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Fractionation and Characterization of Brewers' Spent Grain Protein Hydrolysates

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Protein hydrolysates with a low and high degree of hydrolysis were enzymatically produced from brewers' spent grain (BSG), the insoluble residue of barley malt resulting from the manufacture of wort in the production of beer. To that end, BSG protein concentrate (BPC), prepared by alkaline extraction of BSG and subsequent acid precipitation, was enzymatically hydrolyzed with Alcalase during both 1.7 and 120 min. Because these hydrolysates contained many different peptides, fractionation of the hydrolysates with graded ammonium sulfate or ethanol precipitation was performed to obtain fractions homogeneous in terms of molecular weight (MW) and hydrophobicity. The emulsifying and foaming capacities of the resultant fractions were determined. MW distributions and surface hydrophobicities of fractions with protein contents exceeding 75% were investigated to determine relationships between technofunctional and physicochemical properties. It was found that the emulsifying and foaming properties are determined by different physicochemical properties of the proteins or peptides. Neither MW nor hydrophobicity alone determines the emulsifying and foaming properties of protein hydrolysates. BSG protein hydrolysates with good emulsifying properties contained less than 40% of fragments with MW exceeding 14 500. Moreover, these hydrolysates had a high surface hydrophobicity. BSG protein hydrolysates with good foaming properties contained less than 10% of material with MW lower than 1700. Hydrolysates with good foaming properties showed low surface hydrophobicities, except for protein hydrolysates with higher levels of protein fragments with MW exceeding 14 500 than of such fragments with MW in a 1700−14 500 range.

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

INTRODUCTION

Brewers' spent grain (BSG), the residue left after separation of wort during the brewing process, is rich in protein and fiber. However, to date, its use has mainly been limited to animal feeding. To increase the potential application of such insoluble proteins, they can be hydrolyzed. We reported earlier (*1*) that enzymatic hydrolysis of BSG proteins improves emulsifying and foam-forming properties. Because proteins and peptides do not dissolve in oil, they can only stabilize oil-in-water emulsions (*2*). The physicochemical characterization of the hydrolysates showed the importance of the presence of protein fragments with relatively high molecular weight (MW) (exceeding 14 500) and high surface hydrophobicity for favorable technofunctional properties. The type of enzyme used also had a large impact on the obtained technofunctional properties (*1*). However, these hydrolysates consisted of heterogeneous mixtures of low MW as well as high MW peptides with varying hydrophobicities. We set out to fractionate these hydrolysates to determine relationships between their physicochemical and technofunctional properties.

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Ultrafiltration [100 000, 50 000, and 20 000 MW cutoff (MWCO) (*3*) and 30 000 MWCO (*4*)] of gluten hydrolysates indicated that their contribution to foam stability (FS) decreases as the MW of hydrolysate fractions decreases. It is clear that a minimum three-dimensional structure has to be retained if the resulting hydrolysates are to have foaming and emulsifying properties. Polypeptides with low MW are unable to form stable protein films at the oil−water or air−water interfaces (*5*, *6*). According to Wang et al. (*3*), emulsifying and foaming capacities are associated with surface hydrophobicities. Determination of the emulsifying properties of gluten hydrolysates showed that the most hydrophobic peptides were adsorbed at the interfacial layer, while the hydrophilic peptides were present in the aqueous phase (*7*). Gluten hydrolysates, prepared by limited enzymatic hydrolysis with chymotrypsin and subsequent ultrafiltration (150 000 and 50 000 MWCO), resulted in retentates, which contained peptides that were both hydrophobic and positively charged, whereas the permeates contained hydrophilic and neutral peptides. This resulted in opposite technofunctional properties, probably because amphipathicity of peptides is a major factor for their ability to stabilize interfaces. Permeates had foaming capacity at pH 6.5 but neither foam-stabilizing nor emulsifying properties. The retentates showed better emulsifying and foaming properties at

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pH 4.0 and 6.5 than the hydrolysates (*8*, *9*). Also, the foaming properties of gluten protein hydrolysates depend less upon their solubility than their emulsifying properties (*10*).

The optimum peptide size of hydrolysates generated from soy proteins for emulsifying and foaming properties generally lies between 15 and 35 amino acids (*11*). Proteolysis of SPI followed by ultrafiltration (100 000, 50 000, and 20 000 MWCO) results in permeate peptides with higher emulsifying capacity but lower surface hydrophobicity than those of the peptides in the retentate and the starting material hydrolysates (*12*). However, a high correlation between surface hydrophobicity and emulsifying activity has also been reported (*13*, *14*). Hence, solubility and MW rather than surface hydrophobicity might be the major factors conferring high emulsifying activity to the small peptides of the permeates (*12*). Furthermore, protein solubility has a greater impact on the ability of soy protein hydrolysates to form and stabilize foams than surface hydrophobicity (*15*).

Enzymatic hydrolysis of maize gluten followed by ultrafiltration resulted in a fraction of MW < 5000 with higher emulsifying activity and emulsion stability (ES) than those of the other fractions. Fractions of MW < 5000 and MW > 10 000 showed poor foaming capacity at pH values below 6.0. The FS of these fractions increased with pH, but that of the fraction with intermediate MW (5000−10 000) decreased with increasing pH (*16*). Quinoa protein concentrate had higher emulsifying capacities than the resulting hydrolysate. This could probably be related to the high degree of hydrolysis (DH) (48%). However, the hydrolysate had better ES than the protein concentrate. Despite the higher foaming capacity of hydrolysate than that of the protein concentrate, the protein concentrate showed higher FS. Ultrafiltration (10 000 and 5000 MWCO) of quinoa protein hydrolysate led to permeates that had lower emulsifying and foaming properties than the protein hydrolysate (*17*).

To identify structure−function relationships for β-casein hydrolysates, such hydrolysates were prepared with plasmin. They were further fractionated by ultrafiltration [5000 MW cutoff (MWCO)] and selective precipitation with acid and/or ethanol (EtOH), which resulted in several peptide fractions. The hydrophilic peptides had poor technofunctional properties. The hydrophobic peptides showed interesting foam-forming properties, whereas the amphipathic peptides exhibited good emulsion-forming properties (*18*, *19*). Electrostatic repulsion between emulsion droplets caused by the amphipathic β-casein peptides is probably the main reason for their emulsion-stabilizing properties (*20*). The positive effect of peptides of MW exceeding 2000 on ES was demonstrated with different fractions of casein and whey protein hydrolysates (*21*). Foam formation by casein hydrolysates is independent of the MW of the peptides, whereas foam-forming whey protein hydrolysates seem to contain a percentage of peptides larger than 3000 contributing to this property. The stability of casein hydrolysate foams has been specifically related to peptides with MW exceeding 7000. For foam formation, the presence of amphiphilic peptides might be the most important factor, whereas, for FS, the presence of peptides of relatively high MW seems to be crucial (*22*).

As a sequel to a profound characterization of the hydrolysates resulting from enzymatic hydrolysis of BSG proteins (*1*), the aim of the present work was to obtain homogeneous fractions in terms of MW and hydrophobicity by fractionating the BSG protein hydrolysates by selective precipitation with EtOH and ammonium sulfate (AS). To the best of our knowledge, fractionation of plant protein hydrolysates with graded AS or EtOH precipitation has not been described in the literature. The emulsifying and foaming properties of the resultant fractions were examined. The MW distributions and surface hydrophobicities of fractions with protein contents exceeding 75% were further studied. Finally, the relationships between physicochemical and technofunctional properties of BSG protein hydrolysates were investigated.

MATERIALS AND METHODS

Materials. BSG [25% protein on a dry basis (db)] was from an all-malt mash pilot scale brewery experiment described earlier by Celus et al. (*23*). *Bacillus licheniformis* Alcalase was obtained from Novozymes (Bagsvaerd, Denmark). The main enzyme component of Alcalase is Subtilisin A (Subtilisin Carlsberg). Alcalase (7.8% protein) is a commercial enzyme preparation with high peptidase and limited α-amylase activities. The peptidase activity determined with hemoglobin as a substrate under the experimental conditions described by Brijs et al. (*24*) was 0.32 AU/g. Hyfoama 77 and Versa Whip 510 were enzymatically produced hydrolysates from wheat and soybean protein, respectively, and were from Quest International (Naarden, The Netherlands). SE70M, an enzymatically produced soybean protein hydrolysate, was from DMV International (Veghel, The Netherlands). Denatured ethanol (95%, further referred to as EtOH) was from Brenntag (Mülheim/Ruhr, Germany). All other reagents and chemicals were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade unless specified otherwise.

Enzymatic Hydrolysis of BSG Protein Concentrate (BPC). BPC, prepared by alkaline extraction of BSG followed by acid precipitation (*1*), was incubated with Alcalase. This enzyme was selected on the basis of the results obtained in earlier work (*1*). BPC was hydrolyzed for 1.7 and 120 min, respectively, to obtain hydrolysates with low and high DH, i.e., the ratio of the number of peptide bonds hydrolyzed to the total number of peptide bonds per unit weight present in BPC protein. Because high amounts of hydrolyzed proteins were needed for further experimental work, enzymatic hydrolysis of BPC [150 g (db)] was performed in a water bath (further referred to as "large scale"). A 6.0% (w/v on protein basis) aqueous dispersion of BPC was hydrolyzed for 1.7 and 120 min at pH 9.0 and 60 °C with Alcalase [2.5% enzyme preparation/substrate ratio (w/w of protein)]. During the 120 min hydrolysis, the pH was adjusted by manual addition of 1.0 mol/L NaOH in 30 min time intervals. After the reaction, the mixture was adjusted to pH 6.0 with 2.0 mol/L citric acid and heated in a water bath at 95 °C for 10 min to inactivate the enzyme. Aliquots (50 μL) of the total hydrolysate obtained after enzymatic hydrolysis were withdrawn for colorimetric determination of DH with *o*-phthaldialdehyde (OPA) (*1*). The remaining mixtures were centrifuged (5000*g* for 20 min at 4 °C), and the resulting supernatants were freeze-dried. Sample codes consist of three characters representing the enzyme Alcalase (Alc) and two digits encoding the DH.

Fractionation of BPC Hydrolysates by Ammonium Sulfate Precipitation. On the basis of the outcome of initial experiments, a 3.0% (w/v) aqueous dispersion of the hydrolysates was separated into three fractions by graded AS precipitation. Aliquots of AS were added under continuous stirring to a final concentration of 40% (w/v). The mixtures were then kept overnight at 4 °C. Precipitated material was recovered by centrifugation (10000*g* for 10 min at 4 °C), dispersed in deionized water, dialyzed against deionized water using 6000−8000 MWCO regenerated cellulose dialysis membranes (Spectra/Por, VWR International, Haasrode, Belgium), and freeze-dried to obtain fraction AS_{0-40}. The AS concentration of the supernatant was further increased to 60% (w/v), and the precipitated fraction, referred to as fraction AS_{40-60}, was recovered as described above. The remaining fraction was dialyzed against deionized water (6000−8000 MWCO), freeze-dried, and recovered as fraction AS_{60+}.

Fractionation of BPC Hydrolysates by Ethanol Precipitation. On the basis of the outcome of initial experiments, the hydrolysates (6.0%, w/v) were separated into four fractions by graded EtOH precipitation. Aliquots of EtOH were added under continuous stirring to a final concentration of 70% (v/v). The mixtures were then kept overnight at 4 °C. Precipitated material was recovered by centrifugation (10000*g* for 10 min at 4 °C), dispersed in deionized water, and freeze-dried to obtain fraction $EtOH_{0-70}$. The EtOH concentration of the supernatant was subsequently increased to 80% (v/v) and, in a third step, to 90% (v/v). The corresponding precipitated fractions, referred to as fractions $EtOH_{70-80}$ and $EtOH_{80-90}$, were recovered as described above. EtOH was

removed from the remaining supernatant by rotary evaporation. The resulting fraction was freeze-dried and recovered as fraction $EtOH_{90+}$.

Partial Chemical Composition of Hydrolysates and Their Fractions. Protein contents of the hydrolysates were determined using the Dumas combustion method, an adaptation of the Association of Official Analytical Chemists (AOAC) official method (*25*) to an automated Dumas protein analysis system (EAS, varioMax N/CN, Elt, Gouda, The Netherlands), using 6.25 as the conversion factor. The protein yield is the percentage of supernatant protein weight resulting from the BPC protein mixture. Protein contents of the AS and EtOH fractions were determined using Lowry's colorimetric method (*26*). To that end, the AS fractions were dissolved in 0.1 mol/L sodium phosphate buffer (pH 7.0). The EtOH fractions were dissolved in deionized water. The method was calibrated with a bovine serum albumin standard curve in the corresponding media. The protein yield of the fractions is the percentage of protein weight present in the fraction resulting from the hydrolysate protein weight.

Moisture and ash contents of the AS and EtOH fractions were determined according to AACC methods 44-15A and 08-12 (*27*), respectively. After hydrolysis, monosaccharide compositions of barley, malt, and BSG were determined by the method of Englyst and Cummings (*28*). Arabinoxylan (AX) content was calculated as 0.88 times the sum of xylose and arabinose after correction of the arabinose content for the presence of arabinogalactan peptide based on an arabinose/galactose ratio of 0.7 for barley flour and assuming that all of the arabinose of arabinogalactan peptide is present in the aqueous extract (*29*). Noncellulosic glucose polymer (NCG) was calculated as 0.9 times the glucose content.

Emulsifying Properties. Emulsifying properties were measured by the turbidimetric method of Pearce and Kinsella (*30*), with minor modifications. To prepare the emulsion, 7.0 mL of soy oil (Lesieur, Asnières-sur-Seine, France) and 21.0 mL of protein solution (0.2% protein) in 9.0 mmol/L sodium phosphate buffer containing 35 mmol/L NaCl (pH 7.0; I = 50 mmol/L) (*31*) were homogenized for 60 s at speed 1 and 25 °C in a Waring blender (Torrington, CT). 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) sodium dodecyl sulfate (SDS) solution. The absorbances of the diluted emulsions were measured at 500 nm (A_{500}). The emulsifying activity index (EAI, m^2/g) was calculated as follows:

$$\text{EAI (m/g)} = \frac{2T \times 200}{\phi C} \text{ and } \text{T} = \frac{2.303A_{500}}{L}$$

where A_{500} is the absorbance, L is the light path (10^{-2} m), ϕ is the volume fraction of dispersed phase (0.25), C is the concentration of protein (g/m^3), T is the turbidity, and 200 is the dilution factor. EAI_0 is the emulsifying activity index immediately after emulsion formation, whereas EAI_{30} is the emulsifying activity index after 30 min, and ES is the percentage of emulsion turbidity remaining after 30 min. Determination of the emulsifying properties was performed in duplicate for each sample.

Foaming Properties. Foam was prepared with protein solution (0.6% protein) in 9.0 mmol/L sodium phosphate buffer containing 35 mmol/L NaCl (pH 7.0; I = 50 mmol/L) (*31*), with the whipping method described by Caessens et al. (*19*). A volume of 100 mL of hydrolysate solution was placed in a graduated glass cylinder (diameter of 60 mm) and whipped for 70 s by a propeller rotating at 2000 rpm and 25 °C. The foam-forming ability (F_0, mL/g) is 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. Whereas F_{60} is the foam-forming ability measured at 60 min after the start of whipping per protein weight, FS is the percentage of the initial foam volume remaining after 60 min. The coefficients of variation for a 5-fold determination of F_0 and FS of a standard sample did not exceed 10%.

Size-Exclusion High-Performance Liquid Chromatography (SE-HPLC). 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 containing 0.15% trifluoroacetic acid in Milli-Q water. The column was calibrated with six MW markers: ribonuclease A (MW = 13 700), aprotinin (MW = 6500), insulin chain B (MW = 3500), Ala−Ser−His−Leu−Gly−Leu−Ala−Arg (MW = 824), $(Ala)_5$ (MW = 373), and Ala−Gln (MW = 217) (Sigma-Aldrich). A logarithmic calibration curve was constructed from the MW of the markers and their respective elution times (R^2 = 0.96). Samples containing 0.1% protein were dissolved in the mobile phase, and the elution was monitored at 214 nm.

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. The protein surface hydrophobicities of the BPC hydrolysates and fractions were determined with 1-anilino-8-naphthalene sulfonate (ANS) as the fluorescent probe (*12*). Samples containing 0.1% protein were dispersed in 0.01 mol/L sodium phosphate buffer (pH 7.0), shaken for 16 h at room temperature, and subsequently centrifuged (3000*g* for 10 min at 4 °C). Supernatants were serially diluted with 0.01 mol/L sodium phosphate buffer (pH 7.0) to obtain protein concentrations ranging from 0.01 to 0.16 mg/mL. A 10 μL aliquot of ANS solution [8 mmol/L in 0.01 mol/L sodium phosphate buffer (pH 7.0)] was added to 1.0 mL of each sample (in duplicate). The fluorescence intensity of the protein material was measured with a spectrofluorometer (Fluorolog-3 Model FL3-22, Horiba Jobin Yvon, Edison, NJ) using a 3 nm slit width. Wavelengths of excitation and emission were 390 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 concentration. The initial slope of a plot of fluorescence intensity as a function of the protein concentration was used as an index of protein surface hydrophobicity (S_0) (*12*). The determination of S_0 was performed in duplicate for each sample.

RESULTS

Enzymatic Hydrolysis of BPC Proteins. "Large scale" enzymatic hydrolysis of BPC by Alcalase for 1.7 and 120 min resulted in hydrolysates with low (1.0%) and high (9.0%) DH, respectively. After centrifugation and freeze-drying, 74 and 126 g of material (db) were obtained upon hydrolysis for 1.7 and 120 min, respectively. The 1.0 and 9.0% DH hydrolysates are further referred to as Alc01 and Alc09, respectively. The protein content in the supernatants (58%), determined with the Dumas method, was somewhat lower than reported earlier (*1*). The levels of protein recovered in the supernatants of Alc01 and Alc09 were 49 and 84%, respectively, which is in agreement with and somewhat lower than what was observed for the hydrolysates obtained after hydrolysis in the pH-stat setup for 1.7 and 120 min, respectively (*1*). Next to proteins, Alc01 and Alc09 contained ash [18% (db)], AX [2 and 1% (db), respectively], and NCG [4 and 2% (db), respectively].

Fractionation of BPC Hydrolysates. Because the principles causing precipitation of proteins and peptides by EtOH or AS are different, Alc01 and Alc09 were fractionated by these two graded precipitation processes. Whereas the main effect of EtOH precipitation is the reduction in water activity, AS precipitation is based on "salting in" at low AS concentrations and "salting out" at high AS concentrations. "Salting out" largely depends upon the hydrophobicity of the proteins, whereas "salting in" heavily depends upon surface charge distribution and polar interactions with the solvent (*32*). The hydrolysates were fractionated with AS into three fractions, which were recovered following precipitation at AS concentrations of 40% (AS_{0-40}), between 40 and 60% (AS_{40-60}), and above 60% (AS_{60+}). Apart from this, Alc01 and Alc09 were also fractionated with EtOH into four fractions, precipitating at EtOH concentrations of 70% ($EtOH_{0-70}$), between 70 and 80% ($EtOH_{70-80}$), between 80 and 90% ($EtOH_{80-90}$), and above 90% ($EtOH_{90+}$).

Table 1 shows the protein contents of the AS and EtOH fractions. AS_{40-60} obtained by fractionation of Alc01 and Alc09 and AS_{0-40} resulting from fractionation of Alc01 had high protein contents. Whereas the EtOH fractions resulting from

Table 1. Yields (%), Protein Contents [% (db)], and Protein Yields (%) of Fractions Obtained after Graded AS and EtOH Precipitation of Hydrolysates Resulting from Enzymatic Hydrolysis of BPC with Alcalase for 1.7 min (Alc01) and 120 min (Alc09)[a]

	Alc01			Alc09		
fractions	yield (%)	protein content [% (db)]	protein yield (%)	yield (%)	protein content [% (db)]	protein yield (%)
AS_{0-40}	50	85	73	51	53	46
AS_{40-60}	3	99	6	4	88	6
AS_{60+}	9	66	10	9	57	9
$EtOH_{0-70}$	33	85	39	21	62	22
$EtOH_{70-80}$	22	40	12	18	37	12
$EtOH_{80-90}$	12	85	14	16	67	19
$EtOH_{90+}$	33	76	35	45	59	47

[a] Whereas yields of the fractions are expressed as the percentage of fraction weight resulting from the hydrolysate weight, protein yields of the fractions are expressed as the percentages of fraction protein weight resulting from the hydrolysate protein weight.

Table 2. AX, NCG, and Ash Contents [% (db)] of Fractions Obtained after Graded AS and EtOH Precipitation of Hydrolysates Resulting from Enzymatic Hydrolysis of BPC with Alcalase for 1.7 min (Alc01) and 120 min (Alc09)

	Alc01			Alc09		
fractions	AX content [% (db)]	NCG content [% (db)]	ash content [% (db)]	AX content [% (db)]	NCG content [% (db)]	ash content [% (db)]
AS_{0-40}	1.3	0.5	0.4	0.9	0.5	1.1
AS_{40-60}	2.7	0.6	nd[a]	2.0	0.5	nd[a]
AS_{60+}	8.8	6.1	2.1	6.8	2.8	1.4
$EtOH_{0-70}$	4.5	2.7	7.2	5.0	2.3	6.7
$EtOH_{70-80}$	0.1	0.6	42.4	0.2	0.6	43.0
$EtOH_{80-90}$	0.2	6.3	6.8	0.2	3.3	11.7
$EtOH_{90+}$	0.02	5.3	14.0	0.0	2.1	6.1

[a] nd = not determined.

fractionation of Alc09 had protein contents lower than 75%, the EtOH fractions obtained by fractionation of Alc01 showed protein contents exceeding 75%, with the exception of $EtOH_{70-80}$. **Table 1** also presents the yields and protein yields of the AS and EtOH fractions. The data for the AS fractions indicate that most of the material, including proteins, were present in AS_{0-40}. High levels of material, including proteins, were lost during AS fractionation, because of dialysis against deionized water (6000−8000 MWCO) to remove AS. Whereas EtOH fractionation of Alc09 resulted in $EtOH_{90+}$, which contained 47% of the Alc09 proteins, EtOH fractionation of Alc01 showed similar protein yields of $EtOH_{0-70}$ and $EtOH_{90+}$ (39 and 35%, respectively) (**Table 1**).

Because some AS or EtOH fractions contained low levels of protein, we characterized the nonprotein material. **Table 2** shows the AX, NCG, and ash contents of the AS and EtOH fractions. The $EtOH_{70-80}$ fractions obtained after fractionation of Alc01 and Alc09, which consisted of less than 40% protein material, contained high levels of ash (ca. 43%). In these fractions, 52 and 43% of the ash material of the hydrolysates Alc01 and Alc09, respectively, are recovered. The AX content of $EtOH_{0-70}$ was higher than that of the other EtOH fractions, whereas AS fractionation of Alc01 and Alc09 resulted in AS_{60+} with higher AX content than AS_{0-40} and AS_{40-60}. $EtOH_{70-80}$ contained the lowest level of NCG of all of the EtOH fractions. The NCG content was higher for AS_{60+} than for AS_{0-40} and AS_{40-60}. Because the hydrolysates Alc01 and Alc09 consisted of 4 and 2% NCG, respectively, 14 and 12% of NCG of Alc01 and Alc09, respectively, were recovered in the respective AS_{60+} fractions.

Emulsifying Properties. Figure 1 shows the EAI and ES, measured at pH 7.0, of the hydrolysates Alc01 and Alc09 and their EtOH and AS fractions. The EAI_0 was determined immediately after emulsion formation, whereas EAI_{30} was determined 30 min later. ES is the percentage of emulsion turbidity remaining after 30 min. The emulsifying properties of SE70M, a commercially available product with good emulsifying properties, were determined with the same methods. This resulted in EAI_0 of 55 m^2/g (standard deviation of 3.0%), EAI_{30} of 32 m^2/g (standard deviation of 2.9%), and ES of 59% (standard deviation of 2.4%). Alc01 and Alc09 and most of their AS and EtOH fractions showed EAI_0 values exceeding those of SE70M. Only $EtOH_{0-70}$ and AS_{40-60} obtained by fractionation of Alc01 had somewhat lower EAI_0 values.

Because the $EtOH_{70-80}$ fractions contained ca. 43% ash, the effect of ash on the emulsifying properties was evaluated. The emulsifying properties of Alc09 and SE70M were similar or even decreased when evaluated in 9.0 mmol/L sodium phosphate buffer containing 80 mmol/L NaCl (pH 7.0; I = 100 mmol/L), which corresponds to the addition of 43% NaCl (results not shown). Moreover, the emulsifying properties of $EtOH_{70-80}$, desalted on a Biogel P4 column, were similar or even higher than the emulsifying properties of $EtOH_{70-80}$ (results not shown). This indicates that ash has no effect on the emulsifying properties.

Some fractions resulting from fractionation of Alc01 ($EtOH_{90+}$, AS_{40-60}, and AS_{60+}) and some fractions obtained by fractionating Alc09 ($EtOH_{90+}$, AS_{0-40}, AS_{40-60}, and AS_{60+}) had EAI_{30} and ES values exceeding those of SE70M (32 m^2/g and 59%, respectively). $EtOH_{0-70}$ obtained after fractionation of Alc01 yielded lower EAI_{30} and ES values than those of SE70M. Whereas EAI_{30} values of some fractions obtained by fractionation of Alc01 (AS_{0-40}, $EtOH_{70-80}$, and $EtOH_{80-90}$) and Alc09 ($EtOH_{0-70}$, $EtOH_{70-80}$, and $EtOH_{80-90}$) exceeded 32 m^2/g, their ES values were lower than that of SE70M.

Foaming Properties. Figure 2 shows the foaming properties of Alc01 and Alc09 and their EtOH and AS fractions at pH 7.0. The F_0 and F_{60} values were determined, respectively, 2 and 60 min after the start of whipping, whereas FS is the percentage of foam volume remaining after 60 min. The foam-forming capacities of

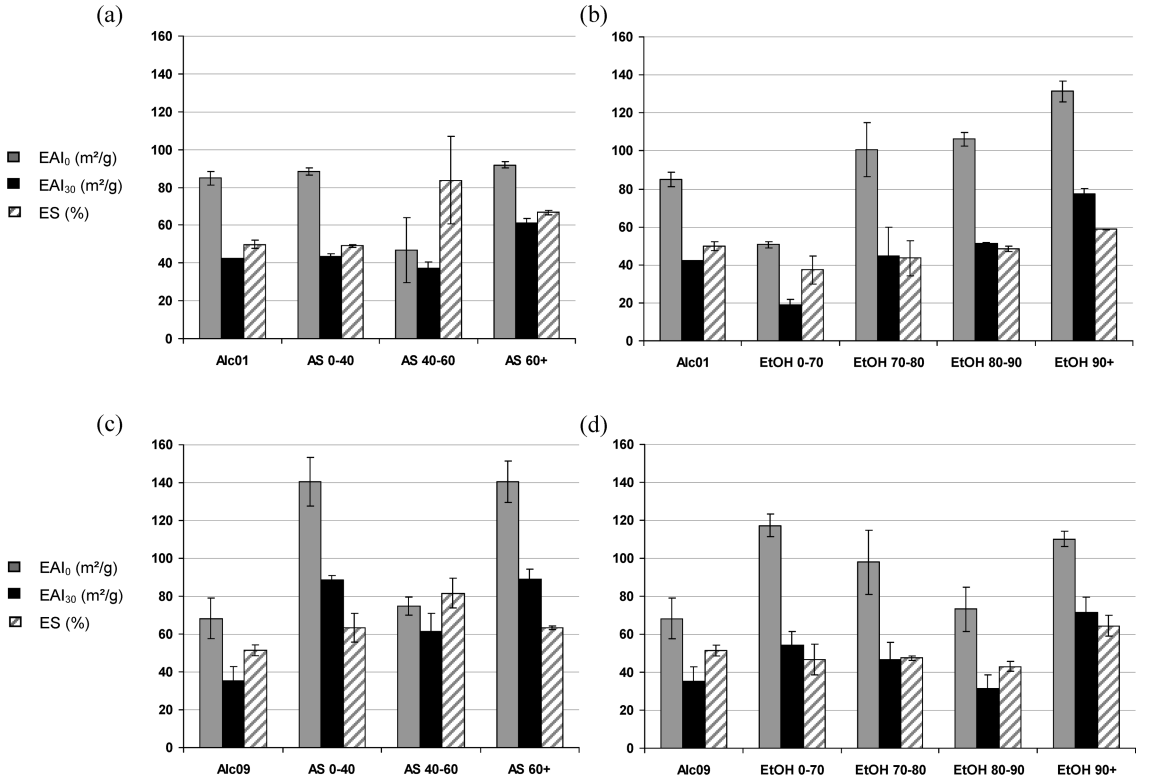

Figure 1. Emulsifying activity index (m^2/g), immediately (EAI_0; unpatterned gray bars) and 30 min after emulsion formation (EAI_{30}; unpatterned black bars), and emulsion stability (ES; %) (patterned bars) at pH 7.0 and 0.2% protein of hydrolysates obtained by enzymatic hydrolysis of BPC with Alcalase for 1.7 min (Alc01; a and b) and 120 min (Alc09; c and d) and their fractions resulting from graded AS precipitation (a and c) and graded EtOH precipitation (b and d). Each of the columns and error bars represents the average and standard deviation of EAI and ES, respectively.

Hyfoama 77 and Versa Whip 510, two commercially available products with high foam-forming capacity, were evaluated for comparison reasons. Hyfoama 77 and Versa Whip 510 showed F_0 values of 100 and 109 mL/g, respectively, while F_{60} of these commercially available products were 66 and 62 mL/g, respectively. The FS of Hyfoama 77 and Versa Whip 510 were 65 and 57%, respectively. Alc01 had a F_0 value comparable to those of the commercially available products, whereas Alc09 had a lower F_0. Some fractions resulting from fractionation of Alc01 (AS_{0-40} and $EtOH_{90+}$) and Alc09 (AS_{0-40}, AS_{40-60}, $EtOH_{0-70}$, and $EtOH_{90+}$) showed F_0 values lower than 100 mL/g.

As for the emulsifying properties, the foaming properties of Hyfoama 77 and Alc09 were similar or even lower in 9.0 mmol/L sodium phosphate buffer containing 164 mmol/L NaCl (pH 7.0; I = 180 mmol/L) than in 9.0 mmol/L sodium phosphate buffer containing 35 mmol/L NaCl. This indicates that ash has no effect on the foaming properties.

FS and F_{60} of $EtOH_{70-80}$ and $EtOH_{80-90}$ resulting from fractionation of Alc01 and Alc09 were higher than those of Hyfoama 77 and Versa Whip 510. All AS fractions obtained from Alc09, AS_{40-60} and AS_{60+} obtained from Alc01, and the $EtOH_{0-70}$ fractions obtained from Alc01 and Alc09 had lower FS and F_{60} than those of the commercially available products. $EtOH_{90+}$ obtained after fractionation of Alc01 and Alc09 and AS_{0-40} resulting from fractionation of Alc01 showed FS values exceeding those of Hyfoama 77 and Versa Whip 510. However, their F_{60} were lower than those of the commercially available products.

SE-HPLC. To relate the emulsifying and foaming properties with the protein or peptide molecular characteristics, fractions with protein contents exceeding 75% (db) were selected and their MW distributions were determined. The SE-HPLC profiles of the fractions obtained after graded AS and EtOH precipitation of Alc01 and Alc09 were divided into three fractions based on their apparent MW (**Figure 3**), as described by Celus et al. (*1*). Fraction I includes the first peak and corresponds to protein fragments with MW exceeding 14 500; fraction II corresponds to protein fragments with MW between 1700 and 14 500; and fraction III corresponds to protein fragments with MW lower than 1700. **Table 3** shows the area percentage of each fraction relative to the total area of the SE-HPLC chromatogram. The fractions that contained less than 75% protein (db) were excluded from **Table 3** because of the presence of high levels of nonprotein material.

The average MWs of both the AS and EtOH fractions decreased with increasing AS and EtOH concentrations. Whereas $EtOH_{0-70}$ consisted of high levels of protein fragments with MW exceeding 14 500, the protein fragments present in $EtOH_{90+}$ had MW lower than 14 500 (**Table 3**).

Surface Hydrophobicity. Figure 4 shows the S_0 of the fractions obtained after graded AS and EtOH precipitation of Alc01 and Alc09. The figure **4** does not show fractions with protein contents lower than 75% (db).

In general, the surface hydrophobicities of both the AS and EtOH fractions decreased with increasing AS and EtOH concentrations. Fractionation of Alc01 resulted in AS_{40-60} with very low S_0.

DISCUSSION

Hydrolysates prepared by enzymatic hydrolysis of BPC with Alcalase with low and high DH were fractionated with graded EtOH and AS precipitation to obtain fractions homogeneous in terms of MW and hydrophobicity. The emulsifying and foaming properties of the fractions were compared to those of commercially available products with good emulsifying or foaming properties. To relate these technofunctional properties with the protein or peptide molecular characteristics, the MW

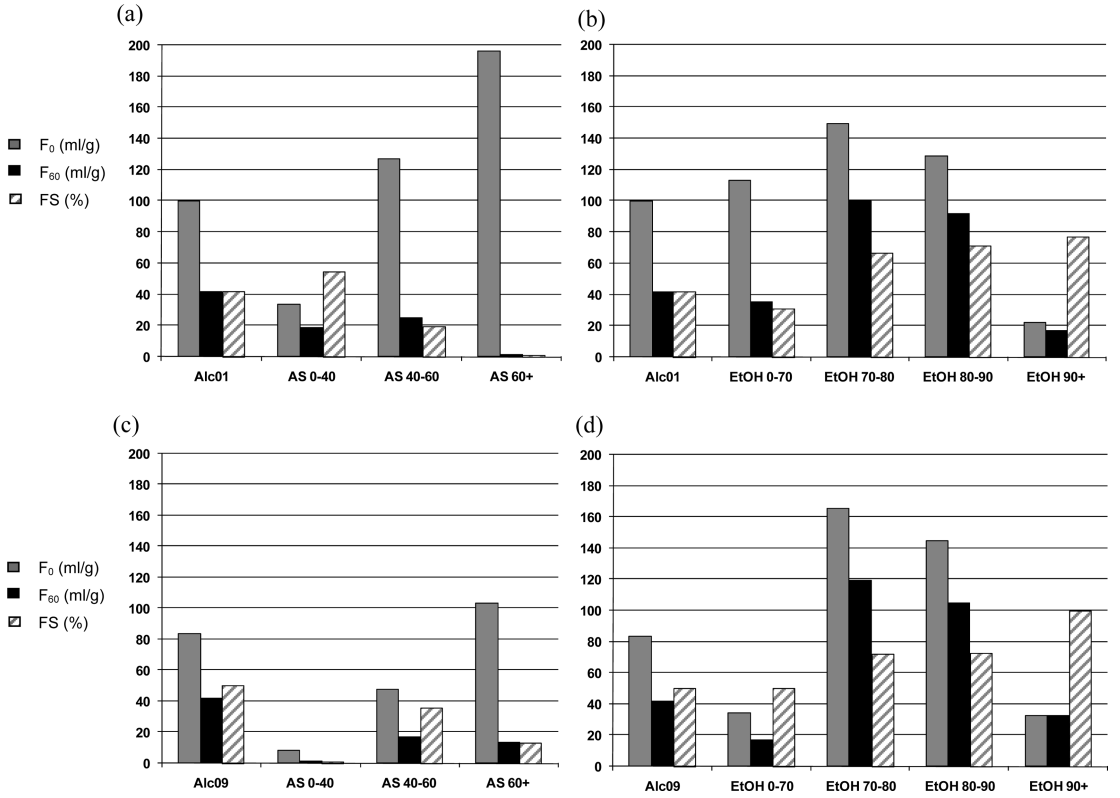

Figure 2. Foam-forming ability (mL/g), 2 min (F_0; unpatterned gray bars) and 60 min (F_{60}; unpatterned black bars) after the start of whipping, and foam stability (FS; %) (patterned bars) at pH 7.0 and 0.6% protein of hydrolysates obtained by enzymatic hydrolysis of BPC with Alcalase for 1.7 min (Alc01; a and b) and 120 min (Alc09; c and d) and their fractions resulting from graded AS precipitation (a and c) and graded EtOH precipitation (b and d).

Table 3. Apparent MW Distribution of Fractions Obtained after Graded AS and EtOH Precipitation of Hydrolysates Resulting from Enzymatic Hydrolysis of BPC with Alcalase for 1.7 min (Alc01) and 120 min (Alc09) and with Protein Contents Exceeding 75% (db)[a]

sample	fraction I	fraction II	fraction III
AS_{0-40} (Alc01)	31	65	4
AS_{40-60} (Alc01)	2	95	3
AS_{40-60} (Alc09)	3	89	8
$EtOH_{0-70}$ (Alc01)	63	37	0
$EtOH_{80-90}$ (Alc01)	nd[b]	95	5
$EtOH_{90+}$ (Alc01)	nd[b]	55	45

[a] The MW distribution is expressed as the area percentage of each fraction relative to the total area of SE-HPLC chromatogram (detection at 214 nm). SE-HPLC profiles are divided into three fractions based on their MW. Fraction I corresponds to protein fragments with MW exceeding 14 500; fraction II corresponds to protein fragments with MW between 1700 and 14 500; and fraction III corresponds to material with MW lower than 1700. [b] nd = not detectable.

distributions and surface hydrophobicities were only determined for fractions with protein contents exceeding 75% (db).

The fractions with lower EAI than SE70M ($EtOH_{0-70}$ and AS_{40-60} obtained after fractionation of Alc01) contained higher levels of protein fragments with MW exceeding 14 500 than of such fragments with MW between 1700 and 14 500 or showed very low S_0. The other AS and EtOH fractions with EAI higher than that of the commercially available product contained less than 40% of protein fragments with MW exceeding 14 500 and had S_0 exceeding 10×10^5. According to Damodaran (*33*), small peptides migrate quickly and adsorb to oil−water interfaces. Wu et al. (*12*) and Kim et al. (*16*) also demonstrated high emulsifying activities of soy and maize protein hydrolysate fractions with LMW, respectively. The minimum hydrophobicity needed to obtain favorable emulsifying properties can probably be ascribed to enhanced interaction between hydrolyzed proteins and lipids at oil−water interfaces as a result of the exposure of hydrophobic groups upon enzymatic hydrolysis, in accordance with the literature. Gluten hydrolysate fractions enriched in hydrophobic peptides indeed show good emulsifying properties (*3*, *7*−*9*). Jung et al. (*14*) and Qi et al. (*13*) demonstrated a high correlation between surface hydrophobicity and emulsifying activity of soy protein hydrolysates. However, Wu et al. (*12*) demonstrated higher emulsifying activity but lower S_0 of soy protein hydrolysate fractions.

Whereas some fractions resulting from fractionation of Alc01 (AS_{40-60} and $EtOH_{90+}$) and Alc09 (AS_{40-60}) had ES and EAI_{30} values exceeding those of SE70M, $EtOH_{0-70}$ obtained by fractionation of Alc01 showed lower ES and EAI_{30} than SE70M. It should be noticed that $EtOH_{0-70}$ contained higher levels of protein fragments with MW exceeding 14 500 than of such fragments with MW between 1700 and 14 500 and/or showed high S_0. Therefore, protein fragments with less than 50% of the protein with MW exceeding 14 500 and/or S_0 lower than 30×10^5 may result in high ES. These results are at variance with some literature data, which propose that proteins form a more stable viscoelastic film at the interface than small MW surfactants (*6*, *33*). However, Kim et al. (*16*) demonstrated higher ES for maize gluten hydrolysate fractions with MW lower than 5000.

The F_0 of some fractions resulting from fractionation of Alc01 (AS_{40-60}, $EtOH_{0-70}$, and $EtOH_{80-90}$) exceeded 100 mL/g. The fractions with F_0 values lower than those of the commercially available products (AS_{40-60} obtained after fractionation of Alc09 and AS_{0-40} and $EtOH_{90+}$ resulting from fractionation of Alc01) contained more than 10% of the protein fragments with MW lower than 1700 or showed S_0 values exceeding 20×10^5. However, $EtOH_{0-70}$ obtained after

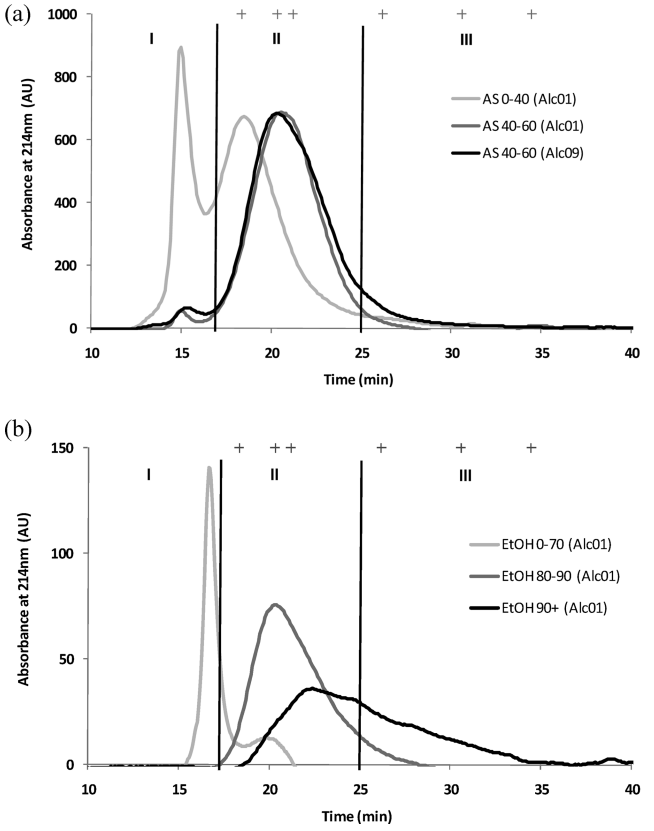

Figure 3. SE-HPLC profiles of fractions obtained after graded AS (a) and EtOH (b) precipitation of hydrolysates resulting from enzymatic hydrolysis of BPC with Alcalase for 1.7 min (Alc01) and 120 min (Alc09) with protein contents exceeding 75% (db). Elution times of MW markers with MW 13 700, 6500, 3500, 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 14 500 (fraction I), protein fragments with MW between 1700 and 14 500 (fraction II), and protein fragments with MW lower than 1700 (fraction III). Absorbance (214 nm) is expressed in arbitrary units (AU).

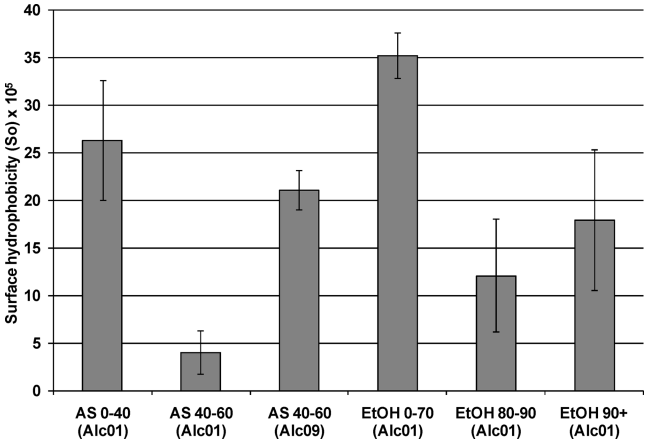

Figure 4. Surface hydrophobicity (S_0) of fractions obtained after graded AS and EtOH precipitation of hydrolysates resulting from enzymatic hydrolysis of BPC with Alcalase for 1.7 min (Alc01) and 120 min (Alc09) and with protein contents exceeding 75% (db). Each of the columns and error bars represents the average and standard deviation of S_0, respectively.

fractionation of Alc01 had S_0 values exceeding 20×10^5 and showed high F_0 values. This could be explained by the higher levels of protein fragments of MW exceeding 14 500 than of such fragments of MW in a 1700−14 500 range. These results are at variance with the literature data, where the increase in foaming capacity of gluten hydrolysate fractions is obviously associated with increased surface hydrophobicity (*3*, *8*, *9*). However, Ortiz and Wagner (*15*) showed a greater impact of protein solubility than of surface hydrophobicity on the foaming properties of soy protein hydrolysates. According to Adler-Nissen and Olsen (*11*), the optimum peptide size of soy proteins lies between 15 and 35 amino acids for good foaming properties, in line with our results.

Whereas fraction $EtOH_{80-90}$ obtained after fractionation of Alc01 showed FS and F_{60} values exceeding those of the commercially available products, fractions resulting from fractionation of Alc01 (AS_{40-60} and $EtOH_{0-70}$) and Alc09 (AS_{40-60}) resulted in low FS and F_{60} values. These results may indicate that S_0 should be between 10×10^5 and 20×10^5 and/or less than 10% of the protein fragments should have MW exceeding 14 500 to exert a favorable foam-stabilizing effect. These results are at variance with research data on gluten hydrolysates (*3*, *4*), which indicated that FS decreases as MW decreases.

In conclusion, fractionation of the BSG protein hydrolysates resulted in homogeneous peptide fractions. It became clear that the physicochemical properties required for emulsifying properties differ from those required for foaming properties. As outlined above, literature on protein hydrolysates is ambiguous about relationships between physicochemical and emulsifying and foaming properties and research reports generally focus on either MW (*11*) or S_0 (*3*, *8*, *9*) in relation to the emulsifying and foaming properties. This paper provides good evidence that neither MW nor hydrophobicity solely determine the emulsifying and foaming properties.

While, for favorable emulsifying activities of the BSG hydrolysate fractions, less than 40% of the proteins/peptides had MW exceeding 14 500, the BSG hydrolysate fractions had favorable foaming activities provided that they did not contain more than 10% of protein fragments with MW lower than 1700. Moreover, favorable emulsifying activities went hand in hand with S_0 exceeding 10×10^5, while good foaming activities were accompanied by S_0 lower than 20×10^5, except for protein hydrolysates with higher levels of protein fragments with MW exceeding 14 500 than of such fragments with MW in a 1700−14 500 range.

ABBREVIATIONS USED

ANS, 1-anilino-8-naphthalene sulfonate; AS, ammonium sulfate; BPC, brewers' spent grain protein concentrate; BSG, brewers' spent grain; DH, degree of hydrolysis; EAI_0, emulsifying activity index (immediately after emulsion formation); EAI_{30}, emulsifying activity index (30 min after emulsion formation); ES, emulsion stability; EtOH, ethanol; F_0, foam-forming ability (2 min after the start of whipping); F_{60}, foam-forming ability (60 min after the start of whipping); FS, foam stability; MW(s), molecular weight(s); MWCO, molecular weight cutoff; OPA, *o*-phthaldialdehyde; S_0, surface hydrophobicity; SE-HPLC, Size-exclusion high-performance liquid chromatography; SDS, sodium dodecyl sulfate.

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