Mandelonitrile lyase from *Ximenia americana* L.: Stereospecificity and lack of flavin prosthetic group

(cyanogenesis/cyanohydrin)

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ABSTRACT A mandelonitrile lyase (EC 4.1.2.10) that catalyzes the dissociation of (S)-(−)-mandelionitrile to benzaldehyde and hydrogen cyanide has been purified to apparent homogeneity from leaves of *Ximenia americana* L. (Olacaceae). The lyase was purified 122-fold with 38% yield by chromatography on carboxymethyl-cellulose and chromatofocusing. The enzyme had a pH optimum of 5.5, with a $K_m$ value of 280 μM. Activity toward 4-hydroxy-(R,S)-mandelonitrile was 77% of that observed with the endogenous substrate; no activity was observed toward the aliphatic substrate acetone cyanhydrin. The enzyme was stable at 4°C and at room temperature for at least 1 month. Native and subunit molecular weights of 38,000 and 36,500, respectively, suggest the enzyme is a monomer. The isoelectric point was pH 3.9 as determined by isoelectric focusing. Staining with periodic acid–Schiff and fluorescein-labeled concanavalin A reagents indicate this enzyme is a glycoprotein. In contrast to (R)-mandelonitrile lyases isolated from *Prunus* species, the *Ximenia* lyase does not appear to be a flavoprotein. A second enzyme that eluted from the chromatofocusing column at pH 4.0 was also active toward mandelionitrile. However, this form accounted for less than 10% of the total activity, and its specific activity was only 6% of that of the major component. Additional physical and kinetic studies suggested this activity may be due to a nonspecific enzyme that is active toward mandelionitrile.

(R)-(−)-Mandelonitrile, produced by hydrolysis of cyanogenic glucosides that occur in members of the family Rosaceae, is reversibly dissociated to benzaldehyde and HCN by the enzyme mandelionitrile lyase, an α-hydroxynitrilase lyase (EC 4.1.2.10) (1). Pfeil and associates (2–4) reported that these enzymes, when isolated from the genus *Prunus*, were flavoproteins and speculated on the role of the flavin cofactor in a reaction not involving oxidation/reduction. Other workers (5–8) have examined the role of the flavin moiety and this has been the subject of considerable debate. Although the (R)-(−)-mandelionitrile lyases of rosaceous species appear to be flavoproteins (1), the analogous enzyme in *Sorghum bicolor* that utilizes 4-hydroxy-(S)-mandelionitrile as a substrate does not contain a flavin cofactor (9). Moreover, lyases in cassava (10) and flax (11), which utilize the aliphatic substrate acetone cyanhydrin, do not appear to be flavoproteins. Thus the (R)+(−)-mandelionitrile lyase of rosaceous species is atypical in its requirement for a flavin cofactor.

The occurrence of the glucoside of (S)-(−)-mandelionitrile in *Ximenia americana* (family Olacaceae) (12) provided an opportunity to examine whether the lyase that acts on the enantiomer of (R)+(−)-mandelionitrile is a flavoprotein. Accordingly the α-hydroxynitrilase lyase of *X. americana* has been purified to apparent electrophoretic homogeneity and its properties have been examined.

MATERIALS AND METHODS

Materials. Leaves of *X. americana* were collected at Fort Desoto Park (Saint Petersburg, FL) and shipped to Davis, California within 24 hr of harvesting. Samples employed in cyanide analysis were lyophilized prior to shipment to Davis. CM-cellulose was purchased from Whatman. Gel filtration media, Polybuffer 74, and Polybuffer Exchanger were obtained from Pharmacia. (R,S)-Mandelonitrile, almond emulsion, fluorescein isothiocyanate-labeled concanavalin A, Schiff’s reagent, Amberlite XAD-4, gel filtration, and SDS/PAGE protein standards were purchased from Sigma. Isoelectric focusing (IEF) PAGplates were obtained from LKB. Acrylamide, N,N'-methylenebisacrylamide, SDS, and N,N',N''-tetramethylmethylenediamine (TEMED) were purchased from Bio-Rad. Thiol reagents, metal salts, and metabolites were from our laboratory collection.

Cyanide Analysis. Cyanide determinations were performed as described (13).

Enzyme Purification. Leaves of *X. americana* (100 g) were homogenized in an ice-cold blender by addition of 2 vol of cold (−20°C) acetone, followed by filtration through a Buchner funnel. This procedure was repeated twice and the final powder was stored at −20°C. The following steps were carried out at 4°C. Protein was extracted by adding acetone powder (30 g) to 35 ml of 0.1 M Mes-KOH (pH 6.3) containing 25% (vol/vol) glycerol and 3 g of Amberlite XAD-4. The slurry was filtered through two layers of cheesecloth and centrifuged for 20 min at 17,000 × g. Subsequent procedures were carried out at room temperature. The supernatant was decanted and chromatographed on a Sephadex G-25 column (2.5 × 41 cm) equilibrated with 50 mM Mes-KOH (pH 6.0) containing 25% glycerol. Fractions (7.0 ml) were monitored for protein by absorbance at 280 nm. Those containing protein were pooled and chromatographed on a CM-cellulose column (1.5 × 5 cm) equilibrated with 50 mM Mes-KOH (pH 6.0) containing 25% glycerol. Fractions (5 ml) were assayed for lyase activity. The active fractions were pooled and dialyzed overnight against 4 liters of 25 mM histidine-HCl (pH 6.2). The dialyzed protein preparation was chromatographed on Polybuffer Exchanger 94 (1.2 × 56 cm), equilibrated with 25 mM histidine hydrochloride (pH 6.2). Material was eluted in Polybuffer 74, pH 3.8. Fractions (6.0 ml) were collected and assayed for lyase activity. Active fractions were pooled and concentrated to 1 ml by ultrafiltration and stored at 4°C.

Mandelonitrile Lyase Assays. Lyase assays were performed by monitoring the decomposition of (R,S)-mandelonitrile. The amount of benzaldehyde produced was measured by

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Abbreviation: IEF, isoelectric focusing.

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Table 1. Purification of mandelonitrile lyase from X. americana

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume, ml</th>
<th>Protein, mg</th>
<th>Activity, units</th>
<th>Specific activity, units/mg</th>
<th>Purification factor</th>
<th>Yield, %</th>
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</thead>
<tbody>
<tr>
<td>Sephadex G-25</td>
<td>89</td>
<td>48.1</td>
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<tr>
<td>CM-cellulose</td>
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<td>1.9</td>
<td>137</td>
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<td>77</td>
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<td>Chromatofocusing</td>
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<tr>
<td>Lyase 1</td>
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<td>5.8</td>
<td>28</td>
<td>8</td>
<td>3</td>
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<tr>
<td>Lyase 2</td>
<td>45</td>
<td>0.15</td>
<td>68</td>
<td>453</td>
<td>122</td>
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<td></td>
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<td>CM, carboxymethyl.</td>
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</table>

CM, carboxymethyl.

**RESULTS AND DISCUSSION**

**Cyanide Analysis.** The cyanide content of leaves of X. americana was determined to be 52 μmol of HCN per g (dry weight). This compares favorably with the value [115 μmol of HCN per g (dry weight)] found in the first report of cyanogenesis in this species (12).

**Purification of Mandelonitrile Lyase.** Attempts to extract mandelonitrile lyase from leaves of X. americana proved to be difficult due to a high concentration of phenolic compounds. Homogenization with aqueous buffers containing a variety of phenolic adsorbents and antioxidants consistently resulted in extremely low yields. However, it was possible to obtain highly active extracts from acetone powders. The inclusion of 25% glycerol in extraction and CM-cellulose buffers was also critical in maintaining enzyme activity, but the presence of glycerol resulted in reduced flow rates at 4°C. Therefore, the purification was carried out at room temperature. Stability studies demonstrated the lyase activities to be stable for up to 1 month at room temperature. Such thermostability has also been observed for the Sorghum lyase (9). Upon application of the desalted Ximenia homogenate to a CM-cellulose column, the lyase activity did not bind and was eluted in the wash fraction (data not shown). This procedure resulted in 19-fold purification with 77% recovery of enzyme activity (Table 1). Subsequent purification by chromatofocusing resulted in the resolution of two lyase activities (Fig. 1). These enzymes eluted in peak fractions having pH values at 4.0 and 3.9 and were designated peaks 1 and 2, respectively. More than 90% of the activity was associated with the latter peak. This simple two-step protocol resulted in a 122-fold purification with a 38% yield for lyase 2 (Table 1). Analysis of lyase 2 by analytical IEF revealed a single protein band at pH 3.9. Assay of the gel yielded a lyase activity peak corresponding to this pH value (Fig. 2). The resolution of two forms of lyase was not unexpected since multiple forms of α-hydroxynitrile lyase have been reported (4, 10, 20, 21). However, the Ximenia lyases demonstrated significantly
different physical and kinetic properties. These data suggested that lyase 1 activity may be due to a nonspecific enzyme that is not involved with cyanogenesis in vivo. The origin and detailed characterization of lyase 1 was not investigated since it comprised less than 10% of the total lyase activity.

Physical Characterization. It is well established that the lyase in rosaceous species is a flavoprotein (1, 4, 20–22); these species contain the glycoside of (R)-(−)-mandelonitrile, called (R)-prunasin (23). A primary goal of this study was to examine whether the lyase of X. americana is also a flavoprotein since this species contains the glucoside of (S)-(−)-mandelonitrile, known as (S)-sambunigrin (12). Prosthetic group analysis failed to detect a flavin moiety. While the absorption spectra for both enzymes showed a maximum at 280 nm, there were no absorption maxima at 380–390 and 460–480 nm, characteristic of the flavoprotein lyases from Rosaceae. The A$_{275}$/A$_{480}$ values for peaks 1 and 2 were 63 and 47, respectively. Furthermore, the intense yellow color, which is characteristic of the flavoprotein lyases from Prunus species (20), was not observed during purification. The data suggest lyase 2 from X. americana does not contain a flavin prosthetic group. Other lyases shown to be devoid of flavin have been isolated from S. bicolor (9), Manihot esculenta (10), and Linum usitatissimum (11). The flax enzyme had a A$_{275}$/A$_{480}$ value in excess of 40 (11). By contrast, the flavoproteins from almond (2) and black cherry (20) seeds exhibited distinct absorbance maxima at 380–390 nm and 460–480 nm. The A$_{275}$/A$_{480}$ value for almonds was reported to be 12.5 (2). Since S. bicolor and X. americana both contain cyanogenic aglycones [(S)-dhurrin in S. bicolor], the flavin moiety does not appear to be associated with the aromaticity of the substrate. It is possible that the flavin prosthetic group is involved in determining the stereospecificity between (R)- and (S)-epimers of mandelonitrile. It would be informative to examine the lyase that acts on 4-hydroxy-(R)-mandelonitrile, the aglycone produced on hydrolysis of the epimer of (S)-dhurrin known as (R)-taxiphyllin (23).

Native molecular weight values for proteins 1 and 2 were 108,000 and 38,000, respectively (data not shown). Subunit molecular weights, as determined by SDS/PAGE, were 29,000 and 36,500, respectively (data not shown). These data suggest that the enzyme in peak 1 exists as a tetramer in native conformation, whereas the major lyase exists as a monomer. The possibility that lyase 1 is derived by partial proteolysis of lyase 2, followed by aggregation cannot be ruled out. Such modifications could account for the observed differences in kinetic properties, as discussed below.

In common with other α-hydroxynitrile lyases (4, 20–22), the X. americana lyase 2 appeared to be a glycoprotein. After SDS/PAGE and subsequent staining with fluorescein isothiocyanate-labeled concanavalin A, the observed fluorescent band had an $R_f$ value corresponding to lyase 2 in silver-stained gels (data not shown). Identical results were obtained by staining with Schiff’s reagent (data not shown). Lyase 2 had a pI value of pH 3.9 as determined by IEF and chromatofocusing. Acidic pI values are characteristic of α-hydroxynitrile lyases (1).

Cations appear not to be required for lyase activity. This was indicated by the observation that lyase 2 was not affected by the metal chelators EDTA (5 mM), α-phenanthonline (0.1 mM), and α,α′-dipyridyl (0.1 mM) (data not shown). Assays performed in the presence of a variety of metal salts [ZnCl$_2$, MnCl$_2$, MgCl$_2$, CuSO$_4$, HgCl$_2$, AgNO$_3$, FeCl$_3$, and Pb(NO$_3$)$_2$; 0.1 mM, final concentration] supported the lack of a metal-ion requirement. However, as reported for other lyases, the Ximenia lyase 2 was inhibited by AgNO$_3$ (50% inhibition at 0.1 mM). This enzyme also exhibited 27% inhibition by reduced glutathione (10 mM, final concentration) and p-chloromercuriphenylsulfonic acid (1 mM, final concentration). These data are consistent with the proposal that a cysteine residue participates in the reaction mechanism of the almond lyase (7).

Kinetic Characterization. Although lyases 1 and 2 shared similar pH optima of 5.5 in 100 mM Mes-KOH, they possessed significantly different $K_m$ values. Lyase 1 had an apparent $K_m$ value of 11 mM and exhibited substrate inhibition (data not shown). However, it was difficult to obtain accurate results at high substrate concentrations due to the nonenzymic breakdown of (R,S)-mandelonitrile (22). By contrast, lyase 2 demonstrated normal hyperbolic kinetics (data not shown), and the $K_m$ value of 280 μM was almost 40 times lower than the $K_m$ observed for lyase 1. Furthermore, the specific activity of lyase 1 was only 6% of that determined for lyase 2 (Table 1).

Recent studies have established the narrow substrate specificity of most β-glycosidases involved in cyanogenesis, particularly with respect to the aglycone moiety (24). This specificity appears to extend to the lyases as well. Studies of substrate specificity revealed lyase 2 to be highly specific for the aromatic ring portion of mandelonitrile. This enzyme showed no activity toward the aliphatic substrate acetone cyanohydrin. Conversely, the flax lyase was active only toward aliphatic cyanohydrins and showed no activity toward (R,S)-mandelonitrile or 4-hydroxy-(R,S)-mandelonitrile (11). Activity of lyase 2 toward 4-hydroxy-(R,S)-mandelonitrile was 77% of the activity observed with the endogenous substrate.

The mandelonitrile employed in these assays necessarily exists as a racemic mixture of (R)- and (S)-enantiomers due to rapid racemization of either enantiomer in aqueous solutions. When 50 μmol of the racemic substrate was added to Ximenia lyase 2 and the reaction was allowed to go to completion, only 25.0 μmol of benzaldehyde was produced. This corresponds to 50% of the original substrate. Subsequent addition of lyase from almond emulsin resulted in the release of 24 μmol of benzaldehyde or an additional 48% of the initial substrate. Although we did not establish which enantiomer was being catabolized by which enzyme, these data clearly indicate extreme specificity with respect to the chirality center of the α-hydroxynitrile. The ability of α-hydroxynitrile lyases to discriminate between the two epimers in a racemic mixture was first described for the Sorghum lyase (9). In the present study we employed almond lyase to further substantiate the stereospecificity of these enzymes.
The *Ximenia* lyase appears to be unique in that among nonflavoprotein lyases it is a glycoprotein (10, 11). The molecular weight and $K_m$ values observed for the *Ximenia* lyase also support the observation of heterogeneity among lyases from different species (2, 22). Indeed, polyclonal antibodies raised against purified lyase from *Prunus lyonii* failed to cross react with lyase from *X. americana* (unpublished data).

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