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Purification and Characterization of Alginate Lyase from *Streptomyces* Species Strain A5 Isolated from Banana Rhizosphere

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To characterize the alginate lyase produced by rhizosphere *Streptomyces*, *Streptomyces* sp. A5 was isolated from banana rhizosphere, and its extracellular lyase was purified to an electrophoretically homogeneous state. The lyase has a molecular mass of 32 kDa by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The optimum temperature and pH were 37 °C and pH 7.5, respectively. Ninety-two percent of the activity was lost after incubation at 70 °C and pH 7.5 for 20 min. The enzyme was inhibited by 0.05 M SDS and 2 mM Hg^{2+}, Cu^{2+}, and Fe^{3+}, but EDTA enhanced the enzyme activity. The K_m value of the lyase was 0.13 mg mL^{-1} with the substrate sodium alginate. The lyase had substrate specificity for polyguluronate units in the alginate molecules. The alginate oligomers prepared by the lyase show growth-promoting activity on the roots of banana plantlets. These results indicated that the encapsulation method using alginate microbeads to inoculate beneficial streptomycete strains might be beneficial to the root growth of banana plantlets.

KEYWORDS: Alginate; alginate lyase; banana; rhizosphere; *Streptomyces*

INTRODUCTION

Alginates are linear polysaccharides composed of (1,4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G), arranged in homopolymeric (MM- or GG-blocks) or heteropolymeric random sequence (MG- or GM-blocks) (*1*). Owing to their gelling ability, stabilizing properties, and high viscosity, alginates extracted from seaweed are widely used as stabilizers, viscosifiers, and gelling agents in the food and beverage, paper and printing, biomaterials, and pharmaceutical industries (*2*).

Recent studies have revealed that alginate hydrolysates exhibit many important bioactivities, such as promoting growth of *Bifidobacteria* spp., accelerating plant root growth, stimulating human keratinocytes, repressing the growth of HeLa cells, improving the function of β-lactogobulin, and enhancing phagocytic activity of macrophages (*2*−*4*). Alginates are degraded by a group of enzymes that catalyze the β-elimination of the 4-O-linked glycosidic bond with formation of unsaturated uronic acid-containing oligosaccharides. Therefore, alginate lyases (EC 4.2.2.3) are crucial in generating oligomeric alginates for innovative endeavors, analyzing alginate fine structure, and protoplasting of red and brown algae (*2*, *5*). The use of alginate lyase for the treatment of alginate polysaccharide buildup in the lungs of cystic fibrosis (CF) patients remains one of the most important goals of studying alginate lyase (*6*). Nowadays, the lyases from many sources, including marine algae, marine mollusks, bacteria, fungi, and bacteriophages, have been characterized (*2*). Although a *Streptomyces coelicolor* gene encoding a putative alginate lyase has been identified, no alginate lyase activity produced by *Streptomyces* strains has been characterized (*7*).

Streptomyces species are well-known saprophytic bacteria in soil and may influence plant growth and protect plant roots against invasion by root pathogenic fungi (*8*). It is well-known that the key to achieving successful results following the introduction of beneficial microbes in soil relies on the survival rate of the inoculated bacteria in a heterogeneous soil environment; the encapsulation method using alginate can be effectively used to protect the plant growth promoting bacteria (PGPB) inoculum from adverse conditions of the soil for their successful establishment in the rhizosphere (*9*, *10*). In Japanese agricultural tradition, marine algae (e.g., *Laminaria japonica*) have been plowed into fields before crop cultivation. The alginate-rich algae could be degraded by alginate lyase-producing soil bacteria, hence releasing oligosaccharides that promote root growth in plants (*11*).

Banana fusarium wilt is regarded as one of the most destructive diseases of banana production, and a cost-effective measure of control for the disease is still not available. Biological control of the pathogenic fungi is a complementary approach for managing fusarium wilt. The introduction of biocontrol *Streptomyces* strains into plantlets is facile in application for banana plantlets derived from tissue culture

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planting material have fewer microorganisms in the banana plant tissue. The encapsulation method using alginate can be effectively used to inoculate biocontrol streptomycetes into banana plants. However, the characteristics of lyase-producing *Streptomyces* from banana rhizosphere are still unknown. Characterization of alginate lyases will enhance and expand the use of these lyases in various industrial, agricultural, and medical fields. In this paper, we describe for the first time the isolation of alginate lyase-producing *Streptomyces* sp. strain A5 from banana rhizosphere and some properties of the lyase.

MATERIALS AND METHODS

Materials. Sodium alginate (1% aqueous solution, 120 mpas) from *Laminaria japonica* was purchased from Qingdao Nanshan Seaweed Co. Ltd., China, and the ratio of M/G was 2.28. Sephadex G-100 was purchased from Amershan Biosciences; DEAE-cellulose was from Sigma Chemical Co. (St. Louis, MO).

Isolation of Alginate Lyase-Producing *Streptomyces* Strains. Roots of banana (*Musa* sp. AAA *cavendish* subgroup. cv. Williams) were dug out carefully and washed in sterilized water to remove soil particles. The washed roots were immersed in 5 mL of sterilized water and stirred for 5 min, and then 100 μL of the supernatant fluid was spread on a Petri dish containing 15 mL of agar medium (1000 mL of water containing 0.1% KNO_3, 0.05% K_2HPO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 0.5% alginate, and 1.5% agar). After incubation at 26 °C for 5 days, *Streptomyces* colonies grown on the agar plates were picked and screened for alginate lyase. Lyase production during the cultivation period was monitored by measuring the viscosity of medium using an Ostwald viscosimeter and the absorbance of uronic acid at 235 nm (*12*).

Identification of Isolate A5. *Streptomyces* sp. A5 was identified by morphological characteristics and 16S rRNA gene sequence analysis. Morphology of the screened strain was studied using light microscopy. The 16S rRNA gene sequence of the *Streptomyces* strain was amplified and sequenced as described previously (*13*).

Purification of Alginate Lyase. For the purification of extracellular alginate lyase, cells of *Streptomyces* sp. strain A5 were aerobically cultured at 30 °C and 150 rpm for 7 days in a liquid alginate medium consisting of 0.1% KNO_3, 0.05% K_2HPO_4, 0.04% $MgSO_4 \cdot 7H_2O$, and 1% alginate. Unless otherwise specified, all operations were carried out at 4 °C. The culture medium was filtered through glass wool to remove streptomycete mycelia, followed by centrifugation to remove any spores and other particles. The addition of 80% ammonium sulfate precipitated the proteins in the culture broth. The resulting precipitate was collected by centrifugation and dissolved in 50 mM phosphate buffer (pH 7.0), and then the solution was dialyzed overnight against the same buffer. The dialyzed sample was lyophilized and applied in 4 mL aliquots to a DEAE-cellulose column (2.5 × 40 cm) previously equilibrated with phosphate buffer (pH 7.0) and eluted with a linear gradient of NaCl (0−2.0 M in a total volume of 2 L) in the same buffer. The active fraction eluted with 0.8 M NaCl was lyophilized, suspended in 1 mL of 50 mM phosphate buffer (pH 7.0), and subjected to a Sephadex G-100 column (2.5 × 40 cm), which had been equilibrated with 50 mM phosphate buffer (pH 7.0) and eluted with the same buffer. The active fraction was concentrated and used as a purified enzyme preparation throughout this study. The lyase activity was assayed with sodium alginate as the substrate by measuring the increase in absorbance at 235 nm due to the formation of a double bond between C-4 and C-5 at the new nonreducing terminus by the β-elimination reaction. Protein was determined by measuring absorbance at 280 nm with bovine serum albumin as a standard. The purity of the purified lyase was confirmed by the SDS-PAGE according to the Laemmli method (*14*). The proteins were visualized using Coomassie brilliant blue R-250.

Alginate Lyase Assays. All of the assays for biochemical characterization were made with the purified enzyme resuspended in 50 mM Tris-HCl buffer (pH 7.5). An aliquot (0.2 mL) of the enzyme solution was added to 2.8 mL of substrate solution of 0.2% sodium alginate in 50 mM Tris-HCl buffer (pH 7.5) incubated previously at 37 °C. At certain time intervals, enzymatic reaction was terminated by heating the mixture in boiling water for 5 min. The change in absorbance at 235 nm of the supernatant was measured (*15*). The lyase activity was quantitatively measured by the thiobarbituric acid method (*16*). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of β-formylpyruvic acid per minute.

Characterization of the Purified Lyase. To determine the optimal temperature of lyase, enzyme reactions were carried out at 23, 30, 37, 50, 60, and 80 °C for 30 min. The thermostability of the lyase was investigated by measuring the residual activity at 50 and 70 °C with the same assay after the enzymes were incubated for 20, 40, 60, and 80 min at various temperatures in Tris-HCl buffer. The pH optimum was examined at 37 °C in the pH range of 6.0−9.0 with Tris-HCl buffer. The effects of metal ions, enzyme inhibitors, and detergents on lyase activity were studied by determining enzyme activity after incubation with different compounds for 1 h at 4 °C as mentioned above. To determine the substrate specificity of alginate lyase, the polymannuronate-rich and polyguluronate-rich substrates were prepared by partial acid hydrolysis of the alginate mentioned previously (*17*), and the substrate specificity of alginate lyase was distinguished according to the method described by Nakagawa et al. (*12*).

Effect of Alginate Lyase-Lysate on Root Growth of Banana. Alginate oligosaccharides were prepared and extracted using the method described by Zhang et al. (*3*). Alginate (5 g) was dissolved in 1 L of 50 mmol/L Tris-HCl buffer (pH 7.5), and 50 units of lyase was added. The reaction was carried out at 37 °C for 24 h, and then the solution was heated to 100 °C for 10 min to stop the reaction. Ethanol was added to the solution until the content of ethanol reached 50% (v/v), and the mixture was centrifuged. The supernatant was filtered through 0.45 μm membranes, lyophilized, and stored at 4 °C. The effect of oligosaccharides on root elongation of banana plantlets was determined using the method described by Xu et al. (*18*). The roots were cut into 0.8−1.2 cm lengths and cultured on B5 medium containing 0.75 mg/mL oligosaccharide mixtures. The roots were incubated at 25 °C under dark conditions.

Statistical Analysis. Data were subjected to analysis of variance (ANOVA), and the mean values of root length were statistically analyzed using Student's *t* test. Differences were considered to be significant when probability was <0.05.

RESULTS

Isolation and Identification of *Streptomyces* Species A5. Thirteen *Streptomyces* strains were isolated and tested to produce alginate lyase. Four of them showed lyase activity as determined by a decrease in viscosity of alginate medium. The lyase activity of isolate A5 was the highest, with a decrease in viscosity of 90%; the remaining three strains showed rates in the range of 30−40%. Isolate A5 grew aerobically on minimal medium with alginate as the sole carbon source. The aerial spore mass is white to pink, the branching substrate mycelium was colorless, mycelia did not fragment into coccoid or bacillary structures, and soluble pigments, including melanin, are not produced. Spore chains are straight, with 10−30 spores per chain. The morphological and cultural characteristics provide evidence that strain A5 should be assigned to the genus *Streptomyces*. A partial 16S rRNA gene sequence (541 bp, accession no. AJ968406) was determined to confirm the identity because some *Nocardioides* spp. or *Kitasatospora* spp. might be misidentified as *Streptomyces* according to morphological characteristics. A search of databases for similar sequences by BLASTN confirmed that the isolate could be grouped in the *Streptomyces* clade. The highest similarity of 96% was shown between the strain A5 and *Streptomyces stramineus* NRRL 12279, *Streptomyces griseorubens* AS 4.1839, *Streptomyces labedae* IFO 15864, *Streptomyces lateritius* IFO 12788, *Streptomyces erythrogriseus*, *Streptomyces griseoflavus*, and *Streptomyces griseoincarnatus*.

Table 1. Purification of Lyase from *Streptomyces* Species A5

fraction	total protein (μg)	total activity (units)[a]	specific activity[b] (units μg^{-1} of protein)	purification (-fold)	yield (%)
supernatant fluid	234	1681	7.18	1.0	100
80% NH_4SO_4	15.1	528	34.97	4.9	31.4
DEAE-cellulose chromatography	6.76	314	46.45	6.5	18.7
Sephadex G-100 chromatography	1.26	128	101.6	14.2	7.6

[a] One unit of lyase activity is defined as the amount of enzyme that increases the absorbance at 235 nm to 0.01 for 1 min. [b] Specific activity = total activity/total protein.

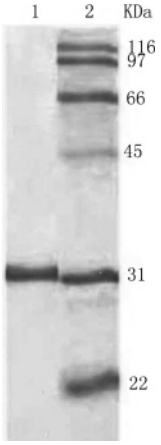

Figure 1. SDS-PAGE of the purified alginate lyase (lane 1); lane 2, standard proteins, sizes indicated on the right.

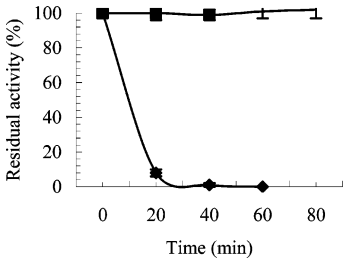

Figure 2. Thermostability of the lyase from *Streptomyces* sp. A5 for different periods of time at different temperatures (50 °C, ■; 70 °C, ◆). Residual activities were calculated with the activity of the nonincubated enzyme sample set as 100%.

The 16S rRNA gene sequence of strain A5 differed from the sequences of other *Streptomyces* species by one or two nucleotides. The results suggested that strain A5 is a member of the genus *Streptomyces*.

Properties of Alginate Lyase. A summary of the purification of lyase from the culture medium of a 7-day-old culture of isolate A5 is presented in **Table 1**. The lyase was purified using the ammonium sulfate precipitation method and DEAE-cellulose chromatography. The lyase was purified 6.5-fold, with a yield of 19%. The second purification step by Sephadex G-100 gel chromatography yielded a homogeneous protein, as shown by SDS-PAGE (**Figure 1**). The overall purification factor was about 14-fold, and the final yield was 8%. The final product had a specific activity of about 102 units μg^{-1} of protein. The molecular mass of the purified alginate lyase was estimated as 32 kDa (**Figure 1**). The enzyme was most active at 37 °C and pH 7.5 in Tris-HCl buffer. Ninety-two percent of the activity was lost after incubation at 70 °C and pH 7.5 for 20 min (**Figure 2**); hence, it is a labile lyase. Divalent metal ions Ba^{2+}, Mn^{2+}, and Mg^{2+} showed no effects on lyase activity at 2 mM (**Table 2**); however, Hg^{2+} and Cu^{2+} exerted a significantly inhibitory effect. At a concentration of 2 mM, Fe^{3+} reduced the activity by 96%. The enzyme was inhibited by the denaturant SDS at a concentration of 0.05 M. At a concentration of 5 mM, EDTA enhanced the enzyme activity by 36%. The substrate specificity of the lyase was evaluated using sodium alginate, polymannuronate [poly(M), 85% purity], and polyguluronate [poly(G), 90% purity] extracted from alginate. The lyase degraded poly(G) more efficiently than the other substrates, indicating a preference for poly(G) (**Table 3**). The kinetics of the alginate lyase was measured using different concentrations of sodium alginate. The K_m and V_{max} values of the lyase were evaluated from a Lineweaver−Burk plot and found to be 0.13 mg mL^{-1} and 1.17 units mL^{-1}, respectively. Sodium alginates showed no significantly different effect on root growth as compared to the control. By contrast, enzymatically depolymerized alginate oligomers have significantly different effects on root elongation compared to alginate ($P < 0.05$) (**Figure 3**). The results suggest that alginate oligomers, but not alginate polyers, are mainly responsible for the root elongation of banana plantlets.

Table 2. Effect of Chemicals on *Streptomyces* Species A5 Alginate Lyase Activity

chemical	concentration	relative activity[a] (%)
control		100
$CaCl_2$	2 mM	86
$BaCl_2$	2 mM	102
$MnCl_2$	2 mM	104
$HgCl_2$	2 mM	22
$MgCl_2$	2 mM	100
$ZnCl_2$	2 mM	85
$CoCl_2$	2 mM	68
$CuCl_2$	2 mM	29
Fe_2Cl_3	2 mM	4
1,10-phenanthroline	1 mM	94
EDTA	5 mM	136
SDS	0.05 M	30
SDS	0.01 M	87

[a] Values are the mean of three independent determinations and expressed as a percentage of control activity (set as 100%).

Table 3. Substrate Specificity of Alginate Lyase from *Streptomyces* Species Strain A5

substrate	specific activity[a] (units/μg)
poly(M)	18 (19%)
poly(G)	128 (133%)
sodium alginate	96 (100%)[b]

[a] One unit of lyase activity is defined as the amount of enzyme that increases the absorbance at 235 nm to 0.01 for 1 min. Specific activity of the lyase was expressed as the units per microgram of protein. [b] The activity of lyase toward sodium alginate was taken as 100%.

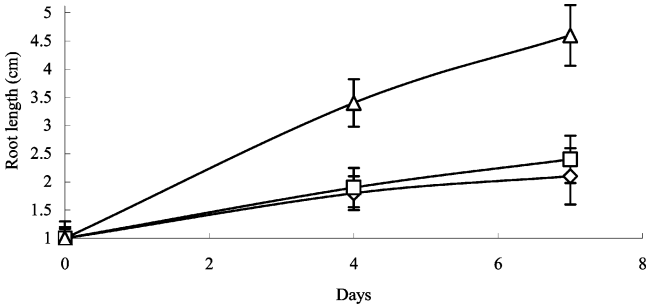

Figure 3. Effect of sodium alginate lyaste mixtures on root elongation of banana plantlets (control, ◇; sodium alginate, □; oligoalginte mixtures, △). Each point represents the mean ± SD ($n = 10$).

DISCUSSION

In this study, we have purified and characterized extracellular polyguluronate specific lyase from the *Streptomyces* sp. strain A5 isolated from banana rhizosphere. Although ORFs similar to that of the extracelluar polyguluronate lyase gene of *Corynebacterium* sp. ALY-1 were found from *Streptomyces coelicolor* genomes (*7*) and *Streptomyces* sp. MET0515 (accession no. AF513503.1), few properties are known about the putative polyguluronate lyase of *Streptomyces* spp. The lyase produced by *Streptomyces* sp. strain A5 is different from that of *Corynebacterium* sp. ALY-1. The lyase of *Corynebacterium* sp. ALY-1 is most active at around pH 7.0 and 55 °C and depends on Mn^{2+}, Mg^{2+}, Ni^{2+}, Ca^{2+}, and Zn^{2+} for optimal activity (*2*), but the metal ions showed slight effect on the lyase activity of *Streptomyces* sp. A5 and the optimum temperature and pH were 37 °C and pH 7.5, respectively. The properties are similar to those produced by marine *Pseudomonas* spp., soil *Bacillus* sp. ATB-1015, *Azotobacter chroococcum*, and *Pseudomonas pyringae* (*2*, *12*, *19*−*21*). Lyase activity of *Streptomyces* sp. A5 increased by 5 mM EDTA, indicating that the enzyme does not require divalent cations; probably, the activity was inhibited by some cations.

The lyase of strain A5 showed specificity for polyguluronate units in the alginate molecules. Guluronate-specific lyases predominantly degrade poly(G) and poly(G/M) alginate substrates and show very low processivity toward poly(M). They have been applied to the preparation of polymannuronate blocks from sodium alginate, and poly(M) block-rich alginate exhibited high antitumor activity and stimulate production of cytokines by human monocytes (*2*). Degradation of alginate by guluronate lyase from *Vibrio* sp. 510 produced a high content of di- to tetrasaccharides (98%) and very low yield of pentasaccharide (2%); the result is similar to that for guluronate lyase from *Corynebacterium* sp. ALY-1 (*3*). It had been reported that oligosaccharide obtained by alginate lyase of *Corynebacterium* sp. had a promoting activity toward lettuce root elongation (*22*). Further studies indicated that an unsaturated oligoguluronate mixture from poly(G) showed a pronounced positive effect on root growth of rice and carrot, but oligomannuronate has no or little positive effect on root elongation (*18*). Therefore, the guluronate specific lyase has the potential to be used in the generation of useful oligosaccharides in agriculture.

In this study, we isolated alginate lyase-producing *Streptomyces* strains from banana rhizosphere and found that oligoalginates produced by isolate A5 promote root growth of banana plantlets. The plant growth-promoting bacteria (PGPB) cells encapsulated by alginate could be protected against environmental stresses and released to the soil gradually when soil microorganisms degrade the polymers (*9*, *10*); the alginate lyase-producing strains should be involved in the process. The influence of alginate lyase-producing *Streptomyces* and depolymerized alginates on physiological processes in plants will be further studied.

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