Carbohydrate Polymers 115 (2015) 677–685

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Screening of complex fucoidans from four brown algae species as procoagulant agents

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ARTICLE INFO

Article history:
Received 26 June 2014
Received in revised form 26 August 2014
Accepted 1 September 2014
Available online 7 September 2014

Keywords:
Hemostasis
Fucoidan
Hemophilia
NMR
Structure
Screening

ABSTRACT

Fucoidans are complex sulfated polysaccharides extracted from brown algae. Depending on the concentration, they have been shown to stimulate and inhibit blood coagulation *in vitro*. Promotion of coagulation is mediated by blocking tissue factor pathway inhibitor (TFPI). We screened fucoidan extracts from four brown algae species *in vitro* with respect to their potential to improve coagulation in bleeding disorders. The fucoidans' pro- and anticoagulant activities were assessed by global hemostatic and standard clotting assays. Results showed that fucoidans improved coagulation parameters. Some fucoidans also activated the contact pathway of coagulation, an undesired property reported for sulfated glycosaminoglycans. Chemical evaluation of fucoidans' complex and variable structure included molecular weight (*Mw*), polydispersity (polyD), structural heterogeneity, and organic and inorganic impurities. Herewith, we describe a screening strategy that facilitates the identification of crude fucoidan extracts with desired biological and structural properties for improvement of compromised coagulation like in hemophilia.

1. Introduction

Fucoidans are highly complex sulfated polysaccharides extracted mainly from brown algae (Berteau & Mulloy, 2003; Pomin & Mourao, 2008). Apart from mediating antiviral, antioxidant and anticancer effects, fucoidans modulate blood coagulation. In contrast to structurally related heparin, however, fucoidans show anticoagulant activity only at high concentrations (Liu et al., 2006). They were shown to be about 10-fold less active than heparin (Cumashi et al., 2007; Liu et al., 2006; Nishino, Nishioka, Ura, & Nagumo, 1994), having an anticoagulant effect above 100 nM (Liu et al., 2006). Therefore, fucoidans are referred to as non-anticoagulant sulfated polysaccharides (NASPs).

Interestingly, fucoidans also exert procoagulant activity by modulating the activity of TFPI, the major physiological inhibitor of the extrinsic coagulation pathway (Crawley & Lane, 2008). Procoagulant activity occurs at much lower concentrations and fucoidans were thus conceived as a novel class of compounds to improve coagulation (Liu et al., 2006; Prasad et al., 2008). Liu and colleagues determined a procoagulant window of 5 nM to 100 nM, which is in the range of 0.1–10 μg/mL depending on the *Mw*. This procoagulant window was recently confirmed in our laboratory (Zhang et al., 2014).

Fucoidans' procoagulant activity may be useful in treating bleeding disorders such as hemophilia A and B, caused by deficient clotting factors FVIII and FIX, respectively (Lillicrap, 2013). *In vitro* assays have shown fucoidans to reverse the prolonged plasma clotting time induced by TFPI at nanomolar concentrations and to

Abbreviations: NASP, non-anticoagulant sulfated polysaccharide; TFPI, tissue factor pathway inhibitor; TF, tissue factor; s.c., subcutaneous; p.o., per os (oral); *Mw*, molecular weight; polyD, polydispersity; OSCS, over-sulfated chondroitin sulfate; *L.j.*, *Laminaria japonica*; *F.v.*, *Fucus vesiculosus*; *U.p.*, *Undaria pinnatifida*; *E.m.*, *Ecklonia maxima*; CAT, calibrated automated thrombography; aPTT, activated partial thromboplastin time assay; CTI, corn trypsin inhibitor; SEC-MALLS, multi-angle laser light scattering; QELS, quasi-elastic light scattering; dRI, differential refractive index; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; TFA, trifluoroacetic acid; NMR, nuclear magnetic resonance; ICP, inductively coupled plasma; MS, mass spectrometry; AES, atomic emission spectroscopy; dPT, dilute prothrombin time; fl, full-length; *Mn*, number average molecular weight; NOE, nuclear Overhauser enhancement.

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http://dx.doi.org/10.1016/j.carbpol.2014.09.001
0144-8617/

accelerate clotting in human hemophilia plasma (Liu et al., 2006; Prasad et al., 2008; Zhang et al., 2014). Procoagulant activity of one fucoidan was demonstrated in bleeding models with hemophilia A mice and dogs after i.v., subcutaneous (s.c.) and oral (p.o.) dosing (Liu et al., 2006; Prasad et al., 2008).

Like most polysaccharides, fucoidans are structurally complex and heterogeneous. Large and disperse *Mw*, complex monosaccharide composition, various sulfation patterns, different linkages and high degree of branching contribute to this complexity (Berteau & Mulloy, 2003; Pomin & Mourao, 2008). Specific structural properties depend on algae species, harvest time, plant parts, location, and extraction procedures (Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2010) and influence fucoidans' pro- and anticoagulant activities (Ale, Mikkelsen, & Meyer, 2011; Morya, Kim, & Kim, 2012). Thus, systematic functional and structural assessment is necessary to identify a fucoidan with the desired procoagulant effect. While biological plasma-based test systems for evaluation of fucoidans' hemostatic effects have been established, their structural characterization poses a challenge. A desired fucoidan extract has procoagulant activity without activating the contact pathway of coagulation, is structurally homogenous, of low *Mw* to potentially increase s.c. and p.o. bioavailability, and contains few impurities. Contamination of heparin, one of the few marketed carbohydrate drugs, by oversulfated chondroitin sulfate (OSCS) (Guerrini et al., 2008; Kishimoto et al., 2008), initially went undetected as pharmacopeial methods to monitor the quality of its activity or structure were lacking (Capila & Linhardt, 2002; Linhardt, 1991). Thus, suitable and efficient strategies and methods to characterize fucoidan extracts are critical.

The aim of this study was to functionally and chemically characterize various fucoidan extracts available from a supplier to evaluate the potential of this naturally sourced substance for improvement of hemostasis. In follow-up studies the most suitable material would then be subjected to fractionation and detailed structural characterization (Zhang et al., 2014).

Pre-selection from a set of commercially available fucoidan extracts for their biological activities identified suitable fucoidans from four algae species: *Laminaria japonica* (*L.j.*), *Fucus vesiculosus* (*F.v.*), *Undaria pinnatifida* (*U.p.*) and *Ecklonia maxima* (*E.m.*). We then analyzed in detail their structural properties, impurities and effects on coagulation *in vitro*, thereby applying a systematic approach to identify the most active, highest quality fucoidan extracts (Fig. 1), and developed fucoidan-specific analytical assays.

2. Material and methods

Details are described in Supplemental information.

2.1. Fucoidans and other reagents

We tested fucoidans from four brown algae species: *L.j.* (Baxter Innovations GmbH, Austria), *F.v.*, *U.p.*, and *E.m.* (Marinova, Australia). For *F.v.*, three different lots were included, which were produced from the same seaweed harvest in two independent runs.

L.j. fucoidan was produced by several rounds of ethanol extraction after addition of EDTA. The other fucoidans were extracted by Marinova's proprietary method using dried and milled seaweed. Extraction was achieved by a cold-water process of targeted filtration without using solvents or excipients. All preparations mainly contain sodium cations.

Porcine heparin, alginate (Sigma-Aldrich, Austria) and oversulfated chondroitin sulfate (OSCS, US Pharmacopeia, USA) were included as controls where mentioned. All other chemicals and reagents were analytical grade.

2.2. Calibrated automated thrombography (CAT) assay

Fucoidans' pro- and anticoagulant activities were monitored by CAT developed by Hemker et al. (2003). This assay is based on the measurement of fluorescence generated by cleavage of a fluorogenic substrate by thrombin over time upon initiation of coagulation by tissue factor. Fucoidans were tested at concentrations of 0.02–300 μg/mL in FVIII-inhibited human plasma (Knappe et al., 2013). Their procoagulant effect was assessed by plotting peak thrombin against the concentration within the inclining part of the profile. The EC_{50} was derived using SigmaPlot 12 software from the resulting sigmoidal curve. To evaluate fucoidans' contact activation, CAT assays were performed in normal human plasma with and without corn trypsin inhibitor (CTI)—a specific inhibitor of the contact pathway initiator factor XIIa.

2.3. Activated partial thromboplastin time assay (aPTT)

The aPTT assay is a standard plasma clotting test in which clotting is initiated by a mixture of contact activators and phospholipids. It primarily assesses the intrinsic pathway of coagulation. aPTT was performed in normal human plasma as previously described (Liu et al., 2006). Assays were performed with an ACL Elite Pro instrument (Instrumentation Laboratory, USA). Samples were run in duplicate. aPTT (s) was plotted against fucoidan concentration (0–60 μg/mL); the concentration with a 50% increase in clotting time over baseline was reported.

2.4. Agarose gel analysis

Fucoidans were analyzed by agarose gel electrophoresis (Vieira, Mulloy, & Mourao, 1991; Volpi & Maccari, 2006). Samples (10–20 μg each) were loaded onto a 0.5% agarose gel in 0.04 M barium acetate buffer (pH 5.3) and run for 2 h at 100 mA in 0.05 M 1,3-diaminopropane-acetate (pH adjusted to 9.0 with acetic acid).

2.5. Average Mw and polyD determination using size-exclusion chromatography and multi-angle laser light scattering (SEC-MALLS)

Fucoidans' *Mw* and polydispersities were measured by an Agilent HPLC System coupled with Wyatt Technology DAWN HELEOS, QELS (quasi-elastic light scattering) and Optilab rEX differential refractive index (dRI) detectors. The change in refractive index/concentration (d*n*/d*c*) value (0.113 mL/g) was determined using a *F.v.* sample.

2.6. Monosaccharide analysis using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Fucoidans (2 mg/mL) were hydrolyzed to monosaccharides using 2 M trifluoroacetic acid (TFA) at 100 °C for 4 h. The monosaccharide compositions were analyzed by a Dionex ICS 3000 system (Dionex, USA) equipped with PAD detector, Dionex guard column CarboPac® PA1 (2 × 50 mm), and Dionex analytical column CarboPac® PA1 (4 × 250 mm).

2.7. Fucose, alginate contents and heterogeneity test using ^{13}C nuclear magnetic resonance (NMR)

Quantitative ^{13}C NMR spectra were obtained with a Bruker Avance III 600 NMR spectrometer at a $^{1}H^{13}C$ frequency of 150 MHz with a dual $^{1}H/^{13}C$-cryoprobe.

Alginate content (% mol alginate/mol total polysaccharide) was calculated based on the fact that alginate contains one carbonyl