Purification of L-asparaginase from a bacteria *Erwinia carotovora* and effect of a dihydropyrimidine derivative on some of its kinetic parameters

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L-Asparaginase shows antileukemic activity and is generally administered in the body in combination with other anticancer drugs like pyrimidine derivatives. In the present study, L-asparaginase was purified from a bacteria *Erwinia carotovora* and the effect of a dihydropyrimidine derivative (1-amino-6-methyl-4-phenyl-2-thioxo, 1,2,3,4-tetrahydropyrimidine-5-carboxylic acid methyl ester) was studied on the kinetic parameters $K_m$ and $V_{max}$ of the enzyme using L-asparagine as substrate. The enzyme had optimum activity at pH 8.6 and temperature 35°C, both in the absence and presence of pyrimidine derivative and substrate saturation concentration at 6 mg/ml. For the enzymatic reaction in the absence and presence (1 to 3 mg/ml) of dihydropyrimidine derivative, $K_m$ values were 7.14, 5.26, 4.0, and 5.22 $M$, and $V_{max}$ values were 0.05, 0.035, 0.027 and 0.021 mg/ml/min, respectively. The kinetic values suggested that activity of enzyme was enhanced in the presence of dihydropyrimidine derivative.

**Keywords:** *Erwinia carotovora*, L-Asparaginase activity, Dihydropyrimidine derivative

L-Asparaginase (E.C. 3.5.1.1) a pyrimidine derivative has long been considered as an important compound in the management of childhood acute lymphoblastic leukemia. Its antileukemic effect is related to the blast cells incapability to synthesize asparagine from aspartic acid. Lymphatic tumor cells require large amounts of asparagine for rapid malignant growth. Therefore, the antileukemic treatment with L-asparaginase aims at depleting the asparagine level in the blood, in order to exhaust its supply. In general, the leukemic treatment involves combination chemotherapy with a number of different anticancer drugs.

L-Asparaginase obtained from *Escherichia coli* and another bacteria *Erwinia carotovora* possess the anti-lymphoma properties and the characteristics of *E. coli* L-asparaginase have been reported earlier.

Pyrimidines, which comprise an integral part of nucleic acids, also show anti-viral, anti-bacterial and anti-cancer activities, depending upon geometry and type of substituents attached to the pyrimidine ring. Conjugation of L-asparaginase with anticancer drugs like dihydropyrimidine derivatives affects its affinity to its substrate L-asparagine. However, the characteristics and effect of dihydropyrimidine derivatives on the L-asparaginase activity from *E. carotovora* have not been studied in detail.

In the present study, attempt has been made to purify L-asparaginase from *E. carotovora* and the effect of a dihydropyrimidine derivative (1-amino-6-methyl-4-phenyl-2-thioxo, 1,2,3,4-tetrahydropyrimidine-5-carboxylic acid methyl ester) has been studied on the enzyme activity by determining the kinetic parameters $K_m$ and $V_{max}$ at variable pH and temperatures.

**Materials and Methods**

All the chemicals were purchased from S. D. Fine-Chem. Ltd., Mumbai, India and were used without further purification unless stated otherwise.

**Bacterial isolates, extraction and purification of L-asparaginase**

*Erwinia carotovora* (MTCC, 1428) was procured from the Institute of Microbial Technology, Chandigarh, India was used as a source of L-asparaginase. The bacterium was cultivated in the nutrient broth (HiMedia Mv 088) containing beef extract (3.0 g), NaCl (5.0 g) and peptone (5.0 g) in 1000 ml distilled water. The medium was sterilized at 15 Lbs for 15 min at 121°C using autoclave.

A slant culture of bacteria (loop full) was transferred in a 100 ml sterile cultivation medium in aseptic condition. The medium was incubated at 25°C, continuously for 7 days. The pH of the culture media was measured every day and if varied, corrected at pH 6.9 with dropwise addition of alkali (0.1 N NaOH). After optimum growth of bacterial culture, nutrient broth was filtered through filter paper.
(Whatman No.1) and filtrate was subjected to centrifugation in cooling centrifuge at 15,000 rpm for 15 min, so as to remove the bacterial cell mass completely. Thereafter, the clear supernatant was collected and treated with ammonium sulphate of 50, 60, 80, and 100% final saturation (w/v). The chilled precipitates from different saturations of ammonium sulphate were collected by cold centrifugation at 20,000 rpm for 15 min and were allowed to settle completely by placing the medium in a refrigerator for overnight. The chilled contents were recentrifuged at 20,000 rpm for 15 min at 4°C and the enzyme was pooled out completely in the form of pellets. The pellets were dissolved in 25 ml of Tris-HCl buffer (0.1 M, pH 8.6) and dialyzed against two changes of Tris-HCl buffer (0.01 M, pH 8.6) for 48 h. The dialyzed enzyme (desalted) was collected and subjected to anion-exchange chromatography.

DEAE-cellulose anion-exchange chromatography

A chromatography column (2 × 40 cm) was packed with DEAE-cellulose and equilibrated with 0.1 M Tris-HCl buffer containing 2 mM MgCl₂ (pH 8.6). The desalted enzyme obtained from dialysis (25 ml) was loaded on to the top of the column and eluted with 1.0 L of linear gradient Tris-HCl buffer composed of NaCl (0-1 M) at room temperature. The flow rate of the column was adjusted to 1 ml/min. The fractions were collected and monitored constantly to determine the protein content in the eluant (280 nm UV/visible Schimadzu). The 15 ml fractions were pooled and assayed for the protein and enzyme activity.

Protein determination

The eluted fractions from DEAE-cellulose anion-exchange column (scanned at 280 nm) were used for quantitative protein determination using bovine serum albumin as a standard and protein content was found to be 0.1 mg/ml. The purity was checked by applying 10% SDS-PAGE and molecular mass was found to be 33.5 kD.

Synthesis of dihydropyrimidine derivative

The dihydropyrimidine derivative 1-amino-6-methyl-L-4-phenyl-2-thioxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylic acid methyl ester was synthesized essentially according to the methods described previously. Briefly, a mixture of benzaldehyde derivative (0.01 mole, 1.06 g) with Z as substituents, 2-Cl, 4-Cl, acetoacetic ester (0.01 mole, 1.30 g) and thiosemicarbazide (0.01 mole, 1.06 g) in absolute ethyl alcohol (15 ml) and catalytic amount of piperidine was refluxed for 4 h. The reaction mixture was cooled and poured over ice. The solid, thus separated was filtered under reduced pressure, dried and recrystallized from methanol to give above product (m p = 218°C, yield = 85%, mol. formula = C₁₂H₁₆N₃O₅S). The product was characterized as 1-amino-6-methyl-L-4-phenyl-2-thioxo, 1, 2, 3, 4-tetrahydropyrimidine-5-carboxylic acid methyl ester (Scheme 1), on the basis of spectral characteristics.

Spectral characterization

¹H NMR (300 MHz, CDCl₃ + DMSO-d₆): δ 4.8 (s, 2H, NH₂), 9.6 (s, 1H, NH), 8.2-7.5 (m, 5H, Ar-H), 5.4(s, 1H, 4-CH), 4.1 (q, 2H, -CH₂CH₃), 2.3 (t, 3H, -OCH₂CH₃), 1.2 (s, 3H, CH₃); IR (KBr): cm⁻¹ 3360 (NH str), 3070 (aromatic C-H str.), 1680 (C=O, str. of ester), 1120 (C = S str.), 1485 (C=C str.), 1180 (C – N vib.) 1080 (C – O str.)

Enzyme activity assay

L-Asparaginase catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. The test organism was grown in nutrient broth containing L-asparagine and pH indicator phenol hypochlorite. During incubation at 25°C, the released ammonia reacts with phenol hypochlorite to form blue color, which indicates the production of L-asparaginase. One unit of L-asparaginase was defined as the amount of enzyme that liberated 1 µmole of ammonia per min under assay conditions.

L-Asparaginase activity was determined in Tris-HCl buffer (pH range 8.0-9.2) and at temperatures 5-55°C. A solution of L-asparagine (6 mg/ml), a
substrate for L-asparaginase was prepared in double-distilled water. A reaction cocktail was prepared by taking 1.0 ml each of the enzyme, asparagine, chromogen solution (0.25% phenol red), Tris-HCl buffer and the volume was adjusted to 5 ml using distilled water. The reaction mixture was incubated at 35°C for 15 min and thereafter, 1.0 ml of 0.66 N H₂SO₄ was added. The level of released ammonia was determined from the ammonium sulphate standard curve by recording the absorbance at 560 nm against the respective blank.

**Determination of kinetic parameters **\( K_m \) and \( V_{max} \)

The \( V_{max} \) and \( K_m \) values were determined in the presence and absence of dihydropyrimidine derivative by increasing the asparagine volume (1.0 to 10.0 ml). In a reaction mixture, the volume of purified enzyme, chromogen, H₂SO₄, distilled water and dihydropyrimidine derivative (1 mg/ml) was fixed at 1.0 ml and Tris-HCl buffer was added to make up a final volume of 5.0 ml. The \( V_{max} \) and \( K_m \) values were determined in absence and presence of the dihydropyrimidine derivative (1-3 mg/ml) using a Lineweaver-Burk double-reciprocal plot.

**Results and Discussion**

L-Asparaginase, an important therapeutic enzyme, has been isolated from a number of sources. However, only *E. coli* and *E. carotovora* asparaginase has shown anti-tumour activity. In the present study, the enzyme was purified from the *E. carotovora* using dialysis and anion-exchange chromatography. The enzyme activity was determined by using L-asparagine as a substrate and found to be 0.31 mg/ml/min (Table 1). The effect of the pH and temperature on the enzyme activity was studied both in the absence and presence of dihydropyrimidine derivative. The activities of the enzyme were determined in a pH range 8.0 to 9.2 at interval of 0.2 and between 5°C to 55°C at interval of 10°C. The optimum pH of the L-asparaginase was found to be 8.6 (Table 2). The enzyme was active between 5°C and 55°C and the optimum temperature was 35°C (Table 3).

Results revealed that in the absence and presence of (1-3 mg/ml) dihydropyrimidine derivative in the reaction medium, the values of \( K_m \) were 7.14, 5.26, 4.0 and 3.22 M respectively, whereas the \( V_{max} \) values were calculated as 0.05, 0.035, 0.027 and 0.021 mg/ml/min (Fig. 1). The decrease in \( K_m \) values with increase in concentration of dihydropyrimidine derivative indicated that L-asparaginase had more affinity towards the substrate (L-asparagine) in the presence of dihydropyrimidine derivative. The decrease in \( V_{max} \) values observed after the addition of dihydropyrimidine derivative indicated the change in microenvironment of the enzyme.

The decrease in \( K_m \) values may be attributed to the fact that dihydropyrimidine derivative might be binding possibly to the one of the monomer of L-asparaginase, a K⁺-dependant oligomeric enzyme, thereby affecting its conformation favourably to

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**Table 1—Activity and purity of L-asparaginase from *Erwinia carotovora* obtained at different steps**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Volume (ml)</th>
<th>Activity (U/ml)</th>
<th>Total activity (U)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broth</td>
<td>100</td>
<td>0.06</td>
<td>6.0</td>
<td>100</td>
<td>1.72</td>
<td>0.03</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>30</td>
<td>0.17</td>
<td>5.1</td>
<td>85</td>
<td>0.84</td>
<td>0.20</td>
</tr>
<tr>
<td>Dialysis</td>
<td>25</td>
<td>0.20</td>
<td>5.0</td>
<td>83</td>
<td>0.77</td>
<td>0.25</td>
</tr>
<tr>
<td>DEAE</td>
<td>15</td>
<td>0.31</td>
<td>4.6</td>
<td>76</td>
<td>0.10</td>
<td>3.10</td>
</tr>
</tbody>
</table>

**Table 2—Effect of pH on L-asparaginase activity in absence and presence of dihydropyrimidine derivative**

<table>
<thead>
<tr>
<th>pH</th>
<th>Enzyme activity (U/ml)</th>
<th>Specific activity (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>0.33</td>
<td>3.3</td>
</tr>
<tr>
<td>8.2</td>
<td>0.46</td>
<td>4.6</td>
</tr>
<tr>
<td>8.4</td>
<td>0.66</td>
<td>6.6</td>
</tr>
<tr>
<td>8.6</td>
<td>0.80</td>
<td>8.0</td>
</tr>
<tr>
<td>8.8</td>
<td>0.60</td>
<td>6.0</td>
</tr>
<tr>
<td>9.0</td>
<td>0.46</td>
<td>4.6</td>
</tr>
<tr>
<td>9.2</td>
<td>0.40</td>
<td>4.0</td>
</tr>
</tbody>
</table>

**Table 3—Effect of temperature on L-asparaginase activity in absence and presence of dihydropyrimidine derivative**

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Enzyme activity (U/ml)</th>
<th>Specific activity (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>05</td>
<td>0.40</td>
<td>4.0</td>
</tr>
<tr>
<td>15</td>
<td>0.46</td>
<td>4.6</td>
</tr>
<tr>
<td>25</td>
<td>0.53</td>
<td>5.3</td>
</tr>
<tr>
<td>35</td>
<td>0.86</td>
<td>8.6</td>
</tr>
<tr>
<td>45</td>
<td>0.50</td>
<td>5.0</td>
</tr>
<tr>
<td>55</td>
<td>0.40</td>
<td>4.0</td>
</tr>
</tbody>
</table>
enhance its activity\textsuperscript{25,26}. The stereochemistry between the aryl group and dihydropyrimidine ring was found to be one of the factors having a pronounced effect on hydrolytic activity. In addition, in an enzyme-dihydropyrimidine derivative bound conformation, the substituted aryl ring (Z = 2-Cl, 4-Cl) might be positioned axially or perpendicular to the dihydropyrimidine ring with a 4-aryl substituted Z, thereby adopting a synperiplanar (relative to C\textsubscript{4}-H) orientation\textsuperscript{27,28}.

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References
2. Roberts J, Bursen G & Joseph M H (1968) J Bacterial 95, 2117-2123

Fig. 1—Effect of dihydropyrimidine derivative on kinetic parameters $K_m$ and $V_{max}$