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

Harald Wajant*, Siegfried Förster, Dirk Selmar, Franz Effenberger, and Klaus Pfizenmaier

Institut für Zellbiologie und Immunologie der Universität Stuttgart, Allmandring 31 (H.W., K.P.), and Institut für Organische Chemie der Universität Stuttgart, Pfaffenwaldring 55 (S.F., F.E.), 70569 Stuttgart, Germany; and Botanisches Institut der Technischen Universität Braunschweig, Mendelsohnstrasse 4, 38092 Braunschweig, Germany (D.S.)

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 catalysis of the aglycon (R)-mandelonitrile was purified to apparent homogeneity. The purified holoenzyme is a homomultimer with subunits of M, = 20,000. At least three isomers 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 p-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; 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. usitatissimum is a homodimer of 82 kDa 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 kDa 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 between 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 ancestral 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. usitatissimum is a homodimer of 82 kDa 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 kDa 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).

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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 amygdalus; PhaMDL, MDL from Phlebodium aureum; SbHNL, HNL from Sorghum bicolor (S)-hydroxy-MDL.

* This work was supported by Bundesministerium für Forschung und Technologie, Germany, grant No. A03U-ZSP Stuttgart and Deutsche Forschungsgemeinschaft grant No. WA 1025/1-1.

* Corresponding author; fax 49–711–685-7484.
between 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 Filicaeae. 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 lyophilized.

**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 hydroxynitrites 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. 1H- and 13C-NMR spectra were recorded at ambient temperature with a Bruker (Bremen, Germany) 300 NMR-spectrometer in DMSO as solvent. For higher resolution, two-dimensional 1H-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 A249 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 5.5) containing 0.084 mM Prunasin from *P. serotina*, 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 hydroxynitrites 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.

**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 40,000g 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 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 A280 reached the initial baseline. 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 H2O 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-C1 (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 5.0. Bound proteins were eluted with 20 mM Tris-C1 buffer (pH 7.5) at a flow rate of 0.4 mL/min. Fractions of 5 mL were collected 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-C1 buffer (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-C1 (pH 7.5). Protein was eluted in a 20-mL linear gradient of 0 to 0.4 M NaCl in 20 mM Tris-C1 (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-C1 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-C1 (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 Vmax was obtained from a Line-weaver-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-C1 (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.,
broad peak. This was probably due to the existence of both cases 168 approximately 20 kD in SDS-PAGE under reducing (Fig. 3) and three well-separated maxima, but the fractions of this part ion exchange, and gel-filtration chromatography (Table I). We found that MDL activity eluted in chromatofocusing, as P. aureum purified isoenzymes, re-chromatographed material was designated as PhaMDL 1s a Multimer of 20-kD Subunits addition to the molecule ion 426(vicianin-H)⁺, typical frag-
ments were detectable, i.e. for negative mode: 131(mande-
lonitrile-H)⁺, 149(Ara-H)⁺, or 290(prunasin residue-H)⁺.

The chemical shifts and assignments of ¹H- and ¹³C-
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.

**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 290(prunasin residue-H)⁺.

As shown in Figure 5 for isoenzyme A, PhaMDL exhibited typical Michaelis-Menten kinetics over a broad range of substrate concentrations. A Lineweaver-Burk plot of the data gave a Vₘₐₓ of 60.1 μmol benzaldehyde mL⁻¹ min⁻¹ and a Kᵥ 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.

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 Vₘₐₓ of 60.1 μmol benzaldehyde mL⁻¹ min⁻¹ and a Kᵥ 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 AgNO₃ (0.2 mM) and iodoacetamide (Table II). Both agents are capable of interacting with the sulfhydryl group of Cys, suggesting that Cys is involved in catalysis of PaMDL. In contrast, PhaMDL was not affected by AgNO₃ 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 1. Purification of PhaMDL

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
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<tbody>
<tr>
<td></td>
<td>mg</td>
<td>units</td>
<td>units/mg</td>
<td></td>
<td>- fold</td>
</tr>
<tr>
<td>Crude extract</td>
<td>190</td>
<td>2,200</td>
<td>11.6</td>
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<tr>
<td>Q-Sepharose FF</td>
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<td>2,100</td>
<td>105</td>
<td>95</td>
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<tr>
<td>Chromatofocusing</td>
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<td>720</td>
<td>480</td>
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<td>41</td>
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<tr>
<td>Gel filtration</td>
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<td>600</td>
<td>3,000</td>
<td>27</td>
<td>260</td>
</tr>
<tr>
<td>Mono Q (pH 7.5)</td>
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<td>600</td>
<td>5,000</td>
<td>27</td>
<td>430</td>
</tr>
<tr>
<td>Mono S (pH 3.9)</td>
<td>0.025</td>
<td>467</td>
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<td>21</td>
<td>1,600</td>
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</table>

All values were determined for pools comprising all three isoenzymes.

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 trimethylsilyl cyanide 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...
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,

Table II. Effects of various reagents on MDL activity

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Inhibition</th>
<th>PaMDL</th>
<th>PhaMDL</th>
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<td>Iodoacetamide</td>
<td>10</td>
<td>93</td>
<td>30</td>
<td>&lt;5</td>
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<td></td>
<td>2</td>
<td>70</td>
<td>&lt;5</td>
<td>&lt;5</td>
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<tr>
<td>PMSF</td>
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<td>DFP</td>
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<tr>
<td></td>
<td>2</td>
<td>80</td>
<td>10</td>
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</tr>
<tr>
<td>Diethyl pyrocarbonate</td>
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<td>79</td>
<td>60</td>
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<td></td>
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<td>AgNO₃</td>
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<td>95</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td></td>
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<td>85</td>
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<tr>
<td>MnCl₂</td>
<td>1</td>
<td>&lt;5&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>1</td>
<td>&lt;5&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>
iodoacetamide, and AgNO₃ 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 Kₘ 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 Kₘ 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.

ACKNOWLEDGMENTS

We thank Dr. Victor Wray (Geellschaft für Biochemische Forschung, Braunschweig, Germany) for recording NMR spectra and Dr. Hans Martin Schiebel (Organisches Institut, Technische Universität Braunschweig, Germany) for performing the mass spectrometric analysis.

Table III. MDL-catalyzed synthesis of (R)-cyano-hydrins from carbonyls and HCN

<table>
<thead>
<tr>
<th>Carbonyl</th>
<th>Yield %</th>
<th>PaMDL</th>
<th>PaMDL</th>
<th>PhaMDL</th>
<th>PhaMDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzaldehyde</td>
<td>82</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Butanale</td>
<td>42</td>
<td>100</td>
<td>55</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Thiophen-2-aldehyde</td>
<td>92</td>
<td>48</td>
<td>99</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>2-Pentanone</td>
<td>17</td>
<td>78</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

Received June 5, 1995; accepted October 10, 1995.

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LITERATURE CITED


Wajant H, Förster S, Böttiger H, Effenberger F, Pfizenmaier K (1995) Acetone cyanohydrin lyase from Manihot esculenta (Cas-
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