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ISOENZYMES OF SOLUBLE STARCH SYNTHETASE FROM *ORYZA SATIVA* GRAINS*

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Key Word Index—*Oryza sativa*; Gramineae; rice; starch synthesis; ADP-glucose: α-1,4-glucosyltransferase; isozymes.

Abstract—Two isoenzymic fractions of soluble ADP-glucose: α-1,4-glucan-4-glucosyltransferase were obtained from developing (non-waxy) rice grains by gradient elution through DEAE-cellulose. After Sephadex G-200 chromatography, fractions I and II were electrophoretically homogeneous and have MW values of 110000 and 69000, respectively. Sodium dodecyl sulfate gel electrophoresis of fraction I produced five bands with MW of 12000, 26000, 50000, 70000, and 105000 while fraction II gave two bands with MW of 12000 and 22000. Fraction II, which contains 1·7% carbohydrate, was active in the absence of added primer while fraction I, which does not contain carbohydrate, required primer.

INTRODUCTION

Starch synthetase (ADP–glucose: α 1,4-glucan-α-4-glucosyl-transferase, E.C. 2.4.1.21) has been considered to play an important role in cereal grain starch synthesis since the discovery of the starch granule-bound [1] and soluble [2] forms of the enzyme. Two isozymes of the soluble enzyme have been found in both waxy [3] and nonwaxy [4] maize seeds, one of which can synthesize an amylopectin-type molecule in the absence of primer. Likewise, two forms of the enzyme were observed in extracts of both waxy and nonwaxy rice grains [5]. One form uses as primer long-chain polysaccharides and large α-glucans while the other utilizes short-chain oligosaccharides more readily. Suggestions have been made [6] regarding the possible roles of the soluble and granule-bound enzymes in the biosynthesis of starch in cereal grains.

Elucidation of the precise role played by the soluble form of starch synthetase requires molecular characterization of this enzyme. Furthermore, studies are needed in order to explain the differences in primer requirements of several forms of the enzyme. The present work was done with these objectives in mind. Two isozymes of soluble starch synthetase from developing rice grains have been isolated and purified. The molecular weights, primer requirements and carbohydrate contents of the isozymic forms were determined.

RESULTS AND DISCUSSION

DEAE–cellulose chromatography of soluble starch synthetase from developing rice grains produced two peaks, both of which were active in the presence of added amylopectin primer (Fig. 1). The faster eluting fraction (fraction I) was not active in the absence of added primer while fraction II was active without adding primer to the solution. When the two fractions were separately chromatographed on Sephadex G-200, fraction I eluted from the column at a volume corresponding to a MW of 110000 ± 10000 while fraction II was found to have a MW of 69000 ± 7000.

The enzymatic activities during the different stages of purification are given in Table 1. The primed activities increased 50- and 60-fold for fractions I and II, respectively, after Sephadex G-200 chromatography compared to the crude extract. The unprimed activity of fraction II after elution through Sephadex was 26·6 times that for the ammonium sulfate-purified enzyme.

Each enzyme fraction obtained from Sephadex chromatography was essentially pure as shown by analytical gel electrophoresis. However, on electrophoresis in

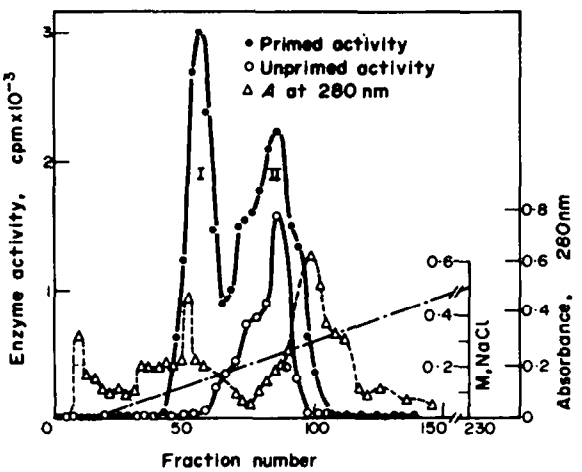

Fig. 1. Elution diagram of soluble starch synthetase from rice grains on DEAE–cellulose chromatography.

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Table 1. Purification of soluble starch synthetase from rice grains.

Fraction	Specific activity (units/mg protein) × 10^3* Primed	Unprimed
Crude extract	0·66	0
$(NH_4)_2SO_4$ cut	1·54	0·37
DEAE-Cellulose		
Fraction I	25·9	0
Fraction II	11·4	2·26
Sephadex G-200		
Fraction I	33·0	0
Fraction II	39·4	9·80

* A unit of enzyme activity is defined as one μmol ^{14}C-glucose transferred from ADP-glucose per min at 37°.

the presence of sodium dodecyl sulfate and β-mercaptoethanol, fraction I gave five bands corresponding to MW values of 12000 ± 2000, 26000 ± 3000, 50000 ± 6000, 70000 ± 7000 and 105000 ± 10000 while fraction II produced two bands with MW of 12000 ± 3000 and 22000 ± 4000. The MW values for the electrophoretic bands are related to one another by whole numbers, within experimental error. This implies that the bands represent aggregates of a monomer having a MW of 12000. The active fractions with MW of 69000 and 110000, which were eluted from Sephadex G-200, should then correspond to a hexamer and decamer, respectively.

Fractions I and II were found to contain 0 and 1·7% carbohydrate, respectively. The carbohydrate present in fraction II probably serves as endogeneous primer since this fraction is active in the absence of added primer. A similar observation applies to granule-bound starch synthetase from developing rice grains after the enzyme was solubilized by sonication in 75% dimethyl sulfoxide. [7]. An enzyme–amylose complex was isolated by sucrose density gradient centrifugation which was active even without added primer, the bound amylose apparently serving as endogeneous primer. Schiefer [8] has reported a similar finding and attributed the unprimed activity of sweet corn starch synthetase to an endogenous primer. The latter could be removed from the enzyme by amylase treatment with concomitant loss of unprimed activity although synthetase activity could be restored by adding a glycogen primer.

The aggregation phenomenon exhibited by the soluble starch synthetase from rice grains was also observed for the enzyme from rice leaves [9]. Interestingly, the subunit MW found for the leaf enzyme is the same, within experimental error, as that observed in the present study for both isozymes in the rice grain and one of the two leaf isozymes has the same MW as fraction II. Although speculations could be made relating the grain and leaf enzymes further studies are needed in order to elucidate the mechanism and implications of the enzymic aggregation observed.

EXPERIMENTAL

Enzyme purification. Rice grains (variety IR8, midmilky stage) were harvested and stored in the freezer until use. Grains (400 g) were ground with dry ice in a Waring blender and macerated with mortar and pestle in an equal wt of sand and 650 ml of pH 8·5 buffer (50 mM Tris-HCl–5 mM dithiothreitol–2 mM EDTA). All operations were done at 0–4°. Macerate was passed through 3 layers of cheesecloth. Residue was further homogenized with the same buffer in a Waring blender and filtered again through cheesecloth. Filtrates were combined and centrifuged at 12000 *g* for 20 min. $(NH_4)_2SO_4$ was added to the supernatant to 40% saturation. After standing for 45 min the supernate was centrifuged at 12000 *g* for 25 min. The ppt. was dissolved in the Tris–HCl buffer and dialyzed overnight. Dialyzate was placed on a DEAE–cellulose column (2·5 × 45 cm) which had been equilibrated with the pH 8·5 buffer. The column was eluted with 1 bed vol. of the buffer followed by a linear gradient of NaCl. Fractions (18 ml) of the eluate were collected, assayed for enzyme and the *A* at 280 nm measured. Suitable fractions were pooled and concentrated by ultrafiltration through a PM-10 filter. The 2 concentrated enzyme fractions from DEAE–cellulose chromatography were separately applied onto a Sephadex G-200 column (2·6 × 60 cm) and eluted with pH 7·5 buffer (50 mM Tris–HCl–0·1 M HCl) using the procedure of ref [10]. MW's of the starch synthetase isozymes were determined using as reference proteins cytochrome C, α-chymotrypsinogen, aldolase, ovalbumin and glyceraldehyde-3-phosphate dehydrogenase. MW's for these proteins given in refs [10] and [11] were used.

Electrophoresis. The method of ref [12] was used for analytical gel electrophoresis. Protein (100 μg) was introduced per gel. Electrophoresis at pH 8·3 was carried out at 2 mA per gel for 30 min, then was increased to 5 mA for 2 hr at 0–4°. Electrophoresed gels were stained with 1% amido black in 7% HOAc for 1 hr at 28° and destained for 18 hr with 7% HOAc. Densitometric tracings at 550 nm were done on the developed gels. SDS gel electrophoresis was done according to ref [13]. Enzyme (100 μg) was applied on each gel (0·5 × 10 cm), after prior incubation of the enzyme with 1% SDS and 1% mercaptoethanol in pH 7 Pi buffer. The wt ratio of SDS to protein was made 3:1 [14]. Electrophoresis was started with a current of 4 mA and increased to 8 mA per gel throughout the 5 hr run at room temp. Gels were then stained with 0·25% coomassie blue in 50% aq. MeOH–HOAc (227:23) for 2 hr at 28° and destained for 10 hr with HOAc–MeOH–H_2O (3:2:35). A densitometric tracing at 550 nm was run for each developed gel. Glyceraldehyde-3-phosphate dehydrogenase, β-galactosidase, phosphorylase a, fumarase, myoglobin and cytochrome C served as marker proteins whose MW values were obtained from ref [13].

Enzyme assay. Starch synthetase activity was measured with and without primer (corn amylopectin, Nutritional Biochemicals Co.) in the reaction mixture. The procedure of ref [15] was essentially followed.

Protein and carbohydrate determination. Soluble protein was measured using the method of ref [16] with bovine serum albumin as standard. Carbohydrate content of each enzyme fraction was determined following the procedure of ref [17] with minor modifications. Purified enzyme fraction (50 μl) was used in the colorimetric estimation of glucose at 630 nm.

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REFERENCES

1. De Fekete, M. A. R., Leloir, L. F. and Cardini, C. E. (1960) *Nature (London)* **187**, 918.
2. Frydman, R. B. and Cardini, C. E. (1964) *Biochem. Biophys. Res. Commun.* **14**, 353.
3. Ozbun, J. L., Hawker, J. S. and Preiss, J. (1971) *Plant Physiol.* **48**, 765.
4. Hawker, J. S. and Downton, W. J. S. (1974) *Phytochemistry* **13**, 893.

5. Tanaka, Y. and Akazawa, T. (1971) *Plant Cell Physiol.* **12,** 493.
6. Tsai, C. Y. (1973) *Bot. Bull. Acad. Sinica* **14,** 125.
7. Perdon, A. A., del Rosario, E. J. and Juliano, B. O. (1975) *Phytochemistry* **14,** 949.
8. Schiefer, S. (1973) *Federation Proc.* **32,** 603.
9. Antonio, A. A., del Rosario, E. J. and Juliano, B. O. (1973) *Phytochemistry* **12,** 1929.
10. Andrews, P. (1965) *Biochem. J.* **96,** 595.
11. Darnall, D. W. and Klotz, I. M. (1972) *Arch. Biochem. Biophys.* **149,** 1.
12. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **21,** 404.
13. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406.
14. Weber, K., Pringle, J. R. and Osborn, M. (1972) in *Methods in Enzymology* (Hirs, C. H. W. and S. N. Timasheff, eds.) Vol. 26, Part C, pp. 3–27. Academic Press, New York.
15. Sanwal, G. G., Greenberg, E., Hardie, J., Cameron, E. C. and Preiss, J. (1968) *Plant Physiol.* **43,** 417.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
17. Yoshida, S., Forno, D. A., Cock, J. H. and Gomez, K. A. (1972) in *Laboratory Manual of Physiological Studies of Rice* pp. 38–39. International Rice Research Institute, Los Baños, Laguna, Philippines.