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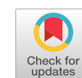
Purification and characterization of a hydroxynitrile lyase from *Amygdalus pedunculata* Pall



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ABSTRACT

Hydroxynitrile lyases (HNLs) are widely used in the asymmetric synthesis of cyanohydrins which are organic compounds used in the production of fine chemicals and pharmaceuticals, because these enzymes exhibit high catalytic efficiency and are very economical. In the present study, seeds of *A. pedunculata* Pall were identified as new potential source of HNLs. The HNL from *A. pedunculata* Pall (APHNL) was purified 138 fold and 4.20% yield with a specific activity of 661 U/mg. SDS-PAGE result showed the enzyme to be present as a monomer and the relative molecular mass determined by MALDI-TOF MS was 61 kDa. APHNL owned highest activity at pH 6.0 and at 60 °C temperature, showing activity up to 80 °C and stable up to 60 °C. APHNL has a *Km* of 0.5 mM, *Vmax* of 665.9 μmol mg^{-1} min^{-1}, *Kcat* of 676.5 s^{-1} and *Kcat*/*Km* of 1353 s^{-1} mM^{-1} using mandelonitrile as substrate. Syntheses of (*R*)-mandelonitrile and (*R*)-2-Hydroxy-2-(3-phenoxy-phenyl)-acetonitrile were carried out using APHNL and molar conversion of (*R*)-mandelonitrile and (*R*)-2-Hydroxy-2-(3-phenoxy-phenyl)-acetonitrile were 90% and 98% with 94% and 93% ee, respectively. These results indicated that APHNL was an excellent biocatalyst and has very high potential for synthesis of enantiopure cyanohydrins.

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1. Introduction

Hydroxynitrile lyases (HNLs) are present in many plants [1], bacteria [2] and an invasive millipede [3]. HNL was first found in almond by Roesnthaler [4] in 1908. To date, HNLs from four plant families (Linaceae, Rosaceae, Euphorbiaceae and Olacaceae) have been widely studied [5]. HNLs are an important type of biocatalyst; these enzymes exhibit optical selectivity and reversibly catalyze the conversion of HCN and aldehydes/ketones to chiral cyanohydrins [6]. Therefore, HNLs, especially HNLs derived from natural products, have been widely studied in the field of asymmetric synthesis. HNLs from different sources have different molecular weights, biochemical characteristics, enantioselectivities and substrate specificities, but can all be used as biological catalysts for the synthesis of optically active cyanohydrins. Optically active cyanohydrins are important synthetic intermediates [7], are easily transformed into β-amino alcohols and α-hydroxyl ketone [8], which provides a solid foundation for asymmetric synthesis. Among the preparation methods of cyanohydrin, enzymatic method is considered to be the most ideal choice because this method meets the requirements of green chemistry. Based on the enantioselectivity of HNLs, HNLs can be divided into two groups [9]: (*R*)-hydroxynitrile lyase evolved from oxidoreductase ancestors such as HNLs from *passiflora* [10] and wild Apricot [11], and (*S*)-hydroxynitrile lyase derived from hydrolases with an α/β-hydrolase fold; these enzymes from cassava [12], *Baliospermum montanum* [13] and *Hevea brasiliensis* [14]. The separation and purification of HNLs is multistep process. The cost of this process is high and the yield of the target enzyme is not high. Although many researchers have purified HNLs, the discovery of better HNLs with high reaction rates and stabilities from unknown biological sources is necessary for sustainable industrial development [15].

A. pedunculata Pall is a deciduous shrub of the almond subgenus of the peach genus in the family Rosaceae and is native to arid regions of northwestern China, Mongolia and eastern Siberia. *A. pedunculata* Pall is a good candidate for desert reclamation because this plant exhibits cold and drought tolerance, has a deep root system, and is adaptable to a wide range of soil types and soil moisture conditions [16], which has great ecological significance for the ecological restoration of barren mountain areas and the collapse of coal mines. In our research group, vegetable oil [17], protein powder [18] and amygdalin were extracted from *A. pedunculata* Pall seeds and the seed shells were processed to activated carbon. The oil mainly contains monounsaturated fatty acids and polyunsaturated fatty acids [17] with a small amount of phenol, tocopherol and phytosterol antioxidant compounds [19,20]. The crude extract from the *A. pedunculata* Pall seeds contains 20–30% protein and is a

Abbreviations: HNL, hydroxynitrile lyase; APHNL, hydroxynitrile lyase from *Amygdalus pedunculata* Pall seeds; PBS, Phosphate buffer; HPLC, High performance liquid chromatograph.

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potential source of plant protein. However, research on APHNL has not been reported.

In the study, the purification of APHNL was carried out by ammonium sulfate precipitation and column chromatography. APHNL was characterized and identified by SDS-PAGE, mass spectrometry and N-terminal sequencing. We studied the enzyme kinetics of APHNL and the effects of pH, temperature and metal ions on activity of APHNL. Using *A. pedunculata* Pall seeds as a source of HNLs is an economically and ecologically valuable substitute for the existing processes and applications.

2. Materials and methods

2.1. Materials

Seeds of *A. pedunculata* Pall purchased from Shenmu county of Shaanxi Province (38°13′–39°29′N 109°40′–110°54′E) in China were used as the source of HNLs and stored at 4 °C after shelling for further use. An AKTA Avant was purchased from GE (Boston, USA). The protein standard for SDS-PAGE was purchased from Thermo Scientific (Massachusetts, USA). Reagents for SDS-PAGE were obtained from BioFroxx (Berlin, Germany). A BCA assay kit was purchased from Biosharp (Hefei, China). All other chemicals used were of analytical grade.

2.2. Extraction of protein

Seeds of *A. pedunculata* Pall were shelled in water at 40 °C and crushed, and then phosphate buffer (20 mM PBS, pH 7.4) was added at 4 °C for 12 h followed by homogenization. The solution was centrifuged at 12000 rpm, the precipitate and grease were removed carefully, and the supernatant was collected.

2.3. Determination of protein content

A BCA assay kit with BSA as a standard was used to measure the protein content according to the Bradford method at 562 nm [21].

2.4. APHNL activity assay

The activity of APHNL was assayed according to the method of Benita kopka [22] with slight modification. The reaction was conducted as follows: 3.95 mL of potassium citrate buffer (pH 5.5), 40 μL of ethanol, 4 μL of madelonitrile and 40 μL of enzyme solution were mixed in a tube at 50 °C and reacted for 1 min, then, the absorbance was measured at 280 nm. As a blank, the same experiment was performed in the absence of APHNL. Under these conditions, the amount of enzyme required to produce 1 μmol of benzaldehyde in 1 min is defined as 1 unit of enzyme.

2.5. Ammonium sulfate precipitation

Highly refined $(NH_4)_2SO_4$ was added to protein extracted from *A. pedunculata* Pall seeds at 0–20%, 20–40%, 40–60%, 60–80%, and 80–100% saturation at 4 °C with uniform stirring. Then, stirring was continued for 30 min to bring the dissolved and aggregated protein to an equilibrium. Then, the mixture was allowed to rest for 2 h followed by centrifugation at 12000 rpm for 20 min; the precipitate was collected. The precipitate was dissolved in phosphate buffer and dialyzed in the same buffer, and 1% $BaCl_2$ was added to the dialysate to confirm the removal of SO_4^{2-}. The enzyme activity was measured in the dialysis bag, and the enzyme was lyophilized until further purification.

2.6. Chromatographic purification

Protein (87 mg) from the 40–60% $(NH4)_2SO_4$ precipitation was dissolved in PBS (20 mM, pH 7.4), and the solution was heated in a water bath for 40 min at 40–45 °C and subsequently centrifuged and filtered through a 0.22 μm membrane for further purification. The processed protein was applied on to a pre-equilibrated HiTrap DEAE Sepharose Fast Flow column (GE, Boston, USA) with start buffer (20 mM PBS, pH 7.4) and eluted with elution buffer (20 mM PBS containing 0.8 M NaCl, pH 7.4) at a flow rate of 3 mL/min. The fractions that were rich in HNL activity were pooled and analyzed.

Hydrophobic chromatography was performed on a HiTrap Phenyl Fast Flow (high sub) column (GE, Boston, USA). The protein purified by ion exchange chromatography was applied on to the pre-equilibrated column with 20 mM PBS containing 2 M $(NH_4)_2SO_4$ (pH 7.4) and eluted with elution buffer (20 mM PBS, pH 7.4) at a flow rate of 3 mL/min; 5 mL fractions were collected. The fractions that exhibited HNL activity were enriched and studied.

2.7. Characterization

2.7.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Using a Laemmli discontinuous buffer system, polyacrylamide gel electrophoresis was performed with a 15% separating gel and a 5% stacking gel [23].

2.7.2. Biological mass spectrometry

After the purified protein was analyzed by SDS-PAGE, the bands from the gel were excised for in-gel digestion and analyzed and identified by matrix-assisted laser desorption and ionization time of flight mass spectrometer (MALDI-TOF MS) and MALDI-TOF MS/MS. Bands from the SDS-PAGE gel containing the target protein were cut into $1mm^3$ strips and treated with 300 μL of 100 mM NH_4HCO_3 and 30% acetonitrile. Then the supernatant was extracted and lyophilized followed by the addition of detergent to perform digestion. 1 μL of the protein hydrolysate target was submitted to the ion source for detection. The results of peptide fingerprinting and tandem mass spectrometry were analyzed against the green plant protein library in SWISS-PROT and the NCBI database by using Mascot (www.matrixscience.com).

2.7.3. N-terminal amino acid sequencing

N-terminal sequence of the protein was determined by the classical Edman degradation technique. The resulting amino acid residues were injected into a high performance liquid chromatograph for on-line analysis; 12 cycles were measured. The result of the N-terminal sequencing was compared by a BLAST search [24].

2.7.4. Determination of amino acid composition

The protein sample was placed in a tube with 6 M HCl and hydrolyzed at 110 °C for 24 h, obtain free amino acids, which were then derivatized with phenyl isothiocyanate. The derivatized amino acids were analyzed by high performance liquid chromatography (HPLC).

Table 1
Purification of APHNL.

Step	Total protein (mg)	Specific activity (U/mg)	Total activity (units)	Yield (%)	Purification (fold)
Homogenate protein	1967 ± 0.7	4.80 ± 0.5	9442	100	1.00
$(NH4)_2SO_4$ (40–60%)	87 ± 0.3	38.8 ± 0.8	3376	35.8	12.4
DEAE FF	2.7 ± 0.2	538 ± 1.8	1453	15.4	112
Phenyl FF	0.6 ± 0.06	661 ± 1.5	397	4.20	138

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