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An exotype alginate lyase in *Sphingomonas* sp. A1: overexpression in *Escherichia coli*, purification, and characterization of alginate lyase IV (A1-IV)

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Abstract

Sphingomonas sp. A1 (strain A1) cells contain three kinds of endotype alginate lyases [A1-I, A1-II, and A1-III], all of which are formed from a common precursor through posttranslational processing. In addition to these lyases, another type of lyase (A1-IV) that acts on oligoalginates exists in the bacterium. A1-IV was overexpressed in *Escherichia coli* cells through control of its gene under the T7 promoter. The expression level of the enzyme in *E. coli* cells was 8.6 U/L-culture, which was about 270-fold higher than that in strain A1 cells. The enzyme was purified to homogeneity through three steps with an activity yield of 10.9%. The optimal pH and temperature, thermal stability, and mode of action of the purified enzyme were similar to those of the native enzyme from strain A1 cells. A1-IV exolytically degraded oligoalginates, which were produced from alginate through the reaction of A1-I, A1-II, or A1-III, into monosaccharides, indicating that the cooperative actions of these four enzymes cause the complete depolymerization of alginate in strain A1 cells.

Alginate is a linear polysaccharide consisting of α-L-guluronate and its C5 epimer, β-D-mannuronate, arranged in three different ways: poly-α-L-guluronate (poly(G)), poly-β-D-mannuronate (poly(M)), and heteropolymeric regions (poly(MG)) [1]. This polymer is produced by brown seaweed and certain bacteria. Alginate from brown seaweed has a wide variety of uses in the food and pharmaceutical industries because of its highly viscous and ion-chelating properties [2]. On the other hand, alginate produced by bacteria, typically by *Pseudomonas aeruginosa*, functions as a biofilm responsible for both chronic pulmonary infections and respiratory difficulty in the lungs of patients with cystic fibrosis [3,4]. This biofilm often protects cells of *P. aeruginosa* from phagocytic cells and/or antibiotics [5–7] and makes the treatment of biofilm-dependent diseases difficult. Therefore, alginate lyases having the ability to depolymerize a highly viscous alginate biofilm are promising as therapeutic agents for these diseases [8]. The enzyme catalyzes the breakdown of alginate through a β-elimination reaction and produces unsaturated oligouronic acids having 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid at the nonreducing terminus [9,10] (Fig. 1).

Alginate lyases have been isolated from various organisms [11,12]. Strain A1[1] cells produce three kinds of endotype alginate lyases [A1-I, A1-II, and A1-III], all of which are formed from a common precursor through posttranslational processing [10]. That is, A1-I (65 kDa) is autocatalytically converted to A1-II (25 kDa) and A1-III (40 kDa). These alginate lyases show different substrate specificities [13,14]: A1-II is active on poly(G) in nonacetylated alginate from brown seaweed and releases unsaturated tri- and tetrasaccharides from alginate as

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[1] *Abbreviations used*: Strain A1, *Sphingomonas* sp. A1; A1-I, alginate lyase I; A1-II, alginate lyase II; A1-III, alginate lyase III; A1-IV, alginate lyase IV; G, α-L-guluronate; M, β-D-mannuronate; TLC, thin-layer chromatography; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBA, thiobarbituric acid; EDTA, ethylenediaminetetraacetic acid.

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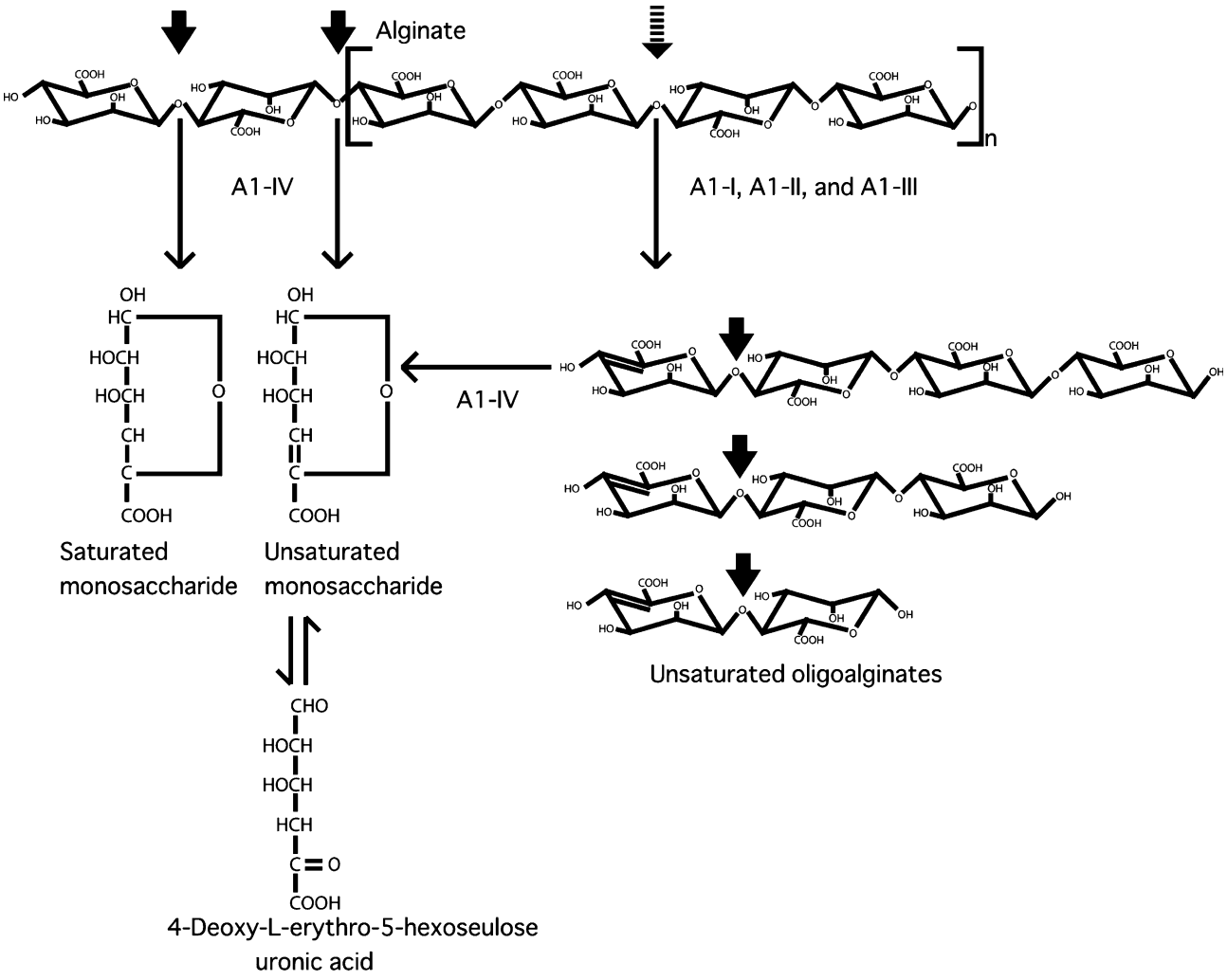

Fig. 1. Alginate depolymerization process. Alginate is depolymerized to 4-deoxy-L-erythro-5-hexoseulose uronic acid through the consecutive reactions of four alginate lyases. Thick and dotted arrows indicate the cleavage sites for endolyases (A1-I, -II, and -III) and the exolyase (A1-IV), respectively. The alginate depolymerization pathway is shown by thin arrows.

the final products. A1-III acts on poly(M) in acetylated alginate (biofilm) produced by *P. aeruginosa*, and finally, degrades the substrate to unsaturated di- and trisaccharides. A1-I has the properties of both A1-II and A1-III, and gives rise to unsaturated di- and trisaccharides [13,14].

Recently, in addition to these alginate lyases, an enzyme (A1-IV) responsible for the degradation of unsaturated oligoalginates produced from alginate through the reaction of A1-III was found to be present in strain A1 cells [15] (Fig. 1). This enzyme A1-IV, formerly designated as an oligoalginate lyase (OAL) [15], also depolymerizes high molecular weight alginate through a β-elimination reaction and releases the monosaccharide from the nonreducing terminus, indicating that the enzyme recognizes both unsaturated and saturated nonreducing saccharides in the alginate molecule.

We have already prepared crystals of endotype lyases, A1-II [16] and A1-III [17], and determined the three-dimensional structure and catalytic amino acid residues of A1-III [18,19]. A1-II, specific for poly(G), was found to be enriched in the β-sheet structure on circular dichroism analysis, and A1-III, active on poly(M), consists of an (α/α)-barrel structure. To obtain crystals of A1-IV to identify the structural features determining the substrate specificity and mode of reaction (endo/exo type) of alginate lyases, it is necessary to prepare a large amount of A1-IV with the properties same as those of the enzyme from strain A1 cells. Here, we construct an overexpression system for the enzyme in *Escherichia coli* cells and compare the properties of the enzyme with those of the native form.

Materials and methods

Materials

Sodium alginate from *Eisenia bicyclis* (average molecular weight, 25,700; viscosity, 1000 centipoise)

was purchased from Nacalai Tesque, Kyoto, Japan. Silica gel 60/Kieselguhr F_{254} thin-layer chromatography (TLC) plates were obtained from E. Merck, Darmstadt, Germany. DEAE- and Butyl-Toyopearl 650 M were purchased from Tosoh (Tokyo, Japan). Sephacryl S-200HR was from Pharmacia Biotech (Uppsala, Sweden) and Bio-Gel P2 was from Bio-Rad Laboratories (Hercules, CA). Restriction endonucleases were obtained from Takara Shuzo (Kyoto, Japan).

Microorganisms

Six *E. coli* strains [BL21(DE3), BL21(DE3)pLysE, BL21(DE3)pLysS, HMS174(DE3), HMS174(DE3)-pLysE, and HMS174(DE3)pLysS] (Novagen, Madison, WI) were used as hosts for the expression of A1-IV.

Construction of a plasmid

To introduce the A1-IV gene into an expression vector, pET3a (Novagen), the polymerase chain reaction (PCR) was performed with pBE11 [20] as a template and two synthetic oligonucleotides as primers. The oligonucleotides were 5′-GGGCATATGAAGAA GCTGGA ACAGCCGCAG-3′ and 5′-CGCGGATCCTTAGAA CGGTTTGGGCAACGTGAT-3′ with *Nde*I and *Bam*HI sites in the 5′ region, respectively. The fragment amplified through the PCR was digested with *Nde*I and *Bam*HI, and then ligated with *Nde*I and *Bam*HI-digested pET3a. The resultant plasmid was designated as pET3a-A1-IV.

DNA manipulations

Subcloning, transformation, and gel electrophoresis were performed as described [21].

Preparation of substrates for A1-IV

The endotype alginate lyases (A1-I, A1-II, and A1-III) were purified from *E. coli* cells having the *a1-I*, *a1-II*, and *a1-III* genes, respectively [14]. Unsaturated oligoalginates (di-, tri-, and tetrasaccharides) were prepared from alginate through the reactions of A1-I, A1-II, and A1-III, and used after purification by gel filtration on a Bio-Gel P2 column (0.9 by 122 cm) previously equilibrated with distilled water [13].

TLC analysis

The unsaturated oligoalginates (di-, tri-, and tetrasaccharides) were separated by TLC using a solvent system of 1-butanol:acetic acid:water (3:2:2, v/v) and visualized by heating the TLC plates at 130 °C for 5 min after spraying with 10% (v/v) sulfuric acid in ethanol. Unsaturated oligoalginates and α-keto acids on the TLC plates were detected with thiobarbituric acid (TBA) [22]. The amounts of unsaturated oligoalginates were determined as follows. Each spot of a saccharide on the TLC plates was densitometrically measured with the NIH1.62 program using standard oligoalginates, of which the weights were determined after freeze-drying. The amounts of oligoalginates determined as described above coincided with those with glucose as a standard. Quantitative analysis of an α-keto acid (4-deoxy-L-erythro-5-hexoseulose uronic acid) was performed using various amounts of 2-deoxy glucose as a standard, since deoxy sugars as well as α-keto acids produced through the enzyme reaction reacted with TBA (Fig. 4E). The molecular masses of α-keto acids, and di-, tri-, and tetrasaccharides were calculated to be 176, 352, 528, and 704 Da, respectively, as described previously [13].

Enzyme assay and protein concentration measurement

Unless otherwise described, A1-IV was assayed using the trisaccharide, a product of the A1-III-reaction, as a substrate. The reaction mixture (10 μl), consisting of 8 nmol trisaccharide, 0.5 μmol Tris–HCl (pH 7.5), and the enzyme (2 μg), was incubated at 30 °C for 10 min and the products formed were analyzed by TLC as described above. The amount of the trisaccharide remaining in the reaction mixture was densitometrically measured using the NIH1.62 program. For determination of the substrate specificity of the enzyme, the trisaccharide of the A1-III-reaction was replaced by the di- and trisaccharides of the A1-I-reaction, and the tri- and tetrasaccharides of the A1-II-reaction. The reaction mixture (0.5 ml), consisting of 700 nmol each oligoalginate, 25 μmol Tris–HCl (pH 7.5), and the enzyme (0.35 mg), was incubated at 30 °C and aliquots (50 μl) of the reaction mixture were withdrawn at appropriate times. Ten-microliter portions of the samples were analyzed by TLC, as described above. One unit of enzyme activity is defined as the amount required to degrade 1 μmol of the di-, tri-, or tetrasaccharide per minute at 30 °C. Protein concentrations were determined by the method of Bradford [23] with bovine serum albumin as a standard, or by measuring the absorbance at 280 nm using a 1-cm path length cuvette, assuming that $E_{280} = 1.0$ corresponds to 1 mg/ml.

Mode of action of A1-IV

The reaction mixture (1 ml), consisting of 0.75 mg high molecular weight alginate, 50 μmol Tris–HCl (pH 7.5), and the enzyme (0.7 mg), was incubated at 30 °C and aliquots (100 μl) of the reaction mixture were withdrawn at appropriate times. Ten-microliter portions of the samples were analyzed by TLC as described above.

Purification of A1-IV from E. coli cells

Unless otherwise specified, all operations were performed at 0–4 °C. *E. coli* cells harboring pET3a-A1-IV were cultured at 28 °C for 3 h ($OD_{600} = 0.5$) in 1.5 L LB medium and then isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM, followed by further incubation for 20 h ($OD_{600} = 3.0$) at 30 °C. The cells were harvested by centrifugation at 5000*g* and 4 °C for 5 min, washed with 20 mM Tris–HCl (pH 7.5), resuspended in the same buffer, and then disrupted ultrasonically (Insonator, Kubota Model 201 M, Tokyo, Japan) at 0 °C and 9 kHz for 20 min. The clear solution (cell extract: 45 ml) obtained on centrifugation at 17,000*g* and 4 °C for 20 min was applied to a DEAE-Toyopearl column (2.7 by 8 cm) previously equilibrated with 20 mM Tris–HCl (pH 7.5). The enzyme was eluted with a linear gradient of NaCl (from 0 to 1 M) in 20 mM Tris–HCl (pH 7.5) (200 ml), 2 ml fractions being collected every 2 min. The active fractions, which were eluted with 0.5 M NaCl, were saturated with ammonium sulfate (30%), and then the enzyme solution was applied to a Butyl-Toyopearl 650 M column (3 by 3 cm) previously equilibrated with 20 mM Tris–HCl (pH 7.5) saturated with ammonium sulfate (30%). The enzyme was eluted with a linear gradient of ammonium sulfate (from 30 to 0%) in 20 mM Tris–HCl (pH 7.5) (100 ml), followed by 20 mM Tris–HCl (pH 7.5) (50 ml), 1 ml fractions being collected every 1 min. The active fractions, which were eluted with 20 mM Tris–HCl (pH 7.5), were combined, concentrated by ultrafiltration with an Amicon model 8200 (Amicon, MA) to about 6 ml, and then applied to a Sephacryl S-200HR column (2.7 by 50 cm) previously equilibrated with 20 mM Tris–HCl (pH 7.5) containing 0.15 M NaCl. The enzyme was eluted with the same buffer, 2.5 ml fractions being collected every 4 min. The enzyme was eluted in fraction numbers 44–48. These fractions were combined and dialyzed against 20 mM Tris–HCl (pH 7.5) overnight. The dialysate was used as the purified enzyme and stored at −20 °C.

Electrophoresis and isoelectric focusing

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [24] and native gradient PAGE were performed as described previously [25]. Isoelectric focusing was performed with an IPGphor system (Amersham Biosciences, Piscataway, NJ), as described in manufacturer's manual.

N-terminal amino acid sequence of A1-IV

The N-terminal amino acid sequence of A1-IV from *E. coli* cells was determined by Edman degradation with a Procise 492 protein sequencing system (Applied Biosystems Division of Perkin–Elmer, Foster City, CA).

Results

Overexpression in E. coli cells and purification of A1-IV

The six *E. coli* strains [BL21(DE3), BL21(DE3)-pLysE, BL21(DE3)pLysS, HMS174(DE3), HMS174 (DE3)pLysE, and HMS174(DE3)pLysS] were transformed with pET3a-A1-IV. Among the transformants obtained, HMS174(DE3) with a plasmid showed the highest expression level (8.6 U/L-culture) of the enzyme (Fig. 2A, lane 3). The expression level of the enzyme in *E. coli* cells was 270-fold higher than that (0.032 U/L-culture) in strain A1 cells. The enzyme was purified 5.45-fold from the *E. coli* HMS174(DE3) cells with an activity yield of 10.9% (Table 1). The purified enzyme was judged to be homogeneous on SDS and native gradient PAGE (Fig. 2).

Characterization of the A1-IV purified from E. coli cells

The properties of the purified enzyme were as follows:

(i) *Molecular weight*. The molecular masses of the enzyme were determined to be 85 kDa by SDS–PAGE (Fig. 2A) and 89 kDa by native gradient PAGE (Fig. 2B), indicating that the enzyme is a monomer.

(ii) *Temperature*, *pH*, and *pI*. The enzyme was most active at pH 7.5–8.5 in 50 mM glycine–NaOH (Fig. 3A) and at 37 °C (Fig. 3B). Fifty percent of

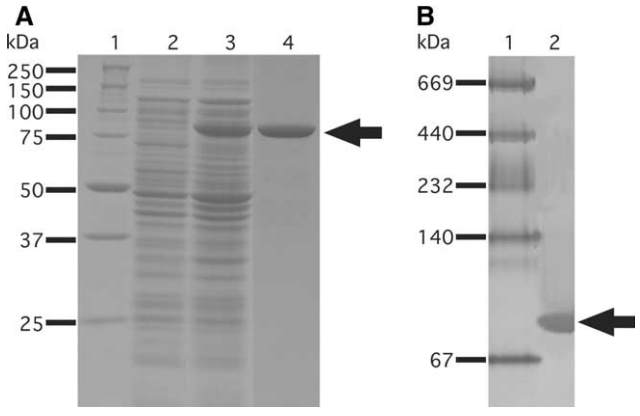

Fig. 2. Electrophoretic profiles of A1-IV. (A) SDS–PAGE. Lane 1, molecular weight standards (from top): 250,000, 150,000, 100,000, 75,000, 50,000, 37,000, and 25,000; lane 2, cell extract of *E. coli* cells harboring pET3a; lane 3, cell extract of *E. coli* cells harboring pET3a-A1-IV; and lane 4, the enzyme (10 μg) purified from *E. coli* cells. (B) Native gradient PAGE. Lane 1, molecular weight standards (from top): thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and bovine serum albumin (67,000); lane 2, the enzyme (30 μg) purified from *E. coli* cells. The arrows indicate the position of A1-IV.

Table 1
Purification of A1-IV from *E. coli* cells

Step[a]	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell extract	586	12.9	0.022	100	1.00
DEAE-Toyopearl 650 M	295	8.0	0.027	62.0	1.23
Butyl-Toyopearl 650 M	89.2	4.4	0.049	34.1	2.23
Sephacryl S-200HR	11.6	1.4	0.12	10.9	5.45

[a] The purification procedures are described in Materials and methods.

the enzyme activity was lost on preincubation at 45 °C for 10 min in 20 mM Tris–HCl (pH 7.5) (Fig. 3C). The p*I* of the enzyme was determined to be 5.5 (data not shown), which is almost identical to the value predicted on determination of the amino acid composition of the enzyme.

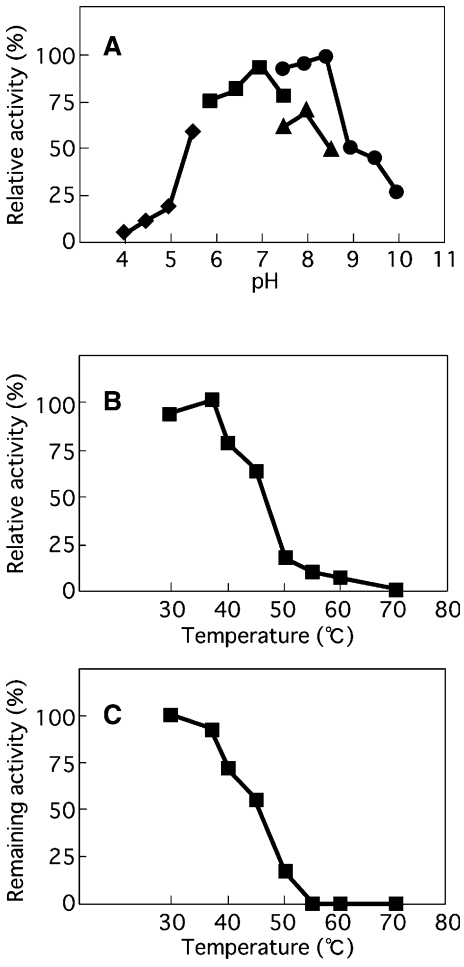

Fig. 3. Effects of pH and temperature on the activity of A1-IV. Experiments were carried out at 30 °C using alginate trisaccharide (8 nmol) as a substrate and the enzyme (2 μg) purified from *E. coli* cells. (A) Effect of pH: reactions were performed at 30 °C for 10 min in the following 50 mM buffers; sodium acetate (◆), potassium phosphate (■), Tris–HCl (▲), and glycine–NaOH (●). The activity at pH 8.5 in glycine–NaOH was relatively taken as 100%. (B) Optimal temperature: reactions were performed for 10 min at various temperatures in 50 mM Tris–HCl (pH 7.5). The activity at 37 °C was relatively taken as 100%. (C) Thermal stability: after preincubation of the enzyme at various temperatures for 10 min, the remaining activity was measured under the conditions specified in Materials and methods. The activity of the enzyme preincubated at 30 °C was relatively taken as 100%.

(iii) *Metal ions and other compounds*. The reaction of the enzyme was performed in the presence or absence of various compounds and then the remaining activity was measured (Table 2). Divalent metal ions (Cu^{2+}, Hg^{2+}, and Zn^{2+}) and *p*-chloromercuribenzoic acid were potent inhibitors of the enzyme. Divalent metal ions (Co^{2+} and Fe^{2+}), iodoacetic acid, and *N*-ethylmaleimide partially inhibited the enzyme activity at 1 mM. Other compounds listed in Table 2 had no significant effect on the enzyme activity.

(iv) *N-terminal amino acid sequence*. The N-terminal amino acid sequence of the enzyme expressed in *E. coli* cells was ^{1}MKKLEQPQS^{9}, which is completely identical to that of the native enzyme from strain A1 cells.

Table 2
Effects of various compounds on A1-IV activity

Compound	Concentration (mM)	Activity[a] (%)
None (control)		100
$AlCl_3$	1	89.9
$CaCl_2$	1	97.3
$CoCl_2$	1	41.2
$CuCl_2$	1	ND[b]
$FeCl_2$	1	33.6
$FeCl_3$	1	71.2
$HgCl_2$	1	ND
$MgCl_2$	1	93.8
$MnCl_2$	1	112
$ZnCl_2$	1	ND
Dithiothreitol	1	127
Glutathione	1	90.1
2-Mercaptoethanol	1	127
Iodoacetic acid	1	56.0
N-Ethylmaleimide	1	20.0
p-Chloromercuribenzoic acid	1	ND
EDTA	1	112
L-Fucose	5	115
D-Galactose	5	97.4
D-Glucose	5	91.5
D-Glucuronic acid	5	106
D-Mannose	5	93.2
L-Rhamnose	5	110
D-Xylose	5	108

[a] Reactions were carried out for 10 min at 30 °C and pH 7.5 (Tris–HCl) in the presence or absence (control) of the above compounds. The activity of the control was relatively taken as 100%.

[b] ND, not detected.

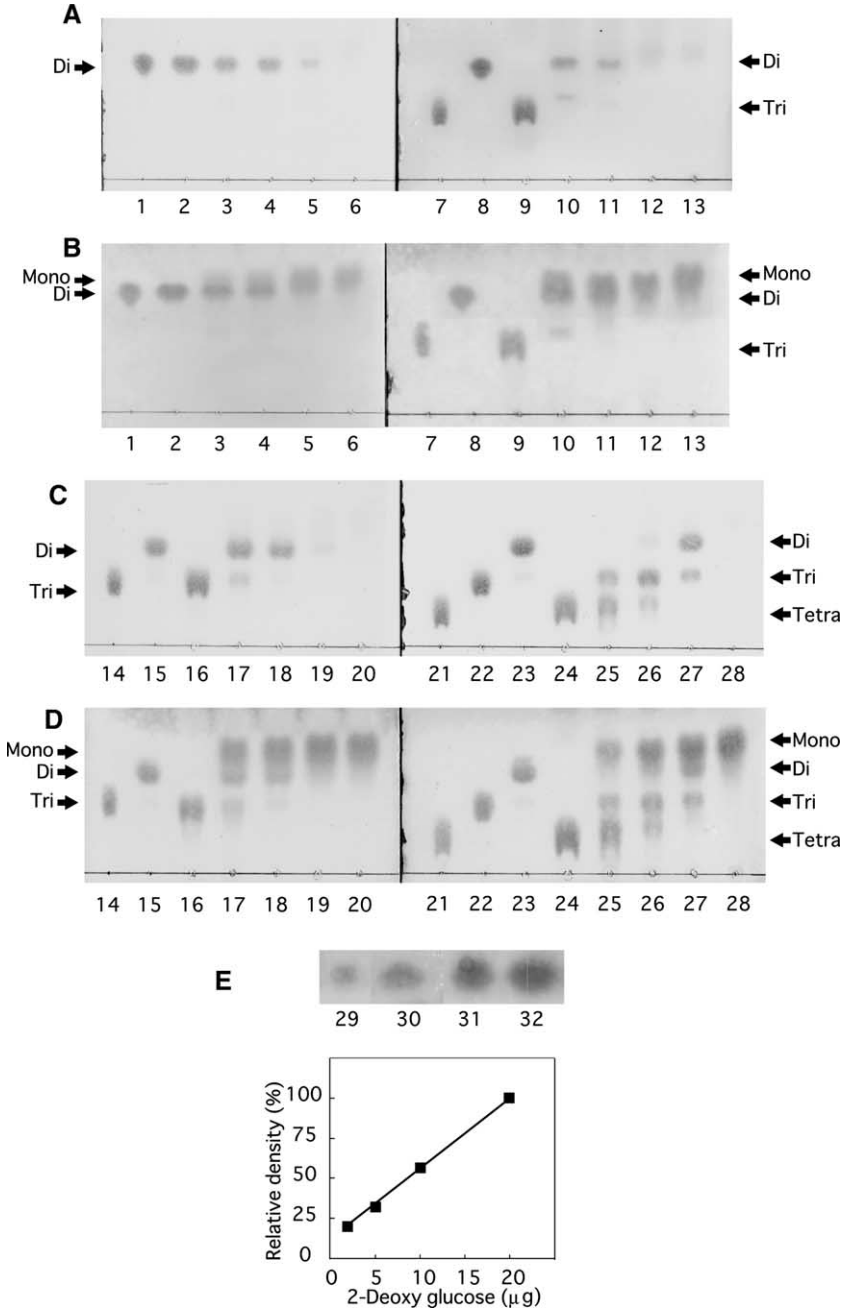

Fig. 4. Substrate specificity of A1-IV. Di- and trisaccharides produced through the reaction of A1-I, and tri- and tetrasaccharides produced through the reaction of A1-II were incubated with the purified enzyme at 30 °C. The reaction mixture (0.5 ml) was composed of 700 nmol each oligoalginate, 25 μmol Tris–HCl (pH 7.5), and the enzyme (0.35 mg), and aliquots (50 μl) of the reaction mixture were withdrawn at the times indicated below. Ten-microliter portions of the samples were analyzed by TLC. (A and C) Oligoalginates in the reaction mixture were detected with sulfuric acid. (B and D) Unsaturated oligoalginates and α-keto acids in the reaction mixture were detected with TBA. Lane 1, authentic disaccharide produced through the reaction of A1-I; lane 2, 0 min; lane 3, 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 60 min; lane 7, authentic trisaccharide produced through the reaction of A1-I; lane 8, authentic disaccharide produced through the reaction of A1-I; lane 9, 0 min; lane 10, 10 min; lane 11, 20 min; lane 12, 30 min; lane 13, 60 min; lane 14, authentic trisaccharide produced through the reaction of A1-II; lane 15, authentic disaccharide produced through the reaction of A1-II; lane 16, 0 min; lane 17, 10 min; lane 18, 20 min; lane 19, 30 min; lane 20, 60 min; lane 21, authentic tetrasaccharide produced through the reaction of A1-II; lane 22, authentic trisaccharide produced through the reaction of A1-II; lane 23, authentic disaccharide produced through the reaction of A1-II; lane 24, 0 min; lane 25, 10 min; lane 26, 20 min; lane 27, 30 min; and lane 28, 60 min. Tetra, Tri, Di, and Mono indicate the tetra-, tri-, di-, and monosaccharides produced from alginate, respectively. (E) Top, various amounts of 2-deoxy glucose (standard) stained with TBA on a TLC plate. Lane 29, 2 μg; lane 30, 5 μg; lane 31, 10 g; and lane 32, 20 μg. Bottom, densitometric calibration. The spots of 2-deoxy glucose on the TLC plate were densitometrically measured with NIH image, the density of 20 μg sugar being relatively taken as 100%.

(v) *Substrate specificity*. We have previously reported that A1-IV could degrade the unsaturated di- and trisaccharides produced from alginate through the reaction of A1-III, which prefers poly(M) of alginate [15]. Here, we examined whether or not the enzyme acts on the unsaturated di-, tri-, and tetrasaccharides produced from alginate through the reactions of A1-I and A1-II. The di-, tri-, and tetrasaccharides produced from alginate through the reactions of A1-I and A1-II were completely degraded (Figs. 4A and C) and the resultant monosaccharides were detected with TBA (Figs. 4B and D). Therefore, the monosaccharides are thought to be nonenzymatically converted to α-keto acids. Fourteen nmol of di- (5 μg), tri- (7.5 μg), and tetrasaccharides (10 μg) was expected to give rise to 28, 42, and 56 nmol of α-keto acids, respectively, if these oligoalginates were completely degraded. As found on quantitative analysis with 2-deoxy glucose as a standard by TBA staining (Fig. 4E), the di- and trisaccharides of the A1-I-reaction were converted to 4 and 6 μg, which are equal to 23 and 34 nmol, respectively, of α-keto acids, and the tri- and tetrasaccharides of the A1-II-reaction were converted to 8 and 10 μg, which are equal to 45 and 57 nmol, respectively, of α-keto acids by A1-IV. The quantities of α-keto acids produced through the enzyme reactions were exactly the same as the theoretical values. A1-IV was most active on the trisaccharides of the A1-I-reaction and hardly degraded the disaccharides of the A1-I-reaction (Table 3). This enzyme exolytically degraded unsaturated oligoalginates because the tetrasaccharides of the A1-II-reaction were converted to disaccharides through trisaccharides during the degradation (Figs. 4C and D; lanes 25–27).

(vi) *Mode of action*. A1-IV acted on not only unsaturated oligoalginates but also a high molecular weight alginate (25.7 kDa) containing M and G residues, and released monosaccharides (Figs. 5A and B). The resultant monosaccharides were also nonenzymatically converted to α-keto acids (Fig. 5B). Although the tailing of this product seems to be due to a disaccharide, the enzyme was found to produce only monosaccharides from alginate molecules, since disaccharides were not detected with sulfuric acid (Fig. 5A). The viscosity of the reaction mixture decreased as the enzyme reaction proceeded (data not shown), indicating that viscous alginate was depolymerized by A1-IV. High molecular weight alginate 7.5 μg (equal to 0.3 nmol) should be converted to α-keto acids (44 nmol) finally. A1-IV degraded 6.5 μg of high molecular weight alginate in 15 min (Fig. 5A; lane 4), produced 5 μg (28 nmol) of α-keto acids (Fig. 5B; lane 4), and finally produced 9 μg (51 nmol) of α-keto acids (Fig. 5B; lane 10), as found on quantitative analysis with 2-deoxy glucose as a standard by TBA staining (Fig. 4E). The final quantities (51 nmol) of α-keto acids produced by A1-IV in the reactions were almost identical to the theoretical values. This enzyme turned out to show exolytic activity toward substrates, i.e., unsaturated oligoalginates and high molecular weight alginate.

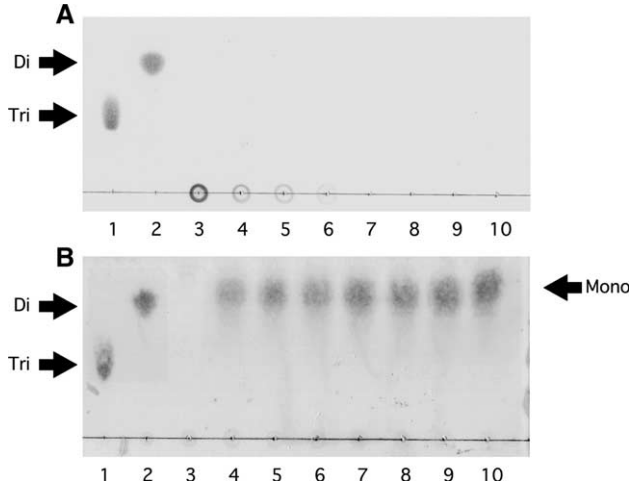

Fig. 5. Mode of action of A1-IV. High molecular weight alginate was incubated with the purified enzyme at 30 °C. The reaction mixture (1 ml) was composed of 0.75 mg high molecular weight alginate, 50 μmol Tris–HCl (pH 7.5), and the enzyme (0.7 mg). Aliquots (100 μl) of the reaction mixture were withdrawn at the times indicated below. Ten-microliter portions of the samples were analyzed on TLC plates. (A) The reaction products were stained with sulfuric acid. (B) The reaction products were stained with TBA. Lane 1, authentic trisaccharide produced through the reaction of A1-III; lane 2, authentic disaccharide produced through the reaction of A1-III; lane 3, 0 h; lane 4, 0.25 h; lane 5, 0.5 h; lane 6, 1 h; lane 7, 3 h; lane 8, 5 h; lane 9, 7 h; and lane 10, 24 h. Tri, Di, and Mono indicate the tri-, di-, and monosaccharides produced from alginate, respectively.

Table 3
Substrate specificity of A1-IV

Substrate	Specific activity (U/mg)
Disaccharide of A1-I-reaction	0.091 (47.9%)
Trisaccharide of A1-I-reaction	0.19 (100%)[a]
Trisaccharide of A1-II-reaction	0.18 (94.7%)
Tetrasaccharide of A1-II-reaction	0.12 (63.2%)

The purified enzyme from *E. coli* cells was incubated at 30 °C and pH 7.5 (Tris–HCl) for 10 min with various substrates.

[a] The activity toward the trisaccharide of the A1-I-reaction was taken as 100%.

Discussion

Overexpression in *E. coli* cells was achieved and a purification system for A1-IV of strain A1 was constructed. The enzyme from *E. coli* cells showed enzymatic properties, except for the effects of a few

compounds, similar to the native form from strain A1 cells. The system enabled us to prepare the large amount of the enzyme necessary for protein crystallization.

A1-IV from *E. coli* cells completely degraded the unsaturated di-, tri-, and tetrasaccharides produced from alginate through the reactions of A1-I, A1-II, and A1-III with different substrate specificities, and the resultant monosaccharides were nonenzymatically converted to an α-keto acid (4-deoxy-L-erythro-5-hexoseulose uronic acid). This indicates that A1-IV can degrade oligoalginates with various M and G compositions. Therefore, A1-IV is thought to cause the complete depolymerization of alginate in strain A1.

Polysaccharide lyases have been classified into 12 families based on their amino acid sequences (Henrissat, B., Coutinho, P., and Deleury, E.; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Alginate lyases that act on poly(M) and poly(G) belong to polysaccharide lyase families 5 and 7, respectively. Since A1-IV exhibits no significant homology with other proteins in protein and DNA databases, it has not been assigned to any family yet. Recently, the complete genome sequences of many bacteria were determined and a protein (Accession No. AB011415) from *Agrobacterium tumefaciens* has been found to exhibit significant identity (55.0%) with A1-IV. Therefore, the two proteins must constitute a new polysaccharide lyase family.

Alginate lyases have been obtained from various organisms, including algae, fungi, marine invertebrates, and bacteria [12]. Alginate lyases so far analyzed prefer either poly(M) or poly(G) and act on alginate endolytically, although there have been a few reports on alginate lyases that depolymerize both poly(M) and poly(G), and show exolytic activity. Alginate lyases from strain A1 (A1-I, 65 kDa) [14], *Alteromonas* sp. strain H-4 (32 kDa) [26], and *Bacillus* sp. ATB-1015 (41 kDa) [27] show activity toward both substrates, and the enzymes from marine bacterium ATCC 433367 (38 kDa) [28] and strain A3 (100 kDa) [29,30] exhibit exolytic activity. However, these lyases are significantly different from A1-IV in molecular weight and sequence similarity. To the best of our knowledge, no alginate lyase has been reported previously that exolytically acts on unsaturated and saturated alginates of various lengths and M/G ratios, except for A1-IV discussed here. Structural analysis of A1-IV together with endotype alginate lyases [16–18] will provide new information on the structure–function relationship of alginate lyases.

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