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Purification and Characterization of Microbial Gellan Lyase

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Gellan lyase was purified from the culture fluid of soil samples incubated in a medium containing gellan as a sole carbon source. The enzyme was a monomer with a molecular mass of 140 kDa and was most active at pH 7.5 and 45°C. The enzyme was highly specific to gellan and lowered the viscosity of the polymer.

Gellan is the exopolysaccharide produced by *Sphingomonas paucimobilis* (8) and consists of a linear repeating tetrasaccharide [→3)-β-D-Glc-(1→4)-β-D-GlcA-(1→4)-β-D-Glc-(1→4)-α-L-Rha-(1→] composed of D-glucose (Glc), D-glucuronic acid (GlcA), and L-rhamnose (Rha) (3). Native gellan has *O*-acetyl and glyceryl residues on the D-glucosyl residue adjacent to the D-glucuronyl residue. Though the deacetylated gellan has recently been commercialized for potential food applications, as has been indicated by Mikolajczak et al. (7), the highly viscous properties of gellan have largely limited its utility, particularly in the food industry. Therefore, methods for depolymerization are now sought not only to prepare low-viscosity and low-molecular-mass gellan for novel physiological and food technological functions but also to exploit new areas for the application of gellan in biopolymer-based industries. There are three reports on gellan-degrading microbial enzymes. One such enzyme is sphinganase, and the other is gellan lyase (4, 7, 9). However, no detailed information on the gellan-degrading enzymes, and especially gellan lyase, has been presented, since the enzymes have not been purified. In this article, we describe for the first time the complete purification of microbial gellan lyase and some of its properties.

Microorganisms degrading gellan. When soil (paddy field) samples were cultured in a liquid medium consisting of 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $Na_2HPO_4 \cdot 12H_2O$, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.01% yeast extract with 1% gellan (molecular mass, 5×10^5; deacetylated; Wako Pure Chemicals Co., Osaka, Japan) serving as a carbon source, sufficient growth of microorganisms was observed at pH 7.2 and 30°C. On gellan (1.5%) plates, the microorganisms in the paddy field sample grew well and completely liquefied the plates after prolonged incubation. The microbial cells initially formed a colony on the surface of the plates, and then some of them sank into the plates, making a pit (data not shown). The pit-forming microorganisms were cultured for the purification of gellan-degrading enzyme.

Effect of cell growth on viscosity of gellan medium. The culture was performed in the 0.5% gellan medium, and the relationship between cell growth and the viscosity of the medium was determined (Fig. 1). The viscosity of gellan at 25°C was determined with a concentric cylinder viscometer (Rotovisco RV-11; Haake Co., Karlsruhe, Germany). The viscosity (η) was calculated (in centipoise) from the following equation: $\eta = k \cdot u \cdot s \cdot E \cdot R$ (k, constant of 0.68; u, spindle speed of 6 rpm; s, indication value; E, sensitivity of 0.3; and R, deceleration ratio of 1). The viscosity of the culture decreased with increasing cell growth and reached the level of water when cell growth reached the stationary phase. No change in viscosity was observed in the medium without cells. These data indicate that gellan was depolymerized by certain microbial cells and that the products were utilized for cell growth.

Enzymes involved in gellan degradation. Cells grown for 72 h in the gellan (1%) medium described above were harvested by centrifugation at 4,000 × *g* and 4°C for 10 min, washed in 20 mM potassium phosphate buffer (KPB) (pH 7.0), and then resuspended in the same buffer. The cells were ultrasonically disrupted at 0°C and 90 kHz for 5 min, and the clear solution resulting after centrifugation at 9,000 × *g* and 4°C for 20 min was used as the enzyme source. Protein was determined by the method of Lowry et al. (6), with bovine serum albumin being used as a standard, or by measuring the A_{280} on the assumption that an E_{280} of 1.0 corresponds to 1 mg/ml. Gellan-degrading enzyme (gellan lyase) was assayed in a mixture (1 ml) composed of 0.05% gellan and 50 mM KPB (pH 7.0). After the addition of the enzyme, the mixture was incubated at 30°C for 30 min. The reaction was terminated by immersing the test tubes in boiling water for 5 min, and 0.2 ml of the mixture was removed for testing by the thiobarbituric acid method (10). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of β-formylpyruvic acid per min. Assays for α-L-rhamnosidase and β-D-glucosidase were accomplished with *p*-nitrophenyl sugar derivatives (Sigma Chemical Co., St. Louis, Mo.) being used as substrates (4). One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min. The mixed culture was performed in gellan medium, and enzyme activities (gellan lyase, α-L-rhamnosidase, and β-D-glucosidase) in both the intracellular and extracellular fractions were determined (Table 1). The gellan lyase activity was observed only in the medium. Little enzyme activity was detected when the gellan in the medium was replaced with glucose. On the other hand, α-L-rhamnosidase and β-D-glucosidase activities were detected both in the medium and within cells when gellan was present in growth medium, and these activities were much higher than those in glucose-grown cells. These results indicate that the gellan-degrading enzymes were inducible in the presence of gellan and that α-L-rhamnosidase and β-D-glucosidase were expressed at higher levels in order to efficiently utilize the gellan depolymerization products.

Purification of gellan lyase. Unless otherwise specified, all operations were carried out at 0 to 4°C. Culture broth (10.5 liters) that was incubated at 28°C for 72 h in gellan (1%)

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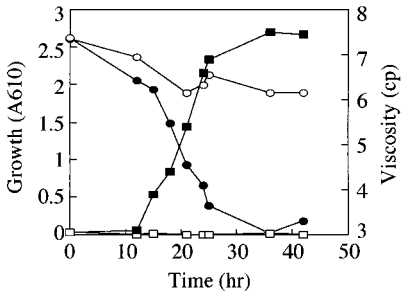

FIG. 1. Effect of cell growth on the viscosity of gellan. The mixed culture was performed in 200 ml of 0.5% gellan medium at 30°C. As a control, 200 ml of 0.5% gellan medium without cells was incubated similarly. For the measurement of cell growth (A_{610}) (squares) and viscosity (circles), 10-ml samples were periodically removed from the media with (solid symbols) and without (open symbols) cells. cp, centipoise.

medium as described above was frozen at −20°C, thawed at room temperature, and then centrifuged at 6,500 × *g* for 10 min. The resultant supernatant was frozen and thawed again as described above, and the supernatant obtained after centrifugation was used as the crude enzyme solution. The crude enzyme solution (7.4 liters) was applied to a DEAE-cellulose (Nacalai Tesque Co., Kyoto, Japan) column (4.7 by 41 cm) previously equilibrated with 20 mM KPB (pH 7.0). The enzyme was eluted with a linear gradient of 0 to 0.7 M NaCl (2 liters) in 20 mM KPB (pH 7.0), and 12-ml fractions were collected every 6 min. The active fractions, which were eluted at 0.3 to 0.4 M NaCl, were combined and saturated with ammonium sulfate (30%). The enzyme solution (420 ml) was applied to a Butyl-Toyopearl 650M (Tosoh Co., Tokyo, Japan) column (2.7 by 17 cm) previously equilibrated with 20 mM KPB (pH 7.0) saturated with ammonium sulfate (30%). The enzyme was eluted with a linear gradient (30 to 0%) of 20 mM KPB (pH 7.0) saturated with ammonium sulfate (500 ml). Fractions (3 ml each) were collected every 5 min. The active fractions, which were eluted at 20 mM KPB (pH 7.0) saturated to 15% with ammonium sulfate, were combined and concentrated to about 3 ml by ultrafiltration with an Amicon model 8200 (Amicon Co., Beverly, Mass.). The concentrate was applied to a Sephadex G-150 (Pharmacia Biotechnology Co., Uppsala, Sweden) column (2.6 by 90 cm) previously equilibrated with 20 mM KPB (pH 7.0). The enzyme was eluted with the same buffer, and 3.1-ml fractions were collected every 30 min. The active fractions eluted between fractions 73 and 77 were combined and concentrated by ultrafiltration as described above. These steps are summarized in Table 2. The preparation at this stage was subjected to native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE as described previously (2, 5). Though the preparation appeared to be almost homogeneous on the native gel, a major protein band and a few faint bands were seen on the SDS gel. Therefore, after PAGE (7.5% gel), the major protein band was electroeluted from the gel in 20 mM KPB (pH 7.0). The eluted protein was homogeneous by SDS-PAGE (Fig. 2A). The reaction catalyzed by the enzyme was monitored by both the thiobarbituric acid method and the increase in A_{235} to confirm that the enzyme purified was in fact a gellan lyase.

TABLE 1. Specific activities of enzymes produced by microbial cells in mixed culture

Enzyme	Sp act (mU/mg)[a]			
	Glucose		Gellan	
	Extra	Intra	Extra	Intra
Gellan lyase	ND	ND	2.30	ND
β-D-Glucosidase	ND	0.063	0.99	1.10
α-L-Rhamnosidase	ND	0.059	3.10	52.0

[a] The mixed cultures were performed in media with glucose and gellan as carbon sources. Extra, extracellular fraction; Intra, intracellular fraction; ND, not detected.

TABLE 2. Purification of gellan lyase

Step[a]	Total protein (mg)	Total activity (mU)	Sp act (mU/mg)	Yield (%)	Purification (fold)
Crude enzyme	1,590	4,120	2.59	100	1.0
DEAE-cellulose	101	2,060	20.4	50.0	7.9
Butyl-Toyopearl 650M	22.5	495	22.0	12.0	8.5
Sephadex G-150	0.30	208	671	5.0	259

[a] Purification procedures are described in the text.

Properties of gellan lyase. The molecular mass of gellan lyase was determined to be 140 kDa by SDS-PAGE (12.5% gel) (Fig. 2A) and gradient PAGE (Fig. 2B) performed according to the guide book from Pharmacia LKB (7a), thus confirming that the gellan lyase purified from the mixed culture was a monomer with a size of 140 kDa. The enzyme was most active at pH 7.5 in sodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (HEPES) buffer (Fig. 3A) and at 45°C (Fig. 3B). The enzyme was stable below 45°C at pH 7.5. Fifty percent of the activity was lost after incubation at 50°C and pH 7.5 for 10 min (Fig. 3C). The reaction was performed in the presence or absence of various metals or other inhibitory compounds, and residual activity was measured. Such divalent metal ions as Ca^{2+}, Mo^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, and Zn^{2+} showed no effects on enzyme activity at 1 mM. Mercury (Hg^{2+}) significantly inhibited the reaction at 0.1 mM. EDTA also inhibited the reaction by 50% at 1 mM. Such sulfhydryl agents as dithiothreitol, glutathione (reduced form), and 2-mercaptoethanol (1 mM each) and *N*-ethylmaleimide (0.1 mM) exhibited no effect on enzyme activity. To examine the substrate

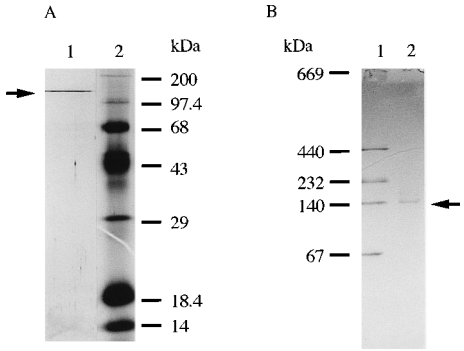

FIG. 2. Electrophoretic profiles of gellan lyase. (A) Gellan lyase electroeluted from a native gel was subjected to SDS-PAGE (12.5% gel) and stained with silver. Lane 1, gellan lyase; lane 2, molecular weight standards consisting of myosin H chain (200,000), phosphorylase *b* (97,400), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen (29,000), β-lactoglobulin (18,400), and lysozyme (14,000). (B) Gellan lyase (2 μg) was subjected to gradient PAGE and stained with Coomasie brilliant blue R-250 after gel filtration and elution with a Sephadex G-150 column. Lane 1, molecular weight standards consisting of thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and bovine serum albumin (67,000); lane 2, gellan lyase. Arrows indicate the gellan lyase.

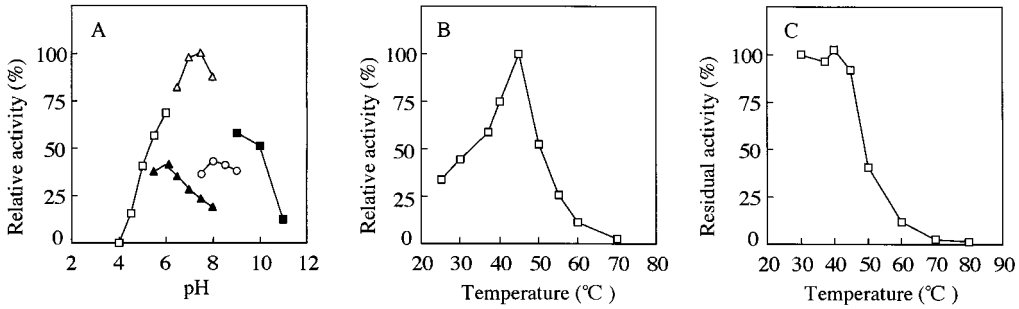

FIG. 3. Effect of pH and temperature on the activity and stability of gellan lyase. Experiments were carried out at 30°C with 0.05% gellan and gellan lyase purified with a Sephadex G-150 column (Table 2). (A) Effect of pH. Reactions were performed at 30°C for 60 min in the following buffers (50 mM each): sodium acetate (□), potassium phosphate (▲), sodium HEPES (△), Tris-HCl (○), and glycine-sodium hydroxide (■). Activity at pH 7.5 in HEPES buffer was relatively defined as 100%. (B) Optimal temperature. Reactions were performed for 30 min at various temperatures in 50 mM sodium HEPES buffer, pH 7.5. Activity at 45°C was relatively defined as 100%. (C) Thermal stability. After preincubation of the gellan lyase for 10 min at various temperatures, residual activity was measured at 30°C for 30 min in 50 mM sodium HEPES buffer, pH 7.5. Activity of the enzyme preincubated at 30°C was defined as 100%.

specificity of this enzyme, the reaction was performed at 30°C for 60 min with each substrate at 0.05%, 50 mM sodium HEPES buffer (pH 7.5), and the enzyme. The enzyme was highly specific to gellan, especially deacetylated gellan, and other gellan-related polysaccharides, such as S-88, welan, rhamsan, and S-198, which were kind gifts from T. J. Pollock, Shin-Etsu Bio, Inc., and alginate were inert as substrates. The viscosity of a 0.5% gellan solution at pH 7.5 and 30°C with and without gellan lyase was examined. Although no change in the viscosity was seen in the absence of the enzyme, the viscosity of the gellan solution in the presence of the enzyme decreased and finally reached a level comparable to that of water (data not shown).

Thus, for the first time, we have purified gellan-specific lyase from a microbial mixed culture, although the microbial strain or symbiotic system for lyase production has not been specified. Two kinds of gellan-degrading enzymes have been posited so far (4, 7, 9). One is sphinganase, and the other is gellan lyase. Sphinganase is reported to be a protein with a size of 110 kDa and acts on many exopolysaccharides, such as welan, S-198, and S-88, other than gellan. Though the gellan lyase has not been purified completely, it is roughly estimated to be a polypeptide with a size of 135 kDa and to have a broad specificity to gellan-related polymers (4). The gellan lyase that we have purified is highly specific to gellan and is thought to be different from the gellan lyase reported by Kennedy and Sutherland (4).

Native gellan is so highly viscous that it is difficult to use in food and biopolymer-based industries. Although chemical methods to produce low-viscosity and low-molecular-mass welan have been developed (1), the enzymatic method described in this study can produce a polysaccharide that contains a gellan backbone and that is more appropriate for industrial purposes. The low-viscosity gellan produced with gellan lyase should prove to be a useful material with novel physiological and food technology functions.

We appreciate the kind gift of polysaccharides (native gellan, S-88, welan, rhamsan, and S-198) from T. J. Pollock, Shin-Etsu Bio, Inc. We thank J. H. Roh, Kyoto University, for gradient PAGE analysis.

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