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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.

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 ($\mathbf{I_{2S}}$ and $\mathbf{G_{2S}}$, respectively) whereas **A** residues can be N-sulfated ($\mathbf{A_{NS}}$), N-acetylated ($\mathbf{A_{NAC}}$) and frequently 6-O-sulfated ($\mathbf{A_{6S}}$) (Bisio et al., 2009; Guerrini et al., 2013; Rabenstein, 2002). Small numbers of N,6-O-sulfated glucosamine ($\mathbf{A_{NS,6S}}$) residues can also possess an additional O-sulfate group at position C-3 ($\mathbf{A_{NS,6S,3S}}$). 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 $\mathbf{A_{NAc}}$ 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 $\mathbf{A_{NAc}}$ 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 ($\mathbf{I_{2S}}$-$\mathbf{A_{NS,6S}}$) 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 Na_2SO_4 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 D_2O and freeze-drying. Spectra were obtained from solutions in D_2O 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, Silberstrei-fen, 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 OrangeTM 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.