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# High Production of β-Glucosidase in *Schizophyllum commune*: Isolation of the Enzyme and Effect of the Culture Filtrate on Cellulose Hydrolysis

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Optimization experiments with response surface statistical analysis were performed with *Schizophyllum commune* to obtain high β-glucosidase yields. The factors in the optimization experiment were the concentrations of cellulose, peptone, and $KH_2PO_4$. Their optimal values were 3.2, 3.0, and 0.2 g/100 ml, respectively. Enzyme assays revealed very high β-glucosidase (22.2 U/ml) and cellobiase (68.9 U/ml) yields. The avicelase yield was low as compared with that from *Trichoderma reesei*. Mixtures of *S. commune* and *T. reesei* culture filtrates caused faster and more extensive saccharification of Avicel than could be achieved by either filtrate alone. A β-glucosidase was isolated and purified from the optimized culture filtrate of *S. commune*. The electrophoretic mobility of the purified β-glucosidase indicated a molecular weight of 97,000. The amino acid composition was similar to that of β-glucosidase from *T. reesei*. The acidic (aspartate and glutamate) residues or their amides or both made up approximately 20% of the protein. The $NH_2$-terminal amino acid of the enzyme was histidine.

The breakdown of cellulose is catalyzed by a multiple enzyme system in *Trichoderma reesei* and *Sporotrichum pulverulentum* (6, 19). The major enzymes of the system are endo-β-1,4-glucanase (cellulase) (EC 3.2.1.4), cellobiohydrolase (exo-cellobiohydrolase) (EC 3.2.1.91), and β-1,4-glucosidase (β-D-glucosidase) (EC 3.2.1.21). Although the last does not actually hydrolyze cellulose, it does play an important role in cellulose hydrolysis. β-Glucosidase hydrolyzes cellobiose, the end product and inhibitor of cellobiohydrolase, thus increasing the rate of cellulose hydrolysis (20, 21). The β-glucosidase yield is low in some strains of *T. reesei* (20, 21), the most commonly examined source of cellulases, although the yield can be increased by carefully controlling fermentation conditions (24). Supplementation of the *T. reesei* complex with external β-glucosidase has been shown to increase saccharification rates due to hydrolysis of the inhibitory cellobiose (21).

In previous work we found that a wood-destroying basidiomycete, *Schizophyllum commune*, is a potent producer of cellulase, xylanase, and β-glucosidase activities in submerged cultures (18; M. Desrochers, L. Jurasek, and M. G. Paice, Dev. Ind. Microbiol., in press). In this paper the β-glucosidase production has been optimized, and some of the properties of one specific β-glucosidase are examined.

## MATERIALS AND METHODS

**Source of enzymes.** *S. commune* Fries strain no. 13 Delmar (ATCC 38548) was used throughout this work. *T. reesei* QM9414 freeze-dried culture filtrate was kindly provided by M. Mandels (U.S. Army Natick Research and Development Command) and was reconstituted to its original concentration as a solution (15.7 mg/ml) in 0.2 M sodium acetate buffer (pH 5.0).

**Optimization of β-glucosidase production.** The optimization procedure was essentially the same as that described earlier (5, 12; Desrochers, Jurasek, and Paice, in press). Three concentrations, those of cellulose (Solka-Floc SW40, Brown Co.), peptone (Difco Laboratories), and $KH_2PO_4$, were varied in this experiment. Two responses, β-glucosidase yields and pH, were determined.

**Medium and inoculation procedure.** The medium for production of β-glucosidase from *S. commune* was derived from the optimization experiment and contained (concentrations [grams per 100 ml] given in parentheses): cellulose (3.2), peptone (3.0), $KH_2PO_4$ (0.2), $Ca(NO_3)_2 \cdot 4H_2O$ (1.5), and $MgSO_4 \cdot 7H_2O$ (0.5). Trace elements were added according to Mandels et al. (11). The growth conditions were the same as those described previously (Desrochers, Jurasek, and Paice, in press). The culture filtrate used for the experiments described in this paper was obtained by centrifugation or filtration of the cultures or both.

**Enzyme assays.** The cellulase complex consisted of at least three different enzymes. β(1 → 4)-D-Glucan 4-glucanohydrolase (EC 3.2.1.4) is referred to here as carboxymethyl cellulase (CM-cellulase) or endoglucanase. β-Glucosidase (EC 3.2.1.21) was assayed as cellobiase or *p*-nitrophenyl-β-glucosidase (PNPGase). β(1 → 4)-D-Glucan cellobiohydrolase (EC 3.2.1.91) was estimated from avicelase or filter paper cellulase assays. The cellulase complex of *S. commune* is stable at 30°C (Desrochers, Jurasek, and Paice, in press); hence, most of the assays were performed at this temperature. Some of the assays were done at 50°C, a temperature

which is tolerated by the *T. reesei* cellulase complex.

**CM-cellulase and filter paper cellulase activities.** Methods for the determination of CM-cellulase and filter paper cellulase activities were the same as those described by Mandels et al. (11). Enzyme dilution in CM-cellulase assays was such that 5 μmol of reducing sugar was produced.

**Cellobiase activity.** Cellobiase activity was measured by the method of Sternberg (20) modified as follows: 100 μl of enzyme solution was added to 100 μl of cellobiose (Sigma Chemical Co.) solution (20 mM cellobiose in 0.2 M sodium acetate buffer [pH 5.0]) and incubated for 30 min at 30°C. Enzyme dilution was such that less than 1 μmol of glucose was produced. To stop the reaction, we boiled the solution for 5 min and then allowed it to cool. Thirty microliters of this solution was added to 3 ml of freshly prepared Statzyme glucose 50 reagent (Worthington Diagnostics), mixed, and incubated for 15 min at 30°C. The optical density at a wavelength of 500 nm was read within 15 min of the end of incubation. Units of cellobiase activity were expressed as micromoles of glucose produced $min^{-1}$.

**PNPGase.** PNPGase was assayed by the technique described by Nisizawa et al. (17), except that 0.1 M sodium acetate buffer (pH 5.0) was used. Enzyme dilution was such that 0.1 μmol of *p*-nitrophenol was produced. Units were expressed as micromoles of *p*-nitrophenol produced $min^{-1}$.

**Xylanase activity.** Xylanase assays were performed as described previously (18). Enzyme dilution was such that 5 μmol of reducing sugar was produced. Units were expressed as micromoles of reducing sugar produced $min^{-1}$.

**Avicelase activity.** Avicelase activity was measured by incubating 1 ml of enzyme solution with 1% Avicel (FMC Corp.) at 30°C in 0.1 *M* sodium acetate buffer (pH 5.0) for 24 h. Enzyme dilution was such that 5 μmol of reducing sugar was produced. The reducing sugars were determined by the DNSA method (13). Units were expressed as micromoles of reducing sugar produced $min^{-1}$.

**Viscometric determination of endoglucanase.** A viscometric determination was performed by the method of Hulme (9) at 30°C over a period of 25 min. Units were defined as microequivalents of β-1,4-glycosidic bonds broken per min during the initial stages of depolymerization.

**Saccharification of Avicel by mixed culture filtrates.** A total of 2 ml of a 6% suspension of Avicel in 0.2 M sodium acetate buffer (pH 5.0) was added to 2 ml of a mixture of *S. commune* culture filtrate and *T. reesei* reconstituted culture filtrate. The suspension was incubated at 30°C on a rocking mixer (Labindustries). After inoculation, the amount of reducing sugar was determined by the DNSA method. Thimerosal (50 mg/liter) was added to all enzyme solutions to prevent microbial growth.

**β-Glucosidase purification.** The enzyme complex in the culture filtrate of the optimized medium was precipitated by adding 3 volumes of ethanol to 1 volume of culture filtrate at −18°C. The precipitate was dissolved in 0.2 M pyridine–acetic acid buffer at pH 5.0 and freeze dried. The freeze-dried crude enzyme complex (1 g) dissolved partially in 15 ml of the above buffer after stirring for 15 min. After centrifugation (10 min at 700 × *g*), the supernatant (13 ml) was applied to a Bio-Gel A (Bio-Rad Laboratories) column (2.5 by 100 cm) equilibrated with the same buffer. The column was eluted with a linear gradient formed by 900 ml of the above buffer and 900 ml of 0.6 *M* pyridine–acetic acid buffer (pH 5.0). The β-glucosidase fractions yielded 80 mg of freeze-dried material that was further purified on a Bio-Gel P200 (Bio-Rad Laboratories) column (1.5 by 90 cm) equilibrated with 0.1 M pyridine–acetic acid buffer (pH 5.0). The β-glucosidase fraction (4 mg) was freeze dried for further analyses.

**Electrophoresis.** Sodium dodecyl sulfate-polyacrylamide gels were prepared by the method of Weber and Osborn (23).

**Amino acid analysis.** Protein samples were hydrolyzed by the method of Moore and Stein (15). Hydrolysis times of 24, 48, and 72 h were used. The protein hydrolysates were analyzed on a Beckman 119CL amino acid analyzer. The numbers of amino acid residues per molecule were calculated with the molecular weight determined by electrophoresis as a guide. The threonine value was obtained by linear extrapolation to zero-time hydrolysis. Half cystine and methionine residues were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation (14). Tryptophan was determined by hydrolysis with methanesulfonic acid (16).

**Amino-terminal amino acid determination.** The amino-terminal amino acid was determined by dansylation followed by hydrolysis by the method of Gray (7). Dansyl amino acids were separated on polyamide plates (26).

## RESULTS

**Culture optimization and enzyme yields in the culture filtrates.** With a medium optimized for endoglucanase production (Desrochers, Jurasek, and Paice, in press) as a center point, a response surface statistical experiment with three factors produced the graphs shown in Fig. 1. The contours of β-glucosidase (PNPGase) yields and pH, after a 9-day incubation, were plotted at three concentrations of $KH_2PO_4$. Although $KH_2PO_4$ did not show any interaction with cellulose or peptone, it showed an effect on β-glucosidase production. The optimum concentration of $KH_2PO_4$ was 0.2 g/100 ml. Optimal concentrations of cellulose and peptone were determined from coordinates of the peak of the β-glucosidase response surface in the central diagram shown in Fig. 1. The figure also shows the pH response surface. The highest β-glucosidase activities were found in cultures whose final pH was near 5.5.

Various enzyme yields found in the *S. commune* optimized culture filtrate are shown in Table 1.

**pH optimum of β-glucosidase activity.** The β-glucosidase (PNPGase) in the culture

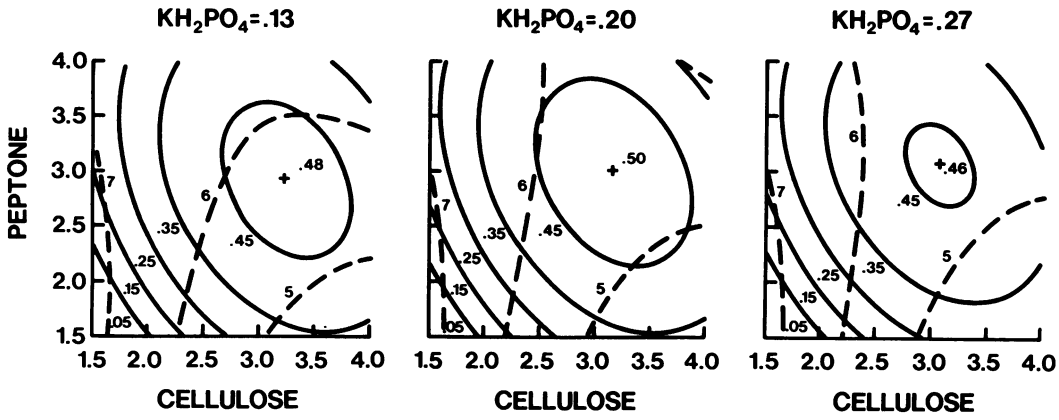


FIG. 1. *Effect of peptone, cellulose, and $KH_2PO_4$ concentrations (grams per 100 ml) on β-glucosidase (PNPGase) yield, expressed in optical density units at an absorbance of 420 nm/μl (solid contour lines), and on the final pH of the culture (broken contour lines). The β-glucosidase optimum was found at the top of the response surface in the central diagram.*

TABLE 1. *Enzyme yields in the optimized S. commune culture filtrate*

| Enzyme | Yield (U/ml) at: | |
|---|---|---|
| | 30°C | 50°C |
| CM-cellulase | 17.9 | |
| Endoglucanase (viscometrically) | 4.3 | |
| Filter paper cellulase | | 0.34 |
| Avicelase | 0.02 | |
| Cellobiase | 31.4 | 68.9 |
| *p*-Nitrophenyl-β-glucosidase | 22.2 | |
| Xylanase | 55.6 | |

filtrate was assayed in McIlvaine buffers to determine the optimum pH at 30°C. As shown in Fig. 2, the β-glucosidase optimum was at pH 5.3.

**Stability of β-glucosidase activity.** The stability of β-glucosidase (PNPGase) in the culture filtrate was measured at different pH values over a period of up to 240 h at 30°C (Fig. 3). The activity was most stable at pH 7.0. The thermostability was measured at pH 7.0 over a period of up to 72 h (Fig. 4). The activity was stable at 4°C. Its half-life was more than 72 h at 30°C and 24 h at 40°C, but was less than 1 hour at 50°C. This latter observation was confirmed by the cellobiase activities at 30 and 50°C (Table 1), where only an approximately twofold rate enhancement was found at 50°C.

**Combining culture filtrates of *S. commune* and *T. reesei* for enhanced saccharification of cellulose.** The enzyme yields of the *S. commune* optimized culture filtrate are shown in Table 1. The filtrate exhibited a high cellobiase (β-glucosidase) yield and a low avicelase yield, probably reflecting a deficiency in cellobiohydrolase. The *T. reesei* reconstituted culture filtrate, on the other hand, was apparently

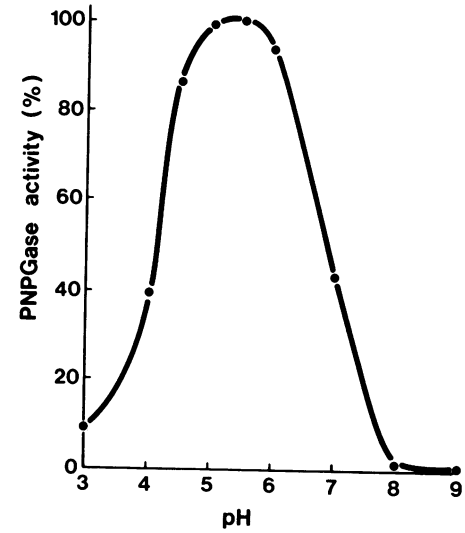


FIG. 2. *Effect of pH on the β-glucosidase (PNPGase) activity of S. commune culture filtrate at 30°C in McIlvaine buffers. The results are given as percentages of the original activity.*

rich in cellobiohydrolase (avicelase, 0.40 U/ml at 30°C; filter paper cellulase, 1.16 U/ml at 50°C) and low in β-glucosidase (cellobiase, 4.26 U/ml at 50°C; PNPGase, 0.48 U/ml at 30°C). Thus, some of the activities of the *S. commune* and *T. reesei* culture filtrates complemented each other, and their mixture was expected to provide a better balanced and more efficient enzyme system.

Indeed, Fig. 5 shows that a mixture consisting of 80% *S. commune* and 20% *T. reesei* culture filtrates produced higher yields of sugar from

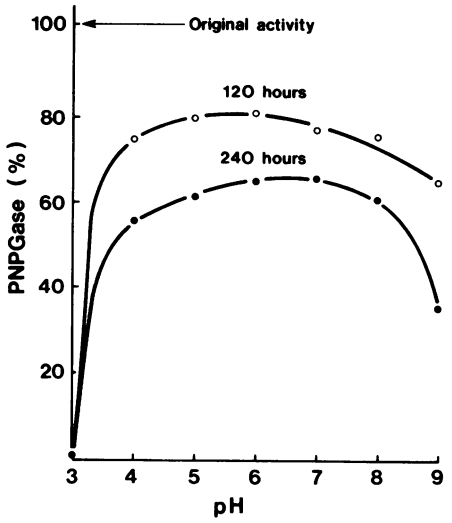


FIG. 3. *Effect of pH on the stability of β-glucosidase (PNPGase) in S. commune culture filtrate.*

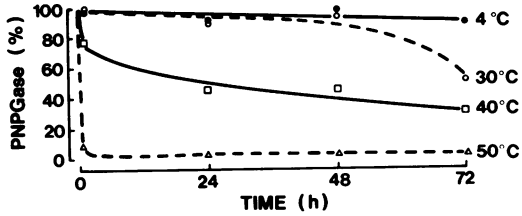


FIG. 4. *Thermostability of β-glucosidase (PNPGase) in S. commune culture filtrate at pH 7.0 over a period of up to 72 h. The results are given as percentages of the original activity.*

Avicel than either of the culture filtrates alone. The optimal ratio of the two filtrates varied with hydrolysis time, shifting toward a higher proportion of *S. commune* filtrate with increasing time.

**β-Glucosidase purification.** The enzyme was purified in two steps. First, the crude enzyme complex was run on a diethylaminoethyl Bio-Gel A column. As shown in Fig. 6, a xylanase was the first enzyme to be eluted. CM-cellulases and β-glucosidase were eluted later, but they did not separate. Further purification on Bio-Gel P-200 produced a β-glucosidase fraction that eluted first and separated from the CM-cellulase as shown in Fig. 7. The β-glucosidase fraction was freeze dried, yielding 2.7 mg of material which showed only one band when subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 8).

**Molecular weight, amino acid analysis, and $NH_2$-terminal determinations.** The electrophoretic mobilities of protein standards on sodium dodecyl sulfate gels were plotted against their molecular weights to establish the standard curve in Fig. 9. The electrophoretic mobility of purified *S. commune* β-glucosidase indicated that its molecular weight is around 97,000.

Results of the amino acid analysis are shown in Table 2. The $NH_2$-terminal amino acid determined by the dansyl procedure for proteins (7) was found to be histidine.

## DISCUSSION

We previously optimized a cellulose-peptone medium with respect to CM-cellulase (endoglucanase) yields (Desrochers, Jurasek, and Paice, in press). With a similar procedure, the medium described in this paper has been optimized for high yields of β-glucosidase. The main difference between the two media is a slightly higher content of cellulose, peptone, and phosphate, as well as a higher final pH, in the new medium. This new medium, as expected, yields more β-glucosidase. The PNPGase yield in the optimized culture is now 22.2 U/ml at 30°C, as compared with 12.5 U/ml in the CM-cellulose optimized culture. The cellobiase yield in the new culture has also approximately doubled. Other enzymic activities (endoglucanase, xylanase, and filter

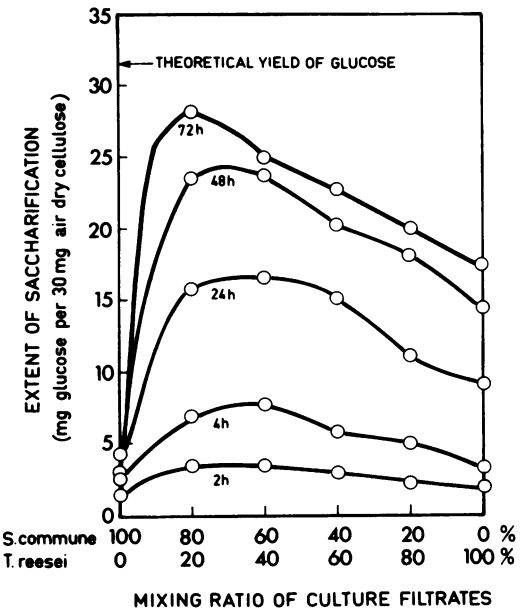


FIG. 5. *Effect on saccharification rates of mixing S. commune and T. reesei culture filtrates. Culture filtrates of S. commune and T. reesei were mixed in various proportions and allowed to saccharify crystalline cellulose (Avicel) in a 3% suspension at 30°C. The mixture of the two filtrates produced a higher saccharification rate than either filtrate alone.*

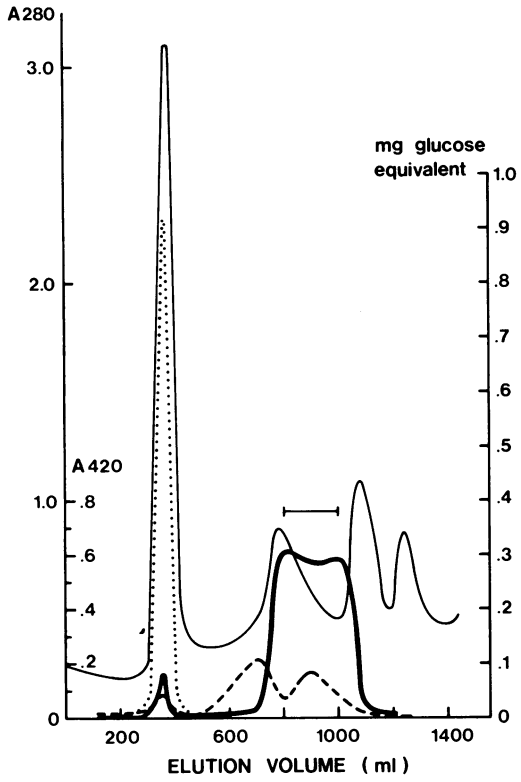


FIG. 6. *Ion-exchange chromatography of a crude enzyme complex from S. commune on diethylaminoethyl Bio-Gel A. The elution was carried out with a linear gradient of 0.2 to 0.6 M pyridine-acetic acid buffer (pH 5.0). The main β-glucosidase fraction was kept for further purification. Thin line, optical density at an absorbance of 280 nm ($A_{280}$); heavy line, β-glucosidase (PNPGase) yield in optical density units at an absorbance of 420 nm ($A_{420}$) per 10 μl; ---, CM-cellulase yield in milligrams of glucose equivalent per 25 μl; and ...., xylanase yield in milligrams of glucose equivalent per 5 μl.*

paper cellulase) in the new culture have decreased markedly, except for avicelase, the activity of which has remained equally low. This shows that not only the overall amount of enzyme, but also the balance of the cellulase components, can be modified by variation of the medium composition.

The synergistic action of the *S. commune* and *T. reesei* cultures (Fig. 5) enhanced the digestion of crystalline cellulose (Avicel) by 60%, as compared with the rate that could be achieved with *T. reesei* filtrate alone. An obvious explanation of the synergism is the effect of supplementary β-glucosidase on cellulose hydrolysis by *T. reesei* filtrates. As observed earlier by Sternberg (20), the enzyme stimulates cellulose saccharification by removing inhibitory cellobiose from the hydrolysate. Alternatively, the synergism can be interpreted as a stimulating effect of cellobiohydrolase on cellulose saccharification by an *S. commune* enzyme complex. Cellobiohydrolase, which seems to be obligatory for fast hydrolysis of highly ordered cellulose, is abundant in *T. reesei* cultures.

β-Glucosidase production by *Schizophyllum commune* Fries strains 845 and 699 was studied by Wilson and Niederpruem (25). Very low β-glucosidase production was reported by these authors. On the other hand, the β-glucosidase yields reported in our paper are exceptionally high. This apparent discrepancy can be explained either by the different media or by the different *S. commune* strains used in Wilson and Niederpruem's work.

We partly characterized a β-glucosidase from *S. commune*. Its molecular weight of 97,000 is within the range of molecular weights of β-glucosidases isolated from different microorga-

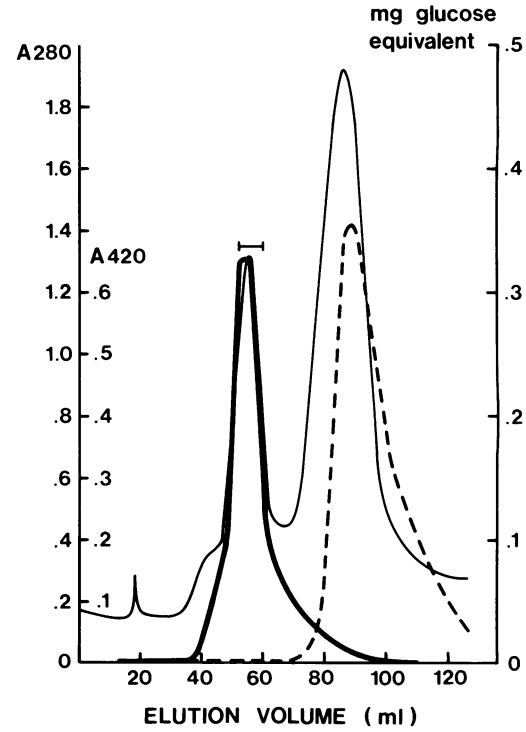


FIG. 7. *Gel filtration on Bio-Gel P200 of a β-glucosidase-rich fraction isolated from an ion-exchange column. The sample was eluted with 0.1 M pyridine-acetic acid buffer (pH 5.0). Thin line, optical density at an absorbance of 280 nm ($A_{280}$); heavy line, β-glucosidase (PNPGase) yield in optical density units at an absorbance of 420 nm ($A_{420}$) per μl, and ---, CM-cellulase yield in milligrams of glucose equivalent per 10 μl.*

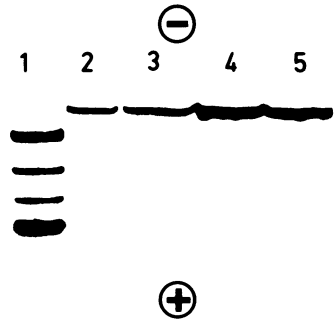


FIG. 8. *Sodium dodecyl sulfate-polyacrylamide gel electropherogram of β-glucosidase. Lane 1, Protein standards (from top): bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome c (last two merged); lanes 2 through 5, β-glucosidase at increasing loadings.*

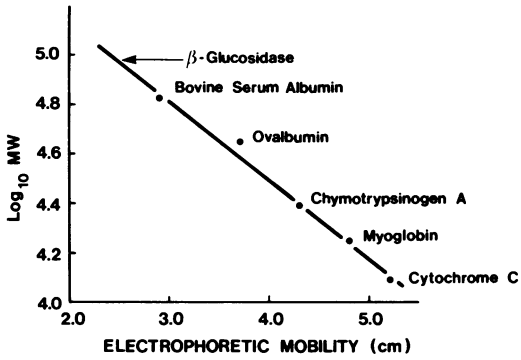


FIG. 9. *Estimation of molecular weight of β-glucosidase from a logarithmic plot of $\log_{10}$ molecular weight (MW) against electrophoretic mobility on a sodium dodecyl sulfate-polyacrylamide gel.*

nisms. A 320,000-molecular-weight β-glucosidase was isolated from *Lenzites trabea* by Herr (8). De Gussem et al. (3) found a fraction with a molecular weight of 65,000 to 69,000 in *Stachybotrys atra*, and Umezurike (22) measured a number of β-glucosidases with molecular weights ranging from 45,000 to 380,000, depending on the age of the culture in *Botryodiplodia theobromae*. The white-rot fungus *Sporotrichum pulverulentum* produces β-glucosidases with molecular weights ranging from 165,000 to 182,000 (4).

The amino acid composition of *S. commune* β-glucosidase is similar to that found in a β-glucosidase from *T. reesei* (2) in that the acidic (aspartate and glutamate) residues make up approximately 20% of the protein hydrolysate. The unique amino-terminal amino acid (histidine) suggests that the β-glucosidase is a single-chain protein. Partial amino acid sequences for β-glucosidases have been published. For example, active site sequences of the enzymes isolated from *Aspergillus wentii* (1) and bitter almonds (10) have been determined by labeling with conduritol-B-epoxide, a substrate analogue. Preliminary results in our laboratory indicate that the epoxide is also an inhibitor of β-glucosidase from *S. commune*. Further work on characterization of the *S. commune* β-glucosidase is in progress.

TABLE 2. *Amino acid composition[a] of β-glucosidase from S. commune*

| Amino acid | Molecular ratios | |
|---|---|---|
| | Avg | Nearest/ Integer |
| Lysine | 16.4 | 16 |
| Histidine | 13.0 | 13 |
| Arginine | 26.6 | 27 |
| Aspartic acid (and amide) | 109.4 | 109 |
| Threonine | 55.7 | 56 |
| Serine | 102.3 | 102 |
| Glutamic acid (and amide) | 74.5 | 75 |
| Proline | 44.2 | 44 |
| Glycine | 86.4 | 86 |
| Alanine | 121.9 | 122 |
| Half cystine | 10.7 | 11 |
| Valine | 74.5 | 75 |
| Methionine | 15.0 | 15 |
| Isoleucine | 49.0 | 49 |
| Leucine | 60.3 | 60 |
| Tyrosine | 28.7 | 29 |
| Phenylalanine | 25.1 | 25 |
| Tryptophan | 11.3 | 11 |

[a] Ratios are based on a molecular weight of 96,000.


## ACKNOWLEDGMENTS

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