In Vitro Synthesis and Processing of Wheat α-Amylase

TRANSLATION OF GIBBERELLIC ACID-INDUCED WHEAT ALEURONE LAYER RNA BY WHEAT GERM AND XENOPUS LAEVIS OOCYTE SYSTEMS

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ABSTRACT

Wheat (Triticum aestivum) RNA was used to program synthesis of the α-amylase protein by Xenopus laevis oocytes. A 41,500-dalton protein was made which was identified as α-amylase by immunoprecipitation with rabbit anti-α-amylase antiserum raised against the purified wheat protein and by its co-migration with authentic α-amylase on sodium dodecyl sulfate polyacrylamide gels. Synthesis of α-amylase was dependent upon injection of RNA extracted from gibberellic acid-induced aleurone layers from wheat. The amount of α-amylase produced was proportional to the amount of RNA injected and reached a plateau within 4 hours after injection. When the same RNA was translated in a wheat germ cell-free translation system, a 43,000-dalton protein was produced. Addition of dog pancreas microsomal membranes to the wheat germ translation system resulted in processing of the α-amylase protein to a form which co-migrated with authentic α-amylase purified from malted wheat and with the protein synthesized in oocytes.

During seed germination, the aleurone layer of wheat becomes a major target for hormone-induced production of several enzymes involved in the breakdown of stored reserves (for review see 15, 43). GA3 has been shown to act on isolated aleurone layers to induce synthesis of large amounts of α-amylase (11, 27). This GA3-induced system can secrete the newly synthesized α-amylase into the incubation medium.

It has been shown previously that α-amylase can be synthesized in vitro in both wheat germ and reticulocyte lysate cell-free translation systems (14, 28, 31). Production of α-amylase in these systems is dependent upon addition of mRNA from GA3-induced aleurone layers. The α-amylase protein synthesized in these systems is approximately 1500 daltons larger than authentic α-amylase and may represent a precursor form of the protein which does not normally accumulate in vivo (28, 31).

There are numerous precedents for the existence of larger forms of secreted proteins. Blobel and associates (6, 19) have shown that an NH2-terminal “signal sequence” directs secretory proteins into the lumen of the ER. This sequence is cleaved co-translationally producing a smaller product. In addition, glycosylation also appears to occur co-translationally as the protein passes through the membrane of the ER. In vitro systems have been developed which are able to cleave signal peptides and glycosylate proteins to the same extent as their in vivo counterparts. These systems consist of stripped microsomal membranes and they act on nascent protein chains as they are being synthesized in in vitro cell-free translation systems (3, 6, 17).

Another system for studying protein processing is Xenopus oocytes. Injection of RNAs into Xenopus oocytes programs translation and processing of RNAs from a variety of heterologous sources. Translation products found in Xenopus oocytes after injection of RNAs of plant origin include phenylalanine ammonia-lyase and flavanone synthase of parsley and storage proteins of maize (16, 25, 37). Animal mRNAs are also translated in Xenopus oocytes as well as mRNAs for secretory proteins from Dictyostelium discoideum and a wide variety of viral proteins (10, 26, 42). Post-translational processing of both plant and animals proteins has been demonstrated (1, 23, 25, 34) and zein proteins have been shown to be compartmentalized within the membrane vesicles of oocytes (16).

To resolve the question of whether or not α-amylase is processed, we employed both of these processing systems: the Xenopus oocyte translation-processing system and a coupled wheat germ translation-dog pancreas stripped microsomal membrane-processing system. We report here the results obtained with these two systems and show that both produce a processed α-amylase indistinguishable from the one made in wheat aleurone layers.

MATERIALS AND METHODS

RNA Preparation. GA3-induced RNA was purified from aleurone layers after 24 h incubation in the presence of 20 mM Na succinate, 20 mM CaCl2, 0.2% (w/v) chloramphenicol, and 2 µM GA3 (Sigma) using the procedure of Click and Hackett (7) as modified by Jacobsen and Zvar (18). RNA from noninduced aleurone layers was prepared in the same way except that GA3 was omitted from the incubation buffer. The RNA was stored in H2O at -70°C after it had been heated to 55°C for 5 min to disperse aggregates.

Antibody Preparation. α-Amylase was purified from malted wheat by the method of Kruger (21). Antiserum was raised in a male New Zealand white rabbit by subcutaneously injecting 25 µg α-amylase and 0.5 ml Freund's complete adjuvant. After 2 weeks, the rabbit was boosted intravenously with 20 µg purified α-amylase. Blood was drawn from the rabbit at biweekly intervals thereafter, allowed to clot at 4°C overnight, and centrifuged to remove the clot. The resulting supernatant was used in the experiments presented here. Five ml serum was used per 10 oocytes.

Experimental Animals. Adult female frogs were obtained from Peter Fraser, 3101 Auburn Rd., Utica, MI 48087; or NASCO, Janesville Ave., Fort Atkinson, WI 53538. The frogs were maintained under laboratory conditions for at least 2 months prior to use. Females were given 500 units human chorionic gonadotropin (E. R. Squibb and Sons, Inc.) at least 3 weeks prior to laparotomy and were used within 3 months. Only animals which had oviposited were used for the isolation of oocytes.

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2 Present address: Armos Corp., 180 Kimball Way, So. San Francisco, CA 94080.
Treatment and Classification of Oocytes. Mature oocytes (stage 6) were obtained by anesthetizing a frog in 1% TMS (ethyl-maminobenzoate methyl sulfonate) (Sigma) for 10 to 20 min followed by surgically removing one to two ovarian lobes. The oocytes were washed in Hepes-modified Barth’s solution (MBS-H) (12) and then transferred to a Petri dish containing fresh MBS-H solution with 10 units K Penicillin G/ml and 10 μg benzyl P Streptomycin (sulfate)/ml.

Stage 6 oocytes (1–1.2 mm in diameter), identified by their unpigmented equatorial band, were separated from the ovarian tissue using watchmaker’s forceps and a 200-μl capillary glass micropipette. Isolated oocytes were then placed in fresh MBS-H and incubated at 19°C for 12 to 24 h prior to use.

Labeling of Oocytes Protein. Groups of 8 to 10 oocytes were microinjected with 50 nl each of a mixture containing 25 mCi/ml [35S]methionine plus 500 μg/ml purified RNA from GA2-induced aleurome layers from wheat. Exceptions are noted in figure legends. Controls included groups of 20 to 30 oocytes injected with either 50 nl 25 mCi/ml [35S]methionine alone, or 50 nl [35S]-methionine (25 mCi/ml) plus purified RNA (175 μg/ml) from uninduced aleurome layers of wheat. In all cases, the oocytes were subsequently incubated 16 to 24 h at 19°C in MBS-H.

Oocyte Analysis. Oocytes which had been microinjected and incubated were homogenized by repeated pipetting in 1.5-ml plastic tubes containing 0.5 ml RIPA buffer (0.15 M NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1% Trasylol [Aprotinin, Sigma]) and centrifuged 1 min in an Eppendorf microfuge. Pellets were resuspended in 0.3 ml RIPA and spun as above. Supernatants from the two spins were combined and centrifuged for 5 min. The resulting supernatants were incubated with premun- nate rabbit serum to remove proteins unrelated to α-amylase which cross-reacted with the rabbit antibodies. Staphylococcus aureus ghosts (Pansorbin, Calbiochem) were then added and the resulting precipitates pelleted through a 1.5 ml sucrose cushion (20). (In some cases pre-precipitations were done with S. aureus ghosts alone. The procedure used is indicated in figure legends.) Supernatants from this centrifugation were incubated with an excess amount of α-amylase antiserum for 15 min at room temperature followed by 30 min on ice. Antiserum was then precipitated with S. aureus ghosts, the pellets washed three times with 1 ml RIPA buffer, and the final pellet resuspended in 60 μl SDS sample buffer. Samples were heated at 90°C for 5 min to dissociate the S. aureus ghosts which were then removed by centrifugation. Fifty μl supernatant was run on 10% SDS-polyacrylamide gels (22). The gels were stained with Coomassie brilliant blue, de- stained in 7.5% acetic acid, prepared for fluorography by soaking in Enhance (New England Nuclear), dried, and fluorographed at −70°C on Kodak X-OMAT R film.

Competition Assays. Anti-α-amylase antiserum in 0.05 ml RIPA was preadsorbed 15 min at 0°C with increasing amounts of unlabeled α-amylase purified from malted wheat. This mixture was then added to homogenized oocytes and treated as previously described.

Quantitation of Precipitated Material. The region of the dried gel corresponding to the position of the α-amylase band on a fluorogram was excised and dissolved in 0.5 ml 30% H2O2. 0.2 ml 60% HClO4. Gel pieces were then counted in 10 ml Aquassure (New England Nuclear) by liquid scintillation spectrophotometry.

Wheat Germ Cell-Free Translation System. Wheat germ was kindly provided by Herb Sanguinetti, General Mills, Vallejo, CA. Extracts were prepared according to Roberts and Patterson (35) and treated with micrococcal nuclease as described by Pelham and Jackson (32). Fifty-μl reactions containing 75 μM K-acetate, 1.7 mM Mg-acetate, 20 mM Hepes, 0.8 mM spermidine, 1 mM ATP, 25 μM GTP, 0.5 mM DTT, 10 μl wheat germ extract, 100 μg/ml total RNA, and 10 to 20 μCi [35S]methionine (Amersham, specific radioactivity > 1000 Ci/mmol) were incubated at 24°C for 1 h. Reactions were stopped with EDTA. Immunoprecipitations were performed as described above. When stripped microsomal membranes were to be used in translation reactions, 4.5 units human placental RNase inhibitor were included in the reactions. In the absence of inhibitor, the wheat germ system is greatly inhibited (13, 38). Addition of inhibitor allows protein synthesis to proceed at half the protein level seen when no microsomes are included. Placental RNase inhibitor was prepared by the method of Blackburn (5) and was the generous gift of Biotec, Inc., Madison, WI.

Preparation of Stripped Microsomes. Microsomal membranes were isolated from canine pancreas as described by Katz et al. (19). Membranes were incubated 10 min with 30 units micrococcal nuclease to remove any endogenous RNA immediately before they were added to wheat germ translation reactions in amounts indicated in figure legends.

RESULTS

Synthesis of α-Amylase in Oocytes. RNA from GA2-induced wheat aleurome layers was injected into Xenopus laevis oocytes along with [35S]methionine to determine whether or not wheat RNA could program translation in oocytes. The oocytes were allowed to incubate for 16 h after which they were homogenized and immunoprecipitated. An initial precipitation with preimmune rabbit serum to remove any background binding between Xenopus and rabbit proteins not due to anti-α-amylase specific sites on the antiserum was followed by an immunoprecipitation with anti-α-amylase antiserum. A fluorogram of the immunoprecipitated products separated on 10% SDS-polyacrylamide gels is shown in Figure 1. Injection of the wheat RNA was responsible for production of the α-amylase protein as no band appears in the 41,500-
the intensity of the α-amylase band increases substantially when the amount of RNA injected was increased from 5 to 25 ng per oocyte (tracks 2 and 3, respectively) indicating that the amount of α-amylase synthesized is proportional to the amount of RNA injected. Increasing the amount of injected RNA above 25 ng per oocyte did not cause an increase in synthesis of α-amylase (data not shown).

We also injected oocytes with RNA from aleurone layers which had been incubated in buffer containing no GA$_3$. This RNA did not program α-amylase synthesis. This result was expected since no α-amylase is produced in cell-free translation systems programmed with RNA isolated from aleurone layers immediately after addition of GA$_3$ (GA$_3$-RNA) or after incubation without GA$_3$ (GA$_3$-RNA) (14, 28, R. Boston, unpublished results).

Figure 1b shows a fluorogram of the total [35S]methionine-labeled proteins found in oocytes injected with [35S]methionine and GA$_3$-RNA, GA$_3$-RNA, or buffer containing no RNA. No band appears darker in the GA$_3$-RNA track at the 41,500-dalton region than in the other tracks. The absence of a predominant band in this region indicates that α-amylase accounts for a small proportion of the proteins being synthesized in the oocyte.

**Competition of Oocyte Product with α-Amylase Isolated from Wheat.** If authentic α-amylase is being synthesized by the oocytes, one would expect purified wheat α-amylase to compete with the oocyte product for binding to the anti-α-amylase antiserum. The results of such a competition experiment are shown in Figure 2 where anti-α-amylase antiserum was first preadsorbed with α-amylase isolated from malted wheat and then used in the immunoprecipitation of the oocyte homogenates. Immunoprecipitated proteins were run on gels, excised, solubilized, and counted by liquid scintillation spectroscopy. All of the binding activity to anti-α-amylase antibody above that of control immunoprecipitations is competed out by the α-amylase from malted wheat. Although the amount of unlabeled α-amylase which must be added to prevent immunoprecipitation of α-amylase synthesized in oocytes is 1 to 10 µg, the amount of α-[35S]amylase is not this large. This is because we added excess antibody to all reactions to insure a high level of precipitation of the α-amylase in the oocyte homogenate.

**Time course of α-Amylase Synthesis in Oocytes.** The time course of α-amylase synthesis after injection of oocytes is shown in Figure 3. Oocytes were injected with GA$_3$-induced RNA, incubated for 0 to 48 h and α-amylase immunoprecipitated after a pre-precipitation with *S. aureus* ghosts. α-Amylase is detected as early as 30 min after injection. By 4 h, the level of α-amylase in the oocytes plateaus and remains constant for as long as 48 h.

As the time of incubation of oocytes is increased, a prominent band of mol wt 58,000 daltons appears. Precipitation of this protein is due to cross-reaction with normal rabbit serum rather than α-amylase specific sites on the antibody as the protein is removed by a precipitation with preimmune serum. In addition, synthesis of this protein is not dependent upon injection of GA$_3$-induced wheat RNA; it appears after long incubation in oocytes injected with RNA from uninduced aleurone layers or with [35S]methionine alone (data not shown).

**Processing of α-Amylase Synthesized in a Wheat Germ Translation System by Stripped Microsomal Membranes.** The protein products synthesized in a wheat germ translation system in the presence (tracks 2 and 3) and absence (track 1) of dog pancreas microsomes are compared in Figure 4. In the absence of microsomal membrane, α-amylase runs as a protein of 43 kd. Low amounts of microsomes produce a doublet with one band migrating as the 43-kd protein and the other as a 41,500-dalton protein. Further addition of microsomes results in disappearance of the larger band leaving only the 41,500-dalton protein. We assume this processing to be due mainly to cleavage of a signal peptide. Dog pancreas microsomes are known to have both glycosylation and signal peptidase activities. In the case of α-amylase, however, carbohydrate accounts for only 0.7% of the weight of the secreted protein, or less than 290 daltons (36, 41). This difference in mol wt probably would not be detectable on our gels.

**DISCUSSION**

Our results indicate that wheat α-amylase contains a signal peptide of approximately 1500 daltons. This peptide is cleaved from an α-amylase precursor by stripped microsomal membranes producing a protein of the same mol wt as α-amylase purified from malted wheat. Blobel and others have shown that secretory
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Our results show that for α-amylase, cleavage of the signal peptide probably accounts for most of the mol wt difference seen between in vivo and in vitro synthesized α-amylase. Stripped microsomal membranes produce a protein co-migrating with α-amylase purified from malted wheat. Likewise, a 41,500-dalton protein is synthesized in oocytes programmed with GA3-induced RNA. Injection of 1 μg/ml tunicamycin along with RNA and [35S]methionine does not affect the mol wt of the protein produced (data not shown). It has been reported that wheat α-amylase is not a glycoprotein (41), whereas barley α-amylase is glycosylated to an extent of 0.7% by weight (36). Our data are consistent with α-amylase having no glycosylation and being processed by cleavage of a 1,500-dalton peptide. Even if wheat α-amylase were glycosylated at the level seen for the barley protein, we would probably not detect the 290-dalton difference with our gel system.

Lane et al. (8, 23) have shown that oocytes programmed with heterologous RNA from secretory proteins are able to translate, process, and secrete these proteins into the incubation medium. RNA for nonsecretory proteins (e.g. globin) produce proteins which remain in the cytoplasm. It is likely that α-amylase is also being secreted from the oocytes in these experiments. Figure 3 shows a time course of α-amylase production and accumulation in oocytes over a 48-h period. The amount of α-amylase in the oocytes remains constant between 4 and 48 h. This could be due to early synthesis of a stable protein whose production stops after 4 h or to constant synthesis degradation rates between 4 and 48 h.

Stability of proteins synthesized in oocytes is quite variable ranging from t1/2 = 0.6 h, for guinea pig milk proteins to t1/2 = 22 h for chicken ovalbumin (24). For α-amylase however, it seems likely that after 4 h, α-amylase is being secreted into the medium preventing an accumulation of the protein in the oocyte.

This hypothesis is also consistent with data from in vivo synthesized α-amylase. Mozer has shown that α-amylase is present in the media containing GA3-induced aleurone layers as early as 5 h after induction (29). RNA isolated from tissue treated in the same way is able to program α-amylase synthesis in a wheat germ cell-free translation system as early as 4 h after induction of wheat aleurone layers (R. Boston, unpublished results). This suggests that as soon as mRNA for α-amylase is synthesized in vivo, it is translated into α-amylase and the α-amylase secreted shortly thereafter. In oocytes, RNA is available for translation immediately after injection, thus eliminating the 4-h lag time necessary for induction of transcription of the α-amylase gene seen in vivo. Attempts to detect α-amylase in the media of injected oocytes incubated for 4 and 8 h have been unsuccessful, although by 16 h, a small amount of α-amylase can be immunoprecipitated. We have not yet looked at later time points, however, nor have we determined the half-life of α-[35S]amylase within oocytes.

α-Amylase is known to exist in several isozymic forms. It is possible that these isozymes are generated by post-transcriptional modification. We have shown here that co-translational processing of α-amylase can occur but we have not examined our in vitro products before and after processing to see whether or not several isozymes are being synthesized and whether the same products are made by the oocyte system as by the wheat germ-dog pancreas microsome system. Our RNA preparation may contain a population of different mRNAs for α-amylase if each isozymeic form of α-amylase is encoded by a unique mRNA rather than being generated by post-translational modifications. Regardless of the origin of α-amylase isozymes, it is clear that all isozymes made and detected in our system can be cleaved from 43,000-dalton proteins to 41,500-dalton proteins.

We have shown that wheat RNA can be translated correctly in the Xenopus system. It has been shown previously that oocytes can
not only translate heterologous RNAs, but also transcribe DNAs from animals and viruses (9, 33). It is likely that this will hold true for plant DNAs. If so, it could prove a very useful system for identifying genes isolated and cloned into bacteria. If an entire gene had been isolated, its injection could program transcription and translation of the protein product (30, 40). This product might be identified by immunoprecipitation as described here or by activity as is the case of the cloned interferon gene (30). We think the oocyte system can be a useful system, not only for isolating and studying animal and virus genes, but also those from plants.

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