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Enzymatic Solubilization of Brewers' Spent Grain by Combined Action of Carbohydrases and Peptidases

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Brewers' spent grain (BSG), a high-volume coproduct from the brewing industry, primarily contains proteins, barley cell wall carbohydrates, and lignin. To create new possibilities for the exploitation of this large biomass stream, the solubilization of BSG by the combined action of carbohydrases (Depol 740 and Econase) and peptidase (Alcalase and Promod 439) was explored. Hydrolysis protocols were optimized with respect to temperature (influencing both microbial contamination and rate of enzymatic hydrolysis), pH, enzyme dose, order of enzyme addition, and processing time. On the basis of this approach, one- and two-step protocols are proposed taking 4−8 h and yielding combined or separate fractions of hydrolyzed oligosaccharides and liberated hydrolyzed protein. Optimized procedures resulted in the solubilization of >80% of the proteinaceous material, up to 39% of the total carbohydrates, and up to 42% of total dry matter in BSG. Of the original xylan present in BSG, 36% could be solubilized. Sequential and simultaneous treatments with the two enzyme types gave similar results. In sequential processes, the order of the carbohydrase and peptidase treatments had only minor effects on the outcome. Depol 740 released more pentoses than Econase and gave slightly higher overall dry matter solubilization yields.

KEYWORDS: Brewers' spent grain; enzymatic solubilization; hydrolysis; carbohydrase; peptidase; Depol 740; Econase; Alcalase

INTRODUCTION

Barley (*Hordeum vulgare* L.) is among the major cereal crops in the world. The primary use of barley is in the production of animal feed, whereas smaller quantities are used for food and beer production (*1*−*3*). An early step in the production of beer involves the germination of barley to synthesize and activate hydrolytic enzymes, a process called malting. The enzymes hydrolyze primarily the barley starch (but also other components) during the mashing process, and the resulting sugar-rich liquor (wort) is fermented to beer. This process yields an abundant coproduct called brewers' spent grain (BSG), which consists of the barley malt residue after separation of the wort. About 200 g of wet BSG is produced per liter of beer [reviewed by Mussatto et al. (*2*)]. BSG is a lignocellulosic material that consists of hemicellulose (∼28%, mainly arabinoxylans), cellulose (∼17%), lignin (∼28%), and protein (∼20%) (*2*, *3*), as well as a few percent of residual starch and starch-derived products (glucose, maltosaccharides).

Traditionally, BSG has been utilized directly as animal fodder (*2*). Because large amounts of BSG are produced continuously worldwide, other exploitation routes are of current interest (*2*). The recovery of carbohydrates and/or proteins from BSG is potentially attractive because this has the potential create new, higher value applications. Several promising compounds have been extracted from BSG (*2*, *4*), such as arabinoxylan or corresponding oligosaccharides (*5*, *6*), the phenolic compounds ferulic and *p*-coumaric acid (*7*, *8*), and lignin (*9*). Novel potential products may include protein-enriched material, prebiotics (*10*, *11*), and hydrolysates to be used, for example, as growth media for (probiotic) bacteria (*12*−*17*) or for the production of xylitol (*18*).

Use of enzymes in the barley grain mashing process step or for solubilization of BSG has previously been investigated to some extent, with the main focus on the use of carbohydrases (*6*, *8*, *19*−*31*). We (*17*) and others (*27*) have recently studied how peptidases may be used to hydrolyze and release most of the protein in BSG. Despite these previous studies, there still is limited knowledge concerning the potential of modern commercial enzyme technology to transform BSG within an industrially acceptable processing time frame. For example, integrated processes, combining several types of enzymatic

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treatments and activities to produce a spectrum of products, have been little investigated.

We have studied enzymatic solubilization of BSG by combining commercial peptidases and carbohydrases under conditions that limit processing time and hence minimize the problem of microbial contamination. Issues addressed include process temperature (affecting both microbial contamination and enzyme speed) and time, pH, choice and order of enzymes, sequential or simultaneous treatments with the different enzymes, and enzyme dosages. The outcome of processes consisting of treatment with the carbohydrases Econase or Depol 740 followed by treatment with Alcalase has been evaluated in more detail.

MATERIALS AND METHODS

Enzymes. The peptidases Alcalase 2.4 L (Novozymes, Bagsvaerd, Denmark) and Promod 439 (Biocatalysts Ltd., Cardiff, U.K.) and the carbohydrate-degrading enzyme preparations Depol 740 L, Depol 686 L, Depol 670 L (all Biocatalysts), and Econase CE (AB Enzymes, Darmstadt, Germany; new name is Rohament CL) were kindly provided by the manufacturers.

Hydrolysis. Brewers' spent grain (BSG) was provided by Ringnes Brewery (Hagan, Norway). Hydrolysis reactions of freeze-dried and milled BSG (mesh size = 1 mm) were in general carried out at 50 or 60 °C for 4 h (one-step processes) or 2 × 4 h (two-step processes) with agitation at 150 rpm in an incubation shaker (Minitron, Infors AG CH-4103, Bottmingen, Switzerland). In the two-step processes the supernatant obtained after the first 4 h incubation was decanted and new buffer and the next enzyme were added, followed by another 4 h of incubation. Reactions were run in buffer (100 mM Na_2HPO_4/NaH_2PO_4) in the pH range from 5.5 to 9. In some experiments, BSG was suspended directly in Milli-Q water resulting in a pH of approximately 5. The initial dry matter concentration was either 3.33 or 6.67% w/v, and reactions were performed in 50 mL tubes. The dry matter concentration in the second step of the two-step reactions was reduced because no corrections were made for the solubilization of dry matter in the first step. In standard experiments, 20 μL of enzyme/g of BSG dry matter (DM) was added. In most cases, buffers were supplied with NaN_3 (0.02% w/v) to avoid variation that could be caused by microbial growth (see below for details).

Microbiological Analyses. Bacterial growth was monitored by plating on Plate Count Agar (casein peptone dextrose yeast agar) (Merck Microbiology, Darmstadt, Germany) at 30 °C under aerobic conditions. *Bacillus* was verified by further growth and enumeration on MYP agar (mannitol−egg yolk−polymyxine−agar) (*32*) (Merck) at 30 °C and by microscopic observations of spores.

Dry Matter and Total Nitrogen Analysis. Reaction mixtures were centrifuged at 10000*g* for 10 min at 4 °C (Beckman Centrifuge Coulter Avanti J-25, Beckman Coulter, Inc., Fullerton, CA), and the supernatants were collected. The pellet fractions were washed twice in dH_2O (Milli-Q, Millipore, Bedford, MA) and subsequently dried (105 °C for at least 16 h, ULM 400, Memmert, Schwabach, Germany). The amounts of DM in the hydrolysates were determined by comparing the amount of DM in the pellets with the DM content of the starting material. The nitrogen contents in the pellet samples were analyzed by Kjeldahl (Kjeltec Auto 1035 sampler system, Tecator, Nerliens, Oslo, Norway) and converted to protein by using a factor of 6.25 (*33, 34*).

Enzyme Activity Measurements. Peptidase activities in the enzyme preparations were determined using casein as a substrate, according to the method of Fujii et al. (*35*) with modifications (*17*). Reactions were conducted at 50 °C, in 20 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, and 0.02% NaN_3, using 0.8% (w/v) casein (Hammarstein, Merck) as substrate. Reactions were stopped by the addition of ice-cold trichloroacetic acid solution [100 mM TCA, 1.9% (v/v) acetic acid, 220 mM sodium acetate]. The amount of acid-soluble peptides was measured spectrophotometrically at 280 nm after removal of the precipitated protein by centrifugation at 20000*g* for 15 min. Activities were expressed as nanokatals per milliliter of enzyme preparation (*17*). Activities were calculated on the basis of a standard solution of tyrosine (0.552 M) (Sigma T-3754).

Table 1. Chemical Composition of BSG from Ringnes

analysis of BSG	mg/g of dry wt	SD
total carbohydrates	459	nd[a]
total starch[b]	78	±2
mixed-linkage β-glucan	4	±2
bound phenolics	7	±1
Klason lignin	126	±1
acid-soluble lignin	90	nd
total protein	234	±14

[a] See **Table 2** for further details; nd, not determined. [b] Total starch includes glucose and maltosaccharides.

Table 2. Anhydrous Sugar Composition of BSG from Ringnes

component	mg/g of dry wt
arabinose	87
galactose	16
glucose	209
xylose	137
mannose	10
total carbohydrates	459

Xylanase activity was determined using 1% birchwood glucuronoxylan (Roth 7500) as substrate in 50 mM sodium citrate buffer at pH 5.0. Reactions were conducted at 50 °C, and dinitrosalicylic acid (DNS, 2-hydroxy-3,5-dinitrosalicylic acid) was used as stopping agent. Spectrophotometric analysis of color was performed at 540 nm.

Total cellulolytic activity at pH 5.0 was measured on filter paper (and expressed as FPU/mL). Both activities were analyzed using the methods described previously (*36*).

Feruloyl esterase (FAE) activity was determined essentially according to the method of Figueroa-Espinoza et al. (*37*) using 4 mM ethyl ferulate (Sigma-Aldrich) in 0.025 M sodium acetate buffer, pH 5.0, as substrate. The activity assay was carried out by incubating 0.05 mL of enzyme sample with 0.45 mL of substrate solution at 50 °C for 10 min, after which the reaction was terminated by the addition of 0.25 mL of 0.1 M glycine−NaOH buffer (pH 10). Released ferulic acid was analyzed spectrophotometrically at 325 nm, using commercial ferulic acid (Fluka) as standard.

Peptide Analysis by Size Exclusion Chromatography. Molecular weight distributions of proteinaceous material in the hydrolysates were determined by gel filtration chromatography as previously described (*17*), using three Superdex Peptide HR 10/300 columns (Amersham Pharmacia Biotech; linear separation range from 100 to 7000 Da) coupled in series to increase resolution, connected to a Dionex Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA). The mobile phase consisted of a 30:70 (v/v) mixture of acetonitrile and 100 mM sodium acetate buffer, with 0.1% trifluoroacetic acid (TFA) at pH 5.5. Peptides were detected by monitoring absorbance at 214 nm.

Starch Analysis. Starch (including glucose and maltosaccharides) was analyzed as total starch, using a standard assay procedure, (Megazyme kit K-TSTA) in which starch and maltosaccharides are quantitatively converted to glucose by digestion with a thermostable amylase and an amyloglucosidase (*38*).

In summary, milled sample (90 mg) was dispersed in 0.2 mL of ethanol (80%) and incubated in 3 mL of α-amylase solution (300 units in 50 mM MOPS buffer, pH 7.0) at 100 °C for 6 min and then equilibrated to 50 °C followed by the addition of 4 mL of sodium acetate buffer (200 mM, pH 4.5) and 0.1 mL of amyloglucosidase (20 units). Samples were incubated at 50 °C for 30 min. The total sample volume was then accurately adjusted to 10 mL (or 100 mL for some samples) using deionized waste and, after centrifugation, aliquots of supernatant (0.1 mL) were analyzed for glucose assay, using glucose oxidase reagent (GOPOD) (20 min of incubation at 50 °C and absorbance measured at 510 nm).

Table 3. Peptidase Activity (Casein)[a]

enzyme[b]	ΔA_{280} (min/μL or mg)	nkat/mL or g	activity (nkat) per g of DM of BSG[c]
Alcalase	0.693	986.8	19.7
Promod 439	0.695	988.0	19.8
Depol 670	0.0068	9.73	0.20
Depol 740	0.0066	9.41	0.19
Depol 686	0.0053	7.76	0.16
Econase	0.0021	2.75	0.06
BSG Ringnes	0.0003	0.39	0.39

[a] Activity was measured at pH 7.5 and 50 °C. [b] All enzymes were formulated as liquids. BSG was measured in suspension. [c] Activities (in nkat/g of DM) of the enzyme loadings (20 μL/g of BSG dry matter).

Table 4. Activity Profile of Carbohydrate-Degrading Enzymes[a]

	activities[b]		
enzyme	CE (FPU/mL)	XYL(nkat/mL)	FAE(nkat/mL)
Econase	77	49000	0
Depol 740	2.5	12300	27

[a] Activities were measured at pH 5 and 50 °C. Data are from Forssell et al. (*6*). [b] CE, cellulase, overall cellulolytic activity on filter paper, expressed in FPU (filter paper unit); XYL, xylanase; FAE, feruloyl esterase.

Mixed-Linkage β-Glucan Analysis. β-Glucan was assayed using a Mixed Linkage kit (K-BGLU) (Megazyme International Ireland Ltd.), modified to use 100 mg of sample weight. This confirmed that residual mixed-linkage β-glucan was a minor component of the BSG (<0.5%).

Monosaccharide and Lignin Analysis of BSG. The BSG substrate was hydrolyzed with 0.4 M H_2SO_4 in an autoclave (AH 23-17) at 0.14 MPa. Subsequently, soluble sugars were quantified in an ion chromatograph equipped with an IC-PAD detector (*39*). Acid-soluble lignin was quantified by measuring UV absorbance at 205 nm. These analyses were done at STFI-Packforsk AB, Stockholm, Sweden.

Klason lignin was determined using 50 mg of sample dispersed in 0.75 mL of 12 M (72%) sulfuric acid and incubated at room temperature (3 h) prior to dilution to 1 M acid concentration. After a further incubation (100 °C for 2.5 h in sealed tubes), the insoluble residue was recovered by filtration (no. 3 glass sinter) under gentle suction and washed free of acid. Klason lignin was determined as the weight gain in each glass sinter after drying to constant weight at 50 °C.

Monosaccharide Composition of Enzymatic Hydrolysates. Solubilized oligosaccharides were hydrolyzed to monosaccharides by treatment with 1 M H_2SO_4 (final concentration) for 2 h at 100 °C with continuous agitation. To 1 g of this hydrolysate were added 2.5 mL of deionized water, 0.2 mL of 0.5 M H_2SO_4, and 8 mL of acetonitrile (LiChrosolv, Merck). After 30 min of mixing, centrifugation (1500*g*,, 15 min), and filtration (0.2 μm MFS-13 PTFE filter, Advance MFS), monosaccharides in 25 μL of the sample were analyzed by use of a HPLC system (Perkin-Elmer) equipped with a refractive index detector (RI) (series 200 refractive index detector, Perkin-Elmer). Separation was achieved using a cation-H-microguard precolumn (30 × 4.6 mm i.d.) and an Aminex HPX-87H cation-exchange column (300 × 7.8 mm i.d.) (Bio-Rad Laboratories, Hercules, CA). The mobile phase was 5 mM H_2SO_4 at a flow of 0.4 mL min^{-1}. The column temperature was 30 °C. Carbohydrates were identified according to their retention times, which were compared to external standards (arabinose, glucose, xylose, and maltose). Due to coelution it was not possible to distinguish galactose or mannose from xylose. However, the content of galactose (2.0%) and mannose (1.2%) in BSG is low (*3*). Detector responses were recorded and analyzed using the Turbochrom Chromatography workstation v4.1 (Perkin-Elmer). Results were corrected for decomposition of the monosaccharides during acidic hydrolysis, according to the method of Englyst et al. (*40*).

Cell Wall Bound Alkali Extractable Phenolic Acids. The phenolic acids were determined following saponification with 4 M NaOH (5 mg/mL for 18 h at room temperature in the dark), according to the method of Waldron et al. (*41*), using *trans*-cinnamic acid as internal standard.

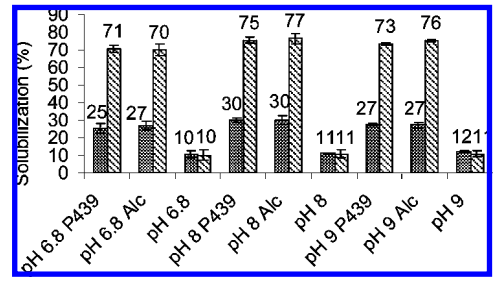

Figure 1. Solubilization of dry matter (% DM, gray bars) and nitrogen (% N, slashed bars) by Alcalase (Alc) and Promod 439 (P439). Conditions: pH 6.8, 8, or 9; 60 °C; 4 h; 3.33% DM; and 20 μL of enzyme/g of DM. Columns lacking the "P439" or "Alc" labels represent control reactions without added enzyme.

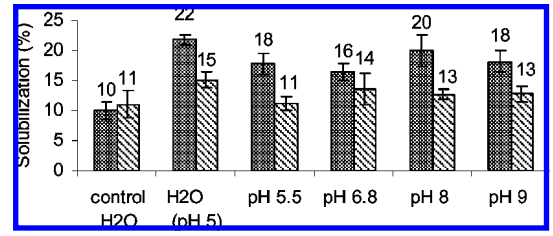

Figure 2. Solubilization of dry matter (% DM, gray bars) and nitrogen (% N, slashed bars) by Depol 740. Conditions: H_2O (i.e., pH ~5.0) and buffer at pH 5.5, 6.8, 8, or 9; 60 °C; 4 h; 3.33% DM; and 20 μL of enzyme/g of DM; control, no enzyme added.

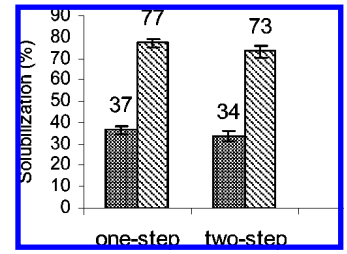

Figure 3. Comparison of one- and two-step reactions with Depol 740 and Alcalase. The figure shows solubilization of dry matter (% DM, gray bars) and nitrogen (% N, slashed bars). The samples were incubated for 4 h with both enzyme preparations present (one-step) or for two subsequent periods of 4 h (two-step), first with Depol 740 and then with Alcalase. Conditions: pH 8; 60 °C; 6.67% DM; and 20 μL of enzyme/g of DM.

High-Performance Anion-Exchange Chromatography (HPAEC) of Oligosaccharides. For qualitative analysis of oligosaccharides, HPAEC was performed on a Dionex Bio LC system (Dionex) with a (4 × 250 mm) PA1 (Dionex) column run at 1.0 mL/min, using the Chromeleon 6.7 software package for process control. Elution was performed using a gradient from 0 to 1 M sodium acetate in 100 mM NaOH, with the following program: 0−15 min, 15 mM NaOH; 15−20 min, 15→100 mM NaOH; 20−69 min, 0→0.4 M NaOAc in 100 mM NaOH; 69−70 min, 0.4→1 M NaOAc in 100 mM NaOH; 70−75 min, 1 M NaOAc in 100 mM NaOH; 75.1−76 min, 1→0 M NaOAc in 100 mM NaOH; 76.1−90 min, 100→15 mM NaOH. The external standards were arabinose, xylose, and glucose (all supplied by Sigma).

Matrix-Assisted Laser Desorption Ionization−Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) Analyses of Oligosaccharides. MALDI-TOF-MS experiments were performed using a procedure described by Verhoef and co-workers (*42*). Hydrolysate samples (approximately 20 μL) were desalted by adding a small spatula of Dowex 50 particles (H^+ form), followed by mixing and centrifugation (1300*g*, 5 min). Two microliters of a 9 mg/mL solution of 2,5-dihydroxybenzoic acid (DHB; Bruker Daltonics, Bremen, Germany) in 30% acetonitrile was applied to a MTP 384 target plate (Bruker Daltonics). One microliter of desalted sample was then mixed into the

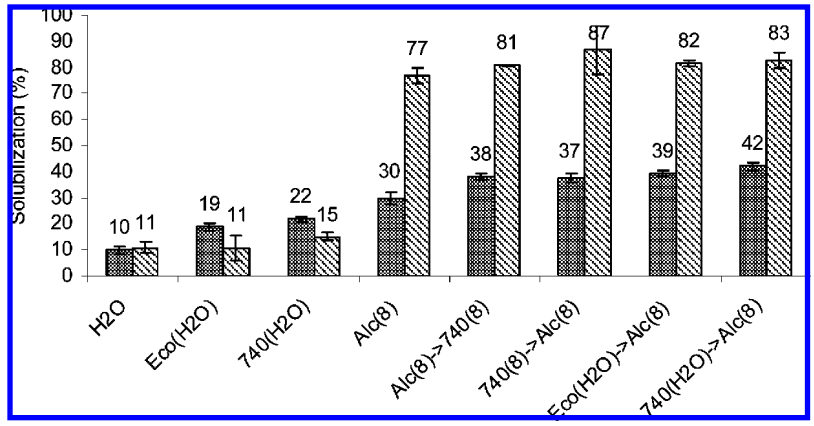

Figure 4. Selected hydrolysis reactions. The figure shows solubilization of dry matter (% DM, gray bars) and nitrogen (% N, slashed bars) after various treatments with enzymes. The incubation time was 4 h or 2 × 4 h for reactions with two enzyme preparations ("->"). Conditions: 60 °C; 3.33% DM; and 20 μL of enzyme/g of DM. Abbreviations; Alc, Alcalase; Eco, Econase; 740, Depol 740; 8/H_2O, pH (H_2O means no buffer added and a pH of ~5). The difference in solubilization yield between the two-step reactions depicted in **Figure 3** and the corresponding two-step reaction shown in this figure [740(8)->Alc(8)] is due to the difference in dry matter concentration.

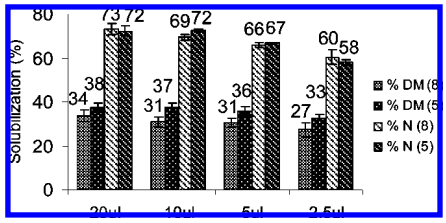

Figure 5. Dose−response effects in two-step hydrolysis. The figure shows solubilization of dry matter (% DM) and nitrogen (% N) in two-step reactions (2 × 4 h, 60 °C, 6.67% DM) with different enzyme doses of Depol 740 [first step; performed at either pH 8 or ~5 (H_2O)] and Alcalase (second step; pH 8). Note that solubilization yields at 20 μL of enzyme dose/g of DM are slightly lower than those shown in **Figure 4**; this is due to the different dry matter concentrations.

DHB droplet, and the droplet was dried under a stream of air. The samples were analyzed with an Ultraflex instrument (Bruker Daltonics) with a Nitrogen 337 nm laser beam operated in positive acquisition mode. The data were collected from averaging 250 laser shots, with the lowest laser energy necessary to obtain sufficient spectrum intensity.

Statistical Analysis. All hydrolysis reactions, as well as all analytical methods described above, were carried out in triplicates. The presented results are the mean of determinations made in triplicates, and the standard deviations are shown as error bars in the figures.

RESULTS AND DISCUSSION

Substrate Properties. Data on the composition of the BSG used in this study are provided in **Tables 1** and **2**. The protein content of the BSG was 23.4 ± 1.4% (*17*), the total carbohydrate content was 45.9%, and the starch content was 7.8 ± 0.2%. Klason lignin accounted for 12.6% and acid-soluble lignin, 9%. The relatively high value obtained for acid-soluble lignin may be due to protein degradation products formed during the acid hydrolysis. Cell wall bound phenolics were a minor component (<1%) of the BSG, and ferulic acid was the major phenolic acid present.

Selection of Temperature for Enzymatic Hydrolysis. Initially, experiments were carried out at 50 and 60 °C. When incubation was performed for 4 h, growth of *Bacillus* sp. (on MYP selective medium) was limited to approximately 3 log cfu/mL, even at 50 °C. At 60 °C, the temperature used in the processes described below, growth was <3 log cfu/mL even after 24 h. To minimize variation possibly caused by microbial contamination, all further hydrolysis experiments were conducted in media containing NaN_3.

The effect of temperature (50 or 60 °C) on the BSG solubilization efficiency of Alcalase and various Depol preparations (740, 686, and 670) was tested in 4 h reactions carried out at pH 6.8 (results not shown). Alcalase showed a consistently higher solubilization yield at 60 °C (approximately 6% in terms of DM yield and 14% in terms of N yield), in line with what has been observed earlier (*17*). The carbohydrases present in the Depol preparations were slightly more efficient in hydrolyzing BSG at 50 °C, but still worked well at 60 °C (yield differences were approximately 2% in terms of DM and 3% in terms of N release). In all further experiments, the incubation temperature was set to 60 °C so that enzyme treatment times could be minimized, and the increased temperature would help prevent possible problems with microbial growth.

Enzymes Used for Hydrolysis of BSG. A comparative study on the efficiency of commercial peptidases for solubilization of proteins in BSG had previously shown that Alcalase was clearly the most effective (*17*). Thus, Alcalase and a similar product containing *Bacillus* peptidases called Promod 439 were used in the present study. According to the manufacturer, optimum conditions for Alcalase are temperatures around 60 °C and pH values between 6.5 and 8.5 (product sheet, Novozymes). For Promod 439 temperatures between 45 and 60 °C and pH values between 6.5 and 9.0 are recommended (product sheet, Biocatalysts). The two peptidase preparations showed equal peptidase activities when tested on a casein substrate (**Table 3**). For hydrolysis of nonproteinaceous compounds several enzyme preparations primarily containing glycoside hydrolases were tested (Depol 670, Depol 686, Depol 740, and Econase). Initial testing consistently showed that Depol 670 and Depol 686 solubilized less material from the BSG than Depol 740 (results not shown). Therefore, only Depol 740 of the Depol preparations was used for further studies. According to the manufacturers, optimal conditions for the enzyme preparations are Depol 740, $T = 40-65$ °C, pH 4−6; Econase, $T = 55-65$ °C, pH 4−5.5.

DM solubilization yields obtained with Econase were consistently 2−3% less than yields obtained with Depol 740. Econase was nevertheless included in this study because enzyme activity measurements showed that Econase and Depol 740 have different carbohydrase profiles (**Table 4**). Econase is derived from a classically modified *Trichoderma reesei* strain, whereas

Table 5. Monosaccharide Composition of BSG Hydrolysates and Solubilization Yields[a]

	sugar content (mg/g of DM) (% of total in BSG)									
	Glc	Xyl[b]	Ara	Ara + Xyl[b]	total	Glc−Xyl−Ara[b] (ratios; sum is 100)	Ara/Xyl[b]	hexose/pentose[b]	soluble DM (%)	protein (mg/g of DM)
BSG	209 (100)	163 (100)	87 (100)	250 (100)	459 (100)	46−35−19	0.53	0.84		234
control (H_2O)[c]	59 (28)	0.6 (0.4)	0	0.6 (0.2)	60 (13)	99−1 −0		96	10	26
Econase (H_2O)	78 (37)	33 (20)	21 (24)	54 (22)	132 (29)	59−25−16	0.63	1.46	19	26
Alcalase (pH 8)	21 (10)	9 (5.8)	6 (6.8)	15 (6.0)	36 (7.9)	58−26−16	0.63	1.34	20[d]	166[d]
total Eco-Alc	99 (47)	42 (26)	27 (31)	69 (28)	168 (37)	59−25−16	0.63	1.43	39	192
Depol740 (H_2O)	68 (33)	42 (26)	29 (33)	71 (28)	139 (30)	49−30−21	0.68	0.96	22	35
Alcalase (pH 8)	18 (8.6)	13 (8.0)	7 (8)	20 (8.0)	38 (8.3)	47−34−19	0.56	0.93	20[d]	159[d]
total 740-Alc	86 (41)	55 (34)	36 (41)	91 (36)	177 (39)	49−31−20	0.65	0.96	42	194

[a] The table shows data for the starting material (BSG), BSG after treatment with water only (control), and BSG after two-step reactions with a carbohydrase and Alcalase. In the two-step reactions BSG was first treated with Econase or Depol740 at pH 5 (BSG dissolved in H_2O), yielding the first hydrolysate, and subsequently treated with Alcalase at pH 8, yielding the second hydrolysate. Conditions: 60 °C, 4 h each step, 3.33% DM, and 20 μL of enzyme/g of DM. For comparison, data on N (protein) and DM solubilization (see **Figure 4**) are included. For qualitative analysis of the reaction products, see **Figures 6** and **7** (and text). [b] Xylose could not be distinguished from galactose and mannose in the assay used for analyzing the hydrolysates. This introduces an error, which, however, is small because of the low galactose and mannose contents of BSG (**Table 2**). The data for BSG are derived from **Table 2**; the figure for xylose was obtained by summing the contents of xylose, galactose, and mannose, as presented in **Table 2**. [c] In the control reaction, the pellet was not washed, meaning that some residual solubilized material was left in the pellet. Thus, the values for the control represent minimum values. [d] Represents the amount of DM/protein released by the Alcalase treatment that follows the carbohydrase treatment in the two-step procedures.

the biological source of Depol 740 is a *Humicola* species. When the enzymes are dosed as volume per gram of substrate, Econase has clearly higher xylanase and cellulase activity as compared to Depol 740. However, in contrast to Depol 740, Econase does not contain detectable feruloyl esterase activity (**Table 4**), and this may limit the accessibility of the substrate for xylanases and cellulases.

Choice of pH. Figure 1 shows the performances of Alcalase and Promod 439 at various pH values. The two peptidases performed equally well and worked best at pH 8, in terms of both DM and N solubilization. We used conditions identical to those used in a previous study on the solubilization of BSG proteins with commercial peptidases (*17*) and, as expected, we obtained similar solubilization levels. **Figure 2** shows the performance of Depol 740 at different pH values. We observed the best DM yield (22%) at pH 5 but only marginally less solubilization at pH 8 (20%). As expected, solubilization of N by Depol 740 was low (11−15%, depending on pH), that is, only slightly higher than the level of N solubilization obtained without any enzyme addition (11%). Econase treatment resulted in slightly lower DM solubilization than Depol 740 (19% at pH 5, 14% at pH 6.8, 19% at pH 8.0). As for Depol 740, DM solubilization by Econase reached a minimum at neutral pH (6.8).

In a previous study, Faulds et al. (*31*) have tested the effect of pH on dry matter solubilization by individual enzyme preparations including Alcalase and Depol 740 (but not Econase). With Depol 740, solublization levels were quite similar to those obtained here, except for some conspicuous differences at pH 9. Dry matter solublization with the peptidases Alcalase and Promod 439 was in the order of 15−20% (pH 7−9) instead of the approximately 25−30% observed in the present study (**Figure 1**). Faulds et al. used a lower incubation temperature (50 °C) which, as discussed above, hardly affects the efficiency of Depol 740 but which clearly reduces the efficiency of Alcalase. The BSG in this previous study came from ale production, whereas the BSG used in the present study came from lager production. Differences between BSG may affect dry matter solublization, for example, due to differences in polymeric cross-linking between the various types of polymers or differences in the conformation and hydrolyzability of the partially denatured protein material.

Factors Affecting the Solubilization Yield. Various process factors with possible influence on the solubilization yield of DM and N were evaluated using Depol 740 for carbohydrate and Alcalase for protein solubilization. Both one-step (4 h) and two-step (2 × 4 h; sequential enzyme treatments) reactions were investigated. The obtained DM and N yields were similar for the two types of processes (**Figure 3**). Thus, simultaneous enzymatic solubilization of peptides and carbohydrates can be carried out with a 4 h reaction time. However, as a result of this combined reaction, a mixture of peptides and carbohydrates is obtained. Because these fractions may find different types of applications (*2, 7, 28, 43−45*) such a separation will often be desirable.

To obtain two separate products from enzymatic hydrolysis of BSG, a series of two-step reactions were performed, combining Alcalase treatment at pH 8 with carbohydrase treatments (Econase and Depol 740) at pH 5 or 8 while varying the order of the enzyme treatments. The different two-step procedures yielded comparable levels of solubilized nitrogen, amounting to yields slightly above 80% (**Figure 4**; note that Alcalase in all cases was applied at pH 8 and that **Figure 1** shows that Alcalase alone solubilizes up to 77% of the nitrogen). With respect to the release of DM, which is around 40%, the data presented in **Figure 4** illustrate that (1) Econase is slightly less effective in solubilizing DM than Depol 740 [Eco(H_2O)→Alc(8) vs 740(H_2O)→Alc(8)] and (2) Depol 740 is more effective in terms of DM solubilization at pH 5.0 than at pH 8.0 [740(8)→Alc(8) vs 740(H_2O)→Alc(8)] (see also **Figure 2**). From a practical point of view, a process in which the first step is conducted in water (i.e pH is approximately 5) with a carbohydrate-degrading enzyme followed by Alcalase treatment at pH 8 seems most favorable, because this procedure yields maximum solubilization and two separate product streams. It should be noted, though, that some of the solubilized nitrogen (up to about 15%) will end up in the carbohydrate fraction, primarily because of solubility in water and, to some extent, also due to enzymatic actions during the carbohydrase treatment (see **Table 5**). The choice between Econase and Depol 740 also depends on the desired product (see below). The two-step procedures solubilized >80% of the protein (which comprises 23.4% of DM in BSG) and approximately 40% of the dry matter. Thus, about half of the total solubilized dry matter

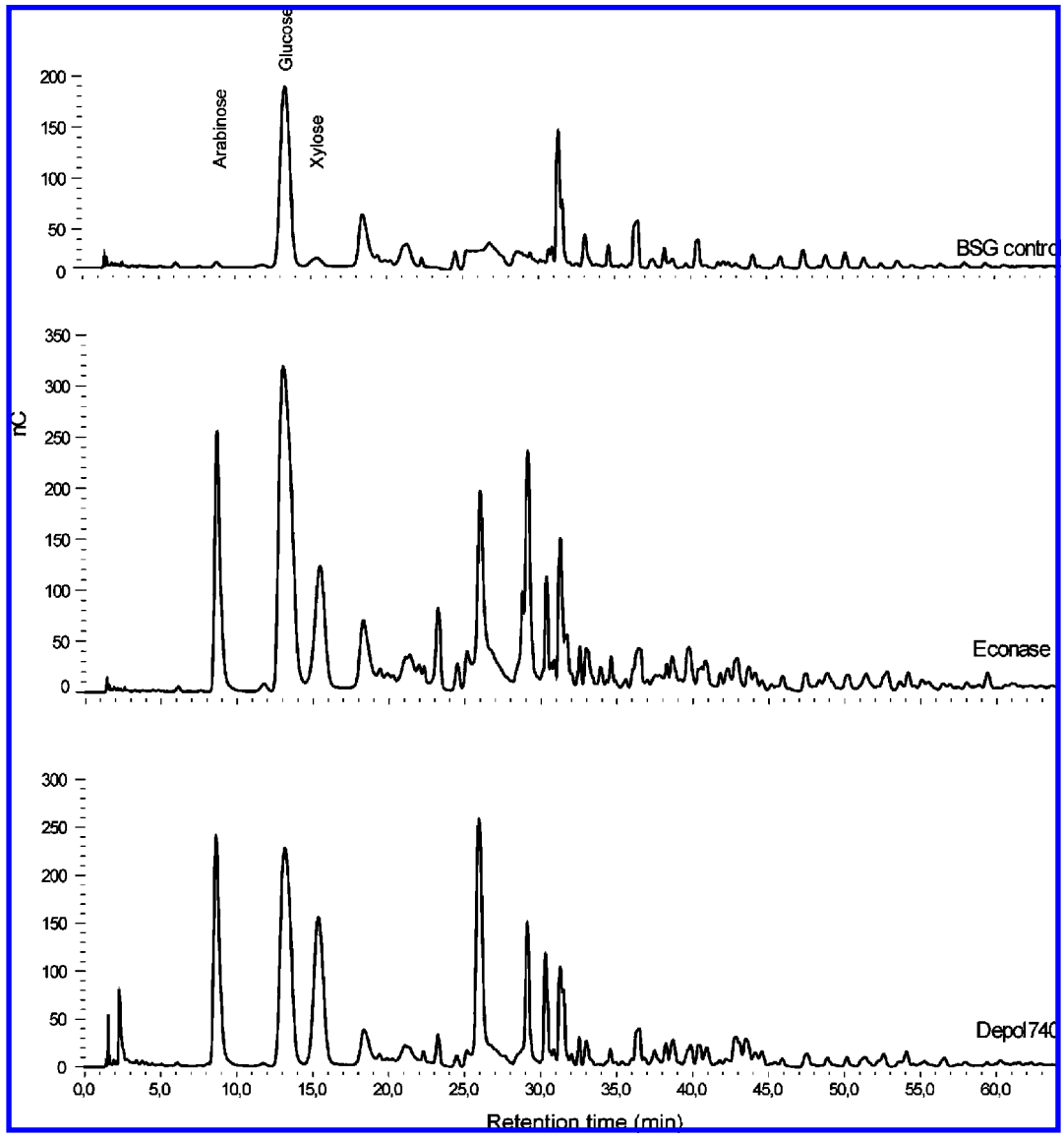

Figure 6. Fingerprints of mono- and oligosaccharides in BSG hydrolysates. The figure shows the results from anion-exchange chromatography of hydrolysates obtained after treating BSG with Econase or Depol 740 at pH 5 (60 °C, 4 h, 3.33% DM, and 20 μL of enzyme/g of DM). The upper panel shows the results for a control reaction (no enzyme added). Monosaccharide peaks were identified using standards for arabinose, glucose, and xylose. Oligosaccharide peaks appear to the right (elute from approximately 25 min and onward). Monosaccharide compositions of the hydrolysates are given in **Table 5**. Note that 99% of the carbohydrates released in the "BSG control" sample consist of glucose.

was protein ($0.80 \times 23.4/0.40 = 47\%$). The sugar content of the total solubilized dry matter was around 42%, as discussed below.

Effects of Enzyme Dosage and Dry Matter Content. To get an impression of the sensitivity of the outcome of the reaction for variation in the enzyme dose and water content, two of the most promising procedures of **Figure 4** were run with different enzyme doses and increased dry matter content (6.67% DM). Depol 740 was added in the first reaction at pH 5 or 8, followed by Alcalase hydrolysis at pH 8. The results (**Figure 5**) show clear, albeit not dramatic, dose−response effects. For example, under optimal conditions, that is, running the Depol 740 treatment at pH 5, a reduction of the enzyme dosages from 20 to 5 μL per gram of DM reduced DM and N yields from 38 to 36% and from 72 to 67%, respectively. Previous studies on the performance of Alcalase alone also showed that dose−response effects are weak (*17*) and that the marginal gain obtained by considerable increases in enzyme dosages is limited. At all dosages, DM solubilization was slightly more effective at pH 5 than at pH 8, confirming the results obtained in reactions run at 3.33% DM. The results also show that solubilization yields for both DM and N are lowered by increasing the dry matter content during the hydrolysis reactions. For example, the process in which the first step is done at pH 5 and the second at pH 8 yielded 38 and 72% solubilizations of DM and N when run with 6.67% DM and the addition of 20 μL of enzyme (**Figure 5**), whereas the solubilization yields increased to 42 and 83% when run with 3.33% DM, respectively (**Figure 4**).

Solubilized Material from Econase versus Depol 740. The carbohydrate compositions of hydrolysates obtained after treatment with Econase or Depol 740 were evaluated by analyzing monosaccharide compositions and oligosaccharide product profiles (**Table 5**; **Figures 6** and **7**). Monosaccharide compositions were analyzed for both hydrolysates, that is, the hydrolysate after carbohydrase treatment and the hydrolysate obtained after the subsequent treatment with Alcalase. The latter hydrolysates contained up to about 22% of the total released

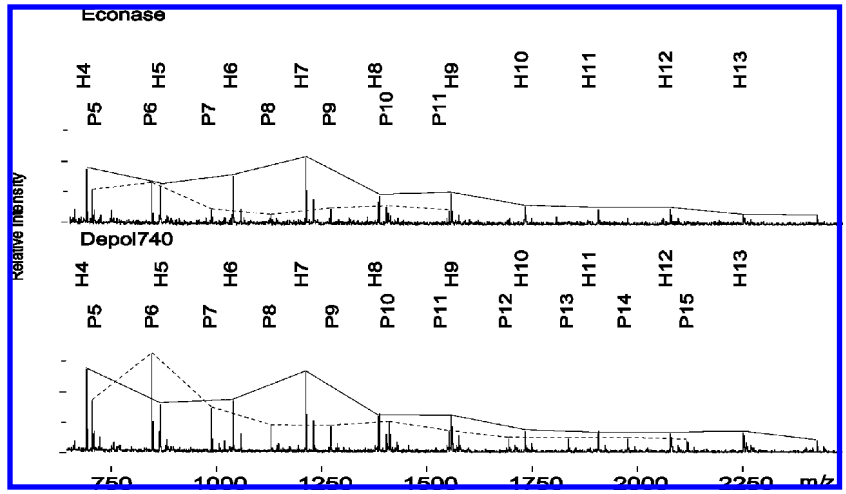

Figure 7. MALDI-TOF-MS analysis of oligosaccharides in BSG hydrolysates. Peaks representing pentose oligosaccharides are connected by the dotted line, whereas peaks representing hexose oligosaccharides are connected by the solid line. Hydrolysates were obtained after hydrolysis of BSG with Econase (top) or Depol 740 (bottom). Reaction conditions were as in **Figures 4** and **6**.

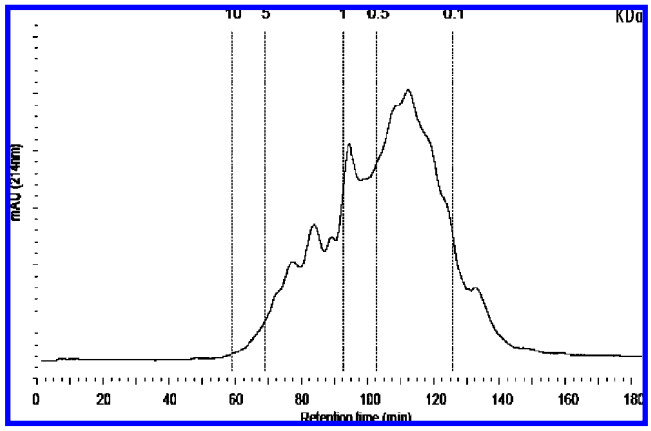

Figure 8. Molecular weight distribution of peptides in a BSG hydrolysate obtained after two-step hydrolysis. The hydrolysate was obtained after hydrolysis with Econase in H_2O (pH ~5) followed by Alcalase treatment at pH 8 using the conditions described in the caption of **Figure 4**. Molecular mass markers, derived from analysis of a standard sample, are indicated in kDa.

monosaccharides, due to a combination of residual carbohydrase activity during the Alcalase treatment and the fact that sugars solubilized by the carbohydrases in the first step are washed out of the pellet in the second step [Alcalase itself is devoid of carbohydrase activity (*31*)]. The results demonstrate that the two carbohydrase preparations act rather differently, as was to be expected on the basis of the activity data in **Table 4**.

Treatment of BSG in the absence of added enzyme led to the solubilization of 13% of the carbohydrates, yielding almost exclusively glucose-based products (**Table 5**). Considering the recalcitrance of cellulose, this glucose probably comes from starch (glucose, maltose, maltosaccharides, and residual starch fragments). The data show that at least 28% of the glucose in BSG (**Table 2**) was solubilized by simply incubating the BSG in water. The majorities of sugars released by the carbohydrase preparations consisted of arabinose and xylose and were thus derived from arabinoxylan (**Table 5**). Although the product profiles for both Depol 740 and Econase are dominated by hydrolysis of hemicellulose, clear differences of the enzymes were visualized in the product pattern. Hydrolysates obtained with Depol 740 contained relatively more pentoses than hydrolysates obtained with Econase (**Table 5**). The HPAEC fingerprint (**Figure 6**) further demonstrated that the two carbohydrase preparations yielded different product profiles, especially in the oligosaccharide parts of the chromatograms. This difference in the oligosaccharide products is further illustrated by the MALDI-TOF-MS-based product analysis depicted in **Figure 7**. Compared to the Econase hydrolysate, the Depol 740 hydrolysate shows more pentose oligomers. The product profile of the control sample almost exclusively showed hexose oligosaccharides (not shown).

Table 4 shows that Econase has higher cellulase activity than Depol 740, which may explain the higher occurrence of glucose and hexose oligomers in Econase hydrolysates. The xylanase activity of Econase was also higher as compared to Depol 740, but despite this, Depol 740 was more effective in releasing arabinoxylans. This may be due to the feruloyl esterase (FAE) activity in Depol 740 (**Table 4** and product sheet, Biocatalysts). FAE removes ferulic acid from arabinose and may also hydrolyze diferulic acid cross-links between arabinoxylan chains (*45*, *46*). Thus, more substrate may become available for the xylanases, which results in increased liberation of pentose sugars. Depol 740 hydrolysates show a slightly higher arabinose to xylose ratio, indicating that the cross-linked regions are relatively rich in arabinose.

Up to 42% of DM was solubilized by the Depol 740−Alcalase process (**Table 5**). In terms of the glucose, xylose, and arabinose fractions, the total solubilization yields for this process were 41, 34, and 41%, respectively. The solubilization yield for arabinoxylan was 36% in the Depol 740−Alcalase process and 28% in the Econase−Alcalase process.

Figure 8 shows a gel fitration-based peptide profile for the hydrolysate obtained after treatment of BSG with Econase and Alcalase. The peptide profile obtained after this and other two-step reactions (carbohydrase first, then Alcalase) did not depend on whether Depol 740 or Econase was used in the first step and was highly similar to the peptide profile obtained after treatment with Alcalase only (*17*).

Concluding Remarks. Although several higher value applications of BSG are conceivable (see ref *2* among others), exploitation of BSG for purposes other than animal feeding (and landfill) so far is limited. This work demonstrated processes based on commercial enzymes that may be used to decompose BSG effectively into carbohydrate- and protein-derived fractions. By the combined action of carbohydrases and peptidases, >40% of the dry matter and >80% of the proteinaceous material in BSG could be hydrolyzed within 8 h under conditions preventing

microbial growth, by simply using water in the first step and pH adjustment to pH 8 in the second step, all at 60 °C. Future work could focus on testing the functionalities of the various types of hydrolysates, for example, the suitability of the carbohydrate fraction as prebiotic food supplement or of the peptide fraction as food or feed supplement.

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