

Enzymic Mechanism of Starch Breakdown in Germinating Rice Seeds¹

12. BIOSYNTHESIS OF α -AMYLASE IN RELATION TO PROTEIN GLYCOSYLATION

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ABSTRACT

The biosynthetic mechanism of α -amylase synthesis in germinating rice (*Oryza sativa* L. cv. Kimmazé) seeds has been studied both *in vitro* and *in vivo*. Special attention has been focused on the glycosylation of the enzyme molecule. Tunicamycin was found to inhibit glycosylation of α -amylase by 98% without significant inhibition of enzyme secretion. The inhibitory effect exerted by the antibiotic on glycosylation did not significantly alter enzyme activity.

In an *in vitro* system using poly-(A) RNA isolated from rice scutellum and the reticulocyte lysate translation system, a precursor form of α -amylase (precursor I) is formed. Inhibition of glycosylation by Tunicamycin allowed detection of a nonglycosylated precursor (II) of α -amylase. The molecular weight of the nonglycosylated precursor II produced in the presence of Tunicamycin was 2,900 daltons less than that of the mature form of α -amylase (44,000) produced in the absence of Tunicamycin, and 1,800 daltons less than the *in vitro* synthesized molecule.

The inhibition of glycosylation by Tunicamycin as well as *in vitro* translation helped clarify the heterogeneity of α -amylase isozymes. Isoelectrofocusing (pH 4-6) of the products, zymograms, and fluorography were employed on the separated isozyme components. The mature and Tunicamycin-treated nonglycosylated forms of α -amylase were found to consist of three isozymes. The *in vitro* translated precursor forms of α -amylase consisted of four multiple components. These results indicate that heterogeneity of α -amylase isozymes is not due to glycosylation of the enzyme protein but likely to differences in the primary structure of the protein moiety, which altogether support that rice α -amylase isozymes are encoded by multiple genes.

In contrast to a long-held belief that α -amylase is synthesized *de novo* in the aleurone layers of germinating cereal seeds such as barley when triggered by gibberellic acid (4, 6, 12), recent experiments using histochemical techniques have demonstrated that the dominant site of formation of α -amylase is the scutellar epithelium (24, 25). Electron microscopic observations clearly show the formation of membranous structures such as RER and Golgi apparatus in this tissue in early germination, suggesting the presence of a potentially active system of protein secretion *in situ* (26).

In work reported in our previous publication (21), we demon-

strated the *in vitro* synthesis of α -amylase directed by poly-(A) containing RNA isolated from the scutellar tissues in order to characterize the nature of α -amylase formation at the molecular level. It was found that the *in vitro* synthesized product had a lower mol wt than the mature secretory form of α -amylase molecule.

During the biosynthesis of many secretory glycoproteins, an extrapeptide (signal peptide) recognizing the ER membrane is co-translationally cleaved as it enters the ER cistern; in completion of the chain elongation, the polypeptide is co-translationally glycosylated (2, 3, 14). Therefore, neither extrapeptide-attached nor extrapeptide-cleaved nonglycosylated precursor forms of secretory glycoprotein synthesized following the above described scheme are normally detectable in short pulse-chase labeling experiments *in vivo*. Although the *in vitro* translation products directed by mRNA are likely to be the precursor forms of secretory glycoproteins containing the signal peptide, no reliable method is available for detecting the nonglycosylated precursor form lacking the signal peptide, making the comparison between these two precursors and the completely mature form difficult.

Recently, it has been shown that the antibiotic TM² specifically inhibits the glycosylation of various types of secretory glycoproteins in mammalian and fungal systems (33, 36). A considerable number of secretory proteins are known to be glycosylated via the lipid-linked pathway, a core region of the oligosaccharide chain having the invariable structure, Man β -1,4-glcNAc- β -1,4-glcNAc linked to an asparagine residue in the tripeptide sequence Asn-[X]-Ser/Thr (40). TM inhibits the formation of the lipid oligosaccharide chain by preventing the synthesis of the lipid-linked glcNAc intermediate (17, 37, 39). This lipid-linked oligosaccharide also exists in plants (18, 35). In this report, the inhibitory effect of TM on protein glycosylation has been examined to characterize the nonglycosylated precursor form of α -amylase in the scutellar epithelium of rice seedlings.

α -Amylase in cereal seeds is known to consist of several isozymes (31), but it is not clear whether these isozymes originate from the posttranslational modification of a single gene product or from different multiple genes. Heterogeneity of α -amylase isozymes in human salivary gland and pancreas is considered to result from posttranslational modification, e.g. glycosylation and deamidation (13). To elucidate the origin of heterogeneity of rice α -amylase isozymes, the inhibition of glycosylation by TM in combination with *in vitro* translation has been determined.

¹ Paper 11 of the series is Reference 26 by Okamoto *et al.* (Plant Physiol, in press, 1982). This research was financially aided by the research grant from Mombusho (Japanese Ministry of Education) and the Mitsubishi Foundation (Tokyo).

² Abbreviations: TM, Tunicamycin; glcNAc, *N*-acetylglucosamine; poly-(A), polyadenylic acid; IgG, immunoglobulin G; Con A, concanavalin A.

MATERIALS AND METHODS

Incubation of Rice Seed Scutellar Tissues. Ten pieces of scutella were freshly dissected from rice seedlings (*Oryza sativa* L. cv. Kimmazé) at the 4-d germination stage as described previously (21). They are free from contamination of endosperm tissues, and were preincubated at 30°C for 3 h in 0.3 ml of a solution containing 10 mM Tris-HCl buffer (pH 7.0), 30 mM CaCl₂, and antibiotics (4), and in the presence or absence of TM (30 µg/ml). After preincubation, the medium was discarded and the scutella washed with the above buffered medium. To determine the incorporation of [³⁵S]Met or [³H]Man into α-amylase, scutella were incubated at 30°C for an additional 2 h in 0.3 ml of the above buffered medium containing [³⁵S]Met or [³H]Man (100 µCi) in the presence or absence of TM (30 µg/ml).

For the measurements of α-amylase activities in the incubation medium or to make α-amylase zymograms, scutella were incubated at 30°C for 1 to 4 h in 0.3 ml of the buffered medium in the presence or absence of TM (30 µg/ml) for an appropriate time. Samples of the incubation medium were then analyzed as described below.

For the staining of either protein or carbohydrate portion of the enzyme molecule, 2,000 pieces of scutella were preincubated in 30 ml of buffered medium in the absence or presence of TM, then further incubated at 30°C for 4 h in 30 ml of a fresh buffered medium as described above (see below).

In Vitro Protein Synthesis. Poly-(A)-rich RNA isolated from the rice scutellum at 4-d germination stage following the method reported previously (21) was translated in a reticulocyte lysate system. The reaction mixture contained 50 µl of reticulocyte lysate (Radiochemical Centre, Amersham, U. K.), 100 µCi of [³⁵S]Met, and 5 µg of RNA in a total volume of 68 µl. After incubation at 30°C for 1 h, the reaction was stopped by the addition of 0.2 µg of pancreatic RNase A.

Immunochemical Methods. α-Amylase labeled *in vivo* or *in vitro* was precipitated by the anti-α-amylase IgG, specific to rice seed α-amylase, essentially following the experimental procedures described previously (21). Either the outside medium obtained after incubating the scutellum for 2 h in the presence of [³⁵S]Met or [³H]Man or the total reaction mixture of the reticulocyte lysate cell-free system was made to 2% (w/v) with SDS, followed by additional incubation at 30°C for 1 h. Then the whole mixture was made to about 3% (v/v) with Triton X-100. After addition of 0.5 ml 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 of anti α-amylase IgG or nonimmune control IgG was added and incubated for an additional 1 h at 30°C. Then 20 µl (packed volume) of protein A-Sepharose gel was added to the mixture and allowed to incubate for 1 h with occasional stirring. The immunoprecipitates bound to protein A-Sepharose were eluted with 50 µl of a solution containing 1% SDS and 50 mM DTT. Eluates were then subjected to the radioactivity measurements, SDS-polyacrylamide gel electrophoresis, Con A-Sepharose column chromatography, or slab gel isoelectrofocusing.

Identification of In Vivo or In Vitro Synthesized α-Amylase. Immunoprecipitates of either [³⁵S]Met or [³H]Man-α-amylase were applied to 10% SDS-polyacrylamide gels, electrophoresed, and fluorographed following the method described previously (21). To separate multiple forms of α-amylase, the immunoprecipitates dissolved in 1% SDS and 50 mM DTT were subject to isoelectrofocusing (pH 4–6) on slab gels according to the procedure described by Ames and Nikaido (1), and subsequently fluorographed.

Protein or Carbohydrate Staining. After 4-h incubation, 30 ml of the incubation medium was collected and centrifuged at 10,000g for 10 min; the supernatant fraction was applied to a column of anti-α-amylase IgG bound to Sepharose 4B (packed volume, 0.3 ml), which was preequilibrated with the buffer medium. The anti-

α-amylase IgG-immobilized Sepharose 4B was prepared according to the procedures described in a brochure supplied by the manufacturer (Pharmacia, Uppsala). After washing the column with 5 ml of 20 mM Tris-HCl buffer (pH 7.0) containing 30 mM CaCl₂ and 0.5 M NaCl, α-amylase was eluted with 3 ml of 0.2 M glycine-HCl buffer (pH 2.3). The eluate was then made to 10% TCA, the resulting precipitate collected, washed in acetone by centrifugation, and finally dissolved in 50 µl of SDS-sample buffer (16). For protein or carbohydrate staining, 5 to 40 µl of the sample solution, respectively, were subject to SDS-gel electrophoresis. After electrophoresis, the gels were stained with Coomassie brilliant blue or with fuchsin-sulfite reagent (PAS) according to Zacharius *et al.* (42).

Con A-Sepharose Column Chromatography. Immunoprecipitates of the [³⁵S]Met-α-amylase were dissolved in a solution containing 1% SDS and 50 mM DTT, diluted with 1 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 1 mM MgCl₂, 1 mM DTT, and 0.05% SDS solution. The solution was then applied to a column of Con A-Sepharose (packed volume, 200 µl), which was preequilibrated with the above buffer solution. After washing with 5 ml of the same buffer, materials bound to Con A-Sepharose were eluted with 3 ml of the buffer containing 0.5 M α-methylmannoside. Protein fractions either bound or unbound to Con A-Sepharose column were adjusted to 10% TCA, and after centrifugation the precipitates obtained were washed twice in acetone, dissolved in SDS-sample buffer (16), and finally applied to the SDS-gel electrophoresis and fluorography.

Enzyme Assay. α-Amylase activity was determined according to the method described previously by Okamoto and Akazawa (24).

Reagents. TM was kindly donated by Dr. G. Tamura, University of Tokyo. [³H]Man, [³⁵S]Met, and rabbit reticulocyte lysate (cell-free translation system) were the products of the Radiochemical Centre, Amersham, U. K., and purchased from Japan Isotope Centre. Protein A-Sepharose and Con A-Sepharose were purchased from Sigma.

RESULTS

Effect of TM on Glycosylation and Secretion of α-Amylase. To examine the effect of TM on the biosynthesis of α-amylase in the scutellar epithelium of rice seeds, freshly dissected scutella were incubated for 2 h in the medium containing [³⁵S]Met or [³H]Man. Under the experimental conditions employed (Fig. 1), the incorporation of [³⁵S]Met into the TCA-insoluble total protein fraction in the whole tissue was not significantly inhibited in the presence of TM (17% inhibition), whereas TM seems to inhibit the secretion of some protein molecules in the medium (57% inhibition). The incorporation of [³⁵S]Met into the immunoprecipitable α-amylase in the medium was inhibited only 22% in the presence of TM. On the contrary, the incorporation of [³H]Man into α-amylase was almost completely inhibited (98%). Overall results indicate that TM totally inhibits the glycosylation of the α-amylase molecule, although the antibiotic does not exhibit a significant effect on the synthesis and subsequent secretion of the enzyme molecule from the scutellar tissue. It can be thus inferred that the glycosylation is not a prerequisite step for the secretion of α-amylase in the rice seedlings.

SDS-Polyacrylamide Gel Electrophoresis of Three Forms of α-Amylase. In our previous investigation (21), we have compared *in vivo* and *in vitro* synthesized α-amylase employing SDS-polyacrylamide gels. On the basis of electrophoretic mobility, the *in vivo* synthesized α-amylase, mature secretory form, was found to be 2,100 daltons larger than the *in vitro* synthesized molecule, which we presume to be the precursor polypeptide containing the extrapeptide. To determine if the precursor forms of secretory glycoproteins are synthesized according to the signal hypothesis, it is desirable to detect the nonglycosylated and processed poly-

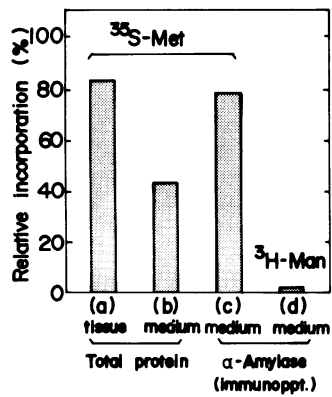


FIG. 1. Effect of TM on incorporation of [^{35}S]Met or [^3H]Man into total protein and α -amylase. a, Incorporation of [^{35}S]Met into TCA-insoluble total protein (scutellar tissue); b, incorporation of [^{35}S]Met into TCA-insoluble total protein (incubation medium); c, incorporation of [^{35}S]Met into immunoprecipitable α -amylase (incubation medium); d, incorporation of [^3H]Man into immunoprecipitable α -amylase (incubation medium). At the end of a 2-h incubation, each group of scutella was homogenized, followed by centrifugation to obtain the supernatant. The incubation medium of each group was also centrifuged to obtain a clear solution. Radioactivities of TCA-insoluble total protein fraction or α -amylase fraction precipitable by anti- α -amylase IgG in either scutellar extracts or incubation medium were then measured as previously described (24). Radioactivity was hardly detectable in the immunoprecipitable α -amylase fraction in tissue extracts regardless of the presence or absence of TM in the incubation mixture.

peptide (*i.e.* extrapeptide removed) as well as the putative precursor polypeptide synthesized in the *in vitro* translation system. Figure 2 shows the fluorogram of the mature secretory form of α -amylase which is labeled with [^{35}S]Met in the absence of TM (a), nonglycosylated α -amylase labeled with [^{35}S]Met in the presence of TM (b), and nonglycosylated α -amylase synthesized in the *in vitro* translation system (c). Each of them was clearly separated as a single band on the SDS-polyacrylamide gel electrophoresis (see d). The difference in mol wt of TM-treated (b) and *in vitro* synthesized (c) α -amylase is 1,800, which would correspond to the mol wt of the extrapeptide. The difference of mol wt of mature (a) and TM-treated (b) α -amylase is 2,900 daltons. It is therefore inferred that after cleavage of the extrapeptide, oligosaccharide chain in mol wt of 2,900 daltons is introduced into the nascent polypeptide chain via the lipid-linked pathway to produce the mature secretory form of α -amylase (mol wt, 44,000).

Is α -Amylase Produced under TM Treatment Absolutely Carbohydrate-Free? In our previous report (21), we have calculated the mol wt of the carbohydrate moiety of rice α -amylase to be 2,100 daltons, based on the result of phenol-sulfuric acid method of Dubois *et al.* (5). However, the method employed cannot detect the amino sugars (*e.g.* glcNAc), although the oligosaccharide chain introduced into glycoprotein molecules via the lipid-linked pathway is known to contain at least two molecules of glcNAc (mol wt, 221) (see above). Therefore, mol wt of the carbohydrate portion in α -amylase is considered to be greater than 2,500 daltons, and the value is roughly equivalent to the mol wt of carbohydrate moiety (2,900) estimated from the difference of electrophoretic mobilities between TM-treated and untreated α -amylase (*cf.* Fig. 2, a and b). However, care must be taken to estimate the mol wt of glycoprotein molecule because of anomalous electrophoretic behaviors frequently encountered.

To further demonstrate that α -amylase synthesized in the scutellar tissues in the presence of TM has no carbohydrate moiety, staining with fuchsin-sulfite reagent (PAS) on polyacrylamide gels, as well as fluorography of [^3H]Man- α -amylase were em-

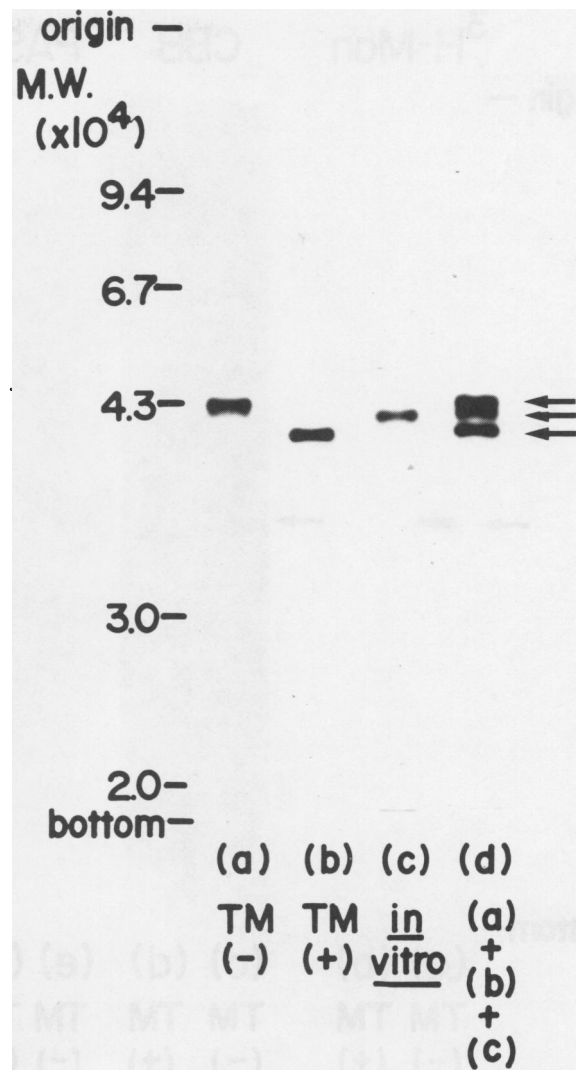


FIG. 2. SDS-polyacrylamide gel electrophoresis of α -amylase synthesized *in vivo* in absence or presence of TM or *in vitro*. a, [^{35}S]Met- α -amylase synthesized *in vivo* in the absence of TM; b, [^{35}S]Met- α -amylase synthesized *in vivo* in the presence of TM; c, [^{35}S]Met- α -amylase synthesized *in vitro* reticulocyte lysate translation system; d, mixture of a, b, and c. a and b show the α -amylase molecules secreted into the incubation medium. The radioactive band of immunoprecipitable α -amylase was hardly detectable in the tissue extracts irrespective of the presence or absence of TM. The values on the left refer to mol wt of marker proteins coelectrophoresed. Upper arrow on the right hand refers to the position of α -amylase synthesized *in vivo* in the absence of TM (mature enzyme of secretory form); middle arrow, the position of enzyme synthesized *in vitro* (precursor I); and lower arrow, the position of enzyme synthesized *in vivo* in the presence of TM (precursor II).

ployed. Figure 3, a shows the fluorogram of [^3H]- α -amylase, and no band was detectable at the position of α -amylase in the TM-treated system. On the other hand, α -amylase synthesized in the presence or absence of TM was stainable with Coomassie brilliant blue (Fig. 3, c and d), although with PAS reagent only the α -amylase produced in the absence of TM could be stained and the one synthesized in the presence of TM was not stainable (Fig. 3, e and f). These results demonstrate that α -amylase synthesized in the presence of TM is free of carbohydrate.

Binding to Con A. Glycoprotein with oligosaccharide chains containing mannose binds to the lectin Con A and are elutable with α -methylmannoside (19). As shown in Figure 4, the mature

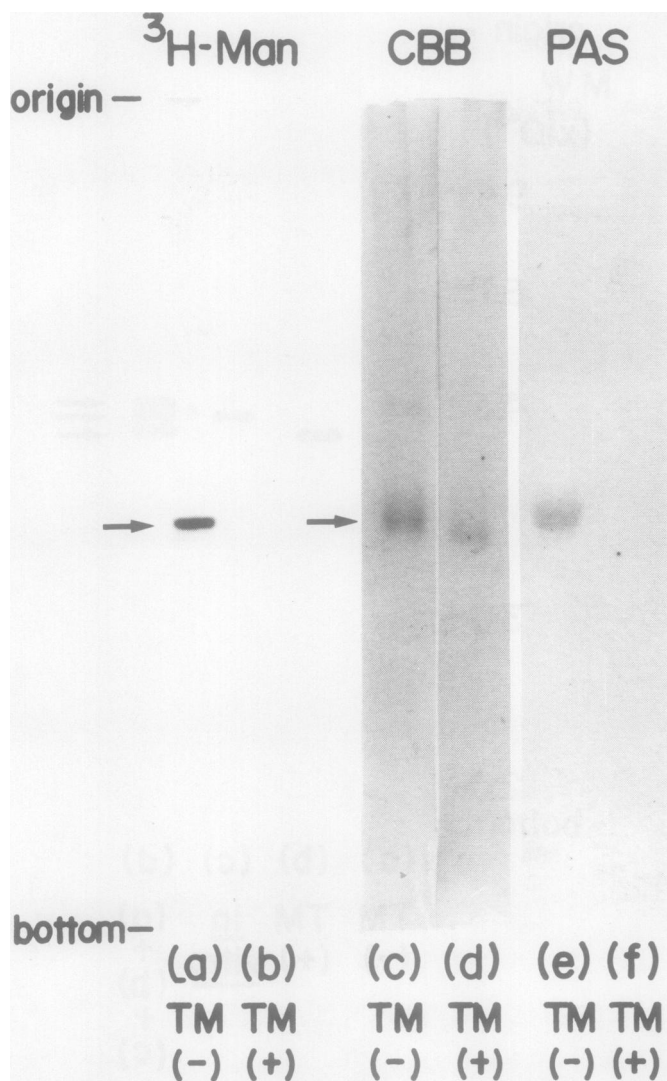


FIG. 3. Effect of TM treatment on glycosylation of α -amylase. a and b, Fluorogram of immunoprecipitable [^3H]Man- α -amylase synthesized *in vivo* in the absence (a) or presence (b) of TM; c and d, Coomassie brilliant blue (CBB) staining of immunoadsorbent α -amylase synthesized *in vivo* in the absence (c) or presence (d) of TM; e and f, carbohydrate (PAS) staining of immunoadsorbent α -amylase synthesized *in vivo* in the absence (e) or presence (f) of TM.

secretory form of α -amylase synthesized *in vivo* in the absence of TM is bound to Con A, whereas that synthesized in the TM-treated system or *in vitro* system cannot be retained on a column of Con A-Sepharose. Therefore, these results provide additional proof that the secretory forms of α -amylase synthesized *in vivo* contain a high-mannose-type oligosaccharide chain in the molecule.

Effect of Inhibition of Glycosylation on α -Amylase Activity. As presented above, glycosylation is not essential to the secretion of α -amylase, and we next examined the effect of TM treatment on α -amylase activity. Within a 2-h incubation period, the reduction in α -amylase activity in the presence of TM was only 17% to 20% (Fig. 5). A prolonged incubation with TM, however, resulted in the decline of α -amylase activity as well as the incorporation of [^{35}S]Met into the α -amylase molecule (data not shown), presumably due to the inhibitory effect of the antibiotic on the biosynthesis of other enzyme system(s) required for the α -amylase synthesis and/or enhancement of the susceptibility of the nonglycosylated form of α -amylase to proteolytic degradation (*cf* 8). Be-

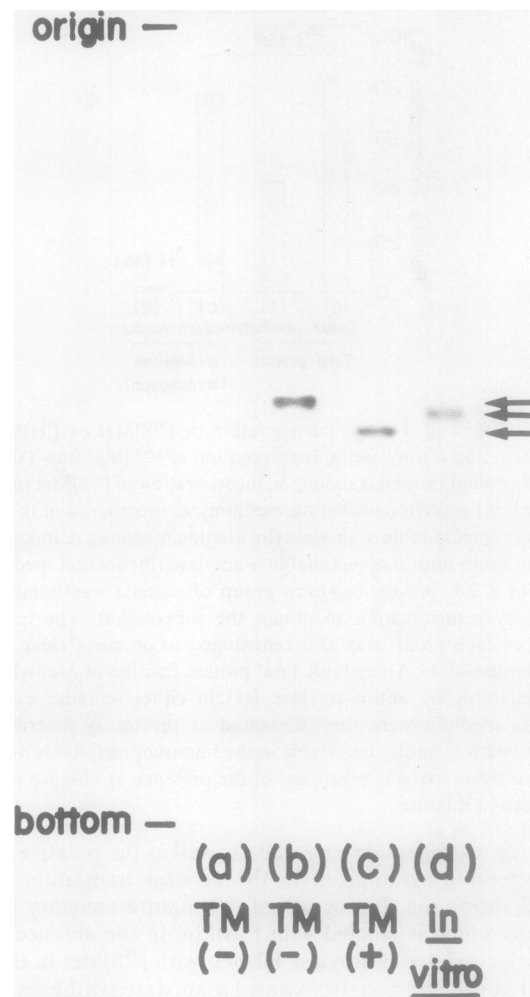


FIG. 4. Binding of [^{35}S]Met- α -amylase to Con A-Sepharose column. Immunoprecipitates of α -amylase with [^{35}S]Met (about 2,000–3,000 cpm) under various experimental conditions were applied to a column of Con A-Sepharose. Fractions passed through the column (a, c, and d) or eluted with α -methylmannoside (b) were subjected to the SDS-gel electrophoresis and subsequent fluorography as described in the text. a and b, [^{35}S]Met- α -amylase synthesized *in vivo* in the absence of TM; c, [^{35}S]Met- α -amylase synthesized *in vivo* in the presence of TM; d, [^{35}S]Met- α -amylase synthesized *in vitro* in reticulocyte lysate translation system. No radioactive band was detectable when α -methylmannoside elutable fractions of the [^{35}S]Met- α -amylase synthesized *in vivo* in the presence of TM or *in vitro* system were applied to SDS-gel electrophoresis and fluorography (data not shown).

cause the inhibition of the secretion of α -amylase by the TM-treatment is only 22% during a 2-h incubation period, it can be concluded that the carbohydrate moiety of α -amylase is not obligatory for the expression of enzyme activity.

Origin of the Heterogeneity (Isozymes) of α -Amylase. It has been reported that rice α -amylase consists of several isozymes (31), and our previous experiments have shown that the scutellar epithelium produces isozymic components (24). To elucidate the nature of the origin of these isozymes at the molecular level, glycosylated and nonglycosylated forms of α -amylase synthesized in the absence or presence of TM were subject to isoelectrofocusing (pH 4–6) followed by an α -amylase zymogram. The results (Fig. 6) show three α -amylase isozymes. Two major bands and one minor band were clearly detectable in the untreated control system (Fig. 6, a). It must be stressed that two bands marked as A and B appear to correspond well with the isozymic bands A and B

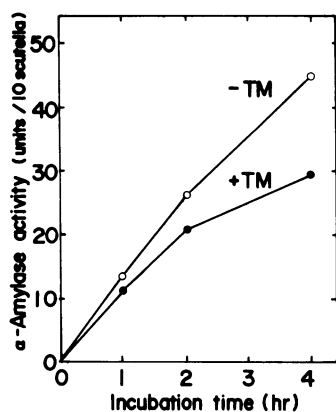


FIG. 5. Effect of TM treatment of scutellum on α -amylase activities in incubation medium. α -Amylase activities in the incubation medium with (●) or without (○) TM pretreatment (3 h) of the scutellar tissues were measured subsequently at the stated incubation periods following the method described previously (24).

derived from the scutellar (and endosperm) extracts (see Fig. 2 of Ref. 24). However, all three bands disappear upon treatment with anti- α -amylase IgG which was raised against purified α -amylase, indicating that the amylolytic activities of the three bands may well correspond to α -amylase isozymes. The carbohydrate-free form of α -amylase secreted from the scutellum in the presence of TM also shows the same multiple enzyme activities as the glycosylated mature forms (c). The mixture of the TM-treated and untreated α -amylase did not cause the alteration of the banding pattern, indicating that the carbohydrate moiety of the α -amylase molecule has no influence on the isoelectric point of the protein molecule (e).

The carbohydrate moiety introduced into the polypeptide chain via the lipid-linked pathway which is subject to inhibition by TM, is known to consist of a high-mannose-type neutral oligosaccharide chain (36). Therefore, results obtained here indicate that the heterogeneity of α -amylase molecules is not due to glycosylation of the enzyme molecule.

Inasmuch as α -amylase is a secretory protein, it is conceivable that the extrapeptide would be cleaved from the precursor molecule during its biosynthesis. There is a possibility that the molecular heterogeneity of carbohydrate-free α -amylase (as demonstrated in Fig. 6, c) is due to different processing events of a single polypeptide. To investigate such a possibility, we further examined the immunoprecipitates of both *in vivo* and *in vitro* labeled α -amylase using isoelectrofocusing. When the immunoprecipitates of TM-treated or untreated α -amylase were subject to isoelectrofocusing, two major and one minor bands were detected (Fig. 7, a and b). The *in vitro* synthesized molecule appears to consist of four different polypeptide chains (c). Therefore, the results suggest possible existence of four mRNAs coding for four different α -amylase isozymes.

DISCUSSION

Many secretory glycoproteins are known to contain oligosaccharides in the molecule introduced via the lipid-linked pathway (36). There are a number of investigations showing that TM inhibits the formation of the lipid-linked oligosaccharide by blocking the transfer of glcNAc phosphate to dolichol phosphate (17, 37, 39). Consequently, the specific inhibitory effect exerted by TM has been utilized to elucidate the mechanism of glycoprotein biosynthesis as well as the role of oligosaccharide chains in the secretory process. Although in some cases nonglycosylated forms of glycoproteins are known to be normally secreted (11, 22, 28), it is reported that secretion of others is markedly curtailed by the

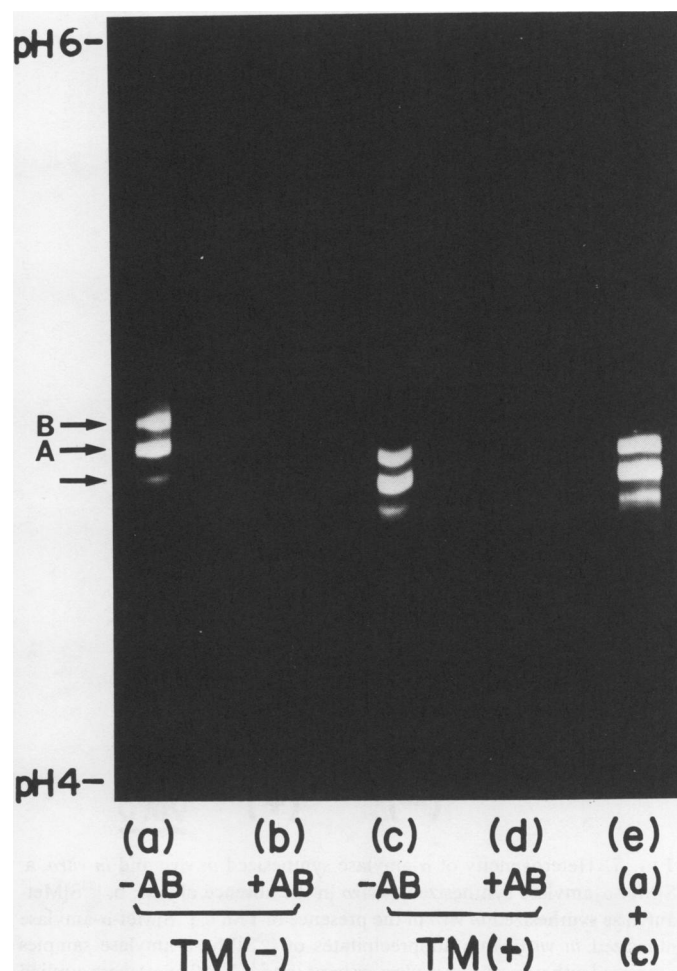


FIG. 6. Heterogeneity of α -amylase zymogram pattern of incubation medium and effect of TM. a, α -Amylase isozymes secreted into incubation medium in the absence of TM (no treatment with anti- α -amylase IgG); b, same as a but treated with anti- α -amylase IgG; c, α -amylase isozymes secreted into incubation medium in the presence of TM (no treatment with anti- α -amylase IgG); d, same as c but treated with anti- α -amylase IgG; e, mixture of a and c (no treatment with anti- α -amylase IgG). Scutella were incubated in the absence or presence of TM for 4 h at 30°C, incubation medium collected, and a 75- μ l aliquot was applied to the isoelectrofocusing on polyacrylamide gel (pH 4–6) following the method reported previously (24). To another aliquot of the incubation medium was added 50 μ g of anti- α -amylase IgG and incubation continued for 1 h at 30°C. After centrifugation, a 100- μ l aliquot of the supernatant was applied to an isoelectrofocusing gel. Electrophoresed samples were then subject to α -amylase zymograms, by incubating with starch gel-coated glass plate (1 h at 30°C).

TM treatment (9, 11, 38). Nonglycosylated forms of human interferon (22) and yeast carboxypeptidase Y (9) synthesized under conditions of TM treatment are secreted and retain their original biological and/or enzyme activities. In the case of α -amylase biosynthesis in barley aleurone layers, TM was shown to inhibit the secretion of α -amylase 60% to 80%, but these results were based only on enzyme activity measurements (32). The experimental findings obtained in the present work show that the inhibition of the glycosylation of α -amylase exerted by TM does not have a significant influence on the secretion and expression of enzyme activity.

The current concept is that the signal peptide is cleaved during biosynthesis of most secretory protein molecules, while being transported to the lumen of the ER (3, 29). This mechanism has

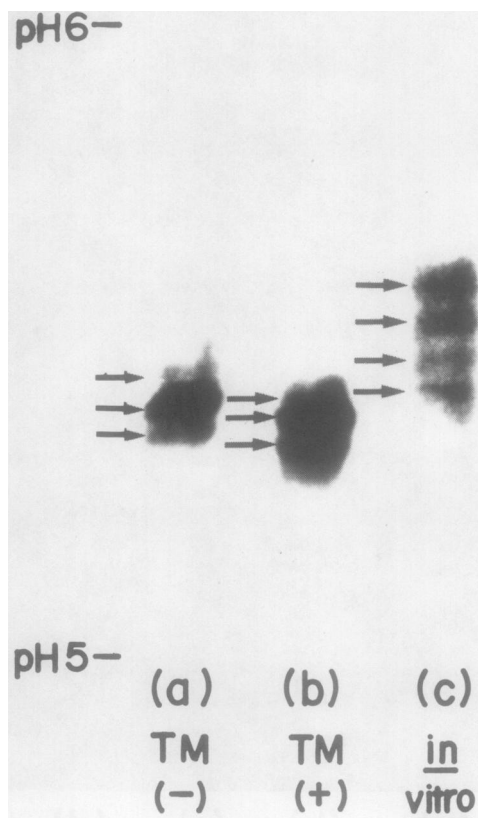
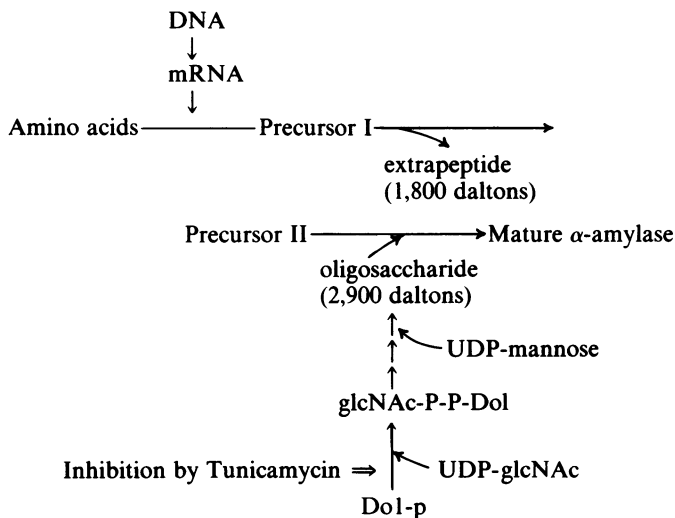


FIG. 7. Heterogeneity of α -amylase synthesized *in vivo* and *in vitro*. a, [^{35}S]Met- α -amylase synthesized *in vivo* in the absence of TM; b, [^{35}S]Met- α -amylase synthesized *in vivo* in the presence of TM; c, [^{35}S]Met- α -amylase synthesized *in vitro*. Immunoprecipitates of [^{35}S]Met- α -amylase samples synthesized either *in vivo* or *in vitro* systems (about 5,000 cpm) were applied to the slab gel isoelectrofocusing (pH 4–6) and subsequently to fluorography. In the figure, only a portion of the fluorogram (pH 5–6) is shown, as no band was detectable in the range of pH 4–5.

been based on studies of a secretory protein not containing a saccharide chain (nonglycoprotein). In a cell-free protein synthesizing system directed by the mRNA of secretory proteins without saccharide chain, it is recognized that the products synthesized are generally larger in mol wt than those synthesized *in vivo*. However, in the case of secretory proteins having glycoprotein structure, the above mechanism is not applicable, as the saccharide chain must be introduced into the nascent polypeptide chain to make the mature form. Therefore, to establish the relationship between precursor(s) and completed mature forms of secretory glycoproteins, it is necessary to detect the processed nonglycosylated molecule in addition to the *in vitro* synthesized nonglycosylated precursor polypeptide. So far, a thorough comparison between these two hypothetical precursors and the final mature form of a secretory glycoprotein have been performed for a viral glycoprotein (30) and peptide hormones (10).

As to the mechanism of α -amylase biosynthesis in the aleurone layers isolated from wheat (27) or barley (23) and in mammalian tissues using rat parotid (7) or dog pancreas (20), it has been found that the *in vitro* synthesized α -amylase has an extrapeptide segment which is destined for cleavage at some stage in the biosynthetic pathway. In all such cases, it has been reported that the *in vitro* synthesized precursor α -amylase is larger in mol wt than that of the *in vivo* synthesized molecule, and no special attention has been given for the glycosylation reaction involved in the maturation of the enzyme molecule. In our present investigation of the biosynthesis of rice seed α -amylase, precursor(s) and mature forms of the enzyme were compared by SDS-polyacrylamide gel electro-

phoresis. From comparisons of the electrophoretic mobilities of precursor forms and the final complete secretory form, and as well as other accompanying data, a possible biosynthetic pathway of α -amylase is formulated as follows:



In the scheme, it can be seen that following transport of the elongating polypeptide chain into the cisternal luminal space of ER the extrapeptide (mol wt, 1,800 daltons) would be cleaved (precursor I), and then a 2,900 daltons mol wt oligosaccharide chain would be added to the nascent polypeptide (precursor II) via the lipid-linked pathway. Indeed, in the present study we were able to detect precursor I in the *in vitro* system directed by mRNA, and precursor II as the processed nonglycosylated form in the *in vivo* system in the presence of TM. It must be realized that these hypothetical precursor forms, detectable on the SDS-polyacrylamide gel, may not exist *in vivo*, as it is presumed that both the cleavage of the extrapeptide and the subsequent glycosylation occur cotranslationally. In fact, a short pulse-chase labeling experiment has failed to detect any of these precursor form(s) of α -amylase under the *in vivo* experimental conditions employed (data not shown).

It has long been thought that the heterogeneity of glycoprotein molecules originates mainly from the posttranslational modification or the glycosylation of the nascent polypeptide chain, as was surmised from the structural variation of the carbohydrate moiety so far found in various glycoproteins (15, 34). It is, in fact, almost impossible to distinguish whether the heterogeneities of glycoprotein molecules observed by gel electrophoresis are due to the difference in the primary structure of protein molecule or in the carbohydrate moieties attached to it. The answer can be determined when the total amino acid sequences are known or the carbohydrate moiety is removed from the glycoprotein. However, determination of the total amino acid sequence of each individual isozyme species is difficult and it is known that the removal of carbohydrate group from glycoproteins by chemical means is impossible without disruption of the protein structure. As an alternative, to clarify the origin of heterogeneity of α -amylase isozymes, the inhibitory effect of TM on protein glycosylation was utilized in the present study. The absence of carbohydrate appears to have caused little conformational change in the protein since the α -amylase retains the original enzyme activity. Consequently, the heterogeneity of α -amylase isozymes appears to result from the different primary structures of the proteins and not to glycosylation. Furthermore, examination of the reaction product in the *in vitro* translation system directed by poly-(A)-containing RNA indicates that the heterogeneity of α -amylase polypeptides demonstrable in the presence of TM is likely to arise from the existence of multiple mRNA species and not to differential cleavage of

peptides from a single translation product. Here, one could argue whether multiple forms of α -amylase detected by electrophoresis are due to artifacts possibly produced during the isolation steps. However, this possibility is not very likely because three molecular species are identifiable in both the intact amylase (Fig. 6) and the completely unfolded polypeptides prepared by immunoprecipitation followed by SDS and heat treatment and isoelectric focusing (Fig. 7).

The precise relationship between the *in vivo* products and the four products detectable in the *in vitro* system is not clear. In the *in vitro* translation, poly-(A)-containing RNA isolated from the total RNA fraction was used instead of polysomal RNA. Therefore, for the purpose of determining whether the *in vitro* synthesized products exactly correspond to those produced *in vivo*, experiments using polysomal mRNA will be needed.

Our overall data support a notion that α -amylase isozymes are encoded by different genes, although the possibility cannot be totally excluded that heterogeneous mRNAs coding for multiple α -amylase isozymes are produced from the primary transcript of a single gene during mRNA processing. It has recently been reported that mouse salivary gland and liver α -amylase mRNA are coded by the same gene and have different 5'-nontranslatable sequences (41). In any event, the specific inhibitory effect of TM on α -amylase glycosylation, combined with *in vitro* translation directed by mRNA will provide a novel experimental system for elucidating the origin of heterogeneity of secretory glycoproteins in plant tissues.

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