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Purification and characterization of a thermostable glucoamylase from the thermophilic fungus *Thermomyces lanuginosus*

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Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) was purified from the culture filtrates of the thermophilic fungus *Thermomyces lanuginosus* and was established to be homogeneous by a number of criteria. The enzyme was a glycoprotein with an average molecular weight of about 57000 and a carbohydrate content of 10–12%. The enzyme hydrolysed successive glucose residues from the non-reducing ends of the starch molecule. It did not exhibit any glucosyltransferase activity. The enzyme appeared to hydrolyse maltotriose by the multi-chain mechanism. The enzyme was unable to hydrolyse 1,6-α-D-glucosidic linkages of isomaltose and dextran. It was optimally active at 70°C. The enzyme exhibited increase in the $V_{max.}$ and decrease in K_m values with increasing chain length of the substrate molecule. The enzyme was inhibited by the substrate analogue D-glucono-δ-lactone in a non-competitive manner. The enzyme exhibited remarkable resistance towards chemical and thermal denaturation.

In recent years there has been considerable interest in the production of glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3), since it is capable of directly hydrolysing starch to glucose. This enzyme was purified to homogeneity from several species of mesophilic fungi (Lineback *et al.*, 1969; Fukui & Nikuni, 1969; Yamasaki *et al.*, 1977*c*) and was shown to be a glycoprotein having a molecular weight in the range 50000–100000. It was also shown to exist in two or more isoenzymic forms in *Aspergillus niger* (Venkataramu *et al.*, 1975), *Penicillium oxalicum* (Yamasaki *et al.*, 1977*a*), *Mucor rouxianus* (Tsuboi *et al.*, 1974) and several other fungi (Takahashi *et al.*, 1978; Iizuka & Mineki, 1977; Razzaque & Ueda, 1978).

Information on the production and purification of glucoamylase from thermophilic fungi, which is expected to be thermostable, is meagre (Subrahmanyam *et al.*, 1977; Taylor *et al.*, 1978). Thermostable immobilized enzyme reactors will have potential applications in industry. Hence studies were initiated in our laboratory on the production of glucoamylase from thermophilic fungi. Our ultimate aim is to develop a stable immobilized glucoamylase reactor for the continuous production of glucose.

We have reported the occurrence of glucoamylase, from the thermophilic fungus *Thermomyces lanuginosus* ML–M, that exhibited remarkable thermal stability (Basaveswara Rao *et al.*, 1979). The present paper describes, for the first time, the purification, characterization, chemical and kinetic properties of this thermostable glucoamylase.

Experimental

Materials

The following biochemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.: glucose oxidase, peroxidase, *o*-dianisidine, DEAE-cellulose (fine grade), acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine, ammonium persulphate, Tris, glycine, Coomassie Brilliant Blue R, Coomassie Brilliant Blue G, phenazine methosulphate, Nitro Blue Tetrazolium, sodium dodecyl sulphate, 2-mercaptoethanol, Blue Dextran, crystalline bovine serum albumin, ovalbumin, α-chymotrypsinogen, lysozyme, aldolase, phosphorylase, catalase, maltose, maltotriose, Amylopectin Azure, D-glucono-δ-lactone (1,2,3,4,5-pentahydroxyhexanoic acid lactone), periodic acid and all substrate analogues. Bio-Gel P-100 was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Soluble starch was from E. Merck, Darmstadt, West Germany. Isomaltose and amylopectin were from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Rabbit liver glycogen was obtained from V. P. Chest Institute, New Delhi, India.

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Isolation and selection of the fungus

Strains of *T. lanuginosus* were isolated from compost or soil by the method described previously (Maheshwari, 1968). The high-yielding strain *T. lanuginosus* ML–M was selected after screening of several of these isolates for the production of glucoamylase.

Growth conditions

T. lanuginosus ML–M was grown in shake cultures at 50°C on a medium containing soluble starch (2%), L-asparagine (0.4%), K_2HPO_4 (0.1%), $MgSO_4,7H_2O$ (0.05%) and Vogel's trace-element solution (0.01%, v/v) (Vogel, 1964). After 48h of growth, the mycelium was filtered by suction and the culture filtrate was stored frozen at −20°C.

Enzyme assay

Glucoamylase was routinely assayed in a total reaction mixture, of final volume 1.0ml, containing 50mM-sodium acetate buffer, pH5.0, 0.2% soluble starch and suitably diluted enzyme, at 50°C for 10min. The reaction was terminated by immersing the tubes in a boiling-water bath for 20min and the amount of glucose was determined by the coupled glucose oxidase/peroxidase method of McComb & Yushok (1958) with minor modifications. After the reaction had been stopped, the tubes were cooled, and 2.5ml of glucose oxidase reagent containing 7.5 units of glucose oxidase, 10.5 units of peroxidase, 250μg of *o*-dianisidine and 50mM-sodium acetate buffer, pH5.0, was added and the mixture was incubated at room temperature (25°C) for 30min. The reaction was stopped by the addition of 2.5ml of 20% (v/v) H_2SO_4. The intensity of the pink colour was measured in a Klett–Summerson colorimeter with a 54 filter.

One unit of the enzyme is defined as the amount that liberates 1μmol of glucose/min under the conditions of assay. Specific activity is expressed as units/mg of protein.

Purification of glucoamylase

Culture filtrates of *T. lanuginosus* (48h old) were used as the source of glucoamylase. All column operations were conducted at room temperature unless otherwise mentioned. The protocol of the purification procedure is as follows.

DEAE-cellulose ion-exchange chromatography. The crude culture filtrate (44ml) was directly applied to a DEAE-cellulose (fine grade) column (1.5cm × 16cm) pre-equilibrated with 50mM-sodium acetate buffer, pH5.0. The column was extensively washed with 250ml of the same buffer. The enzyme was eluted with a linear gradient of 100ml of 50mM-sodium acetate buffer, pH5.0, and 100ml of the same buffer containing 0.3M-NaCl. The flow rate was 30ml/h, and 5ml fractions were collected. The active fractions were pooled and concentrated by dialysis against solid sucrose.

Preparative polyacrylamide-gel electrophoresis. The enzyme obtained in the above step was dialysed against 0.1M-Tris/glycine buffer, pH8.6, and preparative polyacrylamide-gel electrophoresis was performed. A 2ml portion of the sample was applied to a column (1.7cm × 16cm) of polyacrylamide gel (7%, w/v), and electrophoresis was performed at 200V for 6–8h at 4°C. The enzyme was eluted either by slicing the gel into 0.5cm-thick pieces and maceration in 0.2M-sodium acetate buffer, pH5.0, or electrophoretically into a dialysis sac at 10min intervals. The active fractions were pooled, extensively dialysed against distilled water and stored frozen at −20°C.

Alternatively, the enzyme was purified by analytical polyacrylamide-disc-gel electrophoresis. A 100μl portion of the sample obtained in the first step, after dialysis against 1M-Tris/glycine buffer, pH8.6, was applied to the gel tubes (0.5cm × 10cm), and electrophoresis was performed in 1M-Tris/glycine buffer, pH8.6, at 100V for 3–4h. The protein bands were located by staining one of the gels with Coomassie Brilliant Blue G at the end of the electrophoresis run. The remaining gels were cut corresponding to the major protein band (closest to the dye band), which was found to exhibit the enzyme activity. The gel discs were finely macerated with a glass rod, eluted with 0.2M-sodium acetate buffer, pH5.0, extensively dialysed against distilled water and stored frozen at −20°C.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard.

Electrophoresis

Polyacrylamide-gel electrophoresis of the purified glucoamylase was performed at pH8.6 or 4.3 in accordance with the procedures of Davis (1964) and Reisfeld *et al.* (1950), respectively. Gels at a concentration of 7% (w/v) acrylamide were employed, and the proteins were stained with 0.2% Coomassie Brilliant Blue R in 7% (v/v) acetic acid in 50% (v/v) methanol solution. Destaining was carried out with 7% acetic acid in 50% methanol. Approx. 50μg of protein was applied to each gel.

Activity staining of glucoamylase

Glucoamylase on polyacrylamide gels was located by coupling its reaction with glucose oxidase in the presence of the electron acceptors. The gels were polymerized with glucose oxidase (30 units/gel). After electrophoresis, the gels were incubated in 0.5M-sodium acetate buffer, pH5.0, for 30min and then the staining solution, containing 0.4mg of Nitro

Blue Tetrazolium/ml, 0.2mg of phenazine methosulphate/ml and 0.4% soluble starch or maltose in the same buffer, was added. The gels were incubated in dark at 40°C for the appearance of the bands, which generally took 1h. Substrate was omitted from the control gels. After the appearance of the bands, the gels were washed with distilled water and stored in 7% (v/v) acetic acid.

Glycoprotein staining of the gels

Gels were stained for glycoproteins by the periodate–Schiff technique of Zacharius & Zell (1969).

Determination of total carbohydrate

Total carbohydrate content of glucoamylase was determined by the phenol/H_2SO_4 procedure (Dubois *et al.*, 1956), with glucose as the standard.

Paper chromatography of the reaction products

Samples (10μl) containing 10–100μg of sugar were spotted on the base-line of a Whatman no. 1 filter paper and dried. Chromatography was performed with propan-1-ol/ethyl acetate/water (7:1:2, by vol.) as the solvent system for 36–48h at room temperature. Spots were developed in accordance with the procedure of Trevelyan *et al.* (1950).

Determination of the molecular weight of T. lanuginosus glucoamylase

Bio-Gel P-100 gel filtration. The molecular weight of the enzyme was determined by the procedure of Andrews (1965), with a Bio-Gel P-100 column (2cm × 103cm) standardized with the following protein markers: bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 43000), α-chymotrypsinogen (mol.wt. 25000) and lysozyme (mol.wt. 14000). The column was equilibrated with 0.1M-sodium acetate buffer, pH5.0, containing 0.2M-NaCl and eluted with the same buffer. The molecular weight was estimated from a plot of log(mol.wt.) against elution volume.

Analytical polyacrylamide-gel electrophoresis. The molecular weight of the enzyme was determined by analytical polyacrylamide-gel electrophoresis in accordance with the procedure of Hedrick & Smith (1968), with α-chymotrypsinogen (mol.wt. 25000), ovalbumin (mol.wt. 43000), bovine serum albumin (mol.wt. 67000) and bovine serum albumin dimer (mol.wt. 134000) as the standard marker proteins.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The procedure of Weber & Osborn (1969) was used to determine the molecular weight of the enzyme by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The standard protein markers used were: lysozyme (mol.wt. 14000), aldolase (mol.wt. 40000), catalase (mol.wt. 60000), bovine serum albumin (mol.wt. 67000) and phosphorylase *a* (mol.wt. 94000).

Results

Purification of glucoamylase

The balance sheet for the purification of glucoamylase from the culture filtrates of *T. lanuginosus* is given in Table 1. The final enzyme preparation had a specific activity of 80 units/mg of protein. The specific activities of several preparations of the enzyme were in the range 75–90 units/mg. The enzyme was 24-fold purified with 40% recovery. The ratio of activity towards the substrates soluble starch and maltose (S/M ratio) increased during purification, indicating the presence of a contaminating maltase, a disaccharidase, which was eliminated during purification.

The purified enzyme can be stored indefinitely (at least 2 years) at either 4°C or −20°C even in dilute solutions (5μg/ml) without significant loss of the activity. Freezing and thawing or freeze-drying had no effect on the enzyme activity. It was optimally stable in the pH range 6.5–8.0.

Catalytic properties of glucoamylase

Molecular activity of the enzyme was calculated to be $4.76 \times 10^3 min^{-1}$, on the basis of a molecular weight of 57000 and the $V_{max.}$ calculated from the double-reciprocal plot. The purified enzyme showed

Table 1. *Summary of purification of glucoamylase from the culture filtrates of T. lanuginosus ML–M*
Experimental details are given in the text.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification factor	Recovery (%)	Total maltase activity (units)	S/M ratio*
1. Crude culture filtrate	23	76	3.3	1	100	31	2.45
2. DEAE-cellulose ion-exchange chromatography	0.866	40	46	14	53	6	6.6
3. Preparative polyacrylamide-gel electrophoresis	0.375	30	80	24	39.5	3.5	8.57

* Ratio of enzyme activity towards the substrates soluble starch and maltose.

(i) a linear relationship between initial reaction rate and enzyme concentration up to 0.5 μg/ml of reaction mixture, (ii) a linear rate of glucose formation for 30 min when 0.2% soluble starch and enzyme (up to 0.25 μg) were used and (iii) optimal enzyme activity at pH 5.0 at 0.2% soluble starch or 0.4% maltose concentration at 50°C.

Homogeneity of glucoamylase

The final preparation obtained after the purification moved as a single band on polyacrylamide-gel electrophoresis at pH 8.6 and 4.3 (Fig. 1) and also in the presence of sodium dodecyl sulphate. The protein band coincided well with the glucoamylase activity band obtained by using either of the substrates, soluble starch or maltose.

Glycoprotein nature of glucoamylase

The glycoprotein nature of the enzyme was established by staining the gels by using the periodate–Schiff technique, which gave a pink band corresponding to the protein and activity bands of glucoamylase (Fig. 1). The total carbohydrate content of the purified enzyme was estimated to be 10–12%. The composition of the individual sugars is yet to be established.

Action patterns of glucoamylase

To distinguish between α-, β- and gluco-amylases, the reaction products obtained by the action of salivary α-amylase, sweet-potato β-amylase and *T. lanuginosus* glucoamylase on starch were separated and identified by paper chromatography. Glucose was the only product detectable when *T. lanuginosus* glucoamylase acted on starch, indicating its exo-glucohydrolytic nature. On the other hand, four products were identified with salivary α-amylase. Three of them corresponded to standard glucose, maltose and maltotriose, and one spot remained just above the base-line, representing the oligosaccharide fraction. This pattern of product formation is indicative of the endo-glucan hydrolytic nature of α-amylase. Only one product, maltose, was detected with sweet-potato β-amylase, this being due to its exo-maltohydrolytic action.

The enzyme cleaved maltotriose first to maltose and glucose, and the maltose thus formed was subsequently converted into glucose. This sequence of formation of the products from maltotriose favours the multi-chain mechanism (Greenwood & Milne, 1968) as the predominant mechanism operative for the hydrolysis of maltotriose by *T. lanuginosus* glucoamylase.

In order to determine whether the *T. lanuginosus* enzyme possessed transfer activity, it was incubated with maltose, and the products were separated and identified chromatographically. Glucose was the only product detectable at any stage of the reaction. No transfer products were detected. The same result was obtained even when the reaction was performed at high substrate concentration (60% maltose solution).

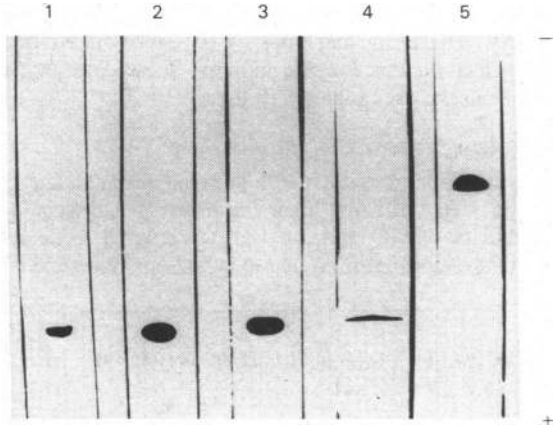

Fig. 1. *Polyacrylamide-gel electrophoresis of purified glucoamylase (50 μg) from T. lanuginosus in 7% (w/v) gels*

Electrophoresis was performed at pH 8.6 (gels 1–4) and pH 4.3 (gel 5) as described in the Experimental section. Gels 1 and 5, stained for protein; gel 2, stained for enzyme activity with soluble starch as substrate; gel 3, stained for enzyme activity with maltose as substrate; gel 4, stained for glycoprotein.

Starch–iodine reaction

The exo-hydrolytic nature of glucoamylase from *T. lanuginosus* was demonstrated by using the starch–iodine reaction, and it was distinguished from the endo-hydrolytic nature of salivary and bacterial α-amylases. The intensity of the starch–iodine colour decreased rapidly within 5 min of the reaction in the case of α-amylases, whereas it declined only slowly in the case of glucoamylase. This was in correlation with the amount of glucose produced during the reaction. It was observed that with α-amylases no glucose could be detected in the reaction mixture even after 40 min incubation, whereas with glucoamylase from *T. lanuginosus* the amount of glucose increased, with a concomitant decrease in the intensity of starch–iodine colour (Fig. 2).

Rates of hydrolysis of different substrates

The initial rates of the reaction with increasing concentration of purified glucoamylase from *T. lanuginosus* for different substrates were compared to establish that the enzyme acts from the non-reducing ends of the substrate molecule. Plots of velocity versus enzyme concentration (Fig. 3) became non-linear at relatively low enzyme concen-

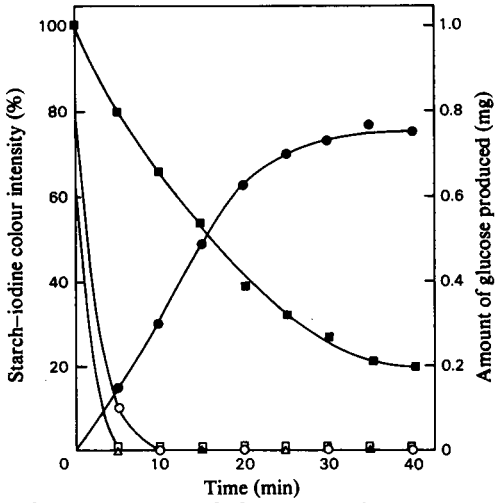

Fig. 2. *Time course of the action of α-amylase and glucoamylase on soluble starch as measured by the decrease in the starch–iodine colour*

The reaction mixture (2.0ml) contained 0.05% soluble starch, 100mM-sodium acetate buffer, pH5.0, and enzyme at appropriate amounts (0.16 unit of salivary α-amylase or 0.18 unit of bacterial α-amylase or approx. 2μg of *T. lanuginosus* glucoamylase). With the α-amylases, 0.5μmol of $CaCl_2$ was also included in the reaction mixture. Incubations for salivary α-amylase (□) and bacterial α-amylase (○) were performed at 37°C and that for glucoamylase (■) at 50°C. The reaction was stopped at different time intervals by the addition of 0.5ml of 0.1M-HCl followed by 2.5ml of freshly prepared iodine reagent containing 50μg of I_2 and 500μg of KI. Colour was developed for 10min at room temperature and its intensity was measured in a Klett–Summerson colorimeter with a 66 filter. The Figure also shows amounts of glucose produced by the salivary α-amylase (△), bacterial α-amylase (▲) and *T. lanuginosus* glucoamylase (●) under the reaction conditions mentioned above.

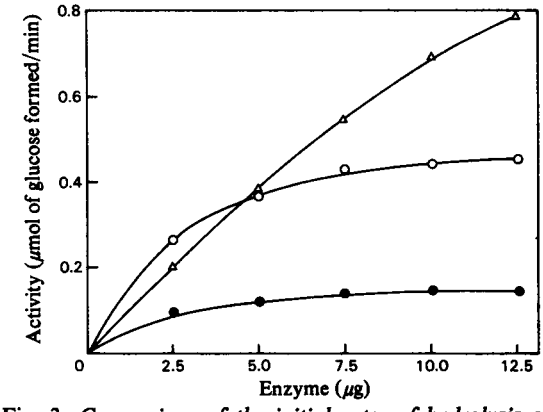

Fig. 3. *Comparison of the initial rates of hydrolysis of different substrates by the T. languginosus glucoamylase*

Reaction mixture (1.0ml) contained 2mg of substrate (●, amylose; ○, amylopectin; △, glycogen), 50mM-sodium acetate buffer, pH5.0, and different concentrations of the enzyme. The reaction was performed at 50°C for 10min. After the reaction had been stopped 50μl of the sample was taken for the determination of glucose.

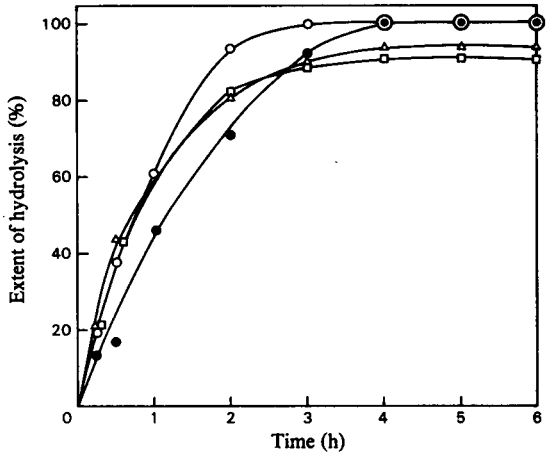

Fig. 4. *Comparison of the extents of hydrolysis (%) of different substrates by T. lanuginosus glucoamylase*

The reaction mixture (2.0ml), containing 20mg of substrate (●, maltose; ○, maltotriose; △, soluble starch; □, glycogen), 50mM-sodium acetate buffer, pH5.0, and enzyme (20μg), was incubated at 50°C, and 10μl portions of the samples were taken at different time intervals for the determination of glucose.

trations (less than 2.5μg) with amylose and amylopectin, whereas with glycogen, which is a highly branched molecule, the non-linearity was observed only at high concentrations of the enzyme (above 7.5μg). Thus the rates of hydrolysis of different substrates fell in the order of amylose < amylopectin < glycogen, indicating the availability to the enzyme of more points of attack in the more numerous non-reducing ends of the branched substrates than in the linear substrates.

Extent of hydrolysis of maltose, maltotriose, soluble starch and glycogen

The percentage conversion of maltose, maltotriose, soluble starch and glycogen into glucose by the purified glucoamylase from *T. lanuginosus* was calculated from the amount of glucose produced and that of the substrates taken originally. All the substrates were hydrolysed rapidly, and the degree of hydrolysis was as follows: 100% for maltose and maltotriose, 94% for soluble starch and 91% for glycogen (Fig. 4).

Molecular weight of T. lanuginosus glucoamylase

The molecular weight of the enzyme was estimated to be 57000 ± 2000 by gel filtration on a Bio-Gel P-100 column. Electrophoresis of the enzyme on sodium dodecyl sulphate/polyacrylamide gels gave a single band with a molecular weight of 55000 ± 1500. Analytical polyacrylamide-gel electrophoresis of the purified enzyme revealed a single protein band at all gel concentrations. The molecular weight of the enzyme was estimated to be 58000 ± 3000 from the plot of slope versus molecular weight.

Effect of temperature on glucoamylase activity

Optimum temperature for the hydrolysis of soluble starch by *T. lanuginosus* glucoamylase in a 15 min reaction was 70°C (inset to Fig. 5). The activation energy of the reaction calculated from the Arrhenius plot (Fig. 5) was 61.5 kJ/mol.

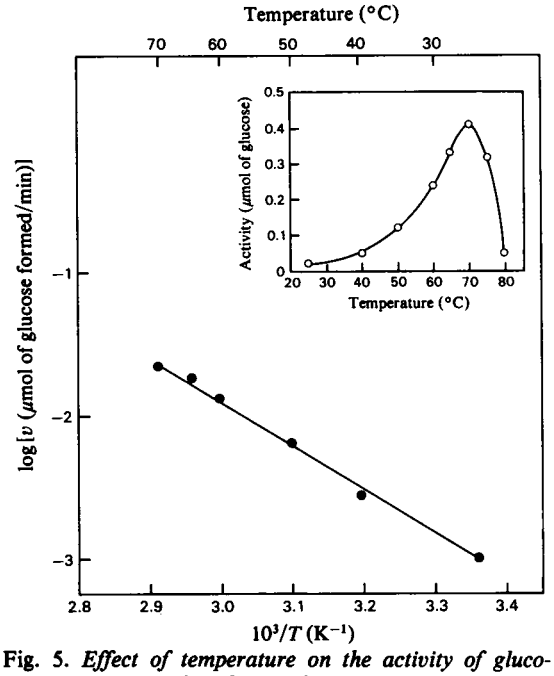

Fig. 5. *Effect of temperature on the activity of glucoamylase from T. lanuginosus*

The reaction mixture (0.9 ml), containing 0.4% soluble starch and 50 mM-sodium acetate buffer, pH 5.0, was preincubated at defined temperatures in the range 25–80°C for 15 min, and the reaction was started by the addition of 0.1 ml of enzyme (100 ng) in the same buffer and was allowed to proceed for 15 min. The reaction was terminated by keeping the tubes in a boiling-water bath for 20 min, and the amount of glucose liberated was determined by the glucose oxidase/peroxidase procedure as described in the Experimental section.

Substrate specificity of glucoamylase

The relative rates of hydrolysis of various substrates by the purified glucoamylase from *T. lanuginosus* are given in Table 2. The enzyme hydrolysed high-molecular-weight substrates such as dextrin, soluble starch, amylopectin and glycogen more rapidly than low-molecular-weight substrates such as *p*-nitrophenyl α-D-glucoside, maltose (Glcpα1–4Glc) and maltotriose (Glcpα1–4Glcpα1–4Glc) [though the enzyme failed to hydrolyse isomaltose (Glcpα1–6Glc) and dextran (α1–6-glucan). Trehalose, turanose, melibiose, melezitose, cellobiose, gentibiose, *p*-nitrophenyl β-D-glucoside, *p*-nitrophenyl α-D-mannoside and *p*-nitrophenyl α-D-galactoside also did not serve as substrates for the *T. lanuginosus* glucoamylase.

Effect of substrate analogues on glucoamylase activity

Among the various analogues tested, D-glucono-

Table 2. *Specificity of the purified glucoamylase from T. lanuginosus for various substrates*

The compounds were divided into two groups because of the large differences in the ease of hydrolysis of various substrates. Different enzyme and substrate concentrations were employed for the determination of their relative rates of hydrolysis. The substrate and enzyme concentrations used for group I (*p*-nitrophenyl α-D-glucoside, maltose, maltotriose and other glucosides) were 10 mg/ml and 0.8 μg/ml of the reaction mixture respectively, and those for group II (dextrin, soluble starch, amylopectin and glycogen) were 2 mg/ml and 0.4 μg/ml respectively. The reaction (in a total reaction mixture of 1.0 ml containing 50 mM-sodium acetate buffer, pH 5.0) was conducted at 50°C for 10 min and was stopped by keeping the tubes in a boiling-water bath for 20 min, and the amount of glucose liberated was determined by the glucose oxidase/peroxidase method as described in the Experimental section. Where *p*-nitrophenyl derivatives were used as substrates, the reaction was terminated by the addition of 0.5 ml of 0.2 M-glycine/NaOH buffer, pH 10.0, and the absorbance was measured at 420 nm. The activity values given in the Table are the μmol of glucose liberated/min per 0.8 μg of enzyme. The rate of hydrolysis of maltose was arbitrarily set at 100 and the relative values for the hydrolysis of the other substrates were calculated on this basis.

Substrate	Activity	Relative rate of hydrolysis
p-Nitrophenyl α-D-glucoside	2.8×10^{-3}	32.5
Maltose	8.6×10^{-3}	100
Maltotriose	4.24×10^{-2}	493
Dextrin	6.39×10^{-2}	743
Starch (soluble)	6.6×10^{-2}	767
Amylopectin	6.87×10^{-2}	799
Glycogen	4.55×10^{-2}	529

δ-lactone alone inhibited the *T. lanuginosus* glucoamylase activity to a significant extent, compounds such as glucose, glucose 6-phosphate, galactose, melibiose, melezitose, gentibiose, trehalose, sucrose and turanose having no effect. Kinetic analysis of D-glucono-δ-lactone inhibition by Lineweaver–Burk and Dixon plots showed that it was of a non-competitive type. The K_i value obtained from these plots was 5.2mM. This may be explained on the basis that the lactone acted as a transition-state analogue as a consequence of its distorted half-chair conformation, in a similar way to that suggested for the lysozyme-catalysed reactions (Ford *et al.*, 1974).

Kinetics of the glucoamylase

The K_m values of *T. lanuginosus* glucoamylase for *p*-nitrophenyl α-D-glucoside, maltose, maltotriose, dextrin, soluble starch and glycogen are given in Table 3. The K_m value decreased with increasing chain length of the substrate molecule. Thus the K_m value decreased from 833μg/ml (2.3mM) for maltose to 556μg/ml (1.1mM) for maltotriose, when the chain length of the substrate molecule was increased by one glucose residue. Also, the K_m values for high-molecular-weight substrates such as dextrin, soluble starch and glycogen decreased by 7-, 18- and 38-fold respectively when compared with the value obtained for maltose as substrate. This could be due to an increased number of interactions between the active site of the enzyme and the substrate molecule, resulting in an increased affinity of the enzyme with increasing chain length of the molecule.

Table 3. *K_m values of glucoamylase from T. lanuginosus for various substrates*

The assay conditions were identical with those described in the Experimental section except that the substrate concentration was varied. The amount of purified enzyme used for the substrates *p*-nitrophenyl α-D-glucoside and maltose was 2μg/ml of the reaction mixture, and that for the substrates maltotriose, dextrin, soluble starch and glycogen was 0.4μg/ml.

Substrate	K_m value (μg/ml)
p-Nitrophenyl α-D-glucoside	833 (2.75mM)
Maltose	833 (2.3mM)
Maltotriose	556 (1.1mM)
Dextrin	114
Starch (soluble)	46
Glycogen	22

Effect of denaturants on glucoamylase activity

The results summarized in Table 4 show that significant denaturation of the enzyme occurred only with relatively high concentrations of guanidinium chloride and with still higher concentrations of urea. After treatment with 8M-urea, gel filtration on a Sephadex G-25 column resulted in complete recovery of the enzyme activity. On the other hand, only 50% of the original enzyme activity was recovered after Sephadex G-25 gel filtration of the guanidinium chloride (4M)-treated enzyme. The glucoamylase was stable to treatment with sodium dodecyl sulphate and exhibited complete activity in the presence of 1.5% sodium dodecyl sulphate.

Thermal stability of glucoamylase

The thermal-stability curves of glucoamylase at different temperatures in the range 50–80°C are given in Fig. 6. The enzyme was completely stable at 50°C for 6h, lost only about 40% activity at 60°C and was rapidly inactivated at 70°C and 80°C. The $t_{\frac{1}{2}}$ (half-life) values of the enzyme calculated were 7.3h at 60°C, 30min at 70°C and 10min at 80°C at pH6.0.

Table 4. *Effect of denaturing agents on glucoamylase activity*

Purified glucoamylase from *T. lanuginosus* (6μg) was incubated for 60min at 30°C in 1ml of a mixture containing 50mM-sodium acetate buffer, pH5.0, and the denaturant at the concentrations indicated. A portion (50μl) was taken and assayed under the conditions described in the Experimental section. The reaction was terminated by the addition of 2ml of Somogyi (1952) copper reagent and diluted to 4ml with distilled water and mixed thoroughly. The tubes were covered with aluminium foil and transferred to a boiling-water bath for 10min, and then cooled in tap water. Then 2ml of Nelson's (1944) colour reagent was added and the intensity of the green colour was estimated in a Klett–Summerson colorimeter with a 54 filter. The residual activities were calculated and are expressed as the fractional percentages of the control (untreated enzyme) values.

Urea		Guanidinium chloride		Sodium dodecyl sulphate	
Concn. (M)	Activity	Concn. (M)	Activity	Concn. (%, w/v)	Activity
2	97	2	68	0.5	100
4	88	3	55	1.0	100
6	77	4	30	1.5	100
8	64	5	20	2.5	100

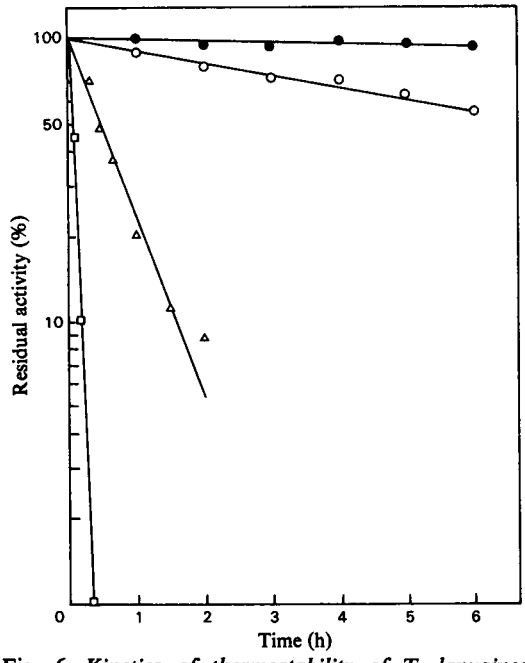

Fig. 6. *Kinetics of thermostability of T. lanuginosus glucoamylase in a semi-logarithmic plot*

The enzyme (2μg/ml) was incubated at defined temperatures in the range 50–80°C (●, 50°C; ○, 60°C; △, 70°C; □, 80°C) in 100mM-potassium phosphate buffer, pH 6.0, in a constant-temperature water bath. Portions of the samples were withdrawn at appropriate time intervals, zero time serving as the control, and were cooled immediately in ice. The residual activities were determined and expressed as the fractional percentages of the control values.

Discussion

The extracellular glucoamylase purified from the thermophilic fungus *T. lanuginosus* exhibited remarkable thermal stability and hydrolysed soluble starch quantitatively to glucose. The enzyme did not show any isoenzymic pattern at any stage of purification.

The enzyme was highly specific for α-D-1,4-glucosidic linkages. The rate of hydrolysis of various substrates increased with increasing chain length of the substrate (Table 2). The relative rate of hydrolysis of glycogen was less than that of soluble starch and amylopectin, probably owing to the steric hindrances provided by the highly branched structure of the molecule. Glucoamylase from other mesophilic fungi was found to catalyse the hydrolysis of α-D-1,6-glucosidic linkages of isomaltose and panose (Hiromi *et al.*, 1966*a*,*b*; Pazur & Kleppe, 1962; Pazur *et al.*, 1977). Purified glucoamylase from *T. lanuginosus*, on the other hand, did not exhibit any activity towards isomaltose, even after prolonged incubation at high concentrations of the enzyme and the substrate.

Glucoamylase from *T. lanuginosus* effected 100% hydrolysis of maltose and maltotriose to glucose, whereas only 94 and 91% conversion was obtained with starch and glycogen respectively. Although these data are consistent with values expected for complete hydrolysis, the small difference observed in the yields of the product in the case of starch and glycogen could be due to the inability of the enzyme to attack α-D-1,6-glucosidic linkages. This is further supported by the observed failure of glucoamylase to cleave dextran (α1–6-glucan) and isomaltose (Glcpα1–6Glc). However, this conclusion is not completely valid because hydrolysis of the α-D-1,6-glucosidic linkages at the branch points is also controlled by the environment of the linkage (Abdullah *et al.*, 1963). Further studies on the action patterns of glucoamylase with other oligosaccharides containing the α-D-1,6-glucosidic linkages are necessary to substantiate this point.

The optimum temperature for the hydrolysis of soluble starch by *T. lanuginosus* glucoamylase was 70°C. This value is higher than that reported for glucoamylase from other sources (Venkataramu *et al.*, 1975; Yamasaki *et al.*, 1977*a*; Taylor *et al.*, 1978), an observation to be expected from the thermophilic nature of the organism. The activities of some enzymes from thermophilic organisms increased abruptly at about 50°C, as shown by the breaks in the Arrhenius plots (Hachimori *et al.*, 1970; Sugimoto & Nosoh, 1971; Middaugh *et al.*, 1976). It has been postulated (Sugimoto & Nosoh, 1971) that this activation may be due to conformational changes in the enzyme molecule occurring at certain temperatures. However, with *T. lanuginosus* glucoamylase no breaks in the Arrhenius plot (Fig. 5) were observed.

Glucoamylase from *T. lanuginosus* was completely stable at 50°C for 6h, lost activity slowly at 60°C and was inactivated rapidly at 70°C and 80°C. The enzyme isolated from several mesophilic fungi, on the other hand, was shown to be highly susceptible to thermal denaturation above 50°C (Venkataramu *et al.*, 1975; Yamasaki *et al.*, 1977*a*,*b*).

Several theories have been postulated in an attempt to explain the thermostability properties exhibited by the enzymes isolated from thermophilic micro-organisms when compared with the corresponding enzymes from mesophilic micro-organisms (Singleton & Amelunxen, 1973). These include protective factors within the cell (Koffler, 1957; Nakamura, 1960; Prasad & Maheshwari, 1978) and higher average degree of hydrophobicity (Ohta, 1967) in proteins isolated from thermophilic sources. In addition, studies with the α-amylase isolated from *Bacillus stearothermophilus* by Campbell and co-

workers (Manning & Campbell, 1961; Manning *et al.*, 1961; Campbell & Manning, 1961) indicated that this protein may possess a unique structure, that of a semi-random- or random-coiled well-hydrated molecule, that was responsible for its extraordinary thermostability. However, thorough investigations of glyceraldehyde 3-phosphate dehydrogenase from *B. stearothermophilus* and other proteins (Singleton *et al.*, 1969; Freeze & Brock, 1970; O'Brien *et al.*, 1973; Tanaka *et al.*, 1971; Chell *et al.*, 1978) led to the conclusion that the inherent thermal stability of proteins isolated from thermophilic micro-organisms was not due to any external factors or extraordinary structural differences but rather to subtle differences in the protein structure. In order to elucidate the mechanism of thermostability of *T. lanuginosus* glucoamylase, the enzyme was isolated from the mesophilic fungus belonging to *Rhizopus* sp. and characterized. The physicochemical properties of the *T. lanuginosus* and *Rhizopus* glucoamylases were compared, and it appeared that the thermostable nature of the *T. lanuginosus* enzyme was due to its distinctly different protein structure from that of the enzyme from the mesophilic organism.

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