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Identification and Characterization of Latex-Specific Proteins in Opium Poppy[1]

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ABSTRACT

Latex from the opium poppy, *Papaver somniferum* L., was analyzed by polyacrylamide gel electrophoresis (PAGE). Two latex-specific bands were identified in protein samples of poppy latex using one-dimensional native PAGE. Second dimension analysis with SDS-PAGE indicates that these proteins have a relative molecular weight of approximately 20 kilodaltons. We have termed these polypeptides the major latex proteins (MLPs). Polyclonal antibodies prepared against the MLPs were used to probe protein gel blots of latex and poppy tissues known to lack laticifers. Laticifer-free tissues showed no reaction with anti-MLP immunoglobulin G indicating that MLPs are found only in poppy latex. MLP distribution was also examined in mature opium poppy tissues by immunocytochemistry. Laticifers were differentially labeled by fluorescein isothiocyanate secondary labeling of anti-MLP immunoglobulin G and could easily be identified in both transverse and longitudinal section. Fractionation studies of isolated latex showed that MLPs are concentrated in the latex cytosol and not in alkaloidal vesicles. Analysis of latex proteins by conventional two-dimensional electrophoresis indicates that the two MLP bands are composed of several distinct polypeptides with similar relative molecular weights. The pIs of these molecules range from 6.0 to 3.5. The role(s) of MLPs in laticifer metabolism has not been determined.

Laticifers are highly specialized internal secretory systems which are present in both primitive and advanced taxa of flowering plants. Although their distribution is limited to about fifteen families, certain latex-bearing plants are of major economic importance due to their ability to synthesize and store useful secondary products such as alkaloids, hydrocarbons, or enzymes.

Latex exuded from the unripe capsule of the opium poppy, *Papaver somniferum* L., is air-dried and harvested as raw opium. Opium poppy latex represents the cytoplasm of a series of fused cells that comprise the mature laticifer system (5). Young laticifer initials contain typical cell organelles including nuclei, plastids, mitochondria, dictyosomes, and ribosomes (19–21, 29). As the laticifer differentiates, numerous membrane-bound vesicles are formed from dilating ER and eventually dominate the cytoplasm of the mature latex vessel (20, 29). These vesicles have been shown to contain the large amounts of phenanthrene alkaloids including morphine and codeine (9, 10).

Opium poppy laticifers are metabolically active at maturity. Isolated latex is able to convert radioactive precursors into phenanthrene alkaloids (3, 6–9, 15). Enzymes of general cellular metabolism have been detected in isolated poppy latex (1), as well as enzymes which may be involved in alkaloid biosynthesis (2, 24–27).

Biochemical investigations of latex metabolism are complicated by the presence of oxidative enzymes which can interfere with enzyme assays when latex is exposed to air. We have chosen a molecular approach to investigate the diversity and distribution of proteins in isolated opium poppy latex. Using PAGE we have identified several abundant, low mol wt polypeptides which are localized in the laticifer cytosol. These MLPs[2] are only detectable in this unusual cell type.

MATERIALS AND METHODS

Latex Collection. Opium poppies, *Papaver somniferum* L. (UNL186) were grown from seed in an incubator at 23°C under a 12 day/night photoperiod with 165 μE m^{-2} s^{-1} of light provided by fluorescent lights (General Electric F20 12CW). After 60 d, plants were transferred to artificially illuminated benches (230 μE m^{-2} s^{-1} General Electric fluorescent lights F40 CW) at room temperature and induced to flower by exposure to a long d (16 h d/8 h night).

Latex was harvested from developing poppy capsules 2 to 7 d after petal drop. Capsules were lanced with a razor blade and the exuded latex immediately collected into 100 μl capillary pipettes, quick frozen in liquid N_2 and stored at −70°C.

Some of the freshly collected latex was fractionated according to the method of Roberts (24). Whole latex was mixed 1:1 (v/v) with ice-cold 50 mM K-phosphate (pH 7.0) and 500 mM mannitol. The mixture was maintained at 4°C and centrifuged for 30 min at 1,000*g*. The supernatant (fraction 1) was removed and the pelleted vesicles lysed in an equal volume of mannitol free phosphate buffer and centrifuged at 123,000*g* for 10 min.The three resulting fractions represent the laticifer cytoplasm (fraction 1), the contents of alkaloidal vesicles (fraction 2) and a membrane pellet (fraction 3). The fractions were examined electrophoretically as described below and an aliquot from each fraction was analyzed by HPLC for phenanthrene alkaloids (13).

Electrophoretic Analysis. For electrophoretic analysis, latex samples were mixed 1:1 (v/v) with Laemmli's (16) sample buffer containing either 5% SDS or 5% NP-40. Samples were analyzed by nondenaturing electrophoresis on 12.5% polyacrylamide gels using the nonionic detergent NP-40 (Sigma Chemical) in place of SDS. Gels were stained with either Coomassie brilliant blue or silver (33). Replicate gel lanes were treated with periodic acid-Schiff reagent (PAS) to identify glycoproteins (11). All protein determinations were performed using the method of Bradford (4).

The 1000*g* supernatant from freshly collected latex was also analyzed by two-dimensional electrophoresis (23). Latex serum

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[2] Abbreviations: MLPs, major latex proteins; NP-40, nonidet P 40; IgG, immunoglobulin G; IEF, isoelectric focusing.

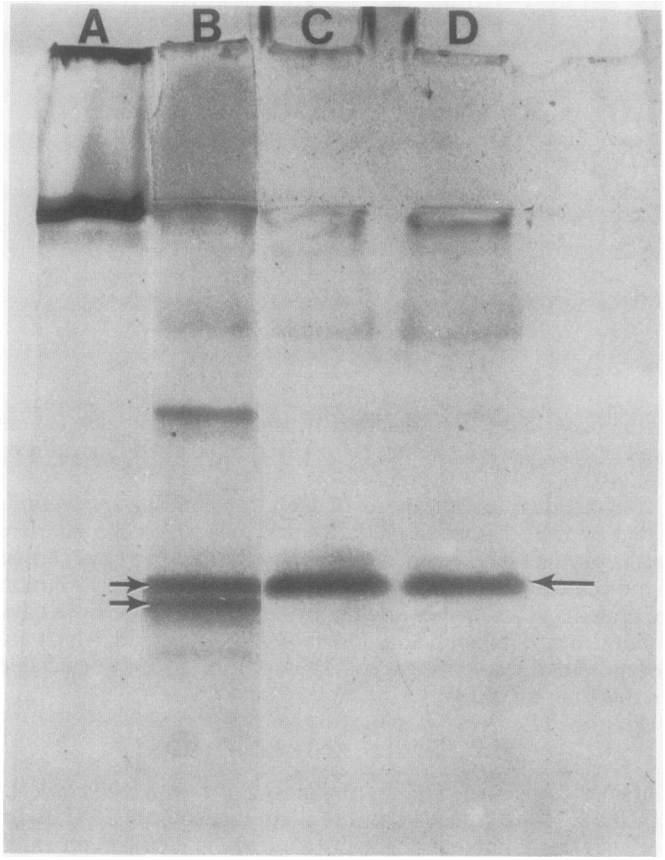

FIG. 1. Comparison of staining patterns of opium poppy latex proteins in a native polyacrylamide gel. Lane A, PAS stain for carbohydrate; lane B, Coomassie blue stain showing the two MLP bands (arrows); lane C (15 μg protein) and lane D (30 μg protein) stained with silver show only top MLP band (arrow).

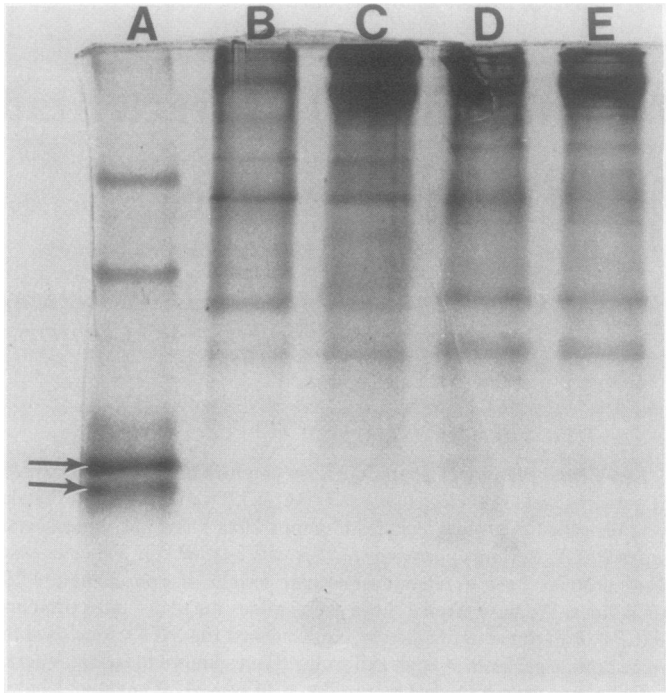

FIG. 2. Coomassie-stained native gel comparing protein samples from opium poppy latex (A), zygotic embryos (B, 20 μg protein; C, 40 μg protein) and from suspension cultures of proembryos (D, 20 μg protein; E, 40 μg protein). MLP bands (arrows) are only seen in latex lane.

was mixed 1:1 with O'Farrell's lysis buffer (23) and 150 μg protein loaded on tube gels for first dimension isoelectric focusing. After focusing, the tube gels were equilibrated for 2 h in SDS sample buffer, and then run in the second dimension on gradient (7.5–17.5% polyacrylamide) vertical slab gels. Two-dimension gels were stained with Coomassie brilliant blue.

Preparation of MLP Antisera. Polyclonal antibodies were prepared against the major latex proteins identified by native gel electrophoresis. Preparative electrophoresis was performed on latex protein samples on NP-40 polyacrylamide gels as previously described. Bands corresponding to the MLPs were excised from the gel, homogenized, and mixed 1:1 (v/v) with Freunds complete adjuvant. The MLP adjuvant mixture was injected subcutaneously at two sites on the back of a female New Zealand white rabbit. Booster injections were given 2 and 3 weeks after the initial injection and the rabbit was bled 6 weeks from the start of the treatment. Serum was clarified by centrifugation of clotted blood and IgGs were purified by affinity chromatography on *Staphylococcus* protein A sepharose column. After serum application, the affinity column was washed with sufficient PBS (pH 7.2) to return the $A_{280\,nm}$ of the eluent to baseline. IgGs were released from the column with 0.1 M citrate buffer (pH 3.0) and precipitated with 50% $(NH_4)_2SO_4$. The pellet was resuspended in borate-buffered saline and extensively dialyzed against additional borate-buffered saline. Dialyzed anti-MLP IgG (0.8 mg protein/ml) was stored at −20°C.

Protein Blot Analysis. Proteins from one-dimensional and two-dimensional polyacrylamide gels were electrophoretically transferred to nitrocellulose filters (30). Filters were washed three times for 30 min in a blocking solution composed of 0.1% NP-40, 1% BSA, and 0.02% NaN_3 in phosphate buffered saline (NBA-PBS). The blots were incubated in antibody diluted 1:100 with NBA-PBS for 12 h, washed three times for 30 min with NBA-PBS, and incubated for 3 h with 2.5 μCi of [^{125}I]protein A in 50 ml NBA-PBS. Filters were washed six times for 15 min in NBA-PBS, rinsed with PBS, and air-dried. All immunological procedures were carried out at 4°C. Control blots prepared from duplicate gels were treated with pre-immune serum rather than anti-MLP. Autoradiographs were exposed on Kodak x-ray film (XAR-5) at −70°C with an intensifying screen.

Electron Microscopy and Immunocytochemistry. Tissues were prepared for and examined by transmission EM as previously described (22).

MLPs were localized in cryosections of mature opium poppy pedicels and capsules by secondary antibody immunolabeling as described by Wilson *et al.* (32). Sections 20 to 25 μm thick were made with an American Optical Cryo-Cut II at −20°C and attached to slides pretreated with 1% gelatin and 0.5% chromium potassium sulfate. Slides with attached sections were rinsed three times for 5 min in blocking solution (1% BSA in PBS) and incubated for 30 min in MLP antiserum (1:10 dilution in PBS) at 37°C.

Slides were washed seven times for 5 min in blocking solution and then incubated 30 min at 37°C in a 1:40 dilution of fluorescein-conjugated goat anti-rabbit IgG (Sigma Chemical). Unbound label was removed by a second series of seven 5-min washes with PBS. Control slides were prepared in an identical manner using preimmune serum in place of anti-MLP.

RESULTS

Extraction of opium poppy latex with sample buffer containing 5% SDS yields less than 0.4 mg protein/ml latex. Sample buffer containing the nonionic detergent NP-40 in place of SDS solubilizes at least 15 times more latex protein (about 6.6 mg/ml latex).

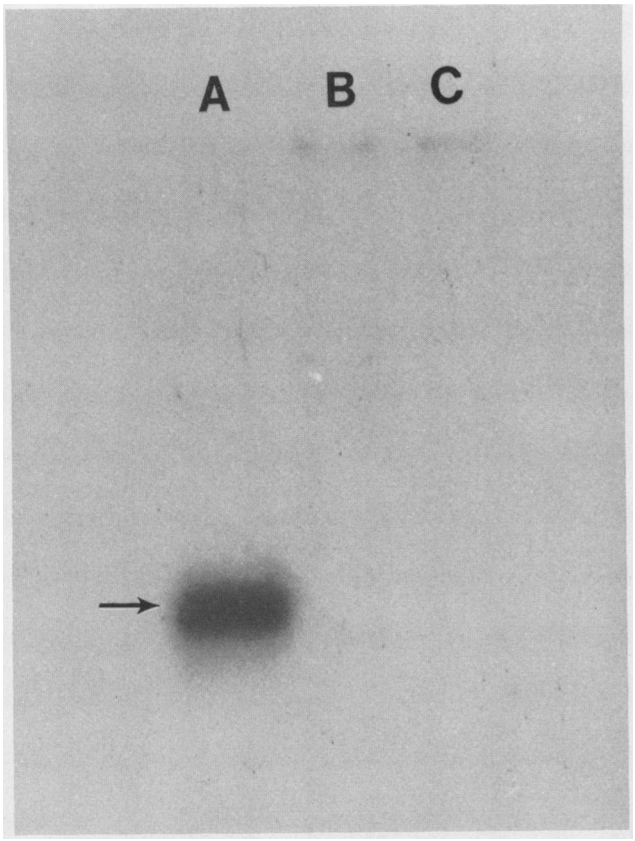

FIG. 3. Autoradiograph of protein gel blot probed with anti-MLP IgG. Only the opium poppy latex lane (A) shows a strong reaction with ^{125}I-protein A. Lanes loaded with protein from zygotic embryos (B) and tissue culture derived proembryos (C) have only weak, nonspecific reactions in areas corresponding to the top of the stacking gel.

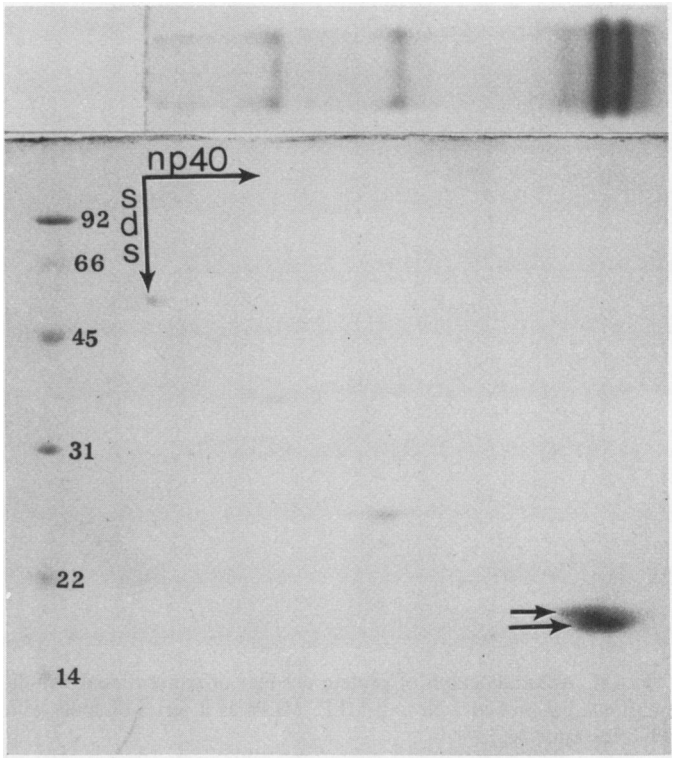

FIG. 4. SDS polyacrylamide gel of protein sample from opium poppy latex that was run on a native gel in the first dimension. MLPs (arrows) appear as two closely migrating spots with an M_r of approximately 20 kD.

Relatively few abundant proteins are resolved in protein samples of opium poppy latex by native polyacrylamide gels (NP-40 PAGE) stained with Coomassie brilliant blue. The most conspicuous latex protein bands appear as a rapidly migrating doublet (Fig. 1, lane B). Based on Coomassie staining these MLPs appear to be present in approximately equal molar amounts. However, only the upper band of the MLP doublet can be visualized by silver staining (Fig. 1, lanes C, D). Neither of the MLP bands show a positive reaction for carbohydrate when stained with periodic acid-Schiff reagent (Fig. 1, lane A).

Analysis of native gels stained with Coomassie blue suggests that the MLPs are latex specific. Polypeptides which co-migrate with the MLPs (Fig. 2, lane A) are not observed in zygotic embryos protein samples (lanes B, C) or protein samples from suspension cultures of proembryos (lanes D, E). Both of these tissues are known to lack laticifers (22, 29).

Immunoblot analysis also indicates that the distribution of MLPs is limited to laticifers. Nondenaturing gels containing protein samples from latex, zygotic embryos, and cultured proembryos were electroblotted onto nitrocellulose. Blots were incubated with anti-MLP and reacted with ^{125}I-labeled *Staphylococcus* protein A. An autoradiograph of an anti-MLP protein blot is shown in Figure 3. The sample lane loaded with latex protein shows a strong signal with the correct electrophoretic mobility (A, arrow). Lanes which contained embryo (B) and proembryo proteins (C) have no detectable reaction except for extremely faint, nonspecific bands in the stacking gel. No antibody binding is seen in control blots probed with pre-immune serum (data not shown).

The apparent mol wt of the MLPs were determined by excising

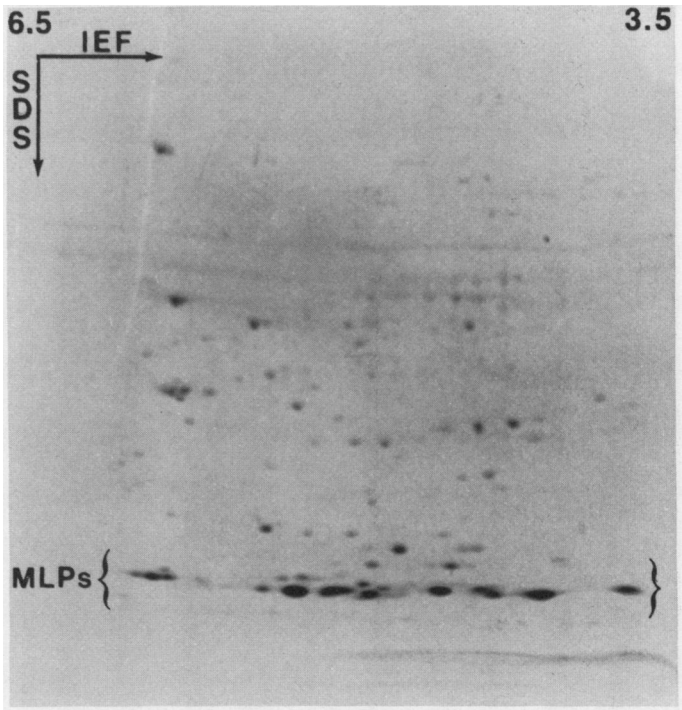

FIG. 5. Conventional two-dimensional gel of opium poppy latex protein sample. First-dimensional isoelectric focusing separates MLPs into several polypeptides (MLPs) with pIs from 6.0 to 3.5. Second dimension SDS-PAGE shows the MLPs to all have M_rs of about 20 kD.

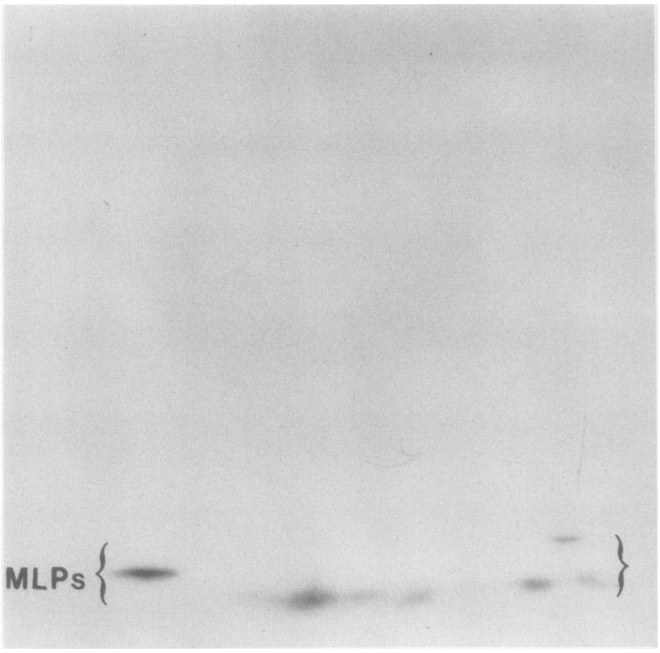

FIG. 6. Autoradiograph of protein gel blot of conventional two-dimensional gel probed with anti-MLP. MLPs as a series of spots with about the same M_r.

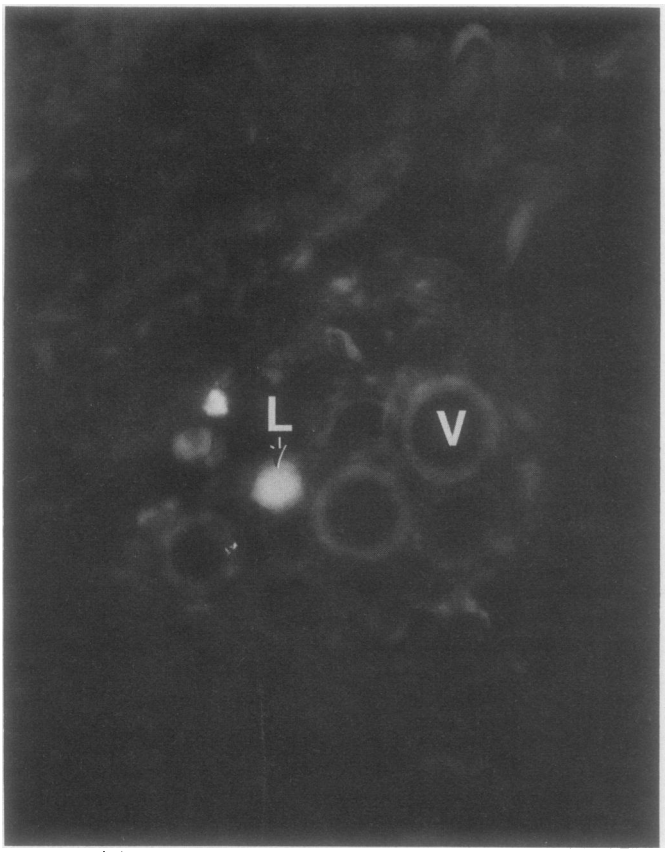

FIG. 7. Transverse section through an opium poppy pedicel showing immunolabeling of laticifer (L) in vascular bundle. Note weak autofluorescence of lignin in vessel (V) (× 220).

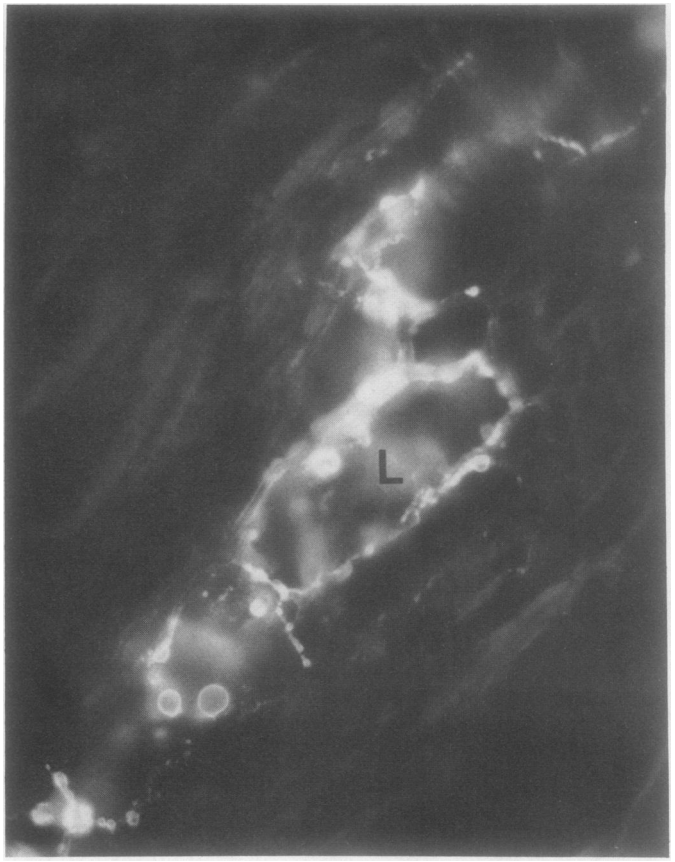

FIG. 8. Longitudinal section from opium poppy capsule showing immunolabeling of laticifer (L) along the periphery of the cell (× 500).

a latex sample lane from a nondenaturing gel running it on an SDS slab gel in the second dimension (Fig. 4). Using this approach, the two MLPs observed by NP-40 PAGE ran as two closely migrating spots with M_r of approximately 20 kD. The identity of the MLP spots were confirmed immunologically in protein gel blots.

MLPs were also analyzed by conventional two-dimensional gel electrophoresis using IEF gels for separation in the first dimension. A large number of spots were seen in Coomassie stained 2-D gels indicating the wide variety of diverse proteins present in the latex cytosol. As expected, the MLPs were the most abundant proteins and displayed a similar M_r (about 20 kD) in the second dimension; however, each of two MLP bands observed with both NP-40 one-dimensional PAGE and NP-40/SDS two-dimensional PAGE was resolved into several polypeptides by the IEF gel (Fig. 5). These MLP polypeptides showed an extremely wide range of isoelectric points ranging from about 6.0 to 3.5 and each reacted with anti-MLP in protein gel blots (Fig. 6).

The distribution of MLP in poppy capsules and pedicels was investigated *in situ* by immunocytochemistry using fluorescein isothiocyanate goat antirabbit secondary labeling. Laticifers are differentially labeled by MLP antiserum and are easily recognized in transverse section among elements of the phloem in vascular bundles (Fig. 7). In longitudinal section, fluorescent labeling is also laticifer specific and appears to be concentrated along the peripheral laticifer cytoplasm (Fig. 8).

Opium poppy laticifers are known to have an unusual cytoplasmic organization in comparison to other types of plant cells. The dominant feature of the poppy laticifer is the presence of numerous alkaloid-rich, membrane-bound vesicles which occupy most of the cell lumen at maturity (Fig. 9). The latex exuded

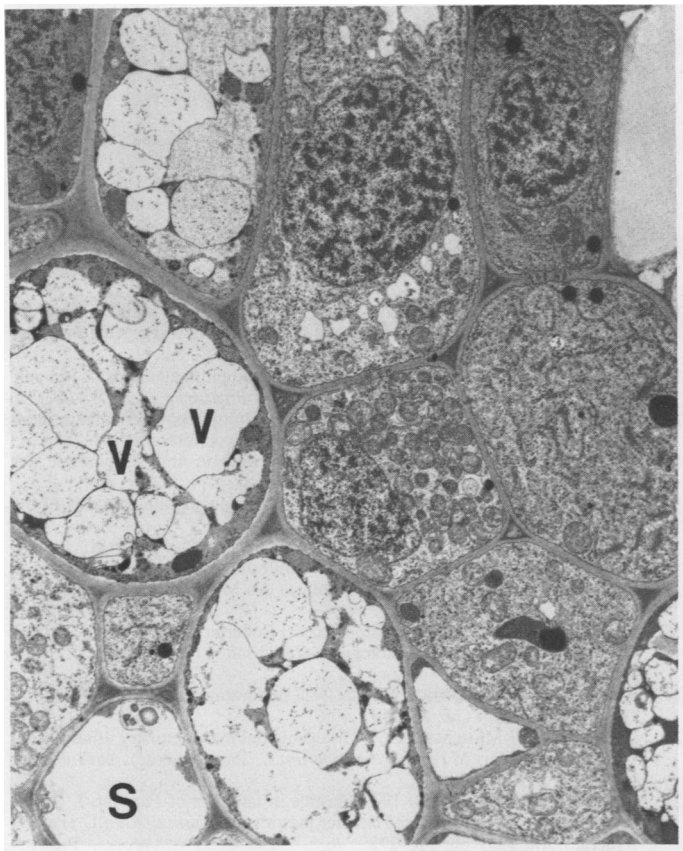

FIG. 9. Electron micrograph of laticifers in the vascular bundle showing numerous membrane-bound vesicles (V) surrounded by a thin layer of cytoplasm, S, sieve tube member.

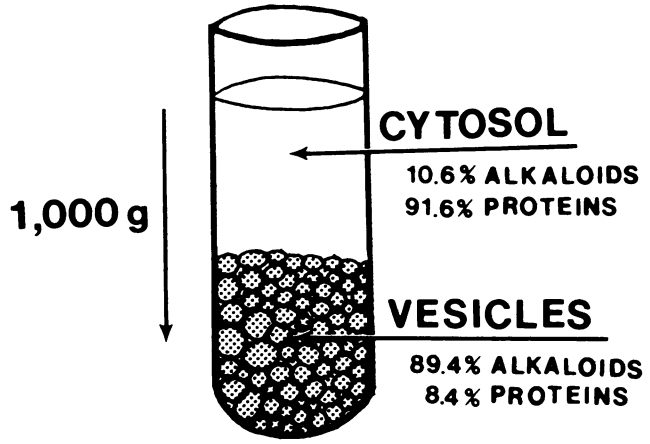

FIG. 10. Diagram illustrating the fractionation of opium poppy latex following a 30-min centrifugation at 1000*g*. Results of alkaloid and protein analysis listed for each fraction.

from a wounded opium poppy capsule or leaf represents the entire cytoplasmic contents of the laticifer and includes alkaloidal vesicles as well as other organelles.

Several investigators (1, 2, 10, 24–27) have shown that the alkaloid-rich vesicle fraction can be separated from the laticifer cytosol by low speed centrifugation (1000*g*) in high osmotic buffer. We examined the distribution of MLPs in freshly fractionated latex using a similar approach. The majority (89.4%) of NP-40 soluble proteins are concentrated in the supernatant of fractionated latex, *i.e.* the laticifer cytosol (Fig. 10). Analysis of the alkaloid content of the supernatant and pellet fractions by

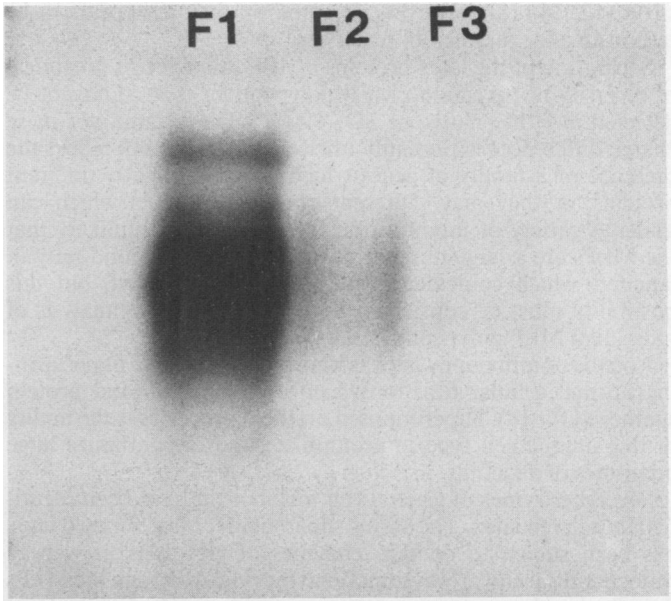

FIG. 11. Autoradiograph of protein gel blot from fractionated opium poppy latex. Strongest reaction with anti-MLP seen in the latex cytosol (F1) with very little reaction seen in the contents of alkaloidal vesicles (F2) or the vesicle membranes (F3).

HPLC revealed that almost 90% of the alkaloids remain associated with the pelleted vesicles (Fig. 10). Thus, most of the alkaloidal vesicles are not disrupted during latex fractionation.

The high amounts of protein in the latex supernatant suggested that the MLPs might also be concentrated in that fraction. To examine this possibility, 20 μg of protein extracts from three latex fractions: (a) latex cytosol, (b) vesicle content, (c) vesicle membrane were subjected to NP-40 PAGE and probed with anti-MLP. Fraction 1 shows the strongest MLP signal (Fig. 11, F1). Thus, it appears that these latex-specific proteins are localized in the laticifer cytoplasm which surrounds the alkaloidal vesicles, and not within the vesicles or the vesicle membranes.

DISCUSSION

Immunocytochemical localization of laticifers has been reported for *Asclepias syriaca* and related species (32). The IgGs used in this study were raised against the serum fraction from ultracentrifuged *A. syriaca* latex. Although individual latex proteins were not identified, laticifer specificity was obtained by preabsorbing the IgGs with undifferentiated callus. It should be noted that 'latex' obtained from nonarticulated laticifers of *A. syriaca* represents vacuolar sap (31), rather than exuded cytoplasm as is the case in opium poppy latex.

Most of the protein in poppy latex has been found to be concentrated in the top fractions of latex separated by sucrose density centrifugation (27). Our data suggests that a significant portion of the proteins in the latex cytosol are present as MLPs. Detailed electron microscopic examination of opium poppy laticifers (19–22, 29) have not revealed any subcellular structures with an obvious storage function. MLPs are probably present as soluble cytoplasmic proteins within the latex cytosol, rather than as a protein body, peroxisome, or some other type of protein-rich inclusion.

MLPs do not appear to have a significant carbohydrate component as indicated by the absence of PAS staining. In addition, the differential staining of the upper MLP band with silver stain suggests the two bands observed in native and SDS gels have different physico-chemical properties. Several investigators have described polypeptides in SDS-PAGE which fail to stain with

silver (12, 14, 28); however, no common feature appears to be responsible for this lack of reactivity.

Separation of the latex proteins by IEF uncovered a great deal of diversity in pIs among MLP polypeptides that could not be detected in either native or SDS-PAGE. The meaning of these charge differences is presently unclear, but they may reflect the presence of a family of genes which code for slightly different proteins, or they may represent one gene product which can undergo variety of modifications. We consider it unlikely that the MLPs are a large number of unique proteins found only in laticifers which coincidently have the same mol wt, but this possibility must be considered until more extensive analysis of individual MLP polypeptides can be performed.

Isolated opium poppy latex is known to be capable of performing normal cellular functions such as respiration and protein synthesis (17, 18). Superimposed on these processes is the ability of this unique cell type to accumulate and/or synthesize large quantities of alkaloids.

Several enzymes of general cell metabolism have been identified in isolated latex. The 1000*g* supernatant of fractionated latex has been shown to contain enzymes of glycolysis as well as mitochondrial and glyoxysomal enzymes (1). Although the MLPs described in the present investigation are also located in the latex supernatant, the relative abundance and limited distribution of the MLPs makes it unlikely that these proteins are general 'housekeeping' enzymes.

Few of the enzymes responsible for alkaloid synthesis have been detected in opium poppy latex. Progress in this area has been limited because of a lack of suitable assays for the individual steps in this complex pathway. One key enzyme, L-3,4 dihydroxyphenylalanine (dopa) decarboxylase has been detected in poppy latex and appears to be localized in the latex serum (26, 27). The specificity of the MLPs for laticifers observed in protein gel blots suggests that these proteins may represent important enzymes of alkaloid synthesis including dopa-decarboxylase.

Future research is being directed toward assessing possible enzymic function(s) of opium poppy MLPs and investigating the appearance of these proteins during laticifer differentiation.

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