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Immobilization of *Aspergillus* Beta-Glucosidase on Chitosan

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β-Glucosidase of *Aspergillus phoenicis* QM 329 was immobilized on chitosan, using the bifunctional agent glutaraldehyde. The most active preparation based on the amount of support contained a 1:2.5 enzyme-to-chitosan ratio (wt/wt). However, the specific activity of the bound enzyme decreased from 10 to 1% with increasing enzyme-to-chitosan ratio. Compared with free β-glucosidase, the immobilized enzyme exhibited: (i) a similar pH optimum but more activity at lower pH values; (ii) improved thermal stability; (iii) a similar response to inhibition by glucose; and (iv) mass transfer limitations as reflected by higher apparent K_m and lower energy of activation.

It has been demonstrated that the rate of saccharification of cellulose by *Trichoderma* cellulases can be significantly increased by supplementation with β-glucosidase obtained from *Aspergillus phoenicis* (13). The purpose of this investigation is to evaluate the usefulness of immobilized β-glucosidase in the saccharification of cellulose. The stimulatory effect of β-glucosidase appears to be due to the hydrolysis of cellobiose, which is an inhibitor of the cellulase enzymes (5, 7, 8, 15). In turn, glucose inhibits β-glucosidase activity (4). High levels of glucose must be tolerated in practical saccharifications of cellulose in which a glucose syrup is the desired end product. Therefore, β-glucosidase activity is strongly inhibited during the reaction, and the level of this enzyme must be increased to obtain maximal rates.

Unlike the cellulase enzymes, β-glucosidase attacks a soluble molecule (cellobiose) and so should be appropriate for immobilization because the substrate and product can diffuse in and out of the supporting matrix. Furthermore, by immobilizing β-glucosidase the same enzyme may be used for subsequent saccharifications. β-Glucosidases from sweet almonds and from *Alcaligenes faecalis* have been immobilized previously on cellulose carbonate (2) and cyanogen bromide-activated cellulose (12), respectively. Also, β-glucosidase from almonds has been used in a hollow fiber reactor to hydrolyze salicin (14). α-Chymotrypsin and acid phosphatase have been immobilized on chitosan (deacetylated chitin) with and without glutaraldehyde (9). This paper describes the immobilization of β-glucosidase on chitosan by glutaraldehyde and compares some of the properties of the immobilized and free enzyme.

MATERIALS AND METHODS

Preparation of enzyme. β-Glucosidase was isolated from culture filtrates of *A. phoenicis* QM 329 and purified by acetone precipitation and kaolin adsorption (13). The enzyme preparation used in these studies had a specific activity of 140 U/mg with cellobiose as substrate.

Immobilization. Chitosan, obtained from Pfanstiehl Laboratories Inc., was dissolved in sufficient 0.5% acetic acid to make a 0.25% chitosan solution (pH 3.3). A known amount of (powdered) β-glucosidase was then dissolved in an aliquot of the chitosan solution. After standing for 30 min at room temperature, an aliquot of 2.5% glutaraldehyde in 0.05 M citrate buffer (pH 4.5) was added such that the final concentration of glutaraldehyde was approximately 0.2%.

The reaction was allowed to proceed for 2 h at room temperature, and then the solution was poured into 6 volumes of acetone with stirring. The immobilized enzyme precipitated and was removed by filtration. The residue was washed with citrate buffer until no activity or protein could be detected in the washings. This material, which was kept moist with buffer, was then used in the following studies.

Enzyme assays. β-Glucosidase activity was measured by the glucose produced from a 7.5 mM solution of cellobiose incubated at 50°C in 0.05 mM citrate buffer at pH 4.8. For soluble enzyme, the assays were carried out in 2-ml reactions and stopped by submerging in boiling water for 5 min. Unless otherwise noted, the immobilized enzyme (samples from 2 mg of enzyme per 25 mg of chitosan) was assayed in screw-cap tubes submerged horizontally in a water bath and shaken reciprocally at 220 strokes/min with a 4.5-cm amplitude; the reaction was stopped by centrifugation and removal of the supernatant, which was submerged in boiling water for 5 min. When cool, glucose was measured by the Glucostat reagent (Worthington Biochemicals Corp., Freehold, N.J.). One unit of activity is the amount of enzyme producing 1 μmol of glucose per min.

Sugar analysis by liquid chromatography. Carbohydrate analyses were performed on a Micromeritic model 7000 liquid chromatograph. The sugars were separated on a Waters μ Bondapak carbohydrate analysis column (3.9 mm ID by 30 cm), using acetonitrile-water (75:25) as eluting solvent, and detected by a LDC Refracto-Monitor detector.

RESULTS

Effect of pH. The pH optimum for both the immobilized and free form of the enzyme was approximately pH 4.5 (Fig. 1). However, the immobilized enzyme showed greater activity at lower pH. This effect may be caused by the microenvironmental pH of the chitosan matrix. At high H^+ concentrations the amino groups of the chitosan would be protonated, thereby attracting hydroxyl ions which would maintain a higher microenvironmental pH than in the bulk solution and thus stabilize the β-glucosidase (2, 11). The similarity on the alkaline side of the optimum cannot be so easily explained.

Effect of temperature. For 30-min assay intervals, the optimum temperature was 15 to 20°C higher for the immobilized enzyme (Fig. 2). Above 75°C the immobilized activity fell due to destruction of the enzyme. The temperature data were replotted in the form of Arrhenius plots (Fig. 3), from which the energy of activation was calculated as 11.95 Kcal/mol for the free enzyme and 7.93 for the immobilized enzyme. A similar decrease in energy of activation has been found with immobilized invertase (3).

A comparison of the thermal stability of the two forms of β-glucosidase is shown in Table 1. The free form of β-glucosidase was stable at 50°C for 40 days, and even after 67 days 85% of the original activity was still retained. However, at 65 and 70°C the free enzyme was rapidly inactivated, whereas at these temperatures the immobilized enzyme was relatively stable (half-life of 10.5 and 1.8 days, respectively). When the log of the activity retained was plotted against time at the temperature used for inactivation, the free enzyme always gave straight-line plots, indicating a first-order reaction. But at temperatures below 80°C for the immobilized enzyme, a straight-line relationship was not consistently found (Fig. 4). A similar situation has been observed for immobilized glucose isomerase (6). A possible explanation for "non-first-order decay" might be that the number of cross-links to the support, to other enzyme molecules, and within

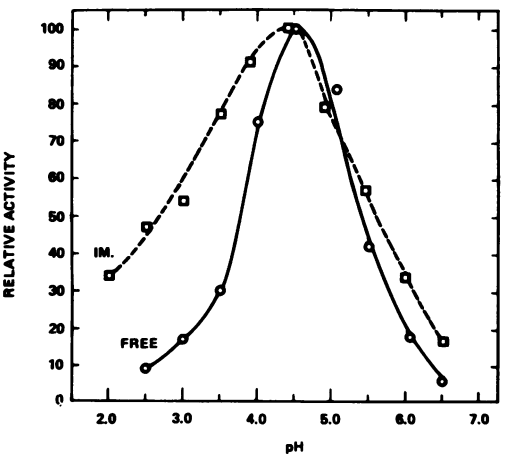

FIG. 1. *Effect of pH on β-glucosidase activity. Activity based on 30-min assays at 50°C using 0.05 M citrate buffer. Symbols:* (○) *Free enzyme;* (□) *immobilized enzyme.*

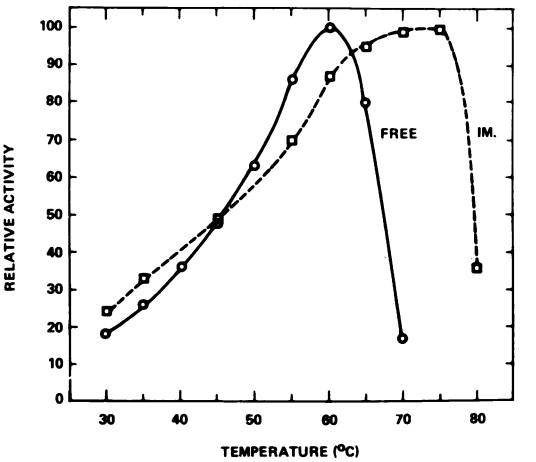

FIG. 2. *Effect of temperature on β-glucosidase activity. Activity based on 30-min assays at pH 4.8 in 0.05 M citrate buffer. Symbols:* (○) *Free enzyme;* (□) *immobilized enzyme.*

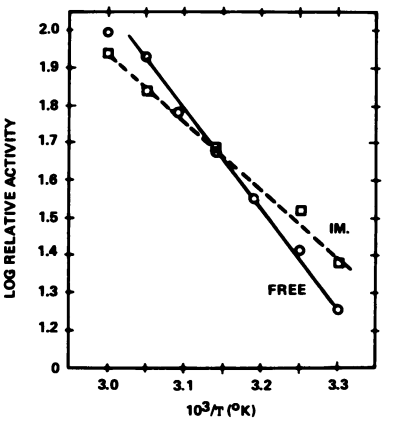

FIG. 3. *Arrhenius plot from data in Fig. 2. Symbols:* (○) *Free enzyme, μ = 11.95 Kcal/mol;* (□) *immobilized enzyme, μ = 7.93 Kcal/mol.*

a single enzyme molecule varied, resulting in heterogeneity in the thermal stability of the enzyme.

Specificity. The immobilized β-glucosidase, like the free enzyme, cleaved a variety of β-glucosidic linkages. The rate of cleavage of both aryl and alkyl glucosides relative to cellobiose was greater with immobilized β-glucosidase than with the free enzyme (Table 2). It was not clear whether this was due to a change in affinity of the enzyme for these substrates or to the rate of mass transfer peculiar to immobilized systems.

Km. The K_m of the free β-glucosidase was 0.8 mM cellobiose, and substrate inhibition occurred above 10 mM (13). The apparent K_m of the immobilized enzyme varied with the activity present in the assay (Fig. 5). With relatively high activity (V_{max} = 0.06 μmol/min) the K_m was about 5.0 mM cellobiose, whereas at lower activity (V_{max} = 0.02 μmol/min) it was 3.7 mM. The activity in the assay was increased by using a larger sample of the same immobilized preparation. Substrate inhibition was observed only above 40 to 50 mM cellobiose.

TABLE 1. *Thermal stability of free and immobilized β-glucosidase*[a]

Temp (°C)	Half-life (h)	
	Free	Immobilized
55	216	
60	8.6	
65	0.5	252
70	0.04	43
75		1.6
80		0.07

[a] Enzymes were incubated at the designated temperatures in 0.05 M citrate buffer, pH 4.8, and then assayed for retention of activity.

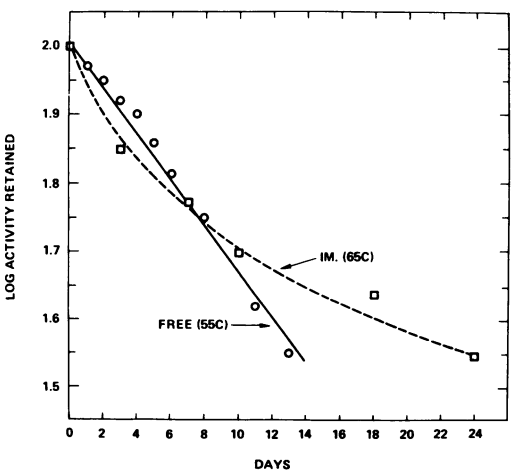

FIG. 4. *Semilog plot of thermal inactivation of β-glucosidase in 0.05 M citrate buffer at pH 4.8. Free enzyme was incubated at 55°C and the immobilized enzyme was incubated at 65°C for the times indicated and assayed under standard conditions. Symbols:* (○) *Free enzyme;* (□) *immobilized enzyme.*

TABLE 2. *Relative activity of free and immobilized β-glucosidase on various β-glucosides*[a]

Substrate	Relative activity	
	Free	Immobilized
Cellobiose	100	100
Cellotriitol	45	53
p-Nitrophenyl-β-glucoside	28	50
Amygdalin	25	55
Salicin	14	21
Methyl-β-glucoside	3	13

[a] Assay conditions: pH 4.8 (0.05 M citrate buffer), 30 min, 50°C, 7.5 mM substrate.

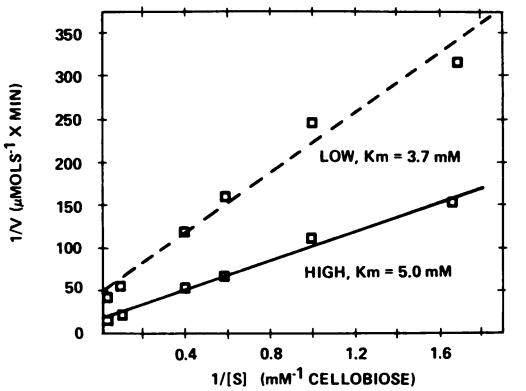

FIG. 5. *Lineweaver and Burk plots to determine apparent K_m of immobilized β-glucosidase at two levels of activity. Samples of immobilized enzyme were taken from the same preparation with approximately three times more sample in the "high"* (——) *determination than in the "low" determination* (-----).

Glucose inhibition. On the basis of a Dixon plot (Fig. 6), the cleavage of cellobiose by free β-glucosidase appeared to be competitively inhibited by glucose. The K_i based on the region of intersection of the three slopes was approximately 1.8 mM glucose. From the K_m and K_i values for the free enzyme, a theoretical rate curve for the hydrolysis of 1.0% cellobiose with various initial concentrations of glucose was calculated, and rates with free and immobilized β-glucosidase were experimentally measured. Experimental data points using free and immobilized β-glucosidase fell about the calculated curve when the velocity of the reaction was measured on the basis of glucose production (Fig. 7). Experimental data points based on the decrease in cellobiose were consistently above

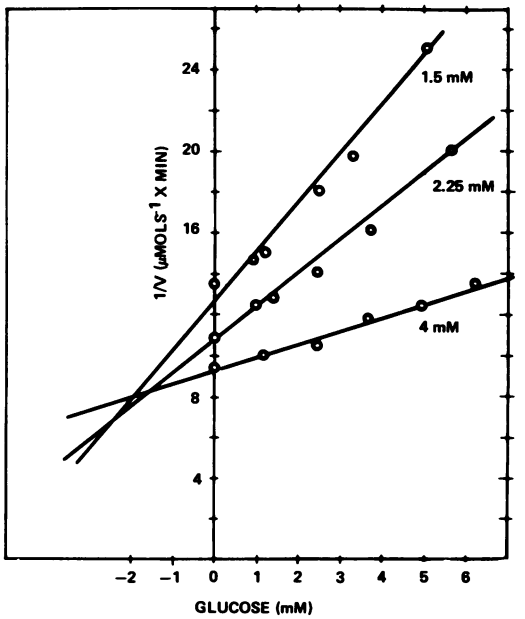

FIG. 6. *Dixon plot for inhibition by glucose of β-glucosidase activity of the free enzyme, using 1.5, 2.25, and 4.0 mM cellobiose. K_i is approximately 1.8 mM glucose.*

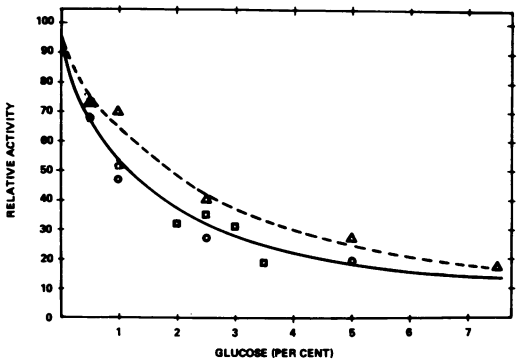

FIG. 7. *Effect of glucose concentration on the hydrolysis of 1% cellobiose by β-glucosidase in 0.05 M citrate buffer, pH 4.8, incubated at 50°C. Solid line represents the calculated curve based on K_m = 0.8 mM cellobiose and K_i = 1.8 mM glucose. Symbols: (○) Free enzyme activity based on glucose production; (□) immobilized enzyme activity based on glucose production; (△) free enzyme activity based on decrease in cellobiose. Glucose production was measured by the Glucostat method; decrease in cellobiose was measured by liquid chromatography.*

the calculated values; this difference might be due to the transglycosylating activity of the β-glucosidase. The purpose of collecting these experimental inhibition points was to confirm the degree of β-glucosidase inhibition that would occur in a practical saccharification in which 10 to 15% cellulose slurries are hydrolyzed and high levels of glucose are produced.

Specific activity. The activity of the immobilized β-glucosidase was determined from the rate at which 50 ml of a 1% cellobiose solution was hydrolyzed at 50°C. A plot of glucose produced versus time gave a straight line from 0% to approximately 60% hydrolysis before any deviation from linearity was noted. It was this linear portion that was used to calculate activity. The results obtained from representative samples are shown in Table 3.

The amount of activity retained on the chitosan varied from 1 to 10%, depending on the enzyme loading used. Less than 3% of the initial enzyme activity was found in the washings collected during the preparation. Free enzyme treated with glutaraldehyde under the same conditions did not lose activity. When the immobilization was carried out in the presence of 5% cellobiose, the retention of activity was somewhat greater. The loss of activity, which may be caused by conformational changes on immobilization and/or increasing mass transfer effects, is being investigated further.

Although β-glucosidase can be immobilized on chitosan at a loading level in excess of 1:1 on a weight basis, this high level of loading does not give a product with high activity. Similar observations have been made with acid phosphatase immobilized on chitosan, and it was suggested that the loss of specific activity may be due to inactivation of the enzyme when it is present in excess (9). Our most active material was prepared from 10 mg of enzyme powder and 25 mg of chitosan cross-linked with 0.2% glutaraldehyde.

In the samples shown in Table 3, the immobilized enzyme was free floating in the solution. However, if this material is to be used in a saccharification, it must be confined. To determine the effect of confinement on activity, a sample of immobilized enzyme was sandwiched between layers of fine-mesh stainless-steel

TABLE 3. *Effect of enzyme-chitosan ratio on the activity of immobilized β-glucosidase*

Enzyme (mg)	Chitosan (mg)	t_{50} (min)[a]	Immobilized material (U/mg)	
			Chitosan	Enzyme
1	25	96	0.58	14.5
2	25	65	0.85	10.7
5	25	64	0.87	4.3
10	25	35	1.59	3.9
20	25	48	1.15	1.5

[a] t_{50}, Time for 50% conversion of cellobiose to glucose.

screening and assayed as described above. It was found that the reaction rate was identical to that of the free-floating sample.

DISCUSSION

The reason for investigating immobilized β-glucosidase is to assess its potential usefulness in the saccharification of cellulose with cellulase enzymes obtained from *Trichoderma*. In this respect, immobilization of β-glucosidase from *A. phoenicis* on chitosan has some favorable aspects, but also presents some problems.

A favorable property is the pH optimum of 4.5 for both the free and immobilized enzyme, which is close to the optimum, pH 4.8, for enzymatic cellulolysis. The optimum temperature for cellulose saccharification is 50°C. At this temperature the free and immobilized enzymes are stable. At higher temperatures the stability of the immobilized enzyme is significantly improved over that of the free enzyme. The degree of inhibition by glucose is about the same for free and immobilized β-glucosidase. Therefore, the kinetics of cellobiose hydrolysis in batch saccharifications where glucose concentrations are high should be similar for the two forms of the enzyme.

The rate of mass transfer of the substrate and products to and from immobilized enzymes presents problems not found with free enzymes. For the substrate to be acted upon by an immobilized enzyme, it must diffuse into the matrix and the products must diffuse out. These diffusional processes often result in a lower concentration of substrate and a higher concentration of product at the enzyme site than in the bulk solution. With increasing enzyme activity, the diffusional problems become more significant. Some of the results presented here can be interpreted as representing mass transfer effects. The apparent lower energy of activation for the immobilized enzyme (7.9 versus 11.9 Kcal/mol) has been interpreted as an indication of diffusional limitation (10). At higher temperatures, the reaction rate of the immobilized relative to the free enzyme is less because of the diffusional limitation of substrate and product. Likewise, the increase in K_m with increasing enzyme activity, as well as the increase in substrate concentration required for substrate inhibition, is another indication of diffusional limitation (10). The increased activity of the immobilized enzyme on aryl and alkyl β-glucosides may also reflect the influence of mass transfer rates in that if the diffusion rates of the various substrates are approximately equal, the concentration of substrate in the immobilized matrix will be higher, and the product lower, the slower the substrate is cleaved. This could result in higher relative activities with these substrates.

Another problem with the use of immobilized β-glucosidase is its low specific activity, varying from 1 to 10% of the free enzyme. At the 10% level, the immobilized material would have to be reused at least 10 times just to compete economically with using free β-glucosidase without recovery.

Gong et al. (4) have demonstrated glucose inhibition of β-glucosidase from *Trichoderma*. The degree of glucose inhibition for *A. phoenicis* β-glucosidase (used in this study) appears comparable with that of the *Trichoderma* system. If there were no inhibition by glucose, the amount of β-glucosidase produced by *Trichoderma* theoretically would be sufficient for the complete conversion of cellobiose to glucose throughout a saccharification in which glucose concentrations of at least 5% are obtained. Glucose inhibition of the β-glucosidase in *Trichoderma* cellulases necessitates the supplementation of this enzyme in such saccharifications (13). It is desirable to keep the cellobiose concentration in the saccharification reaction as low as possible (less than 2 mg/ml) to prevent inhibition of the cellulase enzymes. Inhibition by glucose is more pronounced at low cellobiose concentrations. For example, when the glucose concentration reaches 50 mg/ml and the cellobiose concentration is 2 mg/ml, the calculated β-glucosidase activity is only 5% of maximum. The development of an immobilized β-glucosidase offers a means of raising this enzyme's activity in a reaction while conserving the enzyme by permitting repetitive use.

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LITERATURE CITED

1. **Barker, S. A., S. H. Doss, C. J. Gray, J. F. Kennedy, M. Stacy, and T. H. Yeo.** 1971. β-D-glucosidase chemically bound to microcrystalline cellulose. Carbohydr. Res. **20**:1–7.
2. **Bernath, F. R., and W. R. Vieth.** 1974. Collagen as a carrier for enzymes: materials science and process engineering aspects of enzyme engineering, p. 157–185. *In* A. C. Olson and C. L. Cooney (ed.), Immobilized enzymes in food and microbial processes. Plenum Press, New York.
3. **Dickensheets, P. A., L. F. Chen, and G. T. Tsao.** 1977. Characteristics of yeast invertase immobilized on porous cellulose beads. Biotechnol. Bioeng. **19**:365–375.
4. **Gong, D.-S., M. R. Ladisch, and G. T. Tsao.** 1977. Cellobiase from *Trichoderma viride*: purification, properties, kinetics, and mechanism. Biotechnol. Bioeng.

19:959–981.
5. **Halliwell, G.** 1975. Action of the cellulase complex, p. 319–336. *In* M. Bailey, T.-M. Enari, and M. Linko (ed.), Symposium on the Enzymatic Hydrolysis of Cellulose. The Finnish National Fund for Research and Development, Helsinki.
6. **Havewala, N. B., and W. H. Pitcher, Jr.** 1974. Immobilized glucose isomerase for the production of high fructose syrups, p. 315–328. *In* E. K. Pye and L. B. Wingard (ed.), Enzyme engineering, vol. II. Plenum Press, New York.
7. **Howell, J. A., and J. D. Stuck.** 1975. Kinetics of Solka Floc cellulose hydrolysis by *Trichoderma viride* cellulase. Biotechnol. Bioeng. **17**:873–893.
8. **Maguire, J. R.** 1977. Kinetics of the hydrolysis of cellulose by β-1,4-glucan cellobiohydrolase of *Trichoderma viride*. Can. J. Biochem. **55**:644–650.
9. **Muzzarelli, R. A. A., G. Barontini, and R. Rocchetti.** 1976. Immobilized enzymes on chitosan columns: α-chymotrypsin and acid phosphatase. Biotechnol. Bioeng. **18**:1445–1454.
10. **Pitcher, W. H., Jr.** 1975. Design and operation of immobilized enzyme reactors, p. 151–199. *In* R. A. Messing (ed.), Immobilized enzymes for industrial reactors. Academic Press Inc., New York.
11. **Royer, G. P.** 1975. The kinetics of immobilized enzymes, p. 49–91. *In* H. H. Weetall (ed.), Immobilized enzymes, antigens, antibiotics, and peptides. Marcel Dekker, Inc., New York.
12. **Srinivasan, V. R., and M. W. Bumm.** 1974. Isolation and immobilization of β-D-glucosidase from *Alcaligenes faecalis*. Biotechnol. Bioeng. **16**:1413–1418.
13. **Sternberg, D., P. Vijayakumar, and E. T. Reese.** 1977. β-Glucosidase: microbial production and effect on enzymatic hydrolysis of cellulose. Can. J. Microbiol. **23**:139–147.
14. **Van Dongen, D. B., and D. O. Cooney.** 1977. Hydrolysis of salicin by β-glucosidase in a hollow fiber reactor. Biotechnol. Bioeng. **19**:1253–1258.
15. **Wood, T., and S. I. McCrae.** 1975. The cellulase complex of *Trichoderma koningii*, p. 231–254. *In* M. Bailey, T. M. Enari, M. Linko (ed.), Symposium on Enzymatic Hydrolysis of Cellulose. The Finnish National Fund for Research and Development, Helsinki.