Phytochemistry, 1975, Vol. 14, pp. 949–951. Pergamon Press. Printed in England.

SOLUBILIZATION OF STARCH SYNTHETASE BOUND TO *ORYZA SATIVA* STARCH GRANULES

ALICIA A. PERDON, ERNESTO J. DEL ROSARIO* and BIENVENIDO O. JULIANO

Chemistry Department, International Rice Research Institute, Los Baños, Laguna, Philippines

(*Received* 10 *August* 1974)

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

Abstract—Starch synthetase was solubilized from purified starch granules of ripening grains of rice at the midmilky stage. The procedure consisted of making the granules amorphous and dispersing the amorphous starch by sonication in 75% dimethysulfoxide. A starch synthetase–amylose complex was isolated by discontinuous sucrose density gradient centrifugation, which does not require added primer and can utilize both ADP glucose and UDP glucose. A starch-free protein fraction was obtained by treatment with sodium dodecyl sulfate and β-mercaptoethanol.

INTRODUCTION

Starch synthetase (ADP-glucose: α-1,4-glucan-α-4-glucosyltransferase, E.C. 2.4.1.21) is believed to play a key role in starch biosynthesis [1–3]. In the developing and mature rice grain (*Oryza sativa* L.) the enzyme exists both in a soluble form and in a form bound to the starch granule [4,5]. The soluble form is the major form in which starch synthetase occurs in waxy rice grains, but the bound form is the major form found in nonwaxy grains [5]. The level of bound synthetase tends to be positively correlated with the amylose content of rice starch granule [5].

Previous efforts to solubilize the synthetase bound to the starch granule, while retaining enzymic activity, by grinding, guanidine or urea treatment and amylase digestion have been unsuccessful [6–9]. The present report describes a successful procedure for solubilizing the enzyme from rice starch granules while preserving enzyme activity in a form suitable for characterization.

RESULTS AND DISCUSSION

Washing the acetone powder of rice grains with phosphate buffer–$MgCl_2$ increased the specific activity of the starch synthetase toward ADP glucose and UDP glucose three-fold due to the removal of adhering protein (Table 1). Previous results indicated that the acetone treatment does not reduce the protein content of rice starch granules [10]. The removal of surface protein was incomplete since exhaustive NaOH extraction of protein from IR8 rice starch granules reduces the protein content to 0·5% [11]. Perez *et al.* [10] showed that intact rice starch from leaf sheaths can be freed of most of its protein using SDS and β-mercaptoethanol while retaining 35% of the starch synthetase activity.

The specific activity, particularly toward UDP glucose as substrate, of the purified starch granules increased after grinding in a Wig-L-Bug amalgamator for 30 sec (Table 1). The starch granules became damaged and amorphous by this treatment as evidenced by the swelling in cold water and the lack of birefringence of 99% of the granules under polarized light. Similar increases in specific activity of potato and waxy corn starch granules to ADP glucose have been reported after grinding [6, 7], but grinding had no effect on sweet corn and wrinkled pea starch granules [6]. By contrast, Frydman and Cardini [6] reported loss of starch synthetase activity with UDP glucose as donor on grinding of potato, sweet corn and wrinkled pea starches.

* Permanent address: Chemistry Department, University of the Philippines at Los Baños.

Table 1. Starch synthetase activity (without added primer) and protein content of *Oryza sativa* starch from developing grains at the midmilky stage

Sample description	Starch synthetase activity (unit*/mg protein) ADP glucose	UDP glucose	Protein content (% of starch)
A. Acetone powder of rice grains	1·2	1·1	6·2
B. Residue of A after washing with buffer and distilled water	3·8	3·5	4·8
C. B after grinding in a Wig-L-Bug amalgamator	6·1	11·2	4·8
D. Supernate after sonication of C in buffer—75% DMSO	6·6	11·3	9·0
E. Active fraction (No. 29) after sucrose gradient centrifugation of D	13·1	>16†	7·5‡

* A unit of enzyme activity is defined as μmol ^{14}C-glucose transferred from ADP glucose or UDP glucose per min at 37°.
† Aged sample.
‡ % Of amylose.

About 50% of the protein in the amorphous starch granules was solubilized with sonication in 75% dimethylsulfoxide (DMSO). Either sonication in buffer alone or stirring in 75% DMSO of the amorphous starch granules gave less than one-fifth the value obtained when sonication and DMSO treatments were combined. The strong action of DMSO–water on starch is well known [12] although the effects of sonication on enzymes are not well understood. Sonication is employed in the pharmaceutical industry in dispersing starch-containing tablets.

After separation from starch by centrifugation the extracted protein had apparent specific activities similar to those of the amorphous granules (Table 1). Actual specific activities were higher since the presence of 19% DMSO in the assay mixture for starch synthetase activity of amorphous granules reduced the activity by 50%. Such DMSO concentration has no effect on the activities of glucoamylase and α- and β-amylases [13]. The TCA precipitate of this extract gave a weight ratio of 11 mg starch/mg protein, indicating that both the starch synthetase and starch were solubilized. Disc electrophoresis resulted in three protein bands which stained for carbohydrate. The major band remained in the stacking gel, whereas the two other bands migrated into the gel. These results are indicative of complexes between starch synthetase and starch.

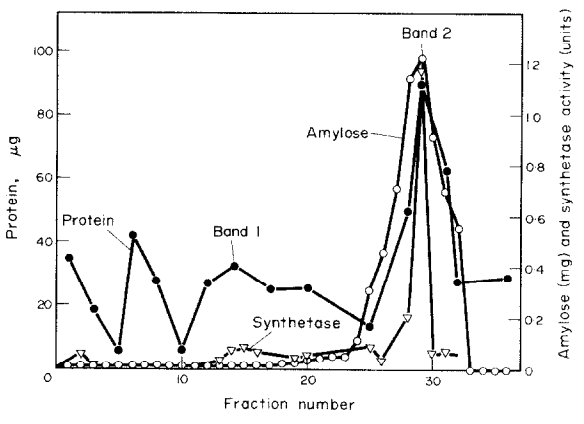

Fig. 1. Distribution of protein, amylose and starch synthetase activity (ADP glucose substrate without added primer) in 0·5-ml fractions obtained from sucrose density gradient centrifugation of solubilized granule-bound rice starch synthetase.

Discontinuous sucrose density gradient centrifugation of the solubilized enzyme gave two opalescent bands (Fig. 1). Band 1 appeared at the boundary of the 45 and 55% sucrose layers and band 2 between the 35 and 45% sucrose layers. In addition, slight precipitates at the bottom and a hazy layer on top were obtained. Band 2 corresponded to peaks in starch synthetase activity (even in the absence of added primer) and protein and amylose content (Fig. 1). Fraction No. 29 of band 2 had higher specific activity than the starting solubilized enzyme (Table 1), suggesting that this active fraction contains starch synthetase complexed with amylose. By contrast, band 1 corresponded to a protein peak but had a low starch synthetase activity even in the presence of added primer. The protein of band 2 had 1·8 times as much lysine as the

protein of band 1 as determined by ion-exchange chromatography. Residual protein in purified rice starch has also been found to correlate positively with the amylose content of the starch [14].

Sodium dodecyl sulfate (SDS) disc gel electrophoresis on 10 μg of band 2 obtained from sucrose gradient centrifugation gave a major protein band corresponding to a MW of about 30000. This band did not stain for carbohydrate using periodic acid-Schiff reagent [15], although the top of the gel stained for carbohydrate. The observed molecular weight corresponds to one of the aggregate forms of soluble starch synthetase from rice leaves which require added primer [16]. Hence, treatment with SDS and β-mercaptoethanol dissociated the enzyme-carbohydrate complexes. Ammonium sulfate fractionation indicated that the enzyme activity was higher in the 0–10% fraction than in the more salt-soluble fractions.

EXPERIMENTAL

Whole developing IR8 rice grains at the midmilky stage were ground at 0° with a mortar and pestle in 0·07 M phosphate buffer (pH 7·5)–0·01 M glutathione – 0·07 MEDTA [17] and sand (1:1:1·5 w/v/w). The brei was passed through six layers of cheesecloth and the filtrate was centrifuged at 10000 g for 20 min at 0°. The ppt. was suspended in 1 liter of cold (1–4°) dist H_2O and centrifuged at 10000 g. The H_2O washing was repeated 4× followed by 5 washings with cold Me_2CO [18]. The final ppt was dried over P_2O_5 at 4°.

The crude starch was further extracted with 0·1 M phosphate buffer (pH 7·2)–0·006 M $MgCl_2$ [19] overnight at 4° and centrifuged at 3000 g. The ppt. was then washed with H_2O. H_2O washing was repeated at least 3× and the starch was freeze-dried. The purified starch was ground in 200-mg lots in a Wig-L-Bug amalgamator for 30 sec and 1 g of the treated starch was sonified in 15 ml 0·05 M HEPES (pH 7·5)–0·001 M DTT–75% DMSO and 4 g of fine glass beads (<3 mm dia.) at 20 kHz with a Sonifier cell disruptor at 80 W setting for 1 hr at 0°. The sonified soln was centrifuged at 34000 g for 30 min at 4°. A portion of the supernatant fluid was precipitated with TCA and the ppt analyzed for total carbohydrates by the anthrone method [20] and for protein [21]. Disc electrophoresis was performed on this extract and the electrophoregram was stained for protein and carbohydrate [15, 22].

The supernatant soln was fractionated by discontinuous sucrose density gradient centrifugation [19]. Two ml of the soln was layered over a 15 ml gradient consisting of 5 ml each of 35, 45 and 55% (w/v) sucrose soln and then centrifuged at 40000 g for 1 hr at 0° in a swing-out rotor. The bottom of each cellulose nitrate tube was punctured with a fine needle and 0·5-ml fractions were collected. These fractions were assayed for starch synthetase activity with ADP glucose or UDP glucose as the glucose donor and without added primer [23] and for amylose content [24]. Protein was assayed after precipitation with TCA followed by dissolving the ppt with NaOH [21]. The MW using SDS gel (7%) electrophoresis [25] and the $(NH_4)_2SO_4$ solubility were estimated on the most active fraction.

REFERENCES

1. De Fekete, M. A. R., Leloir, L. F. and Cardini, C. E. (1960) *Nature* **187,** 918.
2. Badenhuizen, N. P. (1973) *Ann. N.Y. Acad. Sci.* **210,** 11.
3. Preiss, J., Ozbun, J. L., Hawker, J. S., Greenberg, E. and Lammel, C. (1973) *Ann. N.Y. Acad. Sci.* **210,** 265.
4. Murata, T., Sugiyama, T. and Akazawa, T. (1965) *Biochem. Biophys. Res. Commun.* **18,** 371.
5. Baun, L. C., Palmiano, E. P., Perez, C. M. and Juliano, B. O. (1970) *Plant Physiol.* **46,** 429.
6. Frydman, R. B. and Cardini, C. E. (1967) *J. Biol. Chem.* **242,** 312.
7. Chandorkar, K. R. and Badenhuizen, N. P. (1966) *Stärke* **18,** 91.
8. Chandorkar, K. R. and Badenhuizen, N. P. (1967) *Cereal Chem.* **44,** 27.
9. Tanaka, Y., Minagawa, S. and Akazawa, T. (1967) *Stärke* **19,** 206.
10. Perez, C. M., Palmiano, E. P., Baun, L. C. and Juliano, B. O. (1971) *Plant Physiol.* **47,** 404.
11. Briones, V. P., Magbanua, L. G. and Juliano, B. O. (1968) *Cereal Chem.* **45,** 351.
12. Leach, H. W. and Schoch, T. J. (1962) *Cereal Chem.* **39,** 318.
13. Lineback, D. R. and Sayeed, A. L. (1971) *Carbohydrate Res.* **17,** 453.
14. Kongseree, N. and Juliano, B. O. (1972) *J. Agr. Food Chem.* **20,** 714.
15. Zacharius, R. M. and Zell, T. E. (1969) *Anal. Biochem.* **30,** 148.
16. Antonio, A. A., Del Rosario, E. J. and Juliano, B. O. (1973) *Phytochemistry* **12,** 1929.
17. Murata, T. and Akazawa, T. (1968) *Arch. Biochem. Biophys.* **114,** 76.
18. Leloir, L. F., De Fekete, M. A. R. and Cardini, C. E. (1961) *J. Biol. Chem.* **236,** 636.
19. Mitsuda, H., Yasumoto, K., Murakami, K., Kusano, T. and Kishida, H. (1967) *Agr. Biol. Chem.* **31,** 293.
20. McCready, R. M., Guggolz, J., Silveira, V. and Owens, H. S. (1950) *Anal. Chem.* **22,** 1156.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). *J. Biol. Chem.* **193,** 265.
22. Davies, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121,** 404.
23. Sanwal, G. G., Greenberg, E., Hardie, J., Calderon, E. C. and Preiss, J. (1968) *Plant Physiol.* **43,** 417.
24. Juliano, B. O. (1971) *Cereal Sci. Today* **16,** 334.
25. Segrest, J. P. and Jackson, R. L. (1972) in *Methods Enzymol.* (V. Ginsburg, Ed.), Vol. 28B p. 54.