A Simple Method for Isolation of Polymannuronate and Polyguluronate from Alginate Hydrolyzed by Organic Acids

Dong Soo Lee, Myung Kyo Shin[1], Jae Hyeung Pyeun[2] and Jin Woo Lee[3]*

Research Center of Biotechnology & Bioengineering, KBP Co., Ltd., Pyungtaek, Kyunggi 459-050, Korea
[1]*Division of Environment and Energy, Korean Institute of Industrial Technology, Cheonansi, Chungnam 330-825, Korea*
[2]*Faculty of Food Science and Biotechnology, Pukyong National University, Busan, 608-737, Korea*
[3]*Department of Biotechnology, Dong-A University, Busan 604-714, Korea*

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Alginate with a MW of 1,283 kDa was hydrolyzed with 0.4 M organic acids at 100°C for 3 hr. Molecular weights of alginates hydrolyzed with organic acids ranged from 7.5 to 53.2 kDa. There was no significant difference in the molar ratio of mannuronate to guluronate in alginates hydrolyzed with organic acids. Acetic acid was found to be the most effective organic acid for hydrolysis of alginate. The MW of alginate decreased with increasing concentration and reaction time with acetic acid as a hydrolyzing agent. The correlations between the MW of hydrolyzed alginate and concentration of acetic acid as well as reaction time with 0.4 M acetic acid were plotted and the relevant equations obtained in this study. Polymannuronate and polyguluronate were isolated by pH adjustment of alginate hydrolyzed with 0.4 M acetic acid. The molar percentages of mannuronate in polymannuronates isolated from alginate hydrolyzed with 0.4 M acetic acid at 100°C were increasing in proportional to the reaction time such as 75% for 1 hr, 90% for 3 hr, and 98% for 5 hr of reaction time.

Key words : Alginate, hydrolysis, polymannuronate, polyguluronate, organic acids

Introduction

Seaweed alginates consist of β-D-mannuronate and its C-5 epimer, α-L-guluronate [5,14,20]. Alginates are widely used industrially as stabilizers, emulsifiers and gelling agents because of their ability to retain water. The molecular weight (MW) and molar ratio of D-mannuronate to L-guluronate (M/G ratio) are dependent on the algal species and harvest time as well as the location of the polymer in plants [10]. The M/G ratio and MW of alginate affect on properties of alginates [6,17].

The relative molar ratio of mannuronate to guluronate in the alginate hydrolyzed with 2 N sulfuric acid increased with reaction time [10]. Two partitions of polymannuronate with a purity of 80.9% and polyguluronate with a purity of 62.8% were isolated from the alginate hydrolyzed with 8.0% sulfuric acid for 3 hr and sequentially with 2 N sulfuric acid for 2 hr [2]. The MW and viscosity of alginate decreased according to the increased reaction temperature, time and concentration of hydrochloric acid, whereas solubility and emulsifying ability increased [14]. Polymannuronate with a purity of 92% and polyguluronate with a purity of 87% were isolated from alginate hydrolyzed with hydrochloric acid for 2 hr at 100°C [10]. The Nucleosil 10 SB and Zorbax column chromatography were used to separate uronic acid from alginate hydrolyzed with acetic acid [23]. Isolation of polymannuronate and polyguluronate from alginates hydrolyzed with sulfuric acid was conducted by the column chromatography with the specific concentration, velocity and pH of potassium phosphate solution as an elution buffer [9].

Major study on preparation of alginate with low MW and isolation of polymannuronate and polyguluronate for new applications of seaweed alginate has been investigated with inorganic acid such as hydrochloric and sulfuric acids. However, the recent method for isolation of polymannuronate and polyguluronate with inorganic acids resulted in environmental contaminations. An enzymatic method for preparation of homopolymannuronate blocks and strictly alternating sequences of mannuronic and guluronic units has been reported [1,12], but it is a costly process to apply for commercial production of polymannuronate. The new and simple method with organic acids, which have been used as food additives for its safety, for preparation of hydrolyzed alginate with low MW and isolation of polymannuronate and polyguluronate was developed in this

***Corresponding author**
Tel : +82-51-200-7593, Fax : +82-51-200-7505
E-mail : jwlee@mail.donga.ac.kr

study. Isolated polymannuronate as a functional additives and/or foods will be used for new applications of seaweed alginate.

Materials and Methods

Hydrolysis of seaweed alginate by organic acids

Seaweed alginate was kindly donated from the Myengsin Co. Ltd. (Kyungsangnam-Do, Korea). It was prepared from *Macrocystis pyrifera* and used for this study. Alginates with a MW of 1,283 kDa was mixed with preheated acetic acid, citric acid, lactic acid, malic acid, oxalic acid., succinic acid, and tartaric acid. Alginate (30 g) was mixed with 600 ml of 0.4 M organic acid and incubated at 100°C for 3 hr. After that, reaction mixture was neutralized with 1 M $NaHCO_3$ and mixed with same volume of 95% ethanol for precipitation.

Isolation of polymannuronate and polyguluronate from hydrolyzed alginates

Alginate was dissolved in preheated 0.4 M acetic acid. The reaction for hydrolysis of alginate maintained for 5 hr at 100°C. The resulting mixture was centrifuged at 5,000× *g* for 20 min after adjustment of pH to 2.9±0.1. Polymannuronate in supernatant and polyguluronate in precipitate were separated and neutralized with 1M $NaHCO_3$. Each part was mixed with same volume of 95% ethanol for precipitation. Each precipitate was dissolved in distilled water and the above procedure was repeated 2 or 3 times.

Analytic methods

The molar composition of mannuronate and guluronate in alginates hydrolyzed with organic acids was measured with high pressure liquid chromatography (HPLC) using a Spectra Physics P2000 system controller equipped with Whatman partisil 10-SAX anion exchange column (250×4.6 mm i.d.) [9]. The D-mannuronic acid lactone and L-gulucuronic acid lactone used for standard materials were purchased from Sigma-Aldrich (St. Louis, MO). The buffer, 0.02 M KH_2PO_4 (pH 4.6) solution containing 5% methanol, was used for elution.

The MWs of the alginates hydrolyzed with organic acids were measured by the gel chromatography equipped with equipped with a TSK PW_{XL} column (Viscotek, USA) and a RI detector. Pullulan standards (Showa Denko, Japan) with narrow polydispersity and MWs ranging from 5.80×10^3 to 1.60×10^6 were used to plot a calibration curve [8]. Deionized water was used as a mobile phase at a flow rate of 1.0 ml/min. The sample concentration and injection volume were 5.0 mg/ml and 100 µl, respectively. All of the sample solutions were filtered through 0.45 µm-pore-size filters (Adbentec MFS, Inc., Japan) before injection.

Measurement of total sugars in fractionated samples was carried out by the phenol sulfuric acid method [7]. The glucose (Sigma-Aldrich, USA) was used for the calibration curve of total sugar as standard.

Results

Effect of organic acids on hydrolysis of alginate

Organic acids including acetic acid, citric acid, lactic acid, malic acid, oxalic acid, succinic acid and tartaric acid were used for hydrolysis of alginate (Table 1). The reaction temperature and time for hydrolysis of alginate with 0.4 M organic acids were 100°C and 3 hr. Molar percentages of alginates hydrolyzed with organic acids ranged from 76.3 to 83.2%. Molar ratios of mannuronate to guluronate in hydrolyzed alginate ranged from 2.62 to 2.89. The molecular weight of alginate hydrolyzed with organic acid ranged from 7.5 to 53.2 kDa. The molecular weight of alginate hy-

Table 1. Effect of organic acids on hydrolysis of alginate[a]

Organic acid	Composition of hydrolyzed alginate			Molecular weight (kDa)	Molar percentage of hydrolysis (%)
	Mannuronate (%)	Guluronate (%)	M/G ratio		
Acetic acid	72.61	27.39	2.65	7.5	78.7
Citric acid	73.73	26.27	2.81	24.0	78.3
Lactic acid	70.13	29.87	2.35	33.8	76.3
Malic acid	69.87	30.13	2.32	53.2	78.4
Oxalic acid	74.69	25.31	2.95	37.6	83.2
Succinic acid	68.69	31.31	2.19	35.4	78.6
Tartaric acid	71.84	28.16	2.55	33.1	79.2

[a]Reaction temperature and time with 0.4 M of organic acids were 100°C and 3 hr.

Table 2. Effect of acetic acid concentration on hydrolysis of alginate[a)].

Concentration (M)	Composition of hydrolyzed alginate			Molecular weight (kDa)	Molar percentage of hydrolysis (%)
	Mannuronate(%)	Guluronate(%)	M/G ratio		
0	73.2	26.8	2.73	1,283.0	0.0
0.2	73.6	26.3	2.80	204.0	81.4
0.4	74.8	26.2	2.85	102.3	80.6
0.6	73.2	26.7	2.74	62.1	80.3
0.8	72.8	27.2	2.68	32.5	79.8
1.0	72.3	2.4	2.64	17.3	79.4

a) [a]Reaction temperature and time with acetic acid were 100°C and 40 min.

drolyzed with acetic acid was 7.5 kDa whereas that with malic acid was 53.2 kDa. The molar ratio of mannuronate to guluronate (M/G) in partially hydrolyzed alginate with organic acids ranged from 2.19 to 2.95. There was no significant difference in the molar ratio of M/G in hydrolyzed alginates. The molar ratio of M/G in the intact alginate used in this study was 2.68.

Effect of acetic acid concentration on hydrolysis of alginate

Alginate was hydrolyzed with various concentrations of acetic acid, known as the most effective hydrolytic agent in this study, at 100°C for 40 min (Table 2). The molar percentage and Mw of hydrolyzed alginates ranged from 79.4 to 81.4% and from 17.3 to 204.0 kDa, respectively. Generally speaking, the higher the concentration of acetic acid was used to hydrolyze alginate, the lower the MW of hydrolyzed alginate was obtained as shown in Fig. 1. The equation from the correlation between the MW of hydrolyzed alginate and the concentration of acetic acid was Y=776,400-986,700X. The Y and X mean the MW of hydrolyzed alginate and the concentration of acetic acid, respectively.

Effect of reaction time on hydrolysis of alginate

Alginate was hydrolyzed with 0.4 M acetic acid at 100°C for various times (Table 3). Molar percentage and MW of hydrolyzed alginates ranged from 78.6 to 80.7% and from 4.3 to 432.0 kDa, respectively. The molecular weight of alginate lineally decreased with reaction time whereas the molar ratio of M/G in hydrolyzed alginates seemed to be constant. There was no big difference in the molar percentage of hydrolyzed alginates with different reaction times but the correlation between the MW of alginate hydrolyzed with acetic acid and the reaction time was observed as shown in Fig. 2. The equation was Y=506,509-2,901X where Y and X mean the MW of hydrolyzed alginate and the reaction time with

Fig. 1. Correlationship between the molecular weight of partially hydrolyzed alginate at 100°C for 40 min and concentration of acetic acid.

0.4 M acetic acid, respectively. Initial reaction for hydrolysis of alginate up to 60 min was more vigorous than late one.

Isolation of polymannuronate and polyguluronate from hydrolyzed alginates

The process for isolation of polymannuronate and polyguluronate from the alginate hydrolyzed with 0.4 M acetic acid at 100°C was developed in this study with modification of Gaceas' procedure as shown in Fig. 3 [9]. Polymannuronate fractions were dissolved in acidic condition such as in pH 2.9 whereas polyguluronate fractions were precipitated under the same acidic condition. The molar percentage of mannuronate in the polymannuronate isolated from the alginate hydrolyzed with the process developed in this study increased with reaction time for hydrolysis of alginate. On the basis of the high performance liquid chromatographic analysis of D-mannuronate lactone and L-guluronate lactone as shown in Fig. 4, molar percentages of mannuronates in polymannuronates isolated from

Table 3. Effect of reaction time with acetic acid on hydrolysis of alginate[a)]

Reaction time (min)	Composition of hydrolyzed alginate			Molecular weight (kDa)	Molar percentage of hydrolysis (%)
	Mannuronate(%)	Guluronate(%)	M/G ratio		
0	73.2	26.8	2.73	1,283.0	0.0
10	73.4	26.6	2.76	432.0	80.7
20	74.2	26.2	2.83	245.6	80.8
40	74.3	25.7	2.89	105.2	80.4
60	74.0	26.0	2.85	42.7	80.3
120	73.6	26.9	2.74	9.2	79.3
180	72.6	27.4	2.65	7.5	78.7
240	72.4	27.6	2.62	4.3	78.6

a) Reaction temperature and concentration of acetic acid were at 100°C and 0.4 M.

Fig. 2. Correlationship between the molecular weight of partially hydrolyzed alginate at 100°C and reaction time with acetic acid.

alginate hydrolyzed with 0.4 M acetic acid at 100°C were 75% for 1 hr, 90% for 3 hr, and 98% for 5 hr of reaction time as shown in Fig. 5.

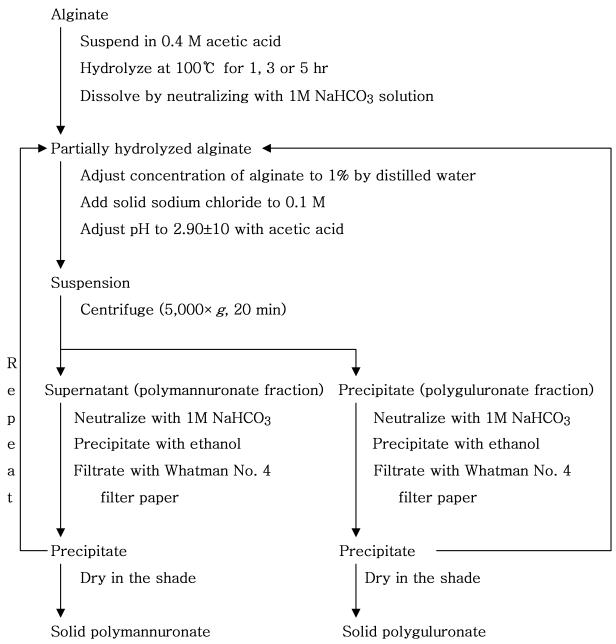

Fig. 3. Process for isolation of polymannuronate and polyguluronate from partially hydrolyzed alginates with 0.4 M acetic acid.

Discussion

The molecular weight of alginate hydrolyzed with acetic acid in this study lineally decreased with increasing concentration of acetic acid and reaction time. The hydrolytic kinetics of alginate with acetic acid showed the 1st order reaction. This result was coincided with the report where the Mw of alginate lineally decreased with increased reaction time and concentration of hydrochloric acid [14]. Hydrolysis of seaweed alginates with various concentrations of sulfuric acid for different reaction time had been reported [13] and rapid hydrolysis of alginate isolated from *Laminaria digitata* with 1 M oxalic acid at 100°C for 1 hr had been observed [11]. Partial hydrolysis due to oxidation of alginates with organic acids as well as inorganic acids resulted in decreasing of their MW such as other polysaccharides.

Alginate is one of the few polysaccharide that can be obtained from both eukaryotes and prokaryotes. Unlike bacterial alginates, seaweed alginates consist of [1→4] linked block copolymers of β-D-mannuronate (polymannuronate) and α-L-guluronate (polyguluronate) [5,20]. In the mechanism of gel formation, which is dimerization of polyguluronate sequences with divalent cations chelated between the alginates chains, the size of divalent cation is one of most important factors. The size of calcium ion other than those of divalent cations allows it to fit into the space formed

Fig. 4. High performance liquid chromatograms of (a) D-mannuronate lactone (M), (b) L-guluronate lactone (G) and (c) mixture of D-mannuronate lactone and L-guluronate lactone (1 : 1) eluted with 20 mM KH_2PO_4 (pH 4.6) containing 5% methanol at a flow rate of 1.0 ml/min.

Fig. 5. High performance liquid chromatograms of polymannuronate isolated from alginates hydrolyzed with 0.4 M acetic acid for (a) 1 hr (b), 3 hr and (3) 5 hr eluted with 20 mM KH_2PO_4 (pH 4.6) containing 5% methanol at a flow rate of 1.0 ml/min.

by guluronate residues between intra and inter molecules of seaweed alginates [18,19]. Among organic acids used in this work, acetic acid which showed strong hydrolyzing effect may be due to characteristics of acetic acid with relatively smaller molecular size and conformational feature of seaweed alginate.

Partial hydrolysis of alginate with acetic acid may occur first at the linkage of alternative residences due to its conformational feature of alginate. Polymannuronate adopts flat ribbon-like 2-fold chain confirmations in the solid state, similar to those found in β-1,4 diequatorially linked polymers such as cellulose [3,21], while polyguluronate adopts a buckled 2-fold chain confirmation [4]. Partial hydrolysis of alginate with acetic acid resulted in the mixture of polymannuronate and polyguluronate. The solubility of alginate at low pH depends on the uronic acid composition of the samples [11]. Difference in solubility due to its composition of uronic acids in polymannuronate and polyguluronate might be possible to separate each fraction.

Some of polysaccharides including alginate affects on cholesterol metabolism in rat [16]. The alginate with higher content of D-mannuronic acid reduced cholesterol levels in liver and blood and increased polyenoic acid in serum lipid of rats [22]. Partially hydrolyzed alginate with hydrochloric acid also reduced cholesterol levels in liver and blood of cholesterol-fed rats [15]. In this work, a new and simple method for isolation of polymannuronate with low MW from alginate hydrolyzed with acetic acid was developed. The more detailed functional study of polymannuronate with low MW will be followed to apply it for the anti-cholesterol agent in the food industry as a food additive.

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초록 : 유기산에 의해 가수분해된 알긴산에서 폴리만뉴론산과 폴리글루론산을 분리하는 간단한 방법

이동수 · 신명교[1] · 변재형[2] · 이진우[3]*
(한국바이오폴리머 생물공학연구소, [1]한국생산기술원 환경에너지본부, [2]부경대학교 식품생명공학부, [3]동아대학교 생명공학과)

분자량이 1,283 kDa인 알긴산을 0.4 M 유기산을 사용하여 100°C에서 3시간 반응시켜 가수분해하였다. 유기산에 의하여 가수분해된 알긴산의 분자량은 7.5에서 53.2 kDa이었다. 유기산으로 가수분해된 알긴산을 구성하는 만뉴론산과 글루론산의 비율은 가수분해하지 않은 알긴산과 비교하여 큰 차이가 없었다. 사용한 유기산 중에서 알긴산을 가수분해하는 가장 효과적인 유기산은 초산이었다. 사용한 초산의 농도 및 반응시간이 증가할수록 가수분해된 알긴산의 분자량은 감소하였다. 본 연구를 통하여 가수분해된 알긴산의 분자량과 초산의 농도 및 반응시간과의 상관관계를 나타내는 관계식을 구하였다. 초산으로 가수분해한 알긴산의 pH를 조절하여 폴리만뉴론산과 폴리글루론산을 분리하였다. 초산과 반응하는 시간이 증가할수록 폴리만뉴론산에 존재하는 만뉴론산의 비율은 증가하였다. 폴리만뉴론산에 존재하는 만뉴론산의 비율은 100°C에서 0.4 M 초산과 1시간, 3시간 및 5시간 반응하였을 경우에 각각 75%, 90% 및 98%이었다.