

## A Soybean Vacuolar Protein (P34) Related to Thiol Proteases Is Synthesized as a Glycoprotein Precursor during Seed Maturation\*

(Received for publication, January 16, 1992)

Andrew Kalinski, Diane L. Melroy‡, Radhey Shyam Dwivedi§, and Eliot M. Herman¶

From the Plant Molecular Biology Laboratory, United States Department of Agriculture, Agricultural Research Service, Beltsville, Maryland 20705

We have examined the synthesis, posttranslational processing, and localization of soybean P34, a member of the papain superfamily. P34 has been identified as a constituent of oil storage organelles or oil bodies isolated from seed lysates and has been assumed to be one of the oil body proteins. Electron microscopic immunocytochemistry with a monoclonal antibody demonstrated that P34 is localized in the protein storage vacuoles but not in the oil bodies. Immunocytochemical observations of partially disrupted seed cells showed that the association of P34 with oil bodies appears to occur as a consequence of cell lysis. *In vitro* synthesis of P34 results in the formation of a 46-kDa polypeptide that increases to 47 kDa due to core glycosylation by canine microsomes. *In vivo* synthesis studies in the presence and absence of tunicamycin, an inhibitor of N-linked glycosylation, indicate that pro-P34 is 47 kDa. Since the cDNA sequence of prepro-P34 contains a single putative glycosylation site in the precursor domain, we conclude that P34, like a few other vacuolar proteins, is synthesized as a glycoprotein precursor. Pulse-chase experiments showed that the processing of pro-P34 to mature P34 occurs in a single step and that this posttranslational cleavage occurs on the carboxyl side of an Asn, which is typical of seed vacuolar proteins. Pro-P34 (47 kDa) is detected in immunoblots of maturing seeds. Analysis of RNA indicates that the P34 genes are expressed only during seed maturation and that the P34 mRNA is related to other thiol protease mRNAs detectable in other organs and plants. Unlike other seed thiol proteases that are synthesized only after seed germination, P34 accumulates during seed maturation.

(Tahio *et al.*, 1983; Chan *et al.*, 1986; Ishidoh *et al.*, 1987, 1989), thiol proteases expressed in slime molds (Williams *et al.*, 1985; Pears *et al.*, 1985; Datta and Firtel, 1987), plant cell proteases including papaya fruit papain (Cohen *et al.*, 1986), barley (Rogers *et al.*, 1985; Koehler and Ho, 1988; Koehler and Ho, 1990), pea (Guerrero *et al.*, 1990), rice (Watanabe *et al.*, 1991), mung bean (Mitsunashi *et al.*, 1986; Akasofu *et al.*, 1989), and kiwi fruit actinidin (Preaekelt *et al.*, 1988; Podivinsky *et al.*, 1989). Sequence comparisons between the protein and deduced sequences show considerable homology in several domains, which indicates that this gene family has been highly conserved. The sequence homology among thiol proteases extends from the 5' to 3' ends of the open reading frames, including not only the mature polypeptide but also the signal sequence and precursor domains (North, 1986).

Although the sequence and structure of members of the thiol protease family is well characterized, the elucidation of the physiological roles of these proteins, especially in higher plants, is still not well understood. The expression of plant thiol protease genes has been shown to be correlated with the onset of physical stress (Schaffer and Fischer, 1988; Guerrero *et al.*, 1990) and the mobilization of vacuolar reserve storage proteins, such as in legume (Baumgartner and Chrispeels, 1977; Baumgartner *et al.*, 1978), rice (Watanabe *et al.*, 1991), barley seeds (Koehler and Ho, 1988; Holwerda *et al.*, 1990), and brassica seedlings (Dietrich *et al.*, 1989). In other instances, the presence of abundant thiol protease(s) does not appear to be well correlated with an obvious physiological function, such as in the case of bromelin, a thiol protease of pineapple plant stems.

Where there is a good correlation between synthesis of thiol proteases and physiological function there still may be considerable complexities involved in thiol protease gene expression. For example barley aleurones express at least three different thiol proteases, termed cysteine proteases EP-A and EP-B (Koehler and Ho, 1990) and aleurain (Rogers *et al.*, 1985), during germination and in response to the phytohormone gibberellin. Two of these thiol proteases (EP-A and EP-B) are secreted from the cell, whereas the other (aleurain) is sequestered within the aleurone cells. The intracellular thiol protease appears to be localized in a vacuole-like subcellular compartment that is distinct from the protein storage vacuoles, termed aleurone grains, that contain seed storage proteins. Aleurain has not yet been shown to possess proteolytic activity, and the functional role and exact characterization of the aleurain-containing organelle remains to be determined.

We have previously shown that isolated soybean oil bodies contain polypeptides of 34, 24, 18, and 17 kDa, which we have termed P34, P24, P18, and P17, respectively (Herman 1987; Herman *et al.*, 1990). Using monoclonal antibodies directed against P34, we observed that it undergoes developmentally regulated processing to a 32-kDa protein during seedling

Thiol proteases belong to the papain superfamily (EC 3.4.22), which are expressed in diverse eukaryotic species (see Bond and Butler (1987) for review). The sequences of cDNA and genomic clones have been determined for many members of this superfamily, including the cathepsins of animal cells

\* Supported in part by Grant 89-37231-4460 from the United States Department of Agriculture Office of Competitive Grants (to E. M. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Biology Dept., Siena Heights College, Adrian, MI 49221.

§ Visiting scientist. Permanent address: Dept. of Botany, Howard University, Washington, D. C. 20059.

¶ To whom correspondence should be addressed: Plant Molecular Biology Laboratory, United States Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705. Tel.: 301-504-5258; Fax: 301-504-5320.

growth as the consequence of the removal of an amino-terminal decapeptide (Herman *et al.*, 1990). We isolated a cDNA clone of P34 and found it was highly homologous to the papain superfamily of thiol proteases (Kalinski *et al.*, 1990). The apparent expression of P34 during seed maturation rather than seed germination sets it apart from other described seed thiol proteases. The apparent localization of a thiol protease-related gene product in the oil bodies was surprising, and as a consequence, we undertook a series of studies to examine the developmental regulation of the synthesis, processing, and localization of P34 in order to better define its potential physiological roles.

#### EXPERIMENTAL PROCEDURES

**Materials**—Soybean plants (*Glycine max* Merr. L cv. Century) used as a source of developmentally staged seeds were maintained as previously described (Herman *et al.*, 1990). Germinating seeds were grown by imbibing dry seeds overnight and sowing the seeds on wet paper towels. The seedlings were maintained in the dark at room temperature as a source of staged cotyledons. Monoclonal antibodies P3E1 and P4B5, which are directed against two different epitopes of P34, were previously described in Herman *et al.* (1990). A cDNA clone encoding the entire open reading frame of P34 was previously described in Kalinski *et al.* (1990). Tomato (*Lycopersicon esculentum* cv. Pik Red), rice (*Oryza sativa* cv. Calrose 76), and barley (*Hordeum vulgare* L. cv. Himalaya) were grown in the greenhouse.

**RNA and DNA Isolation**—Total RNA was isolated as previously described (Kalinski *et al.*, 1990). Poly(A<sup>+</sup>) RNA was fractionated from total RNA on Poly(A<sup>+</sup>) Quik column (Stratagene Cloning Systems). Phagemid DNA containing a P34 cDNA insert was isolated from recombinant bacteria using a boiling method (Holmes and Quigley, 1981). The P34 cDNA insert was released from pBluescript SK(−) phagemid DNA by *EcoRI* and *BamHI* digestions, separated from the vector DNA by electrophoresis on 0.8% agarose gel, and then extracted from the gel (Sambrook *et al.*, 1989). RNA and DNA were quantified spectrophotometrically.

**In Vitro Translation, in Vivo Labeling Immunoprecipitation, Oil Body Protein Isolation, SDS-PAGE,<sup>1</sup> Immunoblots**—mAb P3E1- and P4B4-secreting cell lines were expanded *in vivo* as ascites tumors. Ascites fluid (0.33 ml) was diluted 2:1 with 25 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) and clarified by centrifugation in a Brinkmann microcentrifuge for 5 min. The supernatant was passed over a protein G-Sepharose 4B column (Pharmacia-LKB, Uppsala, Sweden) and washed with TBS. The bound IgGs were eluted with 0.025 M glycine HCl, pH 2.8, neutralized with 0.1 M sodium borate/boric acid buffer, pH 8.6, and dialyzed against 4 liters of borate-buffered saline (10 mM sodium borate, 150 mM NaCl, pH 8.6) overnight at 7 °C. Equal amounts of mAbs P3E1 and P4B5 IgGs were combined based on  $A_{280\text{ nm}}$  and coupled to CNBr-Sepharose 4B with excess IgG in order to obtain maximum amounts of bound antibody, according to the manufacturer's directions.

**In vitro translation of midmaturation (150 mg, fresh weight) soybean seed poly(A<sup>+</sup>) mRNA** was accomplished using a reticulocyte translation kit (*in vitro* express, Stratagene) with (<sup>35</sup>S)methionine, according to the supplied protocol. Canine microsomes (Promega Biotec) (1.8 or 3.6 eq) were added to some of the translation reactions in order to assess cotranslational processing of newly synthesized polypeptides.

**In vivo labeling** was accomplished by incubation of thin slices (1 mm thick) in 25  $\mu$ Ci of carrier-free (<sup>35</sup>S)methionine. Samples were initially pulsed for 1 h and, in some instances, chased by transferring the tissue slices to 4% sucrose, 1% asparagine, 1 mM methionine for varying lengths of time. In order to examine the glycosylation status of the newly synthesized proteins, tissue samples were incubated in 0.05 mg/ml tunicamycin for 30 min, then 25  $\mu$ Ci of (<sup>35</sup>S)methionine was added, and the samples were incubated for 1 h. Parallel controls were incubated in the same solutions without tunicamycin. The *in vivo* labeled samples were placed in microcentrifuge tubes and then frozen with liquid nitrogen and extracted by sonication in a water bath sonicator for 15 min after the addition of 50  $\mu$ l of 6 M urea, 5% (w/v) SDS, 50 mM Tris-HCl, pH 6.8, 5% v/v  $\beta$ -mercaptoethanol (SDS-urea sample buffer). The resulting lysates were removed to a

new microcentrifuge tube, diluted with TBS containing 2% v/v Triton X-100, and then clarified by centrifugation in a Brinkmann microcentrifuge. The labeled polypeptides were recovered by adding 50  $\mu$ l of mAb P3E1/P4B5 Sepharose 4B and tumbling the samples overnight at room temperature. The Sepharose-antibody-P34 complexes were recovered by washing the pellet five times with TBS. After the final wash, the buffer was removed and the Sepharose-antibody-P34 complex was denatured by the addition of 100  $\mu$ l of 8 M urea, 5% (w/v) SDS, 50 mM Tris-HCl, pH 6.8, with 5% (v/v)  $\beta$ -mercaptoethanol.

Oil bodies were isolated from 24-h imbibed soybean seeds by homogenization in 0.1 M Tris-HCl, pH 8.6, using a Polytron apparatus. The homogenate was filtered through several layers of cheese-cloth and then centrifuged for 20 min at 20,000 rpm in a Beckman SW-28 and L2-70 M centrifuge. The oil body pad was resuspended in the homogenization buffer and recentrifuged. The oil body pad was then resuspended in 0.5 M NaCl, 0.1 M Tris-HCl, pH 8.6, and centrifuged as before. The oil body pad was divided into two portions, and the first was resuspended in distilled water and centrifuged as above. The second oil pad fraction was resuspended in 0.1 M Na<sub>2</sub>CO<sub>3</sub> and left for 1 h on ice to remove proteins not embedded in the membrane (Fujiki *et al.*, 1982). The Na<sub>2</sub>CO<sub>3</sub>-extracted oil pad was recovered by centrifugation as before. Both the distilled water and Na<sub>2</sub>CO<sub>3</sub> oil pads were extracted with cold acetone to remove triglycerides. The precipitated protein was dissolved in SDS-sample buffer and analyzed by SDS-PAGE.

Developmentally staged samples for SDS-PAGE-immunoblots were obtained by staging the seeds based on fresh weight (Herman *et al.*, 1990). Cotyledon tissue was ground in SDS-PAGE sample buffer containing 5% (v/v)  $\beta$ -mercaptoethanol at a ratio of 1 mg of tissue to 10  $\mu$ l. SDS-PAGE analysis was accomplished using the discontinuous buffer system and a 12.5% resolving gel. Radioactive polypeptides resolved by SDS-PAGE were analyzed by fluorography after impregnating the gels with Enhance (Du Pont) according to the manufacturer's directions. Immunoblot analysis of P34 was accomplished by electrophoretically transferring the resolved proteins to a nitrocellulose membrane in 20 mM sodium borate, pH 8.6, 20% methanol, 0.05% thioglycolate. Immunolabeling was accomplished by incubation with a 1:10,000 dilution of mAb P4B5 ascites fluid in 1% (w/v) gelatin/TBST overnight at room temperature. Anti-mouse IgG-alkaline phosphatase (Sigma) at 1:1000 dilution in 1% gelatin/TBST (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% (w/v) Tween 20) for 90 min at room temperature was used to indirectly label the bound P4B5 antibodies. The alkaline phosphatase activity was visualized with the chromogenic substrate nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**Northern and RNA Slot Blot Hybridization**—For Northern blot analysis, RNA samples (15  $\mu$ g) were fractionated in an agarose/formaldehyde gel (Lehrach *et al.*, 1977) and transferred onto a nitrocellulose filter. The filter was baked for 2 h at 80 °C and prehybridized in 50% (v/v) formamide, 5  $\times$  SSPE (1  $\times$  SSPE: 0.18 M NaCl, 10 mM Na<sub>2</sub>PO<sub>4</sub>, pH 7.0, 1 mM EDTA), 5  $\times$  Denhardt's, 0.1% (w/v) SDS, and 200  $\mu$ g/ml denatured salmon testes DNA for 5 h at 42 °C. Denatured random-primed labeled P34 cDNA insert (3  $\times$  10<sup>6</sup> cpm/ml) prepared according to Feinberg and Vogelstein (1983) was hybridized to the filter for 19 h at 42 °C. The filter was washed twice for 20 min in 2  $\times$  SSPE, 0.1% (w/v) SDS at room temperature and for 30 min in 0.1  $\times$  SSPE, 0.1% SDS at 42 or 58 °C stringency wash temperatures. Slot blot analysis was performed as described (Sambrook *et al.*, 1989). Prehybridization, hybridization, and posthybridization washes (68 °C) were done as described above for Northern blot.

**Electron Microscopy and Immunocytochemistry**—Developmentally staged soybean seed cotyledons were cut into approximately 1-mm<sup>3</sup> cubes and fixed with 4% formaldehyde and 2% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4, overnight at 7 °C. The tissue pieces were dehydrated in a graded ethanol series and embedded in the hard formulation of LR White resin. Ultrathin sections mounted on nickel grids were blocked with 10% fetal bovine serum in TBST (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% (w/v) Tween 20) for 15 min at room temperature. The sections were then labeled with affinity-purified mAb P4B5. The monoclonal antibody was isolated from ascites fluid by chromatography on an oil body protein-Sepharose 4B column (Herman *et al.*, 1990). Anti-P34 mAbs were eluted with 0.1 M glycine HCl, pH 3.0, and dialyzed against 1000 volumes of TBS. The sections were labeled in 0.02 mg/ml mAb P4B5 IgG<sub>1</sub> diluted in 10% fetal bovine serum/TBST, pH 7.4, for 15 min at room temperature. The grids were then washed with TBST and indirectly labeled with 10 nm colloidal gold particles coupled to rabbit antimouse

<sup>1</sup> The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

IgG. The grids were again washed with TBST, rinsed in water, and stained with 5% aqueous uranyl acetate. The grids were examined and photographed with Hitachi H300 and H500 electron microscopes.

## RESULTS

**P34 Is Found in Purified Oil Bodies and Can Be Selectively Removed from the Oil Body Fraction by Carbonate Extraction**—Oil bodies were purified by centrifugal flotation of seed lysates. The SDS-PAGE of proteins extracted from crude soybean oil bodies isolated from whole seeds, total cotyledon, and axis homogenates are shown in Fig. 1 (lanes 1–3). As previously shown, four major polypeptides, 34, 24 (oleosin), 18, and 17 kDa, are enriched as a consequence of purifying soybean oil bodies (Herman, 1987; Murphy and Cummins, 1989; Tzen *et al.*, 1990). Additional high molecular weight proteins corresponding to the molecular weight of soybean vacuolar storage proteins are also observed. In order to examine whether P34 is embedded in the oil body membrane, isolated oil bodies were treated with 0.1 M sodium carbonate for 1 h on ice. The carbonate treatment of the oil bodies resulted in the selective extraction of P34, whereas the 24- and 18-kDa oleosins remained firmly embedded in the oil body membrane (Fig. 1, lane 4). The selective removal of P34 by carbonate may indicate that P34 is not embedded in the oil body membrane. This would be consistent with the deduced sequence of the P34 (Kalinski *et al.*, 1990), which does not contain any obvious domain for membrane insertion.

**Immunological Detection of pro-P34 in Maturing Soybean Seed**—The open reading frame of P34 indicated that there is a potential coding region encoding 122 amino acids 5' of the experimentally determined amino terminus (Kalinski *et al.*, 1990; Herman *et al.*, 1990), which is homologous to the prepro regions of the deduced sequences of other plant thiol proteases. In order to examine whether there is an accumulation of high molecular weight putative precursors of P34, samples of soybean seed cotyledons at different developmental stages were extracted with SDS-sample buffer. The samples were

fractionated by SDS-PAGE and blotted onto nitrocellulose membranes. Replicate membranes were stained with Amido Black to visualize total proteins or immunolabeled with P3E1 mAb (Fig. 2). As previously shown, the 34-kDa P34 is accumulated during the course of seed development (Herman *et al.*, 1990). A putative pro-P34 polypeptide is immunolabeled with an apparent molecular mass of 47 kDa (Fig. 2B). The size of the putative pro-P34 observed on the immunoblots is similar to the predicted size of the pro-P34 based on its primary structure. This high molecular weight polypeptide is not observed during germination and seedling growth (data not shown), which is consistent with the accumulation of P34 mRNA and its translation to P34 polypeptide during seed maturation.

**P34 Is Localized in Protein Storage Vacuoles**—Biochemical fractionation of soybean seeds and analysis of the proteins found in the oil bodies recovered by centrifugal flotation have shown the presence of four abundant polypeptides of 34, 24, 18, and 17 kDa (Herman, 1987; Murphy and Cummins, 1989; Herman *et al.*, 1990; Tzen *et al.*, 1990). The 24- and 18-kDa polypeptides are members of the oleosin family of integral membrane proteins of the oil bodies. The primary structure of the 34-kDa protein indicates that it belongs to the papain superfamily of thiol proteases, which in plants includes members that are localized in the vacuole or are secreted. The 34-kDa protein does not have a membrane insertion region, which suggests that if it is associated with the oil bodies, it is either bound to the oil body surface or to other proteins such as the oleosins. The biochemical characteristics of P34, synthesis as a prepolypeptide, and its glycosylation, are consistent with the synthesis of a secretory protein destined for secretion or accumulation in the vacuole. This suggests that P34 may not be an authentic oil body protein. In order to resolve the question of the localization of P34, electron microscopic immunocytochemical analysis was performed with immunoadfinity-purified monoclonal antibodies and indirect labeling with colloidal gold second antibody. The purified mAb P4B5 densely labeled the protein storage vacuoles or protein bodies in the storage parenchyma cells (Fig. 3). Segments of endoplasmic reticulum in the cytoplasm surrounding the protein storage vacuoles were also labeled with gold particles, which is consistent with the synthesis of P34 as an N-linked glycoprotein precursor. Oil bodies in the surrounding cytoplasm were not labeled by mAb P4B5 and the colloidal gold second antibody (Fig. 3), which indicates that P34 is not a genuine constituent of the oil bodies. The localization of P34 within the protein storage vacuoles is consistent with the

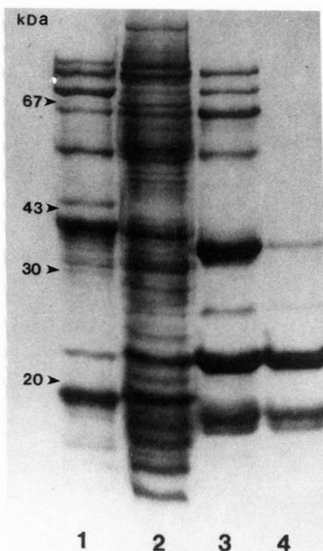


FIG. 1. SDS-PAGE showing the isolation and purification of soybean oil body proteins. Lanes 1 and 2 show the total polypeptide distribution of the cotyledon and axis, respectively. Lane 3 shows the molecular mass distribution of oil body proteins of midmaturation soybean seeds after washing with both low and high salt buffers. Note that the resulting sample is enriched in polypeptides of 34 (P34), 24 (oleosin), 18, and 17 kDa. Lane 4 shows that a 1-h extraction with 0.1 M sodium carbonate appears to selectively remove the P34 band, indicating that this protein is not firmly embedded in the oil body.

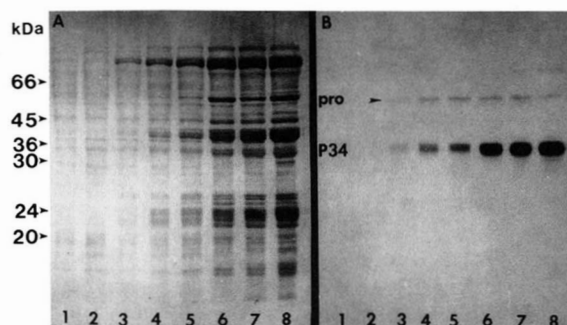


FIG. 2. Accumulation of P34 in maturing soybean seeds. A, total protein distribution of the staged samples on Amido Black-stained nitrocellulose. B, the polypeptides labeled with mAb P3E1 ascites. Each lane in both panels corresponds to the polypeptides extracted from 1 mg of fresh weight of staged cotyledons. Lanes 1–8 are from 12-, 25-, 65-, 100-, 150-, 200-, 250-, and 300-mg fresh weight seeds, respectively.

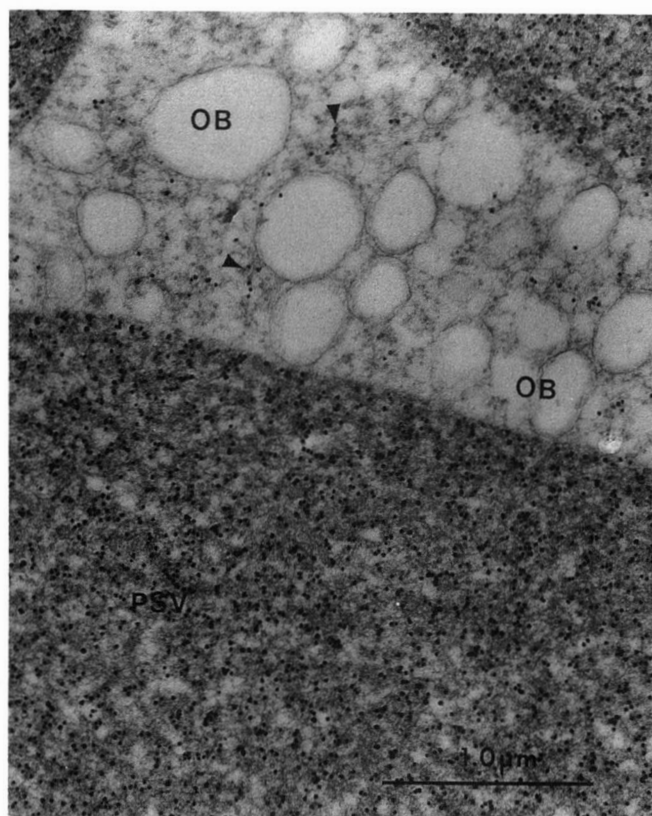


FIG. 3. Immunogold localization of P34 in a midmaturation cotyledon storage parenchymal cell. The matrix of the protein storage vacuoles (PSV) is densely labeled by gold particles. In contrast, there is very little label associated with the oil bodies (OB). Gold particles are also apparently associated with segments of the endoplasmic reticulum (arrowhead). The immunocytochemical localization indicates that P34 is not associated with the oil bodies *in situ*.

biosynthesis and posttranslational data presented in the next sections, which show P34 to be posttranslationally processed by mechanisms typical for vacuolar proteins.

The immunocytochemical observations suggested that P34 may become associated with the oil bodies as a consequence of cellular lysis and/or fractionation. We also conducted immunogold examination of partially disrupted cells on the edge of the tissue blocks. Disruption of these cells is a consequence of mincing the tissue as a preparation for fixation and embedding for electron microscopy. In these cells, the protein storage vacuoles were observed to be disrupted, releasing their contents into the cytoplasm. Immunogold localization of P34 in the disrupted cells with mAb P4B5 shows extensive labeling of the oil bodies in these cells (Fig. 4). Therefore, based on our immunocytochemical observations, we consider the association of P34 with the oil bodies to be the consequence of affinity of this protein for the oil body surface.

**P34 Is Localized in the Golgi Apparatus of Maturing Soybean Seed Cells**—P34 was also localized in the Golgi apparatus and secretion vesicles of midmaturation soybean storage parenchymal cells by indirect immunogold microscopy. Previous studies have shown that storage proteins, lectins, and enzymes sequestered within protein storage vacuoles are also localized within the Golgi apparatus, which mediates the protein deposition (see Herman (1988) for review). Fig. 5, A and B, shows a medial cross-section and an enface section in the *trans* region of the Golgi apparatus of midmaturation soybean cotyledon cells. Indirect immunogold localization is a preferable method to the alternative biochemical experiment using mo-

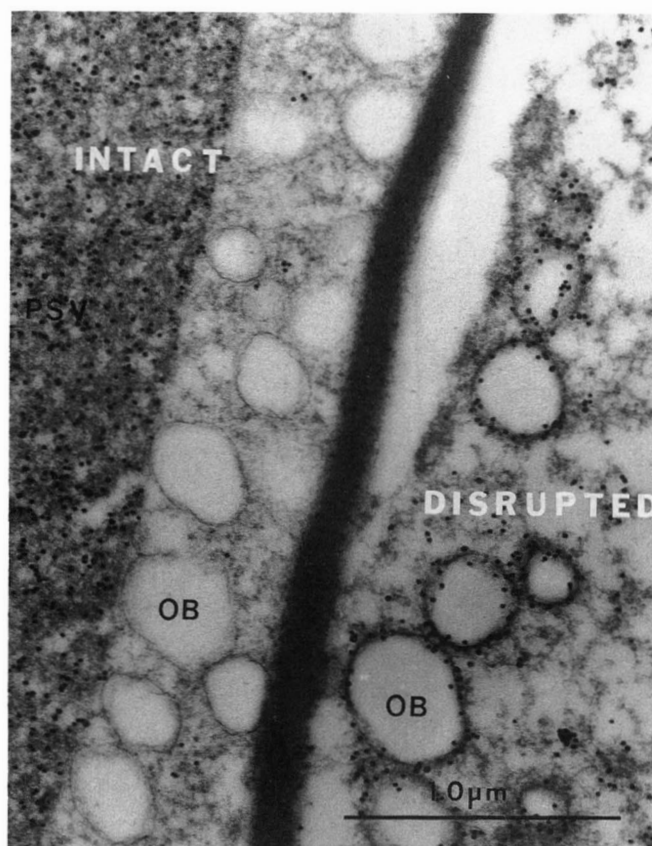


FIG. 4. P34 is apparently redistributed as the consequence of cellular disruption. Two adjacent cells are shown. One is intact; the other is disrupted. In the intact cell, P34 is localized within the protein storage vacuole (PSV) and is not associated with the oil bodies. However, in the disrupted cell, anti-P34 monoclonal antibodies label the oil bodies (OB), indicating that P34 becomes associated with the oil body membrane as the consequence of cell lysis.

nensin inhibition for immature legume seeds, since this inhibitor has been shown to induce the extracellular secretion of legume storage proteins and lectins rather than cessation of transport in the Golgi apparatus characteristic of other systems (see Tartakoff (1983) for review). Additional gold label was observed on small electron-dense vesicles located in the cytoplasm of mid to late maturation storage parenchyma cells (Fig. 5B). The electron-dense vesicles appear to be derived from the Golgi apparatus and would function in transporting proteins to the incipient protein storage vacuoles. Similar small electron vesicles have been shown to contain vacuolar localized soybean seed galactosidase and have been observed to be fused to protein storage vacuoles (Herman and Shannon, 1985; Herman, 1992).

**P34 Remains Associated with the Protein Storage Vacuole during Mobilization of Storage Proteins**—During seed ontogeny, the protein storage vacuoles undergo developmentally regulated and temporal differentiation or subdivision of the central vacuole into numerous small protein storage vacuoles, which are filled with storage proteins and other enzymes, lectins, and defense proteins. After germination, the storage proteins are progressively digested, and the small protein storage vacuoles fuse to reform the central vacuole (for a review, see Herman (1992)). The immunocytochemical localization of P34 was examined during seed germination and seedling growth. This laboratory has isolated P34 and observed its apparent association with oil bodies in imbibed and germinated seeds (Herman *et al.*, 1990). P34 was localized in the protein storage vacuoles during the developmental stage

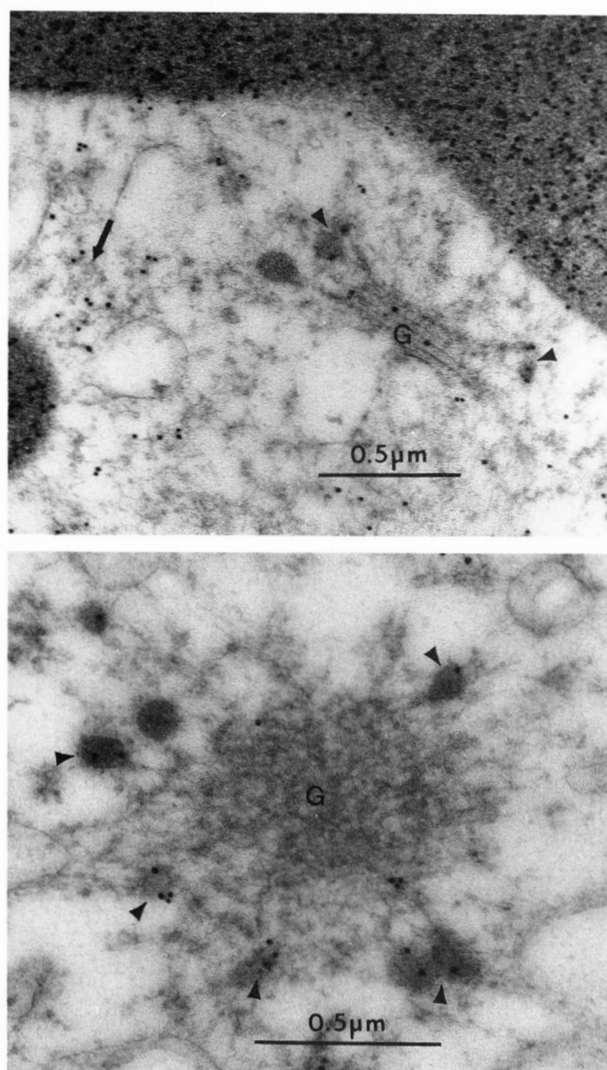


FIG. 5. The immunogold localization of P34 in the Golgi apparatus of midmaturation soybean storage parenchyma cells. A, an immunogold labeled cross-section of a Golgi apparatus (G) is shown. Gold particles are localized on the cisternal membranes and on the secretion vesicles associated with the *trans* face (arrowheads). Gold particles are also shown localized on segments of the endoplasmic reticulum (arrows) and on the matrix of the protein storage vacuoles (PSV). B, an enface section of a Golgi apparatus in the *trans* face region is shown. Immunogold label is localized on the Golgi secretion vesicles (arrowheads).

of storage protein mobilization that is shown in Fig. 6. Very little gold label was apparent in the other cellular constituents, including the oil bodies. However, in cells that contained disrupted protein vacuoles, P34 was redistributed and observed to have a very specific association with the oil bodies (data not shown).

**P34 Gene Expression Occurs in Midmaturation Soybean Seeds**—The expression of mRNAs encoding P34 during the course of soybean seed maturation and seedling growth was examined with slot blot hybridization assays. Identical amounts of total RNA derived from cotyledons (15-, 45-, and 150-mg fresh weight seeds) and 3, 7, and 12 days of seedling growth were blotted onto nitrocellulose membranes. The blot was washed under high stringency conditions in order to assess the abundance of messages specifically encoding P34 and not other members of the papain superfamily of thiol proteases. In order to determine whether similar mRNAs present in other organs and plants would hybridize to P34

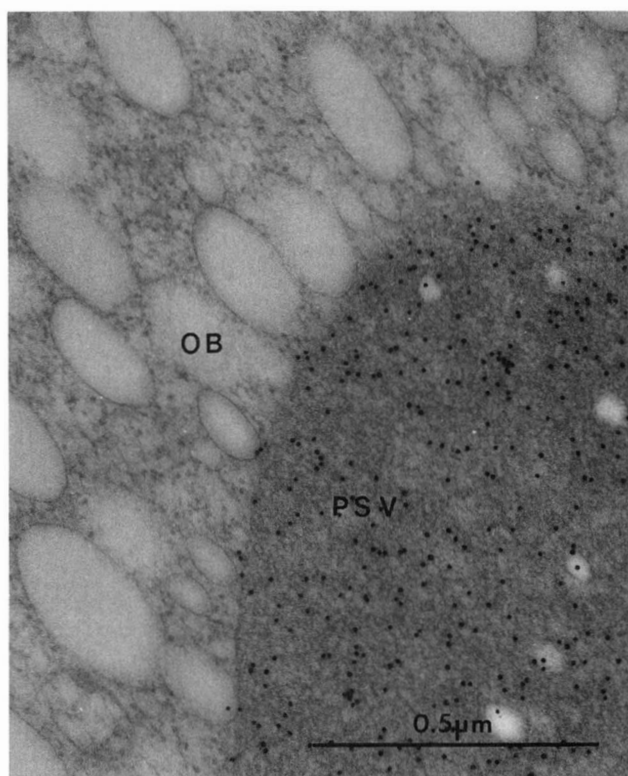


FIG. 6. The immunogold localization of P34 in the protein storage vacuoles (PSV) in a storage parenchyma cell of a 3-day-old germinating soybean seed. The transition of P34 to a 32-kDa polypeptide occurs at this developmental stage as the consequence of the removal of an amino-terminal decapeptide. P34/32 is localized in the protein storage vacuole (PSV), whereas adjacent oil bodies (OB) are devoid of immunogold label.

cDNA under high stringency conditions, total RNA from soybean, tomato, rice, and barley leaves was blotted onto adjacent slots of the nitrocellulose membrane. The resulting hybridization clearly shows that P34 mRNA is accumulated in midmaturation soybean seed cotyledons and is not found prior to the initiation of storage substance accumulation in 15-mg fresh weight seeds or during seedling growth (Fig. 7A). Further, P34 mRNA was not observed in soybean leaves nor was any very closely related mRNA observed in tomato, rice, or barley leaves.

**P34 Message Is Related to Other mRNAs Encoding Thiol Proteases**—The thiol proteases are widely distributed among eukaryotic cells. Southern blot analysis of several plant species has shown, including in soybean (Kalinski *et al.*, 1990), that these genomes contain several thiol protease genes. In order to assess whether similar thiol protease genes are expressed in germinating soybean seeds and mature leaves, Northern blots were probed with P34 cDNA (Fig. 7B). Low and moderate stringency washes (42 and 58 °C, respectively) indicate that there is a similar thiol protease mRNA synthesized during seedling growth in soybean cotyledons. The mRNA for this thiol protease is 1.7 kilobases, slightly larger than that of P34, 1.5 kilobases, indicating that it is encoded by a distinct gene. This protease is likely to be similar to other described proteases, which are expressed during seedling growth that probably mediates the mobilization of storage proteins. The P34 cDNA strongly cross-hybridizes with a 1.7-kilobase mRNA of mature soybean leaves at low stringency. However the P34 cDNA cross-hybridizes to the leaf thiol protease mRNA poorly at moderate stringency (58 °C). This indicates that the thiol protease mRNAs observed during

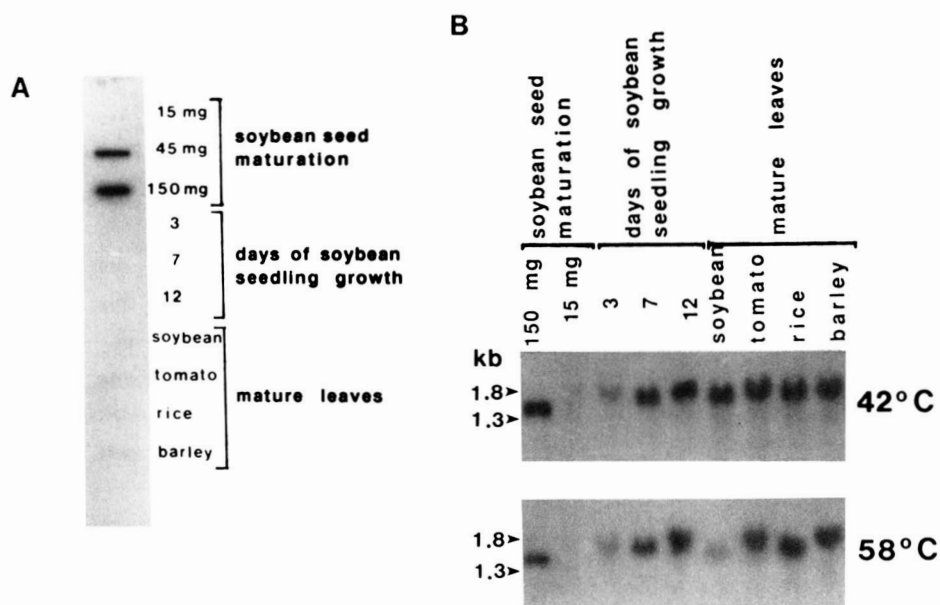


**FIG. 7. Expression of P34 genes.** A, slot blot of RNA (15 µg/lane) isolated from soybean and other plants was hybridized with the soybean seed random-primed labeled P34 cDNA, and the blot was washed at 68°C for 30 min. B, Northern blots of the same RNA samples as above were hybridized with the P34 cDNA and washed under low (42°C) and moderate stringency (58°C) for 30 min. RNA of 0.16 to 1.77 kilobases were used as size markers.

seedling growth and in mature leaves are different mRNAs, although they are similar in size. The P34 cDNA cross-hybridizes to thiol protease mRNAs present in tomato, rice, and barley leaves at low and moderate stringency washes (Fig. 7B). These results indicate that the P34 gene appears to be expressed only during soybean seed maturation. Further, these results demonstrate that P34 is a member of the papain family of thiol proteases expressed in soybean plants.

The tissue-specific distribution of P34 in soybean tissues and organs was also examined by immunoblot analysis. Samples obtained from a soybean plant in the reproductive stage, including late maturation cotyledon, axis, aleurone, seed coat, and pod, as well as stem, roots, and mature leaves, were fractionated with SDS-PAGE and transferred to nitrocellulose membranes. Replicate blots were stained with Amido Black to visualize total protein or probed with immunoaffinity-purified mAb P4B5. A 34 kDa band was immunolabeled only in the samples derived from cotyledons, axis, and aleurone, indicating that expression of P34 is restricted to embryonic tissues (data not shown). The relative intensity of the immunolabeled P34 band in the axis lane was much reduced, as compared with the cotyledon lane, indicating that there appears to be differential amount of P34 accumulation among embryonic tissues.

**In Vitro and in Vivo Synthesis of Prepro and Pro-P34 and Its Posttranslational Processing**—In order to assess the molecular size of newly synthesized P34 and to examine whether it is posttranslationally processed, a series of *in vitro* and *in vivo* synthesis studies were conducted. Poly(A<sup>+</sup>) mRNA isolated from midmaturation soybean seeds was translated in rabbit reticulocyte lysate. Immunoprecipitation with mAbs P3E1/P4B5 coupled to Sepharose 4B beads and size analysis by SDS-PAGE fluorography resulted in the identification of a 46 kDa band (Fig. 8A, lane 1). The size of the isolated polypeptide is similar to the estimated 43-kDa polypeptide, which would result from the translation of the open reading frame of the P34 cDNA clone. Two additional labeled bands (39 and 34 kDa) were immunoprecipitated by P3E1/P4B5 mAb-Sepharose 4B beads (Fig. 8A, lane 1). These additional polypeptide bands may result from translation initiation at methionine codons corresponding to amino acid residue numbers 78 and 120 of the P34 open reading frame (Fig. 9). *In*

*vitro* translation reactions supplemented with canine pancreatic microsomes were undertaken in order to estimate the size of the putative signal sequence identified in the open reading frame of the P34 cDNA clone. However, translation in the presence of microsomes resulted in a net increase in molecular mass of the immunoprecipitated translation product. Fig. 8A, lanes 2 and 3, show the effect of the addition of two different concentrations of canine microsomes supplementing the translation mixture. Note that in lane 2 both 46- and 47-kDa polypeptides were recovered, whereas reactions with twice the microsome concentration of lane 2 resulted in a nearly complete conversion of 46-kDa prepro-P34 to 47-kDa pro-P34. These results are consistent with core N-linked glycosylation at the consensus site (Kornfeld and Kornfeld, 1985) in the precursor segment (Fig. 9). The additional minor polypeptides at 39 and 34 kDa were also observed in translation reactions in the presence of canine microsomes. The 39- and 34-kDa secondary translation products do not shift in molecular weight in response to added canine microsomes (Fig. 8A, lanes 1–3, asterisks). This is consistent with analysis of the primary structure of the P34 cDNA, which shows a single putative asparagine-linked glycosylation site at amino acid residue 70 (Fig. 9, boxed), preceding the codons for methionines 2 and 3. Therefore, these truncated secondary translation products lacking the consensus glycosylation site would not be expected to undergo molecular weight shift by core glycosylation.

This conclusion is supported by *in vitro* transcription/translation of a P34 cDNA clone (Kalinski *et al.*, 1990) in which two labeled immunoprecipitated polypeptides of 39 and 34 kDa were isolated (data not shown). Supplementing the translation reaction with canine microsomes did not alter the molecular weight of either of these two bands, indicating the absence of a cleavable signal sequence or a core glycosylation site on the truncated translation product (data not shown). The capped synthetic mRNA did not appear to direct the synthesis of the entire open reading frame, which we hypothesize to be the consequence of a short 5'-untranslated leader of 54 base pairs, including the T7 polymerization promoter. The synthetic mRNA was experimentally determined to correspond in size to the full length of the cDNA. Therefore, although the P34 cDNA apparently encodes the entire open

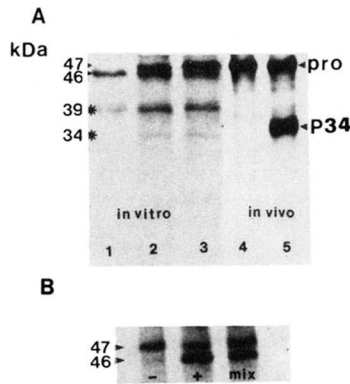


FIG. 8. A, synthesis of P34 *in vitro* and *in vivo*. Analysis of *in vitro* and *in vivo* synthesis of P34 is shown by SDS-PAGE fluorography of polypeptides immunoaffinity-purified with anti-P34 monoclonal antibodies P3E1/P4B5 coupled to Sepharose 4B. Lane 1 shows the *in vitro* synthesis of P34 by rabbit reticulocyte lysate. The major polypeptide is 45 kDa, with additional minor polypeptides 39 and 34 kDa. The other bands correspond to the predicted molecular weight resulting from translation initiation at methionines 2 and 3 in the P34 primary structure. Lanes 2 and 3 show the consequences of the addition of two concentrations of canine microsomes to the reticulocyte reaction mixture. The 45-kDa polypeptide is shifted to 46 kDa, whereas the secondary translation products at 39 and 34 kDa are not shifted in response to added canine microsomes. Lanes 4 and 5 show *in vivo* pulse and chase of immature soybean cotyledons. The pro-P34 has a mass of 46 kDa, which is apparently identical with that of the *in vitro* translation product synthesized in the presence of canine microsomes. A 6-h chase results in the conversion of about half of the labeled pro-P34 to mature P34 (34 kDa). The incomplete processing at 6 h chase indicates that there are no intermediate processing products. B, synthesis of P34 in the presence of tunicamycin. The glycosylation of pro-P34 was verified by metabolic labeling of immature soybean cotyledons in the presence of the core glycosylation inhibitor tunicamycin. Tunicamycin inhibition (lane +) resulted in the accumulation of 45-kDa pro-P34, whereas in control samples, pro-P34 accumulated as a 47-kDa polypeptide (lane -). The difference between the control and inhibited samples is shown in the mixture (lane mix), which is consistent with the predicted effect of the deletion of a single glycan chain attached to the putative site at amino acid 70 in the precursor region of pro-P34.

reading frame, the *in vitro* translation of the synthetic RNA appears to be initiated at the second and third methionines corresponding to residues 78 and 120 in the precursor region.

In order to examine the molecular size of *in vivo* synthesized pro-P34 and its posttranslational product, thin slices of immature soybean cotyledons were labeled with ( $^{35}$ S)methionine. Some identical tissue samples were incubated with a nutrient solution containing unlabeled methionine to chase the newly synthesized P34. The labeled P34 was recovered from denatured seed lysates with mAb P3E1/P4B4-Sepharose 4B beads and analyzed by SDS-PAGE and fluorography. The results of this experiment are shown in Fig. 8A, lanes 4 and 5. The newly synthesized pro-P34 recovered after a pulse with radioactive methionine has a mass of 47 kDa, which is apparently identical with that of the *in vitro* translation product synthesized in the presence of canine microsomes (compare lanes 3 and 4). A 6-h chase results in the conversion of about half of the labeled pro-P34 to mature P34 (34 kDa). The incomplete processing at 6-h chase indicates that there are no intermediate processing products that would be observed in incomplete processing. Similar experiments on seed thiol proteases synthesized by germinating mung bean cotyledons (Mitsushashi and Minamikawa, 1989) and barley aleurones (Koehler and Ho, 1990) appear to have multiple processing steps leading to the formation of the mature protein.

The molecular weight shift observed with *in vitro* transla-

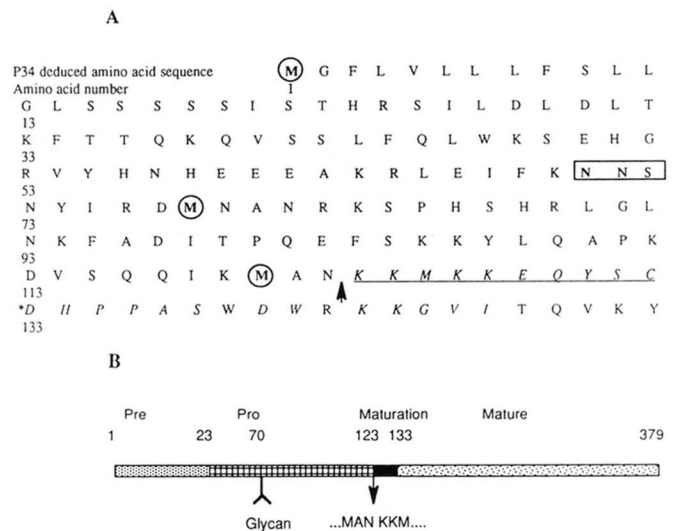


FIG. 9. Summary of the primary structure of the precursor domain of P34, deduced from the cDNA sequence, amino-terminal protein sequence, and *in vivo* and *in vitro* synthesis studies from the results presented here, as well as in Herman *et al.* (1990) and Kalinski *et al.* (1990). *In vitro* translation resulted in translation initiation at the 3 methionines of the precursor domain, which are shown in **boldface**. N-Linked glycosylation occurs at the consensus site at amino acid 70 (boxed), which is part of the precursor segment of P34. Processing of pro-P34 to P34 occurs on the carboxyl side of Asn-122, a processing site typical of seed vacuole proteins. Maturation to P32 during seedling growth occurs on the carboxyl side of cysteine 132. The decapeptide removed is indicated by the underlined sequence. The resulting amino terminus of P32 aligns with amino termini of the mature animal cell cathepsins and the plant thiol proteases papain and actinidin. The experimentally determined amino acid sequence of P34 and P32 is shown in *italics*.

tion in the presence of canine microsomes suggests that core glycosylation of the P34 mRNA occurs *in vitro* on the consensus site at amino acid 70 of the pro domain. In order to examine whether P34 is synthesized as a glycoprotein precursor *in vivo*, as predicted by the sequence and *in vitro* studies, thin slices of midmaturation soybean cotyledons were preincubated in the core glycosylation inhibitor tunicamycin. After the 2-h preincubation in the inhibitor, the incubation medium was supplemented with 25  $\mu$ Ci of ( $^{35}$ S)methionine. Radioactive pro-P34 was recovered from the seed lysates by immunoaffinity with mAb P3E1/P4B5 beads and analyzed by SDS-PAGE fluorography. The result of this experiment is shown in Fig. 8B. Control samples of pro-P34 had an apparent mass of 47 kDa (lane -), whereas pro-P34 synthesized in the presence of tunicamycin is about 2 kDa smaller (lane +). SDS-PAGE fluorography of mixed control and inhibitor samples shows a doublet separated by 2 kDa (lane mix). This result is consistent with the mass of a single high mannose glycan side chain attached to pro-P34.

## DISCUSSION

Herman *et al.* (Herman, 1987; Herman *et al.*, 1990) and others (Murphy and Cummins, 1989; Tzen *et al.*, 1990) have identified P34 as a constituent of the seed oil storage organelle or oil bodies. Remarkably, immunolocalization reveals that the biochemically deduced localization is an artifact of cell lysis and fractionation used in the oil body preparation procedure. Why is P34 purified by the simple expedient of isolating oil bodies? Based on the information we have detailed in this paper, we envision that the affinity of P34 for the oil bodies results from solubilization with the storage proteins and its binding to the oil bodies. Kalinski *et al.* (1991) have

determined the cDNA sequence of isoforms of 24-kDa soybean oleosin, the major oil body membrane protein. The sequence has not revealed any structural features that are obvious candidates for P34's binding to oil bodies. Soybean oil bodies also contain a minor oleosin (18 kDa) of unknown sequence. We cannot exclude the possibility that P34 interacts with the minor, rather than the major, soybean oleosin. Whether the binding of P34 to the oil body membranes is merely fortuitous or whether it is indicative of some aspect of its potential function(s) will require additional investigation.

The posttranslational processing of pro-P34 to mature P34 apparently involves the cleavage on the carboxyl side of Asn at residue 122 (Kalinski *et al.* 1990) of P34, producing the experimentally determined amino terminus (Herman *et al.* 1990). Asn-specific processing has been demonstrated in a wide variety of plant seed vacuolar proteins, such as the 11s superfamily of storage proteins. Although processing on the carboxyl side of Asn is a common form of processing of seed vacuole proteins, processing in many other sites is also known. Except for papain, processing after Asn for maturation of the pro form is not apparently characteristic of other plant thiol proteases (Mitsuhashi and Minamikawa, 1989; Koehler and Ho, 1990). The seed vacuolar-specific mode of processing of P34 provides indirect biochemical evidence in support of the immunocytochemical localization. The processing of other described plant cell thiol proteases has indicated that there may be one or more intermediate products that give rise to the final mature product. In this regard, P34 is quite different in that there is apparently only a single processing site of the precursor during seed maturation. The amino-terminal sequence of P34 exhibits a second processing during seedling growth. Removal of a decapeptide from the amino terminus produces a 32-kDa polypeptide (Herman *et al.*, 1990) whose amino terminus aligns with the amino terminus of other plant and animal thiol proteases.

In this paper, we have shown that P34 is synthesized as a glycoprotein *in vivo* and *in vitro*. The N-glycosylation of pro-P34 likely occurs at the consensus site in the precursor domain. The glycosylation of precursor segments has been shown for a limited number of plant vacuolar proteins. A glycoprotein precursor was first shown for the jack bean lectin concanavalin A (Herman *et al.*, 1985) and has subsequently been shown in wheat and rice lectins (Mansfield *et al.*, 1988), as well as  $\beta$ -glucanase (Shinshi *et al.*, 1988). The glycopeptide excised from pro-concanavalin A is also processed on the carboxyl side of Asn residues (Faye and Chrispeels, 1987). Analysis of the deduced sequences available for plant thiol proteases shows that putative glycosylation sites are present in some of the precursor domains of plant thiol protease genes, such as papain (Cohen *et al.*, 1986), actinidin (Podivinsky *et al.*, 1989), barley cysteine protease (Koehler and Ho, 1990), and  $\gamma$ -oryzain (Watanabe *et al.*, 1991), but this is not a highly conserved feature, since consensus glycosylation sites are absent in the precursor domain of aleurain (Rogers *et al.*, 1985), mung bean (Mitsuhashi and Minamikawa, 1989), and  $\alpha$ - and  $\beta$ -oryzains (Watanabe *et al.*, 1991). Some of these proteins, such as barley aleurain, possess glycosylation sites, but these are located in the mature domain (Holwerda *et al.*, 1990). Those genes that possess glycosylation sites in the precursor domain are not in aligned positions. Why might glycosylation of the precursor be an essential feature for some thiol proteases? Vernet *et al.* (1990) have shown that papain secretion from insect cells is inhibited in the translation of mutated genes that possess deleted consensus glycosylation sites. One interpretation of this observation is that lack of glycosylation in the precursor domain results in misfolding

with consequent recognition and disposal by the protein quality control mechanisms of animal cells in the pre-Golgi region of the endomembrane system (for a review, see Pelham (1989)). Cotranslational core glycosylation may be essential for the cooperative interactions of other enzymes, for example binding protein and protein disulfide isomerase (for a review, see Pelham (1989)), which mediate the folding of the precursor protein into a correct tertiary structure.

P34 is a moderately abundant protein of soybean embryonic tissues; however, the physiological function of P34 remains unknown. Many vacuolar proteins undergo endoproteolytic cleavage upon deposition in the vacuole. A primary processing site is on the carboxyl side of exposed Asn residues. Such cleavage sites are highly conserved in the 11s storage protein family and are present in legume lectins such as concanavalin A (Bowles *et al.*, 1986) and P34 (this paper). Scott *et al.* (1992) have isolated the 11s-processing protease from soybean seeds. Although the processing protease has been inferred to be a thiol protease, the protein Scott *et al.* (1992) have isolated and P34 have different molecular weights, indicating that P34 is not the processing protease. We have considered the possibility that P34 is the protease responsible for degradation of storage proteins during seedling growth. The removal of a decapeptide from the amino terminus of P34 during seedling growth coincides with the initiation of storage protein mobilization. The activation of thiol proteases by removal of small protein segments has been documented in cathepsins (for a review, see Bond and Butler (1987) and Erickson (1989)). However, we still have not shown that the 32-kDa processed form of P34 possesses proteolytic activity. We have also shown in this paper that soybean seeds appear to synthesize a new mRNA related to P34 during seedling growth. This mRNA is likely to encode a protease that functions to mobilize storage proteins. Recent measurements of proteolytic activity in germinating soybean seeds also clearly show that the protease is either activated or newly synthesized after several days of seedling growth (Wilson *et al.*, 1988; Papastoitis and Wilson, 1991). Therefore, it appears that P34 is neither a storage protein processing enzyme nor the endoprotease induced during seedling growth. We do not yet know the physiological function of this protein. Without an obvious functional role, and in its developmental regulation, P34 appears to be different from the other described seed thiol proteases.

## REFERENCES

- Asakofu, H., Yamauchi, D., Mitsuhashi, W., and Minamikawa, T. (1989) *Nucleic Acids Res.* **17**, 6733
- Baumgartner, B., and Chrispeels, M. J. (1977) *Eur. J. Biochem.* **77**, 223-233
- Baumgartner, B., Tokuyasu, K. T., and Chrispeels, M. J. (1978) *J. Cell Biol.* **79**, 10-19
- Bond, J. S., and Butler, P. E. (1987) *Annu. Rev. Biochem.* **56**, 333-364
- Bowles, D. J., Marcus, S. E., Pappin, D. J. C., Findlay, J. B. C., Eliopoulos, E., Maycox, P. R., and Burgess, J. (1986) *J. Cell Biol.* **102**, 1284-1297
- Chan, S. J., San Segundo, B., McCormick, M. B., and Steiner, D. F. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 7721-7725
- Cohen, L. W., Coghlan, V. M., and Dihel, L. C. (1986) *Gene (Amst.)* **48**, 219-227
- Datta, S., and Firtel, R. A. (1987) *Mol. Cell. Biol.* **7**, 149-159
- Dietrich, R. A., Maslyer, D. J., Heupel, R. C., and Harada, J. J. (1989) *Plant Cell* **1**, 73-80
- Erickson, A. H. (1989) *J. Cell. Biochem.* **40**, 31-41
- Faye, L., and Chrispeels, M. J. (1987) *Planta* **170**, 217-224
- Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6-13
- Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982) *J. Cell Biol.* **93**, 97-102
- Guerrero, F. D., Jones, J. T., and Mullet, J. E. (1990) *Plant Mol. Biol.* **15**, 11-26

- Herman, E. M., and Shannon, L. M. (1985) *Plant Physiol.* **77**, 886–890
- Herman, E. M., Shannon, L. M., and Chrispeels, M. J. (1985) *Planta* **165**, 23–29
- Herman, E. M. (1987) *Planta* **172**, 336–345
- Herman, E. M. (1988) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **39**, 139–155
- Herman, E. M. (1992) in *Advances in Structural Biology* (Malhotra, S. K., ed.) Jai Press, Greenwich, CT, in press
- Herman, E. M., Melroy, D. L., and Buckhout, T. J. (1990) *Plant Physiol.* **94**, 341–349
- Holmes, D. S., and Quigley, M. (1981) *Anal. Biochem.* **114**, 193–197
- Holwerda, B. C., Galvin, N. J., Baranski, T. J., and Rogers, J. C. (1990) *Plant Cell* **2**, 1091–1106
- Ishidoh, K., Towatari, T., Imajoh, S., Kawasaki, H., Kominami, E., Katunuma, N., and Suzuki, K. (1987) *FEBS Lett.* **223**, 69–73
- Ishidoh, K., Kominami, E., Suzuki, K., and Katunuma, N. (1989) *FEBS Lett.* **259**, 71–74
- Kalinski, A., Weisemann, J., Matthews, B. F., and Herman, E. M. (1990) *J. Biol. Chem.* **265**, 13843–13848
- Kalinski, A., Loer, D. S., Weisemann, J., Matthews, B. F., and Herman, E. M. (1991) *Plant Mol. Biol.* **17**, 1095–1098
- Koehler, S., and Ho, D. (1988) *Plant Physiol.* **87**, 95–103
- Koehler, S., and Ho, D. (1990) *Plant Cell* **2**, 769–783
- Kornfeld, R., and Kornfeld, S. (1985) *Annu. Rev. Biochem.* **54**, 631–664
- Lehrach, H., Diamond, D., Wozney, J. M., and Boedtke, H. (1977) *Biochemistry* **16**, 4743–4751
- Mansfield, M. A., Peumans, W. J., and Raikhel, N. V. (1988) *Planta* **173**, 482–489
- Mitsuhashi, W., Koshiba T., and Minamikawa, T. (1986) *Plant Physiol.* **80**, 628–634
- Mitsuhashi, W., and Minamikawa, T. (1989) *Plant Physiol.* **89**, 274–279
- Murphy, D. J., and Cummins, I. (1989) *Phytochemistry (Oxf.)* **28**, 2063–2069
- North, M. J. (1986) *Biochem. J. Lett.* **238**, 623–624
- Papastoitis, G., and Wilson, K. A. (1991) *Plant Physiol.* **96**, 1086–1092
- Pears, H. K., Mahbubani, H. M., and Williams, J. G. (1985) *Nucleic Acids Res.* **13**, 8853–8866
- Pelham, H. R. B. (1989) *Annu. Rev. Cell Biol.* **5**, 1–23
- Podivinsky, E., Forster, R. L. S., and Gardner, R. C. (1989) *Nucleic Acids Res.* **17**, 8363
- Preaekelt, U. A., McKee, R. A., and Smith, H. (1988) *Plant Mol. Biol.* **10**, 193–202
- Rogers, J. C., Dean, D., and Heck, G. R. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 6512–6516
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Schaffer, M. A., and Fischer, R. L. (1988) *Plant Physiol.* **87**, 431–436
- Scott, M. P., Jung, R., Muntz, K., and Nielson, N. C. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 658–662
- Shinshi, H., Wenzler, H., Neuhaus, J. M., Felix, G., Hofsteenge, J., and Meins, Jr., F. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 5541–5545
- Tahio, K., Towatari, T., Katunuma, N., Teller, D. C., and Titani, K. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 3666–3670
- Tartakoff, A. M. (1983) *Cell* **32**, 1026–1028
- Tzen, J. T. C., Lai, Y. K., Chan, K.-L., and Huang, A. H. C. (1990) *Plant Physiol.* **94**, 1282–1289
- Vernet, T., Tessier, D. C., Richardson, C., Laliberte, F., Khouri, H. E., Bell, A. W., Storer, A. C., and Thomas, D. Y. (1990) *J. Biol. Chem.* **265**, 16661–16666
- Watanabe, H., Abe, K., Emori, Y., Hosoyama, H., and Arai, S. (1991) *J. Biol. Chem.* **266**, 16897–16902
- Williams, J. G., North, M. J., and Mahbubani, H. (1985) *EMBO J.* **4**, 999–1006
- Wilson, K. A., Papastoitis, G., Hartl, P., and Tan-Wilson, A. T. (1988) *Plant Physiol.* **88**, 355–360