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Enzymatic Hydrolysis of Brewers' Spent Grain Proteins and Technofunctional Properties of the Resulting Hydrolysates

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Brewers' spent grain (BSG) is the insoluble residue of barley malt resulting from the manufacture of wort. Although it is the main byproduct of the brewing industry, it has received little attention as a marketable commodity and is mainly used as animal feed. Our work focuses on one of the main constituents of BSG, i.e., the proteins. The lack of solubility of BSG proteins is one of the limitations for their more extensive use in food processing. We therefore aimed to generate BSG protein hydrolysates with improved technofunctional properties. BSG protein concentrate (BPC) was prepared by alkaline extraction of BSG and subsequent acid precipitation. BPC was enzymatically hydrolyzed in a pH-stat setup by several commercially available proteases (Alcalase, Flavourzyme, and Pepsin) for different times and/or with different enzyme concentrations in order to obtain hydrolysates with different degrees of hydrolysis (DH). Physicochemical properties, such as molecular weight (MW) distribution and hydrophobicity, as well as technofunctional properties, such as solubility, color, and emulsifying and foaming properties, were determined. Enzymatic hydrolysis of BPC improved emulsion and/or foam-forming properties. However, for the hydrolysates prepared with Alcalase and Pepsin, an increasing DH generally decreased emulsifying and foam-forming capacities. Moreover, the type of enzyme impacted the resulting technofunctional properties. Hydrolysates prepared with Flavourzyme showed good technofunctional properties, independent of the DH. Physicochemical characterization of the hydrolysates indicated the importance of protein fragments with relatively high MW (exceeding 14.5 k) and high surface hydrophobicity for favorable technofunctional properties.

KEYWORDS: Brewers' spent grain; proteolysis; technofunctional properties; hydrolysates

INTRODUCTION

Brewers' spent grain (BSG), the extracted residue of barley malt resulting from the manufacture of wort, is the main byproduct of the brewing industry. Although BSG is rich in protein and fiber, its main application has been limited to animal feeding. Recently, attempts have been made to use BSG in human nutrition or in energy production. BSG has also been used in biotechnological processes, for example, as a substrate for the cultivation of microorganisms, as a source of value-added products such as ferulic and *p*-coumaric acids and xylose and arabinose, as a substrate for enzyme production, or as an additive or carrier in brewing (*1*). However, in spite of these possible applications, BSG still finds limited uses. Therefore, the development of new techniques to use this agro-industrial byproduct as a starting material for the production of a wide range of valuable products is of great interest. Some studies on the enzymatic hydrolysis of BSG have been described in literature, e.g., the release of ferulic and *p*-coumaric acids with an esterase and a xylanase (*2*, *3*) and the influence of pretreatments on the polysaccharide hydrolysis (*4*). In the present study, BSG proteins are the main target. To the best of our knowledge, no work was performed on the enzymatic hydrolysis of proteins present in BSG.

To increase the potential applications of insoluble proteins, chemical and enzymatic hydrolysis can be applied. Enzymatic hydrolysis is mild and does not destroy amino acids. The effects on the nutritional properties are therefore limited (*5*, *6*). In addition, enzymes are specific in their action, and this allows controlled processing (*6*). The enzyme used determines which peptides are produced. The hydrolysates formed by various enzymes may have different functionalities (*7*). Protein hydrolysis changes molecular weight (MW), charge and exposure of hydrophobic groups and reactive amino acid side chains. These molecular changes result in altered solubility, viscosity, sensory properties, and emulsifying and foaming behavior (*8*).

Literature data show that the extent to which the functional properties of a protein may be altered depends on the degree to which the protein has been hydrolyzed and the enzyme used.

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For both whey protein and casein, hydrolysis has been reported to improve (*9*, *10*) as well as to reduce (*10*, *11*) emulsion-forming properties. Both decreased (*9*, *11*) as well as increased (*12*) emulsion stabilities (ES) upon hydrolysis of casein using various enzymes have been observed. For β-lactoglobulin, most hydrolysates show improved ES (*7*). The positive effect of peptides larger than 2.0 k on ES was demonstrated with different fractions of casein and whey protein hydrolysates (*13*). Foam formation by casein hydrolysates is independent of the MW distribution of the peptides, whereas foam-forming whey protein hydrolysates seem to contain a portion of peptides larger than 3.0 k that contributes to the property. The stability of casein hydrolysate foams is specifically related to the presence of peptides with MW exceeding 7.0 k. For foam formation, the presence of amphiphilic peptides might be the most important factor, whereas, for foam stability (FS), the presence of peptides of relatively high MW seems to be crucial (*14*).

The emulsifying and foaming behavior of wheat gluten increases after hydrolysis with Alcalase up to a degree of hydrolysis (DH) of 5%. However, more extensive hydrolysis of gluten markedly reduces the emulsifying and foaming capacities (*15*).

The emulsifying behavior of soy and pea protein hydrolysates increases with increasing DH (*16*–*18*). The optimum peptide size for hydrolysates generated from soy proteins to show good emulsifying and foaming properties generally lies between 15 and 35 amino acids (*6*). Enzymatic treatment decreases the foaming capacity of pea protein hydrolysates (*18*). For both pea and soy proteins, the ES and FS decrease with increasing DH (*16*, *18*).

After a profound characterization of the BSG proteins (*19*), the aim of the present study was to enzymatically hydrolyze BSG proteins and to investigate the application properties of the resulting materials. Technofunctional properties of the resulting hydrolysates, such as solubility, color, and emulsifying and foaming behavior, as well as their MW distributions and hydrophobicities, were evaluated. These findings allow controlled processing of BSG proteins towards peptides with improved emulsifying and foaming behavior, which can be valorized as attractive ingredients in the food and beverage industries.

MATERIALS AND METHODS

Materials. BSG (25% protein on a dry basis) was from a pilot scale brewery experiment described by Celus et al. (*19*). *Bacillus licheniformis* Alcalase and *Aspergillus oryzae* Flavourzyme were obtained from Novozymes (Bagsvaerd, Denmark). Pepsin from porcine stomach was from Sigma-Aldrich (Bornem, Belgium). All chemicals and MW markers were from Sigma-Aldrich and were of at least analytical grade.

Preparation of Brewers' Spent Grain Protein Concentrate. BSG protein concentrate (BPC) was prepared by alkaline extraction of BSG (17% w/v) with 0.1 M NaOH at 60 °C. After 60 min of extraction, samples were filtered (180 μm). The proteins in the filtrate were precipitated by acidification to pH 4.0 using 2.0 M citric acid. The protein precipitate obtained after centrifugation at 10 000 g for 10 min at 4 °C, was finally freeze-dried.

Composition of BPC. Moisture and ash contents were determined according to AACC methods 44-15A and 08-12 (*20*), respectively. Protein was determined using the Dumas combustion method, an adaptation of the AOAC official method (*21*) to an automated Dumas protein analysis system (EAS, varioMax N/CN, Elt, Gouda, The Netherlands), using 6.25 as the conversion factor. Fat content was determined by extraction with petroleum ether following acid hydrolysis with 3.0 M HCl (60 min). The remaining fraction was considered to consist of carbohydrate material.

Limited Enzymatic Hydrolysis of BPC by Alcalase. A 6.0% (w/v on protein basis) aqueous dispersion of BPC was hydrolyzed in a pH-stat setup (Titrino, Metrohm, Herisau, Switzerland) for different times and/or with different enzyme concentrations. After the reaction period, the mixture was adjusted to pH 6.0 with 2.0 M citric acid and heated at 95 °C for 10 min to inactivate the enzyme. The mixture was centrifuged (5000 *g*; 20 min; 4 °C) resulting in both supernatant and pellet, which were freeze-dried. The protein yield of supernatant, i.e., the percentage of supernatant protein weight resulting from the BPC protein mixture, including a limited quantity of the proteases, was determined with the previously mentioned Dumas method. Supernatants were used to determine protein solubility, MW distribution, hydrophobicity, color, and emulsifying and foaming properties.

The DH, defined as the ratio of the number of peptide bonds hydrolyzed (h) to the total number of peptide bonds per unit weight present in BPC protein (h_{tot}), was calculated from the amount of base consumed during hydrolysis (*22*), as given below

$$\mathrm{DH}\ (\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times N_b \times 100}{\alpha \times M_P \times h_{tot}} \quad (1)$$

where B is base consumption (mL); N_b is normality of the base; α is average degree of dissociation of the α-NH_2 groups; M_P is mass of BPC protein (g); h represents hydrolysis equivalents [mequivalents (mequiv)/g protein]; and h_{tot} is the total theoretical number of peptide bonds per unit weight present in BPC protein, determined from the amino acid composition of BPC (7.75 mequiv/g protein), determined by Nutrinov (Vezin le Coquet, France).

Limited Enzymatic Hydrolysis of BPC by Flavourzyme and Pepsin. Enzymatic hydrolysis with Flavourzyme and Pepsin was performed in the same way as hydrolysis with Alcalase. Before centrifugation, 50 μL of the total hydrolysate obtained after enzymatic hydrolysis was withdrawn for determination of the DH. The remaining mixture was centrifuged (5000 *g*; 20 min; 4 °C) resulting in supernatant and pellet, which were both freeze-dried.

Whereas the DH of the hydrolysates prepared with Alcalase was calculated with eq 1, the DH of the hydrolysates prepared with Flavourzyme and Pepsin was colorimetrically determined with *o*-phthaldialdehyde (OPA). A high correlation ($R^2 = 0.93$) existed between the determination of DH with eq 1 and with OPA for hydrolysates prepared with Alcalase. The OPA reagent was prepared by combining 7.620 g disodium tetraborate, 200 mg sodium dodecyl sulfate (SDS), 160 mg OPA 97% (in 4.0 mL 95% ethanol), and 176 mg dithiothreitol (DTT) and adding deionized water to a final volume of 200 mL. A leucine concentration range (0–0.2 mg/mL) was used as the standard curve. Total hydrolysate was diluted (1:80) in 1.0% (w/v) SDS. OPA reagent (3.0 mL) was added to all samples (400 μL), and the absorbance (340 nm) was measured after 20 min (*23*). The levels of peptide bonds hydrolyzed (h) were calculated as follows:

$$h(\mathrm{mequiv/gprotein}) = \frac{(\mathrm{leucine\text{-}NH_2 mequiv/gprotein}) - 0.51}{0.77} \quad (2)$$

with 0.77 and 0.51 the slope and intercept, respectively, of a linear plot between h and leucine-NH_2 mequiv/g protein ($R^2 = 0.95$), determined with BPC as substrate.

Sample codes are sequentially composed of three letters representing the enzyme (Alc for Alcalase, Fla for Flavourzyme, and Pep for Pepsin) used and two digits encoding the DH.

Protein Solubility. In order to determine protein solubility as a function of pH, samples (1.0% protein) were dispersed in 10.0 mL deionized water. The pH of the suspensions was adjusted to pH 2.0, pH 4.0, pH 6.0, pH 8.0, and pH 10.0 by using either 0.1 M HCl or 0.1 M NaOH. The suspensions were shaken for 60 min at room temperature and their pH was measured. Protein contents in supernatants (SN_{sol}), obtained after centrifugation (10 000 *g*; 10 min), were determined by Lowry's colorimetric method calibrated with a bovine serum albumin (BSA) standard curve (*24*). Protein solubility was calculated as the percentage of proteins present in SN_{sol}.

Size Exclusion High Performance Liquid Chromatography. Size exclusion high performance liquid chromatography (SE-HPLC) was performed with a Superdex Peptide 10/300 GL-column (GE Healthcare,

Uppsala, Sweden) at 25 °C with a flow rate of 0.5 mL/min and a sample injection volume of 100 μL using an AKTA Explorer 100 (GE Healthcare). The mobile phase was 30% acetonitrile (ACN) with 0.15% trifluoroacetic acid (TFA) in Milli-Q water. The column was calibrated with 6 MW markers: ribonuclease A (MW = 13.7 k), aprotinin (MW = 6.5 k), insulin chain B (MW = 3.5 k), Ala-Ser-His-Leu-Gly Leu-Ala-Arg (MW = 824), $(Ala)_5$ (MW = 373), and Ala-Gln (MW = 217) (Sigma-Aldrich). A calibration curve was made from the MW of the markers and their respective elution times ($R^2 = 0.96$). Aliquots of samples (0.1% protein) were dissolved in the mobile phase, and the elution was monitored at 214 nm.

Reversed Phase High Performance Liquid Chromatography. Reversed phase high performance liquid chromatography (RP-HPLC) was performed on a Vydac 214 TP C4 protein/peptide reversed phase column (Alltech Associates, Inc., Deerfield, Massachusetss) at 50 °C with a flow rate of 0.5 mL/min and a sample injection of 20 μL using an LC-2010 system (Shimadzu, Kyoto, Japan). The elution system consisted of Milli-Q water containing 0.1% (v/v) TFA (solvent 1) and ACN containing 0.1% TFA (v/v) (solvent 2). Proteins were eluted with a linear gradient from 0.0% to 50.0% solvent 2 in 80 min and detected at 214 nm. Aliquots of samples (1% protein) were dissolved in 30.0% ACN with 0.15% TFA in Milli-Q water and filtered through a 0.45 μm membrane (regenerated cellulose, Alltech Associates).

Surface Hydrophobicity. The protein surface hydrophobicities of BPC and the resulting hydrolysates were determined with 1-anilino-8-naphthalene sulfonate (ANS) as the fluorescent probe (*25*). Samples (0.1% protein) were dispersed in 0.01 M sodium phosphate buffer (pH 7.0), shaken for 16 h at room temperature, and subsequently centrifuged (3000 *g*; 10 min; 4 °C). Supernatants were serially diluted with 0.01 M sodium phosphate buffer (pH 7.0) to obtain a range of protein concentrations from 0.01 to 0.16 mg/mL. A 10 μL aliquot of ANS solution (8 mM in 0.01 M sodium phosphate buffer pH 7.0) was added to 1.0 mL of each sample. The fluorescence intensity of the protein was measured with a spectrofluorometer (Fluorolog-3 model FL3-22, Horiba Jobin Yvon, Edison, New Jersey) using a 3 nm slit width. Wavelengths of excitation and emission were 390 nm and 480 nm, respectively. The fluorescence intensity of each control solution (without ANS) was subtracted from that with ANS to obtain the net fluorescence intensity at each protein concentration. The initial slope of a plot of fluorescence intensity as a function of protein concentration was used as an index of protein surface hydrophobicity (S_0).

Color. A colorimeter (model Colorquest 45/0 LAV, CQ/UNI-1600, HunterLab, Reston, Virginia) was used for all color determinations. The instrument was calibrated with a white and black calibration tile. The colorimeter was set to an illuminant condition D_{65} (medium daylight) and a 10° standard observer. Each sample was placed in a clear Petri dish and covered with a white plate. The color was measured as L*, a*, and b* color spaces (CIE 1976). L* is a measure of the brightness from black (0) to white (100); a* describes red–green color with positive a* values indicating redness and negative a* values indicating greenness; b* describes yellow–blue color with positive b* values indicating yellowness and negative b* values indicating blueness. Color measurements were in fivefold, and the coefficients of variation for L*, a*, and b* did not exceed 0.5%, 2.6% and 1.2%, respectively. Color differences (ΔL*, Δa*, Δb*) were evaluated by the difference in L*, a*, and b* between a sample and a reference (BSG). The total color difference (ΔE^*) was calculated as follows (*26*):

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

Emulsifying Properties. Emulsifying properties were measured by the turbidimetric method of Pearce and Kinsella (*27*), with minor modifications. To prepare the emulsion, 7.0 mL of soy oil (Lesieur, Asnières-sur-Seine, France) and 21.0 mL protein solution (0.2% protein for Pepsin and Flavourzyme; 0.07–2.0% protein for Alcalase) in 9.0 mM sodium phosphate buffer containing 35 mM NaCl (pH 7.0) (*28*) were homogenized for 1 min at speed 1 and 25 °C in a Waring blender (Torrington, Connecticut). Immediately and 30 min after emulsion formation, a 50 μL sample was withdrawn from the bottom of the cup and diluted with 10.0 mL of a 0.1% (w/v) SDS solution. The absorbances of the diluted emulsions were measured at 500 nm (A_{500}). The emulsifying activity index (EAI, m^2/g) was determined from the absorbance measured immediately after emulsion formation and calculated as follows:

$$\mathrm{EAI}(m^2/g) = \frac{2 \times T \times 200}{\varphi \times C} \quad (3)$$

and $T = 2.303 \times A_{500} / L$

where A_{500} is the absorbance; L is the light path (10^{-2} m); Φ is the volume fraction of the dispersed phase (0.25); C is the concentration of protein (g/m^3); T is the turbidity; and 200 is the dilution factor. ES was the percentage of emulsion turbidity remaining after 30 min. The coefficient of variation for the determination of EAI and ES did not exceed 10%.

The emulsifying properties of the hydrolysates obtained with Alcalase (Alc13) were determined at different protein concentrations (0.07–2.0% protein) at pH 7.0. The emulsifying properties of the hydrolysates (0.2% protein) were also evaluated in 24 mM sodium acetate (pH 4.0) containing 35 mM NaCl.

Foaming Properties. Foam was prepared with protein solution (0.6% protein) in 9.0 mM sodium phosphate buffer containing 35 mM NaCl (pH 7.0) (*28*) with the whipping method described by Caessens et al. (*29*). A volume of 100 mL of hydrolysate solution was placed in a graduated glass cylinder (diameter 60 mm) and whipped for 70 s by a rotating propeller at 2000 rpm at 25 °C. The foam-forming ability (F_0, mL/g) was defined as the initial foam volume measured at 2 min after the start of whipping per protein weight. Foam volume loss was monitored during 60 min. FS was the percentage of foam volume remaining after 60 min relative to the initial foam volume. The coefficient of variation for the determination of F_0, and FS did not exceed 10%. The foaming properties of the hydrolysates were also determined in 24 mM sodium acetate (pH 4.0) containing 35 mM NaCl.

Table 1. Hydrolysis Conditions with Indication of pH, Temperature (°C), Enzyme Preparation To Substrate Ratio (E/S; % w/w of protein), Time of Hydrolysis (min), and the Resulting Degree of Hydrolysis (DH; %), Sample Code, Protein Contents of Supernatants Resulting From Enzymatic Hydrolysis of BPC, and Protein Yields of SN

enzyme	pH[a]	T[a] (°C)	E/S (%)	time of hydrolysis (min)	DH (%)	sample code[d]	protein content SN[e] (%)	protein yield SN (%)
Alcalase	9.0	60	2.5	1.7	3[b]	Alc03	70	57
Alcalase	9.0	60	2.5	6.7	6[b]	Alc06	74	56
Alcalase	9.0	60	2.5	37	11[b]	Alc11	71	75
Alcalase	9.0	60	2.5	120	13[b]	Alc13	68	83
Flavourzyme	9.0	40	5.0	20	2[c]	Fla02	66	77
Flavourzyme	9.0	40	5.0	60	4[c]	Fla04	68	79
Flavourzyme	9.0	40	5.0	360	6[c]	Fla06	67	92
Pepsin	3.0	60	2.5	3.3	2[c]	Pep02	71	51
Pepsin	3.0	60	2.5	13.3	4[c]	Pep04	73	54
Pepsin	3.0	60	5.0	60	6[c]	Pep06	77	72
Pepsin	3.0	60	5.0	360	8[c]	Pep08	76	76

[a] The optimum pH and temperature (*T*) of the proteases were determined with brewers' spent grain protein concentrate (BPC) as substrate. [b] Degree of hydrolysis (DH; %) calculated by the pH-stat method. [c] Degree of hydrolysis (DH; %) colorimetrically determined with *o*-phthaldialdehyde. [d] Sample code consists of 3 letters representing the enzyme used and 2 digits encoding the DH. [e] Protein yields of SN are expressed as the percentage of SN protein weight solubilized from the BPC proteins by enzymatic hydrolysis.

RESULTS

Composition of BPC. After alkaline extraction and acid precipitation, BSG proteins were concentrated in the BPC fraction. BPC contained 60% proteins, 12% fat, 2.0% ash, and, by difference, 26% carbohydrates (on dry basis). Approximately 17% of the starting BSG material was present in BPC, which contained 41% of the BSG proteins.

Limited Enzymatic Hydrolysis of BPC. For each individual enzyme, the optimum pH and temperature were determined with BPC as substrate. **Table 1** lists these values as well as the DH

following incubation at these optimal conditions in a pH-stat setup for different times and/or with different enzyme to substrate ratios. Hydrolysates with the highest DH (13% and 11%) were obtained after hydrolysis with Alcalase at 2.5% enzyme to substrate ratio for 120 min and 37 min, respectively. With Alcalase, Flavourzyme, and Pepsin, hydrolysates with the lowest DH were obtained after hydrolysis for 1.7 min, 20 min, and 3.3 min at 2.5%, 5%, and 2.5% enzyme to substrate ratios, respectively.

After hydrolysis of BPC, supernatants obtained after centrifugation were recovered, freeze-dried, and weighed. **Table 1** shows that protein contents of the freeze-dried supernatants (% of dry matter), determined with the Dumas method, varied between 66% and 77%. The level of protein recovered in the supernatant after hydrolysis of BPC proteins with Alcalase, Flavourzyme, and Pepsin increased with increasing DH (**Table 1**). The protein content was independent of the percentage of proteins, which became soluble following enzymatic hydrolysis. This pointed to increasing solubilization of nonprotein material with increasing DH. The highest protein yield (92%) was obtained for Fla06. Although Alc03, Fla02, and Pep02 showed comparable DH values, the proteins released in the supernatants varied between 51% and 57% for Pep02 and Alc03, respectively, and were up to 77% for Fla02. This may well indicate differences in MW composition of the protein fraction. Furthermore, the high level of protein materials released in samples Alc11, Alc13, Fla06, Pep06, and Pep08 and their higher DH values may point to the presence of low MW peptides.

Protein Solubility. Figure 1a,**b** shows protein solubilities as a function of pH for BSG, BPC, and the resulting hydrolysates. As expected, BSG proteins are poorly water soluble (less than 8%). The alkaline extraction of BSG proteins and consecutive precipitation of the extracted proteins at pH 4.0 show that BPC proteins evidently have high solubility at alkaline pH values and low solubility at pH values around 4.0. Proteins in BPC had higher solubility at pH 2.0 and pH 5.8 (respectively, 23% and 41%) than those in BSG (**Figure 1a**).

Enzymatic hydrolysis of BPC proteins leads to increased solubility at low pH values and a similar high solubility at alkaline pH conditions. The increased protein solubility may be due to the decrease in MW and to protein unfolding. Both nonpolar and some polar amino acid groups, buried inside protein molecules, could be exposed on the surface of protein molecules after unfolding. These exposed polar amino acids may interact with water molecules through hydrogen bonds and electrostatic interactions, resulting in increased protein solubility (*17, 25*). In general, solubility of the hydrolysates increased with increasing DH. The lowest protein solubility was observed at pH 4.0, except for Pep06 and Pep08 which showed comparable solubilities over the entire pH range (**Figure 1a**,**b**).

Size Exclusion High Performance Liquid Chromatography. Figure 2 shows the SE-HPLC profiles of BPC and hydrolysates with different DH obtained after enzymatic hydrolysis with Alcalase. Elution was monitored at 214 nm. Extinction at this wavelength is mainly caused by peptide bonds. This implies that the level of the smallest peptides is underestimated. Since other simple methods for detection of peptides are not available, extinction at 214 nm is used to determine the level of protein fragments, despite this imperfection (*30*). SE-HPLC profiles were divided into three fractions based on their apparent MW, calculated from the calibration curve (**Figure 2**). Fraction I includes the first peak and corresponds to protein

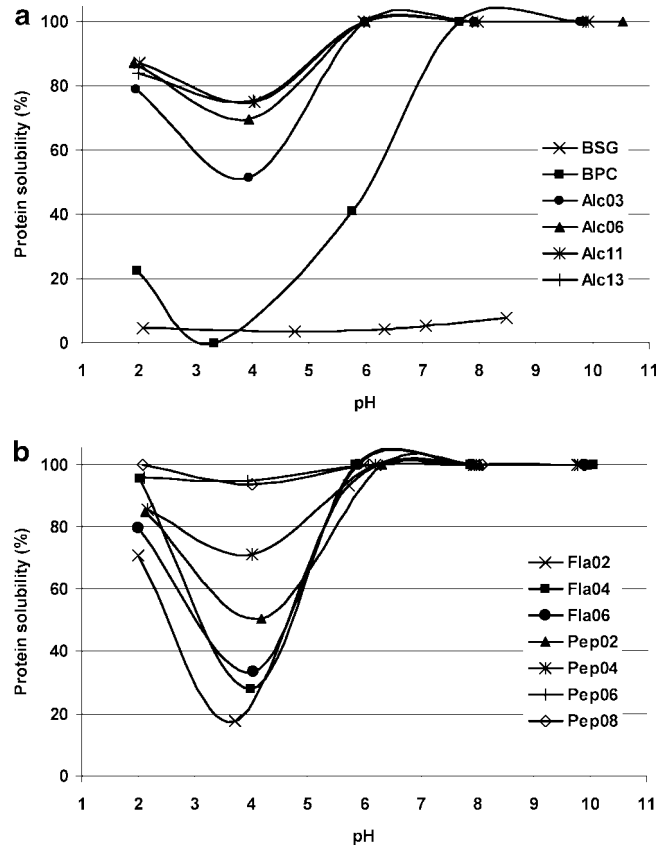

Figure 1. Protein solubility (1.0% protein) as a function of pH for (**a**) Brewers' spent grain (BSG), BSG protein concentrate (BPC), and hydrolysates obtained after hydrolysis with Alcalase (Alc03, Alc06, Alc11, Alc13) and for (**b**) hydrolysates obtained after hydrolysis with Flavourzyme (Fla02, Fla04, Fla06) and Pepsin (Pep02, Pep04, Pep06, Pep08). Sample codes consist of 3 letters encoding the enzyme and 2 digits encoding the DH (**Table 1**).

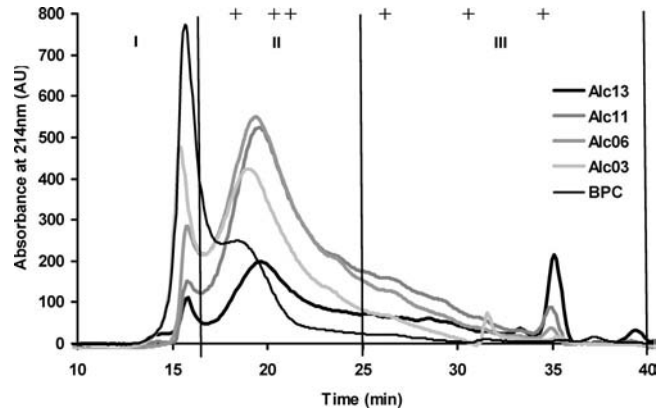

Figure 2. SE-HPLC profiles of brewers' spent grain protein concentrate (BPC) and hydrolysates with different DH obtained after enzymatic hydrolysis with Alcalase (**Table 1**). Elution times of molecular weight (MW) markers with MW 13.7 k, 6.5 k, 3.5 k, 824, 373, and 217 are indicated from left to right with + symbols. SE-HPLC profiles are divided into three fractions: protein fragments with MW exceeding 14.5 k (fraction I), protein fragments with MW between 1.7 k and 14.5 k (fraction II), and protein fragments with MW lower than 1.7 k (fraction III). Absorbance (214 nm) is expressed in arbitrary units (AU).

fragments with MW exceeding 14.5 k, fraction II to protein fragments with MW between 1.7 k and 14.5 k, and fraction III to protein fragments with MW lower than 1.7 k. As expected,

Table 2. Apparent Molecular Weight (MW) Distribution Expressed As Area Percentage of Each Fraction Relative To the Total Area of SE-HPLC Chromatogram (detection at 214 nm) and Area Percentage of Each Fraction Relative to the Total Area of RP-HPLC Chromatogram

	SE-HPLC[b]			RP-HPLC[c]	
sample[a]	fraction I	fraction II	fraction III	fraction A	fraction B
Alc03	21	68	11	36	64
Alc06	9	74	18	60	40
Alc11	4	69	27	93	7
Alc13	9	55	37	76	24
Fla02	44	33	23	nd	nd
Fla04	40	38	22	23	77
Fla06	30	59	11	38	62
Pep02	32	43	25	53	47
Pep04	31	42	26	62	38
Pep06	16	50	33	81	19
Pep08	20	45	35	87	13

[a] Sample codes consist of 3 letters encoding the enzyme and 2 digits encoding the DH. [b] SE-HPLC profiles are divided into 3 fractions (Figure 2). Fraction I corresponds to protein fragments with MW exceeding 14.5 k, fraction II to protein fragments with MW between 1.7 k and 14.5 k, and fraction III to material with MW lower than 1.7 k. [c] RP-HPLC profiles are divided into 2 fractions (fractions A and B) (Figure 3). nd: not determined.

proteins in BPC eluted mainly in fraction I. It should be noticed that the peak MW in fraction I decreased with increasing DH (**Figure 2**).

Table 2 shows the area of each fraction relative to the total area of the SE-HPLC chromatogram. In general, fraction II was the major one, except for samples Fla02 and Fla04 which contained higher levels of protein fragments in fraction I. An increasing DH resulted in decreasing levels of protein fragments in fraction I (**Table 2**). For Alcalase and Pepsin hydrolysates, the levels of protein fragments in fraction III increased with increasing DH. However, hydrolysates obtained after enzymatic hydrolysis with Flavourzyme had a decreased level of protein fragments in fraction III at the highest DH. This is probably the result of a further degradation of the peptide fragments initially formed in fraction III by the exopeptidase activity of Flavourzyme into amino acids and short peptide chains which cannot be detected at 214 nm. It should be noticed that the underestimation of the peptides present in fraction III of Fla06 leads to overestimation of the level of protein fragments present in fractions I and II, since the relative contribution of each fraction to the total area was calculated.

Reversed Phase High Performance Liquid Chromatography. Figure 3 shows the RP-HPLC profiles of BPC and hydrolysates with different DH obtained after enzymatic hydrolysis with Alcalase. As mentioned above, the extinction value at 214 nm underestimates the levels of the smallest peptides. RP-HPLC profiles were divided into two fractions. While fraction A consisted of the protein fragments eluting between 20 and 52 min, fraction B contained the protein fragments that eluted between 52 and 80 min. The separation between fractions A and B was chosen as the time between the two main peak fractions in the RP-HPLC profiles of the hydrolysates. Proteins in BPC eluted mainly in fraction B.

Table 2 shows the area percentage of fractions A and B relative to the total area of the RP-HPLC chromatogram. **Table 2** shows that, with increasing DH, the level of protein fragments present in fraction A increased, while the proportions of those present in fraction B decreased. While the separation in RP-HPLC is primarily based on differences in hydrophobicity, retention times are also influenced by peptide size. The retention time of small peptides (less than 15 residues) seemed to depend only on amino acid composition, while that of larger peptides

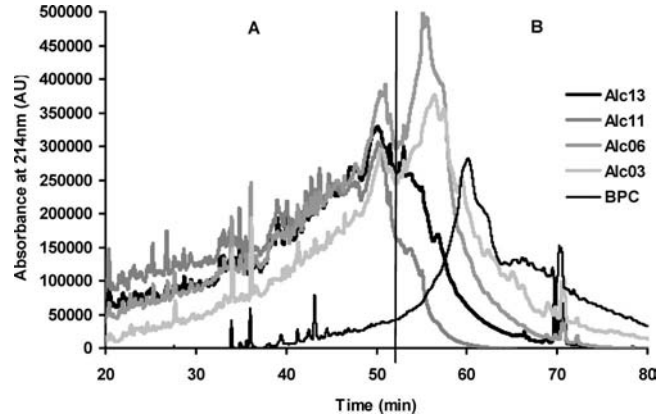

Figure 3. RP-HPLC profiles of brewers' spent grain protein concentrate (BPC) and the hydrolysates obtained after enzymatic hydrolysis with Alcalase with different DH (**Table 1**). RP-HPLC profiles are divided into two fractions (fractions A and B). Absorbance (214 nm) is expressed in arbitrary units (AU).

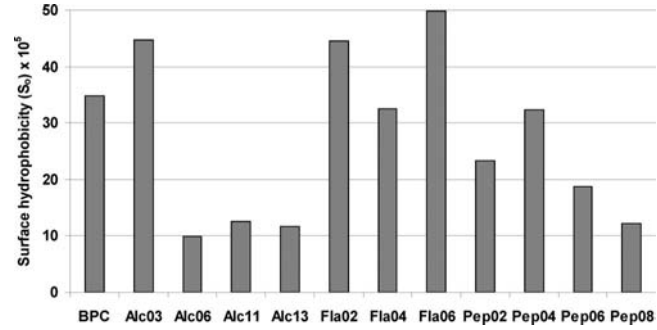

Figure 4. Surface hydrophobicity (S_0) of brewers' spent grain protein concentrate (BPC) and the resulting hydrolysates. Sample codes consist of 3 letters encoding the enzyme and 2 digits encoding the DH (**Table 1**).

was also influenced by factors such as peptide size (*30, 31*). In accordance with van der Ven et al. (*30*), we found a relation between apparent MW of protein fragments and their retention in RP-HPLC. The area percentage of fraction I in SE-HPLC was significantly positively correlated with that of fraction B in RP-HPLC ($R^2 = 0.53$, p value < 0.05), and a significant negative correlation was observed between the area percentage of fraction III in SE-HPLC and that of fraction B in RP-HPLC ($R^2 = 0.55$, p value < 0.05).

Surface Hydrophobicity. The surface hydrophobicity (S_0) of a protein is an index of the number of hydrophobic groups on its surface in contact with the polar aqueous environment. **Figure 4** shows the S_0 of BPC and the resulting hydrolysates measured by an ANS fluorescent probe. Limited enzymatic hydrolysis of BPC with Alcalase and Flavourzyme clearly increased S_0. However, in contrast to the hydrolysates prepared with Flavourzyme, S_0 decreased with increasing DH for the hydrolysates prepared with Alcalase. Hydrolysates prepared with Pepsin had a lower S_0 than BPC, and S_0 decreased with DH. The effect of protein hydrolysis on surface hydrophobicity is well-known (*25*). In a native protein molecule, hydrophobic groups are buried inside the core of the folded structure. Partial hydrolysis exposes some of these groups, resulting in increased surface hydrophobicity. Small peptides could have fewer hydrophobic binding sites than larger peptides. This observation indicated that the surface hydrophobicity of a protein could be decreased by excessive enzymatic hydrolysis. Moreover, S_0 was significantly negatively correlated with DH ($R^2 = 0.41$, p value

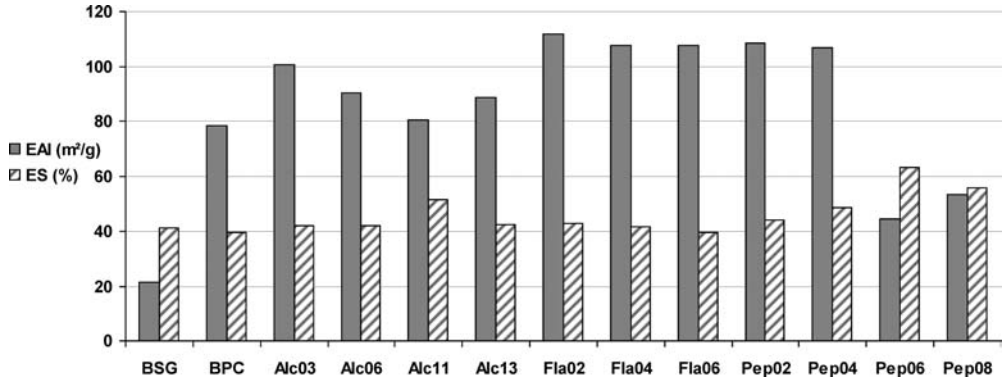

Figure 5. Emulsifying activity index (EAI; m^2/g) (unpatterned bars) and emulsion stability (ES; %) (patterned bars) at pH 7.0 and 0.2% protein of brewers' spent grain (BSG), BSG protein concentrate (BPC), and BPC hydrolysates. Hydrolysate codes consist of 3 letters representing the enzyme used and 2 digits encoding the DH (**Table 1**).

< 0.05). Furthermore, significant positive ($R^2 = 0.52$, p value < 0.05) and negative ($R^2 = 0.47$, p value < 0.05) correlations were found between S_0 and the area percentages of fractions I and III in SE-HPLC, respectively. However, no significant correlation existed between S_0 and the area percentage of fractions A and B in RP-HPLC.

Color. Color parameters L* (brightness), a* (redness), and b* (yellowness) were higher for BPC samples (63.9, 7.1, and 24.1, respectively) than for BSG samples (59.9, 4.6, and 19.7, respectively). Brightness decreased (between 41.2 and 60.6) and redness increased (between 8.4 and 10.9) after enzymatic hydrolysis of BPC, except for Pep02 which showed redness (7.0) comparable to that of BPC. For the hydrolysates prepared with Alcalase and Pepsin, L* decreased as DH increased. Hydrolysates of BSG had a higher yellowness (between 20.4 and 23.1) than the parent material, except for Fla04 which showed a comparable b* value (19.5). However, yellowness of the hydrolysates was lower than that of BPC.

The changes in color parameters L*, a*, and b* are also reflected in the total color difference (ΔE^*), i.e., the difference in color between the sample and BSG, which was lowest for Pep02 (2.6) and highest for Alc13 (19.9). Pep04 showed a ΔE^* (6.7) comparable to that of BPC (6.5). In general, ΔE^* was significantly positively correlated with DH ($R^2 = 0.64$, p value < 0.01). A higher DH went hand in hand with a higher ΔE^*, except for Fla06.

Emulsifying Properties. Figure 5 shows the EAI and ES, measured at pH 7.0, of the protein solutions of BSG, BPC, and the resulting hydrolysates (0.2% protein). BSG had lower emulsifying activity than BPC. Hydrolysis of BPC improved the emulsion-forming ability, except for Pep06 and Pep08. EAI decreased with increasing DH for hydrolysates prepared with Alcalase and Pepsin, while, for hydrolysates prepared with Flavourzyme, EAI was more or less unaffected. Alc03, Fla02, Fla04, Fla06, Pep02, and Pep04 showed comparablely high emulsifying capacities. Although Pep06 and Pep08 had low emulsifying capacities, these hydrolysates resulted in the most stable emulsions (**Figure 5**). For hydrolysates prepared with Alcalase and Pepsin, ES increased with DH. Whereas the results showed a significant negative correlation between EAI and ES ($R^2 = 0.77$, p value < 0.01), EAI and ES were not significantly correlated with DH.

The effect of the protein concentration on these properties was evaluated at pH 7.0 for Alc13. EAI decreased with increasing protein concentration (**Figure 6**). To evaluate the impact of pH on the emulsifying properties, emulsifying activity of the hydrolysates was also determined at pH 4.0. In general, EAI values were lower at pH 4.0 than at pH 7.0,

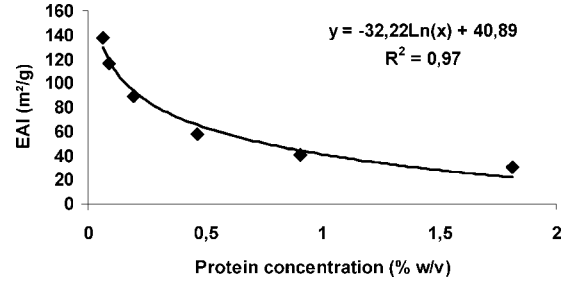

Figure 6. Emulsifying activity index (EAI; m^2/g) for different protein concentrations (%) for hydrolysates prepared with Alcalase (Alc13).

probably due to the lower solubility of proteins at pH 4.0 (**Figure 1**). ES was higher at pH 4.0 than at pH 7.0 (results not shown).

Foaming Properties. Figure 7 shows the foam-forming properties of BSG, BPC, and the hydrolysates at pH 7.0. BSG had negligible foam-forming capacity. Despite its high fat content, BPC formed foams. Hydrolysis of BPC had no impact on or improved foam-forming capacity. FS of the hydrolysates was higher than that of BPC, except for Alc06, Alc11, Alc13, and Pep08. Although the foam-forming abilities of hydrolysates prepared with Alcalase and Pepsin decreased with increasing DH, rather the opposite was observed for hydrolysates prepared with Flavourzyme. The highest foam-forming capacity was observed for Alc03, Alc06, and Pep02. FS decreased as DH increased, except for Flavourzyme. Hydrolysates Alc03, Fla02, and Fla06 yielded foams with a high FS (**Figure 7**). Thus, mild hydrolysis with Alcalase and Pepsin improved foam-forming capacity and FS of BPC.

For the hydrolysates prepared with Alcalase, Flavourzyme, and Pepsin, no significant correlations were found between F_0 and FS nor between F_0 and DH. However, there was a significant negative correlation between F_0 and DH for the hydrolysates prepared with Pepsin ($R^2 = 0.93$, p value < 0.05). FS and DH were significantly negatively correlated ($R^2 = 0.53$, p value < 0.05).

As also performed for the emulsifying properties, foam-forming properties were determined at pH 4.0. Foam-forming capacity and FS were lower at pH 4.0 than at pH 7.0, probably due to the lower solubility of the hydrolysates (results not shown).

DISCUSSION

The present study generated BSG protein hydrolysates with improved technofunctional properties. BPC was prepared by alkaline extraction of BSG followed by acid precipitation. BPC

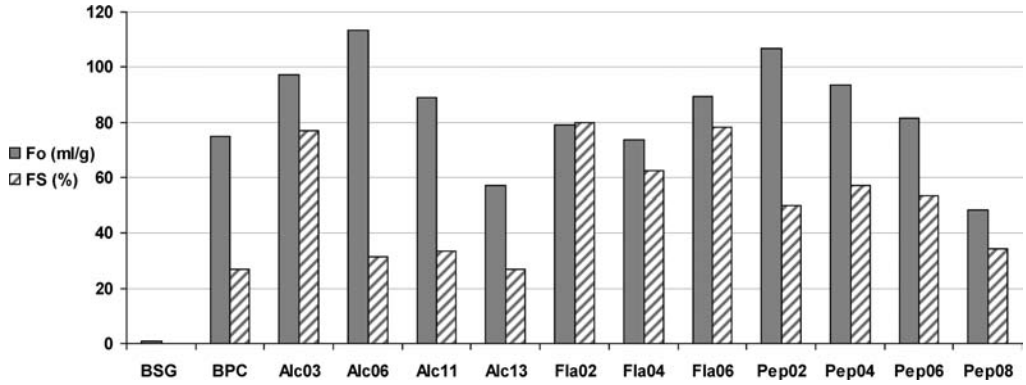

Figure 7. Foam-forming ability (F_0) (mL/g) (unpatterned bars) and foam stability (FS) (%) (patterned bars) at pH 7.0 and 0.6% protein of brewers' spent grain (BSG), BSG protein concentrate (BPC), and BPC hydrolysates. Hydrolysate codes consist of 3 letters representing the enzyme used and 2 digits encoding the DH (**Table 1**).

was enzymatically hydrolyzed in a pH-stat setup with Alcalase, Flavourzyme, or Pepsin for different times and/or with different enzyme to substrate ratios to obtain hydrolysates with different DH. Enzymatic hydrolysis of BPC increased solubility at low pH values. A comparable high solubility was observed at alkaline pH values. Enzymatic hydrolysis of BPC increased emulsion-forming ability. Foam-forming capacity improved (or was comparable) after enzymatic hydrolysis of BPC. Moreover, FS increased.

In general, an increasing DH resulted in increasing protein yields in the supernatant, increasing solubility of the proteins over the entire pH range, lower MW of the protein fragments, and decreased S_0. Furthermore, the type of enzyme used impacted the molecular properties of the hydrolysates. The hydrolysates prepared with Flavourzyme (Fla02, Fla04, and Fla06) showed high protein yields in the supernatant, high levels of protein fragments with MW either exceeding 14.5 k or lower than 1.7 k, and high S_0. Except for Fla06, total color difference with BSG increased with increasing DH.

The above results show that the type of enzyme used is a key factor in determining the emulsifying and foaming capacities of the resulting hydrolysates. Moreover, for hydrolysates prepared with Alcalase and Pepsin the emulsifying and foaming capacities decreased with increasing DH. High levels of protein fragments with MW exceeding 14.5 k are needed to obtain good emulsifying as well as foaming properties. In spite of the high level of protein fragments with MW exceeding 14.5 k, hydrolysates prepared with Flavourzyme showed somewhat lower foam-forming ability but high FS, probably due to the presence of low MW material produced by the exopeptidase activity of Flavourzyme.

Whereas EAI and ES were significantly negatively correlated, F_0 and FS were not. A higher DH resulted in a decreased FS, while EAI, ES, and F_0 were not significantly correlated. Increasing levels of protein fragments with MW lower than 1.7 k resulted in lower EAI ($R^2 = 0.37$, p value < 0.05) and higher ES ($R^2 = 0.40$; p value < 0.05). Whereas for hydrolysates prepared with Alcalase and Pepsin a significant positive correlation between the level of protein fragments with MW exceeding 14.5 k and EAI ($R^2 = 0.92$, p value < 0.05; and $R^2 = 0.99$, p value < 0.01; respectively) was found, Flavourzyme hydrolysates showed no significant correlation between these parameters. An increased S_0 went hand in hand with increased emulsifying capacities ($R^2 = 0.41$, p value < 0.05). This may be due to the enhanced interaction between the hydrolysates and lipids at the oil–water interfaces through the exposed hydrophobic groups formed during enzymatic hydrolysis. However, ES and S_0 were not correlated. Furthermore, foam-forming capacity and FS decreased with increasing levels of protein fragments with MW lower than 1.7 k ($R^2 = 0.41$, p value < 0.05; and $R^2 = 0.41$, p value < 0.05; respectively). A significant positive correlation existed between the level of protein fragments with MW exceeding 14.5 k and FS ($R^2 = 0.56$, p value < 0.01). Whereas foam-forming capacity was not correlated with S_0, FS increased with increasing S_0 ($R^2 = 0.69$, p value < 0.01). EAI decreased with increasing protein concentration. Furthermore, EAI as well as F_0 were lower at pH 4.0 than at pH 7.0.

In conclusion, enzymatic hydrolysis of BPC, prepared by alkaline extraction of BSG followed by acid precipitation, improved emulsifying and foam-forming properties. Physicochemical characterization of the hydrolysates indicated the importance of the presence of protein fragments with relatively high MW (exceeding 14.5 k) and high surface hydrophobicity, as well as of the type of enzyme used to obtain favorable technofunctional properties. Differences in physicochemical and technofunctional properties of hydrolysates obtained after hydrolysis with exoproteolytic and endoproteolytic enzymes are indicated, as are differences between hydrolysates obtained after hydrolysis with endoproteolytic enzymes from different classes. Further fractionation of the hydrolysates will be performed to study the relationships between molecular and technofunctional properties of these hydrolysates.

ABBREVIATIONS USED

ACN, acetonitrile; ANS, 1-anilino-8-naphthalene sulfonate; BPC, brewers' spent grain protein concentrate; BSA, bovine serum albumin; BSG, brewers' spent grain; DH, degree of hydrolysis; DTT, dithiothreitol; EAI, emulsifying activity index; ES, emulsion stability; F_0, foam-forming ability; FS, foam stability; mequiv, mequivalents; MW, molecular weight; OPA, *o*-phthaldialdehyde; RP-HPLC, reversed phase high performance liquid chromatography; S_0, surface hydrophobicity; SE-HPLC, size exclusion high performance liquid chromatography; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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