In Vitro Synthesis of the α and α' Subunits of the 7S Storage Proteins (Conglycinin) of Soybean Seeds

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ABSTRACT

Messenger RNAs (mRNAs), isolated from immature soybean (Glycine max L., Merr.) seeds, that bound to oligo(dT)-cellulose were fractionated by centrifugation in sucrose density gradients containing dimethyl sulfoxide. mRNAs with sedimentation values between 21S and 25S coded for the in vitro translation of polypeptides with electrophoretic mobilities similar to those of the α and α' subunits of the 7S seed storage protein. High pressure liquid chromatographic analyses of the trypsin-induced fragments ("column fingerprinting") verified that the polypeptides produced in vitro were closely related to authentic α and α' subunits.

The fractions of RNA that coded for the in vitro synthesis of these subunits contained three major species of nonribosomal RNA (molecular weights of 1.1, 0.84, and 0.75 × 10^6) sufficiently large to code for the synthesis of the α' and α subunits.

Soybean seeds contain, at maturity, approximately 45% (by dry weight) protein, about 70% of which is in the form of storage proteins. During periods of rapid storage protein synthesis (10) as much as 1.5–4 mg of protein per seed per day may be produced (estimated from Ref. 7). These features led us to consider the developing soybean seed an appropriate system for studies of gene expression in a higher plant.

Soybean storage proteins sediment in sucrose gradients with sedimentation rates of approximately 7S (conglycinin, vicilin) and 11S (glycin, legumin). The 7S protein is composed of three major subunits, α', α and β (21, 22), the amino acid compositions of which have been determined (22). Recently, we reported that proteins synthesized in vitro from polyribosomes and poly(A) + RNA isolated from immature (developing) seeds had electrophoretic mobilities similar to those of the 7S and 11S proteins (2, 4). Here, we demonstrate, by column fingerprinting trypsin-generated fragments, that two polypeptides synthesized in vitro are similar to the α' and α subunits. We have also estimated the mol wt of the mRNAs presumed to code for their translation in vitro.

Received for publication July 5, 1979 and in revised form November 9, 1979

MATERIALS AND METHODS

Purification of mRNA and in Vitro Protein Synthesis. mRNA was purified from polysomes extracted from soybean seeds (cv. Provar, grown in greenhouses or the field) as previously described (2) or by the method described by Hall et al. (9). The latter method is a total nucleic acid extraction method employing SDS and proteinase K (Beckman Chemicals3) digestion. Poly(A) + RNA selection was carried out using two cycles of affinity chromatography on oligo(dT)-cellulose (Collaborative Research and Boehringer-Mannheim Corps.) (11). mRNAs were sized on 5–20% (w/v) sucrose gradients dissolved in 95% DMSO (5) and 4% formamide (v/v) in a Beckman SW-40 rotor centrifuged at 40,000 rpm at 28 C for 44–50 h. Gradients were fractionated with an ISCO sucrose gradient monitor (280 nm) and the RNA contained in individual fractions were precipitated with ethanol after adding sodium acetate to 0.3 M. RNA collected by centrifugation was washed with 70% ethanol to remove residual DMSO, dried in vacuo, and taken up in autoclaved water.

In vitro protein synthesis was carried out with wheat germ extracts as previously described (2) and with mRNA-dependent reticulocyte lysate as described by Pelham and Jackson (18). Reaction mixtures contained [3H]Leucine purchased from Amerham/Searle (Arlington Heights, Ill.).

PAGE and Trypsic Fragment Analysis. Aliquots of the translation reactions were heated in the presence of SDS and β-MCE and subjected to electrophoresis in a discontinuous polyacrylamide slab gel system (14). The gels were 5% acrylamide in the stacking gels and 10 or 12% acrylamide in the separation gels.

Polypeptides synthesized in vitro (labeled with [3H]Leu) were separated by PAGE, and protein bands most similar in mobility to authentic α' and α subunits were located by fluorography (12). The bands were excised from the dried gels, the gels were rehydrated, and PPO was removed with DMSO followed by water washes. Gel segments were recast in 5% polyacrylamide in one-fifth Tris-glycine electrophoresis buffer (14), and electrophoresed onto a bed of hydroxylapatite as described by Ziola and Scraba (27). The hydroxylapatite was transferred to a column and washed extensively with the one-fifth Tris-glycine buffer followed by 10 mM Na phosphate (pH 6.4) to remove residual acrylamide. Washing with 0.5 M phosphate eluted the bound polypeptides. Authentic

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4 Abbreviations: DMSO: dimethyl sulfoxide; β-MCE: β-mercaptoethanol; PAGE: polyacrylamide gel electrophoresis; HPLC: high pressure liquid chromatography; SDG: sucrose density gradient.
\( \alpha' \) and \( \alpha \) subunits, labeled in culture with \(^{14} \text{C}\)Leu (24), were purified in a similar manner. Appropriate amounts of \(^{3} \text{H}\) and \(^{14} \text{C}\)polypeptides were combined, 100 \( \mu \text{g} \) of BSA was added as carrier, and protein was precipitated with trichloroacetic acid. Precipitates were prepared for trypsin digestion as described by Vogt et al. (26) which included performic acid oxidation. Proteins were digested with TPCK-treated trypsin (Sigma, St. Louis, Mo.) and subjected to HPLC on Technicon (Belgium) Chromobeads type C-3 resin. Elution was accomplished with a quadratic gradient containing pyridine and acetic acid (26). Three-ml fractions were transferred to scintillation vials, dried at 100 C, the residue was dissolved in 0.3 ml of 9:1 NCS Soluhibler (Amer sham/ Searle):H2O and counted in a liquid scintillation counter with 5 ml of toluene-PPO-POPOP scintillation cocktail. Tritium cpm were corrected for \(^{14} \text{C} \) cross in the \(^{3} \text{H} \) channel.

### Gel Electrophoresis of the RNAs

Samples of RNA that had been ethanol-purified from sucrose gradients were subjected to electrophoresis in several types of gel systems: (a) 0.6 \( \times \) 9 cm cylindrical gels of 1.8 or 2.2% polyacrylamide and 0.5% agarose (composite gels, ref. 17) containing 0.2% SDS were run in the Tris-acetate, sodium acetate, and EDTA buffer of Loening (13). After electrophoresis, the gels were scanned at 260 nm with a Gilford spectrophotometer with attached gel scanner. RNAs were heated at 60 C for 30 s in electrophoresis buffer prior to loading on the gels to partially disrupt possible RNA-RNA aggregates. (b) 9 \( \times \) \( 10.5 \times 0.12 \)-cm or 9 \( \times \) \( 16 \times 0.12 \)-cm slab gels composed of 4% acrylamide (40:1, acrylamide:methylene bis-acrylamide, ref. 15) in 8 M urea and 0.2% SDS, were run in 0.1 M Tris-borate, 2.5 mM EDTA, and 0.2% SDS (18) at 10–15 v/cm for 10–16 h. RNA was detected after staining with a solution containing ethidium bromide and viewing with a UV light. RNAs were heated in 4.5 M urea in electrophoresis buffer prior to loading on the gel. (c) Agarose slab gels (2%) (dimensions given above) containing 5 mM methylmercury hydroxide (Alfa Div., Ventron Corp., Danvers, Mass.) prepared as described by Bailey and Davidson (1) were electrophoresed in boric acid-sodium borate for varying periods of time at 5–10 v/cm. RNA was visualized after staining with ethidium bromide. Prior to electrophoresis, RNAs were denatured with 10 mM methylmercury hydroxide in the electrophoresis buffer. All operations with methylmercury were conducted in a tested fume hood with precautions consistent with the toxicity of the chemical.

### RESULTS

#### Preparation of mRNA

In the soybean variety under study, the 7S storage proteins began to accumulate once the developing seeds reached about 50 mg/seed fresh weight (4). We chose to use seeds 150–200 mg each because seeds of that size are easy to handle and because the 7S proteins are synthesized rapidly during that period of development. The plants are considered to be in late R4 and R5 stages of development (8). mRNAs prepared from polyribosomes isolated from developing seeds are highly active in \( \text{in vitro} \) protein synthesis reactions (4). We also used the method of Hall et al. (9) for isolating total nuclear acids which combines homogenization in hot SDS and digestion with proteinase K. However, this procedure resulted in the simultaneous extraction of gelatinous, polysaccharide-like materials. By proper seed selection, and oligo(dT)-cellulose and hydroxylapatite chromatography much of this material was eliminated and the resultant RNAs were highly active in \( \text{in vitro} \) translation reactions (3).

Poly(A)-containing RNAs, selected by two passages over oligo(dT)-cellulose, sedimented in DMSO-containing sucrose gradients (Fig. 1). The mRNA activity in fractions 8 through 20 of the gradient, as measured by \(^{3} \text{H}\)Leu incorporation in \( \text{in vitro} \) translation reactions (open bars), corresponded well with the UV absorbance profile of the RNA. The optical density peak in fraction 15 represents a mixture of mRNAs and 18S ribosomal RNA which contaminated these preparations (Fig. 1). The relative amounts of mRNA greater than 18S varied between preparations (4), but we have noted a correlation between mRNA preparations which were highly active (i.e. cpm incorporated in \( \text{in vitro} \) translation reactions per \( \mu \)g RNA) and the amount of mRNA with higher sedimentation rates.

The products of the translation reactions ranged in mol wt from less than 10,000 (for RNAs in fraction 8) to greater than 90,000 for RNAs with high sedimentation values. After PAGE and fluorography of the reaction products that result from the translation of the mRNAs sedimenting between the 18S and 25S ribosomal RNAs, it was apparent that there are similarities between the electrophoretic mobilities of authentic \( \alpha' \) and \( \alpha \) subunits of the 7S protein and several polypeptides synthesized \( \text{in vitro} \) (bands 1 and 2 of Fig. 2). The polypeptides produced in \( \text{in vitro} \) translation reactions in response to mRNAs from other fractions of the sucrose gradients have also been described (4). However, we have chosen to do tryptic fingerprinting of bands 1 and 2 (of Fig. 2) because they appear to be well separated from other polypeptides in that part of the gel, thereby making it easier to excise each of the bands of radioactivity separately.

SDG-purified mRNAs were also translated in mRNA-dependent reticulocyte lysates (18). Although there were several minor differences in the products synthesized by the wheat germ and reticulocyte systems they were not in the region of the gel to which the subunits of either the 7S or the 115S proteins migrated (data not shown). The results support our conclusion that the translation products are not artifacts of the translation system used. We have, therefore, continued to use only the wheat germ translation system.

#### Tryptic Fragment Mapping of the Polypeptides

\(^{3} \text{H}\)Leu-labeled polypeptides from \( \text{in vitro} \) translation reactions with electrophoretic mobilities most similar to the \( \alpha' \) and \( \alpha \) subunits (bands 1 and 2 of Fig. 2) were excised from gels, combined with authentic \(^{14} \text{C}\) labeled \( \alpha' \) and \( \alpha \), and digested with trypsin. A comparison of the elution of \(^{3} \text{H}\) and \(^{14} \text{C}\) from the HPLC column reflects the degree of similarity of the polypeptides so treated; dissimilar polypeptides gave very different elution patterns (Fig. 3D). Figure 3A verified that the \( \alpha' \)-like subunit produced \( \text{in vitro} \) is similar but not identical to authentic \( \alpha' \). While there appear to be differences in several regions of the elution profile from the column, many of the \(^{3} \text{H}\) and \(^{14} \text{C}\)-labeled fragments eluted simultaneously.

Figure 3B shows the result of a similar experiment with the \( \alpha \) subunit demonstrating the relatedness of the \( \text{in vitro} \) synthesized polypeptide to the authentic subunit. Since elution from the column is highly charge-dependent, one would expect differences between authentic and \( \text{in vitro} \)-produced polypeptides, because slight post-translational modifications (i.e. glycosylation) of the proteins occur \( \text{in vitro} \) (21) but would not necessarily occur \( \text{in vitro} \). It is apparent that polypeptides similar to the \( \alpha' \) and \( \alpha \) subunits of the 7S protein were synthesized \( \text{in vitro} \). This technique has been
used to identify precursors of avian myeloblastosis virus coat protein (26), to demonstrate the *in vitro* translation of the small subunit of ribulose bis-phosphate carboxylase (6), and to compare the relatedness of α and β chains of the histocompatibility complex (25).

It became apparent during the experiments described above that the trypsin-induced fragments of authentic α' subunit resemble those of the α subunit. Therefore [3H]Leu-labeled α' and [14C]Leu-labeled α subunits, produced "in culture," were directly compared. The HPLC elution profiles showed few differences (Fig. 3C); many of the [3H- and [14C]-labeled fragments eluted simultaneously from the column demonstrating the similarity between the two subunits. In another experiment in which two apparently unrelated polypeptides, the 11S basic subunits and the 11S acidic subunits, were similarly compared, there was no correspondence in the elution of their peptide fragments (Fig. 3D).

**Electrophoretic Analysis of the RNAs.** The number of polypeptides translated *in vitro* in response to the RNAs in fraction 34 was not great (less than 6 major bands) and α' and α are the highest mol wt polypeptides (Fig. 2). We reasoned that it might be possible to visualize particular species of mRNAs, under appropriate conditions of electrophoresis, which code for the *in vitro* synthesis of these polypeptides. Figure 4 demonstrates the results of three types of analyses. In each experiment, we used RNA which sedimented in sucrose gradients between the 18S and 25S rRNAs (Fig. 1). The first gel system, 2.0% polyacrylamide and 0.5% agarose, separated a broad peak of RNA between the ribosomal species, with an average mol wt of 0.9 × 10^6. A similar RNA fraction electrophoresed in 4% polyacrylamide, 8 mM urea, and 0.2% SDS revealed the presence of two distinct bands of RNA migrating more slowly than 18S RNA with apparent mol wt of 0.75 and 0.84 × 10^5, and a more diffuse band of RNA at about mol wt 1.1 × 10^5. Since neither of these gel systems is a completely denaturing one, several experiments were done with 2% agarose gels containing 5 mM methylmercury hydroxide. Under these conditions, the RNAs were not resolved into two distinct bands, but into a broad band with average mol wt of 0.84 × 10^5. The values for the mol wt of the presumed mRNAs correlate well with the apparent mol wt of the polypeptides synthesized *in vitro*. We estimated, using the PAGE system described under "Materials and Methods," that the α' subunit has an apparent mol wt of 0.84 × 10^5 and α of 0.75 × 10^5. Using the urea-containing gel system described by Swank and Munkres (20) we estimated mol wt of 0.91 × 10^5 and 0.84 × 10^5, respectively, for the two subunits. Other workers estimated lower mol wt values for these proteins (22). Glycosylation of the subunits (23) may cause these discrepancies in electrophoretic mobilities. Given the uncertainties about the mol wt of the polypeptides, the estimated mol wt of the visualized bands of RNA are reasonable for mRNAs coding for the α' and α subunits of the 7S protein.

**DISCUSSION**

We have demonstrated that mRNAs isolated from developing seeds are active in *in vitro* protein synthesis. We obtained, after denaturing sucrose density gradient centrifugation, fractions of mRNA which code for the *in vitro* synthesis of polypeptides with electrophoretic mobilities similar to those of the α' and α subunits of the 7S storage protein. This identification is further strengthened by comparing trypsin-digests of authentic subunits and *in vitro* synthesized polypeptides by HPLC. The differences between the elution profiles shown in Figure 3 (A and B) may reflect post-translational modifications (such as glycosylation) of the 7S protein subunits which occur in the cotyledons but not *in vitro*. Such modification may result in increased or decreased susceptibility of the peptide to trypsin digestion. Additionally, the presence of a post-translational modification on the side chain of an amino acid such as aspartate (23) would most likely result in altered chromatographic mobility. A fragment derived from trypsin-digested seed protein containing such a modification would not coelute from the HPLC column with an equivalent but unmodified fragment synthesized *in vitro* or it may coelute with a nonequivalent fragment and appear to have the wrong 3H/14C ratio. Uhr et al. (25) have examined the relatedness of the products of several alleles of the major histocompatibility complex by a similar method and conclude that "Generally, a 40 to 50 per cent homology by peptide mapping techniques represents an 80 to 90 per cent homology by amino acid sequence." If Uhr et al. (25) are correct, our data indicate that polypeptides closely related to α' and/or α subunits of the 7S storage protein have been synthesized *in vitro* (Fig. 3, A and B) and that the two subunits are themselves closely related (Fig. 3C).

The results of several other types of experiments (data not shown) suggest that band 2 (of Fig. 2) is not the result of incomplete translation of the mRNA for band 1, or due to the cleavage of band 1 to produce band 2. If the latter were the case, one might expect that the relative proportions of the shorter polypeptides would increase with the time of the *in vitro* translation reactions. We have terminated translation reactions at various times (from 15 to 150 min) and found that the shorter (but related) polypeptides were produced early in the incubation indicating direct translation rather than from processing of precursor polypeptides. As shown in Figure 2 (tracks 31 and 34) we were able to effect a degree of separation of the mRNAs for bands 1 and 2 in the
Fig. 3. HPLC analysis of fragments resulting from trypsin digestion of storage protein subunits and polypeptides in vitro. (A and B). $^3$H-labeled polypeptides produced in vitro were excised from polyacrylamide gels (separately) and combined with $\alpha'$ or $\alpha$ subunits (also excised from gels) isolated from cotyledons cultured in the presence of $[^3]$CLeu. Trypsin digestion and fragment separation on Chromobeads Type C-3 resin were done as described by Vogt et al. (24). A: elution of fragments of $^3$C-labeled $\alpha'$ (-----) and a $^3$H-labeled polypeptide (band I, Fig. 2) that had electrophoretic mobility similar to authentic $\alpha'$ (-----). B: elution of fragments of $^3$C-labeled $\alpha$ (-----) and a $^3$H-labeled polypeptide (band 2, Fig. 2) that had electrophoretic mobility similar to authentic $\alpha$ (-----). C: elution of fragments of $^3$C-labeled $\alpha'$ and $^3$H-labeled $\alpha$, both of which were isolated from cultured cotyledons. D: elution of fragments of $^3$C-labeled acidic subunits and $^3$H-labeled basic subunits of the 11S storage protein, each group of subunits isolated from cotyledons labeled in culture.

DMSO-containing sucrose gradients. The results of our experiments suggest that bands 1 and 2 probably result from the translation of different mRNAs.

At first glance, the 7S storage protein does not seem to be as complex as the 11S storage protein of soybean, i.e. the former contains primarily 3 subunits, whereas the latter may be composed of as many as 10 subunits. However, experiments currently being conducted in our laboratories indicate the presence, both in the seed and in in vitro reactions, of additional polypeptides related (by virtue of their mol wt and tryptic fingerprints) to the $\alpha'$ and $\alpha$ subunits of the 7S protein. Recent studies with other seed storage proteins have shown similar heterogeneity of protein subunits. For example, zein, the storage protein of corn, was initially believed to be a two-subunit protein, but was later shown to consist of 12 or more subunits (18). Park et al. (16) demonstrated that the isoforms of zein produced in vitro can be divided into at least three families, differing from one another in the sequence homologies of their mRNAs to different cloned cDNAs made from mixed zein-specific mRNAs. Until similar types of experiments are conducted with the mRNAs for the $\alpha'$ and $\alpha$ subunits of the soybean 7S protein, conclusive information about the relatedness of these mRNAs probably will not be available.

In experiments which were repeated with different RNA preparations, the SDG fractions containing the mRNAs that coded for the in vitro synthesis of the $\alpha'$ and $\alpha$ subunits were shown to contain three species of nonribosomal RNA when subjected to electrophoresis in a gel system containing 8 M urea (Fig. 4). When the same fraction of RNA was electrophoresed under the denaturing conditions of methylmercury hydroxide, a single spreading band of RNA was seen. Whether the discrepancies resulted from the differences in the resolving powers of the gel systems or to differences in the denaturants used remains to be determined.

Acknowledgments—We thank Dr. A. Szalay for helpful instructions (and cautions) in the use of methylmercury-containing gels, and L. Holowach and K. Gabbard for critical reviews of the manuscript.
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