Available online at www.sciencedirect.com
SCIENCE DIRECT
Tetrahedron
ELSEVIER
Tetrahedron 61 (2005) 10908–10916

A new (*R*)-hydroxynitrile lyase from *Prunus mume*: asymmetric synthesis of cyanohydrins

Samik Nanda, Yasuo Kato and Yasuhisa Asano*

Biotechnology Research Center, Toyama Prefectural University, 5180 Kurokawa, Kosugi, Toyama 939-0398, Japan

Received 8 August 2005; revised 31 August 2005; accepted 31 August 2005

Available online 23 September 2005

Abstract—A new hydroxynitrile lyase (HNL) was isolated from the seed of Japanese apricot (*Prunus mume*). The enzyme has similar properties with HNL isolated from other *Prunus* species and is FAD containing enzyme. It accepts a large number of unnatural substrates (benzaldehyde and its variant) for the addition of HCN to produce the corresponding cyanohydrins in excellent optical and chemical yields. A new HPLC based enantioselective assay technique was developed for the enzyme, which promotes the addition of KCN to benzaldehyde in a buffered solution (pH=4.5).

1. Introduction

Hydroxynitrile lyases (HNLs) are widely distributed in nature and believed mainly to be a plant enzyme. There are four classes of HNL reported in the literature till now,[1] among them mandelonitrile lyase (EC 4.1.2.10) has been extensively studied in the area of enzymology and organic synthesis. Hydroxynitrile lyases (HNLs) are one of the key enzymes in cyanogenic plants,[2] catalyzing the final step in the biodegradation pathway of cyanogenic glycosides releasing HCN and the corresponding carbonyl components. HCN released by HNL can serve as a repellent factor to predators or as susceptibility component of plants to fungal attack when produced at high local concentrations.[3] Mandelonitrile lyase, one of the most well studied HNLs, are mainly found in Rosaceae species (Genus: *Prunus*), catalyzes the formation of benzaldehyde and HCN from (*R*)-mandelonitrile.[4] Whereas formation of the cyanohydrin from aldehyde and HCN should also be catalyzed by the same enzyme. Pioneering work by Effenberger, Griengl and others opened a new era in the area of asymmetric cyanohydrin synthesis by HNLs.[5] For example, crude meals from the kernels of almond, apple, cherry, apricots, and plums serve as source of HNLs. To detect new HNLs, it has proved not sufficient simply to screen for the presence of carbonyl compounds or HCN, since the breakdown of cyanohydrins may be catalyzed by enzymes other than HNLs: instead, the catalysis of cyanohydrin formation is a more reliable indicator of the presence of a HNL.[6]

Optically active cyanohydrins are excellent building blocks for the total synthesis of several biologically active natural products.[7] Both the alcohol and the nitrile parts of the cyanohydrin functionality can undergo transformation to a range of groups.[8] There are general methods that proceed without racemization, so that the optical purity is retained. In our previous communication we had reported a few cyanogenic plant species capable of showing mandelonitrile lyase activity[9] from a rich biodiversity of plant kingdom available in Japan. Japanese flowering apricot may be the longest lived of the flowering fruit trees eventually forming a picturesque 20-ft tall tree. It produces Ume fruit round in shape and 1–3 inches in diameter. The ripened fruit is yellowish red in color and have a fleshy covering. In this article, we would like to report our research on a new HNL from the seed of Japanese apricot.

2. Results and discussion

2.1. Enzyme assay

The enzyme was isolated from the seeds of ripened Ume fruit. Crude preparation of enzyme serves the purpose of our study, but we have partially purified the enzyme by 30% $(NH_4)_2SO_4$ precipitation to obtain a homogeneous solution

Keywords: Hydroxynitrile-lyase; *Prunus mume*; Cyanohydrins; Asymmetric synthesis.
Abbreviations: HNL, hydroxynitrile lyase; PmHNL, *Prunus mume* hydroxynitrile lyase; PaHNL, *Prunus amygdalus* hydroxynitrile lyase; DIPE, diisopropyl ether; TBME, *tert*-butyl methyl ether; DBE, di-*n*-butyl ether; DME, 1,2-dimethoxyethane.
* Corresponding author. Tel.: +81 766 56 7500x530; fax: +81 766 56 2498; e-mail: asano@pu-toyama.ac.jp

0040–4020/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2005.08.105

Table 1. Monosubstituted benzaldehydes screened with crude PmHNL

Substrate	Conversion (%)[a]	ee (%)[b]	Configuration[c]
Benzaldehyde	13	93	*R*
2-Chlorobenzaldehyde	37	56	*R*
3-Chlorobenzaldehyde	38	92	*R*
4-Chlorobenzaldehyde	21	99	*R*
4-Bromobenzaldehyde	22	99	*R*
4-Fluorobenzaldehyde	28	84	*R*
2-Methylbenzaldehyde	6.0	61	*R*
3-Methylbenzaldehyde	7.5	87	*R*
4-Methylbenzaldehyde	7.0	95	*R*
2-Methoxybenzaldehyde	6.0	41	*R*
3-Methoxybenzaldehyde	31	92	*R*
4-Methoxybenzaldehyde	17	97	*R*
2-Trifluoromethylbenzaldehyde	72	5	*R*
3-Trifluoromethylbenzaldehyde	91	68	*R*
4-Trifluoromethylbenzaldehyde	90	76	*R*
3-Nitrobenzaldehyde	87	65	*R*
4-Nitrobenzaldehyde	89	71	*R*
4-Benzyloxybenzaldehyde	5.8	98	*R*
4-*N*,*N*-Dimethylaminobenzaldehyde	nd	0	
4-Hydroxybenzaldehyde	nd	5	*R*
3-Phenoxybenzaldehyde	42	>99	*R*
4-Allyloxybenzaldehyde	6.4	98	*R*
4-*tert*-Butyldimethylsilyloxybenzaldehyde	4.8	97	*R*

[a] Conversion (%) was calculated after 30 min of reaction time.[15]
[b] Determined by chiral HPLC.
[c] Confirmed by comparing the retention time with that of optically active standard compounds.

of enzyme. Previously reported HNL assay method[10] mainly involved the decomposition of mandelonitrile in a buffered solution and the formation of benzaldehyde was measured spectrophotometrically. We have developed a new HPLC based assay technique, in which the formation of (*R*)-mandelonitrile from benzaldehyde in citrate buffer (pH=4.5) was monitored by chiral HPLC. At different time intervals aliquots were withdrawn and analyzed by chiral HPLC. It was assumed that at low pH, corresponding chemical reaction was suppressed, so the overall mandelonitrile production only comes from HNL catalyzed addition. However, to obtain accurate results, a blank reaction was performed side by side without enzyme, and the amount of mandelonitrile obtained was deducted from the biocatalyzed reaction product. The HPLC based HNL assay technique has several advantages over the other traditional assay technique. Sensitivity and reproducibility are very high in the HPLC based assay method. Using different dilution of enzyme and varying substrate concentration yields almost similar results in all case. Moreover, cyanohydrins itself are well separated in a chiral column, hence avoiding further derivatization method. Finally, configurational assignment of the product cyanohydrins is also possible with comparing standard compounds (synthesized by chemical methods).

2.2. Reaction condition for substrate screening

Next, our focus is to test a large number of different aldehydes and ketones for possible substrate of PmHNL (*Prunus mume* hydroxynitrile lyase). Generally similar reaction condition as the PmHNL assay was employed for the substrate screening methods. In a typical screening methods substrates were taken in aq buffer (pH 4.5) followed by addition of KCN (1.25 equiv) and enzyme solution (see Section 4 for detailed description). After certain time (see Tables 1–6), reaction mixtures were taken and the formation of products were analyzed with chiral HPLC/GC. The main intention of the study was to test a large number of substrates and find the substrates, which provide better enantioselection. We have attempted an enzymatic transcyanation with acetone cyanohydrin as the cyanide source, but the slow reaction (decomposition of acetone cyanohydrin is slow at lower pH e.g., 4–4.5) rate

Table 2. Heteroaromatic and polycyclic aldehydes screened with crude PmHNL

Substrate	Conversion (%)[a]	ee (%)[b]	Configuration[c]
2-Furancarboxaldehyde	1.2	98	*S*
2-Thiophenecarboxaldehyde	31	88	*S*
2-Pyridinecarboxaldehyde	89	22	*S*
3-Pyridinecarboxaldehyde	90	75	*R*
4-Pyridinecarboxaldehyde	65	41	*R*
2-Quinolinecarboxaldehyde	38	21	*S*
4-Quinolinecarboxaldehyde	73	28	*R*
1-Napthalenecarboxaldehyde	60	93	*R*
2-Napthalenecarboxaldehyde	58	98	*R*
9-Anthral	16	0	

[a] Conversion (%) was calculated after 30 min of reaction time.[15]
[b] Determined by chiral HPLC.
[c] Confirmed by comparing the retention time with that of optically active standard compounds.

Table 3. Disubstituted benzaldehydes screened with crude PmHNL

Substrate	Conversion (%)[a]	ee (%)[b]	Configuration[c]
2,3-Dichlorobenzaldehyde	11	22	*R*
2,4-Dichlorobenzaldehyde	13	78	*R*
2,5-Dichlorobenzaldehyde	8.8	57	*R*
2,6-Dichlorobenzaldehyde	10	12	*R*
3,4-Dichlorobenzaldehyde	7.9	94	*R*
3,5-Dichlorobenzaldehyde	21	92	*R*
2,3-Dimethoxybenzaldehyde	7.0	37	*R*
2,4-Dimethoxybenzaldehyde	11	48	*R*
2,5-Dimethoxybenzaldehyde	9.0	63	*R*
2,6-Dimethoxybenzaldehyde	6.5	32	*R*
3,4-Dimethoxybenzaldehyde	13	78	*R*
3,5-Dimethoxybenzaldehyde	17	97	*R*
Piperonal	34	98	*R*
2,4-Dimethylbenzaldehyde	5.8	86	*R*

[a] Conversion (%) was calculated after 30 min of reaction time.[15]
[b] Determined by chiral HPLC.
[c] Confirmed by comparing the retention time with that of optically active standard compounds.

Table 4. Polysubstituted benzaldehydes screened with crude PmHNL

Substrate	Conversion (%)[a]	ee (%)[b]	Configuration[c]
2,3,4-Trimethoxybenzaldehyde	14	11	*R*
2,4,5-Trimethoxybenzaldehyde	16	28	*R*
3,4,5-Trimethoxybenzaldehyde	24	31	*R*
2,3,4,5-Tetrafluorobenzaldehyde	26	23	*R*
2,3,5,6-Tetrafluorobenzaldehyde	21	12	*R*
2,3,4,5,6-Pentafluorobenzaldehyde	31	0	
2,3,4,5,6-Pentabromobenzaldehyde	18	0	

[a] Conversion (%) was calculated after 1 h of reaction time.[15]
[b] Determined by chiral HPLC.
[c] Confirmed by comparing the retention time with that of optically active standard compounds.

Table 5. Aliphatic methylketones screened with crude PmHNL

Substrate	Conversion (%)[a]	ee (%)[b]	Configuration[c]
2-Butanone[d]	48	72	*R*
2-Pentanone	46	81	*R*
3-Methyl-2-butanone	39	42	*R*
2-Hexanone[d]	48	80	*R*
4-Methyl-2-pentanone[d]	40	88	*R*
3,3-Dimethyl-2-butanone	28	38	*R*
2-Heptanone	39	74	*R*
5-Methyl-2-hexanone	30	76	*R*
2-Octanone	22	67	*R*
2-Nonanone	20	65	*R*
2-Decanone	18	52	*R*
2-Undecanone	21	31	*R*
2-Dodecanone	14	0	
Cyclopropylmethylketone	58	0	
Trimethylsilylmethylketone	62	72	*R*

[a] Conversion (%) was calculated after 3 h by analyzing ^{1}H NMR of the crude reaction mixture.
[b] Determined by chiral GC (β-Dex120).
[c] Confirmed by comparing the retention time with that of optically active standard compounds.
[d] These cyanohydrins are not resolved in chiral GC column, hence ee was determined by preparing the corresponding TMS–ether derivative.

Table 6. Aliphatic aldehydes screened with crude PmHNL

Substrate	Conversion (%)[a]	ee (%)[b]	Configuration[c]
Propanal	48	78	*R*
Butanal[d]	51	84	*R*
Isobutyraldehyde	43	88	*R*
Pivaladehyde	29	92	*R*
Pentanal[d]	36	85	*R*
Hexanal[d]	38	81	*R*
Cyclopentanecarboxaldehyde	51	91	*R*
Cyclohexanecarboxaldehyde	54	94	*R*

[a] Conversion (%) was calculated after 3 h by analyzing ^{1}H NMR of the crude reaction mixture.
[b] Determined by chiral GC (β-Dex325).
[c] Confirmed by comparing the retention time with that of optically active standard compounds.
[d] These cyanohydrins are not resolved in chiral GC column, hence ee was determined by preparing the corresponding TMS–ether derivative.

makes the process not applicable for screening purpose, whereas the use of HCN was avoided due to difficulties in handling. The pH profile of the reaction was investigated and it was found that the enzyme is active mainly at lower pH. Increasing the pH (pH of the final reaction mixture) higher than 5.5 led to yielding products of low optical purity due to the corresponding chemical reaction. At pH 6.5 activities was totally lost, and the product was completely racemic (Fig. 1).

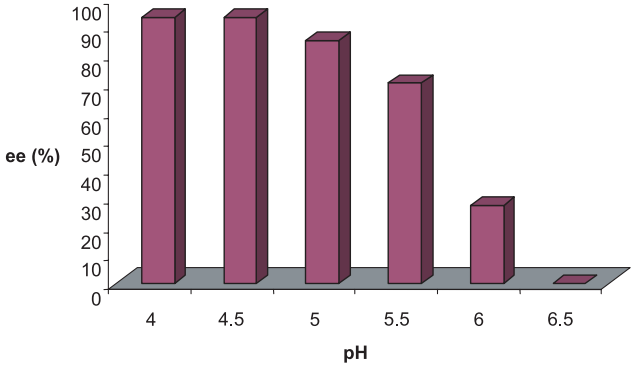

Figure 1. pH effect on ee of PmHNL catalyzed (*R*)-mandelonitrile formation.

The enzyme works nicely in a wide temperature range varying from −10 to 40 °C. The best temperature for the enzymic reaction was found to be 15–25 °C, using benzaldehyde as a substrate. Above 40 °C, the enzymic activity decreased significantly and it produces racemic cyanohydrin (Fig. 2).

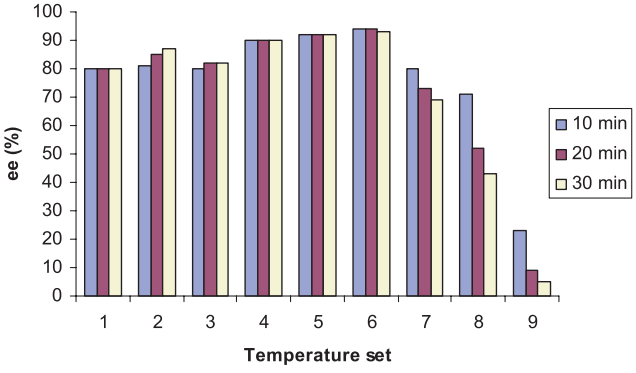

Figure 2. Temperature dependence of ee for (*R*)-mandelonitrile synthesis by PmHNL three sets of data were measured after 10, 20, and 30 min reaction time. Temperature of the reaction was −10 °C (1), 0 °C (2), 5 °C (3), 10–12 °C (4), 18–20 °C (5), 25 °C, (6), 40 °C (7), 50 °C (8), 70 °C (9).

Next, we have screened different organic solvents for the PmHNL activity. It has been reported by several researchers that, defatted powdered HNL meal (almond, apple, cherry) are active in polar but aprotic organic solvents and produces cyanohydrin with high enantioselectivity.[11] Whereas immobilization of HNLs on some support also allowed its use in organic solvent.[12] We have tested PmHNL's activity in several organic solvents by taking the partially purified homogeneous enzyme solution. A biphasic reaction condition (organic solvent/buffer, 10:1) allows us to detect the product by HPLC. The best solvent was diisopropylether (DIPE), *tert*-butylmethylether (TBME) and di-*n*-butylether (DBE). With all the three solvents more than 95% enantioselectivity was observed. However, the rate of the reaction was very slow and it takes 24–36 h to obtain appreciable amount of conversion. HCN in organic solvent and acetonecyanohydrin was used as the cyanating agent (Fig. 3).

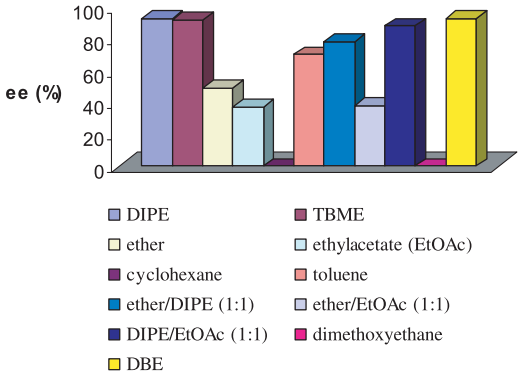

Figure 3. Solvent effect on ee of PmHNL catalyzed (*R*)-mandelonitrile synthesis.

2.3. Substrate specificity

A large number of substrates including aliphatic, aromatic, and heteroaromatic aldehydes as well as aliphatic ketones were tested for PmHNL activity.

2.3.1. Aromatic aldehydes. Aromatic aldehydes (mono, di, and polysubstituted), heteroaromatic aldehydes as well as polycyclic aromatic aldehydes were employed as PmHNL substrates. The results were summarized in Tables 1–5.

It was observed that substrates having *ortho* substituent (irrespective of its electronic nature) are poor substrates in terms of enantioselectivity of the resulting cyanohydrins. We have taken a series of substituted chlorobenzaldehydes and compared their enantioselectivity with PmHNL (Fig. 4).

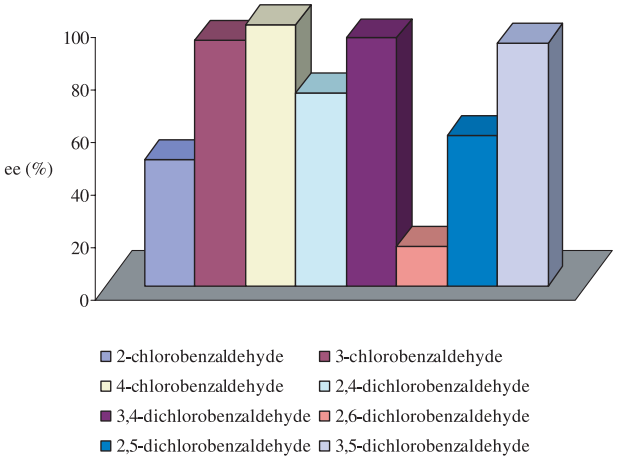

Figure 4. ee comparison of chlorosubstituted benzaldehydes (showing an *ortho* effect).

2.3.2. Aliphatic ketones. Mainly methyl ketones were tested for PmHNL activity. In a typical reaction ketones were taken in citrate buffer (pH 4.0, reaction pH 4.5),

followed by addition of enzyme and aq KCN solution. Generally it takes 3 h for appreciable amount of conversion (detected by TLC). Longer reaction time allows complete conversion but also leads to product having poor optical purity due to the corresponding chemical cyanation. The chemical yield of the obtained cyanohydrins was determined from ^{1}H NMR of crude samples. By comparing the integral values of Me-signals (singlet, at δ 2.2 ppm in the ketone and δ 1.5–1.6 ppm in the cyanohydrin) the relative ratios hence the chemical yield was determined.

The optical purity of the ketone cyanohydrins were measured efficiently by chiral GC (β-cyclodextrin stationary phase). Only three of the synthesized cyanhohydrins need to be derivatized (ethylmethylketone, *n*-butylmethylketone and isobutylmethylketone, as their TMS ether) in order to determine the ee. Remaining all cyanohydrins is well separated by β-cyclodextrin stationary phase, hence eliminating the derivatization protocol as often used by many researchers. The optical purity of the synthesized cyanohydrins is lower when compared with similar HNL from almonds. Methyl ketones having no branching at α-position are good substrates, for example, ethyl, *n*-propyl, *n*-butyl, and *n*-amyl methyl ketones provide cyanohydrin with 72–88% ee. Whereas substrates having branching at α-position give poor ee, for example, *i*-propyl and *t*-butyl methylketone yields cyanohydrin with 42 and 38% ee only. Increasing the chain length has a mild but definite effect on optical purity of the synthesized cyanohydrins. Undecanone (having C9 unit at one end) is the limiting substrate in the series accepted by PmHNL, although ee is low (31%, Table 5), where as dodecanone yields racemic cyanohydrin.

2.3.3. Aliphatic aldehydes. Aliphatic aldehydes were screened for PmHNL activity by similar methods as described earlier for corresponding methyl ketones. Reaction was monitored by TLC and chemical yield was determined with the help of ^{1}H NMR. It seems that aliphatic aldehydes are better substrates than their methyl ketone counterparts. Their reactivities are high probably due to the high reactivity of aldehyde functionality than those of the ketones. It is important to mention that all the aliphatic aldehydes tested so far, provides excellent enantioselection. Thus, it is quite evident that aliphatic aldehydes are better substrates for PmHNL than the corresponding methyl ketones. From the previous discussion we have also noticed, with the branching at α-position in the methyl ketone leads with poor enantioselection (*t*-butylmethyl ketone and isopropylmethyl ketone), whereas the corresponding aldehyde partners, for example, pivaladehyde and isobutyraldehyde both are excellent substrates for PmHNL (Table 6). Thus, presence of methyl moiety in the methyl ketones must play a decisive role for poor enantioselectivity. The optical purity of the product cyanohydrins was determined directly by chiral GC cyclodextrin columns and in some cases cyanohydrins were derivatized to the corresponding trimethylsilylether (OTMS).

2.3.4. Preparative scale asymmetric synthesis of cyanohydrins by PmHNL. Owing to their broad synthetic potential, cyanohydrins have attracted attention as starting materials for the preparation of several important classes of compounds, and it is probable that the use of enantiomerically pure cyanohydrins as building blocks for the production of chiral industrial chemicals will continue to grow. The pioneering work of Becker and Pfeil demonstrated for the first time that the HNL isolated from almond can be employed successfully for the production of (*R*)-cyanohydrins from aldehyde and HCN on a kilogram scale. One should consider four major points in a large scale HNL catalyzed synthesis of cyanohydrins, for example, reaction media, cyanating source, physical state of enzyme

Table 7a. Preparative scale cyanohydrin a synthesis by PmHNL with selected aromatic aldehydes

Substrate[a]	Yield (%)	Time (h)[b]	ee (%)[c]
Benzaldehyde	65	24	95
para-Tolualdehyde	68	42	96
para-Anisaldehyde	78	20	98
4-Fluorobenzaldehyde	60	16	86
4-Chlorobenzaldehyde	78	16	99
4-Bromobenzaldehyde	82	26	99
4-Nitrobenzaldehyde	93	6	71
3-Nitrobenzaldehyde	96	9	64
4-Trifluoromethylbenzaldehyde	88	8	72
2-Furan carboxaldehyde	65	78	96
2-Thiophene carboxaldehyde	82	24	88
3-Pyridine carboxaldehyde	80	12	65
2-Quinlonine carboxaldehyde	78	14	14
4-Quinlonine carboxaldehyde	82	15	21
Piperonal	88	30	97
3-Phenoxybenzaldehyde	68	36	98
3,4-Dichlorobenzaldehyde	52	52	94
3,5-Dichlorobenzaldehyde	58	60	95
3,4-Dimethoxybenzaldehyde	65	48	92
3,5-Dimethoxybenzaldehyde	48	50	93
4-Benzyloxybenzaldehyde	60	72	98
Napthalene-1-carboxaldehyde	70	32	90
Napthalene-2-carboxaldehyde	78	40	96

[a] All reactions were performed with 5 g of substrate, 1.5 equiv of acetonecyanohydrin in biphasic reaction media (total reaction volume is 50 mL, DIPE/aq buffer, 10:1).
[b] The reaction was monitored by TLC.
[c] ee's were determined by chiral HPLC analysis at 254 nm (by applying same condition as described earlier).

and external conditions (temperature and pH). We generally apply a vigorously stirred biphasic (citrate buffer, pH 4.0, and organic solvent) reaction media using transcyanation reaction with acetonecyanohydrin and PmHNL as the biocatalyst. We generally employed DIPE, TBME, and DBE as the organic media for transcyanation reaction. By applying the above reaction condition we are able to synthesize a large number of cyanohydrins with excellent enantioselection from several aromatic aldehydes (Table 7a). It was observed that substrates, which showed poor enantioselectivity in aq media, (e.g., 3-NO_2, 4-NO_2, 3-pyr, 4-CF_3, 2 and 4 quinolinecarboxaldehyde) do not markedly change their activity with change in the reaction media (Table 7a). Moderate to low enantioselectivity was observed in the synthesized cyanohydrins from those highly reactive aldehydes. As all those aldehydes are highly activated due to their structural feature, chemical yield of the corresponding cyanohydrins is very high. The possible reason for the low enantioselectivity can be attributed due to the corresponding chemical cyanation, which occurs side by side with the enzymatic cyanation. Though it was thought that by changing the reaction media from aq to organic solvent, the corresponding chemical reaction may be suppressed.

By employing the above reaction condition, aliphatic methylketones and aliphatic aldehydes were subjected to PmHNL catalyzed cyanohydrin synthesis. The detailed results are shown in Table 7b. In case of aliphatic carbonyl compounds, those compounds were tested only which provides good enantioselection in the earlier screening method (Tables 5 and 6).

It is clear from the above discussion that by applying a vigorously stirred biphasic reaction media (aq buffer/polar aprotic solvent like DIPE), the yield and optical purity of the synthesized cyanohydrins can be enhanced markedly. This method is also applicable for obtaining a large quantity of chiral cyanohydrins for industrial purposes. Further focus will be on the reuse of this new enzyme PmHNL by immobilization methods as reported by other researchers with different HNLs. And currently we are working on those aspects to find out an effective PmHNL system, which allows us to overcome all the associated problems as discussed earlier.

2.4. Structure–activity relationships

Though hydroxynitrile lyase research was widely explored by the researchers, no suitable model except one rough model by Ognyanov et al.[13] was known from which one can predict structure–activity relationship of a given sets of substrate. The model reported by Ognyanov and co-workers is based on computer assisted modeling, and one can have a rough idea of predicting the steric limits of modified substrate for PaHNL (*Prunus amygdalus* Batsch. *Syn*: *Prunus dulcis*) catalyzed cyanohydrin formation. Though one can explain the minimum steric requirements needed to be a perfect HNL substrate by using this model, but one cannot explain the minimum electronic parameters needed to be an ideal HNL substrate. The following structure–activity relationship can be predicted from the above discussion as well as previous results reported by other researchers, although the prediction is purely hypothetical, as no theoretical calculations were performed. The prediction is entirely based on the results obtained by us and several previous researchers. When we compare our enzyme PmHNL with the most widely known (*R*)-HNL, PaHNL (almond); we found that PmHNL is better enzyme than PaHNL with regard to the aromatic substrates. With aliphatic aldehydes PmHNL gives similar results as PaHNL. However, PaHNL is superior when aliphaticmethyl ketones were used as substrate. The following points are predicted based on our research on PmHNL and previous results with PaHNL.

(a) presence of substituents in *ortho* position (irrespective of its electronic nature) always lead to sluggish reaction (poor ee) compared to *meta* and *para* substituents.
(b) electron donating groups such as −Me, reduced the reactivity of the aldehyde, thereby affording lower yields compared to that of the parent compound benzaldehyde. Whereas presence of group having −I (inductive) effect and +R (resonance) effect, for example, halogen, OMe are more reactive than the parent benzaldehyde.

Table 7b. Preparative scale cyanohydrin synthesis by PmHNL with selected aliphatic aldehydes and methyl ketones

Substrate[a]	Yield (%)	Time (h)[b]	ee (%)[c]
Propanal	68	11	94
Butanal	58	14	90
Isobutanal	62	10	94
Pivalaldehyde	52	6	96
Pentanal	57	16	88
Hexanal	60	22	90
Cyclopentanecarboxaldehyde	70	14	94
Cyclohexanecarboxaldehyde	72	12	93
2-Pentanone	60	42	72
2-Hexanone	65	58	83
4-Methyl-2-pentanone	56	48	88
5-Methyl-2-hexanone	49	60	65
Trimethylsilylmethylketone	52	18	78

[a] All reactions were performed with 5 g of substrate, 1.5 equiv of acetonecyanohydrin in biphasic reaction media (total reaction volume is 50 mL, DIPE/aq buffer, 10:1).
[b] The reaction was monitored by TLC.
[c] ee's were determined by chiral GC analysis (by applying same condition as described earlier).

(c) presence of strong electron withdrawing substituents such as $-NO_2$, CF_3 activated the aldehyde so much, that almost in all cases complete conversion was observed within a short time. High reactivity of these aldehydes also caused the low ee due to non-enzymatic cyanation reaction. It was reported by Han et al.[5e] that *para*-trifluoromethylbenzaldehyde when treated with PaHNL, produced cyanohydrin with no enantioselectivity; but the same substrate provides 75% ee when reacted with PmHNL. Where as *meta*-trifluoromethyl benzaldehyde provides 70–80% ee with both the enzyme. The reason for *para*-selectivity of PmHNL for this particular substrate is unclear.

(d) 4-hydroxybenzaldehyde acted as poor substrate for PmHNL. But when the hydroxyl group was protected as ether functionality (–OBn, –Oallyl, –OTBS), the respective aldehydes are excellent substrates for PmHNL in terms of enantioselection.

(e) *N*-containing heteroaryl carboxaldehydes turned out to be poor substrates from an enantioselective point of view, as observed in the case of pyridine and quinoline series aldehydes. Whereas O and S-containing heterocyclic carboxaldehydes are excellent substrates for PmHNL as well as PaHNL.

(f) in the polycyclic aromatic aldehyde series, poor reactivity of 9-anthral suggested that maximum two rings can be accommodated in the PmHNL active site (both of the substrates 1 and 2-napthal provides good selectivity with PmHNL compared to PaHNL as reported by Riva et al.[5f]).

(g) increasing the number of substituents in the aromatic ring often lead to poor enantioselection. Tri, tetra, and penta substituted aromatic aldehydes always provides poor or no enantioselectivity.

(h) both PmHNL and PaHNL accept a broad range of aliphatic aldehydes (acyclic and cyclic) as their substrates, and overall good selectivity was observed. It was observed that, aliphatic cyclic aldehydes, for example, cyclopentane and cyclohecxane carboxaldehyde are excellent substrate compared to their linear counterpart, as both of the aldehydes react fast and provides the corresponding cyanohydrin in good chemical and optical yield.

(i) aliphatic methylketones are also accepted as substrate, but compared to their aldehyde counterpart less enantioselectivity was observed. It is also important to mention that PaHNL is little superior than PmHNL, when aliphatic methyl ketones are used as substrates (in terms of ee).

3. Conclusion

In conclusion, we have found a new (*R*) hydroxynitrile lyase from Japanese apricot (*P. mume*). The new enzyme accepts a broad array of substrates ranging from aromatic, heteroaromatic, bicyclic as well as aliphatic carbonyl compounds and yields the corresponding cyanohydrins with excellent enantioselection. Studies directed towards finding new substrates for PmHNL and predicting an effective model, which can explain the minimum requirements needed to be an efficient HNL substrate are in progress. We are also investigating the enzymological properties of PmHNL in detail.

4. Experimental

4.1. General

Unless otherwise stated, materials were obtained from commercial suppliers and used without further purification. Ripened Ume fruit (*P. mume*) was obtained from local fruit market and stored at 4 °C. All aldehydes used in the experiment are freshly distilled or washed with aq $NaHCO_3$ solution to minimize the amount of free acid, which are supposed to inhibit HNL activity. Mandelonitrile and acetone cyanohydrins were freshly distilled prior to use. Reactions were monitored by TLC, carried out on 0.25 mm silica gel plates (Merck) with UV light, ethanolic vanillin, and phosphomolybdic acid/heat as developing agents. Silica gel 100–200 mesh was used for column chromatography. Yields refer to chromatographically and spectroscopically homogeneous materials unless otherwise stated. NMR spectra were recorded on 400 MHz spectrometer at 25 °C in $CDCl_3$ using TMS as the internal standard. Chemical shifts are shown in δ. ^{13}C NMR spectra were recorded with a complete proton decoupling environment. The chemical shift value is listed as δ_H and δ_C for ^1H and ^{13}C, respectively. Chiral HPLC was performed using chiral OJ-H column (0.46×25 cm, Daicel industries) with water 717 autosampler and UV–vis detector (254 nm). Eluting solvent used was different ratio of hexane and 2-propanol. Chiral GC analysis was performed in a Schimadzu autosampler with cyclodextrins columns as chiral stationary phase (Fused silica capillary column, 30 m×0.25 mm×0.25 μm thickness, β-Dex-120 and β-Dex-325 from SUPELCO, USA) using He as a carrier gas (Detector temperature: 230 °C and injection temperature 220 °C).

4.2. Enzyme extraction from *P. mume*

Ripened Ume fruit was taken and the fleshy cover was removed to obtain the seeds. The upper layer of the seeds was cracked with hammer to give the soft kernels inside. Those kernels were collected and homogenized in a homogenizer at 4 °C, with aq potassium phosphate buffer (10 mM, pH=6.0), to give a milky suspension. The suspension was filtered through four layers of cheese cloth to remove the insoluble part. After that it was centrifuged (18,800 g, 30 min), removal of the residue gives a crude preparation of PmHNL. The crude preparation was fractionated with $(NH_4)_2SO_4$. Proteins precipitating with 30% saturation were collected by centrifugation (18,800 g, 20 min), dissolved in minimum volume of phosphate buffer 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.

4.3. PmHNL assay

In a typical assay reaction 1.0 M of benzaldehyde solution (in DMSO, 40 μL) was dissolved in 400 mM citrate buffer (760 μL, pH=4.0), followed by addition of 100 μL of enzyme solution and 100 μL of 1.0 M KCN solution (total

reaction volume 1 mL). After 5 min, the 100 μL of the reaction mixture was taken out and extracted with 900 μL hexane–2-propanol (9/1), the organic layer was analyzed with chiral HPLC for the formation of (*R*)-mandelonitrile. A blank reaction was also performed without enzyme, and the amount of mandelonitrile obtained was deducted from the biocatalyzed reaction product. One unit of the enzyme is defined as the amount of the enzyme that produces 1 μmol of (*R*)-mandelonitrile under reaction condition in 1 min.

The protein content in PmHNL was measured by Bradford method using Bio-Rad protein assay kit using BSA as the standard.[14] The protein content in a crude extract of PmHNL was found to roughly 10 mg/mL and activity was 120 U/mL as determined by the above method. The enzyme is a flavoprotein (FAD containing), as evident by the yellow color. Absorption spectroscopy also reveals the fact.

4.4. Substrate screening condition using PmHNL

In an Eppendrof tube, 1.0 M solution (40 μL, in DMSO) of the respective carbonyl compound was taken. Citrate buffer (400 mM, pH=4.0), 760 μL was added to it, followed by addition of 100 μL PmHNL and 1.25 M, solution of KCN (dissolved in citrate buffer, pH=4.0). The reaction was monitored by taking a small aliquot of reaction mixture (every 5 min interval, 25 μL). The sample was extracted with ethyl acetate and the organic layer was analyzed with HPLC. The course of the reaction was followed until appreciable amount of conversion was achieved (30 min to 1 h, depending on structural features of the aromatic aldehydes). For the case of aliphatic aldehydes and ketones, the above procedure was little modified, as substantial more amount of sample is needed to analyze the product formation by GC. Aliphatic carbonyl compounds (100 mg) were dissolved in 5 mL of citrate buffer, followed by addition of PmHNL solution (200 U/mmol of substrate) and 1.0 M of KCN solution (2 equiv). For those cyanohydrins, which are well resolved in chiral GC column (comparing from standard synthesized compounds), the course of the reaction was followed by extraction and analyzing the product formation by GC. In other cases, the reaction was followed by TLC, and after appreciable amount of conversation was achieved, the cyanohydrins are derivatized as the corresponding silyl ether (OTMS) and analyzed by chiral GC. It was assumed that in all the cases corresponding chemical cyanation caused by KCN is minimum due to low pH of the reaction medium. Using KCN as a cyanating source, within 1–3 h (depending on the structural features of the carbonyl compounds), appreciable amount of conversion was achieved, hence large number of substrates (approx. 20) can be analyzed in a single day operation.

4.5. Generation of HCN solution in organic solvent

All reaction equipment in which cyanides are used or produced was placed in a well ventilated hood. Proper gloves were worn in when handling cyanides, splash proof goggles and proper mask were also used when dealing with HCN. The solution of HCN in organic solvent (DIPE, TBME, DBE) can be generated as follows. In an aq solution of NaCN/KCN, concd HCl (35%) was dropped at 0 °C, until pH=4.0. The mixture was stirred for 20 min and then extracted with an appropriate organic solvent. The HCN solution was stored in a dark bottle at 0 °C, with little addition of citrate buffer (pH=4.0). The HCN concentration of the obtained solution was determined as reported by Sheldon et al.[12a]

4.6. Preparative scale biphasic synthesis of cyanohydrins using PmHNL

Carbonyl compounds (5 g) were taken in appropriate solvent (DIPE, TBME or DBE, 50 mL) saturated with 5 mL of citrate buffer (pH=4.0). Solution of PmHNL (50 U/mmol of substrate) followed by acetonecyanohydrin (1.5 equiv) was added to the reaction mixture. The reaction was vigorously stirred until the desired conversion was achieved (by TLC). Usual extractive work-up afforded the cyanohydrins.

Acknowledgements

JSPS (Japan Society for Promotion of Science) post doctoral fellowship to S. N. is gratefully acknowledged, this research was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2005.08.105. Spectral (^{1}H and ^{13}C NMR) data for selected compounds and GC/HPLC retention time are available.

References and notes

1. (a) Smitskamp-Wilms, E.; Brussee, J.; Van der Gen, A.; van Scharrenburg, G. J. M.; Sloothaak, J. B. *Recl. Trav. Chim. Pay-Bas.* **1991**, *110*, 209–215. (b) Gerstner, E.; Kiel, U. Z. *Physiol. Chem.* **1975**, *356*, 1853–1857. (c) Xu, L.-L.; Singh, B. K.; Conn, E. E. *Arch. Biochem. Biophys.* **1986**, *250*, 322–328. (d) Yemm, R. S.; Poulton, J. E. *Arch. Biochim. Biophys.* **1986**, *247*, 440–445. (e) Albrecht, J.; Jansen, I.; Kula, M.-R. *Biotechnol. Appl. Biochem.* **1993**, *17*, 191–203. (f) Wajant, H.; Förster, S.; Selmar, D.; Effenberger, F.; Pfizenmaier, K. *Plant Physiol.* **1995**, *109*, 1231–1238. (g) Wajant, H.; Mundry, K.-W. *Plant Sci.* **1993**, *89*, 127–133. (h) Bove, C.; Conn, E. E. *J. Biol. Chem.* **1961**, *236*, 207–210. (i) Kuroki, G. W.; Conn, E. E. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6978–6981. (j) Wajant, H.; Förster, S.; Bottinger, H.; Effenberger, F.; Pfizenmaier, K. *Plant Sci.* **1995**, *108*, 1–11. (k) Hughes, J.; Lakey, J. H.; Hughes, M. A. *Arch. Biochim. Biophys.* **1994**, *311*, 496–502.
2. (a) Hickel, A.; Hasslacher, M.; Griengl, H. *Physiol. Plant* **1996**, *98*, 891–898. (b) Wajant, H.; Effenberger, F. *Biol. Chem.* **1996**, *377*, 611–617. (c) Seigler, D. S. Cyanide and

Cyanogenic Glycosides. In *The Chemical Participants*, 2nd ed.; Rosenthal, G. A., Berenbaum, M. R., Eds.; Herbivores: Their Interactions with Secondary Metabolites; Academic: New York; Vol. 1, pp 35–77. (d) Conn, E. E. *Ann. Rev. Plant. Physiol.* **1980**, *31*, 433–451.

3. (a) Nahrstedt, A. *Plant Syst. Evol.* **1985**, *150*, 35–47. (b) Lieberei, R.; Selmar, D.; Biehl, B. *Plant Syst. Evol.* **1985**, *150*, 49–63. (c) Okolie, P. N.; Obasi, B. N. *Phytochemistry* **1993**, *33*, 775–778. (d) Kakes, P.; Hakvoort, H. *Phytochemistry* **1992**, *31*, 1501–1505. (e) Wajant, H.; Riedel, D.; Benz, S.; Mundry, K.-W. *Plant Sci.* **1994**, *103*, 145–154. (f) Poulton, J. E. Enzymology of Cyanogenesis in Rosaceous Stone Fruits. In *β-Glucosidases: Biochemistry and Molecular Biology*; Esen, A., Ed.; ACS Symposium Series 533; Oxford University Press: New York, 1993; pp 170–190. (g) Wu, H. C.; Poulton, J. E. *Plant Physiol.* **1991**, *96*, 1329–1337.
4. (a) Garcia-Urdiales, E.; Alfonso, I.; Gotor, V. *Chem. Rev.* **2005**, *105*, 313–354. (b) Gregory, R. J. H. *Chem. Rev.* **1999**, *99*, 3649–3682. (c) North, M. *Tetrahedron: Asymmetry* **2003**, *14*, 147–176. (d) Griengl, H.; Schwab, H.; Fechter, M. *Trends Biotechnol.* **2000**, *18*, 252–256. (e) Effenberger, F. *Chimia* **1999**, *53*, 3–10. (f) Johnson, D. V.; Griengl, H. *Adv. Biochem. Eng./Biotechnol.* **1999**, *63*, 32–55.
5. Few recent references on HNL catalyzed cyanohydrin synthesis and screening for new HNL. A detailed referencing can be found in the previous reviews (4a–4e). (a) Kobler, C.; Bohrer, A.; Effenberger, F. *Tetrahedron* **2004**, *60*, 10397–10410. (b) Solís, A.; Luna, H.; Manjarrez, N.; Pérez, H. I. *Tetrahedron* **2004**, *60*, 10427–10431. (c) Avi, M.; Fechter, M. H.; Belaj, F.; Pöchlauer, P.; Griengl, H. *Tetrahedron* **2004**, *60*, 10411–10418. (d) Kobler, C.; Effenberger, F. *Tetrahedron: Asymmetry* **2004**, *15*, 3731–3742. (e) Han, S.; Chen, P.; Lin, G.; Huang, H.; Li, Z. *Tetrahedron: Asymmetry* **2001**, *12*, 843–846. (f) Cruz Silva, M. M.; Melo, M. L.; Parolin, M.; Tessaro, D.; Riva, S.; Danieli, B. *Tetrahedron: Asymmetry* **2004**, *15*, 21–27. (g) Li, N.; Zong, M.-H.; Peng, H.-S.; Wu, H.-C.; Liu, C. *J. Mol. Catal. B: Enzym.* **2003**, *22*, 7–12. (h) Li, N.; Zong, M.-H.; Liu, C.; Peng, H.-S.; Wu, H.-C. *Biotechnol. Lett.* **2003**, *25*, 219–222. (i) Fröhlich, R. F. G.; Zabelinskaja-Mackova, A. A.; Fechter, M. H.; Griengl, H. *Tetrahedron: Asymmetry* **2003**, *14*, 355–362. (j) Hernández, L.; Luna, H.; Ruíz-Terán, F.; Vázquez, A. *J. Mol. Catal. B: Enzym.* **2004**, *30*, 105–108.
6. Hickel, A.; Heinrich, G.; Schwab, H.; Griengl, H. *Biotechnol. Tech.* **1997**, *11*, 55–58.
7. Kruse, C. G. Chiral Cyanohydrins — Their Manufacture and Utility as Chiral Building Blocks. In *The Commercial Manufacture and Applications of Optically Active Compounds*; Collins, A. N., Sheldrake, G. N., Crosby, J., Eds.; Chirality in industry; Wiley: Chichester, UK, 1992; Chapter 14, pp 279–299.
8. (a) Schmidt, M.; Herve, S.; Klempier, N.; Griengl, H. *Tetrahedron* **1996**, *52*, 7833–7840. (b) Brussee, J.; Loos, W. T.; Kruse, C. G.; van der Gen, A. *Tetrahedron.* **1990**, *46*, 979–986. (c) Menendez, E.; Brieva, R.; Rebolledo, F.; Gotor, V. *J. Chem. Soc., Chem. Commun.* **1995**, 989–990.(d) A list of reactions on both the hydroxyl (protected and unprotected) and nitrile functionality and corresponding literature references can be found at Ref. 4b
9. (a) Asano, Y.; Tamura, K.; Doi, N.; Ohmiya, T., *Biosci. Biotech. Biochem.* **2005**, in press. (b) Tamura, K.; Asano, Y. Studies on mandelonitrile lyases from plants. Masters thesis, Toyama Prefectural University, 2003.
10. Hanefeld, U.; Straathof, A. J. J.; Heijnen, J. J. *Biochim. Biophys. Acta* **1999**, *1432*, 185–193.
11. (a) Zandergen, P.; Van der Linden, J.; Brussee, J.; Van der Gen, A. *Synth. Commun.* **1991**, *21*, 1387–1391. (b) Lin, G.; Han, S.; Li, Z. *Tetrahedron* **1999**, *55*, 3531–3540. (c) Kiljunen, E.; Kanerva, L. T. *Tetrahedron: Asymmetry* **1997**, *8*, 1225–1234.
12. (a) van Langen, L. M.; Selassa, R.; Rantwijk, F.; Sheldon, R. A. *Org. Lett.* **2005**, *7*, 327–329. (b) Veum, L.; Hanefeld, U.; Pierre, A. *Tetrahedron* **2004**, *60*, 10419–10425. (c) Effenberger, F.; Ziegler, T.; Förster, S. Degussa. Eur. Patent 276,375, 1987. (d) Kula, M, R.; Stürtz, I, M.; Wandrey, C.; Kragl, U. Forschungszentrum Jülich. Eur. Patent 446,826, 1991. (e) Becker, W.; Pfeil, E. Boehringer. Ger. Patent 1,593,260, 1966. (f) Wehtje, E.; Adlercreutz, P.; Mattiason, B. *Appl. Microbiol. Biotechnol.* **1988**, *29*, 419. (g) Westman, T, L. Monsanto, U.S. Patent 3,649,457, 1968.
13. Ognyanov, V. I.; Datcheva, V. K.; Kyler, K. S. *J. Am. Chem. Soc.* **1991**, *113*, 6992–6995.
14. Bradford, M. *Anal. Biochem.* **1976**, *72*, 248–254.
15. Gerrits, P. J.; Zumbrägel, F.; Marcus, J. *Tetrahedron* **2001**, *57*, 8691–8698.