PURIFICATION AND CHARACTERIZATION OF GLUCOAMYLASE FROM A *RHIZOPUS ORYZAE* MUTANT

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ABSTRACT

Glucoamylase produced by Rhizopus oryzae *mutant 4U2 was purified nearly 3-fold with a yield of 15% by ammonium sulfate fractionation, Sephadex G-100 gel filtration, CM-Sephadex chromatography, and chromatofocusing. The purified enzyme was most active at pH 4.0 and showed a temperature optimum at 55°C. The enzyme was stable for an hour at temperatures up to 40°C and over a pH range of 3.0 to 7.0. It had an isoelectric point (pI) of 8.2 and its molecular weight was approximately 80 kDa as determined by gel filtration on Sephadex G-100 and SDS-PAGE. The K_m of the enzyme for amylopectin and soluble starch were 2.4 and 12.2 mg/mL, respectively. The Vmax for amylopectin and soluble starch were 2.3 and 5.0 mM glucose per mg protein per min, respectively.*

INTRODUCTION

Glucoamylase (EC 3.2.1.3) is an enzyme that hydrolyzes α-1,4-glucosidic linkages and releases glucoses from the non-reducing chain-ends of amylose, amylopectin and glycogen. The enzyme also cleaves α-1,6- and α-1,3-bonds but at a slower action[1] The enzyme has been isolated and purified from *Aspergillus awamori*[2], *A. niger*[2-3], *A. oryzae*[4], *A. terreus*[5], *Candida antarctica*[6], *Mucor rouxianus*[7], *Rhizopus* sp.[8], *R. delemar*[9-10] and *R. oryzae*[11]. Glucoamylase from *Rhizopus* species is widely used for saccharification of starch and amylopectin to glucose[12-13].

Of the thirty eight *R. oryzae* mutants isolated, mutant 4U2 expressed the highest yield of glucoamylase[14]. In this study, we purified and studied the properties of glucoamylase from the mutant.

MATERIALS AND METHODS

Cultivation Conditions

The enzyme production by *Rhizopus oryzae* mutant 4U2 was conducted as described by Suntornsuk and Hang[15]. After 4 days of fermentation, the fungal mycelia were removed by filtration with Whatman No. 4 filter paper, and the filtrate was then clarified by centrifugation at 13,000 x g for 15 min at 4°C. The clear supernatant was used as the crude enzyme.

Enzyme Purification

All subsequent purification steps were carried out at 0-4°C.

Ammonium sulfate fractionation

The clear solution was concentrated 15-fold on an Amicon Model 8400 ultrafiltration unit (Danvers, MA) with a 30 kDa MW-cutoff membrane (Amicon YM 30) and nitrogen gas was used as a pressure source. Protein in the retentate was precipitated by adding solid ammonium sulfate into the following saturation ranges: 0-20%, 20-40%, 40-60% and 80-100%.

The precipitates collected by centrifugation at 23,000 x g for 20 min were dissolved in a minimal volume of 25 mM phosphate buffer, pH 6.8.

Gel filtration

The enzyme obtained from ammonium sulfate precipitation was applied to a Sephadex G-100 column (2.5 x 47.5 cm) previously equilibrated with 25 mM phosphate buffer, pH 6.8. The column was eluted with the same buffer at a flow rate of 30 mL/h.

Ion-exchange column chromatography

The pooled active fraction was introduced onto a Carboxymethyl (CM)-Sephadex C-25 column (2 x 10 cm) pre-equilibrated with 25 mM phosphate buffer, pH 6.8. The column was initially eluted with a two-bed volume of the buffer to wash out unadsorbed proteins. The adsorbed proteins were then eluted with a linear gradient of 0-0.5 M NaCl in the same buffer at the flow rate of 30 mL/h. A salt concentration in fractions was determined by a Chemtrix Type 70 conductivity meter (Hillsboro, OR) with using NaCl solution as a standard.

The most active fractions were combined, concentrated by ultrafiltration using an Amicon UF cell Model 52 (Lexington, MA) with a 10 kDa MW-cutoff membrane (Amicon YM 10) and desalted by a Sephadex G25-80 column (1.5 x 17 cm) earlier equilibrated with 25 mM ethanolamine-acetic acid buffer, pH 9.4. The enzyme was eluted with the same buffer at a flow rate of 30 mL/h.

Chromatofocusing

Polybuffer Exchanger 94 (PBE 94) and Polybuffer 96 were used to purify the enzyme at the final step. Following the directions given by Pharmacia, a PBE 94 column (1.0 x 20.5 cm) was pre-equilibrated with 25 mM ethanolamine-acetic acid buffer, pH 9.4. The pooled salt-free sample was applied to the column and eluted with 1:10 diluted Polybuffer 96 (adjusted to pH 6.0 with acetic acid and degassed) at a flow rate of 30 mL/h. pH of the fractions was also monitored by an Accumet Model 230 pH/ion meter (Fisher Scientific Corp., Pittsburgh, PA).

Analytical Methods

Protein was determined either by measuring the absorbance at 280 nm on a Perkin-Elmer Hitachi 200 spectrophotometer (Tokyo, Japan) or by the protein-dye binding method of Bradford[16]. Glucoamylase activity was assayed as described by Hang and Woodams[17]. One unit of glucoamylase was defined as the amount of enzyme producing 1 μmole glucose/min under the assay conditions.

Enzyme Characterization

Molecular weight determination

Two methods were used to determine the molecular weight of glucoamylase. Gel filtration on Sephadex G-100 was used as described by Andrews[18]. The marker proteins were bovine gamma globulin (158 kDa), bovine serum albumin (68 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B-12 (1,350 dal). The Kav of glucoamylase was used to determine the molecular weight by comparing with the standard proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli[19] on a 0.75 mm thick polyacrylamide slab gel (7 cm x 8 cm) consisting of 7.5% separating and 4% stacking gels by using the minigel system (Mini-Protein II) of Bio-Rad. The molecular weight standards used (Bio-Rad) were myosin (199 kDa), β-galactosidase (120 kDa), bovine serum albumin (87 kDa) and ovalbumin (48.1 kDa).

Isoelectric point determination

The isoelectric point of glucoamylase was determined by measuring the pH of the most active fraction eluted from the chromatofocusing column.

Effect of pH and temperature

The studies on effect of pH and temperature on enzyme activity were performed as the standard assay except using 0.1 M phosphate citrate buffer, pH 3.0-8.0 and a range of temperature at 20-80°C. The experiments on effect of pH and temperature on enzyme stability were carried out by incubating the enzyme solution at pH and temperature ranges of 3.0-8.0 and 20-80°C for 60 min. The enzyme activities were then determined by the standard enzyme assay.

Kinetic studies

The Michaelis-Menten constant (Km) and maximum velocity (Vmax) of purified enzyme for amylopectin and soluble starch were obtained from incubating the enzyme with different substrate concentration at 30°C for 20 min, measuring the reaction velocity and establishing the Lineweaver-Burk double reciprocal plot.

Determination of end product from enzymatic reaction

Thin layer chromatography (TLC) was performed for examining products of starch hydrolysis by the enzyme. Soluble starch, amylopectin and maltose were used as a substrate. A reaction mixture containing 200 μL of 1% substrate dissolved in 0.01 M acetate buffer (pH 4.8) and 200 μL of purified glucoamylase was incubated at 30°C. At a period of incubation, a sample was taken and then spotted onto a silica gel G plate (Fisher Scientific Corp., Pittsburgh, PA). Maltose and glucose standards were also applied onto the plate. The plate was developed by the ascending method with the solvent system of acetonitrile:distilled water (85:15, v/v), and the products were visualized by spraying with sulfuric acid:methanol (1:3, v/v) and heating at 110°C for 10 min[20].

RESULTS AND DISCUSSION

The purification results of glucoamylase are summarized in Table 1. The enzyme was purified approximately 3-fold with a yield of 15% by ammonium sulfate fractionation, Sephadex G-100 gel filtration, CM-Sephadex ion-exchange chromatography and chromatofocusing. Each step slightly improved the purification except chromatofocusing possibly caused by unstability of the enzyme under focusing pH. Ion-exchange chromatography on CM-Sephadex C-25 was the most effective step for the purification. The elution patterns of glucoamylase by Sephadex G-100 gel filtration, CM-Sephadex column chromatography and chromatofocusing are shown in Figs. 1, 2 and 3, respectively. On SDS-PAGE, a single protein band was shown in CM-Sephadex and chromatofocusing fractions, indicating that the enzyme was purified to homogeneity (Fig. 4). The purified enzyme had an active peak, suggesting that only one form of glucoamylase was present. Similarily, a single form of glucoamylase from *R. oryzae* NRRL 395 (the parent strain of mutant 4U2) has been reported[11].

The molecular weight of glucoamylase produced by *R. oryzae* mutant 4U2 was found to be 78 kDa on Sephadex G-100 gel filtration and 82 kDa on SDS-PAGE (Figs. 5 and 6). The result suggests that glucoamylase from *R. oryzae* mutant 4U2 was a monomer. Fungal glucoamylases have also been reported to be monomeric[21]. The molecular weight of this enzyme from the parental strain of *R. oryzae* as determined by SDS-PAGE is 67 kDa[11]. From other species, it varies between 58 and 100 kDa[8,10,22].

Table 1. Purification of glucoamylase from *Rhizopus oryzae* mutant 4U2.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture filtrate	1188.0	90.0	13.2	1.0	100
80% $(NH4)_2SO_4$ fractionation	746.6	35.8	20.9	1.6	63
Sephadex G-100	467.3	20.0	23.4	1.8	39
CM-Sephadex	409.5	6.8	60.2	4.6	35
Chromatofocusing	178.2	4.3	41.4	3.1	15

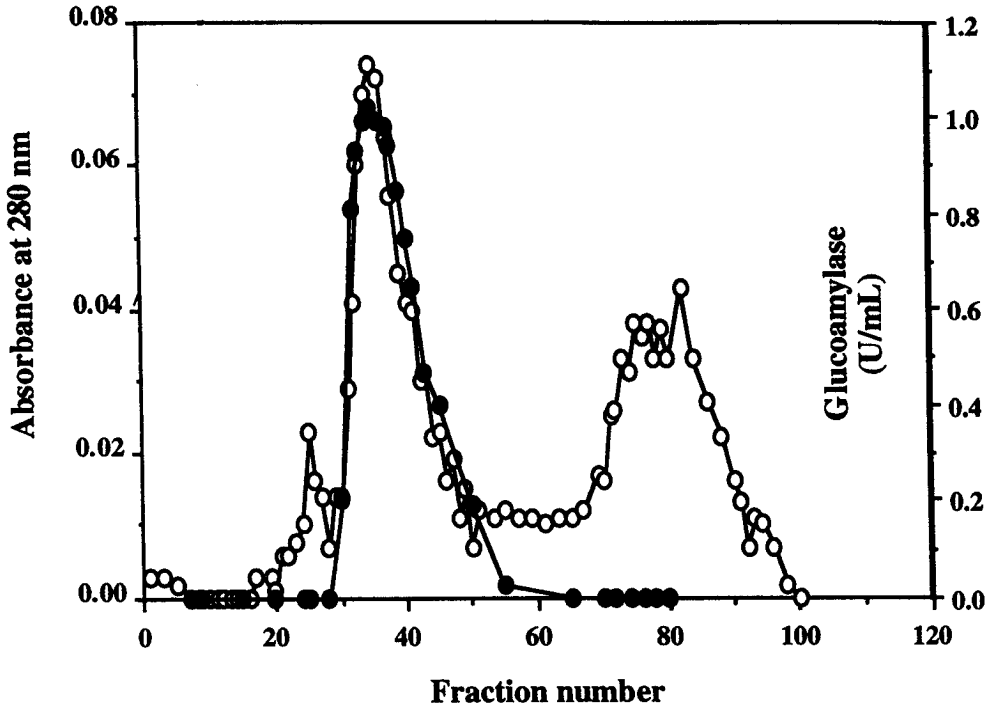

Fig.1. Gel filtration chromatography of glucoamylase from *Rhizopus oryzae* mutant 4U2 on Sephadex G-100. ●, Glucoamylase activity; o, Absorbance at 280 nm.

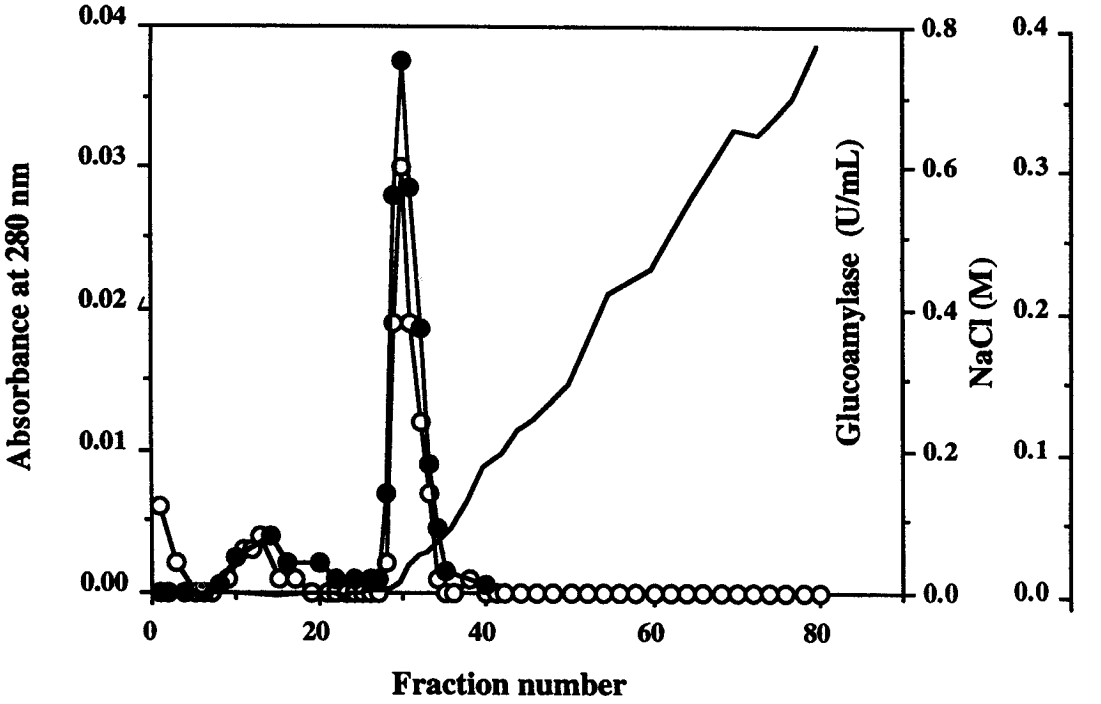

Fig.2 Ion-exchange chromatography of glucoamylase from *Rhizopus oryzae* mutant 4U2 on CM-Sephadex C-25. ●, Glucoamylase activity; o, Absorbance at 280 nm.; — NaCl concentration.

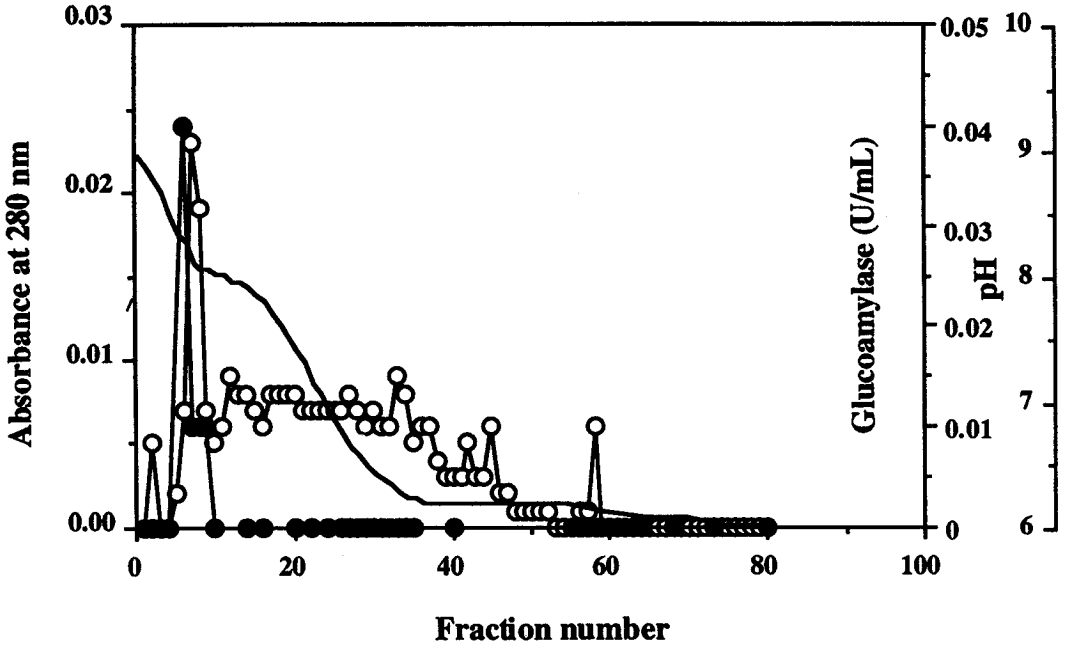

Fig.3 Chromatofocusing of glucoamylase from *Rhizopus oryzae* mutant 4U2 on Polybuffer Exchanger 94. ●, Glucoamylase activity; o, Absorbance at 280 nm.; — pH.

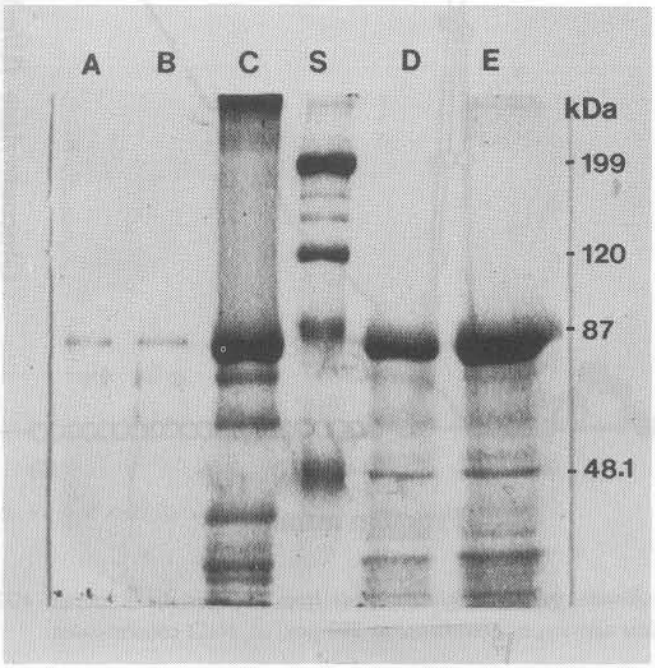

Fig.4 SDS-PAGE profile of glucoamylase from *Rhizopus oryzae* mutant 4U2. Lanes: A, Chromatofocusing eluate; B, CM-Sephadex eluate; C, Sephadex G-100 eluate; D, Concentrated crude filtrate; E, 80% Ammonium sulfate saturation fraction; S, Molecular mass standards (myosin, 199 kDa; β-galactosidase, 120 kDa; bovine serum albumin, 87 kDa; ovalbumin, 48.1 kDa)

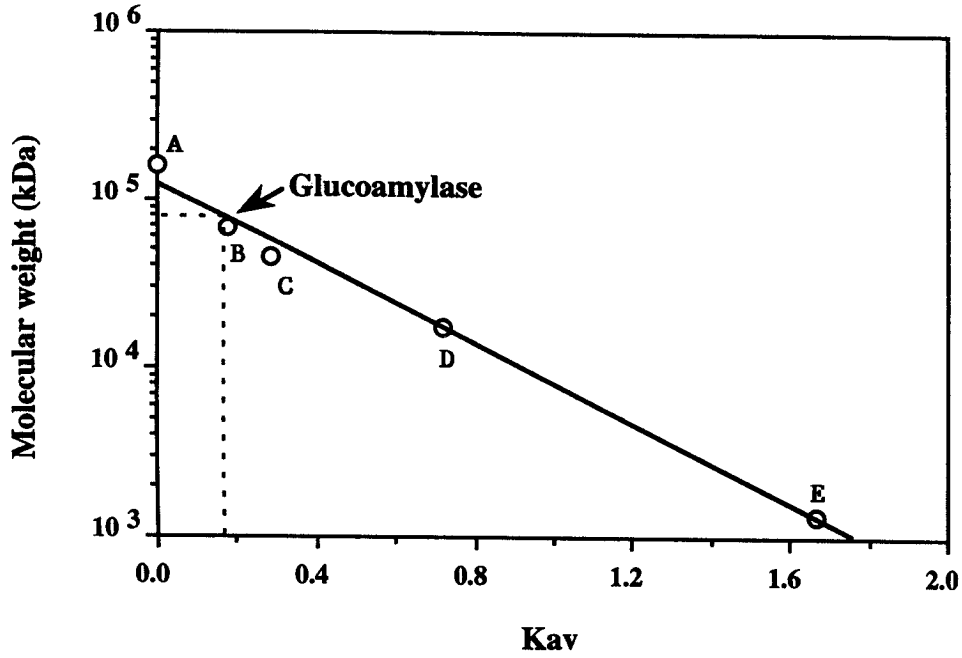

Fig.5 Molecular weight determination of glucoamylase from *Rhizopus oryzae* mutant 4U2 by gel filtration on Sephadex G-100. Protein markers used were: A, Bovine gamma globulin (158 kDa); B, Bovine serum albumin (68 kDa); C, Chicken ovalbumin (44 kDa); D, Horse myoglobin (17 kDa) and E, Vitamin B-12 (1,350 daltons)

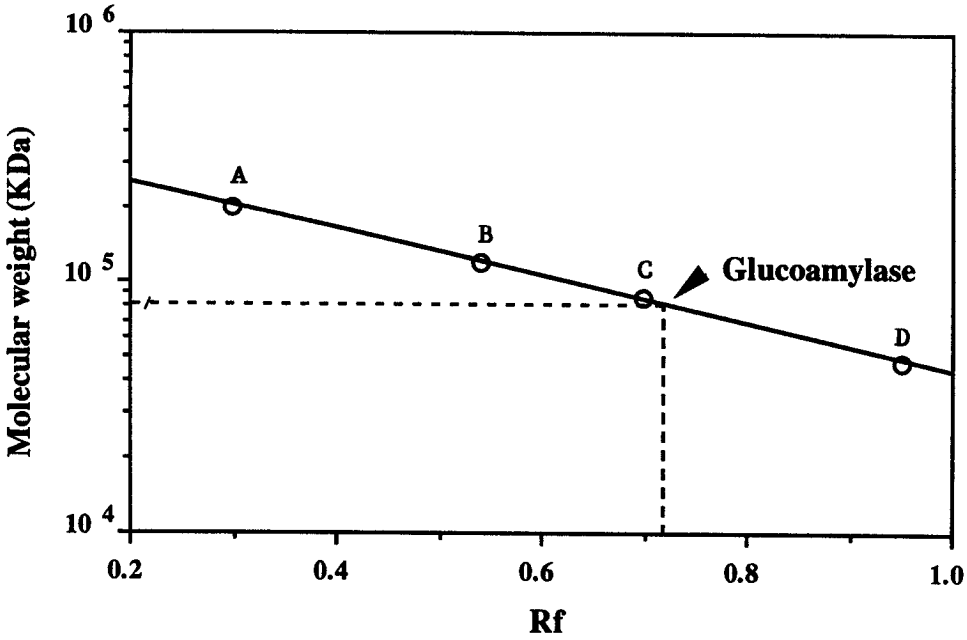

Fig.6 Molecular weight determination of glucoamylase from *Rhizopus oryzae* mutant 4U2 by SDS-PAGE. Protein markers were: A, Myosin (199 kDa); B, β-galactosidase (120 kDa); C, Bovine serum albumin (87 kDa) and D, Ovalbumin (48.1 kDa)

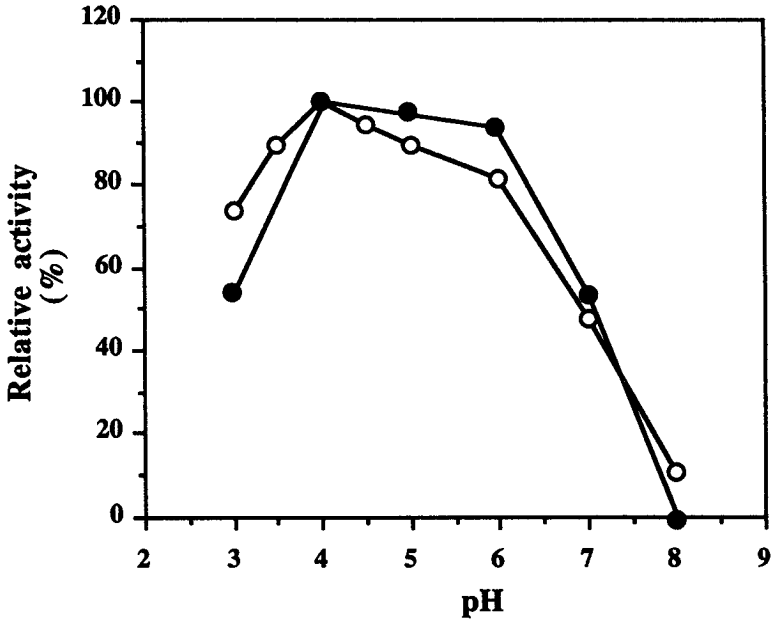

Fig.7 Effect of pH on the activity (o) and stability (●) of glucoamylase from *Rhizopus oryzae* mutant 4U2. Relative activity is expressed in comparison with the activity at pH 4 which is taken as 100%.

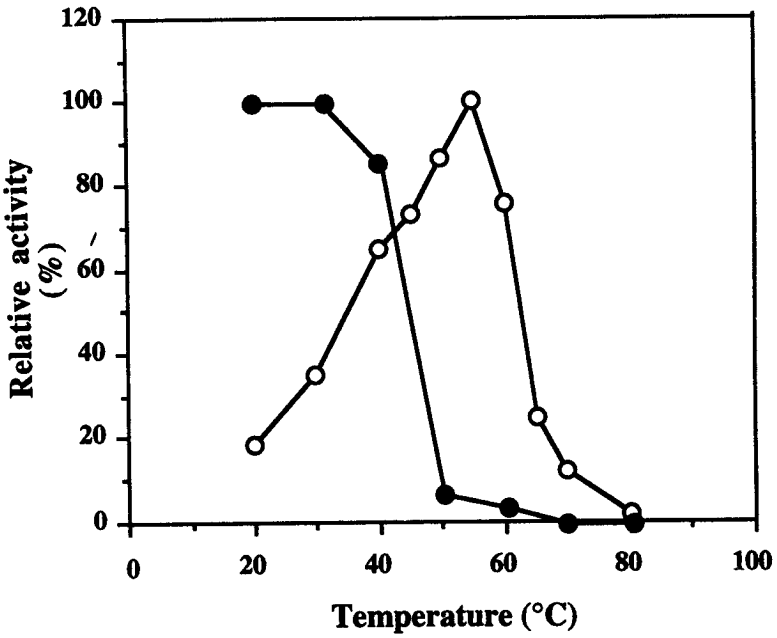

Fig.8 Effect of temperature on the activity (o) and stability (●) of glucoamylase from *Rhizopus oryzae* mutant 4U2. Relative activity is expressed in comparison with the activity at 30°C which is taken as 100%.

The isoelectric point (pI) of the purified glucoamylase was found to be 8.2 by chromatofocusing (Fig. 3). Likewise, the pI of the enzyme from the parent strain has been reported to be 8.7[11]. The pI values for *Rhizopus* glucamylases are between 8.5 and 9.1[4,8]. However, the pIs of glucoamylase from other fungi are acidic, for instance, the pIs of glucoamylase from *Aspergillus niger* and *A. terreus* are 4.2 and 3.4, respectively[23-24].

The enzyme showed an optimal activity at pH 4.0 (Fig. 7) and at 55°C (Fig. 8). The enzyme was stable over a pH range of 4.0-6.0 (Fig. 7) and up to 40°C (Fig. 8). It was considerably unstable, however, at pH below 4.0 and above 6.0, and at temperatures over 40°C. Similarily, the optimal pH and temperature of glucoamylase from the parent strain were 4.8-5.0 and 60°C, respectively[11]. In contrast, the enzyme from the parent was found stable in the pH range of 3.0-8.0. *Rhizopus* glucoamylases are generally stable between pH 3.0-8.0 and show optimal activities between pH 4.0 and 5.0[8,22]. They are also highly active over a broad temperature range of 30-60°C[8,22].

The K_m values for amylopectin and soluble starch were 2.4 and 12.2 mg/mL, respectively. The K_m values of the parental enzyme for both substrates have been reported to be 0.98 and 1.34 mg/mL, respectively[11]. The results suggest that glucoamylase had a higher affinity for amylopectin than soluble starch. The activity of glucoamylase increases with longer chain length of the substrate molecule or larger substrate size[12]. The V_{max} values of the mutant enzyme for amylopectin and soluble starch were 2.3 and 5.0 mM glucose per mg protein per min, respectively. The Vmax values of the enzyme from the parent for amylopectin and soluble starch have been reported to be 782 and 136 μmoles glucose produced per mg protein per min, respectively[11].

As analyzed by TLC, glucose was the sole product of enzymatic hydrolysis of maltose, amylopectin and starch (data not shown). The results confirm that the purified enzyme was a glucoamylase.

Overall, the properties of glucoamylase from *R. oryzae* mutant 4U2 were similar to those of the parental enzyme. This suggests that the structural gene of the enzyme from mutant 4U2 might not be altered. However, a detailed structural analysis of the enzyme and genetic analysis of the mutant needs to be carried out to confirm this conclusion.

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บทคัดย่อ

จากการศึกษาเอนไซม์กลูโคอะไมเลสจากเชื้อกลายพันธุ์ *Rhizopus oryzae* สายพันธุ์ 4 ยู 2 พบว่าสามารถทำให้บริสุทธิ์ด้วยวิธีการตกตะกอนด้วยแอมโมเนียมซัลเฟต โครมาโตกราฟฟีแบบเจลฟิลเตชันและแบบแลกเปลี่ยนอิออน และโครมาโตโฟกัสซิ่ง เอนไซม์ที่บริสุทธิ์มีความเข้มข้นเพิ่มขึ้น 3 เท่า และมีผลได้ 15 เปอร์เซ็นต์ เอนไซม์ดังกล่าวแสดงการทำงานสูงสุดที่พีเอช 4.0 และที่อุณหภูมิ 55 องศาเซลเซียส เอนไซม์มีความคงตัวได้ดีในช่วงพีเอชระหว่าง 3.0 ถึง 7.0 และสูญเสียความสามารถในการทำปฏิกิริยาที่อุณหภูมิเกินกว่า 40 องศาเซลเซียสในเวลา 60 นาที เอนไซม์มีค่าไอโซอิเล็คตริคประมาณ 8.2 จากการศึกษาด้วยวิธีอิเล็คทรอโฟริซิส ภายใต้สภาวะที่ทำให้เสียสภาพด้วยโซเดียมโดเดซิลซัลเฟตและเจลฟิลเตชัน พบว่าเอนไซม์มีน้ำหนักโมเลกุลประมาณ 80 กิโลดาลตัน ค่าคงที่ของไมเคิลลิส-เมนเทนของเอนไซม์ต่ออะไมโลเพคติน และแป้ง มีค่า 2.4 และ 12.2 มิลลิกรัมต่อมิลลิลิตร ค่าความเร็วสูงสุดในการทำปฏิกิริยาของเอนไซม์ต่ออะไมโลเพคติน และแป้ง มีค่า 2.3 และ 5.0 มิลลิโมลาร์กลูโคสต่อมิลลิกรัมโปรตีนต่อนาที