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Immunocytochemical Localization of Mandelonitrile Lyase in Mature Black Cherry (*Prunus serotina* Ehrh.) Seeds[1]

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ABSTRACT

Mandelonitrile lyase (MDL, EC 4.1.2.10), which catalyzes the reversible dissociation of (*R*)-(+)-mandelonitrile to benzaldehyde and hydrogen cyanide, was purified to apparent homogeneity from mature black cherry (*Prunus serotina* Ehrh.) seeds by conventional protein purification techniques. This flavoprotein is monomeric with a subunit molecular mass of 57 kilodaltons. Glycoprotein character was shown by its binding to the affinity matrix concanavalin A-Sepharose 4B with subsequent elution by α-methyl-D-glucoside. Upon chemical deglycosylation by trifluoromethanesulfonic acid, the molecular mass was reduced to 50.9 kilodaltons. Two-dimensional gel analysis of deglycosylated MDL revealed the presence of several subunit isoforms of similar molecular mass but differing slightly in isoelectric point. Polyclonal antibodies were raised in New Zealand white rabbits against deglycosylated and untreated MDL. Antibody titers were determined by enzyme linked immunosorbent and dot immunobinding assays, while their specificities were assessed by Western immunoblot analysis. Antibodies raised against untreated lyase recognized several proteins in addition to MDL. In contrast, antisera raised against deglycosylated MDL were monospecific and were utilized for developmental and immunocytochemical localization studies. SDS-PAGE and immunoblotting analysis of seed proteins during fruit maturation showed that MDL first appeared in seeds shortly after cotyledons began development. In cotyledon cells of mature seeds, MDL was localized primarily in the cell wall with lesser amounts in the protein bodies, whereas in endosperm cells, this labeling pattern was reversed. N-terminal sequence data was gathered for future molecular approaches to the question of MDL microheterogeneity.

Cyanogenesis, the production of the respiratory poison HCN[3] by biological organisms, was first described in plants almost two centuries ago (24). Today, over 2650 cyanogenic plant species distributed throughout 130 families of angiosperms, gymnosperms, and pteridophytes are now documented (26). Although low levels of HCN are probably produced by all plants during ethylene biosynthesis (12), some species exhibit far greater HCN release due to catabolism of cyanogenic glycosides or cyanolipids. The latter phenomenon accounts for numerous cases of acute cyanide poisoning of animals, including humans (23). Additionally, in areas of the world where cyanogenic plants such as cassava and lima beans comprise a major item of the diet, chronic cyanide poisoning and associated pathological conditions still exist (23).

A common feature of cyanophoric plants is that cyanogenic glycoside (or cyanolipid) catabolism occurs at a significant rate only after their tissues have been damaged by herbivory, fungal attack, or mechanical means. Therefore, it is generally assumed that these cyanogens and their catabolic enzymes are localized in the intact plant in different compartments and come into contact with each other upon tissue disruption. Although only limited information is currently available, examples of compartmentation at both tissue and subcellular levels are known (13, 14). To broaden our knowledge of how compartmentation normally prevents premature and suicidal cyanogenesis in undamaged plants, our laboratory is investigating the mature seeds of black cherry (*Prunus serotina* Ehrh.) that accumulate the cyanogenic disaccharide (*R*)-amygdalin. In previous publications (15, 16, 31), we showed that amygdalin is degraded in seed extracts by the sequential action of amygdalin hydrolase, prunasin hydrolase, and MDL. All three glycoproteins have been extensively purified and characterized, but their tissue and subcellular localizations remain undocumented. As part of our long-term goal to elucidate how amygdalin and its catabolic enzymes are compartmentalized in black cherries, MDL was purified to homogeneity. Monospecific polyclonal antibodies raised against this protein (after chemical deglycosylation) were used to monitor its appearance in seeds during fruit maturation and to determine its localization in mature seeds.

MATERIALS AND METHODS

Plant Materials

For enzyme purification, mature black cherry (*Prunus serotina* Ehrh.) fruits were harvested from trees grown at several locations in Iowa City. Black cherry pits, obtained by manually removing the mesocarp and exocarp, were washed, sterilized for 10 min with 10% (v/v) Clorox solution (Clorox Co., Oakland, CA), blotted dry, and stored in sealed containers at 4°C until used. For analysis of MDL appearance in seeds at different developmental stages, whole fruits were collected from a single tree, washed, blotted dry, and stored at −20°C until used.

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[3] Abbreviations: HCN, hydrogen cyanide; MDL, mandelonitrile lyase; IgG, immunoglobulin G; TFMS, trifluoromethanesulfonic acid; IEF, isoelectric focusing; pI, isoelectric point; TBS, Tris-buffered saline.

Chemicals and Chromatographic Materials

Protease inhibitors, (*R,S*)-mandelonitrile, polyvinylpolypyrrolidone, trifluoromethanesulfonic acid, Reactive Red 120-Agarose, Freund's adjuvants, and alkaline phosphatase-conjugated goat anti-rabbit IgG were provided by Sigma Chemical Co. Chromatographic materials were purchased from the following sources: concanavalin A-Sepharose 4B, Pharmacia Fine Chemicals, Piscataway, NJ; DEAE-cellulose, Whatman Chemical Separation Ltd., Kent, UK. Colloidal gold-conjugated goat anti-rabbit IgG (AuroProbe EM GAR G10) was obtained from Janssen Life Sciences Products, Olen, Belgium.

Enzyme Purification

All operations were performed at 4°C. Mature seeds (15 g) were homogenized in a mortar with 2 g polyvinylpolypyrrolidone, 4 g glass beads, and 35 mL of buffer A (0.1 M histidine-HCl, pH 6.2, containing 1 mM PMSF, 5 mM Na_2EDTA, 10^{-7} M pepstatin A, 10^{-6} M tosyl-lysine chloromethylketone and 1 mM iodoacetamide. The homogenate was filtered through four layers of cheesecloth and centrifuged twice at 27,500*g* for 25 min. The final supernatant was dialyzed overnight against 4 L of buffer B (10 mM histidine-HCl, pH 6.2, containing 1% [w/v] NaCl), recentrifuged at 27,500*g* for 10 min, and applied to a concanavalin A-Sepharose 4B column (12 × 1.6 cm) preequilibrated with buffer B. After washing the column with this buffer to remove unbound proteins, bound proteins were eluted by 150 mL of buffer B containing 0.3 M α-methyl-D-glucoside. Fractions (5 mL) displaying MDL activity were pooled, dialyzed overnight against 4 L of buffer C (10 mM histidine-HCl, pH 6.2), and applied to a DEAE-cellulose column (12 × 1.6 cm) preequilibrated with buffer C. After extensive washing with buffer C to remove unbound proteins, elution of bound proteins was accomplished by a linear gradient (150 mL) of 0 to 350 mM NaCl in this buffer. Fractions (5 mL) exhibiting MDL activity were pooled, dialyzed overnight against 4 L of buffer D (20 mM sodium acetate-HCl, pH 5.0), and applied to a Reactive Red 120-Agarose column (12 × 1.6 cm) preequilibrated with buffer D. Unbound proteins were removed by extensive washing with buffer D, while the bound MDL was eluted with a linear gradient (150 mL) of 0 to 1.0 M KCl in this buffer. Fractions (5 mL) displaying MDL activity were pooled and dialyzed overnight against 4 L of 10 mM Tris-HCl buffer, pH 8.8, before being concentrated by ultrafiltration to approximately 4 mL. This concentrate was subjected to nondenaturing PAGE (7.5%) at pH 8.8 according to Weber and Osborn (30) except omitting SDS. The zone containing MDL, easily discernible by its yellow appearance, was excised and electroeluted to yield a homogeneous preparation that was stored in small aliquots (0.5 mL) at −20°C.

Electrophoresis

MDL homogeneity was routinely assessed by one-dimensional (18) and two-dimensional SDS-PAGE (21).

Enzyme Assay

MDL activity was measured spectrophotometrically by monitoring the conversion of (*R,S*)-mandelonitrile to benzaldehyde and HCN by the increase in absorbance at 249.6 nm (31).

Protein Estimation

Protein content was estimated by the Bradford procedure (2) with BSA serving as standard.

Deglycosylation of MDL

Chemical deglycosylation of MDL was performed using TFMS as described by Edge *et al.* (5). The deglycosylated protein was recovered by extraction into aqueous phase followed by overnight dialysis against 4 L of 2 mM pyridine acetate buffer, pH 5.5. The dialyzed preparation was concentrated by ultrafiltration (Amicon Minicon) to approximately 1 mL and stored at −20°C.

Generation of Polyclonal Antibodies against MDL

Before immunization of animals, homogeneous MDL, with or without prior deglycosylation, was subjected to two-dimensional PAGE (IEF range pH 4–6.5, followed by 10% SDS-PAGE). Areas containing MDL were excised and electroeluted into 10 mM Tris-acetate buffer (pH 6.8), giving a final concentration of approximately 100 μg/mL. Preimmune sera were collected from four male New Zealand white rabbits (about 2 kg each) before intradermal injection at multiple sites along the neck, shoulders, and backbone with approximately 150 μg antigen emulsified with Freund's complete adjuvant. Two animals (henceforth referred to as rabbits No. 1 and No. 2) received deglycosylated MDL, whereas rabbits No. 3 and No. 4 were immunized with untreated (*i.e.* glycosylated) MDL. After 21, 35, 49, and 63 d, booster injections (75–100 μg antigen/animal) were administered in Freund's incomplete adjuvant. Antisera were collected 13 d after each booster injection by bleeding from marginal ear veins, prepared by centrifugation, and stored in small aliquots (2 mL) at −20°C to avoid multiple freezing and thawing.

Characterization of Anti-MDL Antisera

Antisera titers were determined by ELISA and dot immunobinding assays (3, 10). Antisera specificities were analyzed by immunoblotting techniques. Crude cherry extracts as well as homogeneous MDL were electrophoresed by SDS-PAGE on 15% gels according to Laemmli (18). Proteins were electroblotted overnight onto nitrocellulose filters at 4°C at 30 V (90 mA) in 25 mM Tris, 200 mM glycine, 20% (v/v) methanol transfer buffer using a Trans-Blot apparatus (Bio-Rad). Nonspecific protein binding sites were blocked by incubating filters for 1 h at 37°C with PBS containing 3% (w/v) BSA (blocking solution). Filters were then incubated in anti-MDL antiserum (diluted 1:5 with blocking solution) for 2 h at 37°C. Unbound primary antibodies were removed by three washes (10 min each) with PBS containing 5% (w/v) nonfat dry milk powder and 0.05% (v/v) Tween 20. The filters were incubated with

alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:1000 with blocking solution) for 2 h at 37°C followed by three washes (10 min each) with distilled water. The locations of antigen-antibody complexes were visualized by color development catalyzed by alkaline phosphatase with 0.005% (w/v) 5-bromo-4-chloro-3-indolyl phosphate in combination with 0.01% (w/v) nitro blue tetrazolium and 4 mM $MgCl_2$. In control experiments, anti-MDL antiserum was either omitted or replaced by rabbit preimmune serum.

For closer analysis of the monospecificity of anti-MDL antiserum from rabbit No. 1, *P. serotina* seed proteins were resolved by two-dimensional PAGE (21). Transblotting, blocking of nonspecific sites, and immunostaining were undertaken as described above. In control experiments, anti-MDL antiserum was replaced by preimmune serum.

Analysis of MDL Levels in Maturing Black Cherry Seeds by Immunoblotting

Black cherry fruits were collected at different developmental stages between June 9 and August 10, 1989 (equivalent to 20–82 DAF), and stored at −20°C until processed. Single fruits were then placed in liquid N_2 and cracked open while still frozen. Seed tissues were removed and homogenized in a mortar at 4°C with 0.5 mL of 25 mM histidine-HCl buffer, pH 6.2, containing 1 mM PMSF, 5 mM Na_2EDTA, 10^{-7} M pepstatin A, 10^{-6} M tosyl-lysine chloromethylketone, and 1 mM iodoacetamide. Extracted proteins were immediately denatured by heating at 100°C for 5 min in the presence of 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 2% (w/v) SDS, and 65 mM Tris-HCl buffer, pH 6.8. After centrifugation for 10 min at 15,000*g*, the supernatants were subjected to SDS-PAGE (5–15% gradient gels) according to Laemmli (18). Resolved proteins were electroblotted onto nitrocellulose as previously described and challenged using anti-MDL antisera.

Immunohistochemical Localization of MDL

Immunohistochemical localization of MDL in black cherry seeds was undertaken according to Smart and Amrhein (28) with the following modifications. Small sections of cotyledon and endosperm tissues (1 mm^3) were fixed for over 24 h at 4°C in PBS (pH 7.2) containing 2% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde, followed by thorough washing with PBS. To cross-link lipids, tissues were postfixed for 1 h at room temperature with 1% (w/v) osmium tetroxide and subsequently washed thoroughly with PBS. The fixed tissues were subjected to series dehydration with acetone and infiltration with Spurr's resin for 24 h at room temperature. After transfer of tissues into fresh Spurr's resin, polymerization was continued for over 24 h at 50°C. Ultrathin sections were cut with a DuPont diamond knife on a Sorvall Porter-Blum MT 2-B Ultramicrotome. Sections were collected on 300-mesh copper grids and exposed to the following treatments at room temperature: (a) incubation for 1 h with blocking buffer (50 mM Tris-HCl, 200 mM NaCl, pH 7.2 [TBS] containing 3% [w/v] BSA and 0.1% [v/v] Tween 20) to block nonspecific binding sites; (b) incubation for 1 h with anti-MDL antiserum (diluted 1:5 with blocking buffer) followed by three rinses with TBS; (c) after blocking for another 30 min with TBS containing 3% (w/v) BSA, grids were incubated for 1 h with goat anti-rabbit IgG conjugated to 10 nm colloidal gold (diluted 1:20 with blocking buffer); (d) after rinsing three times with TBS and once with double-distilled water, grids were poststained with 2% (w/v) uranyl acetate for 20 min (without counter-staining by lead citrate) prior to examination with a Hitachi HU-12 electron microscope at 75 kV. The specificity of labeling was determined on the basis of the following control treatments: (a) sections were incubated with preimmune serum instead of primary antibody; and (b) sections were incubated with secondary antibody without prior exposure to the primary antibody. Immunochemical findings were confirmed by double blind experiments, in which the authors provided Dr. Kenneth Jensen (University of Iowa Botany Department) with grids bearing sections from cotyledons or endosperm tissue as well as samples of immune and preimmune sera. The latter applied immune or preimmune sera to selected grids before returning them to the authors for further processing. For each grid, the tissue source and primary antibody selected by Dr. Jensen were withheld until the authors had predicted both tissue identity and immunochemical treatment. In all cases, the authors' predictions proved correct. Results were recorded on Kodak black and white EM film (4489, Estar thick base). The confidence level for the immunolabeling density and percentage of area coverage were statistically analyzed at the University of Iowa Image Analysis Facility.

Protein Sequencing

Amino-terminal sequence analysis of homogeneous MDL (after nondenaturing PAGE) was performed at the University of Iowa Protein Structure Facility by Edman degradation on an Applied Biosystems 475A protein sequencer.

RESULTS AND DISCUSSION

α-Hydroxynitrile lyases that catalyze the dissociation of α-hydroxynitriles to HCN and an aldehyde or ketone have been extensively purified and characterized from plants accumulating aromatic and aliphatic cyanoglycosides (22). Best known are the (*R*)-(+)-MDL (EC 4.1.2.10) isolated from seeds of members of the Prunoideae and Maloideae. Curiously, these enzymes possess flavin adenine dinucleotide bound close to their catalytic sites, although they do not catalyze a net oxidation-reduction reaction. Often requiring only five to 15-fold purification to reach homogeneity, these glycoproteins are clearly major seed constituents and may serve additional roles, perhaps as storage proteins. Most MDLs exist as multiple forms, but the chemical nature and physiological significance of such microheterogeneity remain largely obscure.

Purification of Black Cherry MDL

Although we had previously reported the extensive purification of *P. serotina* MDL by concanavalin A-Sepharose 4B chromatography and PBE 94 chromatofocusing (31), the final preparation exhibited slight contamination by prunasin hydrolase. Homogeneity was attained here by utilizing DEAE-cellulose and Reactive Red 120-Agarose chromatography and

nondenaturing PAGE. The enzyme was thereby purified 34-fold with 52% recovery of initial activity (Table I). SDS-PAGE indicated a subunit molecular mass of 57 kD. This is in close agreement with earlier findings that the five isozymes of *P. serotina* MDL partially resolved by chromatofocusing were monomeric with individual molecular masses falling between 57 and 59 kD (31). MDLs isolated from other members of the Prunoideae have molecular masses ranging from 58 to 60 kD (22).

Generation of Polyclonal Antibodies against Glycosylated and Deglycosylated MDL

All attempts to completely resolve the black cherry MDL isozymes failed, presumably due to their similar mass and pI (31). Polyclonal antibodies, therefore, were of necessity raised against a mixture of MDL isozymes (henceforth referred to as glycosylated MDL). Because we anticipated that antisera raised against glycosylated MDL would contain antibodies that cross-reacted with other glycoproteins having common or similar glycans (19), a portion of the homogeneous MDL preparation was also subjected to deglycosylation by TFMS before immunization. One-dimensional SDS-PAGE showed that TFMS treatment reduced the apparent molecular mass of MDL from 57 to 50.9 kD, indicating a minimum carbohydrate content of 10.7% (Fig. 1). Upon two-dimensional PAGE, glycosylated MDL was seen as a complex band that tailed extensively toward the anode side (Fig. 2A). After deglycosylation, MDL appeared as seven resolved isoforms (five major, two minor) of similar molecular mass but having slightly different pI values (Fig. 2B, C). While the nature of this structural heterogeneity remains unclear, we believe that our data reflect the existence of isoforms showing minor variations in primary structure that might result from the polyploidic nature of *P. serotina* (4) or from gene duplication. This belief is supported by the partial resolution by chromatofocusing of five forms of native MDL that displayed pIs within an extremely narrow pH range (pH 4.58–4.63) (31). Additionally, multiple forms of MDL are common among other rosaceous plants (1, 8, 9, 22). The alternative possibility, that the observed multiplicity arises from partial proteolysis during purification or TFMS treatment, is deemed less likely. Reasonable precautions were taken to minimize protease action during MDL extraction and purification (see "Materials and Methods"). In this context, the stability of black cherry MDL toward proteases should be noted. Glycosylated MDL was totally stable toward trypsin and V_8 protease for 3 h at 37°C (data not shown). Furthermore, Edge *et al.* (5) showed that TFMS treatment of several proteins for 2.5 h at 0°C caused rapid loss of peripheral sugars but left peptide backbones intact.

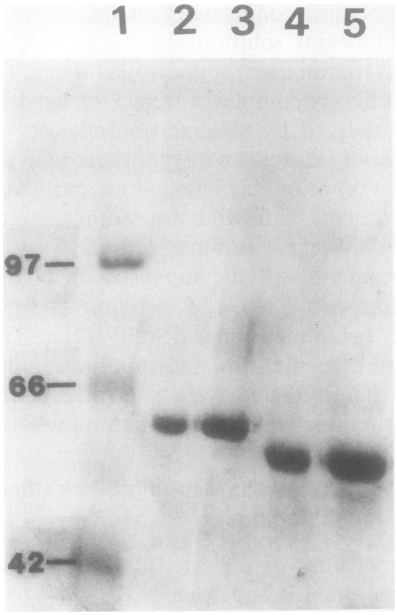

Figure 1. Effect of TFMS deglycosylation on the mol wt of *P. serotina* MDL as shown by one-dimensional SDS-PAGE analysis with staining by Coomassie brilliant blue R-250. Samples electrophoresed were the molecular mass standards phosphorylase B (97 kD), BSA (66 kD), and ovalbumin (42 kD) (lane 1), untreated MDL (lanes 2 and 3), and deglycosylated MDL (lanes 4 and 5).

Characterization of Anti-MDL Antisera

Using ELISAs, the titers of rabbit anti-MDL antisera were approximately 1:50,000. Dot immunoassays, while time-saving and requiring less antigen, were also less sensitive, recording titers of approximately 1:1000. The specificities of anti-MDL antisera were tested by immunoblotting after one- and two-dimensional SDS-PAGE of crude cherry seed extracts and homogeneous MDL preparations. Antisera raised against glycosylated MDL cross-reacted with at least six other polypeptides in addition to MDL (data not shown). This suggested that they contained antibodies directed against the glycan portions of MDL, which recognized other glycoproteins having similar carbohydrate moieties. By contrast, antisera raised against deglycosylated MDL appeared monospecific for MDL, as indicated by intense immunoreaction toward a single polypeptide band of molecular mass corresponding to

Table I. *Purification of P. serotina MDL*

Purification Step	Volume	Protein Content	Activity	Specific Activity	Yield	Purification
	mL	*mg·mL*$^{-1}$	*μkat·mL*$^{-1}$	*μkat·mg*$^{-1}$	%	*-fold*
Crude extract	32.5	18.80	0.625	0.033	100	1
Con A-Sepharose	150.0	1.34	0.092	0.069	67.8	2.1
DEAE-cellulose	68.0	0.25	0.159	0.632	53.3	19.2
Reactive Red 120-Agarose	41.5	0.22	0.254	1.136	51.9	34.4

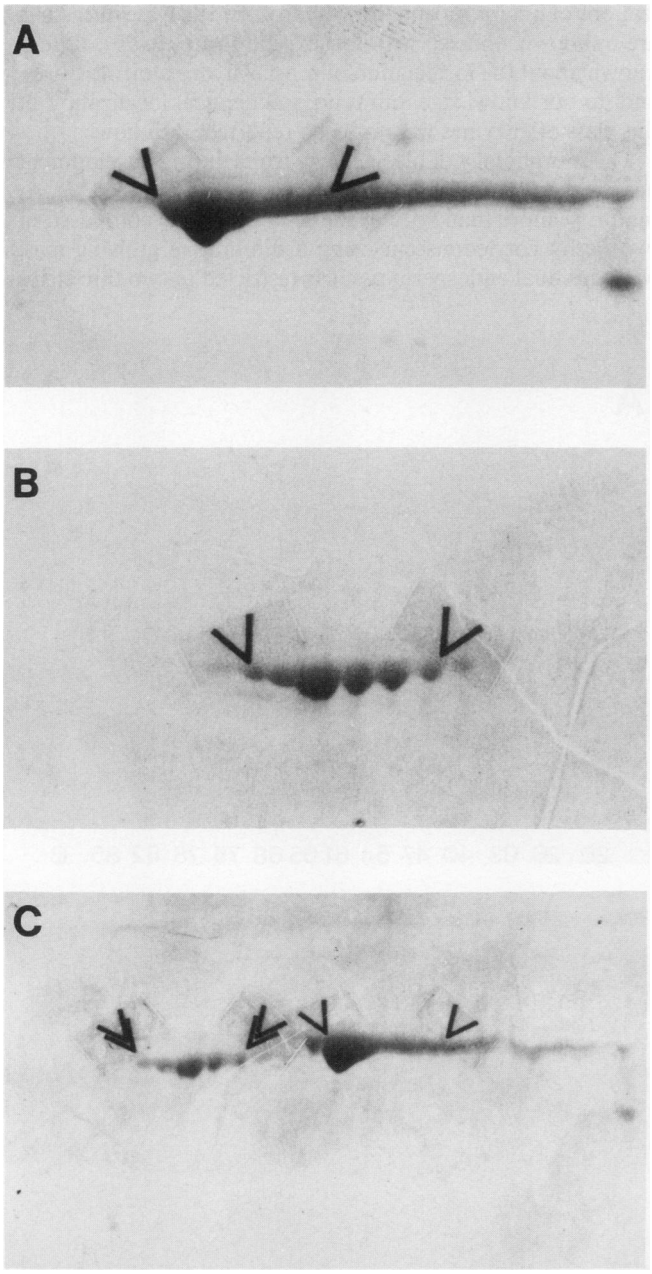

Figure 2. Two-dimensional SDS-PAGE analysis of untreated MDL (A), TFMS-deglycosylated MDL (B), and a mixture of untreated and deglycosylated MDL (C). Protein samples were subjected to IEF (pH range 4.0–6.5) followed by SDS-PAGE (10% gels). Gels were stained with Coomassie brilliant blue. The areas between the arrows shown in plates A and B were excised and electroeluted for use as antigens. In plate C, untreated and deglycosylated MDL are indicated by single and double arrows, respectively.

that of homogeneous MDL (Fig. 3). Identical behavior was shown by antisera raised against native and deglycosylated carrot cell-wall β-fructosidase, a glycoprotein having high mannose and complex oligosaccharide side chains (19). In control experiments in which anti-MDL antiserum was omitted or replaced by preimmune serum, no immunostaining was observed. The monospecificity of the anti-MDL antiserum from rabbit No. 1 was further confirmed by immunoblotting after two-dimensional SDS-PAGE (Fig. 4), indicating its suitability for developmental studies and immunocytochemistry.

MDL Levels during Fruit Development

The appearance of MDL in seed tissues during fruit maturation was monitored by Western immunoblot analysis. Figure 5 shows that MDL was not detectable by either technique within the period 20 to 33 DAF, during which time the seed consisted primarily of liquid endosperm tissue. This flavoprotein began accumulating along with conventional storage proteins approximately 7 d after initiation of cotyledon development.

Immunogold Localization of *P. serotina* MDL

Recognizing that large-scale catabolism of cyanogenic glycosides occurs only after tissue damage, several laboratories have focused their attention upon the modes of compartmentation by which intact plants prevent premature cyanogenesis.

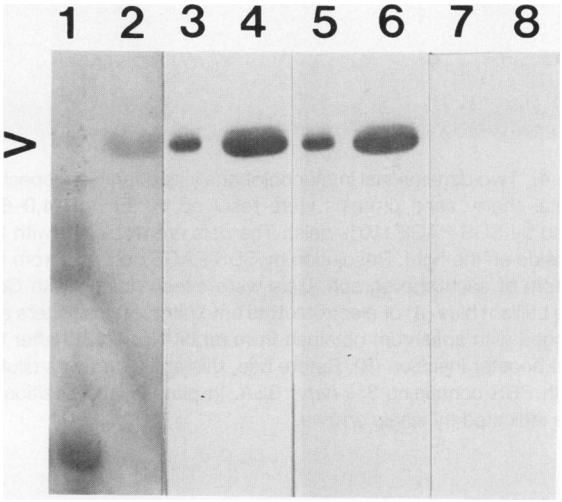

Figure 3. Western immunoblot analysis of specificity of antisera from rabbits immunized with deglycosylated MDL. Total cherry seed proteins (odd-numbered lanes) and homogeneous MDL (even-numbered lanes) were subjected to SDS-PAGE followed by electroblotting onto nitrocellulose filters. Proteins in lanes 1 and 2 were stained with Amido black. Proteins in lanes 3 through 8 were challenged using the following primary antisera (diluted fivefold with PBS containing 3% [w/v] BSA): 3 and 4, antiserum obtained from rabbit No. 1 13 d after second booster injection; 5 and 6, antiserum from rabbit No. 1 13 d after third booster injection; 7, rabbit No. 1 preimmune serum. Lane 8 received secondary antibody only. The position of MDL is indicated by the arrow.

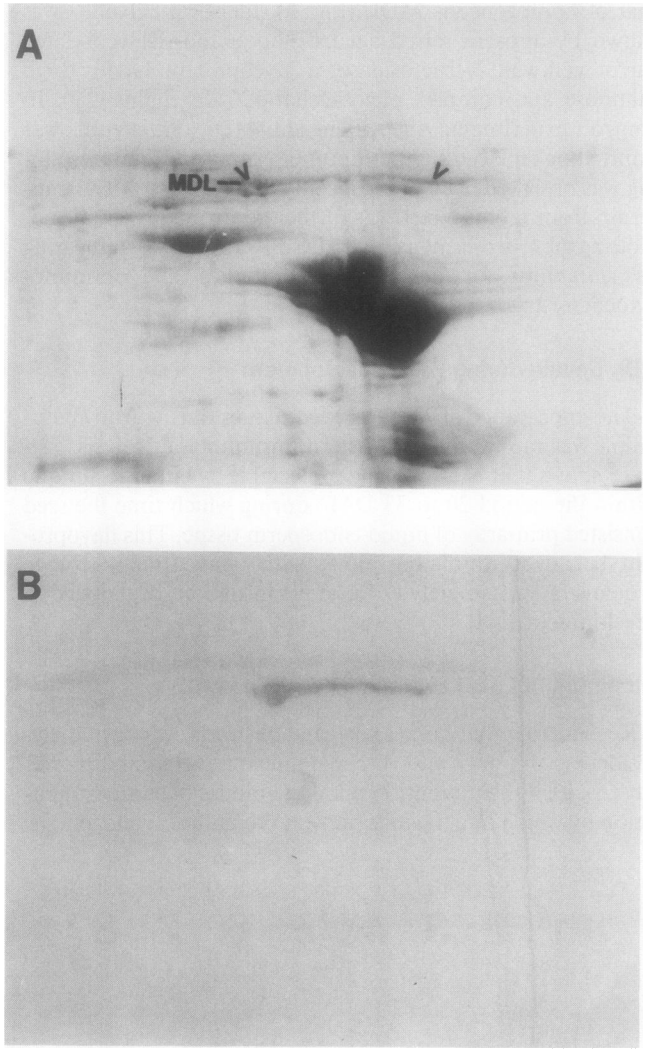

Figure 4. Two-dimensional immunoblot analysis of antisera specificity. Total cherry seed proteins were resolved by IEF (pH 4.0–6.5) followed by SDS-PAGE (10% gels). The gels are displayed with the anode side on the right. Resolution by SDS-PAGE occurred from top to bottom of each photograph. Gels were either stained with Coomassie brilliant blue (A) or electroblotted onto nitrocellulose filters and challenged with antiserum obtained from rabbit No. 1 13 d after the second booster injection (B). Before use, this antiserum was diluted 1:5 with PBS containing 3% (w/v) BSA. In plate A, the position of MDL is indicated by single arrows.

A complete picture is available for only one organism, namely the leaves of 6-d-old light-grown sorghum seedlings, in which the authors demonstrated a clear compartmentation of substrate and catabolic enzymes within different tissues (14). The cyanogenic glycoside dhurrin was located in the vacuoles of epidermal cells, whereas the β-glucosidase and α-hydroxynitrile lyase were present almost exclusively in the underlying mesophyll tissues (25). These enzymes were located in the chloroplast and cytosol, respectively (29). In other species, compartmentation apparently occurs at the subcellular level, with the catabolic enzymes frequently having an apoplastic location. For example, in cassava, cells throughout the entire tuber cross-section possess both cyanogens (predominantly linamarin) and linamarase (13). In common with linamarases from *Phaseolus lunatus* and *Trifolium repens* (6, 11), cassava linamarase resides in the cell wall (20). The subcellular localizations of linamarin and the α-hydroxynitrile lyase in cassava are unknown. Indeed, with few exceptions (6, 7, 27), little is known about the localization of most α-hydroxynitrile lyases, and, to our knowledge, immunocytochemical localization of this class of enzymes has not been reported until now.

The morphology of black cherry fruits during development has been well documented by Labrecque and Barabe (17). At maturity, more than 95% of the seed volume is composed of two fleshy cotyledons enclosing a diminutive growing axis, while residual endosperm tissue is restricted to two thin strips

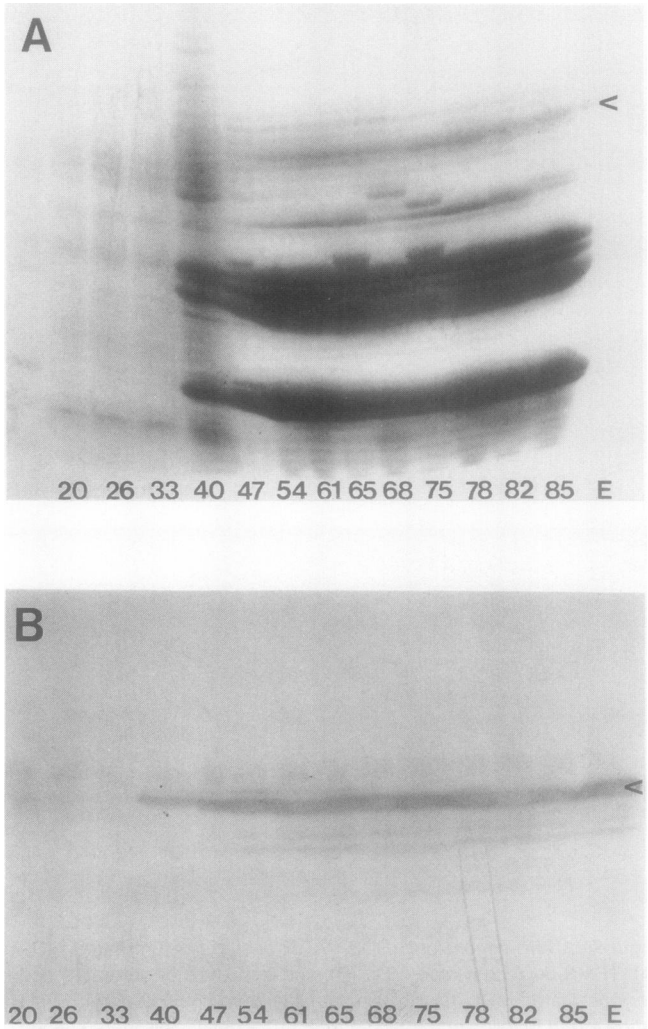

Figure 5. Western immunoblot analysis of *P. serotina* seed proteins during fruit development. At intervals after flowering, proteins were extracted from single seeds as described in "Materials and Methods" and subjected to SDS-PAGE (5–15% gradient gels). Proteins were either stained with Coomassie brilliant blue (A) or electroblotted onto nitrocellulose and challenged with anti-MDL antiserum (B). The number under each lane denotes DAF. The position of MDL is indicated by arrows.

of cells associated with the surrounding testa. Preliminary studies with several types of embedding media revealed that osmium postfixation coupled with Spurr's embedding was optimal for immunocytochemistry of black cherry seed tissues, which are particularly rich in lipid. These procedures allowed fine structural preservation and high retention of antigenicity with only low background staining. By contrast, when seed tissues were embedded in Lowicryl, LR White, or LR Gold resins, difficulties were encountered with respect to infiltration, ultra-thin sectioning, and structural preservation.

Following incubation with anti-MDL antiserum (from rabbit No. 1) and gold-conjugated goat anti-rabbit antibodies, ultra-thin sections from cotyledon tissue showed heavy labeling throughout the cell walls (Fig. 6A, B). Weaker labeling was visible over the protein bodies, while a few randomly distributed gold particles were present over the lipid bodies. By contrast, in sections of endosperm tissue the most intense deposition of gold particles was over the protein bodies (Fig. 6D, E). Cell walls were only weakly labeled, whereas the lipid bodies showed only background levels. In control experiments in which anti-MDL antiserum was replaced by preimmune serum, both cotyledon and endosperm sections exhibited only an extremely weak and irregular background (Fig. 6C, F). Similarly, sections incubated with secondary antibodies alone did not show any immunolabeling (not illustrated).

The above findings were confirmed by multiple experiments, including double blind experiments, and their confidence levels were assessed by statistical analysis (Table II). Student's t tests revealed that the density of immunolabeling shown by cotyledon cell walls was significantly higher than those shown by cotyledonar protein bodies (at $P < 0.05$ level), lipid bodies (at $P < 0.01$ level), and by endosperm cell walls (at $P < 0.05$ level). Furthermore, the protein bodies of endosperm cells showed significantly greater immunolabeling than endosperm cell walls (at $P < 0.05$ level), lipid bodies (at $P < 0.01$ level), and cotyledonar protein bodies (at $P < 0.05$ level).

Two important questions are raised by these immunohistochemical data. First, does the observed tissue-specific localization of MDL reflect the sequestration of different isozymes in cotyledon and endosperm tissues? This possibility could not be confirmed or refuted using the polyclonal antibody raised here because it recognized all MDL isozymes. In theory, this question might be approached by careful dissection of seeds into cotyledon and endosperm fractions followed by partial purification and isozyme analysis. Preliminary experiments, however, indicate that, while one can readily obtain

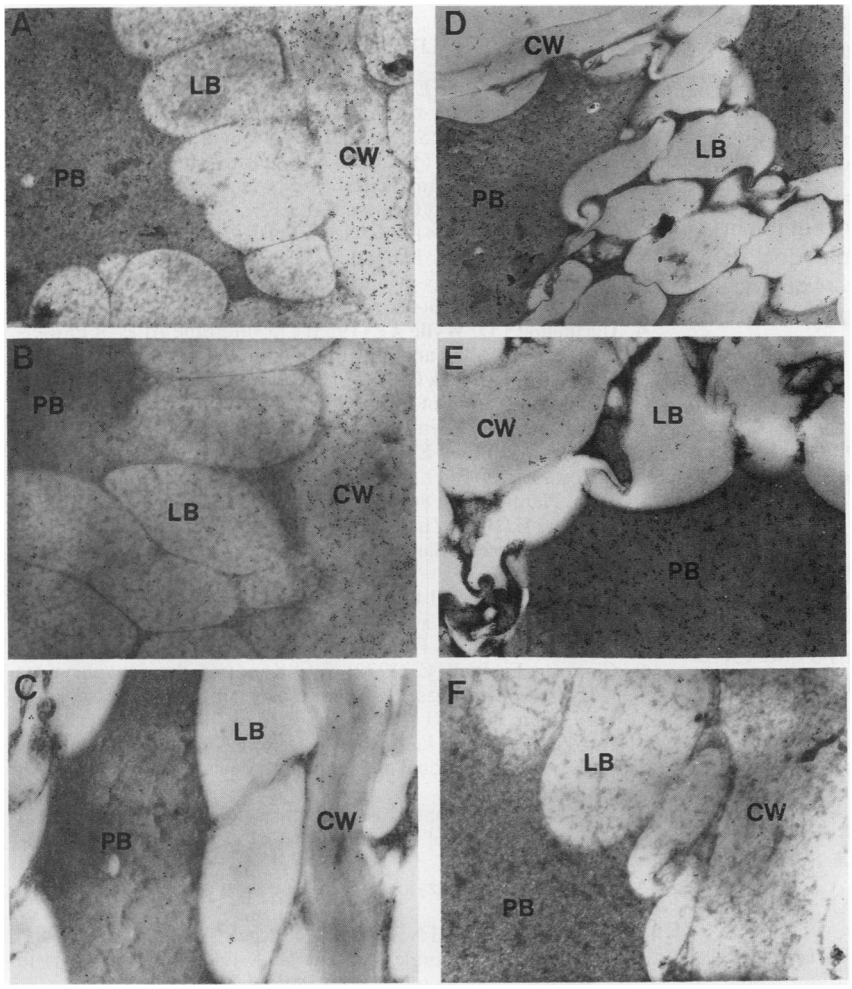

Figure 6. Immunogold electron microscopic localization of MDL in black cherry seeds. Thin sections of cotyledons (A and B) and endosperm (D and E) were treated with anti-MDL antisera. Bound MDL antibodies were localized with immunogold IgG. As controls, cotyledon (C) and endosperm (F) sections were also exposed to preimmune serum before treatment with immunogold IgG. Abbreviations: CW, cell wall; LB, lipid body; PB, protein body. Magnification for micrograph D was ×36,000; all others were ×54,000.

Table II. *Statistics for Colloidal Gold Immunolabeling of MDL in Black Cherry Seeds*

Tissue	Treatment	Subcellular Structure	Samples Counted	Gold Particle Density	Area Coverage
			No.	*No.·cm*$^{-2}$	%
Cotyledon	Anti-MDL antiserum	Cell walls	17	38.04 ± 2.80	4.45 ± 0.39
		Lipid bodies	17	4.85 ± 0.57	0.29 ± 0.05
		Protein bodies	17	14.85 ± 0.97	0.97 ± 0.08
Cotyledon	Pre-immune serum	Cell walls	10	0.95 ± 0.06	0.05 ± 0.02
		Lipid bodies	10	0.58 ± 0.06	0.04 ± 0.02
		Protein bodies	10	1.25 ± 0.07	0.15 ± 0.03
Endosperm	Anti-MDL antiserum	Cell walls	19	9.90 ± 0.99	0.53 ± 0.07
		Lipid bodies	19	3.57 ± 0.30	0.25 ± 0.05
		Protein bodies	19	43.08 ± 3.30	4.58 ± 0.38
Endosperm	Pre-immune serum	Cell walls	9	1.36 ± 0.07	0.15 ± 0.03
		Lipid bodies	9	1.01 ± 0.09	0.09 ± 0.02
		Protein bodies	9	1.53 ± 0.07	0.26 ± 0.04

Table III. *N-Terminal Amino Acid Sequence of P. serotina MDL*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
NH_2-	Leu-	Ala-	Thr-	Thr-	Ser-	Asn-	His-	Asp-	Phe-	Ser-	Tyr-	Leu-	Arg-	Phe-	Ala-	Tyr-

cotyledon tissue free of contamination by endosperm, the reverse is not true. Second, does MDL localized in the cell wall and the protein bodies perform distinct functions? By analogy with cyanophoric species that house cyanogenic β-glycosidases in the cell wall (6, 11), it seems probable that the apoplastic MDL is involved in rapid HCN release upon tissue disruption. Because the cotyledons occupy 95% of the seed volume, the cotyledonar MDL presumably plays the dominant role, but the assistance of the protein body MDL in this process cannot be excluded. Whether the latter MDL also functions as a storage protein is currently unknown but might be judged by ascertaining its fate during seed germination. Its role as such is expected to be minor, given the overwhelming preponderance of conventional storage proteins present in mature black cherry seeds (Fig. 5).

N-Terminal Sequencing

To gain further insight into the nature and physiological significance of the microheterogeneity shown by MDL, we are resorting to molecular approaches. An unresolved mixture of *P. serotina* MDL isozymes was subjected to N-terminal sequencing. The sequence of the first 16 amino acids, apparently common to all isozymes submitted, is given in Table III. Comparison with the amino terminal sequence of *P. lyonii* MDL showed complete identity except at positions 6 and 13, which were occupied by Asp and Ser, respectively (B.K. Singh, L.L. Xu, E.E. Conn, unpublished results); both replacements may represent single nucleotide changes. Using the monospecific antibodies raised here and oligonucleotide probes based on the above sequence, we intend to identify the gene(s) specifically involved in the expression of MDL during black cherry development.

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LITERATURE CITED

1. **Aschhoff H-J, Pfeil E** (1970) Auftrennung und Charakterisierung der Isozyme von D-Hydroxynitril-Lyase (D-Oxynitrilase) aus Mandeln. Hoppe Seyler's Z Physiol Chem **351:** 818–826
2. **Bradford MM** (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
3. **Campbell AM** (1984) Assay techniques. *In* RH Burdon, PH van Knippenberg, eds, Laboratory Techniques in Biochemistry and Molecular Biology. Monoclonal Antibody Technology. Elsevier, Oxford, pp 33–65
4. **Darlington CD, Wylie AP** (1955) Chromosome Atlas of Flowering Plants. Allen and Unwin, London, p 143
5. **Edge ASB, Faltynek CR, Hof L, Keichert LE Jr, Weber P** (1981) Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. Anal Biochem **118:** 131–137
6. **Frehner M, Conn EE** (1987) The linamarin β-glucosidase in Costa Rican wild lima beans (*Phaseolus lunatus* L.) is apoplastic. Plant Physiol **84:** 1296–1300
7. **Gerstner E** (1974) Zur Localisation des Flavinenzyms D-Hydroxynitril-Lyase in Samen von Prunoideen. Naturwissenschaften **61:** 687
8. **Gerstner E, Kiel U** (1975) Eine neue Mandelsaurenitril-Lyase (D-Oxynitrilase) aus *Prunus laurocerasus* (Kirschlorbeer). Hoppe-Seyler's Z Physiol Chem **356:** 1853–1857
9. **Gerstner E, Pfeil E** (1972) Zur Kenntnis des Flavinenzyms Hydroxynitril-Lyase (D-Oxynitrilase). Hoppe-Seyler's Z Physiol Chem **353:** 271–286
10. **Hawkes R, Niday E, Gordon J** (1982) A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem **119:** 142–147
11. **Kakes P** (1985) Linamarase and other β-glucosidases are present in the cell walls of *Trifolium repens* L. leaves. Planta **66:** 156–160
12. **Kende H** (1989) Enzymes of ethylene biosynthesis. Plant Physiol **91:** 1–4
13. **Kojima M, Iwatsuki N, Data ES, Villegas CDV, Uritani I** (1983) Changes in cyanide content and linamarase activity in wounded cassava roots. Plant Physiol **72:** 186–189

14. **Kojima M, Poulton JE, Thayer SS, Conn EE** (1979) Tissue distributions of dhurrin and of enzymes involved in its metabolism in leaves of *Sorghum bicolor*. Plant Physiol **63:** 1022–1028
15. **Kuroki GW, Poulton JE** (1986) Comparison of kinetic and molecular properties of two forms of amygdalin hydrolase from black cherry (*Prunus serotina* Ehrh.) seeds. Arch Biochem Biophys **247:** 433–439
16. **Kuroki GW, Poulton JE** (1987) Isolation and characterization of multiple forms of prunasin hydrolase from black cherry (*Prunus serotina* Ehrh.) seeds. Arch Biochem Biophys **255:** 19–26
17. **Labrecque M, Barabe D** (1984) Developpement du fruit de *Prunus serotina* (Rosaceae). Can J Bot **62:** 195–206
18. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
19. **Lauriere M, Lauriere C, Chrispeels MJ, Johnson KD, Sturm A** (1989) Characterization of a xylose-specific antiserum that reacts with the complex asparagine-linked glycans of extracellular and vacuolar glycoproteins. Plant Physiol **90:** 1182–1188
20. **Mkpong OE, Yan H, Chism G, Sayre RT** (1990) Purification, characterization, and localization of linamarase in cassava. Plant Physiol **93:** 176–181
21. **O'Farrell PH** (1975) High-resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021
22. **Poulton JE** (1988) Localization and catabolism of cyanogenic glycosides. *In* D Evered, S Harnett, eds, Cyanide Compounds in Biology, Ciba Foundation Symposium No. 140. John Wiley & Sons, Chichester, UK, pp 67–91
23. **Poulton JE** (1989) Toxic compounds in plant foodstuffs: cyanogens. *In* JE Kinsella, WG Soucie, eds, Food Proteins. American Oil Chemists' Society, Champaign, IL, pp 381–401
24. **Robinson ME** (1930) Cyanogenesis in plants. Biol Rev **5:** 126–141
25. **Saunders JA, Conn EE** (1978) Presence of the cyanogenic glucoside dhurrin in isolated vacuoles from *Sorghum*. Plant Physiol **61:** 154–157
26. **Seigler DS** (1991) Cyanide and cyanogenic glycosides. *In* GA Rosenthal, MR Berenbaum, eds, Herbivores. Academic Press, San Diego (in press)
27. **Selmar D, Frehner M, Conn EE** (1989) Purification and properties of endosperm protoplasts of *Hevea brasiliensis* L. J Plant Physiol **135:** 105–109
28. **Smart CC, Amrhein N** (1987) Ultrastructural localization by protein A-gold immunocytochemistry of 5-enolpyruvylshikimic acid 3-phosphate synthase in a plant cell culture which overproduces the enzyme. Planta **170:** 1–6
29. **Thayer SS, Conn EE** (1981) Subcellular localization of dhurrin β-glucosidase and hydroxynitrile lyase in the mesophyll cells of *Sorghum* leaf blades. Plant Physiol **67:** 617–622
30. **Weber K, Osborn M** (1969) The reliability of molecular weight determinations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem **244:** 4406–4412
31. **Yemm RS, Poulton JE** (1986) Isolation and characterization of multiple forms of mandelonitrile lyase from mature black cherry (*Prunus serotina* Ehrh.) seeds. Arch Biochem Biophys **247:** 440–445