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Precursor Forms of Pea Vicilin Subunits

MODIFICATION BY MICROSOMAL MEMBRANES DURING CELL-FREE TRANSLATION

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ABSTRACT

Polyribosomal RNA isolated from pea cotyledons at various developmental stages programmed the cell-free synthesis of polypeptides which were recognized by antibodies specific for pea storage proteins. There were quantitative and qualitative changes in the template activity during seed maturation. Most of the polysomal RNA was associated with the membrane fraction, and all of the template for storage protein occurred in this fraction. Using RNA from a stage of seed maturation at which the synthesis of the high-molecular weight vicilin polypeptides predominate, it was found that the major translation products, although antigenically recognizable as storage protein, did not coincide with the authentic vicillin polypeptides on denaturing polyacrylamide gels. The addition during translation of microsomal membranes from dog pancreas or pea cotyledons resulted in the appearance of new polypeptides which did coincide with some of the authentic vicilin polypeptides (in the apparent molecular weight regions of 75,000 and 50,000) and were antigenically recognizable as storage protein. Other translation products related to storage protein were not visibly altered in their electrophoretic mobility by the addition of membranes. Microsomal membranes treated with Triton X-100 were not effective in modifying the cell-free products. The modified vicilin polypeptides and at least two other translation products were protected from proteolytic degradation, suggesting that they were sequestered within microsomal vesicles. Thus, these storage protein components may be synthesized by a mechanism analogous to that described for membrane and secretory proteins (Blobel G, B Dobberstein 1975 J Cell Biol 67: 835-851).

The biosynthesis of storage proteins of legumes and cereals has been studied during seed maturation by *in vivo* pulse labeling (5, 34) and by cell-free translation of polyribosomes and RNA (3, 5, 6, 8, 9, 13, 15, 16, 18, 22, 24, 29, 35, 36). The synthesis and accumulation of individual storage proteins and their component polypeptides occur at well-defined stages during seed development (for review, see ref. 32). The major storage proteins of peas (legumin and vicilin) both consist of a number of component polypeptides. In the mature seed, they are localized in protein bodies.

It is now clear that the storage proteins of many seeds undergo a range of post-translational modifications. These include glycosylation (2, 10, 12) and post-translational cleavage of a high mol wt precursor (9, 33) as well as other undefined changes which result in altered electrophoretic mobility of polypeptides (34) and proteins (14, 26, 28). There is also evidence which suggests that some of the newly synthesized storage protein polypeptides of cereals (6, 8, 18, 24, 36) and legumes (15, 16) are synthesized as short-lived precursors which may undergo modification during or after translation. In the case of the cereals, the precursors appear to be equivalent to the secretory and membrane proteins of mammalian cells in that a "signal" peptide may occur on the polypeptide.

Here, we have examined in more detail the nature of the translation products from an *in vitro* protein-synthesizing system programmed with pea cotyledon RNA and the relationship of these products to the component polypeptides of storage proteins of mature seed. Based upon the results of the cell-free system, we have concluded that the template for storage protein occurs in membrane-bound polysomes and that there are qualitative changes in the mRNA population during seed development. At least some of the vicilin polypeptides are synthesized as precursors *in vitro* and can be modified, by the addition of microsomal membranes from dog pancreas or pea cotyledon, in such a way that they coincide with authentic vicilin polypeptides on SDS-PAGE[1].

MATERIALS AND METHODS

Plant Material. Peas (*Pisum sativum* L.) line PI/G 086 (stock collection, Commonwealth Scientific and Industrial Research Organization, Canberra) selected from cv. Greenfeast were grown in artificially lit cabinets at 25 C as described (25), and pods of a defined age from flowering were used in each experiment. Seeds were harvested, the testa and axis were removed, and the cotyledons were either used immediately, as for pulse-labeling studies, or stored in liquid N_2 prior to use as a source of polyribosomes and RNA.

Polyribosome and RNA Isolation. Total polyribosomes were isolated from cotyledons ground to a fine powder with a mortar and pestle using liquid N_2 as described (16). Membrane-bound and free polyribosomes were prepared in a similar manner, using the high salt buffer without detergents. The extract was filtered and centrifuged at 1,000*g* for 10 min. The membrane-bound polyribosomes were pelleted at 40,000*g* for 30 min. The supernatant was layered over a 2 M sucrose cushion and centrifuged at 264,000*g* for 3 h in the 70 rotor of a Beckman L5-65B ultracentrifuge to yield a pellet of free polysomes. The phenol-SDS-chloroform procedure (16) was used to prepare RNA from total, free, and membrane-bound polyribosomes. After precipitation from ethanol the RNA was dissolved in water and freed of low mol wt DNA and RNA by adding Na-acetate (pH 6.0) to 3 M and, after keeping for 2 h at 0 C, centrifuging out the high mol wt RNA. The latter was washed three times with 70% (v/v) ethanol and

[1] Abbreviations: PAGE, polyacrylamide gel electrophoresis; IgG, immunoglobulin G; DAF, days after flowering; TEA, triethanolamine; M_r, apparent molecular weight measured relative to the mobility of bovine serum albumin (68,000), ovalbumin (45,000) chymotrypsinogen (25,000), and cytochrome *c* (12,400). Values assigned to storage protein subunits are approximate and are used mainly for descriptive purposes.

once with 90% (v/v) ethanol, dried under N_2, and then dissolved in water for translation or prepared for the isolation of poly(A) RNA as described (1) using oligo(dT)-cellulose (P-L Biochemicals, Milwaukee, WI). The bound fraction was precipitated from ethanol (66%, v/v, final concentration) denatured with dimethyl sulfoxide and reapplied to the oligo(dT) column in 0.5 M NaCl. The RNA which bound the second time was designated poly(A) RNA and was precipitated with ethanol and washed as described above.

Isolation of Microsomal Membranes from Dog Pancreas and Pea Cotyledons. Microsomal membranes were prepared from pancreas essentially as described (4), using the EDTA procedure. Briefly, this consisted of chopping the tissue (weight, 27 g) with a motor-driven razor blade in 50 mM TEA-HCl (pH 7.5), 0.25 M sucrose, 50 mM K-acetate, 5 mM Mg-acetate, 2 mM DTT. The volume was gradually increased to 125 ml. The extract was filtered through Miracloth and centrifuged successively at 1,000*g* for 10 min, at 10,000*g* for 10 min, and at 93,000*g* (average) for 90 min. The 93,000*g* pellet was resuspended in 25 mM TEA-HCl (pH 7.5), 0.25 M sucrose, 4 mM DTT by means of a glass rod and hand-operated homogenizer. The yield at this stage was 1664 A_{280} units in a volume of 19.6 ml. An equal volume of 20 mM EDTA, 100 TEA-HCl (pH 7.5) was added and the mixture was centrifuged at 93,000*g* for 60 min over a 0.5 M sucrose cushion in 25 mM TEA-HCl (pH 7.5), 25 mM K-acetate, 2 mM Mg-acetate, 4 mM DTT. The pellet was resuspended in 20 mM Hepes-KOH (pH 7.5) using a glass homogenizer (yield ≅ 469 A_{280} units). The volume was adjusted so that the A_{280} was 50, an equal volume of glycerol was added, and the membrane fraction was stored in 1- to 2-ml aliquots at −20 C. Processing activity was retained for at least 12 months without loss. Microsomal membranes were prepared from pea cotyledons (12 DAF) by a similar method. A range of concentrations of membranes was tested in the cell-free translation system ranging from 0.5 to 5 μl/25 μl assay. The highest concentration resulted in up to 70% inhibition of protein synthesis. The membranes did not possess template activity, as judged by their addition to the cell-free system in the absence of added mRNA.

Pulse Labeling of Pea Cotyledons and Cell-free Protein Synthesis. Pea cotyledons (11 DAF) were pulse-labeled with a mixture of 14 ^{14}C-amino-acids (Radiochemical Centre, Amersham, United Kingdom) and the salt-soluble proteins were extracted and prepared for electrophoresis as described (34).

The wheat germ cell-free system (S-30) was prepared as described (16) using commercial wheat germ (Mungo Scott, Sydney). Each batch of S-30 was optimized with respect to K-acetate, Mg-acetate, and creatine phosphokinase and was reoptimized for the salts when microsomal membranes were added during translation. The incubation mixture (25 μl) contained 5 μl wheat germ S-30, 20 mM Hepes-KOH (pH 7.8), 20 μM spermidine, 2 mM DTT, 1.2 mM β-mercaptoethanol, 8 mM creatine phosphate, 1 mM ATP, 0.1 mM GTP, 8 μg/ml creatine phosphokinase, and 0.125 μCi of a ^{14}C-amino acid mixture containing 16 amino acids (Radiochemical Centre, Amersham). K^+ and Mg^{2+} optima varied for each S-30 but were usually about 130 and 1.5 mM, respectively. Altering the salt concentrations had no qualitative effect on the pattern of products. Microsomal membranes usually were added at the beginning of the translation period; the quantities are indicated in the figure legends. Optimal RNA concentrations were determined for each preparation. They varied from 200 to 400 μg/ml for polyribosomal RNA and from 20 to 60 μg/ml for poly(A) RNA. Reactions were incubated at 25 C for 90 min. In some experiments, microsomal membranes were added post-translationally to cell-free translation reactions which had been terminated by addition of pancreatic ribonuclease (10 μg/ml) for 10 min at 25 C. The post-translational incubation was carried out for a further 60 min at 25 C. Incorporation of radioactive amino acids into protein was determined by taking 3-μl aliquots for trichloroacetic precipitation on small rectangles of 3MM filter paper (23). The remainder of each reaction mixture was frozen or prepared immediately for SDS-PAGE or immunoaffinity chromatography and electrophoresis.

Trypsin Resistance of Cell-free Translation Products. At the end of the translation period, 25-μl aliquots were incubated with various concentrations of chymotrypsin-free trypsin (Worthington) for 30 min at 0 C. The reaction was stopped by the addition of soybean trypsin inhibitor (final concentration, 5 mg/ml), and the samples were precipitated with cold acetone and dissolved in digestion buffer for SDS-PAGE.

Immunoaffinity Chromatography. Large-scale translation mixtures (up to 1 ml) were used for immunoaffinity chromatography. After translation, unlabeled amino acids (the same 16 amino acids as in the ^{14}C-amino acid mixture, each at 50 μM) and unlabeled storage protein (0.5 mg/ml) were added and the ribosomes were removed by centrifugation at 105,000*g* (average) for 2 h. The supernatant was dialyzed against 0.45 M NaCl, 30 mM Na-phosphate (pH 7.4), 0.1% (w/v) casein hydrolysate with four changes during 15 h. Triton X-100 (0.1%, w/v) was included in the dialysis solution when microsomal membranes had been present during translation. Affinity chromatography was performed as described (34) using IgG which was linked to Ultrogel AcA34 (LKB Produkter, Sweden). The IgG had been purified over a column of pea storage proteins also linked to Ultrogel AcA34 (34). The bound and non-bound fractions were concentrated by precipitation with 1.5 volumes methanol and 6 μl/ml glacial acetic acid at −20 C overnight and centrifuged at 10,000*g* for 15 min prior to solubilization for SDS-PAGE.

SDS-PAGE and Fluorography. Samples were precipitated with 5 volumes cold acetone or with 1.5 volumes methanol and 6 μl/ml acetic acid. The protein pellets were dissolved in 0.125 M Tris-HCl (pH 6.7), 2% (w/v) SDS, 5% (w/v) glycerol, 0.01% (w/v) bromphenol blue, 1% (v/v) β-mercaptoethanol and heated to 85 C for 2 min prior to electrophoresis on slab gradient [12.5 to 20% (w/v)] acrylamide gels as described (34). The gels were fixed, stained, destained, and photographed before preparing them for fluorography (19) using Kodak XRP-54 or Ilford Rapid R type S x-ray film. Because of the very different amounts of radioactivity associated with different polypeptides on the same SDS-PAGE, it has been necessary to expose some fluorographs for two time periods.

RESULTS

Translation of Cotyledonary mRNA and Characterization of Cell-free Products. Polyribosomal RNA isolated from pea cotyledons at four successive stages during the storage protein accumulation phase of seed maturation programmed the wheat-germ cell-free translation system and resulted in a 15- to 50-fold stimulation over background incorporation. The radioactive translation products were fractionated by SDS-PAGE and compared with [^{14}C]formaldehyde-labeled polypeptides from protein bodies of mature seeds (Fig. 1A). Comparison of unmodified and [^{14}C]formaldehyde-labeled storage protein polypeptides showed that formaldehyde labeling did not affect their mobility on SDS-PAGE (not shown). The cell-free products ranged in size from M_r = 5,000 to 90,000 with major polypeptides of about 13,000, 22,000, 29,000, 47,000, 52,000, and 76,000. There were qualitative and quantitative changes in the pattern with development, *e.g.* in the 76,000, 52,000, 47,000, and 22,000 regions. At no development stage was there precise correspondence in size between the cell-free translation products (Fig. 1A, tracks 2 to 5) and the vicilin polypeptides found in mature seeds (Fig. 1A, track 1) or with the major pulse-labeled *in vivo* products of immature seeds (Fig. 1B, *cf.* tracks 1 and 2). At 11 DAF, these latter major *in vivo* products are in the 75,000 and 50,000 region (Fig. 1B, track 1) and have been identified as being vicilin components (34).

Immunoaffinity column chromatography (using antibodies

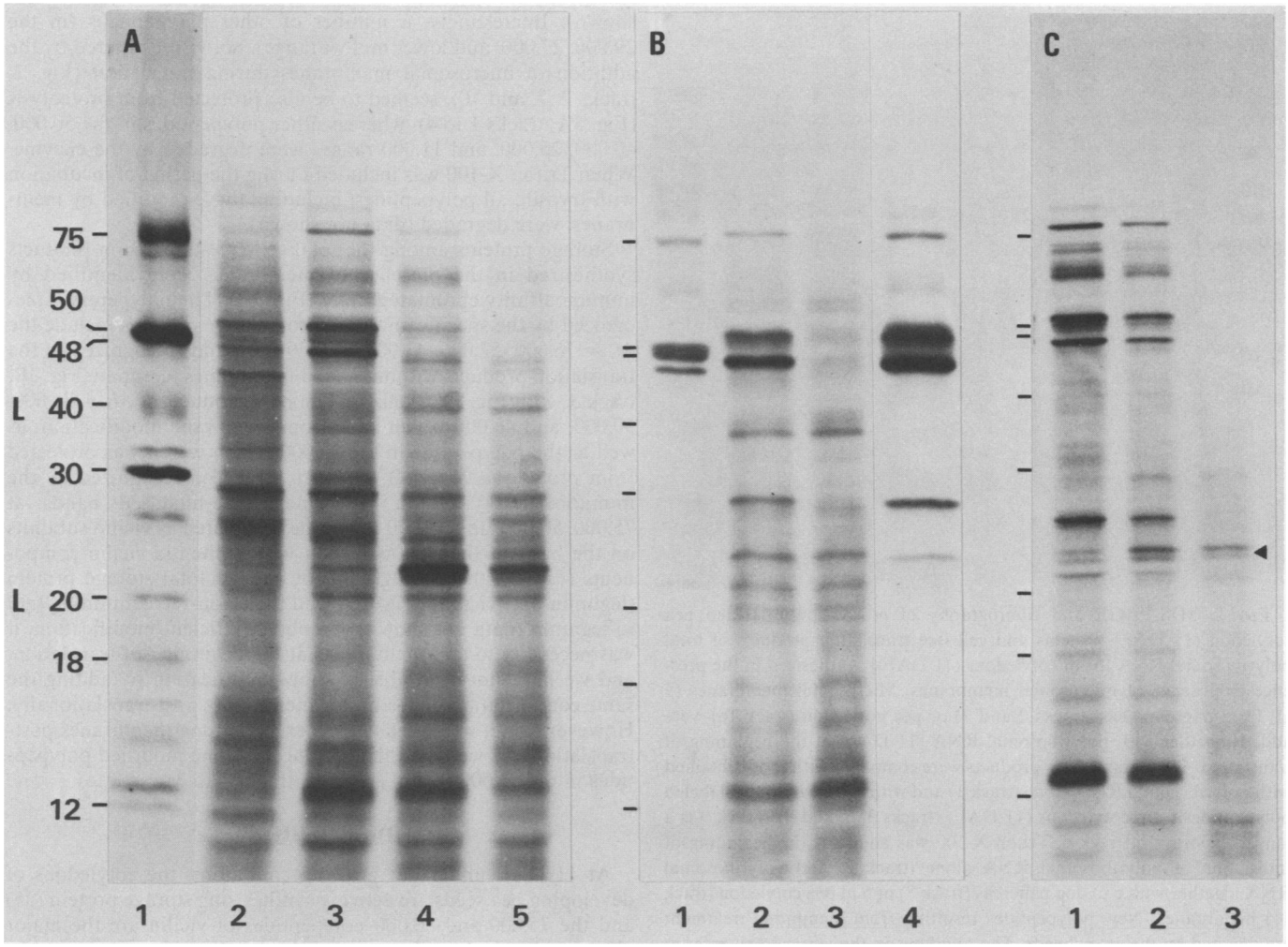

FIG. 1. SDS-PAGE and fluorography of the cell-free translation products of pea polyribosomal RNA and the pulse-labeled *in vivo* products. A, the cell-free products of total polyribosomal RNA isolated from cotyledons at different stages during protein accumulation compared with [^{14}C]-formaldehyde-labeled (34) polypeptides from mature protein bodies. Track 1, the polypeptides from mature protein bodies; tracks 2, 3, 4, and 5, *in vitro* translation products from total cotyledon polyribosomal RNA at 8, 11, 17, and 22 DAF, respectively. The mature legumin polypeptides are at M_r = 40,000 and 20,000; the remaining polypeptides which are assigned M_r values are all vicilin components. B, immunoaffinity chromatography of the translation products of total polyribosomal RNA (11 DAF). Track 2, total cell-free translation products, and the translation products which did not (track 3) and did (track 4) bind to the immunoadsorbent specific for storage protein. Pulse-labeled *in vivo* products synthesized by cotyledons (11 DAF) are shown in track 1 for reference. C, cell-free translation products of total (track 1), membrane-bound (track 2), and free polyribosomal (track 3) RNA from cotyledons (15 DAF). The arrow denotes the 22,000 nonstorage protein doublet. The numbers on the vertical axis refer to $M_r \times 10^{-3}$.

made against pea total storage protein) of the *in vitro* translation products of polyribosomal RNA from cotyledons (11 DAF) yielded two populations of polypeptides, one which did not bind (Fig. 1B, track 3) and one which was bound to the immunoadsorbent (Fig. 1B, track 4). The major bound products were of M_r = 52,000 and 47,000 with other products at M_r = 76,000, 29,000, and 25,000. It is again clear that the polypeptides recognized by antibodies to storage proteins are not precisely coincident with the three major, *in vivo* pulse-labeled vicilin polypeptides in the M_r = 50,000 region nor with the one at M_r = 75,000 (Fig. 1B, compare tracks 1 and 4).

The polyribosomes from cotyledons 15 DAF were fractionated into a membrane-bound and free population followed by RNA isolation and translation (Fig. 1C). The translation products of the membrane-bound polyribosomal RNA showed considerable enrichment for polypeptides of M_r = 76,000, 52,000, and 47,000 which are shown above to be related to storage protein (*cf.* Fig. 1B, track 4), as well as a number of other unidentified polypeptides (Fig. 1C, track 2). The major cell-free product of the free polyribosomal RNA was a doublet of 22,000 (Fig. 1C, track 3, arrowhead) considered to be a nonstorage protein (30). However, the template for this doublet was also present in the membrane-bound fraction (Fig. 1C, track 2).

Fractionation of total polyribosomal RNA by oligo(dT)-cellulose chromatography followed by cell-free translation showed no selective binding of any particular component of the mRNA population. It was also clear that the total mRNA population was represented in the non-poly(A) fraction. Fractionation of the polysomal RNA on the oligo(dT)-column resulted in a 6- to 10-fold increase in template specific activity (data not shown).

Modification of Cell-free Translation Products by Microsomal Membranes. When microsomal membranes from dog pancreas or pea cotyledon were added to the cell-free system at the beginning of translation, three new polypeptide products were formed (Fig. 2, compare track 1 with tracks 2 and 10; see arrowheads) with the same mobility as authentic vicilin polypeptides (Fig. 2, compare tracks 2 and 3, 6 and 7, and 9 and 10). One occurred in the 75,000 region, and the other two occurred in the 50,000 region; one was coincident with the vicilin component at M_r = 50,000, whereas the other coincided with the faster-moving, minor band in this

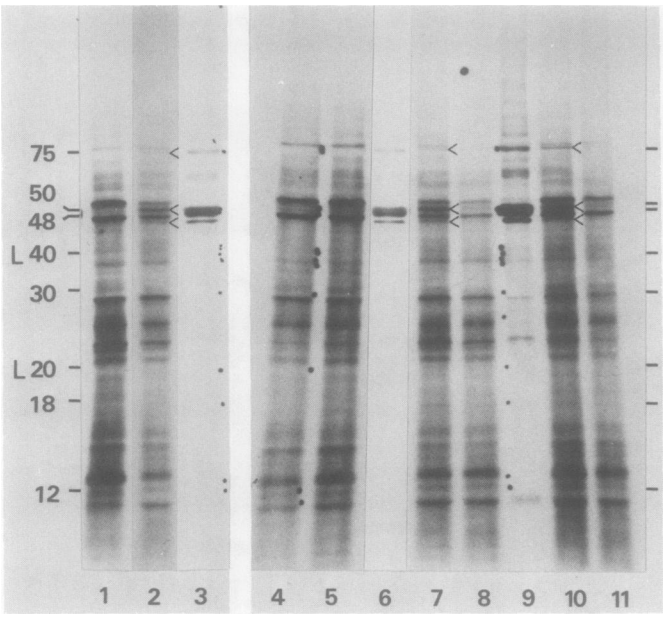

FIG. 2. SDS-PAGE and fluorography of *in vivo* pulse-labeled pea cotyledon (11 DAF) proteins and cell-free translation products of total polyribosomal RNA [from cotyledons (11 DAF)] synthesized in the presence or absence of microsomal membranes. Microsomal membranes (5 μl) from dog pancreas (tracks 2 and 7) or pea cotyledon (track 10) were added together with polyribosomal RNA (11 DAF) at the beginning of translation. These translation products were compared with those obtained with polyribosomal RNA alone (track 1) and with the *in vivo* pulse-labeled polypeptides from cotyledons (11 DAF) (tracks 3, 6, and 9). Track 9 is a longer exposure of track 6. Triton X-100 was added at the beginning of translation of polyribosomal RNA alone (track 5) and polyribosomal RNA together with 5 μl dog pancreas (track 8) or 5 μl pea cotyledon (track 11) membranes. New polypeptides resulting from membrane treatment are denoted by the arrowheads. The numbers on the vertical axis refer to $M_r \times 10^{-3}$.

region at M_r = 46,000. There are no visible changes in any of the other cell-free translation products.

To test whether intact microsomal membranes were required for the appearance of the new polypeptides, we added Triton X-100 to the cell-free translation system both in the presence and absence of microsomal membranes. Although by itself the detergent had no effect on the polypeptide pattern or amount of cell-free products (Fig. 2, tracks 4 and 5), it largely abolished formation of the two new polypeptides in the 50,000 region found in the presence of dog (Fig. 2, compare tracks 7 and 8) or pea (Fig. 2, compare tracks 10 and 11) microsomal membranes. Similarly, the presence of Triton in the translation reaction reduced the formation of the M_r = 75,000 component formed in the presence of dog (or pea) microsomal membranes (Fig. 2, tracks 8 and 11) but did not abolish it altogether.

Protection of Membrane-modified Polypeptides from Proteolysis and Their Identification as Storage Proteins. To test whether modification by membranes also involved sequestration of the modified protein within the microsomal membrane vesicles, the total translation products of pea polyribosomal RNA (11 DAF), which had been synthesized in the presence of microsomal membranes, then were treated with trypsin in a second incubation period. In the M_r = 50,000 region, although all products were digested to some extent, the modified polypeptides (Fig. 3A, arrowheads) were preferentially protected from trypsin. The polypeptides in the 75,000 region are not visible on this fluorograph but, on longer exposures it is clear that the new faster migrating band is the one which survived proteolytic treatment (data not shown). Interestingly, a number of other polypeptides (in the 29,000, 23,000, and lower mol wt range), not visibly altered by the addition of microsomal membranes during translation (Fig. 2, tracks 2, 7, and 10), seemed to be also protected from proteolysis (Fig. 3A, tracks 1 to 4), whereas other polypeptides in the 50,000, 40,000, 25,000, and 11,000 ranges were degraded by the enzyme. When Triton X-100 was included during the period of incubation with trypsin, all polypeptides, including those modified by membranes, were degraded (data not shown).

Storage proteins among the total cell-free translation products synthesized in the presence of membranes were identified by immunoaffinity chromatography (Fig. 3B). The polypeptides recognized by the specific antibodies for storage proteins include the M_r = 76,000, 52,000, 47,000, and 29,000 bands recognized in the translation products without added membranes (compare Fig. 3B, track 3, with Fig. 1B, track 4). The new products at M_r = 75,000, 50,000, and 46,000 which arise from membrane modification, as well as the polypeptide in the 23,000 region which was protected from proteolysis (Fig. 3B, track 3), were also recognized by the immunoaffinity column. The membrane-modified bands at 75,000, 50,000, and 46,000 can all be designated as vicilin subunits on the basis of their co-migration with authentic vicilin components (Fig. 2), their recognition by IgG to total storage protein (legumin plus vicilin) (Fig. 3B), and their non-recognition by IgG to legumin (data not shown). To obtain efficient modification, it was necessary to add membranes at the beginning of translation, and we have not been able to show modification by adding the same concentration of pea or dog membranes post-translationally. However, when we added a large excess of dog membranes post-translationally, we noted the presence of some modified polypeptides in the 50,000 region (Fig. 3C, tracks 1 to 3).

DISCUSSION

At 11 DAF under our growing conditions, the cotyledons of developing pea seeds are actively synthesizing storage protein (25) and the 75,000 and 50,000 polypeptides of vicilin are the major products detected by short-term pulse-labeling of cotyledons (33). When total polysomal RNA from cotyledons (11 DAF) was translated in a wheat germ cell-free system and the products were compared with mature storage protein (16) or with pulse-labeled *in vivo* products (Fig. 1B) by SDS-PAGE, there was little correspondence between the two sets of products. A possible exception is a polypeptide in the 29,000 region of the *in vitro* and pulse-labeled products. Despite the lack of correspondence based upon size, we have previously shown by tryptic peptide mapping (16) that about half the total *in vitro* product is related to mature storage product. We were previously unable to recognize specifically any of the individual *in vitro* products by direct or indirect immunoprecipitation (16) but, here, we have obtained high selectivity and efficient recovery of certain polypeptides, namely those of M_r = 76,000, 52,000, 47,000, 29,000, and 25,000, by using an immunoadsorbent instead of immunoprecipitation from solution.

The profile of *in vitro* translation products changes with seed development in a way similar to that of *in vivo* pulse-labeled products (34). For example, polypeptides in the 50,000 size range are major products 11 DAF but are very much reduced or absent by 17 and 22 DAF (Fig. 1A). The reverse is apparent for polypeptides in the 22,000 range. The period about 15 DAF is transitional in that there is an almost complete spectrum of the translation products found throughout development (*cf.* Fig. 1, A and C). These results contrast in one respect with findings for other legume seeds (5, 15) in which constant patterns were observed for both *in vivo* and *in vitro* translation products during the phase of storage protein accumulation. These results are consistent with those systems in that translatable mRNA for a particular polypeptide was detectable during the synthesis of that protein (5, 15).

Because the storage proteins must be transported through at

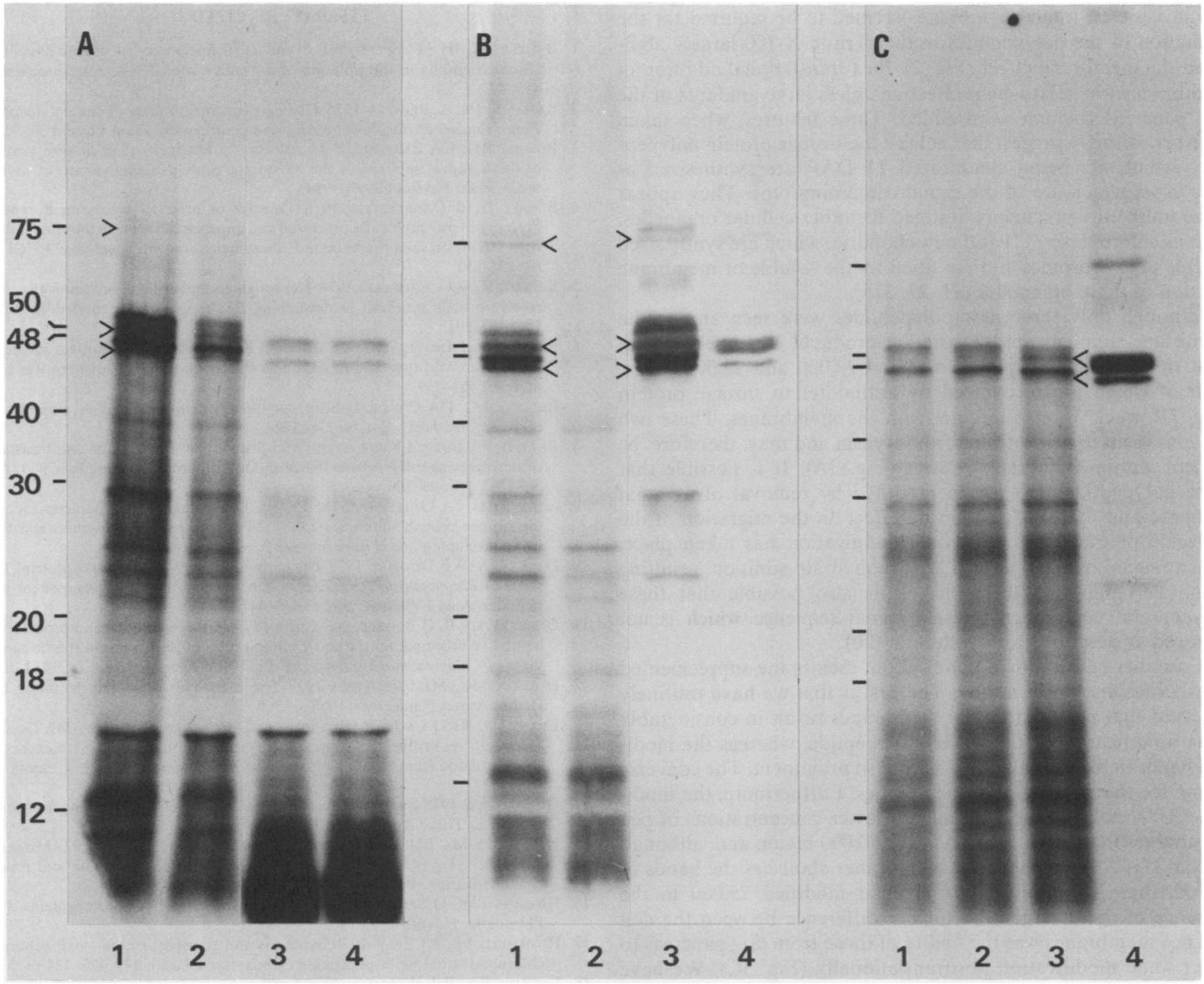

FIG. 3. SDS-PAGE and fluorography of *in vivo* pulse-labeled pea cotyledon (11 DAF) proteins and cell-free translation products of polyribosomal RNA (11 DAF) synthesized either in the presence of, or prior to treatment with, dog pancreas microsomal membranes. Arrowheads denote membrane-modified bands. A, protection of the membrane-modified polypeptides from trypsin digestion. At the end of translation trypsin was added to a final concentration of zero (track 1), 12 (track 2), 40 (track 3), and 80 μg/ml (track 4) and incubated at 0 C for 30 min. The reaction was stopped by adding soybean trypsin inhibitor to 5 mg/ml. B, immunoaffinity chromatography of the cell-free translation products of polyribosomal RNA (11 DAF) synthesized in the presence of dog pancreas membranes. Total cell-free products (track 1) and the products which did not (track 2) and did (track 3) bind to the affinity column containing IgG to total storage proteins. *In vivo* pulse-labeled products (11 DAF) are included for reference (track 4). C, effect of post-translational addition of increasing levels of microsomal membranes (dog) to the cell-free translation products of polyribosomal RNA (11 DAF). At the end of translation, pancreatic ribonuclease was added to 10 μg/ml and zero (track 1), 10 (track 2), and 50 μl (track 3) microsomal membranes were added and incubation continued at 25 C for a further 60 min. *In vivo* pulse-labeled products (11 DAF) are included for reference (track 4). The numbers in the vertical axis refer to $M_r \times 10^{-3}$.

least one membrane before deposition in the vacuole or protein body, we investigated the subcellular localization of the polyribosomes responsible for storage protein synthesis. The proportion of polysomes attached to membranes (as judged by sedimentation behavior) increases from 30% at 8 DAF to about 70% by 11 to 13 DAF (unpublished observations), which is consistent with other seed systems (5–7, 18, 29). Here, it was found that the polypeptides known to be related to storage proteins were synthesized only on RNA from membrane-bound polyribosomes, whereas one set of polypeptides thought to be nonstorage protein in origin was synthesized by both free and membrane-bound polyribosomal RNA. Although it is possible that this reflects the *in vivo* situation, it is more likely that the membrane-bound population was contaminated by free polysomes.

Inasmuch as the cell-free translation products characterized as storage protein were synthesized on membrane-bound polyribosomes and differed in mobility on SDS-PAGE from known storage protein polypeptides, we considered the possibility that they were synthesized as precursors with a fragment equivalent to the signal sequence described for many mammalian intrinsic membrane and secreted proteins (4, 21). The function of such a sequence could be to initiate the appropriate series of steps in the intracellular transport of the newly synthesized storage protein polypeptide. Supplementing the cell-free translation system with stripped microsomal membranes from dog pancreas or pea cotyledons resulted in the appearance of three new polypeptides, all of the vicilin type. One of these migrated to the same position as the 75,000 vicilin polypeptide, whereas the other two migrated to the same positions as the two vicilin polypeptides in the 50,000 region on SDS-PAGE (Fig. 2). These new polypeptides reacted with IgG against total storage proteins (Fig. 3B) and were protected from proteolytic attack by trypsin, suggesting that they were sequestered

within vesicles. Intact membranes seemed to be required for the formation of the polypeptides in that Triton X-100 largely abolished the membrane effect (Fig. 2). Post-translational addition of membranes was relatively ineffective unless large amounts of the dog pancreas fraction were added. These features, when taken together, strongly suggest that at least the storage protein polypeptides which are being synthesized 11 DAF are synthesized as precursors which are of the signal-containing type. They appear to be unlike the precursors destined for other cellular organelles, such as chloroplasts (17) and mitochondria, which are synthesized on free polyribosomes and modified by the soluble or membrane fraction of these organelles (11, 27, 31).

Although only three new polypeptides were seen among the membrane-supplemented translation products, it is likely that at least two other polypeptides, of M_r = 29,000 and 23,000 range, both of which are recognized by antibodies to storage protein (Fig. 3B, track 3), also interact with the membranes. These two proteins seem to be protected from trypsin and may, therefore, be present within membrane vesicles (Fig. 3A). It is possible that these polypeptides have been modified by removal of a signal sequence and that this has had no effect on the migration of the molecule or that more than one modification has taken place, such as signal removal as well as carbohydrate addition, resulting in no net migration difference. It is also possible that these polypeptides contain an internal signal sequence which is not removed as described for ovalbumin (20).

A number of additional points about membrane-supplemented translations are worth noting. The first is that we have routinely observed that pea microsomal membranes result in considerably more modification of the 75,000 polypeptide, whereas the modified bands in the 50,000 region are not so prominent. The converse is true for the dog pancreas membranes. Furthermore, the modified 75,000 polypeptide is formed at lower concentrations of pea membranes than are the bands in the 50,000 region and, although Triton X-100 treatment of the membranes abolishes the bands of 50,000, there is usually some residual modified 75,000 in the presence of the detergent. A further difference between the dog and pea membranes was the ability of those from dog pancreas to effect some modification posttranslationally (Fig. 3C). We have also noted that the membranes from both sources seem less inhibitory of total protein synthesis with freshly prepared wheat germ extracts. The reasons for these differences are not understood at present.

Evidence has been obtained for similar precursors in the storage proteins of barley and maize (6, 8, 18, 24, 36) and Burr *et al.* (8) presented preliminary evidence that the additional sequence was located at the amino-terminus of a zein polypeptide. The storage proteins of French bean have also been shown to be synthesized *in vitro* as polypeptides of different size to those found *in vivo* (15), but it is not known whether these precursors are affected by membranes. Our results contrast with those of Evans *et al.* (13) and Croy *et al.* (9) who have reported that some of the components of pea vicilin are synthesized *in vitro*, in the absence of added membranes, as products with the same mobility as authentic mature subunits. Whether the difference between our data and theirs is due to differences in the resolution of slight variations in polypeptide size or to some more fundamental parameter remains to be resolved. In general, it appears likely that storage proteins of both cereals and legumes may be synthesized on membrane-bound polysomes as precursors which are modified during translation. So far, the data are generally consistent with the signal hypothesis (4), but considerable effort is now required to substantiate this proposal, in particular the presence of a signal sequence needs to be confirmed.

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