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Purification and Characterization of a Novel (*R*)-Mandelonitrile Lyase from the Fern *Phlebodium aureum*[1]

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Using high-performance liquid chromatography and nuclear magnetic resonance we identified vicianin as the cyanogenic compound of *Phlebodium aureum*. The (*R*)-hydroxynitrile lyase involved during cyanogenesis in the catabolism of the aglycon ([*R*]-mandelonitrile) was purified to apparent homogeneity. The purified holoenzyme is a homomultimer with subunits of M_r = 20,000. At least three isoforms of the enzyme exist. In contrast to other hydroxynitrile lyases, mandelonitrile lyase (MDL) from *P. aureum* was not inhibited by sulfhydryl- or hydroxyl-modifying reagents, suggesting a different catalytic mechanism. The enzyme is active over a broad temperature range, with maximum activity between 35 and 50°C, and a pH optimum at 6.5. In contrast to (*R*)-MDLs isolated from several species of the Rosaceae family, (*R*)-MDL from *P. aureum* is not a flavoprotein. The substrate specificity was investigated using immobilized enzyme and diisopropyl ether as solvent. The addition of cyanide to aromatic and heterocyclic carbonyls is catalyzed by this (*R*)-MDL, whereas aliphatic carbonyls are poorly converted.

If cyanogenic plants, which are widely distributed among higher plants, are damaged, HCN is released into the environment. This phenomenon, designated as cyanogenesis, is caused by the catabolism of cyanogenic glycosides or, in some cases, cyanogenic lipids (Seigler, 1991). Catabolism of cyanogenic glycosides is initiated by β-glucosidases, which hydrolyze the cyanogenic glycoside to cyanohydrin (α-hydroxynitrile) and a saccharide. Subsequently, the unstable cyanohydrin decomposes spontaneously or enzymatically by the action of an α-HNL to cyanide and a carbonyl compound (Conn, 1981). Thus, it is suggested that the predominant physiological role of cyanogenesis is protection from predators (Nahrstedt, 1985). However, for seedlings of *Hevea brasiliensis*, it was shown that cyanogenic glycosides also serve as N-storage compounds (Lieberei et al., 1985; Selmar et al., 1988).

To date, HNLs of various angiosperms have been purified and characterized (Poulton, 1988; Kuroki and Conn, 1989; Wajant and Mundry, 1993; Hughes et al., 1994; White et al., 1994). According to their FAD content, HNLs can be divided into two groups: flavoprotein and nonflavoprotein HNLs. Flavoprotein HNLs are exclusively found in two subfamilies of the Rosaceae family (Gerstner et al., 1968), whereas nonflavoprotein HNLs have been described for several families of higher plants (Poulton, 1988; Selmar et al., 1988; Kuroki and Conn, 1989). The flavoprotein HNLs isolated from Rosaceae species are (*R*)-MDLs. They are all glycoproteins of similar size (Poulton, 1988) and are serologically related (Gerstner and Pfeil, 1972). The flavoprotein HNLs do not catalyze a net oxidation or reduction reaction (Jorns, 1979). Therefore, it was proposed that (*R*)-MDL from Rosaceae evolved from an ancestoral flavoprotein, which lost its capability to catalyze oxidation/reduction reactions. According to this hypothesis, the FAD of flavoprotein HNL serves to maintain the structural integrity of the enzyme (Jorns, 1979).

In contrast to the flavoprotein HNLs, which form a homogenous group of enzymes, the nonflavoprotein HNLs form an extremely heterogenous group of proteins. They differ in size, extent of glycosylation, subunit composition, and substrate specificity (Poulton, 1988; Kuroki and Conn, 1989; Hughes et al., 1994). Even acetone cyanohydrin lyases from *Linum usitatissimum* and *Manihot esculenta*, which have the same cyanohydrin as natural substrate, show no common biochemical properties. Whereas the acetone cyanohydrin lyase from *L. usitatissimium* is a homodimer of 82 kD catalyzing the synthesis of (*R*)-cyanohydrins (Xu et al., 1988; Albrecht et al., 1993), MeHNL (EC 4.1.2.37) is a homotrimeric molecule of 90 kD accepting (*S*)-cyanohydrins as substrate (Hughes et al., 1994; Wajant et al., 1995). Furthermore, a serological cross-reactivity can be detected neither between MeHNL and HNL from *L. usitatissimum* (acetone cyanohydrin lyase; EC 4.1.2.37) nor among other nonflavoprotein HNLs (Wajant et al., 1995).

Recently, the genes encoding for the flavoprotein HNL from *Prunus serotina* (Cheng and Poulton, 1993) and the nonflavoprotein MeHNL (Hughes et al., 1994) and SbHNL (EC 4.1.2.11) (Wajant et al., 1994), respectively, were cloned. There are no obvious sequence homologies be-

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Abbreviations: DFP, diisopropyl fluorophosphate; EE, enantiomeric excess; HNL, hydroxynitrile lyase; MDL, mandelonitrile lyase; MeHNL, HNL from *Manihot esculenta* (acetone cyanohydrin lyase); PaMDL, MDL from *Prunus amygdalum*; PhaMDL, MDL from *Phlebodium aureum*; SbHNL, HNL from *Sorghum bicolor* ([*S*]-*p*-hydroxy-MDL).

tween the deduced amino acid sequences of these HNLs. For the HNL from *P. serotina*, limited homologies to other flavoproteins were found (Cheng and Poulton, 1993). For MeHNL, no homologies to proteins with known function were described (Hughes et al., 1994). However, analysis of the SbHNL sequence revealed considerable homologies to Ser carboxypeptidases (Wajant et al., 1994). In particular, SbHNL contains a catalytic triad Ser-Asp-His (Wajant et al., 1994), which is described for three groups of independently evolved Ser proteases (Liao and Remington, 1990). Therefore, Ser proteases are regarded as an example of convergent molecular evolution (Liao and Remington, 1990). Facing the lack of sequence homologies among the three cloned HNLs and taking into account the biochemical heterogeneity of HNLs, it was proposed that HNLs also have independently evolved from several ancestral enzymes (Wajant et al., 1994).

Here we describe the purification of a novel (*R*)-MDL from the fern *Phlebodium aureum*, which contains no FAD. To our knowledge, this MDL is the first HNL isolated from Filitaceae. PhaMDL (EC 4.1.2.10) possesses no common properties with the flavoprotein lyases from Rosaceae, except that it has the same natural substrate, mandelonitrile, which is released from prunasin in *Prunus* sp. and from vicianin in *P. aureum*, respectively. PhaMDL is a multimer of 20-kD subunits and is suitable for synthesis of (*R*)-cyanohydrins in organic media.

MATERIALS AND METHODS

Plant Materials

Phlebodium aureum was cultivated at 22°C under natural light conditions. Directly after harvest, the leaves were frozen in liquid nitrogen, crushed using a mortar and pestle, and lyophylized.

Chemicals and Biochemicals

Except where noted, amino acid-modifying agents were purchased from Sigma. Protein standards for gel filtration were obtained from Pharmacia (catalase, aldolase, BSA, and ovalbumin) or Serva (Paramus, NJ) (carbonic anhydrase and trypsin inhibitor). Protein standards for SDS-PAGE and chromatography resins were also obtained from Pharmacia, and the BCA protein assay kit was obtained from Pierce.

Extraction and Purification of Cyanogenic Glycosides

To remove lipids and other lipophilic substances, the freeze-dried powder was extracted with petrol ether using a soxhlet apparatus. To extract the cyanogenic glycosides, the defatted plant material was suspended in methanol (2 mL/g fresh weight) and homogenized in an Ultra Turrax homogenizer (IKA, Staufen, Germany) (3 × 60 s). After filtration, the methanol extract was evaporated. The dried material was dissolved in methanol/water (30:70), filtered (Spartam 30B [Schleicher & Schüll], 0.45 μm), and applied to an HPLC system, using a preparative RP-18 column (16 mm i.d.; 100 mm long; 15 μm particle size). Separation was performed isocratically with methanol:water (30:70) at a flow rate of 5 mL/min. Cyanogenic glycosides were detected by cyanide liberation after their hydrolysis. For this, after evaporation of methanol, aliquots of each fraction were combined with β-glucosidase (emulsin, Serva, 2 mg/mL in phosphate buffer, pH 5.5) and incubated for 1 h at room temperature. To dissociate the hydroxynitriles produced, NaOH (1 N) was added. After neutralization, cyanide was analyzed using the Spectroquant kit for cyanide (Merck, Darmstadt, Germany). The fraction containing cyanogenic glycosides was freeze dried and purified by a second HPLC, which was run on an analytical column (RP-18, 4 mm i.d.; 250 mm long; 5 μm particle size). Again, separation was performed isocratically with methanol:water (30:70) at a flow rate of 1 mL/min.

Characterization and Identification of Vicianin

For mass spectroscopy, the fast atom bombardment technique with xenon and glycerol as the matrix was used. Mass spectroscopy was performed in positive as well as in negative mode. ^{1}H- and ^{13}C-NMR spectra were recorded at ambient temperature with a Bruker (Bremen, Germany) 300 NMR-spectrometer in DMSO as solvent. For higher resolution, two-dimensional ^{1}H-NMR spectra (correlated spectroscopy) were also obtained.

Assay for MDL Activity

MDL activity was determined by measuring the conversion of (*R*)-mandelonitrile to benzaldehyde and cyanide (Jorns, 1979). The increase of A_{249} caused by the production of benzaldehyde was recorded spectrophotometrically for 1 to 2 min. The slow base-catalyzed, nonenzymatic decomposition of mandelonitrile, determined in a separate control reaction, was subtracted. Assays were carried out in 1 mL of 50 mM sodium acetate (pH 5.3) containing 0.084 mM mandelonitrile. The amount of enzyme that decomposes 1 μmol of mandelonitrile in 60 s under these conditions is defined as 1 unit.

Protein Concentration Determinations

Protein concentrations were determined by a commercial assay (BCA assay, Pierce) with BSA as standard.

Enzyme Purification

PaMDL was purified as described elsewhere (Lauble et al., 1994).

Purification of MDL from *P. aureum*

All steps of enzyme purification, except the fast protein liquid chromatography procedures, were carried out on ice.

Step 1. Anion-Exchange Chromatography on Q-Sepharose FF

Ten to 20 g of freeze-dried powdered plant material of *P. aureum* were extracted three times with 15 volumes of 50

mM sodium acetate (pH 5.7). The suspensions were centrifuged at 40,000*g* for 30 min in a Ti 45 rotor, and the supernatants were collected. The pooled supernatants were loaded at a flow rate of 3 mL/min onto a Q-Sepharose FF filled HR10/10 column (Pharmacia), previously equilibrated with 20 mM sodium acetate, pH 5.7. Subsequently, the column was washed with 20 mM sodium acetate (pH 5.7) at the same flow rate until the A_{280} reached the initial base line. Bound materials were eluted with a 200-mL linear gradient of 0 to 0.4 M NaCl in 20 mM sodium acetate (pH 5.7) at a flow rate of 2 mL/min. Fractions of 5 mL were collected in an ice-cooled rack and assayed for MDL activity, and positive fractions were pooled.

Step 2. Chromatofocusing on a Mono P HR 5/20 Column

Active pools from step 1 were dialyzed for 24 to 28 h with two changes against deionized H_2O and were subsequently applied at a flow rate of 0.5 mL/min onto a Mono P HR 5/20 column (Pharmacia), equilibrated in polybuffer PB 94 (Pharmacia) diluted 1:10 in 25 mM piperazine-Cl (pH 5.0). Bound proteins were eluted at a flow rate of 0.25 mL/min using a linear gradient (192 mL) of polybuffer PB 94 diluted 1:10 in 25 mM piperazine-Cl (pH 4.0), and active fractions were pooled.

Step 3. Gel-Filtration Chromatography on Superdex 200

Protein samples (5–10 mL) were applied to a HiLoad 26/60 Superdex 200 prep grade column (Pharmacia) and eluted with 20 mM Tris-Cl (pH 7.5) at a flow rate of 1 mL/min.

Step 4. Anion-Exchange Chromatography on Mono Q

Fractions from step 3 with high specific activity were pooled and concentrated by application on Mono Q HR 5/5 (Pharmacia), equilibrated in 20 mM Tris-Cl (pH 7.5). Protein was eluted in a 20-mL linear gradient of 0 to 0.4 M NaCl in 20 mM Tris-Cl (pH 7.5) at a flow rate of 1 mL/min.

Step 5. Cation-Exchange Chromatography on Mono S

Fractions from step 4 with high specific activity were pooled, diluted 1:10 with 30 mM sodium citrate (pH 3.9), and loaded at a flow rate of 1 mL/min onto Mono S HR 5/5 (Pharmacia), equilibrated with 30 mM sodium citrate (pH 3.9). Proteins were eluted using a linear gradient from 0 to 0.4 M NaCl in 30 mM sodium citrate (pH 3.9) at a flow rate of 0.5 mL/min.

Gel Electrophoresis

SDS-PAGE was performed with a 1-mm-thick polyacrylamide gel as described by Laemmli (1970). Samples were boiled at 100°C for 5 min in the presence of 1% SDS and 75 mM DTT. Gels were silver stained according to the method of Blum et al. (1987).

Estimation of Apparent Molecular Mass

Apparent molecular masses of two native MDL isoforms were estimated by means of gel-filtration chromatography on a HiLoad 26/60 Superdex 200 prep grade column. Three milliliters of each purified isoform of MDL were loaded onto the column at a flow rate of 0.4 mL/min and eluted at the same flow rate with 20 mM Tris-Cl buffer (pH 7.5) containing 400 mM NaCl. The column was calibrated with the following protein standards: catalase (232 kD), aldolase (158 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), and trypsin inhibitor (20.1 kD).

Effects of Temperature and pH on MDL Activity

Enzyme reactions for temperature experiments were carried out in 100 mM sodium acetate (pH 6.0) containing 0.84 mM (*R,S*)-mandelonitrile. For determination of the pH dependence of MDL activity, the reaction was measured at 23°C. The buffers used for this purpose were 50 mM sodium citrate (pH 3.3–5.2), 50 mM sodium acetate (pH 5.3–6.5), and Tris-Cl (pH 6.5–7.5), each containing 0.84 mM (*R,S*)-mandelonitrile. Reactions were initiated by addition of 20 to 40 ng of MDL (0.37–0.74 unit). The observed activity was corrected for the base-catalyzed spontaneous decomposition of (*R,S*)-mandelonitrile at the appropriate temperature or pH.

Data Analysis of Enzyme Kinetics

The initial velocity for a wide range of substrate concentrations was determined in triplicate in 100 mM sodium acetate (pH 6.0) at 23°C for fixed enzyme concentrations of MDL isoforms. The obtained data were analyzed according to the method of Michaelis and Menten (Palmer, 1981). An accurate estimation of V_{max} was obtained from a Lineweaver-Burk plot (Palmer, 1981). Statistical parameters (confidence interval, SD) were determined using the Sigma Plot software from Jandel Scientific (Carle Madera, CA).

Effects of Additives

The effect of various inhibitors on the activity of purified MDL was examined using (*R,S*)-mandelonitrile as substrate (0.84 mM). The enzyme (approximately 100 ng) was incubated with the inhibitors at various concentrations in 100 μL of 20 mM sodium acetate (pH 6.0) at 37°C for 10 min. Subsequently, the enzymatic reaction was started by addition of 20 μL of enzyme solution to 1 mL of sodium acetate (pH 5.3) containing 0.84 mM mandelonitrile.

UV/Visible Spectra

Spectra were measured in a UVIKON 710 spectrophotometer (Kontron, Neufahrn, Germany), recording 100 nm/min, using a 1-cm path length. The MDLs were analyzed in 5 mM Tris-Cl (pH 7.5).

Synthesis of (*R*)-Cyanohydrins in Organic Media

PaMDL (EC 4.1.2.10) and PhaMDL were immobilized on avicel-cellulose as described elsewhere (Ziegler et al.,

1990). Then, a mixture of immobilized enzyme, appropriate carbonyl (0.5 mmol), and HCN (2.6 mmol) in diisopropyl ether (4 mL) was shaken for 4.5 h at room temperature. To obtain the cyanohydrins, the immobilized enzyme was removed by filtration and the remaining solution was concentrated. Reaction yields were determined by NMR, and optical purity (in percentage EE) was measured by GC.

RESULTS AND DISCUSSION

Identification of (*R*)-Vicianin

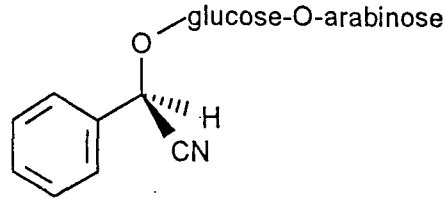
The cyanogenic glycoside, which occurs in high amounts in the leaves of *P. aureum*, was identified by mass spectroscopy and NMR spectroscopy as (*R*)-vicianin, which is the β-vicianoside of (*R*)-mandelonitrile, corresponding to the arabinoside of prunasin (Formula 1). In mass spectra, in addition to the molecule ion ^{426}(vicianin-H)$^+$, typical fragments were detectable, i.e. for negative mode: ^{131}(mandelonitrile-H)$^+$, ^{149}(Ara-H)$^+$, or ^{293}(prunasyl residue-H)$^+$.

The chemical shifts and assignments of ^{1}H- and ^{13}C-NMR analysis of the cyanogenic glycoside isolated from *P. aureum* are identical with those reported for vicianin isolated from the fern *Davallia trichomanoides* by Lizotte and Poulton (1985). These data were confirmed by correlated spectroscopy spectra.

PhaMDL Is a Multimer of 20-kD Subunits

The purification procedure comprises chromatofocusing, ion exchange, and gel-filtration chromatography (Table I). We found that MDL activity eluted in chromatofocusing, as well as in anion-exchange chromatography, as a single broad peak. This was probably due to the existence of various isoforms. The *A* profile of the last purification step (cation-exchange chromatography on Mono S) showed three well-separated maxima, but the fractions of this part of the gradient all possessed comparable specific MDL activities (Fig. 1). In re-chromatography experiments with distinct fractions we found no change in the elution profile (data not shown). These results suggest the presence of at least three isoforms of PhaMDL, designated as PhaMDL A, B, and C. Further details (degree of purification, yields at each step) of a typical enzyme purification of pooled isoenzymes are summarized in Table I. For experiments with purified isoenzymes, re-chromatographed material was used.

The apparent molecular mass of the native isoenzymes A and C, estimated by gel filtration on Superdex 200, was in both cases 168 ± 30 to 40 kD (95% confidence interval) (Fig. 2). All isoenzymes showed a single polypeptide band of approximately 20 kD in SDS-PAGE under reducing (Fig. 3) and nonreducing (data not shown) conditions. Therefore, it was concluded that MDL exists as a multimer of noncovalently associated 20-kD subunits. Isoenzyme A and C eluted as a single highly symmetric peak from the gel-filtration column, indicating that PhaMDL exists in a stable, multimeric state and not in an equilibrium of several unstable forms.



Formula 1

A spectra were determined for isoforms A (Fig. 4) and C (data not shown) of PhaMDL over wavelengths of 230 to 450 nm. Spectra were similar, with a common maximum at 278 nm due to aromatic amino acids. There were no absorption maxima at 389 and 463 nm, which would have been indicative of a flavin prosthetic group, suggesting that PhaMDL is a nonflavoprotein HNL.

Enzymatic Properties of PhaMDL

The temperature and pH dependence of MDL activity of isoforms A and C are similar. Both isoforms were found to be active over a broad range of temperature with maximal activity at 35 to 40°C. Because of the base-catalyzed decomposition of mandelonitrile, pH dependence was only determined below pH 7.5. Activity generally increased from pH 3.5 to 6.2 and leveled off between pH 6.2 and 7 for both isoforms. In all experiments, the spontaneous decomposition of mandelonitrile was determined separately and subtracted from the enzyme plus spontaneous catalyzed rate of benzaldehyde production.

Kinetics properties of PhaMDL were examined using mandelonitrile and purified isoenzymes. Principally we found no significant differences in substrate saturation curves of the three isoforms of PhaMDL (data not shown). As shown in Figure 5 for isoenzyme A, PhaMDL exhibits typical Michaelis-Menten kinetics over a broad range of substrate concentrations. A Lineweaver-Burk plot of the data gave a V_{max} of 60.1 μmol benzaldehyde mL^{-1} min^{-1} and a K_m value of 0.83 mM benzaldehyde. However, at very low substrate concentrations (<0.2 μM) we found a significant deviation from the calculated Michaelis-Menten kinetics (Fig. 5). This behavior can be explained by interaction between several binding sites of the enzyme, which occurs at low substrate concentrations during the binding of substrate.

The influences of various additives on the enzymatic activity of PhaMDL and PaMDL were compared. The flavoprotein HNL PaMDL was strongly inhibited in the presence of $AgNO_3$ (0.2 mM) and iodoacetamide (Table II). Both agents are capable of interacting with the sulfhydryl group of Cys, suggesting that a Cys is involved in catalysis of PaMDL. In contrast, PhaMDL was not affected by $AgNO_3$, and iodoacetamide treatment resulted in only modest inhibition. Likewise, the Ser-modifying agents DFP and PMSF showed a differential effect on enzymatic activity of the two MDLs. Whereas PaMDL was strongly inhibited by both agents, PhaMDL was only poorly or not at all affected by these inhibitors (Table II). However, PhaMDL as well as PaMDL were significantly inhibited by diethyl pyrocarbonate, a reagent capable of reacting with the imidazole group of His, indicating the involvement of this amino acid in catalysis by both MDLs. The other reagents tested, such as

Table I. *Purification of PhaMDL*

All values were determined for pools comprising all three isoenzymes.

Step	Total Protein	Total Activity	Specific Activity	Yield	Purification
	mg	*units*	*units/mg*	*%*	*-fold*
Crude extract	190	2,200	11.6	100	-
Q-Sepharose FF	20	2,100	105	95	9.1
Chromatofocusing	1.5	720	480	33	41
Gel filtration	0.2	600	3,000	27	260
Mono Q (pH 7.5)	0.12	600	5,000	27	430
Mono S (pH 3.9)	0.025	467	19,000	21	1,600

ions and reducing agents, did not affect the activities of the MDLs (Table II).

Use of MDL for the Synthesis of (*R*)-Cyanohydrins in Organic Media

In recent years, several groups have developed procedures for the synthesis of optically active cyanohydrins (Effenberger et al., 1987, 1990; Smitskamp-Wilms et al., 1991). Such cyanohydrins are important building blocks for the synthesis of α-hydroxy acids, α-hydroxy ketones, or β-ethanolamines. In addition to chemical methods, such as the enantio-selective addition of trimethylsilylcyanide to aldehydes in the presence of chiral catalysts (Dalton et al., 1991; Hayashi et al., 1993), the HNL-catalyzed addition in organic media is of special interest (Effenberger et al., 1987, 1990). The use of HNLs in organic media avoids the base-catalyzed racemic addition, which spontaneously occurs in aqueous systems, and allows the conversion of substrates that are poorly soluble in water (Effenberger et al., 1987, 1990).

MDLs from Rosaceae have been successfully used for the synthesis of a wide range of aromatic and heterocyclic (*R*)-cyanohydrins (Effenberger et al., 1987; Smitskamp-Wilms et al., 1991), whereas synthesis of aliphatic (*R*)-cyanohydrins with MDLs from Rosaceae is hampered by the reduced specific activity of these enzymes for the respective carbonyls (Smitskamp-Wilms et al., 1991). However, Albrecht et al. (1993) described the use of HNL from *L. usitatissimum* for these applications. Moreover, SbHNL has been used for the synthesis of (*S*)-cyanohydrins (Effenberger et al., 1990; Smitskamp-Wilms et al., 1991). However, this enzyme allows only the effective synthesis of aromatic (*S*)-cyanohydrins. The usefulness of MeHNL and HNL from *H. brasiliensis* (EC 4.1.2.37), which are (*S*)-HNLs, for the production of aliphatic (*S*)-cyanohydrins is under investigation by several groups (Klempier et al., 1993; Wajant et al., 1995).

To evaluate the biotechnological potency of PhaMDL, we have used immobilized enzyme to catalyze the formation of aromatic, heterocyclic, and aliphatic (*R*)-cyanohydrins (Table III) and compared it with the catalytic properties of PaMDL. Like PaMDL, PhaMDL preferentially accepted aromatic and heterocyclic carbonyls, but aliphatic carbonyls

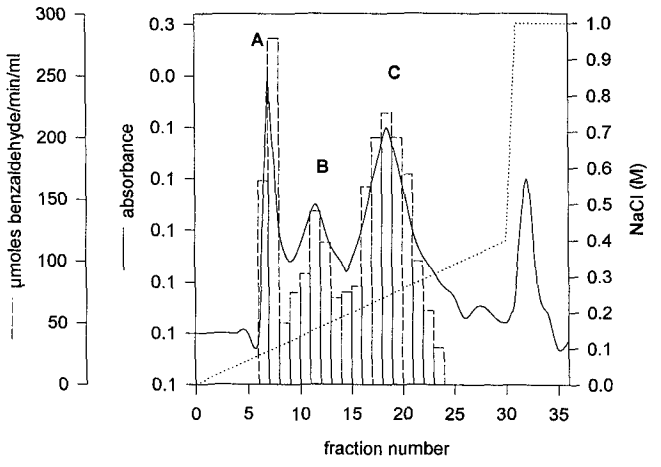


Figure 1. Cation-exchange chromatography of MDL isoenzymes. The MDL-containing fraction obtained after anion-exchange chromatography on Mono S was applied at a flow rate of 1 mL/min on a Mono S HR 5/5 (Pharmacia), equilibrated with 30 mM sodium citrate (pH 3.9). The adsorbed enzyme was eluted using a linear gradient from 0 to 0.4 M NaCl in 30 mM sodium citrate (pH 3.9) at a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected and assayed for MDL activity.

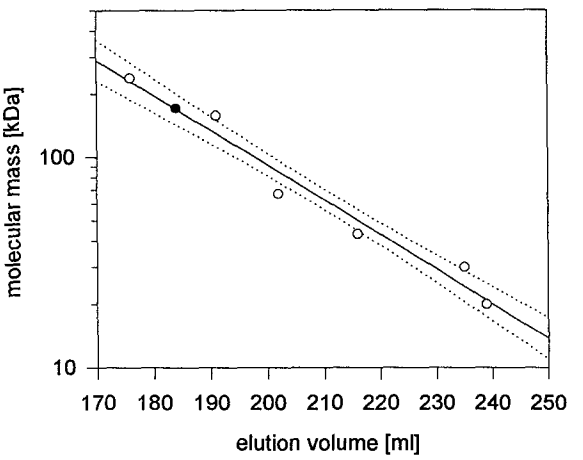


Figure 2. Estimation of native molecular mass of MDL A and C by gel-filtration chromatography on Superdex 200. Three milliliters of purified MDL A and C (10 µg/mL) were loaded onto a HiLoad 26/60 Superdex 200 preparative grade column (Pharmacia), equilibrated in 20 mM Tris buffer (pH 7.5) containing 400 mM NaCl at a flow rate of 0.4 mL/min. Molecular mass markers (○) used were catalase (232 kD), aldolase (158 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), and trypsin inhibitor (20.1 kD). Both isoenzymes, A and C, of MDL eluted in fractions corresponding to 168 kD (●). Dotted lines show the 95% confidence interval.

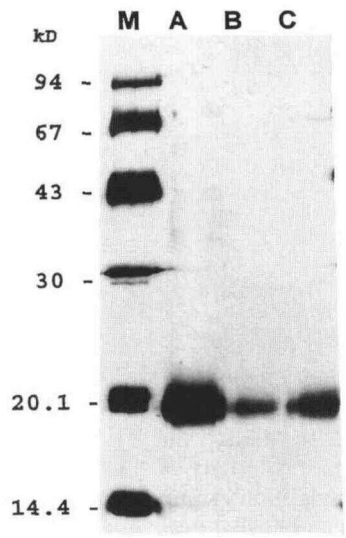


Figure 3. SDS-PAGE of MDL isoenzymes. Twenty microliters (20–60 ng) of peaks A, B, and C (Fig. 1) were reduced by treatment with DTT and separated by electrophoresis on a 17% acrylamide gel. Protein bands were visualized by silver staining. Molecular mass markers (lane M) used were phosphorylase B (94 kD, 64 ng), BSA (67 kD, 83 ng), ovalbumin (43 kD, 147 ng), carbonic anhydrase (30 kD, 83 ng), soybean trypsin inhibitor (20.1 kD, 80 ng), and lactalbumin (14.4 kD, 121 ng).

were not excluded. In contrast to PaMDL, 2-thiophen aldehyde was an efficiently converted substrate for PhaMDL, indicating that use of PhaMDL may be superior to PaMDL in selected applications.

Comparison of PhaMDL with Other HNLs

Molecular size and polypeptide arrangement are quite different from those of (*R*)-MDLs isolated from Rosaceae species as well as from those of (*S*)-MDL from *Ximenia americana* (Kuroki and Conn, 1989). Moreover, various inhibitors affected PhaMDL and PaMDL in a totally different manner (Table II). For PhaMDL, lack of inhibition by DFP, PMSF,

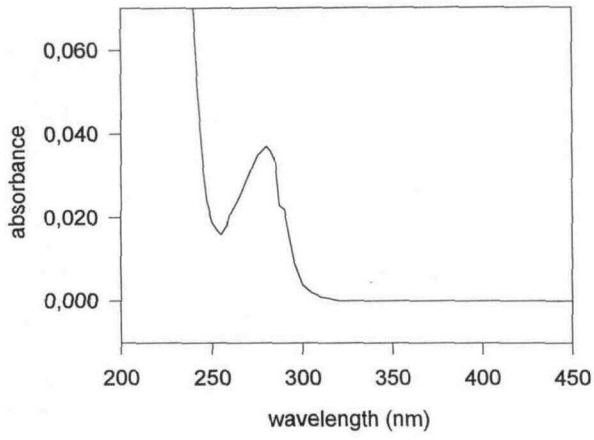


Figure 4. Absorption spectrum of purified PhaMDL. The spectrum was determined for PhaMDL isoenzyme A with a protein concentration of 40 μg/mL in 50 mM sodium acetate, pH 5.7.

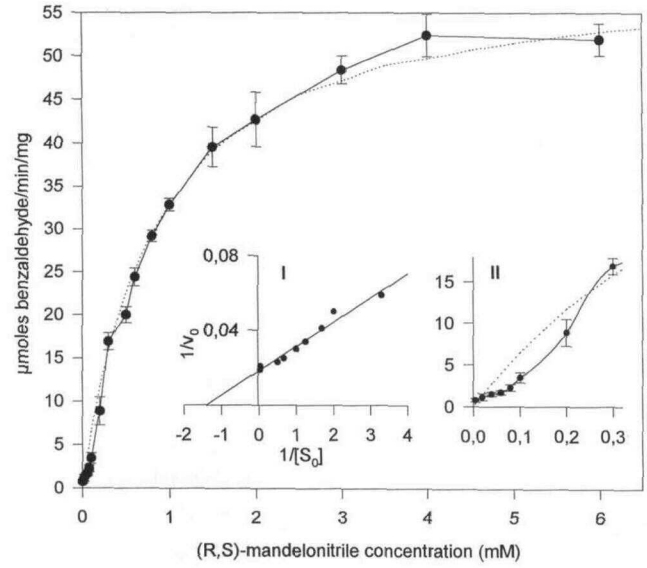


Figure 5. Response of MDL to increasing concentrations of (*R,S*)-mandelonitrile. Original data from triplicates of a typical experiment with fixed amounts of MDL isoform A. Rate values (μmol min^{-1} mg^{-1}) for the dissociation of (*R,S*)-mandelonitrile to benzaldehyde and HCN were plotted against substrate concentration. A Lineweaver-Burk plot of the data gave a V_{max} of 60.1 μmol benzaldehyde mL^{-1} min^{-1} and a K_m value of 0.83 mM benzaldehyde (coefficient of determination value 0.99) (inset I). Inset II, The deviation of experimentally determined rate values from the calculated Michaelis-Menten kinetics in the early interval of the curve (0–0.2 mM).

Table II. *Effects of various reagents on MDL activity*
Comparison of PaMDL and PhaMDL.

Reagent	Concentration	Inhibition	
		PaMDL	PhaMDL
	mM	%	
Iodoacetamide	10	93	30
	2	70	<5
PMSF	2	50	<5
	0.5	25	<5
DFP	2	80	10
	0.5	57	<5
Diethyl pyrocarbonate	2	79	60
	0.5	50	60
$AgNO_3$	1	95	<5
	0.2	85	<5
DTT	5	<5	<5
EDTA	5	<5	<5
EGTA	5	<5	<5
$CaCl_2$	1	<5	<5
$MgCl_2$	1	<5	<5
$MnCl_2$	1	<5	<5
$ZnCl_2$	1	<5	<5
$CuSO_4$	1	<5	<5

Table III. *MDL-catalyzed synthesis of (R)-cyanohydrins from carbonyls and HCN*

Comparison of PaMDL and PhaMDL. Reactions were carried out with immobilized enzyme in diisopropyl ether for 1 to 4.5 h at room temperature. Yields were determined by NMR, and EE of (*R*)-cyanohydrins was measured by GC analysis of *O*-acetyl derivatives. n.d., Not determined.

Carbonyl	Yield		EE	
	PhaMDL	PaMDL	PhaMDL	PaMDL
	%		%	
Benzaldehyde	82	99	99	99
Butanale	42	100	55	90
Thiophen-2-aldehyde	92	48	99	87
2-Pentanone	17	78	n.d.	n.d.

iodoacetamide, and $AgNO_3$ suggest that there is no Cys or Ser involved in catalysis. In contrast, PaMDL was inhibited by all of these compounds, indicating the involvement of Ser and/or Cys in the catalytic mechanism of this enzyme. The inhibition of enzymatic activity by Ser-modifying agents has also been described for SbHNL (Wajant et al., 1994) and MeHNL (Wajant et al., 1995). Therefore, it seems possible that PhaMDL, in contrast to PaMDL, uses a catalytic mechanism different from other HNLs. Furthermore, in opposition to MDLs from Rosaceae, PhaMDL contains no FAD. Differences in the kinetics properties of both enzymes were found as well. We found for PhaMDL a K_m value of 0.83 mM benzaldehyde and deviations from Michaelis-Menten kinetics at low substrate concentrations, whereas for MDLs from Rosaceae, Michaelis-Menten kinetics have been described with a K_m value of 94 μM benzaldehyde (Xu et al., 1986). Such striking differences between HNLs possessing the same natural substrate are also found for acetone cyanohydrin lyases from *L. usitatissimum* and *M. esculenta*. These HNLs differ in subunit composition, molecular size, and stereoselectivity for chiral substrates (Xu et al., 1988; Albrecht et al., 1993; Wajant et al., 1995). Taking into account the lack of sequence homologies among the three cloned HNLs, so far, and the biochemical heterogeneity described above and elsewhere (Wajant et al., 1994, 1995), we believe that convergent evolution of HNL is probable. According to this idea, several species of plants have recruited distinct enzymes (proteases) during evolution to adopt them for the same function: the accelerated release of HCN from cyanohydrins.

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