Isolation and characterization of L-asparaginase from marine actinomycetes

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The marine environment is a potential source of novel actinomycetes, which are a potent source of antibiotics and novel bioactive compounds. Marine actinomycetes were enumerated in sediment samples from Parangipettai and Cochin coastal areas of South India (PDK7 and PDK2). The isolates showed potential L-asparaginase activity. The partially purified L-asparaginase showed a specific activity of 64.07 IU/mg protein, 83-fold pure and yielded 2.18 per cent of protein. The enzyme activity was maximum between pH 8 and 9 and maintained stability at pH 8. The L-asparaginase showed maximum activity at 60°C, and stable up to 80°C. The enzyme has a molecular weight of 140 kDa. It showed cytostatic effect on JURKAT cells (Acute T cell leukemia) and K562 cells (Chronic myelogenous leukemia) at 24 h and cytotoxic effects at 48 h.

Keywords: marine actinomycetes, therapeutic L-asparaginase, anti-lymphoma activity
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Introduction

The marine biosphere is one of the earth’s richest innumerable habitats. Marine microorganisms are considered as untapped sources of metabolites and products with novel properties. They have a diverse range of enzymatic activity and are capable of catalyzing various biochemical reactions. Thus, there is enormous scope for investigations to explore the possibilities of deriving new products of economic importance from potential marine microorganisms. Considering the fact that marine environment is saline in nature it could provide rare and unique microbial products, particularly the enzymes that could be safely used for human therapeutic purpose.

Among the microorganisms, actinomycetes gain special importance as the most potent source of antibiotics and other bioactive secondary metabolites. Cohn first discovered actinomycetes in 1875 and studies on actinomycetes have been numerous since then. Actinomycetes have provided many important bioactive compounds of high commercial value and are routinely being screened for new bioactive compounds. The enzyme, L-asparaginase (L-asparagine amino hydrolase E.C. 3.5.1.1) has been intensively investigated over the past two decades owing to its importance as an antineoplastic agent. Although the enzyme has been found in a variety of bacteria, fungi and actinomycetes and mammals, few of the purified preparations have shown to possess antitumour activity7. Like bacteria, actinomycetes are also a good source for the production of L-asparaginase2,3.

The oncolytic enzyme, L-asparaginase is employed in the treatment of tumour and acute lymphatic leukemia. It is reported to be an effective antilymphoma agent in humans. The enzymes isolated from Escherichia coli (L-asparaginase, EC–2) and Erwinia caratovora are now being used for the treatment of acute lymphoblastic leukemia.

L-Asparaginase enzyme activity and its antilymphoblastic leukemia activity from actinomycetes have not been fully explored. The present investigation deals with isolation and characterization of L-asparaginase from marine actinomycetes and assessment of its antitumour activity in animal cell lines.

Materials and Methods

The marine sediment samples were collected from 3 different sites at a depth of 40 cm, two sites near Parangipettai (Lat. 11° 42’ N; Long 79° 46’ E) and one site near Cochin (Lat. 8° 28’ N; 76° 57’ E) in December 2003 and January 2004. The samples were collected using alcohol rinsed Peterson-grab and were
transferred to new polythene bags using sterile spatula and subsequently transported to the laboratory for further microbiological analysis.

**Enrichment and Isolation of Marine Microorganisms**

One gram of sediment was transferred to conical flask containing 100 mL of sterile seawater complex broth and 100 mL of sterile starch casein broth for the pre-enrichment of samples and incubated at 30°C for 14 d in an incubator cum shaker. A loopful of the inoculum from the pre-enriched seawater complex broth and starch casein broth was streaked onto seawater complex agar (SWC), Ken knight agar (KA), starch casein agar (SCA), glucose asparagine agar (GAA) and the plates were incubated at 30°C for 7 d. Single discrete colonies were isolated and used for identification.

**Identification of Actinomycetes**

Isolated colonies were identified using the standard ISP procedure. The isolates were screened for asparaginase activity as per the method of Gulati et al. The crude enzyme was prepared as per Imada et al. and used in the enzyme assay developed by Benny & Ayyakkannu.

**Purification of L-asparaginase from Marine Actinomycetes**

L-asparaginase was partially purified using the following steps at 4 to 8°C:

**Step 1—Ammonium Sulphate Precipitation**

The crude enzyme prepared was brought to 45 per cent saturation with ammonium sulphate (2.6 g) at pH of 8.4 and kept overnight in cold room. After equilibration, the supernatant was subjected to centrifugation at 4200 g, for 10 min at 4°C. After centrifugation, the supernatant was brought to 85 per cent saturation with ammonium sulphate (5.8 g) and centrifuged at 4200 rpm, at 4°C for 10 min. Then the precipitates were collected separately and stored at 4°C for further purification. The yield was around 65.83%.

**Step 2—Dialysis**

The pre-treated dialysis tubes were used for dialysis of the precipitates collected in step 1. The precipitate was dissolved in 1 M Tris HCl buffer and dialyzed. After dialysis, the samples were used for protein estimation and enzyme assay.

**Step 3—Gel Filtration on Sephadex column**

**Gel Filtration on Sephadex G 50 column**

The dialyzed samples were dissolved in 0.05 M Tris HCl (pH 8.4) buffer and loaded onto pre-equilibrated column with 0.05 M Tris HCl Sephadex G 50 (Hidex-Hi media; 100×1.5 cm column). It was eluted with 0.05 M Tris HCl (pH 8.4) buffer containing 0.1 M KCl. Fractions were collected at the flow rate of 5 mL/30 min and L-asparaginase was assayed by procedure described earlier. Fractions showing high activity were pooled and freeze dried.

**Gel Filtration on Sephadex G 200 column**

Fractions from Sephadex G 50 were loaded on to the pre-equilibrated Sephadex G 200 column (Hidex-Hi media; 100×1.0 cm column) with 0.05 M Tris HCl and eluted with 0.05 M Tris HCl buffer (pH 8.5). Fractions were collected at a flow rate of 5 mL/30 min. Fractions showing high L-asparaginase activity were pooled and lyophilized. The lyophilized samples were used for further characterization and evaluation of antitumour activity.

**Characterization of Purified L-asparaginase**

The lyophilized samples were used for the characterization of L-asparaginase and to optimize its activity. Testing the activity of the enzyme was carried out at wide range of pH (4 to 10) and temperatures (20 to 100°C).

**Electrophoretic Separation of Purified L-asparaginase Fraction**

The lyophilized L-asparaginase purified in Sephadex G 200 filtration was subjected to SDS-PAGE to determine the molecular weight. Of the SDS-PAGE (7.5, 10 and 12% acrylamide gel containing 0.1% SDS) used, 7.5% gave good separation. The BenchMark™ protein molecular marker was used (Invitrogen-Cat. No. 10747-012). After electrophoresis, the gel was washed with buffer and stained with Commassie brilliant blue R-250 (Calbiochem).

**Antitumour Activity**

The antitumour activity of purified L-asparaginase was confirmed in Jurkat cells (acute T cell leukemia) and K562 cells (chronic myelogenous leukemia) of American type culture collection. The L-asparaginase inhibition effects on the leukemic cell types were compared with two MEK inhibitors-Topotecan and PD 184352.
Results and Discussion

Enrichment and Isolation of Marine Actinomycetes on Different Media

The search for therapeutic enzymes throughout the world stimulated a variety of different approaches for the isolation of therapeutic enzyme producing microbial strains. The marine sediment samples were collected from Parangipettai and Cochin. Pre-enrichment was carried out at 40°C for 14 d. Starch casein agar added plates showed 52×10⁴, 65×10⁴ and 45×10⁴ of actinomycetes. The results obtained in the present investigation (Table 1) demonstrate that different kinds of pretreatment methods may be successfully applied to marine sediments to facilitate the isolation of actinomycetes⁶,15-18.

Identification of Actinomycetes

All the isolates produced grey and white colonies without pigmentation and showed fast growth within two days. It was identified by slide culture, morphological characteristics, physiological and enzymatic properties, carbon utilization and nitrogen utilization as *Streptomyces* sp. using the procedure given in ISP⁵. The samples of *Streptomyces* spp. isolated from Parangipettai were designated as PDK 5, 6 and 7 and those from Cochin as PDK 1, 2, 3 and 4, respectively. The isolates showed resistance against tetracycline, ampicillin, penicillin, streptomycin but were sensitive to ciprofloxin, cephalothin, chloramphenical, and erythromycin¹⁹-²¹.

Screening of L-asparaginase Positive Cultures

Out of the isolated *Streptomyces* spp. screened for L-asparaginase activity, PDK7 from Parangipettai and PDK2 from Cochin showed positive results in rapid plate assay method.

L-asparaginase Assay

The isolates from *Streptomyces* spp. were found to possess L-asparaginase, which liberated 1.72 to 3.746 μ mole/mg/mL/h ammonia having specific activity of 0.76 IU/mg. The culture filtrates of sonicated preparations of *Streptomyces* sp PDK7 were used as crude enzyme preparations and showed the L-asparaginase activity. The isolate PDK7 was found to liberate more ammonia than PDK2. Sonicated preparations from 25 strains of *Streptomyces* species were cultured in ST₂ broth showed L-asparaginase and L-glutamine deamidating activity⁹. Similar effects in L-asparaginase of *Vibrio succinogens* were observed by many scientists²². Varying amounts of L-asparaginase (L-asparagine amidohydrolase E.C. 3.5.1.1) were reported in many genera of marine microorganisms⁹. It has been observed that sediment samples from southeast Porto-Novo region harbour contained more potent L-asparaginase producing strains²³ and culture filtrates of the *Streptomyces* sp. isolated from the marine sediments showed L-asparaginase activity²⁴.

Purification of L-asparaginase

L-asparaginase assay was carried out at all purification steps and the results are summarized in Table 2. The supernatant fraction exhibited 70 per cent of L-asparaginase enzyme activity as compared to only 0.5 per cent of activity by the particulate fraction. It can be observed that the ammonium sulphate purified sample showed a specific activity of 0.83 IU/mg, being approximately 1.09-fold pure. In the final step, the enzyme showed a specific activity of 63.07 IU/mg, being approximately 83-fold pure. The final recovery of protein was 2.18 per cent (Figs 1 & 2).

The final purification step in Sephadex G 200 showed a specific activity of 63.07 IU/mg of protein, being approximately 85-fold pure and the final recovery of protein was 2.18 per cent. The marine *Vibrio* sp. showed an enzyme specific activity of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Total count of marine actinomycetes on different pre-enrichment media</th>
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<tbody>
<tr>
<td>Sampling place</td>
<td>Sample</td>
</tr>
<tr>
<td>Parangipettai Sediment</td>
<td>14</td>
</tr>
<tr>
<td>Parangipettai Sediment</td>
<td>14</td>
</tr>
<tr>
<td>Cochin Sediment</td>
<td>14</td>
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</tbody>
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| SWC-Seawater complex agar, SCA-Starch casein agar, KA-Ken knight agar & GAA-Glucose asparagine agar |

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Purification of L-asparaginase from <em>Streptomyces</em> spPDK2</th>
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<tbody>
<tr>
<td>Purification steps</td>
<td>Total protein (mg)</td>
</tr>
<tr>
<td>Crude extract</td>
<td>489.5</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation 45-85%</td>
<td>296.0</td>
</tr>
<tr>
<td>Sephadex G 50 filtration</td>
<td>1.26</td>
</tr>
<tr>
<td>Sephadex G 200 filtration</td>
<td>0.13</td>
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14 IU/mg of protein, approximately 19-fold pure, and the final recovery of protein was 1.6 per cent\textsuperscript{12}. A species of marine micro algae, *Chlamydomonas* was isolated with L-asparaginase enzyme activity of 78 IU/mg of protein and had been purified over 600-fold\textsuperscript{1}. The large-scale production of L-asparaginase enzyme from strain *Citrobacter* sp C6 with an overall purification of 180-fold and a yield of 4.3 per cent was carried out successfully\textsuperscript{25}. The purified L-asparaginase showed anti-lymphoma activity in lymphosarcoma in C3H.

**Characterization of L-asparaginase**

The purified L-asparaginase was characterized for its activity. It exhibited maximum activity between pH 8 and 8.5 with 85% of the activity at physiological pH. At pH 10 it retained only 70% activity. The effect of pH on the stability of L-asparaginase and its activity was also studied by incubating the enzyme at different pH 4, 5, 6, 7, 8, 9 and 10. The enzyme was quite stable at pH 8 and 9, except at pH 10 where it showed maximum loss of activity (Figs 3 & 4). Maximum activity of L-asparaginase was found at
60°C. At 80°C, it showed 85 per cent of its activity while at 90°C it showed maximum loss of activity.

The enzyme was exposed to temperatures ranging from 0-100°C to find out its stability. It was unaffected up to 80°C. However, maximum loss of the activity was evident at 100°C (Figs 5 & 6).

**Electrophoretic Separation of Purified L-asparaginase**

The molecular weight of lyophilized L-asparaginase was determined by 7.5% SDS-PAGE. Only a single band was seen in SDS-PAGE and the mobility of the band corresponded with a molecular weight of 140,000 Da in BenchMark™ protein molecular marker (Fig. 7). Purified L-asparaginase from *V. succinogenes* had a molecular weight of 146,000 and subunit molecular weight of approximately 37,000 in 10% SDS-PAGE. The purified L-asparaginase from *Chalmydomonas* sp. showed a higher molecular weight of 275,000 Da in disc gel electrophoresis.

**Antitumour Activity of Purified L-asparaginase**

In the present investigation, the antilymphoma activity of purified L-asparaginase from marine actinomycetes was confirmed in Jurkat cells (acute T cell leukemia) and K562 cells (chronic myelogenous leukemia) of American Type Culture Collection (ATCC). L-asparaginase showed cytostatic effects on the two leukemic cell lines at 24 h growth inhibition of both the Jurkat and K562 cell lines. It was compared with two MEK inhibitors, Topotecan and PD-184352. Topotecan is widely used in all clinics for leukemia therapy and PD-184352 is also a cytotoxic molecule. At 24 h growth inhibition in the two leukemia cell types, the L-asparaginase GI 50

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Fig. 3—Effect of pH on activity of L-asparaginase

Fig. 4—Effect of pH on stability of L-asparaginase

Fig. 5—Effect of temperature on activity of L-asparaginase

Fig. 6—Effect of temperature on stability of L-asparaginase

Fig. 7—SDS-PAGE of L-asparaginase-7-5% gel
showed cytostatic effects, which were similar to the cytostatic effects of Topotecan inhibitor and higher than PD184352 inhibitor (Figs 8 & 9).

At 48 h growth inhibition in the two leukemic cell lines, L-asparaginase GI 50 showed more inhibition and possessed cytotoxic effects on the cell lines. This effect of purified L-asparaginase on the leukemic cell lines was almost similar to the cytotoxic effects produced by Topotecan inhibitor (Figs 10 & 11). A 60 per cent incidence of complete remission has been reported in a study of almost 6,000 cases of acute lymphoblastic leukemia when the enzyme was administrated intravenously. Ueno et al. obtained the cell cycle arrest and apoptosis of leukemia cells induced by asparaginase and found that the apoptotic cell death of murine leukemia cells was induced by E. coli asparaginase. Further work on the mechanism of L-asparaginase has to be evaluated.

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References