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„Proteomic Analysis of Barley Cultivars: Investigation of Disulfide Interactions and their Influence on Gluten Network Formation for Bread Baking“

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Julia Füreder, BSc

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Abstract

Barley (*Hordeum vulgare*) is an underutilized crop in today's food industry, despite being the fourth most produced crop in the world. Its primary purposes are currently limited to animal feed and malt production. Barley is a rich source of dietary fibre like β-glucans, which can be beneficial for our health. Furthermore, barley shows good adaptability to unfavourable growing conditions, which can be employed to advantage. Due to these benefits, it is anticipated that the use of barley in food products will increase in the future. One way to increase its use would be to incorporate it into bread. This, however, faces challenges due to the unfavourable bread baking properties of barley, which are believed to be caused by the inadequate formation of a gluten protein network, that is crucial for dough quality. The full understanding of the gluten network is still an ongoing process, the foundation in wheat, however, is believed to be disulfide bond formation between high- and low-molecular weight gluten protein subunits (HMW/LMW-GS). To comprehensively analyse the protein composition of three barley cultivars, namely PS3, Golden Promise and Wild Barley, a protocol for Osborne fractionation extraction of barley proteins was established. This protocol separates proteins into albumins, globulins, prolamins and glutelins, based on their solubility in a series of extraction agents. The impact of grain size on the extraction efficiency was examined but found to be only marginal. Furthermore, a comparative analysis was conducted to evaluate three protein quantification methods: the Bradford Assay, the Dumas method, and the Nanodrop. The results indicated that the Bradford Assay yielded the most reliable outcomes. The protein profiles of all three barley cultivars were analysed using 1/2-Dimensional Gel Electrophoresis (1/2D-GE) techniques. A comparison between the protein profiles of the cultivars highlighted differences, particularly in terms of their C-hordein composition. Furthermore, by comparing reducing and non-reducing conditions during the sample preparation process, proteins affected by disulfide bond formation, were examined. Certain protein spots involved in the disulfide network were subjected to Matrix-Assisted Laser Desorption Ionization – Time of Flight mass spectrometry for protein identification. Consequently, it can be proposed that the barley gluten network comprises D-hordeins, acting as the structural backbone, similar to HMW-GS in wheat, while B- and γ-hordeins function as the monomeric units like LMW-GS in wheat. There remain significant gaps in knowledge as to why barley bread has poor baking properties, which are subject for further research.

Zusammenfassung

Gerste (*Hordeum vulgare*) ist eine Kulturpflanze, deren Potential in der heutigen Lebensmittelindustrie nicht komplett ausgenutzt wird, obwohl sie weltweit die am vierthäufigsten angebaute Nutzpflanze ist. Ihre Verwendungszwecke beschränken sich hauptsächlich auf die Tierfutter- und Malzproduktion. Gerste ist eine reichhaltige Quelle an Ballaststoffen wie β-Glucanen, die sich positiv auf unsere Gesundheit auswirken können. Darüber hinaus zeigt Gerste eine gute Anpassungsfähigkeit an ungünstige Anbaubedingungen, was zum Vorteil verwendet werden kann. Aufgrund dieser Vorteile wird erwartet, dass der Einsatz von Gerste in Lebensmitteln in Zukunft zunehmen wird. Eine Möglichkeit, die Einsatzgebiete zu erweitern, wäre die Verwendung in Brot. Dieses Vorhaben steht jedoch aufgrund ihrer ungünstigen Backeigenschaften vor Herausforderungen, die vermutlich auf die unzureichende Ausbildung eines Glutennetzwerks zurückzuführen sind, das entscheidend für die Teigqualität ist. Das vollständige Verständnis der Glutenstruktur ist noch fortlaufend. Es wird angenommen, dass die Basis bei Weizen die Bildung von Disulfidbrücken zwischen hoch- und niedermolekulare Protein-Untereinheiten (HMW/LMW-GS) ist. Um das Proteinprofil von drei Sorten, nämlich PS3, Golden Promise und Wildgerste umfassend zu analysieren, wurde ein Extraktionsprotokoll zur Osborne-Fraktionierung implementiert. Dieses Protokoll trennt Proteine in Albumine, Globuline, Prolamine und Gluteline, auf der Grundlage ihrer Löslichkeit in einer Reihe von Extraktionsmitteln. Der Einfluss der Korngröße auf die Extraktionseffizienz wurde untersucht, erwies sich jedoch als marginal. Darüber hinaus wurde ein Vergleich zwischen drei verschiedenen Methoden zur Quantifizierung durchgeführt, nämlich dem Bradford Assay, der Dumas-Methode und dem Nanodrop. Die Ergebnisse zeigten, dass der Bradford Assay die zuverlässigsten Ergebnisse lieferte. Die Proteinprofile aller drei Kultivare wurden mit 1/2-dimensionaler Gelelektrophorese (1/2D-GE) analysiert. Ein Vergleich der Proteinprofile zeigte Unterschiede zwischen den Sorten, insbesondere in Bezug auf ihr C-Hordein Profil. Darüber hinaus wurden durch den Vergleich von reduzierenden und nicht-reduzierenden Bedingungen während der Probenvorbereitung Proteine identifiziert, die Disulfidbrücken bilden. Bestimmte Proteine, die am Disulfidnetzwerk beteiligt sind, wurden mit Matrix–Assistierte Laser–Desorption–Ionisierung – Flugzeitanalyse Massenspektrometrie (*MALDI-ToF MS*) untersucht. Folglich kann erwogen werden, dass das Gersten-Glutennetzwerk aus D-Hordeinen besteht, die als strukturelles Rückgrat fungieren, ähnlich wie HMW-GS in Weizen, während B- und γ-Hordeine als monomere Einheiten wie LMW-GS in Weizen fungieren. Es gibt immer noch erhebliche Wissenslücken, warum Gerstenbrot schlechtere Backeigenschaften aufweist. Diese Lücken sind Gegenstand weiterer Forschung.

Graphical Abstract

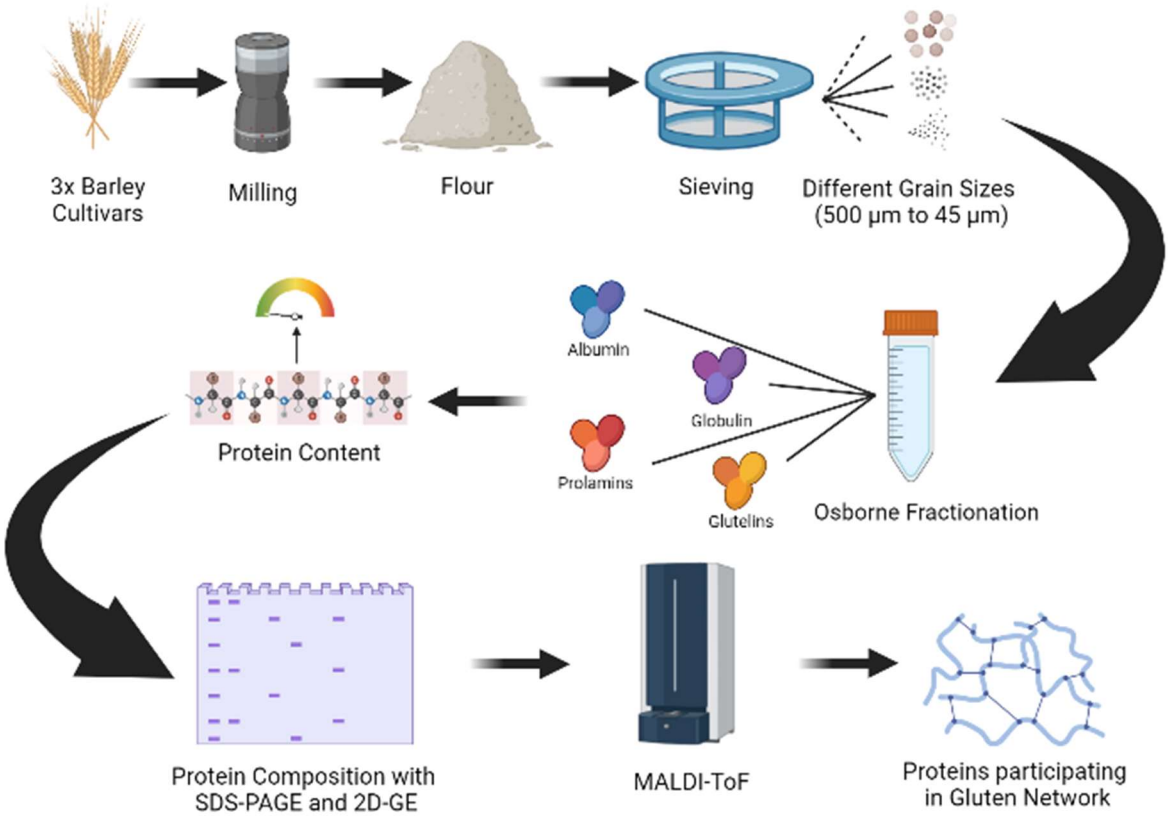

Figure 1: Three different cultivars of barley (PS3, Golden Promise and Wild Barley) are milled and sieved to obtain flour with varying grain size. Afterwards, proteins are extracted using an Osborne fractionation protocol that extracts proteins based on their solubility in 4 different solutions. For analysis of the protein composition, the protein content is measured and 1D - and 2D - gel electrophoresis is done. By comparing reducing and non-reducing conditions, proteins that possibly interact in a disulfide-driven gluten network are identified with MALDI-ToF-MS. Image was created on BioRender.

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Abbreviations

1D/2D-GE	1/2-Dimensional Gel Electrophoresis
BSA	Bovine Serum Albumin
DTE	1,4-Dithioerythrit
DTT	Dithiothreitol
EFSA	European Food Safety Authority
ESI	Electrospray Ionization
GBP	Giga-base Pairs
GP	Golden Promise
GS	Glutenin Subunits
HCCA	α-Cyano-4-Hydroxycinnamic Acid
HCL	Hydrochloric Acid
HMW	High-molecular Weight
LMW	Low-molecular Weight
MALDI	Matrix-assisted Laser Desorption/Ionization
MS	Mass Spectrometry
NON-RED	Non-Reducing
PAGE	Polyacrylamide Gel Electrophoresis
RED	Reducing
RNA	Ribonucleic Acid
RT	Room Temperature
RTOF	Reflector-time of Flight
SDS	Sodium Dodecyl Sulfate
SSP	Subspecies
TOF	Time of Flight
TRIS	Tris(hydroxymethyl)aminomethane
WB	Wild Barley
B-ME	β-Mercaptoethanol

1 Introduction

1.1 Barley's Role in Food

Cultivated barley (*Hordeum vulgare),* has been a staple food ingredient for more than 10,000 years. It was first domesticated from its relative *Hordeum vulgare* subspecies *spontaneum,* which is commonly known as wild barley. This was done by saving seeds from plants that showed favourable traits for the next season or by unconscious domestication as a result of environmental selection. This process, which can be traced back by looking at the genetic origin of specific traits, resulted in a conversion from an unreliable wild species into a promising crop, which has a higher number of seeds and improved seed fertility.[1]

At the moment, barley is after maize, wheat and rice the fourth most produced cereal crop worldwide, which is cultivated in more than 100 countries all over the world[2,3]. It is the second most produced crop in Europe, next to bread wheat (*Triticum aestivum*)[4]. Cultivated barley is a highly adaptable crop that can grow in temperate climates as well as in tropical regions and even in high altitude or mountainous areas. It is also a very important part of insuring global food safety, because in recent years, the wheat production industry has failed to meet the global demand, which triggered price instability, hunger and governmental instability[2,5]. The demand for wheat is expected to increase even further in the next years, due to the growing world population while the environmental conditions needed to cultivate wheat are threatened by climate change simultaneously. In this case, barley plays a vital part to ensure global food safety due to its higher resistance to generally unfavourable growing conditions such as cold, draught and poor soil quality in comparison to wheat[2].

The potential of barley as a cereal for direct human consumption is not fully utilized yet. It is estimated that more than 60 % of the barley yields worldwide are used for animal feed, while the majority of the remaining 40 % is used to produce malt[5]. In order to produce malt from barley, a controlled germination of the grain is elicited, where hydrolytic enzymes are synthesized that largely digest the cell walls, proteins and starch of the endosperm, which makes the grain more friable. After drying, the malt is mashed and the wort is separated[6]. The residues, also called spent grain is mostly used for animal feed. Less than 5 % of the barley crop is used for direct human consumption[7]. The primary use of cereal crops like barley, wheat, oat and rye for direct human consumption is for breads, pastries and porridge. With the advent of bread wheat, barley's use as a bread grain increasingly fell into oblivion since it is not comparable to bread wheat in regard to its taste and the gluten network that is needed to form a light and fluffy

bread[2]. People's preferences for bread grain changed over the years from barley to rye and eventually to wheat. Barley's main purpose nowadays is to infuse wheat flour with dietary fibres. The optimal amount of barley flour to replace wheat flour at the moment is around 20-40 %, otherwise the sensory quality of the bread will decrease.[8] Nonetheless, the implementation of barley in more food products would have sustainability and economic benefits and adds more nutritional value[2]. An illustration of the different uses of barley in today's food industry can be seen in **Figure 2**.

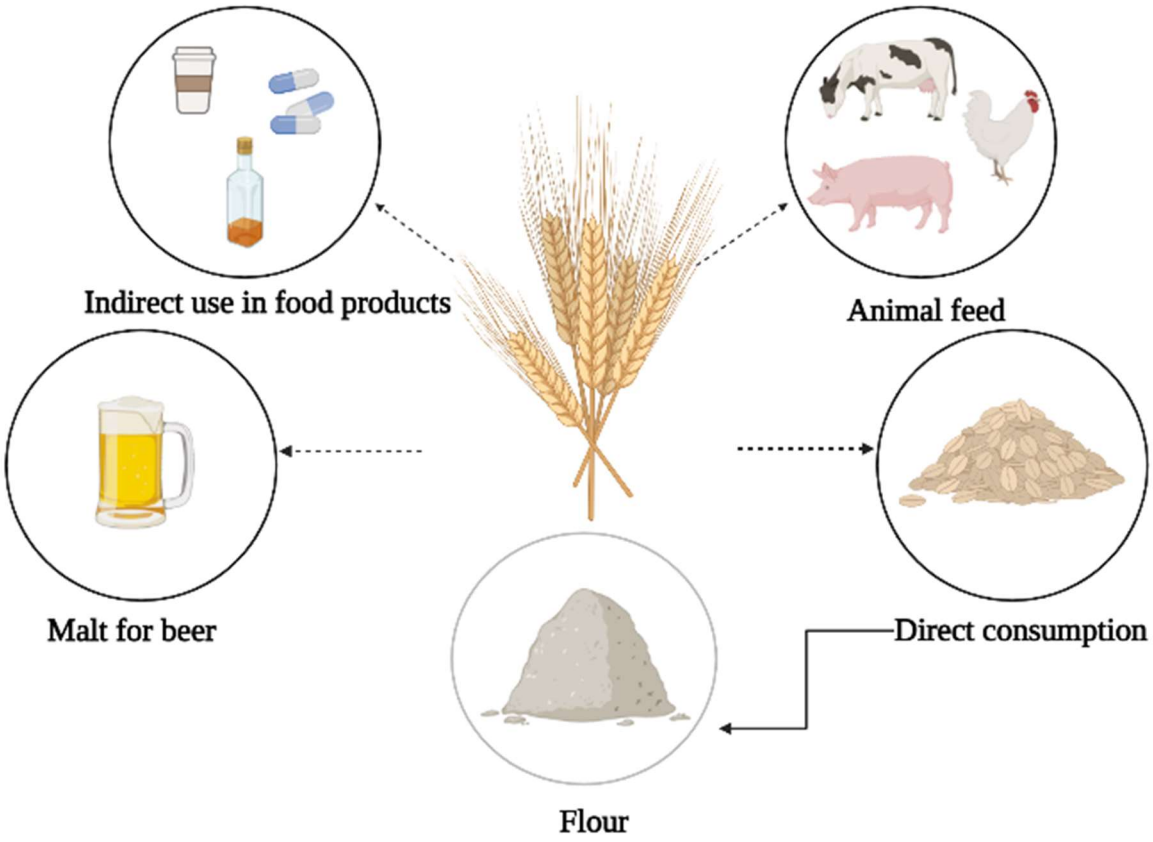

Figure 2: Illustration of the current range of application for barley grains. The majority of the barley yield worldwide is used for animal feed or to produce malt for beer. Additionally, in small parts, it is also used for direct or indirect human consumption in other food products. One possibility to incorporate it into the human diet is in the form of flour. Image was created on BioRender.

1.2 Barley's Health Benefits

The increasing awareness and demand for a healthy and balanced diet paired with the publication of numerous studies about the health benefits of barley has reinforced the popularity of barley as an inexpensive, adaptable, and secure food source. Barley contains a high level of insoluble and soluble dietary fibre like β-glucans (around 4.5 %), which can improve the cardiovascular status by reducing risk factors such as high serum cholesterol levels and postprandial blood glucose levels when it is consumed regularly[2,9].

The serum cholesterol level is an important biomarker for cardiovascular diseases in general. The US Food and Drug administration states a clear relationship between the consumption of foods that show a low level of saturated fat and cholesterol and a decreased risk of coronary heart diseases. Foods that reportedly reduce the serum cholesterol level qualify for a health claim, which can be used on food packaging and in advertisements.[10] A meta-analysis conducted concluded that the consumption of at least 3 grams of β-glucans per day decreases the blood cholesterol level significantly, which is why the regular consumption of β-glucans is strongly recommended by the European Food Safety Authority (*EFSA*)[11,12]. Whole grain barley and dry milled barley qualify as valuable sources of β-glucans. The proposed mechanism suggests that the soluble dietary fibres found in barley increase the viscosity of the meal, which traps bile acids and cholesterol and prevents their reabsorbance. Consequently, there is a need to replace the lost bile acids, which is done by synthesizing them from circulating cholesterol. This process ultimately leads to a reduction in serum cholesterol levels.[13]

Moreover, barley beneficially contributes to the regulation of type 2 diabetes, which is of high relevancy with rising numbers of type 2 diabetes in most developed countries. Bread which mostly contains wheat on the other hand has a high amount of rapidly digestible starch, which makes it unsuitable for diabetic patients due to the increase in postprandial glucose levels[13]. Studies have demonstrated a dose-response effect of β-glucans on the mitigation of postprandial glycaemic response. It is suggested that the high viscosity of the consumed meal induced by β-glucans may slow down the digestion of starch by delaying the diffusion of α-amylase towards its starch substrate and also decelerating the absorbance of glucose by slowing the diffusion of sugars and α-dextrins towards the intestinal epithelium down, which is a result of the starch digestion[10].

Furthermore, barley can support the growth and maintenance of gut microorganisms, because the dietary fibres present in barley brans are the major source of energy for gut microbiota[10]. Barley based foods show the capability of lowering inflammation markers through a gut microbiota mediated mechanism. The intake of barley can increase the plasma concentration of certain gut hormones like glucagon-like peptide-1 (*GLP-1*) and peptide YY (*PYY*), which are associated with metabolic regulation and appetite control, which could have a lowering effect on diseases related to the metabolic syndrome such as obesity and diabetes[14].

Controversially to the positive propulsion of barley in science, hardly any increase of use in food products for direct human consumption was observed. Barley is often praised for its versatility and its safety for providing food with high nutritional value to masses of people. However, in terms of taste and processing properties, it cannot compete with other cereals like wheat or oat.[15]

1.3 Genetics, Structure and Protein Profile

1.3.1 Genetics

Hordeum vulgare has a diploid genome with seven pairs of chromosomes, which adds up to a total of 14 chromosomes. It is part of the *Triticeae* tribe, which evolved over 12 million years ago. There is a high number of different barley varieties and genotypes, including spring and winter barley and two- and six-rowed barley.[4] The size of the barley genome is estimated to be around 5.1 Giga-base pairs (*Gbp*) and it mainly consists of repetitive domains[3,16]. Despite its vast genome, barley is popularly used as a genomic model for cultivated hexaploid wheat, which has an even more complex genome with around 17 Gbp. Many distinct traits that can both be found in barley and wheat can be traced back to the same responsible gene, which has high practical importance in forming a genomic model[3].

1.3.2 Structure

The barley grain consists of the embryo, the aleurone layer, the endosperm and the husk, which is covering the grain and ensures the integrity of the grain. The embryo contains starch, lipids and proteins to sustain the embryo before it germinates. The embryo is surrounded by the aleurone layer, which contains protein, phytin, phospholipids, ribonucleic acid (*RNA*) and carbohydrates. It is responsible for the regulation of enzymes that degrade the endosperm. The husk mainly contains cellulose and a small amount of bitter substances.[17] Barley contains around 8-13 % of proteins on a dry weight basis, which is synthesized in the endosperm and the aleurone layer during the grain development[7,17]. The protein composition of barley is dependent on the cultivar and environmental factors, for example the nitrogen-level during the growing stage. Studies showed that the amount of nitrogen fertilizer applied to the crops during the growing stage had a significant effect on the total protein content[18]. The protein content of barley grains that have been grown in a hot, dry and nitrogen-rich environment show a higher protein content[17]. The proteins present in the barley endosperm form a network that is surrounding starch granules. 75 % of the barley kernel weight consists of the starchy endosperm. Apart from starch, 4-9 % of the weight are β-glucans that can be found in the endosperm cell wall. The concentration of both β-glucans and starch, the total protein concentration, the amount of protein that is bound to the surface, and the thickness of the seed coat and cell walls has an influence on the hardness of the grain and therefore its processing behaviours.[19,20] The barley bran, which is the outer layer of the grain, contains the aleurone, hyaline, testa and pericarp tissue. The main components of the bran include starch, polysaccharides that also include dietary fibre, lignin, proteins, lipids and minerals[21]. The free

lipid content makes around 2-3 % of the barley kernel and the mineral content is 1.5-2.5 %. The structure of the barley grain can be seen in **Figure 3**[21].

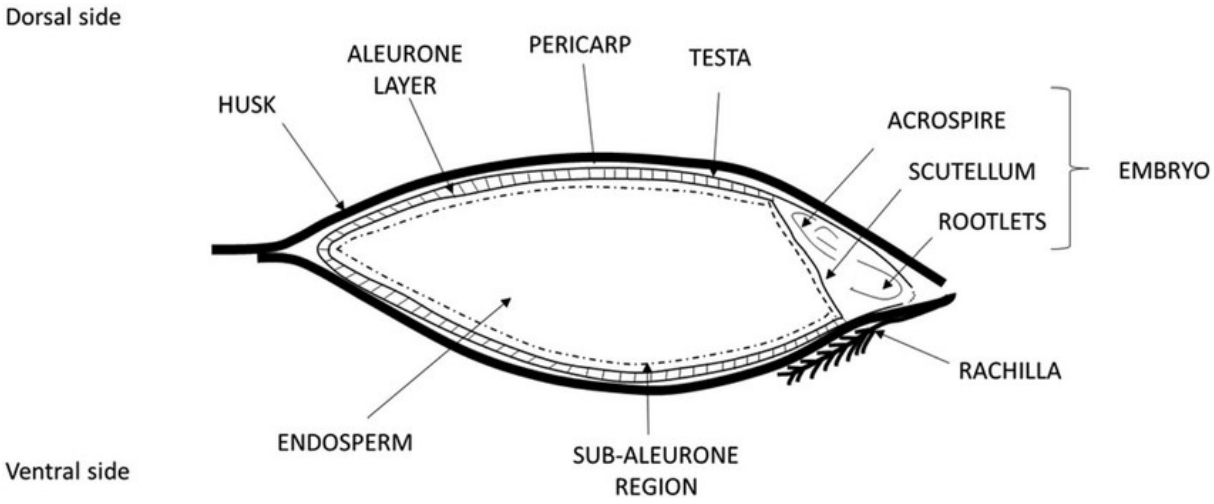

Figure 3: Grain structure of barley as presented in *Filipowska et al., 2021*[22]. The three primary components of the seed are the protective layer, which is called the husk, the energy-providing endosperm and the embryo, that provides nourishment for the developing seed.

1.3.3 Protein Profile

According to Thomas Burr Osborne, proteins in barley can be divided into four different fractions based on their solubility in a series of extraction solvents applied to the plant tissue[23]. The four fractions are the water-soluble albumins, the salt-soluble globulins, the alcohol-soluble prolamins and the weak acid or weak alkali-soluble glutelins. The proteins in the barley endosperm are mostly considered undesirable side products by the brewing industry. Previously published articles about barley mostly focused on the influence and relationship between the prolamin fraction or the total protein content with the respective malt quality. Less studies targeted the characterization of each of the four protein fractions.[18] The following chapters depict a short summary of the current knowledge about function and characterization of these four protein fractions in *Hordeum vulgare*.

1.3.3.1 Albumins and Globulins

Albumin, globulin and glutelin fractions are mainly structural and metabolic proteins in barley[18]. Depending on their molecular structure, they are responsible for different functional properties. Albumin and globulin proteins make the biggest fraction of cytoplasmic proteins, which are enriched in the barley bran and germ[24]. During food processing, the barley bran is often removed, because it is less suitable for various food applications, resulting in the so-called

bran-based pearled flour, which is a mostly unwanted waste product that is repurposed as animal feed. The remaining part is called the pearled grain[18]. Many studies demonstrated that the albumin and the globulin proteins in general cannot be properly separated by extraction only, which is why some protocols work with a combined method for both fractions and they are often treated as one fraction in later existing literature[23,25]. The major barley albumin is called Protein Z with a molecular weight of 40 kDa and a pI around 5.5-5.8. It makes up 5 % of the total albumins. It is heat stable and resistant to enzymatic degradation, which means that it is still present in the final food product, for example beer. It has a vital role for the foam stability of beer.[17]

1.3.3.2 Prolamins

The prolamins as derived from different cereals have specific names. The prolamins from barley, maize and wheat are called hordein, zein and gliadin respectively and they are rich in proline and glutamine residues[7,18,26]. The alcohol-soluble prolamins mostly have a molecular weight of around 30-75 kDa and they add up to 35-55 % of the total protein content. They are categorized based on their amino acid composition and electrophoretic mobility and can be divided into four different groups in barley[6,19]. The first ones are sulfur-rich B-hordeins with a molecular weight of 30-45 kDa, which make up around 70-80 % of the total hordeins. The second group consists of the sulfur-poor C-hordeins, which account for around 10-20 % and have a molecular weight of 45-75 kDa. The last two groups are the D-hordeins and the sulfur-rich γ-hordeins, which make less than 5 % and have a molecular weight of 100 kDa and 35-40 kDa respectively.[27] The B-hordeins are coded for by at least 13 genes, while the C-hordeins are transcribed from 20-30 genes. The γ-hordeins comprise at least 3 isoforms and the D-hordein family is only coded for by a single gene with up to five post-translational modified isomers[26]. The B-hordeins can be further divided into the B1 and B3 subtypes based on their molecular weight and isoelectric points, B1 and B3 are, however the most common ones[26–28]. Further distinction can also be made by separating high-molecular weight (*HMW*) prolamins (D-hordeins) from low-molecular weight (*LMW*) prolamins, including B- and γ-hordeins, although separation into the previously described four groups is more common in barley[26,27]. To be able to comprehensively analyse them, they are often extracted under reducing conditions, as many of them form inter- or intramolecular disulfide bonds and the addition of 1,4-Dithioerythrit (*DTE*), for example, can facilitate their extraction[7,18,29]. In the presence of a reducing agent, glutelin proteins on the other hand, can also be soluble in the alcohol fraction[30].

A summary of the information about hordeins stated in this chapter can be found in **Table 1**.

Table 1: Overview of the four different hordein fractions, belonging to the alcohol-soluble prolamin proteins found in barley and their characteristics like molecular weight and their respective percentages of the total hordeins in barley[6,19,26–28].

Type	***Molecular Weight***	***Percentage of Total Hordeins***	***Characteristics***	***Coding Gene[17]***
B-hordein	30-45 kDa	70-80 %	Sulfur-rich; LMW	*Hor2*
C-hordein	45-75 kDa	10-20 %	Sulfur-poor	*Hor1*
D-hordein	105 kDa	< 5 %	HMW	*Hor3*
γ-hordein	35-40 kDa	< 5 %	Sulfur-rich; LMW	*Hor5*

1.3.3.3 Glutelins

Glutelins are traditionally extracted using a dilute acid or a dilute alkali solution, but recently it is more common to use a detergent or a chaotropic agent like sodium dodecyl sulfate (*SDS*) or urea for their extraction[6]. Due to their poor solubility, drastic conditions are needed for extraction[17]. The glutelin fraction adds up to about 35-45 % of the total protein content of barley[6]. Glutelins contain a high amount of glutamine, proline and glycine and they show good emulsifying properties[17]. It is extremely challenging to purify the glutelin fraction, as they are always contaminated with hordeins. Very few to none of the barley glutelins have been characterized yet. Together with the alcohol-soluble prolamins, they are the major components of the storage protein composite collectively referred to as gluten.[6]

Even though Osborne classification is universally accepted and used in cereal protein studies, a different protein classification system, where proteins are classified by their molecular function, is used in modern proteomics. Based on this system, proteins can be divided into three major groups. The first ones are storage proteins which involves the prolamin super family (B-, C-, D-, γ-hordeins) and storage globulins. The second category includes structural and metabolic proteins like ß-amylases, α-amylases, peroxidases, and lipoxygenases. The third one consists of protective proteins like serpins, barley amylase/subtilisin inhibitor, hordoindolines and hordothionins.[31]

1.4 Significance of the Gluten Structure for Bread Baking

Bread is one of the most popular staple foods all over the world, which rapidly increases satiety after consumption. However, most breads predominantly employ wheat flour, which possesses lower levels of essential nutrients such as vitamins, minerals, and dietary fibre when compared to barley flour. Moreover, wheat flour exhibits higher quantities of rapidly digestible starch, making it less suitable for individuals suffering from diabetes. Recent developments in the food industry reveal a growing preference for bread varieties that offer enhanced nutritional profiles over those produced solely from wheat. In this context, incorporating barley flour in bread production holds promise for delivering bread with improved nutritional value and added health benefits.[13]

Barley has already been successfully incorporated into chemically leavened baked goods like noodles, pancakes, or biscuits. However, its integration into yeast leavened baked goods, such as bread, has faced limited success. The formation of a well-developed gluten network, a crucial factor influencing the processing characteristics of bread dough, was found to be inadequate in barley bread, thus restricting its applications in the food industry.[11,13,32] Consequences of the sparsely formed gluten network in breads purely made from barley flour are collapsing of the bread after cooling, faster firming, lower bread volume and poorer crumb structure than bread made of wheat, which all result in lower sensory qualities[11,32].

1.4.1 Gluten Network in Wheat Dough

In wheat, gluten represents a complex protein mixture consisting of numerous distinct proteins that belong to the prolamin and glutelin protein families. They are also referred to as storage proteins, because they provide carbon, nitrogen, and sulfur to the seed. These proteins are interconnected through both covalent and non-covalent bonds, resulting in the formation of a viscoelastic gel upon the addition of water and sufficient kneading. The resulting structure can maintain a large volume of gas, that gives the resulting product its unique properties like a larger volume, shape, and texture. Gluten is heat stable and it is commonly employed in food processing to enhance texture, flavor, and moisture content. The precise mechanism of the gluten formation process is still unclear.[29,33–37]

Figure 4 provides an illustration of the gluten formation process.

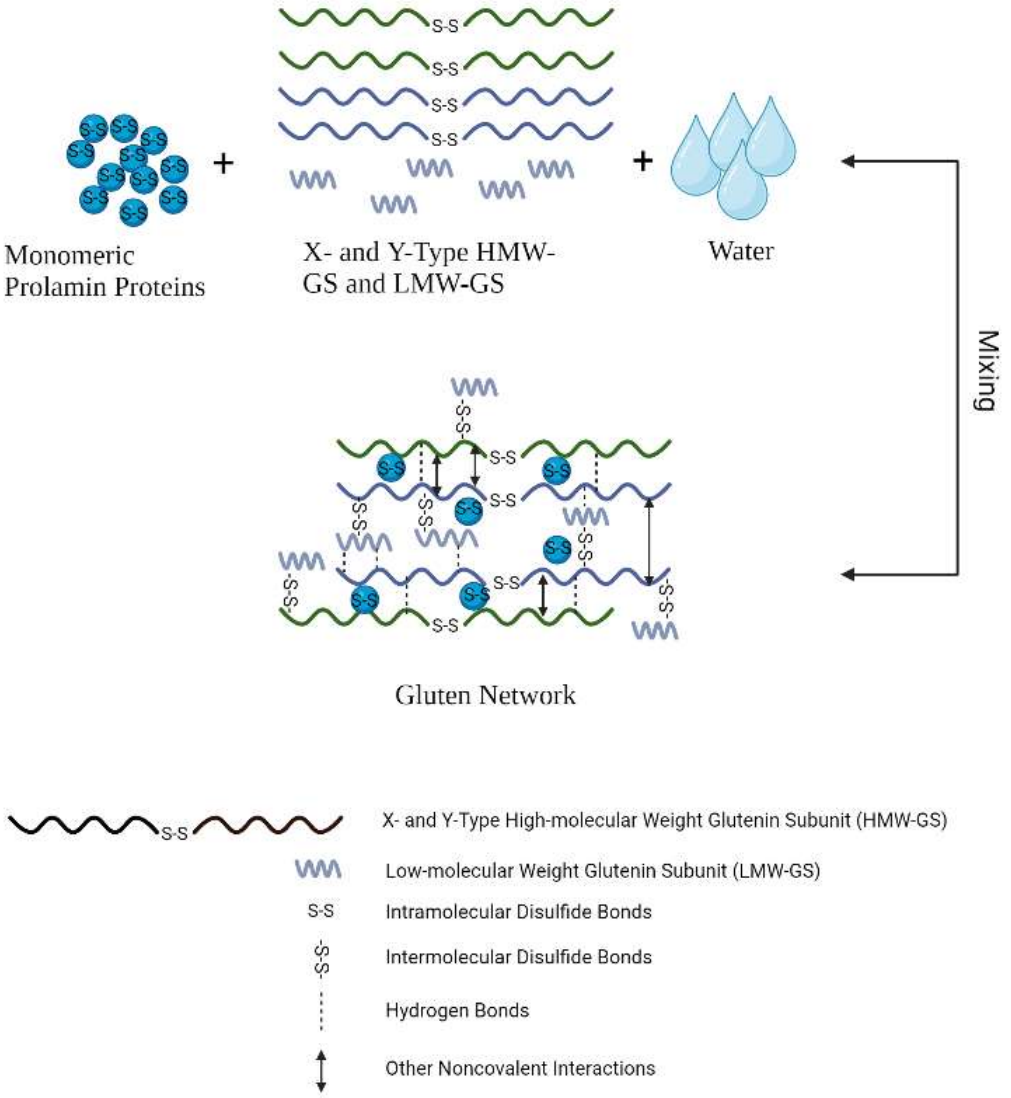

Figure 4: Mechanism behind the formation of the gluten network in wheat dough. By mixing prolamin and glutelin proteins with water and kneading it sufficiently, a gel-like structure is formed due to covalent and non-covalent interactions between the protein fractions, which can hold bigger volumes of gas. Image was created on BioRender.

The key in building the gluten network is the formation of intermolecular disulfide bonds, which are built by cross-linking cysteine amino acid residues. The so-called high- and low-molecular weight glutenin subunits (*HMW-GS* and *LMW-GS*) form the elastic and strong polymer backbone of the wheat gluten structure by connecting two different subunits, x- and y-type HMW-GS and the LMW-GS by disulfide bonds. The x- and y-types are expressed by two different genes and can be distinguished based on their molecular weight and

electrophoretic mobility. The x-types have a molecular weight of about 83-88 kDa, while the y-type has a mass of 67-74 kDa. Moreover, monomeric low-molecular weight proteins, mostly consisting of prolamin proteins, bind to the glutelin backbone without forming disulfide bonds, contributing to the viscosity and extensibility of the dough. Spectroscopic studies proved that noncovalent interactions and hydrogen bonding between the subunits is also important to form the gluten network. Since wheat has a hexaploid genome, there should be 6 different HMW-GS (3 x-types and 3 y-types) in theory, but due to gene silencing, most wheat cultivars only possess 3-5 HMW-GS.[29,33–37]

Mature HMW-GS consist of three structural domains, which are the non-repetitive N-terminal domain, the repetitive central domain, and the C-terminal domain. Most x-type HMW-GS contain four cysteine residues (three in the N-terminal domain and one in the C-terminal), while most y-types contain seven cysteines (five in the N-terminal domain and one each in the other two domains[38]. The structure of these HMW-GS is illustrated in **Figure 5**.

1.4.2 Gluten Network in Barley Dough

In case of barley, the gluten network is mainly composed of hordeins, which belong to the alcohol-soluble prolamin protein category[34]. Research suggests that the high-molecular weight hordein subunit (D-hordein) serves as the structural backbone of the gluten network by forming disulfide bonds with low-molecular weight subunits (B- and γ-hordeins), similar to the HMW-GS in wheat[6,39]. Supporting this notion, D-hordeins can only be extracted at high concentrations of a reducing agent, indicating their role in forming the backbone[6]. A reason for why the gluten network in barley may be less well developed is the fact that the D-hordeins only make less than 5 % of the total hordeins in barley, while the HMW-GS make about 45 % of the total gluten proteins in wheat[29,35]. Additionally, there is only one locus known at the moment (*Hor3*), which encodes the D-hordein gene in contrast to the 3-5 HMW-GS present in wheat[36,37,40]. Furthermore, the D-hordein has one additional central nonrepetitive domain, interrupting the central repetitive domain. It is still unclear which impact this additional domain has on the structure and function of the protein, as well as the baking qualities of barley. In total, D-hordein has more cysteine residues overall than the x- and y-type HMW-GS (five in the N-terminal domain, three in the central nonrepetitive domain and one each in the repetitive and C-terminal domain). The D-hordein is therefore more resembling the y-type HMW-GS in molecular mass (67-74 kDa) and cysteine profile. It is not clear yet, what impact the additional cysteine residues in D-hordein have on its gluten network-forming properties. One possibility is that the cysteines could form intramolecular disulfide bridges, resulting in a weaker dough strength.[40]

In **Figure 5** an illustration for the comparison between x- and y-type HMW-GS and D-hordein can be seen.

Previous studies on the gluten structure in general also showed that high concentrations of plant material like ß-glucans destroys the gluten network by reducing the water availability, which consequently compromises its baking properties[11,32].

Further research is required to comprehensively map and enhance our understanding of the disulfide-driven network of barley flour proteins, particularly concerning its influence on baking properties across various barley cultivars.

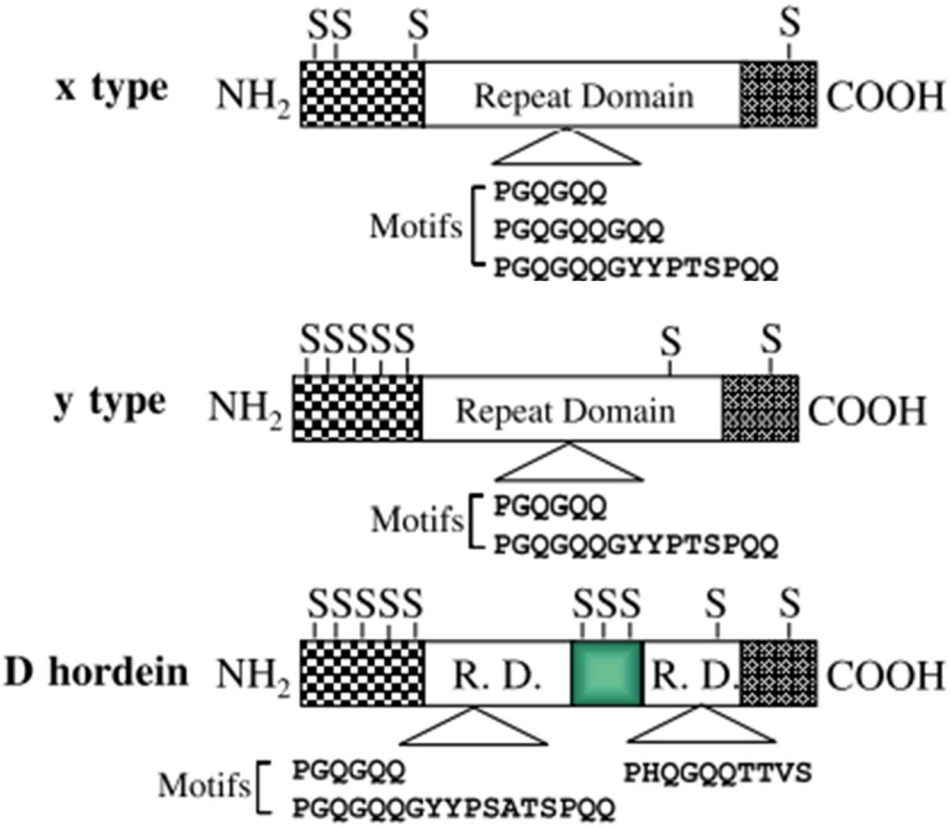

Figure 5: Comparison between the structure of x- and y-type high-molecular weight glutenin subunits in wheat and D-hordein in barley, which are both believed to form the elastic backbone of the gluten structure by covalent binding between cysteine residues. Graphic was presented in *Gu et al., 2003*[1].

1.5 Barley Cultivars

1.5.1 Wild Barley

Wild barley (*Hordeum vulgare* ssp. *spontaneum*) is the progenitor of cultivated barley and is nowadays regarded as a subspecies (*ssp.*) within the same major species (*Hordeum vulgare*). According to archaeological findings, both barley species, wild barley and cultivated barley, were important parts of ancient diets along with Spelt, Emmer and Einkorn[41]. The difference between wild and cultivated barley is morphological and adaptive. Examples for morphological differences are that wild barley is 2-rowed and has a tough and non-brittle awn. Adaptive traits are its well-developed dormancy system and a high drought tolerance.[42] The respective genes for draught resistance and disease resistance in the wild species have been extensively used for gene transfers but hardly any progress has been made to improve nutritional properties[20].

1.5.2 Golden Promise

Golden Promise (*Hordeum vulgare* cultivar *golden promise)* is a 2-row, hulled spring barley cultivar, which is used extensively for malting and whisky production[16]. It shows superior agronomic traits such as a short stature, high salt tolerance and earliness, which made it popular for malting in the first place[43]. The main research interest in Golden Promise at the moment is based on its genetic transformability, which is superior to other cultvars[16]. The Golden Promise variant used in this project was bred so that the starch fraction consists of more than 99 % amylose by reducing the expression levels of certain starch branching enzyme-expressing genes (*SBEIIa, SBEIIb* and *SBEI*) by more than 90 %, which results in a slower digestion of starch in the human intestines and lowers the glycaemic index of meals prepared with this cultivar[44].

1.5.3 PS3

PS3 (*Hordeum vulgare* cultivar *PS3)* is a naked spring barley cultivar, which means that it is lacking the protective hull-layer. Naked barley only makes a very small percentage of the worldwide barley production. One reason for the sparse production of naked barley could be that it is not recommended for malting and is therefore mainly grown for food end-uses, which have a significantly lower demand.[45] The naked barley cultivar used in this project was developed by the organic breeding company Agrologica (Mariager, Denmark). The parental lines of PS3 are called Power and Sec[46].

1.6 Mass Spectrometry

Mass spectrometry is a technique to qualitatively and quantitatively analyse different chemical compounds in a sample. It involves the introduction and ionization of the sample, followed by their separation based on the mass-to-charge ration (*m/z*) using a mass analyser and the detection[47]. Ionization of the analyte is necessary in order to enable its acceleration or deflection through various electric fields. The entire analysis takes place in high vacuum conditions to prevent incidental ion collisions, which could lead to unintended fragmentation or energy dissipation, thereby compromising the accuracy of the analytical results. Ionization can be achieved regardless of the samples aggregate state. Well-known techniques include electrospray ionization (*ESI*) and matrix-assisted laser desorption/ionization (*MALDI*).[48]

Once the sample has been successfully ionized, the ions are separated according to their mass-to-charge ratio, a task which is performed by a mass analyser. This involves the application of variable and/or static magnetic and electric fields. A wide array of mass analysers is currently available, with the most commonly utilized ones being time-of-flight (*ToF*) analysers, orbitrap analysers, quadrupole devices, and ion traps. Hybrid instruments combining multiple analysers can also be employed to achieve enhanced separation capabilities.[49] The benefits of mass spectrometry include its rapidity, the possibility of coupling it with complementary analytical methods such as chromatography, and its inherent selectivity[50].

1.6.1 Matrix-Assisted Laser Desorption/Ionization-Time of Flight

1.6.1.1 Matrix-Assisted Laser Desorption/Ionization

Matrix-assisted laser desorption/ionization involves the ionization of analyte molecules from a solid phase, achieved by embedding the sample in a matrix material that possesses laser-absorbing properties[51]. Typically, the analyte-to-matrix ratio ranges from 1:10,000 to 1:1,000. Evaporation of the solvent leads to co-crystallization of the analyte and the matrix[52]. A laser beam is then directed onto the dried droplet and the laser light is absorbed by the solid phase, resulting in the ionization of all solid-phase molecules[53]. Rather than relying on primary ions as generated during chemical or electron ionization, MALDI utilizes photons to ionize the analyte molecules[52]. MALDI-MS is considered a soft ionization technique, as it induces minimal fragmentation of the compounds[54]. On the other hand, direct exposure of the sample to laser light may trigger photolytic decomposition[52]. Laser-absorbing matrices are predominantly composed of small organic molecules with chromophoric properties, such as aromatic rings[54]. Examples include α-cyano-4-hydroxycinnamic acid (*HCCA*) and cobalt powder embedded in glycerin for enhanced UV light absorbance[52,51]. The selection of an

appropriate matrix largely depends on the sample characteristics and analysis purposes[52]. MALDI is particularly well-suited for the analysis of larger molecules, such as proteins, peptides, and polymers[55,56]. However, prior to analysis, it is necessary to purify the sample in order to eliminate interfering substances like salts and detergents, which complicate accurate measurements. This purification step also makes it possible to concentrate the samples. One purification approach involves using pipette tips containing a stationary phase capable of selectively binding to the analytes, thus enabling their separation from interfering components. However, this technique is restricted to the purification of small sample volumes.[57] Overall, the advantages of MALDI-MS include the ability to analyse larger biological molecules and a wide range of compounds. It yields well-defined spectra due to the generation of fewer multiply charged molecules and supports high sample throughput[54,52]. The mass range covered by MALDI-MS ranges from 10 Da to 500 kDa. Drawbacks include the challenge of identifying a suitable matrix for specific analytes. MALDI-MS can be coupled with various mass analysers, such as the time-of-flight mass analyser. **Figure 6** provides a visual representation of the operating principles of MALDI-MS.[54]

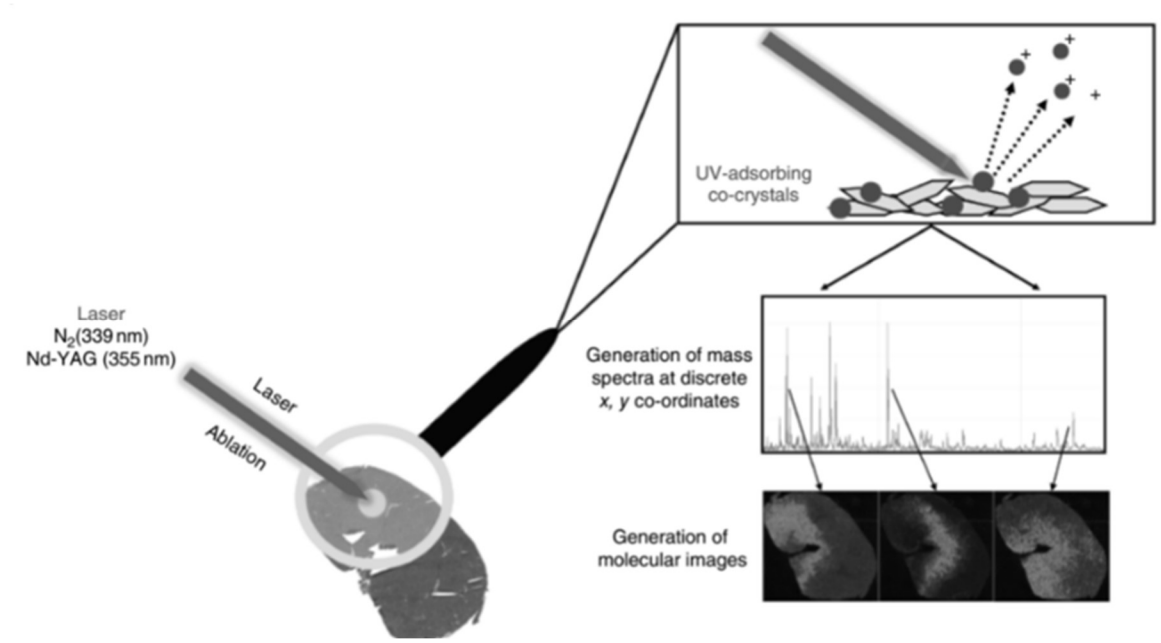

Figure 6: Operating principle of MALDI-MS. The sample is co-crystallized with a laser-absorbing matrix. Afterwards, a laser is directed onto the crystals and all solid components of the sample spot are ionized by absorbing the laser energy[54].

1.6.1.2 Time of Flight

The time of flight mass analyser separates the generated sample ions by their mass-to-charge ratio. It consists of two separate sections. The first one is called the source, which involves a

compact region, where a high-intensity electric field is applied. The second section, referred to as the drift region, extends over a considerably longer distance, where no electric field is applied. **Figure 7** shows the configuration of a linear time of flight mass analyser. The electric field within the source region is defined as $E = U_b * q$, where U_b represents the acceleration voltage and q stands for the ion charge. Before entering the time-of-flight mass analyser, the ions are accelerated within the electric field, acquiring a kinetic energy of $E = \frac{1}{2} m * v^2$, where m represents the ion mass and v represents the velocity of the ion. By equating these two equations, the expression $v = \sqrt{\frac{2*q*U_b}{m}}$ is derived. This means that the flight time of an ion is proportional to the square root of the mass-to-charge ratio $\sqrt{\frac{m}{q}}$. As a result, the measurement of flight time allows the determination of the ion mass.[58] The ability to simultaneously measure multiple masses without the need to filter or separate them beforehand contributes to the exceptional speed of analysis, which is within the scope of a microsecond. Therefore, the use of fast detectors is needed[52,59]. Moreover, the time of flight mass analyser demonstrates excellent suitability for high-mass analysis, enabling the determination of protein masses and the characterization of post-translational modifications[60].

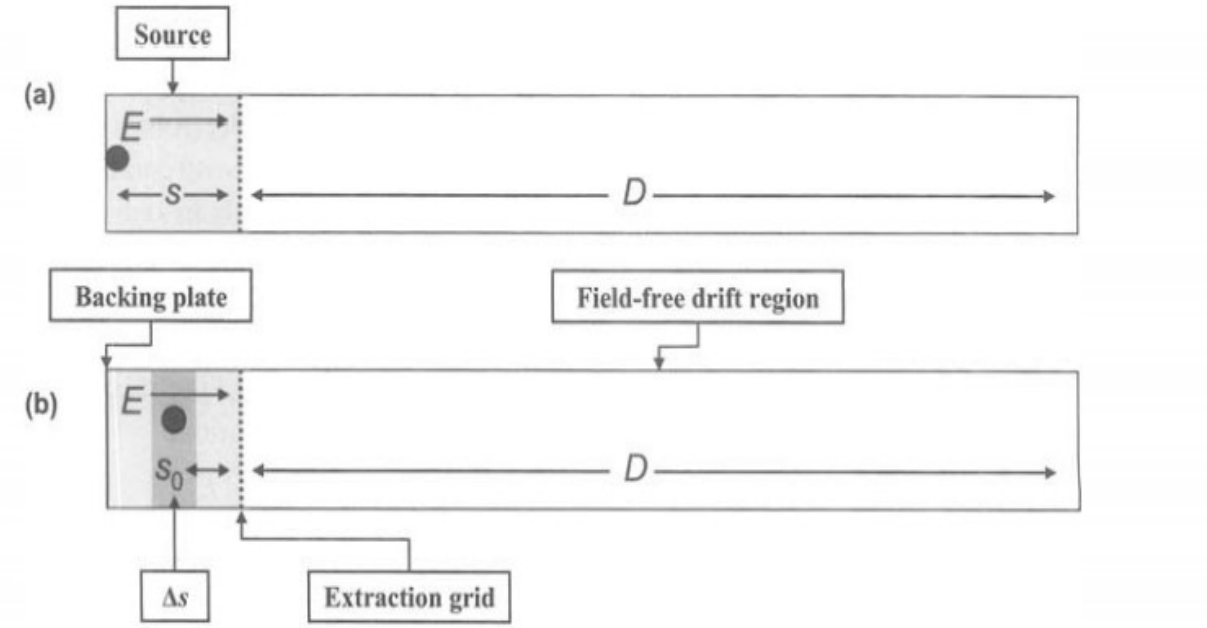

Figure 7: Setup of a linear time of flight mass analyser. In the source, the ions are focused into a compact bundle and given a specific level of kinetic energy. Then, they are separated in the field-free drift region based on their respective mass-to-charge ratio and analysed by measuring the time needed to pass the flight tube[60].

However, a challenge is that ions formed at the beginning of the source tend to spend a slightly longer time within the electric field, resulting in higher kinetic energy compared to ions generated at the end of the source. This discrepancy introduces inaccuracies and contributes to the relatively modest resolution observed in linear time of flight mass analysers[52,60]. To address this limitation, the reflector-time of flight *(RToF*) concept was introduced.

A reflector is an arrangement of multiple electrodes, where each has a distinct voltage level, which generates an electric field characterized by varying strengths, also referred to as a gradient field. In this configuration, ions that reach the end of the drift field experience deceleration, followed by acceleration towards the detector in the opposite direction. Ions with identical mass but a higher kinetic energy have a longer flight time within this field before their trajectory is reversed. This mechanism effectively smooths out the discrepancies in kinetic energy, ensuring that ions with the same mass arrive at the detector simultaneously.[60] The RToF design exceeds the resolution of linear ToF instruments by more than three-fold[52]. **Figure 8** shows the setup of a RToF[60].

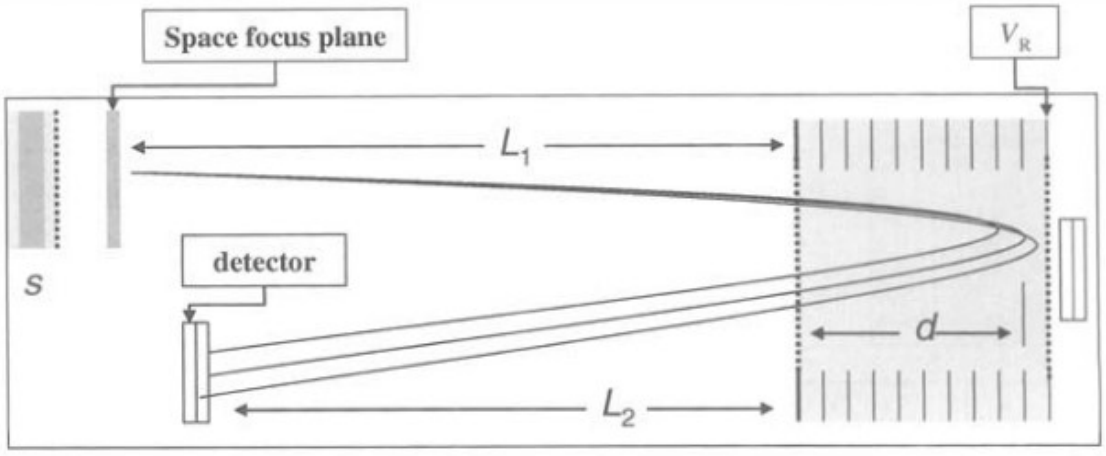

Figure 8: Setup of a reflector-time of flight. In comparison to a linear time of flight analyser, the flight tube is curved to enhance separation of the generated ions by smoothing out discrepancies in their kinetic energy[60].

1.7 Gel Electrophoresis

To ensure precise analytical measurements of proteins, it is necessary to reduce the complexity of the biological sample before analysis. Gel electrophoresis is a suitable technique for protein separation, where negatively charged proteins are separated based on their migration in a gel matrix. The electrophoretic mobility of a protein relies on different factors such as its molecular weight, surface charge (which is pH-dependent), size, and shape. The separation process takes place in a polyacrylamide gel that is made by polymerizing acrylamide monomers with the help of a crosslinking agent. Typically, a discontinuous gel system is used as it increases protein separation. Among various gel systems, the Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (*SDS-PAGE*) system is the most used. It consists of a stacking gel and a resolving gel. The stacking gel, which has a lower pH and a larger pore size compared to the resolving gel, facilitates the concentration of proteins into a thin line before they enter the resolving gel, where they are separated based on their molecular weight. The Laemmli electrophoresis buffer commonly contains a tris(hydroxymethyl)aminomethane - hydrochloride (*tris-HCl*) buffer to stabilize the pH, SDS to denature and unfold protein structures, and a reducing agent that disrupts disulfide bonds. Additionally, a dye like Bromophenol Blue is added to visualize the migration progress.[61]

After separation is completed, the proteins are fixed onto the gel and visualized by staining them. One way to do that is to use Coomassie Brilliant Blue, which is a disulfonated triphenylmethane dye that gives the protein bands a blue colour by interacting with specific amino acids. Other stain techniques that are commonly used are silver staining or staining with Nile red[62].

For enhanced separation capabilities, 2-Dimensional Gel Electrophoresis (*2D-GE*) can be used. In 2D-GE, proteins are separated in two dimensions. The first dimension is based on their isoelectric point (*pI*), which is the pH at which the protein carries a neutral charge. This is achieved by an immobilized pH gradient. The second dimension is based on their molecular weight, applying the same principle as for 1D-GE. By incorporating a second dimension, the resolving power is increased, thereby facilitating the detection of low-abundance proteins.[61]

2 Aim and Hypothesis

Based on the current state of knowledge, as summarized in the introduction, the aim of this master thesis is to develop a method for protein profiling of three barley cultivars (PS3, Golden Promise and Wild Barley). As the protein extraction is a vital step for comprehensive proteome analysis, a suitable extraction method should be found. To simultaneously reduce the complexity of the sample, the extraction is done by applying Osborne fractionation. It is postulated that the grain size of the barley flour has an influence on the extraction efficiency and should therefore be examined.

Furthermore, it is hypothesized that a comparison of the protein profile under reducing and non-reducing conditions on 1D- and 2D-GE will reveal proteins influenced by disulfide bonding. These proteins from the 1D/2D-GE are subjected to trypsin digestion and analysed with MALDI-ToF MS. Further, it should be investigated if these proteins are expected to be involved in the barley gluten network. Moreover, it is postulated that the protein profile of the three different cultivars will differ from each other. The development of protein extraction and fractionation techniques for barley, followed by the characterization of their functional properties, may expand the range of opportunities for utilizing barley protein fractions in both food and non-food applications[18].

3 Materials and Methods

3.1 Materials

In **Table 2**, a list of chemicals, that were used for experimental analysis can be found.

Table 2: List of chemicals for experimental uses. All reagents were of analytical grade. The reference number signifies the product number of the chemical on the provider's website as of July 2023.

Chemical	***Company***	***Purity***	***Reference Number***
1,4-Dithioerythritol	Sigma Aldrich, Austria	≥ 99.0 %	D8255-25G
2-Propanol	Honeywell Riedel-de Haën™, Germany	≥ 99.9 %	603-117-00-0
Acetic Acid	Sigma Aldrich, Austria	≥ 99.0 %	A6283-1L
Acetonitrile	Sigma Aldrich, Austria	≥ 99.9 %	34851
a-Cyano-4-hydroxy-cinnamic Acid	Sigma Aldrich, Austria	500	C8982
Agarose	Sigma Aldrich, Austria	-	R0801
Aluminium Sulfate ~ 14 Hydrate	VWR, USA	≥ 99.9 %	21070.297
Amberlite IRN 150L	Thermo Scientific, USA	-	11375327
Ammoniumhydrogen Carbonate	Sigma Aldrich, Austria	≥ 99.9 %	5.33005
Bovine Serum Albumin	Sigma Aldrich, Austria	≥ 99.0 %	232-936-2
Bromophenol Blue	Merck, Germany	≥ 95.0 %	204-086-2
CHAPS	Sigma Aldrich, Austria	≥ 98.0 %	J67359.14
Coomassie Brilliant Blue G250	Sigma Aldrich, Austria	100	1.12553
Dipotassium Hydrogen Phosphate	Merck, Germany	≥ 99.5 %	A141701845
Ethanol	VWR, USA	96%	20824.365
Glycerol	Merck, Germany	≥ 99.0 %	1.04057.2511
Iodoacetamide	Sigma Aldrich, Austria	≥ 99.0 %	I1149
Ortho-Phosphoric Acid	Merck, Germany	≥ 85.0 %	PX0996
Paraffin Oil	Sigma Aldrich, Austria	-	18512
Peptide Calibration Standard	Bruker Daltonics, Germany	-	8206195
Pharmalyte 3-10	Thermo Scientific, USA	-	10575545
POROS™ 50 R1 and R2 Perfusion Chromatography™ Bulk Media	Thermo Scientific, USA	-	8-0014-40-0993
Potassium Dihydrogen Phosphate	Merck, Germany	≥ 99.5 %	1.04873.1000
Potassium Hydroxide	Merck, Germany	≥ 85.0 %	1.05033.5000
Protein Assay Dye Reagent Concentrate	Bio Rad, USA	-	#5000006
Sodium Chloride	Honeywell Fluka™, Germany	≥ 99.0 %	L1160

Chemical	*Company*	*Purity*	*Reference Number*
Sodium Dodecyl Sulfate	Sigma Aldrich, Austria	≥ 98.5 %	L3771-1KG
Sodium Hydroxide	Sigma Aldrich, Austria	≥ 98.0 %	1.003E+09
Sodium Tetraborate Decahydrate	Sigma Aldrich, Austria	≥ 99.5 %	B3545-500G
Spectra Multicolor Broad Range Protein Ladder	Thermo Scientific, USA	-	26634
Superabsorber	Gerhardt, Germany	≥ 99.5 %	14-0295
Thiourea	Thermo Scientific, USA	≥ 99.5 %	140750050
Trifluoroacetic Acid	Sigma Aldrich, Austria	≥ 99.0 %	T6508
Tris(hydroxymethyl)aminomethan	Merck, Germany	≥ 95.0 %	1.08382.1000
Trypsin	Promega, Sweden	Sequencing Grade	07.07.9002
Urea	Thermo Fisher Scientific, Austria	≥ 99.0 %	XJ3593872

In **Table 3**, a list of all instruments or consumables, that were used can be found.

Table 3: List of instruments for analysis and consumables for experimental use. The model signifies the product name of the instrument or consumable as stated on the provider's website as of July 2023.

Instrument or Consumables	*Company*	*Model*
1 mL Disposable Syringe	Braun, Germany	Omnifix 9161406V
2D-GE Gels	Bio-Rad, USA	Any kD™ Criterion™ TGX Stain-Free™ Protein Gel, 11 cm IPG/prep+1 well
Centrifuge	Eppendorf, Germany	5417 R
Criterion Cell	Bio-Rad, USA	Criterion™ Cell
Disposable Syringe Filter Units with Pore Size 0.22 µm	Advantec MFS, USA	13CP020AN
Disposable Syringe Filter Units with Pore Size 1.2 µm	Sartorius, Germany	Minisart® Syringe Filter SFCA
Dumas Nitrogen Analyser	Gerhardt, Germany	Dumatherm
Dumas Weighing Boat	Gerhardt, Germany	DumaFoil 3.5 x 3.5 cm
Lyophiliser	Christ, Germany	Delta 2-24 LSCplus
GeLoader Pipette Tips	Eppendorf, Germany	GeLoader tips Eppendorf, 0030 001.222
Heater Block	Antylia Scientific, USA	Cole-Parmer™ Stuart™ Block Heater
IEF-Cell	Bio-Rad, USA	Protean IEF-Cell
Low-binding Eppendorf tubes	Eppendorf, Germany	Protein LoBind Eppendorf 0030 108.094 and 0030 108.116
MALDI Target Plate	Bruker Daltonics, Germany	MTP AnchorChipTM 600/384 T F
Milling System	KoMo, Austria	Fidibus 21
Milli-Q Direct Water Purification System	Merck, Germany	-
Molecular Imager	Bio-Rad, USA	ChemiDocTM XRS+ with Image LabTM Software
Molecularporous Membrane Tubing for Dialysis	Fisher Scientific, UK	Standard RC Tubing, MWCO 6-8kDa
Nanodrop	Thermo Fisher Scientific, Austria	Nanodrop One
Needle	Becton Dickinson GmbH, Germany	23G BD 300800
Power Pac	Bio-Rad, USA	Power Pac 200

Instrument or Consumables	*Company*	*Model*
IPG-Strips	Bio-Rad, USA	ReadyStrip™ IPG Strips, 11 cm, pH 3-10
Scalpel	Swann-Morton, UK	Stainless Steel Surgical Scalpels
1D-GE Gels	Bio-Rad, USA	Criterion™ TGX™ Precast Gels, 12+2 Wells, AnykDa
Sieving System	Retsch, Germany	AS 200 Control
Spectrophotometer	BioTek Instruments, USA	Synergy 2
SpeedVac Vacuum Concentrator	Marshall Scientific, USA	Genevac EZ-2 Plus Evaporating System
Thermo Shaker	BioSan A/S, Denmark	TS-100

3.2 Methods

3.2.1 Sample Preparation

Samples of barley cultivars PS3, Golden Promise and Wild Barley were received from the Department of Agroecology at Aarhus University, Denmark. These samples were cultivated in field plots during the summer of 2021 at Aarhus University (Flakkebjerg, Denmark). The grains of Golden Promise were milled using a Fidibus 21 mill (KoMo, Austria) and sieved (Retsch, Germany) into five different grain sizes (500 µm, 250 µm, 125 µm, 63 µm and 45 µm). The PS3 cultivar, which was already received in the form of flour, underwent the same sieving process. In addition to the sieving, analysis was done on the unsieved flour for both cultivars. No sieving was done for the Wild Barley flour, as the sample amount received was too little for sieving. The grain size of the commercially bought wheat flour (Manitoba wheat flour, HavneMøllen, Vejle, Denmark) was already below 45 µm, so no sieving was done on these samples either.

3.2.2 Protein Extraction from Barley Flour

3.2.2.1 Osborne Fractionation

To determine an appropriate method for protein extraction from barley flour, two Osborne fractionation protocols, as described by *Wang et al., 2007*, and *Celus et al., 2006,* were applied with slight modifications[6,18]. The objective was to compare the extraction efficiency and quantity of proteins obtained from each of the four fractions. Both protocols were applied to 250 mg of unsieved and 45 µm grain size barley PS3 cultivar flour and wheat in duplicates.

3.2.2.1.1 Osborne Fractionation as described in *Wang et al., 2007*

In the extraction protocol based on *Wang et al., 2007*, water-soluble albumins were extracted twice at room temperature (*RT*) for 30 minutes each using 1.25 mL of Milli-Q water (Merck, Germany). After addition of the extraction reagent, samples were vortexed (Velp Scientifica, Italy) and put on a thermo shaker (BioSan, Denmark) at 1200 rpm. Afterwards, samples were centrifuged (Eppendorf, Germany) at 2000 g for 5 minutes at room temperature and supernatant was collected. Pellet was redissolved in the extraction agent and the same procedure was repeated. Supernatants from each fraction were combined. For the extraction of salt-soluble globulins, samples were extracted twice at room temperature for 30 minutes each using 1.25 mL of 0.15 M potassium phosphate buffer, pH 8. The same procedure as described for the extraction of the albumins was followed and supernatants were combined. Alcohol-soluble prolamins were extracted three times at 60°C for 30 minutes each using 1.45 mL of 55 % 2-propanol (Honeywell, Riedel-de Haën™, Germany), 12.5 µL of 1 % acetic acid (Sigma

Aldrich, Austria) and 12.5 µL of 1 M DTE (Sigma Aldrich, Austria*)*. In contrast to *Wang et al., 2007*, DTE was chosen as the reducing agent instead of β-Mercaptoethanol (*β-ME*), due to its similarity to Dithiothreitol (*DTT*), which is a stronger reducing agent than β-ME but less toxic[63,64]. The same procedure as described for the extraction of the albumins was followed and supernatants were combined. Alkali or acid-soluble glutelins were extracted three times at room temperature for 30 minutes each using 1.45 mL of a 0.05 M sodium borate buffer, pH 10, 12.5 µL of 1 M DTE and 12.5 µL of 1 % SDS (Sigma Aldrich, Austria). In contrast to *Wang et al., 2007*, DTE was used as the reducing agent instead of β-ME. The same procedure as for the extraction of the albumins was followed and supernatants were combined. An overview of the procedure can be found in **Figure 9**[18].

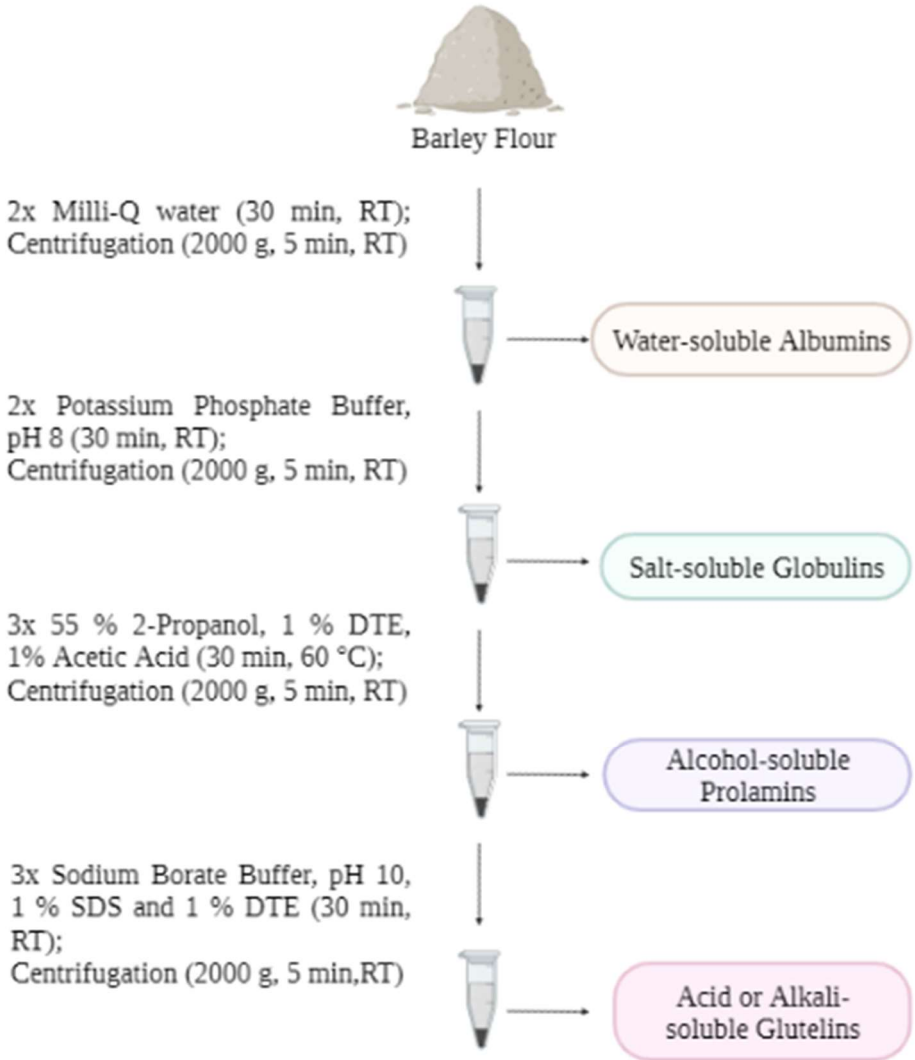

Figure 9: Overview of Osborne fractionation protocol of barley proteins as proposed in *Wang et al., 2007*, with slight alterations[18]. Instead of β-ME, DTE was used as the reducing agent. Image was created on BioRender.

3.2.2.1.2 Osborne Fractionation as described in *Celus et al., 2006*

For the extraction protocol based on *Celus et al., 2006*, the water-soluble albumins were extracted twice at room temperature for 10 minutes each using 1.5 mL of Milli-Q water (Merck, Germany). After addition of extraction reagent, samples were vortexed (Velp Scientifica, Italy) and put on a thermo shaker (Biosan, Latvia) at 1200 rpm. Subsequently, they were centrifuged (Eppendorf, Germany) at 2000 g for 10 minutes at room temperature and supernatant was collected. Pellet was redissolved in the extraction agent and the same procedure was repeated. Supernatants from each fraction were combined. For the extraction of the salt-soluble globulins, samples were extracted twice at room temperature for 10 minutes each using 1.5 mL of 5 % sodium chloride (Honeywell Fluka™, Germany). The same procedure as for the extraction of the albumins was followed and the supernatants were combined. Alcohol-soluble prolamins were extracted three times at 60 °C for 10 minutes each using 1.49 mL of 55 % 2-propanol (Honeywell, Riedel-de Haën™, Germany), and 15 µL of 1 M dithioerythritol (Sigma Aldrich, Austria). In contrast to *Celus et al., 2006*, DTE was used as the reducing agent instead of DTT. Furthermore, 2-propanol was used instead of 1-propanol, based on the more frequent usage of 2-propanol in existing literature[27,65–67]. The same procedure as for the extraction of the albumins was followed and supernatants were combined. Alkali or acid-soluble glutelins were extracted three times at room temperature for 10 minutes each using 1.45 mL of 6 M urea solution, 15 µL of 1 M DTE and 30 µL of 1 % SDS (Sigma Aldrich, Austria). In contrast to *Celus et al., 2006*, DTE was used as the reducing agent instead of DTT, as it has a similar strength, but is less toxic[63,64]. The same procedure as for the extraction of the albumins was followed and supernatants were combined.

An overview of the Osborne fractionation procedure as described in *Celus et al., 2006* can be found in **Figure 10**[6].

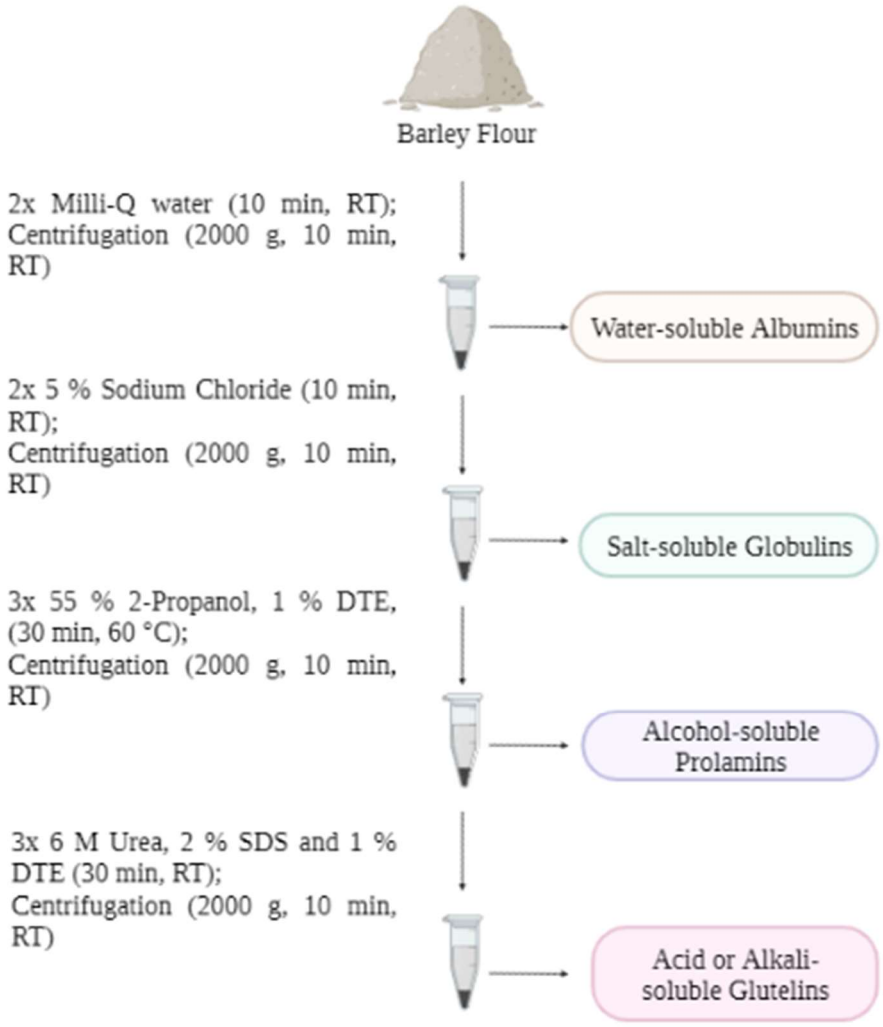

Figure 10: Overview of Osborne fractionation protocol of barley proteins as proposed in *Celus et al., 2006*, with slight alterations[6]. Instead of β-ME, DTE was used as the reducing agent and 2-propanol was used instead of 1-propanol for prolamin extraction. Image was created on BioRender.

3.2.2.1.3 Adaption of Osborne Fractionation extraction protocol

Several articles have reported a high extraction efficiency when using 55 % ethanol for the extraction of the prolamin fraction[7,21,67,68]. Therefore, the extraction protocol described by *Wang et al., 2007* was applied to the unsieved and 45 µm PS3 barley cultivar flour and wheat (each in duplicates) as mentioned in **3.2.2.1.1**. However, the 55 % 2-propanol was substituted with 55 % ethanol. Furthermore, the extraction protocol, in both alterations was applied to the same set of samples to compare reducing versus non-reducing conditions. For the reducing conditions, the extraction protocol was applied as stated in chapter **3.2**. On the other hand, for

the non-reducing conditions, the extraction protocol was applied as stated in chapter **3.2**, without using 1 mol*L^{-1} DTE. An overview of the different versions of the extraction protocol of *Wang et al., 2007* that were applied can be seen in **Figure 11**.

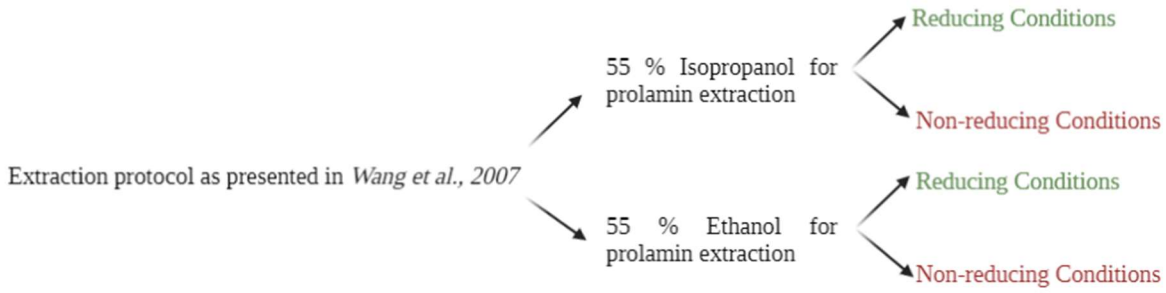

Figure 11: Overview of the alterations carried out for the extraction of proteins from barley flour using the protocol as proposed in *Wang et al., 2007,* to enable comparison between reducing and non-reducing conditions. Due to frequent use in literature, comparison between 55 % 2-propanol and 55 % ethanol, regarding their prolamin extraction efficiency was done as well[7,21,67,68].

3.2.2.1.4 Osborne Fractionation on flour with different grain sizes

The extraction protocol described in *Wang et al., 2007*, using 55 % 2-propanol and under reducing conditions, was applied to different grain sizes of barley flour from the PS3 cultivar. Extraction procedure was conducted on the unsieved barley flour as well as on grain sizes of 500 µm, 250 µm, 125 µm, 63 µm and 45 µm. The grain size that resulted in the highest protein yield was used for further experiments.

3.2.3 Dialysis and Lyophilization

Following the extraction process, the samples were subjected to additional sample preparation steps based on the method suggested by *Idowu et al., 2021*, for protein analysis of sesame proteins[69]. Samples were centrifuged at 6500 g for 30 minutes at room temperature (Eppendorf, Germany) and supernatant of the albumin, globulin and prolamin fraction was filtered through a disposable syringe filter with a pore size of 0.22 µm (Advantec MFS, USA). Glutelin fraction was filtered through a disposable syringe filter with a pore size of 1.2 µm (Sartorius, Germany), due to a higher viscosity. Protein concentration in each fraction was measured using the Bradford Assay. Afterwards, globulin and prolamin fractions were dialyzed against 20 volumes of Milli-Q water using a 6-8 kDa molecular weight cut off membrane (Fisher Scientific, UK) for two days at 4°C, with three daily changes of dialysate to remove the remaining salt from the previous extraction. Due to precipitation, prolamin fraction was afterwards dialyzed against

20 volumes of 50 % ethanol for one day at 4°C, with three changes of dialysate to dissolve the proteins again. All four fractions were lyophilized (Christ, Germany) for three days to remove the liquid. Albumin, globulin and glutelin fraction were resuspended in Milli-Q water, while prolamin fraction was resuspended in Laemmli sample buffer (20 mM tris, 2 % SDS, 20 % glycerol, Bromophenol Blue) to reach a final concentration of 2 mg*mL^{-1} for 1D- and 2D-GE.

3.2.4 Quantification of Protein Content

The protein concentration in the extracts of barley flour in different grain sizes and the unsieved flour was measured using three different methods to identify the most suitable one. The three methods used were the Bradford Assay, the Dumas method and the Nanodrop from Thermo Fisher Scientific, Austria. The samples were diluted to a suitable concentration for the Bradford Assay. This was not possible for the Dumas method, since the values for the diluted samples were below the linear range of the instrument. Therefore, undiluted samples were measured with the Dumas method and the Nanodrop, since no dilution is needed for this photometric measurement. Based on the results obtained from these three methods, the most suitable method for protein content determination was selected.

3.2.4.1 Bradford Assay

For determination of the protein concentration in the barley flour extracts via the Bradford Assay, the samples were diluted 1:5 (albumin and globulin fraction), 1:10 (prolamin fraction) and 1:2 (glutelin-fraction) with Milli-Q water. Blanks of the respective extraction solutions were prepared by diluting it 1:5, 1:10 and 1:2 with Milli-Q water. Starting from a 2 mg*mL^{-1} bovine serum albumin (*BSA*) standard solution (Sigma Aldrich, Austria) in Milli-Q water, 6 calibration standards (0.025 – 0.4 mg*mL^{-1} BSA) were prepared. The concentrated Bradford dye (Bio-Rad, USA) was diluted 1:5 with Milli-Q water. In a 96-well plate, 200 µL of diluted dye were mixed with 10 µL of diluted sample. Samples were incubated for 15 minutes at room temperature and absorbance was measured with a spectrophotometer (Biotek, USA) without lid at 595 nm. Calibration curves with a coefficient of correlation of > 0.98 were used for data analysis. All samples, blanks and calibration standards were measured in biological and technical duplicates.

3.2.4.2 Dumas Nitrogen Analysis

To determine the protein concentration in the barley flour extracts and the solid barley flour samples using the Dumas method, the following steps, based on former work of *Schmidt et al., 2019* were performed[70]. First, two standard samples were prepared. Therefore, a tinfoil

weighing boat (Gerhardt, Germany) was filled with 30 mg of superabsorber (Gerhardt, Germany). On top, 60 µL of 0.5 % tris(hydroxymethyl)aminomethane (*THAM*, Merck, Germany) were added and the weight of the sample was noted. For the preparation of liquid samples, a tinfoil weighing boat was filled with 60 mg of superabsorber (Gerhardt, Germany) and 150 µL of liquid sample were added and the weight of the sample was noted. For the preparation of solid samples, 30 mg of solid sample were added to the tinfoil weighing boat and the weight was noted. Tinfoil boats were folded and stored in a desiccator until measurement. Samples were measured in the Dumatherm (Gerhardt, Germany) and a protein factor of 5.83 was used to calculate the protein content as suggested in *Jones, D.B., 1941*[71]. Samples were measured in biological and technical duplicates.

3.2.4.3 Photometric Measurement using the Nanodrop

For determination of the protein concentration in the barley flour extracts using the Nanodrop (Thermo Fisher Scientific, Austria), a general reference setting based on a 1 mg*mL^{-1} protein solution producing an absorbance at 280 nm of 1.0 with a pathlength is 1 cm was used. Baseline correction at 340 nm was enabled. Blank samples were measured first to determine the background absorbance. To measure the samples, 2 µL of sample were transferred onto the pedestal. Every sample was measured in technical duplicates.

3.2.5 Gel Electrophoresis

3.2.5.1 One-Dimensional Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

One-dimensional sodium dodecyl sulfate – polyacrylamide gel electrophoresis (*1D-GE*) was done based on former work from *Zhao et al., 2017* and *Poulsen et al., 2022*[72,73]. In both, reducing and non-reducing conditions, 15 µL of sample were mixed with 15 µL of Laemmli sample buffer (20 mM tris(hydroxymethyl)aminomethane, 2 % SDS, 20 % glycerol, bromophenol blue, 20 mM DTE for reducing conditions. The samples were vortexed and placed in a heating block (Antylia Scientifix, USA) at 90 °C for 2 minutes and centrifuged at 10,000 rcf (Eppendorf, Germany) for one minute. Additionally, 10 µL of sample and 5 µL of protein ladder (Thermo Scientific, USA) were loaded onto a Criterion™ TGX™ precast gel (Any kDa) (Bio-Rad, USA). Gels were run at 200 V for about 40 minutes in a Criterion™ Cell (Bio-Rad, USA) with a Power Pac 200 (Bio-Rad, USA). Proteins were fixed onto the gel for at least two hours under constant shaking with a fixing solution containing 50 % ethanol (VWR, USA) and 8 % o-phosphoric acid (Merck, Germany) in Milli-Q water. Gels were stained with a Coomassie Brilliant Blue staining solution (5 % (w/v) aluminium sulfate (VWR, USA), 88 % (v/v) Milli-Q water, 2 % (v/v) o-phosphoric acid (85 %) (Merck, Germany), 0.4 % (w/v)

Coomassie Brilliant Blue dye (Sigma Aldrich, Austria), 10 (v/v) % ethanol (VWR, USA)) for 45 minutes under constant shaking and washed with 50 % ethanol and Milli-Q water. Pictures were taken using the Molecular Imager (Bio-Rad, USA). Gels were stored in Milli-Q water at 4 °C until further use.

3.2.5.2 Two-Dimensional Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

Two-dimensional sodium dodecyl sulfate – polyacrylamide gel electrophoresis (*2D-GE*) was done based on former work described in *Larsen, L.B. et al., 2010* and *Poulsen et al., 2022* by preparing the lysis buffer (7 M urea, 2 M thiourea (Thermo Scientific, USA), 1 % DTE and 40 mM tris(hydroxymethyl)aminomethane at pH 7.5) and mixing it with 1 % (w/v) amberlite (Thermo Scientific, USA), before filtering it[73,74]. Then, 2 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sigma Aldrich, Austria) and 2 % (v/v) pharmalyte 3-10 (Thermo Scientific, USA) was added. Then, 50 µL of sample were mixed with 200 µL of lysis buffer and put on a shaker for one hour at room temperature. Samples were centrifuged at 11,700 rcf for 10 minutes at 4 °C. In the IEF-Cell (Bio-Rad, USA), 200 µL of sample were pipetted in and IPG strips (11 cm, pH 3-10; Bio-Rad, USA) were put in with the gel-side facing downwards. Approximately 1 mL of paraffin oil was pipetted on top. Isoelectric focussing was performed for 24 hours. Afterwards, gel strips were washed with Milli-Q water and mixed with 5 mL of equilibration buffer I (6 M urea, 30 % glycerol, 2 % SDS, 0.05 % tris-HCl, 65 mM DTE) for 15 minutes at room temperature. Gels were washed with Milli-Q water and mixed with 5 mL of equilibration buffer II (6 M urea, 30 % glycerol, 2 % SDS, 0.05 % tris-HCl, 270 mM iodoacetamide) for 15 minutes at room temperature. Strips were rinsed with running buffer (25 mM tris(hydroxymethyl)aminomethan, 0.2 M glycine, 1 % SDS) and placed into the polyacrylamide gel (Any kD™ Criterion™ TGX Stain-Free™ Protein Gel, 11 cm IPG/prep+1 well, Bio-Rad, USA). Strips were sealed with 0.5 % agarose (Sigma Aldrich, Austria) in running buffer with bromophenol blue and 5 µL of protein ladder (Thermo Scientific, USA) was loaded. Gels were run at 200 V for about 40 minutes in a Criterion™ Cell (Bio-Rad, USA) with a Power Pac 200 (Bio-Rad, USA). Proteins were fixed onto the gel for at least two hours under constant shaking with a fixing solution containing 50 % ethanol (VWR, USA) and 8 % o-phosphoric acid (Merck, Germany) in Milli-Q water. Gels were stained with Coomassie Brilliant Blue (5 % (w/v) aluminium sulfate (VWR, USA), 88 % (v/v) Milli-Q water, 2 % (v/v) o-phosphoric acid (85 %) (Merck, Germany), 0.4 % (w/v) Coomassie Brilliant Blue dye (Sigma Aldrich, Austria), 10 % (v/v) ethanol (VWR, USA)) for 45 minutes under constant shaking and washed with

50 % ethanol and Milli-Q water. Pictures were taken with the Molecular Imager (Bio-Rad, USA). Gels were stored in Milli-Q water at 4 °C until further use.

3.2.6 Matrix-assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry

The following MALDI-ToF sample preparation method is based on *Larsen, L.B. et al., 2010* and *Poulsen et al., 2022*[73,74]. Protein spots from 1D- and 2D-GE were cut into small pieces using a scalpel and destained with 50 % acetonitrile (Sigma Aldrich, Austria) at 4 °C for 1-14 days. Acetonitrile solution was changed until gel was transparent. Solutions were removed using a 1 mL syringe (Braun, Germany) and a needle (Becton Dickinson GmbH, Germany). Afterwards, 100 % acetonitrile was added until gel turned white. Solution was removed and gel was incubated in 10 mM DTE in 0.1 M ammoniumhydrogencarbonate (Sigma Aldrich, Austria) at 56 °C for 45 minutes. Samples were cooled to room temperature, solution was removed and 55 mM iodoacetamide (Sigma Aldrich, Austria) in 0.1 M ammoniumhydrogencarbonate was added. Samples were incubated for 30 minutes at room temperature in the dark. Solution was removed and 50 % acetonitrile was added. After 15 minutes, solution was removed, and 100 % acetonitrile was added until gel pieces turned white. Solution was removed and 20 µL of 0.1 M ammoniumhydrogencarbonate was added and mixed for 5 minutes. Afterwards, 30 µL of 100 % acetonitrile was added and mixed for 15 minutes. Solution was removed and gels were dried on heating block at 56 °C with open lid for 45 minutes. Aluminium foil was put on top to shield samples from contamination. Then, 50 µL of 12.5 ng*µL^{-1} trypsin (Promega, Sweden) in 50 mM ammoniumhydrogencarbonate were added and samples were incubated on ice for 45 minutes. Excess of trypsin solution was removed and 50 µL of 50 mM ammoniumhydrogencarbonate was added. Samples were incubated at 37 °C over night. Digests were either used directly for further analysis or stored at -20 °C.

To ensure the reliability of the experiment, an in-solution digestion with trypsin was done on a 0.01 mg*mL^{-1} and 0.1 mg*mL^{-1} BSA solution (Sigma Aldrich, Austria) in 6 M urea. To 100 µL of the BSA solution, 5 µL of 200 mM DTE in 25 mM ammoniumhydrogencarbonate was added and incubated for one hour at room temperature. Afterwards, 20 µL of 200 mM iodoacetamide in 25 mM ammoniumhydrogencarbonate were added and incubated for one hour at room temperature in the dark. To consume any leftover alkylating agent, 20 µL of reducing reagent was added. Then, 900 µL of 25 mM ammoniumhydrogencarbonate was added to dilute the urea. For protein digestion, 2.4 µL of 12.5 ng*µL^{-1} trypsin for the lower concentrated BSA standard solution and 24 µL of 12.5 ng*µL^{-1} trypsin for the higher concentrated sample were added. BSA was digested overnight at 37 °C.

To purify and desalt digests, samples were run through a custom-made desalting column. Therefore, digests were acidified with 10 % trifluoroacetic acid (Sigma Aldrich, Austria) until pH 1 was reached. The column was made by packing a 10 µL pipette tip (Eppendorf, Germany) with a C18 membrane (Thermo Scientific, USA), which was cut out using a stamp. The column was activated by adding 10 µL of 100 % acetonitrile and forcing it through the pipette tip using a syringe, followed by a washing step with 10 µL 0.1 % trifluoroacetic acid. All of the sample was transferred onto the tip column and released. Column was washed with 10 µL of 0.1 % trifluoroacetic acid and peptides were extracted from column with 5 µL of saturated a-Cyano-4-hydroxycinnamic acid (Sigma Aldrich, Austria) in 70 % acetonitrile and 0.1 % trifluoroacetic acid. Three droplets were put on MALDI target plate (Bruker Daltonics, Germany) and left to dry in the dark. External calibration was done by using a mixture of 0.5 µL peptide calibration standard (Bruker Daltonics, Germany) and 0.5 µL of saturated a-Cyano-4-hydroxycinnamic acid in 70 % acetonitrile and 0.1 % trifluoroacetic acid.

Samples were measured using the autoflex speed (Bruker Daltonics, Germany) in reflector mode in a *m/z* range of 700 – 3500, positive ionization mode and an average laser intensity of 70 %. The instrument was controlled using flexControl 3.4 (Bruker Daltonics, Germany) and analysis was done in flexAnalysis 3.4 (Bruker Daltonics, Germany) and BioTools 3.4 (Bruker Daltonics, Germany). Peptide Mass Fingerprinting (PMF) was done in Mascot 2.6.1 (Matrix Science, UK). PMF searches were done against UniProtKB database and an in-house built barley database. Viridiplantae was chosen as taxonomy, carbamidomethyl cysteine as fixed modifications and oxidized methionine as variable modifications. Peptide tolerance of up to 0.5 Da, together with a peptide charge of 1 H^+ and number of missed cleavage sites of up to 2 was set. A peptide-spectrum-match was considered as positive when the score was significant and at least 4 peptides were identified.

4 Results

4.1 Protein Extraction from Barley Flour

4.1.1 Comparative Analysis of Protein Extraction Methods

Figure 12 depicts a comparison between the four different Osbourne fractions, extracted with two different extraction protocols respectively. EM1 showed a higher protein concentration in the extracts of the albumin, prolamin and glutelin fraction. EM2 on the other hand, showed a superior extraction efficiency for the globulin fraction. Previous literature has indicated that the water-soluble albumin fraction and the salt-soluble globulin fraction cannot be effectively separated solely through extraction, which is why many protocols work with a combined method for both fractions[23,25]. Therefore, the combined protein concentration of both fractions was used for data analysis, which resulted in an almost equal protein concentration from both extraction methods (1.94 ± 0.16 mg*mL^{-1} for EM1 versus 1.98 ± 0.04 mg*mL^{-1} for EM2). Consequently, further experiments were conducted using EM1.

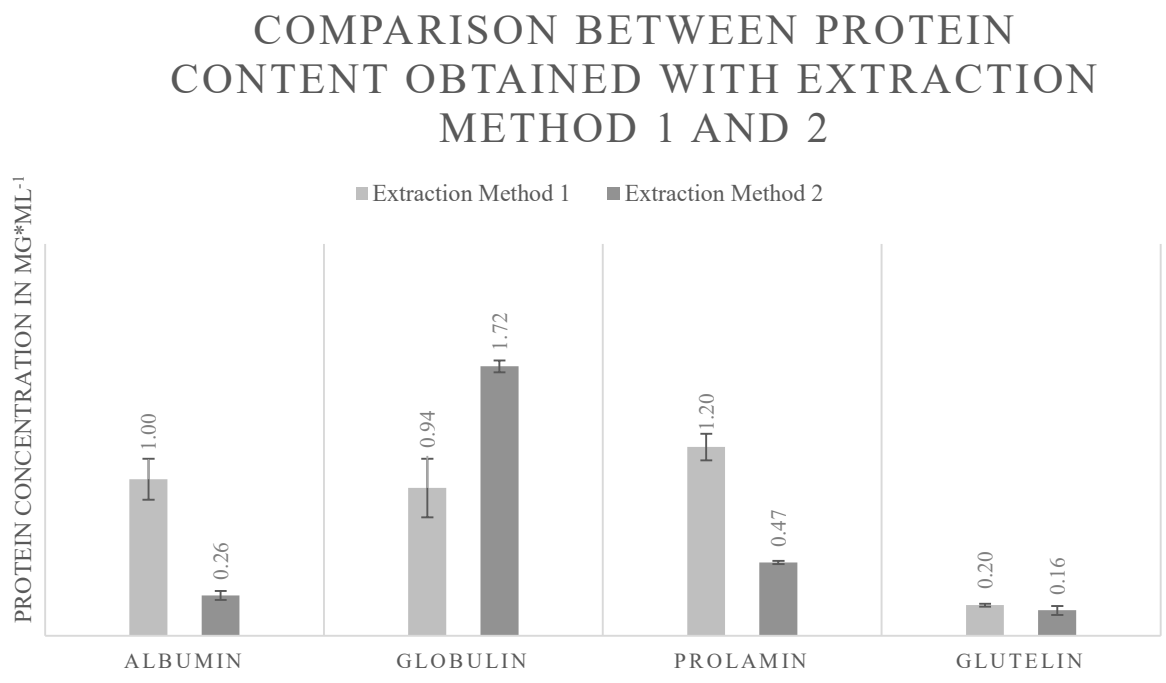

Figure 12: Comparison between the protein concentration in the extracts obtained with the Osborne fractionation protocol proposed by *Wang et al., 2007*, labelled as "extraction method 1" (*EM1*) and the protocol proposed by *Celus et al., 2006*, labelled as "extraction method 2" (*EM2*) in the unsieved PS3 barley cultivar. Due to a higher protein yield with EM1, further experiments were conducted with this extraction method. Protein concentration was measured using the Bradford Assay.

Table 4 gives a short summary of the differences between the two protocols, as stated in **3.2.2.1**.

Table 4: Comparison between extraction method 1 by *Wang et al.,2007* and extraction method 2 by *Celus et al., 2006*.

Protein Fraction	***Extraction Method 1***	***Extraction Method 2***
Albumin	Milli-Q Water	Milli-Q Water
Globulin	Potassium Phosphate Buffer (pH 8)	5 % Sodium Chloride
Prolamin	55 % 2-Propanol, 1% DTE, 1 % Acetic Acid	55 % 2-Propanol, 1 % DTE
Glutelin	0.05 M Sodium Borate Buffer (pH 10), 1 % DTE, 1 % SDS	6 M Urea, 1 % SDS, 1 % DTE

The experimentally obtained distribution of all four fractions from both extraction methods was compared to literature values, which can be seen in **Figure 13**. The albumin and globulin fraction combined accounted for around 10-20 % of the total protein amount. Around 30-50 % are composed of prolamins, while the glutelins make 35-45 %. The diagram shows that the extraction efficiency of the glutelin fraction is insufficient, because the experimentally determined quantity is significantly less than reported in literature. Interestingly, the albumin and globulin extracts show the highest amount of proteins, which should be the smallest fraction according to literature results. The values obtained from EM1 are closer to the proposed values from literature, even though they still differ considerably, especially in the glutelin fraction.

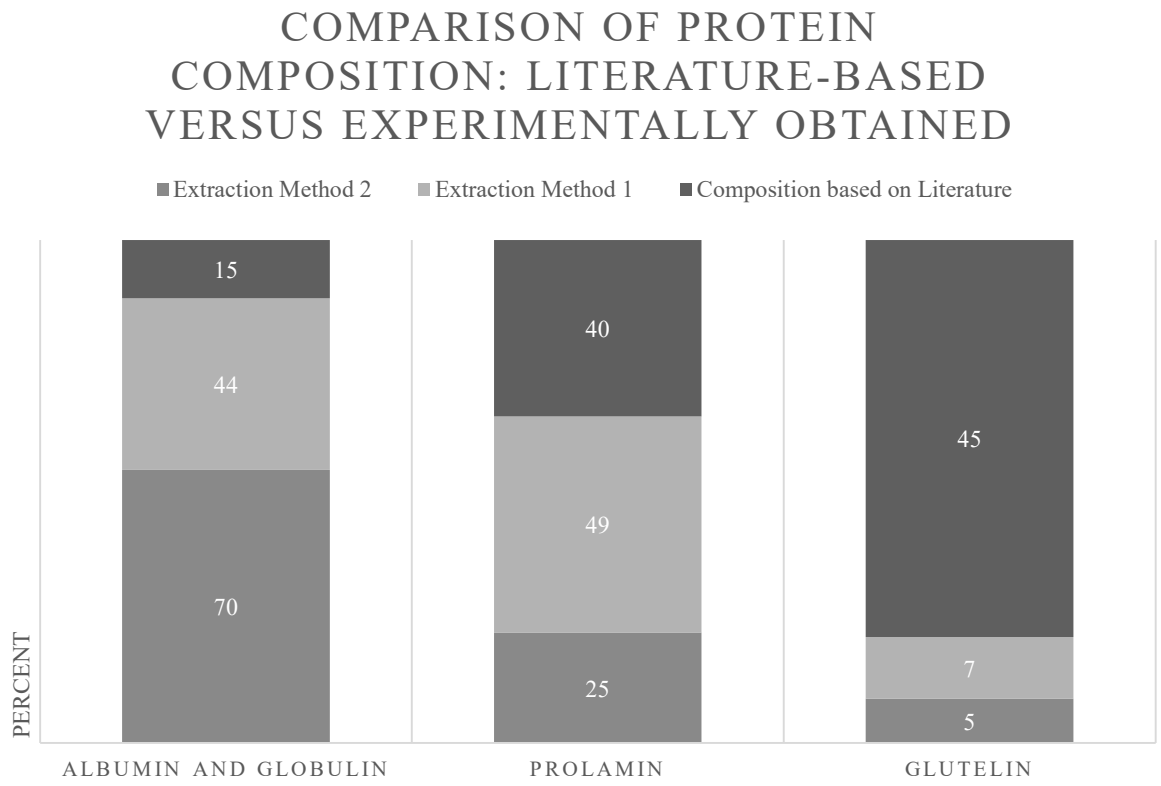

Figure 13: Protein composition of barley flour in percent as proposed in *Jaeger et al., 2021* compared to the experimentally obtained composition by using extraction method 1 and 2 on unsieved PS3 barley flour[17]. Protein concentration was measured using the Bradford Assay. Albumins and globulins represent around 10-20 % of the total proteins according to *Jaeger et al., 2021*, prolamins 30-50 % and glutelins 35-45 %. The experimentally obtained values show a different distribution. Here, the albumin, globulin and prolamin fractions compose a higher percentage of the totally extracted proteins than the glutelins, which is believed to be the largest fraction according to *Jaeger et al., 2021.*

In **Figure 14**, the extraction yields of the alcohol-soluble prolamin fraction, extracted with either 55 % ethanol or 55 % 2-propanol under non-reducing and reducing conditions respectively, are compared. The extraction was done as suggested in *Wang et al., 2007*. The 55 % 2-propanol resulted in a higher amount of proteins in the extracts in both conditions, which is why further extraction of the prolamin fraction was done with 55 % 2-propanol.

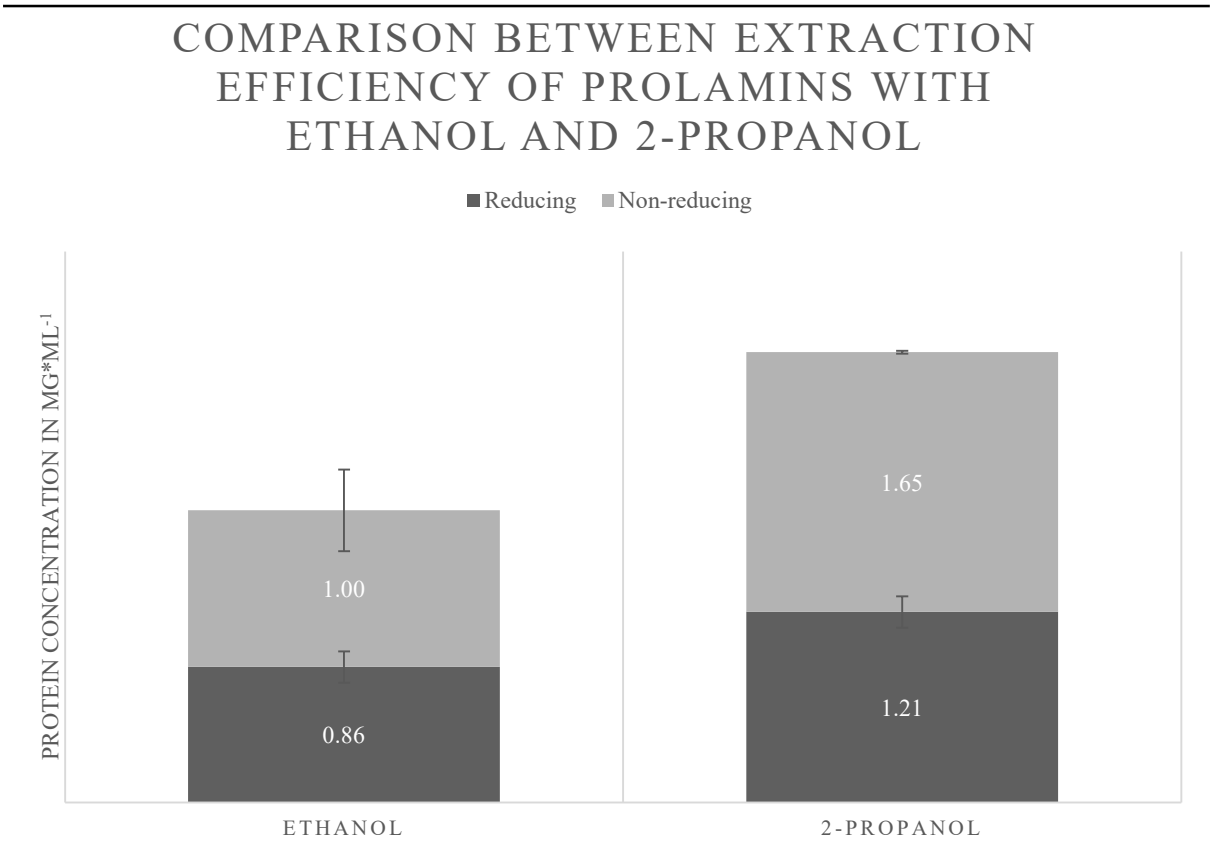

Figure 14: Comparison between the concentration of alcohol-soluble prolamins in unsieved PS3 barley flour extracts obtained by extraction with either 55 % ethanol or 55 % 2-propanol in reducing (1 % DTE) and non-reducing conditions respectively, following the protocol of *Wang et al., 2007*. Protein concentration was measured using the Bradford Assay. The results show that the extraction efficiency of 2-propanol is superior to ethanol and that more proteins were extracted under non-reducing conditions.

4.1.2 Investigation of Grain Size Influence on Extraction Efficiency

The influence of the grain size on extraction efficiency was investigated. The grain sizes used were unsieved barley flour, 500 µm, 250 µm, 125 µm, 63 µm and 45 µm, as previously mentioned. The protein concentration in the extracts was measured using the Bradford Assay. The protein concentration in the albumin and globulin fractions shows a dispersion resembling a normal distribution with a peak at 250 µm. In the prolamin fraction, the 45 µm and the unsieved barley flour both surpass the protein concentration of the 250 µm grain size flour

slightly. Hardly any glutelin proteins were detected in any of the cultivars. The highest glutelin concentration was found in the unsieved and 500 µm grain size flour. The 250 µm barley flour showed the highest yield in the albumin and globulin fraction. A similar result was found in the 45 µm and unsieved flour samples in the prolamin fraction. Therefore, further experiments were done with the 250 µm grain size flour of the PS3 and Golden Promise cultivars. **Figure 15** shows the extraction efficiency of proteins from different grain size flours of the barley PS3 cultivar.

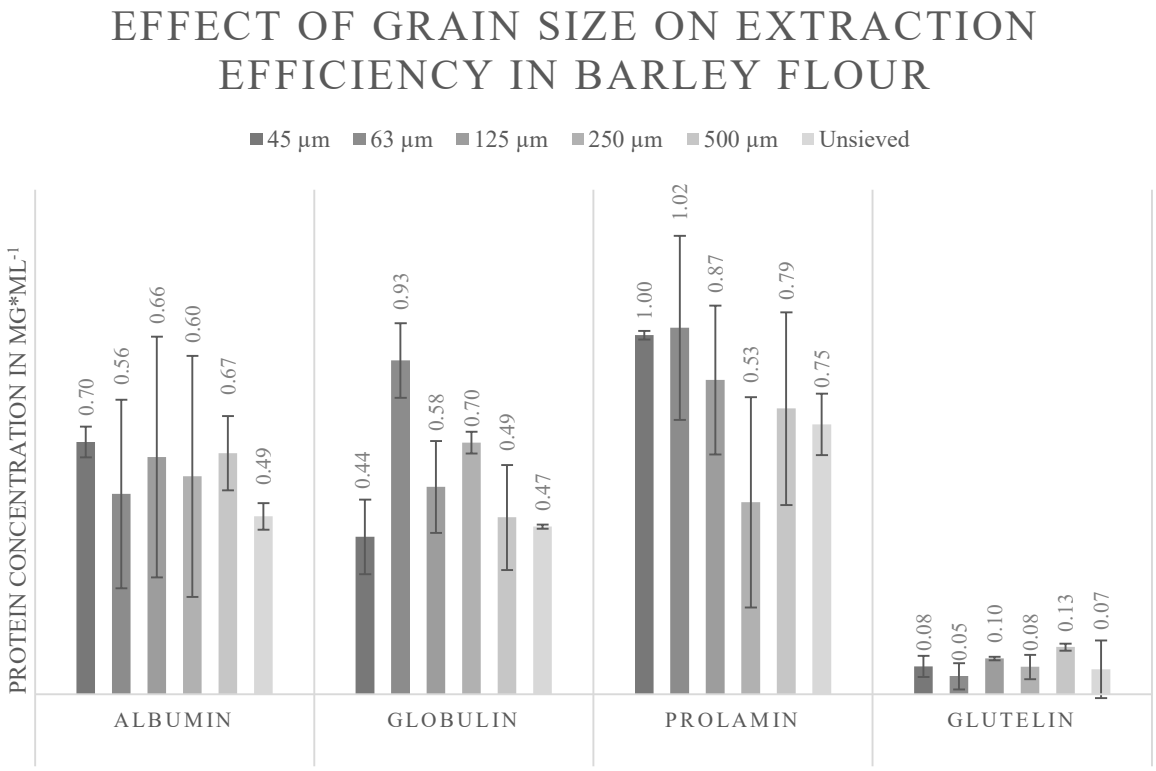

Figure 15: Comparison of extraction efficiency in different grain sizes of PS3 barley flour, ranging from 45 µm to 500 µm. Additionally, unsieved PS3 barley flour was tested. The protein concentration in the extracts was measured using the Bradford Assay.

4.2 Comparative Analysis Between Different Methods for Protein Quantification

In order to use a suitable method for protein quantification in the flour extracts, three different methods were applied, and their results compared. Therefore, the protein concentration in the extracts of barley flour in different grain sizes, as discussed in **4.1.2**, was measured using (i) the Bradford Assay, (ii) the Dumas method and (iii) the Nanodrop (Thermo Fisher Scientific, Austria). The results from (i) can be found in **Figure 15**, (ii) in **Figure 16** and (iii) in **Figure 17**. Furthermore, the nitrogen content of the solid flour before and after extraction was measured using the Dumas method. The results are stated in **Table 5.**

Table 5: Protein content of solid barley flour samples from all three barley cultivars and wheat before and after protein extraction. Biological duplicates of every sample were measured using the Dumas method.

Sample	*Average Protein Content in %*	*Standard Deviation in %*
	Before Extraction	
PS3 - 45 µm Grain Size	10.1	1.5
PS3 - Unsieved	12.3	0.3
Golden Promise - Unsieved	9.9	0.3
Wild Barley - Unsieved	21.8	0.6
Wheat - Unsieved	14.9	1.3
	After Extraction	
PS3 - 45 µm Grain Size	1.2	0.1
PS3 – Unsieved	1.5	< 0.1
Golden Promise - Unsieved	0.7	< 0.1
Wild Barley - Unsieved	1.9	0.3
Wheat - Unsieved	0.3	< 0.1

The highest protein content was measured in the Wild Barley cultivar, followed by wheat. Golden Promise shows the lowest protein content. There is a difference observed between the unsieved PS3 barley cultivar and the 45 µm grain size, which yielded a lower protein content. After extraction, a small amount of proteins was still detected in the pellet. By comparing the amount of proteins in mg in the extracts with the total amount of protein measured in the solid flour in mg, an extraction efficiency of 36 % for unsieved PS3 with extraction method 1 (EM1) versus 27 % with extraction method 2 (EM2) was calculated. The 45 µm PS3 flour resulted in an extraction efficiency of 42 % for EM1 and 27 % for EM 2. Wheat resulted in an extraction efficiency of 45 % for EM1 and 37 % for EM2.

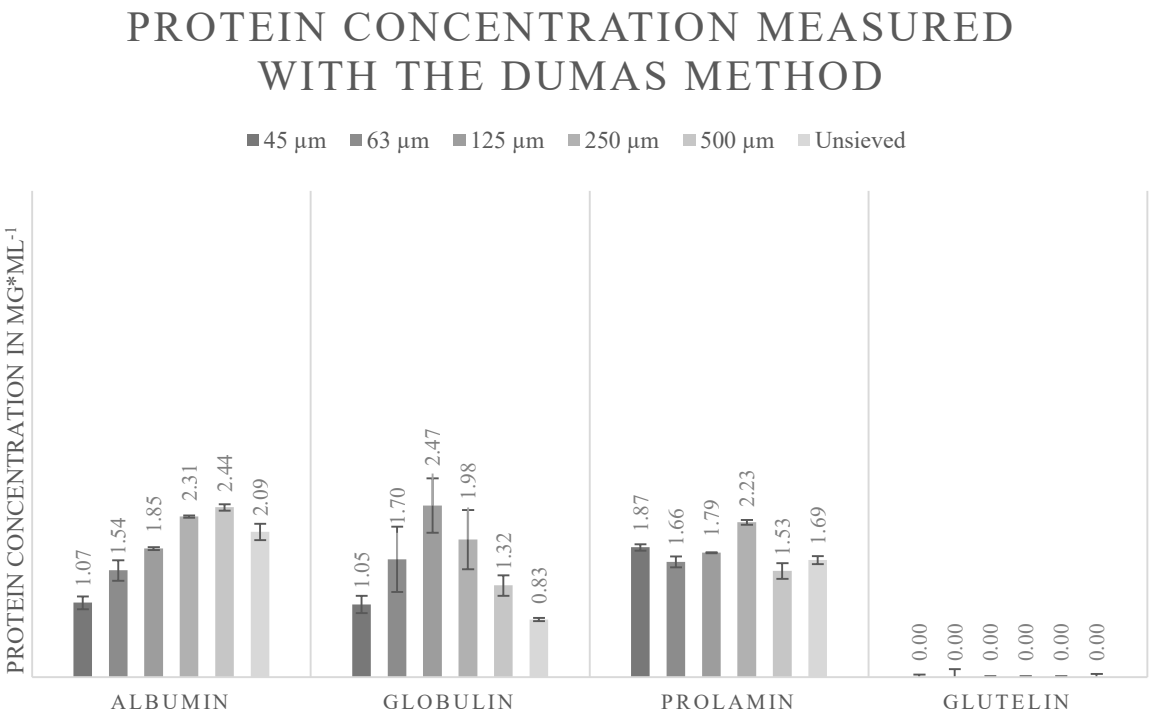

Figure 16: Protein concentration of PS3 cultivar in different grain sizes ranging from 45 µm to 500 µm measured with the Dumas method, using 0.5 % THAM for external calibration. Negative values were set to zero.

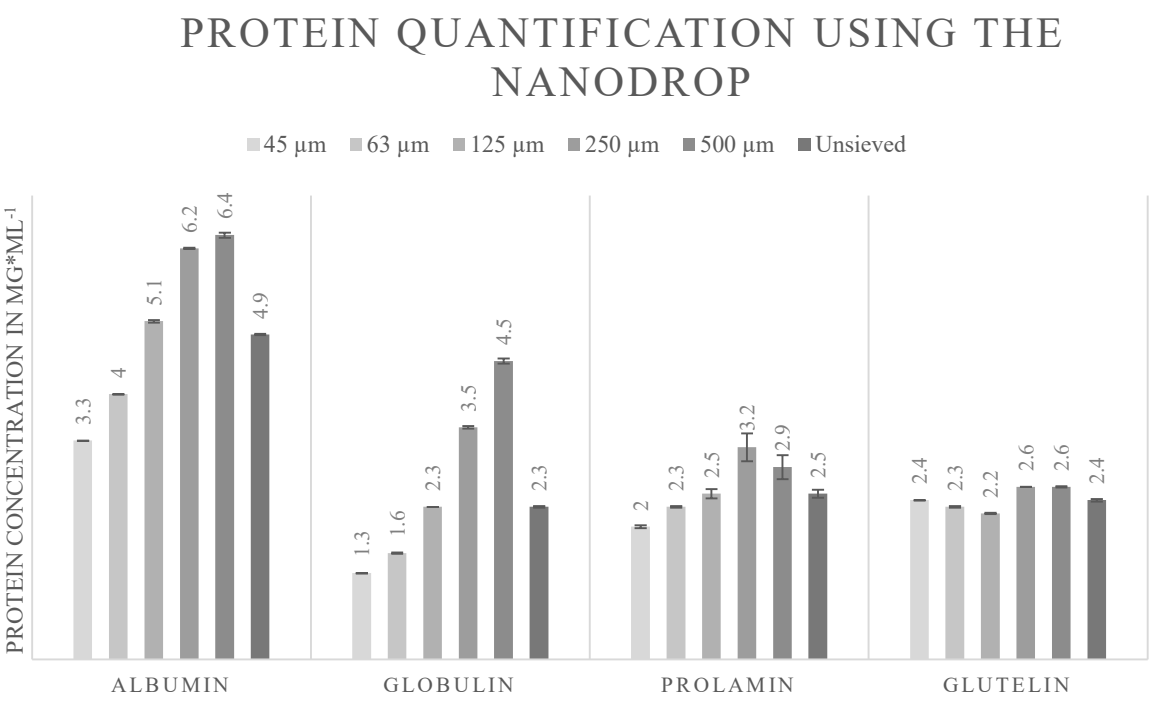

Figure 17: Protein concentration of PS3 cultivar in different grain sizes ranging from 45 µm to 500 µm measured with the Nanodrop.

The Nanodrop measurements yielded the highest protein content in the samples, whereas the Bradford Assay resulted in the lowest concentration. All three methods detected the smallest amount of protein in the glutelin fraction, equally. Interestingly, the results obtained from the Bradford Assay do not show any connection between grain size and protein quantity, unlike the Dumas method and the Nanodrop measurements, which indicated certain patterns for specific fractions. For instance, **Figure 16** demonstrates an increase in concentration from the smallest grain size to 500 µm in the albumin fraction, as well as the albumin and globulin fraction shown in **Figure 17.** Conversely, the globulin fraction **Figure 16** and the prolamin fraction in **Figure 17** show a gradual increase in concentration until reaching either 125 µm or 250 µm, after which the concentration decreases.

Considering the advantages and disadvantages of each method, further experiments were conducted using the Bradford Assay.

4.3 Protein Composition

4.3.1 One Dimensional – Gel Electrophoresis

In **Figure 18** - **Figure 21**, 1D-GE of all four Osborne fractions (albumin, globulin, prolamin and glutelin) are presented. All four fractions of all three barley cultivars and wheat were analysed. The albumin and globulin fraction were both extracted under non-reducing conditions. During the sample preparation for the 1D-GE, two versions of the Laemmli sample buffer were prepared. On of them contained 20 mM DTE (abbreviated in the figures as '+DTE') while the other one did not contain any DTE (abbreviated as '-DTE'). Each cultivar and each fraction were prepared with and without reducing conditions to assess the effect of the reducing agent on the protein profile. The prolamin and glutelin fraction on the other hand were extracted in reducing (abbreviated as 'red') and non-reducing conditions (abbreviated as 'non-red') respectively. Similar as to the water- and salt-soluble fraction, the effect of DTE added to the sample buffer was tested as well, by adding it to the sample buffer or leaving it out.

Figure 18 shows the protein profile of the water-soluble proteins in the previously mentioned barley cultivars and wheat. In total, Golden Promise shows the highest number of individual protein bands, while PS3 shows the lowest. When comparing reducing and non-reducing conditions ('+DTE' versus '-DTE') in the PS3 cultivar and wheat, no difference in the protein profile can be observed. However, in the case of the two cultivars, Golden Promise and Wild Barley, some disparities can be observed, which are highlighted in **Figure 18**. Both cultivars show two additional protein bands (I+II in Golden Promise and III+IV in Wild Barley) under reducing conditions that are not visible under non-reducing conditions. Some protein bands can be found in all eight samples (V), while others are only visible in barley (VI). Additionally, wheat shows two different protein bands at a molecular weight of 40 – 50 kDa (VIII), where at the same molecular weight one (PS3) or three (Golden Promise) protein bands can be observed in barley. Another visible difference between cultivars is that Golden Promise shows a higher intensity of high-molecular weight proteins with a mass of approximately 100 kDa (VII) than the other samples. In all samples, two protein bands at a molecular weight of 35-40 kDa (IX) can be seen, except for PS3, which only shows one.

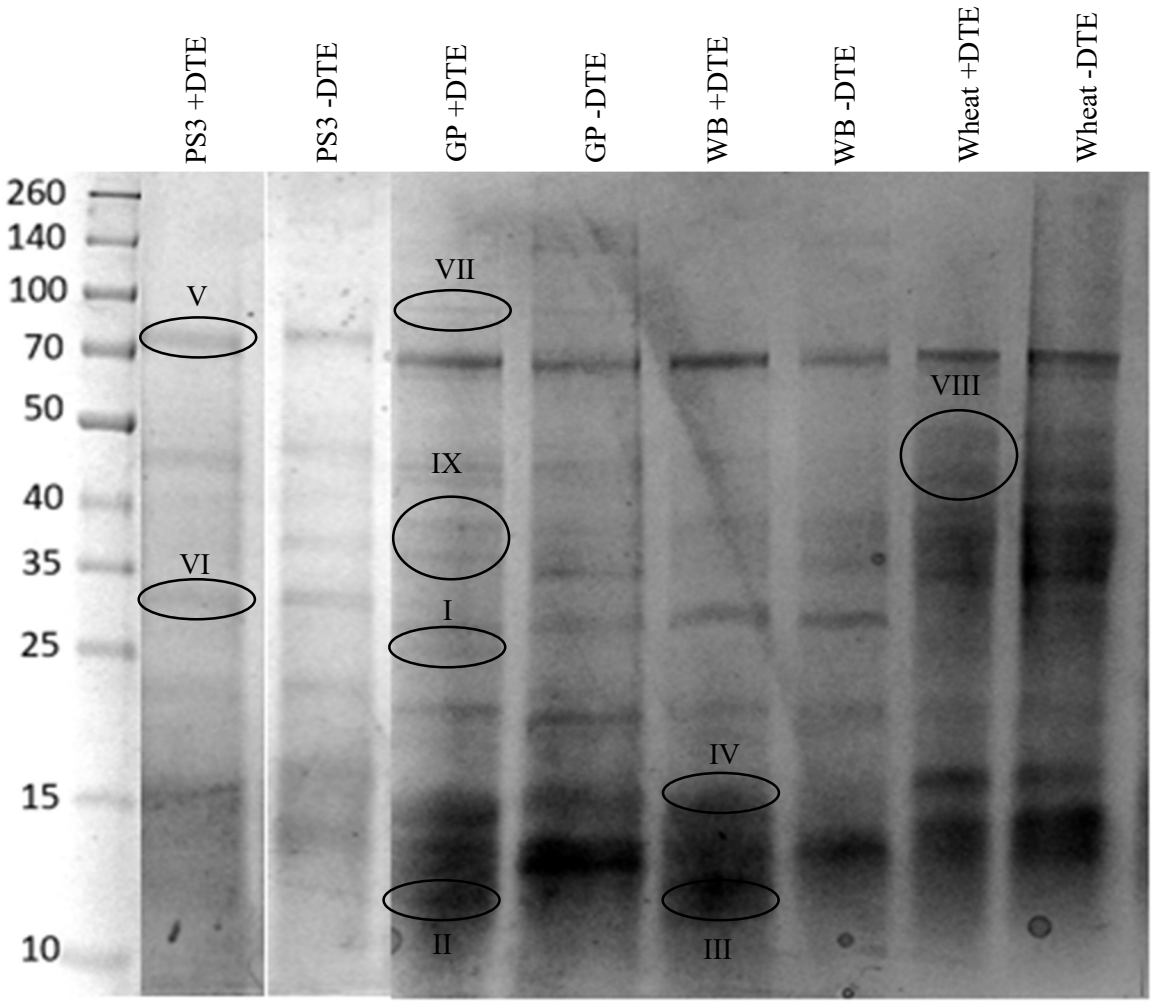

Figure 18: 1D-GE of albumin fraction of barley cultivars PS3, Golden Promise (GP), Wild Barley (WB) and wheat. For reducing conditions, 2 % DTE was added to the sample buffer (+DTE) whereas no DTE was added for non-reducing conditions (-DTE). Proteins were extracted under non-reducing conditions. A protein ladder ranging from 10-260 kDa was used as reference. Proteins I-IV are only visible under reducing conditions. Some protein bands can be found in all eight samples (V), while others are only visible in barley (VI). GP shows the highest intensity of HMW proteins (VII). Wheat shows two different protein bands between 40-50 kDa (VIII), while in barley, one (PS3) or three (GP) are visible. In all samples, two protein bands can be seen in position IX, except for PS3, which only shows one.

The protein profile of the salt-soluble proteins in the previously mentioned barley cultivars and wheat show, in contrast to the albumin fraction, no proteins above a molecular weight of 120 kDa. Identically to the albumin fraction on the other hand, no difference in the protein profile between reducing and non-reducing conditions in the PS3 cultivar can be seen. The greatest difference within the same sample can be observed in the Golden Promise cultivar. Five additional protein bands, two of them with a molecular weight of around 100-140 kDa (XII), one of them with a molecular weight of around 50 kDa (X) and two with a molecular weight of less than 15 kDa (XI) are present in the samples where DTE was added. The Wild Barley cultivar shows a considerably lower protein intensity in non-reducing conditions. However,

apart from the protein bands with a molecular weight of less than 15 kDa (XIII), the protein profile is similar to the reduced sample. The protein bands XIII show three bands that are only present in the samples with DTE, whereas one band can only be seen without DTE. In contrast to the albumin fraction, the globulin fraction shows a difference between the two conditions in wheat. Under reducing conditions, two protein bands are visible (XIV), while only one band is observed in the same position in non-reducing conditions. On the other hand, when no DTE is added, the protein band XV has a higher intensity. When comparing the albumin to the globulin protein profile, only the albumin fraction contains proteins at a molecular weight of 70 kDa. The globulin fraction on the other hand contains more proteins at 50 kDa, which are lacking in the albumin fraction. **Figure 19** shows the protein profile of the salt-soluble proteins in the previously mentioned barley cultivars and wheat.

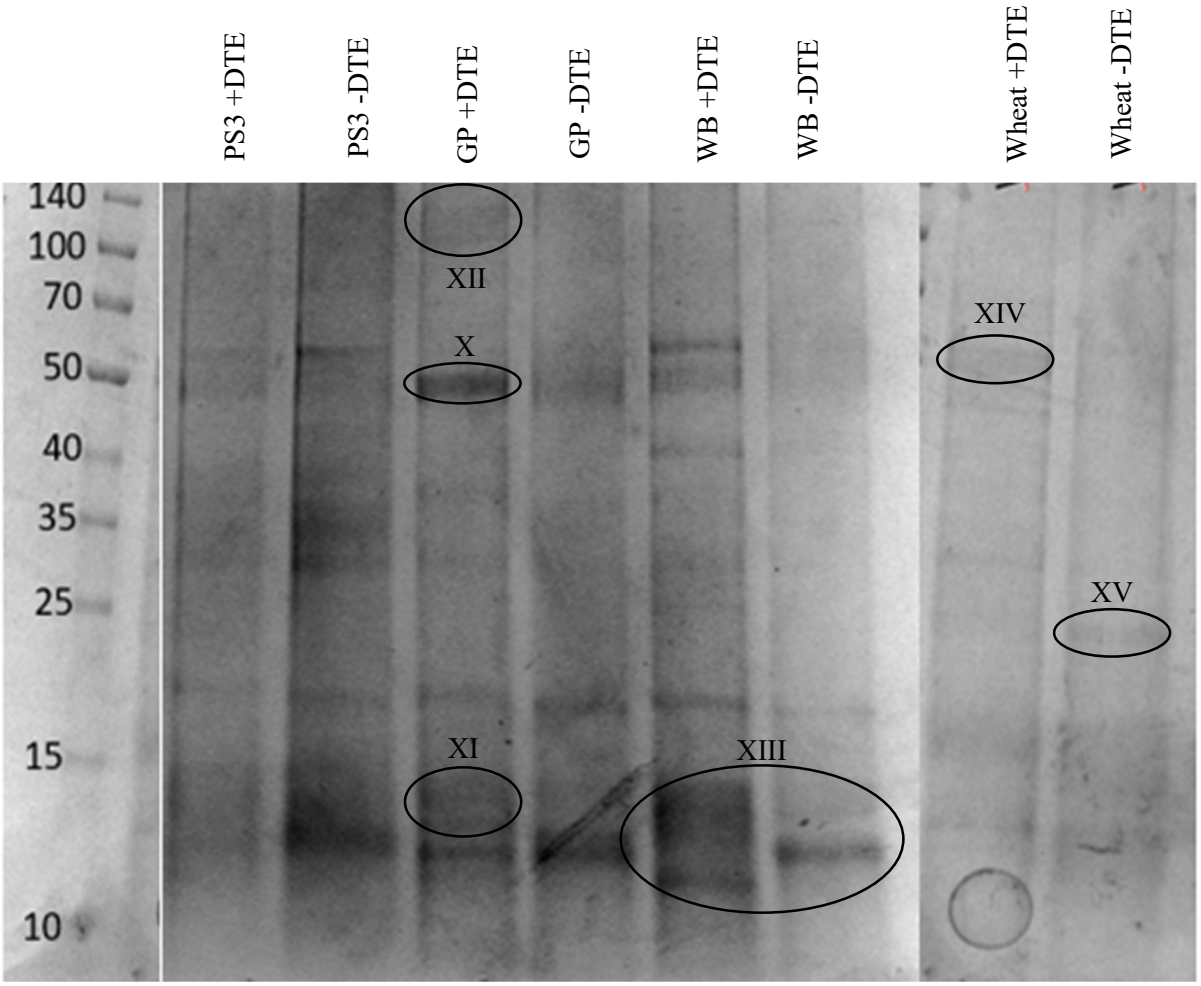

Figure 19: 1D-GE of globulin fraction of barley cultivars PS3, Golden Promise (GP), Wild Barley (WB) and wheat. For reducing conditions, 2 % DTE was added to the sample buffer (+DTE) whereas no DTE was added for non-reducing conditions (-DTE). Proteins were extracted under non-reducing conditions. A protein ladder ranging from 10-260 kDa was used as reference. No proteins above a molecular weight of 140 kDa were present. GP shows five additional protein bands (X-XII) in reducing conditions. Some protein bands can also be seen under reducing conditions in WB (XIII) and wheat (XIV). Wheat shows one band in non-reducing conditions (XV), that cannot be seen under reducing conditions.

Figure 20 and **Figure 21** show the protein profile of the alcohol-soluble and acid/alkali-soluble proteins in the three barley cultivars and wheat. The distribution pattern for all cultivars is the same. The first sample of each cultivar (Well 1, 5, 9) was extracted under reducing conditions with 1 % DTE (abbreviated at 'red'), and additionally, 20 mM DTE was added to the Laemmli sample buffer ('+DTE'). The second sample of each cultivar (Well 2, 6, 10) was extracted under reducing conditions, but no DTE was added to the sample buffer ('-DTE'). The third sample (Well 3, 7, 11) was extracted under non-reducing conditions ('non-red'), but 20 mM DTE was added to the sample buffer. The last sample (Well 4, 8, 12) was extracted under non-reducing conditions and no DTE was added to the sample buffer.

Firstly, it can be stated that there are no protein bands with a molecular weight lower than 25 kDa observed in any of the samples in **Figure 20**. Furthermore, some samples, where no DTE was added (either to the extraction solvent or the sample buffer), show more high-molecular weight proteins (XVI) that are less separated. This is the case in well 2, 4, 5, 6, 8 and 10. Moreover, in wells 2, 4, 6, 8 and 10 an intense black line in the bottom of the well is noticeable (XVII). These samples have in common that no DTE was added to the sample buffer. Another interesting observation is the effect of decrease in reducing agent on the two protein bands, marked as XVIII, in all three barley cultivars. The highest intensity of the protein bands can be observed in the sample, where the concentration of DTE is the highest (Well 1, 5, 9), while the lowest visibility can be seen when no DTE was added during the whole sample preparation process. The same observation can be made for protein band XXIII. In the Golden Promise cultivar, two light protein bands with a molecular weight of more than 70 kDa can be seen (XXIV), in contrast to the other cultivars, which only show one. Additionally, the protein band XXV has a higher intensity in Golden Promise than in the other cultivars. Both the PS3 and Golden Promise cultivar show three and four well-separated protein bands respectively at a molecular weight of around 50 kDa (XIX). The Wild Barley cultivar on the other hand has only two separated protein bands (XX), where the one with a lower molecular weight is significantly higher in intensity. Furthermore, the Wild Barley is missing protein band XXI, which is present in the other two barley cultivars, but shows two faint high-molecular weight bands at a mass of 140 kDa (XXII).

In general, barley prolamins can be divided into four groups, B-hordein (30-40 kDa), C-hordein (45-75 kDa), D-hordein (105 kDa) and γ-hordein (35-40 kDa)[6,19,27]. The high-molecular weight D-hordeins can only be seen under reducing conditions during extraction

except for the Golden Promise cultivar. There it can also be seen under non-reducing extraction conditions. In **Figure 20**, the prolamin proteins from all three barley cultivars are displayed.

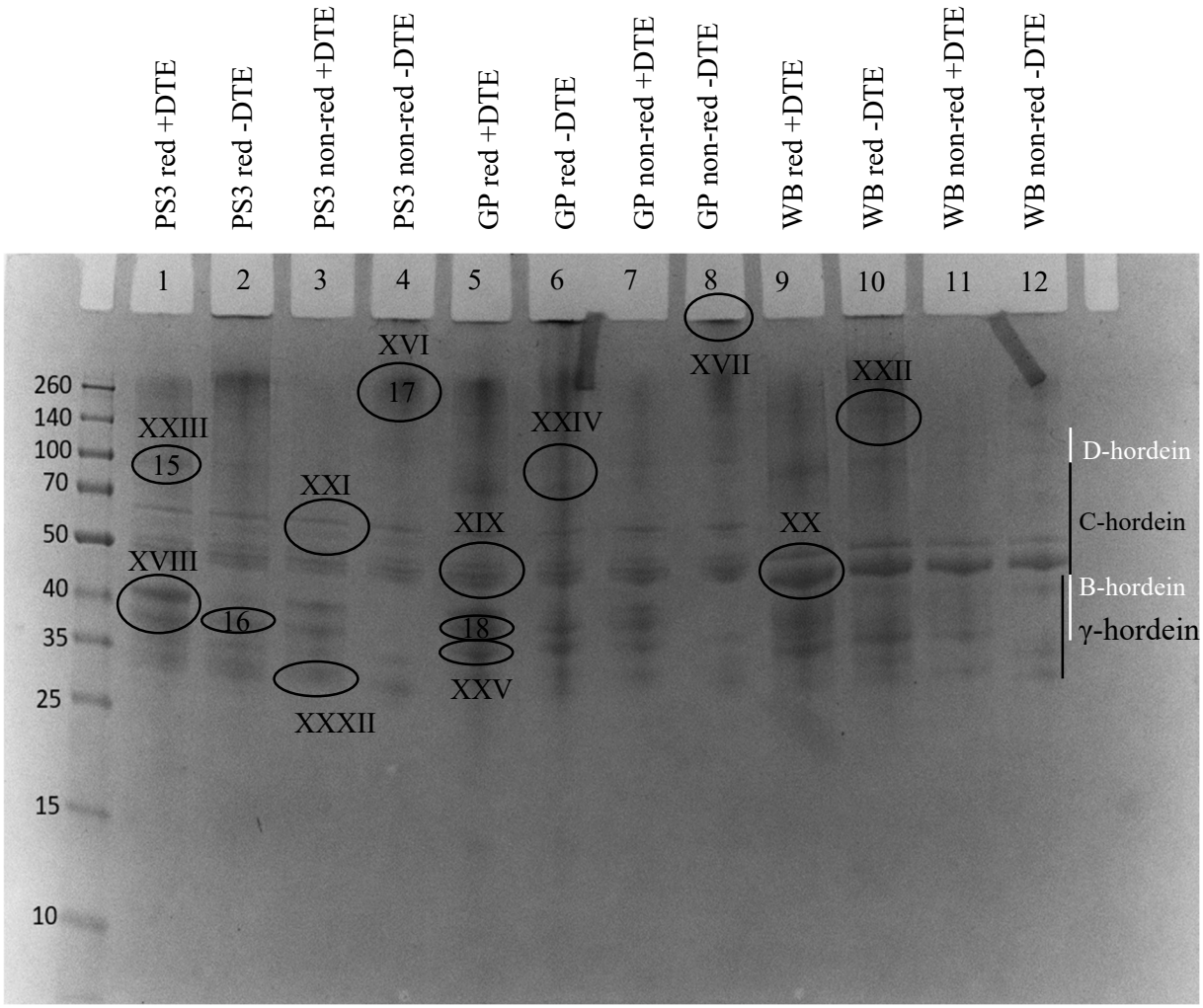

Figure 20: 1D-GE of prolamin fraction of barley cultivars PS3, Golden Promise (GP) and Wild Barley (WB). For reducing conditions, 2 % DTE was added to the sample buffer (+DTE) whereas no DTE was added for non-reducing conditions (-DTE). Samples were extracted both under reducing conditions (red) and non-reducing conditions (non-red). A protein ladder ranging from 10-260 kDa was used as reference. More HMW proteins can be seen in samples, where no DTE was added (XVI). Some wells also show an intense black line at the bottom of the well (XVII). The amount of DTE added during the sample preparation process has an influence on the intensity of some protein bands XVIII and XXIII. GP shows two protein bands (XXIV), while the other cultivars only show one. Protein band XXV has a higher intensity in GP than in the other cultivars. PS3 and GP show three or four protein bands at around 50 kDa (XIX), while WB only has two (XX). Protein band XXI is missing in WB, but it shows one additional band (XXII). Protein bands 15-18 were cut out for MALDI-ToF identification. On the left side, the molecular weight range of the four different hordein subgroups, as discussed in the introduction, are indicated as reference.

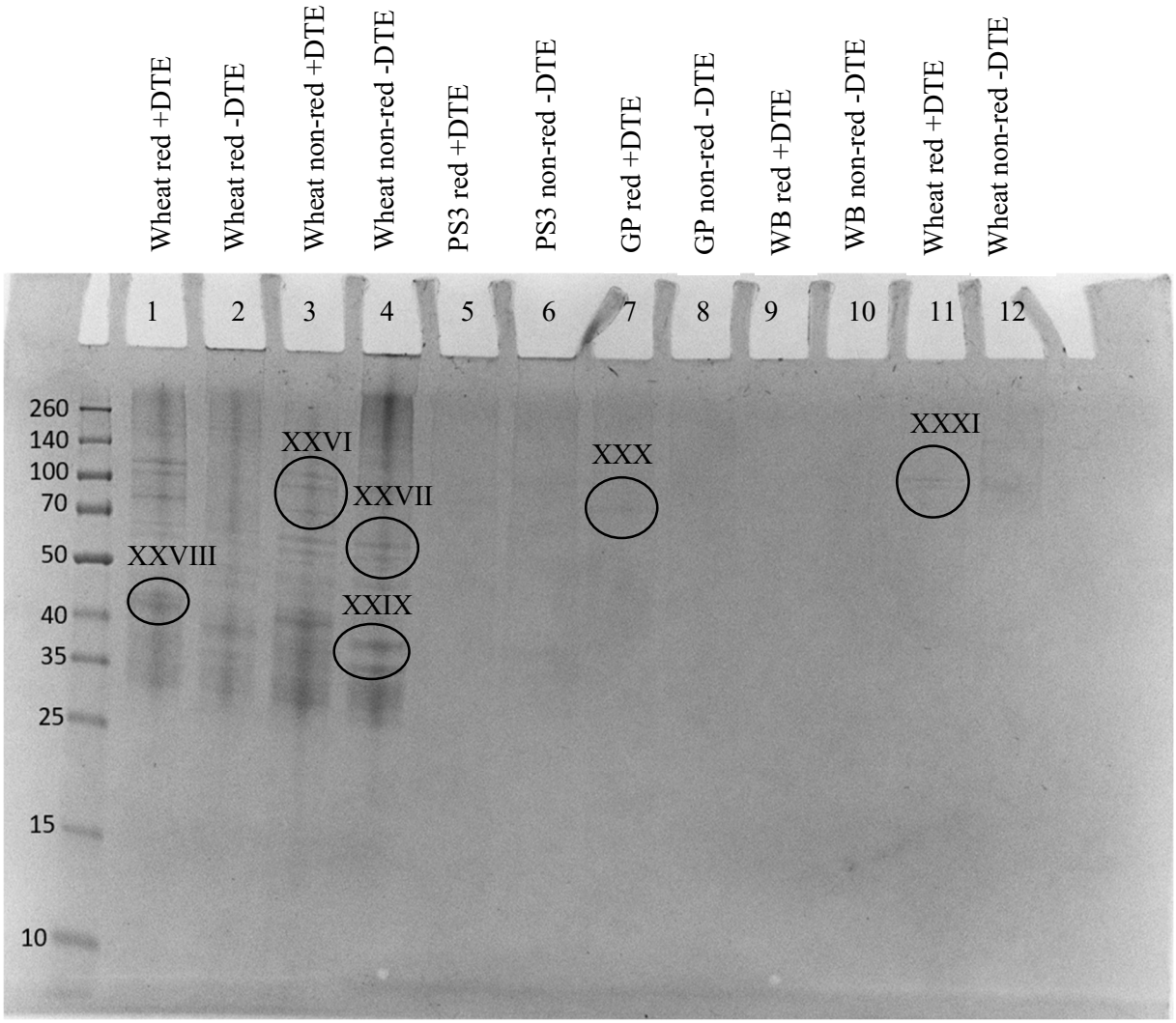

Figure 21: 1D-GE of prolamin fraction of wheat (Wells 1-4) and glutelin fraction of barley cultivars PS3, Golden Promise (GP), Wild Barley (WB) and wheat (Wells 5-12). For reducing conditions, 2 % DTE was added to the sample buffer (+DTE), whereas no DTE was added for non-reducing conditions (-DTE). Samples were extracted both under reducing conditions (red) and non-reducing conditions (non-red). A protein ladder ranging from 10-260 kDa was used as reference. The three protein bands XXVI are only visible under reducing conditions, while bands XXVII are more intense, the less DTE is added. Two protein bands (XXVIII) can only be observed under reducing conditions in the sample buffer, while the protein bands XXIX are only visible under non-reducing conditions in the sample buffer. The only proteins observed in the glutelin fraction (XXX – XXXI) are also present in the prolamin fraction of the respective samples.

Figure 21 shows the prolamin proteins from wheat and the glutelin fraction from all three barley cultivars and wheat. For the wheat samples (Well 1-4), the same sample pattern as for the prolamin proteins from **Figure 20** is applied. By comparing wheat to barley, it is evident that there are more high-molecular weight proteins in wheat. The three protein bands at a molecular weight of 70-100 kDa (XXVI), which disappear when no reducing agent is added to the sample buffer, are noticeable. Furthermore, the protein bands at a molecular weight of about

60 kDa (XXVII), that are more prominent, the less DTE is added. Only one of the two XXVII protein bands can be observed in barley. Two wheat protein bands that are in very close proximity to each other (XXVIII) can only be observed when the sample buffer has a reducing agent in it, whereas the non-reducing sample shows two protein bands at a lower molecular weight (XXIX).

Furthermore, the gel shows the glutelin fraction of all three barley cultivars and wheat in wells 5-12. Hardly any protein bands are visible in this fraction. The protein bands that can be observed (XXX and XXXI), are also present in the prolamin fraction of the respective samples.

4.3.2 Two-Dimensional Gel Electrophoresis

In **Figure 22** - **Figure 27** 2-Dimensional Electrophoresis gels of the prolamin fraction of every barley cultivar in reducing and non-reducing conditions are presented. For reducing conditions, proteins were extracted under reducing conditions with 1 % DTE and the same amount of DTE was also added to the lysis buffer. For non-reducing conditions, no reducing agent was added throughout the sample preparation process. **Figure 22** shows the prolamin fraction of the PS3 cultivar in reducing conditions and **Figure 23** shows the non-reducing conditions.

Some of the proteins can be observed in both conditions (I-IX), while others can only be observed in one of the two variations (X-XIII). Most of the observed proteins in **Figure 22** are located within a pI range of 5-10 and a molecular weight of 25-140 kDa. Correspondingly to the 1D-GE of the same sample, no proteins with a molecular weight of less than 25 kDa can be observed. A considerable amount of proteins show similar properties and are located at a pI of 7 and a molecular weight of 40 kDa (XII). In comparison to the 1D-GE, the protein band at a molecular weight of 100 kDa (XXIII) cannot be observed in the 2D-GE, neither in reducing nor non-reducing conditions. Yet, one protein band at a molecular weight between 100 – 140 kDa and a pI of 8-9 is observed. The amount of proteins that are visible under non-reducing conditions is considerably lower than in **Figure 22**, even though the same amount of protein has been applied to the gel. The unreduced sample shows less proteins at a molecular weight of 40 kDa, which correlates to the disappearance of the two protein bands XVIII in **Figure 20**, when no DTE was added during the sample preparation process. Proteins at a molecular weight of 50 kDa, on the other hand, are still present. Interestingly, no proteins above a molecular weight of 70 kDa can be seen, while there is an intense protein band at a molecular weight of 260 kDa present in the 1D-GE (XVI).

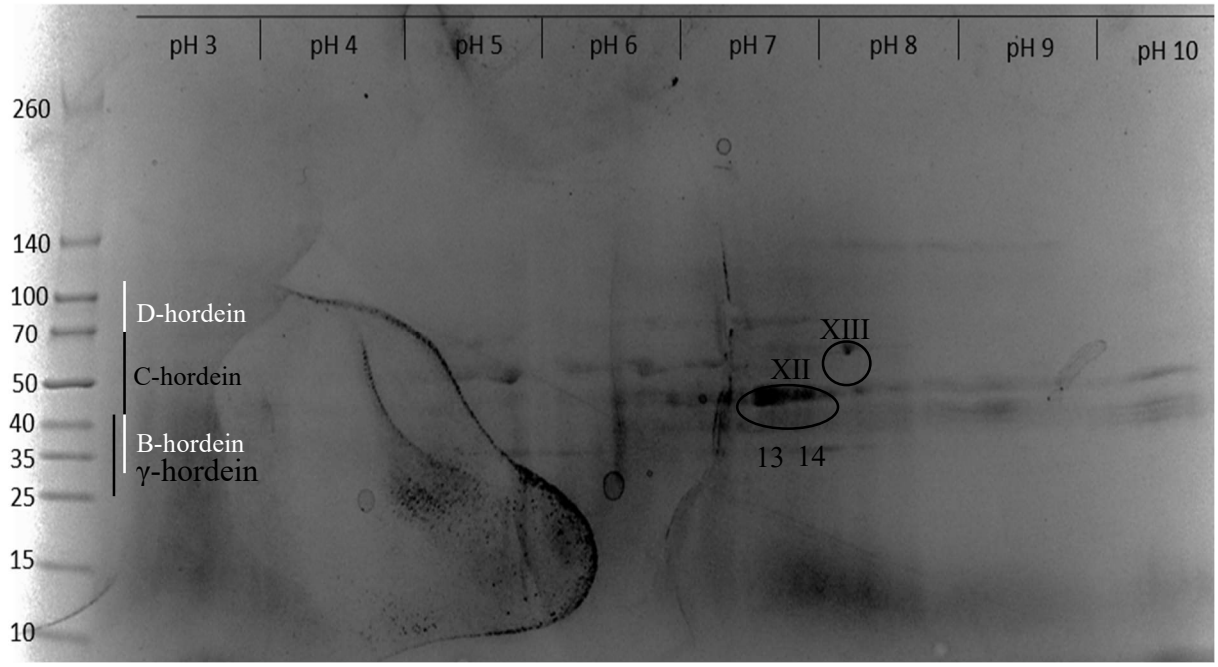

Figure 22: 2D-GE of prolamin fraction of 250 µm grain size barley cultivar PS3, extracted under reducing conditions. 1 % DTE was added to IPG lysis buffer. An IPG strip with a pH range from 3-10 was used. A protein ladder ranging from 10-260 kDa was used as reference. Protein spots XII and XIII can only be observed under reducing conditions. These proteins show similar characteristics in terms of molecular weight and pI. Proteins labelled as 13 and 14 were subjected to identification with MALDI-ToF MS. On the left side, the molecular weight range of the four different hordein subgroups, as discussed in the introduction, are indicated as reference.

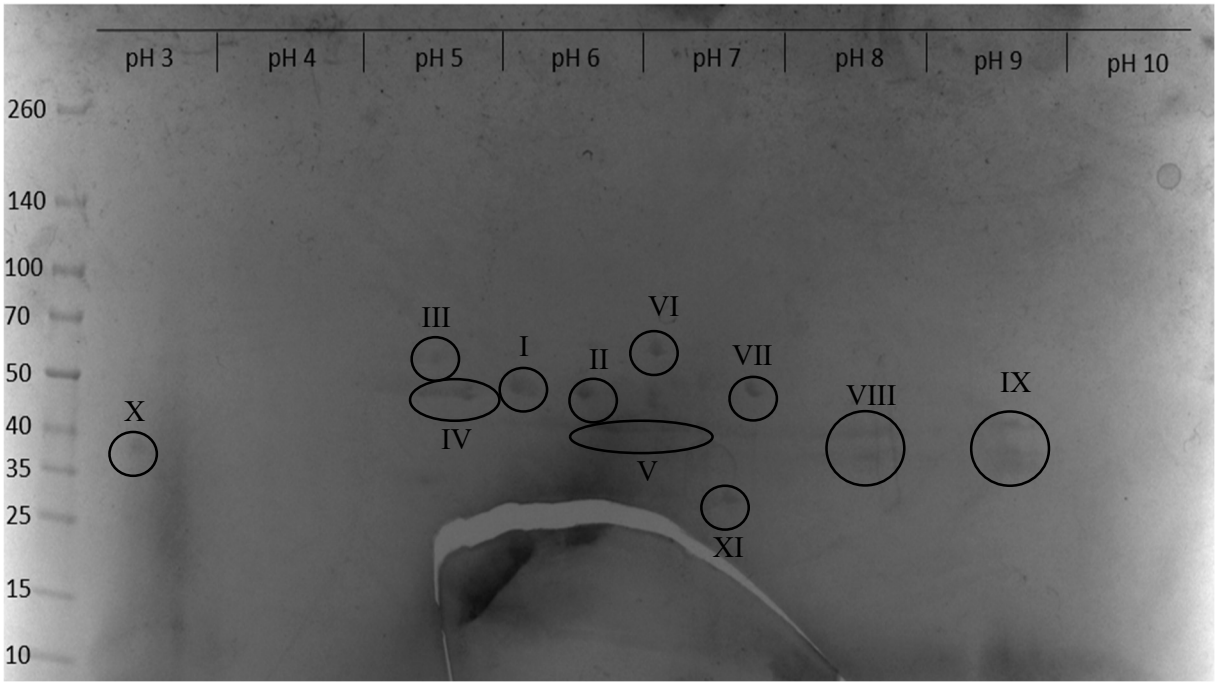

Figure 23: 2D-GE of prolamin fraction of 250 µm grain size barley cultivar PS3, extracted under non-reducing conditions. No DTE was added to IPG lysis buffer. An IPG strip with a pH range from 3-10 was used. A protein ladder ranging from 10-260 kDa was used as reference. Some protein spots (I-IX) can be observed in both conditions, while others (X-XI) can only be observed in non-reducing conditions.

Figure 24 and **Figure 25** show the protein profile of the prolamin fraction of Golden Promise in reducing and non-reducing conditions. Most of the proteins are located in a pI range of 5-10 and a molecular weight of 25 – 100 kDa. The two protein spots with the highest intensity are located between pI 6 and pI 7 at a molecular weight of about 35 kDa (XV and XVI). Furthermore, more low-intensity protein spots with a mass of around 25 kDa (XVII and XVIII) can be seen in Golden Promise, in contrast to the 1D-GE. In comparison to the PS3 cultivar, the observed proteins are more shifted to a pI of 6, instead of 7. In general, the proteins also appear to be better separated. Additionally, there are more proteins observed at a molecular weight of around 35 kDa (XV-XIX) and a higher count of proteins at pI 9 and 10 (XX) in comparison to PS3. When reducing and non-reducing conditions between the same cultivar are compared, it can be stated that all proteins with a molecular weight lower than 40 kDa (XV-XX) cannot be seen under non-reducing conditions, which is in accordance with the observations on the 1D-GE. Based on the 1D-GE, the protein pattern of the Golden Promise and PS3 cultivar is similarly shaped, but the 2D-GE reveals significant differences within these two barley samples.

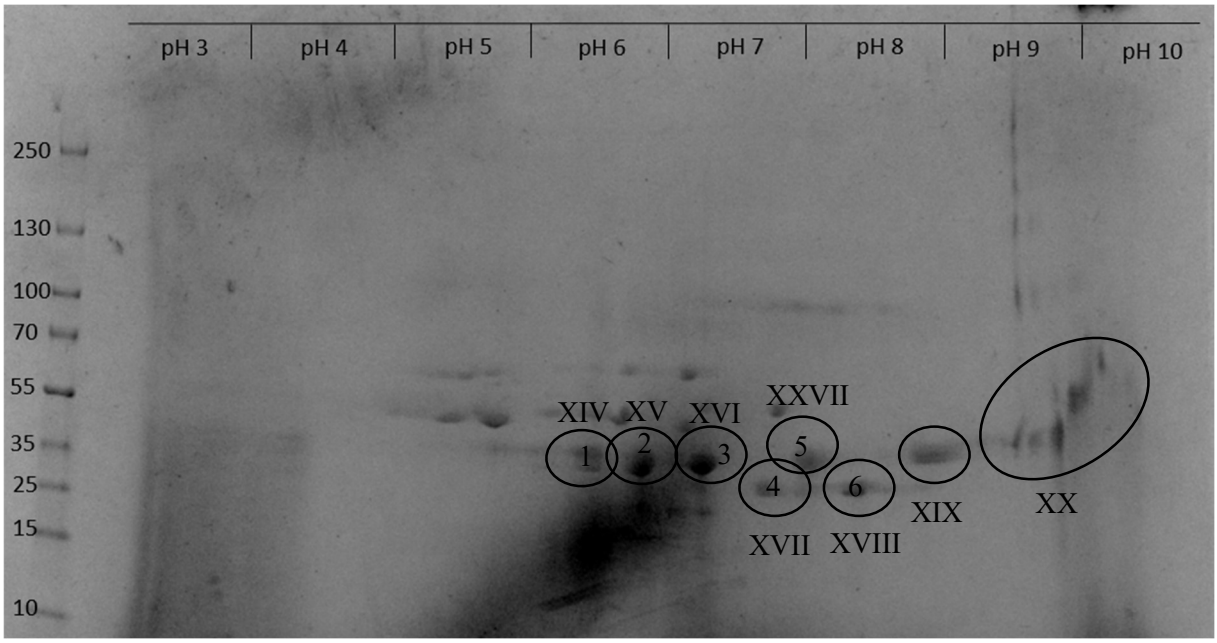

Figure 24: 2D-GE of prolamin fraction of 250 µm grain size barley cultivar Golden Promise, extracted under reducing conditions. To the IPG lysis buffer, 1 % DTE was added. An IPG strip with a pH range from 3-10 was used. A protein ladder ranging from 10-260 kDa was used as reference. Protein spots XV and XVI show the highest intensity. In comparison to the 1D-GE, more low-molecular weight proteins can be seen in the 2D-GE (XVII and XVIII). Additionally, there are more proteins observed at a molecular weight of around 35 kDa (XV-XIX) and a higher count of proteins at pI 9 and 10 (XX) in comparison to PS3. Protein spots (XV-XX and XXIV) can only be seen in reducing conditions. Protein spots 1-6 were subjected to protein identification with MALDI-ToF MS.

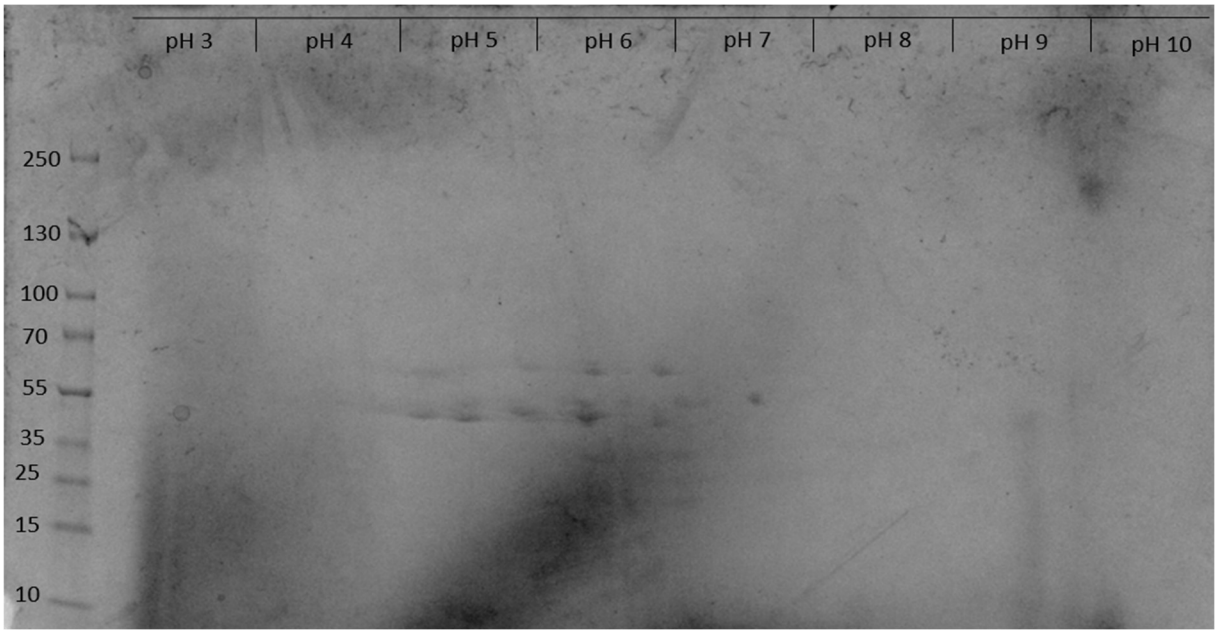

Figure 25: 2D-GE of prolamin fraction of 250 µm grain size barley cultivar Golden Promise, extracted under non-reducing conditions. No DTE was added to IPG lysis buffer. An IPG strip with a pH range from 3-10 was used. A protein ladder ranging from 10-260 kDa was used as reference.

Figure 26 and **Figure 27** show the protein profile of the prolamin fraction of Wild Barley in reducing and non-reducing conditions. Similar to the other two cultivars, the majority of the proteins is concentrated within a pI range of 5-10 and a molecular weight of 25 – 130 kDa. Overall, the protein pattern of Wild Barley is more similar to the pattern of PS3 than Golden Promise. For example, there can be more proteins observed at a pI of 7-8 at the same molecular weight as in the PS3 cultivar (XXII-XXIV). Furthermore, both cultivars show a high-molecular weight protein band at 100 kDa (XXI). The protein spots XXIII correlate with the protein spots XX from the 1D-GE, indicating that there may be more than two proteins in the 1D-GE bands. When comparing the reducing and non-reducing conditions, it is noticeable that some proteins that appear in a band shape in the reducing conditions, are better separated into spots in the non-reducing conditions (XXV). Furthermore, the protein band at 100 kDa (XXI) cannot be observed in the non-reducing gel, which is identical to the other two cultivars. Some spots (XXII, XXVI) are missing under non-reducing conditions or have a significantly lower intensity.

Based on these results, the protein spots 1-6 from **Figure 24**, 7-10 from **Figure 26**, 11-12 from **Figure 27**, 13-14 from **Figure 22** and 15-18 from **Figure 20** were chosen for identification with MALDI-ToF-MS.

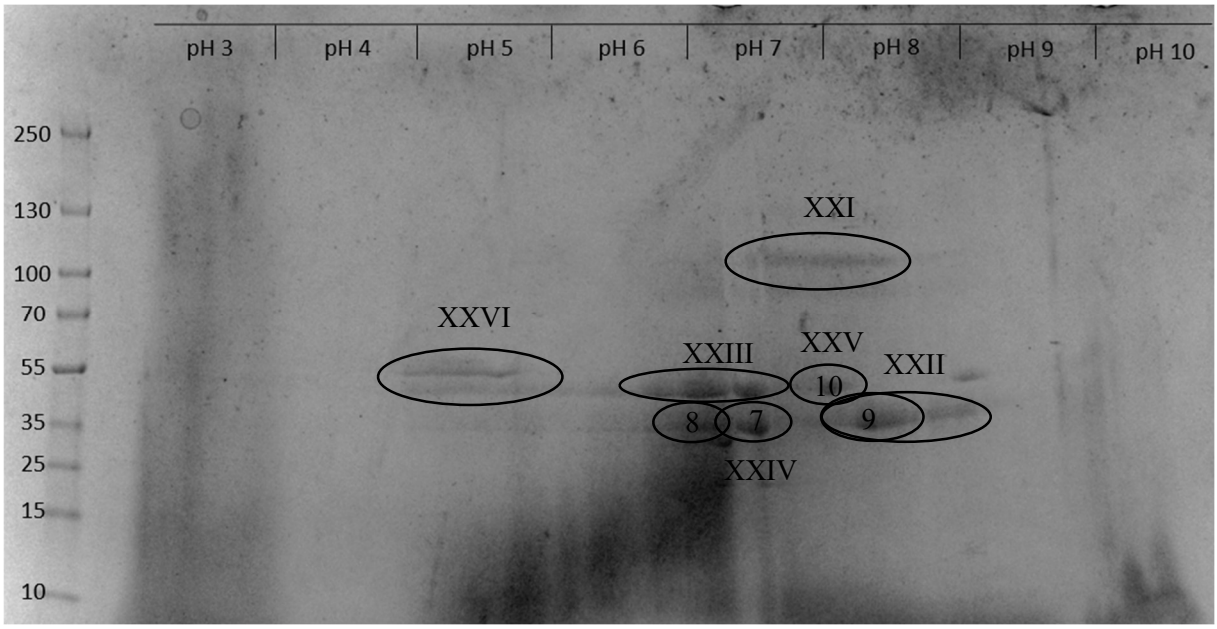

Figure 26: 2D-GE of prolamin fraction of 250 µm grain size barley cultivar Wild Barley, extracted under reducing conditions. To the IPG lysis buffer, 1 % DTE was added. An IPG strip with a pH range from 3-10 was used. A protein ladder ranging from 10-250 kDa was used as reference. WB shows more proteins at a pI of 7-8 at the same molecular weight as PS3 (XXII-XXIV). Both cultivars show the HMW band XXI. The protein spots XXIII can also be seen on the respective 1D-GE. Protein bands XXV are better separated under non-reducing conditions. XXI is only observed under reducing conditions. Some spots (XXII, XXVI) are missing under non-reducing conditions or have a significantly lower intensity. Protein spots 7-10 were subjected to identification with MALDI-ToF MS.

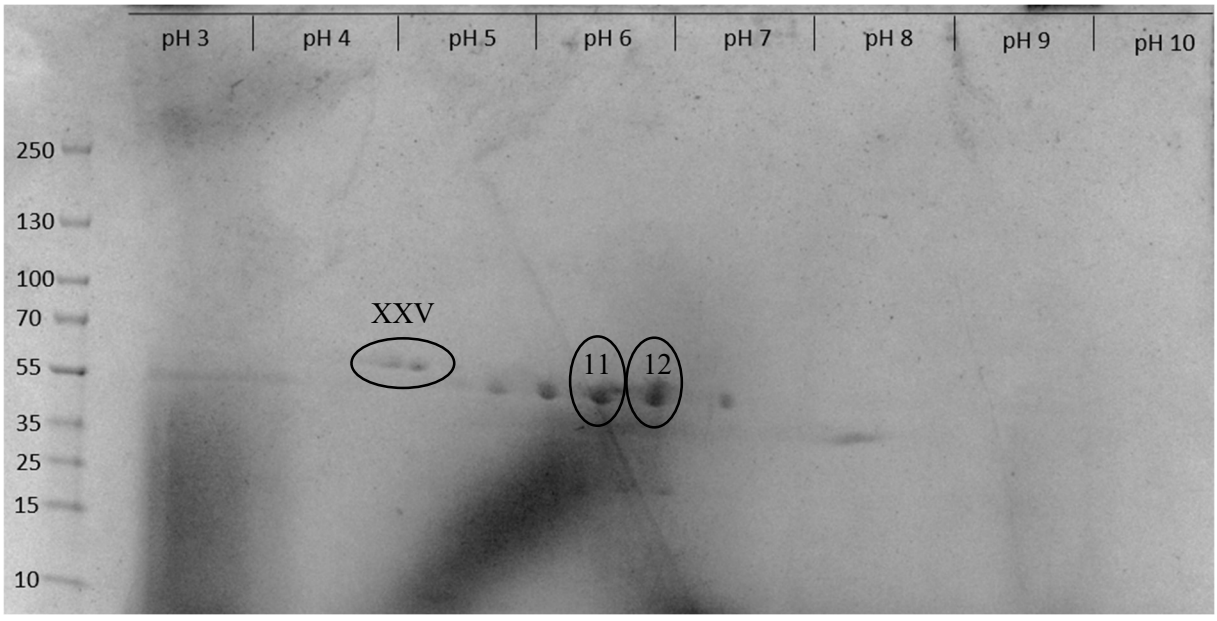

Figure 27: 2D-GE of prolamin fraction of 250 µm grain size barley cultivar Wild Barley, extracted under non-reducing conditions. No DTE was added to IPG lysis buffer. An IPG strip with a pH range from 3-10 was used. A protein ladder ranging from 10-250 kDa was used as reference. Protein bands XXV are better separated under non-reducing conditions. Protein spots 11-12 were subjected to identification with MALDI-ToF MS.

4.4 Proteins in Disulfide Network

The following proteins, displayed in **Table 6** resulted in a significant identification with MALDI-ToF-MS.

Table 6: Significant proteins identified in 1D-GE and 2D-GE samples with MALDI-ToF MS.

Sample ID	*Proteins identified*	*Accession Key*	*Name*	*Database*	*Score*	*Matched Peptides*
1	HOR3_HORVU	P06471	B3-Hordein	Barley	38/38	5
2			No Result			
	F2CWQ7_HORVV	F2CWQ7	Predicted Protein	Barley	68/60	7
3	C76BA_SWEMU	D1MI46	Geraniol 8-Hydroxylase	UniProtKB	58/55	6
4-5			No Result			
6	I6TMW0_HORVU	I6TMW0	B1-Hordein	Barley	63/60	6
	I6SW25_HORVU	I6SW25	B1-Hordein	Barley	62/60	6
	I6TRT2_HORVU	I6TRT2	B1-Hordein	Barley	61/60	6
	I6SJ22_HORVU	I6SJ22	B1-Hordein	Barley	60/60	6
	AT13A_ARATH	Q9SCK0	Autophagy-related protein	UniProtKB	58/58	7
7	F2CWQ7_HORVV	F2CWQ7	Predicted protein	Barley	63/60	7
	FB222_ARATH	Q9M0U2	Putative F-box protein	UniProtKB	60/58	5
8	C76BA_SWEMU	D1MI46	Geraniol 8-hydroxylase	UniProtKB	61/58	8
9	HOR3_HORVU	P06471	B3-Hordein	Barley	39/38	6
	F2CWQ7_HORVV	F2CWQ7	Predicted Protein	Barley	89/60	10
10-14			No Result			
15	CASA1_BOVIN	P02662	Alpha-S1-casein	UniProtKB	84/70	7
16	SAS1_PARBF	P22065	SASP	UniProtKB	81/70	7
17	SAS1_PARBF	P22065	SASP	UniProtKB	70/70	7
18	TOLB_WOLSU	Q7MA16	Tol-Pal system protein TolB	UniProtKB	71/70	7
BSA (0.01)			No Result			
BSA (0.1)	ALBU_BOVIN	P02769	Albumin	UniProtKB	146/58	19

A total of 18 proteins with a significant score were identified. Nine of those were matched against the in-house built barley database, which includes both reviewed and unreviewed proteins from UniProtKB. Nine proteins were identified by searching against the whole UniProtKB database. Some proteins were identified in more samples, for example HOR3_HORVU (sample 1 and 9), protein F2CWQ7_HORVV (sample 3, 7 and 9), protein SAS1_PAREF (sample 16 and 17) and protein C76BA_SWEMU (sample 3 and 8). Other samples resulted in more than one significant hit, for example sample 3 (2 significant proteins identified), sample 6 (5 significant proteins identified), sample 7 (2 significant proteins identified) and sample 9 (2 significant proteins identified). In some samples no protein was identified (sample 2, 4, 5, 10-14, BSA 0.01 mg*mL^{-1}). BSA was only identified in the higher concentrated sample (0.1 mg*mL^{-1}).

The following overview in **Table 7** gives information about the proteins that were found in the respective samples, retrieved from UniProtKB. The pI and molecular mass were calculated on Expasy based on the sequence from UniProtKB. All of the proteins identified with the barley database are unreviewed proteins on UniProtKB, except for B3-Hordein.

Table 7: Overview of the proteins found in the 2D-GE protein spots with a significant score against the in-house built barley database and their characteristics. Information was retrieved from UniProtKB.

Protein	***Organism***	***Amino acids***	***Mass (kDa)***	***pI***	***Re-viewed***	***Evidence***	***Function or Characteristics***
HOR3_HORVU (B3-Hordein)	*Hordeum vulgare*	264	30.195	7.74	Yes	Protein level	Sulfur-rich seed storage protein; belongs to prolamin or glutelin family; eight cysteine residues.
F2CWQ7	*Hordeum vulgare* subsp. *vulgare* (Dom. Barley)	358 (Fragment)	40.566	10.03	No	Transcript level	Function unknown; three cysteine residues.

Protein	*Organism*	*Amino acids*	*Mass (kDa)*	*pI*	*Re-viewed*	*Evidence*	*Function or Characteristics*
I6TMW0 (B1-Hordein)	*Hordeum vulgare*	253	29.127	7.59	No	Transcript level	Nutrient reservoir activity; eight cysteine residues.
I6SW25 (B1-Hordein)	*Hordeum vulgare*	267	30.844	8.14	No	Transcript level	Nutrient reservoir activity; eight cysteine residues.
I6TRT2 (B1-Hordein)	*Hordeum vulgare*	278	32.153	8.14	No	Transcript level	Nutrient reservoir activity; eight cysteine residues.
I6SJ22 (B1-Hordein)	*Hordeum vulgare*	286	32.798	8.68	No	Transcript level	Nutrient reservoir activity; eight cysteine residues.

Table 8 presents an overview of all the proteins that were matched against the UniProtKB database and their characteristics. Information about the proteins was retrieved from UniProtKB. The pI and molecular mass were calculated on Expasy based on the sequence from UniProtKB.

Table 8: Overview of the proteins found in the 2D-GE protein spots with a significant score against the UniProtKB database and their characteristics. Information was retrieved from UniProtKB.

Protein	*Organism*	*Amino acids*	*Mass (kDa)*	*pI*	*Reviewed*	*Evidence*	*Function/Characteristics*
D1MI46	*Swertia mussotii*	495	55.456	8.85	Yes	Protein level	Hydroxylase (Cytochrome P450 family); involved in hydroxygeraniol synthesis, a precursor of sweriamarin.
Q9SCK0	*Arabidopsis thaliana*	603	66.564	9.13	Yes	Protein level	Connects autophagy to plant nutritional status (targets autophagy under nutrient starvation); Involved in autophagosome enclosure and/or vacuolar delivery events.

Protein	*Organism*	*Amino acids*	*Mass (kDa)*	*pI*	*Reviewed*	*Evidence*	*Function/Characteristics*
Q9M0U2	*Arabidopsis thaliana*	132	14.890	9.87	Yes	Predicted	-
P02662	*Bos taurus*	214	24.529	4.98	Yes	Protein level	Important role in the capacity of milk to transport calcium phosphate
P22065	*Para-clostridium bifermentans*	70	7.696	9.05	Yes	Protein level	SASP are bound to spore DNA and protect the DNA backbone from chemical and enzymatic cleavage
Q7MA16	*Wolinella succino-genes*	421	47.680	8.36	Yes	Inferred from homo-logy	Part of the Tol-Pal system. Important for maintaining outer membrane integrity

5 Discussion

The objective of this master thesis was to develop a method for protein profiling of three distinct barley cultivars (PS3, Golden Promise, and Wild Barley) that enables comparison under both reducing and non-reducing conditions. The aim was to identify proteins involved in the formation of a gluten network, which is driven by disulfide bonds, using MALDI-ToF analysis. Comparing the results derived from the experiments on protein extraction and quantification, it is evident that the efficiency of the glutelin extraction is insufficient in comparison to values reported in literature[17]. This may stem from several factors such as a limited solubility of the proteins, the requirement of harsh extraction conditions, or the possibility that glutelin proteins might be dispersed within the other three fractions since it is the last fraction that is extracted[25]. This could also account for the higher percentage of albumin and globulin proteins observed, which should be considerably lower according to literature[17]. Considering the low protein content in the pellet after extraction, it is more likely that the proteins are dispersed within the other fractions than being present in the protein pellet. It was stated in the introduction that the glutelin proteins in barley add up to 35-45 % of the total protein content, while only around 10 % of the proteins are still present in the sample pellet. One way to enhance extraction efficiency of the glutelin proteins would be to extract them before the other fractions, which could, however, also encounter the problem of contamination with prolamin proteins. Regarding the impact of grain size on extraction efficiency, it was found that the efficiency was higher for the 45 µm grain size flour than for the unsieved flour. But overall, the effect of grain size on extraction efficiency was minimal. The greatest glutelin concentration was found in the unsieved and 500 µm grain-sized flour, suggesting that glutelin proteins predominantly exist in molecular structures larger than 500 µm.

Following the comparison of three protein quantification methods, the Bradford Assay was selected for subsequent analysis. The Bradford Assay is a method to determine the quantity of proteins in a sample by using the Coomassie Brilliant Blue dye, which binds to cationic and apolar side chains of amino acid in an acidic solution. Upon binding to proteins, the absorbance wavelength of the dye shifts from 465 nm to 595 nm, with the intensity correlating to the protein concentration, which can be measured photometrically. The Bradford Assay offers simplicity, time efficiency, stability and robustness.[75] However, its specificity to cationic and apolar amino acid side chains can have a negative effect on the accuracy of the results. In the conducted experiments, the overall concentration of proteins was measured rather than the concentration of specific proteins, assuming an even distribution of proteins with varying cationic and apolar

amino acid content. In comparison to the other two methods, the Bradford Assay showed a higher standard deviation than the Dumas or the Nanodrop method, indicating a lower repeatability of the measurement.

The Dumas method involves combustion of a sample in an oxygen flow at 900 °C, where its organic compounds are oxidized. The resulting gases are trapped and eliminated, except for nitrogen and nitrogen oxides, which are later converted into elementary nitrogen. The remaining nitrogen is measured, and by applying a sample-specific factor, the protein content as a percentage of the sample weight can be calculated. This factor is dependent on the amino acid composition of the protein. In this case, a mixture of various different proteins is measured using the Dumas method, which can result in an inaccurate determination of the protein content.[76] Advantages of the Dumas method include speed, the ability to measure the protein content of solid samples and the avoidance of toxic chemicals[77]. However, due to the relatively low concentration of proteins in the extracts, a large sample volume is required in order to stay within the instrument's calibration range. Additionally, the Dumas method measures the whole nitrogen content, in contrast to the nitrogen of proteins, potentially leading to inaccurate results, although this is partly compensated for with the sample-specific protein factor. This could explain why the Dumas method results in a slightly higher protein concentration compared to the Bradford Assay when measuring the same samples. The standard deviation of the Dumas measurements was lower than the Bradford Assay but higher than the Nanodrop measurement. However, the error bars for the Nanodrop method only represent technical replicate variability, unlike the other measurements, where biological replicates where measured, making a direct comparison inaccurate.

The Nanodrop measures the absorbance of the sample at 280 nm and automatically converts it into the protein concentration in $mg*mL^{-1}$. At 280 nm, the absorption is related to the sample's tryptophan, tyrosin and cysteine amino acid composition. The Nanodrop's wide linear range eliminates the need for sample dilution, increasing its practicality. Additionally, only a minimal sample volume of 1-2 µL is needed for measurement. The comparison of all three methods clearly indicates that the Nanodrop yields the highest protein concentration. One possible explanation for this is that the absence of nucleic acid removal from the samples, which can significantly increase the protein concentration. The ratio of the absorbance values at 260 nm and 280 nm is an indicator for the sample purity, since nucleic acids and proteins have absorbance maxima at 260 nm and 280 nm, respectively.[78] In this case, the experimentally obtained values significantly exceeded the maximum recommended value of 0.7, signifying

that the method is unsuitable for the measurement of the protein quantity in these samples. Furthermore, the measurement is based on the amino acid composition and could therefore be inaccurate if multiple different proteins with a varying tryptophan, tyrosin and cysteine concentration are measured. After careful consideration of the advantages and drawbacks of each method, further experiments were conducted using the Bradford Assay, as no further purification of the samples from nucleic acids was done and the Bradford Assay required significantly less sample volume compared to the Dumas method.

Regarding the results obtained through 1D-GE and 2D-GE, **Figure 20** shows the absence of protein bands below 25 kDa. This could potentially be an artifact from the preceding dialysis process. Although a dialysis membrane with a molecular weight cutoff of 6-8 kDa was used, and the GE of the globulin fraction, which was also dialysed and still shows smaller proteins below 25 kDa, the possibility of certain proteins with slightly higher molecular weights migrating through the membrane cannot be completely excluded. However, it is worth noting that barley prolamin proteins are typically situated within the molecular weight range of 30-100 kDa, suggesting the absence of proteins below this molecular weight. While no glutelin proteins have been identified yet, it remains possible that there may exist low-molecular weight variants. The application of dialysis prior to analysis with 1D- or 2D-GE might have removed such proteins, making it uncertain whether proteins with a molecular weight below 25 kDa are generally present or not[27]. The absence of proteins in **Figure 21** can likely be attributed to the insufficient extraction method for the glutelin proteins, as presented in **Figure 13**, or their dispersion in other fractions, as mentioned earlier[25].

Furthermore, the results indicated differences in the protein composition between two analytical methods, 1D-GE and 2D-GE, applied to the same cultivar. For example, the three protein spots labelled as XXIII in the 2D-GE in **Figure 26** correlate with the two protein bands labelled as XX in the 1D-GE in **Figure 20,** based on their respective molecular weight. In the 1D-GE, only two protein bands are observed. The discrepancy in the number of bands between 1D-GE and 2D-GE may be (i) due to the fact that one protein band has a significantly higher intensity, overshadowing a possible second band, or (ii) that the proteins have the same molecular weight, but differ in their isoelectric point.

Moreover, differences in the protein composition among the three barley cultivars were found. Specifically, differences within a molecular weight of 45 - 70 kDa (XXI, XIX, XX, XXI in **Figure 20**), which corresponds to proteins belonging to the C-hordein family, were the most prominent. This suggests that the C-hordein profile of the sample is cultivar-dependent.

By comparing the effect of reducing and non-reducing conditions during extraction on the 1D-GE, the impact of reduction of mostly inter-, but also intramolecular disulfide bridges, formed between cysteine residues in proteins, is investigated. Reduction of disulfide bonds involves converting oxidized cystine (S-S) residues to two sulfhydryl groups of cysteines (S-H). Intermolecular disulfide bonds lead to protein aggregation and contribute to the stability of those aggregates. Reduction of disulfide bonds decreases protein stability, facilitating protein extraction[79]. In contrast to expectations, **Figure 14** shows a higher protein content under non-reducing conditions. It is important to note that this experiment was only conducted once, and these results may also be attributes to technical and biological variability in the analysis. To confirm these results, further repetitions of the experiment are necessary.

Comparing the same samples prepared in four sample preparation variations on the 1D-GE in **Figure 20**, a higher effect on the change of the protein profile is observed when DTE is added during the extraction process rather than solely adding in to the 1D-GE sample buffer. This suggests that DTE may facilitate the extraction of the proteins, resulting in a higher concentration or greater number of proteins present in the samples prepared under reducing conditions, which could be confirmed experimentally. This might also explain why the difference between the albumin and globulin fraction on the 1D-GE are only marginal, since they were both extracted under the same conditions. By comparing the effect of DTE in two samples that were already extracted under reducing conditions, the influence of storage on the potential reformation of disulfide bonds is examined. The results indicate that the effect of DTE may diminish over time, as seen in the prolamin fraction in all three barley cultivars and wheat.

Figure 20 shows that samples extracted under reducing conditions exhibit a higher number of distinct protein bands, especially at a molecular weight below 40 kDa. One possible explanation is that the formation of intermolecular disulfide bridges between proteins can result in protein aggregates with a higher mass. This can be observed in sample 4, which does not contain any reducing agent and shows a high intensity band at 260 kDa, whereas the same band is absent in sample 1, which contains DTE in both extraction process and sample buffer. Sample 1, however, shows two additional protein bands at a molecular weight of 35-40 kDa, that are likely to form intermolecular disulfide bridges. Certain wells (2, 4, 6, 8, 10) even contain an intense black line in the bottom of the well. These samples have in common that no DTE was added to the sample buffer, suggesting that protein aggregates may still be intact and result in a molecular weight that is too high to migrate in the 1D-GE gel, and thus cannot be observed.

This discovery is important to consider when looking at the proteins in the gluten network. As mentioned in the introduction, the key in building the gluten network in wheat is the formation of intermolecular disulfide bonds between LMW-GS and HMW-GS[29,33–35]. This leads to the conclusion that the two protein bands, labelled as XVIII in **Figure 20**, present in all three barley cultivars and wheat, may potentially contribute to the formation of the gluten network. In barley, high-molecular weight hordein subunits (D-hordein), with a molecular weight of about 100 kDa are believed to serve as the structural backbone of the gluten network by forming disulfide bonds with low-molecular weight subunits. D-hordein is homologous to the HMW-GS proteins in wheat, which have a major influence on the gluten network formation and therefore the bread-baking qualities of wheat flour[40]. Supporting this statement, it was reported that D-hordein can only be extracted under high concentrations of a reducing agent[6,34,39]. This was experimentally confirmed by observing the protein band at a molecular weight of 100 kDa (XXIII in **Figure 20**). This band is believed to be D-hordein and is only visible on the gel under reducing conditions during extraction and in the presence of DTE in the sample buffer. In comparison to wheat, it is evident that the concentration of HMW-GS is significantly higher than the D-hordein in barley. This corresponds to the statement that D-hordein only makes 5 % of the total hordein content in barley, while HMW-GS make 45 % of the total gluten protein content in wheat[29,35]. Furthermore, three different protein bands above a molecular weight of 70 kDa can be seen in wheat, indicating a higher diversity of HMW-GS in wheat. These protein bands may correspond to different x- and y-HMW-GS, that are also affected by reducing conditions, thus verifying that they are involved in the gluten network. Unfortunately, it was not confirmed by the company HavneMøllen (Vejle, Denmark), which cultivar was employed to produce the wheat flour used in the experiments, making it challenging to determine the precise HMW-GS type present solely based on literature research.

There is a structural similarity between LMW-GS in wheat and B-hordeins in barley. Existing literature suggests the presence of at least 10 different B-hordeins in barley, that can be divided into two types, B1- and B3-Hordeins, both with a mass of around 30 kDa. On the 2D-GE, multiple spots around this molecular weight can be seen. However, the mass as well as the pI of the B-hordeins overlap with those of γ-hordeins, making it challenging to precisely identify the protein spots based on the 2D-GE. In **Figure 24** and **Figure 25**, it can be observed that all proteins with a molecular weight of less than 40 kDa are only visible in reducing conditions and not under non-reducing conditions. Among the γ-hordeins, there are three different types, γ1-, γ2- and γ3-Hordeins. Literature indicates that γ1- and γ2-Hordeins form intermolecular disulfide bridges, while γ3-Hordein only forms intramolecular disulfide bridges. Based on this

information, protein band XXXII in **Figure 20** can be identified as γ3-Hordein.[80] It is not affected by the presence of DTE, confirming the hypothesis that it does not form intermolecular disulfide bonds. When looking at **Figure 20**, it can be concluded that both B-and γ-hordeins participate in a disulfide network, as their profile changes between reducing and non-reducing conditions.

Some of those protein spots, that are considered to be involved in the gluten network (spot 1-14 in **Figure 22** - **Figure 27**) were chosen for identification with MALDI-ToF MS. Sample 1-6 in **Figure 24**, 7-9 in **Figure 26** and 13-14 in **Figure 22** are only present under reducing conditions, which means that they are forming intermolecular disulfide bonds, making them possible candidates for participation in the barley gluten network. The identified proteins P06471, F2CWQ7, I6TMW0, I6SW25, I6TRT2 and I6SJ22 within these spots are potentially involved in the formation of the gluten network in barley. Samples 10-12 in **Figure 26** and **Figure 27** are present in both conditions, therefore it is assumed that they are not forming intermolecular disulfide bonds. These three protein spots did not result in an identification with MALDI-ToF MS. Among the identified proteins is F2CWQ7_HORVV, which was detected in samples 3, 7 and 9 and is associated with the organism *Hordeum vulgare* subsp. *vulgare* (Domesticated Barley), according to UniProtKB. However, it was also identified in the Wild Barley cultivar. Most of the identified proteins belong to the B-hordein fraction. Four proteins (I6TMW0, I6SW25, I6TRT2 and I6SJ22), which all belong to the B1-Hordein group, show high similarities in their amino acid sequence.

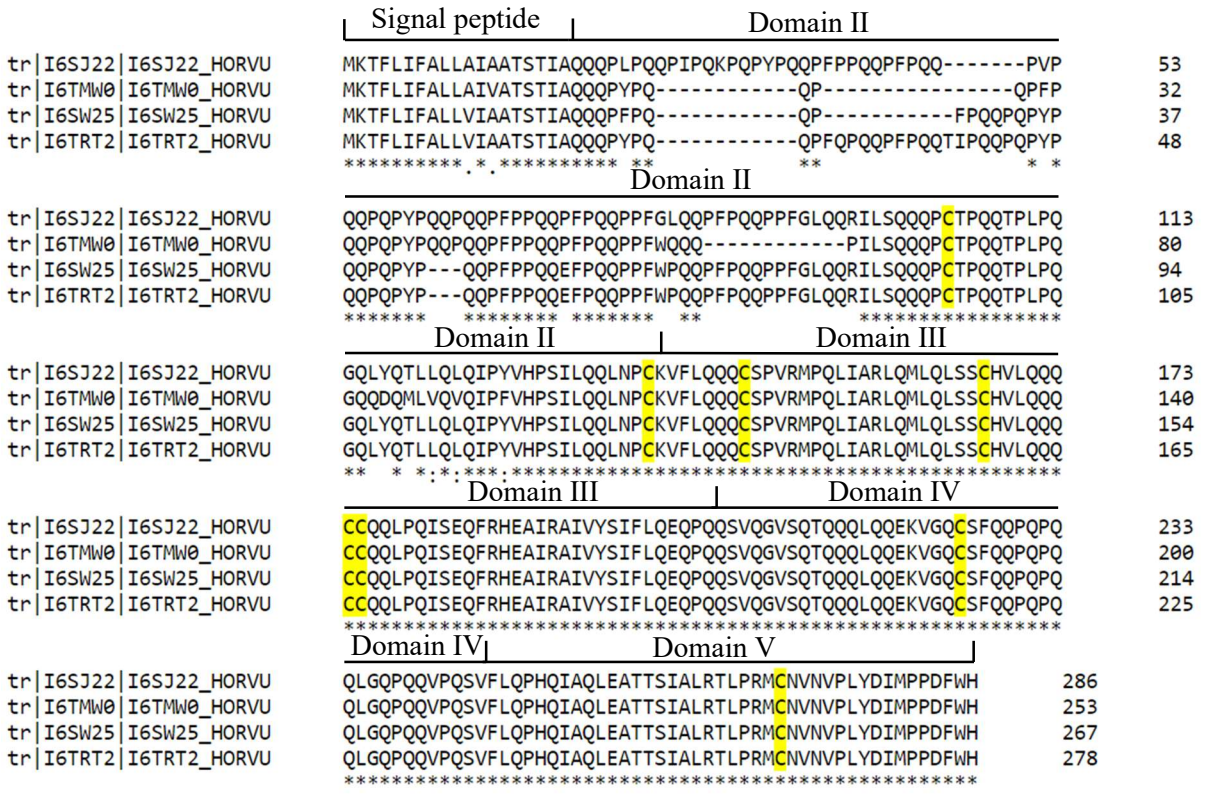

Figure 28: Sequence alignment on UniProtKB of barley B-hordein proteins I6TMW0, I6SW25, I6TRT2 and I6SJ22, that were identified with MALDI-ToF MS in sample 6. An asterisk (*) indicates a fully identical amino acid residue between all groups, a colon (:) indicates strongly similar properties between the groups and a period (.) indicates weakly similar properties. Cysteine residues (C) are marked in yellow. Regarding to Anderson, 2013, the protein sequence of B-hordeins can be divided into five different domains[28]. A short signal peptide, followed by a region of short repeat motifs with slight variations (domain II), a nonrepetitive domain (III), a glutamine-rich domain (IV) and a C-terminal domain (V).

Figure 28 shows a sequence alignment conducted on UniProtKB. Regarding to Anderson, 2013, the protein sequence of B-hordeins can be divided into five different domains[28]. A short signal peptide, followed by a region of short repeat motifs with slight variations (domain II), a nonrepetitive domain (III), a glutamine-rich domain (IV) and a C-terminal domain (V). There are eight cysteine residues in every protein, marked in yellow in **Figure 28** which are able to form inter- and intramolecular disulfide bonds.

A simplified illustration of the five different domains of the B1-Hordein proteins can be found in **Figure 29**. As stated above, the B-hordein structure is similar to LMW-GS in wheat. In the LMW-GS, an additional short nonrepetitive domain separates the signal peptide from the repetitive domain[28]. It is suggested by Anderson, 2013 that six out of eight cysteine residues, marked in red in **Figure 29**, form intramolecular disulfide bonds. The cysteine residues that are likely to form intramolecular disulfide bonds are connected with arrows. Consequently,

cysteine residues 1 and 7 could potentially form intermolecular disulfide bonds. Although the ability of B-hordeins to form intermolecular disulfide bonds has not been experimentally determined yet, it is assumed that they have a similar ability as LMW-GS, given their comparable number and position of cysteine residues.[28] Therefore, it can be concluded from these results that the identified B-hordein proteins are likely to form intermolecular disulfide bonds and potentially participate in the formation of the barley gluten network.

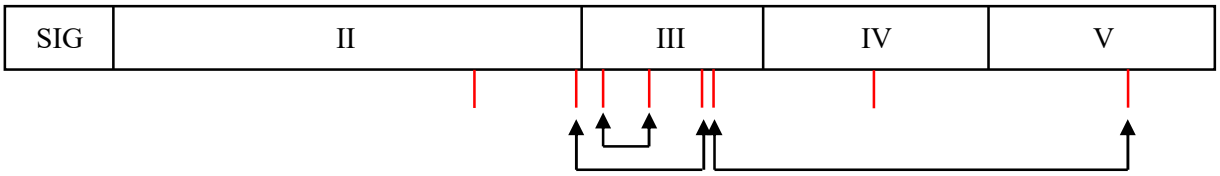

Figure 29: Simplified illustration of the five different structural domains of B-hordeins, as described in Anderson, 2013[28]. These domains are a short signal peptide, followed by a region of short repeat motifs with slight variations (domain II), a nonrepetitive domain (III), a glutamine-rich domain (IV) and a C-terminal domain (V). The eight cysteine residues are marked in red and the ones that are believed to form intramolecular disulfide bonds among themselves are linked by arrows. The remaining cysteine residues can potentially form intermolecular disulfide bonds.

In comparison to other cultivars, the Golden Promise cultivar shows one extra protein band (XXV in **Figure 20**), indicating the potential presence of an additional B- or γ-hordein or glutelin proteins that are dispersed in the prolamin fraction. Golden Promise also shows the highest number of individual proteins below a molecular weight of 40 kDa on the 2D-GE in **Figure 24**. Preliminary baking trials conducted at Aarhus University indicate that Golden Promise has less optimal baking qualities than PS3. This may be due to the low concentration of D-hordein in Golden Promise, as seen in **Figure 20**. Additionally, it is possible that the additional B- or γ-hordeins in Golden Promise form intermolecular disulfide bonds between themselves, hindering a bond formation with D-hordein. To validate this hypothesis, additional data on the baking qualities of both cultivars needs to be collected. In addition to the protein composition, various factors such as the presence of plant material, which can disrupt the protein binding and the reduction of water availability due to a high amount of β-glucans also has an influence on the quality of the gluten network and should be taken into consideration when comparing the baking qualities of different barley cultivars.

Furthermore, six proteins were identified with MALDI-ToF MS when searching against the UniProtKB database that show little association with the sample origin. However, the

identification of proteins from various organisms may also be due to contamination throughout the sample preparation process or false-positive peptide spectrum matches. The predicted masses from UniProtKB do not align with the molecular weight of the proteins observed on the 2D-GE (for example Q9SCK0 from *Arabidopsis thaliana* is predicted to be 66 kDa on UniProtKB but appears at 30 kDa on the 2D-GE, and D1MI46 from *Swertia mussotii* is predicted to be 55 kDa on UniProtKB and is observed at 35 kDa on the 2D-GE). This discrepancy suggests a more likely false-positive identification, contamination or degradation of the proteins. Furthermore, the protein scores are only marginally higher than the cut off value for significant proteins.

In contrast, the standard reference sample of BSA resulted in a significant score of 146/58, indicating successful trypsin digestion. Consequently, this can also be assumed for the samples from the 2D-GE, since the same chemicals and enzyme were used for both sets of samples. Although, it has to be considered that the BSA was digested in-solution in comparison to the in-gel digestion of the barley proteins. Still, the BSA samples can be used as a positive control throughout the workflow. However, the bovine serum albumin could only be identified in the highest concentration (0.1 $mg*mL^{-1}$), approximately ten times higher than the protein concentration in a gel spot. The lowest concentration (0.01 $mg*mL^{-1}$) of BSA, which should correspond to the concentration of a protein spot in a 2D-GE gel did not result in a significant identification. This suggests that the protein concentration in samples 2, 4-5 and 10-14, that did not result in an identification, is too low for analysis with MALDI-ToF MS. Improving peptide extraction from the gel or enhancing enrichment with the desalting column may increase the chances of successful identification or using more sensitive methods like LC-MS.

In general, the score for proteins identified in barley is considerably lower compared to the score obtained for BSA, further increasing the risk of false-positive peptide spectrum matches. Additionally, many of the identified proteins are still unreviewed, meaning that they have only been identified on mRNA level and not yet on protein level. Moreover, an in-silico digest of the four identified B1-Hordeins in sample 6 (I6TMW0, I6SW25, I6TRT2 and I6SJ22) revealed that they mostly result in the same peptides. None of the peptides specific to individual proteins within this group were detected in the mass spectrum, making it uncertain how many of them are truly present in the sample. The high similarity between individual B-hordeins has to be considered for future analysis with mass spectrometry.

6 Conclusion

In the course of this master thesis, an extraction protocol for Osborne fractionation of barley proteins was established. The isolation properties of glutelins through this method was found to be inadequate. Additionally, the effect of grain size on extraction efficiency was examined, revealing only minimal influence within the grain sizes used. Moreover, a comparative analysis was conducted to evaluate three protein quantification methods. The results indicated that the Bradford Assay yielded the most reliable outcomes and was consequently selected for further experiments.

A comparison between the barley protein profiles obtained through 1D-GE and 2D-GE highlighted differences among the barley cultivars used, PS3, Golden Promise and Wild Barley, particularly in terms of their C-hordein composition. Furthermore, by comparing reducing and non-reducing conditions, proteins affected by disulfide bond formation, which serves as the basis for the development of a gluten network, were examined. The main proteins found to interact within the disulfide network were B- and γ-hordeins. Due to their similar molecular weight and pI, identifying them solely based on the GE and literature research is challenging. Certain protein spots involved in the disulfide network were subjected to trypsin digestion for identification using MALDI-ToF MS. Six proteins (P06471, F2CWQ7, I6TMW0, I6SW25, I6TRT2 and I6SJ22), potentially involved in the barley gluten structure formation were identified, all of which belong to the B-hordein subgroup. Consequently, it can be proposed that the barley gluten network comprises D-hordeins, acting as the structural backbone of the gluten structure, similar to HMW-GS in wheat, while B- and γ-hordeins function as the monomeric units similar to LMW-GS in wheat.

Preliminary results obtained from baking quality assessments of PS3 and Golden Promise, conducted at Aarhus University, revealed that Golden Promise exhibited weaker bread baking characteristics. This cultivar also shows the greatest difference in the protein profile between reducing and non-reducing conditions in 1D-GE and 2D-GE. Hence, a prospective area of research involves determining whether the B- and γ-hordeins in Golden Promise form intra- or intermolecular disulfide bonds amongst themselves, hindering bond formation with D-hordein. Overall, the concentration and diversity of HMW proteins in barley is lower than in wheat, which may also contribute to the inferior gluten structure. In addition to the protein composition, other factors such as the presence of plant material, the quantity of β-glucans and other types of protein interactions also influences the quality of the gluten network and should therefore be taken into consideration.

7 Supplementary Material

The following **Table 9**-**Table 14** contain the measured values displayed in **Figure 12** - **Figure 17**.

Table 9: Measured absorbance using the Bradford Assay displayed in **Figure 12**. Samples were measured in biological (BD) and technical (TD) duplicates. Second column shows the average of the blank-corrected measured absorbance from the TD. Third column shows the calculated protein concentration in mg*mL^{-1}. Calibration Curve used for the calculation: y = 1.0684x – 0.0181, R^2 = 0.9934 with a dilution factor of 10. Fourth column shows the average and fifth column the standard deviation of the BD, corrected to their respective sample weight. x̄ is an abbreviation for average and SD for standard deviation.

Fraction	*x̄ Absorbance of TD*	*Concentration in mg*mL^{-1}*	*x̄ Concentration of BD [mg*mL^{-1}]*	*SD of BD [mg*mL^{-1}]*
		Extraction Method 1		
Albumin BD 1	0.086	0.974	1.00	0.13
Albumin BD 2	0.107	1.171		
Globulin BD 1	0.075	0.871	0.94	0.19
Globulin BD 2	0.105	1.152		
Prolamin BD 1	0.113	1.222	1.20	0.08
Prolamin BD 2	0.127	1.353		
Glutelin BD 1	0.005	0.216	0.20	0.01
Glutelin BD 2	0.004	0.207		
		Extraction Method 2		
Albumin BD 1	0.009	0.254	0.26	0.03
Albumin BD 2	0.013	0.291		
Globulin BD 1	0.172	1.775	1.72	0.04
Globulin BD 2	0.178	1.831		
Prolamin BD 1	0.034	0.483	0.47	0.01
Prolamin BD 2	0.035	0.492		
Glutelin BD 1	-0.002	0.155	0.16	0.03
Glutelin BD 2	0.003	0.193		

Table 10: Calculated total protein content in % for **Figure 13**. The concentration in mg*mL^{-1} was multiplied by the sample volume (2.5 mL for albumin and globulin, 4.5 mL for prolamin and 3.75 mL for glutelin) and the percentage of the total amount was calculated. x̄ is an abbreviation for average and SD for standard deviation.

Fraction	*Total amount of protein in mg*	*Percentage of total protein content in %*
	Extraction Method 1	
Albumin and Globulin	4.9	44
Prolamin	5.4	49
Glutelin	0.7	7
	Extraction Method 2	
Albumin and Globulin	5.9	70
Prolamin	2.11	25
Glutelin	0.41	5
	Literature-based values	
Albumin and Globulin	-	15
Prolamin	-	40
Glutelin	-	45

Table 11: Measured absorbance using the Bradford Assay displayed in **Figure 14**. Samples were measured in biological (BD) and technical (TD) duplicates. Second column shows the average of the blank-corrected measured absorbance from the TD. Third column shows the calculated protein concentration in mg*mL^{-1}. Calibration Curve used for the calculation: y = 0.1476x – 0.0053, R^2 = 0.9840 with a dilution factor of 10. Fourth column shows the average and fifth column the standard deviation of the BD, corrected to their respective sample weight. x̄ is an abbreviation for average and SD for standard deviation.

Extraction Solvent	*x̄ Absorbance of TD*	*Concentration [mg*mL^{-1}]*	*x̄ Concentration of BD [mg*mL^{-1}]*	*SD of BD [mg*mL^{-1}]*
		Non-Reducing		
Ethanol BD 1	0.017	1.18	1.00	0.26
Ethanol BD 2	0.011	0.81		
2-Propanol BD 1	0.024	1.66	1.65	0.01
2-Propanol BD 2	0.024	1.64		
		Reducing		
Ethanol BD 1	0.011	0.79	0.86	0.10
Ethanol BD 2	0.013	0.93		
2-Propanol BD 1	0.016	1.14	1.21	0.10
2-Propanol BD 2	0.018	1.28		

Table 12: Measured absorbance using the Bradford Assay displayed in **Figure 15.** Samples were measured in biological (BD) and technical (TD) duplicates. Second column shows the average of the blank-corrected measured absorbance from the TD. Third column shows the calculated protein concentration in mg*mL^{-1}. Calibration Curve used for the calculation: y = 0.5818x + 0.0175, R^2 = 0.9977 with a dilution factor of 5 for albumin and globulin, 10 for prolamin and 2 for glutelin. Fourth column shows the average and fifth column the standard deviation of the BD, corrected to their respective sample weight. x̄ is an abbreviation for average and SD for standard deviation.

Fraction	x̄ *Absorbance of TD*	*Concentration [mg*mL^{-1}]*	x̄ *Concentration of BD [mg*mL^{-1}]*	*SD of BD [mg*mL^{-1}]*
		45 µm		
Albumin BD 1	0.0605	0.67	0.70	0.04
Albumin BD 2	0.0675	0.73		
Globulin BD 1	0.0260	0.37	0.44	0.10
Globulin BD 2	0.0430	0.51		
Prolamin BD 1	0.0410	1.00	1.00	0.01
Prolamin BD 2	0.0400	0.99		
Glutelin BD 1	-0.0025	0.06	0.08	0.03
Glutelin BD 2	0.0115	0.10		
		63 µm		
Albumin BD 1	0.0255	0.37	0.56	0.26
Albumin BD 2	0.0685	0.74		
Globulin BD 1	0.0980	1.00	0.93	0.10
Globulin BD 2	0.0810	0.85		
Prolamin BD 1	0.0310	0.84	1.02	0.26
Prolamin BD 2	0.0520	1.20		
Glutelin BD 1	-0.0105	0.02	0.05	0.04
Glutelin BD 2	0.0045	0.08		
		125 µm		
Albumin BD 1	0.0335	0.43	0.66	0.33
Albumin BD 2	0.0885	0.89		
Globulin BD 1	0.0390	0.49	0.58	0.13
Globulin BD 2	0.0600	0.67		
Prolamin BD 1	0.0260	0.73	0.87	0.21
Prolamin BD 2	0.0430	1.01		
Glutelin BD 1	0.0125	0.10	0.10	0.00
Glutelin BD 2	0.0105	0.10		
		250 µm		
Albumin BD 1	0.0265	0.37	0.60	0.33
Albumin BD 2	0.0815	0.84		
Globulin BD 1	0.0660	0.72	0.70	0.03

Fraction	*x̄ Absorbance of TD*	*Concentration [mg*mL^{-1}]*	*x̄ Concentration of BD [mg*mL^{-1}]*	*SD of BD [mg*mL^{-1}]*
Globulin BD 2	0.0610	0.68		
Prolamin BD 1	0.0020	0.33	0.53	0.29
Prolamin BD 2	0.0260	0.74		
Glutelin BD 1	-0.0005	0.06	0.08	0.03
Glutelin BD 2	0.0115	0.10		
		500 µm		
Albumin BD 1	0.0525	0.60	0.67	0.10
Albumin BD 2	0.0695	0.74		
Globulin BD 1	0.0260	0.39	0.49	0.15
Globulin BD 2	0.0430	0.59		
Prolamin BD 1	0.0180	0.61	0.79	0.27
Prolamin BD 2	0.0400	0.98		
Glutelin BD 1	0.0195	0.12	0.13	0.01
Glutelin BD 2	0.0235	0.14		
		Unsieved		
Albumin BD 1	0.0435	0.52	0.49	0.04
Albumin BD 2	0.0375	0.47		
Globulin BD 1	0.0370	0.46	0.47	0.01
Globulin BD 2	0.0380	0.47		
Prolamin BD 1	0.0230	0.69	0.75	0.09
Prolamin BD 2	0.0300	0.81		
Glutelin BD 1	-0.0135	0.01	0.07	0.08
Glutelin BD 2	0.0195	0.13		

Table 13: Measured nitrogen content in percentage using the Dumas method displayed in **Figure 16**. Samples were measured in biological (BD) and technical (TD) duplicates. Second column shows values that are already blank corrected. Third column shows the calculated protein concentration in mg*mL^{-1} in 150 µL of sample. Fourth and fifth column show the average and standard deviation of biological (BD) duplicates. Negative values were set to value 0. x̄ is an abbreviation for average and SD for standard deviation.

Fraction	*Measured Nitrogen in %*	*Protein Concentration [mg*mL^{-1}]*	*x̄ Protein Concentration [mg*mL^{-1}]*	*SD [mg*mL^{-1}]*
		45 µm		
Albumin BD 1	0.0180	1.01	1.07	0.09
Albumin BD 2	0.0199	1.14		
Globulin BD 1	0.0196	1.13	1.05	0.12

Fraction	*Measured Nitrogen in %*	*Protein Concentration [mg*mL^{-1}]*	*x̄ Protein Concentration [mg*mL^{-1}]*	*SD [mg*mL^{-1}]*
Globulin BD 2	0.0169	0.96		
Prolamin BD 1	0.0363	1.83	1.87	0.05
Prolamin BD 2	0.0379	1.90		
Glutelin BD 1	-0.0018	-0.11	0.00	0.04
Glutelin BD 2	-0.0031	-0.16		
		63 µm		
Albumin BD 1	0.0249	1.43	1.54	0.15
Albumin BD 2	0.0288	1.64		
Globulin BD 1	0.0238	1.36	1.70	0.47
Globulin BD 2	0.0350	2.03		
Prolamin BD 1	0.0340	1.71	1.66	0.08
Prolamin BD 2	0.0316	1.61		
Glutelin BD 1	-0.0010	-0.06	0.00	0.12
Glutelin BD 2	-0.0038	-0.22		
		125 µm		
Albumin BD 1	0.0328	1.87	1.85	0.02
Albumin BD 2	0.0321	1.84		
Globulin BD 1	0.0477	2.75	2.47	0.39
Globulin BD 2	0.0378	2.19		
Prolamin BD 1	0.0348	1.79	1.79	0.01
Prolamin BD 2	0.0360	1.79		
Glutelin BD 1	-0.0020	-0.17	0.00	0.01
Glutelin BD 2	-0.0037	-0.19		
		250 µm		
Albumin BD 1	0.0404	2.30	2.31	0.02
Albumin BD 2	0.0409	2.32		
Globulin BD 1	0.0397	2.28	1.98	0.43
Globulin BD 2	0.0293	1.68		
Prolamin BD 1	0.0442	2.25	2.23	0.04
Prolamin BD 2	0.0437	2.20		
Glutelin BD 1	-0.0030	-0.17	0.00	0.02
Glutelin BD 2	-0.0026	-0.15		
		500 µm		
Albumin BD 1	0.0423	2.41	2.44	0.05
Albumin BD 2	0.0435	2.48		
Globulin BD 1	0.0247	1.42	1.32	0.15
Globulin BD 2	0.0218	1.22		
Prolamin BD 1	0.0285	1.45	1.53	0.11
Prolamin BD 2	0.0312	1.61		

Fraction	*Measured Nitrogen in %*	*Protein Concentration [mg*mL^{-1}]*	*x̄ Protein Concentration [mg*mL^{-1}]*	*SD [mg*mL^{-1}]*
Glutelin BD 1	-0.0030	-0.17	0.00	0.01
Glutelin BD 2	-0.0034	-0.19		
		Unsieved		
Albumin BD 1	0.0353	2.01	2.09	0.12
Albumin BD 2	0.0381	2.17		
Globulin BD 1	0.0146	0.85	0.83	0.02
Globulin BD 2	0.0142	0.81		
Prolamin BD 1	0.0334	1.73	1.69	0.06
Prolamin BD 2	0.0325	1.64		
Glutelin BD 1	-0.0033	-0.19	0.00	0.05
Glutelin BD 2	-0.0021	-0.12		

Table 14: Measured protein concentration using the Nanodrop in mg*mL^{-1} as displayed in **Figure 17**. Samples were measured in technical duplicates. For the measurement, 2 µL of sample were used. x̄ is an abbreviation for average and SD for standard deviation.

Fraction	*Protein Concentration [mg*mL^{-1}]*	*x̄ Protein Concentration [mg*mL^{-1}]*	*SD [mg*mL^{-1}]*
	45 µm		
Albumin TD 1	3.30	3.30	0.00
Albumin TD 2	3.30		
Globulin TD 1	1.29	1.29	0.00
Globulin TD 2	1.29		
Prolamin TD 1	1.98	1.99	0.02
Prolamin TD 2	2.01		
Glutelin TD 1	2.39	2.39	0.00
Glutelin TD 2	2.39		
	63 µm		
Albumin TD 1	3.99	3.99	0.00
Albumin TD 2	3.99		
Globulin TD 1	1.60	1.59	0.01
Globulin TD 2	1.58		
Prolamin TD 1	2.29	2.28	0.01
Prolamin TD 2	2.27		
Glutelin TD 1	2.27	2.28	0.01
Glutelin TD 2	2.29		

Fraction	*Protein Concentration [mg*mL^{-1}]*	x̄ *Protein Concentration [mg*mL^{-1}]*	*SD [mg*mL^{-1}]*
	125 µm		
Albumin TD 1	5.06	5.07	0.02
Albumin TD 2	5.08		
Globulin TD 1	3.56	2.26	0.00
Globulin TD 2	3.53		
Prolamin TD 1	2.46	2.51	0.07
Prolamin TD 2	2.56		
Glutelin TD 1	2.23	2.23	0.01
Glutelin TD 2	2.22		
	250 µm		
Albumin TD 1	6.17	6.17	0.01
Albumin TD 2	6.16		
Globulin TD 1	3.56	3.54	0.02
Globulin TD 2	3.53		
Prolamin TD 1	3.03	3.17	0.21
Prolamin TD 2	3.32		
Glutelin TD 1	2.55	2.55	0.00
Glutelin TD 2	2.55		
	500 µm		
Albumin TD 1	6.37	6.40	0.04
Albumin TD 2	6.43		
Globulin TD 1	4.51	4.48	0.04
Globulin TD 2	4.44		
Prolamin TD 1	3.00	2.87	0.18
Prolamin TD 2	2.75		
Glutelin TD 1	2.65	2.64	0.01
Glutelin TD 2	2.63		
	Unsieved		
Albumin TD 1	4.90	4.90	0.01
Albumin TD 2	4.91		
Globulin TD 1	2.34	2.34	0.01
Globulin TD 2	2.34		
Prolamin TD 1	2.44	2.49	0.06
Prolamin TD 2	2.53		
Glutelin TD 1	2.37	2.35	0.02
Glutelin TD 2	2.34		

8 References

(1) Pourkheirandish, M.; Komatsuda, T. The Importance of Barley Genetics and Domestication in a Global Perspective. *Annals of Botany* **2007**, *100*, 999–1008.

(2) Giraldo, P.; Benavente, E.; Manzano-Agugliaro, F.; Gimenez, E. Worldwide Research Trends on Wheat and Barley: A Bibliometric Comparative Analysis. *Agronomy* **2019**, *9*, 352–370.

(3) Sato, K. History and Future Perspectives of Barley Genomics. *DNA Research* **2020**, *27*, 1–8.

(4) Capettini, F.; Ceccarelli, S.; Grando, S. *Barley: Production, Improvement, and Uses*; Blackwell Publishing Ltd.: Ames, IA, USA, 2010.

(5) Harwood, W. A., Ed. *Barley: Methods and Protocols*; Springer Protocols; Humana Press: New York, NY, 2019.

(6) Celus, I.; Brijs, K.; Delcour, J. A. The Effects of Malting and Mashing on Barley Protein Extractability. *Journal of Cereal Science* **2006**, *44*, 203–211.

(7) Wang, C.; Tian, Z.; Chen, L.; Temelli, F.; Liu, H.; Wang, Y. Functionality of Barley Proteins Extracted and Fractionated by Alkaline and Alcohol Methods. *Cereal Chemistry Journal* **2010**, *87*, 597–606.

(8) Reidzane, S.; Gramatina, I.; Galoburda, R.; Komasilovs, V.; Zacepins, A.; Bljahhina, A.; Kince, T.; Traksmaa, A.; Klava, D. Composition of Polysaccharides in Hull-Less Barley Sourdough Bread and Their Impact on Physical Properties of Bread. *Foods* **2022**, *12*, 155–170.

(9) Henrion, M.; Francey, C.; Lê, K.-A.; Lamothe, L. Cereal B-Glucans: The Impact of Processing and How It Affects Physiological Responses. *Nutrients* **2019**, *11*, 8–22.

(10) Tosh, S. M.; Bordenave, N. Emerging Science on Benefits of Whole Grain Oat and Barley and their Soluble Dietary Fibers for Heart Health, Glycemic Response, and Gut Microbiota. *Nutrition Reviews* **2020**, *78*, 13–20.

(11) Kinner, M.; Nitschko, S.; Sommeregger, J.; Petrasch, A.; Linsberger-Martin, G.; Grausgruber, H.; Berghofer, E.; Siebenhandl-Ehn, S. Naked Barley-Optimized Recipe for Pure Barley Bread with Sufficient Beta-Glucan According to the EFSA Health Claims. *Journal of Cereal Science* **2011**, *53*, 225–230.

(12) Tiwari, U.; Cummins, E. Meta-Analysis of the Effect of β-Glucan Intake on Blood Cholesterol and Glucose Levels. *Nutrition* **2011**, *27*, 1008–1016.

(13) Yu, L.; Ma, Y.; Zhao, Y.; Pan, Y.; Tian, R.; Yao, X.; Yao, Y.; Cao, X.; La Geng; Wang, Z.*; et al.* Effect of Hulless Barley Flours on Dough Rheological Properties, Baking Quality, and Starch Digestibility of Wheat Bread. *Frontiers in Nutrition* **2021**, *8*, 785847.

(14) Nilsson, A.; Johansson-Boll, E.; Sandberg, J.; Björck, I. Gut Microbiota Mediated Benefits of Barley Kernel Products on Metabolism, Gut Hormones, and Inflammatory Markers as Affected by Co-ingestion of Commercially Available Probiotics: A Randomized Controlled Study in Healthy Subjects. *Clinical Nutrition* **2016**, *15*, 49–56.

(15) Baik, B. K.; Newman, W.; Newman, R. K. Food Uses of Barley. In *Barley*; Ullrich, S. E., Ed.; Wiley: New York, NY, 2010; pp 532–562.

(16) Schreiber, M.; Mascher, M.; Wright, J.; Padmarasu, S.; Himmelbach, A.; Heavens, D.; Milne, L.; Clavijo, B. J.; Stein, N.; Waugh, R. A Genome Assembly of the Barley 'Transformation Reference' Cultivar Golden Promise. *Genome Report* **2020**, *10*, 1823–1827.

(17) Jaeger, A.; Zannini, E.; Sahin, A. W.; Arendt, E. K. Barley Protein Properties, Extraction and Applications, with a Focus on Brewers' Spent Grain Protein. *Foods* **2021**, *10*, 1389–1410.

(18) Wang, J. M.; Chen, J.X., Dai, F.; Wu, F. B.; Yang, J. M.; Zhang, G. P. Protein Fractions in Barley Grains as Affected by Some Agronomic Factors and Their Relationships to Malt Quality. *Cereal Research Communications* **2007**, *35*, 129–140.

(19) Holopainen, U. R. M.; Pihlava, J.-M.; Serenius, M.; Hietaniemi, V.; Wilhelmson, A.; Poutanen, K.; Lehtinen, P. Milling, Water Uptake, and Modification Properties of Different Barley (Hordeum vulgare L.) Lots in Relation to Grain Composition and Structure. *Journal of Agricultural and Food Chemistry* **2014**, *62*, 8875–8882.

(20) Elouadi, F.; Amri, A.; El-baouchi, A.; Kehel, Z.; Salih, G.; Jilal, A.; Kilian, B.; Ibriz, M. Evaluation of a Set of Hordeum vulgare subsp. spontaneum Accessions for β-Glucans and Microelement Contents. *Agriculture* **2021**, *11*, 950–1005.

(21) Sardari, R. R. R.; Sutiono, S.; Azeem, H. A.; Galbe, M.; Larsson, M.; Turner, C.; Nordberg Karlsson, E. Evaluation of Sequential Processing for the Extraction of Starch, Lipids, and Proteins From Wheat Bran. *Frontiers in Bioengineering and Biotechnology* **2019**, *7*, 413–427.

(22) Filipowska, W.; Jaskula-Goiris, B.; Ditrych, M.; Bustillo Trueba, P.; Rouck, G. de; Aerts, G.; Powell, C.; Cook, D.; Cooman, L. de. On the Contribution of Malt Quality and the Malting Process to the Formation of Beer Staling Aldehydes: A Review. *Journal of the Institute of Brewing* **2021**, *127*, 107–126.

(23) Osborne, T. B. The Proteids of Barley. *Journal of American Chemical Society* **1895**, 539–567.

(24) Rani, M.; Sogi, D. S.; Gill, B. S. Characterization of Gliadin, Secalin and Hordein Fractions using Analytical Techniques. *Scientific Reports* **2021**, *11*, 23135.

(25) Wilson, C. M.; Shewry, P. R.; Faulks, A. J., Miflin, B. J. The Extraction and Separation of Barley Glutelins and their Relationship to Other Endosperm Proteins. *Journal of Experimental Botany* **1981**, *32*, 1287–1293.

(26) Tanner, G. J.; Colgrave, M. L.; Blundell, M. J.; Howitt, C. A.; Bacic, A. Hordein Accumulation in Developing Barley Grains. *Frontiers in Plant Science* **2019**, *10*, 649–660.

(27) Colgrave, M. L.; Goswami, H.; Howitt, C. A.; Tanner, G. J. What is in a Beer? Proteomic Characterization and Relative Quantification of Hordein (Gluten) in Beer. *Journal of Proteome Research* **2012**, *11*, 386–396.

(28) Anderson, O. D. The B-hordein Prolamin Family of Barley. *Genome* **2013**, *56*, 179–185.

(29) Shewry, P. R.; Field, J. M.; Kirkman, M. A.; Faulks, J.; Audrey, B. J. The Extraction, Solubility, and Characterization of Two Groups of Barley Storage Polypeptides. *Journal of Experimental Botany* **1980**, *31*, 393–407.

(30) Schalk, K.; Lexhaller, B.; Koehler, P.; Scherf, K. A. Isolation and Characterization of Gluten Protein Types from Wheat, Rye, Barley and Oats for Use as Reference Materials. *PLOS ONE* **2017**, *12*, e0172819.

(31) Bahmani, M.; O'Lone, C. E.; Juhász, A.; Nye-Wood, M.; Dunn, H.; Edwards, I. B.; Colgrave, M. L. Application of Mass Spectrometry-Based Proteomics to Barley Research. *Journal of Agricultural and Food Chemistry* **2021**, *69*, 8591–8609.

(32) Gill, S.; Vasanthan, T.; Ooraikul, B.; Rossnagel, B. Wheat Bread Quality as Influenced by the Substitution of Waxy and Regular Barley Flours in Their Native and Extruded Forms. *Journal of Cereal Science* **2002**, *36*, 219–237.

(33) Šmídová, Z.; Rysová, J. Gluten-Free Bread and Bakery Products Technology. *Foods* **2022**, *11*, 480–498.

(34) Biesiekierski, J. R. What is Gluten? *Journal of Gastroenterology and Hepatology* **2017**, *32*, 78–81.

(35) Shewry, P. R.; Franklin, J.; Parmar, S.; Smith, S. J.; Miflin, B. J. The Effects of Sulphur Starvation on the Amino Acid and Protein Compositions of Barley Grain. *Journal of Cereal Science* **1983**, *1*, 21–31.

(36) Anjum, F. M.; Khan, M. R.; Din, A.; Saeed, M.; Pasha, I.; Arshad, M. U. Wheat Gluten: High Molecular Weight Glutenin Subunits-Structure, Genetics, and Relation to Dough Elasticity. *Journal of Food Science* **2007**, *72*, 56-63.

(37) Joye, I. J.; Lagrain, B.; Delcour, J. A. Endogenous Redox Agents and Enzymes That Affect Protein Network Formation During Breadmaking – A Review. *Journal of Cereal Science* **2009**, *50*, 1–10.

(38) Furtado, A.; Bundock, P. C.; Banks, P. M.; Fox, G.; Yin, X.; Henry, R. J. A Novel Highly Differentially Expressed Gene in Wheat Endosperm Associated with Bread Quality. *Scientific Reports* **2015**, *5*, 10446.

(39) Salcedo, G.; Sanchez-Monge, R.; Argamenteria, A.; Aragoncillo, C. Low Molecular Weight Prolamins: Purification of a Component from Barley Endosperm. *Journal of Agricultural and Food Chemistry* **1982**, *30*, 1155–1157.

(40) Gu, Y. Q.; Anderson, O. D.; Londeorë, C. F.; Kong, X.; Chibbar, R. N.; Lazo, G. R. Structural Organization of the Barley D-hordein Locus in Comparison with its Orthologous Regions of Wheat Genomes. *Genome* **2003**, *46*, 1084–1097.

(41) Ullrich, S. E., Ed. *Barley*; Wiley: New York, NY, 2010.

(42) Bothmer, R. von; Sato, K.; Komatsuda, T.; Yasuda, S.; Fischbeck, G. The Domestication of Cultivated Barley. *Developments in Plant Genetics and Breeding* **2003**, *7*, 9–27.

(43) Walia, H.; Wilson, C.; Condamine, P.; Ismail, A. M.; Xu, J.; Cui, X.; Close, T. J. Array-Based Genotyping and Expression Analysis of Barley cv. Maythorpe and Golden Promise. *BMC Genomics* **2007**, *8*, 87–101.

(44) Bohl, M.; Gregersen, S.; Zhong, Y.; Hebelstrup, K.; Hermansen, K. Beneficial Glycaemic Effects of Barley Bread Compared to Wheat Bread in Type 2 Diabetes. *Research Square* [Online early access]. DOI: 10.21203/rs.3.rs-2607823/v1.

(45) Meints, B.; Vallejos, C.; Hayes, P. Multi-use Naked Barley: A New Frontier. *Journal of Cereal Science* **2021**, *102*, 103370.

(46) Agrologica. Golden Baking Barley. https://landsorten.dk/varieties/Gylden%20Bagebyg (accessed June 9, 2023).

(47) Watson, J. T.; Sparkman, O. D. *Introduction to Mass Spectrometry: Instrumentation, Applications and Strategies for Data Interpretation*, 4th ed.; John Wiley & Sons: Chichester England, Hoboken NJ, 2007.

(48) Guodong, C.; Li-Kang, Z.; Birendra N., P. LC/MS: Theory, Instrumentation, and Applications to Small Molecules. *HPLC for Pharmaceutical Scientists* **2007**, 281–346.

(49) Gross, J. H. *Massenspektrometrie - Spektroskopiekurs kompakt*, 3rd ed.; Springer Spektrum: Heidelberg, 2019.

(50) Perry, R. H.; Cooks, R. G.; Noll, R. J. Orbitrap Mass Spectrometry: Instrumentation, Ion Motion and Applications. *Mass spectrometry reviews* **2008**, 661–699.

(51) Altakhis, M.; Pillidge, C. J.; Osborn, A. M.; Torley, P. J.; Kaur, M. Assessment of the Potential Use of MALDI-TOF MS for the Identification of Bacteria Associated with Chilled Vacuum-Packaged Lamb Meat. *Meat Science* **2021**, 108508.

(52) Gross, J. H. *Massenspektrometrie: Spektroskopiekurs kompakt*; Springer Spektrum: Berlin, 2019.

(53) He, Q.; Sun, C.; Liu, J.; Pan, Y. MALDI-MS Analysis of Cancer Drugs: Significance, Advances, and Applications. *Trends in Analytical Chemistry* **2021**, 116183.

(54) Smith, A.; Mosele, N.; L'Imperio, V.; Pagni, F.; Magni, F., Eds. *Integration of Omics Approaches and Systems: Biology for Clinical Applications*, 1st ed.; John Wiley & Sons, Inc.: New York, 2018.

(55) Robinson, K. N.; Steven, R. T.; Bunch, J. Matrix Optical Absorption in UV-MALDI MS. *Journal of the American Society for Mass Spectrometry* **2018**, 501–511.

(56) Hillenkamp, F.; Peter-Katalini, J., Eds. *MALDI-MS in Protein Chemistry and Proteomics*, 2nd ed.; Wiley-VCH Verlag GmbH & Co. KGaA: New York, 2014.

(57) Bonnel, D.; Franck, J.; Mériaux, C.; Salzet, M.; Fournier, I. Ionic Matrices Pre-Spotted Matrix-Assisted Laser Desorption/Ionization Plates for Patient Maker Following in Course of Treatment, Drug Titration, and MALDI Mass Spectrometry Imaging. *Analytical Biochemistry* **2013**, 187–198.

(58) Cole, R. B. *Electrospray and MALDI Mass Spectrometry: Fundamentals, Instrumentations, Practicalities and Biological Applications*. Time-of-Flight Mass Spectrometry, 2nd ed.; John Wiley & Sons, Inc.: New York, 2010.

(59) O. Lay, J. MALDI-TOF Mass Spectrometry of Bacteria. *Mass spectrometry reviews* **2001**, *4*, 172–194.

(60) Cotter, R. J. *Encyclopedia of Genetics, Genomics, Proteomics and Bioinformatics: Time-of-flight Mass Spectrometry*; Wiley-VCH Verlag GmbH & Co. KG: New York, 2005.

(61) Kurien, B. T.; Scofield, R. H. *Protein Electrophoresis: Methods and Protocols*; Springer Protocols; Humana Press; Springer: New York, 2012.

(62) Neuhoff, V.; Stamm, R.; Pardowitz, I. Essential Problems in Quantification of Proteins Following Colloidal Staining with Coomassie Brilliant Blue Dyes in Polyacrylamide Gels, and their Solution. *Electrophoresis* **1990**, *11*, 101–117.

(63) Valetti, C.; Sitia, R. The Differential Effects of Dithiothreitol and 2-Mercaptoethanol on the Secretion of Partially and Completely Assembled Immunoglobulins Suggest that Thiol-mediated Retention Does Not Take Place in or Beyond the Golgi. *Molecular Biology of the Cell* **1994**, *5*, 1311-1324.

(64) German Statutory Accident Insurance. 1,4-Dimercapto-2,3-butandiol in GESTIS Database. https://gestis.dguv.de/data?name=122448 (accessed June 11, 2023).

(65) Lexhaller, B.; Colgrave, M. L.; Scherf, K. A. Characterization and Relative Quantitation of Wheat, Rye, and Barley Gluten Protein Types by Liquid Chromatography-Tandem Mass Spectrometry. *Frontiers in Plant Science* **2019**, *10*, 1530–1546.

(66) Howard, K. A.; Gayler, K. R.; Eagles, H. A.; Halloran, G. M. The Relationship Between D-Hordein and Malting Quality in Barley. *Journal of Cereal Science* **1996**, *24*, 47–53.

(67) Kanerva, P.; Sontag-Strohm, T.; Brinck, O. Improved Extraction of Prolamins for Gluten Detection in Processed Foods. *Agricultural and Food Science* **2011**, *20*, 206–216.

(68) Luo, H.; Harasymow, S.; Paynter, B.; MacLeod, A.; Izydorczyk, M. S.; O'Donovan, J. T.; Li, C. Genetic and Environmental Impact on Protein Profiles in Barley and Malt. *Journal of the Institute of Brewing* **2019**, *125*, 28–38.

(69) Idowu, A. O.; Alashi, A. M.; Nwachukwu, I. D.; Fagbemi, T. N.; Aluko, R. E. Functional Properties of Sesame (Sesamum indicum Linn) Seed Protein Fractions. *Food Production, Processing and Nutrition* **2021**, *3*.

(70) Schmidt, J. M.; Damgaard, H.; Greve-Poulsen, M.; Sunds, A. V.; Larsen, L. B.; Hammershøj, M. Gel Properties of Potato Protein and the Isolated Fractions of Patatins and Protease Inhibitors – Impact of Drying Method, Protein Concentration, pH and Ionic Strength. *Food Hydrocolloids* **2019**, *96*, 246–258.

(71) Jones, D. B. Factors for Converting Percentages of Nitrogen in Foods and Feeds into Percentages of Protein. *United States Department of Agriculture* **1941**, 1–22.

(72) Di Zhao; Le, T. T.; Nielsen, S. D.; Larsen, L. B. Effect of Storage on Lactase-Treated β-Casein and β-Lactoglobulin with Respect to Bitter Peptide Formation and Subsequent in Vitro Digestibility. *Journal of Agricultural and Food Chemistry* **2017**, *65*, 8409–8417.

(73) Poulsen, N. A.; Larsen, L. B. Proteomics. *Encyclopedia of Dairy Sciences*; Elsevier, 2022; pp 933–940.

(74) Larsen, L. B.; Wedholm-Pallas, A.; Lindmark-Månsson, H.; Andrén, A. Different Proteomic Profiles of Sweet Whey and Rennet Casein Obtained After Preparation From Raw Versus Heat-treated Skimmed Milk. *Dairy Science and Technology* **2010**, *90*, 641–656.

(75) Becker, J. M.; Caldwell, G. A.; Zachgo, E. A. *Biotechnology: A Laboratory Course*, 2nd ed; Academic Press, 1996.

(76) Bender, D. A. *A Dictionary of Food and Nutrition*, 4th ed.; Oxford University Press: Oxford, United Kingdom, 2014.

(77) Mihaljev, Ž.; Jakšić, S.; Prica, N.; Ćupić, Ž.; Baloš, M. Comparison of the Kjeldahl Method, Dumas Method and NIR Method for Total Nitrogen Determination in Meat and Meat Products. *Journal of Agroalimentary Processes and Technologies* **2015**, *21*, 365–370.

(78) Matlock, B. Assessment of Nucleic Acid Purity. https://assets.thermofisher.com/TFS-Assets/CAD/Product-Bulletins/TN52646-E-0215M-NucleicAcid.pdf (accessed April 13, 2023).

(79) Wiedemann, C.; Kumar, A.; Lang, A.; Ohlenschläger, O. Cysteines and Disulfide Bonds as Structure-Forming Units: Insights From Different Domains of Life and the Potential for Characterization by NMR. *Frontiers in Chemistry* **2020**, *8*, 280–288.

(80) Rechinger, K. B.; Simpson, D. J.; Svendsen, I.; Cameron-Mills, V. A Role for Gamma 3 Hordein in the Transport and Targeting of Prolamin Polypeptides to the Vacuole of Developing Barley Endosperm. *The Plant Journal for Cell and Molecular Biology* **1993**, *4*, 841–853.