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Effect of enzyme-assisted hydrolysis on brewer's spent grain protein solubilization – peptide composition and sensory properties

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ABSTRACT

This study aimed to valorize brewer's spent grain (BSG) from a side-stream into protein ingredients suitable for human consumption. The impact of protease treatments was studied for solubilizing BSG proteins. Treatment with Protamex or simultaneous co-incubation of Protamex and Flavourzyme solubilized up to 60% of the total protein in BSG, whereas co-incubation with Flavourzyme increased the availability of hydrophobic amino acids (Val, Phe, Ala, Leu, Ile) in extracts. The scale-up of protease treatments demonstrated comparable solubilized protein fractions in 0.1 L and 10 L reaction volumes. Thorough sequence-based peptide analysis by liquid chromatography-ion mobility-mass spectrometry resulted in the identification of 479 and 451 water-soluble peptides in the hydrolysates obtained with Protamex or co-incubation of Protamex and Flavourzyme, respectively. Main cutting sites on BSG proteins were identified between Leu-Gln, Tyr-Phe, Pro-X (Protamex), complementing with a variety of cutting sites mainly next to Gln, Pro, Ile, and Phe when combined with Flavourzyme. Uniform protease activity throughout the entire B-hordein sequence and the formation of peptides with varying sequence lengths did not increase the bitterness of the hydrolysates compared to the BSG sample with water extraction. These results support the characterization of enzymatic treatments in plant-based materials and the production of hydrolysates with desired composition.

1. Introduction

In recent years, there has been a growing interest for food enrichment with high-quality plant-based protein concentrates and isolates, as well as dietary fibers. Various plant sources e.g., legumes, cereals, seeds, or nuts, are widely used as raw materials for production of these ingredients (Sá, Moreno, & Carciofi, 2020). At the same time, enormous amounts of food industry protein- and fiber-rich side-streams, such as brewer's spent grain (BSG), are left unattended and usually discarded or used as a feed. Each year 39 million tons of BSG is generated by breweries worldwide (Lynch, Steffen, & Arendt, 2016). Although BSG is rich in proteins; fibers such as hemicellulose, cellulose, and lignin; lipids; vitamins; and minerals, its wider use is still limited due to its high moisture content and microbiological instability (Lynch et al., 2016; Robertson et al., 2010). Thus, biorefinery concepts with simple fractionation methods would be a promising solution for producing value-added ingredients. Lately, the possibilities of using BSG as activated carbon or sorbent, solid fuel or starting material for biogas production, fertilizer, construction material, and extraction of high-value components (like arabinoxylans, polyphenol, antioxidants, glucose, or proteins) for human nutrition have been considered (Jackowski, Niedzwiecki, & Trusek, 2020; Puligundla & Mok, 2021).

The proteins are one of the most valuable components in BSG, comprising 20–30% of the dry weight (Lynch et al., 2016; Robertson et al., 2010), whereas the storage proteins, hordeins and globulins, have been found to be the most abundant ones in BSG (Bi et al., 2018). Suitable pre-treatment procedures (e.g., chemical, physical, or enzymatic) are necessary for increasing BSG protein extractability (He et al., 2019; Niemi, Martins, Buchert, & Faulds, 2013; Qin, Johansen, & Mussatto, 2018; Rommi, Niemi, Kemppainen, & Kruus, 2018; Yu et al., 2020). Protein yield, as well as the physicochemical properties of pro-

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teins from BSG, can be improved by degrading plant cell wall matrix with carbohydrases or unfolding of proteins and releasing peptides and amino acids with protease treatments (Celus, Brijs, & Delcour, 2007; Treimo, Aspmo, Eijsink, & Horn, 2008, 2009). Protease treatments are especially valuable by specifically solubilizing proteins, increasing protein separation efficiency, and producing high protein products (He et al., 2019). In food industry, the hydrolyzed BSG proteins can be beneficial due to the increased digestibility of the proteins, as well improved techno-functional properties, or presence of potential bioactive peptides (Celus et al., 2007; Cermeño et al., 2019; Connolly, O'Keeffe, Piggott, Nongonierma, & Fitzgerald, 2015). However, one of the limitations for using such hydrolysates in food products may be the sensory characteristics due to the formation of peptides with astringent or bitter off-flavors (Großmann, Merz, Appel, Thaler, & Fischer, 2021; Schlegel et al., 2019).

Usually, the main methods for characterizing protein hydrolysates involve analysis of the degree of hydrolysis of the peptide bonds, applying different separation techniques such as size exclusion chromatography, electrophoretic methods, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), or capillary electrophoresis (Silvestre, 1997). These methods give an overview of the extent of hydrolysis, approximate molecular weight distribution of proteins and peptides, but do not provide specific information about the composition of protein hydrolysates. Essentially, liquid chromatography coupled with mass spectrometry (LC-MS) becomes especially useful for identification of peptide sequences in the hydrolysates (Léonil, Gagnaire, Mollé, Pezennec, & Bouhallab, 2000). The MS-based methods have been applied in determination of bioactive peptides in food proteins and hydrolysates. For instance, the BSG hydrolysates have been efficiently fractionated and specific BSG-derived bioactive peptides exhibiting angiotensin converting enzyme or dipeptidyl peptidase IV inhibitory activities have been identified (Cermeño et al., 2019; Connolly et al., 2015). Additionally, using sensory-guided fractionation techniques together with peptide identification allow to reveal amino acid sequences of the bitter peptides and thus support further development of protein hydrolysates with higher sensory acceptance (Liu, Jiang, & Peterson, 2014). Another advantage of MS-techniques is the possibility of characterizing the specificity of different proteases (Hinnenkamp & Ismail, 2021). However, for enzymatic treatments of BSG proteins, there is still a lack of studies on determination of specific water-soluble peptides and the cleavage sites.

The aim of this study was to obtain solubilized BSG proteins and peptides with acceptable sensory characteristics. The potential treatments for BSG were chosen based on the evaluation of protein solubilization, free amino acid (FAA) content, and sensory attributes such as taste and odor of the extracts. The process reliability was demonstrated by amplifying the enzymatic process from the laboratory-scale (0.1 L) to the semi pilot-scale (10 L). Furthermore, an ion mobility enabled LC-MS/MS system was used to identify the water-soluble peptides. Each peptide was localized on the BSG protein sequence, allowing to assign the cleavage sites of the proteins and the activities of the proteases.

2. Materials and methods

2.1. Materials

The BSG of India pale ale-type beer was obtained from brewery in Freising, Germany. The wet sample was dried by convective drying at 70 °C, ground to the particle size of about 385 μm, and stored in aluminum bags at room temperature until further analyses. Commercial enzymes, Protamex® (declared activity 1.5 AU/g) a serine endoprotease and Flavourzyme® (declared activity 1000 LAPU/g) a mixture of endo- and exopeptidase, were provided by Novozymes (Bagsvaerd, Denmark). Other chemicals were obtained from Sigma-Aldrich (Darmstadt, Germany).

2.2. Composition analyses of BSG

Dry matter and ash content of the BSG sample was determined using a thermo-gravimetrical system (TGA 601, Leco Corporation, St. Joseph, MI, USA) at 105 °C and 950 °C, respectively (Naumann, Schweiggert-Weisz, Haller, & Eisner, 2019). The crude protein content was measured by the Dumas combustion method on a TruMac N system (Leco Corporation, St. Joseph, MI, USA) by applying a conversion factor of N × 6.25. Analyses were performed in duplicates and in accordance with the Association of Official Analytical Collaboration (AOAC) Official Methods 923.03–2003 and 968.06–2003, respectively. The fat content was assessed using an automated Soxhlet apparatus B-811 (BÜCHI Labortechnik AG, Flawil, Switzerland) according to the AOAC 996.01–2000 standard procedure. Soluble and insoluble dietary fiber contents were determined using enzymatic-gravimetry; total dietary fiber was calculated (total dietary fiber = soluble dietary fiber + insoluble dietary fiber) according to the AOAC Official Method 991.43–2016. Total phytic acid content was measured using Phytic Acid Assay Kit from Megazyme (K-PHYT, Bray, Ireland) according to the manufacturer's instructions.

2.3. Solubilization of BSG proteins

For laboratory-scale protein solubilization experiments the ground sample was mixed with deionized water at 1:10 (w/v) ratio in 250 mL Erlenmeyer flasks. The pH was adjusted with 2 M of NaOH to pH 8.5. The pH for enzymatic treatments was set based on the manufacturer's suggestions and according to the pH drop occurring during the incubation. Thereafter, 0.5% Protamex or 0.1% Flavourzyme was added based on dry matter content of BSG and the mixtures were incubated in a shaker incubator (KS 3000 i control, IKA, Staufen, Germany) at 50 °C, 150 rpm, for 3 h. The water extraction at the same temperature, pH, and time as the enzymatic treatments was added as a control. After all treatments, the suspensions were heated for 10 min at 95 °C, cooled down, and centrifuged at 4000 x g for 15 min. The scale-up study was carried out in a 10 L following the same protocol as in laboratory-scale experiments. The collected extracts were used for determination of solubilized protein, FAA, water-soluble peptides, and sensory characteristics.

2.4. Characterization of extracts

2.4.1. Solubilized protein determination

The solubilized protein content in collected supernatants was determined by a Pierce™ Modified Lowry Protein Assay Kit (Thermo Scientific™, Rockford, USA) according to manufacturer's instructions, considering bovine serum albumin as calibration standard. Additionally, the protein (total N) content was determined by Kjeldahl method using N × 6.25 as a conversion factor (ISO 5983–2:2009). Solubilized protein is presented as concentration in mg/mL or % of total protein in BSG calculated by using Eq. (1).

$$\text{Solubilized protein or FAA (\%)} = \frac{\text{Initial volume [mL]} \times \text{protein or FAA content in supernatant} \left[\frac{\text{mg}}{\text{mL}}\right]}{\text{sample mass [mg]} \times \text{protein content [\% db]} \times \text{dry matter [\%]}} \times 100 \quad (1)$$

2.4.2. FAA determination

Analysis of FAA was performed by an Acquity UPLC system (Waters Corp., Milford, MA, USA). The BSG extracts were centrifuged for 5 min at 13,304 × g and filtered through a 0.2-μm PTFE filter (Merck Millipore, Darmstadt, Germany). Before the injection, FAA were derivatized with AccQFluor Reagent (Waters Corp.) according to the manufacturer's procedure. 0.7 μL derivatives were loaded on AccQTag™Ultra columns (2.1 × 100 mm) at 55 °C (flow rate 0.7 mL/min) connected to a PDA detector ($\lambda = 260$ nm). Amino acids were separated using a 10 min gradient from 0.1 to 59.6% B (A, AccQTag Ultra eluent A; B, AccQTag Ultra

eluent B; Waters Corp.). The concentrations of amino acids (mmol/L) were calculated using standard curves. The FAA content is presented as concentration in mg/mL or % of total protein in BSG calculated by using **Equation 1**.

2.4.3. LC-MS/MS analysis of peptides

The supernatants of enzymatically treated samples were mixed with acetonitrile (ACN) at 1:1 ratio to precipitate the proteins, vortexed, centrifuged for 10 min at 18,407 × g, diluted with deionized water (1:9) and transferred into the autosampler vials. Water-soluble peptides were separated using Acquity I-Class Plus UPLC system (Waters, Milford, MA, USA) on a Waters Acquity UPLC HSS T3 1 × 150 mm column and detected using Vion IMS-QTof (Waters, Milford, MA, USA). Eluent A was 0.1% formic acid (FA) in water and 0.1% FA in ACN was used as eluent B. Four consecutive linear gradients for peptide elution were as follows: during the first minute 0–0.5% eluent B, 0.5–40% eluent B over the next 45 minutes, from 51 to 57 minutes 95% eluent B, and to the end of 60-minute run 0.5% eluent B. An injection volume of 1 µL was used. The autosampler and column temperature were set at 8 and 40 °C, respectively. The capillary and cone voltages were 3 kV and 40 kV, respectively. The cone and desolvation gas flows were 50 and 700 L/h, respectively. Source and desolvation temperatures were kept at 120 °C and 500 °C, respectively. The data were acquired in HDMSe acquisition mode over the mass range 50–2000 *m/z* in positive ionization mode.

For the identification of peptides, the sequences of 16 barley (*Hordeum vulgare*) proteins (B-, C-, D-, γ-hordeins, and globulins were taken from the UniProt Database (https://www.uniprot.org). The peptide data were processed with Waters UNIFI (1.9.4.053) using Peptide Map workflow and filtered using the following selection criteria: mass error was between -5 and 5 ppm and the number of matched first gen primary ions was ≥1. The visualization of the results obtained from UNIFI processing was performed using in-house data analysis routines implemented in Python programming language (Python Software Foundation, version 3.5, available at http://www.python.org).

2.4.4. Sensory assessment

The sensory analyses were performed with trained panelists (n = 8; $\bar{x}$(age)=34; 1 male, 7 females), who evaluated enzymatically treated BSG suspensions according to taste and aroma attributes. The evaluation was conducted in a standardized sensory room (ISO 8589:2007) and the panelists had at least two years of experience with sensory analysis. An additional training session was carried out with selected BSG extracts prior testing session to familiarize with the products and specify attributes for the assessment. The samples were evaluated in two parallels. All the samples were coded with a three-digit number, and the order of samples was randomized according to Williams' Latin square design. Sensory attributes (taste and odor) were evaluated on a 0–9 scale, where 0 means none, 1—very weak, 5—moderate, and 9—very strong.

2.5. Statistics

Results are expressed as average ± standard deviation of three or two replicates. Statistical differences among data were calculated based on paired two-tailed *t*-test (Microsoft Excel for 365 MSO Version 2110 Build 16.0.14527.20270). The values were considered significantly different when $p < 0.05$.

3. Results and discussion

3.1. Characterization of raw material

After drying and milling of the raw BSG, the dry matter content was 97.6%. The protein, total dietary fiber, fat, and ash content were 22.5%, 45.8%, 7.2%, and 4.4% (dry basis), respectively (**Supplementary Table S1**). A vast majority of the dietary fibers were insoluble dietary fiber (almost 100%, dry basis). The pH of the BSG sample in 25 °C water suspension was 5.1, which should be considered if pH adjustment for enzymatic pre-treatments is needed. Investigation of antinutrients revealed phytic acid content of 1.7% (dry basis). Depending on the used barley varieties, malting, or brewing processes, the used BSG samples can have different composition (Jin et al., 2022; Robertson et al., 2010). Therefore, it is important to have sufficiently robust processing methods that would be applicable for a wide variety of BSG samples.

3.2. Characterization of solubilized BSG protein extracts

3.2.1. Analysis of protein and FAA content

Table 1 shows solubilized protein (determined by Lowry assay and Kjeldahl method) and FAA content of extracts after enzymatic treatments in 0.1 L as well as 10 L reaction volumes. Lowry assay was applied due to the possibilities of quick and easy protein determination with relatively low sample amounts. In further experiments with selected reaction conditions and larger sample quantities, the protein content was also evaluated by Kjeldahl method.

Based on laboratory-scale screening experiments (data not shown), protease treatment conditions that allowed the highest protein solubilization while using the lowest enzyme dose and incubation time were chosen. One of the tested enzymes was Protamex as this endopeptidase has demonstrated a high degree of BSG protein solubilization as well as great potential for the production of plant-based hydrolysates with lower bitterness (Schlegel et al., 2019; Treimo et al., 2008). The experiments with selected reaction conditions showed that up to 50% of protein (determined by Lowry assay) can be solubilized with 0.5% of Protamex treatment for 3 h in 0.1 L reaction volume (Table 1). Additionally, Flavourzyme (an exopeptidase) was used together with the above-mentioned protease as it could decrease a bitter aftertaste of obtained hydrolysates by removing the N- and C-terminal hydrophobic amino acids from bitter peptides (Fu, Liu, Hansen, Bredie, & Lametsch, 2018; Saha & Hayashi, 2001). Moreover, extensive hydrolysis, higher protein solubility, small-size peptides, and increased FAA content are expected if sequential or simultaneous hydrolysis with endo- and exopeptidases is performed (Hinnenkamp & Ismail, 2021; Vioque et al., 1999). In this study, co-incubation of Protamex and Flavourzyme increased FAA content compared to those of Protamex treatment alone ($p < 0.05$) (Table 1). Flavourzyme treatment alone did not enhance protein solubilization. The FAA content, on the other hand, increased, but remained almost four-fold lower compared to the incubation together with Protamex. Therefore, these results also indicate the need to hydrolyze the BSG protein into peptides with endopeptidases before exopeptidases can be applied. However, studies have demonstrated that Flavourzyme could be used for improving techno-functional properties of BSG proteins and increasing the solubility of proteins at different pH values (Celus et al., 2007). Usually, such enzymatic treatments are performed for protein isolates or concentrates. Here, direct enzymatic treatment of BSG was applied which may also lead to lower enzyme activities due to the limited access of peptidases to the cleavage sites of proteins.

The scale-up experiments in 10 L reaction volumes were conducted similarly to the laboratory-scale experiments. No constant pH control was applied after its initial pH adjustment to 8.5, and after 3 h of incubation, the pH of all samples was around 7.5. The results show that the scale-up process was effective and similar levels of solubilized protein and FAA were observed in both reaction volumes (Table 1). According to the results obtained with Kjeldahl method, the BSG treatment with Protamex or simultaneous co-incubation with Protamex and Flavourzyme led up to 60% of solubilized protein content. Both Lowry assay and Kjeldahl method can be used for the determination of solubilized protein fraction. Lowry assay becomes especially useful if changes of protein content in a larger number of samples in the same data set are examined. For comparative purposes with the data from the literature, standard methods (e.g., Kjeldahl) should be preferred.

Table 1
Solubilized protein and FAA content of BSG extracts after various treatments in 0.1 L and 10 L reaction volumes.

Reaction volume and sample name		Solubilized protein by Lowry assay		Solubilized total protein by Kjeldahl method		FAA	
		mg/mL	%	mg/mL	%	mg/mL	%
0.1 L	Extr.: pH 8.5 3 h	5.5 ± 0.4[a]	25[a]	4.1 ± 0.1[a]	19[a]	0.22 ± 0.01[a]	1.0[a]
0.1 L	0.5% Prot 3 h	11.0 ± 0.5[b]	50[b]	12.6 ± 0.1[b]	57[b]	0.33 ± 0.02[b]	1.5[b]
0.1 L	0.5% Prot + 0.1% Flav 3 h	11.0 ± 0.9[b]	50[b]	12.9 ± 0.1[c]	59[c]	1.17 ± 0.03[c]	5.3[c]
0.1 L	0.1% Flav 3 h	4.2 ± 0.9[c]	19[c]	3.9 ± 0.1[d]	18[d]	0.32 ± 0.02[b]	1.4[b]
10 L	Extr.: pH 8.5 3 h	4.4 ± 0.7[a]	20[a]	3.2 ± 0.0[a]	15[a]	0.31 ± 0.02[a]	1.5[a]
10 L	0.5% Prot 3 h	12.6 ± 0.4[b]	57[b]	12.8 ± 0.0[b]	58[b]	0.42 ± 0.02[b]	1.8[b]
10 L	0.5% Prot + 0.1% Flav 3 h	14.6 ± 0.8[ab]	67[ab]	13.4 ± 0.0[b]	61[b]	1.23 ± 0.10[c]	5.7[c]

The data represents the average ± standard deviation (n = 3 for 0.1 L and n= 2 for 10 L). Different letters within the same column of the same reaction volume are significantly different ($p < 0.05$). FAA, free amino acid; Extr., water extraction; Prot, Protamex; Flav, Flavourzyme.

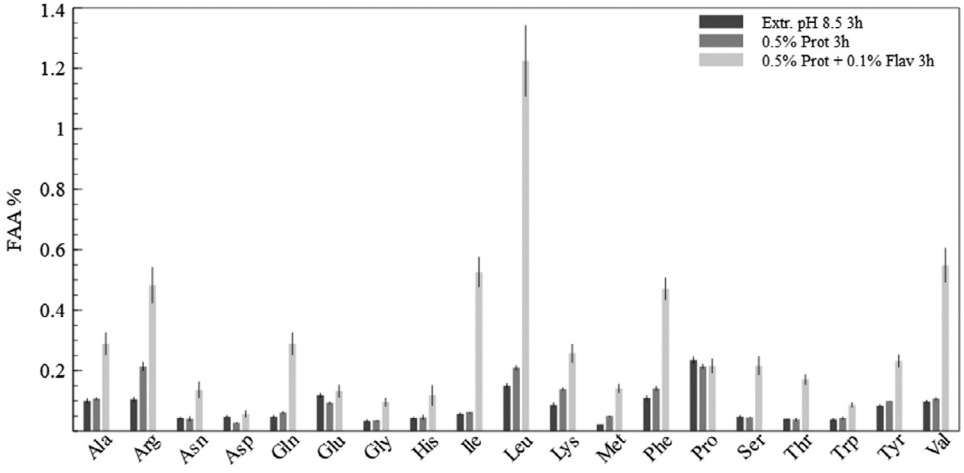

Fig. 1. Free amino acid (FAA) composition (%) after non-enzymatic (Extr. pH 8.5 3 h) and enzymatic treatments (0.5% Prot 3 h and 0.5% Prot with 0.1% Flav 3 h) in 10 L reaction volumes. The data represents the average ± standard deviation (n = 2). The sum of the FAA content in extracts is presented in Table 1. Extr., water extraction; Prot, Protamex; Flav, Flavourzyme.

Based on the dry matter content of the extracts in 10 L scale-up experiments, the protein concentration in water extracted sample was 18.4 ± 0.3%. Applying only Protamex treatment or co-incubation of Protamex and Flavourzyme increased the protein content up to 53.9 ± 0.3% and 46.9 ± 0.3% on a dry matter basis, respectively. After drying, these powders can be used as protein-rich products. However, as the extracts are mixtures of solubilized proteins and fibers, further fiber separation should be considered for increasing the purity of the final product (He et al., 2019; Rommi et al., 2018).

Fig. 1 presents the FAA composition of BSG extracts from the scale-up study. After water extraction of BSG at pH 8.5 for 3 h, the FAA content was 1.5% of the total protein content in BSG (Table 1). In this water extracted BSG sample the dominating amino acids were Pro, Leu, Phe, Glu, Arg, and Ala. Previous research has demonstrated that the most abundant amino acids in BSG and its hydrolysates are Gln, Pro, and Glu (Treimo et al., 2008; Yu et al., 2020). However, the released FAA content is dependent on the brewing processes and therefore varies significantly between different BSG types (Jin et al., 2022). Regarding protease treatment of BSG, the FAA analysis gives additional information about the enzyme activities. As Protamex has mainly high endopeptidase activity then most of the FAA remained comparable to that of the control sample. However, an increase in Lys, Leu, and Arg contents refer to the low exopeptidase activity of this enzyme. As expected, co-incubation of Protamex and Flavourzyme increased the content of most of the hydrophobic amino acids, especially Leu, Ile, Phe, Val, Ala, and also Gln and Arg (Fig. 1). First four from the list are also essential, which supports the further use of these hydrolysates in food products as well.

3.2.2. Analysis of water-soluble peptides

No peptides were detected in the control water-soluble extract of BSG without enzyme treatment. In contrast, 479 and 451 water-soluble peptides were identified in samples treated by Protamex and Protamex together with Flavourzyme, respectively, of which only 109 peptides were found to be common for both samples. Most of the peptides were derived from the C- and B-hordeins, which is in agreement with a higher abundance of these storage proteins in untreated barley grain (Shewry, 1992).

Both enzymatic treatments resulted in a mixture of peptides of varying sequence lengths (Fig. 2). More uniform distribution of smaller peptides in the range from 4 to 16 amino acid long sequences manifested in BSG samples undergone combined treatment with two enzymes. Moreover, there was a clear production of 19, 20, and 40 amino acid long peptides along with the disappearance of 21 and 41 amino acid long ones, indicating the exopeptidase action of Flavourzyme (Fig. 2).

The peptide coverage profiles of B-hordein provide a good overview of the protein regions more accessible by the enzymes (Fig. 3). Similar peptide coverage profiles were plotted also for C-, D-, and γ-hordeins (**Supplementary Figure S1, S2**). The peptide coverage profiles show that the hydrolyzed regions of the proteins are very similar among the two treatments. It can be suggested that the addition of Flavourzyme to the BSG sample resulted in the degradation of peptides obtained after the action of Protamex and no additional regions of hydrolysis were accessed by the enzymes. Nevertheless, certain differences in peptide intensities can be distinguished. Thus, in the sample treated by Protamex only, a larger number of peptides with high intensities was obtained (Fig. 3**A**). These peptides were most likely hydrolyzed to smaller peptides: the number of peptides with lower intensity over the same part of the sequence was higher in the BSG extract after combined treatment containing Flavourzyme as well (Fig. 3**B**). This is in good accordance with the study on enzymatic hydrolysis of BSG proteins, where the level of high molecular weight protein fragments has been decreased after the treatment with Flavourzyme (Celus et al., 2007). Some peptides with

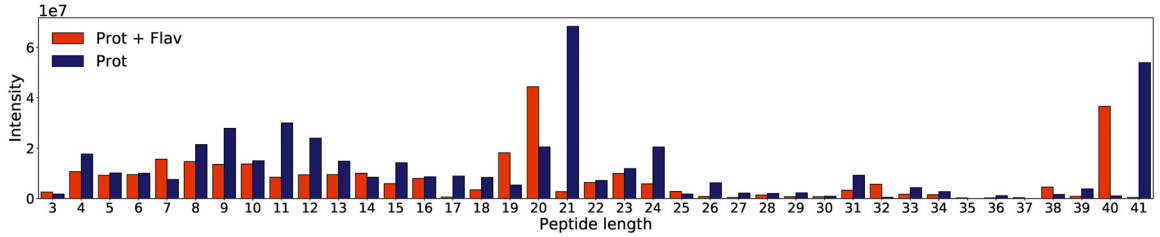

Fig. 2. Distribution of all identified peptides by length in BSG samples treated by 0.5% Prot and 0.5% Prot with 0.1% Flav for 3 h. Prot, Protamex; Flav, Flavourzyme.

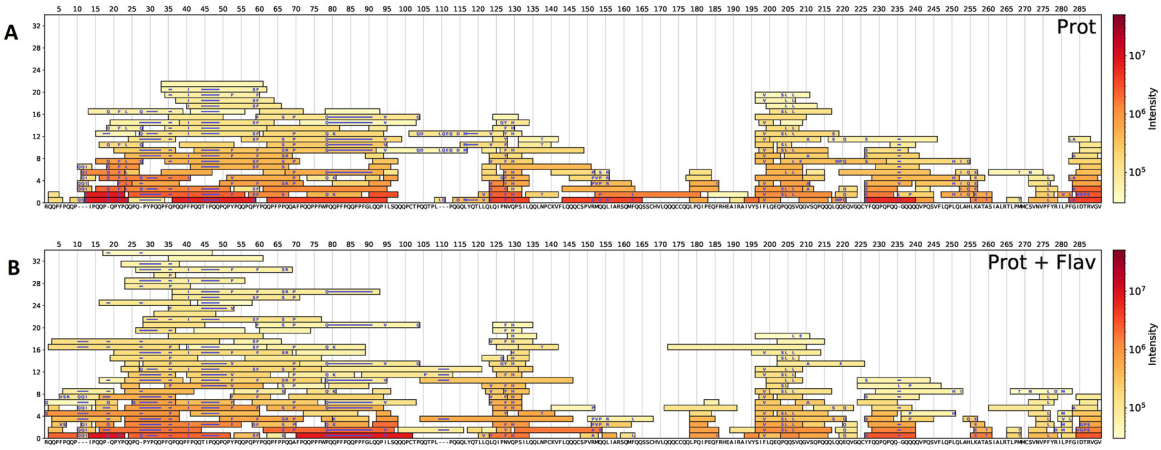

Fig. 3. Peptide coverage profiles of B-hordein in BSG samples treated by (A) 0.5% Prot and (B) 0.5% Prot with 0.1% Flav for 3 h. The intensities of peptides are shown in colors according to the color scale displayed on the right. The sequence of the reference B-hordein is plotted on the horizontal axis. Blue lines and one-letter abbreviations of amino acids on the peptides indicate the differences in the peptide sequences in genetic variants of B-hordein. Thus, the peptides from all genetic variants of B-hordein are shown on one plot. Prot, Protamex; Flav, Flavourzyme.

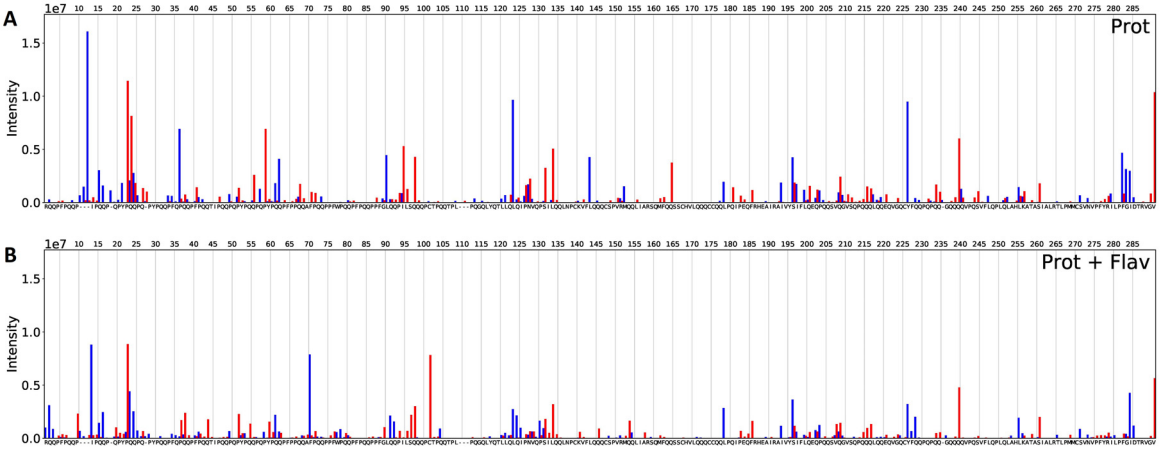

Fig. 4. The enzyme cleavage sites of B-hordein based on the water-soluble peptides identified in BSG samples treated by (A) 0.5% Prot and (B) 0.5% Prot with 0.1% Flav for 3 h. The sequence of B-hordein is plotted on the horizontal axis. Blue bars: summed intensities of the N-terminal amino acids of peptides. Red bars: summed intensities of the C-terminal amino acids of peptides. Prot, Protamex; Flav, Flavourzyme.

high intensity (e.g., B-hordein f70–102, f24–51, f2–10) were also seen on the Fig. 3**B**, which can be attributed to the endopeptidase activity of Flavourzyme directly towards B-hordein.

The difference in the activities of Protamex and Flavourzyme towards B-hordein can be seen in Fig. 4. The hydrolysis of peptides by exopeptidases, that cleave one or two amino acids from the N- and C-terminal end of peptides, was evident in the cleavage sites pattern of B-hordein in the sample treated with two enzymes. Thus, the abundant cleavage sites Leu_{90}-Gln_{91}, Leu_{123}-Gln_{124}, and Tyr_{226}-Phe_{227} in the case of the treatment with Protamex alone (Fig. 4**A**) replaced with a ladder of consecutive cleavages Gln_{91}-Gln_{92} and Gln_{92}-Pro_{93}, Gln_{124}-Ile_{125}, Ile_{125}-Pro_{126}, and Phe_{227}-Gln_{228}, Gln_{228}-Gln_{228}, respectively, when combination with Flavourzyme was used (Fig. 4**B**). The amino acids released during the hydrolysis of the respective peptides were abundant among the FAA measured in the sample treated with Flavourzyme (Leu, Gln, Ile, Phe) (Fig. 1). Moreover, the presence of the endopeptidase activity of Flavourzyme can be supported by the disappearance of Pro_{36}-Gln_{37}, Pro_{59}-Tyr_{60}, Leu_{144}-Gln_{145}, Gln_{164}-Ser_{165} cleavages, as well as the appearance of Phe_{70}-Pro_{71}, Cys_{102}-Thr_{103} ones (Fig. 4**B**).

Similar interesting data visualization has been applied to peptidomics of hydrolysates produced by the same enzymes from bovine

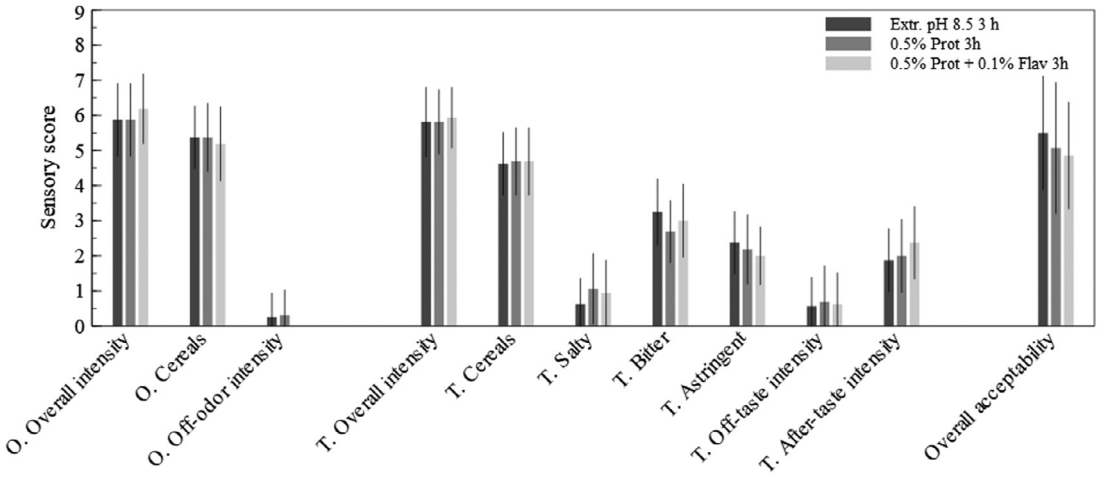

Fig. 5. Sensory analysis (taste – T and odor – O) of BSG extracts after non-enzymatic (Extr. pH 8.5 3 h) and enzymatic treatments (0.5% Prot 3 h and 0.5% Prot with 0.1% Flav 3 h) in 10 L reaction volumes. The data represents the average ± standard deviation (n = 2). Extr., water extraction; Prot, Protamex; Flav, Flavourzyme.

muscle and porcine plasma, where cleavage mainly occurred between Leu and Lys as well as Ile and Ala amino acid residues in Protamex and Flavourzyme treatments, respectively (Fu et al., 2018). Primary sequences of plant-based and animal proteins are different. Barley protein composition is high in Gln and Pro, whereas Lys and Leu contents are relatively low compared to the animal-based proteins. (Ahmad, Imran, & Hussain, 2018; Arendt & Zannini, 2013). Nevertheless, one amino acid in the cleavages Leu-X, Ile-X and X-Ile is identical in BSG and animal proteins. There have been no studies on BSG proteins enzymatic treatment published before including the data on specific water-soluble peptides produced and describing the cleavage sites of used proteases.

The sum of intensities of all identified peptides in BSG extract after hydrolysis with Protamex and Flavourzyme was 1.5-fold lower than that of the sample produced by Protamex treatment only. As shown above (Table 1), FAA content in the sample treated with two enzymes was higher than in the sample with Protamex (1.23 mg/mL vs 0.42 mg/mL). This confirms that the addition of Flavourzyme promotes further degradation of Protamex produced peptides with an extensive release of amino acids and probably also dipeptides, undetectable by peptide mapping approach applied in this study.

3.2.3. Sensory assessment

Production of protein hydrolysates with high nutritional value for further food applications can be limited due to their sensory properties, especially bitter taste. The sensory assessment of enzymatically treated and untreated BSG samples of the scale-up experiment demonstrated a similar effect in taste and odor (Fig. 5). The overall intensity score for odor for all extracts was 6.0 ± 1.0, whereas the most dominant was cereal odor. The score for taste was 5.9 ± 0.9, where cereal taste was the most abundant similarly to odor. The bitter sensation score in untreated BSG was 3.3 ± 0.9 and the astringency score was 2.2 ± 0.2. The bitterness score of Protamex treated sample was 2.7 ± 0.9 and co-incubation with Flavourzyme gave a bitterness score of 3.0 ± 1.0. The sensory assessment of hydrolysates demonstrated that the used enzymes and hydrolysis conditions are not affecting taste or odor profile, which is also in agreement with other studies where sensory properties (e.g., bitterness) of Protamex or Flavourzyme treated hydrolysates remained similar to untreated protein samples (Meinlschmidt, Sussmann, Schweiggert-Weisz, & Eisner, 2016; Schlegel et al., 2019).

The bitter taste of the protein hydrolysates is mostly associated with low molecular weight hydrophobic peptides (Schlegel et al., 2019; Tong et al., 2020). In this study the proteases used at chosen reaction conditions demonstrated uniform protease activity throughout the B-hordein sequence, and peptides with varying sequence lengths and amino acid composition were formed (Fig. 2 **and** 3). Moreover, Protamex treatment alone demonstrated removal of Leu and Arg residues from the peptides (Fig. 1), which can lead to lower bitterness intensity of the hydrolysate. The debittering effect of Leu aminopeptidase and Arg aminopeptidase has been demonstrated for the production of soybean protein hydrolysates as well (Tong et al., 2020). Although co-incubation of Protamex with Flavourzyme effectively released hydrophobic amino acids (e.g., Val, Phe, Ala, Leu, Ile) from the peptides (Fig. 1), no effect on sensory properties of the hydrolysates was detected. Therefore, adding exopeptidases to the mixture could be useful if small size water-soluble peptides or a higher amount of FAA are favored. Additionally, the advantages of Flavourzyme can be more pronounced if other proteases (e.g., Alcalase) and hydrolysates with higher initial bitterness are applied (Meinlschmidt et al., 2016; Vioque et al., 1999).

Moreover, the variations in the BSG origin and especially the content of polyphenols can also contribute to bitterness and astringency of the samples (Soares et al., 2013). Thus, debittering by additional purification or masking should be considered for increasing the quality of the final product.

4. Conclusions

In this study, the simplicity and robustness of BSG protein solubilization and the effect of scaling up the solubilization process were demonstrated. Based on the efficiency of solubilizing proteins, sensory characteristics, and economic aspects regarding enzyme dose and incubation time, Protamex and Flavourzyme treatments were characterized. Up to 60% of the proteinaceous fraction was solubilized without affecting the sensory characteristics of the treated samples. Ion Mobility enabled LC-MS/MS analyses allowed to identify 479 and 451 water-soluble peptides in BSG samples treated with only Protamex or with simultaneous treatment with Protamex and Flavourzyme, respectively. The peptide coverage profiles and analysis of cleavage sites of BSG proteins provided a good overview of the protein regions more accessible by those proteases as well as their respective preferred cleavages. As no additional change (increase or decrease) in bitter sensation was observed during the incubation with used enzymes and reaction conditions, then adding exopeptidase to the mixture, becomes especially useful if smaller peptides and increased amino acid content are preferred. In further investigations, such hydrolysates could be advantageous for increasing nutritional value or improving the techno-functional properties of various protein-enriched food products such as dairy alternatives (e.g., cheese- or yoghurt-like products), meat products, or sports drinks.

Declaration of Competing Interest

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

CRediT authorship contribution statement

Marie Kriisa: Methodology, Investigation, Writing – original draft. **Anastassia Taivosalo:** Methodology, Investigation, Writing – original draft. **Maike Föste:** Writing – review & editing. **Mary-Liis Kütt:** Conceptualization, Supervision, Writing – review & editing. **Maret Viirma:** Investigation. **Reimo Priidik:** Formal analysis, Visualization. **Malgorzata Korzeniowska:** Investigation. **Ye Tian:** Writing – review & editing. **Oskar Laaksonen:** Project administration, Writing – review & editing. **Baoru Yang:** Project administration, Writing – review & editing. **Raivo Vilu:** Conceptualization, Supervision, Writing – review & editing.

Ethical Statement – Studies in humans and animals

Not applicable.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2022.100108.

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