Gellan lyases – novel polysaccharide lyases

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A number of bacterial strains capable of degrading the bacterial exopolysaccharide gellan® have been isolated by standard enrichment procedures. They include several pink-pigmented Gram-negative rod-shaped bacteria. A red-pigmented Gram-positive bacillus earlier found to degrade the exopolysaccharide xanthan from *Xanthomonas campestris* also showed slight gellanase activity. All the Gram-negative bacteria are non-fermentative, motile and amylase-producing. The gellan degradation in each case is due to eliminase-type enzymes (lyases) which appear to be extracellular enzymes cleaving the sequence ... β -D-glucosyl-(1 $\rightarrow$ 4)- β -D-glucuronosyl... in the tetrasaccharide repeat unit of the substrate polysaccharides. Although in some isolates these enzymes appear to be exo-acting, it appears from the loss in viscosity of the alternative substrate deacetylated rhamsan that they are predominantly endoenzymes. The enzyme activity is inducible: it is almost absent from glucose-grown cells. Associated with the 'gellanase' activity, all the Gram-negative bacterial isolates possess intracellular α -L-rhamnosidase and β -D-glucosidase activities apparently located in the periplasm. The enzymes are highly specific and fail to cause significant degradation of most of the other bacterial exopolysaccharides which have been shown to be structurally related to gellan. As well as acting on gellan, they exert similar degradative activity against the chemically deacylated form of polysaccharide S194 (rhamsan gum), which is effectively a gentiobiosylated form of gellan. The enzymes only have relatively slight activity against the natural, acylated gellan-like polysaccharides from the bacteria now designated as strains of *Sphingomonas paucimobilis*.

Keywords: gellan, gellanases, gellan lyases, red-pigmented bacteria

INTRODUCTION

Gellan is the exopolysaccharide produced commercially as a chemically deacylated gelling agent and product from a bacterium designated originally as *Auromonas* (*Pseudomonas*) *elodea*, but now termed *Sphingomonas paucimobilis* (Pollock, 1993). The polysaccharide is one of a series of eight, structurally closely related, bacterial products (Jansson *et al.*, 1983, 1985, 1986a, b; Cairns *et al.*, 1991). These polymers share much of their backbone structure but differ in the nature and location of their side-chains and in the presence or absence of certain acyl groups. The native exopolysaccharide product of *S. paucimobilis* is composed of a linear tetrasaccharide repeating unit sequence (Fig. 1) (Jansson *et al.*, 1983). O-Acetyl and L-glyceryl residues are attached to the D-

glucosyl residue adjacent to D-glucuronic acid (Kuo *et al.*, 1986). All the related polysaccharides from bacterial strains now also considered to be *S. paucimobilis* (Pollock, 1993) possess similar linear structures in which there is at least an identical -(D-glucose-D-glucuronic acid-D-glucose)- trisaccharide sequence with the same anomeric configurations as in gellan (Fig. 1). The main structural differences are in the nature and location of the monosaccharide and disaccharide side-chain groupings and in some of the polymers, the presence of L-mannose as an alternative to L-rhamnose in the main-chain structures. Many of the polysaccharides are acylated, although in most of the polymers the location of the acyl groups has yet to be determined (e.g. O'Neill *et al.*, 1990). An exception is welan gum in which O-acetyl groups have been demonstrated in the 2-position of approximately 85% of the 3-linked glucose residues (Stankowski & Zeller, 1992).

Abbreviation: TBA, thiobarbituric acid.

(a) Gellan gum

(b) Welan gum (S130)

(c) Rhamsan gum (S194)

(d) (S657)

Fig. 1. Structures of gellan, welan, rhamsan and S657 polysaccharide.

The physical properties of the polysaccharide solutions also vary considerably. The aqueous solutions of all the gellan-like polysaccharides are highly viscous and show high thermal stability. In aqueous systems, the *S. paucimobilis* gellan polymer forms highly viscous solutions or weak gels but on deacylation with mild alkali it is capable of forming rigid, brittle gels in the presence of cations such as Mg^{2+} (Baird *et al.*, 1983). The polysaccharide adopts a double helical structure in which the duplex is stabilized by inter-chain hydrogen bonds (Chandrasekaran *et al.*, 1988). It has been suggested that the *O*-acetyl groups on the native polysaccharide only have a weak effect on aggregation of gellan molecules, whereas the *L*-glyceryl residues are detrimental to crystal packing (Chandrasekaran & Thailambal, 1990). In the deacylated form, gellan is marketed as a gelling agent for various food and non-food applications including the replacement of agar in bacterial culture media. Only the deacylated polymer exhibits gelation with properties similar to agar, for which it can substitute in a number of applications (Sutherland, 1994). The exopolysaccharides with related structures have high solution viscosity and stability at high temperatures but do not normally form gels, either before or after chemical deacylation.

Enzymes degrading bacterial and other polysaccharides are useful adjuncts in structural determination and such enzymes have been obtained from a wide variety of bacteria, fungi and bacteriophages. In only a few cases have enzymes degrading microbial exopolysaccharides been obtained from the same species which produce the polysaccharides. The majority of exopolysaccharide-degrading enzymes act through hydrolytic cleavage of the polymers but some enzymes are polysaccharide lyases which act through a β -eliminative mechanism (Linhardt *et al.*, 1986; Sutherland, 1990). A brief report of a lyase-type enzyme acting on gellan, isolated from a bacterium found in garden compost, has appeared (Schmedding *et al.*,

1987) but little information about the bacteria or the enzyme specificity was reported. A study of soil bacterial isolates noted that certain species degraded culture plates solidified with gellan but, apart from noting that the bacteria were Gram-negative gliding bacteria, did not characterize the bacteria or indicate the enzyme specificity (Casida, 1989). Recently, Mikolajczak *et al.* (1994) reported an enzyme activity which they named 'sphinganas' from a *Bacillus* sp. acting in undetermined fashion as an endoglycanase on welan and, to a lesser extent, gellan but not on rhamsan (Fig. 1). We now report the isolation in pure culture of several bacterial strains capable of growth on gellan as sole carbon and energy source and providing a source of novel degradative enzymes (gellanases or gellan lyases). The extracellular polysaccharide lyases yield fragments with a non-reducing terminal unsaturated uronic acid and are associated with intracellular glycosidases.

METHODS

Bacterial isolation. Gellan (0.2%, w/v) was incorporated as sole carbon and energy source into a simple synthetic medium consisting of a salts base and 0.5% (w/v) ammonium nitrate as nitrogen source. Mud or soil from local sites was added to 1 l aliquots of medium in 2 l Erlenmeyer flasks and the mixtures were incubated at 30 °C for 7–10 d with shaking at 200 r.p.m. The supernatant liquid from the flasks was decanted into fresh medium and the process repeated several times. Aliquots were then cultured on plates of the same medium solidified with gellan in which the polysaccharide content had been increased to 0.5% (w/v). Those plates which showed significant depressions around colonies, indicative of gellanase production, were repeatedly subcultured on the same medium then on yeast extract agar plates until pure (Sutherland & Wilkinson, 1965). Finally gellanase activity in the pure cultures was confirmed by further plating on gellan medium plates incubated at 30 °C.

The gellanase-producing bacteria obtained in pure culture were then used to prepare extracellular enzymes, and ultrasonic lysates of the bacterial cells provided sources of intracellular glycosidases. One red-pigmented Gram-variable *Bacillus* species described earlier as a source of xanthan lyase (Sutherland, 1987) was also found to grow slowly as a highly pigmented adherent film on gellan plates and to produce small amounts of degradative enzymes, but was not investigated further. Extracellular enzymes were prepared from all isolates by growth of the bacteria in a synthetic medium containing 0.1% ammonium sulphate and 0.2% gellan, together with the salts used in yeast extract medium. Medium was dispensed in 800 ml aliquots in 2 l Erlenmeyer flasks and cultures were grown for 10–12 d at 30 °C with shaking at approximately 200 r.p.m. Bacteria and any insoluble undegraded gellan were removed by centrifugation at 30000 *g* for 30 min. The clear supernatant fluid was concentrated to a small volume by tangential-flow filtration, re-centrifuged and used as 'enzyme'.

Enzyme isolation and purification. Cell lysates were prepared by ultrasonic treatment or by passage through a French pressure cell (Aminco). The cell lysates were ultracentrifuged at 110000 *g* for 60 min, dialysed in the cold against 10 mM Tris buffer (pH 7.5) and concentrated if necessary by dialysis against polyethylene glycol (molecular mass 6000 Da).

Analytical procedures. Enzyme assays for lyase activity were routinely performed using the thiobarbituric acid (TBA) assay procedure of Weissbach & Hurwitz (1958). Assays for β -D-

glucosidase, α -L-rhamnosidase and other glycosidases were performed using nitrophenyl sugar derivatives as substrates (Sigma). D-Glucose release from exopolysaccharides was monitored using the glucose oxidase procedure. Viscosity changes due to enzyme action were measured using a Brookfield LVTD instrument with the system equilibrated at 30 °C. Monosaccharide constituents of the polymers and of the products of enzyme hydrolysis were identified initially by descending paper chromatography using butan-1-ol/pyridine/water (6:4:3, by vol.) as mobile phase, then HPLC after hydrolysis with 0.25 M H₂SO₄ at 100 °C for 8 h as indicated by Kennedy & Sutherland (1987).

Gellan was purchased from Kelco Division of Merck. Other related polysaccharides were either kindly donated by Dr J. Baird (Kelco) or prepared in our laboratory. The polymers from mutant strains of *S. paucimobilis* (*A. elodea*), kindly provided by Dr I. Sa Correia, Instituto Tecnico Superior, Lisboa, Portugal, were prepared in our laboratory. All the commercial polysaccharides except gellan were dissolved in distilled water and ultracentrifuged to remove particulate material. They were then further purified by extensive dialysis before use. Deacetylation of all polysaccharides was performed by heating the solutions in 0.05 M NaOH at 100 °C for 15 min, neutralization with 0.1 M HCl, dialysis against distilled water and lyophilization.

Preliminary experiments indicated that interaction of the enzyme in the culture supernatants with gellan increased the reducing material present and released material reacting in the TBA test. There was also increase in A_{235} . Optimal activities were observed for preparations from several strains in the pH range 6.5–7.5 and temperatures of 35–40 °C.

RESULTS

Bacterial characteristics

Five isolates were Gram-negative, motile rods which grew relatively poorly on almost all media tested. All five bacteria appeared to be similar in most respects tested but two strains yielded slightly mucoid colonies on solid media, whereas the others appeared to produce little if any extracellular polysaccharide. To avoid the problems of exopolysaccharide in concentrated culture supernatants, the non-mucoid isolates were used for most purposes. Colonies and cell deposits were pink coloured. The pink pigment from the Gram-negative bacteria was cell-associated; it was insoluble in ethanol or petroleum ether but could be extracted with chloroform/methanol (2:1, v/v). Absorption maxima at 490 and 530 nm were recorded. The sixth culture, isolated earlier in our laboratory, was Gram-positive or Gram-variable, and grew well on solid media as a thick, adhesive, brick-red film. Although it grew well on the surface of synthetic medium solidified with agar, its degradation of gellan was very much less marked than that of the Gram-negative isolates, and for this reason it was not extensively studied. In liquid medium it grew as a surface pellicle or as granular material. Culture supernatants, when added to gellan as substrate, released TBA-positive material but caused very little liquefaction of the gels.

The Gram-negative bacteria were strongly amylolytic when tested by growth on starch agar plates for 48 h at 30 °C followed by flooding of the plates with iodine. The production of amylolytic enzymes and of α -D-glucosidase was induced by growth in media containing starch; the

enzymes involved in starch degradation were effectively absent from glucose- or gellan-grown bacterial cultures. There was no detectable degradation of cellulose, xanthan, sodium pectate, or agarose. The bacteria were not proteolytic. They were urease positive, weakly catalase positive, oxidase negative (Kovacs) and showed growth on succinate, lactate, acetate and alanine. Cell lysates of gellan-grown cells were strongly active against the α -L-rhamnoside and the β -D-glucoside but not against other nitrophenyl glycoside substrates tested. Some slight intracellular α -L-rhamnosidase activity was also present in bacterial cells grown on starch or glucose. Concentrated culture supernatants from bacteria grown in the presence or absence of gellan showed weak activity against the same two nitrophenyl substrates. This increased in older cultures as cell lysis occurred. The *Bacillus* sp. lacked amylolytic activity but possessed similar glycosidase activities to the Gram-negative bacteria.

Enzyme isolation

Supernatant fluid from gellan-grown cultures was recovered after centrifugation and concentrated by tangential-flow filtration in a Waters Millipore Minitan equipment (10000 Da molecular mass cut-off). After extensive dialysis, further concentration was achieved using polyethylene glycol (molecular mass 6000 Da). The presence of gellan lyase activity in the concentrated fluid was confirmed using the TBA method. In tests with filters of known permeability, it became clear that the enzyme activity was retained by filters with 100000 Da molecular mass cut-off. This was also true of the α -L-rhamnosidase activity obtained from the cytoplasmic fraction. The attempted partial purification of the proteins was complicated by the presence of partially digested substrate and by the relatively low enzyme yields. The concentrated supernatants were dialysed against 10 mM Tris buffer (pH 7.0) and subjected to chromatography on columns (22 × 1 cm or 85 × 2.5 cm) of the strong anion-exchanger Q Sepharose FF (Pharmacia) using a stepwise gradient of NaCl in Tris buffer at a flow rate of 17.6 ml h⁻¹. After elution of unbound material with three column volumes of Tris buffer, in a typical purification most of the lyase activity eluted at 0.4 M NaCl. Preliminary examination using PAGE indicated the presence of several polypeptides. The major polypeptide, of molecular mass ~ 135 kDa, may possibly be the gellan lyase.

Enzyme specificity and activity

The concentrated and partially purified enzymes from all the Gram-negative isolates behaved similarly. They appeared to be highly specific, acting primarily on gellan and having little if any activity on most of the other, structurally similar, polysaccharides in the series or on the original acylated polysaccharide from which gellan is derived. An exception was the polymer from strain ATCC 31961 (Kelco polymer S194, 'rhamsan'; Fig. 1). Activity against the native polymer was relatively slight, but it was very greatly increased following the removal of any O-acetyl groups present by treatment with alkali. There was linear release of material reacting in the TBA assay from

Fig. 2. Lyase digestion of substrates: action on gellan and deacetylated polysaccharide S194. Enzyme from strain 14.1 was used in this experiment: 5 ml substrate (1 mg ml⁻¹) was mixed with 5 ml 10 mM Tris, pH 7.0, and 5 µl enzyme (10 mg protein ml⁻¹) and incubated at 30 °C. Aliquots (500 µl) were removed and boiled for 5 min. The TBA assay was then performed on triplicate 100 µl samples. ■, Gellan; +, deacetylated S194.

Table 1. Action of lyase enzyme preparations on gellan and related substrates

All results are expressed as a percentage of the gellan result. Typically gellan yielded 10430 pmol product (mg protein)⁻¹ h⁻¹.

Substrate	Enzyme source (strain)				
	2A	11.1	12.1	13.1	14.1
Gellan	100	100	100	100	100
Deacetylated S194	127	96	—	65	85
S657	—	0	0	0	3
S130	—	—	—	0.9	5
MJ200*	43	49	49	18.5	58.5
PA4*	23	19.4	19.8	24	29.9
SB10†	0	0	0	0	2.7
SB30†	0	0	8.4	2.3	0

* Polysaccharides from gellan mutants with no apparent mannose present.

† Polysaccharides containing mannose.

either gellan or deacylated polysaccharide S194 (Fig. 2) over a 10–12 h period. Thereafter, TBA-reacting products continued to be formed but at a lower rate. Also of interest was the action on exopolysaccharides from several mutant strains of *S. paucimobilis* obtained in recent studies by I. Sa Correia and co-workers (unpublished results). These polymers appear to contain mannose, probably L-mannose, in partial replacement for L-rhamnose although the exact structures have not yet been fully characterized. The presence of the relatively uncommon sugar L-mannose in the polysaccharide structures apparently

Fig. 3. Effect of gellan lyase on solution viscosity. Polysaccharide (1 ml), at a concentration of 5 mg ml⁻¹ for deacetylated S194 or 2.5 mg ml⁻¹ for gellan, was mixed with 5 µl enzyme (10 mg protein ml⁻¹) and the viscosity was determined over a period of time in the Brookfield viscometer at 30 °C. ■, Gellan; +, deacetylated S194.

inhibited enzyme action for some but not all of the enzyme preparations (Table 1). The optimal pH of the lyase enzyme preparations from all the Gram-negative bacterial strains were in the range pH 6.5–7.5.

As there was only slow loss of solution viscosity from gellan, the mode of action of the enzymes appeared at first to be exolytic. However, similar experiments using the chemically deacetylated polysaccharide S194 (rhamnogum) as substrate (Fig. 3) indicated a steady loss in viscosity with time, characteristic of an endoenzyme. These results were mirrored by the increasing release with time of characteristic oligosaccharides from both substrates as followed by paper chromatography and by the reduction in the amount of non-diffusible material over a 12 h period (Fig. 4). The poor solubility and low effective viscosity of the gellan caused the initial interpretation. All incubation mixtures contained some polymeric material even after extensive enzyme treatment and this limit digestion product was retained within dialysis sacs when monosaccharides and oligosaccharides were separated. After 25 h incubation under standard conditions, gellan and deacetylated rhamnogum yielded 38% and 40.5% undigested material respectively. On more prolonged incubation, the amount of undigested material fell to 29% in the case of gellan.

Associated with the gellan lyases were β -D-glucosidases and α -L-rhamnosidase activities. As these were predominantly cell-associated, it is probable that there is initial release of the tetrasaccharide repeat units and subsequent degradation by the glycosidases rather than breakdown by some other mechanism. However, from Fig. 2 it can be seen that release of TBA-positive material was linear under standard conditions over 10 h. Glucose release was low over the first 12 h of incubation but continued thereafter up to 64 h (data not shown). This would also appear to confirm that the action of the gellan

Fig. 4. Recovery of macromolecular material after digestion with gellan lyase. Mixtures of 50 ml polymer solution (0.25%, w/v), 50 ml 10 mM Tris buffer, pH 7.0, 1 ml 2% (w/v) sodium azide and 500 μ l enzyme (10 mg protein ml⁻¹) were prepared and incubated at 30 °C. Four flasks of each mixture were set up, and at appropriate times the contents of the flask were boiled for 20 min, cooled and divided into three aliquots, each of which was dialysed against four changes of 2.5 l distilled water. The retentates were then lyophilized and weighed. ■, Gellan; +, deacetylated S194.

Fig. 5. Chromatography of diffusible products from enzyme digests on Biogel P2. The pooled diffusible material from incubation at 30 °C for 25 h was evaporated to dryness under reduced pressure at 50 °C, redissolved in 1 ml deionized water and applied to the Biogel P2 column (54 $\times$ 1 cm). The columns were eluted with water as indicated in Methods and 0.75 ml fractions collected at 10 min intervals. Samples (10–100 μ l) were tested for carbohydrate content using the phenol sulphuric acid assay. In the gellan digest, fractions 13–18, 20–28 and 29–39 were pooled to give pooled fractions 1, 2 and 3, respectively. In the digest of the deacetylated S194 polysaccharide, pools 1, 2 and 3 corresponded to fractions 16–23, 24–31 and 32–39, respectively. (a) Gellan digest. (b) Digest of deacetylated S194 polysaccharide.

lyase in cleaving the main chain of the polysaccharide is the primary activity. The major products of exhaustive enzyme degradation of gellan appeared to be D-glucose and a trisaccharide or tetrasaccharide with an unsaturated uronic acid at the non-reducing terminal. Analogous products were obtained from the other substrate. Paper chromatography or HPLC of digests of either substrate failed to reveal any free rhamnose. The low molecular mass products recovered after dialysis of enzyme digests of gellan or deacetylated S194 polymer could be separated by gel permeation chromatography on a Biogel P2 column (54 $\times$ 1 cm) into three major fractions (Fig. 5). On paper chromatography of fraction 1, there was a major slow-migrating product (R_{Glc} 0.07) which was possibly an oligomer of the repeat unit tetrasaccharide into which a double bond has been introduced at the non-reducing terminal uronic acid. Smaller amounts of other slower-moving material may represent oligomers of this tetrasaccharide. Fraction 2 contained material of R_{Glc} 0.55 as the major constituent. Both these fractions contained relatively large amounts of unsaturated uronic acid as measured by the TBA test. Fraction 3 was mainly composed of free glucose. The profile on Biogel P2 of enzyme digest products obtained from deacetylated S194 resembled that from gellan, but paper chromatography of the material showed it to be considerably more complex. One oligosaccharide purified by preparative paper chromatography of pooled fraction 2 from gellan was found to contain equimolar amounts of D-glucose, L-rhamnose and unsaturated uronic acid. Because of the complexity of the fractions further purification is necessary. Characterization of the oligosaccharide fragments from both substrates is under way. Analysis of acid hydrolysates of the undigested limit material from either polymer revealed no differences from the initial substrates.

Starch degradation

When streaked onto starch agar, incubated and stained with Gram's iodine solution, strains of the Gram-negative gellanase-producing bacteria all showed wide unstained zones indicative of starch degradation. Testing of concentrated culture supernatants from starch-grown bacteria showed that when release of glucose was followed using the glucose oxidase assay, glycogen was the best substrate and pullulan was slightly more degraded than was starch. Similar testing of a series of maltodextrins indicated that maltose and maltotriose were rapidly hydrolysed to glucose but maltopentaose and maltohexaose were more slowly degraded. Isomaltose was only slightly hydrolysed; kojibiose and trehalose were not significantly degraded, but α -nitrophenyl-D-glucopyranoside was rapidly hydrolysed. Comparison of the amylolytic activity of the isolates with that from a preparation of pullulanase from *Enterobacter aerogenes* indicated clear differences in substrate specificity towards both polymeric and oligomeric materials. Chromatographic examination of the breakdown products indicated that while glucose was the only low molecular mass product from glycogen or

pullulan, some maltose and higher malto-oligosaccharides were also present in starch hydrolysates. Degradation of these polymers and oligosaccharides was only seen in cultures induced by growth on starch or glycogen. When cultured on synthetic medium containing 0.1% (w/v) starch and solidified with gellan, differences were seen between the five isolates. Some immediately degraded gellan and starch, while others only degraded gellan after the starch had been utilized.

DISCUSSION

A number of bacterial strains capable of degrading gellan have been isolated by standard enrichment procedures. They include several small, pink-pigmented Gram-negative bacteria and a red-pigmented Gram-positive bacillus earlier found to degrade xanthan. Only the red-pigmented *Bacillus* sp. shows any resemblance to the strain recently reported by Mikolajczak *et al.* (1994) to degrade welan gum, with lesser activity against gellan and none against rhamosan. All the micro-organisms which we have described excrete enzymes that degrade gellan and also possess intracellular α -L-rhamnosidase activity. The gellan degradation in each case is due to eliminase-type enzymes (lyases) which appear to be endo-enzymes as there is linear release of material reacting in the TBA assay, and loss of solution viscosity. Associated with the extracellular gellan lyases are intracellular β -D-glucosidase and α -L-rhamnosidase activities. It appears that there is initial release of the tetrasaccharide repeat units through the eliminative cleavage and subsequent degradation by glycosidases. The major products of the degradation of gellan by the enzyme mixtures appear to be a trisaccharide and a tetrasaccharide, each with an unsaturated uronic acid at the non-reducing terminal. The lyase enzymes appear to be highly specific, acting primarily on the polymer gellan, which is chemically deacetylated during commercial production, and on the deacetylated S194 polysaccharide and having little if any activity on either the native or deacetylated forms of the other polysaccharide structures in the series.

The specificity of the gellan-degrading enzymes is unusual in that only the polysaccharides which have been modified by chemical deacetylation are substrates, and of the eight polysaccharide structures which form the gellan group (Sutherland, 1990, 1994) only two of the seven available for testing are degraded. Other polysaccharide lyases, including many of those active on alginates, have been shown to be strongly inhibited by the presence of the *O*-acetyl groups which are known to be present on D-mannuronosyl residues (Davidson *et al.*, 1977; Kennedy *et al.*, 1992). In contrast, most of the xanthan lyases (Sutherland, 1987) act on xanthan whether or not either acetyl or pyruvate ketal groups are present, although acetan, a polysaccharide with structural similarities to xanthan (Jansson *et al.*, 1993), is not degraded unless the acetyl groups present on the main-chain glucose residues are first chemically removed (I. W. Sutherland, unpublished results). The presence of *O*-acetyl groups can greatly affect the ordered structure adopted by some

bacterial polysaccharides in solution (e.g. Sutherland, 1990, 1994), but it is surprising that such a relatively small substituent on the polysaccharides should so greatly affect susceptibility to the action of certain enzymes. The action of the enzyme on rhamosan gum, in which there is a gentobiosyl side-chain attached to the glucose residue distal to the uronic acid (Fig. 1), indicates that the ordered structure resulting from this substituent is still available for enzyme attack provided all acyl groups have been removed. However, the polymers such as welan and S657, in which the glucosyl residue attached to the reducing terminus of the uronic acid is substituted by a side-chain, are not attacked. The side-chains of these latter two polysaccharides clearly inhibit enzyme-substrate binding and indicate that the binding or cleavage site must extend to the glucose residues on either side of the glucuronic acid. This result agrees with the observation by Crescenzi *et al.* (1987) that through hydrogen bonding, the short side-chains of L-mannose or L-rhamnose mask the uronic acid residues in the conformation adopted in aqueous solutions. This result was also confirmed by Lee & Chandrasekaran (1991) in an X-ray and computer modelling study of gellan and three structurally related polysaccharides in which it was concluded that although all have the same double-helical conformations, the side-chains shielded the carboxylate groups to varying degrees. The shielding was considerable in welan and S-657 polysaccharides, but much less in rhamosan. In aqueous solutions of welan the polysaccharide is very highly ordered and even on heating fails to show the normal order-disorder transition (Chandrasekaran *et al.*, 1994). It was suggested that the complete removal of the side-chains would be required to expose the carboxylate groups entirely, as is seen in gellan. This could perhaps allow the enzyme to cleave at its recognition site, which in the polysaccharides rhamosan and gellan is unsubstituted. Perhaps predictably, polymers in which L-mannose replaces some or all of the L-rhamnosyl residues are not good substrates. These enzymes should prove useful in determining the subtleties of polysaccharide structure in the gellan family of polymers and in the products from mutants of the bacterial strains.

The gellan lyases are newly identified enzymes in a series of polysaccharide lyases isolated mainly from bacteria and bacteriophages, but also from other sources. Such enzymes include alginases (alginate lyases), pectate lyase, xanthan lyase, heparinase, chondroitinase, and a number acting on various other bacterial exopolysaccharides. With the exception of the xanthan lyase, these enzyme all cleave the main chains of uronic acid-containing polysaccharide substrates in which there is a 1,4 α - or β -glycosyl residue linked to the uronic acid (Linhardt *et al.*, 1986). In species that grow on the polysaccharides as carbon substrates, such enzymes appear to provide the bacteria synthesizing them with a mechanism for converting various monosaccharide products to 2-keto-3-deoxy-aldoates which can then be metabolized further to pyruvate and triose phosphate, as was demonstrated by Preiss & Ashwell (1962a, b; 1963a, b) for both alginates and polygalacturonic acid. In the plant pathogen *Erwinia*

chrysanthemi, the five gene products, including pectate lyase, which are involved in pectate degradation and catabolism are subject to a complex regulatory circuit (Reverchon *et al.*, 1991). Although enzyme activity degrading gellan has recently been demonstrated in cultures of a strain of *Bacillus brevis*, there was no indication of the mode of action of the enzyme or the nature of any oligosaccharides produced and the specificity observed was very different from the enzymes we now describe (Mikolajczak *et al.*, 1994). Welan gum, in which there is an α -L-rhamnopyranosyl or α -L-mannosyl residue attached to the main-chain glucose distal to the uronic acid (Fig. 1), was the optimal substrate; rhamsan was not degraded although gellan was. The enzymes also, unlike those which we have found, acted equally on the native acylated polymers and their deacylated derivatives.

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