.4%) was significantly higher compared to fermented PPI (79.5 ± 0.3%) and to the average of the samples produced by the combination of both treatments (76.6 ± 1.3%). The differences in protein contents might be due partial metabolism of the proteins and increase in organic acids such as lactic acid and, in lesser extent, acetic acid [46]. In addition, the ash content could be attributed to the addition of inorganic acid (hydrochloric acid) and sodium hydroxide to adjust the pH for each sample conditions.

Table 4. Chemical composition of untreated and treated pea protein isolates.

Samples	Dry Matter (%)	Protein Content (%) *	Ash Content (%) *
Untreated PPI	96.6 ± 0.3 [a]	84.7 ± 0.1 [a]	5.2 ± 0.5 [a]
Fermented PPI	94.9 ± 0.6 [b]	79.5 ± 0.3 [b]	6.9 ± 0.1 [bde]
P_Hy	92.7 ± 0.7 [c]	84.9 ± 0.1 [a]	5.5 ± 0.1 [ac]
P_HyF	97.6 ± 0.1 [a]	78.3 ± 0.2 [c]	7.2 ± 0.5 [b]
P_FdH	96.7 ± 1.2 [a]	78.0 ± 0.2 [c]	5.9 ± 0.8 [cd]
E_Hy	94.8 ± 1.0 [b]	82.0 ± 0.8 [c]	6.3 ± 0.2 [b]
E_HyF	97.6 ± 0.2 [a]	74.9 ± 0.1 [d]	9.3 ± 0.2 [c]
E_FdH	96.6 ± 0.3 [a]	76.4 ± 0.3 [e]	8.4 ± 0.5 [c]
T_Hy	92.5 ± 0.9 [c]	83.3 ± 0.1 [c]	5.9 ± 0.5 [ab]
T_HyF	97.9 ± 0.5 [a]	75.8 ± 0.3 [d]	7.9 ± 1.1 [e]
T_FdH	96.1 ± 1.9 [a]	76.1 ± 0.2 [d]	7.8 ± 0.8 [e]

Results are expressed as means ± standard deviation (n = 4). Means marked with different letters within one column indicate significant differences between treated samples from each enzyme and the untreated pea protein isolate (PPI) and fermented PPI (Tukey, $p < 0.05$). P: papain; E: Esperase®; T: trypsin; Hy: hydrolysis; HyF: hydrolysis followed by fermentation; FdH: fermentation followed by hydrolysis. * based on the dry matter content.

3.3. Proteolysis of PPI

The SDS-PAGE and gel filtration were performed to observe the effects of the different treatments on the pea proteins. The molecular weight distribution of the untreated PPI and treated samples is shown in Figure 1 and the positions of the main allergens are marked. The untreated PPI showed protein fractions between 97.5 and 6.5 kDa. The fermented PPI did not show major changes in the electrophoretic pattern as previously shown by García Arteaga, Leffler [33] for six lactic fermentations. The enzymatic hydrolysis facilitated significant changes in the molecular weight distribution of the respective samples with an increase in smaller peptides. This was observed in the samples that were only enzymatically hydrolyzed as well as in the samples with combined methods. The protein pattern of the sample treated with papain (P_Hy) only showed bands smaller than 40 kDa—with the exception of one band around 69.1 kDa. This band was degraded by the subsequent fermentation (P_HyF) and only bands smaller than 27 kDa were found. The proteolysis

with Esperase® (E_Hy) and trypsin (T_Hy) resulted in protein fractions below 40 kDa and 34 kDa, respectively. Fermentation of these hydrolysates (E_HyF and T_HyF) did not change the molecular weight distribution, while hydrolysis after fermentation (FdH) resulted in further protein degradation with protein fractions smaller than 27 kDa.

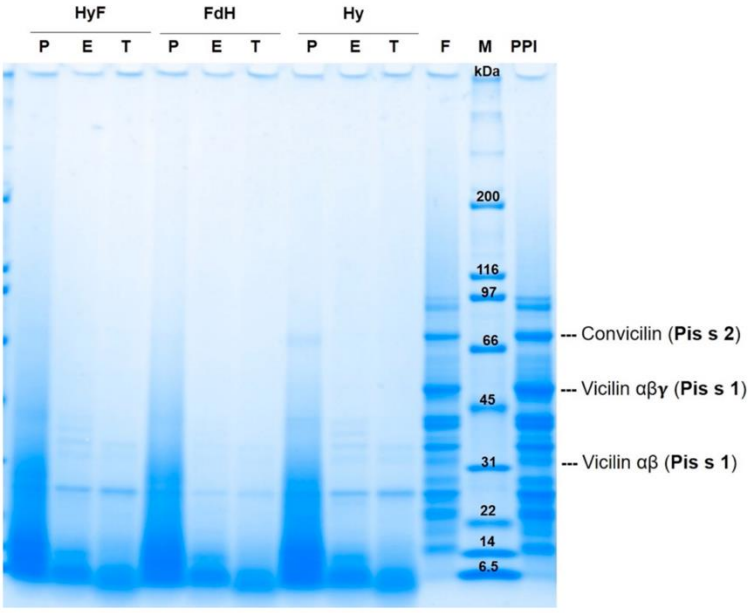


Figure 1. SDS-PAGE of pea protein isolate (PPI) and treated samples using *L. plantarum* and different enzymes and treatments. P: papain; E: Esperase®; T: trypsin; Hy: hydrolysis; F: fermented PPI; HyF: hydrolysis followed by fermentation; FdH: fermentation followed by hydrolysis; M: molecular weight standard, indicated in kilo Dalton (kDa).

Fermentation alone did not lead to large changes in the molecular weight distribution of the respective samples, probably due to the inability of *L. plantarum* to metabolize large polypeptides [47]. Enzymatic hydrolysis enhanced the degradation of large polypeptides into smaller peptides that can be easily metabolized by *L. plantarum* [47]. Furthermore, *L. plantarum* activates peptidases with higher specificity for hydrophobic dipeptides [46]. Proteolysis is known to release hydrophobic amino acids and peptides, which then can be digested by the lactic acid bacteria.

The samples that were first fermented and then enzymatically hydrolyzed showed protein fractions below 26 kDa. One explanation might be that due to the low pH, partial acid hydrolysis occurred during fermentation, and the enzymes then further broke down these hydrolyzed fractions.

3.3.1. Effect of Combined Methods on Pea Protein Allergens

A protein band at 63–80 kDa [4,18,48,49] could represent the Pis s2 allergen. In the present study, a protein band around 70.9 ± 0.9 kDa was found in the untreated PPI and with reduced intensity in the fermented PPI; this fraction could correspond to the Pis s2. The reduction could be due to a reduction in protein solubility (as explained later in Section 3.5.1) rather than to a proteolytic effect of fermentation with *L. plantarum*. Furthermore, P_Hy also showed this allergen fraction with a slightly lower intensity than the untreated PPI. This could explain that papain alone was not able to cleave this fraction.

Protein bands found around 50.1 ± 0.8 kDa and 31.91 ± 0.5 kDa could correspond to the Pis s1 of the mature vicilin (αβγ) and its proteolytic fraction (αβ), respectively. The Pis s1 αβγ was present in the untreated PPI and with less intensity in the fermented PPI. Its proteolytic fraction was present in the untreated PPI, fermented PPI, E_Hy, T_Hy, E_HyF, and T_HyF.

3.3.2. Effect of Combined Methods on the Degree of Hydrolysis

Both trypsin and Esperase® are serine endoproteases, with trypsin having specificity for basic residues, such as lysine and arginine derivatives [50] and Esperase® having a broader specificity, such as for both hydrophobic and hydrophilic residues [51]. The latter might explain the higher DH of all Esperase® treated samples (Table 5). Papain cleaves peptide bonds C-terminal of glycine and cysteine residues among others [52]. Glycine and cysteine residues might interfere with the OPA agent giving unstable and weak signals [40,53]. This effect might have been the reason why papain treated samples showed lower DH compared to other hydrolyzed samples even when the electrophoretic results showed significant changes.

Table 5. Degree of hydrolysis (%) of untreated and treated pea protein isolates.

Samples	DH [%]
Untreated PPI	1.88 ± 0.14 [a]
Fermented PPI	1.32 ± 0.05 [b]
P_Hy	3.73 ± 0.08 [c]
P_HyF	5.48 ± 0.16 [d]
P_FdH	3.92 ± 0.44 [c]
E_Hy	9.57 ± 0.46 [c]
E_HyF	10.76 ± 0.15 [d]
E_FdH	9.98 ± 0.37 [c]
T_Hy	6.86 ± 0.06 [c]
T_HyF	9.22 ± 0.20 [d]
T_FdH	9.26 ± 0.27 [d]

Results are expressed as means ± standard deviation (n = 4). Means marked with different letters within one column indicate significant differences treated samples from one enzyme and the untreated pea protein isolate (PPI) and the fermented PPI (Tukey, $p < 0.05$). P: papain; E: Esperase®; T: trypsin; Hy: hydrolysis; HyF: hydrolysis followed by fermentation; FdH: fermentation followed by hydrolysis.

Furthermore, the combination of fermentation after enzymatic hydrolysis significantly increased the DH value compared to the untreated PPI, the fermented PPI and the enzymatic treated sample. This could be related to the aforementioned ability of *L. plantarum* to take up the smaller peptides released after enzymatic hydrolysis. However, P_FdH and E_FdH did not show significant differences to P_Hy and E_Hy samples, respectively. In the case of P_FdH, this could be due to the higher exposure of cysteine residues interfering with the measurement; in the case of E_FdH, this could be due to protein agglomeration promoted by fermentation, which hides the cleavage site for Esperase®.

3.4. Reaction of Polyclonal Antibodies with PPI

The soluble proteins from all samples were separated by gel filtration and individual fractions analyzed by ELISA using three individual polyclonal rabbit sera raised against PPI. The results from all treated samples showed a compelling degradation towards lower molecular weight proteins (Figure 2A). ELISA analyses of the total protein (Figure 2B) and individual fractions gave a reduced immunogenicity for all samples. In particular, trypsin-treated samples showed a reduced antibody reactivity to background levels (Figure 3A,B). Since the three polyclonal sera used for the ELISA showed different binding profiles for the individual proteins, it can be concluded that the soluble proteins are no longer recognized by the antibodies. The only exception are the eluted fractions containing higher molecular

weight proteins, which are certainly resistant to the treatment applied. The ELISA results for the total protein showed that also the overall signal is significantly reduced in those preparations with the highest degradation. Therefore, the fraction of high molecular weight immunogenic proteins may be lower than suggested by the ELISA values of the high molecular weight fractions.

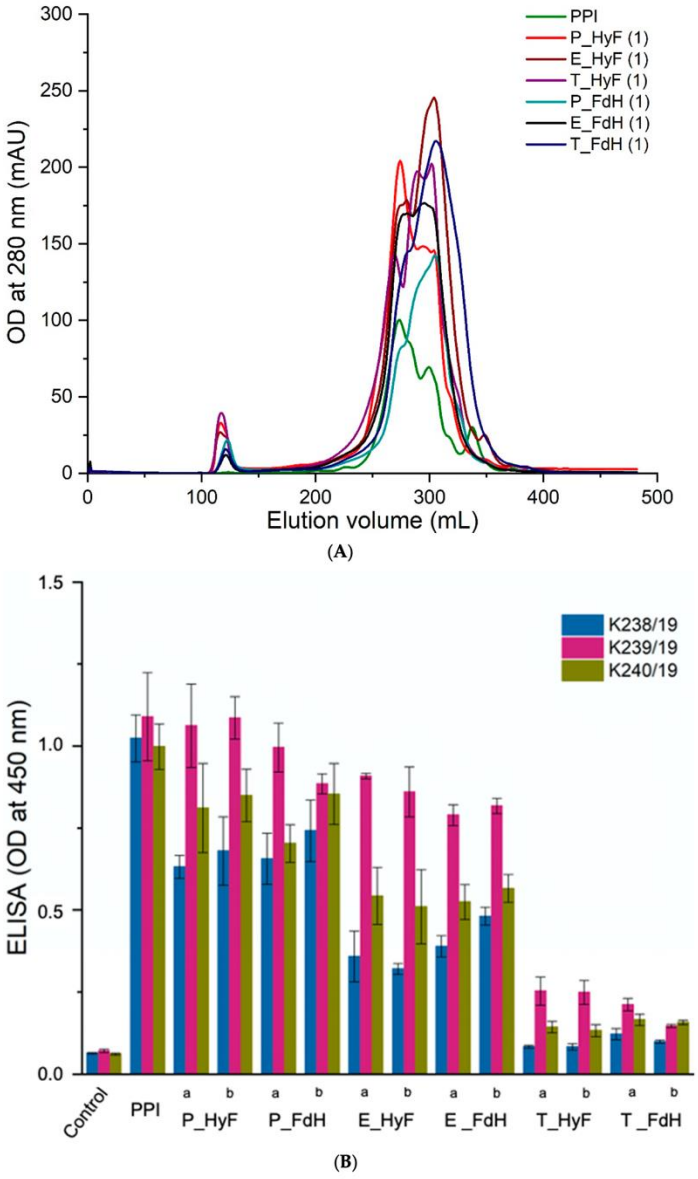
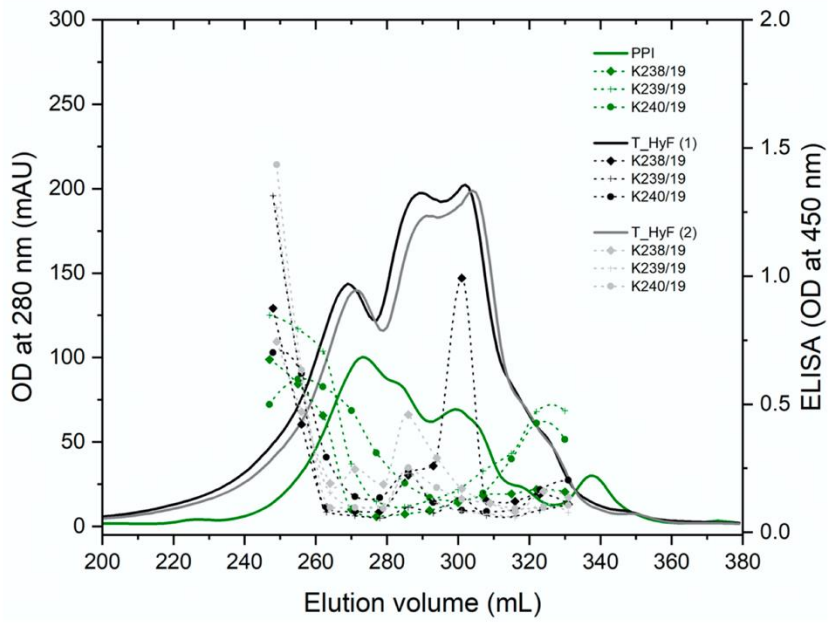
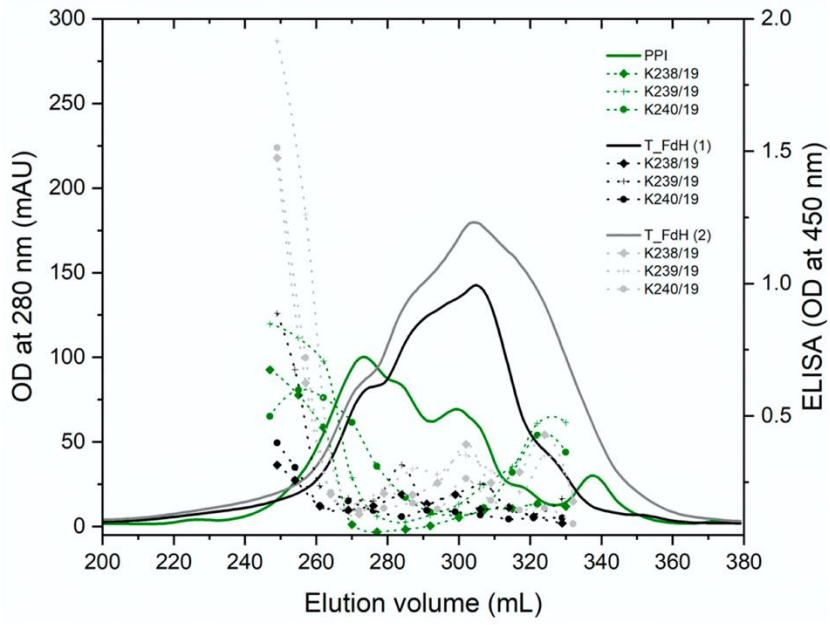


Figure 2. Protein size distribution and ELISA of pea protein isolate (PPI) and its hydrolysates by means of (**A**) gel filtration and (**B**) ELISA of total protein using three immunized rabbit sera. The sample replicates were analyzed independently (a and b). P: papain; E: Esperase®; T: trypsin; Hy: hydrolysis; HyF: hydrolysis followed by fermentation; FdH: fermentation followed by hydrolysis.



(**A**)



(**B**)

Figure 3. Gel filtration and ELISA results from trypsin treated protein isolates by combination methods of (**A**) enzymatic hydrolysis followed by fermentation (T_HyF) and (**B**) fermentation followed by enzymatic hydrolysis (T_FdH).

These SDS-PAGE results in combination with the results from the gel filtration and ELISA show that the combination of enzymatic hydrolysis and fermentation degrades pea proteins to a higher degree. Reduced reactivity with the antibody sera could imply reduction of the allergic potential of pea protein preparations. Fermentation followed by enzymatic hydrolysis was particularly successful, as it seemed to degrade all major potential pea allergens. However, the reduction in allergenicity needs to be confirmed by further immunological studies, such as prick tests.

3.5. Functional Properties

Changes in the molecular weight distribution of proteins cause changes in the exposed hydrophobic and ionizable groups as well as in the ability of the proteins to aggregate, which can influence the functional properties [54]. Therefore, the effect of protein degradation on functional properties were studied in detail.

3.5.1. Protein Solubility

The results of the protein solubility analyses are shown in Table 6; these results correlate strongly with the DH values. At acidic pH (pH 4.5), the untreated and fermented PPI were significantly different from all other samples. Samples treated with Esperase® showed the highest protein solubility of up to 66%, whereas the protein solubility of papain and trypsin treated samples was also significantly increased. The fermentation followed by enzymatic hydrolysis was most effective in increasing solubility at acidic pH.

Table 6. Functional properties of untreated and treated pea protein isolates.

Samples	Protein Solubility [%]		Emulsifying Capacity	Foaming Capacity
	pH 4.5	pH 7.0	(mL/g)	(%)
Untreated PPI	6.98 ± 0.47^{a}	40.26 ± 0.81^{a}	725 ± 8^{a}	840 ± 8^{a}
Fermented PPI	5.72 ± 0.44^{a}	10.72 ± 1.67^{b}	310 ± 13^{b}	807 ± 3^{a}
P_Hy	31.19 ± 1.24^{b}	43.64 ± 1.99^{ac}	465 ± 18^{c}	1234 ± 56^{b}
P_HyF	35.87 ± 1.12^{c}	42.85 ± 1.38^{ac}	398 ± 21^{d}	1190 ± 17^{b}
P_FdH	38.12 ± 1.69^{c}	47.37 ± 4.42^{c}	383 ± 10^{d}	1335 ± 73^{c}
E_Hy	60.01 ± 1.25^{b}	61.52 ± 1.01^{c}	391 ± 10^{c}	1261 ± 67^{b}
E_HyF	63.74 ± 1.46^{c}	74.95 ± 2.65^{d}	300 ± 14^{b}	985 ± 33^{c}
E_FdH	66.55 ± 1.64^{d}	67.28 ± 2.76^{e}	450 ± 4^{d}	1576 ± 22^{d}
T_Hy	42.95 ± 7.04^{b}	50.94 ± 2.19^{c}	670 ± 31^{c}	1993 ± 53^{b}
T_HyF	48.89 ± 1.87^{bc}	52.55 ± 1.20^{c}	664 ± 24^{c}	1934 ± 150^{b}
T_FdH	51.31 ± 0.44^{c}	63.08 ± 2.22^{d}	705 ± 12^{ac}	2575 ± 47^{c}

Results are expressed as means ± standard deviation (n = 4). Means marked with different letters within one column indicate significant differences between treated samples from one enzyme and the untreated pea protein isolate (PPI) and the fermented PPI (Tukey, $p < 0.05$). P: papain; E: Esperase®; T: trypsin; Hy: hydrolysis; HyF: hydrolysis followed by fermentation; FdH: fermentation followed by hydrolysis.

At neutral pH, the fermented PPI showed significant lower protein solubility compared to the untreated PPI. The PPI showed similar protein solubility to P_Hy and P_HyF, whereas the P_FdH was significantly different. The papain and trypsin treated samples showed the highest protein solubility when the fermentation step was followed by enzymatic hydrolysis. Among the samples that were only hydrolyzed or were hydrolyzed and then fermented, a significant difference in protein solubility could not be measured. However, the samples treated with Esperase® were significantly different from each other and from the untreated PPI. The lower solubility of E_FdH compared to E_HyF could be explained by an increase in insoluble aggregates due to acid denaturation during fermentation, which hinders the Esperase® activity to cleave on specific protein sites.

Other studies have shown negative or no effect of fermentation on the protein solubility. This has been attributed to changes in the protein surface, surface charge and the LAB cell

surface, which might promote hydrophobic interactions [33,47,55]. Thus, the improvement in the protein solubility of treated samples is certainly due to enzymatic hydrolysis.

3.5.2. Emulsifying Capacity

Results from emulsifying capacity are shown in Table 6. The untreated PPI showed the highest emulsifying capacity with 725 mL/g, followed by T_FdH with 700 mL/g. In contrast, the fermented PPI and the E_HyF showed the lowest emulsifying capacity with 310 mL/g and 300 mL/g, respectively. The difference among the results of the treated samples could be due to different changes in protein conformation, peptide release, and their interactions with other components such as microbial cells, which could reduce the amphiphilic character of the proteins [56]. Moreover, the ratio albumin/legumin/vicilin, the presence of polar lipids and partial denaturation have also been shown to affect emulsifying capacity [57–60]. Although all treated samples had lower emulsifying capacities than the untreated PPI, the emulsifying capacity of the treated samples is still in a good range to be used as food ingredient. A high DH is known to impair emulsifying capacities [54], and although there was no correlation between the DH value and the emulsifying capacity, the sample with the highest DH (E_HyF) showed the lowest emulsifying capacity.

3.5.3. Foaming Capacity

A foam is a dispersion of air in water. The effect of proteins in foam formation is similar to the one in forming emulsions. Their amphiphilic character allows proteins to interact with the hydrophobic and hydrophilic fractions of air and water, respectively, during whipping, reducing surface tension. Similarly, the foaming capacity depends on different factors such as protein fractions ratio, pH of the solutions, and lipid content [61,62].

The foaming capacities of untreated and fermented PPI were not significantly different with 840% and 807%, respectively. On the other hand, all other treated samples showed a significantly improved foaming capacity, with the highest foaming capacity found in the fermented and subsequently hydrolyzed samples. Of the treated samples, the trypsin samples showed the highest capacities.

3.6. Sensory Analysis

Although products containing pea proteins are increasing, the characteristic pea off-flavors remain a major challenge. In addition to naturally occurring off-flavors, PPI treatment can lead to changes in the flavor and taste profile. It is known that enzymatic hydrolysis can increase the bitterness of protein preparations from legumes, whereas fermentation of legumes promotes the degradation and formation of aroma compounds.

The bitterness of the untreated PPI compared to those treated with papain or trypsin was not significantly different (Figure 4). However, the bitterness ranking was the highest after treatment with Esperase® and was significantly higher than that of the untreated PPI. Although fermentation enhanced further hydrolysis (Section 3.3), the peptidases from *L. plantarum* may not be sufficient to completely cleave hydrophobic residues.

As expected, the untreated PPI was ranked highest for plant-like off-flavor, while this attribute was significantly reduced for all combined samples. The samples fermented prior to enzymatic hydrolysis showed the strongest reduction of the plant-like off-flavor, where T_FdH received the lowest rank.

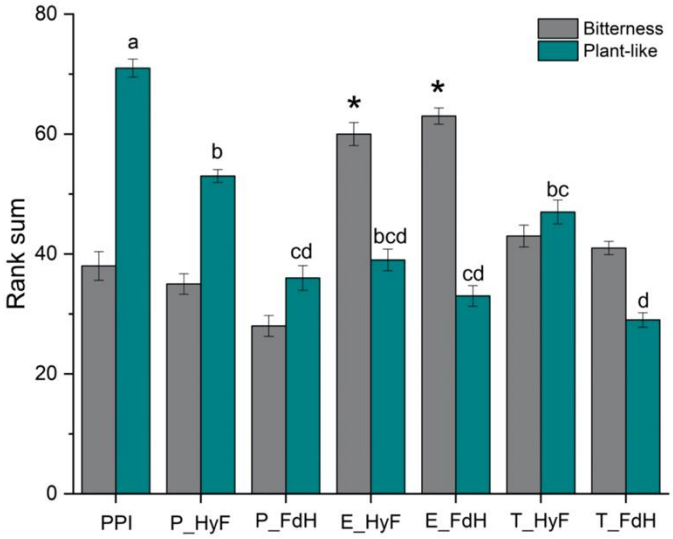


Figure 4. Results are expressed as sum of ranks ± standard deviation (n = 11). Rank sums marked with different letters indicate significant differences (Duncan's, $p < 0.1$). An asterisk (*) indicate significant differences to the untreated PPI (Duncan's, $p < 0.1$). PPI: pea protein isolate; P: papain; E: Esperase®; T: trypsin; HyF: hydrolyzed followed by fermentation; FdH: fermentation followed by hydrolysis.

4. Conclusions

Various studies have investigated the effects of enzymatic hydrolysis and fermentation on pea proteins; however, to the best of our knowledge, a combination of both methods has not yet been investigated. Our study shows that the order of combination of both methods can have a significant impact on the proteins, their immunological and functional properties, as well as the characteristic off-flavors of PPI. The fermentation of PPI followed by enzymatic hydrolysis showed stronger protein degradation and an effect on functionality of the proteins as well as a reduction of off-flavors. The SDS-PAGE and gel filtration showed a significant reduction in the proteins molecular weight by enzymatic digestion. Analyses of the individual size fractions showed a reduced immunogenicity using three different polyclonal sera in ELISA. However, further in vivo tests are required to confirm that treated PPI will be tolerated better by allergic or sensitized individuals at those amounts corresponding to the daily consumption in protein-enriched food. The increase in protein solubility, especially in acidic conditions, suggests that treated pea proteins can be used to increase the protein content in different food products. The reduction of pea off-flavors could allow the increase of protein content without hindering the acceptance by consumers; the application in different products and their acceptance still need to be investigated. The combination of treatments can be a promising method to be used in the food industry to enhance pea protein isolate functionality and neutralize off-flavors, and could significantly lower the allergenicity.

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CHAPTER 4

Screening of Twelve Pea (*Pisum sativum* L.) Cultivars and its Isolates Focusing on the Protein Characterization, Functionality and Sensory Profile[4]

Abstract

The protein composition of peas depends on different genetic and environmental factors. Previous studies have shown that enzymatic hydrolysis and fermentation changed functionality and sensory profile of pea protein isolates from cultivar “Navarro”. This study aimed to screen several pea cultivars to investigate potential differences regarding chemical composition, functional properties, main allergens and flavor. In order to facilitate an adaptation of the results to industrial implementation, all pea protein isolates were spray-dried as this drying procedure is the most common method used in the food industry. The protein yields varied considerably depending on the pea cultivar. Electrophoretic results showed that the potential pea allergens and molecular weight distribution were not significantly different among the isolates. A principal component analysis showed that some isolates were clustered regarding their particle size and functional properties. However, the sensory profiles were slightly different among all isolates with significant differences in the *pea-like* and *bitter* attributes. The study is of high interest to the food industry as it highlights the importance of selecting the appropriate pea cultivar for specific food applications.

4 García Arteaga, V., Kraus, S., Schott, M., Muranyi, I., Schweiggert-Weisz, U., & Eisner, P. (2021). Screening of Twelve Pea (Pisum sativum L.) Cultivars and Their Isolates Focusing on the Protein Characterization, Functionality, and Sensory Profiles. Foods, 10 (4), 758. doi:10.3390/foods10040758

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Screening of Twelve Pea (*Pisum sativum* L.) Cultivars and Their Isolates Focusing on the Protein Characterization, Functionality, and Sensory Profiles

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Abstract: Pea protein concentrates and isolates are important raw materials for the production of plant-based food products. To select suitable peas (*Pisum sativum* L.) for protein extraction for further use as food ingredients, twelve different cultivars were subjected to isoelectric precipitation and spray drying. Both the dehulled pea flours and protein isolates were characterized regarding their chemical composition and the isolates were analyzed for their functional properties, sensory profiles, and molecular weight distributions. Orchestra, Florida, Dolores, and RLPY cultivars showed the highest protein yields. The electrophoretic profiles were similar, indicating the presence of all main pea allergens in all isolates. The colors of the isolates were significantly different regarding lightness (L*) and red-green (a*) components. The largest particle size was shown by the isolate from Florida cultivar, whereas the lowest was from the RLPY isolate. At pH 7, protein solubility ranged from 40% to 62% and the emulsifying capacity ranged from 600 to 835 mL g^{-1}. The principal component analysis revealed similarities among certain pea cultivars regarding their physicochemical and functional properties. The sensory profile of the individual isolates was rather similar, with an exception of the *pea-like* and *bitter* attributes, which were significantly different among the isolates.

Keywords: pea (*Pisum sativum* L.); spray-dry; functional properties; sensory profile; protein characterization; pea allergens

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1. Introduction

Peas (*Pisum sativum* L.) were domesticated around 10,000 years ago. Over the years, evolution and breeding has influenced the number of pea cultivars found today. In Europe, according to the Food and Agriculture Organization database [1], France and Germany were the biggest dry pea seed producers in 2019. The differences among cultivars depend on their cultivated status (wild or cultivated), geographical origin, and usage (fresh or dry) [2]. The study of different cultivars, their breeding, and their inclusion in the genome database is a continuous process [3]. From an agronomic point of view, cultivation factors such as maximum yield security, plant stability, seed percentage, and protein yield are the most important characteristics considered for pea cultivation; however, for industrial food production, factors such as protein content, functionality, taste, and color are also considered [4]. Peas contain high amounts of protein at around 20–35%, low amounts of fat at around 0.5–4.0%, and high amounts of starch at around 30–48% [5–7]. Previous studies have investigated the differences in pea cultivar compositions and have found environmental and genotypic variations as the main factors for the described data discrepancies.

The aroma of the pea seeds also changes significantly depending on the cultivar, harvest year, and processing conditions [8,9].

Vegetarian or vegan diets might lead to protein deficiencies, making peas an interesting protein source for plant-based food products [10]. According to the Global Market Insights report [11], the pea protein market is estimated to grow by 12% compound annual growth rate (CAGR) by 2026. The main proteins in peas correspond to storage proteins. These are divided into globulins and albumins, corresponding to 55–80% and 18–25%, respectively, depending on genetic and environmental factors [6,12,13]. Similar to other legumes, the major globulins in peas are divided into 7S vicilin–convicilin and 11S legumin fractions [14]. The molecular structures and weight distributions are different among these proteins. Legumin is a hexamer with major polypeptide subunits of ~40 and ~20 kDa, which can be bound by disulfide bonds. Vicilin is a trimer (each subunit ~50 kDa) lacking cysteine residues that can undergo post-translational proteolysis, resulting in different fractions. Convicilin is a trimer (~70 kDa) without any translational modification [15,16].

Pea proteins are used as concentrates (40–90% protein) and isolates (>90% protein) in the food industry; however, the extraction of pea protein isolate (PPIs) at laboratory and pilot scales has shown protein contents of around 80–90% [17–19]. These studies have found that depending on the cultivar and the extraction method, the protein solubility and emulsifying and foaming capacity were significantly affected; however, Stone and Karalash [17] concluded that overall, the extraction method has a greater influence than the cultivar. The PPIs investigated in the above-mentioned studies showed higher functionality than commercial isolates. This could be due to a deviating production or drying process. Industrial protein ingredient suppliers usually use spray drying, whereas lyophilization is mainly used for scientific purposes at the laboratory scale. Spray drying might affect the aroma and protein structure, and thus the protein profile, particle size, and functionality [9,20]. Moreover, most authors have investigated cultivars available in their countries. In Germany, the cultivars Astronaute and Salamanca are mainly used because of their high seed yields [21]; however, to our knowledge, only protein preparations of the latter cultivar have been characterized scientifically [22]. A broader screening of European pea cultivars would increase the ability to select a cultivar that fulfills specific product needs.

Another reason for the high popularity of peas as raw materials for protein isolation is that unlike soy, pea proteins do not need to be declared as allergens in Europe. However, two major allergens, namely convicilin (Pis s 2) and vicilin (Pis s 1), have been identified [23]. Pis s 2 corresponds to a 62–67 kDa fraction, whereas Pis s 1 corresponds to 47–50 kDa (mature vicilin-$\alpha\beta\gamma$) and 32 kDa (vicilin-$\alpha\beta$) fractions. These allergens could potentially promote cross-reactions with other legume allergens; thus, recent studies suggest their inclusion in the allergen declaration list [24,25]. The allergenic potential might vary within and among cultivars, as they have shown significant proteomic variations of the same pea cultivar harvested over three consecutive years [16].

The present study aimed to investigate pea cultivars grown in Germany and France, regarding chemical compositions of their flours and isolates, as well as the protein yields, functional properties, aroma profiles, and molecular weight distribution of the PPIs. Among the data assessed, this study aimed to identify PPIs of cultivars showing similar chemical, functional, and sensory properties in order to use them in combination or interchangeably in the food industry, without having significant effects on the final product quality.

2. Materials and Methods

2.1. Materials

The different field pea seeds (*Pisum sativum* L.) were kindly provided by Norddeutsche Pflanzenzucht Hans-Georg-Lembke KG (Holtsee, Germany) and are shown in Table 1. The Broad Range™ Unstained Standard, 4–20% Criterion™ TGX Stain-Free™ Precast Gels, and Coomassie blue R-250 were purchased from Bio-Rad Laboratories GmbH (Feldkirchen, Germany). Sodium dihydrogen phosphate, sodium dodecyl sulfate, and sodium monohy-

drogen phosphate were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). All chemicals used in this study were of analytical grade.

Table 1. List of pea cultivars investigated in this study.

Cultivar	Harvest Year	Place of Cultivation	Cotyledon Color	Admitted in
Navarro	2018	Malchow/Mecklenburg-Vorpommern	yellow	Germany
Dolores	2015	Oderaue/Mecklenburg-Vorpommern	yellow	Germany
Greenwich	2018	Hohenlieth/Schleswig-Holstein	green	Great Britain
Bluetime	2018	Hohenlieth/Schleswig-Holstein	green	Great Britain
Ostinato	2018	Rodez/France	yellow	France
Kalifa	2017	Hohenlieth/Schleswig-Holstein	yellow	Breeding line
Salamanca	2018	Malchow/Mecklenburg-Vorpommern	yellow	Germany, Czech Republic, etc.
Florida	2015	Dreveskirchen/Mecklenburg-Vorpommern	yellow	Germany
RLPY141091	2018	Rodez/France	yellow	Germany
Orchestra	2018	Rodez/France	yellow	France, Germany
Astronaute	2018	Groß Kiesow/Mecklenburg-Vorpommern	yellow	France, Germany, etc.
Croft	2018	Hohenlieth/Schleswig-Holstein	green	Great Britain

2.2. Production of Pea Flour

Peas were dehulled and split using an underflow peeler (Streckel and Schrader KG, Hamburg, Germany). The kernels were separated using a zig-zag airlift system and milled with a pilot plant impact mill with 0.5 mm sieve insertion (Alpine Hoakawa AG, Augsburg, Germany).

2.3. Production of Pea Protein Isolate

The isolation of pea protein was performed according to Tian and Kyle [26] following an alkaline extraction with isoelectric precipitation (AE-IEP) with some changes. An aqueous alkaline extract of the pea flour was prepared in deionized (DI) water at a ratio of 1:5 (w/w) at pH 8.0 using 3.0 mol/L NaOH, which was stirred for 60 min. The protein extract was sieved (0.8 mm) after centrifugation at 8000× g for 20 min at 15 °C (8K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). For isoelectric precipitation, the protein extract was adjusted to pH 4.5 using 3.0 mol/L HCl and left overnight at 4 °C. The precipitated proteins were separated by centrifugation at 8000× g for 20 min at 15 °C and the protein isolate was dispersed in DI water to a dry matter content of 8%. After neutralization to pH 7.0, the isolate dispersion was homogenized at 11,000 rpm for 2 min using an Ultraturrax (IKA®-Werke GmbH and Co. KG, Staufen, Germany) prior to spray drying. The spray drying was performed using a Mini Spray Dryer B-191 (BUCHI Labortechnik GmbH, Essen, Germany) at inlet and outlet temperatures of 180 °C and 80 °C, respectively, as well as with a 95% aspirator output. The spray-dried isolates were used for further analysis. The protein yield was calculated as grams of protein per kilogram of seeds. Due to the limited amounts of pea seeds, the protein extractions and spray drying were performed once. We assumed that the protein extraction and yield values are representative of the process, as other studies have shown low standard deviations in their own extractions [17,18].

2.4. Chemical Composition

The analysis of the chemical compositions of the pea flours and PPIs included determination of the dry matter, ash, protein, starch, and fat contents.

Dry matter and ash contents were determined using thermogravimetric methods (TGA 701, Leco Instruments, Mönchengladbach, Germany). The protein content was determined according to the Dumas combustion method (TruMac N, Leco Instruments, Mönchenglad-

bach, Germany) using the average nitrogen-to-protein conversion factor of N × 6.25. All analyses were performed in duplicate and in accordance with the Association of Official Analytical Collaboration (AOAC) Official Methods [27,28].

The starch content was determined in duplicate using a Starch UV-Test Kit according to the manufacturer's instructions (R-Biopharm AG, Darmstadt, Germany). The fat content was determined according to the Caviezel method [29] with some modifications. In extraction vessels, 2–3 g of the sample was mixed with 1.5 g potassium hydroxide, 5 mL stock solution, and 40 mL 1-butanol. After separation of the derivatized fatty acids by gas chromatography (GC 7890A, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany), the total fat content was determined by summing up all detected methyl esters in relation to an internal standard. Mazola corn germ oil served as the reference. The results are given in fat%, calculated as methyl ester.

2.5. Molecular Weight Distribution Using Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight distribution was analyzed using SDS-PAGE under non-reducing and reducing conditions according to the method used by Laemmli [30], with slight modifications. Briefly, 5 µg/µL protein solution (based on dry matter) was prepared in 1× treatment buffer (50% (*v*/*v*) 2× Tris-HCl treatment buffer, 50% (*v*/*v*) phosphate buffer (pH 7)). The 2× treatment buffer was prepared using 0.125M from the 4× stacking gel buffer (0.5M Tris, adjusted with HCl to pH 6.8), 4% from 10% SDS, 20% glycerol, and 0.02% Bromophenol Blue, while for reduction conditions 0.2M dithiothreitol was added. The samples were heated (95 °C, 5 min) prior to centrifugation at 12,045× *g* for 3 min (MiniSpin, Eppendorf AG, Hamburg, Germany). The supernatants were mixed 1:10 (*v*/*v*) with 1× treatment buffer, from which 3 µL was added into the gel pocket of the Bio-Rad 4–20% Criterion™ TGX Stain-Free™ Precast Gels. The Broad Range™ Unstained Protein Standard was used as the molecular weight marker. The running time was 30 min, followed by staining using Coomassie Brilliant Blue R-250. Finally, gel images were obtained using an EZ Imager (Gel Doc™ EZ Imager—Bio-Rad). Protein bands and their intensities were calculated using Image Lab Software. SDS-PAGE was performed in duplicate, with each sample being prepared two times independently.

2.6. Color

The colors of the protein isolates were measured using the Digi Eye system (VeriVide Limited, Leicester, UK) and a Nikon D90 camera (Nikon Metrology GmbH, Düsseldorf, Germany). The International Commission on Illumination (CIE) L*a*b* method was used to measure the parameters lightness (L*), green-red (a*), and blue-yellow (b*). The white color from the calibration board was used as the white reference for comparison among samples. The total color difference (ΔE^*_{ab}) compared to the white reference board was calculated according to the CIE76 formula (Equation (1)). The color determination was performed in triplicate.

$$\Delta E^*_{ab} = \sqrt{\left(L^*_2 - L^*_1\right)^2 + \left(a^*_2 - a^*_1\right)^2 + \left(b^*_2 - b^*_1\right)^2} \quad (1)$$

2.7. Particle Size

The particle size distribution of all pea protein isolates was determined using a Master-Sizer S Long Bed Version 2.19 equipped with a QS Small Volume Sample Dispersing Unit DIF2021 (Malvern Panalytical Ltd., Malvern, UK). The sample was dispersed in 1-Butanol for 2 min at 3000 rpm before measurements. After another minute, a second measurement was conducted. The measuring range was set at 300 RF 0.05–900 µm. The particle size was based on Mie theory with a refractive index of 1.33, using an index of 0.1 for dispersion media and 1.56 for the dispersed phase, with an imaginary proportion of 0.1.

2.8. Functional Properties

All analyses of functional properties were performed in duplicate.

2.8.1. Protein Solubility

The protein solubility measurements at pH 4.5 and 7.0, respectively, were performed according to Morr and German [31]. The soluble protein content was determined photometrically at 550 nm following the Biuret method [32] using bovine serum albumin (BSA) as the standard for calibration.

2.8.2. Foaming Capacity

The foaming capacity was analyzed at pH 4.5 and 7.0 according to Phillips and Haque [33] using a whipping machine (Hobart N50, Hobart GmbH, Offenburg, Germany). Briefly, 5% (*w*/*v*) dispersions were whipped (580 rpm) for 8 min and the foaming capacities were determined as the relation between the initial and final volume.

2.8.3. Emulsifying Capacity

The emulsifying capacity was determined according to Wang and Johnson [34] and García Arteaga, et al. [35] at pH 4.5 and 7.0. Briefly, 10 mL min^{-1} oil was added to a dispersion (1% *w*/*w*) in a 1 L reactor equipped with an Ultra-Turrax instrument and a conductivity meter. The volume of added oil was used to calculate the emulsifying capacity (mL oil/g sample).

2.9. Sensory Analysis

2.9.1. Sample Preparation

A 2% sample solution (1.7% protein, *w*/*w*) was prepared with tap water for each PPI. The respective samples were adjusted to pH 7.0 with 1 mol/L NaOH and coded using three-digit random numbers.

2.9.2. Sample Evaluation

The sensory evaluation was conducted according to DIN 10967-1-1999 and as described by García Arteaga, et al. [35]. Briefly, a trained panel evaluated attributes regarding retronasal aromas and tastes of the different PPIs. From each sample solution, 20 mL was presented at room temperature in a glass cup and in random order. The sensory evaluation was split into two evaluation sessions. In the evaluation sessions, each panelist evaluated six and seven samples, respectively. The panelists assessed the samples according to the following attributes: *fatty* (2-nonenal); *green* (hexanal); *earthy* (geosmin); *roasty* (2-acetylpyrazine); *pea-like* (2-isopropyl-3-methoxypyrazine); *metallic* ((trans)-4,5-Epoxy-(E)-decenal); *malty*; *nutty* (2,5-dimethylpyrazine). Additionally, panelists assessed the samples according to tastes such as *bitter*, *sweet*, *salty*, *astringent*, *mouth-coating*, and overall intensity. The intensities were scored from 0 (not perceivable) to 10 (very intense).

2.10. Principal Component Analysis

A principal component analysis (PCA) is a multivariate statistical data analysis tool used to simplify the variability of data with a reduced number of dependent variables. A PCA (correlation matrix) was used to evaluate the similarities among isolates regarding their protein content, fat content, color, particle size, and functional properties. A covariance PCA was used to evaluate the aroma and taste. The PCA plots were performed using the software OriginPro 2018b.

2.11. Statistical Analysis

Protein extractions were performed once and the resulting isolates were used for further analyses. Due to the low protein yields, all analyses were performed in duplicate, unless stated otherwise, and the results are expressed as mean values ± standard deviations. Non-parametric statistical analyses were performed due to the low number

of replicates. The Kruskal–Wallis test was used to determine statistical differences among the cultivars. Dunn's test with Bonferroni correction for p-values was used as a test for multiple comparisons. The results of the sensory analysis were analyzed using one-way ANOVA followed by Tukey's post hoc test. A Kendall correlation coefficient was used to determine correlations between physicochemical, functional, and sensory properties. All statistical analyses were performed using OriginPro 2018b and were considered statistically significant at $p < 0.05$. The raw data are available as Mendeley Data [36].

3. Results and Discussion

3.1. Chemical Composition and Protein Yield

Table 2 shows the chemical compositions of the flours and PPIs, as well as the protein yields after spray drying.

3.1.1. Pea Flours

The protein contents of the dehulled pea flours ranged from 21.3% to 27.2%, similar to values obtained by Barac and Cabrilo [18] and Nikolopoulou and Grigorakis [6]. The flour from RLPY cultivar showed the highest protein content (27.2%), whereas the flour from Greenwich had the lowest protein content at 21.3%. The ash and fat contents ranged from 2.5% to 3.6% and from 1.9% to 2.5%, respectively. The flour from the Florida cultivar showed the highest fat content of 2.7%, whereas the flours of Dolores, Ostinato, Kalifa, RLPY, and Orchestra cultivars showed the lowest amounts at 1.9%. The flour from Navarro had the highest starch content, while RLPY had the lowest. The protein and starch contents obtained in this study were within the ranges of different cultivars investigated in other studies [22,37].

3.1.2. Pea Protein Isolates

The protein contents of the pea protein isolates (PPIs) ranged from 83.5% to 90.3%. The PPI obtained from the RLPY cultivar showed the highest protein content, while the one from Navarro showed the lowest. The protein contents were in the same range as in other studies [17,18]; however, other studies obtained higher protein yields (62–89%), probably attributed to the drying technique, as high losses are common during spray drying [38]. It is worth mentioning that protein isolation at industrial scale might result in higher yields when the drying kinetics are correctly determined [39,40]. The highest protein yield was 62.2 g protein kg^{-1} $seed^{-1}$ obtained from the Orchestra cultivar, followed by Florida with 59.2 g kg^{-1}. The lowest protein yields were obtained from Navarro and Greenwich cultivars at 33.8 g kg^{-1} and 34.8 g kg^{-1}, respectively. The ash contents of the PPIs varied from 5.3% to 8.5%, probably due to formation of salts (NaCl) after adjusting the pH during the different process steps. The fat contents ranged from 4.7% to 9.0%, with the Greenwich isolate having the highest fat content and Dolores isolate the lowest. The PPIs without a de-fatting step had higher lipid contents, probably due to the protein–lipid interactions during the extraction; Gao and Shen [41] showed that PPIs extracted after AE-IEP had predominantly hydrophobic β-sheets in their protein structures that could promote these interactions [42]. Furthermore, the increase in fat content might promote lipid–protein interactions in the isolates, which may lead to a higher hydrophobic character of the complexes, resulting in lower protein solubility. Their interaction may also reduce the availability of lipophilic groups, limiting the absorption of fat [43]. The color, aroma, and functionality might be also affected by the fat content, especially after lipid oxidation by lipoxygenase [44].

Table 2. Chemical composition and protein yield of dehulled flour and protein isolates produced from different pea cultivars.

Cultivar	Dry Matter	Protein *	Ash 550 *	Fat *	Starch *	Protein Yield **
	[%]	[%]	[%]	[%]	[%]	[g kg^{-1}]
Dehulled Flour						
Navarro	89.6 ± 0.0	22.1 ± 0.1	2.9 ± 0.0	2.3 ± 0.0	52.6 ± 0.2	-
Dolores	91.4 ± 0.0	26.5 ± 0.2	3.0 ± 0.0	1.9 ± 0.0	44.3 ± 2.5	-
Greenwich	90.9 ± 0.0	21.3 ± 0.2	2.7 ± 0.0	2.5 ± 0.0	48.2 ± 0.3	-
Bluetime	91.5 ± 0.1	22.4 ± 0.4	2.9 ± 0.0	2.2 ± 0.0	40.3 ± 0.5	-
Ostinato	91.2 ± 0.0	25.0 ± 0.2	3.6 ± 0.1	1.9 ± 0.0	47.6 ± 0.5	-
Kalifa	91.4 ± 0.1	24.2 ± 0.1	3.0 ± 0.0	1.9 ± 0.0	46.6 ± 0.1	-
Salamanca	90.8 ± 0.0	22.4 ± 0.1	2.8 ± 0.0	2.0 ± 0.0	49.2 ± 3.3	-
Florida	91.2 ± 0.1	24.8 ± 0.1	2.9 ± 0.0	2.7 ± 0.2	45.0 ± 4.6	-
RLPY 141091	91.3 ± 0.1	27.2 ± 0.0	2.8 ± 0.0	1.9 ± 0.0	32.5 ± 0.8	-
Orchestra	92.2 ± 0.1	26.3 ± 0.2	3.5 ± 0.2	1.9 ± 0.0	35.8 ± 0.3	-
Astronaute	91.2 ± 0.0	22.0 ± 0.0	2.5 ± 0.1	2.0 ± 0.1	45.3 ± 1.1	-
Croft	91.8 ± 0.1	22.5 ± 0.1	2.6 ± 0.0	2.1 ± 0.0	48.0 ± 2.2	-
Protein Isolate						
Navarro	93.0 ± 0.0	83.5 ± 0.4	5.3 ± 0.3	5.9 ± 0.0	-	33.8
Dolores	93.5 ± 0.1	89.5 ± 0.2	5.4 ± 0.1	4.7 ± 0.1	-	54.4
Greenwich	93.8 ± 0.0	83.6 ± 0.4	6.0 ± 0.6	9.0 ± 0.2	-	34.8
Bluetime	94.4 ± 0.0	84.1 ± 0.0	6.4 ± 0.4	8.4 ± 0.3	-	42.2
Ostinato	94.1 ± 0.0	86.0 ± 0.5	7.6 ± 0.4	7.1 ± 0.4	-	38.6
Kalifa	93.0 ± 0.0	86.9 ± 0.9	5.9 ± 0.1	7.0 ± 0.5	-	46.2
Salamanca	93.7 ± 0.6	85.0 ± 0.3	6.1 ± 1.0	8.7 ± 0.6	-	42.2
Florida	92.5 ± 0.0	87.4 ± 1.1	5.6 ± 0.1	7.4 ± 0.7	-	59.2
RLPY 141091	93.4 ± 0.0	90.3 ± 0.0	8.5 ± 0.7	7.3 ± 0.8	-	53.6
Orchestra	92.8 ± 0.3	87.1 ± 0.1	6.7 ± 1.1	6.2 ± 0.9	-	62.2
Astronaute	96.0 ± 0.2	86.4 ± 0.1	5.4 ± 0.1	7.8 ± 0.1	-	42.1
Croft	92.5 ± 0.1	86.7 ± 0.6	6.2 ± 0.1	7.8 ± 0.1	-	47.3

Results are expressed as means ± standard deviations (n = 2). No significant differences were found among cultivars within the same column (Dunn's test with Bonferroni correction, $p < 0.05$). Note: * based on dry matter; ** based on protein content (g of protein/kg of seeds).

3.2. Molecular Weight Distribution

Gel electrophoresis was performed under non-reducing and reducing conditions to reveal differences within the protein composition of the isolates from the different cultivars (Figure 1). The protein fractions ranged from 93 to 6.5 kDa. Three major fractions were identified in both conditions. Under non-reducing conditions, fractions around ~65 kDa, ~53 kDa, and ~45 kDa were most prominent, while under reducing conditions, 53 kDa proteins were absent and the intensity of the ~39 kDa fraction increased. Bands around 86 and 91 kDa may have been due to convicilin precursors and lipoxygenase (LOX), respectively [16,45]. The most visible difference between non-reducing and reducing conditions was observed for all protein isolates around the 50–56 kDa region, which might correspond to legumin [16,18]. Legumin consists of two polypeptides, one acid (Leg α) and one basic (Leg β) subunit, connected via disulfide bonding. These subunits were found at around 37–40 kDa for Leg α and 19–22 kDa for Leg β, with higher intensities under reducing conditions.

Among the different protein fractions, the allergens Pis s2 and Pis s1 were investigated in detail. Table 3 shows the protein band intensities for each of the allergen fractions. These allergens lack cysteine residues, hindering the formation of disulfide bonds [14]. For this reason, the allergen protein fractions were expected to appear under both conditions.

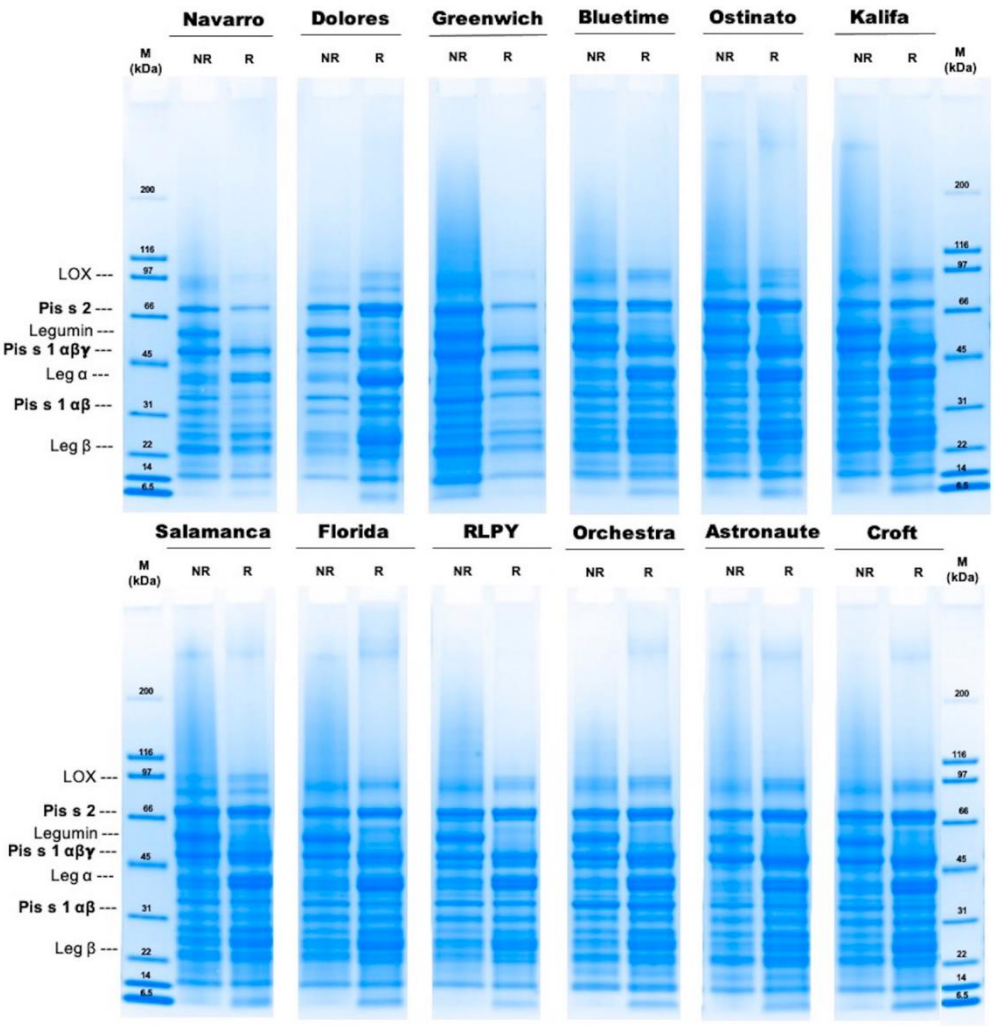

Figure 1. Molecular weight distribution of pea protein isolates from different cultivars, as determined by SDS-PAGE under non-reducing (NR) and reducing (R) conditions. Pis s2, Pis s1 αβγ, and Pis s2 αβ correspond to the allergen fractions from convicilin, mature vicilin- αβγ, and vicilin-αβ, respectively. M: molecular weight standard indicated in kilodalton (kDa).

Convicilin Pis s2. The average molecular weight of the Pis s2 fraction was around 65 kDa, and values were not significantly different among the isolates. The isolate from Orchestra showed the strongest intensities under both conditions. In contrast, the Kalifa isolate and the Navarro isolate showed the lowest intensities under non-reducing and reducing conditions, respectively. The intensity of this protein fraction increased slightly under reducing conditions for all isolates, except for the Navarro and Greenwich isolates, where the intensity of the bands was slightly lower.

Vicilin Pis s1. The mature allergen fraction (vicilin-αβγ) was around 45 kDa under both conditions and was not significantly different among the isolates. Under non-reducing conditions, the Navarro isolate showed the strongest intensity. On the other hand, the RLPY and Florida isolates showed the lowest intensities. Under reducing conditions,

vicilin-αβγ from Astronaute isolate showed the highest intensity, whereas the one from the Greenwich isolate showed the lowest. Vicilin-αβγ can go through post-translational cleavage, resulting in different fractions [46]. From these proteolytic fragments, vicilin-αβ (~32 kDa) was shown to bear a high allergenic potential [23]. Besides the Orchestra isolate, all isolates showed lower intensities of the vicilin-αβ fractions compared to the mature fraction and were not significantly different.

Table 3. Protein band intensities (Int) of globular protein allergens of pea protein isolates, namely convicilin (Pis s2), vicilin αβγ (Pis s1), and vicilin-αβ (Pis s1) from different cultivars as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Cultivar	Protein Band Intensity [Int]					
	Convicilin Pis s2		Vicilin αβγ Pis s1		Vicilin αβ Pis s1	
	NR	R	NR	R	NR	R
Navarro	185 ± 16	146 ± 79	308 ± 34	280 ± 73	81 ± 13	95 ± 50
Dolores	214 ± 5	367 ± 35	166 ± 22	282 ± 43	118 ± 5	97 ± 11
Greenwich	219 ± 47	185 ± 57	229 ± 55	256 ± 36	126 ± 16	113 ± 2
Bluetime	241 ± 17	263 ± 49	236 ± 66	311 ± 39	111 ± 7	127 ± 9
Ostinato	253 ± 31	343 ± 32	252 ± 66	361 ± 11	105 ± 19	116 ± 37
Kalifa	141 ± 6	205 ± 36	285 ± 47	392 ± 46	122 ± 8	153 ± 7
Salamanca	280 ± 2	363 ± 5	233 ± 51	350 ± 20	89 ± 3	88 ± 16
Florida	218 ± 16	283 ± 51	196 ± 88	330 ± 60	94 ± 1	72 ± 6
RLPY 141091	294 ± 13	379 ± 63	149 ± 25	285 ± 78	117 ± 8	106 ± 0
Orchestra	302 ± 50	421 ± 32	212 ± 78	398 ± 19	228 ± 38	180 ± 5
Astronaute	251 ± 34	365 ± 44	293 ± 72	411 ± 20	118 ± 28	99 ± 8
Croft	261 ± 55	372 ± 79	235 ± 79	353 ± 29	93 ± 7	79 ± 20

Results are expressed as means ± standard deviations ($n = 2$). No significant differences were found among cultivars within the same column (Dunn's test with Bonferroni correction, $p < 0.05$). NR: non reducing conditions; R: reducing conditions.

The intensities of the protein and allergen fractions can differ among legume cultivars, while the intensity of the allergen fractions specifically can give an indication of the allergenic potential [14,18,47]. Overall, the Orchestra isolate showed slightly stronger intensities for the potential allergen fractions compared to the other isolates, whereas the isolate from Navarro had the lowest intensities for these fractions. However, the allergen fraction intensities were not significantly different among isolates. It is known that the globulin-to-albumin and legumin-to-vicilin ratios change throughout seed development [48], which could affect the presence and intensity of potential allergens. Even under the same environment, harvesting, and storage conditions, the variation among proteins in pea cultivars can be very large [14].

3.3. Color

Table 4 shows the color values of the samples and the white reference. The lightness (L*) levels among isolates were significantly different; the isolate of Orchestra cultivar showed significantly higher lightness (90.6) than the Bluetime (86.8) isolate. The Greenwich, Bluetime, and Croft isolates showed the lowest a* values, which corresponded to their cotyledon green color; however, only the isolate from Croft was significantly different to the isolates from Salamanca and Astronaute cultivars. In contrast, the isolate from the Navarro cultivar showed higher b* values, suggesting a stronger yellow color. The total color difference (ΔE^*_{ab}) allows for quantification of the colors and allows comparison between samples; the lower the ΔE^*_{ab} value, the whiter the isolate is. All ΔE^*_{ab} values ranged between 19.2 and 23.4. According to the lowest difference from the white reference, the isolates from the Dolores and Greenwich cultivars were most white, while the isolate from Navarro cultivar was least white.

Table 4. CIE lab color results from pea protein isolates from different cultivars and a commercial pea protein isolate.

Cultivar	Pea Isolate CIE Color			
	L*	a*	b*	ΔE^*_{ab}
Navarro	$89.3 \pm 0.4_{ab}$	$2.8 \pm 0.1_{ab}$	$23.7 \pm 0.2_{a}$	$23.4 \pm 0.2_{a}$
Dolores	$88.5 \pm 0.1_{ab}$	$1.9 \pm 0.0_{ab}$	$19.1 \pm 0.2_{a}$	$19.2 \pm 0.1_{a}$
Greenwich	$88.2 \pm 0.1_{ab}$	$0.6 \pm 0.1_{ab}$	$19.2 \pm 0.4_{a}$	$19.3 \pm 0.3_{a}$
Bluetime	$86.8 \pm 0.3_{a}$	$0.9 \pm 0.1_{ab}$	$20.5 \pm 0.7_{a}$	$21.0 \pm 0.7_{a}$
Ostinato	$89.4 \pm 0.3_{ab}$	$3.2 \pm 0.2_{ab}$	$20.5 \pm 0.4_{a}$	$20.4 \pm 0.5_{a}$
Kalifa	$89.5 \pm 0.3_{ab}$	$2.8 \pm 0.0_{ab}$	$20.5 \pm 0.2_{a}$	$20.3 \pm 0.2_{a}$
Salamanca	$88.3 \pm 0.2_{ab}$	$3.3 \pm 0.1_{ab}$	$21.3 \pm 0.3_{a}$	$21.5 \pm 0.3_{a}$
Florida	$88.6 \pm 0.3_{ab}$	$2.7 \pm 0.2_{ab}$	$20.8 \pm 0.2_{a}$	$20.8 \pm 0.3_{a}$
RLPY 141091	$90.1 \pm 0.2_{ab}$	$3.1 \pm 0.1_{ab}$	$22.0 \pm 0.2_{a}$	$22.0 \pm 0.3_{a}$
Orchestra	$90.6 \pm 0.6_{b}$	$2.6 \pm 0.3_{ab}$	$20.9 \pm 0.4_{a}$	$20.3 \pm 0.5_{a}$
Astronaute	$88.2 \pm 0.3_{ab}$	$3.5 \pm 0.1_{a}$	$22.8 \pm 0.3_{a}$	$22.9 \pm 0.3_{a}$
Croft	$87.3 \pm 0.3_{ab}$	$-0.5 \pm 0.0_{b}$	$19.9 \pm 0.2_{a}$	$20.3 \pm 0.3_{a}$

Results are expressed as means ± standard deviations (n = 3). Subscripts with different letters indicate significant differences within the same column (Dunn's test with Bonferroni correction, $p < 0.05$). Note: ΔE^*_{ab}: color difference compared to a white reference.

3.4. Particle Size

Spray drying is one of the most common methods for drying protein solutions on an industrial scale. However, protein structures are to known to be affected by spray drying due to applied temperatures, vaporization, and the air–water interface.

These effects can cause protein denaturation and further aggregation of the exposed hydrophobic regions, which can affect the particle size of the dried proteins [20]. The particle size, in turn, is known to affect the physicochemical properties of proteins [49,50]. The particle sizes of the PPIs, described as the average volume weighted mean ($d_{4,3}$), are shown in Table 5. The average $d_{4,3}$ of the cultivar isolates was 11.9 µm. Of all protein isolates, the Florida protein isolate showed the largest $d_{4,3}$ at 18.8 µm, followed by the Dolores and Croft isolates. The isolate from RLPY showed the smallest $d_{4,3}$ at 7.5 µm, followed by Ostinato and Astronaute isolates. The different particle sizes among the investigated cultivar isolates might lead to differences in physicochemical behavior as a result of different particle morphologies [51].

Table 5. Physicochemical and functional properties of pea protein isolates from different pea cultivars.

	Particle Size	Protein Solubility **		Emulsifying Capacity		Foaming Capacity
Cultivar	$d_{4,3}$	pH 4.5	pH7.0	pH 4.5	pH 7.0	pH 4.5
	[µm]	[%]	[%]	[mL g^{-1}]	[mL g^{-1}]	[%]
Navarro	13.19 ± 0.56	10.3 ± 0.2	51.5 ± 0.9	405 ± 1	600 ± 7	805 ± 0
Dolores	15.81 ± 0.06	7.4 ± 0.0	60.8 ± 2.8	340 ± 7	706 ± 14	808 ± 4
Greenwich	12.82 ± 0.19	8.8 ± 1.3	55.4 ± 3.4	396 ± 2	734 ± 7	839 ± 36
Bluetime	9.20 ± 0.49	7.7 ± 0.2	53.8 ± 2.4	365 ± 1	710 ± 8	915 ± 0
Ostinato	7.86 ± 0.02	8.3 ± 1.9	60.4 ± 1.9	385 ± 14	787 ± 32	959 ± 10
Kalifa	13.55 ± 1.53	7.3 ± 0.0	40.0 ± 2.1	354 ± 1	747 ± 3	911 ± 40
Salamanca	10.15 ± 0.40	5.9 ± 0.6	48.6 ± 3.6	378 ± 11	744 ± 2	835 ± 0
Florida	18.84 ± 1.31	0.9 ± 1.3	41.3 ± 7.1	340 ± 7	781 ± 23	884 ± 14
RLPY 141091	7.53 ± 0.01	2.3 ± 0.6	52.6 ± 2.8	359 ± 5	835 ± 7	874 ± 13
Orchestra	11.31 ± 0.21	1.5 ± 0.0	61.8 ± 6.0	366 ± 1	790 ± 6	835 ± 9
Astronaute	7.94 ± 0.29	6.3 ± 0.3	52.4 ± 0.9	381 ± 7	681 ± 23	858 ± 23
Croft	14.66 ± 1.35	0.0 ± 0.0	43.6 ± 5.1	355 ± 0	790 ± 24	861 ± 6

Results are expressed as means ± standard deviations. No significant differences were found among cultivars within the same column (Dunn's test with Bonferroni correction, $p < 0.05$). The particle size was based on Mie's theory (RI1.33). Note: $d_{4,3}$: volume weighted mean; ** based on protein content.

3.5. Functional Properties

High functional properties of PPIs are desired to increase their usage as ingredients in different plant-based food products. Table 5 shows the results of the functional properties.

3.5.1. Protein Solubility

At pH 4.5, the Navarro isolate showed the highest protein solubility at 10.3%, which was different to the isolate from Florida (0.9%), Orchestra (1.5%), and Croft (0.0%) cultivars. On the other hand, at pH 7.0, the Orchestra isolate showed the highest protein solubility (61.8%), followed by the Dolores (60.8%) and Ostinato (60.4%) isolates. Overall, the protein solubility levels at pH 7 were similar among the isolates. Other studies have shown similar solubilities or even values up to 80% at pH 7.0 [17–19]. The protein solubility level is related to extraction and drying methods; for example, isolates obtained after alkaline extraction and isoelectric precipitation have lower solubility than those obtained after salt-induced extraction [17]. Moreover, in contrast to lyophilization used in previous studies, spray drying leads to higher protein denaturation, increasing hydrophobic protein–protein interactions, and thus reducing overall protein solubility [52]. High protein solubility levels are, however, essential for beverage and dairy-alternative applications; treatments such as proteolysis or the addition of $_{L}$-Arginine and sodium carbonate are known to improve protein solubilities of PPIs [22,35].

3.5.2. Emulsifying Capacity

The isolate from Navarro showed the highest emulsifying capacity at pH 4.5 with 405 mL g^{-1}, while the one from Dolores and Florida showed the lowest. There were significant moderate correlations between the emulsifying capacity at pH 4.5 and both the protein solubility at pH 4.5 ($r = 0.50$) and the protein content ($r = -63$). On the other hand, the emulsifying capacity at pH 7.0 showed a significant positive moderate correlation with the protein content ($r = 0.45$). Thus, at neutral pH, the RLPY isolate showed the highest emulsifying capacity at 835 mL g^{-1} and was highly different from the isolates from Navarro and Astronaute cultivars. Hydrophobic residues are essential to facilitate protein oil interactions [53], however a high number of protein–protein interactions would form aggregates hiding hydrophobic residues, thus hindering the ability to interact with oil. These aggregates might be formed during spray drying, thus increasing particle size. However, no significant correlations were found between the emulsifying capacity and the particle size. Moreover, the vicilin/legumin ratio plays an important role in the formation of emulsions; Barac and Cabrilo [18] showed that the lower the ratio is, the higher the emulsifying capacity of the isolate, especially at neutral pH ranges. Although electrophoretic results showed no significant differences among allergens or overall in the electrophoretic patterns, further quantification of the fractions might be necessary to determine correlations with the functional properties.

3.5.3. Foaming Capacity

At pH 7.0 no foam formation was observed, whereas at pH 4.5 all isolates showed an average foaming capacity of 866%. These results are in contrast to the results of Chao and Aluko [54], who obtained higher foaming capacities the further the pH moved away from the isoelectric point. On the other hand, Gharsallaoui and Cases [55] suggested that close to the isoelectric point (pH 4.5), pea globulins are more surface-active and a reduction in the electrostatic charge of the protein molecules might result in electrostatic repulsion, in turn increasing adsorption. The latter is important for the formation of foam [56] and might explain the foaming capacity at pH 4.5 for the cultivars investigated in this study. Another explanation is that the fat content in the PPIs might have acted as an antifoam agent. In order to destroy a foam film, the hydrophobic particle droplets that emerge from the aqueous phase into the air–water interface are critical [57]. At pH 7, the hydrophobic protein surfaces facilitate the entrance of the fat droplets, leading to defoaming. At pH 4.5,

the hydrophobic side chains of the proteins are hidden, hindering the penetration of the fat droplets in the foam films.

A principal component analysis (PCA) was applied to analyze the relationships among the different cultivars and their colors, protein and fat contents, particle sizes, and physicochemical properties. Figure 2A shows a biplot of principal component (PC)1 and PC2 using the standardized scores for the isolates. The first two components of the PCA explained 57.13% of the total variance. The protein content (−0.44) and emulsifying capacity at pH 4.5 (0.51) had the strongest influence on PC1. On the other hand, the fat content (0.58) and foaming capacity (0.54) had the strongest influence on PC2; moreover, on the negative quadrant of the PC2, the particle size showed a strong influence (−0.41).

The isolate from Navarro cultivar scored the highest for PC1 (1.93), opposite to the isolates from Kalifa, RLPY, and Croft. This is in agreement with the emulsifying capacities shown in Section 3.5. Furthermore, the Dolores isolate scored the highest in the PC2 (−2.30), followed by Navarro (−1.51), as they showed lower fat contents among the isolates. Negative moderate correlations were found between the protein content and the protein solubility (pH 4.5) and emulsifying capacity (pH 4.5). On the other hand, the protein content was significantly positive and moderately correlated with the emulsifying capacity at pH 7.0. The particle size showed no significant correlations to other investigated attributes. When replacing a raw material in an existing product, not only are the composition and functionality important, but the color should be also considered, as it can affect the perception of the product by the consumer; for this reason, the ΔE^*_{ab} of the isolates was included in the PCA. However, the ΔE^*_{ab} showed low influence on any of the components.

The PCA shows two clusters plus two outliers. The isolates from RLPY, Croft, Kalifa, Florida, and Orchestra cultivars formed the first cluster; on the opposite side, isolates from Ostinato, Bluetime, Salamanca, Astronaute, and Greenwich cultivars formed the second cluster. These clusters suggest that the physicochemical characteristics are probably more similar and one cultivar could be replaced with another from the same cluster. On the other hand, the isolates from Navarro and Dolores were found to be further away from all other isolates, which might hinder the replacement of these cultivars. Moreover, the isolates should be chosen by considering the requirements of the final products. For example, the RLPY isolate could be used in applications with neutral pH, such as dairy alternatives, as it is plotted as having higher protein content, high emulsifying capacity (pH 7.0), and moderate protein solubility (pH 7.0); however, its application at low pH values is inappropriate due to its lower protein functionality. On the other hand, the Navarro isolate might be better suited in applications with acidic pH values, such as in plant-based mayonnaise.

3.6. Sensory Analysis

A principal component analysis was applied to analyze relationships between samples and retronasal aroma attributes and taste profiles (Figure 2B). PC1 and PC2 represented 66.03% of the total variance; the following values represent the coefficient values (influence) of the attributes and the scores of the isolates from each cultivar.

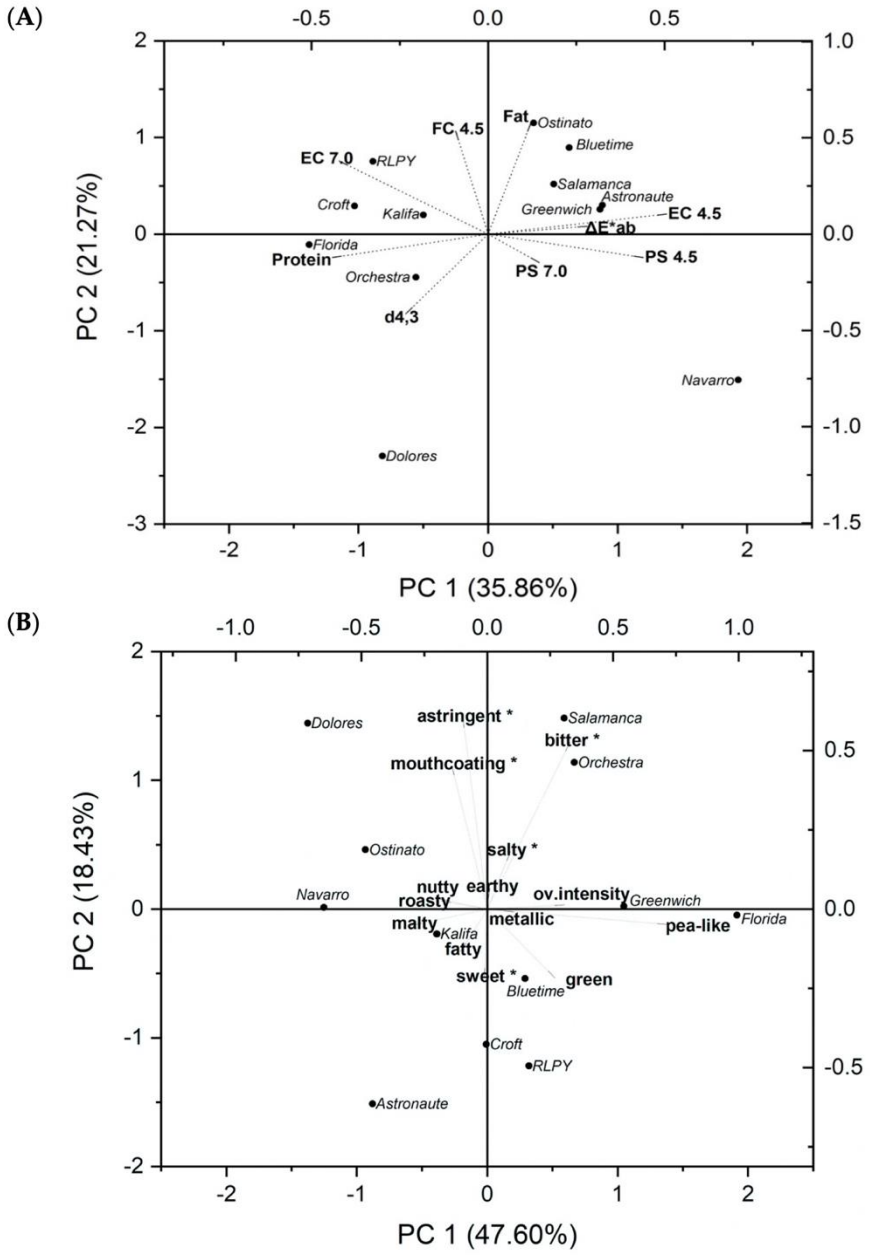

Figure 2. Biplot of (**A**) physicochemical properties and (**B**) sensory profiles of pea protein isolates from different pea cultivars. Attributes with an asterisk (*) refer to taste. PS: protein solubility; EC: emulsifying capacity; FC: foaming capacity; ΔE^*_{ab}: color difference compared to a white reference; $d_{4,3}$: particle size. The numbers represent the pH (7.0 or 4.5) in which the analysis was performed.

Aroma. According to a one-way ANOVA, *pea-like* was the only aroma attribute with a significant difference among isolates and showed the strongest influence on PC1 (0.71), followed by *malty* (−0.30) and *green* (0.27) aromas. For PC2, the *green* aroma showed the strongest influence among all aroma attributes (−0.22). The *metallic*, *earthy*, *roasty*, and *nutty* attributes showed almost no influence on any of the components. The isolates from Dolores (−1.38) and Navarro (−1.25) scored the lowest for PC1, which suggests these isolates were perceived to have the least *pea-like* aroma. In contrast, Florida (1.92) and Greenwich (1.05) isolates scored the highest for this component, indicating a stronger *pea-like* aroma. The *pea-like* aroma is known to be well-perceived because of the low thresholds of 2-isopropyl-3-methoxypyrazine [58], which might explain its strong influence. The PCA showed the isolates from Greenwich, Florida, RLPY, and Croft cultivars as being closer to the *green* attribute, in agreement with the results from the sensory analysis. The *green* aroma originating from hexanal is a characteristic oxidation product of fatty acids, in particular of linoleic and linolenic acids catalyzed by LOX [44]. Higher activity of this enzyme could increase the *green* aroma perception; however, there was only a moderate negative correlation ($r = -0.54$) between the *green* aroma and the LOX band intensities under reduction conditions. Although it has also been mentioned that green cultivars have higher levels of hexanal [8], there was no significant correlation between the a* color and *green* aroma.

Environmental and genetic conditions might affect the production and degradation rates of the aroma compounds, which would result in higher or lower aroma perceptions [8,59]. Using HC/MS analysis, Azarnia and Boye [8] found that the concentrations of volatile compounds depended on the cultivar, crop year, storage, and processing conditions. Specific processing methods such as enzymatic treatment or fermentation might be useful to reduce some of the characteristic off-flavors of pea isolates. However, other aroma compounds might be generated or enhanced and might further increase or decrease consumer acceptance [4,60,61]. Furthermore, methoxypyrazines are very stable during fermentation due to their chemical nature, and therefore are very difficult to remove or reduce [62]. Therefore, pea cultivars low in *pea-like* aroma, such as from Dolores or Navarro, are recommended to be used for production of PPIs with sensory appeal.

Taste. The *bitter* attribute was the only significant taste attribute according to one-way ANOVA. The *bitter* (0.53) and *astringent* (0.60) tastes had the strongest influence on PC2. As shown in the PCA, the PPIs from Salamanca and Orchestra scored highest for bitter taste. In contrast, the PPI from Astronaute scored lowest for *bitter* taste and was significantly less *bitter* than the PPI from Salamanca. The Dolores isolate scored the highest for *astringent* taste, together with Salamanca and Orchestra isolates. Moreover, *salty* and *sweet* tastes had little influence on either component, which suggests that the intensity of these tastes was lower and similar among the samples. A high *bitter* taste for a PPI might hinder its application in food products; thus, several methods have been investigated to reduce the bitterness of legume protein isolates [61,63].

Overall Intensity. The isolate from Florida cultivar showed the highest overall intensity, whereas the isolate from Dolores showed the lowest intensity. However, the overall intensity levels among the PPIs were not significantly different. The overall intensity was moderately correlated with the *pea-like* ($r = 0.66$), *green* ($r = 0.43$), and *malty* ($r = -0.47$) aroma, which suggests that these compounds were characteristic of the isolates, as mentioned previously in the aroma section.

4. Conclusions

Peas are a valuable source of protein and are increasingly used in plant-based products; however, due to the large number of different cultivars, most of them have not been characterized regarding their chemical composition, functional properties, and sensory profiles. In this study, all these aspects were investigated for 12 cultivars grown in Germany and France.

Our study shows that the chemical composition of flour and isolates from the cultivars are slightly different. The main allergen fractions were present in all the PPI and showed no significant differences. The PCA showed two cluster of cultivars regarding the physicochemical and functional characteristics; however, these clusters were not found in the sensory profile PCA. This suggests that although some isolates could be substituted interchangeably for the same products with regard to their similar functionalities, the flavor of these products could be affected. However, only the *pea-like* and *bitter* aromas were significantly different among isolates. The cultivars Salamanca and Astronaute are the most used cultivars in Germany; they showed similarities according to the physicochemical-functional PCA cluster; however, Salamanca isolates had a significant higher bitter taste and slightly higher *pea-like* aroma than Astronaute. These differences should be considered for targeted product developments as they might influence the acceptance by consumers. The usage of cultivars such as Navarro and Dolores should be carefully considered, as their isolates are mostly different to the other cultivars investigated in the present study.

The obtained PPI might be used in the food industry, especially under neutral conditions (pH 7.0), except when foaming is required; however, when designing a food product in the acidic range at pH 4.5, the specific selection of a suitable cultivar might be more important. Differences with laboratory and commercial processing of PPI should be considered; although spray drying was used in this study, larger spray-dryers may affect the physicochemical, functional and sensory properties of the isolates. The results of this study highlighted the importance of a tailored selection of cultivars for protein extraction as well as the suitability of pea cultivars for specific food applications.

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Discussion

Plant-based diets have gained popularity in the last decade. These diets aim to target health and environmental issues. Following plant-based diets can reduce greenhouse gas emissions by 65% compared to high meat diets (Kolbe, 2020) and can reduce the incidence of chronic diseases such as diabetes and cardiovascular disease (Kahleova, Levin, & Barnard, 2017; Kahleova, Tura, Hill, Holubkov, & Barnard, 2018). However, people following plant-based diets are at risk of a deficient protein intake (Bakaloudi et al., 2021). The food industry is looking for high quality protein ingredients that are economically and readily feasible to replace soy and to be adapted in large-scale production. In this context, protein ingredients from pea have been investigated profoundly, because peas are not considered main allergen, they feature a high protein content, its production is more sustainable compared to other pulses and plant-based protein sources. However, pea proteins exhibit poor functional properties and an unpleasant flavor compared to soybean proteins, which limit their overall quality and application potential. Furthermore, recent studies have shown an increase in pea allergy prevalence, which might hinder their application in food products.

Several studies have investigated different methods focusing mainly on the effects on protein functionality and to a lesser extent on effects on the sensory profile. Depending on the legume, further studies have been performed to investigate the allergenicity of modified legume proteins. However, fewer studies have focused on pea proteins compared to soybean proteins. Due to the increasing interest by food industry, the development of pea ingredients with higher product quality is of high interest.

Therefore, the goal of the present dissertation was to elucidate optimal modification techniques of isolated pea proteins in order to increase the quality of the protein isolate regarding

- Functional properties
- Sensory acceptance
- Degradation of potential allergens

Special emphasis was placed on non-thermal treatments such as enzymatic hydrolysis and fermentation, which represent established methods that could be easily implemented.

Functional properties of modified pea proteins

The food industry complies a great number of food products in which different protein ingredients are used. The substitution of animal proteins with plant proteins raises a new level of challenges. Animal proteins have been profoundly studied regarding their interaction with the food

matrix and effects on human health. The main functional properties of animal proteins such as whey, casein and egg proteins include foam capacity, gelling and emulsifying capacity. Because soy was the only plant-based raw material providing protein functionalities that could compete with the ones from animal sources, it was the unrivaled crop used as functional ingredient in industrial scale for the past decades. However, in recent years the demands on plant-based functional ingredients are increasing towards environmental, sensory and health-related requirements. Consequently, increasing research has been performed on the functionality of alternative, regional plant-based proteins especially from pulses. It has been shown, that the extraction method affects protein functionality (Momen, Alavi, & Aider, 2021; Schutyser, Pelgrom, van der Goot, & Boom, 2015). In general, pea proteins have shown reduced functional properties compared to the ones from soybean. Several studies have been conducted to understand and improve functional properties of pea proteins but have been limited on their scope regarding active ingredients such as enzymes or microorganisms as well as time of treatment and further analyses.

Plant-based protein isolates are used since decades to positively influence the texture properties of food products. For example, soy protein was used industrially even before the number of vegan-vegetarian people rose sharply. The main purposes were to

- substitute expensive animal-based protein and meat
- benefit from the adhesive protein properties for food production
- stabilize complex food matrices
- influence and optimize food texture properties and creaminess

Proteins require an optimal interaction with the food matrix for texture and mouthfeel optimization. Therefore, the most relevant functional properties of protein isolates are:

1. Protein solubility
2. Emulsifying capacity
3. Foaming capacity

The protein solubility is considered as a requirement for other properties, such as foaming and emulsifying capacities. Proteolysis most certainly promoted hydrophilic interactions (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016) changing protein structure and electrostatic forces (Lam, Can Karaca, Tyler, & Nickerson, 2016) as partial enzymatic proteolysis improved proteins solubility especially at pH 4.5 (Chapter 1), which is in agreement with studies using pea and different protein legumes (Coscueta, Campos, Osório, Nerli, & Pintado, 2019; Klost & Drusch, 2019; Schlegel, Sontheimer, et al., 2019). On the other hand, fermentation of pea proteins (Chapter 2) showed a slight increase of solubility at pH 4.5 but had a negative effect on the

solubility at neutral pH regardless of the microorganism used. These microorganisms were selected due to potential proteolytic activity but it seems that the extent of proteolysis was not enough or the production of other compounds reduce the ability of protein to interact with water. Other studies have found similar protein solubility results using lactic acid bacteria (MeinIschmidt, Ueberham, Lehmann, Schweiggert-Weisz, & Eisner, 2016; Schlegel, Leidigkeit, Eisner, & Schweiggert-Weisz, 2019). However, in combination with enzymatic proteolysis (Chapter 3), regardless if fermentation was before or after or of the pH value, the protein solubility was higher compared to only enzymatically treated pea proteins. This suggests that the combination of treatments, especially fermentation followed by enzymatic hydrolysis, allowed a different hydrophilic interaction probably to a higher release of smaller peptides, amino acids and probably higher number of carboxyl groups.

Emulsifying capacity showed different results even within same treatments. Single enzymatic treatment of pea proteins improved significantly the emulsifying capacity whereas the combination of enzymes and lactic acid bacteria showed a significant reduction compared to the untreated pea protein isolate. This suggests that the enzymatic proteolysis promotes the redirection of amphiphilic residues to the water-oil phase reducing the interfacial tension; however, lactic acid bacteria and their released compounds affect negatively this interaction probably rising from a change in surface charge, interaction between bacteria cells and proteins (Daeschel & McGuire, 1998; Marín et al., 1997) as well as agglomeration due to by-products interaction.

On the other hand, the foaming capacity was significantly higher on hydrolysates with single enzyme proteolysis or in combination with fermentation, although the latter in a lesser extent. Fermentation by itself showed no effect in foaming capacity compared to the untreated pea protein isolate. The ability of protein to promote good emulsifying and foaming capacities is known to depend on high hydrophobic surface, low electrostatic repulsion and low surface tension (Barac, Pesic, Stanojevic, Kostic, & Cabrilo, 2015; Lam et al., 2016; Zayas, 1997); however, the difference in why the combination of treatments shows a reduced emulsifying capacity but higher foaming capacity is not completely understood. Other studies have shown contradictory results regarding the ability of pea protein to create emulsion depending on the protein molecular size (Barac et al., 2010; Peng et al., 2016). It might also be possible that there is a particular and no-yet studied interaction between fermentation by-products and specific peptides.

These results suggests that the selection of enzymes, microorganisms and experimental design have a great influence on the changes of functionality of pea proteins; however, comparing with others studies, the raw material and protein extraction method has also a great influence in the results with each experiment. The application of pea protein hydrolysates with single enzyme treatment and in combination with fermentation should be assessed in different food such as dairy

alternatives, drinks, protein supplements, and bakery products. These assessments will validate the use of modified pea proteins as food ingredients and also will verify which treatment might suit better to a specific application.

Sensory profile of modified pea proteins

When developing or improving a food product using new ingredients, the most important characteristics for the consumer are the internal factors such as flavor, taste, smell and texture (Chen & Antonelli, 2020). Taste and smell are the main attributes that help the consumer to choose to buy again a product (Liem & Russell, 2019; Nadathur, Wanasundara, & Scanlin, 2017). The legume family is characterized for having flavor compounds like beany, earthy, green and bitter (Boyaci Gunduz, Gaglio, Franciosi, Settanni, & Erten, 2020; Kaczmarska, Chandra-Hioe, Frank, & Arcot, 2018; Trindler, Annika Kopf-Bolanz, & Denkel, 2022). However, aroma and taste profile depend of each individual legume and also within the different cultivars of the same legume, environmental characteristics, harvest time and storage. Peas are widely known for their beany and green off-flavors and bitter taste. These characteristic pea flavors can be masked in food products using a higher sweetness level or additional aromas extending, however, the ingredients list, which now plays an important decision factor for the consumer. Fermentation is an effective and “clean” method to modify the flavor profile of different food matrices.

Microorganisms’ metabolism is responsible for the production of aromatic compounds and by-products which affect the sensory profile of the substrate being fermented (Engels et al., 2022; Y. Wang et al., 2021). Therefore, within this dissertation controlled microbial fermentation with food grade strains was investigated in order to refine the flavor profile of pea protein isolates to be used as fermented ingredients for food applications and beverages.

Fermentation of pea protein isolates with lactic acid bacteria affected the sensory perception (Chapter 2); the sensory profile obtained by the fermentation of each individual strain and duration of the fermentation resulted in significantly different perceptions. Variables investigated were the use of aerobic and anaerobic fermentation strains as well as the different fermentation times such as 24h and 48 h. A principal component analysis showed clusters according to the fermentation times; the 24-h fermented pea protein isolates presented less intensity of attributes, especially *Lactobacillus plantarum,* but longer fermentation increased the intensity of some attributes and reduced preference; this suggests that controlled and rather shorter fermentation is required to obtained an appealing flavor and acceptance. Among the selection of strains, *Lactobacillus plantarum* showed the least pea characteristic off-flavors giving a more “neutral” perception. This is in agreement with others studies which have found that *Lactobacillus plantarum* has the ability

to decrease aldehyde and ketone concentrations responsible for the beany off-flavors (Schindler et al., 2012; Shi, Singh, Kitts, & Pratap-Singh, 2021).

Fermentation has also been used as a debittering method for enzymatic hydrolyzed legumes (Meinlschmidt, Schweiggert-Weisz, & Eisner, 2016; Saha & Hayashi, 2001; Tchorbanov, Marinova, & Grozeva, 2011). Hydrolyzed protein products such as baby and elderly formulas, collagen powders and sport drinks have been in the market for several years and they continue to increase due to recent findings of their health benefits (Etemadian et al., 2021; Moreno-Valdespino, Luna-Vital, Camacho-Ruiz, & Mojica, 2020). However, enzymatic proteolysis is responsible for the production of bitter peptides (Adler-Nissen & Olsen, 1979; Matoba & Hata, 1972) and hinders the acceptance by the consumers; the intensity of bitterness depends on the enzyme, substrate, degree of hydrolysis and the peptides cleaved. Usually, longer times and higher degrees of hydrolysis have shown stronger bitter intensities (Chapter 1). The hydrolysates also showed a decrease in other pea characteristic off-flavors but it might be attributed rather to the increase in bitterness. However, the sensory evaluation was performed with highly-hydrolyzed samples or least-hydrolyzed samples which only included endopeptidases; exopeptidases might promote other profile which might or might not improve the sensory perception of the product. Flavourzyme is an exopeptidase which, in this study, did not promote larger changes in the protein distribution and, therefore, was not selected for further sensory analysis. However, other studies have shown contradictory results regarding an improvement of taste using this enzyme, mainly used as second stage hydrolysis (Meinlschmidt, Schweiggert-Weisz, Brode, & Eisner, 2016; Xia et al., 2022). In contrast, the results obtained in this work from the proteolysis with alcalase are in accordance with different studies (Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016; Schlegel, Sontheimer, et al., 2019; Xia et al., 2022), showing a high degree of hydrolysis and also a high increase in bitterness.

For the combination of enzymatic proteolysis and fermentation (Chapter 3), three enzymes and one lactic acid bacteria were selected according to their effects on proteolysis and effects on sensory profile. In this chapter, the pea off-flavors *pea-like*, *green*, and *beany* were grouped in a "*plant-like*" attribute. The combined methods still showed an increase in bitterness for samples hydrolyzed with Esperase® but all hydrolyzed samples significantly showed a reduced *plant-like* off-flavor. However, the results obtained by the trained panel showed contradictory information compared to Chapter 1. In further studies, the single hydrolyzed and combined methods should be validated by quantitative methods such as e-tongue system or sensoproteomics. The latter aid to investigate the peptide fractions obtained after pea proteolysis, their interaction with aroma compounds and their contribution to new aroma compounds. These studies would allow a deeper understanding of off-flavor formation, mitigation and interaction as well as the effects on protein

modification, consumer perception, and food application (Mittermeier-Kleßinger, Hofmann, & Dawid, 2021).

Immunogenicity of modified pea proteins

Peas are not included in the European allergen list and do not need to be declared as allergens. However, their increasing allergenicity incidence is being evaluated and scientists are recommending their inclusion into the list or a rise in awareness for consumers (Codreanu-Morel, Morisset, Cordebar, Larré, & Denery-Papini, 2019; Dreyer et al., 2014; Hildebrand et al., 2021; Lavine & Ben-Shoshan, 2019).

Different methods have been studied in order to reduce allergenicity of legumes. Heat processing has been highly studied (Comstock, Maleki, & Teuber, 2016; Malley, Baecher, Mackler, & Perlman, 1975; Son et al., 2000; Verma, Kumar, Das, & Dwivedi, 2012); however, Malley et al. (1975) found that pea albumins were highly heat resistant retaining their allergenicity upon cooking or boiling but are partly degraded upon autoclaving at 120ºC for 15 min. Lidzba et al. (2021) obtained inconclusive results after thermal treatment of pea proteins and the reduction of Pis s1 and Pis s2. Hypoallergenic formulations are mainly produced through enzymatic hydrolysis (Kiewiet, Faas, & De Vos, 2018), whereas fermentation has been less studied to reduce immunogenicity, specially using pea proteins.

In the present dissertation, the enzymatic treatment of pea proteins (Chapter 1) showed that Pis s2 was more susceptible for degradation even at short proteolysis times and it also depended on the enzyme used. Pis s1 was more resistant and was highly dependent on the enzyme and treatment time. The 32 kDa protein fraction obtained by proteolysis of the Pis s1, vicilin $\alpha\beta$, was also maintained except for the papain and Esperase® proteolysis for 2 hours. Therefore, the proteolysis of pea proteins varied significantly even when using different enzymes of the same enzyme family, suggesting the importance of the cleavage site. On the other hand, fermentation of pea proteins with different lactic acid bacteria (Chapter 2) did not show significant changes in the molecular weight distribution, which is in accordance with Licandro et al. (2020), Meinlschmidt, Ueberham, et al. (2016), and Schlegel, Leidigkeit, et al. (2019). Presumably the included biomass of bacteria cells and metabolites produced during fermentation or after neutralization of the samples such as sodium lactate (Yen, Chen, Pan, & Wu, 2010) influenced the final product properties. The lack of significant changes in the protein distribution after fermentation suggests the prevalence of the immunogenicity of the allergenic fractions. The usage of precise fermentation is increasingly being investigated. Using this technology, microorganisms are genetically modified to produce specific products (Spinnler, 2021). An efficient way to reduce the

immunogenicity of the protein isolates could be the genetical modification of microorganisms to obtain enzymes that will specifically hydrolyze the allergen fractions in question. This would save time and costs for exploring the most suitable fermentation strain for allergen degradation.

Moreover, the combination of methods (Chapter 3) was most effective in changing the molecular weight distribution, especially for the hydrolysis of fermented pea protein isolates. This thesis proves the ability of *Lactobacillus plantarum* to metabolize smaller peptides (Corsetti & Valmorri, 2011; Kleerebezem et al., 2003) and to promote proteolysis of some protein fractions which can be further hydrolyzed by enzymes.

However, the electrophoretic results should be verified by immunological techniques or proteomic analysis. Allergenomics is a recent term used for the study of allergens using targeted or untargeted proteomics (Picariello, Mamone, Addeo, & Ferranti, 2011). Within the present dissertation, a mass spectrometry proteomic analysis was performed (not published) comparing the flour, pea protein isolate and the 2-h trypsin-hydrolyzed sample as one example of protein modification by enzymatic hydrolysis. The analysis showed the presence of Pis s1 and Pis s2 for all samples and a slight reduction of both potential allergens in the hydrolyzed sample as well as lower amounts in the flour (Table 1).

Table 1. Abundance of allergen fractions identified by mass spectrometry

					LFQ Intensity		
Allergen	**UniProt accesion number**	**Fasta Header**	**Mol. weight [kDa]**	**Unique peptides**	**Pea Flour**	**Pea Protein Isolate**	**2-h Trypsin hydrolysate**
Vicilin	P13918	Vicilin	52.231	77	6.07E+11	2.00E+12	1.60E+12
(Pis s1)	Q702P0	Pis s 1.0102	47.297	7	3.55E+10	4.73E+10	4.75E+10
	Q702P1	Pis s 1.0101	47.278	11	4.73E+11	1.53E+12	1.17E+12
	P02855	Provicilin	31.54	32	1.24E+11	7.02E+11	6.83E+11
	P02856	14kD component	14.039	10	4.96E+10	1.63E+11	2.43E+11
Convicilin	CAB82855	Convicilin	72.062	28	4.00E+11	2.01E+12	1.44E+12
(Pis s2)	P13915	Convicilin	66.989	9	1.83E+10	4.04E+09	1.46E+09
	P13919	Convicilin	46.396	1	5.44E+07	0.00E+00	0.00E+00

LQF Intensity: Label-free quantification

Mol.Weight: molecular weight according to the theoretical values in UniProt database

The color illustrate the intensity from a high (green) to a less (yellow) abundance of proteins

However, there are several limitations of a proteomic analysis of allergens: 1) the identification is limited to the protein sequences found in the database and each pea cultivar could have different sequences; and 2) to perform the LC-MS/MS analysis, a further tryptic digestion was applied, which modifies the primary and secondary structure of the proteins during sample preparation and

might complicate the interpretation of proteomic results. This method seems more useful when an identification of allergens in food matrixes is needed and together with IgE mapping might result in better risk assessment of foods (Di Girolamo, Muraca, Mazzina, Lante, & Dahdah, 2015). Thus, the reduction in immunogenicity should be assessed by methods that not only investigate a reduction in allergen fractions but also measure the degradation of the target epitopes.

Methods such as such as Enzyme-linked Immunosorbent Assay (ELISA), Western-blot, Prick- and Challenge tests are used to understand the results of these modification on the IgE binding capacity of sensitized sera to the treated proteins. In this thesis, an indirect ELISA confirmed the electrophoretic results (Chapter 3) and is also in accordance with the results obtain by Lidzba et al. (2021), who previously studied the effects of flavourzyme, papain and pepsin by means of ELISA and found a reduction especially for Pis s1. Although in the present thesis no specific IgE antibodies for Pis s1 and Pis s2 were available, an overall reduction of immunogenicity of the combined methods was proven and compared to the electrophoretic results; the results showed that mainly large peptides were degraded into smaller ones with exception of few higher molecular weight proteins proven resistant to the applied treatments. These results are also in accordance with the SDS-PAGE results obtained in Chapter 1 showing a reduction in larger protein fraction by means of enzymatic hydrolysis. To our knowledge there is only one study which assessed the allergenicity of fermented pea flour with different microorganisms. Barkholt et al. (1998) showed that only *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Lactococcus raffinolactis* and *Rhizopus microsporus* reduced the antigenicity to 10% compared to the untreated flour. However, it was not mentioned the protein content in the studied samples or the soluble proteins in the SDS-PAGE buffer. For this reason, the alleged reduction in allergenicity might be due to a reduced protein solubility rather than to changes in the protein structure of the epitopes. In order to perform a more accurate investigation regarding the effects of proteolysis on immunogenicity, it might be necessary to use monoclonal antibodies specific against the potential allergen fractions of pea, namely Pis s1 and Pis s2. Lidzba et al. (2021) successfully generated monoclonal antibodies against recombinant pea Pis s1 and proved their efficacy by means of ELISA.

Furthermore, to confirm the results from the ELISA in this thesis, a Western blotting was performed using the same IgE antibodies (not published). Figure 1 shows that the IgE binding of the fermented sample remained similar to the untreated pea protein isolate, while it was reduced for the hydrolyzed and combined hydrolyzed and fermented samples. The Western-blot also shows the further decrease in binding when using fermentation of pea protein isolate followed by enzymatic hydrolysis, especially regarding the Pis s1 allergen fraction. These results confirm the degradation of allergenic fractions shown by SDS PAGE and ELISA in the scientific papers

published in relation to this dissertation. Further tests with sera from sensitized patients and tests directly in patients are necessary to confirm the production of reduced allergenicity or even hypoallergenic pea protein ingredients.

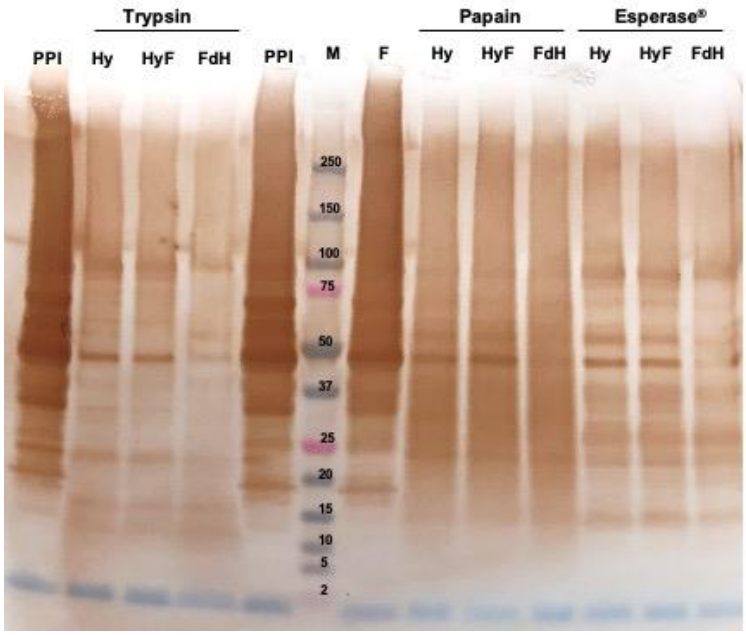

Figure 1 Western Blot of pea protein isolate (PPI) and treated samples using *L. plantarum*, enzymes and combination of both. P: papain; E: Esperase®; T: trypsin; Hy: hydrolysis; F: fermented PPI; HyF: hydrolysis followed by fermentation; FdH: fermentation followed by hydrolysis; M: molecular weight standard, indicated in kilo Dalton (kDa).

Cultivar differentiation

Besides the impact of different treatments on protein functionality, sensory profile and immunogenicity, variation in results might occur within the same legume species. Extrinsic factors such as climate conditions, harvest time and storage are known to affect the overall aspects of proteins from different cultivars (Nikolopoulou, Grigorakis, Stasini, Alexis, & Iliadis, 2007; N. Wang, Hatcher, Warkentin, & Toews, 2010). The first three chapters were performed using the same pea cultivar “Navarro” but there are other pea cultivars used in Germany and the European Union. Hence, the fourth study (Chapter 4) screened the main pea cultivars used by the food industry in Germany and France regarding functionality, sensory profile and allergen fractions of the pea protein isolates. Although the protein content differed significantly, the protein functionality was similar among the cultivars. Interestingly, the characteristic pea off-flavor and *bitter* taste was significantly different among cultivars. Several studies have investigated the differences in protein functionality and aroma profiling using different cultivars and extraction methods (Azarnia et al., 2011; Barac et al., 2010; Cui, Kimmel, Zhou, Rao, & Chen, 2020; Guleria, Dua, & Chongtha, 2009;

Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015); However, the results from the different studies are hardly comparable as the evaluation methods varied. This encourages further studies with a greater number of cultivars or to establish universal methods that are easy and practical to apply in different research groups.

The protein weight distribution of different pea cultivars showed slight differences which disagrees with the electrophoretic and densitometric results from Barac et al. (2010). They showed larger differences in intensity and protein concentration. The allergen differences among pea cultivars have not been investigated so far; however, several studies have investigated the epitopes of different cultivars in a variety of pollen- and food sources (Alché et al., 2007; Dvořáček et al., 2022; Kwaasi, Parhar, Tipirneni, Harfi, & al-Sedairy, 1994; Malalgoda, Meinhardt, & Simsek, 2018). These studies have found cultivar-specific epitopes, which arise the concern of further and deeper research on the most common pea cultivars and their specific epitopes; this would increase the database in which different pea proteins could be compared and would allow a proper cultivar selection for further applications and health claims.

Final conclusions

For the continuous improvement and development of pea protein ingredients, a deeper understanding is necessary on how different technologies affect protein structure and their functionality, sensory profile and immunogenicity. Several studies have been performed in order to investigate the effects of enzymatic hydrolysis and fermentation on pea protein; however, they studied one or two aspects but never functionality, sensory profile and immunogenicity altogether. This thesis combined both treatments in different order for the first time and further studies might profit from this knowledge as the results showed significant differences.

The technologies proved efficient in modifying protein isolates with *L. plantarum* fermentation followed by tryptic proteolysis showing the most appropriate method in order to obtain a pea protein isolate with optimized functional properties, a neutral taste and reduced immunogenicity. Further research should be conducted to understand how harvest and storage time affects protein fractions in the isolates and also how these affect different functionalities and further applied treatments. Also, it would be necessary to understand the changes in other compounds such as specific fatty acid composition, which would might affect functionality and probably the sensory profile and the aroma compounds further extracted. Further quantitative analysis of the volatile and non-volatile compounds would increase the confidence on the results before or after a trained-panel uses a more subjective approach. Although only one chapter (Chapter 3) proved that the combination of technologies was able to reduce the immunogenicity of treated samples with high degrees of hydrolysis by means of indirect ELISA, it also proved that the degree of hydrolysis and electrophoretic results are a valid indication of changes in the epitope of pea proteins. Further studies are necessary to assure a correct labeling of these ingredients as reduced-allergenic or hypoallergenic depending on the reduction in antigenicity.

Furthermore, due to the constant improvements in protein extraction technologies, it is necessary to understand how protein fractions and its allergens will be affected, and thus, the interaction with aroma compounds and their further modifications.

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