Note

Detection of Alginate Oligosaccharides from Mollusks

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Received June 7, 2006; Accepted July 11, 2006; Online Publication, November 7, 2006
[doi:10.1271/bbb.60313]

We attempted in this study to detect alginate oligosaccharides (AO) from mollusks. The samples were collected from turban shells and abalones which were typically seen on brown algae. High-performance liquid chromatography (HPLC) and negative-ion electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) analyses were used to confirm the presence of AO. Samples spiked with AO resulted in observable peaks where the HPLC area was increased. The highest content was estimated to be 401.8 mg/100 g of digestive organ. The product-ion data derived from AO molecular weight were detected at a constant interval by Q-TOF MS/MS analysis. These findings indicate that AO was present in the digestive organs of mollusks.

Key words: alginate oligosaccharides; quadrupole time-of-flight; mass spectrometry; mollusk; detection

Many studies have been reported on alginate-degrading enzymes that catalyze the degradation of alginate polysaccharides, these being isolated from many sources including brown algae, mollusks, and marine bacteria.1) It is well known that almost all enzymes are of the lyase type that catalyze the degradation of alginate polysaccharide by a β-elimination mechanism, a double bond being formed between the C4 and C5 carbons at the non-reducing terminal. We have developed a production method for AO of less than 1,000 molecular weight that decomposed alginate polysaccharide by using alginate lyase which was obtained from a marine bacterium (Pseudoalteromonas sp. No. 1786). AO were found by HPLC, fast atom bombardment (FAB)-MS and nuclear magnetic resonance (NMR) analyses to be mainly eight kinds of mixture composed of disaccharides, trisaccharides, and tetrasaccharides (HPLC pattern of AO: P1, P2, P3, P4, P5, P6, P7 and P8 in order of elution shown in Fig. 1A).2,3) AO differing in molecular weight from our AO have been reported to have a variety of biological activities (e.g., promotion of bifidobacterial growth in skim milk, root growth-promoting activity, production of IFN-γ and suppression of IgE).4–6) We have earlier reported the antihypertensive effects in an animal study and hypotensive effects in clinical studies.7,8) Rapid advancements in MS have recently led to the development of various techniques that are being widely utilized for determining the molecular weight of an unknown substance and/or structural analysis. Especially noteworthy are MS/MS techniques like tandem mass that are increasing the accuracy of analyses. Structural analyses of AO using NMR and MS/MS techniques have also been reported.10,11) Although alginate lyases and the physiological actions of AO have been mentioned, there have been no reports of AO being detected in the natural world. We therefore attempted to detect AO by using HPLC, Q-TOF MS, and Q-TOF MS/MS analyses of the digestive organs of mollusks which were raised on brown algae and from which alginate lyases are reported to have been isolated in the digestive organs.1) We obtained data which indicate the presence of AO in the digestive organs of these mollusks.

Turban shells and disk abalones were purchased from Tsuchiura Gyorui Co., and Ezo abalone from Cosmo Sea Farming. The digestive organs from all the turban shells and abalones were collected, and approximately 10 g of each was suspended in 20 ml of 5% perchloric acid and shaken in a CUTE mixer (CM-1000, Tokyo Rikakikai Co., Tokyo, Japan) at 1,500 rpm for 20 min. After centrifugal separation at 3,500 rpm for 20 min, the supernatant was collected and the precipitate was suspended in 20 ml of 5% perchloric acid before again...
being shaken in the CUTE mixer at 1,500 rpm for 20 min. After centrifugal separation at 3,500 rpm for 20 min, the latter supernatant was mixed with the former supernatant, and the pH value was adjusted to 7.0 by 30% KOH. The supernatant after centrifugation at 3,500 rpm for 20 min was increased to 50 ml in a volumetric flask with deionized water. The sample was centrifuged at 15,000 rpm for 30 min, and then loaded into a Sep-Pak C18 Plus cartridge (Waters Corp., Milford, MA, USA). The eluate was centrifuged in centrifugal filter units (UFC3 BGC 00, Millipore Corp., Billerica, MA, USA) at 5,000 rpm for 60 min., and ethanol was added to a final concentration of 80% to purify the filtrate. This procedure was used to obtain the sample for analysis.

HPLC analysis was carried out with a Waters 600E system under the following analytical conditions: column, TSK-GEL DEAE-2SW 4.6 mm × 250 mm (Tosoh Corp., Tokyo, Japan); column temperature, room temperature; monitor wavelength, 230 nm; mobile phase, deionized water–0.25 M NaCl with a linear gradient (0–40 min); flow rate, 1.0 ml/min; analytical time, 60 minutes.

The elution profiles of AO and a sample from turban shells by HPLC are shown in Fig. 1. Each structure of P1 through P8 is considered as follows. P1, Δ-GulA; P2, Δ-ManA (ManA''); P3, Δ-GulA-GulA; P4, Δ-ManA-GulA; P5, Δ-ManA-ManA (ManA''); P6, Δ-GulA-GulA-GulA; P7, Δ-ManA-GulA-GulA; P8, Δ-ManA-GulA-ManA (ManA'').

Table 1. AO Content in Digestive Organs of Mollusks (mg/100 g)

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<th>Mean ± S.D.</th>
<th>Max.</th>
<th>Min.</th>
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<tr>
<td>Turban shells (n = 12)</td>
<td>167.2 ± 125.9</td>
<td>401.8</td>
<td>14.6</td>
</tr>
<tr>
<td>Disk abalones (n = 12)</td>
<td>67.7 ± 44.2</td>
<td>158.6</td>
<td>28.3</td>
</tr>
<tr>
<td>Ezo abalones (n = 15)</td>
<td>75.9 ± 51.5</td>
<td>201.1</td>
<td>5.2</td>
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</table>

Calculated by using purified P8 as the standard. Sensitivity corrections were necessary for disaccharides of 0.5, trisaccharides of 0.75, and tetrasaccharides of 1.0, because good correlation was shown between the sensitivity and the number of double bonds at the non-reducing terminals.

Table 1 shows the content of AO in the digestive organs of mollusks. It appears that AO was contained in the digestive organs, the content being 167.2 ± 125.9 mg in turban shells, 67.7 ± 44.2 mg in disk abalones, and 75.9 ± 51.5 mg in ezo abalones. However there was wide disparity among the samples of the same species, which may have been caused by food consumption before analysis or differences in alginate lyase activity.

Notwithstanding these disparities, the results of the HPLC analysis suggested the presence of AO in the digestive organs of the mollusks.

However, while HPLC suggested the presence of AO in the digestive organs of the mollusks, there was also a
possibility that other substances could have had the same retention time. Thus, accurate ESI tandem mass spectrometry was carried out in the negative ion mode with a Q-TOF Premier hybrid quadrupole time-of-flight instrument (Waters Corp.), equipped with Z-Spray source technology and LockSpray. Nitrogen was used as the desolvation and nebulizing gas at flow rates of 350 and 100 L/h, respectively, the source and desolvation temperatures being 80 and 180°C, respectively. The capillary voltage was 2.7 kV and the cone voltage was maintained at 40 V. Each sample was diluted with water/methanol (1:1, v/v), and was subjected to MS by infusion at a flow rate of 5 μl/min. The collision-induced dissociation MS/MS analysis was conducted with argon as the collision gas at an indicated analyzer pressure of 3 × 10⁻³ mbar, and the collision energy was adjusted to 12–15 eV to optimize the sequence information for each sample. The mass range was m/z 50–1000, and the resolving power was 10,000 (FWHM).

Figure 2 shows ESI MS data of a sample from turban shells in the negative ion mode. Figure 2A shows the MS data, where the major parent ions at m/z 351.0569, 527.0895, and 703.1229 were produced. This is in accordance with alginate oligosaccharide ions by FAB-MS that have been previously reported. The [M − H]⁻ ions were selected as precursor ions for MS/MS product-ion scan experiments. In the MS/MS data for the disaccharides, the major product ions at m/z 175.0240 (C₆H₁₀O₅, calculated mass of 175.0243) and 193.0346 (C₆H₈O₇, calculated mass of 193.0348) were produced. These ions appear to have been derived from either a monomer having a double bond or not (Fig. 2B). In the MS/MS data for the trisaccharides, the major product ions at m/z 175.0241, 193.0348 and 351.0565 (C₁₂H₁₅O₁₂, calculated mass of 351.0564) derived from cleavage of AO were observed (Fig. 2C). In the MS/MS data for the tetrasaccharides, the major product ions at m/z 175.0242, 351.0562 and 527.0898 (C₁₈H₂₃O₁₈, calculated mass of 527.0884) derived from cleavage of AO were observed at a constant interval (Fig. 2D).

Zhang et al. have reported on the product ion mass spectra of sixteen purified AO samples using either alginate lyase or mild hydrolysis with tandem mass spectrometry (ESI MS/MS, negative ion mode). In the present examination, the product ions derived from AO were consistent with those of Zhang et al. that were previously reported and observed at a constant interval. All m/z values between the actual mass and calculated mass were also similar, having less than a ±2.7 ppm error at the maximum. These results from Q-TOF MS/MS analysis are considered to provide strong evidence for the presence of AO. On the other hand,
Zhang et al. also have said that an internal mannuronate residue produced weak but specific product ions at m/z 307 and/or 483. As neither product ions at m/z 307 nor 483 was observed in the present experiment, there is a possibility that an internal guluronate residue was included, and further investigation should be done. In conclusion, these findings from HPLC and MS/MS analyses indicate the presence of AO in the digestive organs of mollusks.

References