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Purification and Characterization of a Glucoamylase from *Rhizopus oryzae*

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ABSTRACT

Glucoamylase (EC 3.2.1.3) of Rhizopus oryzae *NRRL 395 was purified approximately sevenfold by sequential ammonium sulfate fractionation, Bio-Gel P-100 gel filtration, Q-Sepharose anion exchange and S-Sepharose cation exchange. The pH and temperature optima were 4·8 and 60°C, respectively. Enzyme was stable at temperatures up to 40°C and pH values between 3 and 8. The molecular weight was 67 000 daltons as determined by sodium dodecylsulfate–polyacrylamide gel electrophoresis, and the pI was 8·7 as determined by chromatofocusing. The K_m for amylopectin and soluble starch were 0·98 and 1·34 mg/ml, respectively. The V_{max} for amylopectin and soluble starch were 782 and 136 μmoles of glucose produced per mg of protein per min, respectively. The enzyme activity was inhibited by Hg^{2+}, Pb^{2+} and Cd^{2+}, but not by EDTA.*

INTRODUCTION

Glucoamylase (EC 3.2.1.3) hydrolyzes α-1,4-glucan links in polysaccharides and removes successive glucose units from the non-reducing ends of the chains. Studies have been reported on the isolation, purification and properties of glucoamylase obtained from the culture filtrates of *Rhizopus delemar* (Tsujisaka *et al.*, 1958), *R. nodusus* (Muthukumaran & Dhar, 1983), *Rhizopus* spp. (Takahashi *et al.*, 1978), *Aspergillus awamori* (Yamasaki *et al.*, 1977*a*; Pestana & Castillo, 1985), *A. candidus* (Mahajan *et al.*, 1983;

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Kolhekar *et al.*, 1985), *A. niger* (Barker *et al.*, 1971; Simley *et al.*, 1971; Lineback *et al.*, 1972), *A. phoenicis* (Lineback & Baumann, 1970), *Candida antarctica* (De Mot & Verachtert, 1987), *Mucor rouxianus* (Yamasaki *et al.*, 1977*b*), *Penicillium oxalicum* (Yamasaki *et al.*, 1977*c*) and *Saccharomyces cerevisiae* (Kleinman *et al.*, 1988). Glucoamylase synthesis in *Aspergillus* spp. is known to be accompanied by the formation of transglucosidase, which adversely affects the efficiency of saccharification because of the formation of oligosaccharides from glucose (Solomon, 1978). *Rhizopus* spp. generally do not produce transglucosidase and thus have been widely used in the Amylo-process for converting starch to sugar with an efficiency of up to 100% (Lin, 1969).

Yu & Hang (1989) reported that *Rhizopus oryzae* was capable of simultaneously saccharifying and fermenting corn and other cereals to L(+)-lactic acid in submerged culture. Recently Yu & Hang (1990) found that the mold produced an appreciable quantity of glucoamylase in the culture filtrate. The objective of the present investigation was to isolate, purify, and characterize the glucoamylase of *R. oryzae* grown on rice.

MATERIALS AND METHODS

Materials

Electrophoretic protein standards were obtained from Diversified Biotech (Newton Center, MA). Materials for column chromatography were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Chemicals for polyacrylamide gels and Coomassie Blue were obtained from Bio-Rad Laboratories (Richmond, CA).

Culture

A lactic acid-producing strain of *Rhizopus oryzae* NRRL 395 (C. W. Hesseltine, Northern Regional Research Center, USDA, Peoria, IL) was maintained on potato dextrose agar slants. Spores of the mold were produced on steamed rice by the method of Wang *et al.* (1975).

Substrate

Long grain enriched rice (Comet American Marketing, Houston, TX) was obtained from a local supermarket.

Enzyme production

Ten grams of rice and 10 ml of distilled water were placed in a 250 ml Erlenmeyer flask and sterilized at 121°C for 15 min. After cooling, each flask was inoculated with 10^7 spores and incubated at 30°C for 7 days under stationary conditions. At the end of fermentation, glucoamylase was extracted from the rice medium by adding 100 ml of 0·5% NaCl to each flask and standing at 4°C for 4 h. The mixtures were then centrifuged at 13 000 × *g* for 15 min, at 4°C. The supernatants were designated as the crude extract.

Enzyme purification

Ammonium sulfate fractionation
To 100 ml of crude extract, solid ammonium sulfate was added to 50% saturation at 4°C. The precipitate was discarded and the supernatant was brought to 70% saturation with ammonium sulfate. The precipitate, collected by centrifugation at 13 000 × *g* for 15 min, was dissolved in a minimum volume of 5 mM Tris–HCl buffer, pH 8·0.

Gel filtration on Bio-Gel P-100
The enzyme obtained from ammonium sulfate precipitation was dialyzed against 5 mM Tris–HCl buffer, pH 8·0, at 4°C overnight and then loaded on a Bio-Gel P-100 column (2·5 × 45 cm). The enzyme activity was eluted with 5 mM Tris–HCl buffer, pH 8·0. Fractions of 5 ml were collected at a flow rate of 30 ml/h.

Anion exchange on Q-Sepharose
The active fractions obtained from gel filtration were pooled and loaded on a Q-Sepharose column (2 × 22 cm). The enzyme activity was eluted by applying a linear gradient of Tris–HCl buffer (pH 7·5) up to 150 mM. Fractions of 5 ml were collected at a flow rate of 30 ml/h.

Cation exchange on S-Sepharose
The active fractions from the Q-Sepharose column were pooled and passed through a S-Sepharose column (1·2 × 10 cm). The enzyme activity was eluted with 20 mM potassium phosphate buffer (pH 7·0). Fractions of 5 ml were collected at a flow rate of 30 ml/h.

Determination of isoelectric point
The enzyme obtained from the S-Sepharose column was dialyzed against 25 mM imidazole buffer, pH 9·4 at 4°C overnight and applied to a Polybuffer exchanger 94 (PBE 94) column (1 × 10 cm) previously equilibrated with

25 mM imidazole buffer (pH 9·4). The pH gradient was formed by the addition of 10% Polybuffer 96 (pH was adjusted to 6·0 with acetic acid). Fractions of 2 ml were collected with a flow rate of 20 ml/h.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The molecular weight of glucoamylase was estimated from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), using the method of Laemmli (1970).

Relative mobility (R_f) is defined as the distance migrated by protein divided by the distance migrated by the tracking dye. The reference proteins used were cytochrome c (12 400), lactoglobulin (18 400), carbonic anhydrase (29 000), lactate dehydrogenase (36 000), ovalbumin (43 000), glutamate dehydrogenase (55 000), and phosphorylase b (95 500).

Analytical methods

Standard enzyme assay

Glucoamylase activity was determined by a slight modification of the method of Lemmel *et al.*, (1980). An aliquot (0·5 ml) of a properly diluted enzyme solution was incubated with 0·5 ml of 1% amylopectin solution buffered to pH 4·8 with 0·01 M acetate buffer for 3 min at 55°C. The reaction was stopped by adding 1 ml of dinitrosalicyclic acid reagent and heating the test tube for 5 min in boiling water. After cooling, 10 ml of distilled water was added to the tube and the absorbance at 540 nm was measured. Using glucose as the standard, the amount of glucose produced in the reaction mixture was determined. A unit of glucoamylase activity is defined as the amount of enzyme that liberates 1 μmole of glucose per minute under the assayed conditions. The specific activity of glucoamylase was calculated by dividing the enzyme activity by its protein content.

Protein determination

The protein content of the enzyme solution was measured by the protein-dye binding method of Bradford (1976). Absorbance was measured at 590 nm and bovine serum albumin as the protein standard.

Properties of glucoamylase

The effect of temperature and pH on enzyme activity was studied using the standard method except that a temperature range of 30–60°C and a pH range of 3·0–8·0 (0·1 M phosphate–citrate) were used.

Thermal stability was tested using the standard method except that the enzyme was incubated for 30 min at different temperatures. pH stability was tested by incubating the enzyme at 30°C for 24 h at different pH values.

The Michaelis–Menten constants (K_m) of the enzyme for soluble starch and dextrin were determined at 55°C in 10 mM acetate buffer, pH 4·8 containing different substrate concentrations. The K_m and V_{max} values were derived by Lineweaver–Burk plots of initial velocity for different concentrations of a substrate.

The effect of various chemicals on the enzyme activity was tested using the standard method except that 0·1 ml of the substance in question at 1 mM and 10 mM was added to the reaction mixture. Residual activity was assayed as described above.

RESULTS AND DISCUSSION

Results for purification of *R. oryzae* glucoamylase are summarized in Table 1. The enzyme was purified approximately sevenfold with an overall yield of about 2% by using a combination of ammonium sulfate fractionation, gel filtration, anion exchange Q-Sepharose chromatography, and cation exchange S-Sepharose chromatography. Salt fractionation and gel filtration failed to purify the enzyme. Chromatography on Q-Sepharose resulted in a sixfold increase in specific activity from the crude extract (Fig. 1). Further purification on a S-Sepharose column resulted in only a small increase in specific activity.

Chromatography of the purified enzyme on a Polybuffer exchanger 94 column revealed maximal activity at pH 8·7. The isoelectric point of the enzyme is thus about 8·7. Takahashi *et al.* (1978) reported that the pI of three glucoamylases from a *Rhizopus* sp. were 8·7 for glucoamylase$_1$ and glucoamylase$_2$, and 8·8 for glucoamylase$_3$, respectively. The SDS–PAGE pattern of the purified enzyme showed a single protein band.

TABLE 1
Purification of Glucoamylase from *R. oryzae*

Step	*Protein (mg)*	*Activity (U)*	*Sp. act. (U/mg)*	*Purification (fold)*	*Yield (%)*
1. Crude extract	63	4 473	71	1	100
2. 50–70% $(NH_4)_2SO_4$	38	1 828	49	0·7	41
3. Bio-Gel P-100	29	1 712	59	0·8	38
4. Q-Sepharose	1·8	721	406	5·7	16
5. S-Sepharose	0·15	71	482	6·8	1·6

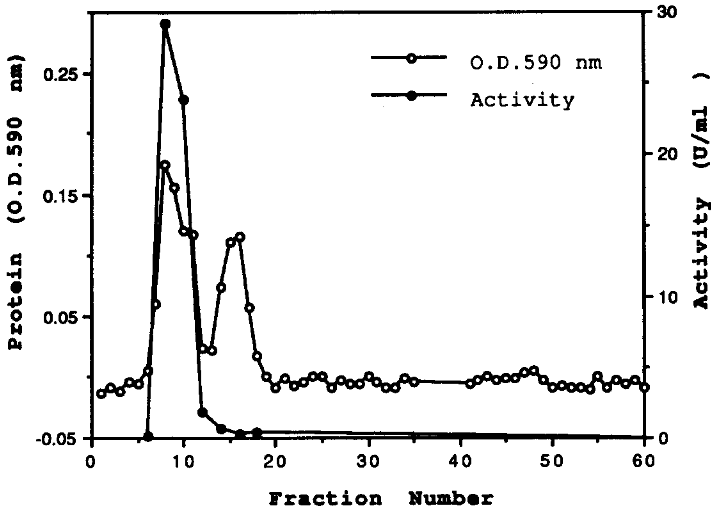

Fig. 1. Anion exchange chromatography of *R. oryzae* glucoamylase on Q-Sepharose.

The molecular weight of *R. oryzae* glucoamylase as determined by SDS–PAGE was found to be 67 000 daltons. The molecular weight of *R. nodosus* glucoamylase was reported to be 71 000 (Muthukumaran & Dhar, 1983) and molecular weights of three glucoamylases from a *Rhizopus* sp. were 74 000, 58 000, and 61 400, respectively (Takahashi *et al.*, 1978).

The optimal pH of *R. oryzae* glucoamylase was found to be 4·8–5·0. The optimal pH for *R. nodosus* glucoamylase was reported to be 4·8 (Muthukumaran & Dhar, 1983).

R. oryzae glucoamylase was stable in the pH range of 3·0–8·0. Glucoamylase from other *Rhizopus* spp. was reported to be stable at similar pH values (Takahashi *et al.*, 1978; Muthukumaran & Dhar, 1983).

The optimal temperature for *R. oryzae* glucoamylase was 60°C. It is thus similar to the glucoamylase of *R. nodosus* (Muthukumaran & Dhar, 1983), but differs from *R.* delemar glucoamylase which had an optimal temperature at 40°C (Tsujisaka *et al.*, 1958).

The enzyme was stable up to 40°C. At 50, 55, and 60°C, the activity was reduced by 22, 44, and 100%, respectively. Takahashi *et al.* (1978) also found that three glucoamylases from a *Rhizopus* sp. were stable at 40°C and lost their activity completely after incubation at 60°C for 5 min. *P. oxalicum* glucoamylase was reported to be stable at 55°C (Yamasaki *et al.*, 1977*a*).

The K_m for amylopectin and soluble starch were 0·98 and 1·34 mg/ml, respectively. The V_{max} for amylopectin and soluble starch were 782 and 136 μmoles of glucose produced per mg of protein per min, respectively.

The relative activity of *R. oryzae* glucoamylase in the presence of various metal ions was as follows: Ba^{2+}, 100%; Ca^{2+}, 100%; Zn^{2+}, 100%; Cd^{2+},

80%; Pb^{2+}, 4%; Hg^{2+}, 0%; EDTA had little effect on the enzyme. These results are in accord with those of Takahashi *et al.* (1978). Lack of inhibition by EDTA indicates that metal ions may not be required for activity.

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