Polysaccharide lyases from gellan-producing *Sphingomonas* spp.

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A number of *Sphingomonas* strains capable of synthesizing the bacterial exopolysaccharide gellan and related polymers were shown to possess constitutive gellanase activity. In each case, the degradation of deacylated gellan was due to extracellular, eliminase-type enzymes (lyases) which cleave the sequence ... β -D-glucosyl 1,4- β -D-glucuronosyl... in the tetrasaccharide repeat unit of the substrate polysaccharides. Deacetylated rhamsan was an alternative substrate but there was little or no action against most other polysaccharides with similar structures. Slight differences were found between the specificities of the lyases from different strains. Activities of gellan lyase preparations were generally low. As well as the extracellular 'gellanase' activity, all the bacteria possessed varying amounts of β -D-glucosidase and β -D-glucuronidase activities apparently located in the periplasm. The products from deacylated gellan and the chemically deacylated form of polysaccharide S194 (rhamsan gum), which is effectively a gentiobiosylated form of gellan, closely resembled those recently obtained by the authors from other, gellan-degrading, non-gellan-producing bacteria. The enzymes had negligible activity against the natural, acylated gellan and rhamsan polysaccharides from bacteria now designated as strains of *Sphingomonas*.

Keywords: gellanases, gellan lyases, polysaccharides, polysaccharases

INTRODUCTION

Gellan is marketed as Gelrite, the chemically deacylated polysaccharide gelling agent obtained from bacteria originally designated as *Auromonas* (*Pseudomonas*) *elodea*, but now termed *Sphingomonas* strains (Pollock, 1993). The polymer is one of eight closely related bacterial products which share much of their backbone structure but differ in the nature and location of their side-chains and in the presence or absence of acyl groups (Jansson *et al.*, 1983, 1985, 1986a, b; Cairns *et al.*, 1991; Stankowski & Zeller, 1992). In its native form, gellan is composed of linear tetrasaccharide repeating units in which O-acetyl and L-glycerol residues are attached to the D-glucosyl residue adjacent to D-glucuronic acid (Fig. 1) (Jansson *et al.*, 1983; Kuo *et al.*, 1986). The structurally related polysaccharides from bacteria now also considered by Pollock (1993) to be *Sphingomonas* strains possess similar linear structures in which there is at least an identical -(D-glucose-D-glucuronic acid-D-glucose)- trisaccharide sequence with the same linkages and anomeric configurations as gellan.

Several of these polymers are acylated, form highly viscous aqueous solutions and show high thermal stability but do not normally form gels. The gelling agent 'gellan' is produced on deacylation with mild alkali and is marketed as Gelrite; it forms brittle gels in the presence of cations such as Mg^{2+} (Baird *et al.*, 1983).

Polysaccharide-degrading enzymes either effect hydrolytic cleavage of the polymers (glycanohydrolases) or act as polysaccharide lyases through a β -eliminative mechanism (Linhardt *et al.*, 1986; Sutherland, 1987, 1990, 1995). Polysaccharide lyases have been obtained from bacteria, fungi and bacteriophages; a few have been found in the same microbial species which produce the polysaccharide substrates. Schmedding *et al.* (1987) briefly described a lyase-type enzyme acting on gellan, but gave no information about the specificity of the enzyme. Mikolajczak *et al.* (1994) reported an enzyme activity, designated 'sphingonase', from a *Bacillus* sp. The material acted in undetermined fashion, possibly as an endoglycanase, on welan, to a lesser extent on gellan but not on rhamsan (Fig. 1) We recently reported the isolation in pure culture of several bacteria growing on deacylated gellan as sole carbon and energy source and providing a

Abbreviations: EPS, exopolysaccharide; TBA, thiobarbituric acid.

(a) Gellan gum

(b) Welan gum (S130)

(c) Rhamsan gum (S194)

(d) S657

(e) S88

(f) S198

(g) NW11

Fig. 1. Structures of gellan and related polysaccharides.

source of novel degradative enzymes (gellanases or gellan lyases) (Kennedy & Sutherland, 1994). These lyases yielded oligosaccharide fragments with a non-reducing terminal unsaturated uronic acid; the bacteria producing them also produced intracellular glycosidases. Following the observation by ourselves (A. M. de Souza & I. W. Sutherland, unpublished results) and others (e.g. Martins & Sá-Correia, 1994) that several cultures of *Sphingomonas* spp. showed reduction in the yield of exopolysaccharide (EPS) on prolonged incubation, we now report the presence of a polysaccharide lyase activity in some of the *Sphingomonas* spp. which synthesize gellan and related polymers. Although preparations were active against deacetylated substrates they generally failed to show significant degradation of the native polysaccharide from which gellan is prepared.

METHODS

Bacterial culture and enzyme isolation. The bacteria used in this study were EPS-producing strains now designated as *Sphingomonas* spp.: ATCC 31461, 31961, 31554 and 31555; and NCIMB 11942. Although all these strains produce polysaccharides with some structural similarity to gellan, strain NCIMB 11942 differed from the others in apparently producing a typical lipopolysaccharide as opposed to sphingolipid. In some cases, spontaneous polysaccharide-negative mutants were isolated in our laboratory. Strain Mj216 (a spontaneous EPS-

derivative of ATCC 31461) was kindly supplied by Dr I. Sá-Correia, Instituto Tecnico, Lisbon, Portugal. Bacteria were cultured on glucose-free semi-synthetic medium consisting of a salts base supplemented with 0.1% (w/v) yeast extract and 0.1% (w/v) casein hydrolysate as nitrogen source (Sutherland & Wilkinson, 1965) and solidified with 0.75% (w/v) gellan. Alternatively, a similar medium containing 1% (w/v) glucose and solidified with 1.2% (w/v) agar was used for routine culture. Those cultures which produced significant depressions around colonies on gellan medium, indicative of gellanase production, were repeatedly subcultured onto the same medium, then onto yeast extract agar plates. Finally, gellanase activity in the pure cultures was confirmed by further plating on gellan medium plates incubated at 30 °C.

The gellanase-producing strains were then used to prepare extracellular enzymes by growing the bacteria in the semi-synthetic medium containing 0.05% gellan or in the same medium with glucose as carbon substrate. Cultures (900 ml aliquots in 2 l Erlenmeyer flasks) were grown for 5–6 d at 30 °C with shaking at about 200 r.p.m. Bacteria were removed by centrifugation at 30 000 *g* for 30 min. The clear supernatant fluid was concentrated by tangential-flow filtration (Millipore Minitan), recentrifuged and dialysed exhaustively against 10 mM Tris buffer, pH 7.5, prior to use as 'enzyme'. In culture supernatants which contained relatively large amounts of EPS, the polymer was removed by tangential-flow filtration as described by Sutherland & Macdonald (1986), although this also caused some loss of enzyme activity. Ultrasonic lysates of the bacterial cells provided sources of intracellular glycosidases. The lysates were ultracentrifuged at 110 000 *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 6 kDa), then stored at -40 °C.

Analytical procedures. Enzyme assays for lyase activity used the thiobarbituric acid (TBA) assay procedure of Weissbach & Hurwitz (1958). Assays for β -D-glucosidase, β -D-glucuronidase and other glycosidases were performed using nitrophenyl sugar derivatives as substrates (Sigma). Viscosity changes due to enzyme action were measured at 30 °C using a Brookfield LVTD instrument. Monosaccharides were identified in acid hydrolysates of the polysaccharides (0.25 M H₂SO₄ for 16 h at 100 °C) or of the oligosaccharide products of enzyme treatment, by descending paper chromatography using butan-1-ol/pyridine/water (6:4:3, by vol.) as mobile phase, then quantified by HPLC as indicated by Kennedy & Sutherland (1987). Oligosaccharides were also compared by paper electrophoresis in pyridine/acetic acid/water (1:10:100, by vol., pH 3.5) at 180 mA for 2 h.

Gellan was purchased from Schweizerhall. Native and deacetylated rhamsan gum were kindly provided by Dr S. Zeller of Kelco, Aero Drive, San Diego, CA, USA. Other related polysaccharides were either prepared in our laboratory or kindly donated by Dr J. Baird (Kelco). The commercial polysaccharides, except gellan and welan, were dissolved in distilled water, ultracentrifuged to remove particulate material, purified by extensive dialysis and lyophilized before use. Deacetylation of the polysaccharides was performed by heating 1% (w/v) 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 concentrated 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 enzyme activity was observed at pH 7.0–7.5 when tested against gellan and over a

broader pH range, 4.5–8.0, when tested against deacetylated rhamnan. The optimum temperatures were 35–40 °C.

RESULTS

Gellan degradation by bacteria

Concentrated supernatants of all five wild-type *Sphingomonas* strains, and the EPS[−] mutant Mj216, showed some degradation of gellan but did not show detectable degradation of cellulose, xanthan, sodium pectate, agarose or other polysaccharides tested using reducing sugar assays and chromatographic examination for the production of oligosaccharides or other breakdown products. Cell lysates of glucose- or gellan-grown cells of the ATCC strains did not show any degradation of gellan, deacetylated rhamnan or related polysaccharides but were strongly active against the β -D-glucoside and the β -D-glucuronide but not against other nitrophenyl glycoside substrates tested, including the α -L-rhamnoside. The lysate of strain NCIMB 11942 failed to degrade gellan but differed in that it possessed strong β -D-glucosidase activity and only weak activity against the nitrophenyl- β -D-glucuronide. On further testing for release of reducing sugar or TBA-reactive material, concentrated culture supernatants from all the bacterial strains grown in the presence or absence of gellan showed activity against acyl-free gellan and deacetylated rhamnan.

Enzyme isolation

Supernatant fluid from gellan-grown cultures was recovered after centrifugation and concentrated by tangential-flow filtration (Waters Millipore Minitan; 10 kDa molecular mass cut-off). After extensive dialysis, further concentration was achieved using polyethylene glycol (molecular mass 6 kDa). The presence of gellan lyase activity in the concentrated fluid was confirmed using the TBA method. The relatively low activities of the enzymes, and the presence of EPS in some preparations, caused some problems in the attempted partial purification of the proteins. The concentrated supernatants from strains Mj216 and NCIMB 11942 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, 68% of the lyase activity eluted at 0.4 M NaCl. The semi-purified enzymes from wild-type strains and from Mj216 and the EPS[−] mutant of NCIMB 11942 now showed much simpler profiles on PAGE and were used for further studies.

Enzyme specificity and activity

The concentrated and partially purified enzymes from all the bacteria behaved similarly but not identically; polysaccharide lyase activities were frequently very low. All preparations appeared to be highly specific, acting on

Fig. 2. Action of gellan lyase preparations from *Sphingomonas* strains Mj216 and NCIMB 11942 on deacetylated gellan and deacetylated polysaccharide S194, measured as release of TBA-reactive material. Substrate (5 ml, 1 mg ml^{−1}) was mixed with 5 ml 10 mM Tris, pH 7.0, and 5 μ l enzyme (1.25 or 1.97 mg protein ml^{−1}) and incubated at 30 °C. Aliquots (500 μ l) were removed and boiled for 5 min. The TBA assay was then performed on triplicate 100 μ l samples. ■, Enzyme from strain NCIMB 11942 acting on deacetylated gellan; +, □, enzyme from strain Mj216 acting on deacetylated gellan (+) and deacetylated S194 (□).

deacetylated gellan and the deacetylated polysaccharide from strain ATCC 31961 (Kelco polymer S194, 'rhamnan') (Fig. 2, Table 1). They generally lacked activity on other, structurally similar polysaccharides in the series, whether in the native acetylated or the deacetylated form. Nor were they active on the original acetylated polysaccharide from which gellan is derived. There was linear release of material reacting in the TBA assay from either gellan or deacetylated polysaccharide over a 10–12 h period. Thereafter, TBA-reacting products continued to be formed but at a lower rate. An exception was seen in the Mj216 preparation, which did show some activity against the batch of native S657 polysaccharide tested, although surprisingly it was inactive against the corresponding deacetylated material. The two strains used for most of the experiments were chosen because of their apparently higher activity and also because one (Mj216) was a mutant derived from a strain typical of *Sphingomonas* spp. in lacking lipopolysaccharide (ATCC 31461), while the other (NCIB 11942) was atypical and apparently possessed LPS (H. Weitz & I. W. Sutherland, unpublished results). Activity of the enzymes was slightly (10%) enhanced by the addition of 5 mM Ca²⁺. Higher concentrations were inhibitory. Other cations at various concentrations failed to cause any stimulation of enzyme activities.

Incubation of enzyme preparations from Mj216 or NCIMB 11942 with aqueous solutions of deacetylated gellan or the chemically deacetylated polysaccharide S194

Table 1. Relative activities of enzymes from *Sphingomonas* strains against gellan and related polysaccharide substrates

Substrate (1 mg ml⁻¹) in 10 mM Tris buffer, pH 7.0, was incubated with enzyme and samples were taken at 0 and 3 h for the TBA reaction.

Substrate	Enzyme source:	
	Strain Mj216	NCIMB 11942
Gellan	100	100
Native gellan	0	0
S194 (rhamsan)	2.4	0
S194 DAC (deacetylated rhamsan)	46.9	40.3
S194 DAC (deacetylated rhamsan)*	40.2	37.1
S88	5.2	0
S88 DAC (deacetylated S88)	4.7	0.5
S130 (welan)	2.7	7.0
S130 DAC (deacetylated welan)	2.4	0
S198	0.6	0
S198 DAC (deacetylated S198)	0	0
S657	10.7	0
S657 DAC (deacetylated S657)	0	1.1
11942	0	0
11942 DAC (deacetylated 11942)	0	0

* Sample prepared by Dr S. Zeller at Kelco.

Fig. 3. Effect of gellan lyase preparations from *Sphingomonas* sp. NCIMB 11942 on solution viscosity. The polysaccharide (2 ml) at a concentration of 5 mg ml⁻¹ for deacetylated S194 or 2.5 mg ml⁻¹ for deacetylated gellan, was mixed with 50 µl enzyme (1.25 or 1.97 mg protein ml⁻¹) and the viscosity was determined over a period of time in the Brookfield viscometer at 30 °C, using spindle speed 0.3. +, Deacetylated gellan; □, deacetylated S194.

(rhamsan gum) as substrates indicated loss in viscosity with time (Fig. 3). These results were mirrored by an increase with time in the release of reducing sugar and characteristic oligosaccharides from both substrates as

Fig. 4. Chromatography on Biogel P2 of diffusible products from digests of deacetylated gellan using enzyme from strain Mj216. 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, centrifuged and applied to the Biogel P2 column. The column was eluted with water as indicated in Methods and fractions collected at 12.5 min intervals. Samples (10–100 µl) were tested for carbohydrate content using the phenol/sulphuric acid assay.

followed by paper chromatography. All incubation mixtures contained some residual non-diffusible, polymeric material even after extensive enzyme treatment. A problem was encountered during attempts to use enzyme preparations from the other strains due to the presence of EPS and its contribution to the measurable viscosity values.

As well as the extracellular polysaccharide lyases, the bacterial strains under examination possessed intracellular β -D-glucosidase and β -D-glucuronidase activities. The products of exhaustive enzyme degradation of deacetylated gellan appeared to be oligosaccharides with an unsaturated uronic acid at the non-reducing terminal. Analogous products were obtained from the deacetylated polysaccharide S194, but glucose was additionally detected, presumably due to digestion of part of the side-chain residues by β -glucosidase. 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 deacetylated gellan could be separated by gel permeation chromatography on Biogel P2 into two major fractions (Fig. 4). On paper chromatography of fraction 1, there was a major slow-migrating product (R_{Glc} 0.07), which was possibly the repeat unit tetrasaccharide into which a double bond has been introduced at the non-reducing terminal uronic acid. On paper electrophoresis, this oligosaccharide had a similar mobility to other tetrasaccharides containing a single uronic acid residue. Smaller amounts of other slower-moving material may represent oligomers of this tetrasaccharide. Fraction 2 contained material of R_{Glc} 0.55 which appeared to contain equimolar amounts of D-glucose, L-rhamnose and unsaturated uronic acid, but has not yet been fully characterized. The profile on Biogel P2 of the products of enzyme digestion of deacetylated S194 resembled that from deacetylated gellan, but paper chroma-

tography of the material showed it to be considerably more complex, especially among the slower-moving products, and lacking the fastest-moving oligosaccharide. The oligosaccharides were all TBA-positive when eluted from preparative paper chromatograms. The chromatographic and electrophoretic mobilities of many of the products were identical to those of the oligosaccharides reported in our earlier study on gellan lyases (Kennedy & Sutherland, 1994). Further characterization of the oligosaccharide fragments from both substrates is in progress. Analysis of acid hydrolysates of the undigested limit material from either polymer revealed no differences from the initial substrates other than the presence of small amounts of TBA-reactive material.

DISCUSSION

Gram-negative bacteria capable of degrading deacylated gellan through the action of eliminase-type enzymes (lyases) have recently been isolated by enrichment culture (Kennedy & Sutherland, 1994). The enzymes differ from those associated with a bacterium recently reported by Mikolajczak *et al.* (1994) to degrade welan gum and to a lesser extent gellan. We now report the presence of polysaccharide lyases in several of the *Sphingomonas* spp. which are used in the commercial production of gellan and polysaccharides of related structure. Intracellular β -D-glucosidase and β -D-glucuronidase activities were also noted but α -L-rhamnosidase activity was absent, in contrast to the gellan-degrading bacteria studied earlier by us (Kennedy & Sutherland, 1994). The specificity of the enzymes from *Sphingomonas* spp. degrading deacylated gellan resembles that of the enzymes from the bacteria studied earlier, but the *Sphingomonas* enzymes show higher activity against gellan than against deacetylated rhamsan. Chromatographic analysis of the products also reveals close similarities. One surprising result was the failure of the enzymes to hydrolyse the EPS from strain NCIMB 11942 if, as suggested by Lobas *et al.* (1994), it is very similar to gellan in chemical structure as well as in physical properties. However, only the composition of this polysaccharide was reported and no structural details have been determined (Anson *et al.*, 1987); this polymer may well show sufficient structural differences to explain the lack of enzyme action.

The enzymes described here correspond to some other polysaccharide lyases, including many of those active on alginates, which have been shown to be strongly inhibited by the presence of the O-acetyl or other acyl groups present on the polymeric substrates (Davidson *et al.*, 1977; Kennedy *et al.*, 1992). O-Acetyl groups can greatly affect the ordered structure adopted by some bacterial polysaccharides in solution (e.g. Sutherland, 1994), but it is surprising that a relatively small substituent on the polysaccharides should affect susceptibility to enzyme action. This is especially true when one considers that the enzymes in our study degraded rhamsan gum, in which there is a gentibiosyl side-chain attached to the glucose residue distal to the uronic acid (Fig. 1). The ordered

structure resulting from this substituent is clearly still available for enzyme attack, provided all acyl groups have been removed. However, despite their similar structures, welan and S657 are resistant. Polymers in which substituent side-chains are on the glucosyl residue attached to the reducing terminus of the uronic acid were not substrates for the enzymes under study; this may therefore indicate that the binding or cleavage site extended to the glucose residues on either side of the glucuronic acid. Crescenzi *et al.* (1987) observed that in the conformation adopted in aqueous solutions, the short side-chains of L-mannose or L-rhamnose mask the uronic acid residues through hydrogen bonding. Lee & Chandrasekaran (1991) used X-ray and computer modelling of gellan and three structurally related polysaccharides to conclude that although all have double-helical conformations, the side-chains shielded the carboxylate groups to varying degrees. The shielding was extensive in welan and S-657 polysaccharides, but low in rhamsan. Complete removal of the side-chains would be required to leave completely exposed carboxylate groups, as is found in gellan. This might allow the enzyme to cleave at its recognition site, which in the polysaccharides rhamsan and gellan, is unsubstituted.

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 EPSs. These enzymes 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, except for xanthan lyase, which attacks the side-chains (Sutherland, 1995). In non-polysaccharide-producing bacterial species, the presence of various polysaccharide lyases enables the bacteria synthesizing them to convert various polysaccharides and the corresponding monosaccharide products to 2-keto-3-deoxy-aldonoates which can then be metabolized further to pyruvate and triose phosphate (e.g. Preiss & Ashwell, 1963).

Although *Pseudomonas aeruginosa* and other alginate-synthesizing *Pseudomonas* spp. possess alginate lyase activity, none can utilize alginate as sole carbon and energy source. A similar situation has now been demonstrated in bacteria of the genus *Sphingomonas* synthesizing gellan and structurally related polysaccharides. This raises the question of a possible role for EPS-degrading enzymes in bacterial strains which excrete EPS. Are these lyases therefore, essentially connected with polysaccharide synthesis and excretion? If so, this is not confined to the production of polysaccharide lyases. *Rhizobium* spp. capable of synthesizing the EPS succinoglycan contain a succinoglycan depolymerase gene among the genes essential for polymer biosynthesis (Glucksman *et al.*, 1993) and 1,4- β -D-endoglucanases (CM-cellulases) have recently been reported in two species of cellulose-synthesizing bacteria, in close association with the polysaccharide biosynthetic genes (Standal *et al.*, 1994; Mattysse *et al.*, 1995). On the other hand, studies on EPS-synthesizing *Escherichia coli* and related bacteria have apparently totally

failed to reveal the presence of enzymes degrading the EPSs which they synthesize (K. Jann, I. S. Roberts and C. Whitfield, personal communications). Surprisingly, no enzyme activity was detected in the cytoplasmic contents obtained from bacterial lysates. This might suggest that the role of the enzymes is in cleavage of polysaccharide chains at the surface of the cell rather than in a periplasmic or intracellular location. In such a location, the enzymes might release excess polysaccharide or cleave material covalently linked to other surface macromolecules.

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