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Messenger RNA for G1 protein of French bean seeds: Cell-free translation and product characterization

(storage protein/peptide mapping in denaturing gels/protein synthesis/modified 5′ termini)

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ABSTRACT **The fraction of poly(A)-containing RNA isolated from ripening bean (*Phaseolus vulgaris*) cotyledons that sedimented at 16 S in linear logarithmic sucrose gradients was at least as active a messenger as viral RNA when added to a cell-free protein-synthesizing system from wheat germ. The major products synthesized *in vitro* were polypeptides of about 47,000 and 43,000 daltons, corresponding to two of the three subunits of G1 protein, the most abundant bean seed storage protein. No trace of the largest (53,000 daltons) subunit was found among the polypeptides synthesized *in vitro*. Proof that the 47,000- and 43,000-dalton polypeptides coded by the 16S RNA were indeed subunits of G1 protein was obtained by immunoprecipitation with monovalent antibody to G1 protein and by electrophoretic mapping of peptides on acrylamide gels after digestion of mixtures of authentic protein and radioactive translation products with protease V8, chymotrypsin, and trypsin. The subunits synthesized *in vitro* were slightly smaller than the native subunits, probably because they lacked the sugar residues present on the holoprotein.**

The biosynthesis of crop seed storage proteins is currently attracting much attention. Cell-free synthesis of ethanol-soluble polypeptides that migrate on electrophoresis with mobilities similar to those of the major polypeptides of the prolamine zein has been accomplished by using polysomes isolated from protein bodies of corn (*Zea mays*) endosperm (1). Larkins *et al.* (2) prepared mRNA from corn endosperm polysomes, which yielded 21,800 and 19,000 molecular weight polypeptides similar to those of zein when translated in a cell-free protein-synthesizing system from wheat germ. Some success has been obtained with cell-free translation of the prolamine hordein (3) from barley (*Hordeum vulgare*) and of globulin from oat (*Avena sativa*) groats (4).

The rapid accumulation of a few types of protein in the cotyledons during ripening of legume seeds identifies this tissue as being superior for studies on plant protein biosynthesis at the molecular level (5–7). In the French bean (*Phaseolus vulgaris*), some 50% of the total protein present in the mature seed is G1 (globulin-1) protein (8). In the cultivar Tendergreen, this protein has three polypeptide subunits with molecular weight of 53,000, 47,000, and 43,000 (9–11); in this article, these subunits will be referred to as α, β, and γ, respectively. Other workers have used different names for the major bean storage protein (10). In a previous study (12), polysomes from growing cotyledons were added to a cell-free translation system from wheat germ and shown to support the synthesis of polypeptide products that comigrated with authentic G1 subunits. The accumulation of G1 protein during growth of bean cotyledons has been studied, and procedures for immunoprecipitation of G1 protein have been detailed (13). We now describe the translation of 16S RNA isolated from a preparation of total poly(A)-containing RNA from young (12–16 mm long) bean cotyledons and show that it contains the messenger for the translation of the β and γ subunits of G1 protein.

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MATERIALS AND METHODS

Preparation of Seed mRNA. Cotyledons were excised aseptically from ripening bean seeds (12–16 mm long) grown in the University of Wisconsin Biotron, frozen in liquid N_2, and stored at −80°. Batches of 50 g of cotyledons were thawed to nearly 0°; then 130 ml of 0.2 M Na borate, pH 9/30 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA)/5 mM dithiothreitol/1% sodium dodecyl sulfate ($NaDodSO_4$) was heated to 100° and added to the cotyledons. The mixture was immediately ground with a Polytron homogenizer (Brinkmann Instrument Co.). After addition of 65 mg of Proteinase K (Beckman), the extract was incubated at 37° for 1 hr; then 10 ml of 2 M KCl was added. The extract was chilled on ice and cleared by centrifugation. RNA was precipitated overnight with 2 M LiCl, washed twice with 2 M LiCl, redissolved in water, and precipitated with ethanol. Poly(A)-containing RNA was obtained by two cycles of oligo(dT)-cellulose chromatography (14). RNA sedimenting at 16 S in a linear logarithmic (0–30%) sucrose gradient (15) was precipitated with ethanol, then purified by a second cycle of sucrose gradient ultracentrifugation and used as mRNA. Electrophoretic analysis of this RNA on agarose/acrylamide gels (data not shown) revealed a diffuse band lying between 25S and 18S ribosomal RNA markers isolated from bean cotyledons; no ribosomal RNA could be detected in the mRNA preparation itself.

Cell-Free Protein Synthesis. The cell-free system was incubated at 30° and contained: 2.5 mM ATP, 0.2 mM GTP, 5 mM phospho*enol*pyruvate, 25 μM of 19 of the 20 genetically coded amino acids (methionine being excluded), 90 mM potassium acetate, 4 mM magnesium acetate, 120 μM spermidine diphosphate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (adjusted to pH 7.6 with NH_4OH), and 0.3 A_{260} unit of wheat germ extract (16, 17) in each 50-μl reaction mixture. The specific activity of [^{35}S]methionine was 20 Ci/mmol; it was used at a final concentration of 20 μM, except in the peptide mapping experiments where its specific activity was 970 Ci/mmol and the concentration used was 0.47 μM. For some experiments, the protein-synthesis reaction mixtures were scaled up to 100 μl. Radioactivity in acid-insoluble products was determined on paper discs (18); the counting efficiency was 70%.

Polypeptide Product Characterization. Isolation of G1 protein, preparation of monovalent antiserum in rabbits, direct immunoprecipitation, and electrophoretic procedures were as

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; $m^7G^{5'}p$, 7-methylguanosine-5′-monophosphate.

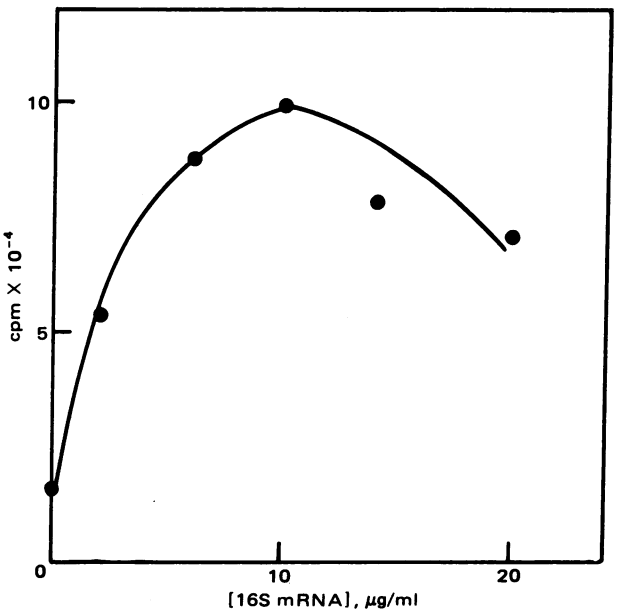

FIG. 1. Saturation curve for G1 mRNA translation. mRNA isolated from bean cotyledons was added in amounts from 0.1 to 1 μg to 50-μl reaction mixtures containing S23 extract from wheat germ and other components detailed in *Materials and Methods*. [^{35}S]Methionine incorporation is shown for 10-μl samples taken after 60 min of reaction at 30°.

described (13). Indirect immunoprecipitation was done by a modification of the procedure described by Dobberstein *et al.* (19). To 90 μl of the protein-synthesis reaction mixture, 10 μl of 1.5 M NaCl, 10% Triton X-100, and 10% Na deoxycholate in 0.1 M sodium phosphate buffer (pH 7.2) was added. After centrifugation for 1 hr at 100,000 × *g*, the supernatant was removed and 5 μl of rabbit anti-G1 IgG (1.6 mg of protein per ml) was added. After incubation for 1 hr at 37°, 7.5 μl of goat anti-rabbit IgG serum (Miles) containing 14.5 mg of antibody per ml was added. The mixture was incubated at 37° for 15 min and then at 4° overnight. The immunoprecipitate was recovered by centrifugation and was subjected to three cycles of washing before dissociation and electrophoresis as described (13).

For peptide mapping (20), authentic G1 protein and radioactive products obtained by cell-free protein synthesis were mixed and digested with chymotrypsin (Sigma), protease V8 from *Staphylococcus aureus* (Miles), or trypsin (Worthington). For digestion with chymotrypsin or protease V8, G1 protein (100 μg) and 20 μl of the cell-free products were mixed and incubated at 37° with 12 μg of enzyme in 66 μl (final volume) of 125 mM Tris·HCl, pH 6.8/10% glycerol/0.5% $NaDodSO_4$/0.0001% bromophenol blue tracking dye. Samples (8 μl) were withdrawn at different time intervals, added to 2 μl of a solution containing 10% 2-mercaptoethanol and 2% $NaDodSO_4$, and boiled for 2 min. They were then applied to lanes of a 20% acrylamide gel that contained $NaDodSO_4$ and an acrylamide:bisacrylamide ratio of 200:1, as described by Knowland (21). Similar procedures were used for digestion with trypsin, except that 50 μg of G1 protein and 20 μl of the cell-free reaction products were incubated with 4.8 μg of protease in 48 μl (final volume) of 67 mM barbital buffer, pH 8.6.

RESULTS

Translation characteristics of seed mRNA

Poly(A)-containing RNA that sedimented at 16 S in linear logarithmic sucrose gradients was an active messenger in the

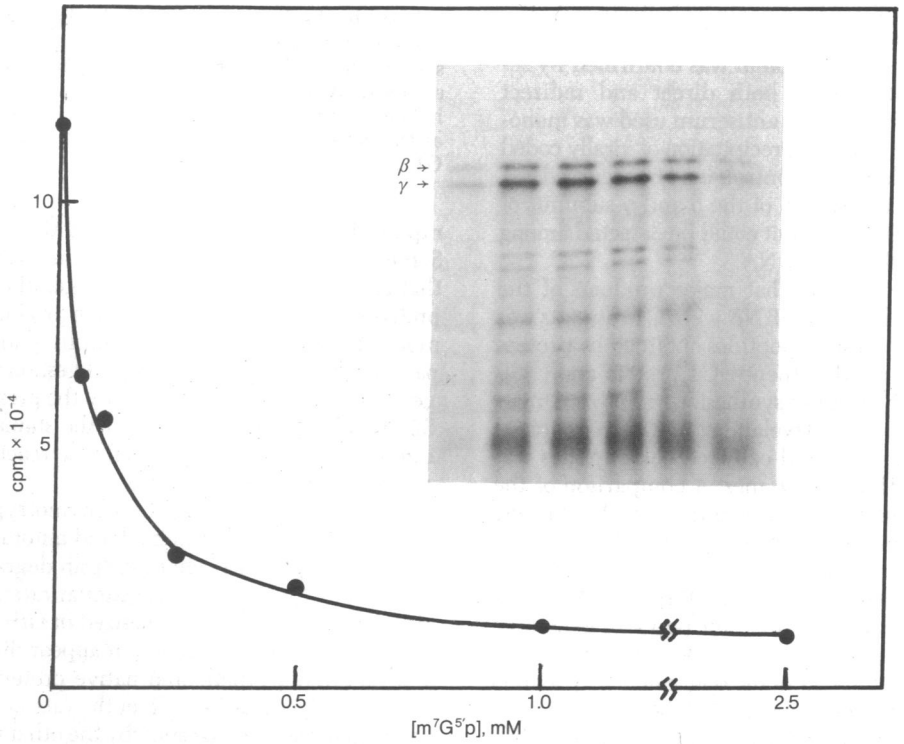

FIG. 2. Inhibition of [^{35}S]methionine incorporation by $m^7G^{5'}p$. Increasing amounts of $m^7G^{5'}p$ were added to cell-free reactions coded by 0.5 μg of G1 mRNA per ml. Trichloroacetic acid-insoluble radioactivity is shown for 10-μl samples of the 50-μl reactions. (*Inset*) Fluorograph of an electrophoretic separation of samples taken from each reaction that, on the basis of acid-insoluble counts, had approximately equal amounts of radioactivity. The lane to the left corresponds to the reaction containing no $m^7G^{5'}p$; the other lanes are in ascending order of $m^7G^{5'}p$ content (the reaction containing 2.5 mM $m^7G^{5'}p$ is omitted).

wheat germ cell-free protein-synthesizing system. Linear kinetics were obtained for the first 45 min of incubation, and near-linear incorporation of [^{35}S]methionine continued for over 75 min. The reaction was saturated with 10 μg of mRNA per ml (Fig. 1); over a 1-hr incubation period at this concentration, each mRNA molecule served as a template for approximately 10 polypeptide chains [assuming that all the protein synthesized was G1 globulin, which contains an average of three methionine residues in each of the three polypeptide subunits (unpublished results)]. At 2 μg/ml, the mRNA was an even more efficient template, each molecule being "read" about 27 times. On this basis, the seed mRNA was a very efficient template, comparing favorably with brome mosaic virus RNA component 4, which we found to be read about 15 times when added to our translation system at 2 μg/ml.

Most eukaryotic mRNAs have a 5′ cap structure (22). Inhibition of amino acid incorporation on addition of 7-methylguanosine-5′-monophosphate ($m^7G^5'p$) to the cell-free translation system is thought to be strongly indicative of the presence of a cap on the mRNA (23). Methionine incorporation was inhibited by about 90% by the presence of 1 mM $m^7G^5'p$ (Fig. 2). The apparent K_i for inhibition of 16S mRNA-directed translation by $m^7G^5'p$ was 90 μM. Electrophoretic analysis of the polypeptide products synthesized *in vitro* showed a similar profile at each concentration of $m^7G^5'p$ tested (Fig. 2, *inset*). Since the effect of this inhibitor was the same for both of the dominant polypeptides, migrating at positions corresponding to molecular weights of 47,000 and 43,000, this suggests that the mRNAs for both of these polypeptides are capped.

Characterization of products synthesized *in vitro*

Electrophoresis of a sample of a protein-synthesis reaction coded by bean 16S RNA, followed by staining (24) and fluorography (25), revealed that polypeptides having similar mobilities to those of the β and γ subunits of G1 protein were major products of the cell-free synthesis (lane 1, Fig. 3). The identity of these polypeptides as subunits of G1 protein was confirmed by selective immunoprecipitation by both direct and indirect techniques (lanes 4–6, Fig. 3). The antiserum used was monovalent for G1 protein (13), and no precipitation of virally coded translation products (added as a control; lane 6) occurred.

Despite the efficient translation of the β and γ subunits of G1 protein, no trace of the α subunit could be detected among the products coded by the 16S mRNA.

Further evidence confirming that major products of the cell-free reactions coded by 16S mRNA were G1 subunits was obtained by coelectrophoresis of peptides obtained by proteolytic digestion of mixtures of authentic G1 protein and [^{35}S]-methionine-labeled polypeptides synthesized *in vitro*. Under conditions similar to those of Cleveland *et al.* (20), very rapid proteolysis of the G1 protein and cell-free polypeptides was obtained with three different enzymes. A comparison of the peptide profiles obtained by autoradiography and by staining (Fig. 4) reveals that many bands can be seen by both techniques for each of the proteolytic enzymes used, indicating that the radioactive peptides have authentic counterparts. Moreover, clearly different maps were obtained for peptides generated by each protease. The α subunit of G1 can be seen in the stained gels; its intensity is similar to that of the β subunit; the γ subunit is less heavily stained. Protease V8 degraded the α subunit more rapidly than the β subunit, which in turn appeared to be slightly more rapidly degraded than was the γ subunit. The α subunit is missing from the map visualized by autoradiography and the β and γ subunits appear to contain similar amounts of radioactivity. However, the β subunit was again found to be more

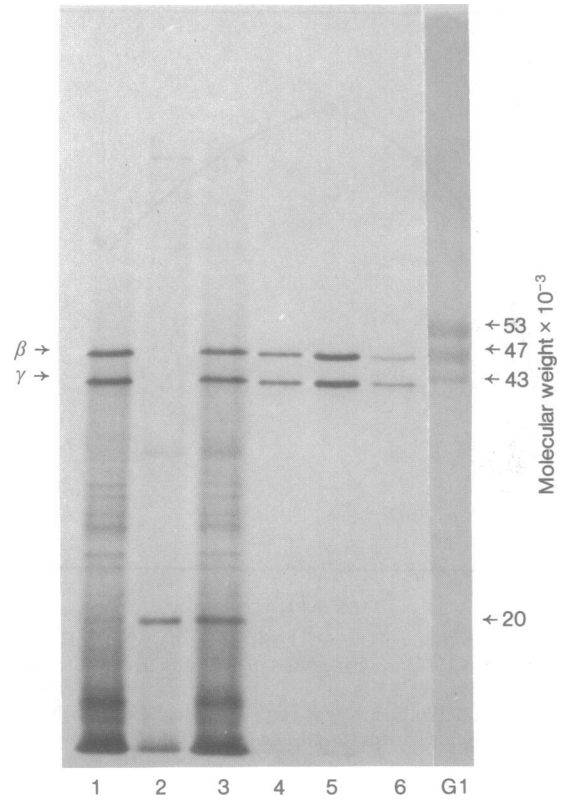

FIG. 3. Fluorography of an electrophoretic separation of polypeptides synthesized *in vitro*. Lane 1 contained 2.5 μl of the total polypeptide products of 50-μl cell-free reaction coded by 0.5 μg of 16S RNA from bean cotyledons. Lane 2 shows 2.5 μl of the products coded by 1 μg of brome mosaic virus RNA; lane 3 shows 5 μl of a mixture of the products shown in lanes 1 and 2. Selective immunoprecipitation of the β and γ subunits of G1 protein from products of reactions shown in lane 1 by the direct technique yielded material giving the electrophoretic profile seen in lane 4; the indirect technique gave the profile shown in lane 5. Indirect immunoprecipitation of the mixed viral and seed mRNA-directed polypeptide products provided material seen in lane 6. Authentic G1 protein was run in the same 13% gel (anode at the bottom); the stained polypeptide subunits are seen in lane G1.

rapidly hydrolyzed by protease V8 than was the γ subunit. Some bands can be seen in the autoradiogram of V8 peptides that are not present in the stained gel; these may result from proteolysis of incomplete translation products and are detected more efficiently by autoradiography than by staining. Both panels shown in Fig. 4 for the V8 digestion were from the same gel, but are of different lanes since the presence of wheat germ S23 peptides in the pictures of the stained gel containing a mixture of a sample of the cell-free reaction and G1 protein resulted in a complex picture.

The panels seen in Fig. 4 for chymotrypsin digestion are of identical lanes for the stained and autoradiographic profiles. As with V8 protease, chymotrypsin degraded the native and synthetic G1 polypeptides at similar rates. In contrast, trypsin degraded the material synthesized *in vitro* much more rapidly than it did the native protein. It appears likely that differences between the synthetic and native proteins, for example, the extent of glycosylation, affect the rate of digestion by trypsin more than they do digestion by the other two proteases tested. Possibly, the cleavage sites are also affected since the correspondence between the stained and autoradiographic profiles obtained for the tryptic peptides is poorer than for peptides obtained by using the other proteases.

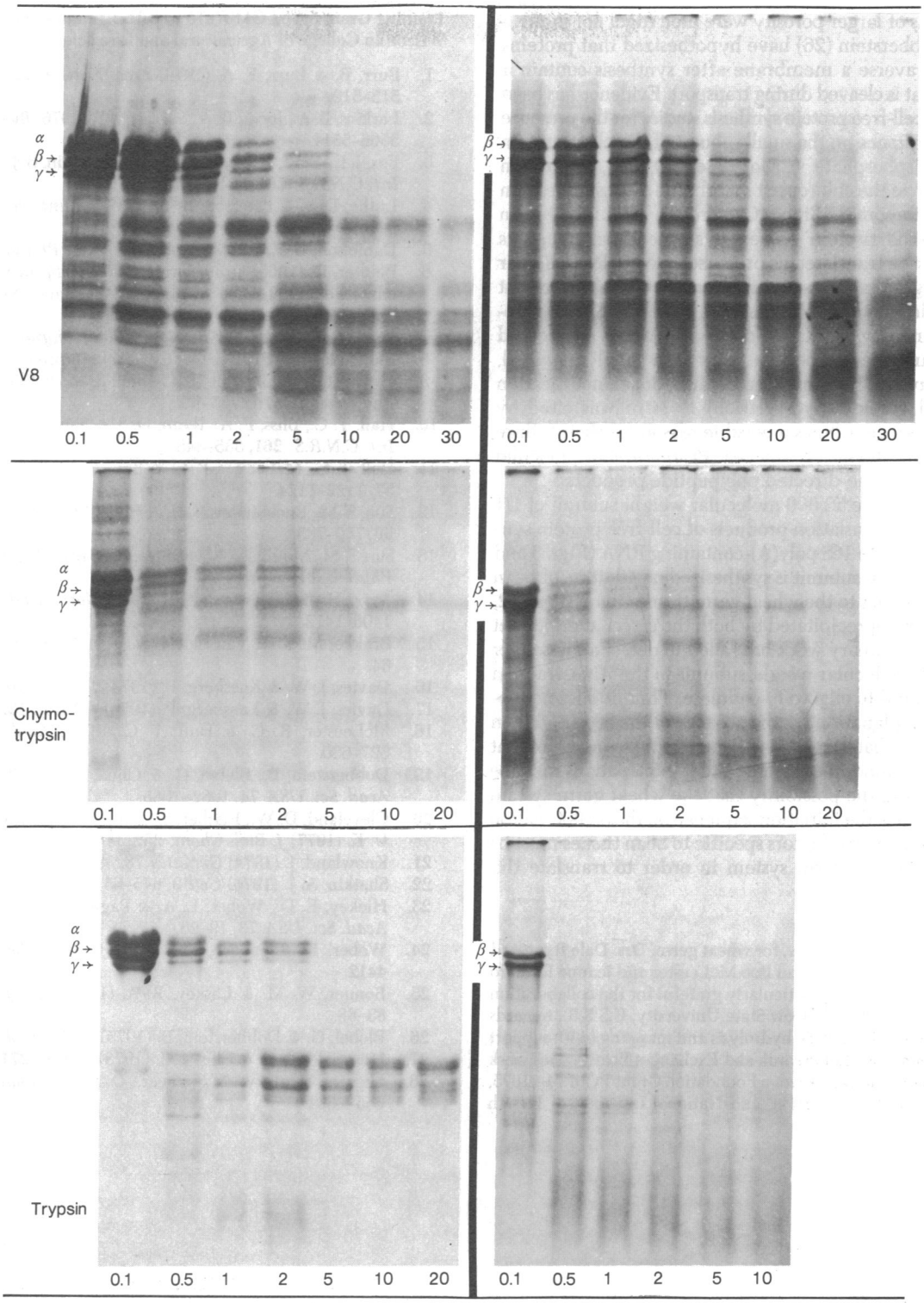

FIG. 4. Peptide maps of authentic G1 protein and of products synthesized *in vitro*. Authentic G1 protein and products of cell-free synthesis directed by mRNA from developing bean cotyledons were digested with protease V8 (from *Staphylococcus aureus*), chymotrypsin, or trypsin. Samples were withdrawn at the times (min) indicated and subjected to electrophoresis on 20% polyacrylamide gels. For each enzyme treatment, the Coomassie brilliant blue-stained peptides are on the left and autoradiograms of the [^{35}S]methionine-containing peptides are on the right. The arrows indicate the positions of the β and γ subunits of G1 protein.

DISCUSSION

The data presented here show that mRNA isolated from cotyledons of the French bean serves as an efficient template for the cell-free synthesis of polypeptides that, on the basis of electrophoretic mobility, immunoprecipitation, and peptide mapping, are similar to the β and γ subunits of the most abundant seed storage protein. Careful inspection of the data shown in Fig. 3 reveals that the polypeptides synthesized *in vitro* migrated slightly faster on electrophoresis than did the native β and γ subunits of G1 protein; this was seen more clearly when these proteins were allowed to migrate further down the

gel or when gels of larger porosity were used (data not shown). Blobel and Dobberstein (26) have hypothesized that proteins that have to traverse a membrane after synthesis contain a presequence that is cleaved during transport. Evidence has been obtained from cell-free protein synthesis studies for the presence of such a presequence on the small subunit of the plant protein ribulose 1,5-bisphosphate carboxylase (19, 27), a protein transported across the chloroplast membrane after synthesis on cytoplasmic ribosomes. Since G1 protein accumulates in membrane-bound protein bodies, it seemed likely that its subunits might be translated as precursor molecules of higher molecular weight than the subunits found *in vivo*. Our present observations detract from this possibility, but the β and γ subunits we obtained by translation of seed mRNA *in vitro* could be smaller than expected because they were not glycosylated, as they are *in vivo* (11). Lack of post-translational modification (which would include glycosylation) *in vitro* was cited by Higgins and Spencer (28) as a possible reason for their failure to detect immunochemically any pea (*Pisum sativum*) globulin among seed polysome-directed polypeptide products.

The absence of the 53,000 molecular weight subunit of G1 protein from the translation products of cell-free protein synthesis directed by the 16S poly(A)-containing RNA (Figs. 3 and 4) is intriguing. This subunit is synthesized on addition of bean cotyledon polysomes to the wheat germ translation system (12, 13) and is immunoprecipitated by both the direct and indirect techniques. Preliminary work has failed to detect a messenger for the 53,000 molecular weight subunit in the RNA fraction that does not bind to oligo(dT)-cellulose. Thus, it does not appear that the explanation for the absence of the 53,000-dalton subunit is simply that its mRNA lacks a poly(A) sequence or that it cannot be immunoprecipitated. Other explanations must be sought, including the possibility that the wheat germ system does not recognize the initiation sequence on the 53,000-dalton subunit mRNA, or that factors specific to bean tissues must be added to the wheat germ system in order to translate this polypeptide.

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