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Purification and properties of a xanthan depolymerase from a heat-stable salt-tolerant bacterial consortium

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SUMMARY

A bacterial consortium (NRRL B-14401) resulting from soil enrichment growth on xanthan gum produces enzymes that can degrade xanthan gum in salt-containing solutions at temperatures up to 65°C. One component that cleaves the backbone linkages of both xanthan gum and carboxymethyl cellulose is called xanthan depolymerase. Two such depolymerase activities were isolated by high performance anion exchange chromatography, and their molecular weights determined by size exclusion chromatography to be 170 000 and 100 000 Da. The 170-kDa protein was purified and its properties studied. Sodium chloride and potassium chloride enhanced the hydrolysis of carboxymethyl cellulose, but decreased the rate of degradation of xanthan gum. The purified enzyme, which was optimally active at pH 6, was less stable to extremes of temperature than crude mixtures of cell-free culture broth; stabilized by its substrate it was active for more than 6 h at 50°C.

INTRODUCTION

The physical properties of xanthan gum persist throughout a variety of temperature extremes and solution conditions; its relative stability in salt solutions leads to its use in drilling fluids made with either brine or fresh water. The polysaccharide demonstrates pseudoplastic behavior in solution, making it a good candidate for a viscosifier of hydraulic fracture fluids for the recovery of natural gas, where it is used to suspend the propping agents that are forced under pressure into the fracture zone. However, once the propping agent is in place, it is desirable to reduce the viscosity of the fracture fluid in order to stimulate the flow of gas out of the fracture zone. Chemical degradation of the polysaccharide with hypochlorite has been used to cause a reduction of viscosity, but this method is potentially hazardous and difficult to control.

The need for an enzymatic viscosity breaker that would function at elevated temperature and salt concentrations led to the discovery of a bacterial consortium that produced the appropriate enzymes for the reduction of xanthan gum solution viscosity [3–5], isolated by soil enrichment growth on xanthan gum. The extracellular enzymes produced by this consortium were shown to retain activity up to 65°C, and to be stabilized by the addition of salt. Analysis of the degradation products of xanthan gum treated with the enzyme mixture showed that pyruvated mannose was released from the side-chain of the polysaccharide, and that the backbone of the polymer was cleaved in a novel manner to yield branched oligosaccharides [5]. Preliminary characterization of the enzymes involved indicated the action of a β-(1→4)-glucanase for backbone cleavage, and a lyase that removed pyruvated mannose to leave unsaturated 4,5-*ene*-glucuronic acid non-reducing ends on some of the oligosaccharides. The lyase has been purified [1]; it is a 33-kDa protein that is stable at 55°C, and moderately stable at 60°C in the presence of sodium chloride. At 65°C the enzyme was rapidly inactivated regardless of the presence of NaCl. In addition to the identification of two depolymerase activities, this report describes the purification and preliminary characterization of a 170-kDa xanthan depolymerase.

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MATERIALS AND METHODS

Consortium maintenance and growth

The bacterial consortium NRRL B-14401 was maintained at 4°C in a buffered growth medium containing 0.15% (w/v) xanthan gum and 2% NaCl as previously described [5]. For the production of the xanthan-degrading enzymes, 250 ml of sterile media was innoculated and cultured at 45°C until the viscosity of the medium was substantially reduced. A 50 ml sample from this culture was used to innoculate 1-L cultures in 2.8-L Fernbach flasks which were incubated at 45°C with shaking.

To determine the rate and amount of lyase and depolymerase produced when cultured under these conditions, a

1-L culture was sampled at 6-h intervals for a total of 96 h, and allowed to continue incubation for a total of 162 h. Total xanthanase activity, cell growth by absorbance at 600 nm, and viscosity of the culture were measured. Enzyme activity was measured by incubating 50 μl of culture supernatant with 200 μl of xanthan (0.1% w/v) in 20 mM sodium phosphate and 50 mM sodium chloride at 45°C for 1 h, and determining the reducing sugar released from the polysaccharide with a Bran and Luebbe AutoAnalzer II configured to measure reducing sugar by an alkaline ferricyanide method (Bran and Luebbe Analyzing Technologies, Buffalo Grove, IL, USA; [7]); control assays without added substrate are used to correct for background reducing sugar content in the enzyme source. Reducing sugar values were expressed as glucose equivalents released in 100 ml of buffered solution. Because xanthan depolymerase can also hydrolyze carboxymethyl cellulose (CMC), this compound was also tested as a substrate to discriminate between xanthan-degrading activity attributable to the lyase, and that due to the action of the depolymerase. Viscosity was measured on a Brookfield LVT1 viscometer (Brookfield, Stoughton, MA, USA).

Purification of xanthan depolymerase

Twelve 2.8-L Fernbach flasks each containing 1 L of medium were innoculated with 50 ml of a 48-h culture, and grown for an additional 48 h at 45°C with shaking. Bacteria were removed by centrifugation (16 000 × *g*, 40 min), and the supernatant concentrated ten-fold in a Pellicon ultrafiltration device equipped with a 10 000 molecular weight cutoff cassette (Millipore, Milford, MA, USA). Following dialysis for 24 h at 4°C against 20 mM sodium phosphate pH 6.0, the concentrate was centrifuged as described and the supernatant pumped (50 ml min^{-1}) at room temperature through a DEAE Acti-mod Spiral Separation module (Lab module 1XCF, 6.25 × 8.125 cm; FMC Corporation, Pine Brook, NJ, USA) equilibrated in 20 mM sodium phosphate, pH 6.0. The module was washed with equilibration buffer, and the enzymes eluted with 1 M NaCl in 20 mM sodium phosphate, pH 6.0. The unbound fraction was passed over the module twice, and bound protein was eluted between runs with 1 M NaCl in 20 mM sodium phosphate, pH 6.0. The salt-eluted fractions containing the total xanthanase activity were pooled and concentrated to 200 ml with a Minitan ultrafiltration device (Millipore Corp.) equipped with a 30 000 molecular weight cutoff membrane. The concentrated enzyme solution was dialyzed for 24 h against 20 mM sodium phosphate pH 6.0, centrifuged at 16 000 × *g* for 1 h, and passed through a DEAE Acti-mod purification module (4.7 × 2.5 cm; FMC Corp.) at 10 ml min^{-1}. The column effluent was monitored at 280 nm; after the sample was applied, the module was washed with equilibration buffer until the absorbance decreased to the starting value. A linear gradient of 0–1 M NaCl in 20 mM sodium phosphate, pH 6.0, was used to elute bound protein. Fractions of 10 ml were collected, and assayed for enzyme activity using 0.1% (w/v) xanthan gum and 2.0% (w/v) carboxymethyl cellulose as substrates. Two fractions showing both xanthanase and CMCase activity were isolated and named depolymerase I and II; the peak fractions that eluted at 0.50 M NaCl from the DEAE Acti-Mod (depolymerase I) were pooled and concentrated in an Amicon stirred cell ultrafiltration device (Amicon, Beverly, MA, USA) with a 30 000 molecular weight cutoff membrane. The concentrate was washed in the Amicon cell with 25 mM sodium acetate pH 5.1, filtered through a 0.45-μm filter, and then pumped through a CM-Acti-Mod module (equilibrated in 25 mM sodium acetate pH 5.1) at 10 ml min^{-1} and room temperature. Bound protein was eluted with a linear sodium acetate gradient, starting with 25 mM at pH 5.1 and increasing to 1 M at pH 7.0. Fractions of 10 ml were collected, and those with xanthanase and CMCase activity pooled and concentrated to 5 ml in the Amicon device just described. Glycerol was added to make a 10% (v/v) solution, and the sample loaded on a 2.5 × 75 cm Bio-Gel A-0.5M gel filtration column (Bio-Rad Laboratories, Richmond, CA, USA) equilibrated in 100 mM sodium phosphate, pH 6.0, flowing at 20 ml h^{-1}. Fractions of 3.3 ml were collected and assayed for enzyme activity by the reducing sugar assay. Fractions with depolymerase activity were pooled and concentrated by Amicon ultrafiltration. Concentrated enzyme was chromatographed in 100-μl aliquots on an Ultraspherogel SEC3000 size-exclusion column (7.5 × 300 mm; Beckman Instruments, Fullerton, CA, USA) at 0.25 ml min^{-1} in 0.1 M sodium phosphate pH 6.0. Column effluent was monitored at 280 nm, and the depolymerase identified by reducing sugar assay.

Determination of molecular weight of xanthan depolymerase

The molecular weight of the purified enzyme was measured by denaturing polyacrylamide gel electrophoresis (PAGE) [12] on 7% acrylamide gels and compared to standard protein mixtures of known molecular weight (Bio-Rad, Richmond, CA, USA). Gels were silver stained with reagents and instructions supplied by the manufacturer of the silver stain kit (Boehringer Mannheim, Indianapolis, IN, USA).

The mobility of the purified enzyme obtained from Ultraspherogel HPLC size exclusion chromatography was compared to the mobility of protein standards of known molecular weight (Sigma, St. Louis, MO, USA). An aliquot of the peak fraction of the second depolymerase (II) from the DEAE Acti-Mod chromatography was also run on the Ultraspherogel column and fractions collected and assayed with xanthan gum and CMC and the amount of reducing sugar measured. The molecular weight of depolymerase II was estimated by comparison of the elution time of the enzymatic activity to the elution times of the protein standards.

Characterization of enzymatic properties of xanthan depolymerase

The salt tolerance of depolymerase I was measured in solutions containing increasing amounts of sodium chloride and either CMC or xanthan gum as substrate. Typically 50 μl of enzyme in 20 mM sodium phosphate, pH 6.0, was added to 200 μl of 2.0% CMC or 0.1% xanthan gum in

100 mM sodium phosphate pH 6.0, and the amount of sodium chloride added ranged from 0 to 0.5 M (final concentration). The same experiment was repeated with potassium chloride, and the range tested from 0 to 0.25 M. The mixtures were incubated for 1 h at 45°C, cooled in an ice bath briefly, and the amount of reducing sugar measured.

To determine the optimum pH of hydrolysis, stock xanthan gum (0.1%) and CMC (2.0%) solutions in 100 mM sodium phosphate plus 100 mM sodium acetate were prepared and titrated to the appropriate pH in the range 4.0–9.0. To 200 μl of substrate, 50 μl of depolymerase was added and the mixture incubated at 45°C for 1 h. The amount of reducing sugar was measured.

Temperature stability was determined by incubating 50 μl aliquots containing depolymerase in 25 mM sodium phosphate pH 6.0, with and without 0.5 M NaCl. The mixtures were incubated at 50°C and at the appropriate time the sample removed and held at 0°C until all samples had been heat treated. To determine residual enzyme activity 200 μl of 2.0% CMC in 100 mM sodium phosphate pH 6.0 was added and the mixtures incubated for 1 h at 45°C before measurement of the reducing sugar.

To determine whether substrate enhanced thermal stability, 50 μl of depolymerase was added to 2.0 ml of 2.0% CMC in 20 mM sodium phosphate and 0.5 M NaCl, pH 6.0, and the mixture incubated at 45°C and 50°C; 100-μl aliquotes were removed at 30-min intervals for a total of 360 min. The aliquots were held at 0°C until all samples had been collected, and the reducing sugar measured.

RESULTS

Time course of xanthanase synthesis

Fig. 1 shows that significant xanthanase activity first appeared at 24 h, and reached a maximum at 36 h. The increase in enzyme activity follows the growth of the consortium as measured by an increase in the absorbance at 600 nm (data not shown). The increase in enzyme activity is commensurate with the decrease in viscosity of the culture medium, which rapidly declined starting around 20 h. Total xanthanase activity gradually declined after 36 h in a linear fashion until no significant activity was measurable, at approximately 120 h. The results when CMC was used as a substrate show a slightly different profile; the CMCase activity reached its maximal rate of hydrolysis at 36 h, but rather than decline from that point, as did total xanthanase activity, the level of CMCase activity remained essentially constant for the next 36 h, after which it gradually declined, essentially disappearing by 120 h. The decline in xanthanase activity after 36 h may indicate that a loss of lyase activity was responsible for the reduction in reducing groups formed during polymer hydrolysis. Whether this apparent loss of lyase activity was due to a reduction of enzyme production or enzyme instability is currently under study. All attempts to isolate the individual bacteria that produce either the lyase or the depolymerase have not yet been successful.

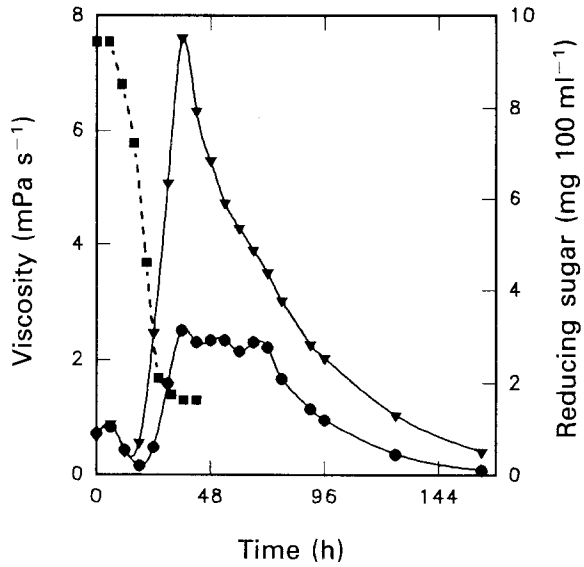

Fig. 1. Time course of xanthanase production by the bacterial consortium NRRL B-14401. Samples of culture medium were assayed for xanthanase activity (▼) and CMCase activity (●) at the times shown and the amount of reducing sugar formed in 1 h at 45°C measured. The viscosity of the medium (■) was measured with a Brookfield LVT1 viscometer at 45°C.

Purification of the 170-kDa xanthan depolymerase

Anion exchange chromatography on the spiral DEAE-Acti-Mod module resulted in the initial separation of total xanthanase from bulk protein that does not adsorb under these conditions, and enhanced the resolution achieved by the second anion exchange step. Chromatography on the microporous membrane-based DEAE module separated the lyase from the depolymerase activity; the latter consisted of two peaks of enzyme activity, named depolymerase I and II, that hydrolyzed both xanthan gum and carboxymethyl cellulose (Fig. 2). Depolymerase I eluted at 0.50 M NaCl, and depolymerase II at 0.55 M NaCl, with overlapping activity between the two peaks. Depolymerase I activity was conservatively pooled to minimize contamination from depolymerase II. Following concentration, depolymerase I was adsorbed to a cation exchange module, where a combination of increasing ionic strength and pH resulted in the release of the enzyme which did not occur by increasing ionic strength alone. Depolymerase I was then purified by size exclusion chromatography, first in preparative fashion followed by an analytical separation on HPLC. Purity of the enzyme after this step was assessed by denaturing PAGE, and is shown in Fig. 3. Based on SDS/PAGE analysis, the enzyme has an apparent molecular weight of 160 000 Da. This value compared favorably with the molecular weight determined by HPLC of 180 000 Da. The average apparent molecular weight is 170 000 Da.

Depolymerase II activity was subjected to HPLC size exclusion chromatography under the same conditions. Based on the elution time of the enzymatic activity, the depolymerase II had an estimated apparent molecular weight of 100 000 Da.

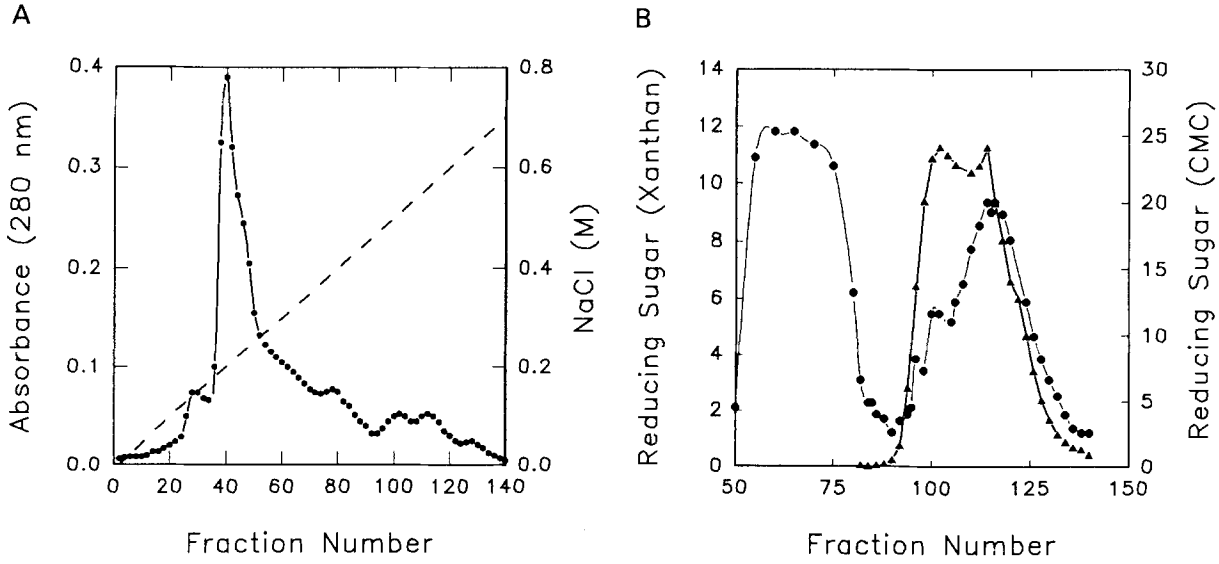

Fig. 2. (A) Chromatography of the xanthanase mixture on DEAE Acti-Mod purification module. Solid line, absorbance at 280 nm of column effluent at the start of the salt gradient; dashed line, NaCl gradient in 20 mM sodium phosphate, pH 6.0. (B) enzyme activity of column fractions. •, Reducing sugar formed using xanthan gum as substrate; ▲, reducing sugar formed using CMC as substrate. No reducing sugar was formed using CMC with fractions 50–80. Fractions 95–105 were pooled for depolymerase I purification.

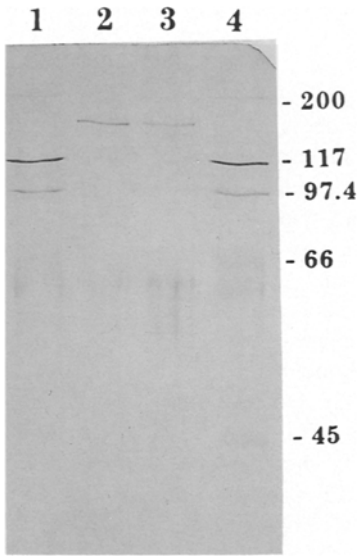

Fig. 3. PAGE of the purified xanthan depolymerase on a 7% gel followed by silver staining. The outside lanes (1,4) have protein standards (molecular weights, in kDa, shown to the right); lane 3 has 20 µl of purified depolymerase; lane 2 has the same sample concentrated five-fold.

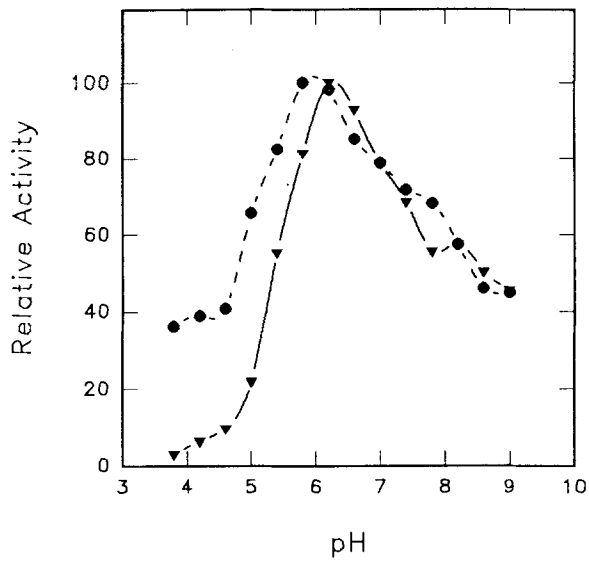

Fig. 4. The effect of pH on xanthan depolymerase activity. The assay contained purified depolymerase and substrate in 100 mM sodium acetate and 100 mM sodium phosphate at the pH values shown; values are % of maximum reducing sugar formed in 1 h at 45°C. Xanthan gum (•) and CMC (▼) were used as substrates.

Properties of the purified enzyme: effects of pH, salts, and heat

Depolymerase I was assayed separately with both xanthan gum and CMC substrates in a mixed buffer of sodium acetate and sodium phosphate to determine the optimal pH for polymer hydrolysis. Fig. 4 shows that the optimal pH is 6.0 for these substrates. The enzyme retains at least 50% of its maximal activity in the range pH 5–8; the decline in enzyme activity is more precipitous in acidic than in alkaline solutions.

The enzyme was either more active or less active in solutions of increasing salt concentrations depending on substrate, as shown in Fig. 5. With xanthan gum as the substrate, the amount of reducing groups formed in 1 h decreased as the concentration of NaCl increased; activity was the highest without any added NaCl, and half-maximal activity was at approximately 0.1 M NaCl. With CMC, at

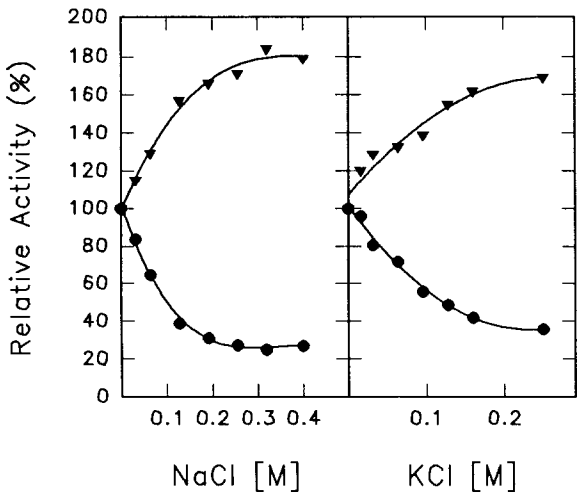

Fig. 5. The effect of salt on xanthan depolymerase activity. Reducing sugar formation in 1 h at 45°C in the presence of either NaCl (left panel) or KCl (right panel) when assayed with xanthan gum (•) or CMC (▼). Reducing sugar formed in 1 h without salt was set to 100%; values plotted were determined by comparison to this control value.

0.4 M NaCl the activity was 75% higher than without salt added to the enzyme. Similar behavior was observed when KCl was substituted for NaCl.

Heat stability of depolymerase I was determined by incubating the enzyme without substrate and subsequently measuring the remaining hydrolytic activity in a 1-h incubation with substrate at 45°C. Solutions with and without 0.5 M NaCl were tested to examine whether salt could stabilize the enzyme, as was seen for the crude enzyme [5] and the purified xanthan lyase [1]. Fig. 6 shows that at 50°C enzyme activity declined rapidly; the addition of NaCl had only a very slight stabilizing effect. To determine whether substrate could protect the enzyme from destabilization by heat, solutions containing depolymerase I and CMC were incubated at 45°C and 50°C for up to 6 h, and aliquots removed at 30-min intervals. Fig. 7 shows that at both 45°C and 50°C, the amount of reducing sugar was still increasing after 6 h, indicating that the depolymerase was still active.

DISCUSSION

The application of xanthanases in the recovery of natural gas requires that the enzymes function in brines at elevated temperatures. A bacterial consortium developed by Cadmus et al. [3–5] produces xanthan gum-degrading enzymes that have the highest heat stability of any reported to date. An earlier study [2] developed salt-sensitive and salt-tolerant xanthanase systems containing enzymes that cleaved all of the linkages in the polysaccharide, but these enzymes did not have the ability to function at the higher temperatures required for practical applications. Hou and coworkers [8, 9] developed a salt-tolerant bacterial consortium called HD1, from which they purified a xanthan depolymerase with a molecular weight of 60 000 Da [10]. This enzyme had a

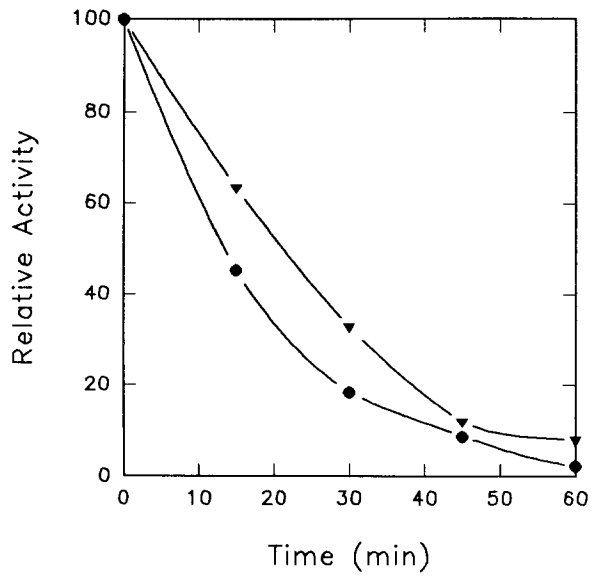

Fig. 6. Heat stability of xanthan depolymerase without substrate. Enzyme was incubated at 50°C in 20 mM sodium phosphate pH 6.0 with (▼) and without (•) 0.5 M NaCl for the time indicated, and removed from heat. CMC was added and the mixture incubated for 1 h at 45°C; reducing sugar was measured and remaining activity determined. Value is % of unheated control.

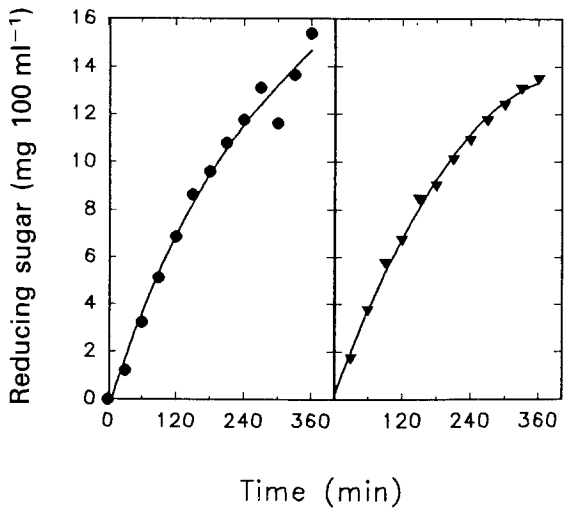

Fig. 7. Heat stability of xanthan depolymerase incubated with substrate. Left panel, amount of reducing sugar formed at the indicated time at 45°C with CMC as the substrate; right panel, incubation of xanthan depolymerase with CMC at 50°C.

temperature optimum of 30°C, and the residual activity after a 20-min incubation at 45°C was only 33% of the maximal activity at 30°C. Depolymerase I, produced by the consortium studied in this report, had a molecular weight of 170 000 Da. and was functional at higher temperatures than the depolymerase from HD1, with enzymatic activity after 6 h at 50°C in the presence of substrate. The heat stability of the purified enzyme without substrate was significantly reduced, and only very slightly stabilized by the addition of salt.

Few enzyme systems that can hydrolyze xanthan gum have been reported [5,6,13]. Some hydrolysis can occur with fungal cellulases if the xanthan under study has been treated to increase random order, such as by heating and removing all salts through extensive dialysis against distilled water [14]. The structure of the polysaccharide is likely to determine how effectively hydrolytic enzymes degrade the molecule, and may account for the decrease in relative depolymerase activity seen when salt is added to solutions containing xanthan gum as substrate, as opposed to those with CMC, where increased hydrolysis is seen. The effect of salt on xanthan gum conformation has been studied [11], and salt induces a more highly ordered structure, which may reduce access to the cellulosic backbone of xanthan by the depolymerase. This phenomenon suggests that the effect of the salt seen in this study has to do with the substrate conformation or the mechanism of substrate binding to the enzyme, which must be different than for the interaction of CMC and the depolymerase.

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