BACTERIAL DEGRADATION OF XANTHAN GUM

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SUMMARY
Various bacterial enzyme complexes that degrade xanthan gum are reviewed. Analyses of degradation products of Corynebacterium sp., Bacillus sp. and mixed cultures indicate enzymic activities specific for each sugar linkage in the polysaccharide. Enrichment culture techniques led to isolation of single and mixed cultures that produce salt-sensitive and salt-tolerant enzymes. A new high-temperature xanthanase complex is described that is stable to 65°C in the presence of salt. The reactions catalyzed by the high-temperature enzyme(s) are (a) removal of side-chain, terminal pyruvated mannose residues through a lyase activity, which leaves an unsaturated D-glucopyranosyluronic acid residue and (b) cleavage of backbone β-(1→4)D-glucosyl linkages to release two branched, O-acetylated oligosaccharides. Pentasaccharide and unsaturated tetrasaccharide formed represent xanthan side chains that are respectively unsubstituted and pyruvated.

INTRODUCTION
Interest in microbial degradation of xanthan gum is directed toward two objectives. The first is to understand the nature of aerobic and anaerobic degradation of xanthan gum under conditions of use, particularly in oil field environments. The second, which is also our own particular interest, is to develop a viscosity breaker for xanthan gum-based hydraulic fracture fluids used to stimulate the flow of natural gas and petroleum from geological formations of low porosity.

This review will cover the various bacterial exoenzyme complexes for degradation of xanthan gum. It will be confined to examples that contain adequate descriptions of products. In addition, a new high-temperature (HT) xanthanase will be described, which can function in the presence of high concentrations of salt at 65°C, a temperature considered moderate for subterranean formations bearing natural gas. As with some of the other xanthanases, the new enzyme complex is produced by a mixed culture. With identification of branched oligosaccharides in the product mixture, glycosidases of bacterial origin have now been found that cleave all of the sugar linkages in xanthan gum.
XANTHAN GUM STRUCTURE AND CONFORMATION

The average repeat unit structure (refs. 1-2) of xanthan gum is given in structure (1):

\[
\begin{array}{c}
\text{3} \quad \text{3} \\
\text{2} \quad \text{2} \\
\text{4} \quad \text{4} \\
\text{4} \\
\end{array}
\]

\[
\begin{array}{c}
\text{Ac-6Mana} \\
\text{Ac-6Mana} \\
\text{GlcA} \\
\text{GlcA} \\
\text{Pyr} \\
\text{Man} \\
\text{Man} \\
\end{array}
\]

Commercial samples of the gum, as well as those prepared in our laboratory from cultures of *Xanthomonas campestris* NRRL B-1459, are fully 0-acetylated on C-6 of the 3-(1-2)-linked D-mannosyl residues. Only about half of the side-chain terminal mannosyl residues, however, bear the pyruvic acetal, i.e., 4,6-0-(1-carboxyethylidene), substituent (ref. 3). A fully pyruvated commercial preparation is available (ref. 4). Studies on solution properties of a similar preparation have been published recently (ref. 5). The reader is referred to that article for a discussion of the influences of sample history, extent of pyruvlation and acetylation, heating and ionic strength on the molecular conformations of xanthan. For our purposes, it is sufficient to note that xanthan gum dissolved in brines of high salinity would be employed in hydraulic fracture fluids. Under these conditions, the polysaccharide likely would be in its compact double helical conformation even at the aforementioned moderate subterranean temperature. Fungal cellulase, it should be noted, catalyzes random cleavage of the 3(1-4)-linked glucosyl main chain when xanthan is in the unordered conformation, but does not attack the ordered helix (ref. 6).

MICROBIOLOGY

Bacteria that secrete inducible enzymes for degrading xanthan gum have been isolated from soil, water, and sewage enrichment cultures. Small samples of soil, etc., are added to broth containing 0.25% xanthan, low levels of nitrogen, buffer and mineral salts. Several weeks' incubation may be required. Experience has shown that environmental conditions for the enrichment culture may be varied in order to produce xanthanases with desired properties; e.g., increased salt content elicited organisms that produced salt-tolerant enzymes; elevated incubation temperature resulted in a xanthanase more stable to heat.

Indications of xanthanase activity in enrichment cultures are obtained first by observing reduction of viscosity, and monitoring this effect by
measuring reducing sugars liberated when cell-free broth is mixed with a buffered xanthan solution.

Pure or mixed cultures are isolated from the enrichment cultures, after it is determined that the xanthanase produced is suitable for further investigation. In some instances, a mixed culture is more stable, is easier to cultivate, and produces significantly higher yields of enzyme(s).

CORYNEBACTERIAL XANTHANASE

A patent issued in 1981 (ref. 7) described isolation of a Corynebacterium sp. (NCIB 11535) from a soil enrichment culture on xanthan gum as sole carbon source. The extracellular xanthanase produced aerobically by the organism during growth in the presence of xanthan gum also depolymerized carboxymethyl cellulose (CMC).

At least nine reaction products were detected by thin-layer chromatography after native xanthan gum in distilled water was incubated with the enzyme at 30°C. Only four products were obtained from the deacetylated polysaccharide and these were characterized. In addition to D-mannose and its pyruvic acetal, two were shown by compositional and methylation analyses, and by analogy with the known structure of xanthan gum, to be linear oligosaccharides (structs. 2 and 3).

\[
\begin{align*}
4\text{-ene-GlcA} & \rightarrow 1-2\text{Man} \rightarrow 3\text{Glc} \rightarrow 1-4\text{Glc} \\
\text{Man} & \rightarrow 1-4\text{GlcA} \rightarrow 1-2\text{Man} \rightarrow 3\text{Glc} \rightarrow 1-4\text{Glc} 
\end{align*}
\]

Light absorbance at 232 nm and a positive thiobarbituric acid test suggested that the tetrasaccharide was terminated by an unsaturated (4-ene-) glucuronic acid residue formed through action of a lyase. Presence of two glucose residues in both of the linear oligosaccharides indicated that the hydrolase component of the enzyme complex attacked \(\beta\)-(1→4)-glucosyl linkages to the glucosyl residues bearing side chains.

The corynebacterial xanthanase did not have good thermal stability; 30% of the activity was lost in one hour at 30°C.

XANTHANASES FROM STRAINS OF BACILLUS

Lesley (ref. 8) reported in 1961 the isolation of a Bacillus by the soil enrichment culture technique with the exopolysaccharide of X. phaseoli as sole carbon source for growth. This polysaccharide is similar structurally to xanthan gum. The inducible, extracellular enzyme activity was "markedly increased by dialysis against distilled water" and was stable during concentration at 40°C. One of the digestion products from the X. phaseoli
polysaccharide was characterized as a UV-absorbing (237 nm) trisaccharide comprising equal amounts of glucose, mannose and an apparent 4-ene-glucuronic acid residue. Component ratios were estimated from colorimetric analyses and from radiochromatographic measurements of hydrolyzed trisaccharide obtained from polysaccharide grown on U-14C D-glucose.

Depolymerase-secreting bacilli were also obtained by use of the X. campestris polysaccharide as sole carbon source (refs. 9-10). No information was given regarding thermal stability or activity in the presence of salt. At least one of the enzymes was active on CMC and, to a lesser extent, on other α-D-glucans. The enzymes were employed to assess structural differences among the similar heteropolysaccharides produced by many species of Xanthomonas (ref. 10). The main structural differences revealed by examination of oligosaccharide products related to amounts of O-acetyl and pyruvic acetal substituents. In addition to small amounts of mannose, glucose and, probably, acetylmannose, the major products were described as linear oligosaccharides that contained 2 moles of glucose per mole of uronic acid. The backbone linkage hydrolyzed by these enzymes would, therefore, be the same as by the corynebacterial glucanase.

Two xanthanases were reported by Cadmus et al. (ref. 11) from different Bacillus strains that could not be keyed to known species. One produced a salt-sensitive enzyme and was easy to cultivate; the other Bacillus produced a salt-tolerant xanthanase that was difficult to cultivate except in the presence of other indigenous or added soil bacteria, which alone did not produce the enzyme.

The salt-sensitive enzymes had pH 5.4 and 45 C optima. Stability was maintained up to 42 C for 30 min and from pH 4.8 to 6.0. The salt-tolerant xanthanase had similar temperature and pH optima; however, in the absence of salt, it was stable only to about 38 C from pH 5.8 to 8.0. It was later shown that the addition of salt to the reaction mixtures increased temperature stability to almost 50 C (ref. 12). Both types of xanthanase(s) could be stored for at least 3 years at -20 C. They were easily concentrated and purified using DEAE cellulose columns, but differed in ease of elution.

**Products**

Enzymolysis products were prepared by adding enzyme to 0.5% solutions of xanthan gum at pH 5.4 and incubating at 42 C until viscosity disappearance. A low-molecular-weight (LMW) fraction was separated from an undigested high-molecular-weight (HMW) portion by use of a nominal 10,000 MW ultrafiltration membrane.
Enzymes from both xanthanase-producing bacilli give the same mixture of products that represent all the possible side-chain sugar residues: mannose, its pyruvic acetal, glucuronic acid and 6-O-acetyl mannose. The latter was characterized unambiguously by mass spectrometry (MS) of its per-0-trideuterioadonitrile. Absence of UV-absorbing products indicated that the apparent enzyme mixture contained only hydrolases. Free glucose was not found. Instead, the HMW fraction separated by membrane filtration contained, to the extent of approximately 90% of its composition, the (1→4)-linked glucosyl backbone.

Hou et al. (ref. 13) reported a similar mixture of products from inducible, extracellular enzyme(s) produced by a Bacillus sp. grown in mixed culture. With these enzymes, unlike the NRRC enzymes, glucose was also liberated. A residual fraction of estimated weight-average molecular weight of 6.0 X 10^4 comprised 13% of the product carbohydrate. This fraction was not characterized further. By including 4% NaCl in the enrichment medium, cultures of salt-tolerant bacteria were isolated from geographically diverse water and soil samples. The enzyme preparation studied was optimally active between 35 and 45 C, but was not active beyond 50 C. Salt effects were not examined.

HIGH-TEMPERATURE XANTHANASE

Several soil samples were used to select producers of xanthanase(s) that would be more suitable for use as a viscosity breaker at the temperatures prevailing in subterranean rock formations. Enrichment media containing 3% NaCl were inoculated with the soil sample and incubated at 45 C. In the mixed culture subsequently isolated, no single microorganism can yet be designated as the enzyme producer.

The enzymes were produced in a low nitrogen broth containing 2% NaCl, xanthan, buffer and trace minerals. Cultures were incubated in shaken flasks at 45 C for 48 h. After centrifugation, the cell-free broth was concentrated and then dialyzed to remove excess salts.

The enzyme(s) were stable at 60 C for 30 min. in comparison to 40 C for other salt-tolerant xanthanases (ref. 11). The optimum temperature for enzyme activity was 50 C. When NaCl (>1%) was added to reaction mixtures, the percent of maximum activity increased from 40 to 80% at 65 C. HT xanthanase was stable over the pH range 5.0-8.5 with maximum stability and activity at pH 5.7. Xanthanase solutions were stable at room temperature for at least 3 days, and a minimum of 3 months at 4 C.

Significant increases of NaCl concentration caused small decreases in activity of this enzyme. In 10% NaCl, the activity at 45 C was reduced to
40% of the maximum. At 1% concentrations, MgCl₂ and CaCl₂ showed no deleterious effects on xanthanase activity.

**Products**

LMW products were separated from HMW material by means of a dialysis apparatus equipped with a membrane of nominal 10,000 molecular weight retention. Thin-layer cellulose chromatography of the LMW fraction revealed 0-acetylated, UV-absorbing oligosaccharide(s) near the origin and a single hexose spot with mobility characteristic of 4,6-0-(1-carboxyethylidene)-D-mannose. Oligosaccharide and pyruvated mannose were separated by column chromatography on Bio-Gel P-2. The oligosaccharide fraction did not contain pyruvic acid and exhibited an absorption spectrum characteristic (refs. 7-8) of unsaturated uronic acid. Methylation analysis of this fraction accorded with a 1:1 mixture of branched oligosaccharides (structs. 4 and 5).

\[
\begin{align*}
\text{Man} & \rightarrow \text{4GlcA} & \rightarrow \text{1-2(Ac-6)Man} & \rightarrow \text{3(GlcP)Glc} \\
4\text{-ene-Glc} & \rightarrow \text{Al-2(Ac-6)Man} & \rightarrow \text{3(GlcP)Glc}
\end{align*}
\]

Prior to methylation, the mixture was carboxy-reduced with NaBD₄. In this way, the 2,3,6-tri-0-methyl glucose derived from glucuronic acid residues was identified by MS as the 6-d₂ compound; the 1-d-1,2,5,6-tetra-0-methyl glucitol derived from di-0-substituted reducing end groups was identified in like manner.

The HMW fragment was 0-acetylated, devoid of pyruvic acetal, and gave a UV spectrum similar to that obtained for the LMW products. Methylation analysis again revealed a 3,4-di-0-substituted glucose reducing end group. The data agreed closely with a structure containing three trisaccharide and two unsaturated disaccharide side chains plus two additional unsubstituted main-chain (1-4)-linked glucosyl residues; MW = 4600, calculated as K⁺ salt. Excess unsubstituted glucosyl residues in xanthan have been reported (ref. 14).

From the above results, it appears that HT xanthanase consists of two enzymes: a lyase that specifically removes pyruvated mannose residues and a new β-(1-4)-glucanohydrolase that cleaves the linkage between the glucosyl residue bearing the side chain and the succeeding unsubstituted glucosyl residue of the main chain. CMC is degraded by the preparation.

Research has led to isolation of both single and mixed aerobic bacterial cultures that degrade xanthan gum and revealed a variety of enzymic activities specific for each sugar linkage in the polysaccharide. Apart from confirming xanthan structure, the work has demonstrated that enrichment culture under
conditions of elevated salinity and temperature can, ultimately, yield isolates that produce degradative extracellular enzymes with enhanced stability and functionality.

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REFERENCES