



A non-hemorrhagic hybrid heparin/heparan sulfate with anticoagulant potential



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ABSTRACT

The structural characterization and the anticoagulant potential of a novel heparin/heparan sulfate-like compound from the heads of *Litopenaeus vannamei* shrimp are described. While it is distinct from either heparin or heparan sulfate, enzymatic depolymerization and nuclear magnetic resonance spectroscopy analyses revealed that this molecule does share some structural features with heparin, such as the high degree of N- and 6-O-sulfation and minor N-acetylation, and with heparan sulfate, in the glucuronic acid content. Its ability to stabilize human antithrombin explains its significant anticoagulant activity in aPTT and Factor-Xa inhibition assays. Interestingly, in contrast to mammalian heparin, the shrimp compound displayed negligible hemorrhagic effect. Together, these findings have particular interest since they reveal a novel molecule with significant anti-Xa activity coupled with low bleeding effects which make the shrimp heparin/HS-like compound a potential alternative for mammalian heparin.

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1. Introduction

Heparin and heparan sulfate (HS) are the most extensively studied glycosaminoglycans (Guerrini et al., 2013) and their structures are qualitatively similar (Casu, Naggi, & Torri, 2010) in that both polysaccharide chains are composed of 1 → 4 linked disaccharide units, consisting of β-D-glucuronic acid (G) or α-L-iduronic acid (I) and α-D-glucosamine (A). Variable modification patterns occur at several positions of the constituent subunit disaccharides. The I and, more rarely, G residues can be O-sulfated at position C-2 (I_{2S} and G_{2S}, respectively) whereas A residues can be N-sulfated (A_{NS}), N-acetylated (A_{NAC}) and frequently 6-O-sulfated (A_{6S}) (Bisio et al., 2009; Guerrini et al., 2013; Rabenstein, 2002). Small numbers of N,6-O-sulfated glucosamine (A_{NS,6S}) residues can also possess

an additional O-sulfate group at position C-3 (A_{NS,6S,3S}). This rare trisulfated glucosamine is a marker for the pentasaccharide motif active for antithrombin (AT), resulting in inhibition of the major coagulation cascade proteases, including Factor Xa (Guerrini et al., 2013).

Both polymers are biosynthesized as proteoglycans through alternate additions of G and A_{NAC} residues to the tetrasaccharide linkage region (β-GlcA-(1,3)-β-Gal-(1,3)-β-Gal-(1,4)-β-Xyl-β1 → O-Ser). Next, a series of sequential N-deacetylation/N-sulfation of A_{NAC} residues, epimerization of some G to I and multiple O-sulfations at different positions take place to modify the polysaccharides (Rabenstein, 2002). Although biosynthesis of heparin and HS chains occurs by the same reactions, the modification degree is different. Heparin undergoes more extensive uronic acid epimerization and sulfation, such that it has a higher iduronic to glucuronic acid ratio and total O-sulfate group content (Casu & Lindahl, 2001; Casu et al., 2010; Lyon & Gallagher, 1998; Molist et al., 1998). In addition, the non-sulfated sequence G-A_{NAC} is predominant in HS, whereas the trisulfated disaccharide (I_{2S}-A_{NS,6S}) is the major repeating structural unit present in heparin (Bisio et al., 2009; Guerrini et al., 2013).

Heparin has been used clinically as the drug of choice in the prevention and treatment of thromboembolic diseases (Alban, 2008).

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However, its use is accompanied by some side effects, frequently requiring monitoring of partially activated thromboplastin time and resulting in other hemorrhagic complications (Nader et al., 2001). In addition, several adverse clinical manifestations and deaths resulting from the use of contaminated heparins have been reported (Blossom et al., 2008; Kishimoto et al., 2008; Rudd et al., 2011). These concerns have stimulated the search for alternative anticoagulants with reduced side effects. Interestingly, compounds structurally related to heparin/HS with variable biological activities have been described in many species of invertebrates (Andrade et al., 2013; Cassaro & Dietrich, 1977; Dietrich et al., 1985; Medeiros et al., 2000; Santos et al., 2007). Previous studies have shown the occurrence of a non-hemorrhagic heparin analog in the heads of the *Litopenaeus vannamei* shrimp, which has anti-inflammatory and anti-angiogenic properties, but is devoid of anticoagulant activity (Brito et al., 2008; Dreyfuss et al., 2010).

Now, we describe the structural features, anticoagulant and anti-hemostatic activities, in addition to the AT stabilization properties of a second distinct heparin/HS from *L. vannamei*, which represents the most commonly farmed shrimp species in Brazil and one of the most commonly farmed in the world (Cahú et al., 2012).

2. Experimental

2.1. Materials

Heparan sulfate from bovine pancreas was a gift from the late Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy). Heparitinases I and II, and heparinase (heparinase I, EC4.2.2.7) were prepared as previously described (Dietrich et al., 1989). Sodium heparin from porcine mucosa was obtained from Laboratory Derivati Organici (Trino Vercellese, Italy). Chondroitin 4-sulfate (CS-4) and chondroitin 6-sulfate (CS-6), extracted from whale cartilage, and dermatan sulfate (DS) extracted from pig skin, were acquired from Miles Laboratories (Elhart, IN, USA).

2.2. Isolation and purification of heparin/heparan sulfate (H/HS) from shrimp heads

The isolation of hybrid H/HS from *L. vannamei* heads was performed as described (Brito et al., 2008). Briefly, 16 kg of shrimp heads were defatted with acetone and submitted to proteolysis at 60 °C for 24 h. Then, the polysaccharides extracted were complexed with ion-exchange resin and eluted with 3.0 M NaCl. The pool of glycosaminoglycans (GAGs) obtained was submitted to acetone fractionation to obtain F-0.5, F-0.7 and F-1.0 fractions. The shrimp H/HS was then purified by ion-exchange chromatography followed by NaCl elution (0.5, 0.8 and 1.0 M). Effluents were analyzed by carbazole assay to detect the presence of GAGs as previously described (Dische, 1947). The compounds eluted with 0.8 M NaCl were desalted by gel filtration through a Sephadex G-25 column by eluting with 10% ethanol. After lyophilization, the purified shrimp H/HS compound (52.8 mg) was analyzed.

2.3. Electrophoresis

The shrimp H/HS was analyzed by agarose gel electrophoresis using two different buffers systems: 0.05 M 1,3-diaminopropane acetate (PDA/Aldrich), pH 9.0, and the discontinuous system barium acetate/PDA. Briefly, aliquots (5 µL) were applied to a 0.5% agarose gel (Bio-Rad) and run for 1 h at 100 V. The GAGs in the gel were fixed with 0.1% N-acetyl-N,N,N-trimethylammonium bromide solution. After 2 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5:5, v/v/v) as described by Dietrich and Dietrich (1976). Subsequently the gel

was destained with a solution containing acetic acid/ethanol/water (0.1:5:5, v/v/v).

2.4. Enzymatic digestion

Enzymatic digestion was performed as previously described (Lima, Hughes, et al., 2013). Briefly, 100 µg of the isolated compound and heparin were incubated with a mixture of heparin lyases (2.5 mIU each) and the disaccharides produced resolved on a 150 × 4.6 mm Phenosphere SAX column (Phenomenex, Torrance, CA, USA) using a NaCl gradient of 0–1 M during 30 min with a 1 mL/min flux and UV detection at 232 nm.

2.5. Molecular weight determination

Samples were analyzed by GPC-HPLC on a 300 × 7.8 mm BioSep SEC™ S-2000 LC Column (Phenomenex, Torrance, CA, USA) using isocratic elution (0.3 M Na₂SO₄ mobile phase) at a flow rate of 1 mL/min and UV detection at 205 nm. The column was previously calibrated with polysaccharides of known molecular weights (1.7, 4, 10, 16 and 20 kDa).

2.6. Nuclear magnetic resonance analysis

For NMR experiments, the samples were deuterium exchanged by repeated dissolution in D₂O and freeze-drying. Spectra were obtained from solutions in D₂O at 25 °C, using TSP as standard ($\delta = 0$). All spectra were obtained with a Bruker 400 MHz, 600 MHz AVANCE II or III NMR spectrometer (Bruker GmbH, Silberstreifen, Germany) with a triple resonance 5-mm probe and in Agilent 600 MHz System with 5-mm Cold Probe. 1D and 2D signal assignments were performed using 1H-(zg, and zgpr) and HSQC (hsqcetgpsi) programs. HSQC was acquired using 8–16 scans, respectively, per series of 2 K × 512 W data points with zero filling in F1 (4 K) prior to Fourier transformation (Lima, Viskov, et al., 2013).

2.7. Purification of antithrombin (AT)

AT was purified from fresh citrated human plasma by affinity chromatography on heparin-sepharose. Briefly, human plasma was loaded onto a heparin-sepharose column, which had been equilibrated with 0.1 M Tris-HCl, 0.01 M sodium citrate and 0.25 M sodium chloride. Unbound and weakly bound proteins were eluted from the column by washing with 0.1 M Tris-HCl, 0.01 M sodium citrate and 0.25 M sodium chloride followed by 0.1 M Tris-HCl, 0.01 M sodium citrate and 0.5 M sodium chloride (5 column volumes). AT was eluted with 0.1 M Tris-HCl, 0.01 M sodium citrate and 2 M sodium chloride, dialyzed against 12.5 mM phosphate buffer and concentrated.

2.8. Differential scanning fluorimetry (DSF)

AT was at 30 nM in the presence of an excess of GAG, based where necessary on weight average molecular weight (Mw). The dye Sypro Orange™ was employed at 100× concentration (5 µL of 5000× concentration dye into 245 µL fresh deionized water). The dye has an excitation wavelength of 300 or 470 nm, and emits at 570 nm when bound to hydrophobic residues. The protein was subjected to a step-wise temperature gradient, from 32 °C to 85 °C in 0.5 °C steps. There was an initial 2 min incubation period at 31 °C and 5 s between each temperature increase to equilibrate. Data were collected for 30 s at each temperature (Uniewicz et al., 2010). First derivatives of the melting curves were employed with Solvitzky–Golay smoothing with a second order polynomial.

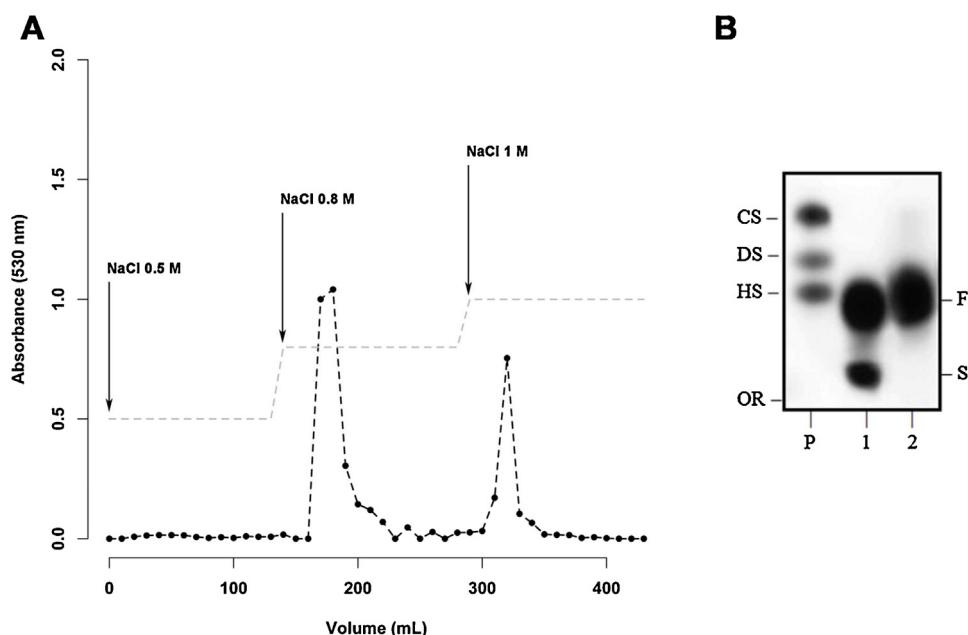


Fig. 1. (A) Purification of the fraction F-0.7 by ion-exchange chromatography on DEAE Sephacel. The column was eluted with a salt gradient represented in the graph by the dotted line; (B) Electrophoretic profile in Ba/PDA system of (1) mammalian heparin and (2) shrimp H/HS obtained by ion-exchange and gel chromatography of F-0.7 on Sephadex G-25. OR-origin; P- pattern containing 5 μ g of chondroitin 4/6 sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS), F- heparin fast moving component; S- heparin slow moving component.

2.9. Anticoagulant activity

The activated partial thromboplastin time (aPTT) assay was performed according to the method of the kit APTTest (Rosario, Argentina). Heparin, heparan sulfate and shrimp H/HS were dissolved in physiological saline to the appropriate concentrations (generating a volume of 10 μ L) and incubated with 90 μ L of plasma at 37 °C during 3 min. Then, 100 μ L of bovine cephalin was added and incubated at 37 °C. After 3 min of incubation, 100 μ L of pre-warmed 0.25 M CaCl_2 solution were added to the mixture and the clotting time was measured in triplicate using Quick Timer Coagulometer (Drake Electronica Commerce Ltd., Sao Paulo, Brazil).

2.10. Assay for the anti-Xa activity

The anti-Xa activity assay was conducted in a 96-well microplate according to the instructions of Biophen Heparin Anti-Xa kit (HYPHEN Biomed, ref: 221010) as follows: 40 μ L of AT was incubated at 37 °C for 2 min in the presence of increasing concentrations of heparin or shrimp H/HS diluted in buffer pH 8.4 (0.05 M Tris, 0.175 M NaCl, 0.0075 M EDTA, containing 0.1% polyethylene glycol). 40 μ L of purified bovine Factor Xa (FXa) was then added to each well, mixed and incubated at 37 °C for exactly 2 min. Then, 40 μ L of a chromogenic substrate for FXa was added and the mixture was incubated for 2 min at 37 °C. Following incubation, 80 μ L of 30% acetic acid was added to stop the reaction and the absorbance was measured against a corresponding blank.

2.11. Residual hemorrhagic effect

The residual hemorrhagic effect of shrimp H/HS compound was analyzed by a modified model of topical scarification in rat tail (Cruz & Dietrich, 1967). After anesthesia with ketamine and xylazine in a 1:1 (v/v) proportion, a scar was made with a surgical blade in the distal portion of the tail. Then, the tail was then dipped vertically in physiological saline solution, dabbed with gauze and dipped again in fresh saline to observe bleeding. The tail was dipped in a solution containing shrimp H/HS or heparin at different concentrations

for 2 min and then washed extensively with saline solution. The treated tail was immersed in new physiological saline solutions for 40 min and the amount of blood was measured by Bradford assay. The results were expressed as the sum of the protein values of each tube minus the amount of protein present before the exposure to the test substance.

3. Results and discussion

3.1. Isolation and purification of shrimp H/HS

The compounds isolated from shrimp heads following proteolysis and fractionation by ion-exchange resin were precipitated with acetone in different proportions to obtain fractions named F-0.5, F-0.7 and F-1.0. The electrophoretic profiles of such fractions in diaminopropane/acetate buffer (PDA) indicated the presence of compounds with migration between heparan and dermatan sulfate, although the F-0.7 and F-1.0 fractions also possess a band corresponding to chondroitin sulfate (data not shown). The analysis of the ^{13}C NMR spectrum shows the presence of low intensity signals (~ 63 ppm e ~ 54 ppm) (data not shown) corresponding to N-acetylated galactosamine residues, which confirms the presence of galactosaminoglycans. The shrimp H/HS was further purified from fraction F-0.7 by anion-exchange chromatography on DEAE–Sephacel using a step-wise salt gradient (Fig. 1A). The fraction eluted with 0.8 M NaCl yielded 70.0% of total uronic acid content and was further desalted by gel chromatography on Sephadex G-25. In the discontinuous barium/PDA buffer, in which mammalian heparin is fractionated in three bands named fast, intermediate and slow moving components (Bianchini et al., 1980; Medeiros et al., 2000), the shrimp H/HS compound showed only one band, which migrates as heparan sulfate and/or as the fast mammalian heparin component (Fig. 1B). Such electrophoretic behavior could indicate that the compound isolated from shrimp has a high disulfated disaccharide content (Volpi, 1993). Similar behavior was also observed in heparin-like compounds from other crustacean species, such as *Penaeus brasiliensis* shrimp (Dietrich et al., 1999) and *Artemia franciscana* brine shrimp (Chavante et al., 2000).

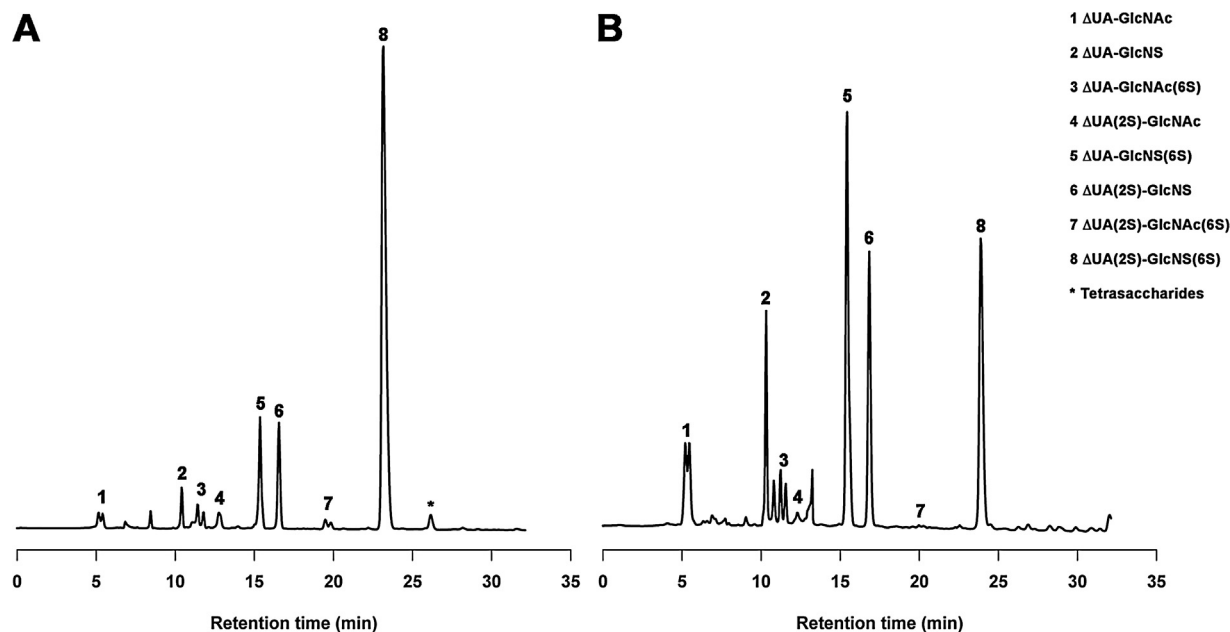


Fig. 2. HPLC separation of degradation products formed from heparin (A) and shrimp H/HS (B) following the combined action of heparinase and heparitinases I and II. (1) Δ UA-ANac; (2) Δ UA-ANS; (3) Δ UA-ANac,6S; (4) Δ UA(2S)-ANac; (5) Δ UA-ANS,6S; (6) Δ UA(2S)-ANS; (7) Δ UA(2S)-ANac,6S; (8) Δ UA(2S)-ANS(6S); * residual undigested tetrasaccharide.

3.2. Enzymatic depolymerization

The disaccharide composition of the shrimp H/HS was determined by enzymatic depolymerization with a mixture of heparinase and heparitinases I and II from *Flavobacterium heparinum*. Upon the combined action of these enzymes, shrimp H/HS yielded a high content of disulfated disaccharide (Δ U-ANS,6S/ Δ U,2S-ANS) (Fig. 2B), contrasting with porcine mucosal heparin, in which the trisulfated disaccharides are predominant (Fig. 2A). Since disulfated disaccharides are mainly formed by heparitinase II action, these results suggest that shrimp H/HS is more extensively degraded by this enzyme, similar to heparin-like polysaccharides from other invertebrate species (Andrade et al., 2013; Dietrich et al., 1985, 1989, 1999; Medeiros et al., 2000). In contrast to heparan sulfates, in which around 50–60% of all disaccharides are Δ U-ANac/ Δ U-ANS (Andrade et al., 2013; Dietrich et al., 1998; Zhang, Xie, Liu, Liu, & Linhardt, 2009), the results show that the molecule purified from shrimp contains just 10.7% of these units. In fact, the high degree of N,6-O-sulfation and minor N-acetylation are the main structural features of shrimp H/HS observed by enzymatic depolymerization. Together, the data suggest the occurrence of an atypical molecule with characteristics of a hybrid heparin and heparan sulfate.

3.3. Molecular weight and structural features of shrimp heparin/heparan sulfate

Shrimp H/HS showed an average molecular weight of 15 kDa, similar to porcine heparin (~16 kDa) and lower than bovine heparan sulfate (~25 kDa). The compound was characterized by $^1\text{H}/^{13}\text{C}$ heteronuclear single-quantum coherence (HSQC)-NMR analysis, as shown in Fig. 3A and B and Table 2. Following H/C signal correlation, the compound isolated from shrimp heads exhibited similar characteristics to signals ascribed to porcine heparin. An interesting observation is the absence of signals corresponding to non-sulfated iduronic acid in the shrimp H/HS spectra. This result suggests a particular mode of shrimp H/HS synthesis which, after the epimerization step, results in all iduronic acid residues being 2-O-sulfated, contrasting with the biosynthesis process that occurs in mammalian heparin and HS. In addition, combined analysis of

the data from the enzymatic depolymerization and HSQC spectra show that the shrimp H/HS polymer consists mainly of disaccharide units containing N,6-sulfated glucosamine linked to glucuronic acid (G-ANS,6S), N-sulfated glucosamine linked to 2-sulfated iduronic acid (I_{2S}-ANS) and N,6-sulfated glucosamine linked to 2-sulfated iduronic acid (I_{2S}-ANS,6S). The monosaccharide content of shrimp H/HS is shown in Table 1. A relatively high content of ANS (72.5%) and 6-O-sulfation (71.5%) together with low degree of ANac (22.5%) report a structure which is very close to mammalian heparin and distinct from heparan sulfate (Casu & Lindahl, 2001; Casu et al., 2010; Lyon & Gallagher, 1998). On the other hand, in HSQC analysis from shrimp H/HS, the signals attributed to G are higher than I_{2S} residues, resembling the higher ratio of glucuronic/iduronic acid, similar to heparan sulfate. These differences have also been observed in mollusk and crustacean heparin-like compounds, which also contain high proportions of G residues (Andrade et al., 2013; Dietrich et al., 1985, 1989, 1999; Medeiros et al., 2000). Taken together, the NMR and enzymatic depolymerization data confirm that the molecule isolated from shrimp shares structural features with both heparin and heparan sulfate, consequently, it is most appropriate to define it as a hybrid of heparin and heparan sulfate. Other interesting data include the presence of about 5% of the rare 3-O-sulfation on N-sulfated glucosamine residues. Such residues are usually undetectable in HS (Guerrini, Guglieri, Naggi, Sasisekharan, & Torri, 2007; Guerrini, Naggi, Guglieri, Santarsiero, & Torri, 2005). However, A_{3S} is found in the high affinity AT-binding site of heparin (Linhardt, Wang, Loganathan, & Bae, 1992) and is strongly associated with the anti-FXa activity of heparin (Guerrini et al., 2007). For this reason, it became of great interest to evaluate the anticoagulant potential of the hybrid shrimp H/HS.

3.4. Anticoagulant activity assays

The anticoagulant activity of the shrimp H/HS compound was investigated by the classical clotting assay aPTT and by determining its capacity to catalyze antithrombin mediated inactivation of factor Xa using porcine heparin (190 IU/mg) as a reference. As shown in Fig. 4, at 5 $\mu\text{g}/\text{mL}$, porcine heparin prolonged the clotting time for more than 240 s, detected by aPTT. The same effect is achieved

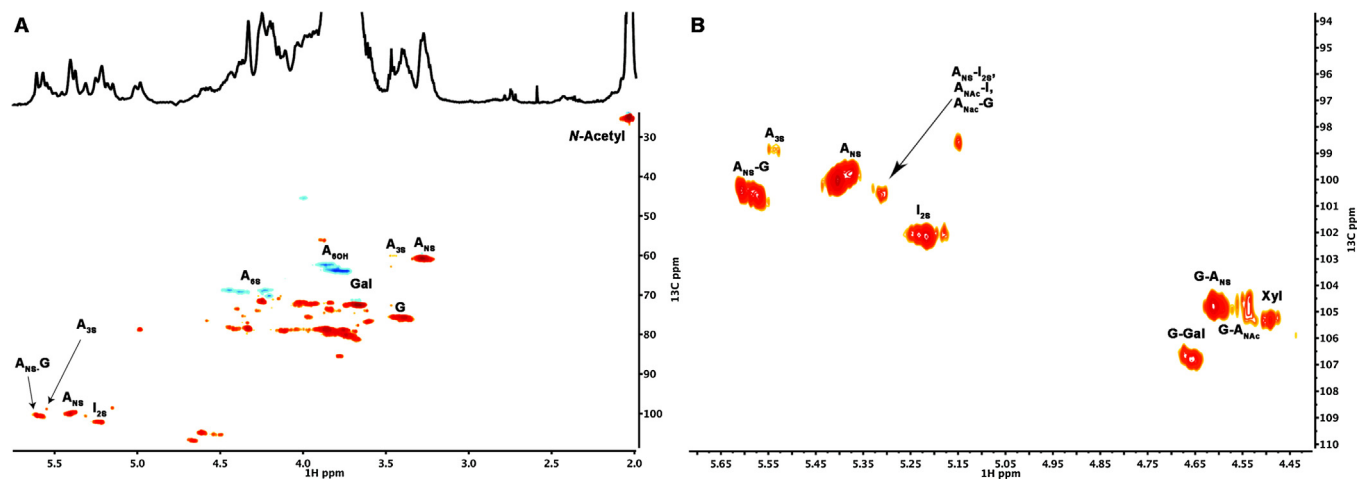


Fig. 3. $^1\text{H}/^{13}\text{C}$ heteronuclear single-quantum coherence (HSQC)-NMR spectrum. (A) Major identified signal used for and (B) Anomeric region. N,6-sulfated glucosamine linked to glucuronic acid (G- $\text{A}_{\text{NS},6\text{S}}$); N-sulfated glucosamine linked to 2-sulfated iduronic acid ($\text{I}_{2\text{S}}\text{-A}_{\text{NS}}$); N,6-sulfated glucosamine linked to 2-sulfated iduronic acid ($\text{I}_{2\text{S}}\text{-A}_{\text{NS},6\text{S}}$). Signals used for composition analysis are A_{NAc} (2.04/25 ppm), A_{NS} (3.46/60.7 ppm), $\text{A}_{3\text{S}}$ (3.45/60 ppm), $\text{A}_{6\text{OH}}$ (3.86/62.3), $\text{A}_{6\text{S}}$ (4.38/69.1 ppm), G (3.38/75.8 ppm), $\text{I}_{2\text{S}}$ (5.20/102.1).

Table 1
Saccharide composition calculated by 2D HSQC spectrum integration (A) and (B) HPLC disaccharide analysis. Glucosamine N-sulfate (ANS); Glucosamine N-acetyl (ANAc); Glucosamine 3-O-sulfate (A3S); Glucosamine 6-O-sulfate (A6S); Glucuronic acid (G); Iduronic acid (I); Iduronic acid 2-O-sulfate (I2S).

(A) Monosaccharide (%)								
A_{NS}	A_{NS}	A_{NAc}	$\text{A}_{3\text{S}}$	$\text{A}_{6\text{S}}$	G	I	$\text{I}_{2\text{S}}$	
	72.5	22.5	5.1	71.5	77.6	n.d.	22.4	
(B) Disaccharides (%)								
	$\Delta\text{UA-ANAc/D0A0}$	$\Delta\text{UA-ANS/D0S0}$	$\Delta\text{UA-ANAc(6S)/D0A6}$	$\Delta\text{UA(2S)-ANAc/D2A0}$	$\Delta\text{UA-ANS(6S)/D0S6}$	$\Delta\text{UA(2S)-ANS/D2S0}$	$\Delta\text{UA(2S)-ANAc(6S)/D2A6}$	$\Delta\text{UA(2S)-ANS(6S)/D2S6}$
Shrimp H/HS	11.02	9.14	4.51	3.15	28.87	17.63	0.00	25.68
Pancreas HS	45.77	17.03	11.08	3.89	7.19	8.79	0	6.23
Lung HS	28.69	16.87	17.99	5.24	10.65	12.66	0	7.90
Heparin (6th Int. Std.)	3.42	2.75	3.71	2.20	9.25	8.10	1.51	69.06

by the shrimp compound at a higher concentration, demonstrating that it is able to inhibit the clot formation via the intrinsic pathway. Next, we examined whether the molecule from shrimp was able to inhibit directly the activity of FXa. As shown in Fig. 5, despite its lower activity, it inhibited the FXa activity in a concentration-dependent manner, reaching approximately 97% of inhibition at about 0.6 $\mu\text{g}/\text{mL}$. The anticoagulant action of heparin and heparin-like saccharides depends their ability to bind to and potentiate the inhibitory activity of AT against plasma proteases. More recently, it has been shown that this activity is strongly correlated with thermal stabilization of AT (Lima, Hughes, et al., 2013), which led us to investigate the effect of the compound on AT stability.

3.5. Thermostabilizing effect of shrimp H/HS compound on AT

The thermostabilizing effect of shrimp H/HS saccharide on AT was evaluated by differential scanning fluorimetry (DSF) and the

results are shown in Fig. 6. Although the strongest effects on protein stability have been achieved by unfractionated heparin (UFH) and AT-binding pentasaccharide (59.25 and 65.25 $^{\circ}\text{C}$, respectively), the shrimp polysaccharide also showed a considerable increase in the thermal stability of AT (57.25 $^{\circ}\text{C}$). The data showed that the

Table 2
 $^1\text{H}/^{13}\text{C}$ major chemical shifts for the shrimp H/HS.

	A_{NAc}	G	$\text{A}_{\text{NS},6\text{S}}$	$\text{I}_{2\text{S}}$	$\text{A}_{\text{NX},6\text{X}3\text{S}}$
1	5.31/100	4.6/104	5.4–5.31/100	5.20/102.1	5.53/98.8
2	–	3.38/75.8	3.46/60.7	4.33/78.6	3.45/60
3	–	–	–	–	–
4	–	–	–	–	–
5	–	–	–	–	–
6	–	–	3.86–4.38/62.3–69.1	–	–
CH3	2.04/25	–	–	–	–

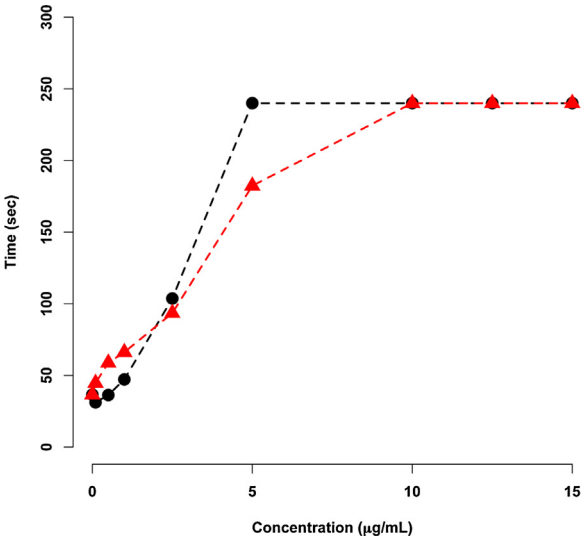


Fig. 4. Anticoagulant activity of heparin and shrimp H/HS by aPTT (activated partial thromboplastin time) assay. (●) Heparin; (▲) Shrimp H/HS.

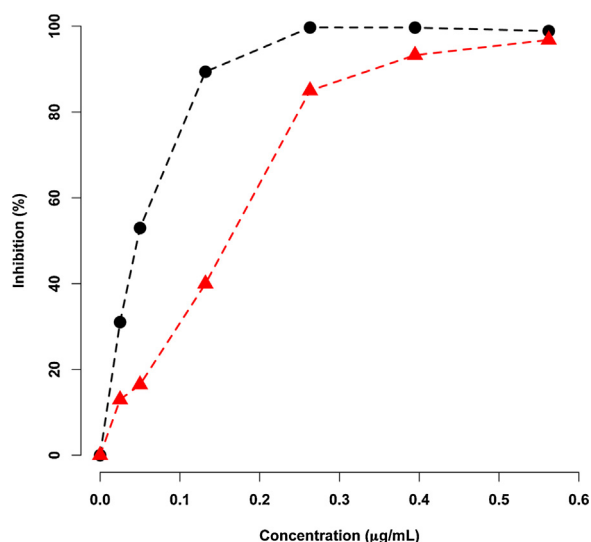


Fig. 5. Anti-Xa activity of mammalian heparin and shrimp H/HS. (●) Heparin; (▲) Shrimp H/HS.

anticoagulant activity of the compound from shrimp results, at least in part, from its ability to bind and stabilize AT. Curiously, crustaceans do not possess blood coagulation cascade similar to that of mammals; a fact that makes the occurrence of molecules that act upon coagulation proteins remarkable and suggests that the shrimp H/HS molecules can modulate the activity of endogenous serine proteases.

3.6. Bleeding time

Owing to its ability to interfere in the hemostatic balance, the clinical use of mammalian heparin is involved with some undesirable effects, such as hemorrhage, therefore, the residual hemorrhagic effect of shrimp H/HS was investigated. As shown in Fig. 7, at 100 µg/mL, porcine heparin has a potent hemorrhagic effect. Interestingly, the molecule isolated from shrimp, despite its anticoagulant potential, had an insignificant effect on bleeding. These results are in agreement with previous findings that there is

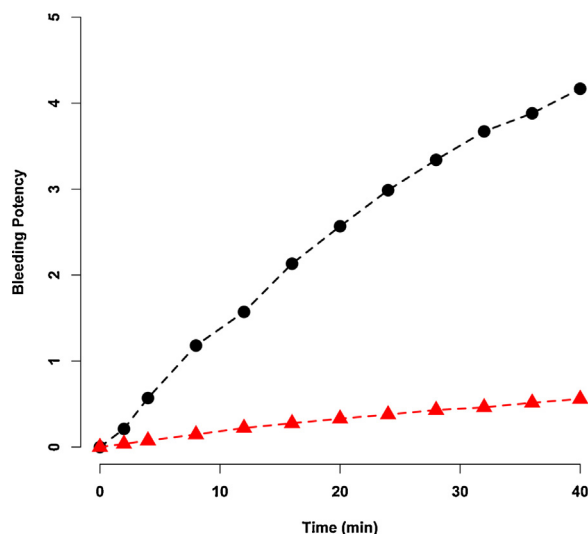


Fig. 7. Bleeding activity of the shrimp H/HS glycosaminoglycan and porcine intestinal mucosa heparin (100 µg/mL) or shrimp H/HS (100 µg/mL) applied topically, and the bleeding potency measured after 2 min following saline solution washing. (●) mammalian heparin, (▲) shrimp H/HS.

no correlation between hemorrhagic activity and anti-Xa activity or inhibition of clot formation (Boucas et al., 2012; Nader, Tersariol, & Dietrich, 1989). In fact, it was earlier proposed that the anti-hemostatic activity of heparin is due to its binding to myosin ATPase molecules of the injured vessels and consequent inhibition of the normal muscular contractility (Cruz & Dietrich, 1967). Thus, it could be speculated that the shrimp H/HS compound is not able to bind to myosin ATP molecules with the same affinity of heparin, so that it must be dislodged after washes with saline solutions, as described in Section 2.

4. Conclusions

The results describe a novel glycosaminoglycan isolated from heads of shrimp *L. vannamei* which possesses a high degree of N,6-sulfation and minor N-acetylation together with the high content of glucuronic acid and an absence of non-sulfated iduronic acid. Despite exhibiting lower anticoagulant activity than heparin, its lower hemorrhagic effect and the fact that it is obtained from shrimp farm waste makes this GAG a suitable candidate as an alternative for mammalian heparin.

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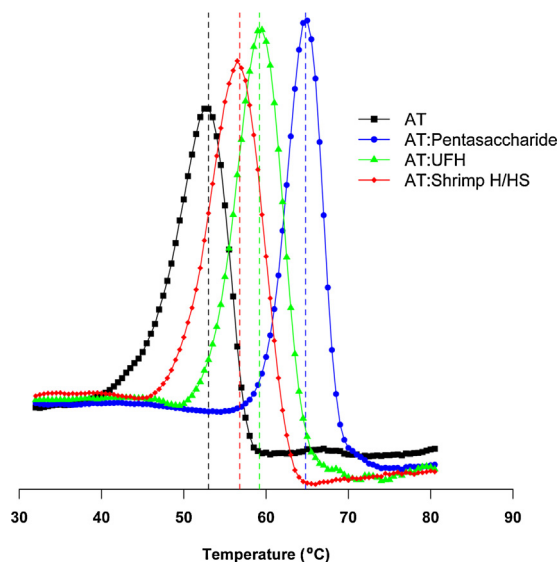


Fig. 6. Thermostabilizing effect of Shrimp H/HS on AT evaluated by differential scanning fluorimetry (DSF). (■) AT; (●) AT:Pentasaccharide; (▲) AT:UFH; (◆) AT:Shrimp H/HS.

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