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Screening for New Hydroxynitrilases from Plants

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We established a simple HPLC method to determine the activity and stereochemistry of the chiral mandelonitrile synthesized from benzaldehyde and cyanide, and applied it to screen for hydroxynitrile lyase (HNL) activity of plant origin. A total of 163 species of plants among 74 families were examined for (*R*)- and (*S*)-HNL activities using the method. We discovered that homogenate of leaves of *Baliospermum montanum* shows (*S*)-HNL activity, while leaves and seeds from *Passiflora edulis*, and seeds from *Eriobotrya japonica*, *Chaenomles sinensis*, *Sorbus aucuparia*, *Prunus mume*, and *Prunus persica* show (*R*)-HNL activity. Partially purified (*R*)-HNLs from *Passiflora edulis* and *Eriobotrya japonica* acted not only on benzaldehyde but also on aliphatic ketone. The enantiomeric excess of (*R*)-methylpropylketone cyanohydrin synthesized from 2-pentanone using homogenate from leaves of *Passiflora edulis* was 87.0%, and that of (*R*)-mandelonitrile synthesized by homogenate from seeds of *Eriobotrya japonica* was 85.0%.

Key words: hydroxynitrile lyase; *Prunus dulcis*; *Baliospermum montanum*; *Passiflora edulis*; *Eriobotrya japonica*

Cyanogenesis is an ability of plants to release hydrogen cyanide in the cells, and is considered a mechanism to protect plants from attack by fungi and predators. Hydroxynitrile lyases (HNLs) are one of the key enzymes in cyanogenesis and catalyze the final step in the biodegration of cyanogenic glycosides.[1)] Cyanogenesis is believed to be distributed in over 2,650 species of higher plants.[2,3)]

Hydroxynitrile lyase (HNL) is a general term referring to four enzymes, mandelonitrile lyase (EC 4.1.2.10), *p*-hydroxymandelonitrile lyase (EC 4.1.2.11), acetone–cyanohydrin lyase (EC 4.1.2.37), and hydroxynitrilase (EC 4.1.2.39). HNLs are used as biocatalysts for the synthesis of optically active cyanohydrins, which are important building blocks in the fine chemical and pharmaceutical industries.[4)] Effenberger *et al.* demonstrated that (*R*)-methylalkylketone cyanohydrin from methylalkylketone is obtained with good chemical yields and in high optically purity using partially purified (*R*)-mandelonitrile lyase from *Prunus dulcis* (Miller) D. A. Webb. (syn: *Prunus amygdalus* Batsch.) (almond).[4,5)] Griengl *et al.* have used (*S*)-specific HNL from *Hevea brasiliensis* (H.B.K.) Muell. Arg. (rubber) in the chiral synthesis of (*S*)-cyanohydrins.[6)] As far as we know, there has been almost no systematic screening for enzymatic activity of (*R*)- and (*S*)-mandelonitrile lyases in plants, although microbial screening is a standard and versatile method to obtain industrially important new biocatalysts.[7)] We found that only a small portion of cyanogenic plants show detectable HNL activity. Especially (*S*)-mandelonitrile lyase has been found only among *Hevea brasiliensis* (rubber),[8)] *Manihot esculenta* Crantz (cassava),[9)] *Ximenia americana* Linn.,[10)] *Sorghum bicolor* Moench,[11)] and *Annona squamosa* Linn.[12)]

In this study, we examined a variety of plants for (*R*)- and (*S*)-mandelonitrile lyase activities using our newly developed HPLC method to determine the activity and the stereochemistry of the chiral mandelonitrile synthesized from benzaldehyde and potassium cyanide. After extensive screening of plants, we discovered producers of (*R*)- and (*S*)-mandelonitrile lyases. They were used to catalyze the synthesis of optically active mandelonitrile and methylpropylketone cyanohydrin from benzaldehyde and 2-pentanone respectively.

Materials and Methods

Materials. Seeds of *Prunus dulcis* (almond), kindly donated by Lotte Co., Ltd., Tokyo, Japan, and Meiji Seika Kaisha Ltd., Tokyo, Japan, were used for extraction of (*R*)-mandelonitrile lyase. Bodies of various plants (leaves, roots, shoots, spikes, and rhizomes) were mainly collected at the Botanic Gardens of Toyama and used for extraction of mandelonitrile lyase. An HPLC

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Abbreviation: HNL, hydroxynitrile lyase

column, CHIRALCEL OJ-H, was purchased from Daicel Chemical Industries Ltd., Osaka, Japan. 2-Pentanone was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. (Trimethylsilyl) diazometane was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, U.S.A. Potassium cyanide was from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other chemicals were from commercial sources and were used without further purification. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a JEOL LA-400 spectrophotometer (Tokyo, Japan).

Enzyme assay. Enzyme activity was assayed by measuring the production of optically active mandelonitrile synthesized from benzaldehyde and cyanide. The standard assay solution contained 300 µmol citrate buffer (pH 3.5–6.0), 50 µmol of benzaldehyde, 100 µmol of potassium cyanide, and 100 µl of the enzyme in a final volume of 1.0 ml. The reaction was started by the addition of 100 µl of the enzyme solution, which was incubated at 25 °C for 1 to 120 min. Aliquots (100 µl) were withdrawn at various reaction times, and the reaction was stopped by the addition of 0.9 ml of organic solvent (hexane:isopropanol = 9:1 by volume), the mandelonitrile formed was extracted, and the supernatant, obtained by centrifugation (15,000 × *g*, 1.0 min at 4 °C), was assayed by HPLC. The amounts of benzaldehyde and (*R*)- and (*S*)-mandelonitrile were determined by HPLC with a CHIRALCEL OJ-H column at 254 nm using a mobile phase of the solvent (hexane:isopropanol = 9:1 volume) at a flow rate 1.0 ml/min. The retention times of benzaldehyde and (*R*)- and (*S*)-mandelonitrile were about 5.8, 16.9, and 21.8 min respectively. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol of optically active mandelonitrile from benzaldehyde per min under the standard assay condition. In the control experiment, boiled enzyme was used. The amount of racemic mandelonitrile formed non-enzymatically in the reaction mixture was subtracted from the amount in the former reaction mixture. The reaction progressed linearly in proportion to the amount of enzyme in the first few minutes.

Preparation of partially purified (R)-mandelonitrile lyase from Prunus dulcis (almond). The surfaces of almonds were sterilized with 0.52% (w/v) sodium hypochlorite and then dried. All the purification steps were performed at 4 °C. Almond (26.0 g) was homogenized with 250 ml of 20 mM ammonia solution in a SMT PH91 process homogenizer (Tokyo, Japan). The homogenate was filtered through four layers of cheese cloth and centrifuged at 28,000 × *g* for 15 min at 4 °C. To remove unnecessary protein, the homogenate was subjected to isoelectric precipitation[11] (at both pH 4.8 and pH 9.2) with 1.0 M acetic acid and 1.0 M ammonia solutions. After all steps of isoelectric precipitation were finished, the extracts were centrifuged at 28,000 × *g* for 15 min at 4 °C. The supernatant, which was adjusted to pH 6.4, was precipitated with ethanol to obtain partially purified enzyme. The precipitate was suspended in 10 ml of 50 mM imidazole–HCl buffer (pH 6.0). The suspension was dialyzed overnight against 2 liters of 10 mM imidazole–HCl buffer (pH 6.0), and the buffer was changed once. The dialyzed preparation was pooled and (*R*)-mandelonitrile lyase activity was measured.

Typical procedure for extraction of HNL from plants. Seeds from plants were cracked with a hammer to release the soft kernels inside. The kernels were collected and crushed in process homogenizer at 4 °C, with 10 mM KPB (potassium phosphate buffer, pH 6.0), to give a milky suspension. The suspension was filtered through four layers of cheese cloth to remove the insoluble part. The suspension was then centrifuged (18,000 × *g*, 20 min at 4 °C), and removal of the residue gave a crude enzyme preparation, which was fractionated with $(NH_4)_2SO_4$. Proteins precipitating with 30% saturation were collected by centrifugation (18,000 × *g*, 20 min at 4 °C), dissolved in minimum volume of 10 mM KPB, pH 6.0, and dialyzed against the same buffer with three changes. After that the dialyzed solution was centrifuged and the supernatant was stored at 4 °C and assayed for HNL activity.

Leaves, roots, shoots, spikes, or rhizomes were placed in four layers of cheese cloth and frozen by soaking in liquid nitrogen. The frozen samples were cracked with a hammer to release small broken pieces of the samples inside. They were made into finer pieces or powder by grinding on mortar and pestle on ice. The finely ground samples were suspended in 10 mM KPB, pH 7.0, and extracted overnight with stirring at 4 °C. The suspension was centrifuged (18,000 × *g*, 20 min at 4 °C), and removal of the residue gave a crude enzyme preparation, which was concentrated about 10 times by centrifugation at 2,000 × *g* and 4 °C with a Centriprep YM-10 (Amicon, Beverly, MA, U.S.A.), and then used for activity measurement.

The homogenates of the following 156 species among 74 families were inactive. L and S stand for the origin of the organ of the plant samples, leaves and seeds respectively. Other organs are specified in parentheses.
Acanthaceae: *Thunbergia grandiflora* (Roxb. ex Rottl.) Roxb. (L);
Aizoaceae: *Mollugo pentaphylla* Linn. (L);
Apocynaceae (2 species): *Nerium indicum* Mill. (L), *Trachelospermum jasminoides* (Lindl.) Lem. (S);
Aquifoliaceae: *Ilex verticillata* (Linn.) A. Gray (L);
Araceae (9 species): *Alocasia macrorrhiza* (Linn.) G. Don (L), *Anthurium andreanum* Linden 'Elizabeth' (L), *Anthurium andreanum* Linden 'Obake' (L), *Anthurium andreanum* Linden cv. (L), *Dieffenbachia amoena* Gentil (L), *Dieffenbachia maculata* (Lodd.) G. Don (L), *Monstera deliciosa* Liebm. (L), *Spathiphyllum* cv. (S), *Typhonodorum lindleyanum* Scott ex Oestr. (L);
Aristolochiaceae: *Aristolochia debilis* Sieb. et Zucc. (L);

Berberidaceae (2 species): *Mahonia japonica* (Thunb. ex Murr.) DC. (L), *Nandina domestica* Thunb. (L, S);
Betulaceae: *Corylus avellana* Linn. (L);
Calycanthaceae (2 species): *Calycanthus floridus* Linn. (L, S), *Chimonanthus praecox* (Linn.) Link "Concolor" (L, S);
Cannaceae: *Canna edulis* Ker-Gawl. (L);
Caprifoliaceae (3 species): *Sambucus adnata* Wall. ex DC. (L), *Sambucus nigra* Linn. (L), *Viburnum wrightii* Miq. (L);
Caricaceae: *Carica papaya* Linn. (L);
Celastraceae: *Euonymus alatus* (Thunb. ex Murr.) Sieb. (L);
Cochlospermataceae: *Cochlospermum vitifolium* (Willd.) Spreng. (L);
Commelinaceae: *Commelina communis* Linn. (L);
Compositae (4 species): *Artemisia princeps* Pamp. (L), *Bidens frondosa* Linn. (L), *Cynara scolymus* Linn. (L), *Matricaria recutita* Linn. (L);
Coriariaceae: *Coriaria nepalensis* Wall. (L);
Cornaceae (2 species): *Benthamidia florida* (Linn.) Spach (L), *Cornus officinalis* Sieb. et Zucc. (L, S);
Cucurbitaceae: *Trichosanthes cucumeroides* (Ser.) Maxim. (L);
Cycadaceae: *Cycas revoluta* Thunb. (L);
Cyclanthaceae: *Carludovica palmata* Ruiz et Pav. (L, S);
Dilleniaceae: *Dillenia indica* Linn. (L);
Ebenaceae: *Diospyros cathayensis* Steward (L, S);
Ericaceae: *Pieris japonica* (Thunb. ex Murr.) D. Don (L);
Euphorbiaceae (9 species): *Acalypha australis* Linn. (L), *Acalypha hispida* Burm. fil. (L), *Acalypha wilkesiana* Muell. Arg. (L), *Alchornea davidii* Franch. (L), *Antidesma bunius* (Linn.) Spreng. (L), *Chamaesyce maculata* (Linn.) Small (L), *Euphorbia grandicornis* Goeb. (L), *Mallotus japonicus* (Thunb.) Muell. Arg. (L), *Sapium sebiferum* (Linn.) Roxb. (L);
Ginkgoaceae: *Ginkgo biloba* Linn. (L);
Goodeniaceae: *Scaevola sericea* Vahl (L);
Hamamelidaceae (2 species): *Liquidambar formosana* Hance (L), *Liquidambar styraciflua* Linn. (L);
Hypoxidaceae: *Curculigo capitulata* (Lour.) O. Kuntze (L);
Juglandaceae (2 species): *Carya ovata* (Mill.) K. Koch (L), *Pterocarya stenoptera* C. DC. (L);
Labiatae: *Mentha arvensis* Linn. (L, S);
Lauraceae (2 species): *Machilus thunbergii* Sieb. et Zucc. (L, S), *Persea americana* Mill. (L);
Leguminosae (22 species): *Acacia baileyana* F. J. Muell. (L), *Aeschynomene indica* Linn. (L), *Albizia julibrissin* Durazz. (L), *Bauhinia variegata* Linn. (L), *Caesalpinia decapetala* (Roth) Alst. var. *japonica* (Sieb. et Zucc.) Ohashi (L, S), *Caesalpinia pulcherrima* (Linn.) Swartz (L), *Cassia mimosoides* Linn. (S), *Cassia siamea* Lam. (L), *Ceratonia siliqua* Linn. (S), *Cladrastis kentukea* (Dum.-Cours.) Rudd (L), *Crotalaria assamica* Benth. (S), *Derris trifoliata* Lour. (L), *Entada phaseoloides* (Linn.) Merr. (L), *Gleditsia japonica* Miq. (L), *Gleditsia triacanthos* Linn. var. *triacanthos* (L, S), *Gleditsia triacanthos* Linn. var. *inermis* Willd. (S), *Pueraria lobata* (Willd.) Ohwi (L), *Robinia psedoacacia* Linn. (L), *Sophora flavescens* Ait. (S), *Sophora japonica* Linn. (L, S), *Trifolium repens* Linn. (L), *Wisteria floribunda* (Willd.) DC. (S);
Liliaceae: *Eucomis autumnalis* (Mill.) Chitt. (L, S);
Linaceae: *Reinwardtia indica* Dumort. (L);
Lythraceae: *Lagerstroemia indica* Linn. (L);
Magnoliaceae (4 species): *Magnolia denudata* Desr. var. *purpurascens* (Maxim.) Rehd. et Wils. (L), *Magnolia virginiana* Linn. (L), *Michelia champaca* Linn. (L), *Liriodendron chinense* Sargent (L),
Malpighiaceae (2 species): *Malpighia glabra* Linn. (L), *Tristellateia australasiae* A. Rich. (L);
Malvaceae: *Abutilon theophrasti* Medik. (L, S);
Marattiaceae: *Angiopteris lygodiifolia* Rosenst. (L);
Marsileaceae: *Marsilea quadrifolia* Linn. (L);
Meliaceae: *Melia azedarach* Linn. (L, S);
Menyanthaceae: *Nymphoides peltata* (Gmel.) O. Kuntze (L);
Moraceae: *Broussonetia kazinoki* Sieb. (S);
Myrtaceae: *Eugenia javanica* Lam. (L);
Nymphaeaceae: *Nuphar japonicum* DC. (L, rhizome);
Nyssaceae (2 species): *Camptotheca acuminata* Decne (L), *Davidia involucrata* Baill. (L);
Oleaceae (3 species): *Jasminum sambac* (Linn.) Ait. (L), *Ligustrum lucidum* Ait. (L, S), *Osmanthus heterophyllus* (G. Don) P. S. Green (S);
Onagraceae: *Ludwigia stipulacea* (Ohwi) Ohwi (L);
Orchidaceae: *Bletilla striata* (Thunb. ex Murr.) Reichb. fil. (L);
Oxalidaceae: *Oxalis corniculata* Linn. (L);
Palmae (2 species): *Butia yatay* (Mart.) Becc. (L, S); *Washingtonia filifera* (Linden ex Andre) H. Wendl. (L);
Passifloraceae (3 species): *Passiflora coccinea* Aubl. (L), *Passiflora ligularis* Juss. (L), *Passiflora quadrangularis* Linn. (L);
Poaceae (5 species): *Arthraxon hispidus* (Thunb. ex Murr.) Makino (L), *Dendrocalamus giganteus* Munro (L), *Imperata cylindrical* Beauv. (L), *Miscanthus tinctorius* (Steud.) Hack. (spikes), *Phyllostachys heterocycla* (Carr.) Mitford f. *pubescens* (Mazel ex Houz. de Leh.) Muroi (shoots);
Pteridaceae (2 species): *Acrostichum aureum* Linn. (L), *Pteris fauriei* Hieron. (L);
Punicaceae: *Punica granatum* Linn. (L);
Ranunculaceae: *Aconitum carmichaelii* Debx. (roots);
Rosaceae (10 species): *Cotoneaster franchetii* Bois (S), *Kerria japonica* (Linn.) DC. (L), *Prunus tomentosa* Thunb. ex Murr. (L), *Pyracantha coccinea* M. J. Roem. (L, S), *Rhaphiolepsis indica* (Linn.) Lindl. ex Ker var. *umbellata* (Thunb. ex Murr.) Ohashi (L), *Rosa hygonis* Hemsl. (L), *Rosa moschata* J. Herrm. (L), *Rosa nitida* Willd. (L), *Rosa rugosa* Thunb. ex Murr. (L), *Spiraea thunbergii* Sieb. ex Blume (L);
Rubiaceae (4 species): *Gardenia jasminoides* Ellis (L),

Gardenia sootepensis Hutch. (L), *Paederia scandens* (Lour.) Merrill (L), *Rubia argyi* (Lev.) H. Hara (L);
Rutaceae (3 species): *Citrus aurantium* Linn. (L), *Murraya paniculata* (Linn.) Jack (L, S), *Poncirus trifoliata* (Linn.) Rafin. (S);
Sapindaceae (2 species): *Cardiospermum halicacabum* Linn. (S), *Koelreuteria paniculata* Laxm. (L);
Saxifragaceae: *Hydrangea quercifolia* Bartr. (L);
Simaroubaceae: *Ailanthus altissima* Swingle (L);
Solanaceae: *Solanum quitoense* Lam. (L);
Sterculiaceae: *Dombeya wallichii* (Lindl.) K. Schum. (L);
Tamaricaceae: *Tamarix chinensis* Lour. (L);
Taxaceae: *Taxus cuspidata* Sieb. et Zucc. var. *nana* Hort. ex Rehder (L);
Taxodiaceae (2 species): *Metasequoia glyptostroboides* H. H. Hu et Cheng (L), *Taxodium disticum* (Linn.) L. Rich. (L);
Theaceae: *Eurya emarginata* (Thunb. ex Murr.) Makino (L, S);
Typhaceae: *Typha latifolia* Linn. (L);
Umbelliferae: *Hydrocotyle sibthorpioides* Lam. (L);
Urticaceae: *Boehmeria platanifolia* Franch. et Savat. (L);
Verbenaceae (3 species): *Clerodendrum macrosiphon* Hook. fil. (L), *Duranta repens* Linn. (L), *Vitex agnus-castus* Linn. (L);
Zingiberaceae: *Elettaria cardamomum* (Linn.) Maton (L);

Protein determination. Protein concentration was determined with a Bio-Rad protein assay Kit (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as a standard, measuring absorbance at 595 nm.[13)]

Results

Activity measurement of (R)-mandelonitrile lyase from Prunus dulcis (almond)

(*R*)-Mandelonitrile lyase from *Prunus dulcis* was used as a typical source of HNL, and its reaction conditions were investigated. It was possible to detect both the enantiomers of mandelonitirle by HPLC with a column CHIRALCEL OJ-H. The retention times were confirmed with commercially available (*R*)- and racemic mendelonitrile. The yields were quantitatively measured by HPLC with the calibration curves. The time course of the formation of (*R*)-mandelonitirile at various pHs from pH 4.0 to 6.5 is shown in Fig. 1. The theoretical yield (100%) of mandelonitrile was 50 mM. The reaction was completed in a few minutes and activity was high at pH 4.5. Since mandelonitirle formed is stable at pH 4.5, as shown in Fig. 1, pH 4.5 was chosen as the reaction condition. Figure 2 shows the changes in enantiomeric

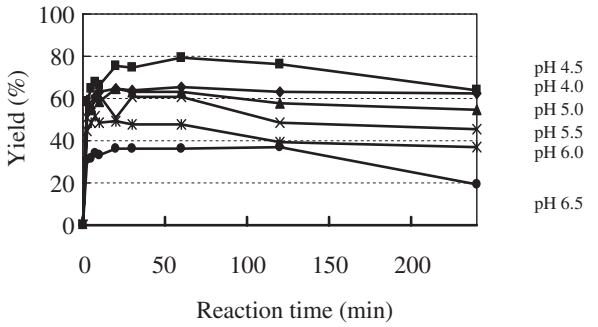

Fig. 1. Effect of Various pHs on (*R*)-Mandelonitrile Synthesis with Partially Purified HNL from *Prunus dulcis* (almond).
The conditions of the enzyme reactions were such and (*R*)-mandelonitrile concentrations were measured as described in "Materials and Methods". pH 4.0 (◆), pH 4.5 (■), pH 5.0 (▲), pH 5.5 (×), pH 6.0 (∗), pH 6.5 (●).

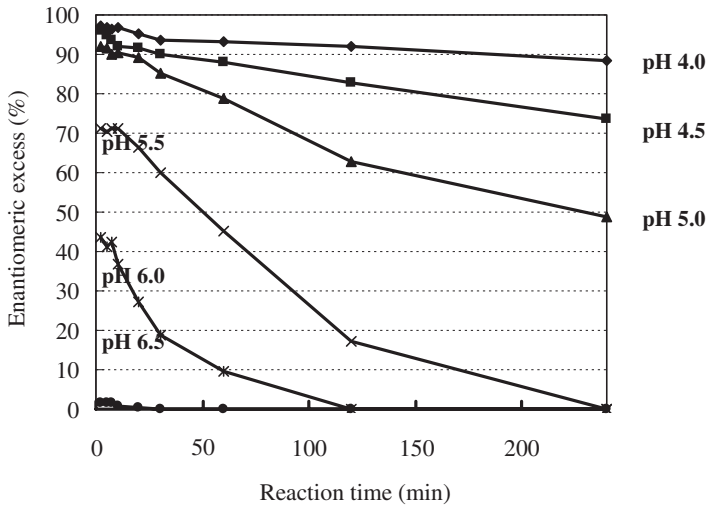

Fig. 2. Effect of Various pHs on the Enantiomeric Excess of (*R*)-Mandelonitrile Synthesis with Partially Purified HNL from *Prunus dulcis* (almond).
The conditions of the enzyme reactions were such and the enantiomeric excess of (*R*)-mandelonitrile was measured as described in "Materials and Methods". The enantiomeric excess of the samples from the experiment in Fig. 1 are shown. pH 4.0 (◆), pH 4.5 (■), pH 5.0 (▲), pH 5.5 (×), pH 6.0 (∗), pH 6.5 (●).

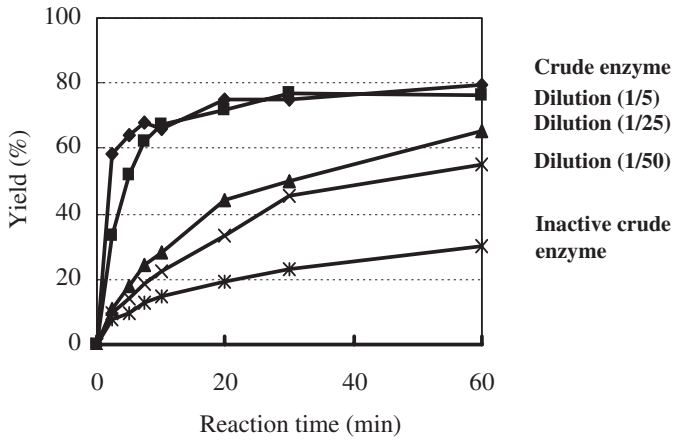

Fig. 3. Time Course of Synthesis of (*R*)-Mandelonitrile at pH 4.5 by Partially Purified HNL from *Prunus dulcis* (almond).
The conditions of the enzyme reactions were such and the (*R*)-mandelonitrile concentrations were measured as described in "Materials and Methods". The enzyme solution (73.5 U/mg) was diluted 1.0 (◆), 1/5 (■), 1/25 (▲), or 1/50 (×) times, or was heat-treated enzyme (∗).

Table 1. HNL Activity Shown by the Homogenates of Various Plants

Species	Family	Organ	Stereochemistry	Activity[a] (units/mg protein of homogenate)
Baliospermum montanum	Euphrobiaceae	Leaves	*S*	0.63
Passiflora edulis	Passifloraceae	Leaves	*R*	16.3
Passiflora edulis	Passifloraceae	Seeds	*R*	12.7
Eriobotrya japonica	Rosaceae	Seeds	*R*	9.70
Prunus mume	Rosaceae	Seeds	*R*	6.88
Prunus persica	Rosaceae	Seeds	*R*	1.69
Chaenomeles sinensis	Rosaceae	Seeds	*R*	1.68
Sorbus aucuparia	Rosaceae	Seeds	*R*	0.70

[a]The homogenate of kernels from *Prunus dulcis* showed 73.5 units/mg of (*R*)-HNL activity under the same condition.

excess of mandelonitrile formed in the same reaction as described in Fig. 1 at various pHs. In a short reaction time, the enantiomeric excess of the product was high at pHs 4.0 and 4.5, although at pHs lower than 5.5, optical purity was soon lost. Since the reaction product, once formed, was kept at neutral pH, racemization and loss of enantiomeric excess was observed in the region of pH 5.0 to 6.0. Figure 3 shows the time course of (*R*)-mandelonitirle formation at pH 4.5, which gave the best result.

In a control experiment, boiled enzyme was used, with which an appreciable amount of racemic mandelonitrile was formed. Hence, in the actual activity measurement, the amount of total racemic mandelonitirile formed non-enzymatically was subtracted from the amount of mandelonitrile formed in the first a few minutes, when the reaction progressed linearly.

Screening for various (R)- and (S)-mandelonitrile lyase producing plants

One hundred seventy-three species of plants originating in 71 families (Rosaceae [15], Euphorbiaceae [10], Leguminosae [22], Passifloraceae [4], Araceae [9] and other families [103]) were collected at the Botanic Gardens of Toyama or other sources, as shown in Table 1. In these plants, it was found that homogenate of *Baliospermum montanum* (Willd.) Muell. Arg. (Euphorbiaceae) leaves showed (*S*)-mandelonitrile activity (0.63 units/mg), while homogenates of leaves and seeds from *Passiflora edulis* (Passifloraceae) (16.3 units/mg (leaves)), seeds from *Eriobotrya japonica* (Thunb.) Lidnley (Rosaceae) (biwa in Japanese) (9.70 units/mg), *Prunus mume* (Sieb.) Sieb. et Zucc. (Rosaceae) (ume in Japanese) (6.88 units/mg), *Prunus persica* (Linn.) Batsch cv. (Rosaceae) (hanamomo in Japanese), *Chaenomles sinensis* (Thouin) Koehne (Rosaceae) (karin in Japanese), and *Sorbus aucuparia* Linn. (Rosaceae) (mountain ash) showed (*R*)-HNL activity. The HNL activity shown by the homogenates of these new sources of HNL was enough to be detected by the HPLC method, although lower than that shown by seeds of *Prunus dulcis* (73.5 units/mg). The stereochemistry of the product was determined by comparing the retention time of (*R*)-mandelonitrile or (*R*)-2-hydroxy-2-methylpentanoic acid methyl ester, synthesized with HNL from *Prunus dulcis*, as a standard.[4)]

Synthesis of optically active methylpropylketone cyanohydrin using (R)-HNLs from Passiflora edulis and Eriobotrya japonica and (S)-HNL from Baliospermum montanum

(*R*)-Methylpropylketone cyanohydrin synthesis using (*R*)-HNL from *Prunus dulcis* (almond) has been reported.[4] The product methylpropylketone cyanohydrin was derivatized to 2-methyl-2-hydroxy pentanoic acid, and then to 2-methyl-2-hydroxy pentanoic acid methyl ester to estimate the chilarity of the product. 2-Pentanone (2 mmol) was mixed with citrate buffer (0.4 M, pH 4.5, 39 ml), to which was added potassium cyanide solution (1.0 M, 5.0 ml), and the mixture was stirred. Then the reaction was started by the addition of 5.0 ml of the enzyme solution from homogenates of the enzyme sources: (*R*)-HNL from leaves of *Passiflora edulis* (46 units), seeds from *Eriobotrya japonica* (16.9 units), *Prunus dulcis* (87.5 units), and (*S*)-HNL from *Baliospermum montanum* (9.5 units), and this was incubated at 25 °C for 4 h. The reaction was started by the addition of 5.0 ml of the enzyme solution, and the reaction mixture was incubated at 25 °C for 4 h, as described by Effenberger *et al.*[4] After 4 h, the reaction mixture was extracted three times with 50 ml ethyl acetate. The organic layer was dried over Na_2SO_4 and concentrated. After the residue had been dried for 12 h *in vacuo*, optically active methylpropylketone cyanohydrins from 2 mmol of 2-pentanone, using HNLs from *Passiflora edulis*, *Eriobotrya japonica*, *Prunus dulcis*, and *Baliospermum montanum*, were obtained in 34.0% yield (76.9 mg), 38.9% yield (85 mg), 46.4% yield (105 mg), and 31.5% yield (71.2 mg) respectively. The compounds were identified as methylpropylketone cyanohydrin, from the following spectral data: ^{1}H-NMR (400 MHz, $CDCl_3$) $\delta = 1.76$ (2H, m), 1.61 (3H, s), 1.55 (2H, m), 1.00 (3H, t, $J = 7.6$ Hz). ^{13}C-NMR (100 MHz, $CDCl_3$) $\delta = 121.95$, 68.64, 43.75, 27.66, 17.60, 13.75.

Synthesis of optically active 2-hydroxy-2-methylpentanoic acid

Concentrated HCl (5 ml) was added to the enzymatically synthesized methylpropylketone cyanohydrin. The mixture was stirred for 7 h at room temperature, heated to 60 °C for 12 h, and then boiled for 5 h. After HCl gas was removed *in vacuo* using an aspirator, the residue was extracted three times with 15 ml each time of diethyl ether. The extracts were dried over Na_2SO_4, and concentrated and dried for 5 h *in vacuo*. Optically active 2-hydroxy-2-methylpentanoic acids, using HNLs from *Passiflora edulis* and *Prunus dulcis*, were obtained as crystals in 27.8% yield (73.5 mg) and 30.8% yield (40.7 mg) respectively from 2 mmol of 2-pentanone, while those synthesized by HNLs from *Eriobotrya japonica* and *Baliospermum montanum* were obtained as colorless oil in 32.6% yield (83.6 mg) and 29.0% yield (77.6 mg) respectively from 2 mmol of 2-pentanone. The compounds were identified as 2-hydroxy-2-methylpentanoic acid from the following spectral data: ^{1}H-NMR (400 MHz, $CDCl_3$) $\delta = 1.70$ (2H, m), 1.46 (3H, s), 1.24 (2H, m), 0.96 (3H, t, $J = 7.1$ Hz). ^{13}C-NMR (100 MHz, $CDCl_3$) $\delta = 181.37$, 74.66, 42.15, 25.91, 16.89, 14.04.

Synthesis of 2-hydroxy-2-methylpentanoic acid methyl ester from 2-hydroxy-2-methylpentanoic acid, and determination of the configuration of the products

To a reaction mixture comprising 13.2 mg of 2-hydroxy-2-methylpentanoic acid (0.1 mmol), 200 µl methanol, and 700 µl toluene, was slowly added 65 µl (Trimethylsilyl) diazomethane (0.13 mmol).[14] Nitrogen gas evolved from the reaction mixture as the methylation reaction proceeded. The reaction was stopped when the color of the mixture changed from yellow to colorless. The reaction mixture was concentrated and dried for 4 h *in vacuo*, and the 2-hydroxy-2-methyl pentanoic acid methyl ester formed was dissolved in ethyl acetate, and analyzed with a Shimazu GC-14B gas-liquid chromatograph, as described below. The column used was Cyclodextrine-β-236M-19 (0.25 mm × 25 m, DF = 0.25, GL Science Inc., Tokyo, Japan). Helium was used as a carrier gas a flow rate of 40 ml/min. H_2 and air were used at 0.6 kg/cm^2 each. The injection port temperature was 220 °C. The oven temperature was 85 °C for 2-hydroxy-2-methylpentanoic acid methyl ester.

Optically active methylpropylketone cyanohydrin was synthesized using partially purified HNL enzymes from *Passiflora edulis*, *Eriobotrya japonica*, and *Baliospermum montanum*. The retention times of (*R*)- and (*S*)-2-methyl-2-hydroxy pentanoic acid methyl ester were about 6.1 and 6.8 min respectively. The specific activities of HNLs, detected in the crude preparation of various plant sources with benzaldehyde as a substrate, are shown in Table 2. The enantiomeric excess of (*R*)-methylpropylketone cyanohydrin synthesized from 2-pentanone using homogenate from leaves of *Passiflora edulis* was 87.0%, and that of (*R*)-mandelonitrile synthesized by homogenate from seeds of *Eriobotrya japonica* was 85.0%. Enantiomeric excess was calculated from the peak areas of (*R*)-2-hydroxy-2-methylpentanoic acid methyl ester, which had been synthesized with *Prunus dulcis* HNL[15] and (*S*)-2-hydroxy-2-methylpentanoic acid methyl ester, both synthesized and detected as one of the racemates.

Table 2. Summary of HNL Activites Shown by Various Plant Sources and Their Application to the Synthesis of Hydroxynitriles

Species		Benzaldehyde		2-Pentanone	
		e.e.	*R/S*	e.e.	*R/S*
Baliospermum montanum	(Leaves)	37.8	*S*	—	—
Passiflora edulis	(Leaves)	69.4	*R*	87.0	*R*
Eriobotrya japonica	(Seeds)	85.0	*R*	24.2	*R*
Prunus dulcis	(Seeds)	89.8	*R*	72.2	*R*

—; no reaction.

Table 3. Comparison of the HPLC and Spectrophotometric Assays for HNL

Evaluation point	HPLC[a]	Spectrophotometeric[16,27]
Sensitivity in quantification	Enough	Not enough and cannot be used for colored samples
Simplicity for assay	Less simple	Simple
Preparation of cyanohydrin as a substrate	Not necessary	Necessary
Determination of configuration	Possible	Not possible

[a]this study.

Discussion

We started screening for new HNLs because we noticed that the number of reported HNLs (distributed in less than 20 species) is much smaller than the number of cyanogenic plants (2,650 species),[2,3] or of cyanogenic glycosides (about 65 kinds). Although these aspects of plant cyanogenesis are interrelated, the number of HNL-producing plants appears to be too small. Therefore, screening and discovery of new HNLs should contribute not only to basic studies of plant cyanogenesis, but also to the development of a new bioprocess for chiral cyanohydrin synthesis.

At least 65 cyanogenic glycosides, 28 non-cyanogenic nitrile glycosides, and 2 fairly stable cyanohydrins are known from higher plants.[1–3] The cyanogenic glycosides are classified into six groups from the biosynthetic pathway of amino acids. These are considered to be synthesized from either phenylalanine, tyrosine, valine/isoleucine, leucine, 2-(2′-cyclopentenyl) glycine, or nicotinic acid.

One of the most well-studied HNLs, madelonitrile lyase (EC 4.1.2.10) produced by *Prunus dulcis* (Miller) D. A. Webb (syn: *Prunus amygdalus* Batsch.) (almond), is (*R*)-specific and catalyzes a reversible reaction acting on (*R*)-mandelonitrile to form benzaldehyde and cyanide.[4,5,16] HNL is a monomeric glycoprotein, requiring FAD as a cofactor, with a M_r of 50,000. Similar enzymes are reported to be distributed among *Prunus laurocerasus*,[17] which produces HNL with an M_r of about 60,000. The M_rs of the enzymes from *Prunus serotina*[18] and *Prunus lyonii*[19] are about 50,000 to 59,000, while the enzyme from *Ximenia americana* is without FAD, with a M_r of about 37,000 to 38,000, and acts on (*S*)-4-hydroxymandelonitrile in 77% mandelonitrile.[10] Hydroxymandelonitrile lyase (EC 4.1.2.11) has been reported to be distributed in *Sorghum vulgare*[20] and *Sorghum bicolor*.[21] The enzyme from *Sorghum bicolor* does not accept aliphatic ketones. An aliphatic ketone- and (*R*)-specific enzyme, acetone cyanohydrin–lyase (EC 4.1.2.37), has been found to be produced by *Linum usitatissimum* (Flax).[22] (*S*)-Specific 2-hydroxyisobutyronitrile acetone–lyase is also called hydroxynitrilase (EC 4.1.2.39), and has been purified from *Heava brasilliensis* (rubber)[8] and *Manihot esculenta* (cassava).[9] The X-ray structures of these HNLs, such as mandelonitrile lyase from *Prunus dulcis*, *p*-hydroxymandelonitrile lyase from *Sorghum bicolor*, hydroxynitrilase from *Hevea brasiliensis*, and *Manihot esculenta*, have been reported.[23–26]

As described here, we developed a new method for measurement of mandelonitrile lyase activity by detecting mandelonitirile formation with HPLC. To assay the enzyme activity, two methods has been used. One is UV spectrophotometric detection of benzaldehyde at 249 nm at normal pH,[27] and the other is colorimetric detection of cyanide in the degradation reaction.[16] However, for the purpose of screening unknown activity determining the stereochemistry of the product, impurities having absorption in the ultraviolet region and interference by the green color of chlorophyll, and sometimes a lack of the both the enantiomers of the substrate, are among the drawbacks in measurement. For this reason, we established a simple method using HPLC. It became clear that the specific activity of almond HNL in the cyanohydrin-forming reaction at pH 4.5 is about five times higher than the degradation reaction at pH 6.0. For example, the specific activity of a partially purified mandelonitrile lyase from almond was calculated to be 73.5 units/mg in the synthetic reaction. The two methods of enzymatic activity measurement, the HPLC method and the established spectrophotometric methods,[16,27] were compared, as shown in Table 3. The features and advantages of the new HPLC methods are as follows: (1) Sensitivity; HPLC is more sensitive than the spectrophotometric method, although both appear to be sensitive enough to measure the activity of partially purified clear HNL samples. However, the spectrophotometric assay has an inevitable drawback in that it cannot be applied to plant homogenates with dark colors such as the green color of chlorophyll. (2) Simplicity for assay; a spectrophotometer is easier to handle than the less distributed apparatus HPLC. (3) Availability of the substrate; the HPLC method has an advantage in that there is no need to prepare cyanohydrin, while in the case of spectrophotometric assay, one must have substrate cyanohydrin. (4) Determination of configuration; this is definitely possible with a chiral column in HPLC, but not possible in the spectrophotometric method.

We investigated for (*R*)- and (*S*)-mandelonitrile lyase activities from the homogenates of various organs of plants, such as leaves, roots, shoots, spikes, and rhizomes of a total of 163 species of plants among 74 families, mainly from the Botanic Gardens of Toyama.

We targeted plants belonging to Rosaceae, Euphorbiaceae, Leguminosae, and Passifloraceae, because many cases of cyanogenesis have reported in these families, but also many other families. We discovered that homogenate of leaves of *Baliospermum montanum* (Euphorbiaceae) shows (*S*)-HNL activity. No cyanogenesis nor any occurrence of cyanogenic glycoside has been reported in *Baliospermum montanum*. The enantiomeric excess of (*S*)-mandelonitrile synthesized using partially purified (*S*)-HNL from *Baliospermum montanum* was about 37.8%. On the other hand, leaves and seeds from the *Rosaceae* plants *Passiflora edulis*, *Eriobotrya japonica*, *Prunus mume*, *Prunus persica*, *Chaenomeles sinensis*, and *Sorbus aucuparia* showed (*R*)-HNL activity. (*R*)-HNL is known to occur in Rosaceae and Linaceae. We found the occurrence of (*R*)-HNL in *Passiflora edulis* (Passifloraceae) for the first time. The role of these HNLs in cyanogenesis ought to be studied further, because cyanogenic glycosides have been isolated from *Passiflora edulis*,[28] and prunasin and amygdalin have been identified in *Prunus mume*.[29] (*R*)-Cyanohydrins are synthesized with the meals of peach (*Prunus persica* L.) and loquat (*Eriobotrya* sp. L.) in micoaqueous organic medium, although the enzyme activity and other properties are not clear.[30]

Thus, we discovered new producers of HNL of plant origin, while less than 20 plants have been reported to be the producers of HNLs in the long history of the enzymology of HNLs. These results show that the newly discovered HNLs have the potential of application in the asymmetric synthesis of optically active cyanohydrins. It is of interest to know the structure and the detailed enzymatic properties of these new enzymes, because (*S*)-specific HNLs such as the one in *Baliospermum montanum* are rarely found, and an (*R*)-specific HNL from *Prunus mume* showed very high stereoselectivity and wide substrate specificity in (*R*)-cyanohydrin formation (data not shown). The substrate specificities and the classification of these HNLs will be made clear in detail in future studies. The enantiomeric excess of the chiral cyanohydrins synthesized in water, as shown in Table 2, can be further improved by optimizing the reaction conditions or by using HNLs an organic medium. Some of these enzymes will soon be purified to homogeneity, the enzymatic properties of the enzymes will be characterized, and cDNA cloning of the gene for the enzyme is in progress.

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Note Added in Proof

While this paper was under reviewing process, our paper on HNL from *Prunus mume* has appeared.[31]

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