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L-Guluronan-specific alginate lyase from a marine bacterium associated with *Sargassum**

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ABSTRACT

The major extracellular alginate lyase activities secreted by a Gram-negative, facultative bacterium associated with actively growing *Sargassum fluitans* have been resolved an examined for substrate specificity. A fraction excluded from Sephadex G-75 was equally active toward (1→4)-β-D-mannuronan, (1→4)-α-L-guluronan, and alginate with the formation of di- and tri-saccharides as apparent limit products and oligo-saccharides indicative of an endolytic mechanism. A second fraction which was included during G-75 filtration was inactive toward D-mannuronan and 4 times more active toward L-guluronan than native alginate. Proton magnetic resonance spectrometry identified the primary product of this enzyme as *O*-(4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid)-(1→4)-*O*-(α-L-gulopyranosyluronic acid)-(1→4)-*O*-α-L-gulopyranuronic acid. The L-guluronan-specific enzyme requires 0.5M NaCl for maximal activity and has been purified as a monomeric protein having an apparent molecular mass of 38 kD and an approximate pI of 4.5. The predominant formation of trisaccharide over the course of a reaction showed a primarily exolytic mechanism, indicating an enzyme activity unique from any previously reported.

INTRODUCTION

Enzymes capable of degrading alginate have been identified in bacteria[1–14] and marine molluscs[15–17]. These enzymes act as glycosiduronic eliminases generally known as alginate lyases, which introduce a 4,5-double bond in either D-mannuronate or L-guluronate upon cleavage of the (1→4)glycosidic linkage of the alginate polymers. The bacterial alginate lyases which have been purified and evaluated to date show a specificity for either blocks of D-mannuronan[8–10,13,14] or L-guluronan[2–4]. Lyases able to depolymerize the heteropolymeric regions of alginate have also been identified[18,19].

Bacteria associated with actively growing tissues of the pelagic species of *Sargassum*, *S. fluitans* and *S. natans*, have been shown to secrete alginate lyases[11,12]. These bacteria are Gram-negative rods, which are either oxidative and oxidase positive or fermentative and oxidase negative. Different morphologies of each were noted by

* Abbreviations: AL, alginate lyase; h.p.l.c., high-performance liquid chromatography; TBA, 2-thiobarbituric acid; PEI, poly(ethyleneimine); SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; n.m.r., nuclear magnetic resonance.

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scanning electron microscopy. Varying specificities and mechanisms were observed for both intracellular and extracellular enzymes from the different isolates. One of the fermentative isolates secreted a single enzyme specific for endolytic degradation of D-mannuronan and the properties of this purified enzyme have been described[13,14]. A second fermentative isolate, SFFB 080483 ALG-G, was further examined on the basis of secreted activity that showed a marked preference for L-guluronan over D-mannuronan[20]. The ability of this organism to degrade L-guluronan with the accumulation of unsaturated di- and tri-saccharides in the medium prompted an examination of alginate-degrading enzymes secreted by this bacterium. The specificities and mechanisms of the different alginate lyase activities secreted by this organism are reported here.

EXPERIMENTAL

Chemicals and reagents. — Chemicals were analytical or h.p.l.c. grade, except as indicated. Deuterium oxide was 99.9996% isotopically pure (Sigma Chemical Co., St. Louis, MO). Water for all aqueous solutions was deionized and glass distilled.

Substrates. — Sodium alginate was purchased as a purified grade (Fisher Scientific Co.) originally obtained from *Macrocystis*. Homopolymeric block regions of alginate, D-mannuronan, and L-guluronan, and heteropolymeric blocks having a high percentage of alternating sequence, were prepared from HCl-hydrolyzed alginate using methods developed by Haug *et al.*[21] and were fractionated by gel filtration chromatography on Sephadex G-50 with 0.5M NaCl as eluant. Proton and ^{13}C-n.m.r. analysis established that the D-mannuronan preparations contained 10–15% of guluronate and L-guluronan contained $<5\%$ of mannuronate[22]. Total uronic acid equivalents were determined with methods of Blumenkrantz and Asboe-Hansen[23], using D-mannuro-no-3,6-lactone (Sigma Chemical Co.) as the standard.

Growth of bacteria. — Bacteria were grown in PESI (Provasoli's enriched sea water) supplemented[24] with 0.27 mg/L KI, and contained alginate as the only carbon source. Defined colonies of marine bacterial isolate SFFB-080483 ALG-G, which had been maintained on PESI with 1% alginate and 1.5% agar, were transferred to the liquid alginate (0.1%) medium and grown until the optical densities at 600 nm (1-cm cell) were 0.15–0.40. These cultures were transferred to 1 L of medium in Fernback flasks, where they were grown with shaking at 120 r.p.m. on a G-10 Gyrotory Shaker (New Brunswick Scientific) at 22°. For isolation of the extracellular enzyme, cells were harvested at early to mid-log phase.

For comparing growth rates and rate of extracellular enzyme production, 1 L of medium was processed at each time interval. Cells were removed by centrifugation at 12 000*g* for 10 min at 2° in a Beckman J2-21 centrifuge. The supernatant was concentrated and dialyzed by tangential flow filtration using a Millipore Pellicon cassette system with a polysulfone membrane (PTCG), which allows retention of proteins larger than 10 kD. The extracellular medium was concentrated further using an Amicon Ultrafiltration Cell with a PM-10 membrane.

Enzyme purification. — The concentrated extracellular medium was extensively dialyzed against 0.1M sodium phosphate buffer, pH 7.0, using the Amicon PM-10 membrane. This dialyzed concentrate was then treated with polyethyleneimine (PEI; purchased from Sigma Chemical Co., St. Louis, MO). A volume of 15 μL of a 10% solution of PEI was added per mL concentrate. The precipitate was removed by centrifugation at 12 000g for 10 min and the resulting supernatant, which retained the enzyme activity, was applied to a Sephadex G-75 superfine column (2.5 × 104 cm) maintained at 4° in 0.1M sodium phosphate, pH 7.0.

Protein was determined on the column fractions by measuring the absorbance at 280 nm or by using the Coomassie Blue binding-assay of Bradford[25]. For determination of specific activity of fractions, the Lowry method[26] as modified by Peterson[27] was used.

Enzyme assays. — The alginate lyase (AL) activity was quantified by spectrophotometric determination of the chromophore generated upon reaction of thiobarbituric acid (TBA) with periodate-treated products[1] using 3-deoxy-D-*manno*-octulosonic acid (Kdo, Sigma Chemical Co.) as the standard. The enzyme reaction was carried out in 0.025M sodium hydrogenphosphate pH 7.5, 0.5M NaCl, 0.10% sodium alginate, for 10 min at room temperature. When assaying the medium directly for extracellular enzyme activity, corrections for endogenous TBA-reactive material were made by subtracting any 548 nm-absorbing material found when periodate was added to the incubation at zero time. One unit of activity generated 1 nmol of unsaturated termini in 1 min at 22°.

Enzyme activity was also monitored by the increase in absorbance at 232 nm[1,13] in 1.00-cm quartz cuvettes using a Guilford Model 2400 spectrophotometer (Guilford Instrument Laboratories, Inc., Oberlin, OH) with a Honeywell chart recorder (Ft. Washington, PA).

Electrophoresis. — SDS-PAGE was performed according to Laemmli[28], using single-dimension, 1.5-mm thick 10% slab gels. The conditions for analysis and protein staining are described in detail in the Hoefer Scientific Instrument catalog (Hoefer Scientific Instruments, San Francisco, CA).

Isoelectric focusing gels were purchased as 1.0 mm-thick prepared gels, Ampholine PAG plates, pH 3.5–9.5 (LKB) and were run by using an LKB Multiphore system according to instructions provided with the gels. The isoelectric point of the enzyme was determined by comparison with standard proteins (Sigma IEF Standards). Enzyme activity in the gel was determined by sectioning the gel into 1-cm slices which were incubated overnight with 200 μL of alginate substrate as already described. Samples (100 μL) of the solutions were withdrawn and assayed for unsaturated products. Proteins were stained with Coomassie Brilliant Blue R-250.

Analysis of alginate lyase-generated products. — Incubation mixtures were prepared which contained 0.1% D-mannuronan or 0.1% L-guluronan and enzyme fractions recovered from the G-75 Sephadex column. At specified times, the mixtures were automatically sampled and the products evaluated by h.p.l.c. as described previously[22]. Preparative quantities of the lyase products were fractionated by gel filtration on a column (5 × 89 cm) of Bio-Gel P-2 eluted with 0.1M NH_4HCO_3. The products were formed by incubating 15 mL of 0.1% L-guluronan in 0.03M sodium phosphate buffer,

pH 7.5, 0.5M NaCl with 0.75 mL containing 244 units of the AL-2 L-guluronan-specific enzyme for 63 h at room temperature.

Proton n.m.r. spectrometry on the major product from the P-2 column was carried out using a Nicolet NT-300 spectrometer at 300 MHz in the Fourier-transform mode. A sample (25 mg) was dissolved in 0.6 mL D_2O (Sigma, 100 atom %) with 3 mg sodium 4,4-dimethyl-4-silapentanoate-*2,2,3,3-d*$_4$ (TSP). The spectrum, recorded at 45°, is the result of 120 acquisitions using a 90° pulse with a 6-s delay.

RESULTS

Growth of G isolate in liquid alginate cultures. — The increase in turbidity measured at 600 nm was used as a measure of growth. Total uronic acid (520 nm) was determined to evaluate the utilization of alginate by the organism, and TBA-reactive products (548 nm) in the medium were measured. These results are summarized in Fig.

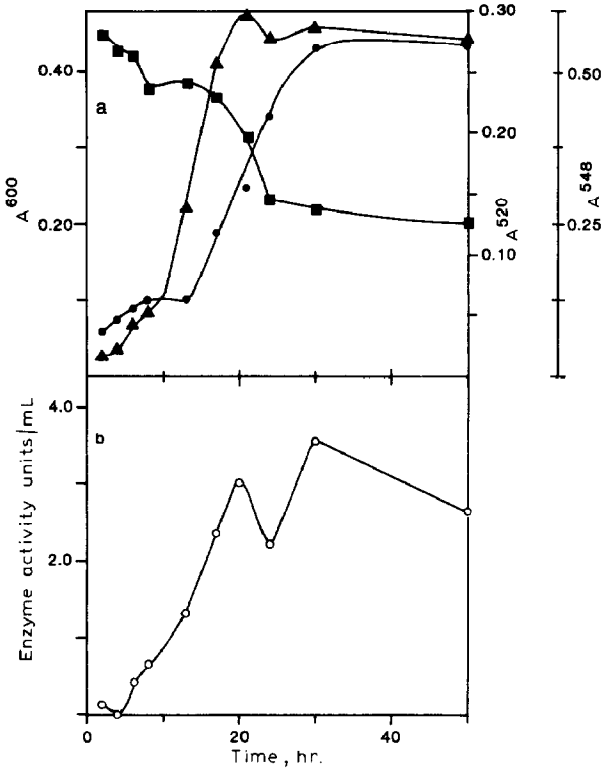

Fig. 1. (a) Growth of ALG-G on alginate. Liquid cultures were assayed at indicated time-points for turbidity (A_{600}, ●) as a measure of growth. Total uronic acid (A_{520}, ■) was determined by the method of Blumenkrantz and Asboe-Hansen[21]. Unsaturated equivalents generated by the lyase reaction were quantified by the periodate-thiobarbituric acid assay (A_{548}, ▲). (b) Appearance of lyase activity. Enzyme activity was measured by quantifying the amount of thiobarbituric acid-reactive products formed in 10 min by fixed amounts of concentrated, dialyzed, extracellular medium. The amount of enzyme activity is reported as that found in 1 mL of the original incubation medium. One unit of activity results in the formation of one nmol of unsaturated termini per min at 22°.

1a. Enzyme activity in the extracellular medium was measured by quantifying the amount of TBA-reactive products formed during 10 min by a fixed amount of concentrated, dialyzed, extracellular medium with 0.1% sodium alginate solution in 0.03M sodium phosphate, pH 7.5 and 0.5M NaCl. The high NaCl concentration was necessary for significant enzyme activity to be measured. The amount of enzyme activity is reported as that found in 1 mL of the original incubation medium, and the appearance of this extracellular activity with time is given in Fig. 1b. The increase in enzyme activity slightly precedes the increase in growth of the organism. Consumption of alginate was incomplete, as indicated by the uronic acid still present in the extracellular media. Significant quantities of TBA-reactive products remain in the medium.

Purification and properties of L-*guluronan lyase.* — After removal of alginate polymers with PEI, the concentrated extracellular AL fraction was subjected to gel filtration on Sephadex G-75. Two principal peaks of enzyme activity were recovered; one eluted with the void volume (AL-1) and contained a large amount of protein; the other was retained (AL-2) and contained a significant amount of enzyme activity. Both fractions showed enhanced activity in the presence of 0.5M NaCl. The purification procedure applied to the enzyme activities in the medium results in a 10-fold increase in specific activity of the G-specific lyase after G-75 Sephadex chromatography (Fig. 2).

Fractions AL-1 and AL-2 were analyzed by SDS-PAGE. Detection with Coomassie Blue (data not shown) indicated the presence of several protein bands in AL-1, ranging from 25 to >60 kD. AL-2 contained a prominent band (>90% of the total stained protein) having an apparent molecular mass of 38 kD, with as many as four minor components.

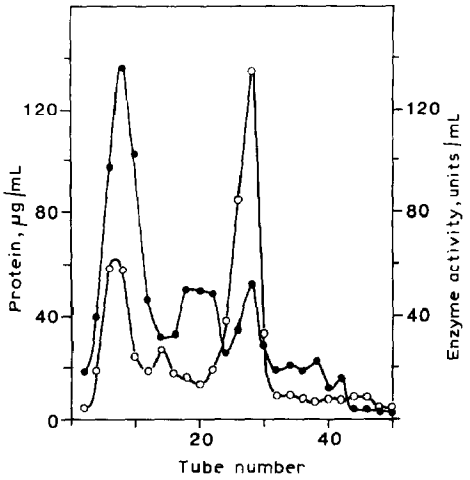

Fig. 2. Elution profile of extracellular alginate lyases from ALG-G on Sephadex G-75. The supernatant solution following PEI treatment was applied to a column of Sephadex G-75 Superfine (2.5 × 104 cm) and eluted with 0.1M sodium phosphate, pH 7.0. Lyase activity is presented as units/mL (○); protein is presented as μg/mL (●). Fractions comprising the peak which was eluted with the void volume were combined as AL-1. Fractions comprising the retained peak, which had a high amount of enzyme activity, were combined as AL-2.

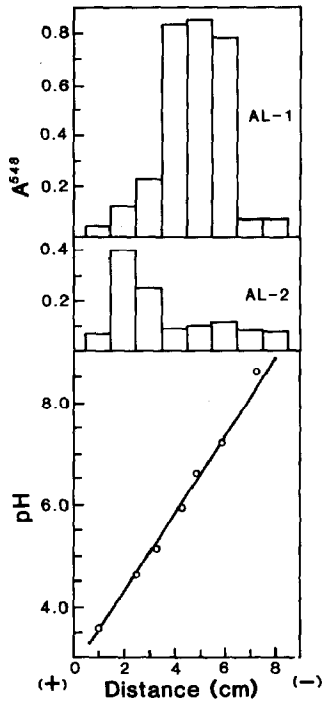

Fig. 3. Isoelectric focusing of AL-1 and AL-2. Fractions from the G-75 Sephadex were subjected to electrophoresis on isoelectric focusing gels, pH 3.5–9.5. Enzyme activity in segments of the gel was determined as described in Material and Methods. The results for AL-1 are shown on the upper panel and for AL-2 in the middle panel. The lower panel defines the pH gradient, with the following standards and their respective pI values: amyloglucosidase, 3.55; trypsin inhibitor, 4.55; beta-lactoglobulin A, 5.13; bovine carbonic anhydrase, 5.65; human carbonic anhydrase, 6.57; myoglobin, 7.16; lactic dehydrogenase, 8.55.

Isoelectric focusing of G-75-Sephadex fractions. — Isoelectric points for the activities were determined by electrophoresis in isoelectric focusing gels. Lyase activities determined in individual gel slices allowed the assignment of pI values relative to those of standard proteins (Fig. 3). AL-1 contained a relatively broad activity peak focusing near neutrality. AL-2 showed a focused activity having a pI of ~4.5, which corresponded to the position of the predominant protein band detected with Coomassie Blue. Measurement of lyase activity of the gel slices indicate that enzyme activity coincides with the protein peaks on the gel.

Substrate specificity of lyase fractions. — The activity of the Sephadex G-75 fractions toward L-guluronan, D-mannuronan, and alginate were compared by evaluating the rate of unsaturated product formation based upon the increase in absorbance at 232 nm. Table I summarizes the results, indicating a marked preference of AL-2 for L-guluronan while AL-1 depolymerizes all three substrates at approximately the same rate. The small amount of absorbance change measured for the action of AL-2 on D-mannuronan may be the result of bonds being broken adjacent to the L-guluronate residues (10–15%) known to contaminate the D-mannuronan substrate.

TABLE I

Comparison of the rate of depolymerization of D-mannuronan, L-guluronan, and alginate by AL-1 and AL-2

Substrate[a]	*Enzyme activity* (*nmol unsaturated termini*[b]*/min/μg protein*[c])	
	AL-1	*AL-2*
Alginate	0.107	0.293
L-Guluronan	0.098	1.120
D-Mannuronan	0.128	0.033

[a] Oligomeric block regions were prepared from partial acid hydrolyzates as described in Materials and Methods. Sodium alginate was from *Macrocystis* (Fisher Scientific Co.). Substrates were present at 0.1% at the start of each reaction. [b] Enzyme activity was determined by measuring changes in absorbance at 232 nm with time. Constant rates were observed over 5 min and reported activities were essentially initial velocities. [c] Protein was determined by the method of Peterson[27].

Product formation by these two lyases was determined by h.p.l.c. Incubation mixtures were prepared and automatically sampled at the indicated times. When AL-2 was incubated with either L-guluronan or native alginate, a single unsaturated product having a retention time corresponding to a guluronic acid tri-saccharide was detected by absorbance at 232 nm. Results are plotted as relative peak area of tri-saccharide product formed (Fig. 4). As in the case of the continuous spectrophotometric assays, the h.p.l.c.

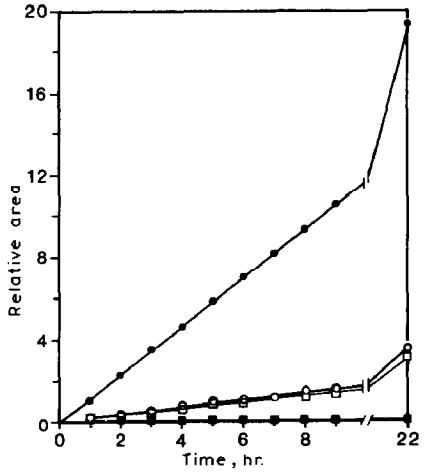

Fig. 4. Product formation determined by h.p.l.c. Incubation mixtures were prepared that contained either 0.1% D-mannuronan or 0.1% L-guluronan. At indicated times the mixture was automatically sampled and evaluated by h.p.l.c. as described in the text. Results are plotted as relative peak area of trisaccharide product formed per mL protein in the enzyme preparation. The retention time of an unsaturated mannuronic acid trisaccharide was 5.30 min, and the retention time of an unsaturated guluronic acid trisaccharide was 5.13 min. Enzyme fraction AL-1 activity was measured with L-guluronan (○) and D-mannuronan (□); AL-2 activity was measured with L-guluronan (●) and D-mannuronan (■).

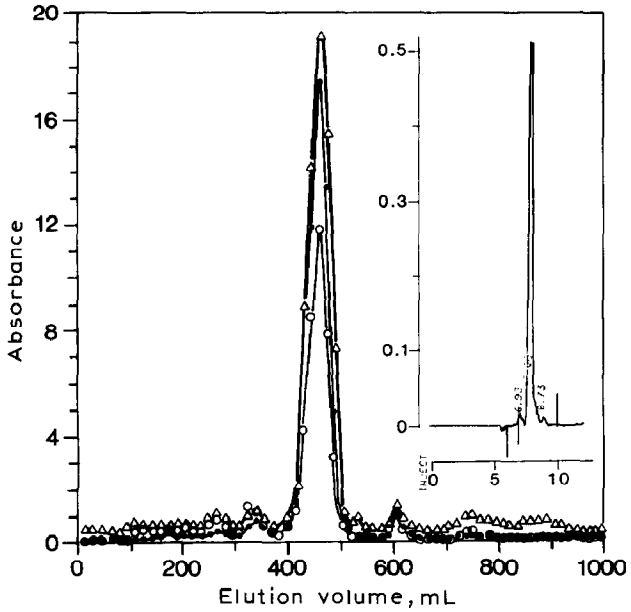

Fig. 5. Purification and h.p.l.c. analysis of limit products. A 15-mL mixture containing 1% L-guluronan in 0.03M sodium phosphate, pH 7.5, 0.5M NaCl was incubated with 280 μg AL-2. The mixture was filtered through a sterile 0.22-μm Millex filter and incubated at room temperature for 63 h. The incubation mixture was then applied to a Bio-Gel P-2 column (5 × 89 cm) and eluted with 0.1M NH_4HCO_3. The elution was monitored for unsaturated termini at 232 nm (●), total uronic at 520 nm (●) and carbonyl groups at 210 nm (△). Fractions from the P-2 column were evaluated by h.p.l.c. on a Zorbax C_{18}-10 μm (9.4 × 250 mm) column developed at room temperature with 10% acetonitrile, 10mM tetrabutylammonium hydroxide, and 0.1M sodium phosphate, pH 6.5. The inset panel is the result given upon analysis of the major peak from the P-2 column.

kinetic assays showed AL-2 to have a marked preference for L-guluronan as a substrate and almost no activity toward D-mannuronan. AL-1 had a similar, lower activity toward both L-guluronan and D-mannuronan. Exhaustive digestion (18 h, 22°) of alginate by AL-2 resulted in the formation of unsaturated guluronic acid trimer constituting 84% of the products detected by h.p.l.c. A small amount of material (7%) preceded the trimer and another small peak (7%) followed the trimer. These peaks had similar, but not identical, retention times as the unsaturated mannuronic acid dimer, trimer, or tetramer which were used as standards.

Purification and characterization of the L-*guluronan-specific enzyme product.* — When the limit product(s) of AL-2 were fractionated on a Bio-Gel P-2 column, a single predominant peak eluted from the column (Fig. 5). Several chromatographic fractions were evaluated by h.p.l.c. The main product was a single component having a retention time of 7.66 min under the conditions of the evaluation (Fig. 5, panel insert).

This predominant P-2 fraction was evaluated by proton n.m.r. spectrometry and the results are presented in Fig. 6. Based upon published shift values[29–33], individual protons were identified as the proton (5.20 p.p.m.) attached to the anomeric carbon

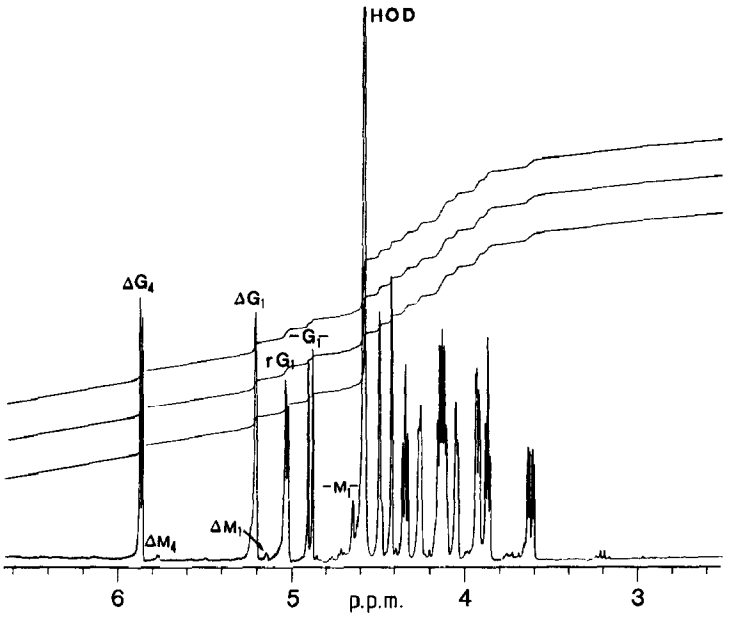

Fig. 6. Proton-n.m.r. analysis of major product formed by the AL-2-catalyzed depolymerization of L-guluronan. The conditions of the analysis are summarized in Table II.

($\triangle G_1$) of the non-reducing terminus, the proton (5.03 p.p.m.) attached to the anomeric carbon (G_1) of an internal residue, and the proton (4.89 p.p.m.) attached to the anomeric carbon (rG_1) at the reducing terminus. Components having shift values of 5.15 and 4.67 p.p.m., for $\triangle M_1$ and M_1, respectively[30], appear to be present in very small amounts. A summary of shift values and relative signal intensities for the guluronic acid product is presented in Table II. The unique proton attached to the unsaturated carbon ($\triangle G_4$) of the non-reducing terminus is a stroichiometric product of the lyase-catalyzed event. Setting this signal to 1.00 and comparing the signals of protons attached to anomeric carbon atoms indicates there are three anomeric carbons in the ratio of one

TABLE II

Proton-n.m.r. analysis of the major product from the AL-2-catalyzed reaction

Chemical shift (p.p.m.)	*Proton position*	*Relative amount*
5.96	H-4	1.00
5.20	Non-reducing H-1	1.00
5.03	Internal H-1	0.90
4.89	Reducing H-1	0.78

The n.m.r. analysis was performed with a Nicolet NT-300 spectrometer at 300 MHz in the Fourier-transform mode. A 25-mg sample of the major limit product purified on Bio-Gel P-2 (Fig. 5) was dissolved in 0.6 mL of D_2O (Sigma, 100 atom %) with 3 mg of sodium 4,4-dimethyl-4-silapentanoate-*2,2,3,3-d*$_4$ (TSP). The spectrum is the result of 120 acquisitions at 45° using a 90° pulse with a 6-sec delay. Chemical shifts and signals were determined from the spectrum shown in Fig. 6.

non-reducing anomeric carbon, one terminal anomeric carbon, and one reducing anomeric carbon. This result is consistent with the structure of a tri-saccharide, *O*-(4-deoxy-α-L-*erythro*-hex-4-enopyranosyluronic acid)-(1→4)-*O*-(α-L-gulopyranosyluronic acid)-(1→4)-*O*-α-L-gulopyranuronic acid, being the primary product generated by the action of this enzyme on an α-L-guluronan.

DISCUSSION

Of several different alginase-secreting bacteria isolated from healthy pelagic *Sargassum* tissues, the ALG-G isolate was unique in its secretion of alginate lyase activities showing a preference for L-guluronan and in its inability to assimilate more than half of the unsaturated products it generated during the eliminative depolymerization of alginate. The depolymerization products accumulated by this bacterium growing on alginate as the sole carbon source included a mixture of dimeric and trimeric unsaturated uronic acid oligosaccharides[20]. The appearance of unsaturated products in the medium paralleled the appearance of the extracellular alginate lyase activity (Fig. 1). Both the levels of the alginate lyase and the accumulated unsaturated products reached a maximum in 20 h and remained constant for the duration of the culture. It would therefore appear that the L-guluronan-preferring lyase activity is responsible for these products, which the bacterium is unable to assimilate, as well as those that are taken up and utilized for growth.

The size fractionation of the extracellular alginate lyases from exponential-phase cultures revealed two different alginate lyase activities, distinguished by both substrate preference and mechanism. The impure larger-molecular-weight fraction included a nearly neutral endolytic activity that showed little or no preference toward D-mannuronan versus L-guluronan versus native alginate. As this fraction contains several protein components, more than one alginate lyase may be present.

The most active extracellular alginate lyase activity was purified by gel filtration to near homogeneity as an L-guluronan-specific enzyme comprised of a single acidic protein subunit having an apparent molecular mass of 38 kD. This activity exhibited a predominantly exolytic mechanism, with the formation of unsaturated trisaccharide throughout the course of the reaction. With L-guluronan as substrate, tri- and disaccharide in the ratio of 12:1 comprised >93% of the limit products detected by h.p.l.c. Proton n.m.r. analysis of the tri-saccharide limit-product established a structure that contained only α-(1→4)-linked resides and which was almost devoid of mannuronate residues. The presence of small signals typical of those found with products generated by D-mannuronan-specific lyase confirms the presence of mannuronic acid in the guluronic acid block-preparations. Previous results indicated that up to 5% of the L-guluronan preparation was mannuronic acid[22]. The ability of this enzyme to depolymerize native alginate suggests the presence of an endolytic activity as well.

L-Guluronan-specific alginate lyases have been characterized from other bacteria. An extracellular endolytic activity which formed di- and tri-saccharide products was isolated from *Klebsiella aerogenes*[4]. An endolytic L-guluronan lyase was purified from a

bacterium isolated from rotting *Fucus* fronds and found to be a monomeric, anionic protein having a molecular weight[8] of 35 kD. While the kinetics of the formation of different products was not monitored as in the case of the AL-2 enzyme under study here, it would appear that the AL-2 enzyme is unique in the nearly exclusive formation of the tri-saccharide throughout the course of the enzyme-catalyzed reaction.

ALG-G will grow on L-guluronan as the sole carbon source and remove substrate in relation to growth without the accumulation of unsaturated oligomers[34]. It would at this point be plausible to expect that the exolytic L-guluronan-specific enzyme, when it generates tri-saccharide devoid of D-mannuronate residues, provides product which is assimilated for growth. The role of this L-guluronan-specific enzyme in the ecological relationship between the producing bacterium and the *Sargassum* host is a matter of further investigation. It is noteworthy that this Gram-negative facultative bacterium has nearly identical properties, including GC fraction of the DNA, general metabolism, and fatty acid composition, with a *Sargassum*-associated isolate (ALG-A) that secretes only an endolytic D-mannuronan-specific lyase[12,35]. It would appear that these bacteria may complement each other with respect to the depolymerization of alginate and the utilization of the lyase-generated products.

The endolytic L-guluronan-specific lyase secreted by *Klebsiella pneumoniae* has been cloned and expressed in *E. coli*[36]. The D-mannuronan-specific lyase from ALG-A has recently been cloned and expressed in *E. coli*[37]. This same approach may now be taken with the ALG-G bacterium to provide quantities of the exolytic L-guluronan-specific enzyme for further study. Collectively, the *Sargassum*-associated alginase-secreting bacteria may constitute an important genetic resource with which to explore relationships between alginate lyase enzyme structure, specificity, and mechanism. In addition they should provide useful reagents for probing the function of alginate in normal and pathogenic relationships, and perhaps for modifying alginate to suit specific industrial applications.

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