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Amylases of the thermophilic fungus *Thermomyces lanuginosus*: Their purification, properties, action on starch and response to heat

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Abstract. **A thermophilic fungus *Thermomyces lanuginosus*, strain IISc 91, secreted one form each of *α*-amylase and glucoamylase during growth. Both enzymes were purified to homogeneity by ion-exchange and gel-filtration chromatography and obtained in mg quantities. *α*-Amylase was considered to be a dimeric protein of ~ 42 kDa and contained 5% (by mass) carbohydrate. It was maximally active at pH 5·6 and at 65°C. It had an activation energy of 44 kJ mol^{-1}. The apparent K_m for soluble starch was 2·5 mg ml^{-1}. The enzyme produced exceptionally high levels of maltose from raw potato starch. At 50°C, the enzyme was stable for > 7h. At 65°C, *α*-amylase was nearly 8-times more stable in the presence of calcium. Addition of calcium increaed the melting temperature of *α*-amylase from 66°C to 73°C. Upon incubation at 94°C, *α*-amylase was progressively and irreversibly inactivated, and converted into an inactive 72 kDa trimeric species.**

Glucoamylase was a monomeric glycoprotein of ~ 45 kDa with a carbohydrate content of 11% (by mass). It effected up to 76% conversion of starch in 24 h producing glucose as the sole product. Its apparent K_m for soluble starch was 0·04 mg ml^{-1} and V_{max} was 660 µmol glucose min^{-1} mg protein^{-1}. It also hydrolyzed maltose. Its activity on maltooligosaccharides increased with the chain length of the substrates. Glucoamylase was stable at 60°C for over 7h. Its activation energy was 61 kJ mol^{-1} Glucoamylase did not show synergistic effect with α-amylase. The properties of *α*-amylase and glucoamylase of *Thermomyces lanuginosus* strain IISc 91 suggest their usefulness in the commercial production of maltose and glucose syrups.

Keywords. Starch; amylase; glucoamylase; thermostable amylases; *Thermomyces lanuginosus*.

1. Introduction

Some filamentous fungi have been demonstrated to be capable of secreting high amounts of protein in the growth medium (van Brunt 1986). Fungi are therefore attracting increasing attention as sources of amylolytic enzymes suitable for the industrial conversion of starch into maltose or glucose. A thermophilic fungus, *Thermomyces lanuginosus* (syn. *Humicola lanuginosa*), was reported to produce glucoamylase (α-1,4-glucan glucohydrolase; EC 3.2.1.3) that was capable of quantitatively converting soluble starch into glucose (Taylor *et al* 1978; Rao *et al* 1981). The enzyme was a glycoprotein with a molecular mass of ~ 57 kDa. It was active at 70°C and was completely stable at 50°C (Rao *et al* 1981). Jensen *et al* (1988) reported a glucoamylase in another strain of *T. lanuginosus* which had a molecular mass of 70–77 kDa. This strain also produced a thermostable α-amylase (α-1,4 glucan glucanohydrolase; EC 3.2.1.1) of molecular mass of 54–57 kDa that required Ca^{2+} for activity (Jensen and Olsen 1992). In spite of the demonstration that *T. lanuginosus*

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produces a potentially useful amylolytic system, its biochemical characterization has been limited.

We observed that the productivity of α-amylase and glucoamylase can vary appreciably in strains of *T. lanuginosus*. A strain, IISc 91, isolated in our laboratory consistently produced higher levels of these two enzymes than other strains of the fungus. In view of the extraordinary degree of variability in thermophilic microorganisms (Johnson 1979) and reports on physicochemical differences in some polysaccharide-degrading enzymes (cellulase and xylanase) from strains of thermophilic fungi (Khandke *et al* 1989; Anand *et al* 1990) the characterization of the amylolytic system of strain IISc 91 was necessary. A chromatographic procedure was evolved for the separation of α-amylase and glucoamylase activities from the culture filtrates of this strain. Both enzymes were purified to homogeneity. Their catalytic and general properties were studied. An anomaly was observed in the determination of the molecular masses of the two enzymes by gel-filtration and electrophoretic methods. The purified enzymes were used to study the quantitative and the qualitative aspects of starch saccharification. The effect of heat on the structure and function of α-amylase was determined. The results are reported in this paper.

2. Materials and methods

2·1 *Organism and culture conditions*

Strains of *T lanuginosus* were from our collection of thermophilic fungal cultures. Strain 1457 was kindly provided by Dr Bo Jensen, University of Copenhagen, Denmark. Other strains were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA, International Mycological Institute (IMI), Kew, Surrey, UK, and Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands. The fungus was grown in a medium containing (wt/vol) 2% soluble potato starch, 0·4% L-asparagine, 0·15 % KH_2PO_4, 0·1% K_2HPO_4, 0·05% $MgSO_4 \cdot 7H_2O$ and 0·01% (vol/vol) of a tracc elements solution (Vogel 1964) in shake flasks at 50°C for 4 days when maximal levels of amylolytic enzymes in culture medium were produced.

2.2 *Enzyme and protein assays*

α-Amylase was assayed based on the decrease in iodine staining of starch. The reaction mixture (total volume 2 ml) containing enzyme solution and 5 mg soluble potato starch in 50 mM sodium acetate buffer, pH 5·0 (henceforth referred to as buffer A) was incubated for 5 min at 50°C. Reaction was terminated by adding 1 ml of 0·5 M HCl. An aliquot (0·25 ml) of this solution was mixed with iodine reagent (0·02% I_2 + 0·2% KI in 0·5 M HCl). After adding distilled water to dilute the colour of the sample, the intensity of the blue colour was measured in a Klett-Summerson type colorimeter using filter no. 66. One unit of α-amylase activity was defined as the amount of enzyme that hydrolyses 1 mg of starch per min at 50°C.

Glucoamylase activity was measured in 50 mM sodium-potassium phosphate buffer (pH 6·0) containing 2·5 mg soluble starch in a total volume of 1 ml. After incubation for 15 min at 50°C, reaction was terminated by boiling for 30 min. The glucose liberated was estimated by the glucose oxidase-peroxidase method (McComb and Yushok 1957).

One unit of glucoamylase was defined as the amount of enzyme that liberates 1 μmol of glucose per min at 50°C.

Protein in culture filtrates was precipitated by 10% trichloroacetic acid and estimated by the method of Lowry *et al* (1951) using bovine serum albumin as standard. Protein in chromatography column effluents was monitored by measuring absorbance at 280 nm.

2.3 *Purification of enzymes*

A representative procedure for the purification of *α*-amylase and glucoamylase from the culture filtrates of *T. lanuginosus* IISc 91 is described below. All steps were carried out at 4°C.

2.3a *Preparation of culture filtrates* (*step* 1): Cultures were suction-filtered through Whatman filter paper no. 1 to remove fungal mycelia. A light-brown solution containing approximately 0·6 mg protein ml^{-1} was obtained (2 litre).

2.3b *Ultrafiltration* (*step* 2): The crude culture filtrate was concentrated using a Minitan acrylic ultrafiltration system (Millipore Corporation) at a flow rate of 100 mlh^{-1}. The brown-coloured concentrated protein solution was clarified by centrifugation at 10,000 *g* for 10 min.

2·3c *Separation of α-amylase and glucoamylase* (*step* 3): The pH of the protein solution (250 ml) was brought to 5·0 using 50mM acetic acid and the protein was chromatographed on a 10 × 1·2 cm column of DEAE-Sephadex A-50 (bed volume 10 ml) that had been pre-equilibrated with buffer A. After washing out the unbound protein with 500 ml of same buffer, proteins bound to the column were eluted at a flow rate of 20 mlh^{-1} with a linear gradient of 0–400 mM NaCI in 400 ml of the above buffer. Five ml fractions were collected. Fractions containing *α*-amylase and glucoamylase activities were pooled separately and lyophilized.

2·3d *Purification of glucoamylase* (*step* 4): The glucoamylase-enriched protein powder (50 mg) was dissolved in 3 ml of buffer A and chromatographed in batches (1·5 ml) on a column (151 × 1·5 cm) of Ultrogel AcA 54 (bed volume 175 ml). One ml fractions were collected at a flow rate of 10 mlh^{-1}. Glucoamylase-enriched solution obtained from this step was brought to pH 8·0 using 50 mM Na_2 HPO_4 and then subjected to ion-exchange chromatography on a column (5 × 1·2 cm) of DEAE-Sephadex A-50 (bed volume 5 ml) at a flow rate of 20 mlh^{-1}. After washing the column with 100 ml of 50mM Na-K phosphate buffer, pH 8·0 (henceforth referred to as buffer B), protein bound to the column was eluted with a linear gradient of 0–400 mM NaC1 in 400 ml of the same buffer. Five ml fractions were collected.

2.3e *Purification of α-amylase* (*step* 5): The *α*-amylase-enriched protein powder obtained from DEAE-Sephadex A-50 (pH 5·0) chromatography was dissolved in 4·0 ml of buffer A and applied on a column (151 × 1·5 cm) of Ultrogel AcA 54 (bed volume 175 ml) in batches of 1 ml. One ml fractions were collected at a flow rate of 10 mlh^{-1}. Active fractions were pooled and the pH of the solution was brought to 8·0 using

50 mM $Na_2 HPO_4$. The protein solution was applied on a column (5 × 1·2 cm) of DEAE-Sephadex A-50 (bed volume 5 ml) which had been pre-equilibrated with buffer B. The column was washed with 100 ml of the same buffer at a flow rate of 20 mlh^{-1} and the adsorbed protein was eluted with a linear gradient of 0–400 mM NaCl (total volume 400 ml) prepared in the same buffer. Five ml fractions were collected. The fractions possessing α-amylase activity were pooled and concentrated by lyophilization. After dissolving the enzyme powder in 1·5 ml of buffer A, it was applied on a column (136 × 1·2 cm) of Bio-Gel P-30 (bed volume 135 ml) and eluted with the same buffer. Two ml fractions were collected at a flow rate of 10 mlh^{-1}. Purified enzyme was stored at – 20°C.

2.4 *Carbohydrate analyses*

The amount of unhydrolysed starch during enzymatic saccharification was estimated after precipitating it with 80% (vol/vol) ethanol. The precipitate was dissolved in 52% (vol/vol) perchloric acid and an aliquot was used for estimation of starch by the anthrone method (Yemm and Willis 1954). Reducing sugars in starch hydrolysates were estimated using alkaline copper sulphate reagent (Somogyi 1952). Total sugar in the starch hydrolysate was estimated by the anthrone method. The carbohydrate content of the purified enzymes was determined by the phenol-sulphuric acid method (Dubois *et al* 1956). The degree of polymerization (DP) of the starch hydrolysis products was determined by dividing the total sugar value with the reducing sugar value. Per cent saccharification of starch was calculated as follows: [mg of glucose × (162/180) × 100]/(mg of initial substrate). (The factor 162/180 normalizes the conversion for the weight gain caused by addition of water to the glycosyl moiety on hydrolysis).

The products liberated by the action of α-amylase on starch were analysed by gel-filtration chromatography on a column (150 × 2·5 cm) of Bio-Gel P-2 using water as the eluant. One ml fractions were collected at a flow rate of 8 mlh^{-1}. Paper chromatography of starch hydrolysate was performed on Whatman no. 3 paper in descending direction using *n*-propanol-ethyl acetate-water (7:1:2, by vol) as solvent system. The sugars were visualized by alkaline $AgNO_3$ method (Trevelyan *et al* 1950).

The end-products of oligosaccharide hydrolysis by α-amylase was analysed on Waters HPLC system using NH_2-Spherisorb column and acetonitrile-water (70:30 by vol) as a solvent at a flow rate of 0·7 ml min^{-1}. Sugars were detected using Waters refractive index detector (R-401) and quantified using standard sugars.

2.5 *Gel electrophoresis of protein*

Native PAGE was performed in 7·5% polyacrylamide gel. SDS-PAGE was carried out in 10% (wt/v) polyacrylamide gel according to the methods of Laemmli (1970) or Lugtenberg *et al* (1975). Gels were stained by Coomassie brilliant blue R or by $AgNO_3$ to visualize protein bands.

Glucoamylase on non-denaturing gel was visualized by coupling its reaction with glucose oxidase in the presence of the electron acceptors (phenazine methosulphate and nitroblue tetrazolium) as given by Rao *et al* (1981). For visualization of α-amylase activity, non-denaturing gel was polymerized with 0·5% soluble potato starch. After electrophoresis, gel was washed successively with distilled water, 500 and 50 mM

sodium acetate buffer (pH 5·0) for 15 min each and placed in I_2-KI solution for 2 h when band containing α-amylase appeared as a clear area against dark blue background.

2.6 *Molecular mass determination*

Molecular mass of amylases was determined by SDS-PAGE, on native PAGE (Ferguson 1964; Hedrick and Smith 1968), by HPLC using a TSK 2000 SW gel-filtration column at pH 5·0 using buffer A, and also by their mobility on an Ultrogel AcA 54 gel-filtration column (151 × 1·5 cm) at pH 8·3 in 100 mM Tris-glycine buffer.

For Ferguson (1964) analysis, purified enzymes and standard proteins were electrophoresed on 10%, 12%, 14%, 16% and 18% (wt/vol) polyacrylamide gels (375 mM Tris-HC1, pH 8·8) with 3% (wt/v) polyacrylamide stacking gel (125 mM, pH 6·8). Electrophoresis was carried out at 20 mA using Tris-glycine buffer (pH 8·3). The relative mobilities of standard proteins and the two amylases on each gel was plotted against gel concentration. The negative slope of the line obtained for each protein was plotted against the logarithm of its molecular mass to obtain the standard curve. The molecular mass of α-amylase and glucoamylase was determined by interpolation of the standard curve.

2.7 *Amino acid composition and N-terminal sequence determination*

Amino acid composition of amylases was determined by the method of Moore and Stein (1963) on an amino acid analyser (Shimadzu, SCL-6B). Tryptophan content of purified enzymes was calculated from ultraviolet spectrum of protein in 0·1 M sodium hydroxide (Bencze and Schmid 1957). N-terminal amino acid sequence of purified enzymes was determined by Edman degradation on an automated gas phase sequenator (Shimadzu model PSQ-1).

2.8 *Thermostability determination*

Purified enzymes (10 µg in 1 ml) were incubated in the respective assay buffers at the required temperatures in a thermostated water bath. Aliquots (100 µl) of the sample were withdrawn at different time intervals, promptly cooled on ice and the residual activity was determined. The half-life ($t_{1/2}$) of inactivation was determined from a semi-logarithmic plot of residual activity as a function of time.

2·9 *Effect of heat*

For studying the effect of heat on structure of α-amylase, enzyme was kept in a boiling water bath at 94°C (at the altitude of Bangalore) for the required time period. Samples were mixed with the buffer for analysis by native or SDS-PAGE.

2.10 *Fluorescence measurements*

A Hitachi 650–60 fluorospectrometer connected to a temperature control accessory was used. The enzyme solution was equilibrated at each temperature for 10 min before

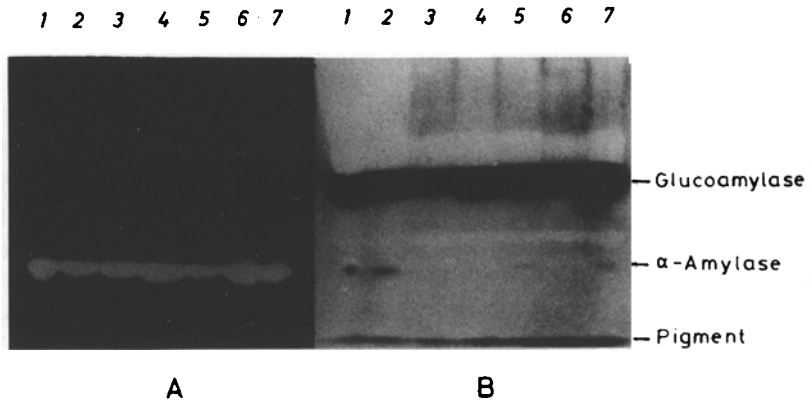

Figure 1. Native PAGE analysis of culture filtrate protein of different strains of *T. lanuginosus*. Protein (25 µg per lane) was electrophoresed. (**A**) *α*-Amylase staining. (**B**) Glucoamylase staining. Lane 1, IISc 91; lane 2, CBS 39562; lane 3, IMI 84400; lane 4, Denmark 1457; lane 5, ATCC 26909; lane 6, IISc 86; lane 7, ATCC 22083·

recording the spectrum. The samples were excited at 280 nm and the emission spectra were recorded between 300 to 400 nm. Melting temperature (T_m) was determined from the plot of $\Delta F/\Delta t$ versus t.

3. Results

3.1 *Strain variability*

Some 20 strains of *T lanuginosus* isolated from compost/soil samples from India and those obtained from culture collections were compared for productivity of *α*-amylase and glucoamylase. Strain IISc 91 produced nearly 2- to 10-fold higher levels of *α*-amylase and glucoamylase than other strains. Its yield was 40–44 units *α*-amylase and 4–5 units glucoamylase per ml of culture filtrate. Both enzyme activities appeared simultaneously and reached their maximum levels on day 4 of culture·

To determine whether strains from different geographical origins produce isoenzyme forms of *α*-amylase and glucoamylase, 4-day-old culture filtrates were lyophilized. The samples were dialyzed overnight against the buffer A at 4°C and analysed by native PAGE. I_2-KI staining of the gel showed that all seven strains of the fungus tested exhibited one glucoamylase and one *α*-amylase of identical mobility (figure 1).

3.2 *Purification of enzymes*

The purification of glucoamylase and *α*-amylase are summarized in tables 1 and 2, respectively. The two enzyme activities were separated by ion-exchange chromatography at pH 5·0 with glucoamylase eluting before *α*-amylase. For both enzymes,

Table 1. Purification of glucoamylase from *T. lanuginosus* IISc 91

Purification step	Vol (ml)	Total activity (U)	Total protein (mg)	Sp. act. (U/mg)	Purification (-fold)	Recovery (%)
Culture filtrate	2000	8000	1240	6·4	1	100
Ultra-filtration	250	7200	705	10·2	1·6	90
DEAE-Sephadex (pH 5·0)	80	5640	105·2	53·6	8·4	71
Ultrogel AcA 54	50	3363	8·2	410	63	42
DEAE-Sephadex (pH 8·0)	60	2376	4	594	93	30

Table 2. Purification of α-amylase from *T. lanuginosus* IISc 91·

Purification step	Vol (ml)	Total activity (U)	Total protein (mg)	Sp. act. (U/mg)	Purification (-fold)	Recovery (%)
Culture filtrate	2000	80,000	1240	64·5	1	100
Ultrafiltration	250	72,250	705	102·4	1·6	90
DEAE-Sephadex (pH 5·0)	80	54,800	97·7	560·9	8·6	69
Ultrogel AcA 54	30	40,440	9·6	4212·5	65·3	51
DEAE-Sephadex (pH 8·0)	30	38,850	5·7	6815·7	105·6	49
Bio-Gel P-30	15	32,445	4·5	7210	112	41

subsequent steps of gel-filtration and ion-exchange chromatography at pH 8·0 were effective in removing the contaminating proteins. Both enzymes were obtained in 4–5 mg quantities from 2 litre culture filtrate. The purified enzymes gave single protein band on SDS-PAGE after $AgNO_3$ staining (figure 2).

3.3 *Catalytic properties*

At 50°C α-amylase and glucoamylase were maximally active at pH 5·6 and 6·0, respectively. In 5 min assay, α-amylase showed maximum activity at 65°C and glucoamylase at 70°C. However, enzymes were routinely assayed at 50°C as this was the optimum temperature of growth of the fungus. From the Lineweaver-Burk plot of the data, the calculated V_{max} for α-amylase was 8000 mg starch depolymerized min^{-1} mg $protein^{-1}$ and the apparent K_m was 2·5 mg ml^{-1}. For glucoamylase, the V_{max} was 660 µmol glucose min^{-1} mg $protein^{-1}$ and K_m was 0·04 mg ml^{-1}. The activation energy calculated from the Arrhenius plot of initial rate of reaction versus temperature was 44 kJ mol^{-1} for α-amylase and 61 kJ mol^{-1} for glucoamylase.

3.4 *Thermal stability*

The thermal stability of purified enzymes was studied by measuring catalytic activity and by studying fluorescence properties at different temperatures. At 50°C, the optimal

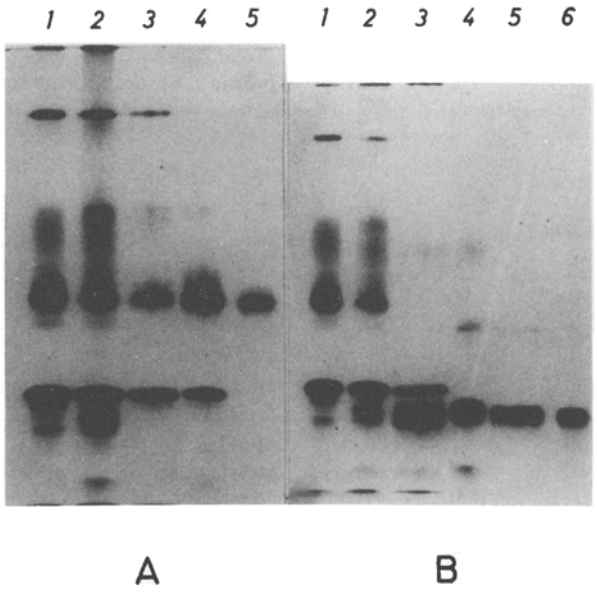

Figure 2. SDS-PAGE analysis of glucoamylase- and α-amylase-enriched protein fractions at different steps of purification. $AgNO_3$ staining. (**A**) Glucoamylase. (**B**) α-Amylase. Lane 1, culture filtrate protein (30 μg); lane 2, ultrafiltration (30 μg); lane 3, DEAE-Sephadex A-50, pH 5·0 (30 μg); lane 4, Ultrogel AcA 54 (20 μg); lane 5, DEAE-Sephadex A-50, pH 8·0 (20 μg); lane 6, Bio-Gel P-30 (10 μg).

growth temperature of *T. lanuginosus*, α-amylase was completely stable for 7h, the maximal time period tested. Glucoamylase was fully stable even at 60°C for 7h. At temperature above 60°C, enzymes were less stable. At 65°C, half-life ($t_{1/2}$) of α-amylase and glucoamylase was 0·6 and 4 h, respectively. At 70°C both enzymes were inactivated to a similar extent, the $t_{1/2}$ being 0·3 h.

Because α-amylases from several mesophilic organisms have been reported to require Ca^{2+} for activity and/or stability (Vihinen and Mantsala 1989), the effect of a chelating compound on its catalytic activity was studied. Ethylenebis (oxonitrilo) tetra-acetate (EGTA) at 10 mM inhibited *T. lanuginosus* α-amylase activity by 60% indicating that Ca^{2+} was required for activity. Ca^{2+} also increased the thermostability of α-amylase, its $t_{1/2}$ increased 8-fold at 65°C and 2-fold at 70°C. Ca^{2+} had no effect on the T_{opt}, of α-amylase and activation energy. At > 60°C, glucoamylase was less stable in the presence of added calcium, its $t_{1/2}$ was 1·5 h at 65°C and 0·2 h at 70°C.

On heating, the emission maximum of the native α-amylase shifted from 336 to 344 nm (data not shown). Similar observations were made for glucoamylase but its emission maximum (348 nm) shifted by 4 nm only (352 nm). First derivative plot of fluorescence data showed that addition of Ca^{2+} increased the melting temperature (T_m) of α-amylase from 66°C to 73°C. When the 90°C heated sample was cooled to 25°C for 30 min, it was observed that the emission maxima returned to the original value. However, the original fluorescence intensity had decreased.

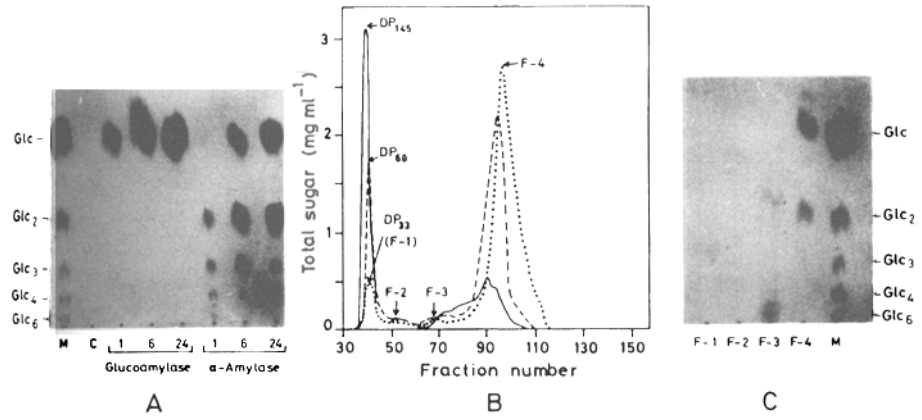

Figure 3. Analysis of products liberated by the action of α-amylase and glucoamylase on raw potato starch. (**A**) Paper chromatography of reaction products. Starch (200 mg) was incubated with α-amylase (4·0 U) or glucoamylase (0·4 U) in 10 ml of buffer A at 50°C for different time periods (1, 6 and 24 h). Abbreviations: A, α-amylase; G, glucoamylase; M, mixture of sugar standards; C, control (unhydrolyzed starch). (**B**) Bio-Gel P-2 chromatography of α-amylase reaction mixtures (2 ml) after 1 h (——), 6h (---) and 24 h (····). (C) Paper chromatography of 24h starch hyclrolysate (25 µg) obtained from Bio-Gel P-2 chromatography.

3.5 *Substrate specificity*

Various α-glucans (starch, amylose, glycogen) were hydrolyzed by the purified enzymes to nearly similar extent. Maltose was hydrolyzed by glucoamylase at approximately one-fourth the rate of soluble starch. It was not hydrolyzed by α-amylase. The enzymes showed very low (3–4% activity relative to that on soluble starch) activity on chitin, pullulan, α- and β-cyclodextrins and were totally inactive on cellulose.

3.6 *Action on starch and maltooligosaccharides*

In a 1 h reaction, α-amylase produced predominantly polysaccharides of average DP_{145} and very little maltooligosaccharides (figure 3). In 6 h, the chain length of polysaccharide decreased to average DP_{68} and glucose appeared with concomitant increase in oligosaccharides. In 24 h, starch hydrolysates contained 12% polysaccharide (F-1, average DP_{33}), 2·8% oligosaccharide (F-2, average DP_{10}), 0·8% maltoheptose (F-3), 80·2% maltose and 4·2% glucose (F-4). The data showed that maltose is the principal final product of raw potato starch hydrolysis by α-amylase. Glucoamylase liberated only glucose from raw potato starch in a time-dependent manner (figure 3A) and there was no evidence of transglycosylated product being formed. The data in table 3 showing its action on maltooligosaccharides are consistent with its exo-acting mode of hydrolysis. Its action on maltose shows that the enzyme has α-glucosidase activity. Activity of glucoamylase on Glc_3–Glc_7 increased with the chain length of substrates.

Table 3. Sugars released from hydrolysis of oligosaccharides by *T. lanuginosus* glucoamylase.

Substrate	Time (min)	Glc	Glc_2	Glc_3	Glc_4	Glc_5	Glc_6	Glc_7
		(%)						
Glc_2	15	46	44					
	30	71	20					
	45	83	10					
	60	91	6					
Glc_3	10	4	7	89				
	20	6	9	85				
	30	8	18	74				
	40	10	21	69				
	60	14	25	61				
Glc_4	10	6	4	20	70			
	20	10	5	28	57			
	30	10	5	32	54			
	40	12	6	38	44			
	60	15	8	45	32			
Glc_5	10	9	2	7	22	60		
	20	11	3	10	27	50		
	30	15	4	16	29	36		
	40	27	10	28	22	13		
	60	30	14	24	12	4		
Glc_6	10	12	3	9	13	27	36	
	20	15	4	10	14	29	30	
	30	18	5	11	17	26	23	
	40	28	18	24	10	8	5	
	60	38	19	22	14	4	0	
Glc_7	10	15	2	7	8	14	19	22
	20	16	4	9	9	16	22	13
	30	17	4	11	12	14	15	10
	40	22	5	10	9	16	19	7
	60	25	4	10	10	12	10	0

3.7 *Saccharification of starch*

To determine if the action of purified enzymes is quantitatively similar to that of the crude culture filtrates, soluble potato starch was incubated with 4-day-old culture filtrates or with purified enzymes for up to 6 h at 50°C (based on estimation of ethanol-precipitable polysaccharide), in amounts equivalent to that in the culture filtrates. After 6 h, the amount of starch degraded by a mixture of *α*-amylase and glucoamylase was 70% and that by culture filtrates it was 63%. The amount of glucose (17 mg) produced from a mixture of *α*-amylase and glucoamylase was quantitatively equivalent to that produced by individual enzymes (2 mg + 16 mg). Similar results were obtained with raw potato starch. These results showed that glucoamylase of *T. lanuginosus* IISc 91 does not act synergistically with *α*-amylase. This point was further brought about by the experimental data in figure 4. Regardless of the amount of glucoamylase used, saccharification of starch was not increased by addition of *α*-amylase.

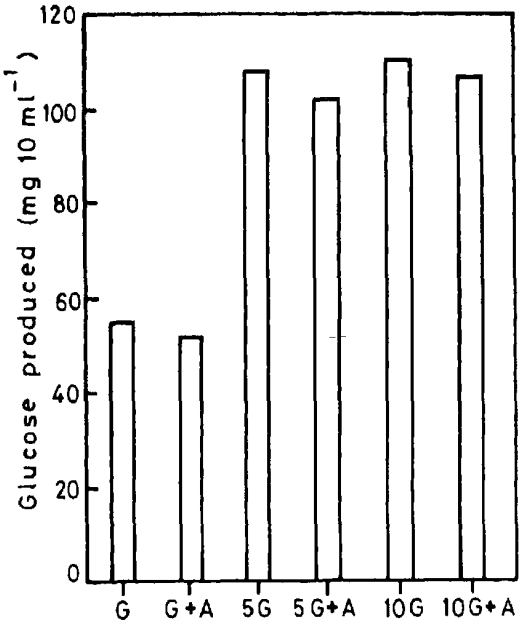

Figure 4. Saccharification of raw potato starch by different ratios of α-amylase and glucoamylase. Starch (200 mg) was incubated with amylases in 10 ml of buffer A for 6 h at 50°C. Abbreviations: G, glucoamylase, 0·4 U; A, α-amylase, 4·0 U.

Table 4. Amino acid composition of α-amylase and glucoamylase from *T. lanuginosus* IISc 91[a].

Amino acid	α-Amylase	Glucoamylase
Aspartic acid[b]	16·25	12·4
Threonine	7·38	6·71
Serine	6·67	10·14
Glutamic acid[c]	8·47	8·91
Proline	3·91	5·95
Glycine	10·06	8·73
Alanine	11·02	14·77
Cysteine	ND[d]	ND
Valine	5·44	4·41
Methionine	1·18	0·24
Isoleucine	3·64	3·69
Leucine	7·51	6·15
Tyrosine	3·55	3·44
Phenylalanine	3·73	3·56
Histidine	2·37	1·90
Lysine	3·42	2·08
Arginine	2·54	3·4
Tryptophan	4·86	3·44

[a]Values are expressed as mole per cent. [b]Includes asparagine. [c]Includes glutamine. [d]ND, not detected.

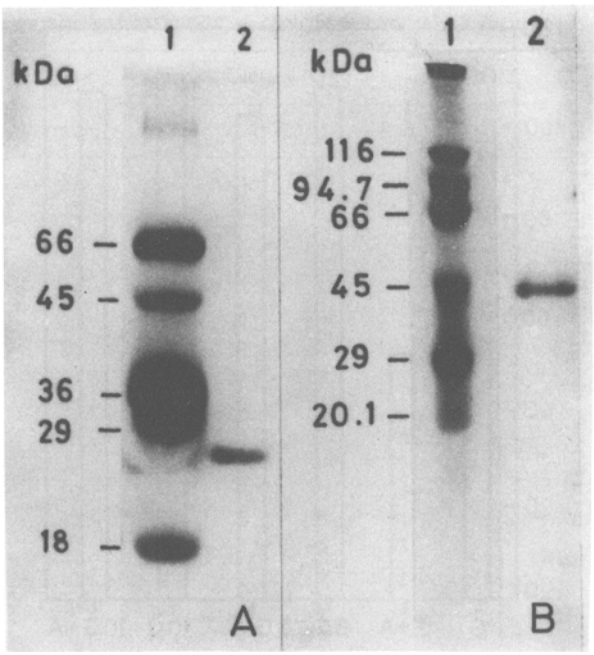

Figure 5. Characterization of α-amylase and glucoamylase by SDS-PAGE. The procedure of Lugtenberg *et al* (1975) was used and gels were stained by Coomassie brilliant blue R. (**A**) α-Amylase (15 µg), (**B**) Glucoamylase (15 µg). The marker proteins (kDa) used were α-lactoalbumin (18), soyabean trypsin inhibitor (20·1), carbonic anhydrase (29), lactate dehydrogenase (36), ovalbumin (45), bovine serum albumin (66), phosphorylase *b* (94·7) and β-galactosidase (116).

The extent to which starch could be hydrolyzed over a longer period of time by α-amylase and glucoamylase was determined. Starch (200 mg in 10 ml) was incubated with glucoamylase (0·4 units) or α-amylase (4·0 units) at 50°C for 24 h in 50 mM sodium acetate buffer (pH 5·6). α-Amylase degraded 90–97% starch (based on estimation of ethanol-precipitable starch) and glucoamylase hydrolysed 62–76% of starch (based on amount of glucose produced). The incubation of reaction mixture in a dialysis bag and the continuous removal of product by dialysis did not increase saccharification of starch by glucoamylase. This indicated that the incomplete saccharification of starch was not due to the product inhibition of glucoamylase by glucose.

3.8 *Molecular properties*

Sugar estimation by the phenol-H_2SO_4, method showed that α-amylase contained 5% (by mass) and glucoamylase contained 11% (by mass) carbohydrate. α-Amylase was rich in Asp/Asn, Glu/Gln, Ala and Gly while glucoamylase was rich in Asp/Asn, Ser and Ala (table 4). Cys was not detected either in α-amylase or in glucoamylase. The first

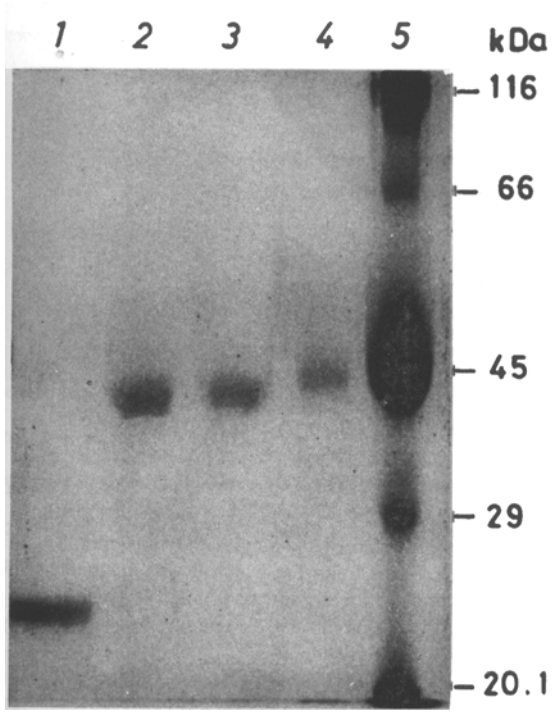

Figure 6. SDS-PAGE analysis of *α*-amylase after different lime of heating. Enzyme was heated to 94°C and 25 µg sample was electrophoresed. Lane 1, unheated; lane 2, 5 min; lane 3, 20 min; lane 4, 90 min; lane 5, protein markers.

21 N-terminal amino acid residues of the two enzymes were determined. *α*-Amylase amino acid sequence was A( ) PDEWKAZSYIFMLTDRFRT. The N-terminal amino acid sequence of glucoamylase was ATGSLDAFAAZPPIAFZGILG. SDS-PAGE (pH 8·3) analysis showed that *α*-amylase and glucoamylase have molecular mass of ~ 24 and 45 kDa, respectively (figure 5). Gel filtration by HPLC at pH 5·0 gave molecular mass of *α*-amylase as ~ 72 kDa and of glucoamylase as ~ 68 kDa. By gel-filtration behaviour on a column of Ultrogel AcA 54 using a 100 mM Tris-glycine buffer of pH 8·3, the molecular mass of both proteins was estimated to be ~ 70 kDa. On the other hand, by Ferguson plot analyses native *α*-amylase and glucoamylase were found to have molecular masses of ~ 42 and ~ 45 kDa, respectively.

3.9 *Effect of heat on α-amylase*

Because industrial starch liquefaction is mediated at high temperatures, it was of interest to study the response of *α*-amylase to high temperature. Whereas the native *α*-amylase migrated on SDS-PAGE as a single band of ~ 24 kDa, the enzyme which

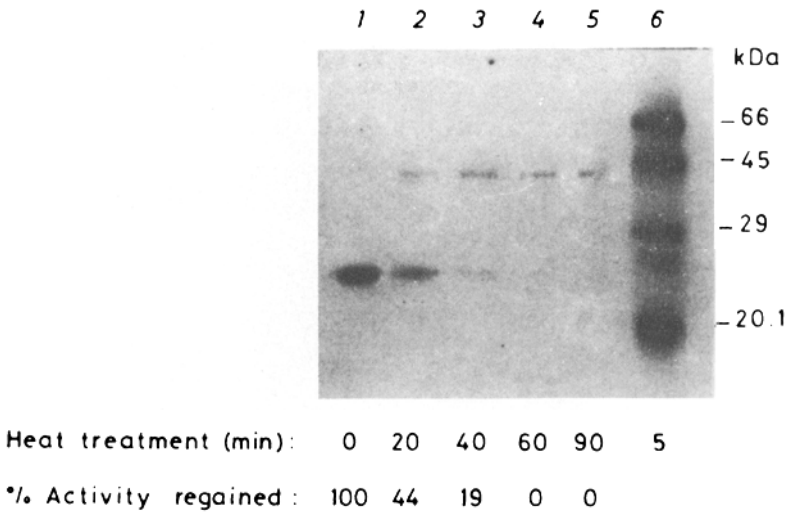

Figure 7. SDS-PAGE analysis of reactivated α-amylase. The enzyme (30 µg in 30 µl of buffer A) was heated for the indicated time following which it was incubated at 37 C to allow reactivation. An aliquot was removed for measurement of enzyme activity.

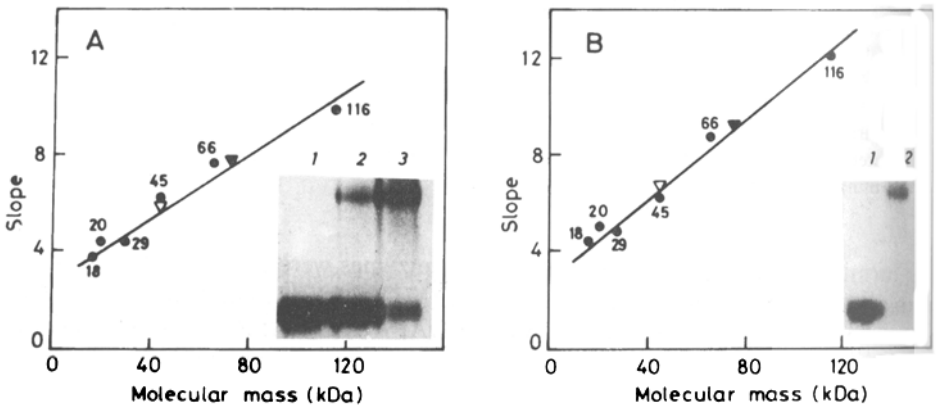

Figure 8. The slope-molecular mass relation of heated α-amylase and marker proteins. The data are based on comparative migrations of sample and standard proteins on native PAGE using gel of different concentrations. (**A**) α-amylase (lane I) heated for 5 min (lane 2) and 20 min (lane 3). (**B**) α-amylase (lane I) heated for 90 min (lane 2). Low molecular mass species (Δ), high molecular mass species (▲).

had been heated to 94°C for 5, 20 and 90 min migrated as single bands of ~ 45 kDa (figure 6), regardless of treatment with 2·5% (by vol) β-mercaptoethanol. The intensity of the protein-stained band of α-amylase decreased with the time of heating. The 5-rnin heated α-amylase possessed 50% of the original activity; that heated for 20 min was inactive but it regained nearly 50% of the original activity upon incubation for 3 h at

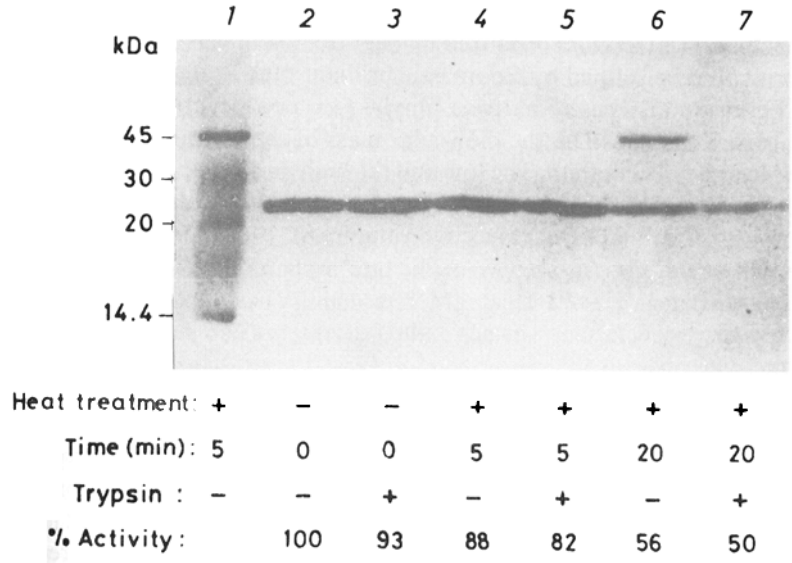

Figure 9. Trypsin sensitivity of heat-treated α-amylase. Enzyme (50 µg in 50 µl buffer A) was heated at 94°C for 5min (lanes 4 and 5) or 20 min (lanes 6 and 7) and immediately kept at 37°C for 30 h. Samples were incubated with or without trypsin (1 µg in 10 µl of 100 mM NH_4HCO_3, pH 8·2) at 37°C for 6 h. Aliquots (20 µl) were removed for measurement of α-amylase activity. For SDS-PAGE analysis 30µg protein per lane was used. Lane I, marker proteins in order of increasing molecular mass: lysozyme, soyabean trypsin inhibitor, carbonic anhydrase and ovalbumin. Lanes 2 and 7, α-amylasc treated as indicated.

37° or 50°C. In contrast, only 5% activity was regained at 0° or 4°C even after 30 h. Addition of Ca^{2+} to reaction mixtures did not affect the extent of reactivation.

The ability to regain enzyme activity decreased with the time of heating (figure 7). The regain of activity at 37°C was associated with the reappearance of a 24 kDa protein on SDS-PAGE (lanes 2 and 3) that corresponded to the native enzyme (lane 1). The intensity of the 24 kDa band appeared to be related to the activity regained. The 60 or 90 min heat treatment produced a form of α-amylase that migrated as ~ 45 kDa (lanes 4 and 5). In these samples, enzyme activity was not detected and the intensity of the 45 kDa protein bands decreased progressively.

On non-denaturing gels also, the 5, 20 and 90 min heated α-amylase exhibited two protein-stained bands (figure 8), of which the higher mobility protein migrated as the native enzyme. This species of protein was catalytically active as determined by I_2-KI staining on the gel or by activity measurement after electroeluting the protein band from the gel. By contrast, the shifted protein band with the lower electrophoretic mobility was inactive.

To probe differences in the tertiary structure of the native and heat-inactivated α-amylase, their resistance to proteolysis by trypsin was tested. As seen in figure 9, neither the native (lanes 2 and 3) nor the 5-min heated-reactivated α-amylase (lanes 4 and 5) were susceptible to trypsin digestion. By contrast, the high molecular mass form produced by 20 min heat-treatment of α-amylase (lane 6) was degraded by trypsin (lane 7).

Structural differences between the native and the heat-treated α-amylase was further indicated by $AgNO_3$ staining and by Schiffs base. Unlike the native α-amylase, the shifted band species (45 kDa) produced by heat treatment was not stained by $AgNO_3$ although it could be stained by Coomassie brilliant blue R and Schiffs base.

Since Ferguson analysis of native α-amylase on non-denaturing gel had given its molecular mass as ~ 45 kDa, the molecular mass of the heat-inactivated protein was evaluated by the same method. The low and the high molecular mass protein species in 5, 20 or 90 min species of heated α-amylase samples were found to have molecular masses of ~ 45 and ~ 72 kDa, respectively (figure 8).

4. Discussion

A comparison of the results of the previous and the present investigations on *T. lanuginosus* shows that not only the amount of amylolytic enzymes secreted vary appreciably among strains (present study), but their composition can also be different. For example, whereas Taylor *et al* (1978) reported that strain K13/1 produced two forms of glucoamylases, in other strains only one form has been reported (Taylor *et al* 1978; Rao *et al* 1981). On the other hand, whereas strain K13/1 apparently lacked α-amylase activity, this enzyme was found in other strains studied. Moreover, differences in the physical properties of enzymes from different strains are evident: the molecular mass of glucoamylase and α-amylase of strain 1457 (Jensen *et al* 1988) are different from that in strain IISc 91 (present study). Dissimilarities in properties of other polysaccharide-degrading enzymes from thermophilic fungi have also been noted (Khandke *et al* 1989; Anand *et al* 1990). Differences in enzymological properties are commonly ascribed to strain difference. An unusually high degree of genetic variability may be a feature of organisms living in the hot habitat (Johnson 1979).

All strains of *T. lanuginosus* examined were found to secrete one form each of glucoamylase and α-amylase and there was no indication of polymorphism at the gene loci for these enzymes in the strains examined. The electrophoretic pattern of the crude culture filtrate protein of strain IISc 91 and the enzyme activity profile in different purification steps suggest that this strain produces only one form each of glucoamylase and α-amylase. This situation is in contrast to other fungal species where multiplicity of forms have been reported (Yamasaki *et al* 1977; Svensson *et al* 1982; Hayashida *et al* 1988; Ono *et al* 1988; Fagerstrom *et al* 1990).

A simple procedure was evolved to obtain pure α-amylase and glucoamylase from the culture filtrates of *T. lanuginosus* strain IISc 91 in reasonable yield. Anion-exchange and gel-filtration chromatography have been used to separate multiple forms of glucoamylase in Aspergillus oryzae (Razzaque and Ueda 1978), *Penieillium oxalicum* (Yamasaki *et al* 1977) and Rhizopus sp. (Takahashi *et al* 1978). In the present study, anion-exchange chromatography was used to cleanly separate α-amylase from glucoamylase with approx 8-fold purification. An effective step was Ultrogel AcA 54 gel-filtration chromatography which separated amylases from major contaminant and pigments without significant loss in enzyme activity. This step alone resulted in up to 65- and 68-fold purification of α-amylase and glucoamylase, respectively. The recovery of glucoamylase (30%) in the present study is comparable to that purified from strain ML-M by anion-exchange chromatography and preparative PAGE by Rao *et al* (1981). However, our purification procedure of glucoamylase is an improvement over

that used by Rao *et al* (1981) as larger volumes of culture filtrates can be processed and pure enzymes can be obtained in mg quantities. Rao *et al* (1981) did not report on the presence of α-amylase. The specific activity of α-amylase of strain IISc 91 was comparable to that of strain 1457. However, the recovery of this enzyme varies; it was 42% in strain IISc 91 and only 0·7% in strain 1457 (Jensen *et al* 1988).

The α-amylase and glucoamylase of *T. lanuginosus* IISc 91 show a combination of properties which make them interesting enzyme systems in applied and basic research. Although not as highly thermostable as some α-amylases from the thermophilic archaebacteria (Koch *et al* 1991; Laderman *et al* 1994) and bacteria (Bealin-Kelly *et al* 1991), the *T. lanuginosus* α-amylase and glucoamylase are the most thermostable enzymes from fungal sources (Vihinen and Mantsala 1989; Fagerstrom *et al* 1990). Both enzymes are active on raw starch. Unlike the enzymes of *Aspergillus* K-27 (Abe *et al* 1988), glucoamylase of strain IISc 91 did not require the presence of α-amylase for maximal hydrolysis of raw starch. Strain IISc 91 α-amylase produced higher levels of maltose (80%) than other α-amylases (Doyle *et al* 1989). By comparison, the highly thermostable α-amylase from *Pyrococcus furiosus* mainly produced maltooligosaccharides (Laderman *et al* 1994). The *T. lanuginosus* α-amylase also has higher affinity for starch than the *Pyrococcus* enzyme. The glucoamylase of *T. lanuginosus* can effect high conversion of starch into glucose without transglycosylation reactions. The incomplete conversion of starch could be due to the inability of the enzyme to attack α-D-1, 6-glucosidic linkages in substrate, as shown by its very low action on pullulan. The measurements of fluorescence emission spectra in presence and absence of calcium showed that it increases the melting temperature of the enzyme. These results were corroborated by increased $t_{1/2}$ values of α-amylase in presence of calcium. In this regard the behaviour of *T. lanuginosus* α-amylase resembles the mesophilic counterparts. The present experiments were, however, insufficient to resolve whether calcium is bound to the enzyme or the mechanism by which it influences activity and stability of α-amylase. It is interesting to note that glucoamylase from the same source was less stable in presence of calcium above 60°C.

The evaluation of the apparent molecular mass of the purified enzymes by size-exclusion and electrophoretic methods gave different results. The molecular mass of α-amylase as determined by gel-filtration chromatography was three times of that obtained by SDS-PAGE (24 kDa), suggesting that the enzyme is a trimer. On the other hand, analysis using native PAGE gave a molecular mass of 42 kDa. Since the migration of proteins on gel-filtration depends on the hydrodynamic volume rather than on the molecular mass of the protein, the gel-filtration could give an anomalous result. The partial amino acid sequence of α-amylase showed the presence of a single N-terminal amino acid (Ala). Based on these considerations, the native α-amylase of our strain of *T. lanuginosus* is considered to be a dimer of two 24 kDa subunits which are held together by non-covalent interactions. The protein could be a heterodimer rather than a homodimer, with the N-terminal of one of the polypeptides being blocked. The final proof will be available from primary structure determination and cross-linking studies. This is the first indication of a dimeric α-amylase in fungi although homodimeric α-amylase in a hyperthermophilic archaebacterium (Laderman *et al* 1994) and aggregated forms in bacteria (Robyt and Ackerman 1973) have been reported. The glucoamylase from strain IISc 91 is considered to be a 45 kDa monomeric protein.

The electrophoretic analysis of *T. lanuginosus* α-amylase that had been incubated at boiling temperature revealed the potential of the protein to dissociate into subunits and

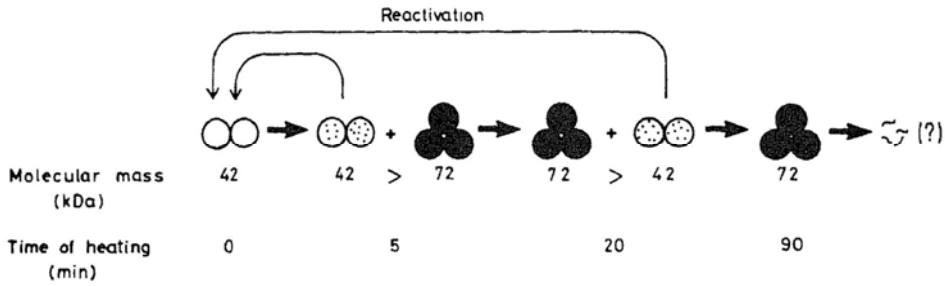

Figure 10. A schematic representation of the observations on the effect of heat on α-amylase. The native dimeric enzyme is shown by open circles, the partially active enzyme by dotted circles and the inactive trimeric species by filled circles.

reassociate to produce a specific oligomeric form. A schematic presentation of the observations and a plausible interpretation is given in figure 10. Whereas the native (~ 42 kDa) enzyme is dissociated by SDS into ~ 24 kDa monomer, the 5 or 20min heated protein is partially denatured and is in an SDS-undissociable form. This conformational state is potentially able to return to the native state in a temperature- and time-dependent process as shown by the regain of the complete enzymatic activity. Since *T. lanuginosus* α-amylase does not contain cysteine residues and β-mercaptoethanol had no effect on the conversion of the native into a partially denatured form, disulphide bonding did not participate in generating the altered conformation state. In addition to the partially denatured dimeric form of α-amylase, a new enzymatically inactive ~ 72 kDa trimeric species was formed by the self-association of subunits. With prolonged heating, the concentration of the dimeric, partially active protein decreased while that of the inactive trimeric protein increased. The susceptibility of the oligomeric protein to digestion by trypsin suggested a distorted tertiary structure. Presumably this form of the protein was prone to fragmentation, as indicated by the loss of protein staining on the gel. To our knowledge, heat-induced structural reorganization in fungal protein has not been reported before.

Jensen *et al* (1988) purified an α-amylase from the culture filtrates of *T. lanuginosus* strain 1457. By the SDS-PAGE method its molecular weight was estimated to be 54–57 kDa. The enzyme glucoamylase of this strain also has a higher molecular weight (70–77 kDa) than of strain IISc 91. We are of the opinion that the difference in the molecular weight of the enzymes purified from the two strains may be real although this was not evident from native PAGE of the crude culture filtrate proteins wherein the enzymes showed identical mobility. This is not surprising as two proteins of different sizes can have the same mass/charge ratio and migrate similarly in native PAGE. On the other hand, since migration of protein on gel-filtration column depends on its hydrodynamic volume, this method may give an anomalous value. For the present we have tended to consider results from analytical PAGE (Ferguson analysis) more approximate. This method was originally developed to resolve similar discrepancy in the molecular mass of phosphorylase *b* (Hedrick and Smith 1968). Differences have been noted also in physicochemical properties of xylanase and cellulase between strains of thermophilic fungi (Khandke *et al* 1989; Anand *et al* 1990). The existence of high spontaneous variability in naturally occurring strains of thermophilic organisms has not been appreciated and exploited (Johnson 1979). Therefore, effort towards

the characterization of enzymes from different strains of thermophilic organisms is justified.

In conclusion, this study has shown that the amylolytic enzymes produced by *T. lanuginosus* IISc 91 may have practical applications in the starch industry on account of the high purity of products formed (glucose or maltose), their thermostability and ability to digest raw starch. In addition, the α-amylase of this strain would be useful in the study of the structural reorganization in protein at high temperature and the thermoinactivation process.

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Dr K Mahalingeshwara Bhat, Institute of Food Research, Reading, UK, performed HPLC analysis of sugars. Prof. S K Podder gave valuable suggestions and comments. Our work was supported by research grants from the Council of Scientific and Industrial Research, and Department of Science and Technology, New Delhi.

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