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Impact of hydrolysis conditions on the detection of mannuronic to guluronic acid ratio in alginate and its derivatives

Jiaojiao Lu[a], Hai Yang[b,c,*], Jie Hao[a], Chengling Wu[a], Li Liu[a], Naiyu Xu[a], Robert J. Linhardt[d], Zhenqing Zhang[a,*]

[a] Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases and College of Pharmaceutical Sciences, Soochow University, Suzhou 215021, Jiangsu, China
[b] Department of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, China
[c] Department of Pharmacy, Qingdao Central Hospital, Qingdao 266042, Shandong, China
[d] Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 8th St., Troy, NY 12180, USA

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ABSTRACT

Alginate is a linear and acidic polysaccharide, composed of (1 → 4) linked β-D-mannuronic acid (ManA) and α-L-guluronic acid (GulA). The ratio of ManA to GulA (*M*/*G*) is one of the most important factors for the application of alginate and its derivatives in various areas. In this work, a robust and accurate method was developed to analyze *M*/*G* using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The impact of hydrolysis conditions on the release patterns of ManA and GulA from alginate and its derivatives was investigated. The release patterns of ManA and GulA need to be considered separately to obtain an accurate *M*/*G*. Several hydrolysis conditions were established that released ManA and GulA completely and maintained these saccharide residues intact. The proper *M*/*G* of alginates from different sources and its derivatives could then be calculated by integration of the corresponding ManA and GulA peaks.

1. Introduction

Alginate is a linear copolymer composed of (1 → 4) linked β-D-mannuronic acid (ManA) and α-L-guluronic acid (GulA). Along its linear chain, there are homo-oligomeric regions of mannuronic acid (M-blocks) and of guluronic acid (G-blocks) as well as hetero-oligomeric regions (MG-blocks) (Haung, Larsen, & Smidsrod, 1966; Haug, Larsen, Smidsrod, & Painter, 1969). Alginate is a natural polysaccharide occurring in the cell wall of brown algae and also formed as a biofilm by some bacteria (Evans & Linker, 1973; Rehm & Valla, 1997; Coster, Stewart, & Greenberg, 1999). The most commercially important alginates are extracted from brown seaweeds and are widely used in different industries as gelling and texturizing agents, stabilizers, drug carriers and excipients (Murata, Sasaki, Miyamoto, & Kawashima, 2000; Stevens, Qandilo, Langer, & Shastri, 2004; Goh, Heng, & Chen, 2012). In addition to molecular weight (M_w), the ratio of mannuronic to guluronic acid (*M*/*G*) is important for the selection of the appropriate application for an alginate (Gacesa, 1988; Sen, 2011), as the *M*/*G* plays a significant role in alginate's physicochemical properties (Murata et al., 2000; Stevens et al., 2004; Pawar & Edgar, 2012). The *M*/*G* of an alginate depends on the algae species from which it was extracted and the harvest time, the harvest location and the processing of the algae (De Vos, De Haan, & Van Schilfgaarde, 1997). Thus, it is important to measure *M*/*G* accurately for determining the appropriate application of an alginate.

Nuclear magnetic resonance (NMR) spectroscopy is a widely used method to detect *M*/*G* (Grasdalen, Larsen, & Smisrod, 1979; Grasdalen, Larsen, & Smisrod, 1981; Rahelivao, Andriamanatonnina, Heyraud, & Rinando, 2013). However, NMR requires substantial amounts of sample and spectra often need to be acquired at high temperature to decrease the viscosity of the alginate solution. Thus, the applications of NMR to measure *M*/*G* of alginate and its derivatives can be challenging for small scale samples or screening many alginate samples (Wang, Yu, Zhao, Guan, & Du, 2005; Zhang et al., 2004).

The hydrolysis of alginate and its derivatives to constituent monosaccharides, followed by separation-based analysis represents another strategy for the analysis of *M*/*G*. High-performance liquid chromatography (HPLC), capillary electrophoresis (CE), gas chromatography (GC), and high-performance anion-exchange

* Corresponding authors. Tel.: +86 512 6588 2593; fax: +86 512 6588 2593.
E-mail addresses: qdzx08@163.com (H. Yang), z_zhang@suda.edu.cn (Z. Zhang).

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chromatography (HPAEC) are such separation techniques and can be efficiently applied in high sensitivity analysis (Voragen, Schols, De Vries, & Pilnik, 1982; Guttman, 1997; Rumpel & Dignac, 2006). However, most of these separation methods require derivatization following the hydrolysis of a polysaccharide to its constituent monosaccharides. Such a derivatization step requires additional assay validation and can introduce impurities. The application of HPAEC using pulsed amperometric detection (PAD) can address these concerns. HPAEC-PAD was first described at the end of the last century (Lee, 1990, 1996). Since then, it has been widely used in the analysis of various carbohydrate products (Andersen & Sorensen, 2000; Cai, Liu, Shi, Liang, & Mou, 2005; Grey, Edebrink, Krook, & Jacobsson, 2009). Recently, a new method using HPAEC-PAD was developed to analyze many different kinds of sugars directly, including ManA and GulA. This method could also be applied to more efficiently measure *M*/*G* without any required monosaccharide derivatization (Zhang, Khan, Nunez, Chess, & Szabo, 2012).

In addition to separation and detection, however, the method used for alginate hydrolysis represents an important factor in accurately determining *M*/*G*. Acidic hydrolysis is the major method used for polysaccharide analysis, however, many sugars are not stable under acidic conditions, and uronic acids are particularly labile at low pH values and at elevated temperatures (Aida, Yamagata, Watanable, & Smith, 2010; Aida et al., 2012). An optimized method to hydrolyze alginate and its derivatives to monosaccharides, while maintaining intact sugars, is critical for the accurate analysis of *M*/*G*.

In this paper, we optimize the acid hydrolysis of alginate and then use HPAEC-PAD to accurately analyze ManA and GulA. We also monitor the release patterns of ManA and GulA under different acidic hydrolysis conditions for alginate and its derivatives. The impact of the hydrolysis conditions on the accurate measurement of *M*/*G* was investigated. The hydrolysis conditions examined include the use of different acids, acid concentrations, sample concentrations, hydrolysis temperatures, and hydrolysis times.

2. Experimental

2.1. Materials

Sodium alginate (AR, 90%, 89 mPa s) extracted from *Macrocystis pyrifera* (*M.p.*) was purchased from Aladdin (Shanghai, China). Sodium alginates extracted from *Sargassum fusiforme* (*S.f.*, 570 mPa s) and *Laminaria japonica* (*L.j.*, 187 mPa s), respectively, were purchased from Haizhilin Corp. (Qingdao, China). M- and G-block were prepared in our laboratory as previously described (Zhang et al., 2006). Trifluoroacetic acid (TFA) (99%) was purchased from Energy Chemical (Shanghai, China). Sodium hydroxide solution (50%) was purchased from Merck (Darmstadt, Germany). Sodium acetate was purchased from Sigma-Aldrich (St. Louis, MO). High-purity water (resistivity ≥18.2 MΩ cm, 25 °C) was used throughout the study. All other chemicals and reagents were of HPLC grade.

2.2. Sample preparation

ManA and GulA were prepared by hydrolysis of M block and G blocks, respectively. Both hydrolysis reactions were carried out at 100 °C for 24 h in 2 M TFA. The hydrolyzates were purified by preparative HPLC using UV detection (210 nm) (data not shown). A semi-preparative Carbopac PA1 (20 × 250 mm, Dionex, Sunnyvale, CA) column was used at room temperature with a flow rate of 10 mL/min. The isocratic mobile phase consisted of 5 mM NaOH and 150 mM NaOAc. Purified ManA and GulA were desalted on a column (2.6 × 30 cm) packed with Sephadex G-10 (GE, Healthcare Bio-Science, Uppsala Sweden). Freshly prepared ManA and GulA were quantified using carbazole assay as previously described (Bitter & Muir, 1962). ManA and GulA standards were used to validate retention times and calibrate responses to PAD.

Table 1
Hydrolysis conditions for alginate, M- and G-block.

Sample concentration	Temperature (°C)	Acid concentration (M)	Time (h)
2.5 mg/mL and 5.0 mg/mL	100	1	2
			4h
			6
			12
			18
			24
		2	2
			4
			6
			12
			18
			24
	120	1	2
			4
			6
			12
			18
			24
		2	2
			4
			6
			12
			18
			24

Specific volumes of 1 M or 2 M TFA were added to 5 mg alginate, M-block, or G-block to afford 2.5 mg/mL or 5 mg/mL solutions. Hydrolysis reactions were carried out at 100 or 120 °C, and aliquots were taken at 2, 4, 6, 12, 18 and 24 h. The different hydrolysis conditions studied are provided in Table 1. All experiments were performed in duplicate. The reactions were monitored by TLC to determine completeness of hydrolysis (Zhang, Xie, Zhang, & Linhardt, 2007). Excess TFA was removed by rotary evaporation. Each hydrolysate was re-dissolved in the same volume of water, and its pH was adjusted to neutral using dilute aqueous NaOH. The final concentration of each solution was diluted to ~50 ppm for HPAEC-PAD analysis.

2.3. HPAEC-PAD analysis

Analysis was performed on a Metrohm 850 Professional system with a 919 IC auto-sampler plus equipped with dual pumps and with PAD (Herisau, Switzerland). Data were acquired and analyzed with MagIC Net 2.4 software (Herisau, Switzerland). A Carbopac PA1 analytical column (4 × 250 mm, Dionex, Sunnyvale, CA) was used at a flow rate of 1 mL/min and a temperature of 30 °C. The isocratic mobile phase consisted of 5 mM NaOH and 150 mM NaOAc. The recording time of data acquisition was 15 min and the injection volume was set to 20 μL.

2.4. NMR analysis

About 5 mg of each alginate was dissolved in 1–2 mL D_2O with 0.02% (w/v) 3-(trimethylsilyl) propionate-d_4 (TMSP) (Cambridge Isotope Labs, USA) as a chemical shift reference. NMR spectra were obtained with a Bruker Avance III NMR spectrometer at a ^1H frequency of 600 MHz for 96 scans. The temperatures were set at 25 °C for M and G blocks and 65 °C for alginate samples.