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HYDROLYSIS OF UNORDERED XANTHAN IN SOLUTION BY FUNGAL CELLULASES

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ABSTRACT

After removal of ions, *Xanthomonas campestris* polysaccharides (xanthan) could be dissolved in distilled water to give the unordered conformation. In this condition, the polymers were susceptible to random degradation by fungal cellulases at 40–50°. The products, including D-glucose, oligosaccharides, and larger fragments, could be separated by gel-permeation chromatography. The pattern of fragmentation depended on the substrate and on the enzyme preparation used. Some of the oligosaccharides have been partially characterised. The system is of particular interest in that the *absence* of salts is required for enzyme activity and the xanthan molecule is only susceptible in the unordered conformation. The cellulase systems provide a means of producing fragments of various d.p. from xanthan preparations in reasonable yield.

INTRODUCTION

Xanthan, the exopolysaccharide from *Xanthomonas campestris* and related bacterial species or pathovars, can be hydrolysed by specific enzymes obtained by enrichment culture. One of these, a *Bacillus* species, produced various enzymes which degraded the polysaccharide to yield monosaccharides and oligosaccharides[1]. More recently, a red-pigmented *Bacillus* species was used as a source of enzymes active on xanthan[2]. These included a (1→4)-β-D-glucanase and a β-D-glucosidase. The β-D-glucanase was an endo-enzyme which also hydrolysed other β-D-glucans, although mainly at lower rates than for xanthan. An exception was carboxymethylcellulose (CMC), which yielded reducing material to almost the same extent as the bacterial polysaccharides. Such enzymes have been used to provide a rapid comparison of different xanthan samples[3].

The structure of the xanthan molecule is essentially that of a substituted cellulose carrying trisaccharide substituents on alternate D-glucosyl residues; *O*-acetyl groups and pyruvic acetals are also present[4,5]. Despite the structural similarity of xanthan to CMC and the earlier observation that the xanthanase from the *Bacillus*

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species hydrolysed CMC, reports of enzymic attack on xanthan by enzymes of the cellulase type are conflicting. In a patent claim, Griffith *et al.*[6] suggested that treatment of xanthan at 50° for 4 h with an enzyme derived from an *Aspergillus* species *increased* the viscosity of the solution. Other enzyme fractions decreased the solution viscosity. The nature of the hydrolytic action was apparently not investigated. Random breakdown of the main chain of xanthan was reported by Rinaudo and Milas[7] in salt-free solution. In this system, the polysaccharide was in the unordered conformation. No hydrolysis of the ordered helical conformation was observed.

A study is now reported of the action of various cellulase preparations on xanthan solutions in the unordered state, obtained through the use of ion-free polysaccharide and enzyme solutions.

MATERIALS AND METHODS

Enzymes. — The commercial enzymes, their microbial sources, and suppliers are listed in Table I, along with their pH and temperature optima. An enzyme preparation used in the study by Rinaudo and Milas[7] was a gift from these authors. The enzyme from a derived non-polysaccharide-producing strain of *X. campestris* was prepared in the laboratory by continuous culture in a semi-synthetic growth medium using standard microbiological procedures[8]. All protein solutions containing ~10 mg.mL^{-1} were deionised by passage of 2-mL aliquots through a column (30 × 1 cm) of Sephadex G50 at 2–4°. The enzymes were checked for activity and protein concentration, and then stored frozen until required.

Substrates. — Xanthans from two commercial sources, Kelzan (Kelco Inc., San Diego, U.S.A.) and Rhodopol (Rhone Poulenc S.A., Paris, France), were used along with material from laboratory strains. The polysaccharides prepared in the laboratory contained pyruvate and acetate (strains 646 and 2182), pyruvate only (strain 556), or acetate only (strain 1128)[3]. An aliquot of Kelzan that had been

TABLE I

SOURCES OF CELLULASE PREPARATIONS

Preparation	*Microbial source*	*pH Optima*[a]	*Temperature optima*[a] *(degrees)*	*Supplier*
A	*Trichoderma viride*	4.0–5.0	40–45	Miles-Kali
B	*Aspergillus niger*	4.0–5.0	40	Rohm
C	*Aspergillus niger*	3.5–5.0	40–45	Rohm
D	*Penicillium emersoni*	4.0–5.0	75–85	A.B.M.
E	*Aspergillus niger*	4.5–5.5	55–65	Novo Industri
F	*Trichoderma viride*	5.5–6.5	50–65	Novo Industri
G	Basidiomycete	—	—	SEAB[b]
H	Basidiomycete	—	—	Sigma

[a]Data provided by suppliers. [b]Kindly provided by Dr. M. Rinaudo.

depyruvylated by mild hydrolysis with trifluoroacetic acid[9] was also used. All polysaccharides were deionised by passage of 0.1% solutions through a column (20 × 2 cm) of Amberlite MB1 mixed-bed resin, and then lyophilised.

Gel-permeation chromatography. — Gel-permeation chromatography of the products of cellulase hydrolysis was performed on columns (56 × 1 cm) of Biogel P30, P6, and P2 (Biorad). The columns were calibrated with Blue Dextran and dextran fractions of molecular weight 70,000, 40,000, and 10,000 (Pharmacia A.B.), and maltotriose. All columns were perfused at a flow rate of 6 $mL.h^{-1}$, and 1-mL fractions were collected using a Pharmacia FRAC 100 system. The carbohydrate content of each fraction was monitored for carbohydrate content by the phenol–sulphuric acid procedure[10].

Analytical procedures. — The analysis of polysaccharides and fragments was performed essentially as described earlier[3]. Protein was measured by the Lowry procedure with bovine serum albumin as standard[11]. D-Glucose was determined by a micromodification of the D-glucose oxidase procedure[12], using a total volume of 635 μL. This yielded an E_{415} of 0.25/μg of substrate and was required to measure the very small amounts released by enzyme action.

Viscometry. — Changes in viscosity following enzyme action were measured by using a Wells–Brookfield LVD instrument fitted with cone CP41. Samples (2 mL) were incubated at 50° or 30°.

Enzyme activities. — The presence of glycosidase activities in enzyme preparations was determined essentially as described by McCleary[13], using nitrophenyl D-glucopyranosides and D-mannopyranosides. Cellulase activity was confirmed using carboxymethylcellulose as substrate, by measuring reducing material released.

Paper chromatography and electrophoresis. — Examination of the products of low molecular weight eluted from the Biogel columns was by paper chromatography with the solvent systems described earlier, and by paper electrophoresis[3]. All monosaccharides and oligosaccharides were detected with alkaline silver nitrate.

EXPERIMENTAL

Establishment of hydrolysis conditions. — Initial attempts to hydrolyse xanthan preparations with commercial cellulases were unsuccessful. At best, very small amounts of reducing material were liberated; there was no appreciable reduction in solution viscosity, and the results were very variable. The enzyme preparations often contained high concentrations of salt in order to preserve their stability under industrial conditions. The report of Rinaudo and Milas[7] that random breakdown of the xanthan molecule could be achieved in salt-free solutions led us to prepare salt-free *substrate* and *enzymes*. Solutions of the polysaccharides were passed through mixed-bed ion-exchange resins to remove all ions and then lyophilised, and the residues were redissolved in glass-distilled water. Similarly, the enzymes used in this study were deionised by passage through Sephadex G50 and maintained frozen in solution until required, after determination and adjustment of the

TABLE II

ENZYME ACTIVITIES OBSERVED[a] IN COMMERCIAL PREPARATIONS

	Activity			
Enzyme preparation	*α-D-Mannosidase*	*β-D-Mannosidase*	*β-D-Glucosidase*	*CM-Cellulase*
A	−	−	+	+
B	+	+	++	+
C	−	±	±	+
D	−	−	−	+
E	+	+	+	+++
F	+	+	++	+++
G	−	±	+	++
H	−	−	±	+

[a]Enzyme solutions (0 1 mg of protein) in 500 μL of 10mM HEPES buffer (pH 6.5) were incubated with 0.1 μmol of nitrophenyl substrate for 60 min at 37° and examined for release of nitrophenol. A 1% CM-cellulose solution (1 mL) was used and loss of viscosity noted after incubation for 60 and 120 min.

protein concentration. Enzyme and polysaccharide solutions were mixed and incubated at 50° or 35°. At the lower temperature, several drops of toluene were added to inhibit bacterial growth. Some of the polysaccharide–enzyme mixtures showed considerable loss of viscosity, but all showed some liberation of reducing sugar. The enzyme preparations were all complex mixtures when examined by polyacrylamide gel electrophoresis, and several different activities could be recognised (Table II) with nitrophenyl glycosides and carboxymethylcellulose as test substrates.

Liberation of products. — Even after prolonged incubation at 50°, the viscosity of some preparations was only slightly diminished by cellulase treatment; amounts of reducing material or of D-glucose released were also small. Typical results are shown in Fig. 1. In Table III, the extent of release of D-glucose from several xanthan preparations is shown. The polymers tested were from three wild-type strains, one of which yielded non-pyruvylated polysaccharide. Material from normal and chemically depyruvylated samples of commercial xanthans was also tested. Attempts to use polymer containing pyruvate, but no acetate, were largely unsuccessful, as the material did not readily dissolve in the absence of ions. It also tended to cause precipitation of enzyme protein.

Fractionation of the products. — The hydrolysis products were initially fractionated on a column of Biogel P30 which had been calibrated with dextran fractions. Each preparation yielded a range of fragments, some xanthan preparations being apparently more susceptible to breakdown than others. The profile of fragments can be seen in Fig. 2 for several, typical reaction-products. The larger material, eluted in fractions 10–20, represented 50% or less of the starting material, with relatively little difference in this part of the profile when different polymers or enzymes were used. This material, when passed through Biogel P6 or P2, eluted at

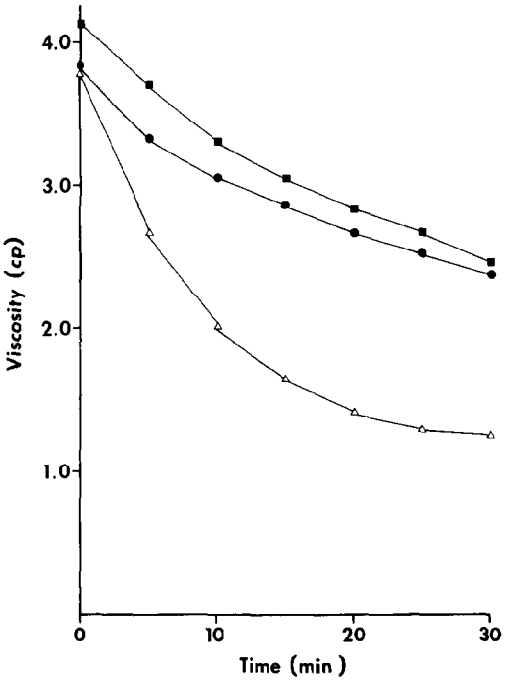

Fig. 1. Reduction of viscosity of xanthan solution by cellulases. A 0.2% solution (2 mL) of deionised xanthan in distilled water was treated with cellulase (20–30 μg of total protein) at 50°, and the viscosity was measured in a Wells–Brookfield cone/plate viscometer (spindle 41 at 60 r.p.m.).

the void volume. The yield of material of lower d.p. was generally ~50% of the original polymer. Exceptions were the enzymes G and H, which tended to yield more material of low molecular weight, and enzyme A, which consistently gave less polysaccharide degradation. Prolonged incubation did not markedly alter the fragmentation patterns.

TABLE III

D-GLUCOSE RELEASE FROM XANTHAN PREPARATIONS

Strain	*Substrate*		D-*Glucose release* (*μg/mg of protein/40 h*)		
	Acetate	*Pyruvate*	*Enzyme*		
			B	*G*	*F*
646	+	+	381.6	44.1	259.4
1128	+	–	37.6	12.7	65.1
2182	+	+	109.6	36.4	126.0
Kelzan	+	+	50.3	14.7	98.6
KDP[a]	+	–	450.4	180.7	350.5
Rhodopol	+	+	60.4	17.1	66.3

[a]Chemically depyruvylated Kelzan.

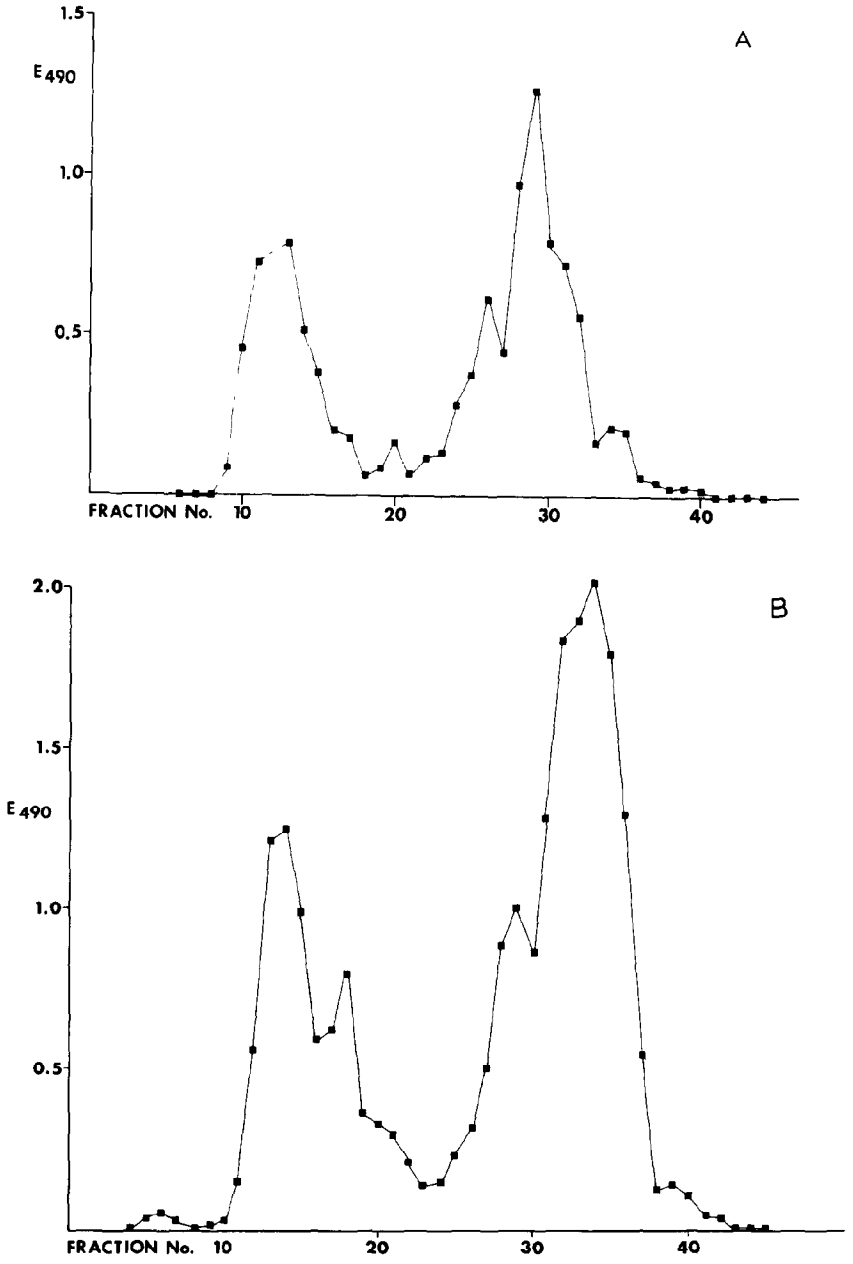

Fig. 2. Chromatography of cellulase-treated xanthans (~10 mg) on a column (56 × 1 cm) of Bio-Gel P30 by elution with distilled water at 5 mL/h. Fractions (1 mL) were collected, and assayed for carbohydrate content by the phenol–sulphuric acid assay: *A*, xanthan 646; *B*, xanthan 2182.

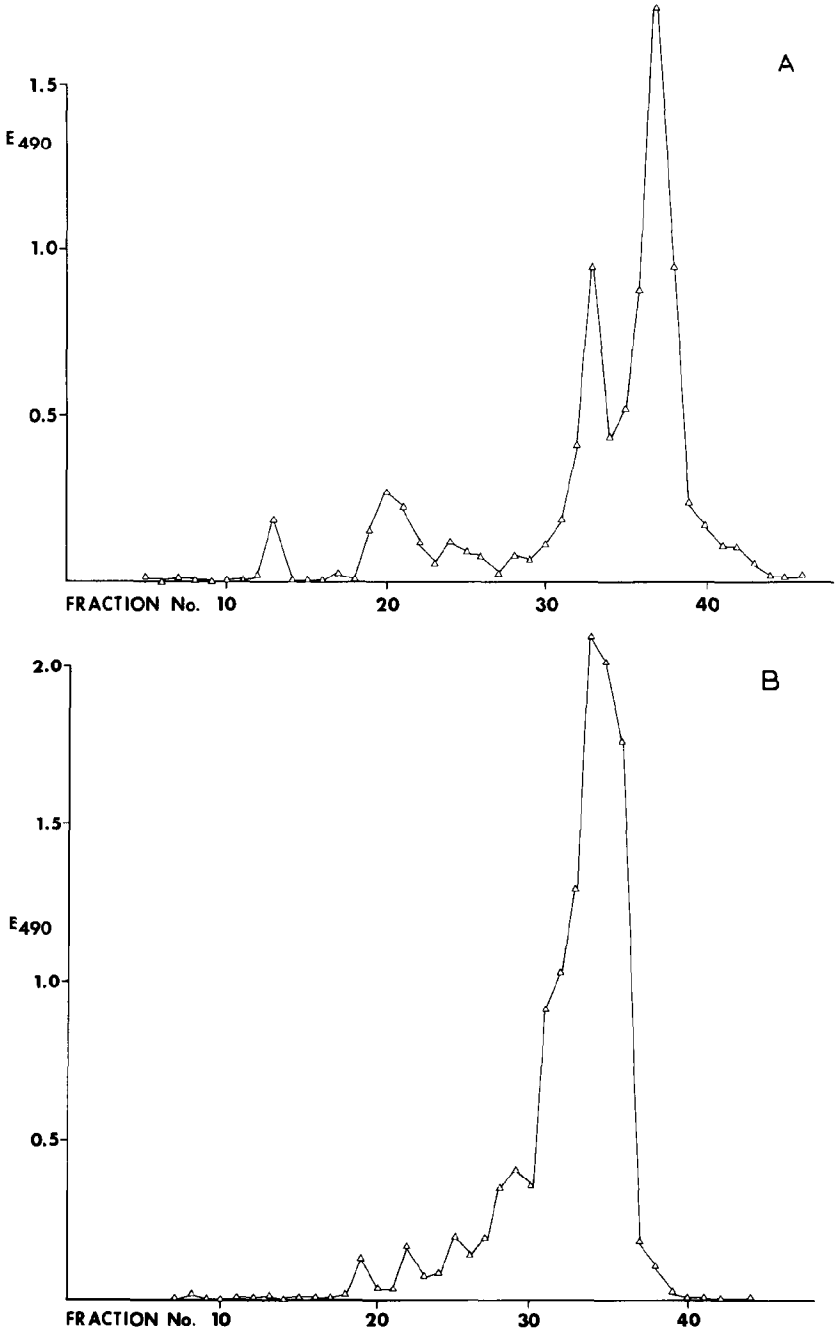

Fig. 3. Chromatography of the material of low molecular weight eluted from Bio-Gel P30 columns (fractions 26–40 in Fig. 2) after cellulase digestion of xanthan. The fractions were combined and lyophilised, and a solution of the residue in water (1 mL) was applied to a column (56 × 1 cm) of Bio-Gel P6 and eluted with distilled water as in Fig. 2: *A*, xanthan 646; *B*, xanthan 2182.

Resolution of the fragments of low molecular weight in the eluates from the P30 column was achieved by using columns of Biogel P6 or P2. The chromatograms thus obtained indicated a mixture of products comprising two major oligosaccharides (Fig. 3) and products of lower molecular weight, which contained (paper chromatography) glucose, some cellobiose, and, for some hydrolysates, traces of mannose. In addition, there were two major slow-moving oligosaccharides with R_{Glc} 0.12 and 0.05 (ethyl acetate–acetic acid–formic acid–water, 18:3:1:4).

Identification of the products. — Hydrolysis of the larger fragments gave D-glucose, D-mannose, and D-glucuronic acid in the expected 2:2:1 molar ratios. Acetate and pyruvate were present in non-stoichiometric amounts, except in the products from strain 1128 where no pyruvate was detected. When these oligosaccharides of relatively high d.p. were treated with a xanthanase preparation, the products were similar to those obtained from the original polysaccharides, when hydrolysed with the same enzyme. The ratio of acetylated to non-acetylated fragments showed some variation. No particular significance should be attached to this finding, as some acetyl groups may be lost during polysaccharide purification and subsequent enzyme treatment.

The material of d.p. 5–15 or less comprised D-glucose, cellobiose, and oligosaccharides. It was significant that, in the neutral products, little if any mannose was detected from most enzyme digests of xanthan. There was a considerable excess of glucose and cellobiose over mannose demonstrable by paper chromatography. The oligosaccharides in the fraction of lower molecular weight apparently comprised oligomers of the repeating unit of xanthan. These had an electrophore-

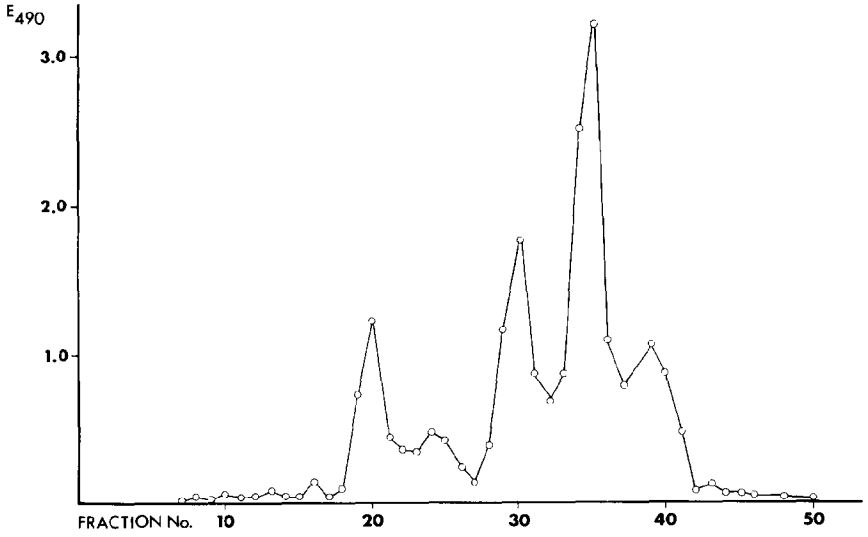

Fig. 4. Chromatography of the material of low molecular weight from a cellulase digest of xanthan 1128 on a column (56 × 1 cm) of Biogel P2 eluted with distilled water at 5 mL/h.

tic mobility of M_{GlcA} ~0.35 in pyridinium acetate buffer (pH 4.5), but moved very slowly (R_{Glc} <0.1) in paper chromatography. The oligosaccharides all contained D-glucose, D-mannose, and D-glucuronic acid in the molar ratios 2:2:1. Chromatography on Biogel P2 indicated various proportions of two oligosaccharides, most probably a pentasaccharide and a decasaccharide, together with monosaccharides (Fig. 4). The terminal reducing sugar in each case was D-glucose. To confirm the existence of terminal, non-reducing β-D-mannopyranosyl groups, the pentasaccharides and decasaccharides were treated at 30° for 16–20 h with a β-D-mannosidase from *Helix pomatia*[13]. This enzyme was not active against oligosaccharides carrying a terminal acetal on the β-D-mannopyranosyl groups. However, the two oligosaccharides from strain 1128, and a small amount of the corresponding non-acetalated material from the other strains tested, released D-mannose. The other products were a tetrasaccharide and an octasaccharide in which the ratios of D-glucose–D-mannose–D-glucuronic acid were 2:1:1. The mobilities in electrophoresis and chromatography were slightly increased compared with the pentasaccharide. Partial, acid hydrolysis (0.1M trifluoroacetic acid, 45 min, 95°) yielded D-glucose, cellobiose, and the aldobiouronic acid 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose.

DISCUSSION

The exopolysaccharide xanthan secreted by *Xanthomonas campestris* strains is susceptible to hydrolysis by endo-(1→4)-β-D-glucanases. The composition of the xanthan and, in particular, the regularity of the trisaccharide side-chains and the presence of acyl groups are likely to affect the action of such enzymes. Two groups of endo-(1→4)-β-D-glucanases, differing in their modes of action, can be recognised. "Xanthanases", also possessing carboxymethylcellulase activity, cause almost total hydrolysis of the polysaccharide to its component, repeating pentasaccharides[2]. The presence or absence of acetyl or acetal groups has little effect on the activity of the xanthanases; they act on the substrate in the salt form, but are inhibited by salt concentrations in excess of 0.1M. The xanthanases described so far do not degrade microcrystalline cellulose or cellulose–dye complexes.

The second type of endo-(1→4)-β-D-glucanase, the subject of this study, is that represented by "cellulases" of bacterial or fungal origin. One report on the action of such enzymes on xanthan indicated *increased* viscosity and improved flow through filters[6]. Rinaudo and Milas[7] used salt-free conditions at 38° to obtain decreased viscosity thought to be due to random breakage of the main chain. The rate of hydrolysis, as measured by viscosity loss, was dependent on the "quality" of the polysaccharide solution; the presence of aggregates or microgels reduced the hydrolysis rate. It was suggested that the rate of hydrolysis might be directly correlated with the degree of local order of the polymer, as indicated by its specific rotary power. No hydrolysis was observed in the ordered state existing in the presence of salts.

The present study confirms these results, various xanthan preparations being hydrolysed by commercial cellulases of fungal origin. Hydrolysis occurred *only* at elevated temperatures in the absence of ions, reflecting the unordered state of the polysaccharide substrate. The major products of enzyme action were D-glucose, cellobiose, and oligomers of the pentasaccharide repeating-unit of xanthan. Release of D-glucose or reducing material was low, even after incubation for 48 h at 45–50° and, of the total available D-glucose in the polymer, only 5% or less was converted into the monosaccharide. Part of the molecule resisted enzyme action; in most of the xanthan preparations tested, ~50% of the material remained as relatively large oligosaccharides (d.p. >25). The other portions of the polysaccharide molecule yielded D-glucose, cellobiose, and oligosaccharides of d.p. 5–15. As the molecular weight of xanthan is normally considered[14] to be in excess of 10^6, it seems probable that all molecules had been partially degraded. If only some of the polysaccharide originally present had been hydrolysed, the material of high molecular weight should have been detectable on gel-permeation chromatography. As it has proved possible[15] to resolve some xanthan preparations into strands with high and low contents of pyruvate, it is possible that some xanthans may behave differently from the material used in this study, some strands being totally resistant to hydrolysis by cellulases.

The results obtained from the action of the cellulases indicate the probable non-uniformity of xanthan molecules. The release of a series of fragments of different size could be due to sites of preferential action of the endo-β-D-glucanases. These sites are most probably regions of the polysaccharide chain from which one or more side-chains are lacking:

$$\rightarrow 4)\text{-}\beta\text{-D-Glc-}[(1\rightarrow 4)\text{-}\beta\text{-D-Glc-}]_5\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc-}(1\rightarrow$$
$$\qquad\qquad\uparrow \qquad\qquad\qquad\qquad\qquad\qquad\uparrow$$

This would enable the enzyme to interact with its substrate in a manner probably impossible where regular, fully acylated, side chains provide steric hindrance. Some support for this theory may be found in the isolation of D-glucose and cellobiose among the hydrolysis products, and in analyses of xanthan from various laboratories. These results (Table IV), if they are an accurate representation of the xanthan structure, indicate the presence of mannose in less than stoichiometric amounts and the possibility that ~10% of the D-glucosyl residues lack substituent side-chains. The size of binding site for enzyme action is not known, but the isolation of small amounts of cellotriose from one non-pyruvylated xanthan indicates the possibility that more than one side chain might be absent from contiguous D-glucose sequences.

Although heterogeneity of exopolysaccharides has not been generally recognised, this is probably a result of the absence of enzymic systems to provide controlled hydrolysis under mild conditions. However, lipopolysaccharide molecules, which, in their synthesis and structure, closely resemble exopolysaccharides, have

TABLE IV

REPORTED ANALYSES OF XANTHANS

Molar ratio	*Reference*
D-*Glucose*–D-*Mannose*	
1:0.97	5
1:0.93; 1:0.83[a]	16
1:0.97; 1:0.90[a]	17
1:0.90	18
1:0.83; 1:0.77[a]	19

[a]Other intermediate values were also reported.

been shown to be heterogeneous[20]. Variations in chain length and in substituents have been observed, several of the antigenic determinants being unevenly distributed. Thus, polysaccharide molecules may not be as faithfully synthesised as was thought. The influence of interruptions in the sequence of polysaccharides on the properties of the molecules has been stressed by Rees *et al.*[21] and could well account for the variations in susceptibility of xanthan preparations to endo-β-D-glucanases observed in this study. In concentrated solutions, failure of the enzymes to cleave the chains may be due to stable side-by-side dimerisation as postulated from hydrodynamic studies[22]. In the hydrolysis of cellulose, synergism between the different components of cellulase systems has been proposed[23]. Hydrolysis of xanthan may also involve synergism, but is most probably similar to that of cellulose, the disordered components being susceptible to hydrolysis[24].

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