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Barley proteins – source and factor of haze formation in beer

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„Je planmäßiger die Menschen vorgehen,

desto wirksamer trifft sie der Zufall.“

Friedrich Dürrenmatt

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1 Abstract

Turbidity gives the first visual impression of beer quality to the consumer. Consumers expect from a filtered beer a clear, bright, non-hazy product which remains so during the shelf life of the product. Hazy products are often regarded as defective and perhaps even potentially harmful. Therefore, haze formation is an important problem in beer production. For breweries not only costs from rejected turbid beers and therefore an “image problem” arises, but also increased costs because of raised use of filter aids have to be considered. Data from leading manufacturer of filter aids showed that the costs of kieselgur consumption can be more than doubled in case of filtration problems due to turbidity. According to experience in haze identification at the “Lehrstuhl für Brau- und Getränketechnologie”, in Weihenstephan, an impact of protein content in barley and different modified malts on haze formation directly after filtration could be observed. This surveillance was the motivation for the intensive study of the influence of barley proteins on haze formation in beer. This work was accomplished with the intention to understand changes over the malting and brewing process in protein content and composition and their influence on haze formation in filtered beer.

This thesis therefore presents an overview of several research studies and analytical methods on haze formation, protein analytic and haze identification. An overall picture of the role of protein haze particles was provided. Some proteins have already been found (protein Z, LTP1) influencing haze formation, but up to now barley proteins have not been followed from barley into the finished beer, in their respect to influence beer turbidity. For this reason special focus lied on changes in protein content and composition from barley to finished beer. It was also investigated how different malt modification changes the protein composition in finished beer and how these differences influence final beer quality, e.g. turbidity directly after filtration. These changes were analytically followed with global nitrogen measurement (Kjeldahl method and determination of free amino nitrogen), a Lab-on-a-Chip technique and 2D-PAGE. Turbidity was measured with a two angle turbidity measurement instrument.

The first approach was to prove the existence of differences in protein composition of beer brewed with 100 % barley raw material to beer brewed with 100 % barley malt.

Differences in the protein composition of the final beer could be revealed and it could be observed that the malting process was the reason of these differences. This was the motivation to find the initial point of changes during malting in protein composition in beer. The first step was a research on the influence of malting (different proteolysis stages) on protein composition in respect to protein haze in beer.

It was possible to show simple and reproducible haze identification methods for the brewing industry to track the source of haze formation. Differences in final beer quality and protein composition of beer brewed with 100 % barley raw material in comparison to beer brewed with 100 % barley malt could be shown. Subsequently malt with different germination states was produced, to find a protein fraction which correlates with haze formation in beer. With this experimental setup a new, not yet identified haze forming fraction of 28 kDa was found in the beer. This fraction could be tracked from barley over the malting process to the finished beer.

2 Zusammenfassung

Die Gewährleistung einer konstant bleibenden Produktqualität über einen längeren Zeitraum hinweg ist eines der Hauptziele der Getränkeindustrie. Denn Biertrinker erwarten von einem gefilterten Bier, dass es bis zum Ende seines Haltbarkeitsdatums seine Klarheit behält. Trübe Biere, oder Biere die Partikel enthalten, hinterlassen unverzüglich einen negativen Eindruck, da sie den Anschein erwecken können, dass eventuell sogar eine potentielle Gefährdung gegenüber des Biergenießers besteht. Brauereien müssen nicht nur mit dem entstandenen Schaden durch das Image-Problem kämpfen, sondern auch mit erhöhten Kosten während der Produktion (Filterhilfsmittel). Das Problem ist, dass selbst einwandfrei filtriertes und biologisch sauberes Bier nach längerer Lagerung allmählich seinen Glanz verliert, bis es schließlich zur Bildung einer sogenannten kolloidalen Trübung bzw. eines Bodensatzes kommt. Dies wird vom Verbraucher nicht akzeptiert und mit einer Qualitätsminderung gleichgesetzt.

Am Lehrstuhl für Brau- und Getränketechnologie hat sich über die Zeit eine Kompetenz zur Trübungsidentifizierung entwickelt. Aufgrund von Beobachtungen über einen längeren Zeitraum und Anfragen aus der Industrie, konnte festgestellt werden, dass Trübungen insbesondere schon nach dem Filter auftreten können, wenn unterschiedlich gelöstes Malz verwendet wurde. Aufgrund dieser Beobachtungen wurde in dieser Arbeit versucht, die Veränderungen der Gerstenproteine über den Mälzungs- und Brauprozess zu verfolgen und so deren Einfluss auf eine Trübungsbildung schon direkt nach der Filtration festzustellen.

In dieser Doktorarbeit wurde daher ein Überblick über sämtliche Forschungsarbeiten zum Thema Trübungsbildung, Trübungsidentifizierung und Proteinanalytik gegeben. Zusätzlich wurde eine allumfassende Darstellung der Rolle von proteinischen Partikeln in der Trübungsbildung im Bier aufgezeigt. Anhand dieser Literaturrecherche kann gesehen werden, dass schon einige spezifische Proteine identifiziert wurden (LTP1, Protein Z), die im Bier trübungsverursachend sind. Bis jetzt wurde aber noch nicht versucht, Gerstenproteine über den Mälzungs- und Brauprozess zu verfolgen und ihren Einfluss auf die Trübungsbildung zu belegen. Aus diesem Grund wurde, in der vorliegenden Arbeit, versucht die Unterschiede in

Proteingehalt und -zusammensetzung von der Gerste, über das Malz, bis hin ins fertige Bier zu erfassen.

Die Vorgehensweise zur Erfassung dieser Unterschiede war folgende. Zuerst wurden die Unterschiede in Proteingehalt und –zusammensetzung zwischen 100 % Gerstenrohfruchtbieren und Allmalzbieren und deren Einfluss auf Bierqualitätsparameter, vor allem Trübungsneigung, untersucht. Aufgrund der Unterschiede, vor allem in Proteingehalt und –zusammensetzung, wurde angenommen, dass vor allem der Mälzungsprozess verantwortlich für diese Abweichungen ist.

Daraufhin wurde Gerste bei unterschiedlichen Bedingungen (Keimtemperatur, Weichgrad und Keimdauer) vermälzt, um aufgrund der nun entstandenen unterschiedlichen Lösungsgrade Rückschlüsse auf eine Trübungsbildung proteinischer Ursache zu erhalten. Mit Hilfe dieses Versuchsaufbaus konnte eine Proteinfraktion von 28 kDa gefunden werden, welche eine erhöhte Trübung schon am Filterauslauf verursacht.

3 Index

4 Preamble

4.1 List of Reviewed Publications

(1) Steiner, E., Gastl, M., Becker, T., 2011. Protein changes during malting and brewing with focus on haze and foam formation: a review. Eur. Food Res. Technol. 232, 191-204.
(2) Steiner, E., Back, W., 2009. A critical review of protein assays and further aspects of new methods in brewing science. Brewing Sci. 62, 90-94.
(3) Steiner, E., Becker, T., Gastl, M.: Turbidity and Haze Formation in Beer – Insight and Overview. J. Inst. Brew. 116 (4), 360–368, 2010
(4) Steiner, E., Auer, A., Becker, T., Gastl, M.: Comparison of beer quality attributes between 100% barley malt and barley adjunct beer focusing on changes in the protein composition. Journal of the Science of Food and Agriculture, 2011 (published online, Oct. 3rd 2011).
(5) Steiner E, Arendt EK, Gastl M, Becker T. Influence of the malting parameters on the haze formation of beer after filtration. Eur Food Res Technol. 2011; 233 (4): 587-97.

4.2 List of Conferences

(1) Steiner, E., Klose, C., Back, W., Arendt, E.K.: Modification of Proteins during Malting and Brewing and their Influence on Filterability; First International Symposium for Young Scientistst and Technologists in Malting, Brewing and Distilling, 2008, Cork
(2) Steiner, E., Becker, T., Gastl, M.: Turbidity and Haze Formation in Beer – Insight and Overview; Second International Symposium for Young Scientists and Technologists in Malting, Brewing and Distilling 2010, Freising
(3) Steiner, E., Arendt, E.K., Becker, T., Gastl, M.: Impact of different malting parameters on the protein composition of malt, wort and finished beer; 2010 MBAA Convention, 2010, Providence, RI

(4) Steiner, E., Auer, A., Gastl, M., Kreisz, S.: Comparison of beer quality attributes between 100% barley malt and barley adjunct beer focusing on changes in the protein composition; 2010 MBAA Convention, 2010, Providence, RI

(5) Steiner, E., Novy, R., Gastl, M., Becker, T.: Influence of silica sol on beer quality parameters. 33rd Congress European Brewery Convention, 2011

(6) Gastl, M., Steiner, E.; Munoz, A., Becker, T., Identification of barley varieties by Lab-on-a-Chip capillary gel electrophoresis. MBAA Annual Conference, 2011

4.3 Thesis Organization & Directions

This thesis is divided into three coherent chapters. Chapter 1 is an introduction which overviews source, formation and main components of beer haze focusing on protein haze. The introduction describes the necessity of this thesis referring to a solid literature research.

Chapter 2 lists the research carried out in this PhD-thesis generated by a number of papers accepted and published in peer-reviewed international journals. This chapter starts with an introduction in beer proteomics (paper 1; Steiner, E., Gastl, M., Becker, T., 2011. Protein changes during malting and brewing with focus on haze and foam formation: a review. Eur. Food Res. Technol. 232, 191-204.). Followed by a register of analyses methods in proteomics (paper 2; Steiner, E., Back, W., 2009. A critical review of protein assays and further aspects of new methods in brewing science. Brewing Sci. 62, 90-94.). Also an overview of haze identification methods is given (paper 3; Steiner, E., Becker, T., Gastl, M.: Turbidity and Haze Formation in Beer – Insight and Overview. J. Inst. Brew. 116(4), 360–368, 2010).

The two research papers (Steiner, E., Auer, A., Becker, T., Gastl, M.: Comparison of beer quality attributes between 100% barley malt and barley adjunct beer focusing on changes in the protein composition. Journal of the Science of Food and Agriculture, 2011; and Steiner, E., Arendt, E.K., Gastl, M., Becker, T.: Influence of the malting parameters on the haze formation of beer after filtration. Eur. Food Res. Technol. show the results generated in this research.

Chapter 3 discusses the overall intention of this thesis in respect to the given results and gives a perspective on research which needs further enhancements and overworking.

5 Introduction

5.1 Colloids and turbidity

During brewing proteins and macromolecules from raw materials undergo several changes. Throughout mashing proteins are solubilized and transferred into the produced wort; in wort boiling proteins are glycated and coagulated and during fermentation and maturation process, proteins aggregate as well, because of low pH (1).
Proteins in beer appear as colloids and are able to cause turbidity in the final product. Therefore it is necessary to understand the influence of the brewing process and the changes proteins are exposed to respectively also the forces which influence particle aggregation. In beer turbidity appears either directly after filtration or after some time in the bottled/filled beer. The turbidity which occurs directly after filtration is linked to a poor filtration (2) and the beer, where haze shows after some time, is referred to as colloidal instable (3-4).

Microscopic particles of one phase dispersed in another are generally called colloidal solutions or dispersions. Most of the industrial produced foodstuffs contain colloids, which determine their rheological property and texture. Colloids are particles within a size range from few nanometers up to some microns and are able to exist between all possible states of aggregation (e.g. aerosols or emulsions) (5).

"*The term 'colloid' is derived from the Greek word 'kolla' for glue. It was originally used for gelatinous polymer colloids, which were identified by Thomas Graham in 1860 in experiments on osmosis and diffusion* (6)*".*

Colloids are defined as follows:
"…The term colloidal refers to a state of subdivision, implying that the molecules or polymolecular particles dispersed in a medium have at least in one direction a dimension roughly between 1 nm and 1 µm, or that in a system discontinuities are found at distances of that order… The name dispersed phase for the particles should be used only if they have essentially the properties of a bulk phase of the same composition... A fluid colloidal system composed of two or more components may be

called a sol, e.g. a protein sol…When a sol is colloidally unstable (i.e. the rate of aggregation is not negligible) the formation of aggregates is called coagulation or flocculation... The rate of aggregation is in general determined by the frequency of collisions and the probability of cohesion during collision. (7).*"*

"Colloids are aggregations of small molecules due to the delicate balance of weak attractive forces (such as the van der Waals force) and repulsive forces. The aggregation depends on the physical environment, particularly the solvent. When the solvent changes, the aggregation may collapse (8)*."*

In solutions particles are exposed basically to three different forces: A gravitational force, which influences the settling/raising of particles, depending on their density relative to the solvent; a viscous drag force, which influences the motion of the particles and the 'natural' kinetic energy of particles and molecules, which causes Brownian motion (6). Colloidal particles are constantly in motion. The irregular movement and collision of particles in liquids is due to the Brownian Motion. Colloidal systems are solutions of large molecules, where the large molecules are the colloidal/Brownian particles. The minimum size of a Brownian particle is about 1 nm and the maximum about 10 µm (9). The Browninan movement is described as *"The movement of particles in a colloidal system such as an aerosol caused by collision with the molecules in the fluid in which the particles are imbedded." (7).* With this movement favorable conditions for collisions between colloids can be created, which leads to enlargement of colloids and therefore to visible particles (10). In Figure 1 size ranges of colloids, particles and other substances and their visibility for human eyes and microscopes are illustrated (11).

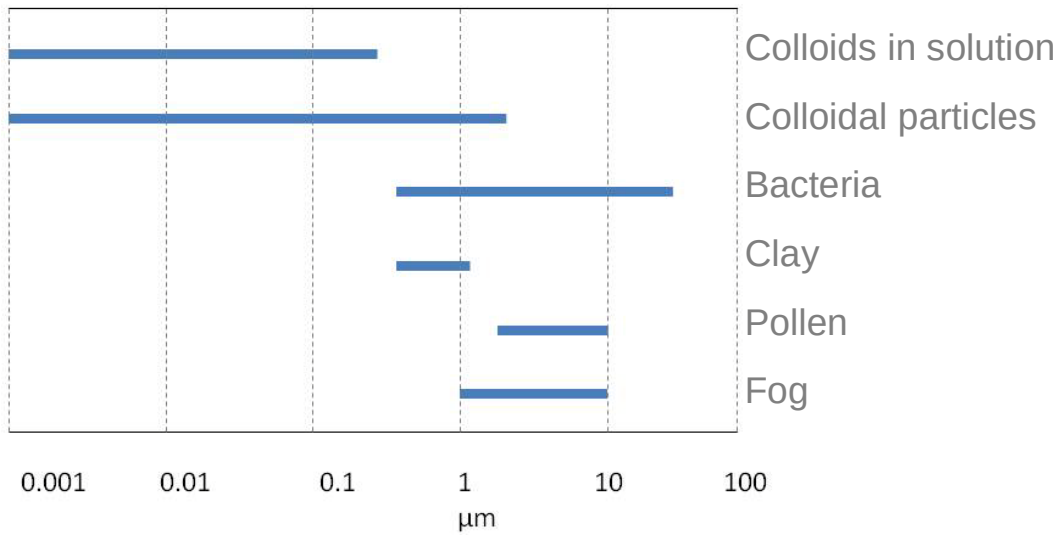

Figure 1: Size ranges of particles, colloids and other substances (11)

There also exist several physical and chemical forces between particles which make them combine and form larger particles (i.e. colloids). These forces can be of different nature (12):

Adhesive forces, which are the attractive forces between different molecules, are caused by forces acting between two substances, such as mechanical forces and electrostatic force. Cohesive forces are intermolecular forces and exist between molecules of the same substances. These forces are for example:

- Electromagnetic forces between opposite charged ions which lead to covalent/ionic bonds and hydrogen bonding.
- The total force between polar and non-polar (but not ionic) molecules is called the van der Waals force, which are intermolecular forces between polar molecules (dipole-dipole). In beer (or in other aqueous solutions) these forces arise because most materials, when dispersed in water, can be ionized to a certain degree or adsorb ions from solutions and therefore become charged (6). Depending on the forces, which exist between macromolecules, colloids and particles and/or between particles and the surrounding liquid, haze is formed in beer.

To describe the turbidity of a solution (beer) on a scientific basis, turbidity measurement is necessary. The basis for turbidity measurement of solutions is the ability of particles to scatter light. In a colloidal dispersion particles exist in the size

range from 1-1000 nm. Particles of this size exhibit a large surface area. Due to this enlarged surface, colloids scatter light and the scattering can be calculated as “turbidity”. When light goes through a colloidal solution at a 90 ° angle a “light scattering” can be observed. This is referred to as *Tyndall Effect (10)*. This can be seen in Figure 2, where the propagation of light in a homogenous media (A) and in a medium containing particles (B) is displayed (13).

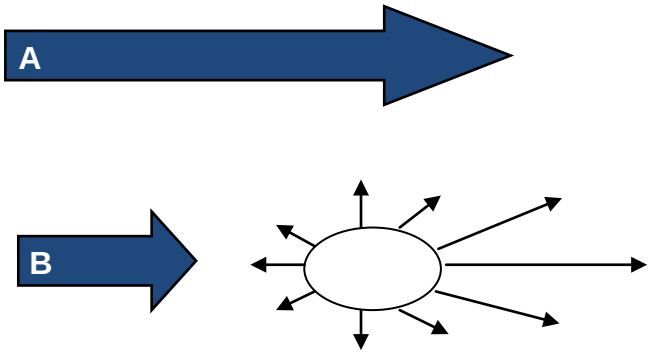

Figure 2: Light propagation in a homogenous medium and a medium containing solid particles

Tyndall was the first to study the phenomenon of the scattering of light by particles in colloidal solution. In 1944-1947 Debye was the first to use light scattering (the measurement of light-scattering intensity) to determine the molecular weight of a macromolecule in dilute solution (8). Figure 3 shows how the intensity of scatter varies as a function of the angle for two particle diameters (14). Small particles (<1 µm) scatter the light with the same intensity in all directions. The scatter of big particles (>1 µm) becomes lopesided.

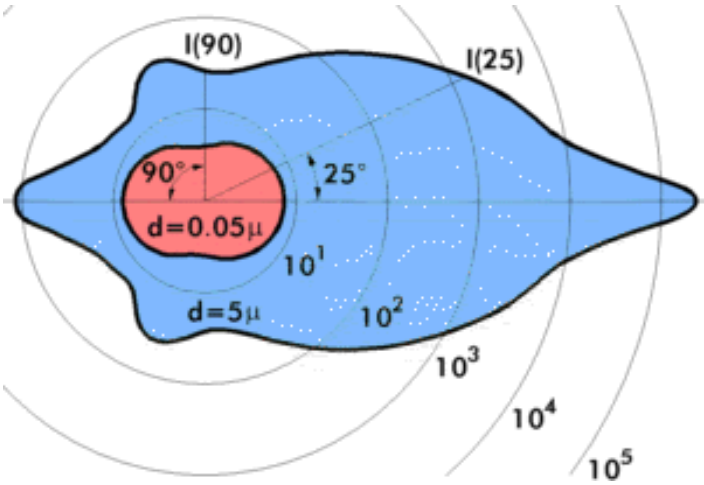

Figure 3: Angle dependency of light scatter of different particle sizes

Turbidity in beer is measured via turbidity photometers which detect the light, scattered by the sample, see also Figure 4 (15).

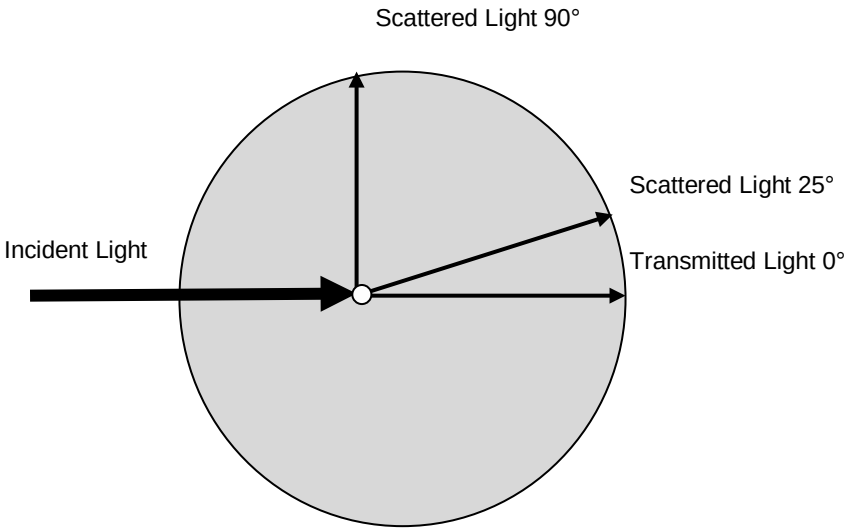

Figure 4: Schematic figure of light scatter

In beer mostly two angles are used. One at 25 ° forward scattering, which indicates bigger particles (> 1 µm) for example yeast cells, and one at 90 ° forward scattering which hints to smaller colloids (< 1 µm) (16). According to MEBAK (17) the specifications for turbidity in beer are for the 25 ° angle: < 0.5 EBC and for the 90 ° angle < 1 EBC.

5.2 Protein structure and function – from barley to beer

"In the first half of the 19th century…Gerardus Mulder was investigating the properties of substances extractable from both animal and plant tissues. He found these to contain carbon, hydrogen, nitrogen, and oxygen and believed them to be "without doubt the most important of the known substances… without them life would be impossible on our planet"… Mulder named these substances "proteins"… from the Greek, meaning "first" or "foremost"…" (18)

In the previous sections the development of colloids and therefore also protein haze in beer, has been described. Several protein functional properties, such as emulsification, foaming, haze formation etc. are closely related to protein solubility (19). In beer mostly simple proteins (e.g. LTP1, protein Z), in contrary to conjugated proteins (nucleoproteins, phosphoproteins, glycoproteins, chromoproteins, lipoproteins and membrane proteins) exist (20). These simple proteins in beer nearly always have a function: positive such as body and mouthfeel and foam formation and negative, such as haze formation. Proteins in beer are derived mostly from barley and are exposed to several "forces" and changes through the malting and brewing process. The changes start during seed development (21-34) and are continued during malting, mashing, wort boiling and fermentation. During malting, barley storage proteins are partially degraded by proteinases into amino acids and peptides that are critical for obtaining high quality malt and therefore high quality wort and beer. During mashing proteins are solubilized and transferred into the produced wort. Proteins are coagulated throughout wort boiling and fermentation and therefore can be separated (3-4, 35-38). The coagulation of proteins during the brewing process is based on the fact, that large protein molecules are sensitive to their surrounding and undergo denaturation, which can result in coagulation when subjected to heat, alcohol, etc.

"Denaturation: The irreversible process in which the structure of a protein is disrupted, resulting in a partial or complete loss of function." "Coagulation: The clotting or precipitation of protein in a liquid into a semisolid compound."

Both, denaturation and coagulation are irreversible (39). Several aspects of the brewing process are affected by soluble proteins, peptides and/or released amino acids. Figure 5 shows an extract of main external effects on the protein content and composition of barley, malt, wort and beer.

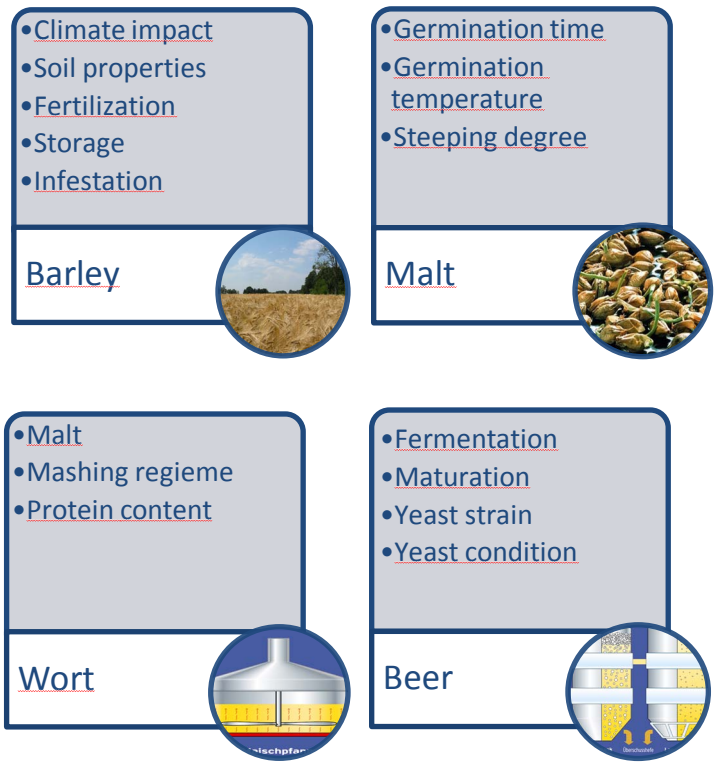

Figure 5: External effects on protein content and composition in barley, malt, wort and beer.

According to the “forces” to which proteins are exposed to during malting and brewing, proteins in beer can have different conformities and therefore they can also have different characteristics and functions in comparison to the barley proteins. According to these changes it is possible that “haze sensitive proteins” are developed. Thus it is important to know basic protein design and how the protein structures can be influenced.

In the following abstracts biological polymers made of proteins and peptides are described more closely. Biological polymers consist of amino acids, nucleotides, or sugars (8). A protein is build up by amino acids which are linked by peptide bonds. A peptide bond is an amide linkage between an amino group of one molecule and the carboxyl group of another. A protein which exhibits catalytic activities is an enzyme (8). Figure 6 shows the main structure levels of a protein (40). The sequence of the amino acid residues in a protein is called the primary structure. The primary structure defines the charge of a molecule. The secondary structure reveals the arrangement of the chain in space, i.e. a local folding. This is a regular geometry of the segments, and is formed as α-helix and β-sheet. These coiled segments (α-helix and β-sheet) are formed due to intramolecular forces. How the secondary structure appears

depends on the bond length and bond angles of the peptide bond, the coplanar arrangement of the atoms involved in the amide groups, the hydrogen bonds between N-H groups and C=O groups to maintain the maximum stability, and the range of the distance in the hydrogen bonds. The tertiary structure is, in contrary to the secondary structure, an overall folding - a three dimensional structure. This overall folding makes the protein compact and globular in shape. The tertiary structure can be divided into so called domains. Domains are peptide chains which can be folded independently from the other segments. When domains are combined differently, proteins with different functions are built. It can be said that the function of a protein depends on its tertiary structure. The tertiary structure (native conformation) can be denatured by forces which cleave hydrogen bridges, ionic or hydrophobic bonds. Quaternary structure is the topology of several globular arranged polypeptide chains aggregated together and resembles the total protein assembly. In contrary to tertiary structure quaternary structure can easily be separated by using an external force such as ultracentrifuge. This shows that the interpeptide chain

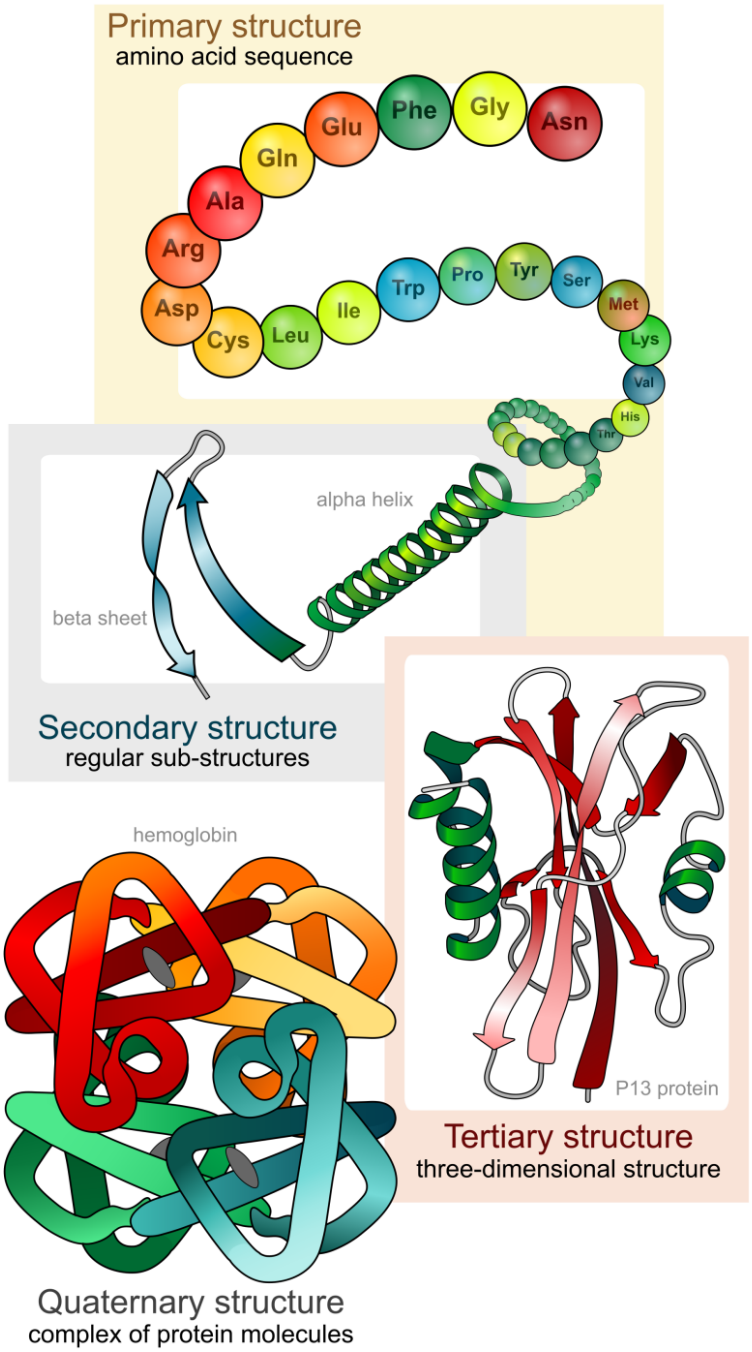

Figure 6: Main protein structure levels

attraction is neither strong (it can easily be separated) nor weak (it sticks together to form an assembly) (20).

A solution such as beer contains a heterogeneous mixture of proteins, i.e.: The sample contains a wide range of molecular species. The proteins in beer can be different in size, may have the same size, but differ in charge because of diverse amino acid substitution. They could also be molecular homogenous and might exhibit conformational heterogeneity. It can therefore be stated that all proteins are polyampholytes and carry an electric charge, which is determined by the amino acid composition, N- and C-terminal amino acids, pH, ionic strength, any post translational changes and the nature of the buffer ions (41). The point at which the charge of the protein is zero is called the isoelectric point. This point serves as characteristic for every protein. Proteins precipitate easily at the isoelectric point which can also be used for protein characterization (42-43). The fact that protein precipitate easily at the isoelectric point is important for haze formation in beer.

6 Motivation

As it is described in the section “introduction” proteins are known to have an influence on turbidity in final beer. From experience in haze identification and requests from the industry it is known that not only colloidal stability but also the, until now, rather neglected turbidity directly after filtration is an issue regarding beer quality. In the knowledge of haze identification it was already apparent that poor malt quality and/or over modified malt could lead to increased protein turbidity after filtration.

Many studies have been conducted on colloidal haze, but no research has been carried out concerning protein haze directly after filtration and on the influence of different malt parameters (i.e. time, temperature, and steeping degree). Since experience showed influence of different malt quality on protein haze after filtration, a literature research was conducted regarding the influence of variation in proteolysis in malt. No studies have been found about the influence of different proteolytic modified malt (under-, over modified malt) on protein composition in final beer. According to these practical investigations the influence of the malting process on the influence of protein composition in the final beer has been taken as initial point for investigations.

To get a fundamental overview on barley proteins and their influence on haze formation in beer, the already well known barley proteome was followed during the malting and brewing process. To gain an overall perception of the influence of barley proteins not only different proteolysis stages were observed but also the influence of malting itself in comparison to barley raw material and exogenous enzymes has been investigated. This thesis deals with the influence of different malting parameters and therefore different malting stages on final protein composition and thus on haze formation in final beer, after filtration.

The overall purpose of this study was to identify proteins/protein fractions and to track their origin from barley raw material into the final beer according to the haze formation process.

7 References

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8 Summary of Results

8.1 Protein changes during malting and brewing with focus on haze and foam formation: a review

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REVIEW PAPER

Protein changes during malting and brewing with focus on haze and foam formation: a review

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Abstract Beer is a complex mixture of over 450 constituents and, in addition, it contains macromolecules such as proteins, nucleic acids, polysaccharides, and lipids. In beer, several different protein groups, originating from barley, barley malt, and yeast, are known to influence beer quality. Some of them play a role in foam formation and mouthfeel, and others are known to form haze and have to be precipitated to guarantee haze stability, since turbidity gives a first visual impression of the quality of beer to the consumer. These proteins are derived from the malt used and are influenced, modified, and aggregated throughout the whole malting and brewing process. During malting, barley storage proteins are partially degraded by proteinases into amino acids and peptides that are critical for obtaining high-quality malt and therefore high-quality wort and beer. During mashing, proteins are solubilized and transferred into the produced wort. Throughout wort boiling proteins are glycated and coagulated being possible to separate those coagulated proteins from the wort as hot trub. In fermentation and maturation process, proteins aggregate as well, because of low pH, and can be separated. The understanding of beer protein also requires knowledge about the barley cultivar characteristics on barley/malt proteins, hordeins, protein Z, and LTP1. This review summarizes the protein composition and functions and the changes of malt proteins in beer during the malting and brewing process. Also methods for protein identification are described.

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Proteins in barley and malt

Barley (*Hordeum vulgare L.*) is a major food and animal feed crop. It ranks fourth in area of cultivation of cereal crops in the world. Barley is commonly used as raw material for malting and subsequently production of beer, where certain specifications have to be fulfilled. These specifications are among others: germinative capacity, protein content, sorting (kernel size), water content, kernel abnormalities, and infestation. Malting includes the controlled germination of barley in which hydrolytic enzymes are synthesized, and the cell walls, proteins, and starch of the endosperm are largely digested, making the grain more friable [1–3]. Proteins in beer are mainly derived from the barley used. The mature barley grain contains a spectrum of proteins that differ in function, location, structure, and other physical and chemical characteristics. Barley seed tissues have different soluble protein contents and distinct proteomes.

The three main tissues of the barley seed are the aleurone layer, embryo, and starchy endosperm that account for about 9, 4, and 87%, respectively, of the seed dry weight [4, 5]. The level of protein in barley is an important determinant in considering the final product quality of beer, for example for cultivar identification or as an indication of malting quality parameters [4], and it is influenced by soil conditions, crop rotation, fertilization, and weather conditions. For malting barley, the balance between carbohydrates and proteins is important, since high protein content reduces primarily the amount of available carbohydrates. Proteins present in barley seeds are important quality

determinants. During malting, barley storage proteins are partially degraded by proteinases into amino acids and peptides which are critical for obtaining high-quality malt and therefore high-quality wort and beer [1, 6, 7].

Germination provides the necessary hydrolytic enzymes to modify the grain, which are, in the case of proteins, endoproteases, and carboxypeptidases. These enzymes degrade storage proteins, especially prolamins (hordeins) and glutelins [8] and produce free amino acids during germination by cleavage of reserve proteins in the endosperm [9]. According to Mikola [10], there exist five seine carboxypeptidases in germinating barley, which have complementary specificities and mostly an acidic pH optimum. All of these carboxypeptidases consist of 2 identical subunits, each compose of two polypeptide chains, cross-linked by disulphide bridges [9, 11, 12]. Barley malt endoproteases (EC.3.4.21) develop multiple isoforms mainly during grain germination and pass through kilning almost intact [8, 13]. Jones [13–17] surveyed those enzymes and their behavior during malting and mashing. Cysteine proteases (EC 3.4.22) are clearly important players in the hydrolysis of barley proteins during malting and mashing. However, it seems likely that they do not play as predominant a role as was attributed to them in the past [15, 16, 18–22]. It has been found out that metalloproteases (EC 3.4.24) play a very significant role in solubilizing proteins, especially during mashing at pH 5.8–6.0 [23]. All current evidence suggests that the serine proteases (EC 3.4.21) play little or no direct role in the solubilization of barley storage proteins [23, 24], even though they comprise one of the most active enzyme forms present in malt [22]. While none of the barley aspartic proteases (EC 3.4.23), that have been purified and characterized, seem to be involved in hydrolyzing the seed storage proteins, it is likely that other members of this group do participate. Jones [17] investigated endoproteases in malt and wort and discovered that they were inactivated at temperatures above 60 °C. Jones et al. [6] examined the influence of the kilning process toward the endoproteolytic activity. These enzymes were affected by heating at 68 and 85 °C, during the final stages of kilning, but these changes did not influence the overall proteolytic activity.

Other proteins are involved in protein folding, such as protein disulfide isomerase (EC 5.3.4.1), which catalyzes the formation of protein disulfide bridges. Due to their heat-sensitivity, proteinases are inactivated when the temperature rises above 72 °C [25–30]. They are almost totally inactive within 16 min [1, 7, 13].

Summarizing the most important factors for the protein composition, as origin in finished beer are barley cultivar and the level of protein modification during malting, which is judged by malt modification which is conventionally measured in the brewing industry as the Kolbach index (soluble nitrogen/total nitrogen*100) [31, 32].

To get an overview of the main proteins in malt and beer, the most studied proteins are described in the next paragraphs. Proteins can be classified pursuant to their solubility. Osborne [33–37] took advantage of this fact and developed a procedure to separate the proteins. Proteins are divided into water-soluble (albumins), salt-soluble (globulins), alcohol-soluble (prolamins), and alkali-soluble (glutelins) fractions [34–36, 38, 39]. Osborne fractionation is a relatively simple, fast, and sensitive extraction–analysis procedure for the routine quantitation of all protein types in cereals in relative and absolute quantities, including the optimization of protein extraction and of quantitative analysis by RP-HPLC. High-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify, and purify the individual components of the mixture.

Not only Osborne fractionation and HPLC but also several other methods exist to separate and identify proteins in barley, malt, wort, and beer. To get an overview over the applications of the described methods in the review, a description follows in the next paragraphs.

Several authors [5, 39–60] characterized barley and barley malt proteins with help of 2D-PAGE. Other authors [25, 26, 29, 30, 32, 41, 61–65] used 2D-PAGE and mass spectrometry to fingerprint the protein composition in beer and to evaluate protein composition with regard to foam stability and haze formation. Klose [39] followed protein changes during malting with the help of a Lab-on-a-Chip technique and validated the results with 2D-PAGE. Iimure et al. [64] invented a protein map for the use in beer quality control. This beer proteome map provides a strong detection platform for the behaviors of beer quality–related proteins, like foam stability and haze formation. The nucleotide and amino acid sequences defined by the protein identification in the beer proteome map may have advantages for barley breeding and process control for beer brewing. The nucleotide sequences also give access to DNA markers in barley breeding by detecting sequence polymorphisms.

Hejgaard et al. [66–73] worked with immunoelectrophoresis and could identify several malt and beer proteins. Shewry et al. [54, 74–78] determined several methods for investigation of proteins in barley, malt, and beer mainly with different electrophoresis methods. Asano et al. [62, 63] worked with size-exclusion chromatography, immunoelectrophoresis and SDS–PAGE. Mills et al. [79] made immunological studies of hydrophobic proteins in beer with main focus and foam proteins. He discovered that the most hydrophilic protein group contained the majority of the proteinaceous material but it also comprised polypeptides with the least amount of tertiary structure.

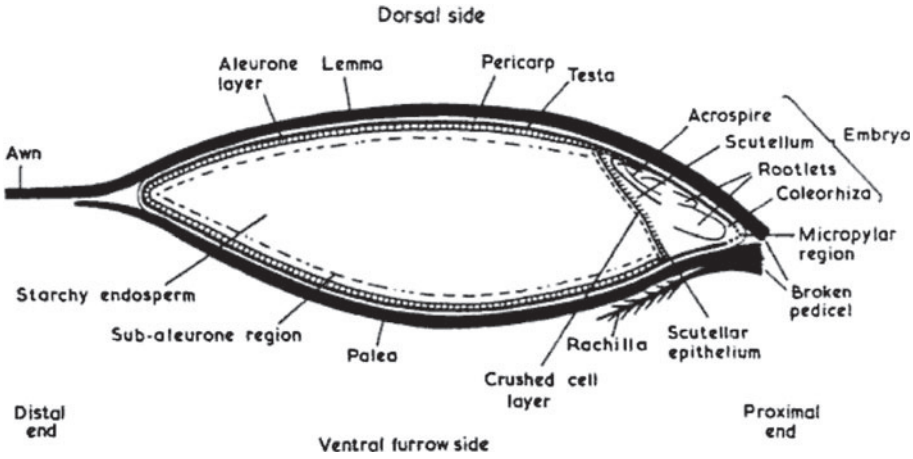

Fig. 1 Shematic longitudinal section of a barley grain [81]

Vaag et al. [28] established a quantitative ELISA method to identify a 17 kDa Protein and Ishibashi et al. [80] used an ELISA technique to quantify the range of foam-active protein found in malts produced in different geographic regions, and using different barley cultivars. Van Nierop et al. [30] used an ELISA technique to follow LTP1 content during the brewing process.

Osman et al. [18–20] investigated the activity of endoproteases in barley, malt, and mash. Hence, protein degradation during malting and brewing is very important for the later beer quality (mouthfeel, foam, and haze stability). It was suggested that estimation of the levels of degraded hordein (the estimation of the levels of hordein degraded during malting truly reflects the changes in proteins during malting and can measure the difference in barley varieties related to proteins and their degrading enzymes) during malting is a sensitive indicator of the total proteolytic action of proteinases as well as the degradability of the reserve proteins. And therefore, it is possible to predict several beer quality parameters according the total activity of all proteinases and the protein modification during malting.

To obtain good results, those separation and identification methods can be combined. Van Nierop et al. [30], for example, used ELISA, 2D-PAGE, RP-HPLC, electrospray mass spectrometry (ESMS), and circular dichroism (CD) spectrophotometry to follow the changes of LTP1 before and after boiling.

Since there exist various methods to separate and identify proteins in this review, an overview over existent proteins in barley, malt, wort, and beer is provided according to only one method, which is Osborne fractionation. These fractions are described more closely in the next sections.

Barley glutelin

About 30% of barley protein is glutelin that dissolves only in diluted alkali [54]. Glutelin is localized almost entirely in the starchy endosperm (Fig. 1), is not broken down later on, and passes unchanged into the spent grains [81, 82].

Glutelin is the least well-understood grain protein fraction. This is partly because the poor solubility of the components has necessitated the use of extreme extraction conditions and powerful solvents which often cause denaturation and even degradation (e.g., by the use of alkali) of the proteins, rendering electrophoretic analysis difficult. Also, because glutelin is the last fraction to be extracted, it is frequently affected by previous treatments and contaminated with residual proteins from other fractions, notably prolamins, which are incompletely extracted by classical Osborne procedures [83]. It has not been possible to prepare an undenatured glutelin fraction totally free of contaminating hordein [3].

Barley prolamin

The prolamin in barley is called hordein and it constitutes about 37% of the barley protein. It dissolves in 80% alcohol and part of it passes into spent grains. Hordein is a low-lysine, high-proline, and high-glutamine alcohol-soluble protein family found in barley endosperm (Fig. 1). It is the major nitrogenous fraction of barley endosperm composing 35–55% of the total nitrogen in the mature grain [1, 84–86]. Hordeins are accumulated relatively late in grain development, first being observed about 22 days after anthesis (when the grain weighs about 33% of its final dry weight) and increasing in amount until maximum dry weight is reached [87]. The major storage proteins in most cereal grains are alcohol-soluble prolamins. These are not single components, but form a polymorphic series of polypeptides of considerable complexity [88]. Hordein is synthesized on the rough endoplasmic reticulum during later stages of grain filling and deposited within vacuoles in protein bodies [89, 90]. Silva et al. [91] ascertained that the exposure of hordeins to a proteolytic process during germination reduces its content and originates in less hydrophobic peptides.

Some malt water–soluble proteins result from the hordein proteolysis. Hordeins are the most abundant proteins in barley endosperm characterized by their solubility in alcohol. These storage proteins form a matrix around the starch granules, and it is suggested that their degradation during malting directly affects the availability of starch to amylolytic attack during mashing [92].

Shewry [75, 77] divided the hordeins according to their size and amino acid composition in four different fractions (A-D), dependent on their size and amino acid composition. A-hordeins (15–25 kDa) seem to be no genuine storage proteins as they contain protease inhibitors and α-amylases. B-hordeins (32–45 kDa) are rich in sulfur content and are, with 80%, the biggest hordein fraction. B-hordeins have a general structure, with an assumed signal peptide of 19 aminoacid residues, a central repetitive domain rich in proline and glutamine residues, and a C-terminal domain containing most of the cysteine residues are encoded by a single structural locus, Hor2, located on the short arm 1 of chromosome 1H(5), 7–8 cM distal to the Hor1 locus which codes for the C-hordeins. C-hordeins (49–72 kDa) are low in sulfur content, and D-hordeins (>100 kDa) are the largest storage proteins and are encoded by the Hor3 locus located on the long arm of chromosome 1H(5) [85, 87, 93, 94].

Cereal prolamins are not single proteins but complex polymorphic mixtures of polypeptides [54]. During malting, disulfide bonds are reduced and B- and D-hordeins are broken down by proteolysis. Well-modified malt contains less than half the amount of hordeins present in the original barley. D-hordeins are degraded more rapidly than their B-type counterparts, and the latter are more rapidly degraded than C-hordeins [3, 95].

Barley albumins and globulins

Many researchers extract a combined salt-soluble protein fraction, because water extracts contain globulins as well as albumins. The two classes of proteins may be separated by dialysis, but there is considerable overlap between the two [83]. Albumins and globulins consist mainly of metabolic proteins, at least in the cereal grains [96] and are found in the embryo and the aleurone layer, respectively [81, 82]. Whereas prolamins are degraded during germination, albumins and other soluble proteins increased during the germination process [92].

Globulins

The globulin fraction of barley is called edestin. It dissolves in dilute salt solutions and hence also in the mash. It forms about 15% of the barley protein. Edestin forms 4 components (α, β, γ, and δ) of which the sulfur-containing β-globulin does not completely precipitate even on prolonged boiling and can give rise to haze in beer. Enzymes and enzyme-related proteins are mainly albumins and globulins [42].

Albumins

The albumin of barley is called leucosin. It dissolves in pure water and constitutes about 11% of the protein in barley. During boiling, it is completely precipitated. α-Amylase, protein Z, and lipid transfer proteins are barley albumins and are important for the beer quality attributes: foam stability and haze formation [97]. Albumins can be further divided into protein Z and lipid transfer proteins as functional proteins

Protein Z

Protein Z belongs to a family of barley serpins and consists of at least four antigenically identical molecular forms with isoelectric points in the range 5.55—5.80 (in beer: 5.1–5.4), but same molecular mass near 40 kDa [1, 55, 67, 68, 98]. Protein Z is hydrophobe and exists in free and bound forms in barley, like α-amylase, and there also exist heterodimers. Protein Z contains 2 cysteine and 20 lysine residues per monomer molecule and is relatively rich in leucine and other hydrophobic residues. Protein Z accounts for 5% of the albumin fraction and more than 7% in some high-lysine barleys [67, 99]. The content of protein Z in barley grains depends on the level of nitrogen fertilization [67, 100]. Protein Z makes up to 20–170 mg/L of beer protein [79]. In mature seeds, protein Z is present in thiol bound forms, which are released during germination [101]. The function of the protein is at present unknown but it is known that it is deposited specifically in the endosperm responding to nitrogen fertilizer, similar to the hordein storage proteins. The synthesis is regulated during grain development at the transcriptional level in dependence of the supply of nitrogen [98, 100, 102, 103]. It is stated that upregulation of transcript levels could be effectuated within hours, if ammonium nitrate was supplied through the peduncle, and equally rapid reduced when the supply was stopped [103]. Finnie et al. [49] investigated the proteome of grain filling and seed maturation in barley. They identified a group of proteins that increased gradually both in intensity and abundance, during the entire examination period of development and were identified as serpins. Also Sorensen [55] and Giese [98] could detect the expression of protein Z4 (a subform of protein Z) only during germination. Protein Z4 has an expression profile similar to β-amylase and seed storage proteins (hordeins).

Three distinct serpin sequences from barley could be found in the databases SWISSPROT and TREMBL: protein Z4, protein Z7, and protein Zx. These different protein

Z forms are thought to have a role as storage proteins in plants, due to their high "Lys" content and the fact that serpin gene expression is regulated by the "high-Lys" alleles lys1 and lys3a [49, 104].

Hejgaard et al. [68] suggest that the precursors of protein Z originate from chromosomes 4 and 7, and thus, they are named protein Z4 and protein Z7. Rasmussen and co-workers [105] were able to estimate the size of protein Z mRNA at 1.800 b. This is sufficient to code for the 46.000 or 44.000 MW precursor peptides found in vitro translations plus leave 400–500 b for the 5′ and 3′ non-coding regions. Doll [106] and Rasmussen [107] suggest that protein Z could be a candidate for modulation of the barley seed protein composition to balance the nutritional quality of the grain. Giese and Hejgaard [98] found out that during germination, protein Z becomes the dominant protein in the salt-soluble fraction in developing barley. The proteins in barley malt are known to be glycated by D-glucose, which is a product of starch degradation during malting [108]. Bobalova et al. [109] investigated in their research the glycation of protein Z and found out that protein Z glycation is detectable from the second day of malting. The role of protein Z in beer is described more detailed in the sections foam and haze formation.

Lipid transfer protein

Lipid transfer proteins (LTPs) are ubiquitous plant lipid-binding proteins that were originally identified by their ability to catalyze the transfer of lipids between membranes. LTPs are abundant soluble proteins of the aleurone layers from barley endosperm. The compact structure of the barley LTP1 comprises four helices stabilized by four disulfide bonds and a well-defined C-terminal arm with no regular secondary structure [110] which is shown in Fig. 2, where a 3D and surface protein of barley LTP native protein (here called 1LIP, red) is shown [111]. In comparison with other plant lipid transfer proteins, the barley protein has a small hydrophobic cavity but is capable of binding different lipids such as fatty acids and acyl-CoA [25, 112, 113]. According to molecular mass, this multigene family is subdivided into two subfamilies, ns-LTP1 (9 kDa) and ns-LTP2 (7 kDa); both located in the aleurone layer of the cereal grain endosperm [56, 114]. LTP1 and LTP2 are expressed in barley grain but only LTP1 has been able to be detected in beer. LTP1 is claimed to be an inhibitor of malt cysteine endoproteases [14, 115]. The role of LTP1 in beer is described more detailed in the sections foam and haze formation.

Protein Z and LTP1

Evans [116, 117] investigated the influence of the malting process on the different protein Z types and LTP1. He discovered that the amount of LTP1 did not change during germination but a significant proportion of the bound/latent protein Z was converted into the free fraction. He claims that during germination, proteolytic cleavage in the reactive site loop converts protein Z to a heat and protease stable forms, and hence, they can survive the brewing process. He ascertained also that kilning reduced the amount of protein Z and LTP1 [66, 118].

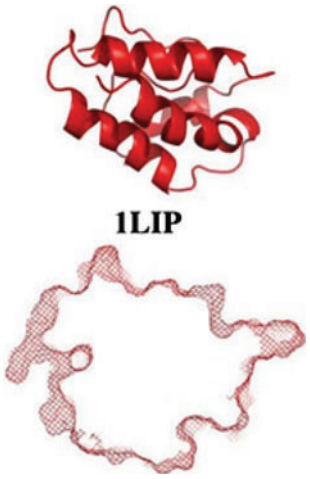

Fig. 2 3D and surface protein of barley LTP native protein (1LIP, *red*) is shown [111]

Evans [116] analyzed feed and malting barley varieties and could not find any differences in the level of protein Z and LTP1. He also ascertained malt-derived factors that influence beer foam stability, such as protein Z4, β-glucan, viscosity, and Kohlbach index. Beer components (protein Z4, free amino nitrogen, β-glucan, arabinoxylan, and viscosity) were correlated with foam stability [117]. Protein Z4, protein Z7, and LTP1 have been shown to act as protease inhibitors [116, 119, 120].

Proteins in wort and beer

Proteins influence the whole brewing process not only in the form of enzymes but also in combination with other substances such as polyphenols. As enzymes, they degrade starch, β-glucans, and proteins. In protein–protein linkages, they stabilize foams and are responsible for mouthfeel and flavor stability, and in combination with polyphenols, they are thought to form haze. As amino acids, peptides, and sal ammoniac, they are important nitrogen sources for yeast [121]. Only about 20% of the total grain proteins are water soluble. Barley water-soluble proteins are believed to be resistant to proteolysis and heat coagulation and hence pass through the processing steps, intact or somewhat modified, to beer [116, 122, 123]. Several aspects of the brewing process are affected by soluble proteins, peptides, and/or

Table 1 Enzymes in barley and barley malt [1, 7, 166, 167]

	Enzyme	Substrate	Product
Cytolysis	β-glucan-solubilase	Matrix linked β-glucan	Soluble, high molecular weight β-glucan
	Endo-β-(1-3) glucanase	Soluble, high molecular weight β-glucan	Low molecular weight β-glucan, cellobiose, laminaribiose
	Endo-β-(1-4) glucanase	Soluble, high molecular weight β-glucan	Low molecular weight β-glucan, cellobiose, laminaribiose
	Exo-β-glucanase	Cellobiose, laminaribiose	Glucose
	Xylanase	Hemicellulose	β-D-Xylose
Proteolysis	Endopeptidase	Proteins	Peptides, free amino acids
	Carboxypeptidase	Proteins, peptides	Free amino acids
	Aminopeptidase	Proteins, peptides	Free amino acids
	Dipeptidase	Dipeptides	Free amino acids
Amylolysis	α-amylase	High and low molecular weight α-glucans	Melagosaccharides, oligosaccharides
	β-amylase	α-glucans	Maltose
	Maltase	Maltose	Glucose
	Limit dextrinase	Limit dextrins	Dextrins
	Pullulanase	α-1,6-D-glucans in amylopectin, glykogen, pullulan	Linear amylose fractions
Other	Lipase	Lipids, lipidhydroperoxide	Glycerine, free fatty acids, fatty acid hydroperoxide
	Lipoxygenase	Free fatty acids	Fatty acid hydroperoxide

amino acids that are released. No more than one-third of the total protein content passes into the finished beer which is obtained throughout mainly two processes; mashing and the wort boiling. Mashing is the first biochemical process step of brewing and completes the enzymatic degradation started during malting. Enzymes synthesized during malting are absolutely essential for the degradation of large molecules during mashing. These enzymes are displayed in Table 1 [1, 7]. The three biochemical basic processes taking place during malting are cytolysis, proteolysis, and amylolysis, which are indicated by β-glucan, FAN, and extract concentration, respectively. In order to get good brews, part of the insoluble native protein must be converted into "soluble protein" during malting and mashing. This fraction comprises a mixture of amino acids, peptides, and dissolved proteins, and a major portion of it arises by proteolysis of barley proteins [23]. During the brewing process, there are three possibilities to discard the (unwanted) proteinic particles. The first opportunity is given during wort boiling, where proteins coagulate and can be removed in the "whirlpool". The second, during fermentation, where the pH decreases and proteinic particles can be separated by sedimentation. The third step is during maturation of beer. During maturation, proteins adhere on the yeast and can be discarded [124].

It has also been demonstrated that yeast proteins are present in beer, but only as minor constituents [73]. Beer contains $\sim$500 mg/L of proteinaceous material including a variety of polypeptides with molecular masses ranging from <5 to >100 kDa. These polypeptides, which mainly originate from barley proteins, are the product of the enzymatic (proteolytic) and chemical modifications (hydrogen bonds, Maillard reaction) that occur during brewing, especially during mashing, where proteolytic enzymes are liable for those modifications [125]. A beer protein may be defined as a more or less heterogeneous mixture of molecules containing the same core of peptide structure, originating from only one distinct protein present in the brewing materials [126]. Jones [13–17] surveyed proteinases and their behavior during malting and mashing. Proteinases are not active in beer anymore; hence, they are inactivated when the temperature rises above 72 °C, which happens already during mashing [1, 7, 13, 25–30].

Proteins influence two main quality aspects in the final beer: 1st haze formation and 2nd foam stability. In the following lines, these quality attributes are described in a more detailed way.

Haze formation

Proteins play a major role in beer stability; hence, they are, beside polyphenols, part of colloidal haze. There exist two forms of haze; cold break (chill haze) and age-related haze [127]. Cold break haze forms at 0 °C and dissolves at higher temperatures. If cold break haze does not dissolve, age-related haze develops, which is non-reversible. Chill haze is formed when polypeptides and polyphenols are bound non-covalently. Permanent haze forms in the same manner initially, but covalent bonds soon form and

Table 2 Distribution of hordeins in barley according to their size [75]

Type	MW (kDa)	% of total hordeins
A	10–16	>5
B	30–46	80–90
C	48–72	10–20
D	>100	>5

insoluble complexes are created which will not dissolve when heated [128]. Proanthocyanidins (condensed tannins) from the testa tissue (seed coat) of the barley grain are carried from the malt into the wort and are also found after fermentation of the wort in the beer. There they cause precipitation of proteins and haze formation especially after refrigeration of the beer, even if it previously had been filtered to be brilliantly clear [129]. Proteins, as the main cause of haze formation in beer, can be divided into two main groups: 1st proteins and 2nd their breakdown products. Protein breakdown products are characterized by always being soluble in water and do not precipitate during boiling. Finished beer contains almost only protein breakdown products [126]. The content of only 2 mg/L protein is enough to form haze [118]. Beer contains a number of barley proteins that are modified chemically (hydrogen bond formation, Maillard reaction) and enzymatically (proteolysis) during the malting and brewing processes, which can influence final beer haze stability. Leiper et al. [130, 131] found out that the mashing stage of brewing affects the amount of haze-active protein in beer. If a beer has been brewed with a protein rest (48–52 °C), it may contain less total protein but more haze-active proteins because the extra proteolysis caused release of more haze causing polypeptides. Asano et al. [62] investigated different protein fractions and split them in 3 categories: 1st high, 2nd middle and 3rd low molecular weight fractions being high molecular weight fractions: >40 kDa, middle molecular weight fraction: 15–40 kDa and low molecular weight fraction: <15 kDa. Nummi et al. [132] even suggested that acidic proteins derived from albumins and globulins of barley are responsible for chill haze formation (Table 2).

Researchers proofed that proline-rich proteins are involved in haze formation [63, 65, 124, 127, 128, 130, 131, 133–137]. Outtrup et al. [138] say that haze-active proteins are known to be dependent on the distribution of proline within the protein. Nadzeyka et al. [127] suggested that proteins in the size range between 15–35 kDa comprised the highest amount of proline. It was also investigated that proline and glutamic acid-rich hordeins, in the size range between 10–30 kDa, are the main initiators causing haze development [63, 74]. β-Amylase, protein Z, and two chymotrypsin inhibitors have relatively high-lysine contents [100]. Barley storage proteins that are available for hydrolysis are all proline-rich proteins [15]. Dadic and Belleau [139, 140] on contrary say that there is no specific amino acid composition for haze-active proteins. Leiper [130, 131] even says that not only the mainly consistence of proline and glutamic acid of the glycoproteins is responsible for causing haze but also that the carbohydrate component consists largely of hexose. It was found out that the most important glycoproteins for haze formation are 16.5 and 30.7 kDa in size. Glycation is a common form of non-enzymatic modification that influences the properties of proteins [109]. Non-enzymatic glycation of lysine or arginine residues is due to the chemical reactions in proteins, which happen during the Maillard reaction [109]. It is one of the most widely spread side-chain-specific modifications formed by the reaction of α-oxoaldehydes, reducing carbohydrates or their derivatives with free amine groups in peptides and proteins, such as e-amino groups in lysine and guanidine groups in arginine [141, 142]. The proteins in barley malt are known to be glycated by D-glucose, which is a product of starch degradation during malting [108]. D-glucose reacts with a free amine group yielding a Schiff base, which undergoes a rapid rearrangement forming more stable Amadori compounds.

Haze-sensitive proteins

Polypeptides that are involved in haze formation are also known as sensitive proteins. They will precipitate with tannic acid, which provides a mean to determine their levels in beer. Proline sites of these polypeptides bind to silica gel hydroxyl groups so that haze-forming proteins are selectively adsorbed, since foam proteins contain little proline and are thus not affected by silica treatment [143]. Removal of haze forming tannoids can be effected using PVPP [143]. To assure colloidal stability, it is not necessary to remove all of the sensitive proteins or tannoids. Identification of a tolerable level of these proteins can be used to define a beer composition at bottling that delivers satisfactory haze stability [94, 99]. To prolong stability of beer, stabilization aids are used. Haze-forming particles are removed with: (a) silica, which is used to remove proline-rich proteins that have the ability to interact with polyphenols to form haze in bright beer, or (b) PVPP, which is used to remove haze-active polyphenols.

Evans et al. [144] investigated the composition of the fractions which were absorbed by silica. This analysis revealed that the mole percentage of proline ranged between 33.2 and 38.0%, and of glutamate/glutamine between 32.7 and 33.0%, consistent with the proline/glutamine–rich composition of the hordeins [144]. Iimure et al. [65] stated in their studies that proteins adsorbed onto silica gel (PAS) are protein Z4, protein Z7, and trypsin/amylase inhibitor pUP13 (TAI), rather than BDAI-1

(α-amylase inhibitor), CMb, and CMe. Lázaro et al. [145] investigated the CM proteins CMa, CMb, and CMe. The CM proteins are a group of major salt-soluble endosperm proteins encoded by a disperse multigene family and act as serine proteinase inhibitors. Genes CMa, CMb, and CMe are located in chromosomes 1, 4, and 3, respectively. Protein CMe has been found to be identical with a previously described trypsin inhibitor. Furthermore, Iimure et al. [64] analyzed proline compositions in beer proteins, PAS, and haze proteins. It was proofed that the proline compositions of PAS were higher (ca. 20 mol%) than those in the beer proteins (ca. 10 mol%), although those of the haze-active proteins such as BDAI-1, CMb, and CMe were 6.6–8.7 mol%. These results suggest that BDAI-1, CMb, and CMe are not predominant haze-active proteins, but growth factors of beer colloidal haze. Serine proteinase inhibitors have also been called trypsin/α-amylase inhibitors, and it has been proposed that some of them might inhibit the activities of barley serine proteinases. However, none have been shown to affect barley enzymes [16]. Robinson et al. [146] identified a polymorphism for beer haze-active proteins and surveyed by immunoblot analysis throughout the brewing process. In this polymorphism, some barley varieties contained a molecular weight band at 12 kDa, while in other varieties, this band was absent. Pilot brewing trials have shown that the absence of this 12 kDa protein conferred improved beer haze stability on the resulting beer. This band was detected by a polyclonal antibody raised against a haze-active, proline/glutamine–rich protein fraction; it was initially assumed that the band was a member of the hordein protein family [144, 147].

Foam formation

Beer foam is an important quality parameter for customers. Good foam formation and stability gives an impression of a freshly brewed and well-tasting beer. Therefore, it is necessary to investigate mechanisms that are behind foam formation. Beer foam is characterized by its stability, adherence to glass, and texture [148]. Foam occurs on dispensing the beer as a result of the formation of CO_2 bubbles released by the reduction in pressure. The CO_2 bubbles collect surface-active materials as they rise. These surface-active substances have a low surface tension, this means that within limits they can increase their surface area and also, after the bubbles have risen, they form an elastic skin around the gas bubble. The greater the amount of dissolved CO_2 the more foam is formed. But foam formation is not the same as foam stability. Foam is only stable in the presence of these surface-active substances [1]. Beer foam is stabilized by the interaction between certain beer proteins, for example LTP1, and isomerized hop α-acids, but destabilized by lipids [30, 148]. The intention is to find a good compromise of balancing foam-positive and foam-negative components. Foam-positive components such as hop acids, proteins, metal ions, gas composition (ratio of nitrogen to carbon dioxide), and gas level, generally improve foam, when increased. Whereas foam negatives, such as lipids, basic amino acids, ethanol, yeast protease activity, and excessive malt modification, decrease foam formation and stability. Free fatty acids, which are extracted during mashing, have a negative effect on foam stability [64, 65, 80, 85, 88, 128–131, 166].

Foam-positive proteins can be divided into high molecular weight proteins (35–50 kDa) and low molecular weight proteins (5–15 kDa) which primary originate from malt but in small amount can also originate from yeast [62, 73, 148]. It is thought that during foam formation, amphiphile proteins surround foam cells and stabilize them by forming a layer. They arrange themselves into bilayers, by positioning their polar groups toward the surrounding aqueous medium and their lipophilic chains toward the inside of the bilayer, defining a non-polar region between two polar ones [149]. There are two main opinions concerning the nature of foaming polypeptides in beer. The first position claims the existence of specific proteins which basically influence foam stability. Those proteins are known as protein Z and LTP1 [150, 151]. The second argument claims the existence of a diversity of polypeptides which stabilize foam; the more hydrophobic their nature, the more foam active they are [122, 152], like hordeins that are rich in proline and glutamine content and exhibit a hydrophobic β-turn-rich structure [74]. Kapp [153] investigated the influence of albumin and hordein fractions from barley on foam stability, because both are able to increase the foam stability. The ability to form more stabile foams seems to be higher by albumins than by hordeins. Denaturation of these proteins causes an increase in their hydrophobic character and also in their foam stability. This confirms the already known opinion that the more hydrophobic the protein, the better is the foam stability [122, 152]. The foams from albumins are more stable than those from hordeins. This may also be the reason for the increased ability of albumin fractions to withstand the presence of ethanol. The foam stability of both albumins and hordeins is increased by bitter acids derived from hops.

Whereas the barley LTP1 does not display any foaming properties, the corresponding beer protein is surface active. Such an improvement is related to glycation by Maillard reactions on malting, acylation on mashing, and structural unfolding on brewing which was ascertained by Perrocheau et al. [25]. During the malting and brewing processes, LTP1 becomes a surface-active protein that concentrates in beer foam [55]. LTP1 is modified during boiling and this modified form influences foam stability [28, 150]. The two

forms have been recovered in beer with marked chemical modifications including disulfide bond reduction and rearrangement and especially glycation by Maillard reaction. The glycation is heterogeneous with variable amounts of hexose units bound to LTPs [112]. The four lysine residues of LTP1 are the potential sites of glycation [112]. Altogether, glycation, lipid adduction, and unfolding should increase the amphiphilic character of LTP1 polypeptides and contribute to a better adsorption at air–water interfaces and thus promote foam stability.

Van Nierop et al. [30] established that LTP1 denaturation reduces its ability to act as a binding protein for foam damaging free fatty acids and therefore boiling and boiling temperature are important factors in determining the level and conformation of LTP1 and so enhance foam stability. Perrocheau et al. [25] showed that unfolding of LTP1 occurred on wort boiling before fermentation and that the reducing conditions are provided by malt extract. Van Nierop et al. [30] showed that the wort boiling temperature during the brewing process was critical in determining the final beer LTP1 content and conformation. It was discovered that higher wort boiling temperatures (102 °C) resulted in lower LTP1 levels than lower wort boiling temperatures (96 °C). Combination of low levels of LTP1 and increased levels of free fatty acids resulted in low foam stability, whereas beer produced with low levels of LTP1 and free fatty acids had satisfactory foam stability. LTP1 has been demonstrated to be foam promoting only in its heat denatured form [55, 150, 154].

Perrocheau et al. [26] investigated heat-stable, water-soluble proteins that influence foam stability. Most of the heat stable proteins were disulfide-rich proteins, implicated in the defense of plants against their bio-aggressors, e.g., serpin-like chymotrypsin inhibitors (protein Z), amylase and amylase-protease inhibitors, and lipid transfer proteins (LTP1 and LTP2). Leisegang et al. [95–97] identified LTP1 as a substrate for proteinase A, which degrades LTP1, but does not influence protein Z and may have a negative influence on beer foam stability. Iimure et al. [32] invented a prediction method of beer foam stability using protein Z, barley dimeric α-amylase inhibitor-1 (BDAI-1) and yeast thioredoxin and confirmed BDAI-1 and protein Z as foam-positive factors and identified yeast thioredoxin as a possible novel foam-negative factor. Jin et al. [155, 156] found out in their research that structural changes of proteins during the wort boiling process are independent of the malt variety. It was discovered that barley trypsin inhibitor CMe and protein Z were resistant to proteolysis and heat denaturation during the brewing process and might be important contributors to beer haze formation. Vaag et al. [28] found a new protein of 17 kDa which seemed to influence foam stability even more than protein Z and barley like LTP1. She could support this theory by the correlation of the content of this so called 17 kDa protein and the foam half-life of lager beers. LTP1 and the 17 kDa protein exhibit some similarities; their tertiary structures are characterized by disulfide bridges, both are rich in cysteine and are modified during heating to a more foam promoting form. Ishibashi et al. [80] agrees that both malting and mashing conditions influence the foam-active protein levels in experimental mashes. Proteinaceous materials in beer have as well been implicated in the stabilization of beer foam. Molecular weight has been reported to be important for foaming potential, while the hydrophobicity of polypeptides has been cited as a controlling factor [62]. Kordialik-Bogacka et al. [157, 158] investigated also foam-active polypeptides in beer. In contrary to Osman et al. [123] in this investigation, it was confirmed that fermentation influences the protein composition of beer and particularly in beer foam.

Yeast polypeptides were also found in beer foam. It was noted that, especially during the fermentation of high gravity wort, excessive foaming may occur, and this may be one of the reasons why beer brewed at higher gravities has a poor head. It was detected that polypeptides of molecular weight about 40 kDa present in fermented wort and foam originated not only from malt but also from yeast cells. Okada et al. [159] studied on the influence of protein modification on foam stability during malting. They found that the foam stability of beer samples brewed from barley malts of 2 cultivars decreased as the level of malt modification increased, but the foam stability of another cultivar did not change. In this research, they defined BDAI-I as an important contributor to beer foam stability.

Conclusion

Proteins do not only influence haze formation; furthermore, they play an important role for mouthfeel and foam stability. These aspects are important for brewers, since consumer judge beer also according to these quality attributes. As it is known, most foam-positive proteins are also haze active, Evans et al. [144] made an investigation to immunologically differentiate between those two protein forms (foam and haze-active proteins) and concluded that no barley variety or growing condition have any significant influence on beer stability. It was also demonstrated in a regression analysis that a prediction of foam stability is not possible, which underlines the complexity of these problems. It is suggested that both foam-active and foam-negative components should be measured and that the amount of hordeins and protein Z4 are somehow related. It was also ascertained that foam and haze-active proteins share some epitopes and that oxygen during the brewing process influence haze stability of beer [147].

Leiper et al. [130, 131] studied beer proteins that are involved in haze and foam formation. All proteins were found to be glycosylated to varying degrees. The size range of the polypeptides which make up the glycoprotein fraction of beer is relatively narrow and the range was found to be from 10 to 46 kDa. The glycoproteins were found to consist of proteins, six carbon sugars (hexoses), and five carbon sugars (pentoses). Beer glycoproteins were found to exist in three forms; those responsible for causing haze, those responsible for providing foam stability, and a third group that appeared to have no role in physical or foam stability. Approximately 25% of beer glycoproteins are involved in foam and foam stability. As 3–7% of beer glycoproteins have been identified as being involved in haze formation, this leaves around 70% of beer glycoproteins that appear to have no role in either physical and/or foam stability. This fraction contains the most abundant beer polypeptide, protein Z, which is glycosylated with both hexoses and pentoses. It has been estimated that about 16 % of the lysine content of protein Z are glycated during the brewing process through Maillard reaction [61, 126].

There are three major groups of proteins in beer. The first consists of a group of proline-rich fragments originating from hordein ranging in size from 15–32 kDa which are involved with haze formation. The second is LTP1 (9.7 kDa in pure form) that is involved in foam stability and the third is protein Z (40 kDa) that appears to have no direct function, but may play a role in stabilizing foam once it has been formed [130, 131]. Several authors [25, 30, 49, 66, 70, 73, 125, 126, 160, 161] investigated haze-active proteins in beer. Two major proteins in beer are claimed to cause haze formation and influence foam stability; protein Z and LTP 1. Protein Z and LTP1 are heat stable and resistant to proteolytic modification during beer production and appear to be the only proteins of barley origin present in significant amounts in beer. It is presumed that protein Z causes haze and is all the same positive for foam stability [70, 73]. LTP1 is claimed not to influence foam stability but the quantity of foam generated [55, 117]. Protein Z is homologous to serine protease inhibitors and these inhibitory properties might be the reason that protein Z is not degraded by proteolytic enzymes during malting and mashing [104, 126, 162, 163]. Curioni et al. [125] showed that glycation of protein Z improved foam stability and might prevent precipitation of protein during the wort boiling step. Both glycation and denaturation increase the amphiphilicity of LTP1 polypeptides and contribute to a better adsorption at air–water interfaces of beer foam [55, 164]. Jin et al. [155, 156] found out in their research that structural changes of proteins during the wort boiling process are independent of the malt variety. It was discovered that barley trypsin inhibitor CMe and protein Z were resistant to proteolysis and heat denaturation during the brewing process and might be important contributors to beer haze formation. It is known that foam-active hydrophobic protein fractions in beer can be hydrolyzed by proteinases leading to a decrease in foam stability.

Besides proteins, other beer constituents such as iso-alpha acids, peptides, amino acids, proteinase, fatty acids, and melanoidins were suggested to influence haze formation and foam properties [154, 165]. The contents of these constituents in beer were influenced by brewing material variables such as barley varieties, malt types, hop usage, yeast strains, and malting and brewing processes.

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8.2 A critical review of protein assays and further aspects of new methods in brewing science

E. Steiner and W. Back

A Critical Review of Protein Assays and Further Aspects of New Methods in BrewingScience

Total nitrogen content of barley, malt and beer was usually measured by Kjeldahl method. In brewing science this method has been used for many years to measure total protein (N x 6.25 = protein concentration) in beer, but it measures nitrogen rather than proteins. Kjeldahl method determines total nitrogen but is prone to interference from non-protein and nitrogen-containing compounds, and fails to detect subtle changes in the protein content of wort and beer.
Many quality attributes (e.g. turbidity, mouthfeel, foam stability) and processability (parameters such as filterability, are affected by the protein composition and content in beer. For example protein Z (M_R 40 kDa) [12] is claimed to be responsible for haze formation and LTP1 [25] (M_R 10 kDa) for foam stability. *Siebert* [23] suggests that a higher amount of prolin results in a higher turbidity.
Therefore, not only to measure the quantitative protein content, but also the qualitative protein composition is important to brewers.
The aim of this review is to describe and compare different methods of protein quantification and qualification. For that reason six different methods have been evaluated.

Descriptors: protein assays, Kjeldahl method, Bradford method, lab-on-a-chip analysis, 2D-PAGE, mass spectrometry

1 Introduction

Beer is a complex mixture of over 450 constituents, and, in addition, it contains macromolecules such as proteins, nucleic acids, polysaccharides and lipids [4].

Proteins and protein structure play a major role in beer and beer quality. Beer contains ~500 mg/L of proteinaceous material, including a variety of polypeptides with molecular masses ranging from 5 to 100 kDa the majority of which lie within the 10–40 kDa size range. These polypeptides, which mainly originate from barley, are the product of the proteolytic and chemical modifications during malting and brewing [5, 12, 20, 25].

Proteins influence the whole brewing process not only in the form of enzymes but also in combination with other substances such as polyphenoles. As enzymes they degrade starch, ß-glucanes and proteins, in protein-protein linkages they stabilize foams and are responsible for mouthfeel and flavour stability and in combination with polyphenoles they are thought to form haze. As amino acids, peptides, sal ammoniac they are important nitrogen sources for yeast. Studies on these aspects have already been done for barley (variety differentiation, development of enzymes during germination etx. [2, 9, 10, 27–29]) and for beer. In beer the main focus was on foam and haze active proteins [15, 16, 21, 22].

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Figures see Appendix

It is important for brewers to know which methods are most appropriated and useful not only for quantitative but also for qualitative protein assays.

2 Materials

Total protein content (Kjeldahl method, Bradford assay), coagulable nitrogen, nitrogen fractionation (precipitation of magnesium sulfate and phosphomolybdenum acid) and free amino nitrogen of freshly collected beer were immediately measured. Samples of the collected beer were freeze dried and prepared for 2D-PAGE and lab-on-a-chip analysis.

2.1 Methods

2.1.1 Kjeldahl method

The standard method for determining protein content of beer is the Kjeldahl method [8, 19]. The standard value of total nitrogen content in beer ranges between 700–800 mg N/L.

Nitrogenous compounds in the beer are digested with hot sulphuric acid in the presence of catalysts to give ammonium sulphate. The digest is made alkaline with sodium hydroxide solution and released ammonia is distilled into an excess of boric acid solution. The ammonia is titrated with standard acid solution.

2.1.2 Nitrogen fractionation

- Precipitation of magnesium sulfate (> 2600 Da). To estimate high molecular weight nitrogen. High molecular weight nitrogen is precipitated with magnesium sulfate and analysed by a

Kjeldahl procedure. Standard value: 130–180 mg/L.

- Precipitation of phosphomolybdenum acid (< 2600 Da).: to estimate middle molecular weight nitrogen. Middle molecular weight nitrogen is precipitated with phosphomolybdenum acid and analysed by a Kjeldahl procedure. Standard value: 160–200 mg/L.

2.1.3 Coagulable nitrogen according to MEBAK [19]. Standard value: 15–25 mg/L

Estimation of high molecular nitrogen. Precipitation of high molecular nitrogen during 5 hours of boiling at 105–108 °C. Digestion of nitrogen with Kjeldahl method.

2.2 Free amino nitrogen in beer by spectrophotometry (IM) according to EBC [8]

The method gives an estimate of amino acids, ammonia and, in addition, the terminal α-amino nitrogen groups of peptides and proteins. Proline is partially estimated at the wavelength used.

2.3 Bradford assay

Bradford assay [3] is a protein determination method which involves the binding of Coomassie Brilliant Blue G-250 to protein. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm, and it is the increase in absorption at 595 nm which is monitored. This assay is very reproducible and rapid. It is virtually completed in approximately two minutes and presents good colour stability for approximately one hour. There is little or no interference from cations such as sodium or potassium, carbohydrates such as sucrose. The use of strongly alkaline buffers develop a small amount of color but the assay may be run accurately by the use of proper buffer controls.

2.4 Lab-on-a-chip

Lab on a chip technique capillary electrophoresis was carried out on the Agilent 2100 bioanalyzer [26]. The principles of these electrophoretic assays are based on traditional gel electrophoresis principles that have been transferred to a chip format. The chip accommodates sample wells, gel wells and a well for an external standard (ladder). Micro-channels are fabricated in glass to create interconnected networks among these wells. During chip preparation, micro-channels are filled with a sieving polymer and fluorescence dye. Once wells and channels are filled, the chip becomes an integrated electrical circuit.

Extraction for lab-on-a-chip technique: Resolve 100 mg of freeze dried sample in 1.5 mL of lysis puffer (2M Urea, 15 % Glycerol, 0,1M Tris, pH 8.8, 0.1M DTT). 4 µL of this solution were denatured using 2 µL of Agilent denaturing solution and heated for 5 min. at 100 °C. After dilution with deionised water, 6 µL were applied to the Protein 80+ LabChip (detection performance between 4.5 and 95 kDa) for analysis in the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). The ladder consisted of reference proteins of 3.5, 6.5, 15, 28, 46, 63 kDa plus the upper and the lower markers of 95 and 1.6 kDa. According to the Agilent manual any peak detected below 5 kDa is named a system peak and is not included in analysis. Results can be shown in an electropherogram or a gel-like image, as known from SDS-PAGE analysis, where the intensity of bands equals the peak heights in the electropherogram.

2.5 2D-PAGE

2D-PAGE (Two dimensional polyacrylamide gelelectrophoresis) [11] was carried out on the Ettan™ IPGphor™ 3 IEF System and the Ettan™ DALT*six* Large Vertical System from GE Healthcare on 12.5 % acrylamide gels.

Extraction for 2D-PAGE was carried out as followed: TCA/Acetone precipitation [7]:

The combination of TCA and acetone is commonly used to precipitate proteins during sample preparation for 2D electrophoresis, and is more effective than either TCA or acetone alone.

Resuspend 300 mg freeze dried sample in 1 mL TCA 10 % in acetone with 20 mM DTT. Precipitate proteins for at least 45 min at –20 ºC. Pellet proteins by centrifugation (15 min) and wash pellet with 1 mL cold acetone containing 20 mM DTT. Remove residual acetone by air drying or lyophilisation. Resolve the pellet in 0.5 mL lysis puffer (9.5 M urea, 1 % (w/v) dithiothreitol (DTE), 2 % (w/v) CHAPS, 2 % (v/v) carrier ampholytes (pH 3–10) and 10 mM Pefabloc® proteinase inhibitor).

High-resolution two-dimensional electrophoresis (2D PAGE) for the separation of complex protein mixtures is a combination of isoelectric focusing (IEF) in the first dimension in presence of urea, detergents and DTT, with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. Proteins are separated according to isoelectric point (pI) and molecular mass (M_R), and quantified according to relative abundance. Depending on the gel size and pH gradient used, 2D PAGE can resolve more than 5.000 proteins simultaneously (~2.000 proteins routinely), and can detect <1 ng of protein per spot. Furthermore, it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or post-translational modifications. This is in contrast to LC-MS/MS based methods, which perform analysis on peptides, where Mr and pI information is lost, and where stable isotope labelling is required for quantitative analysis. An additional strength of 2D PAGE is its capability to study proteins that have undergone some form of post-translational modification (such as phosphorylation, glycosylation or limited proteolysis) and which can be readily located in 2D gels as they appear as distinct ‘spot trains’ in the horizontal and/or vertical axis of the 2D gel. Thousands of proteins can be resolved in a single experiment allowing the major proteins in a sample to be isolated and protein levels in related samples to be compared. In combination with mass spectrometry, the proteins can also be identified.

In combination with advanced image analysis, 2D-PAGE is a powerful methodology for detecting changes in protein composition during development, and to pinpoint most influential proteins different processes.

2.6 Mass spectrometry (MS) [1]

Mass spectrometry is an analytical tool used for measuring the molecular mass of a sample. In proteomics, Matrix Assisted Laser Desorption Ionisation (MALDI) [14] is used for the identification of isolated proteins 2D-PAGE. One method used is peptide mass fingerprinting by means of MALDI-MS. MALDI is based on the bombardment of sample molecules with a laser light to bring about sample ionisation. This procedure usually involves the excision of individual spots from a 2D gel and the enzymatic digestion of proteins within each, before analysing the digest mixture using mass spectrometer. The initial MS spectrum determining the molecular masses of all of the components of the digest mixture, can often provide sufficient information to search a database using just several molecular weights from this peptide map.

With the help of MALDI following analyses and results can be obtained [1]:

It is useful to measure accurate molecular weight, to confirm samples, to determine the purity of a sample, to verify amino acid substitutions, to detect post-translational modifications and to calculate the number of disulphide bridges.

It is helpful for reaction monitoring of enzyme reactions, chemical modification, protein digestion.

MALDI is needed for amino acid sequencing, confirmation of sequences, de novo characterisation of peptides, identification of proteins by database searching with a sequence "tag" from a proteolytic fragment and also for oligonucleotide sequencing: The characterisation or quality control of oligonucleotides. Even protein folding and protein-ligand complex formation can be monitored and the macromolecular structure can be determined.

3 Results and Discussion

The most applied method in protein determination in beer is Kjeldahl method. Although Kjeldahl method has been automated, it still employs toxic and hazardous reagents. The advantage of this method lies in its suitability, reproducibility and the approval for beer and its raw materials analysis. This method, in combination with a fractionation (precipitation of magnesium sulfate and phosphomolybdenum acid) gives an overview of the amount of different protein fractions in beer. If amino acids and/or free amino nitrogen are analysed additionally a general survey of several nitrogenous components in beer is guaranteed. According to this fractionation information of foam stability (middle-molecular-weight proteins, precipitation of phosphomolybdenum acid), mouthfeel (coagulable nitrogen and nitrogen achieved from precipitation of magnesium sulfate) and fermentation (free amino nitrogen) can be obtained.

The disadvantage of these methods lies in the number of analyses. At least five different methods have to be performed to provide an insight into the protein composition of beer. As a matter of fact not only the quantity but also the duration of these methods implies an error source.

Studies on determination of protein content have already been made [6, 13, 18, 24, 30]. In these articles total protein content and Protein composition with SDS-PAGE were measured. In all these articles was indicated that the Bradford assay is recommended for brewing purposes. It is fast, simple, sensitive, reproducible and remarkable lack of response of compoundswhich interfere with other methods. *Siebert and Hii* [13, 24] say, that the Bradford assay is suitable for the detection of high molecular weight proteins, for example foam active proteins.

Several authors compared, for total protein content the Kjeldahl method with the Bradford assay. In all articles it is mentioned that Bradford is more accurate than Kjeldahl method.

Kjeldahl method showed higher nitrogen content as the Bradford assay. With Kjeldahl total nitrogen content is determined, and just with a factor of 6.25, protein content is calculated, it is evident from the "protein" values that most of the measured nitrogen is associated with low molecular weight interfering substances. With the Bradford assay only proteins and not total nitrogen content are analysed. Also, that many proteins are glycosylated and protein assays fail to take account of carbohydrate constituents. This could explain the higher concentration of total protein content in the Kjeldahl assay.

SDS-PAGE requires extraction, gel casting, electrophoretic separation, staining and interpretation. With lab-on-a-chip technique nearly all steps, but extraction, are achieved in one step. This avoids mistakes and is even faster than 'normal' SDS-PAGE. In figure 1 [17] separation of malt with lab-on-a-chip technique can be seen, this method can be easily applied to beer. lab-on-a-chip technique provides fast and reproducible results which can absolutely be compared with SDS-PAGE. Relative amount of protein can be obtained. Therefore it is possible to compare samples from the same raw material.

In figure 2, proteins were separated with help of 2D-PAGE. In region b proteins with a molecular weight of ~43 kDa are shown. Proteins of this size are claimed to influence haze formation. Marked spots in 'Region 1' represent foam active proteins.

4 Conclusion

With several different methods, e.g.: Kjeldahl, fractionation, Bradford assay, information on the protein content of beer and some information of their effects on beer quality parameters can be obtained. Bradford assay is easier, faster and cheaper than Kjeldahl method but not yet established as analysis in brewing science. Bradford assay is recommended for monitoring changes in the protein composition during the brewing process.

To get an insight in protein composition and how proteins influence processability, mouthfeel, foamstability etc., other analyses have to be performed. With the help of a lab-on-a-chip technique a fast overview of several protein components is achieved. To gain knowledge in protein structure and composition 2D-PAGE and MALDI have to be made.

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Appendix

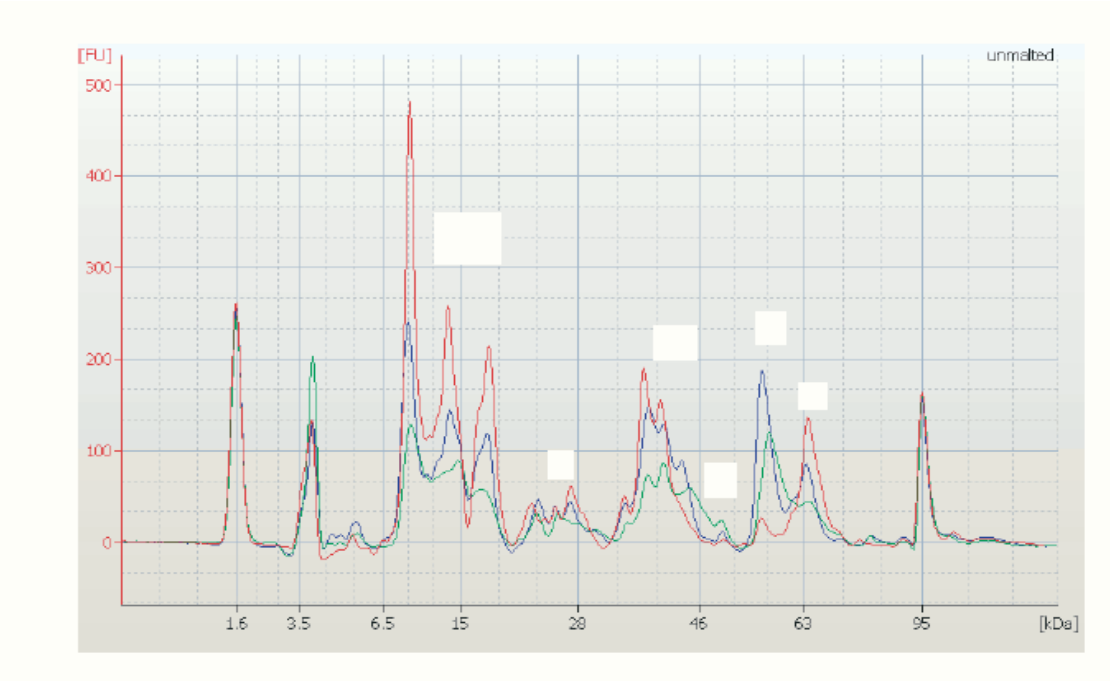

Fig. 1 **Separation of malt with lab-on-a-chip technique [17]**

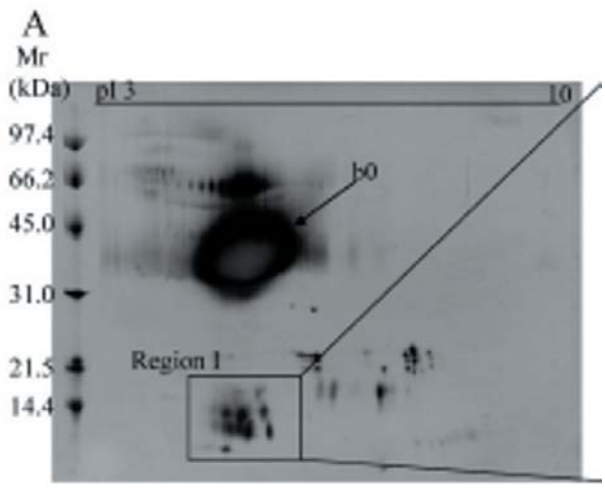

Fig. 2 **2D-PAGE of beer [16]; marked spots in Region 1 represent foam positive proteins**

8.3 Turbidity and haze formation in beer – insights and overview

Turbidity and Haze Formation in Beer – Insights and Overview

Elisabeth Steiner*, Thomas Becker and Martina Gastl

ABSTRACT

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Beer is a complex mixture of over 450 constituents. In addition, it contains macromolecules such as proteins, nucleic acids, polysaccharides and lipids. Proteins influence the entire brewing process with regard to enzymes, which degrade starch, β-glucans and proteins; with protein-protein linkages that stabilize foam and are responsible for mouthfeel and flavour stability; and in combination with polyphenols, thought to form haze. With this complexity, problems in processability are as various as the constituents. Several substances in beer are responsible for haze formation. Organic components such as proteins, polyphenols and carbohydrates (α-glucans, β-glucans) are known to form haze. In addition, inorganic particles such as filter aids and label remains can cause increased turbidity. In this article only non-microbiological induced hazes are described. Many studies have been conducted on the identification of haze and foam active components in beer. Hence the aim of this work was to survey the different possibilities of haze formation and for haze identification. A summary is provided on methods for haze identification including dyeing methods, microscopic analyses and size exclusion chromatography.

Key words: GPC, haze formation, haze identification, microscope, proteins.

INTRODUCTION

Haze formation is an important problem in beer production, as it affects the quality of the end product. Beer consists of various ingredients such as proteins, carbohydrates, polyphenols, fatty acids, nucleic acids, amino acids etc. These ingredients can precipitate and haze is formed. Malted barley contains 70–85% total carbohydrates, 10.5–11% proteins, 2–4% inorganic matter, 1.5–2% fat and 1–2% other substances[20]. Beer haze consists of several components: the most common organic parts are proteins (40–75%), polyphenols (in combination with proteins) and to a smaller proportion carbohydrates (2–15%). There exist two forms of haze: cold break (chill haze) and age-related haze[30]. Cold break haze forms at 0°C and dissolves at higher temperatures. If cold break haze does not dissolve, age related haze, which is irreversible, develops. Chill haze is formed when polypeptides and polyphenols are non-covalently bound. Permanent haze forms in the same manner initially, but covalent bonds are soon formed and insoluble complexes are created that will not dissolve when heated[34]. Haze formation can be due to residual starch, pentosans from wheat-derived adjunct, oxalate from calcium-deficient worts, β-glucan from inadequately modified malt, carbohydrate and protein from autolysed yeast, lubricants from can lids, and dead bacteria from malt[3]. Haze particles can show different appearances. Glenister et al.[15] published a classification for haze particles in beer as follows:

1. Native particles, which originate from the beer by coagulation/precipitation,
2. Process particles, which originate from materials (e.g., filter aids) added during the process,
3. Foreign particles, which enter the beer as accidental contaminants.

These particles can have the shape of flakes, ribbons and grains. Flakes are thin, film-like particles with no regular formation. When flakes precipitate, ribbons are formed. Grains can be mixed up with singular cells and bacteria.

Bamforth et al.[3] also divided haze into several types. Visible haze, seen as "bits" that contain protein and perhaps pentosans, is thought to arise as the skins around foam generated within the package. Visible haze formation can limit the shelf life of products, since the consumer expects a clear beer[35]. There are also the "invisible" hazes, which are also called "pseudo-hazes." These are caused by very small particles (<0.1 μm) that cause high levels of light scatter when measured at 90° to incident[3].

COMPONENTS OF HAZE PARTICLES

Proteins

Beer contains ~500 mg/L of proteinaceous material, including a variety of polypeptides with molecular masses ranging from <5 to >100 kDa. The content of only 2 mg/L protein is enough to form haze[19]. Only about 20% of the total grain proteins are water-soluble. Barley water-soluble proteins are believed to be resistant to proteolysis and heat coagulation and hence pass through the processing steps, intact or somewhat modified, to beer[11,31,36]. These polypeptides, which mainly originate from barley proteins, are the product of the proteolytic and chemical

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modifications that occur during brewing[9]. Only one third of the total protein content passes into the finished beer. Proteins play a major role in beer stability; hence they are, beside polyphenols, part of colloidal haze. Proteins, as the main cause of haze formation in beer, are divided into two main groups: first proteins and second their breakdown products. Protein breakdown products are characterised by always being soluble in water and they do not precipitate during boiling. Finished beer contains primarily protein breakdown products. A beer protein may be defined as a more or less heterogeneous mixture of molecules, containing the same core peptide structure, and originating from only one distinct protein present in the brewing materials[17]. Several aspects of the brewing process are affected by soluble proteins, peptides and/or amino acids that are released. Many studies have been conducted on the identification of haze and foam active proteins. Asano et al.[1] investigated different protein fractions and split them into three categories: high-, middle- and low molecular weight fractions with the following separation: high molecular weight fractions of >40 kDa, middle molecular weight fractions of 15–40 kDa and low molecular weight fractions of < 15 kDa. Nummi et al.[25], suggested that the acidic proteins derived from albumins and globulins of barley are responsible for chill haze formation. Researchers have proven that proline-rich proteins are involved in haze formation[2–4,10,18,22,23,29,30,32–34]. With the help of the wort boiling process, fermentation and maturation, protein particles can be removed. Proteins coagulate during the wort boiling process, thus they can be removed in the whirlpool. The pH decreases during fermentation and proteins can be separated as cold trub. Proteins during maturation adhere onto the yeast and can be discarded with the yeast[3].

Polyphenols

Phenolic components, which also can participate in haze formation, reach the beer through hops and malt. They exert an influence on several beer quality attributes, such as the colloidal stability of beer. Due to defined conditions, for example insertion of oxygen, protein precipitation products can occur.

Proteins and polyphenolic compounds can combine to form soluble complexes. These can grow to colloidal size, at which time they scatter light, and grow even larger, which can lead to sediment formation. The protein/polyphenol ratio has a strong influence on the amount of haze formed; the largest amount occurs when the numbers of polyphenol binding ends and protein binding sites are nearly equal[32].

Glucans

Glucans are polysaccharides that only contain glucose as the structural components and are linked with glycosidic bonds. Barley starch consists of two polysaccharides: amylose (20–30%) and amylopectin (70–80%), which are D-glucose monomers linked together with α-(1-4) and α-(1-6) bonds.

Degraded starch is the main component of beer extract. If starch is not fully degraded, haze can occur. This can happen during the following brewing process steps:

1. During the malting process: When the content of glassy kernels is higher than 3%, filtration and turbidity problems can result.
2. The second step is milling, when barley malt grains are not correctly milled and hence cannot be fully degraded by enzymes. Starch stays in the wort and in the beer and can form haze.
3. In the third step, mashing, starch cannot be degraded due to incorrect or too short temperature rests.
4. If the lauter temperature is too high, a late saccharification can occur that also leads to haze formation.
5. When starch kernels survive the mashing process and reach the wort boiling vessel, agglutination can occur. Starch is not degraded further, reaches the beer and causes turbidity problems.
6. When yeast is stressed (high temperatures, high extract concentration, etc.), the storage polysaccharide glycogen, which similar to starch, can be released. Glycogen, the glucose storage compound of animals, is a more branched version of amylopectin and exhibits more α-1-6-bonds than starch, hence the molecular size of glycogen is larger than that of amylopectin[16,26,27].

The β-glucans are polysaccharides of D-glucose monomers linked by β-glycosidic bonds (β-(1-3) and β-(1-6)) and occur in barley as structural substances in the cell walls. The β-glucan passes from the barley, via malting, mashing and wort boiling, through the fermentation into the finished beer. The β-glucan is known to cause problems in filtration, as it increases viscosity. Speers et al.[37] found out that β-glucan at a size of 300 kDa increases turbidity after filtration.

Inorganic matter

Particles which do not originate from organic sources such as barley, hops, yeast and water are, in this context, called inorganic matter. Inorganic components are often dirt particles, which are present due to poor cleaning and filter aids. These substances are comprised of dust particles, remains of labels, filtration aids, etc. Filter and stabilisation aids can appear in beer as haze, if these particles pass the filters and the trap-filters.

Calcium oxalate

Haze can also be caused by calcium oxalate. Calcium oxalate is formed from oxalic acid and calcium. Oxalic acid already exists in barley and calcium is available from the water. The oxalic acid concentration is dependent on the year of harvest with calcium coming mostly from the brewing water. The solubility product of calcium oxalate in beer is low and therefore it precipitates in the form of crystals. Those crystals can have the form of octahedrons, rosettes, prisms and amorphous forms[16,38,39]. It is important that there is sufficient calcium in the grist to ensure precipitation of the oxalate[8].

Turbidity gives the first visual impression of beer quality to the consumer. Therefore it is necessary to have methods to not only identify haze, but also to infer on the source of the haze. The aim of this research was to show examples for applications in haze identification in beer.

MATERIALS AND METHODS

Beer samples

Commercial beer samples with several different turbidity problems were used in this investigation. The samples were analyzed according to following methods.

Turbidity. Turbidity was measured according to MEBAK Band II; chapter 2.15.1.2[28], with a two angle turbidity measurement instrument (LabScat, Sigrist, Ennetbürgen). The angles were 90° and 25° in forward scattering. Only the data of the 90° angle are shown, since there was no significant difference to the measurement at the 25° angle. The amount of turbidity is expressed in EBC units.

Enzymatic identification of haze particles. The purpose of enzymatic haze identification is the specific degradation of the turbidity in beer with enzymes. Table I shows the enzymes used for enzymatic haze identification. A 100 mL aliquot of turbid beer sample per enzyme was degassed and transferred into a 180 mL glass bottle with a swing stopper. Enzymes were added and the beers were incubated for at least 12 h at the analysis temperature. The turbidities of the samples without enzymes, and with enzymes after 12 h of incubation, were measured according to MEBAK volume II; chapter 2.15.1.2[28].

Concentration of haze particles. Commercial samples with increased turbidity were degassed and filtered by membrane-filtration (cellulose nitrate filter, 50 mm, 1.2 µm and filter station, Sartorius, Göttingen). The membranes were transferred into 14 mL plastic tubes, washed with 3 mL distilled water and the membrane was discarded. This 3 mL of distilled water, enriched with particles, was again concentrated in a centrifuge, the supernatant was discarded and the particles were transferred into 200 µL of distilled water.

Microscopic analyses. The aim of microscopic haze identification is the visualization of the haze particles. An Axioskop 50 microscope, (Zeiss, Göttingen), a Sony camera DSC-S75 (Sony, Tokyo) and a magnification of 400X, were used. Identification of haze particles was carried out using several different adjustments of the microscope and various dyes.

Viewing options are as follows.

1. Transmitted light (for transparent and liquid samples). The beam of light goes from below through the objective to the ocular.
2. Reflected light (for solid samples). The beam of light goes through the objective directly onto the object. The reflected light goes back through the objective into the ocular
3. Polarisation (crystalline objects). The microscope is equipped for polarization work. The beam of light is crossed because of two polarization filters. Crystalline objects are able to turn the level of polarized light and thus appear white upon a black background. Particles which interfere with this passage of polarized light appear as bright objects in a dark field.
4. Fluorescence: Filter G365 (Zeiss, Göttingen). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. The light goes through the objective onto the object. The specimen is illuminated with light of a specific wavelength (or wavelengths), which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different colour than the absorbed light). The autofluorescence of the haze particles can be analyzed. Phenolic particles (such as ferulic acid) have a blue-green fluorescence.
5. Particle dyeing[12–14] involves adding 1.5 mL distilled water to 1 mg dye. The particles and the dye are mixed together directly onto the microscope slide. Table II shows the dyeing materials used.
6. Haze identification with membrane filtration. Commercial samples with increased turbidity (90° angle > 1 EBC; 25° angle >0.5 EBC) can be degassed and filtered with the help of a membrane filtration unit (cellulose nitrate, filter 50 mm, 1.2 µm, and filter station, Sartorius, Göttingen) and dried. An area of 1 cm^2 of the dried membrane is cut out of the membrane and placed onto a microscope slide and viewed with immersion oil (Immersol 518 N, Zeiss, Göttingen). Membrane and oil have the same refraction index, thus the membrane becomes transparent and particles can be seen using transmitted light microscopy.

Gel permeation chromatography (GPC). With gel permeation chromatography, particles are separated according to their size. Gel permeation chromatography was used to separate glucans. Glucans can be detected using a photometric analysis with iodine. Glucans react with iodine to form blue complexes. The analysis is a modification of the method in MEBAK volume II 2.3.2[28]. Äk-

Table I. Enzymes used for enzymatic haze identification.

Enzyme	Company	EC-Nr.	Degradation	Analysis temperature [°C]	Amount
Pepsin	Sigma	3.4.23.1	Proteins	40	0.1 g
Amyloglucosidase	Sigma	3.2.1.3	Dextrins, starch	20	30 µL
Ultraflo®Max	Novozymes	3.2.1.73	β-glucan	20	30 µL

Table II. Dyes used for the staining of haze particles.

Dye	Company	Material stained	Colour
Eosin Yellow	ICN, Aurora	Proteinaceous material	Stains slightly pink
Thionine	ICN, Aurora	Jellied and precipitated material out of polysaccharides	Stains neutral polysaccharides purple and acidic slightly pink
Methylene Blue	Merck, Darmstadt	Adsorbing substances, fibres, tannins and polyphenols	Stains dark blue
Iodine dilution; 1N	J. T. Backer, Deventer	Starch-containing particles and PVPP	Stains starch-containing particles a blue-purple colour and PVPP a strong orange colour

taprime (Amersham Biosciences, Freiburg) and a Superdex™200 (10–600 kDa) column (Amersham Biosciences, Freiburg) were used. The eluent was a phosphate buffer (0.05 M disodium hydrogen phosphate mixed with 0.05 M potassium dihydrogen phosphate at pH 7.0). The flow rate was 2.2 mL/min. The first fraction was collected after 90 min. A total of 31 fractions were collected at a fraction size of 11 mL. The photometer was a Cadas 200 (Dr. Lange, Berlin; λ = 578 nm). Chemicals for the measurement were according to MEBAK volume II 2.3.2[28].

Sample preparation: A 40 mL sample was precipitated in 120 mL ethanol, stirred for 10 min and centrifuged at 9,000 rpm for 10 min (Sigma 6K15, Sigma Laborzentrifugen GmbH, Osterode). The residue was dissolved in 10 mL buffer and separated with the help of gel permeation chromatography.

The collected samples were measured with a photometer. The reference measurement was comprised of 6 mL phosphate buffer (pH 4.5), 4 mL phosphate buffer (pH 7) and 0.5 mL dissolved iodine solution. The samples contained 6 mL phosphate buffer (pH 4.5), 4 mL sample and 0.5 mL dissolved iodine solution. After the addition of the iodine solution, measurements were taken after 30 sec. Interpretation was carried out with the help of a spreadsheet program.

Particles were separated according to size exclusion. High molecular weight particles eluted first, thus a separation of glycogen (50 kDa) and amylopectin (5–10 kDa) was possible. Figure 1 shows a typical evaluation of a sample with turbidity problems related to yeast management and brewhouse problems.

RESULTS

To examine haze particles which can occur in beer, particles were collected and concentrated according to Material and Methods and stepwise analyses were conducted. Examples are shown for each "haze-initiator" using the following techniques:

i. Turbidity measurement
ii. Enzymatic haze identification
iii. Microscopic haze identification
iv. Verification of the source of glucan by gel permeation chromatography.

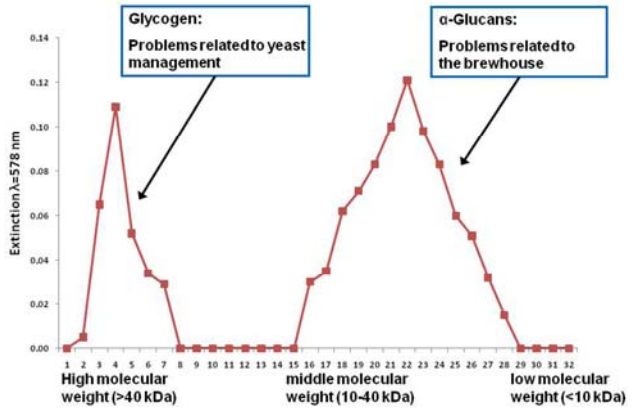

Fig. 1. Gel permeation chromatography (Superdex® column with separation of particles from 10–600 kDa) of a beer with glycogen and α-glucan turbidity[16].

Numerous samples from different breweries were analyzed. To provide an overview over different haze formations in beer, five different sources of haze formation are presented.

1. Haze caused by proteins
2. Haze caused by α-glucans
3. Haze caused by calcium oxalate
4. Haze caused by inorganic matter (filter aids)
5. Haze caused by inorganic matter (labels, glass particles, etc.)

Haze caused by proteins. In Fig. 2, enzymatic haze identification is shown. The difference between bar 1 and 2 indicates the effectiveness of the enzyme. The highest turbidity difference was in the sample treated with the enzyme pepsin, which degraded the protein haze.

According to the analysis, proteins were the source of haze formation in the beer. The beer turbidity was first measured at room temperature. A decrease in the turbidity occurred in the second measurement of the beer sample, which had already been incubated for 12 h with enzymes. Corresponding to this decrease, and the assumption that the particles were comprised of proteins, the particles were stained with Eosin Yellow and Methylene Blue (Fig. 3 and 4) to confirm the results of the enzymatic identification. Protein haze often appears as transparent flakes,

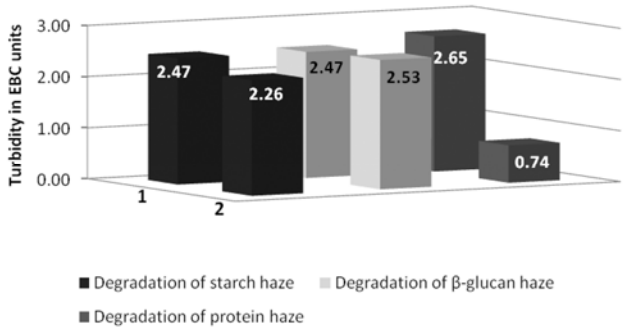

Fig. 2. Compilation of protein haze by the use of enzymatic degradation. From left to right the bars show the degradation of starch/β-glucan/protein haze. Bar 1 shows the turbid sample and bar 2 shows the sample treated with enzyme after 12 h of reaction time.

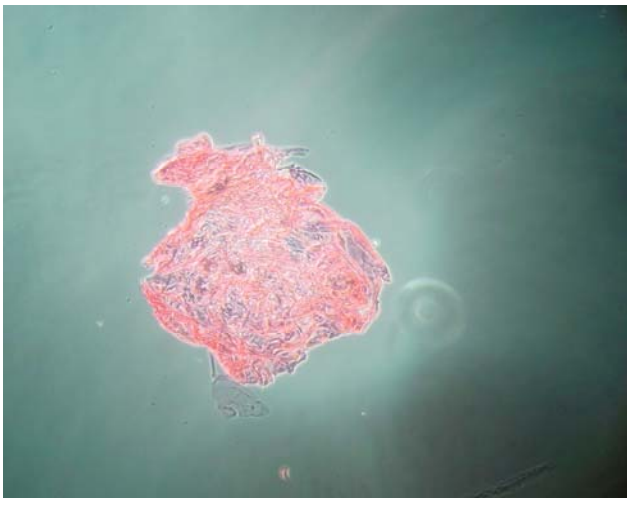

Fig. 3. Protein haze particle, stained with Eosin Yellow.

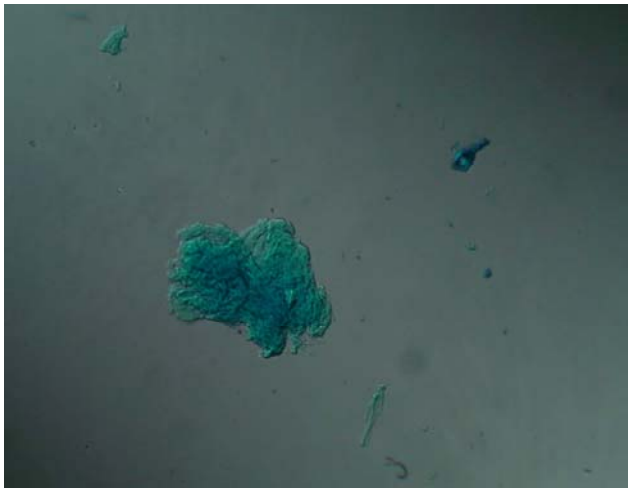

Fig. 4. Protein-phenolic haze particle stained with Methylene Blue.

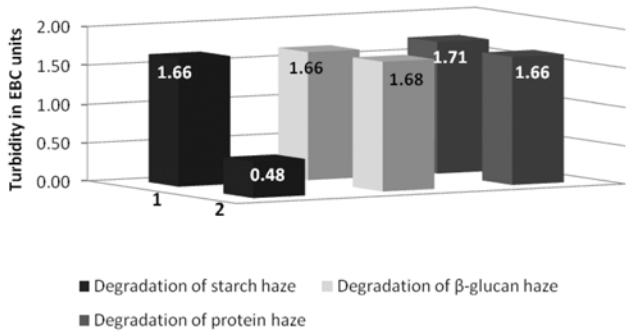

Fig. 5. Compilation of starch haze by the use of enzymatic degradation. From left to right the bars show the degradation of starch/β-glucan/protein haze. Bar 1 shows the turbid sample and bar 2 shows the sample treated with enzyme after 12 h of reaction time.

which look somewhat fragile. The combination of protein and phenolic compounds in haze particles, which Siebert et al.[34] already discussed in 1996, are shown in Fig. 4. The protein parts stain green and the more phenolic parts stain blue. The formation of protein haze often lies in a poor clarification of the beer from the yeast[5–7,21,24].

Haze caused by α-glucans. In Fig. 5, enzymatic degradation of another beer haze is illustrated. Bar 1 shows the turbid sample and bar 2 shows the sample treated with enzyme after 12 h of reaction time. The difference between bar 1 and 2 indicates where the enzyme was most effective. In this case, starch was identified as the haze-forming substance. This is seen in the decrease in turbidity in the sample where amyloglucosidase had been added, since amyloglucosidase is an enzyme which degrades starch.

Starch can be identified microscopically with an iodine-solution and Thionine. A distinction between glycogen and starch can be carried out using size exclusion chromatography. Figure 6 shows the identification of starch particles with Thionine and Fig. 7 with an iodine solution. In Fig. 6 it can be seen that Thionine has dyed only polysaccharides. A protein flake in the middle of the picture remains unstained. Figure 7 shows dark blue

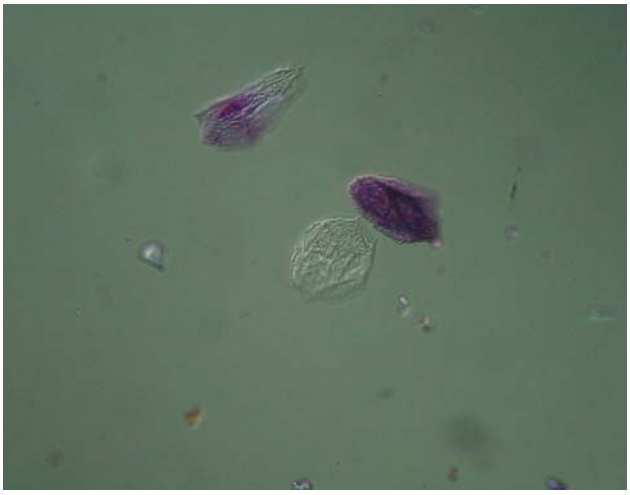

Fig. 6. Haze made of polysaccharides stained with Thionine.

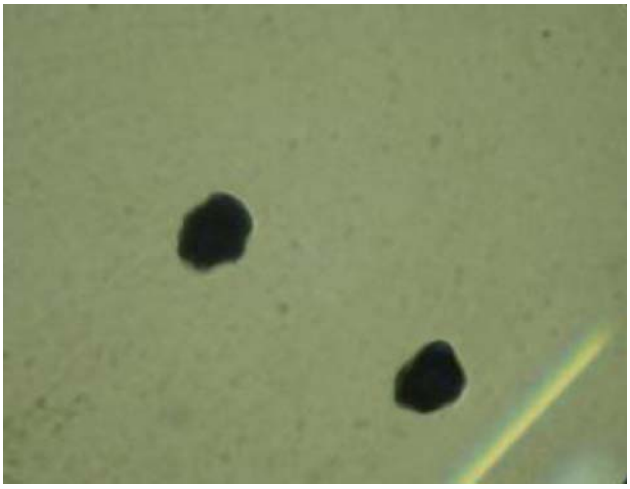

Fig. 7. Starch granulates stained with iodine solution.

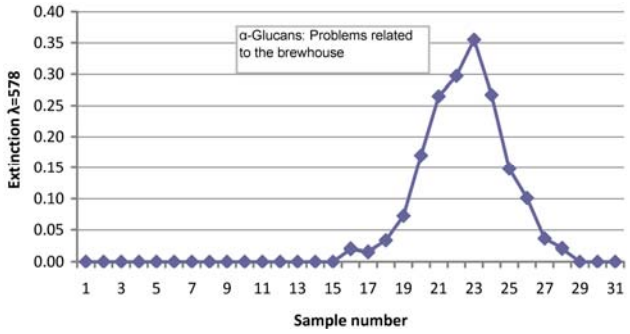

Fig. 8. Gel permeation chromatography, beer sample with haze made of starch components.

starch granulates. Figure 8 shows the verification of the starch induced haze formation by gel permeation chromatography. The haze, in this case, was clearly identified as starch-induced and problems in the brewhouse are suggested.

Haze caused by calcium oxalate. Figures 9–11 show the identification of calcium oxalate in beers with turbidity problems and no indication of organic haze. The iden-

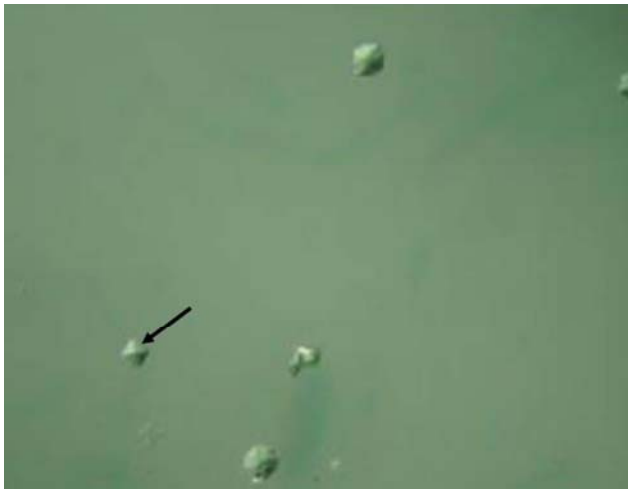

Fig. 9. Calcium-oxalate dihydrate in transmitted light beam. A pyramidic crystal is marked with an arrow.

Fig. 10. Calcium-oxalate monohydrate in a polarized light beam.

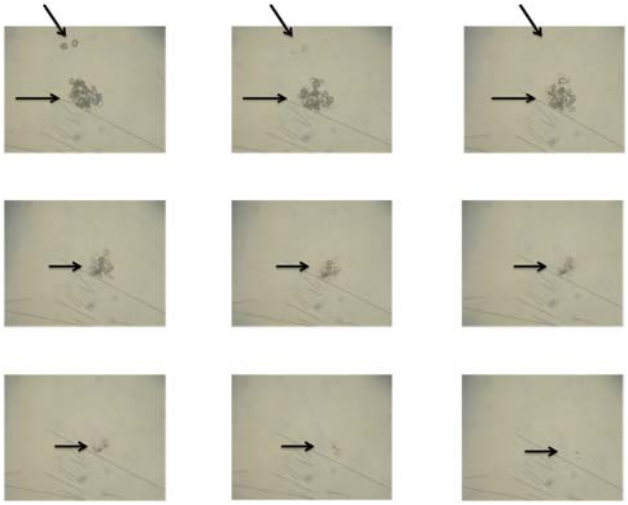

Fig. 11. Solubilisation of calcium-oxalate monohydrate with sulphuric acid.

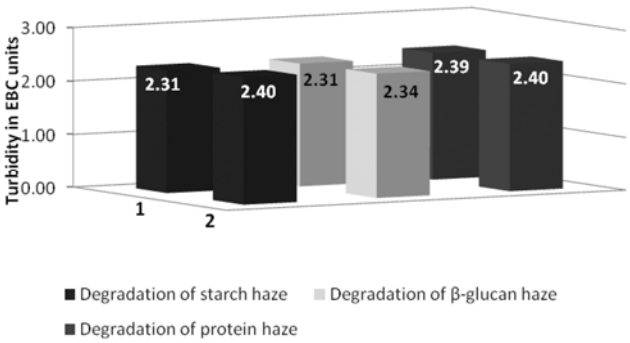

Fig. 12. Enzymatic haze identification, no organic haze identified. From left to right the bars show the degradation of starch/β-glucan/protein haze. Bar 1 shows the turbid sample and bar 2 shows the sample treated with enzymes after 12 h reaction time.

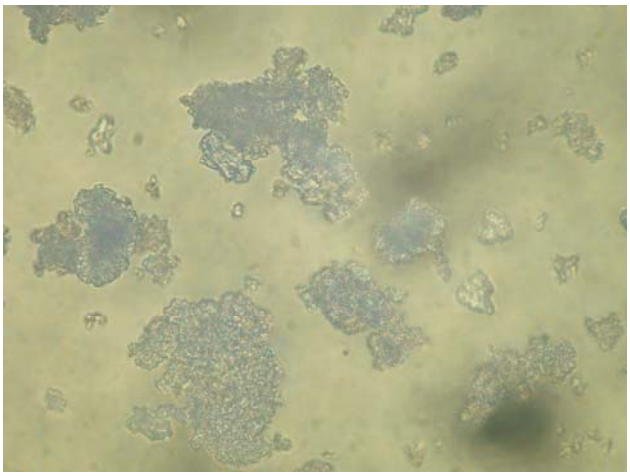

Fig. 13. PVPP particles in a transmitted light beam.

tification of calcium oxalate can be carried out in three different ways. Calcium oxalate dihydrate has a very characteristic crystalline structure (Fig. 9). Calcium oxalate monohydrate, in a polarized light-beam (Fig. 10), appears white on a black background and can be dissolved with sulphuric acid. The structure of calcium oxalate dihydrate resembles pyramids, but calcium oxalate, in the form of calcium oxalate monohydrate can also appear as needles (picture not shown). The dissolution of calcium oxalate can be seen in Fig. 11. The arrows indicate where calcium oxalate was being dissolved with sulphuric acid.

Haze caused by inorganic matter (filter aids). Figure 12 shows enzymatic haze identification without any indication of organic haze. Bar 1 shows the turbid sample and bar 2 shows the sample treated with enzymes after 12 h of reaction time. In this case there was no difference between the bars.

Microscopic identification was carried out and the results are shown in Figs. 13–17. The haze particles can be identified as PVPP (polyvinylpolypyrrolidone) a stabilization aid, and kieselguhr, a filtration aid. These particles indicate a problem with the filtration technology. Figure 13 shows PVPP-particles under transmitted light, Fig. 14 shows a PVPP particle stained with iodine solution, Fig.

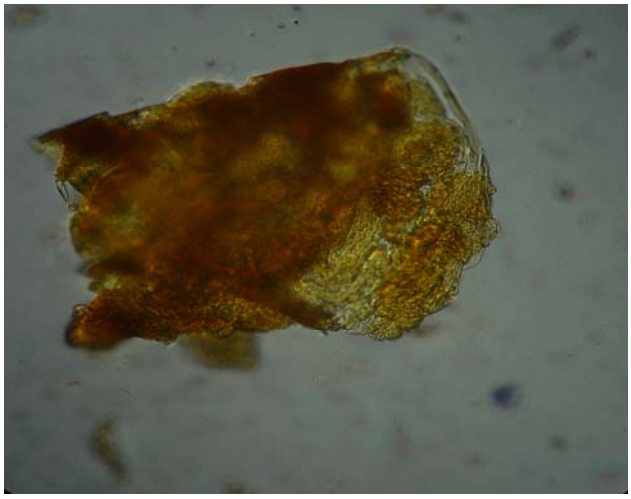

Fig. 14. PVPP particle stained with iodine solution.

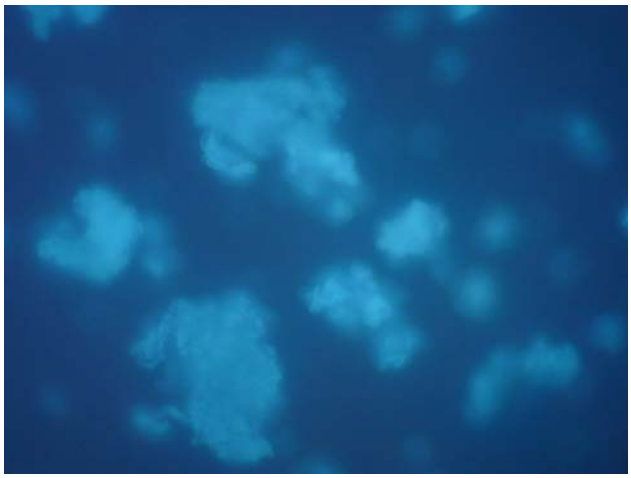

Fig. 15. PVPP particles exhibiting auto fluorescence.

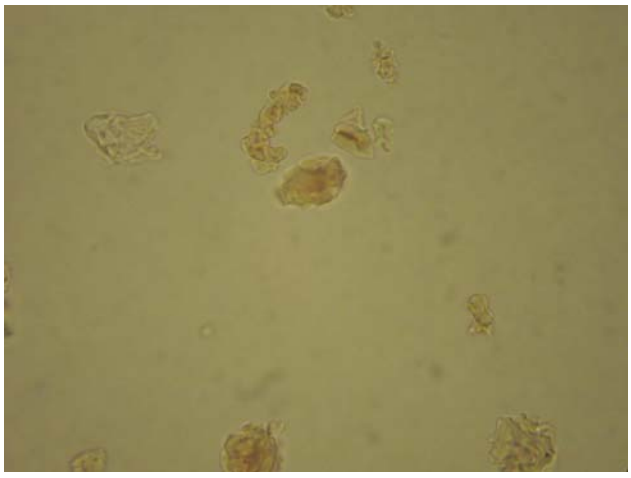

Fig. 16. PVPP particles on a membrane with immersion oil in a transmitted light beam.

15 shows PVPP-particles exhibiting auto-fluorescence and Fig. 16 shows PVPP identified on a membrane, where it resembles protein particles. Figure 17 shows Kieselguhr particles in transmitted light. Kieselguhr particles appear geometrical in form and can resemble ladders, circles, quadrates etc. Haze caused by filter aids is mostly due to a poorly conducted filtration.

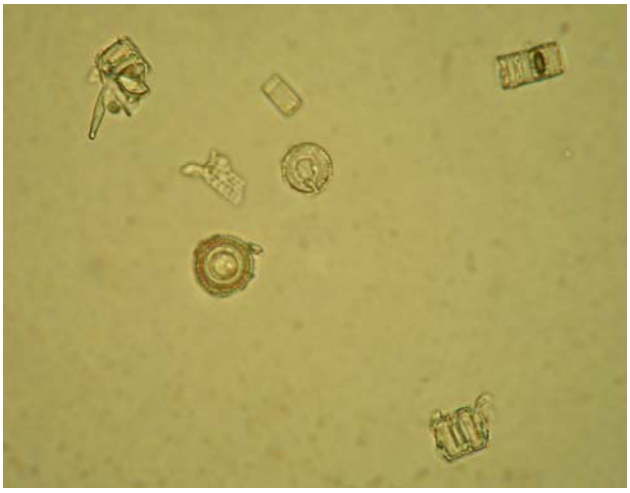

Fig. 17. Kieselguhr particles in a transmitted light beam.

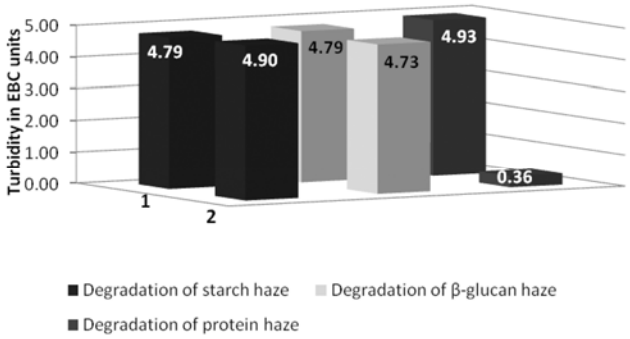

Fig. 18. Compilation of protein haze by the use of enzymatic degradation. From left to right the bars show the degradation of starch/β-glucan/protein haze. Bar 1 shows the turbid sample and bar 2 shows the sample treated with enzymes after 12 h of reaction time.

Haze caused by inorganic matter (labels, glass particles, etc.). Figure 18 shows the enzymatic haze identification of a beer with turbidity problems. It was clearly indicated, that proteins induced the haze formation. Bar 1 shows the turbid sample and bar 2 shows the sample treated with enzymes after 12 h of reaction time. The highest turbidity difference was in the sample treated with the enzyme pepsin, which degrades protein haze. Figures 19–22 show particles microscopically identified in this beer. Figure 19 shows a particle in reflected light which was an aluminium particle. Figure 20 shows a ribbon-like label particle that exhibited auto-fluorescence. The latter is shown in Fig. 21. Particles which have clear cut edges and exhibit auto-fluorescence are often label-remains. Figure 22 shows a glass particle. The turbidity occurred in all of the bottled beers. All of these particles came from a defective bottle washing machine and induced the precipitation of protein material in the bottled beer.

CONCLUSIONS

Turbidity gives a first visual impression of the quality of the beer to the consumer. Therefore it is necessary to

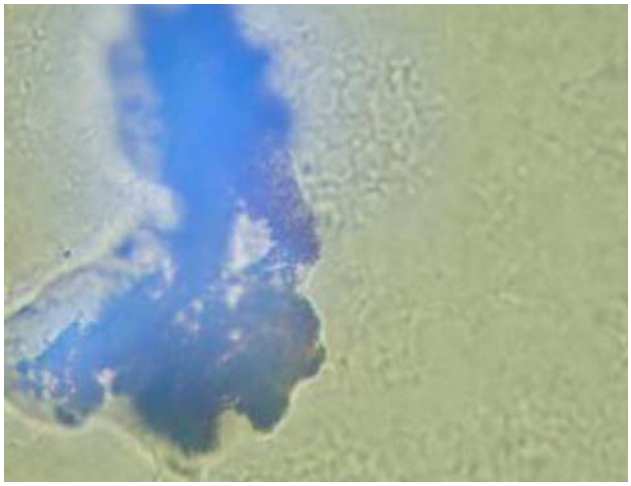

Fig. 19. Aluminium particle in a reflected light beam.

Fig. 20. Small piece of label in a transmitted light beam.

Fig. 21. Small piece of label exhibiting autofluorescence.

have methods not only to identify the haze, but also to infer on the source of haze formation. In this research a simple, reproducible and low cost analysis procedure, which can be carried out with basic laboratory equipment, was developed. With this experimental setup, it was demonstrated, that the components of haze-particles in beer can easily be determined and technological factors during the brewing process of haze formation can be tracked step by step. It is possible in most cases to identify the components of haze, and also the source of haze formation, as demonstrated by the examples in this paper.

Fig. 22. Glass particle in a transmitted light beam.

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8.4 Comparison of beer quality attributes between beers brewed with 100% barley malt and 100 % barley raw material

Research Article

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Comparison of beer quality attributes between beers brewed with 100% barley malt and 100% barley raw material†

Elisabeth Steiner,* Andrea Auer, Thomas Becker and Martina Gastl

Abstract

BACKGROUND: Brewing with 100% barley using the Ondea® Pro exogenous brewing enzyme product was compared to brewing with 100% barley. The use of barley, rather than malt, in the brewing process and the consequences for selected beer quality attributes (foam formation, colloidal stability and filterability, sensory differences, protein content and composition) was considered.

RESULTS: The quality attributes of barley, malt, kettle-full-wort, cold wort, unfiltered beer and filtered beer were assessed. A particular focus was given to monitoring changes in the barley protein composition during the brewing process and how the exogenous OndeaPro® enzymes influenced wort protein composition. All analyses were based on standard brewing methods described in ASBC, EBC or MEBAK. To monitor the protein changes two-dimensional polyacrylamide gel electrophoresis was used.

CONCLUSION: It was shown that by brewing beer with 100% barley and an appropriate addition of exogenous Ondea® Pro enzymes it was possible to efficiently brew beer of a satisfactory quality. The production of beers brewed with 100% barley resulted in good process efficiency (lautering and filtration) and to a final product whose sensory quality was described as light, with little body and mouthfeel, very good foam stability and similar organoleptic qualities compared to conventional malt beer. In spite of the sensory evaluation differences could still be seen in protein content and composition.

Keywords: barley; brewing; enzymes; protein composition; 2D-PAGE

INTRODUCTION

Interest in brewing beer directly from barley and exogenous enzymes has increased in recent years. This has been as a result of decreased acreage of quality barley for brewing, poor harvests due to climate change, a focus on brewing costs, particularly in certain markets, such as China, and a focus on decreasing the energy usage (CO_2 emissions) of the brewing process. As a consequence malt prices have increased and are likely to increase further in the future. These factors have renewed interest in exogenous enzyme solutions in order to raise efficiency and optimise raw material usage for brewing beer. One obvious prospect is to investigate the use of barley as an alternative to malt. The potential advantages of using barley as the brewing raw material would be to save energy (i.e. kilning stage) and water (steeping and germination stages) during malting, as well as to avoid malting losses due to respiration and the removal of acrospires and rootlets.

During the 1970s much research was carried out into investigating the potential of barley brewing.[1–16] Initial investigations began with brewing mashes that used a small proportion of malt grists that were predominantly unmalted barley. Due to the lower enzyme activity present in barley, suitable methods for enzyme replacement were required. Milling and mashing also had to be adapted to obtain good-quality worts. As no commercially successful beer products resulted from this activity, little research has been done on brewing with 100% barley raw material since this time. The following problems when brewing with 100% barley raw material and exogenous enzymes were identified. The use of unmalted barley resulted in low extract yields, high wort viscosity, a decrease in the rate of lautering, and the formation of an undesirable haze, as well as negative impacts on beer flavour, compared to beer brewed with 100% malted barley.[17,18] Undesirable impacts on flavour attributes, including bitterness, astringency and acridity,[19] were observed. However, there were also benefits, because of a lower dimethyl sulfide content (formed during the malting process).[20] During the brewing process concentrations of exogenous enzymes can lead to inhibition of the enzymes[17] and adapted mashing protocols such as extended protein rests were required because the temperature optima of the exogenous enzymes used lead to a longer duration of mashing.[21] Allen[16] even mentioned improvements in beer quality.

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The implications for the substitution of malt with barley as the primary raw material in the brewing process requires an understanding of the chemical and enzymatic modifications occurring during malting and the influence of these modifications on brewing process efficiency and final beer quality. Barley malt is the main raw material and the main starch source traditionally used for brewing worldwide. The aim of malting is to produce enzymes in the germinating cereal grain that cause certain changes in chemical constituents of barley in preparation for brewing. In effect, malting is the controlled germination of barley, followed by a termination of this natural process by the application of heat (kilning) to produce the required flavour and colour. The kilning process produces flavour and colour compounds, which are important in consumers' appreciation of the final character of the beer.[17]

A few of the enzymes required for brewing, such as β-amylase, are already present in the barley, but the majority of enzymes have to be accumulated or synthesised after barley germination. These enzymes include α-amylase, proteases, cellulases and β-glucanases. In good-quality final malt all the enzymes needed for the conversion of starch, non-starch polysaccharides and proteins into their yeast-usable components are present. When barley is used for brewing, exogenous enzymes have to be added to efficiently achieve similar chemical changes during the mashing process. During germination, enzyme synthesis will modify the endosperm of the grain. This is completed during mashing where starch is degraded into fermentable sugars, which will be converted into alcohol by the yeast during fermentation. Therefore, the enzymes produced during malting are essential for the degradation of large molecules during mashing.

Three basic biochemical processes take place during malting and mashing: amylolysis, cytolysis and proteolysis. These are necessary for the efficient production of wort and are described as follows.[22–24]

1. *Amylolysis* describes the degradation of starch into fermentable sugars and is characterised in terms of the extract recovered (water soluble malt/barley components) and its fermentability. Amylolysis is important as the simple sugars in the wort can be fermented to alcohol by yeast. The following enzymes take part in amylolysis: α-amylase, β-amylase, α-glucosidase (or maltase) and limit dextrinase (or pullulanase, see also Table 1).
2. *Cytolysis* describes the breakdown of cell walls during the malting process. Indicators for cytolysis are friability, β-glucan content and viscosity. The following enzymes take part in cytolysis: β-glucan-solubilase, endo-β-(1–3) glucanase, endo-β-(1–4) glucanase, exo-β-glucanase and xylanase (see also Table 1).
3. *Proteolysis* is a modification of grain protein into high-, middle- and low-molecular-weight forms and amino acids. The Kolbach index, soluble nitrogen and free amino nitrogen (FAN) give a first impression of the solubilisation of the malt proteins. The following enzymes take part in proteolysis: endo-proteases (primarily cysteine and metallo), carboxypeptidase, dipeptidase (see also Table 1, and the references therein).

The interactions between the actions of the three biochemical processes influence the chemical composition and process efficiency of brewing. Extract yield is one of the most important barley-malt quality attributes as it is one of the primary economic determinants of how much beer can be produced from a ton of barley or malt. Extract is essentially the amount of material, mostly soluble sugar substances, which can be recovered into the wort. In general, if there is a normal malt amylolytic enzymatic activity, extract will indicate the sugar content and therefore the later alcohol percentage.[25] Increased proteolytic activity increases the starch availability and could also produce, given the circumstances, higher extract values. The proportion of extract that can be fermented by yeast is called apparent attenuation limit, degree of attenuation or fermentability percentage of the wort. Wort attenuation depends on the availability of fermentable sugars and on the yeast remaining in contact with the wort.[24,25] By changing the duration and temperature of the malting process the composition of the carbohydrates as well as the fermentability of the wort can be influenced, thus obtaining various types of beer.[26]

Cell wall modification is critical to the process efficiency and economy (extract) of brewing. When cell walls are not modified sufficiently, yield losses result and there can be an increase in undesirable high molecular non-starch polysaccharides (such as β-glucans and pentosans), which cause lautering and, later, beer filtration problems. High-molecular-weight β-glucans are responsible for difficulties in beer filtration, precipitate formation,[27] haze formation in beer and possibly reduced extraction efficiency in the brewing industry.[28] Shearing forces during the brewing process could lead to a cross-linking of the molecules and thus to the formation of a so-called gel.[24,29] Due to the negative effects on lautering and filtration, brewers strive to minimise the content of β-glucan in wort and beer. However, on the positive side, β-glucan may enhance foam stability[30] even though literature exists which could not prove this fact.[31,32]

The protein and protein-derived components are important in wort because of their effect on the organoleptic character of the beer and their importance for yeast nutrition. These effects include foam quantity and foam stability, richness of taste, formation of active flavour compounds (Maillard products), haze stability, and the progress of yeast fermentation. During malting, barley storage proteins are partially degraded by proteinases into amino acids and peptides that are critical for obtaining high-quality malt and therefore high-quality wort and beer. During mashing, proteins are solubilised and transferred into the produced wort. Throughout wort boiling proteins are glycated. The most important factor is the protein composition, whose origin in finished beer is impacted by barley cultivar and the level of protein modification during malting. This is judged by malt protein modification, conventionally measured in the brewing industry as the Kolbach index [(soluble nitrogen/total nitrogen) $\times$ 100].[24,25]

In beer several different protein groups, originating from barley, barley malt and yeast, are known to influence beer quality.[24,29,30,33–36] Some of them play a role in foam formation and body and mouthfeel; others are known to form haze and have to be precipitated to guarantee haze stability, since turbidity gives a first visual impression of the quality of beer to the consumer. A certain amount of protein is required in the beer, since a certain amount of FAN, is necessary for yeast nutrition. Low levels of FAN can result in low yeast propagation and therefore to unwanted byproducts of the fermentation, such as diacetyl. Therefore, FAN values must be sufficiently high to ensure that a lack of nitrogenous yeast nutrients does not limit fermentation.[37] Low-molecular-weight nitrogenous compounds also play a central role in the colour and flavour development of malt following the Strecker reaction.[25] Conversely, a high FAN concentration can lead to undesired off-flavours via the Maillard reaction.[24,25] Beers with high FAN content tend to produce high colours (due to

Table 1. Enzymes in barley and barley malt[22,24,29,60–66]

Enzyme and method of lysis	EC Number	*De novo* enzymes	Substrate	Product
Cytolysis				
β-Glucan-solubilase	EC 3.2.1.4	–	Matrix linked β-glucan	Soluble, high molecular weight β-glucan
Endo-β-(1 → 3) glucanase	EC 3.2.1.39	*De novo* synthesised	Soluble, high molecular weight β-glucan	Low molecular weight β-glucan, cellobiose, laminaribiose
Endo-β-(1 → 4) glucanase	EC 3.2.1.4	*De novo* synthesised	Soluble, high molecular weight β-glucan	Low molecular weight β-glucan, cellobiose, laminaribiose
Endo-β-(1 → 3), (1 → 4)-glucanase	EC 3.2.1.73	*De novo* synthesised	Soluble, high molecular weight β-glucan	Low molecular weight β-glucan, cellobiose, laminaribiose
Exo-β-glucanase	EC 3.2.1.58	–	Cellobiose, laminaribiose	Glucose
Xylanase	EC 3.2.1.8	–	Hemicellulose	β-D-Xylose
Proteolysis				
Endopeptidase	EC 3.4.2	*De novo* synthesized	Proteins	Peptides, free amino acids
Dipeptidase	EC 3.4.13.11	*De novo* synthesised	Dipeptides	Free amino acids
Amylolysis				
α-Amylase	EC 3.2.1.1	*De novo* synthesised	High and low molecular weight α-glucans	Melagosaccharides, oligosaccharides
β-Amylase	EC 3.2.1.2	–	α-Glucans	Maltose
Limit dextrinase	EC 3.2.1.142	*De novo* synthesised	Limit dextrins	Dextrins
Other				
Lipase	EC 3.1.1.5	–	Lipids, lipid hydroperoxide	Glycerine, free fatty acids, fatty acid hydroperoxide
Lipoxygenase	EC 1.13.11.33	–	Free fatty acids	Fatty acid hydroperoxide

the Maillard reaction). In addition, the lack of high- and middle-molecular-weight proteins leads to poor foam formation/stability and little body and mouthfeel,[26,38,39] so an optimal amount of protein modification is required.

The OndeaPro® exogenous enzyme formulation (Novozymes A/S, Krogshoejvej 36, 2880 Bagsvaerd, Denmark) enables the production of wort similar in quality to the worts produced with malt. To gain an appropriate viscosity (for good lautering and filtration efficiency), fermentable sugars and FAN (for yeast nutrition) a mixture of specific enzymes is needed. OndeaPro®, contains exogenous β-glucanase and xylanase to reduce viscosity by hydrolysing non-starch polysaccharides, a mixture of α-amylase and pullulanase (limit dextrinase) to hydrolyse starch into fermentable sugars, proteases to provide FAN, and lipase to degrade triglycerides to ensure low haze formation in wort.

Application

The advantages and disadvantages of using 100% barley for brewing are summarised below.[16,19,24,25,40,41] The advantages are:

1. Energy consumption by not malting, particularly the avoidance of the energy intensive kilning stage, results in a reduced carbon footprint.
2. Barley is substantially cheaper than malt due to the costs (i.e. energy, water, capital) and losses (respiration, removal of rootlets and acrospires ~15% loss) involved in the malting process.
3. Beer taxes in some countries (e.g. Japan, Kenya) are linked to the percentage of malt used for brewing, so that less malt usage results in reduced taxes and cheaper beer.
4. More consistent beer batches can be produced when barley raw material is used.
5. Facile stabilisation according to the lower nitrogen concentration in beer brewed with barley raw material, which potentially leads to a better ageing stability (i.e. reduced haze and flavour staling precursors).
6. The respiration loss during malting (approx. 12%) needs to be considered. More starch is available in barley raw material.
7. Similar gelatinisation temperatures for barley or malt starch.

The disadvantages are:

1. Milling is harder for barley than for the more friable malt and results in a higher degree of abrasion on the mill rollers for grist production.
2. As barley is harder and more difficult to mill, a higher percentage of fine material in the grist can lead to problems during lautering.
3. Problems during lautering and filtration may occur as a result of higher contents of β-glucan and pentosans.
4. A reduced wort FAN–amino acids content may adversely affect yeast nutrition resulting in lower yeast growth and vitality which could result in poor fermentation performance.
5. Brewing with 100% barley raw material can lead to lower extract yield and insufficient final attenuation.
6. The washing of barley inherent in the steeping stage of malting removes dirt, microbes and extraneous material.
7. As barley is not kilned, there is a reduction in the formation desirable Maillard products (aromatic compounds, colour) which changes the flavour of the beer produced.
8. Beer brewed with 100% barley raw material has been described as having little body or mouthfeel.

Task

The basis of the barley brewing process is the replacement of malt by barley. The complement of hydrolytic enzymes for starch, protein, non-starch polysaccharide, etc., normally accumulated in the malt during the course of malting are replaced with exogenous enzymes typically obtained from microbial sources, to bypass the

Table 2. Parameters used in the brewing process

	Method 1		Method 2	
Parameter	Barley	Malt	Barley	Malt
Milling	Barley and malt were milled twice in a two roller mill		Barley and malt were milled twice in a two roller mill	
Water	40 L		40 L	
Temperature/time	61.9 °C/30 min; 71.9 °C/60 min; 75 °C/5 min		54 °C/30 min; 64 °C/60 min; 78 °C/30 min	
Lauter halt	10 min		10 min	
Total time lautering	122 min	90 min	90 min	90 min
Boiling time	70 min		70 min	
Bitter units	20 Bitter units		20 Bitter units	
Whirlpool halt	20 min		20 min	
Final amount of wort	56 L	52 L	53 L	49 L
Enzymes used in Ondea® Pro (50 g 10 kg^{-1} barley)	β-Glucanase: low viscosity Xylanase: low viscosity Lipase: good haze stability α-Amylase: provision of fermentable sugars Pullulanase: provision of fermentable sugars Protease: provision of free amino acids			

Two mashing methods are described: one with a protein rest, and one without a protein rest. Infusion mashing was used in both methods, for both the barley and the malt. Important values and enzyme dosage are also given. Ten kilograms of barley or malt were used in each method.

malting process. During mashing, enzymes[26] convert starch into limit/dextrins and yeast fermentable sugars. A proportion of the proteins are converted to peptides and amino acids, while β-glucan/pentosans (non-starch polysaccharides) are hydrolysed to lower-molecular-weight components that do not interfere with filtration-based brewing processes. In this investigation the OndeaPro® exogenous brewing enzyme formulation (Novozymes) provided a suitable mixture of enzymes for barley brewing which is added at the start of the mashing process. In this trial the process efficiency and the quality of beer produced from 100% barley was compared to traditionally brewed beer that was produced with barley malt. The overall purpose of this study was to determine the effects of barley raw material and exogenous enzymes in comparison to 100% malt on the efficiency of the brewing process (mashing, lautering, fermentation) and to determine the final quality of the produced beer.

EXPERIMENTAL

Materials

Barley and malt (type: 'Pilsner'), CV Marthe, was harvested 2007, malted by conventional malting practices to produce high quality malt by Weyermann® GmbH & Co. KG Brau-, Röst- und Caramelmalzfabrik (Bamberg, Germany). The OndeaPro® exogenous brewing enzyme formulation (α-amylase, β-glucanasen, xylanase, proteinase, pullulase, lipase) was provided by Novozymes.

Methods

Brewing

Brewing was conducted at the 60 L pilot scale with fermentation being carried out in 50 L cylindro-conical tanks at 12 °C with yeast, type W134. Maturation was achieved within 2 weeks at 0 °C. Brewing and fermentation were carried out at the Institute of Brewing and Beverage Technology, Lehrstuhl für Brau- und Getränketechnologie, Weihenstephan, TU München. Brewing was carried out as summarised in Table 2. Barley or malt were milled twice with a gap of 0.2 mm in a two roller mill (MIAG, Braunschweig, Germany) To gain the same effects of modification as in malting exogenous enzymes (Ondea® Pro) are used, which are also listed in Table 2. Ondea® Pro is a mixed enzyme product and many of the existing functionalities of the enzymes used in the conventional process are built into this product. The Ondea® Pro enzyme dosage for mashing was 50 g 10 kg^{-1} barley.

Total protein content (determined by the Kjeldahl method and Bradford assay), coagulable nitrogen, and free amino nitrogen of freshly collected wort and beer were measured immediately. Samples of the collected wort and beer were freeze dried and prepared for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Malt, barley, wort and beer analysis

Common malt, wort and beer analyses were done according to standard MEBAK procedures.[42]

Kjeldahl nitrogen

The Kjeldahl method according to MEBAK[42] is the standard method for determining protein content of beer.[42,43] The standard value of total nitrogen content in beer brewed with barley malt ranges between 700 and 800 mg N L^{-1}.

Coagulable nitrogen

Coagulable nitrogen was measured according to MEBAK.[42] Standard values are in the range of 15–25 mg L^{-1} for barley malt.

Free amino nitrogen

Free amino nitrogen (FAN) in wort and beer wort was measured by spectrophotometry international method (IM) according to EBC.[43] The method gives an estimate of amino acids, ammonia and, in addition, the terminal α-amino nitrogen groups of peptides and proteins. Proline is partially estimated when the 570 nm

wavelength is used. The standard value of FAN in beer brewed with barley malt ranges between 200 and 250 mg L^{-1} in wort and 100–120 mg L^{-1} in beer.

Bradford assay

The Bradford total protein assay is a protein determination method which involves the binding of Coomassie brilliant blue G-250 to protein.[44] This assay was used to determine the concentration of the extracted samples for 2D-PAGE.

Two-dimensional polyacrylamide gel electrophoresis

2D-PAGE was used to enable a more detailed comparison of the changes in protein composition between barley, malt, wort and beer. 2D-PAGE[45] was carried out on the Ettan™ IPGphor™ 3 IEF System and the Ettan™ DALTsix Large Vertical System (GE Healthcare Europe GmbH, Munich Commercial Center, Oskar-Schlemmer-Str. 11, 80807 München, Germany) on 12.5% acrylamide gels. Two hundred milligrams of milled barley and freeze-dried wort and beer were precipitated with trichloroacetic acid (TCA)/acetone according to Damerval *et al*.,[46] by mixing lysed or disrupted sample in 2 mL 10% TCA in acetone with 20 mmol L^{-1} dithiothreitol (DTT) to precipitate proteins with incubation for at least 45 min at −20 °C. The precipitated suspention was centrifuged for 15 min (14.000 g), the supernatant decanted and the pellet washed with a further 2 mL cold acetone containing 20 mmol L^{-1} DTT. Residual acetone was removed by air drying or lyophilisation. After precipitation the samples were solubilised in 1 mL urea lysis buffer (containing 9.5 mol L^{-1} urea, 1% (w/v) dithiothreitol (DTT), 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3–10)) for malt samples and 0.5 mL urea lysis buffer for freeze-dried wort/beer samples. Protein concentration was measured by the Bradford method using bovine serum albumin as a standard.

A 350 µg sample were applied for in gel rehydration to the gel strips. Passive rehydration was carried out overnight. Isoelectrofocusing (IEF) was carried out using 18-cm IPG 3–10 NL strips (ReadyStrip; GE Healthcare) and an Ettan IPGphor 3. The running conditions were as follows: initial IEF (1 h, 500 V), gradient (8 h, 1000 V); gradient (3 h, 8000 V), hold (2 h 40 min; 8000 V), gradient (3 h; 10 000 V); hold (1 h; 10 000 V).[45] The second dimension was carried out on an Ettan DALTsix Electrophoresis Unit (220 V), gel sizes of 20 × 25 cm, a gel thickness of 1.0 mm and total acrylamide concentration of 12.5%. Sodium dodecyl sulfate (SDS)-PAGE was started with 5 mA per SDS gel (100 V maximum setting) for ∼2 h. Continue with 15 mA per SDS gel (200 V maximum setting) for ∼16 h overnight or with a higher current for faster runs. The run was terminated when the bromophenol blue tracking dye had migrated off the lower end of the gel.

Gels were fixed for 3 h in 50% ethanol and 3% phosphoric acid, washed three times for 20 min in water pre-incubated for 1 h in 34% methanol, 3% phosphoric acid and 17% (w/v) ammonium sulfate solution. Coomassie blue (G-250; 0.35 g) was added per litre of solution and stained for 4–5 days. Gels were washed a few times in water to remove background stain scanned and analysed with Delta2D from DECODON (DECODON GmbH, Greifswald, Germany).

Modified raible assay

Filterability of the beer was measured by the modified 'raible assay' carried out according to Kreisz.[47] Demanded values for beers brewed with barley malt having a good filterability are: F_{spez} [hl/m^2h] = 5.5–9.

Sensory evaluation

Sensory evaluation was carried out according to MEBAK.[42,48–50] Sensory evaluation of fresh and forced beers were performed according to the Deutsche Landwirtschafts Gesellschaft (DLG) scheme [with a score from 5 (the best) to 1 (the worst)][42] and a stale taste according to Eichhorn [score from 1 (not aged) to 4 (extremely aged) in half scores]. The acceptance describes the subjective impression of ageing by the panellist (100% = not aged).

Aromatic compounds

The analysis is based on the various water vapour distillation methods published by MEBAK.[42]

RESULTS AND DISCUSSION

In this research to assess the validity of brewing beer from barley two different mashing schemes were compared. The mashing schemes were selected according to their relevance to commercial brewing practice. The first method (Method 1) was an abridged two-stage mash method (61.9 °C then 71.9 °C) that is commonly used by the brewing industry, because of time and cost management. The second protocol (Method 2) was a programmed infusion mashing method that uses mash temperatures of 54 °C, then 64 °C to assure appropriate temperature optima for the OndeaPro® exogenous enzymes used. Table 2 shows the differences between the two mashing schemes. Conventional wort and beer analyses were conducted to compare the wort/beer quality and production efficiency of the brews using either 100% barley raw material, or 100% barley malt by the two mashing methods. These different mashing methods showed the differences between a method that is conventionally used by the brewing industry with a method that is appropriate for brewing with 100% barley raw material and exogenous enzymes. Method 1, which does not have a 'protein rest' (mash of 54 °C) is not suitable for the protease component of the OndeaPro® product, since a the exogenous protease is rapidly inactivated at higher mash temperatures.

Malt

Table 3 shows the results of conventional malt and barley quality assessment of the barley and malt used in the brewing trials. The commercially desired range for each malt quality parameter is also provided. Brewers and maltsters gain an appreciation for the quality of malt by proteolytic and cytolytic attributes. The Kolbach index, soluble nitrogen and FAN indicate the degree of solubilisation of the barley protein due to proteolysis as a result of malting. The Kolbach index and FAN of the malt were slightly lower than desired, but soluble nitrogen was within the desired range. Such results warn brewers that such a malt could produce wort that was slow to ferment, a product of unwanted aroma compounds (e.g. diacetyl), and may have a poor final attenuation due to the lack of yeast nutrition (FAN). As cytolysis is the breakdown of cell walls during the malting process, indicators for malt cytolysis are friability, β-glucan content and viscosity. All results met the desired values which pointed towards good cytolytic solubilisation of the malt.

Wort and beer

Extract yield and final attenuation

The extract yield (Table 4) is one of the most important malt quality attributes.[25] The concentration was measured in terms of

Table 3. Malt and barley analyses for the malt, used in the trials

Analyses	Unit	Desired values	Results for barley	Results for malt
Water content	%	<14	13.3	4.5
Extract	% dm	>81	ND	78.2
Viscosity (8.6%)	mPa s	<1.56	ND	1.44
Viscosity, 65 °C (8.6%)	mPa s	<1.60	ND	1.49
Friability	%	>85	ND	96.1
Whole kernels	%	<2	ND	0.8
Saccharification	minutes	<15	ND	5–10
Final attenuation	% app.	81–84	ND	81.70
Colour	EBC	3–5	ND	2.50
Boiled wort colour	EBC	4.5–7	ND	4.10
pH value		5.9–6.0	ND	5.69
Crude protein	% wfr.	<11.5	11.50	11.20
Soluble nitrogen	g kg^{-1} malt dm	6.50–7.50	ND	6.76
Kolbach index	%	39–42	ND	37.70
Free amino nitrogen	g kg^{-1} malt dm	1.30–1.60	ND	1.25
β-Glucan, 65 °C	mg L^{-1}	<350	ND	164
α-Amylase	ASBC wfr.	>40	ND	43

Table 4. Global wort and beer analyses brewed with two different mashing methods (method 1, without protein rest; and method 2, with protein rest) and either 100% barley or 100% barley malt

		Method 1		Method 2	
Analysis	Desired values	Barley	Malt	Barley	Malt
Wort gravity (°P)	11.3–12.3	10.2[a]	11.9	10.7	13.0
Final Attenuation (% app.)	81–84	64[a]	81	78	80
Alcohol content (% vol.)	4.3–5.8	3.21[a]	4.76	3.93	4.78
Colour of wort (EBC)	7–11	8.9	9.9	8.7	9.5
Colour of beer (EBC)	4	4.02	4.67	4.20	6.13[a]
pH value, wort	5.3–5.6	5.4	5.1	5.4	5.2
pH value, beer	4.3–4.6	4.72	4.66	4.70	4.49
Viscosity, wort (mPa s)	<1.6	2.09[a]	1.52	1.59	1.61
Viscosity, beer (mPa s)	<1.6	2.30[a]	1.51	1.45	1.57
β-Glucan wort (mg L^{-1})	<200	2650[a]	174	85	142

[a] Results which differ considerably.

grams of solids per 100 grams of wort. Wort obtained by congress mash normally has an apparent attenuation limit (AAL) of ~80%. The AAL depends on the complete hydrolysis of starch and on a sufficient amino acid supply for the yeast. Given the chosen parameters for the mashing regime, a wort gravity of 11.3–12.3 °P and an AAL of 81–84% for the wort made with malt should be produced. In Table 4 it can be seen that the wort gravity and also AAL for the barley worts are lower. These differences were due to the different mashing regimes (Method 2 was the more intense mashing regime) and to the temperature optimum of the exogenous enzymes. For Ondea® Pro only a mashing regime starting at 54 °C is suitable, because of the temperature optimum of specific enzymes. Only the brews made of barley malt reached the demanded values. Both values, extract and final attenuation, and therefore also alcohol content were too low. This showed us, as already has been stated by other researchers,[7,26] that even when exogenous enzymes were used the yield was still a little too low.

Viscosity and β-glucan content

Viscosity gives an impression of the process efficiency (lauter and filtration characteristics) of beer during the process. Wort and beer viscosity is influenced by the macromolecules present. Generally, low viscosity is considered advantageous for the filtration process. Narziß[51] considered for congress mashing <1.53 mPa × s as really good viscosity, 1.53–1.57 mPa × s as good, 1.58–1.61 mPa × s as satisfactory, 1.62–1.67 as poor, and >1.67 as bad.

During malting and mashing β-glucan is enzymatically hydrolysed into predominantly smaller oligosaccharides.[27] The data in Table 4 show that when beer was brewed with Method 1 and barley raw material and exogenous enzymes that wort viscosity and β-glucan content were higher (viscosity barley wort = 2.09 and malt wort = 1.52) and the final attenuation was lower than in all malt beer. The mashing method without protein rest was not suitable for brewing with exogenous enzymes; hence the enzymes could not work properly and therefore could not degrade certain substances (such as β-glucan, high-molecular-weight proteins, etc.) during the mashing, which led to increased viscosity, due to increased β-glucan content. This could also be confirmed with the data of Table 2, where the differences in the processability are shown. In Table 2 the lauter time for barley wort produced with Method 1 was 122 min in comparison to 90 min for the other wort lauter times. In contrast to the results given in the literature,[40] good lauter and filtration characteristics for barley raw material beer could be achieved. With the optimum mashing programme and exogenous enzymes (Method 2), no significant differences in the lauter and filtration processing could be seen. The mash in Method 2 was optimised for the exogenous enzymes to reduce wort β-glucan content and, as a consequence, improve filterability. These peculiarities could be based on the pilot scale used, where no shear forces were applied. Therefore the β-glucan molecules were not able to cross-link and no 'gel' was formed. Even though the viscosity was too high the filter was not blocked because of the 'missing' gel. It can be concluded that the results for β-glucan and viscosity for the barley brews were more satisfactory in terms of beer processability with Method 2.

Total nitrogen and coagulable nitrogen

Total nitrogen values were obtained from the sum of all nitrogenous compounds present and were determined by the Kjeldahl method. The nitrogenous constituents of wort included amino acids, peptides, proteins, nucleic acids and their degradation products.[24,29,52] Table 5 shows total nitrogen content as well as coagulable nitrogen. Even though mashing regime 2, using raw barley, included a protein rest, total nitrogen content was still higher in beer brewed with barley malt. Interestingly, coagulable nitrogen was slightly higher in beers made of barley raw material.

Table 5. Wort and beer analyses brewed with two different mashing methods (method 1, without protein rest; and method 2, with protein rest) and either 100% barley, or 100% barley malt

		Method 1		Method 2	
Sample	Desired value	Barley	Malt	Barley	Malt
Free amino nitrogen (mg L^{-1})					
Kettle-full-wort	200–250	46	157	81	206[a]
Cold-wort	–	57	160	84	213[a]
Unfiltrate	100–120	10	64	19	112[a]
Filtrate	–	10	63	21	112[a]
Coagulable nitrogen (mg L^{-1})					
Kettle-full-wort	–	134	294	76	37[a]
Cold-wort	–	76	142	48	12[a]
Unfiltrate	–	31	29	31	19[a]
Filtrate	15–25	24	25	21	17[a]
Total nitrogen (mg L^{-1})					
Kettle-full-wort	900–1100	444	822	722	1050[a]
Cold-wort	–	549	869	734	1063[a]
Unfiltrate	700–800	336	613	449	775[a]
Filtrate	–	317	605	425	758[a]
Foam stability according to NIBEM (s)					
Beer	>300	332[a]	283	332[a]	221
Filterability (F_{spez} h L/m^2 h)					
Beer	>5.5	7.49	6.19	7.70	6.20
Stability (warm days)					
Beer	>15	>15	5	>15	>15

[a] Results which differ considerably.

Table 6. Ageing indicators of beer brewed with two different mashing methods (method 1, without protein rest; and method 2, with protein rest) and either 100% barley or 100% barley malt

	Method 1				Method 2			
Thermal aging indicators [µg L^{-1}]	Barley	Barley aged	Malt	Malt aged	Barley	Barley aged	Malt	Malt aged
	9.2	23	11	47	20	51	7	20
Oxygen ageing indicators (µg L^{-1})	10	12	16	22	1	2	2	43
Total amount ageing indicators (µg L^{-1})	20	36	28[a]	70[a]	113	212	78[a]	351[a]

[a] The results indicated with a show the higher values of ageing indicators in the beers brewed with 100 % malt.

This effect might be explained by the germination process, where nitrogenous compounds were already degraded and therefore fewer high-molecular-weight proteins (i.e. coagulable nitrogen) passed into the finished beer. Thus, beer made of barley raw material showed more coagulable nitrogen.

The effects of a low nitrogen content are described in the sections 'Free amino nitrogen and wort/beer colour' and 'Sensory evaluation', respectively.

Free amino nitrogen and wort/beer colour

The typical FAN levels recommended for optimum yeast nutrition is between 120 and 150 mg 100 g^{-1}.[24,29] Wort colour is a consequence of the products formed by Maillard reaction from FAN and reducing sugars.

The increased FAN content in the beer brewed with mashing method 2 and barley malt, in Table 5, was due to the more intense mashing method and the already degraded proteins in the malt. A higher FAN content induced an increase in the beer colour based on the Maillard reaction, which can be seen in Table 4. Also, an increase in ageing indicators due to Strecker aldehydes was observed (Table 6). A low FAN content could be limiting for yeast nutrition, which was indicated by the the diacetyl aroma in the beers brewed with barley raw material (Table 7 and Table 8).

Malting includes the controlled germination of barley in which hydrolytic enzymes are synthesised and the cell walls, proteins and starch of the endosperm are largely digested, making the grain more friable.[23,24,53] During malting, nitrogenous substances are released from the cell walls and are then degraded. This occurs as a result of enzyme action and the substances are used as nutrition for the growing seed. When malt was used, more nitrogenous substances were already free, which could become soluble and be degraded during the mashing process, which explained the higher nitrogen values in wort and beer brewed with malt and a mashing method with protein rest, which was because of the latter.

Table 7. Sensory profile according to DLG of beer brewed with two different mashing methods (method 1, without protein rest; and method 2, with protein rest) and either 100% barley or 100% barley malt

Method and sample	Aroma	Taste	Mouthfeel	Carbonation	Bitterness	Grade
Method 1						
Barley	3.38	3.50	3.13	3.75	4.00	3.58
Barley, aged	3.50	3.25	3.50	4.00	3.87	3.59
Malt	3.63	3.75	3.63	4.00	4.13	3.83
Malt, aged	3.87	3.50	3.63	3.87	3.75	3.72
Method 2						
Barley	3.43	3.50	4.14	4.07	3.93	3.74
Barley, aged	2.86	2.93	3.79	4.00	3.43	3.28
Malt	4.50	4.29	4.43	4.07	4.14	4.30
Malt, aged	3.57	3.57	4.07	3.86	3.79	3.72

Table 8. Sensory profile according to Eichhorn of beer brewed with two different mashing methods (method 1, without protein rest; and method 2, with protein rest) and either 100% barley or 100% barley malt

				Acceptance (%)					
Method and sample	Smell	Taste	Bitter taste	100	80	60	40	20	0
Method 1									
Barley	1.00	1.00	1.00	x	–	–	–	–	–
Barley, aged	1.75	2.00	1.37	–	–	x	–	–	–
Malt	1.00	1.00	1.00	x	–	–	–	–	–
Malt, aged	1.63	1.75	1.63	–	–	x	–	–	–
Method 2									
Barley	1.00	1.00	1.00	x	–	–	–	–	–
Barley, aged	2.29	2.29	2.00	–	–	x	–	–	–
Malt	1.00	1.00	1.00	x	–	–	–	–	–
Malt, aged	2.00	2.21	1.71	–	–	x	–	–	–

Values marked with an x show the acceptance of the tasting panel according to ageing.

Sensory evaluation and foam stability

Beer flavour must be suitable for the type of beer and is characterised by aroma and palatefulness, the liveliness (sparkle) and the bitter taste. The beer aroma depends on yeast strain (fermentation by-products), hop varieties and sulfur content.[22,24,29] Beer foam is, like the sensory impression, an important quality parameter for customers. Good foam formation and stability gives an impression of a freshly brewed and well-tasting beer. Beer foam is characterised by its stability, adherence to the glass, and texture.[54]

Table 6 shows the values of ageing indicators. It can be seen that beer produced with 100% barley malt showed higher values than beer brewed with 100% barley raw material. Even though the values in ageing indicators were higher in 'malt', 'raw material' showed no beneficial ageing stability (Tables 6, 7 and 8). Acceptance of the compared aged beers was the same. The reason of the higher content of ageing indicators has been described in the section 'Free amino nitrogen and wort/beer colour'.

As expected from the low FAN content a low diacetyl effect in the beers brewed with 100% barley raw material could be observed. A low FAN content may limit yeast nutrition and therefore may lead to poor fermentation, resulting in increased diacetyl in beer.

Of interest was the rating in 'mouthfeel'. Beers produced with barley raw material showed less body and mouthfeel than beers brewed with barley malt, which was already mentioned in former studies.[24,29] Less body and mouthfeel could be due to lower total nitrogen content in beers brewed with 100% barley raw material, since middle- to high-molecular-weight proteins influence body and mouthfeel. For example, more soluble nitrogen in the beer leads to a better body and mouthfeel. The slightly increased values in coagulable nitrogen of the beers brewed with barley raw material seemed to have no influence on body and mouthfeel.

The difference in foam formation was interesting. Beer made with 100% raw barley showed a better foam stability (Table 5), even though the total nitrogen was lower. This could be explained by the higher β-glucan and coagulable nitrogen content in the beers brewed with raw material.

Still, light beers, with little body and mouthfeel and very good foam stability and similar organoleptic qualities compared to a 'normal beer' were produced.

Protein composition

In this investigation a comparison between brewing with 100% barley raw material and exogenous enzymes and brewing with 100% barley malt was made. As it could be seen in the standard beer analyses, the biggest differences could be seen in the nitrogen analyses. By analysing the protein composition with 2D-PAGE not only the applicability of barley raw material in brewing could be demonstrated, but also changes in the different protein compositions. Huge differences in the protein composition with the help of 2D-PAGE could be shown in: (1) raw material

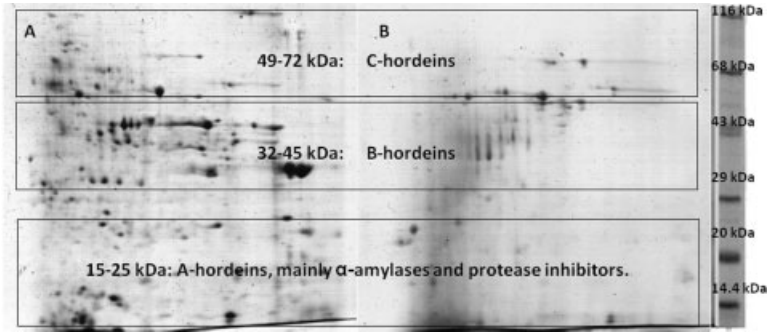

Figure 1. 2D-PAGE of the used barley raw material (A) and the malt (B). The degradation of the different hordein fractions can be followed from the left side (barley raw material) to the right side (barley malt).

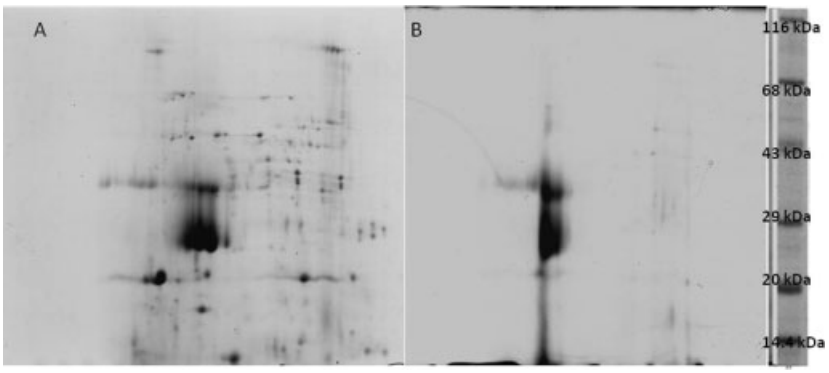

Figure 2. Image A shows a 2D-gel of a kettle-full-wort brewed with 100% barley raw material and image B shows a 2D-gel of a kettle-full-wort brewed with 100% barley malt. Both worts were brewed with a mashing program which includes a protein rest.

(malt), (2) wort, and (3) beer, produced from 100% raw material or 100% barley malt. From barley raw material to barley malt the degradation of hordeins could be seen (Fig. 1). Well-modified malt contains less than half the amount of hordeins present in the original barley,[53,55] which can be seen in Fig. 1. Hordeins were degraded and converted during malting into soluble peptides and amino acids to provide substrates for the synthesis of proteins in the growing embryo during malting. The degradation of hordeins during malting is also necessary to allow enzymes access to the starch, thus facilitating its complete hydrolysis.[23,24,29,56,57] In Fig. 1 the differences in the protein composition of barley raw material and barley malt were visualised. Because of degradation of nitrogen, proteins and development of enzymes the spot pattern is quite different. Only in the region between 40 and 50 kDa some similarities can be seen. Because of the classification (A–D hordeins) of Shewry[58,59] it is assumed that the proteins in the region between 40 and 50 kDa were B-hordeins. A-hordeins (15–25 kDa) contain protease inhibitors and α-amylases. B-hordeins (32–45 kDa) are rich in sulfur content and are with 80% the biggest hordein fraction. C-hordeins (49–72 kDa) are low in sulfur content and D-hordeins (>100 kDa) are the largest storage proteins. The degradation of the different hordein fractions can be followed in Fig. 1, from the left side (barley raw material) to the right side (barley malt).

Figure 2A, which indicates the influence of the malting process, shows a two-dimensional gel of a kettle-full-wort brewed with 100% barley raw material and Fig. 2B shows a two-dimensional gel of a kettle-full-wort brewed with 100% barley malt. Both worts were brewed with a mashing programme which includes a protein rest. Fig. 2A shows many more protein spots than Fig. 2B, which could be because the malting process was not performed. Proteins need to be extracted from the cell walls, and solubilised and degraded, which could lead to a different protein composition. Even during the fermentation the picture of the different protein composition does not change (Fig. 3). Fig. 3A shows many more protein spots than Fig. 3A, which could be for the same reasons (the missing malting step) as in the wort. In wort and beer, made of barley raw material, more dissolved protein compounds could be found. This could be because of the malting process that was not performed and the difference in the enzyme composition.

The differences in the protein composition should explain the differences in body and mouthfeel and foam stability. To gain more detailed information in the protein composition an identification of the proteins should be made.

CONCLUSION

To gain an insight into the sensory and analytical differences of beer made of barley raw material and beer made of barley malt several analyses were carried out. Analytical investigation showed that the biggest differences were in the nitrogen content, as well as in the protein composition. All of these differences could be explained due to the missing malting process. Even though differences could be seen analytically, differences in sensory evaluation were not as significant as expected. With 100% barley raw material and exogenous enzymes it is possible to produce beer, which is not significantly different to beer produced with 100% barley malt. Only a diacetyl effect, as a result of the low FAN concentration when missing the protein rest, was a negative aspect in the beer brewed with 100% barley. Interestingly, beer made of 100%

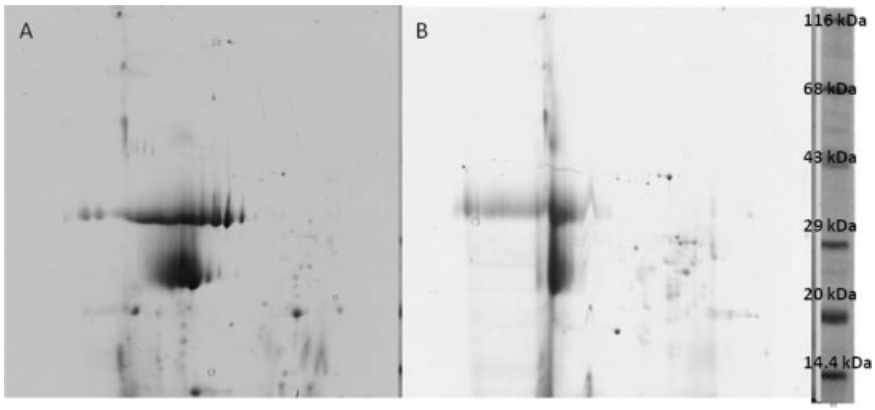

Figure 3. Image A shows a 2D-gel of a beer brewed with 100% barley raw material and image B shows a 2D-gel of a beer brewed with 100% barley malt. Both beers were brewed with a mashing program which includes a protein rest.

barley raw material showed a better foam stability, even though nitrogen content was lower. Differences in the protein composition could explain the differences in sensory evaluation, e.g. body and mouthfeel and foam stability. Identification of these differences should be further investigated, since these differences could be the factors which influence good foam formation and less body and mouthfeel in barley raw material beers.

In 1971 Pfenninger *et al.*[7] stated that it is possible to make beers with up to 50% barley raw material which has similar organoleptic qualities to all malt beers. It can be seen that OndeaPro® from Novozymes, a mixture of enzymes suitable for brewing, produces mostly the requested specifications for the resulting 100% barley raw material beer. With the following enzymes (which already exist in barley malt): β-glucanase and xylanase for low viscosity, α-amylase, pullulanase and protease for provision of fermentable sugars and free amino nitrogen, and lipase, to degrade triglycerides and thus ensure low haze formation in wort, all enzyme classes which occur in malting and brewing were covered. In the production of beers, fulfilling the required qualities, as in beers brewed with barley malt, is possible when brewing with barley raw material and exogenous enzymes under the appropriate mashing regime. Further investigation should be carried out to determine the influence of the quality of the barley used as raw material.

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8.5 Influence of the malting parameters on the haze formation of beer after filtration

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ORIGINAL PAPER

Influence of the malting parameters on the haze formation of beer after filtration

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Abstract Malting changes the chemical and enzymatical composition of barley. During malting, enzymes are synthesized, cell walls (pentosans, proteins, etc.) degraded and starch becomes available for enzymatic attack. The progress of germination defines the final beer quality and processability in several aspects: mouthfeel, foam and haze formation (different proteins), processability (viscosity caused by certain substances, like β-glucan), fermentation progress (FAN, sugar content), etc. The objective of this research was to study the influence of different modified malt on turbidity in beer after filtration. This was achieved by analyzing selected malts at different germination stages and afterward studying their influence on the final beer composition, focusing on protein content and composition. Protein fractions were analyzed using a Lab-on-a-Chip technique, which separates the proteins—based on their molecular weight—by capillary electrophoresis. This analysis was supported by the use of two-dimensional gel electrophoresis (2D-PAGE). Additionally, common malt and beer analyses and turbidity and filterability measurements were performed. The protein composition could be followed from malt to beer with both the Lab-on-a-Chip technique and 2D-PAGE. No differences in protein composition could be seen in the final protein composition of the beer. However, it could be observed, with Lab-on-a-Chip technique, that high amounts of a protein fraction with a size of 25–28 kDa caused increased turbidity in the beer.

Keywords Malt · Proteins · Haze · Lab-on-a-Chip technique · 2D-PAGE

Abbreviations

EBC	European Brewing Convention
MEBAK	Mitteleuropäische Brautechnische Analysenkommission

Introduction

Beer is a complex mixture of more than 450 constituents, and in addition, it contains macromolecules such as proteins, nucleic acids, polysaccharides and lipids. In beer, several different proteins exist; these are on the one hand important for foam formation and mouthfeel, and on the other, they influence haze formation and colloidal stability of the final product. These polypeptides, which originated mainly from barley proteins, are the product of the enzymatic (proteolysis) and chemical modifications (hydrogen bonds, Maillard reaction) that occur during malting and brewing [1]. During malting, barley storage proteins are partially degraded by proteinases into amino acids (yeast nutrition) and peptides (foam formation, body and mouthfeel), which are critical for obtaining high-quality malt and therefore high-quality wort and beer. In the mashing process, proteins are solubilized and transferred into the wort. Throughout wort boiling, proteins are glycated and also precipitated, and therefore, it is possible to separate the precipitated proteins from the wort as hot trub. During fermentation, the pH drops and causes the

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proteins to aggregate, thus making it possible to separate them. Beer proteins may be defined as a more or less heterogeneous mixture of molecules containing the same core of a peptide structure, originating from only one distinct protein present in the brewing materials [2]. Proteins influence two main quality aspects in the final beer: foam stability and haze formation. Beer foam is characterized by its stability (head retention), adherence to glass (foam cling) and texture [3]. Foam occurs on dispensing the beer as a result of the formation of CO_2 bubbles released by the reduction in pressure. Beer foam is stabilized by the interaction between certain beer proteins, for example LTP1, and isomerized hop α-acids, but destabilized by lipids [3, 4].

Haze can be described in different forms, which includes proteins, polyphenols and glucans (β-glucan, α-glucan and glycogen). These components can precipitate and haze is formed. Haze formation can be attributed to barley proteins, which are the product of the proteolytic and chemical modifications during the brewing process. Residual starch can also cause haze formation, either when the content of glassy kernels is higher than 3%, or when barley malt grains are not properly milled, or during the mashing process when starch cannot be degraded due to incorrect or too short temperature rests. β-Glucan can cause turbidity, when malt is inadequately modified. Other haze-forming substances are carbohydrates (glycogen) and protein from autolysed yeast, lubricants from can lids and dead bacteria from malt [5]. Due to the diversity of origin of haze formation in beer, amylolytic, cytolytic and proteolytic aspects are included and described. The main focus will be placed on protein haze.

Proteins are one of the primary causes of haze formation in beer and are divided into two main groups: proteins and their breakdown products. Protein breakdown products are always soluble in water and do not precipitate during wort boiling. As little as 2 mg/L of protein is enough to form haze in beer [6]. There are two forms of colloidal haze: cold break (chill haze) and age-related haze [7]. Cold break haze is formed at 0 °C and dissolves at higher temperatures. If cold break haze does not dissolve, age-related haze will develop, which is nonreversible. Chill haze is formed when polypeptides and polyphenols are bound noncovalently, whereas permanent haze forms in the same manner initially, but covalent bonds soon form and insoluble complexes are created, which do not dissolve when heated [8]. The cause of storage haze has been identified as the interactions between haze-active proteins and certain polyphenols [9–11].

Kreisz [12] defined filterability in his studies, as haze formation at the filter outlet and rise of pressure at the filter inlet. In the present research, this haze formation, after filtration, will be examined regarding protein turbidity. In our experience with haze identification in beer, increased turbidity directly after filtration is often correlated with proteins and poor malt quality. It is known that yeast and bacteria in the size range between 10 and 45 μm, and also, their excreted polysaccharides but also high molecular weight components in beer like β-glucans and α-glucans can cause haze after filtration [12–14]. To our best knowledge, no research has been carried out on the influence of proteins on filterability, i.e., haze formation at the filter outlet and rise of pressure at the filter inlet. In this paper, insights on the changes in the protein content and protein composition from barley to beer are given. The main focus is placed on the malting process and how malting influences the final protein composition in beer with regard to filterability, primarily to haze formation directly after filtration.

Response surface methodology (RSM), with a face-centered design, was only used to set up the experimental design. This experimental setup was done to investigate the influence of three malting parameters (germination time, degree of steeping and germination temperature) on the quality of final beer, in regard to protein composition. The experimental design allows an overview of a wide range of malting parameters (from under-to-over-modified malt).

2D-PAGE and Lab-on-a-Chip technique were used to monitor the changes in the protein composition from malt to beer. This was done by visualization of the changes in the protein spot pattern (2D-PAGE) and separation of protein fractions obtained by Lab-on-a-Chip technique. Several authors [7, 9, 15–18] claim that proteins of a certain size range (LTP1 at 10 kDa, protein Z at 40 kDa, hordeins in the size range between 15 and 30 kDa) are haze-forming proteins. With the Lab-on-a-Chip technique, an overview of potential haze-forming protein fractions with their relative concentration was provided.

Experimental

Materials

Barley, variety Marthe, was harvested in 2007 and delivered by Weyermann® GmbH & Co. KG Brau-, Röst- und Caramelmalzfabrik, Bamberg, Germany, to the Institute of Brewing and Beverage Technology. Malting was carried out in a micromalting pilot plant in 1 kg batches. RSM was applied to determine the impact of three predictor factors (germination time, degree of steeping and germination temperature) on the protein composition in beer. A face-centered cube design with double replicated factorial was constructed using the software package Design Expert by StatEase (Stat-Ease Corporation, Minneapolis, USA). The center point was repeated three times. RSM was not applied to determine the relative contributions of the three

Table 1 Malting parameters defined by RSM

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9
Germination time [d]	5	5	7	7	5	5	7	6	7
Germination temperature [°C]	18	12	18	12	12	18	12	15	18
Steeping degree [%]	48	48	48	48	42	42	42	45	42

Sample 8 shows the parameters for the reference malt. The other samples show the extreme points

factors (germination time, degree of steeping and germination temperature) on proteolysis. The objective of this experimental setup was only to find extreme values in the malting regime and to guarantee differences in the malt quality. Variations in germination time between 5 and 7 days, germination temperature between 12 and 18 °C and steeping degree between 42 and 48% were applied (Table 1). Nine combinations with different times and temperatures were selected, and a duplicate analysis of the center points was carried out. Brewing was carried out using a microscale equipment (10 L) at the Institute of Brewing and Beverage Technology in Freising (Germany). The fermentations were performed in 10-L cylindroconical tanks, at 12 °C with pure culture yeast (W134). Maturation was carried out over a 2-week period at 0 °C. These brewing trials were performed in duplicate. Filtration was carried out via modified Raible assay according to Kreisz [12].

Methods

The following methods were used to monitor the changes in the protein content and protein composition. Total protein content (Kjeldahl method, Bradford assay), coagulable nitrogen (MEBAK II 2.9.2),) and free amino nitrogen (MEBAK II 2.8.4.1.1) of freshly collected beer were immediately measured. Samples of the collected beer were freeze-dried and prepared for 2D-PAGE and Lab-on-a Chip analysis. Common malt, wort and beer analyzes were done according to MEBAK [19].

Lab-on-a-Chip technique: capillary gel electrophoresis

Lab-on-a-Chip Technique capillary electrophoresis was performed on the Agilent 2100 Bioanalyzer (Agilent, Böblingen, Germany). The principles of these electrophoretic assays are based on traditional gel electrophoresis principles which have been transferred to a chip format. The chip accommodates sample wells, gel wells and a well for an external standard (ladder). The microchannels are fabricated in glass to create interconnected networks among these wells. During chip preparation, the microchannels are filled with a sieving polymer and fluorescence dye. Once wells and channels are filled, the chip becomes an integrated electrical circuit and proteins are able to be separated based on their charge.

Extraction for Lab-on-a Chip technique For analysis of the total protein content, wort and beer samples are extracted in an ultrasonic bath at room temperature for 5 min with 400 mL 2 M urea solution (2 M Urea, 15% Glycerol, 0.1 M Tris, pH 8.8, 0.1 M DTT) and centrifuged for 15 min. The total protein content was determined by the Bradford assay, and all samples were diluted to 2 mg/mL. Four microliters of this solution was denatured using 2 µL of Agilent denaturing solution and heated for 5 min at 100 °C. After dilution with deionized water, 6 µL was applied to the Protein 80 + Lab Chip (detection performance between 4.5 and 95 kDa) for analysis in the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The ladder consisted of reference proteins of 3.5, 6.5, 15, 28, 46 and 63 kDa plus the upper and the lower markers of 95 and 1.6 kDa. According to the Agilent manual, any peak detected below 5 kDa is named a system peak and is not included in analysis. Results can be shown in an electropherogram or a gel-like image, as known from SDS–PAGE analysis, where the intensity of bands equals the peak heights in the electropherogram.

Two-dimensional gel electrophoresis (2D-PAGE)

2D-PAGE was carried out on the Ettan™ IPGphor™ 3 IEF System and the Ettan™ DALTsix Large Vertical System from GE Healthcare (Freiburg, Germany) on 12.5% acrylamide gels. The protein concentration was measured by the Bradford method using bovine serum albumin as a standard.

Two hundred milligrams of milled barley and freeze-dried wort and beer were separately precipitated with TCA/acetone prior to extraction. The combination of TCA and acetone is commonly used to precipitate proteins during sample preparation for better resolution of 2D-PAGE. TCA/acetone precipitation is carried out as follows:

1. Suspend lysed or disrupted sample in 10% TCA in acetone with 20 mM DTT.
2. Precipitate proteins for at least 45 min at −20 °C.

3. Pellet proteins by centrifugation (15 min) and wash pellet with cold acetone containing 20 mM DTT.
4. Remove residual acetone by air drying or lyophilization.
5. After precipitation, the samples are solubilized in 1 mL urea lysis buffer (containing 9.5 M urea, 1% (w/v) dithiothreitol (DTE), 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3–10)) for malt samples and 0.5 mL urea lysis buffer for freeze-dried samples.

2D-PAGE Three hundred and fifty micrograms of the sample was applied for in gel rehydration to the gel strips. Passive rehydration was done over night. Isoelectrofocusing (IEF) was carried out using 18-cm IPG 3–10 NL strips (ReadyStrip. GE Healthcare, Germany) and an Ettan IPGphor 3 (GE Healthcare). The running conditions were as follows: initial IEF (1 h, 500 V), gradient (8 h, 1,000 V); gradient (3 h, 8,000 V), hold (2 h 40 min; 8,000 V), gradient (3 h; 10.000 V); hold (1 h; 10.000 V). Prior to the second-dimension separation (SDS–PAGE), it is essential that the IPG strips are equilibrated to allow the separated proteins to fully interact with the SDS. The IPG strips were incubated for 15 min in the buffer containing 50 mM Tris–HCl (pH 8.8), 2% (w/v) SDS, 1% (w/v) dithiothreitol (DTT), 6 M urea and 30% (w/v) glycerol. This is followed by a further 15-min equilibration in the same solution containing 4% (w/v) iodoacetamide and traces of bromophenol blue instead of DTT.

Second dimension was carried out on an Ettan DALTsix Electrophoresis Unit (220 V), gel sizes of 20 × 25 cm, a gel thickness of 1.0 mm and total acrylamide concentration of 12.5%. SDS–PAGE is started with 5 mA per SDS gel (100 V maximum setting) for approximately 2 h. Continue with 15 mA per SDS gel (200 V maximum setting) for approximately 16-h overnight or higher current for faster runs. Terminate the run when the bromophenol blue tracking dye has migrated off the lower end of the gel.

Gels were fixed for 3 h in 50% ethanol and 3% phosphoric acid, washed 3 times for 20 min in water, and pre-incubated for 1 h in 34% methanol, 3% phosphoric acid and 17%(w/v) ammoniumsulfate solution; 0.35 g of Coomassie Blue (G-250) was added per 1 L solution and stained for 4–5 days. Gels were washed a few times in water to remove background stain scanned and analyzed with Delta2D from DECODON (DECODON GmbH, Greifswald, Germany).

Modified Raible assay

Raible assay was carried out according to Kreisz [12].

Required values for good filterability: F_{spez} [hL/m^{2}h] = 5.5–9.

Results and discussion

The impact of the malt quality on the chemical composition of beer was evaluated. Special focus lied on the protein content and protein composition. To give an overview of the influence of varied malt qualities, results are discussed also according to their amylolytic, cytolytic and proteolytic specifications, which cannot be seen totally independent of each other. In Tables 2 and 3, sample 8 is marked in red. Sample 8 is made with standard malting attributes according to MEBAK specifications and serves as reference malt.

Polyphenols

Phenolic compounds also play a role in haze formation and reach the beer through hops and malt. They have an influence on several beer quality attributes, such as astringency and colloidal stability of beer. Haze-active polyphenols are monomers, dimers, trimers, and higher polymers of the proanthocyanidins epicatechin, catechin and gallocatechin [10, 20]. Due to defined conditions, for example, insertion of oxygen, protein precipitating polyphenolic products can occur.

In Table 3, differences in the content of polyphenols, anthocyanogens and tannoids could be observed. Samples 1, 3 and 6 which also showed raised turbidity after filtration exhibited slightly increased values in total polyphenols and tannoids. Also, sample 4 showed higher values but did not show an increase in turbidity. The content of anthocyanogens varied in all the samples but could not be correlated with haze formation.

Since the influence of polyphenols is known in correlation with colloidal instability [5, 8, 10, 11, 17, 20–25], it could be possible that differences in colloidal stability would occur, which is not discussed further since this study only concentrates on haze formation directly after filtration.

Amylolytic and cytolytic specifications

Amylolysis is the degradation of starch into fermentable sugars and is defined over the final attenuation and extract. A potential extract of more than 80% is the standard value for barley malt used for brewing purposes. The extract values ranged usually at levels between 80 and 81% dm.

To achieve extract levels of 80–81%, a minimum of 5-day germination time, 12 °C germination temperature and a 42% steeping degree is necessary. Wort attenuation depended on the availability of fermentable sugars and on the yeast remaining in contact with the wort. The concentration was measured in grams of solids per 100 grams of wort. Barley wort obtained by congress mash normally

Table 2 Measured values of the analyzed malt samples

Analyses	Demanded values	Method	Samples								
			(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Water content [%]	3–6	MEBAK I 4.1.4.1	4.9	4.9	4.5	4.5	4.8	4.9	4.7	4.6	4.7
Extract [% d.m.]	>81	MEBAK I 4.1.4.2.2	80.1	81	80.1	81	80	80.1	80.8	80.8	80.6
Viscosity (8.6%) [mPas]	<1.56	MEBAK I 4.1.4.4.1	1.41	1.50	1.41	1.45	1.67	1.46	1.52	1.41	1.43
AAL [% app.]	81–84	MEBAK I 4.1.4.10	86	83	84	81	79	82	80	83	83
Color [EBC]	3–5	MEBAK I 4.1.4.2.8.2	3.4	2.4	4.5	3.2	2.3	2.5	2.6	3.2	2.9
pH	5.9–6.0	MEBAK I 4.1.4.2.7	5.8	5.9	5.8	5.8	6.0	6.0	5.9	5.9	6.0
Crude protein [% d.m.]	9–11	MEBAK I 4.1.4.5.1.1	9.0	9.2	8.8	8.9	9.2	9.5	9.2	9.3	9.2
Soluble nitrogen [mg/100 g malt d.m.]	650–750	MEBAK I 4.1.4.5.2	715	738	697	816	598	617	613	696	676
Kolbach index [%]	39–42	MEBAK I 4.1.4.5.3	49.7	50.1	52.1	57.9	39.3	41.9	41.6	46.8	45.9
Free amino nitrogen [mg/100 g malt d.m.]	130–160	MEBAK I 4.1.4.5.5	173	178	167	203	131	142	128	162	154
β-glucan VZ 65 °C [mg/L]	<350	MEBAK I 4.1.4.9.2	50	639	221	123	717	466	606	290	205

Sample 8 is framed red and shows the values of the standard malt (6/15/45)

Table 3 Measured values of the analyzed beer samples

Analyses	Demanded values	Method	Samples								
			(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
pH	4.3–4.6	MEBAK II 2.14	5.0	4.9	4.9	4.9	4.9	4.9	5.0	4.9	4.9
Color [EBC]	7–11	MEBAK II 2.13.2	4.3	5.8	5.6	4.1	4.1	4.3	3.2	3.9	4.1
Viscosity (8.6%) [mPas]	<1.6	MEBAK II 4.1.4.4	1.50	1.56	1.49	1.65	1.53	1.43	1.66	1.51	1.50
Total nitrogen [mg/L]	700–800	MEBAK II 2.5.2.1	812	810	817	584	550	862	633	691	585
Coagulable nitrogen mg/L	15–25	MEBAK II 2.9.2	14	21	13	18	11	13	15	16	18
Total amount polyphenols [mg/L]	150–200	MEBAK II 2.17.1	213	174	238	185	171	205	166	179	167
Anthocyanogens [mg/L]	50–70	MEBAK II 2.17.2	60	51	88	58	82	57	75	69	58
Free amino nitrogen [mg/100 mL]	10–12	MEBAK II 2.8.4.1.1	110	110	120	90	70	170	110	130	80
ß-glucan [mg/L]	<350	MEBAK II 2.5.2	16	371	5	371	249	165	666	168	114
Tannoids PVP/L	0–60	MEBAK II 2.17.3	81	48	90	46	45	78	52	58	51

Sample 8 shows the values of the beer brewed with standard malt

has an AAL (apparent attenuation limit) of 80%. The AAL depends on the complete hydrolysis of starch and on a sufficient amino acid supply for the yeast. Standard values for AAL range between 81 and 84%. For the used malts, the AAL values varied between 79 and 86%. Like extract, AAL showed a minimum at 5-day germination time, 12 °C germination temperature and 42% steeping degree (Table 2). Samples with low AAL correlated with low FAN content (Table 2). These samples were malted with low temperatures (12 °C) which could lead to less-modified malts and therefore also to decreased cytolytic degradation. Less-modified malt could lead to proteolytic and cytolytic induced haze formation. According to the amylolytic specifications, no haze formation should be induced.

Cytolysis is the breakdown of cell walls during the malting process. Indicators for cytolysis are friability, β-glucan content and viscosity. β-Glucan is responsible for difficulties in beer filtration, precipitate formation, haze formation in beer and possibly reduced extraction efficiency in the brewing industry. Shearing forces during the brewing process could lead to a cross-linking of the molecules and thus to the formation of a so called gel [14]. Due to the negative effects on lautering and filtration, minimum β-glucan content is desired. Appropriated barley malt standard values suggest β-glucan content lower than 300 mg/L. The samples showed β-glucan values between 50 and 717 mg/L for the wort produced by isothermal mashing 65 °C and 16–666 mg/L for the finished beers obtained from the brewing trials. β-Glucan is degraded during malting. In the malting process, the strongest impact on β-glucan content is caused by germination temperature and time. The longer the germination period the more β-glucan is degraded. This effect can be seen in the results, e.g., samples 1 and 3 (Table 2 for malt analyses and Table 3 for beer analyses).

Viscosity gives a forecast of the processability (lauter and filtration characteristics) of beer during the process. Wort and beer viscosity are influenced by the macromolecules—e.g. β-glucan—present. Generally, low viscosity is considered advantageous for the filtration process. Values for viscosity range between 1.41 and 1.67 mPa × s for the wort produced by isothermal mashing 65 °C (Table 2) and 1.49–1.66 mPa × s for the finished beers produced during the brewing trials (Table 3). Viscosity in barley wort and beer is mainly dependent on β-glucan content, which can be seen in Tables 2 and 3. If β-glucan content is high, viscosity values will also raise e.g. samples 2, 5 and 7. Nondegraded β-glucan leads to poor proteolytic specifications, due to cell wall degradation, and could also lead to β-glucan-induced turbidity in beer.

Proteolytic specifications

Proteolysis is the modification of grain protein into high-, middle- and low molecular weight forms and amino acids. Kolbach index, soluble nitrogen and free amino nitrogen (FAN) give a first impression of the solubilization of the malt proteins. Nitrogen compounds in worts are fundamental for brewing processes and beer quality and stability. Total nitrogen values are obtained from the sum of all nitrogenous compounds present and are determined by the Kjeldahl method. The nitrogenous constituents of wort include amino acids, peptides, proteins, nucleic acids and their degradation products. Appropriate barley malt standard values suggest FAN content for worts, to assure yeast nutrition, between 130 and 160 mg/100 g malt d.m., for FAN content in beer 100–120 mg/L and for total nitrogen in beer between 700 and 800 mg/L. Wort color is a consequence of the products formed by Maillard reaction out of FAN and reducing sugars [14]. Depending on the step of the Maillard reaction, uncolored or colored products are formed. The color of malt and beer is mostly attributed to melanoidins, product of the final phase of the Maillard reaction [26]. Standard values range between 3 and 5 EBC for worts produced by isothermal mashing 65 °C and 7 and 11 EBC for beer [19]. The influence of the FAN content on the wort and beer color can be seen in Tables 2 and 3. In Table 2, sample 3, which was the most solubilized malt sample (7-day germination time, 18 °C germination temperature and 48% steeping degree), showed the highest amount in FAN and also in wort color. Similar results could be observed in Table 3, for beer.

The Kolbach index represents the proportion of total nitrogen present in the malt that is soluble and is the most frequently used parameter for the evaluation of proteolysis. A balanced composition of high molecular weight proteins (foam stability, body and mouthfeel) and low molecular weight proteins (yeast nutrition) should be guaranteed. Standard values for barley malt are between 38 and 42% [19, 27].

Tables 2 and 3 show analyses values for proteolytic attributes. As it was already mentioned in the section cytolytic specifications, values are strongly dependent on germination time and temperature along with steeping degree. Steeping degree exerted the highest influence on proteolytic attributes. This effect was already described by several authors [14, 27, 28]. For example, sample 5 with the following malting parameters: 5-day germination time, 12 °C germination temperature and 42% steeping degree (5712742) showed lower values in FAN content and Kolbach Index than sample 2. Even though only the degree of steeping was varied, warmer malting conditions lead to a displacement toward root and acrospire growth, which can lead to a lower Kolbach index [14, 28]. This effect can be followed in Table 2. Sample 3 (7/18/48) was the malt sample with the best solubilized malt and showed a Kolbach index of 52.1, whereas sample 4 (7/12/48) had a Kolbach index of 57.9.

Protein content and protein composition

Malt

With Lab-on-a-Chip technique, changes in protein degradation were followed. The electropherograms generated with the Lab-on-a-Chip technique (Fig. 1a) revealed protein molecular weight regions of 5–8 kDa, 9–20 kDa, 21–28 kDa, 30–32 kDa, 33–45 kDa, 48–65 kDa and 66–94 kDa. In Fig. 1a, variations between a over modified—sample 3—(7-day germination time, 18 °C

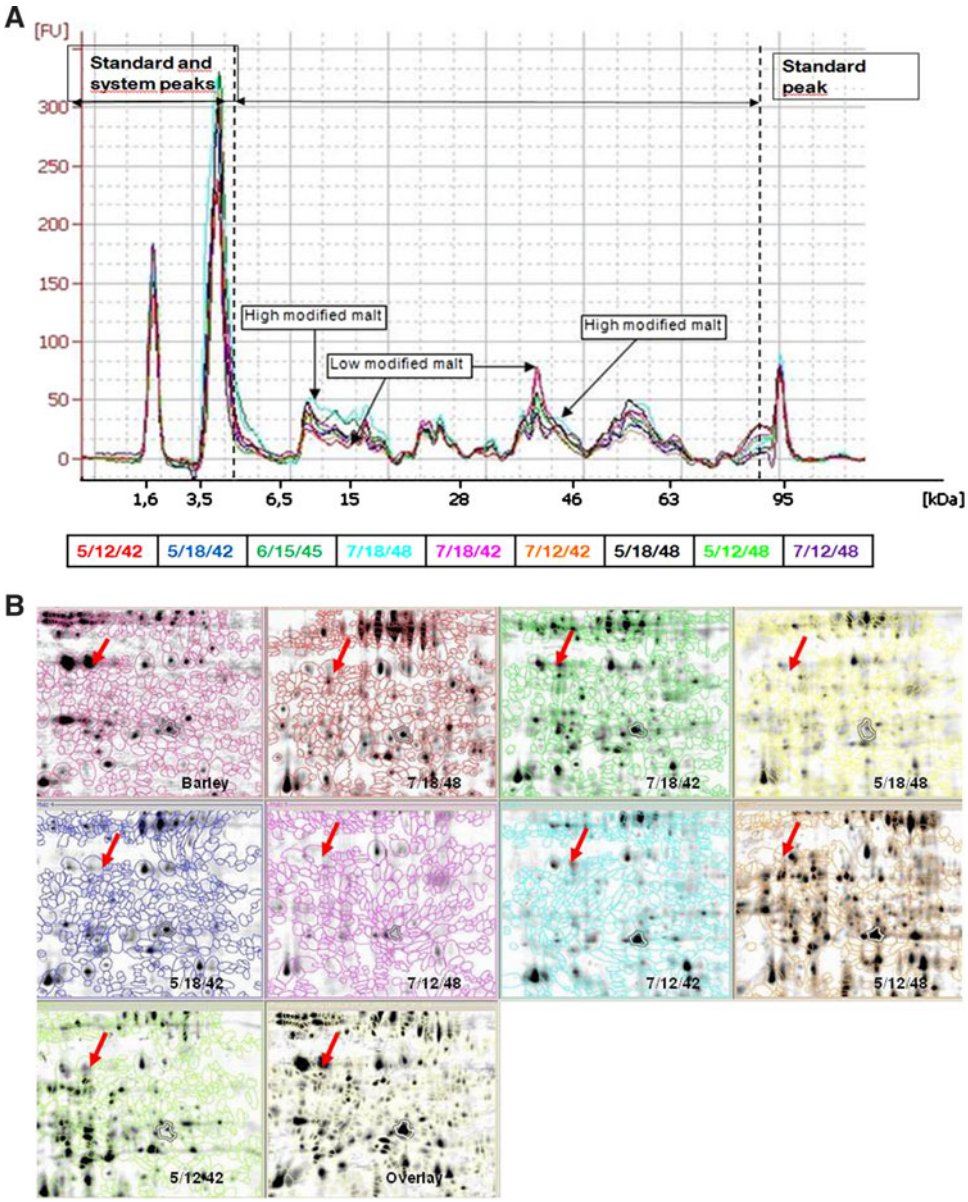

Fig. 1 **a** Electropherogram of protein fractions in different malted samples. Analysis is made with Lab-on-a-Chip technique. In the table below, the malt samples are listed with the color they have in the electropherogram. **b** 2D-PAGE of malt. In Fig. 1b, the same details of barley and different malts are shown. The size range lies between 20 and 40 kDa. The same protein in barley and different malts is marked with an *arrow*. Differences in the appearance can be seen

germination temperature and 48% steeping degree) and an under modified—sample 5—malt (5-day germination time, 12 °C germination temperature and 42% steeping degree) are marked with arrows. This is also shown in relative concentrations in Table 4. The high-modified malt samples (samples 1–4) showed higher values in low molecular weight proteins (9–20 kDa) than in high molecular weight proteins (66–94 kDa). The high-modified malt had already undergone several cytolytic and proteolytic changes, which lead to degradation of macromolecules. Therefore, high-modified malt contained a higher content of low molecular weight proteins. On the contrary, low-modified malt contained a higher content of high molecular weight proteins.

In addition to Lab-on-a-Chip technique, which reveals protein fractions with relative concentrations, 2D-PAGE was carried out. With 2D-PAGE, it is possible to show differences in protein composition, since proteins are separated according to charge and size and are displayed in single spots. With 2D-PAGE, it was therefore expected to receive more information on protein composition according to size and isoelectric point. Figure 1b shows 2D-gels of proteins from malts produced under different malting parameters. This figure shows the proteins in the size range between 20 and 40 kDa and pH between 4 and 7. Hence, the main differences lied between 20 and 40 kDa which is the size range of B-hordeins [29, 30]. This is also the size range for the most haze-active and haze-forming proteins which are rich in proline and glutamic acid [16, 17, 31–38]. The highest influence on proteolysis exerted steeping degree, as it was already described in the section

Table 4 Relative concentrations [ng/μL]of protein fractions of malt

Malt: relative concentration [ng/μL]									
kDa	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9
5–8	272	366	225	150	92	119	101	225	185
9–20	186	290	320	210	276	265	239	307	305
25–28	86	91	94	91	120	98	98	122	81
30–32	7	20	25	5	19	21	25	22	22
33–45	156	180	176	181	163	101	187	197	143
48–65	94	170	168	161	125	186	150	212	146
66–94	4	35	2	13	10	50	29	40	2

"proteolytic specifications". In the gels with 48% steeping degree, more protein spots could be seen in the selected area. This indicated an intensified degradation of proteins into smaller protein fractions and also the development of enzymes. The influence of germination and malting on enzyme development was already studied by several authors [36, 39–44] and should not be a part of this study.

Lab-on-a-Chip technique and 2D-PAGE were carried out to illustrate changes in protein composition during malting. With a demonstration of the differences in protein composition already of the malt, differences in protein composition of the beer were expected. According to these results, it was assumed that differences could also be seen in the final beer. Beers using raw barley and barley malt in different modification stages were brewed to confirm these results.

Beer

With regard to the differences in protein concentration of the malt samples, differences in the protein composition of the finished beer were expected. Whereas in the fractionation of malt proteins with Lab-on-a-Chip technique various peaks could be seen, in the fractionation of beer proteins only 4 peaks remained. These peaks were at 5–8 kDa, 9–18 kDa, 25–28 kDa and 42–45 kDa. Differences were visible in the relative concentration of the proteins at 25–28 kDa, 9–18 kDa and 42–45 kDa (Fig. 2; Table 5). To gain more detailed information on protein composition, 2D-PAGE was carried out. As opposed to the malt samples, no differences in the protein composition of beer could be seen, and therefore, no gels are shown.

During mashing proteins, mainly albumins and globulins are extracted, and during wort boiling and fermentation, high molecular weight proteins can be separated. This could be the reason for the similarity of the protein composition in the final beer.

In Table 6, values for turbidity (90° and 25° angle) and filterability are shown. Filterability was very good for all beer samples, and no significant differences could be obtained. Whereas different turbidity values were obtained (Table 6, increased turbidity is marked), a closer look was taken on the relation between relative concentrations of protein fractions. The increase in turbidity could be due to β-glucan and/or proteins. Since only in sample 7 (7/12/42) a high β-glucan content was observed, it was assumed that the increased turbidity was not caused by β-glucan. On closer examination, coherences between increased turbidity and a higher concentration of the protein fraction at 28 kDa could be seen. The highest content of this fraction was in the samples 1, 3, 5 and 6 (Table 5), which are the samples with the highest turbidity value.

This study showed that with the same barley and different malting parameters, no differences in the protein composition occurred, but the protein content varied (Fig. 2). During malting and brewing, several changes occur in the barley proteins. In this study, a protein fraction with a molecular mass of 28 kDa could be observed influencing turbidity and also very over-modified malt caused increased turbidity. This can be attributed to a number of factors. Leiper et al. [16, 17] studied the influence of the mashing process (mashing with different temperature halts). These researchers were able to show that if a beer has been brewed including a protein halt in the mashing program (48–52 °C), less total proteins could be found, but the remaining proteins were haze proteins, due to increased proteolysis. Leiper et al. [4, 16, 17, 31, 32, 35, 38] mentioned the influence of proteins in a size range between 15 and 30 kDa as causative agents for haze. In the present study, this thesis could be supported, since proteolysis already takes place in malting and is continued during the mashing process. This is shown in Table 6, where brewing with extremely over-modified malt (samples 1 and 3), i.e., increased proteolysis, caused raised turbidity.

In the following abstract, possible explanations, found in the literature for a haze-forming protein fraction at 25–28 kDa, are given. In the literature, only studies on colloidal haze could be found. Since, in our expertise, protein haze after filtration always lead to colloidal haze, it

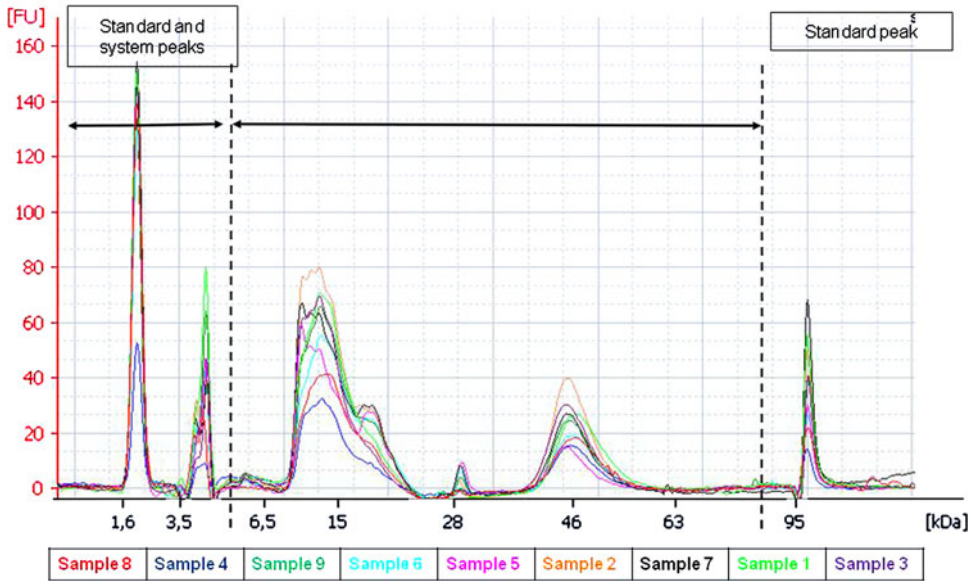

Fig. 2 Electropherogram of protein fractions in beer, made out of malt with different malting parameters. Analysis is made with Lab-on-a-Chip technique. In the table below, the malt samples are listed with the color they have in the electropherogram

Table 5 Relative concentrations [ng/μL] of protein fractions in beer

Beer: relative concentration [ng/μL]									
kDa	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9
5–8	272	5	116	150	92	119	101	123	185
9–20	2,892	1,153	1,344	1,185	583	177	1,160	189	1,570
25–28	62	31	36	4	36	52	30	29	32
33–45	1,249	509	637	680	144	277	439	563	830

Table 6 Measured values of turbidity and filterability in the analyzed beer samples

Turbidity	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9
25°	1.64	0.53	1.48	0.44	1.51	1.86	0.33	0.34	0.26
90°	1.12	0.69	1.11	0.53	0.71	0.76	0.44	0.43	0.44
Filterability [hL/m^{2}h]	8.44	7.6	6.75	8.18	6.67	7.77	6.73	6.97	8.69

Samples with increased turbidity are marked with a red box

was assumed that the same haze-forming proteins already exist in the protein turbidity directly after filtration.

Asano et al. [45] investigated different protein fractions and divided them into 3 categories: high-, middle- and low molecular weight fractions with following separation: high molecular weight fractions: >40 kDa, middle molecular weight fraction: 15–40 kDa and low molecular weight fraction: <15 kDa. It was found that the most important glycoproteins for haze formation are in the size range of 16.5–30.7 kDa. Nadzeyka [7] investigated that proteins in the size range between 15 and 35 kDa contained the highest amount in proline. A literature research revealed that proline- and glutamic acid-rich hordeins, in the size range between 10 and 30 kDa, are the main initiators for haze development [9, 15]. A number of researchers could show that proline-rich proteins are involved in haze formation [5, 7, 9–11, 16, 17, 46–49]. Sheehan et al. [33], Jin et al. [32, 33] and Leiper et al. [16, 17] investigated heat stable proteins and found that haze-forming glycoproteins in this size range (15–30 kDa) exist. They established glycoproteins at 30, 25–29 and 30.7 kDa, respectively, which caused haze formation at increased concentrations. Jones et al., Poulle et al. and Zhang et al. claimed that a cysteine endoproteinase (EC 3.4.22) at 30 kDa exists and the maximal activity is in the acidic pH range. It is possible that this protein with a molecular weight of 25–28 kDa is an endoproteinase with proline in the hydrolytic site, since proline-rich proteins could cause increased turbidity [35,

37, 38]. Osman et al. [50–52] reported in his studies that it is possible that this protein fraction at 25–28 kDa is a "new" protein which is formed from specific proteins originating from the malt. This can be explained by the structural changes, including glycation by the Maillard reaction during malting. Acylation during mashing and structural unfolding during the brewing have also been claimed to be a reason.

Conclusion

Turbidity gives a first visual impression of the quality of beer to the consumer. Therefore, it is necessary to have methods not only to identify haze but also to infer what the source of haze formation is. This research was performed to investigate the influence of the malting parameters (germination time and temperature and steeping degree) on haze formation in beer directly after filtration. Special emphasis was placed on protein analyses.

According to the amylolytic, cytolytic and proteolytic specifications differences in the protein content and protein composition of finished beer were expected. The variations in protein composition and protein fractions gained by 2D-PAGE and Lab-on-a-Chip technique in relation to proteolytic analyses lead to the assumption that differences in protein fractions and composition could also be visible in finished beer. With this research and experimental setup, it was possible to show the influence of malting on haze-forming protein fractions in beer, already directly after filtration. The study of Leiper et al. [16, 17] could be supported, since it could be shown that very over-modified malt lead to increased turbidity already after filtration.

The aim of this study was also to find a protein fraction which correlates with malting and haze formation in beer. A haze-forming fraction of 25–28 kDa was found, especially in the beers brewed with under-modified malt. The nature of this 25–28 kDa protein fraction is still unclear; therefore, it is necessary to identify this protein with specific methods, like MALDI-TOF, IC-MS, etc.

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15 Conclusion and Outlook

Haze formation is an important quality aspect to the consumer. Therefore it is in the interest of both, brewers and consumers, to minimize haze formation in beer. In previous studies specific proteins and their influence on haze formation have been investigated. To continue this research, the influence of malting on haze formation in beer has been evaluated. To prevent haze, its formation mechanisms and also haze forming substances have to be known.

The overall purpose of this study was to investigate barley proteins and their “vita” into the finished beer. To give a résumé, the questions, asked in the motivation, are again outlined: Is it possible to find a procedure to identify different sources of haze formation? Where are the differences in protein composition in beers brewed with 100 % barley raw material and 100 % barley malt? How do the malting process and the proteolytic stage effect protein composition in finished beer? Is there a way to identify new proteins/protein fractions which influence haze formation?

These issues were addressed by following approaches:

1) A further development of the already existing methods for haze identification, to find a simple and reproducible approach of haze identification.
2) Investigation of differences in protein composition and therefore in haze formation between beer brewed with 100 % barley and beer brewed with 100 % barley malt have been investigated.
3) Examinations of influences of different malting parameters (germination time and temperature and steeping degree) on haze formation in beer have been examined.

To identify different sources of haze in beer a procedure was developed. With an approach of 4 steps (turbidity measurement, enzymatic and microscopic haze identification and verification of the source of glucan by GPC), it was possible to find a simple and reproducible procedure to investigate haze and the source of haze in beer. With this basis it was possible to identify protein haze in beer and to focus on the source of barley protein haze, which was accomplished with the two other approaches. A good survey - to get an impression on the importance of malting, on

final beer quality focusing on haze formation – is the comparison of beer brewed with 100 % barley raw material and exogenous enzymes and beer brewed with 100 % barley malt.

Barley lacks of basic degradation processes (proteolysis, cytolysis, and amylolysis) which are accomplished during malting and mashing. The basis of the process of barley brewing is the replacement of malt by barley and addition of enzymes obtained from other sources, so that the malting process can be bypassed. The traditional source of enzymes used for the conversion of cereals into beer is barley malt. Ondea Pro® from Novozymes, a mixture of enzymes suitable for brewing with barley raw material, was used during the mashing process, as replacement for enzymes activated and synthesized during malting.

In beer produced with 100 % barley raw material neither haze formation after filtration, nor colloidal instability did occur. As differences could be seen in protein composition between barley malt and barley raw material, the question was raised, when do these differences appear and how do they influence haze formation? To answer this question, 9 extreme points from a response surface methodology were investigated to determine the impact of three predictor factors (germination time, degree of steeping, and germination temperature) on the protein composition in final beer. It was assumed that during the first stages of germination several proteins influencing haze formation were produced. This was also the starting point for the investigations that malting (different proteolytic solubilization degree) influences final protein composition in beer. With this study the protein content and protein composition could be followed and differences could be observed. Beers brewed with the same barley, malted under different parameters did not show any differences in final protein composition but in protein content. A variation at 28 kDa, from different proteolytic solubilization, showed the biggest influence on haze formation. Beers which had an increased concentration of the fraction at 28 kDa showed more haze formation, already directly after filtration. The variations in protein composition and protein fractions gained by 2D-PAGE and Lab-on-a-Chip technique lead to the assumption, that differences in protein fractions and composition could also be visible in finished beer. A protein fraction at 28 kDa could be observed in beer. It can be stated, that all beers with increased concentrations of this fraction showed increased turbidity.

Due to the presented results, we must ask the following questions:

A fraction at 28 kDa could be found influencing haze formation in all malt beers. The nature of this 25-28 kDa protein fraction is still unclear; therefore it is necessary to identify this protein with specific methods, like MALDI-TOF, IC-MS, etc. But what about beer brewed with 100 % barley raw material? No significant differences in haze formation, or colloidal stability were obtained even though differences could be seen in the A-hordeins. Either, it is certain that only the exogenous enzymes make the differences in final protein composition, or are these proteins decomposition products from the different modification processes such as glycosylation?

Whatever the reason for these differences is, it was shown that brewing with 100 % barley raw material leads to differences in final protein composition, while brewing with malt independently of the malting parameters does not have influence on final protein composition but on protein content. Further investigation should be made including more varieties, even feed barley, to gain a broader view on different protein compositions.

It is not only important what protein/protein fraction leads to haze formation or colloidal instability but also how it can be influenced during the malting and brewing process. It is suggested to find a method to extract and enrich different protein fractions from wort and beer (e.g. membrane adsorption chromatography) and add these fractions during different stages in the brewing process. With the addition of these fractions it should be possible not only to prove the theory about the increase/decrease of the 28 kDa fraction and its influence on haze formation, but also to find process steps to influence this fraction.

Condition of yeast and yeast propagation can also have influence on clarification of the beer and therefore on haze formation and colloidal stability. In this research only one pure culture yeast strain was used. Further investigations could be carried out using different yeast strains under different conditions. This could be also important for example for wheat beer, where colloidal haze is desired.

This research should serve as initial point for further investigations. It was shown that 2D-PAGE and Lab-on-a-Chip technique are two powerful tools in proteome analysis to follow protein changes during the malting and brewing process.

Summarizing it can be said that with these investigations it was possible to find coherences between malting (different proteolytic stages) and haze formation in beer. With this work a solid basis on further research has been established.

10 Appendix

10.1 Table of figures

11 Curriculum Vitae

Persönliche Daten

Elisabeth Wiesen (geb. Steiner); geboren am 31. 01. 1981, Judenburg; Österreich; verheiratet

Schulbildung

1987-1992: Volksschule; Knittelfeld

1992-1999: Gymnasium; BG/BRG Knittelfeld

1999: Matura

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1999-2005 Studium Lebensmittel- und Biotechnologie an der Universität für Bodenkultur in Wien

2003 Auslandssemester an der TU Stockholm; Schwerpunkt Biotechnologie

2005 Diplomarbeit am Lehrstuhl für Technologie der Brauerei I – TU-München/Weihenstephan; Thema: Ursachenfindung der Trübungspartikeln in alkoholfreien Bieren

Seit 2007 Promotion am Lehrstuhl für Brau- und Getränketechnologie, TU-München/Weihenstephan. Thema: Barley proteins – source and factor of haze formation in beer

Berufserfahrung

2000 Obersteirische Molkerei reg. Gen. M b H

2001 Austria Email AG

2002 AMA; Agrarmarkt Austria

2003 Advanced Polymer Engineering G m b H

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