Molecular identification of a polyM-specific alginate lyase from *Pseudomonas* sp. strain KS-408 for degradation of glycosidic linkages between two mannuronates or mannuronate and guluronate in alginate

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Abstract: An alginate lyase gene of a newly isolated *Pseudomonas* sp. strain KS-408 was cloned by using PCR with the specific primers designed from homologous nucleotide sequences. A partial protein sequence of KS-408 alginate lyase was homology-modeled on the basis of the crystal structure of A1-III alginate lyase from *Sphingomonas* sp. strain A1. The proposed 3-D structure of KS-408 alginate lyase shows that Asn-198, His-199, Arg-246, and Tyr-253 residues are conserved for the catalytic active site. The recombinant KS-408-1F (with signal peptide) and KS-408-2F (without signal peptide) alginate lyases with the $(His)_6$ tag consist of 393 (44.5 kDa) and 372 (42.4 kDa) amino acids with isoelectric points of 8.64 and 8.46, respectively. The purified recombinant KS-408 alginate lyase was very stable when it was incubated at 40 °C for 30 min. Alginate oligosaccharides produced by the KS-408-2F alginate lyase were purified on a Bio-Gel P2 column and analyzed by thin-layer chromatography, fast-protein liquid chromatography, and electrospray ionization mass spectrometry. ^{1}H NMR data showed that the KS-408-2F alginate lyase cleaved the glycosidic linkages between two mannuronates (mannuronate-β(1–4)-mannuronate) or mannuronate and guluronate (mannuronate-β(1–4)-guluronate), indicating that the KS-408 alginate lyase is a polyM-specific lyase.

Key words: Pseudomonas sp. strain KS-408, alginate lyase, alginate oligosaccharides, polyM-specific lyase.

Résumé : Un gène codant une alginate lyase nouvellement isolée de *Pseudomonas* sp. souche KS-408 a été cloné par PCR à l'aide d'amorces spécifiques conçues à partir de séquences homologues de nucléotides. La séquence protéique partielle de l'alginate lyase de KS-408 a été modélisée sur la base son homologie avec l'alginate lyase A1-III de *Sphingomonas* sp. souche A1, à partir la structure cristalline de cette dernière. La structure proposée en 3-D de l'alginate lyase de KS-408 montre que les résidus Asn-198, His-199, Arg-246 et Tyr-253 sont conservés au sein du site actif catalytique. Les alginate lyases recombinantes KS-408-1F (avec peptide signal) et KS-408-2F (sans peptide signal) comprenant une étiquette $(His)_6$ tag comportent respectivement 393 (44,5 kDa) et 372 (42,4 kDa) acides aminés et des points isoélectriques de 8,64 et 8,46. L'alginate lyase recombinante KS-408 était très stable lors d'une incubation à 40 °C pendant 30 minutes. Les alginates produits par l'alginate lyase KS-408-2F ont été purifiés sur une colonne Bio-Gel P2 et analysés par TLC, FPLC et ESI MS. Les données en ^{1}H RMN montrent que l'alginate lyase KS-408-2F a clivé les liens glycosides entre deux mannuronates (mannuronate-β(1–4)-mannuronate) ou entre un mannuronate et un guluronate (mannuronate-β(1–4)-guluronate), indiquant que l'alginate lyase KS-408 est une lyase spécifique de poly-M.

Mots-clés : Pseudomonas sp. souche 408, alginate lyase, alginates, lyase spécifique de poly-M.

[Traduit par la Rédaction]

Introduction

Alginate, an acidic polysaccharide, is composed of (1–4)-linked β-D-mannuronate (M) and its C5-epimer α-L-guluronate (G). It is produced by brown algae and some bacteria belonging to the genera *Azotobacter* and *Pseudomonas* (Wong et al. 2000). Alginates are widely used in the food, cosmetics, and pharmaceuticals and for biotechnological purposes, such as cell immobilization (Kim et al. 2011).

Enzymatically depolymerized alginates with low molecular weights (average M_r = 1800) have been known to exhibit useful biological activities. They promote the growth of bifidobacteria and the elongation of barley roots (Akiyama et al. 1992; Yonemoto et al. 1993). In terms of the biological effects on mammalian cells, alginate oligosaccharides (average M_r = 2000) enhanced the growth of human endothelial cells and keratinocytes (Kawada et al. 1999). Recently, Iwamoto et al. (2005) have reported that alginate oligosaccharide of a

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specific size stimulated secretion of cytotoxic cytokines from human macrophage. Some alginate polymers (average M_r = 230 000) were reported to have antitumor effects and to enhance phagocyte activity of macrophages (Fujihara and Nagumo 1992).

Various alginate lyases have been identified in algae, bacteria, and marine organisms (Wong et al. 2000; Yamamoto et al. 2008; Kim et al. 2009, 2011; Park et al. 2011). These alginate lyases catalyze β-elimination reactions by releasing unsaturated oligosaccharides with 4-deoxy-α-L-*erythro*-hex-4-enopyranuronosyl uronate at their nonreducing end (Kim et al. 2011). Alginate lyase can be used as a therapeutic agent against *Pseudomonas aeruginosa* infection, and it can degrade the acetylated alginate-rich mucoid layer to enhance susceptibility of *P. aeruginosa* to antibiotics (Ramsey and Wozniak 2005; Alkawash et al. 2006).

Alginate lyases have their own substrate specificity, and they can be classified based on their preferential cleavage site on alginate substrate (Yamasaki et al. 2004). Many alginate lyases possessing a preference for polyG block or polyM block have been reported. PolyG-specific alginate lyase was cloned from *Sphingomonas* sp. strain A1. Some polyM-specific alginate lyases were also cloned and characterized from *P. aeruginosa* and *Azotobacter* sp. (Cote and Krull 1988; Xiao et al. 2006). They depolymerize alginate to unsaturated di- and tri-saccharides. The alginate lyase of *P. aeruginosa* can degrade acetylated alginate. In addition to endolytic alginate lyases, exolytic alginate lyase were found from *Sphingomonas* sp. strain A1 and *Agrobacterium tumefaciens* C58 (Miyake et al. 2003; Ochiai et al. 2006). They are responsible for the complete degradation of alginate oligosaccharides into constituent monosaccharides.

Recently, we have cloned and characterized a polyG-specific alginate lyase from a marine bacterium, *Streptomyces* sp. strain ALG-5, that degrades G-rich region of alginates (Kim et al. 2009). It releases unsaturated di-, tri-, and tetrasaccharides from alginate. We have also obtained the recombinant oligoalginate lyase that exolytically degraded alginate into unsaturated monosaccharides (Park et al. 2011). Enrichment of the pools of the recombinant alginate lyases with different substrate specificities is valuable for expanding the utilization of alginate. Hence, it is of interest to have a polyM-specific alginate lyase that can be used for cooperative degradation of alginate into constituent monosaccharides, in combination with polyG-specific and (or) exolytic alginate lyases. Herein, we report on the cloning of an alginate lyase gene from a newly isolated *Pseudomonas* sp. strain KS-408 to obtain a polyM-specific recombinant alginate lyase. The recombinant KS-408 alginate lyase was prepared, purified, and characterized, and its basic properties were compared with other alginate lyases. The degradation products were isolated and their structures were analyzed by thin-layer chromatography (TLC), electrospray ionization tandem mass spectrometry (ESI MS/MS), and ^{1}H nuclear magnetic resonance (NMR) spectroscopy.

Materials and methods

Materials

Alginates (250 and 3500 centipoises) and TLC silica gel 60 were purchased from Sigma (USA). Bio-Gel P-2 gel was purchased from Bio-Rad. Ni-Sepharose, His-Buffer kits, and HiTrap desalting column were purchased from GE Healthcare (USA). TLC standards and poly-G-specific alginate lyase (ALG-5 lyase) were prepared based on the previous study of Kim et al. (2009). Restriction enzymes and T4 DNA ligase were purchased from NEB (England), and LA Taq polymerase was purchased from Takara (Japan). Plasmid purification, PCR product purification, and DNA extraction in the agarose gel were performed using a QIAquick Plasmid purification kit, QIAquick PCR purification kit, and QIAquick gel extraction kit (QIAGEN, Germany), respectively. PCR for DNA amplification was performed using Biometra TPersonal (Whatman, Germany).

Cloning and expression of alginate lyase gene from KS-408

The genomic DNA of *Pseudomonas* sp. strain KS-408 was used as the template for amplification of the alginate lyase gene. The alginate lyase primers were designed on the basis of homolog sequences from *algX* and *algI* gene sequence in alginate biosynthesis cluster from various *Pseudomonas* species; *Pseudomonas fluorescens* NCIMB10525 (AF522790), *Pseudomonas syringae* (FI142977), *Pseudomonas* sp. strain QD3 (AL161336.1), *P. fluorescens* Pf0-1 (NC007492), and *P. fluorescens* Pf-5 (NC004129). The forward primer was derived from the AlgX gene sequence as 5pro-algXF: 5′-GTCGAAGCGAAAATTTGCACACGCAACGTATTCCC-3′; the reverse primer was derived from the AlgI gene sequence as 3pf-algIR: 5′-GCGATAGCGTTGCCCGCTCARRTAGTA-CAAGCCGAG-3′. The PCR products were ligated with pGEM-T easy vector. The nucleotide sequence was blasted by using BlastX program and the open reading frame (ORF) was searched by using NCBI's ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

The signal peptide cleavage site was predicted using SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP-3.0/). To clone the alginate lyase full gene into the expression vector (referred to as KS-408-1F), the forward primer 5pf-alg1F (*Eco*RI) 5′-GGAATTCGATGCAAAAGTTATTGCTCCCTG GCC-3′ and the reverse primer 3pf-alg1R (*Xho*I) 5′-GCTCGAGGCTGCCTTTGTTGCCTTTTTCATGC-3′ were used. On the other hand, the primers; 5pf-alg2F (*Eco*RI) 5′-GGAATTCAGCGGCGCCACTGCGTCCACCCCAGG-3′ and 3pf-alg1R (*Xho*I) 5′-GCTCGAGGCTGCCTTTGTTGCCTTT TTCATGC-3′ were used for cloning the KS-408 lyase gene without the signal peptide region (referred to as KS-408-2F). Two alginate lyase genes, KS-408-1F and KS-408-2F, were ligated into pET21b(+) expression vector. The recombinant *Escherichia coli* BL21 (DE3) cells harboring the pET-KS-408 plasmids were cultured on Luria–Bertani medium supplemented with 50 μg/mL ampicillin for 2–3 h up to an OD_{600} = 0.4–0.6 in a shaking incubator at 180 r/min and 37 °C. The cells were incubated at 15 °C for 30 min to adapt to cold environment at static condition, and then cultured for 24 h at 15 °C to express the KS-408 alginate lyase gene induced by the addition of 1 mmol/L IPTG (isopropyl-β-D-thiogalactopyranoside).

Homology modeling of KS-408 alginate lyase

A homology model for KS-408 alginate lyase was constructed based on the X-ray crystallographic structure of

Sphingomonas sp. strain A1 alginate lyase A1-III (pdb code: 1hv6). Homology modeling was carried out using the Modeller 7v7 program (http://salilab.org/modeller/). Superimposition of the KS-408 alginate lyase model on that of A1-III alginate lyase was visualized by using Python Molecule Viewer (PMV version 1.5.4, http://mgltools.scripps.edu/).

SDS–PAGE gel electrophoresis and immunoblotting

The cells were boiled with Laemmli buffer at 95 °C for 10 min, sonicated, and loaded into two sets of SDS – polyacrylamide gels. One gel was visualized with Coomassie brilliant blue R-250 and the other was transferred onto nitrocellulose membrane. The membrane was blotted with polyclonal antibody against hexahistidine (H-15, Santa Cruz Biotechnology Inc., USA) and peroxidase-conjugated anti-rabbit IgG (Jackson Immunoresearch, USA), and then visualized with CN/DAB (4-chloronaphthol/3,3′-diaminobenzidine) solution (Pierce, USA).

Purification of KS-408 alginate lyase

The cells were harvested and sonicated in 50 mmol/L potassium phosphate buffer (pH 7.2) containing 300 mmol/L NaCl, 10 mmol/L imidazole, 10% (*v*/*v*) glycerol, 0.5% (*v*/*v*) Triton X-100, 1 mg/mL lysozyme, and 1 mmol/L PMSF (phenylmethylsulphonyl fluoride) by using ultrasonicator Vibra Cell CX400 (Sonics & Materials Inc., USA). The cell homogenate containing the expressed $(His)_6$-tagged KS-408 alginate lyase was loaded on the Ni-Sepharose column equilibrated with 20 mmol/L phosphate buffer (pH 7.2) and 0.5 mol/L NaCl. The column was washed with the same buffer containing 20 mmol/L imidazole, and then the bound protein was eluted with the same buffer containing 300 mmol/L imidazole. The protein fraction eluted with 300 mmol/L imidazole was proven to have alginate lyase activity by the thiobarbituric assay (TBA) method. Excess imidazole and NaCl were then removed using HiTrap desalting column by using 20 mmol/L phosphate buffer and 0.15 mol/L NaCl. Protein concentration was calculated by measuring absorbance at 595 nm using the Bradford method.

Substrate preparation

For the alginate lyase activity assay, sodium alginate and acetylated alginate with high molecular weight, polyM block, polyG block, and polyMG block were used. Alginates designated with polyM block, polyG block, and polyMG block were prepared by acid hydrolysis methods according to Haug et al. (1966). Acetylated alginate was obtained from *P. aeruginosa* ATCC 39324 cultured on *Pseudomonas* isolation agar (Difco, USA) plate. Acetylated alginate was purified according to the protocol of May and Chakrabarty (1994). The MG ratio of the purified substrate was determined according to the standard test method ASTM F2259 (ASTM F2259-10, ASTM International 2010). The degree of *O*-acetylation in acetylated alginate was determined according to the protocol of McComb and McCready (1957).

Assay of alginate lyase activity

The purified enzyme was added into 1 mL of 20 mmol/L phosphate buffer (pH 7.2) containing 0.2% (*m*/*v*) sodium alginate and incubated at 37 °C for 10 min. The reaction was stopped by heating in boiling water for 10 min. The lyase activity was assayed by measuring the increase in absorbance at 235 nm due to the formation of a double bond between C-4 and C-5 at the nonreducing terminus by the β-elimination reaction. The resulting deoxy sugar was determined by the TBA method (Kim et al. 2009). The degradation products were analyzed by TLC and fast-protein liquid chromatography (FPLC). The degraded products on TLC were separated twice with a developing solvent of 1-butanol – acetic acid – water (2:1:1, by volume). The degradation products were visualized by dipping into 10% (*v*/*v*) sulfuric acid in ethanol. The degradation products were also analyzed by FPLC equipped with a Superdex peptide 10/100 GL column (GE Healthcare, USA). The degraded unsaturated oligoalginates were detected by measuring the absorbance at 235 nm with UV detector.

Analysis of substrate specificity and stability of KS-408 alginate lyase

For investigation of substrate specificity, 20 μg of purified KS-408 alginate lyase and 0.2% (*m*/*v*) substrates as a final concentration in 20 mmol/L phosphate buffer (pH 7.2) were incubated in 1 mL of reaction mixture for 10 min at 37 °C, and then the reaction mixture was boiled for 10 min to inactivate the enzyme. To evaluate the enzyme stability over temperature ranges from 20 to 70 °C, 50 μg of KS-408 alginate lyase in 0.5 mL of 20 mmol/L phosphate buffer (pH 7.2) was incubated for 30 min at various temperatures (20, 30, 37, 40, 50, 60, 70 °C) before further incubation for 10 min at 37 °C with the substrate. To determine optimal pH, 50 μg of KS-408 alginate lyase was incubated for 10 min at 37 °C in appropriate buffers (20 mmol/L citrate buffer (pH 3–6), 20 mmol/L phosphate buffer (pH 6–9), 20 mmol/L Tris–HCl buffer (pH 6–10), and 20 mmol/L glycine buffer (pH 9–11)) before further incubation with the substrate. To evaluate the effect of metal ion on enzyme activity, 50 μg of KS-408 alginate lyase was incubated with 2 mmol/L salt solution (NaCl, $ZnCl_2$, $CaCl_2$, $MgCl_2$, $HgCl_2$, KCl, $CoCl_2$, $MnCl_2$, $BaCl_2$) in 20 mmol/L phosphate buffer for 10 min, and then incubated with 0.2% (*m*/*v*) alginate substrate at 37 °C for 10 min.

Structural analysis of KS-408 alginate lyase-degrading products

A 0.4% (*m*/*v*) alginate was digested by KS-408 alginate lyase and the degradation fractions were analyzed using FPLC (Park et al. 2011). The unsaturated uronate fractions were detected by measuring the absorbance at 235 nm. For the determination of the molecular mass of each fraction, ESI MS (6410 Triple Quadrupole LC-MS, Agilent, USA) was used. The purified oligosaccharides from Bio-Gel P-2 column were dissolved in a small volume of methanol–water (1:1, *v*/*v*), and then injected onto the LC MS. The flow rate of the electrospray source was 5 μL/min. MS was performed in negative mode with an ion spray voltage of 4 kV and source temperature of 350 °C.

^{1}H NMR of alginates degraded by KS-408 alginate lyase

A 0.4% (*m*/*v*) alginate in 20 mL of buffer (10 mmol/L phosphate, pH 7.2) was mixed with enough of the purified enzyme and then incubated at 37 °C until the substrate was digested completely. To confirm whether the substrate was digested completely, the reaction mixture was withdrawn pe-

riodically and analyzed by FPLC. After the alginate substrate and reaction products were lyophilized, they were dissolved with D_2O and then lyophilized again. The lyophilized samples were dissolved with D_2O and then analyzed by high-field ^1H NMR spectroscopy, using an ECX-NMR 400 Hz JEOL spectrometer (JEOL, USA) following the protocol of Grasdalen (1983). A 20 μL volume of TMSP (0.75% 3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionic acid, Na salt in D_2O) was add to the NMR sample tube as an internal standard. Data collection was carried out at an elevated temperature, 80 °C. Sample spinning, proton spectral width, number of scans, relaxation delay, proton pulse angle, and acquisition time were 15 Hz, –5 – 15 ppm, 64, 5 s, 90°, and 4.098 s, respectively. NMR spectrum of the alginate product from degradation by KS-408 lyase was compared with that from degradtion by ALG-5 lyase (Kim et al. 2009).

Interpretation of ^1H NMR spectra

The molar fraction of the monomer units (F_G, F_M), the diads (F_{GG}, F_{MM}, F_{GM}, F_{MG}), and the G-centered triads (F_{GGG}, F_{MGM}, F_{GGM}, F_{MGG}) of substrates were calculated in accordance with Grasdalen (1983) and Zhang et al. (2004).

In the case of alginate-lyase-degraded oligoalginates, unsaturated 4-deoxy-L-*erythro*-hex-4-enepyranosyluronate (Δ) is produced on the nonreducing end by a β-elimination reaction. The specific resonance signals and their amounts from the reducing ends (G_{red} and M_{red}) and the nonreducing ends (Δ) can be identified and calculated from the NMR spectra of the degraded alginate samples (Heyraud et al. 1996). If GGG, GGM, GMG, and GMM sequences are cleaved by a polyG-specific lyase, G_{red} on the reducing end and ΔG (G↓GG and G↓MG) or ΔM (G↓GM and G↓MM) on the nonreducing end can be detected, respectively. In the case of a polyM-specific lyase, MMM, MGM, MMG, and MGG are cleaved to M_{red} on the reducing end, and ΔM (M↓MM and M↓GM) or ΔG (M↓MG and M↓GG) on the nonreducing end can be detected. To calculate the molar fraction of G and M units from the enzyme-catalyzed degradation product, the intensity (I) of total G and M was divided by the sum of Δ (calculated from the intensity of Δ-4-G and Δ-4-M), G, and M. The averaged degree of polymerization (DP_n) was estimated from $DP_n = (I_{\underline{\Delta}\text{-}1\text{-}G} + I_{\underline{\Delta}\text{-}1\text{-}M} + I_{\underline{G}\text{-}1} + I_{\underline{M}\text{-}1} + I_{\underline{G}red} + I_{\underline{M}red}) / [(I_{\underline{\Delta}\text{-}1\text{-}G} + I_{\underline{\Delta}\text{-}1\text{-}M} + I_{\underline{G}red} + I_{\underline{M}red}) / 2]$ (Ertesvåg et al. 1998).

Results and discussion

Cloning and heterologous expression of KS-408 alginate lyase

The 16S rDNA analysis of the isolated bacteria possessing alginate-degrading activity revealed that the closest relatives of the strain KS-408 were *Pseudomonas* species (99%). The isolated alginate-degrading bacterium was named *Pseudomonas* sp. strain KS-408. To clone the alginate lyase gene of *Pseudomonas* sp. strain KS-408, PCR was performed with specific primers designed based on homologous nucleotide sequences. The PCR product was ligated into pGEM-T easy vector and the nucleotide sequence was determined. The size of the PCR product using primers 5pro-algXF and 3pf-algIR was 1464 bp with an 1110 bp ORF that starts with an ATG codon at position 81 and terminates with a TGA codon at position 1190. The nucleotide sequence of KS-408 alginate lyase gene was deposited in the GenBank database (accession No. JN802116). The putative protein deduced from the ORF region consisted of 369 amino acids with a molecular mass of 41.7 kDa. The sequence of ORF region was blasted with other proteins in GenBank database and elucidated to have a high sequence identity with alginate lyase precursors or polyM-specific lyases. The analysis of BlastP and ClustalW revealed that the deduced amino acid sequence of the KS-408 alginate lyase showed 94% identity with the alginate lyase precursor of *P. fluorescens* SBW25 and *Pseudomonas* sp. strain QD03, 85% identity with polyM lyase from *P. fluorescens* Pf-1, and 80% identity with polyM lyase from *P. fluorescens* Pf-5. There was a highly conserved hydrophilic NNHSYW motif in the center of the protein and a semiconserved hydrophobic WLEPFCTLY region in the C-terminus, which indicated that the KS-408 alginate lyase is similar to the periplasmic protein AlgL from *P. aeruginosa* and *Pseudomonas* sp. strain QD03 (Schiller et al. 1993; Xiao et al. 2006). *Pseudomonas* sp. strain QD03 showed lyase activity on the polyM block and acetylated alginate made from pathogenic bacterial biofilm.

The cloned gene was inserted into pET21b(+) vector to express the alginate lyase protein. The recombinant *E. coli* harboring the KS-408 alginate lyase gene was cultured, and the expressed protein was analyzed by using SDS–PAGE and immunoblotting. The full-sequence protein (referred to as KS-408-1F) was deduced to have 393 amino acid residues, with a theoretical molecular mass of 44.5 kDa and isoelectric point of 8.64. The protein without signal peptide (from Met-1 to Ala-21), referred to as KS-408-2F, consisted of 372 amino acid residues with a theoretical molecular mass of 42.4 kDa and isoelectric point of 8.46. From SDS–PAGE and immunoblotting profile after induction with IPTG, both KS-408 alginate lyases with $(His)_6$ tag showed thick bands (Fig. 1, lanes 2 and 4), indicating that the addition of IPTG clearly induced the expression of the recombinant protein (Fig. 1). The expressions of the recombinant alginate lyases were confirmed by immunoblotting with polyclonal antibody and peroxidase-conjugated anti-rabbit IgG.

Homology modeling of KS-408 alginate lyase

Homology modeling of *Pseudomonas* sp. strain KS-408-2F alginate lyase was constructed based on the crystal structure of A1-III polymannuronate-specific lyase with ΔMG trimer (1hv6.pdb) from *Sphingomonas* sp. strain A1. A 3-D structure model was obtained by using Modeller 7v7 program after protein sequence alignment by position-specific iterated blast. Only 211 amino acid sequence from Phe-103 to Lys-314 could be aligned with A1-III alginate lyase (from Tyr-98 to Lys-306) with 29% sequence identity. Protein sequence alignment of KS-408-2F and A1-III was carried out based on position-specific iterated Blast. The constructed partial 3-D model of KS-408-2F alginate lyase was superimposed on the crystal structure of A1-III (Fig. 2). Although only partial protein sequence has very low homology, the active site of KS-408 alginate lyase could be predicted. The active site consisting of Asn-198, His-199, Arg-246, and Tyr-253 was strictly conserved. Based on the above results, KS-408 alginate lyase may be included with the polysaccharide lyase family 5.

Fig. 1. SDS–PAGE and immunoblotting analysis of KS-408 alginate lyases. The recombinant KS-408 alginate lyases were separated on 12% (*m*/*v*) SDS–PAGE. The proteins on SDS–PAGE gel were visualized by staining with Coomassie brilliant blue R-250 (left panel). Over-expressed KS-408 alginate lyases on a nitrocellulose paper were detected by immunoblotting method (right panel). Lanes: M, protein marker; 1 and 2, KS-408-1F recombinant cells induced without and with IPTG, respectively; 3 and 4, KS-408-2F recombinant cells induced without and with IPTG, respectively; 5 and 6, purified KS-408-1F and KS-408-2F, respectively. Immunoblotting was performed with anti-6xHis antibodies.

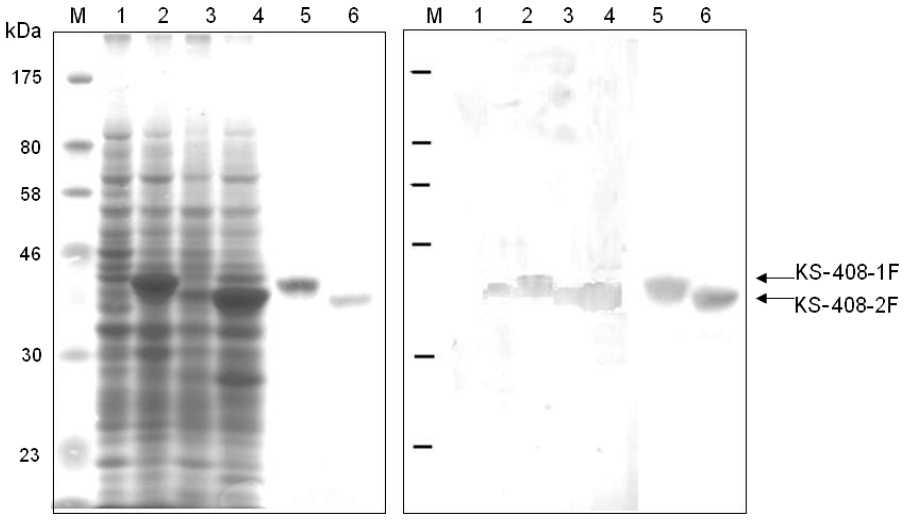
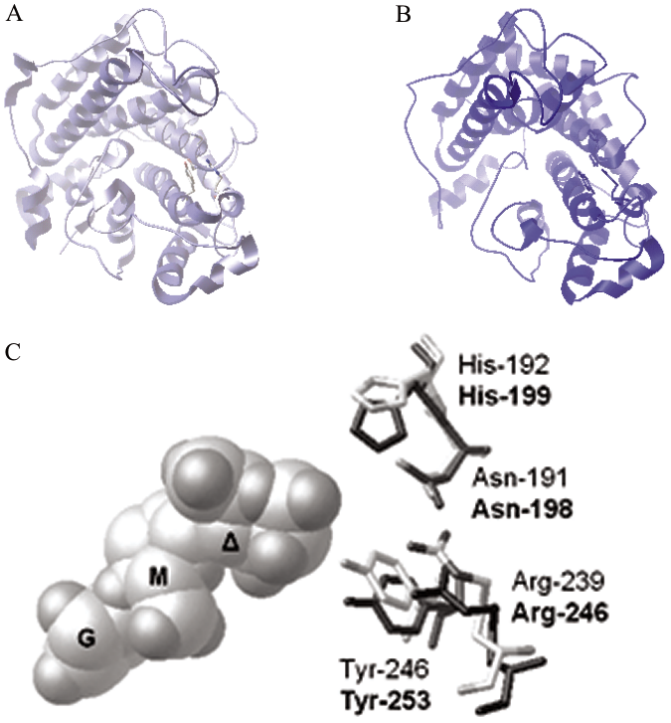


Fig. 2. Homology model of KS-408 alginate lyase constructed on the crystal structure of A1-III alginate lyase (1hv6.pdb) from *Sphingomonas* sp. strain A1. (A) Crystal structure of A1-III alginate lyase, (B) constructed homology model of KS-408-2F alginate lyase, (C) superimposition of KS-408-2F alginate lyase (black) and A1-III alginate lyase (grey) with trisaccharide. Conserved amino acid residues in the active site are indicated.



Partial purification of KS-408 alginate lyase

After the recombinant *E. coli* was cultured with IPTG induction, KS-408 alginate lyases with $(His)_6$ tag were purified by a Ni-Sepharose affinity column chromatography (Table 1).

Table 1. Purification of alginate lyase from recombinant *Escherichia coli* BL21 using a Ni-Sepharose column.

	KS-408-1F[a]		KS-408-2F[b]	
	Yield (%)	Activity (U/mg)[c]	Yield (%)	Activity (U/mg)[c]
Crude lysate	100.0	4.0	100.0	5.1
Purified protein	10.1	51.6	13.4	63.2

[a]KS-408-1F was expressed with full sequence of alginate lyase with $(His)_6$ tag fusion peptide.

[b]KS-408-2F was expressed without signal peptide of alginate lyase with $(His)_6$ tag fusion peptide.

[c]Unit represents the amount of enzyme (mg) that can change 1.0 of the absorbance of 235 nm for 1 min.

The recombinant alginate lyases (KS-408-1F and KS-408-2F) were purified with a yield of 10%–13% from total lysates. The specific activities of the purified alginate lyases were enhanced 12.5- to 13-fold more than those of crude lysates.

Characterization of KS-408 alginate lyase enzymes

Based on the fact that both of alginate lyases KS-408-1F and KS-408-2F have almost same specific activities at given pH and temperature but the expression level of KS-408-2F was higher than that of KS-408-1F, we employed KS-408-2F alginate lyase for further study. The effect of pH on the activity of the purified KS-408-2F alginate lyase is shown in Fig. 3. The KS-408-2F alginate lyase was highly active at pH of 8 (potassium phosphate buffer), whereas at the pH of Tris–HCl buffer inhibited the activity. The activity was very low in an acidic environment (below pH 6). The effect of temperature on enzyme stability was also investigated. The activity of KS-408-2F alginate lyase was well maintained at moderate temperature between 20 and 30 °C; however, it significantly lost activity after 30 min incubation at temperature higher than 40 °C (data not shown).

The enzymatic activity of the KS-408-2F alginate lyase

Fig. 3. Effect of pH on the activity of KS-408-2F alginate lyases. The purified enzyme was incubated in 20 mmol/L buffer solution for 10 min before adding 0.4%(*m*/*v*) sodium alginate solution. Enzyme activity was assayed by measuring the absorbance at 235 nm. The data represent the average value of three measurements.

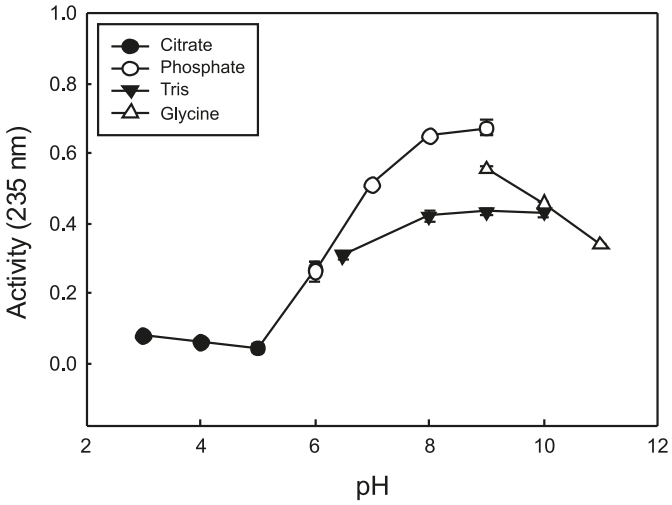


Fig. 4. Effect of metal ion on the relative activity of KS-408-2F alginate lyase. Control reaction was carried out in the absence of ion solution. The purified enzyme was incubated with metal ion in 20 mmol/L phosphate buffer for 10 min before adding 0.4% substrate solution.

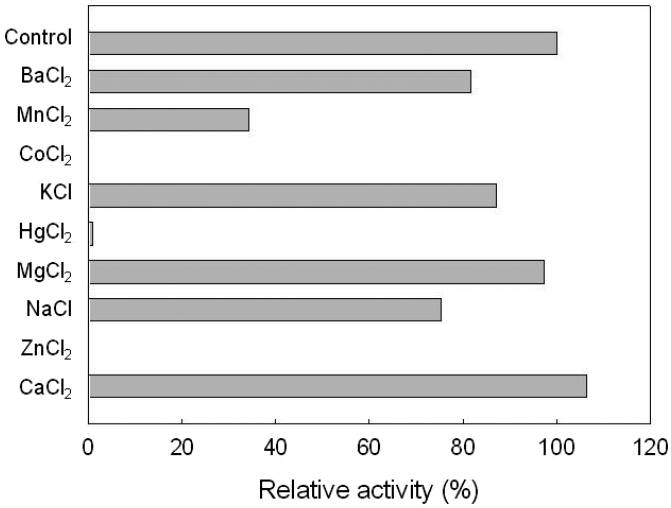


Table 2. The chemical composition of guluronate and mannuronate of used substrates.

Designation	F_G	F_M	F_{GG}	$F_{GM,\ MG}$	F_{MM}	F_{GGM}	F_{MGM}	F_{GGG}	DP_n[a]
Alginate (Sigma)	0.38	0.62	0.11	0.27	0.35	0.06	0.21	0.05	30–40[b]
PolyG block	0.87	0.13	0.87	0.01	0.11	0.01	0.01	0.86	19
PolyM block	0.11	0.89	0.01	0.10	0.79	0.01	0.09	0.00	19
PolyMG block	0.42	0.58	0.02	0.41	0.16	0.02	0.36	0.00	23
Acetylated alginate (d.a.[c] = 0.28)	0.11	0.89	0.00	0.13	0.73	0.00	0.16	0.00	30–40[b]

[a]DP_n indicates the average degree of polymerization.

[b]Alginate (cps = 3500) and acetylated alginate (*Pseudomonas aeruginosa* ATCC 39324) were degraded to a DP_n = 30–40 by mild acid hydrolysis.

[c]d.a. is the degree of O-acetylation.

was evaluated in the presence or absence of various metal ions. As shown in Fig. 4, Zn^{2+}, Hg^{2+}, and Co^{2+} strongly inhibited the lyase activity, while Ba^{2+}, Mn^{2+}, Na^{+}, and K^{+} partially inhibited the lyase activity. The presence of 1 mmol/L Mg^{2+} and Ca^{2+} did not affect the activity of the KS-408-2F alginate lyase.

To investigate substrate specificity, five structurally different alginates were used as the substrate (Table 2). Figure 5A showed the relative activities of KS-408-2F alginate lyase toward alginate, polyM block, polyG block, polyMG block, and bacterial alginate. In the case of 10 min incubation at 37 °C, KS-408-2F lyase showed higher degrading activity for polyM block (100%), polyMG block (58.0%), and bacterial alginate (57.6%) than for alginate (28.8%) and polyG block (1.7%). Interestingly, although both the acetylated alginate and polyM block substrates have a similar level of mannuronate, the relative activity for acetylated alginate was rather lower than that for polyM block, indicating that acetylation of mannuronate reduced the effectiveness of KS-408-2F alginate lyase. Substrates completely degraded by KS-408-2F alginate lyase were separated on TLC with Biogel P-2 column-purified unsaturated oligouronates, dimer, and trimer as standards. When the starting points of each TLC plate were compared, undegraded alginate and polyG block remained at the starting points (Fig. 5B). Although acetylated alginate purified from mucoid *P. aeruginosa* was degraded by KS-408-2F alginate lyase (Fig. 5A, analyzed by TBA method), the degradation products from the acetylated alginate could not be detected on both the starting point nor on the developing area by dipping the TLC plate in 10% sulfuric acid – ethanol reagent (data not shown). The degraded oligomeric products of the polyM block were separated by using FPLC and Biogel P-2 gel filtration chromatography. The fractions at the elution times of 16.7 and 15.6 min were subsequently analyzed by ESI MS (Fig. 5C). Each fraction was injected onto the LC MS with mobile-phase 10 mmol/L ammonium acetate – methanol (1:1, *v*/*v*), and ESI MS was performed in negative mode ($[M\text{-}H]^-$). From the ESI MS data, the peaks of 16.7 and 15.6 min were determined to be a dimer ($[M\text{-}H]^- = 351$) and a trimer ($[M\text{-}H]^- = 527$), respectively. Based on the above results, we assumed that the KS-408-2F alginate lyase is a polyM-specific lyase that showed higher preference for the polyM block as the substrate.

Analysis of KS-408-2F alginate lyase-catalyzed alginate degradation patterns by ^{1}H NMR

The degradation profiles of alginate by the KS-408-2F alginate lyase or polyG-specific ALG-5 alginate lyase were

Fig. 5. Substrate specificity of KS-408-2F alginate lyase. (A) 0.2% (*m*/*v*) each substrate was digested with 20 μg of KS-408-2F alginate lyase for 10 min at 37 °C. Enzyme activity was assayed by the thiobarbituric assay method. Ac-alginate means acetylated alginate obtained from *Pseudomonas aeruginosa* ATCC 39324. (B) 1% each substrate was completely digested by adding enzyme. The completion of digestion was confirmed by fast-protein liquid chromatography (FPLC). Lanes: S, purified unsaturated uronate, dimer, and trimer; 1, alginate; 2, polyM block; 3, polyG block; 4, polyMG block. (C) FPLC profile of degraded polyM block (thin-layer chromatography sample) by KS-408-2F alginate lyase.

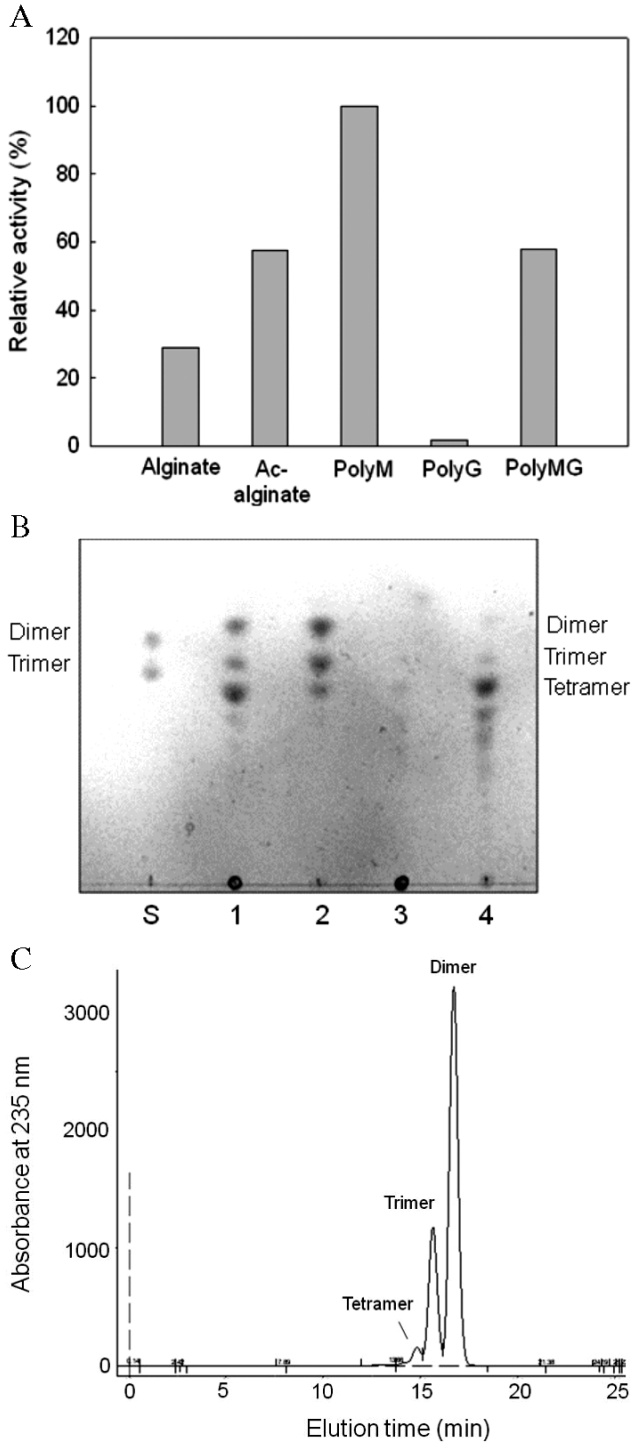


Table 3. Composition of alginate before and after treatment with KS-408 lyase.

Alginate	F_G	F_M	F_{GG}	F_{MM}	DP_n
PolyM block					
Before KS-408	0.11	0.89	0.01	0.79	19
After KS-408	—[a]	0.25	0.00	0.08	2–3
PolyG block					
Before KS-408	0.87	0.13	0.87	0.11	19
After KS-408	0.84	0.10	0.81	0.05	>12
PolyMG block					
Before KS-408	0.42	0.58	0.02	0.16	23
After KS-408	0.28	0.19	0.00	0.09	4

[a]No resonance signals caused by the internal G (G-1) could be recorded.

compared by ^{1}H NMR spectroscopy (Fig. 6). There were some different chemical shifts of signals between the products of degradation by KS-408-2F alginate lyase or ALG-5 lyase. $M_{red\text{-}\beta}$ reducing end signal (4.91 ppm) newly appeared after alginate degradation by KS-408-2F alginate lyase, while $G_{red\text{-}\beta}$ reducing end signals (4.90–4.86 ppm) appeared after alginate degradation by ALG-5 lyase. The ratios of intensity from internal G signal (5.07 ppm) and sequential G signal (4.47 ppm) were different when alginates were degraded by KS-408 lyase or ALG-5 lyase. Therefore, KS-408-2F alginate lyase is expected to produce a mannuronate-derived reducing end by cleaving the alginate like these ways, M↓MM, M↓MG, M↓GG, or M↓GM, while polyG-specific ALG-5 lyase produced guluronate reducing end.

PolyM, polyG, or polyMG blocks were degraded with KS-408-2F alginate lyase, and then analyzed by NMR spectroscopy to confirm the substrate specificity of KS-408-2F alginate lyase (Fig. 7 and Table 3). As shown in Fig. 7B, the internal G signal (5.07 ppm) of polyG block was not changed in the absence or presence of KS-408-2F alginate lyase. On the contrary, the internal M signals (4.71–4.68 ppm) of polyM and polyMG blocks were decreased after complete degradation of both blocks by KS-408-2F alginate lyase (Figs. 7A and 7C), clearly indicating that KS-408-2F alginate lyase is polyM-specific alginate lyase. When the M—M bond in polyM block was cleaved, an M reducing end (M_{red}) and ΔM on the nonreducing end were produced. In the NMR spectrum of polyG block, there were very low peaks from the unsaturated ends (5.90–5.75 ppm) and a decrease in DP_n (from 19 to >12) of polyG block after the degradation of polyG block by KS-408-2F alginate lyase, because some mannuronate moiety was in the polyG block (F_M = 13%).

We investigated the preference of KS-408-2F alginate lyase for the M—G bond or G—M bond in a polyMG block. The polyMG block used in this study had mainly a MG or GM ($F_{MG} + F_{GM}$ = 82%, Table 2) fraction with a small portion of the GG fraction. When the M–G bond in polyMG block was cleaved, the degradation products would be M_{red} on the reducing end and ΔM or ΔG on the nonreducing end. Contrarily, when G—M bond in polyMG block was degraded, the degradation products would be G_{red} on the reducing end and ΔG or ΔM on the nonreducing end. In the NMR spectrum (Fig. 7C), the signals from MM, MG, and MGM decreased, as expected, when the polyMG block was degraded by KS-408-2F alginate lyase. Interestingly, the resonance signal arising from $M_{red\text{-}\beta}$ (4.91 ppm) was larger than that from

Fig. 6. Comparison of ^{1}H NMR (400 MHz) spectra of alginate degradation before and after treatment with *Pseudomonas* sp. strain KS-408-2F alginate lyase or *Streptomyces* sp. strain ALG-5 lyase. The reaction was allowed to proceed to completion by adding enzyme repeatedly. The signal originated from the underlined residue. The numbers denote proton causing the signal. M or G without underline indicates neighbor residue. *Streptomyces* sp. strain ALG-5 lyase was obtained by Ni-Sepharose column as described previously (Kim et al. 2009).

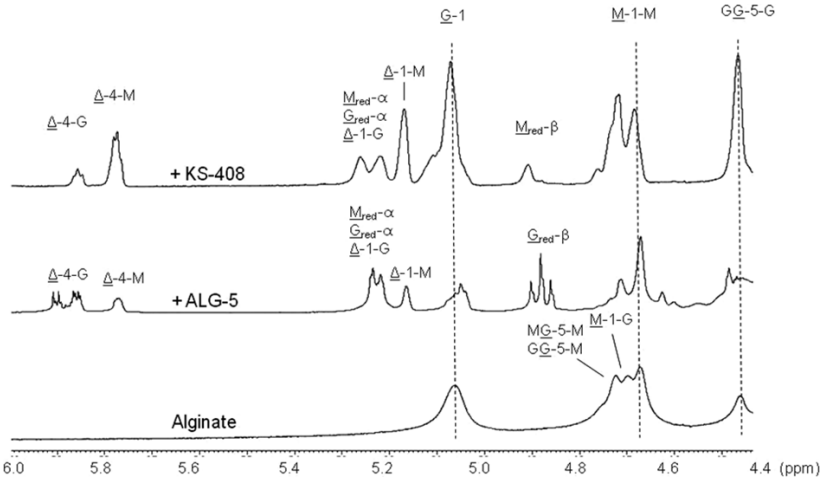
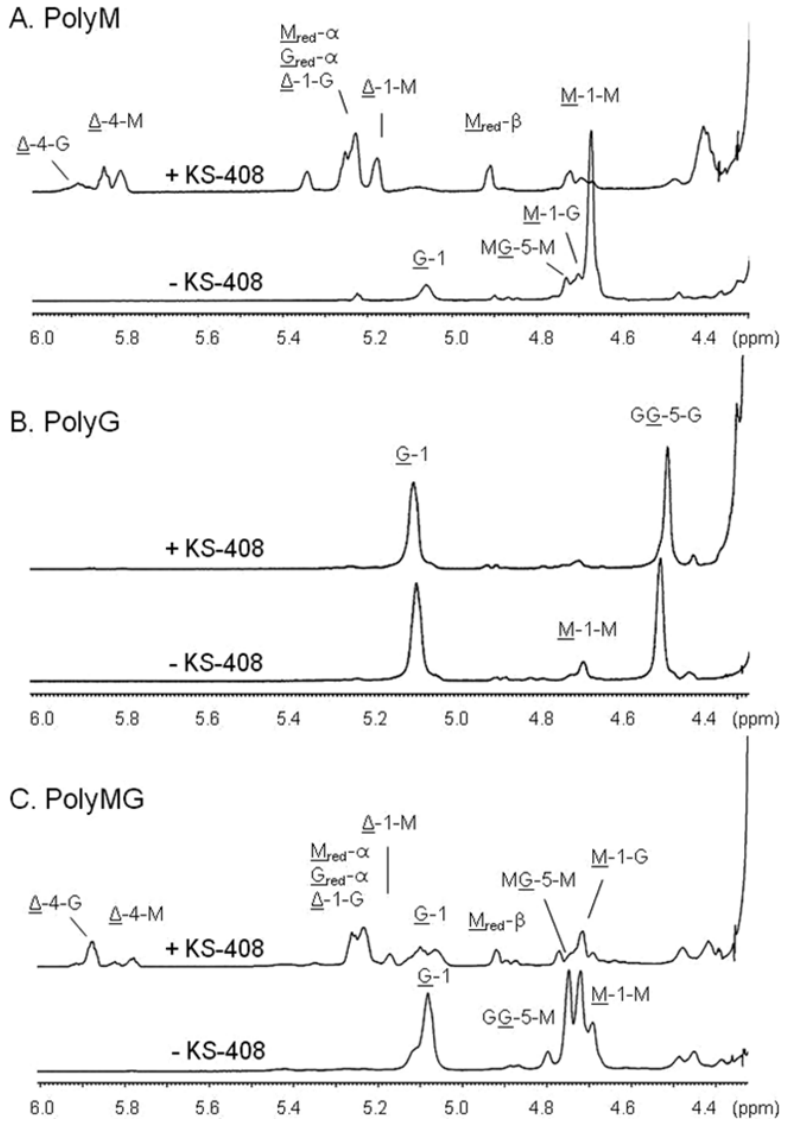


Fig. 7. ^{1}H NMR (400 MHz) spectra of alginate substrates before and after treatment with *Pseudomonas* sp. strain KS-408-2F alginate lyase. (A) PolyM block, (B) polyG block, and (C) polyMG block. The signal originated from the underlined residue. The numbers denote proton causing the signal. M or G without underline indicates neighbor residue.



$G_{red\text{-}\beta}$ (4.90–4.86 ppm, Fig. 6), clearly showing that KS-408 alginate lyase cleaved M—G bonds preferentially, compared with G—M bonds. This result also supports that KS-408 alginate lyase is a polyM-specific lyase that preferentially degrades the glycosidic linkages of M—M and M—G.

Recently, macroalgae have attracted much attention as renewable biomass for the production of biofuels and biochemicals. To date, laminaran and mannitol in algae have been considered as carbohydrate sources (Horn et al. 2000). Actually, alginate constitutes up to 30% of dry mass of brown seaweeds. Therefore, it is of interest to saccharify alginate polysaccharide into the constituent monosaccharides by using alginate lyase as the biocatalyst to use them as carbon source for the production of biofuels and biochemicals (Shin et al. 2010; Ryu and Lee 2011). A combined use of alginate lyase with a different mode of action is advantageous on the saccharification of alginate (Rahman et al. 2010). The saccharification bioprocess of alginate to alginate monosaccharides is under development by using polyM-specific KS-408-2F alginate lyase in combination with polyG-specific ALG-5 lyase and exolytic MJ-3 oligoalginate lyases.

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References

Akiyama, H., Endo, T., Nakakita, R., Murata, K., Yonemoto, Y., and Okayama, K. 1992. Effect of depolymerized alginates on the growth of *Bifidobacteria.* Biosci. Biotechnol. Biochem. **56**(2): 355–356. doi:10.1271/bbb.56.355. PMID:1368312.

Alkawash, M.A., Soothill, J.S., and Schiller, N.L. 2006. Alginate lyase enhances antibiotic killing of mucoid *Pseudomonas aeruginosa* in biofilms. APMIS, **114**(2): 131–138. doi:10.1111/j.1600-0463.2006.apm_356.x. PMID:16519750.

ASTM International. 2010. ASTM F2259-10. Standard test method for determining the chemical composition and sequence in alginate by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. ASTM International, West Conshohocken, Penn., USA. Available from http://www.astm.org/Standards/F2259.htm.

Cote, G.L., and Krull, L.H. 1988. Characterization of the extracellular polysaccharides from *Azotobacter chroococcum.* Carbohydr. Res. **181**(1): 143–152. doi:10.1016/0008-6215(88)84030-8.

Ertesvåg, H., Erlien, F., Skjåk-Bræk, G., Rehm, B.H.A., and Valla, S. 1998. Biochemical properties and substrate specificities of a recombinantly produced *Azotobacter vinelandii* alginate lyase. J. Bacteriol. **180**(15): 3779–3784. PMID:9683471.

Fujihara, M., and Nagumo, T. 1992. The effect of the content of D-mannuronic acid and L-guluronic acid blocks in alginates on antitumor activity. Carbohydr. Res. **224**: 343–347. doi:10.1016/0008-6215(92)84123-A. PMID:1591771.

Grasdalen, H. 1983. High-field, ^{1}n.m.r spectroscopy of alginate: sequential structure and linkage conformations. Carbohydr. Res. **118**: 255–260. doi:10.1016/0008-6215(83)88053-7.

Haug, A., Larsen, B., and Smidsrød, O. 1966. A study of the constitution of alginic acid by partial acid hydrolysis. Acta Chem. Scand. **20**: 183–190. doi:10.3891/acta.chem.scand.20-0183.

Heyraud, A., Gey, C., Leonard, C., Rochas, C., Girond, S., and Kloareg, B. 1996. NMR spectroscopy analysis of oligoguluronates and oligomannuronates prepared by acid or enzymatic hydrolysis of homopolymeric blocks of alginic acid. Application to the determination of the substrate specificity of *Haliotis tuberculata* alginate lyase. Carbohydr. Res. **289**: 11–23. doi:10.1016/0008-6215(96)00060-2. PMID:8805773.

Horn, S.J., Aasen, I.M., and Østgaard, K. 2000. Ethanol production from seaweed extract. J. Ind. Microbiol. Biotechnol. **25**(5): 249–254. doi:10.1038/sj.jim.7000065.

Iwamoto, M., Kurachi, M., Nakashima, T., Kim, D., Yamaguchi, K., Oda, T., et al. 2005. Structure–activity relationship of alginate oligosaccharides in the induction of cytokine production from RAW264.7 cells. FEBS Lett. **579**(20): 4423–4429. doi:10.1016/j.febslet.2005.07.007. PMID:16055120.

Kawada, A., Hiura, N., Tajima, S., and Takahara, H. 1999. Alginate oligosaccharides stimulate VEGF-mediated growth and migration of human endothelial cells. Arch. Dermatol. Res. **291**(10): 542–547. doi:10.1007/s004030050451. PMID:10552212.

Kim, D.E., Lee, E.Y., and Kim, H.S. 2009. Cloning and characterization of alginate lyase from a marine bacterium *Streptomyces* sp. ALG-5. Mar. Biotechnol., **11**(1): 10–16. doi:10.1007/s10126-008-9114-9. PMID:18553118.

Kim, H.S., Lee, C.-G., and Lee, E.Y. 2011. Alginate lyase: structure, property and application. Biotechnol. Bioprocess. Eng. **16**(5): 843–851. doi:10.1007/s12257-011-0352-8.

May, T.B., and Chakrabarty, A.M. 1994. Isolation and assay of *Pseudomonas aeruginosa* alginate. Methods Enzymol. **235**: 295–304. doi:10.1016/0076-6879(94)35148-1. PMID:8057902.

McComb, E.A., and McCready, R.M. 1957. Determination of acetyl in pectin and in acetylated carbohydrate polymers. Anal. Chem. **29**(5): 819–821. doi:10.1021/ac60125a025.

Miyake, O., Hashimoto, W., and Murata, K. 2003. An exotype alginate lyase in *Sphingomonas* sp. A1: overexpression in *Escherichia coli*, purification, and characterization of alginate lyase. Protein Expr. Purif. **29**(1): 33–41. doi:10.1016/S1046-5928(03)00018-4. PMID:12729723.

Ochiai, A., Hashimoto, W., and Murata, K. 2006. A biosystem for alginate metabolism in *Agrobacterium tumefaciens* strain C58: molecular identification of Atu 3025 as an exotype family PL-15 alginate lyase. Res. Microbiol. **157**(7): 642–649. doi:10.1016/j.resmic.2006.02.006. PMID:16545947.

Park, H.H., Kam, N., Lee, E.Y., and Kim, H.S. 2011. Cloning and characterization of a novel oligoalginate lyase from a newly isolated bacterium *Sphingomonas* sp. MJ-3. Mar. Biotechnol. In press. doi:10.1007/s10126-011-9402-7. PMID:21826589.

Rahman, M.M., Inoue, A., Tanaka, H., and Ojima, T. 2010. Isolation and characterization of two alginate lyase isozymes, AkAly28 and AkAly33, from the common sea hare *Aplysia kurodai.* Comp. Biochem. Physiol. B, **157**(4): 317–325. doi:10.1016/j.cbpb.2010.07.006. PMID:20708706.

Ramsey, D.M., and Wozniak, D.J. 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. Mol. Microbiol. **56**(2): 309–322. doi:10.1111/j.1365-2958.2005.04552.x. PMID:15813726.

Ryu, M., and Lee, E.Y. 2011. Saccharification of alginate by using exolytic oligoalginate lyase from marine bacterium *Sphingomonas* sp. MJ-3. J. Ind. Eng. Chem. **17**(5–6): 853–858. doi:10.1016/j.jiec.2011.08.001.

Schiller, N.L., Monday, S.R., Boyd, C.M., Keen, N.T., and Ohman, T.E. 1993. Characterization of the *Pseudomonas aeruginosa*

alginate lyase gene (*algL*): cloning, sequencing, and expression in *Escherichia coli.* J. Bacteriol. **175**(15): 4780–4789. PMID: 8335634.

Shin, J.W., Choi, S.H., Kim, D.E., Kim, H.S., Lee, J.H., Lee, I.S., and Lee, E.Y. 2010. Heterologous expression of an alginate lyase from *Streptomyces* sp. ALG-5 in *Escherichia coli* and its use for preparation of the magnetic nanoparticle-immobilized enzymes. Bioprocess Biosyst. Eng. **34**(1): 113–119. doi:10.1007/s00449-010-0452-4. PMID:20640446.

Wong, T.Y., Preston, L.A., and Schiller, N.L. 2000. Alginate lyase: review of major sources and enzyme characteristics, structure–function analysis, biological roles, and application. Annu. Rev. Microbiol. **54**(1): 289–340. doi:10.1146/annurev.micro.54.1.289. PMID:11018131.

Xiao, L., Han, F., Yang, Z., Lu, X.-Z., and Yu, W.-G. 2006. A novel alginate lyase with high activity on acetylated alginate of *Pseudomonas aeruginosa* FRD1 from *Pseudomonas* sp. QD03. World J. Microbiol. Biotechnol. **22**(1): 81–88. doi:10.1007/s11274-005-7713-4.

Yamamoto, S., Sahara, T., Sato, D., Kawasaki, K., Ohgiya, S., Inoue, A., and Ojima, T. 2008. Catalytically important amino-acid residues of abalone alginate lyase HdAly assessed by site-directed mutagenesis. Enzyme Microb. Technol. **43**(6): 396–402. doi:10.1016/j.enzmictec.2008.06.006.

Yamasaki, M., Moriwaki, S., Miyake, O., Hashimoto, W., Murata, K., and Mikami, B. 2004. Structure and function of a hypothetical *Pseudomonas aeruginosa* protein PA1167 classified into family PL-7: a novel alginate lyase with a β-sandwich fold. J. Biol. Chem. **279**(30): 31863–31872. doi:10.1074/jbc.M402466200. PMID:15136569.

Yonemoto, Y., Tanaka, H., Yamashita, T., Kitabatake, N., Ishida, Y., Kimura, A., and Murata, K. 1993. Promotion of germination and shoot elongation of some plants by alginate oligomers prepared with bacterial alginate lyase. J. Ferment. Bioeng. **75**(1): 68–70. doi:10.1016/0922-338X(93)90181-7.

Zhang, Z., Yu, G., Guan, H., Zhao, X., Du, Y., and Jiang, X. 2004. Preparation and structure elucidation of alginate oligosaccharides degraded by alginate lyase from *Vibrio* sp. 510. Carbohydr. Res. **258**: 187–197.