Journal of Molecular Catalysis B: Enzymatic 101 (2014) 40–46

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Purification, immobilization and characterization of (*R*)-hydroxynitrile lyase from *Prunus amygdalus turcomanica* seeds and their applicability for synthesis of enantiopure cyanohydrins

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ARTICLE INFO

Article history:
Received 28 June 2013
Received in revised form
25 December 2013
Accepted 28 December 2013
Available online 7 January 2014

Keywords:
Hydroxynitrile lyase
Prunus amygdalus turcomanica Lincz.
Eupergit supports
Enantiopure cyanohydrins

ABSTRACT

A hydroxynitrile lyase (HNL) was purified from wild almond seeds (*Prunus amygdalus turcomanica* Lincz.) for the first time. Native and subunit molecular masses of the HNL were determined as 100 and 25 kDa, respectively indicating that the enzyme is a homotetramer. The purified enzyme was immobilized onto Eupergit CM and Eupergit C 250 L supports and their lyase and carboligation (synthetic) activities were characterized in terms of optimal pH, temperature and kinetic parameters. While the optimal pH of the free HNL for the lyase activity was 6.0, it was 5.5 for both of the immobilized HNLs. Optimal temperature was determined as 25 °C for all HNL preparations. For mandelonitrile cleavage, the apparent K_m – V_{max} values were 0.38 mM – 197.0 U mg protein^{-1} for the free HNL, 1.30 mM – 26.0 U mg protein^{-1} for HNL immobilized onto Eupergit CM (HNL-Eup CM) and 0.95 mM – 17.5 U mg protein^{-1} for HNL immobilized onto Eupergit C 250 L (HNL-Eup C 250 L), respectively. For the carboligation activity, the optimal pH was measured as 4.0 and optimal temperature was determined as 5 °C for all of the HNL preparations. For mandelonitrile synthesis, the apparent K_m – V_{max} values were 14.0 mM – 2.70 U mg protein^{-1} for the free HNL, 41.0 mM – 0.49 U mg protein^{-1} for HNL-Eup CM and 38.0 mM – 0.54 U mg protein^{-1} for HNL-Eup C 250 L, respectively. All of the HNL preparations were employed for the synthesis of mandelonitrile, 2-chloromandelonitrile, 3,4-dihydroxymandelonitrile and 2-hydroxy-4-phenyl butyronitrile in a biphasic *tert*-butyl methyl ether–citrate buffer (pH 4.0) medium. The results showed that the immobilized HNL preparations were better than the free HNL in the synthesis of abovementioned cyanohydrins except 2-chloromandelonitrile with higher yields and enantiopurities.

1. Introduction

Hydroxynitrile lyases (HNLs, EC 4.1.2.x) catalyze the cleavage of cyanohydrins to produce highly toxic hydrocyanic acid (HCN) and aldehydes or ketones. This reaction is the final step of cyanogenesis and serves to protect organisms from attacks by fungi and predators [1]. According to their flavoprotein (FAD) content, HNLs can be divided into two groups: FAD containing HNLs and non-FAD containing HNLs. FAD containing HNLs have been solely detected in two subfamilies of the *Rosaceae* family while non-FAD containing HNLs have been found in several families of higher plants [2].

On the other hand, HNLs can be able to stereoselective addition of HCN to aldehydes or ketones to produce enantiopure cyanohydrins *in vitro* conditions (Fig. 1) [3]. Recently, researchers have been focused on HNLs catalyzed synthesis of enantiopure cyanohydrins since these compounds are key intermediates in the production of important chemicals, including α-hydroxy carboxyclicacids, α-hydroxyaldehydes, α-hydroxyketones, β-amino alcohols, α-fluoro cyanides which are key synthons for the preparation of pharmaceuticals, agrochemicals and other many biologically active compounds [4]. A variety of cyanogenic plant seeds or leaves have been used as HNL sources for the synthesis of enantiopure cyanohydrin and HNLs are classified into two groups as (*R*)-HNL and (*S*)-HNL depending on their stereoselectivies in the synthesis of enantiopure cyanohydrin [5]. Up to now, (*R*)-HNLs have been detected in the different sources such as *Prunus amygdalus* [6], *Prunus laurocerasus* [7], *Prunus serotina* [8], *Prunus lyonii* [9], *Prunus mume* [10], *Phlebodium aureum* [11], *Passiflora edulis* [12], *Eriobotrya japonica* [13] whereas (*S*)-HNLs have been found in *Ximenia americana* L. [14], *Sorghum bicolor* [15], *Manihot esculenta* [16], *Hevea brasiliensis* [11], *Baliospermum montanum* [17,18].

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http://dx.doi.org/10.1016/j.molcatb.2013.12.019

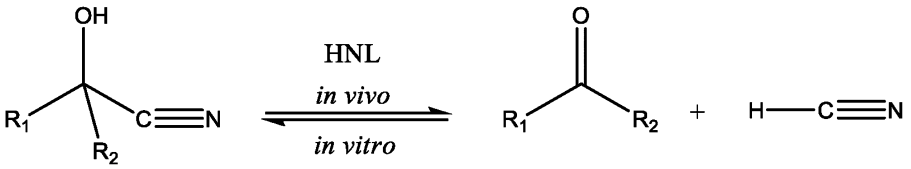


Fig. 1. The cleavage or synthesis of cyanohydrins by HNL.

The enantiospecificity of HNLs towards a various range of substrates make them appealing catalysts in the preparation of enantiopure cyanohydrins. However, their recovery and reusability problems in free form limited substantially their uses for industrial purposes. Therefore, the development of immobilization techniques for HNLs can be a solution to enhance their recovery and reusability [19]. Wehtje et al. [20] covalently immobilized HNL purified from bitter almonds onto porous silica via different spacer arms and was used in the production of (*R*)-mandelonitrile. They reported that (*R*)-mandelonitrile was synthesized with 86% yield and 92% enantiomeric excess by the immobilized HNL. Van Langen et al. [21] immobilized *P. amygdalus* (*R*)-oxynitrilase as a cross-linked enzyme aggregate (CLEA) and reported that immobilized HNL showed highly effective carboligation activity in microaqueous conditions. Cabirol et al. [22] prepared the immobilized forms of HNLs from *P. amygdalus*, *M. esculenta* and *H. brasiliensis* in sol–gels. They also examined the immobilized HNLs for synthesis of enantiopure cyanohydrins. Tükel et al. [23] investigated the lyase and carboligation activities of partially purified *P. pseudoarmeniaca* HNL in free and immobilized forms. They reported that free and immobilized *P. pseudoarmeniaca* HNL preparations were very attractive catalysts for (*R*)-mandelonitrile synthesis.

P. amygdalus turcomanica Lincz. (a wild almond) is a dwarf, flowering tree and grows naturally in the various regions of Turkey. The ripened fruits of *P. amygdalus turcomanica* Lincz. are fleshy and greenish. As far as we know, fruits and seeds of *P. amygdalus turcomanica* Lincz. have not been used in the industrial productions of any products. In this study, we focused to purify a new HNL from *P. amygdalus turcomanica* Lincz. (*Pat*HNL) seeds for the first time. Subsequently, the purified HNL was immobilized onto Eupergit CM and Eupergit C 250 L supports and their lyase activities were characterized in terms of optimum pH, temperature and kinetic parameters. The free and immobilized HNL preparations were used for the synthesis of industrially important enantiopure cyanohydrins such as mandelonitrile, 2-chloromandelonitrile, 3,4-dihydroxymandelonitrile, 2-hydroxy-4-phenylbutyronitrile.

2. Materials and methods

2.1. Materials

Mature fruits of *P. amygdalus turcomanica* Lincz. were collected in July, in the county Nizip, Turkey. HiPrep Phenyl FF (high sub, 16/10) column (1 cm × 20 cm) was purchased from GE Healthcare. (*R*/*S*)-mandelonitrile, 2-chlorobenzaldehyde, 3,4-dihydroxy benzaldehyde, 3-phenylpropionaldehyde, (*R*)-mandelonitrile, Eupergit CM, Eupergit C 250 L, bovine serum albumine (BSA) and Sepharose 6B were supplied from Sigma–Aldrich (St. Louis, MO, USA). Ammonium sulphate ($(NH_4)_2SO_4$), citric acid, potassium cyanide (KCN), dimethylsulfoxide (DMSO), acetone, benzaldehyde, *tert*-butyl methyl ether (TBME), hexane, 2-propanol and acetic acid were obtained from Merck (Darmstadt, Germany). Nucleocel Delta S chiral column (4.6 mm × 250 mm) was purchased from Macherey–Nagel GmbH & Co. KG (Düren, Germany).

2.2. Methods

2.2.1. Purification procedure

Homogenization of almond seeds: Fleshy covers of the almond fruits were removed and their hard seeds were stored at 5 °C until use. The hard seed covers of almond seeds were cracked with a hammer and 200 g of soft kernels were homogenized in prechilled acetone for 2 min. The slurry was filtered and the filtrate was discarded. Then, 200 mL of chilled acetone were added onto the residue and homogenized as mentioned above. This procedure was repeated for 3 times. The resulting powder was dried in an incubator at 5 °C and stored at −20 °C until use.

Preparation of crude HNL extract: Five grams of the powder were added into 50 mL of phosphate buffer (50 mM, pH = 6.2) and the mixture was mixed at 4 °C for 1 h. Subsequently, the mixture was centrifuged at 8000 × *g* for 15 min at 4 °C. The supernatants collected together were used as crude HNL source for further purification steps.

$(NH_4)_2SO_4$ fractionation: The crude HNL solution was fractionated by 0–20%, 20–30% and 30–40% (w/w) saturation of solid $(NH_4)_2SO_4$.

Hydrophobic interaction chromatography: Proteins precipitated in 30–40% $(NH_4)_2SO_4$ saturation were dissolved in a minimum volume of phosphate buffer (50 mM, pH = 6.2) and loaded onto HiPrep Phenyl FF (high sub, 16/10) column (1 cm × 20 cm) pre-equilibrated with phosphate buffer (50 mM, pH = 7.0) containing 40% $(NH_4)_2SO_4$ (w/v). The bound proteins were eluted with phosphate buffer (50 mM, pH = 7.0) containing 40–0% $(NH_4)_2SO_4$ as gradient at a flow rate of 2.0 mL min^{-1}. The eluants fractionated at a volume of 3.5 mL were monitored for protein content at 280 nm and the protein fractions were assayed for HNL activity. The fractions showing HNL activity were pooled for next purification step.

Gel filtration chromatography: The pooled fraction was loaded onto Sepharose 6B column (1.5 cm × 27 cm) pre-equilibrated with phosphate buffer (50 mM, pH = 7.0). The elution of proteins was performed by phosphate buffer (50 mM, pH = 7.0) and fractionated at a volume of 3.5 mL. The fractions were monitored for protein content at 280 nm and the protein fractions were assayed for HNL activity.

2.2.2. SDS-PAGE analysis

The purity and subunits of *Pat*HNL were analyzed according to Laemmli [24] on 15% polyacrylamide gel by a vertical mini gel apparatus (Bio-Rad) by using reference protein markers (Fermentas SM1811) at 200 V for 1 h. The protein bands were visualized according to Blum et al. [25].

2.2.3. Molecular mass determination of PatHNL by HPLC

The molecular mass of *Pat*HNL was determined by using high-performance liquid chromatography (HPLC) equipped with Biosuite 250 HR size exclusion column (5 μm, 7.8 mm × 300 mm). The detection of proteins was achieved by using diode array detector at 220 nm. The mobile phase was a phosphate buffer (100 mM, pH 7.0) containing 0.3 M NaCl with the flow rate of 1.0 mL min^{-1} at 30 °C. The molecular mass of reference proteins were 26.6 kDa (triosephosphate isomerase), 36.5 kDa (lactate dehydrogenase),

48.5 kDa (fumarase), 58.5 kDa (pyruvate kinase), 90 kDa (lactoferrin), 116 kDa (β-galactosidase) and 180 kDa (α2-macroglobulin).

2.2.4. Immobilization of PatHNL

The immobilization procedure was performed according to Mateo et al. [26]. One gram of each support was treated with 9.0 mL of the purified *Pat*HNL solution (1.0 mg protein mL^{-1} prepared in a phosphate buffer (1.0 M, pH = 7.0)). The immobilization process was performed at 25 °C for 24 h. The immobilized *Pat*HNL preparations were collected by filtration and rinsed with pure water to remove unbound or weakly bound protein molecules. This rinsing procedure was continued until no protein was detected in the filtrates. The immobilized HNL preparations were stored at 5 °C until use. Lowry protein assay was applied to determine protein amount in the filtrates [27].

2.2.5. HNL assay

The lyase activities of HNL preparations were spectrophotometrically determined according to Tükel et al. [23]. Briefly, 2.85 mL of citrate buffer (50 mM, pH = 5.5) and 0.1 mL of HNL solution were mixed at 25 °C for 2 min. The reaction was initiating by adding 50 μL of (*R*/*S*)-mandelonitrile solution (30 mM in absolute ethanol). After 10 min, 0.5 mL of aliquots withdrawn and diluted to 3.0 mL with acetate buffer (50 mM, pH = 4.0). The amount of formed benzaldehyde was measured at 250 nm. The same experiment without HNL was performed as blank. One unit of HNL activity was defined as the amount of HNL produced 1.0 μmol benzaldehyde in 1 min under the assay conditions.

2.2.6. Characterization of PatHNL

2.2.6.1. Lyase activity. The lyase activities of HNL preparations were measured in 50 mM of acetate buffers (pH = 4.0–5.5), citrate buffer (pH = 6.0) and phosphate buffers (pH = 6.5 and 7.0). The optimum temperatures of all the HNL preparations were investigated at different temperatures ranging from 10 to 40 °C.

The apparent maximum reaction rate (V_{max}) and the apparent Michaelis–Menten constant (K_m) of free and immobilized preparations were determined by applying the HNL assay for various mandelonitrile concentrations (0.1–2.0 mM). The activities of free and immobilized preparations were measured at their optimum conditions. Enzyme Kinetics Module programme (Sigma-Plot 12.0) was used for determining the apparent V_{max} and the apparent K_m values of HNL preparations.

The storage stabilities of all HNL preparations were investigated at room temperature and 5 °C.

2.2.6.2. Carboligation activity. The effects of pH and temperature on the carboligation activities of free and immobilized *Pat*HNL were tested for (*R*)-mandelonitrile synthesis. The effect of pH was investigated in a biphasic TBME–citrate buffer with different pH values (400 mM, 3.5–6.0). To determine the optimum temperature, the carboligation activities of free and immobilized HNLs were measured in the temperature range of 5–25 °C in biphasic TBME–citrate buffer at pH 4.0. The apparent V_{max} and the apparent K_m values of free and immobilized *Pat*HNL preparations for the carboligation activity were determined for the synthesis of mandelonitrile by using various benzaldehyde concentrations. The carboligation activities of all the *Pat*HNL preparations in the characterization and kinetic experiments were measured by using HPLC described in Section 2.2.8 after 2 h reaction time.

2.2.7. Chemical synthesis of mandelonitrile, 2-chloromandelonitrile, 3,4-dihydroxy mandelonitrile and 2-hydroxy-4-phenylbutyronitrile

The chemical synthesis of mandelonitrile, 2-chloromandelonitrile, 3,4-dihydroxymandelonitrile and 2-hydroxy-4-phenylbutyronitrile used as standards for chiral HPLC analyses were achieved according to Cabirol et al. [22]. The characterizations of the synthesized cyanohydrins were performed by using chiral HPLC, FTIR and NMR analyses. ^{1}H NMR and ^{13}C NMR spectra in DMSO-d6 were recorded on a Bruker Ultrashield TM NMR 300 MHz spectrometer with tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ) were expressed in parts per million (ppm), multiplicity (s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet) and coupling constants (J) were expressed as Hertz (Hz). A FTIR instrument (Perkin Elmer Spectrum RX/FTIR system) was used to determine the functional groups after the pellet was prepared in KBr. HPLC analyses were performed on Nucleocel Delta S chiral column at 220 nm.

2.2.7.1. (R/S)-mandelonitrile. ^{1}H NMR δ (ppm): 4.51 (s, 1H, H_5C_6-CHOH-CN), 5.51 (s, 1H, H_5C_6-CHOH-CN), 7.38–7.42 (m, 5H, H_5C_6-CHOH-CN).

^{13}C NMR δ (ppm): 63.2 (H_5C_6-CHOH-CN), 119.2 (H_5C_6-CHOH-CN), 128–135 (H_5C_6-CHOH-CN).

IR (film) cm^{-1}: 3411, 3064, 2250, 1697, 1494, 1455, 1433, 1284, 1193, 1086, 1041, 1026.

2.2.7.2. (R/S)-2-chloromandelonitrile. ^{1}H NMR δ (ppm): 3.5 (s, 1H, H_4ClC_6-CHOH-CN), 5.78 (s, 1H, H_4ClC_6-CHOH-CN) 7.26–7.65 (m, 4H, H_4ClC_6-CHOH-CN).

^{13}C NMR δ (ppm): 60.9 (H_4ClC_6-CHOH-CN), 117.9 (H_4ClC_6-CHOH-CN), 128–133 (H_4ClC_6-CHOH-CN).

IR (film) cm^{-1}: 3395, 3067, 2923, 2250, 2058, 1957, 1921, 1805, 1694, 1593, 1568, 1476, 1443, 1417, 1278, 1245, 1191, 1126, 1056, 1035.

2.2.7.3. (R/S)-3,4-dihydroxy mandelonitrile. ^{1}H NMR δ (ppm): 3.4 (s, 1H, $HOCHOCH_3C_4$-CHOH-CN), 4.35 (s, 1H, $HOCHOC\ C_4H_3$-CHOH-CN), 4.35 (s, 1H, $HOCHOCC_4H_3$-CHOH-CN), 5.2 (s, 1H, $HOCHOCC_4H_3$-CHOH-CN), 7.0 (d, 1H, $HOCHOCC_4H_2H$-CHOH-CN), 7.0 (d, 1H, $HOCHOCC_4H_2H$-CHOH-CN), 7.1 (s, 1H, $HOCHOCC_4H_2H$-CHOH-CN).

^{13}C NMR δ (ppm): 60.1 ($HOCHOCC_4H_3$-CHOH-CN), 118.2 ($HOCHOCC_4H_3$-CHOH-CN), 124.2–128.8 ($HOCHOCC_4H_3$-CHOH-CN), 140.1–140.3 ($HOCHOCC_4H_3$-CHOH-CN).

IR (film) cm^{-1}: 3395, 3067, 2923, 2250, 2058, 1957, 1921, 1805, 1694, 1593, 1568, 1476, 1443, 1417, 1278, 1245, 1191, 1126, 1056, 1035.

2.2.7.4. (R/S)-2-hydroxy-4-phenylbutyronitrile. ^{1}H NMR δ (ppm): 2.14 (H_5C_6-CH_2-CH_2-CHOH-CN), 2.55 (t, H_5C_6-CH_2-CH_2-CHOH-CN), 3.65 (s, H_5C_6-CH_2-CH_2-CHOH-CN), 4.22 (t, H_5C_6-CH_2-CH_2-CHOH-CN), 7.27–7.29 (H_5C_6-CH_2-CH_2-CHOH-CN).

^{13}C NMR δ (ppm): 28.4 (H_5C_6-CH_2-CH_2-CHOH-CN), 35.2 (H_5C_6-CH_2-CH_2-CHOH-CN), 63.2 (H_5C_6-CH_2-CH_2-CHOH-CN), 118.4 (H_5C_6-CH_2-CH_2-CHOH-CN), 126–142 (H_5C_6-CH_2-CH_2-CHOH-CN).

IR (film) cm^{-1}: 3338, 3026, 2929, 1773, 1664, 1497, 1453, 1132, 1029.

2.2.8. HNL catalyzed synthesis of (R)-mandelonitrile, (R)-2-chloro mandelonitrile, (R)-3,4-dihydroxymandelonitrile and (R)-2-hydroxy-4-phenylbutyronitrile

To 200 μL of the free HNL (2 mg mL^{-1}) or 50 mg of immobilized HNL preparations, 500 μL of citrate buffer (400 mM, pH = 4.0), 100 μL of aldehyde solution (1.0 M in DMSO) and 1.0 mL of TBME were added at 5 °C. The reaction was initiated by adding 200 μL of HCN solution (1.0 M in TBME) prepared according to Bhunya et al. [28]. Subsequently, 100 μL of sample withdrawn from TBME phase was diluted to 500 μL with hexane/2-propanol mixture (96/4, v/v) and analyzed by a HPLC equipped with Nucleocel Delta S chiral column. The mobile phase was hexane/2-propanol mixture (96/4,

Table 1
The summary of purification steps of *Pat*HNL.

Purification step	Total protein (mg)	Total activity (U)	Specific activity[a] (U mg protein^{-1})	Yield (%)	Purification fold
Crude HNL extract	248	703	2.8	100	1
30–40% $(NH_4)_2SO_4$ saturation	115	528	4.6	75	1.6
Phenyl sepharose 6 fast flow hydrophobic interaction chromatography	8.1	146	18	21	6.4
Sepharose 6B gel filtration chromatography	3.6	137	40	19	14

[a] Lyase activity.

v/v) containing 0.1% acetic acid at the flow rate of 1.0 mL min^{-1} at 25 °C. The cyanohydrin and aldehydes samples were detected at 220 nm. Enantiomeric excess (ee) of the synthesized cyanohydrins were calculated from the formula given below:

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

2.2.9. Reusability of HNL-Eup CM and HNL-Eup C 250 L for (R)-mandelonitrile synthesis

The reusability of HNL-Eup CM and HNL-Eup C 250 L for (*R*)-mandelonitrile synthesis were investigated in a batch type reactor for 10 cycles. To 50 mg of immobilized *Pat*HNL preparations, 600 μL of citrate buffer (400 mM, pH = 4.0), 100 μL of benzaldehyde solution (1.0 M in DMSO) and 1.0 mL of TBME were added at 5 °C. The reaction was initiated by adding 200 μL of HCN solution (1.0 M in TBME) and performed at 5 °C for 2 h. The separation of immobilized *Pat*HNL preparations from the reaction mixture was achieved by filtration. The formed products were analyzed by chiral HPLC mentioned before. After each experiment, the immobilized *Pat*HNL preparations were washed with 1 mL of citrate buffer (400 mM, pH = 4.0). For the next operation, the fresh reaction medium was loaded onto the reactor and these procedures were repeated 10 times.

3. Results and discussion

In this study, a new HNL was purified from *P. amygdalus turcomanica* seeds applying the purification steps presented in Table 1. The purified HNL is a homo-tetramer with a subunit relative molecular mass of 25 kDa on SDS-PAGE (Fig. 2). A broad band for the purified *Pat*HNL in SDS-PAGE may be due to the glycosylation of the enzyme. The similar result was also reported in the literature [29].

The estimated molecular mass of native HNL was determined as 100 kDa by HPLC (data not shown). In the literature, the molecular mass values of (*R*)-HNLs purified from various sources, varied from 15 to 180 kDa [12]. Jansen et al. reported the molecular mass of *P. amygdalus* HNL as 72 kDa and the enzyme as a monomer. In the same report, the molecular mass of *S. bicolor* HNL was 180 kDa and the enzyme was a heterotetramer [30]. Wajant et al. reported that the molecular mass of *P. aureum* HNL was reported to be 108 kDa and the enzyme had multimers in the native form and molecular mass of each monomer was approximately 20 kDa [31].

The amounts of bound protein were determined as 86 and 93% of the initial loading protein (9 mg) per gram of Eupergit CM and Eupergit C 250 L supports, respectively. HNL-Eup CM and HNL-Eup C 250 L showed 13 and 9% of the lyase activity of the free HNL, respectively. These activity decreases upon immobilization may be due to diffusion limitations and/or decrease in mobility of the HNL.

The lyase activity of all the HNL preparations were measured in the different pH values ranging from 4.0 to 7.0 and the result are shown in Fig. 3A. The relative activities of all HNL preparations increased in the pH range of 4.0–5.5. Both immobilized HNL preparations had a maximum activity at pH 5.5 whereas the free HNL had its maximum activity at pH 6.0. At pH 7.0, the relative activities were determined as 60, 79 and 19% of the maximum activities for the free HNL, HNL-Eup CM and HNL-Eup C 250 L, respectively.

Fig. 3B shows the lyase activities of free and immobilized HNL preparations investigated at 10–40 °C. The relative activities of all HNL preparations increased at 10–25 °C and they had a maximum activity at 25 °C. The relative activities of all HNL preparations decreased rapidly above 30 °C and determined as 19, 38 and 19% for the free HNL, HNL-Eup CM and HNL-Eup C 250 L, respectively at 40 °C. These declines may be due to the increasing rate of spontaneous cleavage of mandelonitrile at the mentioned temperature.

The lyase activities of all the HNL preparations were determined at (*R*/*S*)-mandelonitrile concentrations of 0.1–2.0 mM under their optimal conditions. By using the Enzyme Kinetics Module,

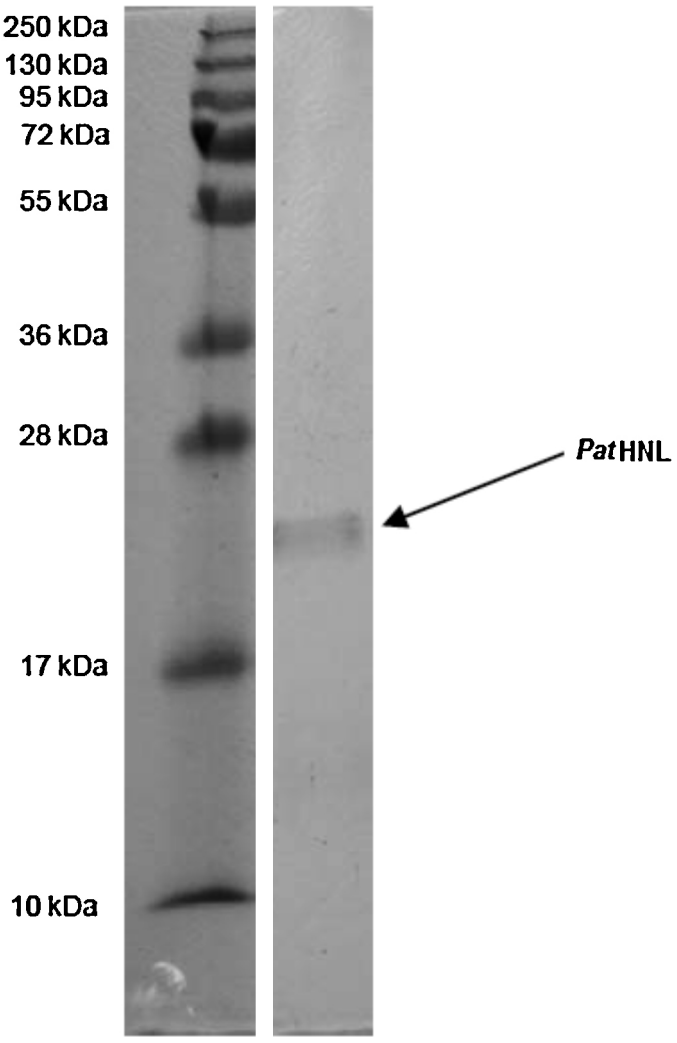


Fig. 2. SDS-PAGE of the purified *Pat*HNL.

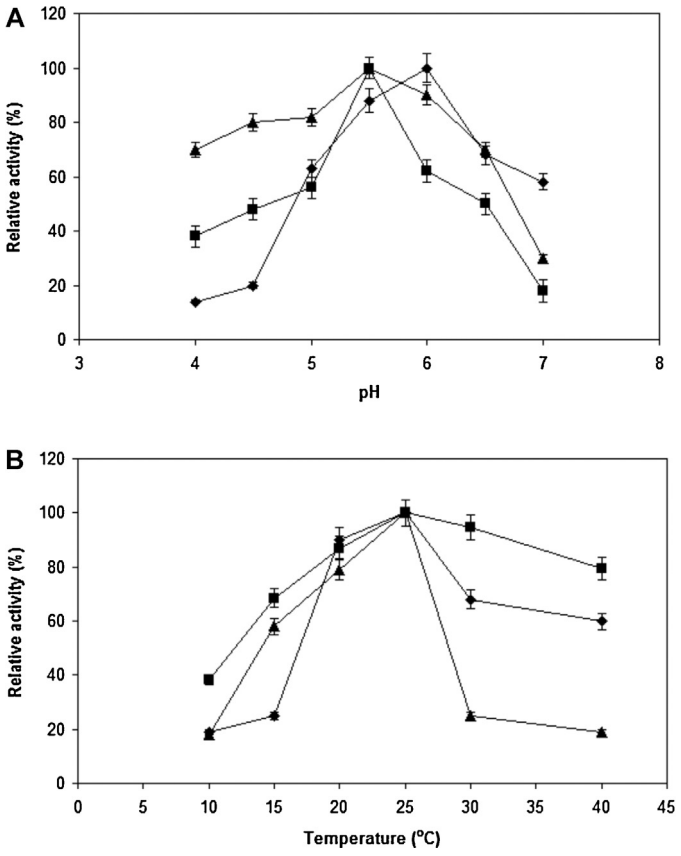


Fig. 3. (A) The effect of pH on the lyase activities of free and immobilized *Pat*HNL preparations (free HNL (♦), HNL-Eup CM (■) and HNL-Eup C 250 L (▲)). (B) The effect of temperature on the lyase activities of free and immobilized *Pat*HNL preparations (free HNL (♦), HNL-Eup CM (■) and HNL-Eup C 250 L (▲)).

the apparent K_m – V_{max} values of the free HNL, HNL-Eup CM and HNL-Eup C 250 L preparations were determined as 0.38 mM – 197.0 U mg protein^{-1}, 1.30 mM – 26.0 U mg protein^{-1} and 0.95 mM – 17.5 U mg protein^{-1}, respectively. The apparent catalytic efficiency values (k_{cat}/K_m) of free HNL, HNL-Eup CM and HNL-Eup C 250 L preparations were 9.52×10^8, 3.66×10^7 and 3.36×10^7 s^{-1} M^{-1}, respectively. In the literature, K_m values of *P. aureum*, *E. japonica* expressed in *Pichia pastoris*, *E. japonica* HNLs were reported as 0.83, 0.47, 0.55 mM, respectively towards mandelonitrile [23].

At room temperature, the relative activity of free HNL decreased rapidly and depleted at the end of 15 days storage time while the immobilized HNLs were very active at the same conditions with retained initial activities of 67 and 83% for HNL-Eup CM and HNL-Eup C 250 L, respectively. At the end of 30 days storage time, the corresponding retained activities were 48 and 71% for HNL-Eup CM and HNL-Eup C 250 L, respectively. At 5 °C, a nearly linear decrease was observed in the relative activity of free HNL depending on the storage time. After 30 days storage time, the relative activity of free HNL was completely lost. However, the rates of activity decrease in the immobilized preparations were slower than that of the free HNL. At the 30 days storage time, the relative activities were measured as 78 and 83%, respectively for HNL-Eup CM and HNL-Eup C 250 L at 5 °C (Fig. 4). These results showed that the storage stability of *Pat*HNL was positively influenced by immobilization.

The obstruction of spontaneous cyanohydrin formation is a key importance to achieve the synthesis of cyanohydrin with high ee value in HNL-catalyzed syntheses. This difficulty can be overcome by performing the enantiopure cyanohydrin synthesis at low pHs (4.0–5.0) and temperatures (0–5 °C). The effects of pH on the carboligation activities of the free HNL, HNL-Eup CM and HNL-Eup C

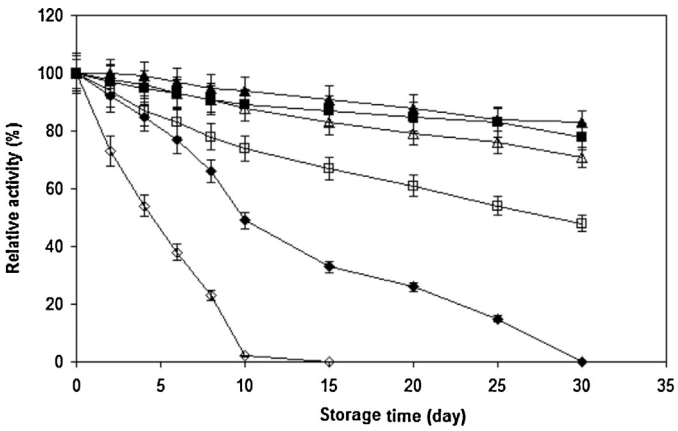


Fig. 4. The storage stabilities of free and immobilized *Pat*HNL preparations at room temperature (free HNL (◊), HNL-Eup CM (□) and HNL-Eup C 250 L (△)) and at 5 °C (free HNL (♦), HNL-Eup CM (■) and HNL-Eup C 250 L (▲)).

250 L were tested in a biphasic TBME–citrate buffer with a different pH values in the range of 3.5–6.0 at 5 °C. As shown in Fig. 5A, the carboligation activities of *Pat*HNL preparations and accordingly ee value of (*R*)-mandelonitrile were affected markedly from the reaction pH and the highest carboligation activity was determined at pH 4.0 for all the *Pat*HNL preparations. Furthermore, the highest ee value for (*R*)-mandelonitrile was determined at both pH 3.5 and 4.0 as >99%. Therefore, the optimal pH value for the carboligation activity was selected as 4.0 for all the *Pat*HNL preparations.

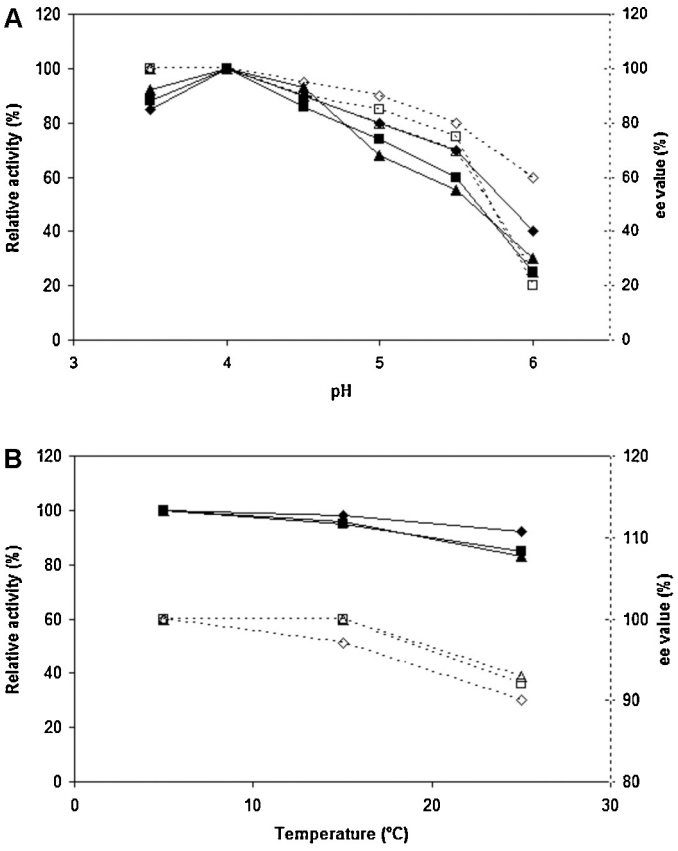
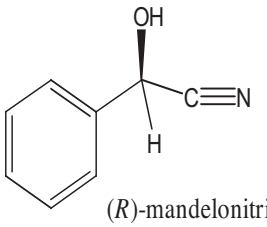
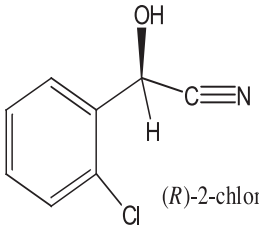
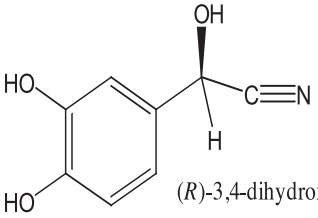
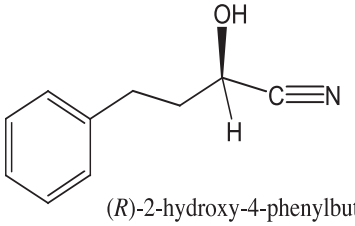


Fig. 5. (A) The effect of pH on the carboligation activities of *Pat*HNL preparations (free (♦), HNL-Eup CM (■) and HNL-Eup C 250 L (▲)) and ee value of *Pat*HNL preparations (free (◊), HNL-Eup CM (□) and HNL-Eup C 250 L (△)). (B) The effect of temperature on the carboligation activities of *Pat*HNL preparations (free (♦), HNL-Eup CM (■) and HNL-Eup C 250 L (▲)) and ee value of *Pat*HNL preparations (free (◊), HNL-Eup CM (□) and HNL-Eup C 250 L (△)).

Table 2
The results of enantiopure mandelonitrile, 2-chloromandelonitrile, 3,4-dihydroxymandelonitrile and 2-hydroxy-4-phenylbutyronitrile syntheses catalyzed by free and immobilized *Pat*HNL preparations.

Enzyme	Product	Yield (%)	ee (%)
Free HNL	 (*R*)-mandelonitrile	91	93
HNL-Eup CM		100	97
HNL-Eup C 250 L		100	96
Free HNL	 (*R*)-2-chloromandelonitrile	60	45
HNL-Eup CM		90	2
HNL-Eup C 250 L		100	1
Free HNL	 (*R*)-3,4-dihydroxymandelonitrile	92	>99
HNL-Eup CM		100	>99
HNL-Eup C 250 L		100	>99
Free HNL	 (*R*)-2-hydroxy-4-phenylbutyronitril	87	94
HNL-Eup CM		90	>99
HNL-Eup C 250 L		88	90

The effect of temperature on the carboligation activities of the free HNL, HNL-Eup CM and HNL-Eup C 250 L was examined for 5, 15 and 25 °C in biphasic TBME–citrate buffer at pH 4.0. As shown in Fig. 5B, the highest carboligation activity and enantioselectivity were determined at 5 °C for all the *Pat*HNL preparations. In our previous study, we showed that the yield and enantiopurity of (*R*)-mandelonitrile catalyzed by free and immobilized *P. pseudoarmeniaca* HNL preparations were affected markedly from the reaction pH and temperature and the best conditions were pH of 4.0 and temperature of 5 °C [23].

The apparent K_m – V_{max} values of the free HNL, HNL-Eup CM and HNL-Eup C 250 L were 14.0 mM – 2.70 U mg protein^{-1}, 41.0 mM – 0.49 U mg protein^{-1} and 38.0 mM – 0.54 U mg protein^{-1}, respectively towards benzaldehyde. K_m value of *E. japonica* HNL towards benzaldehyde was reported to be 11.5 mM [31].

All of the HNL preparations were evaluated for the syntheses of (*R*)-mandelonitrile, (*R*)-2-chloromandelonitrile, (*R*)-3,4-dihydroxy mandelonitrile, (*R*)-2-hydroxy-4-phenyl butyronitrile and the results are demonstrated in Table 2. After 3 days reaction time, (*R*)-mandelonitrile synthesis was achieved with yield-ee% values of 91–93, 100–97, 100–96%, respectively for the free HNL, HNL-Eup CM and HNL-Eup C 250 L. Wehtje et al. reported (*R*)-mandelonitrile acquired with 86% yield and 92% ee in a packed bed reactor by using HNL immobilized onto modified porous silica [20]. Cabirol et al. demonstrated that free *P. amygdalus* HNL (*Pa*HNL), *Pa*HNL encapsulated into aqua gels and *Pa*HNL immobilized as CLEA catalyzed the synthesis of (*R*)-mandelonitrile with yield-ee% values of 98–97, 97–97 and 97–99%, respectively [22]. Nanda et al. obtained 65% yield and 95% ee for the synthesis of (*R*)-mandelonitrile by using crude *P. mume* HNL after 24 h reaction time [10].

The free and immobilized HNLs were tested to obtain (*R*)-2-chloromandelonitrile which is used for the preparation of (*R*)-2-chloromandelic acid, an oral anticoagulant, and precursor of antithrombotic agent clopidogrel [21]. The free HNL catalyzed the reaction with 60% yield and 45% ee values at the end of 3 days reaction time. While both immobilized HNLs catalyzed the same reaction with 100% yield, however, the formed product was nearly racemic. These findings revealed that the spontaneous addition rate of HCN to 2-chlorobenzaldehyde was higher than those of the free HNL and immobilized HNLs catalyzed reactions. This situation may be explained that *ortho* substituent probably causes a steric hindrance for the addition of HCN to 2-chlorobenzaldehyde by the free and immobilized HNL preparations, therefore, the spontaneous addition rate plays a dominant role. The same results were reported

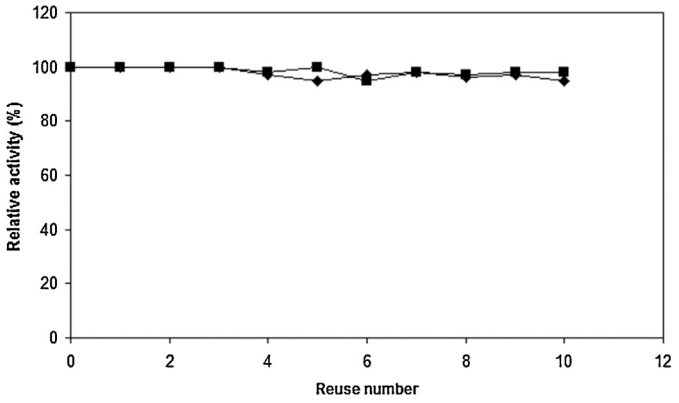


Fig. 6. The reusability HNL-Eup CM (♦) and HNL-Eup C 250 L (■) for (*R*)-mandelonitrile synthesis.

by Nanda et al. [10]. However, the opposite result was also reported in the literature [21].

In the synthesis of (*R*)-3,4-dihydroxymandelonitrile, a chiral intermediate for noradrenaline synthesis, the obtained yields were 92, 100 and 100%, respectively for the free HNL, HNL-Eup CM and HNL-Eup C 250 L preparations. The ee% values of (*R*)-3,4-dihydroxymandelonitrile were determined as >99% for all the *Pat*HNL preparations.

(*R*)-2-hydroxy-4-phenylbutyronitrile is a key intermediate for the preparation of (*R*)-2-hydroxy-4-phenylbutyric acid, an important building block for the synthesis of ACE inhibitors [32]. The synthesis of (*R*)-2-hydroxy-4-phenylbutyronitrile was resulted in 87, 90, 88% yields for the free HNL, HNL-Eup CM and HNL-Eup C 250 L catalyzed reactions, respectively. The corresponding ee% values were obtained as 94, 99 and 90%.

The reusability tests for HNL-Eup CM and HNL-Eup C 250 L were investigated for (*R*)-mandelonitrile synthesis in biphasic TBME–citrate buffer (pH 4.0) at 5 °C. Both HNL-Eup CM and HNL-Eup C 250 L preparations were successfully reused 10 times without loss of activity (Fig. 6) and easily separated from the reaction mixture at the end of the each reuse. Furthermore, the enantioselectivity of both HNL-Eup CM and HNL-Eup C 250 L preparations was fully protected at the end of the each cycle. Tükel et al. [23] reported that partially purified *P. pseudoarmeniaca* HNL immobilized onto Eupergit C and Eupergit C 250 L supports were protected 96 and 90% of their initial activities, respectively at the end of the 10 reuses in the synthesis of (*R*)-mandelonitrile and ee value of (*R*)-mandelonitrile was 99% after 10 reuses for both operations. Gröger et al. [33] reused cross-linked and subsequently poly(vinyl alcohol)-entrapped (*R*)-oxynitrilase 20 times in a biphasic citrate buffer (pH 4.5) – TBME/hexane mixture for the synthesis of (*R*)-mandelonitrile and reported that the immobilized (*R*)-oxynitrilase almost protected its initial activity and enantioselectivity after 20 reuses.

4. Conclusion

*Pat*HNL was purified 14-fold with a 19% purification yield from the seeds of *P. amygdalus turcomanica*. After gel filtration and SDS-PAGE analyses, the enzyme was determined to be homotetramer in native form. The lyase activity of 13 and 9% was recovered when the *Pat*HNL was immobilized onto Eupergit CM and Eupergit C 250 L supports, respectively. The immobilized *Pat*HNL preparations produced enantiopure (*R*)-mandelonitrile, (*R*)-3,4-dihydroxymandelonitrile and (*R*)-2-hydroxy-4-phenyl butyronitrile with a higher yield and enantiopurity than the free HNL. The immobilized *Pat*HNL preparations were successfully reused 10 times without loss of activity and enantioselectivity.

Acknowledgement

This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) with the project number of 109T427.

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