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SOME PROPERTIES OF A GLUCOAMYLASE PRODUCED BY THE THERMOPHILIC FUNGUS *Humicola lanuginosa**

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ABSTRACT

The thermophilic fungus *Humicola lanuginosa* elaborates two glucoamylases. Some properties of one of these enzymes (glucoamylase II) were investigated. The enzyme was found to have a higher pH optimum than reported for other fungal glucoamylases, and to be very thermostable. In particular, it displayed unusual resistance to heat in the presence of its substrate. It appeared to be capable of completely degrading starch. However, the possibility that traces of contaminating alpha amylase assist in degradation could not be ruled out.

INTRODUCTION

Because of their ability to hydrolyse starch and malto-oligosaccharides ("malto-dextrins") to glucose, the glucoamylases [(1 → 4)-α-D-glucan glucohydrolases, EC 3.2.1.3] are important in the commercial production of D-glucose from starch. The enzymes of choice in industry are the thermostable enzymes obtained from fungi, as these permit rapid reactions at high temperatures. In this paper, we show that the thermophilic fungus *Humicola lanuginosa* produces two glucoamylases. Some properties of one of these enzymes, namely glucoamylase II, have been studied.

EXPERIMENTAL

Materials and general methods. — AnalaR soluble starch was obtained from Hopkin and Williams Ltd., London, England and maltose (extra pure) from B.D.H. Chemicals, Poole, Dorset, England. *O*-(2-Diethylaminoethyl)cellulose, DE23, was obtained from Whatman Ltd., Maidstone, Kent, England. Alpha Amylase (Stat-Pack) and Aquacide II were purchased from Calbiochem, La Jolla, California, U.S.A. Carbohydrate contents of substrates were determined by hydrolysis[1] to D-glucose in 1.5M sulphuric acid for 3 h at 100°. D-Glucose was determined with D-glucose oxidase by using the GOD-Perid reagent obtained from Boehringer Mannheim GmbH, Mannheim, W. Germany, a correction factor for loss of glucose being applied[2].

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

Enzyme-assay procedures. — Glucoamylase activity was determined by the addition of enzyme (0.1 ml) to 1.0% starch or maltose (0.5 ml) and 0.2M sodium citrate buffer (0.4 ml) at the appropriate pH. Incubations were carried out for 20 min at 37° and the reaction was stopped by the addition of 0.6M perchloric acid (1 ml). D-Glucose was determined by a modification of the GOD-Perid method. Portions of the mixture were diluted to 1 ml with water and the GOD-Perid reagent (5 ml) added. After incubation for 25 min in the dark at 20–25°, the absorbance was read at 640 nm. The presence of perchloric acid did not affect the activity of the glucose oxidase under these conditions. One unit of activity was defined[3] as the amount of enzyme that catalyses the liberation of 1 mg of D-glucose in 30 min. Protein concentration was determined by measuring absorbance at 260 and 280 nm and applying the formula derived by Warburg and Christian[4]. Alpha amylase activity was detected by using an amylopectin labelled with blue dye (Amylopectin Azure) as substrate[5]. This is hydrolysed by alpha amylase to give soluble, blue products. The method used was that recommended by Calbiochem for use with their alpha amylase Stat-Pack.

Electro-focusing. — This was performed on poly(acrylamide) plates in the pH range 3–7 or 3.5–9.0, as described by Matthew *et al.*[6] with modifications in the gel ingredients as formulated by Karlsson *et al.*[7] Glucoamylase activity was detected as follows. Whatman No. 1 paper was cut to fit the plate exactly and impregnated with a 1% solution of starch or maltose in 0.2M sodium citrate buffer, pH 5.5. The plate was overlaid with the damp paper and incubated in a humid, air-tight box for 40 min at 37°. The paper was removed, dried, and sprayed with GOD-Perid reagent. Areas of enzymic activity, where starch or maltose had been converted into D-glucose, showed up as green spots on a white background.

Partial purification of enzymes. — *Humicola lanuginosa* K13/1 was maintained on starch agar slopes containing Oxoid peptone L34 (5.0 g), Oxoid yeast extract L21 (2.0 g), dipotassium hydrogenphosphate (0.40 g), magnesium sulphate heptahydrate (0.05 g), sodium chloride (0.10 g), ferric chloride hexahydrate (0.01 g), soluble starch (10 g), and Oxoid agar No. 3 (15 g) per litre. The pH was 6.8. After incubation for 6 days at 37°, one quarter slopes were used to inoculate Aureomycin F.I. development medium (50 ml/250 ml conical flask). The medium contained sucrose (30 g), Staley's Hi Pro Con milled soya meal (20 g), molasses (2.0 g), sodium chloride (5.0 g), calcium carbonate (4.0 g), ammonium sulphate (2.0 g), and corn-steep liquor (6.0 g) per litre. The pH was adjusted to 6.6–6.8. The organism was grown for 30 h at 37° with an agitator speed of 220 rev/min (2-inch throw), and was then transferred at an inoculum level of 3% to starch C fermentation medium (50 ml/250 ml conical flask, total volume 5L). The medium contained Oxoid tryptone L42 (20 g), Oxoid yeast extract L21 (20 g), and soluble starch (5.0 g) per litre. The pH was adjusted to 6.5–7.0. After incubation for 3 days at 50° with an agitator speed of 220 rev/min (2-inch throw), mycelia were removed by filtration on a Büchner funnel with Whatman No. 52 paper.

The filtrate (3,460 ml) was brought to 80% saturation with solid ammonium sulphate and kept overnight at 4°. The precipitate was removed by centrifugation at

12,000*g* for 30 min at 5°, dissolved in 10 mM citrate–phosphate buffer, pH 7.8 (180 ml), and dialysed against the same buffer (3L) at 4° overnight. A precipitate formed in the dialysis bag and was removed by centrifugation at 18,000*g* for 30 min at 5°. The supernatant was adjusted to pH 5.5 with 0.2M citric acid and polyethylene glycol 6,000 (PEG) was added slowly with stirring to give a concentration of 20%. The mixture was stirred for a further 10 min, kept for 20 min at 4° and removed by centrifugation at 18,000*g* for 30 min at 5°. The precipitate was dissolved in 10 mM citrate–phosphate buffer, pH 7.8 (45 ml). Additional PEG was added to the supernatant to increase the concentration to 45%. The precipitate thus obtained was dissolved in 10 mM citrate–phosphate buffer, pH 7.8 (45 ml).

Both enzyme fractions were subjected to batch-wise adsorption onto DEAE-cellulose. DE23 was pre-equilibrated with 10 mM citrate–phosphate buffer, pH 7.8 and added to the enzyme solutions (~40 ml, 200 mg of protein) in the proportion of 1 g of damp DE23 to 20 mg of protein. The mixture was stirred for 30 min at 4° and the DE23 removed by filtration on a Buchner funnel with Whatman No. 52 paper. The cake was washed twice by resuspending in buffer, stirring, and filtering. The DE23 was successively eluted with 0.1M, 0.3M, 0.5M and 1.0M sodium chloride in 10 mM citrate–phosphate buffer, pH 7.8 (2 × 30 ml) as already described. The fraction containing maximum activity (0.3M sodium chloride for both enzyme fractions) was dialysed against 10 mM citrate–phosphate buffer, pH 7.8, concentrated with Aquacide II, and redialysed.

RESULTS

Two enzyme fractions were obtained by treatment with PEG and DE23. The fraction precipitating at 0–20% PEG exhibited both glucoamylase and α-D-glucosidase activities. The glucoamylase (glucoamylase I) had a pH optimum of 4.9 and the α-D-glucosidase an optimum pH of 3.3. This fraction was not investigated further. The enzyme precipitating at 20–45% PEG, namely glucoamylase II, was subjected to further study.

The specific activity of glucoamylase II on starch was 4.1 U/mg protein after PEG fractionation and 12.5 U/mg protein after treatment with DE23. No alpha amylase activity could be detected in the fraction. Electrofocusing revealed the presence of a single starch- and maltose-hydrolysing enzyme having an isoelectric point of pH 4. The ratio of activities maltose:starch at pH 6.6 was 1:14.6.

Effect of pH on activity. — Glucoamylase II had an optimum pH of 6.6, as shown in Fig. 1. The enzyme was shown to be completely stable in the pH range 5–10 for 24 h at room temperature. At pH 4 and pH 3.5, losses of activity of 5 and 14%, respectively, occurred.

Heat stability. — Pre-incubation of the enzyme at various temperatures for 30 min at pH 6.6 showed it to be completely stable up to 50°. About 4% of activity was lost at 60° and 48% at 70° (see Fig. 2). The enzyme showed remarkable heat stability in the presence of starch. After boiling for 10 min in 0.5% starch at pH 6.6,

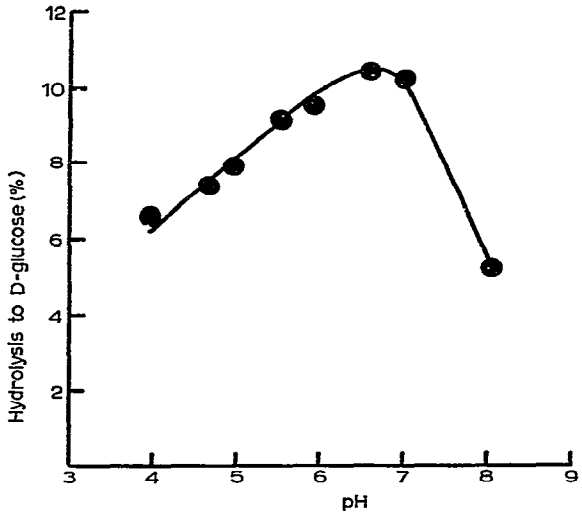

Fig. 1. pH Optimum for the action of glucoamylase II on starch. The mixture, containing 0.72 U of enzyme, was as given in the text. Final concentrations of buffers were 40mM sodium acetate (pH 3.5–5.5) and 40mM sodium phosphate (pH 5.6–8.1).

28–31% of activity was retained (see Figs. 3 and 4). The amount of activity remaining was dependent on pH, as shown in Fig. 4, maximum activity being retained at pH 6.6. The optimum temperature for enzyme activity was 65–70° (see Fig. 5).

Hydrolysis of starch. — The enzyme was able to hydrolyse starch completely to D-glucose, as shown in Fig. 6. Hydrolysis up to 75% took place with ease and the remainder with more difficulty. Although alpha amylase could not be detected under normal conditions of assay (15 min), the enzyme was allowed to act on Amylopectin

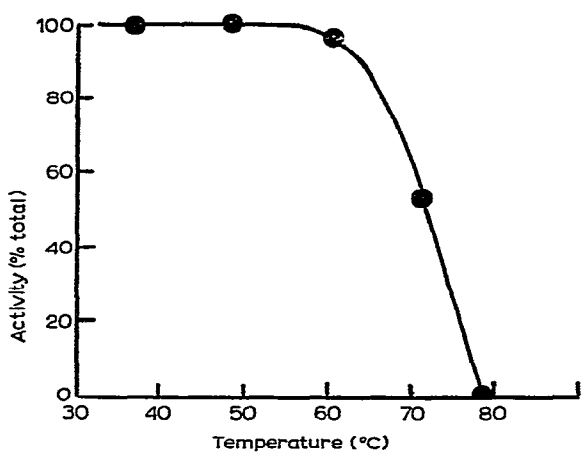

Fig. 2. Temperature-stability of glucoamylase II. The enzyme (0.1 ml, 0.72 U) was incubated with 0.2M sodium citrate buffer, pH 6.6 (0.4 ml) for 30 min at the temperatures indicated, cooled rapidly to 37° and the activity determined against 0.5% starch, as described in the text.

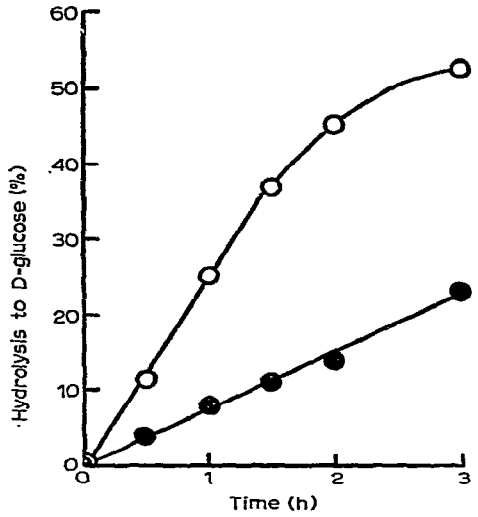

Fig. 3. Resistance of glucoamylase II to heat-inactivation in the presence of starch. A standard mixture containing starch and glucoamylase II (0.54 U) at pH 6.6 (see text) was boiled for 10 min and cooled in ice (1 min). Residual activity was determined at 37°. Untreated digest, O—O; pre-boiled digest, ●—●.

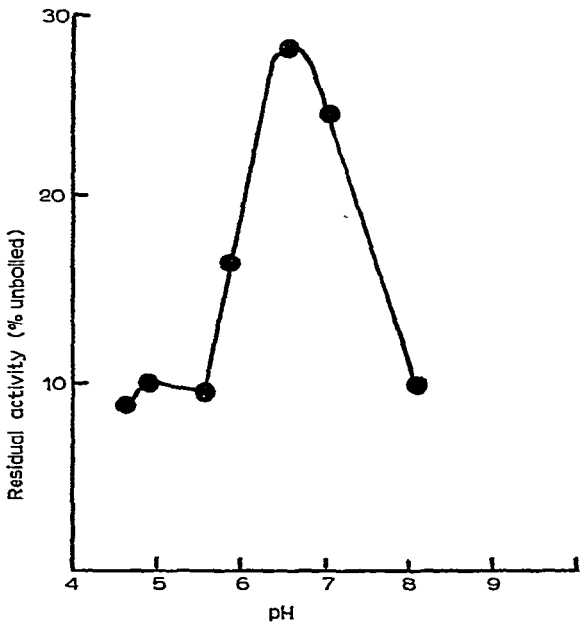

Fig. 4. Resistance of glucoamylase II to heat-inactivation in the presence of starch: dependence on pH. Standard mixtures containing starch and glucoamylase II (0.54 U) buffered at various pH values were boiled for 10 min and cooled in ice (1 min). Residual activity was determined by incubation for 30 min at 37°.

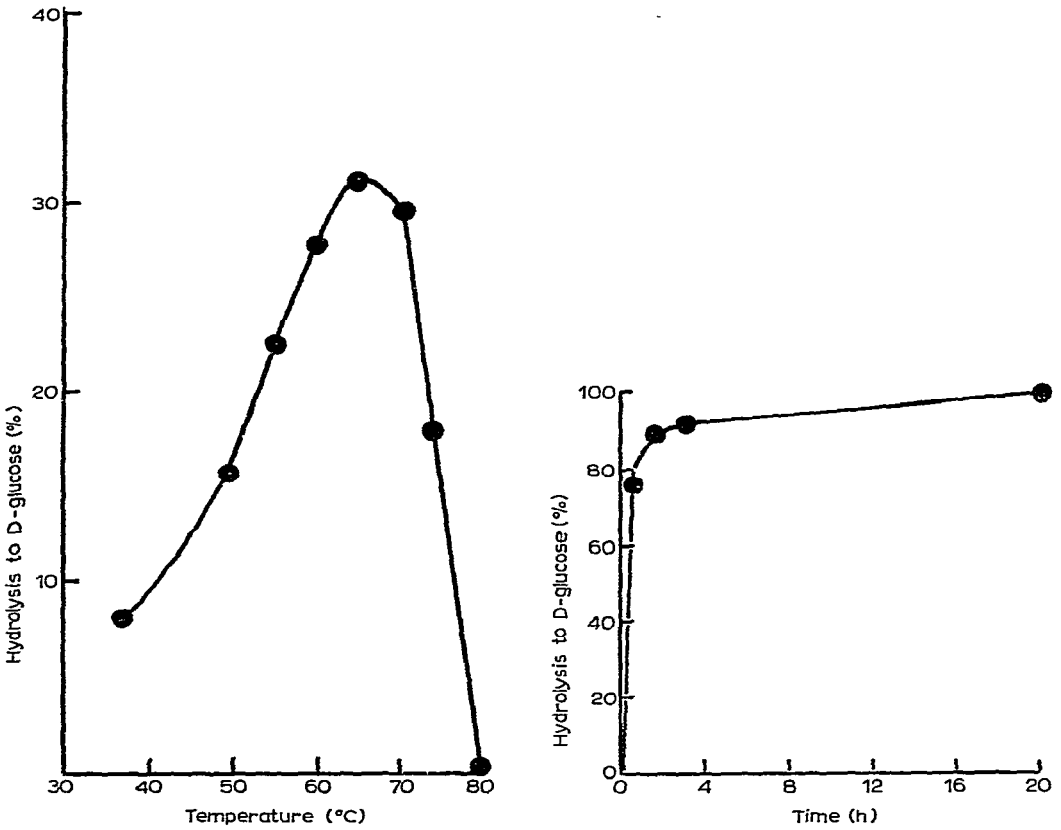

Fig. 5. Temperature optimum for the action of glucoamylase II on starch. Enzyme (0.1 ml, 0.60 U) and 0.2M sodium citrate, pH 6.6, (0.4 ml) were pre-incubated for 5 min at the temperatures shown. Starch (0.5 ml, 1%), similarly pre-heated, was added and the mixtures were incubated for 20 min. Activity was determined as described in the text.

Fig. 6. Hydrolysis of 0.1% soluble starch by glucoamylase II. Glucoamylase II (0.1 ml, 3.6U) was incubated with 0.2% starch (0.5 ml) and 0.2M sodium citrate buffer, pH 6.6 (0.4 ml) at 37°. D-Glucose produced was determined as described in the text and calculated as a percentage of that released by acid hydrolysis.

Azure for 18 h to see if any activity could be found over the prolonged period required to bring about complete conversion of starch into D-glucose. Under these conditions, a minute amount of alpha amylase (1.5×10^{-3} S.U./ml) was detected.

DISCUSSION

Although glucoamylases from fungi have been extensively studied, little information exists on the polysaccharide-degrading enzymes of thermophilic fungi. *Humicola lanuginosa* glucoamylase II differs from other fungal enzymes reported in having a neutral pH optimum. Members of the *Aspergillus* species produce en-

zymes[8-14] that have optimal activity in the pH range 4.1–5.0. Such fungi as *Endomyces bispora*[15], *Endomycopis fibuligera*[16], and *Cephalosporium charticola*[17] have optimum pH values of about 5.5. The glucoamylase II of *Humicola lanuginosa* has a pH optimum of 6.6.

The fungal glucoamylases described here have optimum temperatures ranging from 52–70°. Glucoamylase II comes within this range, with a temperature optimum of 65–70°. However, it appears to be more resistant to heat than other fungal glucoamylases. Some of the most thermostable glucoamylases studied have been those from *A. niger*[10]. These, like glucoamylase II, are stable to heating for 30 min up to a temperature of 60°. However, at 70° the enzymes of *A. niger* retain only about 20% of their activity, whereas glucoamylase II retains about 50%. The enzyme also seems remarkably resistant to heat in the presence of its substrate. Boiling for 10 min destroys only about 70% of the original activity (Figs. 3 and 4).

Starch is completely hydrolysed by glucoamylase II, as shown in Fig. 6. It is not known whether the minute amount of alpha amylase present is sufficient to assist the hydrolysis, as postulated for fungal glucoamylases by Marshall and Whelan[2,18] or whether the enzyme acts independently of alpha amylase, as claimed for a glucoamylase of *A. niger* by Gasdorf *et al.*[19].

Mangallam *et al.*[20] recently investigated the thermophilic fungi *Terula thermophila, Mucor miehe, Humicola lanuginosa,* and *Sporotrichum thermophile* and demonstrated that they produce glucoamylases. The work reported in this paper indicates that the properties of glucoamylases from thermophilic fungi may differ in some respects from those of other fungi. It would, therefore, appear that the thermophiles may well merit further investigation.

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