APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1986, p. 37–44
0099-2240/86/070037-08$02.00/0

Vol. 52, No. 1

Purification and Properties of a Novel Xanthan Depolymerase from a Salt-Tolerant Bacterial Culture, HD1

CHING T. HOU,* NANCY BARNABE, AND KATHY GREANEY

Corporate Research Sciences Laboratory, Exxon Research and Engineering Co., Annandale, New Jersey 08801

Received 18 November 1985/Accepted 31 March 1986

A novel xanthan depolymerase (endo-β-1,4-glucanase) was isolated from a salt-tolerant bacteria culture (HD1) grown on xanthan. The depolymerase was purified 55-fold through chromatography on ion-exchange and molecular sieve columns, including high-performance liquid chromatography. The purified enzyme fraction was homogeneous as judged by polyacrylamide gel electrophoresis. The molecular weight of this enzyme is 60,000. Optimum pH and temperature for xanthan depolymerase activity were around 5 and 30 to 35°C, respectively. The enzyme was not stable at a temperature higher than 45°C. The activation energy calculated from an Arrhenius plot was 6.40 kcal (26.78 kJ). The enzyme molecule contains no sugar moiety. The amino acid composition of the enzyme protein was determined. Xanthan depolymerase cleaves the endo-β-1,4-glucosidic linkage of the xanthan molecule, freeing reducing groups of some sugars and decreasing viscosity of the polymer solution. Only the backbones of β-1,4-linked glucans with side chains or other substituents were cleaved. No monosaccharide was produced by the action of this enzyme. The oligosaccharide(s) in the low-molecular weight fraction consisted of 15 to 58 monosaccharide units. The enzymic reaction resulted in the decrease in weight-average molecular weight of xanthan from 6.5×10^6 to 8.0×10^5 in 0.5 h. This enzyme alone could not degrade xanthan to a single or multiple pentasaccharide unit(s). Results suggest that there may be regions inside the xanthan molecule that are susceptible to the attack of this enzyme. Xanthan depolymerase activity was not inhibited by many chemicals, including thiols, antioxidants, chlorinated hydrocarbons, metal-chelating agents, and inorganic compounds, except ferric chloride and arsenomolybdate. Many biocides were tested and found not to be inhibitory. Conditions used in enhanced oil recovery operations, i.e., the presence of formaldehyde, $Na_2S_2O_4$, 2,2-dibromo-3-nitrilopropionamide, and an anaerobic environment, did not inhibit xanthan depolymerase activity.

Xanthan is an anionic extracellular polysaccharide produced by *Xanthomonas campestris* NRRL B-1459 (17). The high viscosity of this polymer solution is relatively insensitive to temperature, ionic strength, shear, and pH. For this reason, xanthan finds commercial use as a viscosity-enhancing agent for aqueous solutions. The primary structure of xanthan was established by Jansson et al. (12). It consists of a main chain of β-1,4-linked D-glucose units, as in cellulose, but with a three-sugar side chain attached to alternate glucose residues. Pyruvic acetal, i.e., 4,6-O-(1-carboxyethylidene), substituents are on the terminal *d*-mannosyl residues of some of these side chains. The molecular weight of xanthan varies from 3×10^6 to 15×10^6, depending on the methods and conditions used in the determination of molecular weight.

It was reported that xanthan is inert to the attack by microbes or available enzymes (13). Rinaldo and Milas (16) were the first to show partial hydrolysis of xanthan by cellulase only in the absence of salt where xanthan is in the unordered conformation. More recently, Cadmus et al. (4) reported the biodegradation of xanthan by a *Bacillus* sp. in the presence of salt. The xanthanase they obtained was a mixture of enzymes that attacked all of the side chain linkages in the xanthan molecule, including the one involving (1→3)-linkage of acetylated mannose to the glucosidic backbone. They found no endocellulase activity in their cultures. Sutherland (20) described an enzyme system hydrolyzing the polysaccharides of xanthomonas species. The β-glucanohydrolase hydrolyzed both β-1,3- and β-1,4-linked polymers with side chains or other substituents.

In recent enhanced oil recovery field tests with xanthan as the viscosity-controlling agent, xanthan was found to be degraded by microbial (enzymic) activity (2). Since then, the biodegradation of xanthan, and methods for its prevention, have become important research areas. The study of partial degradation of xanthan into polymers with different molecular weights is also important for more precise investigation of xanthan conformational properties.

In a previous paper (C. T. Hou, N. Barnabe, and K. Greaney, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, O69, p. 247), we described the isolation of a mixed culture, culture HD1, which utilized xanthan as its carbon source in the presence of salt. Extracellular enzyme(s) produced by culture HD1 degraded the xanthan molecule. In this paper, we describe the purification of a novel depolymerase which breaks the endo-β-1,4-glucosidic linkage of xanthan molecules. We elected to study this particular enzyme because it causes the initial loss of viscosity of xanthan solutions. Product identification as well as physicochemical properties of the purified enzyme were studied.

MATERIALS AND METHODS

Bacterial strains. A salt-tolerant, xanthan-utilizing mixed culture was isolated from a soil sample of Linden, N.J. (Hou et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1985). The culture was maintained on a 2% salt-containing nutrient agar which contained glucose, mannose, and xanthan as the carbon and energy sources (Hou et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1985). For the production of xanthan-degrading enzymes, the culture was grown at 30°C aerobically in a 2.8-liter shake flask containing 1 liter of medium

* Corresponding author.

with the following composition (per liter): 2.5 g of xanthan, 0.5 g of $(NH_4)_2SO_4$, 0.8 g of yeast extract, 0.4 g of peptone, 1.5 g of KH_2PO_4, 0.7 g of K_2HPO_4, 2.0 g of $NaNO_3$, 20 g of NaCl, and 10 ml of trace metals solution. The composition (per liter) of the trace metals solution was 0.5 mg of $CuSO_4 \cdot 5H_2O$, 1 mg of H_3BO_3, 0.7 mg of $MnSO_4 \cdot H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, and 1 mg of MoO_3. The inoculum, about 2%, was a 3-day-old shake-flask culture of the same medium. The flasks were shaken at 200 rpm at 30°C. Xanthan-degrading enzyme(s) was harvested after 3 to 4 days of incubation when the maximum yield was reached (Hou et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1985).

Chemicals. Xanthan was obtained from Kelco Co. (Keltrol, no. 16573; Clark, N.J.). Chemicals were reagent grade and were used without further purification. Biocides were commercial grade obtained from available sources. Bio-Gel A-0.5m, DEAE-cellulose, and DEAE-Bio-Gel and Bio-Gel P-2 were purchased from Bio-Rad Laboratories (Richmond, Calif.). Thin-layer precoated cellulose plates were obtained from Pierce Chemical Co. (Rockford, Ill.).

Enzyme assays. Enzyme assays with either viscosity or reducing sugar measurements were conducted by incubating a 1.25 ml of a xanthan stock solution and a small amount of enzyme(s) at 25°C. Xanthan stock solution was prepared by dissolving 0.188% xanthan, 0.4 mM $MgSO_4$, and 0.03 mM $MnSO_4$ in 0.05 M sodium acetate buffer (pH 5.4). At certain time intervals, samples were taken for either viscosity or reducing sugar assay. Viscosity was measured at 25°C with a low-shear 30 rheometer (Contraves Industrial Products Ltd., Middlesex, U.K.) at a shear rate of 1.3 s^{-1}. The rheometer was equipped with an Endocal refrigerated circulating bath (Neslab Instruments, Inc., Portsmouth, N.H.). Reducing sugars, calculated as glucose, were determined by the method of Somogyi (19). Initial tests on time profile studies of these assay methods with concentrated cell-free broth of strain HD1 showed that both reactions took place immediately. The differences in viscosity and reducing sugars between two time intervals (1.0 to 2.0 min change in viscosity (centipoise) 0.5 min to 1.5 min for change in reducing sugars) were measured. Enzyme activity was expressed as change in viscosity (centipoise) per minute (depolymerase) or micromoles of reducing sugar group released (as glucose) per minute (for both endo- and exoglucanases). Specific enzyme activity was expressed per milligram of protein.

α-Mannosidase (or β-mannosidase) activity was assayed by incubating 0.25 ml of 0.1 M citrate buffer (pH 4.5), 0.25 ml of 0.01 M *p*-nitrophenyl-α,D-mannopyranoside (or *p*-nitrophenyl-β,D-mannopyranoside), and 50 μl of enzyme solution at 25°C for 5 min. After the reaction, 2 ml of 0.2 M borate buffer (pH 9.8) was added to stop the reaction. The amount of nitrophenyl liberated was measured at 405 nm and calculated with the molar extinction coefficient for nitrophenyl, 18.5×10^3.

Analytical methods. Thin-layer chromatography was performed for the identification of monosaccharides. The solvent system was pyridine-ethyl acetate-acetic acid-water (5:5:1:3, vol/vol). Sugars on thin-layer chromatography plates were detected by spraying the plates with *p*-anisidine-phthalic acid reagent (18).

Fluorescent derivatives of xanthan were prepared by isocyanide coupling (5) of fluoresceinamine to the carboxy groups of xanthan. A method developed by Holzwarth (10) was followed for the molecular weight distribution studies.

Acrylamide gel electrophoresis was conducted in 10% gel. Gels were stained with Coomassie brilliant blue (15). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in a 12% polyacrylamide gel according to the method of Weber and Osborn (22). The enzyme solution was preincubated for 3 min at 100°C in a small amount of 100 mM sodium phosphate buffer (pH 7.0), containing 1% sodium dodecyl sulfate. The molecular weight marker proteins were soybean trypsin inhibitor (19,900), carbonic anhydrase (28,800), ovalbumin (42,600), bovine serum albumin (67,600), phosphorylase B (95,500), and β-galactosidase (131,800).

A glycoprotein test was conducted with fuchsin-sulfite stain of the polyacrylamide gel according to the method of Zacharius et al. (23).

Amino acid analyses were performed with a Beckman model 120B amino acid analyzer (Beckman Instruments Inc., Fullerton, Calif.) after 24-, 48-, and 72-h hydrolyses of 5-mg samples of protein according to the procedure of Moore and Stein (7).

High-performance liquid chromatography (HPLC) was performed with a Varian model 5000 HPLC apparatus (Varian Associates, Inc., Florham Park, N.J.) which had been equipped with a Varian refractive index detector RI-3 and a Perkin-Elmer LC 75 UV detector (The Perkin-Elmer Corp., Norwalk, Conn.). A Synchropak AX300 column (Varian) was used for protein separation. Two types of columns, Aminex HPX-87C and Aminex HPX-42A (Bio-Rad), were used for monosaccharide and oligosaccharide analyses, respectively. HPLC analyses of carbohydrates were performed at 85°C.

Separation of reaction products. Degradation products of xanthan formed by the enzyme fraction recovered from HPLC (see below) were studied. Enzyme protein (2.5 mg) was incubated with 144 mg of xanthan in 80 ml of 0.05 M sodium acetate buffer (pH 5.4) at 35°C. At certain time intervals, samples were taken and boiled for 5 min to deactivate the enzyme. Samples were then separated into low-molecular-weight fraction (LMWF) and high-molecular-weight fraction (HMWF) by passing them through an ultrafiltration unit (Amicon Corp., Lexington, Mass.) with PM10 membrane (10,000-molecular-weight cutoff). The LMWF was concentrated with a rotary evaporator and desalted with a Bio-Gel P-2 column (Bio-Rad). The desalted LMWF was again concentrated and was either assayed for total carbohydrate content by the phenol-sulfuric acid method (6) or analyzed for monosaccharide by thin-layer chromatography and HPLC. The HMWF was subjected to molecular weight distribution studies by the fluorescent-derivative method (10).

RESULTS

Purification of enzyme(s). All steps were performed at 4°C. Unless otherwise stated, the buffer solution was 0.05 M sodium phosphate buffer, pH 7.0.

(i) Step 1. Concentration. After 3 days of growing culture HD1 on xanthan at 30°C, cells were removed by centrifugation at 10,000 × *g* for 20 min. The supernatant (30 liters, collected from several batches) was concentrated to 500 ml in an Amicon model DC-10 ultrafiltration unit with a hollow-fiber H10P10-20 cartridge. It was then washed with 0.05 M phosphate buffer (pH 7.0) with three replacements of volume.

(ii) Step 2. DEAE-cellulose column chromatography. The concentrated and washed fraction from step 1 was applied to a DEAE-cellulose column which had been equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). The column (10.5 cm in diameter, 33 cm long) was washed with 3 liters of the

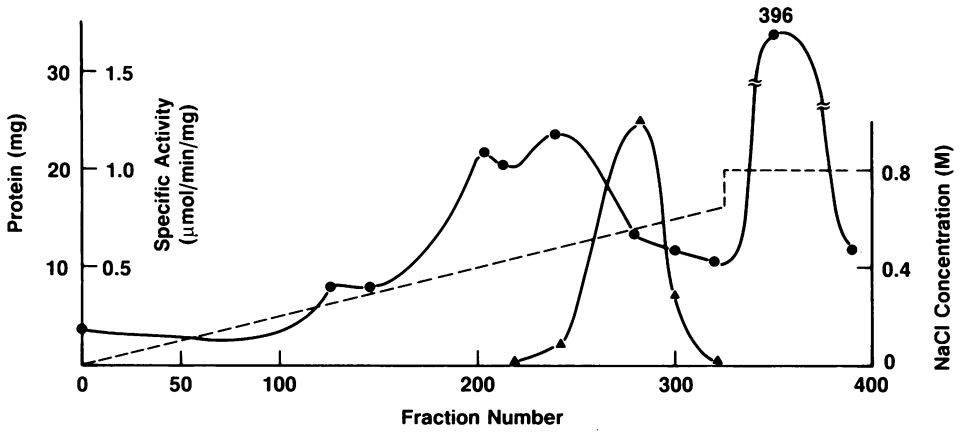

FIG. 1. DEAE-cellulose column chromatography of xanthan-degrading enzyme(s) from culture broth of HD1. Concentrated cell-free culture broth of HD1 grown on xanthan was applied. Fractions (20 ml) were collected. Symbols: ●——●, protein; ▲——▲, specific enzyme activity by reducing sugar assay; -----, NaCl concentration.

same buffer and then was eluted by a linear gradient of 0 to 0.6 M NaCl in the same buffer. Final elution of the column was carried out with the same buffer containing 0.8 M NaCl. Fractions (20 ml) were collected. Gradient fractions 225 to 320 contained enzyme activities for xanthan degradation (both reducing sugar production and viscosity reduction) (Fig. 1). Very small amounts of α- and β-mannosidase activities were found in the wash fraction (specific activities, given in nanomoles per minute per milligram, were 0.42 for α-mannosidase and 0.44 for β-mannosidase). Fractions 225 to 320 were combined, concentrated, and washed with 0.05 M phosphate buffer (pH 7.0) with an Amicon hollow-fiber concentration model DC 2 with an H1P10-20 cartridge (10,000-molecular-weight cutoff).

(iii) Step 3. DEAE-Bio-Gel column chromatography. The Amicon filter-concentrated fraction (190 ml) obtained from step 2 was applied to a DEAE-Bio-Gel column (5.6 cm in diameter by 27 cm) which had been equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). The column was washed with 1 liter of the same buffer containing 0.3 M NaCl. It was then eluted with a linear gradient of 0.3 to 0.7 M NaCl in the same buffer. Each 15-ml fraction was collected. The NaCl gradient elution of the DEAE-Bio-Gel column chromatography is shown in Fig. 2. Fractions 75 to 115 showed high enzyme activity assayed by the production of reducing sugars. These fractions also had the ability to decrease viscosity of xanthan solutions. These fractions were combined and concentrated in an Amicon ultrafiltration unit with a PM10 membrane.

(iv) Step 4. HPLC. The Amicon filter-concentrated fraction obtained from step 3 was applied to an HPLC with a Synchropak AX300 column. The mobile phase was 0.05 M sodium phosphate buffer (pH 7.0). The column was eluted with a programmed pattern of NaCl gradient. Protein patterns separated by HPLC are shown in Fig. 3. The protein peaks were collected, and their enzyme activities were tested. The activities for viscosity reduction and for release of reducing sugar groups were found in protein peak 5 of the HPLC separation. Several runs were performed to collect a sufficient amount of protein peak 5 fraction for the following studies. A summary of enzyme purification is listed in Table 1. A 55-fold purification was achieved.

Purity and molecular weight of enzyme. The purified enzyme fraction obtained from step 4 HPLC exhibited a single-protein band in polyacrylamide gel electrophoresis (Fig. 4). The molecular weight of the xanthan depolymerase estimated by calibrated Bio-Gel A-0.5m column was 60,000. Electrophoresis in a 12% polyacrylamide gel in the presence of sodium dodecyl sulfate also gave a single-protein band with a molecular weight of 60,000, indicating that the enzyme consists of a single subunit. Attempts to apply a glycoprotein stain to the enzyme on polyacrylamide gel showed that this enzyme contains no sugar moiety.

Time profile of xanthan depolymerase activity. Control

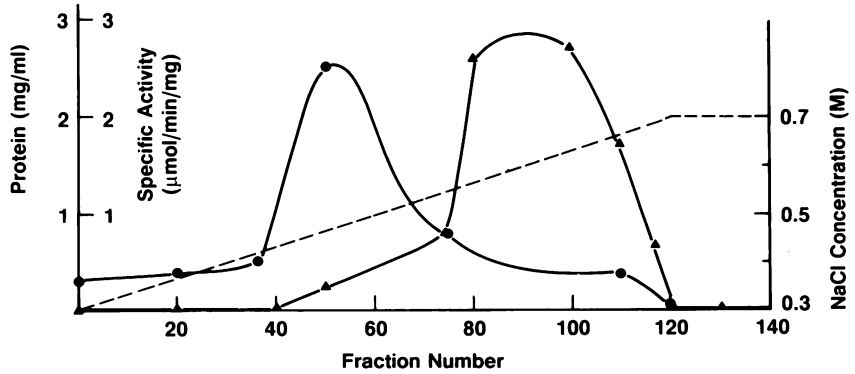

FIG. 2. DEAE-Bio-Gel column chromatography of xanthan-degrading enzyme(s). Enzyme fraction obtained from step 2 was used. Fractions (15 ml) were collected. Symbols: ●——●, protein; ▲——▲, specific enzyme activity by reducing sugar assay; -----, NaCl concentration.

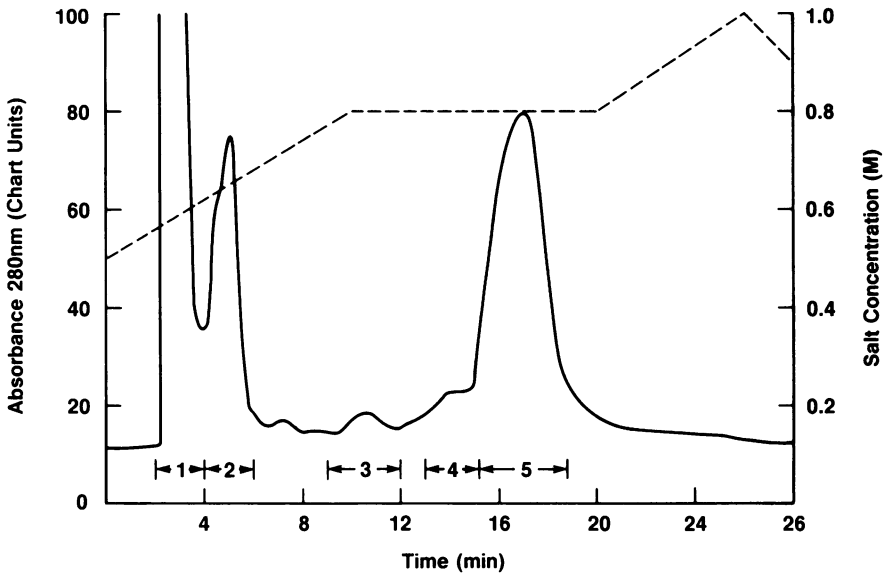

FIG. 3. HPLC ot xanthan-degrading enzyme. Enzyme fraction obtained from step 3 was used. A 0.5-ml sample was applied in each run to a preprogrammed salt-gradient pattern. ——, A_{280}; -----, salt concentration.

experiments with either heat-killed or no enzyme showed no degradation of xanthan as measured by viscosity and reducing sugar assays. Time profile studies of xanthan degradation by the purified enzyme fraction are shown in Fig. 5. Enzyme activity was followed by both release of reducing sugar groups (Fig. 5A) and solution viscosity changes (Fig. 5B). The release of reducing sugar groups was linear for the first 5 min and tapered off after that. A similar reaction pattern was observed in the viscosity assay. For these reasons, enzyme activity was measured during the initial 3 min of reaction for either reducing sugar assay or viscosity assay. At 20 h of incubation, an additional amount of enzyme was added to the reaction mixture. No further release of reducing sugars or further decrease in solution viscosity was observed.

Identification of reaction products. The reaction products at 0, 0.5, 1, 5.5, and 18 h of incubation were studied. They were separated into LMWF and HMWF according to the method described previously. Thin-layer chromatography assay of these LMWFs showed that no monosaccharide was produced from xanthan by the action of xanthan depolymerase. This was confirmed by monosaccharide analyses with an Aminex HPX-87C column with HPLC. The oligosaccharide in LMWF was further assayed with HPLC with an Aminex HPX-42A column. A peak detected on this column indicated the molecular weight of the oligosaccharide at greater than that of DP15 (15 monosaccharide units, the upper limit of resolution for this HPLC column). The oligosaccharide(s) (smaller than 10 pentasaccharide units or 10,000 molecular weight) in LMWF was not further identified. However, because it passes through an Amicon PM 10 membrane but elutes at the void volume on an Aminex HPX-42A column, the oligosaccharide(s) in the LMWF is estimated to consist of 15 to 58 monosaccharide units. Total carbohydrate in these LMWFs was less than 5% of the substrate xanthan. The HMWFs of 0.5- and 18-h samples were assayed for molecular weight distribution by the fluorescent-derivative method (10; Hou et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1985). The weight-average molecular weight of xanthan decreased from 6.5×10^6 to 8.0×10^5 and 7.5×10^5, respectively, by the action of the purified enzyme fraction, confirming our observation in the time profile studies (Fig. 5) that this enzyme cannot further degrade the HMWF.

Effect of pH. The effect of pH on xanthan depolymerase activity was studied in the pH range 3.5 to 9.0 with different

TABLE 1. Purification of xanthan-degrading enzyme(s) from xanthan-grown culture HD1

Step	Total protein (mg)	Enzyme activity					
		Reducing sugar assay			Viscosity assay		
		Sp act[a]	Total U[b]	Recovery (%)	Sp act[c]	Total U[d]	Recovery (%)
1. Amicon concentrated cell-free broth	4,858	1.6	7,772	100	12.2	59,267	100
2. DEAE-cellulose column chromatography	603	2.0	1,206	15.5	10.2	6,150	10.4
3. DEAE-Bio-Gel column chromatography	30	19	570	7.3	168.6	5,058	8.5
4. HPLC	7	77	540	6.9	657	4,599	7.7

[a] Specific activity is expressed as units per milligram of protein.
[b] One unit is expressed as 1 μmol of reducing sugar (calculated as glucose) produced per min.
[c] Specific activity is expressed as units per milligram of protein.
[d] One unit is expressed as a change in solution viscosity of 1 cP/min.

buffer solutions at 0.05 M (sodium acetate buffer, pH 3.5 to 6; sodium phosphate buffer, pH 6 to 7; and Trishydrochloride buffer, pH 8 to 9). Enzyme activity was measured at 25°C with a rheometer, according to the method described above, with 100 μg of enzyme. The optimal pH for enzyme activity was found to be around 5 (Fig. 6).

Effect of temperature. Temperature dependence of the activity of xanthan depolymerase was studied in the range of 5 to 60°C. The reaction mixture was equilibrated at test temperature for 5 min before the addition of enzyme to start the reaction. Reaction rates were compared. The optimum temperature for xanthan depolymerase activity was found to be around 30 to 35°C (Fig. 7). The activation energy calculated from the Arrhenius plot of log velocity versus the reciprocal of absolute temperature was 6.40 kcal (26.78 kJ)/mol.

The stability of the enzyme at different temperatures was also studied. The enzyme was incubated at various temperatures for 20 min. Activity was then assayed at 25°C. The relative percentages of activity after incubating the enzyme at different temperatures were 100 (25°C), 100 (30°C), 100 (35°C), 100 (40°C), 33 (45°C), 16 (50°C), and 0 (60°C).

Michaelis constant. The effect of xanthan concentrations on the enzyme activity in pH 5.4 buffer solution was studied at 35°C. The K_m value calculated from Lineweaver-Burk plots of log velocity (viscosity change) versus reciprocal of substrate concentration was 1.9×10^{-7} M.

Amino acid analyses. The amino acid composition of xanthan depolymerase is presented in Table 2. The results are an average of three runs with different times of hydrolysis (24, 48, and 72 h). It was surprising to note that no sulfur-containing amino acids were detected in this enzyme.

Substrate specificity. Possible substrates including polysaccharides with known or unknown structures were tested for substrate specificity of xanthan depolymerase. Enzyme activity was measured by the release of reducing sugars. Solution viscosity assay was also performed where applicable. ZNS 63008K was a viscous heteropolysaccharide with unknown structure obtained from Kelco Co. Xanthan depolymerase has a very narrow substrate specificity (Table 3). It hydrolyzed only β-1,4-linked polymers with side chains or other substituents. The enzyme did not hydrolyze unsubstituted cellulose. The following polysaccharides, whose structures are not known, were not substrates for xanthan depolymerase: KID97 (high-temperature-stable oil field biopolymer, anionic heteropolysaccharide produced by an *Alcaligenes* sp. [Kelco]); KIA 108 and KIA 112 (anionic heteropolysaccharides [Kelco]); Gelrite (polysaccharide consisting of uronic acid, rhamnose, and glucose [Kelco]); mansan (from *Rhinocladiella mansoniei* NRRL Y-62720); indican (polysaccharide consisting of glucose, rhamnose, and galacturonic acid); cryptocan (polysaccharide consisting of mannose, glucuronic acid, and xylose); and a polysaccharide (consisting of glucose and rhamnose) from methanol-grown *Methylocystis parvus* OBBP (11).

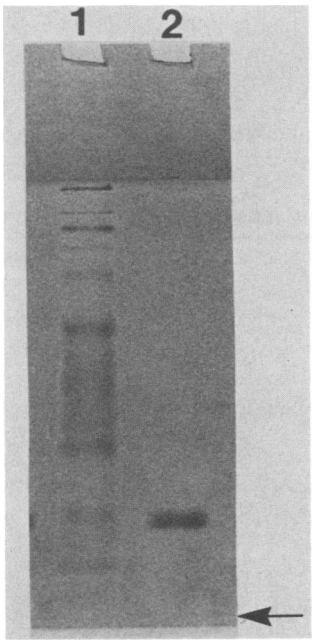

FIG. 4. Polyacrylamide gel electrophoresis of xanthan depolymerase (endo-β-1,-4-glucanase). Lane 1 received 15 μg of concentrated crude enzyme fraction. Lane 2 received 5 μg of purified enzyme fraction obtained from fraction 5 of HPLC. Arrow indicates marker front.

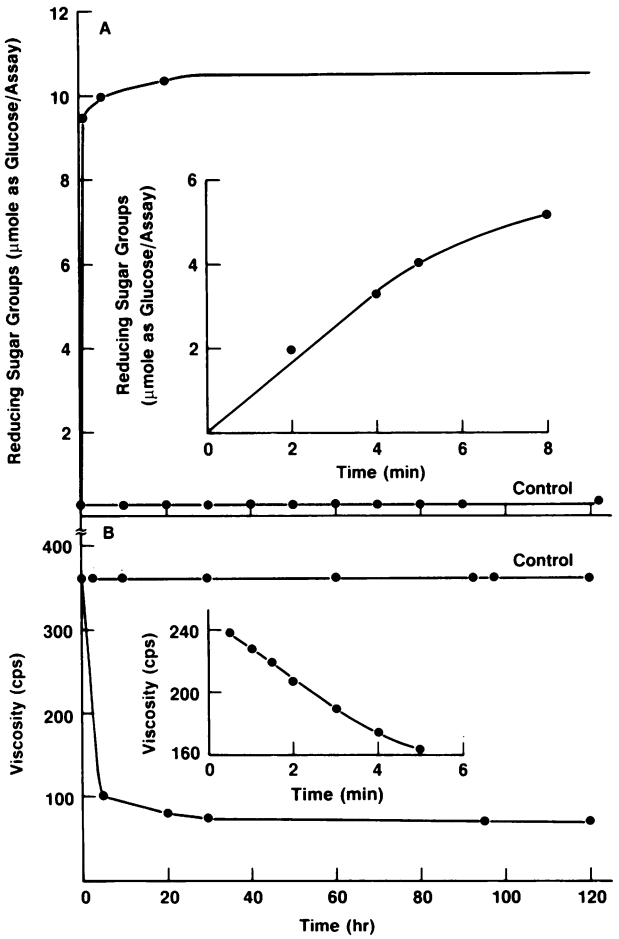

FIG. 5. Time course of degradation of xanthan by purified enzyme fraction from step 4 purification. The reactions were conducted as described in the text. Enzyme activity assays were followed by both the release of reducing sugar groups (A) and the decrease in solution viscosity (B). Controls had no addition of enzyme fraction. Inserts were data obtained from experiments of shorter incubation time. The viscosity assays for the inserts of (B) were conducted at 25°C in the measuring cup of contraves rheometer.

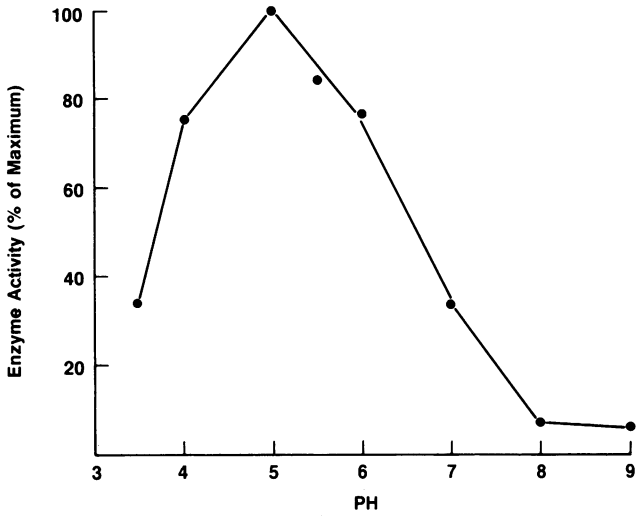

FIG. 6. Effect of pH on activity of xanthan depolymerase. Activity was measured at 25°C by following the decreases in solution viscosity. The buffer solutions (0.05 M) used were pH 3 to 6, sodium acetate buffer; pH 6 and 7, sodium phosphate buffer; pH 8 and 9, Trishydrochloride buffer.

Inhibition studies. Various chemicals, including thiols, metal ions, and metal-chelating agents, were tested for inhibition of xanthan depolymerase activity. These chemicals (at 1 mM concentration unless otherwise stated) were added into xanthan stock solutions. Enzyme activity was assayed viscosimetrically. The enzyme activity was inhibited strongly by ferric chloride and arsenomolybdate (Table 4). Inhibition by thiol reagents and metal-chelating agents was not significant. The enzyme activity was not inhibited by the following chemicals: NaCl; KCl; ferrous sulfate (5.5 mM); KOH (5.0 mM); NaOH (5.0 mM); ammonium sulfate (5.0 mM); Trizma (Sigma Chemical Co. [St. Louis, Mo.] brand of tromethamine) (5.0 mM, pH 8.5); Triton X-100 (4%); formaldehyde (5.5 mM); citric acid (5.5 mM); $Na_2S_2O_4$ (0.2%); antioxidants (100 parts per million [ppm]) such as sodium dithionite and sodium hypochlorite; chlorinated hydrocarbons (100 ppm) such as chloroform, chlorobenzene, *p*-chlorobenzoic acid, α-chloro-3,4-dihydroxyacetophenone, and chlorophenol. The following biocides (100 ppm) also failed to inhibit xanthan depolymerase activity: Busan 30, Busan 77, Busan 85, Busan 110, Busan 1030, sodium omadine, Dow antimicrobial 7287 (2,2-dibromo-3-nitrilopropionamide) (Dow Chemical Co., Midland, Mich.), MBT 3522 (methylene bisthiocyanate), Kathon WT (8.6% 5-chloro-2-methyl-4-isothiazoline-3-one and 2.6% 2-methyl-4-isothiazoline-3-one), 2,2-dibromo-3-nitrilopropionamide, Dowicide B (sodium trichlorophenate), and Dowicide 75 [67.5% 1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane chloride]. Xanthan depolymerase activity was not affected by an anaerobic environment.

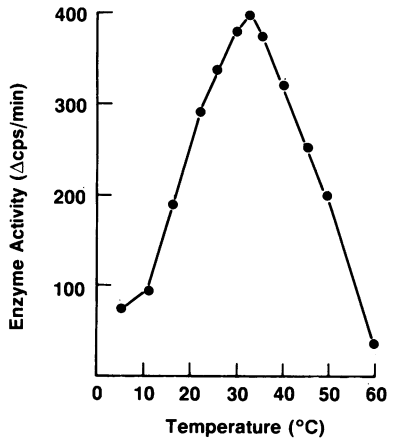

FIG. 7. Effect of temperature on the activity of xanthan depolymerase. The reaction mixture was equilibrated at test temperature for 5 min before the addition of enzyme to start the reaction. Their reaction rates (as change of solution viscosity [centipoise] per minute) were compared.

TABLE 2. Amino acid composition of xanthan depolymerase[a] from xanthan-grown culture HD1

Amino acid	No. of residues/ enzyme molecule
Aspartic acid	52
Threonine	40
Serine	40
Glutamic acid	36
Proline	16
Glycine	32
Alanine	40
Valine	16
Isoleucine	28
Leucine	32
Tyrosine	16
Phenylalanine	8
Lysine	16
Histidine	4
Arginine	16
Tryptophan[b]	48

[a] The molecular weight of xanthan depolymerase was estimated as 60,000 daltons.
[b] Tryptophan was determined spectrophotometrically.

DISCUSSION

Reports on xanthan-degrading enzymes are rare. Lesley (14) reported an enzyme from a *Bacillus* sp. acting on

TABLE 3. Substrate specificity of xanthan depolymerase

Substrate	Major structure	Enzyme activity (μmol of reducing sugar/min)[a]
Kelzan[b]	βG1 → 4G / M--	3.45 (26 cP/min)
Carboxymethyl-cellulose	βG1 → 4G	2.40
Cellulose	βG1 → 4G	0
Cellulose acetate	βG1 → 4G	0
D-(+)-Cellobiose	βG1 → 4G	0
ZNS 63008K		4.93 (13 cP/min)
Scleroglucan	$\beta G_6 1 \rightarrow 3G$ \ βG1	0
Actinomyces viscosus	βGlu1 → 4 βG1 → 4G	0
Starch	αG1 → 4G	0
Dextran	αG1 → 6G	3.0
D-(+)-Trehalose	αG1 → 1G	0

[a] A total of 40 μg of enzyme was used per assay. Numbers in parentheses were by viscosity assay where applicable.
[b] Kelzan, Kelco brand of xanthan gum.

Xanthomonas "phaseoli" exopolysaccharide. This enzyme released a complex mixture of fragments including a trisaccharide, thought to contain equal amounts of D-glucose, D-mannose, and an unsaturated uronic acid. The high hydrolysis rate for xanthan by a fungal cellulase reported by Rinaldo and Milas (16) could not be observed by Sutherland (20) or Hou et al. (unpublished data). Recently, Sutherland (21) reported that commercial cellulases hydrolyze xanthan at elevated temperature and in the absence of ions. The release of D-glucose or reducing material was only 5% or less of the total available D-glucose in the polymer. The products of xanthan digestion by crude enzyme preparations were complex. Because the products included both glucose and mannose, both glucosidases and mannosidases appear to be present. Difficulties have been encountered by other workers in separating the components of β-glucanase (endo enzyme)/β-glucosidase (exo enzyme) preparations degrading cellulose because of the multiplicity of enzymes present (1, 8, 9). Likewise, it has not yet proved possible to obtain any homogeneous protein from the xanthan-degrading systems (4, 20).

In this study, we have purified xanthan depolymerase to homogeneity as judged by polyacrylamide gel electrophoresis (Fig. 4). This is the first report on a purified xanthan-degrading enzyme. The low recovery at step 2 of enzyme purification (DEAE-cellulose column chromatography) may be attributed to the presence of many hydrolytic enzymes in the concentrated cell-free culture broth and to the synergetic effect among these enzymes. The purified enzyme acted on xanthan, resulting in the release of reducing sugar groups and the decrease in solution viscosity. However, the enzyme could not degrade the xanthan molecule completely (Fig. 5). It releases reducing sugar groups but produces no monosaccharide. It cleaves xanthan polymer from a weight-average molecular weight of 6.5×10^6 to 8×10^5 in 0.5 h. These results indicated that the purified enzyme fraction is an endo-β-1,4-glucanase which cleaves β-1,4-glucosidic linkages of xanthan from inside the molecule. The fact that this enzyme alone could not degrade xanthan to a more uniform single or multiple pentasaccharide unit(s) may indicate that xanthan is not a homogeneous molecule built from pentasaccharide units. Alternatively, there may be several regions inside the polymer molecule that are susceptible to the attack of this enzyme due to lack of trisaccharide side chain at that specific region(s).

The optimum pH and temperature for the purified xanthan depolymerase activity were around 5 and 30 to 35°C, respectively. The enzyme has a relatively low K_m value of 1.95×10^{-7} M for xanthan. Since the xanthan molecule can be attacked by the enzyme at multiple sites either simultaneously or sequentially, the K_m value calculated from Lineweaver-Burk plots may represent different meaning.

The enzyme was not stable at a temperature higher than 45°C, although it showed better stability in its crude form (Hou et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1985). These results resemble those obtained by Cadmus et al. (4) for xanthanase. However, xanthanase is a mixture of enzymes that attacked all of the side chain linkages in xanthan but not the endo-β-1,4-glucosidic linkage. The partially purified β-glucanohydrolase from a *Bacillus* sp. (20) cleaved both endo-β-1,4-linkage and endo-β-1,3-linkage. However, our xanthan depolymerase was inactive against unsubstituted cellulose. It is unique that this enzyme hydrolyzed only the backbone of β-1,4-linked glucans with side chains or other substituents. Certain arrangements of these substituents are probably necessary for the substrates to adapt the correct configuration for hydrolysis. It was surprising to note that dextran (α-1,6-linkage) was hydrolyzed by this enzyme.

The xanthan depolymerase activity was inert to many chemicals, including thiols, antioxidants, chlorinated hydrocarbons, metal-chelating agents, and inorganic compounds except ferric chloride and arsenomolybdate. Biocides, including those used in enhanced oil recovery operations, such as formaldehyde and 2,2-dibromo-3-nitrilopropionamide, were not inhibitors. Anaerobic environments and $Na_2S_2O_4$ also failed to inhibit xanthan depolymerase activity. Therefore, other means have to be developed to exclude this enzyme or the microbes which produce this enzyme from the enhanced oil recovery operation. Xanthan depolymerase could provide a useful tool in structure studies of xanthan and other similar types of polysaccharides.

TABLE 4. Inhibition of xanthan depolymerase activity

Inhibitor (1 mM)	Inhibition (%)
Thiol reagents	
Iodoacetic acid	0
Iodoacetamide	0
N-Ethylmaleimide	40
p-Hydroxymercuribenzoate	40
5,5′-Dithio-bis-2-nitrobenzoic acid	0
Metal-binding agents	
Sodium azide	0
α,α′-Bipyridyl	30
Thiourea	20
8-Hydroxyquinoline	0
EDTA	0
1,10-Phenanthroline	0
Thiosemicarbazide	20
Imidazole	30
KCN	20
Metals	
Ferric chloride (5.0 mM)	100
Arsenomolybdate (4%)	100

LITERATURE CITED

1. **Beldman, G., M. F. Searle-van-Leeuwen, F. M. Rombouts, and F. G. J. Voragen.** 1985. The cellulase of Trichoderman viride: purification, characterization and comparison of all detectable endoglucanases, exoglucanases and β-glucosidases. Eur. J. Biochem. 146:301–308.
2. **Bragg, J. R., S. D. Maruca, W. W. Gale, L. S. Gall, W. C. Wernau, D. Beck, I. M. Goldman, A. I. Laskin, and L. A. Nasland.** 1983. Control of xanthan-degrading organisms in the Loudon pilot: approach, methodology, results. Society of Petroleum Engineering of AICHE.
3. **Bredderman, P. J.** 1974. Tryptophan analysis of proteins in 6M guanidine hydrochloride: modification for more general application. Anal. Biochem. **61**:298–301.
4. **Cadmus, M. C., L. K. Jackson, K. A. Burton, R. D. Plattner, and M. E. Slodki.** 1982. Biodegradation of xanthan gum by *Bacillus* sp. Appl. Environ. Microbiol. **44**:5–11.
5. **DeBelder, A. N., and K. O. Wik.** 1975. Preparation and properties of fluorescein-labelled hyaluronate. Carbohydr. Res. **44**:251–257.
6. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
7. **Duggan, E. L.** 1963. Measurement of amino acids by column

chromatography. Methods Enzymol. **3**:492–504.
8. **Eriksson, K. E., and B. Petterson.** 1975. Extracellular enzyme system utilized by the fungus *Sporotrichium pulverulentum (Chrysosporium lignorum)* for the breakdown of cellulose. 1. Separation, purification and physico-chemical characterization of five endo-1,4-β-glucanases. Eur. J. Biochem. **51**:193–206.
9. **Halliwell, G., and M. Griffin.** 1978. Affinity chromatography of the cellulase system of *Trichoderma Koingii*. Biochem. J. **169**:713–715.
10. **Holzwarth, G.** 1978. Molecular weight of xanthan polysaccharide. Carbohydr. Res. **66**:173–186.
11. **Hou, C. T., L. I. Laskin, and R. N. Patel.** 1978. Growth and polysaccharide production by *Methylocystis parvus* OBBP on methanol. Appl. Environ. Microbiol. **37**:800–804.
12. **Jansson, P., L. Kenne, and B. Lindberg.** 1975. Structure of extracellular polysaccharide from *Xanthomonas campestris*. Carbohydr. Res. **45**:274–282.
13. **Kelco Co.** Xanthan gum, 2nd ed., p. 9. Kelco Co., Clark, N.J.
14. **Lesley, S. M.** 1961. Degradation of the polysaccharide of *Xanthomonas phaseoli* by an extracellular bacterial enzyme. Can. J. Microbiol. **7**:815–825.
15. **Raymond, S.** 1962. A convenient apparatus for vertical gel electrophoresis. Clin. Chem. **8**:455–470.
16. **Rinaldo, M., and M. Milas.** 1980. Enzymic hydrolysis of the bacterial polysaccharide xanthan by cellulase. Int. J. Biol. Macromol. **2**:45–48.
17. **Rogovin, S. P., R. F. Anderson, and M. C. Cadmus.** 1961. Production of polysaccharide with *Xanthomonas campestris*. J. Biochem. Microbiol. Tech. Eng. **3**:51–63.
18. **Schweiger, A.** 1962. Trennung einfacher Zucker auf zellulose-schichten. J. Chromatogr. **9**:374–376.
19. **Somogyi, M.** 1945. A new reagent for the determination of sugars. J. Biol. Chem. **160**:61–69.
20. **Sutherland, I. W.** 1982. An enzyme system hydrolyzing the polysaccharides of xanthomonas species. J. Appl. Bacteriol. **53**:385–393.
21. **Sutherland, I. W.** 1984. Hydrolysis of unordered xanthan in solution by fungal cellulases. Carbohydr. Res. **131**:93–104.
22. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
23. **Zacharius, R. M., T. E. Zell, J. H. Morrison, and J. J. Woodlock.** 1969. Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. **30**:148–152.