Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-018-8886-8

GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Proteomic profiling of barley spent grains guides enzymatic solubilization of the remaining proteins

Xuezhi Bi[1] · Lijuan Ye[1,2] · Ally Lau[1] · Yee Jiun Kok[1] · Lu Zheng[1] · Daniel Ng[1] · Kelly Tan[1] · Dave Ow[1] · Edwin Ananta[2] · Christina Vafiadi[3] · Jeroen Muller[2]

Received: 3 October 2017 / Revised: 14 February 2018 / Accepted: 18 February 2018

Abstract

Within the brewing industry, malted barley is being increasingly replaced by raw barley supplemented with exogenous enzymes to lessen reliance on the time-consuming, high water and energy cost of malting. Regardless of the initial grain of choice, malted or raw, the resultant bulk spent grains are rich in proteins (up to 25% dry weight). Efficient enzymatic solubilization of these proteins requires knowledge of the protein composition within the spent grains. Therefore, a comprehensive proteomic profiling was performed on spent grains derived from (i) malted barley (spent grain A, SGA) and (ii) enzymatically treated raw barley (spent grain B, SGB); data are available via ProteomeXchange with identifier PXD008090. Results from complementary shotgun proteomics and 2D gel electrophoresis showed that the most abundant proteins in both spent grains were storage proteins (hordeins and embryo globulins); these were present at an average of two fold higher in spent grain B. Quantities of other major proteins were generally consistent in both spent grains A and B. Subsequent in silico protein sequence analysis of the predominant proteins facilitated knowledge-based protease selection to enhance spent grain solubilization. Among tested proteases, Alcalase 2.4 L digestion resulted in the highest remaining protein solubilization with 9.2 and 11.7% net dry weight loss in SGA and SGB respectively within 2 h. Thus, Alcalase alone can significantly reduce spent grain side stream, which makes it a possible solution to increase the value of this low-value side stream from the brewing and malt extract beverage manufacturing industry.

Keywords Barley spent grain · Proteomics · Enzymatic solubilization · Hordein · Mass spectrometry

Introduction

Malted barley is the major substrate used in brewing industry as a source of starch and protein and to provide the hydrolytic enzymes necessary for the degradation of the barley's storage proteins and carbohydrates to produce the wort, which is then fermented. It is also used in the food industry for the production of malt extract, an attractive ingredient due to its nutritional and sensorial qualities. However, malting is a time-consuming process with high cost in water and energy. The brewing industry is thus moving towards the replacement of malted barley with non-malted raw barley in which industrial enzymes are added to assist or replace the enzymes that are generated during germination of the barley grains.

Barley spent grain (BSG) is the high volume and low-value side stream from malt extract beverage or beer industry, with a global annual production of approximately 38.6 million tons (Mussatto 2014). This by-product is rich in carbohydrates, proteins, and minerals (White et al. 2008). In the last decade, many efforts have been made to valorize spent grain, which is mainly used today as an animal feed, with a focus on the industrial exploitation of the residual carbohydrates, proteins, or phenolic compounds in BSG for human diet (Ktenioudaki et al. 2012; Stojceska et al. 2008), energy production (Xiros

Xuezhi Bi and Lijuan Ye contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-018-8886-8) contains supplementary material, which is available to authorized users.

✉ Xuezhi Bi
bi_xuezhi@bti.a-star.edu.sg

[1] Bioprocessing Technology Institute, Agency for Science, Technology and Research, 20 Biopolis Way, #06-01 Centros, Singapore 138668, Singapore

[2] Present address: Nestlé R&D Center (Pte) Ltd, 29 Quality Road, Singapore 618802, Singapore

[3] Nestlé Research Center, CH-1000 Lausanne 26, Lausanne, Switzerland

Published online: 17 March 2018

and Christakopoulos 2009), and microorganism or enzyme production (Dhillon et al. 2012; Souza et al. 2012). A major development includes optimization to the mashing process by addition of industrial enzymes to assist the barley hydrolysis in extraction. Furthermore, the substitution of malted barley with raw barley and industrial enzymes will significantly reduce water consumption and wastewater generation resulting from the malting process, as 4.5–5 m^3 of wastewater is generated on average for every tone of malt produced.

Although the gross composition of BSG has been investigated intensively (Gupta et al. 2010; Robertson et al. 2010), the identity and quantity of the protein components of BSG remain unclear. Besides fibers (cellulose, hemicellulose, and lignin), proteins are the second most abundant content in spent grain biomass and the poor water solubility of spent grain proteins is the leading reason for limited applications of BSG. Previous efforts made to enhance protein solubility have mostly focused on enzyme selection (Celus et al. 2007), enzymatic solubilization conditions (Treimo et al. 2009), or physicochemical pretreatment (Niemi et al. 2012), whereas the protein components of BSG have not been well characterized. Hence, a better understanding of the BSG proteins will facilitate the development of new exploitations of this agro-industrial by-product. Moreover, evaluation of the remaining insoluble protein content in spent grains also gives an indication of the extraction efficiency of the mashing process that will affect the nutritional value of the wort for use in production of malt extract food and beverages, as well as the free amino nitrogen content necessary for optimum yeast fermentation in brewing.

In the present study, proteomic analysis was carried out on spent grains derived from two commonly applied processing techniques using (1) malted (i.e., germinated) barley and (2) non-malted raw barley. Industrial enzymes were applied to solubilize the remaining proteins in the by-product and reduce the side stream. BSG proteins were identified and quantified by liquid chromatography-mass spectrometry (LC-MS), and protein integrity was assessed by gel electrophoresis. These enabled a knowledge-based selection of proteases for enzymatic hydrolysis and establishment of an efficient enzymatic strategy for solubilization of the remaining proteins in the spent grains. Among five shortlisted proteases examined, Alcalase was found to significantly solubilize the remaining proteins and reduce the total dry weight of the spent grains.

Materials and methods

Materials

Spent grain samples from processes using malted barley (SGA) and non-malted barley supplemented with a proprietary mix of exogenous enzymes (SGB) were provided by Nestlé R & D Singapore; aliquots of grain samples were milled in liquid nitrogen, lyophilized at − 86 °C in Martin Christ Alpha 2–4 freeze dryer (Osterode, Germany) and stored at − 20 °C. Mass spectrometry grade Trypsin Gold and sequence grade chymotrypsin were purchased from Promega (Madison, USA); proteinase K, papain, Alcalase 2.4 L, Neutrase, and protease S were from Sigma-Aldrich (St. Louis, USA).

1D and 2D gel electrophoresis

For 1D sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, 100 mg of lyophilized spent grain sample was extracted with 1 ml of SDS extraction buffer (4% SDS, 100 mM dithiothreitol (DTT), 100 mM Tris-HCl pH 7.5) at 95 °C for 10 min and clarified at 21,100×*g* for 10 min at 4 °C. Protein concentration was determined using RC DC Protein Assay (Bio-rad, Hercules, USA), and 30 μg of extracted proteins were resolved in a precast 12% TGX gel (Bio-Rad, Hercules, USA) and subsequently stained with 0.1% Coomassie R-250.

For 2D gel electrophoresis, interfering pigments and polysaccharides were first extracted from 100 mg of lyophilized spent grain sample with 1 ml 10% trichloroacetic acid (TCA) in acetone at − 20 °C for duration. The grains were washed thrice with 1 ml acetone each and pelleted at 21,100×*g* for 10 min at 4 °C. The grain pellet was dried in a vacuum concentrator (Labconco, Kansas City, MO, USA) at 4 °C, extracted with volume UTC buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-base) for 1 h at 37 °C with mixing, and clarified at 21,100×*g* for 30 min at 10 °C. Protein concentration was determined; 400 or 800 μg of protein extract were separated by 2D gel electrophoresis on 18 cm pH 3–10 non-linear Immobilized pH Gradient strip (Bio-Rad) followed by 12% SDS-PAGE, and Coomassie-stained (0.1% (*w*/*v*) Coomassie Brilliant Blue R250, 50% (*v*/*v*) methanol and 10% (v/v) acetic acid in H_2O) for identification or silver-stained for visualization as performed previously (Bi et al. 2006).

Protein identification by NanoLC-MS/MS

Major 1D gel bands and 2D gel spots were excised and washed three times with 25 mM ammonium bicarbonate in 50% acetonitrile, dehydrated with 100% acetonitrile, reduced with 20 mM DTT in 50 mM ammonium bicarbonate at 56 °C for 25 min, alkylated with 55 mM iodoacetamide in 50 mM ammonium bicarbonate at room temperature for 30 min in the dark, and digested with 10 ng/μl trypsin or chymotrypsin in 25 mM ammonium bicarbonate at 37 °C overnight. Digested samples were subsequently extracted first with 20 mM ammonium bicarbonate, then with 50% (*v*/*v*) acetonitrile and 5% (*v*/*v*) formic acid in H_2O, dried in vacuum concentrator (Labconco), and dissolved in 1% (*v*/*v*) formic acid and 2% (*v*/*v*) acetonitrile.

LC-MS/MS analysis was performed using a nanoAcquity HPLC (Waters, Milford, USA) coupled to LTQ Orbitrap Elite MS (ThermoFisher Scientific, Waltham, MA, USA) according to previously described method (Lee et al. 2015) with minor modifications. Briefly, eluted peptides were separated online in a Waters nanoACQUITY UPLC BEH130 C18 column (1.7 μm, 75 μm × 200 mm) at 35 °C using mobile phases A 0.1% formic acid' and B 0.1% formic acid in acetonitrile on a gradient of 5–40% B in 50 min and 40–97% B in 3 min at a flow rate of 0.3 μl/min. Mass spectra were acquired using nanoelectrospray in positive ionization mode at 1.8 kV. The LTQ Orbitrap Elite was operated in a top 15 peak data dependent survey scan at a resolution of 120,000 from 350 to 1800 m/z and CID MS/MS with normalized collision energy of 35.

MS raw files were searched against barley (*Hordeum vulgare* L.) database extracted from NCBI (38,557 relevant entries, downloaded on 6 Feb 2017) using Sequest HT algorithm in Proteome Discoverer version 1.4 and Peaks Studio 8.0 (Bioinformatics Solutions Inc., Waterloo, Canada). The appropriate enzyme (trypsin or chymotrypsin) as used for the sample was selected. A maximum of two missed cleavage sites were allowed with peptide length ranging from 6 to 44 amino acids. The peptide and fragment ion mass tolerances were limited to ± 10 ppm and ± 0.6 Da respectively. Maximum modifications per peptide were set at three, with dynamic modifications of oxidation on methionine and deamidation on asparagine and glutamine, and static modification of carbamidomethylation on cysteine defined in the search parameters. Positive protein identification required at least two unique peptides and a maximum 1% false discovery rate (FDR).

Shotgun proteomics and label free quantitative analysis

One hundred micrograms of protein extracted using SDS extraction buffer were reduced, alkylated, and tryptic-digested on 30 k molecular weight cut-off regenerated cellulose membrane (filter-assisted sample preparation method (Wisniewski et al. 2009). The LC-MS/MS was carried out as described in previous section except for a change to gradient from 5 to 40% B in 105 min and 40–90% B in 5 min. Three biological replicates were performed for the LC-MS/MS-based shotgun proteomics analysis.

Quantitative proteomics software package MaxQuant (version 1.5.2.8) (Cox and Mann 2008) was used to perform label-free quantification (LFQ) on the three biological replicates datasets. Fixed modification of carbamidomethylation to cysteine residues and variable modifications of oxidation (to methionine residues) and acetylation (to the protein N-terminus) were specified and search against the same barley (*H. vulgare* L.) database as above and a built-in common contaminants database. MS/MS tolerance for FTMS was set at 10 ppm and MS/MS tolerance for ITMS was set at 0.5 Da tolerance. Minimum peptide length is 7 and minimum number of peptides is 1 with a minimum ratio count of 1 for quantification of protein. Peptide peaks were matched between runs with a matching time windows of 0.7 min and an alignment time windows of 20 min. Proteins which are identified by shared peptides are grouped in one protein group. Protein group identifications were filtered at 1% FDR using a reverse decoy strategy. Quantification of protein groups was based on razor and unique peptides. MaxQuant LFQ algorithm extracts the isotope patterns of each peptide and matches these isotopic patterns across different runs (Cox et al. 2014). Total peptide signal within each run was then normalized to allow comparisons between runs. A maximal list of peptides was used to generate a matrix of protein ratios between SGA and SGB and a least square regression was used to calculate the best estimate for the protein ratios. A "LFQ" normalized "area" is derived. The "LFQ" intensities of all the protein groups identified were summed to derive total protein amount and corresponding protein percentage was calculated by dividing the "LFQ" value of the protein group with the summed value. The SGB/SGA ratio of individual protein was obtained by dividing the LFQ intensity of the protein in SGB over SGA.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaino et al. 2016) with the dataset identifier PXD008090 and https://doi.org/10.6019/PXD008090.

Protease hydrolysis of spent grains

The accession numbers of the main proteins were input to perform virtual cleavage by ExPASy PeptideCutter tool ENREF_33 (Gasteiger et al. 2005). Proteases were screened according to the number of predicted cleavage site. Proteases that have most cleavage numbers were used to hydrolyze the barley spent grain. Two commercial proteases, Alcalase 2.4 L and Neutrase, along with two proteases with broad specificity, papain and protease S, were also selected for hydrolysis together with proteinase K.

Enzymatic hydrolysis using proteinase K, Alcalase 2.4 L, Neutrase, papain, or protease S was carried out at laboratory scale. Enzyme activity was determined and aligned for all of the enzymes with enzymatic assay of Protease as defined by Sigma-Aldrich, where one Sigma unit was defined as activity required to hydrolyze casein to produce color equivalent to 1.0 μmole of tyrosine per minute at pH 7.5 at 37 °C (color by Folin & Ciocalteau's reagent). Aliquot of 100 mg milled and freeze-dried spent grain powder was topped up to 1 ml with deionized water and solubilized by 24 U of protease per gram of SGA and SGB at 60 °C with agitation at 800 rpm for 1.5 h. A reaction without enzyme was used as negative control. After enzymatic treatment, hydrolysates were centrifuged at 21,100×*g* for 10 min at 4 °C and the pellets washed once with deionized water. To calculate dry weight loss, washed pellets were weighed in a desiccator after drying at 105 °C

until weight remained constant. To determine hydrolysis efficiency, remaining proteins in the washed pellets were extracted with SDS extraction buffer at 95 °C for 15 min, and protein content quantified by RC DC Protein Assay (Bio-Rad) before analysis by SDS-PAGE.

Three replicates were performed for each enzymatic hydrolysis reactions unless otherwise stated, and Student's *t* test (p value < 0.05) was applied for paired samples.

Results

Label-free quantitative (LFQ) shotgun proteomic profiling

The proteome compositions of malted spent grains (SGA) and non-malted spent grains (SGB) were determined and compared by LC-MS/MS-based shotgun proteomics profiling. A total of 1346 and 1146 protein groups were confidently detected (≥ 2 unique peptides at 1% FDR) in SGA and SGB respectively when the NCBInr database was used for the search with taxonomy restricted to barley (*H. vulgare* L.) (Supplemental Table S1).

As shown in Table 1 and Supplemental Table S2, the most abundant proteins identified in SGA and SGB were storage proteins, with B-hordeins and embryo globulins accounting for the majority of storage proteins found in both spent grains. In general, SGB contained over twice more storage proteins than SGA. The lower amount of storage proteins remaining in SGA was likely the result of proteases, de novo synthesized during malting, that aided in solubilization of the proteins (Celus et al. 2006). This was supported by the detection of barley cysteine protease EP-B2 with at least seven unique peptides in SGA but barely detectable in SGB (7% LFQ intensity of SGA) (Supplemental Table S1); EP-B2 is an efficient enzyme at solubilizing hordeins-like gluten (Gass et al. 2006). This is consistent with the previous finding that a major gibberellic acid-induced barley aleurone cysteine proteinase digested hordein during the malting process (Giese and Hejgaard 1984).

The second most abundant category of protein identified in both spent grains was enzymes involved in carbohydrate metabolism (Table 1), such as α- and β-amylases which are both major enzymes involved in amylolysis during malting and mashing (Steiner et al. 2012). Unlike β-amylase which is synthesized during grain development, α-amylase is synthesized during barley germination (Giese and Hejgaard 1984) or malting. Hence, the relatively easier removal of exogenously added industrial enzymes compared to de novo produced enzymes during filtration to separate BSG from the wort likely accounted for the ten times less α-amylase present in SGB compared to SGA.

Several pathogenesis-related proteins remained in both spent grains. Serpin (serine proteinase inhibitor) Z4 acts as an inhibitor to protect barley seed storage proteins from breaking down by endogenous and exogenous serine proteases during development (Roberts et al. 2003). While serpin Z4 is reported to be inhibitory to serine proteases in barley malt (Specker et al. 2014), specific inhibition of malt serine proteases during mashing has shown no effect on the soluble protein content in the resulting wort (Jones 1999). However, serpin Z4, present at 2.36 times higher in SGB, in an active form could potentially affect the activity of exogenous proteases supplemented in the mashing of the raw barley grains. A number of α-amylase/trypsin inhibitors, CMa, CMb, CMc, and CMd, were also identified in both spent grains. CM proteins do not inhibit malt α-amylase but have been shown to be active against a malt serine protease (Jones and Fontanini 2003). They are presumed to function primarily in defense against pests due to their inhibitory effect on exogenous α-amylases in insects. Plant toxin alpha-hordothionin which plays an important role in antifungal and antibacterial activities (Bohlmann and Apel 1991) was also identified as an abundant protein in both spent grains.

Other proteins, which survived mashing, were housekeeping proteins, such as elongation factors and histone 4, and chaperones like heat shock cognate 70 kD protein and protein disulfide-isomerase.

Complementary 2D gel electrophoresis proteomic profiling of BSG

To remove major interfering substances of downstream analyses such as oligosaccharides, polysaccharides, lignin, phenolic component, and fat (McCarthy et al. 2013), as well as small degraded protein fragments in the spent grain, 10% TCA in acetone solution was used to clean-up the milled and lyophilized spent grains prior to protein extraction and subsequent resolution by 2D gel electrophoresis. Spots of high intensity were excised for in-gel digestion followed by LC-MS/MS analysis and the identities are annotated in Fig. 1 and Supplemental Fig. S1. The major proteins were β-amylase, hordeins, and embryo globulin. All three proteins were associated with multiple spots at different isoelectric points or different MWs on the silver-stained gels; such presentation was likely a result of post-translational modifications and protein degradation during the mashing process (Ostergaard et al. 2004).

Embryo globulin with a predicted MW of 72.2 kDa, for example, was associated with the largest number of spots (> 10 for SGB sample) with different MWs on the gels (Fig. 1, Table 2), indicating variable degradation. β-amylase could exist in an inactive bound form or a released mature and active form following removal of its N- and C-terminal propeptides; the latter has a predicted MW of 55.1 kDa and pI of 5.99 (P82993). Hence, spot 44, found only in SGB, likely corresponded to the inactive bound form of β-amylase while spot 28, found in both SGA and SGB, corresponded to the

Table 1 Comparison of top 25 abundant proteins of SGA and SGB

Protein category	Protein	Accession No.	Relative amount in SGA (%)	Relative amount in SGB (%)	SGB/SGA (LFQ intensity ratios)
Storage protein—hordein	B1 hordein	AFM77743.1	2.22	4.11	2.67
	B3 hordein	AFM77746.1	4.03	7.35	2.63
	D hordein	BAA11642.1	0.79	1.46	2.65
	γ-1 hordein	P17990.1	1.00	2.01	2.90
	γ-3 hordein	P80198.1	[b]0.18	0.72	5.68
Storage protein—globulin	Embryo globulin	AAA32936.1	3.67	7.15	2.81
	[a]Triticin [100%,0.0,87%]	BAK04052.1	1.24	1.85	2.15
	[a]Vicilin-like seed storage protein [73%,0.0,90%]	BAK04746.1	[b]0.62	0.75	1.74
Carbohydrate metabolism	α-amylase type B isozyme	P04750.2	1.14	[c]0.08	0.10
	β-amylase	P16098.1	5.30	5.57	1.51
	Beta-glucosidase	ACF07998.1	[b]0.50	0.72	2.08
	Chitinase	JC5918	0.67	[c]0.47	1.01
	Fructose-bisphosphate aldolase	ACO44685.1	0.94	[c]0.67	1.03
	Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	P08477.1	1.28	1.08	1.21
	Granule-bound starch synthase 1, chloroplastic/amyloplastic	P09842.1	1.18	1.25	1.52
	[a]Pyruvate, phosphate dikinase 2 [100%,0.0,98%]	BAJ92764.1	[b]0.45	0.78	2.49
	Starch branching enzyme I	AAP72268.1	[b]0.53	0.80	2.17
	Sucrose synthase	S24966	1.69	1.73	1.47
Pathogenesis related	Alpha-amylase/trypsin inhibitor CMa	P28041.2	[b]0.56	0.72	1.85
	Alpha-amylase/trypsin inhibitor CMb	P32936.2	1.13	1.16	1.48
	Alpha-amylase/trypsin inhibitor CMd	P11643.2	0.86	0.74	1.23
	Alpha-hordothionin	P01545.1	0.88	1.25	2.06
	Barperm1	AAB71680.1	0.95	[c]0.40	0.61
	Protein synthesis inhibitor I	P22244.2	[b]0.31	0.71	3.31
	Serpin-Z4	P06293.2	0.69	1.14	2.36
	Trypsin inhibitor CMc	P34951.2	0.87	1.21	2.01
Housekeeping	[a]ATP synthase subunit beta, mitochondrial-like [100%,0.0,96%]	BAJ90283.1	0.69	[c]0.38	0.79
	Elongation factor 1-alpha	CAA90651.1	1.34	1.20	1.30
	[a]Elongation factor 2-like [100%,0.0,99%]	BAJ87342.1	0.81	[c]0.53	0.96
	[a]Histone H4 [100%,8e-68,100%]	BAK08280.1	0.90	0.94	1.49
Chaperone	[a]Heat shock cognate 70 kDa protein 4 [100%,0.0,98%]	BAK00257.1	0.95	[c]0.66	1.00
	Protein disulfide-isomerase	P80284.2	0.86	1.64	2.77

[a] Annotated by BLASTp against NCBInr database with tribe restricted to Triticeae [% Cover, Expect value,% Identity]

[b,c] Relative protein amount not within top 25 most abundant proteins of SGA and SGB respectively

active processed form. Proteases activated during malting are known to process and release β-amylase (Celus et al. 2007); this corroborated with our observed absence of bound β-amylase in malted SGA. In contrast, most B- and C-hordeins 2D spots matched their predicted intact MWs. Note that C-hordein contains few lysine or arginine residues, and hence were only identified as a major protein in 2D-PAGE analysis following chymotrypsin digestion (Fig. 1) but not in tryptic peptide-based LFQ shotgun analysis.

Identified proteins from the 2D gels with experimental and theoretical MW and pI values of all spots excised and assessed are listed in Table 2.

Selection of proteases based on proteomics profiling for solubilization of remaining proteins in BSG

The malted SGA and non-malted SGB contained the same major protein families such as hordeins and embryo globulin,

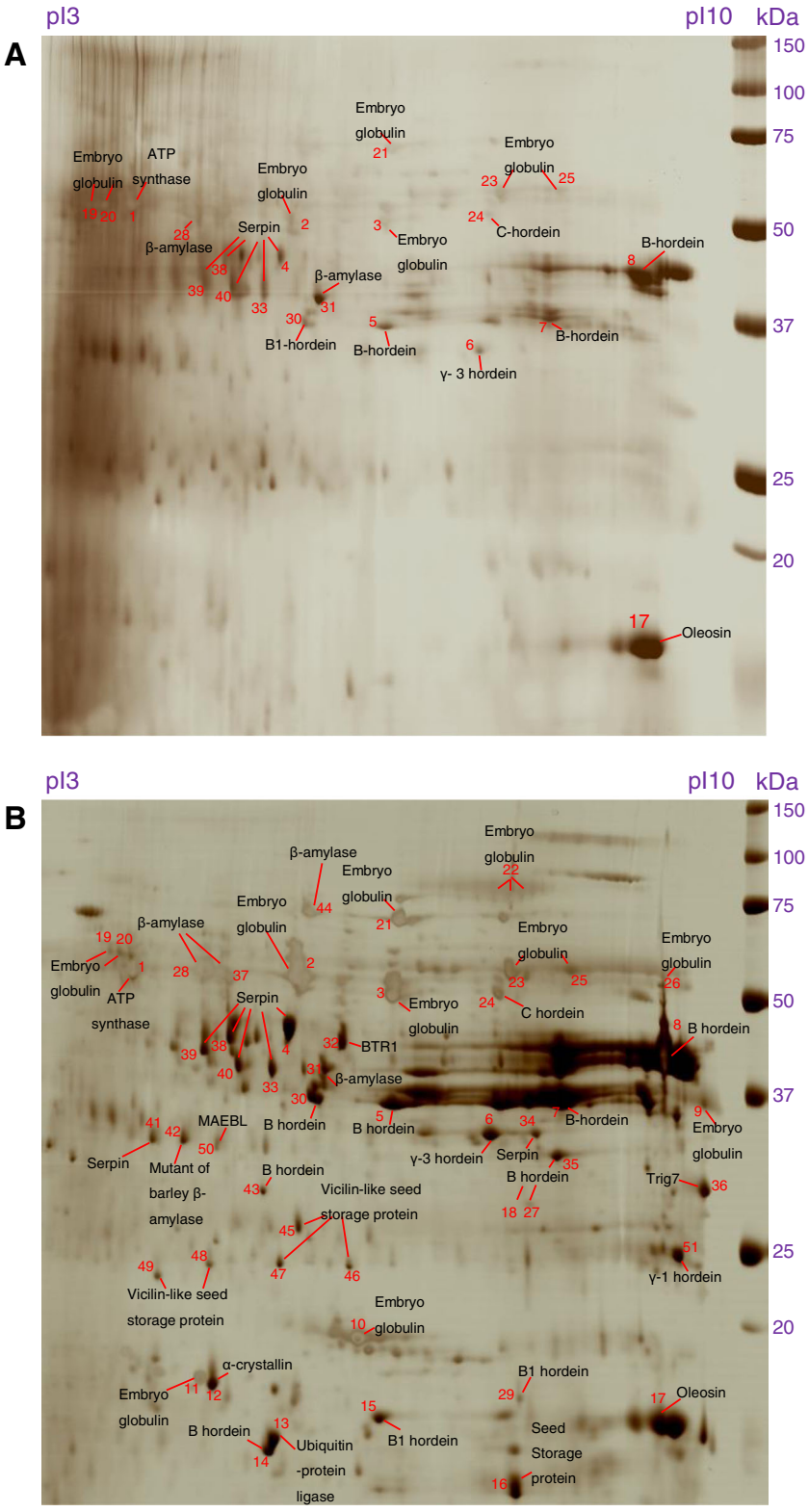

Fig. 1 2D gel electrophoresis of proteins extracted from spent grains, silver-stained. First dimension IEF pH 3–10NL, second dimension 12% SDS-PAGE. Major protein spots were identified by LC-MS/MS. **a**: Spent grain A. **b** Spent grain B

which are coincidentally insoluble in water when intact. Thus, based on the sequences of these proteins, the use of proteases with relevant cleavage efficiency can overcome this insolubility problem. Such targeted enzyme selection saves time and effort by avoiding the need to screen all proteases available in the market.

Hordeins, the most abundant proteins in spent grain, contain a high percentage of proline and glutamine (up to 35%), which

Table 2 LC-MS identified 2D gel spots from SGA and SGB

Spot No.	Protein name	Accession No.	Theo. MW (kDa)/pI	Exp. MW (kDa)/pI	Sequest protein score	No. of peptides (MS/MS)
1	ATP synthase	BAJ90283.1	59.3/6.24	60/4.5	77.78	14
2	Embryo globulin	AAA32936.1	72.2/7.27	65/5.5	72.32	13
3	Embryo globulin	AAA32936.1	72.2/7.27	60/6	26.34	9
4	Serpin z-type	CAA66232.1	43.2/5.94	50/5.5	23.78	7
5	B hordein	AFM37567.1	29.9/7.31	37/6	106.07	4
6	γ-3 hordein, partial	CAA51204.1	32.8/7.09	30/6.5	45.89	3
7	B hordein	ACU09489.1	30.1/7.91	37/7	76.83	4
8	B hordein	ACU09489.1	30.1/7.91	45/8	104.32	5
9	Embryo globulin	AAA32936.1	72.2/7.27	37/8	73	10
10	Embryo globulin	AAA32936.1	72.2/7.27	20/6	27.2	5
11	Embryo globulin	AAA32936.1	72.2/7.27	15/5	108.5	8
12	ACD_ScHsp26_like, Alpha crystallin domain	BAK06674.1	17.5/5.69	15/5	47.08	6
13	Ubiquitin-protein ligase	BAJ85100.1	17.1/7.34	10/5.5	17	3
14	B hordein	AFM37567.1	29..9/7.31	10/5.5	49.43	4
15	B1 hordein	CAA25509.1	20.6/7.24	10/6	5.74	2
16	Seed storage protein, partial	AAB71679.1	3.4/9.32	10/6.5	62.19	2
17	Oleosin	BAJ87238.1	18.5/9.69	10/8	109.29	5
18	B hordein	ACU09489.1	30.1/7.91	25/6.5	23.35	3
19	Embryo globulin	AAA32936.1	72.2/7.27	70/4.5	119.29	13
20	Embryo globulin	AAA32936.1	72.2/7.27	70/4.5	144.3	14
21	Embryo globulin	AAA32936.1	72.2/7.27	75/6	33.68	4
22	Embryo globulin	AAA32936.1	72.2/7.27	80/6.7	56.23	10
23	Embryo globulin	AAA32936.1	72.2/7.27	60/6.7	66.82	7
24	C hordein	P02864.1	3.3/6.23	50/6.6	27.72	3
25	Embryo globulin	AAA32936.1	72.2/7.27	60/7	91.84	10
26	Embryo globulin	AAA32936.1	72.2/7.27	60/8	205.16	11
27	B hordein	ACU09489.1	30..1/7.91	25/6.7	245.86	4
28	β-amylase	AGY14538.1	59.5/6.01	60/5.1	69.87	13
29	B1 hordein	CAA25509.1	20.6/7.24	15/6.6	136.36	3
30	B hordein	AFM37567.1	29.9/7.31	37/5.75	212.26	4
31	β-amylase	AGY14530.1	59.5/5.91	40/5.75	76.17	16
32	BTR1	BAJ97491.1	38.1/6.39	45/5.75	47.44	10
33	Serpin z-type	CAA66232.1	43.2/5.94	40/5.5	453.93	18
34	Serpin z-type	CAA66232.1	43.2/5.94	30/6.75	148.68	10
35	B hordein	ACU09489.1	30.1/7.91	30/7	114.2	4
36	Trig 7	ACI00278.1	29.5/9.67	30/8	215.51	8
37	β-amylase	AGY14530.1	59.5/5.91	55/5.8	59.78	15
38	Serpin z-type	CAA66232.1	43.2/5.94	45/5.75	89.37	14
39	Serpin Z7	Q43492.2	42.8/5.68	45/5.7	102.75	12
40	Serpin z-type	CAA66232.1	43.2/5.94	40/5	337.08	14
41	Serpin z-type	CAA66232.1	43.2/5.94	35/4.9	34.05	6
42	β-amylase mutant	1B1Y_A	56.2/6.11	35/5.1	11.95	4
43	B hordein, truncated	ACU09492.1	30.0/7.31	30/5.5	36.37	3
44	β-amylase	AGY14538.1	59.5/6.01	75/5.7	30.67	10
45	Vicilin-like seed storage protein	BAK04746.1	76.9/5.82	28/5.6	416.62	7
46	Vicilin-like seed storage protein	BAK04746.1	76.9/5.82	25/5.8	171.93	5

Table 2 (continued)

Spot No.	Protein name	Accession No.	Theo. MW (kDa)/pI	Exp. MW (kDa)/pI	Sequest protein score	No. of peptides (MS/MS)
47	Vicilin-like seed storage protein	BAK04746.1	76.9/5.82	25/5.5	68.1	3
48	Vicilin-like seed storage protein	BAK04746.1	76.9/5.82	25/5.3	37.79	6
49	Vicilin-like seed storage protein	BAK04746.1	76.9/5.82	25/5	21.26	2
50	MAEBL	BAJ86520.1	33.9/8.27	30/5.3	101.3	13
51	γ-1 hordein	P17990.1	34.7/8.02	25/8	190.12	7

are hydrophobic and insoluble in water. Therefore, digestion with a general protease or enzymes with preferred cleavage at proline and glutamine are required to break down these proteins into smaller fragments before they become soluble in water. Subtilisin A from *Bacillus* (Doucet et al. 2003), prolyl endoprotease from *Aspergillus niger* (Montserrat et al. 2015; Stepniak et al. 2006), and barley cysteine protease EP-2B are potential choices based on the literature (Gass et al. 2006). However, except for subtilisin A, the other two enzymes are not readily available or economically viable for industrial food processing. Hence, only Alcalase (commercial form of subtilisin) activity on spent grains was further examined.

β-amylase contains high percentage of glycine (10.5%) and alanine (7.7%) while barley embryo globulin (BEG1) contains up to 13–15% arginine and glutamic acid; therefore, different non-specific proteases such as proteinase K can be used to hydrolyze these proteins. Moreover, in silico digestion with ExPASy PeptideCutter tool (https://web.expasy.org/peptide_cutter) indicated that proteinase K has the highest number of cutting sites for the most abundant proteins in BSG (Gasteiger et al. 2005). As protein solubilization does not only rely solely on the number of cleavage sites but also on factors such as accessibility and binding capacity, the performances of other food grade proteases with broad specificity, such as Neutrase, papain (papaya proteinase I), and protease S, were also examined. Neutrase is recommended by Novozymes for its activity on gluten (a general name for cereal storage proteins, including hordeins). Papain was selected as it can cleave peptide bonds formed by glycine and members of the papain family has been reported to completely degrade hordeins (Simpson 2001). Protease S, a type of serine protease, was selected because of its thermostability with broad specificity for native and denatured proteins.

Solubilization efficiency of proteases on BSG proteins

Figure 2a showed the SDS-PAGE profile of proteins extracted from SGB after treatment with the five proteases; the negative control consisted of SGB treated with water without enzyme. Most of the protein bands present in the untreated and negative controls were absent in the Alcalase-treated and the proteinase K-treated samples. The molecular weight of proteins decreased to a lesser extent after treatment with protease

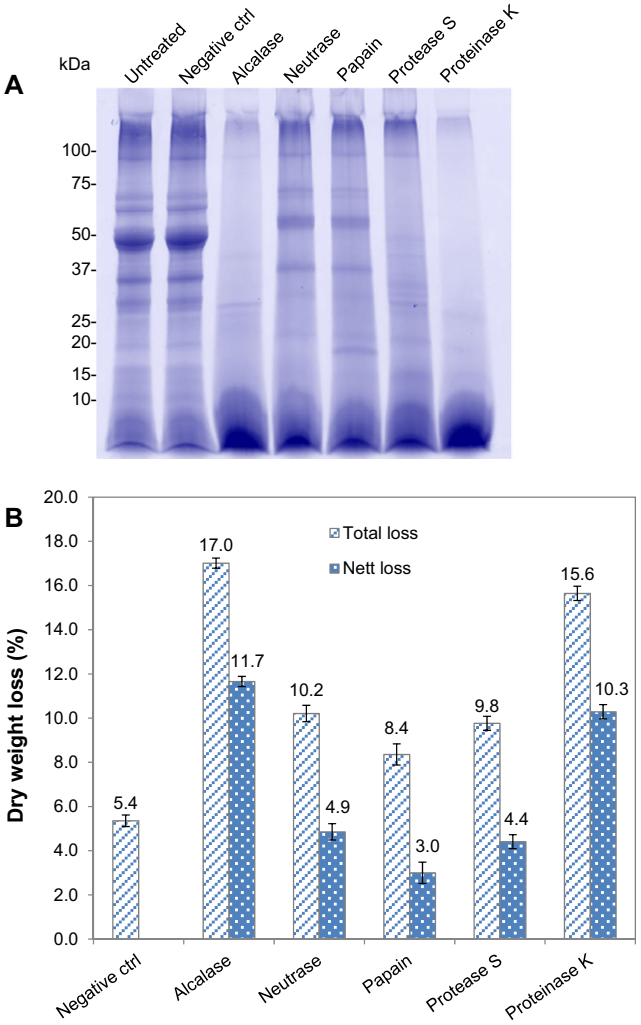

Fig. 2 Alcalase and proteinase K were the most efficient at solubilizing SGB among five proteases screened. **a** 1D SDS-PAGE of proteins extracted from SGB after treatment with different proteases, and **b** corresponding total and net dry weight loss. Hydrolysis was carried out with enzyme dosage of 24 U/g spent grain for 1.5 h in deionized water. Net weight loss was calculated from the difference between total weight loss in enzyme-treated sample and in negative control, where no enzyme was added. Error bars represent one standard deviation of triplicate data. ctrl control

S, Neutrase, and papain. The predominant bands in the enzyme-treated samples occurred below 10 kDa, thus indicating that remaining proteins in the spent grain had been enzymatically hydrolyzed into small fragments. Alcalase and proteinase K digestion resulted in the best solubility of BSG, followed by protease S, while Neutrase and papain had the weakest solubilization capability among the five proteases. Some of the major protein bands identified in the untreated and negative control were β-amylase, B3 hordein, serpin, and embryo globulin (Supplemental Fig. S2).

Net dry weight losses of SGB following protease digestion (Fig. 2b) were consistent with hydrolytic efficiency of the five enzymes as demonstrated by SDS-PAGE result. Among the proteases, Alcalase was the most efficient at solubilizing SGB, with 11.7% net weight loss, and this is also consistent with previous reports on proteases screened for protein solubilization of brewer's spent grain (Treimo et al. 2008). Alcalase showed the same effective solubilization of SGA, with 9.2% net weight loss (Fig. 3). The detection of dry weight loss in negative water control of both SGA and SGB samples (~6%) suggested the presence of residual enzymatic activity in the spent grains. As no significant changes in BSG proteins were observed between untreated and negative water control samples (Figs. 2a and 3a), these dry weight losses were not a result of protease activity but remaining carbohydrase activity present in the spent grains, such as β-amylase (Lundgard and Svensson 1987), β-glucanase (Loi et al. 1987), and xylanase (Li et al. 2005) activities detected in our study (data not shown). This is consistent with reported observation of up to 13% solubilization of the carbohydrates by residual enzymes in spent grains, yielding mainly glucose-based products (Niemi et al. 2012; Treimo et al. 2008).

Discussion

To the best of our knowledge, this is the first report of comprehensive proteome profiles comparing BSG derived from malted barley (SGA) and enzymatically treated raw barley (SGB) via complementary shotgun proteomics and 2D gel electrophoresis approaches. Evaluation of the remaining insoluble protein content in BSG will enable a knowledge-based selection of commercial enzymes to increase solubility of BSG proteins, and the choice of proteases used has been shown to affect the technofunctional properties, such as emulsifying and foaming properties, of BSG protein hydrolysates (Celus et al. 2007). These enzymes can also be potentially use in the mashing process to improve nutritional or physicochemical properties of downstream malt extract beverages and beers. Other relevant proteome profiling studies reported are mainly associated with the brewing industry where the proteomes of various barley cultivars and corresponding barley malts are compared to analyze malt qualities (Jin et al. 2013; Ostergaard et al. 2002), or the proteomes of wort or beer are characterized to evaluate the presence of immunogenic barley proteins and polypeptides (Colgrave et al. 2012; Picariello et al. 2012).In this study, we have shown that, with the exception of β-amylase, the most abundant proteins found in both spent grains were storage proteins—hordeins and globulins. Hordein content may be underestimated, as it does not contain many lysine or arginine residues in general, and thus not easily identified and quantified using tryptic peptide-based LFQ shotgun proteome analysis. Hordein C, for example, was only identified as a major protein in 2D-PAGE analysis (Fig. 1) following digestion with chymotrypsin, an enzyme that is often used as an alternative to trypsin for MS identification of hordeins (Colgrave et al. 2012;

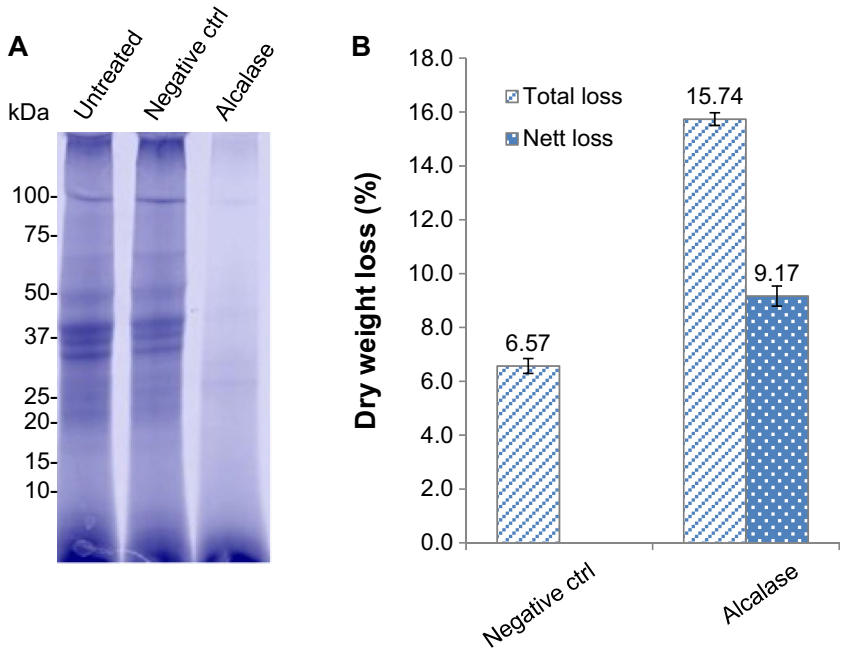

Fig. 3 Alcalase was similarly effective at solubilizing proteins in SGA. **a** 1D SDS-PAGE of proteins extracted from SGA after Alcalase treatment, and **b** corresponding total and net dry weight loss. Error bars represent one standard deviation of triplicate data. ctrl control

Flodrova et al. 2012; Schalk et al. 2017). SGB appeared to contain higher protein content, especially hordeins, than SGA (Table 1 and Fig. 1) but our initial assessment found similar amount of total SDS extractable protein content within both spent grains (~21% of dry weight, data not shown). This suggests that the endogenous malt proteases have higher efficiency at breaking down hordeins and other barley proteins but a significant portion of the resulting fragments or polypeptides may remain insoluble and not extractable. Industrial processes using malted and/or enzymatically treated raw barley will thus similarly benefit from further optimization strategy to improve protein solubilization efficiency.

To this end, we screened five proteases for their ability to solubilize and extract the remaining proteins or their breakdown fragments in spent grains. These enzymes were selected based on their potential efficiency at digesting high abundant proteins or large fragments identified from the proteome profiling of the spent grains, as well as economic viability (cost and availability of food grade enzyme preparation). Such targeted enzyme selection saves time and effort by avoiding the need to screen all proteases available in the market. Alcalase was found to solubilize hordeins and other remaining proteins in the spent grains most efficiently at a dose of 24 U/g spent grain for 1.5 h (Figs. 2 and 3). The same dosage of Alcalase has been reported by Treimo et al. (2008, 2009) to solubilize much more proteins in brewer's spent grains (25–30% gross dry weight loss) but required prolonged enzymatic hydrolysis treatment (5–18 h) and digestion at much lower loading of 3.33% (*w*/*v*) freeze-dried spent grain in enzyme favorable pH-controlled buffers. A lower loading which translates to higher water usage and energy cost will be less environmentally sustainable, and the use of pH-controlled buffers may not be industrially feasible. Moreover, if residual spent grain enzymatic activity (10–13% dry weight loss in negative control samples) is to be excluded, the resultant net solubilization ascribed to added enzymes is only 12–20%. Though this is higher than the observed solubilization efficiency of 9.2–11.7% in this study, the treatment parameters established in this study, a much shorter enzymatic treatment of 1.5 h and higher loading of 10% (*w*/*v*) dried spent grain in water, are more feasible for industrial implementation in terms of lower energy cost and water consumption, as well as ease of integration with any standard mashing process. For industrial implementation where "fresh" spent grains containing ~67% moisture are used, an equivalent loading can be achieved by mixing "fresh" spent grain and water at 1:2 (*w*/*v*) ratio with an optimized amount of Alcalase (to be determined empirically for different mashing processes). An incubation period of a few hours at 50–60 °C subsequently will help reduce spent grain volume without an excessive increase in cost or processing time. Alcalase may also be used directly in the mashing process though its effect on the mash, in terms of consequent sensorial qualities of end-products for example, will need to be examined. Other commercial enzymes, such as carbohydrases, have also been used in combination with Alcalase to further digest BSG components in another study that yielded a 25–30% dry weight loss (Robertson et al. 2011). The corresponding reduction in BSG volume in addition to enhance extraction of its organic components via enzymatic strategy will also help reduce the cost associated with handling and disposal of this side stream.

In summary, a comprehensive proteome profiling of spent grains derived from malted barley and raw barley supplemented with industrial enzymes enabled (i) a knowledge-based selection of proteases for enzymatic hydrolysis and (ii) establishment of an efficient enzymatic strategy for the solubilization of remaining proteins in the spent grains. Similar protein solubilization efficiency and spent grain side stream reduction by Alcalase treatment were achieved for both types of spent grains. Hence, Alcalase treatment of BSG or its implementation in upstream mashing process will enhance the extraction of more protein nutrients from the substrate and translate to a reduction in side stream mass and volume that will help reduce the cost associated with handling and disposal.

Acknowledgments This research was supported by Biomedical Research Council (BMRC), Agency for Science, Technology and Research and a joint research collaboration fund between Bioprocessing Technology Institute, Agency for Science, Technology and Research, Singapore, and NESTEC LTD, Switzerland.

Authors' contributions X Bi designed and supervised the study. L Ye, A Lau, L Zheng, and K Tan performed the experiments. L Ye, X Bi, A Lau, YJ Kok, and D Ng analyzed the data and wrote the manuscript. J Muller, C Vafeiadi, D Ow, and E Ananta gave valuable suggestions during experimental design and revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

References

Bi X, Lin Q, Foo TW, Joshi S, You T, Shen HM, Ong CN, Cheah PY, Eu KW, Hew CL (2006) Proteomic analysis of colorectal cancer reveals alterations in metabolic pathways: mechanism of tumorigenesis. Mol Cell Proteomics 5(6):1119–1130. https://doi.org/10.1074/mcp.M500432-MCP200

Bohlmann H, Apel K (1991) Thionins. Annu Rev Plant Physiol Plant Mol Biol 42(1):227–240. https://doi.org/10.1146/annurev.pp.42.060191.001303

Celus I, Brijs K, Delcour JA (2006) The effects of malting and mashing on barley protein extractability. J Cereal Sci 44(2):203–211. https://doi.org/10.1016/j.jcs.2006.06.003

Celus I, Brijs K, Delcour JA (2007) Enzymatic hydrolysis of brewers' spent grain proteins and technofunctional properties of the resulting hydrolysates. J Agric Food Chem 55(21):8703–8710. https://doi.org/10.1021/jf071793c

Colgrave ML, Goswami H, Howitt CA, Tanner GJ (2012) What is in a beer? Proteomic characterization and relative quantification of hordein (gluten) in beer. J Proteome Res 11(1):386–396. https://doi.org/10.1021/pr2008434

Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M (2014) Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol Cell Proteomics 13(9):2513–2526. https://doi.org/10.1074/mcp.M113.031591

Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26(12):1367–1372. https://doi.org/10.1038/nbt.1511

Dhillon GS, Kaur S, Brar SK (2012) In-vitro decolorization of recalcitrant dyes through an ecofriendly approach using laccase from *Trametes versicolor* grown on brewer's spent grain. Int Biodeterior Biodegrad 72(Supplement C):67–75. https://doi.org/10.1016/j.ibiod.2012.05.012

Doucet D, Otter DE, Gauthier SF, Foegeding EA (2003) Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. J Agric Food Chem 51(21):6300–6308. https://doi.org/10.1021/jf026242v

Flodrova D, Ralplachta J, Benkovska D, Bobalova J (2012) Application of proteomics to hordein screening in the malting process. Eur J Mass Spectrom (Chichester) 18(3):323–332. https://doi.org/10.1255/ejms.1184

Gass J, Vora H, Bethune MT, Gray GM, Khosla C (2006) Effect of barley endoprotease EP-B2 on gluten digestion in the intact rat. J Pharmacol Exp Ther 318(3):1178–1186. https://doi.org/10.1124/jpet.106.104315

Gasteiger E, Hoogland C, Gattiker A, Se D, Wilkins MR, Appel RD, Bairoch A (2005) Protein identification and analysis tools on the ExPASy server. In: Walker JM (ed) The proteomics protocols handbook. Humana Press, Totowa, NJ, pp 571–607

Giese H, Hejgaard J (1984) Synthesis of salt-soluble proteins in barley. Pulse-labeling study of grain filling in liquid-cultured detached spikes. Planta 161(2):172–177. https://doi.org/10.1007/BF00395478

Gupta M, Abu-Ghannam N, Gallaghar E (2010) Barley for brewing: characteristic changes during malting, brewing and applications of its by-products. Compr Rev Food Sci Food Saf 9(3):318–328. https://doi.org/10.1111/j.1541-4337.2010.00112.x

Jin Z, Mu YW, Sun JY, Li XM, Gao XL, Lu J (2013) Proteome analysis of metabolic proteins (pI 4-7) in barley (*Hordeum vulgare*) malts and initial application in malt quality discrimination. J Agric Food Chem 61(2):402–409. https://doi.org/10.1021/jf3034418

Jones BL (1999) Malt endoproteinases and how they affect wort soluble protein levels. In: Proceedings of the 9th Australian Barley Technical Symposium, Pathways into the 21st Century. Melbourne, Australia. Australian Barley Technical Symposium Inc., Warwick, Qld, Australia, pp 2.39.31–2.39.38

Jones BL, Fontanini D (2003) Trypsin/alpha-amylase inhibitors inactivate the endogenous barley/malt serine endoproteinase SEP-1. J Agric Food Chem 51(19):5803–5814. https://doi.org/10.1021/jf030075x

Ktenioudaki A, Chaurin V, Reis SF, Gallagher E (2012) Brewer's spent grain as a functional ingredient for breadsticks. Int J Food Sci Technol 47(8):1765–1771. https://doi.org/10.1111/j.1365-2621.2012.03032.x

Lee KG, Kim SS, Kui L, Voon DC, Mauduit M, Bist P, Bi X, Pereira NA, Liu C, Sukumaran B, Renia L, Ito Y, Lam KP (2015) Bruton's tyrosine kinase phosphorylates DDX41 and activates its binding of dsDNA and STING to initiate type 1 interferon response. Cell Rep 10(7):1055–1065. https://doi.org/10.1016/j.celrep.2015.01.039

Li Y, Lu J, Gu G (2005) Control of arabinoxylan solubilization and hydrolysis in mashing. Food Chem 90(1):101–108. https://doi.org/10.1016/j.foodchem.2004.03.031

Loi L, Barton PA, Fincher GB (1987) Survival of barley (1→3, 1→w4)-β-glucanase isoenzymes during kilning and mashing. J Cereal Sci 5(1):45–50. https://doi.org/10.1016/S0733-5210(87)80008-5

Lundgard R, Svensson B (1987) The four major forms of barley β-amylase. Purification, characterization and structural relationship. Carlsb Res Commun 52(4):313–326. https://doi.org/10.1007/bf02907173

McCarthy AL, O'Callaghan YC, Piggott CO, FitzGerald RJ, O'Brien NM (2013) Brewers' spent grain; bioactivity of phenolic component, its role in animal nutrition and potential for incorporation in functional foods: a review. Proc Nutr Soc 72(1):117–125. https://doi.org/10.1017/S0029665112002820

Montserrat V, Bruins MJ, Edens L, Koning F (2015) Influence of dietary components on *Aspergillus niger* prolyl endoprotease mediated gluten degradation. Food Chem 174:440–445. https://doi.org/10.1016/j.foodchem.2014.11.053

Mussatto SI (2014) Brewer's spent grain: a valuable feedstock for industrial applications. J Sci Food Agric 94(7):1264–1275. https://doi.org/10.1002/jsfa.6486

Niemi P, Faulds CB, Sibakov J, Holopainen U, Poutanen K, Buchert J (2012) Effect of a milling pre-treatment on the enzymatic hydrolysis of carbohydrates in brewer's spent grain. Bioresour Technol 116:155–160. https://doi.org/10.1016/j.biortech.2012.04.043

Ostergaard O, Finnie C, Laugesen S, Roepstorff P, Svennson B (2004) Proteome analysis of barley seeds: identification of major proteins from two-dimensional gels (pI 4-7). Proteomics 4(8):2437–2447. https://doi.org/10.1002/pmic.200300753

Ostergaard O, Melchior S, Roepstorff P, Svensson B (2002) Initial proteome analysis of mature barley seeds and malt. Proteomics 2(6):733–739. https://doi.org/10.1002/1615-9861(200206)2:6<733::AID-PROT733>3.0.CO;2-E

Picariello G, Mamone G, Nitride C, Addeo F, Camarca A, Vocca I, Gianfrani C, Ferranti P (2012) Shotgun proteome analysis of beer and the immunogenic potential of beer polypeptides. J Proteome 75(18):5872–5882. https://doi.org/10.1016/j.jprot.2012.07.038

Roberts TH, Marttila S, Rasmussen SK, Hejgaard J (2003) Differential gene expression for suicide-substrate serine proteinase inhibitors (serpins) in vegetative and grain tissues of barley. J Exp Bot 54(391):2251–2263. https://doi.org/10.1093/jxb/erg248

Robertson JA, Castro-Marinas L, Collins SR, Faulds CB, Waldron KW (2011) Enzymatic and chemical treatment limits on the controlled solubilization of brewers' spent grain. J Agric Food Chem 59(20):11019–11025. https://doi.org/10.1021/jf202814j

Robertson JA, I'Anson KJA, Treimo J, Faulds CB, Brocklehurst TF, Eijsink VGH, Waldron KW (2010) Profiling brewers' spent grain for composition and microbial ecology at the site of production. LWT-Food Sci Technol 43(6):890–896. https://doi.org/10.1016/j.lwt.2010.01.019

Schalk K, Lexhaller B, Koehler P, Scherf KA (2017) Isolation and characterization of gluten protein types from wheat, rye, barley and oats for use as reference materials. PLoS One 12(2):e0172819. https://doi.org/10.1371/journal.pone.0172819

Simpson DJ (2001) Proteolytic degradation of cereal prolamins—the problem with proline. Plant Sci 161(5):825–838. https://doi.org/10.1016/S0168-9452(01)00482-4

Souza DT, Bispo AS, Bon EP, Coelho RR, Nascimento RP (2012) Production of thermophilic endo-beta-1,4-xylanases by *Aspergillus fumigatus* FBSPE-05 using agro-industrial by-products. Appl Biochem Biotechnol 166(6):1575–1585. https://doi.org/10.1007/s12010-012-9563-5

Specker C, Niessen L, Vogel RF (2014) In vitro studies on the main beer protein Z4 of *Hordeum vulgare* concerning heat stability, protease

inhibition and gushing. J Inst Brew 120(2):85–92. https://doi.org/10.1002/jib.118

Steiner E, Auer A, Becker T, Gastl M (2012) Comparison of beer quality attributes between beers brewed with 100% barley malt and 100% barley raw material. J Sci Food Agric 92(4):803–813. https://doi.org/10.1002/jsfa.4651

Stepniak D, Spaenij-Dekking L, Mitea C, Moester M, de Ru A, Baak-Pablo R, van Veelen P, Edens L, Koning F (2006) Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. Am J Physiol Gastrointest Liver Physiol 291(4):G621–G629. https://doi.org/10.1152/ajpgi.00034.2006

Stojceska V, Ainsworth P, Plunkett A, İbanoğlu S (2008) The recycling of brewer's processing by-product into ready-to-eat snacks using extrusion technology. J Cereal Sci 47(3):469–479. https://doi.org/10.1016/j.jcs.2007.05.016

Treimo J, Aspmo SI, Eijsink VG, Horn SJ (2008) Enzymatic solubilization of proteins in brewer's spent grain. J Agric Food Chem 56(13):5359–5365. https://doi.org/10.1021/jf073317s

Treimo J, Westereng B, Horn SJ, Forssell P, Robertson JA, Faulds CB, Waldron KW, Buchert J, Eijsink VG (2009) Enzymatic solubilization of brewers' spent grain by combined action of carbohydrases and peptidases. J Agric Food Chem 57(8):3316–3324. https://doi.org/10.1021/jf803310f

Vizcaino JA, Csordas A, Del-Toro N, Dianes JA, Griss J, Lavidas I, Mayer G, Perez-Riverol Y, Reisinger F, Ternent T, Xu QW, Wang R, Hermjakob H (2016) 2016 update of the PRIDE database and its related tools. Nucleic Acids Res 44(22):11033. https://doi.org/10.1093/nar/gkw880

White JS, Yohannan BK, Walker GM (2008) Bioconversion of brewer's spent grains to bioethanol. FEMS Yeast Res 8(7):1175–1184. https://doi.org/10.1111/j.1567-1364.2008.00390.x

Wisniewski JR, Zougman A, Nagaraj N, Mann M (2009) Universal sample preparation method for proteome analysis. Nat Methods 6(5):359–362. https://doi.org/10.1038/nmeth.1322

Xiros C, Christakopoulos P (2009) Enhanced ethanol production from brewer's spent grain by a *Fusarium oxysporum* consolidated system. Biotechnol Biofuels 2(1):1754–6834. https://doi.org/10.1186/1754-6834-2-4