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Short communication

Is isolation and characterization of heparan sulfate from marine scallop *Amussium pleuronectus* (Linne.) an alternative source of heparin?

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ABSTRACT

The present study describes the isolation, purification and structural characterization of heparan sulfate (HS) from marine scallop. Enzymatic depolymerization of the isolated HS, followed by polyacrylamide gradient gel electrophoresis (PAGE), confirmed its presence of HS is by metachromatic activity and agarose gel electrophoresis. The purity of HS is determined by the following treatment with heparin lyases though PAGE. ^{1}H NMR spectroscopy revealed that the marine scallop HS is enriched in both glucuronic and sulfated iduronic acid residues. The result of this analysis clearly demonstrates that the isolated glycosaminoglycan (GAG) is HS. Marine scallop HS has contents of carbon, hydrogen, nitrogen and sulfated disaccharide and an elevated average sulfation level. The activated partial thromboblastin time (APTT) and prothrombin time (PT) of marine scallop HS, are lower than standard bovine intestinal HS. Moreover, marine scallop HS shows equal degree of sulfation to HS standard.

1. Introduction

Heparin, a linear polysaccharide comprised of highly sulfated $1 \rightarrow 4$ linked uronic acid-$(1 \rightarrow 4)$-D-glucosamine repeating disaccharide units, has been used clinically as an anticoagulant for more than half a century. Commercial manufacturing of heparin relies on either porcine (or) bovine intestinal (or) bovine lung tissue as raw material. The appearance of mad cow disease has limited the use of bovine heparin. Moreover, it is not easy to distinguish bovine and porcine heparin, this makes it difficult to ensure the species source of heparin. Non-animal sources of heparin, such as chemically synthesized, enzymatically synthesized, or recombinant heparins, are currently not available for pharmaceutical purposes. These concerns have motivated us to look for alternative, non-mammalian sources of heparin (Saravanan, Vairamani, & Shanmugam, 2010). The HS is of considerable interest because of its complexity and diversity of structure, which appear to be cell-specific and possibly differentiation-specific, together with their propensity for interaction with a wide range of extracellular proteins (Hernaiz, Hyun-Ok, Gunay, Toida, & Linhardt, 2002).

The marine environment is an exceptional reservoir of bioactive natural products, many of which exhibit structural features not found in terrestrial natural products. Research on pharmacological properties of marine natural products has led to the discovery of many potentially active agents considered worthy of clinical application (Saravanan et al., 2009). The marine molluscs show extensive species diversity and their bi-products have received much attention from the beginning of 20th century. Among the molluscs, the pharmacological activities ane other properties are 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 (Saravanan & Shanmugam, 2010). Marine scallop *Amussium pleuronectus* is an Indian bivalve mollusc of great local economic importance. Scallops are widely distributed from the intertidal area to ~7000 m. Thus, we have turned our attention towards this scallop which is a potential alternative source of heparin. Hence, an attempt has been taken to isolate and characterize the HS from the marine scallop *A. pleuronectus* using enzyme digestion and the structure of the HS is resoluted through ^{1}H NMR and its APTT and PT assays are also studied.

2. Results and discussion

After defatting with organic solvents and extraction by proteolytic treatment, 8.3 g of crude GAGs and 6 mg of purified HS from marine scallop is recorded. The disaccharide profile such as 51.2% of uronic acid, 38.8% of hexosamine and 20.4% of sulfate content are also observed.

The marine scallop HS recorded the 22.49% of carbon, 3.89% of hydrogen and 2.02% of nitrogen whereas the standard HS observed 22.80% of carbon, 3.08% of hydrogen and 2.08% of nitrogen. Likewise the commercial heparin showed the carbon, hydrogen and

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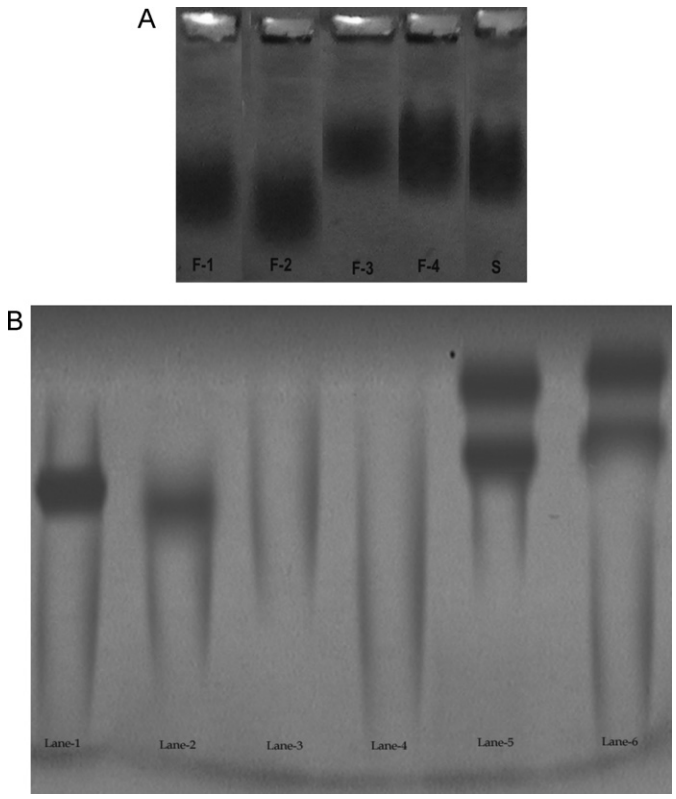

Fig. 1. (A). Agarose gel electrophoresis of DEAE-Cellulose fractioned sample. F, fraction number; S, standard HS. (B). PAGE analysis of bovine and purified marine scallop HS. Lane 1 – bovine HS; Lane 2 – marine scallop HS; Lane 3 – bovine HS treated with heparin lyase-I; Lane 4 – marine scallop HS treated with heparin lyase-I; Lane 5 – marine scallop HS treated with heparin lyase-III; Lane 6 – bovine HS treated with heparin lyase-III.

nitrogen content as 13.0–24.6%, 2.0–5.3% and 0.6–4.5% respectively (Kavanagh & Jaques, 1973). The variation in the carbon, hydrogen and nitrogen may be due to the variation of disaccharide pattern present in the heparin complex isolated from different sources (Pugazhendi, 2002).

The marine scallop purified HS has only one band in agarose gel electrophoresis, which migrates fastly when compared with mammalian HS component Fig. 1(A). This is an indication of its smaller molecular weight. Fig. 1(B) PAGE illustrates that some structural features of the marine scallop are analyzed with heparinases prepared from *Flavobacterium heparinum*. Since the heparinase acts on glucosamino-iduronidic linkages where the glucosamine is *N*-sulfated and the uronic acid has a sulfate at the *C*-2 position (Dietrich et al., 1989). PAGE analysis of marine scallop HS showed a molecular mass of ~15,000 Da (Lane 2) which is compared with standard bovine HS (Lane 1). After treated heparin lyase III, the marine scallop HS and standard HS showed two bands (Lanes 5 and 6). Heparin lyase I acts on both HS and heparin, in contrast heparin lyase III is specific for its action on HS and used to confirm the presence of HS and distinguish it from heparin. From this isolated GAG from marine scallop is HS.

Furthermore, the ^{1}H NMR spectrum (see Fig. 2(A)) and the agarose gel and the PAGE show that marine scallop *A. pleuronectus* HS is free from impurities which can be compared with standard bovine HS (Fig. 2(B)). Pharmaceutical heparin is typically prepared from bovine or porcine intestinal mucosa or beef lung (Casu, 1985). However, heparin samples with peculiar structures and properties have been purified from several species of molluscs (Dietrich et al., 1985).

The activity of APTT and PT of purified HS from marine scallop *A. pleuronectus* sample is found to be 135 IU/mg and 100 IU/mg

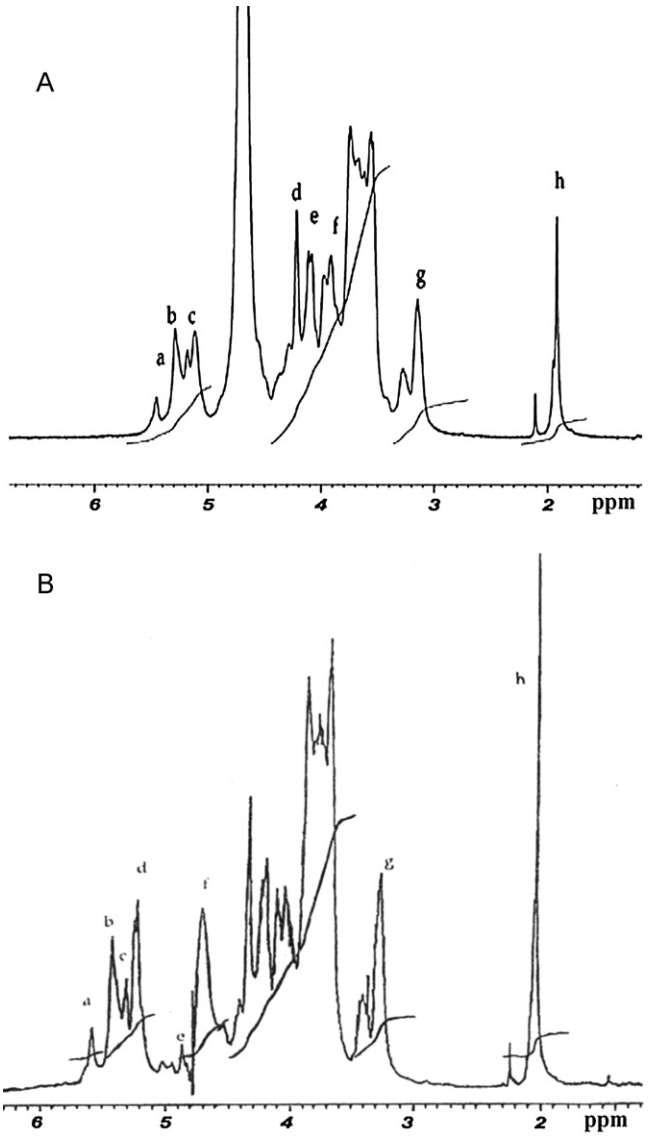

Fig. 2. (A) ^{1}H NMR spectra of HS from *A. pleuronectus*. (B) ^{1}H NMR spectra of standard bovine HS. Labelled peaks are assigned as: (a) H-1 of GlcNpS → GlcAp; (b) H-1 of GlcNpAc → IdoAp; (c) H-1 of IdoAp; (d) H-1 of GlcAp; (e) H-5 of IdoAp; (f) H-4 of IdoAp; (g) H-2 of GlcAp; and (h) methyl of GlcNAc.

respectively. The anticoagulant activity of HS is tested *in vitro* by APTT and PT assays and the values compared with those of standard HS (~170 IU/mg). APTT and PT are related to the intrinsic coagulation phase in plasma. HS has effected the APTT and PT assays, this has been 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 (Linhardt, Wang, Loganathan, & Bae, 1992). In summary the APTT and PT activity of purified HS is 135 IU/mg and 100 IU/mg and has a molecular weight of 15,000 Da. These results suggest that HS isolated from marine scallop *A. pleuronectus* are effective *in vitro* and should be tested *in vivo* in a further study.

3. Experimental

3.1. Collection of animals

Samples which have been collected from Mudasalodai landing center (south east coast of India) are brought to laboratory, washed

in tap, distilled water and then shells are removed. The tissues of the whole body are defatted and used for further extraction.

3.2. Isolation of HS

The isolation of HS was carried out by the method of Hernaiz et al. (2002) with suitable modification. Amberlite IRA-900 (1.5 × 50 cm) and DEAE-Cellulose (1.5 × 50 cm) column chromatography was used in place of strong anionic exchange (SAX) resin.

The four DEAE-Cellulose fractions affording ethanol precipitates are applied to a Sephadex G-100 column (1.5 × 90 cm) (Saravanan & Shanmugam, 2010) equilibrated with a solution of 200 mM NaCl and eluted at 0.33 ml/min. GAG-containing fractions are identified by metachromatic activity (Grant, Linhardt, Fitzgerland, Park, & Langer, 1984) (confirmation test) and UV detection at 525 nm. The metachromatic positive fractions are combined, desalted by dialysis against distilled water.

3.3. Characterization of HS

3.3.1. Enzymatic depolymerization of bovine and marine scallop HS

The standard bovine and purified marine scallop HS are depolymerized by the method of Hernaiz et al. (2002).

3.3.2. Determination of disaccharide profile

The uronic acid, hexosamine and sulfate contents are estimated by following the methods of Bitter and Muir (1962), Wagner (1979) and Terho and Haritiala (1971) respectively.

3.3.3. Elemental analysis

The carbon, hydrogen and nitrogen contents are analyzed with the help of CHN operating mode the PE 2400 Series II CHNS/O Analyzer.

3.3.4. Agarose and gradient polyacrylamide gel electrophoresis

Agarose gel electrophoresis of the HS is performed as previously described (Patel, Ehrlich, Stivala, & Singh, 1980). The average molecular masses of purified HS is analyzed by polyacrylamide linear gradient resolving gels prepared and run as described previously (Edens, Al-Hakim, Weiler, Fareed, & Linhardt, 1992). The average molecular mass of isolated HS is determined and compared with standard bovine lung HS. Then the band is observed under gel documentation system and molecular weight was determined through the standard (Sigma).

3.3.5. ^{1}H NMR analysis

^{1}H NMR spectroscopy (Varian BRUKER-500 MHz) is performed using conditions described previously (Sudo et al., 2001).

3.3.6. Blood coagulation assays

APTT and PT measurements are performed using a kits obtained from Instrumentation Laboratory (Lexington, USA).

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