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Molecular characterization of a novel oligoalginate lyase consisting of AlgL- and heparinase II/III-like domains from *Stenotrophomonas maltophilia* KJ-2 and its application to alginate saccharification

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Abstract−Molecular identification and development of a novel recombinant alginate lyase as the biocatalyst for alginate saccharification are prerequisite for bioethanol fermentation from brown seaweed biomass. We identified and characterized a novel oligoalginate lyase for complete degradation of alginate from *Stenotrophomonas maltophilia* KJ-2 that grow on alginate as the sole carbon source. KJ-2 oligoalginate lyase consisted of AlgL- and heparinase II/III-like domains. The recombinant KJ-2 oligoalginate lyase exhibited substrate preference toward polymannuronate and alginate as well as oligoalginate. The recombinant KJ-2 oligoalginate lyase completely degraded alginate into unsaturated uronate monomer most efficiently at pH 7.5 and 37 °C. Interestingly, AlgL-like recombinant proteins showed more like endolytic activity. The recombinant KJ-2 oligoalginate lyase was a novel oligoalginate lyase consisting of AlgL- and heparinase-like domains and could be used as a candidate for biocatalyst selection to saccharify alginate for bioethanol production from brown seaweed.

Keywords: Alginate, AlgL-like Domain, Alginate Lyase, Heparinase II/III-like Domain, Oligoalginate Lyase, *Stenotrophomonas maltophilia* KJ-2

INTRODUCTION

Alginate is a polysaccharide consisting of α-L-guluronate (G) and its C5 epimer, β-D-mannuronate (M) monomers [1]. In terms of polymer structure, alginate is a complex copolymer containing three different kinds of blocks, polymannuronate (polyM), polyguluronate (polyG) and polymannuronate-co-guluronate (polyMG) [2]. Biological synthesis and degradation of alginate in cell is very complex. Transport and consecutive degradation of alginate in *Sphingomonas* sp. A1 have been extensively studied at the molecular level. A cell-surface pit, ATP-binding cassette transporter and relevant membrane proteins responsible for the alginate import were identified based on proteomics [3]. Cytoplasmic alginate lyases A1-I, A1-II and A1-III were elucidated to catalyze the cleavage of glycosidic bonds in the transported alginate and release di- and trisaccharides in an endolytic manner [4]. Alginate lyase A1-IV was responsible for the complete degradation of oligoalginate into monosaccharides in *Sphingomonas* sp. A1 [5].

Alginate lyase is the key enzyme for alginate degradation. It catalyzes the β-elimination breakage of the glycosidic bond of alginate [6]. Alginate lyase can be classified into polyM-, polyG-, and polyMG-specific lyase based on substrate specificity, and endolytic and exolytic lyase on the basis of mode of cleavage. Various endolytic alginate lyases have been identified and characterized [7-12]. Some exolytic oligoalginate lyase capable of degrading alginate and oligoalginate into monosaccharides was also cloned and characterized, and the crystal structure of exolytic alginate lyase Atu 3025 from *Agrobacterium tumefaciens* was elucidated [13-18]. Recently, recombinant exolytic alginate lyase of *Sphingmonas* sp. MJ-3 was successfully used for the preparation of unsaturated uronate monosaccharides from alginate [19]. Endolytic alginate lyase from *Streptomyces* sp. ALG-5 was immobilized onto magnetic nanoparticle and re-used for the production of alginate oligomers [20].

Alginate is the most abundant carbohydrate in brown seaweed, up to 40% of dry cell weight, and is readily extracted using a simple process [21]. Alginate has attracted much attention as a renewable bioresource for bioethanol production using metabolic engineered microbial cell platforms [22]. To produce bioethanol from brown seaweed alginate, the development of an efficient saccharification method is of importance, and alginate lyase can play a key role in alginate saccharification. In cellular alginate metabolism, many different alginate lyases play together for the efficient alginate degradation. In a similar manner, a synergistic catalysis by endolytic polyM-, polyG-, and polyMG-specific lyases together with exolytic alginate lyases can be expected in an alginate saccharification process when these alginate lyases are used in combination. Thus, novel recombinant alginate lyases continuously need to be identified and developed as the biocatalyst for alginate saccharification. Recently, we isolated *Stenotrophomonas maltophilia* KJ-2 that grow on alginate as the sole carbon source to identify a novel alginate lyase that can be used for complete degradation of alginate [10]. In this study, a novel oligoalginate lyase possessing AlgL- and heparinase II/III-

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‡This article is dedicated to Prof. Hwayong Kim on the occasion of his retirement from Seoul National Univerisity.

like domains with different alginate lyase activities was cloned and characterized to evaluate the possibility of using the alginate lyase as a biocatalyst for alginate saccharification.

MATERIALS AND METHODS

1. Chemicals

Alginate of brown algae was purchased from Sigma (USA). Homopolymeric blocks of polyM, polyG and polyMG were prepared from alginate as described previously [9, 23].

2. Cloning, Subcloning and Heterologous Expression of KJ-2 Alginate Lyase Relevant Genes

An oligoalginate lyase gene was cloned from *S. maltophilia* KJ-2 (KTCC 32000) by using the methods previously described [10,17]. The homologous sequence of putative alginate lyase protein (GenBank no. CAQ46078) and heparinase II/III family protein (GenBank no. YP_002028454) from *S. maltophilia* K279a and *S. maltophilia* R551-3 were used as the primers. The forward (5KJ2HepMF (*Nde*I)) and reverse (3KJ2alg1R (*Eco*RI)) primers were used for cloning of the full sequence of KJ-2 oligoalginate lyase without signal peptide into a pColdI vector. After the full nucleotide sequence was determined, the primers with restriction sites and no signal sequence were designed for the subcloning of AlgL- and heparinase II/III-like fragments in pET-21b(+) and pColdI vectors, respectively. The forward primers (5KJ2HepMF, 5KJ2-oligo1000F) and the reverse primers (3KJ2HepMR, 3KJ2alg1R) were used for AlgL- and heparinase II/III-like domains, respectively (Table 1). A PCR was performed using pfu-X DNA polymerase (SolGent, Korea) for KJ-2 oligoalginate lyase and the AlgL-like fragment, and LA-tag DNA polymerase (Takara, Japan) for the heparinase II/III-like fragment, respectively. The PCR products were ligated to a T-blunt vector (SolGent, Korea), and then transferred to pColdI (Takara, Japan) or pET-21b(+) vector (Novagen, USA). The nucleotide sequence of the KJ-2 oligoalginate lyase was registered to GenBank (Accession no. KC430928).

The recombinant *Escherichia coli* HMS174 (DE3) was used as the hosts for expression the genes of KJ-2 oligoalginate lyase, AlgL- and heparinase II/III-like fragments. For the recombinant HMS strain, 34 μg chloramphenicol/ml and 20 mg/ml rifampicin were supplemented to LB medium. The cells were cultured at 15 °C for 24 h to express the genes by adding 1 mM IPTG.

3. Purification of KJ-2 Oligoalginate Lyase and SDS-PAGE Analysis

The cells were harvested and sonicated in 100 mM potassium phosphate buffer (pH 7.2) using an ultrasonicator (Bandelin-Sonoplus HD 2070, Germany). The cell homogenate containing the recombinant KJ-2 oligoalginate lyase with His-tag was loaded on a Ni-Sepharose column (Amersham Biosciences, USA) equilibrated with 100 mM potassium phosphate buffer (pH 8.0) and 600 mM KCl. The KJ-2 oligoalginate lyase was eluted with 50 mM phosphate buffer containing 500 mM imidazole. The target fraction was desalted using a HiTrapTM desalting column (Amersham Biosciences, USA). The recombinant proteins were analyzed on a 12% SDS-polyacrylamide gel as previously described [17].

4. Assay of the KJ-2 Oligoalginate Lyase, AlgL- and Heparinase II/III-like Protein Activities

The purified KJ-2 oligoalginate lyase, AlgL- and heparinase II/III-like proteins were incubated at 35 °C in 0.5 ml of 20 mM potassium phosphate buffer (pH 7.4) containing 0.08-2.0% (w/v) sodium alginate. The alginate lyase activity was determined by measuring the absorbance at 235 nm [4] or by using the 2-thiobarbituric acid (TBA) method at 548 nm [24], with 2-deoxy-D-glucose as a standard. The production of mono- and oligosaccharides by alginate lyase was also analyzed by TLC and FPLC [17]. An FPLC equipped with a Superdex peptide 10/300 GL column (GE Healthcare, USA) and UV detector was used with 0.2 M bicarbonate buffer (pH 7.2).

5. Analysis of Alginate Degradation Products by FPLC and ESI-LC-MS

The degraded alginate products were separated using BioGel P2 gel filtration chromatography (1.5×36 cm, BioRad, USA), as described

Table 1. Primers used for cloning and sub-cloning of KJ-2 oligoalginate lyase, AlgL- and heparinase-like alginate lyases from *Stenotrophomonas maltophilia* KJ-2

Primer name	Sequence (5′→3′)
For KJ-2 oligoalginate lyase sequencing	
5KJ2-alg1F	5′-ATGAGGTTGCARCCGCTGTYCG-3′
3KJ2-alg1R	5′-TCATTTKCCGTCACCCTTGCTGC-3′
5KJ2-algMF	5′-GTTCCTGCTGTTCGCCAACG-3′
For cloning of KJ-2 oligoalginate lyase to pColdI vector	
5KJ2HepMF(*Nde*I)	5′-CATATGGCGCCCGCTGCCGCTGCGCGGCAG-3′
3KJ2alg1R(*Eco*RI)	5′-GGAATTCTCATTTTCCGTCACCCTTGCTG-3′
For cloning of KJ-2 AlgL-like domain to pET-21b(+)	
5KJ2HepMF(*Nde*I)	5′-CATATGGCGCCCGCTGCCGCTGCGCGGCAG-3′
3KJ2HepMR(*Xho*I)	5′-GCTCGAGGAAGGGCCTGGCCCTGTTC-3′
For cloning of KJ-2 heparinase-like domain to pColdI	
5KJ2-oligo1000F(*Nde*I)	5′-GCATATGGAGGGCCTGCAGGTGGC-3′
3KJ2alg1R(*Eco*RI)	5′-GGAATTCTCATTTTCCGTCACCCTTGCTG

R, Y and K mean A+G, C+T and G+T, respectively
Under lined nucleotides indicate the restriction sites

previously [9]. The mixture was eluted with 0.2 M NH_4HCO_3 at 0.3 ml/min flow rate, and the fractions were collected as a 2 ml/tube. The alginate oligosaccharides and monosaccharide were detected by measuring absorbance change at 235 nm. Isolated alginate degradation products were lyophilized for ESI-LC-MS analysis.

The molecular mass of alginate oligosaccharides and monosaccharide was determined using electrospray-ionization mass spectroscopy (6410 Triple Quadrupole LC-MS, Agilent, USA). Each fraction dissolved in methanol : water (1 : 1, v/v) was used for LC-MS analysis with the electrospray source at a 0.5 ml/min flow rate. Ammonium acetate : methanol (1 : 1, v/v) was used as the mobile phase. MS was operated in negative mode with an ion spray voltage of 4 kV. The source temperature was 350 °C.

RESULTS AND DISCUSSION

1. Identification and Cloning of a Novel Oligoalginate Lyase Gene from *S. maltophilia* KJ-2

S. maltophilia KJ-2 was isolated from Korean-style salted fish paste. It could grow on an agar plate containing alginate as the sole carbon source, indicating that *S. maltophilia* KJ-2 possesses an exolytic oligoalginate lyase that completely degrades alginate for cell growth. To clone a gene of KJ-2 oligoalginate lyase, we designed and conducted PCR-identification and cloning based on the fact that the oligoalginate lyases of *Sphingomonas* sp. A1 possesses the highly conserved domain of heparinase II/III-like family protein in spite of low identity [5,14,17]. The PCR primers were designed from homologous nucleotide sequences of heparinase II/III family protein gene in the alginate degrading enzyme cluster of *S. maltophilia* R551-3 and *S. maltophilia* K279a from the GenBank genome sequence database. The putative alginate lyase protein (Smlt2602) of *S. maltophilia* K279a and heparinase II/III family protein (Smal_2067) of *S. maltophilia* R551-3 shared 93.5% amino acid sequence identity.

We isolated the genomic DNA of *S. maltophilia* KJ-2 and conducted PCR with the specific primers listed in Table 1. The KJ-2 oligoalginate lyase gene consisting of 2,229 bp (742 amino acids) was cloned to a T-blunt vector for sequencing and then the sequence

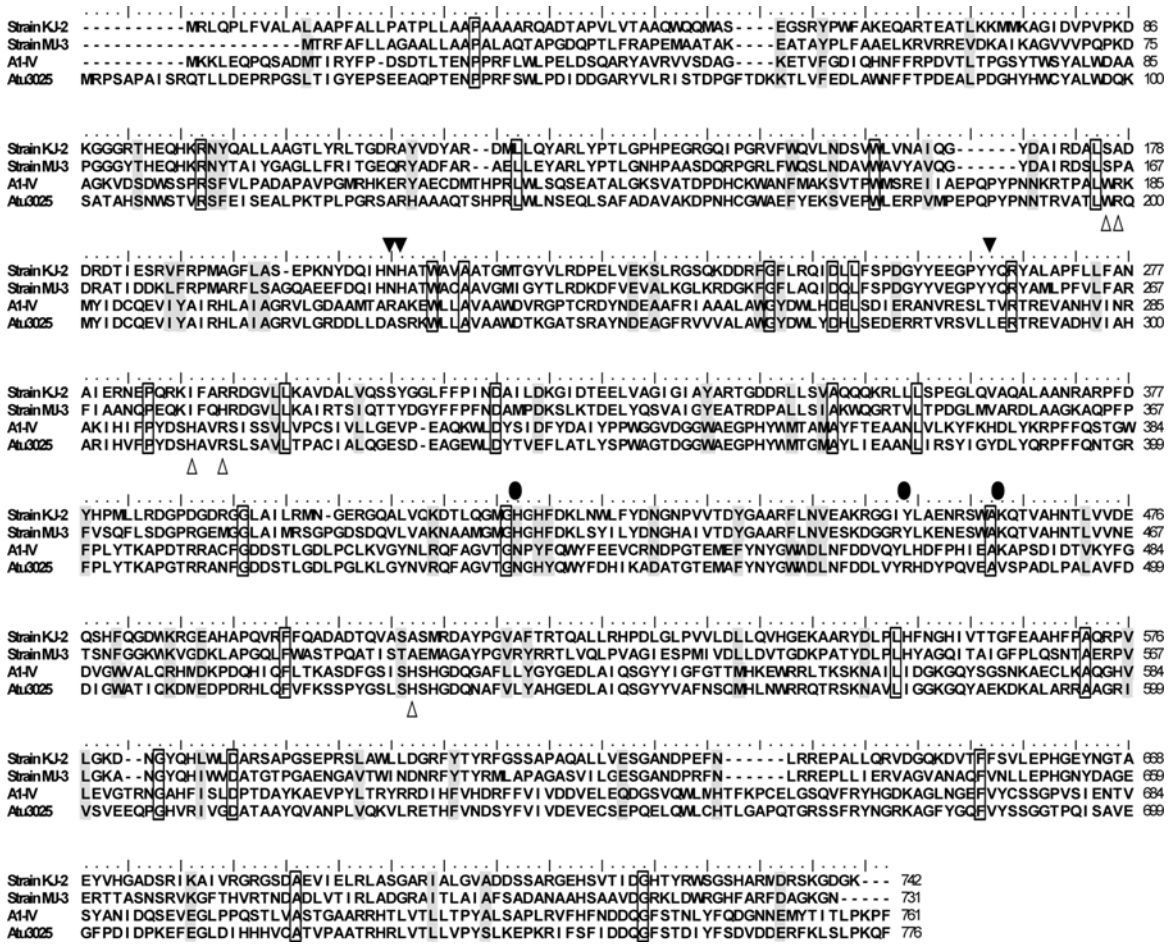

Fig. 1. Multiple sequence alignment analysis of KJ-2 oligoalginate lyase with other oligoalginate lyase sequences of strain MJ-3 alginate lyase (*Sphingomonas* sp. MJ-3 oligoalginate lyase, AEM45874), A1-IV (*Sphingomonas* sp. A1 oligoalginate lyase, BAB03319) and Atu3025 (*Agrobacterium tumefaciens* strain C58 exolytic alginate lyase, AAK90358). Outlined and shadowed amino acid sequences indicate identical and similar amino acid residues. Closed triangle and circle symbols above the sequences indicate the possible catalytic sites of AlgL-like domains and heparinase II/III-like domain, respectively. Open triangles under the sequences represent the candidates for catalytic sites for exolytic alginate degradation of A1-IV and Atu3025 [5,16].

was determined. The nucleotide sequence of the KJ-2 oligoalginate lyase was submitted to GenBank (Accession no. KC430928). For protein expression, the PCR product (2,163 bp) of KJ-2 oligoalginate lyase without signal peptide was also cloned.

2. Multiple Sequence Alignment Analysis

The KJ-2 oligoalginate lyase amino acid sequence was analyzed by multiple sequence alignment with other oligoalginate lyases (Fig. 1). The amino acid sequence of KJ-2 oligoalginate lyase showed 51.7, 9.8, 8.6% sequence identity with oligoalginate lyases of *Sphingomonas* sp. MJ-3, *Sphingomonas* sp. A1 (A1-IV) and *A. tumefaciens* strain C58 (Atu3025). Thus, we expected that the primary structure of KJ-2 oligoalginate lyase was quite different from other oligoalginate lyases, especially A1-IV and Atu3025.

When the AlgL-like conserved domain of KJ-2 oligoalginate lyase (amino acid sequence from 42 to 271) was compared with the amino acid sequence (from 10 to 291)- of A1-III polyM lyase (1HV6) part, sequence identity was 14.1%. Although the sequence identity was low, the potential catalytic amino acids such as Asn^{207}, His^{208} and Tyr^{264} were conserved (indicated as closed triangle in Fig. 1). From this analysis, we expected that KJ-2 oligoalginate lyase might possess an endolytic alginate lyase activity.

A part of heparinase II/III-like domain (amino acid sequence form 388 to 674) of KJ-2 oligoalginate lyase exhibited approximately 12.9% sequence identity with heparinase II (2FUQ) from *Pedobacter heparinus* as the template (amino acid sequence form 371 to 685) [25]. Based on the homology model of KJ-2 oligoalginate lyase constructed using 2FUQ as the template, catalytically important amino acids such as His^{418}, Tyr^{455} and Lys^{464} (closed circle in Fig. 1; His^{406}, Lys^{446}, Tyr^{468} in 2FUQ) are conserved in the heparinase II/III-like domain of KJ-2 oligoalginate lyase. Based on the multiple sequence alignment, we concluded that the cloned gene from *S. maltophilia* KJ-2 encoded an oligoalginate lyase consisting of AlgL- and heparinase II/III-like domains that might have alginate-degrading activity.

3. Subcloning of AlgL- and Heparinase II/III-like Proteins and Heterologous Expressions

The DNA fragments of AlgL-like domains (amino acid sequence from 27 to 376) and heparinase II/III-like domains (amino acid sequence from 360 to 742) were subcloned using PCR with the specific primers (Table 1). The division of N-terminal and C-terminal domains was determined using homology modeling based on A1-III alginate lyase from *Sphingomonas* sp. A1 (1HV6) and heparinase II from *P. heparinus* (2FUQ). The KJ-2 oligoalginate lyase was divided by N-terminal alpha/alpha-barrel domain and C-terminal two-layered beta-sheet domain. To subclone the AlgL- and heparinase II/III-like proteins, the corresponding specific forward and reverse primers with restriction sites were designed based on the DNA sequence of KJ-2 oligoalginate lyase, and were used for PCR (Table 1). For cloning into the pColdI vector, we used the primer containing the stop codon. After conducting PCR subcloning, we obtained 1,062 bp and 1,164 bp PCR products containing the N-terminal AlgL-like fragment and the C-terminal heparinase II/III-like fragment of KJ-2 oligoalginate lyase, respectively.

Genes of KJ-2 oligoalginate lyase, AlgL- and heparinase II/III-like proteins were expressed in *E. coli* HMS174. The band for KJ-2 oligoalginate lyase with fusion partner with molecular mass of 81.5 kDa appeared in the SDS-PAGE (Fig. 2). AlgL-like protein encoding 360 amino acids and heparinase II/III-like recombinant proteins encoding 379 amino acids were successfully expressed in *E. coli*. The bands corresponding to the AlgL- and heparinase II/III-like proteins with fusion partner appeared at 40.4 and 44.6 kDa, respectively. The expression level of heparinase II/III-like protein was higher than that of AlgL-like protein (Fig. 2).

The alginate-degrading activities of KJ-2 oligoalginate lyase, AlgL-

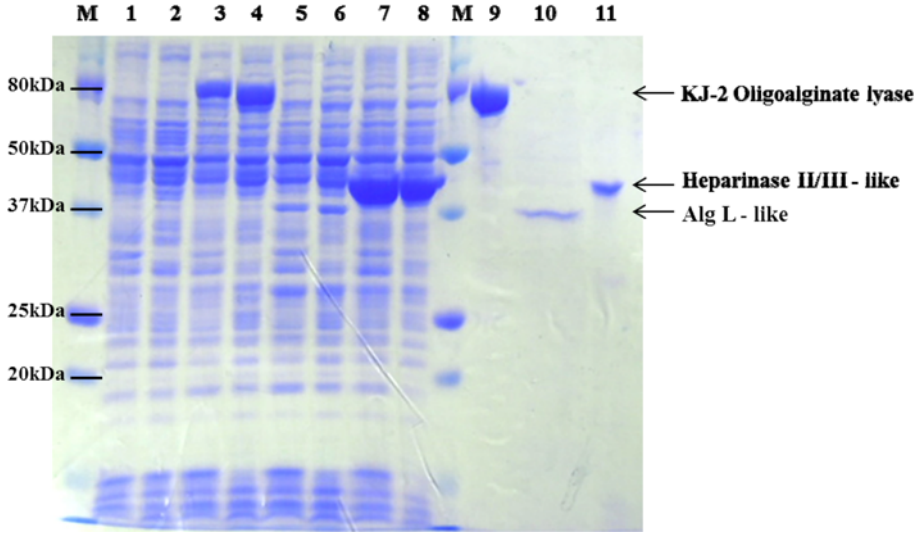

Fig. 2. SDS-PAGE analysis of the recombinant KJ-2 oligoalginate lyase, AlgL- and heparinase II/III-like proteins in *E. coli* HMS174 (DE3). The recombinant alginate lyases were expressed with 1 mM IPTG induction. Lane M, marker; 1 & 2, whole cell and lysate of *E. coli* HMS174 (DE3) as a control; 3 & 4, whole cell and lysate expressing KJ-2 oligoalginate lyase; 5 & 6, whole cell and lysate expressing AlgL-like recombinant protein; 7 & 8, whole cell and lysate expressing heparinase II/III-like recombinant protein; 9, purified protein of recombinant KJ-2 oligoalginate lyase; 10, purified protein of AlgL-like recombinant protein; 11, purified protein of heparinase II/III-like recombinant protein.

and heparinase II/III-like proteins were determined by measuring the amount of the released unsaturated uronic acid from alginate. As expected, the recombinant KJ-2 oligoalginate lyase exhibited the highest alginate-degrading activity, followed by AlgL-like protein. Initial production rates of reducing sugar by the recombinant KJ-2 oligoalginate lyase and AlgL-like protein were 412 and 10 μg/ml·min, respectively. Interestingly, the heparinase II/III-like protein also showed alginate-degrading activity, although the activity was very low, 3.81×10^{-3} μg/ml·min. Based on the above results, we expected that the KJ-2 oligoalginate lyase was most probably a novel oligoalginate lyase consisting of two different domains of AlgL- and heparinase II/III-like lyases possessing different alginate lyase activities.

4. Characterization of Recombinant KJ-2 Oligoalginate Lyase

Since KJ-2 oligoalginate lyase showed much higher alginate-degrading activity than those of AlgL- and heparinase II/III-like recombinant proteins, the recombinant KJ-2 oligoalginate lyase was further characterized. To analyze the effect of pH and temperature together with substrate specificity, the recombinant proteins were purified to homogeneity using a Ni-Sepharose column chromatography. The pH effect on the activity of KJ-2 oligoalginate lyase was analyzed in the range of 3.5-10.5 using various buffers such as sodium acetate, potassium phosphate, Tris-HCl, and sodium citrate buffers. The pH optimum was determined to be 7.5, and the KJ-2 oligoalginate lyase was active in the range from 7.0 to 9.0 (Fig. 3(a)). The temperature effect on the activity of KJ-2 oligoalginate lyase at various temperatures from 20 up to 70 °C was investigated. With respect to temperature, maximum activity was obtained at 37 °C (Fig. 3(b)).

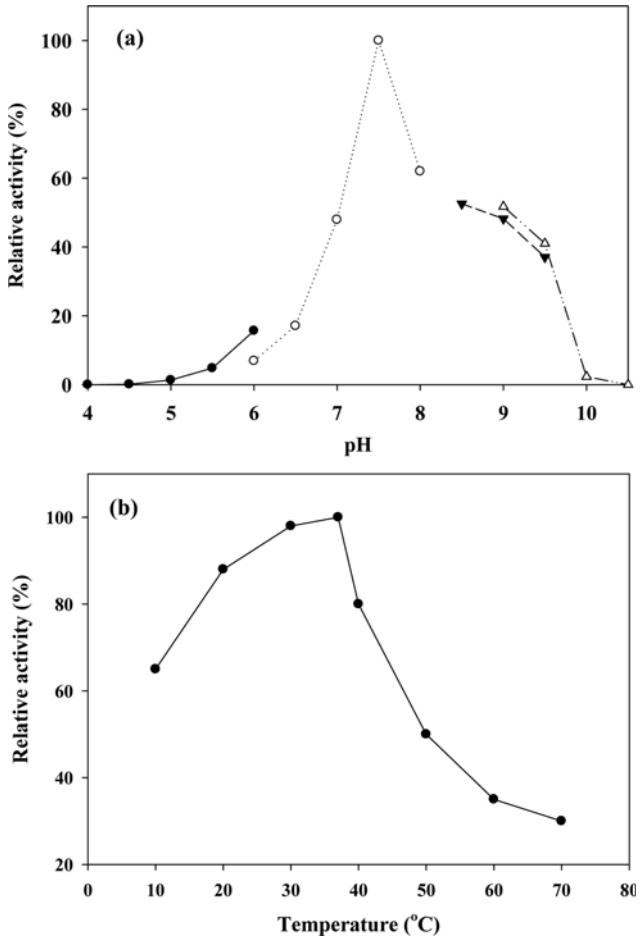

Fig. 3. Effects of reaction (a) pH and (b) temperature on the recombinant KJ-2 oligoalginate lyase activity. The activity was measured by the TBA method. (Symbol: ◆, sodium acetate buffer; ○, potassium phosphate buffer; ▼, Tris-HCl buffer; △, sodium citrate buffer).

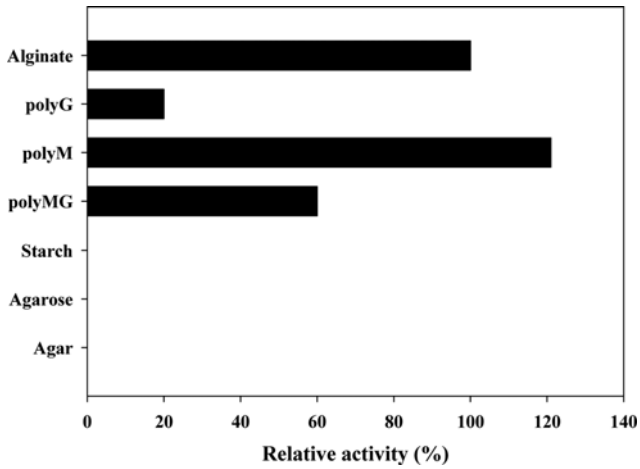

Fig. 4. Substrate specificity analysis of the recombinant KJ-2 oligoalginate lyase.

The substrate specificity of the recombinant KJ-2 oligoalginate lyase was also analyzed using alginate, polyM, polyG, and polyMG blocks. The recombinant KJ-2 oligoalginate lyase exhibited the highest activity to polyM block, followed by alginate (Fig. 4). The recombinant KJ-2 oligoalginate lyase showed higher degradation activity toward polyM block than alginate polymer because the degree of polymerization of polyM block prepared by partial acid hydrolysis of alginate was lower than alginate. The recombinant KJ-2 oligoalginate lyase did not show any degrading activity toward starch, agarose and agar.

5. Alginate Saccharification using the Recombinant KJ-2 Oligoalginate Lyase and Analysis of Degradation Products

A 2.0% (w/v) alginate was saccharified using the recombinant KJ-2 oligoalginate lyase, and the alginate degradation products were analyzed by FPLC (Fig. 5). In the course of 2.0% (w/v) alginate degradation by the recombinant KJ-2 oligoalginate lyase, various peaks with different elution volumes were detected (Fig. 5(a)). Elution volumes of mono-, di-, and tri-saccharide were 17.9, 16.7, and 15.7 ml, respectively. The m/z values of the corresponding peaks were determined to be 175, 351 and 527 for mono-, di- and trisaccharide, respectively, when the peaks were analyzed using ESI-MS with a negative mode ($[M-H]^-$). To confirm the activity of KJ-2 oligoalginate lyase toward oligosaccharides, alginate was degraded to oligomers by the recombinant ALG-5 endolytic alginate lyase [9] (Fig. 5(b)), and these oligomeric products were further degraded by KJ-2 oligoalginate lyase. The oligomeric products such as trimers and tetramers were degraded to dimers and monomers (Fig. 5(c)). As alginate degradation reaction progressed further, almost

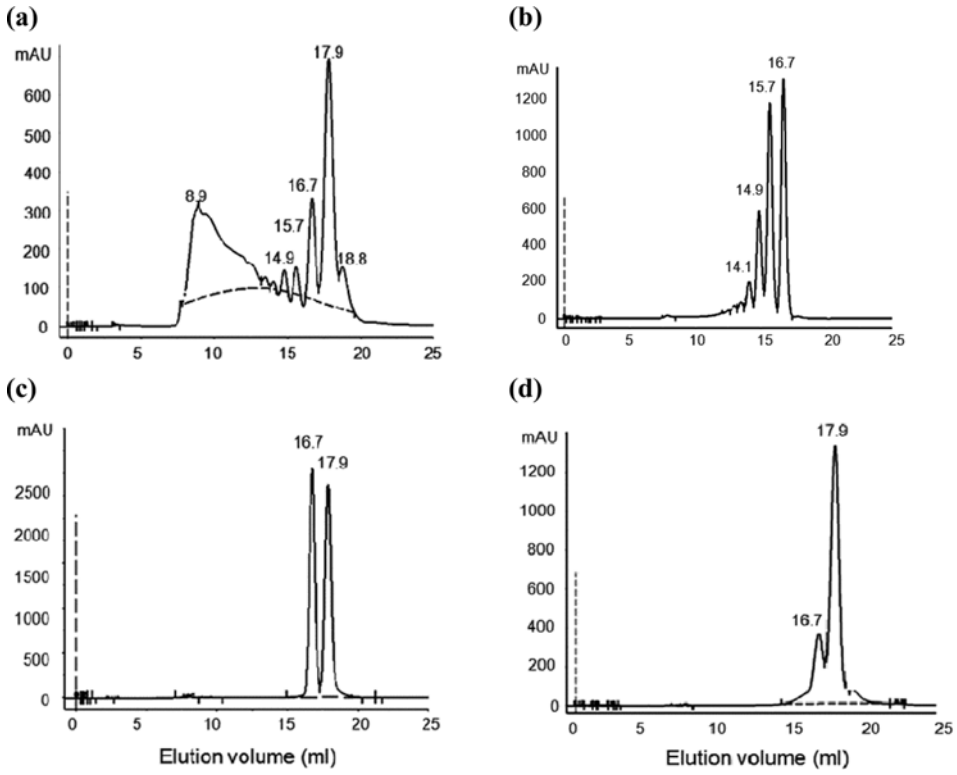

Fig. 5. FPLC analysis of alginate degradation products by the recombinant KJ-2 oligoalginate lyase. (a) Degradation products generated in the course of degradation of 2.0% (w/v) alginate by the recombinant KJ-2 oligoalginate lyase. (b) Degradation products of 2.0% (w/v) alginate depolymerized by the recombinant ALG-5 endolytic polyG-specific alginate lyase. (c) The recombinant KJ-2 oligoalginate lyase was added to sample (b) and then the degradation products generated in the course of further degradation by the recombinant KJ-2 oligoalginate lyase were analyzed by FPLC. (d) FPLC analysis of degradation products after complete degradation of sample (c) by the recombinant KJ-2 oligoalginate lyase. The dotted line represents the injection time. The reaction mixtures of (a), (b) and (c) were diluted five-fold and reaction mixture of (d) was diluted ten-fold before sample injection. Absorption peaks were detected at 235 nm. Elution volumes of 14.8, 15.7, 16.7 and 17.9 ml were tetramer, trimer, dimer and monomer, respectively.

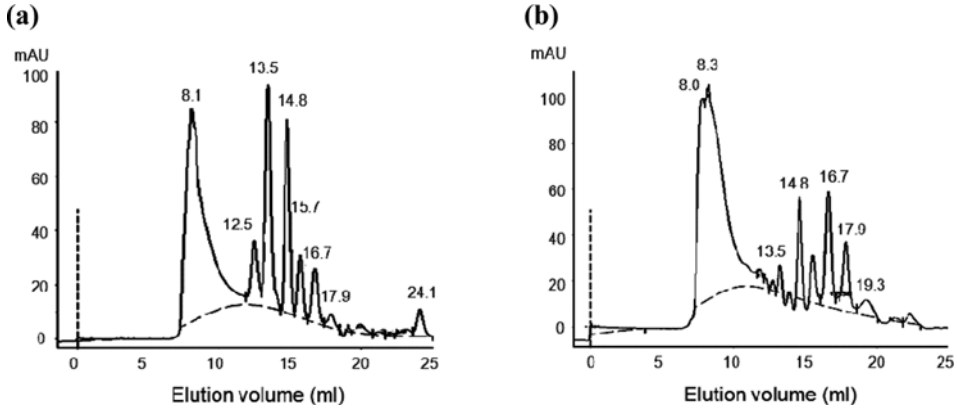

Fig. 6. FPLC analysis of alginate degradation products prepared by using the AlgL-like or heparinase II/III-like recombinant proteins. (a) Degradation products generated in the course of degradation of 1.0% (w/v) alginate by the AlgL-like recombinant protein. (b) Degradation products generated in the course of degradation of 1.0% (w/v) alginate by the heparinase II/III-like recombinant protein. Elution volumes of 12.5, 13.5, 14.8, 15.7, 16.7 and 17.9 ml were hexamer, pentamer, tetramer, trimer, dimer and monomer, respectively. Elution volumes of 19.3 and 24.1 ml were unknown peaks.

all peaks except monomer peaks disappeared (Fig. 5(d)), indicating that the KJ-2 oligoalginate lyase could degrade oligoalginate into monosaccharides.

The alginate degradation products produced by using the AlgL-like or heparinase II/III-like recombinant proteins were also analyzed by FPLC (Fig. 6). Interestingly, oligomers such as dimer, trimer and tetramer were generated together with monomer, indicating that heparinase II/III-like recombinant proteins exhibit exolytic

alginate lyase activity. Compared with the KJ-2 oligoalginate lyase, the subcloned AlgL-like or heparinase II/III-like domains could not depolymerize alginate completely into unsaturated monosaccharides even after long reaction time of 48 h due to low lyase activity. The degradation patterns of AlgL-like or heparinase II/III-like recombinant proteins were quite different. As shown in Fig. 6(a), only small amount of monosaccharide was generated by AlgL-like recombinant protein. The relative ratio of monomer to oligomers generated by AlgL-like recombinant protein was much lower than that by heparinase II/III-like recombinant protein, indicating that AlgL-like recombinant protein exhibited more like endolytic alginate lyase activity than heparinase II/III-like recombinant protein. This result was somewhat anticipatable based on the multiple sequence analysis that the amino acid sequence of the active site of A1-III endolytic polyM-specific lyase was expected to be highly conserved in AlgL-like domain in the KJ-2 oligoalginate lyase. On the contrary, relatively higher amount of monosaccharide, compared with that generated by AlgL-like recombinant protein, was generated by heparinase II/III-like recombinant protein (Fig. 6(b)), suggesting that the heparinase II/III-like recombinant protein showed more like exolytic alginate lyase activity.

Recently, the oligoalginate lyases of *Sphingomonas* sp. A1 (A1-IV) and *A. tumefaciens* strain C58 (Atu3025) have been characterized [5,14,15], and the 3-D structure of Atu3025 was analyzed by X-ray crystallography [16]. When the amino acid sequence of the KJ-2 oligoalginate lyase was compared with those of A1-IV and Atu3025, the sequence identity was below 10%, indicating that the KJ-2 oligoalginate lyase was quite different from A1-IV and Atu3025. Alginate lyase A1-IV and Atu3025 only showed exolytic activity. The oligoalginate lyases of A1-IV and Atu3025 have been shown to possess only heparinase II/III-like domain in the C-terminal region, while other polyM/polyG/polyMG-specific alginate lyases do not have that domain [4,7,8,11,12]. In contrast, the oligoalginate lyase of *Sphingomonas* sp. MJ-3 has been appeared to possess AlgL-like domain (from aa 163 - aa 350) and heparinase II/III-like domain (from aa 381 to aa 506) [17]. Hence, we expected that the MJ-3 oligoalginate lyase might possess novel activity. However, the recombinant proteins of the subcloned AlgL- and heparinase II/III-like domains of the MJ-3 oligoalginate lyase did not show any alginate-degrading activity. Thus, we demonstrated that the KJ-2 oligoalginate lyase is a novel oligoalginate lyase consisting of two different domains of AlgL- and heparinase II/III-like alginate lyases possessing different lyase activities.

As to the mode of action, the KJ-2 oligoalginate lyase degraded alginate exolytically and thus completely generated unsaturated monosaccharides from alginate, while the recombinant protein of AlgL-like domain degraded alginate in more likely endolytic mode. In the sense of catalysis, the activity of KJ-2 oligoalginate lyase consisting of AlgL- and heparinase II/III-like domains was much higher than those of the AlgL- and heparinase II/III-like recombinant proteins. Herein, one interesting point is that how two independent domains of the AlgL- and heparinase II/III-like domains in the KJ-2 oligoalginate lyase could enhance alginate degrading activity. Further investigation is required to understand how a synergistic catalysis by AlgL-like and heparinase II/III-like proteins can play a role in alginate degradation by the KJ-2 oligoalginate lyase.

CONCLUSION

We identified a novel recombinant KJ-2 oligoalginate lyase, cloned, heterologously expressed in *E. coli* and then characterized. The recombinant KJ-2 oligoalginate lyase is a novel oligoalginate lyase consisting of AlgL- and heparinase-like domains with different lyase activities. The recombinant KJ-2 oligoalginate lyase completely degraded alginate into monosaccharides. We also showed that the recombinant proteins of AlgL- and heparinase II/III-like domains exhibited an alginate-degrading activity, although their activities were rather low. The recombinant KJ-2 oligoalginate lyase can be used as a candidate for biocatalyst selection to saccharify alginate for further applications, for example, bioethanol production.

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