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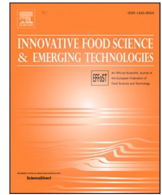

Brewers' spent grain proteins: The extraction method determines the functional properties

Yi Ling Chin [a,b,c], Julia Katharina Keppler [a], Somayeh Taghian Dinani [a], Wei Ning Chen [b,c], Remko Boom [a,*]

[a] Laboratory of Food Process Engineering, Wageningen University & Research, PO Box 17, 6700 AA Wageningen, the Netherlands
[b] Food Science and Technology Programme, Nanyang Technological University, 62 Nanyang Drive, 637459, Singapore
[c] School of Chemistry, Chemical Engineering and Biotechnology, Nanyang Technological University, 62 Nanyang Drive, 637459, Singapore

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ABSTRACT

Brewers' spent grains (BSG) are a major byproduct from the brewing industry that are currently discarded or used as animal feed. This study systematically explored the effects of BSG protein extraction methods using alkali, ethanol or enzymes on the protein's structure, composition and function. Proteins extracted at pH 12 were partially unfolded and glutelin-rich while those extracted by 55% ethanol containing 2-mercaptoethanol were highly aggregated and hordein-rich. Enzymatic-assisted extraction resulted in peptides below 10 kDa that had no distinct structural elements. Being completely water-soluble, these peptides gave good emulsifying properties (activity: 83 m^2/g protein; stability: 35 min) and a high antioxidant activity. On the other hand, alkali-extracted and ethanol-extracted proteins were mostly insoluble but exhibited high water holding capacities (2.5–4.0 g/g) that enabled gelation. The results highlighted that selection of the extraction method is critical, as the composition, structure and function of proteins are modified, which affects its potential applications.

Industrial relevance: As a major side stream from the production of beer, brewers' spent grains are present in huge amounts. These residues are a promising source of alternative proteins, but before separating them, it is important to first consider an extraction method that does not compromise on its functionality. This study offered insights into the influence of extraction methods on the structure and function of the resulting protein concentrates as well as its future applications.

1. Introduction

Fuelled by the need to reduce environmental waste, the recovery of valuable components from agricultural residues and by-products from food processing has become increasingly relevant. Coupled with the shift towards alternative sources of proteins in recent years, it would be desirable to recover proteins from food waste residues. Brewers' spent grains (BSG), a by-product of the beer brewing industry, make up 85% of brewing waste and largely consist of insoluble proteins (20%), fibres (70%) and an abundance of phenolic compounds (Mussatto, Dragone, & Roberto, 2006). As the mashed barley residues that are generated after starch is converted to sugars, BSG is a major side stream that that can be further exploited for other applications. Annually, an estimated 39 million tonnes of BSG is generated worldwide (Macias-Garbett, Serna-Hernández, Sosa-Hernández, & Parra-Saldívar, 2021), yet most of this waste is currently discarded or used as animal feed due to microbial instability caused by its high moisture content (Mussatto et al., 2006).

Utilisation of the BSG protein fraction is not straightforward as the proteins that remain in BSG after wort removal are by definition insoluble. These proteins are mostly storage proteins known as hordeins and glutelins. Hordeins, which in their native form are alcohol and alkali-soluble, make up about 35–55% of the total barley grain proteins and can be further subdivided into A, B, C, D and γ hordeins based on their electrophoretic mobility and amino acid compositions (Celus, Brijs, & Delcour, 2006). Glutelins, which are in native form also alkali- but not alcohol-soluble, constitute 23% of the total barley proteins. Although BSG typically originates from barley, there are differences in terms of protein extractability as a result of the brewing process. During the mashing process of beer brewing, a complex is formed between the residual high molecular weight aggregates composed of proteins and carbohydrates (gelprotein) in the malt and the glutelins that form an impenetrable layer on BSG (Moonen, Graveland, & Muts, 1987). Celus

* Corresponding author.
E-mail addresses: yiling.chin@wur.nl (Y.L. Chin), julia.keppler@wur.nl (J.K. Keppler), wnchen@ntu.edu.sg (W.N. Chen), remko.boom@wur.nl (R. Boom).

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et al. (2006) showed that mashing likely induces the formation of disulfide bonds, resulting in strong protein aggregation. Therefore, protein extraction from a byproduct like BSG is more challenging than from unprocessed barley due to the physical and thermal processing history. More extreme extraction strategies may thus be necessary to separate the proteins from its matrix.

Alkaline extraction is one of the most common ways to fractionate BSG proteins (Connolly, Piggott, & FitzGerald, 2013). At an alkaline pH, the lignocellulosic BSG matrix is partially broken up and proteins in the system attain a net negative charge. The increased repulsion within and between polypeptide chains lead to enhanced protein solubilisation. Adjustment of the pH of the system to the isoelectric point of the proteins, causes the proteins to become insoluble and precipitate. The preference for alkali extraction of protein stems from its high extraction yield of about 82–85% (Vieira et al., 2014). However, this method comes at the expense of changes in the protein's structure, composition and function (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015; Yang, Zamani, Liang, & Chen, 2021), which were not extensively investigated in previous studies but are critical when deciding on suitable applications for these proteins. Furthermore, alkalis are capable of solubilising other components in a lignocellulosic matrix including hemicellulose and lignin (Vieira et al., 2014), resulting in a compositional change of the fractions which may influence its functionality.

Owing to the limited solubility of BSG proteins, another existing method is via enzymatic action, which involves the use of proteases and carbohydrases directly on BSG to open its matrix and release peptides, or to extract proteins by alkali first followed by enzymatic hydrolysis (Celus, Brijs, & Delcour, 2007; Connolly et al., 2019). The resulting protein hydrolysates, containing 66–77% protein and a yield as high as 92% (Celus et al., 2007), showed increased solubility and improved emulsifying and foaming properties when compared to the alkali-extracted protein concentrate. However, the use of enzymes could be costly and difficult to scale industrially. A third option, as demonstrated by Celus et al. (2006), is to use an aqueous alcohol mixture containing a reducing agent to reduce the disulfide bridges between hordeins and solubilise these proteins. A resulting extraction yield of about 12% was obtained but the functional properties of the resulting BSG protein concentrate were not examined. In addition, food-grade reagents were not employed in the abovementioned literature, thereby restricting its applications in food.

Several studies showed that the choice of extraction method significantly affects the protein's composition, structure and function. Yang et al. (2021) demonstrated that alkaline extraction of pea protein had more profound impact on the protein conformation than salt extraction and altered its gelling properties. Similar results were found by Hadnađev et al. (2018) with hempseed meal protein isolates, in which the structural differences in the protein secondary structure impacted the water retention capacity. From this aspect, it is important to select a suitable protein extraction method based on its desired functionality and intended application before optimising the extraction process to attain desirable yields.

To the best of our knowledge, few studies adopted aqueous alcohol protein extraction for BSG and no work has yet been performed to compare BSG proteins obtained by different extraction techniques. While there are other recent technologies such as ultrasound, microwave or pulsed electric field that aim to improve extraction yields or reduce extraction time, the focus of this work is to examine how solvents modify the protein's structure and composition. Given the existing information, we hypothesise that different ways of extraction will produce protein concentrates that vary in composition, structure and functional properties. Therefore, the objective of this work is to systematically explore how the extraction method influences the composition, structure and function of protein from BSG. The proteins were extracted separately using alkaline extraction and aqueous ethanol. A commercial BSG protein hydrolysate was compared. Proximate analysis of the various samples was performed, and their structures were analysed by spectroscopy and electrophoresis. The physicochemical and technofunctional properties were also evaluated. Finally, potential food applications pertaining to the composition, structure and function of BSG-derived protein fractions were discussed.

2. Materials and methods

2.1. Materials

BSG was kindly supplied by Asia Pacific Breweries Pte Ltd. (Singapore) and stored in polyethylene bags at −20 °C before freeze-drying (FreeZone 2.5, Labconco, Kansas, US) at −50 °C, 0.01 mbar for 5 days. The freeze-dried BSG was milled in a rotor mill (Pulverisette 14, Fritsch, Idar-Oberstein, Germany) and passed through a 400 μm air-jet sieve (e200LS, Hosokawa Alpine, Augsburg, Germany). Barley protein isolate (BPI), obtained by pH-adjusted enzymatic extraction, was kindly supplied by EverGrain LLC (St. Louis, USA). Soybean oil containing 92 g fat per 100 ml was purchased from a local supermarket (The Netherlands). Acetone and hexane (100%) were purchased from Actu-All Chemicals B.*V.* (Oss, The Netherlands). Ethanol (≥ 96%) was purchased from VWR Chemicals (Amsterdam, The Netherlands). Folin-Ciocalteu reagent and Pierce™ BCA Protein Assay Kit were purchased from Thermo Fisher Scientific (Waltham, USA). 2× Laemmli sample buffer, running buffer, Precision Plus Protein Dual Color Standards and Bio-safe Coomassie Stain were purchased from Bio-Rad Laboratories (Hercules, USA). 8-anilinonapthalene-1-sulfonic acid ammonium salt (ANSA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Alfa Aesar (Ward Hill, USA). All other reagents were purchased from Sigma-Aldrich (St Louis, USA). For all experiments, Milli-Q water was used.

2.2. Preparation of defatted and dephenolised BSG (dBSG)

Milled BSG was defatted with hexane in a SOXTHERM® unit (SOX416, Gerhardt, Germany) and left to evaporate overnight in a fume hood. Phenolics were removed according to the protocol described by Meneses, Martins, Teixeira, and Mussatto (2013). Briefly, an acetone-water mixture (60:40, *v*/v) was added to defatted BSG at a solid-liquid ratio of 1:20 (*w/v*) and incubated at 60 °C for 30 min with agitation. The mixture was centrifuged at 4500*g* for 20 min at 20 °C followed by vacuum filtration. The residue was dried in a vacuum oven (VD53, Binder, Tuttlingen, Germany) to a dry matter content of about 90% and termed as dBSG.

2.2.1. Alkali-extracted BSG protein concentrate (A-BPC)

To prepare A-BPC, dBSG was dispersed in Milli-Q water at a solid-liquid ratio of 1:20 and pH was adjusted to 12 using 6 M NaOH. The extraction was carried out for 24 h at 20 °C and 40 rpm (SB3 rotator, Stuart, UK). The solid-liquid mixture was separated by centrifugation at 10,000*g* for 20 min at 20 °C. The supernatant was collected and pH was adjusted to 4 using 6 M HCl for isoelectric precipitation of proteins. A second centrifugation step at 10,000*g* for 20 min at 4 °C was applied to collect the precipitates before neutralising the pH and freeze-drying the precipitate (Epsilon 2-10D LSCplus, Martin Christ, Germany) at −20 °C, 0.01 mbar for 3 days.

2.2.2. Preparation of ethanol-extracted BSG protein concentrate (E-BPC)

To prepare E-BPC, dBSG was added to 55% ethanol containing 0.5% 2-mercaptoethanol at a solid-liquid ratio of 1:10. The mixture was incubated at 60 °C for 1 h in a shaking water bath at 200 rpm (SW22, Julabo, The Netherlands) and centrifuged at 10,000*g* for 20 min at 20 °C. The supernatant was collected and rotary evaporated (RC600, KNF, USA) at 40 °C. Precipitates were collected by centrifugation at 10,000*g* for 20 min at 4 °C, washed several times with water and freeze-dried.

2.3. Compositional analysis

The nitrogen content was determined with the Dumas combustion method (FlashSmart™ Elemental Analyzer, Thermo Scientific, US), with a protein conversion factor of 5.83 (Jones, 1931). The lipid content was determined using hexane in a SOXTHERM® unit. Ash content was determined by gravimetric method in a furnace at 550 °C for 24 h (AAF1100, Carbolite, UK). The fibre content was determined by difference. The total phenolic content was determined by the Folin-Ciocalteu method as described previously (Chin, Chai, & Chen, 2022), with samples dispersed at pH 12 (protein concentration of 10 mg/ml) and expressed as gallic acid equivalent (GAE).

2.4. Molecular weight estimation of protein by gel electrophoresis

The molecular weight ranges of the protein extracts were estimated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions in a vertical Bio-Rad mini-gel electrophoresis unit. Sample buffer (65.8 mM Tris-HCl pH 6.8, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue) was mixed with 2-mercaptoethanol at a ratio of 19:1 (*v/v*). All samples were dispersed in the reducing sample buffer, ensuring that the final protein concentration was 2 mg/ml. After a centrifugation step at 10,000*g* for 10 min to separate insoluble parts, the supernatant was heated at 95 °C for 10 min and centrifuged at 10,000*g* for 5 min. Then, 15 μl of the supernatant and 5 μl of Precision Plus Protein Dual Color Standards were loaded on a 12% Mini-PROTEAN® TGX™ Precast gel (Bio-Rad Laboratories, Hercules, USA) in different lanes. The electrophoresis was carried out at 200 *V* for approximately 40 min. The gel was washed three times with MilliQ water and stained with the Bio-safe Coomassie Stain. Excess stain was removed with MilliQ water. The GS-900 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, USA) was used for gel imaging.

2.5. Protein secondary structure by Fourier-transform infrared (FTIR) spectroscopy

The secondary structure of the proteins was investigated using an ATR-FTIR (ALPHA II-Platinum ATR, Bruker, USA). About 20 mg of sample was placed on the crystal cell of the spectrometer at room temperature before being pressed. The FTIR spectrum for each sample, measured in at least triplicates, was recorded at a resolution of 4 cm^{-1} and averaged over 32 scans across the spectral range of 400–4000 cm^{-1}. For interpretation of the results, baseline correction and vector normalisation were applied on the OPUS 8.1 software (Bruker, USA). The secondary structure in the range of 1600–1700 cm^{-1} was processed using the derivative option on the software with 9 smoothing points. The peaks in the second derivative spectra were assigned as follows: β-turns (1662–1684 cm^{-1}), α-helix (1655–1658 cm^{-1}), random coils (1640–1650 cm^{-1}), intramolecular β-sheets (1629–1632 cm^{-1}) and intermolecular β-sheets (1619–1621 cm^{-1}), respectively (Keppler, Heyn, Meissner, Schrader, & Schwarz, 2019).

$$\text{EAI}\ (\text{m}^2/\text{g protein}) = \frac{2 \times 2.303 \times A_0 \times \textit{dilution factor}}{C \times \varnothing \times 10{,}000}, \text{ where } \textit{dilution factor} \text{ is } 100,\ C = \text{weight of protein per unit volume} \left(g/ml\right),\ \Phi = \text{oil fraction of emulsion.} \tag{3}$$

2.6. Surface hydrophobicity

The surface hydrophobicity was measured using ANSA as a fluorescence probe. Samples were dispersed in 10 mM phosphate buffer (pH 7) and incubated at 60 °C for 1 h at 500 rpm (ThermoMixer C, Eppendorf, Germany) before being centrifuged at 4000*g* for 30 min. The protein concentration in the supernatant was determined by the BCA assay and diluted with phosphate buffer to a range between 0.005 mg/ml to 0.3 mg/ml. To 4 ml of diluted samples, 20 μl of 8 mM ANSA in 10 mM phosphate buffer (pH 7) was added and left in the dark for 15 min. The fluorescence intensity of the samples was measured at the excitation wavelength of 390 nm and the emission wavelength of 470 nm (RF-6000, Shimadzu, Japan). The net fluorescence intensity was calculated by subtracting the fluorescence of proteins without ANSA from the protein samples with ANSA at each protein concentration.

2.7. Water and oil holding capacities (WHC) and nitrogen solubility index (NSI)

Dried samples with known masses (M_0) were added to water or oil respectively at a solid-liquid ratio of 1:50 (*w/v*) in pre-weighed tubes and left to mix at 40 rpm for 20 h (SB3 rotator, Stuart, UK). Subsequently, the samples were centrifuged at 4800*g* for 30 min at 20 °C. The supernatant was carefully removed with a pipette and the mass of the wet pellet was recorded (M_1). The WHC was calculated according to Eq. (1). The wet pellet was then freeze-dried to remove the water and its protein content was determined using Dumas. The amount of solubilised protein was calculated by difference (Eq. (2)).

$$\text{WHC (g water/g dry sample)} = \frac{M_1 - M_0}{M_0} \tag{1}$$

$$\text{NSI}\ (\%) = \frac{\textit{Protein content}_{\textit{initial sample}} - \textit{Protein content}_{\textit{dry pellet}}}{\textit{Protein content}_{\textit{initial sample}}} \times 100\% \tag{2}$$

2.8. Emulsifying properties

Samples were dispersed in 10 mM phosphate buffer (pH 7) at a protein concentration of 0.1% (w/v). Then, 5 ml of soybean oil was added to 15 ml of each sample and homogenised at 20,000 rpm for 1 min (Ultra Turrax T18 Digital, IKA, Germany). In the next step, 50 ml of each emulsion was pipetted from the bottom of the container at 0 and 10 min after homogenisation, then diluted with 5 ml of 0.1% SDS solution. The absorbances of the diluted emulsions (A_0 and A_{10}, for 0 and 10 min respectively) were read at 500 nm (DR 6000, Hach, US), and used to calculate the emulsifying activity index (EAI) and emulsion stability index (ESI) according to Eqs. (3) and (4).

$$\text{ESI}\ (\textit{min}) = \frac{A_0 \times \Delta t}{A_0 - A_{10}}, \text{ where } \Delta t = 10\ \textit{min} \tag{4}$$

2.9. Gelling capacity

The thermal gelation behaviour of BSG proteins was determined by the lowest gelling concentration according to Yang et al. (2021) with some modifications. Protein-rich samples (A-BPC, *E*-BPC and BPI) between the protein concentrations of 2% to 20% (*w/v*) were dispersed in 10 mM phosphate buffer (pH 7) and allowed to hydrate for at least 3 h with vigorous agitation. The samples were subsequently heated at 95 °C for 1 h and cooled immediately by ice bath and stored at 4 °C overnight.

The lowest gelling concentration was defined as the concentration at which the sample did not fall or slip when the tube was inverted.

2.10. Antioxidant activity

In view of the possible differences in solubility of antioxidants, two antioxidant assays were selected to eliminate possible influences of solvent choice on measured antioxidant activity.

2.10.1. 2,2′-9-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

An ABTS assay was performed by solubilising 10 mg of ABTS in 2.6 ml of 2.45 mM potassium persulfate solution to yield a final ABTS concentration of 7 mM. The concentrated ABTS solution was stored in the dark at room temperature for 16 h and then diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Samples were diluted to a protein concentration of 0.1 mg/ml using 55% ethanol (adjusted to pH 12). Then, 300 μl of the supernatant was mixed with 900 μl of the diluted ABTS and stored in the dark at room temperature for 1 h. The absorbance was read at 734 nm. Gallic acid was used as a standard.

2.10.2. 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

A DPPH assay was performed according to Xu, Wei, Xue, and Huang (2022) with slight modifications. DPPH solution (1.75×10^{-4} M) was freshly prepared in methanol and samples were diluted to a protein concentration of 0.3 mg/ml using 55% ethanol (adjusted to pH 12). Subsequently, 500 μl of the supernatant was mixed with 500 μl of DPPH solution and the absorbance was measured at 517 nm after 1 h. Gallic acid was used as a standard.

2.11. Statistical analysis

All experimental data were analysed using SPSS Statistical Software (Version 28, IBM, US). A descriptive Duncan's test was used to evaluate the statistical significance between samples at a significance level of 95% ($P \leq 0.05$). All reported results are shown as mean ± standard deviation and all tests were done at least in duplicate.

3. Results and discussion

3.1. Composition

The composition of BSG, dBSG and its derived fractions are shown in Table 1. Our results agree well with values reported previously in literature (Connolly et al., 2013; Mussatto et al., 2006), in which the majority of BSG was comprised of proteins and fibres with a small amount of lipids. After defatting and removal of the phenolics, the lipid content of dBSG was significantly reduced in comparison with the initial BSG ($P \leq 0.05$). However, the protein content of dBSG was not changed significantly ($P > 0.05$) and the overall distribution of components in dBSG was roughly similar to the initial BSG, in which proteins and fibres form the bulk of the material. This also suggests that the effects due to the high fibre content in dBSG should not be neglected.

Table 1
Compositions of BSG, dBSG, A-BPC, *E*-BPC and BPI on a dry basis. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. Fibre was calculated from the other components by mass balance. Different small upper letters in the same row indicate significant difference at $P \leq 0.05$.

Composition (%)	BSG	dBSG	A-BPC	E-BPC	BPI
Protein	22.7 ± 0.5[c]	24.2 ± 0.5[c]	73.1 ± 2.4[b]	74.1 ± 0.7[b]	78.6 ± 0.04[a]
Lipid	9.0 ± 0.1[a]	1.4 ± 0.3[c]	4.8 ± 1.4[b]	5.2 ± 0.3[b]	1.3 ± 0.1[c]
Phenolics*	0.09 ± 0.000[c,d]	0.03 ± 0.002[d]	2.5 ± 0.07[b]	0.2 ± 0.009[c]	2.7 ± 0.06[a]
Ash	3.9 ± 0.05[b]	3.5 ± 0.02[c]	0.6 ± 0.1[d]	0.2 ± 0.001[e]	4.7 ± 0.02[a]
Fibre	64.3	70.9	19	20.3	12.7

* Phenolics were first quantified as mg GAE/ml and converted to mg GAE/g BSG. This is then converted into a percentage (1 mg/g = 0.1%).

In A-BPC and *E*-BPC, protein content was significantly increased to >70% ($P \leq 0.05$), which indicates that the extraction methods were effective for enrichment in proteins. We observed an increase in lipid content relative to dBSG, which may be related to the comparatively lower amount of fibre present.

The Folin-Ciocalteu assay showed that A-BPC and BPI had a significantly higher phenolic content than the other samples ($P \leq 0.05$). In both cases, alkaline pH and enzymatic hydrolysis could have released the insoluble-bound phenolics from cell wall materials such as hemicellulose (Madhujith & Shahidi, 2009) and subsequently could have led to covalent or non-covalent interactions between proteins and phenolics, resulting in this higher phenolic content. This agrees well with previously published work, in which alkali-extracted protein and enzymatically hydrolysed proteins were relatively high in phenolics (Connolly et al., 2013). It is worth mentioning that proteins are reactive to Folin-Ciocalteu reagent (Everette et al., 2010) and may contribute to the perceived phenolic content.

3.2. Molecular weight estimation by SDS-PAGE

Fig. 1 shows the SDS-PAGE gel profile of samples under reducing conditions. The objective of determining the molecular weights of the proteins is to verify their identities with existing literature, which may provide some insight into their structure. In BSG, about six bands at 100 kDa, ~60 kDa, 50 kDa, ~30 kDa, ~22 kDa and 10–15 kDa were evident. These bands in the 10–100 kDa range could represent a small amount of residual water- and salt-soluble proteins, namely the albumins and globulins, left in BSG after the malting and mashing process (Celus et al., 2006). Most of the albumins and globulins are likely to have solubilised in the wort during the brewing process. It was reported previously that

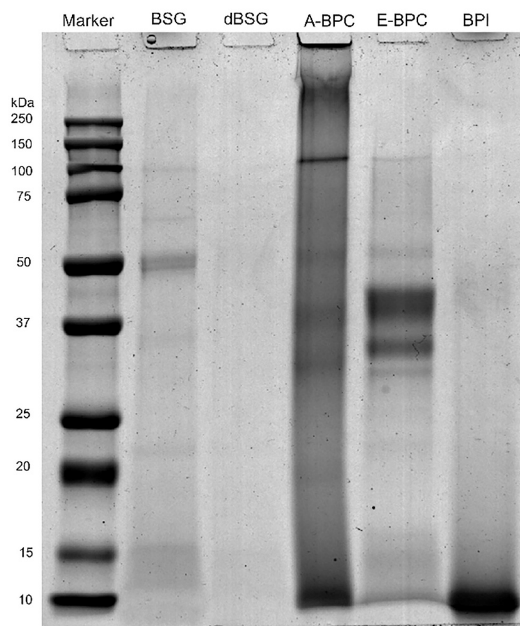

Fig. 1. SDS-PAGE of different samples under reducing conditions. BSG, dBSG, A-BPC, *E*-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively.

barley albumins and globulins are present in the range of 21–58 kDa and 16.5–53 kDa respectively (Linko, Lapvetelainen, Laakso, & Kallio, 1989). A small amount of hordeins is also expected due to the breakdown of storage proteins during malting, which explains the presence of a faint band at 100 kDa.

After defatting and dephenolisation, the bands initially present in BSG were no longer seen in dBSG. This is because the residual proteins were washed away and therefore the majority of the proteins present in dBSG were insoluble and trapped within the matrix. Acetone, which is typically used for protein precipitation, could also have caused aggregation of proteins such that their solubility is reduced. Without prior extraction or pretreatment, these proteins would not solubilise in the buffer and therefore no bands were present for dBSG. The A-BPC and *E*-BPC protein extracts revealed proteins that were slightly more soluble in the buffer (see Section 3.5), so bands were present again in the gel.

In A-BPC, a distinct band at 100 kDa, plus some faint bands in the range of 30–50 kDa and around 20 kDa could be observed against a smeared background throughout the lane. These bands could represent D-hordeins or glutelins, B-hordeins and A hordeins (Celus et al., 2006). The band smearing suggests that prolonged exposure of the protein aggregates at high pH may have led to protein fragmentation with varying degrees of hydrolysis (Wang, Li, Liu, Ren, & Qu, 2016). Another possibility is that some proteins were associated with other alkali-soluble components such as phenolics or cell wall polysaccharides like hemicellulose. The formation of cross-linked, high molecular weight complexes is confirmed by the presence of a band at the top of the lane. It is known that protein-phenol or protein-polysaccharide interactions can be induced under the applied alkaline conditions (Mohamed, Hojilla-Evangelista, Peterson, & Biresaw, 2007). Sęczyk, Świeca, Kapusta, and Gawlik-Dziki (2019) observed that the addition of phenolics to white bean proteins not only produced a band at the bottom of the well but also resulted in smears below the interface between the stacking and resolving gels.

For *E*-BPC, distinctive bands could be seen that are representative of typical hordein subunits. The bands at 100 kDa, 55–80 kDa, 35–50 kDa and below 15 kDa likely represent D-hordeins, C-hordeins, B-hordeins and A-hordeins respectively (Wang et al., 2010). These bands were also found in A-BPC, albeit at different intensities. In particular, the 100 kDa band was more prominent and the 35–50 kDa band was less intense in A-BPC than in E-BPC. This likely indicates that A-BPC is composed of hordeins and glutelins, while E-BPC is enriched in hordeins. Both hordeins and glutelins can be solubilised under alkaline conditions, and previous studies have also shown that hordeins cannot be completely separated from glutelin (Celus et al., 2006), therefore the presence of hordeins in A-BPC is expected.

Lastly, the bands for BPI were diffuse and in the lower molecular weight range, as the enzymatic hydrolysis produced short-chain peptides and amino acids. This is expected from using the proprietary enzymatic formulation in its production and is aligned with the results of previous studies showing a high proportion of peptides after hydrolysis (Celus et al., 2007; Connolly et al., 2019).

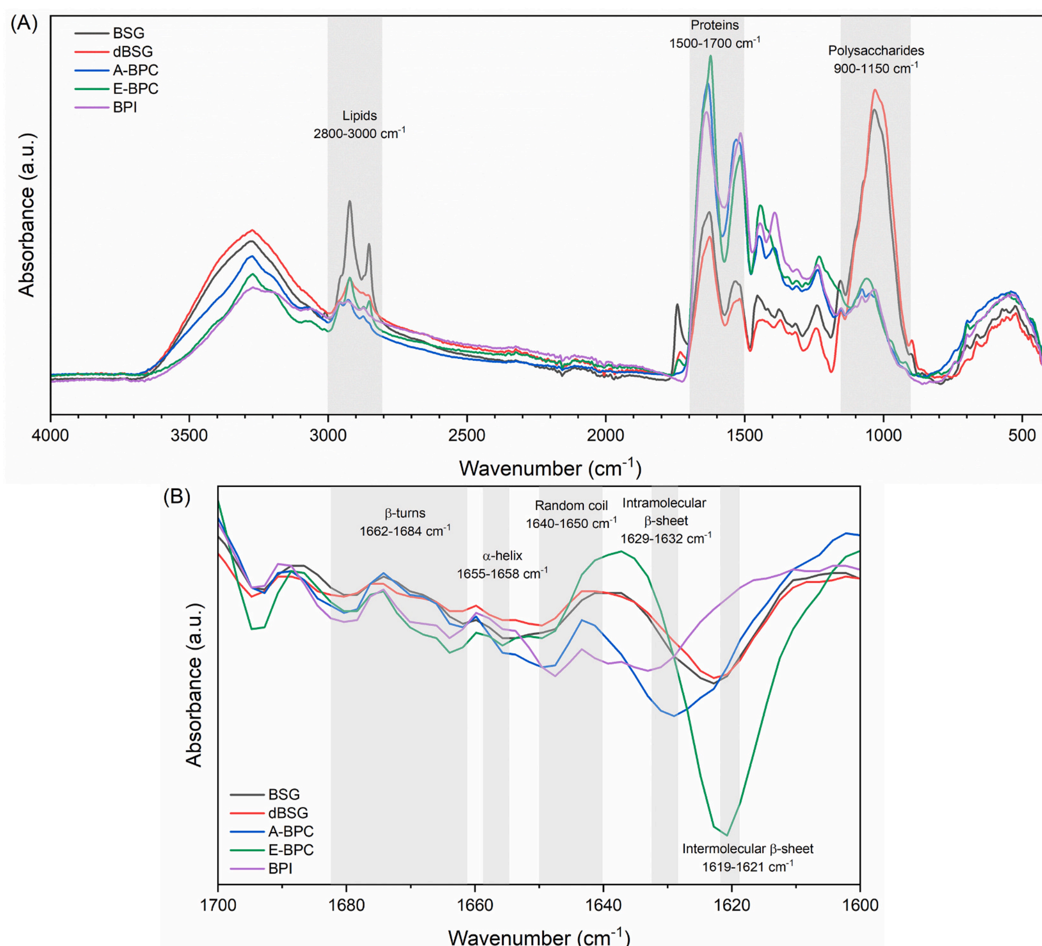

Fig. 2. (A) Full FTIR spectra from 400 to 4000 cm^{-1} of dried samples, (B) FTIR spectra of the second derivative of the amide I region. BSG, dBSG, A-BPC, *E*-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Composition and protein secondary structure by FTIR- spectroscopy

FTIR analysis was used to compare the structural differences resulting from the different extraction methods. Typically, absorption bands at 900–1150 cm^{-1}, 1500–1700 cm^{-1} and 2800–3000 cm^{-1} are assigned to the respective functional groups of C—O stretching, C═O stretching and N—H bending, as well as C—H stretching. Each wavenumber region is indicative of carbohydrates, proteins or lipids, although not necessarily limited to those. In the full spectra (Fig. 2A), the absorbance was higher for A-BPC, *E*-BPC and BPI in the amide I and II region (1500–1700 cm^{-1}) as compared to the lipid and polysaccharide regions (2800–3000 cm^{-1} and 900–1150 cm^{-1}, respectively). Conversely, the absorbance was higher in the polysaccharide region than the lipid and protein regions for BSG and dBSG. This is in line with the composition analysis in Table 1: the protein is the major component in A-BPC, E-BPC and BPI while the fibre content is highest in BSG and dBSG.

With the second derivative of the amide I region (Fig. 2B), the secondary structure of the proteins can be determined. The samples possessed a mixture of β-turns (1662–1684 cm^{-1}), α-helix (1655–1658 cm^{-1}), random coils (1640–1650 cm^{-1}) and intra- and intermolecular β-sheets (1629–1632 cm^{-1} and 1619–1621 cm^{-1}, respectively). The proteins that were initially present in BSG and dBSG were already aggregated as shown at 1621 cm^{-1}, where intermolecular aggregates are typically found. The thermal processes in beer brewing typically unfold the protein and reduce native barley protein structural elements such as intramolecular β-sheets and α-helices, while they increase the intermolecular β-sheets associated with aggregate formation (Hadnađev et al., 2018). This is supported by Celus et al. (2006), who concluded that the mashing process encourages the formation of disulfide bonds which results in protein aggregation.

Between A-BPC, *E*-BPC and BPI, significant differences can be seen. BPI did not show distinct structural elements since these proteins were enzymatically hydrolysed into peptides. E-BPC had the most intermolecular aggregates out of all samples, indicating severe protein unfolding in the presence of the reducing agent followed by subsequent aggregation during precipitation. In A-BPC, these aggregates were absent and a distinct signal at 1630 cm^{-1} was observed, indicative of intramolecular β-sheets. A similar observation was also made by Jia, Sethi, van der Goot, and Keppler (2022), who found a high signal intensity corresponding to intramolecular β-sheets in covalently and non-covalently modified sunflower proteins. Since alkaline extraction is likely to induce partial protein unfolding (Jiang, Chen, & Xiong, 2009), more phenol-binding sites are exposed. Under alkaline conditions, bound phenolics are released from cell wall polysaccharides and can bind to these sites, resulting in a change in protein secondary structure. However, one should exercise caution in interpreting results from heterogeneous samples because also non-protein material could induce a signal at this wavenumber.

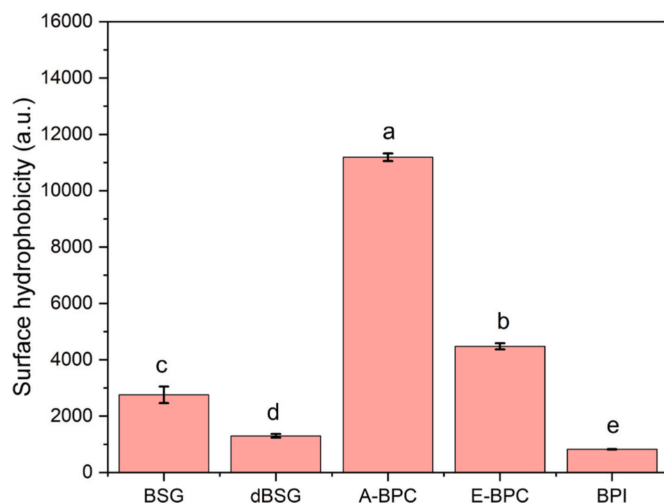

Fig. 3. Surface hydrophobicity of soluble fraction by ANSA. BSG, dBSG, A-BPC, E-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. Different small letters indicate significant difference at $P \leq 0.05$.

3.4. Surface hydrophobicity of the soluble fraction by ANSA

The protein surface hydrophobicity is a measure of the number of hydrophobic groups on a protein's surface in contact with a polar aqueous environment. In a native protein, these hydrophobic groups are mostly buried inside the core of the folded protein. From Fig. 3, A-BPC had the highest surface hydrophobicity in its soluble fraction, which could arise due to partial protein unfolding and exposure of hydrophobic groups. This is probably a result of conjugation or complexation of proteins to phenolic compounds, as supported by previous studies which indicate that protein unfolding occurs when proteins interact with phenolics (Cao & Xiong, 2017; Jiang, Zhang, Zhao, & Liu, 2018; Xu, Han, Huang, & Xu, 2021). The phenolics attached to the protein could also have a higher number of aromatic rings with a low number of hydroxyl groups, contributing to an increase in surface hydrophobicity. The increased surface hydrophobicity can also be caused by a different protein composition in A-BPC as compared to the other samples. From SDS-PAGE (Section 3.2), we can deduce that A-BPC contains both hordeins and glutelins, both of which are proteins that have large numbers of non-polar amino acids, whereas *E*-BPC is composed of only hordeins. This difference could result in A-BPC having larger surface hydrophobicity. It is important to mention that all fractions except BPI displayed low overall solubility (see Section 3.5), therefore only the soluble fraction was used for determination of surface hydrophobicity and is not representative of the whole sample.

In the case of BPI, hydrophobic groups are also exposed due to the enzymatic hydrolysis, but the smaller peptide size means that there are fewer hydrophobic binding sites, resulting in BPI having the lowest surface hydrophobicity (Bamdad, Wu, & Chen, 2011; Celus et al., 2007). For *E*-BPC, BSG and dBSG, the FTIR signal gave evidence that these proteins form aggregates in a hydrophilic, aqueous environment (Fig. 2B, at 1621 cm^{-1}), and therefore the hydrophobic groups were hidden and interacted minimally with the ANSA fluorescent probe under aqueous conditions. Generally, it can be concluded that different extraction methods resulted in proteins of varied compositions and structures, which will impact their functional properties.

3.5. Nitrogen solubility index

The protein solubility is a critical factor that impacts most functional properties of a protein. According to Fig. 4A, only about 10% of BSG proteins are soluble in water. These are likely residual proteins that remained in the interstitial pores of the spent grains after lautering, since BSG proteins should be insoluble as it is the insoluble residue from mashing. In line with the results from SDS-PAGE, defatting and dephenolisation removed most of these water-soluble proteins, resulting in an even lower NSI of dBSG.

Protein extraction by alkaline and ethanolic methods doubled the NSI for both A-BPC and E-BPC relative to BSG, although the protein solubility in water remained relatively poor (27% and 18%, in A-BPC and *E*-BPC respectively) as compared to pea protein isolates with solubilities ranging from 46 to 91% (Stone et al., 2015). The NSI is limited due to the high incidence of hydrophobic amino acids in hordeins and glutelins (Wang et al., 2010). A-BPC had a higher NSI than E-BPC despite having a higher surface hydrophobicity of the soluble fraction in A-BPC (Fig. 3). This is likely because E-BPC was highly aggregated (Fig. 2B) and therefore does not solubilise well anymore. The relatively higher protein

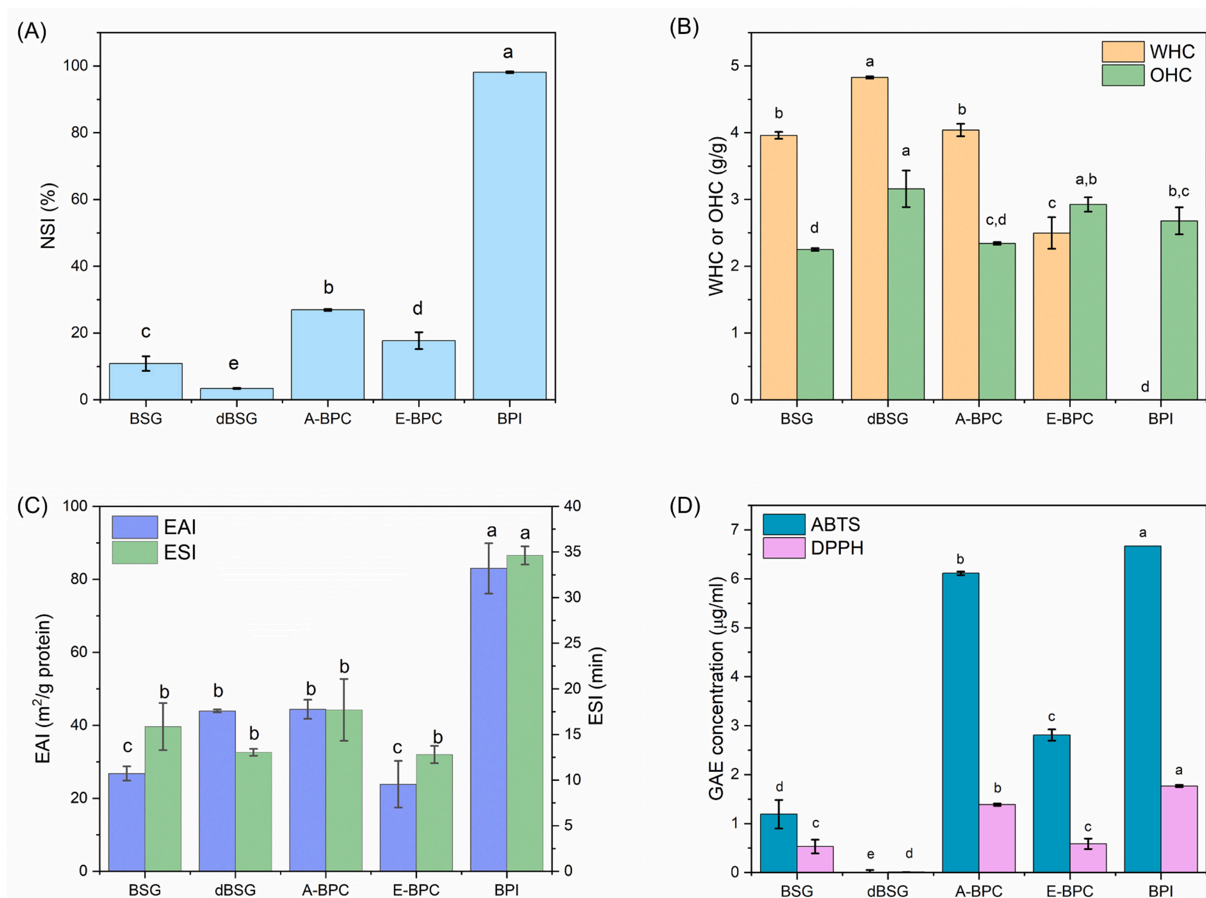

Fig. 4. (A) Nitrogen solubility index (NSI) of all samples dispersed in water; (B) Water holding capacity (WHC) and oil holding capacity (OHC) of all samples; (C) Emulsifying activity index (EAI) and emulsifying stability index (ESI) of all samples; (D) Antioxidant activity based on ABTS and DPPH assay. BSG, dBSG, A-BPC, *E*-BPC and BPI refer to brewers' spent grains, defatted and dephenolised BSG, alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. Different small letters indicate significant difference at $P \leq 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

solubility could also be due to protein-phenol interactions that result in partial unfolding of the protein to expose hydrophobic and hydrophilic groups on its surface, or the obstruction of charged amino groups on protein by phenolics that lead to a shift in its isoelectric point (Xu et al., 2022), thus improving the protein solubility. In addition, other studies have shown that in the absence of phenolics, the protein solubility can already be enhanced through a pH shift from alkaline to neutral as a result of conformational changes at extreme pH conditions (Jia et al., 2022).

Of all the protein-rich fractions, BPI had the highest NSI ($P \leq 0.05$), and in fact was completely soluble in water. This can be ascribed to the reduction in molecular weight into short-chain peptides after enzymatic hydrolysis. Most of their tertiary structure is probably lost, such that the residual chains can reorient to expose the more hydrophilic amino acids, enabling solubility to be enhanced strongly. Polar amino acids that are exposed can interact with water molecules through hydrogen bonding and electrostatic interactions, leading to increased protein solubility.

3.6. Water and oil holding capacity

The ability to bind water and oil depends on the polar and nonpolar side chains of the protein respectively, as well as the protein or aggregate conformation and surface hydrophobicity. Fig. 4B shows the WHC and OHC of BSG, dBSG and its derived fractions. BPI was completely water soluble after hydrolysis (Fig. 4A) and thus was not capable of holding water as a solid, but the exposure of some hydrophobic groups after hydrolysis enabled BPI to hold some oil. A-BPC and E-BPC have a WHC of about 4 g/g and 2 g/g respectively, which is relatively similar to the values obtained by Wang et al. (2010). As a partially unfolded protein, A-BPC has exposed polar amino acid side chains allowing it to hold water better than E-BPC. Furthermore, A-BPC contains high molecular weight glutelins (Fig. 1) which can form a network that can hold more water than E-BPC which only contains hordeins. Moreover, interactions between proteins and phenolics result in conformational changes that expose hydrophilic groups and increase their accessibility for interacting with water molecules, resulting in an increase in WHC. Conversely, E-BPC forms strong protein aggregates according to the FTIR results and is better able to hold oil. This explains the higher OHC of E-BPC than A-BPC ($P \leq 0.05$). Since E-BPC is rich in hordeins which contains many hydrophobic amino acids such as proline, it is expected to have a higher oil uptake. Our results generally show comparable water and oil holding capacities to those of protein isolates from pea (Stone et al., 2015). Despite the low protein content of BSG and dBSG, both samples showed a high WHC and OHC, which is attributed to the presence of fibres to hold water and oil (Wang, Suo, de Wit, Boom, & Schutyser, 2016). We conclude that, of all protein-rich fractions, A-BPC has the highest WHC and OHC ($P \leq 0.05$), which is a useful property in applications involving food structuring such as meat analogues, as they influence the mouthfeel and texture of the product.

3.7. Emulsifying properties

When proteins are able to rapidly diffuse to, adsorb onto and unfold at an oil-water interface and form a viscoelastic film around the oil

droplet, they can help to stabilise emulsions (Wang et al., 2010). Emulsification requires an interplay between protein amphiphilicity, solubility, and water and oil holding capacities (Wang et al., 2010). Fig. 4C illustrates the emulsifying properties of BSG, dBSG and its derived fractions. In general, all fractions showed an EAI ranging from 24 to 83 m^2/g and ESI ranging from 13 to 35 min. These values compare relatively well to soy protein isolates (26–41 m^2/g) and egg albumin (49 m^2/g), but are still poorer than for sodium caseinate and β-lactoglobulin (166 and 153 m^2/g respectively) (Pearce & Kinsella, 1978).

BPI exhibited a significantly higher emulsifying activity and stability than the other samples ($P \leq 0.05$). This can be attributed to BPI having a lower molecular weight and a significantly higher protein solubility ($P \leq 0.05$), that enables it to migrate and adsorb easily from the bulk phase to the oil-water interface and provide better emulsification. Similar findings were made by Celus et al. (2007) and Chin et al. (2022) regarding the improved emulsion-forming abilities of hydrolysed proteins.

With partial protein unfolding in A-BPC, hydrophobic and hydrophilic groups were exposed, resulting in high WHC and OHC and thus an amphiphilic property was expected. However, an overall poor protein solubility of 27% likely prevented the migration of proteins to the oil-water interface (Malik & Saini, 2017). Soluble proteins in A-BPC could also form crosslinks with phenolics, which may hinder the ability for proteins to rearrange at the interface and form an interfacial film (Salgado, Molina Ortiz, Petruccelli, & Mauri, 2012). It is also worth mentioning that the surface hydrophobicity measurement in Section 3.4 was only applied on the soluble fraction and is not representative of the whole sample, which was used for emulsification.

Between A-BPC and *E*-BPC, E-BPC showed poorer emulsification. The reason is two-fold: firstly, A-BPC has a higher protein solubility than E-BPC, which enables more proteins to diffuse to the oil-water interface. Additionally, the soluble portion of A-BPC showed higher surface hydrophobicity, in which the exposed hydrophobic groups can orient more readily towards the oil phase while the polar groups are directed towards the water phase to lower surface free energy, resulting in an increase in emulsifying activity. Secondly, A-BPC showed a stronger ability to attract water and oil phases, evident from its higher WHC and OHC than *E*-BPC (Fig. 4B). Conjugation of proteins with phenolics lead to protein unfolding which exposes hydrophobic and hydrophilic groups, which in turn improves surface activity (Cao & Xiong, 2017; Jiang et al., 2018; Xu et al., 2021). These results also corroborate with Stone et al. (2015), who showed that alcohol-soluble wheat proteins, analogous to E-BPC, has poorer emulsifying properties than alkali-extracted pea protein isolates.

Besides emulsification, foaming is also an important functionality for proteins. However, good solubility is a pre-requisite for good foaming behaviour, and the presence of insoluble fibres in A-BPC and *E*-BPC will likely destabilise the lamellae between foam bubbles, making it difficult for foams to form. Particle-stabilised foams were previously reported to be possible (Dickinson, 2010), but this is only for particles in the size range of a few nanometres to tens of micrometres, which is not the case in this study. Therefore, we did not evaluate foaming properties in detail even though some interfacial activity might be possible.

3.8. Gelation

According to Fig. 5, the LGC for A-BPC and *E*-BPC was 6% and 20% respectively, whereas BPI was not able to gelate across the entire concentration range selected. Barley proteins were previously reported to form a gel layer ('oberteig') on top of the spent grains after the malting and mashing steps in beer brewing. This gel layer was said to be composed of high molecular weight proteins and carbohydrates (Moonen et al., 1987). Our SDS-PAGE results similarly show that high molecular weight fractions were present in A-BPC and *E*-BPC but were absent in BPI (Fig. 1). In addition, BPI was completely water soluble (Fig. 4A) and unable to bind water (Fig. 4B), which are pre-requisites for

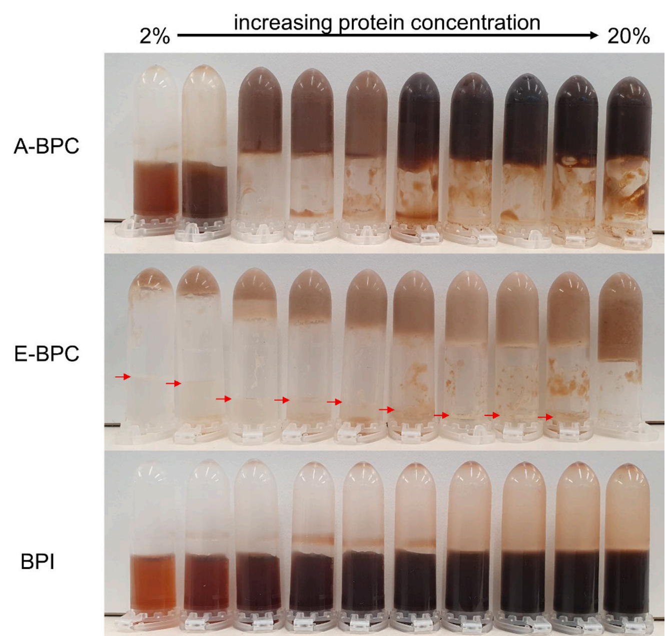

Fig. 5. Gelation of BSG proteins obtained by different extraction methods. A-BPC, *E*-BPC and BPI refer to alkali-extracted BSG protein concentrate, ethanol-extracted BSG protein concentrate and barley protein isolate, respectively. The protein concentration increases from left (2%, *w/v*) to right (20%, w/v), with an increment of 2%. The red arrows in E-BPC indicate the excess liquid at the bottom of the tube when inverted.

forming a gel (Banerjee & Bhattacharya, 2012). A previous study by Kotlar, Ponce, and Roura (2013) also showed that no gelation occurred for protein hydrolysates up to a concentration of 16%, which agrees well with our results.

The limited protein solubilities of A-BPC and E-BPC imply that these fractions result in dispersions of protein particles with only a small amount of proteins being solubilised. When subjected to thermal treatment without agitation, the particles sedimented over time in a swollen state, leading to particle jamming (Berghout, Boom, & van der Goot, 2015). A-BPC showed a better gelling capacity than E-BPC, as illustrated by a lower LGC. This could be a result of the alkaline extraction, in which hemicellulose (arabinoxylan) could be co-extracted and contributed to improved gelation as these biopolymers retain water very easily (Carvajal-Millan et al., 2007). Furthermore, the presence of phenolics in A-BPC increased the solubility and WHC of the proteins which in turn influenced its gelation, as previously demonstrated in sunflower protein isolate by Malik and Saini (2017). Proteins that are crosslinked with phenolics may be hindered from protein-protein interactions and are present as swollen flocs with an open structure, therefore a low protein concentration of A-BPC is sufficient to form a gel. In addition, as hypothesised earlier in this study, A-BPC likely contains more glutelins than E-BPC, and previous work concluded that glutelins are involved in the formation of gel-like aggregates (Moonen et al., 1987). For E-BPC, a self-supporting gel could be formed at protein concentrations as low as 2%, but due to the poor WHC and likely poor swelling capacity, syneresis resulted in an excess layer of liquid on top of the gel. When the protein concentration was increased to 20%, the layer of particle extended throughout the total sample, resulting in a single gel layer. Given that thermal gelation is necessary for meat analogue formation, A-BPC proves to be a relatively suitable candidate due to its low LGC in addition to its high WHC and OHC as previously discussed.

3.9. Antioxidant activity of the soluble fraction

Fig. 4D depicts the antioxidant activity based on DPPH and ABTS

assays. Both assays test the ability of compounds to act as free radical scavengers or hydrogen donors. Since DPPH is only soluble in alcoholic media and ABTS is soluble in both water and ethanol, there is a consistently poorer antioxidant activity of all fractions measured with the DPPH assay. The choice of solvent may affect the measured antioxidant activity, but the results of both antioxidant assays correlate strongly, which suggests that the assays are appropriate. Similar trends in antioxidant activity across all samples were observed in both assays, with dBSG having the least antioxidant activity and BPI and A-BPC having the most ($P \leq 0.05$). Due to the presence of a small amount of free phenolics, BSG showed minor antioxidant activity. After dephenolisation, free phenolics were removed and the antioxidant activity in dBSG was lowered.

As previously mentioned, insoluble-bound phenolics that were originally present in BSG were released by enzymatic hydrolysis or alkaline conditions, resulting in a higher total phenolic content present in BPI and A-BPC (Section 3.1). Additionally, in BPI, the presence of bioactive peptides contributes to more radical scavenging activity (Connolly et al., 2019). Based on the typical amino acid composition found in barley, antioxidant amino acids such as tyrosine, methionine, histidine, lysine and tryptophan are present and available for redox reactions (Chin et al., 2022; Xu, Chen, & Liu, 2017). Since BPI is completely soluble in water, coupled with the small peptide size after hydrolysis (Fig. 1), these protein hydrolysates have more exposed antioxidant groups than non-hydrolysed fractions which are hidden away from solution by folding and aggregation (Bamdad et al., 2011). Therefore, BPI has greater accessibility and diffusivity to scavenge free radicals than A-BPC. Furthermore, antioxidative peptides may exert a strong synergistic effect with other antioxidants such as phenolics (Saito et al., 2003), which may explain for the significantly higher antioxidant activity in BPI as compared to A-BPC.

Despite the low phenolic content in E-BPC, there was still some antioxidant activity. This observation was also made previously by Chanput, Theerakulkait, and Nakai (2009) regarding barley hordeins, and can be attributed to the presence of some hydrophobic amino acids which have antioxidant activity (Bamdad et al., 2011).

4. Conclusion

We examined the influence of different protein extraction methods on the composition, conformation and functional properties of BSG extracted proteins. Alkaline extraction resulted in proteins that had a more open structure and were less aggregated, but may have phenolics and polysaccharides bound to the proteins. Ethanolic extraction resulted in proteins that were highly aggregated. The low-molecular weight peptides and amino acids from the enzymatically assisted extraction no longer had distinct structural elements. The highest protein solubility was found in BPI, which allowed better emulsification and may be relevant in applications where emulsions are concerned. The proteins in BSG and its non-hydrolysed protein extracts are primarily insoluble, and therefore perform better in those applications where solubility is not a pre-requisite, such as in the formation of protein gels. In particular, A-BPC showed good gelation combined with a high WHC and antioxidant activity, of which the former two qualities are important for meat analogue applications. Despite the poorer WHC caused by aggregation, E-BPC demonstrated its ability to form gels readily at low concentrations. In the brewing industry, BSG is typically discarded, but we showed that the protein concentrates could be promising for a range of food structuring applications. Further studies into the gelling properties of BSG proteins such as gel strength and morphology should be examined in the future when exploring opportunities to valorise BSG.

CRediT authorship contribution statement

Yi Ling Chin: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Julia Katharina Keppler:** Writing – review & editing, Conceptualization. **Somayeh Taghian Dinani:** Writing – review & editing, Supervision, Conceptualization. **Wei Ning Chen:** Supervision, Resources, Project administration. **Remko Boom:** Writing – review & editing, Supervision, Project administration, Conceptualization.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2024.103666.

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