Polysaccharide Lyases

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To my Family and Friends.

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I would like to thank Ian Sutherland, for his kindness and unlimited patience. To Hugh, for his support through some interesting but difficult moments and to all my friends who encouraged and supported me on this voyage of self discovery.

DECLARATION

I declare that this thesis has been composed by myself and that the work presented herein is my own. Any collaborative work has been duly acknowledged.

Abstract

The enzymes which cleave polysaccharides in an eliminative fashion are known as polysaccharide lyases. The eliminative cleavage caused the formation of an unsaturated uronic acid at the **non** reducing terminus, which can be detected using the thiobarbituric acid assay (TBA). The work presented here is a study of two polysaccharide lyases; one which degrades alginate (*alginate lyase*) and the other which degrades gellan (*gellan lyase*).

Alginate lyase was isolated in mucoid and non-mucoid strains of *Azotobacter vinelandii* and *Azotobacter chroococcum,* as a constitutive, intracellular lyase, which was located in the periplasm. The lyase was mannuronate specific, endo-acting producing a series of oligosaccharides. Separation by membrane filtration and anion exchange chromatography indicated that there was more than one *alginate lyase* present within the cell. When applied to an affinity column (poly-D-mannuronic acid linked to Sepharose), Av UW *alginate lyase* eluted between 36-90mM NaCl. On a Q Sepharose FF. column, both lyase activities eluted with 0.2M NaCl (step gradient). The greatest increase in purity of the Ac 184 *alginate lyase* was observed in the unbound material from the Q Sepharose FF. column.

Av UW *alginate lyase* activity was affected by the presence of cations in the assay mixture; a higher concentration of monovalent cations were required than divalent cations. The presence of chelators (EDTA and EGTA) affected lyase action, enhancing activity. With Ac 184 *alginate lyase,* Ca^{2+} had an inhibitory effect as did both chelators and Na^{+} did not enhance enzyme activity. The *alginate lyase* present in the mucoid strain of *A. vinelandii* Av Ax17.78 did not appear to be involved in encystment but may play a part during cyst germination.

The novel enzyme, *gellan lyase*, an inducible, extracellular enzyme was principally located in the spent culture medium, of red pigmented Gram negative bacteria. The gellan-degrading bacteria were isolated from soil or mud from a local environment and formed pits in gellan-solidified media. The optimum incubation conditions for the lyase were 37-40°C and pH 7.0 in 10mM TRIS for 3h. The release of reducing sugar mirrored the release of TBA products and no free glucose was detected (*glucose oxidase* assay). The enzyme was endo-acting, producing a series of oligosaccharides. Gellan, (Gelrite®) was the best substrate for *gellan lyase* but deacetylated rhamsan (S194) could also be used. The other members of the gellan family of polysaccharides were unsuitable substrates as were the acetylated forms of gellan and rhamsan.

When gellan was the substrate, divalent cations at concentrations above 1mM inhibited crude lyase activity and monovalent cations had no effect . Similar results were observed with divalent cations using the semi-purified *gellan lyase,* but monovalent cations enhanced its activity. When deacetylated rhamsan was the substrate both monovalent and divalent cations enhanced enzyme activity, but EDTA and EGTA were inhibitory.

Separation of the supernatant by membrane filtration indicated that there was more than one *gellan lyase;* one lyase had a nominal molecular mass between 50-100kDa and the other had a nominal molecular mass greater than 100kDa. After purification by anion exchange (Q Sepharose FF., Pharmacia), where the >100kDa lyase eluted between 0.35-0.4M NaCl and gel filtration chromatography (AcA34, Pharmacia), the largest protein in the >100kDa fraction had a molecular mass of 150kDa, when separated by SDS-PAGE.

The other *gellan lyases* studied were isolated from a spontaneous, non-mucoid mutant of *Sphingomonas paucimobilis* NCIB 11942 and from the non mucoid strain *S. paucimobilis* MJ216. As with the lyase isolated from the red pigmented bacteria, gellan and deacetylated rhamsan were the best substrates. The lyase was endo-acting, as shown by the steady loss of viscosity. The lyase activity also eluted from Q Sepharose FF. at 0.35M NaCl.

Abbreviations

BSA	Bovine Serum Albumin
BHB	β-hydroxybutyric acid
cAMP	cyclic adenosine 5' mono phosphate
DP	Degree of polymerisation
DAC	Deacetylated
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)
EPS	Extra cellular polysaccharide
Glc	Glucose
GlcA	Glucuronic acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HV	high viscosity
kDa	kilo Daltons
LV	Low viscosity
MES	2-[N-Morpholino]ethanesulfonic acid
NCIB	National Collection of Industrial Bacteria
nmr	nuclear magnetic resonance
PHB	poly hydroxybutyric acid
PAGE	poly acrylamide electrophoresis
SDS	sodium lauryl sulphate
TBA	Thiobarbituric Acid
TRIS	[hydroxymethyl]aminomethane
UHQ	Ultra High Quality
U.V.	ultra violet

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Chapter 1 INTRODUCTION

Polysaccharides are chains of sugar building blocks found as simple homopolymers comprised of single monosaccharides or as complex heteropolymers consisting of two or more monosaccharides. The structure of the heteropolymers can be further complicated by the addition of side chains. Polysaccharides serve a wide variety of functions in industry as thickeners, suspending or stabilisers in aqueous systems (Table 1). They can produce gels or act as emulsion stabilisers, lubricants or friction reducers. These properties allow polysaccharides to control and modify the rheological properties of aqueous systems. Thickeners include starch, guar gum, locust bean gum, xanthan, gum arabic, carboxymethyl cellulose, alginate and methyl cellulose. Polysaccharide gelling agents include starch, alginate, carrageenan and methyl cellulose. Some polysaccharides can act as either thickeners or gelling agents. Traditionally, plant and algal polysaccharides were used but in the search for novel physical properties, microbial polysaccharides have been found to replace them.

Microbial polysaccharides have regular structures which reflect their synthesis from repeating units formed at the cytoplasmic membrane and their subsequent polymerisation and excretion from the microbial cell (Ielpi *et al.* 1981, 1983, 1993). As well as alginate, cellulose and starch, an early example of an industrially produced microbial polysaccharide was xanthan gum. Xanthan was first isolated from *Xanthomonas campestris* in the 1950's by the US Department of Agriculture and has wide applications in the food industry as well as the oil industry. Alginate is used widely throughout the food industry (Table 2), as is curdlan in Japan; both have other industrial applications. One of the more recently isolated bacterial polysaccharides is gellan (Kang & Veeder 1982) from *Sphingomonas paucimobilis*

(Pollock, 1993). It and a related family of polysaccharides, have potentially wide applications in the food industry as gelling or structuring agents, as an agar substitute and in agriculture and in enhanced oil recovery.

Acidic polysaccharides are found in all environments. In plants, pectins, partially branched polysaccharides containing homopolymeric blocks of 1→4 α-linked methyl galacturonic acid in the unbranched portion of the polymer or pectate (de-esterified pectin) are important structural molecules which affect plant shape and act as a protective barrier. Alginic acid, a linear copolymer of 1→4 β-linked β-D-mannuronic acid and α-L-guluronic acid acts as an anti-desiccant and as a structural polymer in seaweed. The polysaccharide acts to prevent desiccation at low tide as well as providing rigidity or flexibility respectively to the fronds and stipes of the seaweed. In animals, acidic polysaccharides are found in the extracellular matrix in the form of chondroitin or hyaluronate. Both are similar in structure; hyaluronate is a non-sulphated glycosaminoglycan comprised of N-acetyl D-glucosamine and D-glucuronic acid. These complex acidic polysaccharides are often linked to core proteins as proteoglycans, an example of which is heparin.

The structure of acidic polysaccharides has been studied using chemically and enzymatically depolymerised polysaccharides to yield oligosaccharides. Enzymatic degradation is highly specific for the linkage cleaved, is carried out under mild conditions and is therefore preferable to chemical degradation which often requires harsh conditions and is much less specific in the linkages broken (Linhardt *et al.*, 1985).

Table 1 Industrial Applications of Polysaccharides.

Market and Applications	Properties	Polysaccharides
ADHESIVES (also see Paper)		
Latex	Rheology	Cellulosic derivatives
Tile mortars (Cement)	Fluid loss/rheology	Methylcellulose
Wallpaper	Thickening	Algin, starch, modified starch
AGRICULTURE		
Flowable pesticides	Suspension-drift control	Xanthan gum
Liquid Fertilizers	Suspension	Xanthan gum
Liquid feed supplements	Suspension	Xanthan gum, guar gum
CERAMIC, REFACTORIES, WELDING RODS	Slip agent Suspension	Algin Xanthan gum
CLEANERS, POLISHES	Abrasive, suspension acid and Base stability	Xanthan gum
DETERGENTS	Antiredeposition	Carboxymethylcellulose
EXPLOSIVES		
Ammonium nitrate slurries	Water resistance	Guar gum, xanthan gum hydroxypropyl guar gum
Package gels	$Ca(NO_3)_2$ compatibility	Guar gum hydroxypropyl guar gum xanthan gum
FIRE FIGHTING	Foam stabilization ignition retardation	Guar gum, guar derivatives and xanthan gum
INK (FLEXO, GRAVURE, JET) LITHOGRAPHY	Rheology Low-Viscosity- High-Solids	Gum arabic
METAL WORKING		
Refractory Coatings	Suspension	-
OILFIELD		
Drilling muds	Viscosity, suspensions	Xanthan gum, cellulose, ethers
Enhanced Oil Recovery	Viscosity	Xanthan gum
polymer flooding		
Fluid-loss additives	Fluid loss properties	Carboxymethylcellulose
Slimulation	Suspension, fluid loss	Hydroxypropyl guar gum
Hydraulic fracturing	viscosity reduction by chemical /enzymatic breakers crosslinkable	Hydroxyethyl cellulose Xanthan gum Carboxymethylcelluose
Acidizing	Suspension, stability to strong acids at elevated temperatures	Xanthan gum
PAINT		
Latex Trade Sales	Rheology-suspensions	Hydroxyethyl cellulose methylcellulose (microbial biopolymer)
Maintenance coatings		
Industrial coatings		
PAPER		
Wet end binder	Formation beater aid	Modified Starch, guar derivatives locust bean gum and karaya gum
Coatings	Rheology	Algin, Carboxymethylcelluose
Surface sizes	Film forming, water-loss, printing	Starch, modified starch algin
Particle board	Extend glue	
Corrugated board		
Photography	Antistatic coating extention	sodium cellulose sulfate

Sandford *et al.* (1984)

Table 2

Polysaccharides Used in the Food Industry.

	Dry foods	Canned foods	Glassed foods	Bakery products	Dairy products	Frozen foods	Salad dressings	Bever -ages	Soft drinks	Brewing	Confect -ionary	Pharma -ceuticals	Cosmetics	Detergents	Pet foods
Microbial															
Xanthan Gum	X	X	X	X	X	X	X	X			X	X	X		X
Seaweed															
Alginate	X	X	X	X	X	X	X			X	X	X	X		X
Agar						X	X				X	X	X		
Carrageenan		X			X				X		X		X		X
Plant															
Guar gum				X	X	X						X			X
Locust Bean Gum					X							X			X
Psyllium Husks												X			
Quince Seed											X				
Gum Arabic				X				X	X	X	X	X	X		
Gum Tragacanth		X	X				X		X		X	X	X		
Cellulosics															
Carboxymethyl cellulose		X	X	X	X		X	X			X	X	X	X	X
Hydroxypropyl cellulose		X		X							X				
Hydroxyethyl cellulose				X				X							
Microcrystalline cellulose	X											X	X		

Sandford et al. (1984)

Polysaccharide-Degrading Enzymes

Polysaccharide-degrading enzymes fall into two categories:

i) Polysaccharide hydrolases act on polysaccharides to give different types of oligosaccharides by cleaving the glycosidic bonds (glycosyl-oxygen bond) between the sugar residues by the addition of water.

ii) Polysaccharide lyases (eliminases) are enzymes (EC 4.2.2.-) which act on glycosidic linkages present in acidic polysaccharides containing uronic acid residues. Polysaccharide lyases cause an eliminative rather than a hydrolytic reaction resulting in the formation of unsaturated oligosaccharides, by breaking the oxygen-aglycone bond, with the loss of water. This is known as eliminative cleavage and the unsaturated uronic acid product is at the **non-reducing** terminus of the oligosaccharide (Fig. 1). This mechanism was first suggested by Linker *et al.* (1955), when an unusual unsaturated sugar formed by the action of a microbial polysaccharase acting on hyaluronic acid was isolated and characterized. The bacterial *hyaluronate lyase* produced a disaccharide, which had been modified during the cleavage process, causing the loss of one molecule of water from the uronic acid portion of the repeat unit. This resulted in the formation of a disaccharide containing a 4,5 unsaturated uronic acid - a new type of sugar acid.

Lyases are mainly of microbial origin and can be found in a wide range of prokaryotes and eukaryotes including pathogenic and non-pathogenic bacteria and fungi (Table 3). They can be classified according to the monosaccharide residue in the polymer and the nature of the glycosidic linkage which is attacked.

As well as microbial polysaccharide lyases, a number of bacteriophage-borne lyases have been isolated. Parolis *et al.* (1988) isolated a a phage-borne enzyme which could degrade the capsular polysaccharide of *Klebsiella* K14. The enzyme, present in the tail spikes of the bacteriophage, facilitates the penetration of the virus

particles into the bacterial cell. Phage-borne enzymes resemble the enzymes of microbial origin in exhibiting a high degree of substrate specificity. The depolymerisation product is often the repeat or specific unit of the capsular polysaccharide. In the case of *Klebsiella* K14, it is a hexasaccharide. A number of other phage-borne lyases have been reported for other *Klebsiella* capsular polysaccharides; these include *Klebsiella* K5 (van Dam *et al.* 1985) and K64 (Ravenscroft *et al.* 1987). Davidson *et al.* (1977) reported the properties of a phage-borne *alginate lyase* isolated from an *Azotobacter vinelandii* phage.

A number of polysaccharide lyases have been identified for the polysaccharides shown in Table 4. These include polysaccharides which range from simple repeating homopolymers, to repeating heteropolymers, to proteoglycans of complex structure. Most polysaccharide lyases cleave the repeated backbone structure but *xanthan lyase* differed from the other lyases in Table 4, since it cleaved the trisaccharide side chain at the β-D-mannosyl-D-glucuronic acid linkage. Both hydrolases and lyases exhibit a high degree of specificity towards their macromolecular substrates. When the structure of bacterial substrates resembled polymers from eukaryotes (e.g. hyaluronic acid), some of the enzymes acted on substrates from either source.

Various methods have been used to determine both types of enzyme activity, including measurement of changes in viscosity, liberation of reducing material or specific oligosaccharide products. Lyases have been studied using the absorption of U.V. light by the unsaturated uronic acid at 232nm or the production of a chromogen in the thiobarbituric acid test (Weissbach & Hurwitz, 1958).

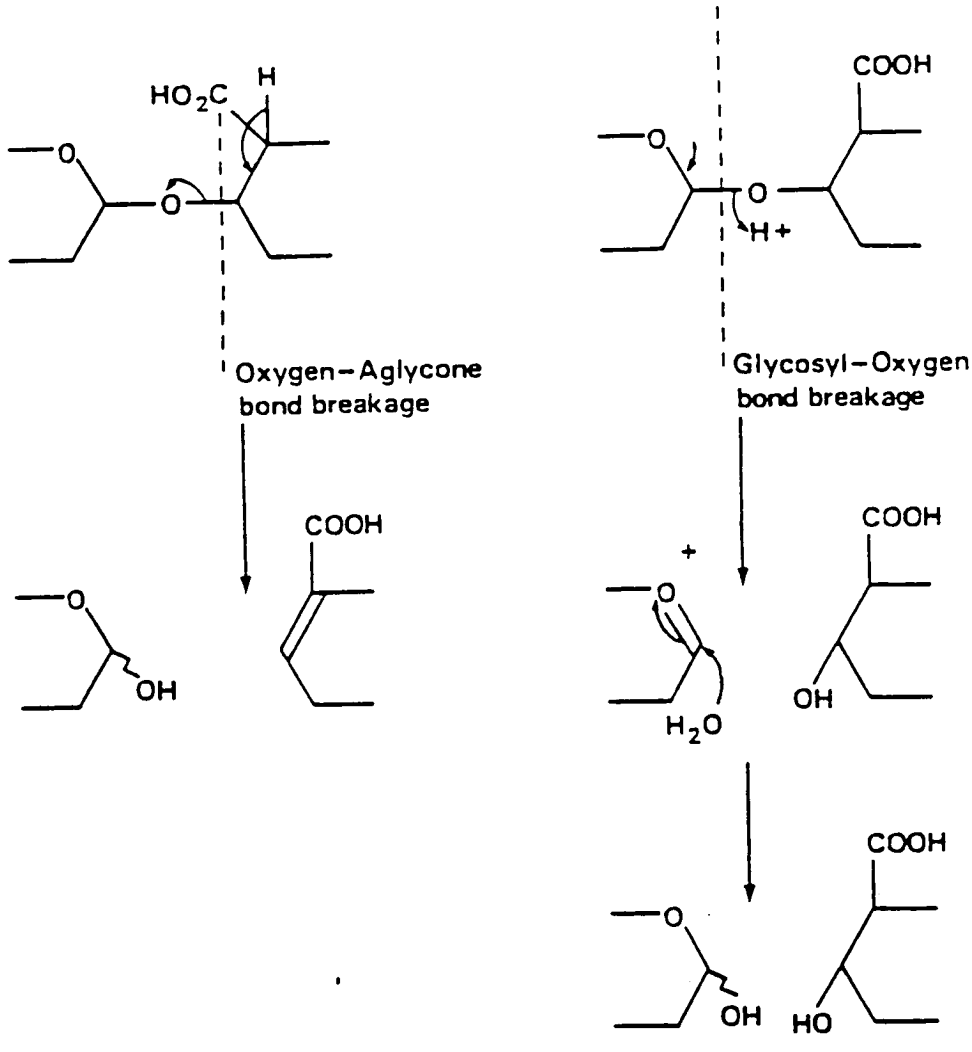
General Eliminase Mechanism

The main characteristic of lyase enzymes is the cleavage of polysaccharides containing uronic acids by the β-elimination of water, thus creating an unsaturated uronic acid at the non reducing end of the glycosidic linkage. Polysaccharides susceptible to eliminative cleavage contain a carboxylate group adjacent to the glycosidic linkage (Fig. 2). The free electron group increases the acidity of the proton on the carbon. The proton can either be removed chemically or enzymatically.

When Albersheim *et al.* (1960) chemically removed the proton from pectin with alkali, unsaturated oligosaccharides were formed. These types of chemical elimination reactions are known as concerted elimination (Gould, 1959) and it is likely that the lyases utilise these properly oriented leaving groups through a similar concerted mechanism. When the proton is removed enzymically from the polysaccharide, this may also result in direct eliminative cleavage forming a β-unsaturated uronic acid on the non reducing side and a hemiacetal group on the reducing side or the reaction may occur in a stepwise manner to produce the same end product.

Markovitz *et al.* (1959) found that tritium was not released in the spent medium from tritiated hyaluronate after enzyme treatment and suggested that bacterial *hyaluronate lyase* attacked the bond between oxygen and C4 of uronic acid resulting in a β-elimination. Ludowieg *et al.* (1961) also suggested that action of bacterial *hyaluronate lyase* resulted in the accumulation of an unsaturated disaccharide whereas testicular *hyaluronidase* resulted in the formation of a tetrasaccharide which could be further modified by the bacterial *hyaluronate lyase* to produce both an

Fig. 1 General Illustration Of Enzyme Catalysed Eliminative Polysaccharide Cleavage



Eliminative Cleavage **Hydrolytic Cleavage**

Linhardt *et al.* (1986)

Table 3 Some Eukaryotic and Prokaryotic Sources of Polysaccharide Lyases

Alginate lyase	*Azotobacter vinelandii*	Kennedy *et al.* 1992
	P .mendocina	Hacking *et al.* 1983
	P. putida	Sutherland & Keen 1981
	P.maltophilia	Sutherland & Keen 1981
	B. circulans	Hansen & Nakamura 1985
	Beneckia pelagia	Pitt & Raisbeck 1978
	Pseudomonas spp	Min *et al.* 1977
	Bacillus circulans	Larsen *et al. 1993*
	Alteromonas spp	Preston *et al.* 1985
	Haliotis spp	Nakada & Sweeny 1967
	Littorina sp	Elykova & Favorov, 1974
	Turbo cornutus	Muramatsu *et al.* 1977
	Pelvetia canaliculata	Madwick *et al.* 1973b
	Dendryphiella salina	Wainwright & Sherbrock-Cox 1981
Pectate lyase	*Erwinia carotovora*	Chatterjee *et al.* 1995
	E. chyrsanthemi	Brooks *et al.* 1990
	Xanthomonas campestris	Beaulieu *et al.* 1991
	Aspergillus niger	Bussink *et al.* 1991
	Neurospora crassa	De Lourdes *et al.* 1991
	P. fluorescens	Liao, 1991
	P. marginalis	Nikaidou *et al.* 1993
	Fusarium oxysporum	Martinez *et al.* 1991
	Streptomyces nitrosporeus	Sato & Kaji, 1980

Table 3 continued

Hyaluronate lyase	*Streptococcus dysgalactiae*	Markovitz *et al.* 1989
	Staphylococcus aureus	Greiling *et al.* 1975
	Clostridium perfringens	Ludowieg *et al.* 1961
Xanthan lyase	*Bacillus* sp	Sutherland, 1987
Chondroitin lyase	*Proteus vulgaris*	Yamagata *et al.* 1968
	P.fluorescens	Yamagata *et al.* 1968
	Bacteroides thetaiotaomicron	Yamagata *et al.* 1968
	Flavobacterium heparinum	Gu *et al.* 1993
	Arthrobacter aurescens	Gu *et al.* 1993
Heparin lyase	*Flavobacterium heparinum*	Desai *et al.* 1993
Gellan lyase	Unclassified Bacteria	Kennedy & Sutherland, 1994
	Sphingomonas paucimobilis	Kennedy (This thesis)

Table 4 Sources of Polysaccharides Lyases

Plant	**Enzyme Commission Number**
Alginate lyase	4.2.2.3
Pectin lyase	4.2.2.10
Pectate lyase	4.2.2.2
Oligogalacturonic lyase	4.2.2.6
Exopolygalacturonate lyase	4.2.2.5
Animal	
Hyaluronate lyase	4.2.2.9
Chondroitin lyase	4.2.2.4, 4.2.2.5
Heparin lyase	4.2.2.8
Heparan lyase	
Microbial (unclassified Lyases)	
Bacterial	
Alginate lyase	4.2.2.3
Emulsan	
Hyaluronate lyase	4.2.2.9
Gellan lyase	
Xanthan lyase	
Bacteriophage	
Heteropolysaccharide lyase	
Alginate lyase	
Hyaluronate lyase	
Klebsiella K14 lyase	
Klebsiella K5 lyase	
Klebsiella K64 lyase	

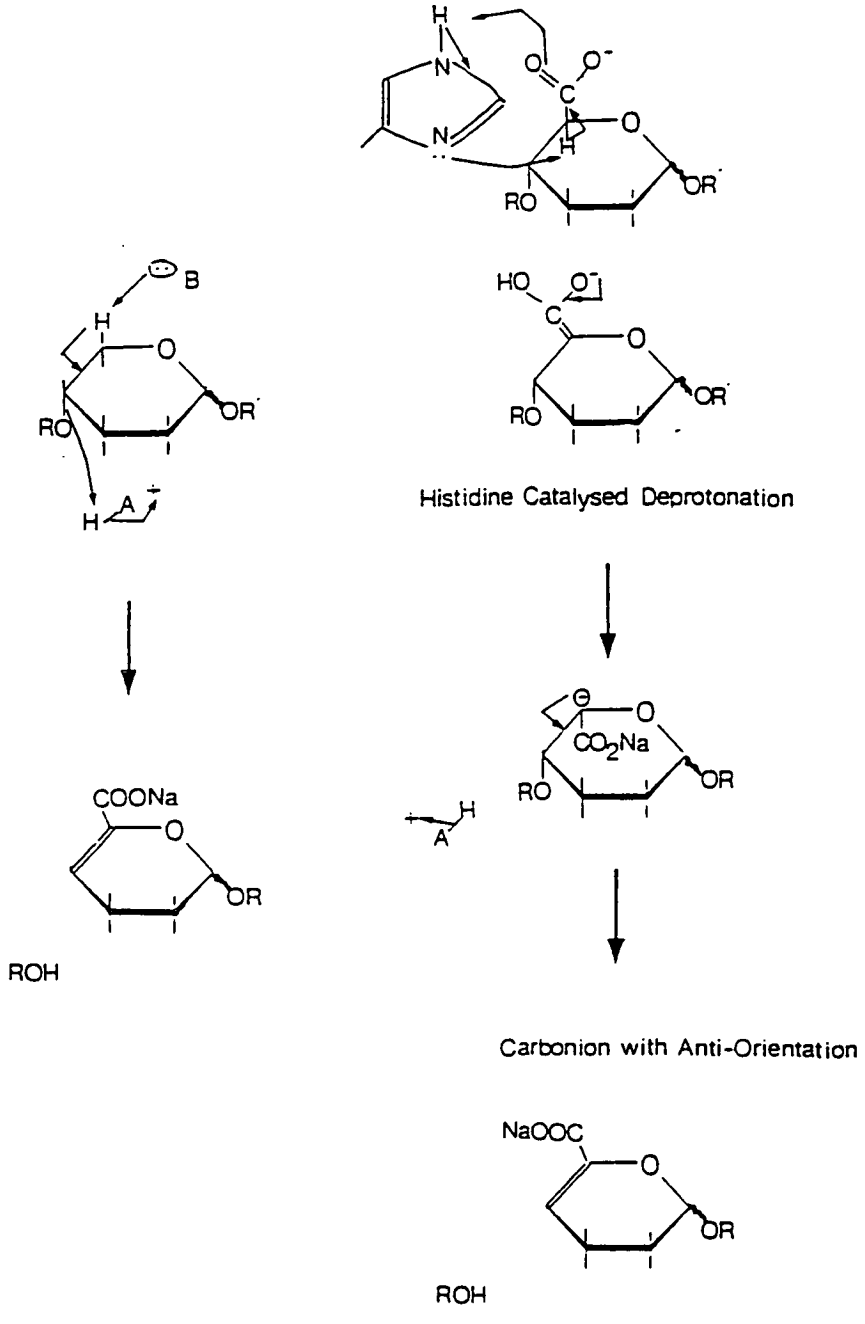
unsaturated disaccharide and a saturated disaccharide. This was due to the fact that the testicular hyaluronidase was a glycosidase which cleaved the endo-N-acetyhexosaminic bonds of hyaluronate to give a series of oligosaccharides of which a tetrasaccharide was the predominant fraction. The testicular hyaluronidase was active against chondroitin A and C as well as hyaluronate whereas the bacterial enzyme was only active on hyaluronate. Heavy water was used by Ludowieg *et al.* (1961), to determine site of action of bacterial *hyaluronate lyase*. The accumulated disaccharide was analysed for ^{18}O; negligible amounts were present. This confirmed the proposal by Markovitz *et al.* (1959) that cleavage was restricted to the bond between the C4 of the glucuronic acid to the oxygen bridge, with the accompanying loss of a proton from the C5.

The geometric relationship between the C4 (aglycone-oxygen) carbon-oxygen bond and the C5 carbon hydrogen bond is significant. The bonds can be diaxial (in an anti relationship), as in pectin or pectate, chondroitin B and some linkages in heparin and alginate. Alternatively the bonds can be in an axial-equatorial relationship as in hyaluronate, chondroitin A & C and certain linkages in heparan and alginate. When the monosaccharides within a polysaccharide have bonds which are in the axial-equatorial relationship, direct eliminative cleavage does not occur chemically since the internal energy is low because the bonds are staggered. The energy required to remove the C5 proton cannot be met by concerted cleavage of the C4 carbon-oxygen bond because of the incorrect orientation.

Greiling *et al.* (1975) demonstrated the involvement of histidine in the catalytic activity of *hyaluronate lyase* and proposed its involvement in the elimination of the axial-equatorial-related leaving groups in hyaluronate. The predominant amino acids in *Staphylococcus aureus hyaluronate lyase* are lysine and histidine (Rautela & Abramson, 1973). The role of histidine was demonstrated using the site specific reagents N-Tosyl-L-phenylalanine-Chloromethyl Ketone (TPCK) and N-A-P-Tosyl-

L-lysine-Chloromethyl Ketone (TLCK). The decrease in the level of lyase activity was attributed to the fact that TPCK and TLCK reacted with histidine residue and therefore only half of the histidine residues were available to the lyase. Histidine would supply the energy to convert the glucuronate into the tautomeric carbeniate structure which, by splitting the glycosamidic bond and formation of a double bond between C4 and C5, could then be transformed to the more stable oxoniate structure. Thus histidine would act as both proton acceptor and proton donor. The uronate may also exist as a tautomeric carbeniate formed by the C5 proton shifting to the carboxylate group. Tautomerisation to carbanion with an *anti* orientation to the C4 carbon-oxygen bond would allow its elimination. The involvement of a carbanion intermediate in the axial-equatorial elimination mechanism is substantiated by the analogous conversion of glucuronide to iduronide residue in heparin biosynthesis by a mammalian C5 epimerase (Jacobsson *et al.*, 1978). When tritiated water was incorporated into the C5 position of the resulting iduronide residue, it suggested that epimerisation may proceed via a mechanism similar to axial-equatorial elimination involving a carbanion intermediate. In epimerisation, the intermediate collapses with the breakage of the C4 carbon-oxygen bond.

Fig. 2 Two Types Of Eliminative Cleavage Anti-Concerted and Stepwise



Anti-Concerted Elimination (Chemical Elimination)

Stepwise Elimination (Enzymatic Elimination)

Linhardt *et al.* (1986)

General Properties of Polysaccharide Lyases

Lyases appear to be mainly enzymes of low to intermediate molecular mass (20-100kDa) and are monomeric giving the same value when measured under reducing and non-reducing conditions. The eukaryotic *chondroitin, heparin, hyaluronate* and *pectate lyases* are all glycoproteins. Most lyases are cationic with the exception of *pectate lyase* from *Erwinia chrysanthemi* (Stack *et al.*, 1980;) and *Streptococcus nitrosporeus* (Sato & Kaji, 1980). Most polysaccharide lyases have activity optima above pH 6; *hyaluronate lyase* has the lowest (4) and *pectate lyase* has the highest (9.5) pH optimum. Most lyases act exolytically and have no minimum size requirement for their substrate. *Pectate lyase* has an absolute requirement for Ca^{2+} ions, but other lyases are affected to a greater or lesser extent by the presence and concentration of divalent cations. The effect of divalent cations can be explained in two ways:

1) the Ca^{2+} is required as a cofactor;

2) the Ca^{2+} binds to the acidic polysaccharide to enhance or reduce the action of the enzyme.

The different nature of the two eliminative mechanisms would not allow the lyase to act on substrates exhibiting both diaxial and axial-equatorial orientation. The exceptions are *chondroitin lyase A, B* and *C*, which are larger than most lyases, having molecular mass greater than 100kDa. As they are monomeric, their larger size suggests the presence of multiple catalytic domains.

Lyase-Producing Organisms and their Characteristics

Organisms which produce polysaccharide lyases ought to have access to their polysaccharide substrate or smaller molecules having the correct linkages

(Gooday,1979). The organisms can be subdivided into pathogenic and non-pathogenic bacteria and have distinct biological properties associated with each group. Plant and animal pathogenic bacteria produce extracellular constitutive lyases which are not under catabolite regulation. Isoenzymes are produced capable of acting in a concerted fashion (Leaback & Walker, 1973) on the acidic polysaccharide substrate under a variety of pH conditions and are often accompanied by corresponding hydrolases (Gooday, 1979). *Erwinia chrysanthemi* cause soft rot disease in most plants. The bacteria produce several pectin degrading enzymes, including *exo-poly-α-D-galacturonidase (hydrolyase)* and *exopolygalacturonic lyase (pectate lyase)* of which there can be up to five isozymes (PelA, B, C, D, E). *Pectate lyase* cleaved the internal α-1,4-galacturonosyl linkages (Collmer & Keen, 1986).

Non-pathogenic bacteria (Buchanan & Gibbon, 1974) include soil isolates, isolates from infected animal or plant material or from the gut or rumen. High concentrations of substrate are available to these isolates in all such locations indicating that the bacteria use their lyase to release carbon for growth rather than for invading tissue. Non-pathogenic bacteria grow better on their polysaccharide substrate than pathogenic bacteria and produce an intracellular lyase which is often located in the periplasmic space of Gram negative cells. This enables the bacteria to utilise the oligosaccharide products rather than secreting them into the environment. Multiple lyases are also produced, each acting on closely related polysaccharides or oligosaccharides.

Other reported sources of polysaccharide lyases include fungal lyases which act endolytically but are limited to two higher classes of fungi and are involved in the depolymerisation of plant polysaccharides (Gooday, 1979). The bacteriophage which infects *Streptococcus* spp. produces a *hyaluronate lyase* (Benecherit *et al.*, 1978).

An *alginate lyase* is produced by the bacteriophage 5 which infects *A. vinelandii* (Davidson *et al.*, 1977). Hollingsworth *et al.* (1984) isolated a phage-borne heteropolysaccharide lyase which degrades the polymer of *Rhizobium trifolii* Strain 4S to yield an octasaccharide product. A number of bacteriophage have been isolated which degrade the polysaccharide produced by *Klebsiella* sp. (Parolis *et al.*, 1985; van Dam *et al.*, 1985; Ravenscroft, 1987). The isolation of a hepatopancreatic *alginate lyase* from *Abalone* revealed the presence of such enzymes in marine gastropods (Nakada & Sweeny, 1967).

An explanation is needed as to why examples of eliminative cleavage rather than hydrolysis of polysaccharides occurs in some bacteria. The unsaturated oligosaccharide residues created during eliminative cleavage are Michael receptors and are subject to nucleophilic attack (House, 1971). This may cause a toxic reaction in higher animals and additional enzymes would be required to obtain metabolites capable of entering the carbon pool (Chatterjee *et al.*, 1985). These enzymes would have to be constitutive to prevent the build up of toxic unsaturated sugars. Bacterial eliminative cleavage may be preferable to hydrolysis, since few bacteria grow on acidic polysaccharides as the sole carbon source. The inability of higher organisms to metabolise unsaturated sugars (Warnick & Linker, 1972) may thus reduce competition for this carbon source. Plants recognise unsaturated sugars as foreign and mount a defence which causes the plant to produce low molecular weight antimicrobial agents called phytoalexins (Davis *et al.*, 1984). Bacteria which produce *hyaluronate lyases* are often animal pathogens. Hyaluronate is poorly antigenic (Ingham *et al.*, 1984). Antibodies have been raised to the unsaturated carbohydrate products of this and other lyases (Caterson *et al.*, 1985).

Both chemical and enzymic eliminative cleavage are base catalysed, whereas hydrolysis occurs under acidic conditions. Bacteria able to ferment the acidicpolysaccharide increased the alkalinity of the medium and therefore favoured eliminative cleavage (Hsu & Vaughn, 1969). *Pectate lyases* have their pH optima around 8.8 whereas *pectate hydrolyases* have optima at pH 4.5. Cleavage of acidic polysaccharide by a lyase may also have a lower energy pathway than that of hydrolases. Hydrolytic enzymes must accommodate both the water molecule and the acidic polysaccharide within the active site. The highly ordered association between the acidic polysaccharide and water may interfere with its addition to the glycosidic linkage.

ALGINATE

Originally, alginate was extracted from brown seaweeds (Phytophaceae) in which it constitutes the main carbohydrate and represents 10-30% total carbohydrate. On the west coast of America alginate is isolated from *Macrocystis* spp.; in northern Europe it is isolated from *Laminaria* spp and *Ascophyllum* spp. These alginates are isolated from natural beds; only in China has the cultivation of seaweed for the alginate industry occurred. Large bundles of stipes grow out of holdfasts which anchor the algae to the rocks. From the stipes grow the fronds. Alginate can be isolated from different parts of the plant but the proportion of uronic acid residues varies with the age of the plant, the season and also the part of the plant from which it was isolated (Table 5) (Haug *et al.*, 1974).

Alginate is a linear heteropolymer comprised of β 1→4 linked β-D-mannuronic acid and its C5 epimer α-L-guluronic acid (Fig. 3). Hirst *et al.* (1964) demonstrated the presence of both uronic acid residues by partial acid hydrolysis. Haug *et al.* (1966,

1967a, b) also used mild acid hydrolysis to describe three types of polymeric sequences: poly-β-D-mannuronic acid (poly M); poly-α-L-guluronic acid (poly G); and poly MG which contained both types of uronic acid. The two uronic acids are structurally similar but adopt different chair conformations, so that the carboxyl group is in the equatorial position (Fig. 3). In brown algae the main skeletal and structural polysaccharide is cellulose, a β1→4 glucan organised in crystalline microfibrils. The cell wall component of brown algae is in the form of a framework of cellulose microfibrils embedded in a 3-dimensional continuous alginate network consisting of calcium-bridged poly G blocks and entangled poly M chains.

The shape of the poly M chain is similar to that found in other β 1→4 linked hexoses. The mannuronic acid is in the C1 conformation and is therefore di-equatorially linked. Poly M is a flat ribbon-like molecule (Fig. 4). The conformation is stabilised by the formation of an intramolecular hydrogen bond between the hydroxy group on C_3 of one unit and the ring oxygen atom C_5 of the next sugar unit (Atkins *et al.*, 1971). The chains are bonded into sheets by means of hydrogen bonds formed between the hydroxyl group of the carboxyl group and the oxygen atom on carbon 3 in the sugar unit parallel chains and between the axial hydroxyl group on the carbon 2 and the oxygen atom of the sugar group in the anti-parallel chains.

The shape of poly G is different to that of poly M; poly G is a buckled chain in which the guluronic acid is in the ^1C conformation and is therefore diaxially linked. The chain conformation is stabilised by an intra-molecular hydrogen bond between the hydroxyl group on carbon 2 and the oxygen atom of the carboxyl group in the adjacent units. The inter-chain bonds are more complicated than those found in poly M and involve water molecules. Each water molecule is in such a position that it functions twice as a hydrogen bond donor and twice as an acceptor (Atkins *et al.*,

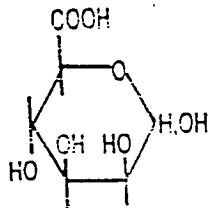
1971). These observations confirmed the prediction of Rees (1972) that the poly M regions will form an extended "ribbon" structure comparable to cellulose, whereas poly G would form a buckled chain. Further confirmation was obtained by X-ray diffraction analysis of the partial hydrolysis products of alginate (Atkins *et al.*, 1973a, b), by ^{1}H-n.m.r. (Penman & Sanderson, 1972) and by ^{13}C-n.m.r. (Grasdalen *et al.*, 1977).

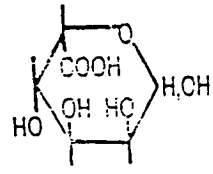
Table 5 Concentration of Homopolymeric Sequences in Alginate.

	% D-Man	% L-Gul	Reference
Macrocystis pyrifera (Sigma)			
High Viscosity	45.3	54.7	Krull & Cote, 1992
Low Viscosity	78.6	21.4	Krull & Cote, 1992
Fucus sp intracellular	PolyM		Larsen & Haug, 1971
Ascophyllum nodosum intracellular	PolyM		Larsen & Haug, 1971

	% PolyM	% PolyG	% Mixed	
A. nodosum	38	20	41	Morris *et al.*, 1980
Laminaria hyperborea	20	49	30	Morris *et al.*, 1980
L. hyperborea stipes	18	58	22	Morris *et al.*, 1980
M. pyrifera	40	41	18	Morris *et al.*, 1980

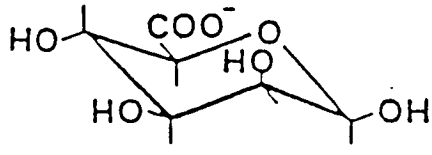
Fig. 3 The Structure of β-D-Mannuronic and α-L-Guluronic Acid.





β-D-Mannuronic Acid

α-L-Guluronic Acid



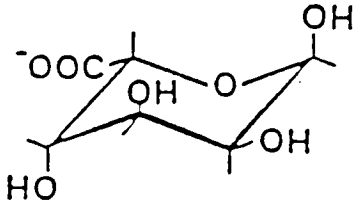
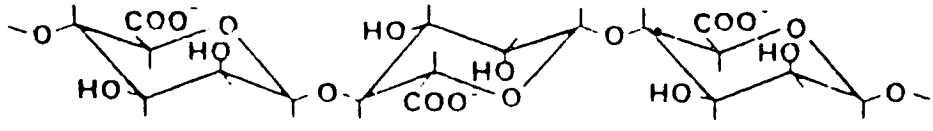
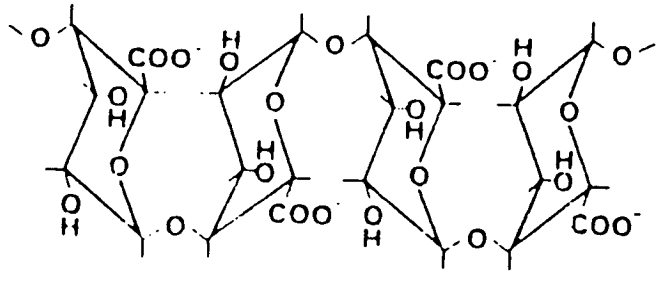


Fig 4. The Chain Conformation of Poly M and Poly G.



Poly M



Poly G

Gelation of Alginate

One of the most industrially important properties of alginate is its ability to form thermo-stable gels in the presence of divalent cations, especially Ca^{2+} and Sr^{2+}. The physical properties of the gel depend on the ratio of uronic acids within the polymer chains. The gel resembles a solid in that it retains a shape, resists stress and consists of 100% water. A gel is defined as a system which owes its characteristic properties to a cross linked network of polymer chains which form at the gel point (Hermans, 1949). Originally, it was suggested that the cross linking was caused by simple ionic bridging of two carboxyl groups on adjacent polymer chains with Ca^{2+} or by chelation of single Ca^{2+} by OH^- or CO_3^- groups on polymer chains. Although these bonds may play a role in gelation they are not energetically sufficient to account for gelation of alginate (Rees, 1969). Circular dichroism studies have shown that the Ca^{2+} reacts preferentially with poly G residues (Morris *et al.*, 1973). Poly MG does not play a direct role in the gelation process except to join the associated segments and provide a 3-dimensional network. The interaction of the poly G with Ca^{2+} fits the "egg box" model described by Grant *et al.* (1973).

The ratio of D-mannuronate to L-guluronate (M:G) can be used to predict the types of gel formed in the presence of divalent cations. Regions rich in D-mannuronate form weak, flexible gels (*e.g.* the alginate isolated from *Macrocystis pyrifera*), whereas regions rich in L-guluronate form rigid brittle gels (Penman & Sanderson, 1972; Rees, 1972) (e.g. alginate isolated from *Laminaria hyperborea*). Rigid, brittle gels have a tendency to undergo syneresis, whereas the elastic gels have less of a tendency to water loss.

The M:G ratio of alginate depended on the algal origins; alginate preparations from *Phaeophyceae* have a ratio ranging from 0.4-1.8 depending on the part of plant

from which it was isolated. *A. vinelandii* (Haug & Larsen, 1974) and *P. aeruginosa* (Evans & Linker, 1973) produce alginates with a M:G ratio greater than 3. This variation causes the physiochemical properties of bacterial alginate to be different from algal alginate. Haug & Larsen (1971), reported that the M:G ratio in alginate was influenced by the Ca^{2+} concentration of the growth medium for *A. vinelandii* strain E. They proposed that Ca^{2+} enhanced the action of the C5 epimerase which converted mannuronate to guluronate and facilitated the action of the *alginate lyases*. Obika *et al.* (1993) also demonstrated that the range of M:G ratio changed dramatically depending on the concentration of Ca^{2+} in the growth medium and suggested that the M:G ratio was a function of the Ca^{2+} concentration in the medium.

Several bacterial sources of alginate have been found. Originally, *Azotobacter vinelandii* (Gorin & Spencer, 1966) and *Pseudomonas aeruginosa* (Linker & Jones, 1966) were reported as producing alginate-like polysaccharides. Later the presence of alginate in cultures of *P. aeruginosa* was reported by Evans & Linker (1973); Linker & Evans (1984) and Dunne & Buckmire (1985). Govan *et al.* (1981) isolated alginate from strains of *Pseudomonas fluorescens, Pseudomonas putida* and *Pseudomonas mendocina* grown on media containing carbenicillin. Both Fett *et al.* (1986) and Osman *et al.* (1986) isolated alginate from the plant pathogen *Pseudomonas syringae* pathovar *glycinea* and indicated that the polysaccharide was an essential factor in the infection process.

A. vinelandii produces alginate as exopolysaccharide but also as a structural component in cyst formation (Sadoff 1975). The cysts act as a resting stage and allow the bacterium to survive under adverse environmental conditions. *A. vinelandii* strains are usually mucoid when isolated and remain so under laboratory conditions.

Some non- mucoid strains, incapable of encystment, have been isolated (Sutherland 1972).

Strains of *P. aeruginosa* do not normally produce alginate unless they have been isolated from sputum samples from cystic fibrosis (CF) patients. The secretion of mannuronate-rich alginate by mucoid strains of *P. aeruginosa* has been correlated with their relative virulence as opportunistic pathogens associated with CF (Pederson *et al.*, 1989). Hacking *et al.* (1983) produced a stable mucoid strain of *P. mendocina* after successive treatments with chemical mutagens.

The structure of bacterial alginate may be the same as or similar to algal alginate but there are also important differences. Bacterial alginates are normally *O*-acetylated on the D-mannuronate residues (Davidson *et al.*, 1977; Skjåk-Bræk *et al.*, 1985). The degree of acetylation depends on the amount of D-mannuronate present in the polymer (Skjåk-Bræk *et al.*, 1986). Sherbrock-Cox *et al.* (1984) showed that some mannuronic acid residues may be di-*O*-acetylated and ^{1}H-nmr confirmed that the D-mannuronate may be substituted in either the O-2 or O-3 position with occasional O-2,3-di-substitution (Skjåk-Bræk *et al.*, 1986). The function of the O-acetyl groups was to prevent the conversion of the mannuronate residues into guluronate residues by a mannuronan C5 epimerase (Skjåk-Bræk *et al.*, 1986). Since acetylation of the polymer is an intracellular process and epimerisation extracellular, the bacterium was able to control the composition and physical properties of the exopolysaccharide. The function of O-acetyl groups in *Pseudomonas* spp. was less well defined due to lack of information about their distribution and about the epimerisation system in these bacteria.

Alginate from *A. vinelandii* (Gorin & Spencer, 1966) closely resembles that of algae since the guluronic acid residues are arranged into blocks of varying lengths

(Larsen & Haug, 1971). In contrast, the alginate produced by *Pseudomonas spp* **never** contains poly G blocks; some species produce polymers which contain only poly M; others produce alginate with both mannuronic acid and guluronic acid residues. The guluronic acid residues are always flanked by mannuronic acid residues in such *Pseudomonas* alginates.

Biosynthesis of Alginate

Lin & Hassid (1966a, b) first described a putative pathway for alginate synthesis for the alga *Fucus gardneri*, based on measurable enzyme activities and the isolation and identification of several nucleotide sugars. In 1975, Pindar and Bucke, proposed a similar pathway for *A. vinelandii* (Fig. 5) and Banerjee *et al.* (1983) described a pathway for *P. aeruginosa*. Although the basic pathway was established there was controversy concerning the routing of the intermediates into the pathway and in the final stages of modification, particularly of the D-mannuronate residues. Banerjee *et al.* (1983) were unable to detect phosphomannose isomerase (PMI) or phosphomannomutase activity but both enzymes are required in the putative pathway for alginate biosynthesis in mucoid *P. aeruginosa*. They demonstrated the involvement of enzymes involved in the Entner-Doudoroff pathway in the synthesis of alginate from glucose or gluconate and that glyceraldehyde-3-phosphate was the precursor for the biosynthesis of the polymer. Lynn & Sokatch (1984) demonstrated the extensive incorporation of C-6 specifically labelled glucose in *P. aeruginosa* and *A. vinelandii*. Only a small amount of C-1 and C-2 glucose was incorporated, suggesting that the synthesis of uronic acid monomers occurred preferentially from C-4, C-5 and C-6 glucose by condensation of triose phosphates. Darzins *et al.* (1985) used a cloned gene encoding PMI activity to restore alginate synthesis in an alginate minus mutant, showing that PMI activity was essential for alginate synthesis. In bacteria, the epimerisation of D-mannuronate to L-guluronate occurs as a post-polymerisation modification. The initial polymeric product is poly M which is subsequently modified to produce mature alginate. In *A. vinelandii* (Skjåk-Bræk & Larsen, 1985) and *P. aeruginosa* (Piggott *et al.*, 1981), this is carried out by an extracellular mannuronan-C5-epimerase. The enzyme catalysed a Ca^{2+}-dependent

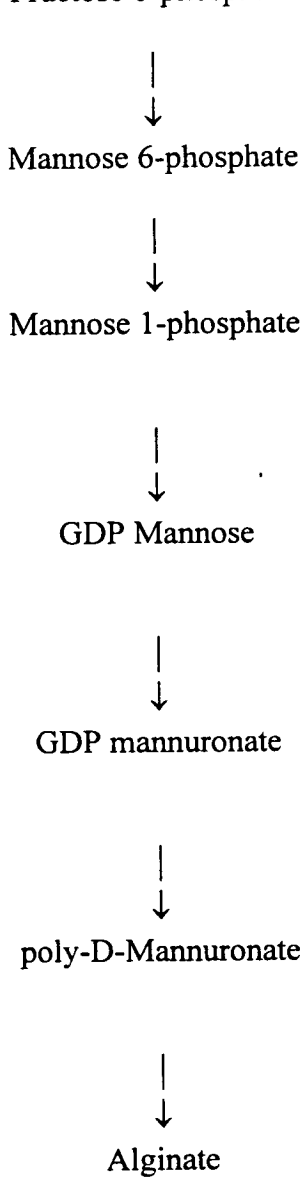
conversion of mannuronic acid to guluronic acid at the polymer level. The epimerase from *A. vinelandii* was unable to catalyse the reverse reaction and convert L-guluronate to D-mannuronate. A similar mechanism has been demonstrated in brown seaweed but the step at which this occurs is less clear. Lin & Hassid (1966b) isolated GDP-L-guluronate in cell-free extracts of *F. gardneri* and proposed that epimerization occurred at the nucleotide level and that both uronic acids were incorporated into alginate during polymerisation. Madgwick *et al.* (1973b) isolated a soluble C5 epimerase in the brown alga *Pelvetia canaliculata*. The carbazole test was used to determine the conversion of β-D-mannuronate (in the absence of borate) to α -L-guluronate, which has a higher extinction coefficient under the same conditions. The change in the ratio of extinction coefficients was proportional to the conversion of residues. Paper electrophoresis confirmed this, L-guluronate was present in the hydrolysed polymer, after 5h there was four times more guluronate in the sample than at the start. Ishikawa & Nisizawa (1981) were able to isolate a C5 epimerase from five species within three Orders of brown algae; *Eisenia bicyclis, Spatoglossum pacificum, Ishige okamurai, I. sinicola* and *Hizikia fusiformis.* The carbazole test was used to determine epimerase activity in all five algal species. Epimerase activity was higher (five times) in the growing part of the frond than in the older part of the frond. It therefore appeared that C5 epimerase, like that isolated in bacteria was present in a wide range of algae.

Quillet & de Lestang-Bremond (1985) proposed that GDP-L-guluronate is synthesised directly from D-sorbitol 6-phosphate via GDP-L-gulose. D[U-^{14}C]-sorbitol 6-phosphate was injected into the tips of young, growing *Sargassum muticum*; this was converted into radioactive L-guluronate within a few hours. The

alginate is then synthesised from GDP-mannuronate and GDP-guluronate and the polymer level epimerase is able to fine tune the structure of the polysaccharide

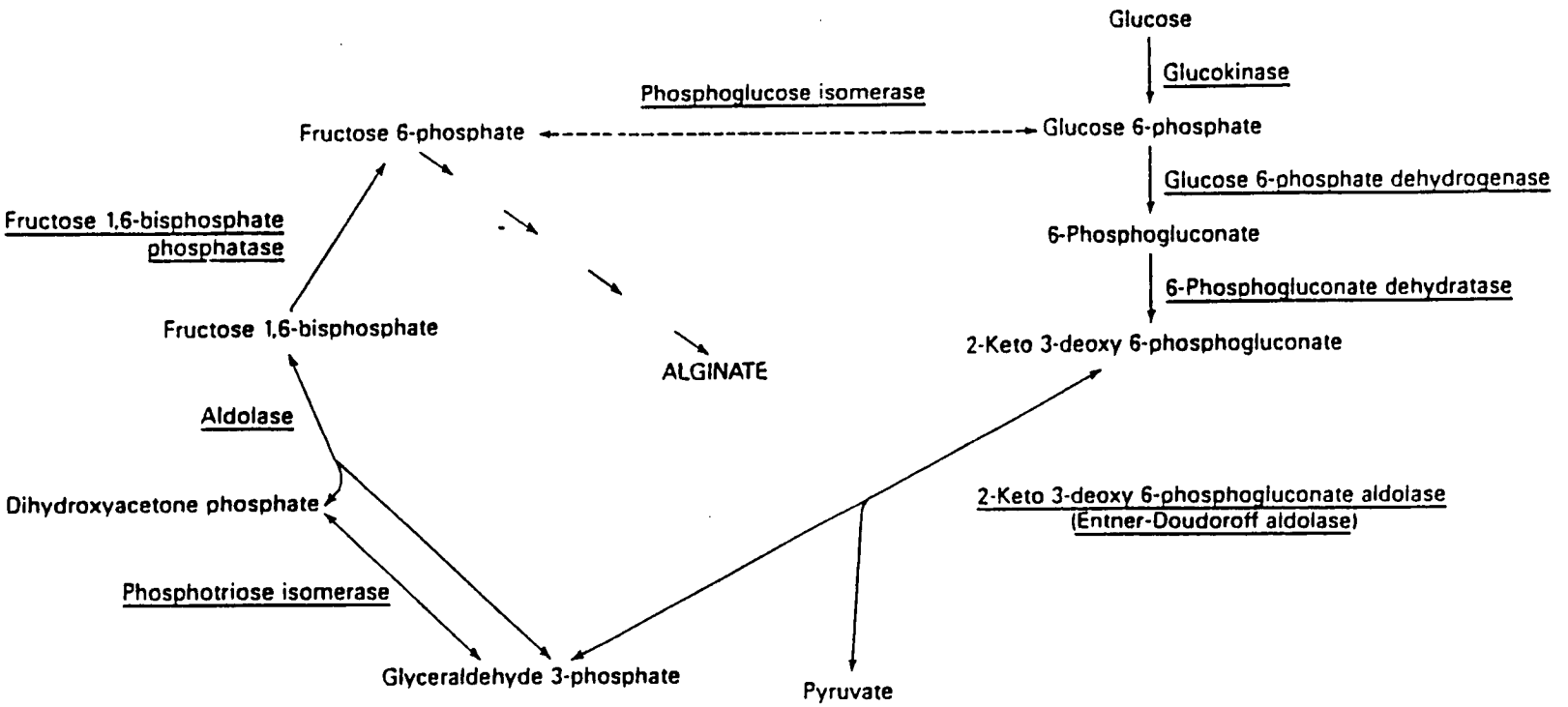
Pindar and Bucke's pathway was based on the detection of enzymes in *A. vinelandii*. Sucrose was converted to glucose and fructose by invertase. After phosphorylation, the hexoses were converted to mannose 6-phosphate by the appropriate hexose 6-phosphate isomerase. Mannose 6-phosphate is converted to mannose 1-phosphate by phosphomannomutase and then to GDP-mannose. Poly M is produced by the subsequent sequential action of GDP-mannose dehydrogenase and alginate polymerase. The extracellular C5-epimerase selectively epimerises mannuronate to guluronate. This assumes the conversion of the carbon source directly to fructose-6-phosphate and no involvement of three-carbon intermediates. This did not correlate with radiotracer data, in which the specific activity of the alginate produced by *A. vinelandii* and *Pseudomonas* spp. was greater than the precursor (Lynn & Sokatch, 1978; Wingender *et al.*, 1985; Anderson *et al.*, 1987). It has been estimated that approximately 80-90% of the glucose is channelled into alginate via the Entner-Doudoroff pathway (Fig. 6), and the remainder by the pathway proposed by Pindar and Bucke (1975). When glucose is metabolized via the Entner-Doudoroff pathway, only the 4, 5, and 6 carbons of the precursor are available for alginate synthesis because of the action of *2-keto-3-deoxy-6-phosphogluconate aldolase*. The rest of the molecule is lost to yield pyruvate. Therefore, when glucose is used as a carbon source, no more than 50% conversion to alginate can be expected. When D-fructose is phosphorylated directly and is not metabolized via the Entner-Doudoroff pathway, about 90% of the carbon source can be converted to alginate.

Fig. 5 Pathway For Alginate Biosynthesis



(Pindar & Bucke, 1975)

Fig. 6 Metabolism of Glucose via the Entner Doudoroff Pathway (solid lines). The dotted line Indicates the Route of Conversion of Glucose into Alginate.



Banerjee *et al.* 1983

Regulation Of Alginate Biosynthesis

Deavin (1976) studied the production of alginate by *A. vinelandii* in batch culture and found that the yield of alginate was dependent on the initial concentration of phosphate. Later (Horan *et al.* 1981), examined the effect of both phosphate and carbon source on alginate production and showed that when phosphate was exhausted, alginate production increased markedly. However, when sucrose was the carbon source, alginate was produced at a low rate during exponential growth. Jarman (1979) suggested that control of alginate biosynthesis might be via *phosphomannose isomerase* (PMI).

The alginate produced by the opportunistic pathogen *P. aeruginosa* has been widely studied. There have been a number of reviews describing the regulation of alginate biosynthesis in *P. aeruginosa* (see May *et al.*, 1991; May & Chakrabarty, 1994). The appearance of mucoid alginate in the sputum samples of CF patients is due to environmental factors including the prolonged antibiotic therapy. The alginate is an important virulence factor in pulmonary infections of CF patients by *P. aeruginosa*. The non-mucoid pathogen quickly undergoes a series of adaptive changes and becomes mucoid, producing large amounts of alginate which has anti-phagocytic properties (Schwarzmann & Boring, 1971), acts as a physical barrier to macrophages (Simpson *et al.*, 1988), and blocks immunodeterminants for opsonic antibodies (Baltimore & Mitchell, 1980; Marrie *et al.*, 1979). The production of viscous alginate in the CF lung may cause obstruction of the airways and interfere with the natural mechanisms of clearing the lungs (Govan & Harris, 1986). Alginate can also act as an adhesin to the cilia of the epithelial cells of the respiratory tract (Baker & Marcus, 1982; Ramphal *et al.*, 1987). The adherence of *P. aeruginosa* strains is proportional to the amount of alginate coating them (Geers & Baker, 1987),

which suggested that the volume of alginate produced by *P. aeruginosa* played an important role in infection.

In vitro, the mucoid *P. aeruginosa* reverted to the non-mucoid phenotype. This caused problems when the biosynthetic pathway and the genes which control it were studied; however, Darzins & Chakrabarty (1984) chemically mutagenised a non-mucoid strain to produce a stable alginate-producing strain.

The loci of the genes which regulated alginate biosynthesis and controlled expression have been demonstrated (Fig. 7). The genes cluster at three regions of the chromosome, at 34min (alginate biosynthetic region), at 9min (a regulatory region) and at 67-69min (mutations conducive to alginate over-production and mucoid phenotype (*muc*)). The *muc* genes were responsible for the primary control of alginate expression (Fyfe & Govan, 1980). Flynn & Ohman (1988) isolated two closely linked genes *alg*S and *alg*T within the *muc* region. The cloned *alg*S gene complemented the spontaneous *alg*S mutants only when integrated into the chromosome (i.e. *cis* complementing), whereas a plasmid-borne *alg*T gene was able to complement *alg*T mutants (i.e. *trans* complementing). *alg*S was thought to be the genetic switch for induction of *P. aeruginosa* alginate in the CF-affected lung. *alg*N prevented *trans* complementation by *alg*T and was a negative regulator of alginate biosynthesis.

Phosphomannoseisomerase-guanosinediphosphomannose pyrophosphorylase (PMI-GMP) is a bifunctional enzyme encoded by the *alg*A gene which catalyses the first and third steps of alginate biosynthesis. PMI is favoured in the forward direction and is essential for alginate biosynthesis as it traps fructose-6-phosphate from the metabolic pool. The reverse reaction to GMP is favoured when GTP is necessary.

May *et al.* (1994) identified three important catalytic regions of the PMI-GMP protein:

i). Arg19 and Lys20 assist in the binding of GTP bringing the neighbouring glycine-rich region closer to the nucleotide binding site;

ii). Lys 175 is essential for binding the GMP substrate mannose-1-phosphate;

iii). Proteolytic cleavage of PMI-GMP showed that the carboxyl terminus is essential for PMI activity but not for GMP activity.

algA mapped to the 20kb alginate cluster at 34min. Darzins *et al.* (1985) demonstrated that there was no DNA sequence homology between the *P. aeruginosa* *algA* gene and the *E. coli manA* gene at nucleotide level; however DNA-DNA hybridisation revealed homology of *P. aeruginosa* *algA* gene with other *Pseudomonas* spp. and with *A. vinelandii*. Within the biosynthetic cluster at 34min there are a number of genes which modify alginate structure. These include *algF*, *algG* and most importantly *algL*. Bacterial alginates are different from algal alginates in that they are *O*-acetylated. The presence of acetyl groups increases the water-binding capacity of the alginate. This may be of significance to mucoid *P. aeruginosa* within the dehydrated environment of the CF infected lung. *algF* gene has been shown to code for an alginate acetylase (Shinabarger *et al.*, 1993; Franklin & Ohman, 1993). The AlgF protein is not membrane-bound but is found in the periplasm; since acetyl CoA cannot cross the cytoplasmic membrane, it is unlikely to be the substrate for the epimerase.

algG is involved in the epimerisation process (Chitnis & Ohman, 1990). *algG* encodes a periplasmic epimerase enzyme which introduces guluronic acid residues into the acetylated polymannuronate chain. Franklin *et al.* (1994) sequenced the *algG* gene. The polymerase enzymes were present in very low levels even in *P. aeruginosa*

strains that produced large amounts of polymer. The *alg*8, *alg*44, a*lg*77 and *alg*60 genes were found at the biosynthetic cluster at 34min and probably encode the proteins responsible for the polymerisation and export of the polymer from the cell. *alg*8 was sequenced and its product identified as a very hydrophobic 55kDa polypeptide. This indicated that it could be membrane bound. The Alg8 protein has not been demonstrated in maxicell analysis or by hyperproduction of the protein. *alg*44 encoded a 41kDa polypeptide which also was very hydrophobic. Hyperexpression of the gene from the *tac* promoter resulted in the appearance of a 41kDa membrane-bound protein. The enzymes involved in polymerisation of bacterial polysaccharides have normally been located in the cell membrane; since the *alg*8 and *alg* 44 gene products are membrane bound proteins, they could be involved in the process of alginate polymerisation.

*alg*L encodes the periplasmic *alginate lyase* and is controlled by the alginate regulatory gene *alg*B. Schiller *et al.* (1993), reported that *alg*L lay between *alg*G and *alg*A within the biosynthetic cluster at 35min on the *P. aeruginosa* chromosome. The DNA sequence predicted the protein would have a molecular weight of 41kDa and would include a 27 hydrophobic amino acid signal sequence, indicating a periplasmic protein. When AlgL was expressed in *E. coli,* it had a molecular mass of 39 kDa. The *alg*L gene may also be transcriptionally regulated since lyase activity appears well into stationary phase, long after alginate transcripts are produced. Both Schiller *et al.* (1993) and Murata et al. (1993) reported that AlgL was regulated by osmolarity. High salt concentrations that stimulated *alginate lyase* also stimulated *alg*D and alginate biosynthesis (Schiller *et al.*, 1993). Berry *et al.* (1989) suggested that AlgL and alginate biosynthesis could be co-regulated. Goldberg & Ohman (1987) showed that *alg*B mutants produced less alginate and less *alginate lyase* than in mucoid

strains of the *P. aeruginosa* again suggesting that both were co-regulated. Wozniak & Ohman, (1991) showed that AlgB was a positive transcriptional regulator of *alg*D, the first gene in the biosynthetic cluster.

Why should a gene which degrades the polymer be found within the biosynthetic gene cluster? May & Chakrabarty (1994) reiterated the fact that *P. aeruginosa* cannot grow on its own polymer as sole carbon source. The lyase is thus of limited use to the bacteria and the following possible roles were suggested:

i). AlgL protein might act as an editing protein to control the length of the polymer or to provide short oligosaccharide chains that prime the polymerisation reaction. *alg*L mutants are still mucoid and therefore AlgL is not essential for alginate biosynthesis;

ii). The role of AlgL in the CF lung may be in the detachment of the biofilm to allow extended colonisation of the lung. The lyase may allow the alginate to be released from the cell surface to provide and extend the protective layer around the infecting bacteria.

*alg*B mutants produced less alginate than mucoid strains but more than non-mucoid strains (Goldberg & Ohman, 1987), which suggested that *alg*B was a regulatory gene rather than a structural gene for alginate biosynthesis. Goldberg & Dahnke (1992) reported the nucleotide sequence, the presence of a single ORF and a gene product of 50kDa. The ORF was similar to the regulatory component of a family of prokaryotic regulatory proteins including NtrC from *Rhizobium meliloti*, in a number of conserved regions (Szeto *et al.*, 1987).

These "two-component regulatory systems" were involved in the adaptation or repression of diverse genes to environmental or cellular signals (Nixon *et al.*, 1986). Goldberg & Dahnke (1992) demonstrated that *alg*B activated the transcription of

algD gene and that *algB* was transcribed at higher levels in mucoid strains than in non-mucoid strains and that increased expression of these genes was responsible for the increased production of alginate. *algC* encoded the enzyme *phosphomannomutase*. The *algC* promoter contains an σ_{54} (RpoN) recognition sequence for *RNA polymerase* binding. *algC* is transcribed in both mucoid and non-mucoid strains of *P. aeruginosa*.

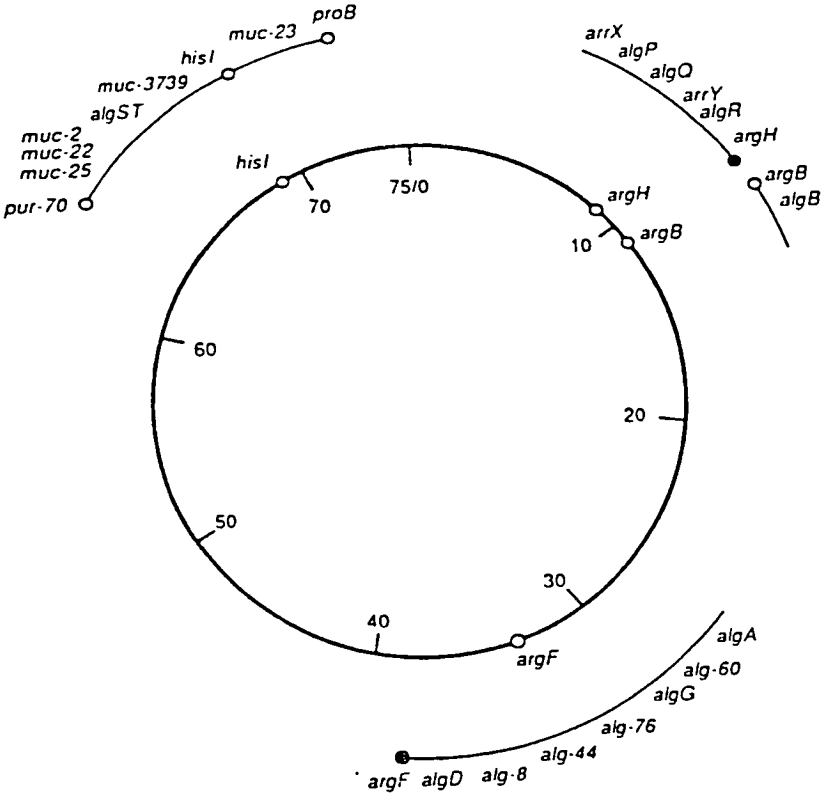
algD encoded *GDP mannose dehydrogenase* (Deretic *et al.*, 1987a) and is the key enzyme of the alginate biosynthetic pathway. It catalysed the double oxidation step of GDP-mannose to GDP-mannuronic acid. The overproduction of this enzyme may channel sugar intermediates into alginate production (Deretic *et al.*, 1987b; Roychoudhury *et al.*, 1989). The *algD* nucleotide sequence contained direct repeats which extended 500 base pairs upstream of the translation start site (Deretic *et al.*, 1987). The *algD* promoter also contained a σ_{54} recognition sequence for *RNA polymerase* binding. The transcription of *algD* gene appeared to be under environmental activation and controlled by at least two regulatory genes *algR* and *algQ* (May *et al.*, 1994). *algR* gene was found to be homologous with a class of transcriptional regulators which respond to environmental stimuli (Deretic *et al.*, 1989). *algQ* functioned as an attenuator of alginate biosynthesis by acting co-operatively with *algR*. *algQ* induced the non-mucoid strain to switch back to mucoidy. *algP* gene product was a highly basic regulatory protein. The predicted amino acid sequence was similar to histone H1 protein from the sea urchin, *Lytechinus pictus*. The proposed role of AlgP histone-like protein was to activate the *algD* promoter by controlling the amount of supercoiling, since supercoiling is necessary for activation of the *algD* promoter. The *alg76* gene nucleotide sequence was determined and transcription of *alg76* appeared to initiate from its own

promoter. This promoter sequence was similar to the *alg*D promoter, suggesting that the *alg*76 promoter was controlled and regulated in the same manner. The Alg76 protein (54kDa) was hyperproduced from the *tac* promoter and contained a 33 amino acid sequence which was cleaved during export of the protein from the cytoplasm. The protein was located in the periplasmic space but was tightly associated with the cytoplasmic membrane. The function of the Alg76 protein has not been determined but it may be involved in polymerisation or export of the polymer or in both processes.

*alg*60 encoded a 55kDa polypeptide, the hyperexpression of which resulted in a 3.5 fold increase in *GDP mannose dehydrogenase* (GMD) activity. This suggested that Alg60 either directly regulated GMD expression or catalysed the biosynthetic step which used a feedback inhibitor of GMD enzyme activity as substrate. Hyperproduction of the Alg60 protein inhibited the mucoid mode of growth.

Fig. 7 Genetic Organisation Of The Loci Involved

Expression of Mucoidy in *P. aeruginosa.*



Alginate biosynthetic region at 34min, transcriptional regulation at 9min and the *muc* locus located at 67-69min.

Deretic *et al.*, 1991

ALGINATE LYASES

Enzymes which depolymerize alginate have been obtained from a variety of organisms including marine molluscs, *Littorina* sp. (Elyakova & Favorov, 1974), from the mid gut of Wreath shell (*Turbo cornutus*) (Muramatsu *et al.*, 1977) and hepatopancreas of abalone (*Haliotis rufescens* and *H. corrugata*) (Nakada & Sweeny, 1967), echinoderms (Epply & Lasker, 1959), bacteria (Kashiwabara *et al.*, 1969; Preiss & Ashwell, 1962), brown algae (Madgwick *et al.*, 1973a, b; Shirawa *et al.*, 1975) and aquatic fungi (Wainwright & Sherbrock-Cox, 1981).

Eppley & Lasker (1959) reported the presence of *alginase* (*alginate lyases*) in the sea urchin *Strongylocentrotus purpuratus*, after measuring the change in substrate viscosity. As enzyme activity was detected in both the gut wall and in the contents of the intestine and the pH optima of both sources were different, they proposed that two enzymes were present. The enzyme from the gut wall had an optimum of pH 4.0 and the enzyme from the intestine contents had a pH optimum of 7.2-7.3. They suggested that one enzyme, isolated from the bacteria within the intestine, was responsible for the degradation of the algal blades; the other enzyme present in the gut wall aided this digestion.

Bacterial *alginate lyases* have been isolated from several members of the alginate-producing *Pseudomonas* genus, including *P. mendocina* (Hacking *et al.*, 1983), *P. putida* and *P maltophilia* (Sutherland & Keen, 1981). *A. vinelandii* also produces an *alginate lyase* (Jarman, 1979). *Alginate lyases* have also been isolated from other non-alginate-producing bacteria including, *Beneckea pelagia* (Pitt & Raisbeck, 1978), *Alteromonas* sp. (Preston *et al.*, 1985), *Bacillus circulans* (Hansen & Nakamura, 1985) and a group of unclassified bacterial isolates from the receptacles of brown algae (Doubet & Quatrano, 1982).

Table 6, (Sutherland, 1995) shows many of the wide range of bacterial alginate lyases isolated and their properties. *Alginate lyases* are specific for the types of polymer blocks and uronic acid residues which they cleave. Generally, *alginate lyases* are defined as *mannuronate lyases*, unable to cleave poly G or as *guluronate lyases*, inactive against poly M (Østgaard *et al.*, 1993). More accurately they should be defined as poly M lyases or as poly G lyases depending on the type of block the enzyme is able to degrade (Haugen *et al.*, 1990). These terms do not identify the actual bond cleaved as there are four types of bonds, M-M, G-G, M-G and G-M. Haugen *et al.* (1990) demonstrated that the extracellular *guluronate lyase* from *K. pneumoniae* attacked the G-G linkages in alginates with high G content, *L. hyperborea* alginate (73.5% guluronate) and mainly G-M linkages in *L. digitata* (60% mannuronate) alginate. Also fragments with a degree of polymerisation (DP) < 10 produced by the *K. pneumoniae* enzyme had a G at the reducing end whereas with *Haliotis* (*mannuronate lyase*) enzyme, both M and G residues were detected at reducing termini. *Alginate lyases* isolated from marine molluscs appear generally to be endo-enzymes acting on mannuronate residues, whereas bacterial *alginate lyases* cleave guluronate residues.

All the enzymes so far reported which act on alginate, are lyases (EC 4.2.2.3) which cleave the glycosidic bond by elimination, and cause the formation of an unsaturated acid, 4-deoxy-L-*erythro*-L-hex-5-ulosuronic acid at the non-reducing end of the oligosaccharide (Fig. 8). *Alginate lyase* can be produced constitutively as in *Pseudomonas* spp. (Jarman, 1979) or *A. vinelandii* (Kennedy *et al.*, 1992) or induced by the presence of alginate in the growth medium as is the case for non-alginate-synthesising micro-organisms.

An intracellular lyase produced by *P. mendocina* is capable of acting on its own polysaccharide, to reduce the viscosity of the fermentation broth (Hacking *et al.*, 1983). This is one of the limiting factors in the manufacture of bacterial alginate by fermentation. *A. vinelandii* also produced an intracellular *alginate lyase* but, as with the *Pseudomonas* sp., *A. vinelandii* cannot use alginate as the sole carbon source for growth (Hacking *et al.*, 1983). *B. pelagia* produces an inducible, extracellular lyase (Pitt & Raisbeck, 1978) and is capable of growth on alginate as the sole carbon source. A strain of *Klebsiella pneumoniae* also produces an inducible, extracellular guluronate-specific *alginate lyase* (Boyd & Turvey, 1977). Østgaard *et al.* (1993) showed that *K. pneumoniae* lyase had an absolute requirement for Ca^{2+}. Aerotolerant and pathogenic bacterial isolates from *Sargassum* tissue, classified as *Alteromonas* spp., produce cell-associated and extracellular *alginate lyases* when grown on alginic acid (Preston *et al.*, 1985). Several unclassified marine isolates produce both extracellular and cell-associated enzymes when induced on alginate (Doubet & Quatrano, 1982).

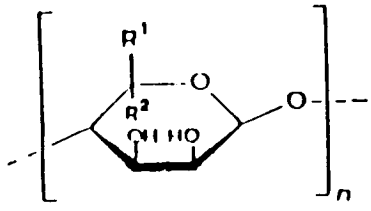
Table 6

The Location and Properties of Some Alginate lyases

Source	M_r	Location	Specificity	Ion effects	Reference
Azotobacter vinelandii	-	Periplasm	polyM		Kennedy *et al.* (1992)
Azotobacter vinelandii phage	30-35kDa	Phage and lysate	polyM		Davidson *et al.* (1977)
Azotobacter chroococcum	-	Periplasm	polyM		Kennedy *et al.* (1992)
Bacillus circulans 1351	34kDa	Extracellular	polyM/polyG	Ca^{2+} on polyM	Larsen *et al.*(1991)
Bacillus circulans JBH2	40kDa	Extracellular	polyM		Hansen *et al.* (1984)
Beneckea pelagia		Extracellular	polyG		Sutherland & Keen (1981)
Klebsiella aerogenes type 25	28kDa	Extracellular	polyM/G		Boyd & Turvey (1977,1978); Caswell *et al.* (1989)
Pseudomonas aeruginosa	40.89kDa	Periplasmic	?polyM		Boyd *et al.* (1993)
Pseudomonas aeruginosa	-	Periplasmic	polyM	'High salts'	Linker & Evans (1984)
Pseudomonas sp.		Intracellular	polyG		Min *et al.* (1977)
Pseudomonas sp.		Intracellular	polyG (DP 15)		Min *et al.* (1977)
Pseudomonas sp. OS-ALG-9	46.36kDa	Intracellular	polyM		Maki *et al.* (1993)
Pseudomonas maltophilia		Intracellular	polyM(acetylated)		Sutherland & Keen (1981)
Vibrio harveyi AL128	57kDa	Extracellular	polyG	Na^{+} (300-1000 mM)	Kitamikado *et al.* (1992)
Vibrio alginolyticus ATCC17749	47kDa	Extracellular	polyM	Ca^{2+} (5-10 mM)	Kitamikado *et al.* (1992)
Marine pseudomonad	50kDa	Extracellular	polyG		Davidson *et al.* (1976)
Species unknown	25kDa	Intracellular	? polyM (non-acetylated)		
Species unknown	40kDa	Extracellular	polyG (non-acetylated)		Takeshita *et al.* (1993)
Species unknown (Marine)	38kDa	Extracellular	polyG (non-acetylated)	Na^{+}(500mM)	Brown & Preston (1991)

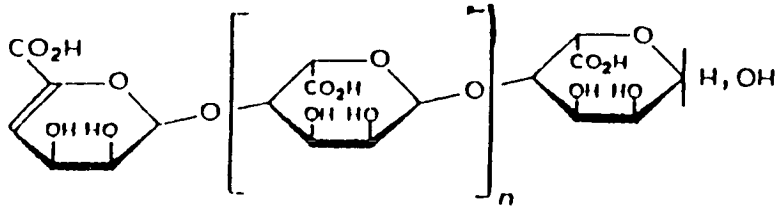
Sutherland 1995

Fig. 8 The formation of unsaturated uronic acid.

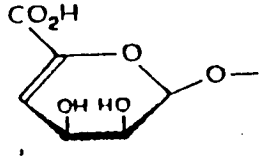


1. R1 = COOH, R2 = H

2. R1 = H, R2 = COOH



3. n = 0, 1, 2



4. Unsaturated residue at the non reducing end

Isolation and Purification of *Alginate lyase*

Several *alginate lyases*, from a number of sources, have been either partially purified or purified to homogeneity. Preiss & Ashwell (1962) isolated an *alginate lyase* from *Pseudomonas* sp. using streptomycin precipitation followed by $(NH_4)_2SO_4$ precipitation and application to a DEAE-cellulose column. The *alginate lyase* from *Pseudomonas* sp. No.9 was partially purified by gel permeation chromatography after cell disruption. Two lyase fractions were obtained with different substrate specificities, one guluronate-specific (SP1) the other mannuronate-specific, (SP2) and markedly different molecular weights. The SP1 was 38kDa and SP2 was 25kDa (Min *et al.*, 1977a, b). The higher-molecular weight enzyme was more thermostable. Both enzymes exhibited endolytic action and were partially inhibited by EDTA. They acted on poly G or poly MG residues and had very little activity on poly M.

An *alginate lyase* from the aqueous extract of the mollusc hepatopancreas (*Littorina*) was purified to homogeneity by $(NH_4)_2SO_4$ fractionation and ion exchange chromatography. During purification it appeared that up to six *alginate lyase* isoenzymes may be present in the crude extract, the most basic of which had a mass of 40kDa and acted endolytically on poly M (Elykova & Favorov, 1977). Two lyases isolated from abalone had different isoelectric points and different substrate specificity after partial purification. The extracellular lyase from *K. aerogenes* acted mainly on poly G. The intracellular enzymes of the marine bacteria acted mainly on poly M; the extracellular enzyme acts preferentially on poly G and both acted on MG segments. The extracellular lyase from *Alteromonas* sp. is endolytic and acts preferentially on poly G and the intercellular enzyme acts on either poly M or poly G.

The constitutive, endo-acting guluronate-specific *alginate lyase* from *P. alginovora* eluted from a DEAE Sepharose ion-exchange column as a single peak and when run on a SDS PAGE, a single band was observed with a mass of 28kDa. This was within the range of molecular mass of *alginate lyases* found by Doubet & Quatrano (1984) of 35kDa and 29kDa by Romeo & Preston (1986). Boyen *et al.* (1990) found that the addition of 5% glycerol was essential to maintain enzyme activity and that divalent cations were required to stabilise the enzyme in dilute solutions. The enzyme was stable between pH 6.0 and 7.8 and was inactive below pH 5.5; the optimum activity was at pH 7.5.

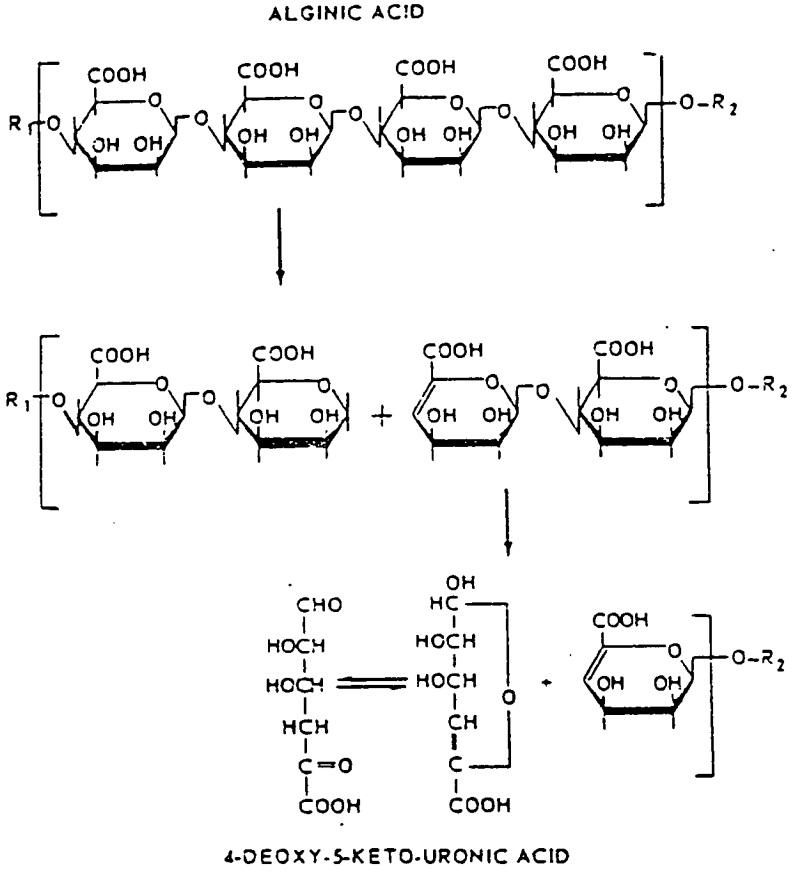
Yonemoto *et al.* (1991) isolated an organism (A1) from a ditch in a paddy field and later identified as *Flavobacterium* sp. (Murata *et al.* 1993), which produced three *alginate lyases*; these were identified as A1-I, A1-II and A1-III, when grown on alginate as the sole carbon source. Initially they were identified as A1-I, A1-II-1 and A1-II-2, since A1-I and A1-II-1 were essentially the same (both had a mass of 60kDa) (Yonemoto *et al.* 1993) but were different from A1-II-2. A1-II-2 was re-designated A1-II. All the lyases were monomeric and most active between pH 7.5-8.5 and at 70°C. A1-I had a molecular mass of 60kDa, A1-II (25kDa) and A1-III (38kDa). A1-I can act on both algal and bacterial alginate (Yonemoto *et al.* 1991), A1-II was only active on algal alginate (Hisano *et al.* 1993a) and A1-III was highly active against *O*-acetylated bacterial alginate (Hisano *et al.*, 1993b).

The mechanism of *alginate lyase* action

Preiss & Ashwell (1962) first suggested a tentative mechanism for the enzymatic degradation of alginate (Fig. 9). The polymer was cleaved by an endo-acting alginase and resulted in the formation of a series of oligosaccharides

containing unsaturated uronic acid on the non-reducing end of the chain. The unsaturated oligosaccharides were further degraded to form 4-deoxy-L-*erythro*-hexosulose uronic acid. Feingold & Bentley (1987) postulated that the initial step of the epimerisation would require the abstraction of the C5 proton by a nucleophilic group of the enzyme to form a carbanion; the carbanion could regain a proton either with retention or inversion of configuration. Gacesa (1987) proposed an extension of the carbanion mechanism, to the lyase enzymes. For the lyase, two amino acid residues, AA1 and AA2 are postulated. AA1 neutralised the carboxylate ion of the uronate residue; AA2 catalysed the removal (abstraction) of the C5 proton forming an enolate carbanion which may be stabilised by resonance which is followed by the β-elimination of the C4. The same carbanion was formed in the epimerase reaction, but is discharged using another amino acid residue (AA3) to return the hydrogen at the C5 with inversion of configuration. β-D-mannuronate assumes the $^4C_1$ conformation and α-L-guluronate adopts the $^1C_4$ conformation. The unsaturated product produced by the action of lyase, four atoms, O, C5, C4 and C3 must be in the same plane as the molecule assuming one of the two possible half chair conformations either $^2H_1$ as is the case of β-D-mannuronate or the $^1H_2$ conformation of α-L-guluronate. For a *mannuronate lyase* product the $^2H_1$ conformation is advantageous, since the equatorial alignment for the sugar residues avoids the diaxial interaction for the oxygen atoms at C1 and C4 in the $^1H_2$ conformation.

Fig. 9 Enzymatic Degradation of Alginate.



Preiss & Ashwell, 1962

The effect of divalent and monovalent cations on *alginate lyase* activity.

Boyen *et al.* (1990) showed that the *alginate lyase* activity from *P. alginovora* was enhanced in the presence of either monovalent or divalent cations. A higher concentration of monovalent (60mM K^+ or Na^+) than divalent cation (30-40mM Mg^{2+}) was required. Stokke *et al.* (1993) reported that for alginate beads gelled with divalent cations, the polymer chains which leached out of Ca^{2+} and Sr^{2+} beads had a lower guluronate content compared to the starting material, triads of guluronate were practically absent in the leached out Sr^{2+} gel beads and a significant loss of triads was observed in the Ca^{2+} gel beads. The industrial applications of alginate gels are diverse; the formation of gel under mild conditions has led to its use in biotechnology and biomedicine to encapsulate or immobilise enzymes and living cells (Smidsrød & Skjåk-Bræk, 1990). Soon-Shiong *et al.* (1992) used implanted immobilised Langerhans Islets to reverse diabetes mellitus on spontaneously diabetic dogs up to 8 months old.

Applications of *alginate lyase*

Alginate lyases can be used in conjunction with cellulases to produce protoplasts from marine algae. Butler *et al.* (1989) determined the experimental conditions required to produce a high yield of protoplasts from *Laminaria* sp. Their isolation involved the chelation of Ca^{2+} by EGTA followed by the incubation of tissue in the presence of commercial cellulases and a *mannuronate lyase* from *Haliotis tuberculata*. The purified *guluronate lyase* from *P. alginovora* (Boyen *et al.*, 1990) was as effective as the abalone *alginate lyases* in releasing large numbers of protoplasts from *Laminaria saccharina* or *L. digitata*. The highest yield of protoplasts was released in the presence of both types of lyase. *Alginate lyases* have

also been used to study the structure of alginate (Boyd & Turvey, 1978; Romeo & Preston, 1986).

The alginate produced in the CF lung makes treatment of the infection more difficult. The normal treatment for bacterial infections is antibiotic therapy. The mucoid nature of the polymer prevents the antibiotic from eradicating the bacterium and only contains the infection. Therefore novel methods of eradicating the mucoid form of the bacterium must be sought. One approach could be use of an alginate-degrading enzyme in conjunction with antibiotic therapy. This combined treatment would remove the alginate from the surface of the bacterium and in turn make it more susceptible to the antibiotic. The *alginate lyase* must therefore be able to degrade the *O*-acetylated alginate produced by *P. aeruginosa* within the CF lung.

Alginates of low viscosity and molecular weight are used within the food industry to alter consistency and texture. Murata *et al.* (1992), used the flocculated cells of bacterial strain A-1 (Yonemoto *et al.* 1991), in a bioreactor system. When A-1 was grown on alginate as the sole carbon source the cells flocculate and these cells were packed into a column.

The cloning of *alginate lyase*

Although information has been accumulated about the purification and properties of *alginate lyase,* little has been published on the molecular aspects of *alginate lyase*, in comparison with the numerous reports on *pectin* and *pectate lyases.* Caswell *et al.* (1989) first cloned the *alginate lyase* gene from *K. pneumoniae*, using the cosmid vector pMMB33 and transduced it into *E. coli. K. pneumoniae* contains a cryptic plasmid (PG1) but the *alginate lyase* gene (*aly*) was not encoded on it (Gacesa *et al.*, 1987). A genomic library was prepared by digesting the DNA with

*Sau*3A and ligating the fragments into the *Bam*H1 site of a cosmid vector. Four clones were obtained, which expressed *alginate lyase* in *E. coli*. However all were unstable due to the loss of insert DNA. DNA from one clone PG1A was isolated and purified on $CsCl_2$ gradient and transformed into *E. coli*. After repeated purification and retransformed with cosmid DNA from the *aly* expressing clones (pSP1), the sub-clone pRC5 was obtained. They contained a 12kb insert and the location of the *alginate lyase* gene was determined using Tγδ mutagenesis. Eight lyase-positive and seven lyase-negative transconjugants were used to analyse the restriction digest fragment patterns. The clustering of the lyase-negative Tγδ inserts suggested that the *aly* gene was contained within a 1.95kb *Hind*III fragment of the pSP1 cosmid. Further subcloning showed that when the 1.95kb fragment was shot gun cloned to the *Lac*Z gene of plasmid vector pHG327 and transformed with JM107, only the clones containing the 1.95kb fragment were lyase positive. In *K. pneumoniae,* most of the lyase activity was secreted into the growth medium, when alginate was present in the medium. However, lyase activity was also detected in both the periplasmic and the cytoplasmic fractions of the cell. The *alginate lyase* from *E. coli* (pSP1) was produced constitutively and had a similar location pattern to *K. pneumoniae*, suggesting that *E. coli* recognised the lyase as an extracellular protein and excreted it into the growth medium. The enzyme from the *K. pneumoniae* and the *E. coli* transformants had a mass of 28kDa and a pI of 8.9. Isoelectric focusing showed the presence of some other minor protein bands derived from a single gene as a result post-translational processing. Gacesa & Goldberg (1992) used the 1.95kb fragment located on pRC5 (Caswell *et al.*, 1989) to introduce the *aly* into mucoid and non-mucoid strains of *P. aeruginosa. Alginate lyase* was expressed and had the same molecular weight, pI and substrate specificity as that found in *K. pneumoniae.*

Expression of *alginate lyase* altered the morphology of the mucoid strains, the amount of polymer produced was lower as was its molecular weight than the control. Baron *et al.* (1994) sequenced the *aly* gene from *K. pneumoniae.* The product was a 32kDa protein with a pH 7.6 optimum.

Brown *et al.* (1991) cloned the *alxM* gene encoding an extracellular D-mannuronic acid-specific *alginate lyase* from a marine bacterium isolated as an epiphyte on the brown alga *Sargassum fluitans*. One isolate, SFFB 080483 Alg A (ATCC 433367), secreted a homogenous, endolytic mannuronic acid-specific *alginate lyase* (Romeo & Preston, 1986). This bacterium was used to construct a genomic library using *Sau*3A-digested chromosomal DNA and ligated into the dephosphorylated *Bam*H1 site of pUC18, transformed into *E. coli* TC4 and ampicillin-resistant colonies selected. Twelve transformants were able to express the *alginate lyase*; pUC18 could not depolymerise alginate. The most active transformant pAL-A3 was used to construct a restriction map. The plasmid contained a 4.1kb insert; the origin of the insert was confirmed by Southern blotting. The pattern of hybridisation to the chromosomal digest was in keeping with a single copy of the gene in the parent strain. As with the parental strain, the *alginate lyase* from the transformed *E. coli* strain was specific for D-mannuronic acid. The enzyme was unable to act on alginate rich in poly G but was more active against poly MG alginate. The level of *alginate lyase* produced by the transformed *E. coli* was significantly higher in the periplasmic fraction than that in the parent strain.

Malissard *et al.* (1993) sequenced pAL-A3. *Alginate lyase* was expressed as a single protein of 30kDa under non reducing SDS-PAGE. When β-mercaptoethanol was present, it gave rise to two polypeptides, one had a molecular weight of 20kDa and the other was 10kDa. The N terminal region of the 20kDa protein had the

sequence GVEFSNP and the 10kDa protein had a ragged KDKEMXXADV N terminus. When the 855 nucleotide sequence was isolated a 285 amino acid protein was produced. The first 20 amino acids formed a signal sequence. The G1-P7 and K178-187 peptide stretches were identical to those found in the N terminal regions of the 20kDa and 10kDa fragments respectively. Both proteolytic cleavage of the surface loop and chemical cleavage of the disulphide bridge occured between C169-C183 of the 30kDa protein and resulted in the formation of the two fragments. The ragged N terminus was evidence that a surface loop had been cleaved.

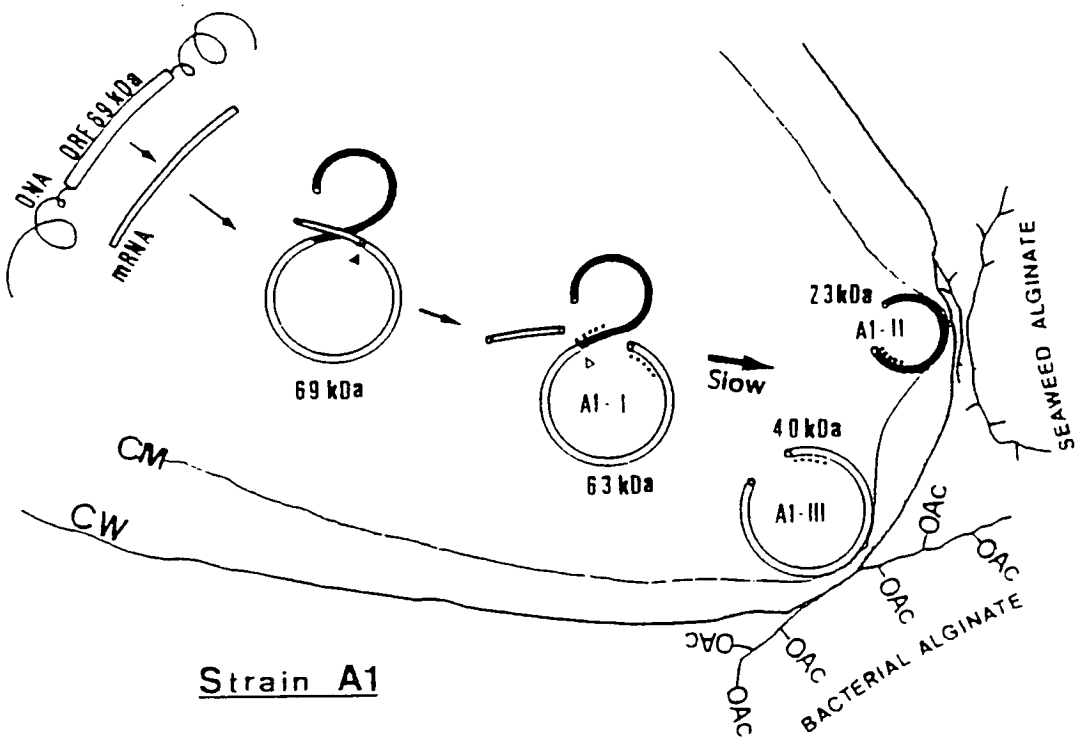
Yonemoto *et al.* (1992) cloned an inducible, intracellular (cytoplasmic) *alginate lyase* from a *Flavobacterium* sp. (A1) (Yonemoto *et al.*, 1991) which produced three types of *alginate lyases*, A1-I, A1-I-1 and A1-I-2. A1-I and A1-I-1 have similar properties and mass 60kDa, A1-I-2 had a mass of 25kDa. Genomic DNA from A1 was digested with *Eco*R1, ligated into the dephosphorylated *Eco*R1 site of plasmid pKK223-3, transformed into *E. coli* DH1 and transformants selected for ampicillin resistance. *E. coli* DH1 does not produce *alginate lyase* nor did it depolymerise alginate. Three transformants expressed *alginate lyase*, ALY1, ALY2 and ALY4. The plasmid DNA from ALY1 and ALY2 was of similar size (11.5kb), and ALY4 was much larger. Plasmid DNA from ALY1 (pALY1) was isolated, purified and transformed into *E. coli* DH1 and contained a 7.0kb insert. The plasmid DNA from ALY2 had the same restriction enzyme digest pattern ALY1. The transformed DH1 (pALY1) produced *alginate lyase* constitutively and this was present in equal amounts in the cytoplasmic and the periplasmic fractions. The addition of IPTG to the culture of *E. coli* DH1 (pALY1) did not affect the production of *alginate lyase* which suggested the gene cloned was under the control of its native promoter. To identify the gene product from the cloned gene, cell extracts were

prepared from A1 and DH1 (pALY1) and chromatographed. A1 gave two peaks, P1 the unabsorbed fraction and P2 eluted at 0.02 M NaCl. P1 corresponded to A1-I and P2 to A1-I-1 , A1-I-2 eluted with A1-I-1 (Yonemoto *et al.*, 1991). The lyase from DH1 (pALY1) was found in the unabsorbed fraction, indicating that the gene cloned directs the synthesis of A1-I alginate lyase.

When a 7.0kb DNA fragment was digested with restriction endonucleases and inserted into *E. coli* DH1 containing plasmid pKK223-3, *alginate lyase* was expressed, indicating that it was under the control of its own promoter. Further subcloning showed that *alginate lyase* was located on a 2.2kb fragment. The expressed protein had a mass of 60kDa. The nucleotide sequence of the 7.0kb *Xho*1-*Bal*1 fragment was determined (Yonemoto *et al.*, 1993). The ORF consisted of 1,866bp which could encode for a polypeptide of 622 amino acids. The amino acid sequence of the N-terminal region was determined for A1-I. The sequence of the first 22 amino acids of A1-I (His-Pro-Phe-Asp-Gln-Ala-Val-Val-Lys-Asp-Pro-Thr-Ala-Ser-Tyr-Val-Asp-Val-Lys-Ala) (Hisano *et al.*, 1993b) was consistent with that of A1-III, whereas that of A1-II (Ala-Pro-Ala-Ala-Ala-His-Ser-Ser-Ile-Asp-Leu-Ser-Lys-(X)-Lys-Leu-Gln-Ile-Pro-Val) (Hisano *et al.*, 1993b) matches the 22 amino acid sequence located in the internal region of A1-I. A1-I was synthesised as a precursor with a molecular mass of 69,153Da and is processed to mature A1-I by cleaving the bond between $_{54}$Ala and $_{55}$His and removing a polypeptide with mass 5,472Da. Murata *et al.* (1993) then described a possible cascade system for the generation of *alginate lyase* (Fig. 10), where the 69kDa precursor was processed to form a mature 63kDa protein. The peptide bond between $_{413}$Ser and $_{414}$Ala is subsequently hydrolysed to generate both A1-II (23kDa) and A1-III (40kDa).

Maki *et al.* (1993) cloned and identified the *alginate lyase* gene (*aly*) from *Pseudomonas* sp. OS-ALG-9. It produced both intracellular and extracellular *alginate lyase.* Kinoshita *et al.* (1991) purified and characterised an intracellular *alginate lyase*. The *aly* gene was isolated from a library constructed with the cosmid vector pHC79 and *Sau*3A digest of genomic DNA. Subcloning experiments located the *aly* gene to a 2.3kb *Hpa*1 fragment. The nucleotide sequence revealed an ORF of 1365bp. Further subcloning revealed the *aly* gene was located on a 1.4kb fragment and was only expressed when the gene was in the correct orientation relative to the other genes within the biosynthetic cluster. The amino acid sequence was determined (398 residues) and the predicted mass of the enzyme was 50,620Da. A hydrophobic sequence was located at the beginning of the sequence which was cleaved during export of the protein from the periplasm. The actual mass of the protein expressed was 46,361Da.

Fig. 10 The Processing Route For the Generation of A1-I, A1-II and A1-III.



▲ and △ , processing sites of 69kDa and 63kDa, respectively: ■■ and oo, regions with identical N-terminal amino acid sequences.

Murata *et al.* 1993

Gellan

Gellan is a novel bacterial exopolysaccharide produced by *Sphingomonas (Pseudomonas) paucimobilis* (Pollock, 1993). The polymer was originally designated S60 (Kang & Veeder, 1982, 1983). The bacterium was isolated from plant tissue (*Elodea*) and initially designated as an unidentified *Pseudomonas* sp. It was then named *Pseudomonas elodea* and deposited in the American Type Culture Collection (ATCC 31461), but was later re-named *Auromonas elodea*. More recently Pollock (1993) re-classified the Gram negative rod-like bacterium as *Sphingomonas paucimobilis* due to its yellow pigment, apparent lack of lipopolysaccharide and its non-fermentative nature.

The original polymer was called S60 or PS60 (Kang & Veeder, 1982, 1983) but is now known as gellan or Gelrite® (generic name). It is produced commercially by an aerobic fermentation process and is available in several forms; as K9A50 a low-acetyl, non-clarified form of gellan for industrial use, as Kelcogel® gellan for foods and industrial products and Gelrite® for microbiological media, plant tissue culture and pharmaceutical industry usage. Both Kelcogel and Gelrite are low acetyl, clarified products

When the bacterium was grown under aerobic fermentation conditions between 28-32°C and pH 6-8, the secreted extracellular polysaccharide formed a highly viscous aqueous solution. This native (acetylated) polysaccharide was recovered by heating the fermentation liquid to 95°C and precipitation with 2 volumes of propan-2-ol. The product contained 50% carbohydrate and 50% insoluble material including protein (10-15% total weight). It could be deacetylated by heating with KOH. The product was precipitated with propan-2-ol and clarified by filtration.

Dreveton *et al.* (1994) studied the effect of fermentor hydrodynamics on gellan fermentation kinetics and rheological properties of the culture broth using different types of mechanical mixers with and without oxygen supplementation. The rheological properties of a polysaccharide solution depend largely on its intrinsic physico-chemical characteristics (molecular weight, polydispersity and degree of substitution) all of which are related to the conditions within the fermentor. Generally the increase in the viscosity of the culture medium during polymer production is one of the most important influences within the fermentor. It limits polymer production, as dissolved oxygen content is decreased, respiration (metabolic turn over) is reduced and cell growth slows. Gellan production was partially related to growth as indicated by Lobas *et al.* (1992), but it continued at a slower rate beyond exponential phase. Therefore, factors which affected cell growth would also affect gellan production. The only time when gellan production stopped at the same time as growth, was under oxygen-limited conditions. Good gellan production was observed in medium with no organic nitrogen substrate. The choice of nitrogen source was important as it influenced the rheological properties of the culture medium. Growth rates were lower on nitrate compared to ammonia or organic nitrogen, but yields of gellan were higher.

Gelation of Gellan

The clarified, low acetyl product is marketed by Kelco as Gelrite® (Kang *et al.*, 1982; Shungu, 1983) as an agar substitute for microbial media. The gelation properties of gellan resembled those of agar and kappa carrageenan gels. Like kappa carrageenan, gellan required cations to be present for gelation to occur, whereas agar does not. A gel strength equivalent to that of agar could be achieved with half the

concentration of gellan. Media solidified with agar, kappa carageenan and gellan are normally resistant to enzymatic degradation (Table 7). The main difference between the native and deacetylated polymer is the gel texture formed on gelation. The native polymer when heated in the presence of cations, formed a soft, elastic gel. The low-acetyl polymer yielded a firm, non-elastic or brittle gel (Kang *et al.,* 1982; Kang & Veeder, 1983). Gellan requires the presence of a salt for gelation to occur. Both the type and concentration of cation affect the strength of the gel. Both mono and divalent cations are able to cause gelation, but a lower concentration of divalent cations is required for gelation than monovalent cations. At calcium concentrations above 0.1% the gel strength decreases and thermoreversablity of the gel is lost. The setting point is dependent on the cation and polymer concentration and can vary between 35-50°C. Gellan melts at 90-100°C.

The native anionic polysaccharide contained 3-4.5% O-acetyl groups as the O-glycosidically linked ester, 11% uronic acid (total weight of polysaccharide), glucose and rhamnose. The molar ratio was L-rhamnose:D-glucose (3:2). The negative specific rotation (-45%) for the deacetylated polysaccharide indicated that most of the glycose units were β-linked. Gellan is a linear polymer comprising of D-glucuronic acid, D-glucose and L-rhamnose. The glucose→rhamnose residues are 1,4 linked (Moorhouse *et al.*, 1981). The rhamnose→glucose residues were also initially reported as being 1,4 linked but were later found to be α 1,3 linked. The acetyl groups are regularly spaced, one acetyl group per 2 repeat units. The acetyl group is thought to be linked to the O-6 of the 1,3 linked glucose residue (Kuo *et al.*, 1986). The other substituent found in gellan is a L-glyceryl group on the O-2 of the same residue as the acetyl group. The glyceryl group is thought to be present on every 1,3 linked glucose residue (Kuo *et al.*, 1986). The structure of the polymer has been

determined by both n.m.r. (Jansson *et al.*, 1983) and mass spectroscopy (O'Neill, *et al.*, 1983). ^{13}C n.m.r. suggested acetylation at C6 in either of the glucose rings (Jansson *et al.*, 1983). Martins & Sá Correia (1991) proposed a pathway for gellan biosynthesis and suggested that the control of gellan synthesis took place at the nucleotide sugar phosphate level, and that feedback inhibition of the nucleotide sugars could play a role in control of gellan biosynthesis.

Gellan belongs to a group of polysaccharides whose structure contain the same carbohydrate backbone as a repeated unit.

...3)-β-D-Glc*p*(1→4)-β-Glc*p*A(1→4)-D-β-Glc*p*(1→4)-α-L-Rha*p*(1→...

Within the group, the modifications of the gellan structure include partial replacement of the 4 linked α-L--rhamnosyl residue in the main chain and/or the addition of mono or diglycosyl side chains (Fig 11). X ray diffraction studies have been used to examine the differences in the two types of gel. Carroll *et al.* (1982, 1983) showed a three fold single helix with axial projections of the chemical repeat unit of 0.94nm. Deacetylation enhanced the packing of the polymer. The increase in crystallisation is consistent with transition to a harder more brittle gel. Acetyl groups inhibit crystallisation, reducing the size and strength of the junction zones. Dehydration of the gellan fibres do not alter the molecular structure but does disrupt the lateral packing (Miles *et al.*, 1984). Chandrasekaran *et al.* (1988) demonstrated that gellan formed a half staggered, parallel double helix with three fold, left handed polysaccharide chains in solution. Native gellan has one or more substitutions (Kuo *et al.*, 1986) acetyl or glyceryl, at the first glucose molecule, a glyceryl group at O-2 and an acetyl group of half occupancy at O-6. Chandrasekaran & Thailambal (1990)

proposed native gellan would form a double helix like gellan. However glyceryl groups would not allow the gellan type of intermolecular aggregations (Chandrasekaran *et al.*, 1988), which are required for strong gelation. The acetyl and glyceryl groups do not interfere with double helix formation but do alter ion binding and to some extent, the gellan-like packing of molecules in the unit cell (Chandrasekaran *et al.*, 1992). The gellan structure is stabilised by the double helix-K^+-water-K^+-double helix interaction. In the presence of Ca^{2+} ions, the direct cross links between the double helices give rise to a much stronger Ca^{2+} double helix interactions.

The polymer gives high viscosities at low concentrations 40-80cP at 0.1% solution and 1000-2000cP at 0.5%. This viscous property is maintained over a wide range of pH and temperature (Miles *et al.*, 1984). The polymer solutions exhibit shear thinning behaviour (Moorhouse *et al.*, 1981), which is suggestive of the breakdown of a weak network or gel structure. This behaviour in microbial polysaccharide solutions has been associated with and attributed to the existence of an ordered or locally rigid chain conformation. X ray diffraction fibre studies have shown that the helical conformation is retained in solution and accounts for the local rigidity of the polymer chain.

Gellan degradation.

The *first r*eport of bacterial degradation of gellan, was a brief report by Schmedding *et al.* (1987), who isolated a bacterium from garden compost which had a lyase type enzyme. Little information about the bacterium or the enzyme specificity were reported. Casida (1989) reported that a Gram negative gliding bacteriun was able to degrade medium solidified with gellan, but did not identify the bacterium or

the enzyme specificity. Recently Mikolajazak *et al.* (1994) reported the presence of an enzyme "sphinganase" from a *Bacillus* sp. The bacillus was most similar to *B. brevis* and its enzyme acted in an unspecified manner as an endoglucanase on Welan (S130) and to a lesser extent on gellan but not on rhamsan (S194).

Industrial Applications of Gellan.

Gellan has many useful properties which may be of commercial importance. It can be used as a thickening, suspending and stabilising agent in aqueous systems, e.g. as an additive to textile printing pastes or formulating low drift herbicides; in the food industry, in salad dressings, thickened puddings and adhesive compounds. Sanderson & Clark (1983) carried out successful preliminary studies to determine the industrial applications of gellan as a gelling agent in the food industry. The deacetylated polysaccharide can be moulded and used as an air freshener matrix, as a gelling agent for gel electrophoresis or as mounting block for electron microscopy. Both acetylated and deacetylated polymers can be used as suspending agents for barium in radiology, in confectionery and as impression materials in tool making and dentistry. Kang *et al.* (1982) found that when gellan was used as a gelling agent in microbial media, bacterial growth compared well to the agar equivalent.

Kang et al. (1982) investigated the use of gellan and the series of eight structurally related bacterial polysaccharides. The polymers share the same repeated backbone structure but differ in the nature and location of their side chains and the presence or absence of certain acyl groups. The L-rhamnose in the backbone can be substituted by L-mannose. Jansson & Widmalm (1994) reported that welan (S130) contains such random substitutions. The backbone is composed of the repeated

tetrasaccharide backbone comprising of glucose, glucuronic acid and rhamnose in the ratio 2:1:1.

The physical properties of the polysaccharide solutions also vary. All are viscous and thermally stable. Native gellan when heated forms a soft elastic gel in the presence of cations whereas the deacetylated polymer forms a hard brittle gel. The type and concentration of ions present affects the strength of the gel. A lower concentration of divalent cations is required to cause gelation than monovalent cations. The native polysaccharide adopted a double helical structure and is stabilised by hydrogen bonds; it was suggested that the *O*-acetyl groups did not interfere with helix formation but that the glycerate groups interfered with the aggregation of gellan molecules. Light scattering and intrinsic viscosity measurements gave a mass of 500kDa for the deacetylated polymer (Grasdalen & Smidsrød, 1987).

Table 7 Enzymes which do not affect Gellan

Enzyme	**Source**
Pectinase	Rohm and Haas
Amylase(a,)	Miles Laboratories
Cellulase	Enzyme Development Corp.
Algin lyase	Non-commercial source a
"Galactomannanase"	Non-commercial source b
Rhozyme HP150	Rohm and Haas
Alcalase 1.5 AU	Novo Laboratories
Papain	Miles Laboratories
Rhozome A-4	Rohm and Haas
Esperase	Novo Laboratories
Maxazyme	Enzyme Development Corp.
Proteinase T	ABM Chemicals Ltd.
Prolase	ABM Chemicals Ltd.
Lipase (wheat germ)	Calbiochem
Lipase (pancreas)	Calbiochem
Lipase M	Enzyme Development Corp.

a Obtained from *Alginovibrio aquatilis*

b Obtained from *Xanthomonas campestris* NRRL

Kang *et al.* 1982

Fig. 11 The Structure of the Gellan Family of Polysaccharides.

a) Gellan gum

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1-

b) Welan gum (S130)

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1-

↑ **1↑3**

O.CO.CH$_3$ **α-L-Rha*p*** or **α-L-Man*p***

c) Rhamsan gum (S194)

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4-α-L-Rha*p*-(1-

1↑6

ß-D-Glc*p*(1→6) α-D-Glc*p*

d) (S657)

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1-

1↑3

α-L-Rha*p*(1→4)α-L-Rha*p*

e) (S88)

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1-

1↑3 or **α-L-Man*p***

α-L-Rha*p*

f) (S198)

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1-

1↑3 or **α-L-Man*p***

α-L-Rha*p*

g) (NW11)

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4-α-L-Man*p*-(1-

h) (PS-P4)

-3-ß-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1-

Chapter 2. MATERIALS AND METHODS

2.1 Stock Cultures

Alginate lyase-producing bacteria

Azotobacter vinelandii Ax 17 78	(Alg^+)	Dr W. Page
Azotobacter vinelandii UW	(Alg^-)	Dr. T Jarman
Azotobacter chroococcum 184	(Alg^-)	Dr W. Page
Azotobacter chroococcum 221	(Alg^-)	Dr W. Page
Azotobacter chroococcum 109	(Alg^-)	Dr W. Page
Azotobacter chroococcum 34	(Alg^+)	Dr W. Page
Azotobacter chroococcum 46	(Alg^+)	Dr W. Page
Azotobacter chroococcum 186	(Alg^+)	Dr W. Page
Alteromonas atlantica T9		
Alteromonas atlantica 511		

Gellan lyase-producing bacteria

Sphingomonas paucimobilis NCIB 11942	
Sphingomonas paucimobilis MJ 216	Dr. I. Sá Correia
Isolates 2A, 11.1, 12.1, 13.1 and 14.1	

The gellan lyase-producing bacteria 2A, 11.1, 12.1, 13.1 and 14.1 were isolated in this laboratory from aquatic environments. Pure cultures were obtained by streaking out single colonies onto Yeast Extract medium (YE) (Sutherland and Wilkinson, 1965) containing 2% (w/v) glucose. These colonies were then tested on YE salts containing 0.8% gellan. Liquefaction of the gellan medium was used to identify the production of a gellan-degrading enzyme.

2.2 Media

2.2.1 YE Basal Medium

(Sutherland & Wilkinson 1965)

	g l-1
X 10 Salts Solution	100ml
Yeast Extract	1
Casein Hydrolysate	1
d.H_2O	900ml

X10 Salts Solution

	g l-1
Na_2HPO_4	100
KH_2PO_4	30
K_2SO_4	10
NaCl	10
$MgSO_4 .7H_2O$	2
$CaCl_2 .2H_2O$	0.01
$FeSO_4 .7H_2O$	0.001

The bacteria were grown on YE Basal Medium containing 0.15% gellan (Gelrite, Schweizerhall). 1% gellan and 2% agar were used to produce solid media.

2.2.2 Burks Nitrogen-Free Medium

(Norris, 1959)

	g l-1
K_2HPO_4	0.65
KH_2PO_4	0.16
$MgSO_4 .7H_2O$	0.2
NaCl	0.2
$CaCl_2 .2H_2O$	0.066
$NaMoO_4 .2H_2O$	0.0025
$FeSO_4 .7H_2O$	0.005

The medium was adjusted to pH 7.2 with NaOH and 1% mannitol or 2% glucose added after autoclaving.

2.3 Culture Conditions

2.3.1 Alginate lyase-producing bacteria

The *Azotobacter* spp. were grown at 30°C in 250ml conical flasks containing 100ml of Burks nitrogen free medium (BM), containing 1% mannitol or 2% glucose as carbon source. This was used to inoculate 1l of BM in a 2l Erlenmeyer flask. The culture was grown for an appropriate length of time and the cells were harvested by centrifugation. The supernatant was discarded and cells were stored frozen at -20°C.

2.3.2 Gellan lyase-producing bacteria

A single colony from solid medium was inoculated into 250ml conical flasks containing 100ml YE basal medium plus 0.15% gellan as the sole carbon source and grown at 30°C. This pre-culture was used to inoculate 1l basal medium in 2l conical

flasks. Five or more of these flasks were used routinely to isolate the enzyme after shaking on an orbital shaker for an appropriate length of time. The cells were pelleted by centrifugation and the supernatant was retained.

2.3.3 Induction of cyst formation

(Page & Sadoff, 1975)

Azotobacter spp cells were grown at 30°C, and harvested after 48h and 72h by centrifugation in sterile 250ml pots. The cells were washed with sterile BM salts, then resuspended in sterile 1l BM containing 0.3% β-hydroxy butyric acid (cyst induction medium). The cyst-induced culture was harvested after 24h, the remaining cyst induced cultures were left for an appropriate length of time.

The cells were harvested at 10,000g for 20min at 4°C. The supernatant was discarded and the cell pellets frozen. The cysts were washed with 50mM TRIS buffer, pH 7.8 containing 3mM EGTA. The cysts were broken by sonication and spun at 40,000rpm for 30min. The pellet was discarded and the supernatant dialysed against running tap water for 4h. The supernatant was concentrated against polyethylene glycol 6,000 (PEG 6000). The concentrated supernatant was spun in the microfuge and the volume made up to 4ml with 200mM HEPES pH 7.6.

2.3.4 Abortive Cyst formation

Abortive cysts were formed when the cyst induction medium was free from $CaCl_2.2H_2O$. They were then treated in the manner described above.

2.4 Isolation of *gellan lyase*

The cells were pelleted from the liquid medium by centrifugation at 10,000g in 250ml Sorvall pots. The cells were washed with 10mM TRIS pH 7.0, then frozen slowly at -20°C. The spent medium was retained and concentrated in a Pellicon (Millipore) tangential flow system, with a 10kDa mass (nominal) cut off membrane. When the volume was reduced to approximately 500ml, the system was flushed with 1.5l of distilled water. This filtrate (c. 2l) was kept on ice, and concentrated using a Minitan (Millipore) tangential flow system with a 100kDa cut off membrane. The filtrate was retained and further concentrated using the 10kDa cut off membrane. The reduced fractions had a total volume of 200ml, one having a nominal mass greater than 100kDa and the other being nominally less than 100kDa. Both fractions were centrifuged at 10,000g in 250ml Sorvall pots. The deposit was discarded and the "supernatant" in Visking tubing (Medicell International) was dialysed against 10mM TRIS HCl pH 7.0, then concentrated against PEG 6000. The volume was reduced to 5ml and centrifuged in a microfuge (Sorvall) at 15,000rpm (12 x 3g). The deposit was discarded and the supernatant used as the enzyme source. This was kept frozen at -20°C, in 1.5ml aliquots and thawed when required.

2.5 Cell Fractionation

2.5.1 Preparation of Total Cell Lysate.

Frozen cell pellets were thawed quickly and resuspended in 10mM TRIS buffer, pH 7.0. The cells were sonicated with 4 x 1min. bursts alternating with 1min. rests. The cells were kept on ice throughout, to prevent denaturation of the protein. The broken cells were spun in the ultracentrifuge at 100,000g for 2h to pellet

membranes. The supernatant was used as 'cytoplasm'; the membranes were resupended in TRIS buffer and both fractions were stored at -20°C.

2.5.2 Preparation of Periplasm and Cytoplasm.

(Osborn *et al.*, 1972)

REAGENTS

750mM Sucrose in 10mM TRIS buffer pH 7.8

1.5mM EDTA pH 7.5

5mg ml^{-1} Lysozyme

200mM HEPES pH 7.6

The cells were harvested from the medium by centrifugation at 10,000g for 20min at 4°C. The pots were wiped dry and the cells resuspended in 100-200ml of cold (4°C) sucrose solution. Lysozyme was added to a final concentration of 100µg ml^{-1} and left stirring in an ice bath for 2min. While stirring, 2 volumes of cold EDTA were added over 10min. The sphaeroplasts were harvested at 10,000g for 10min. The supernatant was decanted off as the periplasmic fraction (**periplasm**) and the pots dried. The sphaeroplasts were resuspended in 20-30ml cold distilled water and broken by sonication (4 x 1min bursts alternating with 1min rests). The broken sphaeroplasts were pelleted by ultracentrifugation (100,000g for 2h). The pelleted membranes were resuspended in HEPES buffer and frozen and the supernatant (**cytoplasm**) was retained.

The periplasmic fraction and the cytoplasmic fraction were dialysed against distilled water stirring at 4°C for 36h to remove the sucrose and concentrated against PEG 6000. The contents of the dialysis sacs were washed out with 10mM TRIS pH 7.3 and microfuged. All the fractions were aliquoted and frozen at -20°C.

2.5.3 Preparation of Cytoplasm and Periplasm.

(Ames *et al.*, 1984)

The original method for 2ml cultures was adapted for larger volumes as follows: the cells were spun down and resuspended in 0.1 vol of d.H_2O. 0.2 vols of chloroform were added, vortexed and incubated at room temperature for 15min. An equal volume of 10mM TRIS (pH 8.0) was added and the cells deposited by centrifugation (10,000g) for 30min. The supernatant was carefully decanted off and the cell pellet discarded. The supernatant (**periplasm**) was dialysed against running tap water, then concentrated against PEG 6000 at 4°C. The samples were then frozen and thawed as necessary.

2.6 Lyase Determination

Gellan Lyase **Assay Mixture**

	Volume (µl)
0.1% gellan	500
10mM TRIS pH 7.3	500
Enzyme	2-200

Alginate Lyase **Assay Mixture**

	Volume (µl)
0.5% alginate (Sigma)	500
50mM MES pH 5.2 or 6.8	500
Enzyme	2-200

All reagents were made up in 50mM TRIS buffer pH 7.0.

Colourimetric Assay Mixture

	µl
Glucose oxidase	20
Peroxidase	10
ADTS	150
50mM TRIS pH7	470
Enzyme	1-100

The free glucose in the lyase assay mixture was determined by monitoring the release of this sugar from gellan by enzymes associated with lyase activity. The lyase activity was stopped by boiling the reaction mixture for 5min before setting up the colorimetric assay mixture. The assay mixture was incubated at 37°C for 1h and read at 415nm. A standard curve was included containing between 0-6µg of glucose. A control containing no ADTS was included as the reagent blank.

2.6.2. Alkaline Phosphatase Assay

Reagents

100mM	Glycine pH 10.5
25mM	ρ Nitrophenyl phosphate
100mM	Acetic acid

The sample (10-200µl) in a final volume of 200µl was added to 600µl 100mM glycine, finally 100µl 25mM ρ-nitrophenyl phosphate was added. The assay mixture was incubated at 30°C for 3h. The reaction was stopped by adding 20µl 100mM acetic acid and read immediately at 405nm. The molecular extinction coefficient of ρ -nitrophenyl phosphate is 18.5. (cm^2 μmol^{-1})

2.7 Analytical Determination

2.7.1 Reducing Sugar Determination

(Park & Johnson, 1949)

Solution 1	0.05% Potassium ferricyanide
Solution 2	0.53% Na_2CO_3, 0.065% KCN
Solution 3	0.15% Ferric Ammonium Sulphate
	0.1g $100ml^{-1}$ Triton X100 in 0.05N H_2SO_4

To 200µl of sample, 200µl of Solution 2 was added, followed by 200µl Solution 1. The assay mixture was boiled for 15min, in tubes of the same diameter. The assay mixture was cooled in ice water and 1ml of Solution 3 was added and allowed to stand at room temperature for 15min. The samples were read at 690nm against a water blank. A standard curve (0-10µg) was included using 1mg ml^{-1} glucose.

2.7.2 Uronic acid Determination

(Blumenkrantz & Asboe-Hansen, 1973)

Solution 1	12.5mM sodium tetraborate in c. H_2SO_4
Solution 2	0.15% m-hydoxyphenyl in 0.5% NaOH

In glass tubes of a similar diameter, in an ice water bath, 1.2ml of Solution 1 was added to 200µl of sample. The tubes were vortexed and boiled for 5 min., The assay mixture was allowed to cool in cold water. 20µl of solution 2 added and vortexed and read at 520nm. A standard curve (0 -20µg) was included using a 1mg ml^{-1} solution.

2.7.3 Acetyl Determination

(Hestrin, 1949)

Reagents

Solution 1	2M hydroxylamine HCl (stored in the cold)
Solution 2	3.5M NaOH
Solution 3	cHCl (SG 1.8), diluted 1:2 with distilled water
Solution 4	0.37M $FeCl_3 .6H_2O$ in 0.1M HCl
Standard solution	4mM acetyl choline in 1mM acetate buffer pH 4.5

To 200µl of sample were added 400µl freshly mixed Solutions 1 and 2 (1:1). The mixture was vortexed and left at room temperature for 2 min before the addition of 200µl Solution 3, further vortexing, addition of 200µl Solution 4 and mixing. Samples were read at 540nm against a suitable control and standard curves containing 10-80µg acetyl.

2.7.4 Total Carbohydrate Determination

(Dubois *et al.*, 1956)

Solution 1	5% aqueous phenol
Solution 2	c. H_2SO_4

To 200µl of sample, 200µl of Solution 1 was added and mixed well. To the bottom of the tube, 1ml sulphuric acid was quickly added and again mixed well. The tubes were left at room temperature for 15min and read at 490nm. A standard curve was included (0-18µg) using 1mg ml^{-1} glucose.

2.7.5 Protein Determination

Protein concentration was determined using the Biorad Microassay Procedure according to Bradford (1976). The samples and standards were prepared in duplicate and read at 595nm within 5-60min. A standard curve of protein concentration 0-12μg was included.

2.7.6 Deacetylation of Polymers

Polymers were deacetylated by boiling (15min) in the presence of 0.05M NaOH, then dialysed against running tap water (48h), frozen and lyophilised.

2.8. Protein Purification Techniques

2.8.1 Ammonium Sulphate Fractionation

The concentrated spent medium ("supernatant") was used to separate *gellan lyase* from other contaminating proteins. Either the periplasm or cytoplasm were used for isolation of *alginate lyase*. Aliquots of supernatant and pelleted material were kept at each of the different cuts of $(NH_4)_2SO_4$ ('low in heavy metals for enzyme work'). Appropriate amounts of solid $(NH_4)_2SO_4$ were added over 1h with stirring in the cold. The samples were centrifuged at 36,590g for 30min to pellet any precipitate. The pellets were redissolved in 10mM TRIS buffer (pH 7.0) and dialysed in the cold against two changes of $d.H_2O$. Samples were frozen for later determination of lyase activity and protein concentration.

2.8.2 Protein Purification by Molecular Weight Determination

10ml of sample were placed in a stirred cell (Amicon) with an appropriate molecular weight cut off membrane (10k-100kDa). The samples were stirred on ice

and the proteins passed through the membrane using compressed air. Aliquots were retained from each fraction and lyase activity and protein concentration determined.

2.8.3 Affinity Chromatography

Purification of *alginate lyase* using affinity chromatography was carried out on a 4ml (5 x 1cm) column of polymannuronate linked to Sepharose. A linear gradient (0-0.4M NaCl) was applied after the removal of unabsorbed material; 1ml fractions were collected at 8min intervals. The column was regenerated by applying 2M NaCl and re-equilibrating with 20mM TRIS pH 7.0.

2.8.4 Ion Exchange Chromatography

Alginate lyase was initially purified on a 15ml (20 x 1cm) DEAE-Sepharose column. The column was washed with 20mM TRIS pH 7.0 and adsorbed material was eluted using a linear 0-0.5M NaCl gradient. Fractions (1ml) were collected at 8min intervals. Later experiments used a 170ml Q-Sepharose FF. (Pharmacia) column.

Preliminary experiments to purify *gellan lyase* were carried out on an 18ml Q-Sepharose FF. anion exchange column (23.5 x 1cm). The proteins were eluted with 10mM TRIS (pH 7.0) at a flow rate of 16ml h^{-1} and fractions (1.2ml) collected at 4.5min intervals. A stepped gradient of NaCl was applied and fractions collected.

A longer Q Sepharose FF. (170ml) column was poured (85 x 2.5cm) and eluted with 10mM TRIS (pH 7.3). The flow rate was determined as 17.6ml h^{-1} and 2.5ml fractions were collected at 5.5min intervals. Unbound material was eluted with 3 column volumes of 10mM TRIS (pH 7.3). Bound material was eluted using a stepped gradient. The column was regenerated with 1M NaCl in 10mM TRIS (pH 7.3) and

then washed with 10mM TRIS pH 7.3 before the next sample was loaded. Fractions were collected at all stages and the absorbance at 280nm monitored. The protein peaks were pooled, dialysed exhaustively against 10mM TRIS (pH 7.3) and then concentrated against PEG 6000. Protein concentration was measured and TBA activity assayed.

2.8.5 Gel filtration chromatography on Ultragel AcA34.

An 86 x 0.6cm column was poured, containing 24ml AcA34 (Pharmacia) which separates protein with a nominal mass of 20k-400kDa. The sample (100µl) was applied to the column and the protein eluted with 10mM TRIS pH 7.3. Fractions (100-400µl) were collected at 12min intervals (flow rate 0.5-2 ml h^{-1}). Protein concentration was estimated using the Biorad Micro Assay procedure.

2.8.6 Gel filtration chromatography on Biogel P2.

The breakdown products of enzyme action were concentrated by rotary evaporation and 1ml of this fraction was applied to a 40ml Biogel P2 (51 x 1cm) column; fractions (1ml) were collected at 14min intervals (LKB pump setting X1, 6) and resulted in a flow rate of 4ml h^{-1}. The material was eluted from the column using d.H_2O and fractions were tested for carbohydrate content.

2.9 Production of Acid-Hydrolysed Gellan.

Acid-hydrolysed gellan was prepared by boiling a 1% solution of gelrite in the presence of 0.025M H_2SO_4 for 1h on a magnetic stirrer/hotplate. The hydrolysed gellan was allowed to cool, neutralised and dialysed for 48h against running tap

water, frozen at -20°C and then lyophilised. The resultant powder was then added to the YE medium.

2.10 Viscometry.

The viscosity of polysaccharide solutions was measured in a Brookfield (model DV-II) viscometer. Sample (2ml) was placed in the cup and allowed to equilibrate to 30°C (20min). An appropriate amount of enzyme was added and the change in viscosity measured over a number of hours.

2.11 Analysis of Breakdown products by Paper Chromatography

Aliquots of substrate were enzymatically degraded in the presence of sodium azide (0.02%). The digests were incubated at 30°C; the reaction was stopped by boiling the sample for 15min. The digested material was put into dialysis sacs, which had been boiled in 2% $NaHCO_3$,1mM EDTA, rinsed with distilled water, boiled in 1mM EDTA, then washed inside and out with water. The contents of the sacs were exhaustively dialysed against two changes of d.H_2O over 36h. The two diffusate fractions were pooled, evaporated on a rotary evaporator at 55°C and resuspended in a minimal volume of Ultra High Quality Water (UHQ) (Elga). They were microfuged for 15min in the cold and frozen at -20°C. The retentate was frozen, freeze-dried, and weighed. The diffusate was applied to paper chromatograms along with sugar standards (1µl, 0.1M) glucose, glucuronic acid and rhamnose. The chromatogram was run in one of two descending solvent systems:

i) Butan-1-ol:pyridine:water	6:4:3	(v:v:v)
ii) Ethyl acetate:acetic acid:formic acid:water	18:3:1:4	(v:v:v)

After 36 or 72h, the chromatograms were removed, dried and developed using the method of Trevelyan *et al.* (1950). The chromatograms were sequentially run through the reagents in a trough, allowed to dry in air and photocopied to provide a permanent record and intensify spots where necessary.

2.12 Sugar analysis by HPLC

The analysis of neutral sugars was carried out on acid hydrolysates of polysaccharide. The polymer (20mg) was hydrolysed overnight in 0.25M H_2SO_4 at 100°C in a sealed ampoule. The hydrolysates were neutralised with regenerated Amberlite IR410 HCO_3^- resin, followed by Amberlite MB1 resin. The liquid was removed; the resin washed with UHQ water and the pooled solutions dried under reduced pressure. After re-solution in a small volume of UHQ water, the solution was microfuged and filtered through a 0.45μm membrane. The neutral sugars in the hydrolysates were analysed on an SCX lead cartridge (30 x 0.75cm microbore column, Brownlee Laboratories Inc.) using a flow rate of 0.2ml min^{-1} UHQ water. The standards run included glucose, rhamnose and gentibiose.

2.13 Molecular Mass Determination by HPLC.

20μl of polymer solution (c. 10mg ml^{-1}) which had been dialysed and filtered (0.45μm) was injected into a Gilson HPLC system and run on the TSK G4000PW or TSK G1000PW column with refractive index monitoring. When the TSK G4000PW column was used, dextran or pullulan of known molecular weight was used as standard. For the TSK G1000PW column, monosaccharide, disaccharide and tetrasaccharide standards were used.

2.14 Paper Electrophoresis.

Samples were applied 1.5cm apart to Whatman No 1 or 3MM paper. The paper was soaked with half-strength buffer (40:400:4000, pyridine:acetic acid:water), blotted dry, placed in the electrophoresis tank and run at 200mA. The electrophoresis strip was dried, examined under short wave U.V., then developed with silver nitrate.

2.15 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out following the method of Laemmli (1970). The 1mm thick resolving gel was either poured as a linear gradient or as a single concentration of acrylamide; this was allowed to polymerise and a 4.5% stacking gel was poured on top and a comb placed in it. The gel was either denaturing or non-denaturing. The running buffer was made as follows:

Running Buffer

	g l-1
TRIS base	3.0
Glycine	14.4
+/-SDS	1 0

10μg of protein was mixed with sample buffer and loaded into the wells. A mixture of molecular weight markers were included for reference. The samples for denaturing gels were boiled for 3min and microfuged for 3min to pellet any insoluble material. The samples for non-denaturing gels were mixed with loading buffer and then microfuged for 3min. The gel was run at 10mA (constant current) for 1h to allow the proteins to stack in gel, followed by 20mA until the blue dye front was within 1cm of the bottom of the gel.

After electrophoresis was complete, the gel was stained with silver (Biorad Bulletin 1089). The gel was then photographed and dried down on 3MM chromatography paper.

Chapter 3. RESULTS AND DISCUSSION

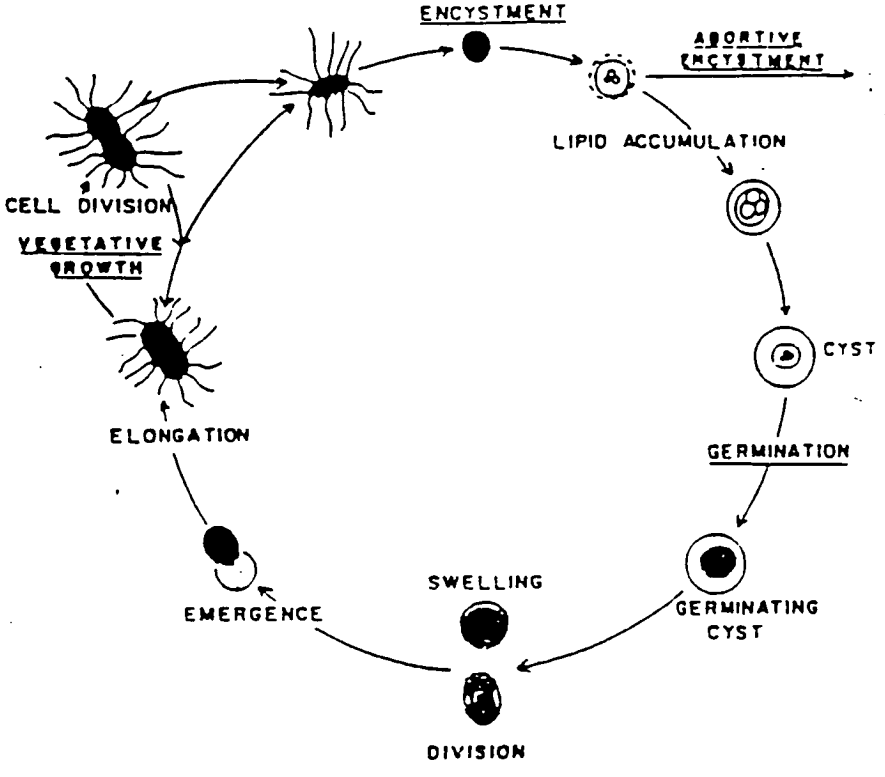
The family of bacteria *Azotobacteraceae* consists of two genera, *Azotobacter* and *Azomonas* which can be isolated from soil and water. They are primarily characterised as non-symbiotic, obligate aerobic, chemoheterotrophs whose differentiating characteristic is the ability to fix molecular nitrogen in nitrogen-free medium, with an organic carbon compound as the energy source. The bacteria are motile, by peritrichous or polar flagella, or non-motile. Although they are strict aerobes, they are able to grow and fix nitrogen under reduced oxygen pressure. Most strains are catalase positive, capsulated and produce slime. Poly-β-hydroxybutyrate (PHB) is the storage polymer. Water-insoluble and water-soluble fluorescent pigments are produced by some species. In the family of *Azotobacteriaceae* the GC content of the DNA ranges from 52-67 mol% (Tm).

The genus *Azotobacter* is distinguished from the other genus in the family because of its ability to form cysts (Fig. 12) after exponential growth or upon induction with specific reagents (Jurtshik & Harper, 1968). *A. vinelandii* and *A. chroococcum* are normally motile but non-motile strains of *A. vinelandii* have been isolated. *A. vinelandii* cells are round-ended rods occurring singly or in pairs. The excretion of a water soluble yellow-green fluorescent pigment occurs in *A. vinelandii*. Rhamnose can be used as the sole carbon source by *A. vinelandii* only. *A. chroococcum* cells are pleiomorphic, blunt, oval-ovoid or coccus-shaped. Young cells are usually found in pairs and are motile by peritrichous flagella. Cysts and capsular slime are produced. A non-diffusible black or brown-black pigment is produced by ageing cells.

Electron microscopy has shown that cysts are modified vegetative cells surrounded by thick coats of intine and exine. Encystment in *A. vinelandii* is

complete within 36h after induction and results in the formation of large optically refractile bodies with hexagonally crystalline outer coat (exine). Lin & Sadoff (1969a), showed that the cyst intine and exine fractions were rich in protein, carbohydrate and lipid. The central body, exine and intine layers also contain high concentrations of Ca^{2+} and lower concentrations of Mg^{2+}. Cyst formation is a simple model for studying cell differentiation in bacteria. Under suitable conditions *A. vinelandii* and *A. chroococcum* cysts can regenerate to form viable vegetative cells. When *A. vinelandii* was grown in glucose, mannitol or sucrose as carbon source, less than 0.1% of the cell population was made up of cysts (Jurtshik & Harper, 1968). The degree of encystment was dependent on the amount of poly-β-hydroxybutyrate (PHB) in the cell. The presence of carbon source in the medium inhibited encystment (Winogradsky, 1938) and abortive cysts were formed as a result of catabolite repression. Abortive encystment could also be caused by an early synthetic block (Lin & Sadoff, 1968) resulting in the formation of an incomplete cyst coat and release of a viscous polymer. This polymer produced in the absence of cations had a lower guluronate content than the normal cyst coat material (Page & Sadoff, 1975). Abortive encystment could not be reversed or prevented by addition of cAMP. Lack of Ca^{2+} also caused formation of abortive cysts.

Fig. 12 Schematic Diagram of the Life Cycle of *A. vinelandii*



Sadoff, 1975

Once encystment began, the vegetative bacteria lost their flagella, became spherical, the cell walls thickened and the developing cysts became more refractile. The cyst consists of a central body, an inner coat (the intine) and an outer coat, the exine (Winogradsky, 1938). The central body is mainly composed of lipid (PHB). Cysts can germinate in aerated medium at 30°C. The first evidence of germination occurs when the cysts become less refractile under phase microscopy. Germination is a slow process, lasting 4-6h in glucose-supplemented medium. The central body swells and occupies the intine (Loperfido & Sadoff, 1973). When the central body has swollen into the exine the cyst coat ruptures and a non-motile, peanut-shaped dividing cell emerges. The cell becomes motile prior to the post germination division.

The exine enables the cyst to resist desiccation but is not an impenetrable barrier as germination substrates can pass through it and respiration begins immediately after the cysts are added to aerated medium (Loperfido & Sadoff, 1973). Exposure of cysts to chelating agents caused them to rupture and the contents of the central body were released (Goldschmidt & Wyss 1966). Page & Sadoff (1975) showed that both EDTA and EGTA caused cysts to lyse to a comparable extent, causing a loss of optical refractility and decrease in the number of cysts. Since EGTA is less selective in binding Mg^{2+}, Ca^{2+} is thought to be the cation involved in maintaining the structure of the cyst coat. By adding Ca^{2+} to cysts grown in sub-optimal Ca^{2+} concentration (0.31mM), encystment was maximally stimulated at 24h and Ca^{2+} had to be added to the medium before the formation of the exine was completed at 36h.

During encystment the composition of the alginates in the intine and exine changed. Lin & Sadoff (1969b) reported that the exine and intine fraction of *A.*

vinelandii contained 33 and 44% total carbohydrate respectively; of this, uronic acid accounted for 40% of the exine carbohydrate and 72% intine carbohydrate. On a dry weight basis, this accounted for 32% of the intine and 13% of the exine cyst cell coat. Mannuronic and guluronic acids were the only uronic acids present in the exine and intine fractions (Sadoff 1975). They existed as homo- and hetero-polymers, the proportions of which were different in the intine and the exine. Exine was rich in poly G and the intine rich in poly M. The shift in the uronic acid composition was reflected in the properties of the polymers. The buckled chain conformation of poly G-rich exine lends itself to strengthening the outer structure. The poly M-rich intine creates a more flexible, intracellular or primary wall tissue.

In *Azotobacter* spp., guluronate is produced by the action of a Ca^{2+}-dependent C5 epimerase acting on D-mannuronate (Haug & Larsen, 1971). Therefore, the control of synthesis or activity of this enzyme is critical during encystment. The absence or removal of Ca^{2+} from the medium would inhibit the action of this enzyme and abortive cysts would be formed. The abortive cysts rupture because of the removal of Ca^{2+} from the outer cell wall. Clearly, both Ca^{2+} and Mg^{2+} are required for encystment to occur and cysts contain four times as much Ca^{2+} as do vegetative cells.

The effect of encystment on *alginate lyase* activity

The formation of cysts was induced by the addition of 0.3% β-hydroxybutyrate (BHB) to BM. The experiments were carried out using strain Av Ax17.78 (EPS^+) since EPS^- strains do not form cysts. The cells were grown in BM for 72h before encystment was induced according to the method described in 2.3.3. Samples of cysts were removed at 24 and 48h. All fractions were tested for lyase and *alkaline*

phosphatase activity and the protein concentration determined (Table 8). The level of *alginate lyase* activity decreased as time elapsed following cyst induction. The level of lyase activity observed after 48h in the encystment medium was approximately half of that observed 24h after induction. There was no significant difference in the protein concentration in the encysted cells. The spent medium was tested after cyst induction, to determine if the *alginate lyase* had been excreted, leached or exported into the medium. This did not appear to be the case, since after concentrating the spent medium, no lyase activity was observed although protein was certainly present in the sample (results not shown).

Having established that *alginate lyase* activity decreased greatly during encystment, the effect of cyst germination on *alginate lyase* was studied. Strain Av Ax17.78 was grown as previously described and cyst formation induced. The cysts were then allowed to germinate for up to 96h, samples were removed at appropriate time intervals and the TBA activity, *alkaline phosphatase* activity and the protein concentration determined (Fig. 13).

These results confirmed that the level of *alginate lyase* activity in Av Ax17.78 decreased during encystment. A 13% reduction in activity was observed after 2h in the encystment medium; this corresponded to a specific activity of 203ρmole ml^{-1} mg $protein^{-1}$ in 72h cells falling to 177ρmole ml^{-1} mg $protein^{-1}$ after 2h in the encystment medium. After 72h post-induction, there was a 87% reduction in activity. As germination proceeded, the level of *alginate lyase* increased from 7ρmole ml^{-1} mg $protein^{-1}$ (2h post induction) to 86ρmole ml^{-1} mg $protein^{-1}$, 96h post-germination. At this stage (96h) the germinated cysts had not achieved an *alginate lyase* level equivalent to that of the 72h cells (203ρmole ml^{-1} mg $protein^{-1}$). The level of *alkaline*

phosphatase and protein concentration did not vary significantly during encystment and germination.

The loss of lyase activity was also observed when abortive cysts (2.3.4) were formed in Ca^{2+}-free BM (Fig. 14). During the vegetative cell growth cycle, the amount of *alginate lyase* increased from a specific activity 5ρmole ml^{-1} mg $protein^{-1}$ to 533ρmole ml^{-1} mg $protein^{-1}$ between 48h and 72h. During encystment, *alginate lyase* decreased from 468ρmole ml^{-1} mg $protein^{-1}$ after 24h post-induction, to 4ρmole ml^{-1} mg $protein^{-1}$ in the next 24h. After a further 24h, no change was observed in *alginate lyase* activity. After 24h post-germination no *alginate lyase* activity was observed. The level of protein fell steadily throughout the process of encystment and germination.

The effect on *alginate lyase* activity of lanthanum as a calcium analogue was determined during encystment. Av Ax17.78 was inoculated into BM in 2l flasks and grown for 48h at 30°C. This was used to inoculate BM in 2l flasks and BM containing 50μM lanthanum acetate. The cells were grown at 30°C, pelleted by centrifugation and later broken by sonication. The results for the control flasks are shown in Fig. 15a and for lanthanum-containing medium in Fig. 15b. Although the curves are similar it should be noted that the amount of *alginate lyase* present in the lanthanum-treated cells decreased between 24h and 72h, whereas in the control cells *alginate lyase* increased between 24-48h and fell between 48-73h.

The final experiment in this series was designed to show if the presence of chloramphenicol in the encystment medium had any effect on *alginate lyase* activity during encystment and post germination. Chloramphenicol inhibits protein synthesis by binding to the 50S subunit of the 70S type ribosomes, it inhibits peptidyl transferase and thus prevents peptide bond formation. Av Ax17.78 was grown in BM

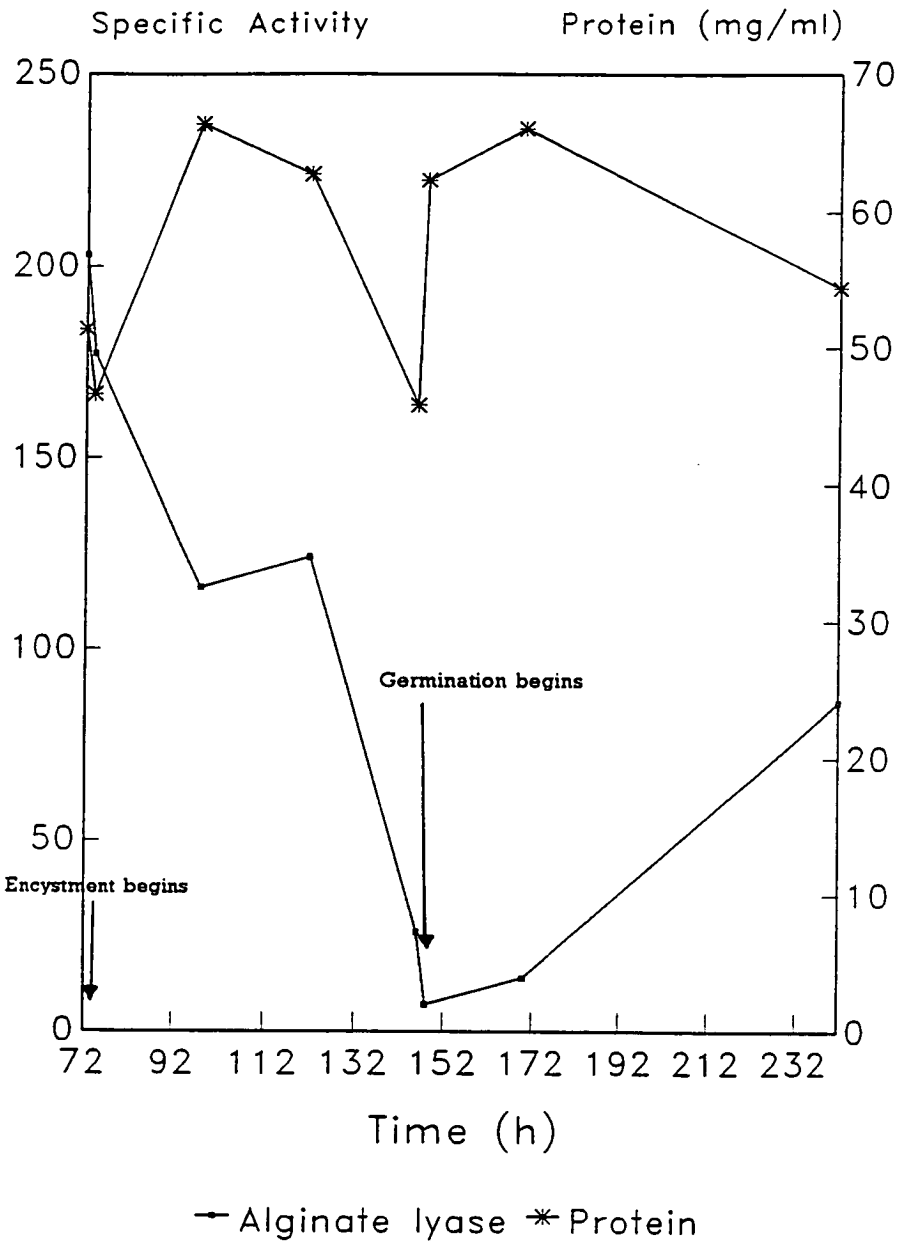
in the presence of 1% mannitol for 72h, a sample was removed at 24h and another allowed to grow for a further 24h. The cells (72h) were harvested and resuspended as described (2.3.3) but after 2h in the encystment medium, chloroamphenicol was added to a final concentration of 100μg ml^{-1} to half the flasks. Samples were removed at 24h intervals and enzyme levels monitored. To half the flasks which had chloroamphenicol present in the encystment medium, more chloroamphenicol was added during germination (100μg ml^{-1}). The result of this experiment is shown in Table 9. The increased level of *alkaline phosphatase* and the low level of protein observed in the 96h control cells would suggest that cell lysis had occurred and protein had been lost to the growth medium. The level of *alginate lyase* activity in the cells decreased as the cells aged. As expected, lyase activity also decreased as encystment proceeded. When chloroamphenicol was present in the encystment medium, lyase activity remained constant in the first 24h period and then activity doubled in the next 24h period. During germination very low levels of lyase activity were detected.

Table 8 The Effect of Encystment on *Alginate Lyase* Production in Av Ax17.78.

Cell Source	*Alginate lyase* Specific Activity	*Alkaline Phosphatase* Specific Activity	Protein gml^{-1}
72 h cells	383	0.9	11.1
24 h cysts	248	1	4.91
48 h cysts	133	5	5.32

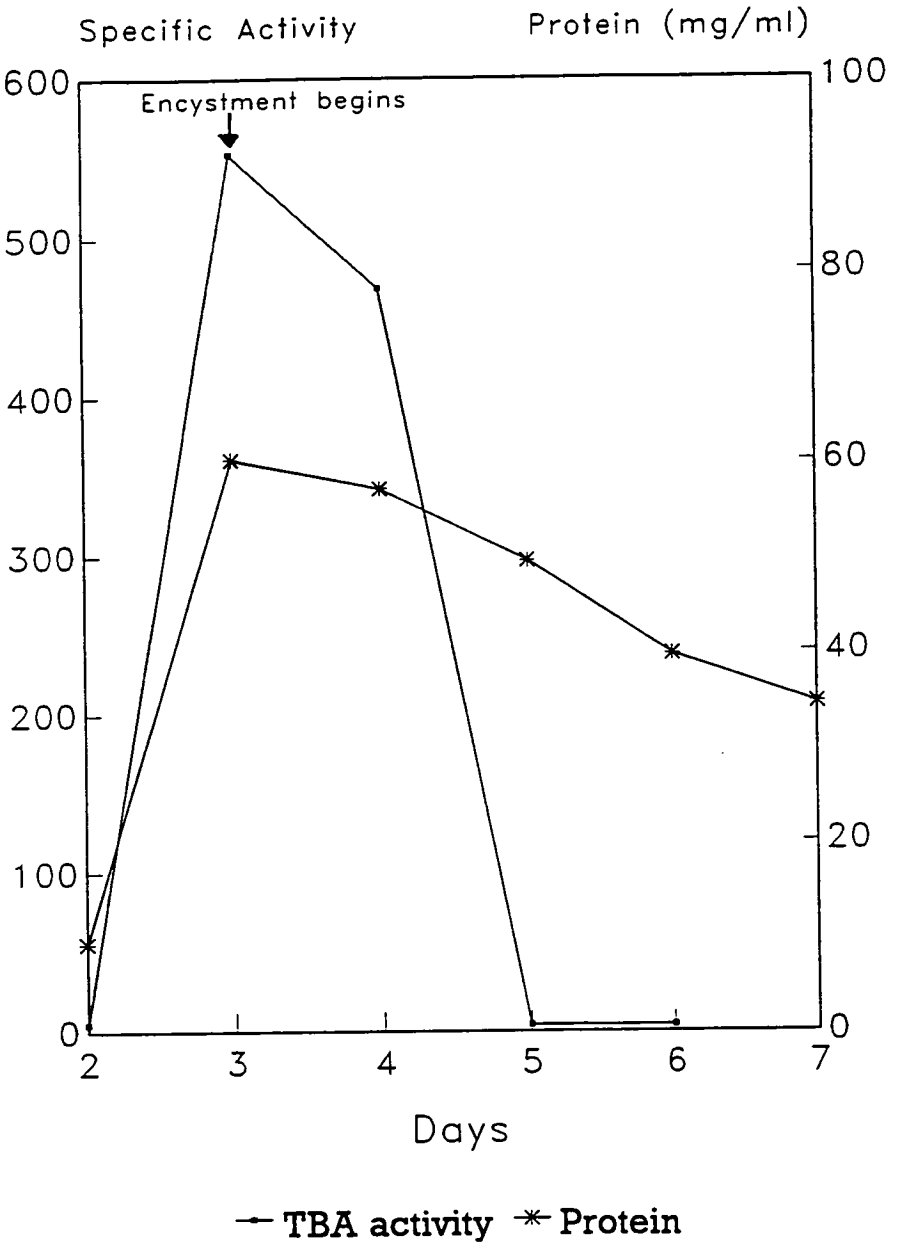
The bacteria were grown at 30°C for 72h. Cyst formation was induced by the addition of 0.3 β-hydroxy butyrate to washed cells. After sonication, the cell debris was removed by centrifugation. The fractions were tested for *alginate lyase* (Specific Activity ρ moles ml^{-1} mg $protein^{-1}$) and *alkaline phosphotase* activity (Specific Activity μ moles ml^{-1} mg $protein^{-1}$).

Fig. 13 The Effect of Encystment and Cyst Germination on Av Ax17.78 *Alginate lyase.*



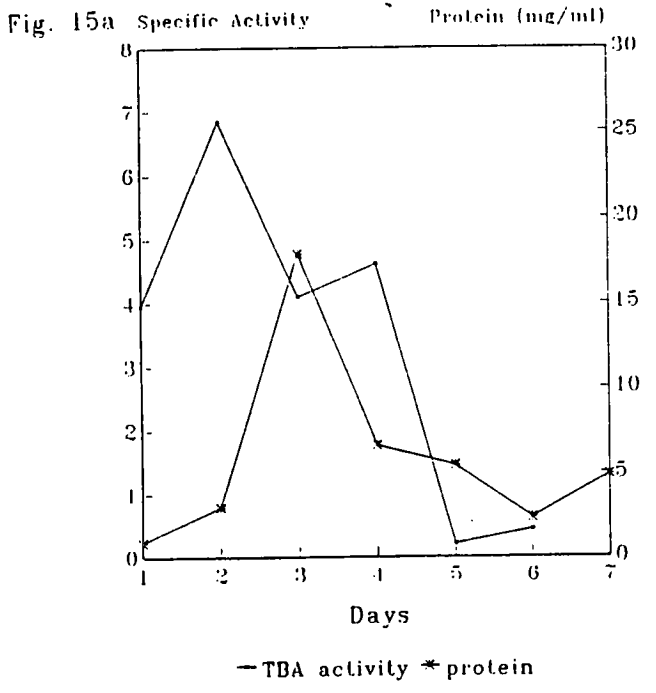
Av AX17.78 was grown in BM with glucose. The cells were harvested by centrifugation and washed. The formation of cysts were induced by the addition of 0.3% β-hydroxybutyrate to BM salts (no carbon source). Samples were removed until 78h post-induction, after which the cysts were harvested, washed and resuspended in medium containing 1% glucose. Samples of germinating cysts were harvested until 90h post-germination. The protein concentration and specific activity (ρmoles ml^{-1} mg $protein^{-1}$) of *alginate lyase* in the sample was determined

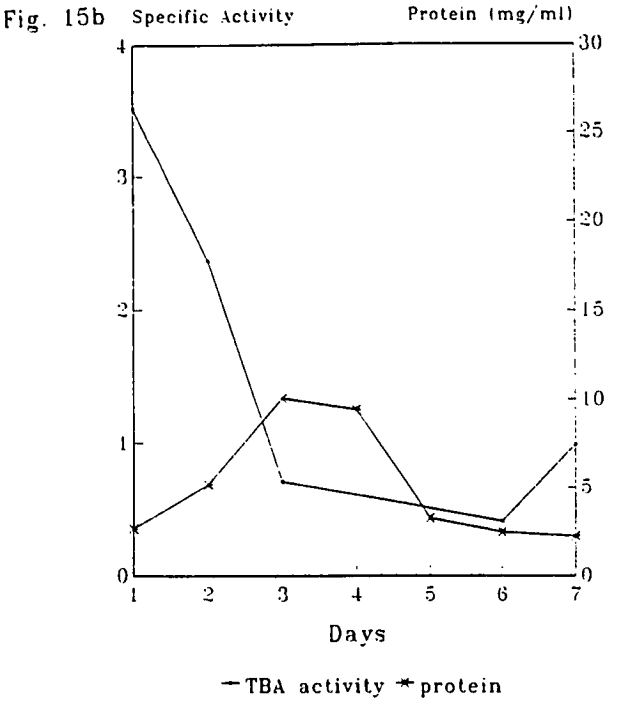
Fig. 14 The Formation of Abortive Cysts in Av Ax17.78 and its Effect on *Alginate lyase*.



Av Ax17.78 was grown in BM with glucose (1%). The cells were harvested by centrifugation and washed. The formation of abortive cysts were induced by the addition of 0.3% β-hydroxybutyrate to calcium free Burks salts. The protein concentration and specific activity (pmoles ml^{-1} mg $protein^{-1}$) of *alginate lyase* in the sample was determined.

Fig. 15 The Effect of Lanthanum on Alginate Lyase Activity During Encystment of Av Ax17.78.





Av Ax17.78 was grown in BM (1% glucose) with lanthanum acetate (50μM). The cells were harvested by centrifugation and washed. The formation of cysts were induced by the addition of 0.3% β-hydroxybutyrate BM salts ((50μM lanthanum, no carbon source). Fig. 15a shows the control experiment (calcium was present in the medium) and Fig. 15b show the effect of lanthanum. The protein concentration and specific activity (ρmoles ml^{-1} mg $protein^{-1}$) of *alginate lyase* in the sample was determined

A. vinelandii has a generation time of 2.5-3h when grown in BM with glucose at a concentration of 0.2-1% (Wilson & Knight, 1952). When grown in sublethal concentrations of butan-1-ol, growth was slow but encystment occurred within 120h (Wyss *et al.*, 1969). When BHB was added to washed cells grown exponentially in glucose-containing BM, complete and synchronous encystment occurred within 48h. BHB is a normal metabolite of *A. vinelandii* and in the presence of BHB at 30°C, encystment was complete within 72h. Its polymeric form, PHB, is accumulated during vegetative cell growth and is thought to play an important role in the regulation of glucose metabolism by reducing the inhibitory levels of reducing pyridine nucleotide factors (Senior & Dawes 1971). It can also be used as an endogenous carbon source or as a promoter of endotropic encystment (Stevenson & Socolofsky 1972).

The fact that BHB can be metabolised to acetate has been used to study biochemical changes which occur in the encysting cells. Glucose is normally metabolised by the Entner-Doudoroff pathway. During encystment the metabolic enzymes become mobilised to oxidise the acetate formed from BHB metabolism and to synthesise cyst components. This involves a glyoxylate shunt pathway and gluconeogenesis. Hitchens & Sadoff (1973) found that after induction by 0.2% BHB, cyst formation was complete after 36h. Analysis of metabolic enzymes showed that *glucose-6-phosphate dehydrogenase* was lost immediately and decreased linearly for 9h. *BHB dehydrogenase* was induced immediately; an initial peak was observed at 6h but it was maximally induced at 21h. This bimodal effect was also observed in enzymes involved in the glyoxylate shunt and gluconeogenesis. *Isocitrate lyase* and *malate synthase* are produced at low levels in vegetative cells but on induction of cyst formation they are rapidly induced and peak at 3h and again at 21h. The final

cell division occurs 3h post induction. The enzymes, *fructose 1,6 diphosphate* aldolase and *fructose 1,6 diphosphatase* exhibit the same bimodal action but in a temporal relationship.

After induction, DNA synthesis proceeded at a decreasing rate for 3h, by which time a 15-18% increase in total DNA occurred (Sadoff, 1973, Sadoff *et al.*, 1971). This represented a complete replication of the chromosome and used endogenous carbon rather than that derived from BHB metabolism. The final cell division occurred at 4h and therefore each pre-cyst contains a full complement of DNA. The rate of RNA synthesis decreased on induction and the overall RNA synthesis stopped by 12h. The carbon skeletons for RNA synthesis are derived from BHB and the main product is ribosomal RNA. Protein synthesis is thought to occur throughout encystment and cell components were produced from BHB. A high rate of protein synthesis was maintained for 9h and then decreased to 40% of the initial rate until cyst maturation was complete.

Encystment involves the sequential turning on and off of specific genes during development. Encystment-specific proteins appear to be synthesised at the expense of non-essential proteins which are degraded to provide amino acids for the synthesis of new proteins (Ruppen *et al.*, 1983, Sadoff *et al.*, 1979). Su *et al.* (1987) studied protein synthesis at different times during the encystment of *A. vinelandii* (ATCC 12837) by pulse labelling with ^{35}S methionine, in an attempt to identify the genes which were inactive during vegetative growth but become activated to produce encystment-active proteins. Su *et al.* (1987) studied the enzymes involved in lipid synthesis, since the changes in membrane lipids are among the first discernible differentiation-specific events occurring after induction of cyst formation. The membranes of vegetative *A. vinelandii* cells contain phospholipids, whereas cysts

have a bilayer of alkylresorcinols and alkylpyran-2-ones (Reusch *et al.*, 1981). Pulse labelling showed that early encystment proteins began to be synthesised at 2h and reached a peak at 12h after initiation of encystment. The concentration of early proteins began to decrease at 16h when intermediate proteins specific to differentiation began to be synthesised. The synthesis of these proteins stopped at 20h post-induction and the labelling pattern of protein remained constant for the remaining four days of encystment. When chloroamphenicol (100mg ml^{-1}) was added to the culture it blocked the synthesis of early cyst proteins. Addition between 0-4h after the addition of BHB to vegetative cells, blocked the whole encystment process. There was also no shift of membrane proteins when chloroamphenicol was present. However, there was no effect if it was added 6h or more after initiation of encystment. Hitchens & Sadoff (1973) reported that encysting cells became resistant to chloramphenicol (100μg ml^{-1}) upon induction. This was not due to the chloramphenicol binding to the ribosomes but to a change in the membrane permeability of encysting cells which prevented it entering the cell.

After cyst induction by BHB, protein synthesis decreased sharply but resumed within 4h, producing a different set of proteins from the vegetative cells. Other biochemical studies indicated that encystment reached an important transition point between 8-12h post-induction. DNA synthesis stopped at 4h after induction but net RNA synthesis continued 12h into the encystment process.

A. vinelandii cyst germination takes place over 8h when supplemented with 1% glucose and can be equally divided into two distinct phases "germination" and "outgrowth". Cysts could not be induced to germinate by heat or cold shock or freezing. Respiration, RNA and protein synthesis began soon after germination commenced. When glucose was added to BM, the cysts began to respire immediately

and produce CO_2 (Loperfido & Sadoff, 1973). As the rate of respiration and RNA synthesis increased sharply between 4-5h, cysts must have a full complement of enzymes for glucose metabolism, ATP formation and the synthesis of macromolecules. Germination of cysts was blocked if protein synthesis, RNA synthesis or ATP formation was blocked (Loperfido & Sadoff, 1973).

"Outgrowth" began 4h into germination and was signalled by DNA synthesis and N_2 fixation, both of which continued at high rates until the vegetative cell emerged from the cyst coat. The rate of respiration and RNA synthesis increased to a level greater than the germination level (Sadoff, 1975). Outgrowth terminated at 8h and the cell emerged.

Loperfido & Sadoff (1973), reported that chloroamphenicol (33μg ml^{-1}), dinitrophenol (0.1mM) and streptomycin (330μg ml^{-1}) blocked the initial increase in respiration and cyst germination. The synthesis of protein and RNA began immediately on the addition of glucose to the cyst suspension. The rate of protein synthesis was detected by the incorporation of ^{14}C-leucine, which was constant for 5h, after which it increased threefold and remained at this level until the germinated cell emerged. RNA synthesis was carried out at a much lower level for the first 3.5h after which there was a seven fold increase by 5h and again remained at this level until the vegetative cell emerged. The initiation of outgrowth was inferred by the onset of DNA synthesis which occurred 5h after the addition of glucose.

Most reports of *alginate lyase* enzymes, apart form that associated with *A. vinelandii* phage (Davidson *et al.*, 1977), relate to extracellular enzymes. They may cleave the polymer to produce fragments which could be utilised as carbon and energy source and feed into the tricarboxylic acid cycle (TCA) as described by Preiss & Ashwell (1962). Hacking *et al.* (1983) postulated that the role of *alginate lyase* in

A. vinelandii was to degrade alginate during encystment, thus making mannuronate residues more accessible to the action of the C5 epimerase and permitting the conversion of mannuronate to guluronate required during encystment. This would appear not be the case in Av Ax17.78 since the level of *alginate lyase* decreased during encystment. The *alginate lyase* was apparently switched off during encystment, since it was not involved in the encystment process. This would be consistent with the switch from the normal complement of metabolic enzymes to the production of those present in the cyst.

The *alginate lyase* described in this study was located in the periplasm and would **not** normally be expected to come into contact with the substrate. Strain Av Ax17.78 cannot grow on its own alginate as sole carbon and energy source. Therefore the *alginate lyase* in Av Ax17.78 **cannot** function as a means of utilising an external polymeric carbon and energy source.

The induction of *alginate lyase* in *Azotobacter* spp grown on alternative carbon sources.

The four strains of *Azotobacter* spp.; Av UW, Av O, Ac 184 and Av Ax17.78 were grown in 100ml BM supplemented with 1% glucose at 30°C on an orbital shaker (200 rpm). The OD_{600} was monitored and plotted against time (Fig. 16a, b). Since the mucoid strain, Av Ax17.78 produced copious amounts of alginate, measuring optical density was not an accurate method of determining growth rate. Greater accuracy was obtained by performing viable counts. At 21.5h there were 9.44 x 10^2 c.f.u.ml^{-1} and at 45.75h there were 1.70 x 10^8 c.f.u.ml^{-1}.

Strains Av UW and Ac 184 were pre-adapted in 100ml BM supplemented with the following carbon sources, 1% glucose, 1% glutamate, 1% glycerol and 0.1%

glucose & 0.1% alginate. The cells were pelleted aseptically and transferred to fresh medium supplemented with the appropriate carbon source. Av UW grew well on glucose and glycerol, but poorly on glutamate Strain Ac 184 grew well on 1% glucose and 1% glutamate and poorly on 1% glycerol. Neither strain grew on alginate after the glucose had been depleted (Fig. 17a, b).

Pre-adapted cultures, grown in 1% glucose and in 1% glycerol (Av UW) were inoculated into fresh flasks of the same medium (1l) and grown for 48h at 30°C. The 1% glucose and 1% glutamate-grown, Ac 184 cells were treated in a similar manner but grown for 24h. The Av UW total cell extracts had a specific activity of 676ρ moles ml^{-1} mg $protein^{-1}$ and 933ρmoles ml^{-1} mg $protein^{-1}$, glucose:glycerol grown cell respectively. The glucose and glutamate grown Ac 184 had a specific activity of 10,527ρmoles ml^{-1} mg $protein^{-1}$ and 56ρmoles ml^{-1} mg $protein^{-1}$ respectively. For both strains glucose was used as the carbon source for production of bacteria for alginate lyase activity. It was also noted that Ac 184 *alginate lyase* was much more active than the Av UW lyase

Different concentrations of glucose were used to determine the optimum concentration for growth of Av UW. Over 24h, at 30°C in BM Av UW grew best between 0.4-1% glucose (Fig. 18). Fig. 17 demonstrates that Av UW was unable to grow on alginate as the sole carbon source but we did not know if alginate induced *alginate lyase*. Av UW was grown in BM (0.1% glucose), in 0.1% glucose and alginate (0.05, 0.1, 0.2%) and alginate alone. One set of flasks were harvested at 10.25h and the other at 26.25h. Total cell extracts were prepared, *alginate lyase* activity and protein concentration were determined (Table 10). It appeared that the presence of alginate in the medium did not induce *alginate lyase* since the 26.25h cells had lower lyase activity compared to the cells grown in glucose. Similar levels

of activity were observed in the 10.25h sample but the bacteria were still growing on glucose in the medium. The level of lyase activity in the alginate only flask (10.25h), could be accounted for by the cells using stored PHB as a carbon source.

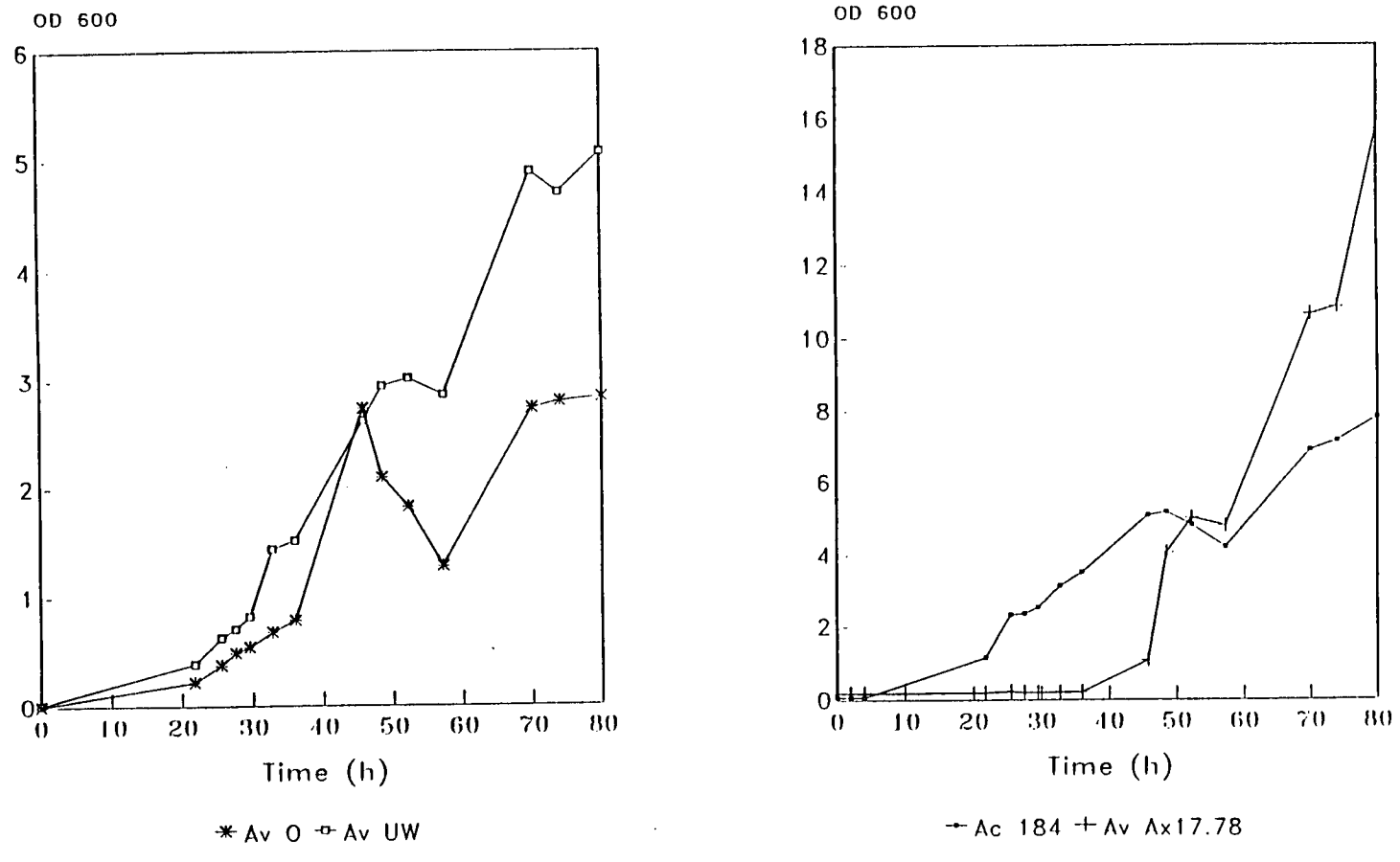
Table 9 The Effect of Chloroamphenicol on *Alginate Lyase* During Encystment and Cyst Germination.

	Alginate Lyase pmole ml^{-1} mg $protein^{-1}$	*Alkaline Phosphatase* µmole ml^{-1} mg $protein^{-1}$	Protein mg ml^{-1}
48h cells	481	22	80.0
72h cells	107	22	24.4
96h cells	166	112	12.56
24h cysts	425	47	8.8
48h cysts	85	13	15.0
72h cysts	149	40	12.4
24h cysts*	108	14	80.0
48h cysts*	103	12	42.4
72h cysts*	232	7	18.6
24h cells	14	3	24.6
24h cells*	16	6	36
24h cells**	14	9	28.5
24h cells***	12	4	37.7
96h cells	19	57	26.5
96h cells*	95	8	60.5
96h cells**	12	16	29.5
96h cells***	7	8	55.0

* Chloroamphenicol added during encystment.
** Chloroamphenicol added during germination.
*** Chloroamphenicol added during encystment and germination.

Fig. 16

Growth Curves of *Azotobacter* spp.



The bacteria Av O, Av UW, Ac 184 and Av Ax 17.78 were grown in 100ml BM (1% glucose) at 30°C on an orbital shaker (200rpm). Samples were removed and the optical density measured at 600nm.

Fig. 17 Growth of Av UW and Ac 184 on Alternative Carbon Sources.

Fig. 17a OD 600

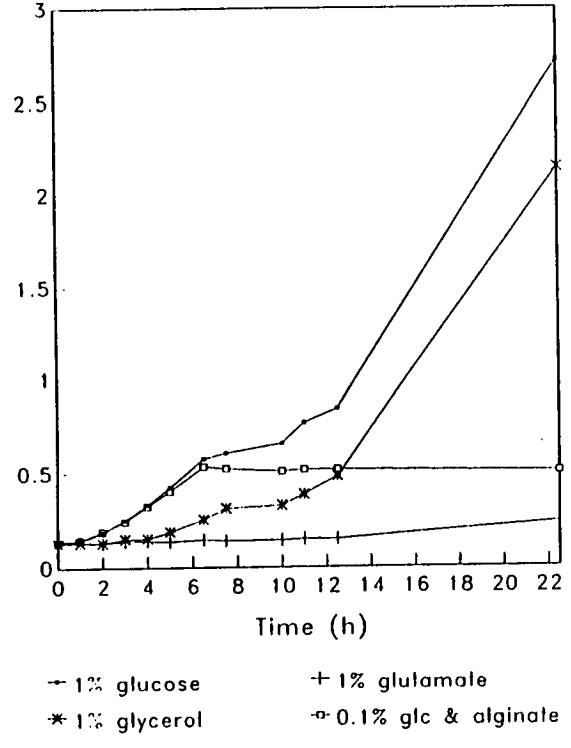
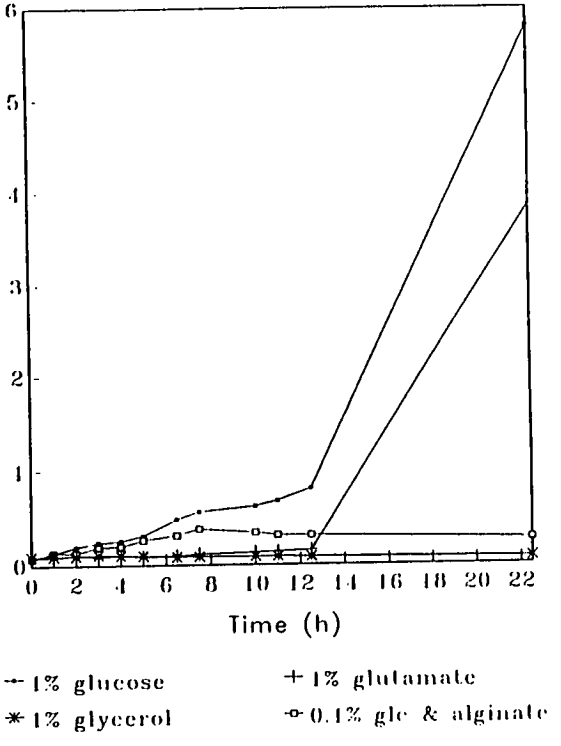
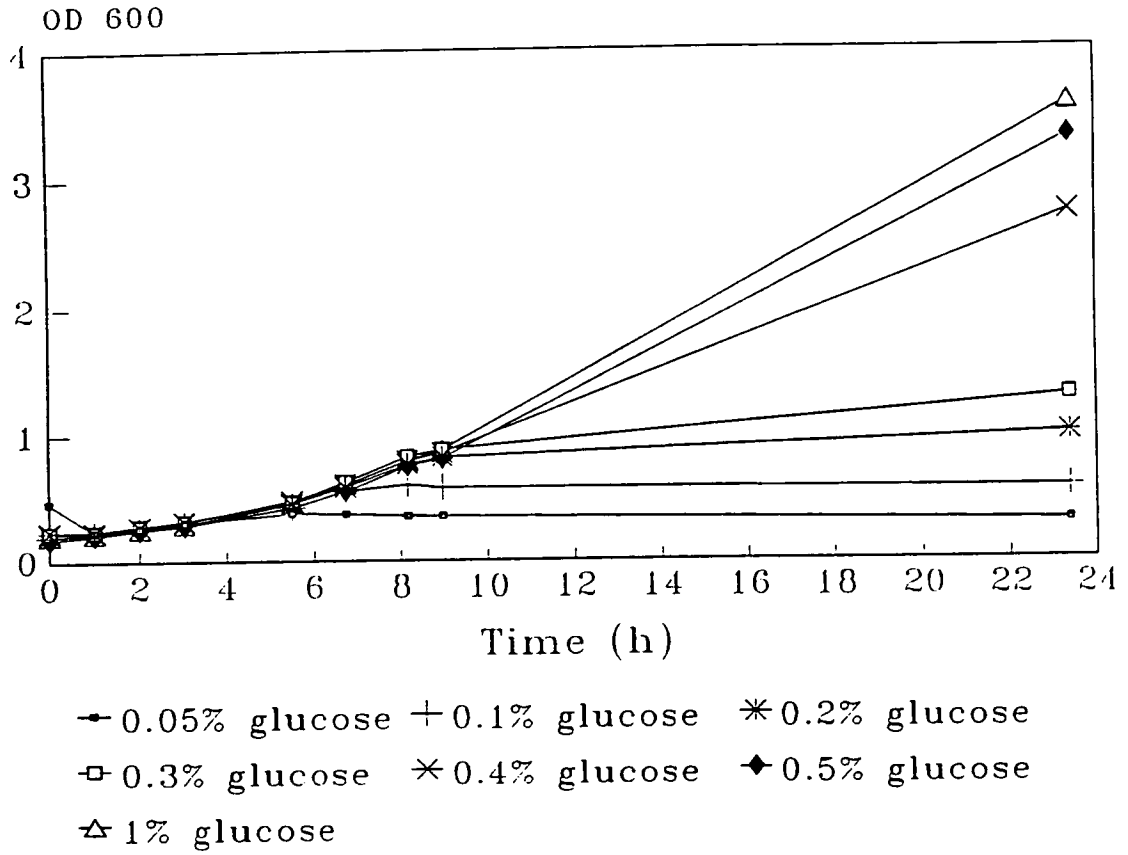


Fig. 17b OD 600



The bacteria, Av UW and Ac 184 were grown in 100ml BM supplemented with one of the above. Samples were removed and the optical density measured at 600nm

Fig. 18 Growth of Av UW on Various Concentrations of Glucose.



The bacterium Av UW was grown in 100ml BM containing different concentrations of glucose at 30°C on an orbital shaker (200rpm). The optical density was measured at 600nm.

Table 10 Induction of Av UW *Alginate Lyase* by the presence of alginate in the growth medium.

Total Cell Extract 10.25h

Growth Conditions	*Alginate Lyase* (ρmole ml-^{1} mg protein^{-1})	Protein (mg ml^{-1})
0.1 % Glucose	404	27.48
0.1 % Glucose + 0.5 % alginate	630	32.25
0.1 % Glucose + 0.1 % alginate	515	26.75
0.1 % Glucose + 0.2 % alginate	420	20.5
0.1 % alginate	451	2.35

Total Cell Extract 26.25h

Growth Conditions	*Alginate Lyase* (ρmole ml-^{1} mg protein^{-1})	Protein (mg ml^{-1})
0.1 % Glucose	418	27.62
0.1 % Glucose + 0.05 % alginate	380	22.88
0.1 % Glucose + 0.1 % alginate	464	28.25
0.1 % Glucose + 0.2 % alginate	203	25.5
0.1 % alginate	115	0.045

Table 11 Isolation of *Alginate Lyase* from *A. chroococcum* Total Cell Extracts.

Strain	*Alginate lyase* (ρmole ml^{-1} mg $protein^{-1}$)	Protein (mg ml^{-1})	EPS
A. chroococcum 34	1239	45.0	+
A. chroococcum 46	2467	7.1	+
A. chroococcum 186	2035	17.5	+
A. chroococcum 184	1502	19.4	-
A. chroococcum 109	180	4.6	-
A. chroococcum 221	1705	20.7	-

Production and Isolation of *Alginate Lyase* in *Azotobacter* spp.

Professor W. Page (University of Alberta, Canada) supplied a number of *A. chroococcum* strains which were either capsulated or non-capsulated. The cells were grown at 30°C for 5d in 2l flasks, harvested and broken. The total cell extracts were assayed for *alginate lyase* activity and the protein concentration determined (Table 11). No lyase activity was present in the spent medium; both the capsulated and non-capsulated strains had intracellular *alginate lyase* activity. The protein concentration of the samples varied considerably from sample to sample. Since Ac 184 was the type strain for this bacterial species, it was used for the remainder of this investigation. The optimal incubation conditions for the *alginate lyase* assay were determined as follows: temperature 30°C, an overnight incubation at pH 5.8 (50mM MES buffer) and a substrate concentration of 5mg ml^{-1}. The alginate lyase of Av Ax17.78 had a pH optimum of 7.2 whereas the lyase of Av UW had a pH optimum of 5.6, otherwise the incubation conditions were the same for all strains.

Preliminary experiments showed that the constitutive *alginate lyase* activity from Av Ax17.78, Av UW, Av O and Ac 184 was located in the periplasm, in both exopolysaccharide (EPS^+) and non-exopolysaccharide (EPS^-) producing strains. The thiobarbituric acid (TBA) assay was routinely used to determine lyase activity. It is dependent on the oxidation of the uronic acid by periodate to form β-formyl pyruvic acid which reacts with TBA to form a pink chromophore. It became obvious early in the experimental work that alginate produced by the EPS^+ strains interfered with the TBA method by giving artificially high readings in the experimental assay and the enzyme-free control. In order to avoid this problem, after the initial experiments, EPS^- strains were used to isolate *alginate lyase*.

The cells were fractionated according to the procedure of Osborn *et al.* (1972); *alkaline phosphatase* was used as a periplasmic marker enzyme. Strain Av O (EPS^-) was grown on BM (1% mannitol) at 30°C for 48h. Sphaeroplasts were prepared and the specific activity of the *alginate lyase* from both the periplasmic and the cytoplasmic fractions determined. The periplasmic fraction had four times as much activity (74pmoles ml^{-1} mg $protein^{-1}$) as the cytoplasmic fraction (17pmoles ml^{-1} mg $protein^{-1}$). The purity of the cytoplasmic fraction was determined by assaying for *alkaline phosphatase* activity. Very low levels of activity were detected in the cytoplasm (9µmoles ml^{-1} mg $protein^{-1}$) whereas 485µmoles ml^{-1} mg $protein^{-1}$ activity was detected in the periplasmic fraction.

The release of the periplasmic contents of Av UW and Ac 184 was determined using the method of Ames *et al.* (1984). The optimum concentration of chloroform required to release the periplasmic proteins was determined. Various percentages of chloroform were added to resuspended cells. Both strains were grown at 30°C for 48h (Ac 184) and 72h (Av UW). Ac 184 was resuspended in 0.025 volume and Av UW in 0.004 volume before the chloroform was added. When Av UW was treated with chloroform, low levels of *alginate lyase* were detectable in the periplasmic fraction (Table 12). However, the higher levels of *alginate lyase* were detected in the cytoplasmic fraction. The implication of this was that either 20% chloroform was insufficient to effectively permeabilise the outer cytoplasmic membrane and release the periplasmic contents or that the *alginate lyase* was located in the cytoplasm.

Esterase activity was determined using ONP-acetate. This enzyme is thought to be located in the cytoplasm and was therefore used as a marker enzyme to test the integrity of the inner cytoplasmic membrane. No activity was detected in the periplasmic fraction but esterase activity was detectable in the cytoplasmic fraction

along with some other enzymes which are located in the cytoplasm (α-D-*galactosidase* and α-D-*mannosidase*). It would therefore appear that the periplasm was not contaminated with cytoplasmic proteins. Both inner and outer cytoplasmic membrane were intact and 20% chloroform was apparently insufficient to permeabilise the outer cytoplasmic membrane.

With strain Ac 184, 0.1% chloroform appeared to be sufficient to release the periplasmic contents of the cell. This fraction had a specific activity of 3,078pmole ml^{-1} mg $protein^{-1}$ (Table 12). A high level of *alginate lyase* activity was detected in the 5-20% chloroform-treated cells, but when the tests were made for the same cytoplasmic enzymes, the 5-20% chloroform-treated cells were positive within 1h whereas the material from 0.1% chloroform treatment had only turned slightly yellow after 4h.

The Av UW cytoplasmic fractions were pooled and loaded onto the Q Sepharose FF. column and eluted with 10mM TRIS pH 7.3. The purification scheme can be found in Table 13; there was a 2.5 fold purification in Fraction 2 (Fr 2). This was part of the gentle slope after the unabsorbed protein had eluted from the column. The total amount of protein in this sample was 0.58mg, and the *alginate lyase* activity was 200 (total activity) and may therefore be an artificial result. The elution profile of the 0.2M NaCl step is shown in Fig. 19. Most of the *alginate lyase* activity was detected in Fr 6, and was equivalent to a 9.4-fold increase in purity of the enzyme.

The Ac 184 0.1% chloroform periplasm and cytoplasmic fractions were loaded separately onto the Q Sepharose FF. column and eluted with a step NaCl gradient. The elution profile of the periplasmic proteins can be found in Fig. 20a. As well as monitoring the OD_{280}, an aliquot from the periplasmic fractions was removed and the

TBA activity in the fractions determined. (Fig. 20b). Fig. 21a and 21b show the elution profile of the cytoplasmic proteins under the same conditions. The separate protein peaks were pooled for both periplasmic and cytoplasmic fractions and tested for *alginate lyase* activity and protein concentration.

The purification scheme for both the 0.1% chloroform periplasmic and cytoplasmic fractions can be found in Table 14. The main purification step was achieved by passing the crude protein extract down the Q Sepharose FF. column; the greatest increases in purity in both cases were found in the unbound material. A 2.8-fold increase in the enzyme activity in the periplasmic fraction and a 3.5-fold increase in the cytoplasmic fraction were obtained.

Isolation of *Alginate Lyase* From Av UW and Ac 184 Total Cell Extracts.

Alginate lyase was isolated from cells grown at 30°C on BM supplemented with 1% glucose as sole carbon source. The cells were grown for 96h and samples removed at 24h intervals. Cells were pelleted by centrifugation and broken as described in 2.2. The fractions were then tested for lyase activity and the protein concentration determined. The results are shown in Table 15. The Ac 184 *alginate lyase* is clearly much more active than the Av UW *alginate lyase*.

Purification of *alginate lyase* from Av UW and Ac 184 by ammonium sulphate precipitation.

Av UW and Ac 184 were grown in BM (1% glucose), for 36h, harvested, and total cell extracts prepared. After ultracentrifugation the cytoplasm was precipitated with ammonium sulphate. At each step, TBA activity and protein concentration were determined. The results can be found in Table 16. A 2.7-fold increase in purity was

obtained at 40% $(NH_4)_2SO_4$ saturation with Av UW. Ac 184 obtained a 1.2 fold purification at 40% $(NH_4)_2SO_4$ saturation and a 1.2-fold purification at 50% $(NH_4)_2SO_4$ saturation.

A 500µl aliquot of Av UW 40% saturated insoluble fraction was loaded onto the affinity column and eluted with a 200ml 0-0.4M NaCl linear gradient. 1ml fractions were collected and the OD_{280} monitored (Fig. 22). The tubes containing protein were pooled to give three fractions. The protein concentration and TBA activity were determined, the results of which can be seen in Table 17. Fraction 2, which eluted between 36-90mM NaCl and was the most active fraction, it was purified 9.5-fold by this step.

Table 12 Release of the Periplasmic contents of Av UW and Ac 184.

% Chloroform	Av UW Periplasm *Alginate lyase* (ρmoles ml^{-1} mg $protein l^{-1}$)	Protein (mg ml^{-1})
0.1	4413	0.12
0.5	413	0.08
1	1149	0.06
2	2463	0.11
5	2777	0.30
10	335	0.72
20	632	0.66

% Chloroform	Av UW Cytoplasm *Alginate lyase* (ρmoles ml^{-1} mg $protein^{-1}$)	Protein (mg ml^{-1})
0.1	4943	34.0
0.5	5161	31.0
1	12175	18.0
2	3852	34.25
5	4856	21.0
10	19057	6.0
20	3827	23.25

% Chloroform	Ac 184 Periplasm *Alginate lyase* (ρmoles ml^{-1} mg $protein^{-1}$)	Protein (mg ml^{-1})
0.1	3078	0.22
0.5	1436	0.38
1	552	0.74
2	2188	1.56
5	8423	8.45
10	5827	19.25
20	5228	21.62

% Chloroform	Ac 184 Cytoplasm *Alginate lyase* (ρmoles ml^{-1} mg $protein^{-1}$)	Protein (mg ml^{-1})
0.1	2252	1.24
0.5	3234	0.72
1	2155	0.21
2	3155	1.75
5	9130	8.6
10	4156	0.22
20	6595	2.52

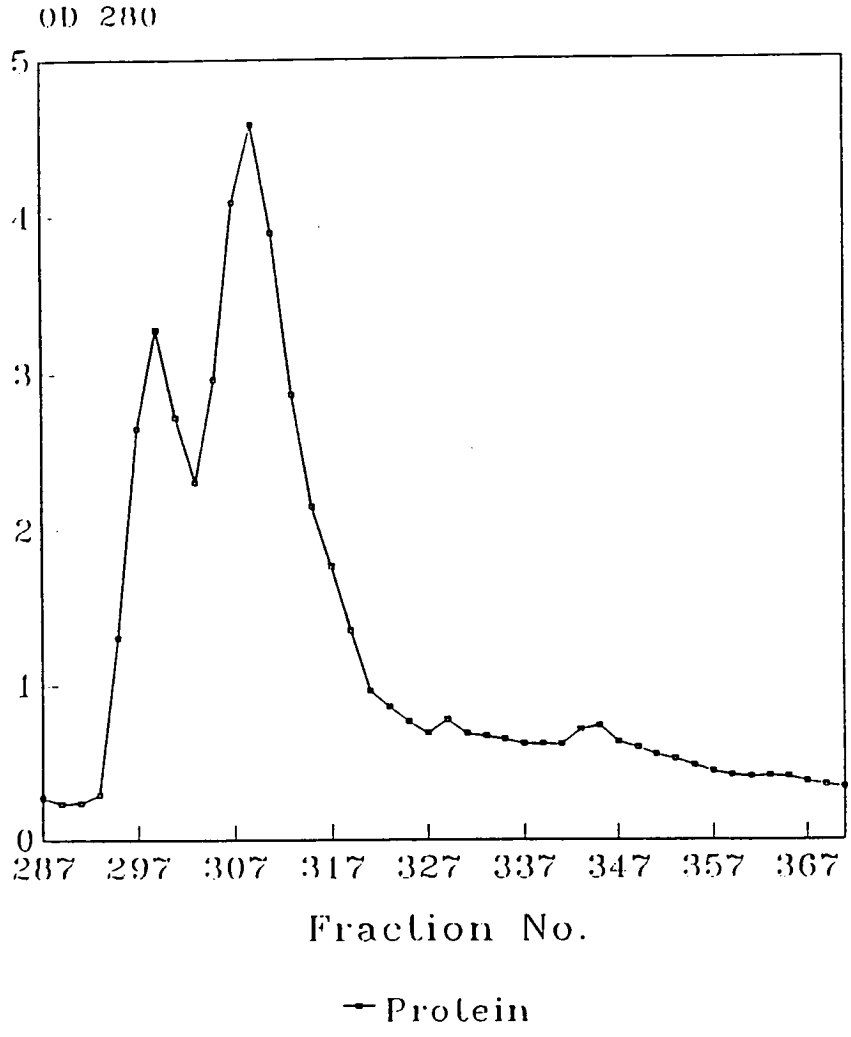
After centrifugation the cells were resuspended in 0.004 volumes (Av UW) and 0.25 volumes (Ac 184). The periplasmic contents were released from the cells by the addition of various concentrations of chloroform. The cytoplasmic contents were released by sonication. Both samples were dialysed against $d.H_2O$, concentrated against PEG 6000. The protein concentration (Biorad micro assay) and the*alginate lyase* activity (TBA assay) in the fractions were determined.

Table 13 Purification Scheme for Av UW *Alginate lyase* After Elution from Q Sepharose FF.

Fraction No.	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity (ρmole ml^{-1} mg protein)	Purification (Fold)
Pool	68270	501.619.0	26.4	136	1	
1	784	38.35	1.3	3.0	20	0.2
2	200	0.6	0.2	2.9	345	2.5
3	0	0.46	0.33	1.4	0	-
4	1028	29.6	4.0	7.45	34	0.2
5	7390	47.23	7.05	6.7	156	1.2
6	22682	17.71	13.62	1.3	1.280	9.4
7	0	79.44	16.38	4.85	0	-
8	0	4.56	3.38	1.35	0	-
9	0	4.14	2.3	1.8	0	-

All the fractions obtained from the Av UW chloroform treated cytoplasm were poled (26.4ml) and applied to the Q Sepharose FF. Unadsorbed material was eluted from the column with 10mM TRIS pH 7.3. A step NaCl gradient was applied (0.2, 0.35, 0.7 and 1M). The fractions were pooled as follows: Fr. 1, tubes 30-110 (unadsorbed),Fr. 2, tubes 111-250 (unadsorbed), Fr. 3, tubes 279-287 (0.2M NaCl), Fr. 4, tubes 294-303 (0.2M NaCl), Fr. 5, tubes 304-327 (0.2M NaCl), Fr. 6, tubes 328-370 (0.2M NaCl), Fr. 7, tubes 482-520 (0.35M NaCl), Fr. 8, tubes 543-567 (0.35M NaCl), Fr. 9, tubes 667-669 (0.7M NaCl).

Fig. 19 The Elution Profile of Fr. 2 from the Chloroform Treated Av UW Cytoplasm with 0.2M NaCl.



0.2M NaCl was applied to the Q Sepharose FF. column between tubes 279-370. The fractions were pooled as in Table 13.

Fig. 20a Elution Profile of Ac 184 (0.1%) Chloroform Periplasm From Q Sepharose FF.

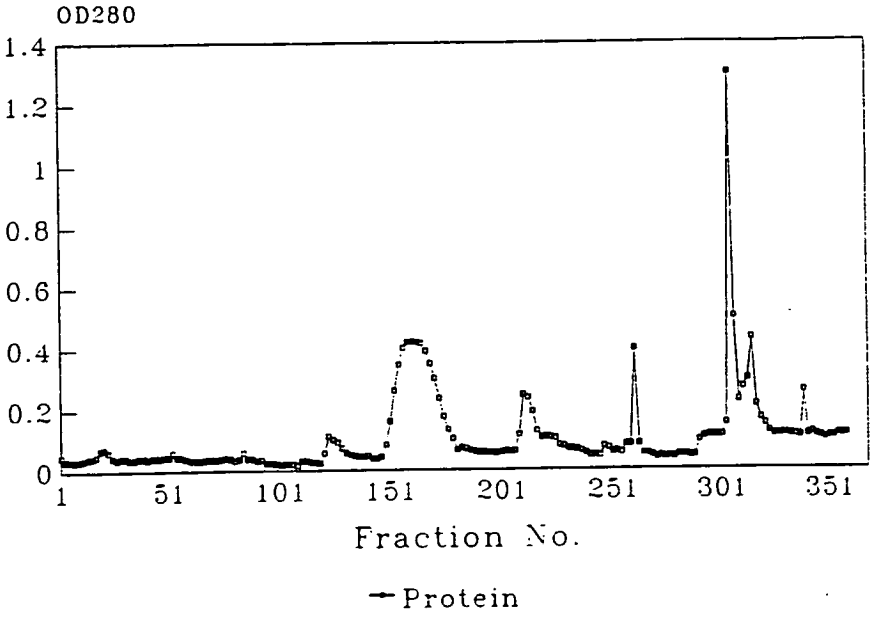
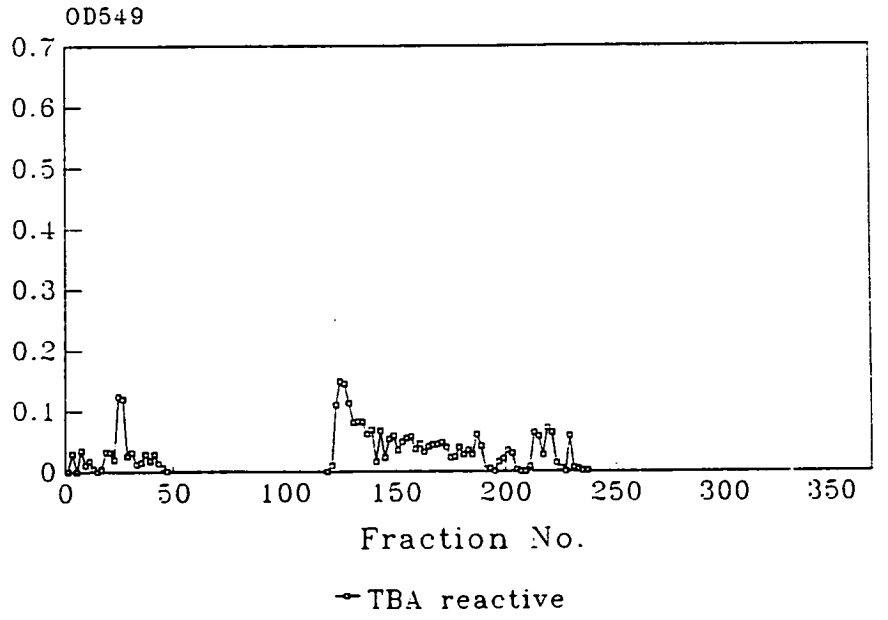


Fig. 20b Elution Profile of TBA ACtive Fractions from Ac 184 (0.1% Chloroform) Periplasm from Q Sepharose FF.



5ml, Ac184 periplasm was applied to the Q Sepharose FF.column and eluted with a step gradient. The unbound material was eluted with 10mM TRIS pH 7.0. Seven protein peaks eluted from the column in total. Fr. 1 (24-28. unabsorbed), Fr. 2. (122-145. 0.2M NaCl), Fr. 3 (149-181. 0.2M NaCl). Fr. 4. (182-207. 0.4M NaCl), Fr. 5 (208-227. 0.4M NaCl), Fr. 6. (259-263. 0.4M NaCl)Fr. 7. (301-325. 1M NaCl).

Fig. 21a Elution Profile of Ac 184 (0.1%) Chloroform Cytoplasm From Q Sepharose FF.

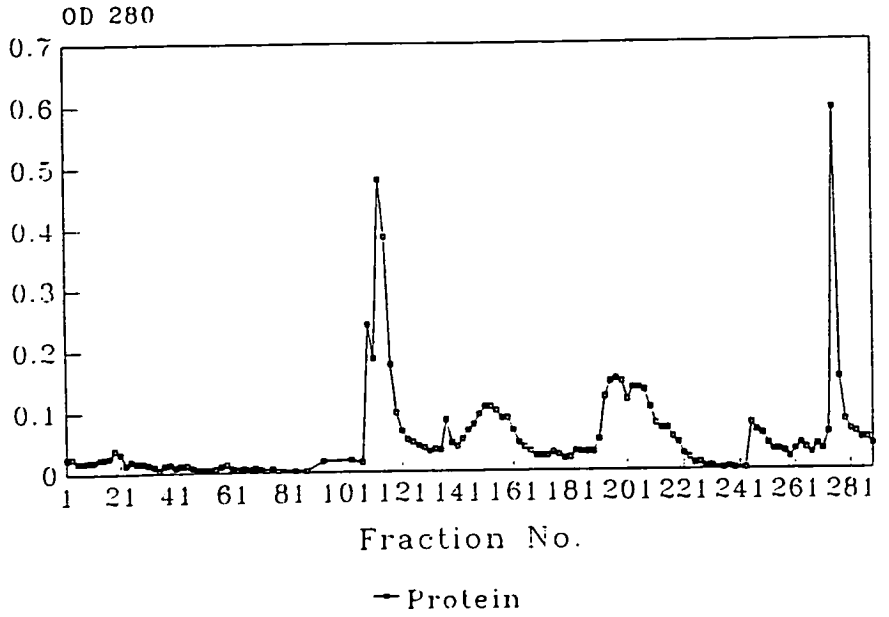
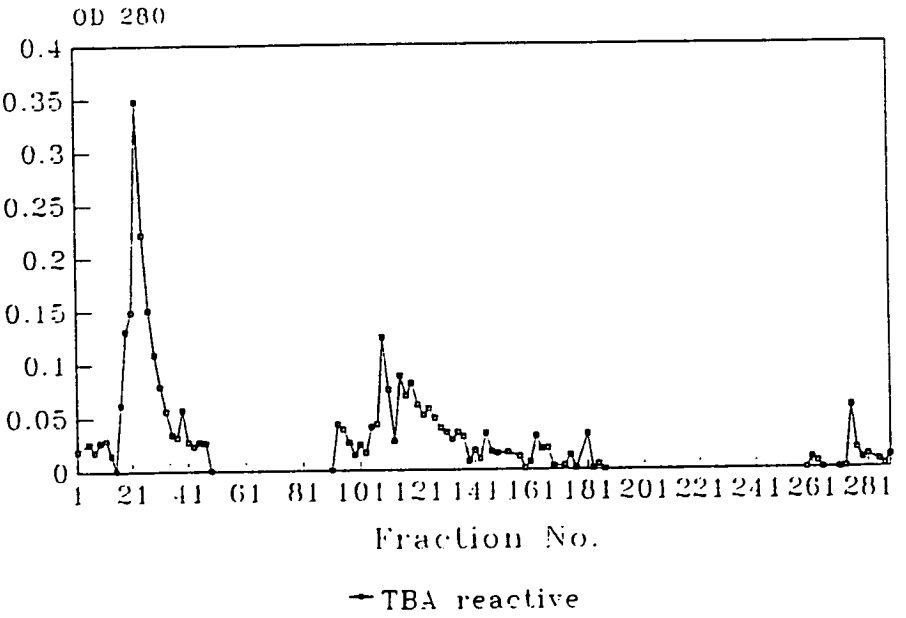


Fig. 21b Elution Profile of TBA Active Fractions from Ac 184 (0.1% Chloroform) Cytoplasm from Q Sepharose FF.



2.5ml, Ac184 cytoplasm was applied to the Q Sepharose FF.column and eluted with a step gradient. The unbound material was eluted with 10mM TRIS pH 7.0. Five protein peaks eluted from the column in total. Fr. 1 (17-31. unabsorbed), Fr. 2, (106-121, 0.2M NaCl), Fr. 3 (143-161. 0.2M NaCl), Fr. 4,.(191-220. 0.4M NaCl), Fr. 5 (274-279, 1M NaCl).

Table 14 Purification Scheme for Ac 184 (0.1% Chloroform) Periplasmic and Cytoplasmic Fractions After Separation on Q Sepharose FF.

Periplasm

Fraction No.	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity (ρmole ml^{-1} mg protein^{-1})	Purification (Fold)
Original	2065	1.12	0.22	5	17208	1
1	511	0.01	0.01	1.05	48667	2.8
2	0	0.08	0.05	1.4	0	-
3	895	0.26	0.10	2.5	3442	0.2
4	350	0.07	0.04	1.7	5282	0.3
5	470	0.14	0.07	1.95	3302	0.2
6	207	0.04	0.02	1.5	5750	0.3
7	30	0.12	0.13	0.9	263	-

Cytoplasm

Fraction No.	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity (ρmole ml^{-1} mg protein^{-1})	Purification (Fold)
Original	6982	3.1	1.24	2.50	2252	1
1	376	0.05	0.03	1.6	7833	3.5
2	0	0.84	0.84	1.0	0	-
3	14	0.02	0.03	0.85	636	0.3
4	538	0.67	0.29	2.3	800	0.3
5	43	0.02	0.06	0.35	1972	0.9

Table 15 The Isolation of *Alginate lyase* from AvUW and Ac 184 Total Cell Extracts.

Av UW

Time	*Alginate lyase* (ρmoles ml^{-1} mg $protein^{-1}$)	Protein (mg ml^{-1})
24	31	14.3
48	21	23.4
72	69	20.3
96	55	40.0

Ac184

Time	*Alginate lyase* (ρmoles ml^{-1} mg $protein^{-1}$)	Protein (mg ml^{-1})
24	3646	2.1
48	9971	10.9
72	9888	8.4
96	11033	7.7

% Saturation Ammonium Sulphate	Av UW Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
0	124650	390	13.0	30	319	1.0
30 soluble	59479	223.85	7.72	29	265	0.8
30 insoluble	3414	18.5	6.25	3	182	0.6
40 soluble	1716	71.50	2.75	26	24	0.08
40 insoluble	67956	80.25	26.75	3	847	2.7
50 soluble	-	11.00	26.75	22	-	-
50 insoluble	4449	56.64	18.88	3	78	0.2
60 soluble	0	2.66	0.14	19	-	-
60 insoluble	537	16.59	5.53	3	32	0.1
70 soluble	-	0.65	0.05	13	-	-
70 insoluble	-	0.29	0.29	1	-	-

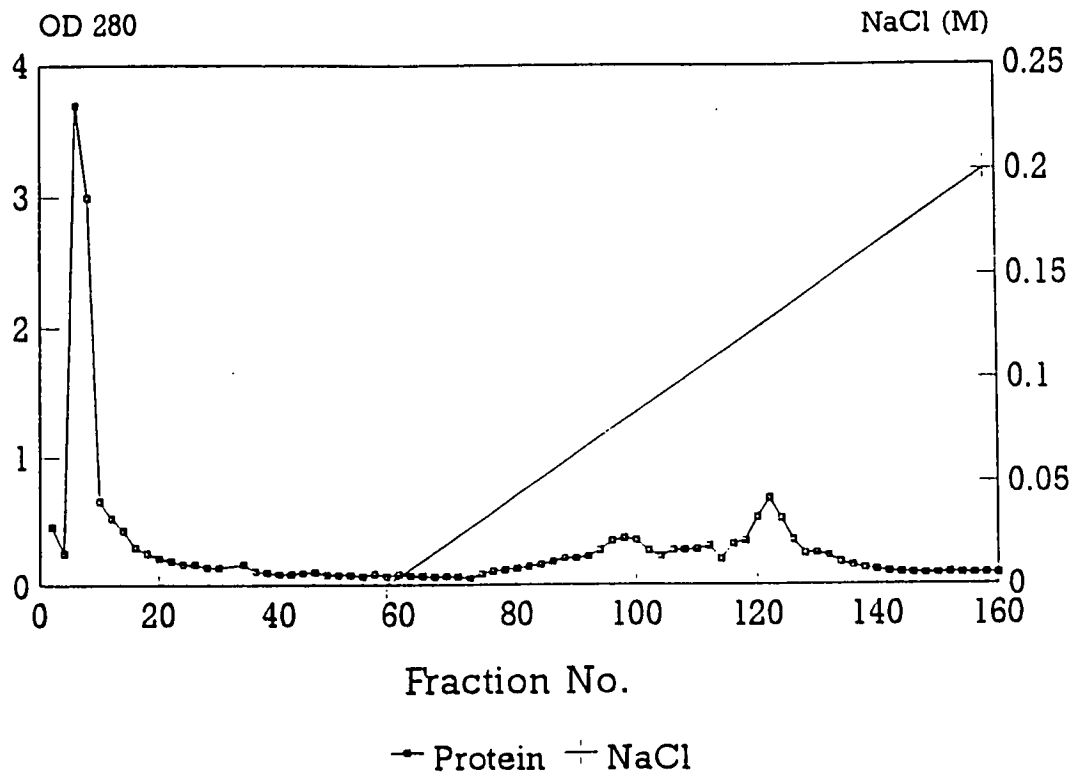
% Saturation Ammonium Sulphate	Ac 184 Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mgml^{-1})	Volume (ml)	Specific Activity	Purification Fold
0	24605	72.1	2.06	35	341	1.0
30 soluble	8432	32.64	0.96	34	258	0.7
30 insoluble	114	8.78	8.78	1	13	0.04
40 soluble	5766	13.95	0.45	31	413	1.2
40 insoluble	951	17.40	5.80	3	54	0.2
50 soluble	1375	4.95	0.18	27.5	278	0.8
50 insoluble	3042	7.59	2.53	3	400	1.2
60 soluble	-	1.20	0.05	24	-	-
60 insoluble	1677	6.93	2.31	3	242	0.7
70 solub	-	1.02	0.05	20.5	-	-
70 insoluble	15	0.36	0.36	1	42	0.1

Specific Activity (ρmole ml^{-1} mg protein^{-1})

Solid ammonium sulphate was added at 4oC to the total cell extracts, until the required saturation was reached. The material was stirred for 1h before cetrifugation. An aliquot of the two fractions were retained and after dialysis were tested for *alginate lyase* activity and the protein concnetration determined.

Fig. 22

The Elution Profile of the 40% Insoluble Ammonium Sulphate Fraction from Av UW from the Affinity Column.



500µl of the Av UW 40% ammonium sulphate insoluble fraction was applied to the affinity column. The unbound material was eluted with 10mM TRIS pH 7.0. A linear gradient was applied to the column (200ml), 0-0.4M NaCl. The elution of protein was monitored at 280nm and the fractions pooled as follows: Fr. 1, (5-21), Fr. 2 (76-201), Fr. 3 (103-143). Fr. 2 had most of the *alginate lyase* activity.

Table 17 Purification Scheme for AV UW (40% Ammonium Sulphate Insoluble Fraction) After Affinity Chromatography.

Fraction	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
Original	11326	13.37	26.75	0.5	847	1
1	215	0.24	0.1	2.42	890	1
2	5467	0.67	0.69	0.98	8088	9.5
3	2939	1.97	1.7	1.16	1490	1.7

Specific Activity ρmole ml^{-1} mg protein^{-1}

The fractions were run on a 7.5 -15% SDS-PAGE and can be seen in Fig 23; the protein profile was complex, multiple bands were present in all the tracts.

Ac 184 total cell extract (30ml) was loaded onto the Q Sepharose FF. column and eluted as described in 2.8.4. The elution profile was monitored at 280nm (Fig. 24) and the fractions containing protein were pooled and assayed for *alginate lyase* (Table 18). A 39-fold purification step was achieved in Fr. 2 (unabsorbed material). Each of the fractions were subsequently divided into two further fractions, > 100kDa and < 100kDa (Amicon cell). A 15-fold increase in purity was achieved in Fr. 1, <100kDa fraction and a 4-fold increase in purity was achieved in Fr. 3, > 100kDa.

Purification of *alginate lyase* by Affinity Chromatography.

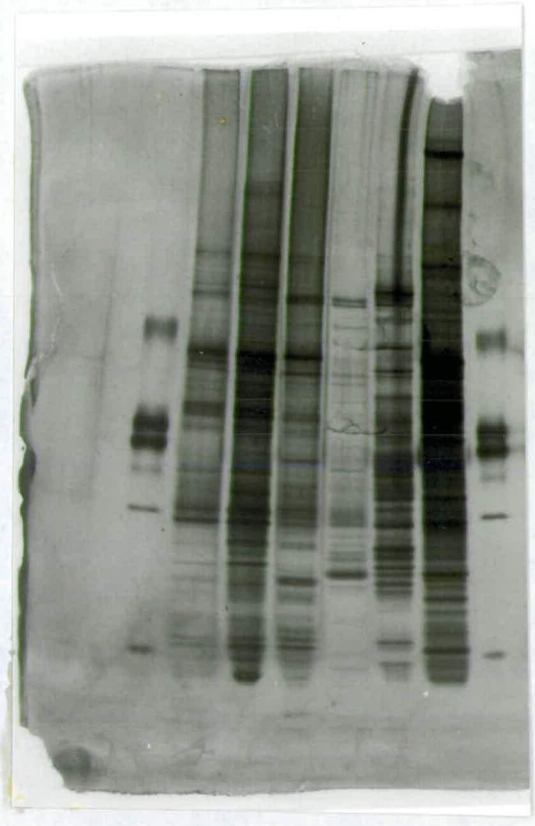
Av Ax17.78 total cell extract was loaded onto the affinity column (poly-D-mannuronate linked to Sepharose). The bound material was eluted with a linear gradient (2.8.3.). The elution profile is shown in Fig. 25; three fractions were obtained after the protein peaks had been pooled. Most *alginate lyase* activity was present in Fr 2.

Av UW concentrated periplasm was applied to the affinity column and the bound material eluted with a linear 200ml 0-0.5M NaCl gradient (Fig. 26). The purification scheme for the pooled fractions can be found in Table 19. Av UW eluted between 0.03-0.12M NaCl and a three-fold purification of the enzyme was achieved .

Ac 184 periplasm was applied to the column under the same conditions. Fig 27 shows the elution profile and the purification scheme for the pooled fractions can be found in Table 20. By passing Ac 184 periplasm through the column a 16-fold increase in purity was achieved in Fr. 1. Fr 3, eluted between 0.22-0.36M NaCl and a 17.84 fold increase in purity was achieved by this step. Fig. 28 shows the separation

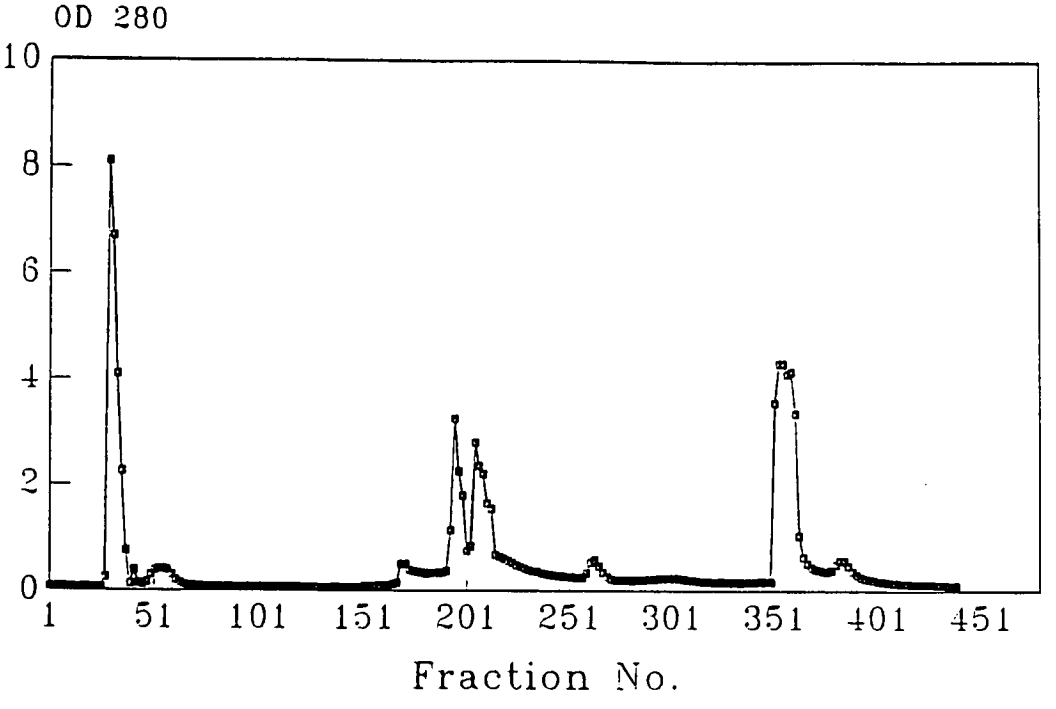
of the fractions after affinity chromatography by SDS-PAGE (7.5-15% acrylamide). Fr. 2 & 3 contained most of the lyase activity. Fr. 2 had a specific activity of 1458ρ mole ml^{-1} mg $protein^{-1}$ and Fr. 3 1038ρmole ml^{-1} mg $protein^{-1}$. Fr. 3 from the Ac 184 contained the most lyase activity (4044ρmole ml^{-1} mg $protein^{-1}$) after separation on the affinity column.

Fig. 23 Separation of the Av UW (40% ammonium sulphate fractionation) Proteins by SDS-PAGE.



Lanes 1 & 8, Standards	BSA, (66kDa),Trypsinogen (Da Trypsin inhibitor Lysozyme
Lane 2	Av UW control
Lane 3	Av UW 40% soluble
Lane 4	Av UW 40% insoluble
Lane 5	Av UW 40% insoluble Fr. 1 (Table 19)
Lane 6	Av UW 40% insoluble Fr. 2(Table 19)
Lane 7	Av UW 40% insoluble Fr. 3 (Table 19)

Fig. 24 The Elution Profile of Total Cell Extracts from Ac 184 on Q Sepharose FF.



30ml of total cell extract was applied to the Q Sepharose FF. column and eluted with a Step NaCl gradient . Fr. 1, (Tubes 26-29 unabsorbed), Fr 2 , (Tubes 40-67, unabsorbed), Fr. 3, (Tubes 163-191, 0.2M NaCl), Fr. 4, (Tubes 192-204, 0.2M NaCl), Fr. 5, (Tubes 205-229, 0.2M NaCl), Fr. 6 (Tubes 254-276, 0.2M NaCl), Fr. 7, (Tubes 350-376, 0.4M NaCl), Fr 8, (Tubes 377-400, 0.4M NaCl).

Table 18 Purification Scheme of Ac 184 Total Cell Extracts from Q Sepharose FF. Eluted Fractions.

Fraction No.	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
Original	13096551	1410	47.0	30	9288	1
1	360968	10.24	2.56	4	29391	3.1
2	230482	0.64	0.32	2	360128	38.7
3	5213	1.76	0.49	3.6	2961	0.3
4	230622	27.17	7.15	3.8	8488	0.9
5	547312	32.60	8.15	4	16788	1.8
6	196383	10.00	2.38	4.2	19638	2.1
7	193652	53.12	13.28	4	3643	0.4
8	82261	15.54	4.20	3.7	5293	0.6

>100kDa

Fraction No.	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
1	2276856	7.65	2.32	3.3	36190	1.2
2	3792	0.01	0.01	1.1	344800	0.9
3	17625	1.44	0.60	2.4	12240	4.1
4	103795	11.47	4.59	2.5	9049	1.1
5	297500	17.75	7.10	2.5	16760	1.0
6	64590	3.19	1.14	2.8	20247	1.0
7	57096	46.82	13.77	3.4	1219	0.3
8	44799	8.55	2.95	2.9	5239	1.0

Table 18 continued

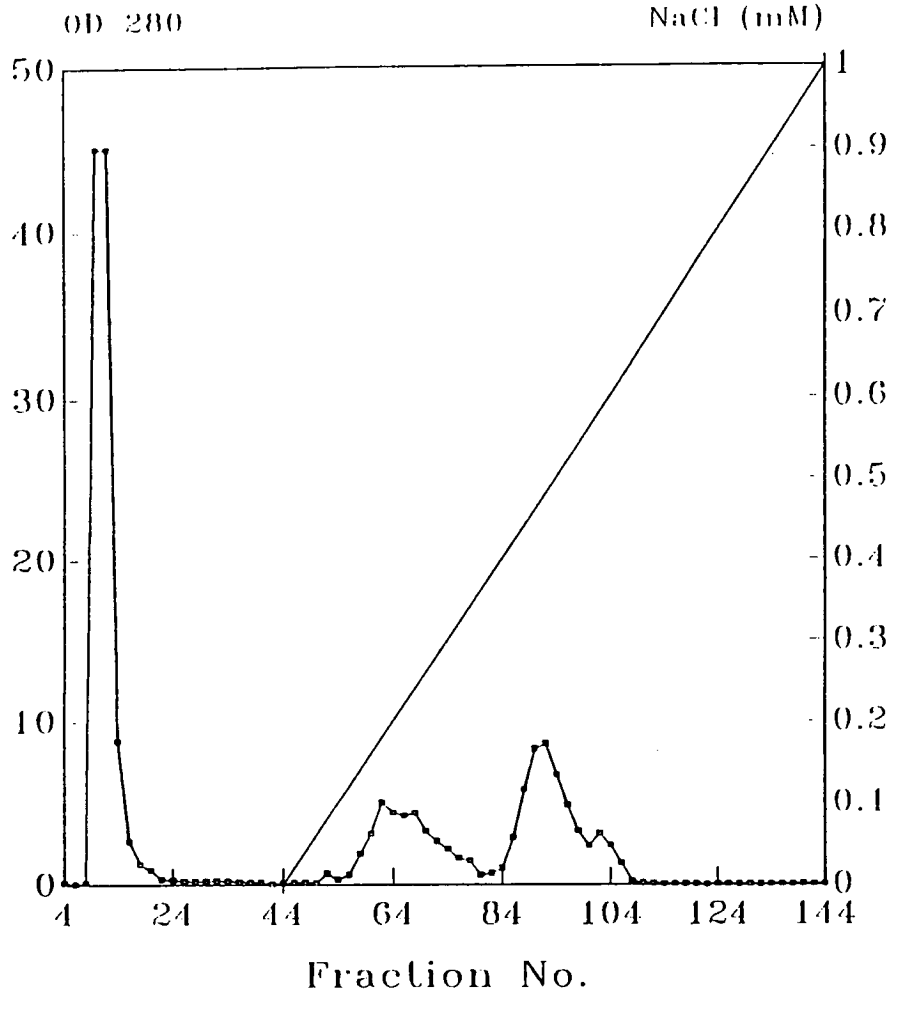
<100kDa

Fraction No.	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
1	46722	0.1	0.11	0.9	467220	15
2	1305	0.16	0.48	0.34	81593	0.2
3	516	0.21	0.05	4.3	2457	0.8
4	64881	32.59	5.82	5.6	1990	0.2
5	63984	4.11	1.11	3.7	15567	0.9
6	5274	2.0	0.54	3.6	2637	0.1
7	34477	5.99	0.82	7.3	5755	1.5
8	1705	1.26	0.28	4.5	1353	0.2

Specific Activity ρmole ml^{-1} mg protein^{-1}

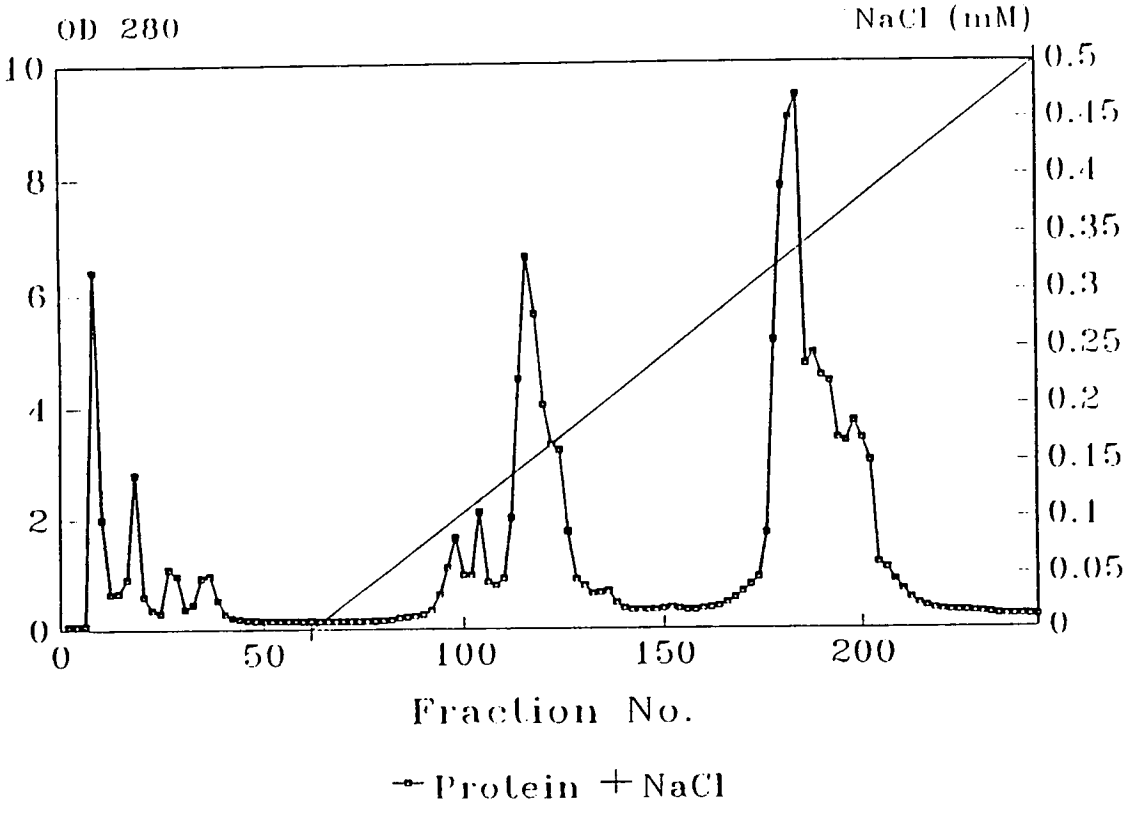
The eight fractions isolated in Fig. 24 were further purified by passing each fraction through the 100kDa membrane (Amicon cell). This resulted in two fractions, one with a nominal molecular mass of >100kDa and one <100kDa. The isolated fractions were tested for *alginate lyase* activity and the protein concentration was determined.

Fig. 25 The Elution profile of Av Ax17.78 from the Affinity Column



3ml of total cell lysate (Specific Activity 2797pmoles ml^{-1} mg $protein^{-1}$) was applied to the affinity column (polymannuronate linked to Sepharose). The fractions were eluted with a linear 100ml, 0-1M NaCl gradient: Fr, 1, (tubes 8-23, unabsorbed), Fr. 2 (tubes 55-81), Fr. 3 (82-108). Fr. 2 had the majority of the *alginate lyase* activity.

Fig. 26 The Elution profile of Av UW from the Affinity Column.



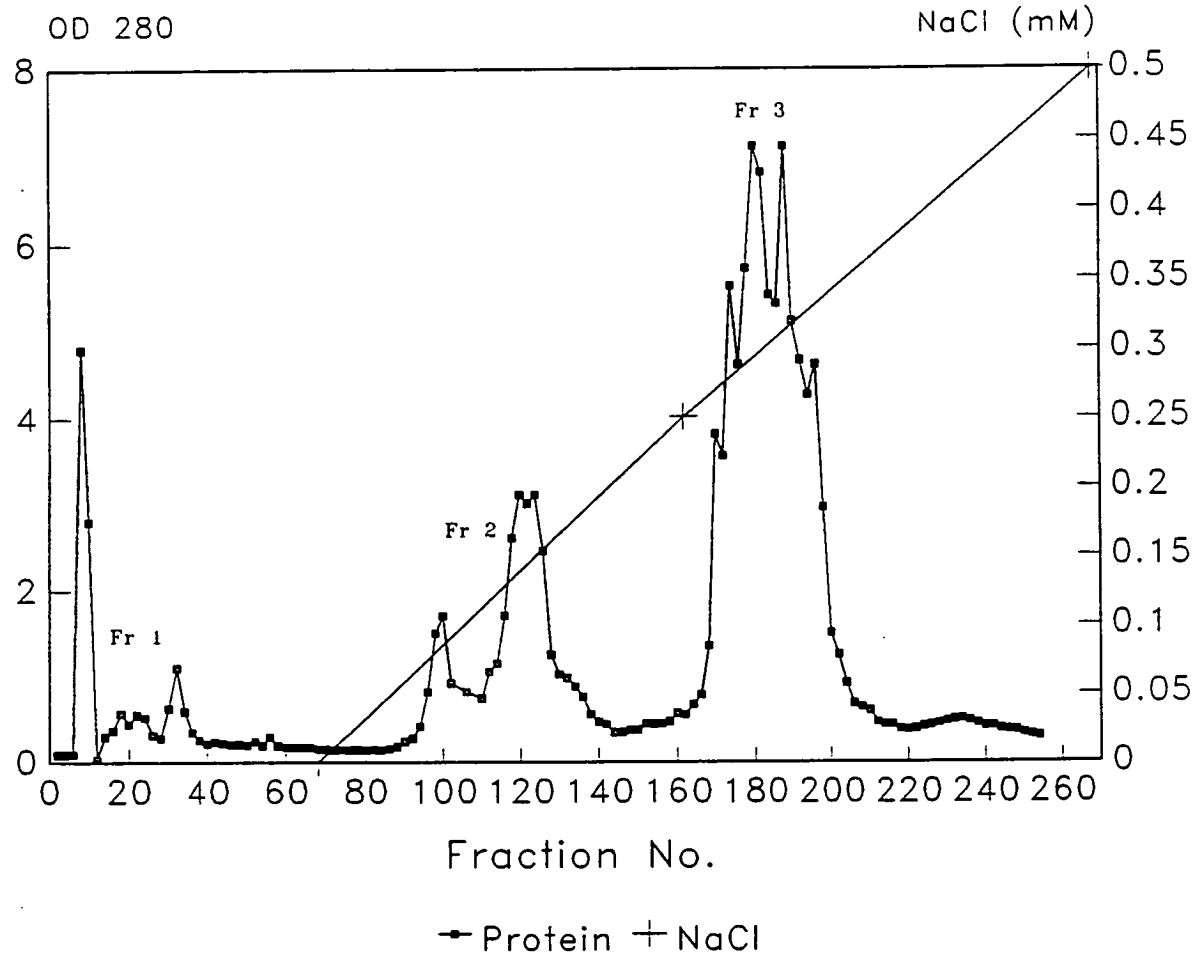
800µl Av UW was applied to the affinity column and eluted with a linear 0-0.5M (200ml) NaCl gradient. The fractions eluted as follows, Fr. 1 (tubes 6-12, unabsorbed), Fr. 2, (tubes 13-38, unabsorbed), Fr. 3 (tubes 94-101), Fr. 4 (tubes 108-136) Fr. 5 (tubes 160-210). Fr. 3 contained the most *alginate lyase* and eluted between 0.03-0.12M NaCl.

Table 19 Purification Scheme for Av UW by Affinity Chromatography.

Fraction	Tube No.	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
Original		19061	43.2	54.0	0.8	443	1
1	6-12	217	3.17	3.52	0.9	68	0.2
2	13-38	2376	8.4	10.5	0.8	283	0.6
3	94-107	4024	3.05	5.55	0.6	1319	3.0
4	108-136	238	2.42	1.22	1.2	98	0.2
5	160-210	420	1.47	1.28	1.2	2.86	0.6

Specific Activity ρmole ml^{-1} mg protein^{-1}

Fig. 27 The Elution Profile of Ac 184 from the Affinity column.



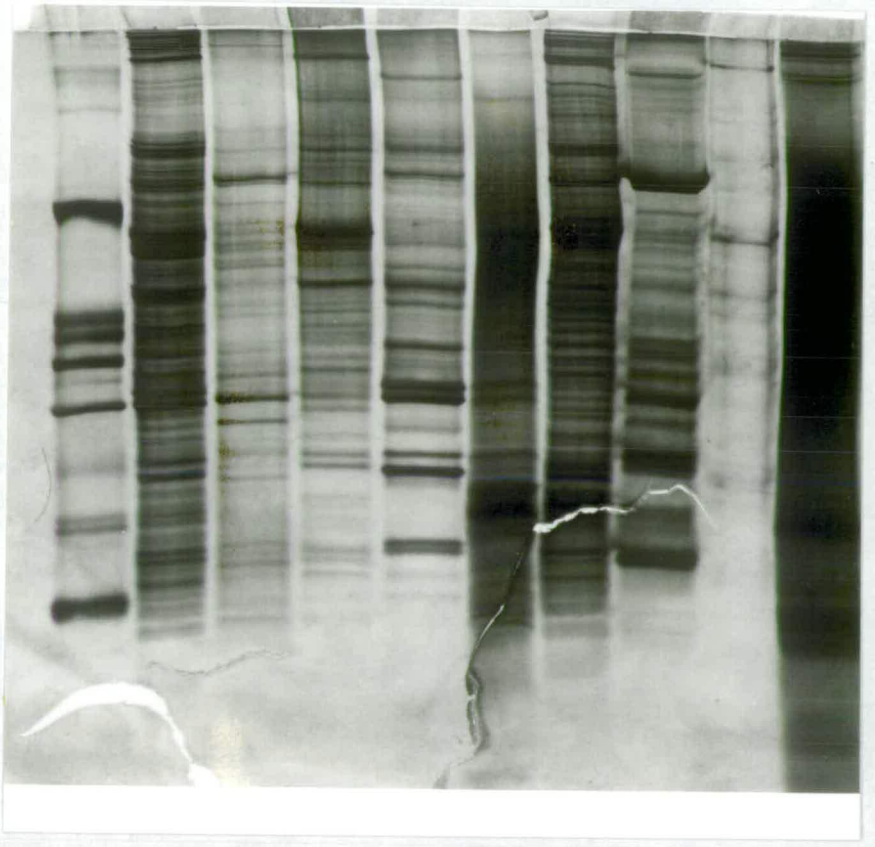
1.5ml Ac 184 was applied to the affinity column and eluted with a linear 0-0.5M (200ml) NaCl gradient. The fractions eluted as follows, Fr. 1 (tubes 7-58. unabsorbed), Fr. 2, (tubes 90-150), Fr. 3 (151-210). Fr. 3 contained the most *alginate lyase* and eluted between 0.22-0.36M NaCl.

Table 20 Purification Scheme of Ac 184 by Affinity Chromatography.

Fraction No.	Total Activity (mole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
Original	61758	44.62	29.75	1.5	1384	1
1	19247	0.85	1.3	0.65	22644	16.36
2	10261	10.4	13.0	0.80	986	0.71
3	7409	0.3	0.4	0.75	24697	17.84

Specific Activity ρmole ml^{-1} mg protein^{-1}

Fig. 28 Separation of Av UW and Ac 184 by SDS-PAGE After Purification By Affinity Chromatography.



Lane 1 BSA 66kDa, Ovalbumin 45kDa, Glyceraldhyde-3-phosphate dehydrogenase 36kDa, Trypsinogen 24kDa, Trypsin Inhibitor 20.1 kDa, Lactoglobin 18.4kDa Lysozyme 14.kDa

Lane 2 Av UW control Lane 3 Av UW Fr. 1 Lane 4 Av UW Fr. 2
Lane 5 Av UW Fr. 3 Lane 6 Av UW Fr. 4
Lane 7 Ac 184 control Lane 8 Ac 184 Fr. 1 Lane 9 Ac 184 Fr. 2
Lane 10 Ac 184 Fr. 3.

The denatured proteins (5-10μg total protein) were separated on a 7.5-15% acrylamide gel (SDS) at 20mAmp., until the dye front had reached the bottom of the gel. The gel was stained with silver.

The Effect of Cations on Av UW and Ac 184 *Alginate Lyase* Activity.

The samples obtained from Table 15 were used to determine the effect of Na^+ and Ca^{2+} on *alginate lyase* activity. Both Na^+ and Ca^{2+} enhanced *alginate lyase* activity in Av UW (Table 21). Maximum enhancement by Na^+ was achieved with 150mM. Ca^{2+} maximally enhanced Av UW lyase activity at 10mM. As well as Na^+ and Ca^{2+}, the effect of the following ions was determined with Av UW enzyme: Mg^{2+}, Mn^{2+}, Fe^{3+}, Mo^{4+} and 1% glycerol. $Fe^{3+}$and Mo^{4+} and 1% glycerol interfered with the TBA assay, therefore no effect could be determined. No lyase activity was detected between 1-20mM Mg^{2+} but activity was observed between 50 and 100mM (Fig. 29) with maximal enhancement at 100mM. Mn^{2+} enhanced lyase activity at 50mM, but this was only 12 times greater than that of the control assay.

Na^+ did not enhance lyase activity in Ac 184 as it did in Av UW. The level of activity observed did not double in the presence of Na^+ (1-200mM). Ca^{2+} appeared to be inhibitory to Ac 184 lyase at concentrations above 10mM; below this it did not have an effect on lyase activity.

The effect of EDTA and EGTA on *alginate lyase* activity was then examined. Both EDTA and EGTA are calcium chelators; originally EGTA was thought to specifically chelate calcium. It is now thought to preferentially chelate Ca^{2+} but will chelate other ions. EDTA is less selective. Since *alginate lyase* activity was enhanced by Ca^{2+} in both organisms it seemed probable that both EDTA and EGTA would affect the activity of *alginate lyase*.

When Av UW *alginate lyase* (Fig. 30) was incubated with 0.5-2mM EDTA, the specific activity remained at the same level. At concentrations above 5mM, the *alginate lyase* activity was enhanced maximally at 50mM. Twice as much lyase activity was observed between 0.5-10mM EGTA; maximum stimulation was

achieved with 50mM. EGTA enhanced lyase activity to a lower extent suggesting that Ca^{2+} does affect the *alginate lyase* in strain AV UW.

Fig. 31 shows the effect of both EDTA and EGTA on the activity of Ac 184 *alginate lyase*. EDTA did not enhance lyase activity in Ac 184; between 0.5-10mM and 150-200mM enzyme activity was reduced to 75%. EGTA increased the specific activity between 1-10mM with a maximum stimulation in the presence of 5mM.

To complete the study, the effect of Na^{+}, Ca^{2+}, EDTA and EGTA on *K. pneumoniae alginate lyase* was examined. The results are shown in Fig. 32. The maximum enhancement of enzyme activity occurred in the presence of 200mM Na^{+}. Ca^{2+} also stimulated an increase in enzyme activity between 10-50mM. The effect of both EDTA and EGTA was much more dramatic; both chelators inhibited enzyme activity in *K. pneumoniae* between the concentrations of 0.5-200mM.

Table 21 The Effect of Ions on Av UW and Ac 184 *Alginate lyase*.

Ion	Na^+	
Concentration (mM)	Av UW *Alginate lyase* (ρmole ml $^{-1}$ mg protein^{-1})	Ac184 *Alginate lyase* (ρmole ml $^{-1}$ mg protein^{-1})
0	119	32821
1	-	31525
5	-	33644
10	450	33249
20	723	40161
50	5608	52626
75	8246	41886
100	8565	49288
150	10312	55093
200	6454	33217

Ion	Ca^{2+}	
Concentration (mM)	Av UW *Alginate lyase* (ρmole ml $^{-1}$ mg protein^{-1})	Ac184 *Alginate lyase* (ρmole ml $^{-1}$ mg protein^{-1})
0	124	29975
1	-	27412
5	-	26890
10	8238	19408
20	6852	15312
50	3817	6359
75	2569	-
100	1398	870
150	10312	55093
200	6454	33217

The assays were carried out under optimal conditions. The 72h sample was used in the Av UW assay and the 48h Ac 184 (Table 15).

Fig. 29 The Effect of Divalent Cations on Av UW *Alginate Lyase*.

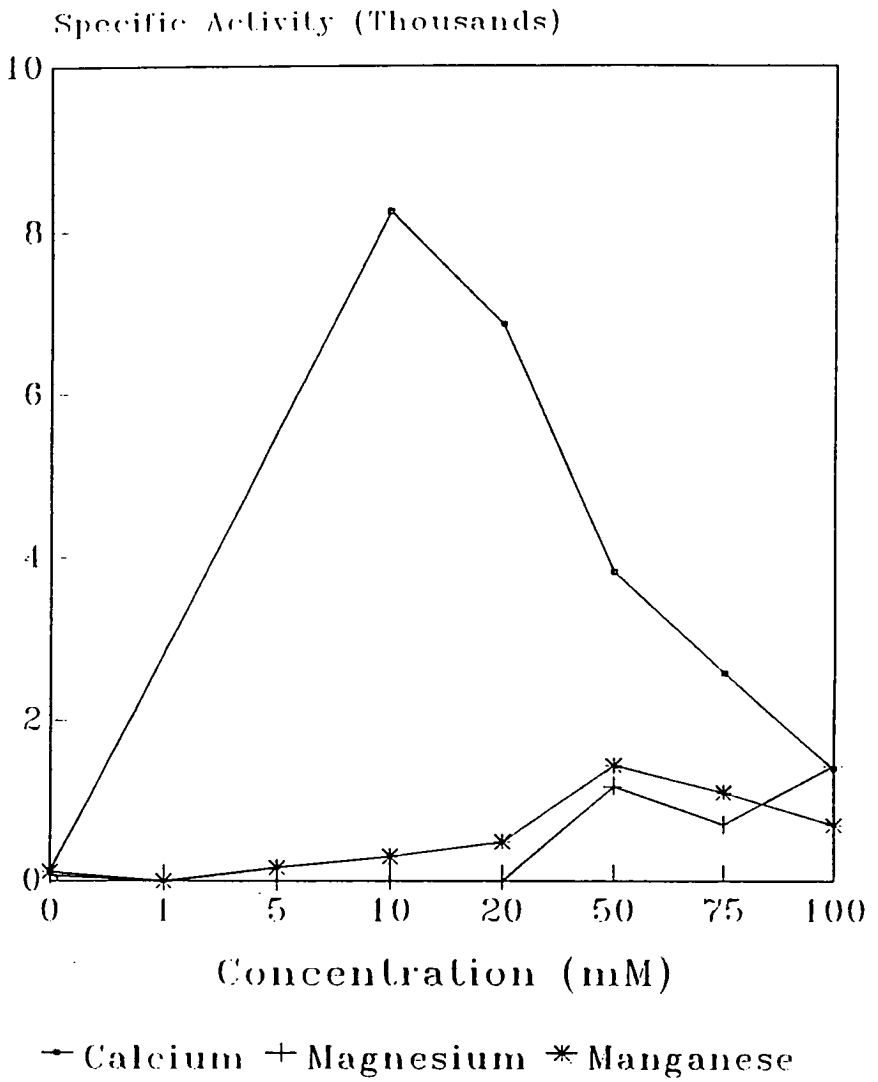


Fig. 30 The Effect of EDTA and EGTA on Av UW *Alginate Lyase*.

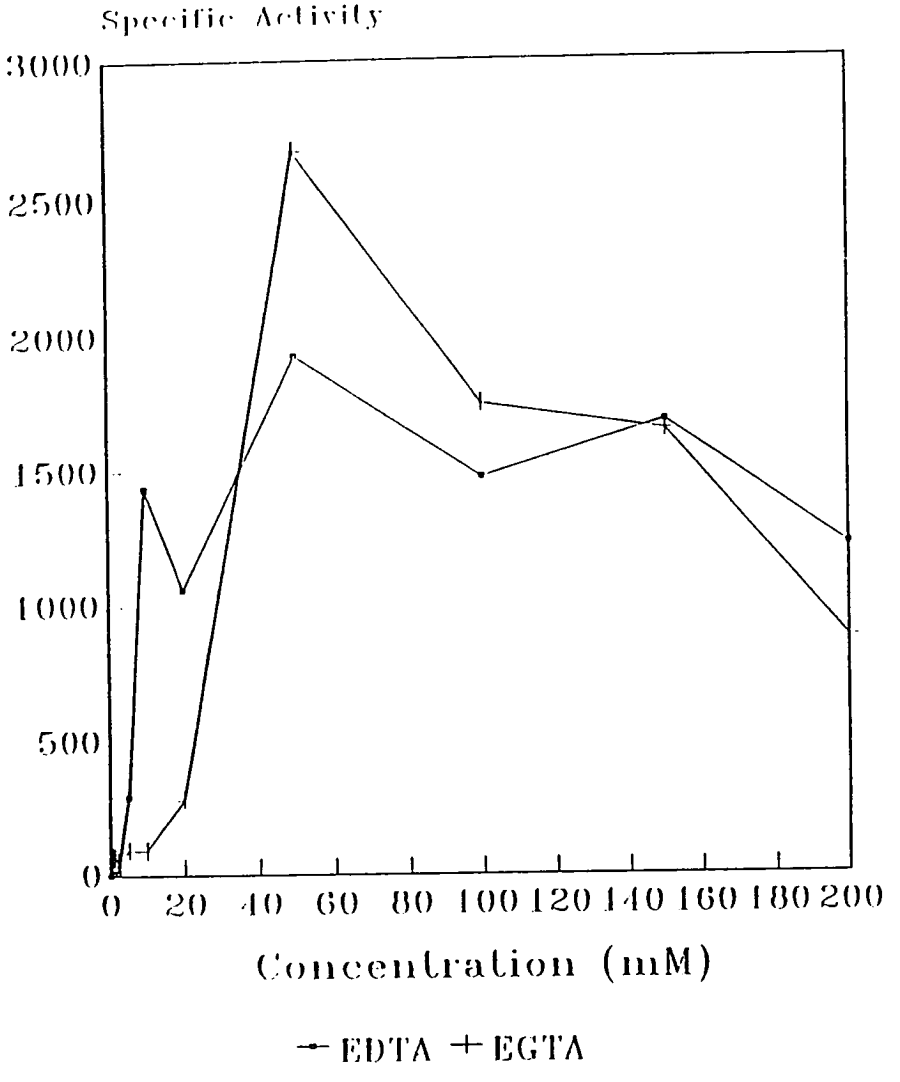


Fig. 31 The Effect of EDTA and EGTA on Ac 184 *Alginate Lyase*.

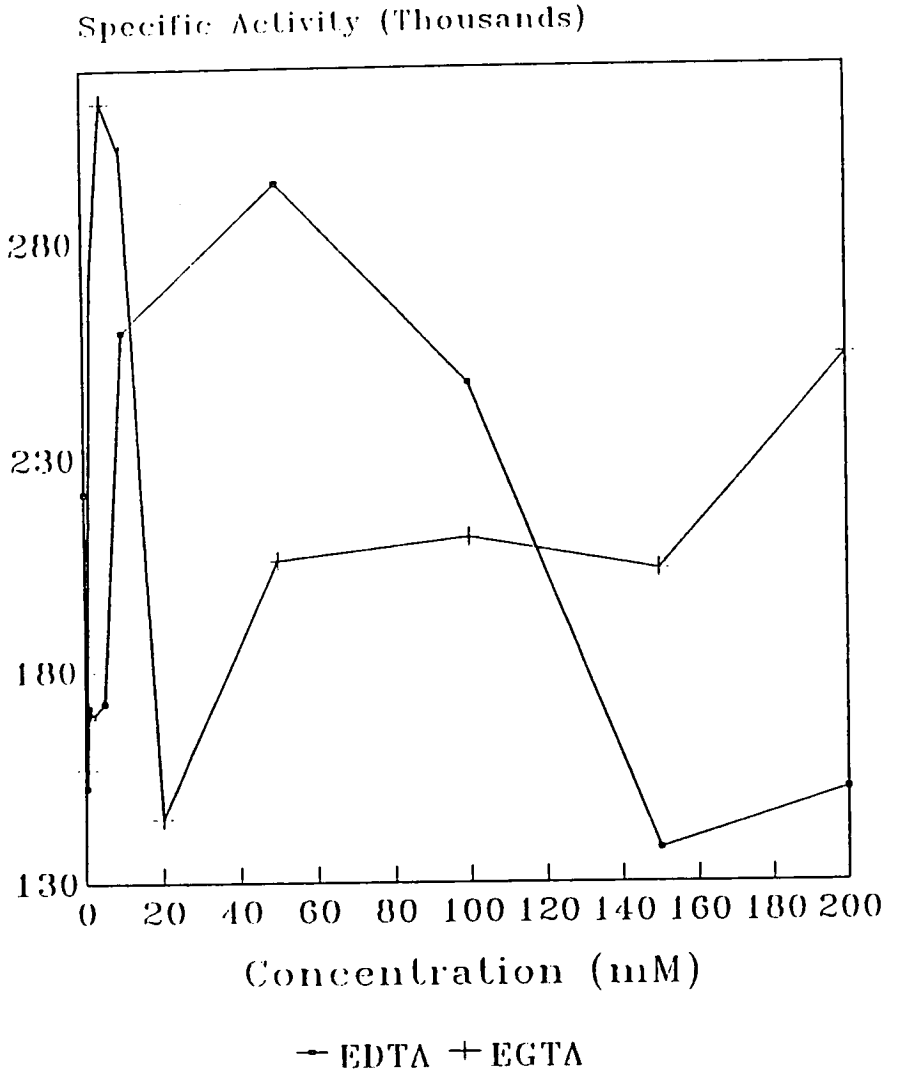
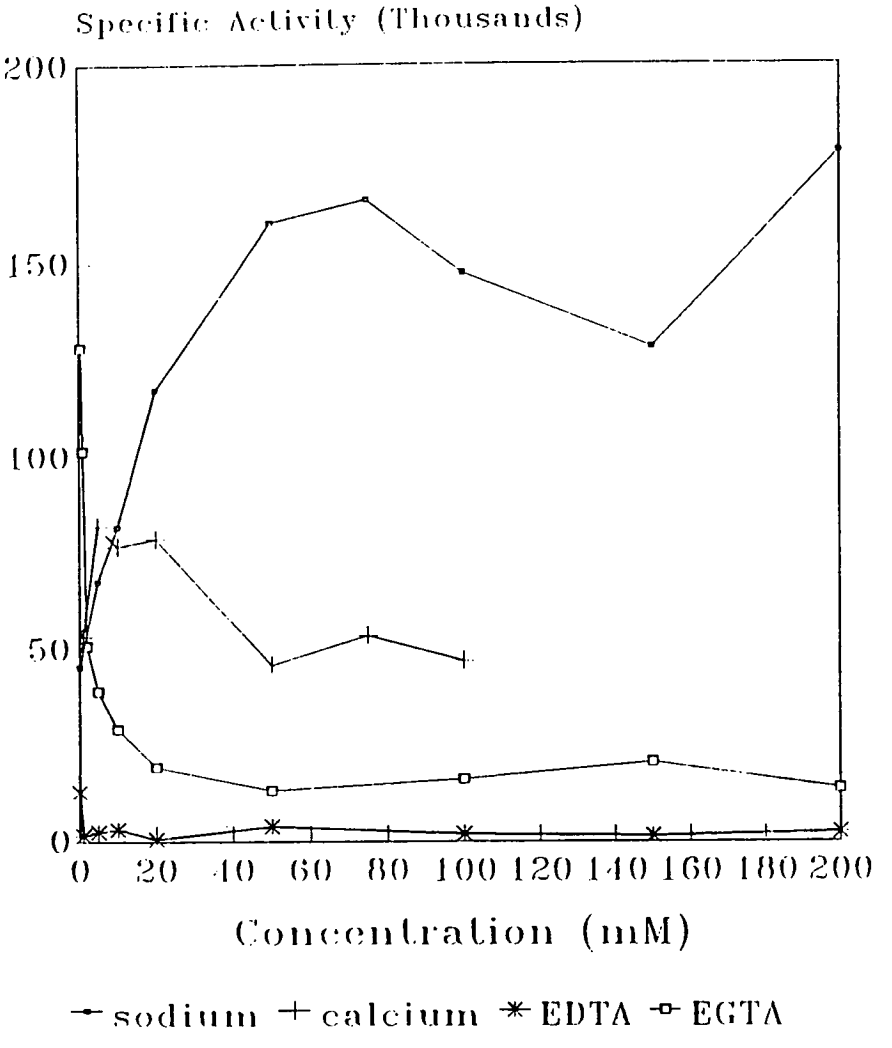


Fig. 32 The Effect of Cations and Chelators on *K. pneumoniae Alginate Lyase.*



The *alginate lyases* isolated from *Pseudomonas* sp. (Preiss & Ashwell, 1962) were enhanced maximally by the addition of 50mM KCl. At concentration higher than this, the enzyme activity was inhibited. The lyase activity in the heptopancreas of the mollusc *Littorina* sp (Elyakova & Favorov, 1974) was also affected by the presence of ions, both monovalent and divalent cations were tested; Cu^{2+}, Zn^{2+} and Hg^{2+} inhibited enzyme activity whereas Na^{+} and K^{+} activated *alginate lyase* at concentrations less than 0.2M, above this concentration they became inhibitory. The divalent cations, Ca^{2+} and Co^{2+} also activated the enzyme but only at concentrations less than 10mM. Boyd & Turvey (1977) studied the effect of various salt concentrations on *alginate lyase* activity and showed that, as with other polysaccharide lyase systems, monovalent cations enhanced enzyme activity at a concentration of 0.3M, whereas divalent cations such as Mg^{2+} enhanced activity at a lower concentration (approximately 0.1M). Higher concentrations of divalent cations inhibited enzyme activity or caused the alginate to precipitate.

Stevens & Levin (1977) reported that the *alginate lyase* from *Alginovibrio aquatilis* required the presence of cations for enzyme activity. Cs^{+}, Rb^{+}, K^{+}, Na^{+} and Li^{+} showed decreasing enzyme enhancement. Enzyme activity was enhanced with increasing size and molecular weight of the cation added and followed the order of metals found in the periodic table of elements. The activity of the purified *alginase* was linearly related to the ionic radius of the added cations and also to the log of the atomic weight of the cations. This was of interest since Williams & Eagon (1962) observed similar monovalent cation requirement for an *alginase* isolated from *Agrobacterium alginicum*. They reported the following ions in decreasing order of enzyme enhancement, NH_4^{+}, Na^{+}, K^{+}, Li^{+} and Rb^{+}. Cs^{+} was not studied. The order of enzyme activation by the alkaline metal ions probably reflects a combined

geometric and electrostatic role that is greater with the larger ions such as Cs^+, with small hydration radius than it is with smaller ions such as Li^+ with a much larger hydration radius. As the radius of these ions increases, the ratio of the surface area to the electrostatic charge of the ion increases, which results in a more weakly oriented shell of water molecules surrounding the ion (Kay & Evans, 1966, Podolsky 1958). Baxter (1959) postulated that the activation of halophilic enzymes by monovalent cations is the result of the reduction of intermolecular repulsions, which allows the enzyme to assume a catalytic configuration. Baxter (1959) also concluded that the lower activation with catalysts of largest hydration radius such as Li^+ may be due to steric hindrance of the enzyme substrate complex. It was also suggested that the requirement for monovalent cations may reflect the marine origin of *A. aquatilis*.

Wainwright & Sherbrock-Cox (1981) reported that the marine fungus *Dendryphiella salina* frequently isolated from seaweed, sea water and coastal sand and salt marsh soils was able to depolymerise sodium alginate. Alginate degradation in the two species was affected differently by NaCl. *D. salina* was stimulated by 1-3% NaCl but inhibited by higher concentrations, whereas *Dendryphiella arenaria* was inhibited by 1% NaCl. Depolymerisation was enhanced by the addition of Mg^{2+}, Ca^{2+} and NaH_2PO_4

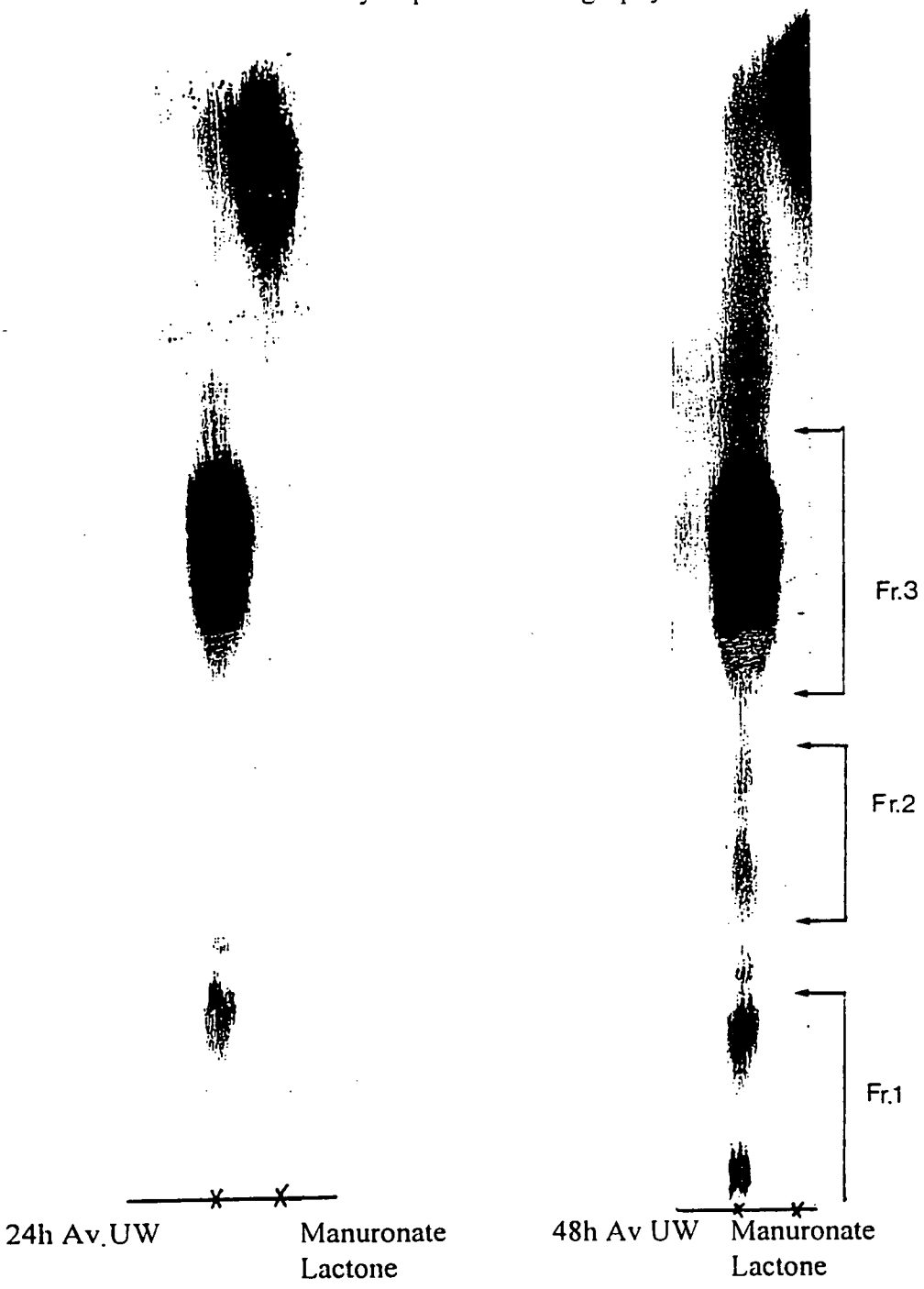
Aasen *et al.* (1992) observed that the removal of cations led to a significant loss of alginate lyase activation. The *alginate lyases* from pseudomonads (Preiss & Ashwell, 1962) and *Abalone* heptopancreas (Nakada & Sweeny, 1967) have been shown to be dependent on high salt concentration, the high concentration being necessary to give alginic acid the proper configuration for enzyme activity and to disrupt the water structure around the alginate molecule (Nakada & Sweeny, 1967).

Geddie (1992) reported that the presence of O-acetyl groups on the mannuronate residues in native bacterial polysaccharide had an effect on both the uptake and the selectivity of bacterial alginate for divalent cations such as Ca^{2+} and Sr^{2+}. The capacity for Ca^{2+} binding and selectivity for this ion was greatly reduced by the presence of *O*-acetyl groups. The interaction of algal alginate with monovalent ions has been studied using circular dichroism studies.

The determination of breakdown products of Av UW and Ac 184 *alginate lyase*

The breakdown products of Av UW and Ac 184 *alginate lyase* were determined using the following polysaccharides: *M. pyrifera*, Av Ax17.78, Av Ax17.78 DAC, PsB, PsB DAC, poly M and G-M blocks. The assay mixtures were incubated for 24h and 48h. at 30°C. The residual polymer was precipitated, the supernatant dried and resuspended in 1ml of d.H_2O. The samples were applied to No. 1 Whatman paper and run by descending chromatography in butan-1-ol:pyridine:water (6:4:3). After staining, only the Av UW 24/48h G-M blocks (Fig. 33) contained breakdown products. A preparative chromatogram run in the same solvent system revealed 3 spots, which were eluted with d.H_2O. The spots were dried and resuspended in d. H_2O; Fr 1 ran closest to the origin and Fr 3 ran farthest away from it. When the fractions were chemically analysed (Table 22), Fr 2 contained most of the unsaturated uronic acid (0.57μmoles). Fr 1 contained undegraded polymer and large oligosaccharides, Fr 2 and Fr 3 contained decreasingly smaller oligosaccharides.

Fig. 33 Separation of Av Uw Breakdown Products by Paper Chromatography.



The assay was set up using Av UW (Specific Activity 1132pmole ml^{-1} mg $protein^{-1}$ *M. pyrifera* alginate). After 24 and 48h the reaction was stopped by boiling the sample. Residual polymer was precipitated with acetone, microfuged and the pellet discarded. The supernatant was dried and resuspended in 100µl. 10µl of sample was applied to No. 1 Whatman paper and run in the butanol:pyridine:water (6:4:3) system for 24h. When the chromatogram was dry it was stained with silver to reveal the material.

Table 22 Chemical Analysis of the Breakdown Products of G-M blocks by Av UW *Alginate lyase*.

Fraction	Unsaturated Uronic Acid	Uronic Acid
1	0.02	0.01
2	0.57	0.05
3	0.11	0.06

The fractions 1, 2, and 3 were isolated from a preparative paper chromatogram which had run in the butanol:pryidine:water (6:4:3) for 24h. Each end of the paper was removed and stained with silver to locate the migrated material. The material was eluted from the paper with d.H_2O. Fr. 1 ran closest to the origin and Fr. 3 furthest from it.

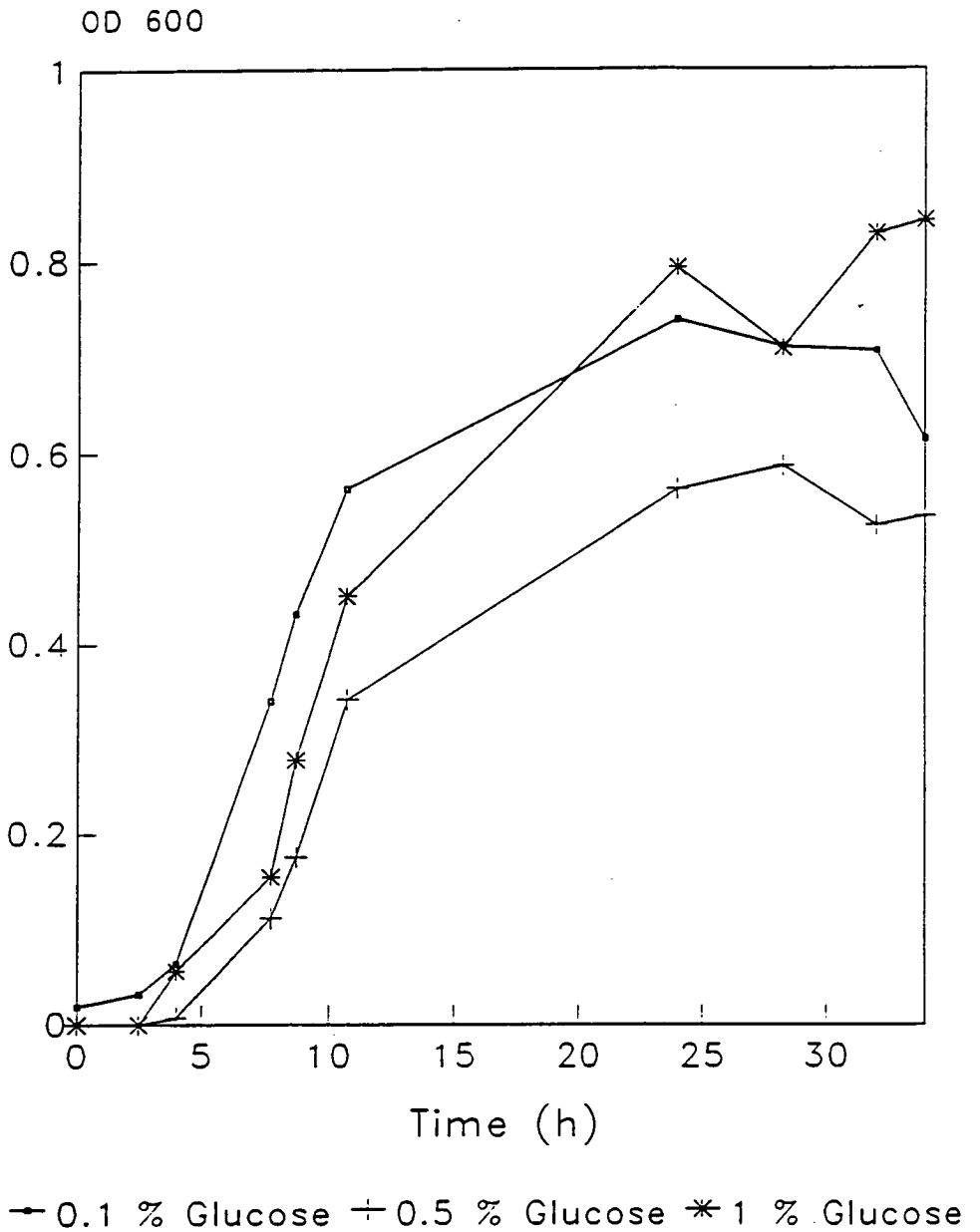
Unsaturated uronic acid μmole ml^{-1} μg $protein^{-1}$
Uronic Acid μmole ml^{-1} μg $protein^{-1}$

The breakdown products from both sources of *alginate lyase* were examined using *Laminaria hyperborea* and *Macrocystis pyrifera* (high viscosity) alginate. The assay mixture was incubated at 30°C for 0h, 18h and 43h at which time it was removed and boiled to denature the enzyme. The contents were exhaustively dialysed against d.H_2O. The breakdown products were chemically analysed (Table 23) and analysed by descending paper chromatography. The results from the polymer degradation are shown in Fig. 34. The amount of residual polymer decreased by 10% (43h) when Av UW *alginate lyase* was present and *L. hyperborea* alginate was the substrate and a 13% (43h) reduction was observed when *M. pyrifera* alginate was the substrate. The amount of residual polymer present in both substrates was reduced by 40 and 45% (*L. hyperborea* to *M. pyrifera* respectively), when Ac 184 *alginate lyase* was the enzyme. This indicated that the Ac 184 *alginate lyase* was more active than the Av UW *alginate lyase*.

When Ca^{2+} was added to the alginate, gelation occurred. This accounted for the discrepancies in the amount of residual polymer. Theoretically, there should have been 250mg of polymer in the time zero controls but, after freeze drying, they contained 316.3 and 307.9mg respectively for the *M. pyrifera* and *L. hyperborea* alginate. The alginate isolated from the retentates of Ac 184 *alginate lyase* (0h) contained 233.1 and 211.9mg (*M. pyrifera* and *L. hyperborea*).

The results of the chemical analysis of the retentate and the diffusate from each polymer (Av UW *alginate lyase*) are shown in Table 23a. The total carbohydrate present in *L. hyperborea* retentate decreased by 20% (0-18h) and by 14% overall. Between 0-18h, the *M. pyrifera* retentate, reduced by 11% and reduced by a further 3% at 43h. This implied that over a relatively short period of time (less than 18h) *L. hyperborea* alginate was the better substrate for Av UW *alginate lyase*. Unsaturated

Fig. 46 Growth Curves of Strain 13.1 on Various Concentrations of Glucose.



The bacteria were grown in YE (0.1, 0.5 and 1% glucose) at 30°C on an orbital shaker. Samples were removed and the optical density measured at 600nm.

uronic acid was released linearly from the *L. hyperborea* and *M. pyrifera* retentates. By 43h, the amount of uronic acid present in the retentates had increased by 11% and decreased by 4% (*L. hyperborea* to *M. pyrifera* respectively). The uronic acid content of the diffusate could not be determined due to interference with the assay. *Glucose oxidase* was used to determine if any "free" glucose was present in the diffusate (the bacteria were originally grown on glucose).

Very little alginate (*M. pyrifera* and *L. hyperborea*) was degraded by Av UW *alginate lyase*. Chemical analysis of both diffusates confirmed this. The initial carbohydrate present in both diffusates was 5 and 10μmole (*M. pyrifera* to *L. hyperborea* respectively); after 43h, this had increased by 22% in the *L. hyperborea* sample and decreased by 45% in the *M. pyrifera* sample. Low levels of unsaturated uronic acid were detected in both diffusates. At 0h, there were 6μmole; at 18h, 8-11μ mole; and 13μmole unsaturated uronic acid were present at 43h. The low levels of total carbohydrate and unsaturated uronic acid present in the diffusate implied that either these substrates were unsuitable substrates for Av UW *alginate lyase* or that the degraded fragments were too large to pass through the dialysis sac or the enzyme was not very active.

The chemical analyses of Ac 184 *alginate lyase* retentates and diffusates are shown in Table 23b. The retentate samples showed a marked decrease in the amount of total carbohydrate. Between 0-18h, the amount of total carbohydrate decreased by 14% (*L. hyperborea*) and by a further 8% at 43h. When *M. pyrifera* alginate was the substrate there was a 36% reduction in total carbohydrate between 0-18h and a further 14% reduction between 18-43h. The amount of unsaturated uronic acid in both retentates increased whereas the amount of uronic acid decreased.

The Ac 184 *alginate lyase* diffusate contained much higher levels of unsaturated uronic acid than the Av UW diffusates. The control diffusates of both enzymes contained similar levels of unsaturated uronic acid. However, there was a 28-fold increase in the level of unsaturated uronic acid at 18h (*L. hyperborea*) and 39-fold increase (*M. pyrifera*), compared to a 2-fold increase with the Av UW *alginate lyase*. Between 18-43h the amount of unsaturated uronic acid in the *M. pyrifera* diffusate increased 3-fold. However the *L. hyperborea* alginate had only increased by 8.9% in the same time period. This indicated that the size of fragments in the retentates of Ac 184 degraded polymers was smaller. This hypothesis was supported by the higher levels of unsaturated uronic acid present in the diffusate and would suggest that the Ac 184 *alginate lyase* sequentially cleaved the polymer into smaller fragments. The oligosaccharides produced by Av UW *alginate lyase* were larger than those produced by Ac 184 *alginate lyase*.

Table 23a

The Chemical Analysis of the breakdown Products of Av UW *Alginate Lyase* when *L. hyperborea* and *M. pyrifera* are the alginate source

Av UW Diffusate *L. hyperborea* alginate

Time (h)	Total Carbohydrate	Glucose Oxidase	Unsaturated Uronic acid	Volume (ml)
0	15.0	0.94	9.80	0.68
18	15.28	0.78	14.27	0.8
43	15.69	2.36	16.9	0.8

Av UW Diffusate *M. pyrifera* alginate

0	6.2	1.4	8.27	0.79
18	3.05	0.3	10.6	0.75
43	3.33	0.55	15.98	0.82

Av UW Retentate *L. hyperborea* alginate

Time (h) mg retentate	Total Carbohydrate	Uronic Acid	Unsaturated Uronic	Retentate (mg)
0	1.004	1.288	0.22	307.9
18	0.912	1.596	0.672	273.1
43	0.96	1.596	1.478	277.0

Av UW Retentate *M. pyrifera* alginate

0	1.012	1.648	0.118	316.3
18	0.888	1.724	0.5	317.3
43	1.0	1.816	1.24	276.7

Total Carbohydrate	µmole glucose ml diffusate^{-1}/mg retentate^{-1}
Glucose Oxidase	µmole glucose ml diffusate^{-1}/mg retentate^{-1}
Unsaturated Uronic acid	µmole unsaturated uronic acid ml diffusate^{-1}/mg retentate^{-1}
Uronic Acid	µmole uronic acid ml diffusate^{-1}/mg retentate^{-1}

Table 23b The Chemical Analysis of the breakdown Products of Ac 184 *Alginate Lyase* when *L. hyperborea* and *M. pyrifera* are the alginate source

Ac 184 Diffusate *L. hyperborea* alginate

Time (h)	Total Carbohydrate	Glucose Oxidase	Unsaturated Uronic acid	Volume (ml)
0	91.11	46.11	8.33	0.88
18	48.61	0.14	222.40	0.95
43	19.58	0.47	328.59	0.7

Ac 184 Diffusate *M. pyrifera* alginate

0	6.55	0.49	14.42	0.65
18	11.67	0.1	368.36	0.99
43	20.83	0.11	950.47	1.15

Ac 184 Retentate *L. hyperborea* alginate

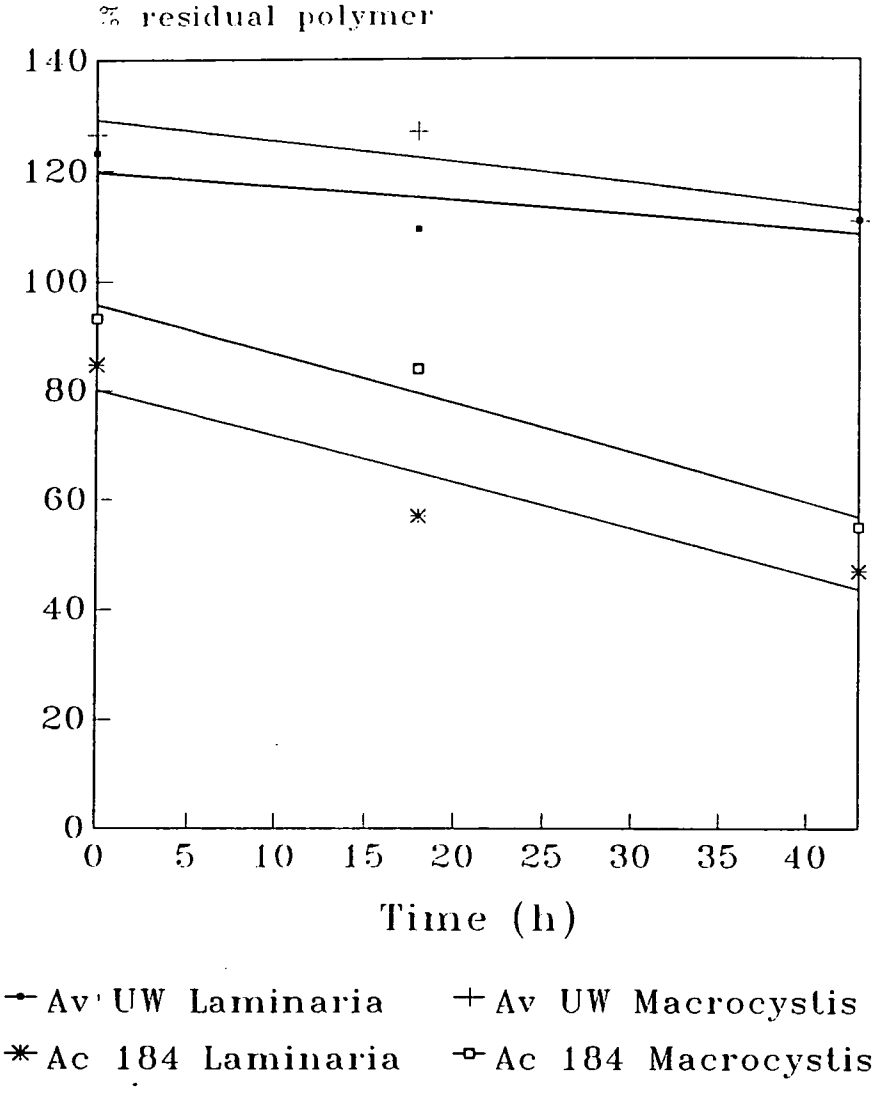
Time (h)	Total Carbohydrate	Uronic Acid	Unsaturated Uronic	mg retentate
0	1.12	2.29	0.25	211.9
18	1.44	2.72	6.24	142.4
43	1.58	2.76	5.54	117.3

Ac 184 Retentate *M. pyrifera* alginate

0	1.72	3.03	0.11	233.1
18	1.22	3.05	7.76	209.6
43	1.44	3.15	15.69	137.4

Total Carbohydrate	µmole glucose ml diffusate^{-1}/mg retentate^{-1}
Glucose Oxidase	µmole glucose ml diffusate^{-1}/mg retentate^{-1}
Unsaturated Uronic acid	µmole unsaturated uronic acid ml diffusate^{-1}/mg retentate^{-1}
Uronic Acid	µmole uronic acid ml diffusate^{-1}/mg retentate^{-1}

Fig. 34 Percentage Residual Polymer after Av UW and Ac 184 Alginate Lyase Degradation.



To determine this, aliquots of the diffusate were analysed by descending paper chromatography. The diffusate was applied to Whatman No. 1 paper and run in the butan-1-ol:pyridine:water for 48h. After developing with silver reagent (Trevelyan 1959) (Fig. 35a, b), the R_{Glc} values were determined (Table 24). Since unsaturated uronic acids fluoresce at 233nm, the chromatogram was examined under short UV light and their R_{Glc} was determined (Table 25). When Av UW *alginate lyase* was the enzyme source, all the samples (*L. hyperborea*) were similar after running for 48h (Fig. 35a). There was little or no material at the base line, the first spot became less intense with time and became slightly more mobile. Spot 5 was the most mobile oligosaccharide. The differences in the *M. pyrifera* alginate were more obvious. Spot 2 became less intense with longer incubation while Spot 3 increased. Spots 2 and 3 appeared to have similar mobility to glucuronic acid and mannuronate. The breakdown products of Ac 184 on both types of alginate were more complex (Fig. 35b).

To determine the size of the fragments produced by Ac 184 digests, the 18 & 43h fractions were combined and applied to a Biogel P2 column. Fractions were collected and the amount of total carbohydrate determined. The OD_{490} was plotted against fraction volume (Fig. 36, 37). When appropriate fractions were pooled, this resulted in two peaks for the *M. pyrifera* sample and three peaks for the *L. hyperborea* sample. The peaks were rotary evaporated to dryness and re-dissolved in UHQ water.

A sample of each peak was injected into the HPLC (Gilson) with the TSK 1,000 column and eluted with UHQ water. The elution profile was monitored and the elution time of each peak was determined. The standards used as controls were

glucose, maltose and maltotetraose. The DP of the standards was plotted against time (Fig. 38) and the DPs of the peaks were estimated.

Fig. 39 shows the elution profile for each of the peaks: set a) *M. pyrifera* alginate, Pk 1 eluted as a single peak (DP 5), Pk 2 contained at least two components which could not be separated (DP 2); set b) *L. hyperborea* alginate, Pk 1 DP 6, Pk 2 DP 5, Pk 3 contained at least two components which could not be separated (DP 4). To determine which of the oligosaccharides contained uronic acid, each of the peaks was applied to No. 1 Whatman paper and electrophoresed (2.14). The results show the spots which fluoresced were either just behind the origin or on the origin. The spots which ran ahead of both glucuronic acid and mannuronate did not fluoresce.

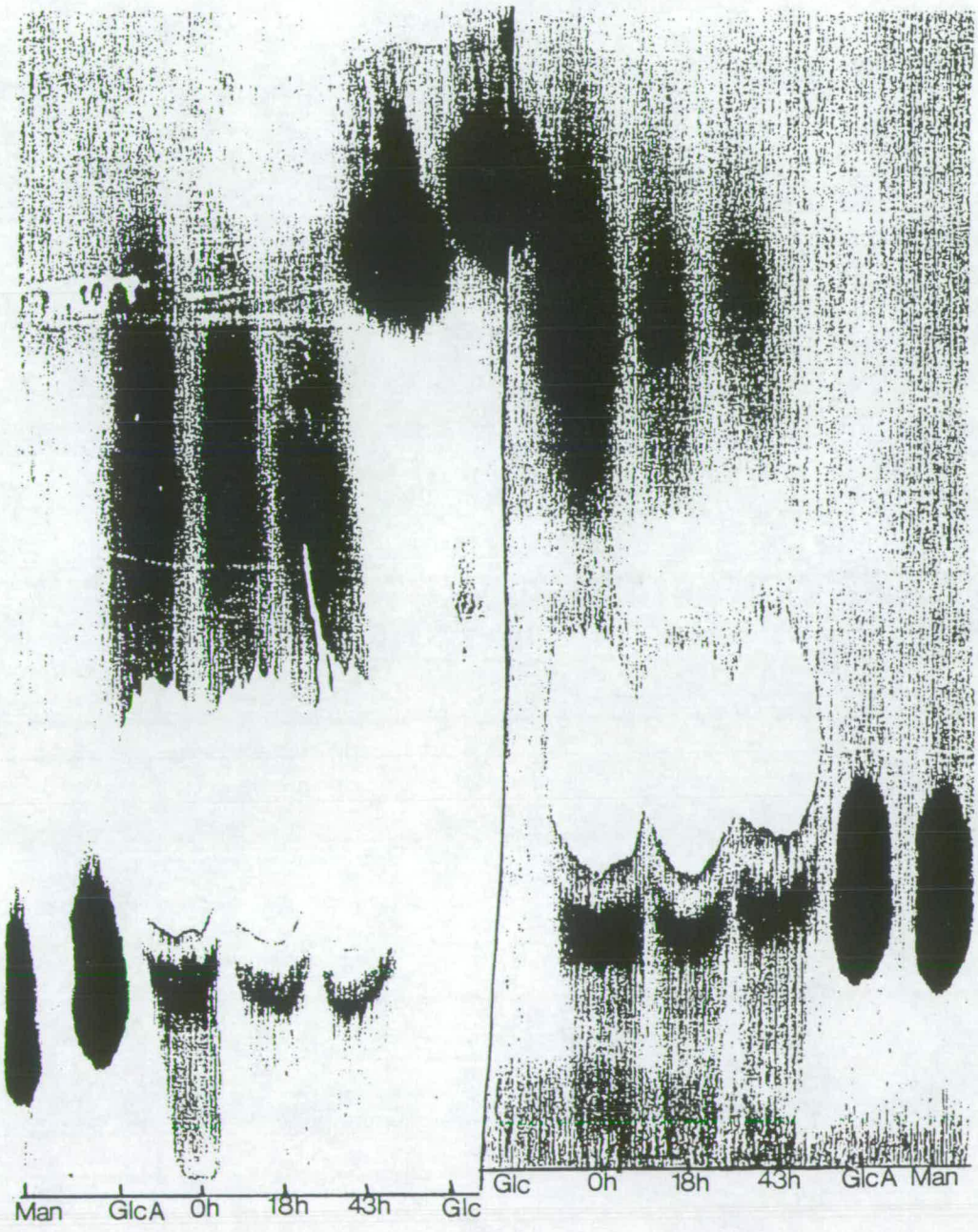
One would therefore conclude that these fragments were quite large and would not migrate far under these conditions. *L. hyperborea* alginate is marketed (BDH) as an alginate rich in L-guluronate residues. This was confirmed by NMR (Table 26) (see Geddie, 1992). The polymer contains 66.5% guluronate and 33.5% mannuronate, of the guluronate residues 55.8% were found as diads and only 10.7% found as mixed diads either GM or MG. This would indicate that the *alginate lyase* from Av UW exhibited an increased specificity for guluronate residues. This confirms the result of the preliminary experiment where Av UW *alginate lyase* degraded the G-M blocks to the greatest extent.

To determine if the DP of the fragments decreased with incubation time, both Av UW and Ac 184 *alginate lyase* were incubated at 30°C with *M. pyrifera* low viscosity alginate. At appropriate intervals an aliquot was removed, boiled and the contents dialysed against d.H_2O. At the same time, a 3ml aliquot was removed and tested for TBA activity; the results can be found in Fig. 39. Table 27 shows the results for Av UW. The amount of residual polymer decreased slightly and low levels

of unsaturated uronic acid were present in the diffusate. The 99h sample showed an unexpected increase in unsaturated uronic acid and total carbohydrate content. Table 28b contains the results of chemical analysis of Ac 184 diffusate and shows that the amount of unsaturated uronic acid increased throughout the experiment (Fig. 40).

The samples were injected into the HPLC and eluted as previously described; examples of the elution profile and DP can be found in Fig. 41 and 42. Fig. 41 shows the typical elution profile for Av UW a, b, and c, have a DP of 5 and d had a DP 4. All were single peaks. The peak whose profile is shown as 'e' (99h) was atypical. It was a complex peak made up of at least two components with DP 6 and DP 4. Fig 42 shows the typical elution profile for Ac 184; all the fractions produced an elution profile comprised of a single peak. The peaks had a DP of 5 up until 143h, when the DP was 4. Paper electrophoresis indicated that most of the material remained behind the recalculated origin, as did the fluorescent spots.

Fig. 35a Separation of the *M. pyrifera* and *L. hyperborea* Breakdown Products of Av UW *Alginate Lyase* by Paper Chromatography.

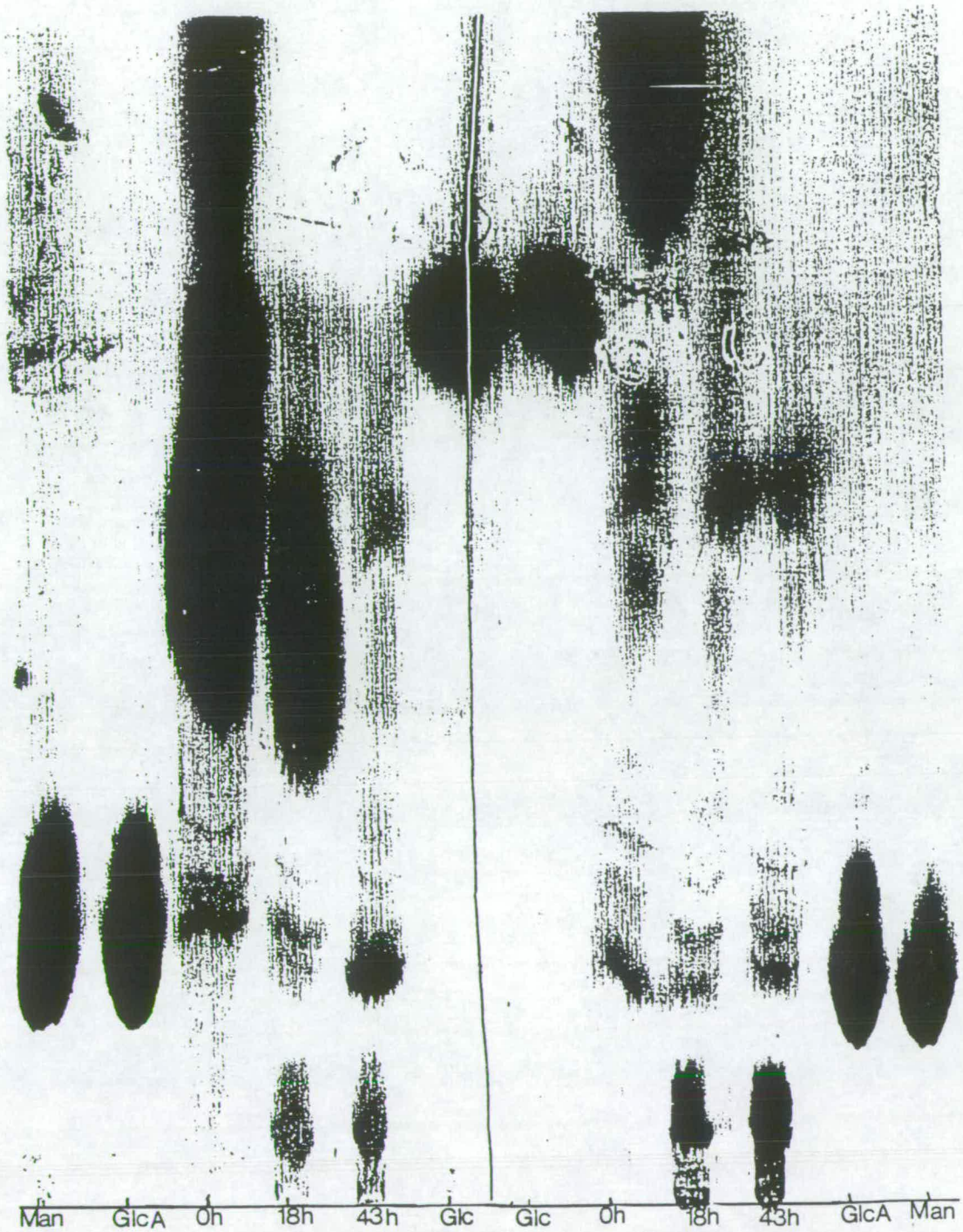


L. hyperborea alginate

M. pyrifera alginate

20µl of each sample and 2µl of standard (Mannuronate lactone, glucuronic acid and glucose 0.1M) were applied to No. 1 Whatman paper and run in the butan-1-ol:pyridine:water system (6:4:3) for 48h. The chromatogram was stained with silver to reveal the material. The samples were applied in the following order: Mannuronate lactone, glucuronic acid, *L. hyperborea* 0h, 18h, 43h, glucose, glucose, *M. pyrifera* 0h, 18h, 43h, glucuronic acid and mannuronate lactone.

Fig. 35b Separation of the *M. pyrifera* and *L. hyperborea* Breakdown Products of Ac 184 *Alginate Lyase* by Paper Chromatography.



L. hyperborea alginate *M. pyrifera* alginate

20µl of each sample and 2µl of standard (Mannuronate lactone, glucuronic acid and glucose 0.1M) were applied to No. 1 Whatman paper and run in the butan-1-ol:pyridine:water system (6:4:3) for 48h. The chromatogram was stained with silver to reveal the material. The samples were applied in the following order: Mannuronate lactone, glucuronic acid,*L. hyperborea* 0h, 18h, 43h, glucose, glucose, *M. pyrifera* 0h, 18h, 43h,glucuronic acid and mannuronate lactone.

Table 24 The Relative Mobilities of Av UW and Ac 184 *Alginate Lyase* Degraded Fragments.

Av UW

	$R_{glc.}$
Glucose	1.00
Glucuronic acid	0.26
Mannuronate lactone	0.23

	1	2	3	4	5
M. pyrifera 0h	0.10	0.23	0.29	0.66	0.90
M. pyrifera 18h	0.11	0.24	0.29	0.75	0.88
M. pyrifera 43h	0.12	0.26	0.32	0.78	0.90
L. hyperborea 0h	0.10	0.22		0.70	0.82
L. hyperborea 18h	0.09	0.21		0.68	0.84
L. hyperborea 43h	0.09	0.20		0.68	0.86

Ac 184

	$R_{glc.}$
Glucose	1.00
Glucuronic acid	0.31
Mannuronate lactone	0.30

	1	2	3	4	5	6	7	8
M. pyrifera 0h			0.27	0.35	0.41	0.69	0.85	1.36
M. pyrifera 18h	0.05	0.11	0.30	0.36		0.66		1.02
M. pyrifera 43h	0.02	0.11	0.30	0.37		0.67	0.81	
L. hyperborea 0h			0.31	0.36	0.42		0.79	1.37
L. hyperborea 18h	0.02	0.11	0.28	0.31	0.38	0.66		
L. hyperborea 43h	0.03	0.11	0.26	0.30	0.37		0.78	

Table 25 The Relative Mobilities of the UV fluorescent Material Produced by Av UW and Ac 184 *Alginate Lyase*.

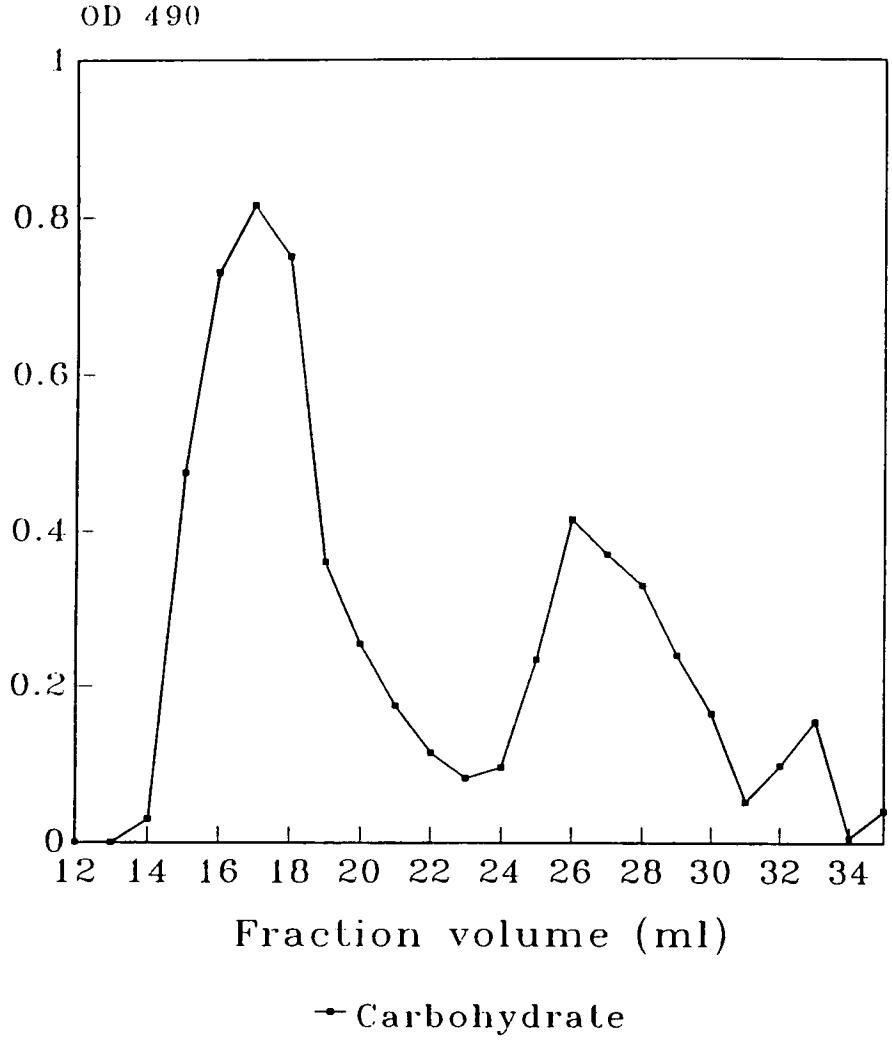
Av UW

				$R_{glc.}$			
Glucose	1.00						
M. pyrifera 0h	0.04	0.18					
M. pyrifera 18h	0.04	0.16					
M. pyrifera 43h	0.04	0.19					
L. hyperborea 0h	0.04	0.15					
L. hyperborea 18h	0.03	0.14	0.19				
L. hyperborea 43h	0.03	0.16	0.19				

Ac 184

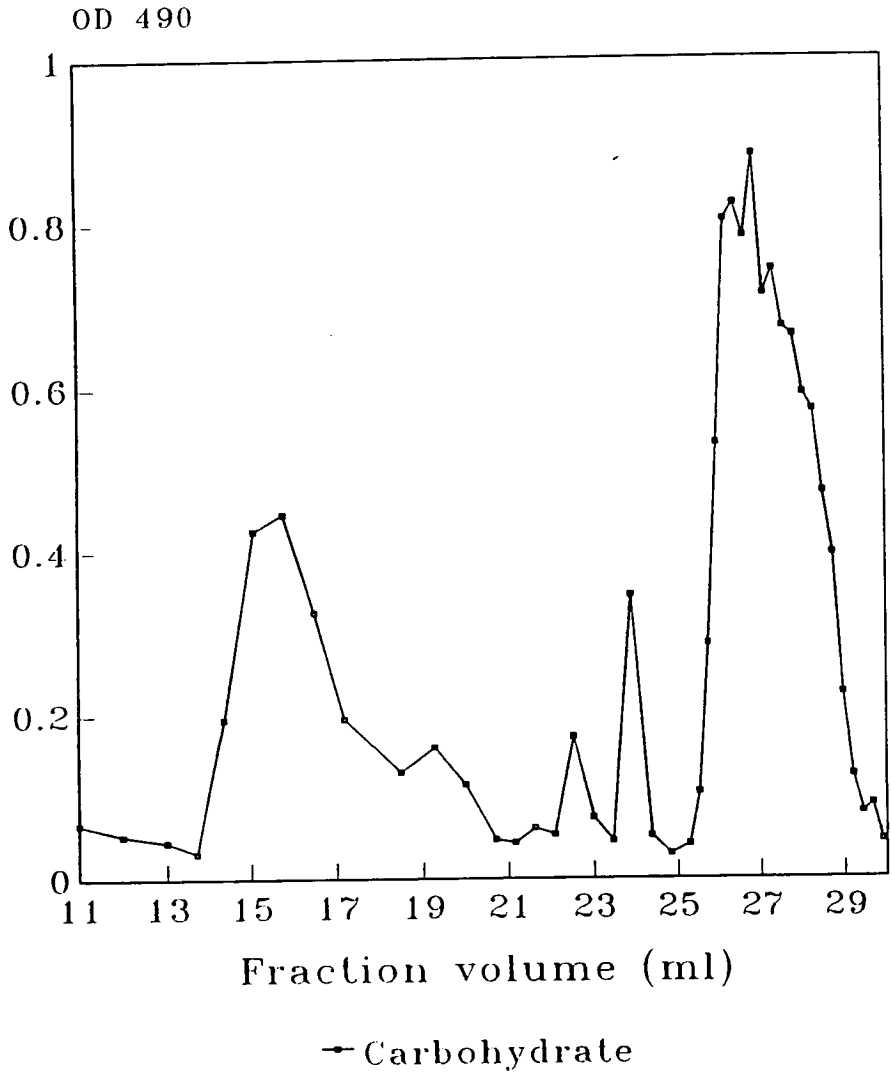
Glucose	1.00						
	1	2	3	4	5	6	7
M. pyrifera 0h	0.05			0.30	0.36		0.75
M. pyrifera 18h	0.05		0.25	0.30	0.39		0.72
M. pyrifera 43h	0.04		0.27	0.32	0.38		0.75
L. hyperborea 0h	0.07		0.26	0.30	0.36	0.45	
L. hyperborea 18h	0.07	0.24	0.27	0.31	0.36		0.70
L. hyperborea 43h	0.07	0.23		0.30	0.36		0.69

Fig. 36 The Elution Profile of Ac 184 *M. pyrifera* Pooled Breakdown Products From Biogel P2.



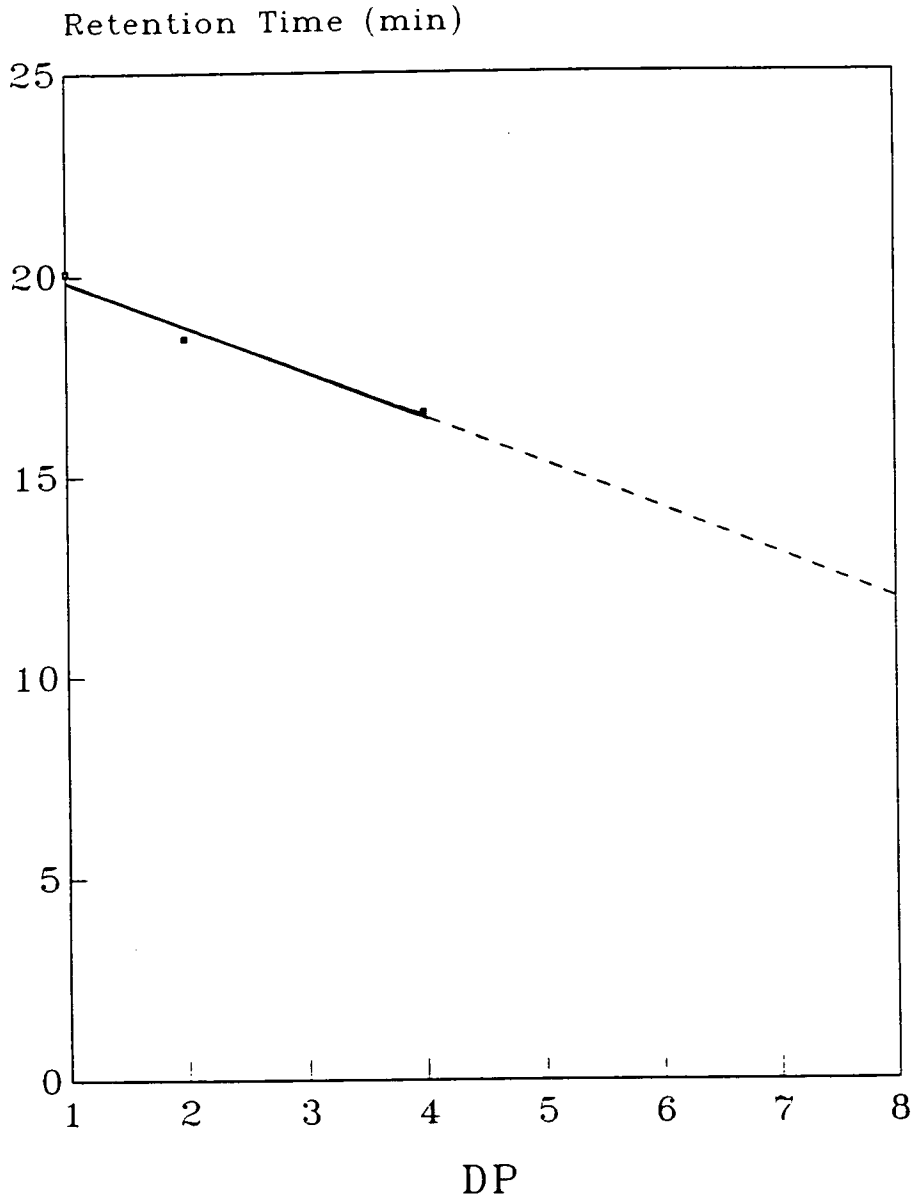
1.5 ml of pooled 18 & 43h sample was applied to the Biogel P2 column and eluted with d.H_2O. 1ml fractions were collected at 14 min intervals. The fractions were tested for total carbohydrate (Dubois, 1956).

Fig. 37 The Elution Profile of Ac 184 *L. hyperborea* Pooled Breakdown Products From Biogel P2.



1.5 ml of pooled 18 & 43h sample was applied to the Biogel P2 column and eluted with d.H_2O. 1ml fractions were collected at 14 min intervals. The fractions were tested for total carbohydrate (Dubois, 1956).

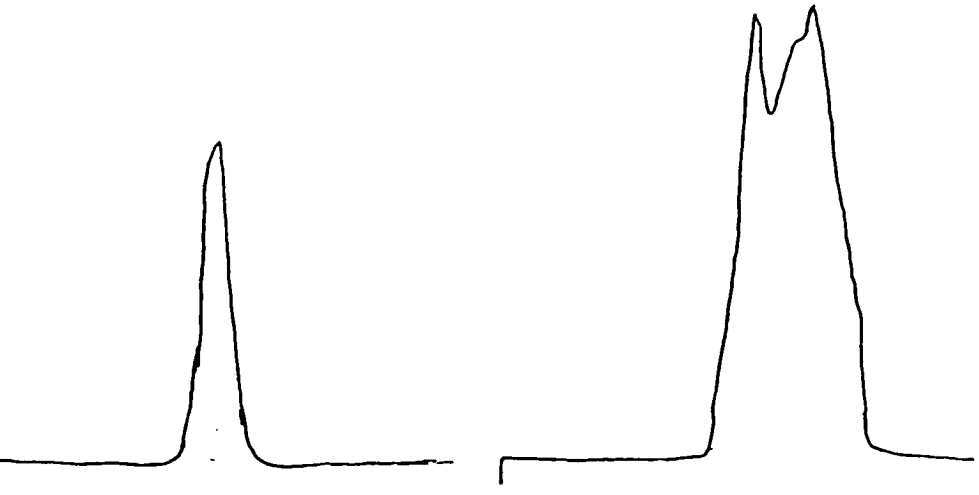
Fig. 38 Standard Curve for the Estimation of the Degree of Polymerisation.



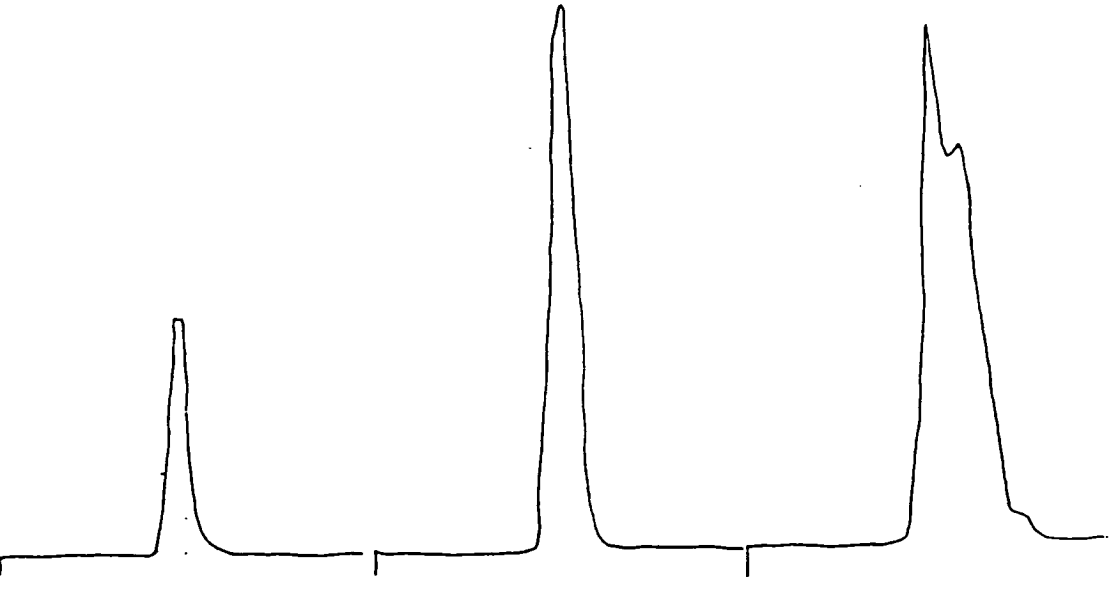
20µl of 0.1M glucose, maltose and maltotetraose were injected as standards.

ig. 39 The Elution Profile of *M. pyrifera* and *L. hyperborea* by HPLC.

et a



et b



et a *M. pyrifera,* Pk 1 & Pk 2.
et b *L. hypoborea* Pk 1, Pk 2 and Pk 3.

0µl of sample was injected into the HPLC with the TSK1,000 column. *M. pyrifera,*
k 1 eluted at 14.92min (DP 5) & Pk 2 eluted at 19.90min (both componentsDP 2).
hypoborea Pk 1 eluted at 14.14min (DP 6), Pk 2 eluted at 15.51min (DP 5)and Pk 3
uted at 15.98min (DP 4).

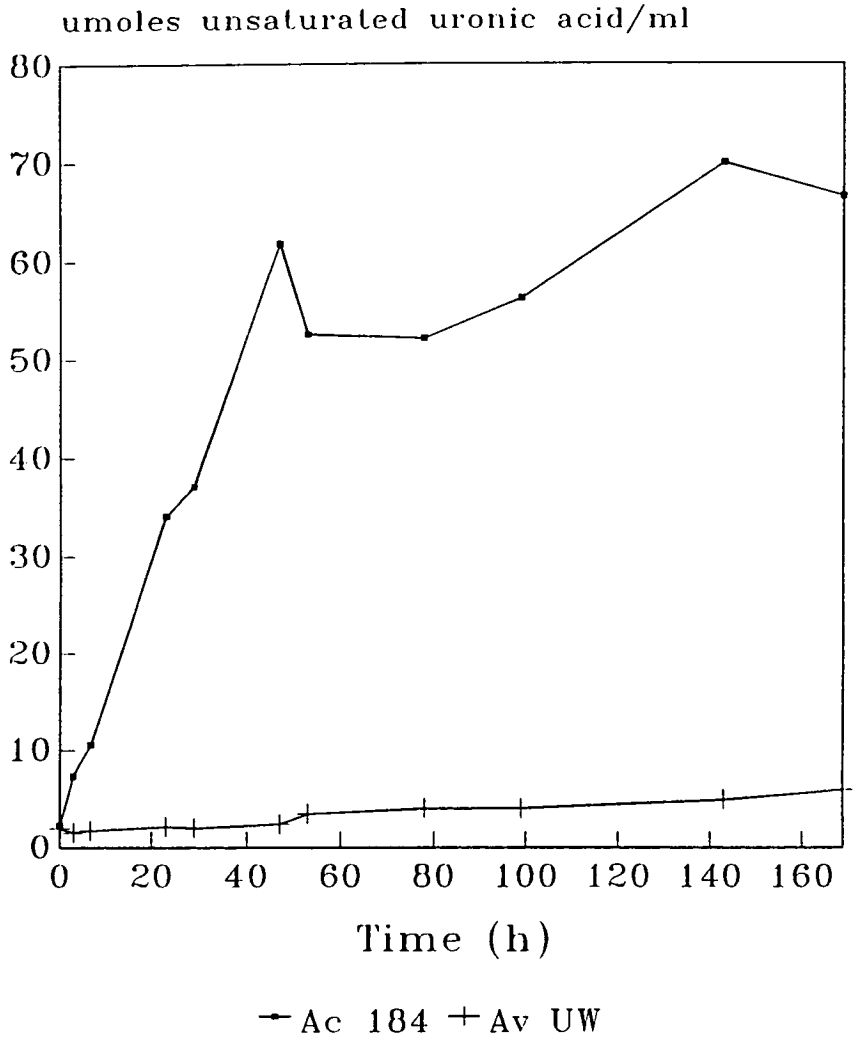
Table 26 Composition and diad frequencies of some of the bacterial and algal alginates

Source	F_G	F_M	F_{GG}	F_{MM}	$F_{GM\,,MG}$	Acetyl
L. hyperborea	0.665	0.335	0.558	0.228	0.107	0
M. pyrifera	0.41	0.59	0.24	0.42	0.17	0
A. vinelandii 73	0.561	0.439	0.372	0.25	0.189	11%
A. vinelandii 206	0.08	0.92	0.03	0.87	0.05	24%
P. aeruginosa B	0.16	0.84	0	0.68	0.16	37%

The analyses were by NMR using a Joel FX-100 spectrophotometer run at 90°C and at 100Mhz as indicated by Skjak-Braek *et al.* (1986).

Geddie (1992)

Fig. 40 The release of TBA Reactive Material from *M. pyrifera* Alginate by Av UW and Ac 184 *Alginate lyase*.



A 3ml aliquot of material was removed from a separate bottle during the incubation and 100µl tested for lyase activity using the TBA test.

Table 27 Chemical Analysis of the Breakdown Products of Av UW *Alginate Lyase* with *M. pyrifera* (Low Viscosity) alginate

Time (h)	Total Carbohydrate	Unsaturated Uronic Acid	Uronic Acid	Sample (ul)	Retentate (mg)
0	4.06	1.85	3.17	700	35.1
3	65.42	3.97		640	36.3
7	3.59	4.94		460	38.1
23	5.33	2.54	2.80	400	32.8
29	3.98	3.36	3.38	580	34.0
47	4.5	4.41	1.72	540	35.4
53	3.54	4.89	2.66	580	34.1
72	7.92	7.84	5.93	600	31.0
78	4.96	4.25		560	38.0
99	51.33	387.60		660	31.9
143	7.6	15.48	5.99	570	29.6
169	6.69	11.59	4.79	500	32.6

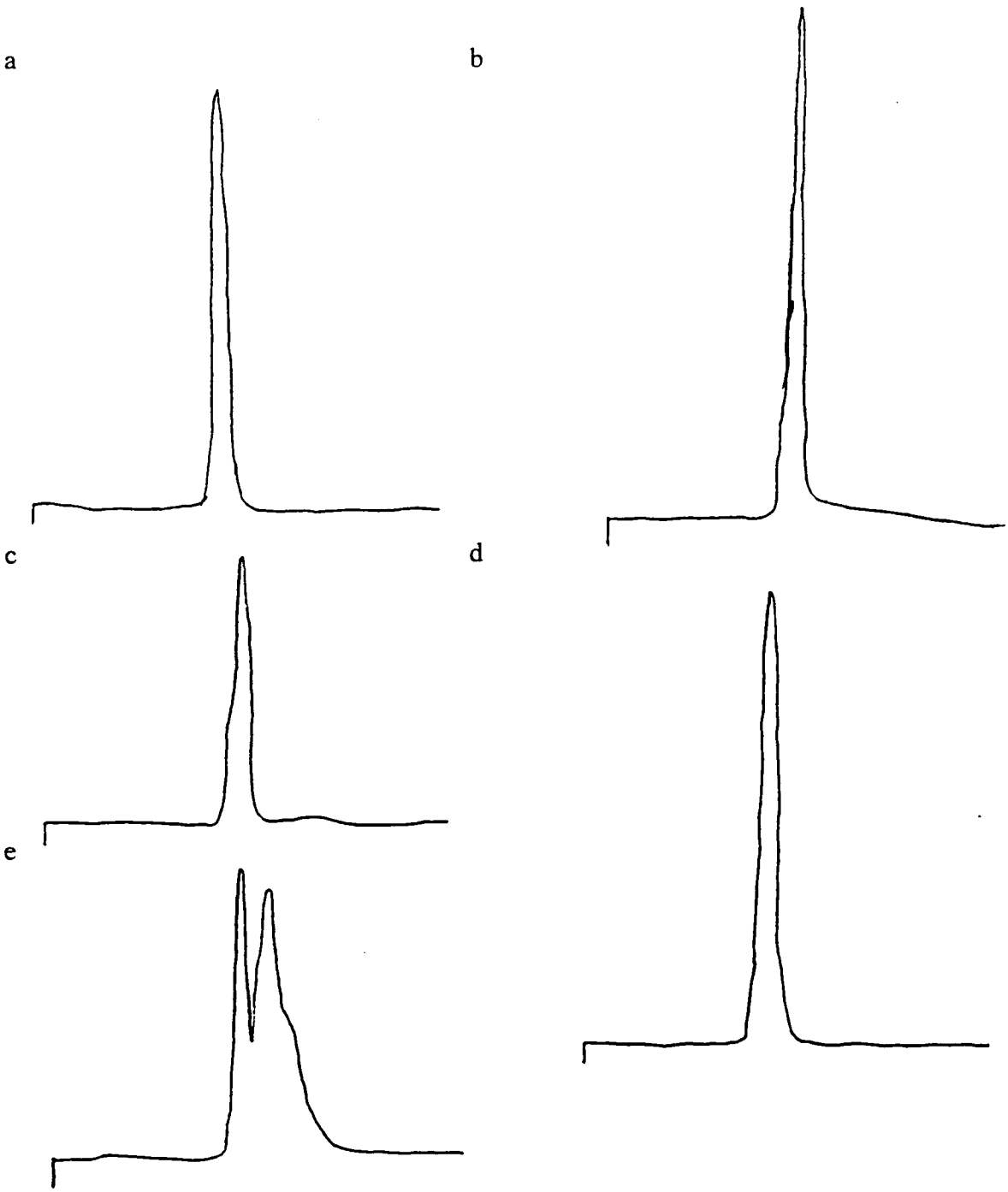
Total Carbohydrate — µmole glucose sample-1
Unsaturated uronic acid — µmoles Glucuronic acid sample^{-1}
Uronic Acid — µmoles Glucuronic acid sample^{-1}

Table 28 Chemical Analysis of the Breakdown Products of Ac 184 *Alginate Lyase* with *M. pyrifera* (Low Viscosity) alginate

Time (h)	Total Carbohydrate	Unsaturated Uronic Acid	Uronic Acid	Sample (ul)	Retentate (mg)
0	36.40	9.31	6.27	840	35.1
3	6.2	8.75	2.16	700	36.3
7	5.35	20.53	3.22	680	38.1
23	14.64	53.49	4.55	570	32.8
29	10.80	99.58	7.08	720	34.0
47	7.54	124.81	6.97	610	35.4
53				520	34.1
72	14.00	167.67	5.82	600	31.0
78	9.02	229.85	7.89	580	38.0
99	7.82	268.79	8.53	640	31.9
143	7.17	348.99	3.52	600	29.6
169	6.38	246.51	3.05	560	32.6

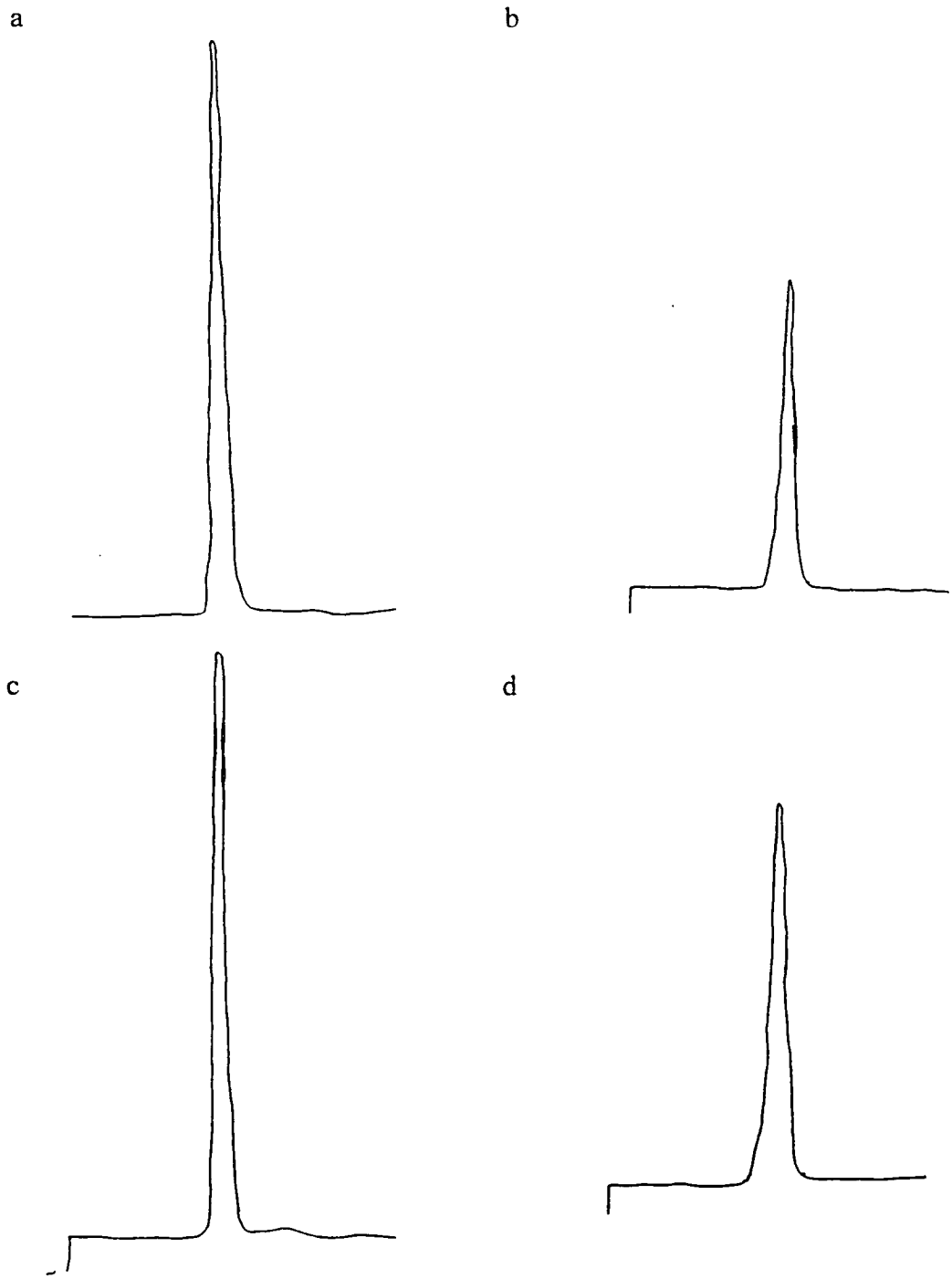
Total Carbohydrate µmole glucose sample-1
Unsaturated uronic acid µmoles Glucuronic acid sample^{-1}
Uronic Acid µmoles Glucuronic acid sample^{-1}

Fig. 41 The Elution Profiles from HPLC of *M. pyrifera* Breakdown Products After Degradation by Av UW *Alginate Lyase*.



a, 7h incubation under standard conditions, eluted at 14.4min and has DP6.
b, 47h incubation under standard conditions, eluted at 14.44min and has DP6.
c, 78h incubation under standard conditions ,eluted at 14.68min and has DP6.
d, 143h incubation under standard conditions, eluted at 14.4min and has DP6.
e, 99h incubation under standard conditions, eluted at 14.4 & 16.25 min and has DP6 & 4.

Fig. 42 The Elution Profiles from HPLC of *M. pyrifera* Breakdown Products by After Degradation by Ac 184 *Alginate Lyase*.



a, 7h incubation under standard conditions, eluted at 14.97min and has DP5.
b, 47h incubation under standard conditions, eluted at 14.94min and has DP5.
c, 78h incubation under standard conditions ,eluted at 15.02min and has DP5.
d, 143h incubation under standard conditions, eluted at 16.34min and has DP4.

Preiss & Ashwell (1962) first separated the breakdown products of alginate degradation by paper chromatography. The components gave a strong positive reaction when sprayed with TBA reagent (Warren, 1960). After a 24h incubation a qualitative change in the chromatographic profile occurred and the intensity of the leading spot increased. The most rapidly moving spot was assumed to be a monosaccharide end product and the slower moving components as a series of unsaturated oligosaccharides. Boyd & Turvey (1977) found that when the *alginate lyase* of *K. pneumoniae* acted on poly G, a series of oligosaccharides were formed, prolonged action resulted in the formation of three products A, B & C on TLC plates. Further separation was carried out using Biogel P4. The uronic acid test in the eluted fractions showed that products were present in the ratio 1:1:0.1, A:B:C. Each of the products was tested with the TBA reagents (Weissbach & Hurwitz, 1959) and found to be positive and therefore contained an unsaturated uronic acid. The degree of polymerisation indicated a di, tri, and tetrasaccharide A,B,C respectively, thought to be Δ-G, Δ-GG and Δ-GGG, where 'Δ' was the unsaturated group and G was the 4 linked α-L guluronic acid.

While examining the role of *O*-acetyl goups and the mannuronan C-5 epimerase Sjkåk-Bræk *et al.* (1985), used the guluronate-specific *alginate lyase* from *K. pneumoniae* to produce oligosaccharides from *A. vinelandii* alginate. A major peak eluted close to the void volume of a Biogel P4 column. This fraction contained mainly homopolymeric blocks of mannuronate (95%) with a DP of 40. A series of small oligosaccharides eluted next, while one fraction contained low molecular weight material. FR II-V contained increasing amounts of guluronate and had progressively shorter chain lengths 11, 8, 6 and 3 respectively and Fr III-V contained

95% guluronate and Fr II contained 12% mannuronate more than half of which were heteroglycoside linkages. No acetyl groups were found in this fraction.

Substrate Specificity of Av UW and Ac 184 *alginate lyase*

A wide range of alginates from a variety of sources were prepared and incubated with Av UW and Ac 184 *alginate lyase.* Fig. 43 shows the result of the incubation of alginate lyase with alginate of increasing mannuronate content and Table 29 shows the results of incubation with various algal and bacterial alginates.

Preiss & Ashwell (1962) found that an *alginase*, isolated from bacteria grown on alginate as the sole carbon source, exhibited a high degree of specificity. It was unable to degrade hyaluronate, chondroitin A, B, or C, polygalacturonic, D-mannuronic acid or L-guluronic acid. The inability of the bacteria to metabolise either D-mannuronic acid or L-guluronic acid indicated that they were not intermediates in the formation of 4 deoxy-5-ketouronic acid. When the same bacteria were grown on polyguluronate as the sole carbon source the cell extracts were able to degrade short poly G chains. The enzyme was less active against poly MG blocks and least active against alginate rich in mannuronate.

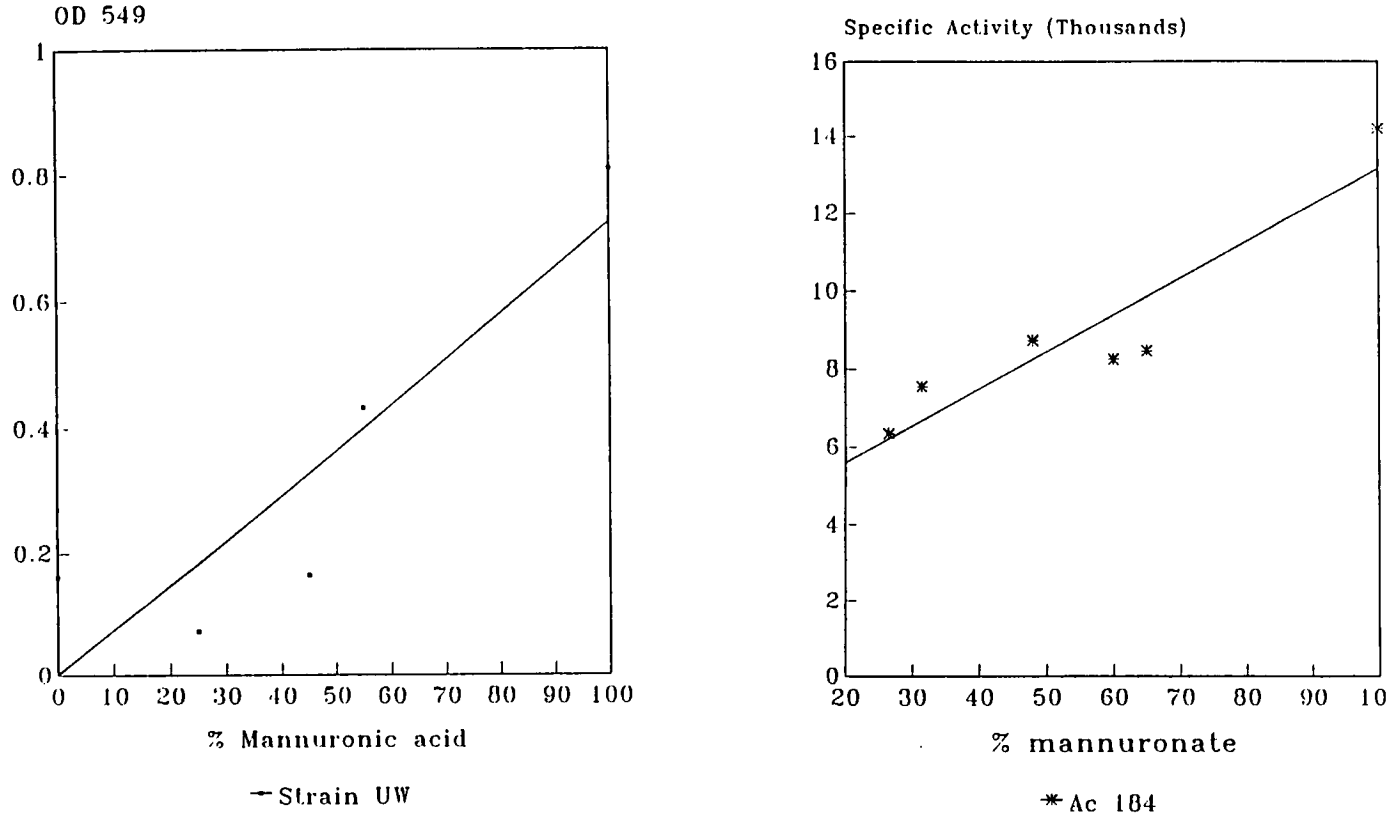
Isolation of *alginate lyase* from *Alteromonas atlantica*

Two isolates of *A. atlantica* were grown in sea water supplemented with yeast extract (0.1%), casein hydrolysate (0.1%) and 0.7% glucose and 0.03% sodium alginate (*L. hyperborea*) as the carbon source. The cells were grown for 7d at 30°C and the spent medium was concentrated by tangential flow filtration. Total cell extracts were prepared and the various fractions were tested for TBA activity and protein concentration. From the results (Table 30), it can be seen that the *alginate*

lyase from both strains of *A. atlantica* was cell-associated and exhibited a preference for the *M. pyrifera* high viscosity alginate. Thus, mannuronate residues are preferred. The two sources of *M. pyrifera* alginate differ in the viscosity of the dissolved polymer. The viscosity of the solution is determined by the length of the polymer chain (DP). Although the *alginate lyases* from both strains were slightly active against the *L. hyperborea* alginate, which is rich in guluronate residues, the alginates still contain 10% M/G and G/M. One cannot therefore conclude whether the enzyme was specific for either MM, M/G or G/M.

Substrate specificity was further examined using a number of other sources of alginate including acetylated and deacetylated bacterial alginate, the results of which can be found in Table 31. The assays were set up using 50mM MES pH 6.8 as the buffer and incubated at 30°C for 16h.

Fig. 43 The Effect of Mannuronate Content on Av UW and Ac 184 *Alginate Lyase*.



The substrate specificity of Av UW and Ac 184 *alginate lyase* were tested using semi-purified enzyme. The assays were incubated at 30°C for 7h. An aliquot was removed and the TBA assay was performed in triplicate. It should be noted that the vertical axis on the Av UW graph is expressed as absorbance at 549nm whereas the vertical axis of the Ac 184 graph is expressed as µmole unsaturated uronic acid min^{-1}.

Table 29 Substrate Specificity of Av UW and Ac 184 *Alginate Lyase.*

Enzyme	Av UW		Ac 184	
Source of Alginate	*Alginate Lyase* (pmole ml^{-1} mg $protein^{-1}$)	% *M. pyrifera* Specific Activity	*Alginate Lyase* (pmole ml^{-1} mg $protein^{-1}$)	% *M. pyrifera* Specific Activity
a) Algal				
M. pyrifera	1102	100	22722	100
b) Bacterial				
Pseudomonas	404	36.7	377	1.7
Azotobacter	151	13.7	5681	25.0
P. aeruginosa	28	2.5	nt	
Pseudomonas DAC	1171	106	6107	26.9
Bacterial DAC	346	31.4	6393	28.1
MG1	295	26.7	4839	21.3

nt not tested

Table 30 Location of *Alginate lyase* activity in *Alteromonas atlantica*

A. atlantica 511-T

	Source of Alginate			
	L. hyperborea	*M. pyrifera* High Viscosity	*M. pyrifera* Low Viscosity	Protein mg ml^{-1}
Spent Medium	545	2,494	940	1.22
Total cell Extract	1,912	15,042	7,957	0.32
Membranes	67	nd	nd	0.34

A. atlantica T9

	Source of Alginate			
	L. hyperborea	*M. pyrifera* High Viscosity	*M. pyrifera* Low Viscosity	Protein mg ml^{-1}
Spent Medium	10	nd	nd	0.47
Total cell Extract	2,393	15,840	5,928	0.26
Membranes	nd	41	nd	0.82

nd no enzyme activity detected

Specific Activity ρ moles ml^{-1} mg $protein^{-1}$

Table 31 Substrate Specificity of *A. atlantica alginate lyase*

	Specific Activity	
	A. atlantica	*A. atlantica*
Substrate	511-T	T9
L. hyperborea	7,759	4,310
M. pyrifera (HV)	20,159	3,341
M. pyrifera (LV)	14,788	7,866
A. vinelandii 206	nd	nd
A. vinelandii 206 DAC	73,607	53,987
Pseudomonas B	21,884	13,362
Pseudomonas B DAC	5,836	nd
P. aeruginosa	2,718	916
P. aeruginosa DAC	3,514	809
Poly mannuronate	1,392	nd
Pseudomonas sp DAC	nd	nd
73 bacteriophage digested	9,947	nd
Azotobacter sp Run 73 DAC	nd	nd

Specific Activity	ρmole ml^{-1} mg $protein^{-1}$
LV	Low Viscosity
HV	High viscosity
DAC	Deacetylated polymer
nd	no enzyme activity detected

Isolation of Gellan Lyase Producing bacteria.

Several bacterial strains were isolated in pure culture from soil or mud, from local sites, in a chemically-defined medium containing 2% gellan. The transfer was repeated several times and colonies selected from medium solidified with gellan (2.3.2). The colonies which were able to degrade gellan formed pits in the medium. The bacteria were routinely maintained on media solidified with agar and medium solidified with gellan. All the bacteria were red pigmented Gram negative motile rods, catalase positive, oxidase negative and produced an amylase. The red pigment was cell-associated, could be extracted with chloroform:methanol (2:1) and had absorption maxima at 490 and 530nm. Generally all the red pigmented bacteria behaved in a similar manner.

All the bacteria were resistant to kanamycin (5μg Multidisc 724E, Oxoid), but only strains 2A and 11.1 were resistant to 30μg (Mastring-S, M26). All were resistant to penicillin at 1.5 I.U. (Multidisc 724E, Oxoid), resistant to 2μg ampicillin (Multidisc 724E, Oxoid) but sensitive to 25μg ampicillin (Mastring-S, M26). All were able to grow on gellan as the sole carbon and energy source and provided a novel degradative enzyme - a *gellanase* or *gellan lyase*.

The bacteria grew poorly on almost all media tested. They were grown on YE supplemented with 1% glucose (Fig. 44). Strain 13.1 grew best in medium containing 1% glucose (OD_{600} 0.859 after 8h) with a generation time of 2h.

Strain 14.1 was grown on YE (0.3% acid-hydrolysed gellan) (500kDa). The OD_{600} (Fig. 45) was monitored and viable counts performed at appropriate time intervals. The spent culture medium was concentrated and lyase activity was detected using the TBA reagent (Table 32). When gellan was the substrate (control), similar activities were observed at 17h and at 113.5h. However, when acid-hydrolysed gellan

was the substrate, the same level of activity was not observed until 67h incubation. By 119h similar levels of activity were observed with both substrates. α-L-*Rhamnosidase* activity was detected in the final spent culture medium after 1h incubation at 30°C and α- and β-*glucosidase* after an overnight incubation.

The growth of 13.1 was determined on medium containing various concentration of glucose (Fig. 46) and subsequently 1% glucose was routinely used. Growth of strain 13.1 on some intermediates of the Entner-Doudoroff pathway and the TCA cycle was determined: on gluconate, α-oxoglutarate and glutamate (1%) as the sole carbon source (Fig. 47). The levels of lyase produced in the following media were determined: 0.1% gellan, 2% gluconate, 2% gluconate & 0.1% gellan, 2% glutamate, 2% glutamate & 0.1% gellan, 0.8% glucose, 0.8% glucose & 0.1% gellan and Luria broth (LB). Table 33 shows that the bacteria grew best on 2% gluconate & 0.1% gellan with the 0.1% gellan culture only producing a third of this weight and the glucose grown cells only producing half g wet weight l^{-1} of the gellan-grown cells.

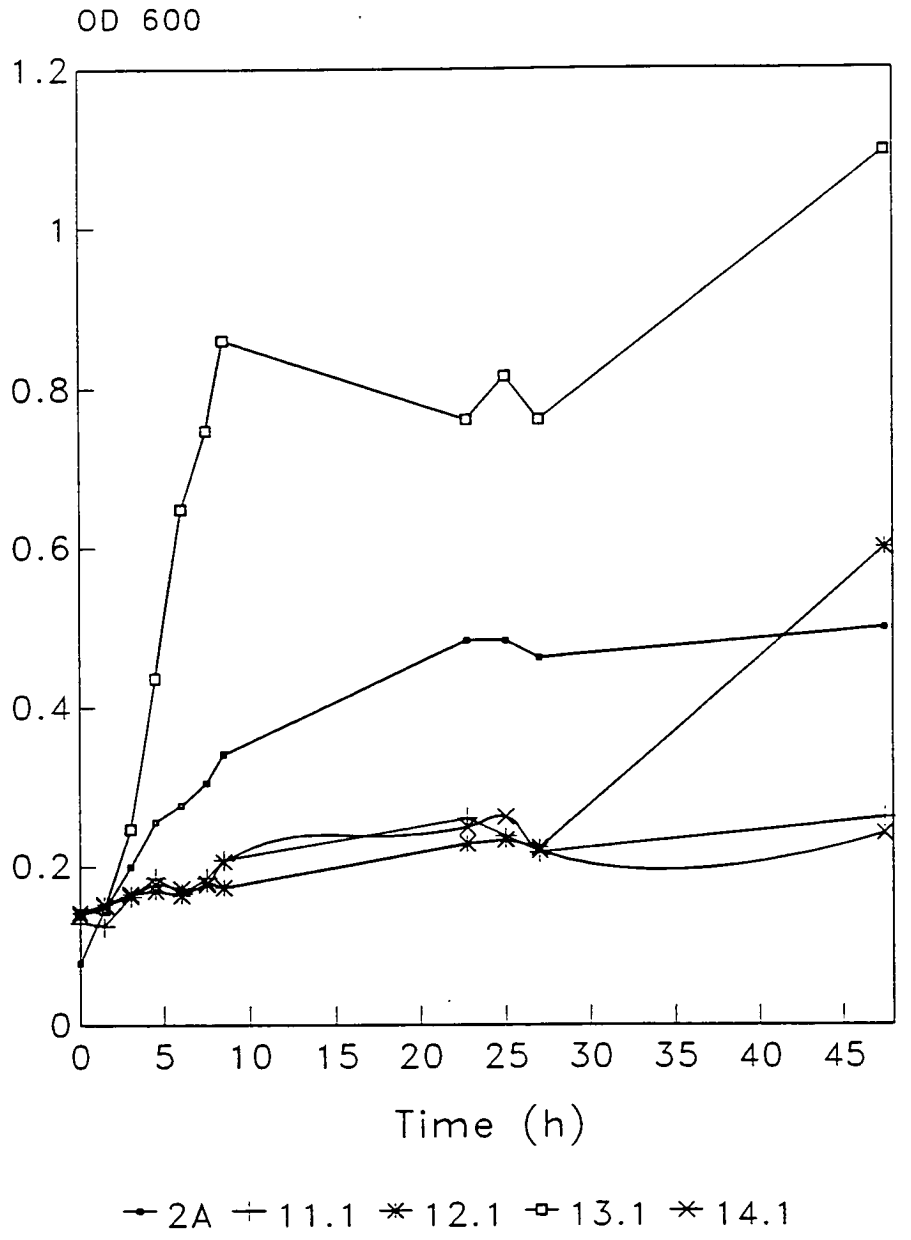
Gellan lyase activity was detected in the supernatant of the 0.1% gellan-grown cells, only 4% of this activity was detected in the 0.8% glucose & 0.1% gellan-grown cells. 11% of that level was detectable in the 2% gluconate & 0.1% gellan grown-cells and 25% in the 2% glutamate & 0.1% gellan-grown cells. However, in the cytoplasmic and periplasmic fractions, *gellan lyase* activity could be detected under all the growth conditions. The level of enzyme activity was higher in the periplasmic fraction than in the cytoplasmic fraction. The *gellan lyase* was located in the periplasm, exported out of the cell where the highest levels of activity were detected. Since the medium supplemented with alternative carbon sources failed to increase

enzyme activity, *gellan lyase* was isolated from bacteria grown on gellan as the sole carbon source

The effect of gellan concentration on growth (13.1) was determined. The percentages (%) of gellan tested were: 0.05, 0.1 and 0.2. A glucose (2%) grown culture was included as a control. The highest OD_{600} (0.85) was achieved on 0.2% gellan (generation time of 2.5h). By comparison, 13.1 grew least well on 2% glucose. When 14.1 was grown on YE+0.2% gellan, an average of 1.5g wet weight l^{-1} was routinely obtained. Some of the 13.1 growth experiments were also carried out on 1/10 YE medium but very poor growth was observed in this medium. If 13.1 was a *Flavobacterium* sp., which can have a vitamin B requirement, this would explain its poor growth on 1/10 YE medium.

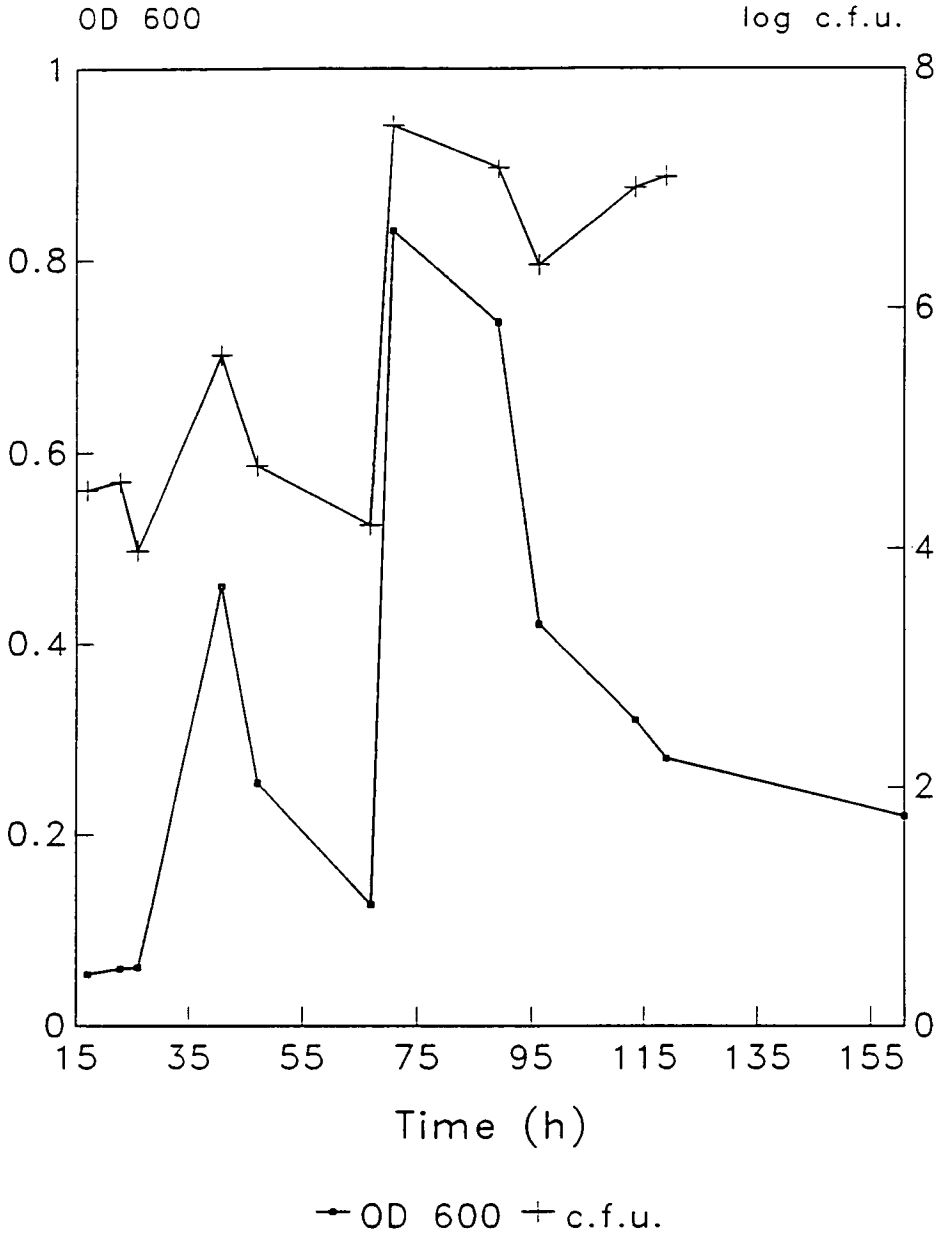
The optimal incubation conditions for the intracellular 13.1 *gellan lyase* were determined; temperature optimum was between 37-40°C, pH 7.0 in 10mM TRIS for a 3h incubation period. When incubated under the above conditions with 10mM MES the pH optimum was 5.2. Either the enzyme has two different pH optima depending on the type of buffer present or more than one enzyme is present in the intracellular fraction. The same incubation conditions were required for the extracellular *gellan lyase* 2A. After incubation with 2A *gellan lyase,* no free glucose was detected using the *glucose oxidase* test. The amount of reducing sugar released by the action of *gellan lyase* mirrored the released of TBA products. It could therefore be assumed that *gellan lyase* was not an a *endoglucosidase* but that it cleaved the repeat backbone unit of gellan at some other sugar residue.

Fig. 44 Growth Curves of Gellan-Degrading Bacteria on 1% Glucose.



The bacteria were grown in YE (1% Glucose) at 30°C on an orbital shaker. Samples were removed and the optical density measured at 600nm.

Fig. 45 Growth Curve and Viable Count For Strain 14.1 on 0.3% Acid Hydrolysed Gellan.



The bacteria were grown in YE (0.3% acid hydrolysed gellan) at 30°C on an orbital shaker. Samples were removed and the optical density measured at 600nm. Viable counts were also performed after serial dilutions were made. The bacteria were plated out on YE agar (1% glucose).

Table 32 *Gellan lyase* Activity in the Supernatant of Strain 14.1 when Grown on Gellan and Acid Hydrolysed Gellan.

Control Gellan Grown Supernatant.

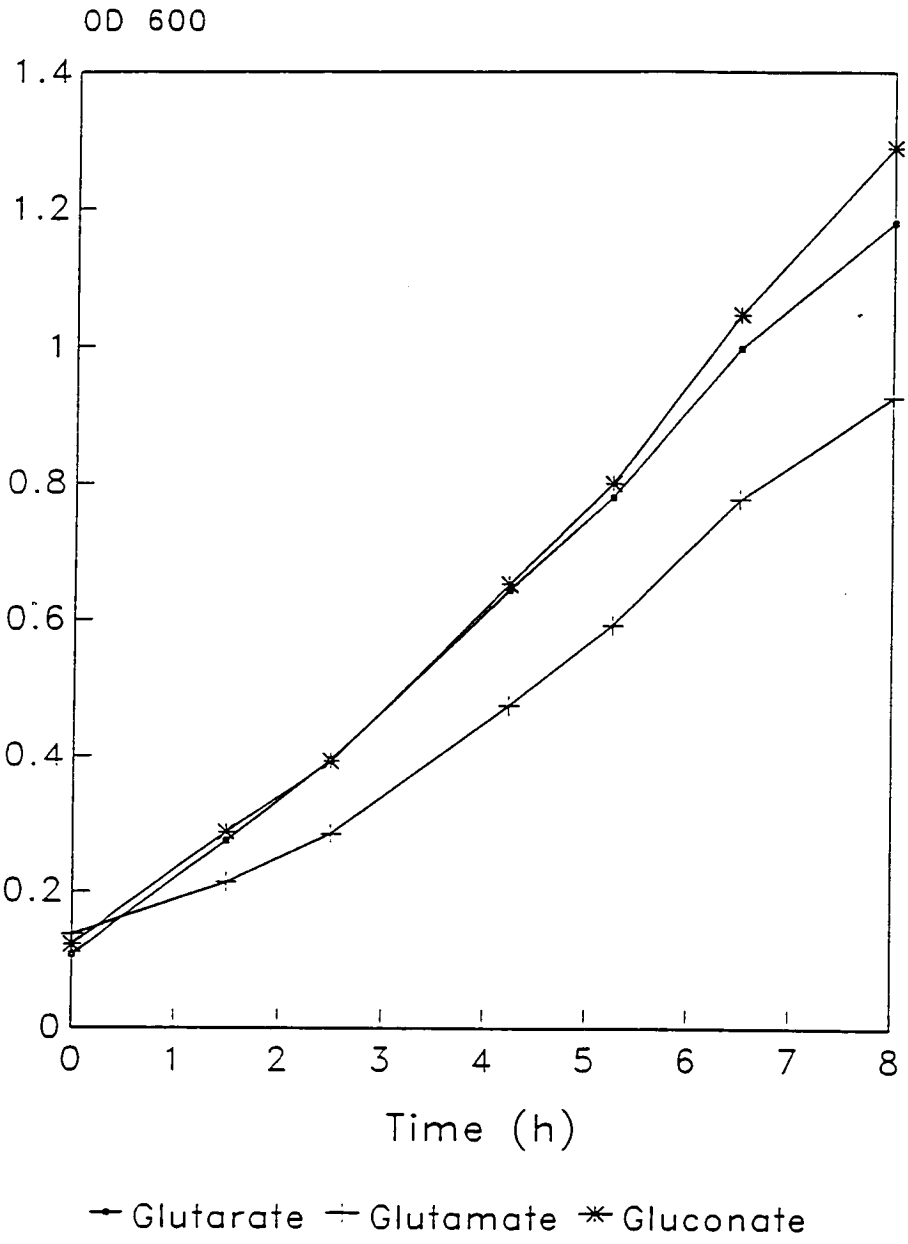
Time (h)	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity
17	574	0.25	0.02	16.4	2333
41	442	0.83	0.06	13.0	532
113.5	10093	4.4	0.71	6.2	2294
119	3532	2.3	0.38	6.1	1536
161	1320	5.44	0.68	8	242

0.3% Acid Hydrolysed Gellan Supernatant

Time (h)	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity
17	131	1.18	0.37	3.2	111
41	5527	2.34	0.34	6.9	2362
113.5	4829	4.4	0.71	6.2	1097
119	3068	2.3	0.39	6.1	1334
161	1216	5.44	0.68	8	223

Specific Activity ρmole ml^{-1} mg protein^{-1}

Fig. 47 Growths curves of Strain 13.1 0n Glutarate, Glutamate and Gluconate.



The bacteria were grown in YE (1% glutarate, glutamate and gluconate) at 30°C on an orbital shaker. Samples were removed and the optical density measured at 600nm.

Table 33 Growth of Strain 2A on 0.1% Gellan Supplemented with Various Carbon Sources

Growth Conditions	gl^{-1} wet weight	Supernatant	Periplasm	Cytoplasm
0.1% Gellan	2.35	3885	5435	522
0.1% Gellan+2% Gluconate	7.18	-	393	185
0.1% Gellan+2% Glutamate	4.54	996	17	415
0.1% Gellan+0.8% Glucose	2.42	188	45	156
2% Gluconate	2.58	560	840	55
2% Glutamate	1.35	-	1273	678
0.8% Glucose	1.09	-	28	14
LB	2.43	-	-	19

Specific Activity pmole ml^{-1} mg $protein^{-1}$

1l of YE supplemented with one of the above were grown at 30°C for 5d. The cells were pelleted by centrifugation and the supernatant concentrated by tangential flow filtration. The protein concentration and *gellan lyase* activity were determined

Induction and Location of *gellan lyase*.

Routinely, all bacteria were grown in batch culture. As the *gellan lyase* was produced the solidified medium liquefied. The supernatant was concentrated by tangential flow filtration, total cell extracts were prepared and the supernatant and cytoplasmic fractions were tested for lyase activity and *glycosidase* activity.

Bacterium 12.1 was grown on gellan (0.1%), glucose (0.7%) and starch (0.1%) The supernatant was concentrated and total cell extracts were prepared. The highest level of extracellular *gellan lyase* was detected in the gellan grown cells (Table 34), although a low level of *gellan lyase* was detectable within the glucose and starch grown cells. The level of activity detected in the glucose-grown cells was only 15% of that detected in the gellan-grown cells and only 4% in the starch-grown cells compared to the gellan-grown cells. An α-L-*rhamnosidase* was associated with the cytoplasmic fraction of the total cell extract of the gellan-grown cells; no activity was detected in either the glucose- or starch-grown cells. The glucose-grown cells did have α-D-*glucosidase* activity associated with the cytoplasmic fraction.

To determine the approximate molecular weight of the 12.1 *gellan lyase*, the supernatants from all three cultures were separated into two nominal fractions, one greater than 100kDa and the other less than 100kDa by tangential flow filtration in the Minitan using the 100kDa membranes. In all cases, most of the lyase activity was associated with the fraction exceeding 100kDa (Table 34). The three supernatant fractions of the gellan-grown cells were tested for the presence of various *glycosidases*. Assays were made for the following enzymes using ONP-linked sugars: α-L-*rhamnosidase*, α-D-*mannosidase*, α-D-*glucosidase*, β-D-*glucosidase* and β-D-*glucuronidase*. An α-L-*rhamnosidase* was associated with the <100kDa fraction. An α-D-*glucosidase* was detected in all the fractions of the glucose-grown

bacteria. In the cytoplasmic fraction the *gellan lyase* was associated with the >100kDa fraction, as was the α-D-*rhamnosidase* activity.

Bacterium 2A was grown YE medium (0.25% gellan). Sphaeroplasts were prepared according to the method of Osborn *et al.* (1972) and were broken by sonication to yield four fractions: supernatant, periplasm, cytoplasm and membranes. Throughout the experiment an average of 1.86g wet weight of cells per litre of medium was routinely obtained. After 72h growth, there was a significant decrease (11%) in residual polymer then a linear decrease to 28% at 96h and 42 % at 120h.

The results of the lyase test are shown in Fig 48; the maximum amount of TBA-reactive material was isolated in the 48h supernatant fraction but the periplasm had a higher concentration of TBA reactive material than the cytoplasm. Some lyase activity is associated with the membrane fraction. After overnight incubation, the supernatant fractions had slight α-L-*rhamnosidase* and β-D-*glucosidase* activity, whereas α-D-*glucosidase* activity was detected within 10min in the 48-96h samples. The periplasmic fraction had β-D-*glucosidase* activity within 0.5h and only slight α-L-*rhamnosidase* and α-D-*glucosidase* activity after overnight incubation. After overnight incubation the cytoplasmic fraction showed β-D-*glucuronidase*, α-L-*rhamnosidase* and α-D-*glucosidase* activity . No 'free' glucose was detected in any of the samples.

Growth on other carbon sources was determined as was the ability to induce *gellan lyase*. The following substrates were used at a concentration of 1%: L-alanine, sodium gluconate, α-oxoglutarate and sodium glutamate. Strain 14.1 could not grow on any of these substrates but the other bacteria (2A, 11.1, 12.1, 13.1) appeared to do so and *gellan lyase* activity was detected.

Gellan lyase was isolated from strain 13.1 grown in glucose (2%) and gellan (0.05, 0.1 and 0.2%) grown cells. The results can be found in Table 35. The level of lyase in the supernatant increased as the concentration of gellan in the growth medium increased. Maximum specific activity of 3,296ρmoles ml^{-1} mg $protein^{-1}$ was detected in the 24h sample, no lyase activity was detected in the 2% glucose control. The same was true for the sample taken at 48h. The cytoplasmic fractions contained some lyase activity but at much lower level than that of the supernatant. The 24h 0.2% gellan-grown sample had a specific activity of 457ρmoles ml^{-1} mg $protein^{-1}$ which was c. 8 times lower than that found in the supernatant. The 48h cytoplasmic fraction contained 4.8 times less activity than its corresponding supernatant sample. Very low activity was observed in the membrane fraction. It was therefore assumed that the *gellan lyase* in 13.1 was an inducible extracellular enzyme, although low levels of the enzyme could be detected within the cell.

After 3.5h at 37°C, slight β-D-*glucosidase* activity was detected in either the 24 or 48h spent culture medium. The complement of intracellular enzymes was more complex. The ONP-α-L-rhamnopyranoside turned yellow as soon as the enzyme was added to the assay mixture and within 30min the following enzymes were detectable: α-D- and β-D-*glucosidase*; after 1.5h, α-D-*mannosidase*. There appeared to be α-L-*rhamnosidase* activity associated with the membrane fraction, since nitrophenol was released within 30min incubation. After 2.5h there was slight β-D-*glucosidase* activity.

A similar study to that described above was carried out on the other *gellan lyase*-producing bacteria grown on YE + 0.2% gellan. The spent culture medium was dialysed against 10mM TRIS pH 7.0 and concentrated against PEG 6000. The 48h spent culture medium had most *gellan lyase* activity (Table 36). 13.1 *gellan lyase*

was the most active and 12.1 was the least active. Lower levels of activity were found in the cytoplasmic fraction and no activity could be detected in the membrane fraction. *Glucosidase* activity was determined in the supernatant and in the cytoplasmic fractions. After 1h β-D-*glucosidase* was present in supernatant fraction of 12.1 and after 2.5h it was present in the 13.1 supernatant. Within 15min, both 13.1 and 14.1 were positive for α-L-*rhamnosidase* and β-D-*glucosidase*.

Table 34 Location and Induction of 12.1 *Gellan Lyase.*

Supernatant

Growth		Specific Activity	
	Control	>100KDa	<100KDa
Gellan	11774	11775	3333
Glucose	1925	1045	226
Starch	470	411	222

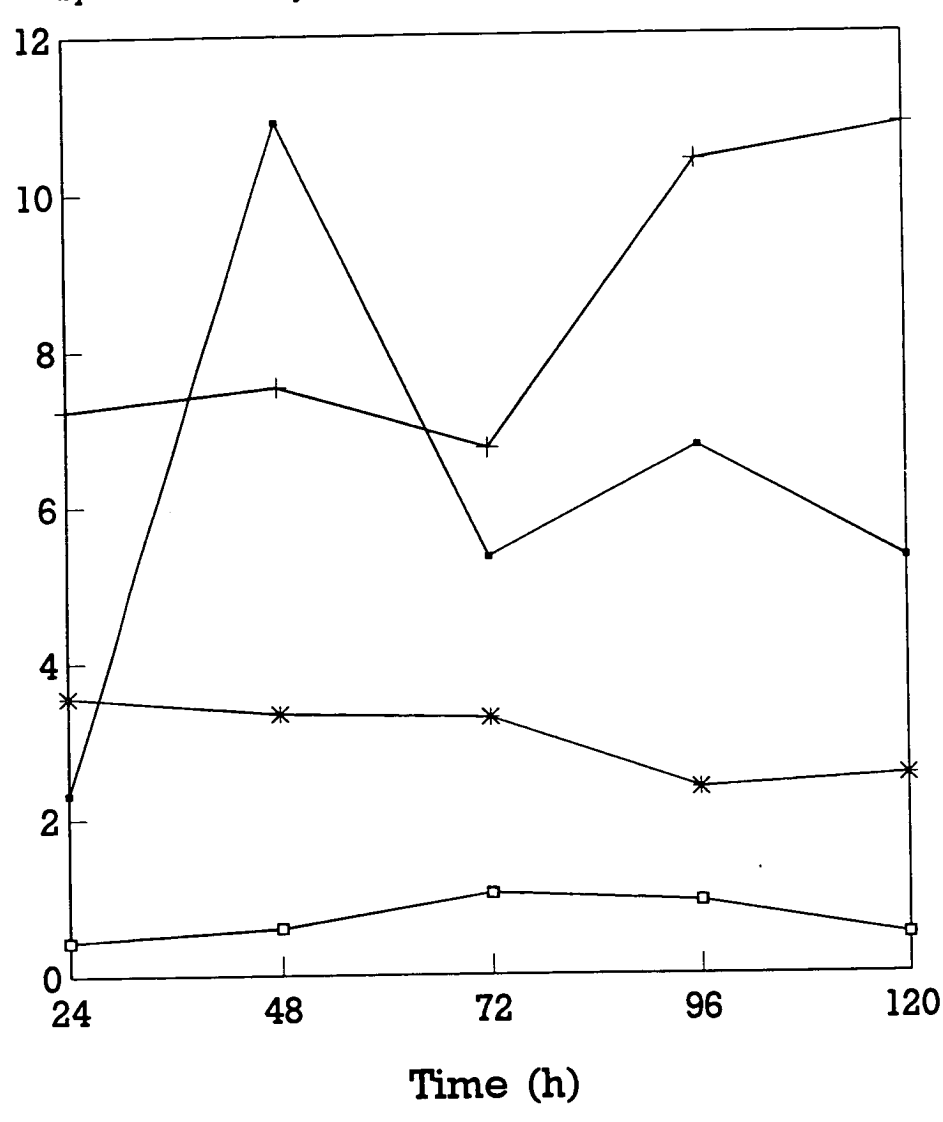
Cytoplasm

Growth		Specific Activity	
	Control	>100KDa	<100KDa
Gellan	8257	8226	580
Glucose	134	110	-
Starch	202	52	25

Specific activity pmole ml^{-1} mg $protein^{-1}$

Strain 12.1 was grown on 0.1% gellan, 0.7% glucose or 0.1% Starch at 30°C for 5d. The cells were harvested and total cell extracts prepared. The spent culture medium (supernatant) was concentrated by tangential flow filtration (10kDa membranes). The concentrated supernatant and cytoplasmic fractions were further concentrated using the 100kDa membranes, resulting in two fractions, one >100kDa and one <100kDa. The protein concentration was determined and the *gellan lyase* activity tested for.

Fig. 48 Location of *Gellan Lyase* in Strain 2A.



Bacterium 2A was grown in YE (0.25% gellan) for a maximum of 5d. at 30°C. The cells were harvested at 24h intervals and sphaeroplasts were prepared according to the method of Osborn *et al.* (1972). Once the periplasm was isolated the sphaeroplasts were broken by sonication. All the fractions were tested for *gellan lyase* activity and the protein concentration was determined.

Table 35 Isolation and Location of 13.1 *Gellan Lyase.*

24h Harvested Cells

Growth Condition	Cell Fraction *Gellan lyase* (Specific Activity)		
	Supernatant	Cytoplasm	Membrane
0.05 % Gellan	1500	517	64
0.10 % Gellan	2393	82	30
0.20 % Gellan	3296	457	2
2.00 % Glucose	0	2	0

48h Harvested Cells

Growth Condition	Cell Fraction *Gellan lyase* (Specific Activity)		
	Supernatant	Cytoplasm	Membrane
0.05 % Gellan	472	27	11
0.10 % Gellan	258	65	0
0.20 % Gellan	3230	678	164
2.00 % Glucose	0	0	1

Specific Activity ρmole $ml^{-1}mg\ protein^{-1}$

Strain 13.1 was grown in YE plus one of the following; 2% glucose, 0.05% gellan 0.1% gellan or 0.2% gellan for either 24h or 48h. The supernatant was concentrated by tangential flow filtration (10kDa membranes) and total cell extracts were prepared by sonication. The membrane fraction was isolated after ultracentrifugation. The protein concentration and *gellan lyase* activity in all the fractions were determined.

Table 36 Isolation and Location of *Gellan Lyase* in 11.1, 12.1, 13.1 and 14.1.

Cell Fraction	Specific Activity Bacterium 11.1	12.1	13.1	14.1
Supernatant	1978	851	7671	1354
Cytoplasm	163	38	136	687
Membrane	-	-	-	-

Specific Activity ρmole ml^{-1} mg $protein^{-1}$

After 48h growth in YE (0.2% gellan), the cells were harvested and the supernatant concentrated. Total cell extracts were prepared by sonication and the membrane fraction isolated after ultracentrifugation. Gellan lyase activity and protein concentration were determined in each of the cell fractions.

Substrate Specificity of *Gellan lyase*.

A number of polymers were tested as substrates for *gellan lyase*. The polymers were either isolated in our laboratory or provided by Dr. Baird (Kelco). Dr. I Sá Correia (Instituto Tecnico Superior, Lisbon, Portugal) provided mutant strains of *S. paucimobilis*. Both acetylated and deacetylated polymers were used as substrates. The polymers were dissolved in d.H_2O and ultracentrifuged to remove insoluble material. The polymers were deacetylated according to the method 2.7.7. The results can be found in Table 37.

The enzymes isolated from the gellan-degrading bacteria exhibit a high degree of specificity for their substrate, acting primarily on deacetylated gellan. *Gellan lyase* was also active against the deacetylated form of rhamsan but inactive against the acetylated form of the polymer. In order to confirm this result, Dr. Baird (Kelco) also provided a sample of deacetylated rhamsan. 14.1 *gellan lyase* was slightly more active on the deacetylated Kelco polymer (115%) than our deacetylated polymer. The enzymes were slightly active against native gellan; once the O-acetyl groups had been removed there was a linear release of TBA reactive material (Fig. 49) by 14.1 *gellan lyase*, when either gellan or deacetylated rhamsan was the substrate.

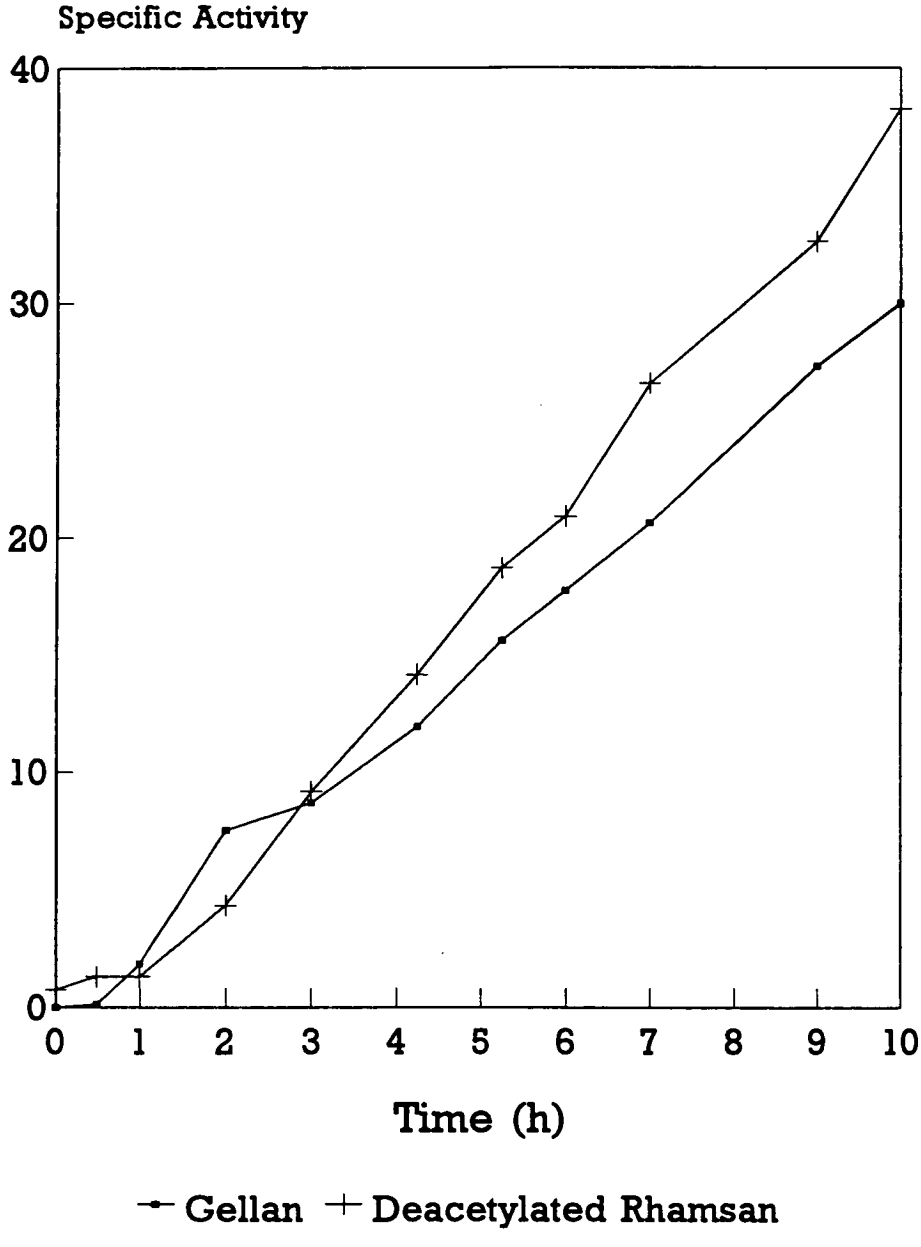
The substrate specificity of 14.1 *gellan lyase* was tested after partial purification by gel filtration on AcA34 (Pharmacia). The substrates tested under standard conditions were gellan, acid hydrolysed gellan and deacetylated rhamsan. TBA reactive material was released linearly over the first 6h (Table 38). After 3h incubation, gellan proved to be the best substrate followed by acid hydrolysed gellan and then deacetylated rhamsan.

Table 37 Substrate Specificity of Gellan-Degrading Bacteria.

	Enzyme Source				
Substrate	2A	11.1	12.1	13.1	14.1
	% Control Specific Activity				
Gellan	100	100	100	100	100
Rhamsan DAC	127	96	-	65	85
S657	2	0	0	0	3
S130	-	-	-	0.9	5
S198	-	-	-	-	2.4
S198 DAC	-	-	-	-	15.5
S88	-	-	-	-	6
S88 DAC	-	-	-	-	7
MJ200	43	49	49	18.5	58.5
PA4	32	2	19.8	24	29.9
SB10	0	7.7	0	0	2.7
SB30	0	19	8.4	2.3	0

The results are expressed as percentage of the gellan results. Typically gellan yielded 10430 ρmole ml^{-1} mg $protein^{-1}$.

Fig. 49 The Release of TBA Reactive material from Gellan and Dacetylated Rhamsan.



Specific Activity ρmole ml^{-1} mg $protein^{-1}$

5ml of substrate (1mg ml^{-1}) was incubated at 30°C in the presence of 5µl 14.1 *gellan lyase* (10mg ml^{-1} protein). 500µl aliquots were removed and boiled for 5min. Lyase activity was determined using 100µl (triplicate) in the TBA assay.

Table 38 Determination of Substrate Specificity of Semi-Purified 14.1 *Gellan Lyase*.

Substrate	Incubation Time (h) 1.5	3	6
	(Specific Activity)		
Gellan	13764	30678	57521
Acid Hydrolysed Gellan	9037	22741	40874
Deacetylated Rhamsan	8784	19723	25878

Specific Activity pmole ml^{-1} mg $protein^{-1}$

The semi-purified enzyme (>100kDa) was further purified by applying it to the gel filtration column. This was used as the enzyme source, its specificity was tested using 20 µl of enzyme (0.58mg ml^{-1} protein), in equal volumes (500µl) of 10mM TRIS pH 7.3 and 1mg ml^{-1} substrate. The assay mixture was incubated at 30°C, aliquots were removed at appropriate times and boiled for 5min. The TBA reagents were used to determine lyase activity.

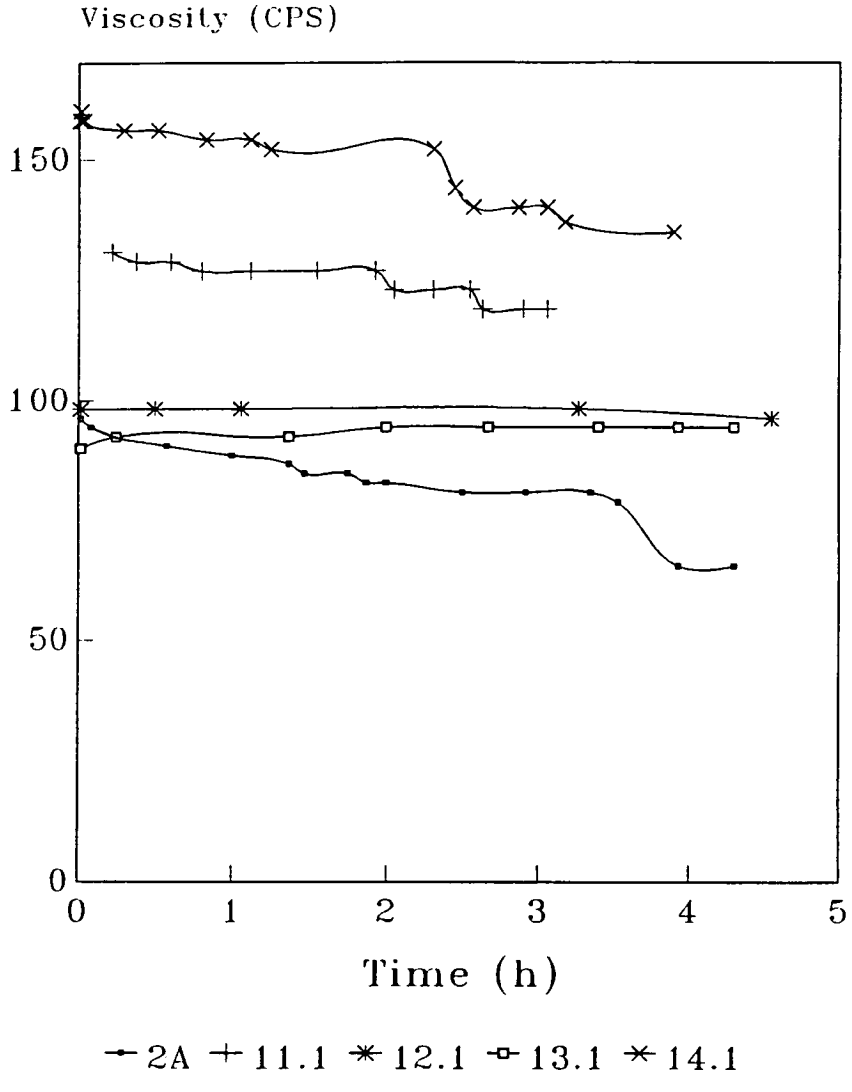
Determination of Lyase Activity by Viscosity Measurements.

Lyase activity can be determined by measuring the loss of viscosity from a polysaccharide solution. Viscosity measurements can also indicate the mode of action of the enzyme. If the enzyme is exo-acting viscosity loss is slow. The enzyme moves sequentially along the polymer cleaving the specific glysosidic linkages. If the enzyme is endo-acting the loss in viscosity in more rapid as the enzyme randomly cleaves the glycosidic linkages within the polymer.

A series of experiments were carried to determine the effect of the action of the gellan-degrading enzymes on the viscosity of the polymer. 2ml of a 0.3% solution of gellan was warmed to 30°C in the cup of the LDII viscometer (Brookfield). Enzyme was added and thoroughly mixed and the viscosity was measured at a constant shear rate over a period of time (Fig. 50). At the end of the experiment a 100µl aliquot was removed and tested for the presence of unsaturated uronic acid (Table 39). The addition of 11.1, 12.1 and 14.1 preparations caused loss of viscosity.

The effect of 14.1 *gellan lyase* on the viscosity of both gellan and deacetylated rhamsan was determined (Fig 51). When gellan was the substrate, there was a slow loss of viscosity and the mode of action of the lyase appeared to be exolytic. However when deacetylated rhamsan was the substrate there was a steady loss of solution viscosity with time. This is characteristic of endo-acting lyases. This was reinforced by the results obtained from the analysis of the breakdown products of *gellan lyase* where a series of oligosaccharides were produced by the action of the lyase.

Fig. 50 The Effect of Gellan Degradation on Viscosity.



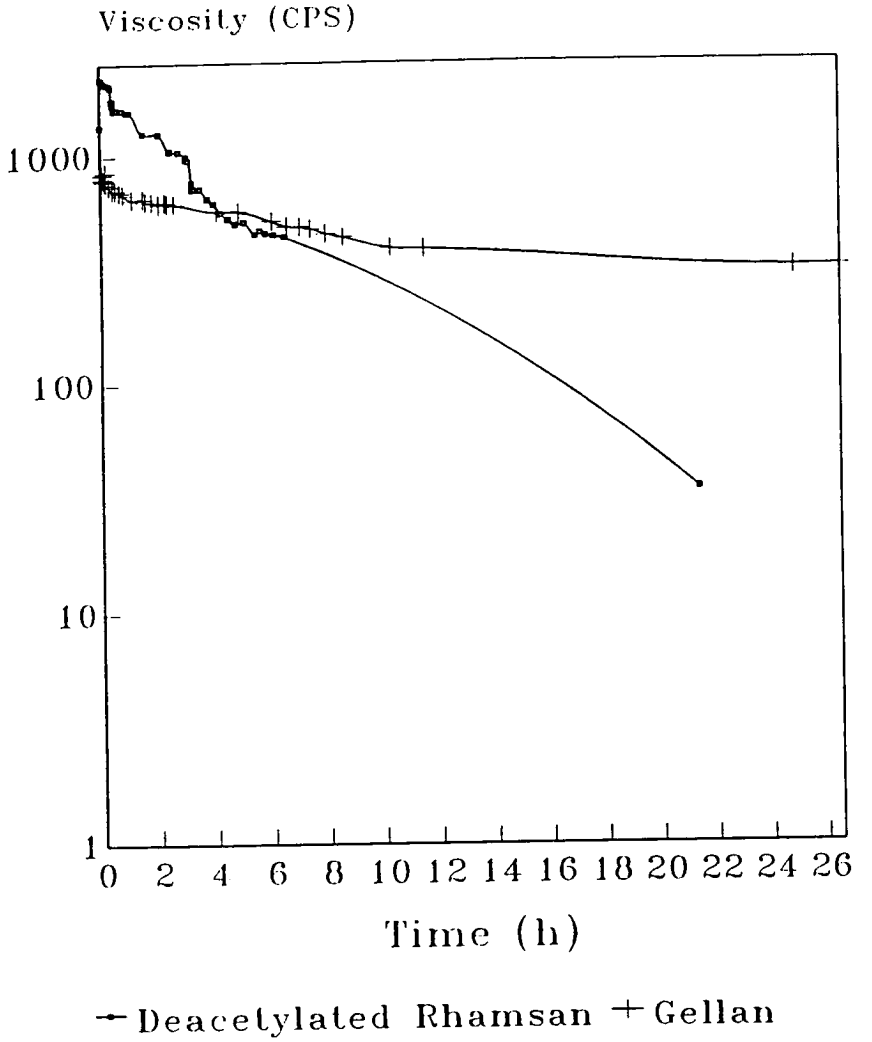
2ml of gellan solution (3mg ml^{-1}), was warmed to 30°C before a volume of enzyme was added (Table 39). The viscosity of the solution was measured at a constant shear rate (Table 39) over a number of hours.

Table 39 Determination of *Gellan Lyase* Activity in the Viscosity Experiment.

Assay Conditions	Enzyme Source 2A	11.1	12.1	13.1	14.1
Shear rate	1.2	1.2	0.6	0.6	1.2
Volume used (µl)	10	50	10	10	10
TBA Activity (OD_{549})	0.504	0.470	0.319	0.425	0.403

The viscosity experiments were carried out at 30°C at constant shear rate (see above) using 2ml of gellan (3mg ml^{-1}). When the polymer solution had warmed to temperature a volume of enzyme (see above) was added and the change in viscosity monitored over a number of hours. To ensure that the decrease in viscosity was due to the action of *gellan lyase,* an aliquot was removed at the end of the experiment and tested with the TBA reagents.

Fig. 51 The Effect of Strain 14.1 *Gellan Lyase* on the Viscosity of Gellan and Deacetylated Rhamsan.



1ml of polysaccharide (gellan 2.5mg ml^{-1}, 5mg ml^{-1} deacetylatated rhamsan) was mixed with an equal volume of 10mM TRIS pH 7.3 at 30°C. 5µl of 14.1 *gellan lyase* was added and the change in viscosity monitored over a number of hours.

The breakdown Products of Gellan Degradation.

The breakdown products from the various gellan-degrading enzyme preparations were isolated. Fig. 52 shows the percentage of residual polymer after enzymatic degradation. 14.1 *gellan lyase* was the most active enzyme, after 120h, 86% of the polymer had been degraded. 11.1 *gellan lyase* was the least active, with 56% remaining (120h). Similar levels of total carbohydrate, reducing sugar and uronic acid were found in each of the retentate samples (Table 40). However, the amount of unsaturated uronic acid present in each of the retentates varied from 3- 4% of total carbohydrate to 9-13%.

The results of chemical analysis of the diffusate samples, can be found in Table 41. The amount of total carbohydrate, unsaturated uronic acid, reducing sugar and uronic acid present in the diffusate increased with time. Low levels of glucose were present in all the diffusates of 11.1, 12.1 and in the 0-3h samples of 2A and 13.1. No glucose was detected in the 14.1 diffusate. All samples were analysed by paper chromatography. The chromatograms were stained with silver and the R_{glc} calculated (Table 42). The first product of gellan degradation appeared within 3h and had a mobility compared to glucose of between 0.08 (2A) and 0.16 (12.1). The intensity of these products increased from 3h. After 21h, depending on the enzyme source, another fraction appeared which had a mobility of between 0.18 (13.1, 72h) and 0.27 (14.1, 96h). Although this fraction had a similar mobility to glucuronic acid it was not believed to be a monosaccharide. In the 14.1, 120h diffusate, the two products were replaced with a product which had a mobility of 0.95. Most of the fractions contained material which had a similar mobility to glucose and probably accounts for the presence of free glucose in the diffusate. Some fractions had rhamnose as well.

Preparative chromatograms were set up and run in the butan-1-ol:pyridine:water system. The appropriate sections were cut out and eluted with UHQ water and chemically analysed (Table 43). Fr. 1 contained material which ran closest to the origin and the remaining fractions ran in sequence according to their number. 2A resolved into three fractions, Fr. 1 corresponded to the material which had a R_{glc} between 0.08-0.1 and contained the largest amounts of unsaturated uronic acid (88%), reducing sugar (83%) and uronic acid (96%). The final spot, Fr. 3 had a R_{glc} 1 but contained unsaturated uronic acid (12%), reducing sugar (13%) uronic acid (4%). 11.1 resolved into five fractions, again Fr. 1 (R_{glc} 0.07), contained 96% of the total carbohydrate, 82% of the reducing sugar, 95% of the uronic acid and 99% of the unsaturated uronic acid. Fr. 3 (R_{glc} 0.71) contained the other 1% of unsaturated uronic acid. The three fractions from 12.1 had the following mobilities, R_{glc} 0.09, 0.35 and 0.6. Unsaturated uronic acid was present in all three fractions, 28%, in Fr. 1, 42% in Fr. 2, and 30% in Fr. 3 (% total carbohydrate). All the unsaturated uronic acid was present in Fr. 1 (R_{glc} of 0.16) of the 13.1 digest. In 14.1, the unsaturated uronic acid was divided equally between Fr. 1 (R_{glc} 0.07) & Fr. 2 (R_{glc} 0.19).

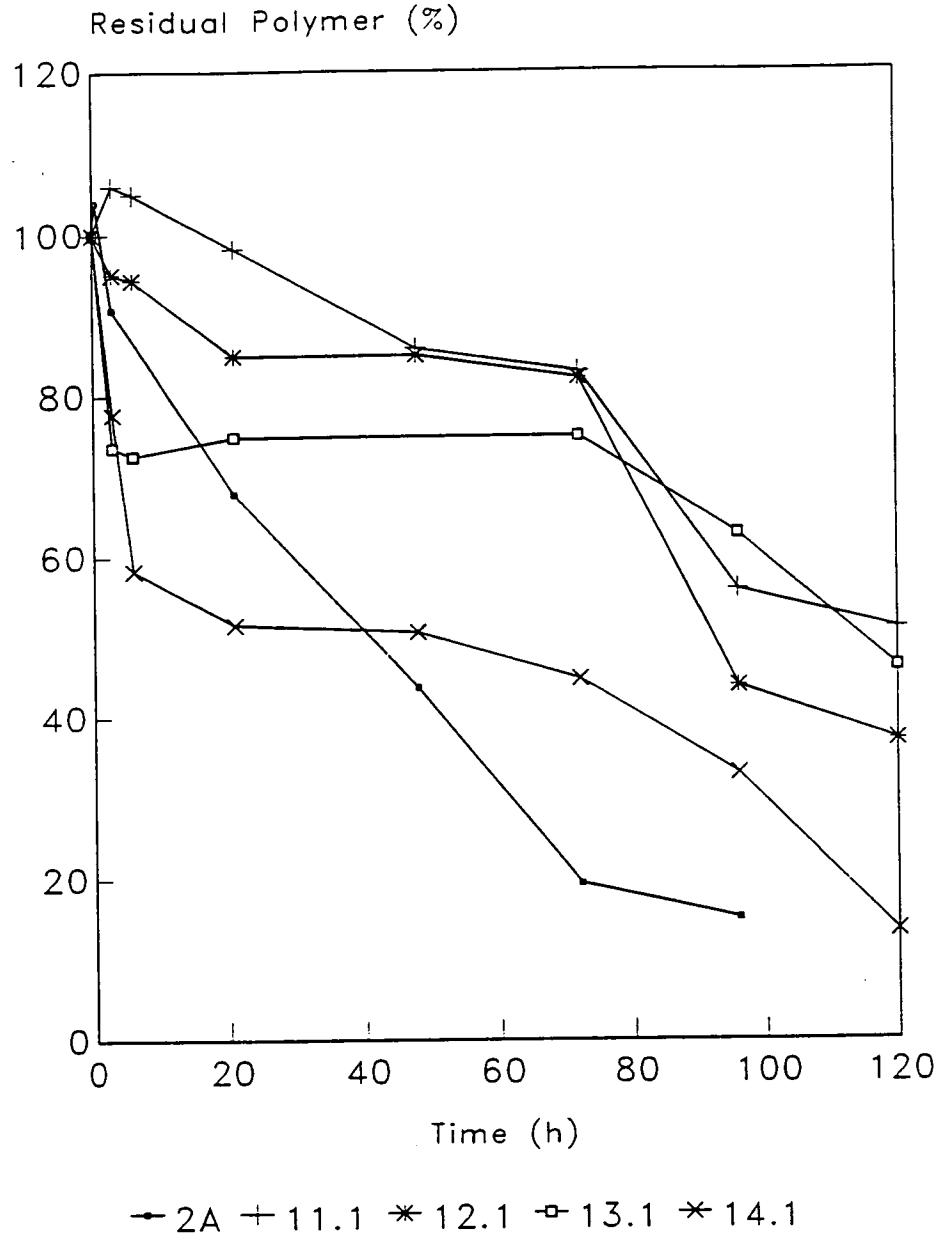
The *gellan lyase* probably cleaves the polymer into large fragments and these are subsequently cleaved to form smaller oligosaccharides. This was confirmed by the 14.1 sample taken after 120h incubation, where the largest and least mobile fragments disappeared and were replaced by a more mobile fraction R_{glc} 0.95. This suggests that the end product of 14.1 degradation was this fragment.

1ml aliquot of the pooled fractions were applied to the Biogel P2 column and separated on the basis of molecular weight. The elution profile (Fig. 53), for each of the enzyme sources, was determined by measuring the amount of carbohydrate in each of the fractions. The fractions which contained carbohydrate were pooled, dried

resuspended in 1.5ml. 11.1, 12.1 and 14.1 resolved into two peaks and 13.1 into three peaks. The elution profiles of 11.1 and 12.1 were similar, the major peak eluted first, followed by a secondary smaller peak. The profiles of 13.1 and 14.1 were different; they eluted as a double peak, which was not separated (13.1) but were resolved into two individual peaks in 14.1. In both cases the first peak was smaller than the second peak.

An aliquot of each peak was applied to No. 1 Whatman paper and run for 24h in butan-1-ol:pyridine:water (6:4:3). The paper was treated in the usual manner and stained with silver. The (R_{glc}) of each of the components was determined (Table 44). The most surprising observation was the complexity of each of the peaks, each being made up of a series of oligosaccharides. 13.1 Pk 1 had three components whereas others had up to 8 components. Only 11.1 and 14.1 Pk 2 contained material which had similar mobility to rhamnose but most of the peaks contained material which ran alongside glucose. Chemical analyses of these peaks are shown in Table 45. Generally most of the unsaturated uronic acid was present in Pk 1, the exception was 13.1 where it was present in Pk 2.

Fig. 52 The Percentage Residual Polymer After Degradation by the Gellan-Degrading Bacteria.



The assay mixture was set up as follows: 100ml substrate (1mg ml^{-1}), 30ml 10mM TRIS pH 7.0, 0.5ml 2% sodium azide, 0.2ml enzyme and incubated at 30°C. At an appropriate time point a flask was removed and boiled for 5min. The assay mixture was dialysed against 4l $d.H_2O$ (2 X 2l). The retentates were frozen and lyophilised. The diffusate was rotary evaporated and resuspended in UHQ water.

Table 40 Chemical Analysis of the 48h Retentate after Degradation By the Gellan-Degrading Bacteria.

Sample	Total Carbohydrate	Unsaturated Uronic Acid	Uronic Acid	Reducing Sugar
11.1	2.26	0.06	0.61	0.75
12.1	1.74	0.08	0.71	0.84
13.1	1.35	0.12	0.77	0.90
14.1	2.06	0.28	0.58	0.96

Total Carbohydrate	µmole Glucose ml^{-1} mg $retentate^{-1}$
Unsaturated Uronic Acid	µmole formyl pyruvate ml^{-1}mg $retentate^{-1}$
Uronic Acid	µmole Glucuronic Acid ml^{-1}mg $retentate^{-1}$
Reducing Sugar	µmole Glucose ml^{-1}mg $retentate^{-1}$

Table 41 Chemical Analysis of The Diffusate After Gellan Degradation.

Enzyme Source 2A

	Incubation Time (h)							
	0	0.5	3	21	48	72	96	120
Unsaturated Uronic Acid	0	5.18	20.25	55.69	88.55	104.14	157.46	
Reducing Sugar	3.06	3.33	10.3	15.83	15.0	21.39	39.17	
Free Glucose	0.01	0.03	-	-	-	-	-	
Enzyme Source 11.1								
Unsaturated Uronic Acid	0	5.86	8.28	21.03	35.86	68.62	140.69	205.75
Uronic Acid	0.03	0.08	0.77	2.06	3.22	7.72	13.79	30.39
Reducing Sugar	5.83	3.06	2.22	7.5	13.33	25.28	47.22	63.89
Free Glucose	0.09	0.03	0.04	0.24	0.03	0.12	0.02	0.03
Total Carbohydrate	3.44	1.89	3.89	13.39	15.00	5.83	8.75	12.0
Enzyme Source 12.1								
Unsaturated Uronic Acid	0	8.85	14.48	50.34	68.28	124.83	125.75	136.09
Uronic Acid	1.03	1.03	1.03	1.67	4.12	12.89	17.64	16.86
Reducing Sugar	8.61	3.05	8.61	10.28	22.22	55.56	45.97	53.89
Free Glucose	0.23	0.08	0.19	0.49	0.21	0.12	1.08	1.68
Total Carbohydrate	5.39	3.22	8.83	26.33	30.11	64.72	98.33	93.89
13.1								
Unsaturated Uronic Acid	29.31	121.72	92.41	119.54	183.44	247.12	618.96	389.65
Uronic Acid	8.34	27.19	24.93	34.20	33.99	42.65	36.47	62.22
Reducing Sugar	20.55	43.33	42.22	65.00	73.33	78.89	156.01	111.55
Free Glucose	0.58	1.00			0.02			
Total Carbohydrate	19.72	68.61	130.55	195.55	380.00	248.89	203.33	364.44

Table 41 continued

	Incubation Time (h)							
	0	0.5	3	21	48	72	96	120
14.1								
Unsaturated Uronic Acid	6.39	4.85	125.06	250.11	380.46	686.21	445.98	772.99
Uronic Acid	0	1.03	4.12	38.11	36.26	37.60	48.42	36.26
Reducing Sugar	10.97	32.44	42.77	94.4	147.78	211.11	126.11	240.00
Total Carbohydrate	8.33	28.61	52.78	116.67	220.00	203.00	166.00	314.44

Unsaturated Uronic Acid	µmoles formyl pyruvate ml^{-1}
Uronic Acid	µmoles Glucuronic acid ml^{-1}
Reducing Sugar	µmoles Glucose ml^{-1}
Total Carbohydrate	µmoles Glucose ml^{-1}
Free Glucose (*Glucose oxidase*)	µmoles Glucose ml^{-1}

Table 42 The Mobilities of the Diffusate Components After Gellan Degradation by the Gellan-Degrading Bacteria.

2A			R_{glc}		
Glucose	1				
Glucuronic Acid	0.20				
Rhamnose	1.70				
Mannose	1.21				
Incubation Time (h)					
0	-				
0.5	0.12				
3	0.08				
21	0.11	0.19	0.32	0.61	0.94
48	0.10	0.18			0.97
72	0.09	0.17			0.96
96	0.09	0.18			0.99

11.1			R_{glc}		
Glucose	1				
Glucuronic Acid	0.26				
Rhamnose	1.50				
Incubation Time (h)					
0					1.54
3	0.18				
6	-				
21	-				
48	0.13	0.25	0.96	1.23	
72	0.14	0.25	0.98	1.24	1.52
96	0.14	0.27	0.98	1.22	1.46
120					

12.1			R_{glc}		
Glucose	1				
Glucuronic Acid	0.29				
Rhamnose	1.52				
Incubation Time (h)					
0				1.12	
3	-				
6	-				
21	0.16				
48	0.14				
72	0.15				
96	0.14		0.88		
120	0.14			0.95	

Table 42 continued

13.1					R_{glc}			
Glucose	1							
Glucuronic Acid	0.25							
Rhamnose	1.62							
Incubation Time (h)								
0	-							
3	0.13							
6	0.13							
21	0.12							
48	0.11	0.20					0.92	1.21
72	0.10	0.18					0.91	1.27
96	0.10	0.18	0.24	0.64	0.82	0.93	1.17
120	0.11	0.2				0.91	1.18

14.1					R_{glc}			
Glucose	1							
Glucuronic Acid	0.23							
Rhamnose	1.56							
Incubation Time (h)								
0					1.04			1.34
3	0.13				1.05			1.35
6	0.11				1.04			1.35
21	0.10				1.04			1.34
48	0.11	0.21	0.63			1.12		
72	0.12	0.23	0.64	0.90			1.21	1.53
96	0.10	0.22	0.65			1.17		
120				0.95			1.28	

20µl of sample and 2µl of each of the standards were applied to No. 1 Whatman paper and run in the butanol:pyridine:water (6:4:3) for 48h

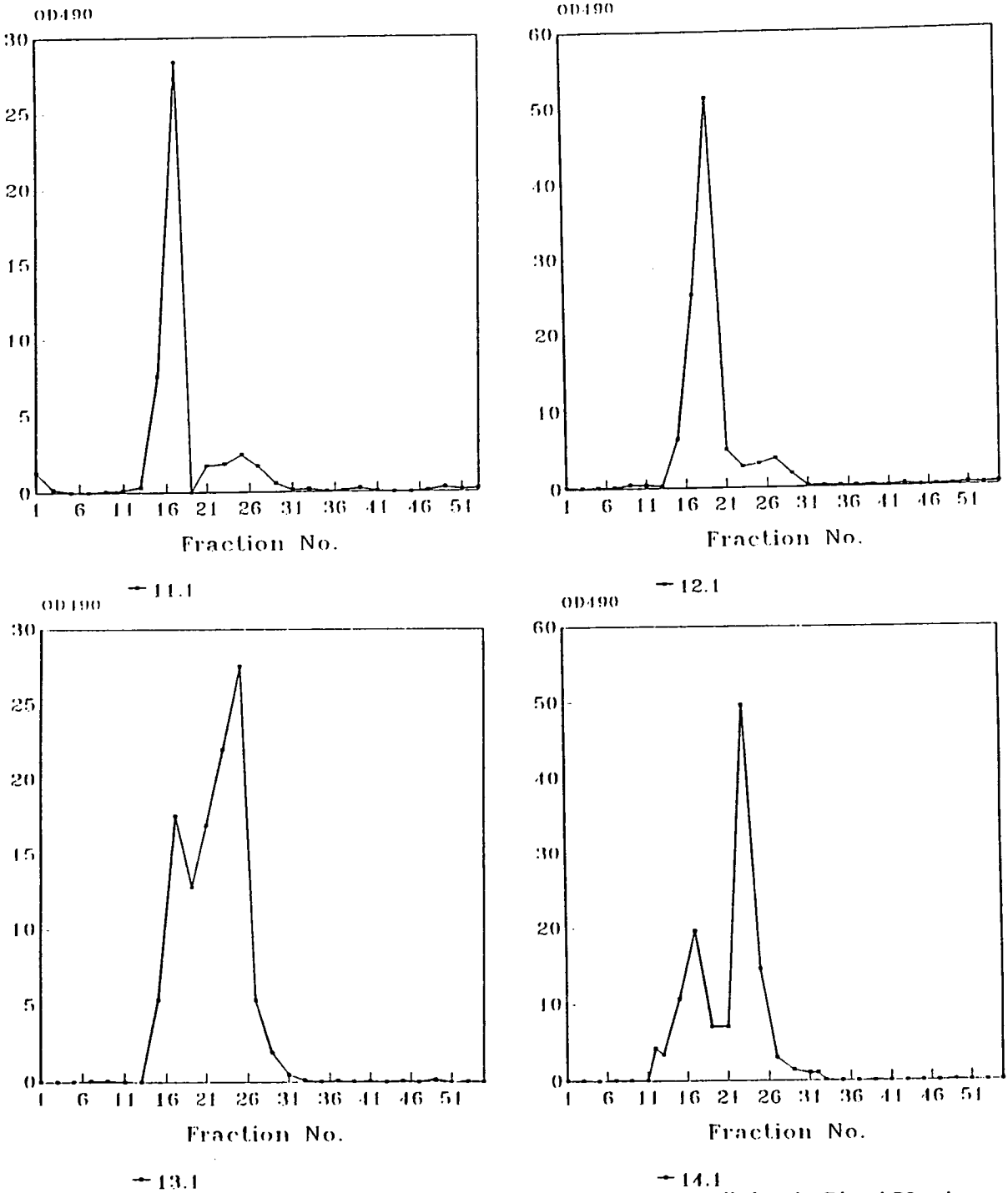
Table 43 Chemical Analysis of the Fractions from the Preparative Chromatogram.

Fraction		Mobility (R_{glc})	Total Carbohydrate	Unsaturated Uronic Acid	Uronic Acid	Reducing Sugar
2A	Fr. 1	-	307.35	46.87	102.78	-
	Fr. 2	-	1.98	-	5.02	-
	Fr. 3	-	41.04	1.98	16.11	-
11.1	Fr. 1	0.07	102.78	23310	50.86	33.60
	Fr. 2	0.29	2.8	-	-	1.30
	Fr. 3	0.71	1.72	221	0.68	1.50
	Fr. 4	0.85	-	-	0.46	1.40
	Fr. 5	1.00	-	-	1.57	3.30
12.1	Fr. 1	0.09	4.55	531	1.68	1.60
	Fr. 2	0.35	5.22	786	-	0.60
	Fr. 3	0.60	1.61	551	0.88	2.1
13.1	Fr. 1	0.16	8.06	16,276	5.29	3.9
	Fr. 2	0.66	7.22	-	-	2.9
	Fr. 3	0.88	2.0	-	0.52	2.6
	Fr. 4	0.45	4.44	-	0.93	4.7
	Fr. 5	1.02	20.83	-	-	13.2
14.1	Fr. 1	0.06	100.0	61103	23.82	20.5
	Fr. 2	0.19	11.89	62620	5.74	6.8
	Fr. 3	0.60	24.17	351	-	8.9
	Fr. 4	0.83	19.44	648	-	16.0
	Fr. 5	1.12	3.38	338	0.74	2.4

Total Carbohydrate	µmole glucose ml diffusate^{-1}
Unsaturated Uronic Acid	µmole formyl pyruvate ml diffusate^{-1}
Uronic Acid	µmole glucuronic acid ml diffusate^{-1}
Reducing sugar	µmole glucose ml diffusate^{-1}

200µl of diffusate was applied to No. 1 Whatman Paper and run in the butanol:pyridine:water (6:4:3) for 72h. The ends of the paper were removed and developed with silver. The material was eluted with UHQ water and the fractions chemically analysed.

Fig. 53 The Elution Profiles of the Diffusable Breakdown Products from Biogel P2.



1ml of the pooled samples from the preparative chromatogram were applied to the Biogel P2 column and separated on the basis of molecular weight. The eluted material (1ml at 10 min intervals) 100µl was tested for carbohydrate (Dubois, 1956) plotted against fraction number. The fractions were pooled as follows: 11.1, Pk 1 tubes 14-19, Pk 2 tubes 20-30, 12.1, Pk 1 tubes 14-23, Pk 2 tubes 24-30, 13.1 Pk 1 tubes 15-17, Pk 2 tubes 18-23, Pk 3 24-31, 14.1 Pk 1 tubes 12-20, Pk 2 tubes 21-30.

Table 44 The Mobilities of the Diffusable Breakdown Products of Gellan-Degradation after Separation on Biogel P2.

		R_{glc}							
Glucose		1							
Glucuronate		0.27							
Rhamnose		1.49							
11.1	Pk 1		0.16	0.26	0.39	0.62	0.93		
	Pk 2		0.11	0.24	0.45	0.63	0.89	1.10	1.45
12.1	Pk 1	0.04	0.16	0.28		0.62	0.85		
	Pk 2			0.28	0.48	0.70	0.93	1.18	
Glucose		1							
Glucuronate		0.58							
Rhamnose		1.54							
13.1	Pk 1		0.14				0.69		1.12
	Pk 2	0.04		0.22	0.45		0.71	0.96	
	Pk 3	0.05		0.22	0.36	0.56	0.69	1.00	1.32
14.1	Pk 1	0.04	0.16	0.28	0.4	0.48	0.7	1.0	
	Pk 2	0.04	0.18	0.28		0.50	0.68	0.93	1.14 1.54

10µl of sample and 2µl of standard was applied to No. 1 Whatman paper and run in the butanol:pyridine: water (6:4:3) system for 24h. The chromatogram was stained with silver and the R_{glc} determined

Table 45 Chemical Analysis of the Pooled Peaks from the Biogel P2 Gel Filtration Column.

Sample		Total Carbohydrate	Unsaturated Uronic Acid	Uronic Acid	Reducing Sugar
11.1	Pk 1	42.2	2.19	10.9	7.0
	Pk 2	15.3	0.23	7.7	6.8
12.1	Pk 1	40.0	4.25	6.8	13.5
	Pk 2	12.9	0.40	1.0	8.6
13.1	Pk 1	14.7	0.77	9.3	2.9
	Pk 2	39.7	8.50	21.0	15.8
	Pk 3	49.4	1.32	10.8	-
14.1	Pk 1	21.1	6.59	41.7	25.3
	Pk 2	78.9	1.51	16.4	46.4

Total Carbohydrate	µmole glucose ml diffusate^{-1}
Unsaturated Uronic Acid	µmole formyl pyruvate ml diffusate^{-1}
Uronic Acid	µmole glucuronic acid ml diffusate^{-1}
Reducing Sugar	µmole glucose ml diffusate^{-1}

Release of TBA reactive material From Gellan and Deacetylated Rhamsan.

Once it was realised that 14.1 *gellan lyase* could act on the deacetylated rhamsan (S194), its breakdown products were determined. As a control, gellan was used as substrate. The retentate was freeze dried, weighed and the percentage residual polymer determined (Fig. 54). Within the first 11h, both polymers were degraded by 14.1 *gellan lyase* by approximately 50%. The deacetylated rhamsan was not further degraded but gellan was degraded by a further 12% in the next 24h. Therefore at the end of the experiment 57% of the deacetylated rhamsan remained but only 38% of the gellan. Chemical analysis of the residual polymer (Table 46) showed that both the unsaturated uronic acid and reducing sugar in the retentate increased with time. Partially acid hydrolysed polymer was applied to Whatman No. 1 paper and run in butan-1-ol:pyridine:water (6:4:3). Glucose, mannose and rhamnose were present in the hydrolysed material as well as unhydrolysed material. There were no gentobiosyl groups in the deacetylated rhamsan fraction but there was some material which ran ahead of mannose. The intensity of the material which ran ahead increased with time (Fig. 55).

The partially hydrolysed polymer was analysed for neutral sugars by HPLC; glucose and rhamnose were present, and eluted 13.35 and 15.34min respectively. At 0h, the gellan sample contained twice as much glucose as rhamnose (expected ratio of 3:1). The concentration of rhamnose remained constant but the concentration of glucose fell by 4%.

The 0h deacetylated rhamsan sample contained only glucose and rhamnose; glucose accounted of 83% of the sample and rhamnose 17% (expected ratio 5:1) At 3h, 17% of the sample eluted at 10.5min. This fraction had disappeared by 11h and was replaced by material which eluted at 17.32min. At 25h, the concentration of this

material (5% at 17.32) had increased by 22 to 27%. When the same hydrolysed retentates were applied to 3MM Whatman paper and electrophoresed they contained material which ran with glucuronic acid (R_{glcA} 1) and material which had a R_{glcA} of 0.75. The deacetylated rhamsan (11 & 25) contained material which had an R_{glcA} of 0.55. The tetrasaccharides which were used as standards had R_{glcA} between 0.57-0.84, depending on whether they were acetylated or not.

Diffusate was applied to paper chromatograms and run in the butan-1-ol:pyridine:water (6:4:3) (Fig. 56). All the gellan samples contained glucose and mannose. The 3-25h samples contained material which ran ahead of mannose. After 3h incubation, two large oligosaccharides of similar mobility to glucuronic acid appeared, as well as material which ran between glucose and glucuronic acid. The intensity of these spots increased with time and the concentration of the largest oligosaccharide decreased. Following enzyme action on gellan or deacetylated rhamsan, rhamnose was seen in all the diffusates and there appeared to be more mannose than glucose. A series of oligosaccharides were produced, the largest of which disappeared with time. Very little material ran between glucose and glucuronic acid between 0-11h but material was present in the 25h sample.

The results of chemical analysis of the diffusate can be found in Table 47. When testing for uronic acid, material present in the samples caused them to turn brown instead of pink. The control samples contained reducing sugar >97% and between 2-3% unsaturated uronic acid. 50% of the total carbohydrate in the gellan sample (3-25h) was accounted for by reducing sugar and 3-7% as unsaturated uronic acid. The deacetylated rhamsan samples (3-25h) contained 47-34% of the total carbohydrate as reducing sugar and only 5-10% of this carbohydrate as unsaturated

uronic acid. It would appear from these results that the gellan reaction had reached completion whereas the action on deacetylated rhamsan was incomplete.

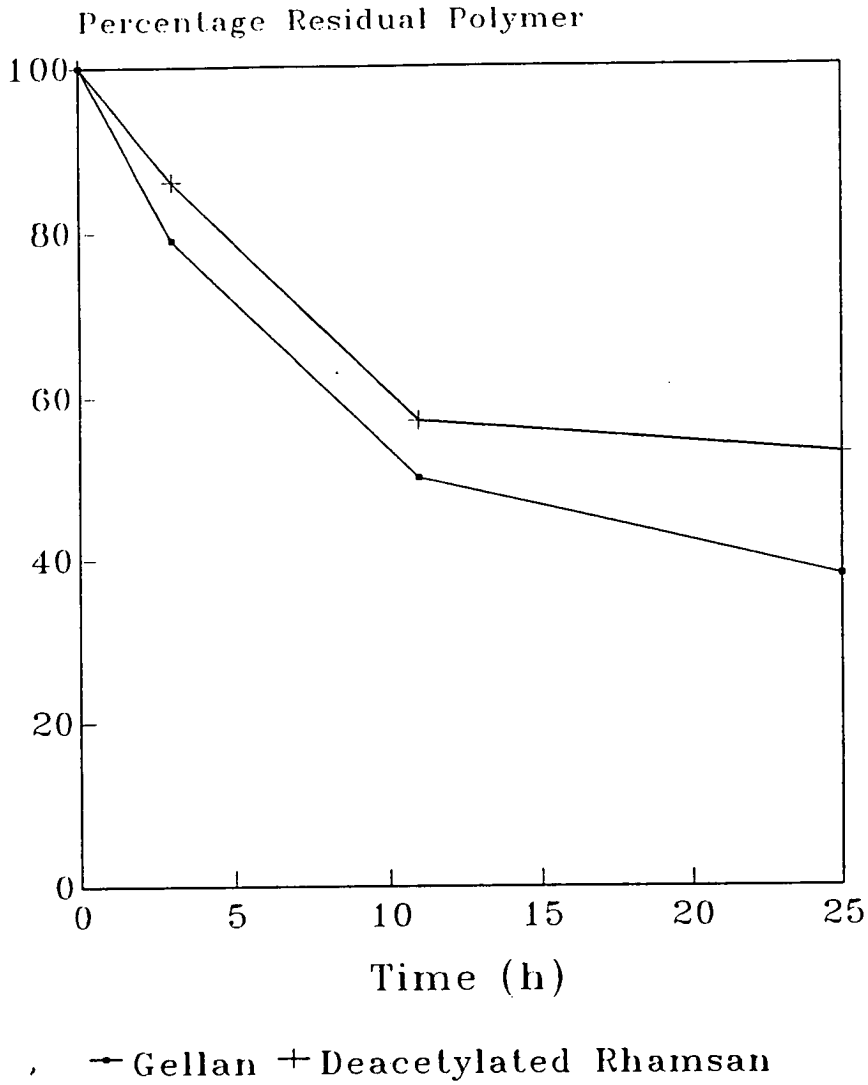
The diffusate fractions (3-25h) were pooled, dried and resuspended in 5ml of UHQ water. 1ml aliquots were applied to the P2 column and eluted with $d.H_2O$. (Fig. 57). Each polymer gave three fractions. Fr. 1 eluted first and Fr. 3 last. The fractions were chemically analysed (Table 48). Unsaturated uronic acid accounted for 3% of the total carbohydrate in each of the gellan fractions. Fr. 3 of the deacetylated rhamsan sample contained 6% unsaturated uronic acid. When each of the fractions was run on paper chromatogram a series of spots appeared. Only Fr. 3 of each sample contained glucose, but all three contained a series of oligosaccharides. The R_{glc} was determined (Table 49) for each. Preparative chromatograms were prepared of Fr. 3. from both substrates. Three spots eluted from the gellan sample (labelled G1, 2, and 3 in the order of movement from the baseline). G3 had similar mobility to glucose. Four spots were eluted from the deacetylated rhamsan (S1, 2, 3 and 4). S3 ran beside the glucose standard and S4 ran as rhamnose.

Naringinase (Sigma) is a commercially available enzyme which possesses both α-L-*rhamnosidase* and β-D-*glucosidase* activity. α-L-*rhamnosidase* are not common enzymes but since the repeated gellan backbone contains α-L-rhamnose it was decided to investigate the effect of *naringinase* on the gellan family of polysaccharides. The polysaccharides studied had either glucose or rhamnose as a side- chain on either 1,3 linked glucose residue or the 1,4 linked glucose residue (Fig. 11). Young *et al.* (1989) found that *naringinase* is a monomer with a molecular mass of 90kDa.

The deacetylated polymers S88, S198, S130 and S657 were incubated at 37°C for 7 days in the presence of 2.5 units of *naringinase* ml of polymer^{-1}. The amount of

residual polymer was determined and only 25-35% of the polymer was degraded after 7 days incubation. *Naringinase*-treated polymer was not a substrate for partially purified 14.1 *gellan lyase*. No TBA reactive material was released, neither would the *naringinase*-treated polymer gel in the presence of Ca^{2+} or Mg^{2+}. The *naringinase*-treated polymers were acid hydrolysed; all contained mannose, glucose, rhamnose and gentibiose as well as oligosaccharides. The oligosaccharides present in all the polymers had R_{glc} 0.7 and 1.2.

Fig. 54 The Percentage Residual Polymer After Degradation by 14.1 *Gellan Lyase*.



The assay mixture was set up as follows: 50ml substrate (2.5mg ml^{-1}), 50ml 10mM TRIS pH 7.0, 1ml 2% sodium azide, 0.5ml enzyme. The flasks were incubated at 30°C for an appropriate interval. The flasks were boiled for 15min and the contents poured into dialysis sac and dialysed against 10l (4 x 2.5l) $d.H_2O$. The retentates were lyophilised and weighed. The diffusates were rotary evaporated and resuspended in UHQ water.

Table 46 Chemical Analysis of the Residual Polymer After Degradation by 14.1 *Gellan lyase*.

Substrate Gellan

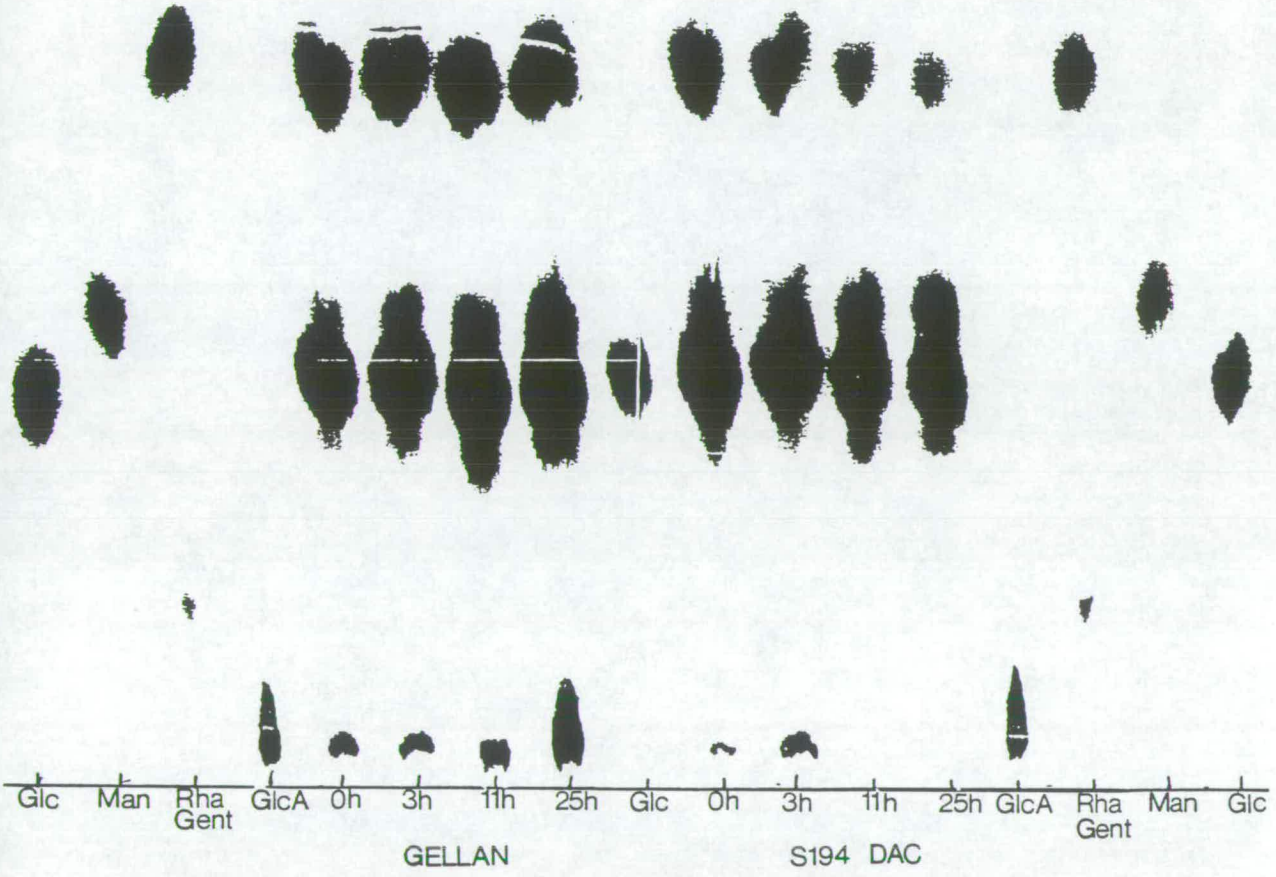
Time (h)	Uronic Acid	Reducing Sugar	Unsaturated Uronic Acid	Total Carbohydrate
0	0.4	0.03	0.01	4.4
3	0.5	0.17	0.03	3.4
11	0.4	0.33	0.11	4.2
25	0.25	0.24	0.10	2.0

Substrate Deacetylated Rhamsan

Time (h)	Uronic Acid	Reducing Sugar	Unsaturated Uronic Acid	Total Carbohydrate
0	0.4	0.032	0.01	3.57
3	0.52	0.018	0.03	3.76
11	0.39	0.43	0.07	2.8
25	0.32	0.48	0.08	3.4

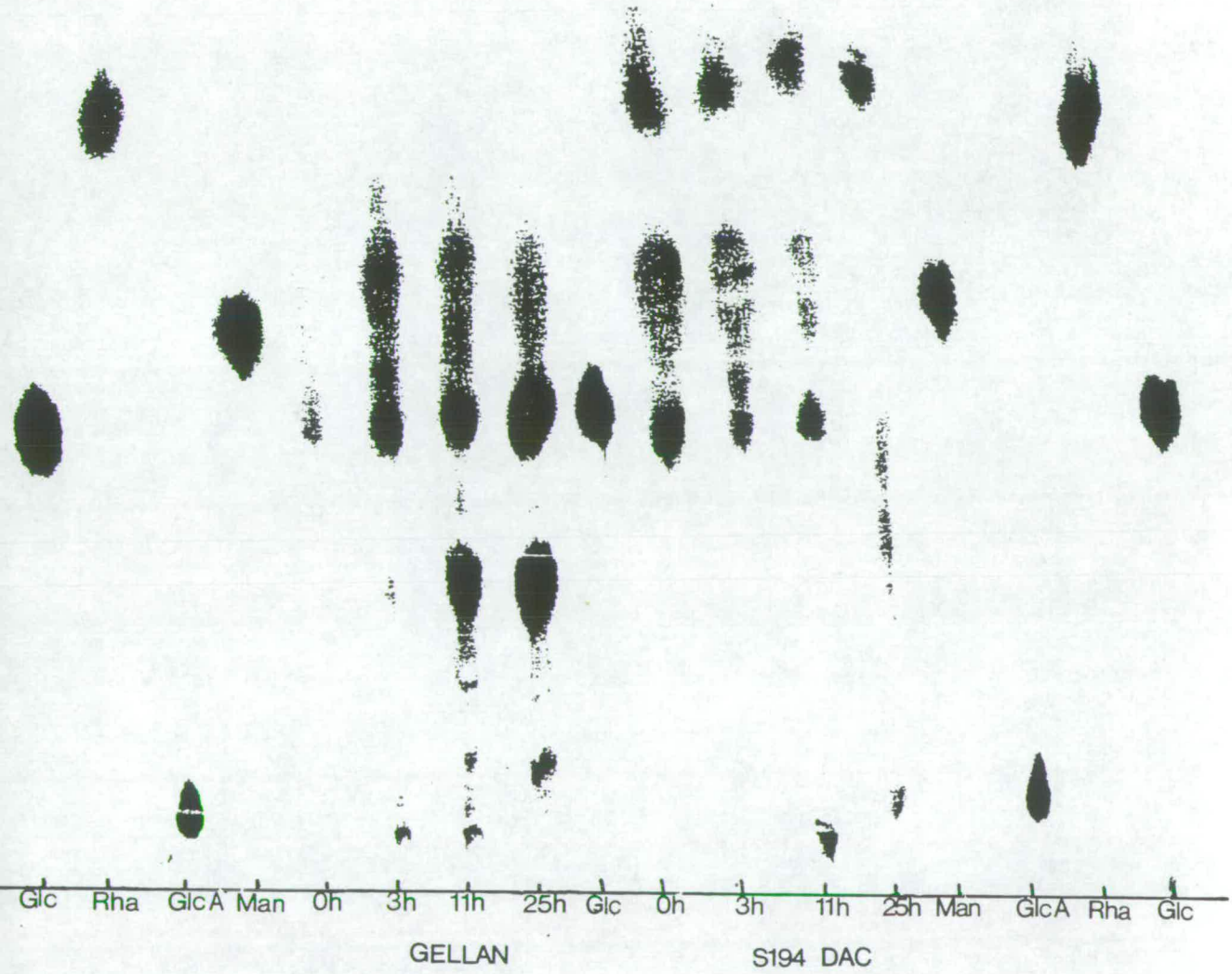
Total Carbohydrate	µmole glucose mg retenate^{-1}
Unsaturated Uronic Acid	µmole formyl pyruvate mg retentate^{-1}
Uronic Acid	µmole glucuronic acid mg retentate^{-1}
Reducing Sugar	µmole glucose mg retentate^{-1}

Fig. 55 The Separation of the Partially Hydrolysed Gellan and Deactylated Rhamsan Breakdown Products by Paper Chromatography.



10µl of partially acid hydrolysed residual polymer (0h, 3h, 11h, 25h) and 2µl of the following standards glucose, rhamnose, mannose gentibiose and glucuronic acid (0.1M) were applied to No. 1 Whatman paper and run in the butanol:pyridine:water (6:4:3) system for 36h.

Fig. 56 The Separation of the Diffusable Gellan and Deacetylated Rhamsan Breakdown Products by Paper Chromatography.



10µl of each of the diffusates and 2µl of the following standards: glucose, mannose, rhamnose, gentibiose and glucuronic acid were applied to No. 1 Whatman paper and run in the butanol:pyridine:water (6:4:3) for 36h. The chromatograms were stained with silver and the R_{glc} calculated.

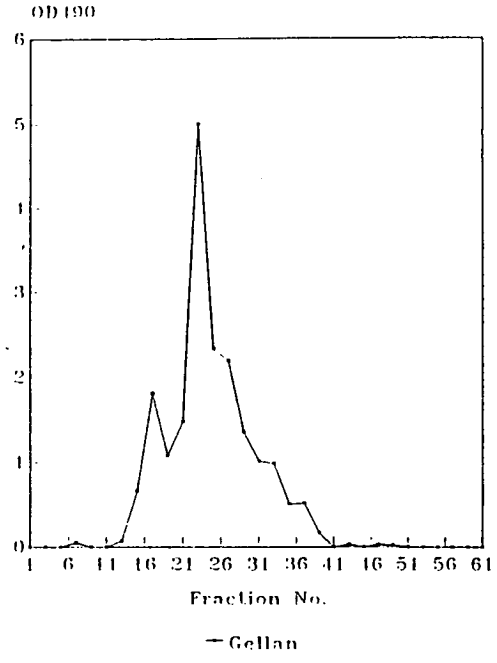
Table 47 Chemical Analysis of Diffusable Breakdown Products of Gellan and Deacetylated Rhamsan by 14.1 *Gellan Lyase*

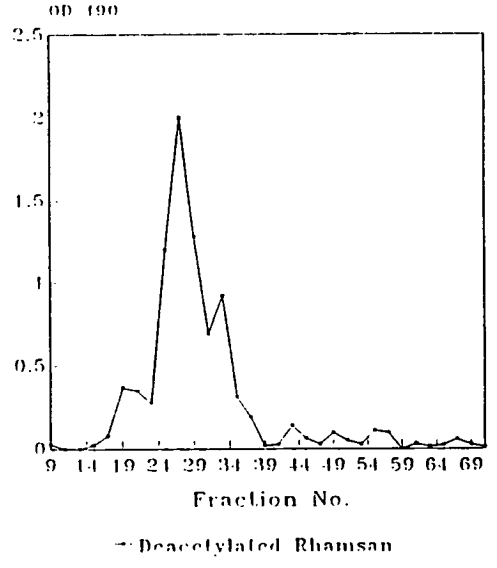
Substrate	Gellan				
Time (h)	Uronic Acid	Reducing Sugar	Unsaturated Uronic acid	Total Carbohydrate	Free Glucose
0	-	4.44	0.09	2.78	0.04
3	9.89	10.55	1.45	19.44	0.03
11	-	33.55	2.21	65.00	-
25	-	41.11	5.75	80.00	-

Substrate	Deacetylated Rhamsan				
Time (h)	Uronic Acid	Reducing Sugar	Unsaturated Uronic acid	Total Carbohydrate	Free Glucose
0	-	5.00	0.09	5.11	1.52
3	8.55	9.44	0.99	21.11	0.2
11	-	18.89	2.39	50.00	-
25	-	6.67	2.00	19.44	-

Total Carbohydrate	µmole glucose ml diffusate^{-1}
Unsaturated Uronic Acid	µmole formyl pyruvate ml diffusate^{-1}
Uronic Acid	µmole glucuronic acid ml diffusate^{-1}
Reducing Sugar	µmole glucose ml diffusate^{-1}
Free Glucose (Glucose Oxidase)	µmole glucose ml diffusate^{-1}

Fig. 57 Separation of the Diffusable Gellan and Deactylated Rhamsan Breakdown Products on Biogel P2 Gel Filtration Column.





A 1ml aliquot of the pooled fractions (3-25h) was applied to the Biogel P2 column and eluted with d.H_2O. 0.75ml fractions were collected at 10min intervals. the fractions were tested for carbohydrate content (Dubois 1956) and plotted against fraction number. The following fractions were pooled: gellan, Fr. 1, 13-18, Fr. 2, 20-28 and Fr. 3, 29-39; deacetylated rhamsan, Fr. 1, 16-23, Fr. 2, 24-31 and Fr. 3, 32-39.

Table 48 Chemical Analysis of Gellan and Deacetylated Rhamsan Diffusate After Separation on the Biogel P2 Column.

Substrate	Gellan				
Fraction No.	Uronic Acid	Reducing Sugar	Unsaturated Uronic Acid	Total Carbohydrate	Free Glucose
1	19.76	19.4	2.67	90.28	-
2	-	83.3	5.38	187.50	-
3	-	20.8	0.48	16.22	0.41
Substrate	Deactylated Rhamsan				
Fraction No.	Uronic Acid	Reducing Sugar	Unsaturated Uronic Acid	Total Carbohydrate	Free Glucose
1	9.68	14.4	0.48	54.44	-
2	-	25.6	0.47	88.89	-
3	-	22.2	3.59	58.30	0.17

Total Carbohydrate	µmole glucose ml diffusate^{-1}
Unsaturated Uronic Acid	µmole formyl pyruvate ml diffusate^{-1}
Uronic Acid	µmole glucuronic acid ml diffusate^{-1}
Reducing Sugar	µmole glucose ml diffusate^{-1}
Free Glucose (Glucose Oxidase)	µmole glucose ml diffusate^{-1}

Table 49 The Relative mobility of the Components of Gellan and Deactylated Rhamsan after Separation on Biogel P2.

Substrate Gellan

Fraction No.	R_{glc}							
1	0.07	0.11	0.20			1.04		
2		0.12	0.20	0.56	0.82		1.19	
3					0.74	1.02	1.17	1.46

Substrate Deactylated Rhamsan

Fraction No.	R_{glc}							
1	0.06							
2	0.05	0.12		0.26				
3	0.07	0.12	0.17	0.25	0.72	0.97	1.13	

Gellan Lyase purification.

Purification of *Gellan Lyase* by Ammonium Sulphate Precipitation.

The supernatants from 2A, 11.1 and 13.1 were concentrated in the Pellicon system with the 10kDa membrane. The concentrated supernatant was used for ammonium sulphate fractionation. The lyase activity was determined as was the protein concentration in both the soluble and insoluble fractions (Table 50). In some fractions lyase activity was detectable but the protein concentration was too low to measure. Ammonium sulphate precipitation of the 2A and 11.1 showed most of the lyase was present in the 80% $(NH_4)_2SO_4$ soluble fraction, whereas for strains 14.1 & 13.1 low levels of lyase activity were detected in the 20%$(NH_4)_2SO_4$ insoluble fractions but most of the enzyme was present in the 60% insoluble fraction. 0.5ml of this fraction was applied to the Q Sepharose FF. column. A step NaCl gradient was applied and the fractions which contained protein pooled (Fig 58). The purification scheme can be found in Table 51. 14.1 *gellan lyase* eluted from the column in the presence of 0.3M NaCl.

Determination of Molecular Mass by Membrane Filtration

The stirred Amicon cell (10ml) was used to separate the proteins by molecular weight using three molecular mass cut off membranes. $(NH_4)_2SO_4$ fractions from strain 11.1 (Table 49) were used. The 80% soluble fraction contained a *gellan lyase* between 50-100kDa. In some of the other fractions *gellan lyase* could be detected in the >100kDa fraction. Since there appeared to more than one *gellan lyase* present, we introduced another step into the routine purification procedure (Table 52). Up until now, the supernatant was concentrated by tangential flow filtration with the 10kDa

cut off membrane (Pellicon).This initial step was retained but was followed by filtration with the 100kDa cut off membranes in the Minitan (fraction >100kDa), after which the fraction <100kDa was concentrated with the 10kDa membranes *in situ.* The final step was to concentrate the fractions against PEG 6000.

Protein Purification by Ion Exchange Chromatography.

Aasen *et al.* (1992) described a method for the one step purification and concentration of the extracellular *alginate lyase* from *K. pneumoniae*. The lyase was produced in low concentrations in the spent medium, much of the product was lost when conventional concentration and purification techniques such a ammonium sulphate precipitation were used. The method used Q Sepharose FF, an anion exchanger to purify the protein. The *alginate lyase* passed through the column while contaminating proteins bound to the matrix. Unlike the *alginate lyase* (*K. pneumoniae*) which passed through the column, the *gellan lyase* bound to the matrix. 14.1 *gellan lyase* was applied to the Q Sepharose FF. column and eluted with a step NaCl gradient The elution profile can be found in Fig 59a and the elution profile of the TBA reactive material can be found in Fig 59b. Fr. 3 contained most of the TBA reactive material and eluted with 0.4M NaCl. This fraction had a specific activity of 19,683ρmoles ml^{-1} mg $protein^{-1}$. In a later experiment *gellan lyase* was found to elute between 0.35-0.4M NaCl

An aliquot of the partially purified lyase was re-applied to the column and eluted with a continuous linear gradient (0-0.5M NaCl) (Fig 60a). The elution profile of the lyase-active fractions can be found in Fig 60b; three fractions eluted between 0.26-0.36M NaCl. A sample of semi-purified lyase was further purified by passing it

through the 100kDa membrane. The lyase was present in both the >100kDa fraction (1.5-fold purification) and in the <100kDa fraction (0.4-fold increase in purity).

Estimation of Molecular Mass by Gel Filtration.

The determination of molecular mass was carried out using the AcA34 column (40-300kDa) according to the protocol (2.8.5). The column was calibrated with β-amylase (200kDa), alcohol dehydrogenase (150kDa), BSA (66KDa) and ovalbumin (45kDa). The semi-purified 14.1 *gellan lyase* (>100KDa) was applied to the column and eluted with 10mM TRIS pH 7.3. The fractions which had TBA activity were pooled (Fig. 61). The protein eluted as a broad based single peak and within this, a narrow based TBA reactive peak eluted. Problems arose when this procedure was repeated; the estimate of molecular mass varied from 96kDa-280kDa. When these fractions were examined by SDS-PAGE the protein profiles were identical (Fig. 62). The largest major band ran between Myosin (205kDa) and phosphorylase B (97kDa) and had a molecular mass of 150kDa.

Table 50 Purification of the Gellan-Degraders Gellan Lyase by Ammonium sulphate Precipitation.

Enzyme Source 2A % Saturation Ammonium Sulphate	Total Activity (pmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
Control	518500	119.0	0.28	425	4375	1
20% soluble	358975	41.5	0.1	415	8650	2
20% insoluble	59600	8.0	0.08	`100	7450	1.7
40% soluble						
40% insoluble	31515	12.5	0.38	33	2513	0.6
60% soluble	84042	-	-	406	-	-
60% insoluble	69902	201.32	14.38	14	347	0.1
80% soluble	1512150	0.3	0.002	425	504166	115
80% insoluble	1275	12.58	0.74	17	101	-

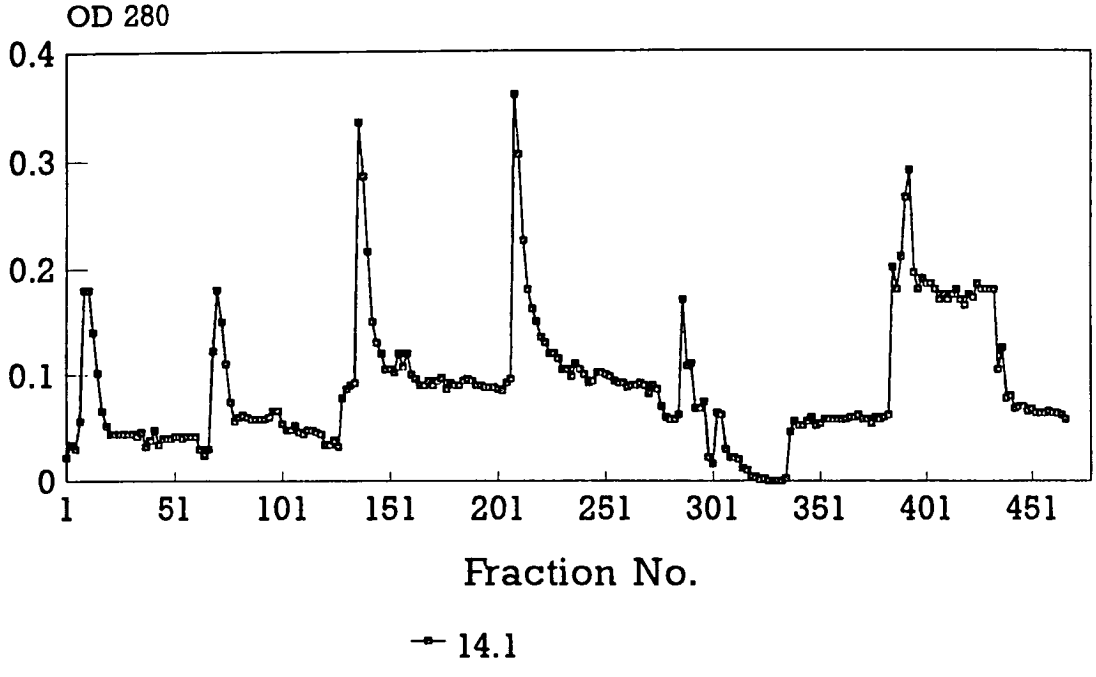
Enzyme Source 11.1 % Saturation Ammonium Sulphate	Total Activity (pmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
Control	289050	213.75	8.55	25	1352	1
20% soluble	65580	66.0	3.3	20	993	0.7
20% insoluble	-	-	-	2	-	-
40% soluble	26370	27	1.5	18	9760	7.2
40% insoluble	-	14.64	7.32	2	-	-
60% soluble	21850	16.87	1.35	12.5	1295	0.9
60% insoluble	11134	6.46	0.68	9.5	1723	1.3
80% soluble	904	-	-	10	-	-
80% insoluble						

Table 50 continued

Enzyme Source 13.1 % Saturation Ammonium Sulphate	Total Activity (pmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
Control	36810	366.0	1.12	30	1095	1
20% insoluble	859	0.19	0.1	1.9	4520	4.1
20% soluble	30915	9.18	0.34	27	3367	3.0
40% soluble	3008	5.17	0.22	23.5	582	0.5
40% insoluble	6561	3.18	0.6	5.3	2063	1.9
60% soluble	1066	0.82	0.04	20.5	1300	1.2
60% insoluble	1607	3.42	2.44	1.4	470	0.4
80% soluble	10106	13.53	0.66	20.5	746	0.7
80% insoluble	238	4.27	0.61	7	55	-

Enzyme Source 14.1 % Saturation Ammonium Sulphate	Total Activity (pmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
Control	-	106.25	4.24	25	-	-
60% soluble	6516	11.4	5.7	2	571	-
60% insoluble	538896	38.4	9.6	4	14033	-

Fig. 58 The Elution Profile of the 60% Ammonium Sulphate Insoluble Fraction from Q Sepharose FF.



0.5ml 60% Ammonium sulphate fraction was applied to the Q Sepharose column and eluted with a step NaCl gradient (0.1, 0.2, 0.3, 0.4 and 0.75M). The gradient was applied at fractions 62, 129, 201, 296 and 385. The fractions which contained protein were pooled as follows Fr. 1 tubes 8-16, Fr. 2 tubes 74-76, Fr. 3 tubes 136-165, Fr. 4 tubes 207-249, Fr. 5 tubes 286-292 and Fr. 6 tubes 387-401. Fr. 4 contained most *gellan lyase*.

Table 51 Purification Scheme for the 60% Ammonium Sulphate Insoluble Fraction of 14.1 *Gellan lyase*.

% Saturation Ammonium Sulphate	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification Fold
Control	67362	4.8	9.6	0.5	14037	1
Fr. 1	85	0.79	0.06	108	-	-
Fr. 2	-	0.06	0.1	0.59	-	-
Fr. 3	720	0.36	0.54	0.67	2002	0.1
Fr. 4	89028	0.8	0.3	2.67	111285	7.9
Fr. 5	-	-	-	0.1	-	-
Fr. 6	-	0.02	0.02	1.19	-	-

Specific Activity ρmole ml^{-1} mg protein^{-1}

0.5ml of 60% ammonium sulphate insoluble fraction was applied to the Q Sepharose FF. column and eluted with a step NaCl gradient , 0.1M NaCl was applied at Fr. 62, 0.2M NaCl, Fr. 129, 0.3M NaCl , at Fr. 201, 0.4M NaCl at Fr 296 and 0.75M at Fr, 385. The fractions which contained protein were pooled as follows: Fr. 1 tubes 8-16, Fr. 2 tubes 74-76, Fr. 3 tubes 136-165, Fr. 4 tubes 207-292, Fr. 5 tubes 286-292 and Fr. 6 tubes 387-401. The protein concentration and gellan lyase activity were determined in the pooled fractions.

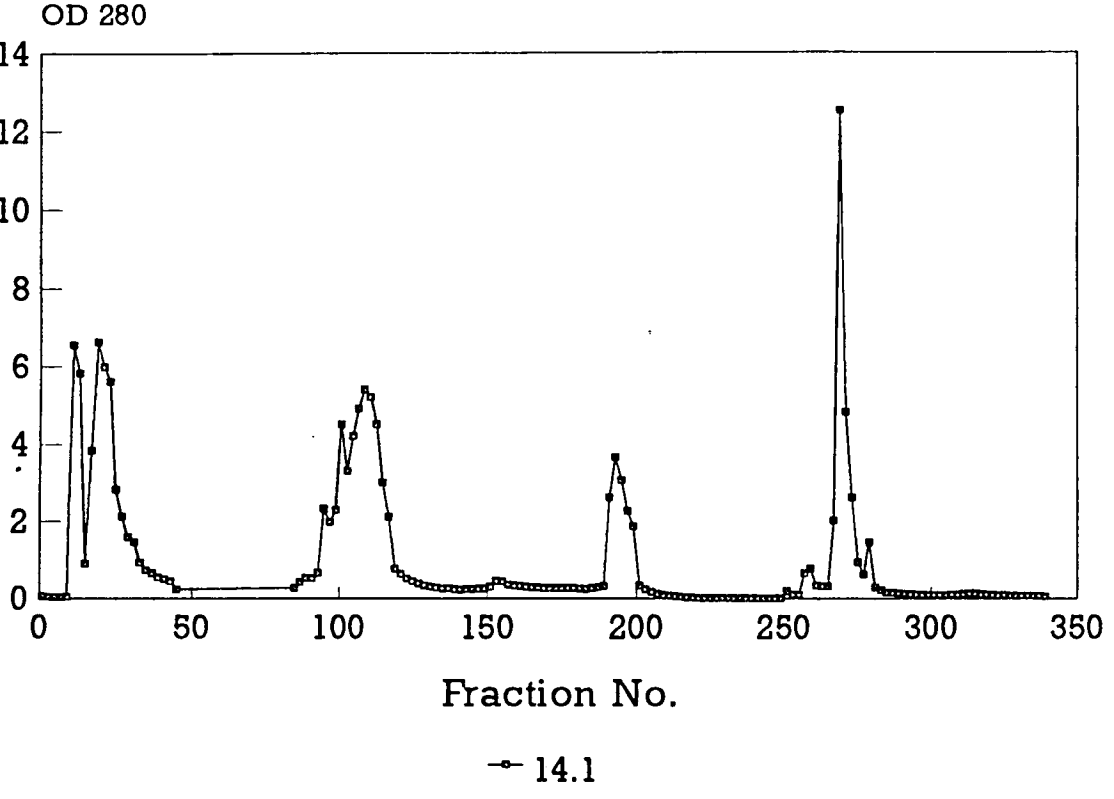
Table 52 Determiation of Molecular Weight of 11.1 *Gellan Lyase* by Membrane Filtration.

	Nominal Molecular Weight					
	≥30KDa	<30KDa	>50KDa	<50KDa	>100KDa	<100KDa
			Specific Activity			
Control	1,553	1,679	1,268	1,640	3,198	2,490
20% soluble	848	33	794	1,232	3,414	1,418
40% soluble	2,551	-	3,207	5,365	1,183	3,120
60% soluble	1,733	-	3,229	651	18,886	6,352
80% soluble	16,680	-	17	4,000	-	55,286
20% insoluble	1,823	2,886	1,319	4,230	2,707	2,739
40% insoluble	1,364	2,247	1,340	3,849	4,027	2,041
60% insoluble	452	4,200	2,467	1,274	21,010	1,197
80% insoluble	7,193	-	1,469	-	1,255	17,619

Specific Activity ρmole ml^{-1} mg $protein^{-1}$

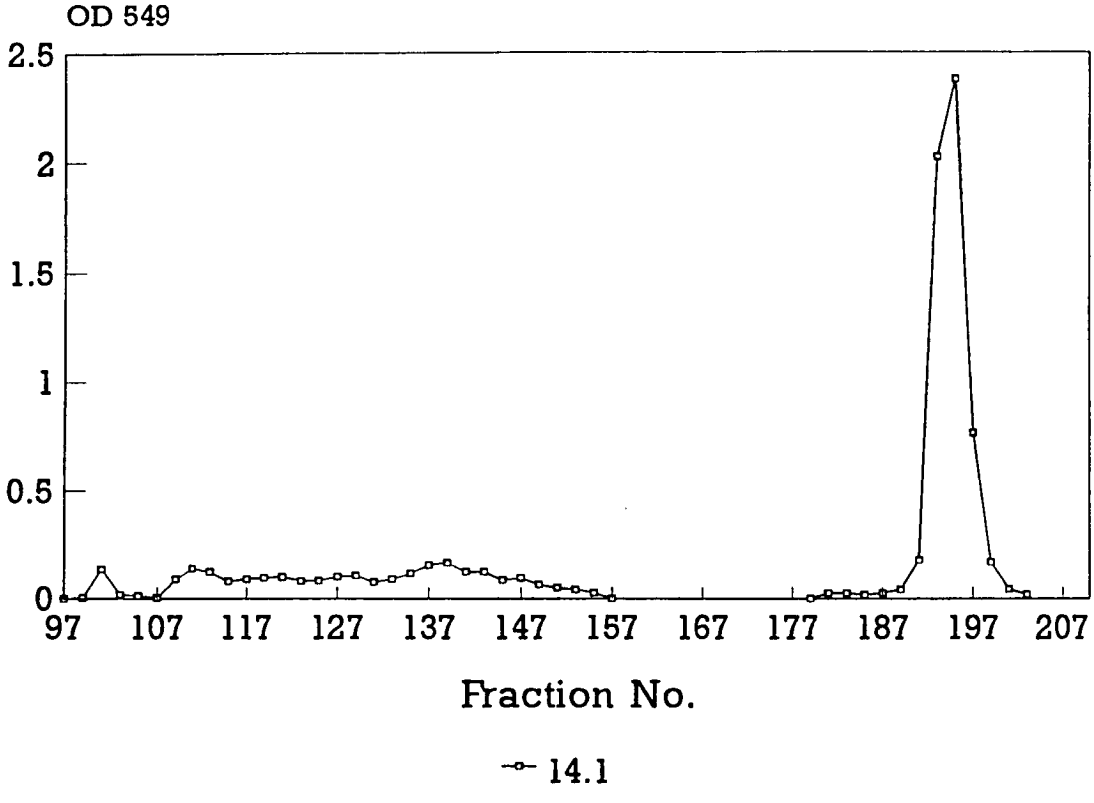
The samples obtained from the ammonium sulphate cuts of 11.1 *gellan lyase* were separated according to their nominal molecular weight using the stirred Amicon cell (10ml). Membranes with different nominal molecular cut off sizes were used (30, 50 and 100kDa). The fractions were tested for gellan lyase using the TBA reaction and the protein concentration was determined.

Fig. 59a The Elution Profile of 14.1 *Gellan Lyase* from Q Sepharose FF.



5ml, 14.1 *gellan lyase* was applied to the Q Sepharose FF. column and eluted with a step NaCl gradient, (0.2, Fr 75 0.4, Fr. 150 and 1M, Fr. 250). 1.2ml fractions were at 4.5min intervals ($16mlh^{-1}$). The elution profile was monitored at 280nm and the fractions containing protein were pooled. Fr. 1 contained the unabsorbed material (tubes 10-46), Fr. 2 eluted with 0.2M NaCl (tubes 89-120), Fr. 3 with 0.4M NaCl (tubes 188-202) and Fr. 4 with 1M NaCl (tubes 250-280). Fr. 3 contained the most *gellan lyase* activity.

Fig. 59b The Elution Profile of TBA Reactive Material from the Q Sepharose FF.



5ml, 14.1 *gellan lyase* was applied to the Q Sepharose FF. column and eluted with a step NaCl gradient, (0.2, Fr 75 0.4, Fr. 150 and 1M, Fr. 250). 1.2ml fractions were at 4.5min intervals ($16mlh^{-1}$). The elution profile was monitored at 280nm and the fractions containing protein were pooled. Fr. 1 contained the unabsorbed material (tubes 10-46), Fr. 2 eluted with 0.2M NaCl (tubes 89-120), Fr. 3 with 0.4M NaCl (tubes 188-202) and Fr. 4 with 1M NaCl (tubes 250-280). Fr. 3 contained the most *gellan lyase* activity.

Fig. 60a The Elution Profile of the Semi-Purified 14.1 Gellan lyase from Q Sepharose FF.

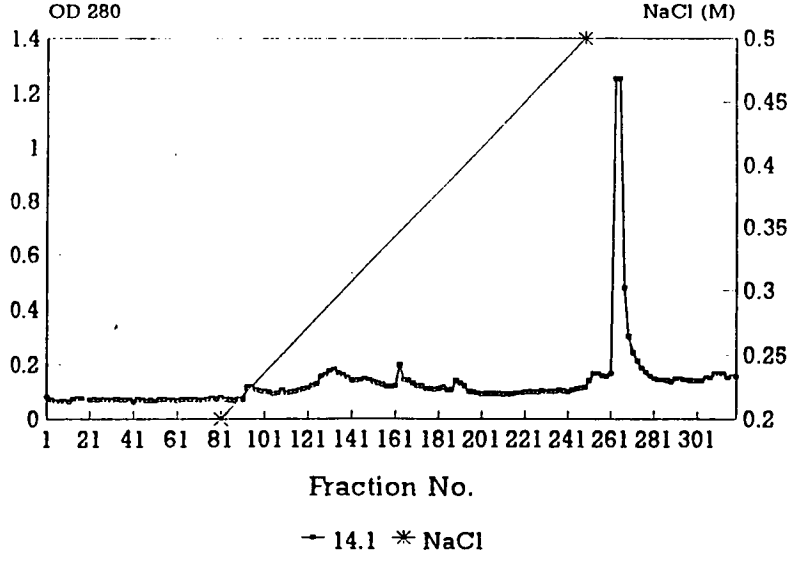
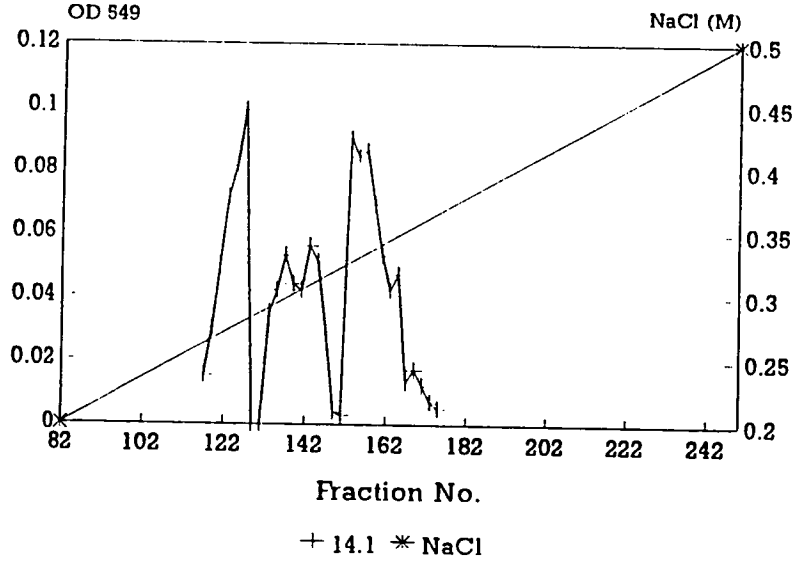
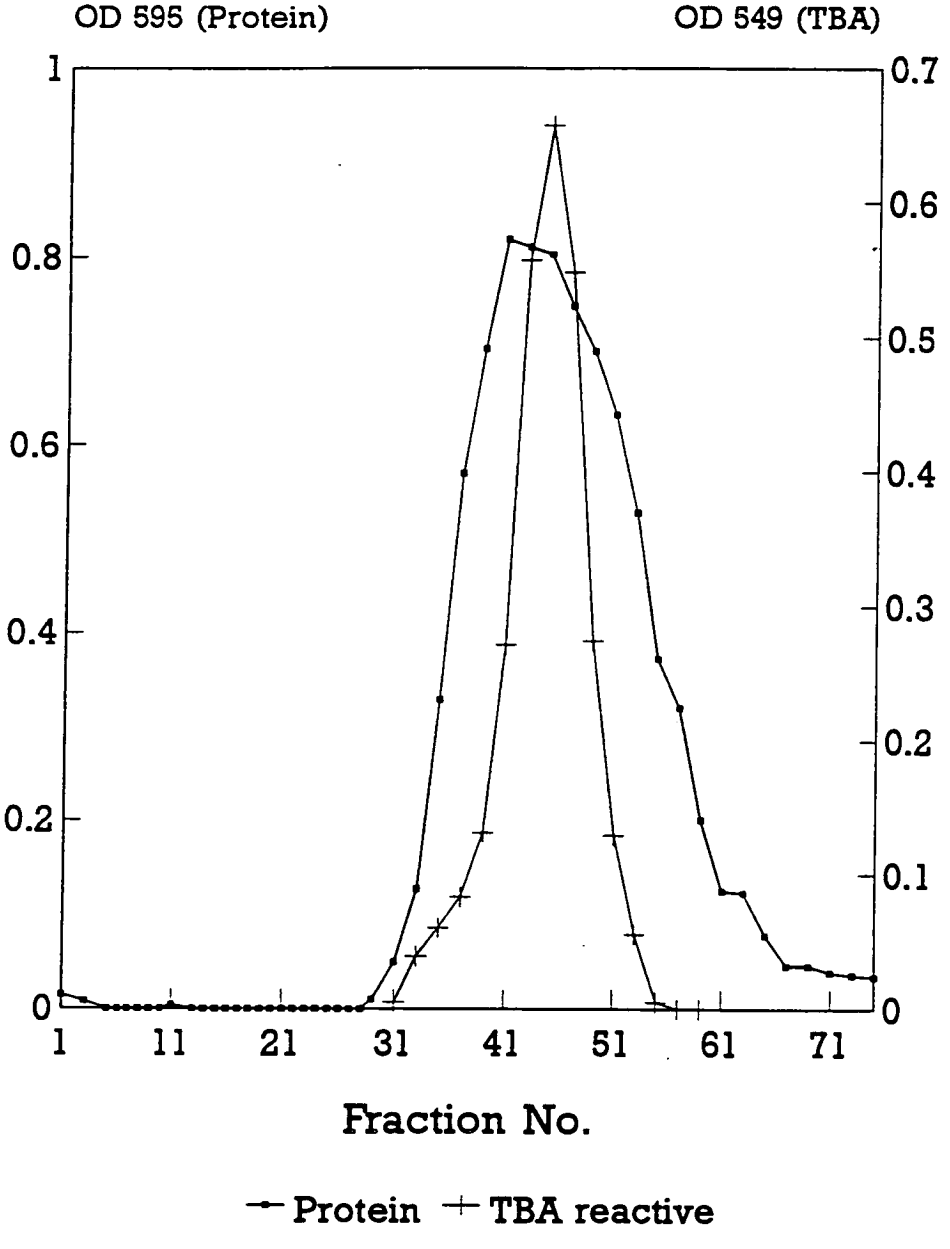


Fig. 60b The Elution Profile of TBA Reactive Material from Q Sepharose FF.



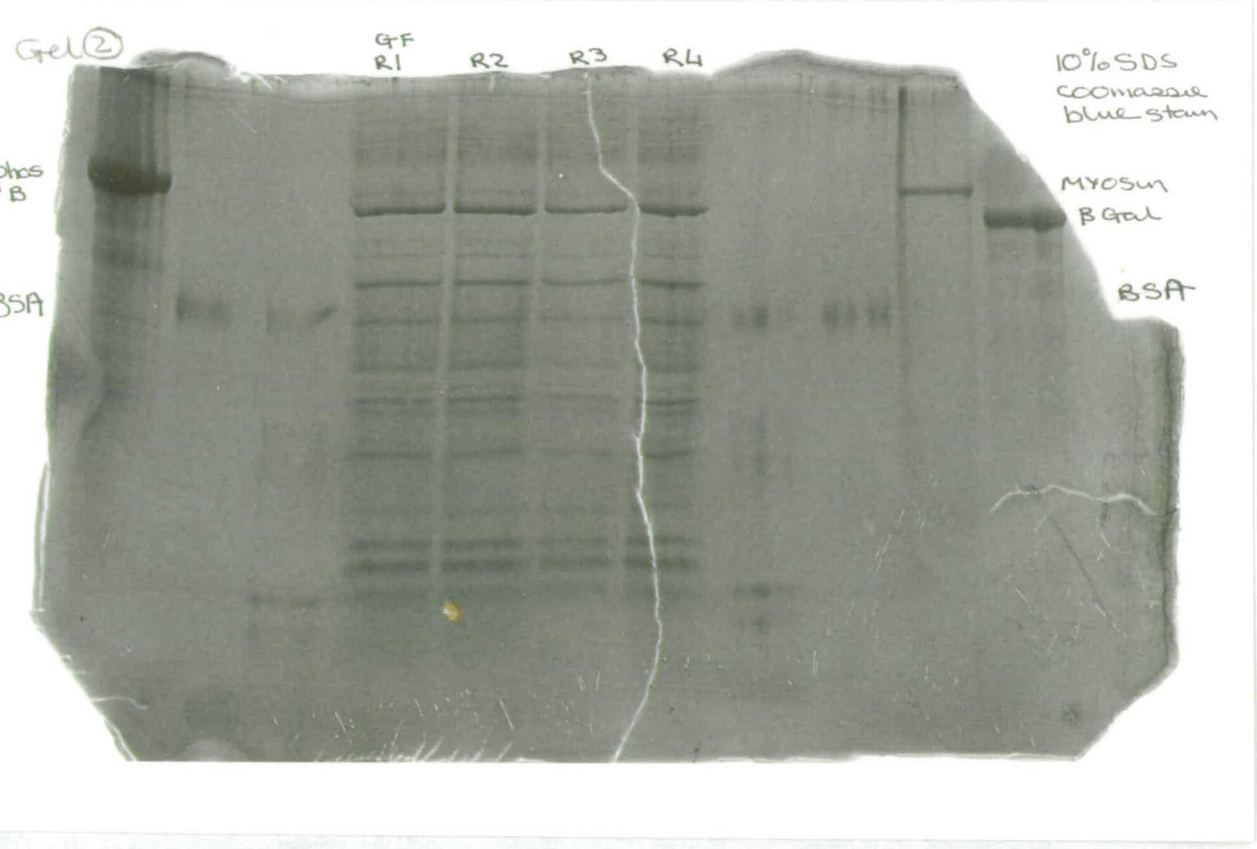
1ml of semi-purified 14.1 *gellan lyase* (Fr. 3) was applied to the Q Sepharose FF. column and eluted with a 200ml linear 0-0.5M NaCl gradient. The elution profile was monitored at 280nm. Three fractions containing TBA reactive material were obtained Fr. 1, tubes 119-127, Fr. 2, tubes 133-149, Fr. 3, tubes 151-175 and Fr. 4 tubes 259-281(1M NaCl) which did not contain any TBA reactive material.

Fig. 61 The Estimation of Molecular Weight of Semi-Purified 14.1 *Gellan Lyase* by Gel filtration.



100µl of semi-purified 14.1 *gellan lyase* was applied to the AcA34 gel filtration column and eluted with 10mM TRIS pH 7.3. The eluted fractions were tested for Protein content (Biorad micro assay) and TBA reactive material. The protein eluted as a single broad based peak and within it an narrower TBA reactive peak eluted. One fraction was pooled containing tubes 31-53.

Fig. 62 The Separation of 14.1 Gellan Lyase by SDS-PAGE after Gel Filtration.



10µg of total protein isolated from four different runs on the gel filtration column were separated on a 10% SDS gel. The estimate molecular mass varied between 96-280kDa (gel filtration column). After separation at 20mAmp the gel was stained with Coomassie blue and revealed identical pattern in each lane. The largest protein present ran between Myosin (205kDa), β galactosidase (116kDa), phosphorylase B(97) and BSA (66kDa) and had a molecular mass of 150kDa.

Effect of Ions on Enzyme Activity

Cations are required for the gelation of gellan. The type and valency of the cation also affect the strength and type of gel formed. Higher concentrations of monovalent cation are required to produce the same gel strength as divalent cations. Cations can also be used to enhance lyase activity and in some cases it is an absolute requirement (*pectate lyase*). Cations can act either as a cofactor or by altering the conformation of the substrate and therefore make it more accessible to the enzyme.

Table 53 shows the effect of monovalent ions and divalent cations on crude 14.1 *gellan lyase*. Low concentrations of Ca^{2+} (<1mM) had little effect on lyase activity. At concentrations higher than this, enzyme activity was inhibited by both Ca^{2+} and Mg^{2+}. NaCl had no effect on enzyme activity up to concentrations of 75mM; above this it had an inhibitory effect. Both EDTA and EGTA inhibit lyase activity; at 1mM lyase activity was halved

The effect of cations on the semi-purified 14.1 *gellan lyase* (Table 54) was tested on both gellan and deacetylated rhamsan. When gellan was the substrate, the 14.1 *gellan lyase* was inhibited by divalent cations. The size of the ion (Mg^{2+} was the smallest and Sr^{2+} the largest) or the concentration of the ion did not affect the result. Monovalent cations enhanced enzyme activity up to concentrations of 200mM (90% of the control activity) and 100mM for Na^{+} and K^{+}. When deacetylated rhamsan was the substrate, both monovalent and divalent cations enhanced lyase activity to some extent, apart from K^{+} which appeared to have the greatest inhibitory affect.

The pH optimum was also determined for the 14.1 *gellan lyase*. Again there appeared to be more than one pH optimum, the first at pH 6.3 and the other at pH 7.3. The 14.1 lyase was most active between 35-40°C on both gellan and deacetylated

rhamsan and as expected, the level of activity observed with deacetylated rhamsan was half of that observed when gellan was the substrate.

While maintaining stock cultures, it was noted that the prolonged incubation of *S. paucimobilis* strains NCIB 11942 (Anson *et al.* 1987) and strain MJ216, formed depressions in the media around the colony of gellan-solidified plates. Both Lobas *et al.* (1992) and Lobas *et al.* (1994) had noticed that non-mucoid strains of *S. paucimobilis* sunk into the media. NCIB 11942 is a mucoid strain, since alginate interfered with the TBA reaction for *alginate lyase* so it was expected that gellan would interfere in the same way. A spontaneous non-mucoid mutant of NCIB 11942 was selected. MJ216 is also a non-mucoid strain. *Gellan lyase* was isolated from the culture supernatant of both strains. The optimal assay conditions were as follows: temperature 30°C, 16h at pH 7.7 for NCIB 11942 in 10mM TRIS, but MJ216 had optima at pH 4.3 and pH 7.0. Neither strain was able to degrade its own polymer nor the polymers from the other strains in the family of gellan-producing bacteria in the native or deacetylated form. Gellan was the best substrate for both enzymes (100%); as with the *gellan lyases* from the red pigmented bacteria, the *gellan lyases* isolated from NCIB 11942 (46%) and MJ216 (54%) used deacetylated rhamsan as substrate. The release of TBA reactive material was monitored. MJ216 released material linearly over a 9h period, whereas NCIB 11942 released considerable material in the first hour and then maintained a steady rate. Viscosity determinations showed that the enzyme was endo-acting as there was a steady loss of viscosity (Fig. 63). The concentrated extracellular *gellan lyase* from NCIB 11942 was applied to the Q Sepharose FF. column and eluted with a step NaCl gradient (Fig. 64).The most active *gellan lyase* fraction was fraction 4 (Table 55) which eluted with 0.35M NaCl.

Table 53 The Effect of Cations and Chelators on 14.1 *Gellan Lyase*

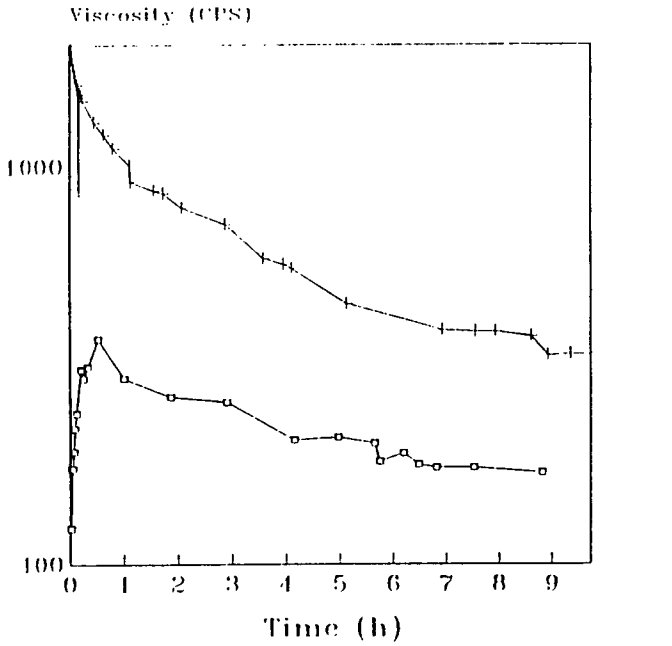
Concentration (mM)	% Control Activity Ion Ca^{2+}	Mg^{2+}	Na^{+}	EDTA	EGTA
0	100	100	100	100	100
0.1	99				
0.3	97				
0.7	99				
1		82	103	46	57
1.7	66				
2		61	117	47	65
3.3	58				
5		51	121	94	48
6.7	46				
10		39	115	94	52
16.7	29				
20		37	103	69	63
33.3	21				
50	37	27	131	4	43
66.7	33				
75		20	135		33
100		15	82		29
150		9	95		
200		5	61		

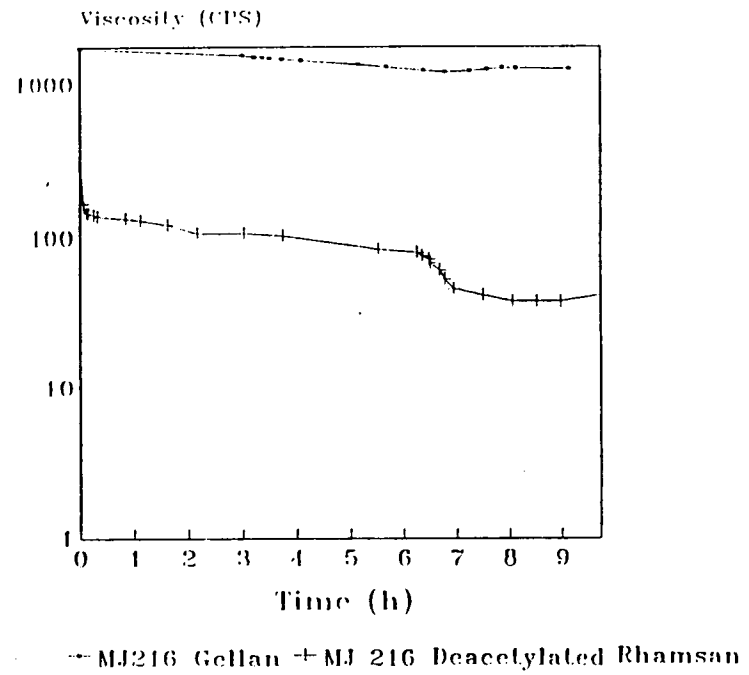
Table 54 The Determination of the Effect of Cations on Semi-Purified *Gellan lyase.*

Concentration (mM)	Gellan substrate					
	Divalent Ion		% Control	Monovalent Ion		
	Mg^{2+}	Ca^{2+}	Sr^{2+}	Li^{+}	Na^{+}	K^{+}
0	100	100	100	100	100	100
0.1	79	98	85	148	145	110
0.5	84	102	104	137	108	112
1	76	117	82	168	104	178
5	67	92	72	114	106	160
10	50	82	57	131	132	128
20	64	72	78	132	134	121
50	23	61	52	196	135	118
75	26	45	72	199	120	110
100	18	41	42	203	107	105
150	10	35	37	115	96	35
200	5	27	37	91	60	39

Substrate	Deactylated Rhamsan Substrate					
Concentration (mM)	Divalent Ion		% Control	Monovalent Ion		
	Mg^{2+}	Ca^{2+}	Sr^{2+}	Li^{+}	Na^{+}	K^{+}
0	100	100	100	100	100	100
0.1	140	80	146	145	211	70
0.5	198	119	162	124	194	67
1	137	112	109	103	157	49
5	198	113	145	115	136	60
10	187	140	220	143	164	99
20	119	119	138	142	231	104
50	108	93	147	141	195	65
75	114	106	145	160	146	73
100	93	81	135	138	167	97
150	57	45	104	128	138	88
200	18	25	68	98	148	49

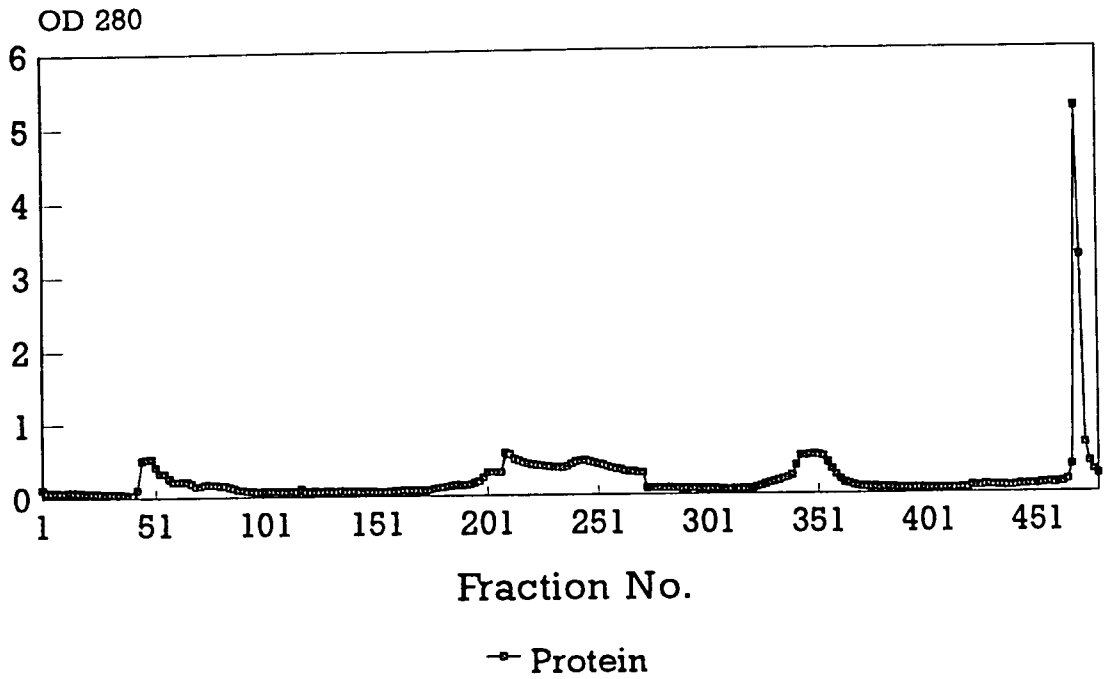
Fig. 63 The Effect of NCIB 11942 and MJ216 *Gellan Lyase* on the Viscosity of Gellan and Deacetylated Rhamsan.





2ml of substrate (3mg ml^{-1} gellan and 12.5mg ml^{-1} deactylated rhamsan) were warmed to 30°C. 10µl of enzyme was added to the substrate and the viscosity monitored at 0.6 shear rate for a number of hours.

Fig. 64 The Elution Profile of NCIB 11942 *Gellan Lyase* from Q Sepharose FF.



10ml NCIB 11942 concentrated supernatant was applied to the Q Sepharose FF. column and eluted with a step NaCl gradient. 0.2M NaCl was applied at Fr. 151, 0.35M NaCl at Fr. 301, 0.75M NaCl at Fr. 421 and 1M NaCl at Fr. 571. Five fractions containing protein were pooled after elution, Fr. 1 (unabsorbed, tubes 42-90), Fr. 2 (0.2M NaCl, tubes 177-205), Fr. 3 (0.2M NaCl, tubes 206-269), Fr. 4 (0.35M NaCl, tubes 324-375), Fr. 5 (0.75M NaCl, tubes 451-499). Fr. 4 contained the most TBA reactive material.

Table 55 Purification Scheme for NCIB 11942 After Separation on Q Sepharose FF.

Fraction No.	Total Activity (ρmole sample^{-1})	Total Protein (mg)	Protein (mg ml^{-1})	Volume (ml)	Specific Activity	Purification (Fold)
Original	43760	19.7	1.97	10	2221	1
Fr. 1	336	1.75	0.25	7	209	0.1
Fr. 2	67	0.42	0.17	2.5	160	0.1
Fr. 3	210	1.8	0.18	10	116	0.05
Fr. 4	7495	4.75	0.5	9.5	1577	0.7
Fr. 5	180	0.45	0.05	9	400	0.2

Specific Activity ρmole ml^{-1} mg protein^{-1}

10ml NCIB 11942 concentrated supernatant was applied to the Q Sepharose FF. column and eluted with a step NaCl gradient. 0.2M NaCl was applied at Fr. 151, 0.35M NaCl at Fr. 301 and 0.75M NaCl at Fr. 421 and 1M NaCl at Fr. 571. Five fraction containing protein were pooled after elution, Fr. 1 (unabsorbed, tubes 42-90), Fr. 2 (0.2M NaCl, tubes 177-205), Fr. 3 (0.2M NaCl, tubes 206-269), Fr. 4 (0.35M NaCl, tubes 324-375), Fr. 5 (0.75M NaCl, tubes 451-499). Fr. 4 contained the most TBA reactive material.

Chapter 4. General Discussion.

The properties of several eukaryotic and prokaryotic *alginate lyases* have been described with respect to substrate specificity, partial purification, purification to homogeneity and the effect of cations on enzyme activity. *Alginate lyases* are usually described as mannuronate specific or guluronate specific but this does not reflect the actual residue cleaved, M-M, M-G, G-M or G-G. Most *alginate lyases* so far reported do not act on acetylated alginate. One of the *alginate lyase* so far isolated, which can degrade acetylated alginate is from the *Flavobacterium* sp A1, (Yonemoto *et al.* 1991). The A1-III *aly* gene was expressed in *Bacillus subtilis* as an extracellular enzyme and had the same properties as those found in the *Flavobacterium* sp. (Hisano *et al.* 1994). The *alginate lyase* eluted from a S Sepharose column with 0.1M NaCl but did not bind to Q Sepharose FF. One of the potentially important uses for *alginate lyase,* in the treatment of cystic fibrosis, requires that the lyase must be able to act on the highly acetylated alginate produced by *P. aeruginosa.*

Romeo & Preston (1986), isolated, purified and characterised the properties of an *alginate lyase* produced by an unclassified bacterium. Separation of the protein on Mono Q (HPLC) resulted in a 27-fold increase in purity of the enzyme. The enzyme was eluted from the column with 0.4M NaCl and had a molecular mass of 29kDa. Divalent cations enhanced enzyme activity at lower concentrations (0.01-0.1M Ca^{2+}) than monovalent ions (0.3-0.4M Na^{+}). $Ca^{2+}$was better than Mg^{2+} and Na^{+} was better than K^{+} in enhancing enzyme activity.

Linker & Evans (1984) described an *alginase* from a mucoid *P. aeruginosa,* which had a temperature optimum between 20-40°C at pH 6.2. The enzyme was mannuronate specific but was inactive against native polymers but was able to degrade deacetylated polymers. A series of oligosaccharides was produced (dimer-

pentamer). The presence of low level *alginase* activity in non-mucoid strains was reported.

The *alginate lyase* from Av UW and Ac 184 exhibit a number of similarities to the *alginate lyase* described here but there were also differences. Like the enzyme isolated from several other strains of bacteria the enzyme was affected by both monovalent and divalent cations. The lyase activity from Av UW was enhanced by 10mM Ca^{2+} or 150mM Na^{+}; the enzyme activity was also enhanced in the presence of 50mM EDTA & EGTA. The lyase from Ac 184 was unaffected by the Na^{+} but Ca^{2+} was inhibitory above concentrations of 10mM. EGTA enhanced enzyme activity between 1-10mM but EDTA reduced the specific activity of the lyase. It is not known if ions affect the conformation of the polysaccharide and therefore make it more accessible for the enzyme or if the cations are required as a cofactor. This can only be confirmed by nmr studies. EDTA and EGTA might play a role in chelating excess cations or removing bound divalent cations from the polymer. The effect of cations on the previously described guluronate-specific *alginate lyase* from *K. pneumoniae* was determined (Boyd & Turvey 1977), confirming that monovalent (200mM) cations were required at a higher concentration than divalent cations (10-50mM). The effect of EDTA and EGTA were determined and it was found that both chelators inhibited lyase activity.

When chloroform was used to permeablise the outer membrane and release the periplasmic contents, Ac 184 was more susceptible to chloroform treatment (Ames *et al.* 1984) than Av UW. When the periplasmic fraction was applied to the Q Sepharose FF. column it was unadsorbed and a 2.8-fold increase in purity was obtained. The combined periplasmic and cytoplasmic fraction was applied to the column; a 2.5-fold purification was achieved. The remaining lyase activity eluted

from the column with 0.2M NaCl. This was a complex fraction comprising several protein peaks.

Both sources of *alginate lyase* were periplasmic, mannuronate-specific, endo-acting, producing a series of oligosaccharides as the result of enzymatic degradation of the polymer. The oligosaccharides produced by the action of Av UW *alginate lyase* had a DP of 5. Ac 184 lyase was more active than the Av UW lyase and consequently a series of oligosaccharides were produced. The DPs of the Ac 184 oligosaccharides, were 6, 5 and 4, when *L. hyperborea* was the substrate and a 5 and 4 when *M. pyrifera* was the substrate.

To date **all** the polysaccharide lyases isolated have been found to be monomeric giving the same molecular weight under reducing and non reducing conditions. Malissard et al (1993) reported that the monomeric *alginate lyase* protein (30kDa) was cleaved in the presence of β mercaptoethanol to form a 20kDa and 10kDa protein. There was no evidence to suggest the *alginate lyase* from Av UW or Ac 184 or the *gellan lyase* from the unclassified bacterium 14.1 were not also monomeric proteins.

The evidence gathered from the nucleotide sequences of several bacteria (Boyd *et al.*, 1993; Maki *et al* 1993; Baron *et al* 1993), has shown that alginate lyase was located in the periplasm. The deduced amino acid sequence showed the presence of a hydrophobic signal sequence which is indicative of periplasmic location. When the nucleotide sequences from the *alginate lyases* were compared to nucleotide sequences in the databases no homology was obtained even to the *pel* genes (pectate lyases). Boyd *et al.* (1993) suggested that *alginate lyase* was located in the periplasm since they were able to detect activity after osmotically shocking *P. aeruginosa.*

They were able to detect lyase activity in cell free extracts of both *P. aeruginosa* and *E. coli* but were unable to distinguish it on SDS-PAGE.

When Maki et al. (1993) tested the substrate specificity of pAL205 in *E. coli* JM109, it was able to act on alginate containing guluronate and mannuronate but lower activity was observed when poly G was the substrate.

Depolymerisation of alginate has been described by in a number of papers. Yonemoto *et al.* (1993), described how the alginate lyase from strain A1 degraded the alginate from *E. bicicylis* into oligosaccharides with a DP of 10 (MW 1,800 by gel filtration). The oligosaccharide contained guluronate residues since it gelled in the presence of divalent cations. The depolymerised alginate was able to act as a oligosaccharine which was able to promote cell differentiation in plant cells.

Unlike traditional sources of polymers (plant), where seasonal variations cause variations in the polymer properties, microbial polysaccharides can be produced by fermentation processes and therefore the properties of the polymer can be carefully controlled. The polysaccharide gellan is one polysaccharide in the family of polysaccharides produced by the bacterium *S. paucimobilis,* other members of this family include Welan (S130), Rhamsan (S194), S657, S88 and S198. All were developed by Kelco for industrial applications. Gellan was developed as a food additive, Welan for application in the oil industry, Rhamsan for agriculture and S198 as a viscosifier for water based lubricants. Gelrite® the low acetyl gellan produced by Kelco is also used as an agar substitute.

There have been a number of reports on the ability of bacteria to degrade gellan. The first was by Casida (1989), in which certain bacteria isolated from soil could hydrolyse gellan, in gellan solidified media. The bacteria were Gram negative rod-like, gliding bacteria and formed either small concave dips in the medium or sank

into the medium. Lobas *et. al.* (1992) noted that some variant colonies of *S. paucimobilis* E1 (ATCC 31461) were able to sink into gellan solidified medium. Strain E1 was unable to produce extracellular polysaccharide and unable to grow on gellan as the sole carbon source. When strain E1 was grown in shaken cultures in the presence of gellan the solution viscosity of the medium fell. A.M. De Souza (unpublished work) also noted reduced EPS yields following prolonged incubation of *S. paucimobilis.* Lobas *et. al.* (1994) also reported that a EPS-producing strain of *S. paucimobilis* P4 sank into and liquefied the gellan matrix. The most comprehensive study to date was carried out by Mikolajczak *et. al.* (1994). This report described an enzyme which was able to degrade a number of polysaccharides in the gellan family and was named *sphinganase*. The enzyme was produced extracellularly and was an endo-acting glucanase. The pH optimum was determined as 7.5 in MOPS buffer. The *sphinganase* eluted from the column (DEAE Sepharose) as a single peak with 0.2-0.3M NaCl. SDS-PAGE indicated the presence of a major band with a molecular mass of approximately 110kDa as well as several other smaller fainter bands. Subsequent purification steps removed these bands and showed that the 110kDa protein was in fact *sphinganase*. The bacterium which produced the enzyme was a pink, spore-forming Gram positive rod which was isolated from soil in medium supplemented with welan (S130). The enzyme was able to degrade a number of polymers in the gellan family. Welan was the best substrate followed by gellan, S198, S7 and S88. It was unable to cleave either rhamsan or xanthan. Deacetylation of the polymer had no effect on the enzyme's ability to degrade the polymer. The random distribution of L-mannosyl and L-rhamnosyl residues in the polysaccharide S130 was reported by Jansson and Widmalm (1994).

Here we report the presence of a highly specific gellan-degrading enzyme which has been designated *gellan lyase* (*gellanase*). *Gellan lyase* was isolated from pink Gram negative bacteria which had been isolated locally from an aquatic environment. The only alternative polysaccharide substrate in this family of polymers, which can act as substrate for this enzyme, is deacetylated rhamsan. The enzyme was isolated as an extracellular enzyme and was found to be endo-acting, due to the rapid loss in viscosity and the series of oligosaccharides which accumulated as the breakdown products of enzyme action. The enzyme is a lyase, since the products of enzymatic degradation react with TBA reagents caused the formation of a pink chromophore (Weissbach & Hurwitz 1959). When the enzyme reaction was followed at 233nm, there was an increase in absorption at this wavelength. This is also indicative of the action of a lyase (Preiss & Ashwell 1962).

Both welan and rhamsan contain the repeated tetrasaccharide present in the backbone, rhamsan has a gentobiosyl group on the first glucose residue (1,3 linked) of the backbone (Peik *et al.*, 1983), whereas welan has either an α L mannose or α L-rhamnose residue (in the ratio of 1:2) linked to the second glucose residue (1,4 linked) in the repeated tetrasaccharide (Peik *et al.*, 1983). Rhamsan does not contain any L-glyceryl groups (Jansson *et al.*, 1986) but is *O*-acetylated (4-13%) In solution, both form viscous solutions. Since both have the same half staggered double helix as gellan (Lee & Chandrasekan, 1991) it was thought that the inability to gel in the presence of cations was due to the presence of these side chains. Lee & Chandrasekan (1991) demonstrated that the side-chain of welan folded in towards the glucuronate residue. Since the side-chain of welan is bulkier than either the glycerate or acetyl groups found in native gellan, it is thought that the side-chains act to shield the glucuronate residue thus reducing its access to the cations necessary for gelation

to occur. The double helices in rhamsan could not associate with each other because of the large flexible side-chains. These side-chains prevent the formation of carboxylate-cation-carboxylate interaction even though the carboxylate groups are not shielded. For the same reason the side-chains prevent other side-chains from other helices associating with each other. Therefore the aggregation of the polymer may be random.

Gellan shows a structural change from the ordered state (double helix) at low temperature to the disordered state at (single stranded) at higher temperature. The transition temperature is 35^{o}C (Milas *et. al.*, 1990). The double helix is composed of two parallel left handed helices (Chandrasekaran *et. al.*, 1988). The gel-sol transition of gellan was observed at 316^{o}K. The transition temperature was dependent on the molecular weight of the polymer and the polymer concentration (Moritaka *et al.*, 1991).

The final *gellan lyase* which was studied came from a yellow pigmented bacterium NCIB 11942 isolated by Anson *et al.* (1987); it had 99% homology to *Pseudomonas paucimobilis.* The bacterium produced a polysaccharide which had L-rhamnose and D-glucose in the molar ratio 0.66:1.0, 16% glucuronic acid and 10% acetate. The polymer gelled in the presence of some cations and belonged to the gellan family (Fig. 11). Another strain derived from MJ216 also yielded lyase activity. Interestingly the substrate specificities of these enzymes closely resembled those of the other *gellan lyase* systems which have been used in this study.

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Alginases from *Azotobacter* species

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Alginate lyases (alginases) have been prepared from strains of *Azotobacter vinelandii* and *Azotobacter chroococcum* in which they were located in the periplasm. The enzymes are present in wild-type strains of each species and in mutants failing to encyst or produce bacterial alginate. The lyases have been partially purified by ion exchange chromatography and by affinity chromatography on a matrix prepared from poly-D-mannuronic acid. Although several bacterial and algal alginate preparations were degraded by the enzymes, highest activity was found on poly-D-mannuronic acid or on algal alginates with high mannuronic acid content. The major product from enzymes of either bacterium was an unsaturated uronic acid, when either alginates or poly-D-mannuronic acid were used as substrates. When tested against a series of algal alginates of increasing D-mannuronic acid content, the enzyme activity was highest against alginates of high D-mannuronic acid content, indicating that the enzymes are endo-D-mannurono-lyases. The alginases from the two bacterial species are not identical in their substrate specificity although both show the same generalized type of action.

Introduction

Alginases are of widespread occurrence, being found in marine gastropods, in bacteriophage, in various marine micro-organisms (Doubet & Quatrano, 1984; Sutherland & Keen, 1981; Brown & Preston, 1991) and in some soil *Bacillus* species (Hansen *et al.*, 1984). Although few of these enzymes have been purified, they have been shown to be endo- or exo-enzymes with specificity towards β-D-mannuronosyl or α-L-guluronosyl linkages. Without exception, the alginases described from these sources have been lyases (eliminases), yielding products with an unsaturated uronic acid at the non-reducing terminus. Although some of the bacteria produce exopolysaccharide, none form alginate. Bacterial alginate synthesis is a feature of certain *Pseudomonas aeruginosa* isolates, plant pathogenic and other *Pseudomonas* spp. and of *Azotobacter vinelandii* and *Azotobacter chroococcum* (Evans & Linker, 1973; Osman *et al.*, 1986; Cote & Krull, 1988). In the *Azotobacter* species, alginate synthesis is essential for the formation of the desiccation-resistant microcysts produced when the bacteria are subjected to nutrient deprivation and other physiological conditions. The microcysts of *A. vinelandii* contain three types of alginate differing in D-mannuronic acid : L-guluronic acid ratios and in *O*-acetylation, while *A. chroococcum* strains produce both alginate and other polysaccharides (Page & Sadoff, 1975). Gacesa (1987) has suggested that alginate lyases and the epimerases which at the polymer level convert D-mannuronosyl residues to L-guluronosyl residues have a common mechanism of action, although this has been disputed by Feingold & Bentley (1987).

Several of the bacteria that synthesize alginate-like exopolysaccharides also produce alginate lyases but cannot use the polymers as sole carbon and energy source. There is no evidence to suggest that the bacterial alginate serves as a carbon and energy reserve for the bacterial cells which produce this polysaccharide. As relatively few bacteria synthesize polysaccharides and the specific polysaccharases which degrade them, investigation of the alginases from *Azotobacter* species may provide information on the characteristics and role of such enzymes.

Methods

Bacteria and culture. Various strains of *A. vinelandii* (NCIB 8789, Wyss, 206 and UW) and *A. chroococcum* (NCIB 8002 and 8003, Ac 34, 46, 184 and 186) were used for the major experiments. Strains UW and 206 do not encyst. A range of other strains tested initially were either laboratory strains or exopolysaccharide-negative (EPS⁻) mutants. Several of the strains (Ac) were kindly provided by Professor W. Page (University of Alberta, Canada) (Page, 1986). Two strains of *Beijerinckia indica* were also examined. The bacteria were grown in

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Abbreviations: EPS, exopolysaccharide; TBA, thiobarbituric acid.

Table 1. *Composition and diad frequencies of some of the bacterial and algal alginates used in this study*

The analyses were by NMR using a Jeol FX-100 spectrometer run at 90 °C and at 100 MHz as indicated by Skjak-Braek *et al.* (1986).

Source	F_G	F_M	F_{GG}	F_{MM}	$F_{GM,MG}$	Acetyl
Laminaria hyperborea	0·665	0·335	0·558	0·228	0·107	0
Macrocystis pyrifera	0·41	0·59	0·24	0·42	0·17	0
Azotobacter vinelandii 73	0·561	0·439	0·372	0·25	0·189	11%
Azotobacter vinelandii 206	0·08	0·92	0·03	0·87	0·05	24%
Pseudomonas aeruginosa B	0·16	0·84	0	0·68	0·16	37%

Norris's (1959) modification of Burk's nitrogen-free medium supplemented with either 0·1% (w/v) mannitol or 2% (w/v) glucose, or in a similar medium containing 0·1% (w/v) casein hydrolysate and 0·1% (w/v) yeast extract as sources of fixed nitrogen and with 1% (w/v) mannitol as carbon source. Cultures were grown in Erlenmeyer flasks with less than half the nominal volume of medium and shaken at 200–300 r.p.m. at 30 °C on an orbital shaker. Bacteria were harvested by centrifugation, washed twice with water and used as a source of enzymes. Total lyase activity was measured in the supernate of ultrasonic lysates of the bacteria after ultracentrifugation at 100000 *g* for 30 min, concentration against polyethylene glycol (mol. mass 6000 Da) and dialysis against water. Alginates (10–20 mg) were dissolved in water and incubated with samples of lysates or other enzyme preparations. No increase in lyase activity was obtained when 0·1% (w/v) sodium alginate was added to the medium and the bacteria were incapable of growth on alginate as sole carbon and energy source.

Cysts of *Azotobacter* were induced by harvesting cells grown for 48 h in Norris medium with mannitol as carbon substrate, centrifuging in sterile centrifuge bottles, washing aseptically and resuspending in Norris nitrogen-free medium containing β-hydroxybutyric acid (0·3%, w/v) as carbon source. The cultures were then shaken at 30 °C. Complete and fairly synchronous encystment was achieved within 48 h. The cysts were then broken by sonication, ultracentrifuged and the supernatant was tested for enzyme activity. Abortive cysts were obtained by transferring cells into Ca^{2+}-free Norris medium and incubated under the same conditions as were used for obtaining normal cysts (Sadoff, 1975).

Enzymes and assay procedures. Enzymes were released by lysis of the bacterial cells either by ultrasonic treatment using 4 × 1 min exposures (MSE ultrasonic generator) or by liberation of the periplasmic contents according to the procedure of Osborn *et al.* (1972). The enzymes were subsequently purified by conventional techniques. Cysts were lysed by treatment with EDTA or EGTA in Tris/HCl buffer (pH 7·8) followed by ultrasonic treatment. Lyase activity was measured using the thiobarbituric acid (TBA) assay technique (Weissbach & Hurwitz, 1958) for unsaturated uronic-acid-containing products. Alkaline phosphatase was assayed using *o*-nitrophenyl phosphate as substrate and the nitrophenol released was measured at 405 nm. Enzymes were isolated and purified by standard techniques, except that an affinity matrix was prepared by coupling poly-D-mannuronic acid to activated Sepharose. This was performed essentially according to the procedure described by Skjak-Braek & Larsen (1985) for the purification of an alginate epimerase from *A. vinelandii*. Protein was measured by the Bradford (1976) procedure using a kit from Bio-Rad and the method was as described by the manufacturers. Elution from the affinity matrix and from DEAE-Sepharose columns was achieved by using a linear gradient of increasing NaCl concentration (0–0·5 M). Protein was monitored at 280 nm.

Substrates. The substrates tested were homopolymeric sequences of poly-D-mannuronic acid and poly-L-guluronic acid, algal alginates of known mannuronic acid:guluronic acid ratio, and some bacterial alginates. The bacterial alginates were prepared in the laboratory, as were the homopolymeric sequences. Algal alginates were either purchased from Sigma or were gifts from various workers. The polymannuronic acid, polyguluronic acid and 'mixed' blocks were prepared from commercial algal alginate from *Laminaria* by partial acid hydrolysis using the procedure of Penman & Sanderson (1972). Deacetylated bacterial polysaccharides were prepared by treatment with 0·1 M-ammonia at 60 °C for 30 min, dialysis and lyophilization. The detailed composition of the major substrates used, where known, is given in Table 1. The composition and frequency of occurrence of adjacent residues of mannuronic acid or guluronic acid (MM or GG diad frequency respectively) were determined by the NMR techniques of Skjak-Braek *et al.* (1986). Other algal alginates were of known mannuronic acid content but not of known sequence or diad frequency. Acetyl determination was by the procedure of Hestrin (1949). Products of enzyme degradation were recovered in the diffusate from dialysis of digest mixtures and characterized by HPLC using SAX columns in a Gilson system with refractive index monitoring and 0·005 M-H_2SO_4 as eluant (mobile phase). Descending paper chromatography with a mobile phase composed of ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.) was also used. The sugars and oligosaccharides were revealed with alkaline silver nitrate reagent. The uronic acid content of oligosaccharides was determined by the procedure of Blumenkrantz & Asboe-Hansen (1973).

All assays were performed in triplicate and repeated approximately ten times, as were the purification procedures for which representative examples are shown.

Results

Occurrence and location of alginate lyase activity

Preliminary tests on a number of wild-type and EPS$^-$ strains of *Azotobacter vinelandii* and *A. chroococcum* revealed that all possessed some alginate lyase activity as measured by increased release of TBA-positive material following incubation of cell lysates with algal alginate from either *Laminaria* or *Macrocystis*. Extracts of *Beijerinckia indica* NCIB 8005 and 8597 were inactive. Some strains of each *Azotobacter* species consistently produced more enzyme activity than others under

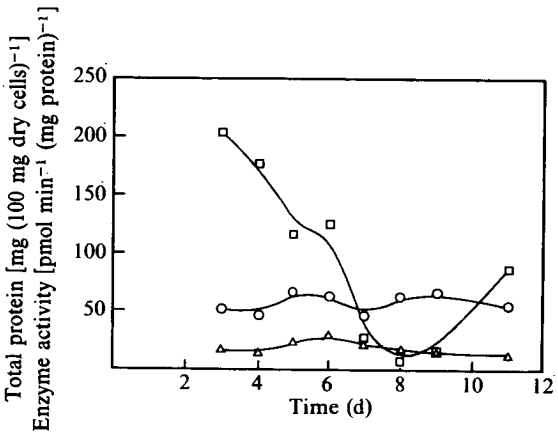

Fig. 1. Production of alginate lyase in *A. vinelandii* during encystment. Cells were transferred to medium containing β-hydroxybutyrate at 3 d and started to encyst after a further 6–12 h. Cysts were allowed to germinate after 7·5 d, by addition of 1 % glucose to the medium. □, Alginate lyase; △, alkaline phosphatase; ○, total protein.

standardized culture conditions. Enzyme activity also depended on the growth medium used. It was high in bacteria grown in either Norris medium or semi-synthetic medium with glucose or mannitol as carbon substrate. However, lysates of cells grown in medium with sodium glutamate or casein hydrolysate as the major carbon source or in peptone, revealed little lyase activity. As there appeared to be no significant difference in yield between alginate$^+$ and alginate$^-$ strains, the latter were used for ease of handling in all subsequent studies and were grown in glucose-containing media.

Tests on the wild-type and EPS$^-$ *A. vinelandii* strains clearly revealed that almost all the alginate lyase activity (93%) was located in the periplasm, the remainder being extracellular or cytoplasmic. Extracellular enzyme activity was greatest in older cultures (>5 d) in which considerable cell lysis had occurred. No significant lyase enzyme activity was associated with the cytoplasm. Similar results were obtained for the corresponding fractions from *A. chroococcum*. The enzyme activity in *A. vinelandii* rapidly reached a maximum in the exponential phase of cultures grown in medium with a fixed nitrogen source. Thereafter it declined. In nitrogen-free medium, activity was again high in the exponential phase but did not decline greatly during the early stationary phase. Activity was highest in cells grown on glucose or mannitol as carbon source. Enzyme activity decreased rapidly following induction of cyst formation in the bacteria and reappeared when germination of the cysts was promoted by addition of fresh glucose or mannitol (Fig. 1). This was in contrast to alkaline phosphatase and total protein levels, which remained relatively constant throughout the morphological changes. During encystment, some alginate lyase activity was initially found at a low level in the culture supernatant but this activity disappeared as the cysts matured. Enzyme activity was detected in lysates of abortive cysts induced in Ca^{2+}-deficient medium; the specific activity remained constant and was close to that found in periplasmic material of vegetative cells. The substrate specificity of any alginase activity associated with cysts or abortive cysts did not appear to differ in any of the respects tested from that found in vegetative cells. The pH optimum of the *A. vinelandii* alginate lyase was 6·8 and the temperature optimum was 30 °C. Alkaline phosphatase, which is known to be a periplasmic enzyme in *Pseudomonas aeruginosa* (Cheng *et al.*, 1970) was used as a control to ensure that there was no significant contamination of other fractions by periplasmic material.

Enzyme purification

The enzyme activity was initially precipitated from cell lysates or from periplasmic preparations by addition of ammonium sulphate to 70% saturation. Slight differences were seen between *A. vinelandii* and *A. chroococcum* after the protein was redissolved; the lyases precipitated with 40% and 50% saturation ammonium sulphate respectively. Further purification was attempted using ion exchange chromatography on DEAE-Sepharose (Fig. 2) and using affinity chromatography on a poly-D-mannuronic-acid-based adsorbent (Fig. 3). Although both chromatographic systems yielded some purification as judged by PAGE and by increased specific activity, neither provided pure enzyme. Despite the relatively complex protein elution patterns observed on the affinity matrix, almost all the lyase activity was found in fractions eluting with 30–35 ml of the buffer gradient. The larger amounts of protein eluting with 100–140 ml of buffer were inactive. Some further purification was obtained by pooling the active fractions obtained from ion exchange chromatography and re-running on the affinity column. The failure to obtain pure enzyme from the affinity column was probably because of interaction between bacterial proteins and the polyanionic matrix. The partially purified enzyme preparation from both *Azotobacter* species showed the same temperature optima of 30 °C. pH optima were at pH 6·8 and 7·2 respectively for *A. chroococcum* and *A. vinelandii* material.

Substrate specificity

The partially purified enzymes were also used to determine substrate specificity. For this, a range of algal alginates of known mannuronic acid content was used,

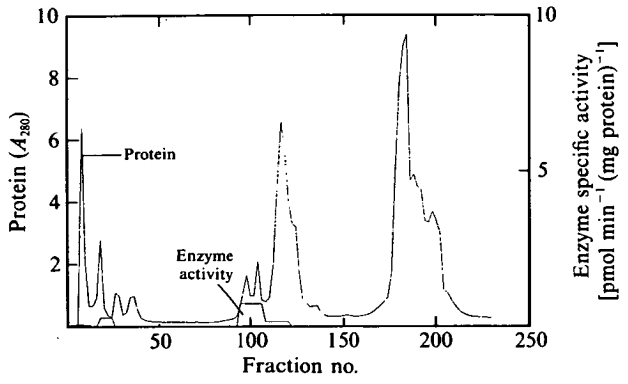

Fig. 2

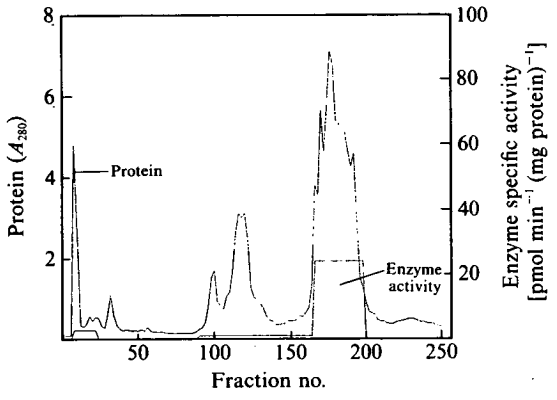

Fig. 3

Fig. 2. Chromatography of *A. vinelandii* (strain UW) periplasm on DEAE-Sepharose. Concentrated periplasmic material (800 µl, containing about 5 mg protein) was loaded onto a 20 × 1 cm column run in 20 mM-Tris/HCl (pH 7·0) with a gradient of 0·5 M-NaCl applied at fraction 62; 1 ml fractions were collected.

Fig. 3. Affinity chromatography of *A. chroococcum* (strain 184) periplasmic material. Concentrated periplasmic material (800 µl, containing about 5 mg protein) was loaded onto a 5 × 1 cm column of polymannuronic acid-Sepharose and run with 20 mM-Tris/HCl (pH 7·0). A gradient of 0·5 M-NaCl was started at fraction 68 and 1 ml fractions were collected.

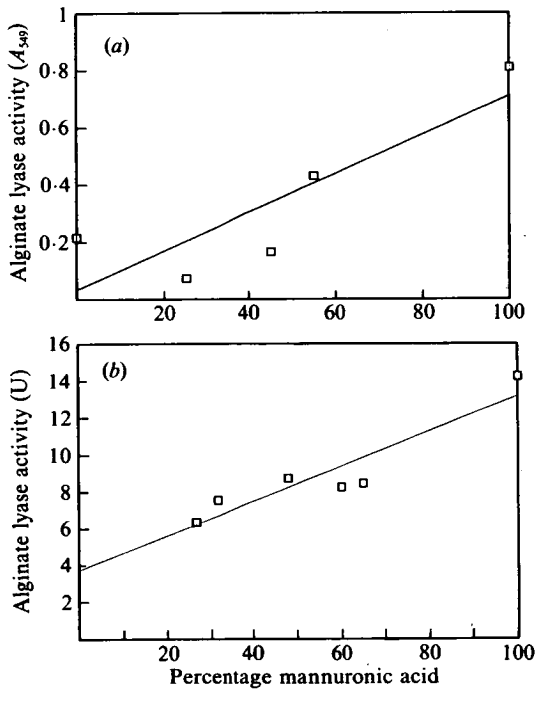

Fig. 4. (*a*) Substrate specificity of partially purified *A. vinelandii* (strain UW) enzyme. Solutions of alginate (10 mg ml^{-1}) were mixed with about 1 mg semi-purified alginate lyase and incubated at 30 °C for 7 h. Samples (100 µl) were withdrawn in triplicate and assayed by the TBA reaction. Note that on the vertical axis lyase activity is shown as A_{549}. (*b*) Substrate specificity of partially purified *A. chroococcum* (strain 184) enzyme. Experimental methods as in (*a*). Note that on the vertical axis lyase activity is shown as units, where 1 unit (U) is the activity that liberates 1 µmol unsaturated uronic acid min^{-1}. The lines are the best fit to the means of triplicate measurements that varied by 1% or less.

together with poly-D-mannuronic and poly-L-guluronic acid preparations. Naturally, acetylated and chemically deacetylated bacterial alginates were also tested. Preliminary experiments indicated that the activity of enzymes prepared from either *Azotobacter* strain was greater against some of the algal alginates than against bacterial products from either *A. vinelandii* or *P. aeruginosa*. Under standard conditions, the highest enzyme activity was observed when the substrates were non-acetylated algal alginates of high D-mannuronic acid content (Fig. 4*a*, *b*).

Lyase preparations from either of the *Azotobacter* species were much more active against an algal alginate preparation than against the native acetylated alginates from *A. vinelandii* or *P. aeruginosa*. The highly acetylated polysaccharides from *Pseudomonas* or *Azotobacter* were relatively resistant to enzyme action. Even when the bacterial alginates had been chemically deacetylated, the algal material was normally the favoured substrate but removal of the *O*-acetyl groups clearly enhanced activity considerably. The relative activities of typical semi-purified preparations from each *Azotobacter* species are indicated in Table 2. Very high activity was found when the substrate was an *A. vinelandii* alginate which had previously been subjected to treatment with an alginate lyase from *Klebsiella aerogenes* having specificity towards guluronosyl linkages (Boyd & Turvey, 1978). This substrate differed from the native alginates in being of lower molecular mass, as were the polymannuronic acid, polyguluronic acid and 'mixed block' samples prepared by the partial acid hydrolysis procedure of

Table 2. *Relative activities of Azotobacter alginases on algal and bacterial alginates*

Alginate	*A. vinelandii*	*A. chroococcum*
(*a*) *Algal*		
Laminaria hyperborea	0·78	0·53
Macrocystis pyrifera	1·0	1·0
M blocks	0·61	0·42
M-G blocks	0·91	0·15
(*b*) *Bacterial*		
Pseudomonas aeruginosa B	0·165	0·28
Pseudomonas aeruginosa B (deac.)	0·4	0·90
Azotobacter vinelandii 206	0·22	0·205
Azotobacter vinelandii 206 (deac.)	0·67	0·41
Azotobacter vinelandii 73	0·26	0·23
Azotobacter vinelandii 73 (deac.)	0·84	0·39

Penman & Sanderson (1972). No detectable difference in specificity was observed for lyase preparations from abortive cysts or from cysts.

The products of alginate degradation were all of low molecular mass. The major product from all substrates was the unsaturated uronic acid deriving from the action of the lyase on the non-reducing terminal D-mannuronosyl residues. This fragment had an $R_{\text{ManA lactone}}$ value of 0·67 on paper chromatography. Some material with a relative mobility of 0·76 was observed in the digests from one of the acetylated bacterial alginates, and this appears to be the corresponding acetylated uronic acid derivative as analysis of the fraction revealed the presence of uronic acid and acetate in approximately equal amounts. In some of the alginates digested for a short time only, small amounts of some slower-moving oligosaccharides were also detected. Digests of 'mixed block' sequences of the two uronic acids consistently yielded small amounts of several oligosaccharides in addition to the unsaturated uronic acid. All these oligosaccharides were TBA-positive and the ratio of TBA-positive material to total uronic acid indicated that they were probably disaccharides or trisaccharides in which the unsaturated uronic acid formed the non-reducing terminus.

Discussion

Occurrence and nature of the alginate lyases

The alginases found as periplasmic enzymes in *A. vinelandii* and *A. chroococcum* are present in vegetative bacteria and are lost on encystment, but are rapidly resynthesized on cyst germination. The enzymes from both species show specificity towards sequences of mannuronosyl residues. In this, they resemble an enzyme from an *A. vinelandii* phage (Davidson *et al.*, 1977) and enzymes from several *Pseudomonas* species. The exact specificity is not entirely clear as the alginases may hydrolyse either ··· ManA–GulA ··· or ··· ManA–ManA ··· linkages or only the latter. The mode of action of enzymes degrading alginate is further complicated by the differences in distribution of monosaccharide residues in different substrates. Thus, as can be seen in Table 1, alginates with the same or very similar mannuronic acid : guluronic acid ratios can have very great differences in monosaccharide sequence and in the frequency of adjacent residues.

Specificity of the alginate lyases

The enzymes from both *Azotobacter* species are alginate lyases, i.e. they are among the relatively large group of polysaccharide lyases which cleave their substrates by β-elimination mechanisms (Linhardt *et al.*, 1986). Further, the terminal non-reducing product formed from either D-mannuronosyl or L-guluronosyl residues through the action of an alginate lyase is identical. It is 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate. For one enzyme, the guluronate-specific lyase from *Klebsiella aerogenes*, the initial rate of reaction depends on the concentration of L-guluronosyl residues in solution (Haugen *et al.*, 1990). When tested against substrates with different distributions of the two component uronic acids, initial reaction rates were considerably lower, indicating that the *Klebsiella* enzyme may either attack one type of linkage or may attack different types at different rates.

The method of preparation of so-called homopolymeric blocks is not sufficiently precise to exclude all heterologous residues and they certainly do contain a small number of heterologous residues. Activity may also be greatly influenced by the nature of residues adjacent to the site of chain cleavage and there may indeed be a requirement for a more heterogeneous structure at or near the site of cleavage in macromolecular substrates. The presence of *O*-acetyl groups on D-mannuronosyl residues appears to be inhibitory to the action of lyase preparations from both species of *Azotobacter*. These substituents are known from the work of Skjak-Braek *et al.* (1986) to be on the 2 and 3 positions, with some 3–11% of the mannuronosyl residues in some bacterial alginate samples being doubly acetylated. Boyd & Turvey (1978) demonstrated that cleavage of alternating sequences (mixed blocks) can be accomplished using enzymes with either polymannuronic acid specificity or polyguluronic acid specificity. The products obtained from cleavage of alginate chains by the different lyases also show variation. Some only produce trimer (Preston *et al.*, 1991), while others form a wider range of products (e.g. Davidson *et al.*, 1977). The enzymes from both *Azoto-*

bacter species yielded the unsaturated uronic acid monomer as the major product and formed only small amounts of higher oligosaccharides from 'mixed block' material. From the relative activities of the preparations from the two bacterial species, although they have the same specificity in general terms, they are not identical in their reactions. This may reflect the role of residues distal to the site of cleavage of the substrate.

Apart from the alginate lyase associated with *A. vinelandii* phage (Davidson *et al.*, 1977), most of the enzymes of this type that have been described are extracellular enzymes secreted by a diverse range of micro-organisms (e.g. Boyd & Turvey, 1978; Hansen *et al.*, 1984; Sutherland & Keen, 1981). As such, the enzymes can probably interact readily with their macromolecular substrates, which are then degraded to provide fragments that can be utilized as carbon and energy sources, feeding into the citric acid cycle as indicated by Preiss & Ashwell (1962). Few of the enzymes have been purified and extensively characterized, exceptions being the polyguluronic-specific lyase from *Klebsiella aerogenes* (Caswell *et al.*, 1989) and the mannuronic-specific lyases from two marine bacteria (Davidson *et al.*, 1976; Romeo & Preston, 1986).

Possible role of the Azotobacter alginate lyase

The alginate lyases described in the present study differ in that they are located in the periplasm where they would not normally be expected to come into contact with their substrate. They cannot function in enabling the bacteria to utilize alginate as an external polymeric source of carbon and energy and indeed the bacteria are unable to grow on alginate. Their role is more likely to be in differentiation and encystment, where very great changes occur in the bacterial surface polymers (Sutherland & Mackenzie, 1977). Alginate with differing composition is found in various locations inside and outside the *Azotobacter* cysts (Sadoff, 1975). However, no change in enzyme specificity was noted following encystment (results not shown). The alginate lyases may play a role in the removal of preformed alginate during differentiation, although our studies show that their activity on the normal highly acetylated EPS is relatively low. Alginate$^-$ strains of *A. vinelandii* fail to encyst, but no alginase$^-$ mutants have yet been reported, so it is not clear whether such mutants are also incapable of encystment or of undergoing some type of morphogenesis.

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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 → 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

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4-α-L-Rha*p*-(1-

(b) Welan gum (S130)

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1-
↑ ↑3
O.CO.CH_3 α-L-Rha*p* or α-L-Man*p*

(c) Rhamsan gum (S194)

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4-α-L-Rha*p*-(1-
↑6
ß-D-Glc*p*(1→6) α-D-Glc*p*

(d) (S657)

-3-ß-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Rha*p*-(1-
↑3
α-L-Rha*p*(1→4)α-L-Rha*p*

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 'sphinganase' 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, recentrifuged 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 H_2SO_4 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, Istituto 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

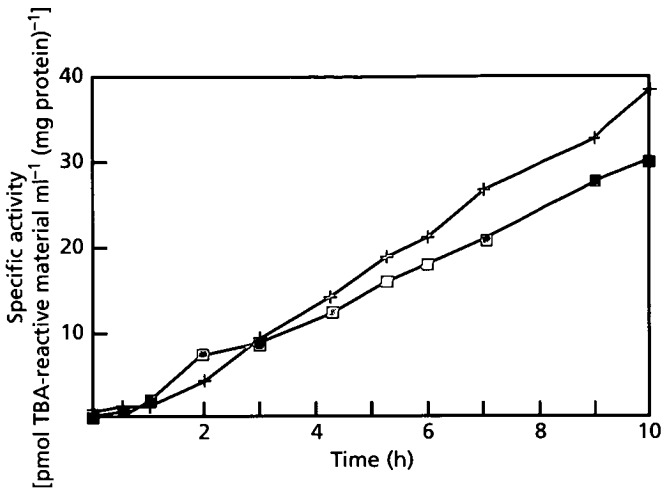

Fig. 2. Lyase digestion of substrates: action on gellan and deacetylated polysaccharide S194. Enzyme from strain 14.1 was used in this experiment: 5 ml substrate (1 mg ml^{-1}) was mixed with 5 ml 10 mM Tris, pH 7·0, and 5 µl enzyme (10 mg protein ml^{-1}) and incubated at 30 °C. Aliquots (500 µl) were 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

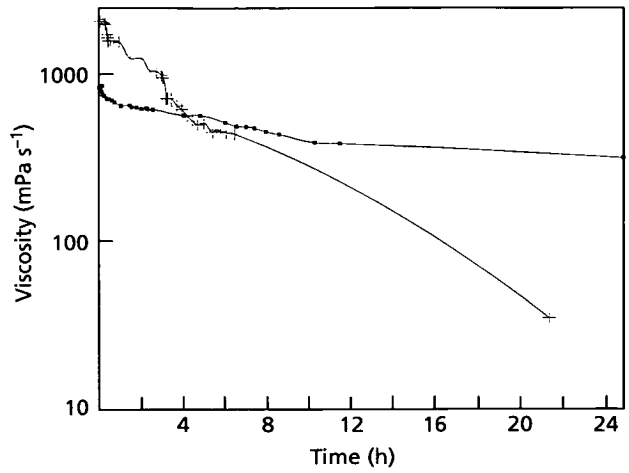

Fig. 3. Effect of gellan lyase on solution viscosity. Polysaccharide (1 ml), at a concentration of 5 mg ml^{-1} for deacetylated S194 or 2·5 mg ml^{-1} for gellan, was mixed with 5 µl enzyme (10 mg protein ml^{-1}) and the viscosity was determined over a period of time in the Brookfield viscometer at 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 (rhamsan gum) 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 rhamsan gum 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

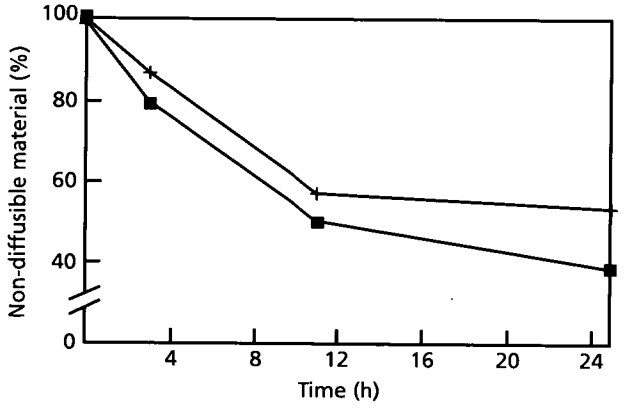

Fig. 4. Recovery of macromolecular material after digestion with gellan lyase. Mixtures of 50 ml polymer solution (0·25%, w/v), 50 ml 10 mM Tris buffer, pH 7·0, 1 ml 2% (w/v) sodium azide and 500 µl enzyme (10 mg protein ml^{-1}) were prepared and incubated at 30 °C. Four flasks of each mixture were 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.

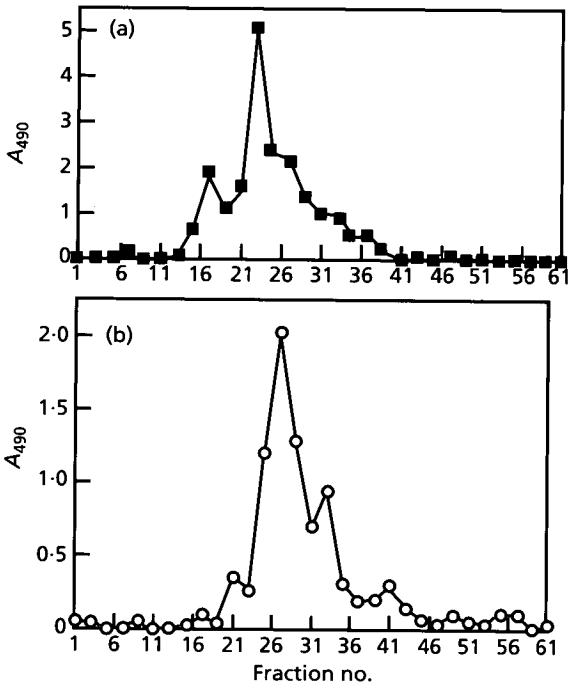

Fig. 5. Chromatography of diffusible products from enzyme digests on Biogel P2. The pooled diffusible material from incubation at 30 °C for 25 h was evaporated to dryness under reduced pressure at 50 °C, redissolved in 1 ml deionized water and applied to the Biogel P2 column (54 × 1 cm). The columns were eluted with water as indicated in Methods and 0·75 ml fractions collected at 10 min intervals. Samples (10–100 µl) 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 deacylated 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 deacylated S194 polymer could be separated by gel permeation chromatography on a Biogel P2 column (54 × 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 deacylated 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 rhamsan. 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 deacylated during commercial production, and on the deacetylated S194 polysaccharide and having little if any activity on either the native or deacylated 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 rhamsan 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 rhamsan. 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 rhamsan 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-aldonoates 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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