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BIOENERGY AND BIOFUELS

Molecular cloning, purification, and characterization of a novel polyMG-specific alginate lyase responsible for alginate MG block degradation in *Stenotrophomas maltophilia* KJ-2

Su In Lee · Sung Hee Choi · Eun Yeol Lee · Hee Sook Kim

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Abstract A gene for a polyMG-specific alginate lyase possessing a novel structure was identified and cloned from *Stenotrophomas maltophilia* KJ-2 by using PCR with homologous nucleotide sequences-based primers. The recombinant alginate lyase consisting of 475 amino acids was purified on Ni-Sepharose column and exhibited the highest activity at pH 8 and 40 °C. Interestingly, the recombinant alginate lyase was expected to have a similar catalytic active site of chondroitin B lyase but did not show chondroitin lyase activity. In the test of substrate specificity, the recombinant alginate lyase preferentially degraded the glycosidic bond of polyMG-block than polyM-block and polyG-block. The chemical structures of the degraded alginate oligosaccharides were elucidated to have mannuronate (M) at the reducing end on the basis of NMR analysis, supporting that KJ-2 polyMG-specific alginate lyase preferably degraded the glycosidic bond in M–G linkage than that in G–M linkage. The KJ-2 polyMG-specific alginate lyase can be used in combination with other alginate lyases for a synergistic saccharification of alginate.

Keywords Alginate · Alginate lyase · PolyMG-specific alginate lyase · Saccharification · *Stenotrophomas maltophilia* KJ-2

S. In Lee · H. S. Kim (✉)
Department of Food Science and Biotechnology, Kyungsung University,
Busan 608-736, Korea
e-mail: hskim@ks.ac.kr

S. H. Choi · E. Y. Lee (✉)
Department of Chemical Engineering, Kyung Hee University,
Gyeonggi-do 446-701, Korea
e-mail: eunylee@khu.ac.kr

Introduction

In post-petroleum era, bioenergy from biomass is a promising alternative. Recently, micro- and macroalgae are considered as cost-competitive biomass for the production of biodiesel, bioethanol, and biohydrogen (Beer et al. 2009; Chisti 2008; Vasudevan and Briggs 2008). Brown seaweed is a kind of macroalgae. Laminaran and mannitol from brown seaweed have been used for bioethanol fermentation (Horn et al. 2000). The most abundant carbohydrate in brown seaweed, however, is alginate as a major component of cell wall matrix. Interestingly, although alginate constitutes 14–37 % of seaweed biomass, alginate has not been considered as carbon source or starting material for biorefinery. Alginate monomers are not easily fermented to bioethanol. Very recently, genes for alginate-related metabolic enzymes and transport systems have been recruited to *Escherichia coli* host, resulting in successful production of bioethanol from alginate by the metabolically engineered *E. coli* (Wargacki et al. 2012).

Alginate is a polysaccharide consisting of β-D-mannuronate (M) and α-L-guluronate (G). The enzyme that degrades alginate is alginate lyase. Alginate lyase catalyzes a β-elimination of glycosidic bonds and produces unsaturated oligosaccharides with double bonds at the non-reducing end (Campa et al. 2004; Matsubara et al. 2000; Albrecht and Schiller 2005; Clementi 1997). A number of alginate lyases from various organisms have been identified, cloned, purified, and characterized (Kim et al. 2011; Wong et al. 2000). Alginate lyases are classified into different polysaccharide lyases (PLs) families based on structurally related catalytic and carbohydrate-binding module in the carbohydrate-active enzymes (CAZY) database (Cantarel et al. 2009). In terms of the mode of degradation, alginate lyase can be classified into endolytic

and exolytic alginate lyases. Endolytic alginate lyase releases unsaturated di-, tri-, and tetra-saccharides as major products by the cleavage of glycosidic bonds inside alginate polymer (Alkawash et al. 2006). Exolytic alginate lyase further degrades oligomeric alginate into unsaturated uronate monomer (Hashimoto et al. 2000; Ochiai et al. 2006; Park et al. 2012).

We are interested in developing biocatalytic way for the production of bioenergy and biochemicals based on alginate and other difficult-to-ferment sugars (Ryu and Lee 2011). In the case of alginate saccharification, alginate lyase is the key enzyme. Use of endolytic and exolytic alginate lyases in combination is advantageous for an efficient saccharification of alginate. Alginate consists of three blocks such as polyM-, polyG-, and polyMG-blocks. The M- and G-unit are distributed randomly in heteropolymeric polyMG-block, and their relative content is highly dependent on alginate sources. Therefore, it is also of interest to use in combination of various endolytic alginate lyases possessing different substrate specificities for a synergistic degradation of alginate consisting of three different blocks.

A polyMG-specific alginate lyase preferentially degrades the heteropolymeric random sequence of polyMG-block. Although a number of endolytic polyM- and polyG-specific lyases have been identified, there have been a few investigations on polyMG-specific lyases so far because polyMG lyase is very rarely found in nature (Yamasaki et al. 2004; Gimmestad et al. 2009). Recently, we have obtained endolytic polyM-, polyG-specific lyases, and exolytic oligoalginate lyase from various microorganisms (Kim et al. 2009; Park et al. 2012; Kam et al. 2011; Shin et al. 2011). In order to expand the pools of the recombinant alginate lyases with different substrate specificities for the saccharification of alginate, herein, we report on the identification and characterization of a polyMG-specific alginate lyase from *Stenotrophomas maltophilia* KJ-2. Interestingly, the homology-modeled structure of the KJ-2 polyMG-specific alginate lyase shares a similar fold structure and a catalytic active site with chondroitin B lyase. The substrate specificity was analyzed, based on the results from ESI MS/MS and NMR analysis of the structure of degradation products. The basic catalytic properties of recombinant KJ-2 polyMG-specific alginate lyase were also characterized.

Materials and methods

Screening and cultivation of alginate-degrading marine bacteria

Alginate-degrading marine bacteria were isolated from salted guts from hairtails (*Trichiurus haumela*), a traditional Korean food. The fermented soup was diluted with deionized water serially and then smeared on M9 agar plate containing 0.8-% (*w/v*) alginate. The plates were incubated at 20 °C for 10 days and stored at 4 °C as a master plate. Several colonies with colors grew on the plate. Among the alginate-degrading bacteria, one colony was chosen for this study. The isolated bacterium was cultured in M9 media or LB broth with alginate to analyze the effect of alginate on the cell growth.

Chemotaxonomic and molecular characterization of the isolated strain KJ-2

The isolated strain KJ-2 was isolated by the standard dilution-plating technique at 25 °C on M9 agar plate with 0.8-% (*w/v*) alginate. The Gram staining was conducted by using the Difco Gram staining kit according to the manufacturer's instruction.

The 16 S ribosomal DNA sequence of the isolated strain was determined by the procedure described in the previous work (Kim et al. 2009). The 16 S rDNA was amplified from the genomic DNA by PCR using the bacterial primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1518r (5′-AAGGAGGTGATCCANCCRCA-3′). The PCR product was sequenced using a BigDye terminator sequencing method. The 16 S rRNA gene was analyzed and aligned with multiple sequence data in GenBank database by using BLAST algorithm and CLUSTAL W program.

Cloning and expression of alginate lyase gene from KJ-2

From 16 S rDNA analysis, alginate-degrading bacteria KJ-2 was identified to *Stenotrophomonas maltophilia*. From NCBI Genome Project (http://www.ncbi.nlm.nih.gov/genomes/), two complete genomes, *S. maltophilia* strain K279a (Welcome Trust Sanger Institute) and strain R551-3 (DOE Joint Genome Institute), could be found. There were more than 90-% protein sequence identities between alginate lyase operons of both strains. So, we could design the PCR primers for cloning the genes of alginate lyase operon. The alginate lyase primers were designed on the basis of homolog sequences from TonB gene and heparinase II/III like protein gene sequence in alginate-degrading cluster from *S. maltophilia* K279a (NC_010943) and *S. maltophilia* R551-3 (NC_011071). The forward primer was derived from TonB gene sequence, as 5KJ2-TonBMF2: 5′-CGCGCTTCTTGTTGATCGAACTCTGC-3′, and the reverse primer were derived from heparinase-like protein gene sequence, as 3KJ2-Hepa-MR: 5′-GCAACGAACAGCGGTTGCAACCTCAT-3′. The PCR products were ligated with T-blunt vector (Solgent, Korea). The nucleotide sequence was analyzed by using BlastX program, and the open reading frame was searched by using ORF finder

(http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The genomic DNA of *S. maltophilia* KJ-2 was used as the template for amplification of alginate lyase gene. The signal peptide cleavage site was predicted using SignalP 3.0 server (Bendtsen et al. 2004). In order to clone the alginate lyase gene into expression vector, the forward primer 5KJ2-ManF (*Nde*I); 5′-CATATGGCAAGCTGGCTCGTGCATGAC-3′ and the reverse primer 3KJ2-ManR (*Xho*I); 5′-CTCGAGTCGATCAACTCCGGTGCTGAC-3′ were used. KJ2 alginate lyase gene was ligated into pET-21b(+) expression vector. The recombinant *E. coli* BL21 (DE3) harboring the pET-21b(+)/KJ-2 recombinant plasmid was cultured on an LB medium supplemented with 50-μg/ml ampicillin for 2–3 h up to OD_{600}=0.4–0.6 in a shaking incubator at 180 rpm 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 KJ-2 alginate lyase gene induced by the addition of 0.7-mM IPTG.

Purification of the recombinant KJ-2 alginate lyase and analysis of its substrate specificity

The cells were harvested and sonicated in lysis buffer (50-mM potassium phosphate buffer (pH 7.2), 300-mM NaCl, 10-mM imidazole, 10-% (*v/v*) glycerol, 0.5-% (*v/v*) Triton X-100, 1-mg/ml lysozyme, 1-mM PMSF). The cell homogenate containing the expressed $(His)_6$-tagged *S. maltophilia* KJ-2 alginate lyase was loaded on Ni-Sepharose column (Amersham Biosciences, USA) equilibrated with 50-mM phosphate buffer (pH 7.2) and 0.5-M NaCl. The column was washed with the same buffer containing 50-mM imidazole, and the *S. maltophilia* KJ-2 alginate lyase protein was eluted with the same buffer containing 300-mM imidazole. The active fraction was desalted using HiTrap™ desalting column (Amersham Biosciences, USA). The substrate specificity of *Stenotrophomas* sp. KJ-2 alginate lyase was determined by using alginate, polyM-, polyG-, and polyMG-block substrates as described in the reference (Haug et al. 1966). MG ratio was calculated from ^{1}H-NMR spectra by using the method described in the references (Ertesvåg et al. 1998; Grasdalen 1983).

SDS-PAGE analysis and immunoblotting

The proteins were separated on 12 % (*w/v*) SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The blotted membrane was incubated with rabbit 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).

Assay of alginate lyase activity

The enzyme purified by Ni-Sepharose affinity column was added into 1 ml of 20-mM phosphate buffer (pH 7.2) containing 0.2-% (*w/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 double bond between C-4 and C-5 at the non-reducing terminus by the β-elimination reaction. The resulting deoxy sugar was determined by the TBA method (Park et al. 2012; Hurwitz and Weissbach 1959). The degradation products were analyzed by TLC and FPLC. The degraded products on TLC were separated twice with a developing solvent of 1-butanol:acetic acid:water (2:1:1, *v/v/v*). The degradation products were visualized by dipping into 10-% (*v/v*) sulfuric acid in ethanol. FPLC equipped with Superdex peptide 10/100 GL column (GE Healthcare, USA) was also used with 0.2-M bicarbonate buffer (pH 7.2). The degraded unsaturated oligoalginates were detected by measuring the absorbance at 235 nm with UV detector.

Analysis of substrate specificity and stability of KJ-2 alginate lyase

For investigation of substrate specificity, 10-μg purified KJ-2 alginate lyase and 0.2-% (*w/v*) substrates as a final concentration in 20-mM Tris buffer (pH 8.0) were incubated in 1-ml reaction mixture for 10 min at 30 °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, 10-μg KJ-2 alginate lyase in 0.5 ml of 20-mM Tris–HCl buffer (pH 8) was incubated for 30 min at various temperature (20, 30, 40, 50, 60, and 70 °C) before further incubation for 10 min with the substrate at 30 °C. To determine optimal pH, 10-μg KJ-2 alginate lyase was incubated for 10 min at 30 °C in appropriate buffers, 20-mM acetate buffer (pH 3–6), 20-mM phosphate buffer (pH 6–9), 20-mM Tris–HCl buffer (pH 6–10), and 20-mM carbonate buffer (pH 9–11) before further incubation with the substrate. To evaluate the effect of metal ion on enzyme activity, 2-μg KJ-2 alginate lyase was incubated with 2-mM salt solution (NaCl, $ZnCl_2$, $CaCl_2$, $MgCl_2$, $HgCl_2$, KCl, $CoCl_2$, $MnCl_2$, $BaCl_2$) in 0.5 ml of 20-mM Tris–HCl buffer (pH 8) for 10 min and then incubated with 0.5 ml of 0.4-% (*w/v*) alginate substrate at 30 °C for 10 min.

Structural analysis of KJ-2 alginate lyase-degrading products

A 0.4-% (*w/v*) alginate was digested by KJ-2 alginate lyase, and the degradation fractions were analyzed using FPLC

(Park et al. 2012). The unsaturated uronate fractions were detected by measuring the absorbance at 235 nm. For the determination of the molecular mass of each fraction, electrospray-ionization mass spectroscopy (ESI-MS, 6410 Triple Quadrupole LC-MS, Agilent, USA) was used. The purified oligosaccharides from Bio-Gel P-2 column (1.5× 360 cm) were dissolved in small volume of methanol:water (1:1, *v/v*) and then injected to the LC/MS. Flow rate of electrospray source was 5 μl/min. MS was performed in negative mode with ion spray voltage of 4 kV and source temperature of 350 °C.

^{1}H-NMR of alginates degraded by KJ-2 alginate lyase

A 0.4-% (*w/v*) alginate in 20 ml of buffer (10-mM phosphate, pH 7.2) was mixed with enough amount of 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 periodically and analyzed by FPLC. After the alginate substrate and reaction products were lyophilized, they were dissolved with D_2O and then lyophilize again. The lyophilized samples were dissolved with D_2O and then analyzed by high-field ^{1}H nuclear magnetic resonance (NMR) spectroscopy, using an ECX-NMR 400-Hz JEOL spectrometer (JEOL, USA) according to the reference (Grasdalen 1983). TMSP (20 μl, 0.75-% 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid, sodium salt in D_2O) was added to the NMR sample tube as an internal standard. Data collection was carried out at an elevated temperature of 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 from alginate-degrading product by KJ-2 lyase was compared with those from ALG-5 lyase and KS-408 lyase (Kim et al. 2009; Kam et al. 2011).

Interpretation of ^{1}H-NMR spectra

The molar fractions of monomers (F_G and F_M), dimers (F_{GG}, F_{MM}, F_{GM}, and F_{MG}), and trimers (F_{GGG}, F_{MGM}, F_{GGM}, and F_{MGG}) of the substrates used in this study are the same with the substrates used in previous paper (Table 1, Kam et al. 2011). The interpretation of NMR spectra for polyM-and polyG-specific alginate lyase-degraded oligoalginates was interpreted based on the previous study (Kam et al. 2011). In NMR analysis of the oligoalginates produced by KJ-2 polyMG-specific alginate lyase, ΔM signal (M↓GMG or GM↓GM) can be detected when MGMG and GMGM sequences are cleaved behind M residue (M_{red} signal), whereas ΔG signal (MG↓MG or G↓MGM) can be detected when MGMG and GMGM sequences are cleaved behind G residue (G_{red} signal).

Substrate preparation

For alginate lyase activity assay, sodium 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). The MG ratio of the purified substrate was determined according to the standard test method ASTM F2259 (ASTM F2259-10, Standard test method for determining the chemical composition and sequence in alginate by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, 2010, http://www.astm.org/Standards/F2259.htm).

Materials

Alginates (250 and 3,500 cps), pectin, starch, and chondroitin B were purchased from Sigma (USA). TLC silica gel 60 plate and Bio-Gel P-2 gel were purchased from Merck (Germany) and Bio-Rad (USA), respectively. Ni-Sepharose™, His-Buffer kits, and HiTrap™ desalting column were purchased from GE Healthcare (USA). Recombinant polyG-specific alginate lyase (referred to as ALG-5 lyase) and polyM-specific alginate lyase (referred to as KS-408 lyase) were prepared according to the previous papers (Kim et al. 2009; Kam et al. 2011). Restriction enzymes and T4 DNA ligase were purchased from

Table 1 The chemical compositions of various substrates used in this study. F_G, F_M, F_{GG}, $F_{GM,MG}$, F_{MM}, F_{GGM}, F_{MGM}, and F_{GGG} represent the fractions of corresponding monomers, dimers, and trimers in the substrates

Substrate	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

[a] DP_n indicates the average degree of polymerization

[b] Alginate (cps=3,500) and acetylated alginate (synthesized by *Pseudomonas aeruginosa* ATCC39324) were degraded to the corresponding substrates with DP_n from 30 to 40 by diluted acid hydrolysis

NEB (England). PfuX DNA polymerase and T-blunt vector was purchased from Solgent (Korea). Plasmid purification, PCR product purification, and DNA extraction in the agarose gel were performed using DNA-spin™ plasmid purification kit (Intron Biotechnology, Korea), Expin™ PCR purification kit, and Expin™ gel extraction kit (GeneAll Biotechnology, Korea), respectively. PCR for DNA amplification was performed using Biometra TPersonal (Whatman, Germany).

Results

Cloning and heterologous expression of KJ-2 alginate lyase

Based on the 16 S rDNA analysis (GenBank ID: JN942761) of the isolated KJ-2 strain with alginate-degradation activity, *S. maltophilia* species (99 %) was the closest relatives. The isolated KJ-2 was designated *S. maltophilia* strain KJ-2 (KCTC32000), and its genomic DNA was used for the cloning of alginate lyase by PCR with specific primers designed based on homologous nucleotide sequences. The resulting open reading frame (ORF) consisted of 1,428 bp with deduced amino acid size of 475 (49.9 kDa). The DNA and amino acid sequences were deposited to GenBank with accession number of JN942762 and AFC88009, respectively.

The putative KJ-2 alginate lyase gene was inserted into pET-21b(+) vector to obtain the recombinant protein to characterize its property. Previously, polyM- and polyG-specific alginate lyases without signal peptide exhibited higher degradation activity than those with signal peptide (Kim et al. 2009; Park et al. 2012). When the putative KJ-2 alginate lyase gene fused with $(His)_6$-tag and without signal peptide is to be expressed, the molecular mass of the putative KJ-2 alginate lyase protein would be 47.8 kDa with 454 amino acids. The expressed recombinant protein was analyzed by using SDS-PAGE and immunoblotting (Fig. 1). A band corresponding to the putative KJ-2 alginate lyase clearly appeared in the SDS-PAGE after induction with 0.7-mM IPTG. When the gene was expressed at 37 °C, the expression level was high, but there was little recombinant protein in lysate (data not shown). The soluble protein in lysate increased when the gene was expressed at 15 °C. The expressions of the recombinant alginate lyases were reconfirmed in immunoblotting profile blotted with polyclonal antibody and peroxidase-conjugated anti-rabbit IgG.

Partial purification and characterization of the recombinant KJ-2 alginate lyase

The recombinant KJ-2 alginate lyase was purified by Ni-Sepharose affinity column chromatography (Fig. 1). First, the effects of pH, temperature, and Ca^{2+} concentration on

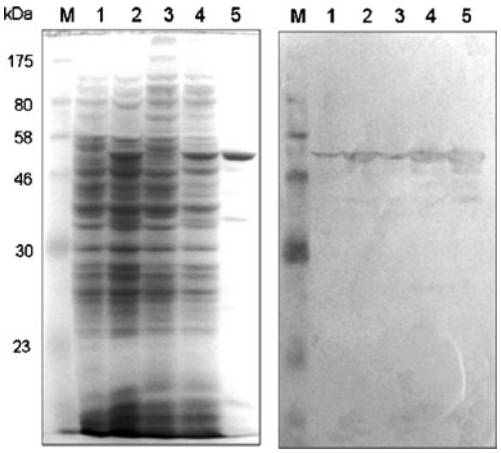

Fig. 1 SDS-PAGE and immunoblotting analysis of KJ-2 alginate lyase. The proteins of the recombinant cells with KJ-2 alginate lyases gene were separated on 12-% (*w/v*) SDS-PAGE. The proteins on SDS-PAGE gel were visualized by staining with Coomassie Blue R-250 (*left*). Overexpressed KJ-2 alginate lyase on a nitrocellulose paper was detected by immunoblotting method (*right*). *Lane M* protein marker, *lanes 1* and *2* recombinant cells induced without and with IPTG, *lanes 3* and *4* lysate of the recombinant cells induced without and with IPTG, *lane 5* partially purified recombinant KJ-2 alginate lyase using Ni-sepharose

the alginate-degrading activity and enzyme stability of the purified KJ-2 alginate lyase were investigated. For the given range of pH from 3 to 11, the enzyme exhibited a stable and high activity at pH between 8 and 9 (Fig. 2a). In order to analyze temperature effects on activity, the purified KJ-2 alginate lyase was reacted in 20-mM Tris–HCl buffer (pH 8.0) for 20 min with 0.2-% (*w/v*) alginate solution at the indicated temperature. Optimum temperature was 40–50 °C. In terms of enzyme stability, the purified enzyme was incubated in 20-mM Tris–HCl buffer at 20–70 °C for 30 min before alginate-degrading reaction was performed with the substrate solution for 10 min at 30 °C. The KJ-2 alginate lyase showed maximum activity at 40 °C (Fig. 2b). However, the KJ-2 alginate lyase significantly lost its activities at temperatures higher than 40 °C, indicating that the practical optimum reaction temperature is 40 °C.

Effects of metal ion and EDTA on the activity of KJ-2 alginate lyase were investigated (Table 2). Some divalent cations such as Co^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, and Zn^{2+} inhibited the lyase activity. The lyase activity was increased at high concentration of Na^{+} and K^{+}, more than 50 mM, and the presence of 1-mM Ca^{2+} exhibited a positive effect on the lyase activity. Interestingly, the lyase activity was completely disappeared when 1-mM EDTA was present in the reaction solution. The effect of Ca^{2+} on the activity of KJ-2 alginate lyase was further analyzed (Fig. 3). The recombinant KJ-2 alginate lyase was incubated with 2-mM EDTA in buffer (pH 8.0) for 10 min for depriving the enzyme activity fully, and then, various amounts of $CaCl_2$ were added and incubated for another 10 min. As the concentration of $CaCl_2$

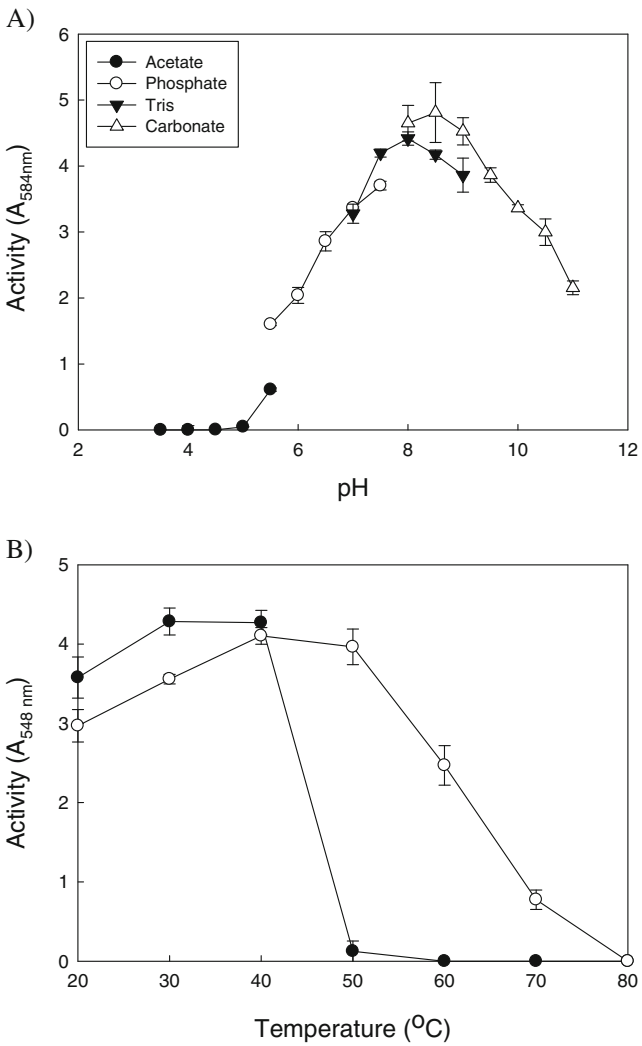

Fig. 2 Effect of pH and temperature on the activity of the purified KJ-2 alginate lyase. The alginate lyase activity was measured by TBA method (A_{548nm}). **a** Effect of pH was investigated by using various buffer solutions. **b** To determine temperature optimum (*open circle*), 10-μg purified KJ-2 alginate lyase was incubated in 20-mM Tris–HCl buffer (pH 8.0) for 10 min before the alginate degradation reaction was performed with 0.2-% (*w/v*) alginate solution at the indicated temperature. To investigate temperature stability (*closed circle*), the purified enzyme was incubated in 20-mM Tris–HCl buffer (pH 8.0) for 30 min before the reaction was performed with the substrate solution for 10 min at 30 °C. Experiments were conducted three times

increased to 4 mM, the lyase activity increased from zero activity to the original activity of the recombinant alginate lyase in the absence of EDTA and $CaCl_2$. This result clearly indicated that the recombinant KJ-2 alginate lyase requires a certain divalent cation such as Ca^{2+} for the activity.

Substrate specificity analysis

Nine structurally different substrates including polyMG-blocks were used to investigate substrate specificity (Table 1; Fig. 4a). The KJ-2 alginate lyase showed higher degrading activity toward alginate and polyMG-block than polyM- and polyG-blocks (Fig. 4a). The activity for polyMG was twice higher than alginate. The KJ-2 lyase exhibited little activity on pectin, starch, agar, and agarose. Interestingly, the KJ-2

Table 2 Effects of various chemicals on KJ-2 alginate lyase activity

Chemical	Concentration (mM)	Activity[a] (%)
None		100
NaCl	1	107
	50	107
	100	120
KCl	1	109
	50	123
	100	99
$BaCl_2$	1	83
$CaCl_2$	1	124
$CoCl_2$	1	27
$CuCl_2$	1	27
$FeCl_2$	1	76
$HgCl_2$	1	17
$MgCl_2$	1	92
$MgSO_4$	1	86
$MnCl_2$	1	26
$ZnCl_2$	1	1
EDTA	0.1	58
	1	0
DTT	1	103

The purified enzyme (2 μg) was incubated with 2-mM metal ion in 0.5 ml of 20-mM Tris buffer (pH 8.0) for 10 min at 30 °C before adding 0.5 ml of 0.4-% (*w/v*) sodium alginate solution

[a]Activity in the absence of any chemicals was taken as 100 %

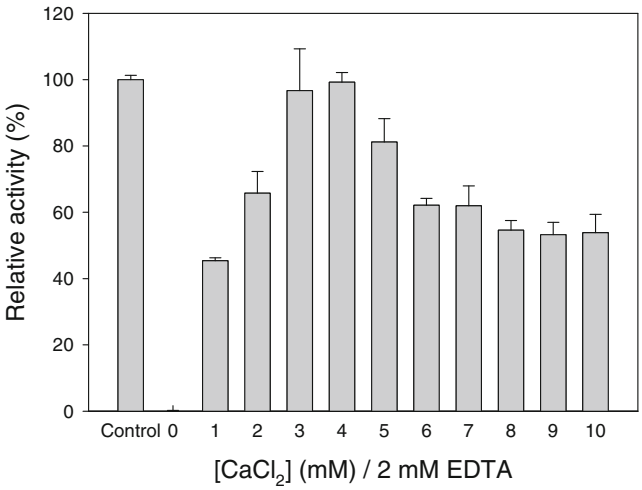

Fig. 3 Effect of Ca^{2+} on the activity of KJ-2 alginate lyase. A 20-μg purified KJ-2 alginate lyase was incubated with 2-mM EDTA in buffer (pH 8.0) for 10 min for depriving enzyme activity, and then, $CaCl_2$ solution with indicated concentration was added and incubated for 10 min. After the addition of alginate solution, the reaction was performed for 10 min at 30 °C. *Control* indicates the activity when the reaction mixture was incubated without EDTA and $CaCl_2$. Experiments were conducted three times

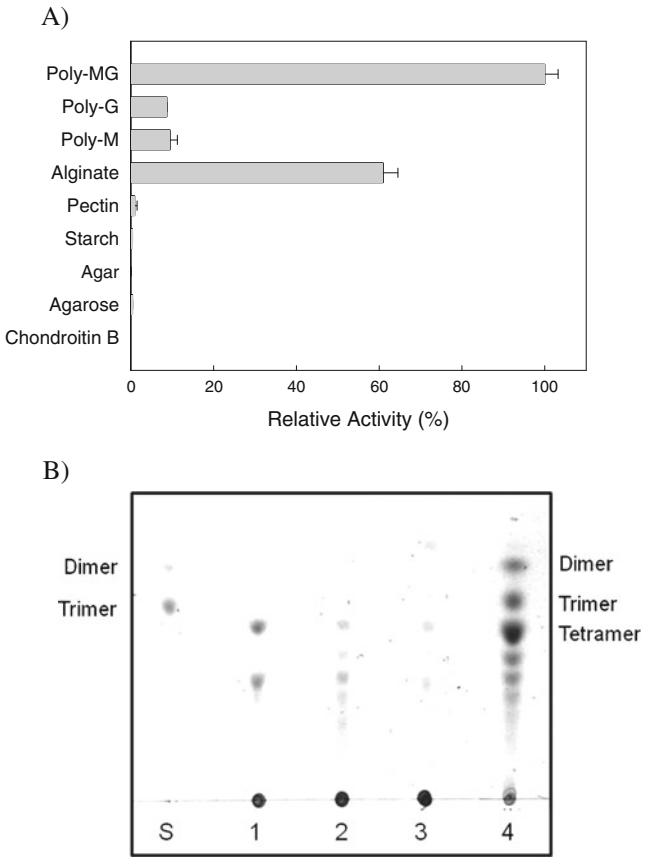

Fig. 4 Substrate specificity of KJ-2 alginate lyase. **a** 0.2 % (*w/v*) of each substrate was digested with 10 μg of KJ-2 alginate lyase for 10 min at 30 °C. Enzyme activity was assayed by TBA method. Experiments were conducted three times. **b** 0.4 % (*w/v*) of each substrate was completely digested by the enzyme. The completion of digestion was confirmed by TLC. *S* purified unsaturated uronate, dimer and trimer, *1* alginate, *2* polyM-block, *3* polyG-block, *4* polyMG-block

lyase did not show any activity toward chondroitin B, although the lyase resembles chondroitin B lyase based on their 3-D structure and homology modeling (see Fig. 7 and "Discussion" section).

The degradation products of alginate, polyM-, polyG-, and polyMG-blocks by the recombinant KJ-2 lyase were analyzed on TLC with Biogel P-2 column-purified unsaturated oligouronates, dimer, and trimer as standards (Fig. 4b). The degradation product mixture of polyMG-block consisted of dimer, trimer, and tetramer exhibited the corresponding spots on TLC plate, whereas most of the reaction mixture from alginate, polyG-, and polyM-blocks remained at original spot. Based on the aforementioned results, we assumed that the KJ-2 alginate lyase is a polyMG-specific lyase that showed higher preference to polyMG-block as the substrate. The degraded oligomeric products of polyMG-block were separated by Biogel P-2 gel filtration chromatography and confirmed by using FPLC and ESI-MS (data not shown).

^{1}H-NMR analysis of substrate degradation patterns by KJ-2 alginate lyase

The degradation profiles of alginate by the KJ-2 alginate lyase were compared with polyM- and polyG-specific alginate lyases by using ^{1}H-NMR spectroscopy (Fig. 5). In the case of polyM-specific lyase, $M_{red\text{-}\beta}$ reducing end signal (4.91 ppm) newly appeared after alginate degradation. $G_{red\text{-}\beta}$ reducing end signals (4.90–4.86 ppm) appeared after alginate degradation by polyG-specific alginate lyase. In contrast, there were mixed signals of $M_{red\text{-}\beta}$ reducing end signal and $G_{red\text{-}\beta}$ reducing end signals after alginate

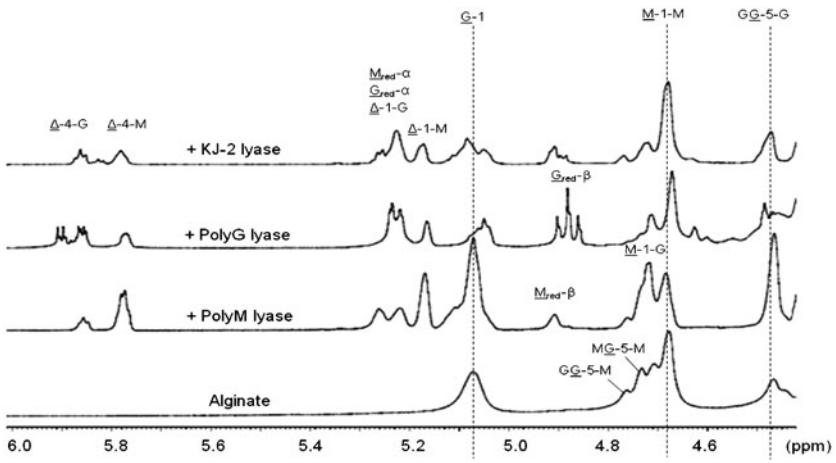

Fig. 5 Comparison of ^{1}H-NMR (400 MHz) spectra of alginate degradation before and after treatment with polyM-, polyG-, and KJ-2 polyMG-specific alginate lyases. PolyM- and polyG-specific lyases were originated from *Pseudomonas* sp. KS-408 and *Streptomyces* sp. ALG-5, respectively (Kam et al. 2011; Kim et al. 2009). The reaction of alginate degradation was allowed to proceed to completion by adding enzymes repeatedly. The signals originated from the underlined residues. The numbers denote protons causing the corresponding signals. M or G without underline indicates neighbor residue

degradation by KJ-2 alginate lyase. The peaks for polyM-block (M-1-M peak, 4.68 ppm) and polyG-block (GG-5-G peak, 4.47 ppm) remained. Therefore, KJ-2 alginate lyase is expected to produce mixed reducing ends derived from mannuronate reducing end and guluronate reducing end due to a polyMG-specific lyase activity.

We prepared polyM-, polyG-, or polyMG-blocks for the degradation experiments using KJ-2 alginate lyase to confirm the substrate specificity (Table 1). Each substrate was degraded with KJ-2 lyase and then analyzed by NMR spectroscopy. When polyG-block was treated with KJ-2 alginate lyase, there was almost no change in NMR spectrum (Fig. 6b). This indicates that KJ-2 alginate lyase do not have polyG-specific lyase activity. Little change in NMR spectrum occurred when polyM-block was treated with KJ-2 alginate lyase. There was a little decrease in MG-5-M (4.74 ppm) and M-1-G (4.71 ppm), indicating that KJ-2 alginate lyase cleaved the glycosidic bonds behind M or G residue in MGM fraction (F_{MGM}) of polyM-block (Fig. 6c). However, when polyMG-block was treated with KJ-2 alginate lyase, drastic changes occurred in NMR spectrum (Fig. 6a). The internal G signal (5.09 ppm) of polyMG-block decreased after treatment with KJ-2 alginate lyase, indicating that KJ-2 alginate lyase degraded polyMG-block in front of internal G residue to generate Δ residue. The peak intensities of M-1-G (4.71 ppm) and MG-5-M (4.74 ppm) also decreased due to the degradation. When M–G or G–M bonds in polyMG-block were cleaved, M reducing end (M_{red}) or G reducing end (G_{red}) were produced. Figure 6a clearly showed that KJ-2 alginate lyase preferred to cleave M–G bond rather than G–M bond based on high peak intensity of M reducing end (M_{red}). However, when excess amount of KJ-2 alginate lyase was added to polyMG substrate, substantial signal of G reducing end (G_{red}) was also detected (data not shown). All of these results supported that KJ-2 alginate lyase is a polyMG-specific lyase that preferentially degrades the glycosidic bond of M–G linkage in polyMG-block.

Discussion

When the amino acid sequence of KJ-2 polyMG-specific alginate lyase was retrieved in Superfamily database (Wilson et al. 2007), KJ-2 alginate lyase is predicted to be included in the pectin lyase-like protein superfamily and chondroitin B lyase family. The KJ-2 alginate lyase exhibited sequence identity of 27.8 and 30.1 % with

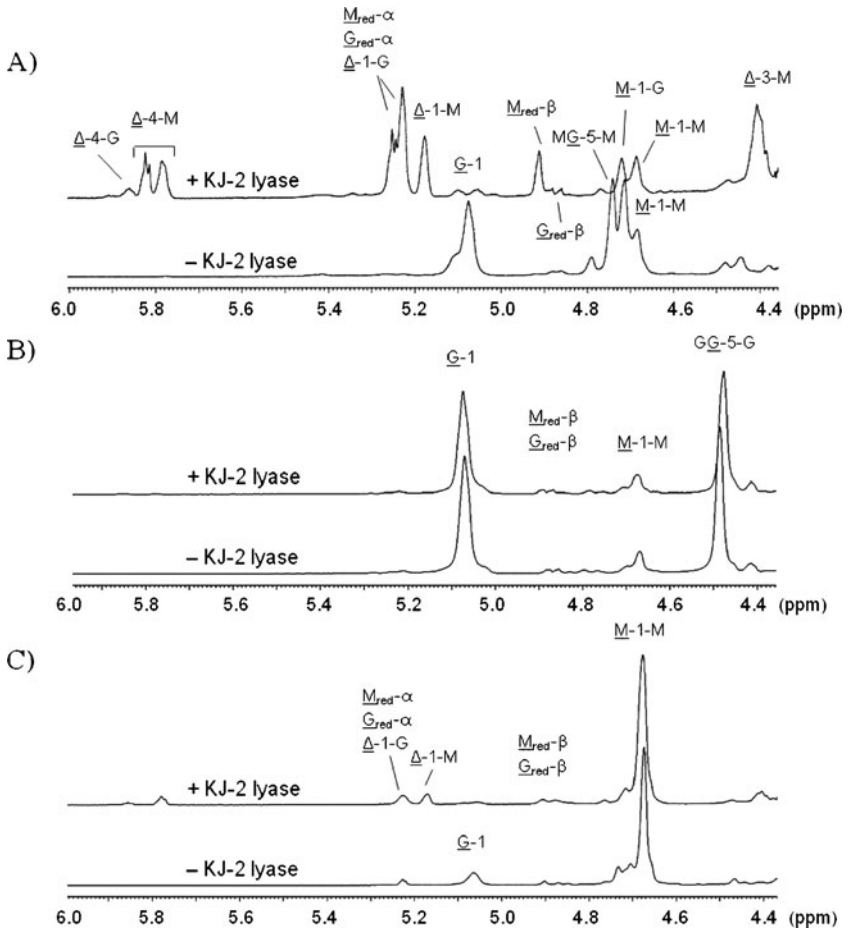

Fig. 6 ^{1}H-NMR (400 MHz) spectra of alginate substrates before and after treatment with *S. maltophilia* KJ-2 polyMG-specific alginate lyase. **a** PolyMG-block, **b** polyG-block, and **c** polyM-block. The signals originated from the underlined residues. The numbers denotes protons causing the corresponding signals. M or G without underline indicates neighbor residue. Panel above **a** shows the enlarged signal of $M_{red-\beta}$ and $G_{red-\beta}$

Azotobactor vinelandii AlgE4A module and *Pedobacter heparinus* chondroitin B lyase, respectively (Fig. 7). Chondroitin B lyase cleaves the β-(1–4)-linkage of dermatan sulfate in a random manner, yielding 4,5-unsaturated dermatan sulfate disaccharides as the product. The structure of AlgE4A consists of right-handed parallel β-helix made of four parallel β-strands with 12 complete turns and an amphipathic α-helix near the N-terminus like in polysaccharide lyase such as pectate lyase C (Rozeboom et al. 2008). On the contrary, chondroitin B lyase possesses β-helix turns made of three parallel β-strand and short turns (Michel et al. 2004).

A homology model of KJ-2 alginate lyase was constructed based on the crystal structure of chondroitin B lyase from *Pedobacter heparinus* (data not shown). When the constructed active site of KJ-2 alginate lyase was superimposed on the crystal structure of chondroitin B lyase, the catalytic active site was similar with each other. In the catalysis mechanism of chondroitin B lyase, Ca^{2+} ion binds the carboxylate of the substrate to enhance the efficiency of proton abstraction by lowering the pK_a of H5-proton. When Ca^{2+} ion was removed, chondroitin B lyase lost its activity. In the case of the KJ-2 alginate lyase, it also lost lyase activity when Ca^{2+} ion was removed by EDTA (Table 2; Fig. 3). However, KJ-2 alginate lyase did not show any activity toward chondroitin substrate (Fig. 4a), although the predicted structure of KJ-2 alginate lyase based on homology modeling is similar with that of chondroitin B lyase. This discrepancy needs to be elucidated.

Up to date, a number of endolytic polyM- and polyG-specific lyases have been identified (Kim et al. 2011). However, there have been a few reports on polyMG-specific lyases so far. *Pseudomonas aeruginosa* PA1167 polyMG-specific lyase revealed to be included in the PL-7 family (Yamasaki et al. 2004). It exhibited preferential β-elimination activity towards polyMG-block (100 %) and then followed by polyM-block (16.8 %) and polyG-block (1.83 %). The KJ-2 alginate lyase showed similar patterns of substrate specificity, even though they did not share high sequence similarity. Recently, new polyMG-specific alginate lyases (AlyA1, AlyA2, and AlyA3) involved in cyst germination in *A. vinelandii* were identified and classified as PL-7 family (Gimmestad et al. 2009). Based on NMR study, the *A. vinelandii* polyMG-specific lyase was expected to cleave the glycosidic bond in G–M linkage. Ca^{2+} ion is required for the activity of *A. vinelandii* polyMG-specific lyase. The KJ-2 and *A. vinelandii* polyMG-specific alginate lyases have the same requirement of Ca^{2+} ion for their activity, but their structures do not share much in common. Interestingly, the KJ-2 polyMG-specific alginate lyase shares much structural similarity with chondroitin B lyase based on comparative homology modeling analysis, and it

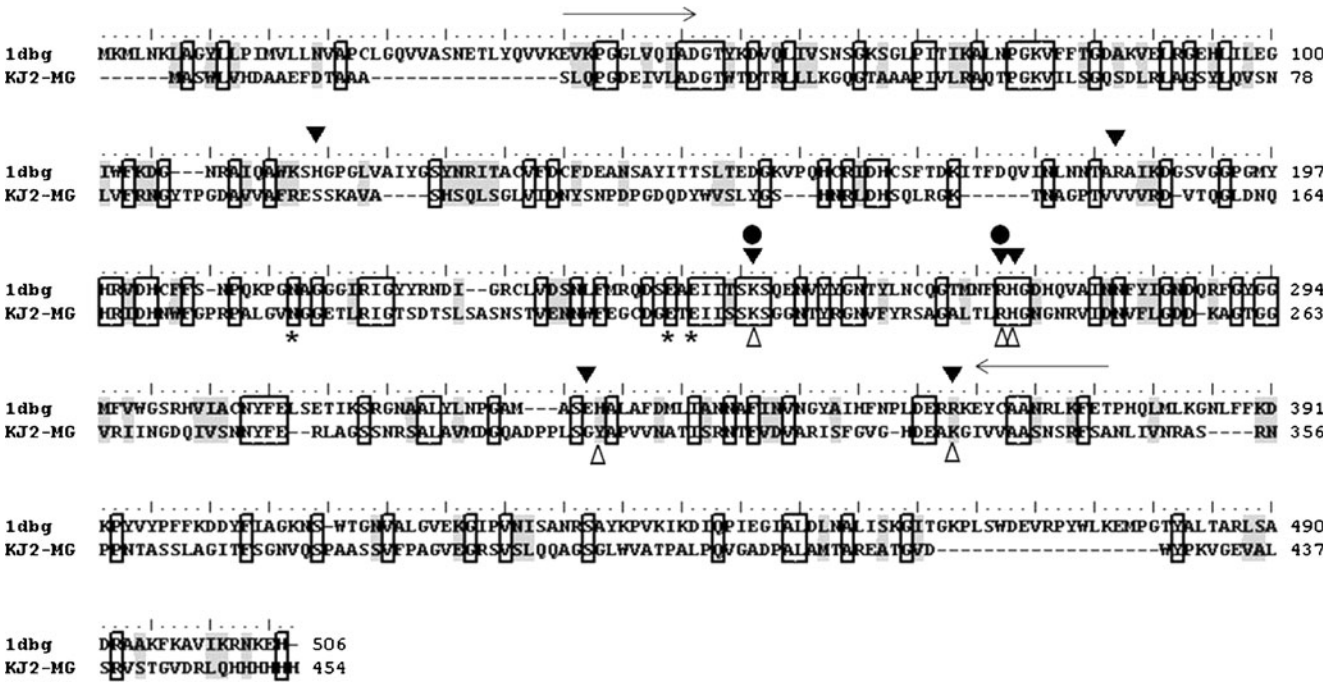

Fig. 7 Protein sequence alignment of *S. maltophilia* KJ-2 polyMG-specific alginate lyase and chondroitin B lyase (1dbg.pdb) from *Pedobacter heparinus* using position-specific iterated blastp for the construction of homology modeling. Identical amino acid residues are outlined, and amino acid residues above 70-% consensus are boxed in pale shade. The *asterisk*, *filled circle*, and *filled inverted triangle* indicate the proposed calcium binding site, catalytic site, and substrate interacting site for chondroitin B lyase, respectively. *Δ* indicates the residue that can play an important role in KJ-2 alginate lyase. Alignment from 18 aa to 345 aa (indicated by *arrows*) was carried out by using position-specified iterated blastp (PSI-blastp) for homology modeling

does not exhibit chondroitin lyase activity but polyMG-specific lyase activity. The structure and function relationship of the KJ-2 polyMG-specific alginate lyase needs to be elucidated by using X-ray crystallography.

As a conclusion, in this study, we identified, cloned, and characterized a novel polyMG-specific alginate lyase from *S. maltophilia* KJ-2. The KJ-2 polyMG-specific alginate lyase was elucidated to preferably degrade the glycosidic bond in M–G linkage of MG blocks in alginate. In order to develop an efficient saccharification of alginate for the production of biofuels and biochemicals, various recombinant alginate lyases possessing different substrate specificities need to be developed because alginate consists of three blocks such as polyM-, polyG-, and polyMG-blocks. The KJ-2 polyMG-specific alginate lyase can be used in combination with other alginate lyases for a synergistic saccharification of alginate.

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