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Development of an Analysis Method for 4-Deoxy-L-erythro-5-hexoseulose Uronic Acid by LC/ESI/MS with Selected Ion Monitoring

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This study describes a simple and rapid analytical quantitative method for measuring 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) using liquid chromatography-electrospray ionization-mass spectrometry (LC/ESI/MS). For a chromatographic condition, Shodex IC NI-424 column (4.6 mm i.d. x 100 mm, 5 µm) for anion analysis and an isocratic elution of 40 mM ammonium formate buffer including 0.1% formic acid (pH 3.75) at a flow rate of 0.5 mL/min was used. The column temperature was set to 40°C. In the analysis of DEH produced by exo-type alginate lyase (AlyFRB) from *Falsirhodobacter* sp. alg1, a peak was detected with a retention time of 3.207 min. The prepared calibration curves for DEH analysis using the selected ion monitoring (SIM) mode of a mass spectrometer revealed a good linear relationship (correlation factor: 0.9998) within the test range (0.1-100 µg/mL). The limits of detection (S/N = 3) and quantification (S/N = 10) for DEH in SIM analysis were 0.008 and 0.027 µg/mL, respectively. Using the developed condition of LC/ESI/MS analysis, separation and detection of alginate unsaturated oligosaccharides were also tested. In an analysis time of about 13 min, this method was able to separate and detect an alginate unsaturated disaccharide, a trisaccharide, and a tetrasaccaride produced by poly(β-D-mannuronate) lyase, respectively. The analysis method established in this study will contribute to the quantitative and qualitative analysis of DEH, and the activity measurement of exo-type alginate lyase.

Keywords: Alginate, 4-Deoxy-L-erythro-5-hexoseulose uronic acid, *Falsirhodobacter* sp. alg1, Exo-type alginate lyase, Liquid chromatography-electrospray ionization-mass spectrometry.

Alginate lyases are enzymes that cleave the glycosidic 1→4 O-linkage in the alginate molecule *via* β-elimination reaction of the 4-O-glycosidic bond [1]. Endo-type alginate lyase depolymerizes alginate into oligosaccharides with a double bond at the non-reducing terminal [1]. The alginate unsaturated oligosaccharides (AUOs) which are produced are processed by an exo-type alginate lyase to yield monosaccharides which further undergo non-enzymatic conversion into 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) [2] (Figure 1). Among the enzymatic decomposition products of alginate, DEH is considered to be an especially key product of biofuel production from alginate using a metabolically engineered recombinant microorganism [3,4]. DEH is a rare deoxy sugar of unknown physiological function, but it has an α-keto acid structure in its molecule like α-ketoglutaric acid known to be an excellent antioxidant substance [5,6]. Consequently, DEH has the possibility of being usable as a valuable physiologically active substance derived from seaweed polysaccharide. From this scientific background, studies regarding the search for exo-type alginate lyase have been actively investigated in the research fields of bioengineering and marine biochemistry [7-11].

In the analysis of the enzymatic decomposition products of alginate, a TLC method using a coloring reagent such as thiobarbituric acid (TBA) [12-14], and sulfuric acid-ethanol is often used [12-17]. In the case of TBA staining, β-formylpyruvic acid produced from DEH and AUOs by periodic acid oxidation, reacts with TBA to form a condensation product having λ_{max}549 nm [1,2]. Accordingly to theory, the TBA reagent is able to simply and specifically detect the enzymatic decomposition products. Sulfuric acid is used as a detection reagent for organic compounds in the TLC analysis. However, the qualitative analysis of DEH in the products using TLC is difficult, because a purified standard of DEH, and exo-type alginate lyases are not commercially available.

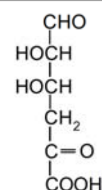

Figure 1: 4-Deoxy-L-erythro-5-hexoseulose uronic acid.

In preceding papers [9,11], we reported the isolation of *Falsirhodobacter* sp. alg1 strain as a novel alginate degrading bacterium. A novel exo-type alginate lyase (AlyFRB) possessed by the alg1 strain is an enzyme capable of depolymerizing alginate and producing only DEH without intermediate products [11]. LC/MS or

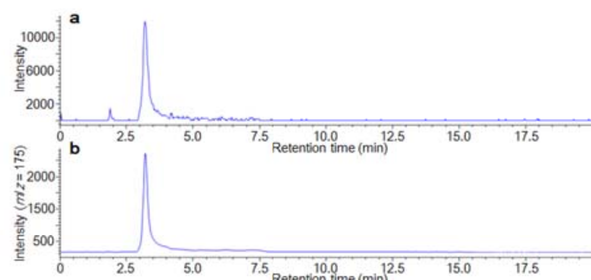

Figure 2: Representative chromatograms of DEH. a: TIC of DEH, b: SIM chromatogram at *m/z* 175. 10 μL of DEH solution (100 μg/mL) was injected into the LC-MS system.

Table 1: The evaluation of precision and specificity of the developed LC/MS condition for DEH analysis.

t_R (min)	t_R-RSD (%)	Area	Area-RSD (%)	k'^a
3.207	0.082	40594	4.020	0.690

The peak of DEH was detected using a mass spectrometer. The analytical data of t_R, t_R-RSD, Area, and Area-RSD in the table were calculated from the SIM chromatograms obtained by a 10 μL injection (n = 5) of DEH (100 μg/mL). [a]Capacity factor.

LC/MS/MS analysis is considered to be the most effective method for separation and identification of DEH. In this study, we prepared DEH using AlyFRB, and developed a simple and rapid analytical method of DEH by LC/ESI/MS. In addition, we also examined the separation and detection of AUOs using the developed condition for DEH analysis. Alginate and its related molecules including DEH and AUOs, have a carboxyl group in the uronic acid unit, and they have a negative electric charge. In this study, an anion chromatography column with quaternary ammonium as the functional group and a formic acid buffer were used for chromatography.

An unsaturated monosaccharide produced by exo-type alginate lyase converts non-enzymatically to the most stable 5-keto-substructure, i.e. DEH [2]. DEH does not absorb at 235 nm due to the transfer of the unsaturation to the ketone group [12,18]. Figure 2 shows the total ion chromatogram (TIC) and selected ion monitoring (SIM) chromatogram of DEH in the negative mode of LC/ESI/MS analysis. The SIM measurement is suitable for identification and sensitive detection of target compound in sample, because it measures only the ion having a specific *m*/*z*. In the SIM chromatogram, the *m*/*z* 175 corresponds to the mass number of the deprotonated ion of DEH [7]. In the chromatography using Shodex IC NI-424 for anion chromatographic column and a 40 mM ammonium formate buffer containing 0.1% formic acid as a mobile phase, a peak of DEH was detected with a retention time (t_R) of 3.207 min. The smaller peak with a retention time of 1.898 min indicates that of components eluted in the void volume (Figure 2a). The residual standard deviation of the retention time (t_R-RSD) was 0.082%, and that of the peak area (Area-RSD) value was 4.020% (Table 1). This suggests that the LC/ESI/MS condition for DEH analysis established in this study has a high rate of reproducibility. A calibration curve was prepared by plotting the detected peak area of DEH in the SIM chromatogram and concentrations of DEH. The correlation factor of the calibration curve obtained from the SIM chromatogram for DEH analysis was higher than 0.999 (Table 2). The results indicate that the LC/ESI/MS condition with an IC NI-424 column show high linearity within the range of concentration. The LODs (S/N = 3) and LOQs (S/N = 10) for DEH analysis were 0.008 and 0.027 μg/mL, respectively (Table 2).

The poly(β-D-mannuronate) lyase (EC 4.2.2.3) used in this study cleaves the 1→4 bond between β-D-mannuronate and α-L-guluronate or β-D-mannuronate, and produce unsaturated

Table 2: Linear regression data, LOD and LOQ for DEH analysis.

Regression equation	Correlation factor	Linearity range (μg/mL)[a]	LOD (μg/mL)[b]	LOQ (μg/mL)[c]
y=403.14x+19.637	0.9998	0.1–100	0.008	0.027

The peak of DEH was detected using a mass spectrometer in SIM mode. [a]Concentrations of DEH. [b]Limit of detection (S/N = 3). [c]Limit of quantification (S/N = 10).

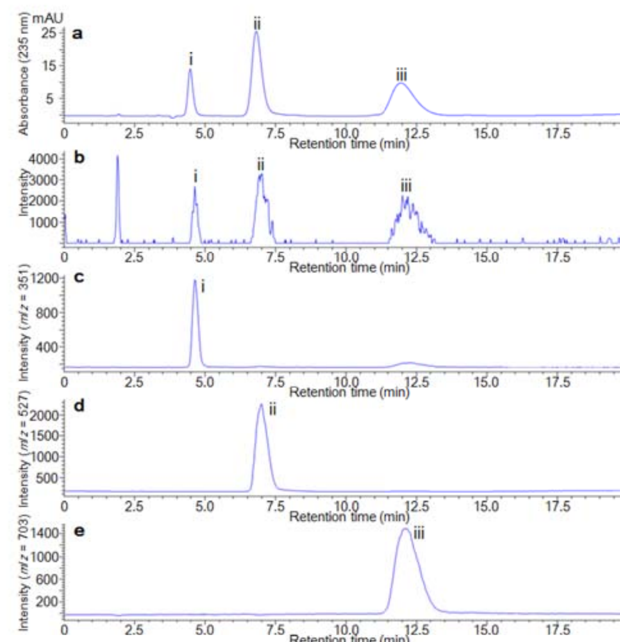

Figure 3: Representative chromatograms of AUOs. a: UV chromatogram of AUOs, b: TIC of AUOs, c: SIM chromatogram at *m/z* 351, d: SIM chromatogram at *m/z* 527, e: SIM chromatogram at *m/z* 703. 10 μL of AUOs solution (375 μg/mL) was injected into the LC-MS system. i, an unsaturated disaccharide; ii, an unsaturated trisaccharide; iii, an unsaturated tetrasaccharide.

Table 3: The evaluation of precision and specificity for AUOs analysis.

AUOs	t_R (min)	t_R-RSD (%)	Area	Area-RSD (%)	k'^a	Rs[b]	α[c]
Disaccharide	4.636	0.036	7644	3.467	1.443	-	-
Trisaccharide	6.992	0.059	31216	1.979	2.684	4.284	1.860
Tetrasaccharide	12.084	0.083	39451	2.354	5.367	6.365	2.000

Each peak of AUOs was detected using a mass spectrometer. The analytical data of t_R, t_R-RSD, Area and Area-RSD in the table were calculated from the SIM chromatograms obtained by a 10 μL injection (n = 5) of AUOs (375 μg/mL). [a]Capacity factor. [b]Resolution factor. [c]Separation factor.

oligosaccharides ranging from 2 to 4 residues with 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid at their non-reducing end and β-D-mannuronate at the reducing end [1]. The AUOs which were prepared using poly(β-D-mannuronate) lyase and sodium alginate of high mannuronate type, were characterized by LC/ESI/MS with the developed condition for the DEH analysis. In a UV chromatogram at 235 nm, the characteristic three peaks were separated and detected from AUOs by HPLC with Shodex IC NI-424 column and a 40 mM ammonium formate containing 0.1% formic acid as a mobile phase (Figure 3a). Figure 3b shows a TIC of AUOs in negative mode with LC/ESI/MS analysis. The peak at the retention time of 1.898 min shows that of components eluted in the void volume (Figure 3b). Each separated oligosaccharide was identified using a mass spectrometer in SIM mode. Figure 3c shows the SIM chromatogram at *m*/*z* 351. The ion *m*/*z* 351 coincides with the mass number of a deprotonated form of an unsaturated disaccharide [15]. The retention time (4.636 min) of the peak which was detected in the SIM chromatogram is completely in accord with that of peak i in TIC. Therefore, it was demonstrated that the peak i indicated an alginate unsaturated disaccharide. Figures 3d and 3e

show the SIM chromatograms at *m/z* 527 and *m/z* 703 in the LC/ESI/MS analysis, respectively. Each value of *m/z* corresponds to a mass number of a deprotonated molecule $[M-H]^-$ of an unsaturated trisaccharide or an unsaturated tetrasaccharide [15]. From complete agreement of the retention time at 6.992 min in the TIC and SIM chromatogram, peak ii in TIC was identified as an alginate unsaturated trisaccharide. Peak iii with a retention time of 12.084 min detected in TIC was shown to correspond to an alginate unsaturated tetrasaccharide. The unsaturated oligosaccharides with 5 or more residues from AUOs prepared in this study were not detected. To validate the precision, the t_R, peak area, k', Rs, and α for each of AUOs were calculated, respectively (Table 3). The t_R-RSD was less than 0.084%, and the Area-RSD value was less than 3.468% (Table 3). The value of Rs is a parameter indicating the separation of adjacent peaks. Values of more than 1.5 refer to the complete separation of adjacent peaks. The values of Rs obtained in this study were 4.284 and 6.365 (Table 3). Thus, it was suggested that the developed method for DEH analysis can be used for separation of AUOs and confirmation of their degree of polymerization.

To the best of our knowledge, this is the first research report on the quantitative analysis method of DEH using LC/ESI/MS. Kim *et al.* [7] have identified DEH from alginate enzymatic degradation products prepared by an exo-type alginate lyase using LC/Q-TOF MS with ACQUITY C18 column and gradient elution of two kinds of mobile phase (0.1% formic acid and 0.1% formic acid in acetonitrile). In our developed LC/MS condition, it is a more convenient method because an isocratic elution of ammonium formate buffer was used. In recent other studies [19,20], there are several reports on detection of DEH using GC/MS in addition to TLC method. In those reports [19,20], the methoxyaminated DEH was detected with a retention time of about 30 min by GC/MS with a fused silica capillary column (DB-5 ms). In the case of our LC/MS method, the derivatization of DEH is unnecessary, and it can be detected DEH directly in an analysis time within 4 min. Therefore, the LC/MS method is more suitable for detection of DEH than the GC/MS method. Furthermore, because of the difference in retention time, it is possible to simultaneously separate and detect from DEH to alginate unsaturated tetrasaccharide within 13 min, so it can be applied to analysis such as activity measurement of exo-type alginate lyase. In conclusion, the developed method in this study, that is, LC/ESI/MS analysis using the SIM mode is expected to be utilized for qualitative and quantitative analysis of DEH as a rapid and sensitive method as an alternative to the TLC method.

Experimental

Materials: Poly(β-D-mannuronate) lyase (from *Flavobacterium* sp.) (EC 4.2.2.3) was purchased from Sigma-Aldrich Co. Sodium alginate of high mannuronate type (IL-6M, the ratio of mannuronate to guluronate: about 2.56) was kindly donated by Kimica Co. Ammonium formate, formic acid and ultrapure water for LC/MS were purchased from Wako Pure Chemical. All other reagents used in this study were of analytical grade.

Preparation of DEH using AlyFRB: DEH was prepared by enzymatic degradation of AUOs using AlyFRB from *Falsirhodobacter* sp. alg1 according to the preceding report [11]. The solution of alginate degradation was filtered using Amicon Ultra-4 centrifugal unit (3000 nominal molecular weight limit, Merck Millipore). The filtrate was freeze-dried using lyophilizer FDU-2200 (Eyela), and the obtained lyophilized powder was used as DEH. The obtained DEH was confirmed to be homogeneous by TLC with developing solvent of *n*-buthanol:acetic acid:water (3:2:2, *v/v*) [11]. DEH was stored at -80°C until use.

Preparation of AUOs using poly(β-D-mannuronate) lyase: Sodium alginate (1 g) was dissolved in 100 mL of ultrapure water, and the sodium alginate solution was used as a substrate. Poly(β-D-mannuronate) lyase (5 mg) was suspended in 1 mL of ultrapure water, and the enzyme suspended solution (100 μL) was added to the substrate every 8 hours. After incubation at 37°C for 3 days, the reaction was stopped by heating the solution to 100°C for 5 min. Degradation of sodium alginate was confirmed from the increase in absorbance at 235 nm. The solution of alginate degradation was filtered by Amicon Ultra-15 centrifugal filter unit (3000 nominal molecular weight limit, Merck Millipore). The filtrate was freeze-dried using lyophilizer FDU-2200, and the obtained lyophilized powder was used in LC/ESI/MS analysis as AUOs. AUOs were stored at -30°C until use.

LC/ESI/MS analysis: The separation and detection of DEH and AUOs was carried out using 6120 Quadrupole liquid chromatograph-mass spectrometer (LC-MS) with a 1260 Series HPLC system consisting of 1260 variable wavelength detector, 1260 quaternary pump, 1260 autosampler, and 1260 thermostatted column compartments (Agilent Technologies Inc.). The UV detector was set at 235 nm. The ionization method of the sample was tested *via* the ESI mode in negative mode. The peaks of DEH and AUOs were detected using a mass spectrometer in SIM mode. In the SIM analysis, the *m/z* was set to 175 (corresponding to the molecular mass of the deprotonated form of DEH) [7], 351 (corresponding to the molecular mass of the deprotonated form of unsaturated disaccharide) [15], 527 (corresponding to the molecular mass of the deprotonated form of unsaturated trisaccharide) [15], and 703 (corresponding to the molecular mass of the deprotonated form of unsaturated tetrasaccharide) [15]. Other experimental conditions for the mass spectrometer were as follows: dry gas, 12.0 L/min; nebulizer, 0.241 MPa; dry temperature, 250°C; vaporizer, 200°C; scan from *m/z* 100 to 1000. The column used in this research was Shodex IC NI-424 column (4.6 mm i.d. x 100 mm, Showa Denko). Elution was performed at a flow rate of 0.5 mL/min with a 40 mM ammonium formate buffer including 0.1% formic acid (pH 3.5) as a mobile phase. The column oven was set at 40°C. The DEH and AUOs were each dissolved in the mobile phase.

Validation of the developed method: The precision test was demonstrated by replicative injections (n = 5) of DEH and AUOs solutions, respectively. Measurements of t_R, Area, k', Rs, and α were used to assess the reproducibility of the developed method. The parameters were defined using these standard formulas:

$$k' = (t_{Rn} - t_0) / t_0,$$
$$Rs = 2(t_{Rn+1} - t_{Rn}) / (W_{n+1} + W_n),$$
$$\alpha = k'_{n+1} / k'_n.$$

In these formulas, t_0 is the retention time of the components eluted in the void volume (t_0 = 1.898 min) and W_n is the width of the peak at baseline. The subscript n represents the order of elution of AUOs. LOD and LOQ for DEH analysis under the present chromatographic conditions were determined at S/N of 3 and 10, respectively.

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