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β-Glucosidase of *Trichoderma*: Its Biosynthesis and Role in Saccharification of Cellulose

DAVID STERNBERG

Food Science Laboratory, U.S. Army Natick Development Center, Natick, Massachusetts 01760

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The extracellular β-glucosidase of *Trichoderma viride* generally is present in low levels when the organism is cultured on cellulose because it is inactivated under the acid conditions which develop in the medium while the other enzymes of the cellulase complex are more stable. With the appropriate pH control, inactivation of β-glucosidase is prevented and the activity of this enzyme increases during growth. In the saccharification of crystalline cellulose, or of cellulose at low concentrations, much of the glucose produced is the result of the cleavage of cellobiose by β-glucosidase. However when high concentrations (10%) of pretreated cellulose are saccharified, significant quantities of glucose are produced by action of enzymes other than β-glucosidase.

Trichoderma is the best known source of an extracellular cellulase capable of solubilizing crystalline cellulose (15). The production of cellulase enzymes is under study with the objective of developing a practical process for the saccharification of waste cellulose (7, 11, 20). The predominant sugars found in saccharification reactions are glucose and cellobiose; minor amounts of xylose and other sugars are present depending on the amount of hemicellulose present in the substrate. Several investigators (1, 2, 4-6, 8, 18, 21, 22) have suggested that the action of cellulases involves the concerted action of (i) endoglucanase(s), which randomly attacks the internal β-1,4-linkages, (ii) cellobiohydrolase, which cleaves off cellobiose units from the nonreducing ends of the glucan, and (iii) β-glucosidase, which hydrolyzes cellobiose to glucose. This concept is consistent with the observation that cellobiose and glucose are the only sugars in significant quantities arising from the enzymatic saccharification of cellulose. It would be desirable to have the ability to control the glucose-cellobiose ratio in the syrup depending on the application of the syrup. Furthermore, cellobiose has been found to inhibit both cellobiohydrolase and endoglucanase (4, 8, 21), and thus decrease the rate of cellulose saccharification. To pursue these problems as they apply to practical saccharifications (i.e., 10 to 20% substrate concentrations), it is necessary to determine how much glucose arises from the cleavage of cellobiose by β-glucosidase and how this varies with the nature and concentration of the cellulose saccharified. The approach used here is to limit the activity of β-glucosidase in crude cellulase preparations from *T. viride*, to determine the effect of this enzyme on glucose-cellobiose ratios and on the rate of hydrolysis of cellulose of varying susceptibilities.

MATERIALS AND METHODS

Cultural. The organism used throughout this study was an enhanced cellulase mutant of *Trichoderma viride* QM 9414. Stock cultures were stored at 24 to 27 C on potato dextrose agar slants. The medium was the one developed by Mandels and Reese (13) with 0.75% Solka-Floc BW 200 as the carbon source, and 0.2% Tween 80 and 0.075% proteose peptone as additives to increase enzyme production (15, 16). For citrate-buffered media 0.011 M citric acid-Na_3 citrate at pH 5.4 was incorporated in the medium. Flask cultures were grown in 2,800-ml Fernbach flasks containing 1 liter of medium and set on a rotary shaker at 250 rpm at 27 to 28 C.

Enzyme assays. Filter paper activity (FP'ase) was measured by the release of reducing sugar from Whatman no. 1 filter paper, and carboxymethyl cellulase (CMC'ase) by the increase in reducing power of a 0.5% CMC solution (type 50T, Hercules Powder Co., Wilmington, Del.) as described previously (11). β-Glucosidase was measured by incubating an appropriate amount of enzyme in 2 ml of a 10 mM solution of cellobiose at 50 C for 30 min; the reaction was stopped by submersion in boiling water for 5 min, and after cooling glucose was measured by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.). All enzyme activities are expressed in units as micromoles of glucose (or glucose equivalents) per minute. Extracellular protein was precipitated in 5% trichloroacetic acid and measured by the Folin method (10). All assays were performed at pH 5.0 in 0.05 M citrate buffer containing 0.01% merthiolate as a preservative. All assays are for extracellular enzymes.

Substrates. The various forms of cellulose used were: (i) dewaxed cotton sliver, a highly crystalline,

fibrous cellulose; (ii) Avicel PH 102 (FMC Corp., Newark, Del.), a microcrystalline cellulose having an average particle size of about 40 μm; (iii) Solka-Floc SW 40 (Brown Co., Berlin, N.H.), a purified wood cellulose which has been hammer-milled and passed through a 40-mesh screen; (iv) Solka-Floc BW 200, same as (iii) except ball-milled and passed through a 200-mesh screen; (v) Sweco-270, prepared from Solka-Floc SW 40 by milling in a Sweco vibratory mill for several days and passed through a 270-mesh screen; (vi) NP 200, ball-milled newspaper passed through 200-mesh screen; (vii) Walseth cellulose, an amorphous form of cellulose prepared by the method of Walseth (19) except that Solka-Floc BW 200 was the starting material; (viii) Whatman no. 1 filter paper.

Saccharifications. Extracellular enzyme was used for all saccharifications. The saccharification of 0.1% cellulose was carried out in test tubes containing 5-ml reaction mixtures and incubated at 50 C; the tubes were shaken frequently and at 15-min intervals one of each series was removed and immersed in a boiling water bath for 5 min to stop the reaction. The saccharification of 10% cellulose was accomplished in either 125-ml flasks containing 50 ml of reaction mixture or 50-ml flasks containing 10 ml of reaction mixture. These reactions were carried out at pH 5.0 and at 50 C with constant rotary shaking at 150 rpm.

Analysis of sugars. Total sugars solubilized from cellulose were measured by the phenol-H_2SO_4 method (3) after the removal of solids by filtration or centrifugation. The amount of glucose in the same solutions was determined by the glucostat method with the modification that δ-gluconolactone (0.4 mg/ml) was added to the solution immediately before starting the assay; this concentration of gluconolactone was sufficient to inhibit the β-glucosidase activity contaminating the glucostat reagent. Qualitative analyses of sugars were made by paper chromatography after the method of Mandels and Reese (13).

RESULTS

Enzyme production and stability. When the basal cellulose medium was inoculated with spores there was about a 2-day lag before extracellular enzymes and protein reached detectable levels; then they accumulated until the cellulose was exhausted (Fig. 1). In the medium without citrate buffer the pH dropped to around 2.7, and β-glucosidase activity remained low. When the medium was buffered with citrate so as not to fall below pH 5.0, β-glucosidase activity accumulated in the medium.

The cellulase enzymes were partially inactivated at pH levels which occur in the medium during growth (Fig. 2). Most of the β-glucosidase activity was lost between pH 3.4 and 3.0, whereas CMC'ase and FP'ase were relatively stable until the pH was adjusted below 3.0.

Cellulose saccharification. Nojirimycin is a potent inhibitor of glucosidases (17). When low concentrations (0.1%) of various cellulosic substrates were saccharified in the presence of nojirimycin (0.001%) the total amount of sugar was not affected but the amount of glucose was greatly reduced (Fig. 3). Chromatograms of these hydrolyzates revealed two major components corresponding to glucose and cellobiose (small amounts of xylose were also detected in SW 40, BW 200, and NP 200 hydrolysates). Therefore most of the sugar produced in the presence of nojirimycin appeared to be cellobiose. Assuming that glucose and cellobiose were the predominant soluble sugars, cellobiose levels were estimated by subtracting glucose from total sugar values for the Walseth hydrolysis, and the glucose values from the cellobiose plus nojirimycin hydrolysis were subtracted from the glucose values of the Walseth plus nojirimycin hydrolysis to give the amount of glucose arising other than from cellobiose cleavage (Fig. 4). In digests without nojirimycin cellobiose accumulated and then fell while glucose concentration rose. With nojirimycin a low level of glucose was produced early and remained constant while a high concentration of cellobiose was maintained. When a cellulase preparation in which β-glucosidase has been selectively inactivated at low pH was used to saccharify Walseth cellulose, the results (not shown) were the same as those with the addition of nojirimycin. These results with low cellulose concentrations indicated that most of the glucose present in digests arose from β-glucosidase action.

When high concentrations (10%) of cellulose were saccharified, the glucose and "cellobiose" patterns were quite different (Fig. 5). In these saccharifications two cellulase preparations with identical FP'ase and CMC'ase activities were used, but in one preparation the β-glucosidase was acid inactivated so the difference between the two preparations for this enzyme was about ninefold. With Avicel more glucose was present in the saccharification mixture containing high β-glucosidase activity, but with BW 200 glucose production was about the same with the two preparations. Furthermore, the amount of glucose produced from BW 200 with the low β-glucosidase preparation exceeded the amount produced from the hydrolysis of 3% cellobiose with the same enzyme. (A 3% cellobiose level was chosen because this was about the highest "cellobiose" concentration calculated for the BW 200 hydrolysate.) Although the difference in β-glucosidase activity for the two preparations was ninefold, the difference in glucose production from 3% cellobiose was only twofold.

Using two cellulase preparations, one with

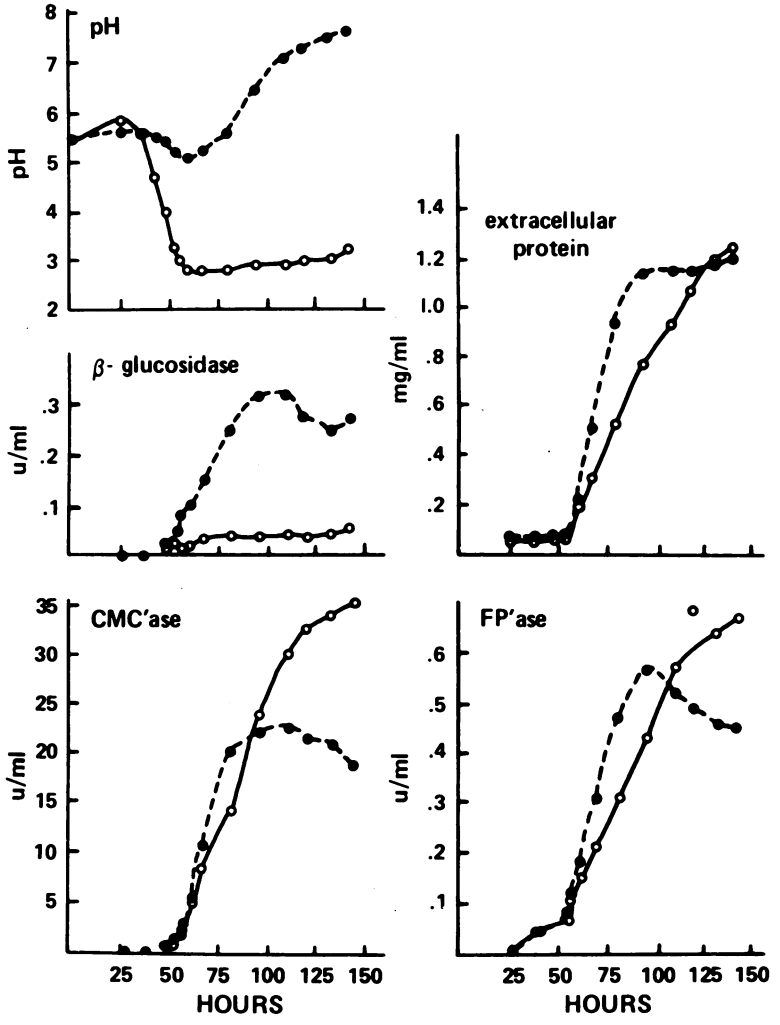

FIG. 1. *Enzyme and extracellular protein production and pH profiles in shake-flask cultures of Trichoderma QM 9414 on 0.75% Solka-Floc BW 200. Symbols: ○, Unbuffered medium; ●, medium buffered with 0.011 M citrate.*

high and the other with low β-glucosidase activity, 10% cellulose suspensions were hydrolyzed for 28 h and the supernatant fluids analyzed (Table 1). With the more crystalline cellulose (cotton sliver and Avicel) much more glucose was produced from the cellulase preparation having high β-glucosidase activity. With more susceptible cellulose (BW 200 and Sweco 270) the amounts of glucose produced from the two cellulase preparations were in closer agreement. The glucose-"cellobiose" ratio increased with increasing susceptibility of cellulose in the low β-glucosidase cellulolysis, whereas this ratio decreased with the high β-glucosidase activity series. Total hydrolysis by the low β-glucosidase and high β-glucosidase preparations was about equal for milled cellulose, but with more crystalline substrates the stimulation by high β-glucosidase increased, reaching 42% for the fibrous cotton.

Cellobiase activities. The K_m for β-glucosidase activity in crude culture filtrates was about 1.5 mM cellobiose (Fig. 6). No substrate inhibition was evident in the crude preparation, whereas purified β-glucosidase from *T. viride* exhibited substrate inhibition above 7.5 mM cellobiose (Fig. 7). After acid inactivation of β-glucosidase in a crude cellulase preparation, cellobiase activity was present but the affinity for cellobiose was low (K_m greater than

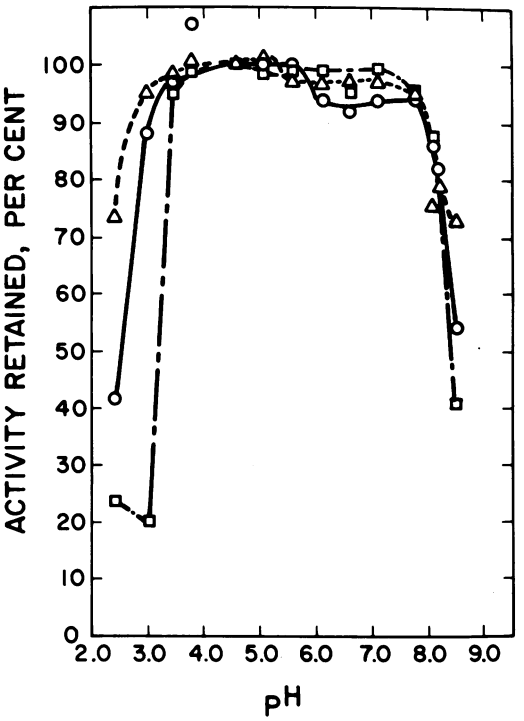

FIG. 2. *pH inactivation of cellulase enzymes. Samples of a culture filtrate were adjusted to pH levels indicated, incubated for 24 h at 27 C, adjusted to pH 5.0, and assayed for enzyme activity. Symbols: ○, FP'ase; △, CMC'ase; □, β-glucosidase.*

50 mM). The K_m and the substrate inhibition of the purified β-glucosidase agree with values reported by Berghem and Pettersson (2).

DISCUSSION

β-Glucosidase activities of filtrates of *Trichoderma* cultured on cellulose are low because of the acid conditions that develop in the medium during the time of maximum cellulase production. When the medium is buffered to about pH 5.0, extracellular β-glucosidase activity increases. Mandels et al. (14) have shown that by adjusting the pH downward in a starved culture, between pH 4.0 and 3.0, 90% of the β-glucosidase activity is lost, whereas FP'ase and CMC'ase remain stable.

When 0.1% cellulose is saccharified with a cellulase preparation with high β-glucosidase activity, most of the soluble product is glucose. When the glucosidase inhibitor nojirimycin is added, most of the soluble product appears to be cellobiose. Based on the analyses of Walseth hydrolyzates, it appears that 90% of the glucose is produced from the cleavage of cellobiose by β-glucosidase. The same value is obtained when β-glucosidase is inhibited by nojirimycin and when it is acid inactivated. This result supports the endoglucanase-cellobiohydrolase-β-glucosidase model of cellulolysis, which proposes that the primary soluble product is cellobiose and that glucose arises from the action of β-glucosidase on cellobiose. However when higher concentrations of milled cellulose are saccharified, it appears that a considerable amount of glucose arises from some process other than the cleavage of cellobiose because the amount of glucose produced from a low β-glucosidase cellulase preparation is about the same as with a cellulase preparation having high β-glucosidase activity. Moreover when low β-glucosidase cellulase is used, more glucose arises from the hydrolysis of milled cellulose than from cellobiose. Therefore, it appears that there is another route for glucose production besides the cleavage of cellobiose by β-glucosidase. The contribution of this other process to glucose production decreases with more crystalline sub-

TABLE 1. *Hydrolysis of cellulose substrates (10%) of differing susceptibilities, comparing preparations having high and low β-glucosidase incubated at 50 C for 28 h*

Substrate	Glucose (mg/ml)		Total sugar (mg/ml)		Cellobiose[a] (mg/ml)		Glucose-cellobiose ratio		Glucose (%)		Stimulation[b] (%)
	A[c]	B[d]	A	B	A	B	A	B	A	B	
Cotton sliver	2.1	6.4	6.9	9.8	4.8	3.4	0.43	1.90	30.4	65.3	42
Avicel PH102	6.7	16.4	22.7	26.7	16.0	6.3	0.42	1.59	29.5	61.4	18
BW 200	16.9	25.0	45.1	48.1	28.2	23.1	0.60	1.08	37.5	52.0	7
Sweco 270	32.2	36.2	70.0	73.5	37.8	37.3	0.85	0.97	46.0	49.3	5
Cellobiose (3%)	12.1	19.5	30.0	30.0	17.9	10.5					

[a] Cellobiose level estimated by subtracting glucose value from total sugar.

[b] Stimulation of hydrolysis by extra β-glucosidase for total sugar: B − A/A × 100.

[c] A, Low β-glucosidase cellulase preparation; FP'ase, 0.5 U/ml, CMC'ase, 25 U/ml, β-glucosidase, 0.05 U/ml.

[d] B, High β-glucosidase preparation; same as "A" except β-glucosidase = 0.26 U/ml.

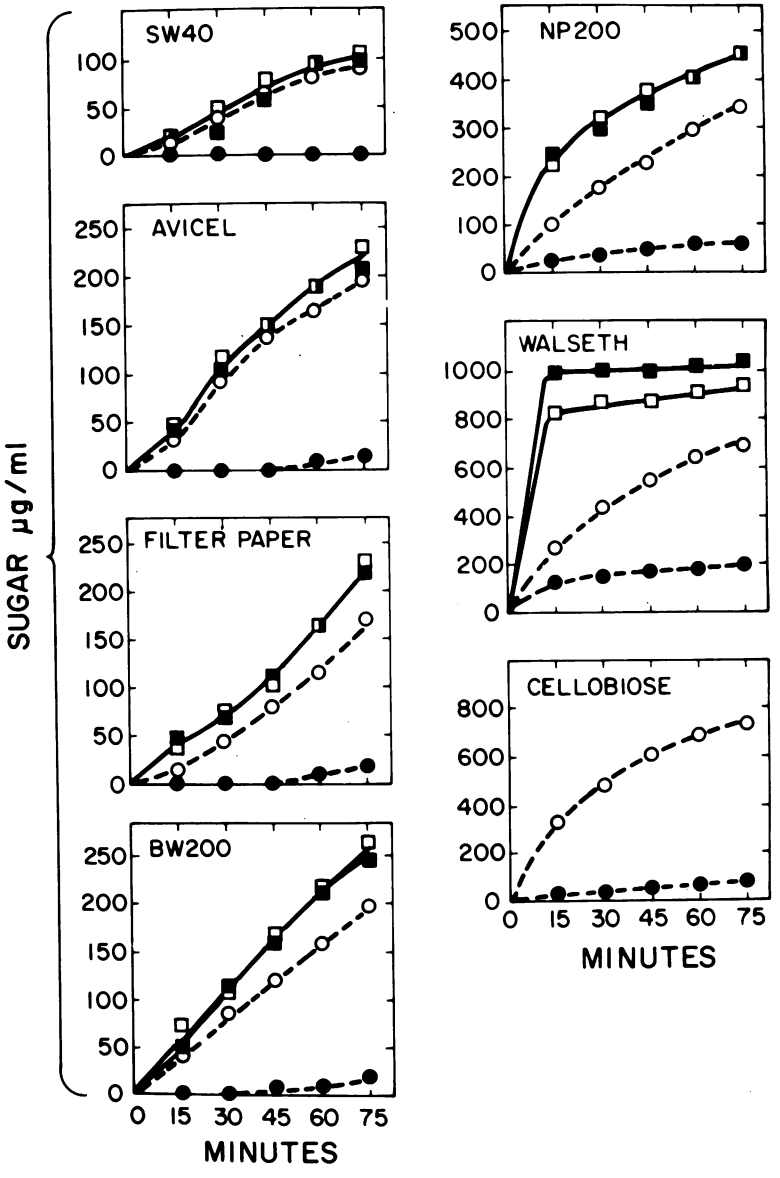

FIG. 3. *Effect of nojirimycin on saccharification of various cellulose substrates. Cellulose concentration = 1 mg/ml; nojirimycin concentration = 0.01 mg/ml. Symbols: ○, Glucose; □, total sugar. Open symbols, without nojirimycin; closed symbols, with nojirimycin. Substrates described in text.*

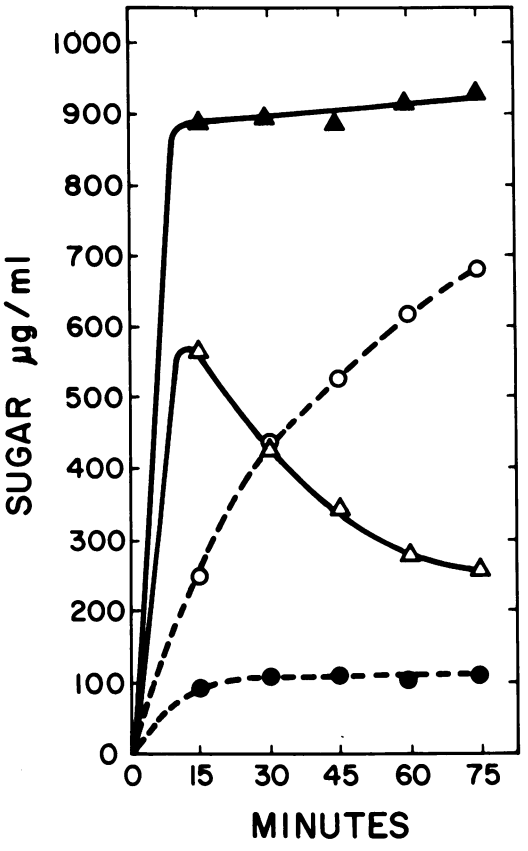

FIG. 4. *Glucose and calculated cellobiose levels in Walseth cellulose (1 mg/ml) hydrolysates. Symbols: ○, glucose; △, cellobiose. Open symbols, without nojirimycin; closed symbols, with nojirimycin (0.01 mg/ml).*

strates, i.e., with cotton sliver and Avicel, significantly more glucose is produced when β-glucosidase activity is higher. Even with so-called high β-glucosidase activity, the *Trichoderma* cellulase preparations acting on 10% cellulose gave a saccharification product ranging only from 65 to 50% glucose after 28 h. Therefore, under the conditions used here, the amount of β-glucosidase in *Trichoderma* cellulase appears to be insufficient if pure glucose is the desired saccharification product.

On the basis of kinetic patterns it appears that in crude cellulase preparations there is a mixture of cellobiase activities. One has a relatively high affinity for cellobiose, is acid labile, and is inhibited at cellobiose concentrations above 7.5 mM; the other has a low affinity for cellobiose, is more acid stable, and is not inhibited by high cellobiose concentrations. The latter enzyme appears to be responsible for much of the hydrolysis of the 3% cellobiose solutions with low β-glucosidase cellulase (Table 1 and Fig. 5). The low affinity of this enzyme for cellobiose (K_m greater than 50 mM) indicates that it may be a glucanase and perhaps is responsible for much of the glucose pro-

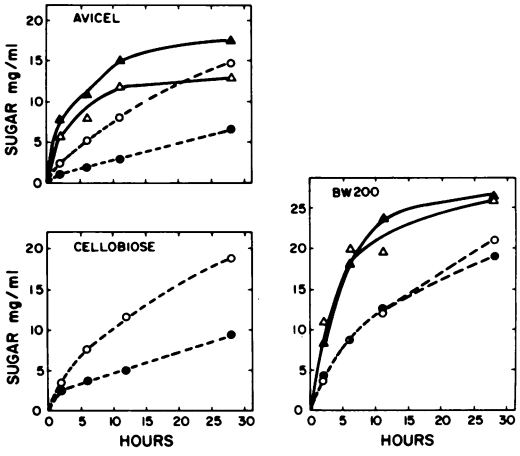

FIG. 5. *Effect of β-glucosidase activity on the hydrolysis of 10% cellulose and 3% cellobiose. Symbols: ○, Glucose; △, cellobiose (calculated). Open symbols, high β-glucosidase preparation: FP'ase = 0.56 U/ml, CMC'ase = 22 U/ml, β-glucosidase = 0.36 U/ml. Closed symbols = low β-glucosidase preparation: same enzyme activities as above except β-glucosidase = 0.04 U/ml.*

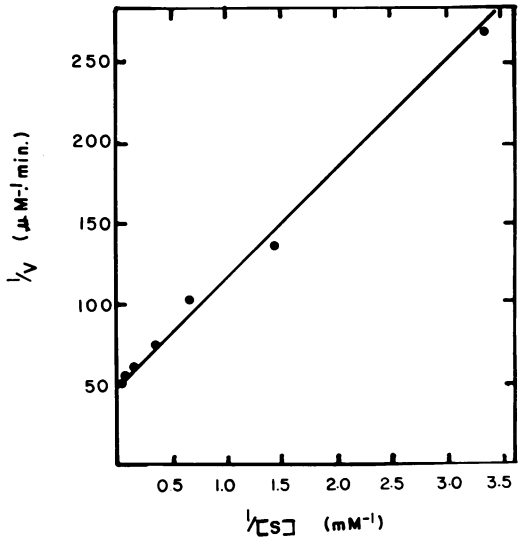

FIG. 6. *Lineweaver Burk plot of β-glucosidase in a crude culture filtrate with cellobiose as the substrate.*

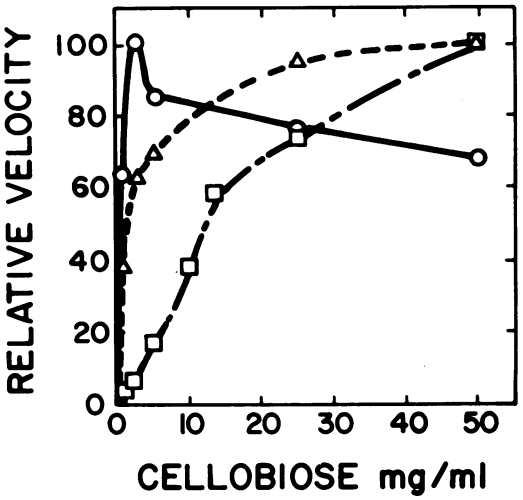

FIG. 7. *Relative velocity of glucose production versus cellobiose concentration. Symbols: ○, β-glucosidase purified from T. viride QM 9414; △, crude cellulase preparation having high β-glucosidase activity; □, crude cellulase preparation having low β-glucosidase activity.*

duced from the saccharification of high concentrations of cellulose with cellulose preparations with little or no β-glucosidase activity.

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