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Enzymatic Solubilization of Proteins in Brewer's Spent Grain

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Brewer's spent grain (BSG) is an abundant, protein-rich coproduct from the beer industry. There is a growing interest in increasing and diversifying the exploitation of BSG and related coproducts for economic and environmental reasons. In this paper, we report on a study of the solubilization of proteinaceous material from BSG using several commercial peptidase preparations. Our data show that Alcalase is the most effective peptidase for solubilization of BSG proteins, with an ability to release up to 77% of total protein. The peptides produced by Alcalase had lower average molecular weight than peptides produced by the less effective enzymes. Processes that combined peptidase treatment with carbohydrate-degrading enzyme preparations such as Depol740 increased the solubilization of dry matter (from 30 to 43% under optimal conditions). However, such additional treatment had little effect on the solubilization of protein. The choice of enzyme dosage depends on the desired hydrolysis time and was assessed through several experiments. Protein solubilization was consistently better at pH 8.0 as compared to pH 6.8. Maximum protein solubilization at pH 8.0 within 4 h required the use of 10−20 μL Alcalase per g of dry matter. However, a considerable degree of solubilization (64%) and hydrolysates with high protein content could be obtained using doses down to only 1.2 μL. Amino acid composition analyses showed that Alcalase treatment solubilizes proline and glutamine (constituents of barley hordein) slightly more efficiently than the other amino acids in BSG.

KEYWORDS: Brewer's spent grain; hydrolysis; protein solubilization; peptidase; Alcalase

INTRODUCTION

Barley (*Hordeum vulgare* L.) is a major animal feed crop, with smaller amounts used for food and malting. It ranks fourth in terms of the agricultural area used for cultivation of cereal crops in the world (*1*). Malting is a process in which the barley grains are made to germinate so that endogenous hydrolytic enzymes are synthesized. The malt is heat dried and used in the mashing step in beer production, where grain's starch is converted into sugars, principally maltose (*2*). Brewer's spent grain (BSG) is a low-value coproduct from the beer industry, consisting of the barley malt residue after its separation from the wort. BSG is a lignocellulosic material consisting primarily of cellulose (17%), noncellulosic polysaccharides (28%, mainly arabinoxylans), lignin (28%), and a relatively large amount of protein (around 20%) (*2, 3*). The chemical composition of BSG may vary with barley variety, time of harvest, characteristics of hops and other adjuncts added during or before the mashing, and the brewing process itself, that is, the conditions used for malting and mashing (*4*). So far, BSG has mainly been utilized as animal fodder (*2*), but there is an interest in finding other applications for this abundant, protein-rich byproduct. For example, the protein fraction is of interest because the protein supply is of major importance in fish, animal, and human nutrition. Outputs from BSG may include higher-value materials for feed and food production, including, for example, protein-enriched materials or growth media for probiotic bacteria (*5–10*). Other valuable products (e.g., ferulic acid) have been enzymatically extracted from BSG mainly by use of carbohydrate-degrading enzymes (*11–16*). Protein recovery from BSG (or during barley grain mashing) by physical, chemical, and enzymatic treatments has been studied (*17–21*), but so far, pure biochemical studies on the use of peptidases for this purpose are rare (*22*). Enzymatic hydrolysis of BSG proteins by commercial peptidases has recently been described using protein fractions obtained after alkaline extraction of BSG and subsequent acid precipitation. The technofunctional properties of the extracted proteins have also been characterized (*23*).

In the present study, we have studied the use of commercial peptidases to solubilize proteins directly from BSG, that is, without using extractions and only using mild conditions. The goal was to solubilize BSG proteins to the largest possible extent by enzymatic hydrolysis and to produce nitrogen-rich hydrolysates that could find applications in the feed, food, or fermentation industry. Proteolytic hydrolysis has the advantage of extracting the proteins in a gentle manner, with minimal risk

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of nondesirable side reactions such as chemical modifications. We have addressed issues such as the choice of enzyme, pH, enzyme dose, and processing time. Furthermore, the effects of destructuring pretreatments with carbohydrate degrading enzymes on the subsequent protein solubilization were evaluated. The outcome of the hydrolysis reactions was analyzed in terms of the degree of solubilization of nitrogen and dry matter (DM), the degree of protein hydrolysis, peptide profiles, and amino acid compositions. Our results show that it is possible to enzymatically extract 80% of the proteinous material in BSG.

MATERIALS AND METHODS

Enzymes. The following commercial peptidases were utilized in this work: the bacterial peptidases Alcalase 2.4L, Neutrase 0.8L, and Protamex (a mix of Alcalase and Neutrase; all three preparations were from Novozymes, Bagsvaerd, Denmark), the plant proteolytic enzymes Papain, Bromelain, and Actinidin (Biochem Europe, Mons, Belgium), cod stomach pepsin (Hov Bio AS, Gimsoysand, Norway), pepsin from hog (pepsin A; Fluka, BioChemika, Sigma-Aldrich, Steinheim, GE), and cheese rennet standard 190 (a mix of 63% chymosin and 37% bovine pepsin; Chr. Hansen, Hørsholm, Denmark). Furthermore, we used a mix of carbohydrate-degrading enzymes containing equal amounts (volume) of Celluclast 1.5L, Pectinex Ultra SP-L, Ultraflo L, and Viscozyme L (all from Novozymes, Bagsvaerd, Denmark). Depol740L was from Biocatalysts (Cardiff, United Kingdom). All commercial enzyme preparations were received as gifts from the manufacturers.

Peptidase Activity. The commercial enzyme preparations used were difficult to quantify with respect to actual peptidase content since they were not purified proteins. For this reason, we determined the proteolytic activity using casein as a substrate, as described by Fujii et al. (*24*), with modifications. Then, we compared our enzyme preparation in a standard assay. Activities were determined at 50 °C in 20 mM TrisHCl (pH 7.5), 5 mM $CaCl_2$, 0.8% (w/v) casein (Hammarstein, Merck), and 0.02% NaN_3. After 60 min of incubation, the reaction was stopped by the addition of an equal volume of an ice-cold trichloroacetic acid (TCA) solution [100 mM TCA, 1.9% (v/v) acetic acid, and 220 mM sodium acetate]. The increase in absorbance was linear with time during the 60 min incubation period. The mixture was allowed to stand at room temperature for 30 min before the white precipitate (insoluble protein) was removed by centrifugation at 20000*g* for 15 min in a microcentrifuge. The amount of acid-soluble peptides was measured spectrophotometrically at 280 nm. Control reactions lacking the enzyme were included and used for baseline correction of the result obtained. Activities were expressed as nkat/mL enzyme preparation (**Table 1**), where an enzyme had an activity of 1 katal if it enabled the reaction to proceed at a rate of 1 mol of liberated tyrosine equiv/s. Activities were calculated using a standard solution of tyrosine (0.552 M) (Sigma). Activities (nkat/g DM) of the enzyme loadings (μL or mg/g DM) used in the hydrolysis experiments are shown in **Table 1**. Note that activities toward casein at pH 7.5 will not necessarily correspond to proteolytic activities toward BSG proteins under the hydrolysis conditions used in this study.

Hydrolysis Reactions. The substrate BSG, obtained from Ringnes brewery (Hagan, Norway), was freeze-dried and milled. Initially, we carried out hydrolyses without pH adjustment or pH control, in an attempt to minimize the need for addition of chemicals. Because BSG yields rather acidic solutions (approximately pH 5), used were the following peptidases with acidic pH optima (and low temperature optima): cod stomach pepsin, pepsine from hog, cheese rennet, and Actinidin. Hydrolyses were carried out using a concentration of 3.33% DM w/v in 50 mL tubes at 30 °C for 18 h with agitation at 150 rpm. We used high enzyme doses (20 μL or 20 mg/g DM; 200 μL in case of cheese rennet) to find values for maximum solubilization of BSG. Because the cheese rennet preparation was not as concentrated as the other enzyme preparations, a bigger volume (200 μL) of this preparation was used in the experiments.

In a second series of experiments, we used protease preparations with neutral or slightly basic pH optima (Alcalase, Neutrase, Protamex, Papain, and Bromelain). Hydrolyses with pH control were carried out in buffer (100 mM Na_2HPO_4/NaH_2PO_4) generating a constant pH of 6.8 ± 0.05. Reactions were run using the conditions described above, at 50 and 60 °C, with an enzyme load of 20 μL or 20 mg/g DM. During all tests, buffers were supplied with NaN_3 (0.02% w/v) to avoid microbiological problems. Some samples were pretreated with carbohydrate-degrading enzymes at 50 °C for 6 h with agitation at 150 rpm, using enzyme loadings of 20 μL/g DM of either an enzyme mix (equal volumes of Celluclast, Pectinex Ultra, Ultraflo, and Viscozyme to produce a mixture with broad carbohydrate-degrading activity) or Depol740.

Table 1. Peptidase Activity (Casein)[a]

enzymes	ΔA_{280}/min/μL or mg	nkat/mL or g	activity (nkat) per g DM BSG[d]
Protamex[b]	1.193	1629.61	32.59
Alcalase	0.693	985.84	19.72
Neutrase	0.493	728.33	14.57
Papain[b]	0.292	385.28	7.706
Bromelain[b]	0.260	344.08	6.882
Ultraflo	0.0070	9.84	0.197
cod stomach pepsin[b]	0.0066	9.45	0.189
Depol740	0.0066	9.41	0.188
Viscozyme	0.0055	7.99	0.160
pepsin from hog[b]	0.0024	4.00	0.080
Celluclast	0.0026	3.46	0.069
Pectinex	0.0013	1.76	0.035
Rennet St190	0.0006	0.88	0.176
Actinidin[b]	0.0001	0.20	0.004
BSG Ringnes[c]	0.0003	0.39	0.389

[a] Activity was measured at pH 7.5 and 50 °C. Note that some of the enzymes, for example, the fish pepsins, most likely are not stable at this temperature. The first five enzymes are all expected to be stable at this temperature under the conditions of the assay. [b] Solid enzyme was dissolved in buffer; results are reported per g or mg. [c] BSG was dissolved in buffer. [d] Activities (in nkat/g DM) of the enzyme loadings used in standard hydrolysis experiments (20 μL or 20 mg/g BSG DM; 200 μL in case of cheese rennet); see the text for further details.

For Alcalase, the effects of enzyme dosage (1.2−20 μL/g DM, corresponding to 1.18−19.72 nkat/g DM) and reaction pH (6.8 or 8.0) were addressed in more detail using the experimental conditions described above. A pH of 8.0 was selected because Alcalase has an alkaline optimum (data sheet, Novozymes). Because the buffering capacity of the phosphate buffer is low at pH 8, the pH dropped to approximately 7.6 during the reaction.

To get a more detailed impression of the progress in the proteolytic reaction, to study dose−response effects, and to measure the degree of hydrolysis (DH) for Alcalase 2.4L, we adopted the pH-stat technique (*25*). Hydrolysis reactions were carried out at pH 8.0 with 3.33% DM w/v in 100 mL Erlenmeyer vessels with magnetic stirring (150 rpm) at 60 °C in a water bath. To ensure initial pH control and to create conditions comparable to the reactions described above, reactions were run in buffer (100 mM Na_2HPO_4/NaH_2PO_4, pH 8, supplied with 0.02% NaN_3). The buffering capacity of the phosphate buffer at pH 8.0 was so low ($pK_a = 6.8$) that its presence hardly affects the progress of the pH-stat experiment. The enzyme dose was varied from 0.2−20 μL (0.20−19.72 nkat)/g DM. A PHM290 pH-Stat Controller (Radiometer Copenhagen, Radiometer Analytical, Lyon, France) equipped with a burette for addition of the base (0.5 M NaOH) and a pH electrode for the pH control (set point pH 8.0) was used in the experiments. The StatSheet version 1.3 X9827 (Radiometer Copenhagen) software package was used for data analysis. Hydrolysis was followed up to 18 h by recording the base consumption.

During the pH-stat experiments, titration reflected cleavage of peptide bonds, since the latter generated a pH drop as a result of liberation of free carboxyl (−COOH) residues. This phenomenon can be used to estimate the DH (% of peptide bonds cleaved) in enzymatic hydrolysis. The equation is $DH = \beta \times N_b \times (1/M_p) \times (1/\alpha) \times (1/h_{tot}) \times 100\%$ (*25*), where β is the consumption of base (mL), N_b is the concentration of base (0.5 M), M_p is the mass of protein (0.234 g protein/g DM, calculated from Kjeldahl measurements), α is the average degree of

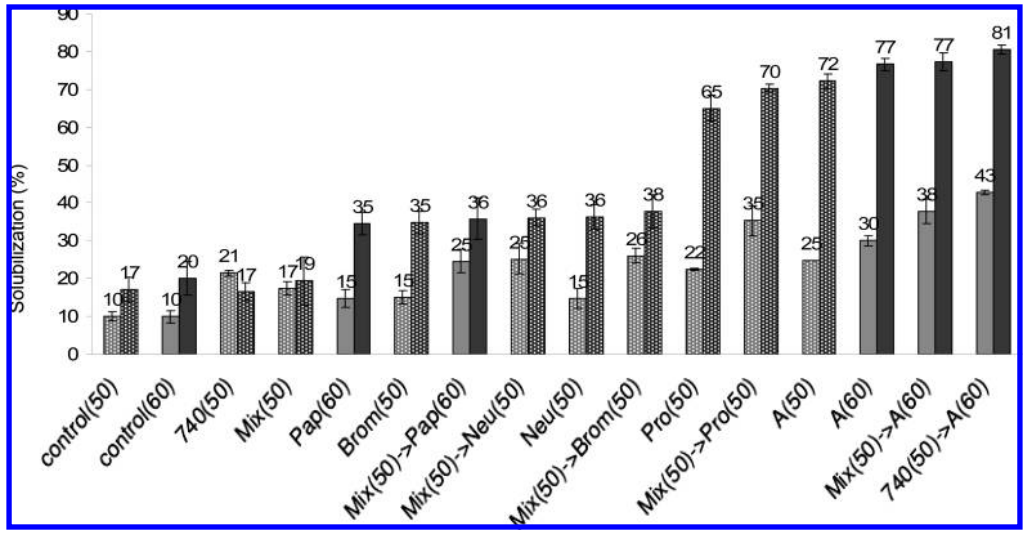

Figure 1. Solubilization of BSG with different enzymes. Hydrolysis was carried out at pH 6.8 for 18 h. "740" and "Mix" indicate that the material was pretreated by incubation for 6 h at 50 °C with Depol740 or a mix of carbohydrases containing equal volumes of Celluclast, Pectinex, Ultraflo, and Viscozyme. The figure shows solubilization of % DM (lighter columns, left in each pair) and % BSG nitrogen solubilized (darker columns, right in each pair). Dotted columns represent reactions performed at 50 °C, while uniform columns represent reactions performed at 60 °C. Total enzyme doses were 20 μL or 20 mg/g DM in each step. Abbreviations: 50 or 60, hydrolysis at 50 or 60 °C, respectively; Pap, Papain (7.71 nkat/g DM); Neu, Neutrase (14.57 nkat/g DM); A, Alcalase (19.72 nkat/g DM); Brom, Bromelain (6.88 nkat/g DM); Prot, Protamex (32.59 nkat/g DM); and control, no enzymes added.

dissociation of the α-NH groups ($1/\alpha = 1.08$ at 60 °C and pH 8) (*25*), and h_{tot} is the total number of peptide bonds per mass unit (9.0 mmol/g, calculated from amino acid analyses).

DM Analysis. The hydrolysates were centrifuged at 10000*g* for 10 min at 4 °C (Beckman Centrifuge Coulter Avanti J-25, Beckman Nerliens Meszansky AS, Oslo, Norway), and the supernatants were collected. The pellet was washed twice in dH_2O (MilliQ, Millipore, Oslo, Norway) and dried for 16 h at 105 °C (ULM 400, Memmert, Schwabach, Germany). The samples were cooled in an exsiccator and weighed. The amount of DM in the pellets was calculated by comparing the weights of the empty tubes with that of the tubes with the pellets after drying. The amount of DM in the supernatants was determined by comparing the amount of DM in the pellets with the DM content of the starting material.

Total Nitrogen. The nitrogen content was quantified using the Kjeldahl method. The BSG samples were ground to ensure uniform particle size (variations in particle size of samples can significantly affect reproducibility in the nitrogen analysis; results not shown). The samples were measured on a Kjeltec Auto 1035 sampler system (Tecator, Nerliens, Oslo, Norway) as described by Adler-Nissen (*25*). The amount of nitrogen in the supernatants was determined by comparing the amount of nitrogen in the pellets with the nitrogen content of the starting material. The protein content was calculated from nitrogen using a conversion factor of 6.25 (*26*).

Size Exclusion Chromatography. Molecular weight distributions of proteinaceous material in the hydrolysates were determined by gel filtration chromatography, using a Superdex Peptide HR 10/300 column (Amersham Pharmacia Biotech; linear separation range from 100−7000 Da) coupled to a Gilson Unipoint high-performance liquid chromatography (HPLC) system (Gilson, Middleton, WI). The mobile phase consisted of a 30:70 (v/v) mixture of acetonitrile and 100 mM Na-acetate buffer with 0.1% trifluoroacetic acid (TFA) at pH 5.5. The flow rate was 0.5 mL min^{-1}. The sample size was 50 μL sterile filtered (0.2 μm, Millex GP, Millipore, Molsheim, France) hydrolysate from reactions with 3.33% DM BSG w/v. Peptides were detected by monitoring the absorbance at 214 nm. The column was calibrated using Bestatin ($M_w = 344.8$, Sigma), vitamin B12 ($M_w = 1355$, Amersham), L-cystein hydrochloride monohydrate ($M_w = 175.6$, Sigma), and AEBSF ($M_w = 239.7$, Sigma) as reference samples. This yielded a linear correlation ($R = 0.99$) between the retention time and the log of molecular mass of peptides in the range of 105−1355 Da.

Amino Acid Composition Analysis. Amino acid analyses were performed at the Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, according to standard EU methods (*27, 28*). Insoluble materials (pellet fractions) of BSG were analyzed after hydrolysis with Depol740 (50 °C, 18 h, and pH 6.8), Alcalase (60 °C, 18 h, and pH 6.8), and pretreatment with Depol740 (50 °C, 6 h, and pH 6.8) and further hydrolysis with Alcalase (60 °C, 18 h, and pH 6.8). In brief, a sample quantity corresponding to 10 mg of nitrogen (N) was oxidized with a performic acid/phenol mixture, hydrolyzed with 6 M HCl for 23 h, pH-adjusted to 2.2, diluted with 0.2 M sodium citrate loading buffer, pH 2.2 (Biochrom Ltd., Cambridge, United Kingdom), and microfiltered (0.45 μm Spartan membrane filter, Schleicher & Schuell, Dassel, Germany) prior to analysis on a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd.) equipped with a Oxidized Feedstuff High Performance PEEK Column (Biochrom Ltd.). Amino acids were detected and quantified photometrically at 440 and 570 nm after postcolumn derivatization with ninhydrin. Data were analyzed against appropriate external standards (Sigma Chemical, St. Louis, MO) using the Chromeleon Chromatography Management Software (Dionex Corp., Sunnyvale, CA).

Statistics. All hydrolysis reactions, as well as all analyses described above, were carried out in triplicate. The presented results are the mean of the replicates, and the standard deviations are shown as error bars in the figures. The significance of differences in the experiments was determined by the two-sample *t* test provided in the Excel software package (Microsoft Excel 2000, Microsoft Corp., Redmond, WA).

RESULTS AND DISCUSSION

Enzymatic Solubilization. The BSG raw material had a protein content of 23.4 ± 1.4% and a low pH (approximately 5). Because of the low pH, hydrolysis had to be carried out with enzymes that were active at low pH, or the pH had to be adjusted. Initially, four different peptidases with low pH and temperature activity optima (cod stomach pepsin, pepsin from hog, cheese rennet, and Actinidin) were used in reactions without pH control, carried out at 30 °C. Enzymes were added in excess to identify maximum solubilization of BSG. These reactions generally gave low yields in terms of solubilized DM (10−13%; control, 9%). Solubilization levels were only a few percent higher than the levels obtained when adding no enzyme at all. Consequently, hydrolysis at low pH was not investigated any further.

Figure 1 shows the degrees of solubilization of DM and nitrogen obtained after running reactions in buffer at pH 6.8

with several commercial peptidase preparations. Hydrolysis was carried out directly on freeze-dried BSG or on material that had been pretreated with carbohydrate-degrading enzymes. Incubation temperatures were adapted to the presumed or known temperature optima of the enzyme preparations. Temperatures below 50 °C were not considered because of microbiological problems that one would expect at ambient temperatures in an industrial setting (i.e., without the use of bacteriostatic agents such as sodium azide). Initially, high enzyme dosages of 20 μL or 20 mg/g DM were used (see **Table 1** for dosage in terms of activity). **Figure 1** shows that peptidases alone could solubilize up to 30% of the DM and that DM solubilization could be increased to almost 43% by pretreatment with carbohydrate-degrading enzymes. These optimum results in terms of DM were obtained using Alcalase alone or Alcalase in combination with Depol740 at 60 °C, respectively. Alcalase in combination with mix pretreatment showed significantly lower DM yield ($P = 0.06$), as compared with Depol740 pretreatment.

The nitrogen data clearly show that the Alcalase treatment was the most efficient treatment for protein solubilization. Alcalase alone solubilized 77% of the protein (which comprises 23.4% of DM in BSG), while solublizing 30% of the DM. Thus, the solubilized DM had a protein content of (0.77 × 23.4)/30 = 60%. Pretreatment with Depol740 increased the degree of protein solubilization to 81%, but because of the solubilization of nonproteinaceous material, the protein content of the solubilized material was only 44% in this case [(0.81 × 23.4)/43]. Generally, the data on solubilization of nitrogen (**Figure 1**) show that pretreatment with carbohydrate-degrading enzymes (Depol740 or mix) had only minor effects on protein solubilization. Assuming that the carbohydrase preparations did degrade some material (as shown by the increase in solubilized DM), the minimal effect on protein release indicates that the protein fraction is not strongly associated or bound to the carbohydrate fraction of BSG. Only pretreatment with Depol740 did significantly ($P = 0.0001$) increase the solubilization of nitrogen by Alcalase, albeit to a minor extent. The Depol740 preparation contains ferulic acid esterase activities (data sheet, Biocatalysts) (*29*) and some peptidase activity (**Table 1**) that might be beneficial for protein solubilization. The mix preparation was made from several commercial enzyme mixtures (Celluclast, Pectinex, Ultraflo, and Viscozyme) and contained a range of carbohydrate-degrading activities aimed at degrading the hemicellulose and cellulose of BSG. However, both Depol740 and mix were only able to solubilize a limited part of the BSG lignin−cellulose−hemicellulose complex. This is consistent with the other findings, which underline the need for a physical and/or chemical pretreatment of lignocellulose before efficient enzymatic hydrolysis can take place (*30*). The somewhat better performance of Depol740 as compared to mix indicates that ferulic acid esterase activities are important for degrading BSG (*31*).

The peptidase dosages used were similar in terms of volume or mass, not in terms of the amount of activity added (as measured toward casein at 50 °C; **Table 1**). Nevertheless, comparison of the results obtained with peptidase treatments clearly shows that Alcalase is the best enzyme, also when enzyme dosages are considered in terms of activity. Protamex is considerably more active toward casein than Alcalase (**Table 1**) but is less efficient than Alcalase when used on BSG. Protamex contains a mixture of Alcalase and Neutrase, and the data obtained for Neutrase clearly show that this enzyme has very low activity on BSG (only 36% N solubilization, as

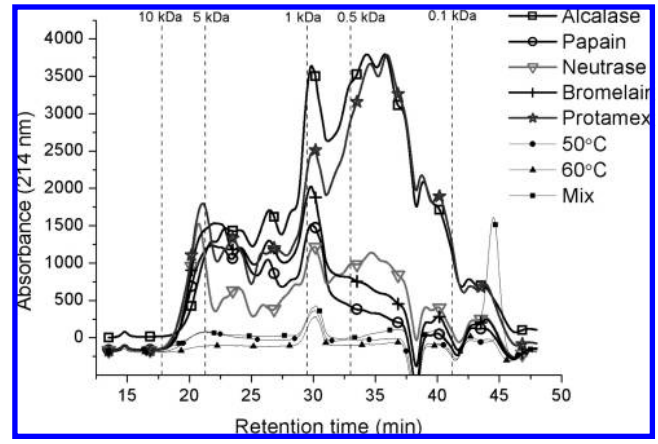

Figure 2. Molecular weight distribution of peptide material in BSG hydrolysates. Peptides were separated on a Superdex Peptide HR 10/300 column. The sample size was 50 μL of hydrolysate from reactions with 3.33% w/v, and absorbance was measured at 214 nm. Hydrolysis conditions: pH 6.8, 18 h, and 50 °C (60 °C for Alcalase and Papain). The picture shows three control reactions: no enzyme, 50 °C; no enzyme, 60 °C; and treatment with mix only at 50 °C. See the legend to **Figure 1** for further details.

opposed to 72% for Alcalase, despite the fact that the amount of nkats used was only slightly lower for Neutrase than for Alcalase; **Table 1** and **Figure 1**). The plant enzymes Bromelain and Papain showed very low degrees of N solubilization (about 35% as compared to 72−77% for Alcalase and 17−20% with no enzymes added). In terms of nkat, the dosages of these enzymes were up to three times lower than the Alcalase dosage. However, considering the highly nonlinear dose−response relationships for peptidase treatments in this type of systems (e.g., see below), dosage effects alone cannot explain the low degree of solubilization obtained with Bromelain and Papain.

Molecular Weight Distributions. The supernatants obtained after hydrolyses were analyzed by gel filtration HPLC. The results (**Figure 2**) clearly show that the highest degree of protein solubilization was obtained by the Alcalase and Protamex treatments and that hydrolysis with these enzymes produced relatively more of the smallest peptides. Analysis of samples obtained after pretreatment with mix or Depol740 followed by peptidase treatment yielded chromatograms that were essentially identical to those shown in **Figure 2** (results not shown).

Dose−Response Curves and DH. Dose−response studies for Alcalase were conducted at two different pH values. Reactions were conducted at 60 °C, and the incubation time was 4 h. The results (**Figure 3**) show clear dose−response effects, which are most prominent for reactions carried out at the optimum of the two tested pH values, pH 8.0. It should be noted that the protein solubilization yield was only modestly reduced when decreasing Alcalase loads. For example, using a dose as low as 1.2 μL (1.18 nkat)/g DM still yielded 64% protein solubilization, and the resulting hydrolysate had a protein content as high as 65% [(0.64 × 23.4)/23].

Alcalase hydrolysis of BSG was further investigated using the pH-stat method to measure the DH (*25*). **Figure 4** shows the hydrolysis progress curves for the first 4 h in hydrolysis reactions with different concentrations of Alcalase at 60 °C, pH 8.0, as well as the end point DH obtained after 18 h. The curves for 10 (9.86 nkat/g DM) and 20 μL (19.72 nkat/g DM) of enzyme were identical, indicating enzyme saturation. After 18 h, there were no significant differences in solubilization between the reactions with 5 (4.93 nkat/g DM), 10 (9.86 nkat/g DM), and 20 μL (19.72 nkat/g DM) Alcalase. The maximum

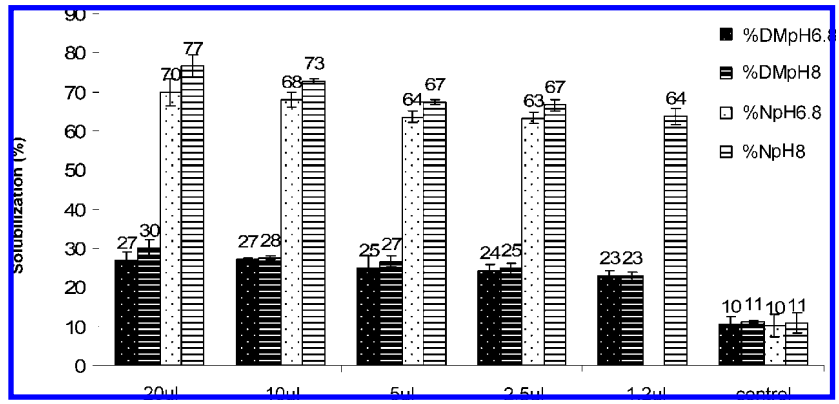

Figure 3. Solubilized % DM (darker columns) and % nitrogen (lighter columns) after 4 h of hydrolysis with different doses of Alcalase. Running conditions: pH 6.8 (dots) and 8 (stripes), at 60 °C, 3.33% DM w/v BSG. Control, no enzyme added. The enzyme dosages vary from 1.2 (1.18 nkat/g DM) to 20 μL (19.72 nkat/g DM). The percentage of solubilized nitrogen in the pH 6.8 reaction with 1.2 μL of Alcalase was not determined.

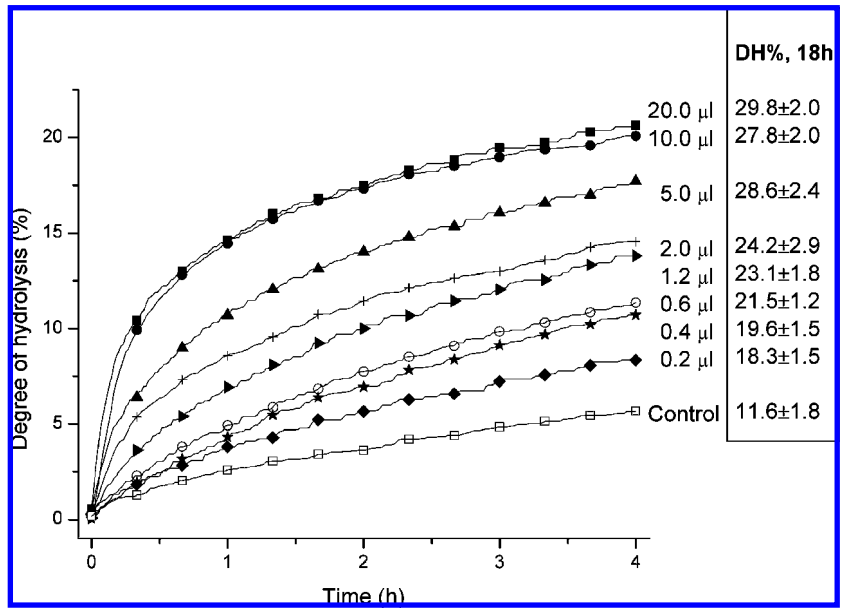

Figure 4. pH-stat progress curves for hydrolysis of BSG with different amounts of Alcalase at pH 8. Enzyme doses are expressed as μL/g DM BSG (expressed in nkat/g DM; dosages vary from 0.20 to 19.72). The DH (%) is expressed as the percentage of peptide bonds cleaved (*25*). The panel to the right shows DH % obtained after 18 h.

DH was about 29%, suggesting an average peptide length of 3−4 amino acids (based on the average mass of an amino acid residue, 119.4 Da, this corresponds to an average peptide mass in the order of 400 Da) (*32*). This fits quite well with the gel filtration results that show major peaks in the molecular mass range of 100−1000 Da (**Figure 2**).

Figure 4 also shows that there was some hydrolysis of peptide bonds in the control reaction without added enzymes. This could be due to the presence of active endogenous enzymes in BSG (*2*), which seem to be present according to the proteolytic activity measurements with the casein assay (**Table 1**). Additionally, some proteinaceous material may dissolve in an enzyme-independent manner during the hydrolysis reactions.

Amino Acid Composition Analysis. Figure 5 shows the amino acid compositions (g/100 g detected amino acids) of BSG and the insoluble residues (pellet fractions) after hydrolysis of BSG with different enzymes. The amino acid composition of the hydrolysis residue after treatment with Depol740 was essentially identical to BSG. Prolamins are globular storage proteins found mainly in cereals. The barley prolamin is called hordein and comprises 35−55% of the total barley grain proteins (*33*). Hordein contains high levels of the amino acids proline and glutamine. These amino acids are also highly represented in BSG, constituting about 25−30% of the amino acids. Alcalase treatment solubilizes proline and glutamine more efficiently than the other amino acids (relative decrease in these amino acids in **Figure 5**). This naturally leads to an increase in the relative amount of several other amino acids after enzyme hydrolysis.

Concluding Remarks. Enzymatic treatments of biomass such as BSG may be complicated by the growth of microorganisms during the incubation. However, under the optimal conditions coming out of the dose−response studies depicted in **Figures 3** and **4** (T = 60 °C; pH 8.0, only 4 h incubation times), reactions carried out in the absence of sodium azide did not result in a detectable increase in the viable cell number (log cfu/g) of *Bacillus* (Treimo, J. Unpublished observations; a further analysis of the microbiological properties of European BSG's will be published elsewhere). Obviously, the occurrence of microbiological problems depends on the starting material, but there is no doubt that the use of a temperature as high as 60 °C combined with a rather high pH and short incubation times will generally reduce problems associated with microbial growth.

To some extent, different methods (physical, chemical, and enzymatic) have been studied to extract proteins from BSG or from barley grains during mashing (*2, 17–21*). However, the

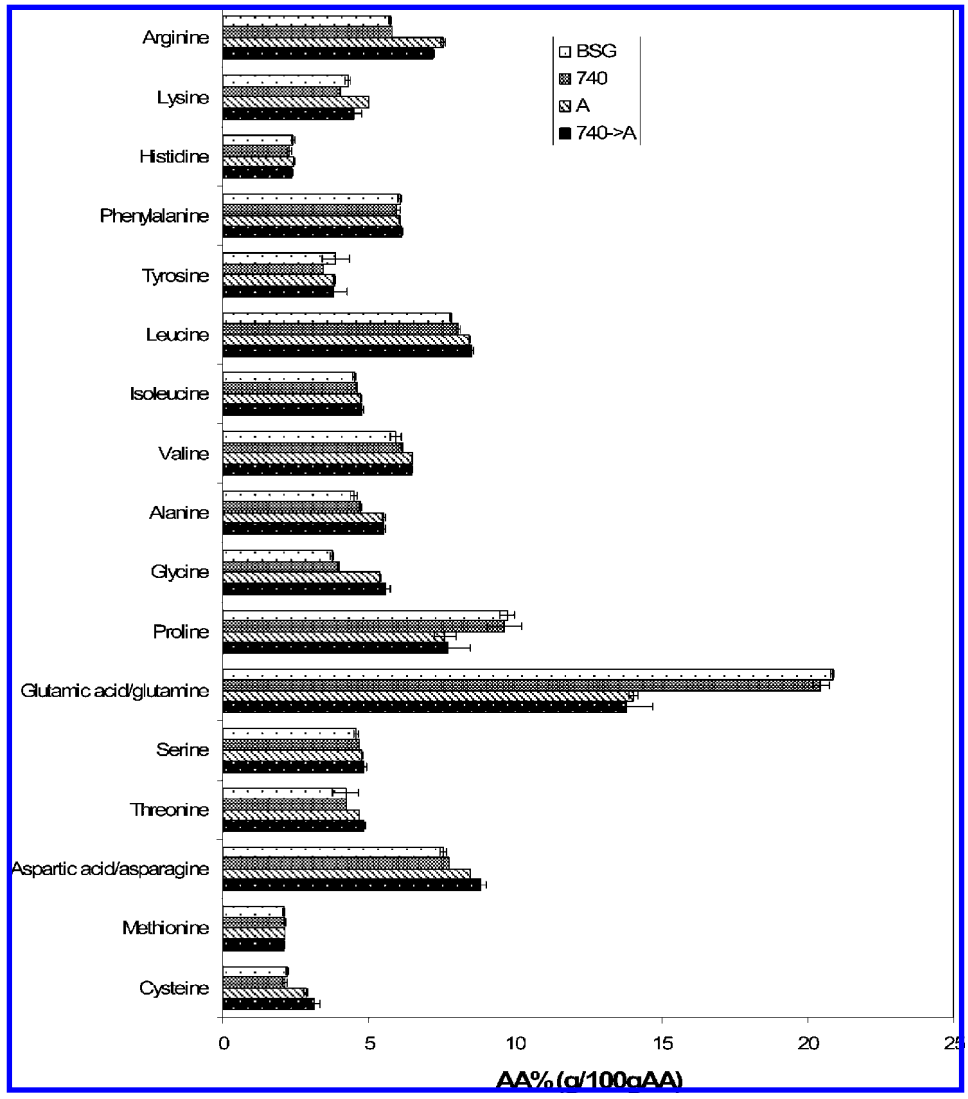

Figure 5. Amino acid composition analyses. The figure depicts results for BSG and insoluble material (pellet) obtained after 18 h of hydrolysis with different enzymes. Relative amounts of amino acids are shown (g per 100 g detected amino acids). Abbreviations: BSG, raw material; A, BSG residue after hydrolysis with Alcalase at 60 °C; 740, BSG residue after hydrolysis with Depol740 at 50 °C; and 740→A, BSG residue after pretreatment with Depol740 at 50 °C and subsequent hydrolysis with Alcalase at 60 °C.

use of peptidases for specific solubilization of BSG proteins has hardly been investigated. Celus et al. (*23*) have recently explored enzymatic hydrolysis of BSG proteins by peptidases, after alkaline extraction and subsequent acid precipitation, and characterized the technofunctional properties. In their study, already extracted proteins were hydrolyzed further by peptidases. In a study from 1978, Prentice and Refsguard (*22*) tested a peptidase from *Streptomyces griseus* provided by Sigma (Type VI, Sigma Chemical Co., St. Louis, MO). Using long incubation times (days), they were able to solubilize up to 87% of the nitrogen in BSG. The present results show that a large fraction (up to 80%) of the proteinaceous material in BSG can be solubilized with commercial industrial peptidase preparations, in particular Alcalase, using a simple enzymatic hydrolysis step. Despite the complexity of the starting material, the degree of nitrogen solubilization was hardly dependent on pretreatments with carbohydrate-degrading enzymes. It is doubtful whether the small increase in protein solubilization caused by Depol740 treatment is worth the extra processing step and enzyme costs, and the relative lower protein content of this hydrolysate. Thus, protein-rich hydrolysates (more than 60% protein) may be produced efficiently using only peptidase treatment.

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