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Effect of pH on the Solubilization of Brewers' Spent Grain by Microbial Carbohydrases and Proteases

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The potential for enzymatic solubilization of brewers' spent grain by carbohydrases and proteases was examined over a broad pH range (pH 3.2−11.2). Enzymes from *Trichoderma* (Depol 686) were most efficient at a lower pH, while enzymes from the *Humicola* preparation (Depol 740) were the best performer over the whole range. Profiling of key glycoside hydrolase, esterase and protease activities across the pH range demonstrated that solubilization of spent grain by the *Trichoderma* enzymes corresponded to the range of maximum activities. This was not the case with the *Humicola* enzymes, where maximum solubilization of the substrate occurred at pH 9.1, at which pH the determined activities were low. Protease activity in Depol 740 was associated with a high solubilization, but inhibition of proteolytic activity resulted in only a 5% decrease in spent grain solubilization. These results suggest that while enzymes can be used to exploit agro-industrials byproduct, the use of high pH increases the extent of hydrolysis and an unidentified factor produced by *Humicola* improves the enzyme-catalyzed solubilization of lignocellulosic material.

KEYWORDS: Spent grain; byproduct; arabinoxylan; xylanase; feruloyl esterase; protease

INTRODUCTION

The plant cell wall consists of a scaffold of long cellulose microfibrils embedded in a matrix of interacting polysaccharides, such as xyloglucan, arabinoxylan, pectin, etc. A stable network is formed by these polysaccharides' binding to the surface of the cellulose microfibrils through hydrogen bonds between hydroxyl groups and via van der Waals forces between the sugar rings. Further network strengthening may be formed through oxidative cross-linking of phenolic acids esterified to the side chains of arabinoxylans and pectins or through calcium or borate diester cross-linking in pectins. Cell walls also contain low levels of structural proteins, which help to reinforce the wall structure.

Accessibility to lignocellulosic tissues is the main technological block to improved utilization of plant-derived materials. Cleavage of load-bearing polysaccharides is a rapid and simple means of achieving wall loosening and, thus, further access to the wall matrix for the carbohydrate-degrading enzymes. From chemical composition data, the efficient deconstruction of lignocellulosic biomass will require a complex mixture of cellulases, hemicellulases, proteases, and lignin-degrading enzymes that break down polymeric material into shorter fragments, which are then hydrolyzed further by various enzymes to form metabolizable soluble components.

Fundamental to any application is to improve accessibility of enzymes to their substrates. The complexity of this biomass dictates the enzyme profile microorganisms require to break down the plant cell wall for metabolic benefit. Microbial colonization of forage cell walls is quite rapid; however, the rate and extent to which the fiber is degraded is determined by factors such as microbial accessibility, the physical and chemical nature of the substrate, and enzyme kinetics. The use of plant-degrading fungi and bacteria or commercially available multienzyme preparations requires the production of enzymes with different modes of activity working synergistically to open up the complex heterogeneous cell wall matrix for efficient and complete saccharification. We have previously shown that multienzyme preparation from *Humicola insolens*, Ultraflo L, is able to release a high proportion of the wall-bound phenolic acids from brewers' spent grain (BSG) and wheat bran, two byproducts from the agro-food industry amounting to an annual worldwide production of millions of tons of biomass. However, total solubilization of this material is limited due to physical accessibility and molecular arrangements of the polysaccharides (*1*–*3*).

In this paper, we describe the effect reaction pH has on the solubilization of BSG by glycoside hydrolase, carbohydrate-acting esterase, and protease-containing multienzyme mixtures and how this solubilization is related to individual enzyme activities across this pH range. The selection of enzymes used to integrate a biomass-solubilization process is also obviously important, because different enzyme

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preparations have different spectra of activities. We also discuss how other factors in these fungal enzyme preparations aid plant cell wall disassembly.

MATERIALS AND METHODS

Material. Brewers' spent grain was obtained from a local brewery (Adnams, Southwold, U.K.) and lyophilised. The composition of lyophilised BSG was carbohydrate (51% w/w, of which 18% Ara, 34% Xyl, 38% Glc, 6% GlcA, 1.7% Gal, 1.2% Man); phenolic acids (0.61%, of which 28% *p*-coumaric, 57% ferulic, 11% diferulic acid); protein (17.6%); Klason lignin (20.1%); and lipid (5.24%, of which 27% palmitic, 55.6% linoleic, 9.4% oleic, 1.1% steric, 5.1% linolenic, 1% eicosenoic acid), as previously reported (*4*). Wheat arabinoxylan (medium viscosity) and azo-casein were purchased from Megazyme International Ltd. (Bray, Ireland). All other chemicals were obtained from Sigma-Aldrich (Poole, Dorset, U.K.). All buffers used were of analytical grade or higher.

Enzymes. Alcalase was generously donated by Novozymes (Bagsvaerd, Denmark). Depol 740, Depol 686, Depol 692 L, Promod 439, and Promod 24 L were kind gifts from Biocatalysts Limited (Cefn Coed, Wales, U.K.). Enzymes were desalted through PD-10 columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) into 50 mM $NaPO_4$ (pH 7.0) prior to use.

Solubilization of BSG. The following buffers were prepared: sodium citrate (50 mM, pH 3.2 and 4.1), McIlvaine's buffer (50 mM, pH 5.4), MOPS (50 mM, pH 6.0), sodium phosphate (50 mM, pH 7.0), TrisHCl (50 mM, pH 8.0), and sodium carbonate (50 mM, pH 9.1 and 10.2). A 50 mg portion of BSG (dry weight) was incubated with 1 U of protease (Promod 24 L, Promod 439, or Alcalase) or 100 U of xylanase (Depol 740, Depol 686) in the above buffers for 4 h at 50 °C, 100 rpm. After incubation, the insoluble residue was recovered by centrifugation and dried at 70 °C overnight to constant weight.

Enzyme Assays. Xylanase and cellulase activities were assayed using the dinitrosalicylic acid (DNS) method (*5*), as adapted by Bailey (*6*). For the pH range experiments, 1% (w/v) solutions of arabinoxylan (wheat endosperm) or CM-cellulose was prepared in sodium citrate (50 mM, pH 3.2 and 4.1), McIlvaine's buffer (50 mM, pH 5.4), MOPS (50 mM, pH 6.0 and 6.5), sodium phosphate (50 mM, pH 7.0 and 7.5), Tris/Cl (50 mM, pH 8.0 and 8.5), and dodium carbonate (50 mM, pH 9.1−11.2). Reactions were performed at 50 °C for 10 min. Control samples involved adding DNS reagent prior to the addition of the enzyme. Respective standard curves of xylose or cellobiose were prepared accordingly. The absorbance at 550 nm was determined using a Thermomax platereader (Molecular Devices Ltd., Wokingham, U.K.). α-Arabinofuranosidase, β-xylosidase, and β-glucosidase activities were determined against the corresponding *p*-nitrophenol derivatives (1 mM) in the above buffers. Reactions (50 °C, 5 min) were stopped by the addition of Na_2CO_3 (1 M), and absorbance was determined at 410 nm. Feruloyl esterase activities were assayed using methyl ferulate (MFA) and methyl *p*-coumarate (MpCA), and the release of the acids was measured by HPLC as previously described (*7*). Reactions were stopped by the addition of 0.4 vol of glacial acetic acid. The amount of free acid released was quantified against standard curves. For the pH optimum experiments, hydroxycinnamate methyl esters were prepared in the above buffers, and the hydrolysis of substrate was followed at 50 °C using the continuous spectrophotometric assay (*8*). Nonspecific protease activity was determined using azo-casein in the buffer range 3.2−11.2 at 50 °C, as per the manufacturer's instructions (Megazyme). Released dye was determined at 450nm. Activity was calculated using the formula in the manufacturer's instruction booklet, dependent on the origin of the protease sample.

One unit of activity (1 U) is defined as the mass (mg) or milliliters of enzyme preparation releasing 1 μmol of product per minute under the defined conditions. All assays were prepared and analyzed in duplicate.

Inhibition of Protease and Esterase Activities in Depol 740. A protease inhibitor cocktail (Sigma Aldrich Catalogue no. P2714), containing AEBSF, EDTA, bestatin, E-64, leupeptin, and aprotinin was prepared in deionized water as per the manufacturer's instructions. For solubilization experiments, the inhibitor cocktail and water (0−50 μL) were mixed with Depol 740 (100 U xylanase-equivalent activity) and left at 4 °C overnight. This preparation was added to BSG (50 mg) in 50 mM $NaCO_3$ (pH 9.1) and incubated at 50 °C for 4 h. Residual BSG was determined as above. The inhibition of protease activity against Azo-casein was determined by adding the protease inhibitor cocktail (0−20 μL) with Depol 740 (4 μL) in $NaCO_3$, pH 9.1 (80−96 μL), leaving at 50 °C for 30 min, and assaying as described above. Inhibition of activity of Depol 740 against MFA and MpCA was determined by mixing the inhibitor cocktail (0−50 μL) with Depol 740 (50 μL) in $NaCO_3$ (pH 9.1), leaving for 5 min at 50 °C, and determining activity using the spectrophotometric method as described above.

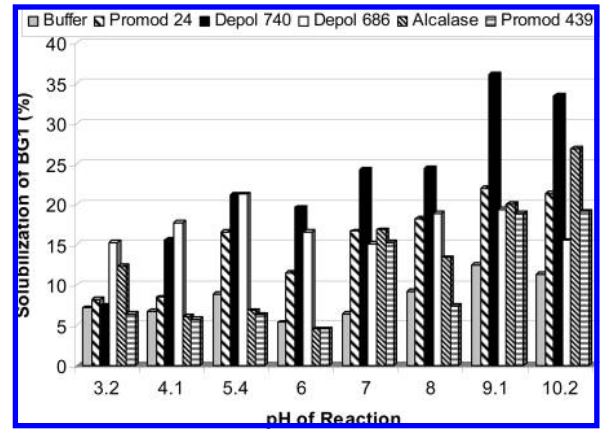

Figure 1. The effect of pH on the solubilization of BSG by carbohydrase and protease mixtures after incubation at 50 °C for 4 h.

RESULTS AND DISCUSSION

Solubilization of Spent Grain with Commercial Carbohydrases and Proteases. In previous studies on enzyme hydrolysis of cereal byproduct, we have used a standard dose of 2 U of xylanase-equivalent activity added to 20−50 mg substrate (*1–3, 9*). Before considering the effect of enzymes on the solubilization of BSG, it was prudent to see if the extent of solubilization could be improved by adding a larger dosage of enzyme. A dose−response was set up for the xylanase-rich preparations (Depol 740) and for the protease preparation (Promod 24) at pH 7.0, 50 °C, 4 h. The range used for proteases was limited to 1−10 protease enzyme units based on the activity of the supplied preparation of Promod 24. Because the activity of xylanase in the *Humicola* preparation (Depol 740) was higher, a greater range of xylanase activity was examined. The protease dose−response showed very little change in solubilization over the 1−10 U range, so it was decided to look at only 1 U protease-equivalent enzyme in subsequent experiments. For the xylanase-equivalent results, addition of xylanase activity above 100 U resulted in no significant improvement in solubilization. Therefore, it was decided to use the equivalent of 100 U of xylanase in those assays.

Over a pH range of 3.1−10.2, a proportion of BSG was solubilized by the addition of buffer alone (**Figure 3**), reaching 12% solubilization at higher pHs. At the lower pH range, slightly more biomass was solubilized in McIlvaine's buffer (pH 5.4). It is possible that endogenous enzymes still present in the spent grain remain active at this pH. The literature shows that proteases are still present in spent grain (*10*), where the cysteine and aspartic proteases were most active at low pH values, and metalloproteases were active only at high pH (*11*). It is also possible that endogenous

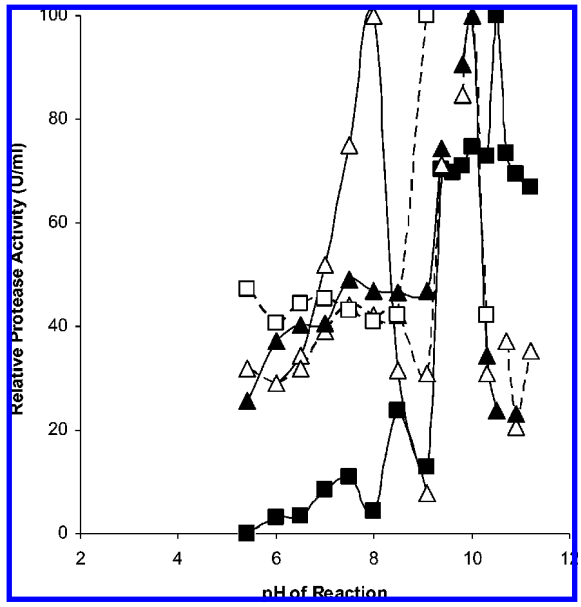

Figure 2. Protease activity of various enzymes as a function of pH at 50 °C. Values are expressed as relative protease activity, with 100 representing the maximum activity. Key to enzymes: –△–, Promod 24; --△--, Alcalase; ▲, Promod 439; ■, Depol 740; □, Depol 686.

carbohydrate-acting enzymes are also still active. At the extreme alkaline range of this study (pH > 9), it is possible that some form of cell wall loosening may occur because the alkaline conditions will slowly hydrolyze more labile linkages, such as the ester-linked phenolic acids. This, in theory, should facilitate greater access of enzymes into the spent grain.

When considering the addition of the enzyme preparations, the *Humicola* sp. preparation Depol 740 was the best all-round enzyme mixture for solubilizing BSG across the pH range, with 36% of BSG being solubilized in 4 h by the equivalent of 100 U of xylanase. Depol 686, from *Trichoderma*, had a similar solubilization effect across the pH range but was clearly the most effective enzyme at the more acidic range (pH 3.2−5.4). Alcalase, from *Bacillus licheniformis* was the most effective of the protease preparations examined, solubilizing 26% of the spent grain biomass at pH 10.2. Promod 439, also from *B. licheniformis*, was not as effective in solubilizing BSG. Promod 24 from *Bacillus subtilis* is a neutral protease and, as such, works more efficiently at a pH range of 7−8, as compared to the alkaline proteases of Alcalase and Promod 439, pH 9−10.

Activity Profiling of Proteases and Carbohydrases As a Function of pH. To understand further the activity of the various enzymes in these multienzyme preparations across the pH range used in this study, protease activity in Alcalase, Promod 24, Promod 439, Depol 740, and Depol 686 and glycoside hydrolase and feruloyl esterase activity profiles of Depol 686 and Depol 740 as a function of pH were determined. The three protease preparations examined were devoid of contaminating main-chain carbohydrase-type activity, so this could not be responsible for the solubilization observed. **Figure 2** shows the relative protease activity in the enzyme preparations against azo-casein over a pH range of 5.4−11.2. It is clear that Promod 24 is a neutral protease and Alcalase, Promod 439, and Depol 740 contain predominantly alkaline proteases. The *Trichoderma*-derived Depol 686 displays maximum proteolytic activity around pH 6. Although there is no nonspecific proteolytic activity at neutral

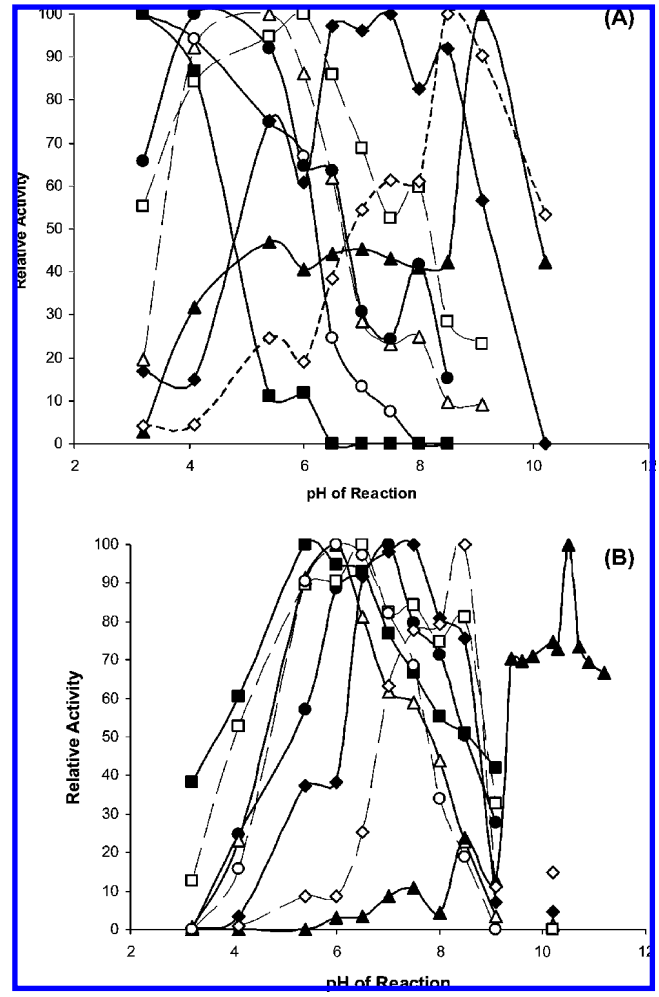

Figure 3. Activity profile of (**A**) Depol 686 and (**B**) Depol 740 as a function of pH. Values are expressed as relative activity, that is, as a percentage of the maximum activity over the pH range. Key to activities: □, xylanase; ▲, protease; △, β-glucosidase; ○, β-xylosidase; ■, α-arabinofuranosidase; ●, cellulose; ◆, feruloyl esterase; and ◇, coumaroyl esterase.

pH for Depol 740, Alcalase and Promod 439 exhibit significant proteolytic activity across the experimental range. It must be remembered that the values shown in **Figure 3** represent relative activities with respect to the maximum activity recorded for each preparation and that the activity in Alcalase and Promod 439 at pH 7 was much greater on azo-casein than Promod 24.

Comparative maximum activities for Depol 686 and 740 are shown in **Figure 3**. The *Trichoderma*-derived Depol 686 (**Figure 3A**) shows optimum activities at a lower pH than the *Humicola*-derived Depol 740 (**Figure 3B**). Profiling of the *H. insolens* preparation, Ultraflo L gave values similar to Depol 740 (results not shown). Protease and esterase activities are the dominant activities at the higher pH for both preparations. Side-chain glycoside hydrolase activities (e.g., β-glucosidase, β-xylosidase, α-arabinofuranosidase) tail off significantly around neutrality for Depol 686, and cellulase activity is absent above pH 7.5. A second, smaller xylanase peak is evident at pH 8, with the main activity between pH 4 and 5.5. Feruloyl esterase activity is also present in Depol 686, which is rare for a *Trichoderma*-derived enzyme preparation. It is possible that feruloyl esterase activity may be a side-activity of another esterase; for example, acetylxylan esterase. There is a shift in the glycoside hydrolase profiles with Depol 740 toward pH 6−8 (**Figure 3B**), which is

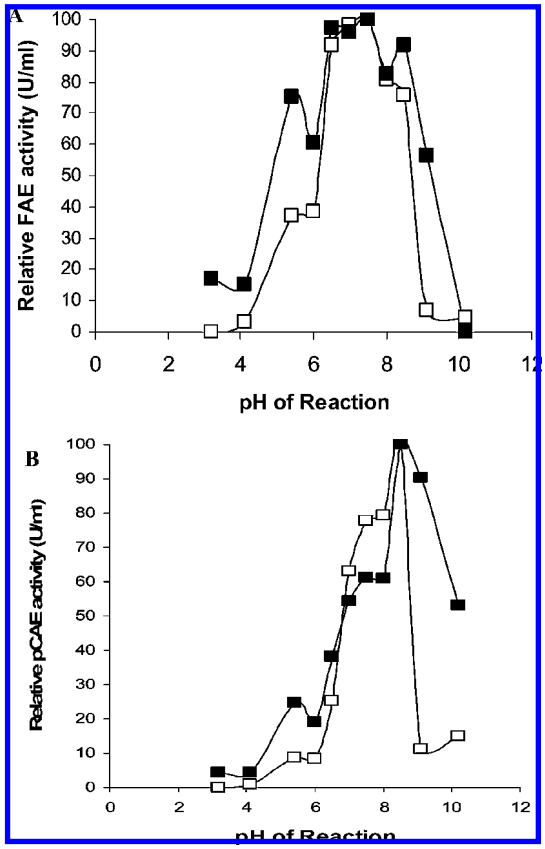

Figure 4. Esterase profiles for Depol 686 (□) and Depol 740 (■) against (**A**) MFA and (**B**) MpCA as a function of pH. Values are expressed as relative activity; that is, as a percentage of the maximum activity over the pH range.

Table 1. Maximum Activities Recorded for Multienzyme Preparations, as per **Figures 3**[a]

enzyme activity	Depol 740	Depol 686	Alcalase	Promod 439	Promod 24
protease	2.18	12.3	6454	6645	15.75
xylanase	9176	8047	ND	ND	ND
β-glucosidase	9.19	146	—	ND	ND
β-xylosidase	0.77	36	—	ND	ND
α-arabinofuranosidase	0.42	94	—	ND	ND
cellulase	1677	5388	ND	ND	ND
feruloyl esterase	3.02	0.84	—	ND	ND
p-coumaroyl esterase	16.84	5.11	—	ND	ND

[a] Values are expressed as units per milliliter of preparation after PD-10 desalting; —, activity not determined; ND, activity not detected.

consistent with reported pH optima for isolated enzymes. There is a clear separation of activity against methyl ferulate (MFA) from that against methyl *p*-coumarate (MpCA), suggesting that the activities are due to different enzymes present in the *Humicola* preparation. Comparison of activities against hydroxycinnamate methyl esters used previously to categorize feruloyl esterases into four classes (*12*) clearly shows that *p*-coumaroyl esterase activity in Depol 686 and Depol 740 has a narrow activity range with an optimum at pH 8, whereas feruloyl esterase activity is slightly broader with a maximum around pH 7.5 (**Figure 4**). This is consistent with previous results which showed that the Humicola multienzyme preparation, Ultraflo, contains predominantly type-B feruloyl esterase activity with a smaller amount of type A (*1*). Values for the maximum activities determined in the enzyme preparations studied are shown in **Table 1**.

Humicola sp. are known to produce at least five endoglucanases, two cellobiohydrolases (CBH), a β-glucosidase (*13*), a cellobiose dehydrogenase (*14*), a β-xylosidase (*15*), two GH11 xylanases (*16*), a GH10 xylanase, a GH 51 α-L-arabinofuranosidase, and a GH43 enzyme capable of releasing arabinose from doubly substituted xylose (*17*). The endoglucanases show optimal activity between pH 7 and 8.5, CBH I (Cel7A) has a pH optimum at pH 5.5, and CBH II (Cel6A) peaks around pH 9 (*13*). From the activity profile of Depol 740 (**Figure 3B**), whereas CBH II could be active at pH 9 in the multienzyme matrix, either other necessary activities for the exposure of cellulose and subsequent release of cellodextrans are low or the enzymes are not active at alkaline pH. In the release of arabinose and xylose from wheat endospermic arabinoxylan, it is postulated that the GH43 acts first to remove the 1,3-α-L-arabinofuranosyl residue from doubly substituted residues, then the GH51 enzyme attacks the 1,2 single substitution (*18, 19*). The GH51 and GH43 arabinose-releasing enzymes act synergistically at pH 6 on wheat arabinoxylan, but not at pH 5, where it is believed the GH51 arabinofuranosidase does not attack the arabinoxylan bonds. The exact conformation of arabinoxylan in BSG is not yet established, so it is not known if such a synergistic reaction is also occurring in our reactions.

These comparative profiles show that although BSG solubilization by the *Trichoderma*-derived Depol 686 is probably related to the maximum activities of its component enzymes, for the *Humicola*-derived Depol 740, solubilization appears to be more related to protease and feruloyl esterase activities rather than glycoside hydrolases because at the point of maximum solubilization (~pH 9.1), very little activity of the glycoside measured hydrolases remained.

Effect of Selective Removal of Protease and Feruloyl Esterase Activities on the Solubilization of Spent Grain by Depol 740. To determine if protein or phenolic acid cleavage was the key to the effective disassembly of BSG, a commercial protease inhibitor cocktail containing AEBSF, EDTA, bestatin, E-64, leupeptin, and aprotinin was used to knock out protease and esterase activities in Depol 740. Inhibition was monitored at pH 9.1 (50 °C), the pH previously shown to have the greatest BSG solubilization effect in the presence of the multienzyme mix. Inhibitor cocktail was preincubated with the enzyme preparation prior to the addition to the substrate, as described in Materials and Methods. An addition of 20 μL of the cocktail was sufficient for the complete inhibition of azo-casein-hydrolyzing activity in Depol 740, with 10 μL being sufficient to abolish activity against both MFA and MpCA.

With protease and feruloyl esterase activity knocked out, inhibitor-cocktail-treated Depol 740 (equivalent to 100 U of xylanase activity) was incubated with BSG (50 mg) for 4 h at 50 °C, and the nonsolubilized residual material was recovered. Inhibition of these activities in Depol 740 resulted in a 5% decrease in solubilization of BSG, as compared to uninhibited enzyme (**Figure 5**). Although we do not see a great pH-influenced deconstruction of BSG, with 60% of the substrate remaining still insoluble after a 4 h treatment, our results suggest a slightly alkaline pH may help deconstruction of spent grain. Because BSG contains a substantial quantity of lignocellulose, the presence of phenolic acid cross-links, and lignin will also impose a constraint on degradation. We have previously demonstrated that Ultraflo released ~70% of the ferulic acid in BSG over a 24 h period but solubilized only 30% of the biomass (*2*). Inhibition of the esterase

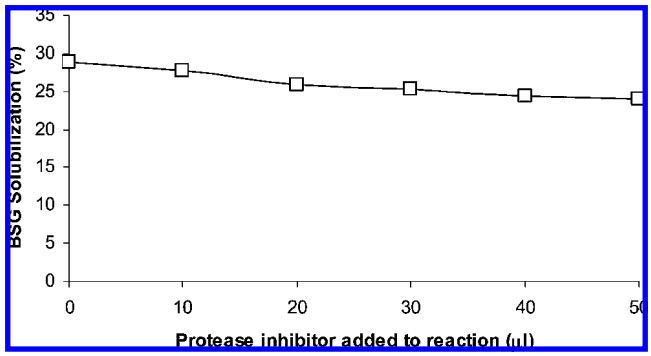

Figure 5. The effect of protease-inhibitor-treated Depol 740 on the solubilization of BSG.

activity by AEBSF and PMSF resulted in a 13-fold decrease in ferulate release (*1*). This agrees with the results in this study, where inhibition of protease/esterase activity made little difference in the solubilization of BSG by Depol 740.

The role of endogenous proteases must also be considered, but at pH values of 8 or greater, none of the exopeptidases, cysteine, and aspartic proteases present in malted barley are active, although the metalloproteases still retain activity (*20*). At higher pHs, some proteins begin to unfold as hydrogen bonds are broken, and this would result in a slight restructuring of the spent grain matrix, thus allowing better accessibility for the carbohydrases. Thus, protein breakdown appears essential for improved degradation by the glycoside hydrolases and carbohydrate-acting esterases present in multienzyme preparations, and our results suggest that proteases resistant to inhibition by the protease inhibitor cocktail may be present in the Depol 740 preparation.

The high solubilization of BSG by Depol 740 at high pH, even with low activities against soluble substrates (**Figure 3**), suggests that (1) an enzyme activity not measured is responsible, (2) the enzymes present display a shifted pH profile on "solid" substrates, or (3) the enzyme preparation contains noncatalytic "disruptins". Many *Humicola* glycoside hydrolases have been shown to contain CBMs (*22*). Alternatively, an expansin-type protein (*23*) similar to that isolated from *Trichoderma reesei* (*24*) may be present in the Depol 740 preparation. Both of these proteins can disrupt hydrogen bonding among cellulose microfibrils or between cellulose and other cell wall polysaccharides, allowing better access of the hydrolytic enzymes to their substrates and, thus, increased solubilization.

It is worth noting that although lignin accounts for 20% of the dry biomass of spent grain, a high proportion of the remaining 80% of the biomass remains inaccessible to enzyme solubilization. This is probably due to the fact that lignin intimately associated with the polysaccharide and wall-bound protein in the matrix (*21*).

In conclusion, the results of these experiments suggest that a combination of proteolytic and glycolytic activity is required to deconstruct spent grain. Although protein and phenolics play some role in limiting degradation by the *Humicola* preparation Depol 740, microorganisms overcome deficiencies in enzyme activities by producing "disruptin" domains, such as binding modules or as yet unidentified noncatalytic proteins. The role of as yet unidentified enzyme activities and protein denaturation as well as other physical pretreatments or cotreatments with enzymes to disrupt this lignified matrix requires further consideration.

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