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Purification and Characterization of A Novel (*R*)-Hydroxynitrile Lyase from *Eriobotrya japonica* (Loquat)

Techawaree UEATRONGCHIT,[1] Ai KAYO,[2] Hidenobu KOMEDA,[2]
Yasuhisa ASANO,[2,†] and Aran H-KITTIKUN[1,†]

[1]*Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*
[2]*Biotechnology Research Center, Toyama Prefectural University, Imizu, Toyama 939-0398, Japan*

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A hydroxynitrile lyase was isolated and purified to homogeneity from seeds of *Eriobotrya japonica* (loquat). The final yield, of 36% with 49-fold purification, was obtained by 30–80% $(NH_4)_2SO_4$ fractionation and column chromatography on DEAE-Toyopearl and Concanavalin A Sepharose 4B, which suggested the presence of a carbohydrate side chain. The purified enzyme was a monomer with a molecular mass of 72 kDa as determined by gel filtration, and 62.3 kDa as determined by SDS-gel electrophoresis. The N-terminal sequence is reported. The enzyme was a flavoprotein containing FAD as a prosthetic group, and it exhibited a K_m of 161 μM and a k_{cat}/K_m of 348 s^{-1} mM^{-1} for mandelonitrile. The optimum pH and temperature were pH 5.5 and 40 °C respectively. The enzyme showed excellent stability with regard to pH and temperature. Metal ions were not required for its activity, while activity was significantly inhibited by $CuSO_4$, $HgCl_2$, $AgNO_3$, $FeCl_3$, β-mercaptoethanol, iodoacetic acid, phenylmethylsulfonylfluoride, and diethylpyrocarbonate. The specificity constant (k_{cat}/K_m) of the enzyme was investigated for the first time using various aldehydes as substrates. The enzyme was active toward aromatic and aliphatic aldehydes, and showed a preference for smaller substrates over bulky one.

Key words: hydroxynitrile lyase; mandelonitrile lyase; *Eriobotrya japonica*; flavoprotein; cyanohydrins

Hydroxynitrile lyase (HNL) is one of the enzymes involved in the catabolism of cyanogenic glycosides in higher plants. This enzyme catalyzes the decomposition of cyanohydrins into the corresponding aldehydes or ketones and HCN for plant defense against predators and microorganisms.[1)] The reverse reaction of HNL, synthesis of chiral cyanohydrins, has attracted the attention of scientists and industry. Chiral cyanohydrins, alcohols containing a cyano group attached to the same carbon atom, are versatile building blocks in the synthesis of large numbers of biologically active compounds in fine chemicals, pharmaceuticals, veterinary products, crop-protecting agents, vitamins, and food additives.[2)] HNL activity was detected for the first time in kernels of *Prunus dulcis (amygdalus)* or almond by Friedrich Wöhler in 1837, cleaving the cyanohydrins to aldehyde and HCN.[3)] The synthesis of chiral cyanohydrins by HNL from almond was first reported by Rosenthaler.[4)]

Recently, we developed a new screening method using chiral HPLC to determine the activity and stereoselectivity of HNL.[5)] We discovered several novel sources of HNL among the 163 plant species in 74 families examined. (*S*)-HNL activity was found in a homogenate of the leaves of *Baliospermum montanum*, while (*R*)-HNLs were detected in homogenates of the leaves and seeds of *Passiflora edulis*, and the seeds of *Eriobotrya japonica*, *Chaenomles sinensis*, *Sorbus aucuparia*, *Prunus mume*, and *Prunus persica*.

Eriobotrya japonica (Thunb.) Lindley, also known as Japanese medlar, is an evergreen tree of the Maloideae subfamily of the Rosaceae family that is native to southeastern China, long since introduced to Japan, hence its name. It is cultivated as well in tropical and subtropical countries.[6)] Its golden fruit, the loquat, is round or oval in shape and has a sweet taste. The fruit is eaten fresh or is made into preserves, jam, jelly, juice, and nectar after removal of the seeds as waste.[7)] The seeds of *Eriobotrya* L. have been reported to be a source of (*R*)-hydroxynitrile lyase for the synthesis of cyanohydrins,[8)] but research on the enzymatic synthesis of chiral cyanohydrins synthesis has been carried out mostly using an excess amount of crude enzyme and a long reaction time to obtain high enantiomeric excess (*e.e.*) and conversion. Crude enzyme or seed powder has

† To whom correspondence should be addressed. Yasuhisa ASANO, Fax: +81-766-56-2498; Tel: +81-766-56-7500 ext 530; E-mail: asano@pu-toyama.ac.jp; Aran H-KITTIKUN, Fax: +66-7421-2889; Tel: +66-7428-6363; E-mail: aran.h@psu.ac.th
Abbreviations: HNL, hydroxynitrile lyase; *Ej*HNL, hydroxynitrile lyase from *Eriobotrya japonica*

been used directly for the reaction without purification and characterization of the enzyme.[8–10] In the case of HNL from *E. japonica* (*Ej*HNL), lack of information and understanding of the characteristics of purified enzyme have led to limited enzyme applications.

To understand the characteristics of *Ej*HNL, we attempted the isolation, purification, and characterization of HNL from seeds of *E. japonica* and its application in the synthesis of cyanohydrins.

Materials and Methods

Materials. Seeds of *E. japonica* were purchased from the National Federation of Agricultural Cooperative Associations (Nagasaki, Japan) and stored at 4 °C. All chemicals used in the experiments were purchased from commercial sources and were used without further purification. Silica gel TLC plates (Merck, NJ) were used to follow the chemical reaction. Silica gel 100–200 mesh (Wako Pure Chemical Industries, Osaka) was used in column chromatography. NMR spectra were recorded with a JEOL LA-400 spectrometer (Tokyo) at 25 °C in $CDCl_3$ using TMS as the internal standard. Chemical shifts were shown as δ_H and δ_C for ^1H and ^{13}C NMR spectra respectively. Chiral HPLC was performed using a chiralcel OJ-H column (Diacel Chemical Industries, Osaka) with a SPD-10A VP UV-vis detector (Shimadzu, Kyoto) at 254 nm. The eluting solvent was 90% hexane and 10% isopropanol by volume. A chiral GC analysis was performed using a Gas Chromatograph GC-14B (Shimadzu) with a fused silica capillary β-Dex-325 column (Supelco, PA) and He as a carrier gas (Detector temperature, 230 °C; injection temperature, 220 °C).

Crude enzyme extraction. Seeds of *Eriobotrya japonica* were sterilized by soaking in 0.1% (v/v) sodium hypochlorite and rinsed with de-ionized water. All purification steps were carried out at 4 °C. Sterilized seeds were homogenized with 3% polyvinylpyrolidone in 10 mM potassium phosphate buffer, pH 6.0 (100 ml/100 g of fresh seeds) in a SMT Process Homogenizer, PH91 (SMT, Tokyo). The homogenate was filtered through four layers of cheesecloth and centrifuged at $28{,}000 \times g$ for 30 min. The supernatant was used as the crude enzyme extract.

Ammonium sulfate precipitation. The crude extract was fractionated by 0–30, 30–80% saturation of ammonium sulfate. The precipitate fractionated at 30–80% was collected by centrifugation at $28{,}000 \times g$ for 30 min. The precipitate was dissolved and dialyzed overnight against an excess volume of the same buffer. Precipitate formed during dialysis was removed by centrifugation, and the supernatant was collected.

DEAE-Toyopearl 650M column chromatography. The enzyme solution was loaded onto a DEAE-Toyopearl 650M column (26 mm × 10.5 cm, 50 ml) equilibrated with 10 mM potassium phosphate buffer, pH 6.0, and eluted with a linear gradient of NaCl (0–0.5 M). Fractions of 2 ml were collected. The protein profile was monitored by measuring absorbance at 280 nm, the protein fractions were assayed for HNL activity, and the active fractions were pooled for further analysis.

Concanavalin A Sepharose 4B column chromatography. The enzyme solution was purified on a Concanavalin A Sepharose 4B column (5 mm × 5 cm, 1 ml) equilibrated with 0.5 M NaCl in 10 mM potassium phosphate buffer, pH 6.0, and eluted with linear gradient of 0–1 M of α-D-methylglucoside. Fractions of 1 ml were collected. The protein profile was monitored by measuring the absorbance at 280 nm, and the protein fractions were assayed for HNL activity.

Activity assay. HNL activity was measured by monitoring the decomposition of (*R,S*)-mandelonitrile to benzaldehyde by the method described by Willeman,[11] with a slight modification. The conversion of (*R,S*)-mandelonitrile to benzaldehyde was followed by continuously measuring the increase in absorbance at 280 nm. The reaction was performed in a quartz cell. The enzyme solution was added to a 50 mM sodium citrate phosphate buffer, pH 5.5, containing 2 mM mandelonitrile in a total volume of 1 ml. Then the reaction mixture was mixed gently, and the reaction was followed for 5 min by spectrophotometer at 280 nm (U-3210 spectrophotometer, Hitachi, Tokyo). The linear change in absorbance for the initial 1 min was used in the calculation. The slope of absorbance was determined and subtracted with a blank. By $\varepsilon_{280} = 1.4\,\text{mM}^{-1}\,\text{cm}^{-1}$, the enzyme activity was calculated.

One unit of HNL activity (1 decomposition unit) was defined as the amount of enzyme that converted 1 μmol of mandelonitrile to benzaldehyde in 1 min under standard assay conditions. In a previous study of *Ej*HNL by our group, 1 unit of HNL activity (1 synthetic unit) was defined as the amount of enzyme that produced 1 μmol of optically active mandelonitrile from benzaldehyde per min under the described assay conditions.[5] The decomposition activity of *Ej*HNL was about 1.3 times more than the synthetic activity.

Protein assay. Protein concentrations were measured using a Bio-Rad protein assay kit with BSA as a standard (Bio-Rad Laboratories, Hercules, CA).[12]

Gel electrophoresis. SDS–PAGE and Native PAGE were used to analyze the molecular mass and homogeneity of the enzyme respectively. Gel electrophoresis was performed with a 1-mm thick polyacrylamide gel.[13] Standard protein markers for SDS–PAGE consisted of phosphorylase *b* (97,400 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da), and lysozyme (14,400 Da).

Estimation of apparent molecular mass. The molecular mass of *Ej*HNL was estimated by gel-filtration chromatography on a Superdex-200 column HR 10/30 (10 mm × 30 cm, 24 ml). The purified enzyme was loaded onto the column and eluted at a flow rate of 0.2 ml/min with 10 mM potassium phosphate buffer, pH 6.0, containing 0.15 M NaCl. The standard protein markers used for calibration were as follows: thyroglobulin (670,000 Da), γ-globulin (158,000 Da), ovalbumin (44,000 Da), myoglobin (17,000 Da), and vitamin B2 (13,500 Da).

N-Terminal sequence. The N-terminal sequence of the purified enzyme (40 µl) was sequenced (30 cycles) with a HP G1005A Protein Sequencing system from APRO Science (Tokushima, Japan).

Prosthetic group analysis. Absorption spectra were measured with a PharmaSpec UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan), using a 1-cm cell path length. The enzyme was analyzed in 10 mM potassium phosphate buffer, pH 6.0.

Kinetic parameters. The initial velocity of the enzymatic decomposition of racemic mandelonitrile was determined in 50 mM sodium citrate buffer, pH 5.5, according to the standard reaction assay at various substrate concentrations.

Effects of pH and temperature. The optimum pH and temperature for activity were assayed according to the standard method at pH 3.5–6.5 and temperatures of 10–80 °C. Stability was monitored after 60 min incubation at various pH levels (3–9, 30 °C) and temperatures (0–80 °C, pH 6.0).

Effect of additives. The effects of various additives on the purified enzyme were examined. The enzyme solution was incubated with various additives in 10 mM potassium phosphate buffer, pH 6.0, at 30 °C for 60 min. The remaining activity of the enzyme was assayed according to standard procedures.

Synthesis of cyanohydrins. The reaction mixture was prepared in a micro-tube: 1.0 M of carbonyl compound (in DMSO, 40 µl) was added to sodium citrate buffer (400 mM, pH 4.0, 760 µl), followed by the addition of enzyme solution (25 units, decomposition units) and KCN solution (1.0 M, 100 µl). The reaction was monitored by taking a small aliquot of the reaction mixture (100 µl) and extraction with organic solvent (90% *n*-hexane, 10% isopropanol by volume for HPLC, ethyl acetate for GC). The organic layer was analyzed by chiral HPLC (for aromatic compounds) and chiral GC (for aliphatic compounds).

To calculate the kinetic parameters of the enzymatic synthesis of cyanohydrins, the initial velocity of the synthesis of chiral cyanohydrins was determined under the conditions described above at various aldehyde substrate concentrations.

Preparation of cyanohydrins for HPLC and GC standard. The carbonyl compound (1 eq.) was dissolved and stirred vigorously in acetic acid. This was followed by the addition of an aqueous solution of KCN (3 eq.) into the mixture. The reaction was monitored by TLC with UV light or iodine vapor as a developing agent. After the reaction was completed, the reaction mixture was neutralized with $NaHCO_3$, extracted with ethyl acetate, dried with anhydrous Na_2SO_4, and evaporated in a vacuum. The cyanohydrins after purification by silica gel column chromatography were characterized by ^{1}H and ^{13}C-NMR spectroscopy and used as standards for HPLC and GC. All cyanohydrins isolated were colorless to slightly yellow oils.

NMR data for standard cyanohydrins.

2b: 2-hydroxy-2-(4-methoxyphenyl)acetonitrile (*p*-anisaldehyde cyanohydrin)

$C_9H_9O_2N$; ^{1}H NMR δ_H ($CDCl_3$): 7.4 (d, 2H, Ar-*H*), 6.9 (d, 2H, Ar-*H*), 5.4 (d, 1H, C*H*CNOH), 3.8 (s, 3H, CH_3O-Ar), 2.6 (d, 1H, O*H*). ^{13}C NMR δ_C ($CDCl_3$): 160.8, 128.3, 127.5, 118.8, 114.5, 63.4, 55.4

2c: 2-hydroxy-2-(thiophen-2-yl)acetonitrile (2-thiophene carboxaldehyde cyanohydrin)

C_6H_5ONS; ^{1}H NMR δ_H ($CDCl_3$): 7.0–7.5 (m, 3H, Ar-*H*), 5.7 (s, 1H, C*H*CNOH), 2.7 (s, 1H, O*H*). ^{13}C NMR δ_C ($CDCl_3$): 148.0, 128.1, 127.4, 127.2, 118.2, 59.36

2d: 2-hydroxy-2-(naphthalen-1-yl)acetonitrile (1-naphthaldehyde cyanohydrin)

$C_{12}H_9ON$; ^{1}H NMR δ_H ($CDCl_3$): 7.5–8.1 (m, 7H, Ar-*H*), 6.1 (d, 1H, C*H*CNOH), 2.7 (d, 1H, O*H*). ^{13}C NMR δ_C ($CDCl_3$): 134.1, 131.1, 130.4, 130.0, 129.1, 127.4, 126.6, 125.7, 125.1, 122.9, 118.0

2e: 2-hydroxy-2-(naphthalen-2-yl)acetonitrile (2-naphthaldehyde cyanohydrin)

$C_{12}H_9ON$; ^{1}H NMR δ_H ($CDCl_3$): 7.4–8.0 (m, 7H, Ar-*H*), 6.0 (d, 1H, C*H*CNOH), 2.6 (d, 1H, O*H*). ^{13}C NMR δ_C ($CDCl_3$): 135.2, 133.7, 131.8, 128.0, 127.6, 127.5, 127.3, 127.0, 126.0, 125.1, 118.2, 63.3

2f: 2-(benzo[*d*][1,3]dioxol-6-yl)-2-hydroxyacetonitrile (piperonaldehyde cyanohydrin)

$C_9H_7O_3N$; ^{1}H NMR δ_H ($CDCl_3$): 7.0 (dd, 2H, Ar-*H*), 6.8 (dd, 1H, Ar-*H*), 6.0 (s, 2H, -OCH_2O-), 5.4 (d, 1H, C*H*CNOH), 2.6 (d, 1H, O*H*). ^{13}C NMR δ_C ($CDCl_3$): 148.9, 148.4, 129.1, 120.7, 118.6, 108.6, 107.2, 101.6, 63.5

2g: 2-hydroxybutanenitrile (propionaldehyde cyanohydrin)

C_4H_7ON; ^{1}H NMR δ_H ($CDCl_3$): 4.4 (t, 1H, C*H*CNOH), 2.9 (s, 1H, O*H*), 1.8 (qd, 2H, CH_2), 1.1 (t, 3H, CH_3). ^{13}C NMR δ_C ($CDCl_3$): 119.8, 62.5, 28.6, 8.9

2h: 2-hydroxy-3-methylbutanenitrile (isobutyraldehyde cyanohydrin)

C_5H_9ON; ^{1}H NMR δ_H ($CDCl_3$): 4.3 (m, 1H, C*H*CNOH), 2.8 (s, 1H, O*H*), 2.1 (m, 1H, C*H*), 0.9 (m,

Table 1. Purification of Hydroxynitrile Lyase from *Eriobotrya japonica*

Purification step	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg)	Yield (%)	Purification (fold)
Crude extract	840	1000	0.8	100	1
30–80% $(NH_4)_2SO_4$	522	140	3.7	62	4
DEAE-Toyopearl 650M	369	38.3	9.7	44	12
Concanavalin A Sepharose 4B	303	7.5	40.9	36	49

6H, C*H*$_3$). ^{13}C NMR δ_C ($CDCl_3$): 118.4, 67.0, 33.0, 17.6, 17.1

2i: 2-hydroxy-3,3-dimethylbutanenitrile (pivalaldehyde cyanohydrin)

$C_6H_{11}ON$; ^{1}H NMR δ_H ($CDCl_3$): 4.1 (d, 1H, C*H*CNOH), 2.4 (d, 1H, O*H*), 1.0 (s, 9H, C*H*$_3$). ^{13}C NMR δ_C ($CDCl_3$): 118.4, 70.7, 35.4, 24.9

2j: 2-cyclohexyl-2-hydroxyacetonitrile (cyclohexanecarboxaldehyde cyanohydrin)

$C_8H_{13}ON$; ^{1}H NMR δ_H ($CDCl_3$): 4.2 (t, 1H, C*H*CNOH), 2.5 (d, 1H, O*H*), 1.8 (m, 10H, C*H*$_2$), 1.2 (m, 1H, C*H*). ^{13}C NMR δ_H ($CDCl_3$): 119.2, 66.4, 42.2, 30.9, 28.1, 27.7, 25.9, 25.4

Results

A hydroxynitrile lyase from seeds of *Eriobotrya japonica* (*Ej*HNL) was purified to homogeneity. The results for a typical preparation are summarized in Table 1.

Crude enzyme with 840 units of activity was extracted from 2 kg of seeds. Ammonium sulfate precipitation and salting out were used to concentrate the crude enzyme solution before column chromatography. *Ej*HNL was precipitated between 30 and 80% saturated salt solution. Approximately 62% of the activity remained, and 4-fold purification was obtained by the salt precipitation step.

The re-dissolved enzyme solution was further purified by anion-exchange chromatography on a column of DEAE-Toyopearl 650M. Proteins absorbed on the column were eluted by increasing the linear gradient from 0 to 0.5 M NaCl. Fractions having HNL activity were combined for further purification. Specific activity increased approximately 3-fold.

An active pool of DEAE-Toyopearl fractions was dialyzed against 10 mM potassium phosphate buffer, pH 6.0. Prior to being loaded onto the affinity column, Concanavalin A Sepharose 4B, the enzyme solution was mixed with NaCl to obtain a concentration of 0.5 M. After loading and washing, the column was eluted with 0–1 M of α-D-methylglucoside as a linear gradient. One major symmetrical peak of protein was eluted at approximately 0.2 M of α-D-methylglucoside. Large amounts of contaminated proteins were removed. By this step, purification 49-fold with a yield of 36% was obtained. The enzyme was interacted with a concanavalin A column, indicating that it was a glycoprotein.

The native molecular mass of purified *Ej*HNL was estimated by HPLC on a Superdex 200 filtration column. From logarithmic plots of molecular mass *vs*. retention time of standard proteins, the molecular mass of the native enzyme was estimated to be 72 kDa. The purified enzyme appeared as one band on native-PAGE gel (Fig. 1A) with a molecular mass of 62.3 kDa from the SDS–PAGE gel (Fig. 1B). Hence, it was concluded that the purified enzyme consisted of a monomer of a single subunit. A thick purified band in SDS–PAGE might have been caused by the glycosidic properties of the enzyme.

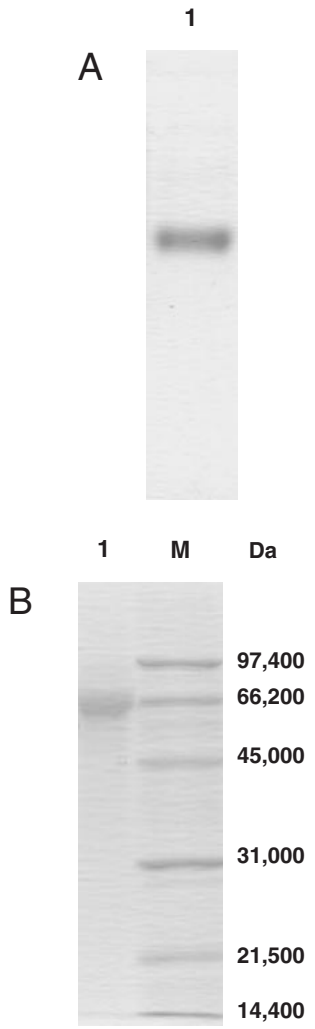


Fig. 1. Native-PAGE (a) and SDS–PAGE (b) of Purified *Ej*HNL. M, Low molecular weight standard marker; lane 1, purified *Ej*HNL.

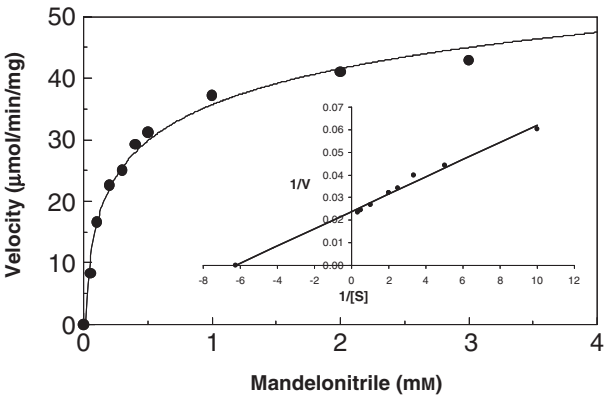


Fig. 2. Substrate Saturation Curve for Mandelonitrile with *Ej*HNL.
The assay conditions are described in "Materials and Methods." The concentration of mandelonitrile was varied as indicated. The inset is a Lineweaver-Burk plot. A V_{max} of 46.5 μmol/min/mg, a K_m value of 161 μM, and k_{cat} 56 s^{-1} and k_{cat}/K_m 348 s^{-1} mM^{-1} were obtained.

The yellow color of *Ej*HNL was also observed during purification, and the enzyme exhibited maximum absorption spectra at 278, 389, and 454 nm, as seen in the absorption spectra of a typical FAD containing protein. The N-terminal sequence of the purified *Ej*HNL was L-A-T-P-S-E-H-D-F-S-Y-S-K-S-V-V-X-A-T-D-L-P-Q-E-E-V-Y-D. X represents an unidentified amino acid. Fifty-seven percent identity in 28 amino acids was found to overlap with the sequence of the FAD-containing (*R*)-hydroxynitrile lyases from *Prunus serotina*[14] and *Prunus dulcis*,[15] but was different from non-FAD hydroxynitrile lyases. Therefore, *Ej*HNL was identified as the FAD-containing HNL.

A substrate saturation curve for purified *Ej*HNL was determined using mandelonitrile. As shown in Fig. 2, the Michaelis-Menten kinetics were typical and exhibited a K_m of 161 μM, a k_{cat} of 56 s^{-1}, and a k_{cat}/K_m of 348 s^{-1} mM^{-1}.

The effect of pH on enzyme activity is shown in Fig. 3. The optimum pH for *Ej*HNL using the natural substrate mandelonitrile was found to be approximately 5.5. At pH 3.5, activity was totally inhibited, but a rapid increase in activity was found at increasing pH levels up to the optimum. At higher pH than optimum, an increase in the spontaneous decomposition of the substrate was observed, and hence the actual optimum pH was difficult to determine.[5] After incubation at various pH for 60 min, excellent stability was found at pH 3–9 (data not shown). At pH 5.5, the optimum temperature was 40 °C for mandelonitrile as for substrate (Fig. 4). Temperatures lower and higher than the optimum caused a regression in activity. Besides the heat-inactivation effect, high temperatures were found to be a cause of spontaneous decomposition of the substrate, which affected the enzymatic activity. Temperature stability was studied, and *Ej*HNL was found to be active over a broad range of temperatures (0–60 °C) after incubation for 60 min. At 70 and 80 °C, the residual activities of the enzyme after incubation were 68 and 28%, respectively (data not shown). At 4 °C in 10 mM potassium phosphate buffer (pH 6.0), *Ej*HNL was stable for at least 30 d without any significant loss of activity.

Next the influences of various additives on the activity of *Ej*HNL were determined (Table 2). The rate of degradation of mandelonitrile due to *Ej*HNL was unaffected by 1 mM of $ZnCl_2$, $MnCl_2$, $MgCl_2$, or $PbCl_2$, and by 10 mM of Na_2EDTA, while 1 mM of $FeCl_3$ caused 50% inhibition. These results suggest that metal ions were not required for the activity. The enzyme was significantly inhibited by a reducing agent and a cysteine modifying agent. β-Mercaptoethanol caused 19% inhibition at 10 mM, iodoacetic acid caused 53% inhibition at 1 mM, while $CuSO_4$, $HgCl_2$, and $AgNO_3$ caused complete inhibition. The serine modifying agent phenylmethylsulfonylfluoride (PMSF) inhibited the enzymatic activity by 59% at 1 mM, while the histidine inhibitor

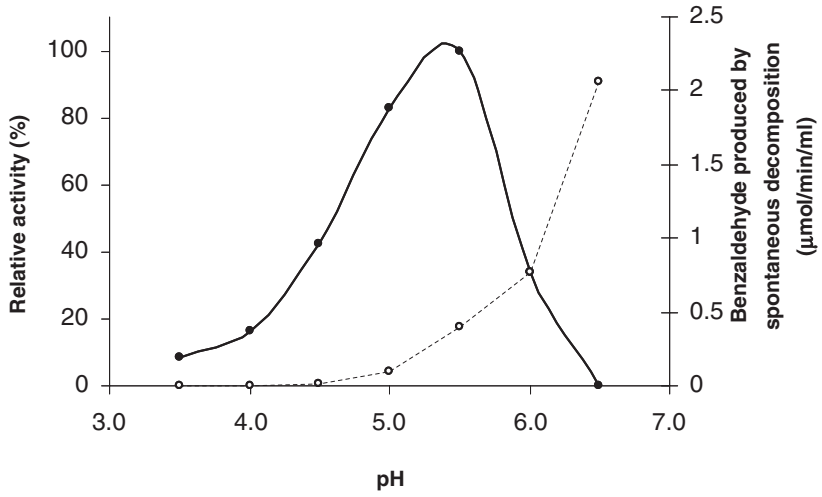


Fig. 3. pH Profiles of Purified *Ej*HNL and Spontaneous Decomposition of Mandelonitrile.
●, Enzyme activity; ○, spontaneous decomposition of mandelonitrile.

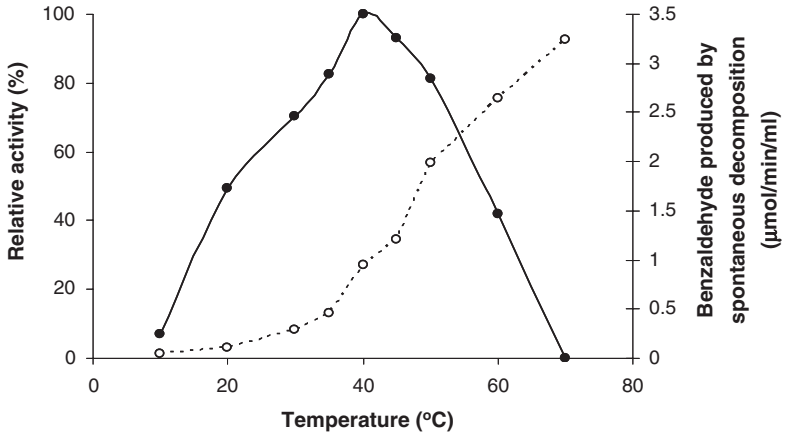


Fig. 4. Temperature Profiles of Purified *Ej*HNL and Spontaneous Decomposition of Mandelonitrile.
●, Enzyme activity; ○, spontaneous decomposition of mandelonitrile.

Table 2. Effects of Various Additives on the Activity of Purified *Ej*HNL

Additive	Concentration (mM)	Inhibition (%)
β-Mercaptoethanol	10	19
Iodoacetic acid	1	53
PMSF	1	59
DEP	1	36
Na_2EDTA	10	0
$MnCl_2$	1	0
$ZnCl_2$	1	0
$MgCl_2$	1	0
$CuSO_4$	1	100
$HgCl_2$	1	100
$PbCl_2$	1	0
$AgNO_3$	1	100
$FeCl_3$	1	50

diethylpyrocarbonate (DEP) caused 36% inhibition at 1 mM. These results indicate that cysteine, serine, and histidine residues are located near the active site of the enzyme and that they participate in the catalysis.

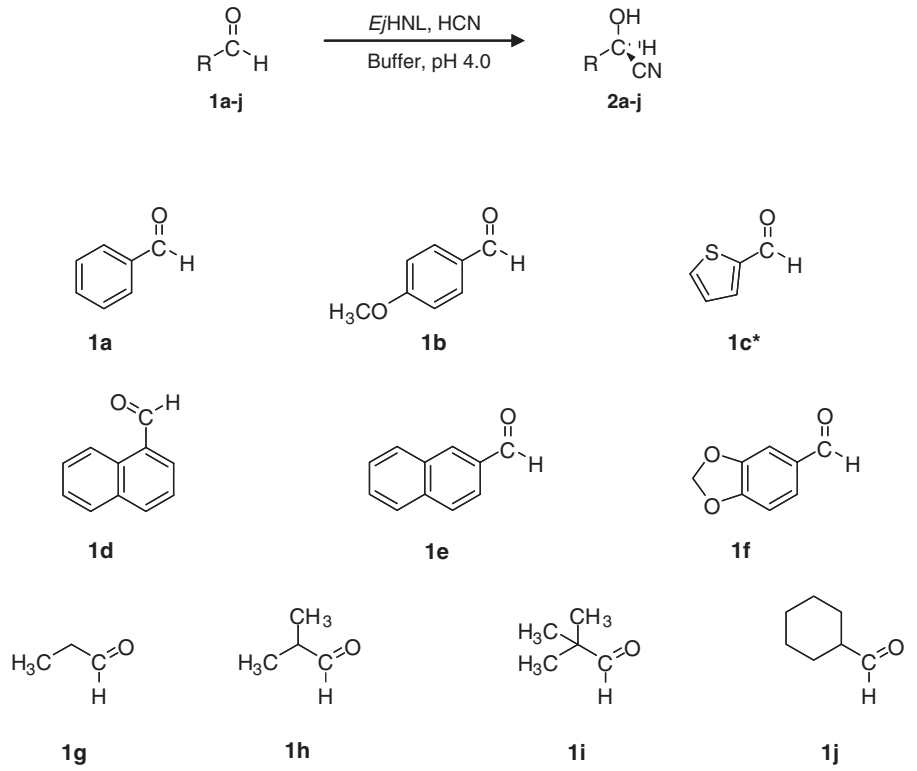
Synthesis of chiral cyanohydrins from selected aromatic and aliphatic aldehydes (Scheme 1) by *Ej*HNL was investigated, as shown in Table 3.

A specificity constant (k_{cat}/K_m) was used to compare the relative rate of the enzyme acting on substrates. For aromatic aldehydes, high specificity constants were obtained for 2-thiophenaldehyde, benzaldehyde, and 2-naphthaldehyde at 160.5, 148.4, and 101.3 s^{-1} mM^{-1} respectively. The specificity constants for bulky aromatic substrates, *p*-anisaldehyde and piperonal, were 50.8 and 29.2 s^{-1} mM^{-1} respectively, while 1-naphthaldehyde was not catalyzed by the enzyme. In contrast to the report on the substrate specificity of HNL from *Eriobotrya* L. by Lin *et al.*,[8] that aliphatic aldehyde (trimethylacetaldehyde) was an unacceptable substrate for the enzyme, we found that *Ej*HNL acted on propionaldehyde, pivaldehyde, and isobutyraldehyde with specificity constants of 19.4, 14.3, and 9.6 s^{-1} mM^{-1} respectively. Cyclohexanecarboxaldehyde was a poor substrate for *Ej*HNL. The enantiomeric excess (*e.e.*) of chiral cyanohydrins on different substrates was studied (Table 3). The *e.e.* was in the range of 3.9–89.5%. Two factors might have affected the *e.e.* value: the specificity of the enzyme for the substrates used, and the rate of spontaneous chemical reaction. A high *e.e.* for chiral cyanohydrins can be achieved with biphasic systems of buffers and water-immiscible organic solvents, and the spontaneous chemical reaction might have been suppressed by these systems.[5] These results will be described elsewhere.

Discussion

HNL was originally classified based on enantioselectivity into two groups. (*R*)-selective HNL catalyzes the formation of (*R*)-cyanohydrins and derived almost entirely from oxidoreductase ancestors such as HNLs from *Rosaceae* and *Linum usitatissimum*, except that an (*R*)-selective HNL from *Arabidopsis thaliana* is derived from an α/β-hydrolase fold.[16,17] (*S*)-selective HNL, derived from hydrolases with an α/β-hydrolase fold, catalyzes the formation of (*S*)-cyanohydrins such as HNLs from *Hevea brasiliensis* and *Manihot esculenta*.[16]

However, according to the EC number based on the chemical reaction of the enzyme, HNL can be classified into four groups. First, mandelonitrile lyase (EC 4.1.2.10) is a (*R*)-HNL that converts the natural substrate (*R*)-mandelonitrile to benzaldehyde and prussic acid. Mandelonitrile lyases from the Rosaceae family (Prunoideae and Maloideae subfamilies) are glycoproteins with posttranslational modifications, and they contain FAD as a prosthetic group. Several plants containing this type of HNL have been reported, including *Prunus amygdalus*,[18] *P. serotina*,[19] *P. mume*,[5] *P. persica*,[5,20] *P. avium*,[20] *P. domestica*,[20] *P. laurocerasus*,[21] *P. lyonii*,[22] *Passiflora edulis*,[5] *Chaenomles sinensis*,[5] *Pouteria sapota*,[20] *Cucumis melo*,[20] *Cydonia oblonga*,[20]



Scheme 1. Synthesis Reaction of Asymmetric (*R*)-Cyanohydrins Catalyzed by (*R*)-*Ej*HNL and Aldehyde Substrates. *(*S*)-configuration was assigned according to Cahn-Ingold-Prelog.[43]

Table 3. Cyanohydrin Synthesis and Substrate Specificity of *Ej*HNL on Aromatic and Aliphatic Aldehydes

Entry	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	*e.e.* (%)	Configuration
1a	Benzaldehyde	11.5	1700	148	65	*R*
1b	*p*-Anisaldehyde	3.5	582	50.8	81	*R*
1c	2-Thiophenaldehyde	35.7	1840	161	90	*S*[a]
1d	1-Naphthaldehyde	—	—	—	24	*R*
1e	2-Naphthaldehyde	1.1	1160	101	88	*R*
1f	Piperonal	2.6	334	29.2	89	*R*
1g	Propionaldehyde	99.9	223	19.4	36	*R*
1h	Isobutyraldehyde	41.8	110	9.6	38	*R*
1i	Pivaldehyde	39.3	164	14.3	14	*R*
1j	Cyclohexane carboxaldehyde	—	—	—	4	*R*

[a]The (*S*)-configuration was assigned according to Cahn-Ingold-Prelog.[43]

and *Phlebodium aureum*.[23] Secondly, *p*-hydroxymandelonitrile lyase (EC 4.1.2.11) is a (*S*)-HNL that catalyzes the decomposition of (*S*)-mandelonitrile and (*S*)-4-hydroxymandelonitrile as natural substrates. *p*-Hydroxymandelonitrile lyases have been investigated in plant extracts of *Baliospermum montanum*,[5] *Sorghum bicolor*,[24] *Annona cherimola*,[20] *Annona squamosa*,[20] and *Ximenia Americana*.[25] Thirdly, acetone cyanohydrin lyase (EC 4.1.2.37) is a (*R*)-HNL that catalyzes the degradation of acetone cyanohydrin in *Linum usitatissimum*.[26] Finally, hydroxynitrilase (EC 4.1.2.39), a (*S*)-HNL catalyzing the decomposition of acetone cyanohydrin and 2-butanone cyanohydrin, was found in *Hevea brasiliensis*[27] and *Manihot exculenta*.[28]

Previous studies of the purification and characterization of hydroxynitrile lyases investigated only certain characteristics, such as molecular mass, optimum pH and temperature, the effects of additives and inhibitors, storage effects, *etc*. A description of all characteristics, including substrate specificity based on activity, was left incomplete. Seeds of *Eriobotrya japonica* were found to be a source of HNL.[5] Ground seeds of *Eriobotrya L.* were used directly to synthesize chiral cyanohydrins without further purification and to study the characteristics of the enzyme.[8] Lack of understanding of the true characteristics of purified enzymes limits their application. Therefore, purification and full characterization of HNL from *Eriobotrya japonica* (*Ej*HNL) was carried out, not only for basic studies of the enzyme, but also for the development of new methods for the enzymatic synthesis of chiral cyanohydrins.

Seeds of *Eriobotrya japonica* were extracted in a buffer containing polyvinylpyrrolidone for protection of the enzyme from phenolic compounds in the crude plant extract that form complexes by hydrogen bonding with peptide bond oxygen or by covalent modification of amino acid residues, causing inactivation of the enzyme.[29] The purified *Ej*HNL had a monomer of a single subunit. Although no chemical identification was done, the enzyme was considered to be a glycoprotein, since it was absorbed to a Concanavalin A Sepharose 4B

column, which binds specifically to the molecules containing sugar residues. Moreover, a broad single band of purified enzyme was observed on SDS–PAGE, indicating that the enzyme is a typical glycoprotein. Similar thick bands of glycoprotein on SDS–PAGE have been reported in *N*-glycanase from rice seeds,[30] α-glucosidase from *Schizosaccharomyces pombe*,[31] and glycoprotein produced by *Phoma tracheiphila*.[32] The glycoprotein HNL was observed in *Prunus dulcis*, *Prunus laurocerasus*, *Prunus serotina*, *Prunus lyonii*, *Sorghum bicolor*, and *Ximenia Americana*,[22,25,33–35] while non-glycosylated HNLs were found in *Hevea brasiliensis*, *Manihot esculenta*, *Linum usitatissimum*, and *Phlebodium aureum*.[23,28,29,33] HNLs isolated from Rosaceae (Prunoideae and Maloideae subfamilies) are glycoproteins with molecular masses of 50–80 kDa.[16] Furthermore, several isozymes of HNL from *Prunus* species have been isolated, differing slightly in molecular mass, isoelectric point, or carbohydrate side chains, *etc.*[34] *Prunus dulcis* containing three or four isozymes, *Prunus laurocerasus* containing three isozymes, and *Prunus serotina* containing five isozymes have been reported.[33] In this study, a single purified isozyme of *Ej*HNL was observed according to the following criteria on the purified enzyme: The results of column chromatography showed a single symmetrical peak of purified enzyme. Single values for molecular mass, optimum pH, optimum temperature, and N-terminal amino acid sequence were observed.

The absorption spectra and N-terminal sequence of the purified *Ej*HNL were similar to FAD-containing HNLs. Moreover, in current work in our group on cloning of the gene encoding *Ej*HNL, we found a conserved FAD-binding region in the gene encoding the enzyme (data not shown). Therefore, *Ej*HNL is most likely a FAD-containing HNL. The unique characteristic of HNLs isolated from the Prunoideae and Maloideae subfamilies of Rosaceae is the presence of FAD. A previous study found that the FAD is bound near the active site.[36] This prosthetic group does not play a role in redox reactions, but it is essential for catalysis, and it provides structural stability to the enzyme. Removal of FAD causes inactivation of the enzyme.[14,37,38]

The Michaelis-Menten constant (K_m) of *Ej*HNL for mandelonitrile was 161 μM. A lower K_m value characterizes higher affinity between substrate and enzyme.[39] HNLs from *Prunus serotina* (K_m of 172 μM), *Prunus dulcis* (K_m of 290 μM), and *Sorghum bicolor* (K_m of 790 μM) exhibited higher K_m values than *Ej*HNL, while a K_m of 93 μM was observed in *Prunus lyonii*.[22,34]

The optimum pH for *Ej*HNL using the natural substrate mandelonitrile was found to be approximately 5.5. The optimum pH varies slightly for different substrates or conditions. The optimum pH for FAD-containing HNLs for the decomposition of mandelonitrile was in the range of 5.0–7.0.[22,33,34] A non-FAD-containing HNL (*Phlebodium aureum* and *Ximenia americana* HNL) catalyzing the same reaction also showed optimum pH of 5.0–6.5,[23,25] while the optimum pH values for *Linum usitatissimum*, *Manihot esculenta*, and *Hevea brasiliensis* HNL were around pH 5.0–6.0 with acetone cyanohydrin as a substrate.[22,28,33] The optimum temperature of the purified *Ej*HNL was 40 °C, while the HNL obtained from *Phlebodium aureum* had an optimum temperature of 35–40 °C on the same substrate.[23] It was difficult to determine the exact optimum pH and temperature of HNL from the decomposition of the substrate due to the effect of the spontaneous decomposition of the substrate at higher pH and temperature. Excellent stability of *Ej*HNL over a wide range of pH levels (pH 3–9) and temperatures (0–60 °C) was observed. HNLs from *Prunus dulcis* and *Sorghum bicolor* have been reported to be stable over a wide pH range,[35] while HNL from *Linum usitatissimum* showed stability at pH 6–11, but was less stable under acidic conditions.[26] HNL from *Hevea brasiliensis* was stable for several h at 30 °C, but above 70 °C was inactivated rapidly.[40] HNL from *Sorghum bicolor* showed good stability against heat inactivation; the activity remaining after incubation (60 °C, 60 min) was higher than 60%, and complete inactivation was observed at 70 °C (30 min).[35]

Metal ions were not required for enzyme activity. The enzymatic activity was inhibited by some heavy metal ions that might react with essential sulfhydryl groups, causing inactivation of the enzyme. Similar results as to metal ions have been obtained for HNL from *Ximenia americana*, *Phlebodium aureum*, *Sorghum bicolor*, and *Prunus serotina*.[23,25,31,41] The cysteine, serine, and histidine amino acid residues might be located near the active site and might be involved in the catalysis. In a similar HNL from *Prunus amygdalus*, an FAD containing HNL, the identified substrate binding site and catalytic site of the enzyme consisted of His-497, Ser-496, Tyr-457, and Cys-328. They formed hydrogen bonding interactions with the hydroxyl group of the substrate. His-497 also acts as a protonating or deprotonating residue to the carbonyl compound and HCN in the catalysis reaction.[42] Based on the inhibition experiment, it is likely that these residues are also located around the active site of *Ej*HNL. The occurrence or non-occurence of these residues around the active site of *Ej*HNL should be made clear by our future primary structure elucidation.

The substrate specificity of *Ej*HNL for the synthesis of chiral cyanohydrins was investigated by measuring the initial velocity of the enzymatic reaction toward the selected aldehydes, and the configuration of the chiral cyanohydrins was assigned according to Cahn-Ingold-Prelog.[43] Previous studies on chiral cyanohydrin synthesis focused on enantiomeric excess (*e.e.*) and the conversion of products, neglecting to measure the initial velocity of the reaction, which is the basis of enzymology.[44–46] Even though several two-phase systems are used to produce chiral cyanohydrins, a buffer system was employed in this study to avoid the effects of

organic solvents on the stability and activity of the enzyme and effects of the partitioning of substrates between organic and aqueous phases. All aldehydes were dissolved in DMSO to increase the solubility of the substrates in buffer, and KCN was used as a source of cyanide to avoid working with highly toxic HCN. The reaction was performed at pH 4.0, at which the chemical addition of cyanide to aldehydes was mostly suppressed. *Ej*HNL catalyzed the synthesis of cyanohydrins from both aliphatic and aromatic aldehydes. It can be inferred that *Ej*HNL preferred aromatic to aliphatic substrates, suggesting that the active site of the enzyme is located near the hydrophobic region, and suggesting greater specificity for small substrates than bulky substrates. These results indicate potential use of *Ej*HNL in the synthesis of cyanohydrins. The enzyme characterized here can be categorized as a mandelonitrile lyase (EC 4.1.2.10) because it was (*R*)-HNL active toward mandelonitrile and its N-terminal sequence was similar to that of the mandelonitrile lyases reported in this group. HNL from *Prunus dulcis*, *Prunus mume*, *Manihot esculenta*, and *Hevea brasiliensis* have shown broad specificity for aliphatic and aromatic substrates,[47,48] while HNL from *Linum usitatissimum* had a narrow range of substrates.[26] HNL from *Ximenia americana* showed fairly good specificity toward aromatic aldehydes.[25] Specificity accepting more bulky substrates might be improved by genetic engineering. (*S*)-HNLs from *Hevea brasiliensis* and *Manihot esculenta* have been engineered by replacing bulky amino acids, which constrict the entrance to active sites, with less bulky amino acids, leading to changes in the specificity of the enzyme accepting larger substrates.[47] The *e.e.* of chiral cyanohydrins synthesized by *Ej*HNL can be further improved by optimizing the reaction conditions to minimize non-enzymatic reactions in organic media. It is of interest to determine the entire structure of *Ej*HNL and to engineer an enzyme suitable for the synthesis of cyanohydrins.

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