Appl Microbiol Biotechnol (1988) 28:380–386

Applied and Microbiology Biotechnology

Purification and properties of an extracellular β-glucosidase from the cellulolytic thermophile *Clostridium stercorarium*

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Summary. *Clostridium stercorarium* cultures grown on cellobiose contain both an extracellular and a cell-bound β-glucosidase activity. A substantial portion of the cell-bound enzyme could be extracted by osmotic shock, suggesting a periplasmic localization. The β-glucosidase present in culture supernatants was purified to homogeneity. It was found to be identical in all aspects tested with the cell-bound β-glucosidase. The enzyme exists as a monomer with an apparent molecular weight of 85.000 (SDS-PAGE) and a pI of 4.8. It shows optimal activity as pH 5.5 and 65° C. Thiol groups are essential for enzyme activity. In the presence of reducing agents and divalent cations the half-life of the purified enzyme was more than 5 h at 60° C. The enzyme hydrolyses at different rates a wide range of substrates including aryl-β-glucosides, cellobiose, and disordered cellulose. K_m values were determined as 0.8 mM for *p*-nitrophenyl-β-glucoside (PNPG) and 33 mM for cellobiose. The cellular localization and the substrate specificity pattern are consistent with a dual role of the *C. stercorarium* β-glucosidase in cellulose saccharification: (1) Cleavage of cellobiose formed by exoglucanase and (2) degradation of cellodextrins produced by endoglucanase action.

Introduction

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyses the hydrolysis of β-glucosidic linkages between aryl, alkyl, or saccharide groups (Woodward and Wiseman 1982). Microbial β-glucosidases show great variation in their patterns of substrate specificity. Many β-glucosidases are capable of producing glucose from cellobiose and cellooligosaccharides and are therefore designated as cellobiases. Some fungal β-glucosidases also show 1,4-β-D-glucan glucanohydrolase activity removing glucose residues from the nonreducing ends of β-glucan chains (Wood and McCrae 1982; Schmid and Wandrey 1987). Furthermore, several bacteria possess cellobiose phosphorylase (EC 2.4.1.20), which carries out the inorganic phosphate dependent phosphorolysis of β-glucosidic bonds yielding α-D-glucose-1-phosphate.

Enzymes capable of hydrolysing cellobiose and cellodextrins are considered as essential components of microbial cellulase sytems. They convert the cellobiose and cellodextrins formed during the enzymatic degradation of cellulose by β-glucanases to fermentable glucose. As β-glucanases are generally subject to product inhibition, an adequate level of β-glucosidase is required for efficient breakdown of cellulose. β-Glucosidases differ from the β-glucanase components of the cellulase system by their cellular localization: β-Glucanases are mostly extracellular enzymes, whereas β-glucosidases are generally cell-bound (Stoppok et al. 1982). β-Glucosidase may therefore be the rate-limiting enzyme in cellulase preparations obtained from culture filtrates (Coughlan 1985).

Product inhibition and thermal inactivation of cellulase constitute two major barriers to the realization of enzymatic cellulose hydrolysis as a commercial process. For that purpose, the cellulases of the thermophilic anaerobic bacteria *Clostridium thermocellum* and *Clostridium stercorarium* may offer significant advantages (Johnson et al. 1982; Madden 1983). From *C. thermocellum* a β-glucosidase (Ait et al. 1982), a cellobiose phos-

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phorylase (Alexander 1968), as well as a cellodextrin phosphorylase (Sheth and Alexander 1969) have previously been isolated. We have recently reported the presence of a β-glucosidase in culture supernatants of *C. stercorarium* (Bronnenmeier and Staudenbauer 1988). In the present paper we describe the purification and some properties of this enzyme.

Materials and methods

Organisms and culture conditions. *Clostridium stercorarium* NCIB 11745 was obtained from the National Collection of Industrial Bacteria, Aberdeen, UK. Bacteria were grown under anaerobic conditions in GS-2 medium with cellobiose as carbon source (Johnson et al. 1981).

Concentration of culture supernatant. Culture supernatant (2.3 l) was concentrated in a Minitan crossflow system (Millipore Corp.) using PTGC filters with nominal molecular weight limit of 10.000. Ultrafiltration was carried out at room temperature employing a flow rate of 1.5 l per hour at a pressure of 0.8 bar. The retentate (64 ml) was desalted by five cycles of ultrafiltration following a two-fold dilution with 20 mM Tris-HCl pH 6.0. The concentrated enzyme solution was stored at −20° C. Protein concentration was determined by the method of Sedmark and Grossberg (1977).

Preparation of cell extract. All operations were performed at 0—4° C. Cells (7 g) were suspended in 2 volumes of 40 mM Tris-HCl buffer, pH 7.0, and disrupted by two passages through an Aminco French pressure cell at 16000 lb/in^2 after addition of DNase I (20 μg/ml). Debris were removed by centrifugation at 20000 *g* for 40 min. To the supernatant 10 ml/g protein of a 10% (wt/vol) solution of streptomycin sulfate in Tris buffer was added. After stirring for 45 min, the precipitate was removed by centrifugation at 20000 *g* for 20 min. Aliquots (2.5 ml) of the streptomycin-treated extract were desalted by passage through a prepacked Sephadex G-25M PD10 column equilibrated with 40 mM Tris-HCl, pH 7.0.

Anion exchange chromatography. The chromatographic system consisted of a Pharmacia FPLC system equipped with a Mono Q HR 5/5 column (0.5 × 5 cm) (Pharmacia, Uppsala, Sweden). The column was equilibrated with 20 mM Tris-HCl buffer at pH 6.0. Elution was effected with a 20-ml linear gradient (0.0—0.4 M NaCl) in equilibration buffer at a flow rate of 1 ml/min. Repeated runs were performed by looping the method with the Pharmacia gradient programmer GP-250.

Gel filtration chromatography. Gel filtration was carried out on a Pharmacia Superose 12 HR 10/30 column (1 × 30 cm) connected to a Pharmacia FPLC system. The total bed volume (Vt) of the column was 24 ml. Enzyme fractions were loaded on the column previously equilibrated with 50 mM Na-succinate buffer, pH 5.8, containing 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 0.4 ml per min. The elution volumes (Ve) of the enzymes were calculated from the peaks of activity. The void volume (Vo) of the column was determined using Blue Dextran. Partition coefficients ($K_{a\,v}$) were obtained from the relationship $K_{a\,v} = (Ve\text{-}Vo)/(Vt\text{-}Vo)$. Molecular weights were estimated employing a calibration curve prepared by plotting the partition coefficients of standard proteins versus the logarithm of their molecular weights.

Enzyme assays. β-Glucosidase activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucopyranoside (PNPG). Assay mixtures (1 ml) containing 4 mM PNPG in 40 mM citrate-phosphate buffer, pH 6.0, were incubated for 30 min at 60° C. Reactions were stopped by addition of 2 volumes 1 M Na_2CO_3. The optical density of the liberated *p*-nitrophenol was measured at 395 nm. One unit of activity is defined as the amount of enzyme liberating 1 μmol *p*-nitrophenol per min.

The activity on cellobiose and other disaccharides was determined by measuring the release of glucose. Assay mixtures (0.5 ml) containing 40 mM substrate in 40 mM citrate-phosphate buffer, pH 6.0, were incubated for 30 min at 60° C. Reactions were stopped by heating for 5 min in a boiling water bath. 0.5 ml of the glucose oxidase reagent solution from a glucose assay kit (Sigma Diagnostics No. 510) were added and the amount of glucose produced determined spectrophotometrically. One unit of cellobiase activity is defined as the amount of enzyme producing 1 μmol glucose per min.

Glucohydrolase activity was determined by measuring the release of reducing sugar from cellulose. Reaction mixtures containing 0.5% (w/v) substrate in 40 mM citrate-phosphate buffer, pH 6.0, were incubated for 30 min at 60° C. Reducing sugars were determined with the 3,5-dinitrosalicylic acid reagent (Aiba et al. 1983). One unit of glucohydrolase corresponds to the release of 1 μmol of glucose per min.

Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing. SDS-PAGE was performed in 10% polyacrylamide slab gels in the presence of SDS according to Laemmli (1970). Enzyme activity was detected in situ by activity staining with 4-methylumbelliferyl-β-D-glucoside as described by Schwarz et al. (1987). Molecular weights were determined with reference to prestained protein standards.

Analytical ultrathin-layer isoelectric focusing in polyacrylamide gels on polyester sheets was performed on an LKB Multiphor 2117 apparatus as described previously (Bronnenmeier and Staudenbauer 1988).

Chemicals. Cellobiose, laminaribiose, *p*-nitrophenyl-β-D-glycosides, arbutin (hydroquinone-β-glucopyranoside), salicin (2-[hydroxymethyl]phenyl-β-D-glucopyranoside), and molecular weight markers for SDS-PAGE were obtained from Sigma. Molecular weight standards for gel filtration were from Pharmacia. Cellulose MN 3000, Avicel TG 104 and chemicals for SDS-PAGE and IEF were purchased from Serva (Heidelberg, FRG). Phosphoric acid-swollen Avicel was prepared by the method of Wood (1971).

Results

Enzyme purification

The purification of the β-glucosidase present in culture supernatants of *C. stercorarium* is summarized in Table 1. The β-glucosidase can be clearly separated from other components of the cellulase system by Mono Q anion exchange chromatography (Fig. 1). β-Glucosidase activity is eluted at 0.12 M NaCl, whereas β-glucanases exhibiting both CMCase and Avicelase activity are eluted between 0.3—0.4 M salt (Bronnenmeier

Table 1. Purification of β-glucosidase from culture supernatants

Fraction	Volume (ml)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	2300	15.0	118	0.127	1.0	100
Ultrafiltration concentrate	64	13.4	90	0.148	1.2	89
Mono Q	8.0	6.6	2.14	3.08	24.3	44
Superose 12	3.2	3.8	0.12	31.6	249.3	25

and Staudenbauer 1988). Further purification of β-glucosidase was achieved by FPLC gel filtration on Superose 12 (Fig. 2). On this column the enzyme migrates as a single peak with an apparent molecular weight of 105.000.

Analysis of the β-glucosidase fractions by SDS-PAGE revealed a single protein band with a molecular weight of 85.000. The identity of this protein band with β-glucosidase was established by activity staining with 4-methylumbelliferyl-β-D-glucoside as substrate (data not shown). The chromatographic behaviour during FPLC gel filtration therefore indicates a monomeric structure of the enzyme under non-denaturing conditions.

Cellular localization

As shown in Table 2, only about 20% of the total β-glucosidase activity of *C. stercorarium* is present in the cell-free supernatant. The remaining activity is found associated with the cells. The finding that more than 60% of the cell-bound β-glucosidase activity could be released by osmotic shock suggests a periplasmic localization of the enzyme.

The electrophoretic mobility of the cell-associated enzyme in SDS-PAGE was indistinguishable from that of the purified extracellular enzyme. Furthermore, an isoelectric point of 4.8 was determined for both forms of the enzyme by narrow-range isoelectric focusing (data not shown).

Optimum pH and chemostability

The pH profile of β-glucosidase activity is shown in Fig. 3. Both the extracellular and the cell-associated form of the enzyme exhibited a broad pH optimum between pH 5—7. Optimal activity was observed around pH 5.5. Omission of inorganic phosphate from the buffer had no effect on PNPG cleavage (data not shown).

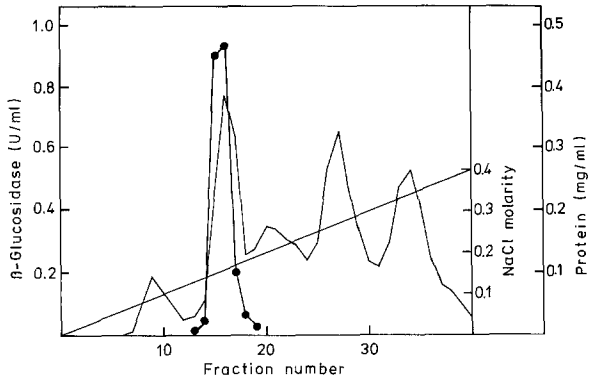

Fig. 1. FPLC anion exchange chromatography of crude *C. stercorarium* cellulase. Concentrated culture supernatant (11.2 mg protein) was applied to a Pharmacia Mono Q HR 5/5 anion exchange column equilibrated with 20 mM Tris-HCl, pH 6.0, and eluted with a 20 ml linear gradient (0.0—0.4 M NaCl). The method was looped 8 times. Fractions (8 × 0.5 ml) were collected and assayed for protein (–) and β-glucosidase activity (●)

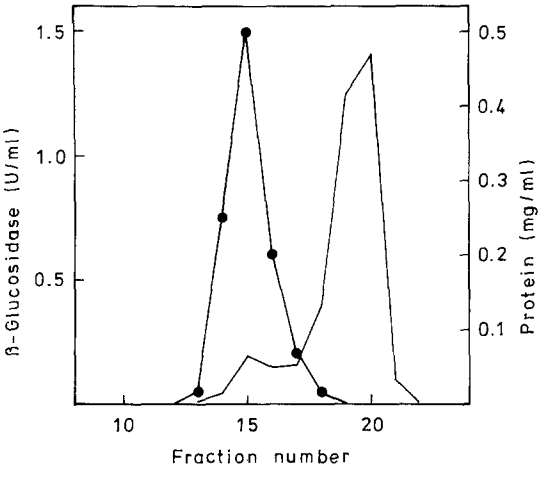

Fig. 2. FPLC gel filtration of β-glucosidase. Peak fractions from the Mono Q column were concentrated in a Centricon centrifugal microconcentrator and loaded on a Superose 12 HR 10/30 equilibrated with 50 mM sodium succinate buffer, pH 5.8, containing 100 mM NaCl. The column was eluted with equilibration buffer at a flow rate of 0.4 ml/min. The method was looped 4 times. Fractions (4 × 0.4 ml) were assayed for protein (–) and β-glucosidase activity (●)

Table 2. Cellular localization of β-glucosidase

Fraction	Activity (U)	Protein (mg)	Specific activity (U/mg)
Culture supernatant	15.0	118	0.127
Cell extract	53.6	444	0.121
Shock fluid[a]	33.9	69	0.494
Extract of shocked cells	19.7	384	0.051

[a] Osmotic shock was carried out by the procedure of Ait et al. (1979)

The effects of various chemical on enzyme activity are summarized in Table 3. Enzyme activity was stimulated by the presence of divalent cations and dithiothreitol, whereas $HgCl_2$ and *p*-hydroxymercuribenzoate had a strong inhibitory effect. Addition of DTT provided protection against inactivation by thiol-specific inhibitors. The enzyme was insensitive against non-ionic detergents such as Brij 35, but completely inhibited by SDS. Nevertheless, the inactivation of *C. stercorarium* β-glucosidase by SDS was reversible, since after SDS-PAGE the enzyme could be reactivated upon removal of the detergent.

Table 3. Effect of chemicals and inhibitors on enzyme activity

Compound(s) added	Relative activity[a] (%)
None	100
DTT (10 mM)	112
$CaCl_2$ (10 mM)	109
$MgCl_2$ (10 mM)	142
EDTA (100 mM)	92
$HgCl_2$ (1 mM)	1
$HgCl_2$ (1 mM) + DTT (10 mM)	65
Iodoacetate (10 mM)	88
p-Hydroxymercuribenzoate (1 mM)	10
p-Hydroxymercuribenzoate (1 mM) + DTT (10 mM)	91
SDS (0.01%)	60
SDS (0.05%)	0
Brij 35 (2%)	99

[a] Assays were carried out with purified extracellular enzyme

Temperature optimum and thermostability

The temperature-activity profiles of the extracellular and the cell-associated β-glucosidase were nearly identical. Both forms of the enzyme showed optimal activity at 65° C (Fig. 4). The enzyme activity declined very sharply at temperatures above 75° C.

Comparison of the thermal stability of the purified extracellular enzyme to that of crude lysates showed a striking difference (Fig. 5). While the crude enzyme was markedly thermostable, exhi-

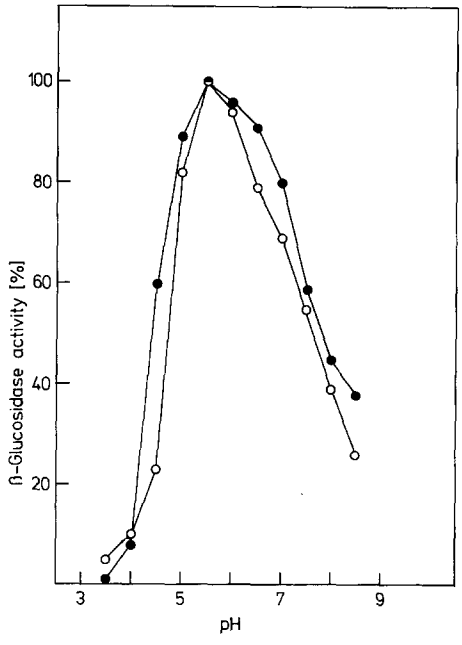

Fig. 3. pH profile of β-glucosidase activity. Incubations were carried out for 10 min at 60° C in 40 mM citrate-phosphate buffer. ●, purified extracellular enzyme; ○, cell extract

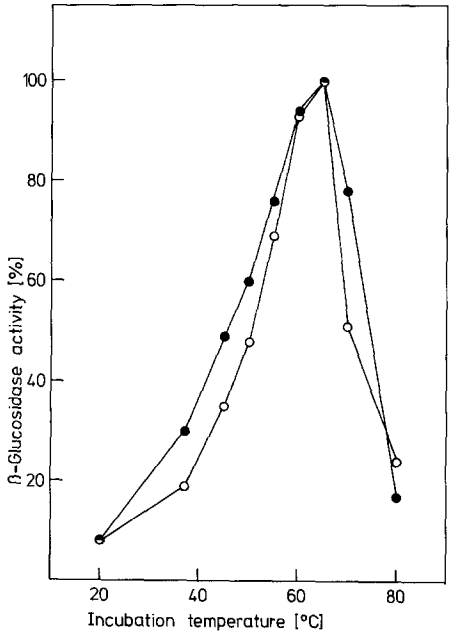

Fig. 4. Effect of temperature on β-glucosidase activity. Incubations were carried out for 10 min at the indicated temperatures under standard assay conditions. ●, purified extracellular enzyme; ○, cell extract

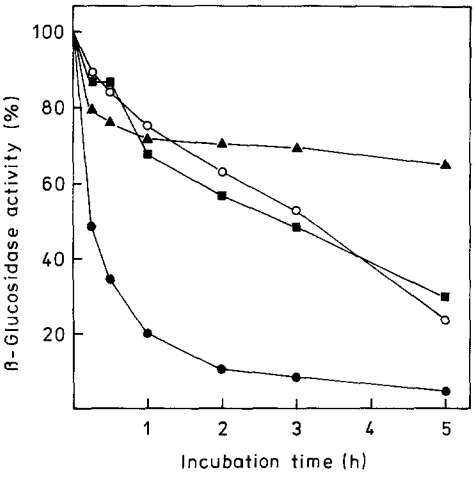

Fig. 5. Thermal stability of β-glucosidase activity. Enzyme preparations were incubated at 60° C in 40 mM citrate-phosphate buffer, pH 5.8. At the times indicated samples were withdrawn for the determination of β-glucosidase activity. Residual activity was expressed as the percentage of the untreated control. ●, purified extracellular enzyme; ■, extracellular enzyme plus 10 mM $MgCl_2$; ▲, extracellular enzyme plus 10 mM $MgCl_2$ and 10 mM DTT; ○, cell extract

biting a half-life of about 3 h at 60° C, the purified enzyme was rapidly inactivated at this temperature. However, the thermostability of the purified enzyme could be increased by addition of divalent cations (Mg^{2+} or Ca^{2+}). Addition of dithiothreitol (DTT) had a similar stabilizing effect (data not shown). Optimal protection against thermal inactivation was achieved by adding both $MgCl_2$ and DTT to the purified enzyme. Under these conditions the half-life at 60° C in the absence of substrate was increased to more than 5 hours.

Substrate specificity

Both forms of the enzyme were capable of hydrolysing a wide range of β-glucosidic substrates (Table 4). Highest activity was observed with aryl-β-glycosides such as arbutin. The enzyme appears to be a genuine β-glucosidase since little or no activity was detectable with other aromatic β-glycosides.

Under optimal conditions cellobiose is hydrolysed at about one third of the rate of PNPG cleavage. The enzyme appears to be quite specific for β-1,4-linkages, since the β-1,3-linked laminaribiose was cleaved at a markedly lower rate. Interestingly, the enzyme exhibited a low but significant activity with amorphous cellulosics. No enzyme activity towards microcrystalline cellulose or carboxymethylcellulose could be detected.

K_m values were determined from Lineweaver-Burk plots, both for the purified enzyme and for the β-glucosidase activity present in crude lysates. The calculated values were 0.8 mM for PNPG and 33 mM for cellobiose, respectively. Identical K_m values were obtained for both forms of the enzyme.

Discussion

C. stercorarium grown on cellobiose produces a β-glucosidase, which is partially released into the culture medium. The major portion of the β-glucosidase activity remains cell-bound. Release of the enzyme by osmotic shock indicates a periplasmic localization. In this respect the *C. stercorarium* β-glucosidase is similar to the enzyme from *C. thermocellum* (Ait et al. 1979). Trapping in the space between the cell wall and the cytoplasmic

Table 4. Activity of β-glucosidase toward various substrates

Substrates	Relative rate of hydrolysis (%)	
	Extracellular enzyme	Cell extract
p-Nitrophenyl-β-D-glucoside	100	100
Salicin (2-[hydroxymethyl]phenyl-β-D-glucoside)	88	103
Arbutin (4-[hydroxyphenyl]-β-D-glucoside)	244	289
p-Nitrophenyl-β-D-xyloside	18	14
p-Nitrophenyl-β-D-mannoside	0	0
Cellobiose	32	30
Laminaribiose	10	12
Cellulose MN 300	16	15
Avicel	0	0
Avicel, acid-swollen	8	5

membrane has also been observed for *Trichoderma reesei* β-glucosidase (Nanda et al. 1982). This decreases the effectiveness of culture filtrates in the saccharification of cellulosics and necessitates their supplementation with β-glucosidase from other sources.

Sensitivity of the enzyme to sulfhydryl-blocking agents indicates the presence of essential thiol groups. Similar conclucions have been reached for other β-glucosidases (Ait et al. 1982; Ohmiya et al. 1985; Patchett et al. 1987). Reducing agents protected the enzyme against thermal inactivation possibly by reducing oxidized thiol groups. Thermostability was further increased by addition of divalent cations. A stabilizing role of divalent cations has been previously observed for other exoenzymes from thermophilic bacteria (Tsukagoshi et al. 1984).

Under optimal conditions the thermostability of *C. stercorarium* enzyme was comparable to that of the β-glucosidases of *C. thermocellum* (Ait et al. 1982) and thermophilic fungi (Breuil et al. 1986), but significantly lower than that reported for the endo-β-1,4-glucanase of *C. stercorarium* (Creuzet and Frixon 1983). The limited thermostability is consistent with the notion that β-glucosidase is generally the least stable component of cellulolytic enzyme systems (Hägerdal et al. 1980).

Enzyme action did not require the presence of inorganic phosphate. The *C. stercorarium* enzyme differs in this respect from the β-glucosidases of *Cellulomonas* (Schimz et al. 1983) and *Erwinia* (Barras et al. 1984) which were shown to be phospho-β-glucosidases. It should be noted, however, that an enzyme of the latter type has also been identified in *C. stercorarium* extracts (unpublished results).

On the basis of substrate specificity the *C. stercorarium* β-glucosidase may be considered as a broad-specificity β-glucosidase. The K_m values indicate that the enzyme has a higher affinity for the artificial substrate PNPG than for cellobiose. This preference for aryl-β-glucosides has also been observed for the β-glucosidases of *C. thermocellum* (Ait et al. 1982) and *Caldocellum saccharolyticum* (Patchett et al. 1987).

The presence of both β-glucosidase and cellobiose-phosphorylase in thermophilic cellulolytic clostridia indicates two pathways of cellobiose degradation. Phosphorolysis is energetically advantageous and might constitute the primary route of cellobiose utilization in anaerobic bacteria. Hydrolysis might become operative only at higher cellobiose concentrations in order to relieve inhibitory effects of cellobiose on other cellulolytic enzymes.

An interesting property deserving further study is the ability of the *C. stercorarium* β-glucosidase to attack disordered cellulose such as phosphoric acid-swollen Avicel. This glucohydrolase activity indicates that the *C. stercorarium* β-glucosidase might also function in the degradation of higher-molecular-weight cellodextrins produced by the action of endo-β-glucanases. It should be noted, that the size of the *C. stercorarium* β-glucosidase is about twice that reported for the enzymes from *C. thermocellum* (Ait et al. 1982) and *Caldocellum saccharolyticum* (Patchett et al. 1987). The increased size of the *C. stercorarium* β-glucosidase might correlate with an extended role in cellulose saccharification.

Acknowledgements. We are grateful to Millipore GmbH for providing us with a Minitan tangential flow system. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 145), from the Fonds der Chemischen Industrie, and from the Dr. Otto Röhm Gedächtnisstiftung GmbH.

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Received November 25, 1987/Accepted January 21, 1988