Electron Microscopic Observation of Calcium-Acetylated Seaweed Alginate Gel

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Abstract Seaweed alginate was acetylated by activated-carbon immobilized *Pseudomonas syringae* in a fluidized bed, up-flow reactor. The acetylation degree of seaweed alginate was about 30%. Calcium-acetylated seaweed alginate gel bead was made and compared to calcium-seaweed alginate gel bead by the scanning electron microscopy (SEM). Structural difference of two gel beads may results from increased viscosity and decreased affinity of acetylated seaweed alginate for calcium ion. On the basis of interior and exterior structure of calcium-acetylated seaweed alginate gels and property of acetylated seaweed alginate, it seems that acetylated seaweed alginate is used for the supporter for electrophoresis and packing materials for liquid chromatography and gel filtration.

Key words: Seaweed alginate, Acetylation, Acetylated seaweed alginate, Gel bead, Immobilization, Activated carbon, Scanning electron microscopy (SEM)

Introduction

Seaweed alginates are unbranched, block copolymers of β-D-mannuronic acid and its epimer, α-L-guluronic acid [11, 23]. The ratios of mannuronic acid to guluronic acid (M/G) indicate the properties of the resulting gels. Alginates with low M/G ratios produce strong and brittle gels, whereas alginates with high M/G ratios form elastic gels [15]. The mechanism of gel formation is believed to proceed by dimerization of polyguluronate sequences with divalent cations, such as Ca^{2+}, which chelated between the chain, the so-called 'Egg-box' model [12-14,16,17].

Seaweed alginate is one of the more widely used polysaccharides. It is used as an emulsifier, a stabilizer, and a thickener in both the food and chemical industries because of its ability to retain water as well as viscosifying, and stabi-

lizing properties. An alginate with a high molecular weight and a low ratio of M/G is recommended for the cultivation of immobilized anchorage-dependent cells [22]. Alginate gels also have potential as an implantation material for hormone-producing cells in bio-artificial endocrine pancreas because of their low content of toxic, pyrogenic and immunogenic materials, and defined pore sizes and mechanical stability [10]. Recently, alginates rich in mannuronate residues have been reported to have chemotactic activity for macrophages and antitumor activity [6].

The microbial acetylation of seaweed alginate was developed by resting cells system of *P. syringae* [4,7]. The acetylation of seaweed alginate by *P. syringae* was found to due to the separated system of biosynthesis and acetylation in this strain [8]. Acetylation of seaweed alginate increases the viscosity of resulting polymer and the flexibility of their gels which can produce a strong, thermoreversible-gel network. Acetylation also decreases its affinity for calcium ions [24]. The altered properties may be explained by both the increase in the total molecular weight of the resulting polymer and the reduction of the net negative charge [9,23].

To develop the support for electrophoresis or packing materials for liquid chromatography and gel filtration with improved properties, calcium-seaweed alginate and calcium-acetylated seaweed alginate gel beads were made and their structures were compared by the scanning electron microscope.

Materials and methods

Bacterial strain and culture conditions

Pseudomonas syringae subsp. *phaseolicola* ATCC 19304 was obtained from the American Type Culture Collection (ATCC, Rockville, USA). This strain was used for acetylating seaweed alginate. Cultures were maintained at 4°C on a modified Dworkin and Foster (DF) agar [5]. The pH of the medium was between 6.7 and 6.9 prior to sterilization. After growth for at 30°C for 3 - 5 days, slants were stored

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at 4°C. Cultures transferred every 4 weeks. Starter cultures were prepared by inoculating *P. syringae* ATCC 19304 from agar slants to 50 ml of modified DF liquid medium in 250 ml Erlenmeyer flasks. Cultures were incubated for 30 - 35 h at 30°C and 180 rpm on a NBS Model G25-KC rotary shaker (New Brunswick Scientific Co., Inc., Edison, USA), prior to use.

Acetylation of seaweed alginate by activated carbon immobilized *P. syringae* in a fluidized bed, up-flow reactor

Preparation of carbon immobilized *P. syringae* ATCC 19304 [8] and acetylation of seaweed alginate by immobilized cells in fluidized bed, up-flow reactor were described in previous report [7]. A continuously fed bioreactor containing carbon immobilized *P. syringae* cells was used for the continuous acetylation of seaweed alginates. The feed was 1.5 g/l of seaweed alginate from *Macrocystis pyrifera* and 1.0% (w/v) gluconic acid in 0.01 M phosphate buffer (pH 6.0) and the feed rate was 0.02 h^{-1}. Temperature was maintained at 25°C and aeration was 0.4 standard liter/min (SLPM). Air was filtered through a 6 inch glass wool-packed tube and a sterile Whatman Hepa-vent filter (Whatman Inc., Clifton, USA). The air flow was regulated with an in-line flow meter. The reactor was a 700 ml Kontes Airlift Bioreactor (Kontes Life Sciences Products, Vineland, USA) containing 25 g of carbon catalyst and working volume of 500 ml. Samples were withdrawn with time throughout operation and acetylated alginate was quantified by chemical assay.

Purification of acetylated seaweed alginate

Acetylated seaweed alginate produced in the immobilized cell reactor was mixed with an equivalent volume of iso-propanol. This mixture was incubated for 12 h at room temperature and then centrifuged at $8,000 \times g$ for 40 min in a Sorval Superspeed Model RC-5B centrifuge (Du Pont Co., Wilmington, USA). The precipitate was washed with iso-propanol and redissolved in distilled water. It was dialyzed against 500 volumes of distilled water for 48 h to remove all low molecular weight compounds and then concentrated in a Buchi Model R110 rotary evaporator (Buchi Lab., Flawil, Switzerland). After concentration, it was stored in a freeze-dried state.

Preparation calcium-acetylated seaweed alginate gel beads

Seaweed alginate and acetylated seaweed alginate were individually dissolved in distilled water to a final concentration of 1% (w/v). The solution was dispensed dropwise from a syringe with a 18 gauge needle from a height of about 30 cm into 0.1 M $CaCl_2$ solution. Alginate beads were left to harden in 0.1 M $CaCl_2$ solution for 1 h. The $CaCl_2$ solution was replaced with fresh solution, prior to observation with scanning electron microscopy (SEM). Diameter of calcium-

seaweed alginate and calcium-acetylated seaweed alginate gel beads was about 1.5 mm.

Scanning electron microscopy of calcium-acetylated seaweed alginate gel beads

Calcium-seaweed alginate and calcium-acetylated seaweed alginate gel beads were prepared by washing with 0.1 M cacodylate buffer (pH 7.2) and fixing for 1 h with 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer at room temperature. Fixed samples were washed three times with same buffer, and post-fixed for 1 h with 0.8% (v/v) osmium tetraoxide. After washing with distilled water, they were treated 0.5% (w/v) with uranium acetate. Samples were washed again with distilled water, and then dehydrated with a graded series of ethanol washes. Dehydrated samples were finally dried in a Denton DCP-1 Critical Drying Apparatus (Denton Vacuum Inc., Cherry Hill, USA) and coated with P_2O_5 using a Edward S150 Sputter Coater (Edward Vacuum Inc., Wilmington, USA). The samples were visualized in a Cambridge S-260 scanning electron microscope (Leica Co., Deerfield, USA).

Analytic methods

Standard curves for alginate and acetyl were prepared from sodium alginate and glucose pentaacetate (Sigma Chemical Co., St. Louis, USA), respectively. Seaweed alginate used in this experiment was isolated from *M. pyrifera* and the viscosity of 2% (wt/vol) solution is 1,400 CP. Alginate concentrations were determined according to the method described by Blumenkarantz and Asboe-Hansen [1]. The degree of acetylation was measured according to the method described McComb and McCready [2].

Results

Acetylation of seaweed alginate

Acetylation of seaweed alginate reached its highest yield (about 90% of seaweed alginate was acetylated.) 3 days after startup. Then the rate of acetylation decreased linearly [7]. The average degree of acetylation of seaweed alginate to prepare calcium-acetylated alginate gel was 30%. The presence of acetyl groups in the seaweed alginate was confirmed by 1H-NMR spectroscopy [9]. Partly overlapping peaks of acetyl protons in NMR spectrum of the acetylated seaweed alginate suggested the presence of either di-acetylated units and/or two mono-acetylated units [9,24]. The presence of acetyl groups on seaweed alginates increased the weight molecular weight (M_w) by 7% and decreased the ability of seaweed alginate to bind with calcium [9].

Comparison of calcium-alginates gel beads

Calcium-seaweed alginate and calcium-acetylated seaweed alginate gel beads observed by scanning electron microscopy (SEM). The surface structure of two kinds of gel beads

appeared different from each other. At low magnification, the surface of calcium-acetylated seaweed alginate gel bead looked like more smooth than calcium-seaweed alginate gel bead which showed some wrinkles on the surface (Fig. 1a and b). Its outer shape was more bumpy and uneven than that of calcium-acetylated seaweed alginate gel bead. Observation of each gel bead at higher magnifications showed that there was basic difference in their surface structure (Fig. 2a and b). The surface of calcium-acetylated alginate gel bead was a condensed structure with a regular fold whereas that of calcium-seaweed alginate did not show any regular fold but something looked like irregularly engraved lines.

Observation of each gel beads surface with more high magnification (Fig. 3a and b) indicated that the surface structure of two gel beads was basically different from each other. The surface shape of calcium-acetylated seaweed alginate gel bead was based on regular folding structure, but that of
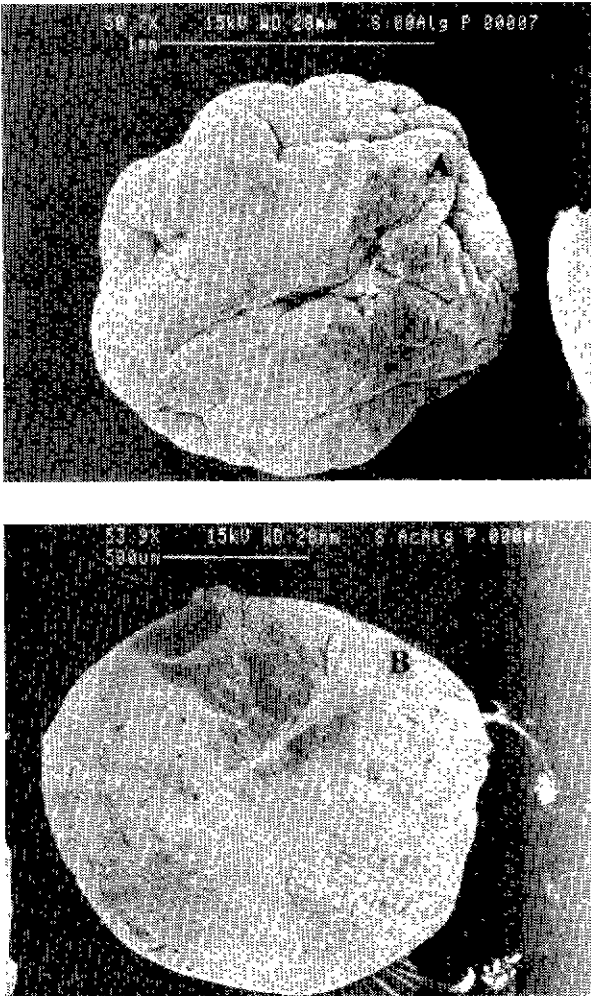

Fig. 1. Scanning elctron micrographs of the surface structure of (a) calcium-seawecd alginate gel and (b) calcium-acetylated seaweed alginate gel.
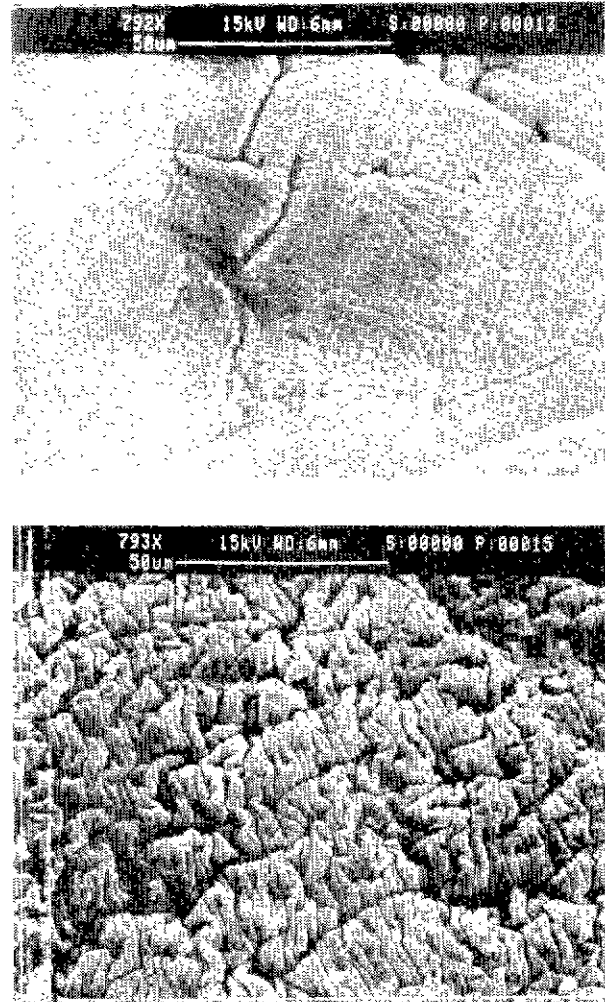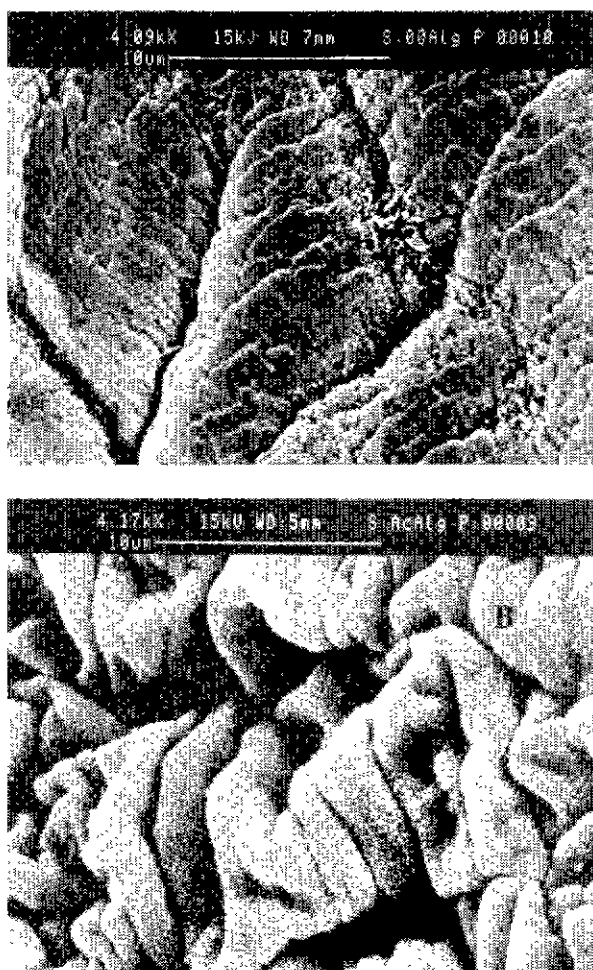

Fig. 2. Scanning elctron micrographs of the fine surface structure of (a) 47calcium-seaweed alginate gel and (b) calcium-acetylated seaweed alginate gel

calcium-seaweed alginate gel bead did not show any regular structure.

The interior of calcium-acetylated seaweed alginate gels showed regular pore holding structure whereas calcium-alginate gels do not show any regular porous structure (Fig. 4a and b).

Discussion

Unlike seaweed algiante, the presence of di-O-acetylated residues in bacterial alginates has been reported [3,21]. Acetylation of seaweed alginate by *P. syringae* ATCC 19304 could be explained by the fact that the ability of this cells acetylated similar substrate, i. e., seaweed alginate from *Macrocystis pyrifera*, which is comprised of 60% mannuronic acid and 40% guluronic acid [18]. Seaweed alginates
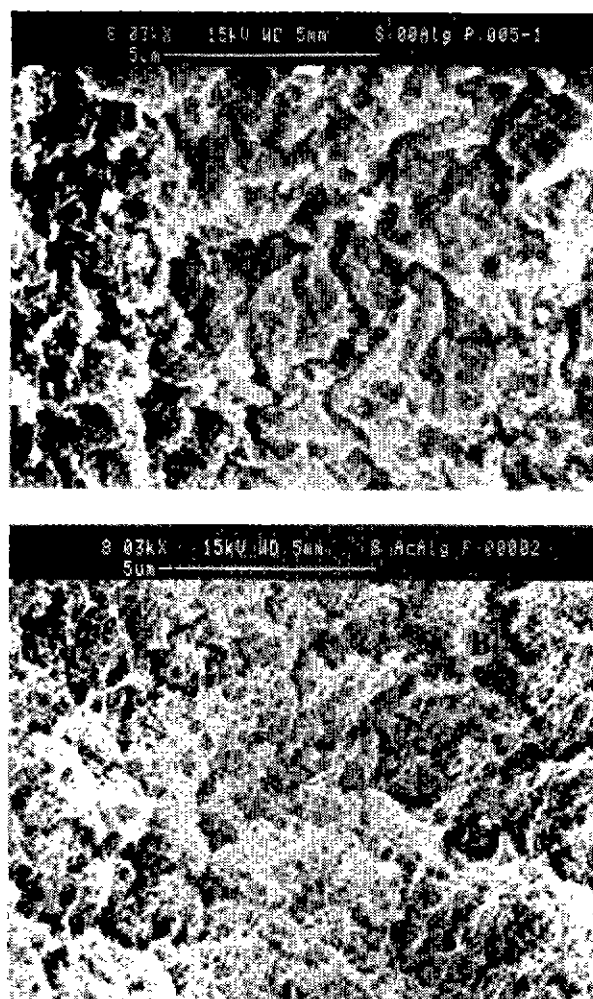
Fig. 3. Scanning elctron micrographs of finer surface structure of (a) calcium-seaweed alginate gel and (b) calcium-acetylated seaweed alginate gel.

Fig. 4. Scanning elctron micrographs of the interior structure of (a) calcium-seaweed alginate gel and (b) calcium-acetylated seaweed alginate gel.

added at the beginning of cultivation or the logarithmic growth phase of *P. syringae* ATCC 19304 are acetylated [7]. Acetylation in the seaweed alginate occurs almost entirely in the O-2 and O-3 position of mannuronic acid residues [8]. Seaweed alginate acts as a feedback inhibitor for alginate biosynthesis in this strain [7].

Gellation is a process by dimerization of polyguluronate sequences with divalent cations, such as Ca^{2+}, which chelated between the chain, the so-called 'Egg-box' model [12-14,16,17]. Gels with low M/G ratios exhibit high porosity, low shrinkage during gel formation, and do not swell after drying. Gels with high M/G ratios shrink more and have low porosity after gel formation. Seaweed alginate has normally more guluronate than bacterial alginate in their composition. The more gulucuronate in alginate can makes the more strong gel with divalent cations.

The role of acetyl groups in bacterial polysaccharides, such as xanthan, is normally stabilization of the ordered confor-

mation [19,20,25]. Acetylation of seaweed alginate increased the viscosity of resulting polymer and decreased its affinity for calcium ion [23], which results in enhancing the swelling ability of calcium gels made from resulting polymers [8, 24]. Acetylated seaweed alginate with site-specifically C-2 and/or C-3 of mannuronate and high portion of guluronate makes strong gel with low affinity for calcium ion [7] which resulted from acetylation of seaweed alginate.

Because of their low content of toxic, pyrogenic and immunogenic materials, and defined regular pore size and mechanical stability, calcium-acetylated seaweed alginate gel could be used as a supporter for electrophoresis and packing material for liquid chromatography and gel filtration like curdlan gel as a promising supporter for electrophoresis. The pore size of gel beads may be controlled by concentration of salt and/or acetylated seaweed alginate.

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