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Effect of glycosylation on the biocatalytic properties of hydroxynitrile lyase from the passion fruit, *Passiflora edulis* – a comparison of natural and recombinant enzymes

Aem Nuylert[a], Yuko Ishida[a,b] and Yasuhisa Asano*[a,b]

Abstract: A hydroxynitrile lyase from the passion fruit, *Passiflora edulis* (*Pe*HNL) was isolated from the leaves and showed high stability in biphasic co-organic solvent systems for cyanohydrin synthesis. Cyanohydrins are important building blocks for the production of fine chemicals and pharmaceuticals. Thus, to enhance production yields of *Pe*HNL for industrial applications, we cloned and expressed recombinant *Pe*HNL in *E. coli* BL21 (DE3) and *P. pastoris* GS115 cells without a signal peptide sequence. The aim of the present study is to determine the effect of *N*-glycosylation on enzyme stability and catalytic properties in microbial expression systems. *Pe*HNL from leaves (*Pe*HNL-N) and *P. pastoris* (*Pe*HNL-P) was glycosylated, whereas that expressed in *E. coli* (*Pe*HNL-E) was not. The enzymes *Pe*HNL-N and *Pe*HNL-P showed much better thermostability, pH stability, and organic solvent tolerance than the deglycosylated enzyme *Pe*HNL-E and the deglycosylated mutant N105Q from *P. pastoris* (*Pe*HNL-P-N105Q). The glycosylated *Pe*HNL-P also efficiently performed transcyanation of (*R*)-mandelonitrile with a 98% enantiomeric excess in a biphasic system with 2-isopropyl ether. These data demonstrate the efficacy of the present methods for improving enzyme expression and stability for industrial application based on *N*-glycosylation.

Introduction

Hydroxynitrile lyase (HNLs; EC 4.1.2.10, 4.1.2.11, 4.1.2.46 and 4.1.2.47) is a key enzyme in plant cyanogenesis and catalyzes the final step in the cyanogenic glycoside degradation pathway. This enzymatic reaction catalyzes the decomposition of cyanohydrin compounds into aldehydes or ketones and releases HCN, which is considered toxic for herbivores and microbes.[1] Due to reverse reactions of HNLs, the enzyme catalyzes the synthesis of optically pure cyanohydrin intermediates which can be converted into α-hydroxy acids, primary and secondary β-hydroxy amines, aziridines, α-hydroxy aldehydes or ketones, α-hydroxy esters, α- and β-amino alcohols, 3-ethanolamines, α-aminonitriles, and α-azidonitriles.[2]

The passion fruit *Passiflora edulis* is known for its cyanogenic glycosides, which include passicoriacin, epipassicoriacin, epitetraphyllin B, cyanogenic-β-rutinoside, amygdalin, prunasin, mandelonitrile rhamnopyranosyl-β-D-glucopyranoside, and sambuni-grin.[3] This plant species also has HNL (*Pe*HNL) catalyzed reactions that are involved in the synthesis of cyanohydrins. *Pe*HNL from leaves is a glycoprotein that lacks flavin adenine dinucleotide as a prosthetic group and is the smallest known HNL, with a molecular mass of 14.4 kDa.[4]

The advantages of biphasic systems comprising buffer and immiscible organic solvents include high efficiency, prevention of substrate or product inhibition, cost effectiveness, and ease of downstream processing following extraction of the product and the enzyme in organic and aqueous phases, respectively.[5] However, added solvents can destabilize the enzyme, leading to lower activities.[6] In a previous study, *Pe*HNL was stable in biphasic systems of water and an organic solvent and showed high stereoselectivity in the synthesis of (*R*)-mandelonitrile from benzaldehyde and cyanide, with a 98.6% enantiomeric excess.[4b] Although these data indicate the great potential of *Pe*HNL as an industrial biocatalyst, it is difficult to collect sufficient amounts of passion fruit leaves and purify *Pe*HNL in industrial quantities. Thus, to enhance production yields, *Escherichia coli* and *Pichia pastoris* have been used as expression systems although recombinant proteins from expression systems vary in post-translational modifications such as glycosylation.[7]

N-glycosylation is a eukaryote post-translational modification that covalently links saccharides to the peptide backbone via a nitrogen of the asparagine residue in the tri-peptide sequence Asn-X-Ser/Thr (where X can be any amino acid except proline).[8] Recombinant proteins expressed in *Pichia pastoris* are often *N*-glycosylated with a high proportion of mannoside. In a recent study in *P. pastoris*, glycosylation played roles in improvement of enzymatic activity and molecular stability under various pH, temperature, and solvent conditions.[9] Accordingly, glycosylation sites of *Rhizopus chinensis* lipase expressed in *P. pastoris* played key roles in secretion, catalytic activity, thermostability, and solvent stability in the presence of *n*-hexane or isooctane.[10] Moreover, expression of glycosylated *Penicillium purpurogenum* β-glucuronidase in *P. pastoris* produced an enzyme of significantly higher thermal stability than that following expression in *E. coli*.[11] Similarly, induction of recombinant elastase *N*-glycosylation sites in *P. pastoris* enhanced bipeptide systhesis in a biphasic system with dimethylsulfoxide (50% v/v), compared with that in wild type cells.[12]

In this study, full-length *Pe*HNL cDNA was cloned from young passion fruit leaves using rapid amplification of cDNA ends (RACE). Subsequently, recombinant *Pe*HNL was expressed in *E. coli* BL21 (DE3) and *P. pastoris* GS115 cells, and was then purified and characterized. To elucidate roles of *N*-glycosylation at Asn105, we expressed mutant *Pe*HNLs with Gln105 and compared their catalytic properties and enzyme stabilities with those of native and recombinant enzymes.

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Supporting information for this article is given via a link at the end of the document.

Results and Discussion

Cloning of HNL cDNA from *Passiflora edulis*

Prior to expression of recombinant enzymes, full-length *Pe*HNL cDNA was cloned and its sequence was determined using RACE with degenerate primers that were designed on the basis of N-terminal amino acid sequences of the purified enzyme, and with gene-specific primers and cDNA from a young leaf. The open reading frame of *Pe*HNL comprised 444 bp encoding 147 amino acid residues including a 26 amino acid signal peptide (Accession No. LC115048; Figure S1). The predicted N-terminal amino acid sequence from the cDNA was identical to that of purified *Pe*HNL from leaves.[4b] Moreover, the predicted mature protein (no signal peptide) had a molecular mass of 14066.92 Da, an isoelectric point of 5.20. The mass of *Pe*HNL from leaves was confirmed using SDS-PAGE analysis (Figure 1A).[4b] (*R*)-*Pe*HNL is smaller than (*R*)-HNLs from *Eriobotrya japonica*, *Prunus amygdalus*, *Linum usitatissimum*, and *Phlebodium aureum*, which have molecular masses ranging from 62 to 108 kDa.[13] Although *Pe*HNL has low sequence homology with previously characterized HNLs, it has 37%, 32%, and 28% amino acid identity with boiling-stable proteins from aspen *Populus tremula* (accession number, 1SI9_A)[14], putative At3q1720 from the *Arabidopsis thaliana* genome (accession number, 1Q4R_A)[15], and olivetolic acid cyclase from *Cannabis stavia* (accession number, 5B08A)[16], respectively (Figure S2). The predicted mature enzyme had an *N*-glycosylation site on the asparagine residue at 105 (Figure S1).

Purification of *Pe*HNLs

To investigate the properties of *Pe*HNL, native *Pe*HNL was purified from leaves (*Pe*HNL-N) and compared with recombinant *Pe*HNL without posttranslational modification (*Pe*HNL-E), recombinant *Pe*HNL with posttranslational modification (*Pe*HNL-P), and *Pe*HNL with an asparagine to glutamine substitution at amino acid 105 (*Pe*HNL-P-N105Q), which were expressed and purified using bacterial and *Pichia* expression sytems.

The purification steps for natural HNL from the leaves of *P. edulis* (*Pe*HNL-N) are summarized in Table 1. A total of 12200 activity units of crude *Pe*HNL-N enzyme were extracted from 1500 g of leaves. Subsequently, *Pe*HNL-N was purified to 13.4 fold with a 0.04% recovery yield and specific activity of 134 U·mg^{-1} after ammonium sulfate fractionation and chromatography through six columns (DEAE-Toyopearl, Butyl-Toyopearl, MonoQ 10/100 GL, Resource Phe, Hydroxyapatite type I, and Con A Sepharose 4B).

To express proteins in *E. coli*, cDNA fragments were cloned into the pColdI vector, which adds an N-terminal His-tag. The resulting recombinant enzyme from *E. coli* (*Pe*HNL-E) was purified to 22.6 fold with a 1.17% recovery yield using a Ni Sepharose 6 Fast Flow column followed by monoQ HR10/100. The specific activity of *Pe*HNL-E was 150 U·mg^{-1} (Table 1), and was slightly higher than those of *Pe*HNL-N, *Pe*HNL-P, and *Pe*HNL-P-N105Q.

Table 1. Purification summary of *Pe*HNLs

Purification step	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification fold
***Pe*HNL-N**				
Crude (leaves 1,500g)	12200	9.96	100	1.00
20-40% $(NH_4)_2SO_4$	5320	11.9	43.6	1.20
DEAE Toyopearl	4220	12.2	34.5	1.23
Butyl Toyopearl	2560	16.8	32.3	1.69
MonoQ HR10/100	360	39	4.5	3.91
Resource PHE	42.2	52.4	0.53	5.26
Hydroxyapatite type II	14.7	58.7	0.18	5.90
Con A Sepharose 4B	3.4	133.6	0.04	13.4
***Pe*HNL-P**				
Cell-free extract (1L)	1065	0.74	100	1.00
20-60% $(NH_4)_2SO_4$	540	1.06	50.7	1.44
Butyl Toyopearl	527	2.37	49.5	3.20
DEAE Toyopearl	146	17.6	13.7	23.8
Resource PHE 1st	63.5	36.8	5.97	49.8
MonoQ HR10/100	14.7	59.6	1.40	80.0
Resource PHE 2nd	3.62	134	0.34	182
***Pe*HNL-P-N105Q**				
Cell-free extract (1L)	263	0.22	100	1.00
20-60% $(NH_4)_2SO_4$	118	0.94	44.8	4.30
Butyl-Toyopearl	86.5	3.25	32.8	14.7
DEAE-Toyopearl	13.8	16.4	5.25	74.5
Resource PHE	2.10	140	0.80	636
***Pe*HNL-E**				
Cell-free extract (1L)	5440	6.64	100	1.00
Ni sepharose	2270	47.8	51	7.2
MonoQ HR10/100	64.1	150	1.17	22.6

Molecular masses and glycosylation statuses of native *Pe*HNL, recombinant *Pe*HNLs, and mutant *Pe*HNL

The proteins *Pe*HNL-N, *Pe*HNL-P, *Pe*HNL-P-N105Q, and *Pe*HNL-E had molecular masses of about 14, 17, 13, and 13 kDa, respectively, in SDS-PAGE analyses (Figure 1A). In subsequent experiments, *Pe*HNL-N and *Pe*HNL-P were reacted with periodic acid-Schiff (PAS) reagent and eluted through a Con A Sepharose 4B column (Figure 1B). These experiments indicated total carbohydrate contents of 7% and 30% in *Pe*HNL-N and *Pe*HNL-P, respectively. These data were consistent with the higher molecular mass of *Pe*HNL-P compared with *Pe*HNL-N (Figure 1A), likely reflecting the high mannose-type glycosylation that is commonly observed in *Pichia pastoris*.[17] In contrast, PAS staining experiments did not show glycosylation of *Pe*HNL-P-N105Q, suggesting that the Asn105 in *Pe*HNL is the only glycosylation site in the present *Pichia* expression system.

Kinetic paramaters of native *Pe*HNL, recombinant *Pe*HNLs, and mutated *Pe*HNL

To investigate enzyme kinetics of *Pe*HNL-N and recombinant *Pe*HNLs, the synthesis of (*R*)-mandelonitrile from benzaldehyde and potassium cyanide was monitored in the presence of native and recombinant *Pe*HNLs using HPLC-chiral column analyses. In these experiments, K_m, V_{max}, K_{cat}, and K_{cat}/K_m values of *Pe*HNL-N were 11.2 ± 0.0 mM, 217 ± 2.7 μmol min^{-1} mg^{-1}, 50.9 ± 2.6 s^{-1}, and 4.56 ± 0.36 mM^{-1}.s^{-1}, respectively, and were comparable to those of *Pe*HNL-P. However, K_m, V_{max}, K_{cat}, and K_{cat}/K_m values of *Pe*HNL-E were 26.1 ± 0.3 mM, 164 ± 1.8 μmol min^{-1} mg^{-1}, 38.4 ± 0.4 s^{-1}, and 1.47 ± 0.05 mM^{-1}.s^{-1}, respectively, and these values were similar to those of *Pe*HNL-P-N105Q, which lacks the potential *N*-glycosylation site (Table 2). K_m values of *Pe*HNL-E and *Pe*HNL-P-N105Q were two times higher

than those of *Pe*HNL-N and *Pe*HNL-P. Moreover, V_{max}, K_{cat}, and K_{cat}/K_m of *Pe*HNL-N and *Pe*HNL-P were 1.3, 1.3, and 2.5 times higher, respectively, than those of *Pe*HNL-E and *Pe*HNL-P-N105Q (Table 2). These results indicate decreased K_m and increased V_{max}, K_{cat}, and K_{cat}/K_m values following glycosylation of *Pe*HNL, suggesting that glycan participates in protein conformation changes that increase activity without reducing substrate affinity for benzaldehyde and KCN. Accordingly, previous studies suggested that glycans interact and form covalent bonds with the protein structure.[11, 18] However, future protein structure and glycan analyses are warranted to investigate the related active site mechanisms.

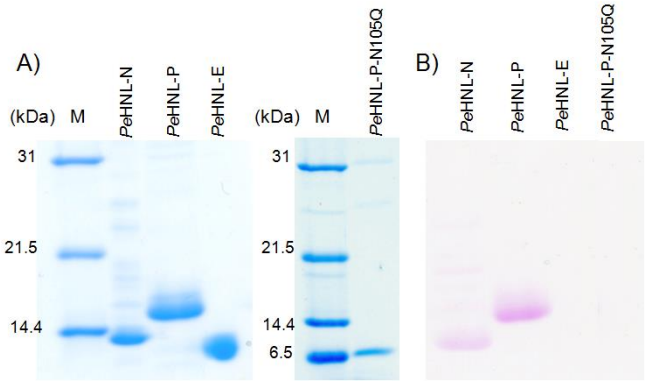

Figure 1. SDS-PAGE analysis and periodic acid-Schiff staining of native and recombinant *Pe*HNLs; A, Coomassies Brilliant Blue staning; B, Periodic acid-Schiff (PAS) staining; M, molecular marker; *Pe*HNL-N, native *Pe*HNL isolated from leaves; *Pe*HNL-P, recombinant *Pe*HNL expressed in the *Pichia* expression system; *Pe*HNL-P-N105Q, recombinant *Pe*HNL lacking the glycosylation site at Asn 105 and expressed in the *Pichia* expression system; *Pe*HNL-E, recombinant *Pe*HNL expressed in the bacterial expression system.

pH, temperature, and organic solvent stabilities of native *Pe*HNL and recombinant *Pe*HNLs

Industrial biotechnology generally requires stable enzymes with sustained activity under varying pH, temperature, and organic solvent conditions.[19] Previous studies indicate that glycosylation improves enzyme stability.[9, 20] Accordingly, glycosylation of β-glucuronidase in *P. pastoris* provides greater stability between pH 4.0 and 7.0 than that in *E. coli* .[11, 21] Thus, to determine the effects of glycosylation on *Pe*HNL stability, we determined enzyme activities under various conditions of pH, temperature, and organic solvent.

In analyses of the effects of pH, activities of all *Pe*HNLs were stable over the range from pH 3.5 to 10.0, although the activity of *Pe*HNL-E was reduced to 60% and 70% at pH 7.0 and 10.0, respectively (Figure 2A). These results suggest that glycosylation does not increase pH stability of *Pe*HNL. Whereas, the one glycosylation site of hydroxynitrile lyase isoenzyme 5 from almonds (*Pa*HNL5) played a key role in stability at low pH when expressed in *P. pastoris.*[22] To avoid nonselective synthesis of chiral cyanohydrins, low pH conditions are necessary for enantioselective synthesis of cyanohydrins.[19a] Thus, the present data warrant further consideration of *Pe*HNLs as suitable biocatalysts for synthesis of pharmaceutical and agrochemical compounds. This tolerance of lower pH may allow control over the synthesis of unstable cyanohydrins by reducing spontaneous chemical degradation.

Table 2. Kinetic parameters of native and recombinant *Pe*HNLs

	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1}.s^{-1})
*Pe*HNL-N	11.2±0.0	217±2.7	50.9±2.6	4.56±0.36
*Pe*HNL-P	13.0±0.1	222±1.7	52.1±1.3	4.00±0.17
*Pe*HNL-P-N105Q	19.8±0.0	169±0.4	39.7±1.6	2.00±0.16
*Pe*HNL-E	26.1±0.3	164±1.8	38.4±0.4	1.47±0.05

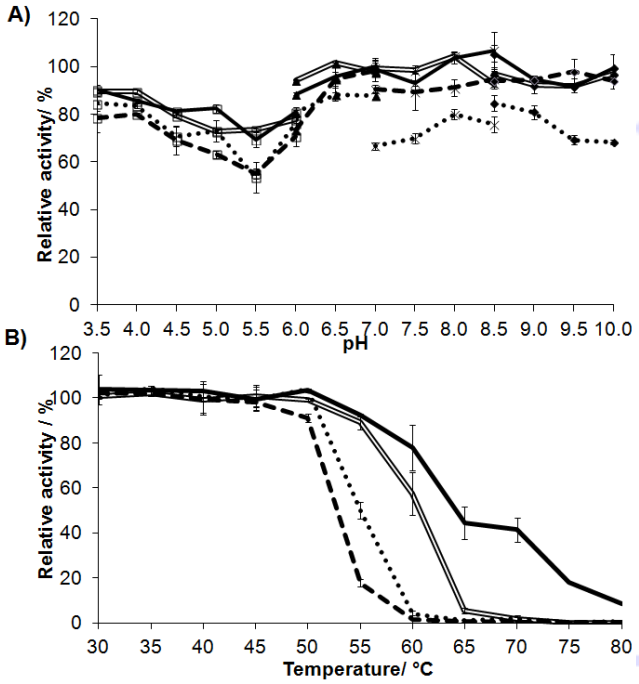

Figure 2. pH (A) and temperature stability (B) of native and recombinant *Pe*HNLs; Solid line *Pe*HNL-N; double line, *Pe*HNL-P; dashed line, *Pe*HNL-P-N105Q; dotted line, *Pe*HNL-E; □, citrate buffer (40 mM); ▲, phosphate buffer (40 mM); ×, Tris-HCl buffer (40 mM); ♦, glycine-sodium hydroxide buffer (40 mM). After incubation of enzymes at various pH or temperatures for 1 h, the remaining enzyme activity was estimated by monitoring the synthesis of (*R*)-mandelonitrile from benzaldehyde and potassium cyanide using HPLC with a chiral column. Data are presented as means ± SD (n = 3).

In further analyses, all *Pe*HNLs showed 100% activity at temperatures of 30°C to 50°C following 1 h preincubation. However, enzyme activities of *Pe*HNL-E and *Pe*HNL-P-N105Q were decreased at 55°C and lost at 60°C. Although both *Pe*HNL-P and *Pe*HNL-N had 90% activity at 55°C, *Pe*HNL-P activity was reduced to 60% at 60°C and lost at 70°C, whereas *Pe*HNL-N had 80% activity at 60°C and enzyme products remained detectable at 80°C (Figure 2B). In direct comparisons at 60°C, *Pe*HNL-P and *Pe*HNL-N had between 60% and 80% remaining activity, whereas *Pe*HNL-E and *Pe*HNL-P-N105Q were almost inactive (Figure 2B). In addition, *Pe*HNL-N had 40% remaining activity at 70°C, whereas *Pe*HNL-P did not. These results suggest that *N*-glycosylation contributes to the thermostability of *Pe*HNL-P and *Pe*HNL-N. Similarly, the hyperthermostability of *Pe*HNL-N may reflect specific glycan compositions of passion fruit enzymes, which differ from those in *Pe*HNL-P from the *P. pastoris* expression system. Meldgaard and Svendsen analyzed irreversible thermal denaturation using *Bacillus*

amyloliquefaciens (1,3-1,4)-β-glucanases, *B. macerans* (1,3-1,4)-β-glucanases, and chimeric enzymes. In agreement with the present data, thermostabilities of these enzymes increased with glycosylation, although these effects were multifactorial.[23] Fonseca *et. al.* also showed that glycoproteins are more stable than their corresponding non-glycosylated forms, owing to steric interactions between sugar residues and protein structures and effects of glycosylation on the protein energy landscape.[24]

According to our previous work, pH and temperature were important parameters for controlling the enantiomeric purity of cyanohydrin products, and the choice of organic solvent was essential to activity and enantioselectivity of enzymes.[4b] Thus, in the present study, we investigated stabilities of native and recombinant *Pe*HNLs following fractionation using organic solvents in biphasic systems comprising 400 mM citrate buffer (pH 4.0) with ethyl acetate (EA), diethyl ether (DEE), methyl-*t*-butyl ether (MTBE), diisopropyl ether (DIPE), dibutyl ether (DBE), or *n*-hexane (Hex) at a volume ratio of 50:50. Following incubation at 10°C for 12 h, *Pe*HNL-N and *Pe*HNL-P stabilities were comparable, with remaining enzyme activities of 60%–94%. In contrast, *Pe*HNL-P-N105Q and *Pe*HNL-E activities varied between 70% and 0% in all biphasic systems apart from those with DEE and DIPE. Interestingly, both of non-glycosylated *Pe*HNL-E and *Pe*HNL-P-N105Q lost the activities by 50% in aqueous buffer with shaking at 1,500 rpm at 10 °C for 12 h (Figure 3) indicating that the presence of glycan could prevent the self-degradation at 10 °C of this enzyme. In the aqueous system, water is such major deleterious reactions as deamidation of Asn/Gln residues and hydrolysis of peptide bonds.[25] Therefore, the enzyme could be more stable in organic solvent than in water.[26] This hypothesis is supported by the evidence that the non-glycosylated enzyme, *Pe*HNL-P-N105Q and *Pe*HNL-E were found to be more stable in the presence of Hex or DBE than in the absence of an organic solvent.The stability of the enzyme in organic solvents depends on the hydrophobicity of the organic solvent. In general, enzymes only need a thin layer of water on the surface of the protein to retain their catalytically active conformation. Moreover, specific activities of the glycosylated enzymes *Pe*HNL-N and *Pe*HNL-P were significantly higher than those of the unglycosylated enzymes *Pe*HNL-P-N105Q and *Pe*HNL-E under conditions of buffer alone and with EA or MTBE. However, no differences in remaining activity were identified between the 4 HNLs in biphasic systems containing DBE or Hex (Figure 3). To investigate the relationship between glycan composition and thermostability and stability of *Pe*HNL in organic solvents, further mass spectrometric analyses of pure *Pe*HNL-N and *Pe*HNL-P glycan structures are required after digestion using endoglycosidases and endopeptidases.

Effect of biphasic systems on (a) enantiomeric excess of synthesized cyanohydrin and (b) transcyanation by native and recombinant PeHNLs

*Pe*HNL-N was reportedly stable in biphasic systems of water and organic solvent, and catalyzed the synthesis of (*R*)-mandelonitrile from benzaldehyde and cyanide, with a 98.6% enantiomeric excess.[4b] All of organic solvent used in this study have the positive values of the logarithm of the partition coefficients (log *P*) which gave a high ee (88-99%) for the products, while the negative log *P* yielded the product cyanohydrins with low ee (2-30%).[27] The biphasic system with ethyl acetate (EA; log *P* 0.67), diethyl ether (DEE; log *P*

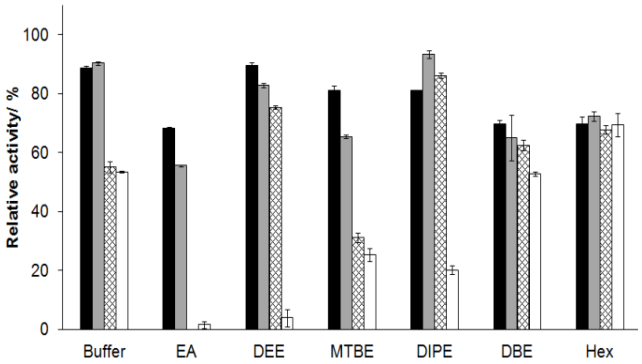

Figure 3. Effects of organic solvents on the stability of *Pe*HNLs (2.34 U of each partially purified); After 12-h incubation at 10°C in each biphasic system at a citrate buffer/organic solvent ratio of 50:50, remaining enzyme activities of each *Pe*HNL were measured using HPLC with a chiral column. Solid bar, *Pe*HNL-N; Grey bar, *Pe*HNL-P; Cross bar, *Pe*HNL-P-N105Q; Open bar, *Pe*HNL-E; EA, ethyl acetate; DEE, diethyl ether; MTBE, methyl-*t*-butyl ether; DIPE, 2-isopropyl ether; DBE, dibutyl ether; HEX, hexane. Data are presented as means ± SD (n = 3).

0.85), methyl-*t*-butyl ether (MTBE; log *P* 1.4), 2-isopropyl ether (DIPE; log *P* 1.9), dibutyl ether (DBE; log *P* 2.9) and hexane (HEX; log *P* 3.5) have been studied on HNLs from *Hevea brasiliensis* (*Hb*HNL),[28] *Manihot esculenta* (*Me*HNL),[28b, 28c] *Sorghum bicolor* (*Sb*HNL),[28b, 28c] *Prunus amygdalus* (*Pa*HNL)[29] and *Eriobotrya japonica* (*Ej*HNL).[30] Thus, to further investigate the effects of glycosylation on enzyme activities, we determined enantiomeric excesses (ee) and transcyanation of (*R*)-mandelonitrile by *Pe*HNLs in biphasic systems. *Pe*HNLs in biphasic systems containing EA, DEE, MTBE, DIPE, or DBE and an equal volume of 400 mM citrate buffer pH 4.0 produced (*R*)-mandelonitrile from benzaldehyde and acetone cyanohydrin with ee of 94%, whereas enzymes from biphasic systems containing citrate buffer and Hex produced (*R*)-mandelonitrile with ee of 50%–35%. The lack of clear differences in ee between the other biphasic systems (Figure 4) suggest that glycosylation of *Pe*HNL does not effect chiral specificity.

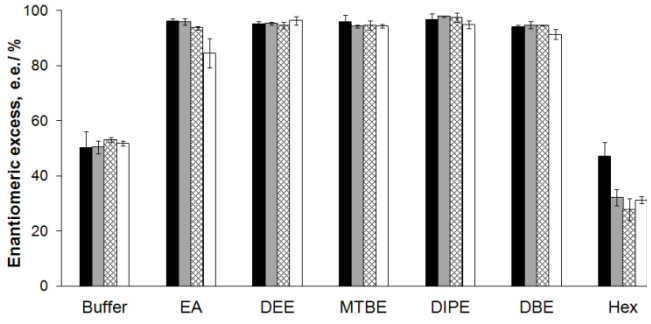

Figure 4. Effects of organic solvents on ee in (*R*)-mandelonitrile products at 12 h after the start of incubation with *Pe*HNLs (2.34 U of each partially purified); (*R*)-mandelonitrile was synthesized using partially purified *Pe*HNLs from benzaldehyde (250 mM) and acetone cyanohydrin (900 mM) in biphasic systems at a citrate buffer/organic solvent ratio of 50:50 Reactions were performed at 10°C to avoid decomposition of product. Solid bar, *Pe*HNL-N; Gray bar, *Pe*HNL-P; Cross bar, *Pe*HNL-P-N105Q; Open bar, *Pe*HNL-E; EA, ethyl acetate; DEE, diethyl ether; MTBE, methyl-*t*-butyl ether; DIPE, 2-isopropyl ether; DBE, dibutyl ether; HEX, hexane. Data are presented as means ± SD (n = 3).

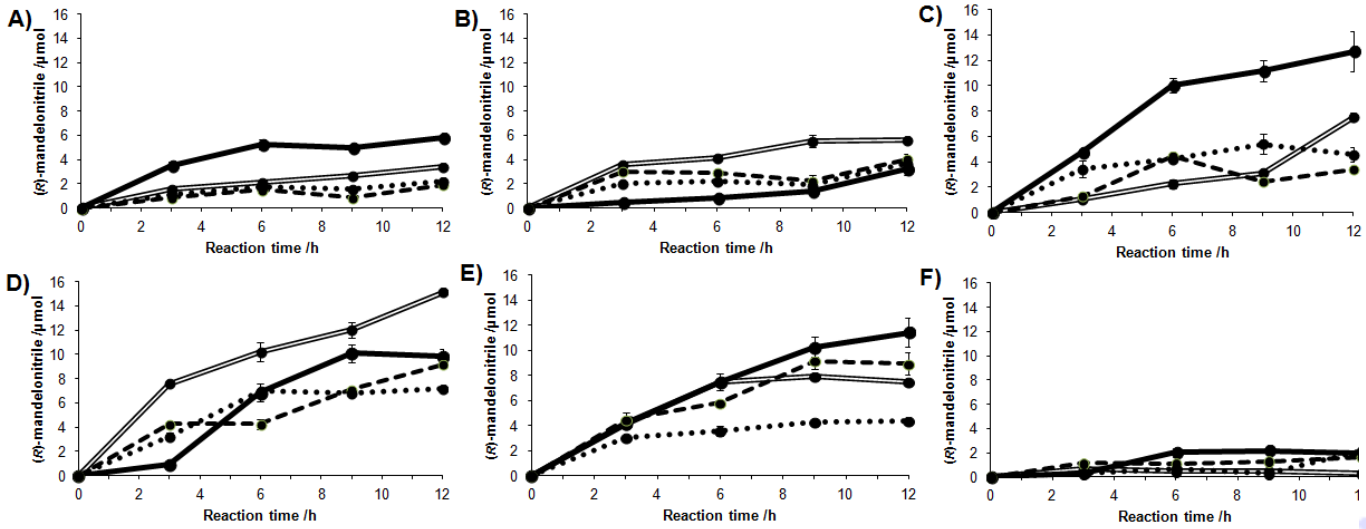

Figure 5. Time course of (*R*)-mandelonitrile synthesis following transcyanation reactions with *Pe*HNLs in biphasic systems; reactions and analyses were perform as described in Figure 4. Products were collected at 3, 6, 9, and 12 h after the start of incubation; A), ethyl acetate; B), diethyl ether; C), methyl-*t*-butyl ether; D) isopropyl ether; E), dibutyl ether; F), *n*-hexane; Solid line, *Pe*HNL-N; double line, *Pe*HNL-P; dashed line, *Pe*HNL-P-N105Q; dotted line, *Pe*HNL-E. Data presented as means ± SD (n = 3).

In further experiments, the relationship between cyanohydrin production and glycosylation of *Pe*HNL was investigated by incubating substrates with each *Pe*HNL, and periodically collecting and measuring quantities of (*R*)-mandelonitrile. Since an enantiomeric purity or ee of 98% of target chiral compound is minimum acceptable level for commercial process[31], we expected that to achieve the highest ee of (*R*)-mandelonitrile and highest productivity of reaction. In most biphasic systems, (*R*)-mandelonitrile production increased with incubation times. Specifically, tested *Pe*HNLs produced 6, 12, and 11 μM (*R*)-mandelonitrile after incubation for 12 h in 50 μL of buffer/EA, buffer/MTBE, and buffer/DBE biphasic systems, respectively (Figures 5A, 5C, and 5E). In 50-μL biphasic buffer/DEE and buffer/DIPE systems, *Pe*HNL-P preferentially synthesized 5- and 15-μM (*R*)-mandelonitrile, respectively, after incubation for 12 h (Figures 5B and 5D), strongly indicating the influence of organic solvent on *Pe*HNL activity. Moreover, MTBE was a suitable solvent for *Pe*HNL-N, as indicated by its stability relative to other *Pe*HNLs in this system, whereas HNL from *Prunus amygdalus* (*Pa*HNL) had the lowest initial catalytic activity in the buffer/MTBE biphasic system.[29] DEE, DIPE, and DBE were reportedly suitable solvents that allowed high production of cyanohydrins by HNLs from *Eriobotrya japonica* (*Ej*HNL)[30], *Arabidopsis thaliana* (*At*HNL)[32], and wild type *Pe*HNL.[4b] The glycosylated enzyme can improve the stability of *Pe*HNL when used in the biphasic system, however the productivity of (*R*)-mandelonitrile was depended on the type of organic solvent.[28a, 29] Hexane was not likely an appropriate organic solvent for the biphasic system under the present experimental conditions (Figure 5F). In our previous study, the production of cyanohydrin in a biphasic system was related to the partition coefficient for benzaldehyde between the buffer and the organic solvent. Non-enzymatic reactions are minimized by extraction and seperation of benzaldehyde into the organic phase of biphasic systems with high partition coefficients, and the HCN remains in the aqueous phase and slowly releases acetone cyanohydrin.[4b] In the present study, the Hex system gave the lowest partition value for benzaldehyde, likely accelerating the non-enzyme reaction significantly.[30]

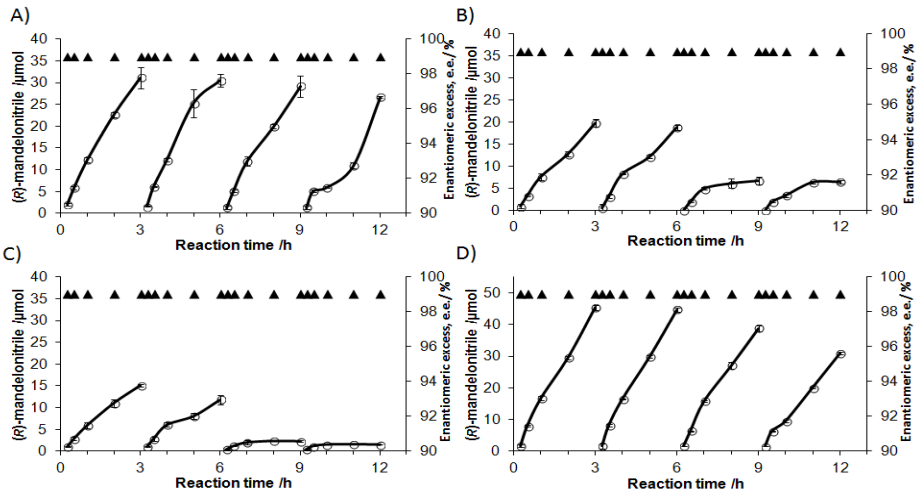

Figure 6. The reusability of *Pe*HNL-P (A), *Pe*HNL-P-N105Q (B), *Pe*HNL-E (C) and *Pe*HNL-N (D) in in synthesis of (*R*)-mandelonitrile from benzaldehyde and acetone cyanohydrin. ○, amount of (*R*)-mandelonitrile. ▲, enantiomeric excess, %. The reaction were performed in biphasic system of buffer (pH 4.0; 50% v/v) and DIPE or MTBE (50% v/v) at 10°C containing benzaldehyde (250 mM), acetone cyanohydrin (900 mM), and enzyme (5 U).

Reusability of native and recombinant *Pe*HNLs

The reusability among *Pe*HNLs in synthesis of (*R*)-mandelonitrile using biphasic systems were compared. The suitable organic solvent for *Pe*HNL-N is MTBE while that for *Pe*HNL-P, *Pe*HNL-P-N105Q and *Pe*HNL-E are DIPE (Figure 3 and 5). The ee value of (*R*)-mandelonitrile was greater than 98.5% in each experimental condition (Figure 6). After four cycles of reusability experiment, the glycosylated *Pe*HNLs, *Pe*HNL-N and *Pe*HNL-P, showed the production of (*R*)-mandelonitrile in the range of 30.8-45 μM and 26.7-31 μM, respectively (Figure 6A and 6D). In contrast, the non-glycosylated *Pe*HNLs, *Pe*HNL-P-N105Q and *Pe*HNL-E, in the first cycle of reusability experiment showed the production of 20 μM and 15 μM of (*R*)-mandelonitrile, respectively, which were lower than that by the glycosylated *Pe*HNLs. After the two cycles, the activity of non-glycosylated *Pe*HNLs was significantly decreased in biphasic system of DIPE (Figure 6B and 6C). These results is likely caused by the organic solvent stability of *Pe*HNL-P and *Pe*HNL-N, which showed the remaining activity of 90% and 80% in biphasic systems of DIPE and MTBE for 12 h incubation at 10°C, respectively (Figure 3.), while *Pe*HNL-P-N105Q and *Pe*HNL-E lost 15% and 80% of the activity in biphasic systems of DIPE at the same condition, respectively.

Conclusions

The present data warrant consideration of *Pe*HNL in the synthesis of important industrial intermediates. In this study, *Pe*HNL cDNA was successfully cloned and expressed in *E. coli* BL 21 (DE3) and *P. pastoris* GS115 cells. The wild type enzyme and the glycosylated enzyme that was expressed in *P. pastoris* showed much better thermostability, pH stability, and organic solvent tolerance than non-glycosylated *Pe*HNLs that were expressed in *E. coli* and the N105Q mutant that lacked the glycosylation site. These data indicate that the glycosylation system in *P. patoris* is desirable when used as an expression host for *Pe*HNL. Finally, synthesis of (*R*)-mandelonitrile and reusability in biphasic systems with *Pe*HNLs were significantly affected by glycosylation and the type of organic solvent. This study confirms the key roles of *N*-glycosylation in enzyme activity, kinetic parameters, and the stability of *Pe*HNL.

Experimental Section

Materials

Leaves of *P. edulis f. flavicarpa* were collected at the Botanic Gardens of Toyama (Toyama Prefecture, Japan) and stored at −20°C until use. Benzaldehyde (redistilled, 99.5%) and *racemic* mandelonitrile were purchased from Sigma Aldrich. All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Kanto Chemical (Tokyo, Japan) and were used without further purification. Restriction endonuclease and the ligation reaction mixture were obtained from Takara Bio (Kusatsu, Japan).

HNL activity assays

Enzyme activities were measured using the methods reported in our previous study [4a]. Briefly, enzyme samples (0.5-5 U/mL) were added to 0.5 mL of 400 mM citrate buffer (pH 4) containing 25 mM benzaldehyde and 50 mM potassium cyanide, mixed and incubated at 25°C for 5 min. Subsequently, 100 μL aliquots of reactant were transferred to 900-μL mixtures of *n*-hexane:2-propanol with a volume ratio of 85:15, and mixed vigorously and centrifuged at 15000 × *g* for 5 min at 4°C. Ten μL aliquots of organic phase were then analyzed using an HPLC system (717 plus Autosample, Waters, Milford, MA USA; LC-6A liquid chromatograph pump, Shimadzu, Kyoto, Japan; column heater, Sugai U- 620, Japan; SPD-10A VP UV-vis detector, Shimadzu, Kyoto, Japan) equipped with a CHIRALCEL OJ-H column (particle size, 5 μm; 4.6 mm i.d. × 250 mm; Diacel, Osaka, Japan) under the following conditions: mobile phase, *n*-hexane:2-propanol with a volume ratio of 85:15 [4b]; flow rate, 1 mL·min^{-1}; absorbance, 254 nm; column oven temperature, 30°C. Benzaldehyde, (*R*)-mandelonitrile, and (*S*)-mandelonitrile retention times were 4.9, 10.2, and 12.7 min, respectively. Enzyme activity was calculated from the linear curve of the reaction in the first 5 min. One unit of HNL activity was defined as the amount of enzyme that produces 1 μmol of optically active mandelonitrile from benzaldehyde and potassium cyanide per min.

Microorganisms

E. coli DH5α (Toyobo, Osaka, Japan), JM 109 (Takara Bio), and BL21 (DE3),(Thermo Fisher Scientific, Waltham, MA, USA) cells, and *P. pastoris* GS115 cells were used as hosts for amplification of plasmid DNAs and recombinant protein expression.

Cloning of *Pe*HNL cDNA

Young leaves were directly collected from plants, immediately frozen in liquid nitrogen, and ground in Plant RNA Reagent (Thermo Fisher Scientific). After isolation of total RNA, first-strand cDNA was synthesized using a SMART RACE cDNA Amplification kit, with a 5'-Full RACE Core Set (Takara Bio, Kusatsu, Japan), a GeneRacer Kit, and SuperScript II and III Reverse Transcriptases (Thermo Fisher Scientific). Degenerate and gene-specific primers were designed according to N-terminal amino acid sequences[4b], and partial cDNA fragments were amplified using PCR (Table S1). PCR products were gel-purified using a Wizard SV PCR and Gel Clean-Up System (Promega, Madison, WI, USA) and ligated into the *Eco*RV recognition site of pBluescript II SK (+) (Agilent Technologies, Santa Clara, CA, USA). DNA sequences were determined using a 3500 Genetic Analyzer (Thermo Fisher Scientific), assembled and analyzed using ATGC and Genetyx Ver. 12 (Genetyx, Tokyo, Japan) respectively. To avoid PCR-derived sequence error, the full-length cDNA sequence was determined using 16 independent clones. The *N*-glycosylation site was predicted using NetNGlyc 1.0 software (http://www.cbs.dtu.dk/).

Construction of expression vectors of in recombinant *Pe*HNL for expression in *E. coli* and *P. pastoris*

To express recombinant *Pe*HNL in *E. coli* cells (*Pe*HNL-E), insert DNA was PCR-amplified using 8F and 8R gene-specific primers (Table S1) and *Pe*HNL cDNA as a template. The products were then double-digested using *Sac*I and *Xba*I restriction enzymes, followed by gel-purification using a NucleoSpin (Macherey-Nagel, Germany). Purified insert DNA was ligated into the corresponding site of pCold I DNA (Takara Bio) using a DNA Ligation Kit (Mighty Mix; Takara Bio). The constructed vector was designated pColdI-*Pe*HNL.

To express recombinant *Pe*HNL in *P. pastoris* GS115 cells, insert DNA was PCR-amplified using 9F and 9R gene-specific primers (Table S1). After digestion using *Eco*RI and *Apa*I, gel-purified insert DNA was ligated into corresponding sites of pPICZαA (Thermo Fisher Scientific). The constructed vector pPICZαA-*Pe*HNL was then linearized by digesting with *Sac* I and eletroporated into *P. pastoris* GS115 cells using an Easyselect Pichia Expression Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

To determine the effects of glycosylation on the molecular and catalytic properties of the enzyme, the potential *N*-glycosylation site (asparagine residue at 105) was replaced with glutamine.

Accordingly, the pPICZαA-*Pe*HNL plasmid was mutated using a QuikChange Lightning Site-Directed Mutagenesis Kit (Aligent Technologies) and 10F and 10R gene-specific primers (Table S1), yelding the vector pPICZαA-*Pe*HNL-N105Q.

Expression and purification of recombinant *Pe*HNLs in bacterial and *Pichia* expression systems

E. coli BL21 (DE3) cells harboring pColdI-*Pe*HNL were inoculated into 5 mL of Luria-Bertani (LB) broth medium containing ampicillin (50 μg mL^{-1}) and incubated overnight at 37°C with shaking at 300 rpm. Subsequenlty, 5-mL aliquots of starter culture were transferred into LB broth (500 mL) containing ampicillin (50 μg·mL^{-1}) in a 2-L Erlenmeyer flask and cultured at 37°C with shaking at 150 rpm for 6 h, followed by a cold-shock at 15°C. After 2 h, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and cells were cultured at 15°C for 24 h with the same shaking rate. Cells were then harvested by centrifugation at 8500 × *g* for 15 min and resuspended in 20 mM potassium-phosphate buffer (KPB; pH 7.4) containing 0.5 M sodium chloride and 25 mM imidazole. Cells were then lysed by sonication, and lysates were centrifuged at 15000 × *g* for 15 min at 4°C to remove debris. Supernatants were loaded onto Ni Sepharose 6 Fast Flow (GE Healthcare, Little Chalfont, UK) columns (i.d. 25 mm; column volume, 30 mL) and eluted with a linear gradient of 25–500 mM imidazole in 20-mM KPB (pH 7.4) containing 0.5 M sodium chloride at a flow rate of 0.5 mL·min^{-1}. Fractions containing the highest enzymatic activity were pooled, dialyzed, concentrated, and loaded onto a MonoQ 5/50 GL (GE Healthcare) HPLC column. Recombinant enzyme was eluted with a linear gradient of 0–200 mM sodium chloride in 20 mM KPB (pH 6.0) at a flow rate of 0.5 mL·min^{-1}. Expression of the recombinant enzyme was monitored using SDS-PAGE[33] and enzyme activity was measured as described above.

Single *P. pastoris* colonies were transformed with pPICZαA-*Pe*HNL or pPICZαA-*Pe*HNL-N105Q and inoculated into 5 mL of YPD (1% yeast extract, 2% peptone, and 2% dextose) broth containing Zeocin (100 μg·mL^{-1}; Thermo Fisher Scientific). Inoculums were then incubated at 30°C overnight with shaking at 300 rpm. Five mL starter cultures were then transferred to YPD broth (500 mL) and cultivated at 30°C in 2-L baffled flasks. After 48 h incubation, cells were harvested by centrifugation and resuspended in 500 mL expression medium, which comprised buffered minimal methanol medium (BMMH) containing 100-mM potassium phosphate buffer (pH 6.0) 1.34% yeast nitrogen base without amino acid, 4 × 10^{-5}% biotin, 0.004% histidine, and 0.5% methanol as an inducer every 24 h. After 96 h of induction, cells were then harvested by centrifugation at 5000 × *g* for 15 min, resuspended in 20-mM KPB (pH 6.0), and lysed using a Multi-Bead Shocker (Yasui Kikai, Osaka, Japan) with 0.5 mm diameter glass beads. After centrifugation at 15000 × *g* for 15 min at 4°C to remove debris, supernatants were fractionated using 20%–60% saturated ammonium sulfate. The recombinant enzyme was then harvested by centrifugation at 18800 × *g* for 20 min at 4°C and dissolved in 120 mL of 20-mM KPB (pH 6.0). After addition of ammonium sulfate to 30% saturation, solutions containing crude enzyme were centrifuged to remove debris and loaded onto a Toyopearl Butyl-650M (Tosoh; i.d., 25 mm; column volume, 40 mL). Proteins were eluted with a gradient of ammonium sulfate from 30% to 0% in the same buffer and the active fraction was dialyzed against 20-mM KPB (pH 6.0), loaded onto a Toyopearl DEAE-650M (Tosoh; i.d. 25 mm; column volume, 20 mL) column, and eluted with a linear gradient of sodium chloride from 0 to 200 mM at a flow rate of 0.5 mL min^{-1}. After addition of ammonium sulfate to 30% saturation, active fractions were centrifuged at 15000 × *g* for 15 min at 4°C and supernatants were loaded onto a Resource Phe column (volume, 1 mL; GE Healthcare). Proteins were eluted with a gradient of ammonium sulfate from 30% to 0% in the same buffer at a flow rate of 0.5 mL·min^{-1}. Active fractions were pooled, concentrated, and desalted using a centrifugal filtration device (Amicon Ultra-15; 10,000 NMWL, EMD Millipore, Billerica, MA, USA), and proteins were loaded onto a MonoQ 5/50 GL column and eluted with a linear gradient of sodium chloride from 0 to 200 mM in 20 mM KPB (pH 6.0) at a flow rate of 0.5 mL·min^{-1}. The positive fraction was re-purified using a Resource Phe as described above. Protein elution was monitored at 280 nm in each purification step, and protein fractions were analyzed using SDS-PAGE. All purification steps were performed at 4°C.

Purification of *Pe*HNL from leaves

Leaves of *Passiflora edulis* (1500 g wet weight) were collected at the Botanic Garden of Toyama and native *Pe*HNL was purified as described previously.[4b] Proteins were fractionated with 20%–40% saturated ammonium sulfate and collected by centrifugation at 18800 × *g* for 20 min at 4°C. Harvested proteins were dissolved in 100 mL of 20 mM KPB (pH 6.0) and dialyzed against the same buffer three times. The resulting fractions were loaded onto a DEAE Toyopearl-650M column (i.d., 25 mm; column volume, 50 mL) and eluted with a linear gradient of sodium chloride from 0 to 100 mM in 20 mM KPB (pH 6.0) at a flow rate of 0.5 mL min^{-1}. After addition of ammonium sulfate to 30% saturation, positive fractions were loaded onto a Butyl-Toyopearl (Tosoh; i.d., 25 mm; column volume, 25 mL) column. Proteins were then eluted stepwise with 1330, 860, 420, and 0 mM ammonium sulfate in 20 mM KPB (pH 6.0) at a flow rate of 0.5 mL·min^{-1}. Active fractions were pooled, concentrated, and desalted using a centrifugal filtration device (Amicon Ultra-15; 10,000 NMWL, EMD Millipore) and then loaded onto a MonoQ 10/100 GL and eluted with a linear gradient of sodium chloride from 0 to 150 mM in 20 mM KPB (pH 6.0) at a flow rate of 1.0 mL min^{-1}. Positive fractions were repurified using Resource Phe as described above. Subsequently, active fractions were concentrated in fresh 10 mM KPB (pH 6.0) using a centrifugal filtration device (Amicon Ultra-15; 10,000 NMWL, EMD Millipore) and loaded onto a Ceramic Hydroxyapatite type I column (i.d., 7 mm; column volume, 2 mL; BioRad, Hercules, CA, USA). Proteins were then eluted with a gradient of 10 to 500 mM KPB (pH 6.0) at a flow rate of 0.5 mL min^{-1}. After concentration and replacement of buffer with 20 mM Tris-HCl (pH 7.4) containing 0.5-M sodium chloride using a centrifugal filtration device, enzymes were loaded onto a HiTrap Con A 4B (1 ml; GE Healthcare) and eluted with a linear gradient of 0–100 mM α-D-methlyglucoside in the same buffer at a flow rate of 0.3 mL min^{-1}.

Periodic acid-Schiff (PAS) staining

After separation of each *Pe*HNL using SDS-PAGE, glycosylation of purified *Pe*HNLs was detected using periodic acid-Schiff (PAS) staining with a GelCode Glycoprotein Staining Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Measurement of kinetic parameters

The initial velocity of the enzymatic synthesis of benzaldehyde to (*R*)-mandelonitrile was assayed in 400 mM sodium citrate buffer, pH 4.0, according to method as described above at various substrate concentrations.

Measurement of pH and temperature stability

To determine pH stability, each *Pe*HNL was preincubated at 30°C in the range of pH 3.5 to 10 (40 mM) for 1 h. On the other hand, in determination of thermostability the enzyme was preincubated in 20 mM potassium-phosphate buffer, pH 6.0 in the range of 30°C to 80°C for 1 h. The remaining activity of the enzyme was measured as described above.

Measurement of organic solvent stability

Each organic solvent (0.75 mL) ethyl acetate (EA), diethyl ether (DEE), methyl-*t*-butyl ether (MTBE), 2-isopropyl ether (DIPE), dibutyl ether (DBE), and hexane (HEX) was mixed with equal volumes of 400 mM citrate-phosphate buffer (0.75 mL; pH 4.0) and equilibrated with shaking at 1500 rpm for 60 min. Native or purified recombinant *Pe*HNLs were added to the citrate-phosphate buffer phase, gently mixed without disturbing the interface between the aqueous and organic phases. The enzyme activity at time zero were measured under the satuated solvent in aqueouse phase and then incubated for 12 h at 30°C with shaking at 1500 rpm. Remaining enzyme activities were analyzed as described above.

To investigate transcyanation reactions in biphasic systems, organic phases containing benzaldehyde (250 mM) were mixed with 400 mM citrate buffer (pH 4.0) containing 2.34 U of each partially purified *Pe*HNL in a total volume of 1.5 mL in 2.0-mL micro-tubes. Reactions were initiated by adding acetone cyanohydrin (900 mM), and the mixture was incubated at 10°C with shaking at 1500 rpm in an incubator shaker (BioShaker M-BR-022UP, Taitec Corporation, Tokyo, Japan). Aliquots of sample (50 µL) were collected from the organic phase at various times and analyzed using HPLC with a chiral column as described above, and ee were determined according to relative concentrations (mM) of the two enatiomers using Eq. (1):

$$\text{ee (\%)} = \frac{[R]-[S]}{[R]+[S]} \times 100 \qquad (1).$$

Reusability

In the studies on the stability and transcyanation of *Pe*HNL in biphasic system, MTBE was selected for *Pe*HNL-N while DIPE was chosen for *Pe*HNL-P, *Pe*HNL-P-N105Q and *Pe*HNL-E. All enzymes were used in the batch transcyanation reaction of benzaldehyde (250 mM) and acetone cyanohydrins (900 mM) in biphasic system of 400 mM citrate buffer, pH 4.0 (0.75 mL) and MTBE or DIPE (0.75 mL) in 2.0-mL micro-tube. Reactions were initiated by addition of each *Pe*HNL (5.0 U) into each biphasic system and and incubated at 10°C with shaking at 1500 rpm. After 3 h incubation, the buffer phase containing the enzyme was recovered and dialyzed against the 400 mM citrate buffer, pH 4.0 (100 mL of buffer; 3 h at 4°C) and then was re-used for the next batch reaction under the same condition. Amount of (*R*)-mandelonitrile produced was measured as described above.

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Keywords: Hydroxynitrile lyase • *Passiflora edulis* • enzyme catalysis • glycosylation • enzyme stability

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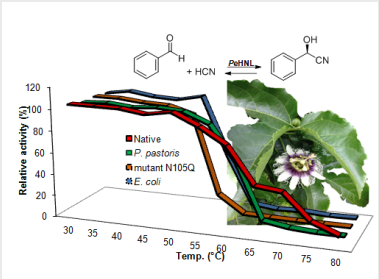
FULL PAPER

Hydroxynitrile lyase from *Passiflora edulis*, (*Pe*HNL) showed great stability in the mandelonitrile synthesis. Here we report the key roles of *N*-glycosylation in the enzyme activity, kinetic parameters, and the stability of *Pe*HNL.



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Effect of glycosylation on the biocatalytic properties of hydroxynitrile lyase from the passion fruit, *Passiflora edulis* - comparison of natural and recombinant enzymes

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