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Purification and Properties of an Extracellular Agarase from *Alteromonas* sp. Strain C-1

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A marine bacterial strain isolated from the Bay of San Vicente, Chile, was identified as *Alteromonas* sp. strain C-1. In the presence of agar, this strain produced high levels of an extracellular agarase. The production of agarase was repressed by glucose, with a parallel decrease in bacterial growth. The enzyme was purified to homogeneity by anion-exchange chromatography and gel filtration, with an overall yield of 45%. The enzyme has a molecular weight of 52,000, is salt sensitive, and hydrolyzes agar, yielding neoagarotetraose as the main product, with an optimum pH of about 6.5.

Agar has been defined as a family of polymers sharing a common backbone of neoagarobiose [*O*-3,6-α-anhydro-L-galactopyranosyl (1→3) O-β-D-galactopyranose] bound through β-1,4 bonds (10, 20). The majority of naturally occurring agars contain substituent groups such as sulfate, methoxyl, and pyruvate (3), which affect the properties of the polymer (12).

Agarolytic enzymes have been reported for several bacterial genera, including *Cytophaga* (25), *Pseudomonas* (13), *Streptomyces* (22), and *Vibrio* (2). Most of these bacteria have been isolated from marine environments, although a few species isolated from rivers (1), soil (21), and sewage (26) have also been described.

Agarolytic bacteria can be divided into two groups according to their effect on solid agar (1). Bacteria in group 1 soften the agar, forming a depression around the colonies, while those in group 2 cause extensive liquefaction of the agar. Although a number of species belonging to these two groups have been reported, extensive studies to explain their distinct patterns of agar degradation have not been carried out.

Pseudomonas atlantica is the only organism for which the enzyme system involved in agar hydrolysis has been studied in detail. In this species, three enzymes seem to be involved in the degradation process (18).

The broad objective of our work is to contribute to a better understanding of the different mechanisms of agar hydrolysis used by marine bacteria.

In our laboratory, we have isolated a few agarolytic strains from the southern Pacific coast. In this article, we describe the purification and characterization of an extracellular agarase from *Alteromonas* sp. strain C-1.

Isolation and identification of agarolytic bacteria. For the screening of agarolytic bacteria, samples of seawater from the Bay of San Vicente (region VIII, Chile) were collected and spread on agar plates of medium A, containing 0.25% casein hydrolysate, 0.05% yeast extract, 2.5% NaCl, 0.06% NaH_2PO_4, 0.5% $MgSO_4$, 0.002% $FeSO_4 \cdot 7\ H_2O$, 0.01% $CaCl_2$, and 1.5% agar (pH 7.25). The plates were incubated at 25°C for 48 h. Colonies that formed depressions or pits on the agar surface were picked up and purified further by the same plating method.

Bacteria were identified by using *Bergey's Manual of Systematic Bacteriology* (19). Staining, morphology, and motility were determined as described by Cowan (9). Oxidation and fermentation tests were done in MOF medium as recommended by Leifson (16), in the absence of agar. Anaerobic conditions were obtained by using Anaerocult A (Merck). The type of flagellum was determined by negative staining with uranyl acetate and electron microscopy as described by Cole and Popkin (8). Other biochemical tests were carried out essentially as described by Stolp and Gadkary (24) and Stanier et al. (23).

Enzyme assays. Agarase activity was determined by the method of Dygert et al. (11) as follows. A 4.5-ml amount of 0.05% agar in 50 mM sodium phosphate (pH 6.5) was heated at 100°C for 2 min and cooled to 30°C. The reaction was started by adding 0.5 ml of the enzyme, and 0.5-ml aliquots were taken at intervals and mixed with 0.5 ml of copper reagent to stop the reaction. Then 0.5 ml of neocuproine-HCl was added and incubated in a boiling-water bath for 5 min. Reducing sugar production was monitored as apparent release of D-galactose. One unit of agarase activity was defined as the amount of enzyme that released 1 μmol of galactose per min under the above conditions.

Enzyme purification. All steps were done at 4°C. An overnight culture of isolated colonies of strain C-1 was prepared in the medium described above and used to inoculate 4 liters of fresh medium containing 0.15% agar. The cells were grown in an orbital shaker (Lab-line) at 140 rpm and 25°C to the stationary phase. Phenylmethylsulfonyl fluoride was added to a final concentration of 0.1 mM, and the cells were centrifuged at 7,000 × *g* for 30 min. The supernatant was brought to 75% saturation with solid ammonium sulfate over 1 h, stirred gently for 1 h, and centrifuged at 7,000 × *g* for 30 min. The pellet was resuspended in 40 ml of 20 mM Tris-HCl (pH 7.5)–0.1 mM EDTA (buffer A) containing 0.1 mM phenylmethylsulfonyl fluoride and dialyzed against the same buffer. The dialysate was loaded on a DEAE-cellulose column (21 by 2.4 cm) equilibrated with buffer A. The column was washed with the same buffer, and the protein was eluted with a 500-ml linear gradient of 0 to 0.5 M NaCl. Fractions of 6 ml were collected and pooled on the basis of activity (73 ml); this pool was diluted with 3 volumes of buffer A, absorbed on a small DEAE-cellulose column (3 by 1 cm), and eluted with 0.4 M NaCl in buffer A. The concentrated protein (9 ml) was loaded on a Sephadex G-75 column (60 by 2.5 cm) equilibrated with buffer A. Elution was carried out with the same buffer. Fractions with activity were pooled and concentrated in a small DEAE-

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TABLE 1. Biochemical and physiological characteristics of *Alteromonas* sp. strain C-1

Characteristic tested	Result
Anaerobic growth	−
Oxidase	+
Catalase	+
Production of indole	−
Production of H_2S	−
Arginine dihydrolase	−
Urease	−
NO_3^- reduced to NO_2^-	+
Starch, agar, carboxymethyl cellulose, and glycogen hydrolysis	+
Gelatin, chitin, and alginic acid hydrolysis	−
Tween 80 esterase	−
Sodium required for growth	+
Utilization of D-glucose, D-galactose, D-mannose, D-xylose, maltose, raffinose, glycerol, and mannitol	+
Utilization of D-fructose, L-arabinose, D-ribose, saccharose, lactose, inulin, dulcitol, and inositol	−
Utilization of malate and succinate	+
Utilization of tartrate, molonate, citrate, acetate, pyruvate, and benzoate	+
Mol% G+C	47

cellulose column as described above. The enzyme was stable when stored at 4°C in 0.4 M NaCl for more than 6 months.

Protein determination. The amount of protein in columns was determined by measuring the A_{280}. The amounts of protein in pooled fractions were estimated by the method of Lowry et al. (17), with bovine serum albumin as the standard.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the procedure of Laemmli (15) with 7.5% acrylamide gels. Proteins were stained with Coomassie brilliant blue R-250.

Hydrolysis products analysis. The hydrolysis products of agar were analyzed by thin-layer chromatography (TLC) on Silica Gel G plates and developed with *n*-butanol-ethanol-H_2O (3:2:2, by volume). Sugars were visualized by spraying the plates with a naphthoresorcinol reagent (27).

G+C content. The G+C content was determined by high-pressure liquid chromatography by the method of Kumura et al. (14). Genomic DNA was prepared by the procedure of Ausubel et al. (4).

Strain properties and identification. The isolated strain had the following characteristics. It produced pits on the agar surface with irregular borders, showing haloes of clearing. The agar on the plate was liquefied by this strain after a week of incubation.

Strain C-1 had the following taxonomic characteristics: gram-negative rods, 0.7 by 2.3 μm; obligately aerobic; motile by means of a polar flagellum; catalase and oxidase positive; indol and arginine dihydrolase negative. Other physiological and biochemical characteristics are given in Table 1. From these results and based on the G+C content, strain C-1 was assigned to the genus *Alteromonas*.

Effect of carbon source on production of agarase by strain C-1. Figure 1 shows the growth curve of strain C-1 and the production of extracellular agarase. The enzyme was induced by agar in the culture medium, reaching a maximum during the stationary phase. Activities of less than 2% of the induced level were found when cells were grown in a medium containing galactose or glucose as the sole carbon source.

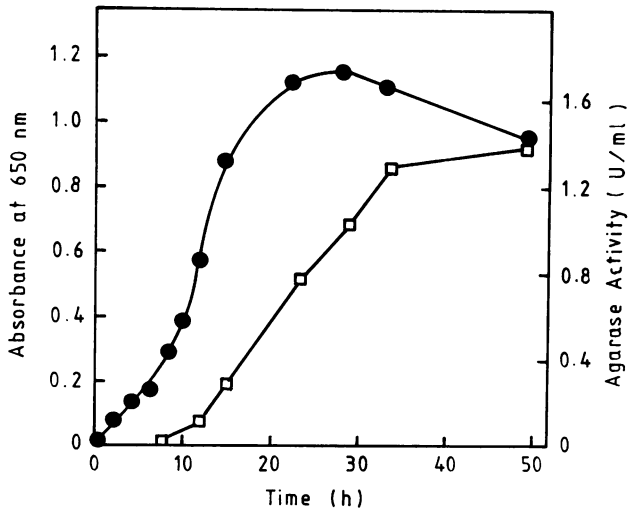

FIG. 1. Growth (A_{650}, ●) and agarase activity (□) of *Alteromonas* sp. strain C-1.

In medium supplemented with 0.1% agar, both the production of agarase and cell growth were impaired when glucose was added. In contrast, no effect was observed in the presence of galactose.

Purification and properties of the agarase from strain C-1. Two chromatographic steps were included in this procedure. The enzyme eluted as a single species in the initial DEAE-cellulose chromatography step. Additional purification of the enzyme was achieved by gel filtration on Sephadex G-75. Table 2 summarizes the results of the each step of the purification. The enzyme was purified about 30-fold, with a final specific activity of 234 U/mg. The overall yield of the purification was 45%.

SDS-PAGE of the purified preparation showed a single band (Fig. 2). The molecular weight corresponded to 52,000, based on the mobility of protein standards.

In order to compare our results with those described for *P. atlantica*, we used the conditions described by Morrice et al. (18) to determine the optimal pH for the activity of the enzyme. Optimal activity was observed at pH 6.5.

The enzyme was stable stored in buffer containing Tris-HCl (pH 7.5) and 0.4 M NaCl for more than 6 months at 4°C. The stability of the enzyme was examined at different temperatures in 20 mM Tris-HCl, pH 7.5. The enzyme activity remained constant after 1 h of incubation at temperatures below 30°C. However, at 35°C, the enzyme was rapidly inactivated, and only 50% of the activity remained after 20 min (results not shown).

TABLE 2. Purification of agarase from *Alteromonas* sp. strain C-1

Step	Vol (ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%)
Cell-free culture fluid	3,750	8,625	1,162.0	7.4	100
Ammonium sulfate (75% saturated)	72	5,760	53.8	107.0	67
DEAE-cellulose	73	4,163	29.8	140.0	48
Sephadex G-75	41	3,896	16.6	234.0	45

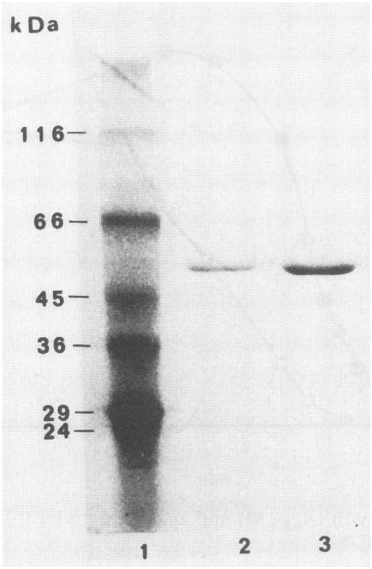

FIG. 2. SDS-PAGE of purified agarase. Lane 1, molecular mass standards; lanes 2 and 3, purified agarase (3 and 6 μg, respectively).

When enzyme activity was measured in the presence of different salts (NaCl, KCl, and NH_4Cl), significant inhibition was observed. It is remarkable that in all cases, the inhibition occurred at salt concentrations below 0.1 M (50% inhibition at 0.1 M salt).

To determine the hydrolysis products of agar with the purified enzyme, a solution of 0.1% agar was digested with 2.3 U of agarase per ml. The reaction was stopped by heating at 100°C for 2 min, and the products were analyzed by TLC. The predominant product of agar hydrolysis comigrated with neoagarotetraose (results not shown).

Agarolytic bacteria produce visible changes on agar because of the cleavage of polysaccharide chains, ranging from softening of the gel to agar pitting and extensive liquefaction (1). The efficiency of agar hydrolysis may be related to the properties of the enzyme system involved in each case or to an effect of the relative concentrations of the enzymes.

In an attempt to explore these possibilities, we started an analysis of the agarases utilized by agar-liquefying bacteria. We describe here the purification and some properties of an extracellular agarase from a bacterial strain isolated from the southern Chilean coast. This strain was identified as *Alteromonas* sp. strain C-1 by several biochemical and physiological tests and from its G+C content (5).

High levels of the extracellular agarase were induced when agar was included in the culture medium. The enzyme was purified to homogeneity basically in two chromatographic steps, in a high yield.

The molecular mass of the agarase from *Alteromonas* sp. strain C-1 was 52 kDa, close to that predicted from the sequence of the *agrA* gene, which codes for the β-agarase I of *P. atlantica* (6). Similarly, the agarase from *Alteromonas* sp. strain C-1 hydrolyzed agar to neoagarotetraose.

However, the enzyme from strain C-1 differs in several properties from the agarases of other species. First, its specific activity was higher than that described for the extracellular agarases from *P. atlantica* (18), *Vibrio* sp. strain AP-2 (2), and *Streptomyces coelicolor* (7) (44, 52, and 3 U/mg, respectively). Its pH activity profile is narrower than that of the β-agarase from *P. atlantica*, with an optimum pH of about 6.5. Furthermore, the enzyme from strain C-1 was rapidly inactivated at temperatures above 30°C, in contrast to the agarases from *P. atlantica* and Vibrio sp. strain AP-2, which are stable at up to 40°C. Perhaps the most striking property of this enzyme is the inhibition of its activity by salts such as NaCl and KCl. This effect has not been observed with other agarases.

Additional work will be required to elucidate the agarolysis mechanism of strain C-1, since it remains to be determined whether other enzymes are involved in that process and their localization.

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