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RESEARCH ARTICLE

Glycosaminoglycans (GAG) from Backwater Clam *Marcia opima* (Gmelin)

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ABSTRACT

Heparin was isolated from whole clam tissue *Marcia opima*. Purification of multimilligram quantities of heparin from this tissue sample permitted a thorough examination of its anticoagulant activity characterization, which was partially purified by fractionation by anion exchange chromatography using DEAE cellulose column. The isolated crude and partially purified fractionated sample showed metachromatic shift while using azure-A. The sample also exhibited prominent anticoagulant activity. Mobility was analyzed by two different buffer systems using agarose gel electrophoresis. The fractionated molluscan GAG was also found to have similar peaks as that of standard heparin when assessed by the FT-IR spectrum. The bivalve GAG was subjected to fractionation for further purification and its chemical components were analyzed. The fractionated clam heparin also showed substantial *in-vitro* anticoagulant activity compared to that of commercial heparins.

Keywords: *Glycosaminoglycans, agarose gel, FT-IR*

Glycosaminoglycans (GAGs) are a family of linear anionic polysaccharides that are typically isolated as proteoglycans linked to a protein core. Heparin and heparan sulfate have been the subject of intensive study because of their well-recognized ability to bind many different proteins that regulated a varity of important biological processes. Heparin and heparan sulfate GAGs are comprised of alternating 1-4 linked glycosamine and uronic acid residuces. Heparin, a sulfated glycosaminoglycan present in several mammalian and other vertebrates tissues has been widely used in medicine for more than 75 years because of its anticoagulant, antithrombotic and antilipaemic activites. The heparins like compounds are also present in some invertebrates. Still date the heparin is prepared from terrestrial mammalian tissues for commercial use [1, 2, 3, 4, 5]. There is an increased demand, but the resource are depleting. So, it is the right time to look for alternative sources for heparin production especially from marine organisms. Marine molluscs are promising source for heparin production [6, 7, 8].

Heparin had been commonly used for prevention and treatment of venous thrombosis [9]. Normally the heparin administered intravenously is of high molecular weight ranging between 15,000 to 30,000 Da but, this is not helpful in treating the patients with deep vein thrombosis (DVT), pulmonary embolism (PE) and coronary thrombolysis. Since molecular weight is one of the limitations that would prevent the drug action against specific target sites, presently low molecular weight heparin (LMWH) has been used for its higher potency in treating DVT, PE and coronary thrombolysis [10]. It was also found quite successful in administrating LMWH through subcutaneous mode to treat DVT [11]. Another added advantage is that LMWH can be given orally due to its greater bioavailability [12].

Present paper describes the extraction, partial purification and validation of heparin isolated from *Marcia opima* and its mobility was analyzed by agarose gel electrophoresis using two different buffer systems.

MATERIALS AND METHODS

Heparin sodium salts (140 USP units/mg), Azure-A (CL No 318), DEAE cellulose, CPC (Cyteyl pyridinium chloride) and Agarose (standard low EEO). All other reagents used were analytical grade.

Extraction of heparin

The clam was collected from the mouth of the Vellar estuary, Tamilnadu, India (11^o 29" N; 79^o 47" E) by hand picking. The shells were removed, 1 kg of the whole meat was ground with one lit of 0.9 M NaCl in a

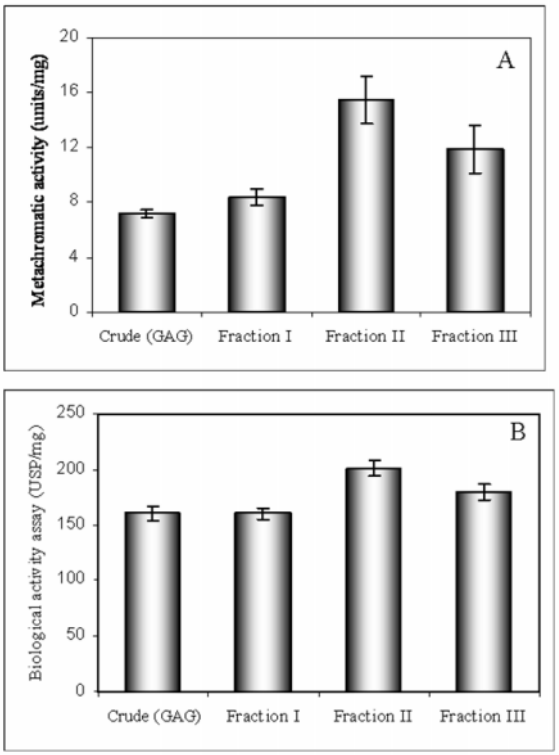

Fig. 1 A. Metachromatic activity assay and, B. Biological anticoagulant assay

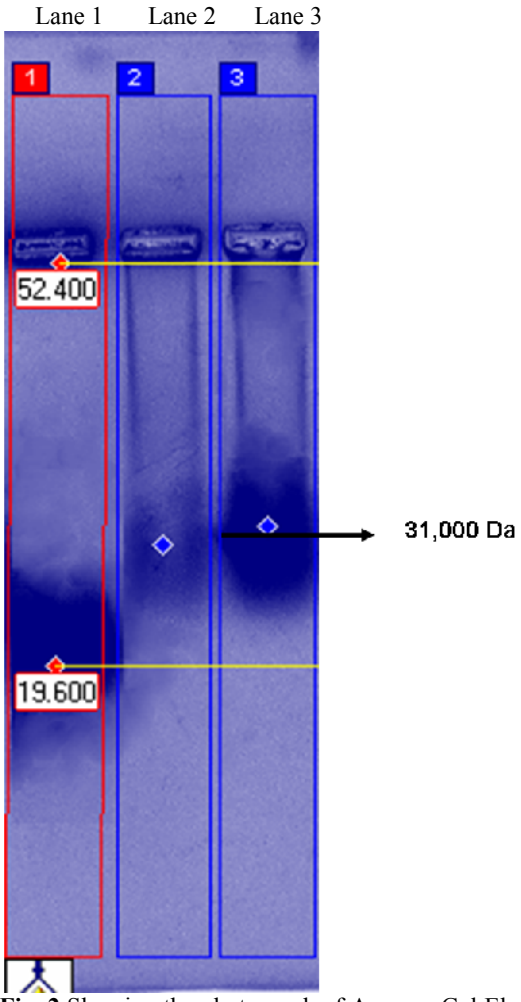

Fig. 2 Showing the photograph of Agarose Gel Electrophoresis
Lane 1 - Standard heparin sodium salt
Lane 2 -10 µl of Isolated GAG sample
Lane 3 -30 µl of Isolated GAG sample

blender and 0.4 M sodium sulfate (3.5 l/kg of tissue) was added to it. The whole content was incubated in a water bath at 55°C for 1 h 30 min and was maintained at pH 11.5 using 10% NaOH solution. Then the pH of the solution was reduced to 7.7 using aluminium sulfate and was heated to 95°C for one hour. After the above process the solution was allowed to cool over night, the samples were centrifuged and the supernantant was collected. Cyteyl pyridinium chloride (3 % of 0.8 M NaCl) was added to the supernatant until a complete white precipitation of the complex appeared at 40°C incubation for a period of 24 hrs. The sample was subjected to centrifugation at 3000 rpm for 90 min and thus the crude heparin complex was obtained. The precipitate was redissolved in 2 M NaCl at 40°C to dissociate CPC salt from heparin and 2 volumes of 95 % methanol was used to precipitate the crude heparin. Hexoamine and uronic acid was determined by using acid hydrolysis (4 M Hcl 100°C for 6 hours) by Tsuji (1970).

Heparin fractionation

Standard heparin sodium salt and isolated molluscan heparin were passed through a DEAE cellulose column (1.5 x 50 cm), with a flow rate of 12 mL/h. The three different molar solutions of NaCl (1.0 M, 2.0 M and 3.0 M) were used for fractionating the sample. The samples were collected for every hour from the column. All the fractionated samples were dialyzed exhaustively against distilled water using dialysis membarane-50 and the samples were lyophilized and subjected to the following assays.

Uronic acid, Hexosamine and other elements estimation

Uronic acid was determined colorimetrically by the Bitter and Muir (1962) method. 5 ml of sulphuric acid reagent (0.025 M sodium tetraborate in concentrated sulphuric acid) was taken in each tube stored at 4°C and 1 mL of fractionated sample was carefully layered over the acid. The tubes were shaken gently and then vigorously with constant cooling. After cooling, the tubes were heated for 10 min in a boiling water bath and again

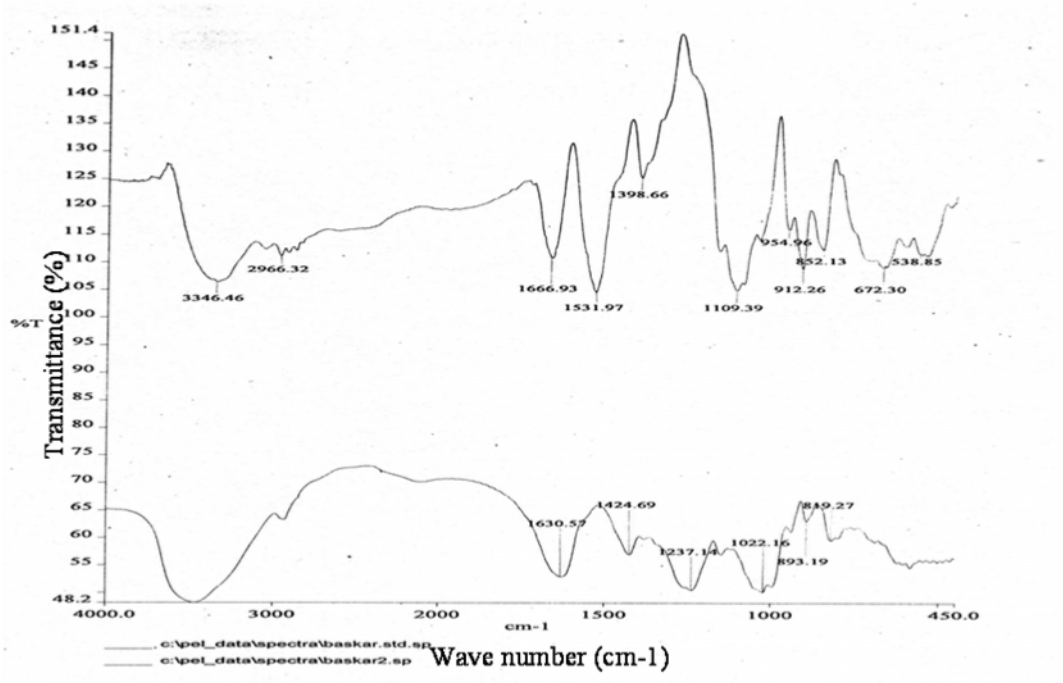

Fig. 3 Infrared spectra of fractionated –III GAG compared with Standard heparin
A - Standard heparin sodium salt
B - Fractionated (III) GAG from *Marcia opima*

cooled to room temperature. 0.2 mL of carbozole (0.125% in ethanol) reagent was added and heated in a boiling water bath for 15 min. The optical density was measured at 530 nm. Glucuronolactone was used as a standard (4-40 µg/mL) and the solution was saturated with benzoic acid.

Hexosamine content was determined colorimetrically after hydrolysis with 2 M HCl for 2 h at 100°C using the method of Tsuji (1970). The elements carbon, hydrogen, nitrogen and sulphur in tested samples were analyzed using micro-elemental analyzer Carb EPBA mode 1106. The amount of sodium was measured by the Flamephotometer systronics MK III.

Metachromatic activity assay

Lyophilized heparin samples were dissolved in double distilled water and aliquots (5 µl) were removed over the three different molar eluted salt solutions and were mixed with 10 ml of 0.02 g/L of azure-A dye solution separately and the absorbance was measured at 620 nm within 30 min [15].

Biological anticoagulant assay

Crude and fractionated heparin sample were determined by comparing the concentration necessary to prevent the clotting of sheep plasma using USP (The United State Pharmacopoeia-1995) method [16].

Agarose gel electrophoresis

The crude isolated GAG samples (10 µl and 30 µl) were loaded on an Agarose gel (0.5% w/v) to perform electrophoresis. This experiment was helped to find out the nature of the isolated product. The presences of GAGs were analyzed by using two different buffer systems such as 1,3-Diaminopropane buffer (pH 9) and Acetate buffer (pH 3.6) for one hour at 120 V. After the electrophoresis, the gel was fixed in 0.1% N-cetyl-N,N,N-trimethylammonium bromide for 12 h. The gel was dried and stained with 0.1% toluidine blue solution (acetic acid, ethanol and water in the ratio of 0.1:5:5 v/v). After staining, the gel was washed in destaining solution (acetic acid, ethanol and water in the ratio 0.1:5:5 v/v) and the result was documented.

FT-IR Spectroscopy

IR spectroscopy of solid sample was tested using Perkin-Elmer-FT-IR instrument (USA). Fraction-III sample was mixed with 500 µg of dried potassium bromide and then compressed to prepare a salt-disc (3 mm diameter). The disc was analyzed from 450 to 4000 cm^{-1}.

RESULTS AND DISCUSSION

Heparin and heparin like compounds, which are present in some invertebrate molluscs, showed high anticoagulant activity and share most of the structural properties with mammalian heparins. Similarly, heparin has been prepared from a number of different species including humans [17], clams [6, 1], shrimp [18] and seaweeds [19, 20].

The fractionated heparin samples were analyzed for metachromatic property against azure-A and were compared with the standard heparin sodium salt. The meta-

Table. 1. Heparin activity from *Marcia opima*

Sample	Azure-A Metachromatic	activity (units/mg)	Biological activity assay (USP* units/mg)
Crude GAG	7.14		160
Fraction I (1.0 M NaCl)	8.25		160
Fraction II (2.0 M NaCl)	15.37		200
Fraction III (3.0 M NaCl)	11.88		180

*USP- United State Pharmacopoeia

Table. 2. Biochemical evaluation of standard heparin, crude and fraction II samples

Elemental analysis (%)	Crude GAG	Fraction II	Standard Heparin
Hexosamine	23.6	29.8	28.8
Uronic acid	23.9	28.4	24.8
Carbon	28.3	26.9	26.0
Hydrogen	4.7	3.7	3.4
Sulphur	7.3	10.2	11.6
Sodium	8.2	11.1	12.0
Nitrogen	1.8	2.4	2.6

chromatic activity of the crude extract was 7.14 units/mg (Table. 1), and the fraction II was showing the proper metachromatic shift. (Fig. 1). These activities were found to be similar to that of scup viscera and turkey [21, 4].

Elementary microchemical analysis of the standard heparin and Fraction II showed progressive decrease in the three elements namely Na, S and N by about 9%, while considering from the standard to Fraction II, but carbon and hydrogen levels have been increased, however with in the range reported by [22] Kavanagh and Jaques (1973). The hexosamine and uronic acid values of the crude, fraction II and standard heparin are with in the range of commercial heparin as reported by earlier workers for heparins isolated from a variety of mammalian tissues (Table. 2) [22, 23, 24].

The *M. opima* showed anticoagulant activity of 160 USP units/mg (Table.1). Dietrich et al., (1989) showed the anticoagulant activity of heparin from two species of molluscs *Donax striatus* and *Tivela mactriodes* as 180 units/mg and 220 units/mg respectively. The process of fractionation helped to enhance the activity of the crude heparin by removal unwanted salt contaminants [6]. Figure 1 showed that the fraction II had a maximum activity of 200 USP units/mg. Such finding was also recorded by Mariana and Barbara (1999).

Electrophoresis mobility of the sample on agarose gel was visualized with toluidine blue staining. There was a variation in mobilities among the various isolated products. The toluidine blue was only bound to the sulfated polysaccharide, but was not found to bind with other compounds [2]. The investigation showed that the isolated product has a molecular size of 31,000 (Fig. 2 lane 2 and 3). The electrophoretic migration of sulfated polysaccharides using two different pH buffer systems in 1, 3-diaminopropane (pH 9) and acetate buffer (pH 3.6) depend on the structure of the polysaccharide, which forms a complex with the diamino buffer [6]. Thus the difference in electrophoretic mobility of the various GAGs is a first indication of distinctive structure of these polysaccharides. The high concentration (30 µl) samples were separated as crispy band (Fig. 2, lane 3). These molecules were compared with standard heparin sodium salt (Fig. 2, lane 1). Dietrich et al., (1989) and Mariana and Barbara (1999) [25] showed that the sulfated polysaccharide had different electrophoretic mobility for different buffer system, depending on the structure of the polysaccharide.

IR spectroscopy of fractionated heparin showed the presence of hydrogen bonds along with acid or amine salt, ionized compound and aliphatic tertiary amine salt. The absorption was read between 450 and 4000 cm^{-1} and the absorption ratios were observed at 1237 cm^{-1} and 819–893 cm^{-1} which attributed to the stretching of S=O bond, C–O–S bonds [26]. In addition the intensity of bands at 1631 cm^{-1} and 1425 cm^{-1} were due to the stretching or deformation vibration of C–O–H bands and were suggesting the presence of combined carboxylate with amine and sulphate [27]. The standard heparin also showed closer peak intensities of similar configuration (Fig. 3). Thus the present investigation showed that *M. opima* could be a possible of heparin in a future.

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