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Note

Heterogeneity of the glucoamylase components of the raw-starch-digesting amylase from *Chalara paradoxa**

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There have been several reports on the non-cooking saccharification of raw starch by amylases produced by micro-organisms[1-4]. The first enzyme in this category, raw-starch-digesting amylase, was reported by Ueda[1], who noted that preparations of glucoamylase from *Aspergillus awamori* and *Rhizopus* sp.could digest corn starch almost entirely to glucose without cooking. Kainuma *et al.*[5] reported an amylase in this category produced by a fungus, *Chalara paradoxa*, isolated from the pith of the sago palm. We have reported on the culture conditions for the production of the raw-starch-digesting amylase[6], the purification[7], properties[7,8], and raw-starch-digestion ability[9,10] of the glucoamylase, and its applications to the alcohol fermentation of raw starch[11,12].

In the purification process, several glucoamylase components were found with different isoelectric points[7]. We now report further on the properties of the glucoamylase components A1–A3 prepared[7] from the culture filtrate of *C. paradoxa*.

The three glucoamylase components (A1–A3) with different isoelectric points were purified[7] by affinity chromatography, using α-cyclodextrin (cyclomaltohexaose) and chromatofocusing. Each was a glycoprotein that was stained by the perchloric

TABLE I

Neutral sugar contents of *C. paradoxa* glucoamylases A1–A3

Glucoamylase[a]	*Proportion* (%)			
	Xylose	*Mannose*	*Galactose*	*Glucose*
A1 (6.8%)	2	60	18	20
A2 (7.2%)	1.5	65	15.5	18
A3 (9.2%)	4	67	16	13

[a] The percentages in brackets are the sugar contents of the glycoproteins.

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TABLE II

Amino acid composition of *Chalara paradoxa* glucoamylases A1–A3

Amino acid	*Molar percentage* (%)		
	A1	*A2*	*A3*
Aspartic acid	8.8	8.7	8.7
Threonine	10.9	11.2	11.1
Serine	16.0	16.0	15.7
Glutamic acid	7.6	7.5	7.5
Proline	5.2	4.8	5.3
Glycine	8.5	8.4	8.5
Alanine	10.4	10.4	10.2
1/2 Cystine	1.0	1.0	1.1
Valine	5.5	5.5	5.7
Methionine	0	0	0
Isoleucine	3.3	3.4	3.5
Leucine	8.8	8.8	8.8
Tyrosine	5.2	5.3	5.2
Phenylalanine	3.5	3.5	3.6
Lysine	2.6	2.6	2.7
Histidine	0.5	0.5	0.5
Arginine	2.1	2.3	2.1

acid–Schiff reagent[13] in gel isoelectric focusing. The sugar contents[7] of A1–A3 were 6.8, 7.2, and 9.2%, respectively, and the sugars present are shown in Table I. The amino acid compositions of A1–A3 were similar (Table II), and they were rich in threonine, serine, and alanine. They had the same N-terminal sequence (Ala–Thr–Leu–Asp–Glu–Phe–Val–Asn–Thr–Glu–) up to the 10th amino acid sequence and the terminal Ala–Thr–Leu–Asp– was the same as that[14] of the glucoamylase of *Aspergillus niger*. Although the complete amino acid sequences of these glucoamylases have not yet been determined, high homology with other fungal glucoamylase is to be expected.

There were no apparent differences between A1–A3 in the immunodiffusion precipitin test and in the peptide maps obtained after treatment with proteases. Hayashida and Flor[15] reported that the heterogeneity of the glucoamylase of *Aspergillus awamori* raw-starch-digesting amylase was the result of protease digestion during the culture. Takahashi *et al.*[16] also reported the heterogeneity of the glucoamylase of *Rhizopus* sp. caused by limit hydrolysis. However, for *C. paradoxa*, there has been no detection of smaller glucoamylases that would be derived from digestion by protease.

The difference of the molecular weights of A1–A3 might be due to the different sugar contents. The role of the sugars in A1–A3 in the digestion of raw starch remains to be determined.

EXPERIMENTAL

Scanning electron microscopy. — *Chalara paradoxa* was cultured on potato-dextrose agar plates for 5 days at 30°. Small agar chips with *C. paradoxa* were cut out and treated with a series of EtOH–water mixtures (EtOH content, 50→100%), then dried with a critical point drier. After coating with gold, the *C. paradoxa* mycelia were observed with a JEOL JSM-880 scanning electron microscope (Fig. 1.). After culture for a few days, the fungus gave many cylindrical oidia which had a length of ~ 10 μm and diameter of ~ 5 μm. The color of the colony became progressively darker and, after a few days, was almost black.

Glucoamylase. — The culture of *C. paradoxa* for the production of amylase was performed as described[6]. The alpha-amylase was removed from the culture filtrate by hydrophobic column chromatography on butyl-Toyopearl 650M (TOSO Co.)[9]. Purified A1–A3 were isolated as described[7].

Polyacrylamide gel isoelectric focusing[17]. — A 4% gel (pH range 2–4) prepared with Servalyt 2-4T (Serva) was used. Each glucoamylase gave a single band which produced a red color with the perchloric acid–Schiff reagent[13], indicative of a glycoprotein.

Amino acid analysis. — Each glucoamylase (5 mg) was hydrolysed for 16 h at 110° in 6M HCl (5 mL). The amino acids released were identified with a Hitachi 835 high-speed amino acid analyser and the results are give in Table II.

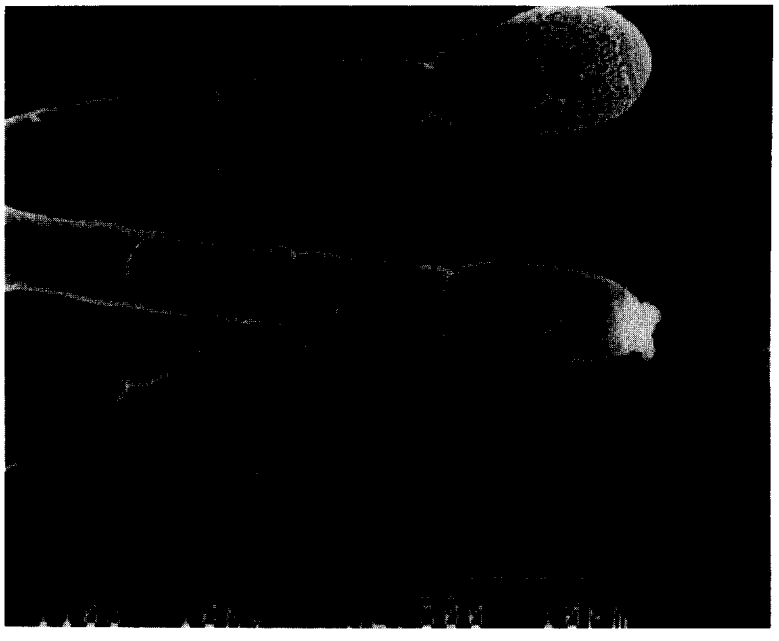

Fig. 1. Scanning electron micrograph of *Chalara paradoxa* on potato dextrose agar.

Peptide mapping. — Each glucoamylase (1 mg) was hydrolysed with a 1/50 molar ratio of *Staphylococcus aureus* V8 protease or by chymotrypsin for 20 h at 37° in 75mM Tris–HCl (pH 6.7) containing 0.05% of sodium dodecyl sulfate (SDS). The hydrolysate was analysed by SDS-PAGE using a 15% gel[18]. The peptide maps of the glucoamylase A1–A3 were similar.

N-Terminal amino acid sequences. — The N-terminal amino acid sequence of each glucoamylase was determined with a 470A peptide sequencer (Applied Biosystems Inc.). The sequence for A1–A3 was Ala–Thr–Leu–Asp–Glu–Phe–Val–Asn–Thr–Glu–. The 11th amino acid in A1 was Arg.

Immunodiffusion. — Rabbit antiserum against glucoamylase A1 was produced by intravenous injection of 1 mL of the enzyme solution twice weekly for 6 weeks, and blood was taken 7 days after the last injection. Antiserum was prepared by precipitation with ammonium sulphate. Diffusion of glucoamylases and rabbit antibodies was carried out by the method of Ouchterlony[19], using 1.5% agar in 50mM phosphate buffer (pH 6.8) containing 0.15M NaCl for 2 days at 4°. The gel was stained with Coomassie Brilliant Blue R250 and air-dried at room temperature. Glucoamylase A1 antiserum gave a single sharp precipitin line with each of the glucoamylases A1–A3.

Neutral sugar composition. — Each glucoamylase (3 mg) was hydrolysed for 10 h at 110° in 6M HCl (5 mL). The trimethylsilylated neutral sugars in the hydrolysate were analysed on a glass column (3 mm × 3 m) packed with Silicone GS-101-5% Uniport HP 60/80 (GL Science Co.) at 190→250° with a Shimadzu GC 6A gas chromatograph. The results are shown in Table I.

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