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# Isolation and Characterization of Low Molecular Weight Glycosaminoglycans from Marine Mollusc *Amussium pleuronectus* (Linne) using Chromatography

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**Abstract** The glycosaminoglycan (GAG) heparin is a polyanionic sulfated polysaccharide most recognized for its anticoagulant activity. In the present study, the GAGs were extracted from bivalve mollusc *Amussium pleuronectus*. The crude GAGs were fractionated by ion-exchange (DEAE-cellulose and Amberlite IRA-900 & 120) chromatography. The recovered active fractions (as determined by metachromatic assay) were confirmed by agarose gel electrophoresis and the active fractions were purified in Sephadex G-100 column. Fractionated and purified GAG molecular weight was determined through gradient polyacrylamide gel electrophoresis. The structural characterization of low molecular weight GAG was analyzed by Fourier transform infrared spectroscopy. The activated partial thromboplastin time of purified GAG is 95 IU/mg and has molecular weight 6,500–7,500 Da. The disaccharide compositional analysis on the GAG sample was sulfated like porcine intestinal mucosal heparan sulfate, and it contains equivalent amount of uronic acid and hexosamine. The results of this study suggest that the GAG from *A. pleuronectus* could be an alternative source of heparin.

**Keywords** Glycosaminoglycans · DEAE-cellulose · Amberlite IRA-900&120 · Sephadex G-100 · APTT · *Amussium pleuronectus*

## Introduction

Heparin and low molecular weight heparin (LMWH) are heterogeneous glycosaminoglycans (GAGs) prescribed as anticoagulants [1]. Heparin has molecular mass range between 3,000 and 40,000, which is biosynthesized as a proteoglycan consisting of a small core protein to which polysaccharide side chains are attached. It is composed of repeating disaccharide units of uronic acid: α-L-iduronic (or) β-D-glucuronic acid and β-D-glucosamine residues linked by α-(1→4) bonds [2]. Only bovine lung or porcine intestine tissues are currently used as raw materials to prepare commercial, pharmaceutical heparins. But the appearance of bovine spongiform encephalopathy, 'mad cow disease', and its apparent link to the similar prion-based

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Creutzfeldt–Jakob disease in humans has limited the use of bovine heparin. Moreover, it is not easy to distinguish bovine and porcine heparins, making it difficult to ensure the species source of heparin [3]. Porcine heparin also has problems with its use, associated with religious restrictions among members of the Muslim and Jewish faiths [4]. Heparin exhibits anticoagulant activity primarily from its binding to the serine protease inhibitor, antithrombin. Unfractionated heparin (UFH) is an effective and relatively safe antithrombotic agent. However, it has a limitation that has prompted the search for new antiscoagulants with improved efficacy and safety. Among the newer anticoagulants, LMWHs are the most evaluated. The advantages of LMWH over UFH include its greater bioavailability, subcutaneous administration, more specific mechanism of action [5], low risk of bleeding [6], reduced or absent effect on bones during long-term use [7], and the possibility of outpatient treatment [8]. Since molecular weight affects heparin biological activity, accurate determination of the molecular weight of heparin becomes important. However, classical techniques for molecular weight determination are problematic for low molecular weight polyelectrolytes, which would permeate the membranes used for membrane osmometry or not soluble in solvents appropriate for vapor phase osmometry and are weak scatterers of light [9]. Further LMWHs are of different chain length, molecular weight distribution, and different physiochemical characteristics that result from their diverse methods of preparation, which make them non-interchangeable. The variations in molecular composition and pharmacological properties of LMWHs are reflected in clinical trails that reported differences in clinical efficacy and safety [10].

Marine organisms are a rich source of structurally novel and biologically active metabolites. So far, many chemically unique compounds of marine origin with different biological activities have been isolated and a number of them are investigated and/or being developed as new pharmaceuticals [11]. The marine molluscs show extensive species diversity and their byproducts have received much attention from the beginning of the 20th century. Among the molluscs, some have pronounced pharmacological activities or other properties useful in the biomedical area. It is surprising that some of these pharmacological activities are attributed to the presence of polysaccharides, particularly those that are sulfated [12]. Hence, an attempt has been made to isolate and characterize the GAG from the marine mollusc *Amussium pleuronectus* using chromatography. The recovered different fractions were confirmed by agarose gel electrophoresis, pooled and purified by gel chromatography using Sephadex G-100 column. The fractionated and purified GAG molecular weight was determined through gradient polyacrylamide gel electrophoresis and the structure of the low molecular weight GAG was determined through Fourier transform infrared (FT-IR) spectroscopy and its APTT assay was also studied.

## Materials and Methods

### Collection of Animals

*A. pleuronectus* were collected from Mudasalodai landing center along Parangipettai (lat. 11° 29′; long. 79° 46′ E) coast (south east coast of India) and brought to the laboratory. The whole tissue was dissected out, ground, defatted, and used for further extraction.

### Extraction of GAGs

The procedure of Holick et al. [13] was adopted for the extraction of GAGs from the collected tissues. The defatted tissues were ground and mixed with 0.4 M $Na_2SO_4$. The

mixture was incubated at 55 °C for 1 h and 30 min (pH 11.5). After incubation, $Al_2(SO_4)_3$ crystals were added to reduce the pH to 7.7 and again incubated to 95 °C for 1 h, and centrifuged (2,500 ×*g*) for 1 h and 30 min at 4 °C. The retentate was recovered, cetylpyridinium chloride (CPC) was added (0.1% *w*/*v*), and the mixture was allowed to stand for 3 h at 4 °C; centrifugation was performed (2,500 ×*g*) at 4 °C for 15 min and the precipitate was recovered and washed two times with 0.1% CPC solution and recovered each time by centrifugation. Finally, the recovered precipitate was dissolved in 2.5 M NaCl and the crude GAG was recovered by methanol (85% *v*/*v*) precipitation. After standing overnight at 4 °C, the crude GAG precipitate was recovered by centrifugation (2,500 ×g) at 4 °C for 15 min.

## Fractionation of GAGs

### *Ion-exchange Chromatography*

The GAGs extracted from the tissue of the clams (1.0 g) were then subjected to ion-exchange column chromatography using DEAE-cellulose. The column was first eluted with distilled water and then eluted with two different molar concentrations of NaCl (1.5–2.0 M); the flow rate of the column was 8 ml/h and the active fractions were collected [14]. Active fractions (tested by metachromatic assay) were pooled, dialyzed (molecular weight cut off between 12,000 and 14,000), freeze dried, and used for molecular weight determination. Then the fractionation of GAGs (1.0 g) was also done using anionic resin on a column of Amberlite IRA-900 ($Cl^-$) [15]. The sample was recovered by stepwise elution with 0.4 M NaCl and 0.8 M NaCl, and the flow rate of the column was 1 ml/min. Active fractions were combined, dialyzed, and freeze dried.

## Conversion of GAGs as Heparin Sodium Salts

The freeze-dried GAGs were converted into heparin sodium salts by using cationic resin (Amberlite IRA-120 in $Na^+$ form, LOBA CHEMIE, India) column [16]. The elute was collected by precipitation with 2.0 vol of acetone; the collected precipitate was dried under vacuum. The recovered white powder of GAG complex was used for further analysis.

## Metachromatic assay

Azure-A assay was performed to estimate the level of sulfo group substitution of the fractionated and purified GAGs. Metachromatic activity is expressed as the negative slope of the standard curve of absorbance at 620 nm vs. heparin concentration (μg/ml) in a dye solution of 0.02 g/l [17].

## Purification of GAGs

### *Gel Chromatography*

GAGs were purified on a 5×90-cm column of Sephadex G-100 (Sigma). The elution rate was approximately 60 ml/h and 15-ml fractions were collected. The activity of all the fractions was tested through metachromatic assay. The active fractions were pooled and extensively dialyzed against distilled water and freeze dried [18].

*Agarose and Gradient Polyacrylamide Gel Electrophoresis*

Agarose gel electrophoresis of the sulfated GAG in the discontinuous buffer barium acetate/1,3-diaminopropane acetate was performed as previously described [19]. The average molecular masses of crude and purified GAGs were analyzed by polyacrylamide linear gradient resolving gels prepared and run as described previously [20]. The average molecular mass of isolated GAGs were determined by comparing with banding ladder of standards prepared from bovine lung polysaccharide marker which was added to identify the bands. Then the bands were observed under gel documentation system and molecular weight was determined through the molecular marker (Sigma).

*FT-IR Analysis*

FT-IR spectroscopy of purified GAG sample of *A. pleuronectus* relied on a Bio-Rad FT-IR-40 model, USA. The sample (10 mg) was mixed with 100 mg of dried KBr and compressed to prepare a salt disc (10 mm diameter) for reading the spectrum further.

Clotting Assays

The assay was carried out using heparan sulfate as standard. Fractionated and purified GAGs were dissolved in saline at various concentrations. Normal human plasma (90 μl) was mixed with 10 μl of a solution of GAG (0–2 mg) and heparin sulfate (0–100 μg). APTT measurements were performed using a kit obtained from Instrumentation Laboratory (Lexington, USA). The plasma (100 μl) containing various concentrations of GAG and heparin sulfate was incubated at 37 °C for 1 min. Bovine cephalin (100 μl) was then added and incubated at 37 °C. After 3 min of incubation, 100 μl of pre-warmed 0.25 M $CaCl_2$ solution was added to the mixture and the clotting time was measured and compared with standard; the activity was expressed as IU/mg.

Carbohydrate Content

*Determination of Uronic Acid*

The uronic acid content was estimated by following the method of Bitter and Muir [21].

*Determination of Hexosamine*

The hexosamine content was determined following the method of Wagner [22].

*Determination of Sulfate Content*

Terho and Haritiala [23] method was used to determine the sulfate content.

## Results and Discussion

Isolation of GAG

The amount of crude GAG was estimated as 17.2 g/kg of tissue in *A. pleuronectus*. After purification using gel chromatography, the yield was found to be 48 mg/kg.

**Table 1** Yield, metachromatic activity, molecular weight, and APTT activity of GAGs extracted from *A. pleuronectus*.

| Yield | | Metachromatic activity | | Column type | Molecular weight (Da) | Source | Activity (IU/mg) |
|---|---|---|---|---|---|---|---|
| Crude (IU/kg) | Purified (IU/kg) | Crude (IU/mg) | Purified (IU/mg) | | | | |
| 28,000 | 172,000 | 28 | 172 | DEAE-cellulose | 30,000 | Human blood | 72 |
| | | | | Amberlite IR-900 | 15,000 | | 84 |
| | | | | Sephadex G-100 | 6,500 | | 95 |

## Metachromatic Assay

The activity and yield of GAG in the crude and purified sample by metachromatic dye method is shown in Table 1.

## Purification of GAGs

In gel chromatography, among the collected fractions, two fractions (fractions III and IV) showed maximum absorbance in 530 nm (Fig. 1), which were pooled, their molecular weight determined, and its structure analyzed by FT-IR spectroscopy. Furthermore, anticoagulant activity of the purified GAG was also determined.

## Electrophoretic Migration and Molecular Weight of GAG

The agarose gel electrophoresis of the mollusc GAG in the discontinuous buffer barium acetate/diaminopropane is shown in Fig. 2a.

The mollusc purified GAG has only one band, which migrates the fastest when compared with mammalian heparin component. This is an indication of its smaller molecular weight. The MW of crude GAGs were found to be 51,000 and 47,000 Da. The MW of fractionated and purified GAGs are depicted in Table 1 and are shown in Fig. 2b.

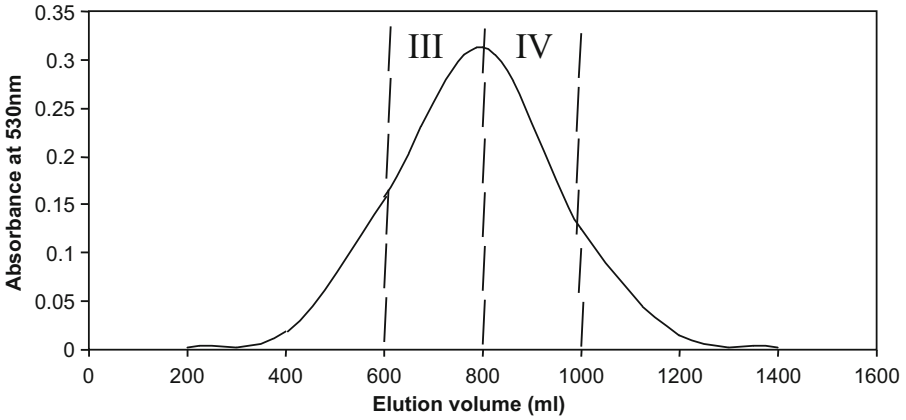



**Fig. 1** Gel chromatography of purified GAGs of *A. pleuronectus*

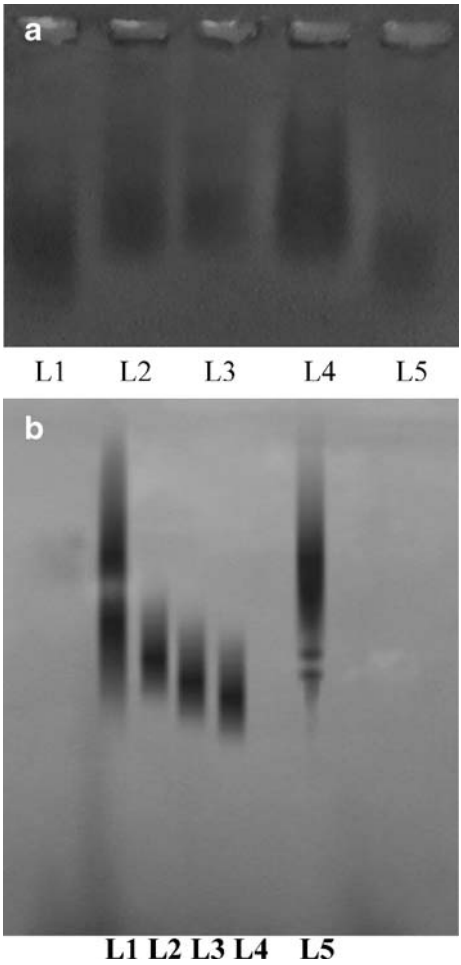



**Fig. 2** **a** Agarose gel electrophoresis of GAG. **b** Gradient polyacrylamide gel electrophoresis

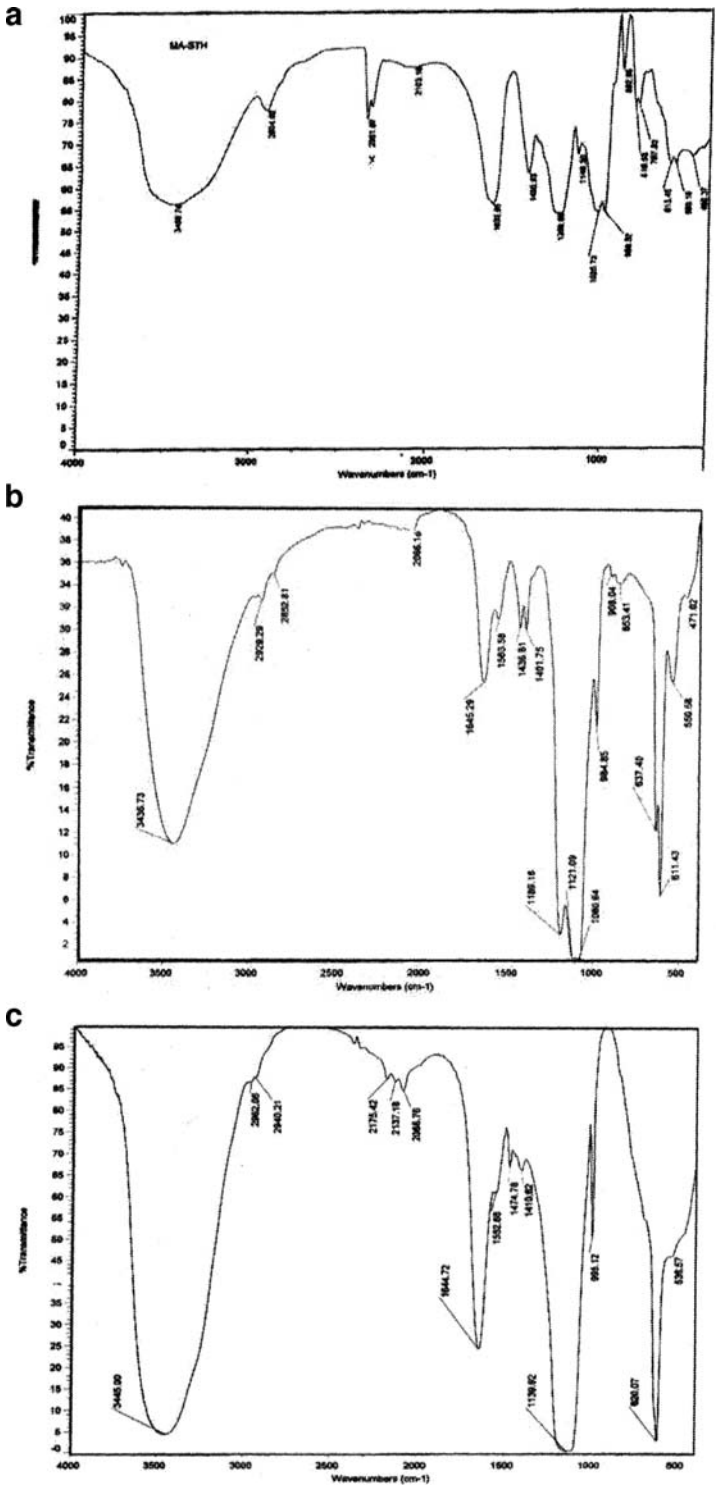
FT-IR Spectrum

FT-IR spectrum of the crude and purified GAGs of *A. pleuronectus* was obtained and compared with the standard (Fig. 3). In the case of FT-IR spectrum of crude sample, the sulfate band started from 1,139.92 $cm^{-1}$ and down to 995.12 $cm^{-1}$. The acetyl amino group is represented by a band in 1,474.78 $cm^{-1}$ and the carboxylic group at 1,552.66 $cm^{-1}$. The most striking characteristic feature of the spectra of GAG is a band at 1,256.56 $cm^{-1}$, which represents the sulfate group as also reported at 1,257.08 $cm^{-1}$.

APTT Assay

The activity of APTT of fractionated and purified GAG from *A. pleuronectus* sample was revealed in Table 1. The activity of APTT of the fractionated sample showed 72 IU/mg in

**Fig. 3** **a** FT-IR spectrum of standard heparan sulfate. **b** FT-IR spectrum of crude GAGs of *A. pleuronectus*. **c** ▶ FT-IR spectrum of purified GAGs of *A. pleuronectus*


a
MA-STH
Wavenumbers (cm-1)
b
%Transmittance
3436.73
2928.29
2852.81
2066.19
1645.29
1563.58
1438.91
1401.75
1180.16
1121.09
1060.64
994.85
959.04
853.41
637.40
611.43
559.58
471.62
Wavenumbers (cm-1)
c
%Transmittance
3445.99
2962.86
2940.21
2175.42
2137.18
2066.76
1644.72
1552.80
1474.78
1418.82
1139.82
995.12
826.07
636.67
Wavenumbers (cm-1)

DEAE-cellulose and 84 IU/mg in Amberlite column, whereas the purified sample showed 95 IU/mg in Sephadex G-100 column. The results of disaccharide profile such as uronic acid, hexosamine, and sulfate contents were found to be 58.2%, 42%, and 25.76% in the purified GAGs, respectively. It is well known that some natural polysaccharides and the derivatives of others possess certain biological activities such as anticoagulation, fibrinolysis, antiviral, and antitumoral effects. In an attempt to improve biological activity, GAG was obtained from *A. pleuronectus*; it was subjected to fractionation and purified by ion-exchange and gel chromatography, respectively and the resulting derivative examined for its anticoagulant and molecular weight determination.

The anticoagulant activity of GAG was tested in vitro by APTT assay and the values compared with those of standard heparin sulfate (~170 IU/mg). APTT is related to the intrinsic coagulation phase in plasma. GAG had effect on the APTT assay, this being expected because sulfate groups are necessary to provide anticoagulant effects and anticoagulant activities of polysaccharides; these are not only dependent on the sulfate content but also on the position of the sulfate groups [24]. In this respect, polysaccharides with lower anticoagulant activity than heparin could exhibit a potent antithrombotic effect with less hemorrhagic risk [25]. The independence of heparin's metachromatic activity on its molecular weight indicate a direct relationship between metachromasia and molecular weight for heparin fragments ranging from 5,000 to 31,000. Metachromatic activity persists with a product as small as tetrasaccharide, while the disaccharide shows no activity at all. Dietrich et al. [19] also found that the disaccharide is without activity, but reported that the tetrasaccharide has only 7% of heparin's activity while the hexosaccharide retained full activity.

The metachromatic activity of GAGs in crude and purified sample of *A. pleuronectus* was estimated to be 28 and 172 IU/mg, respectively, which are still higher than that of *K. opima* 7.142 and 11.883 IU/mg of crude and fractionated sample [26]. The yield from the purified sample of *A. pleuronectus* was found lower than that of tauttog viscera (13,570 IU/kg), scup viscera (15,461 IU/kg), flounder viscera (10,789 IU/kg), and scallop viscera (9,254 IU/kg). LMWHs have a lower reactivity to platelets, which correlates inversely to platelet-rich plasma (PRP). However, LMWH fractions with low and high antithrombotic activity reacted equally with platelets in PRP depleted of antithrombin, suggesting the formation of heparin–antithrombin complexes that protects platelets from aggregation [13].

Currently, UFH is not orally administrated because UFH molecules are not absorbed from the gastrointestinal (GI) tracts presumably because of their size and ionic repulsion from negatively charged epithelial tissue [27]. Long-term UFH administration has shown to increase osteoclastic activity and bone resorption, which may be related to the dosage rather than duration of exposure to UFH [28].

## Conclusion

The APTT activity of purified GAG is 95 IU/mg and has a molecular weight of 6,500–7,500 Da. These results suggest that GAG isolated from *A. pleuronectus* are effective in vitro and should be tested in vivo in a further study.

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