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AMYLOLYTIC ENZYMES FROM *Aspergillus hennebergi* (*A. niger* GROUP): PURIFICATION AND CHARACTERIZATION OF AMYLASES FROM SOLID AND LIQUID CULTURES

DIDIER ALAZARD AND JACQUES F. BALDENSPERGER*†
ORSTOM Department of Microbiology, IRCHA, B.P. 1, 91710 Vert-le-Petit (France)
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ABSTRACT

Two glucoamylases (I and II) were produced during solid-state culture of *Aspergillus hennebergi* (*A. niger* group) on cassava meal, whereas one glucoamylase and one alpha-amylase were synthesized by the mould in liquid culture. These glucoamylases were acidic proteins with thermotolerant activities. Glucoamylase I was not a glycoprotein, but glucoamylase II and the glucoamylase from liquid cultures contained 15% of sugars. The alpha-amylase was significantly less thermotolerant and of smaller molecular weight. The influence of culture conditions on the production of different amylases by the same *Aspergillus* strain on the same substrate is discussed.

INTRODUCTION

Amylolytic enzymes derived from filamentous fungi have been extensively investigated because of their importance in the starch industry for the production of sugar syrups and crystalline sucrose. Glucoamylase and alpha-amylase have been found in several genera of fungi, but the genus *Aspergillus* is that currently most used for commercial production. Numerous reports[1–5] have been published on the purification of glucoamylase from Takamine Diazyme, a commercial enzyme preparation isolated from a semi-solid fermentation of *A. niger* by an alcohol-precipitation procedure. Glucoamylase consists of two isoenzymes which are glycoproteins[6]. Submerged-culture filtrates of *A. niger* contain the same isoenzymes, and also an alpha-amylase[7–9].

Although considerable information is available concerning the purification and the physicochemical and enzymic properties of these enzymes, few comparative studies have been published[10–12] on fungal amylases formed in various culture conditions. We have reported[13] on the influence of the culture conditions on the amylase activities of *A. hennebergi* (*A. niger* group) grown on cassava meal, and now describe the purification of different amylases produced by the mould in liquid and solid cultures.

*To whom enquiries should be addressed.
†Present address: Orstom, B.P. 81,97201, Fort-de-France, Martinique.

MATERIALS AND METHODS

Micro-organism and cultures. — A strain of *A. hennebergi* Blochwitz (*A. niger* group) was used. Submerged and solid cultures on cooked cassava meal were carried out as reported previously[13,14]. Cultures were grown at 35° and harvested after 25 and 48 h for submerged and solid-state culture, respectively.

Preparation of crude extracts. — All operations were performed at room temperature unless otherwise stated.

(*a*) *Solid-state fermentation.* Solid culture (25 g) was suspended in water (~400 ml) and homogenised with an Ultra-Turrax blender. The pH of the suspension (4.5) was adjusted to 9.0 with 0.5M NaOH. After stirring for 1 h, the homogenate was centrifuged at 4,000*g* for 20 min. This process was repeated, and the supernatant fractions were combined and concentrated to ~20 ml *in vacuo* at 35°. This treatment did not cause any significant loss in enzyme activity. After centrifugation at 30,000*g* for 20 min, the crude concentrate was dialysed overnight against 0.1M Tris-HCl buffer (pH 7.2) at 4°.

(*b*) *Liquid fermentation.* Broth culture (1.2 l) was homogenised, and the pH was raised from 5.0 to 7.0 with 0.5M NaOH. The extraction and concentration steps were then carried out as in (*a*).

Under these optimised conditions, 70 and 80% of enzyme activity were recovered for the submerged and solid culture, respectively.

Ion-exchange chromatography. — Each concentrated, crude extract (20 ml) was applied to a column (2 × 20 cm) of DEAE-Sephadex A50, previously equilibrated with 0.1M Tris-HCl buffer (pH 7.2). A linear gradient 0→0.5M NaCl in the same buffer was used for elution. Appropriate active fractions were combined, concentrated by using an Amicon ultrafiltration cell fitted with a PM10 membrane, dialysed against Tris-HCl buffer, and then re-chromatographed on DEAE-Sephadex A50.

Analytical methods. — Proteins were determined by the absorbance at 280 nm or by the method of Lowry *et al.*[15], using bovine serum albumin as standard. Reducing sugars were determined as glucose, using a Technicon Auto Analyser and the alkaline ferricyanide reagent. D-Glucose was specifically determined by using D-glucose oxidase and peroxidase (Sigma Technical Bulletin No. 510). Carbohydrate content of glycoproteins was determined by the phenol–sulfuric acid method[16] with D-glucose as standard.

Assay of amylolytic activity. — Amylase activities were based on the release of D-glucose from 1% of soluble starch (Prolabo) in 50mM citrate buffer at the appropriate pH and temperature (final volume, 5 ml). One unit of glucoamylase activity was defined as the amount of enzyme required to release 1 μmol of D-glucose (or glucose equivalent) per min at 60° and pH 4.5. For convenience, alpha-amylase activity was also expressed as μmol of glucose equivalent released at 50° and pH 5.0. Specific activity was defined as units per mg of protein.

Polyacrylamide gel electrophoresis. — Disc gel electrophoresis was carried out[17] in glass tubes at pH 8.3 in 7.5% acrylamide gels with a current of 3 mA per gel.

Proteins were stained with Coomassie Brilliant Blue. To locate amylase activities, gels were dipped in iodine–iodide solution (0.2% of I_2 + 2% of KI) after 60-min incubation at 50° in starch solution (0.5% of soluble starch in 0.05M citrate buffer at pH 4.5). Amylase activities were revealed as clear zones in a bluish background. Glycoproteins were stained[18] in gels with the periodic acid–Schiff reagent.

Isoelectric focusing. — Determination of isoelectric points was performed[19] in 5% polyacrylamide gels with a Pharmalyte carrier ampholyte, pH range 2.5–5.0 (Pharmacia). Lyophilised samples (~5 g of protein) were dissolved[20] in a sample buffer.

Determination of molecular weights. — The method[21] involved determination of elution volume from a column (1.1 × 120 cm) of Biogel P-150 equilibrated with 0.1M Tris-HCl buffer (pH 7.2) and calibrated with bovine serum albumin (mol. wt. 68,000), ovalbumin (43,000), soybean trypsin inhibitor (21,000), and cytochrome C (12,500). The void volume was determined with Dextran Blue (2,000,000). The standards were layered on the column, and eluted with the same buffer at 6 ml/h (1.2-ml fractions).

RESULTS

Influence of pH on the yield of extracted amylase. — By varying the pH of a homogenised suspension obtained by blending mycelia from solid or liquid cultures, the optimum extraction-yield of amylase activity was determined at pH 9.0 and 7.0 for solid and liquid cultures, respectively. The extraction procedure was therefore chosen as described in Materials and Methods, after comparison of different buffer efficiencies and the effects of surfactants (Tween, Triton X100).

Purification of amylolytic enzymes. — The procedure developed for the isolation of enzymes from solid-state culture is shown in Table I. The elution pattern in DEAE-Sephadex chromatography is shown in Fig. 1. Two active fractions were eluted, name-

TABLE I

PURIFICATION OF AMYLASES FROM *Aspergillus hennebergi* IN SOLID CULTURE

Purification steps	*Volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Purification factor (fold)*	*Yield (%)*
Crude product (25 g wet-wt.)	400	1515	6820	4.5		
Crude extract (pH 9)	20	232	5255	22.6	1	100
Chromatography (DEAE-Sephadex A50)						
Glucoamylase I	7	17.5	1040	59.5	2.6	19.8
Glucoamylase II	6.6	24.7	2260	91.5	4.0	43.0
Re-chromatography (DEAE-Sephadex A50)						
Glucoamylase I	3.4	5.4	522	96.6	4.3	9.9
Glucoamylase II	3.7	12.5	1481	118.5	5.2	28.2

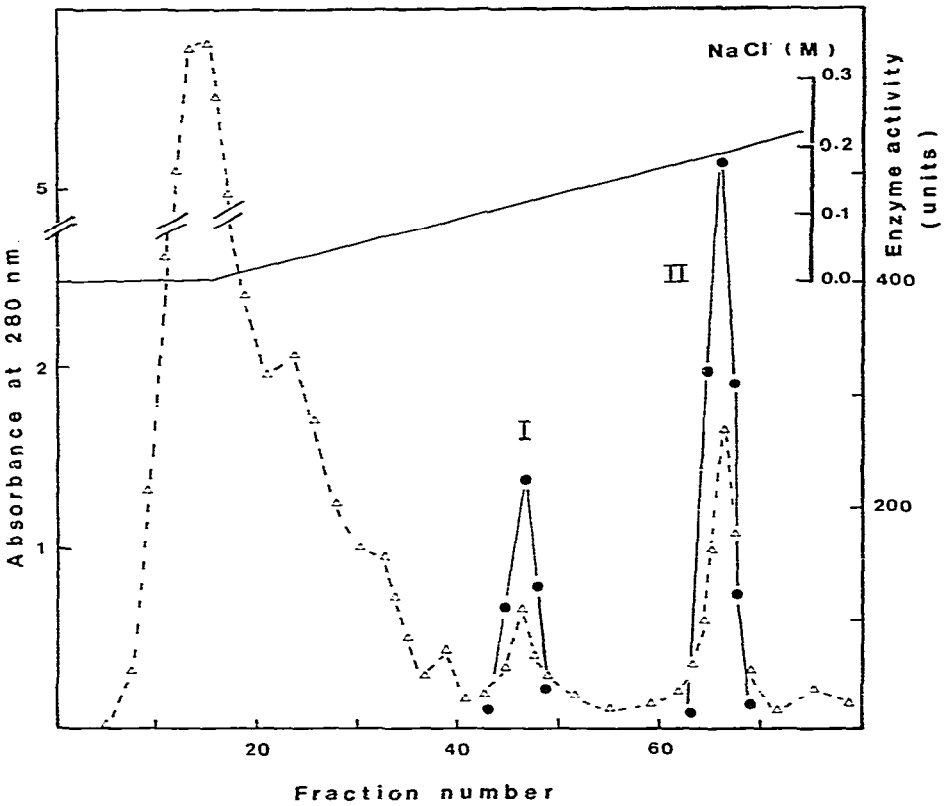

Fig. 1. First ion-exchange chromatography on a column (2 × 20 cm) of DEAE-Sephadex A50 of the crude extract from the solid-state culture of *A. hennebergi*: elution with a linear gradient 0→0.5M NaCl in 0.1M Tris-HCl buffer (pH 7.2) at 25 ml/h (5-ml fractions); 280-nm absorbance, --△--; amylase activity, —·—; NaCl elution gradient, ——.

ly, glucoamylase I at ~0.1M NaCl and glucoamylase II at ~0.2M NaCl, designated according to their electrophoretic mobilities[22]. Each fraction consisted of glucoamylases, as indicated by the 1:1 ratio between the reducing sugars assayed by the ferricyanide procedure, and D-glucose determined by the D-glucose oxidase–peroxidase method. The liquid-culture extract also exhibited two amylolytic peaks in chromatography on DEAE-Sephadex A50 (Fig. 2). The first peak consisted of alpha-amylase, as indicated by the low ratio (1:5) of D-glucose formed to the total reducing-sugars. The latter peak consisted of glucoamylase. The results of the purification experiments are shown in Table II. After re-chromatography on DEAE-Sephadex A50, the four active fractions were found to be homogeneous in polyacrylamide gels stained for proteins (Fig. 3). When gels were stained with the periodate–Schiff reagent, only glucoamylase II from solid culture and glucoamylase from liquid culture gave positive reactions, indicating the presence of carbohydrate in the enzyme molecule (Fig. 3). However, for alpha-amylase and glucoamylase I, a weakly coloured band

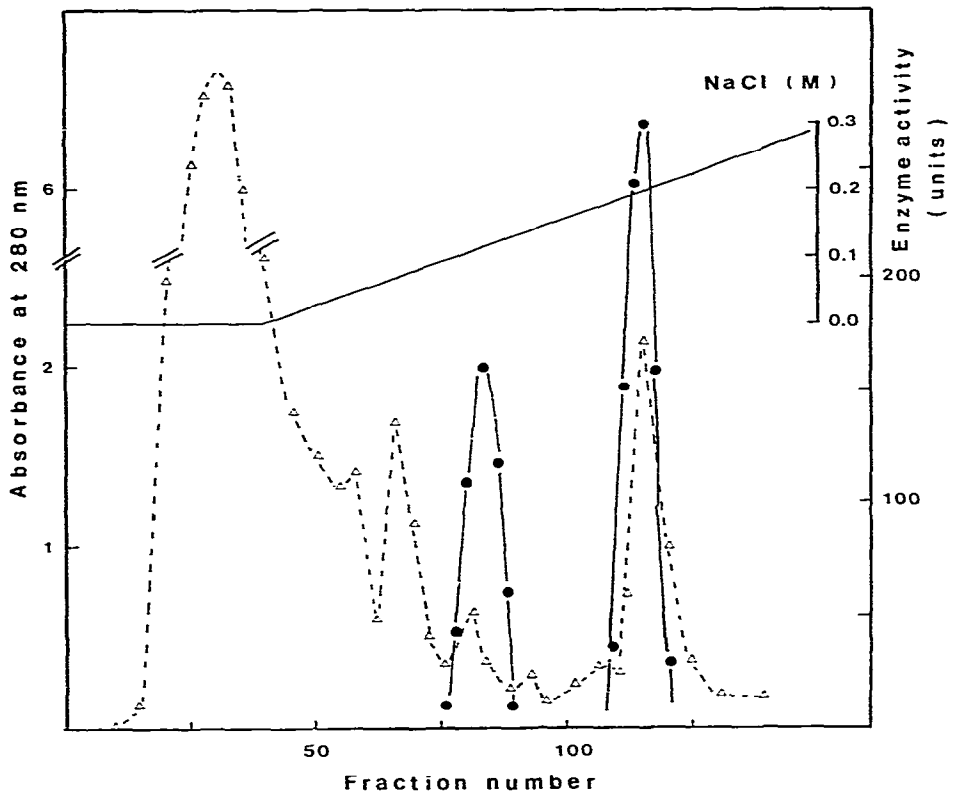

Fig. 2. First ion-exchange chromatography on DEAE-Sephadex A50 of the crude extract from the submerged culture of *A. hennebergi* (see Fig. 1 for details).

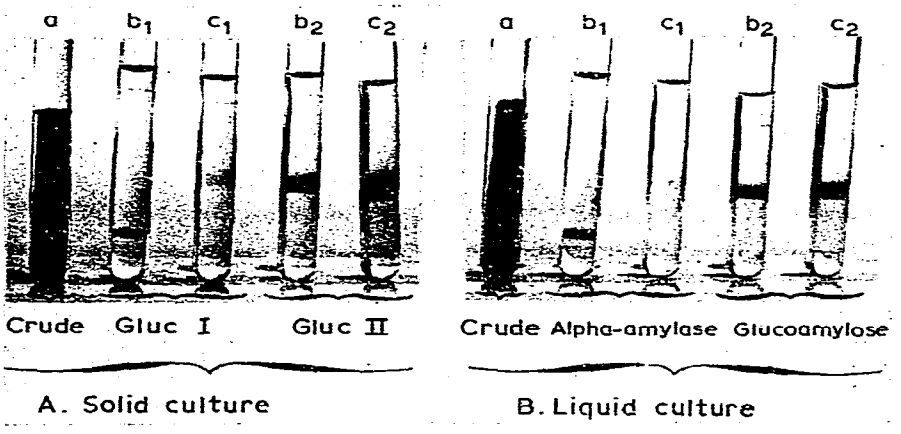

Fig. 3. Disc gel electrophoresis of amylases from solid (A) and liquid cultivation (B) of *A. hennebergi*: ~0.1 mg of protein was used in each tube with staining for amylolytic activity (a, crude extracts), for proteins with Coomassie Blue (b_1 and b_2, purified enzymes), and for carbohydrate with periodate–Schiff reagent (c_1 and c_2, purified enzymes).

TABLE II

PURIFICATION OF AMYLASES FROM *Aspergillus hennebergi* IN LIQUID CULTURE

Purification steps	*Volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Purification factor (fold)*	*Yield (%)*
Broth culture	1200	1900	5420	2.85		
Crude extract (pH 7)	20	359.5	3832	10.6	1	100
Chromatography (DEAE-Sephadex A50)						
Alpha-amylase	5.6	15.4	1654	107.4	10.1	43.1
Glucoamylase	4.8	28.7	1285	44.8	4.2	33.5
Re-chromatography (DEAE-Sephadex A50)						
Alpha-amylase	3.4	9.5	1185	124.7	11.8	30.9
Glucoamylase	2.0	9.4	1066	113.4	10.7	27.8

was also observed in the upper portion of the gels. This contamination, detected by the periodate–Schiff reagent, was not observed when gels were stained for proteins by the Coomassie reagent. Each concentrated, crude extract showed two active bands situated at the same location as that of purified fractions stained for proteins.

Properties of the enzymes. — The molecular weights of the amylases were determined by gel filtration on a calibrated column of Biogel P-150. For the purified enzymes from solid culture, values of 60,000 $\pm$2,000 and 72,000 $\pm$2,500 were obtained for glucoamylase I and II, respectively. For liquid culture, the alpha-amylase had a molecular weight of $\sim$50,000, and that of glucoamylase was 70,000 $\pm$2,000 which was similar to that for glucoamylase II.

Isoelectric focusing of each purified preparation in carrier ampholytes covering the pH range 2.5–5.0 yielded a single protein band at pH $\sim$3.9, indicative of acidic proteins.

Only glucoamylase II from solid culture and glucoamylase from liquid culture were identified in gels as glycoproteins (Fig. 3). The carbohydrate content of these fractions, determined as glucose by the phenol–H_2SO_4 method, was 15%.

The effect of pH on enzyme activities was tested in 0.05M citrate buffer in the pH range 3.0–7.0. The results are shown in Fig. 4. For glucoamylases produced in both conditions of growth, similar curves were obtained with a pH range of maximal activity between 4.0 and 5.0, whereas the pH optimum of alpha-amylase was 5.5.

The temperature optima determined by a 15-min incubation were 70° for the three glucoamylases and $\sim$50° for alpha-amylase (Fig. 5). On heating in 0.05M citrate buffer at the appropriate pH for 15 min at various temperatures, each glucoamylase retained full activity up to 65°, but alpha-amylase lost its activity above 60° (Fig. 6).

DISCUSSION

Our studies showed that the *A. hennebergi* amylase system consisted of two

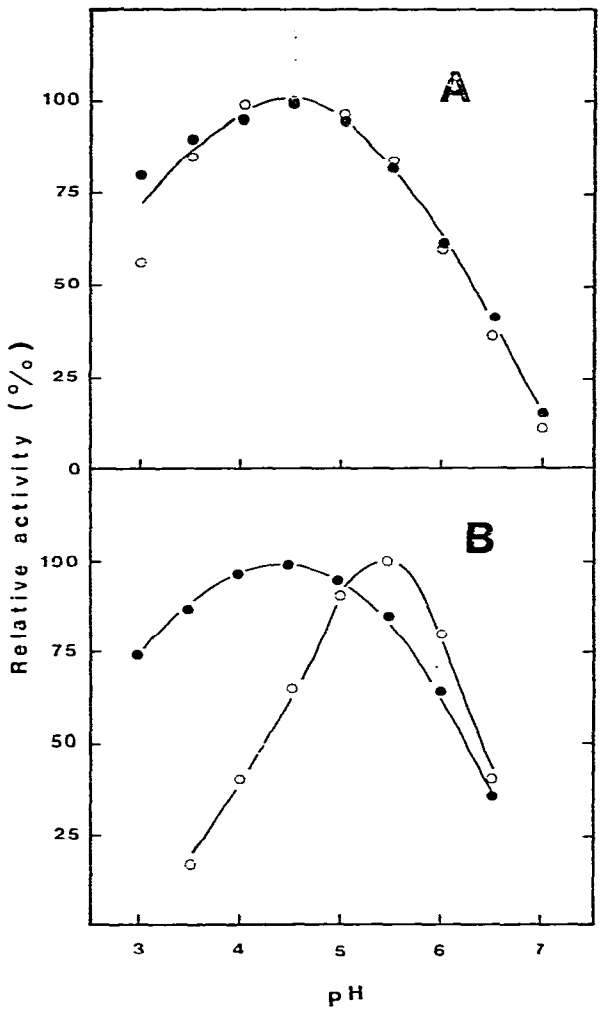

Fig. 4. Effect of pH on amylase activities. Reactions involved 15 μg of enzyme for 15 min, using soluble starch (1%) in the appropriate 0.05M citrate buffer: A, glucoamylase I (—○—) and glucoamylase II (—●—) from solid culture; B, alpha-amylase (—○—) and glucoamylase (—●—) from liquid culture.

main enzymes in both cultivation methods. As reviewed by Ueda[23], the number of amylases and their properties depend on the species and the culture conditions of the moulds. Our data accord with this statement since, although they had similar electrophoretic mobilities in polyacrylamide disc gels, the two amylase systems were not identical in solid and liquid cultures of the same *Aspergillus* strain on the same substrate.

Of the total amylase activity, ~80% was recovered by two successive washings of the solid culture at pH 9.0; in contrast to the finding of Miah and Ueda[11], only two glucoamylases and no alpha-amylase were detected in the crude extract. The two

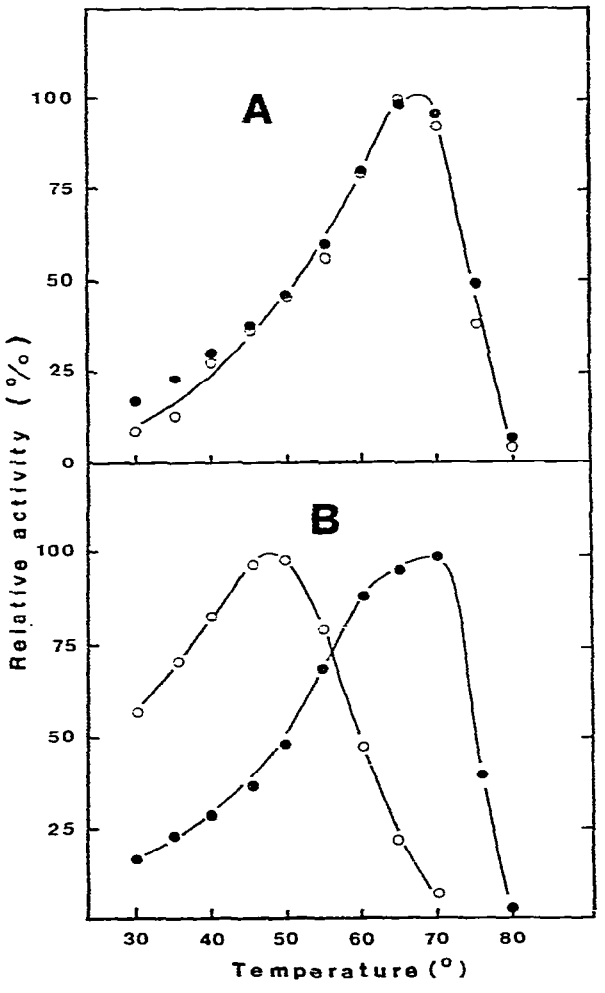

Fig. 5. Effect of temperature on enzyme activities. Amylase activities against 1% of soluble starch were measured for 15 min at the appropriate pH (0.05M citrate buffer) and the temperatures indicated: A, glucoamylase I (—○—) and glucoamylase II (—●—) from solid culture; B, alpha-amylase (—○—) and glucoamylase (—●—) from liquid culture.

glucoamylases (I and II) were separated by chromatography on DEAE-Sephadex A50 and were obtained in the ratio 1:2 with respect to activity. A similar ratio has been reported[11] for wheat-bran, solid culture of *A. oryzae*. On disc gel electrophoresis of the purified enzymes from solid cultures, glucoamylase I gave a sharp band that stained for protein but not glycoprotein. In contrast, glucoamylase II migrated as a wide band in the middle of the gels that stained for protein and glycoprotein, and it was found to contain 15% of sugars.

Some properties of glucoamylases I and II were similar (*e.g.*, acidic nature, optimum pH, thermal stability), and glucoamylase I might be generated by degradation of glucoamylase II during mould cultivation or enzyme extraction, resulting in

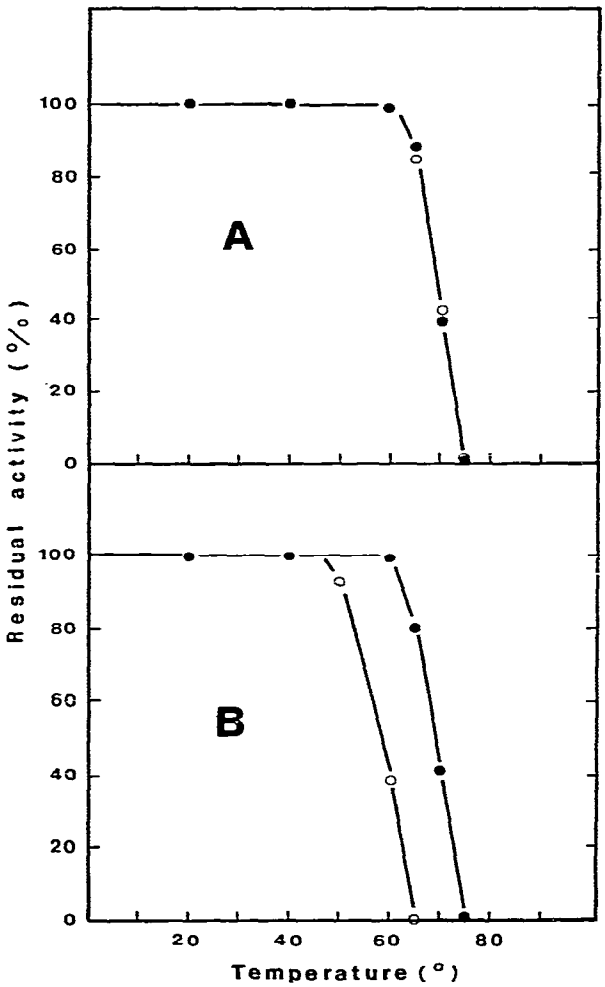

Fig. 6. Temperature stability of amylases. Enzyme solutions (0.5 mg of protein/ml) in 0.05M citrate buffer at the appropriate pH were incubated for 15 min at various temperatures, and the residual activity was then determined: A, glucoamylase I (—○—) and glucoamylase II (—●—) from solid culture; B, alpha-amylase (—○—) and glucoamylase (—●—) from liquid culture.

the loss of the carbohydrate moiety and in the decrease of the molecular weight from 72,000 to 60,000. A similar observation was reported by Hayashida[24], who demonstrated the formation of two types of glucoamylase by *in vitro* proteolysis of the native enzyme. It is unlikely that the two forms of glucoamylase arise as artifacts of the purification procedure, as the 1:2 ratio between glucoamylases I and II was constant in all the crude extracts, regardless of the pH of extraction from the solid culture. Our data are more in agreement with the assumption[8] that glucoamylase isoenzymes are produced by different genes.

Submerged cultures of *A. niger* on corn meal produce[9,25] both glucoamylases and alpha-amylase. Two amylase activities were separated by chromatography on DEAE-Sephadex A50 of the crude extract from liquid culture of our strain of *A. hennebergi* grown on cassava meal. The second peak, eluted with 0.2M NaCl, was a glucoamylase which, after gel electrophoresis, was shown to be a glycoprotein. The molecular weight, carbohydrate content, isoelectric point, optimum pH, and thermal stability of the glucoamylase from liquid culture were very similar to those of glucoamylase II from solid culture.

Barton *et al.*[8] reported that alpha-amylase was produced only when starch was the carbon source of *A. niger*. The first peak eluted with 0.12M NaCl from a column of DEAE-Sephadex A50 loaded with the crude extract from liquid culture of *A. hennebergi* on cassava meal was an alpha-amylase. Its characteristics were significantly different from those of glucoamylases; thus, it was not a glycoprotein, its molecular weight was 50,000, its pH optimum was 5.5, and its activity was destroyed above 60°. According to Barton *et al.*[8], the presence of alpha-amylase may account for the high level of glucoamylase accompanying the growth of *A. niger* on starch, as glucoamylase synthesis is not induced by polymeric glucans. This observation is apparently inconsistent with the lack of alpha-amylase in the crude extract from solid cultures containing two glucoamylases. However, Gasdorf *et al.*[9] have reported an active endo-amylase activity of glucoamylase I free of alpha-amylase. This endo-amylase activity may account for induction of glucoamylase synthesis during growth of *A. hennebergi* on cassava meal. Moreover, even if alpha-amylase was produced in solid-state cultures, it is possible that it was destroyed before harvest, as the pH had decreased to 2.5 after 24 h of growth. Lineback *et al.*[7] have reported that the level of alpha-amylase was drastically decreased in low-pH conditions of cultivation.

Thermotolerance is a well-documented characteristic of glucoamylases produced by black *Aspergillus*, and our data accord with previous reports[3,25]. The three glucoamylases from our strain were stable for 15 min up to 65°. Unlike Ramachandran *et al.*[25], we determined the thermostability by incubating the purified enzymes in the absence of substrate.

The glucoamylases described herein have optimum temperatures ranging around 70°; this is convenient for industrial use, as operation can be at sufficiently high temperature to discourage microbial contamination. However, the reason for the production of two glucoamylases by *A. hennebergi* grown in solid-state cultures and for the lack of alpha-amylase, as compared to the same strain cultivated in submerged conditions, remains unclear. Further studies to elucidate this phenomenon are in progress.

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