

Biosynthesis of Rice Seed α -Amylase: Proteolytic Processing and Glycosylation of Precursor Polypeptides by Microsomes

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ABSTRACT Microsomes prepared from the rice seed scutellum were incubated in wheat germ extracts (S-100 fraction) to direct the synthesis of α -amylase, a secretory protein subject to proteolytic processing (cleavage of the *N*-terminal signal sequence) as well as glycosylation during its biosynthesis. The characterization and identification of the immunoprecipitable products synthesized were performed by SDS gel electrophoresis and subsequent fluorography. The molecular weight of the α -amylase synthesized by the microsomes was found to be identical with that of the mature secretory form of the enzyme on the basis of electrophoretic mobilities. A significant portion of the enzyme molecules synthesized was shown to be segregated into the microsomal vesicles and protected against digestion by endo- β -*N*-acetylglucosaminidase, indicating that both proteolytic processing and glycosylation of the precursor polypeptide chains take place in the microsomes. The modification of the polypeptide chains was further examined by disrupting the microsomal membranes with Triton X-100. Detergent treatment of the microsomes prior to protein synthesis caused an inhibition of both proteolytic processing and glycosylation of the polypeptide chains, leading to the synthesis of the unprocessed nascent (precursor I), processed but nonglycosylated nascent (precursor II) forms, in addition to the mature form of α -amylase. Furthermore, the results of time-sequence analysis of the inhibitory effect of Triton X-100 on the modification of the polypeptide chains have led us to conclude that both proteolytic processing and subsequent glycosylation occur in the microsomes during the biosynthesis of α -amylase.

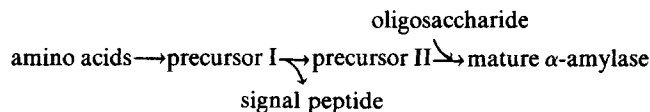
We have demonstrated that, in the early germinating stages of rice seeds, α -amylase molecules are synthesized in the scutellar epithelium and secreted into the endosperm tissues (1, 18). The α -amylase is a typical secretory protein, and it is known to be subject to glycosylation during its biosynthesis (1, 15). Ultrastructural studies have shown that, at the onset of germination, rough endoplasmic reticulum (RER) and Golgi bodies are prominent and well developed in the scutellar epithelium cells (19), indicating that these organelles are possibly engaged in the synthesis, intracellular transport, and secretion of the enzyme molecules.

In recent years, the mechanism of biosynthesis of both secretory and membranous glycoproteins has been thoroughly studied in mammalian system. It is now well accepted that the NH_2 -terminal signal peptide is co-translationally cleaved after it enters the cisternal space of endoplasmic reticulum (ER) (4–

7). The proteolytic processing and glycosylation of the elongating polypeptide chain have been reported to be directed by the microsomal membranes in various experimental systems (21, 23). Although these two reactions are known to be closely coupled with chain elongation and the vectorial segregation of polypeptide chains (3, 11, 13, 20, 21, 23), the nature of their precise relationship has not been well characterized with any of the secretory or membrane glycoproteins.

Previously, we have reported that the translation of poly(A)-mRNA isolated from rice seed scutellum results in the formation of the unprocessed nascent form of α -amylase (17). Subsequently, the formation of the processed nascent form of the enzyme was demonstrated by inhibiting the glycosylation of the precursor with tunicamycin (TM), a specific inhibitor of protein glycosylation via the dolichol pathway (15). The unprocessed nascent and processed nascent forms of α -amylase,

detectable on SDS gel electrophoresis, have been designated as precursor I and precursor II, respectively. We have therefore suggested the following scheme for the biosynthesis of α -amylase (1, 15):



More recently, we have shown that upon completion of polypeptide chain elongation of precursor molecules already associated with the polysomes, (a) unprocessed nascent, (b) processed nascent, and (c) processed and glycosylated polypeptide chains of α -amylase are present, and that the glycosylation is preceded by proteolytic processing during biosynthesis (16). In the present report we present data during that detergent-sensitive elements, probably membranous components in the microsomes, can direct the proteolytic processing and subsequent glycosylation reaction of precursor molecules of rice seed α -amylase.

MATERIALS AND METHODS

Isolation of Microsomes: 2 g of scutellar tissues dissected from 4-d-old germinating rice seeds (*Oryza sativa* var. Kimmaze) were incubated for 1 h at 30°C in 10 mM Tris-HCl buffer (pH 7.0) containing 30 mM CaCl₂ (18). To examine the effect of TM, we bathed scutella for 3 h at 30°C in the above buffer solution containing TM (30 μ g/ml). At the end of incubation, tissues were thoroughly rinsed with distilled water and homogenized in a small mortar using 6 ml of 0.2 M Tris-HCl buffer (pH 8.0) containing 60 mM KCl, 50 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 M sucrose and yeast RNA (3 mg/ml). All the operations were performed at 0°C. The homogenates were then passed through four layers of cheese-cloth and centrifuged at 10,000 g for 10 min, and the supernatant fraction was layered over a discontinuous gradient solution consisting of 5 ml each of 27% and 38% (wt/wt) sucrose solutions also containing 0.2 M Tris-HCl buffer (pH 8.0), 60 mM KCl and 50 mM MgCl₂, and centrifuged at 100,000 g in a Beckman SW 25.3 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). After centrifugation for 2 h, the fraction banding at the interface of the two sucrose solutions was removed with a syringe, diluted with 15 ml of 20 mM HEPES-KOH buffer (pH 7.5) containing 120 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, and 0.25 M sucrose, and centrifuged again at 100,000 g for 30 min in a Beckman SW 25.3 rotor. The microsomal pellets obtained were suspended in the above buffer and stored at -80°C until used.

Protein Synthesis by Microsomes: Protein synthesis directed by microsomes was performed using the translation system of wheat germ extracts. The wheat germ S-100 (100,000 g supernatant) fraction was prepared from the S-30 wheat germ fraction according to the procedure described by Sai-ming et al. (22). The reaction mixture (50 μ l) contained 10 μ l of wheat germ S-100 fraction, 20 mM HEPES-KOH buffer (pH 7.5), 2 mM DTT, 1 mM ATP, 20 μ M GTP, 8 mM creatine phosphokinase (40 μ g/mM MgCl₂), 100 mM KCl, 2.5 mM MgCl₂, 5 μ Ci of ³⁵S-Met, 19 amino acids except Met and 1.2 A₂₆₀ units of microsomes. The mixture was incubated at 20°C for the time periods indicated. The measurement of ³⁵S-Met incorporation into the TCA-insoluble total protein and immunoprecipitates, SDS gel electrophoresis and subsequent fluorography were carried out essentially following the procedures as described previously (17).

Digestion of Oligosaccharide Moiety of α -Amylase with Endo- β -H: After incubation for 40 min, an equal volume of 0.2 M Na-citrate buffer (pH 6.5) containing 10 mM MgCl₂ and 10% sucrose was added to the reaction mixture. One-half of the resulting mixture was removed and made 1% of Triton X-100. 20 mU of endo- β -N-acetylglucosaminidase (Endo- β -H) (Seikagaku Kogyo Co., Tokyo) was added to the detergent-treated and untreated samples. The glycosidase digestion was carried out for 30 min at 35°C, and the reaction terminated by cooling the tubes in an ice-bath. The digested samples were then subjected to immunoprecipitation, SDS gel electrophoresis, and fluorography.

Detergent Treatment during Protein Synthesis: To disrupt the microsomal membranes, Triton X-100 (final concentration of 1%) was added to the reaction mixture of the protein-synthesizing system before the start of incubation or during the incubation at indicated time periods. As shown in the figure legends, the reaction was tested in the presence or absence of aurintricarboxylic acid (ATA).

RESULTS

α -Amylase Synthesis by Microsomes

Microsomes prepared from rice seed scutellum were incubated with wheat germ S-100 fractions having low initiation activity in protein synthesis. The time-course of ³⁵S-Met incorporation into the immunoprecipitable α -amylase is shown in Fig. 1 (A and B). As can be seen, microsomes directed the synthesis of α -amylase up to ~30 min, indicating that ³⁵S-incorporation into the unlabeled polypeptide chains already present in the microsomal fractions is completed within this time period.

As shown in Fig. 2, α -amylase synthesized in the microsomes was identical to the mature secretory form on the basis of their electrophoretic mobilities, suggesting that the microsomes catalyze both proteolytic processing and glycosylation of the elongating polypeptide chains of the enzyme (Fig. 2, lanes a, b, and c). Numerous glycoproteins of either secretory or membranous nature have been shown to be glycosylated in the microsomes via the dolichol pathway, which is specifically inhibited by TM (23). In the present work, when the microsomes were prepared from the TM-treated scutella, the electrophoretic mobility of the α -amylase synthesized in vitro was identical with that of precursor II (Fig. 2, lane d). It is likely

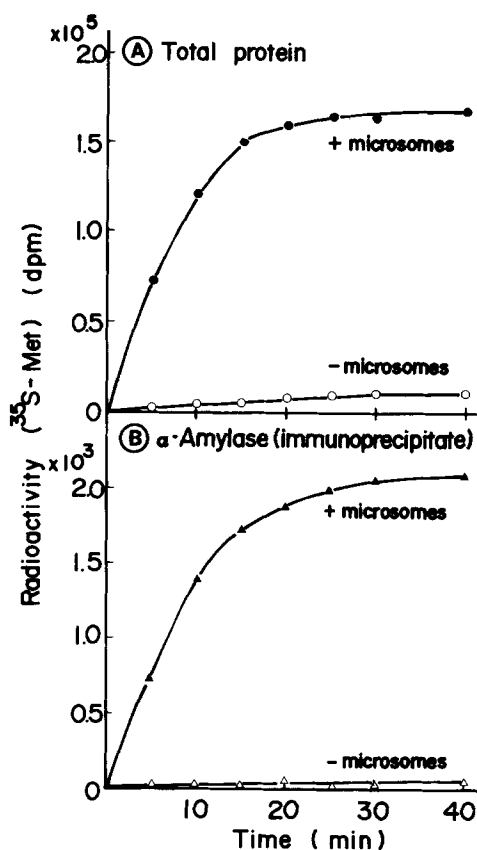


FIGURE 1 Synthesis of protein (A) and α -amylase (B) on microsomes. Protein synthesis was determined in the presence (●, ▲) or absence (○, △) of microsomes according to the procedure described in text. At indicated time intervals, aliquots of the reaction mixture were removed and subjected to radioactivity measurements of total protein (A) or immunoprecipitable α -amylase (B). ³⁵S-Met incorporated into α -amylase was calculated by subtracting the radioactivities of the labeled immunoprecipitates using a nonimmune control IgG from those obtained using the anti- α -amylase IgG.

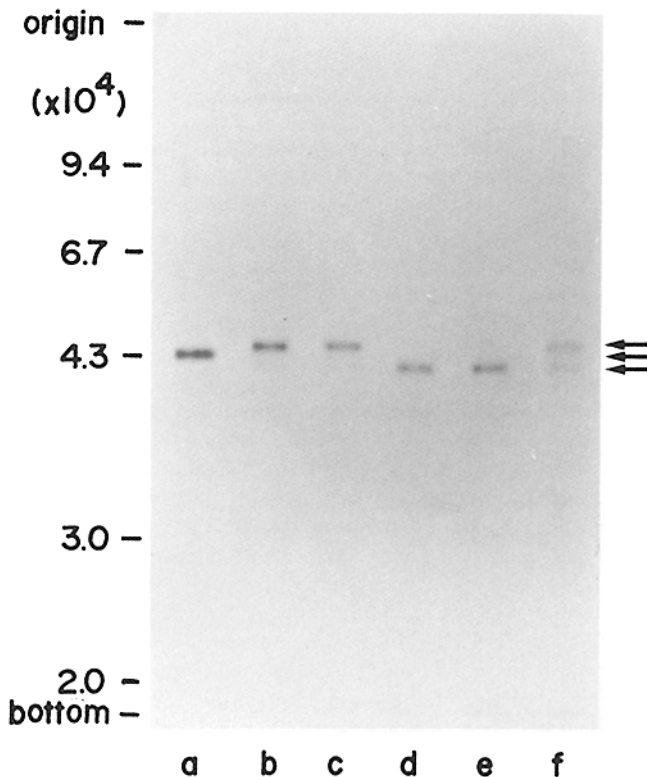


FIGURE 2 SDS gel electrophoresis and fluorography of α -amylase synthesized on microsomes. mRNA-dependent synthesis of α -amylase in the wheat germ (S-30 fraction) translation system and in vivo synthesis of α -amylase using intact scutellar tissues were performed as described previously (17). (a) Immunoprecipitate of α -amylase directed by poly(A)-mRNA in the wheat germ translation system. (b) Immunoprecipitate of α -amylase secreted from scutellar tissues. (c) Immunoprecipitate of α -amylase directed by microsomes. (d) Immunoprecipitate of α -amylase directed by microsomes prepared from scutellum pretreated with TM. (e) Immunoprecipitate of α -amylase directed by microsomes (c) treated with Endo- β -H in the presence of Triton X-100 as described in text. (f) Immunoprecipitate of α -amylase directed by microsomes (c) treated with Endo- β -H in the absence of Triton X-100.

that the glycosylation of the nascent polypeptide chain via the dolichol pathway occurs in the microsomes.

To examine the topology of the completed α -amylase molecules we tested the effects of Endo- β -H, which is known to split the chitobiosyl core moiety of the Asn-(glcNAc)₂-(Man)₃,¹ leaving behind the Asn-linked (glcNAc) (24). When the labeled α -amylase molecules associated with the microsomes were treated with Endo- β -H in the presence of Triton X-100, the labeled product showed an electrophoretic mobility similar to the processed nascent form of α -amylase (precursor II) (Fig. 2, lane e). The Endo- β -H-treated product of the α -amylase polypeptide is not entirely free from carbohydrate, and its molecular weight is presumed to be slightly larger than that of precursor II. However, it is not possible to determine molecular weight differences of a few hundreds, only on the basis of the gel electrophoretic mobilities. In the absence of Triton X-100, the α -amylase molecules largely remain resistant to the Endo- β -H digestion (Fig. 2, lane f). Thus, overall results tend to indicate that a large portion of the α -amylase molecules is segregated into the luminal space of the microsomal vesicles, being protected from the Endo- β -H attack by the microsomal membranes.

¹ Asn, asparagine. glcNAc, N-acetylglucosamine. Man, mannose.

Temporal Sequence of Proteolytic Processing and Glycosylation in Microsomes

The nature of the temporal events in α -amylase synthesis occurring on the microsomal membranes was examined by testing the Triton X-100 effect. Triton X-100 at 1% is expected to solubilize the microsomal membranes completely and prevents both proteolytic processing and glycosylation. As shown in Fig. 3, Triton X-100 inhibited the incorporation of ³⁵S-Met into α -amylase approximately 50%, and the addition of ATA, a specific inhibitor of the initiation of protein synthesis, also caused an inhibition of the ³⁵S-Met incorporation into α -amylase; during 40–50-min incubation, 12% and 18% lowering of the ³⁵S-Met incorporation occurred in the absence or presence of Triton X-100, respectively. Therefore, it can be assumed that some 15% of the α -amylase formation directed by the microsomes in the absence of ATA is ascribed to the enzyme molecules synthesized *de novo* in addition to the completed polypeptide chains of α -amylase previously associated with the microsomes.

α -Amylase synthesized by the microsomes in the presence of Triton X-100 was examined by SDS gel electrophoresis and fluorography (Fig. 4). Without the detergent treatment, two major bands were observed, regardless of the addition of ATA to the reaction mixture (Fig. 4, lanes b and c). Their electrophoretic mobilities correspond to precursor I and precursor II, respectively; additionally a very faint band of the mature enzyme (glycosylated form) was detectable. On the other hand, the major product in the absence of detergent was the mature glycosylated form of the enzyme (Fig. 4, lane a). It can be inferred that the disruption of the microsomal membranes by detergent treatment causes an inhibition of both the proteolytic processing and the glycosylation; alternatively, we cannot exclude a possibility that the enzymes catalyzing the two events are inactivated by the detergent.

In an attempt to characterize the nature of the proteolytic processing and glycosylation more precisely, we examined the Triton X-100 effect time-sequentially. Although α -amylase

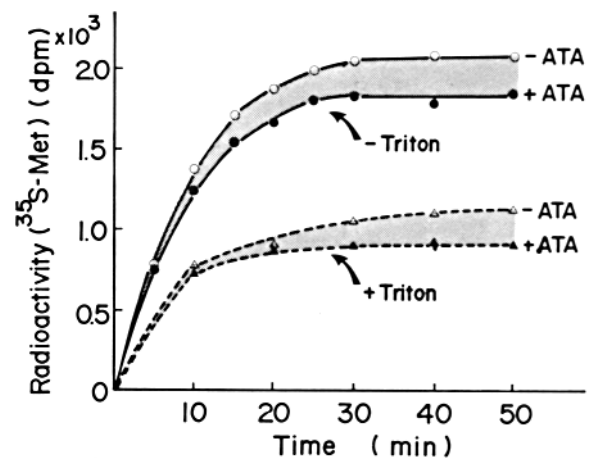


FIGURE 3 Effect of Triton X-100 and ATA on α -amylase synthesis in microsomes. Protein synthesis was performed according to the procedure described in Materials and Methods section, in the absence (—) or presence (---) of 1% Triton X-100 and in the absence (○, △) or presence (●, ▲) of 50 μ M ATA in the reaction mixture. At each indicated time interval, aliquots of the reaction mixture were moved and subjected to immunoprecipitation and subsequent radioactivity measurements. The incorporation of ³⁵S-Met into the immunoprecipitable α -amylase was calculated as described in Fig. 1.

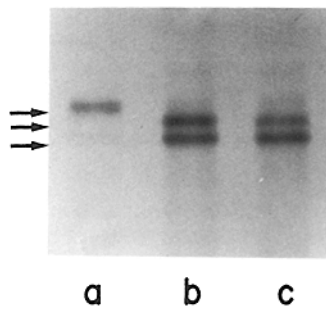


FIGURE 4 SDS gel electrophoresis and fluorography of α -amylase synthesized on microsomes in the presence of Triton X-100. Reaction mixtures as shown in Fig. 3 were subjected to immunoprecipitation after 40-min incubation. Immunoprecipitates were subsequently subjected to the SDS gel electrophoresis and fluorography. (a) Immunoprecipitate of α -amylase directed by microsomes without Triton X-100 and without ATA. (b) Immunoprecipitate of α -amylase synthesized in the presence of Triton X-100 and without ATA. (c) Immunoprecipitate of α -amylase synthesized in the presence of both Triton X-100 and ATA.

synthesis is significantly inhibited (~50%) in the presence of Triton X-100 (cf. Fig. 3), indicating that chain elongation is affected by the detergent, we have employed Triton X-100 treatment throughout. Other detergents, e.g., Nonidet P-40 and deoxycholate, at the same concentrations, were found to prevent protein synthesis more markedly than Triton X-100 (data not shown). Although the wheat germ S-100 fraction exhibited a slight initiation activity in the absence of ATA, the latter was not supplemented to the reaction system, since it was shown that the reagent partially inhibited both processing and glycosylation (data not shown).

As presented in Fig. 5, when Triton X-100 was added to the reaction mixture before the start of protein synthesis, the polypeptide chains already subjected to the proteolytic processing and glycosylation were not completed to produce the mature glycosylated form (Fig. 5, lane a), and in the absence of Triton X-100 only the mature form of α -amylase was detected (Fig. 5, lane e). On the other hand, when the polypeptide chains were allowed to elongate for 8–16 min in the absence of Triton X-100, they were subjected to partial modification during the step; consequently, the amount of precursor I decreased in proportion to the increase of the mature glycosylated form (Fig. 5, lanes b and c) (cf. 16). Therefore, if the initiation of α -amylase polypeptide synthesis did not occur in the present experimental system in the absence of Triton X-100, all the polypeptide chains should have been proteolytically processed to precursor II during the 16-min chain elongation period. However, proteolytically unprocessed precursor I was also detectable (see Fig. 5, lane c), which might conceivably be ascribed to a small extent of initiation reaction. When Triton X-100 was added at 24 min, the main product was the mature glycosylated form (Fig. 5, lane d).

The overall results thus indicate that detergent-sensitive components in microsomes, most likely membrane components, direct the proteolytic processing and subsequent glycosylation of the α -amylase molecules.

DISCUSSION

It is now well accepted that the proteolytic processing and glycosylation of secretory as well as membrane proteins are co-translationally directed by the ER during the transport of polypeptide chains across the membranes (2, 3, 8, 11, 12, 13, 20, 21, 23). In many investigations dealing with the mechanism of processing and glycosylation of secretory proteins, heterologous systems rather than the polysomal or microsomal protein-synthesizing system have been widely used (9), as potent

RNase activities associated with the preparations were found to prevent the effective isolation of the intact polysomes and/or microsomes (23). Indeed, experimental systems employing microsomes have been restricted to those with low RNase activities. Among a few investigations on plant protein synthesis, a study by Higgins and Spencer (10) is noteworthy. They examined the modification of precursor form of vicillin, a storage protein of the pea cotyledon, produced by the polysomal RNA employing the homologous system containing microsomal membranes.

In our investigation, a cell-free homologous system has been employed to elucidate the mechanism of biosynthesis of rice seed α -amylase, with particular attention to the temporal events occurring in the ER of the scutellar epithelium cells. Anatomically, the scutellar tissues dissected from the rice seed largely consist of scutellum proper, comprising a major part of the tissue, and the single-layered epithelium cells located in the outermost part of the scutellum, where the synthesis and secretion of α -amylase molecules are believed to occur (1, 19). Since it is technically difficult to isolate the rough microsomes from the epithelial cells per se, the microsomal fractions were prepared from the whole scutellar tissues using the method described in the Materials and Methods. Although the activities of RNase present in the rice scutella were not determined, the fact that polypeptide chains located at the 5'-end side of mRNA and the unprocessed nascent chains were completed to produce precursor I makes it unlikely that the polysomes associated with the microsomes had been subjected to degradation by RNase to any great extent.

Although the completion of polypeptide chains associated with the microsomes was found to yield the mature form of α -amylase (cf. Fig. 2), most importantly, the prevention by Triton X-100 treatment of polypeptide processing, as shown in Figs. 4 and 5, strongly indicate that both processing and glycosylation are directed by detergent-sensitive membranous elements of the microsomes.

A number of investigations have been reported concerning the relationship among chain elongation, proteolytic processing and glycosylation of membrane proteins, e.g., vesicular stomatitis virus (VSV) glycoprotein (20) and human chorionic gonadotropin (3); the effect of Triton X-100 on microsomal membranes during polypeptide chain elongation had also been tested. However, in none of these studies has the time-sequence analysis of the detergent effect failed to give a precise picture of the temporal sequence of the processing and glycosylation

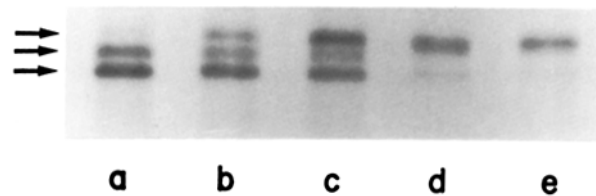


FIGURE 5 Effect of detergent treatment on proteolytic processing and glycosylation of polypeptides synthesized on microsomes. 20% Triton X-100 was added to the reaction mixture to a final concentration of 1% at the indicated time intervals, i.e., 0 (a), 8 (b), 16 (c), and 24 min (d), and incubation continued up to 40 min. Triton X-100 was not added to the reaction mixture in lane e. At the termination of incubation for 40 min, samples were withdrawn and then subjected to the immunoprecipitation and fluorography. In lanes d and e, reaction mixtures containing approximately one half of the radioactivities of lanes a, b, and c were applied to the SDS gels.

during polypeptide chain elongation. Although we must stress that the mechanism disclosed by employing a cell-free microsomal system may not be a direct representation of the events occurring *in vivo*, it appears evident that detergent-sensitive components in the microsomes can direct the proteolytic processing and the subsequent glycosylation of the rice seed α -amylase molecules.

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