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STRUCTURAL STUDIES OF ALGINIC ACID, USING A BACTERIAL POLY-α-L-GULURONATE LYASE*†

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ABSTRACT

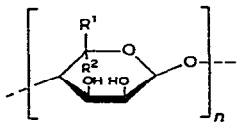
An extracellular poly-α-L-guluronate lyase from *Klebsiella aerogenes* degrades those blocks from alginate which contain both mannuronic and guluronic acid residues (poly-MG blocks) to a mixture of oligosaccharides. From an analysis of these products, it is concluded that poly-MG blocks do not have a strictly alternating sequence of the two uronic acid residues. Enzymic degradation of various samples of algal alginate to leave the poly-M blocks intact has shown that these blocks have a uniform chain-length, estimated at 24 residues.

INTRODUCTION

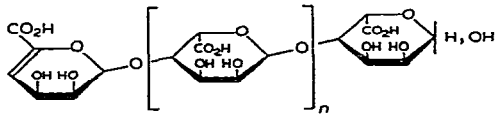
Although it is generally accepted that alginic acid from brown algae consists of a copolymer of three main 1,4-linked structural blocks, poly-β-D-mannuronic acid (poly-M, **1**), poly-α-L-guluronic acid (poly-G, **2**), and blocks in which the two uronic acids occur together (poly-MG)[1,2], detailed information is still lacking on two important points. The first of these is the length of the blocks, and the second is the fine structure of poly-MG blocks. The second point has recently been investigated by both chemical[3] and enzymic methods[4]. In a previous paper[5], we described the isolation, from *Klebsiella aerogenes* type 25, of a poly-α-L-guluronate lyase that degraded poly-G blocks by a random action, to give a mixture of unsaturated oligouronides of general structure **3**, but had no action on poly-M blocks. The enzyme caused degradation of whole alginate to a mixture of products of high and low molecular weight and had extensive action on poly-MG blocks. We have now examined the products of action of the enzyme on poly-MG blocks, from which some structural features of these blocks can be deduced. The action on whole alginate has also been examined, and the large, undegraded fragments have been isolated; from these, the M-block lengths have been estimated.

*Dedicated to Dr. Elizabeth Percival.

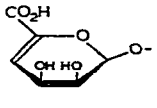
†Studies of Alginate-Degrading Enzymes: Part II. For Part I, see Ref. 5.



1 $R^1 = CO_2H, R^2 = H$
2 $R^1 = H, R^2 = CO_2H$



3 $n = 0, 1, 2$



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For convenience in discussing the structures of various products, the following abbreviations will be used: M– and –M– indicate non-reducing-end and 1,4-linked β-D-mannuronic acid residues; –G and –G– indicate reducing-end and 1,4-linked α-L-guluronic acid residues; and Δ– the unsaturated residue **4**, produced at the non-reducing end of a chain by lyase action.

RESULTS AND DISCUSSION

In a previous paper[5], we found that the alginolyase gave unsaturated di-, tri-, and tetra-saccharides (**3**, $n = 0, 1, 2$) from poly-G, but it was not established whether these were true end-products, or whether further degradation could occur. The action of the enzyme on the oligosaccharides G–G, G–G–G, G–G–G–G, and Δ–G–G–G was investigated. In no case was any appreciable action apparent at an enzyme concentration sufficient to cause extensive degradation of poly-G blocks in less than 1 h. Although not degraded, G–G–G was an inhibitor of enzyme action on poly-G blocks. Table I shows the effect of added trisaccharide on the rate of degradation of poly-G blocks, from which it is concluded that the trisaccharide is probably a competitive inhibitor. The products of enzyme action, established previously, therefore represent true end-products.

TABLE I

INHIBITION OF ENZYME ACTION BY TRIGULURONIC ACID

Substrate conc. (10^{-4}M *guluronate*)	*Rate of increase in absorbance at 230 nm* ($10^{-4}.sec^{-1}$)		
	Inhibitor conc. (10^{-7}M)		
	0	*3*	*6*
0.66	4.60	3.76	2.50
0.99	5.70	4.17	3.25
1.32	6.70	6.17	3.52

Action on poly-MG blocks. — As noted previously[5], poly-MG blocks were readily degraded by the enzyme, to give unsaturated oligosaccharides. Examination of the products by t.l.c. suggested that four components were present, each having an appreciable R_F value consistent with oligosaccharides containing 2–5 units. Gel filtration on a column of Biogel P-4 established that there was no material excluded

by the gel and enabled four fractions (*A–D*, in order of elution) to be isolated. The elution volumes of these fractions were consistent with their containing penta-, tetra-, tri-, and di-saccharides, respectively. In p.c., fractions, *A*, *B*, and *C* migrated as discrete bands, but fraction *D* contained two components, D_1 and D_2 (in approximate ratio 4:1). D_1 had R_{GLCA} 0.52, which is similar to that (0.53) of the disaccharide, Δ–G, isolated previously[5]. Component D_2 had R_{GLCA} 0.31, suggesting that it might be a saturated disaccharide, since a component having a similar mobility has been reported[6], and identified as the diuronide M–M. It was noted previously that gel filtration on Sephadex G-10 separated the disaccharide G–G from salts, but that Δ–G was eluted from this gel admixed with salts. Accordingly, fraction *D* was separated into components D_2, eluted before salts, and D_1, eluted with salts but subsequently desalted on a charcoal column. Fraction D_2 was not detected by absorption at 230 nm, indicating that it did not contain the unsaturated residue **4**, but it was detected by the reagent for uronic acids[7]; fraction D_1 was detected by both methods. Oxidation of each fraction with periodate and subsequent reaction with thiobarbituric acid, to form[8] the chromophore with absorption at 550 nm, confirmed that fractions *A*, *B*, *C*, and D_1 each contained the unsaturated residue, but that D_2 did not.

In order to further characterise some of these oligosaccharides, fractions *C*, D_1, and D_2 were each lactonised, and the products were reduced with sodium borohydride and then hydrolysed with acid[5,9]. The resulting mixtures of aldoses and alditols were examined by electrophoresis in borate buffer, as described previously[5]. Fraction *C* gave glucitol, mannose, and non-migrating material; fraction D_1 gave glucitol and non-migrating material; fraction D_2 gave glucitol and mannose. Fraction D_1, which had a similar R_F value to that of Δ–G, would be expected to give glucitol and degradation products from the unsaturated unit (non-migrating in borate buffer) if it had this structure. The saturated disaccharide D_2, which should give an alditol (from the reducing end) and an aldose, can, therefore, be assigned the structure M–G. It must have arisen from the non-reducing end of the poly-MG blocks, which have a relatively short chain-length. The products from fraction *C* show that this must have contained the trisaccharide Δ–M–G, but the fraction might also contain Δ–G–G, since, after this treatment, internal G-units give 1,6-anhydro-L-gulose (non-migrating in borate buffer). Examination of fraction *C* by ^{13}C-n.m.r. spectroscopy suggested that fraction *C* was a mixture of these two trisaccharides, and the presence of two components was subsequently confirmed by p.c.[10]. Visual estimation of the intensities of the zones in p.c. suggested that Δ–M–G and Δ–G–G were present in the ratio 4:1. Fractions *A* and *B* were not examined further, since it seemed probable that they were mixtures and only small quantities were isolated. The yields of the various oligosaccharides, which were estimated from the areas under the peaks obtained from the Biogel P-4 column and as indicated above, are given as molar ratios in Table II.

Some important conclusions regarding both the specificity of the enzyme and the structure of poly-MG blocks can be drawn from the data in Table II. All of the evidence obtained here and previously[5] places an L-guluronic acid residue at the reducing end of the oligosaccharides, in agreement with the postulate that the enzyme

TABLE II

OLIGOSACCHARIDES FROM ENZYME-DEGRADED POLY-MG BLOCKS

Fraction	*Oligosaccharide*	*Molar ratio*
D_1	Δ–G	8
D_2	M–G	2
C	Δ–M–G	4
	Δ–G–G	1
B	Δ–X–X–G[a]	2
A	Δ–X–X–X–G[a]	2

[a]X may be either an M or G residue.

is specific for the L-guluronosyl linkage, and thus leads to partial structures for fractions *A* and *B* (Table II). The sample of poly-MG blocks used in these experiments contained 55% of mannuronic acid and 45% of guluronic acid. In order to obtain this ratio from the oligosaccharides listed in Table II, it is necessary to assume that some, if not all, of the Δ-residues must have arisen from 4-linked M-residues in the original blocks. Thus, although the enzyme is specific for the L-guluronosyl linkage, the aglycon residue may be either M or G, both of which give rise to the Δ-unit on lysis. An endo-poly-α-L-guluronate lyase from a pseudomonad has recently been shown to have a similar specificity, although it gives different end-products[11]. The structure of poly-MG blocks was originally thought to be a strictly alternating sequence of M- and G-residues, with any homopolymeric sequences present only at chain ends[12]. Simionescu *et al.*[3], on the basis of kinetic studies of the hydrolysis of poly-MG blocks, deduced a more random structure, with –M–M–G– sequences relatively common. Recently, Min *et al.*[4] used an enzymic method and concluded that both –M–M–G– and –G–G–M– sequences are present in poly-MG blocks. Our results support this conclusion in that, even if we assume that all of the Δ-units in Table II arise from M-residues in the original blocks, a minimum of six M–M sequences and one G–G sequence must be present per 53 uronic acid residues. If, as seems probable in view of the isolation of Δ–G–G, some Δ-units have arisen from G-residues, the proportion of homopolymeric sequences is greater, and strictly alternating M–G regions may not be the preponderant structural feature of these blocks.

Action on whole alginates and M-rich blocks. When the enzyme was allowed to act to completion on a number of whole alginates and M-rich fractions from alginate, the products included some unsaturated oligosaccharides, but larger fragments were also present. In view of the complete degradation of both poly-G blocks and poly-MG blocks by the enzyme, it seemed probable that these larger fragments were intact poly-M blocks. Gel filtration of the products on Sephadex G-25 separated a fraction, which was excluded by the gel, from unsaturated oligosaccharides. P.c. of the oligosaccharides indicated components having the mobilities of Δ–G, Δ–G–G, and at least one other component. Gel filtration on columns of Sephadex G-100 was used to estimate the chain lengths of these poly-M blocks (Table III). The columns were

calibrated with Dextrans T-10 and T-20, with long-chain poly-G blocks (c.l., 80) and with short-chain poly-M (c.l., 25).

TABLE III

CHAIN LENGTHS OF POLY-M BLOCKS LEFT AFTER ENZYMIC DEGRADATION OF ALGINATE SAMPLES

Source of alginate	*Chain length (uronic acid residues)*
Commercial sample I	25
Commercial sample II	23
A. nodosum (acid-purified)[a]	23
A. nodosum (Ca^{2+} complex)[a]	24
A. nodosum growing tips	21–30
A. nodosum fruiting bodies	24
Poly-M blocks	23

[a]Samples purified in different ways, see text.

It should be noted that the estimated chain-lengths are not considered absolute, but are strictly relative to one another, and that the values obtained are nearer number-average than weight-average, as the method of detection was by absorption at 230 nm, which gives a measure of the unsaturated residue at the end of each chain.

In addition to two commercial alginate samples (I and II in Table III), alginates extracted from mature *Ascophyllum nodosum* and purified either by acid precipitation or by formation of the calcium complex were examined. Three samples rich in mannuronic acid were examined, one from growing tips of *A. nodosum*[13], which contained 75% of mannuronate, another from fruiting bodies of *A. nodosum*[13], which contained 90% of mannuronate, and a sample of poly-M blocks. Table III shows the remarkable uniformity in the lengths of the poly-M blocks left after enzymic degradation of the other blocks. Only in the case of alginate from growing tips of *A. nodosum* was the elution profile from the column broader than normal, showing greater polydispersity in this fraction. This uniformity in chain lengths of the poly-M blocks suggests a biological control of the length of such blocks. It is known that brown algae contain an epimerase that can convert M-residues into G-residues within the polymeric chain[14], and it is tempting to suggest that, during biosynthesis of alginate in algae, this enzyme comes into play when the poly-M blocks that are being biosynthesized exceed a critical chain-length, estimated by this study to be ~24 units.

EXPERIMENTAL

General methods. — The methods of t.l.c., p.c., and paper electrophoresis in borate buffer have been described[5,10]. The detection of unsaturated oligouronic acids by absorption at 230 nm or with the thiobarbituric acid reagent[8], of total uronic acids[7], and of neutral sugars with the phenol–sulphuric acid reagent[15] have been discussed previously[5]. The procedure for converting uronic acids into aldoses within

oligosaccharides by lactonisation and reduction of the lactones with sodium borohydride, followed by acid hydrolysis, has also been described[5]. Oligosaccharide fractions from columns were desalted either on a column[5] of Sephadex G-10 or, for the unsaturated disaccharide, on charcoal–Celite[16].

Enzyme and substrates. — Preparation of the enzyme from *Klebsiella aerogenes* and determination of its activity have been described[5]. Also described previously are the preparation of polymeric blocks from alginate, of the unsaturated tetrasaccharide Δ–G–G–G[5], and of the saturated oligoguluronic acids[10]. Commercial alginate samples I and II are those described as Samples IV and VII in Ref. 17. Alginate was extracted from whole *Ascophyllum nodosum* (collected in July 1974) by stirring the fronds in 0.2M HCl (5 vol.) for 6 h. The insoluble material was then macerated in 0.2M HCl and stirred overnight. The insoluble material was then extracted with aqueous 3% sodium carbonate for 6 h, and the filtered extract poured into ethanol (3 vol.). The precipitate was collected, and redissolved in water, and the solution was dialysed for 48 h and freeze-dried. The crude alginate was purified either by acid precipitation[13] at pH 2.1 or *via* the calcium complex[18]. Alginates from growing tips of *A. nodosum* (collected in July 1974) and from the intercellular substance of fruiting bodies (collected in early April 1974) were extracted as described before[13].

Action of the enzyme on oligosaccharides. — Each of the oligosaccharides G–G, G–G–G, G–G–G–G, and Δ–G–G–G (10 mg) was separately dissolved in water (1 ml) and incubated at 36° with 0.2M phosphate buffer (pH 7.0, 1 ml) and enzyme solution (1 ml, 40 units) in thermostatted cuvettes (1 cm) in a Unicam SP800 spectrophotometer. The absorbance at 230 nm was plotted for 2 h. The oligosaccharides G–G, G–G–G, and Δ–G–G–G showed no observable increase, whereas G–G–G–G showed an increase of 0.025 during the first 6 min; the absorbance thereafter remained constant. Poly-G blocks incubated under the same conditions showed increases of 0.107 (6 min), 0.272 (16 min), and 1.050 (1 h).

Solutions in 0.05M phosphate buffer (pH 7.0) of trisaccharide G–G–G (1.1 mg per ml) and poly-G blocks (0.24 mg per ml) were mixed in various proportions with enzyme (40 units) in a total volume of 3 ml. The absorbance at 230 nm was plotted as above. The results are given in Table I.

Action on poly-MG blocks. — Poly-MG blocks (150 mg) and sodium chloride (0.5 g) in 0.05M phosphate buffer (pH 7.0, 75 ml) were incubated at 36° with enzyme (900 units) under a layer of toluene. The absorbance at 230 nm was measured on samples (0.2 ml) diluted to 5 ml, until it remained constant (~76 h), and the digest was then freeze-dried. The products were desalted, and then fractionated on Biogel P-4 as described previously[5]. The void volume of the column was 150 ml, and four fractions were eluted: *A* (268–294 ml), *B* (296–320 ml), *C* (322–344 ml), and *D* (350–386 ml). Fractions *A*, *B*, and *C* were desalted on Sephadex G-10, and the four fractions were examined by p.c. Fractions *A* (R_{GLCA} 0.02), *B* (0.06), and *C* (0.17) appeared to be homogeneous, but fraction *D* separated into two components, D_1 (R_{GLCA} 0.52) and D_2 (0.31). Fraction *D* was applied to a column (185 × 1.9 cm) of Sephadex G-10 (swollen in distilled water), and the column was eluted with distilled

water; fractions were monitored by absorption at 230 nm and by the periodate–thiobarbituric acid method. A disaccharide (D_2) eluted at 400–500 ml did not absorb at 230 nm, but was detected by the other method. Disaccharide D_1 (540–610 ml) was eluted with salts and was detected by both methods. The solution was concentrated, and desalted on charcoal–Celite. P.c. indicated that D_1 was pure, but that D_2 contained a trace of D_1. Each oligosaccharide was examined by the periodate–thiobarbituric acid method and, with the exception of D_2, each gave the chromophore absorbing at 550 nm. D_2 gave a slight absorbance at 550 nm (0.06), compared with an equal weight of D_1 which gave an absorbance of 1.07. Oligosaccharides C, D_1, and D_2 were each lactonised, reduced with sodium borohydride, and hydrolysed with acid. The products were examined by paper electrophoresis. Fraction C was also examined by ^{13}C-n.m.r. spectroscopy[10].

Enzymic degradation of alginates. — The alginate (50 mg) and NaCl (0.2 g) were incubated at 35° in 25mM phosphate buffer (pH 7.0, 20 ml) with enzyme (200 units) under a layer of toluene. After 24 h, more enzyme (400 units) was added. Samples (0.5 ml) were withdrawn at intervals, diluted with water (5.5 ml), and monitored at 230 nm. When the absorption was constant (~48 h), showing that enzyme action had ceased, the digest was concentrated at 30° to 2 ml. A small sample was examined by p.c., and the remainder was loaded on a column (2 × 70 cm) of Sephadex G-25, previously equilibrated with aqueous 1% NaCl. Elution was continued with this solvent, and fractions (5 ml) were collected and monitored at 230 nm. On a typical column, material eluted near the void volume of the column (130 ml) was clearly separated from oligosaccharides (210–320 ml). The excluded material (poly-M blocks) was freeze-dried.

A portion (~25%) of the foregoing product was mixed with Procion-orange dextran[19] (5 mg; mol. wt., 2×10^6) and D-mannuronic acid (5 mg) in aqueous 1% NaCl (0.5 ml), and applied to a column (1.5 × 97 cm) of Sephadex G-100 (preswollen and packed in aqueous 1% NaCl). The column was eluted with aqueous 1% NaCl, and fractions (2 ml) were collected. Elution of the orange dextran was monitored by absorption at 490 nm, of poly-M by absorption at 230 nm, and of mannuronic acid by the method for total uronic acid. From the elution volumes of the three peaks, the distribution coefficient, K_d, was calculated[20]. The distribution coefficients of dextran T-10 (mol. wt., 10^4), dextran T-20 (mol. wt., 2×10^4), long-chain poly-G blocks (chain length, 80), and poly-M blocks (chain length, 25) were determined on the same column and used to plot a graph of log (chain length) against K_d. From this graph, the chain lengths of the residual poly-M blocks were calculated.

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