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Oxidative degradation of the sulfated polysaccharide isolated from sea cucumber *Holothuria nobilis*

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ABSTRACT

In this study, a sulfated polysaccharide with a high molecular weight was isolated from sea cucumber *Holothuria nobilis*. It is a fucosylated chondroitin sulfate and being named as HOP. We investigated the effects of several processing variables on the oxidative degradation of HOP using fractional factorial design (FFD) and central composite design (CCD). Moreover, the conditions of the hydroxyl free radical-induced degradation were optimized using response surface methodology (RSM). Our data showed that $R_{(H_2O_2-HOP)}$, reaction pH and H_2O_2 flow rate could significantly ($P < 0.05$) affect the degree of hydrolysis (DH) of HOP. The optimum conditions with Fe^{2+} were found as follows: $R_{(H_2O_2-HOP)}$ of 0.53; reaction pH of 6.91; H_2O_2 flow rate of 0.40 mL/min; reaction time of 2 h; reaction temperature of 30 °C; and HOP concentration of 4 mg/mL. Under these optimum conditions, the DH of HOP was 94.173 ± 0.232 (%), which well matched the value (94.152%) predicted by the RSM model. The preliminary structural characterization of *o*-HOP was analyzed. The results showed that *o*-HOP consisted of β-D-glucuronic acid, β-D-N-acetyl-galactosamine, α-L-fucose and sulfate groups. The specific rotation of *o*-HOP was -43.2°. Furthermore, the sulfation patterns of fucose residues in *o*-HOP were 2,4-O-disulfated fucose, 3-O-sulfated fucose, 4-O-sulfated fucose and non-sulfated fucose, which were consistent with HOP. In addition, we found that the *in vitro* antitumor activity of the degraded HOP fraction (*o*-HOP) was higher than that of HOP against human gastric carinoma SGC-7901 cells.

1. Introduction

As a fucosylated chondroitin sulfate (Fcs) isolated from sea cucumber, HOP consists of alternating residues of β-D-glucuronic acid and N-acetyl-β-D-galactosamine with sulfated fucose branches [1,2]. In different types of sea cucumbers, the patterns and proportions of sulfate in fucose branches are different [3]. Moreover, fucosylated polysaccharide sulfate (HOP) has been purified from sea cucumber *Holothuria nobilis* in our laboratory. In our previous study, we have investigated the structural characterizations of HOP by IR, ^1H and ^{13}C NMR spectra and methylation analysis as well as its anticoagulant activities by prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT) and fibrinogen (FBG) analysis [4].

Data showed that HOP possesses good anticoagulant activity [4]. However, previous studies have shown that polysaccharides with large molecular weights can cause a number of problems in the application, such as high viscosity and low permeability into cells [5,6]. Moreover, more accurate information about its structural characterizations could not be acquired due to the high molecular weight of HOP. Oligosaccharides of HOP must be prepared for further NMR analyses in order to explore advanced structures. Fortunately, a low-molecular-weight derivative can minimize these limitations [7].

There are many methods to prepare the oligosaccharides. Previous studies have reported the polysaccharide depolymerization through physical, chemical and enzymatic treatments, such as ^{60}Co irradiation [8], hermetical microwave degradation [9], acid hydrolysis [10], free-radical depolymerization [11] and enzymatic depolymerization [12]. Among these procedures, mild acid hydrolysis can easily induce partial desulfation and loss of sulfated fucose branches of HOP [3,11]. Mild acid hydrolysis can also lead to an impaired anticoagulant activity of HOP [13]. Moreover, physical method is less desired due to its poor cost efficiency. In addition, enzymes are more sensitive to the reaction environment [11]. In contrast, free-radical depolymerization selectively breaks

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glucuronic acid units in the HOP backbone, with no effects on sulfated fucose branches [11], and only inexpensive reagents are required, resulting in its suitability for large-scale applications.

The degree of hydrolysis (DH) of HOP can be affected by many factors, such as metal ions, H_2O_2–polysaccharide ratio, H_2O_2 flow rate, reaction pH, reaction time, reaction temperature and polysaccharide concentrations. Response surface methodology (RSM) is a useful statistical and mathematical technique for the investigation of complex processes, including fermentation, hydrolysis and chemical reactions [14,15]. Fractional factorial design (FFD), central composite design (CCD) and RSM can provide sufficient information with reduced number of experimental runs, which is the main advantage [16]. Therefore, RSM is less arduous and time-consuming compared with those classical methods [17].

In this study, we, for the first time, attempted to optimize the hydroxyl free radical-induced degradation conditions of HOP using RSM. A two-step design, including FFD and CCD, was used to optimize the degradation reaction. FFD was used to screen the most important factors of the degradation reaction. CCD was used to optimize the most important factors in order to maximize the DH of HOP [18]. In addition, we also compared the anticancer activities of HOP and the degraded HOP fraction (*o*-HOP) against human gastric carinoma SGC-7901 cells *in vitro*.

2. Materials and methods

2.1. Materials

Sea cucumber *H. nobilis* were purchased from a local market in Jiangsu Province, China. DEAE-Sepharose Fast Flow and Sephadex G-100 resin were provided from GE Healthcare (Beijing, China), and Bio-Gel P4 resin was obtained from Bio-Rad. Papain (1.5–10 units/mg), the derivatization reagent 1-phenyl-3-methyl-5-pyrazolone (PMP), the carbohydrate standards, including D-mannose, L-fucose, L-arabinose, D-galactose, D-galactosamine, D-glucosamine, D-glucuronic acid, D-galacturonic acid, lactose and chondroitin sulfate A (bovine trachea), were supplied by Sigma (Shanghai, China).

2.2. Collection and physicochemical properties of native HOP

The crude polysaccharides in the body walls of sea cucumber *H. nobilis* were obtained previously in our laboratory [4,8,1]. Briefly, the body wall of sea cucumber *H. nobilis* was ground into powder. The powder was treated with 6% NaOH at 6 °C for 24 h. Subsequently, proteins associated with the polysaccharides were digested with 1% papain of pH 6.5 at 55 °C for 5 h and then removed from the supernatant using 15% trichloroacetic acid. Finally, the crude polysaccharides in the residual supernatant were precipitated with 95% ethanol at 4 °C overnight and obtained by freeze-drying. For the separation and purification of native HOP, anion-exchange chromatography DEAE-Sepharose Fast Flow column (1.2 cm × 20 cm) and gel filtration chromatography Sephadex G-100 column (1.5 cm × 100 cm) were used separately. These two columns were packed according to the instruction manual. And the constant-flow pump and automatic sampling instrument were provided by Hu Xi (Shanghai, China). Then the crude polysaccharides applied onto the anion-exchange chromatography were eluted by a linear gradient of 0–2 M NaCl solution at a flow rate of 1.0 mL/min [19,20]. The eluted fractions were monitored by metachromatic property using 1,9-dimethylmethylene blue (DMB) at 525 nm [21] and phenol/sulfuric assay at 490 nm [22]. Native HOP of high purity was collected after a further purification of the fractions on the gel filtration chromatography. The molecular weight of HOP was determined as 135.8 kDa by high-performance liquid chromatography (HPLC) gel permeation chromatography on Agilent PL aquagel-OH MIXED-H column (Agilent 1100 system, Palo Alto, CA, USA) with Dextran T-4.32, 12.6, 60.6, 110, 289 and 500 as standards. It was consistent with the previous findings by our group [4].

Table 1
Factors and levels of FFD applied in degradation of HOP.

Independent variables	Variable name	Coded levels		
		−1	0	+1
X_1	*R*	0.2	0.4	0.6
X_2	pH	6	7.5	9
X_3 (mL/min)	H_2O_2 flow rate	0.2	0.5	0.8
X_4 (h)	Time	2	4	6
X_5 (°C)	Temperature	20	30	40
X_6 (mg/mL)	HOP concentration	2	4	6

R, H_2O_2 to HOP ratio.

2.3. Free-radical depolymerization with different metal ions

Hydroxyl radicals (•OH) inducing the HOP degradation were produced using the Fenton-type oxidative system. Various metallic catalysts were used to investigate the free-radical depolymerization, including manganese (II) chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$), copper (II) chloride dihydrate ($CuCl_2{\cdot}2H_2O$), iron (II) dichloride ($FeCl_2$) and cuprous (I) chloride (CuCl). Briefly, 320 mg HOP was dissolved in 80 mL of distilled water, and 100 μL of 0.25 mM metal salt solution was added into 5 mL polysaccharide solution and maintained at 30 °C. An aqueous solution of H_2O_2 (0.033%, w/v) was then added at a flow rate of 12 mL/h during a period of 2 h. The pH of the reaction mixture was maintained at pH 7.5 using 1 N NaOH solution. The reaction was terminated using $NaBH_4$ solution [11,23]. Subsequently, the mixture was concentrated, desalted by ultrafiltration with a 1000-Da cutoff membrane and freeze-dried. Depolymerized HOP fractions were obtained with a yield ranging from 75%-95%.

The depolymerized fractions were eluted by HPLC (Agilent 1100 system, Palo Alto, CA, USA) from an Agilent PL aquagel-OH MIXED-H column (Agilent, USA) using 0.2 M NaCl as the elution buffer. The molecular weights of the obtained depolymerized fractions were determined by a refractive index detector. The same experiment was performed in triplicate using the same batch of HOP.

2.4. FFD

Many factors can affect the depolymerization of polysaccharides, such as the *R* value (H_2O_2–polysaccharide ratio), H_2O_2 flow rate, reaction pH, reaction time, reaction temperature and HOP concentrations. The FFD was used to identify the most important variables for the depolymerization in order to minimize the number of experiments [18]. The variables were coded according to the following equation (Eq. (1)):

$$\chi_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where χ_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the central point, and ΔX_i is the step change value. Table 1 lists the variable values for FFD.

The DH of HOP was calculated as follows (Eq. (2)):

$$\text{DH}\ \ (\%) = \frac{A - B}{A} \times 100 \quad (2)$$

where *A* is the molecular weight of HOP, and *B* is the molecular weight of oligosaccharide prepared *via* free radical-induced degradation. Moreover, the DH of HOP (*Y*) represents the response function. FFD and statistical analysis were performed using Minitab 16.0. Table 2 shows the experiment design and its corresponding