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Partial Purification and Characterization of the mRNA for α-Amylase from Barley Aleurone Layers[1]

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ABSTRACT

The poly(A)-containing mRNA from barley aleurone layers pretreated with gibberellic acid has been purified by phenol-chloroform extraction and repeated oligo[d(pT)]-cellulose chromatography. This RNA has been translated in both the wheat germ and reticulocyte lysate *in vitro* translation systems with greater than 50% of the synthesized protein being α-amylase. The mRNA for α-amylase has been further purified by dimethylsulfoxide-formamide-sucrose density gradient centrifugation and by gel electrophoresis. By these methods, its molecular weight has been determined to be 580,000.

In barley aleurone layers, the synthesis and secretion of several hydrolases begins as early as 6 h after the addition of GA_3 (6, 17, 18). One of these hydrolases, α-amylase, accounts for more than 60% of all *in vivo* protein synthesis in these tissues after 10–12 h of hormone treatment. The induction of this enzyme is inhibited by the addition of cordycepin, an RNA synthesis inhibitor in these tissues (9). Higgins *et al.* (8) have shown that during the first 10 h after addition of GA_3, the level of translatable mRNA for α-amylase increases to a maximum level and remains constant thereafter. However, they showed that only 12–25% of *in vitro* synthesized peptides were α-amylase (8). There is also evidence that the addition of GA_3 increases synthesis of poly(A) RNA in these tissues and the incorporation of added radiolabeled nucleosides into RNA in aleurone layers (12).

Poly(A) RNA was purified from barley aleurone layers after treatment with GA_3 for 17 h. This RNA has been used as a template in two optimized cell-free protein synthesis systems. The polypeptide corresponding to α-amylase accounts for a majority of the protein synthesis in both systems and it is larger than the *in vivo* synthesized protein. Further, the poly(A) RNA has been actionated by use of DMSO[3]-formamide sucrose density gradient centrifugation and by various gel electrophoresis processes. The mRNA for α-amylase is the dominant mRNA in these tissues, and these methods permitted an estimated mol wt to be assigned to this RNA.

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[3] Abbreviation: DMSO: dimethylsulfoxide.

MATERIALS AND METHODS

Sources of Seed and Chemicals. Barley seeds (*Hordeum vulgare* L. cv. Himalaya, 1974 crop) were supplied by the Department of Agronomy, Washington State University, Pullman, Washington, and were stored at 5 C. GA_3 and Sigmacell type 50 cellulose were purchased from Sigma. [4,5-^{3}H]L-Leucine (100 Ci/mmol) was obtained from Amersham Corporation, proteinase K was supplied by Beckman Instruments, and oligo[d(pT)]-cellulose T-1 was supplied by Boehringer-Mannheim Corporation.

Preparation and Treatment of Aleurone Layers. Embryoless half-seeds were surface sterilized and imbibed for 3 days (9). The half-seeds were then incubated for 17 h in 20 mM Na-succinate buffer (pH 5) containing 10 mM $CaCl_2$ and 2.5 μM GA_3 in a reciprocating metabolic shaker at 25 C. At approximately 8 h after initiation of incubation, the buffer solution was replaced with fresh hormone-containing solution.

RNA Extraction and Fractionation. Total RNA was extracted using procedures outlined by Higgins *et al.* (8) with extensive modifications. Routinely, 5,000 deembryonated seeds were used in a single isolation. The tissues were frozen in liquid N_2 and ground to a powder in a precooled Waring blender. A buffer containing 100 mM sodium glycinate (pH 9.5), 100 mM EDTA, 100 mM NaCl, 1% SDS, 1% polyvinylpolypyrrolidone, 5 mM 2',3'-AMP, 10 mM mercaptoethanol, and 100 μg/ml proteinase K was added to the powder. The homogenate was centrifuged, and the resulting supernatant was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v). One-tenth volume 1 M Na-acetate (pH 5.0) and enough acetic acid to lower the pH to 5.0 were added to the final aqueous phase, and the RNA and contaminating carbohydrate were precipitated with two and one-half volumes of ethanol.

From this crude RNA preparation, poly(A)-containing RNA was selected as follows. The RNA was collected as a pellet by centrifugation and resuspended into 20 mM Tris-HCl (pH 7.6), 0.5% SDS using a glass homogenizer. The resulting soluble material was heated to 60 C for 5 min and then rapidly cooled. An equal volume of 1 M Na-acetate was added, and the entire sample was applied to a blank cellulose column (Sigmacel, type 50). This step was necessary to remove a large quantity of carbohydrate which bound nonspecifically to cellulose in high salt and eluted off in low salt. The entire eluant was then applied to an oligo[d(pT)]-cellulose column, and the poly(A)-rich RNA was eluted with 10 mM Tris-HCl (pH 7.6). This RNA was precipitated overnight in 0.3 M ammonium acetate and 70% ethanol. This solution was centrifuged and the resulting RNA pellet dried under vacuum. The pellet was resuspended into 10 mM Tris-HCl (pH 7.6), 0.5% SDS, and 50% formamide, heated to 60 C for 5 min and cooled on ice. This solution was diluted 20-fold with 0.5 M NaCl, 10 mM Tris-HCl (pH 7.6), and 0.5% SDS and applied to a second oligo[d(pT)]-cellulose column. The poly(A)-rich RNA was again eluted off with the low salt buffer and precipitated in ethanol overnight.

DMSO-Formamide-Sucrose Density Gradient Centrifugation. Using methods described by R. Beachy (personal communication), the poly(A)-selected RNA was centrifuged, dried, and resuspended into 100 μl deionized H_2O. To this solution, 400 μl of a solution containing 95% DMSO, 4% deionized formamide, and 1% 1 M Tris-HCl (pH 7.4) containing 1 M LiCl and 100 mM EDTA (v/v/v) was added. This was heated to 60 C for 5 min, cooled, and applied to a sucrose density gradient. This gradient was prepared the previous day and consisted of successive layers of 5, 10, 15, and 20% sucrose in 95% DMSO, 4% formamide, and 1% buffer. The gradient was centrifuged in an SW 40 rotor at 40,000 rpm at 28 C for 48 h. The gradients were fractionated using an Isco automatic fractionator coupled with an UV monitor to determine *A* at 280 nm. Each fraction collected was ethanol precipitated overnight, centrifuged at 10,000*g* for 30 min, washed three times with 70% ethanol and 0.3 M ammonium acetate, and finally dried.

***In Vitro* Protein Translation Reactions.** The reticulocyte lysate system was prepared and used essentially as described by Pelham and Jackson (15). The final assay mixture of 25 μl contained 40 mM Hepes (pH 7.6), 80 mM K-acetate, 1 mM Mg-acetate, 0.5 mM spermidine, 2 mM DTT, 20 μg wheat germ tRNA, 60 μM amino acids minus leucine, 6.7 μM leucine, 50 μCi [^{3}H]leucine, and an energy source consisting of 1 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 3 mM phosphocreatine, and 20 μg/ml phosphocreatine kinase. The wheat germ extract was prepared as described by Bruening *et al.* (4). For the wheat germ *in vitro* translation system, the following concentrations of reagents were used: 41 mM Hepes (pH 7.6), 149 mM K-acetate, 10 mM KCl, 2.2 mM DTT, 2.24 mM Mg-acetate, 0.39 mM spermidine, 50 μM $CaCl_2$, 10 μM EDTA, 60 μM amino acids minus leucine, 6.7 μM leucine, 50 μCi [^{3}H]leucine, and the same energy source as above. All reactions were initiated by the addition of between 50 and 200 ng of RNA and were continued for 90 min. Trichloroacetic acid precipitable cpm for each assay were determined as described by Bruening *et al.* (4).

Each assay was electrophoresed on an SDS-polyacrylamide slab gel system which separates proteins on the basis of mol wt (13). An acrylamide to bis ratio of 30:0.174 was used, and the acrylamide concentrations in the separating gel and stacking gel were 12.5 and 5%, respectively. After electrophoresis, the gels were prepared for fluorography and exposed to Kodak X-omat R film for 1–2 days before development (3).

Methyl Mercury Agarose Gel Electrophoresis. Agarose gel electrophoresis using methylmercuric hydroxide as the denaturing agent was used (1). The agarose concentration in the standard slab gel was 2%. Electrophoresis of the RNA was carried out for 4 h at 80 v at room temperature. After electrophoresis, the gel was placed in a tray of H_2O and two to three drops of ethidium bromide (1 mg/ml) were added. After 1 h, the gel was rinsed with H_2O and photographed over an UV light box.

RESULTS

Comparison of *in Vivo* and *in Vitro* Protein Synthesis. The polypeptide products of a wheat germ cell free translation assay using poly(A) RNA from GA_3-treated tissues and the *in vivo* labeled proteins synthesized at a similar time are compared on an SDS-polyacrylamide gel (Fig. 1). The protein patterns observed for the labeled proteins remaining in the tissue and secreted from the tissues are similar. However, the *in vitro*-synthesized α-amylase is larger than the *in vivo*-labeled α-amylase protein. This confirms a similar report made in wheat aleurone cells (14) and suggests that the *in vitro* product is a possible unprocessed precursor of α-amylase. In both systems, the α-amylase polypeptide is by far the dominant protein synthesized.

DMSO-Formamide-Sucrose Density Gradient Centrifugation of Poly(A)-containing RNA. In Figure 2 the profile of *A* at 280 nm of the poly(A)-containing RNA is shown. A single large *A* peak of RNA is present at 17S. No shoulders on the 17S peak are observed at locations where either 18S or 25S rRNA sediment on parallel gradients indicating that almost all of the rRNA has been removed from the poly(A) RNA. The large *A* region at the top of the gradient apparently represents small wt, UV-absorbing material which remains associated with the RNA even after repeated ethanol precipitations and oligo[d(pT)]-cellulose selections.

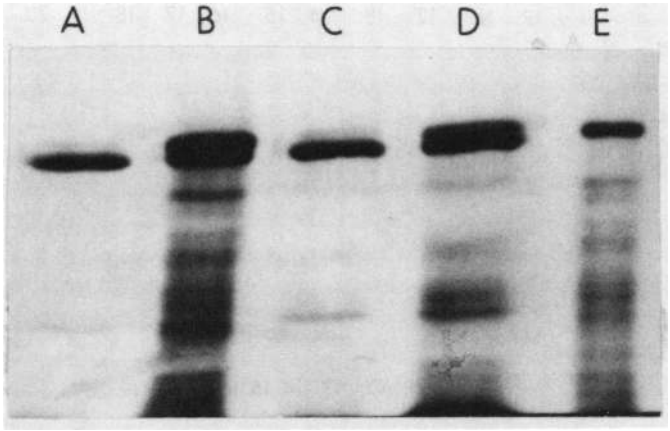

FIG. 1. Fluorogram of SDS-polyacrylamide gel of [^{3}H]leucine-labeled *in vivo* and *in vitro* protein translation products using GA_3-treated aleurone layers. In channel E, the results of a wheat germ *in vitro* translation assay are shown. In channel A, the *in vivo* labeled proteins secreted from GA_3-treated aleurone layers are shown and in channel B, a combination of the *in vivo* and *in vitro* proteins are shown. Similarly, in channel C the *in vivo* labeled proteins remaining in the tissues are shown, and in channel D, a combination of these *in vivo* labeled proteins and the *in vitro* protein translation products are shown.

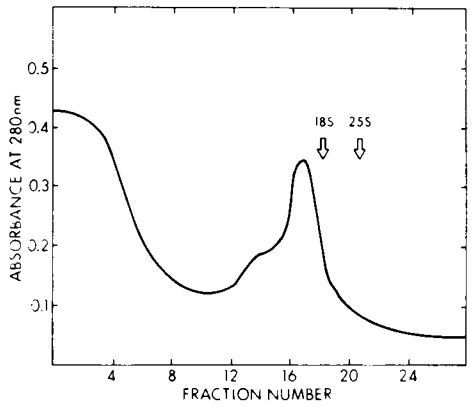

FIG. 2. Profile of *A* at 280 nm of a DMSO-formamide-sucrose gradient fractionation of twice-selected poly(A) RNA from GA_3-treated barley aleurone layers. The location of sedimentation of 18S and 25S rRNA on similar gradients are shown by arrows.

The RNA fractionated by this method was used as a template in the wheat germ (Fig. 3A) and reticulocyte lysate (Fig. 3B) *in vitro* protein synthesis systems. Nearly identical patterns of polypeptides on SDS-polyacrylamide gels are seen. A large majority of the protein synthesized is of mol wt 45,000 when the initial poly(A) material and when fractions 16–18 of the gradient were used. This band has been identified as barley α-amylase by immunoprecipitation using anti α-amylase I_gG made in rabbits to purified barley α-amylase (data not shown). Both systems synthesize larger and smaller mol wt polypeptides than α-amylase but all in greatly reduced quantities when compared to the amount of α-amylase synthesized. The relative quantity of α-amylase synthesized in fractions 16–18 coincides closely with the *A* peak of RNA present on the gradient.

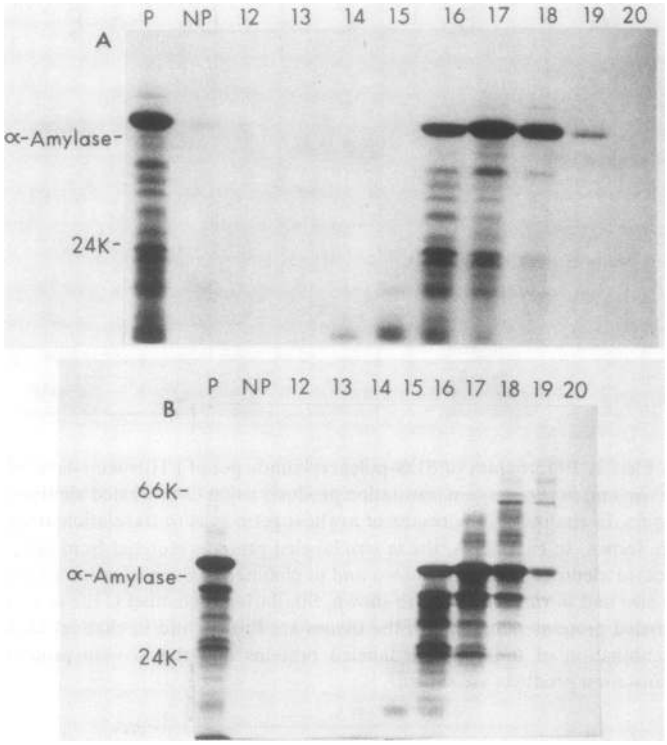

FIG. 3. Fluorograms of SDS-polyacrylamide gels of the *in vitro* protein translation products from assays using fractionated RNA from a DMSO-formamide sucrose density gradient. The assays used wheat germ extract (A) and reticulocyte lysate (B). The results using unfractionated poly(A) RNA are shown in lanes P and using nonpoly(A) RNA in lanes NP. Fraction number refers to the gradient fractions which were assayed. Locations of two protein standards and α-amylase produced *in vivo* are shown by lines.

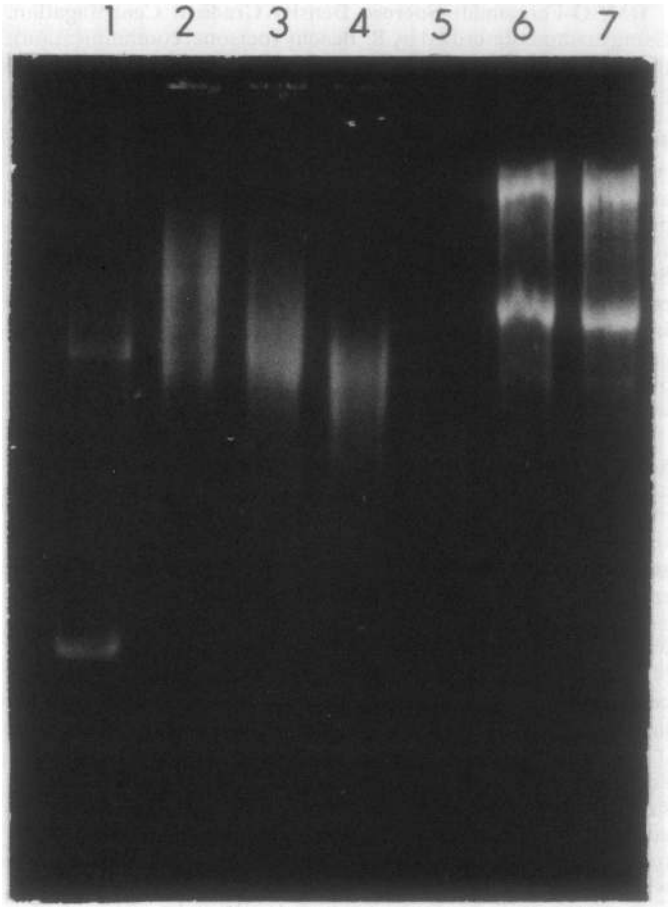

FIG. 4. A methyl mercury gel of RNA fractions from a DMSO-formamide-sucrose density gradient and various RNA standards. In lane 1, two RNAs from subgenomic particles of tobacco mosaic virus of mol wt 600,000 and 300,000 are shown. Lanes 2–5 show gradient fractions 18-15, respectively, and, in lanes 6 and 7, 18S and 25S rRNA standards are shown.

Methyl Mercury Gels of the RNA from Sucrose Gradients. To determine more accurately the mol wt of the mRNA for α-amylase, a denaturing methyl mercury gel system was used. The RNAs from fractions 15–18 of the DMSO-formamide-sucrose density gradients were separated on the basis of size by such means (Fig. 4). The predominant RNA species in the fractions containing translatable α-amylase mRNA migrates at a location consistent with the migration of an RNA species of mol wt 580,000. The presence of this RNA is proportional to the translatable activity for the mRNA for α-amylase in these tissues, and no other mRNA species is present in all fractions which contain such activity. Therefore, this RNA represents α-amylase mRNA. A different gel system using nondenaturing conditions and polyacrylamide gel electrophoresis shows a very similar profile of RNA migration (data not shown). The relative breadth of this band of RNA when compared to the bands for the rRNAs and viral RNAs is probably due to heterogeneity in the size of the poly(A) tail on the RNA (9). Again, as suggested in the *A* profile from the DMSO-sucrose density gradient, no detectable amounts of either rRNAs are present in this preparation of poly(A) RNA.

DISCUSSION

Indirect evidence has been published which suggests that GA_3 controls the transcription of the mRNA for α-amylase (6, 8, 9, 18). RNA synthesis inhibitors such as cordycepin inhibit the induction of the enzyme (9), and the synthesis of poly(A) RNA increases in GA_3-treated aleurone layers (12). Finally, the level of translatable mRNA for α-amylase increases after the addition of GA_3 to aleurone layers (8). However, no direct evidence for this hypothesis exists at the level of mRNA. There also has been evidence published showing that ABA inhibits the GA_3-induced synthesis of α-amylase in these tissues (5, 11). The mechanism of this inhibition by ABA is not well understood. Ho and Varner (10) suggested that translational control of α-amylase synthesis maybe involved. Hybridization studies using complementary DNA to α-amylase mRNA will allow quantitation of the level of mRNA for α-amylase in the various hormone-treated tissues. This will provide a greater understanding of the mechanism of action by both GA_3 and ABA in these tissues. The purpose of this work is to lay the groundwork for such studies on the control of the synthesis of α-amylase in barley aleurone layers.

The *in vitro* pattern of protein synthesis in these tissues using poly(A) RNA isolated from aleurone layers treated for 17 h with GA_3 closely mimics the pattern of *in vivo* protein synthesis occurring at a similar time (Fig. 1). A single protein, α-amylase, accounts for a majority of the protein synthesis occurring in both cases. However, the α-amylase polypeptide synthesized *in vitro* is larger than the α-amylase synthesized *in vivo*. This suggests that processing of the initial translation product occurs *in vivo*. Since α-amylase is a secreted protein, the processing may occur as the polypeptide is transported across a membrane as suggested by the signal hypothesis (2).

The RNA profiles in Figure 4 also demonstrate that the RNA species corresponding to the translatable mRNA for α-amylase is the predominant poly(A) RNA present in these tissues. This

suggests that the effect of GA_3 on these tissues is to increase greatly the quantity of mRNA for α-amylase by either transcriptional control or by processing of precursor RNA. These data also suggest that the relative quantity of this mRNA is sufficient to account for the relative amount of synthesis of α-amylase observed *in vivo*.

The mRNA for α-amylase has an apparent mol wt of 580,000. This corresponds to an RNA of about 1,650 nucleotides in length. Assuming an average poly(A) tail size of 100 nucleotides, this RNA potentially codes for a polypeptide of mol wt 56,000. The α-amylase secreted by aleurone layers has a mol wt of approximately 42,000 (16), and the *in vitro* translation product has a mol wt of approximately 45,000. Thus, only about 20% of the mRNA consists of nontranslated regions.

In the past, it has been shown that several charge isozymes of α-amylase are produced in aleurone layers (7). It is not known whether these isozymes represent posttranslational modifications of the same gene product or represent different gene products. The data presented here do not rule out either possibility. However, since the *in vitro* product is larger than the *in vivo* product, processing of the initial *in vivo* translation product is probably occurring. This posttranslational processing may account for the isozymes observed. In any case, the mRNA for α-amylase as referred to in the text may well represent several different mRNAs for the isozymes of α-amylase of barley aleurone layers.

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