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Purification and Characterization of a Pyruvated-Mannose-Specific Xanthan Lyase from Heat-Stable, Salt-Tolerant Bacteria

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A xanthanase complex secreted by a consortium of heat-stable, salt-tolerant bacteria includes a lyase that specifically removes terminal pyruvated β-D-mannose residues from the side chains of xanthan gum. The enzyme was purified to homogeneity from the culture broth following ion-exchange chromatography and gel permeation chromatography. It consists of a single subunit of molecular weight 33,000. The enzyme is stable to 55°C for more than 6 h in 20 mM sodium phosphate buffer (pH 5.0) containing 0.25 M NaCl. Optimal enzyme activity was observed at 0.05 M NaCl and a pH of 5. The enzyme has a pI of 3.7. It does not remove unsubstituted terminal β-D-mannose residues from xanthan side chains nor does it hydrolyze *p*-nitrophenyl-β-D-mannose. Treatment of xanthan with purified lyase results in a polysaccharide containing side chains terminating in an unsaturated 4,5-*ene*-glucuronic acid.

Xanthan gum is an extracellular polysaccharide produced by the bacterium *Xanthomonas campestris* NRRL B-1459 (12). The primary structure of xanthan consists of a cellulosic backbone chain with trisaccharide side chains attached α-(1→3) to alternating β-(1→4)-linked glucosyl residues (10). The side chains terminate with β-D-mannosyl residues, which may bear a pyruvic acetal [i.e., 4,6-*O*-(1-carboxyethylidene)]; the degree of pyruvation depends on the bacterial strain and growth conditions (13). This polysaccharide is widely used in the food industry as a viscosifying agent. It has desirable rheological properties; solutions with low concentrations of xanthan have high viscosity at low shear rates and dramatic shear thinning at higher shear rates. The increased viscosity of solutions containing xanthan is remarkably stable to extremes in pH, temperature, and the addition of salts. The latter properties suggest that xanthan gum would be highly useful as a viscosifier of hydraulic fracture fluids that are used to stimulate the flow of natural gas from underground regions of low porosity. A viscous fluid is required to maintain proppage such as sand in suspension during the fracturing phase of the operation; however, once the proppage is in position, the viscosity of the carrier needs to be reduced for the gas to flow freely out of the underground reservoir. An enzymatic viscosity breaker capable of functioning at 55 to 65°C and in moderately high concentrations of salt potentially would be of great usefulness in this application, replacing hypochlorite, which is environmentally hazardous, as a viscosity breaker.

Cadmus et al. (1) described the isolation of a salt-tolerant bacterium that produced an enzyme complex capable of degrading xanthan gum at temperatures up to 48°C in the presence of 4% NaCl. The degradation products formed after 24 h of incubation included D-glucuronic acid, D-mannose, pyruvated mannose, 6-*O*-acetyl-D-mannose, and a high-molecular-weight glucan; therefore, this bacterium produces enzymes capable of hydrolyzing the linkages between all of the residues of the side chains. However, no UV-absorbing saccharides were formed during this process, which demonstrated that the bacterium did not produce a lyase under these conditions (16). These results of xanthan degradation studies were initially interpreted as indicating that this bacterium did not produce a depolymerase activity, because the β-(1→4)-linked glucan backbone remained intact. Recent longer-term experiments have shown that a depolymerase activity was present in that culture (16) which was capable of hydrolyzing the glucan backbone of the biopolymer and reducing the viscosity of the medium. A xanthan depolymerase from a salt-tolerant bacterial consortium (HD1) has been purified (7, 9), but the enzyme was not stable at temperatures above 45°C. Pyruvated mannose was found as a degradation product, as were glucuronic acid, mannose, acetylated mannose, and glucose (8); however, it was not reported whether the 4,5-*ene* of glucuronic acid was formed during enzymatic degradation by the HD1 culture. Recently, Cadmus et al. (3) described the isolation of a bacterial consortium which produced a xanthanase complex that was functional up to 65°C in the presence of 3% NaCl. The products of degradation consisted of pyruvated mannose and branched oligosaccharides derived from hydrolysis of the backbone of the polysaccharide. The release of the pyruvated mannose led to the formation of unsaturated 4,5-*ene* glucopyranosyluronic acid residues, indicative of the action of a lyase. This report describes the purification of the lyase from the mixed-culture medium and the characterization of the properties of this enzyme in purified form. The lyase can be used to prepare modified xanthan gum bearing an unsaturated sugar on the nonreducing end of its side chains.

MATERIALS AND METHODS

Bacterial cultures. The heat-stable, salt-tolerant mixed culture (NRRL B-14401) that produces xanthan lyase has been described previously (2, 3). Stocks of this mixed culture, originally isolated from soil, were maintained at 4°C in liquid broth. For the production of xanthan lyase, bacteria were grown in broth containing (all wt/vol) 0.15% xanthan, 2% NaCl, 0.05% $(NH_4)_2SO_4$, 0.025% yeast extract, 0.025% tryptone, and 0.03 M potassium phosphate buffer, pH 6.5, at 45°C with shaking (150 rpm) for 3 to 4 days until the viscosity of the broth was greatly reduced (3). For routine enzyme purification, three or four Fernbach flasks containing 750 ml of medium each were cultured at 45°C as just described and then centrifuged to remove cells (20,000 × *g*, 20 min, 4°C).

Chemicals. The xanthan used for the growth of the lyase-producing mixed culture was obtained from Kelco Co., San Diego, Calif. (Kelzan grade), and used without further purification. For assaying lyase activity, a xanthan preparation high in pyruvated-mannose content was used (Flocon Biopolymer 4800M, lot 239-75; gift from Pfizer Chemicals Division, New York, N.Y.). Prior to its use in enzyme assays, the high-pyruvate xanthan (HP-xanthan) was purified by the following treatment. The xanthan slurry was dialyzed extensively against distilled water. Following dialysis, methanol was added to the solution of HP-xanthan to 50% (vol/vol). The solution was then centrifuged at 16,000 × g for 60 min at 4°C. The HP-xanthan is soluble in this concentration of methanol, while substantial impurities are removed. Next, ethanol is added to make a solution that is 66% (vol/vol) in ethanol, which precipitates the xanthan. The congealed xanthan was sieved out of the mixture and redissolved in water. After a second cycle of ethanol precipitation and resuspension in water, the solution was extensively dialyzed against distilled water and subsequently freeze-dried. The amount of pyruvate in the HP-xanthan was 6% by weight.

Electrophoresis-grade reagents and Dowex ion-exchange resin were purchased from Bio-Rad Laboratories, Richmond, Calif.; standard molecular weight markers, from ISS/Enprotech, Hyde Park, Mass.; the isoelectric focusing system (Phastgel Electrophoresis system) and liquid chromatography resins (Ultrogel AcA 34 and 44, DEAE-Sepharose fast flow), from Pharmacia LKB, Piscataway, N.J.; and *p*-nitrophenyl-β-D-mannopyranoside (pNP-man) and β-mannosidase (snail acetone powder suspension), from Sigma Chemical Co., St. Louis, Mo.

Enzyme assays. Xanthan lyase removes the terminal pyruvated-mannose residues from xanthan. The resulting increase in the amount of reducing sugar forms the basis for the measurement of its activity. Lyase activity was routinely measured in a volume of 0.5 ml containing 0.1% (wt/vol) HP-xanthan in 20 mM sodium phosphate–50 mM NaCl, (pH 5.0) and a source of lyase. After 1 h at 45°C, the samples were cooled in an ice bath for several minutes and then analyzed for reducing sugar in an automatic analyzer with use of an alkaline ferricyanide reagent. The amount of reducing sugar detected was expressed relative to a standard glucose reference. Since xanthan depolymerase also contributes to a slight increase in reducing sugar levels, fractions from various stages of the purification were also assayed with a 2% (wt/vol) solution of carboxymethyl cellulose in 20 mM sodium phosphate, pH 6.0, at 45°C. Xanthan lyase has no activity toward carboxymethyl cellulose.

An alternative assay, which is specific for the lyase, is based on an increase in A_{251} due to the formation of 4,5-*ene*-glucuronic acid residues. This assay was used to determine initial reaction rates as a function of temperature. Solutions of 0.1% (wt/vol) HP-xanthan and an aliquot of enzyme were separately equilibrated to the appropriate temperature and rapidly mixed, and the A_{251} was continuously measured for up to 20 min in a temperature-regulated environment. A reference cell containing xanthan without a source of enzyme was used to correct for any intrinsic A_{251}.

The ability of purified xanthan lyase to hydrolyze pNP-man was examined by incubating 0.5 mg of pNP-man with 10 μg of lyase in 16 mM sodium phosphate (pH 5.0) in a volume of 1 ml at 25°C for 2 h. At that time, 2 ml of 0.1 M Na_2CO_3 was added and the A_{400} of the solution was measured. As a positive control, 2.5 U of β-mannosidase was desalted on a PD-10 desalting column (Pharmacia LKB) equilibrated in 0.1 M sodium acetate, pH 4.0. Approximately 0.2 U of the desalted enzyme was incubated with 0.5 mg of pNP-man in 1.0 ml and processed as just described.

Analytical methods. The viscosity of reaction mixtures containing xanthanases and xanthan was measured with a Brookfield model LVTDV-I digital viscometer (Brookfield, Stoughton, Mass.) equipped with a temperature-controlled, small-sample adapter. Protein concentrations were measured by the bicinchoninic acid method (Pierce BCA assay; Pierce Chemical Co., Rockford, Ill.); bovine serum albumin was used as a reference protein. The thiobarbituric acid test for unsaturated hexose was performed as described by Weissbach and Hurwitz (18). The amount of pyruvate was determined by the enzymatic method of Duckworth and Yaphe (4).

Isolation of degradation product and determination of structure. To determine the enzymatic specificity of the purified lyase, 70 mg of HP-xanthan was dissolved in 20 ml of water and buffered to pH 7 with 0.1 M ammonium formate. A 100-μl aliquot of purified lyase containing 6 μg of enzyme was added, and the reaction mixture was incubated at 45°C for 18 h. The reaction mixture was then transferred to an Amicon 8050 stirred ultrafiltration cell (Amicon Corp., Danvers, Mass.) equipped with a PM 10 membrane. The solution that passed through the membrane was collected and freeze-dried. The residue was dissolved in 3 ml of water. A 2-ml aliquot of this sample was reduced with sodium borohydride and permethylated by the Hakomori method (6); the methylsulfinyl carbanion reagent was prepared as described by Sjöberg (15). The product was then analyzed by capillary gas chromatography-mass spectrometry (GC-MS) on cross-linked methylsilicone (film thickness, 25 m by 0.2 mm by 0.11 μm). The GC temperature was programmed to run at 130°C for 3 min after injection and increase at 5°C/min up to 165°C, followed by a 10-min hold. Helium was used as a carrier at 0.75 ml/min. Reduced and permethylated mannose was used as a reference.

A second large-scale reaction contained 60 mg of HP-xanthan in 20 ml of water buffered to pH 6.8 with 0.1 M ammonium formate and 100 μl of purified xanthan lyase (6 μg of protein). Following incubation at 45°C for 18 h, the reaction mixture was again passed through a 10,000-molecular-weight cutoff membrane and subsequently freeze-dried. The residue was dissolved in 10 ml of water, and a 3-ml portion was redried in a screw-capped test tube to facilitate the hydrolysis step. This residue was dissolved in 1 ml of 2% trifluoroacetic acid (TFA) and heated at 120°C for 1 h. The reaction mixture was then passed over a 1-ml column of Dowex anion-exchange resin to remove the TFA, and the eluant solution containing the hydrolyzed product was reacted to form the peracetylated aldononitrile (PAAN) derivatives as described previously (14). The PAAN derivatives were analyzed by GC-MS as described above, except that the temperature profile started at 160°C with a 3-min hold and then increased 5°C/min up to 185°C, followed by a 10-min hold.

RESULTS

Lyase purification. The cell-free culture broth was concentrated 10-fold and washed once or twice with 20 mM sodium phosphate–50 mM NaCl, pH 6.0, in an Amicon TCF-10 membrane dialysis apparatus equipped with an Amicon XM-50 membrane of nominal 50,000-molecular-weight cutoff. The amount of lyase retained on this membrane varied, depending on the degree of washing of the medium per-

TABLE 1. Purification of xanthan lyase from heat-stable, salt-tolerant bacteria

Step	Total protein (mg)	Total units[a]	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Cell-free culture broth[b]	157	14.2	0.09	100	1
Amicon XM-50 concentation	70	9.5	0.14	67	1.5
DEAE-Sepharose chromatography	7.0	3.6	0.51	25	5.7
Ultrogel AcA 44 chromatography	0.7	2.4	3.43	17	38

[a] One unit is expressed as 1 mmol of reducing sugar (calculated as glucose) produced per min at 45°C.
[b] Typical values obtained from a 3-liter culture grown for 4 days at 45°C.

formed at this step, and ranged from 75 to 90% of the total lyase activity. A membrane of nominal 30,000-molecular-weight cutoff was also tested, but the results were essentially the same (10 to 25% of the enzyme would pass through the membrane) and the amount of time required to concentrate the enzyme increased. The solution retained on the XM-50 membrane was filtered through two layers of glass-fiber filter paper, placed in dialysis tubing, and dialyzed against 20 mM sodium phosphate–50 mM sodium chloride, pH 6.0, for 48 h to equilibrate the concentrated enzyme solution for ion-exchange chromatography. The enzyme concentrate was applied at room temperature to a DEAE-Sepharose Fast Flow column (2.5 by 15 cm) equilibrated with 20 mM sodium phosphate in 50 mM NaCl, pH 6.0, and developed at 1.5 ml/min. The column eluant was monitored at A_{280}, and 7.5-ml fractions were collected. After application of the enzyme concentrate and extensive washing with equilibration buffer, the column was developed with a linear gradient of 0.05 to 0.6 M NaCl in 20 mM sodium phosphate, pH 6.0, in a total volume of 500 ml. Total xanthanase activity was measured by the reducing-sugar assay. Fractions were likewise assayed with 2% (wt/vol) carboxymethyl cellulose as the substrate to identify xanthan depolymerase activity. Lyase activity eluted at approximately 0.3 M NaCl, and the depolymerase activity eluted at approximately 0.45 M NaCl.

Fractions containing lyase activity were pooled and concentrated in an Amicon 8050 stirred ultrafiltration cell with a PM 10 membrane to a volume of <5 ml. Urea (1.8 g) was added to the concentrate, and the volume was adjusted to 5 ml with 20 mM sodium phosphate (pH 6.0) to yield a solution 6 M in urea. This solution was filtered through a 0.45-μm filter and applied to an Ultrogel AcA 44 gel permeation column (2.5 by 98 cm) equilibrated with 20 mM sodium phosphate–50 mM NaCl, pH 6.0, containing 6 M urea. The addition of urea to the buffer improves the resolution of lyase, apparently by eliminating the tendency for the proteins to aggregate at this stage. The column was developed at 20 ml/h at room temperature and monitored at A_{280}, and 3-ml fractions were collected. Lyase activity was located by incubating 100-μl aliquots of the column fractions with 400 μl of 0.1% xanthan in 20 mM sodium phosphate for 1 h at 45°C and measuring the reducing sugar as described previously. The lyase proved to be active in 1.2 M urea, so dialysis of the column fractions prior to assay was not required. Fractions containing lyase were pooled and concentrated in the Amicon 8050 cell. The enzyme was washed with 20 mM sodium phosphate–50 mM NaCl, pH 5.0, and

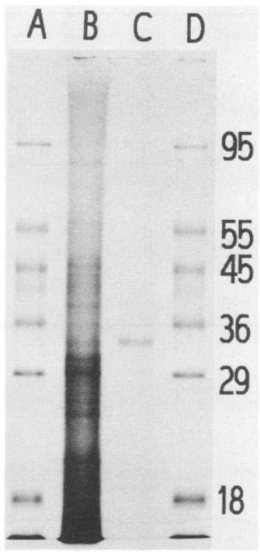

FIG. 1. SDS-PAGE on 10% polyacrylamide of concentrated cell-free culture medium (lane B), purified xanthan lyase (lane C), and protein molecular weight standards (lanes A and D; molecular weights, 10^3, are given on the right) with silver staining.

was either stored at 4°C with 0.02% NaN_3 or frozen. A summary of the purification is presented in Table 1. The yield of xanthan lyase from the concentrated medium retained on XM-50 was calculated to be 17%; this value may be somewhat lower than in actuality due to the presence of xanthan depolymerase in the early stages of the purification. While the contribution of the depolymerase to the reducing sugar formed in 1 h at 45°C is thought to be rather minimal, relative to the contribution of the lyase, the two enzymes are thought to work synergistically, with the depolymerase hydrolyzing the backbone of the biopolymer and increasing the accessibility of the lyase to the pyruvated-mannose residues that are apparently less accessible in highly ordered xanthan structures. At this time there are no known mechanisms to inhibit the depolymerase specifically in order to measure the activity of the lyase in the cell-free culture broth under the same conditions as the purified lyase, which is free of depolymerase activity.

Purity of xanthan lyase. The purity of the lyase preparations following the gel permeation chromatography step was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels (11) followed by silver staining (5). The results obtained from the stained gel showed that the purified lyase was homogeneous, with a molecular weight of 33,000, compared with the relative mobilities of the protein standards (Fig. 1). To determine whether the enzyme exists as a monomer under nondissociating conditions, 150 μg of purified lyase in 1 ml of 50 mM Tris HCl (pH 7.5) containing 100 mM KCl was chromatographed on an Ultrogel AcA 34 column (1 by 115 cm) equilibrated in the same buffer. The sample was applied to the column in 5% glycerol and developed at a flow rate of 20 ml/h. The elution volume for the purified lyase, when compared with those of proteins of known molecular weight, gave a value of 33,000, which indicated that the lyase exists as a monomer in solution. The lyase was also examined by isoelectric focusing in a polyacrylamide gel with a pH gradient of 3 to 10. After the focusing run, the gel was silver

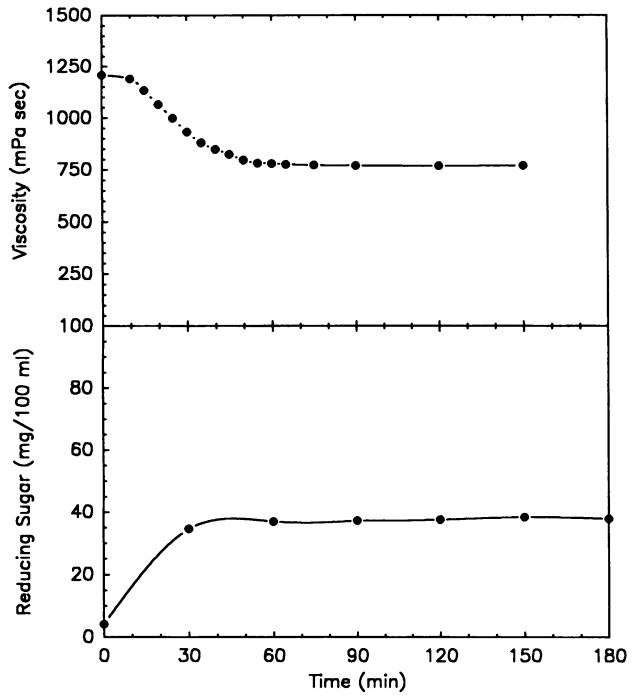

FIG. 2. Time course of degradation of xanthan gum. (Upper) Change in viscosity of a solution of xanthan and purified xanthan lyase. (Lower) Change in amount of reducing sugar during the incubation of xanthan with purified xanthan lyase (same conditions as in upper panel). The reducing power concentration is expressed as glucose produced in 1 h at 45°C.

stained to reveal a single band of pI 3.7, relative to proteins of known pI.

Properties of purified xanthan lyase. The effect of purified lyase on the solution viscosity of xanthan was examined. A stock solution of 0.3% HP-xanthan in 20 mM sodium phosphate (pH 6) was diluted with water to yield a solution of 8 ml (initial viscosity of 1,240 mPa/s at 45°C and a spindle speed of 1.5 rpm at a shear rate of 1.98/s). Purified lyase (12 μg in 200 μl of 20 mM sodium phosphate, pH 6.0) was added to the sample cup maintained at 45°C, and viscosity measurements were recorded for a total of 3 h. The upper panel of Fig. 2 shows that, under these conditions, the addition of purified lyase to a solution of xanthan resulted in a rapid decrease in the viscosity to about 60% of the starting value within 60 min; the viscosity then remained at this level.

To compare the results of the viscosity measurements with the degradation of xanthan gum, a 4-ml reaction mixture identical in composition to the sample in the viscosity experiment was incubated at 45°C, and 0.5-ml aliquots were removed every 30 min and cooled to 0°C for the 3-h sampling period. The amount of reducing sugar in the sample was measured as described previously. The lower panel of Fig. 2 shows that the amount of reducing sugar measured when the pure lyase was added to the HP-xanthan rose rapidly and reached a maximum level in 60 min. This time frame was the same as that for the loss of viscosity with the pure lyase and indicated that the xanthan had been depleted of pyruvated-mannose residues accessible to the lyase. The increase in reducing sugar resulting from the addition of lyase to HP-xanthan also follows the same time course as the production of unsaturated hexose, which was measured by the thiobarbituric acid assay. When analyzed simultaneously for reducing sugar and unsaturated hexose, assays containing HP-xanthan treated with lyase at 45°C showed an initial steady increase in both values, which subsequently reached a constant value for the duration of the measurements (data not shown).

Effect of pH and salt. The effect of pH on purified xanthan lyase activity was studied in the pH range 3.4 to 7.0 by adding a 25-μl aliquot containing 0.4 μg of lyase to 225 μl of HP-xanthan (0.1%, wt/vol) in buffer containing 25 mM sodium acetate, 25 mM sodium phosphate, and 25 mM sodium chloride. The results are shown in Fig. 3A. Maximal release of reducing sugar from xanthan occurred at a pH of 5.0, with the activity of the enzyme decreasing rapidly as the pH decreased from pH 5.0 to 4.0. Cadmus et al. (3) reported a pH optimum of 5.8 for the concentrated cell-free culture supernatant; at a pH of 5.8, the purified lyase was 73% as active at releasing pyruvated mannose as at pH 5.0. The optimal value of pH 5.8 for the concentrated cell-free culture supernatant may be a compromise value between the lyase and depolymerase, which will be determined after the purification of the depolymerase.

The effect of concentration of NaCl on the release of reducing sugar by xanthan lyase was also examined. Stock solutions of 0.1% HP-xanthan in 20 mM sodium phosphate, pH 5.0, with 0 to 2.0 M NaCl were incubated with 0.4 μg of lyase in a volume of 0.5 ml at 45°C for 1 h, and the amount

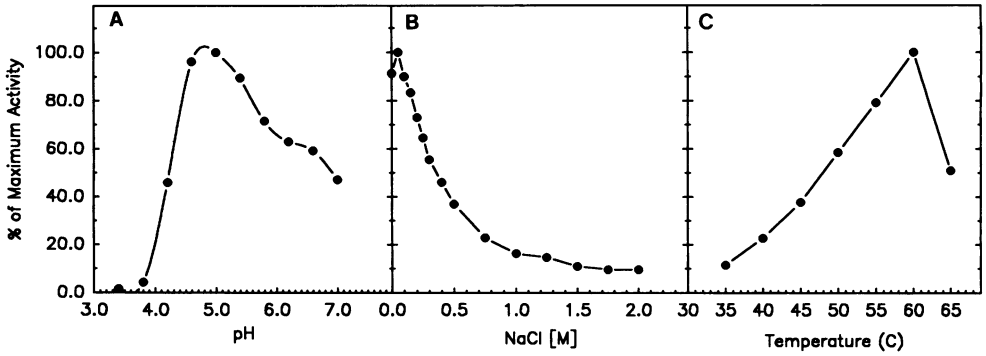

FIG. 3. (A) Effect of pH on purified xanthan lyase activity (20 mM sodium phosphate buffer, 50 mM NaCl; 1 h at 45°C). (B) Effect of NaCl concentration on xanthan lyase activity (20 mM sodium phosphate, pH 5.0; 1 h at 45°C). (C) Effect of temperature on initial rate of the formation of unsaturated 4,5-*ene*-glucuronic acid by purified xanthan lyase (20 mM sodium phosphate, pH 5.0, 50 mM NaCl). Lyase activity is expressed as the relative activity (percentage of maximal activity).

of reducing sugar released from xanthan was measured. The results shown in Fig. 3B indicate that the maximum release of reducing sugar occurred at an NaCl concentration of 0.05 M. The relative activity was reduced by 50% in solutions containing 0.4 M NaCl. Only 16% of the maximal activity remained in solutions containing 1.0 M NaCl; activity could still be detected in solutions as high as 2.5 M in NaCl.

Effect of temperature on lyase activity and stability. To determine the dependence of the initial rate of the reaction on temperature, the change in A_{251} was measured in a thermostatically controlled spectrophotometer. A stock solution of 0.1% HP-xanthan in 20 mM sodium phosphate, pH 5.0, was incubated to the desired temperature; an 0.8-ml aliquot was added to a quartz cuvette and mixed with 0.2 ml of purified xanthan lyase (12 μg), and the cuvette was placed in the temperature-regulated cuvette holder of the spectrophotometer. The absorbance was measured for 20 min against a reference without lyase. As shown in Fig. 3C, the lyase activity increased linearly between 45 and 60°C, with the highest activity being exhibited at 60°C. That the activity declined by 50% at 65°C relative to 60°C indicated a lack of stability at temperatures above 60°C.

The stability of xanthan lyase to elevated temperature was also studied, as was the effect of NaCl on heat stability. Aliquots containing lyase in 20 mM sodium phosphate, pH 5, with and without 0.25 M NaCl and without any xanthan substrate were incubated at 55, 60, and 65°C in 0.5-ml capped polyethylene microcentrifuge tubes for various lengths of time up to 6 h; individual capped tubes were removed from the water bath at 30-min intervals and transferred to a 0°C bath. Upon completion of the heating period, 10-μl aliquots of the heat-treated lyase were added to 490-μl amounts of 0.1% HP-xanthan in 20 mM sodium phosphate containing 50 mM NaCl, pH 5. The reaction mixtures were incubated for an additional hour at 45°C, and the amounts of reducing sugar were determined. The results depicted in Fig. 4 reveal that at 55°C xanthan lyase is stable with or without NaCl in the solution. At 60°C a large difference is seen; lyase in buffer without NaCl is unstable to 6-h treatment, with its activity reduced by 50% in <1 h. When exposed to 60°C in buffer with 0.25 M NaCl, however, the enzyme still retains 90% of its control (i.e., no 60°C exposure) activity after 1 h and 65% of its control activity after 6 h of 60°C exposure. At 65°C, the lyase is unstable; after 1 h, the enzyme is inactivated whether or not NaCl is present, although it appears that 0.25 M NaCl does marginally improve stability during the first hour of 65°C exposure. The presence of a reducing agent (10 mM dithiothreitol) both with and without 0.25 M NaCl neither enhanced nor reduced thermal stability of the purified lyase.

Determination of xanthan lyase specificity. The low-molecular-weight product(s) resulting from the extensive treatment of HP-xanthan with purified xanthan lyase was reduced with sodium borohydride and permethylated. Capillary GC analysis of the product gave a single peak of 9.3-min retention; permethylated mannitol, by contrast, emerges at 4.0 min. Electron impact MS gave a single major fragment ion of high mass, *m/z* 263 (M-59), derived from the loss of the carbinol methoxy group from the methyl ester of tetra-*O*-methyl-(4,6-*O*-carboxyethylidene)-D-mannitol. Chemical ionization MS with isobutane as the reagent gas gave the expected protonated molecular ion, MH^+, of *m/z* 323 and lacked a permethylated-mannitol signal of *m/z* 266 which would have been present if any unsubstituted mannose existed in the low-molecular-weight fraction recovered after lyase treatment. A portion of this fraction was also treated with TFA to remove the pyruvate moiety and then reacted to form the PAAN derivative. When analyzed by GC-MS, the treated PAAN fraction from xanthan gave the same retention time (6.3 min) and MS spectrum as the PAAN of D-mannose. The combined results of the experiments demonstrate that the lyase was specifically recognizing and releasing only pyruvated-mannose residues, in agreement with the results reported by Cadmus et al. (3). That the purified enzyme did not hydrolyze pNP-man when incubated at 25°C for 2 h, during which time authentic β-mannosidase extensively hydrolyzed pNP-man, showed that the lyase is not a general mannosidase.

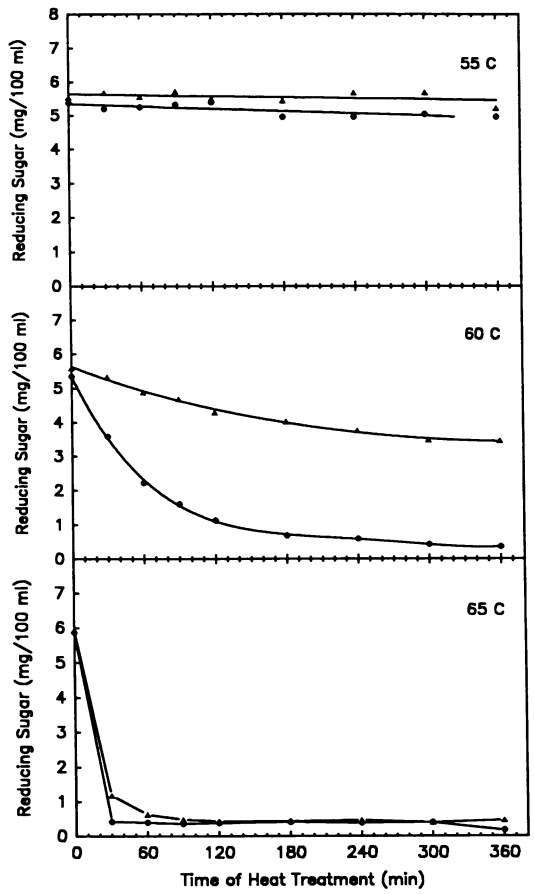

FIG. 4. Effect of NaCl on stability of xanthan lyase exposed to high temperature. Lyase was initially incubated at different temperatures for the indicated time in 20 mM sodium phosphate buffer, pH 5.0, with (▲) or without (●) 0.25 M NaCl. Results shown are amounts of reducing sugar formed after the heat treatment period measured subsequently in a separate 1-h assay containing 0.1% HP-xanthan in 20 mM sodium phosphate–50 mM NaCl, pH 5.0, at 45°C. The reducing power concentration is expressed as glucose produced in 1 h at 45°C. (Top) 55°C; (middle) 60°C; (bottom) 65°C.

DISCUSSION

Very little literature exists on the characterization of xanthan lyases. Sutherland (17) reported on some of the properties of xanthan lyases produced by a *Bacillus* species, a *Corynebacterium* species, and a mixed bacterial culture grown at 30°C. These lyase preparations were specific in their site of hydrolysis, at the β-D-mannosyl-(1→4)-β-D-

glucuronic acid junction, but were apparently not restricted to only the pyruvated form of mannose. No data were reported on the ability of the lyases produced by the bacteria to function at elevated temperatures or salt concentrations. The salt-tolerant culture HD1 (7) did produce pyruvated mannose as a degradation product, but whether this was by a lyase or a β-mannosidase was not reported. The optimal temperature for the xanthanase complex made by HD1 was 30 to 35°C.

The xanthan lyase produced by the bacterial consortium discovered by Cadmus et al. (3) has thermal stability greater than that of any xanthan lyase reported previously. In purified form it is capable of withstanding exposure to 60°C for extended periods of time in the presence of 0.25 M NaCl, which stabilizes the enzyme in some unknown manner. It is interesting to note that, whereas such salt concentrations as 0.25 to 0.5 M enhance thermal stability of the lyase, its enzymatic activity is optimal in 0.05 M NaCl when assayed at 45°C. The apparent molecular weight of the lyase, measured here at 33,000, is in the 30,000 to 33,000 range reported for a lyase from a *Bacillus* species (17). The *Bacillus* enzyme had a pH of optimal activity of 7.25, which is different than the pH optimum of 5.0 for the lyase reported here.

The ability of the purified lyase to act on intact xanthan indicates that this enzyme can be used for preparing modified xanthan, bearing terminal unsaturated 4,5-*ene*-glucuronic acid residues, and to investigate its properties for potential food or industrial applications. The purified lyase may also be useful as a tool to investigate the chemical structure of other polysaccharides of interest to determine whether pyruvated mannose is present. This enzyme could also serve as a model to help gain additional insight into those features that confer salt tolerance and heat stability to an enzyme by studying the composition and structure of this lyase, relative to xanthan lyases that do not have the ability to withstand elevated temperatures and salt concentrations.

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