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Histological and Analytical Evaluation of Glycosaminoglycan from the Clam *Katelysia opima*

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Abstract: Glycosaminoglycan (GAG) of heparin family was localized in the tissues of bivalve *Katelysia opima*. Different histological staining procedures like alcian blue, aldehyde fuchsin and periodic acid-Schiff's reagent (PAS) were followed to test the presence of GAG in various tissues of this bivalve mollusk. The alcian blue with two different pH and combination of alcian blue-aldehyde fuchsin staining solutions indicated the presence of sulfated polysaccharide. The GAG was found in the tissues like gill, palp, foot, mantle and adductor muscle in an order of decreasing pattern. Crude GAG was isolated from these tissues by cetyl pyridinium chloride (CPC) precipitation method and the obtained product was tested for its anticoagulant property along with standard heparin. The isolated GAG was also compared with standard heparin through FT-IR to find its relevant functional groups. The bivalve GAG was subjected to fractionation for further purification and its chemical components were analyzed. The subsequent comparison with the commercial heparin showed that *K. opima* could be a potent source of heparin for commercial use.

Key words: *Katelysia opima*, glycosaminoglycans, localization, section staining, elements analysis, FT-IR

INTRODUCTION

The biological functions of proteoglycans primarily result from the structurally dominant GAG chains emanating from the protein core of the molecule (Hardingham and Fosang, 1992; Jia *et al*., 2001). Proteoglycans (PGs) are heterogeneous macromolecules consisting of a core-protein connected via a tetrasaccharide to linear polysaccharide glycosaminoglycan (GAG) chains. GAGs consist of disaccharide repeat unit (usnally hexouronic acid and N-acetylhexosamine), which can be modified by N-sulfation epimerization of glucuronic to iduronic acid and O-sulfation at several different sites (Andre *et al*., 2006). As a result, GAGs can have extremely variable structures and it is believed that structural differences are responsible for highly specific interactions of GAGs with other macromolecules (Lindahl *et al*., 1998; Iozzo, 1998). Heparan sulfate, which is sulfated less than heparin, is nearly ubiquitous in the animal kingdom and is often a cell surface marker, but heparin is found ouly in the phyla Chordata, Mollusca and Arthropoda (Nader *et al*., 1999).

Their strategic location and highly charged nature make them important biological players in cell-cell and cell-matrix interactions that take place during normal and pathological events, related to the cell recognition, adhesion, migration and growth (Gallagher, 1989; Lindahl *et al*., 1998; Iozzo, 1998; Conrad, 1998; Perrimon and Burnfield, 2000; Kresse and Schonnher, 2001; Turnbull *et al*., 2001; Silva, 2002). Mollelo *et al*. (1963) in a light microscopic study of the developing antler of mule deer (*Odocoileus hemoinus*) suggested GAGs are important molecules involved in the calcification of chondrocytes. A large number of animal species contain GAGs and the mollusks are particularly a rich source of these polysaccharides (Hovingh and Linker, 1982, 1993; Chatziioannidis *et al*., 1999; Lopes-Lima *et al*., 2005). A compound named mactin had been isolated from *Mactrus pussula* and

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Cyprinia islandica (Frommahagen *et al*., 1953). The reported chemical analyses and anticoagulant activities of this glycosaminoglycan were undistinguishable from those of heparin. Among several invertebrates the presence of heparin-like compounds were most probably observed in certain mollusks (Dietrich *et al*., 1985; Pejiler *et al*., 1987; Yeong *et al*., 1996).

Bovine lung and porcine intestine tissues are currently the ouly raw materials used to prepare commercial heparin (Linhardt and Gunay, 1999). Since the terrestrial sources are limited, it becomes necessary to look for alternate sources of heparin especially from marine sources to meet the growing demand. Based on the fact, the present investigation was carried out to analyze the distribution pattern of GAG in the tissues of *K. opima*, which was later used as a source for the GAG of biological significance. This was followed by extraction, fractionation and validation of the property along with commercial heparin.

MATERIALS AND METHODS

Preparation of Tissue Sections Embedded in Paraffin Wax

The clam was collected from the mouth of the Vellar estuary, Tamilnadu, India (11°29 N; 79°47 E) by hand picking. The histological survey of the organs of *K. opima* was performed to determine the cellular location of sulfated polysaccharides. The shells were opened and the tissues were amputated gently from the gill, palp, adductor muscle, foot and mantle edge were dissected from *K. opima*. The tissues were fixed overnight in 10% phosphate buffered neutral formalin (Bullock *et al*., 1976). Sections with thickness of 6 μm were cut on a microtome and layered over glass slides after embedding in paraffin wax (Koshiishi *et al*., 1999). Serial sections of all these organs were stained with different histological staining solutions such as periodic acid-Schiff's (PAS) reagent, alcian blue (pH 2.5 and 1) and aldehyde fuchsin. The combination of staining solutions such as aldehyde fuchsin/alcian blue (pH 2.5), aldehyde fuchsin/alcian blue (pH 1) and PAS/alcian blue with pH 1 or pH 2.5 were helpful in differentiating the type of polysaccharide occurring in the tissues of *K. opima* (Spicer, 1960; Lev and Spicer, 1964; Bancroff and Cook, 1994). Alcian blue, PAS and aldehyde fuchsin staining can produce staining patterns that allow differentiation between cells containing sulfated glycosaminoglycans such as heparin and biogenic amines.

The above stained sections were examined and characterized under bright field microscopy. The whole of the histochemistry of the polysaccharides, mucopolysaccharides and mucoproteins is bound up with the periodic acid-Schiff reaction (Jockson and Hudson, 1937).

Isolation and Fractionation of the Glycosaminoglycans

To dry defatted *K. opima* bivalve sample, 0.4 M sodium sulfate (3.5 L kg^{-1} of tissue) was added. 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. Cetyl pyridinium chloride (CPC- 3% in 0.8 M NaCl) was added to the supernatant until a complete white precipitation of the complex appeared after incubation at 40°C for a period of 24 h. The sample was subjected to centrifugation at 3000 rpm for 90 min and thus the crude heparin complex was obtained. The precipitate was dissolved 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.

The isolated crude molluscan heparins were partially purified by Minitan Ultra-filtration System. Two types of filter packets, one with a molecular retention rate of 30,000 (PTTK OMT 05) and another with a rate of 10,000 (PTGC OMT 05) were used separately for filtration. The purified sample was dissolved in distilled water and drawn into the Minitan holder by a peristaltic pump at the rate of 100 mL min^{-1}. The filtrate and the active compound in these solutions were precipitated and separated by adding two volumes of methanol. Precipitated samples were dissolved in double distilled water and the samples were lyophilized and subjected to the activity check by metachromatic and biological activity assays.

Analytical Methods

Uronic acid was determined colorimetrically by the Bitter and Muir (1962) method. Five milliliter 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 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 um. Glucuronolactone was used as a standard (4-40 μg mL^{-1}) 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 *et al.* (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 crude and two different fraction samples were mixed with 10 mL of 0.02 g L^{-1} of azure-A dye solution separately and the absorbance was measured at 620 nm within 30 min (Grant *et al.*, 1984).

Biological Anticoagulant Activity Assay

The anticoagulant activities of crude and fractionated heparin samples were determined by comparing with the concentration necessary to prevent the clotting of sheep plasma using USP (United State Pharmacopoeia) method.

FT-IR Spectrophotometer Analysis

IR spectroscopies of solid samples were tested using Perkin-Elmer-FT-IR instrument by Toida *et al.* (1999), which helped to analyze different amino and hydroxyl group of these sample molecules. Standard and Fraction II sample (100 μg) were mixed with 500 μg of dried potassium bromide separately and then compressed to prepare a salt-disc (3 mm diameter). These discs were subjected to IR spectral analysis.

High Performance Size Exclusion Chromatography (HPLC)

The Fraction II sample was analyzed by high performance size exclusion chromatography on a TSK column (G3000 SW. XL). Fractionated samples were prepared at a concentration of 10 mg mL^{-1} of 0.3 M sodium sulfate (mobile phase) thoroughly mixed and centrifuged for 30 min at 3000 rpm. Then 30 μL of supernatant was injected into the HPLC system (LKB-Pharmacia). The sample was run at steady state flow of 0.5 mL of mobile phase per minute and at an absorbance of 232 nm of UV detector (Rice *et al.*, 1985).

RESULTS AND DISCUSSION

Paraffin wax sections of 6 μm thickness of the tissue samples was prepared using standard histochemical techniques. GAGs could be stained by a number of dyes, such as alcian blue, aldehyde fuchsin and PAS (Cowman *et al.*, 1984; Rice *et al.*, 1987). Heparin is a highly sulfated polysaccharide, specific stains such as alcian blue of pH 1 and 2.5 and aldehyde fuchsin were used to identify sulfated polysaccharides in the tissues of mantle edge, palp, gill, foot and adductor muscle of the clam. The intensity of sulfated polysaccharides present in tissues is in the order of gill>palp>foot>mantle>adductor (Fig. 1). Ulrich *et al.* (2001) showed the presence of heparin in the labial palp, ctenidia, siphons, pallinm, intestine and foot of northern quahog clam

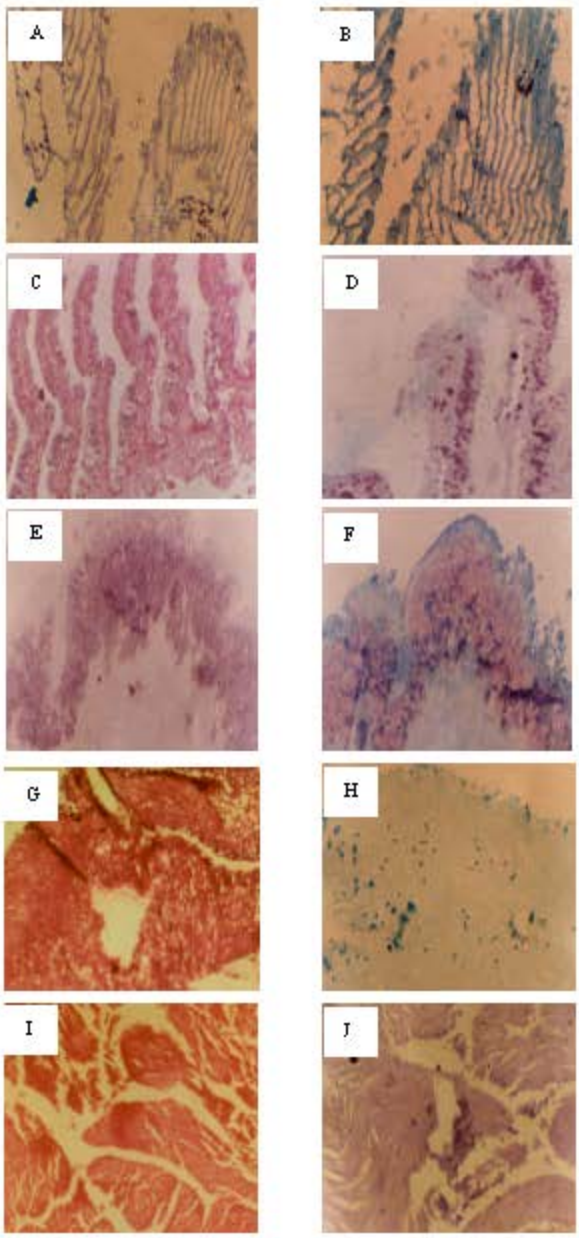

Fig. 1: Sections of the bivalve *Katelysia opima* prepared in paraffin wax; (A and B) sections of gill stained with alcian blue pH 1.0 (x 150) and aldehyde fuchsin/alcian blue pH 2.5 (x 150); (C and D) sections of palp stained with PAS (x 100) and aldehyde fuchsin/alcian blue pH 2.5 (x 150); (E and F) sections of foot stained with aldehyde fuchsin (x 100) and aldehyde fuchsin/alcian blue pH 2.5 (x 100); (G and H) sections of mantle stained with PAS (x100) and alcian blue pH 1.0 (x 100); (I and J) sections of adductor muscle stained with PAS (x 100) and aldehyde fuchsin (x 100)

Table 1: Histochemical reaction of the tissues of *Katelysia opima*

	Colour intensity					
Treatments	Mantle	Palp	Foot	Gill	Adductor muscle	Reaction
PAS	3	2	3	2	3	Red to purple
Alcian blue (pH 2.5)	2	2	3	2	2	Blue
Alcian blue (pH 1.0)	2	3	3	3	3	Strongly blue
Aldehyde fuchsin	3	2	3	3	3	Strongly purple
Alcian blue	2 P*	1 P	3 P	2 P	2 P	Most cells are bluish
(pH 2.5)/PAS	1 B*	1 B	2 B	1 B	2 B	purple
Alcian blue	1 P	2 P	2 P	1 P	2 P	Cells are bluish
(pH 1.0)/PAS	2 B	2 B	3 B	3 B	1 B	purple
Aldehyde fuchsin/	3 P	3 P	3 P	3 P	1 P	Most cells are purple
Alcian blue (pH 2.5)	1 B	1 B	3 B	1 B	2 B	some cells are blue

*B: Blue, *P: Purple

Table 2: Analytical values of commercial heparin and tested samples

	Samples		
Treatments	Standard heparin	Crude	Fraction II
Azure-A metachromatic activity (units mg^{-1})	20.00	7.14	15.37
Biological activity assay (USP* units mg^{-1})	140.00	70.00	120.00
Elemental analysis (%)			
Carbon	26.00	28.30	26.90
Hydrogen	3.40	4.70	3.70
Nitrogen	2.60	1.80	2.40
Sulphur	11.60	7.30	10.20
Sodium	12.00	8.20	11.10
Hexosamine (%)	28.80	23.60	29.80
Uronic acid (%)	24.80	23.90	28.40

*USP: United State Pharmacopoeia

Mercenaria mercenaria by histological localization. The tissues of African snail *Achatina fulica* highly stained with alcian blue and PAS indicated the presence of sulfated polysaccharide (Jia *et al*., 2001). The results obtained in the present study indicate that the GAGs in the *K. opima* clam are primarily located inside granules (Fig. 1) (Table 1). Cells in mammals usually secret proteoglycans into the extracelllular environment on exposure to outer stress. Cells with storage granules concentrate proteoglycans along with other secretary products. These proteoglycans typically contain highly sulfated forms of GAGs including chondroitin sulfate, heparin sulfate and heparin (Jia *et al*., 2001).

The crude GAG showed 7.14 units mg^{-1} of azure-A metachromatic activity and 70 USP units mg^{-1} of biological activity (Table 2). Among the marine mollusks, *Spisula solidissima* and *Cyprina islandica* showed enhanced anticoagulant activity ranging from 70-120 USP units mg^{-1} (Frommhagen *et al*., 1953). Burson *et al*. (1956) showed the activity ranging from 130-150 USP units mg^{-1} in these species. Dietrich *et al*. (1985) showed the anticoagulant activity of heparin from two species of mollusks *Donnax striatus* and *Tivela mactriodes* as 180 units mg^{-1} and 220 units mg^{-1}, respectively. The process of fractionation helped to enhance the activity of the crude heparin by removal of unwanted salt contaminants (Pejiler *et al*., 1987). Table 2 showed that the Fraction II had a maximnm activity of 120 USP units mg^{-1} such findings were also recorded by Pereira and Mulloy (1999). According to Barlow *et al*. (1964) the difference in the activity of heparin is a function of the amount of non-anticoagulant impurity carried over to the isolated product.

Two fractions were obtained by ultrafiltration method. Fraction I had molecular weight above 30,000 Da and Fraction II below 30,000 Da. The fractionation process helped to improve the activity of the isolated or unfractionated heparin (UFH). Since the maximum activity was exhibited by Fraction II (120 USP units mg^{-1}; Table 2), the other fraction was not used for further analyses. This Fraction II was compared with the standard heparin by IR spectrophotometer. Fraction II showed absorption bands for the carboxylic group at 1615 cm^{-1} and for acetyl group at 1375 cm^{-1}, which was similar to that of standard heparin (Fig. 2). The elution profile of the high performance size exclusion

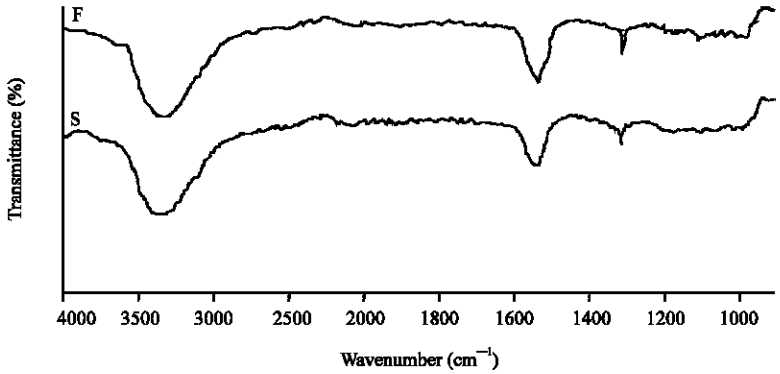

Fig. 2: Infrared spectra of Fraction II and standard heparin; S: Standard heparin sodium salt F: Fraction II heparin from *Katelysia opima*

chromatography is obtained for the Fraction II. The result was compared with standard heparin and found to show similar retention time with a single peak. Subramanian *et al*. (1997) identified the heparin activity from seaweeds such as *Acanthophora spicifera* and *Hypnea valentiae* using HPLC with refractive index detector and found two peaks for both the samples. 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 Kavanagh and Jaques (1973). The values of elemental composition of heparin-like anticoagulant derived from *K. opima* in the present study closely matches with the values of commercial heparin. There are minor variations in hexosamine and uronic acid contents in the samples (Table 2). 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 (Jaques *et al*., 1967; Kavanagh and Jaques, 1973).

The decrease in percentage of Na, S and N from standard to Fraction II would cause the progressive decrease in activity from 140-120 units mg^{-1} (Table 2). Scott and Clayton (1953) obtained three fractions with sulphur content 9.2, 6.3 and 1.2% and found that the fraction with sulphur content 9.2% showed highest anticoagulant activity. Brimacombe and Webber (1964) supported that the degree of sulphation and molecular size contribute equally to the anti-coagulant activity. In *K. opima* heparin activity seems to require a large composition of N, S and Na which takes place when the molecular weight decreases. The molluscan heparin activity is similar to that of commercial heparin, which indicates that *K. opima* is also relatively a potent source of heparin. The hexosamine and uronic acid values of Fraction II and standard heparin in the present study were with in the range of values of commercial heparin (Jaques *et al*., 1967; Kavanagh and Jaques, 1973; Achilleas *et al*., 1999). The analytical values clearly indicate that Fraction II is similar to the commercial heparin. Their activity differs according to molecular size and elemental composition (Wladimir *et al*., 2000). According to Lang (1985) the activity doubles if the polydispersity in the molecular size is decreased.

In conclusion, this study demonstrates that the major glycoconjugate of clam organs is a GAG with a novel repeating unit composed of disaccharide sequences. Jia *et al*. (2001) reported that mucus from African snail *Achatina fulica* contains a heparin like compound. Such background secretion could be a means of introducing proteases to the mucus coat to facilitate digestion or provide a more substantial barrier between an organism and a pathogen. Immunohistochemical technique was helpful in pinpointing heparin to a specific cellular location.

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