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Synthesis of a Possible Precursor of α-Amylase in Wheat Aleurone Cells[1]

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ABSTRACT

α-Amylase from wheat aleurone (*Triticum aestivum*) was synthesized in a S-150 wheat germ readout system using polysomes, and a messenger RNA-dependent reticulocyte lysate system using polyadenylic acid [poly(A)]-enriched RNA. The product was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, precipitation by specific λ-globulin for α-amylase, and proteolysis. Two immunoprecipitated products were synthesized from the readout system, the predominant species migrating coincidentally with authentic α-amylase on sodium dodecyl sulfate-polyacrylamide gels. A putative precursor, 1,500 daltons larger, was evident but was less abundant. The relationship between the two polypeptides was established by proteolytic analysis using *Staphylococcus aureus* V8 protease. At least nine fragments were generated and were identical in both species. The poly(A)-enriched RNA synthesized only the putative precursor in the reticulocyte lysate system. Attempts to process the precursor to the mature size of α-amylase failed. These findings are discussed in connection with the signal hypothesis (proposed for the transport of proteins across membranes) and the mode of secretion of α-amylase in aleurone cells.

Gibberellins are known to induce *de novo* synthesis of α-amylase in cereal aleurone layers, however, the mechanism by which α-amylase is released from the cereal aleurone layers is not understood (8, 28, 29 and refs. cited therein). Using conventional biochemical techniques, a high percentage of the cellular α-amylases are found in a particulate fraction which might function as secretory organelles (18–20, 36). These observations do not prove that the secretion of α-amylase is membrane-mediated. Inasmuch as the synthesis of α-amylase occurs on polysomes (16) associated with the rough ER (11, 29, 46), vesiculation of the ER membranes during homogenization of the aleurone layers could explain the particulate association of α-amylase.

A soluble mode of secretion for α-amylase has been proposed (27), in contrast to secretory organelles. By autoradiographic (6) and immunohistochemical studies (29), no evidence was found for the participation of secretory organelles in mediating α-amylase release. Jones (28) concluded that gibberellic acid may selectively change the permeability of the plasma membrane, thereby affecting the secretion of α-amylase.

Recently (3) a new hypothesis has been proposed which explains how polypeptides may be transported across membranes. This paradigm, the signal hypothesis (3), postulates that the mRNAs of polypeptides destined to be secreted or packaged contain a set of codons on the 5′-terminal which code for a unique sequence of 15 to 30 hydrophobic amino acids (signal) (13). The emergence of this signal during translation triggers the attachment of the ribosome to the membrane and directs the growing chain vectorially across the membrane. The signal is then removed from the polypeptide (precursor) by proteolytic cleavage during or after displacement.

The signal hypothesis has been tested successfully in vertebrates (4, 24, and refs. cited therein) and bacteria (24). In plants, a precursor for the small subunit of RuDP carboxylase has been demonstrated (14, 22) but apparently shares few properties in common with the signal hypothesis (22). In light of the growing acceptance of the signal hypothesis, we studied the α-amylase product synthesized from two *in vitro* translation systems. Although this protein previously had been synthesized in a wheat germ translation system (21), it was not clear whether the final product was a precursor of α-amylase or α-amylase itself. We show here that a putative precursor of α-amylase which is 1,500 daltons larger than the secreted form, may exist. Both the putative precursor and secreted form of α-amylase are present in a readout system when polysomes are used as mRNA source, while only the putative precursor is synthesized in a mRNA-dependent reticulocyte lysate translation system. Treatment of putative precursor and secreted form of α-amylase with micrococcal protease generates at least nine fragments which are identical in both forms.

MATERIALS AND METHODS

Tissue Preparation and Incubation. Fifty g of deembryonated wheat seeds (*Triticum aestivum* cv. Chinese Spring and Anza) were surface-sterilized using 1% hypochlorite and imbibed in sterile H_2O for 36 hr. Aleurone layers were isolated by gently crushing the seeds using a sterile pestle and removing the starch by washing repeatedly in sterile H_2O. The layers were then incubated in 10^{-5} M GA_3 buffer solution (9) with gentle shaking for 15 to 21 hr at room temperature.

Purification of α-Amylase. α-Amylase was purified using a modified procedure of Chen and Jones (7). Protein secreted from half-seeds was heat-treated and fractionated by ammonium sulfate (7). α-Amylase was removed from the bulk protein by affinity chromatography (43) on a cycloheptaamylose Sepharose 6B column (8 × 1.5 cm). After washing the column with 5 volumes of 50 mM Na-acetate (pH 5.5) containing 1 mM $CaCl_2$, the enzyme was eluted using a buffer containing 6 mg/ml of β-cycloheptaamylose. Fractions containing enzyme activity were pooled and dialyzed against 50 mM Tris-HCl (pH 7.8) containing 50 mM NaCl and 1 mM $CaCl_2$. α-Amylase was resolved from the β-cycloheptaamylose by passing the enzyme through the Sephadex A-50 column (10 × 1 cm), with subsequent elution of the enzyme with 50 mM Tris-HCl (pH 7.8) containing 0.5 M NaCl and 1 mM $CaCl_2$. Fractions

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were collected, dialyzed against the acetate buffer described above, and stored at −12 C. ^{3}H-labeled α-amylase was obtained by incubating 200 aleurone cells in 10 ml of medium containing 20 μCi/ml of [^{3}H]leucine (56 Ci/mmol) after the 6th hr of incubation with GA. Aleurone layers were labeled for an additional 16 hr at which time the medium was collected and α-amylase purified as described above.

Isolation of Polysomes. Aleurone layers were washed four times with sterile H_2O and blotted dry between two layers of sterile paper towels. Polysomes were isolated using the procedure described by Larkins *et al.* (32) except that buffer A contained 200 mM Tris-HCl (pH 8.8), 200 mM sucrose, 35 mM Mg-acetate, 200 mM KCl, 25 mM Na_2EGTA, and 1 mM DTT. Aleurone layers were frozen with liquid N_2 and pulverized to a fine powder using a mortar and pestle. Buffer A (120 ml) was then added and the sample ground for an additional 5 min. After thawing, the homogenate was made 1% (w/v) with respect to Triton X-100 and DOC,[4] filtered through four layers of cheesecloth and then centrifuged at 35,000*g* for 10 min. The resulting supernatant fluid was layered over 3 ml of buffer B (50 mM Tris-HCl [pH 8.5], 200 mM KCl, 30 mM Mg-acetate, 5 mM Na_2EGTA, and 1.8 M sucrose) and centrifuged at 150,000*g* for 3.5 hr. The polysomal pellets were washed three times with sterile H_2O. In some experiments, the detergents were deleted; a membrane and free polysome fractions were collected by centrifuging at 35,000*g* for 15 min. The membranes were resuspended, treated with detergents, and the polysomes isolated as described above.

Preparation of IgG Specific for α-Amylase. Antibodies against α-amylase (cv. Chinese Spring) were produced in female rabbits. After control serum was obtained, a single injection containing an equal volume of protein and Freunds' complete adjuvant was given above the scapula in 4 successive weeks at a dosage of 70 μg α-amylase/kg. IgG was partially purified using ammonium sulfate precipitation and chromatography on DEAE-Sephadex (23).

Isolation of Poly(A)-containing RNA. Polysomes were resuspended in 10 mM Tris-HCl (pH 7.5) containing 0.5% (w/v) SDS and 0.5 M NaCl. The suspension was centrifuged at 12,000*g* for 10 min and the RNA passed through a 1-ml oligo(dT)-cellulose column (1), washed with 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl, and eluted with sterile H_2O. The poly(A)-enriched RNA was made 200 mM with respect to NaCl and then precipitated with 2 volumes of 95% (v/v) ethanol.

***In Vitro* Protein Synthesis: Readout System.** A S-150 fraction was prepared from wheat germ by centrifuging the S-30 fraction (37) at 150,000*g* for 1 hr. The S-150 fraction was divided into small aliquots and stored frozen at −70 C. Before use, the S-150 fraction was treated with micrococcal nuclease using the procedure of Pelham and Jackson (39). Protein synthesis was carried out using 100-μl samples containing 20 mM HEPES-KOH (pH 6.8), 1 mM DTT, 2.5 mM Mg-acetate, 90 mM KCl, 1 mM ATP, 0.1 mM GTP, 8 mM creatine phosphate, 6 μg creatine phosphokinase, 25 μCi [^{14}C]leucine (330 Ci/mol) or 5 μCi [^{35}S]methionine (770–1,100 Ci/mmol) and 80 to 150 μg polysomes. The reaction was run at 30 C for 60 min.

***In Vitro* Protein Synthesis: Initiation System.** A rabbit reticulocyte lysate was prepared by the procedure of Evans and Lingrel (15), and treated with micrococcal nuclease before use (39). The reaction mixture (100 μl) contained 70 μl lysate, 10 mM HEPES-KOH (pH 7.0), 1 mM DTT, 1.5 mM ATP, 0.5 mM GTP, 18.5 mM creatine phosphate, 37 μg creatine phosphokinase, 0.5 mM glucose-6-P, 2.5 mM Mg-acetate, 80 mM KCl, 25 μM each of 19 amino acids, 0.04 OD_{260} wheat germ-deacylated tRNA, 25 μM hemin, and 5 μCi [^{35}S]methionine. Two to 3 μg of poly(A)-containing RNA were added and the reaction allowed to proceed for periods up to 90 min at 27 C.

Analysis of the Products from the *in Vitro* Protein Synthesis. The reactions were terminated by placing them on ice. Total [^{35}S]methionine incorporation was measured by precipitating a 5-μl aliquot with 2 ml chilled 5% (w/v) trichloroacetic acid. The precipitates were incubated successively for 5 min each at 0, 90, and 0 C in 5% (w/v) trichloroacetic acid, and were then collected on glass fiber filters. The filters were washed extensively with 5% (w/v) trichloroacetic acid, dried under vacuum, and counted in a Packard liquid scintillation spectrometer.

The total reaction products were prepared for SDS-PAGE by precipitating an aliquot in 5% (w/v) trichloroacetic acid and placing it on ice for at least 10 min. After centrifugation, the precipitate was collected, washed with H_2O, and then resuspended in 0.125 M Tris-HCl (pH 8.3), 1% (w/v) SDS, 5% (w/v) mercaptoethanol, and 5% (v/v) glycerol. The samples were placed in a boiling H_2O bath for 2 min before analysis by SDS-PAGE.

To test for specific α-amylase synthesis, a double immunoprecipitation procedure was used. Samples from the readout system were made 1% each with Triton X-100 and DOC and centrifuged at 100,000*g* for 1 hr to remove the ribosomes and membranes, whereas it was not necessary to centrifuge the reactions from the mRNA-dependent system. Rabbit anti-α-amylase IgG (20 μg) was added to the supernatant fluid and incubated at room temperature for 30 min. Goat anti-rabbit IgG (Calbiochem) was added and the mixture placed in a refrigerator overnight. The immunoprecipitate was collected as described by Rhoads *et al.* (40). After centrifugation, the upper layer was removed and the top of the lower phase repeatedly washed with phosphate-buffered saline (40). The bottom layer was then carefully removed and the immunoprecipitate washed several times. The immunoprecipitate was resuspended in SDS sample buffer (32), heated and analyzed by SDS-PAGE.

SDS-Polyacrylamide Slab Gel Electrophoresis. The products from the *in vitro* translation system were resolved on 10% gels (1 mm thick) in the buffer system described by Laemmli (32). The duration of the electrophoresis was normally about 7 hr, 1 hr longer than it took the tracking dye to reach the bottom of the gel. The gels were routinely stained with Coomassie blue (17) and fluorographed (5, 35) by exposure to Kodak Royal RP X-Omat film for 24 hr to 2 weeks.

Limited Proteolysis of α-Amylase. The *in vitro* translated α-amylase was treated *in situ* with *Staphylococcus aureus* V8 protease and resolved on 15% gels (10) as follows. Trichloroacetic acid precipitate or immunoprecipitates of the reaction mixtures with the addition of 2 μg authentic α-amylase were resolved on 10% gels, stained, and dried. The radioactive α-amylase was then located by autoradiography and removed from the gel using a razor blade. The sliced gel was rehydrated in SDS sample buffer (32) for 10 min and then positioned on top of a slab gel containing a 15% acrylamide resolving gel. Purified α-amylase (5 μg) in SDS buffer containing 20% glycerol was layered over the sliced gel followed by the addition of 1 μg protease. Electrophoresis was initially run at 5 mamp until the tracking dye neared the end of the stacking gel, at which time the current was turned off. After 30- or 50-min digestion, electrophoresis was resumed. The fragments generated by proteolytic treatment were resolved, stained for protein, and fluorographed.

RESULTS

Purification of Secreted Form of α-Amylase. Assuming no loss of α-amylase activity during the heat treatment, α-amylase constitutes about 3 to 6% of the total protein present in the GA_3 incubation medium. Using the purification protocol outlined under "Methods," an apparently homogeneous preparation is ob-

[4] Abbreviations: DOC: sodium deoxycholate; SDS-PAG: sodium dodecyl sulfate-polyacrylamide gel; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IgG: immuno λ-globulin; poly(A): polyadenylic acid.

tained with about a 16- to 33-fold purification. This purified preparation contained at least four activities as resolved by PAGE under nondenaturing conditions.

Analysis by SDS-PAGE (Fig. 1) revealed that the purified enzyme has an apparent mol wt of about 42,000 daltons, in agreement with the published value (45). A positive periodic-Schiff reaction was also observed on SDS-gels, coincident with α-amylase, suggesting the enzyme is a glycoprotein. Similar results have been observed for the enzyme found in barley aleurone layers (29).

Properties of Specific IgG to α-Amylase. Antibodies specific against α-amylase were generated by injecting the purified enzyme from cv. Chinese Spring. We were unable to obtain sufficient amounts of this cultivar to complete this study; in subsequent investigations, the Anza cultivar was used. The IgG preparation from Chinese Spring reacts with the Anza-derived protein, giving a single line in Ouchterlony double diffusion tests (Fig. 2). Analysis of the activities derived from the two cultivars by rocket immunoelectrophoresis (not shown) shows that the two activities shared common antigenic determinants. Two bands were evident in both preparations—a large faint band and a smaller but more intense second band. The enzyme derived from Anza was judged to be only about half as reactive as that from Chinese Spring when the areas under the peaks were compared. Two immunodistinct α-amylases have also been observed in barley aleurone layers (26, 29).

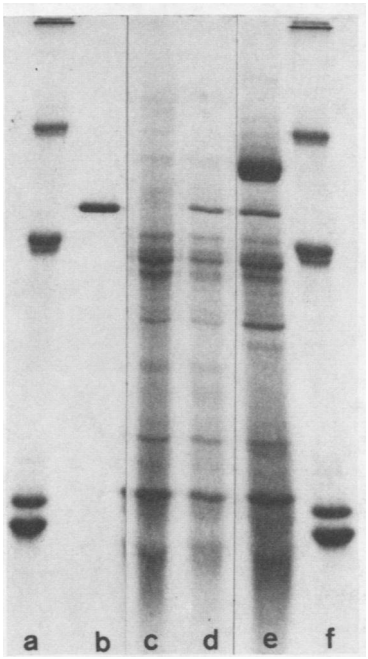

FIG. 1. SDS-PAGE patterns of protein fractions collected during purification of α-amylase. a and f: Protein standards: BSA (mol wt, 68,000); yeast alcohol dehydrogenase (mol wt, 37,000); lysozyme (mol wt, = 14,300); and Cyt *c* (mol wt, 11,700); b: 5 μg purified α-amylase; c: 20 μg void fraction of cycloheptaamylose-Sepharose chromatography; d: 20 μg 30 to 60% $(NH_4)_2SO_4$ precipitate; e: 20 μg heat-treated medium.

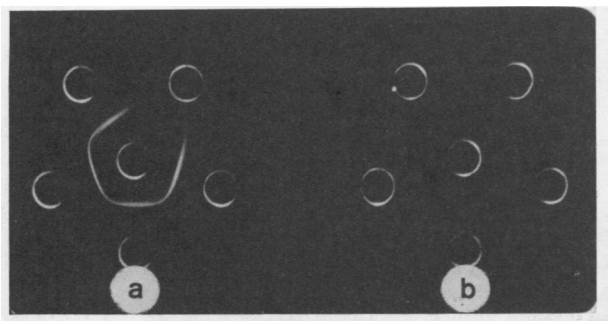

FIG. 2. Immunodiffusion test of α-amylase from cv. Chinese Spring and Anza. Purified α-amylase from Chinese Spring and Anza cultivars was tested against monospecific anti-α-amylase produced by injection into rabbits of α-amylase from Chinese Spring (a) and control serum (b).

Polysome Profiles from Wheat Aleurone. Polysome profiles of the wheat aleurone show a high proportion of the ribosomes (greater than 75%) in polysome form, with a maximum in the hexamer to heptamer region for the free polysomes and in the heptamer-octomer region for the membrane-bound polysomes (Fig. 3). There was always present in both fractions a second group of peaks near the bottom of the gradient. It is not known whether these peaks reflect higher order polysomes or co-sedimentation of membrane-type material which is present in both fractions even after detergent treatment. Tests with ribonuclease were not performed to resolve this problem. Only about 10% of the polysomes (as measured at OD_{260}) was found in the membrane fraction.

Polysome Readout System. In this system, nascent chains associated with the polysomes are completed. Very little initiation of mRNA occurs as indicated by the slight inhibition (18%) of amino acid incorporation by 20 μM aurintricarboxylic acid, a specific inhibitor of initiation when used at low concentrations. The rate of incorporation falls rapidly after 15 min of incubation at 30 C; further incubations up to 2 hr increase the total amount of incorporation by 10%. The optimum conditions for the readout system were similar to those reported previously (33, 44). One exception is the relatively low pH of 6.8 for maximum amino acid incorporation as compared to the values reported elsewhere (33, 44).

Analysis of Products of Readout System. Centrifugation of the 10 mM EDTA-treated reaction mixtures at 100,000*g* for 1 hr pelleted about 25% of the radioactive incorporated material. The majority of this radioactive material sedimented at 20,000*g*. Analysis of these fractions by SDS-PAGE indicated little difference in the polypeptide pattern between the bound and the released products.

When the products of the readout system are tested against the IgG specific for α-amylase, only a single radioactive band is observed on SDS-PAG (Fig. 4). This polypeptide appears to migrate more slowly than authentic α-amylase (Fig. 4b). However, mixing the two preparations results in a single polypeptide band (Fig. 4c). The apparent difference in migration of authentic and *in vitro* translated α-amylase is due to the larger quantity of authentic protein on these gels. Analysis of the total products from the readout system suggests that α-amylase is one of the major polypeptides synthesized in this system (Figs. 4d and 5, a and c). Neither the addition of control IgG to the reaction products nor monospecific anti-α-amylase IgG to control reaction mixtures revealed any polypeptides on SDS-PAG.

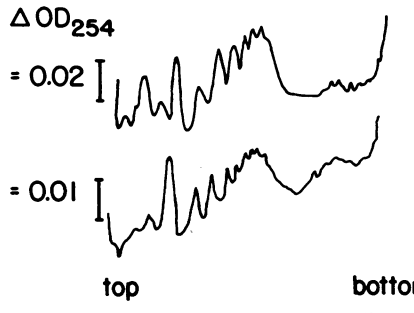

FIG. 3. Polysome profiles of wheat aleurone cells. Free (upper) and membrane-bound (lower) polysomes were isolated by sedimentation. Resuspended polysomes were layered onto a 15 to 60% (w/v) sucrose gradient and centrifuged for 50 min at 48,000 rpm at 4 C in a SW 56 rotor. Gradients were analyzed by a ISCO UA5 UV monitor.

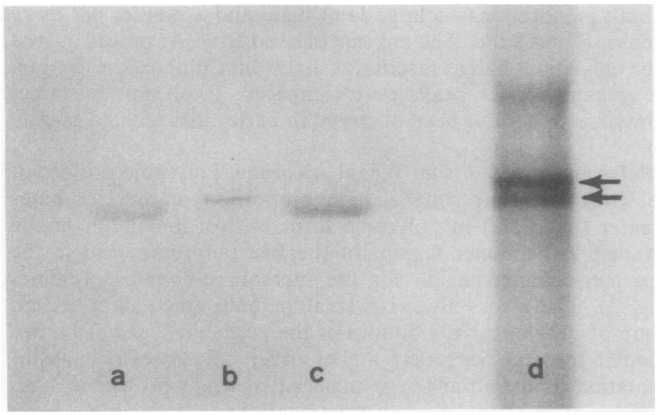

FIG. 4. Fluorography of SDS-PAG containing reaction products from wheat germ readout system. Total polysome fraction was used as mRNA source and incubated with a S-150 wheat germ fraction. Reaction mixture was divided into two parts, one treated with IgG specific for α-amylase and the remainder precipitated with 5% (w/v) trichloroacetic acid. a: Authentic ^{3}H-labeled α-amylase; b: antibody precipitate of α-amylase; c: mixture of a and b; d: trichloroacetic acid precipitate. About 50% of d is shown indicating doublet band (arrows) present in the total reaction mixture. The smaller polypeptide co-migrates with authentic α-amylase while the larger species is the putative precursor of α-amylase.

Analysis of the synthesized polypeptide by protease treatment *in situ* on SDS-PAG and fluorography shows the generation of at least nine distinct fragments in a characteristic pattern (Fig. 5a). All fragments coincided with the products obtained with authentic α-amylase. Thus, based on: (*a*) migration coincident with authentic α-amylase, (*b*) immunoprecipitation by monospecific IgG, and (*c*) generation of identical proteolytic fragments with authentic enzyme, we conclude that the 42,000-dalton polypeptide synthesized by the readout system is α-amylase.

According to the signal hypothesis (3), both the precursor and processed form of the polypeptides destined to be secreted or packaged can be synthesized in the readout system. When the amount of IgG-precipitated α-amylase is increased 4-fold on SDS-PAG, a less intense radioactive band is observed. This polypeptide is about 1,500 daltons larger than the major band that migrates coincidentally with authentic α-amylase (Fig. 5b), and may be the precursor of the secreted form. Analysis of the total reaction products reveals a major polypeptide with the same mol wt as the putative precursor (Figs. 4d and 5, a and c). The putative precursor is present as judged by the intensity of the bands in the same relative amounts as the secreted form.

To test for the possible relationship between the two polypeptides, the bands were treated with *S. aureus* V8 protease and the fragments resolved by SDS-PAGE (Fig. 6, b and c). The fragments generated are identical in both forms, supporting but not proving the existence of a precursor form of α-amylase.

mRNA-dependent System. Because the reticulocyte lysate is free of ER membranes which are implicated in the processing of polypeptides destined to be secreted or packaged (4), we chose it as the translation system for further study of poly(A)-enriched RNA.

Analysis of Reaction Products of mRNA-dependent System. When the reaction products are tested against the monospecific IgG against α-amylase, a single polypeptide is evident on SDS-PAG (Fig. 7a). The polypeptide is slightly larger (1,500 daltons) than the primary IgG-precipitated product synthesized from the readout system (Fig. 7b) and corresponds to the putative precursor observed in the readout system. This polypeptide accounts for 23 to 28% of the total incorporated radioactivity. Mixing the monospecific precipitated products from both the mRNA-dependent and readout system readily reveals the discrepancy in mol wt between the two forms (Fig. 7c). The pattern of proteolytic fragments of the polypeptide from mRNA-dependent system (Fig. 6d) is identical to that obtained from the putative precursor of readout system (Fig. 6c).

Analysis of the total reaction products on SDS-PAG reveals only a single major polypeptide which migrates identically with the IgG-precipitated product (Fig. 4d). The pattern of total polypeptides resolved on SDS-PAG shows marked differences compared to that obtained from the readout system. This difference could be due to selective loss of mRNA by fractionation on the oligo(dT)-cellulose column, differences in initiation and/or elongation rates (2) of mRNAs, or both.

We have attempted to demonstrate the processing of the presumptive precursor *in vivo*. The signal hypothesis (3) predicts that the processing activity resides in the RER and this has been substantiated (25, 42). Microsomal membranes enriched in RER were obtained from aleurone layers incubated for 15 and 21 hr in GA_3 by layering the 12,000*g* supernatant fluid over 1.5 M sucrose. The membranes were collected by centrifugation at 150,000*g* for 3 hr and stripping them of ribosomes by treating with EDTA (4, 30). Addition of these membranes during or after translation in the mRNA-dependent system failed to catalyze the conversion of

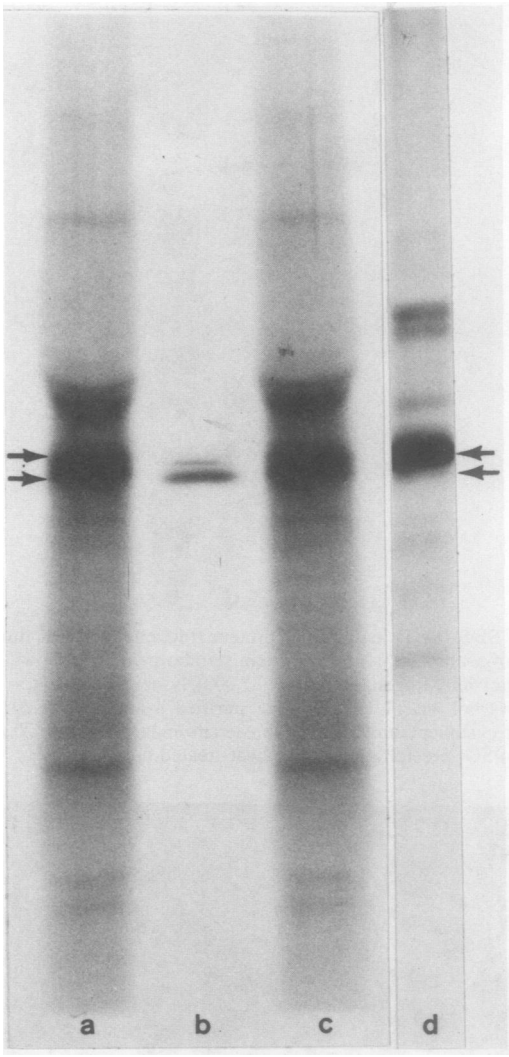

FIG. 5. Fluorography of SDS-PAG containing reaction products from readout and mRNA-dependent system. For details, see legend to Figure 4. a and c: Trichloroacetic acid precipitates of readout system; b: antibody precipitation of α-amylase from readout system; d: trichloroacetic acid precipitate from mRNA-dependent system. Arrows indicate positions of putative precursor and secreted form of α-amylase.

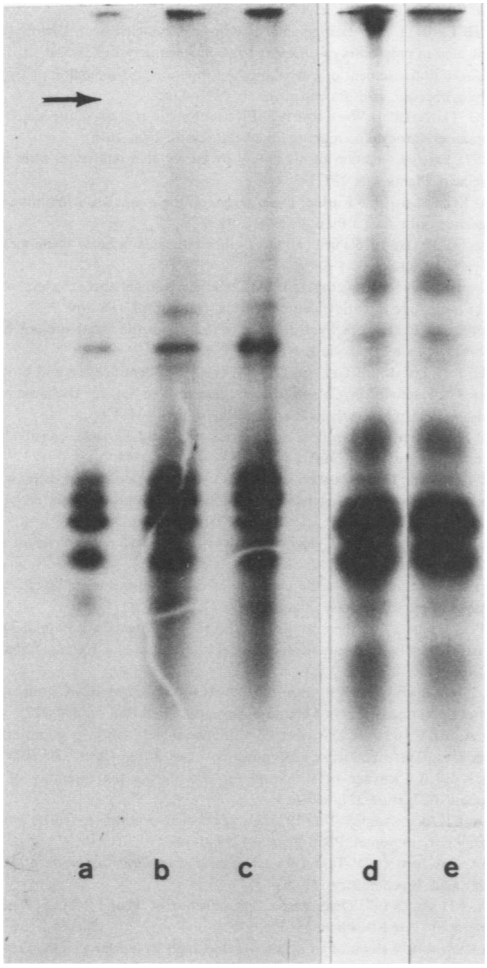

FIG. 6. Limited proteolytic digestion of putative precursor and secreted form of α-amylase. Trichloroacetic acid and antibody precipitates were resolved by SDS-PAGE, dried, and exposed directly to x-ray film. Bands containing putative precursor were sliced from gel containing trichloroacetic acid precipitate (Fig. 5, a and c) while the secreted form of α-amylase was obtained from both the trichloroacetic acid and antibody precipitate sections of the gel. Sliced gels were then treated *in situ* with *S. aureus* V8 protease, stained with Coomassie blue, dried, and exposed to x-ray film for fluorography. Limited proteolytic digestion patterns of a: secreted form of α-amylase from antibody precipitate of readout system; b: secreted form of α-amylase from trichloroacetic acid precipitate of readout system; c and e: putative precursor of α-amylase from trichloroacetic acid precipitate of readout system; d: putative precursor of α-amylase from antibody precipitate of mRNA-dependent system. Parts a to c were incubated with protease *in situ* on polyacrylamide gels for 30 min while parts d and e were incubated for 50 min. Part a contains only about half as much radioactivity as parts b and c, so only the major fragments are evident in this photograph. Three faint fragments are observed on x-ray film and are coincident with fragments observed in parts b and c. Arrow indicates position of untreated α-amylase.

precursor to secreted form. Addition of 0.03 to 0.15 OD_{280}/ml to reaction mixture caused about a 25 to 65% reduction in the incorporation.

DISCUSSION

The signal hypothesis accounts for the selective synthesis of polypeptides on membranes and their transport (3). The hypothesis predicts that the initial product destined to be secreted or packaged should be larger in mol wt (based on amino acid composition alone) than the secreted form. The extra segment of amino acids (the signal) in the precursor form contributes to the

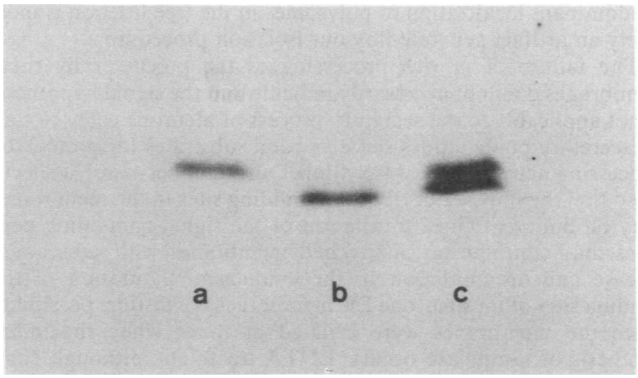

FIG. 7. Fluorography and SDS-PAGE of reaction products from initiation system. Poly(A)-enriched RNA was used to program a mRNA-dependent reticulocyte lysate. For other details, see legend to Figure 4. a: Antibody precipitate of α-amylase from initiation system; b: antibody precipitate of α-amylase from readout system; c: mixture of a and b.

binding of the ribosome to the membrane and transport. If the signal hypothesis can be extended to higher plants, then the mode of secretion of α-amylase consists of membrane-mediated events analogous to the pattern established in animal cells (38). α-Amylase is synthesized on RER (29), transported to the lumen of ER and glycosylated (12). The proteins are either transferred to the Golgi where they accumulate in secretory vesicles (19, 20) or are packaged directly by RER (46) with subsequent discharge across the plasma membrane.

The data presented suggest that a possible precursor for α-amylase which is about 1,500 daltons larger in mol wt than authentic α-amylase (Figs. 5b and 7a) may exist and be synthesized in the mRNA-dependent system. In the readout system in which polysomes were used as the mRNA source, evidence was presented for the occurrence of both the putative precursor and the secreted form. The close relationship between the two forms is indicated by their precipitation by monospecific IgG and the identical pattern of fragments obtained from proteolytic treatment. The presence of both forms in the readout system is predicted by the signal hypothesis in that ribosomes which had just initiated translation should contain unprocessed nascent chains while those near the 3'-end of mRNA should be devoid of them as a result of proteolytic processing (3).

One anomaly in our results is the selective precipitation of the putative precursor in the readout system: only trace amounts are detected while analysis of the total reaction products indicates that the precursor and secreted form are present in about equal quantities (Fig. 4d). It is clear that the monospecific IgG can recognize the putative precursor as indicated by the precipitation of the polypeptide in the mRNA-dependent system (Fig. 7a). IgG precipitation of a noncentrifuged or 20,000*g* centrifuged readout reaction mixture yields a radioactive polypeptide pattern on SDS-PAG almost identical to the pattern of total reaction products and 20,000*g* pellet. This observation suggests that α-amylase, particularly the putative precursor, is complexed with membranes. Such an association would be accounted for by the signal hypothesis (3) and would explain the contamination of the polysome fraction by membranes (see below).

Under our isolation conditions, about 90% of the total polysomes reside in the free polysomal fraction. This probably does not reflect the *in vivo* condition. The free polysome fraction contained membrane-type material in spite of the detergent treatment and at least 50% of the OD_{260} units of the free polysome fraction sediments at 20,000*g* for 10 min. In addition, we could detect no major differences in the polysomes synthesized in the readout system when either free or membrane-bound polysomes were tested. Jones and Chen (29), who used immunohistochemical methods, observed that α-amylase is synthesized on RER. The

predominant localization of polysomes in the free fraction is most likely an artifact generated by our isolation procedure.

The failure of *in vivo* processing of the precursor by these membranes does not necessarily indicate that the signal hypothesis is not applicable to the secretory process of aleurone cells. Not all presecretory polypeptides serve as good substrates for proteolytic processing activity (25). Also, Blobel and Dobberstein (3) speculated that the number of ribosome-binding sites in the membranes may be limiting. Thus, translation of the signal-containing peptides may continue on unattached membranes with subsequent release and accumulation in the cytoplasm. Saturation of the binding sites of the aleurone ER membranes is a distinct possibility since the membranes were isolated at times when maximum synthesis of α-amylase occurs. EDTA treatment, although completely dissociating the ribosome subunits, only partially dissociates the large subunit from the membrane (30, 41). Saturation of the binding sites would account not only for our ability to demonstrate *in vitro* processing of the precursor, but also for the localization of α-amylase in the cytoplasm of GA_3-treated aleurone cells (29), which is the primary evidence for a soluble mode of secretion.

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