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Stabilization of hydroxynitrile lyases from two variants of passion fruits, *Passiflora edulis* Sims and *Passiflora edulis* forma *flavicarpa* by C-terminal truncation

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Abstract: As the synthesis of chiral compounds generally requires a broad range of substrate specificity and stable enzymes, screening for better enzymes and/or improvement of enzyme properties using molecular approaches is necessary for the sustainable industrial development. Here, we report the discovery of unique hydroxynitrile lyases (HNLs) from two species of passion fruits, *Passiflora edulis* forma *flavicarpa* (yellow passion fruit, *Pe*HNL-Ny) and *Passiflora edulis* Sims (purple passion fruit, *Pe*HNL-Np) isolated and purified from passion fruit leaves. These are the smallest HNLs (comprising 121 amino acids). Amino acid sequences of both enzymes are 99% identical; there is a difference of one amino acid in a consensus sequence. Purple passion fruit *Pe*HNL-Np has Ala residue at position 107 and is non-glycosylated at Asn105. As it was confirmed that natural and glycosylated *Pe*HNL-Ny showed superior thermostability, pH stability, and organic tolerance than the *Pe*HNL-Np, we speculated that protein engineering around the only one glycosylation site, Asn105 located at the C-terminal region of *Pe*HNL-Ny might contribute to stabilization of *Pe*HNL. Therefore, we focused on the improvement of stability of the non-glycosylated *Pe*HNL by truncating its C-terminal region. The C-terminal truncated *Pe*HNLΔ_{107} was obtained by truncating 15 amino acids from its C-terminal and was expressed in *Escherichia coli*. *Pe*HNLΔ_{107} expressed in *E. coli* was not glycosylated, and showed improved thermostability, solvent stability, and reusability similar to the wild-type glycosylated form of *Pe*HNL expressed in *Pichia pastoris*. These data reveal that the lack of the high flexibility region at the C-terminal of *Pe*HNL might be the possible reason for improving the stability of *Pe*HNL.

Introduction

Hydroxynitrile lyases (HNLs, EC 4.1.2.10, 4.1.2.11, 4.1.2.46, and 4.1.2.47) act during the final step of the cyanohydrin biodegradation pathway.[1] In cyanogenic plants, HNL plays a role in the defense mechanism of plants by acting decomposition of stored cyanogenic glycosides into aldehydes and hydrogen cyanide for protection against microbes and herbivores. Correspondingly, it was discovered that an arthropod (millipede) synthesizes mandelonitrile as a chemical for defense in order to produce hydrogen cyanide by catalysis of a HNL.[2] As the reversible reaction of HNLs, the enzyme also catalyzes the synthesis of optically pure cyanohydrins which are important synthetic intermediates for fine chemical and pharmaceutical industries.[3] Mainly, HNLs have been discovered and characterized from cyanogenic plants, and they include the (*R*)-selective ones derived from *Prunus amygdalus* (*Pa*HNL),[4] *Prunus serotina* Ehrh. (*Ps*HNL),[5] *Prunus mume* (*Pm*HNL),[6] *Prunus armeniaca* L. (*Pars*HNL),[7] *Prunus communis* (*Pc*HNL),[8] *Linum usitatissimum* (*Lu*HNL),[9] *Phlebodium aureum* (*Pha*HNL),[10] *Arabidopsis thaliana* (*At*HNL),[11] *Eriobotrya japonica* (*Ej*HNL),[12] *Passiflora edulis* f. *flavicarpa* (*Pe*HNL),[13] *Davallia tyermannii* (*Dt*HNL),[14] and *Nandina domestica* (*Nd*HNL),[15] and the (*S*)-selective ones derived from *Hevea brasiliensis* (*Hb*HNL),[16] *Manihot esculenta* (*Me*HNL),[17] *Sorghum bicolor* (*Sb*HNL),[18] and *Baliospermum montanum* (*Bm*HNL).[19] Among the HNLs of plant origin, *Sb*HNL,[18] *Ej*HNL,[12] *Pe*HNL,[13c] *Pa*HNL,[20] *Pc*HNL,[8] and *Pm*HNL[21] are glycosylated proteins, whereas, *Lu*HNL, *Bm*HNL, *At*HNL, *Nd*HNL, *Hb*HNL, *Me*HNL, and *Pha*HNL are not glycosylated. Furthermore, *Ej*HNL, *Pa*HNL *Pc*HNL, and *Pm*HNL contain flavin adenine dinucleotide (FAD), in contrast to the *Lu*HNL, *Pha*HNL, *At*HNL, *Pe*HNL, *Bm*HNL, *Hb*HNL, *Me*HNL, *Sb*HNL, and *Nd*HNL which do not contain FAD. Moreover, the cupin fold-containing HNLs derived have also been discovered from bacteria, *Granulicella tundricola* (*Gt*HNL)[22] and *Acidobacterium capsulatum* (*Ac*HNL).[23]

Two major passion fruit sub-species, *Passiflora edulis* Sims, which yields purple fruits and *Passiflora edulis* f. *flavicarpa,* which gives yellow ones are used in commercial passion fruit products such as concentrated juice, jam, nectar, etc. Whether the yellow passion fruit is a mutant of the purple variety *P. edulis* or its hybrid with other species is not elucidated.[24] Both species contain large quantities of the cyanogenic compound, especially prunasin representing 80 % of cyanogenic glycosides.[25] First, the HNL from *Passiflora edulis* f. *flavicarpa* (*Pe*HNL-Ny) was isolated and purified in our laboratory,[13a, 13b] and its cDNA was successfully cloned into *E.*

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coli and *P. pastoris*.[13c] Glycosylated *Pe*HNL-Ny showed high stability at low pH and in organic solvents that are always required for cyanohydrin synthesis for industrial use. Therefore, we expected that *P. edulis* Sims might also be a valuable resource for discovering new HNLs.

We recently reported the X-ray crystallographic analysis of *Pe*HNL-Ny, and found that *Pe*HNL belongs to the dimeric α+β barrel (DABB) superfamily consisting of a central β-barrel in the middle of the dimer, and the high flexibility region was observed in the rich hydrophobic residues at a residue 107 which lies beyond the C-terminal.[26] We also found that the wild-type glycosylated form of natural *Pe*HNL and the one produced in *P. pastoris Pe*HNL both of which have one glycosylation site at Asn105 located near the C-terminus, showed significantly higher stability than the enzyme without glycosylation produced in *E. coli* which lack the glycosylation system.[13c] Because of the drawback of the time-consuming process of production of glycosylated *Pe*HNL in *P. pastoris*, we aimed to improve the stability of *Pe*HNL expressed in *E. coli* using a targeted mutagenesis approach. The flexible regions are potential targets and thus have been selected for molecular engineering to improve stability of the enzyme.[27] This region can be evaluated with the highest B-factor, a value determined on the basis of atomic displacement obtained from X-ray data.

In this study, we looked for a novel *Pe*HNL from purple *P. edulis* cultivated in Thailand, purified the enzyme and cloned its cDNA for expression of *Pe*HNL-Np, and discovered one mutation among the C-terminal region which affects the stability of *Pe*HNLs upon comparison of its structure and activity. Taken together, the finding of the natural diversity of *Pe*HNLs among the sub-species of passion fruits, and our previous results obtained using molecular engineering of *Pe*HNL-Ny, we speculated that the presence of the glycan at the C-terminal might significantly contribute to the stability of *Pe*HNLs. We improved the activity of *Pe*HNL by reducing the length of the enzyme at the C-terminus using site-directed mutagenesis. The engineered enzymes were purified, and their improved properties were characterized and compared with those of the wild-type enzymes. The substrate specificity of the synthesized enzymes and the enantiomeric excess of the cyanohydrins synthesized in biphasic systems were determined.

Results and Discussion

*Pe*HNL is present in two species of passion fruits

The HNL from *P. edulis* f. *flavicarpa* (yellow passion fruit, *Pe*HNL-Ny) has been discovered, characterized, and gene coding for it was successfully cloned and expressed to produce the recombinant *Pe*HNL-Ny.[13] Recently, we solved the *Pe*HNL-Ny structure and proposed its catalytic mechanism.[26] Here, we report the discovery of HNL from another commercial passion fruit species, *P. edulis* SIM (purple passion fruit, *Pe*HNL-Np), which is cultivated on a large scale in Thailand. A large amount of *P. edulis* SIM leaves (5000 g) was collected at a plantation in Chaing-Mai, Thailand. The HNL activity from the leaf extract was determined by (*R*)-mandelonitrile ((*R*)-MAN) synthesis using HPLC equipped with a chiral column. *Pe*HNL-Np levels (2.42 U/g leaf sample) detected in leaves were approximately 3.3-fold less than in *P. edulis* f. *flavicarpa* (8.13 U/ g leaf sample. *Pe*HNL-Np was also purified to near homogeneity using several rounds of column chromatography as summarized in Table S1. The specific activity for the synthesis of (*R*)-mandelonitrile from benzaldehyde and potassium cyanide (KCN) was 120 U/mg, which was marginally lower than that from *Pe*HNL-Ny (136 U/mg). *Pe*HNL-Np has a molecular mass of 14 kDa, same as *Pe*HNL-Ny, as revealed by SDS-PAGE; moreover, they have 99% identity. At position 107, *Pe*HNL-Np from purple passion fruit has an Ala residue, whereas that from yellow passion fruit has a Thr residue (Figure S2). Therefore, the purple passion fruit *Pe*HNL cannot be glycosylated on the nitrogen atom of the Asn residue of nascent polypeptide in the recognition tripeptide sequence Asn-X-Ser/Thr.[28] Periodic acid Schiff (PAS) staining of the purified *Pe*HNL-Np confirmed that it is not a glycoprotein (Figure S1). Using various approaches such as the use of *N*-glycosylation and *N*-glycan-processing inhibitors and site-directed mutagenesis of *N*-glycosylation site, glycans in plant glycoproteins have long been shown to play a major role in the folding, processing, and secretion of proteins from the endoplasmic reticulum (ER) and the Golgi apparatus.[29] For example, treatment of suspension-cultured carrot (*Daucus carota*) cells with tunicamycin, an antibiotic which inhibits Asn-linked glycosylation, yielded unglycosylated carrot cell-wall β-fructosidase, and the unglycosylated enzyme was easily degraded during the last stages in the secretory pathway or immediately after its arrival in the cell wall.[30] The *N*-glycan is also known to protect the protein from proteolytic degradation and is responsible for thermostability, solubility, and biological activity of concanavalin A (Con A), the lectin from jack bean seeds.[31]

The amount of glycosylated *Pe*HNL-Ny in the whole leaves of the yellow passion fruit was 3.3-fold higher per mg of the total leaf protein than that of non-glycosylated *Pe*HNL-Np in the leaves of purple passion fruits. It seems likely that the presence of glycan affected the transport of *Pe*HNL in the yellow passion fruit cells. The yellow passion fruit grows faster and has a greater resistance to soil fungi, whereas the purple one is more resistant to cold injury.[24] Thus, yellow and purple passion fruits may have been adopted to their environment and evolved to control the amounts of HNLs by glycosylation, according to their survival strategies.

Biochemical properties of two *Pe*HNLs

To compare enzyme kinetics of two natural *Pe*HNLs, the rate of synthesis (*R*)-mandelonitrile from benzaldehyde and KCN was measured using HPLC-chiral column analyses. The V_{max}, k_{cat}, and k_{cat}/K_m values of glycosylated *Pe*HNL-Ny were 220 μmol min^{-1} mg^{-1}, 50.4 s^{-1}, and 3.70 mM^{-1} s^{-1}, respectively, and these values were higher than those of non-glycosylated *Pe*HNL-Np (150 μmol min^{-1} mg^{-1}, 35.7 s^{-1}, and 1.86 mM^{-1} s^{-1}, respectively,) (Table 1.). The K_m of *Pe*HNL-Ny (13.7 mM) was slightly lower than that of *Pe*HNL-Np (19.2 mM), which corresponds with our previous report that the glycan on *Pe*HNL enhances the catalytic efficiency without reducing the substrate affinity for benzaldehyde and KCN.[13c] Also, we proved that those kinetic parameters of natural *Pe*HNL were caused by the presence of glycan.

Table 1. Kinetic parameters of native and C-truncated mutant *Pe*HNLs.

Enzyme	Spec. act. (U mg^{-1})	V_{max} (µmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
*Pe*HNL-Ny	136	220±0.50	13.7±0.01	50.4±0.01	3.70±0.01
*Pe*HNL-Np	120	150±3.20	19.2±0.03	35.7±0.04	1.86±0.01
*Pe*HNL$_{121p}$	110	145±3.27	25.2±0.46	33.6±0.50	1.33±0.01
*Pe*HNL$_{121y}$	122	168±2.37	26.3±0.14	36.4±0.50	1.40±0.32
*Pe*HNLΔ$_{117}$	134	228±0.67	23.4±0.14	53.4±0.16	2.30±0.52
*Pe*HNLΔ$_{112}$	167	270±0.80	25.0±0.16	63.1±0.20	2.53±0.30
*Pe*HNLΔ$_{109}$	118	167±0.37	26.1±0.11	39.0±0.10	1.50±0.05
*Pe*HNLΔ$_{107}$	112	158±0.40	26.6±0.13	37.2±0.10	1.40±0.06
*Pe*HNLΔ$_{105}$	60	103±0.20	33.0±0.12	24.1±0.04	0.73±0.01
*Pe*HNLΔ$_{102}$	n.d.	n.d.	n.d.	n.d.	n.d.

The purified *Pe*HNLs were prepared from various sources; Ny and Np were purified from natural yellow and purple passion fruit leaves, respectively; the subscript number 121p and 121y represented to the whole amino acid sequences of purple and yellow passion fruit, respectively, which were expressed and purified from recombinant in *E. coli*; the C-truncated mutant *Pe*HNLs (*Pe*HNLΔ) with a subscript number corresponds to the total *Pe*HNL length after truncated at C-terminal. The kinetic parameters were obtained by monitoring synthesis of (*R*)-MAN by an HPLC at 254 nm with conducting experiments in triplicates. n.d. = not determined

We determined the kinetic parameters using both purified recombinant *Pe*HNLs lacking glycosylation expressed in *E. coli*. The K_m, V_{max}, k_{cat}, and k_{cat}/K_m values of *Pe*HNL$_{121y}$ were estimated to be 26.3 mM, 168 µmol min^{-1} mg^{-1}, 36.4 s^{-1}, and 1.85 mM^{-1} s^{-1}, respectively, and these values were similar to those of *Pe*HNL$_{121p}$ (25.2 mM, 145 µmol min^{-1} mg^{-1}, 33.6 s^{-1}, and 1.33 mM^{-1} s^{-1}, respectively) (Table 1). Thus, the difference of one amino acid at position 107 of *Pe*HNL appears not to affect the affinity and catalytic efficiency of *Pe*HNL.

The effect of pH and temperature on the synthesis (*R*)-mandelonitrile was studied using the purified natural *Pe*HNLs. *Pe*HNL-Ny and *Pe*HNL-Np exhibited the highest (*R*)-MAN synthetic activity at pH 4.0 with >95% *ee* (Figure. 1A). The *ee* of 99% (*R*)-MAN was obtained at pH 2.5-3.5 for both *Pe*HNLs, but it obviously decreased at pH higher than 4.5 due to a rapidly increasing the spontaneous non-enzymatic reaction yielding the racemic of both enantiomers. The pH stability analyses revealed that both natural *Pe*HNLs were broadly stable over the range of 3.5 to 10 after preincubation at 30°C for 1 h without substrate (Figure. 1C).

The optimum temperature range of both *Pe*HNLs for the (*R*)-MAN synthetic activity was 20 to 25 °C (Figure. 1B). The *ee* of the product by the two natural *Pe*HNLs were more than 95% at low temperatures between 5°C to 20°C, and low *ee* products were obtained when the temperature was increased, due to the acceleration of the non-enzymatic reaction. In addition, the activity of *Pe*HNL-Ny remained above 80% after 1 h incubation at 60°C (Figure. 1D), while *Pe*HNL-Np retained only 40% of activity under the same condition. Obviously, the thermostability was significantly improved by glycosylation in *Pe*HNL-Ny. Furthermore, glycosylated *Pe*HNL-Ny was more stable in the presence of an organic solvent in a biphasic system than non-glycosylated *Pe*HNL-Np toward all the organic solvents tested (Figure. S3). This evidence supports that the glycosylated *Pe*HNL is more stable than non-glycosylated *Pe*HNL.[13c] Recently, we solved the structure of *Pe*HNL and discussed that a glycosylation site on Asn105 of *Pe*HNL might stabilize the high flexibility region at C-terminus, which could improve *Pe*HNL stability.[26] Thus, we hypothesized that reduction in the high flexibility region of *Pe*HNL by the deletion of part of C-terminal region increases the stability of non-glycosylated *Pe*HNL.

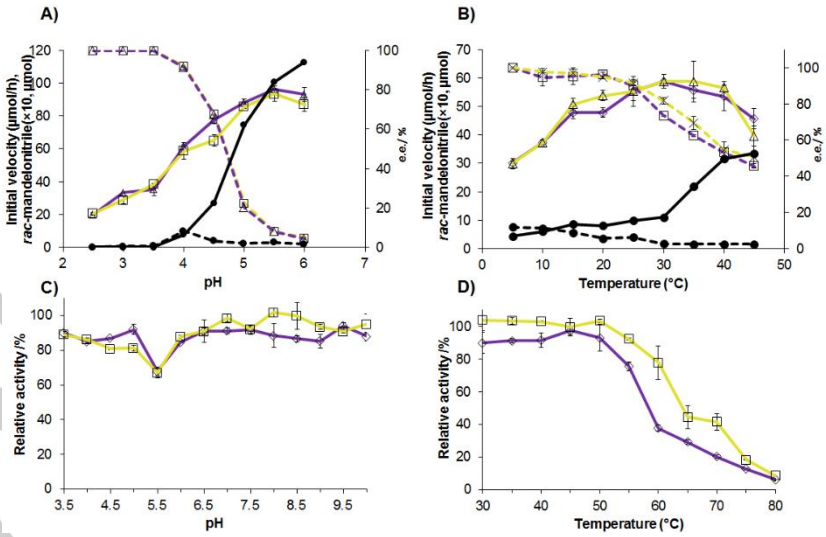

Figure 1. The comparison of biochemical properties of two *Pe*HNLs; *Pe*HNL-Ny (yellow line) and *Pe*HNL-Np (purple line) and *rac*-mandelonitrile formed by non-enzymatic reaction (black line). A) Effect of pH and B) temperature on initial velocity (solid line) and enantiomeric excess (dashed line). C) pH stability and D) temperature stability after incubation of enzymes at various pH or temperatures for 1 h. Remaining 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 mean ± SD (n = 3)

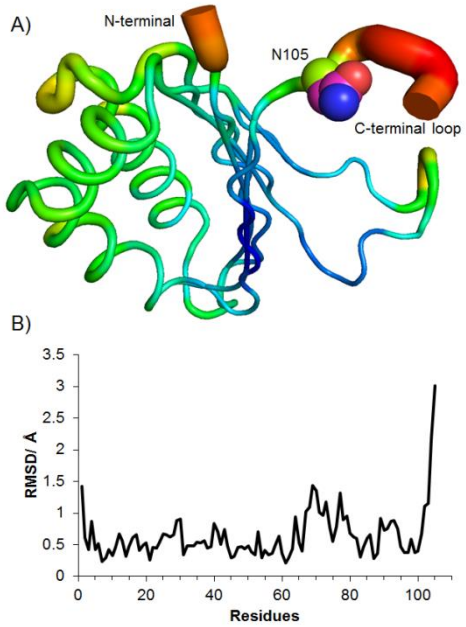

Figure 2. Flexibility in *Pe*HNL. A) A B-factor putty drawing of wild-type *Pe*HNL (PDB ID:5XZQ) structure in the nucleocapsid-like particle. Regions colored red also have a large diameter, which corresponds to higher B factors and are associated with higher structural flexibility. A consensus sequence (N105) for *N*-glycosylation site on *Pe*HNL is shown as cyan spheres. B) Root mean square deviation (RMSD) between the crystal structure of wild-type and *Pe*HNLΔ_{107} (PDB ID: 5XZT).

Identification of flexible regions in *Pe*HNL

The secondary structure of wild-type *Pe*HNL (PDB ID:5XZQ) consists mainly of four antiparallel β-sheets (β1, 6-14 aa; β2, 44-47 aa; β3, 62-68 aa; β4, 94-103 aa), three α-helices (α1, 20-36 aa; α2, 71-79 aa; α3, 81-93 aa), and a special loop at the C-terminal that mainly contains hydrophobic amino acid residues ($_{104}$INETFPYTWALPNKYVVT$_{121}$).[26] During structural analysis, we missed the C-terminus region (residues 110-121) because of the poor electron density, indicating that this region has high flexibility and does not form a stable structure.[26] Generally, the B-factor, which can evaluate the flexibility of an individual residue in a protein, is used for explaining the behavior of atomic electron densities from X-ray data.[27a] The available C-terminal region of wild-type *Pe*HNL crystal structure showed remarkably high B-factor values at the residues E106, T107, F108, and P109, which were 90, 70.5, 72.6, and 82.5, respectively (Figure. 2A). In agreement with the root mean squared deviation (RMSD) at the C-terminal region of the similarity between two superimposed atomic coordinates of *Pe*HNL wild-type and *Pe*HNLΔ_{107} (PDB ID: 5XZT)[26] structure also showed a high RMSD, with an average value above 1.281Å (Figure. 2B). The RMSD value of this region is much higher than that of other regions, indicating that the C-terminus region is highly flexible, which might be responsible for the instability of *Pe*HNL.

Expression and purification of C-terminal truncation mutants

The deletion of unnecessary amino acid or domains at the N- or C-terminus of some enzymes have been used for improving the enzymatic properties and/or enzyme stability, such as endo-β-1,4-glucanase,[32] amylopullulanase,[33] keratinase,[34] and amylase.[35] To further study the roles of the C-terminal loop of *Pe*HNL, truncation mutants were constructed lacking the chosen amino acid residues from this region. Five to 18 residues (4.1 to 14% of the total *Pe*HNL length) were deleted from the C-terminus. Up to 17 amino acid residues of *Pe*HNL could be removed (*Pe*HNLΔ_{105}) without abolishing its activity. A 6xHis tag was attached to the N-terminus of truncation mutants and they were heterologously expressed in *E. coli* BL21 (DE3) cells. The yields of all C-terminal truncation mutants were similar, except *Pe*HNLΔ_{105}, which showed the lowest *Pe*HNL production. The truncation mutants were purified using Ni-sepharose 6 Fast Flow and a Mono Q 5/50 column. The purified *Pe*HNLΔ_{117}, *Pe*HNLΔ_{112}, *Pe*HNLΔ_{109}, *Pe*HNLΔ_{107}, and *Pe*HNLΔ_{105} had molecular masses of approximately 14.9, 14.4, 14.3, 14.2, and 14.2 kDa, respectively, in SDS-PAGE analyses (Figure S4). The molecular masses of these mutants as determined by high-performance gel chromatography on Superdex 200 10/30 were 27.6, 26.8, 26.8, 26.6, and 26.4 kDa, respectively, indicating that the native enzyme was active as a homodimer.

Analysis of specific activity and kinetic parameters of C-terminal truncation mutants

The C-terminal truncation mutants affected the mandelonitrile synthesis activity of *Pe*HNL as shown in Table 1. The specific activity of *Pe*HNLΔ_{117} and *Pe*HNLΔ_{112} were 134 U mg^{-1} and 167 U mg^{-1}, respectively, which were marginally higher than that of wild-type *Pe*HNL$_{121}$. In contrast, the specific activity of *Pe*HNLΔ_{105} was 60 U mg^{-1}, significantly (2-fold) lower than that of wild-type *Pe*HNL$_{121}$; *Pe*HNLΔ_{102} activity could not be detected. These results indicated that the 15 amino acid deletion from C-terminus did not affect *Pe*HNL activity. Next, we determined kinetic parameters of the C-terminal truncation mutants for the synthesis of mandelonitrile from benzaldehyde and KCN. The V_{max}, k_{cat}, and k_{cat}/K_m of *Pe*HNLΔ_{117} and *Pe*HNLΔ_{112} were 228±0.67 and 270±0.80 μmol min^{-1} mg^{-1}, 53.4±0.16 and 63.1±0.20 s^{-1}, 2.30±0.52 and 2.53±0.30 mM^{-1} s^{-1}, respectively,

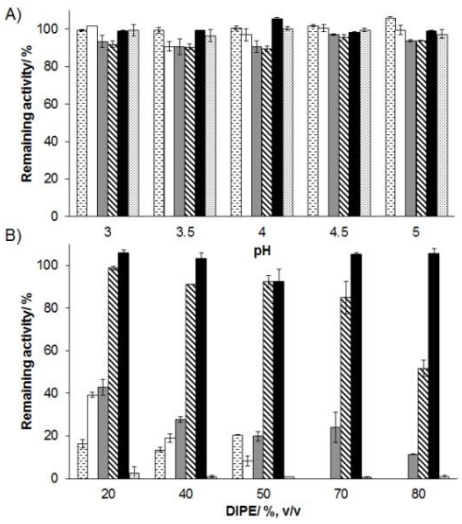

Figure 3. The stability profile of wild-type and C-terminal truncated *Pe*HNL variants. A) Stability at low pH. After incubation in reaction mixtures with varying pH values for 1 h, the remaining activity was measured as described in Figure 1. B) Solvent stability, the remaining activities of *Pe*HNL mutants were measured after incubation in each biphasic system in a citrate buffer (200 μL)/organic solvent (various solvent phase content) at 10 °C for 12 h using HPLC with a chiral column. Dashed bar: *Pe*HNL$_{121y}$. Open bar: *Pe*HNLΔ_{117}. Gray bar: *Pe*HNLΔ_{112}. Cross bar: *Pe*HNLΔ_{109}. Solid bar: *Pe*HNLΔ_{107}. Dot bar: *Pe*HNLΔ_{105}. Data are presented as means ± SD (n = 3)

while the V_{max}, k_{cat}, and k_{cat}/K_m of other C-terminal truncation mutants were similar to those of the wild-type, except for *Pe*HNLΔ$_{105}$, which had the lowest values. The K_m values of wild-type, *Pe*HNLΔ$_{117}$, *Pe*HNLΔ$_{112}$, *Pe*HNLΔ$_{109}$, and *Pe*HNLΔ$_{107}$ were 26.3±0.14, 23.4±0.14, 25.0±0.16, 26.1±0.11, and 26.6±0.13 mM, respectively (Table 1). Thus, truncation of 5-15 amino acid residues from C terminal did not affect the affinity of *Pe*HNL for benzaldehyde.

C-terminal truncation mutant *Pe*HNLΔ$_{107}$ improved *Pe*HNL stability

To prove that the flexible region at C-terminus of *Pe*HNL contributes to enzyme stability, we further investigated the resistance of the wild-type enzyme and truncation mutants to low pH, temperature, and organic solvent. A low pH is necessary for the enantioselective synthesis of cyanohydrins because of the spontaneous unselective addition of HCN to the carbonyl group occurs as a background reaction at elevated pH.[6, 36] We analyzed the stability of C-terminal truncated mutants of *Pe*HNL at low pH (3.0 to 5.0). All *Pe*HNLs were stable over the pH range tested (Figure 3A). Although stability under most pH condition was comparable, a marginal difference in the stability was observed among the *Pe*HNLs. Wild-type, *Pe*HNLΔ$_{117}$, and *Pe*HNLΔ$_{107}$ showed > 95% remaining activity at low pH, which was slightly higher than the remaining activities of *Pe*HNLΔ$_{112}$, and *Pe*HNLΔ$_{109}$ (90%) under same pH conditions for 1 h incubation. It appears that the deletion of 5-17 amino acid residues from C-terminus does not increase the stability of *Pe*HNL under the pH range tested.

Thermostability analyses revealed that the partial deletion of 5 to 13 amino acid (*Pe*HNLΔ$_{117}$, *Pe*HNLΔ$_{112}$, and *Pe*HNLΔ$_{109}$) from C-terminus decreased the thermostability at 45°C compared with that of wild-type for 1 h preincubation. Surprisingly, only *Pe*HNLΔ$_{107}$ exhibited >80% and >60% remaining activity at 50°C and 55°C, respectively, which was highest for 1 h preincubation (Figure 4A). *Pe*HNLΔ$_{107}$ also showed >40% remaining activity after 2 h incubation at 50°C, in contrast to the wild-type and other C-terminal truncated variants that were rapidly inactivated (Figure 4B). These results suggest that the deletion of high flexibility region of 15 amino acid at C-terminal likely contributes to the thermostability of *Pe*HNL.

Previously, we found that diisopropyl ether (DIPE) is a suitable organic solvent for cyanohydrin synthesis by non-glycosylated *Pe*HNL expressed in *E. coli* when used in a biphasic system; however, the wild-type non-glycosylated *Pe*HNL expressed in *E. coli* has low stability in an organic solvent.[13c] In this study, we compared the solvent stability of the wild-type and C-terminus truncated mutants of *Pe*HNL in a biphasic system of buffer and different concentration of DIPE. The stabilities of the variants were monitored after incubation at 10 °C for 12 h with shaking at 1,500 rpm and the residual activity of enzyme were compared with the initial activity in the aqueous phase saturated with organic solvents before incubation. The highest solvent stability was shown by *Pe*HNLΔ$_{107}$ in all concentrations of DIPE (20−80%), with more than 90% residual activity. On the other hand, activity of the wild-type, *Pe*HNLΔ$_{117}$, *Pe*HNLΔ$_{112}$, and *Pe*HNLΔ$_{105}$ enzymes reduced to 90% to 50% as the DIPE concentration increased from 20% to 50% v/v, and these enzymes were almost inactivate at a high DIPE concentration of 70% to 80% v/v (Figure 4B). These results demonstrated that deleting 15 amino acid from the C-terminus (*Pe*HNLΔ$_{107}$) improved the thermostability and solvent stability of *Pe*HNL.

***Pe*HNLΔ$_{107}$ catalyzes the synthesis of cyanohydrins**

We tried to improve the stability of non-glycosylated *Pe*HNL expressed in *E. coli* by truncation of its C-terminus, it showed potential for cyanohydrin synthesis in a biphasic system with an organic solvent. It has been reported that biphasic systems could improve the enantiomeric excess of the cyanohydrin product and minimized the non-enzymatic cyanation reaction upon extraction and separation of an aldehyde into the organic phase in a biphasic system.[13b, 13c, 37] In this study, we synthesized cyanohydrins in the presence of either the wild-type form *Pe*HNL or *Pe*HNLΔ$_{107}$ (10 U) in biphasic systems containing several aldehydes at high concentration (250 mM) in DIPE (0.75 mL) and an equal volume of KCN (300 mM) in citrate buffer (400 mM, pH 3.5) used as a substrate. *Pe*HNL showed a preference for several aromatic aldehydes other than bulky aromatic aldehydes such as 3-methoxybenzaldehyde (1f), 1-naphthaldehyde (1i) and 2-

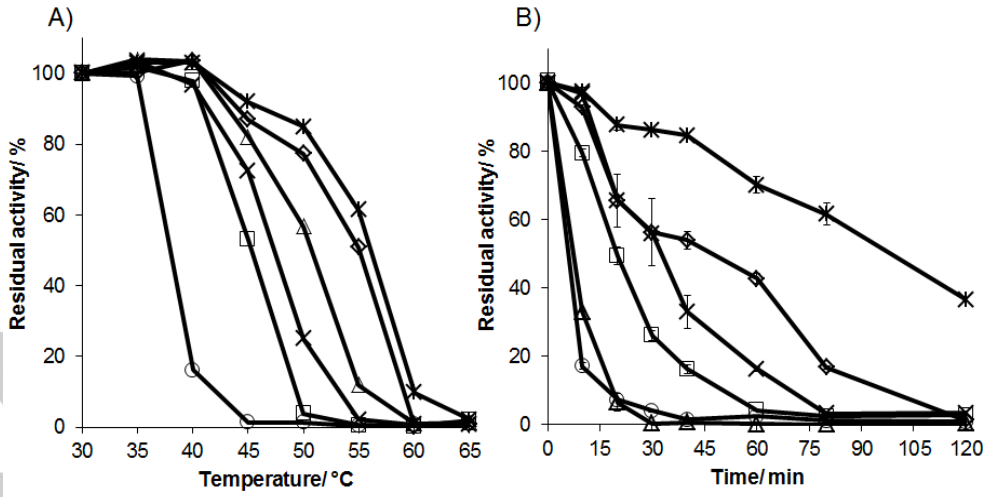

Figure 4. A) Thermostability at different temperatures, B) thermostability at 50 °C of C-terminal truncated variants of *Pe*HNL$_{121y}$ (◊), *Pe*HNLΔ$_{117}$ (□), *Pe*HNLΔ$_{112}$ (Δ), *Pe*HNLΔ$_{109}$ (×), *Pe*HNLΔ$_{107}$ (ж) and *Pe*HNLΔ$_{105}$ (○). Activity of the enzyme was detected by incubating the enzyme for 1 h at different temperatures and then the remaining activity was measured as described in Figure 1. Data are presented as means ± SD (n = 3)

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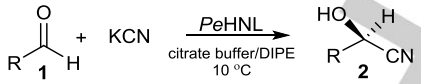
naphthaldehyde (1j) which proved to be poor substrates for *Pe*HNL. The maximum conversion and more than 99% *ee* were obtained in less than 30 min for the synthesis of (2*S*)-thiophen-2-yl-hydroxyacetonitrile (2g). Compared to the wild-type *Pe*HNL$_{121y}$, *Pe*HNLΔ$_{107}$ showed good *ee* (>90%) and the over 90% conversion after 3 h, which are higher than that of the wild-type *Pe*HNL$_{121y}$ for the synthesis of (*R*)-mandelonitrile (2a), (*R*)-2-methylmandelonitrile (2b), (*R*)-2-chloromandolonitrile (2d) and (*R*)-3-chloromandolonitrile (2e). The substrate specificity toward aldehyde or substituted benzaldehydes for *Pe*HNL is the major intermediate cyanohydrins for the preparation of bioactive drugs such as duloxetine (2g, used for treatment of depression and general anxiety disorder),[38] semi-synthetic cephalosporins (2a, antibacterial agents),[39] and clopidogrel (2d, anticoagulant).[40] Thus, *Pe*HNL is a good potential biocatalyst that can be used for industrial application.

Table 2. Conversion and enantioselectivity of purified wild type *Pe*HNL$_{121}$ and C-truncated *Pe*HNLΔ$_{107}$ toward different aldehydes.



1a:Ph; **1b**:2-Me-Ph; **1c**:3-Me-Ph; **1d**:2-Cl-Ph; **1e**:3-Cl-Ph; **1f**:3-MeO-Ph; **1g**:2-thiophene; **1h**:2-furan; **1i**:1-naphthalene; **1j**:2-naphthalene

Sub. 1[R=]		t=0.5 h		t=3 h		t=24 h	
		Conv. [%]	*ee* [%]	Conv. [%]	*ee* [%]	Conv. [%]	*ee* [%]
1a	*Pe*HNL$_{121}$	72.2	95.5	87.7	90.7	90.5	60.5
	*Pe*HNLΔ$_{107}$	76.4	97.1	92.2	93.4	94.1	75
1b	*Pe*HNL$_{121}$	48.3	92.0	81.2	82.7	95.6	65.4
	*Pe*HNLΔ$_{107}$	55.5	93.5	91.1	91.0	96.5	78.8
1c	*Pe*HNL$_{121}$	28.0	53.3	46.0	30.1	87.4	15.6
	*Pe*HNLΔ$_{107}$	29.5	67.5	59.0	58.1	90.3	38.5
1d	*Pe*HNL$_{121}$	60.8	76.6	85.6	60.0	99.5	45.5
	*Pe*HNLΔ$_{107}$	69.1	95.1	93.7	90.7	99.5	90.5
1e	*Pe*HNL$_{121}$	46.5	2.5	50.4	2.5	97.2	7.23
	*Pe*HNLΔ$_{107}$	54.2	99.8	89.0	99.4	97.4	98.9
1f	*Pe*HNL$_{121}$	30.7	22.0	72.5	9.10	96.6	5.70
	*Pe*HNLΔ$_{107}$	32.6	30.5	72.2	26.0	96.6	16.8
1g	*Pe*HNL$_{121}$	82.2	95.5	97.7	90.7	n.t.	n.t.
	*Pe*HNLΔ$_{107}$	86.4	99.1	99.2	96.4	n.t.	n.t.
1h	*Pe*HNL$_{121}$	52.4	90.3	56.5	70.0	64.1	26.0
	*Pe*HNLΔ$_{107}$	54.0	93.6	58.0	78.0	66.8	17.0
1i	*Pe*HNL$_{121}$	30.3	16.4	34.4	4.8	60.1	1.6
	*Pe*HNLΔ$_{107}$	30.6	18.3	34.2	13.6	60.1	3.4
1j	*Pe*HNL$_{121}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	*Pe*HNLΔ$_{107}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not determined; n.t. = not tested

Reusability of *Pe*HNLΔ$_{107}$

The reusability of *Pe*HNLΔ$_{107}$ has been investigated for the production of (*R*)-MAN synthesis in biphasic batch reaction systems. Wild-type *Pe*HNL and *Pe*HNLΔ$_{107}$ showed no significant differences in the production of (*R*)-MAN in the range of 50-55 µM for the first cycle of reaction. After four cycles in the reusability experiment, the production of (*R*)-MAN of wild-type *Pe*HNL was significantly decreased in the DIPE biphasic system, whereas the production of *Pe*HNLΔ$_{107}$ was almost the same as that in the first cycle of the reaction (Figure 5A, 5B). These results are in agreement with the high solvent stability of *Pe*HNLΔ$_{107}$ in contrast to that of the wild-type form which lost more than 50 % activity upon incubation in a biphasic system for 6 h (Figure S5). Thus, the C-terminal truncated *Pe*HNLΔ$_{107}$ without glycosylation which was expressed in *E. coli* could improve the stability and reusability similar to that shown by the glycosylated wild-type *Pe*HNL expressed in *P. pastoris*.[13c]

Conclusion

The natural and glycosylated form of *Pe*HNL from the yellow passion fruit showed much higher thermostability, pH stability, and the tolerance to organic solvents than *Pe*HNL from the purple passion fruit, indicating the important role of the glycosylation. The glycan present at one glycosylation site located on a high flexibility C-terminal region might stabilize the structure and thus reduce the structural flexibility of *Pe*HNL. This hypothesis has been proved by generation of a C-terminal truncated form of *Pe*HNL lacking 15 amino acid residues, and then expressing it in *E. coli* resulting in an enzyme lacking in glycosylation. The non-glycosylated and C-

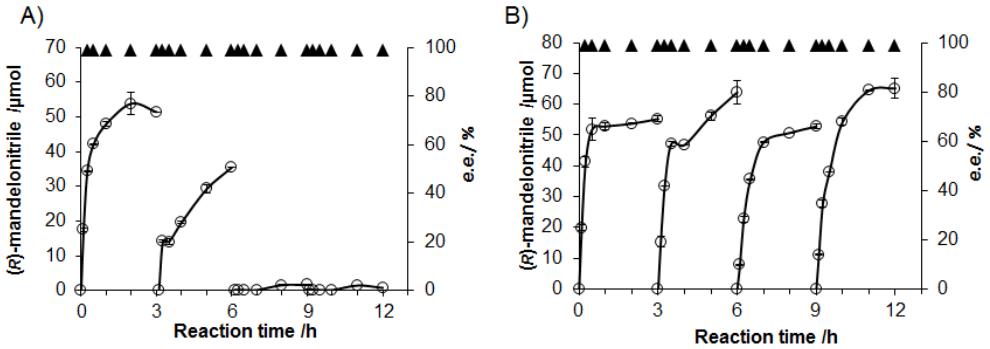


Figure 5. Reusability of A) *Pe*HNL$_{121}$, B) *Pe*HNLΔ$_{107}$ during the production of (*R*)-mandelonitrile from benzaldehyde and potassium cyanide. ○: Amount of (*R*)-mandelonitrile. ▲: Enantiomeric excess [%].

terminal truncated mutant *Pe*HNLΔ$_{107}$ could improve the stability and reusability, as effectively as the glycosylated natural *Pe*HNL and expressed in *P. pastoris*.

Experimental Section

Materials and chemicals

Leaves of *Passiflora edulis* Sims and *Passiflora edulis* forma *flavicarpa* were collected from the Thung Roeng Royal Project Development Center in Chiang Mai, Thailand, and the Botanic Garden of Toyama, Japan, respectively, and stored at -20 °C. Benzaldehyde (redistilled, 99.5 %) and (*rac*)-mandelonitrile were purchased from Sigma-Aldrich. Other chemicals used in the experiments were purchased from chemical sources and used without further purification.

Assays to determine the synthetic activity of HNL

Enzymatic activity was quantified by determining (*R*)-mandelonitrile synthesis using benzaldehyde and KCN as substrates following our previous study.[13a] The reaction was initiated by adding KCN (100 mM) in citrate buffer (pH 4.0, 400 mM) into the reaction mixture (0.5 mL) containing benzaldehyde (50 mM; 20 µL of 1250 mM benzaldehyde dissolved in dimethyl sulfoxide (DMSO)) and enzyme sample (0.5-5 U mL^{-1}) which was incubated at 25 ºC for 5 min. The reaction was stopped by transferring aliquots (100 µL) of the reaction mixture to 900 µL of n-hexane/propan-2-ol mixture (85:15), mixed vigorously, and centrifuged at 15000 × g for 5 min at 4 ºC. The organic layers containing benzaldehyde, (*R*)- and (*S*)-mandelonitrile were analyzed using chiral HPLC as described in our previous study.[13c] One unit of HNL activity was defined as the amount of enzyme that produced 1 µmol of (*R*)-mandelonitrile from benzaldehyde and KCN per minute.

Purification of PeHNLs from leaves

The *Pe*HNL from *P. edulis* forma *flavicarpa* was purified as described previously[13c]. Here we describe a procedure to purify *Pe*HNL-Np. Briefly, *P. edulis* Sims leaves were frozen using liquid nitrogen and ground with mortar and pestle. Extraction and protein fractionation with ammonium sulfate was performed in the same manner as previously reported.[13c] After dialysis of the active ammonium sulfate fraction, proteins from the extract were loaded onto the Toyopearl DEAE -650M column (Tosoh, 50 mm i.d., column volume 150 mL) and eluted with a linear gradient of sodium chloride from 0 to 500 mM in potassium phosphate buffer (KPB, pH 6.0, 20 mM). The active fractions were added ammonium sulfate (30 % saturated) and loaded onto a Butyl-Toyopearl column (Tosoh, 25 mm i.d., column volume 50 mL). Bound proteins were eluted stepwise with ammonium sulfate (1330, 860, 420, and 0 mM) in KPB (pH 6.0, 20 mM). Active fractions were combined, dialyzed thrice against KPB (pH 6.0, 20 mM) and then loaded onto the 2nd Toyopearl DEAE-650M column (25 mm i.d., column volume 20 mL). Bound protein was pre-washed with 50 mM sodium chloride in KPB (pH 6.0, 20 mM) and eluted with a linear gradient of sodium chloride from 50 to 100 mM in the same buffer. Active fractions were pooled, concentrated, desalted by using a centrifugal filtration device (Amicon Ultra-15, 10000 NMWL, EMD Millipore) and then loaded onto a Mono Q 5/50 GL column (1 mL, GE Healthcare). Proteins were then eluted with a linear gradient of sodium chloride from 0 to 100 mM in KPB (pH 6.0, 20 mM) at a flow rate of 0.8 mL.min^{-1}. Active fractions were pooled, concentrated and further purified using the Superdex 200 10/300 GL column (GE Healthcare). Purified *Pe*HNL-Np was monitored using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[41] and glycosylation was detected using Periodic acid-Schiff (PAS)[42] staining using a GelCode Glycoprotein Staining Kit (Thermo Fisher Scientific) according to the manufacturer`s instructions.

Cloning of Pe*HNL-Np cDNA*

Total RNA was isolated from a young leaf of *P. edulis* Sims after 3 months of cultivation and reverse transcribed. As purified *Pe*HNL-Np showed a molecular mass of 14000 Da which is almost the same as *Pe*HNL-Ny, we first tried to identify the *Pe*HNL-Np gene from *P. edulis* Sims using degenerate and gene-specific primers as previously reported.[13c]. DNA sequences were determined using a 3500 Genetic Analyzer (Thermo Fisher Scientific) and assembled and analyzed using ATGC and Genetyx Ver. 12 (Genetyx, Tokyo, Japan), respectively.

B-factor and root mean squared deviation analysis

The B-factor of wild-type *Pe*HNL (PDB ID:5XZQ) was extracted from the pdb structure using the B-FITTER software.[27a] The root mean squared deviation (RMSD) of the similarity between the structures of the two *Pe*HNLs, wild-type *Pe*HNL and *Pe*HNLΔ$_{107}$ (PDB ID: 5XZT) have been calculated by the Molecular Operating Environment (MOE, Chemical Computing Group Inc Canada).

Bacterial strain and construction of the C-terminal truncated Pe*HNL*

E. coli JM 109 (Takara Bio) and BL21 (DE3) (Thermo Fisher Scientific) cells were used as hosts for amplification of plasmid DNAs and recombinant protein expression, respectively. *Pe*HNL from *P. edulis* forma *flavicarpa* was cloned and expressed using the pColdI expression vector (pColdI-*Pe*HNL) as previously described.[13c] Wild-type pColdI-*Pe*HNL was truncated at the C-terminus of each variant using QuikChange® Lightning Mutagenesis Kit (Agilent Technologies, USA) and the truncation primers shown in Table S2. PCR products were treated with 10 U of *Dpn*I at 37 °C for 1 h, and then used for transformation of *E. coli* JM 109 using the heat shock method. Sequences were confirmed using the 3500 Genetic Analyzer.

Gene expression, purification, and determination of molecular mass of wild-type and C-terminal truncated Pe*HNL*

E. coli BL21(DE3) cells harboring recombinant plasmids were cultured in Luria–Bertani (LB, 5 mL) broth containing ampicillin (50 µg ml^{-1}) and incubated overnight at 37 °C with shaking at 300 rpm. The pre-culture starter was transferred into LB broth (500 mL) containing ampicillin (50 µg ml^{-1}) in an Erlenmeyer flask (2 L) and cultured at 37 °C with shaking at 150 rpm until A_{600} reached 0.6, followed by a cold-shock at 16 °C. After 2 h, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM, and cells were cultured at 16 °C for 24 h with the same rate of shaking. Protein extraction and purification were performed exactly as described previously.[13c] Superdex^{TM} 10/300 GL (GE Healthcare) was used to calculate the molecular mass of the purified recombinant wild-type *Pe*HNL and its truncated form based on their relative mobility as compared with values for standard marker proteins as follows: glutamate dehydrogenase (290,000 Da), lactate dehydrogenase (142,000 Da), enolase (67,000 Da), adenylatekinase (32,000 Da), and cytochrome *c* (12,400 Da).

Measurement of kinetic parameters

Kinetic parameters of purified wild-type and C-terminal truncation mutants of *Pe*HNL were assayed by monitoring synthesis of (*R*)-mandelonitrile in sodium citrate buffer (pH 4.0, 400 mM), according to the

method described above, at various concentrations (0.5-100 mM) of benzaldehyde (followed by mixing with total 20 µL of 12.5-2500 mM benzaldehyde dissolved in DMSO). The kinetic parameters of the enzyme (k_{cat} and K_m) were calculated by non-liner least-squares curve fitting against plots of the initial velocity vs substrate concentrations using the Michaelis-Menten equation.

Effect of pH on enzyme activity and stability

Two natural purified *Pe*HNLs were used to determine optimum pH for (*R*)-mandelonitrile synthesis. The reaction was performed at 25 °C between pH 2.5 to pH 6.0 (400 mM) for 5 min. To determine enzyme stability, each *Pe*HNL was preincubated at 30 °C between the range of pH 3.5 to pH 10.0 (40 mM) for 1 h. The remaining activity of the enzyme was measured as described above.

Effect of temperature on enzyme activity and stability

Optimum temperature required for enzyme activity was examined by incubating the reaction mixture at temperatures ranging from 5 °C to 45 °C in citrate buffer (pH 4.0, 400 mM) for 5 min. For determination of thermostability, the enzyme was preincubated in potassium phosphate buffer (pH 6.0, 20 mM) over the range of 30 to 80 °C for 1 h. The remaining activity of the enzyme was measured as described above.

Effect of organic solvent on stability and cyanohydrin synthesis in a biphasic system

Organic solvent stability of two natural *Pe*HNLs were compared using different organic tests as described in detail by Nuylert *et al*.[13c] In the study on cyanohydrin synthesis of C-terminal truncation *Pe*HNL mutants, diisopropyl ether (DIPE) was selected for synthesis of cyanohydrins in a biphasic system, organic solvent phases containing various aldehyde (250 mM) and mixed with citrate buffer (pH 3.5, 400 mM) containing each purified C-terminal truncated *Pe*HNL mutants (10 U) in total volumes of 1.5 mL in 2.0 mL microcentrifuge tubes. Reactions were initiated by adding potassium cyanide (300 mM), and the mixtures were incubated at 10 °C with shaking at 1500 rpm in an incubator shaker (BioShaker M-BR-022UP, Taitec Corporation, Tokyo, Japan). Aliquots of samples (10 µL) were collected from the organic phase at various time-points and analyzed using HPLC at condition as described in detail by Dadashipour *et al*.[2a] Alternatively, for chiral high performance liquid chromatography (HPLC) analysis of the conversion of 2-chlorobenzaldehyde, 3-chlorobenzaldehyde and 4-chlorobenzaldehyde were performed with a CHIRALCEL OD-H column (Diacel, Osaka, Japan) under the following condition: mobile phase n-hexane/propan-2-ol/trifluoroacetic acid with a volume ratio of 96:4:0.2, flow rate 1 mL min^{-1}, absorbance 228 nm, column oven temperature 25 °C. *ee* values were determined according to relative concentration (mM) of the two enantiomers using Equation (1):

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

Reusability

The reusability of recombinant purified *Pe*HNL, wild-type (*Pe*HNL$_{121y}$), and C-terminal truncated *Pe*HNLΔ$_{107}$ expressed in *E. coli* was compared in the cyanohydrin synthesis reaction batch containing benzaldehyde (250 mM) and KCN in biphasic systems of citrate buffer (pH 3.5, 400 mM, 0.75 mL) and DIPE (0.75 mL) in a microcentrifuge tube (2 mL). A reaction was also started by addition of each *Pe*HNL (5 U) the same as described above. After 3 h of incubation, the buffer phase containing the enzyme was recovered and dialyzed against citrate buffer (pH 3.5, 400 mM) using a centrifugal filtration device. The exchanged buffer containing *Pe*HNL was then reused for the next batch of reactions under the same conditions. The amount of (*R*)-mandelonitrile produced was estimated as described above.

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Keywords: enzyme catalysis • hydroxynitrile lyase • *Passiflora edulis* • C-terminal truncation• enzyme stability

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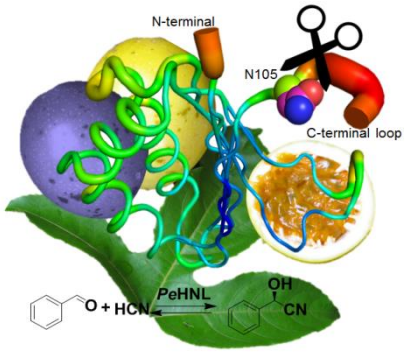
Layout 1:

FULL PAPER

*Pe*HNL of the yellow passion fruit showed much better thermostability, pH stability, and organic tolerance than the *Pe*HNL purple passion fruit. The non-glycosylated and C-truncated mutant *Pe*HNLΔ_{107} could improve the stability and reusability properties as well as the glycosylated natural *Pe*HNL and expressed in *P. pastoris*.



*A. Nuylert, F. Motojima, C. Khanongnuch, T. Hongpattarakere, and Y. Asano**

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Stabilization of hydroxynitrile lyases from two variants of passion fruits, *Passiflora edulis* Sims and *Passiflora edulis* forma *flavicarpa* by C-terminal truncation