Extraction and Separation of Polysaccharides from Laminaria japonica by Size-Exclusion Chromatography

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Received 20 November 2013; revised 27 April 2014

A large number of studies have suggested that polysaccharides, such as fucoidan and laminarin, in various seaweeds have significant biological properties. A different distribution of molecular weights is a prominent sign of many polysaccharides. Therefore, a simple, fast and reliable high-performance size-exclusion chromatography (HPSEC) method was proposed to separate fucoidan and laminarin from Laminaria japonica. After evaluating the different separation conditions for HPSEC, such as the type of mobile phase and flow rate, an acid extraction method was established and optimized by a systematic investigation of the influencing factors. Under the optimal conditions, 169.2 and 383.8 mg g\(^{-1}\) of fucoidan and laminarin, respectively, were extracted. This method is suitable for the extraction and separation of polysaccharides with good reproducibility of the retention time, acceptable linearity, small relative standard deviation and low detection limits.

Introduction

Seaweeds are promising plants that have been used increasingly as important sources of animal feed, human food, fertilizers and pharmaceutical products. As the oldest type of plants on earth, seaweeds are not only easily renewable, which has made them tenacious and prolific, but also possess high levels of vitamins, minerals, amino acid and polysaccharides. Among these, polysaccharides have attracted considerable attention for their many biological activities. In contrast to red seaweeds, whose soluble polysaccharides are sulfated galactans, carrageenans and agar, the soluble polysaccharides in brown seaweeds are mainly laminarin, fucoidans and alginates (1).

Fucoidan and laminarin are believed to be the main water-soluble polysaccharides in brown seaweeds. Fucoids (Figure 1A), a special type of sulfated fucan, are found in almost all brown seaweeds examined thus far but are absent in red seaweeds, green seaweeds or other plants (2). Fucoids have been reported to have potential functions on cerebral ischemia, Alzheimer’s disease, cardiovascular disorders, renal disease and several cancers (3–6). Fucoids are soluble in both water and acid solutions. The reported molecular weights of fucoids are between 100 and 1,600 kDa (7). The other major sugar in seaweed is laminarin, which is composed of glucose monomers joined by \(\beta\)-1,3-glycosidic bonds with \(\beta\) (1, 6) branching (8). Laminarin has two types of chains (G or M) according to their reducing end. G chains are terminated with a glucose residue, whereas M chains are terminated with a mannitol residue (Figure 1B). Depending on the degree of polymerization, the molecular weight of laminarin is \(\sim\)5,000 Da (7). Laminarin exhibits promising immunoregulation, antibacterial and antioxidation ability and plays a role in improving intestinal health (8–10).

The evidence shows that the molecular weight of water-soluble polysaccharides can alter the bioactivity to a certain extent (11). Several studies have examined the molecular weight of polysaccharides because the molecular weight plays an important role in the exploration of bioactivity. Moreover, a large molecular weight is a characteristic of polysaccharides, and several mature measurement techniques have been used. These include light scattering, size-exclusion chromatography (SEC), intrinsic viscosity and sedimentation analysis in analytical ultracentrifugation methods (12). Therefore, the use of using molecular weights of laminarin and fucoidan to separate crude polysaccharides appears feasible.

The traditional method for the separation of fucoidan and laminarin is to extract these two polysaccharides according to their solubilities in different solvents at different temperatures or time (13). Alternatively, in this study, SEC combined with refractive index (RI) detection was applied to separate laminarin and fucoidan from a crude polysaccharide solution according to their distinct molecular weights. This method overcomes some of the problems associated with the traditional methods, namely the disposal of solvents, lengthy and complex procedures. SEC is a relatively easy and commonly used solution for molecular weight characterization. For SEC, the molecular weight range of the column should cover the molecular weight distributions of the chosen polysaccharides. Others like high resolution, low adsorption and stability in a wide pH range are also important. By thorough consideration, Waters Ultrahydrogel\(^{TM}\) WATO 11530 SEC column matches these demands and was selected for separation of these polysaccharides. The types of mobile phases and the flow rate of the mobile phase were optimized to ensure the high-separation efficiency of these polysaccharides. Moreover, the factors affecting extraction, such as the solvent types, extract time, extract temperature and sample-to-solvent ratios, were investigated.

Experimental

Materials

The seaweed species, Laminaria japonica, was purchased in a supermarket in Korea. The fucoidan and laminarin standards were obtained from Sigma-Aldrich (St Louis, MO, USA). Ethyl ether, acetonitrile, methanol, phosphoric acid (\(\geq\)85%), sulfuric acid (95%), sodium nitrate, sodium phosphate dibasic dodecahydrate and sodium phosphate monobasic were supplied by DUKSAN Pure Chemical Co., Ltd. (Ansan, Korea). Hydrochloric acid was acquired from Daejung (Gyeonggi-do, Korea). Distilled
water was filtered using a vacuum pump (Division of Millipore, Waters, USA) and a filter (HA-0.45; Division of Millipore, Waters, USA). The samples were filtered through a filter (0.45 μm, Minisart RC 15; Goettingen, Germany) before being injected into the HPLC system.

**Extraction of polysaccharides preparation**

The extraction of fucoidan and laminarin were carried out by mixing 1.0 g of ground *L. japonica* powder with 30.0 mL solvent in a flask. The suspensions were heated with constant stirring. After cooling to room temperature, the suspensions were centrifuged. To obtain the crude polysaccharide, ethanol was used for precipitation at an ethanol-to-filtrate ratio of 3:1. The sediments were twice washed with acetone and ether, sequentially. The depurated sediments were dried at 60°C and the polysaccharides were obtained for further analysis. The polysaccharides were re-dissolved in the solvent. The extraction solution was filtered (0.45 μm) before being injected into the HPLC system. Each sample was injected three times to evaluate the precision and accuracy of the analysis. The optimal conditions including the mobile phase, kinds of solvents, solvent concentration, time, temperature and sample-to-solvent ratio were examined systematically.

**HPLC analysis**

The HPLC system consisted of a YL9112 Isocratic pump (Young Lin Co., Anyang, Korea), RI detector (RI750F, Young Lin Instrument Co., Anyang, Korea) and integrated data system (Clarity Chromatography Software, version 2.3, DateApex, EU). Injection valves with 200 μL sample loops were used. HPLC was performed using a Waters Ultrahydrogel™ WATO 11530 size-exclusion column (300 × 7.8 mm i.d.) and a Waters Ultrahydrogel™ WATO 11565 guard column (40 × 6 mm i.d.) from Waters (Milford, MA, USA). The mobile phase was used as the isocratic elution at room temperature. The flow rate and injection volume were set to 0.6 mL min⁻¹ and 10.0 μL, respectively.

**Results**

**Optimization of chromatographic separation conditions**

The effect of mobile phase on the separation efficiency

In this study, buffers with different pH values, nitrate with various concentrations, water and different percentage of organic solvents (column limit: ≤ 20%) were all evaluated to identify the most suitable mobile phase (Table I). The retention factor and resolution were selected as the evaluation parameters. For phosphate buffer, only one peak was observed when standard mixtures of the two polysaccharides were injected, regardless of the pH. The resolutions using acetonitrile and methanol as the mobile phases were relatively high, but the retention factors of fucoidan were < 1. The retention factors of the two polysaccharides and resolutions were good when sodium nitrate solutions were applied as the mobile phases. On the other hand, the resolution using water as the mobile phase was better, showing that the peaks can be differentiated successfully using water compared with sodium nitrate. Figure 2 shows the chromatograms using different mobile phases. The peak shapes using water as the mobile phase were better than the others. Moreover, the peak areas of fucoidan and laminarin were larger using water than the other mobile phases under the same conditions. Therefore, water was selected as the mobile phase to separate the polysaccharides for further analysis.

The effect of the flow rate of mobile phase on the separation efficiency

To decrease the diffusion effect of polysaccharides on the chromatographic column, flow rates of 0.4, 0.6 and 0.8 mL min⁻¹ were evaluated. The results showed that when the flow rate was 0.4 mL min⁻¹, the diffusion effect was relative larger and the retention time was too long. At the same time, the retention factor of fucoidan, whose molecular weight is larger, was very low, even though the diffusion effect was small when the flow rate was 0.8 mL min⁻¹. Based on the above-mentioned

Table I

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Retention time (min)</th>
<th>Retention factor (k)</th>
<th>Resolution (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fucoidan</td>
<td>Laminarin</td>
<td>Fucoidan</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>pH = 6</td>
<td>17.9 ± 0.1</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>pH = 7</td>
<td>17.3 ± 0.0</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>pH = 8</td>
<td>17.5 ± 0.3</td>
<td>1.69</td>
</tr>
<tr>
<td>NaNO₃ (mol L⁻¹)</td>
<td>0.01</td>
<td>13.7 ± 0.1</td>
<td>16.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>13.6 ± 0.4</td>
<td>18.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>13.0 ± 0.0</td>
<td>19.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>13.2 ± 0.1</td>
<td>17.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>18.0 ± 0.0</td>
<td>2.06</td>
</tr>
<tr>
<td>Water</td>
<td>13.8 ± 0.0</td>
<td>23.5 ± 0.1</td>
<td>1.13</td>
</tr>
<tr>
<td>Acetonitrile 5%</td>
<td>10.0 ± 0.1</td>
<td>17.2 ± 0.2</td>
<td>0.53</td>
</tr>
<tr>
<td>10%</td>
<td>10.3 ± 0.2</td>
<td>17.1 ± 0.3</td>
<td>0.59</td>
</tr>
<tr>
<td>15%</td>
<td>10.4 ± 0.1</td>
<td>17.9 ± 0.0</td>
<td>0.60</td>
</tr>
<tr>
<td>Methanol 5%</td>
<td>10.5 ± 0.5</td>
<td>18.2 ± 0.0</td>
<td>0.62</td>
</tr>
<tr>
<td>10%</td>
<td>11.2 ± 0.3</td>
<td>18.6 ± 0.1</td>
<td>0.73</td>
</tr>
<tr>
<td>15%</td>
<td>10.3 ± 0.1</td>
<td>18.0 ± 0.2</td>
<td>0.58</td>
</tr>
</tbody>
</table>

The mean value of three determinations ± SD. Mobile phase flow rate: 0.6 mL min⁻¹ and column temperature: 35°C.
factors, 0.6 mL min$^{-1}$ was found to be the most suitable choice for further discussion.

**Optimization of extraction conditions**

The accurate choice of extraction solvent is extremely important for extracting fucoidan and laminarin from brown seaweeds. As shown in Figure 3, the amounts of fucoidan and laminarin extracted with 0.1 mol L$^{-1}$ HCl were higher than those extracted with water or other acids. Therefore, hydrochloric acid was used as the solvent in the following optimization.

The solvent concentration is another factor that can affect the extraction efficiency. The acid concentration affects the hydrolysis ratio of polysaccharides. Figure 4 shows that the amounts of both fucoidan and laminarin extracted were relatively small when the acid concentration was extremely low. At the same time, a higher acid concentration resulted in a smaller amount of fucoidan detected because of hydrolysis. Therefore, a 0.10-M hydrochloric acid solution was more suitable as the extract solvent.

Heating was carried out from 40 to 90°C to determine the optimal temperature. As shown in Figure 5, the amounts of fucoidan and laminarin extracted increased until the temperature reached 80°C, and the levels of the two polysaccharides began to decrease with increasing temperature.

The sample-to-solvent ratio is also another critical factor affecting the amounts of fucoidan and laminarin extracted. A series of sample-to-solvent ratios were examined, as shown in Figure 6. No obvious changes in the amounts of fucoidan and laminarin extracted were observed, and the level of these polysaccharides decreased with the increasing ratio. A lower sample-to-solvent ratio appeared to be more suitable for extraction. Therefore, a sample-to-solvent ratio of 1:30 was considered the appropriate extraction ratio.

From the variation trends of two polysaccharides shown in Figure 7, it can be concluded that time plays a critical role in breaking the glucosidic bonds. The amount of laminarin
increased after heating for 4 h, and the amount began to decrease thereafter, while at the same time, the amount of fucoidan increased instantly and the growth rate decreased gradually. As a result, 4 h was found to be sufficient under economic considerations.

Discussion

**Optimization of chromatographic separation conditions**

SEC is an excellent method for separating macromolecules according to their size or molecular weight. The mobile phase should be chosen carefully considering the existence of polar ionic groups in the stationary phase used in SEC. This suggests that there is no sample loss for SEC chromatography, but for aqueous SEC columns, the elution efficiency can be affected by a range of effects, such as molecular adsorption, ion exclusion and aggregate formation (14). For SEC chromatography, the following basic conditions should be satisfied: (i) the sample should be soluble in the selected mobile phase and the performance of the SEC stationary phase is not destroyed, (ii) there are no interactions between the sample and stationary phase and (iii) the response signals generated by the detector should be strong enough (15). The flow rate of the mobile phase can more or less affect the diffusion of the polysaccharides in the chromatographic column.

**Optimization of extraction conditions**

Three traditional methods are used to extract polysaccharides: heating water extraction method, diluted acid extraction method and dilute alkali extraction method. In general, most polysaccharides can be extracted with hot water or dilute alkali solutions (16). On the other hand, in the present experiment, dilute alkali solutions should not be used as the solvent to avoid dissolving an interferential polysaccharide, such as alginate.

Both the acid concentration and temperature affect the hydrolysis ratio of polysaccharides. A lower temperature can only make the extracellular polysaccharides dissolve in the solvent, which means that it was difficult to prepare polysaccharides exposed to the solvent. On the other hand, glucosidic bonds will break if the temperature is too high. From a combination of theory and experimental data, 80°C was selected as the optimal temperature. As important as temperature, time has an intense effect on the polysaccharides decomposition ratio.

**Validation of the proposed method**

The linearity, precision, limit of detection (LOD) and other characteristics were estimated under optimized experimental conditions to evaluate the proposed method. Calibration curves of two polysaccharides were constructed by the relative peak area of fucoidan and laminarin versus their concentrations with at least nine points. The linearity of the analytes was obtained over the concentration range as shown in Figure 8. As a result, the correlation coefficients of both polysaccharides were >0.99 (fucoidan: $y = 69.507x + 60.293, R^2 = 0.9976$; laminarin: $y = 98.317x + 50.027, R^2 = 0.9977$). The comparative peak area calculated from five replicate extractions under the optimized conditions from one brown seaweed sample was obtained to
estimate the reproducibility from the relative standard deviation (RSD) percentage. The RSDs of fucoidan and laminarin were 4.9 and 6.7%, respectively. The LOD was determined based on a signal-to-noise ratio of 3 for fucoidan and laminarin under the optimal conditions. The LODs for fucoidan and laminarin were 0.15 and 0.21 mg mL$^{-1}$, respectively. The results showed that the proposed method is suitable for analyzing the polysaccharides from seaweeds.

**Conclusion**

A simple SEC method for separating polysaccharides in brown seaweeds was proposed and validated. To develop the chromatogram, a range of mobile phases were examined. Hydrochloric acid (0.10 mol L$^{-1}$) was used to extract fucoidan and laminarin from *L. japonica* with a sample-to-solvent ratio of 1:30 for 4 h at 80°C. The acceptable validation parameters and the simple procedure suggested that the high-performance size-exclusion chromatography method is a suitable method for separating soluble polysaccharides in brown seaweeds based on the molecular weight.

**Acknowledgments**

This study was supported by the Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology (No. 2014002046).

**References**