Xanthan Lyases – Novel Enzymes Found in Various Bacterial Species

By IAN W. SUTHERLAND

Department of Microbiology, Edinburgh University, West Mains Road, Edinburgh EH9 3JG, UK

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Xanthan lyases, cleaving the terminal β -mannosidic linkage of the side-chain of the exopolysaccharide xanthan from *Xanthomonas campestris*, have been obtained from several sources. These include a *Bacillus* species, a *Corynebacterium* species and a mixed culture. The lyases were initially associated with endo- β -glucanases cleaving the main chain of xanthan. Partial purification of the enzymes was achieved and the *Bacillus* preparation was separated by FPLC into material free of endoglucanase and glycosidase activities. The lyase was active on polysaccharides with and without acetate and pyruvate. The optimal size of the substrate appeared to be in the range of degree of polymerization (DP) 25–35, i.e. 5–7 repeat units of the polysaccharide. No activity was found against xanthan modified by reduction of the carboxyl groups or by the addition of amine or hydroxyethyl groups. The combined action of the lyase and the endoglucanase yielded a series of oligosaccharides, each with a side-chain terminating in an unsaturated uronic acid and containing the molar ratio of D-glucose to D-mannose, 2:1.

INTRODUCTION

Lyases, enzymes generating unsaturated uronic acid residues in the breakage of polysaccharide chains, have been recognized for various bacterial exopolysaccharides (EPS). Thus, alginate lyases have been isolated from a number of bacterial species as well as from marine invertebrates. These enzymes differ in their specificity, some being poly- α -L-gulonate lyases (Davidson *et al.*, 1976; Boyd & Turvey, 1977), while others are poly- β -D-mannuronate lyases (Davidson *et al.*, 1977; Currie & Turvey, 1982). Similarly, hyaluronate lyases acting on the polymer synthesized by *Streptococcus* species and enzymes with similar action for various bacterial EPS have been identified. These include some from heterologous bacteria and others from bacteriophage. In all the polymers cleaved by such lyases, the uronic acid residues are present in the main chain of linear molecules. Bacterial EPS containing uronic acids are of frequent occurrence, the charged carbohydrate residues either forming part of the main chain or being side-chain components.

Xanthan, the EPS from *Xanthomonas campestris* strains, contains one molecule of D-glucuronic acid as the subterminal residue of a trisaccharide side-chain attached to cellobiose as a pentasaccharide repeat unit (Jansson *et al.*, 1975; Melton *et al.*, 1976). A number of hydrolytic enzymes degrading xanthan have been identified, mainly endo- β -glucanases cleaving the cellulosic main chain (Holt, 1978; Sutherland, 1982; Hou *et al.*, 1986). Under certain restricted conditions, fungal cellulases will also hydrolyse xanthan (Rinaudo & Milas, 1980; Sutherland, 1984). A mixed culture containing enzymes cleaving the side-chain sugars and leaving the cellulosic main chain of xanthan intact has also been reported (Cadmus *et al.*, 1982).

In an early study on enzymic degradation of xanthan, Lesley (1961) reported the presence of unsaturated oligosaccharides among the products; Holt (1978) also indicated that the fragments generated by enzymes from a *Corynebacterium* species showed absorption at 235 nm indicative of the action of a lyase. The present study reports the preparation, partial purification and some

Abbreviations: DP, degree of polymerization; EPS, exopolysaccharide(s); TBA, thiobarbituric acid.

properties of a xanthan lyase from a *Bacillus* strain earlier found to secrete an endo- β -glucanase and a β -D-glucosidase (Sutherland, 1982), and its comparison with similar enzymes from other isolates.

METHODS

Bacteria and culture conditions. The three bacterial systems used were a *Bacillus* species described earlier (Sutherland, 1982); a *Corynebacterium* species (Holt, 1978); and a mixed culture (X23) isolated by enrichment, which contained a yellow-pigmented Gram-negative rod as the dominant micro-organism. Enzymes were only produced in significant yield in the mixture. All cultures grew in synthetic medium containing 0.1% (w/v) xanthan as the carbon source (Sutherland, 1982). After approximately 6 d incubation in shaken 2-litre Erlenmeyer flasks at 30 °C, the cells were removed by centrifugation at 50000 g. The supernatant fluids containing endo- β -glucanase, lyase and other enzymes were concentrated in a tangential-flow filtration cell (Waters) using a non-cellulosic membrane system with 10000 M_r cut-off. The final concentrated solution (about 300 ml from 10 l supernatant fluid) was washed from the system with 0.02 M-Tris buffer, pH 7.2, centrifuged and reduced to about 10 ml by dialysis against polyethylene glycol (M_r 6000). This was used as the starting material for studies of the xanthan lyases. Preparations from each source showed similar activity, but differed in their content of glucosidases and mannosidases. No endoglucanase or lyase activities were detectable in either the pure or mixed cultures grown in the absence of xanthan, indicating that all the systems were inducible.

Enzyme assays. Substrates were xanthan polysaccharides obtained from commercial producers or prepared from different *X. campestris* pathovars in our laboratory. Some were chemically modified by deacetylation, depyruvylation, addition of amine groups, or reduction of the carboxyl groups (Bradshaw *et al.*, 1983). Polysaccharides from *Acetobacter xylinum* strains were kindly supplied by Dr E. Weng and Dr A. Misaki. Lyases were assayed by determination of thiobarbituric acid (TBA) positive material (Weissbach & Hurwitz, 1958). The presence of glycosidase activities in enzyme preparations was determined using *p*-nitrophenyl D-glucopyranosides and D-mannopyranosides. Cellulase (endo- β -glucanase) activity was confirmed using carboxymethylcellulose as substrate in a Brookfield LVT cone-plate viscometer at 30 °C. Neutral sugars in hydrolysates of the polysaccharides or oligosaccharides were determined by HPLC using the procedure outlined by Kennedy & Sutherland (1987). Hydrolysis was with 0.25 M-H₂SO₄ at 100 °C for 8 h. Acyl groups in intact polysaccharides and oligosaccharides and uronic acid were determined by micromethods described previously (Sutherland, 1984). Xanthan fragments of varying size prepared by cellulase hydrolysis (Sutherland, 1984) and those from the combined action of lyases and endoglucanases were separated on a Biogel P2 column (56 × 1 cm) using water as eluant at a flow rate of 0.6 ml min⁻¹, and by paper chromatography in ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.).

FPLC. Concentrated eluates from fractionation on DEAE-Sepharose were applied to an Ultropac TSK 545 DEAE ion-exchange column (150 × 7.5 mm) (LKB) and eluted with a complex salt gradient in 50 mM-HEPES buffer, pH 7.6, using a Gilson system. Protein was determined by the Lowry procedure.

NMR spectroscopy. NMR spectroscopy and FAB spectroscopy were kindly carried out by Dr L. Parolis (Rhodes University, Grahamstown, S. Africa) and Dr A. J. Clarke-Sturman (Shell Research, Sittingbourne, UK) respectively.

RESULTS

Production and purification of xanthan lyases

Enrichment cultures in our laboratory yielded a number of mixed bacterial cultures which, when grown in media with xanthan as sole carbon source, produced enzymes degrading the polysaccharide. Supernatant fluids from such cultures caused loss of viscosity of xanthan and carboxymethylcellulose solutions and the liberation of TBA-positive material from the former. It was further seen that absorption at 235 nm increased, indicative of release of fragments containing unsaturated uronic acids. In most cases, attempts to obtain pure cultures with comparable activities were unsuccessful. However, an earlier study (Sutherland, 1982) yielded a red-pigmented *Bacillus* species which grew in xanthan-containing media, produced extracellular enzymes with endoglucanase and glycosidase activities, and released fragments which were TBA-positive. Similar enzymes and products were found using the *Corynebacterium* supernatants and those from mixture X23. The endoglucanase (xanthanase) and lyase activities from each preparation could be partially purified by chromatography on DEAE-Sepharose. This removed most of the α - and β -D-glucosidase and α - and β -D-mannosidase activities. The material from either of the two pure cultures could be purified further by FPLC. Monitoring of the eluate at 280 nm indicated that two major components were separated (Fig. 1); these were

Fig. 1. Separation of lyase and endoglucanase. Material was applied to an LKB DEAE column and eluted with a complex gradient of increasing ionic concentration (programme ACB3) in 50 mM-HEPES pH 7.6. The eluate was monitored at 280 nm.

Table 1. *Lyase action on different xanthans*

All results represent the mean of 6–10 duplicate estimations.

Source of xanthan	Lyase activity [pmol TBA (mg protein) ⁻¹ per 16 h]
<i>X. campestris</i> pathovar:	
<i>campestris</i> 646	27.1
<i>phaseoli</i> 1128	33.0
<i>phaseoli</i> X45	19.2
<i>oryzae</i> 61	19.4
<i>oryzae</i> 71	29.0
<i>oryzae</i> 79	18.0
<i>oryzae</i> 86	18.8

identified as lyase activity eluting at 17.3 min and endoglucanase at 21.4 min respectively in the case of the *Bacillus* material. Through repeated use of the FPLC column, enough lyase activity, free from endoglucanase, was obtained to perform tests on its specificity. Enzyme from the *Corynebacterium* species and from mixture X23 behaved similarly but yields were insufficient for extensive testing. Insufficient material was available for M_r determination by PAGE, but FPLC on a size-exclusion column (TSK 4000 PW) indicated a value in the range 30 000–33 000. The lyase was free of all mannosidase activity. The pH optimum of the lyase was approximately 7.25 and all assays were performed at this pH.

Specificity of the lyases

When tested against a range of different xanthan preparations, lyase activity as measured by release of TBA-positive material varied considerably (Table 1). Of particular interest was the finding that three preparations from strains of *X. campestris* pv. *oryzae*, 71, 79 and 86, which are not readily hydrolysed by 'xanthanases', yielded comparable amounts of TBA-positive material to the other xanthans. As it was anticipated that the presence or absence of acyl groups, pyruvate on the terminal β -mannosyl residue, and acetate on the internal α -mannosyl residue, might affect lyase activity, xanthans with differing acyl substituents were also tested (Table 2). The results indicated that the lyase functions on either acylated or non-acylated polymers.

As the lyases were always found to be associated in cultures with endoglucanases, in their natural environment they might well function after preliminary degradation of the polysaccharides to soluble oligosaccharides. This aspect of specificity was examined by using a

Table 2. *Effect of acyl substituents on lyase activity*

Source of xanthan	Acetate (%)	Pyruvate (%)	Lyase activity (nmol TBA per 16 h)
<i>X. campestris</i> pathovar:			
<i>campestris</i> 646	4.5	4.4	18.2
<i>gummisudans</i> 2182	10.0	4.7	8.2
<i>phaseoli</i> 556	1.1	6.0	16.4
<i>phaseoli</i> 1128	7.7	1.7	16.4
<i>phaseoli</i> 1128 DAC*	0	1.3	4.9

* This preparation was chemically deacetylated.

Table 3. *Effect of substrate size on lyase activity*

DP	No. of repeat units	TBA release (nmol)
5-15	1-3	3.1
25-35	5-7	6.2
40-55	8-11	5.7
70-90	14-18	5.3
> 90	> 18	4.4

series of oligosaccharides prepared by the action of fungal cellulases on unordered xanthans (Sutherland, 1982) (Table 3). Other possible substrates included modified xanthans. The D-glucuronosyl residues could be converted chemically to D-glucosyl residues, yielding a poorly soluble macromolecule. This was not a substrate for either the lyases or the endoglucanases. Neither were xanthans that had been modified through the addition of amine and hydroxyethyl groups to free hydroxyl residues, although these alterations did not totally inhibit endoglucanase action. Another source of possible substrates were *A. xylinum* strains forming polymers which are thought to show some resemblance to part of the xanthan structure (Tayama *et al.*, 1986). No detectable TBA-positive material was found from any of the preparations tested, after prolonged exposure to the xanthan lyases.

Nature of the products

Although the addition of lyase free of endoglucanase to native xanthans cleaved terminal mannose residues with attendant increase in absorption at 235 nm, such polymeric products were only produced in low yield and were not readily amenable to characterization. Instead, the oligosaccharides obtained by the action of lyase on material of DP < 20, or by the combined action of lyase and endoglucanase on xanthans, were examined. Typically two to five products were found on preparative paper chromatography of the diffusible products following combined action of these enzymes on polymeric substrates. Two of the products were identifiable as D-mannose and acetylated mannose. Two oligosaccharides, designated A22 and A21 (R_{Glc} 0.35 and 0.15 respectively; $R_{\text{Cellobiose}}$ 0.96 and 0.41), were detected and purified. A further slower-moving oligosaccharide on paper chromatography (A20) had an R_{Glc} of 0.06. All three oligosaccharides contained D-glucose, D-mannose and (unsaturated) uronic acid in the molar ratio 2:1:1. The fastest-moving fragment, A22, also contained 1 mol of acetate. FAB and NMR spectroscopy confirmed the identity of A22 and A21 as tetrasaccharides of M_r 704 and 662 respectively, with the structures indicated in Fig. 2. A22 was the acetylated version of A21. The third oligosaccharide, A20, was believed to be the corresponding octasaccharide and was also acetylated, but the acetyl content was non-stoichiometric, representing a molar ratio of about 0.7. Thus the octasaccharide fraction is probably a mixture of acetylated and non-acetylated material. Detailed studies on the oligosaccharide structures will be published elsewhere. The production of the two tetrasaccharides depended on the substrate xanthan used. No A22 was obtained when non-acetylated polysaccharide was the substrate. Some xanthans with possibly aberrant composition or structure yielded further oligosaccharides, the identity of which has not yet been fully established.

Fig. 2. Fragments resulting from the action of lyase on xanthan.

DISCUSSION

Enzymes degrading bacterial EPS, such as the uronic-acid-containing types of the different *Klebsiella* serotypes, have been obtained from heterologous bacteria or from bacteriophage. Most of these enzymes are hydrolytic in their mode of action, cleaving the backbone or main chain of the macromolecule. They have provided considerable structural information about polysaccharides composed of repeating units (e.g. Hisamatsu *et al.*, 1980). No hydrolytic enzymes have yet been found for block copolymers such as bacterial or algal alginate, and less information is provided from the lyases acting on these substrates (Davidson *et al.*, 1976, 1977). However, lyases for the EPS composed of repeating units have also been reported, including one of the first such enzymes characterized, that degrading the *Streptococcus pneumoniae* type VIII polysaccharide (Becker & Pappenheimer, 1966). It is one of a number of lyases, 4,5-transeliminases of bacterial or bacteriophage origin, now known to cleave the main chain of bacterial EPS (e.g. van Dam *et al.*, 1985).

Enzyme systems degrading xanthan, the EPS from strains of *Xanthomonas campestris*, have been obtained from pure and mixed cultures. Most of these enzyme preparations have been mixtures of glucanases and glycosidases (Cadmus *et al.*, 1982; Hou *et al.*, 1986). The products of enzyme action have mainly been 'limit' oligosaccharides of relatively high M_r , or monosaccharides and the cellulosic backbone of xanthan, although one report did suggest the possible action of a lyase (Lesley, 1961). Xanthan can also be degraded to a limited extent through the action of fungal cellulases from *Aspergillus niger* and *Trichoderma viride* on unordered polysaccharide in dilute solution (Sutherland, 1984). The enzymes only act slowly, yielding products of relatively high M_r together with oligosaccharides and some glucose. The action is inhibited by salts, which promote the ordered structure of the polysaccharide.

The only substrates identified for the xanthan lyases were EPS of the xanthan type or high- M_r oligosaccharides derived from them by main-chain cleavage, i.e. composed of the characteristic pentasaccharide repeating unit, with or without associated acyl groups. Certain *Acetobacter xylinum* polysaccharides have been reported to contain structural similarities to xanthan (Tayama *et al.*, 1985, 1986), but no enzyme activity on these was detected. Activity was also absent if the xanthan molecule had been modified by various chemical procedures. The action of

the lyase was apparently unaffected by the presence or absence of a 4,6 pyruvate ketal group on the terminal β -mannosyl side-chain residue of xanthan. This was surprising, as the ketal would be expected to alter the charge on that portion of the molecule and possibly also its configuration in solution. However, few xanthan preparations are fully pyruvylated (Jansson *et al.*, 1975), therefore the enzyme has probably adapted to act on both ketalated and non-ketalated side-chains.

The xanthan lyases reported here are unusual in that they cleave the side-chain of a bacterial polysaccharide, whereas other lyases studied previously all appear to act on the main chain of their polysaccharide substrates. Despite this, a common feature of xanthan and other polysaccharides degraded by lyases is their site of action, a 4-linked uronosyl residue. In xanthan the actual site of attack is a β -D-mannosyl 1,4- β -D-glucuronosyl side-chain terminus. The enzymes, unlike the endoglucanases with which they are associated, only appear to act on xanthans and provide useful information on the fine structure of these widely used bacterial polysaccharides.

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