Relationships between Guluronate Contents in Alginates Determined by ^1H -NMR Spectroscopy and Their Average Molecular Weights

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The relationships between guluronate content (G%) in alginates determined by ^1H -nuclear magnetic resonance (^1H -NMR) spectroscopy and their weight average molecular weight (WAMW) were examined to evaluate the reliability of the ^1H -NMR method. The partially hydrolyzed alginates with WAMW of 15k, 30k, 45k, 60k and 100k were prepared from alginates with WAMW of 150–200k by autoclaving. The G% in the partially hydrolyzed alginates determined by ^1H -NMR spectroscopy were independent on their WAMW, when the G% was less than 70%. The ^1H -NMR spectroscopic method using partially hydrolyzed alginates prepared by hydrolysis in an autoclave might be a convenient and reliable for the determination of G% in alginates.

Key words: alginate, guluronate content, ^1H -NMR, HPLC, weight average molecular weight, hydrolysis

Alginate, a polysaccharide of brown algae, has been used in various industries, *e.g.* textile, dyeing, paper, food and pharmaceutical.¹⁾ Alginate is a linear co-polymer of D-mannurate (M) and L-guluronate (G), and is known to be composed of homo-polymer blocks of M and G, and hetero-polymer block.^{1,2)}

The physical and chemical properties of alginates, *e.g.* viscosity, solubility, interaction with metals, and the properties of gels, are closely related to their M/G ratio and the relative amounts of homo- and hetero-polymer blocks^{3–6)} besides the average degrees of polymerization (average molecular weights).

In 1962, the determination of the uronic acid composition in alginate was attempted⁷⁾ by the complete hydrolysis with sulfuric acid followed by chromatographic separation of uronates on an anion-exchange column and orcinol reaction. This method was modified by Gacesa *et al.*,⁸⁾ Bouffar-Roupé and Heyraud,⁹⁾ Anzai *et al.*,¹⁰⁾ and Heyraud and Leonard.¹¹⁾ In 1974, Haug *et al.*¹²⁾ reported another method to determine the M/G ratio by quantification of homo-polymer blocks (M-block and G-block) and hetero-polymer block (MG-block); partial hydrolysis with diluted hydrochloric acid (HCl) followed by fractional precipitation by changing pH. Moreover, the methods using ^1H ^{13–15)} and ^{13}C -nuclear^{16–18)} magnetic resonance (NMR) spectroscopy, circular dichroism,¹⁹⁾ and infrared spectroscopy²⁰⁾ were reported. The standard method to measure the M/G ratio, however, has not yet been established.

Recently, many reports^{4–6,20–29)} have been published on the composition and the sequence of uronate residues in alginates using ^1H and ^{13}C -NMR spectroscopy. In these reports, the NMR spectra of alginates were measured after partial hydrolysis of intact alginates because solutions of intact alginates are too viscous to obtain desirable resolution of signals. None of these reports, however, discussed the following problems on the determination of M/G ra-

tio in alginates by ^1H -NMR spectroscopy. (1) The accuracy of integrated values (area) of signals; the areas of signals in spectra are measured by the routine integral program of spectrometers, but the integral values are affected by the drift of the base line. It might sometimes be difficult to adjust the start and the end of integral curve horizontal. (2) The spectrum must be measured in D_2O , in which the huge signal of HDO reduces the S/N ratio of the signals of alginate. The pulse techniques to reduce the huge signal, *e.g.* homogated decoupling (HMG), have been used, but there was no report which mentioned about power of irradiation pulse (irradiation attenuation). (3) No report can assure that the M/G ratio in the partially hydrolyzed alginate coincide with that of intact alginate.

In this paper, we report the relationships between guluronate contents (G%) in partially hydrolyzed alginates and their weight average molecular weights (WAMW) to evaluate the reliability of ^1H -NMR method. The ^1H -NMR spectra were measured by a 270 MHz spectrometer, and the spectra were transferred to digital images and processed by an image analyzing software to measure the area of signals.

Materials and Methods

Preparation of Sodium Alginates with WAMW of 150–200k

Sodium alginates with WAMW of 150–200k (ALG-1~6), were prepared from commercially available sodium alginates, “Sigma Medium Viscosity” (Sigma Chemicals, St. Louis, USA), “Manugel GMB” and “Manucol DMF” (Kelco, California, USA) and “Wako 500-600cP” (Wako Pure Chemicals, Osaka, Japan).

ALG-1: the suspension of Manucol (10 g) in 200 ml of 0.5 M acetic acid was heated in a boiling water bath for 2 h. The reaction mixture was adjusted to pH 1.0 by addition

of 10% HCl and the precipitate was collected by centrifugation (4000 rpm, 15 min). The precipitate was dissolved by addition of 1 M Na₂CO₃ (pH 8–9), and the solution was adjusted to pH 3.0 with 10% HCl. The precipitate was separated by centrifugation (6000 rpm, 15 min) and the pH of the supernatant was adjusted to 1.0. The precipitate was collected by centrifugation and was dissolved in 1 M Na₂CO₃ (pH 8–9). The equal volume of ethanol was added to the solution and the precipitate was collected by centrifugation (6000 rpm, 15 min). The precipitate was washed twice with 50% ethanol and dried at 60°C for 48 h. ALG-2: the suspension of Manucol (10 g) in water (200 ml) was heated in an autoclave at 130°C for 15 min. Equal volume of ethanol was added to the solution and the precipitate was collected by centrifugation. The precipitate was washed twice with 50% ethanol and dried at 60°C for 48 h. ALG-3: Sigma Medium Viscosity was heated in an autoclave at 130°C, 15 min. ALG-3 was prepared by the same method as ALG-2. ALG-4: the suspension of Wako 500–600cP (10 g) in water (200 ml) was heated in an autoclave at 130°C for 20 min. The pH of the solution was adjusted to 1.0 with 10% HCl and the precipitate was collected by centrifugation (6000 rpm, 15 min). The precipitate was dissolved in 1 M Na₂CO₃ (pH 8–9), and the solution was adjusted to pH 3.0 with 10% HCl. The equal volume of ethanol was added to the solution and the precipitate was collected by centrifugation (6000 rpm, 15 min). The precipitate was washed twice with 50% ethanol and dried at 60°C for 48 h. ALG-5: the suspension of Manugel (10 g) in water (200 ml) was heated in an autoclave at 130°C for 15 min and the solution was freeze dried. ALG-6: Manugel was heated in an autoclave at 130°C for 20 min. ALG-6 was obtained by the same method as ALG-2.

Preparation of the Test Samples and Reference Samples

The test samples with WAMW of 15k (in the range of 13–20k for 6 samples), 30k (26–35k), 45k (39–52k), 60k (62–64k) and 100k (97–115k) were prepared from 6 alginates mentioned above. The suspension of each sample (100 mg) in water (2 ml) was heated in an autoclave at 115–130°C for 15–120 min; the conditions were selected according to the results of preliminary examinations. The resulting solutions were evaporated to dryness or lyophilized (method 1).

One series of the reference samples were prepared by heating 6 alginates (100 mg) in 1 M HCl (10 ml) in a boiling water bath. After cooling, the solution was diluted with equal volume of water and adjusted to pH 8–9 with 1 M NaOH. To the solution, equal volume of ethanol was added, and the resulting precipitate was collected by centrifugation (2500 rpm, 5 min). The precipitate was washed twice with 50% ethanol, and dried at 60°C for 48 h (method 2). Another series of the reference samples were prepared by heating the suspension of 6 alginates (100 mg/2 ml water) in an autoclave followed by the addition of the equal volume of ethanol. The following procedure was the same as method 2 (method 3).

Determination of the Weight Average Molecular Weight (WAMW) by HPLC

A high performance liquid chromatograph (HPLC) sys-

tem equipped with a Hitachi L-7100 pump, a Hitachi L-7200 autosampler, a Hitachi L-3300 RI detector and a Shimadzu C-R7A data processor was used. An Ashahipak GS-520 HQ or a GS-620 HQ (each 7.6 mm i.d. × 300 mm, Showa Denko, Tokyo) column was equipped. The column was eluted with 0.3 M NaNO₃ at a flow rate of 1.0 ml/min at 60°C. The chromatograms were processed by the routine software supplied for C-R7A to calculate the weight average molecular weight (WAMW) using pullulan as a molecular weight standard. Acetone was used for the calibration of the retention time.

NMR Spectrometry

The ¹H-NMR spectra were recorded with a JEOL GX-270 spectrometer at 80°C, using 45° pulse, 32k data point, 4.096 sec acquisition, 5.000 sec pulse delay and 16 scans. The homogated decoupling (HMG) pulse sequence was used to reduce the signal of HDO (IRFIN=5561.3 Hz and IRATN=300). The sample (15–17 mg) was dissolved in 0.6 ml of D₂O containing 0.1% of acetonitrile as an internal standard (δ 2.06 for methyl group), and the insoluble materials were filtered off.

Measurement of Peak Area

The expanded spectrum (δ 4.3–5.5) drawn in the area of 20 × 12 cm was transferred to a digital image (72 dpi, about 300kB as a TIFF image) using an image scanner (GT-5000ART, Epson) linked to a Power Macintosh 7100/80AV. The image was processed by “NIH image ver. 1.58”; “skeletonize” treatment, draw straight base line, and then the measured the area of the signals of A: δ 4.95–5.17 (H-1 of G at δ 5.05), B: δ 4.55–4.82 (H-5 of G at δ 4.73 and 4.70, H-1 of M at δ 4.67 and 4.65) and C: δ 4.40–4.50 (H-5 of G at δ 4.45), using “Include Interior Holes” and “Adjust Area” option, as shown in Fig. 1. The guluronate contents (G%=[(area of A)/(area of B+C)] × 100) and the guluronate-guluronate diad frequency (GG%=[(area of C)/(area of B+C)] × 100) were calculated according to Grasdalen.¹⁵⁾

Results and Discussion

Measurement of the Signal Areas of Spectrum by Digital Image Processing

The signal area measurement by the routine program of a spectrometer are affected by the drift of base line, and it is sometime difficult to adjust the start and the end of the integration curve horizontal. We tried to use digital image processing software for the measurement of the signal areas, and an on-line software “NIH image” was shown to be useful for our purpose. The resolution of the digital image was tested from 24 dpi to 144 dpi, and the digital images of 72–144 dpi showed same results in measuring the signal areas. (see Fig. 1).

Condition of NMR Measurement

The huge signal of HDO causes the decrease of S/N ratio of the signals, and the side bands signals of HDO sometime overlapped the signals of alginate. One method to eliminate the huge signal of HDO is the replacement of the exchangeable protons in the alginate molecule with deuterium by repeating addition of D₂O and freeze drying (D₂O

Fig. 1. Process to measure the signal areas of a digital image of the spectrum.

Signal assignment (see reference 15): δ 4.45 (H-5 of guluronate), δ 4.65 and 4.67 (H-1 of mannuronate), δ 4.70 and 4.73 (H-5 of guluronate), δ 5.05 (H-1 of guluronate).

Guluronate content:

$$G\% = \frac{(\text{area of A})}{(\text{area of B} + \text{C})} \times 100.$$

Guluronate-guluronate diad frequency:

$$GG\% = \frac{(\text{area of C})}{(\text{area of B} + \text{C})} \times 100.$$

exchange). The other method is the application of pulse techniques, *e.g.* homogated decoupling (HMG) and water eliminated FT (WEFT), to diminish the signal of HDO. The D₂O exchange method takes a long time for sample preparation, and the pulse techniques might be acceptable for the routine work. The pulse irradiated to diminish the signal of HDO in HMG mode is known to affect the intensities of the signals near to the HDO signal (δ 4.23 at 80°C). Therefore, we first examined the effect of the irradiation power on the area of signals by changing the attenuation values (IRATN). The area of the nearest signal of alginate (at δ 4.45) was not affected when the IRATN was larger than 250. The G% and GG% measured in these conditions were same as those obtained in the normal spectra of the D₂O exchanged samples. In the HMG mode, the signal of HDO was almost diminished but the base line near the irradiation point was not horizontal. The variation coefficients of the G% and GG% values were both found to be less than 3% in three measurements of the same sample.

Relationships between Guluronate Contents and Guluronate-guluronate Diad Frequency and WAMW

There was no study which could assure that the G% determined by ¹H-NMR spectroscopy of the partially hydrolyzed alginates coincided with that of the intact alginates. So, we tried to examine the relationships between the G% and GG% and WAMW.

Since the first report of Penman *et al.*¹³⁾ on ¹H-NMR analysis of alginates, the samples were hydrolyzed with diluted HCl to lower the viscosity, but there was no information on the average molecular weights. The partial hydrolysis with HCl was shown to be difficult to control the degrees of hydrolysis, therefore, we used hydrolysis in an autoclave. At first, we prepared the starting alginates (ALG-1 ~ 6) with WAMW of 150 ~ 200k, which must be the upper limit of WAMW to obtain the desirable resolution and S/N ratio in the 270 MHz ¹H-NMR spectra.

The partially hydrolyzed alginates were prepared by three different methods, and the hydrolysis in an autoclave might be a convenient method without ethanol precipitation and/or neutralization.

The relationships between G% and GG% and WAMW of the 6 alginates and their hydrolysates are summarized in Table 1. The G% of ALG-1 ~ 5 are independent of the WAMW of the test samples. But the G% of the partially hydrolyzed alginates prepared from ALG-6 decreased as the WAMW decreased. The G% of ALG-6 was 89.9%, while those of 15 ~ 45k were about 80%.

The GG%, which represent the frequency of guluronate-guluronate (GG) diad, showed clear dependence on the WAMW of the test samples. The GG% increased as the WAMW of the test sample decreased. In the case of ALG-1, the GG% of the test sample of 15k was about two times larger than that of 100k, while in the case of ALG-6, the GG% of the test sample of 15k was more than 10% more than that of the starting sample.

The G% in partially hydrolyzed alginates obtained three different methods are shown in Table 2. The G% of ALG-1, 2 and 3 obtained by the method 2 and 3 were almost the same as those obtained by method 1, but the G% of ALG-4, 5 and 6 were significantly greater than those of samples obtained by method 1. The GG% of samples obtained by the method 2 and 3 were greater than those of samples obtained by method 1, except for ALG-1.

Many investigators have reported^{14,16,17)} that the G-M bond of alginates can more easily be hydrolyzed than other bonds, and the G-G bond is the most stable. Our results might also be explained by these facts. The decrease of WAMW may cause the increase of relative frequency of the GG diad (GG%), and the decrease of the frequency of other diads. The GG% obtained by the method 2 and 3 indicated the loss of the mannuronate rich oligomer by 50% ethanol treatment. The loss must be greater in the samples with low WAMW.

These results indicate that ¹H-NMR method can detect the change in the balance of the diads frequency in the partially hydrolyzed alginates, but the calculated G% is not affected by the changes in the diad frequency when the samples are prepared by hydrolysis in an autoclave and the WAMW of the samples are in the range of 15 ~ 200k. The independency of G% from the WAMW implied that the G% determined by ¹H-NMR spectroscopy using the partially hydrolyzed alginate obtained by hydrolysis in an autoclave might be reliable for alginate with the G% lower

Table 1. Relationships between guluronate content and guluronate-guluronate diad frequency and weight average molecular weight of six sodium alginates with different guluronate content

WAMW ³⁾	ALG-1		ALG-2		ALG-3	
	G(%) ¹⁾	GG(%) ²⁾	G(%)	GG(%)	G(%)	GG(%)
15k ⁴⁾	22.8±0.6	9.8±0.1	37.4±0.4	18.1±0.4	46.6±0.7	22.9±0.8
30k ⁵⁾	22.9±0.6	7.2±0.2	38.4±0.4	16.1±0.2	47.0±0.8	19.9±0.6
45k ⁶⁾	23.7±0.6	6.9±0.2	38.5±0.5	15.0±0.5	47.4±0.4	19.9±0.6
60k ⁷⁾	23.4±0.6	6.1±0.4	39.0±1.2	14.5±0.6	46.7±0.6	18.6±0.3
100k ⁸⁾	23.0±1.0	5.0±0.4	38.2±0.2	12.9±0.3	47.2±0.3	16.9±1.1
Starting alginate ⁹⁾	22.8±1.4	4.5±0.7	39.4±0.4	10.4±0.9	46.9±0.5	16.8±1.5

WAMW	ALG-4		ALG-5		ALG-6	
	G(%)	GG(%)	G(%)	GG(%)	G(%)	GG(%)
15k	56.4±0.8	38.3±0.1	69.6±2.6	48.8±0.5	80.2±2.0	56.8±0.5
30k	56.5±1.0	36.5±0.2	70.1±0.5	47.7±0.4	80.3±0.5	53.5±0.8
45k	57.2±0.6	35.1±0.6	68.5±0.9	45.9±0.3	79.9±1.3	53.0±0.4
60k	57.9±0.7	33.4±0.8	69.6±1.1	45.1±0.5	81.8±0.3	51.4±0.3
100k	57.6±1.7	31.4±0.5	71.0±0.3	43.0±0.3	82.6±0.9	50.0±0.2
Starting alginate	57.6±0.3	31.3±0.6	71.6±1.4	41.5±0.5	89.9±2.5	44.8±0.4

Average±SD of triplicate measurements.

¹⁾ Guluronate content in percentage; calculated as $G\% = [A/(B+C)] \times 100$. A=signal area of $\delta 4.95 \sim 5.17$, B=signal area of $\delta 4.55 \sim 4.82$ and C=signal area of $\delta 4.40 \sim 4.50$ (see Fig. 1).²⁾ Guluronate-guluronate diad frequency in percentage; calculated as $GG\% = [C/(B+C)] \times 100$.³⁾ Weight average molecular weight measured by GPC-HPLC.⁴⁾ WAMW for 6 alginates were in the range of 13–20k.⁵⁾ 26–35k.⁶⁾ 39–52k.⁷⁾ 62–64k.⁸⁾ 97–115k.⁹⁾ WAMW of the starting alginates were ALG-1; 173k, ALG-2; 193k, ALG-3; 173k, ALG-4; 187k, ALG-5; 148k and ALG-6; 159k.**Table 2.** Guluronate contents and guluronate-guluronate diad frequency in alginates prepared by different methods of partial hydrolysis

	Method 2 (HCl+EtOH) ¹⁾		Method 3 (Autoclave + EtOH) ²⁾		Method 1 (Autoclave)		WAMW ³⁾
	G% ³⁾	GG% ⁴⁾	G%	GG%	G%	GG%	
ALG-1	21.4±0.7	8.1±0.2			22.9±0.6	7.2±0.2	30 K
			24.0±0.8	7.7±0.2	23.7±0.6	6.9±0.2	45 K
ALG-2	38.6±0.5	19.6±0.3			38.4±0.4	16.1±0.2	30 K
			39.5±1.1	17.8±0.9	38.5±0.5	15.0±0.5	45 K
ALG-3	47.9±0.6	27.3±0.7			47.0±0.8	19.9±0.6	30 K
			48.5±0.5	24.7±0.2	47.4±0.4	19.9±0.6	45 K
ALG-4	60.6±0.8	41.5±0.8			56.5±1.0	36.5±0.2	30 K
			61.2±0.2	40.8±0.2	57.2±0.6	35.1±0.6	45 K
ALG-5	79.8±0.7	57.2±1.3			70.1±0.5	47.7±0.4	30 K
			76.8±1.6	55.3±0.6	68.5±0.9	45.9±0.3	45 K
ALG-6	86.8±1.1	62.2±0.3			80.3±0.5	53.5±0.8	30 K
			83.6±1.1	62.7±0.7	79.9±1.3	53.0±0.4	45 K

Average±SD of triplicate measurements.

¹⁾ The WAMW of the samples prepared by the HCl hydrolysis followed by 50% EtOH precipitation were 25–32k.²⁾ The WAMW of the samples prepared by the hydrolysis in autoclave followed by 50% EtOH precipitation were 36–48k.³⁾ Guluronate content (see caption of Table 1).⁴⁾ Guluronate-guluronate diad frequency (see caption of Table 1).⁵⁾ Weight average molecular weight determined by GPC-HPLC.

than 70%. In this study, the WAMW dependency of G% was examined using alginates with WAMW of 150–200k as starting alginates, however, this method must be applic-

able for the G% determination of alginates with higher WAMW.

The GG% in the test samples with WAMW of 100k pre-

pared from ALG-1~5 were almost same as those of the starting alginates. These results implied the possibility that these samples have the same frequency of GG diad as those of the starting alginates. Therefore, the frequency of GG diad in the test sample with WAMW of 100k might reflect that of the intact alginate. For the discussion on the frequency of other diads, the studies using a spectrometer with higher magnetic field must be needed because of insufficient resolution obtained by a 270 MHz spectrometer.

Thus, the ¹H-NMR method using the partially hydrolyzed alginate prepared by the hydrolysis in an autoclave might be a convenient and reliable method for the determination of the G% in alginate. Moreover, it will be noted that the consideration on the molecular weight dependency must be indispensable for the frequency analysis of diad and triad in alginates by the NMR method.

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