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Structure and anticoagulant activity of fucosylated glycosaminoglycan degraded by deaminative cleavage

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ABSTRACT

Fucosylated glycosaminoglycans (FGs) are complex glycosaminoglycans that exhibit potent anticoagulant activity. To study the relationship between molecular size and biological activity, oligosaccharides with (2,5)-anhydro-D-talose units at new reducing ends were prepared by hydrazine deacetylation and nitrous acid depolymerization. The product chemical structures were analyzed by one- and two-dimensional NMR methods. Additionally, anticoagulant activities were evaluated by clotting assay and chromogenic substrate cleavage. The results demonstrated that under mild deacetylation and deaminative cleavage conditions, both products were relatively homogeneous and sulfated fucose branch types and sulfate substituents remained stable. These depolymerized FGs with different molecular sizes had potent intrinsic anticoagulant activities, which were similar to those that were obtained by free-radical depolymerization with similar molecular weights. Decreasing molecular weight may weaken activity but not significantly affect factor Xase and heparin cofactor II (HCII)-mediated thrombin inhibition.

1. Introduction

Fucosylated glycosaminoglycans (FGs) extracted from sea cucumbers have a chondroitin sulfate E-like structure that contains large numbers of sulfated α-L-fucopyranose branches linked to β-D-glucuronic acid residue position 3 (Mourão et al., 1996; Wu, Xu, Zhao, Kang, & Ding, 2010a, 2010b; Wu et al., 2012; Yoshida, Minami, Nemoto, Numata, & Yamanaka, 1992), while the FG fucose branches may have distinguishable sulfate substitution patterns and proportions (Wu et al., 2012). Chondroitin sulfate derivatives have high anticoagulant and antithrombotic properties (Buyue & Sheehan, 2009; Sheehan & Walke, 2006). However, FGs also exhibit undesirable effects such as inducing factor XII activation and platelet aggregation (Fonseca et al., 2010; Li & Lian, 1988). To minimize side effects, its low molecular weight derivative depolymerized FG was prepared, which retained antithrombotic and anticoagulant activities without causing platelet aggregation in human platelet-rich plasma (Suzuki, Kitazato, Takamatsu, & Saito, 1991; Yoshida et al., 1992). Furthermore, pharmacological research has indicated that the depolymerized product and its native FG share common mechanisms for factor tenase inhibition in the intrinsic pathway and thrombin inhibition by activating heparin cofactor II (HCII) (Buyue & Sheehan, 2009; Sheehan & Walke, 2006). The literature and our preliminary studies also confirmed that low-molecular-weight fragments exhibit a better antithrombotic–hemorrhagic ratio than unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH) (Kitazato, Kitazato, Sasaki, Minamiguchi, & Nagase, 2003; Sheehan & Walke, 2006; Wu et al., 2010b). Therefore, to further study the relationship between molecular size and FG biological activity, depolymerized derivatives with different structural characteristics should be prepared.

Based on these facts, searching for a proper protocol to efficiently prepare depolymerized FGs may be necessary. However, a limited number of techniques for molecular reduction have

Abbreviations: GAG, glycosaminoglycan; FG, fucosylated glycosaminoglycan; DFG, depolymerized fucosylated glycosaminoglycan; UFH, unfractionated heparin; LMWH, low-molecular-weight heparin; DD, deacetylation degree; GalNAc4S6S, 4,6-O-disulfated N-acetylgalactosamine; GlcUA, glucuronic acid; GalN, galactosamine; anTal, anhydro-D-talose; Fuc3S, 3-O-sulfated fucose; Fuc4S, 4-O-sulfated fucose; Fuc2S4S, 2,4-O-disulfated fucose; APTT, activated partial thromboplastin time; HCII, heparin cofactor II; EC_{50}, half maximal effective concentration.

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been described. Unlike heparin and chondroitin sulfate, FG cannot be directly digested by polysaccharide degrading enzymes such as heparin lyases or chondroitin ABC lyase, possibly because their sulfated fucose branches may be linked to position 3 of GlcA residues (Luo et al., 2013; Mourão et al., 1996). Free radical depolymerization is the first commonly used method for degraded sample preparation (Kitazato, Kitazato, Nagase, & Minamiguchi, 1996; Suzuki et al., 1991). Recently, the ^{60}Co irradiation method was reportedly used to prepare depolymerized FG with a M_w between 2.5 and 7.6 kDa (Wu et al., 2013). In our previous study, we developed hydrogen peroxide-mediated free radical FG depolymerization in the presence of cupric ion (Wu et al., 2010a), which produced FG fragments with no obvious sulfate or fucose branch loss. These preparation processes may produce different products with different structures, molecular weights, anticoagulant activities, and pharmacological properties. Thus, a need still remains to develop efficient and different methods to prepare low-molecular-weight fragments for chemical structure analysis and structure–activity relationship research. Here, we investigated the application of deaminative cleavage for preparation of depolymerized FG (DFG).

Deaminative cleavage with nitrous acid is a successful chemical method for heparin and heparan sulfate depolymerization because of its advantages such as high selectivity, mild depolymerization conditions and no preferential side chain cleavage (Petitou, Casu, & Lindahl, 2003). This method has been used for LMWH production such as dalteparin, nadroparin and reviparin (Linhardt & Gunay, 1999). However, because the hexosamines in FG are all N-acetylated hexosamine residues, FG cannot be directly depolymerized by nitrous acid. To apply the deaminative cleavage method to prepare depolymerized FG, it may be necessary to partially remove the FG acetyl group. Hydrazinolysis was a commonly used method for glycosaminoglycan (GAG) deacetylation such as chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) (Guo & Conrad, 1989; Shaklee & Conrad, 1984). Because of these fucose branches, the FG structure is more complex than CS, DS and HS structures. Therefore, the current work sought to investigate the deacetylation and deaminative cleavage for FG depolymerization and to characterize the product structures and anticoagulant activities.

2. Experimental design

2.1. Materials

FG (purity: 99.9%, average molecular weight: 69.930 kDa, sulfate/carboxyl groups: 3.54) was isolated and purified from the sea cucumber *Thelenota ananas*. Isolation, purification and characterization of this glycosaminoglycan were performed as previously described (Wu et al., 2010a, 2010b). Hydrazine hydrate (containing about 64 wt.% hydrazine in water) was obtained from Aladdin Reagent (Shanghai, China). Anhydrous hydrazine was obtained from hydrazine hydrate according to the method of Takasaki, Mizuochi, and Kobata (1982). Hydrazine sulfate and sodium nitrite were purchased from DamaoChem., Ltd. (Tianjin, China). Deuterium oxide (D_2O) containing 0.05 wt.% trimethylsilyl-propionic acid (TSP) sodium salt was obtained from Sigma (St. Louis, MO, USA). Activated partial thromboplastin time (APTT) assay kits (Lot No. 10917752) and Tris–HCl (Lot No. 20110726) were obtained from TECO Medical Instruments Co., Germany and Amresco Co., USA, respectively. A chromogenic assay kit for measuring factor VIII: C in concentrates (Lot No. 12101-PK:11) and HCII assay reagents including HCII (Lot No. 09041F), human thrombin IIa (Lot No. 100628A) and thrombin chromogenic substrate (Lot No. 11204-1-PK:1) were purchased from Hyphen Biomed (France). Human coagulation factor VIII was obtained from Shanghai RAAS Blood Products, Ltd., China. All of the other chemicals and reagents used were of analytical grade.

2.2. FG deacetylation

FG deacetylation was performed using Fukuda's modified method that has been described previously (Fukuda, Kondo, & Osawa, 1976; Shaklee & Conrad, 1984). Briefly, dried FG (60 mg) and 1.45 ml hydrazine hydrate containing 1% hydrazine sulfate were added in a reaction tube. The tube was flushed with nitrogen, sealed and incubated at 90 °C for 12 h on a magnetic stirrer at 250 rpm. After the reaction, the solution was added to ethanol (quadruple the solution volume). When several drops of saturated sodium chloride were added, a white precipitate was formed. The precipitate was collected by centrifugation and dissolved in distilled water. This precipitation and dissolution procedure was repeated 4 times to remove the hydrazine and hydrazine sulfate. The resulting solution was dialyzed against flowing tap water for 2 d and distilled water for 1 d with a 3500 Da molecular weight cut-off and subsequently lyophilized.

To optimize the reaction conditions, a series of experiments were performed based on the above representative protocol with varied reaction parameters (time, catalyst type, temperature and concentration of hydrazine), and each experiment was performed at least in duplicate. In the protocol, the sample deacetylation degree (DD) was calculated using the two methyl peak area ratios that were approximately 1.36 and 2.06 ppm in the ^{1}H NMR spectrum, which were from the N-acetylgalactosamine (GalNAc) methyl group and from the fucose sulfate methyl group, respectively. The N-deacetylated FG yield was calculated using the N-deacetylated and native FG sample weights.

2.3. Deaminative FG cleavage

The deaminative cleavage protocol was designed according to the literature (Bienkowski & Conrad, 1985; Shively & Conrad, 1976). The pH 4 nitrous acid reagent was prepared by addition of 0.5 M H_2SO_4 to 5.5 M $NaNO_2$ until pH 4 was reached. In total, 1 ml ice-cold 20 mg/ml N-deacetylated FG solution was added to 2 ml pre-cooling nitrous acid reagent in a reaction tube. The reaction was performed for different times in an ice bath, and the excess nitrous acid was destroyed by addition of 0.5 M NaOH until pH 8 was reached. Finally, the sample was dialyzed with a 1000 Da molecular weight cut-off and lyophilized as described above.

Different reaction times (4, 6, 8, 10, 15, 20, 25, 30 min) and reaction temperatures (room temperature and an ice bath) were investigated using 35% N-deacetylated FG according to the above representative protocol. The deaminative cleavage protocol was then investigated using native FG (M_w 69.930 kDa) and the depolymerized sample (M_w 13.710 kDa) by free radical method, and DFG samples of different M_w (5021–22240 Da) were prepared using deaminative cleavage for structure and activity measurements.

2.4. NMR analysis

NMR analyses were performed at 300 K with a 500 MHz Bruker Advance DRX 500 spectrometer (Bruker BioSpin GmbH) that had been equipped with a ^{13}C/^{1}H dual probe in the FT mode as described previously (Gao et al., 2012). The native, N-deacetylated and depolymerized FG samples were dissolved in deuterium oxide (99.9% D) at 10–20 mg/ml and lyophilized thrice to replace the exchangeable protons with deuterium.