Functional Heterologous Expression and Characterization of Mannuronan C5-Epimerase from the Brown Alga *Saccharina japonica*

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Abstract

Brown algae produce alginate that has various ratios and diverse sequences of two uronic acids, β-D-mannuronic acid and α-L-guluronic acid, compared with those of alginate produced by bacteria. This diversity of alginate in brown algae is caused by mannuronan C5-epimerases (MC5Es), which catalyze the conversion of β-D-mannuronic acid to α-L-guluronic acid. Although several bacterial MC5E enzymes have been well characterized, to date, there exists no information on the biochemical properties of eukaryotic MC5E. In this study, MC5E expression was detected in a brown alga *Saccharina japonica* sporophyte by immunoblot analysis. We also searched for MC5E mRNA from *S. japonica* by RT-PCR and revealed eight partial amino acid sequences, SjC5-I to -VIII. We focused on the highest frequency clone, SjC5-VI, and elucidated its full-length cDNA and putative gene structure. The translated SjC5-VI protein consists of 499 amino acids, with the N-terminal 21 amino acids predicted as a secretion signal sequence. Functional recombinant SjC5-VI (rSjC5-VI) was successfully expressed as a secreted protein using an insect-cell expression system, and we determined the optimal temperature, pH, and NaCl concentrations to be 35°C, 7.0–8.2, and 300 mM, respectively, using the Ca^{2+}-induced gel-formation assay. In addition, Ca^{2+} enhanced gelation by 1.7-fold following rSjC5-VI activity. Furthermore, ^{1}H-NMR spectroscopy of rSjC5-VI-treated polyM revealed alternate epimerization of β-D-mannuronic acid to

α-L-guluronic acid. To the best of our knowledge, this is the first report on the characterization of MC5E activity in eukaryotes.

1. Introduction

Alginate is a linear heteropolysaccharide consisting of β-D-mannuronic acid (M) and α-L-guluronic acid (G) [1-3]. Sequences of these uronic acids in alginate are comprised of three blocks consisting of continuous M or G residues (an M- or G-block) and randomly arranged M and G residues (an MG-block). Alginate from kelp is used in a variety of industries, including food processing, textile, paper, cosmetics, and pharmaceuticals [4]. Alginate is also the most abundant marine biomass and low-cost material [5,6]. Its extensive application is supported by its remarkable properties and functions: 1) it is safe and exhibits low toxicity levels [7]; 2) it displays viscous behavior in solution [4]; 3) it undergoes gelation in the presence of divalent cations; alginate with high G content exhibits increased affinity for divalent metals, such as Ca^{2+}, Sr^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+}, and Cd^{2+}, and strong gelation in accordance with the egg-box model [8-10]; and 4) alginate-formed gel is stable at high temperatures [11].

Brown algae and some kinds of bacteria, namely *Pseudomonas* spp. and *Azotobacter vinelandii*, are major organisms having an alginate biosynthesis system [1,12-15]. Biosynthetic pathways in brown

algae and bacteria are fundamentally considered to be common, except that alginate produced by bacteria is partially acetylated (Fig. 1) [15,16]. Fructose-6-phosphate derived from fructose is a starting material for alginate synthesis, and is enzymatically converted to mannose-6-phosphate, mannose-1-phosphate, GDP-mannose, and GDP-mannuronate, in this order. GDP-mannuronate is polymerized to poly-mannuronate, followed by conversion by mannuronan C5-epimerase (MC5E) of certain forms of M to G. The M/G ratio in alginate is dependent upon MC5E activity and attributable to its mechanical properties and affinity for divalent metal ions. In some bacteria, but not brown algae, alginate is partially acetylated at O-2 and/or O-3 following epimerization [17].

MC5E structure and function are well characterized only in bacteria, i.e., *Pseudomonas aeruginosa* and *A. vinelandii*, which produce alginate as a major component of biofilm. *P. aeruginosa* has one MC5E (AlgG) [18], which is localized in the periplasm. In contrast, seven MC5Es (AlgE1–7) are found as secreted proteins in *A. vinelandii* [19,20]. The epimerization activities of AlgG and AlgE1–7 have been studied, revealing that each enzyme is capable of various patterns of epimerization of M to G. AlgE7 was the only variant identified as a bifunctional enzyme having not only MC5E activity, but also alginate lyase activity [21]. Previous studies reported that alginates produced by *Pseudomonas* spp., such as *P. aeruginosa, P. mendocina, P. putida, and P. fluorescens*, lack G-blocks, while *A. vinelandii*

produced alginate showing high G-block content [17,22]. This difference is considered to be closely related to the number of MC5Es and their respective epimerase activities, as the presence of G-blocks ~~cause alternations in~~ determine alginate physicochemical properties. The rigidity of alginate M/G blocks was reported to increase based on the order MG < MM < GG [23-25]. Also, alginate with G-blocks promotes gel formation in the presence of divalent metals [8-10].

In brown algae, the M/G ratio and the composition of blocks consisting of these residues varies based on several factors, including species, portion (blade, stipe, and rhizoid), season, growth conditions, and habitat [26-28]. These results implied great diversity in how alginate epimerization occurs in brown algae. Therefore, much attention has been focused on expression and enzymatic characterization of brown algal MC5E(s). Several approaches to elucidate brown algal MC5Es at the gene level have been reported. Genomic analysis of brown algae *Ectocarpus siliculosus* and *Saccharina japonica* revealed 45 and 105 MC5E-candidate genes, respectively [15,29]. Furthermore, in *Laminaria digitata*, six different mRNAs for MC5Es were fully or partially cloned, and multiple bands were detected by Southern blot analysis using a specific probe for *L. digitata* MC5E [30]. *De novo* transcriptome analysis of *Undaria pinnatifida* gametophytes suggested that 31 genes might encode MC5E [31]. Intriguingly, these results suggested the potential existence of multiple MC5E genes in brown algae. Therefore, it is possible that brown algae can

produce alginate that has a variety of M/G sequences and ratios compared to bacterial alginate. Determining the roles of these alginate sequences through the activity of MC5E isozymes is a key to understanding the variations in alginate biosynthesis in brown algae. However, no biochemical characterization of purified brown algal MC5E currently exists [32], and previous attempts to produce *L. digitata* MC5E using either *Escherichia coli* or *Pichia pastoris* as expression systems failed [30].

Here, we report the successful expression of recombinant MC5E from brown alga *S. japonica* and its biochemical characterization. This is the first functional analysis of the purified eukaryotic MC5E enzyme.

2. Material and methods

2.1. Materials

Sodium alginate 80–120 cP was purchased from Wako Pure Chemical Industries (Osaka, Japan). PolyM and polyG was prepared from alginate using the method described by Gacesa and Wusteman [33]. Other reagents were purchased from Wako Pure Chemical Industries unless indicated otherwise.

2.2. Vectors and host cells

Cloning vectors pTac-1 and pCR-XL-TOPO were purchased from BioDynamics (Tokyo, Japan) and Life Technologies (Carlsbad, CA, USA), respectively. *E. coli* DH5α (Takara, Shiga, Japan) or TOP10 (Life Technologies) cells were used as the host strain.

The pFastBac1 vector and *E. coli* DH10Bac (Life Technologies) were used to produce recombinant baculovirus with the Bac-to-Bac expression system (Life Technologies). Recombinant protein was expressed in Sf9 insect cells (Life Technologies). PSFM-J1 Medium Wako (Wako Pure Chemical Industries) was used for the cell cultures.

2.3. Protein extraction from *S. japonica*

S. japonica sporophyte was harvested at the shore of Hakodate Bay, Hokkaido, Japan, in June 2012, and measured ~70 cm in length. After freezing in liquid nitrogen, the blade, stipe, and rhizoid were crushed with a grater, followed by protein extraction using a phenol-extraction protocol reported previously [34].

2.4. Preparation of anti-MC5E antibodies

A peptide, N-CNGVSIQGNEVY-C, resembling a conserved amino acid sequence from *L. digitata* MC5Es ManC5-E1–E6 (GenBank accession nos. AJ496449–AJ496454) [30], was conjugated to keyhole limpet hemocyanin (KLH; Sigma-Aldrich, St. Louis, MO, USA). Antibodies were prepared by

injecting rabbits with the KLH-conjugated peptide. Anti-MC5E antibody was purified by affinity chromatography using an immobilized antigen formyl-cellulofine column (Seikagaku Kogyo, Tokyo, Japan).

2.5. SDS-PAGE analysis

SDS-PAGE was conducted using a 10% gel containing 0.1% SDS according to the method described by Porzio and Pearson [35]. After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, and the background of the gel was destained with 5% methanol and 7% acetic acid. Protein Marker, Broad Range (2–212 kDa; New England Biolabs, Ipswich, MA, USA) was used as a molecular weight marker.

2.6. Western blot analysis

Samples were electroblotted and transferred onto a nitrocellulose membrane (Atto, Tokyo, Japan) after separation by SDS-PAGE. The anti-MC5E antibody was used as the primary antibody, followed by administration of horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (Sigma-Aldrich). To detect the His-tag, horseradish peroxidase-conjugated anti-His-tag antibody (Anti-His-tag mAb-HRP-DirecT, Medical and Biological Laboratories, Nagoya, Japan) was used. Signal

was detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

2.7. Total RNA or genomic DNA extraction from *S. japonica*

S. japonica sporophyte (~70 cm) was harvested at the shore of Hakodate Bay, Hokkaido, Japan, in June 2007. Thallus was applied to isolate protoplasts as described previously [36,37]. Total RNA was extracted from protoplasts using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was extracted from protoplasts using an ISOHAIR kit (Nippon Gene, Tokyo, Japan) according to manufacturer instructions.

2.8. Cloning of *S. japonica* MC5E cDNAs

First-strand cDNA was synthesized by incubating 50 ng of total RNA and 50 pmol of oligo dT primer with PrimeScript Reverse Transcriptase (Clontech, Mountain View, CA, USA) at 42°C for 90 min.

A partial cDNA sequence for MC5E was amplified using the primers C5Dg-F and C5Dg-R (Table 1), which were designed based on the conserved amino acid sequences from *L. digitata* MC5Es. RT-PCR was conducted using TaKaRa *Ex Taq* DNA polymerase (Takara) under the following conditions: 30 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 90 s. Amplified DNA was sub-cloned

into plasmid vector pTac-1 and sequenced using a 3130xl Genetic Sequence Analyzer (Applied Biosystems, Foster City, CA, USA).

5′ - and 3′ -rapid amplification of cDNA ends (RACE) was carried out using the specific primers C5-3F1 and C5-3F2 for 3′ -RACE and C5-5F1 and C5-3F2 for 5′ -RACE (Table 1) with the SMART RACE cDNA Amplification Kit (Clontech) according to manufacturer instructions. Sub-cloning and DNA sequencing was conducted as previously described.

The full-length cDNA of SjC5-VI was amplified using a specific primer set for C5Full-F and C5Full-R (Table 1) specific to the 5′ - and 3′ -untranslated regions with Phusion High-Fidelity DNA Polymerase (New England Biolabs) under the following conditions: 30 cycles at 98°C for 15 s, 60°C for 30 s, and 72°C for 60 s. Amplified DNA was treated with A-attachment mix (Toyobo, Osaka, Japan), sub-cloned into the pTac-1 vector, and sequenced as previously described. The sequence data is available from the DNA Data Bank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL), and GenBank with accession number AB299380.

2.9. Genomic PCR for the structural gene

Genomic PCR was carried out using the same method as that described for full-length cDNA amplification, except that genomic DNA was used as the template and the elongation time was set to 10

min. Amplified DNA was sequenced after sub-cloning into a pCR-XL-TOPO vector (Life Technologies). The sequence data is available from the DDBJ, EMBL, and GenBank with accession number LC053765.

2.10. Computational analysis of the signal peptide

The presence of a signal peptide and its cleavage sites were predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) [38].

2.11. Baculovirus expression and purification of recombinant MC5E

The cDNA for SjC5-VI was used to express the functional enzyme in insect cells using a modified pFastBac1 vector designed to fuse a His-tag at the C-terminus of the expressed protein as previously described [37]. The nucleotide sequence encoding the predicted signal peptide in the N-terminal sequence (residues 1–21) was replaced in order to express as secreted recombinant proteins with a honeybee mellitin signal sequence (MKFLVNVALVFMVVYISYIYA) [39,40]. Additionally, the recombinant protein was designed to have both a c-myc-tag (EQKLISEEDL), as well as the 8xHis-tag at the C-terminus. The DNA was amplified using the primers C5-pFB-infF and C5-pFB-infR (Table 1), and cloned between *BamH*I/*Xho*I restriction sites in the modified pFastBac1 vector [37] using the In-Fusion HD Cloning Kit (Takara). Bacmid DNA was prepared from the transformed DH10Bac cells using the

plasmid containing the gene sequence for the recombinant enzyme. Recombinant baculovirus was produced by the Bac-to-Bac system according to manufacturer instructions and stored at 4°C until use.

Sf9 cells were infected with recombinant baculovirus, with a multiplicity of infection of ~10. Cells were cultured in a spinner flask agitated by pump impeller rotation at 17°C for 5 days. The medium was retrieved by centrifugation at 3,000 × *g* for 15 min, and was dialyzed against a buffer containing 20 mM NaPi (pH 8.0) and 0.1 M NaCl. After ammonium sulfate precipitation at 80% saturation, precipitated proteins were dissolved in a buffer containing 10 mM imidazole (pH 7.4) and 0.1 M NaCl, and 300 µL of Ni-NTA agarose resin (Qiagen) was mixed in a conical tube. After incubation on a rotating wheel for 30 min at 4°C, resin suspension was loaded into a disposable column. Resin was washed with a 20-fold volume of 30 mM imidazole (pH 7.4) and 0.1 M NaCl. Protein was eluted with 150 mM imidazole (pH 7.4) and 0.1 M NaCl. After SDS-PAGE analysis, fractions containing the target protein were unified and dialyzed at 4°C for 8 h against 10 mM imidazole-HCl (pH 7.4) and 0.1 M NaCl. Protein concentration was determined by Bradford assay [41] using bovine serum albumin BSA as a standard.

Ca^{2+}-induced gel precipitation assay—Enzyme reactions were carried out in a buffer containing 10 mM imidazole-HCl (pH 7.4), 0.5 M NaCl, 0.1 mg/mL BSA, 0.25% polyM, and 0.01 mg/mL rSjC5-VI at 20°C for 1 h unless described differently in the text. $CaCl_2$ was then added to each sample to a 100 mM final

concentration and mixed. The generated gels were precipitated by centrifugation at 12,000 × *g* for 15 min, the precipitates were washed with ethanol three times, and dissolved in distilled water after drying. The amount of precipitated alginate was measured by the carbazole assay [42,43] using the purchased sodium alginate as a standard.

2.12. ^{1}H-NMR spectroscopy of alginate

PolyM (0.25%) was incubated with rSjC5-VI (0.01 mg/mL) in buffer containing 10 mM imidazole-HCl (pH 8.0), 0.5 M NaCl, and 0.1 mg/mL BSA at 20°C for 12 h. BSA was added to stabilize for enzyme in its reaction and to lessen the loss of protein on tube and tip during manipulation. Then four volumes of cold ethanol was added to the reaction mixture. After centrifugation at 12,000 × *g* for 15 min, precipitates were washed with ethanol three times and dried up at 37ºC. Then, it was dissolved to a concentration of 5 mg/mL in deuterium oxide at pH 7.3. Untreated PolyM with rSjC5-VI was also prepared by the same method without rSjC5-VI. ^{1}H-NMR spectra were measured on a Varian Inova 500 NMR Spectrometer (500 MHz; Agilent Technologies, Santa Clara, CA, USA) equipped with a Varian triple resonance probe at 25°C. Proton chemical shifts were referenced against an internal standard of sodium 2,2-dimethyl-2-silapentane-t-sulfonate-*d*6, and the peaks were assigned according to previous reports [44-47]. Distribution of M and G units and the average length of G were evaluated as follows

[44,48-50]: the monomeric, dyadic, and triadic were calculated using the equations and *F* means fraction of subscript monomeric, dyadic, or triadic units

$F_G + F_M = 1$

$F_G = F_{GG} + F_{GM} = F_{GG} + F_{MG}$

$F_M = F_{MM} + F_{GM} = F_{MM} + F_{MG}$

$F_{MG} = F_{GM}$

$F_{GG} + F_{MM} + F_{GM} + F_{MG} = 1$

$F_G = F_{GGG} + F_{GGM} + F_{MGG} + F_{MGM}$

$F_{GGM} = F_{MGG}$

$F_{MG} = F_{GM} = F_{GGM} + F_{MGM}$

The average length of G ($N_{G>1}$) was calculated by the equation $N_{G>1} = (F_G\text{-}F_{MGM}) / F_{GGM}$.

3. Results

3.1. Immunoblotting analysis of *S. japonica* MC5E

No information was available regarding the *S. japonica* MC5E gene sequence and protein expression profile. However, the gene and translated amino acid sequences of six *L. digitata* MC5E

isoforms (ManC5-E1–E6) had been reported [30], indicating that their catalytic and neighboring residues were highly conserved. Specifically, the C-terminal residues 305–324 (HGIIASKRCNGVSIQGNEVY) from ManC5-E1 are conserved among all *L. digitata* MC5Es. In this study, we created an antibody against a 12-residues synthetic peptide from this C-terminal region and performed western blot analysis on proteins derived from the blade, stipe, and rhizoid of a *S. japonica* sporophyte.

As shown in Figure 2, multiple signals were detected in each sample. A 100-kDa protein was detected in all samples, with indistinguishable expression levels. Two proteins of 52- and 56-kDa were also detected; however, their expression levels differed between samples. These proteins were highly expressed in the rhizoid, but exhibited lower expression levels in the blade and stipe. Additionally, 66- and 80-kDa proteins were also detected in the rhizoid; however, these expression levels were not as strong in the blade and stipe. These results provided evidence of multiple MC5E genes in brown algae, similar to previous reports [30,31,51]. Additionally, our data suggested that these expression patterns observed in *S. japonica* were altered dependent upon the algal portion. Thus, several MC5E isozymes expressed in *S. japonica* were immunochemically identified.

3.2. cDNA cloning of *S. japonica* MC5E

Next, we obtained the putative amino acid sequences of the *S. japonica* MC5Es. A degenerate primer set (Table 1) for RT-PCR of the *S. japonica* MC5E gene was designed based on the common amino acid sequence found in *L. digitata* MC5Es. Amplified cDNAs were sub-cloned, and 50 recombinant plasmids were randomly picked and sequenced. Eight partial nucleotide sequences and their deduced amino acid sequences (SjC5-I–VIII) were revealed (Supplementary Fig. 1). Interestingly, the synthesized peptide sequence (CNGVSIQGNEVY) for the antibody previously described was conserved among all SjC5 variants. For this study, we have focused on SjC5-VI, because the frequency with which we found this DNA sequence was the highest (17/50) among all sequenced cDNA clones. To determine the full open reading frame (ORF) of SjC5-VI, 5′ - and 3′ -RACE was performed using specific primers (Table 1). A 1597-bp cDNA was ultimately obtained by RT-PCR using an additional primer set for the 5′ - and 3′ -untranslated regions. SjC5-VI consisted of 499 amino acids, with the 21 N-terminal residues predicted to be a secretion peptide signaling sequence (Fig. 3), and having a molecular weight calculated at 52,391 Da. According to BLAST analysis, the SjC5-VI amino acid sequence shared identity with those of brown algal MC5Es (Fig. 3). Of these, the highest identity (87%) was with *L. digitata* ManC5-E1 (LdManC5-E1). For bacterial MC5Es, the highest sequence homology was 15%, observed against AlgE4 from *A. vinelandii*. The Asp284 residue from SjC5-VI also constitutes a catalytic residue in

LdMacC5-E1 [30], and was conserved in a similar location as other brown algal MC5Es. Although its corresponding residue, Asp152, was located in the N-terminal region of AlgE4, its neighboring residues were relatively conserved between brown algal MC5Es and AlgE4. Of these, residues corresponding to Tyr149 and His152 were considered catalytic residues in AlgE4, with these residues also conserved in brown algae at Tyr281 and His286 in SjC5-VI. However, another identified catalytic residue, Asp178 in AlgE4, was replaced with a serine residue (Ser310 in SjC5-VI) at the corresponding location in all brown algal MC5Es.

3.3. SjC5-VI structural gene analysis

To determine SjC5-VI gene structure, genomic PCR was performed using similar primers to those previously mentioned (Table 1) for amplification of the full SjC5-VI ORF. The gene is 6803 bp and contains seven exons and six introns agreeing with the GT/AG rule (Fig. 4). This strongly supports that SjC5-VI was derived from *S. japonica* and not from bacteria containing an alginate biosynthesis system.

3.4. Baculoviral expression and purification of rSjC5-VI

Recombinant SjC5-VI (rSjC5-VI) was expressed as a secretion protein fused with an 8xHis-tag using Sf9 insect cells. We purified ~0.8 mg protein by Ni-affinity chromatography from 1-L medium after

culturing at 17°C for five days (Fig. 5A). Purified protein was cross-reacted with anti-His-tag and anti-MC5E antibodies (Fig. 5B).

3.5. MC5E enzyme activity of rSjC5-VI

A Ca^{2+}-induced gel precipitation assay was performed to evaluate the enzyme activity associated with epimerization from M to G in alginate. We first determined the validity of this method under conditions involving various concentrations of polyM and/or polyG (Fig. 6). In the presence of Ca^{2+}, alginate gelation was enhanced by increasing of the ratio of polyG to polyM. This indicated that intermolecular binding occurred through the formation of egg-box structures via Ca^{2+}-binding to G residues. Thus, alginate gelation was closely associated to G-content. Next, we used polyM as a substrate for rSjC5-VI in order to determine substrate-concentration dependence (Fig. 7A). In each tested condition, the amount of precipitated alginate increased following incubation with rSjC5-VI. This result indicated that gelation was enhanced by changes in the M/G ratio. The optimum *in vitro* polyM concentration was determined at 0.25%, with the amounts of precipitated gels reducing in the presence of ≥1% polyM (Fig. 7A). This is likely caused by the rise in reaction-mixture viscosity, limiting the movement of enzymes driven by Brownian motion. Therefore, the catalytic conditions used to investigate MC5E enzyme function included conditions of 0.25% polyM.

Figure 7B shows the results of a time-dependent assay at 20°C. The amount of precipitate increased sharply for the first 2 h, and reached the maximum level after 12 h.

The optimum temperature and pH were determined at 35°C and ~7.0–8.2, respectively (Fig. 8A and 8B). Addition of NaCl enhanced enzyme activity up to 300 mM, after which activity gradually declined (Fig. 8C). However, ~40% of the maximum activity still remained even in the presence of 1.0 M NaCl.

We also studied the effects of EDTA, metal ion (Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}, and Li^{2+}), or 2-mercaptoethanol (Fig. 8D). Of these, Ca^{2+} administration resulted in a 1.7-fold increase in rSjC5-VI activity, while other reagents did not fundamentally change enzyme activity.

The epimerization from M residue to G residue using polyM as a rSjC5-VI substrate were investigated by ^{1}H-NMR spectroscopy analysis of the products. ^{1}H-NMR spectroscopy following rSjC5-VI catalysis revealed evidence of M-to-G epimerization (Fig. 9). Prior to the enzyme reaction, the peak associated with MM-1 was substantial, while that of G-1 was smaller. After incubation with rSjC5-VI, the peak areas of G-1 and MG-1 increased, while that of MM-1 decreased. Additionally, GGG-5, MGG-5, G-3, and G-4 peaks appeared in the spectra following incubation. These data represented evidence that M residues were partially epimerized to G residues by rSjC5-VI.

Substrates become products so the chemical compostion should be referred to the reaction mixture. The chemical composition of substrates before and after incubation with rSjC5-VI were calculated (Table 2). As shown in Table 2, M and MM frequencies in polyM used as a substrate reduced from 0.91 to 0.48 (F_M), and from 0.83 to 0.05 (F_{MM}), respectively. However, F_G, $F_{GM,MG}$, and F_{MGM} increased following incubation with rSjC5-VI from 0.09, 0.08, and 0.07, to 0.52, 0.44, and 0.41, respectively. Nevertheless, dyads and triads containing at least two consecutive G residues, such as F_{GG}, F_{GGG}, $F_{GGM,}$ and F_{MGG} exhibited minimal increases. Additionally, the average length of G residues ($N_{G>1}$) increased slightly from 3.0 to 4.1. These results suggested that the main function of rSjC5-VI is alternately epimerized consecutive M residues into G residues (Fig. 10).

4. Discussion

First of all, we used an *E. coli* expression system with a cold-shock expression vector (induction at 15°C) to express functional SjC5-VI. Since *S. japonica* typically lives at temperatures ≤20°C, we expected native proteins to be more stable at lower temperatures. In this study, high-level expression of recombinant protein was confirmed by SDS-PAGE, although the expressed protein was insoluble (data not shown). Next, two SjC5-VI recombinant protein variants were then expressed in Sf9 insect cells using

a baculovirus expression system at 17°C, 23°C, or 28°C. One variant was a full-length protein and the other had an N-terminal truncation, lacking the predicted signal-peptide region. Under all conditions for both variants, each recombinant protein was expressed and localized to the cytoplasm, but was still insoluble, as in the case of bacterial expression (data not shown). In addition, even in the presence of the predicted signal peptide, we did not detect any recombinant protein as a secreted protein in a medium by western blot analysis. Finally, the 21 N-terminal residues from SjC5-VI were replaced with those of the signal sequence for honeybee melittin, resulting successful expression and secretion of functional rSjC5-VI (Fig. 5). The effect of varying culture temperatures on Sf9 cells was critical to obtaining rSjC5-VI as a soluble protein. The previously expressed proteins were insoluble protein aggregates in medium during culture at temperature ranges of 23–28°C (data not shown). Thus, our constructed baculoviral low-temperature expression system appears to be the only way to address the problem of heterologous expression of functional brown algal MC5Es.

Interestingly, rSjC5-VI displayed activity comparable to that observed at the habitat temperature (10–20°C) of *S. japonica* in nature, and at physiological pH and NaCl concentrations (Fig. 8A and B). These properties should be involved in the environmental conditions enabling rSjC5-VI epimerase activity, however, the *in vivo* localization of brown algal MC5Es, e.g., primary cell wall, middle lamella,

or outside of cells, is unclear. In *L. digitata*, MC5E proteins were found in the medium during protoplast culture [30], and MC5E activity was detected in the protoplast culture medium. Our results on temperature-, pH-, and NaCl-dependence, and the effects of divalent cations (Fig. 8), indicated that rSjC5-VI is capable of functioning in protoplast culture media, such as sea water.

Here, SjC5-VI was identified as a MC5E that incorporates an alternating M/G sequence into alginate (Figs. 9 and 10). Members of the order Laminariales have three distinct parts: blade, stipe, and rhizoid, each with different physical properties. The blade is generally flexible and soft, while the stipe and rhizoid are stiffer than the blade. Considering that SjC5-VI cDNA was isolated from protoplasts derived from the blade of *S. japonica*, it is, therefore, logical that cDNA encoding an MC5E capable of introducing MG-blocks into alginate by incorporating alternating M/G sequences was the most frequent clone. This is because alginate containing larger proportions of MG-blocks show lower rigidity than polyM- and polyG-blocks [23-25]. However, alginate from the blade of brown algae contains not only MG-blocks, but also polyM- and polyG-blocks [44]. Therefore, SjC5-VI may be able to promote G-block incorporation into alginate if unidentified MC5E(s) that recognize M moieties in the alternating sequence created by SjC5-VI specifically perform epimerization. This is considered a potentially effective procedure for G-blocks incorporation, rather than by epimerization into the sequence from one of the ends.

An M-blocks constitutes an unepimerized region in alginate, suggesting that unidentified polyM-binding protein(s) may protect against MC5E attack *in vivo*; however, this remains unclear.

In this study, the expression of multiple MC5Es in *S. japonica* was revealed using a specific antibody (Fig. 2B). These molecular weights were estimated to be 52–100 kDa on SDS-PAGE. According to genomic analysis of *E. siliculosus* [52], the calculated molecular weights of amino acid sequences from the predicted MC5E genes were in the range of 49–92 kDa. Our study also agreed with these views. Interestingly, isozyme expression patterns were different among blade, stipe, and rhizoid (Fig. 2B). This result is thought to be closely related to the fact that M/G ratios of alginates varied dependent on the portion of brown algae. Expression of the smaller-sized bands (~52, ~56, ~66, and ~80 kDa) seemed to vary by tissue (Fig. 2B). Specifically, the 52- and 56-kDa proteins were abundant in stipe, indicating that these proteins might be key enzymes involved in producing alginate with high G content. Therefore, MC5E epimerization through multiple pathways is relevant to the sequence diversity associated with M and G conformations in alginate from brown algae. However, given that proteins estimated at 100 kDa by SDS-PAGE reacted with the antibody for all tissue samples, this suggests that this protein may be a MC5E that is indispensable for alginate synthesis in brown algae. A MC5E AlgG-deleted *P. fluorescens* strain predominantly produced alginate dimers instead of polymers [53].

However, deficient AlgG activity resulting from point mutations did not disturb production of alginate polymers. This suggested that AlgG is required for not only epimerization, but also alginate polymer formation. Thus, it was hypothesized that AlgG formed a complex with other periplasmic proteins, such as AlgK, in order to facilitate the applicable alginate synthesis [53]. Therefore, the 100-kDa protein found in *S. japonica* may be essential to synthesize the alginate polymer, although its specific function remains unidentified.

Further algal MC5E research with this heterologous expression system may offer clues to address the remaining questions concerning alginate biosynthesis in brown algae, even after completion of brown algal genomic sequencing [29,52]. Genomic analysis of brown algae provided important insights into brown algal biology and evolutionary biology. However, candidate genes for alginate lyase were not observed in the *Ectocarpus* genome sequence [51]. Alginate-producing bacteria *P. aeruginosa* or *A. vinelandii* have a gene encoding alginate lyase located within a cluster of alginate-biosynthesis genes [54]. A possible explanation for the lack of alginate lyase in brown algae is that one or more MC5Es may exhibit functions similar to those of alginate lyase, as described previously [51]. During the lyase reaction at the M moiety at subsite +1 in the enzyme, the carboxylate anion is neutralized by a positively charged amino acid in the enzyme active site, leading to the abstraction of the C-5 proton by a general base [21].

Proton donation to the glycosidic oxygen from a catalytic amino acid then occurs to complete the lyase reaction. However, in the epimerase reaction, according to the proposed catalytic mechanism of AlgE4 [55], the protonated carboxylate of the M moiety in subsite +1 forms a hydrogen bond with Asp152 (or Asp178). Tyr149 is then deprotonated by Arg195 and abstracts the C-5 proton, which is replaced by a proton donated to C-5 from the other side of the substrate by His154. Of these residues, Tyr149, Asp152, and His154 were all conserved in the predicted catalytic domain of SjC5-VI (Fig. 3). Thus, these enzyme reactions appear very similar. Indeed, AlgE7 from *A. vinelandii* was identified as a bifunctional enzyme, exhibiting multiple catalytic mechanisms related to MC5E and alginate lyase, likely resident within the same active site [56]. These findings implied that *S. japonica* MC5Es potentially exhibited catalytic mechanisms necessary for alginate degradation. To address this hypothesis, each target protein should be expressed and investigated not only for epimerization, but also alginate degradation. The rSjC5-VI used in this study exhibited no alginate degradation activity based on analysis using thin-layer chromatography and the measurement of UV absorption of reaction products (data not shown).

5. Conclusions

Our results revealed that multiple MC5Es are expressed in brown alga *S. japonica*, and their expression patterns seem to vary dependent on the portion of algae. Furthermore, eight partial cDNAs for MC5Es were found. Of these, recombinant SjC5-VI were successfully expressed in insect cells. Purified recombinant SjC5-VI alternately epimerized M to G. This is the first report on the characterization of eukaryotic MC5E using purified protein. Our represented method is the only way for heterologous expression of functional brown alga MC5E so far. In the future, enumerating the expressed brown alga MC5Es and their characterization will clarify alginate epimerization at the molecular level in brown algae.

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Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Contributions

AI conceived and designed the study and wrote the paper. AI, AS, and YT performed DNA/RNA extraction, DNA cloning, and construction of the protein expression system. AI, MO, and TO performed the western blot analysis and enzyme assays. TM and MT performed NMR analysis.

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Figure legends

Fig. 1. Biosynthetic pathway of alginate and the alginate structure. (A) A proposed biosynthetic pathway for alginate in brown algae and bacteria. Catalytic enzymes are boxed. (B) The alginate components and their (1,4)-linked structures.

F6P = fructose 6-phosphate; M6P = mannose 6-phosphate; M1P = mannose 1-phosphate; GDP-Man = GDP-mannose; GDP-ManA = GDP-mannuronic acid.

Fig. 2. Western blot analysis of MC5E in *S. japonica*. (A) SDS-PAGE protein bands and (B) western blot using anti-MC5E antibody depicting the extracted protein from the blade (Blade), stipe (Stipe), and rhizoid (Rhizoid). Marker denotes protein ladder.

Fig. 3. Amino acid sequence homology between brown algal and bacterial MC5Es. Identical SjC5-VI residues are boxed in yellow. Arrowheads denote the predicted signal-peptide cleavage site. The dotted lines represent the antigen sequence used for anti-MC5E antibody production. Closed circles represent the putative catalytic Asp in *L. digitata* ManC5-E1 [30] and its corresponding residue in *A. vinelandii* AlgE4. Open circles show the conserved amino acids surrounding the catalytic Asp. Asterisks in black show four catalytic residues,

Tyr149, Asp152, His154, and Asp178, in AlgE4 [55]. Asterisks in red show the corresponding amino acids to the catalytic residues of AlgE4. Sequence names: SjC5-VI, *S. japonica* SjC5-VI (present study); LdManC5-E1, *L. digitata* ManC5-E1 (GenBank accession no. AJ496449); LdManC5-E6, *L. digitata* ManC5-E6 (GenBank accession no. AJ496454); EsMEP5, *E. siliculosus* MEP5 (GenBank accession no. CBJ33482); EsMEP5, *E. siliculosus* MEP6 (GenBank accession no. CBJ33483); EsMEP7, *E. siliculosus* MEP7 (GenBank accession no. CBJ33481), AlgE4, *A. vinelandii* MC5E (GenBank accession no. Q44493).

Fig. 4. Genomic structure of SjC5-VI. Exons and introns are shown by boxes and lines, respectively. Red and blue boxes show untranslated and translated regions, respectively. Initiation or termination codons are indicated by arrows.

Fig. 5. Purification of rSjC5-VI. (A) Schematic drawing of rSjC5-VI. Honeybee mellitin signal sequence (HMSS; MKFLVNVALVFMVVYISYIYA). A triangle shows the predicted cleavage site in the folded protein. (B) SDS-PAGE and western blot of purified rSjC5-VI. Marker and rSjC5-VI represent the protein ladder and purified rSjC5-VI, respectively. Western blot for purified rSjC5-VI was conducted using the anti-His-tag antibody (Anti-His-tag) and anti-MC5E antibody (Anti-MC5E), respectively. Arrows show the position of rSjC5-VI.

Fig. 6. Ca^{2+}-induced alginate gel precipitation assay. PolyM and polyG were mixed to various concentrations as indicated, where total concentration of each residue was 0.25%. Ca^{2+}-induced gels were precipitated by centrifugation and their concentration determined and calculated as a percentage of added polyM and/or polyG. Assays were performed in triplicate, and the data were shown as mean ± SD.

Fig. 7. PolyM concentration- and time-dependence for Ca^{2+}-induced gel formation following rSjC5-VI catalysis. (A) Determination of the optimal polyM concentration necessary for rSjC5-VI catalysis. Reactions were conducted in a solution containing 10 mM imidazole-HCl (pH 7.4), 0.5 M NaCl, 0.1 mg/mL BSA, 0.01 mg/mL rSjC5-VI, and 0.1–10% polyM at 20°C for 12 h. Inset shows gel precipitation following Ca^{2+} addition before (the open column) and after rSjC5-VI catalysis (the shaded column). Data are represented as a percentage of added polyM. The increments of the precipitates after the reaction were plotted as "ΔPrecipitated alginate". (B) Determination of the optimal reaction time for rSjC5-VI catalysis. Reactions were conducted in a solution containing 10 mM imidazole-HCl (pH 7.4), 0.5 M NaCl, 0.1 mg/mL BSA, 0.01 mg/mL rSjC5-VI, and 0.25% polyM at 20°C for each indicated time. The increments of Ca^{2+}-induced gel precipitation after the reaction were calculated and plotted. Assays were performed in triplicate, and the data were shown as mean ± SD.

Fig. 8. Biochemical characterization of rSjC5-VI. (A) The effects of temperature on rSjC5-VI catalysis. Assays were conducted in a solution containing 10 mM imidazole-HCl (pH 7.4), 0.5 M NaCl, 0.1 mg/mL BSA, 0.25% polyM, and 0.01 mg/mL rSjC5-VI for 1 h at the indicated temperature. (B) The effects of pH on rSjC5-VI catalysis. Assays were conducted in a buffer containing 10 mM sodium acetate (pH 4.2–5.3), 10 mM PIPES-NaOH (pH 6.1), 10 mM HEPES-NaOH (pH 7.0), 10 mM imidazole-HCl (pH 7.3 and 8.1), 10 mM Tris-HCl (pH 8.9), or 10 mM glycine-NaOH (pH 10.0), 0.5 M NaCl, 0.1 mg/mL BSA, 0.25% polyM, and 0.01 mg/mL rSjC5-VI at 20°C for 1 h. (C) The effects of NaCl concentration on rSjC5-VI catalysis. Assays were conducted in a solution containing 10 mM imidazole-HCl (pH 7.4), 0.1 mg/mL BSA, 0.25% polyM, 0.01 mg/mL rSjC5-VI, and 100–1000 mM NaCl at 20°C for 1 h. (D) The effects of EDTA, various divalent cations, and 2-mercaptoethanol on rSjC5-VI catalysis. Assays were conducted in a solution containing 10 mM imidazole-HCl (pH 7.4), 0.5 M NaCl, 0.1 mg/mL BSA, 0.25% polyM, 0.01 mg/mL rSjC5-VI, and 1 mM EDTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $SrCl_2$, 1 mM $BaCl_2$, 1 mM LiCl, or 5 mM 2-mercaptoethanol for 1 h at 20°C. In all experiments, assays were performed in triplicate, and the data were shown as mean ± SD. The increments of the Ca^{2+} precipitates after each reaction were calculated and plotted.

Fig. 9. ^{1}H-NMR analysis of polyM products following rSjC5-VI catalysis. ^{1}H-NMR spectra of polyM (A) before and (B) after incubation with rSjC5-VI. PolyM (0.25% relative to polyG) was incubated in a solution containing 10 mM imidazole-HCl (pH 7.4), 0.5 M NaCl, 0.1 mg/mL BSA, and 0.01 mg/mL rSjC5-VI at 20°C for 12 h. *G-1* refers to the anomeric proton of G residues. *GGM-5*, *GGG-5*, and *MGG-5* refer to the H-5 proton of the underlined G residue in the triads of GGM, GGG, and MGG, respectively. *MG-1* and *MM-1* refer to the anomeric proton of the underlined M residue in the dyads of MG and MM, respectively. *G-3* and *G-4* refer to the H-3 and H-4 proton of G residues, respectively.

Fig. 10. Schematic drawing of the SjC5-VI epimerization process. SjC5-VI preferentially epimerizes M residues (blue) in M-consecutive sequences to G residues (red) alternately. M residues next to G residues undergo minimal epimerization by SjC5-VI. As a result, MG-repeating sequences are rich in SiC5-VI-epimerized products, and M- and G-consecutive sequences appear locally.

Supplementary Fig. 1. Predicted amino acid sequences of SjC5-I–VII. Identical residues are boxed in yellow. The arrows indicate positions corresponding to used primers. A dotted line represents the antigen sequence for the anti-MC5E antibody. The frequency with which the specific sequence occurred within the 50 randomly picked colonies is shown at the end of each sequence.

Table 1. Primer sequences used in this study.

Name	Sequence
C5Dg-F	5′ -TGGMGNMGNAAYAARATGCA-3′
C5Dg-R	5′ -CNARNCCNGTRTTNCCNGACAT-3′
C5-3F1	5′ -CGGCTTCGACCCCCACGACGACAGCGAC-3′
C5-3F2	5′ -GACAACTACGTCCACGACAACGGCGACG-3′
C5-5R1	5′ - CGTCGCCGTTGTCGTGGACGTAGTTGTC-3′
C5-5R2	5′ - GTCGCTGTCGTCGTGGGGGTCGAAGCCG-3′
C5Full-F	5′ -CCGAGAAAGGCAGGGCGAGAGAGAGGCG-3′
C5Full-R	5′ -CAAGCAAGTGTGTAGCGTCTTA-3′
C5-pFB-infF	5′ - CATCTATGCCGGATCCGCAAACACGGCTTCGAGGAAC-3′
C5-pFB-infR	5′ -TAAGCTTTTGCTCGAGGCAAACAGGCTTGTAGGCAC-3′

Introduced restriction sites are underlined.

Table 2. Distribution of M and G units by rSjC5-VI.

	F_G	F_M	F_{GG}	F_{MM}	$F_{GM,MG}$	F_{GGG}	F_{MGM}	$F_{GGM,MGG}$	$N_{G>1}$
Before [a]	0.09	0.91	0.02	0.83	0.08	0.01	0.07	0.01	3.0
After [b]	0.52	0.48	0.08	0.05	0.44	0.06	0.41	0.03	4.1

[a] Substrate before incubation with rSjC5-VI.

[b] Substrate after incubation with rSjC5-VI at 20°C for 12 h.

M = β-D-mannuronic acid; G = α-L-guluronic acid.

Fig. 1

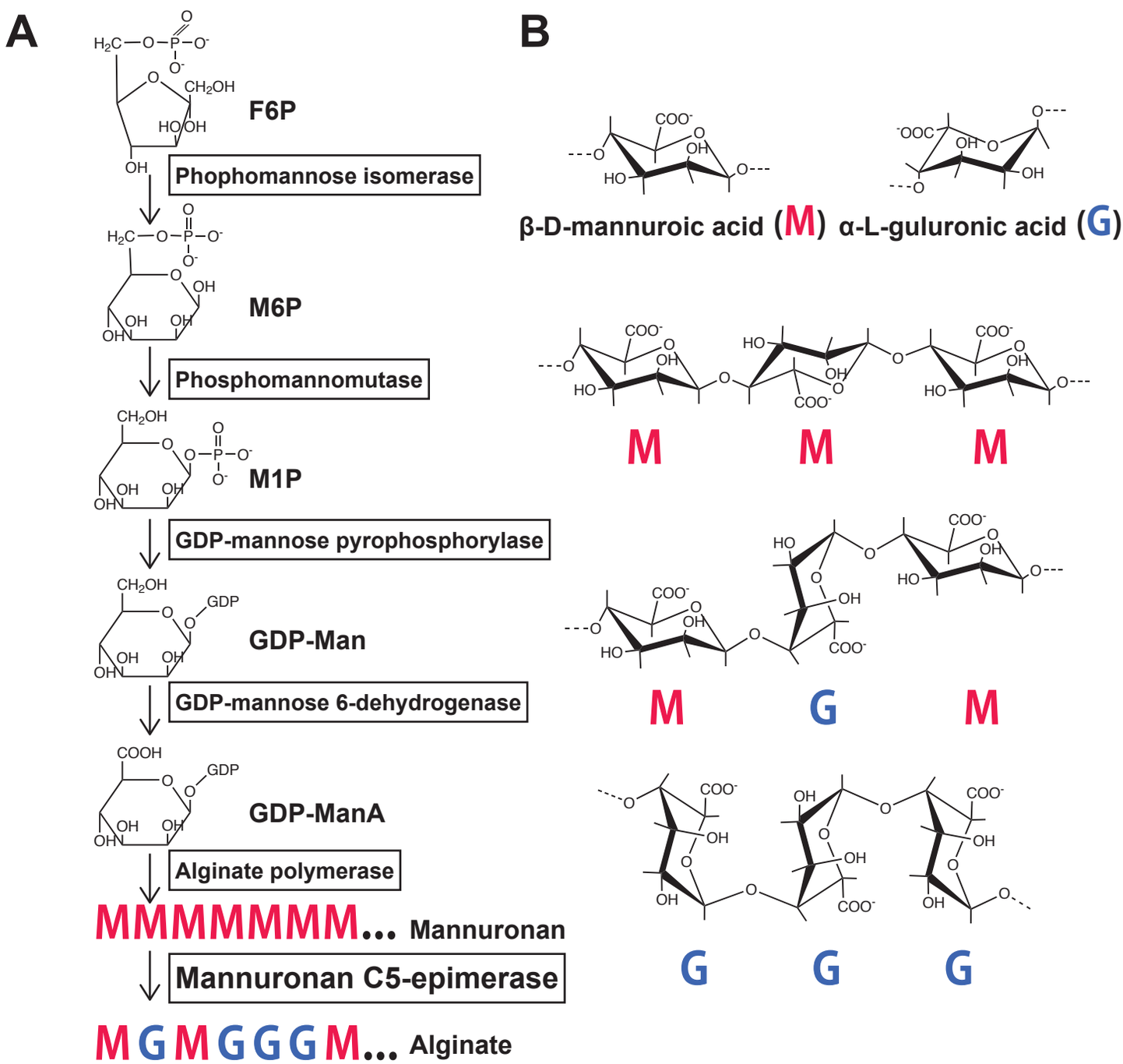

A
F6P
Phophomannose isomerase
M6P
Phosphomannomutase
M1P
GDP-mannose pyrophosphorylase
GDP-Man
GDP-mannose 6-dehydrogenase
GDP-ManA
Alginate polymerase
MMMMMMM... Mannuronan
Mannuronan C5-epimerase
MGMGGGM... Alginate
B
β-D-mannuroic acid (M)
α-L-guluronic acid (G)
M M M
M G M
G G G

Fig. 2

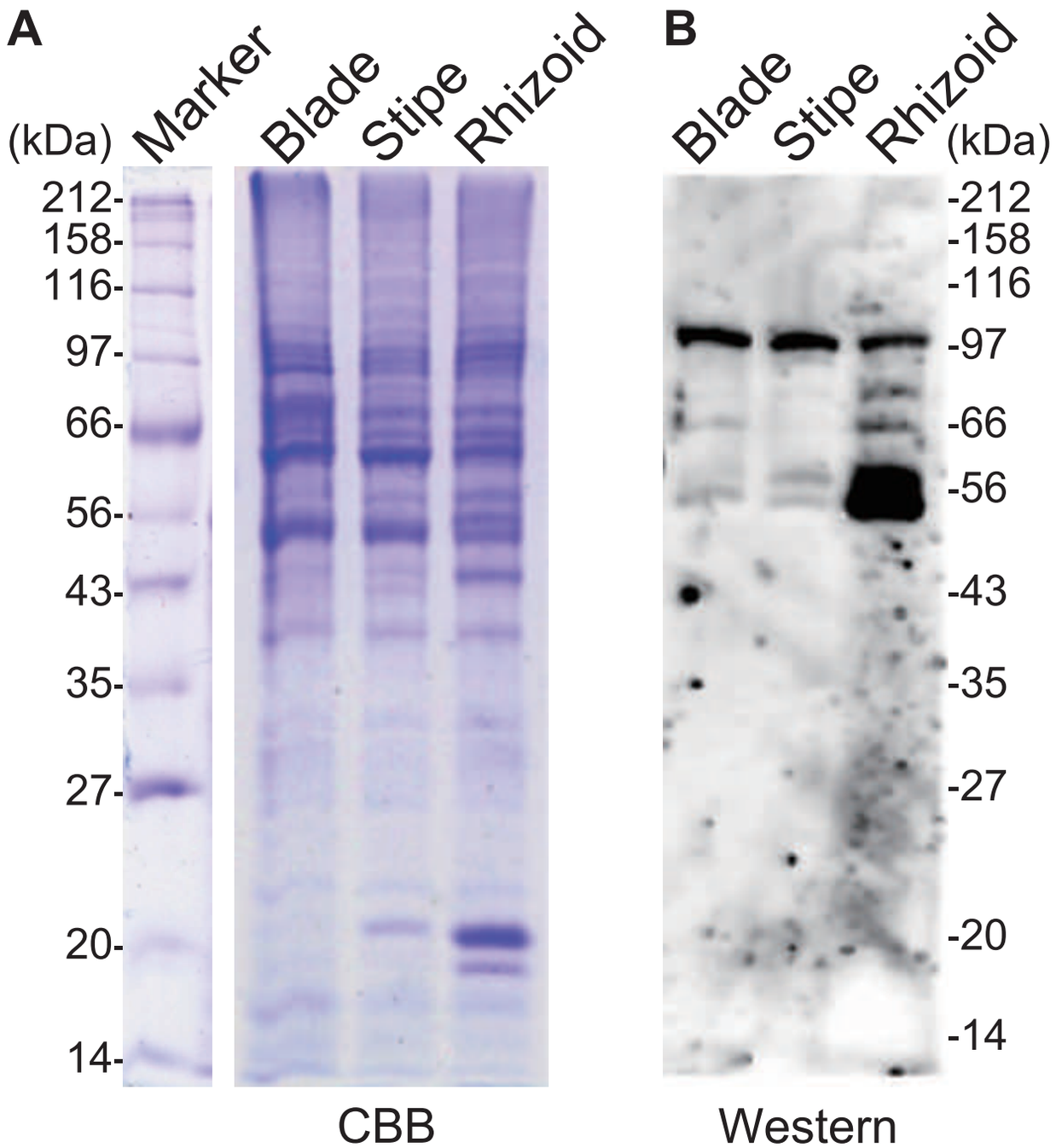

A
Marker
Blade
Stipe
Rhizoid
(kDa)
212-
158-
116-
97-
66-
56-
43-
35-
27-
20-
14-
CBB
B
Blade
Stipe
Rhizoid
(kDa)
-212
-158
-116
-97
-66
-56
-43
-35
-27
-20
-14
Western

Fig. 3

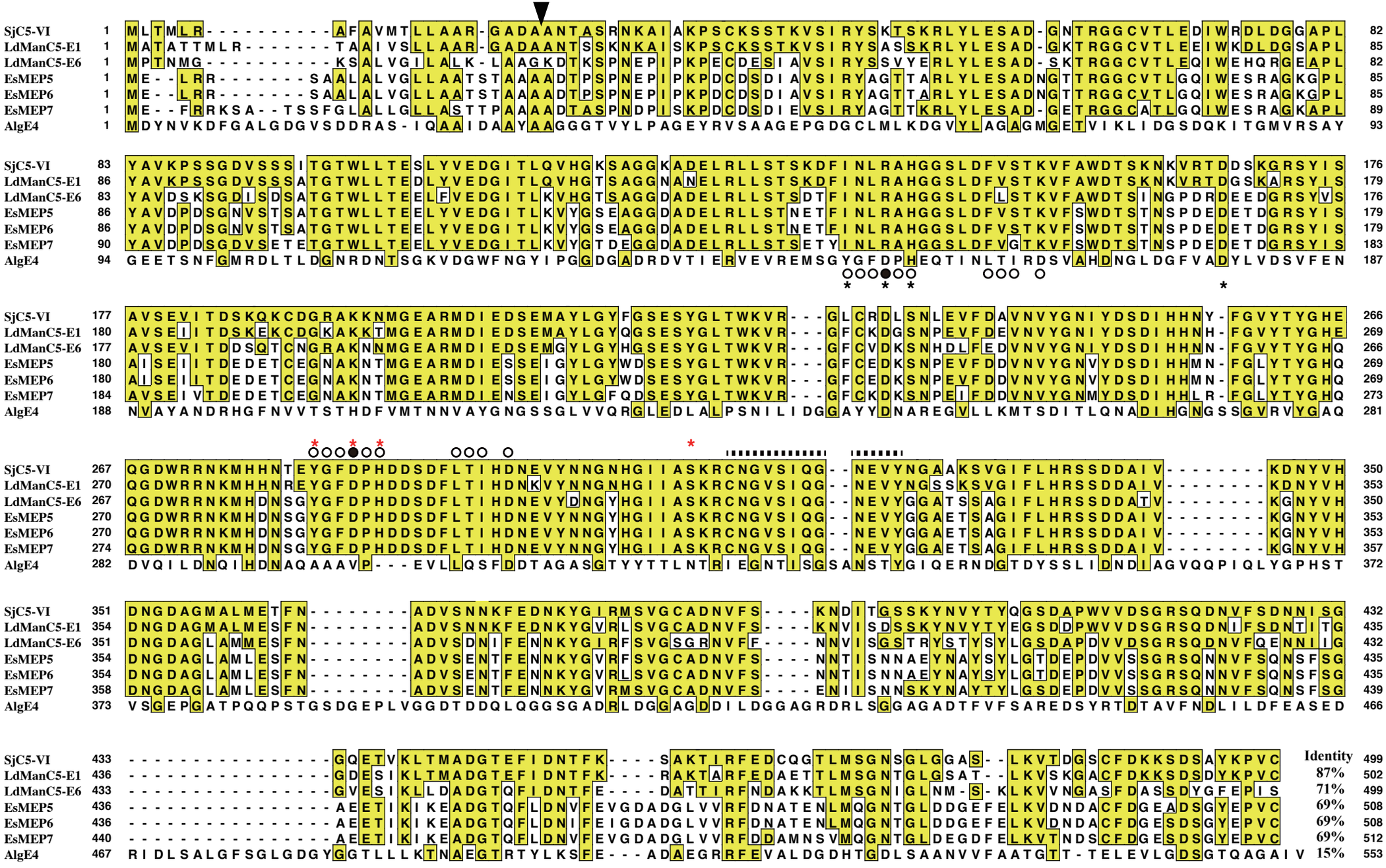

SjC5-VI
LdManC5-E1
LdManC5-E6
EsMEP5
EsMEP6
EsMEP7
AlgE4
Identity
87%
71%
69%
69%
69%
15%

Fig. 4

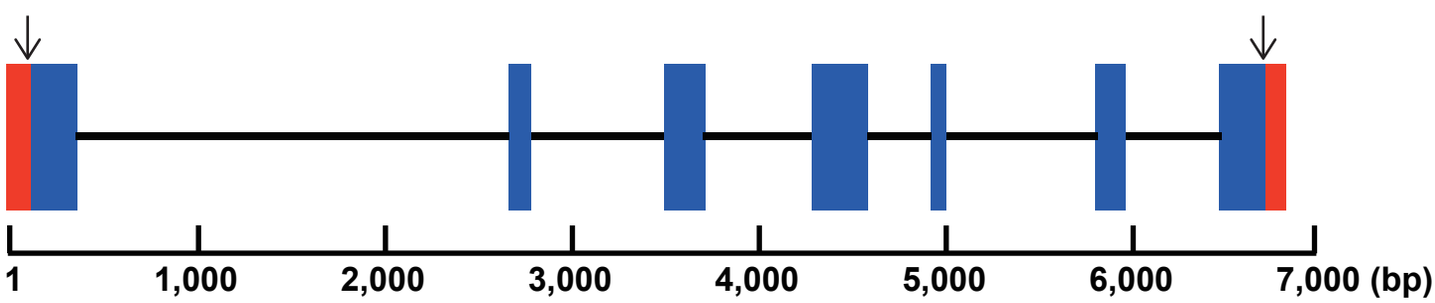


Fig. 5

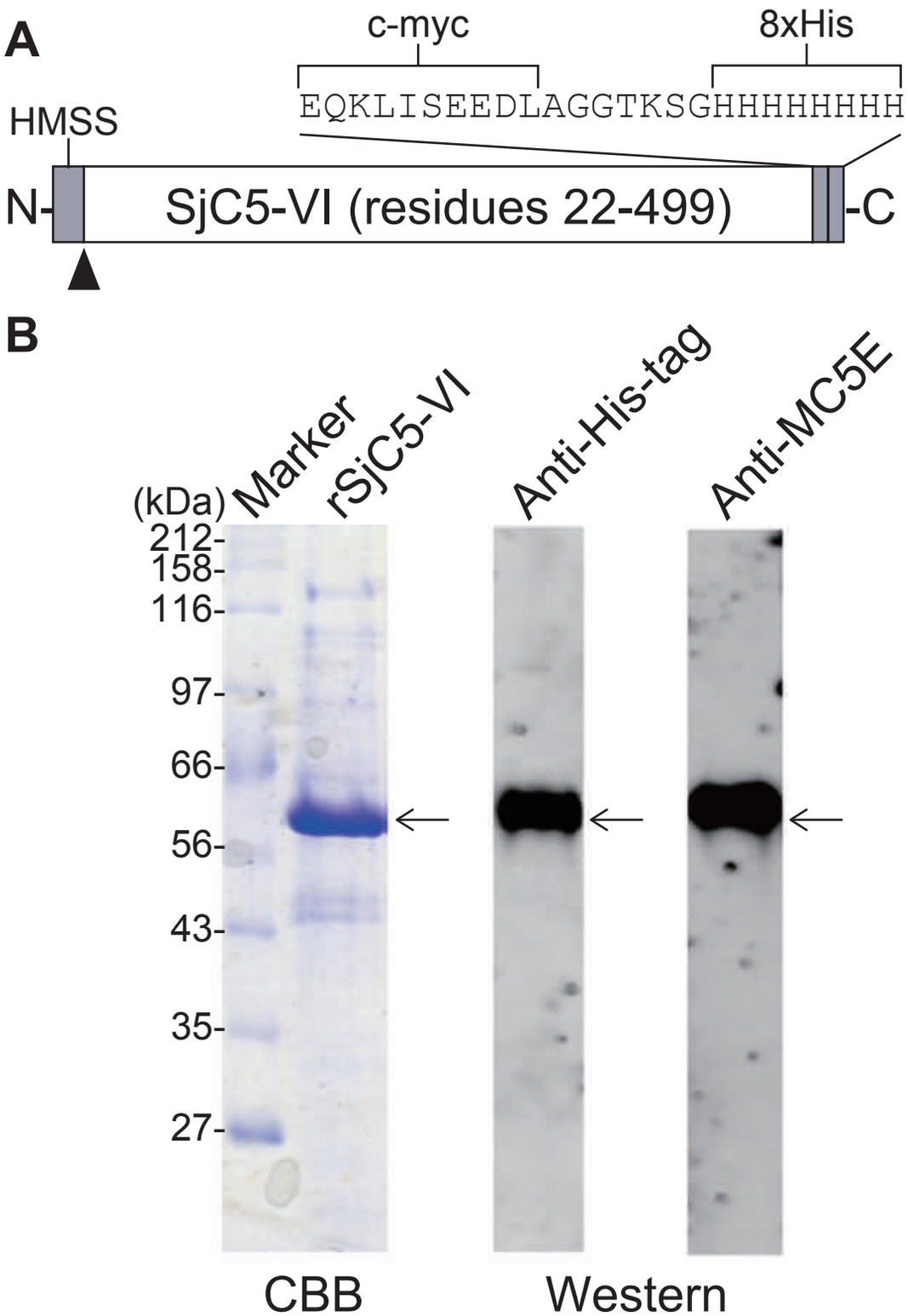

A
c-myc
8xHis
EQKLISEEDLAGGTKSGHHHHHHHH
HMSS
N-
SjC5-VI (residues 22-499)
-C
B
Marker
rSjC5-VI
Anti-His-tag
Anti-MC5E
(kDa)
212-
158-
116-
97-
66-
56-
43-
35-
27-
CBB
Western

Fig. 6

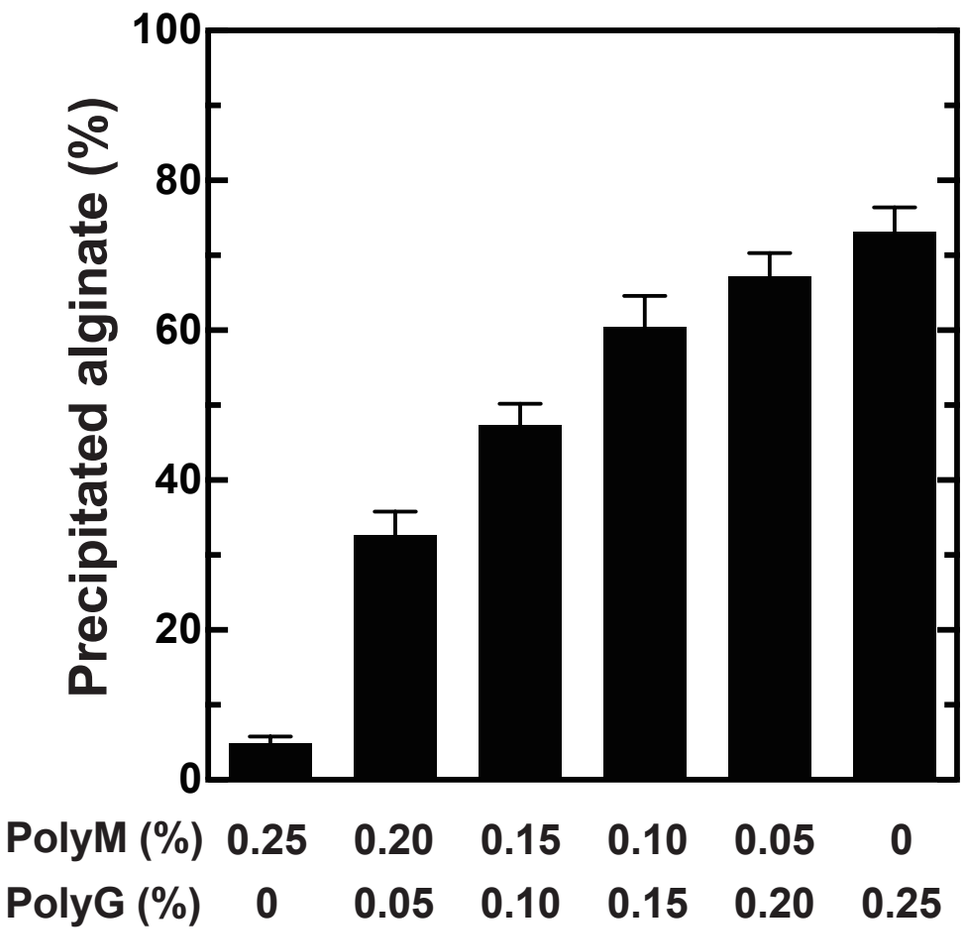

Precipitated alginate (%)
100
80
60
40
20
0
PolyM (%) 0.25 0.20 0.15 0.10 0.05 0
PolyG (%) 0 0.05 0.10 0.15 0.20 0.25

Fig. 7

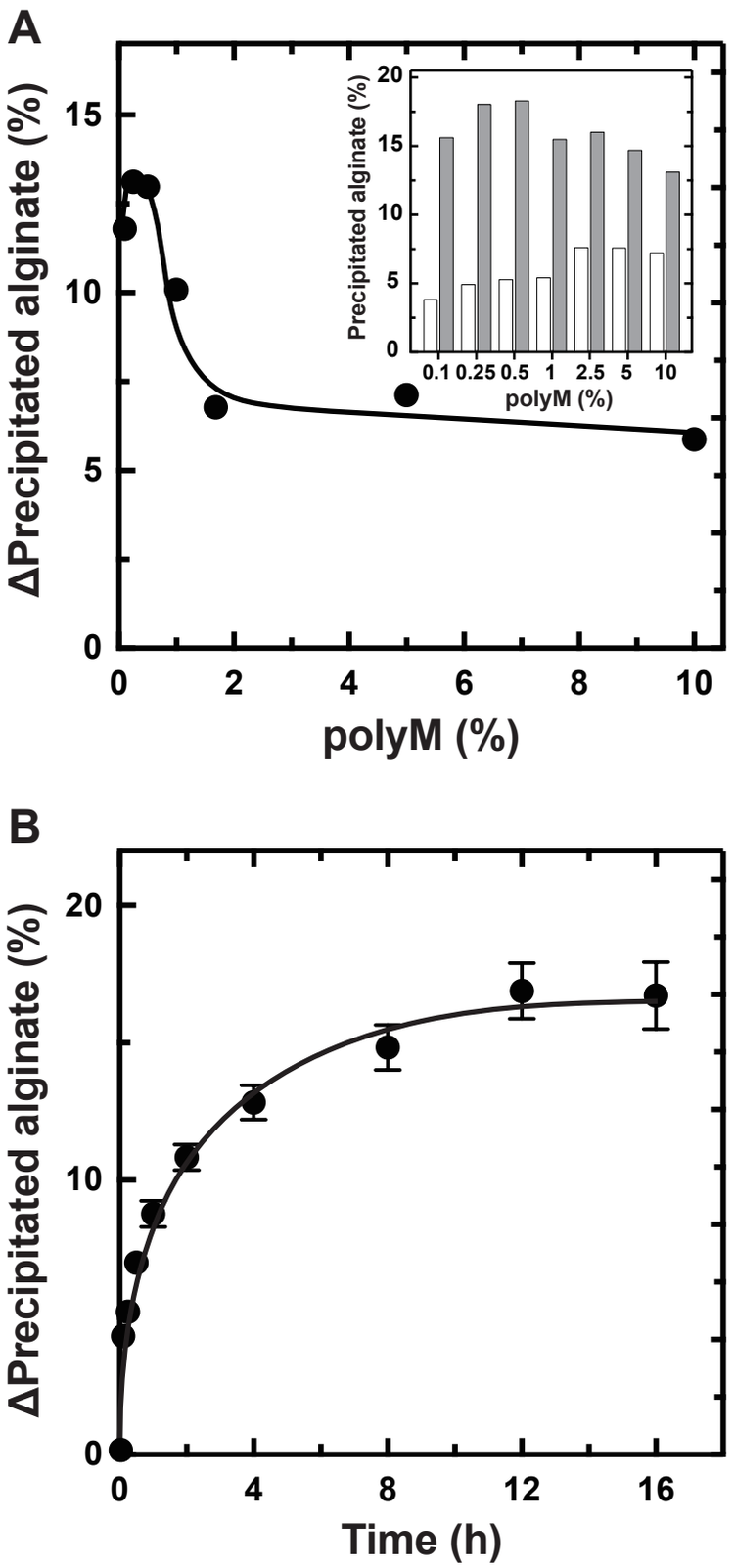

A
ΔPrecipitated alginate (%)
polyM (%)
Precipitated alginate (%)
polyM (%)
0.1 0.25 0.5 1 2.5 5 10
B
ΔPrecipitated alginate (%)
Time (h)

Fig. 8

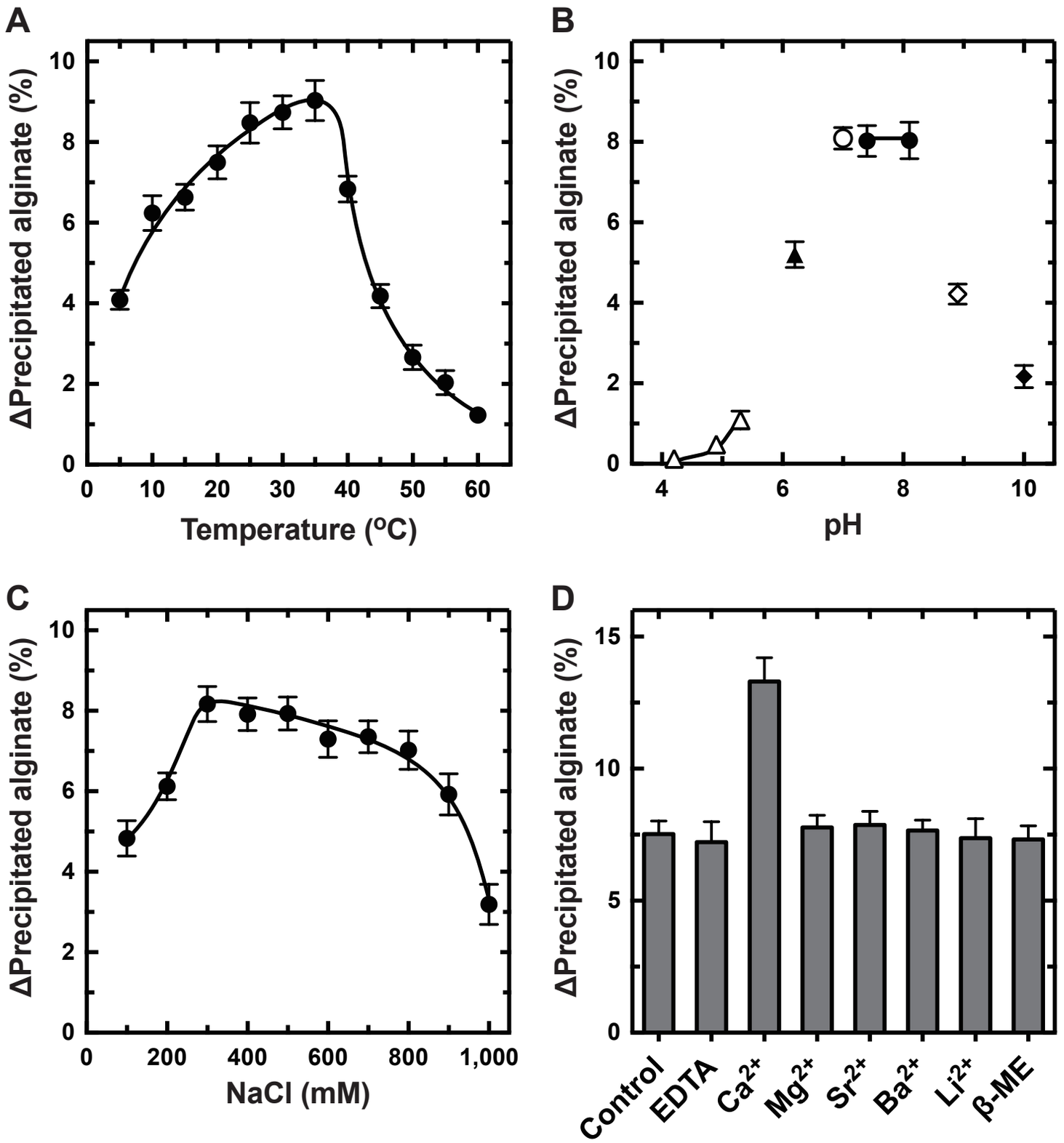

A
ΔPrecipitated alginate (%)
Temperature (°C)
B
ΔPrecipitated alginate (%)
pH
C
ΔPrecipitated alginate (%)
NaCl (mM)
D
ΔPrecipitated alginate (%)
Control
EDTA
Ca2+
Mg2+
Sr2+
Ba2+
Li2+
β-ME

Fig. 9

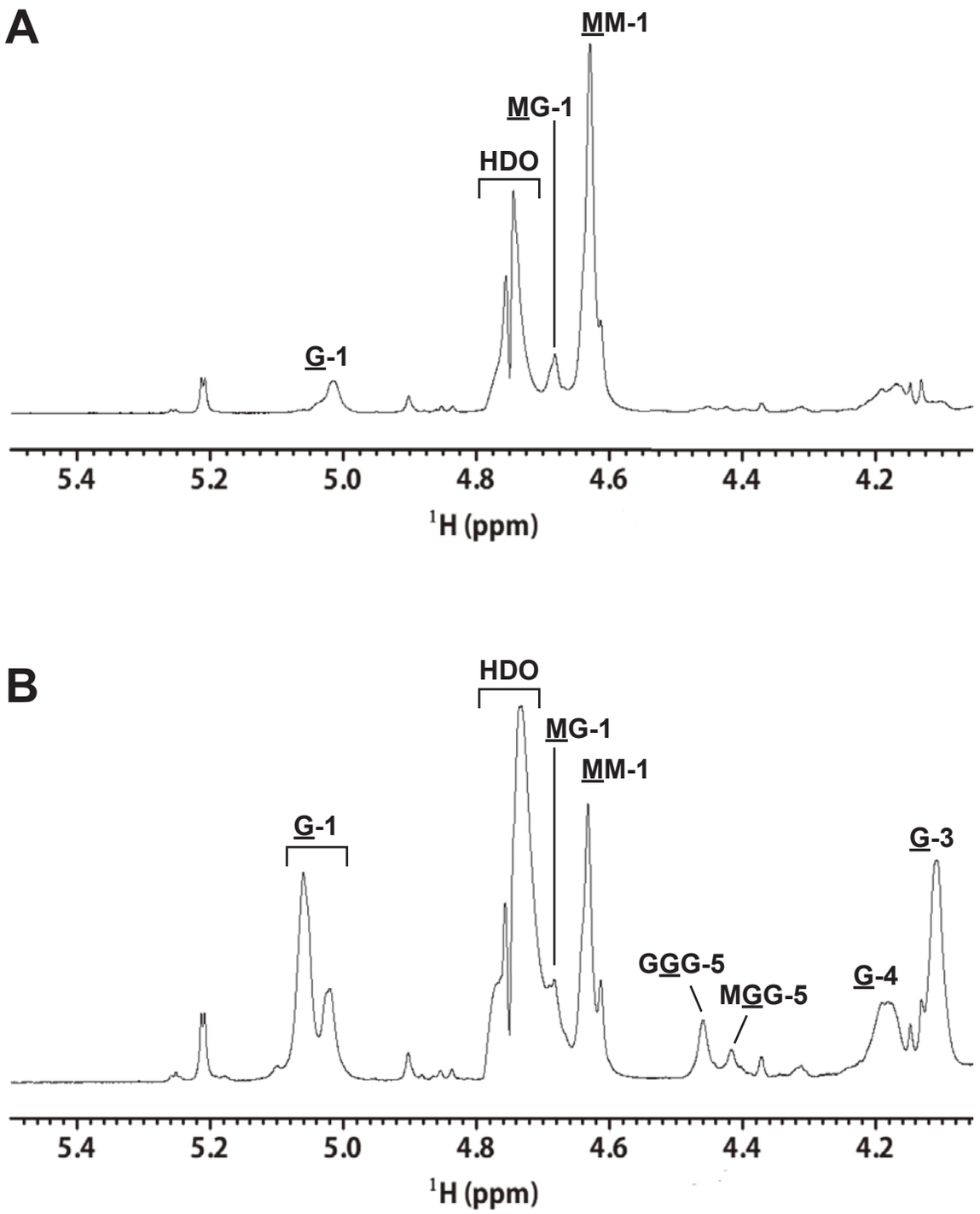
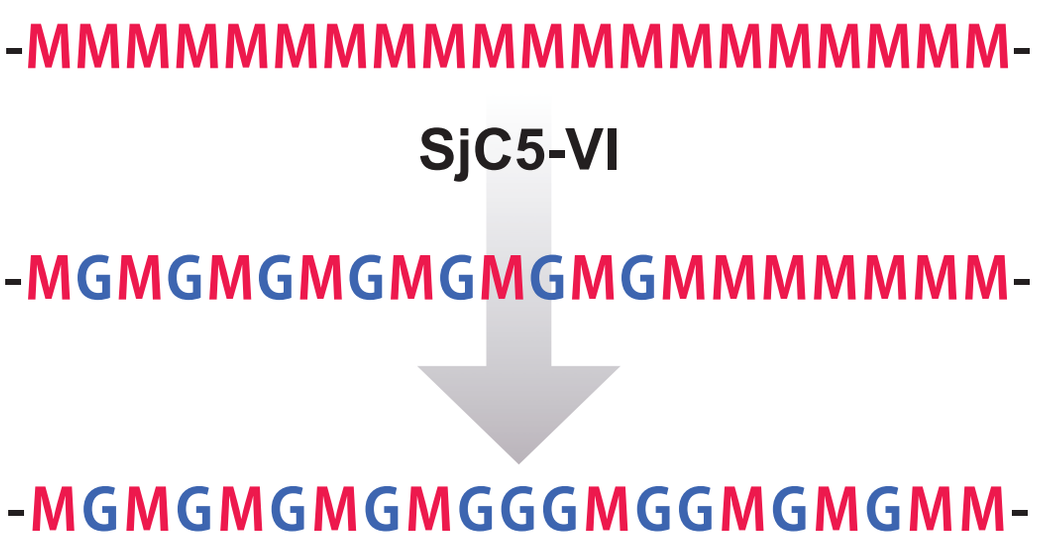

A
MM-1
MG-1
HDO
G-1
5.4
5.2
5.0
4.8
4.6
4.4
4.2
1H (ppm)
B
HDO
MG-1
MM-1
G-1
G-3
GGG-5
MGG-5
G-4
5.4
5.2
5.0
4.8
4.6
4.4
4.2
1H (ppm)

Fig. 10


-MMMMMMMMMMMMMMMMMMMMMM-
SjC5-VI
-MGMGMGMGMGMGMGMGMMMMMMM-
-MGMGMGMGMGGGMGGMGMGMM-

Supplementary Fig. 1