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# Apparent Processing of a Soybean Oil Body Protein Accompanies the Onset of Oil Mobilization[1,2]

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## ABSTRACT

**The membrane surrounding the oil body contains several different specific polypeptides. To study the biosynthesis and post-translational modification of these polypeptides we have prepared monoclonal antibodies against purified oil bodies of soybean (*Glycine max*). Three of the five monoclonals selected recognize a molecular mass 34 kilodalton protein (P34). Epitope mapping of CNBr and proteolytic fragments of P34 indicates that two of the anti-P34 monoclonal antibodies are directed at different epitopes. P34 is accumulated during seed maturation at the same time as the reserve proteins and oil. SDS/PAGE-immunoblots of germinating soybean seed cotyledons indicate that the protein is initially present as a molecular mass 34 kilodalton polypeptide and is processed to molecular mass 32 kilodalton on the fourth through sixth days of seedling growth simultaneously with the onset of oil mobilization. A comparison of reduced and carboxymethylated oil body proteins with nonreduced proteins by SDS/PAGE indicates that P34 exists *in vivo* as a dimer of molecular mass 58 kilodalton. Comparing the amino terminal sequences of P34 and P32 indicates that their difference is at least in part due to the removal of the amino terminus of P34. The amino terminal sequences of P34 and P32 were aligned to show that the transition of P34 to P32 was accompanied by the removal of a hydrophilic decapeptide (KKMKKEQYSC) at the amino terminus of P34. Hopp-Woods hydrophilicity analysis of the deleted amino terminus of P34 shows that it is more hydrophilic and charged than the sequence of the protein which immediately follows.**

The reserve oils of seeds are packaged into discrete organelles termed oil bodies which consist of a triglyceride core bounded by a monolayer of phospholipids (18) into which are embedded a few distinct polypeptides (12, 16). The major oil body membrane proteins, termed oleosins, of maize (16 kD oleosin; 15), soybean (24 kD oleosin; 4), and rape (20 kD oleosin; 13) share common biochemical properties. Their amino acid compositions indicate that each of these proteins is enriched in neutral amino acids and is relatively deficient in sulfur amino acids. A nearly complete deduced amino acid sequence of maize 16 kD oleosin has been published (17). Subsequently a tryptic peptide fragment of the 20 kD rapeseed oleosin has been shown to be homologous to maize 16 kD oleosin (14) suggesting that the major oil body proteins of both monocotyledon and dicotyledon plants may have a common ancestor. In addition to the major protein, each oil body examined has been shown to contain a number of minor polypeptides (4, 12, 15, 16). Soybean oil bodies, for example, have 34, 18, and 17 kD polypeptides (4). Very little is known about the characteristics or function of the minor oil body proteins.

As a part of an ongoing research program on soybean oil bodies we have elicited monoclonal antibodies against soybean oil bodies. By modifying the selection protocols we have obtained specific probes appropriate for a variety of assays. Using the specific antibodies directed against the 34 kD polypeptide P34, we observed that it undergoes developmentally regulated processing. In this paper we show that P34 is processed to a molecular mass 32 kD protein (P32) during seedling growth and that this processing apparently results from the removal of the first 10 amino acids of P34 from its amino terminus.

## MATERIALS AND METHODS

### Plant Material

Soybeans (*Glycine max* Merr. cv Forrest and Century) were maintained in a greenhouse as described previously (4). Maturing soybean seeds harvested from pods were staged based on the fresh weight of the seed (11). Germinating seeds were obtained by sowing seeds in moist vermiculite and incubating the seeds in the dark at ambient room temperature.

### Monoclonal Antibodies and Immunological Assays

#### *Immunization and Fusion*

Soybean oil bodies were isolated as described by Herman (4) from cotyledons of soybeans germinated for 2 d. Oil bodies were fixed for 1 h at room temperature with 1.0% (v/v) glutaraldehyde in 0.1 M K-phosphate buffer (pH 7.2). Free aldehyde groups were blocked with 1 M Tris buffer (pH 8.6), and the oil bodies were extracted twice with ethanol and resuspended in phosphate-buffered saline (PBS, 10 mM Na-phosphate [pH 7.2], and 0.15 M NaCl). The fixation and extraction procedure was chosen to eliminate epitopes which would be sensitive to treatments required for immunocyto-

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chemistry. Isolated oil bodies were diluted with four volumes of PBS and emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemical Co.[4]). BALB/c, female mice were injected subcutaneously with 50 μL (approximately 1 μg protein) of the emulsified antigen at multiple sites. After 2 weeks, mice were boosted interperitoneally (I.P.) with approximately 200 μL (approximately 8 μg protein) of oil bodies emulsified in an equal volume of Freund's incomplete adjuvant (Sigma Chemical Co.). Mice were boosted three times in this manner. Test bleeds (approximately 50 μL) were obtained from the tail vein, and production of antibodies to the oil bodies was determined by ELISA (see below), probing with goat antimouse antibodies conjugated to horseradish peroxidase (Kierkegaard and Perry, Gaithersburg, MD). Strongly positive immunological activity against oil bodies was observed prior to fusion. Three days prior to fusion, mice were given a final I.P. injection with antigen prepared as described above. On the day of fusion, two mice were sacrificed and the spleens removed. Spleenocytes were fused with log-phase NS-1, myeloma cells (7) at a ratio of spleenocytes to myeloma cells of 4:1, by the method of Galfré and Milstein (3). Cells were plated into ten 96-well μL plates, and hybridomas were selected on hypoxanthine, aminopterin and thymidine (HAT) medium. After 12 d on HAT medium, cells were re-fed with hypoxanthine-thymidine (HT) medium. Medium was assayed for antibody production by ELISA when the majority of the wells had cells at 40 to 50% confluence. Positive wells were diluted 1000-fold and plated on 96-well plates. Positive cells from the second round plating were expanded into 24-well microtiter plates (miniclones), and the medium was retested for positive antibody production by immunoblotting against total oil body membrane proteins and by immunocytochemistry. Hybridomas were cloned by the soft agar method of Civin and Banquerigo (1). The antibodies secreted by the isolated clones were isotyped by double diffusion precipitation assays using the anti-isotype antibodies from a commercial kit (Miles).

### Immunological Assay Protocols

*Dot-Blot Screening.* Dot-blot analyses were conducted using a dot-blotting apparatus (Bio-Rad) and were performed according to the manufacturer's recommendations. Oil bodies were applied to nitrocellulose sheets, the nitrocellulose blocked with 3% (w/v) bovine serum albumin (Sigma) and 1:3 dilutions of culture supernatant were reacted with the antigen. Specific binding of antibodies was detected using goat antimouse antibodies conjugated to alkaline phosphatase. Alkaline phosphatase was assayed with chromogenic substrates NBT[5] and BCIP (Sigma).

*ELISA.* ELISA assays were performed by coating 96-well plates with an excess of oil bodies. Fifty μL of oil body suspension was incubated overnight at 37°C and saturating humidity. Plates were washed with TBS. In some instances, the bound oil bodies were fixed with 1% (v/v) glutaraldehyde in PBS for 1 h at room temperature and then washed with PBS containing 0.1 M glycine to block excess aldehyde groups. In other instances the bound oil bodies were extracted with ethanol to remove ethanol soluble material. These plates were used to assess the consequences of aldehyde fixation and lipid extraction on immunoreactivity of the antibodies. Protein binding sites on the 96-well ELISA plates were blocked for 1 h with BLOTTO (6). After blocking, culture supernatants were diluted 1:1 with blocking buffer, and 100 μL were transferred to the ELISA plates and incubated 2 h at room temperature. Following incubation, the wells were washed twice with TBST. Fifty microliters of secondary antibody, goat antimouse conjugated to peroxidase (Bio-Rad) diluted 1:400 in blocking buffer, were added and the plates incubated for 1 h. Following secondary antibody incubation, the plates were washed five times with TBST. Peroxidase activity was detected using 50 μL of ABTS (Kirkegaard and Perry Labs) substrate solution. ABTS substrate was prepared by diluting 1 part of 40 mM ABTS in 9 parts 0.1 M citrate-glycine buffer (pH 4.0) and then adding distilled water to a final substrate concentration of 0.1% (v/v). Plates were evaluated after 1 h at room temperature using an ELISA reader (Bio-Rad) interfaced to a Macintosh PC running Bio-Rad MacReader 1.0 software. Culture supernatants were harvested with a 96-well transfer apparatus (Costar) and the ELISA plates washed with an automatic ELISA plate washer (Bio-Rad).

*Immunoblots.* SDS/PAGE of isolated oil body proteins or total cotyledon proteins was performed with 11% resolving gels and 5% stacking gels using the Laemmli discontinuous buffer system (9). Gel-fractionated proteins were blotted onto nitrocellulose paper in 25 mM borate (pH 8.2) 20% (v/v) methanol buffer containing 0.05% thioglycolate in a tank electroblot apparatus (Hoeffer) operated at 1 A at 15°C for 90 min. The nitrocellulose blots were blocked with either BLOTTO-Tween or 3% (w/v) gelatin in TBST. The blots were then immunolabeled with 1:25 to 1:200 dilutions of the culture supernatants in either BLOTTO-Tween or 1% (w/v) gelatin in TBST. Immunolabeling with culture supernatants was usually done overnight at room temperature. Immunolabeling nitrocellulose blots with ascites antibodies was accomplished by 1:10,000 dilution in either gelatin TBST or BLOTTO-Tween for 90 min at room temperature. In some instances the blots were incubated in Hoeffer multiple channel apparatus to compare the immunolabeling pattern of different cell lines on the same antigen. In some experiments replicate blots were labeled in parallel with rabbit anti-24 kD oleosin serum (4) diluted 1:5000 in either 1% gelatin-TBST or BLOTTO-Tween overnight at room temperature. Antimouse-IgG-alkaline phosphatase or antirabbit IgG-alkaline phosphatase (Sigma) diluted 1:1000 for 90 min at room temperature was used as an indirect label. Alkaline phosphatase activity was visualized with the chromogenic substrates BCIP and NBT (Sigma).

Developmentally staged samples for SDS-PAGE-immunoblots were obtained by staging maturing soybean seeds by

[4] The mention of vendor or product does not imply that they are endorsed or recommended by U.S. Department of Agriculture over vendors of similar products not mentioned.

[5] NBT, nitro blue tetrazolium; BCIP, 5-bromo-4 chloro-3-indoyl phosphate; TBS(T), Tris buffered saline (containing 0.05% [v/v] Tween-20; MAb(s), monoclonal antibody(ies); BLOTTO, 5% nonfat dry milk in TBS; ABTS, 2,2'-azino-di-(3-ethyl-benzothiozolin-sulfonate).

fresh weight (11). Cotyledons were excised from the seeds and ground in a mortar at a ratio of 1 mg of tissue to 10 μL of SDS sample buffer containing 5% (v/v) mercaptoethanol, then heated in a 100°C water bath for 5 min. The samples were then centrifuged for 5 min in a Brinkman microfuge, and 5 μL of the resulting supernatant were used in each gel lane. Samples are prepared as constant weight in sample buffer in order to show the changes in the relative quantity of antigens in the total cotyledon mass. Samples from cotyledons of germinating seeds after 1 to 9 d of seedling growth were obtained by grinding three cotyledons from three different seedlings in a total of 4.5 mL of SDS-sample buffer containing 5% (v/v) mercaptoethanol. The samples were heated to 100°C for 5 min and centrifuged for 10 min at 10,000 rpm in a Sorvall RC-2b centrifuge and SS-34 rotor. A 10 μL aliquot (0.0066 cotyledon) from each sample was analyzed by SDS-PAGE-immunoblots. Samples prepared in a constant volume were used to show changes in the quantity of antigens on a per cotyledon basis.

### *Hybridoma Clones and Purification of Antibodies*

The hybridoma cell lines were expanded as *in vivo* ascites tumors or *in vitro* in tissue culture with RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum. Ascites from cell lines secreting monoclonal antibodies P3E1 and P4B5 (see below) were immunoaffinity purified on a Sepharose 4b column prepared by coupling 20 mg of carboxymethylated oil body proteins in 5% (w/v) SDS, 50 mM sodium borate (pH 8.2) to CNBr-activated Sepharose 4b. The excess SDS was removed and the remaining active functional groups on the Sepharose inactivated by exhaustively washing the column with TBST. Anti-mouse IgM-Sepharose 4b columns (Sigma) were used to purify MAbs P2D3 and P1A9. Ascites supernatants were diluted 1:2 with TBS and applied to the column, recycling the solution five times. The column was washed with TBS until $A_{280}$ nm was zero. The antibodies were eluted with 0.1 M glycine, 0.15 M NaCl (pH 3). The eluate was immediately neutralized with 1 M Tris-HCl (pH 7.4). The immunoaffinity purified IgMs were dialyzed against two changes of 1000 volumes of PBS prior to coupling to cynogen bromide activated Sepharose 4b column material.

### Carboxymethylation of Oil Body Proteins

Oil bodies were isolated from imbibed soybean seeds as previously described (4). Oil bodies were extracted with acetone and with 2:1 chloroform:methanol to remove organic soluble material. The oil bodies were then pressed dry in several layers of Whatman No. 3 paper and immediately dissolved in 6 M Guanidine-HCl, 1 mM EDTA. DTT was added to a final concentration of 10 mM, and the solution incubated at 37°C for 1 h to reduce the proteins. In order to block sulfhydryl groups, sodium iodoacetate was added from a 0.1 M stock solution to a final concentration of 25 mM, and the sample was then incubated for 1 h. The reaction was terminated by adding mercaptoethanol to a final concentration of 1%. The sample was then dialyzed exhaustively against 50 mM ammonium bicarbonate containing 0.01% (v/v) thiodiglycol. After dialysis the sample was lyophilized.

### CNBr and Proteolytic Cleavage and Epitope Mapping

The single dimension peptide mapping protocol of Cleveland *et al.* (2), modified for immunoblotting, was used for epitope mapping. Carboxymethylated oil body proteins were dissolved in 70% formic acid. CNBr was added to 10 mg/mL and the sample incubated overnight. The reaction was then terminated with the addition of 10 volumes of distilled water and the sample lyophilized. CNBr fragments were dissolved at 1 mg/mL in SDS sample buffer. Stock solutions of the proteases *Staphylococcus* V8 and thermolysin (Sigma) of 1 mg/mL in distilled water were added to 1 mg/mL solutions of carboxymethylated oil body proteins in SDS sample buffer to give a final protein to protease ratio of 1:20. The samples were incubated at 30°C for 1 h and the reaction terminated by boiling the samples. Protein fragments derived from CNBr and protease cleavage were fractionated on 18% SDS/PAGE and blotted onto nitrocellulose paper. Replicate lanes of each sample were blocked with BLOTTO-Tween, incubated in 1:5000 dilution of P3E1 or P4B5 ascites for 1.5 h in BLOTTO-Tween, followed by indirect labeling with 1:1000 dilution of antimouse IgG-alkaline phosphatase (Sigma) for 1 h. Alkaline phosphatase activity was visualized as above.

### Immunoaffinity Purification of Oil Body Proteins with the IgM Monoclonals

MAbs P2D3 and P1A9 (IgMs) were coupled to CNBr activated Sepharose-4b according to Pharmacia's directions. Isolated oil bodies were solubilized in TBST containing 1% w/v *n*-octyl-β-D-glucopyranoside (Sigma) for 1 h at room temperature. The solubilized proteins were separated from undissolved oil bodies by centrifugation at 30,000 rpm in a Beckman SW-41 rotor for 1.5 h at 4°C. The top oil pad was removed by freezing it onto a spatula cooled with liquid nitrogen. Solubilized proteins were passed over the MAb-Sepharose-4b column, washed until the $A_{280}$ nm was zero, then eluted with 0.1 M glycine, 0.15 M NaCl (pH 3.0). Samples of the eluted proteins were analyzed by fractionation on 11% SDS/PAGE, blotting on nitrocellulose, and probing the blot with either anti-P34 MAb P3E1 or anti-24 kD oleosin polyclonal serum (4).

### Total Amino Acid Analysis and Aminoterminal Protein Sequencing

Carboxymethylated oil body proteins were dissolved in SDS sample buffer and fractionated on 3 mm preparative SDS/PAGE using Tris-borate running buffer (25 mM Tris, 20 mM borate, 0.1% w/v thioglycolate). The fractionated proteins were electrophoretically transferred to two layers of PDVF membranes (Immobilon-P, Millipore) in borate-methanol-thioglycolate transfer buffer. After the transfer the blot was rinsed three times with 18+ Mohm water, briefly stained with amido black stain, and then destained with water. The blot was air dried and individual pieces of bands of interest were excised for amino acid composition and amino terminal sequencing (10). Amino acid composition and amino terminal protein sequencing were done at the Biotechnology Instrumentation Facility, University of California, Riverside,

and the Chemistry Department, University of Maryland, College Park. Hopp-Woods hydrophilicity analysis of the sequence data was accomplished using DNA inspector IIe running on a Macintosh PC.

## RESULTS

### Properties of Monoclonal Antibodies Elicited against Oil-Body Proteins

#### *Hybridoma Selection, Cloning, and Specificity*

Cell lines secreting antibodies specific for oil body proteins were screened initially by ELISA and dot blot assays. Positive cell lines were diluted into 96-well plates and rescreened by the same methods. Strongly positive cell lines were further screened by SDS/PAGE-immunoblot assays in a multiple channel apparatus. Two categories of antibody secreting cell lines were apparent. Some were IgM antibodies which were strongly positive for whole oil bodies in ELISAs and dot blots but which did not immunolabel any polypeptide bands on immunoblots. These antibodies recognized alcohol-extracted as well as aldehyde-fixed oil bodies by ELISA indicating that they were directed at polypeptide components rather than at an alcohol-soluble oil. The two antibody secreting cell lines in this category were designated P1A9 and P2D3 (Table I). The second category of antibodies were IgGs which were positive on ELISAs and dot blot assays but which also recognized a molecular mass 34 kD polypeptide on immunoblots (see below). These cell lines were called P3E1, P4B5, and P2C11 (Table I).

To determine which oil body protein the IgM MAbs recognized, native oil body proteins were solubilized with octyl glucoside. The solubilized proteins were separated from insoluble material by centrifugation and then passed over MAb P2D3 or P1A9 Sepharose-4b columns. After washing the unbound proteins from the column the specifically bound proteins were eluted by pH 3.0 buffer. SDS/PAGE-immunoblot analysis of the protein eluted from the P2D3 and P1A9 columns showed that both MAbs bound the 24 kD oleosin. Figure 1 shows the results obtained from this experiment with MAb P2D3. We conclude that both P2D3 and P1A9 are directed against an epitope of 24 kD oleosin which is abolished by denaturing with SDS. We have not yet been able to establish whether MAbs P2D3 and P1A9 are directed at the same or at different epitopes of 24 kD oleosin.

#### *Epitope Mapping*

The epitope specificity of the three monoclonal cell lines which are positive for the denatured P34 was examined on immunoblots of partial hydrolysis products of reduced and carboxymethylated oil body membrane proteins (Fig. 2). Based on the amino acid composition (Table II) cyanogen bromide should cleave P34 into a total of five pieces assuming that methionine is not present on either the amino or carboxyl terminus. Eighteen percent SDS/PAGE-immunoblots of CNBr fragments of oil body proteins were probed with MAbs P3E1 and P4B5 (lanes 1 and 2 of Fig. 2). The MAbs P3E1 and P4B5 each labeled the undigested P34. In addition, MAb P3E1 labeled a 15.5 kD fragment and MAb P4B5 a 13 kD fragment. The size of these two fragments indicates that together they account for most of the P34 molecule. This result indicates that there is a methionine residue about midway in the P34 polypeptide and that the P3E1 and P4B5 MAb epitopes are located on opposite sides of this methionine.

Carboxymethylated oil body proteins were partially digested with Staph. V8 and thermolysin proteases and the fragments were separated on 18% SDS/PAGE and transferred to nitrocellulose membranes. In the peptide maps of Staph. V8 digested proteins MAbs P3E1 and P4B5 both labeled the undigested polypeptide fragments. MAbs P3E1 and P4B5 strongly labeled 23, 21, and 19 kD fragments (Fig. 2, lane 3). Slightly smaller Staph. V8 fragments of 15.5 and 13 kD were labeled with P3E1 or P4B5, respectively, but not both (Fig. 2, lanes 3 and 4). Thermolysin digestion patterns also show that MAbs P3E1 and P4B5 are directed at separate epitopes (Fig. 2, lanes 5 and 6). MAb P3E1 strongly labeled two fragments of 21 and 19 kD and MAb P4B5 strongly labeled two fragments of 19 and 17 kD. Epitope analysis of Staph. V8 hydrolysis products indicates that MAb P3C11 appears to be directed at the same epitope as P3E1 (data not shown).

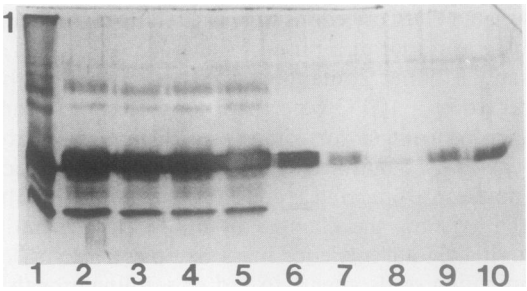


**Figure 1.** SDS-PAGE-immunoblot of solubilized oil body proteins passed over an MAb P2D3-Sepharose-4b column, washed, and eluted with low pH. The nitrocellulose blot was probed with anti-24 kD oleosin antiserum. Lanes 1 through 7 show nonspecifically bound 24 kD oleosin progressively washed from the column. Lane 8 shows that no further immunoreactive material could be washed from the column. Lanes 9 and 10 show polypeptides immunoreactive with anti-24 kD oleosin antibodies eluted at low pH. Parallel experiments with anti-P34 MAbs showed no specific elution of P34 (data not shown).

**Table I.** *Summary Table of Specificity and Subclass of Monoclonal Antibody Clones*

| Antibody | Subclass | Specificity |
|---|---|---|
| P3E1 | IgG1 | Native/denatured P34 |
| P2C11 | IgG1 | Native/denatured P34 |
| P4B5 | IgG1 | Native/denatured P34 |
| P1A9 | IgM | Native P24 |
| P2D3 | IgM | Native P24 |

#### *Other Characteristics of the Affinity of the Monoclonal Antibodies*

*Urea Resistance.* MAbs P3E1 and P4B5 were surveyed for the ability to bind to antigen on immunoblots in the presence

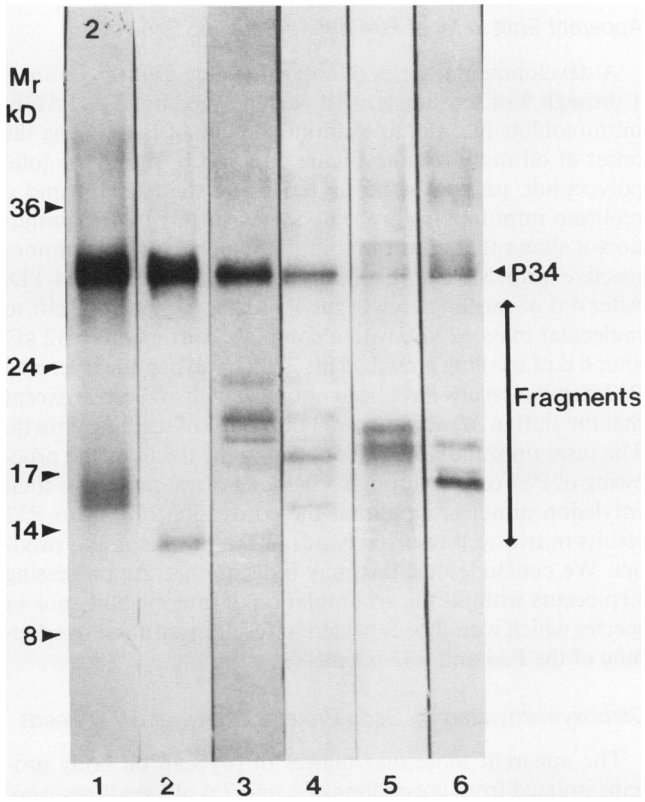


**Figure 2.** SDS/PAGE epitope map of fragments of P34 using MAbs P3E1 and P4B5. Lanes 1 and 2 show CNBr fragments. Lanes 3 and 4 show Staph. V8-generated P34 peptide fragments. Lanes 5 and 6 show thermolysin-generated fragments. Lanes 1, 3, and 5 were probed with MAb P3E1. Lanes 2, 4, and 6 were probed with MAb P4B5. This result indicates that MAbs P3E1 and P4B5 are directed at two different epitopes on the P34 polypeptide.

of varying concentrations of urea. This is useful because urea is frequently used to solubilize membrane proteins. Both MAbs were found to bind antigen in the presence of 2 M urea without diminished label intensity and some residual binding was still detected at 4 M urea (data not shown).

*Cross-Reactivity.* Extracts of imbibed *Glycine tobacia, clandestina, canescens, falcaia, tomentella, soja,* and *max* seeds were fractionated on SDS/PAGE and transferred to nitrocellulose membranes. Immunolabeling of these blots each yielded 34 kD molecular mass immunoreactive bands with either MAbs P3E1 or P4B5, indicating that this protein is conserved in these closely related species (data not shown).

## Characteristics of P34 Polypeptide and Developmental Processing

### *Amino Acid Analysis*

The P34 band from electroblots of preparative SDS/PAGE was analyzed for amino acid composition (Table II). Based on $M_r$ = 34,000 from SDS/PAGE, the best fit was calculated to be 317 amino acids totaling 34,201 D. The amino acid analysis indicates that the protein has 11 sulfur containing amino acids, 7 cysteines and 4 methionines per polypeptide. The protein also contains abundant glycine, alanine, and leucine. The overall hydropathic index (GRAVY) by the Kyte-Doolittle method (8) is −0.69, which is more typical of soluble proteins than of membrane proteins of similar estimated chain length.

### *P34 Appears as a Dimer in Unreduced Samples*

The relatively high cysteine content (seven residues per subunit) suggested the possibility that P34 may be cross-linked by disulfide bridges. In order to examine this possibility, the relative molecular mass of P34 was examined with and without sulfhydryl reduction. Denatured oil body proteins from imbibed seeds were compared with reduced and carboxymethylated proteins by SDS/PAGE. In nonreduced samples of oil body proteins the P34 band had an apparent molecular mass of 58 kD (Fig. 3), indicating that P34 may be configured as a dimer in the oil body. The P34 polypeptide is the only one of the oil body polypeptides that shifts in $M_r$ to a larger apparent molecular mass under nonreducing conditions.

### *Accumulation of P34 during Seed Maturation*

The developmental accumulation of P34 was examined with SDS/PAGE-immunoblots during seed maturation (Fig. 4). Soybean (cv Century) seeds were staged by fresh weight; the molecular mass distribution of the polypeptides at each stage of seed maturation (Fig. 4a) and immunolabeling of P34 by MAb P3E1 (Fig. 4b) were examined. Note that P34 first appears in seeds of about 65 mg fresh weight and is accumulated during middle to late maturation of the soybeans. Mature oil bodies are accumulated in the cytoplasm of storage parenchyma cells during the same developmental period. Therefore P34, like P24 (4), accumulates with approximately the same developmental pattern as oil bodies.

**Table II.** *Amino Acid Composition of P34*

| Amino Acid | P34[a] |
|---|---|
| | *residues/molecule* |
| Asx | 33 |
| Glx | 38 |
| Ser | 26 |
| Gly | 38 |
| His | 9 |
| Arg | 7 |
| Thr | 19 |
| Ala | 25 |
| Pro | 9 |
| Tyr | 20 |
| Val | 16 |
| Met | 4 |
| Cys | 7 |
| Ile | 19 |
| Leu | 18 |
| Phe | 9 |
| Lys | 20 |

[a] Calculated based on 34,000 kD.

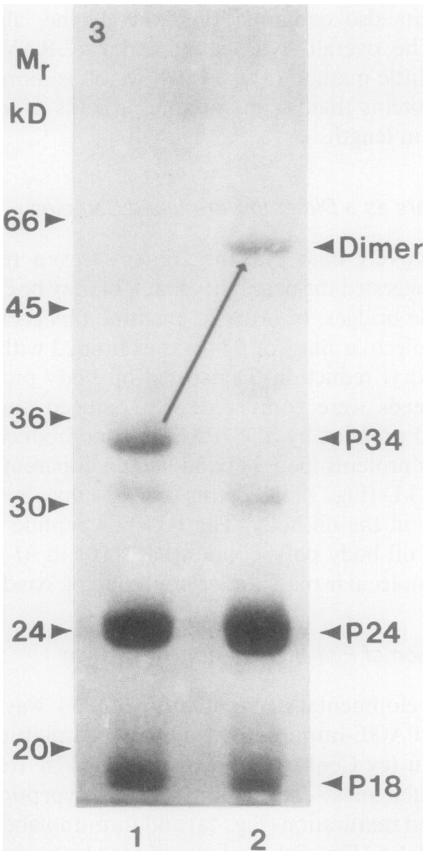


**Figure 3.** A SDS-PAGE comparison of reduced and unreduced oil body protein isolated from imbibed seeds is shown. The reduced sample has molecular mass 34, 24, and 18 kD polypeptides. In the unreduced sample the molecular mass 34 kD is absent, and the polypeptide is apparently shifted to molecular mass 58 kD (arrow). In contrast, the other polypeptides in the unreduced sample do not exhibit a $M_r$ shift.

### *Apparent Shift in $M_r$ of P34 during Seedling Growth*

A developmental series of cotyledons (cv Century) from d 1 through 9 of seedling growth was analyzed by SDS/PAGE-immunoblots in order to examine the fate of P34 during the onset of oil mobilization. Figure 5, a and b, shows the total polypeptide pattern in an amido black stained blot and a replicate immunoblot probed with MAb P3E1. The immunoblot shows that after imbibition the polypeptide immunoreactive with MAb P3E1 has a molecular mass of 34 kD. After 4 d of seedling growth the P34 band appears to shift to molecular mass 32 kD, with a complete conversion to 32 kD after 6 d of seedling growth. This 32 kD polypeptide is termed P32. Similar results have been obtained with cv Forrest except that the shift in $M_r$ occurs on d 3 through 5 of seedling growth. The inset immunoblot (Fig. 5c) shows the detail of the processing of P34 on d 3 through 5 in a cv Forrest sample of total cotyledon proteins. Note that the conversion of P34 to P32 results in two well resolved bands of the precursor and product. We conclude that this may indicate that the processing step occurs without the accumulation of intermediate mol wt species which would be expected to result in a diffuse overlapping of the P34 and P32 bands.

### *Carboxymethylated Oil Body Proteins from 1 and 7 d Seeds*

The apparent molecular masses of soybean oil body proteins isolated from cotyledons of 1 and 7 d old seedlings were compared. Total oil body proteins were reduced and carboxymethylated prior to fractionation by SDS/PAGE. The 1 and 7 d old samples were run in alternating lanes in order to directly compare differences in polypeptide $M_r$. The side by side comparison of 1 and 7 d oil body proteins clearly shows that the P34 of 1 d seeds is completely replaced by P32 in the 7 d sample (Fig. 6). Further, these results indicate that P34 is the only protein which undergoes such an $M_r$ shift during this developmental period.

### *Amino-Terminal Sequence of P34 and P32*

The amino terminal sequences of both P34 and P32 were determined and compared. In order to minimize false glycine

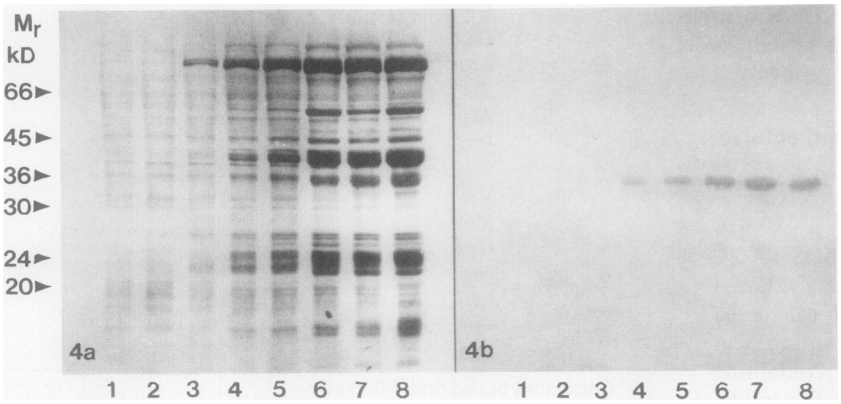


**Figure 4.** Accumulation of P34 during seed maturation. A, The total polypeptide distribution of the staged samples on an amido black stained nitrocellose blot; B, the polypeptides labeled with anti-P34 MAb P3E1 ascites. Each lane of the gels in both panels is the equivalent to the polypeptides extracted from 1 mg fresh weight of staged cotyledon tissue. The P34 polypeptide is first observed at 65 mg fresh weight and is apparently accumulated throughout seed maturation. Lanes 1 through 8 on both panels are from 12, 25, 65, 100, 150, 200, 250, and 300 mg fresh weight seeds, respectively.

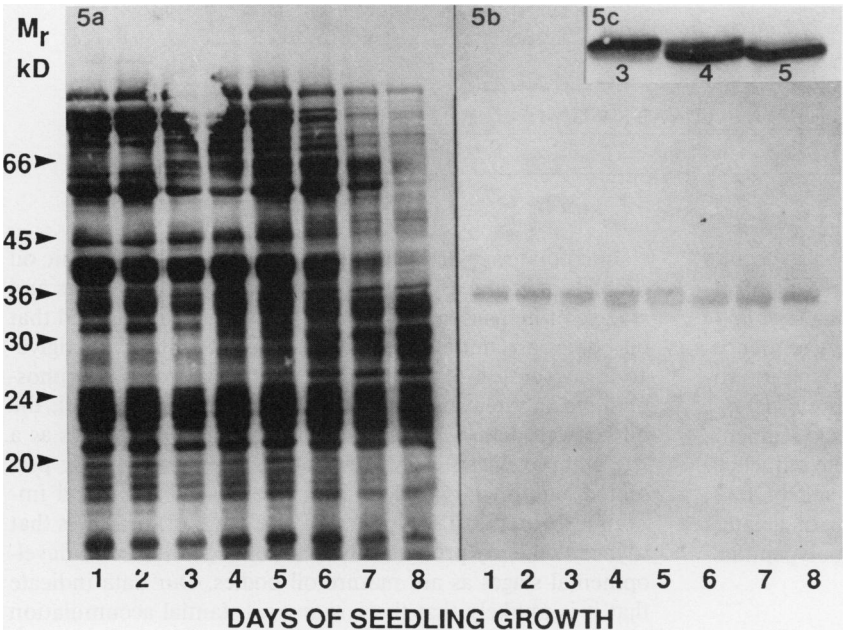


**Figure 5.** Shift in $M_r$ of P34 to P32 during seedling growth. A, The polypeptide distribution on an amido black stained nitrocellulose blot of cv Century cotyledons from a seedling growth series of 1 through 8 d; B, the bands immunoreactive with the MAb P3E1 ascites. On d 1 through 3 the MAb labels a molecular mass 34 kD band, on d 4 through 6 a molecular mass 34 and 32 kD doublet was labeled, and on d 7 and 8 only a molecular mass 32 kD was labeled. The inset SDS-PAGE-immunoblot in panel C shows the detail of the apparent change in molecular mass of P34 to 32 kD during d 3 through 5 in a cv Forrest sample.

measurements a Tris-borate SDS/PAGE running buffer and Borate-methanol transfer buffer were used. The first 18 amino acids of P34 were determined and verified for samples from two different cultivars. The first 15 amino acids were determined for P32. The results of these sequence determinations are shown in Table III.

The amino terminal sequence for P34 carries a high positive charge, with six basic and two acidic amino acids among the first nine residues. In contrast, the amino terminal sequence of P32 has two acidic and no basic amino acids among the first nine. The amino terminal sequence H-P-P-A-S-A of P32 is found within P34 demonstrating that P34 is the precursor for P32. This is also indicated by the immunoreactivity of P32 with MAbs P3E1 and P4B5. However, the amino acid preceding the sequence -H-P-P-A-S-A does not match in the two cv Century samples, with a W (tryptophan) in P34 and D (aspartic acid) in P32. The signals on the chromatograms were 11.5 pmol for W and 20 pmol for D, which were sufficient to identify each respective amino acid. However the amino acid sequence of P32 does properly align with a P34 sample from cv Forrest. The sequence of a cDNA clone of P34 isolated from an expression vector library shows a D in the position in question and only a single -H-P-P-A-S- sequence (6a).

It appears that in a fraction of the P32 sequenced, the aspartic acid amino terminus was missing and that the P32 sequence began with the histidine shown at position 2. Cycles 2, 4, 7, and 10 of the P32 amino acid sequence contain a small amount of the amino acid of the following cycle. This may indicate that the processing step which converts P34 to P32 produces two different amino termini corresponding to amino acids 1 and 2 on P32. The 10 amino acids which are removed from P34 to form P32 correspond reasonably closely in molecular mass to the 2 kD difference estimated on SDS/PAGE-immunoblots. Although the removal of this decapeptide appears to be sufficient to account for processing to P32

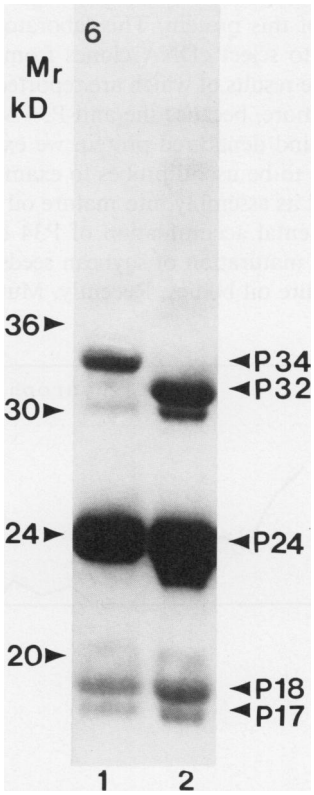


**Figure 6.** A SDS-PAGE comparison of the molecular mass distribution of carboxymethylated oil body proteins from 1 and 7 d old seedling cotyledons is shown. The 34 kD (P34) band present in 1 d old oil bodies (lane 1) is absent in 7 d old oil bodies and is replaced by a 32 kD (P32) polypeptide (lane 2).

**Table III.** *Amino Terminal Sequence of Purified P34 and P32*

| | |
|---|---|
| K-K-M-K-K-E-Q-Y-S-C-D-X-P-P-A-S-X-D- | P34 (Forrest) |
| K-K-M-K-K-E-Q-Y-S-C-W-H-P-P-A-S-A-W- | P34 (Century) |
| D-H-P-P-A-S-A-D-W-G-K-K-G-V-I | P32 (Century) |

X = no amino acid called on that cycle.

it is possible that carboxyterminal processing may also occur. We have not yet examined P34 and P32 for an identical carboxyterminal amino acid. The first 10 amino acids of P34 are highly charged compared to the -H-P-P-A-S-A sequence that follows. Hopp-Woods hydrophilicity plot (5) of the amino terminus of P34 using a 6 amino acid averaging length clearly shows the hydrophilic character of the 10 amino acid segment which is removed compared to the less hydrophilic sequence which follows (Fig. 7). The apparent cleavage site in P34 between amino acid 10 and 11 is at a position of greater hydrophobicity than the adjacent segments of the polypeptide.

## DISCUSSION

We have developed MAb probes for our studies on the ontogeny of soybean oil bodies. The MAb cell lines which we have isolated provide probes for several different types of studies. The $IgG_1$ anti-P34 MAbs have already proven to be useful for examining developmentally regulated expression and processing of this protein. This laboratory has recently used the MAbs to select cDNA clones from an expression vector library, the results of which are reported in Kalinski *et al.* (6a). Furthermore, because the anti-P34 MAbs recognize both the native and denatured protein we expect that these MAbs will prove to be useful probes to examine the biosynthesis of P34 and its assembly into mature oil bodies.

The developmental accumulation of P34 occurs simultaneously with the maturation of soybean seeds and the accumulation of mature oil bodies. Recently, Murphy *et al.* (13) claimed that rapeseed oleosin is inserted onto cytoplasmic oil droplets after the accumulation of both the triglycerides and storage proteins is largely complete. They have proposed that oil bodies are initially accumulated as an emulsified triglyceride suspension of small droplets surrounded by the phospholipid monolayer. Following the accumulation of oil, the oil body proteins would be inserted onto the oil bodies as a late embryogenesis event. In contrast, the data we have presented here on P34 and in a previous biochemical and immunocytochemical study on 24 kD oleosin (4) indicates that soybean oil body proteins are accumulated at the same developmental stages as are mature oil bodies. Our data indicate that it is unlikely that there is any substantial accumulation of naked oil droplets in soybean seeds.

The present results provide the basis for predicting information about the deduced sequence from cDNA cloning of P34. The sequence data of P34 and P32 can be used to determine the correct open reading frame of the clones. Further, the CNBr fragmentation pattern predicts that there should be a methionine close to the midpoint of the P34 polypeptide.

No function of the apparent conversion of P34 to P32 has been elucidated. It is tempting to hypothesize that the removal of the P34 amino terminus is correlated with a particular developmental event involving the oil bodies. The processing does occur simultaneously with the initiation of glyoxysome-mediated oil mobilization; however, the present results do not provide evidence of linkage between these events. The removal of a highly charged amino terminal sequence could

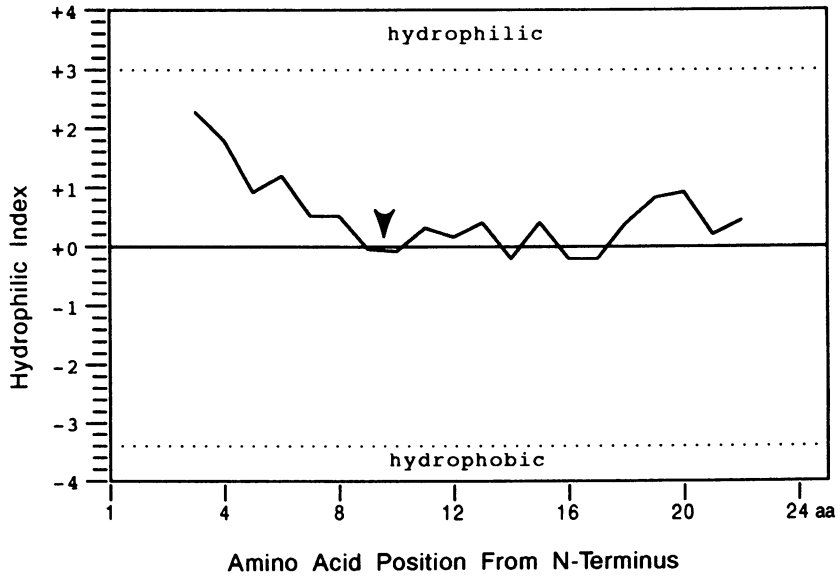


**Figure 7.** A hydrophilicity plot of the 25 amino acids sequenced in P34 and P32 and reconstructed as a single polypeptide. A moving window averaging of 6 amino acids was used to plot the Hopp-Woods hydrophilicity. Note that the first 10 amino acids are hydrophilic while the following segment of the polypeptide has a hydrophilicity index close to 0. The apparent processing site of conversion of P34 to P32 is indicated by an arrow, which corresponds to the minimum hydrophilicity of this part of the polypeptide.

have a specific function, such as recognition, or activation of an enzyme precursor, or alternatively it may simply be the result of hydrolysis of the exposed portion of the polypeptide by cytoplasmic proteolytic enzymes. Based on the hydrophilicity analysis of the amino terminus of P34 it would be expected that this portion of the molecule should be exposed to the aqueous cytoplasm and therefore more accessible to a processing enzyme. We are currently considering the possibility that the amino terminal processing of P34 could be mediated by one of the cytoplasmic aminopeptidases which have been characterized in many different plants. Only P34 shifts $M_r$ at this developmental stage, and it is the only one of the soybean oil body proteins that is not blocked on the amino terminus (our unpublished results). We have synthesized the peptide corresponding to the first 10 amino acids of P34, which are apparently removed during processing. We intend to use this polypeptide to elicit an antibody for use as an immunocytochemical probe to compare the ultrastructure of cells before and after P34 processing.

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