*Carbohydrate Research*, 159 (1987) 255–261
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands



# PURIFICATION OF THE GLUCOAMYLASE COMPONENTS OF *Chalara paradoxa* BY AFFINITY CHROMATOGRAPHY AND CHROMATOFOCUSING*

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(Received April 19th, 1986; accepted for publication, September 11th, 1986)

## ABSTRACT

The six major glucoamylase components (A1–A6) in the culture filtrate of *Chalara paradoxa*, which had different isoelectric points, were isolated electrophoretically pure by affinity chromatography on α-CD(cyclomaltohexaose)–Sepharose 6B and positive pH-gradient chromatofocusing using CM-Sepharose CL-6B and Polybuffer 74. The isoelectric points were: A1, 3.80; A2, 3.75; A3, 3.70; A4, 3.65; A5, 3.60; and A6, 3.55. A1–A3 had molecular weights of 76,000, 77,000, 78,000, respectively, were most active at pH 5.0–6.0 and 50°, and were stable in the pH range 4.5–8.0 and up to 45°. Each of the purified glucoamylases A1–A3 attacked soluble starch to give β-D-glucose.

## INTRODUCTION

Several raw starch-digesting amylases, which are produced from *Aspergillus awamori*[1], *Streptococcus bovis*[2], and *Bacillus circulans*[3], have been described. Kainuma *et al.*[4,5] reported a new amylase in this category from a strain of the black mould *Chalara paradoxa* grown on the pith of Sago palm and which was more active than those previously reported. Ishigami *et al.*[6] reported the purification of the enzyme with raw-starch adsorption and DEAE-cellulose column chromatography, and detected several amylase components (A1–A6) having different isoelectric points. We now report the purification of these components.

## EXPERIMENTAL AND RESULTS

*Materials.* — The crude amylase from *Chalara paradoxa* was prepared, as described by Ishigami *et al.*[5], in large-scale cultivation at Meiji Seika Co. Ltd. (Japan) and precipitated with ethanol. Polyethylene glycol 6,000 (Koch–Light),

*Studies on the Novel Raw Starch-Digesting Amylase obtained from *Chalara paradoxa*, Part 8.
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0008-6215/87/$ 03.50 

TABLE I

PURIFICATION OF AMYLASE COMPONENTS FROM *Chalara paradoxa* CULTURE FILTRATE BY AFFINITY CHROMATOGRAPHY ON α-CD-SEPHAROSE 6B AND CHROMATOFOCUSING

| *Purification step* | *Total protein (mg)* | *Total activity (U)* | *Specific activity (U/mg)* | *Recovery (%)* |
|---|---|---|---|---|
| 1 Crude enzyme | 11,290 | 118,545 | 10.5 | 100 |
| 2 PEG 6,000[a] ppt. | 4,405 | 53,300 | 12.1 | 45 |
| 3 DE-52 cellulose | 1,855 | 24,485 | 13.2 | 21 |
| 4 α-CD–Sepharose 6B | 486 | 10,972 | 43.1 | 18 |
| 5 Chromatofocusing | | | | |
| peak I | 11 | 471 | 43.6 | — |
| peak II | 13 | 556 | 42.8 | — |
| peak III | 13 | 534 | 41.7 | — |

[a]Polyethylene glycol.

Servalyt (Serva Feinbiochemica), and Polybuffer 74 (Pharmacia) were commercial products. α-CD–Sepharose 6B was prepared by the method of Vretbalt[7]. All other chemicals were AR grade.

*Enzyme assay.* — Amylase activity was assayed as follows. A mixture of aqueous 2% soluble starch (0.25 mL), 0.1M acetate buffer (0.25 mL, pH 5.0), and enzyme (0.5 mL) was incubated at 40° for 30 min. The reducing sugar released was then determined by the Somogyi–Nelson method[8] with D-glucose as the standard.

*Purification of the amylase components (Table I).* — The purification was carried out at 4°. Fractions from the column were assayed for protein by measuring the absorbance at 280 nm. For measurements of specific activity, the protein was determined by the method of Lowry *et al.*[9] with bovine serum albumin as the standard. A solution of crude enzyme preparation (50 g) in distilled water (1 L) was centrifuged at 5,000*g* and to the stirred supernatant solution was slowly added aqueous 50% polyethylene glycol 6,000 to 15%. The mixture was stirred for 30 min, the precipitate was collected by centrifugation, and to a solution in 25mM acetate buffer (500 mL, pH 5.0) was added DE-52 cellulose (50 g). The mixture was stirred slowly for 2 h, and insoluble material was collected, washed several times with 25mM acetate buffer (pH 5.0), stirred slowly for 2 h with the same buffer (250 mL) containing 0.3M NaCl, and then filtered. The filtrate was applied to a column (1.5 × 7.5 cm) of α-CD–Sepharose 6B equilibrated with 25mM acetate buffer (pH 5.0). After washing of the column with the same buffer, almost all of the amylase activity remained but it was eluted with the same buffer containing 10 mg/mL of α-CD; 10-mL fractions (50 mL/h) were collected (Fig. 1). Fractions containing the enzyme activity were combined and dialysed against 25mM acetate buffer (pH 5.0).

Isoelectric focusing of the product at this stage revealed six major components (A1–A6) with the following isoelectric points: A1, 3.80; A2, 3.75; A3,

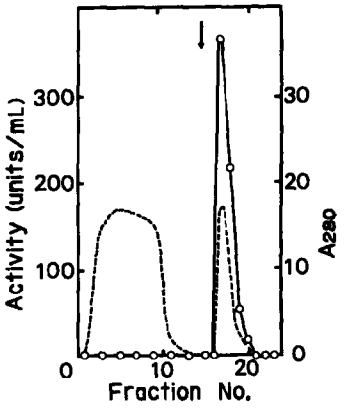


Fig. 1. Affinity chromatography of *Chalara paradoxa* amylase after elution from DE-52 cellulose (see EXPERIMENTAL AND RESULTS). α-CD was added to the buffer at ↓. Fractions 17–19 were combined: —○—, amylase activity; ----, absorbance at 280 nm.

3.70; A4, 3.65; A5, 3.60; and A6, 3.55 (Fig. 2). Further purification was effected by chromatofocusing[10,11]. To the above solution was added Polybuffer 74 to 10%, and the mixture was then applied to a column (2.5 × 20 cm) of CM-Sepharose CL-6B equilibrated with 25mM acetate. The adsorbed materials were eluted with aqueous 10% Polybuffer 74 (5-mL fractions at 25 mL/h). More than 6 peaks with amylase activity were detected (Fig. 3). The material in the major peaks A1–A3 was combined for further study.

*Polyacrylamide gel electrophoresis (PAGE).* — Isoelectric focusing was performed according to the procedure of Righetti and Drysdale[12] in a pH gradient prepared with Servalyt (pH 2–4). The gel was stained for protein using Coomassie

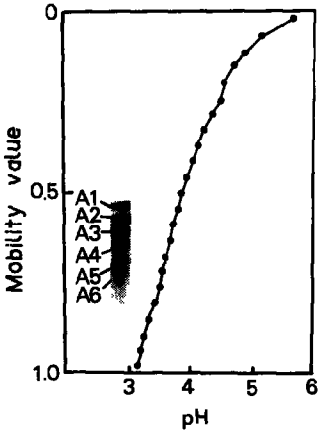


Fig. 2. Isoelectric focusing in a polyacrylamide gel of the fraction from the α-CD–Sepharose 6B column. A 4% acrylamide gel containing 1% of Servalyt 2-4T was used at 200 V for 16 h. The amount of each protein was ~30 μg.

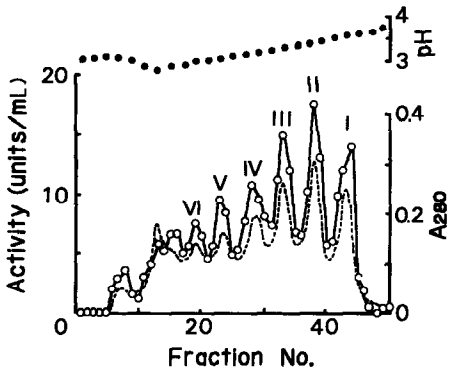


Fig. 3. Chromatofocusing of *Chalara paradoxa* amylase after affinity chromatography on α-CD–Sepharose 6B (see EXPERIMENTAL AND RESULTS): —○—, amylase activity; ----, absorbance at 280 nm; ●, pH. Fractions 43–44, 38–39, 33–34, 28, 23, and 19 corresponded to peaks I–VI, respectively.

Brilliant Blue R-250 or for amylase activity (zymogram). Each of the purified amylase components gave a single protein band (Fig. 4a) and a single active band (Fig. 4b); peaks I–VI correspond to A1–A6.

Electrophoresis in the presence of sodium dodecyl sulfate (SDS)[13] was carried out using a 7.5% gel with cross-linked cytochrome c (MW-MARKER, Oriental Yeast Co., Japan) as standard protein markers: monomer (12,400), dimer (24,800), trimer (37,200), tetramer (49,600), and hexamer (74,400). The molecular weights of A1–A3 were estimated to be 76,000, 77,000, and 78,000, respectively (Fig. 5).

*Paper chromatography.* — Hydrolysates of soluble starch obtained using the purified components were analysed by ascending paper chromatography in 1-butanol–pyridine–water (6:4:4). Each hydrolysis involved aqueous 1% soluble starch and 0.5 U of enzyme for 60 min at 40°. Each reaction was stopped by heating at ~100° for 5 min, and an aliquot (25 μL) was spotted on to Toyo Filter Paper

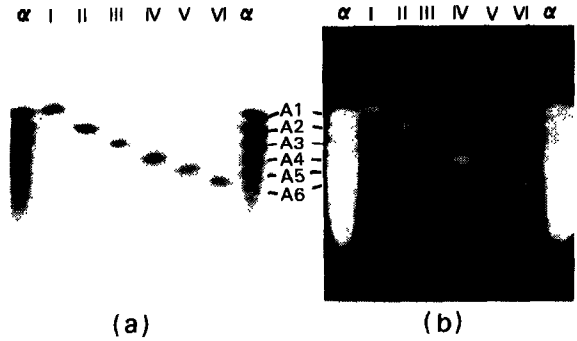


Fig. 4. Isoelectric focusing of the material in peaks I–VI in Fig. 3 (see EXPERIMENTAL AND RESULTS). Gel (a) (on 10–20-μg samples) was stained with Coomassie Brilliant Blue R-250. Gel (b) (on 2–5-μg samples) was an amylase zymogram; α is the fraction obtained after affinity chromatography on α-CD–Sepharose 6B.

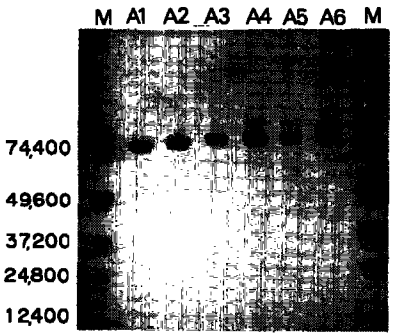


Fig. 5. SDS-PAGE of the amylase fractions (30–50 μg) obtained after chromatofocusing; M contains the molecular weight markers.

No. 50 and irrigated at 60°. Detection was effected with alkaline silver nitrate[14]. Glucose was the sole product of low molecular weight.

*Determination of anomeric configuration.* — The method of Robyt and French[15] was employed with a digital polarimeter DIP-181 (Nihon Bunko Co., Japan) and a 5-mL cell. In order to accelerate the mutarotation, the pH was brought to 10.1 by the addition of anhydrous sodium carbonate (7 mg) to the cell. For A1–A3, there was an increase in the optical rotation, indicating that the product was β-D-glucose.

*Properties of the amylase components.* — *(a) Effect of pH.* The optimum pH of each component was determined by assaying the activity under the standard conditions except that 0.1M acetate buffer (pH 3.5–6), 0.1M phosphate buffer (pH

TABLE II

EFFECT[a] OF VARIOUS CHEMICALS ON THE ACTIVITIES OF *Chalara paradoxa* AMYLASE COMPONENTS

| *Chemical* | *Conc. (mM)* | *Relative activity (%)* | | |
|---|---|---|---|---|
| | | *A1* | *A2* | *A3* |
| None | — | 100 | 100 | 100 |
| $HgCl_2$ | 2 | 29 | 47 | 57 |
| $FeCl_2$ | 2 | 100 | 88 | 86 |
| $FeCl_3$ | 2 | 84 | 85 | 69 |
| $ZnCl_2$ | 2 | 98 | 104 | 118 |
| $CuCl_2$ | 2 | 94 | 98 | 100 |
| $MgCl_2$ | 2 | 118 | 95 | 123 |
| $CoCl_2$ | 2 | 97 | 81 | 108 |
| $CaCl_2$ | 2 | 95 | 89 | 109 |
| $MnCl_2$ | 2 | 100 | 97 | 91 |
| PCMB[b] | 0.2 | 110 | 87 | 113 |
| Iodoacetamide | 2 | 84 | 100 | 86 |

[a]Expressed as a percentage of the original activity. [b]*p*-Chloromercuribenzoate.

TABLE III

$K_m$ VALUES OF *Chalara paradoxa* AMYLASE COMPONENTS

| *Substrate* | $K_m$ (%) | | |
|---|---|---|---|
| | *A1* | *A2* | *A3* |
| Soluble starch | 0.015 | 0.022 | 0.025 |
| Oyster glycogen | 0.014 | 0.018 | 0.02 |
| Short-chain amylose ($\overline{\text{d.p.}}$ 23)[a] | 0.021 | 0.032 | 0.04 |

[a]Average degree of polymerisation.

6–8), and 0.1M borate buffer (pH 8–10) were used together with 0.018 U of each amylase component. For the determination of the pH stabilities, the amylase components were incubated at 40° for 60 min in 50mM buffers of various pH's followed by assay of the residual activity.

*(b) Effect of temperature.* Thermostabilities were determined by pre-incubating at various temperatures (30–60°) for 60 min in 50mM acetate buffer (0.5 mL, pH 5.0). After rapid cooling, the residual activities were assayed under the standard conditions. The properties of A1–A3 were similar. They were most active at pH 5.0–6.0, stable at pH 4.5–8.0 at 40° for 60 min, had optimal activity at 50°, and were stable up to 45°.

*Effect of metal ions and chemical reagents.* — Each purified enzyme (0.018 U) was incubated with 2mM metallic chloride (40°, 30 min) and the residual activities were determined under the standard conditions. The results in Table II show that only $HgCl_2$ was an effective inhibitor which diminished the activities of A1–A3 to 29%, 47%, and 57%, respectively.

*Michaelis constants.* — The apparent $K_m$ values for soluble starch, short-chain amylose, and oyster glycogen, measured under the standard reaction conditions at substrate concentrations of 0.005–0.05% and estimated from Lineweaver–Burk plots, are shown in Table III. The $K_m$ values of A1–A3 for glycogen, which were the smallest for the three substrates, were 0.014%, 0.018%, and 0.02%, respectively.

## DISCUSSION

In a previous paper[6], the glucoamylase produced by *Chalara paradoxa* was purified on DEAE-cellulose and shown to contain more than six active components by isoelectric focusing on polyacrylamide gel. In the present paper, the components were separated by affinity chromatography on α-CD–Sepharose 6B and positive pH-gradient chromatofocusing[10,11]. Almost all of the amylase activity was adsorbed on the α-CD–Sepharose 6B and could be eluted with a solution of α-CD (Fig. 1). This technique has also been used for preparation of beta-amylase. As shown in Fig. 3, the six amylase components (A1–A6), obtained after affinity chromato-

graphy, were separated by positive pH-gradient chromatofocusing with CM-Sepharose CL-6B and Polybuffer 74.

The purified amylase components A1–A3 released only β-D-glucose from soluble starch, indicating them to be glucoamylases. A1–A3 had similar pH optima, pH stabilities, thermostabilities, and optimum temperatures. However, the effects of metal ions and chemical reagents and the $K_m$ values for various substrates were slightly different (Tables II and III). Furthermore, the molecular weights estimated by SDS polyacrylamide gel electrophoresis and the isoelectric points were different. For A1–A3, the isoelectric points were 3.80, 3.75, and 3.70, respectively, and the molecular weights were 76,000, 77,000, and 78,000, respectively. The components A1–A6 were all glycoproteins, and the sugar contents of A1–A3 were 6.8%, 7.2%, and 9.2%, respectively (determined by the phenol–sulfuric acid method; data not shown). A1–A6 are probably not isozymes, but multiple forms arising from variations in charged groups in the molecule.

ACKNOWLEDGMENT

This work was supported by a grant (BCP-86-V-1-9) from the Biomass Conversion Project, Ministry of Agriculture, Forestry, and Fisheries of Japan.

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