Plant Physiol. (1981) 68, 1314–1318
0032-0889/81/68/1314/05/$00.50/0

Enzymic Mechanism of Starch Breakdown in Germinating Rice Seeds

10. *IN VIVO* AND *IN VITRO* SYNTHESIS OF α-AMYLASE IN RICE SEED SCUTELLUM

Received for publication March 25, 1981 and in revised form June 24, 1981

SUSUMU MIYATA, KAZUO OKAMOTO, AKIRA WATANABE, AND TAKASHI AKAZAWA
Research Institute for Biochemical Regulation, School of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

ABSTRACT

Scutellar tissues were dissected from germinating rice seeds and the incorporation of [^{35}S]methionine into the α-amylase molecule was examined by *in vivo* and *in vitro* assay systems. Immunoprecipitation with monospecific anti-α-amylase immunoglobulin G raised against the purified enzyme preparation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography were used to identify α-amylase and its possible precursor molecule. Using freshly prepared scutellar tissues, it was demonstrated that α-amylase is synthesized *de novo* in the scutellar epithelium and secreted into endosperm. The synthesis of α-amylase directed by the polyadenylic acid-containing ribonucleic acid isolated from the scutellar tissues was also established using the translation system of either wheat germ extract or reticulocyte lysate. The immunoprecipitable product obtained in the *in vitro* translation system was smaller in molecular weight than that synthesized *in vivo* on the basis of mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Results are discussed in relation to the processing of the nascent polypeptide precursor of the enzyme molecule and the introduction of the oligosaccharide chain to the cleaved polypeptide to make up the mature form of α-amylase.

It is a long-held belief that α-amylase is synthesized *de novo* in the aleurone layers of germinating seeds of starch-bearing cereals, such as barley, when triggered by gibberellic acid (2, 5, 7, 10). In contrast, it has recently been found, using the histochemical technique, that the dominant site of the formation of amylases and other hydrolases is the scutellar epithelium (18, 20). Gibbons (8) obtained a similar result with barley α-amylase using the immunohistochemical method. Electron microscopic observations of their sections of the scutellar epithelial cell of the rice seed has clearly shown that the developmental formation of both RER and Golgi apparatus occurs at the onset of germination, which suggests that there is an enhancement of the synthesis of secretory proteins in the epithelial cell and the subsequent secretion of the newly synthesized protein molecules outside of the cell (manuscript in preparation). To enable more explicit demonstration of α-amylase synthesis *in situ*, it is desirable to investigate the nature of the enzyme synthesis at the molecular level. Previously, several lines of experimental results have shown that the poly(A)[1]-containing RNA isolated from the aleurone layers of barley (10, 15) and wheat (21) can direct the synthesis of α-amylase molecules in cell-free translation systems. In both cases, possible precursors of α-amylase molecules are produced. As a first step in a molecular approach to the mechanism of the synthesis of α-amylase in scutellum, we have initiated a study of the *in vivo* synthesis of α-amylase, together with the *in vitro* synthesis of the enzyme using poly(A)-containing RNA isolated from the scutellar tissues.

[1] Abbreviations: poly(A), polyadenylic acid; IgG, immunoglobulin G.

MATERIALS AND METHODS

Purification of α-amylase. α-Amylase was purified according to the procedures reported previously (17) with slight modification. Rice seeds (*Oryza sativa* L. var Kimmaze) were germinated in a dark chamber for 7 days at 30°C. In our previous study, only endosperm tissues were used for the enzyme purification, but in the present work whole seeds were used. Methods for $(NH_4)_2SO_4$ precipitation, DEAE-Sephadex A-50, and Sephadex G-100 column chromatography were the same as before, and in the final step heat treatment, as well as affinity chromatography, replaced the previous isoelectric focusing column chromatography. After Sephadex G-100 column chromatography, the eluates were heated at 70°C for 15 min, and the treated samples were centrifuged at 10,000*g* for 15 min. The supernatant fraction collected was concentrated and dialyzed against a 50 mM Na-acetate buffer (pH 5.3) containing 3 mM $CaCl_2$; the dialysate was finally applied to a column of Cyclohepta-amylose-substituted epoxy-activated Sepharose 6B (24), which was equilibrated with the above buffer. Proteins nonspecifically bound to the column were removed by washing with the above buffer containing 0.3 M NaCl, and α-amylase was eluted with a solution of Cyclohepta-amylose (8 mg/ml) in the above dialyzing buffer.

Preparation of Anti-α-Amylase IgG. The rabbit anti-α-amylase IgG was obtained according to the procedure reported previously (19) and its immunochemical specificity was tested by means of double immunodiffusion on an agar plate as well as by immunoelectrophoresis.

Preparation of Scutellar Tissues. Rice seeds sterilized by soaking in a 1% NaOCl solution for 20 min were rinsed several times in sterile water and germinated in an autoclaved Petri dish in a dark chamber at 30°C for 4 days. Scutellar tissues, mainly consisting of scutellum and scutellar epithelium, but free from endosperm or aleurone layers, were dissected from the seedlings under sterile conditions.

Isolation of Poly(A)-Containing RNA. Twenty g scutellar tissues were frozen in liquid N_2 and ground to a fine powder. To the powder was added 50 mM Tris-HCl buffer (pH 9.0) containing 1% SDS and RNA was extracted into the aqueous phase by vigorous stirring with an equal volume of 90% phenol and subsequent centrifugation. The aqueous phase was treated two more times with 90% phenol and the RNA was precipitated by adding ethanol to the aqueous phase at a final concentration of 70%. The precip-

itate collected was suspended in distilled H_2O and the RNA fraction was reprecipitated by the addition of LiCl to a final concentration of 2 M in an ice bath. This procedure was effective in removing a large amount of the contaminating carbohydrate component. After washing the fraction in 70% ethanol, the resulting precipitate was dissolved in 20 mM Tric-HCl buffer (pH 7.5) containing 0.05% SDS and 0.5 M NaCl. The solution was applied to a column of oligo(dT)-cellulose (1) and the poly(A)-containing RNA was eluted with 20 mM Tris-HCl buffer (pH 7.5) containing 0.05% SDS. The poly(A)-containing RNA, precipitated by 1 M LiCl-70% ethanol solution, was dissolved in distilled H_2O.

***In Vivo* Protein Synthesis.** Five pieces of scutella, freshly prepared as above, were incubated in 0.3 ml of a buffered medium containing 10 mM Tric-HCl buffer (pH 7.0), 30 mM $CaCl_2$, 10 units/ml of Penicillin G, and 10 to 50 μCi of [^{35}S]Met. After incubation for the indicated time at 30°C, the whole tissues were removed, thoroughly rinsed, homogenized in a small mortar using 2 ml of the buffered medium, and finally subjected to centrifugation at 15,000*g* for 20 min. The incubation medium was also collected and centrifuged at 10,000*g* for 5 min.

In order to measure the incorporation of [^{35}S]Met into the trichloroacetic acid-insoluble protein fraction derived from either scutellar extracts or incubation medium, trichloroacetic acid solution was added to each fraction so as to make its final concentration 10%. The precipitate formed was sedimented by centrifugation and washed several times in 10% trichloroacetic acid and twice in acetone. The final precipitate was resuspended in SDS-sample buffer (11), heated and analyzed by means of SDS-polyacrylamide gel electrophoresis. For the radioactivity measurements in the trichloroacetic acid-insoluble fraction, an aliquot of the supernatant obtained from either scutellar extracts or incubation medium was placed on a filter paper disc, after thorough washing in 5% trichloroacetic acid solution, ethanol, and ether and subjected to the liquid scintillation counting.

***In Vitro* Protein Synthesis.** The poly(A)-containing RNA fraction isolated from the scutellar tissues was subjected to the *in vitro* translation system using either reticulocyte lysate or wheat germ extracts. The reaction mixture contained 50 μl of reticulocyte lysate cell-free system (Radiochemical Centre), 160 μCi [^{35}S]Met and 5 μg RNA in a total volume of 68 μl. The translation system using the wheat germ extracts was essentially the same as described by Roberts and Paterson (23); the reaction mixture containing 80 μCi [^{35}S]Met and 2.5 μg RNA in a total volume of 32 μl. In both cases, after incubation at 30°C for 1 h, the reaction was stopped by adding 0.2 μg pancreatic RNase A to the incubation mixture.

Immunoprecipitation of α-Amylase Synthesized in the *in Vivo* or *in Vitro* Assay Systems. Aliquots of the supernatant fraction from either scutellar extracts or the incubation medium obtained as above, as well as the reaction mixture of wheat germ or reticulocyte lysate cell-free systems, were made to 2% (w/v) with SDS, heated at 80°C for 3 min and then made to about 3% (v/v) with Triton X-100. After addition of 20 mM Tris-HCl buffer (pH 7.5) containing 0.1% Triton X-100 and 0.14 M NaCl to the mixture, 50 μg each of anti-α-amylase IgG or nonimmune control IgG was added and incubation was continued for 1 h at 30°C. Ten μl (packed volume) of protein A-Sepharose gel (25) was added to the mixture and allowed to incubate for an additional 1 h at 30°C with occasional stirring. Finally, the mixture was applied to a small column to collect the protein A-Sepharose gel with the bound immunoprecipitates. After thoroughly washing the column with the above Tris buffer, 1 M NaCl, and distilled H_2O in a stepwise manner, the immunoprecipitates were eluted with a SDS sample buffer. Eluates were then used for SDS-polyacrylamide gel electrophoresis or radioactivity measurements.

SDS-Polyacrylamide Slab Gel Electrophoresis. The purified preparations of α-amylase and [^{35}S]Met-labeled protein fractions, obtained from either the *in vivo* or *in vitro* systems, were analyzed by SDS-polyacrylamide slab gel electrophoresis, basically following the procedure of Laemmli (11). The acrylamide concentrations in the separating and stacking gel were 10 and 5%, respectively. For the mol wt estimation, standard protein molecules were co-electrophoresed. After electrophoresis, the gel was stained with fuchsin-sulfite reagent according to Zacharius *et al.* (26) or with Coomassie brilliant blue. Alternately, the gels were prepared for fluorography using Enhance as recommended by the manufacturer (12) and was exposed to an X-ray film (Fuji Rx) for 5 days.

Protein and Carbohydrate Analysis. To remove contaminating Cyclohepta-amylose, the purified preparation of α-amylase was precipitated and washed several times with 10% trichloroacetic acid solution, twice with acetone, dried, dissolved in 1 N NaOH, and finally subjected to protein and carbohydrate analysis. Protein content was analysed according to the method of Lowry *et al.* (13) using BSA as a standard. The phenol-sulfuric acid method of Dubois *et al.* (6) was employed for the carbohydrate analysis using glucose as a standard.

Chemicals. The following commercial products were used: [^{35}S]Met and rabbit reticulocyte lysate cell-free translation system from Radiochemical Centre. Amersham; protein A-Sepharose and pancreatic ribonuclease A from Sigma; Enhance (autoradiography enhancer) from New England Nuclear; mol wt marker proteins from the Pharmacia; and penicillin G from Meiji Seika Co. Ltd., Japan.

RESULTS AND DISCUSSION

To measure the incorporation of [^{35}S]Met into the newly synthesized α-amylase molecule, it is essential to precipitate the enzyme protein by the specific antiserum raised against the enzyme molecule. For this purpose, α-amylase was purified from germinating rice seeds in 7.2% yield. In the previous paper (17), yield was only 2% because of enzyme inactivation due to the use of the isoelectric focusing column chromatographic technique. The purified preparation of α-amylase was utilized to immunize rabbits. The prepared anti-α-amylase IgG was monospecific to α-amylase as shown from the result of Ouchterlony double immunodiffusion as well as the immunoelectrophoresis (Fig. 1, A

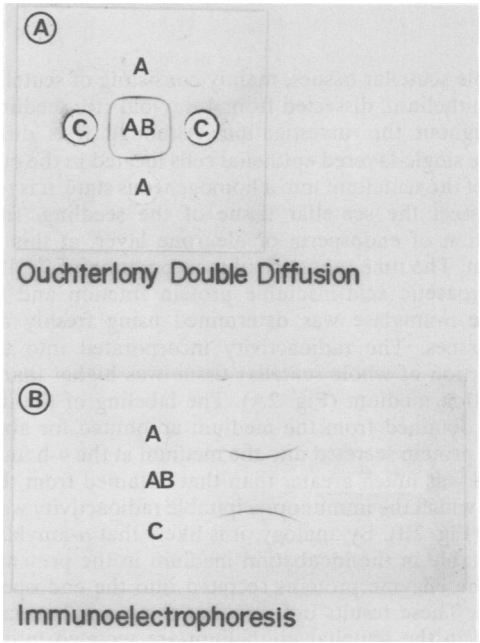


FIG. 1. Ouchterlony double immunodiffusion (A) and immunoelectrophoresis (B). Experimental procedures employed were essentially the same as reported previously (19). (A), purified α-amylase; (C), crude extracts prepared from 7-day-old germinated rice seeds; (AB), anti-α-amylase IgG.

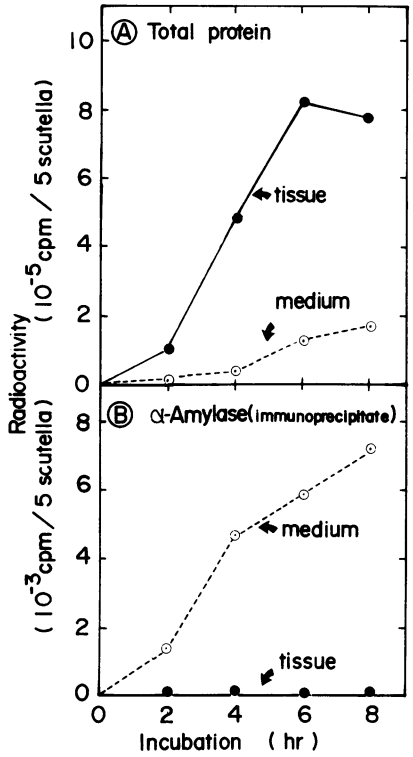


FIG. 2. Incorporation of [^{35}S]Met into (A) trichloroacetic acid-insoluble total protein fraction and (B) α-amylase molecules. (●——●), scutellar tissue extracts; (⊙-----⊙), secretion into medium. After incubation in the medium containing 10 μCi [^{35}S]Met at 30°C for the indicated time, the tissues and the medium were subjected to the experimental procedures described in the text. The radioactivity incorporated into the trichloroacetic acid-insoluble total protein fraction and α-amylase molecules was then measured. For the calculation of [^{35}S]Met incorporated into the α-amylase molecules, the radioactivity of labeled immunoprecipitates obtained using the nonimmune control IgG was subtracted from that obtained using the anti α-amylase IgG.

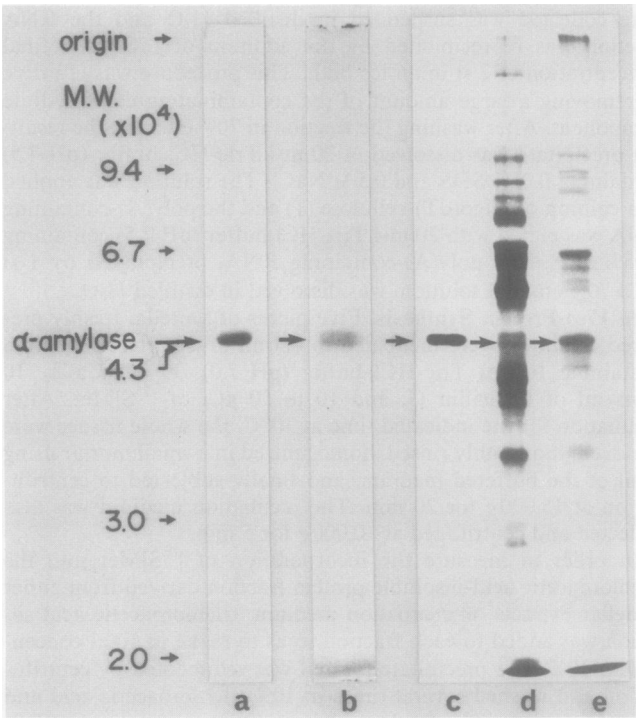


FIG. 3. SDS-slab gel electrophoresis and fluorogram of [^{35}S]Met-labeled immunoprecipitable α-amylase, total proteins in scutellar extracts and those secreted into the incubation medium. (a), Purified α-amylase stained with Coomassie brilliant blue; (b), purified α-amylase stained with fuchsin-sulfite reagent; (c), immunoprecipitate of α-amylase secreted into the incubation medium; (d), trichloroacetic acid-insoluble total proteins in scutellar extracts; and (e), trichloroacetic acid-insoluble total proteins in incubation medium. For the analysis of the labeled products, five pieces of scutella were incubated in a medium containing 50 μCi [^{35}S]Met at 30°C for 4 h, and the whole tissues and incubation medium were subjected to the experimental procedures including immunoprecipitation as described in the text. The band of α-amylase immunoprecipitate in the tissue extract was not detectable in these conditions. The numbers refer to the mol wt of marker proteins coelectrophoresed. Arrows indicate the position of α-amylase.

and B).

The whole scutellar tissues, mainly consisting of scutellum and scutellar epithelium, dissected from 4-day-old rice seedlings were used throughout this investigation. Although it is difficult to separate the single-layered epithelial cells located in the outermost boundary of the scutellum into a homogeneous state, it is relatively easy to dissect the scutellar tissue of the seedling, free from contamination of endosperm or aleurone layer, at this stage of germination. The time sequence of incorporation of [^{35}S]Met into the trichloroacetic acid-insoluble protein fraction and the IgG precipitable α-amylase was determined using freshly dissected scutellar tissues. The radioactivity incorporated into the total protein fraction of whole scutellar tissue was higher than that in the incubation medium (Fig. 2A). The labeling of the immunoprecipitate obtained from the medium accounted for about 15% of the total protein secreted into the medium at the 4-h incubation period, and was much greater than that obtained from the tissue extracts, in which the immunoprecipitable radioactivity was barely detectable (Fig. 2B). By analogy, it is likely that α-amylase molecules detectable in the incubation medium in the present system represent the enzyme proteins secreted into the endosperm of a whole seed. These results indicate that the α-amylase molecules synthesized in the scutellar epithelium are secreted into the endosperm as a secretory form. The fluorogram presented in Figure 3 shows that the electrophoretic mobility of the immunoprecipitable [^{35}S]Met-labeled product in the incubation medium (Fig. 3c) is identical with that of the purified α-amylase preparation (mol wt, 44,000) (Fig. 3a), which indicates that α-amylase is synthesized *de novo* in the scutellar tissue. There are a number of labeled protein components detectable both in the tissue extracts (Fig. 3d) and in the incubation medium (Fig. 3e). The difference between the two patterns of labeled products indicates that the proteins present in the medium are not merely ascribed to leakage of contents from the physically damaged scutellum. α-Amylase appears to be the major protein molecule secreted into the medium although it is not as prominent as that of the enzyme secretion into the medium of the GA_3-treated aleurone layers of barley (16). Since it is presumed that there exists an endogenous GA_3 in the scutellar tissues, we have not examined the effect of GA_3 on the incorporation of [^{35}S]Met into α-amylase. However, exogenously added GA_3 showed little effect on the α-amylase activity both in the tissue extracts and in the incubation medium (data not shown).

If α-amylase synthesized in the scutellar epithelial cells is secreted into the endosperm following a mechanism similar to that established in other secretory proteins (3), the nascent polypeptide chain of α-amylase produced in the *in vitro* translation system directed by the poly(A)-containing RNA isolated from the scutellar tissues is expected to have an extrapeptide segment, which is eventually cleaved at a time when the polypeptide molecule enters the cisternal space of the ER. In a preliminary experiment using the wheat germ system, we observed that the electrophoretic

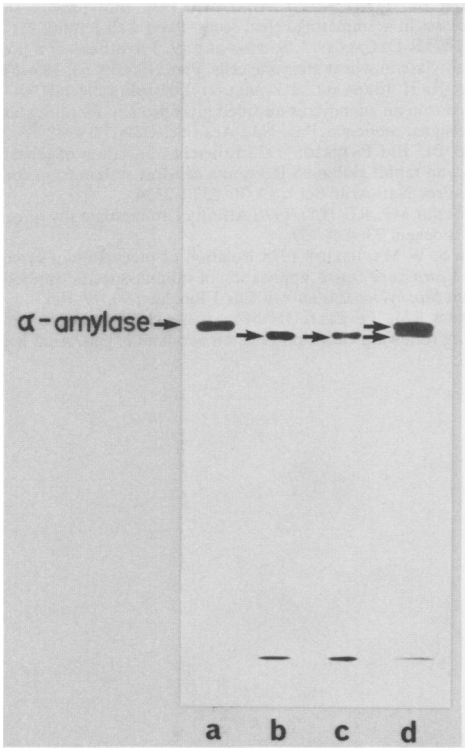
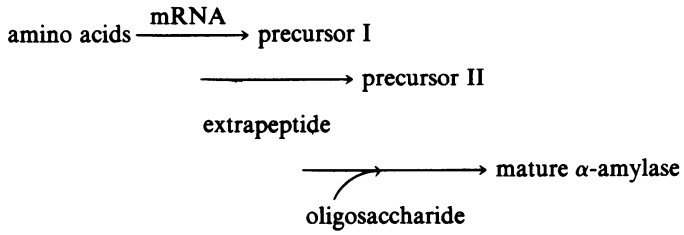


FIG. 4. Fluorogram of SDS-polyacrylamide gel electrophoresis of immunoprecipitates of *in vivo* and *in vitro* labeled α-amylase molecules. (a), Immunoprecipitate of *in vivo*-labeled α-amylase (same as Fig. 3c); (b), immunoprecipitate of *in vitro*-labeled α-amylase (wheat germ translation system); (c), immunoprecipitate of *in vitro*-labeled α-amylase (reticulocyte lysate translation system); and (d), mixture of (a) and (b). A single band was observed when mixture of (b) and (c) was applied (data not shown). Upper arrows indicate the position of *in vivo*-synthesized α-amylase, a mature enzyme of secretory form. Lower arrows indicate the position of *in vitro*-synthesized α-amylase, a possible precursor. Other experimental details are described in the text.

mobility of the immunoprecipitable products is close to that of authentic α-amylase. Because there is a possibility that the wheat germ extracts contain a trace proteolytic activity for cleaving the extrapeptide from the nascent polypeptide, reticulocyte lysate system was also tested. Results of Figure 4 show that the immunochemically identifiable α-amylase molecules obtained in the two different *in vitro* translation systems are identical in size but 1,100 daltons smaller in the mol wt than that synthesized *in vivo*.

Results obtained in the present investigation contradict those of the previous reports dealing with the *in vitro* synthesis of α-amylase using aleurone layers of wheat (21) and barley (15), rat parotid (9), and dog pancreas (14). In all these latter cases, the mol wt of α-amylase synthesized in the cell-free system was found to be larger than that of the enzyme synthesized *in vivo*. We cannot conclude that rice α-amylase lacks a signal sequence based on these previous data alone. α-Amylase is generally a glycoprotein and, in fact, the purified rice seed α-amylase preparation was stainable on the polyacrylamide gel by the fuchsin-sulfite reagent (Fig. 3b). The carbohydrate content in the enzyme molecule was determined to be 4.8% (glucose as a standard). This value is roughly equivalent to 2,100 daltons in the mol wt. Comparing the molecular weights of *in vivo* and *in vitro* synthesized α-amylase is complicated by the fact that the mature enzyme molecule contains the carbohydrate moiety but would lack an extrapeptide sequence, whereas *in vitro* synthesized α-amylase lacks carbohydrate but would contain extrapeptide. Collectively, our results suggest a likely mechanism for α-amylase biosynthesis in rice seed that involves two precursor forms, I and II. It can be schematized as follows:



It is inferred that after the co-translational cleavage of the extrapeptide from the nascent polypeptide (precursor I), an oligosaccharide with a mol wt of approximately 2,000 is introduced into the second precursor (II) to produce the mature enzyme, the final secretory form of α-amylase. In support of such a scheme, it has been established that adenovirus-encoded glycoprotein (19,000 mol wt) contains 25% carbohydrates by weight (22). The *in vitro* synthesized product directed by the viral mRNA, which corresponds to precursor I, was 3,000 daltons smaller than the glycosylated protein normally synthesized *in vivo*, but 2,500 daltons larger than the nonglycosylated protein, corresponding to precursor II, which was synthesized *in vivo* in the presence of Tunicamycin, an inhibitor of protein glycosylation.

It has recently been found by histochemical (18, 20) and/or immunohistochemical means (8) that the formation of amylase occurs around the single layered scutellar epithelial cells in starch-bearing cereal seeds other than rice, such as in barley, wheat, rye, and maize. It is, therefore, likely that the mechanism of the *de novo* synthesis of α-amylase in the scutellar epithelium, as demonstrated here, will not be restricted to rice but rather applicable to other cereals as well. The biochemical mechanism of glycosylation of rice seed α-amylase molecule is now being investigated in this laboratory.

Acknowledgments—The authors wish to record their thanks to Doug Graham for his kind help during the preparation of manuscript and to Mikio Nishimura for invaluable advice throughout this investigation.

LITERATURE CITED

1. AVIV H, P LEDER 1972 Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA 69: 1408–1412
2. BEWLEY JD, M BLACK 1978 Control processes in the mobilization of stored reserves. *In* Physiology and Biochemistry of Seeds, Vol 1, Development Germination and Growth. Springer-Verlag, Berlin/Heidelberg, New York, pp 245–279
3. BLOBEL G, B DOBBERSTEIN 1975 Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J Cell Biol 67: 835–851
4. BRAWERMAN G 1974 The isolation of messenger RNA from mammalian cell. Methods Enzymol 30: 605–612
5. CHRISPEELS MJ, JE VARNER 1967 Gibberellic acid-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol 42: 398–406
6. DUBOIS M, KA GILLES, JK HAMILTON, PA REBERS, F SMITH 1956 Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356
7. FILNER F, JE VARNER 1967 A test for *de novo* synthesis of enzymes: density labeling with H_2O^{18} of barley α-amylase induced by gibberellic acid. Proc Natl Acad Sci USA 58: 1520–1526
8. GIBBONS GC 1979 On the localization and transport of α-amylase during germination and early seedling growth of *Hordeum vulgare*. Carlsberg Res Commun 44: 353–366
9. GORECKI M, EP ZEELON 1979 Cell-free synthesis of rat parotid preamylase. J Biol Chem 254: 525–529
10. HIGGINS TJV, JA ZWAR, JV JACOBSEN 1976 Gibberellic acid enhances the level of translatable mRNA for α-amylase in barley aleurone layers. Nature 260: 166–169
11. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680–685
12. LASKEY RA, AD MILLS 1975 Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. Eur J Biochem 56: 335–341

13. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with Folin phenol reagent. J Biol Chem 193: 265–276
14. MACDONALD RJ, AE PRZYBYLA, WJ RUTTER 1977 Isolation and *in vitro* translation of the messenger RNA coding for pancreatic amylase. J Biol Chem 252: 5522–5528
15. MOZER TJ 1980 Partial purification and characterization of the mRNA for α-amylase from barley aleurone layers. Plant Physiol 65: 834–837
16. MOZER TJ 1980 Control of protein synthesis in barley aleurone layers by the plant hormones gibberellic acid and abscisic acid. Cell 20: 479–485
17. OKAMOTO K, T AKAZAWA 1978 Purification of α- and β-amylase from endosperm tissues of germinating rice seeds. Agric Biol Chem 42: 1379–1384
18. OKAMOTO K, T AKAZAWA 1979 Enzymic mechanism of starch breakdown in germinating rice seeds. 7. Amylase formation in the epithelium. Plant Physiol 63: 336–340
19. OKAMOTO K, T AKAZAWA 1979 Enzymic mechanism of starch breakdown in germinating rice seeds. 8. Immunohistochemical localization of β-amylase. Plant Physiol 64: 337–340
20. OKAMOTO K, H KITANO, T AKAZAWA 1980 Biosynthesis and excretion of hydrolases in germinating cereal seeds. Plant Cell Physiol 21: 201–204
21. OKITA TW, R DECALEYS, L RAPPAPORT 1979 Synthesis of a possible precursor of α-amylase in wheat aleurone cells. Plant Physiol 63: 195–200
22. PERSSON H, H JÖRNVALL, J ZABIELSKI 1980 Multiple mRNA species for the precursor to an adenovirus-encoded glycoprotein: Identification and structure of the signal sequence. Proc Natl Acad Sci USA 77: 6349–6353
23. ROBERTS BE, BM PATERSON 1973 Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. Proc Natl Acad Sci USA 70: 2330–2334
24. SILVANOVICH MP, RD HILL 1976 Affinity chromatography of cereal α-amylase. Anal Biochem 73: 430–433
25. WERNER S, W MACHLEIDT 1978 Isolation of precursors of cytochrome oxidase from *Neurospora crassa*: application of subunit-specific antibodies and protein A from *Staphylococcus aureus*. Eur J Biochem 90: 99–105
26. ZACHARIUS RM, TE ZELL, JH MORRISON, JJ WOODLOCK 1969 Glycoprotein staining following electrophoresis on acrylamide gels. Anal Biochem 30: 148–152