

Biodegradation of Xanthan Gum by *Bacillus* sp.

MARTIN C. CADMUS,* LINDA K. JACKSON, KERMIT A. BURTON, RONALD D. PLATTNER, AND MOREY E. SLODKI

Agricultural Research, Northern Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, Peoria, Illinois 61604

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Strains tentatively identified as *Bacillus* sp. were isolated from sewage sludge and soil and shown to elaborate extracellular enzymes that degrade the extracellular polysaccharide (xanthan gum, polysaccharide B-1459) of *Xanthomonas campestris* NRRL B-1459. Enzyme production by one strain was greatly enhanced when the strain was incubated in a mixed culture. Products of degradation were identified as D-glucuronic acid, D-mannose, pyruvylated mannose, 6-O-acetyl D-mannose, and a (1→4)-linked glucan. These products correlate with the known structure of the gum. The complexity of the product mixture indicated that the xanthanase was a mixture of carbohydrases. The xanthanase complexes were similar to one another in temperature stability, pH and temperature optima, degree of substrate degradation, and enzymolysis products. Differences in pH stability, salt tolerance, recoverability, and yields of enzyme were observed.

Xanthan gum (polysaccharide B-1459), an extracellular polysaccharide, is produced by *Xanthomonas campestris* NRRL B-1459 (17), a microorganism causing vascular disease in cabbages, cauliflower, and rutabagas (5). This heteropolysaccharide has considerable industrial significance and numerous applications (7, 14). The structure of the gum consists of a linear backbone of β -(1→4)-linked D-glucose residues (linked as in cellulose), which has three-unit-long side chains appended on alternate residues (Fig. 1). D-Mannose residues directly appended to the backbone bear O-acetyl substituents on the C-6 position (13, 15). Pyruvic acetal, i.e., 4,6-O-(1-carboxyethylidene), substituents are on the terminal D-mannosyl residues of some of these side chains; their occurrence varies depending on the bacterial strain and the fermentation conditions (2, 18).

Xanthan gum is a highly stable polysaccharide not easily degraded by most microorganisms, although in the unordered conformation it is to some degree subject to attack by cellulase (M. Rinaudo and M. Milas, Chem. Abstr. 92:17,6420, 1980). The potential use of large quantities of the gum in the recovery of crude oil has raised the question of whether xanthan decomposes once it is injected into underground rock formations. We found that the stability of this heteropolysaccharide may be affected when certain microorganisms are in contact with it over periods ranging from 1 month for high concentrations of soil organisms to 1 year or more for solutions of xanthan merely exposed to laboratory air (unpublished data). Some of these solutions, however, were apparent-

ly unaffected when stored in tightly closed containers (unsterile) at room temperature for 10 years. These observations suggested that xanthan-degrading organisms are not abundant in nature.

In addition to increasing the rate of biodegradation, several potential uses for xanthanases can be envisioned. Isolated enzymes can alter xanthan and related polysaccharide structures for subsequent chemical or biological modifications. The enzymes may also be used to confirm existing xanthan structures and show a certain specificity for related polysaccharides produced by different species of microorganisms. Xanthanases may also be useful in altering the viscosity of suspensions injected into underground oil-bearing formations (16).

MATERIALS AND METHODS

Microorganisms. A pure culture of a salt-tolerant strain of *Bacillus* sp. strain K11 (NRRL B-4529) was isolated from soil inside a decaying tree trunk (Iowa City, Iowa). A more prolific enzyme-producing mixed culture consisting of strain K11 and one other isolate (strain K17) was designated 11 + 17 (NRRL B-4530). Strain K17, isolated from the same soil sample, was tentatively identified as a *Flavobacterium* sp. (NRRL B-14010).

Another xanthanase-producing strain, *Bacillus* sp. strain 13-4 (NRRL B-4533), was isolated from sewage sludge (Peoria, Ill.). *Xanthomonas campestris* NRRL B-1459S-4L was used to produce xanthan gum (1).

The following species of *Bacillus* (Agricultural Research Culture Collection) were tested for xanthanase activity: *B.adius*, *B. cereus*, *B. circulans*, *B. firmus*, *B. lentus*, *B. licheniformis*, *B. megaterium*, *B. pasteuriana*, *B. polymyxa*, and *B. subtilis*.

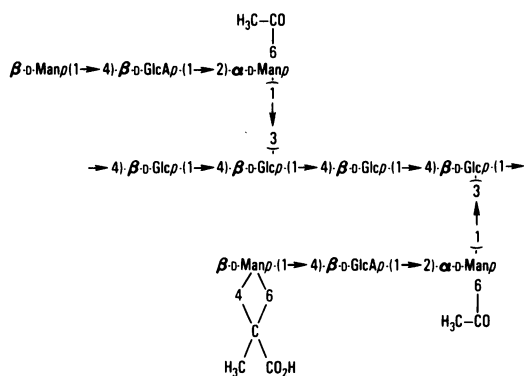


FIG. 1. An average repeating unit of the extracellular polysaccharide (xanthan gum) produced by *X. campestris* NRRL B-1459 (15).

In addition to strain K17, known bacteria tested for association with strain K11 included *Arthrobacter globiformis*, *Arthrobacter simplex*, *Arthrobacter viscosus*, *Alcaligenes faecalis*, *Achromobacter xylosoxidans*, *Agrobacterium radiobacter*, *Brevibacterium linens*, *Flavobacterium aquatile*, *Micrococcus luteus*, and *X. campestris*.

Media. Enrichment broth (EB) contained the following ingredients (per liter): 3.0 g of xanthan, 0.5 g of $(\text{NH}_4)_2\text{SO}_4$, 0.2 g of yeast extract, 0.2 g of tryptone, 0.2 g of peptone, 0.5 g of KH_2PO_4 , 0.5 g of K_2HPO_4 , and 2.5 ml of Speakman salt solution B (22). An enrichment broth containing a high level of salt (EBS) contained the following (per liter): 3.0 g of xanthan, 0.5 g of $(\text{NH}_4)_2\text{SO}_4$, 0.8 g of yeast extract, 0.4 g of tryptone, 1.5 g of KH_2PO_4 , 0.7 g of K_2HPO_4 , 40 g of NaCl, and 2.5 ml of salt solution B.

Stock agar slant medium (KGM) contained the following (per liter): 1.0 g of D-glucose, 1.0 g of D-mannose, 1.0 g of xanthan, 0.5 g of $(\text{NH}_4)_2\text{SO}_4$, 0.8 g of yeast extract, 0.4 g of tryptone, 1.5 g of KH_2PO_4 , 0.7 g of K_2HPO_4 , 18 g of agar, and 2.5 ml of salt solution B. Maintenance broth (KB) contained no glucose or mannose and 2.5 g of polysaccharide per liter; otherwise, it was the same as KGM.

Two test media were used for xanthanase production. One (KT) was used for strain 13-4 enzyme production and, except for containing no NaCl and 2 g of xanthan per liter, was the same as EBS. The other test medium (KTS) contained the following (per liter): 2.0 g of xanthan, 0.5 g of $(\text{NH}_4)_2\text{SO}_4$, 1.8 g of tryptone, 1.5 g of KH_2PO_4 , 0.7 g of K_2HPO_4 , 40 g of NaCl, and 2.5 ml of salt solution B.

Crude culture broth containing xanthan (1) could be substituted for purified gum (160 to 180 g/liter) without the yields of xanthanases being affected.

Isolation and strain characteristics. Soil and sewage samples (0.5 g), obtained from various sources and locations, were added to 20-ml portions of EB and EB plus 4% NaCl, which were incubated at 30°C on a rotary shaker for about 1 month. At the end of this period, certain broths displayed extensive loss of viscosity.

We isolated xanthanase-producing strain 13-4 from sewage sludge by streaking the fermented EB on EB agar plates. As a pure culture, it grew slowly on

standard laboratory media but grew faster on KGM agar that contained xanthan. The gram-positive organism was a sporeforming motile rod that formed short and long chains of random curvature. The strain was designated *Bacillus* sp. strain NRRL B-4533 (13-4).

The salt-tolerant (4% NaCl) enzyme producer (strain K11), isolated from a mixture of soil and decaying tree stump, was difficult to separate from other microorganisms owing to its relatively slow growth rate and its apparent affinity for growth in proximity to other organisms. The pure culture consisted of gram-positive, sporeforming, motile rods similar in size and shape to strain 13-4 cells; however, growth on KGM agar was much slower, and the colonies were mucoid. This strain was designated *Bacillus* sp. strain NRRL B-4529 (K11).

It was difficult to distinguish the species of some of the strains because of the variability in certain tests and the inability of the *Bacillus* strains to grow in some of the media. However, both xanthanase-producing strains appeared to key-down to being most like *Bacillus lentus* (8, 9).

Culture conditions. Stock cultures of the xanthanase-producers were maintained on KGM agar slants. We prepared fresh cultures at monthly intervals by incubating the inoculated media for 3 to 4 days at 30°C; the cultures were then stored at 4°C. Lyophilized culture stocks were also prepared (10) because some strains were difficult to maintain on agar media beyond 6 months.

Culture broths were prepared from stock slants by inoculating two loopfuls of strain 13-4 cells into 12 ml of KB or two loopfuls of culture 11 + 17 cells into 12 ml of KB plus 4% NaCl in Erlenmeyer flasks (50 ml) and then incubating the flasks on a rotary shaker until the viscosity diminished and abundant cell growth was evident (ca. 4 to 5 days for strain 13-4 and 10 to 15 days for the salt-tolerant cultures). We could maintain all cultures in this broth by transferring 1 ml to fresh medium at weekly intervals; broths were also stored at 4°C for up to 2 months and at -20°C for 1 year without significant loss in viability. Lyophilized cultures were easily regenerated in shaking flasks containing KB medium.

Xanthanase-producing cultures were grown in Erlenmeyer flasks (300 ml) containing 50 ml of medium, or Fernbach flasks (2,800 ml) containing 1,000 ml of medium. These flasks (third stage) were inoculated (5% [vol/vol]) with a 3-day-old inoculum (second stage) that had been taken (10% [vol/vol]) from KB or KB plus NaCl. All flasks were shaken at 200 rpm on a rotary shaker (2-in. [5.08-cm] stroke) at 30°C. The maximum production of the enzyme was reached in 3 or 4 days; further incubation resulted in diminished enzyme activity. Twenty-liter fermentors (4) were also suitable for the production of larger quantities of the enzyme.

Enzyme recovery. Fermentation broths were cooled to about 5°C, and the cells were removed with a Sharples supercentrifuge run at 65,000 × g. Volumes of extracellular enzyme-containing supernatant broth greater than 10 liters were concentrated in an Amicon DC-10 hollow fiber filtration assembly to approximately 3 liters and then dialyzed against 0.05 M sodium acetate buffer (pH 5.4) with the same apparatus. Further concentration and dialysis with the buffer were accomplished with the Amicon TCF-10 appara-

tus equipped with a PM-30 membrane. The concentrate was centrifuged at $20,000 \times g$ for 20 min to remove any remaining cells and debris and then stored under toluene at 4°C.

Enzyme purification was accomplished by ion-exchange chromatography. Approximately 100 mg of protein was added to a glass column (diameter, 2.5 cm; length, 50 cm) that was packed with DEAE-cellulose (Whatman DE-52) equilibrated with 0.05 M sodium acetate buffer (pH 5.4) at 50 ml/h. Peaks were detected by UV light absorption at 280 nm. The xanthanase from strain 13-4 was observed as a well-defined peak and was easily separated from other proteins in about 6 h. The salt-tolerant enzyme (from strain K11) was eluted with 0.25 M buffer over a 16-h linear program; the active material was collected, concentrated, and recycled to achieve separation. Active enzyme fractions were concentrated, dialyzed against 0.05 M buffer, and stored under toluene at 4°C.

Enzyme assay. To prepare a stock substrate solution, we vigorously stirred 425 ml of distilled water with a magnetic stirrer and added 0.94 g of xanthan slowly to avoid polysaccharide clumping; 50 ml of 0.5 M sodium acetate buffer (pH 5.4), 50 mg of $MgSO_4 \cdot 7H_2O$, and 2.5 mg of $MnSO_4 \cdot H_2O$ were added, and the mixture was diluted to 500 ml. The mixture was filtered through sintered glass (medium frit) before storage at 4°C under toluene.

We prepared the reaction mixture by adding 1.0 ml of the enzyme solution to 4.0 ml of the stock substrate solution and incubating the mixture in a 42°C water bath. After a suitable time, we terminated the enzymatic reaction by heating the mixture in a 100°C bath for 10 min or by adding 1 drop of 10 N NaOH. Activity was measured by the amount of reducing sugar (not exceeding 12 mg/100 ml), calculated as D-mannose, liberated from xanthan during enzymolysis.

A unit of xanthanase was defined as 1 μ mol of apparent mannose liberated per min at 42°C and pH 5.4.

Analytical procedures. Viscosities were measured at 25°C with a model LVF Brookfield viscometer at 30 rpm. Pyruvic acid was determined by the enzymatic method of Duckworth and Yaphe (3), as described by Jeanes et al. (15). Reducing sugars, calculated as mannose, were determined in an automatic analyzer (25) by the potassium ferricyanide method (12). Glucose was analyzed by a glucose-oxidase procedure. We detected the sugars on thin-layer chromatographic plates (precoated cellulose on plastic [E. Merck AG]) by spraying the plates with *p*-anisidine-phthalic acid reagent (19), and heating them at 100°C; *O*-acetyl was detected by the Hestrin procedure (11). The solvent system (6) for thin-layer and paper chromatograms was pyridine-ethyl acetate-acetic acid-water (5:5:1:3). Sugars, as per-*O*-acetylated aldonitriles (PAAN), were also analyzed by gas chromatography (GC) and GC-mass spectrometry (MS) on a neopentyl glycol succinate liquid phase that was temperature programmed from 170 to 210°C at a rate of 20°C/min and was held at the upper limit (20).

The structure of the high-molecular-weight fraction (HMWF), i.e., the incompletely degraded polysaccharide, was determined by methylation analysis (2, 21) after carboxyl reduction (24). The permethylated product was partially hydrolyzed by a brief period of formolysis (30 min at 100°C). Formic acid was re-

moved in a rotary vacuum evaporator, and hydrolysis was completed with Stellner reagent (23) (12 h at 80°C, dilution [1:1] with distilled water, and continued heating for 4 h at 80°C). Sulfate ion was removed with ion-exchange resin (21), and PAAN derivatives were prepared from the dried, methylated sugars (20). GC of methylated sugars as PAAN derivatives was performed on a butanediol succinate liquid phase that was temperature programmed from 165 to 210°C at a rate of 2°C/min and was held at the upper limit (20).

Whole and partially degraded polysaccharides were recovered by precipitation with alcohol. Lower-molecular-weight products of enzymolysis were separated from the high-molecular-weight material and protein with a PM-10 membrane in the Amicon TCF-10 membrane dialysis apparatus.

RESULTS AND DISCUSSION

Yields of strain K11 xanthanase were low in comparison with those of the mixed culture from which strain K11 was isolated. Consequently, an additional 21 colony types were selected from the mixed culture, none of which exhibited enzyme activity. Subsequently, each colony type was grown along with strain K11. All of the mixed cultures gave positive results; i.e., to some degree, all increased the rate of degradation of xanthan. One isolate (strain K17), when grown with strain K11, rapidly degraded xanthan and yielded almost twice the amount of xanthanase yielded by strain K11 alone. This mixed culture was designated NRRL B-4530 (11 + 17) and was more stable, both in broths and slants, over long periods. The enzymes isolated from K11 and 11 + 17 cultures were identical.

Ten previously identified microorganisms belonging to various genera commonly found in soil were obtained from our culture collection. Nine of these, when grown in the presence of strain K11, elicited a similar enhancement of xanthanase production by strain K11. This effect indicates an association between strain K11 and other microorganisms that was not limited to a single genus or species. Nevertheless, isolates from the original soil source elicited higher enzyme yields in conjunction with strain K11 than did the previously identified cultures.

Known species of *Bacillus* were tested for xanthanase activity. Almost all grew well on KGM agar, but none degraded xanthan after 3 months of incubation in enrichment media.

Stability of xanthanases. Crude and purified enzyme preparations in 0.05 M sodium acetate buffer (pH 5.4) could be stored for at least 3 months at 4°C with no loss of activity; frozen solutions (-20°C) retained 90% of the original activity after 2 years. Lyophilized preparations were stable at room temperature for 1 year.

Some enzyme activity was lost during chromatography on DEAE-cellulose at room temperature (18 to 25°C). These losses may have

TABLE 1. Xanthanase purification with DEAE-cellulose^a

Xanthanase from:	Crude broth (U/ml)	Crude concentrate			Purified prepn			Enzyme recovery		
		Activity (U/ml)	Protein (mg/ml) ^b	Sp act (U/mg)	Activity (U/ml)	Protein (mg/ml) ^b	Sp act (U/mg)	Total on column (U)	Total recovered (U)	% Recovered
Strain 13-4	0.34	7.32	5.46	1.34	1.86	0.247	7.54	148.80	137.84	92
Culture 11 + 17	1.95	21.57	1.18	18.62	1.68	0.007	237.52	496.71	420.02	85

^a Results are expressed as averages of three runs. One unit of xanthanase is defined as 1 μ mol mannose liberated per min at 42°C and pH 5.4.

^b Protein was estimated from the ratio of absorbance at 280 and 260 nm.

been attributable to normal handling during purification. Typical enzyme unit values at various stages of recovery are listed in Table 1. For strain 13-4, recoveries ranged from 82 to 98%, with specific activity increasing nearly sixfold. The strain K11 enzyme recoveries were 81 to 89%; specific activity increased to 13-fold.

The temperature stability of the enzymes in a buffered solution (0.05 M buffer, pH 5.4) is illustrated in Fig. 2. During the 30-min testing periods, strain 13-4 xanthanase was stable up to 42°C over a temperature range of 30 to 60°C, but strain K11 began to lose activity at 38°C; 10% was lost at 42°C. Additional tests of strain K11 at 42°C showed that the enzyme was stabilized completely when 0.25% xanthan substrate was present.

To test for stability at various pHs, we found it necessary to dialyze xanthanases from strains K11 and 13-4 against distilled water for 24 h to remove the existing buffer. The dialyzed preparations were added to various buffered (0.2 M) solutions in the pH range of 2 to 9 to a final concentration of 0.04 M. Figure 3 shows that the salt-tolerant enzyme (from strain K11) was stable over the pH range of 6.0 to 7.5 (25°C), whereas the strain 13-4 enzyme was stable over the pH range of 4.8 to 6.0. These results suggest that buffering the strain K11 enzymes at pH 6 may reduce activity losses during recovery and

storage. The presence of 0.5% substrate did not affect the stability of the enzymes during this 24-h period; however, it did provide stability for an additional 24 h.

Characteristics of xanthanases. To determine the optimum pH for activity, we removed the buffer from the xanthanase solutions by dialysis and then buffered the assay mixtures at several pHs. The reducing power was measured after incubation at 42°C for 15- and 30-min periods. Both enzymes exhibited maximum activity at pH 5.4 (Fig. 4). No activity was observed below pH 4 or above pH 8.

Figure 5 shows the optimum temperature for xanthanase activity as determined after 15- and 30-min periods in assay mixtures buffered (0.05 M) at pH 5.4. It can be seen that the enzymes from strains 13-4 and K11 had temperature optima of 42 and 48°C, respectively. When strain K11 was incubated with 0.1 M sodium chloride, however, the optimum temperature was reduced to 42°C. Subsequent assays for both enzymes were carried out at 42°C and pH 5.4.

The extent of xanthan gum degradation in the presence of various enzyme concentrations is given in Fig. 6. Enzyme solutions of 200 U/ml were added in 0.05- to 0.6-ml portions to substrate solutions containing 150 mg of xanthan in

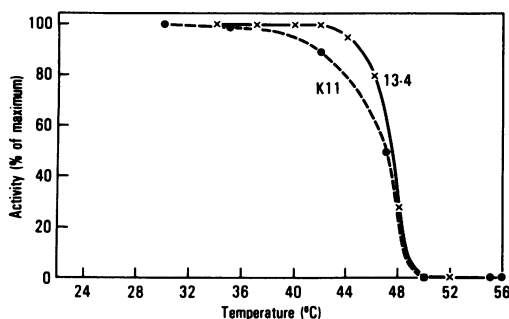


FIG. 2. The effect of temperature on the stability of xanthanases (0.05 M buffer, pH 5.4, 30 min; assay at 42°C, pH 5.3, 15 min).

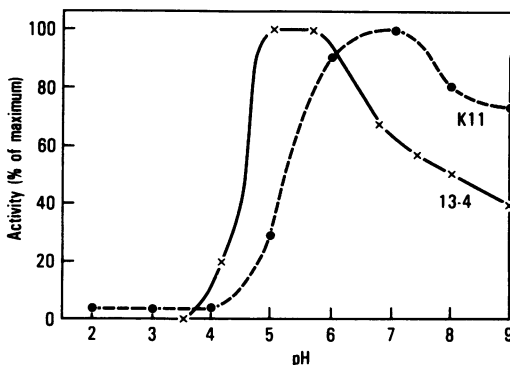


FIG. 3. The effect of pH on the stability of xanthanases (0.04 M buffer, 25°C, 24 h; assay at pH 5.5, 42°C, 30 min).

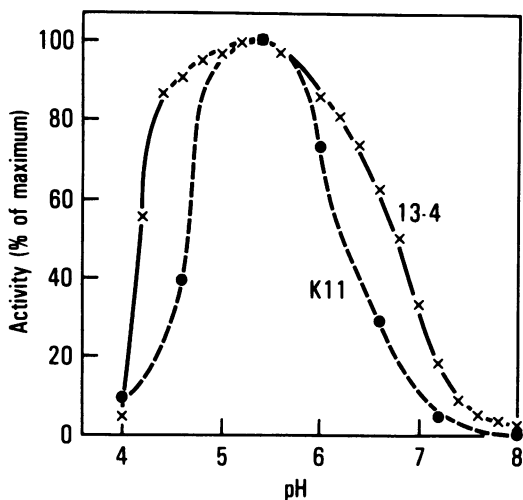


FIG. 4. Optimum pH of xanthanase activity (0.05 M buffer; assay at 42°C, 15 min).

100 ml of 0.05 M sodium acetate buffer (pH 5.4). The extent of degradation was measured after 15 min of incubation at 42°C. After the conversion of 8% of the substrate, determined on the basis of the total reducing sugar calculated as mannose, there was no longer a direct relationship between enzyme concentration and amount of degradation. Accordingly, enzyme activity could not be accurately determined when degradation products exceeded this level.

No differences were observed in the characteristics of crude and purified enzymes.

Products of degradation. Xanthan gum (0.15%) was degraded by the enzymes for 24 h at 37°C. The reaction mixtures were concentrated, and the products from 13-4, K11, and 11 + 17 xanthanases were compared on thin-layer chromatography plates. Figure 7 shows that identical

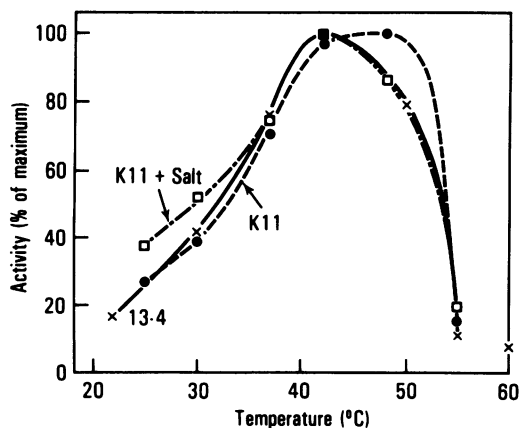


FIG. 5. The relationship of xanthanase action to temperature (0.05 M buffer; assay at pH 5.4, 15 min).

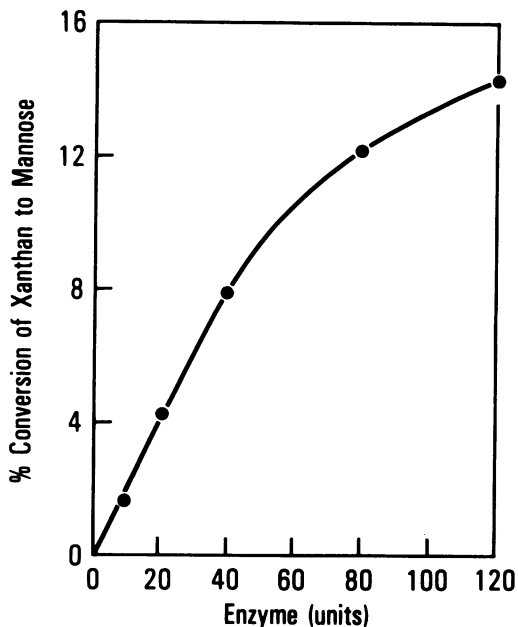


FIG. 6. Degradation of xanthan gum by increasing amounts of enzyme (0.05 M buffer; assay at pH 5.4, 42°C, 15 min). The reducing power is expressed as mannose equivalents.

products were formed by all three enzyme preparations. The degrees of substrate degradation were also similar, and degradation yielded a maximum of 35 to 40% reducing sugar, calculated on the basis of the initial polysaccharide concentration. This information suggested that the actions of the enzymes were similar, even though some variance was noted in other characteristics.

The xanthanase from culture 11 + 17, which was produced in the highest amount, was investigated further as follows. Xanthan (2 g) was dissolved in 200 ml of warm (50°C) distilled water in an Erlenmeyer flask (500 ml) and cooled to 37°C, and 1 drop of 2.5 N HCl was added to give a pH of 5.0. Approximately 1,000 U of enzyme in 20 ml of 0.025 M sodium acetate buffer (pH 5.4) was added, and the reaction mixture was incubated on a rotary shaker at 37°C under toluene. The initial viscosity of 1,750 mPa·s (5-min incubation) was reduced to 10 mPa·s at 18 h. An additional gram of xanthan was added to increase viscosity to 540 mPa·s; in 48 h, this viscosity was reduced to 38 mPa·s. Enzymatic degradation of xanthan gum in solutions containing more than 1% gum was very slow, perhaps owing to an inhibitory substrate complex or a physical problem related to the high viscosity. The final pH of the reaction mixture was 5.3; the reducing sugar (as mannose) was 0.52%, thus indicating a 38.1% con-

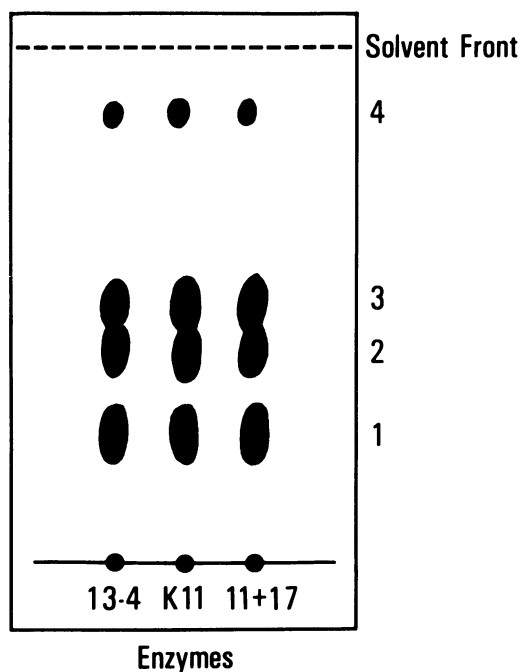


FIG. 7. Thin-layer chromatography (cellulose) of the LMWF (2.5 h in pyridine-ethyl acetate-acetic acid-water [5:5:1:3]). Spot 1, Uronic acid; spot 2, pyruvylated hexose; spot 3, hexose; spot 4, acetylated hexose.

version. The mixture was filtered (Amicon PM-10 membrane) to remove protein and undigested polysaccharide. Lyophilization of the filtrate yielded 2.09 g of low-molecular-weight material. A high-molecular-weight biopolymer precipitated from the retentate with alcohol (yield, 1.18 g) could not be digested further with fresh xanthanase. Similar results were obtained for two additional preparations. The salt content of the filtrate fraction was less than 1% and did not interfere with thin-layer chromatography analyses of sugar components.

The *p*-anisidine-phthalic acid spray revealed four distinct spots (Fig. 7) when the components of the low-molecular-weight fraction (LMWF) were separated on thin-layer chromatography plates. Spots 1 and 3 had the respective mobilities and colors for D-glucuronic acid and D-mannose. The second spot ran slightly behind the solvent front, and the fourth migrated close to the solvent front.

We separated the products of enzymolysis in the LMWF on thick chromatographic paper (Whatman 3M) by descending chromatography (7 h), using the same solvent system. Separated compounds were eluted with distilled water, filtered (Millipore filters with 0.45- μ m pores) to remove paper fibers, and lyophilized. The pyru-

TABLE 2. Analyses of the LMWF

Product	Hexose ^a	Glucuronic acid ^a	<i>O</i> -acetyl ^b	Pyruvic acid ^c
1		+		
2	+			+
3	+			
4	+		+	

^a Determined with *p*-anisidine-phthalic acid spray (19).

^b Determined by a qualitative test with Hestrin reagents (11).

^c Determined by the method of Duckworth and Yaphe (3).

vic acid content was 2.9% in xanthan gum, 3.4% in the LMWF, and 8.8% in the product isolated from spot 2; all other fractions were negative.

The rapid migration rate and color of spot 4 observed in thin-layer chromatography suggested an *O*-acetylated hexose; we confirmed the limitation of *O*-acetyl to this component by spraying the thin-layer chromatography plate with Hestrin reagent (11). Treatment of the LMWF with 0.05 M NaOH for 30 min to bring about de-*O*-acylation eliminated spot 4. Derivatization of the LMWF to form PAAN and the subsequent analysis of the PAAN by GC revealed the presence of a single peak with the retention time of the D-mannose derivative. Because the glucuronic acid and pyruvylated mannose components would not have been detected by this procedure, the single peak probably represented the original free and *O*-acetylated mannoses present in the mixture. We confirmed this by substituting hexadeuterioacetic anhydride (98 atom % D) for acetic anhydride in the derivatization procedure. Chemical ionization MS with isobutane as the reagent gas gave protonated mass ions with *m/e* values of 400 and 403 that respectively corresponded to mono-*O*-acetyl-tetra-*O*-trideuterioacetyl-D-mannonitrile and its pertrideuterioacetylated analog in a 1.0:0.8 molar ratio. Electron impact MS gave, in addition to mass fragments expected for a per-*O*-trideuterioacetyl hexonitrile, primary fragments with *m/e* values of 148 (CH₂OCOCH₃⁺·CHOCOD₃⁺), 223 (CH₂OCOCH₃·CHO·COCD₃⁺·CHOCOD₃⁺), etc., expected to arise from 6-*O*-acetyl-2,3,4,5-tetra-*O*-trideuterioacetyl-D-mannonitrile. In other words, primary fragment ions that were 3 atomic mass units less than the corresponding per-trideuterioacetylated mannose were observed, in about equal intensity, for all ions predicted to include C-6. Intense analogous signals were not observed with fragment ions not predicted to include C-6. It is noteworthy that the derivatization procedure did not perturb the preexisting *O*-acetyl group.

The results of the above tests of the LMWF components obtained after enzymolysis are summarized in Table 2. Although we have not confirmed the exact structure of each of the four components, our results indicate that the components are the same as those previously identified (13, 15) as constituting the side chains of the xanthan gum molecules (Fig. 1).

The structure of the HMWF was examined by methylation analysis (2, 21) after carboxyl reduction (24). Hydrolysis before methylation indicated that D-glucose comprised the bulk (>90%) of the HMWF; D-mannose comprised the remainder. The results of GC-MS showed that the main component in the hydrolyzate of the permethylated HMWF was 2,3,6-tri-*O*-methyl-D-glucose, which could have arisen from the β -(1 \rightarrow 4)-linked backbone of the xanthan gum. Other methylated sugars detected were 2,3,4,6-tetra-*O*-methyl-D-mannose, 3,4,6-tri-*O*-methyl-D-mannose, and 2,3-di-*O*-methyl-D-glucose. These sugars occurred in amounts of 10% or less and could have arisen from incomplete separation of the HMWF from the enzyme(s) or the LMWF. No 2,6-di-*O*-methyl-D-glucose was detected. If side chains were still present after enzymatic digestion, a 2,6-di-*O*-methyl-D-glucose arising from a 1,3,4-tri-*O*-substituted branch point should have been detected in the methylated HMWF.

The presence of 2,3,6-tri-*O*-methyl-D-glucose in the HMWF, the lack of glucose in the LMWF, the absence of 2,6-di-*O*-methyl-D-glucose in the permethylated HMWF, and the composition of the LMWF indicate considerable cleavage of side chains from the cellulosic backbone and their fragmentation. From these results, it appears that the xanthanase was a mixture of enzymes that attacked all of the side chain linkages in xanthan gum, including the one involving (1 \rightarrow 3)-linkage of acetylated mannose to the glucosidic backbone. Because the β -(1 \rightarrow 4)-linked backbone remained intact, an endocellulase type of activity is probably absent.

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