Abstract

Seeds of Abrus precatorius (rosary pea) were identified as a new and potential source of hydroxynitrile lyase. Hydroxynitrile lyase (HNL) from the seeds of this plant was purified up to 9 fold with specific activity of 577 Unit mg⁻¹ protein by using ion exchange and gel filtration chromatographic techniques. The purified enzyme was a heteromer with estimated native molecular mass 205 kDa and its subunits showed two bands of molecular mass of 42.0 and 36.5 kDa in SDS-PAGE, respectively. The enzyme exhibited maximum activity at 30°C with 0.1 M sodium citrate buffer (pH 5). It has \( K_m \) of 13 mM and \( V_{max} \) of 625 Unit mg⁻¹ of protein with mandelonitrile as a substrate. In 120 mL of reaction mixture containing 25 ml of benzaldehyde (substrate) and 6.75 mg of purified enzyme produced 17.5 g of mandelonitrile.

Key words: Abrus precatorius, Cyanohydrins, Hydroxynitrile lyase, Mandelonitrile

Introduction

Hydroxynitrile lyases (HNL, EC 4.1.2.10, EC 4.1.2.11, EC 4.1.2.37, EC 4.1.2.39) are the versatile group of enzymes that catalyse enantioselective synthesis and cleavage of cyanohydrins (Conn 1980; Lieberi et al. 1985) as per following reaction:

\[
\text{RCHO} + \text{HCN} \underset{\text{HNL}}{\overset{\text{HNL}}{\longrightarrow}} \text{RCH(CN)} \text{OH} \\
\text{Aldehyde} \quad \text{Hydrogen cyanide} \quad \text{Cyanohydrin}
\]

Plants are the major sources of HNLs as these enzymes liberate HCN from naturally occurring cyanohydrins in plant systems and this provides general protection against the bacterial and fungal infections (Albers and Hamann, 1934) and it is also used as a nitrogen source by some plants (Goischmidt et al. 2001). HNLs have been reported, purified and characterized from almond (Prunus amygdalus), flax (Linum usitatissimum), apple (Pyrus malus), apricot (Prunus armeniaca), cherry (Prunus serotina), peach (Prunus persica), capulin (Prunus capuli) and rubber tree (Hevea brasiliensis) etc. (Ingrid et al. 2001; Klaus et al. 1997; Tuncel et al. 1995; Asano et al. 2005; Ueatrongchit et al. 2010; Forster and Wajant 1996). In the present investigation some plants of Himachal Pradesh were screened with an objective to find new source of HNL. The catalytic ability of HNLs to form carbon-carbon bond has made these enzymes as important biocatalysts for the synthesis of a range of cyanohydrins in organic chemistry (Pouchlauer 1998). Cyanohydrins or their derivatives are finding wide applications in pharmaceuticals, agrochemicals and cosmetics since these can be readily converted into a myriad of \( \alpha \)-hydroxy carboxylic acids, \( \alpha \)-hydroxy ketones and \( \beta \)-amino acids (Gregory 1999).

Materials and Methods

Source of chemicals

Potassium cyanide (KCN), ethyl acetate, benzaldehyde used in the present study were purchased from SD Fine Chemical Ltd., India. Mandelonitrile was purchased from Merck India Ltd. and other chemicals were of analytical grade procured from various commercial sources.
**Plant material**

A number of plants *Abrus precatorius* (seed), *Carissa carandas* (seed), *Eriobotrya japonica* (seed), *Ficus elastica* (leaves), *Pinus gerardiana* (seed), *Praunus amygdalus* (seed), *Praunus armeniaca* (seed), *Praunus domestica* (seed), *Praunus serotina* (seed) and *Pyrus malus* (seed) were collected from various places in Himachal Pradesh, India and screened for HNL activity.

**Preparation of crude HNL**

Crude HNL was prepared following the method described by Han (Han *et al.* 2001). The seeds of *Abrus precatorius* were soaked in water for 96 h at room temperature. The presoaked seeds or leaves were ground in mortar- pestle with chilled ethyl acetate and air dried at 4 ºC. The dried powder material was termed as ‘meal’. The crude enzyme was prepared by suspending this meal in distilled water (7.4 g/100mL), adjusting its pH to 7.4 with 1N NH4OH. The suspension was incubated overnight at 4 ºC and then its pH was adjusted at 5.4 with 50 % acetic acid. To suppress proteolysis, protease inhibitor phenylmethanesulphonylfluoride (PMSF; 10 mg/300mL) was added to the enzyme preparation. The protein concentration was determined by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard.

**Assay of hydroxynitrile lyase (HNL) activity**

The activity of HNL was measured according to the procedure of Willemen *et al.* (2000). The assay mixture (5 mL) contained 0.1 M sodium citrate buffer (pH 5) and 1 mg crude, enzyme protein and 250 μmole of mandelonitrile (benzaldehyde cyanohydrin). It was incubated at 30 ºC for 30 min and the reaction was quenched by adding 5 mL trichloroacetic acid (TCA). The reaction mixture was centrifuged at (10,000 x g) in sigma ultracentrifuge and the absorbance of the supernatant was recorded at 280 nm. One unit of HNL activity was defined as the amount of enzyme which catalyses the release of 1 μmole of benzaldehyde in 1 min under the assay conditions. Since (*Abrus precatorius*) rosary pea had maximum HNL activity among various plant materials tested, it was used for further purification and characterisation.

**Purification of HNL of rosary pea**

All steps of protein purification were performed at 4 ºC and 0.1 M sodium citrate buffer (pH 5.0) was used as a buffer throughout the purification process. Centrifugation at (15,000 x g) was carried out for 15 min at 4 ºC. The crude enzyme was prepared from 200 g seeds of rosary pea by following the procedure described above.

**Ion exchange chromatography**

The crude enzyme (21.06 mg protein in 130 mL) was loaded onto DEAE-Sepharose column (9 x 2 cm, bed volume 25 mL). The column was washed with start buffer (0.1 M sodium citrate buffer pH 5). The protein was eluted using elution buffer (0.1 M sodium citrate buffer having 1M NaCl, pH 5) at flow rate of 0.5mL/min and fractions of 4 mL each were collected. The fractions rich in HNL activity were pooled and concentrated by lyophilization.

**Gel filtration chromatography**

The pooled and lyophilized fractions of ion exchange chromatography were applied to Sephacryl S-100 column (1.6 x 60 cm) using AKTA Prime Liquid Chromatography System. The concentrated protein (2.5 mL) having 6.7 mg of protein was loaded onto pre-equilibrated (sodium citrate buffer pH 5) Sephacryl S-100 column and the protein was eluted using sodium citrate buffer (pH 5) containing 0.15 M NaCl at a flow rate of 0.3 mL/min and fractions of 4 mL each were collected. Protein fractions rich in HNL activity were pooled and subjected for electrophoresis.

**Optimization of reaction conditions for assay of HNL activity**

To optimize reaction conditions for assay of HNL activity of rosary pea, enzyme reactions were carried using various buffer systems [phosphate buffer, sodium citrate buffer and sodium acetate buffer of 0.1M ionic strength], buffer pH (3-8) and temperature (15º-60ºC).

**Synthesis of mandelonitrile**

Mandelonitrile was synthesized by using the protocol of Rhodium (Rhodium, 1991). In a flat bottom flask, 7.26 g of KCN was added to 75 mL of ethyl acetate and kept overnight on magnetic stirrer, then 15 mL of benzaldehyde mixed with 10 mL of ethyl acetate and 15 mL (3894.6 Unit) of rosary pea hydroxynitrile lyase (0.45 mg/mL protein) was added to it and stirring was continued for 24 h at 4ºC. The reaction mixture was filtered and the residues were washed with ethyl acetate. The filtrate was dried and concentrated in vacuum. The mandelonitrile was quantified using HPLC system equipped with Inertsil ODS-2 (4.6 x 150 mm) column, having mobile phase acetonitrile in water (65 % v/v) at the flow rate of 1.0 mL/min.

**Result and Discussion**

**Screening of some plants material for HNL activity**

In the present studies, ten different plant materials were screened for hydroxynitrile lyase activity (Table 1) and out of these, the seeds of rosary pea (*Abrus precatorius*) exhibited maximum HNL activity (64 units/ mg of protein).

<table>
<thead>
<tr>
<th>Name of Plant</th>
<th>Plant part used</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Abrus precatorius</em></td>
<td>Seed</td>
<td>64</td>
</tr>
<tr>
<td><em>Carissa carandas</em></td>
<td>Seed</td>
<td>30</td>
</tr>
<tr>
<td><em>Eriobotrya japonica</em></td>
<td>Seed</td>
<td>36</td>
</tr>
<tr>
<td><em>Ficus elastica</em></td>
<td>Leaves</td>
<td>35</td>
</tr>
<tr>
<td><em>Pinus gerardiana</em></td>
<td>Seed</td>
<td>32</td>
</tr>
<tr>
<td><em>Praunus amygdalus</em></td>
<td>Seed</td>
<td>59</td>
</tr>
<tr>
<td><em>Praunus armeniaca</em></td>
<td>Seed</td>
<td>16</td>
</tr>
<tr>
<td><em>Praunus domestica</em></td>
<td>Seed</td>
<td>35</td>
</tr>
<tr>
<td><em>Praunus serotina</em></td>
<td>Seed</td>
<td>10</td>
</tr>
<tr>
<td><em>Pyrus malus</em></td>
<td>Seed</td>
<td>22</td>
</tr>
</tbody>
</table>

**Purification of HNL of rosary pea**

The purification of HNL of rosary pea (*Abrus precatorius*) was carried out by using ion exchange and gel filtration chromatographies. The ion exchange chromatography was performed using DEAE Sepharose column. The fractions (6 to 10) showing high HNL activity were pooled together and were concentrated to 2.5 mL by lyophilization (Fig. 1). This step resulted in 2.3 fold purification with a yield of 64 % of enzyme (Table 2).

The lyophilized sample was loaded on Sephacryl S-100 column.
Figure 1: Ion exchange chromatography of crude enzyme preparation on DEAE-Sepharose column (9 x 2 cm, bed volume 25 mL) at a flow rate of 0.5 mL/min and fractions of 4 mL collected each.

Figure 2: Gel filtration chromatography of DEAE-Sepharose fractions on Sephacryl S-100 column (1.6 x 60 cm) at a flow rate of 0.3 mL/min and fractions of 4 mL collected each.

Figure 3: (A) SDS-PAGE of purified hydroxynitrile lyase of rosary pea. Lane 1 was loaded with following protein molecular mass standards: phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and soyabean trypsin inhibitor (20 kDa), lysozyme (14.3 kDa). Crude enzyme (lane 2), ion exchange chromatography fractions (lane 3) and gel filtration chromatography (lane 4) were applied on to the gel. (B) Native-PAGE of purified hydroxynitrile lyase of rosary pea. Lane 1 were loaded with following molecular mass standards: thyroglobuline (660 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (66 kDa). Crude enzyme (lane 2), ion exchange chromatography fraction (lane 3) and gel filtration chromatography fraction (lane 4) having higher HNL activities were applied on to the gel.
activity of activity (548 Unit/mg). With an increase in pH, a decrease in HNL in the reaction (Fig. 4). At pH 5 the enzyme exhibited maximum

The pH of sodium citrate buffer (0.1 M) was varied from 3.0 to 8.0 followed by 0.1 M phosphate buffer (246 Unit/mg) as depicted.

Among the three types of buffers tested, maximum HNL activity (548 Unit/mg) was recorded in 0.1 M sodium citrate buffer pH 5. Fraction numbers 11 and 14-16 (Fig. 2) showed high HNL activity. In this step, HNL of rosary pea was purified up to 9 fold with a yield of 26 % of enzyme. The fractions having higher HNL activity were pooled. SDS polyacrylamide gel electrophoresis of the purified fractions showed the presence of two distinct protein bands which indicated the heteromeric nature of HNL. The upper band of 42 kDa and lower band of 36.5 kDa were found in SDS-PAGE, whereas the Native-PAGE showed a single band of 205 kDa (Fig. 3 A, B).

Characterization of purified HNL of rosary pea

The different reaction parameters like buffer system, ionic strength of buffer, pH optimization, and reaction temperature were optimized for the purified HNL of rosary pea.

Buffer System

Among the three types of buffers tested, maximum HNL activity (548 Unit/mg) was recorded in 0.1 M, sodium citrate buffer followed by 0.1 M, sodium acetate buffer (295 Unit/mg) and 0.1 M, phosphate buffer (246 Unit/mg) as depicted.

pH optimum

The pH of sodium citrate buffer (0.1 M) was varied from 3.0 to 8.0 in the reaction (Fig. 4). At pH 5 the enzyme exhibited maximum activity (548 Unit/mg). With an increase in pH, a decrease in HNL activity of Abrus precatorius was observed.

Figure 4: Effect of pH of 0.1 M sodium citrate buffer on HNL activity of Abrus precatorius.

Temperature optimum

The activity of purified HNL was assayed at different temperatures (5º-70°C). The optimum temperature for HNL activity was recorded at 30ºC (Fig. 5). With an increase in temperature, enzyme showed drastic decrease in its activity. At 45ºC or above the enzyme activity was completely lost.

\[ V_{\text{max}} = 625 \mu \text{mol/min/mg protein and } K_M \text{ of 13 mM of mandelonitrile.} \]

Synthesis of cyanohydrins using HNL of rosary pea

The enzymatic reaction using 15 mL (3894.6 Unit) of enzyme and (35 mL) substrate (benzaldehyde) yielded a yellowish (24 mL) oily substance which had density similar to mandelonitrile (Merck). Further the NMR spectroscopy of the product formed was also performed at Punjab University, Chandigarh, which confirms the synthesis of mandelonitrile. The product formed was recemic in nature its steriospecificity was determined by using Crown pack (+) column. In the reaction mixture, 0.147 mole (15 mL) of benzaldehyde was used and finally 0.132 mole (17.5 g) of mandelonitrile was recovered which corresponded to 90 % molar conversion. Yield of the mandelonitrile was 39 g/mg of enzyme used in the reaction mixture.

Figure 5: Effect of temperature on HNL activity of Abrus precatorius in 0.1 M sodium citrate buffer, pH 5.0.

The major aim of present work was to screen some indigenous plants growing in Himachal Pradesh (Sub Himalayan region) for HNL activity and to find out some new sources of HNL. There were 10 different plant species tested for HNL activity in the present study. Among these 10 plants, Prunus amygdalus (Ingrid et al. 2001), Prunus armeniaca (Tuncel et al. 1995), Prunus malus, Manihot esculenta (Kiljunen and Kanerva 1996; Hughes et al. 1994; Jane et al. 1998), Ficus elastic (Hasslacher et al. 1996), Prunus serotina (Zihua and Poultan 1999) have earlier been reported to possess HNL activity. Out of 10 plants, Abrus precatorius emerge as a good source of HNL (577 units/mg of seed protein) in the present study. The HNL activity of Abrus precatorius was higher compared to earlier reported work [e.g. 188 units/mg of protein in Sorghum vulgare (Seeley et al. 1966), 111.2 units/mg of protein in Prunus lycii (Xu et al. 1986), 34.1 units/mg protein in Linum usitatissimum (Xu et al. 1988) and 258 units/mg in Prunus amygdalus] for the source of this enzyme.

HNL of Abrus precatorius could be purified in two-steps. The results of purification of HNL from seeds of Abrus precatorius showed a 9 fold purification and 26 % of yield. This means that Abrus precatorius seeds were rich in HNL proteins comprising about 11 % of total extractable seeds proteins. The recovery (yield)

Table 2: Purification of hydroxynitrile lyase of Abrus precatorius

<table>
<thead>
<tr>
<th>Purification Stages</th>
<th>Protein Activity (mg)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (Units)</th>
<th>Total Activity (Units)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude sample</td>
<td>0.162</td>
<td>24.32</td>
<td>64</td>
<td>1573.2</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>DEAE ion exchange</td>
<td>1.34</td>
<td>6.71</td>
<td>150</td>
<td>1016.7</td>
<td>64</td>
<td>2.3</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>0.03</td>
<td>0.722</td>
<td>577</td>
<td>416.74</td>
<td>26</td>
<td>9</td>
</tr>
</tbody>
</table>

Figure 6: Graph showing the purification of HNL from Abrus precatorius seeds.

**Table 2:** Purification of hydroxynitrile lyase of Abrus precatorius

**Characterization of purified HNL of rosary pea**

The different reaction parameters like buffer system, ionic strength of buffer, pH optimization, and reaction temperature were optimized for the purified HNL of rosary pea.

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The pH of sodium citrate buffer (0.1 M) was varied from 3.0 to 8.0 in the reaction (Fig. 4). At pH 5 the enzyme exhibited maximum activity (548 Unit/mg). With an increase in pH, a decrease in HNL activity of Abrus precatorius was observed.

**Temperature optimum**

The activity of purified HNL was assayed at different temperatures (5º-70°C). The optimum temperature for HNL activity was recorded at 30ºC (Fig. 5). With an increase in temperature, enzyme showed drastic decrease in its activity. At 45ºC or above the enzyme activity was completely lost.

\[ K_M \text{ and } V_{\text{max}} \text{ of HNL of rosary pea} \]

A Line Weaver- Burk plot (1/v versus 1/[S]) of velocity data of HNL catalyzed reaction at various concentrations of substrate [S] i.e. mandelonitrile was drawn. HNL of Abrus precatorius showed a
of 26 % of HNL activity at the end of purification showed that there was appreciable loss of enzyme during purification. Such loss in enzyme activity is usually observed during purification (Ho et al. 2004). The low yield of enzyme activity may be due to physical or physiological (inactivation) losses during purification. Since enzyme was fairly stable at 0-4 °C after purification, therefore recovery of 26 % of enzyme activity may be due to physical loss of enzyme during various steps followed for purification. Previously, 4.3 fold purification with 60 % yield (Xu et al. 1986) in Prunus lyonii and 1.71 fold purification with 50 % yield in Linum usitatissimum (Albrecht et al. 1993), 151 fold purification with 3 % yield (Gray and Conn, 1989) and 122 fold purification with 38 % yield (Albrecht et al. 1993). in Xemia americana have been reported. The electrophoretic studies (SDS PAGE) of the purified enzyme revealed that HNL of Abrus precatorius comprised two polypeptides of 36.5 and 42 kDa. Native PAGE indicated the presence of a single band of 205 kDa (Fig.3 B). This confirms the polymeric nature of Abrus precatorius HNL. Molecular mass diversity of the enzyme has been reported earlier in Manihot esculenta (28.5 kDa) (Jane et al. 1998), Linum usitatissimum (42 kDa) (Xu et al. 1988), Prunus lyonii (59 kDa) (Xu et al. 1986), Phleobodium aureum (20 kDa) (Wajant et al. 1995), Prunus serotina (57 kDa) and in Phleobodium aureum (20.1 kDa) (Wajant et al. 1995) using SDS-PAGE.

HNL of Abrus precatorius also exhibited some distinct kinetic properties as compared to that of other plants. The $K_m$ and $V_{max}$ values of HNL of Abrus precatorius was 13 mM of mandelonitrile as a substrate and 625 μmol/ min/mg of protein respective with mandelonitrile as substrate, whereas $K_m$ and $V_{max}$ of HNL reported from different sources such as Phleobodium aureum (0.83 mM benzaldehyde and 60.1 μmol/ min/mg protein) (Wajant et al. 1995), Prunus lyonii (93mM mandelonitrile and 450 μmol/ min/mg of protein) (Xu et al. 1986) and Linum usitatissimum (2.5 mM benzaldehyde and 1.11 μmol/ min/mg of protein). These data indicated that HNL of rosary pea seemed to have fairly higher affinity for mandelonitrile and had higher $V_{max}$ in comparison to earlier reported HNLs. Thus, it holds good potential for its application in the synthesis of cyanohydrins. The purified enzyme was eventually used in the synthesis of mandelonitrile from benzaldehyde and a significant 90 % molar conversion was obtained. The conversion rate was much higher as compared to previously reported HNLs, e.g. 73 % in case of HNL of Prunus capuli and 61 % molar conversion by HNL of Mamanca americana (Sdis et al. 1998).

Present study revealed that HNL of rosary pea had best activity at 0.1 M of sodium citrate buffer (pH 5). It indicated that HNL was stable and active in acidic conditions. The higher value of $V_{max}$ showed the affinity of HNL towards its substrate i.e. mandelonitrile. The above data confirmed the high potential of rosary pea seeds for large scale production of hydroxynitrile/ cyanohydrins.

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