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Enzyme-assisted extraction of anticoagulant polysaccharide from *Liparis tessellatus* eggs

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ABSTRACT

This study aimed to recover a heparin-like anticoagulant polysaccharide from *Liparis tessellatus* eggs (PLE) by using enzyme-assisted extraction technique. Extraction experiments were carried out using three different enzymes (Alcalase®2.4L, Flavourzyme®500MG, and Protamex®) under different conditions of temperature (45, 50, and 55 °C), pH (6.5, 7.0, and 7.5), incubation time (24, 36, and 48 h), and enzyme to substrate ratio (*E*/*S* = 0.5, 1.0, and 1.5%, w/w), which were combined according to a D-optimal design. Statistical analysis of extraction results allowed identifying the variables with greater influence on the extraction yield, and selecting the conditions that maximize the PLE extraction. The best extraction results were achieved when using the Protamex® enzyme in an *E*/*S* ratio of 1.34% (w/w), pH 6.60, 47.40 °C, during 26.50 h. Under these conditions, a polysaccharide yield of 2.10% (w/w) was obtained. Clotting time measurements, activated partial thromboplastin time, and prothrombin time for evaluation of the anticoagulant properties of PLE were determined and showed increasing activities in correlation with the concentrations used. In the final step, the heparin-like nature of PLE was confirmed by digestion with heparinases I, II, and III, which showed ΔDiHS-0S, ΔDiHS-6S, ΔDiHS-diS1, and ΔDiHS-diS2 at compositions of 0.04, 0.03, 0.35, and 0.24 mol/g, respectively.

1. Introduction

Liparis tessellatus or 'cubed snail fish' is a marine, demersal fish usually found in the cold waters of Korea, Japan, China and Russia [1]. This fish has low lipid content (0–0.10%) [2] and its eggs show high carbohydrate content (19.50 ± 1.1%). Among the carbohydrates present in the membrane of the *L. tessellatus* eggs, mucopolysaccharides are one of the most important since they have anticoagulant properties [3] with potential for application in the treatment of some diseases. Cardiovascular diseases, for example, occur due to the obstruction in the heart and blood vessels as a result of thrombosis [4,5], and require anticoagulant therapy.

In the last years, marine organisms have been considered a potential source to obtain bioactive compounds and efforts have been directed in order to identify marine species rich in such compounds, as well as in establishing technologies for their extraction from these sources. Some studies have demonstrated the possibility of extracting polysaccharides with anticoagulant properties from marine organisms such as the green seaweed *Caulerpa cupressoides* [5], and tissues of *Styela plicata* [6]. *L. tessellatus* eggs contain polysaccharides with anticoagulant properties, but the conditions that allow obtaining maximum extraction of these compounds from the eggs' membranes need still to be established.

Several technologies can be used for the extraction of components from natural sources, including solid–liquid extraction, microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluids extraction, enzyme-assisted extraction, solid-state fermentation, and high pressure processes [7,8]. Among such techniques, solid–liquid extraction using hot water and enzyme-assisted extraction are the most commonly utilized for the polysaccharides recovery. However, when compared to the enzyme-assisted extraction, the hot water technology present certain limitations because sugar degradation may occur decreasing the extraction yield. Additionally, high energy spent is required due to the use of high temperatures. Enzyme-assisted extraction is

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considered a milder, more convenient, efficient and environment-friendly technology, with lower investment costs and energy requirement than solid–liquid extraction, and has been demonstrated to be effective in improving the yield of the target compound [9].

Based on the abovementioned reasons, the objective of the present study consisted in selecting the conditions of enzyme-assisted extraction to maximize the recovery of anticoagulant polysaccharides from *L. tessellatus* eggs. The extraction experiments were carried out using different enzymes under different conditions of temperature, pH, incubation time, and enzyme to substrate ratio, which were combined according to a D-optimal design. The yield of polysaccharides from *L. tessellatus* eggs was taken as response of the statistical design. Finally, the anticoagulant properties and characteristics of the recovered product were determined.

2. Materials and methods

2.1. Materials and chemicals

Liparis tessellatus eggs (LTE) were purchased from a fresh market in Tongyeong, Korea. The fish eggs were washed with distilled water and freeze-dried to be stored. Alcalase®2.4L, Flavourzyme®500MG, and Protamex® enzymes were obtained from Novozymes, Denmark. D-glucose, chondroitin-6-sulfate, bovine serum albumin (BSA), phenol, Folin-ciocalteau reagent, dimethyl methylene blue (DMMB), and heparinases I, II, and III were purchased from Sigma–Aldrich Chemicals (MO, USA). The unsaturated disaccharide standards for HPLC: ΔDiHS-0S (ΔUA1 → 4GlcNAc), ΔDiHS-NS (ΔUA1 → 4GlcNS), ΔDiHS-6S (ΔUA1 → 4GlcNAc (6S)), ΔDiHS-diS1 (ΔUA1 → 4GlcNS (6S)), ΔDiHS-diS2 (ΔUA(2S)1 → 4GlcNS), and ΔDiHS-triS (ΔUA(2S)1 → 4GlcNS(6S)) were provided from Seikagaku Co. (Tokyo, Japan). The heparin standard for anticoagulation assay was donated by the Geoje Baek General Hospital (Geoje, Korea). All other chemicals were of analytical grade.

2.2. Extraction of polysaccharide from L. tessellatus eggs (PLE)

Ground, freeze-dried LTE samples were initially defatted with acetone (samples were fully soaked in acetone and placed in a shaking incubator at 150 rpm (25 °C) during three days, the solvent being changed to fresh one every day. The solid residues after this process were separated by centrifugation (3000 rpm, 15 min) using an MF 600 Plus large capacity centrifuge (Hanil Science Industrial, Korea). The defatted LTE samples were then washed with deionized water, centrifuged again, and the precipitates were lyophilized for further use in the extraction experiments.

For the enzyme-assisted extraction, 2 g of defatted and lyophilized LTE were added to 20 mL of 50 mM phosphate buffer adjusted to different pH values (6.5, 7.0 or 7.5). Then, the enzyme (Alcalase®2.4L, Flavourzyme®500MG or Protamex®) was added to the mixture to obtain an enzyme to substrate ratio of 0.5, 1.0 or 1.5% (w/w), and the reaction was maintained at 45, 50 or 55 °C, during 24, 36 or 48 h. After extraction, the reaction medium was heated at 90 °C for 20 min to deactivate enzymes and centrifuged (3000 rpm, 15 min) to separate the supernatant, which was then deproteinated (by 60% ammonium sulphate saturation), dialyzed against deionized water, and concentrated under vacuum at 40 °C to °Brix 9. Four volumes of anhydrous ethanol were added to the concentrate in order to precipitate the polysaccharides and the mixture was maintained overnight at 25 °C. The precipitated polysaccharide was then separated by centrifugation (4000 rpm, 30 min), and washed repeatedly with anhydrous ethanol until clear precipitates were obtained. The extracted polysaccharides were freeze-dried and weighed up to constant mass. The PLE yield was calculated using the Eq. (1), where M_{PLE} is the mass of PLE (in g) recovered after ethanol precipitation, and M_{LTE} is the mass of LTE (in g) used in the experiment. All the experiments were carried out in triplicate and mean values are reported.

$$\text{PLE yield } (\%) = \frac{M_{PLE}}{M_{LTE}} \times 100 \quad (1)$$

2.3. Statistical experimental design

A D-optimal experimental design was used to evaluate the effects of four quantitative variables (extraction temperature, pH, incubation time, and enzyme/substrate (*E*/*S*) ratio), and one qualitative variable (enzyme) on the enzyme-assisted extraction of PLE. Thirty six randomized experimental runs were carried out. The conditions of the variables used in each experimental assay are given in Table 1. The values of the variables that maximized the extraction results were determined using regression equations and 3D-response surface plots. Design Expert (Trial 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, USA) was the software used for data analysis.

2.4. Analytical methods

Total carbohydrate content was estimated by the phenol-sulphuric acid method using D-glucose as standard [10]. Protein content was measured by the Bradford assay using BSA as standard [11]. Sulphates were assessed by metachromatic assay using DMMB with chondroitin-6-sulfate as standard [12].

The anticoagulant effect of PLE was evaluated using the activated partial thromboplastin time (APTT) and prothrombin time (PT) assays using an ACL 10000 Automated Coagulation Analyser (MYCO Instrumentation, Inc., USA). Briefly, blood samples were drawn by venipuncture at the antecubial fossa of forearm of healthy donors ($n = 8$), mixed with 0.109 M trisodium citrate, and centrifuged (2000 rpm, 10 min) to obtain platelet poor plasma. Then, 100 μL of this plasma were incubated with PLE at varying concentrations (25–100 μg/mL) and supplemented with calcium chloride to initiate clotting on it. APTT and PT were recorded on the coagulometer (time in seconds were automatically recorded by the machine in the first appearance of fibrin clot formation). Heparin was used as standard, and phosphate buffered saline solution was used as control.

To determine the unsaturated disaccharide composition of the anticoagulant polysaccharide, PLE was subjected to enzymatic degradation using heparinases I, II, and III from *Flavobacterium heparinum* (Sigma–Aldrich Chemicals, Missouri, USA) [13] and analyzed using SAX-HPLC with a Phenomenex SAX 80 column (250 mm × 4.60 mm), mobile phase (Eluent A: 0.2 M NaCl in 2.5 mM sodium phosphate buffer, pH 3.5; Eluent B: 1.2 M NaCl in 2.5 mM sodium phosphate buffer, pH 3.5) at a gradient flow rate of 1.0 ml/min, at a detection wavelength of 232 nm. The peaks detected on PLE were compared with the chromatogram obtained for unsaturated disaccharide standards using the same SAX-HPLC conditions. Standard curves were prepared for the six common unsaturated disaccharide standards to determine the relative composition of PLE.

3. Results and discussion

3.1. Selection of the extraction conditions for maximum PLE recovery

The PLE yield strongly varied (from 0.10% to 1.96%) according to the conditions used for extraction (Table 1). The type