Glycosidases induced in *Aspergillus tamarii*

Secreted α -D-galactosidase and β -D-mannanase

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An α -D-galactosidase (EC 3.2.1.22) and a β -D-mannanase (EC 3.2.1.78), which were secreted into the growth medium when *Aspergillus tamarii* was cultivated in the presence of galactomannan, were purified by a procedure including chromatography on hydroxyapatite and DEAE-cellulose columns. Each of these enzymes showed a single protein band, corresponding to their respective activities, on polyacrylamide-gel electrophoresis. Both enzymes were shown to be glycoproteins containing *N*-acetylglucosamine, mannose and galactose, with molar proportions of 1:6:1.5 for α -D-galactosidase and 1:13:8 for β -D-mannanase. M_r values as determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and by the electrophoretic method of Hedrick & Smith [(1968) *Arch. Biochem. Biophys.* **126**, 155–164] were 56000 and 53000 respectively. The α -D-galactosidase differed markedly from the mycelial forms I and II studied in the preceding paper [Civas, Eberhard, Le Dizet & Petek (1984) *Biochem. J.* **219**, 849–855] with regard to both its kinetic and structural properties.

In the preceding paper (Civas *et al.*, 1984) we studied two internal α -D-galactosidases, namely α -galactosidases I and II, induced in *Aspergillus tamarii* when raffinose was used as carbon source. We also noted the existence of a third α -D-galactosidase (α -galactosidase III) as well as a β -D-mannanase, which are secreted into the culture medium when the galactomannan of *Trifolium repens* (white clover) or guar galactomannan was substituted for raffinose.

Comparative studies on intra- and extra-cellular glycoenzymes have been published by Rudick *et al.* (1979) for α -glucosidases of *Aspergillus niger*. The invertases (β -D-fructofuranosidases) of *Phytophthora megasperma* have also been studied in three races of the same fungus (Ziegler & Albersheim, 1977; West *et al.*, 1980). Furthermore, extensive studies on cell-bound and secreted enzymes have been done on yeast invertases (Gascon & Lampen, 1968; Gascon *et al.*, 1968; Babczinski & Tanner, 1978).

The present paper describes the purification and some properties of β -D-mannanase and α -D-galac-

tosidase III, in order to compare the structural similarities, if any, of these extracellular enzymes and to compare the kinetic properties of α -galactosidase III with those of the mycelial α -galactosidases I and II.

Experimental and results

Materials

o-Nitrophenyl α -D-galactoside, melibiose, raffinose and stachyose and the galactomannans of *Trifolium repens* (white clover), *Medicago sativa* [lucerne (alfalfa)], *Ceratonía siliqua* (locust bean) and *Cyamopsis* sp. (guar) were obtained or prepared as described in the preceding paper (Civas *et al.*, 1984). The glucomannan of *Orchis intacta* (salep) was prepared by the procedure of Daloul *et al.* (1963), and the mannan from *Phytelephas macrocarpa* (ivory nut) was prepared by the procedure of Le Dizet (1963).

Hydroxyapatite was prepared by a modification (Levin, 1962) of the method of Tiselius *et al.* (1956). DEAE-cellulose was from Whatman Biochemicals (Maidstone, Kent, U.K.); it was precycled before use and stored at 4°C in 20mM-sodium acetate buffer, pH4.1. Concanavalin A–Ultrogel was

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provided by the Institut Biologique Français (Villeneuve La Garenne, France). Coomassie Brilliant Blue R-250 and G-250 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine albumin was from Koch-Light Laboratories (Colnbrook, Bucks., U.K.), egg albumin was from Calbiochem (Los Angeles, CA, U.S.A.), phosphorylase *a* and pepsin were from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), and trypsin was from Armour Laboratories (London E.C.1, U.K.). Nycoline [poly(ethylene glycol) 6000] was obtained from Lyon-Schmidt (Noisy Le Sec, France).

Aspergillus tamarii (IP 1017-10) was provided by the Service de Mycologie of the Institut Pasteur (Paris, France). The Raulin medium used for mycelial cultivation was supplemented by the addition of 1g of the white-clover galactomannan/l, and autoclaved for 20 min at 120°C before use. Each Roux bottle (125 ml) was inoculated with 1 ml of aqueous suspension of conidia. Six days at 29°C were necessary to achieve maximal enzymic activity for α -D-galactosidase III and β -D-mannanase. The mycelia were removed by filtration through cheesecloth, and the filtrate was used as a crude extract.

Assay of enzyme activity

The assay for α -galactosidase activity on *o*-nitrophenyl α -D-galactoside, melibiose, raffinose and stachyose was as described in the preceding paper (Civas *et al.*, 1984). The standard assay procedure for β -D-mannanase activity was by incubation of 100 μ l of suitably diluted enzyme with 100 μ l of salep glucomannan (0.1%, w/v) and 50 μ l of McIlvaine (1921) buffer (0.2 M- Na_2HPO_4 /0.1 M-citric acid, pH 4.8) at 37°C. Reducing sugars liberated by enzymic hydrolysis were determined by the Somogyi-Nelson (Somogyi, 1945; Nelson, 1944) micro-dosage technique (Williams *et al.*, 1978). Specific activity was expressed as μ mol of mannose liberated/min per mg of protein at 37°C.

Protein was determined by the technique of Sedmak & Grossberg (1977), with the use of Coomassie Brilliant Blue G-250, with bovine albumin as the protein standard.

Gel-electrophoresis methods and the procedure for g.l.c. have been given previously (Civas *et al.*, 1984).

Thin-layer chromatography

Products of the enzymic hydrolysis of the galactomannans and mannan were detected by t.l.c. on silica-gel-coated plastic sheets F 1500 (Schleicher und Schüll, Dassel, Germany). The solvent system was propan-1-ol/ethanol/ethyl acetate/acetic acid/pyridine/water (7:3:3:2:2:3,

by vol.). Chromatograms were subsequently developed with a solution of β -naphthylamine (Petek, 1962), followed by treatment with 2% (v/v) H_2SO_4 in acetone.

Purification

The crude extract (an average volume of 1250 ml) was placed in a dialysis bag in contact overnight with poly(ethylene glycol) powder. The concentrated solution (25–50 ml) was dialysed against water for 3–4 h and centrifuged at 13000g for 20 min at 4°C.

Column I. The clear supernatant (75–100 ml) was applied to a column (180 mm $\times$ 22 mm) of hydroxyapatite equilibrated with 5 mM-potassium phosphate buffer, pH 5.7. The column was eluted at a flow rate of 75 ml/h. β -D-Mannanase activity, representing 20% of total β -D-mannanase activity, was eluted by washing with 5 mM buffer. After washing with 20 mM-potassium phosphate buffer at the same pH, the major part of the β -D-mannanase activity (56%) was eluted with 50 mM-potassium phosphate buffer (F1). The average pooled volume of active fractions was 400 ml. α -Galactosidase III was subsequently eluted with 100 mM buffer (F2).

Column IIa. The pooled β -D-mannanase (F1) was concentrated before dialysis in an Amicon ultracentrifugation apparatus [PM 10 Diaflo membrane; 1.5 atm (150 kPa)]. After dialysis, this fraction was applied to a column (100 mm $\times$ 22 mm) of DEAE-cellulose equilibrated with 20 mM-sodium acetate buffer, pH 4.1. After washing with 50 mM buffer, β -D-mannanase activity was eluted with 100 mM-sodium acetate buffer, pH 4.1. The β -D-mannanase so obtained showed a single protein band on polyacrylamide-disc-gel electrophoresis (Fig. 1).

Column IIb. The α -D-galactosidase activity eluted from the first column of hydroxyapatite (F2) was contaminated with minor β -D-mannanase activity and β -D-fructosidase. The dialysed eluate was applied to a column (140 mm $\times$ 22 mm) of DEAE-cellulose equilibrated with 20 mM-sodium acetate buffer, pH 4.1. The column was eluted at a flow rate of 24 ml/h, in a stepwise manner with increasing concentrations (50 mM and 100 mM) of sodium acetate. α -D-Galactosidase III was eluted with 100 mM buffer containing 30 mM-NaCl (F2b).

Column IIb. Fraction 2b was applied to a second column (100 mm $\times$ 22 mm) prepared and eluted as above; the enzyme III was eluted with 100 mM-acetate buffer containing 30 mM-NaCl, and showed a single protein band on polyacrylamide-disc-gel electrophoresis (Fig. 1).

Table 1 summarizes the purification procedure beginning with 1280 ml of crude extract.

Table 1. Purification of α -galactosidase III and β -D-mannanase

Enzyme activities were tested under standard conditions as described in the Experimental and results section. The substrate was 20mM-*o*-nitrophenyl α -D-galactoside for α -galactosidase, and saleg glucomannan (0.1%, w/v) was used for β -D-mannanase.

β -D-Mannanase	Volume (ml)	Total activity (μ mol of mannose/min)	Total protein (mg)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
Crude extract	1280	20	7.0	2.9	100	1
Column I (hydroxyapatite)						
Concentration on PM-10	21	11.2	0.8	13.7	56.2	4.8
Column IIa (DEAE-cellulose)						
Concentration on PM-10	18.7	1.7	0.07	26	8.3	9.0
α -D-Galactosidase		(μ mol of galactose/min)				
Crude extract	1280	382.4	7.0	54.6	100	1
Column I (hydroxyapatite)	96	199.2	0.7	274.1	52	5
Column IIb (DEAE-cellulose)	46	112.2	0.2	446.0	29.3	8.2
Column IIIb (DEAE-cellulose)	53.6	87.2	0.2	452	22.8	8.3

Fig. 1. Polyacrylamide (7.5%)-gel electrophoresis of α -D-galactosidase III and β -D-mannanase

(1) Crude extract. (2) α -Galactosidase III: column IIIb (DEAE-cellulose). (3) β -Mannanase: column IIa (DEAE-cellulose).

Determinations of M_r

For both enzymes, α -D-galactosidase III and β -D-mannanase, electrophoretically pure enzyme was examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Each gave a single protein band, of M_r 56000 ± 1000 and 53000 ± 1000 respectively. When examined by the method of Hedrick & Smith (1968), these values were confirmed, suggesting a monomeric structure for both enzymes.

Glycoprotein nature of the enzymes

Both enzymes were stained by periodate/fuchsin reagent on polyacrylamide gels (Kapitany & Zebrowski, 1973). They were also retained by concanavalin A-Ultrogel. The enzymes were eluted from concanavalin A-Ultrogel by the addition, with stirring, of 0.2M- α -methyl glucoside.

By g.l.c., the composition of the carbohydrate moieties, consisting of *N*-acetylglucosamine, mannose and galactose, was estimated to be 1:6:1.5 for α -galactosidase III and 1:13:18 for β -D-mannanase (molar proportions).

Optimum pH

For α -D-galactosidase III the effect of pH on enzymic activity was examined with either *o*-nitrophenyl α -D-galactoside or raffinose as substrate. The pH-activity profile showed a maximum at pH4.8.

The β -D-mannanase showed optimum activity at pH4.5 as determined with saleg glucomannan.

Stability and conservation

Both enzymes were stable at 4°C for several months without any apparent loss of activity. However, α -galactosidase III lost 60% of its activity on freeze-drying, whereas for β -D-mannanase

Fig. 2. Inactivation of hydrolytic activity as a function of temperature and time (a) α -Galactosidase III: samples of electrophoretically pure enzyme ($1.8 \mu\text{g/ml}$) were incubated at 37°C , 45°C (○), 50°C (●) and 55°C (△). At the indicated times, samples ($50 \mu\text{l}$) were withdrawn, and residual hydrolytic activity was assayed with *o*-nitrophenyl α -D-galactoside as substrate. The ordinate represents residual activity. (b) β -D-Mannanase: samples of electrophoretically pure enzyme ($1.7 \mu\text{g/ml}$) were incubated at 37°C , 45°C (○), 50°C (●), 55°C (△) and 60°C (▲). At the indicated times, samples ($50 \mu\text{l}$) were withdrawn, and residual hydrolytic activity was assayed with saleg glucomannan as substrate. The ordinate represents residual activity.

ase the loss was 11%. Figs. 2(a) and 2(b) show that α -D-galactosidase and β -D-mannanase are both resistant to heat treatment. The extracellular α -galactosidase III was much more resistant to heat denaturation as compared with the mycelial forms I and II already described (Civas *et al.*, 1984). This resistance, however, cannot only be attributed to its glycosylation, since the mycelial forms were also glycosylated.

Effect of cations

Cu^{2+} , Fe^{2+} , Mn^{2+} , Co^{2+} , Cd^{2+} , Zn^{2+} , Ca^{2+} and Mg^{2+} showed no apparent effect on α -galactosidase III activity over the concentration range $10 \mu\text{M}$ – 10mM . Under the same conditions, 100% inhibition was obtained with Ag^+ and Hg^{2+} .

No significant inhibition was observed for β -D-mannanase with the above cations, but a slight activation was observed with Fe^{2+} and Ca^{2+} . EDTA over the concentration range $10 \mu\text{M}$ – 10mM has no effect on the enzyme activity.

Substrate specificity

The effect of varying the substrate concentration on the reaction rate was studied with *o*-nitrophenyl α -D-galactoside, raffinose and stachyose for α -galactosidase III. The apparent Michaelis constant (K_m) and maximum velocity (V_{max}) for each substrate were calculated by Lineweaver-Burk plots, and are summarized in Table 2.

The affinity for substrate and the rate of galactose liberation decreased with increasing chain length of the substrate. Very slight hydrolysis was observed with melibiose, even at a substrate concentration of 0.2M , but it was a competitive inhibitor, with a K_i value of $1.0 \times 10^{-2} \text{M}$.

D-Fucose, L-fucose and L-arabinose inhibited α -D-galactosidase III activity when used at a concentration of 130mM . D-Galactose, which has been generally shown to be a powerful competitive inhibitor of α -galactosidases, exhibited mixed-type inhibition with α -galactosidase III. The same kind of inhibition has also been observed for an α -D-

Table 2. Specificity of α -D-galactosidase III in McIlvaine buffer, pH 4.8, at 37°C

Hydrolysis of *o*-nitrophenyl α -D-galactoside was assayed by incubating 50 μ l of enzyme, 50 μ l of substrate and 25 μ l of McIlvaine buffer, pH 4.8, at 37°C. After 5 min the reaction was stopped by the addition of 3 ml of 0.2M- Na_2CO_3 . The liberated *o*-nitrophenol was determined at 420 nm. For raffinose or stachyose, the incubation medium consisted of 100 μ l of enzyme, 100 μ l of substrate and 50 μ l of McIlvaine buffer, pH 4.8. The incubation time was 15 min with raffinose and 45 min with stachyose at 37°C. Galactose liberated was determined by a micro-dosage technique adapted from the Somogyi-Nelson method. The enzymic reaction was stopped by the addition of 0.5 ml of solution A (Somogyi, 1945) diluted to 50% (v/v). After incubation at 100°C in a boiling-water bath for 20 min, the assay tubes were allowed to cool and 0.25 ml of solution B (Nelson, 1944) was added, followed by 3 ml of water. After 10 min the stabilized blue coloration was read spectrophotometrically at 650 nm against suitably prepared blanks. The results were plotted in Lineweaver-Burk double-reciprocal form.

Substrate	K_m (mM)	V_{max} (μ mol of galactose liberated/min per mg of protein)	K_i (mM)
<i>o</i> -Nitrophenyl α -D-galactoside	1.3	505.0	
Melibiose	—	—	10
Raffinose	26.3	80.0	
Stachyose	33.3	1.1	

Table 3. Specificity of β -mannanase in McIlvaine buffer, pH 4.5, at 37°C

Hydrolysis of the glucomannan of *Orchis intacta* and the various galactomannans was assayed by incubating 100 μ l of substrate, 50 μ l of McIlvaine buffer, pH 4.5, and 100 μ l of enzyme at 37°C for 10 min, and liberation of reducing sugars was measured by a micro-dosage technique adapted for the Nelson-Somogyi method (see Table 2).

	Ratio of constituent sugar (glucose/mannose)	K_m (g/100 ml)	V_{max} (μ mol of mannose equivalent liberated/min per mg of protein)
Glucomannan of <i>Orchis intacta</i>	1:3	86.9×10^{-4}	26.8
	(galactose/mannose)		
Galactomannans			
<i>Ceratonia siliqua</i>	1:4	2.0×10^{-2}	43.5
<i>Cyamopsis</i> sp.	1:2	4.0×10^{-1}	50.0
<i>Trifolium repens</i>	1:1.1	5.5×10^{-1}	62.5

galactosidase isolated from *Mortierella vinacea* (Suzuki *et al.*, 1970).

T.l.c. showed that α -galactosidase III liberated galactose (R_F 0.58) from the pentasaccharide (R_F 0.10):

and also gave a tetrasaccharide (R_F 0.23). For the galactomannans, the initial rate of hydrolysis was highest with lucerne galactomannan, where the ratio mannose/galactose was 1.0, the rate of hydrolysis decreasing as this ratio increased.

The affinity of β -D-mannanase for various galactomannans decreased with increasing substitution of the polymannoside chain. The K_m values obtained with glucomannan and galactomannans are summarized in Table 3. As the M_r values of the substrates were not exactly known, their stock solutions were made up by percentage weight (w/v). β -(1,4)-Mannobiose (R_F 0.45), β -(1,4)-man-

notriose (R_F 0.28) and α -Gal(1 $\rightarrow$ 6) β -Man(1 $\rightarrow$ 4) β -Man (R_F 0.24) were the main products obtained after hydrolysis of the galactomannan of *C. siliqua*. With the mannan of *P. macrocarpa*, which is particularly insoluble, we obtained complete solubilization within 24 h at 37°C with stirring: 60 mg of mannan, 4 ml of enzyme solution (8 μ g/ml) and 1 ml of McIlvaine buffer, pH 4.5. A hydrolysate applied to a column of Sephadex G-15 allowed us to separate 87% mannobiose, 7% mannotriose and trace amounts of mannose. The endo-mechanism of attack of β -D-mannanase clearly showed a similarity with the action pattern of β -mannanase isolated from *Bacillus subtilis* (Emi *et al.*, 1972).

Discussion

When *A. tamarii* was cultivated in the presence of galactomannan of *T. repens*, the mycelial α -galactosidase I and α -galactosidase II were still produced, but in considerably diminished amounts (see Table 4), α -galactosidase II especially being detectable only in trace amounts. α -Galactosidase

Table 4. *Distribution of α -D-galactosidase activities in *Aspergillus tamarii**

Cells were grown in the mineral-salts medium with the indicated carbon source for 64h and 140h respectively. *o*-Nitrophenyl α -D-galactoside was used as substrate to test the enzyme activity, which is expressed in μmol of galactose liberated/min.

α -Galactosidase	Raffinose-containing culture		Galactomannan-containing culture	
	Mycelia	Culture fluid	Mycelia	Culture fluid
I } II }	1255	0	10.3	0
III	0	0	48.5	382.5

I thus produced was purified by the procedure described in the preceding paper (Civas *et al.*, 1984). On polyacrylamide-gel electrophoresis, in the presence and in the absence of sodium dodecyl sulphate, it showed a protein band identical with that for α -galactosidase I produced in the presence of raffinose. The same K_m and V_{max} values for *o*-nitrophenyl α -D-galactoside, melibiose and raffinose were also obtained in both cases.

Replacement of raffinose by galactomannan resulted mainly in the production of an α -galactosidase III and a β -D-mannanase, which were secreted in the growth medium. Nevertheless 11% of the total α -galactosidase III activity and 5% of β -D-mannanase activity were still detected in the mycelia. The lack of any β -D-mannosidase activity, inside or outside the cells, led us to the conclusion that α -galactosidase III and β -D-mannanase were solely responsible for galactomannan hydrolysis. In this respect, *A. tamarii* is different from *A. niger*, where a β -D-mannosidase has previously been identified and isolated (Elbein *et al.*, 1977; Bouquelet *et al.*, 1978).

We observe that α -galactosidase III is a monomeric glycoprotein with an M_r of 56000; it contains *N*-acetylglucosamine, mannose and galactose in the molar proportions 1:6:1.5. There is no glucose in carbohydrate moiety, and the proportions of constituent sugars are quite different from those in the mycelial α -galactosidase I. Galactose is always detected in the carbohydrate moieties of these α -galactosidases and of the β -D-mannanase. Rudick *et al.* (1979) have isolated a cell-bound α -glucosidase from *A. niger* and have shown that it was a glycoprotein containing mannose, glucosamine and glucose, whereas the extracellular α -glucosidase has been shown to be a glycoprotein with a single asparagine-linked oligosaccharide chain containing only glucosamine and mannose. The glucosamine/mannose ratio observed was similar to that found for other extracellular glycosidases isolated by Rudick *et al.* (1979) from the culture fluids of *A. niger*. Since the medium that they used for cultivation contained mannose, yeast extract and guar flour, it is not

possible to know whether the enzymes were induced or not.

Studies on invertases isolated from the growth medium of three different races of a plant-pathogen fungus (Ziegler & Albersheim, 1977) have shown that their carbohydrate moieties contain different percentages of mannose and glucosamine. These authors have suggested that these carbohydrate moieties could be involved in determining the specificity of host-pathogen interactions. The invertases extracted from the mycelia of two strains of the same fungus were also glycoproteins containing essentially mannose and glucose in different ratios and smaller amounts of galactose (West *et al.*, 1980). However, these authors could not establish any relationship between the mycelial and secreted forms of the invertases examined.

The mycelial α -galactosidases I and II studied in the preceding paper (Civas *et al.*, 1984) are large- M_r glycoenzymes (M_r 265000 and 254000 respectively). They show certain analogies to the α -galactosidase isolated from *Saccharomyces carlsbergensis*, existing as a dimeric molecule (M_r 300000) containing mannose, glucose and glucosamine (Lazo *et al.*, 1977, 1978). The yeast strain studied by those authors secreted α -galactosidase when melibiose, raffinose or stachyose was present in the growth medium (Lazo *et al.*, 1981); the α -galactosidase localized inside the cell appeared to have the same characteristics as the extracellular form.

There have also been extensive studies on yeast invertases, which have led to the characterization of a glycosylated form (M_r 270000) associated with the cell wall (Neumann & Lampen, 1967, 1969) and an internal form (M_r 135000) containing less than 3% carbohydrate (Gascon & Lampen, 1968; Gascon *et al.*, 1968). Babczinski & Tanner (1978) have characterized an invertase (M_r 190000) associated with the cell membrane; its formation is completely inhibited by tunicamycin.

The structural differences evidenced for the mycelial and secreted α -galactosidases of *A. tamarii* are accompanied by different kinetic proper-

ties. An examination of the kinetic data shows that for raffinose and stachyose, whereas α -galactosidase I and α -galactosidase III have almost identical affinities, $V_{\max}$ values obtained with α -galactosidase III are significantly lower. Furthermore, the mycelial α -galactosidases I and II showed high affinities for melibiose, whereas this substrate was only very slightly hydrolysed by α -galactosidase III, and was also a competitive inhibitor for that enzyme.

Examination *in vitro* of the degradation of the various galactomannans showed that the affinity of β -D-mannanase diminished considerably as the substitution of the polymannoside chain increased. Mannobiose, mannotriose and galactomannobiose were the main products of the hydrolysis of the galactomannans of *T. repens* and *C. siliqua*. The galactomannobiose produced by β -D-mannanase action was hydrolysed by α -galactosidase III, which was also able to remove galactose residues from the undegraded galactomannans. The α -galactosidase III thus differs markedly from the α -galactosidases I and II examined, which did not show any activity on the various galactomannans.

Examination of inhibition of α -galactosidase activity by monosaccharides, with *o*-nitrophenyl α -D-galactoside as substrate, showed dissimilarities in the type of inhibition, depending on whether the enzyme was mycelial or secreted. Thus glucose and mannose uncompetitively inhibited α -galactosidases I and II, but showed no effect on α -galactosidase III over the same concentration range. Non-competitive inhibition was observed for α -galactosidases I and II with galactose, whereas a mixed type of inhibition was noted for α -galactosidase III.

Since the secreted and mycelial forms were glycosylated, in order to understand the importance of the glycan fraction contained in these glycoenzymes, *Hind*III and *Bam*HI genomic DNA fragments from *A. tamarii* were prepared. Cloning of these fragments in *Escherichia coli* HB101 with plasmid pBR322 and cosmid pHC79 was performed as described by Bolivar *et al.* (1977) or Hohn (1979). Tetracyclin-sensitive and ampicillin-resistant clones were stored in glycerol at -20°C . If the DNA is inserted in the reading frame of the *coli* genome, expression might occur. Since *E. coli*, the prokaryotic host, lacks a eukaryotic protein-glycosylation system, the enzymes so produced would be unglycosylated. These enzymes could be extracted and purified for comparison of their properties with those of the uncloned enzymes.

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