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Antithrombotic activities of fucosylated chondroitin sulfates and their depolymerized fragments from two sea cucumbers

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ABSTRACT

Fucosylated chondroitin sulfate (FCS), a glycosaminoglycan extracted from the body wall of sea cucumber, is a promising antithrombotic agent. The chemical structures of FCS*c* isolated from sea cucumber Cucumaria frondosa and its depolymerized fragment (dFCSc) were characterized for the first time. Additionally, anticoagulant and antithrombotic activities were evaluated *in vitro* and *in vivo*. The results demonstrated that dFCS*c* exhibited better antithrombotic-hemorrhagic ratio than native FCS*c* on the electrical induced arterial thrombosis model in rats. Compared to FCS*t* obtained from Thelenota ananas, FCS*c* possessed different sulfation patterns but similar antithrombotic effects. Therefore, sulfation pattern of FCS might not affect anticoagulation and antithrombosis as much as molecular weight may. Our results proposed a new point of view to understand the structure-activity relationship of FCS as alternative agents.

1. Introduction

Thrombotic diseases are reported to contribute to 30% of early deaths globally (Mackman, 2008; Wang et al., 2014; Zhao et al., 2012). Arterial or venous blood clots can cause acute coronary syndrome (ACS) or venous thromboembolisms which are the 3rd leading cause of cardiovascular-event associated deaths (Mega & Simon, 2015). Many antithrombotic drugs including antiplatelets and anticoagulants are available for treating coagulopathies, and heparin (HP) is one of the most commonly prescribed drugs effective for reducing arterial and venous thrombosis in patients with cardiovascular disease. However, these compounds can cause unwanted bleeding that may limit their use and a contaminated batch of HP caused more than 100 deaths and many adverse reactions in the US and Germany (Pan et al., 2010). Low-molecular-weight heparins (LMWH) are also available and have less associated unwanted bleeding (Quinlan, McQuillan, & Eikelboom, 2004) and these drugs complement the diverse additional anticoagulants on the US market, but better drugs are always desired.

Fucosylated chondroitin sulfate (FCS) isolated from sea cucumbers is an important glycosaminoglycan (GAG), which had a chondroitin sulfate-like core backbone consisted of repeating units of β-1,4-glucuronic acid (→4GlcAβ1→) and β-1,3-*N*-acetylgalactosamine (→3GalNAcβ1→) with α-fucose branches linked to the *O*-3 position of GlcA residues (Fig. 1). The extent of GalNAc sulfation varied among sea cucumber species (Kariya, Sakai, Kaneko, Suzuki, & Kyogashima, 2002; Vieira, Mulloy, & Mourão, 1991; Wu et al., 2015; Yoshida, Minami, Nemoto, Numata, & Yamanaka, 1992). FCS isolated from *Ludwigothure agrisea* was 53% 6-sulfated GalNAc units (Vieira & Mourão, 1988), and the polysaccharide from *Stichopus japonicas* contained all 4,6-disulfated GalNAc residues (Nagase et al., 1995). Sulfation patterns of fucose branches differed between species and/or among preparations (Chen et al., 2011; Fonseca, Santos, & Mourão, 2009; Luo et al., 2013; Matsuhiro, Osorio-Román, & Torres, 2012; Mourão et al., 1996; Wu et al., 2012; Wu, Xu, Zhao, Kang, & Ding, 2010; Ye, Xu, & Li, 2012) and fucose side chains were non-, 3-, 4-, 2,4- or 3,4-disulfation patterns.

Sulfation patterns and structures of FCS accounted for anticoagulant and antithrombotic activity (Buyue & Sheehan, 2009; Chen et al., 2013; Fonseca et al., 2010; Fonseca & Mourão, 2006; Mourão et al., 2001; Mourão, Giumaraes, Mulloy, Thomas, & Gray, 1998; Pacheco, Vicente, Zancan, & Mourão, 2000; Zancan & Mourão, 2004; Zhao et al., 2013). Mammalian chondroitin sulfate without

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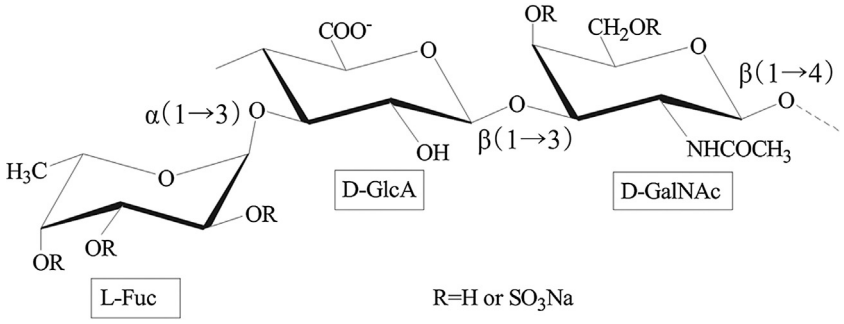

Fig. 1. The repeating units of FCS. FCS had a core backbone consisting of repeating units of β-D-GlcA and β-D-GalNAc with sulfated α-L-Fuc branches mainly linked to the O-3 position of GlcA residues.

fucosylated side chains had no anticoagulant or antithrombotic activity, and modifications such as desulfation or defucosylation eliminated this activity (Mulloy, Mourão, & Gray, 2000; Wu et al., 2012).

Chen's group compared anticoagulant activity of FCSs from four sea cucumber species with different sulfation patterns, and polysaccharides containing 95.9% 2,4-disulfated fucose branches had more anticoagulant effects (Chen et al., 2011). Additionally, FCS could reduce venous and arterial thrombosis in a rat model but unwanted bleeding was a problem (Fonseca et al., 2009). Depolymerization was considered effective for reducing bleeding side effect. Low-molecular-weight FCSs had antithrombotic activity with less bleeding than both UFH and LMWH (Kitazato, Kitazato, Nagase, & Minamiguchi, 1996; Wu et al., 2010; Yang, Wang, Jiang, Lv, &, Zhang et al., 2015; Yang, Wang, Jiang, & Lv, 2015; Zhao et al., 2015).

In this study, FCS*c* was isolated from Cucumaria frondosa, and its low molecular weight fragments were prepared with Cu^{2+} catalytic free-radical depolymerization. Physicochemical properties were analyzed by comparing this to FCS*t* from Thelenota ananas. Detailed structural information was characterized by disaccharide composition and nuclear magnetic resonance (NMR) analysis. Furthermore, anticoagulant and antithrombotic activity were evaluated *in vitro* and *in vivo* to understand the relationship between molecular weight, sulfation patterns, and biological activity of FCS.

2. Materials and methods

2.1. Reagents and animals

LMWH and HP from porcine intestinal mucosa were purchased from Sigma (St. Louis, MO). Q-Sepharose Fast Flow resin was purchased from GE Healthcare (Uppsala, Sweden). Antithrombin (AT), bovine FXa, human thrombin (FIIa), heparin cofactor II (HCII), chromogenic substrate S-2765 and S-2238 were purchased from Adhoc International Technologies Co., Ltd, (Beijing, China). All other chemicals and reagents were of analytical grade.

Male Wistar rats (250–270 g) and male Kunming mice (18–22 g) were purchased from Experimental Animals and Animal Experiments Center (Qingdao, China). Animals were maintained in a temperature-controlled room (24 ± 1 °C) and had free access to food and water before experiments and were acclimatized for at least one week. All experiments were performed in accordance to internationally accepted guidelines on laboratory animal use.

2.2. Extraction and purification of native FCS

Fucosylated chondroitin sulfates, FCS*c* and FCS*t*, were extracted from the body wall of the sea cucumbers C. frondosa and *T. ananas* respectively by methods (Chen et al., 2011) with some modifications. Briefly, the body wall of the sea cucumbers (100 g) was minced and digested with 0.5% papain (containing 5 mM EDTA and 5 mM cysteine, pH 5.7) at 60 °C for 12 h. The digested mixture was centrifuged and four volumes of ethanol were added into the supernatant. After centrifugation, the precipitate was dissolved in water. Then, 1 M HCl was added until a pH of 2.5 was obtained followed by centrifugation to remove acidic albumen. Three volumes of ethanol containing 2 M KAc were added into the supernatant and the polysaccharide was precipitated and collected by centrifugation. Crude polysaccharide was fractionated on a Q-Sepharose Fast Flow column, and eluted with a step-wise gradient of 0, 0.75, 1.5 and 2.1 M NaCl. FCS fractions eluted by 1.5 M NaCl were purified on a Sephacryl S-300 column with 0.1 M NH_4HCO_3. Finally, FCS fractions were pooled, dialyzed and lyophilized.

2.3. Preparation of low molecular weight FCS fragment (dFCS) by Cu^{2+} catalytic free-radical depolymerization

dFCS was obtained by controlled chemical depolymerization of native FCS induced by free radical as previously described (Wu et al., 2010) with modifications. Briefly, the native FCS (100 mg) was dissolved in 100 mL of 10% H_2O_2 solution (containing 1 mM $CuAc_2$, 100 mM K_2HPO_4, pH 7.5). The reaction was stirred at 35 °C for 3 h and stopped by addition of 10 mg of $EDTANa_2$. After centrifugation, four volumes of ethanol were added into the supernatant. The crude product was collected by centrifugation and washed with ethanol.

2.4. Molecular weight analysis

Molecular weight (Mw) and molecular weight distribution of FCSs were determined by high performance gel permeation chromatography (HPGPC) coupled with eighteen-angle laser scattering (MALLs) (Wu et al., 2010). This assay was performed on an Agilent 1260 LC system equipped with a Shodex OHpak SB-804 HQ (Showa Denko, Tokyo, Japan) at 30 °C with a flow rate of 0.6 mL min^{-1}. Signals were measured using a refractive index detector and eighteen-angle laser scattering.

2.5. Composition analysis

Aldohexuronic and acetamidoxyhexose contents of native FCSs and dFCSs were estimated by published methods (Blumenkrantz & Asboe-Hansen, 1973; Reissig, Storminger, & Lrloir, 1955). Sulfate content was measured with a $BaCl_2$-Gelatin method (Dodgson & Price, 1962) and monosaccharide composition was assessed using a 1-phenyl-3-methyl-5-pyrazolone (PMP)-HPLC method (Wang et al., 2012).

Disaccharide composition analysis was performed by mild acidic hydrolysis and enzymatic degradation (Mourão et al., 1996). The identification and quantitation of each disaccharide were