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Overexpression in *Escherichia coli,* Purification, and Characterization of *Sphingomonas* sp. A1 Alginate Lyases

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A bacterium *Sphingomonas* sp. A1 produces three kinds of alginate lyases [A1-I (66 kDa), A1-II (25 kDa), and A1-III (40 kDa)] from a single precursor, through posttranslational processing. Overexpression systems for these alginate lyases were constructed in *Escherichia coli* cells by controlling of the lyase genes under T7 promoter and terminator. Expression levels of A1-I, A1-II, and A1-III in *E. coli* cells were 3.50, 3.04, and 2.13 kU/liter of culture, respectively, and were over 10-fold higher than those in *Sphingomonas* sp. A1 cells. Purified A1-I, A1-II, and A1-III from *E. coli* cells were monomeric enzymes with molecular masses of 63, 25, and 40 kDa, respectively. The depolymerization pattern of alginate with A1-I and A1-II indicated that both enzymes cleaved the glycosidic bond of the polymer endolytically and by β-elimination reaction. A1-II preferred polyguluronate rather than polymannuronate and released tri- and tetrasaccharides, which have unsaturated uronyl residues at the nonreducing terminal, from alginate as the major final products. A1-I acted equally on both homopolymers and produced di- and trisaccharides as the final products. © 2000 Academic Press

Key Words: **alginate; alginate lyase; overexpression; *Sphingomonas;* T7 promoter.**

Alginate is a linear polysaccharide produced by brown seaweeds and certain bacteria. The polymer is composed of β-D-mannuronate and the C5 epimer α-L-guluronate, arranged in three different ways: poly-β-D-mannuronate (polyM),[2] poly-α-L-guluronate (polyG), and heteropolymeric (polyMG) regions in which there is a random arrangement of the monomers (1). Although algal alginate is widely used in food and pharmaceutical industries due to its ability to chelate metal ions and form a highly viscous solution (2), recently, polymers and oligosaccharides with novel physicochemical and physiological functions are sought by biopolymer-based industries in order to expand the application areas of polysaccharides (3). However, it is not easy to obtain novel polysaccharides with more excellent and safe properties than existing ones through screening of microorganisms. The enzymatic modification and molecular design of edible polysaccharides such as alginate, gellan, and xanthan are, therefore, considered to be practical and promising ways to yield novel biopolymers. In fact, through the depolymerization of polysaccharides with polysaccharide lyases, we have prepared various oligosaccharides having physiological properties such as an elicitor for plant cells (4) and growth factors for bifidobacteria (5) and human epithelium cells (6).

On the other hand, mucoid cells of *Pseudomonas aeruginosa* also produce alginate, a capsule-like exopolysaccharide responsible for both chronic pulmonary infections and respiratory difficulties in the lungs of patients with cystic fibrosis (CF) (7,8). The bacterial alginate is highly acetylated in mannuronyl residues (9) and seems to play an important role in the adherence of *P. aeruginosa* to target cells (10). For the clinical treatment of the bacterial infectious diseases, large amounts of antimicrobial agents have been used to repress the bacterial growth in the CF lung. However, the appearance of drug-resistant bacteria and toxicity of the agents, especially for older people, decrease the usefulness of the treatment. Then, we proposed that

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[2] Abbreviations used: A1-I, alginate lyase I of *Sphingomonas* sp. A1; A1-II, alginate lyase II of *Sphingomonas* sp. A1; A1-III, alginate lyase III of *Sphingomonas* sp. A1; CF, cystic fibrosis; PCR, polymerase chain reaction; polyG, polyguluronate; polyM, polymannuronate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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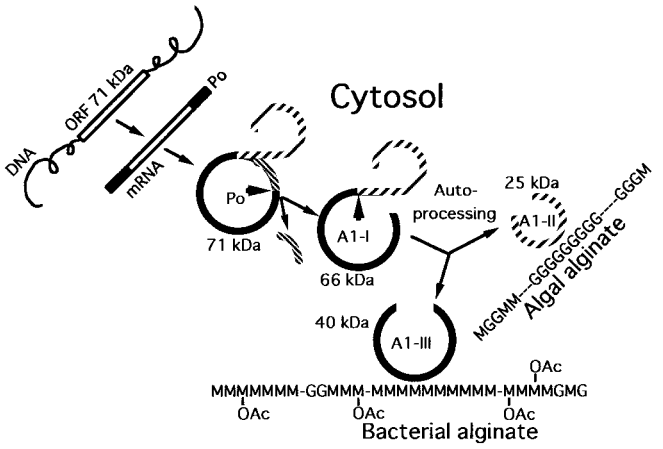

FIG. 1. Biosynthesis of alginate lyase in *Sphingomonas* sp. A1. Po, a precursor form of A1-I and comes to be A1-I through posttranslational processing by removing N-terminal peptide (5 kDa). The resultant A1-I is successively autoprocessed into A1-II and A1-III. M and G, mannuronyl and guluronyl residues, respectively.

the use of alginate-depolymerizing enzymes together with antimicrobial agents may be feasible for the therapy of CF and other infectious diseases caused by *P. aeruginosa*.

Alginate lyase cleaves the glycosidic bonds in alginate by the β-elimination reaction. The bacterium *Sphingomonas* sp. A1 produces three types of cytoplasmic alginate lyases [A1-I (66 kDa), A1-II (25 kDa), and A1-III (40 kDa)] from a common precursor, through posttranslational processing (Fig. 1) (11). A1-I is autoprocessed into A1-II and A1-III (12) with different substrate specificities (11), indicating that A1-I has three independent active sites for a protease and two alginate lyases in a molecule. Although A1-III efficiently liquefies acetylated alginates produced by mucoid cells of *P. aeruginosa* derived from the lungs of CF patients, A1-II preferred nonacetylated alginate produced by brown seaweeds (11). Before the modification and molecular design of edible alginate with alginate lyases (A1-I, A1-II, and A1-III) and the application of alginate lyases (A1-I and A1-III) to the treatment of CF caused by *P. aeruginosa*, the overproduction of them and clarification of their mode of action are required. We have already constructed the overexpression system of A1-III in *Bacillus subtilis* (13). However, it is difficult to optimize the culture condition of *B. subtilis* for production of A1-III, since the formation of the enzyme is significantly influenced by the time of inducer addition and aeration of the culture, in addition to the complicated medium for *B. subtilis*. Here, we describe overexpression systems for these three kinds of alginate lyases, A1-I, A1-II, and A1-III, in *Escherichia coli* cells, purification of them, and comparison of them with respect to their mode of action.

MATERIALS AND METHODS

Materials

Sodium alginate (MW 25,600; 1000 cp) from *Eisenia bicyclis* was purchased from Nacalai Tesque Co. Ltd. (Kyoto, Japan). PolyM and polyG were kind donations from Dr. T. Muramatsu (Nagasaki University, Nagasaki, Japan). Restriction endonucleases, DNA-modifying enzymes, and *E. coli* DH5α-competent cells were purchased from Takara Shuzo Co. (Kyoto, Japan) and Toyobo Co. (Tokyo, Japan). Expression vectors of pET-3a and pET-17b were from Novagen (Madison, WI). Silica gel 60/Kieselguhr F_{254} thin-layer chromatography (TLC) plates were obtained from E. Merck (Darmstadt, Germany). Bio-Gel P2 was from Bio-Rad Laboratories (Hercules, CA), Butyl-Toyopearl 650M and Toyopearl HW65 were from Tosoh Co. (Tokyo, Japan), and SP–Sepharose and SP–Sephadex were from Pharmacia Biotech. Co. (Uppsala, Sweden).

Microorganisms

Six kinds of *E. coli* strains [BL21(DE3), BL21(DE3)pLysE, BL21(DE3)pLysS, HMS174(DE3), HMS174(DE3)pLysE, and HMS174(DE3)pLysS] purchased from Novagen were used as hosts of expression of alginate lyases.

Construction of Plasmids

To subclone A1-I, A1-II, and A1-III genes into the expression vectors, polymerase chain reaction (PCR) was performed by using pALY1-3 (14) as a template and two synthetic oligonucleotides (Table 1) with *Nde*I and *Bam*HI sites added at the terminal as primers. The A1-I, A1-II, and A1-III genes amplified by PCR were digested with *Nde*I and *Bam*HI and ligated with the *Nde*I- and *Bam*HI-digested expression vectors (pET3a and/or pET17b). The resultant plasmids containing A1-I, A1-II, and A1-III genes were designated pET3a–A1-I, pET17b–A1-II, and pET3a–A1-III, respectively.

Sequence and Manipulations of DNA

The nucleotide sequences of A1-I, A1-II, and A1-III genes amplified by PCR were determined by dideoxy-chain termination method using automated DNA sequencer Model 377 (Applied Biosystems Division of Perkin–Elmer, Foster City, CA) (15). Subcloning, transformation, and gel electrophoresis were performed as described previously (16).

Assays for Alginate Lyase and Protein Concentration

The enzyme was incubated at 25°C in 1 ml of a reaction mixture containing 0.1% alginate and 50 mM Tris–HCl buffer, pH 7.2, and the activity was deter-

TABLE 1

Oligonucleotides Used as Primers for PCR

Sequence of oligonucleotides	Annealing sites
5′-CTGCATATGCACCCCTTCGACCAGGCCGTCGTG-3′	5′-region of *a1-I*
5′-CCGGATCCTCAGCTCGAGTGCTTTACGTGGAG-3′	3′-region of *a1-I*
5′-GGCGCCGCATATGGCCCCGGCAGCGGCGCATTCGTCG-3′	5′-region of *a1-II*
5′-CGGGATCCTCAGCTCGAGTGCTTTACGTCGAG-3′	3′-region of *a1-II*
5′-CTGCATATGCACCCCTTCGACCAGGCCGTCGTG-3′	5′-region of *a1-III*
5′-CCGGATCCTCAGGACACCGGCGCCTGCGCTGC-3′	3′-region of *a1-III*

Note. CATATG, *Nde*I site; GGATCC, *Bam*HI site.

mined by monitoring the increase of the absorbance at 235 nm arising from the double bond formed in the reaction products. One unit (U) of the enzyme activity was defined as the amount of enzyme required to produce the increase of 1.0 in absorbance at 235 nm per minute. Protein content was determined by the method of Lowry *et al.* (17), with bovine serum albumin as a standard, or by measuring absorbance at 280 nm assuming that $E_{280} = 1.0$ corresponds to 1 mg/ml.

Purification of A1-I, A1-II, and A1-III

E. coli cells producing A1-I, A1-II, and A1-III were grown in 15 liters of LB medium (16) at 30°C for 8 h and then isopropyl-β-D-thiogalactopyranoside was added to the cultures at a concentration of 1 mM, followed by continuous culture for 16 h. The cells were harvested by centrifugation at 13,000*g*, 4°C for 5 min, washed in 5 mM Tris–HCl buffer, pH 7.2, and then resuspended in the same buffer to disrupt them ultrasonically (Insonator, Kubota Model 201M, Tokyo, Japan) at 0°C, 9 kHz for 40 min. The clear solution obtained after centrifugation at 15,000*g*, 4°C for 20 min was dialyzed against 5 mM Tris–HCl buffer, pH 7.2, and used as cell extracts.

For purification of A1-I, proteins in cell extracts were precipitated with ammonium sulfate (35–60% saturation), recovered, and then dissolved in 35% saturated ammonium sulfate solution. The solution was applied to a Butyl-Toyopearl 650M column (2.6 × 10 cm) equilibrated with 50 mM Tris–HCl buffer, pH 7.5, saturated with ammonium sulfate (30%). The enzyme was eluted with a linear gradient of ammonium sulfate (from 30 to 0%) in 50 mM Tris–HCl buffer, pH 7.5 (210 ml), and 15-ml fractions were collected every 5 min. Active fractions, which were eluted with 21–8% saturated ammonium sulfate, were dialyzed against 20 mM Tris–HCl buffer, pH 7.5, containing 0.5 mM EDTA. The enzyme solution was applied to a SP–Sepharose column (1.6 × 10 cm) equilibrated with 50 mM Tris–HCl buffer, pH 7.5. The enzyme was eluted with a linear gradient of NaCl (from 0 to 0.4 M) in 50 mM Tris–HCl buffer, pH 7.5 (210 ml), and 1.5-ml fractions were collected every 0.5 min. Active fractions eluted with about 0.15 M NaCl were used as a purified A1-I enzyme.

For purification of A1-II, the cell extract was applied to a SP–Sephadex column (5.5 × 30 cm) equilibrated with 20 mM Tris–HCl buffer, pH 7.5, and proteins were eluted stepwise with 20 mM Tris–HCl buffer, pH 7.5, containing 0.4 M NaCl. The enzyme fraction was dialyzed against 50 mM Tris–HCl buffer, pH 7.5, containing 30% saturated ammonium sulfate, and then applied to a Butyl-Toyopearl 650M column (2.6 × 10 cm) equilibrated with 30% saturated ammonium sulfate in 50 mM Tris–HCl buffer, pH 7.5. The enzyme was eluted with a linear gradient of ammonium sulfate (from 30 to 0%) in 50 mM Tris–HCl buffer, pH 7.5 (34 ml), and 1.5-ml fractions were collected every 0.5 min. Active fractions, which were eluted with 28–25% saturated ammonium sulfate, were dialyzed against 20 mM Tris–HCl buffer, pH 7.5. The enzyme was applied to a SP–Sepharose column (1.6 × 10 cm) equilibrated with 20 mM Tris–HCl buffer, pH 7.5, and eluted with a linear gradient of NaCl (from 0 to 0.4 M) in 20 mM Tris–HCl buffer, pH 7.5 (21 ml), and 1.5-ml-fractions were collected every 0.5 min. Active fractions, which were eluted with about 0.18 M NaCl, were used as a purified A1-II enzyme.

The A1-III was purified as follows. The cell extract after the saturation with ammonium sulfate (30%) was applied to a Butyl-Toyopearl 650M column (2.6 × 10 cm) equilibrated with 30% saturated ammonium sulfate in 20 mM Tris–HCl buffer, pH 7.5. The enzyme was eluted with the same buffer and active fractions were pooled. The enzyme solution was dialyzed against 5 mM sodium phosphate buffer, pH 6.0, and then applied to a SP–Sepharose column (1.6 × 10 cm) equilibrated with 5 mM sodium phosphate buffer, pH 6.0. The enzyme eluted stepwise with 20 mM sodium phosphate buffer, pH 7.5, was used as a purified preparation of A1-III.

TABLE 2

Purification of A1-I, A1-II, and A1-III from *E. coli* cells

Step[a]	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
A1-I					
Cell extract	8,010	52,478	6.55	100	1.0
Ammonium sulfate (35–60%)	3,249	32,680	10.1	62.3	1.5
Butyl-Toyopearl 650M	435	19,530	44.9	37.2	6.9
SP–Sepharose	198	14,478	73.1	27.6	11.2
A1-II					
Cell extract	10,695	45,659	4.27	100	1.0
SP–Sephadex	1,243	38,369	30.9	80.4	7.2
Toyopearl HW-65	244	23,994	98.3	52.5	23.0
SP–Sepharose	189	20,534	109	45.0	25.5
A1-III					
Cell extract	3,467	31,894	9.20	100	1.0
Butyl-Toyopearl 650M	2,067	25,420	12.3	79.7	1.3
SP–Sepharose	431	19,380	45.0	60.8	4.9

[a] Purification procedures are described under Materials and Methods.

Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done as described previously (18).

N-terminal Amino Acid Sequences of A1-I, A1-II, and A1-III

N-terminal amino acid sequences of the enzymes were determined by Edman degradation using Procise 492 protein sequencing system (Applied Biosystems Division of Perkin–Elmer).

Thin-Layer Chromatography

Alginate-depolymerization products were analyzed by TLC using a solvent system of 1-butanol:acetic acid: water (3:2:3, v/v/v). The products were visualized by heating the TLC plate at 130°C for 5 min after spraying with 10% (v/v) sulfuric acid in ethanol. Unsaturated saccharides on TLC plates were detected by thiobarbituric acid spray (19).

Depolymerization of Alginate by A1-I and A1-II

Reactions were performed at 30°C in a mixture consisting of 0.5% sodium alginate and purified enzymes. The alginate depolymerization products were periodically analyzed by TLC. PolyM and polyG (0.5%) were also used as a substrate.

Purification of Alginate-Degrading Products

The alginate-degrading standard markers were prepared by gel filtration using a Bio-Gel P2 column (0.9 by 122 cm) previously equilibrated with distilled water as described (20).

RESULTS AND DISCUSSION

Overexpression of A1-I, A1-II, and A1-III in E. coli Cells

After the construction of plasmids (pET3a–A1-I, pET17b–A1-II, and pET3a–A1-III), the accuracy of nucleotide sequences of amplified A1-I, A1-II, and A1-III genes were confirmed by DNA sequencing. In order to select the best host, six kinds of *E. coli* strains were transformed with each plasmid of pET3a–A1-I, pET17b–A1-II, and pET3a–A1-III. As a result, the transformant of BL21(DE3)pLysE with pET3a–A1-I, that of BL21(DE3)pLysE with pET17b–A1-II, and that of HMS174(DE3)pLysS with pET3a–A1-III showed the highest alginate lyase activities of A1-I, A1-II, and A1-III (3.50, 3.04, and 2.13 kU/liter of culture), respectively. The expression levels of the lyases in the transformants with pET3a–A1-I, pET17b–A1-II, and pET3a–A1-III were over 10-fold higher than those in *Sphingomonas* sp. A1 (21).

Purification and Characterization of A1-I, A1-II, and A1-III from E. coli Cells

A1-I, A1-II, and A1-III were purified 11.2-, 25.5-, and 4.89-fold from cell extracts of *E. coli* transformants with activity yields of 27.6, 45.0, and 60.8%, respectively (Table 2). All of the lyases purified through two to three purification steps were homogeneous on SDS–PAGE gel (Fig. 2). As the properties of A1-III expressed in *B. subtilis* have been characterized (13,20), those of A1-I and A1-II were mainly investigated here.

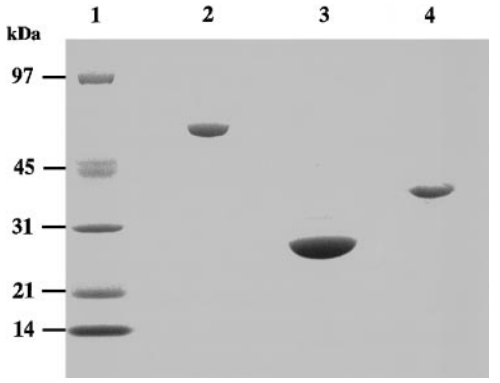

FIG. 2. Electrophoretic profile of A1-I, A1-II, and A1-III. The purified A1-I, A1-II, and A1-III from *E. coli* cells were subjected to SDS–PAGE. Lane 1, molecular weight standards (from top): phosphorylase *b* (97,400); ovalbumin (45,000); carbonic anhydrase (31,000); trypsin inhibitor (21,500); and lysozyme (14,400). Lane 2, A1-I; lane 3, A1-II; lane 4, A1-III.

Molecular mass. A1-I, A1-II, and A1-III were monomeric enzymes with molecular masses of 63, 25, and 40 kDa as determined by SDS–PAGE (Fig. 2) and by gel permeation chromatography (Sephacryl S-200HR) (data not shown). These values were in good agreement with those calculated from their DNA sequences.

pH and temperature. A1-I and A1-II were most active at pH 8.0 in 50 mM Tris–HCl buffer, and at 45 and 70°C, respectively. About 50% activity of A1-I and A1-II was lost after incubation in Tris–HCl buffer (pH 7.2) for 10 min at 45 and 50°C, respectively.

Metal ions and others. The reaction was performed in the presence or absence of various compounds and residual activity was measured (data not shown). A divalent metal ion (Hg^{2+}) partially (40%) inhibited the reactions of A1-I and A1-II at 1 mM. Other divalent metal ions such as Ca^{2+}, Co^{2+}, Mg^{2+}, and Mn^{2+} had no effect on both enzyme activities at 1 mM. Thiol reagents, such as dithiothreitol, glutathione (reduced form), 2-mercaptoethanol, and iodoacetic acid, and a chelator of EDTA (1 mM each) revealed no significant effect on both enzyme activities. Any sugars (L-fucose, D-galactose, D-glucose, D-glucuronic acid, D-mannose, L-rhamnose, and D-xylose) showed no effects on reactions of A1-I and A1-II at 10 mM.

N-terminal amino acid sequence. N-terminal amino acid sequences of A1-I, A1-II, and A1-III were determined to be HPF, PAAAH, and MHPFD, which corresponded to ^{2}HPF^{4}, ^{3}PAAAH^{7}, and ^{1}MHPFD^{5} of predicted ones of A1-I, A1-II, and A1-III from base sequences, respectively. This result shows that N-terminal regions of A1-I and A1-II were modified by *E. coli* aminopeptidase, while A1-III was expressed without modification.

Substrate specificity. Although the effect of acetylation in alginate molecule on the alginate lyase activities has been slightly investigated (11), the detailed substrate specificity of A1-I and A1-II was determined by incubating enzymes at 30°C in a mixture containing 50 mM Tris–HCl buffer, pH 7.2, and various substrates (0.5%) (Fig. 3). A1-I acted on both polyM and polyG, while A1-II preferably depolymerized polyG rather than polyM. Both enzymes were inert on polysaccharides (gellan, xanthan, and pectin) containing the uronyl residues.

Action mode. Formation of various kinds of alginate-depolymerization products was observed in the early stage of reaction by A1-I and A1-II, (Figs. 4A and 4B), thus indicating that both enzymes function in an endolytic manner. After prolonged reaction, three spots corresponding to di-, tri, and tetrasaccharides were detected as the final products of both enzyme reactions (Figs. 4A and 4B). In the case of A1-I, tetrasaccharide was finally converted into disaccharide (data not shown), indicating that A1-I uses tetrasaccharide as a minimum substrate and yields di- and trisaccharides as the final products, while the disaccharide released by A1-II was extremely low in amount and the tetrasaccharide was not further degraded by A1-II. The final products from alginate by A1-I and A1-II were coincident with those from polyM and polyG by A1-I and from polyG by A1-II, respectively (Fig. 3). Therefore, A1-II acts on the pentasaccharide as a minimum substrate and releases tri- and tetrasaccharides as the major final products. The action mode of A1-I was found to be similar to that of A1-III, which produces di- and trisaccharides as the final products and acts on tetrasaccharide as a minimum substrate (20). However, A1-II was found to be different from A1-III in mode of action and substrate specificity. All of the products of A1-I and A1-II reactions were confirmed to have unsaturated saccharides at the nonreducing ter-

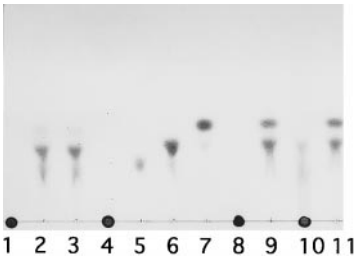

FIG. 3. Substrate specificity of A1-I, A1-II, and A1-III. The reaction was performed at 30°C for 24 h in a mixture consisting of 0.5% polyG or polyM and purified A1-I, A1-II, and A1-III (100 mU). The reaction products were analyzed by TLC. Lane 1, polyG; lane 2, reaction mixture of polyG and A1-I; lane 3, reaction mixture of polyG and A1-II; lane 4, reaction mixture of polyG and A1-III; lane 5, alginate–tetrasaccharide; lane 6, alginate–trisaccharide; lane 7, alginate–disaccharide; lane 8, polyM; lane 9, reaction mixture of polyM and A1-I; lane 10, reaction mixture of polyM and A1-II; lane 11, reaction mixture of polyM and A1-III. Alginate–polymers remain at origin on the TLC plate.

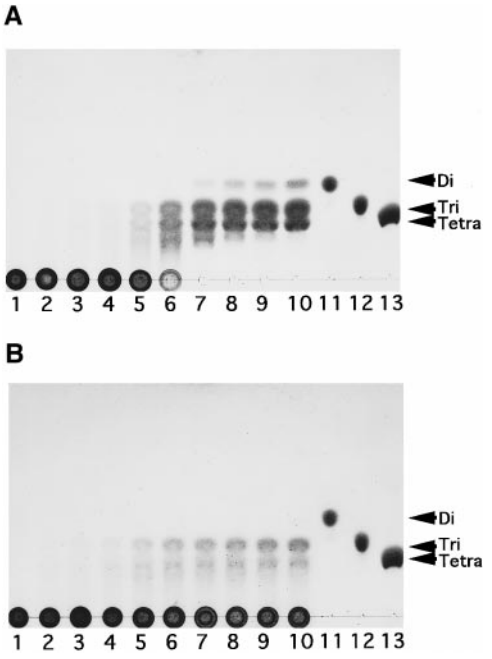

FIG. 4. Degradation of alginate by A1-I and A1-II. The reaction was performed at 30°C in a mixture consisting of 0.5% sodium alginate and purified A1-I (A) or A1-II (B) (100 mU). The reaction products were periodically analyzed by TLC. Reaction times: lane 1, 0 min; lane 2, 10 min; lane 3, 20 min; lane 4, 30 min; lane 5, 60 min; lane 6, 3 h; lane 7, 6 h; lane 8, 9 h; lane 9, 12 h; lane 10, 24 h; lane 11, alginate–disaccharide; lane 12, alginate–trisaccharide; and lane 14, alginate–tetrasaccharide.

minal, since these products reacted with TBA reagent (data not shown). The result indicates that both enzymes function as lyase, but not hydrolase.

Thus, in this study, we have constructed overexpression systems of alginate lyases (A1-I, A1-II, and A1-III) of *Sphingomonas* sp. A1 and characterized the enzymes. These alginate lyases can cleave the glycosidic linkage in alginate and produce di-, tri, and tetrasaccharides with unsaturated uronyl residues at the nonreducing terminal. Because A1-II is specific to polyG just like a *Klebsiella pneumoniae* alginate lyase (22) and A1-III is specific to polyM (20), A1-I consisting of A1-II and A1-III can act on both polymers. Generally, almost all alginate lyases elucidated so far are known to be active on mannuronyl linkage of alginate (23) with the exception of extracellular alginate lyases from *Bacillus circulans* 1351 (24) and *Klebsiella aerogenes* type 25 (25) depolymerizing both of polyG and polyM. Therefore, A1-I is the first cytoplasmic enzyme acting on both polyM and polyG, endolytically.

The easy and massive preparation of purified alginate lyases will facilitate preparation of oligo-alginates, their application to medical uses, and the clarification of their relationship between structure and function by X-ray crystallographic study. In fact, we have already determined the three-dimensional structure of A1-III (26) and crystallized A1-II (27). This structural information is useful for the determination of different substrate specificities of A1-II and A1-III. The determination of the crystal structure of A1-I having three independent active sites for a protease and two alginate lyases is in progress.

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