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Sulfation of citrus pectin by pyridine-sulfurtrioxide complex and its anticoagulant activity

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ABSTRACT

Citrus pectin (CP) was sulfated by the pyridine–sulfur-trioxide complex in dimethyl sulfoxide (DMSO). Monosaccharide composition analysis revealed a decrease in the GalA content after sulfation. A decrease in the average molecular weight (MW) and a fall in particle size of the pectin gave additional proof of pectin degradation during the sulfation reaction. Structural characterization by IR and NMR spectra indicated sulfation occurred mainly at positions C-2, C-3 of the GalA (located in backbone region of the CP). Anticoagulant assays demonstrated that sulfated CP (TBA-3) could prolong activated partial thromboplastin time and thrombin time, with an activity of 51.96 IU/mg and 15.2 IU/mg, respectively. Further investigation on coagulation factors indicated TBA-3 could achieve inactivation of thrombin with both heparin cofactor II and antithrombin. Our results indicated sulfated pectin might be a promising anticoagulant ingredient with excellent activity and simple monosaccharides, and could be a possible substitute for the limited heparin.

1. Introduction

Heparin and its derivatives are well-known sulfated polysaccharides and have been used as a clinical drug for many years. It has been reported that heparin possesses a wide range of biological activities, e.g., anti-inflammatory, and anti-metastasis effects (Melo, Pereira, Fogue, & Mourão, 2004; Mulloy, Hogwood, & Gray, 2010; Wei et al., 2004; Young, 2008). However, the high risk of bleeding has limited its use in other therapeutic applications. Other disadvantages of heparin and its derivatives stem from their animal origin and the lack of a plentiful and reliable supply (Groth, Grünewald, & Alban, 2009). The relevance of the latter problem was recently made public by the "heparin scandal" or the sale of counterfeit heparin (Guerrini et al., 2008). The adulteration of heparin with oversulfated chondroitin sulfate was the pivotal event that initiated research to ultilize natural sulfated polysaccharides or to develop safe chemically sulfated polysaccharides.

The natural sulfated polysaccharides, such as sulfated fucans and sulfated galactans obtained from marine invertebrates, have been reported to show excellent biological activities (Chen et al., 2011; Vitor & Mourão, 2008). However, these polysaccharides are usually found in species that have limited numbers. The second approach was to chemically sulfate a non-sulfated polysaccharide found in nature. For example, the sulfation of glucan was shown to effectively promote its antithrombotic activity *in vivo* (Alban, Jeske, Welzel, Franz, & Fareed, 1995, Alban, Schauerte, & Franz, 2002; Martinichen-Herrero et al., 2005).

Pectin, also known as pectic polysaccharides, is mainly composed of galacturonic acid. Several distinct regions have been identified within the pectin: homogalacturonan (HG, α-(1-4)-D-galacturonic acid (GalA) units having various degree of methyl-esterification), rhamnogalacturonan I (RGI, (1-2)-α-L-Rha-(1-4)-α-D-GalA disaccharide units), and rhamnogalacturonan II (RGII, a HG backbone with complex side chains) (Maxwell, Belshaw, Waldron, & Morris, 2012; Pelloux, Christine, & Mellerowicz, 2007). The pectin is readily available in nature. It has been used in food mainly as a gelling agent in jams and jellies as well as a stabilizer in fruit juices and milk drinks (Cipriani et al., 2009). Furthermore, it has shown various biological activities and has been used in pharmaceutical applications (Srivastava & Malviya, 2010). The simple backbone structure and high content of GalA in pectin would makes its production uncomplicated and would also make it easier to establish quality control parameters. Thus, its chemical derivations, such as amide, formed by reacting carboxylic acid with ammonia,

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have shown a great potential for industry use. (This sentence is not clear. How amide formation is relevent to sulfation?).

The chemically sulfated pectin has been reported to have anticoagulant activities (Nadiezda, 2012; Cipriani et al., 2009; Vityazev et al. 2010). However, the conventional sulfation reaction is performed under harsh conditions using chlorosulfonic acid as the sulfating agent (Vityazev et al. 2010). These harsh condition produces severe changes in the pectin structure, and alters the bioactivity of the sulfated derivatives. It was very clear that a milder and more energy efficient method was required.

In our previous study, we sulfated squid ink polysaccharides with pyridine-sulfurtrioxide complex dissolved in dimethyl sulfoxide (DMSO) and discovered that the polysaccharides molecules underwent less degradation and showed good anticoagulant activities (Chen et al., 2010). In the present study, the same mild system (pyridine-sulfurtrioxide complex in DMSO) was used to derivatized citrus pectin (CP). The composition of the sulfated CP was determined by high-performance liquid chromatography (HPLC), and its detailed structure was investigated by infrared (IR) and NMR spectroscopy. The anticoagulant properties of the sulfated CP, was measured by activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), inhibition of thrombin by heparin cofactor II (HC II) and antithrombin (AT).

2. Experimental protocol

2.1. Material

The CP was obtained from Danisco (*Copenhagen*, Denmark) and stored at −20 °C until used. A TSK G4000 PWXL column was obtained from TOSOH BIOSEP (Tokyo, Japan) and Sephacryl S-300 gel was from Amersham Biosciences (Uppsala, Sweden). The monosaccharides D-mannose (Man), L-fucose (Fuc), rhamnose (Rham), L-arabinose (Ara), D-galactose (Gal), D-galactosamine (GalN), D-glucosamine (GlcN), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), and disaccharide lactose (Lac), and sulfating agent pyridine-sulfurtrioxide were obtained from Sigma–Aldrich (St. Louis, MO, USA). Thiobarbituric acid (TBA) and DMSO were obtained from Jiangsu Pharmacia (Jiangsu, China). The derivatization reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Sinopharm Chemical Reagent (Shanghai, China). The sulfation reagent was prepared by dissolving 6 g pyridine-sulfurtrioxide in 12 mL DMSO.

2.2. Sulfation of the citrus pectin

2.2.1. Sulfation of pectin by pyridine-sulfurtrioxide complex

2 g of CP was dissolved in 50 mL of distilled water, and fractionally precipitated with absolute ethanol and 80% (v/v) ethanol. The process was repeated twice and the final precipitate taken up in acetone (20 mL). The dispersed sample in acetone was sonicated for 10 min, centrifuged (3000 *g*, 15 min), dried by nitrogen gas, and labeled as Sample 1.

The sulfation reaction was performed as follows: Sample 1 (200 mg) was suspended in dry DMSO (10 mL) by stirring for 30 min at 80 °C and then followed with the addition of 4 mL sulfation reagent. The reaction mixture was incubated at 80 °C with stirring for 1, 2, and 3 h. At each timed interval, the reaction mixture was cooled to room temperature in an ice bath, then dialyzed against distilled water (Spectra/Por membrane, MWCO. 1000) for 72 h. The dialyzate was freeze-dried and weighed. About 230 mg of sulfated CP was obtained from each reaction mixture having reaction times of 1 h, 2 h and 3 h. These CP samples were labeled DMSO-1, DMSO-2, and DMSO-3, respectively.

2.2.2. Sulfation of pectin-TBA salt by pyridine-sulfurtrioxide complex in DMSO

Commercial CP (200 mg) was dissolved in 50 mL of distilled water and passed through an ion-exchange column (50 mL, Amberlite IR-120H1). The eluant was collected and the pH adjusted to 6.0–6.5 with TBA and lyophilized to obtain the TBA salt of CP.

The pectin TBA salt (200 mg) was suspended in dry DMSO (10 mL) and stirred at room temperature for 30 min. The reaction was started by adding 6 mL sulfation reagent. The reaction mixture was incubated at 80 °C with stirring for 1, 2, and 3 h. At each timed interval the reaction mixture was poured into ice-cold water (200 mL), and dialyzed against distilled water (Spectra/Por membrane, MWCO. 1000) for 72 h. The dialyzate was freeze-dried and weighed. About 260 mg of each sulfated CP sample, prepared with three different reaction times, were obtained and labeled TBA-1, TBA-2, and TBA-3 respectively.

2.3. Determination of chemical compositions

The monosaccharides composition of the chemically modified CP was determined by the PMP-HPLC method (Strydom, 1994). In brief, the polysaccharide (typically 1 mg) was hydrolyzed with 2 mol/L TFA at 110 °C under nitrogen for 8 h with added lactose as the internal standard. The monosaccharide hydrolyzate was dried under vacuum and then derivatized with 450 μL PMP solution (0.5 mol/L in methanol) and 450 μL NaOH solution (0.3 mol/L) at 70 °C for 30 min. The reaction was stopped by neutralizing with 450 μL of 0.3 mol/L HCl and then extracted three times with chloroform (1 mL). HPLC analyses were performed on an Agilent ZORBAX Eclipse XDB-C18 column (5 μm, 4.6 mm × 150 mm) at 25 °C n on a Alliance 2695 separations module (Waters) linked simultaneously to a 2489 UV detector, the detector wavelength was 250 nm. The mobile phase was 0.05 mol/L KH_2PO_4 (pH 6.9) with 15% acetonitrile (solvent A) and 40% acetonitrile (solvent B) in water. The solvent B gradient used was from 8% to 19% for 25 min.

The sulfate content was determined by turbidimetry, according to Dodgson's method (Dodgson & Price, 1962).

2.4. Determination of average particle size

The average particle size and distribution of pectin in solution were determined by LS-230 Coulter (Beckman Coulter, California, USA). The solutions were diluted to a concentration of 0.5 mg/mL with deionized water, and all measurements were carried out at 25 °C.

2.5. NMR spectroscopy and IR spectroscopy

For NMR analysis, the chemically sulfated CP or native polysaccharide (50 mg) were co-evaporated twice by lyophilizing with D_2O (99.8%) before final dissolution in 500 μL high-quality D_2O (99.96%) containing 0.1 μL acetone. The ^{13}C NMR experiments were carried out at 150 MHz. The observed ^{13}C chemical shifts were relative to internal acetone (31.1 ppm). The polysaccharides (0.5 mg) were prepared as KBr pellets and their IR spectra were acquired a Perkin–Elmer 1000 FT-IR spectrometer at room temperature.

2.6. Anticoagulant assay

Healthy human blood was donated by a 26 year old man and all procedures were performed according to the "Guidance for the Use of Human Blood" published by Zhejiang Provincial Government. The collected human blood was mixed with a solution of 3.8% sodium citrate. The plasma was separated by centrifugation at 3000 × *g* for 10 min. The anticoagulant assays, including activated partial thromboplastin time (APTT) (assay kit from Organon-Tecknica, Fresnes, France), thrombin time (TT) (5 NIH U/mL