Three Forms of α -Glucosidase from Suspension-cultured Rice Cells

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Received March 22, 1985

Three forms of α -glucosidase (EC 3.2.1.20), designated as I, II, and III, have been isolated from suspension-cultured rice cells by a procedure including fractionation with ammonium sulfate, CM-cellulose column chromatography, and preparative disc gel electrophoresis. The three enzymes were homogeneous by polyacrylamide disc gel electrophoresis. α -Glucosidase I was secreted in the culture medium during growth, α -glucosidase II was readily extracted from rice cells with the buffer alone, and α -glucosidase III required NaCl to be solubilized. The molecular weights of the three enzymes were 96,000 (I), 84,000 (II), and 58,000 (III). The three enzymes readily hydrolyzed maltose, maltotriose, maltotetraose, amylose, and soluble starch. α -Glucosidase I possessed strong isomaltose-hydrolyzing activity and hydrolyzed isomaltose about three times as rapidly as α -glucosidase III. The three enzymes produced panose as the main α -glucosyltransfer product from maltose. Half the maltose-hydrolyzing activities of the three enzymes were inhibited by 11.25 ng of castanospermine. The inhibition was competitive.

The enzyme α -glucosidase is ubiquitous in higher plants. It has been suggested that α -glucosidase forms a part of the non-phosphorylolytic pathway for the breakdown of starch, and functions in seed germination by hydrolyzing the oligosaccharides produced by α - and β -amylases.^{1,2)} However, many α -glucosidases from various origins readily hydrolyze soluble starch to glucose, and α -glucosidases from sugar-beet seeds hydrolyze soluble starch at a faster rate than maltose.^{3,4)} This soluble-starch-hydrolyzing activity suggests that the enzymes may even hydrolyze starch to glucose without the preceding action of α - and β -amylases in the plant tissue. Thus the physiological function of α -glucosidase in plant tissue is not yet clear. Tissue culture is often useful in studying the various physiological phenomena of higher plants, and may be useful in making the physiological function of α -glucosidase clear. However, there are only a few reports on α -glucosidase in cultured plant cells.^{5~8)} Furthermore, there are few α -glucosidases which have been isolated in highly purified forms from cultured plant

cells with their properties examined in detail. In our previous paper, we reported that two α -glucosidases were isolated in highly purified forms from soybean callus and that they appeared to be the most common type of α -glucosidase in plants.⁸⁾ When rice cells are grown in R-2 medium⁹⁾ with shaking, they produce two α -glucosidases in the cell as in soybean callus. In addition to the two enzymes, the rice cells secrete a large amount of another α -glucosidase in the culture medium during growth. Suspension-cultured rice cells produce at least three forms of α -glucosidase, which are different from soybean callus. The three enzymes readily hydrolyzed soluble starch and produced panose as the main α -glucosyltransfer product from maltose. This property is different from that of many α -glucosidases in plants. Therefore, it is of much interest to purify the three enzymes from suspension-cultured rice cells and investigate their properties in detail.

This paper deals with the purification and properties of the three α -glucosidases produced by suspension-cultured rice cells.

MATERIALS AND METHODS

Materials. Amylose (EX-I, $\overline{\text{Dpn}}$ about 18), maltose (HHH), maltotriose, maltotetraose, and isomaltose (Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) and soluble starch (Ishizu Pharmaceutical Co., Ltd., Osaka, Japan) were obtained from commercial sources. Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) was kindly given to us by Dr. A. D. Elbein, Department of Biochemistry, Health Science Center, The University of Texas, San Antonio, U.S.A.

Cell culture. Rice cells (*Norin No. 16*) were kindly given to us by Dr. K. Ojima, Faculty of Agriculture, Tohoku University, Sendai, Japan, and have been maintained in this laboratory since 1982. The cells were grown in R-2 medium⁹ for 15 days with gentle shaking (88 strokes per min) at 25°C in the dark. The culture supernatant and the cells were stored at -20°C.

Analytical techniques

Enzyme assays. α -Glucosidase activity was measured by observing the amount of glucose liberated from the substrate by the method of Papadopoulos *et al.*,¹⁰ as modified by Dahlqvist.¹¹ One unit of α -glucosidase activity is defined as the amount of enzyme which liberates 1 $\mu\text{mol/min}$ of glucose from 1 mg maltose in 0.5 ml of 0.05 M acetate buffer, pH 4.5, at 37°C.

Measurement of protein. Protein was measured by the method of Warburg *et al.*¹² The protein profiles in column chromatography were followed by measuring the absorbance of eluates at 280 nm.

Disc gel electrophoresis. Electrophoresis of the enzyme was done in a polyacrylamide gel, pH 4.0, by the method of Reisfeld *et al.*¹³ After the run, the gel was stained for proteins with 1% amido black 10 B in 7% acetic acid.

Paper chromatography. The sugar solution was spotted on Toyo No. 50 filter paper, and the paper was developed three times at room temperature by the ascending method with a solvent system of 1-butanol-pyridine-water (6:4:3, v/v). After drying, the products were detected by the silver nitrate dip method.¹⁴

Estimation of molecular weight. The molecular weight of the purified α -glucosidases was estimated by gel filtration with Sephadex G-200.

Liquid column chromatography. For identification of transglucosylation products, the reaction mixture of α -glucosidase with maltose was subjected to a JLC 6-AUH liquid chromatograph (Nihon Denshi, Akishima, Japan). Orcinol dissolved in conc. sulphuric acid (1.5 g/liter) was used as the assay reagent.

Methylation analysis. Methylation was done as described by Hakomori¹⁵ and as modified by Sanford and

Conrad.¹⁶ The methylated oligosaccharides were hydrolyzed in 2 M trifluoroacetic acid at 120°C for 1 hr in screw-capped tubes. The methylated sugars were identified by thin-layer chromatography.¹⁷

RESULTS

I. Purification of α -glucosidases

The culture supernatant (Fraction I) was concentrated by an Ultrafiltration System (model MC-4; Bioengineering Co., Ltd., Tokyo, Japan). Rice cells (2,300 g) were suspended in 1.6 liters of 0.025 M acetate buffer, pH 4.5 (buffer A), and disrupted by sonication (at 20 kHz, model 5202 PZT, Ohtake Works, Tokyo, Japan) for 15 min at 0°C. Complete disruption of the cells was confirmed by light microscopy. The homogenate was centrifuged at $8,000 \times g$ for 10 min and the supernatant (Fraction II) was collected. The precipitate of cell wall debris was washed, suspended in 500 ml of buffer A containing 2 M NaCl, and stirred overnight. The suspension was centrifuged at $8,000 \times g$ for 10 min and the supernatant (Fraction III) was collected.

Step 1. Fractionation with ammonium sulfate. Fractions I, II, and III were brought to 0.9 saturation with ammonium sulfate. Each precipitate was collected by centrifugation, dissolved in buffer A, and dialyzed overnight against buffer A.

Step 2. First CM-cellulose column chromatography. The dialyzed solutions were separately put on CM-cellulose columns (1.6 cm diameter, 10 cm length) equilibrated with buffer A. The columns were first eluted with the same buffer to wash off the unadsorbed protein, and then with a linear gradient of 0~0.3 M NaCl also in the same buffer (Fig. 1). The eluates having α -glucosidase activity were concentrated by a Micro-Ultrafiltration System (model 8 MC; Amicon Corporation, Lexington, Mass., U.S.A) and dialyzed overnight against buffer A.

Step 3. Preparative disc gel electrophoresis. The concentrated solutions were used for preparative disc gel electrophoresis, pH 4.0. The running column (4.0 cm diameter, 15 cm length) was composed of 80 ml of separating

FIG. 1. CM-Cellulose Column Chromatography of α -Glucosidases I, II, and III from Suspension-cultured Rice Cells.

Protein applied was 980 mg (I), 1070 mg (II), or 850 mg (III); flow rate was about 60 ml/hr; fraction volume was 4 ml.

●—●, absorbance at 280 nm; ○---○, maltose-hydrolyzing activity; ---, NaCl concentration; (I), α -glucosidase I; (II), α -glucosidase II; (III), α -glucosidase III.

TABLE I. PURIFICATION OF THREE α -GLUCOSIDASES FROM SUSPENSION-CULTURED RICE CELLS

	Total protein (mg)	α -Glucosidase activity		
		Total activity (units)	Specific activity (U/mg prot.)	Yield (%)
1. Ammonium sulfate fractionation				
I	1010	11.15	0.011	100
II	2840	22.00	0.008	100
III	1260	19.64	0.016	100
2. Dialysis				
I	980	10.79	0.011	96.8
II	1070	14.92	0.014	67.8
III	850	13.54	0.016	68.9
3. First CM-cellulose column chromatography				
I	147	9.38	0.064	84.1
II	143	10.00	0.070	45.5
III	73	9.08	0.124	46.2
4. Preparative disc gel electrophoresis and second CM-cellulose column chromatography				
I	6.2	1.34	0.216	12.0
II	4.5	1.77	0.393	8.0
III	3.3	1.64	0.497	8.4

I, α -glucosidase I; II, α -glucosidase II; III, α -glucosidase III.

gel and 18 ml of concentrating gel. The gels and the electrode buffer were prepared by the method of Reisfeld *et al.*¹³⁾ Electrophoresis was done at 43 mA for 20 hr with the cathode in the lower chamber. After electrophoresis, the gel was cut into 0.5 cm sections and crushed in buffer A. The active fractions were combined.

Step 4. Second CM-cellulose column chromatography. After removing insoluble materials by filtration, the enzyme solutions were put on the CM-cellulose columns as described above. After washing off the unadsorbed materials with buffer A, the columns were eluted with the same buffer containing 0.3 M NaCl. The eluates were dialyzed overnight against buffer A and concentrated by a Micro-Ultrafiltration System. The purified enzymes from Fractions I, II, and III will be called α -glucosidases I, II, and III, respectively. A summary of the purification procedure is given

FIG. 2. Disc Gel Electrophoresis of α -Glucosidases I, II, and III from Suspension-cultured Rice Cells.

The purified enzyme protein was studied by polyacrylamide disc gel electrophoresis at pH 4.0. Electrophoresis was at a constant current of 3 mA per tube as described in MATERIALS AND METHODS.

I, α -glucosidase I (8.4 μ g); II, α -glucosidase II (10.0 μ g); III, α -glucosidase III (10.3 μ g).

in Table I. The purified enzymes were homogeneous by polyacrylamide gel electrophoresis (Fig. 2). Their protein bands coincided with α -glucosidase activity.

II. Properties of enzymes

1) **Molecular weight.** The molecular weights of the three enzymes were estimated to be 96,000 (I), 84,000 (II), and 58,000 (III) from gel filtration with Sephadex G-200 (Fig. 3).

2) **pH Optimum and pH stability.** The pH optima of the three α -glucosidases were found to be 4.0~4.5 (I), 4.0 (II), and 4.0 (III). After 20 hr preincubation at 30°C with 0.05 M McIlvaine's buffer, the three enzymes were stable in the pH range of 4.0~7.0.

3) **Temperature optimum and heat stability.** The temperature optima of the three α -glucosidases were found to be 55°C (I) and 50~55°C (II, III) (Fig. 4). After 15 min preincubation with 0.05 M acetate buffer, pH 4.5, the three enzymes were stable at temperatures up to 45°C (I, III) and 55°C (II) (Fig. 5).

4) **Substrate specificity.** The activity of the three α -glucosidases on various substrates was examined and the relative rates of hydrolysis are listed in Table II. For enzyme assays we measured the amount of glucose liberated from substrate as described in MATERIALS AND METHODS. Therefore the hydrolysis values of maltose and isomaltose were twice as high as

FIG. 3. Estimation of the Molecular Weights of α -Glucosidases I, II, and III.

Gel filtration on a column of Sephadex G-200 was done as described in MATERIALS AND METHODS.

I, α -glucosidase I; II, α -glucosidase II; III, α -glucosidase III; (1), ovalbumin; (2), bovine serum albumin; (3), γ -globulin.

FIG. 4. Effect of Temperature.

Enzyme activity was estimated with α -glucosidases I, II, and III at various temperatures separately.

●—●, activity of α -glucosidase I; ●—●, activity of α -glucosidase II; ○—○, activity of α -glucosidase III.

FIG. 5. Heat Stability.

After 15 min incubation with 0.05 M acetate buffer, pH 4.5, at various temperatures, residual α -glucosidase activity was measured under standard assay conditions.

●—●, α -glucosidase I; ●—●, α -glucosidase II; ○—○, α -glucosidase III.

TABLE II. SUBSTRATE SPECIFICITY OF THE THREE α -GLUCOSIDASES FROM SUSPENSION-CULTURED RICE CELLS

	Relative rate of hydrolysis (%)		
	α -Glucosidase I	α -Glucosidase II	α -Glucosidase III
Maltose*	100	100	100
Isomaltose*	42.2	35.0	15.5
Maltotriose	117.7	104.0	118.6
Maltotetraose	132.6	139.0	109.7
Amylose	51.0	39.5	45.1
Soluble starch	45.6	57.6	58.4

* The value was calculated from half of the amounts of glucose measured.

FIG. 6. Influence of Castanospermine on the Hydrolysis of Maltose by α -Glucosidases I, II, and III.

I, α -glucosidase I; II, α -glucosidase II; III, α -glucosidase III; ○—○, without castanospermine; ●—●, with 11.25 ng of castanospermine.

those of the other substrate for the same degree of hydrolysis. In Table II the values of maltose and isomaltose were calculated from half the amounts of glucose measured. The three α -glucosidases from rice cells hydrolyzed maltotriose and maltotetraose much faster than maltose, but hydrolyzed amylose (Dpn. about 18) slower. α -Glucosidase I hydrolyzed isomaltose about three times as fast as α -glucosidase III. The Michaelis constants for maltose of the three enzymes were estimated to be 1.848 mM (I), 2.523 mM (II), and 1.848 mM (III) (Fig. 6). When the reaction of the three α -

FIG. 7. Paper Chromatograms of Transglucosylation Products of α -Glucosidases I, II, and III from Maltose. Reaction mixture (2 ml) containing 1.0 ml of 10% maltose, the enzyme preparation (α -glucosidase I, 0.057 unit; α -glucosidase II, 0.039 unit; α -glucosidase III, 0.050 unit), and 0.2 ml of 0.5 M acetate buffer, pH 4.5, was incubated at 37°C. At various times, 0.025 ml of the reaction mixture was chromatographed as described in MATERIALS AND METHODS.

glucosidases with maltotriose was investigated by gas-liquid chromatography, the product was α -glucose (data not shown).

5) *α -Glucosyltransfer products.* α -Glucosidases I, II, and III were incubated with 5% maltose and the products were studied by paper chromatography. Two unknown substances were detected in each case on the chromatograms (Fig. 7). On the basis of similarities in the elution pattern in liquid chromatography, the main substance (X_2) is presumed to be panose (Fig. 8). Methylation of substance X_2 gave rise to 2,3,4,6-tetra-Me-Glc, 2,3,6-tri-Me-Glc, and 2,3,4-tri-Me-Glc. Substance X_2 was hydrolyzed into glucose and maltose by *Aspergillus awamori* α -glucosidase,¹⁸⁾ which hydrolyzed phenyl α -maltoside into glucose and phenyl α -glucoside. These results mean that substance X_2 is panose.

6) *Inhibitor.* To identify the effect of metal ions, enzyme solutions (0.4 ml) were mixed with 0.02 ml of 0.1 M of the ion and incubated at 37°C for 30 min; then, 0.1 ml of 1% maltose was added. The resulting mixture was kept at 37°C for 120 min. Na^+ , K^+ , Ba^{2+} , Ca^{2+} , Co^{2+} , Mn^{2+} , Mg^{2+} , Zn^{2+} , and EDTA had no effect, but Hg^{2+} inhibited the activities of the three enzymes completely. There are two kinds of α -glucosidase that are involved in the processing reaction in the biosynthesis of the oligosaccharide portion of the N-linked

glycoprotein.¹⁹⁾ Elbein *et al.* reported that castanospermine inhibits the processing reaction of the oligosaccharide portion of the influenza viral hemagglutinin.²⁰⁾ This kind of α -glucosidase has not been reported in plants. Therefore, it would be interesting to see how effectively castanospermine can inhibit the three enzymes. When 11.25 ng of castanospermine were mixed in 0.5 ml of the reaction mixture, half of the maltose-hydrolyzing activity was inhibited. The inhibition was essentially competitive when maltose was the substrate (Fig. 6).

DISCUSSION

Three α -glucosidase were isolated in highly purified form from suspension-cultured rice cells. α -Glucosidase I was secreted into the culture medium during growth. α -Glucosidase II was readily extracted from rice cells with buffer alone, whereas α -glucosidase III required NaCl to be solubilized. Therefore we think that α -glucosidase III is bound to the cell wall *via* ionic interactions. The three enzymes seem to be in different parts of the rice cell.

The three α -glucosidase from suspension-cultured rice cells readily hydrolyzed maltose, maltotriose, maltotetraose, amylose, and soluble starch. They are similar to the two α -glucosidases from rice seeds²¹⁾ in substrate

FIG. 8. Liquid Chromatogram of Transglucosylation Products from Maltose.

The reaction mixture in Fig. 5 was incubated at 37°C for 48 hr and 0.2 ml of the reaction mixture was mixed with 4.8 ml of 0.13 M sodium borate, pH 7.5. The mixture was assayed by liquid chromatography using a long (0.8 ϕ $\times$ 50 cm) and a short column (0.8 ϕ $\times$ 15 cm) packed with LC-R-3 quaternary ammonium ion-exchange resin.

1, panose; 2, maltotriose; 3, maltose; 4, isomaltotriose; 5, isomaltose; 6, glucose.

(S), standard; (I), α -glucosidase I; (II), α -glucosidase II; (III), α -glucosidase III.

specificity. However, the three enzymes from suspension-cultured rice cells hydrolyzed isomaltose faster than the two enzymes from rice seeds. The isomaltose-hydrolyzing activity of α -glucosidase I was the strongest of the three enzymes, and α -glucosidase I hydrolyzed isomaltose about three times as fast as α -glucosidase III. One of the two α -glucosidases from sugar-beet seeds readily hydrolyzes isomaltose, but the other does only slightly.⁴⁾ Many α -glucosidases have been reported in plants. However, only α -glucosidase from sugar-beet seeds readily hydrolyzes isomaltose.⁴⁾ α -Glucosidases from *Aspergillus* species^{18,22~25)} readily hydrolyze isomaltose. α -Glucosidase I hydrolyzed isomaltose faster than the enzymes from *A. awamori*.¹⁸⁾ α -Glucosidase I is similar to the enzyme from sugar-beet seeds⁴⁾ in its strong isomaltose-hydrolyzing activity; there have been few reports of this kind of α -glucosidase in plants.

The α -glucosidases from suspension-cultured rice cells and rice seeds differed in their α -glucosyltransfer activity patterns. The three enzymes from suspension-cultured rice cells produced panose as the main α -glucosyltransfer product from maltose while that of the two enzymes from rice seeds²¹⁾ was maltotriose. Most of the α -glucosidases tested in plants readily hydrolyzed soluble starch and produced maltotriose as the main α -glucosyltransfer product from maltose.⁴⁾ Therefore, the three enzymes from suspension-cultured rice cells appear to be a rare kind of α -glucosidase in plants.

The molecular weights of the three enzymes were estimated to be 96,000 (I), 84,000 (II), and 58,000 (III) from gel filtration with Sephadex G-200. The molecular weights of α -glucosidases II and III are close to those of the two enzymes from rice seeds.²¹⁾ Since the molecular weight of α -glucosidase I is the largest of the three, we think that α -glucosidase I may be different from α -glucosidases II and III, and may not be a modified protein of the two enzymes in the culture medium after the secretion.

Acknowledgment. We are grateful to Professor K. Kawazu, Okayama University, for his useful suggestion on methylation analysis.

REFERENCES

- 1) R. R. Swain and E. E. Dekker, *Biochim. Biophys. Acta*, **122**, 87 (1966).
- 2) T. Nomura, Y. Kono and T. Akazawa, *Plant Physiol.*, **44**, 765 (1969).
- 3) S. Chiba, S. Inomata, H. Matsui and T. Shimomura, *Agric. Biol. Chem.*, **42**, 241 (1978).
- 4) Y. Yamasaki and Y. Suzuki, *Planta*, **148**, 354 (1980).
- 5) I. Igaue, C. Kitajima, M. Terasawa and F. Kurasawa, *Niigata Agricultural Science*, **26**, 89 (1974).
- 6) H. Pierrot and J. E. van Wielink, *Planta*, **137**, 235 (1977).
- 7) T. Asamizu, Y. Inoue and A. Nishi, *Plant Cell Physiol.*, **22**, 469 (1981).
- 8) Y. Yamasaki and H. Konno, *Agric. Biol. Chem.*, **49**, 129 (1985).
- 9) K. Ohira, K. Ojima and A. Fujiwara, *Plant Cell Physiol.*, **14**, 1113 (1973).
- 10) N. M. Papadopoulos and W. C. Hess, *Arch. Biochem. Biophys.*, **88**, 167 (1960).
- 11) A. Dahlqvist, *Biochem. J.*, **80**, 547 (1961).
- 12) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1942).
- 13) R. A. Reisfeld, U. J. Lewis and D. E. Williams, *Nature (London)*, **195**, 281 (1962).
- 14) N. E. Welker and L. L. Campbell, *J. Bacteriol.*, **86**, 681 (1963).
- 15) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).
- 16) P. A. Sanford and H. E. Conrad, *Biochemistry*, **5**, 1508 (1966).
- 17) A. Chapman, E. Li and S. Kornfeld, *J. Biol. Chem.*, **254**, 10243 (1979).
- 18) Y. Yamasaki, Y. Suzuki and J. Ozawa, *Agric. Biol. Chem.*, **41**, 2149 (1977).
- 19) L. S. Grinna and P. W. Robbins, *J. Biol. Chem.*, **254**, 8814 (1979).
- 20) Y. T. Pan, H. Hori, R. Saul, B. A. Sanford, R. J. Molyneux and A. D. Elbein, *Biochemistry*, **22**, 3975 (1983).
- 21) Y. Yamasaki and Y. Suzuki, *Agric. Biol. Chem.*, **44**, 707 (1980).
- 22) S. Sugawara, Y. Nakamura and T. Shimomura, *Bull. Agric. Chem. Soc. Jpn.*, **23**, 156 (1959).
- 23) Y. Tsujisaka and J. Fukumoto, *Nippon Nôgeikagaku Kaishi*, **37**, 668 (1963).
- 24) J. H. Pazur and T. Ando, *Arch. Biochem. Biophys.*, **93**, 43 (1961).
- 25) M. J. Rudick and A. D. Elbein, *Arch. Biochem. Biophys.*, **161**, 281 (1974).