Isolation and characterization of 37 kDa heparinase from the purple fluid of *Dolabella auricularia*

P. Abirami., M. Arumugam1, T.T. Ajithkumar & T. Balasubramanian
Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608 502, India.

1E-mail: aru_hep@yahoo.com

Received 31 March 2010; revised 3 June 2010

Present study was to explore the heparinase and antiligcosidase activity of the purple fluid of *Dolabella auricularia*. Heparinase activity was found as 56.42 IU/ml, further the activity was confirmed by APPT test by the reduction of coagulation time with the addition of purple fluid of about 7±0.2. This purple fluid was dialyzed and purified by anion exchange column (DEAE-cellulose). Amount of protein in crude and purified sample was 2mg/ml and 7.6mg/ml. Apparent molecular weight of the purified heparinase was revealed by SDS-PAGE as 37 kDa. Effects of various factors such as temperature and pH were determined and recorded the maximum activity at 30ºC-50ºC and 7 respectively. Antibacterial activity was also observed against four pathogens and activity was observed maximum only in *P. aeruginosa*, *S. aureus*. Antiligcosidase activity exhibits about 120.9IU/ml which has been reduced when compared to that standard. Present study suggests the purple fluid of *D. auricularia* has immense uses in pharmaceutical industries.

Keywords: Nudibranch heparinase; *Dolabella auricularia*; purple fluid; DEAE-cellulose.

Introduction

Heparin is a sulphated mucopolysaccharide belonging to the family glycosaminoglycan that has several biological activities. Heparin may be hydrolyzed with acid only under vigorous condition which results in considerable caramalization, especially of the glucoronic acid, and extensive splitting of heparin. Thus, the products are mono (or) disaccharides from which all the sulphated groups are removed. It was proved that heparin has the ability to induce some bacteria to produce a series of enzymes, which include glucuronidase, sulphoesters, sulphamidase, heparinase and heparatinase. Heparinase is an $\alpha_{1}$-4 eliminase, which acts specifically on the glycosidic linkage between N-sulphated D-glucosamine and sulphated D-glucoronic acid (L-iduronic acid) present in heparin. Various uses of heparinase (heparin lyase) are the elucidation of the structure of heparin, blood de-heparinization and enzymatic assays for heparin.

Sea hares are much attracted due to their biologically active compounds such as Auripyrones A and B (cytotoxin), Aurilide, Dolabellanin A, C, E and P (antimicrobial factor), Dolabellanin C (antineoplastic factor), Dolastatins (antitumour factor), Dolastatins-10 (antineoplastic factor). Sea hare *D. auricularia* had been selected as a candidate species for the present study since it protects itself from predators by having effective defense mechanism i.e., by releasing their dark purple fluid to escape from their enemies. This sea hare *D. auricularia* is also reffered as “eared sea hares”. Present study has been undertaken to explore the nature and bioactive compounds especially on heparinase enzyme from the purple fluid.

Materials and Methods

The *D. auricularia* specimens were collected from the Gulf of Mannar, south east coast of India where the availability of this species is more. The purple fluid was obtained by disturbing the animals and was frozen at -20ºC for further analysis.

The purple fluid was screened for heparinase activity through determining the reduction of heparin by metachromatic assay. It was further confirmed by performing APTT assay. Metachromatic properties of azure-A have long been used to assess the presence of heparin. In the present study, azure-A has been used anti-neoplastic to find out the degradation of heparin...
by heparinase enzyme and the retention time of the blood was measured through APTT assay.

To the extracted purple fluid, solid ammonium sulphate was added up to 80% saturation, which was stirred for 30 minutes and allowed to stand overnight at 4°C. Precipitate was collected by centrifugation at 6000 rpm for 15 minutes, dissolved in 100mM Tris – HCl buffer and dialyzed against the same buffer overnight (4°C). Dialyzed enzyme solution was loaded onto a DEAE-cellulose column (5cm × 20cm) equilibrated with Tris-HCl buffer maintained at pH-9. Heparinase enzyme was eluted with linear gradient of NaCl (0-1M in 100mM Tris-HCl buffer) at the flow rate of 0.5ml/h. The eluted fractions were assayed for enzyme activity. This partially purified enzyme used to investigate the effects of temperature, pH, antimicrobial and antiglycosidase activity.

Amount of protein in crude as well as purified samples was determined by following the earlier method with Bovine Serum Albumin used as a standard.

The effect of temperature on the enzyme activity was examined by incubating the reaction mixture (enzyme + substrate) at various temperatures (30-100°C) for 1 hour and then the residual activity (%) was assayed with standard assay conditions. Aliquots were withdrawn at desired time intervals to test the remaining activity. The un-heated enzyme was considered as control (100%).

The effect of pH on the enzyme activity was examined with four different buffers namely Citrate buffer (pH 4, 5), Phosphate buffer (pH 6, 7, 8), Tris-HCl buffer (pH 9) and Glycine buffer at pH 10 and heparin as a substrate. Stability of the enzyme was evaluated by incubating enzyme solution at different pH (4-10) for 1 hr and the residual activity was assayed by the standard assay condition.

SDS-PAGE was performed to estimate the molecular weight of the purified protein using 5% stacking gel and 12% resolving gel. Molecular weight was estimated by comparing the relative mobility of proteins with different standard molecular weight marker (29 - 205 kDa).

Four species of pathogenic bacteria Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella paratyphi, Vibrio cholerae were used to assess the antibacterial activity of the purple fluid. Pathogens were swabbed on the surface of the Muller Hinton agar plates and the discs (9 mm diameter) impregnated with 50 µl of purple fluid were placed on the surface of the media and incubated at 37°C for 24 hours.

For sugar estimation an alternative to Nelson somogyi method is the di-nitrosalicylic method. The amylase inhibitory activity was measured by reducing sugars method slight modification. The amount of enzyme inhibitory activity was expressed in terms of inhibition of enzyme activity in units (i.e., one unit is the amount of enzyme which release 1 µM glucose).

**Results**

The heparinase activity of D. auricularia’s purple fluid was first screened by metachromatic assay using azure-A dye. Heparinase activity was estimated by measuring the activity against standard heparin (140 USP/mg) and in control. Thus the heparinase activity of the purple fluid of D. auricularia was showed as 56.42 IU/mg. Heparinase activity was also examined by measuring the coagulation time with chicken blood. The coagulation time for the blood with purple fluid was 7 ± 0.2 minutes whereas the test carried out without purple fluid is 25 ± 0.2 minutes for chicken blood. It clearly indicates the heparinase act on the heparin which exhibits the heparinase activity.

Enzyme purification and characterization were performed at room temperature (28±2°C). Purification of heparinase enzyme was performed with ammonium sulphate followed by DEAE-cellulose column chromatography. The molecular weight of the protein was estimated as 37kDa (Fig. 1). The amount protein

![Fig. 1—Molecular weight determination by SDS – PAGE gel electrophoresis](image)

Lane A. Standard Molecular markers (29 - 205 kDa), Lane B. D. auricularia purified sample.
content was higher (7.6mg/ml) in purified sample than that of crude (2mg/ml).

The enzyme was found active at varies temperatures between 30°-50°C and the optimum temperature was 40°-50°C. Enzyme shows about 60 percent activity up to 60°C and it loses completely at 100°C (Fig. 2). Purple fluid was found to be active at wide ranges of pH (7-9) and it show optimum activity at pH-7.0. The activity was decreased at pH 4-6 and pH 10. The 80 percent enzyme activity exhibits at both pH 6 and 8, it retains less than 50 percent activity at pH 4 (Fig. 3).

Antibacterial activity of purple fluid of *D. auricularia* was observed maximum against *Pseudomonas aeruginosa* and *Staphylococcus aureus* with the zone of inhibition 1.2cm and 1cm respectively. Lowest activity was found against *Salmonella paratyphi*, *Vibrio cholerae* with the zone of inhibition 0.9cm and 0.8cm (Table 1). The standard amylase activity was 125 IU/ml. The purple fluid of *D. auricularia* exhibits only 120.9 IU/mg activity of the commercial enzyme.

**Discussion**

Heparin-like anticoagulant substance is known to occur in many marine mollusks—*Busycon undatum*, *Katelysia opima*, *Anomalacardia brasilina*, *Tivela mactroides* and *Donax strictus*. Though the presence of such anticoagulants in various mollusks has been reported, their exact function in mollusks is still not known. It is not possible for the compound to act as anticoagulant, since mollusks are devoid of coagulant system as found in mammals. Since, heparin is an acid mucopolysaccharide binds tightly to many proteins, which in turn changes several of its physical characteristics, including its isoelectric point.

Even though they have wide range of significance in pharmacological activities like antitumor, antineoplastic, cytostatic, antimicrobial etc., but there was no elaborate study on heparinase and α-glucosidase activity of the purple fluid of the *D. auricularia*.

The results of heparinase activity revealed that the purple fluid of *D. auricularia* against the standard heparin. For instance, the blood did not exhibit any coagulation when heparin was added. However, when purple fluid sample was added at 100 µl, the blood showed coagulation after 7 min. But the blood retains as fluid without addition of sample with heparin for more than 900sec. Similar studies were noticed that the blood retains as fluid for 1350 sec while treating with purple fluid of *B. leachii*.

The purple fluid of the *D. auricularia* did not exhibit any hemolytic activity. Earlier, sea hare *Aplysia* and *Dolabella* species have been reported to contain some other biologically active substances, including antibacterial factors, haemagglutins, cytotoxins and chemical defensive substances. Purple fluid of the *D. auricularia* exhibited the broad antibacterial spectrum against some of the tested pathogens. It shows highest activity against *P. aeruginosa* and minimum activity was observed against *V. cholerae*. The earlier studies also found the similar antibacterial activity of the *Aplysia* sp. and *Dolabella* sp. Also the protein isolated from the ink

---

**Table 1**

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Zone of inhibition (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>1.4</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>0.8</td>
</tr>
<tr>
<td><em>S. paratyphi</em></td>
<td>0.6</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>0.2</td>
</tr>
</tbody>
</table>
of sea hare *Aplysia californica* inhibited the growth of Gram positive and Gram negative bacteria, including marine bacteria (*V. harveyii* and *S. aureus*) and pathogenic bacteria (*S. aureus*, *S. pyogenes*, and *P. aeruginosa*) it also inhibited the growth of yeast and fungi, with different efficacies.  

Molecular weight of the heparinase of the purple fluid was estimated as 37 kDa. Most of the substances which are present in the purple fluid are of low molecular weight. Similarly, aplysianin compound was isolated which was active against tumor cells at the molecular weight of 66 kDa. Also, noticed that some sea hares contain low molecular weight substances with antimicrobial and antitumor activities.  

Heparinase was purified by two step purification method such as ammonium precipitation followed DEAE-cellulose column chromatography. The specific activity of the heparinase from purple fluid was high. Effect of temperature and pH was tested on heparinase activity of the purple fluid. In *D. auricularia* heparinase was found to be more active between 40°-50°C at pH 7. This characterization is needful to the pharmacological industry because when it used as drug related forms since temperature and pH were play very important role. But there is no such study available on the effect of these parameters on heparinase enzyme.  

Amylase inhibitors have received considerable attention in recent year because of their importance for the determination of amylase isoenzyme activities, the purification of amylase or hyperlipemia. In the present study, the standard amylase was found to be 125 IU/ml but the amylase with the addition of purple fluid of *D. auricularia* exhibits only 120.9 IU/ml activity.

**Conclusion**  
*D. auricularia* species discharges purple fluid when it is disturbed. The fluid contains bioactive compounds which acts against potential pathogens since the defensive mechanisms of the sea hares are well developed. Hence, further attempts on this purple fluid may reveal bioactive compounds present naturally.

**Acknowledgement**  
Authors thank authorities of Annamalai University, Tamilnadu, India for necessary facilities. We are also thankful to Ministry of Earth and Sciences, Govt. of India for financial assistance under the “Drugs from the Sea Program”.

**References**  


