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Carbohydrate Research 304 (1997) 125–132

CARBOHYDRATE RESEARCH

Some properties and action mode of (1 → 4)-α-L-guluronan lyase from *Enterobacter cloacae* M-1

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Received 9 January 1997; accepted 7 April 1997

Abstract

An intracellular alginate lyase was purified from *Enterobacter cloacae* M-1 by successive fractionation on Q Sepharose FF, SP Sepharose FF, and Sephacryl S-200 HR. The purified enzyme gave a single band on SDS–PAGE and isoelectric focusing. The enzyme easily degraded polyguluronate and produced unsaturated oligoguluronic acids with a wide range of dp. The major end product of the enzyme reaction on polyguluronate was unsaturated triuronic acid. The pattern of oligoguluronic acids (dp 2–9) generated with the enzyme was investigated by fluorophore-assisted carbohydrate electrophoresis. The enzyme was not capable of degrading oligoguluronic acids having a dp < 4. The degradation rate of heptaguluronic acid by this enzyme remarkably increased, compared with that of hexaguluronic acid, and heptaguluronic acid had a single preferential point of cleavage by this enzyme. On the basis of the cleavage pattern of oligoguluronic acids, the number of subsites was estimated to be seven for this enzyme. The catalytic site of the enzyme is located between the second and the third subsites from the non-reducing end.

Keywords: Alginate lyase; Polyguluronate lyase; *Enterobacter cloacae*; Substrate specificity; Fluorophore-assisted carbohydrate electrophoresis

1. Introduction

Alginate is an acidic polysaccharide composed of (1 → 4)-linked α-L-guluronic acid (GulA) and β-D-mannuronic acid (ManA), arranged in three types of block structures, polyguluronate [poly(GulA)], polymannuronate [poly(ManA)] and hetero-polymeric random sequences [poly(ManA/GulA)] [1].

Alginate lyase has recently attracted the attention, because the enzymatic degradation of alginate expands a potential use of this polysaccharide [2,3].

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0008-6215/97/$17.00
PII S0008-6215(97)00230-9

Additionally, some of the alginate-derived oligouronic acids are gaining biochemical interest due to their physiological activities on plant growth [4,5]. As alginate is a hetero-polysaccharide, the choices of enzyme sources and reaction conditions affect the end products. Thus, the elucidation of the substrate specificities of alginate lyases toward various kinds of oligouronic acids is important for the preparation of desired oligouronic acids by enzymatic degradation of alginate. The alginate-degrading enzymes, polymannuronate lyase [(1 → 4)-β-D-mannuronan lyase, EC 4.2.2.3] and polyguluronate lyase [(1 → 4)-α-L-guluronan lyase, EC 4.2.2.11], cleave the glycosidic linkage of alginate by a β-elimination reaction and produce oligouronic acids with a 4,5-unsaturated uronic acid (4-deoxy-L-*erythro*-hex-4-enopyranuronic acid) residue at the non-reducing end [2]. Many alginate lyases were purified, and their physico–chemical properties were characterized [2]. In such studies, some investigators elucidated the substrate specificities of the enzymes toward each of the three types of blocks, and end-products of the reaction were identified using NMR [6], TLC [7], HPLC [8,9], and paper chromatographic techniques [10,11]. However, little is known of the action mode of alginate lyase toward oligomeric substrates [12,13], because it is difficult to prepare various kinds of oligouronic acids which are used as substrates for such studies. In addition, the analysis of alginate-derived oligouronic acids by the methods described above requires relatively large amounts of such oligosaccharides.

We purified extracellular endo-alginate lyase from *Enterobacter cloacae* M-1 and characterized physico–chemical properties of the enzyme [7]. However, the activity of the enzyme obtained from 8 L of culture supernatant was insufficient to elucidate the substrate specificity of the enzyme, and the purified enzyme was very unstable. During the study described above, we found that *E. cloacae* M-1 produced a high activity of an alginate-degrading enzyme in the cell-free extract. Moreover, we have recently developed a method for the preparation of alginate-derived oligouronic acids [14], and also reported that fluorophore-assisted carbohydrate electrophoresis (FACE) was a useful method for the analysis of oligouronic acids [15].

In this study, therefore, intracellular alginate lyase was purified from *E. cloacae* M-1, and its physico–chemical properties were characterized. In addition, we aimed to clarify the action mode of the enzyme toward various kinds of oligoguluronic acids by using FACE.

2. Experimental

Substrate.—Sodium alginate (Duck Algin 350-M; ManA/GulA ratio 0.94) was prepared at Kibun Food Chemifa. Poly(GulA), poly(ManA/GulA), and poly(ManA) were prepared from sodium alginate by partial acid hydrolysis [1]. Circular dichroism analysis [16] showed that the poly(GulA) contained 79% GulA, poly(ManA/GulA) contained 42% GulA, and the poly(ManA) contained 92% ManA. Authentic oligo(GulA)s (dp 1–9) were prepared from poly(GulA) as described previously [14]. Reduced oligo(GulA)s were prepared by the reduction of each parent oligo(GulA) with $NaBH_4$. After the reduction, reaction products were purified by Q Sepharose FF (Pharmacia, Uppsala, Sweden) column chromatography.

Assay of the enzyme activity.—A reaction mixture consisted of 0.1 mL 1% sodium alginate in 50 mM Tris–HCl buffer (pH 7.5) containing 2 mM $CaCl_2$ and 0.1 mL of enzyme solution. The mixture was incubated at 35 °C for 30 min, and then the reaction was stopped by heating at 100 °C for 5 min. The amount of unsaturated uronic acid liberated into the mixture was colorimetrically measured by the thiobarbituric acid (TBA) method [17]. One unit of enzyme activity was defined as the amount of enzyme required to liberate the equivalent of 1 μmol of unsaturated uronic acid per min [18].

Protein determination.—Protein concentration was measured using a BCA-protein assay kit (Pierce, Rockford, IL) [19] with bovine serum albumin as a standard. Protein in column eluates was routinely monitored by the absorbance at 280 nm.

Preparation of a crude enzyme.—*E. cloacae* M-1 [7] was cultured in a 10-L jar fermenter in a medium (7.2 L) composed of 1% sodium alginate, 0.6% polypeptone, 0.3% yeast extract, 0.5% potassium dihydrogen phosphate, and 0.05% magnesium sulfate heptahydrate. The seed culture (800 mL) was inoculated into the medium, and the cultivation was carried out at 35 °C with agitation at 350 rpm and an air-flow rate of 1.5 L/min.

The culture broth at 12 h was centrifuged at 10,000 × *g* for 20 min to harvest the bacterial cells. Then, the wet cells (63 g) were brought to a volume of 100 mL by addition of 20 mM Tris–HCl buffer (pH 7.5) containing 0.02% NaN_3, and then disrupted with an ultrasonicator (INSONATOR Model 200M, Kubota, Tokyo, Japan) at 9 KHz for 30 min. The homogenate was centrifuged at 10,000 × *g* for 20 min to remove cell debris, and the supernatant was

dialyzed against 20 mM Tris–HCl buffer (pH 8.5). The dialyzed solution was centrifuged at 40,000 × *g* for 60 min, and the supernatant was used as a crude enzyme (intracellular alginate lyase).

Enzyme purification.—A half volume (100 mL including 680 units) of the crude enzyme solution was passed through a Q Sepharose FF (Pharmacia) column (50 × 270 mm ID) equilibrated with the same buffer, and the column was eluted with the same buffer at a flow rate of 87 mL/h. The eluate was fractionated into 14.5-mL portions. The fractions containing alginate lyase activity (tube numbers, 30–38) were pooled, and applied to a SP Sepharose FF (Pharmacia) column (16 × 200 mm ID) equilibrated with the same buffer. The enzyme was eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer (total volume, 280 mL) at a flow rate of 90 mL/h. The eluate was fractionated into 2.9-mL portions. The active fractions (tube numbers, 43–48) were combined, and concentrated to 5 mL by ultrafiltration with a YM-3 membrane (Amicon, Beverly, MA). Then, the concentrate was dialyzed against 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl. The dialyzed enzyme solution was applied to a Sephacryl S-200 HR (Pharmacia) column (32 × 700 mm ID) equilibrated and eluted with the same buffer, at a flow rate of 30.8 mL/h. The eluate was fractionated into 5.1-mL portions. The active fractions (tube numbers, 60–65) were collected, and the purity was examined by SDS–PAGE and IEF.

SDS – PAGE and IEF.—SDS–PAGE was performed on a 12.5% polyacrylamide gel by the method of Laemmli [20]. For the determination of molecular weights, a molecular weight standards kit (SDS–PAGE, low range, Bio-Rad, Hercules, CA) was used. Proteins in the gel were stained with Coomassie brilliant blue (CBB) R-250. IEF was performed by using Ampholine PAG plate pH 3.5–9.5 (Pharmacia) as described in the Pharmacia laboratory manual. An IEF calibration kit (broad pI kit, pH 3–10, Pharmacia) was used for the estimation of the pI value. Proteins were stained with CBB R-250.

HPLC.—The molecular weight of the purified enzyme was estimated by gel filtration on a Ultrospherogel SEC 3000 column (75 × 300 mm ID, Beckman, Fullerton, CA) equilibrated with 50 mM sodium phosphate buffer (pH 7.0), containing 150 mM NaCl. For molecular weight determinations, gel filtration calibration kits (high- and low-molecular weight kits, Pharmacia) were used.

N-terminal amino acid sequence.—The N-terminal amino acid sequence of the enzyme was analyzed by Edman degradation with an automated protein sequencer (model 477A; Applied Biosystems, Foster City, CA) after the protein had been blotted on a PVDF membrane (Millipore, Bedford, MA).

Substrate specificity.—The purified enzyme was incubated with poly(ManA), poly(ManA/GulA), and poly(GulA) to elucidate the substrate specificity. In each case the reaction mixture was composed of an equal portion of a 1% solution of substrate in McIlvaine buffer (buffer system consisting of 0.1 M citric acid–0.2 M sodium monohydrogen phosphate, pH 8.0) and the enzyme solution (1 unit/mL). The enzyme reaction was carried out at 30 °C. A small portion of the reaction mixture was taken at the desired time, and then heated at 100 °C for 5 min. Unsaturated uronic acids in the reaction mixture were measured by the TBA method, and the sugar composition of each mixture was analyzed by TLC.

Mode of action.—Oligo(GulA)s were subjected to enzymatic degradation to analyze the cleavage pattern of the purified alginate lyase. The reaction mixture consisted of an equal portion of oligo(GulA) (4 mM, dp 2–9) and the enzyme solution in McIlvaine buffer (pH 8.0). The enzyme reaction was carried out at 30 °C. Five μL of the reaction mixture was taken at the desired time, and the enzyme reaction was immediately stopped by adding 100 μL EtOH. The mixture was dried by a centrifugal vacuum evaporator, and the sugar composition in the mixture was analyzed by FACE.

TLC.—TLC was performed on a plate of Silica Gel 60 (Merck, Darmstadt, Germany) with 4:6:1 1-butanol–formic acid–water. The sugars on the plate were detected by heating at 150 °C with conc. H_2SO_4, or by heating at 100 °C with the TBA reagent [21].

FACE.—Oligouronic acids were labelled with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS, Molecular Probes, OR) and analyzed by polyacrylamide gel electrophoresis on a 30–40% acrylamide gel as described in our previous paper [15].

3. Results and discussion

Purification of endo-alginate lyase from E. cloacae M-1.—The results of the enzyme purification are summarized in Table 1. Through these procedures, the intracellular alginate lyase of *E. cloacae* M-1 was purified 107-fold from the crude enzyme solution with a yield of 28%. The purified enzyme was subjected to both SDS–PAGE and IEF analyses. The

Table 1
Summary of the purification of *endo*-alginate lyase from *E. cloacae* M-1

Steps	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude enzyme	1732	680	0.4	100	1
Q Sepharose FF	25.7	266	10.4	39	26
SP Sepharose FF	6.8	214	31.5	31	79
Sephacryl S-200	4.5	193	42.9	28	107

purified enzyme gave a single band on SDS–PAGE (Fig. 1A) and isoelectric focusing (Fig. 1B).

Properties of the purified alginate lyase.—The molecular mass of the purified enzyme was 39,000 Da, as estimated by SDS–PAGE (Fig. 1A), and 31,000 Da, as estimated by gel filtration (data not shown). These results indicate that the native alginate lyase existed as a single polypeptide chain. The pI of the enzyme was 8.9 (Fig. 1B). This value is largely in agreement with pIs of intracellular and extracellular polyguluronate lyases from *Klebsiella aerogenus* [22,23] and extracellular alginate lyase from *E. cloacae* [7].

The optimum pH was 7.5 at 35 °C with McIlvaine buffer (pH 4 to 8), 50 mM Tris–HCl (pH 7 to 9), and Atkins–Pantin (buffer system consisting of 0.2 M boric acid containing 0.2 M KCl–0.2 M Na_2CO_3, pH 7.5 to 11). The optimum temperature was 40 °C in 50 mM Tris–HCl (pH 7.5). The enzyme was stable between pH 6 to 9 and up to 20 °C. The buffer

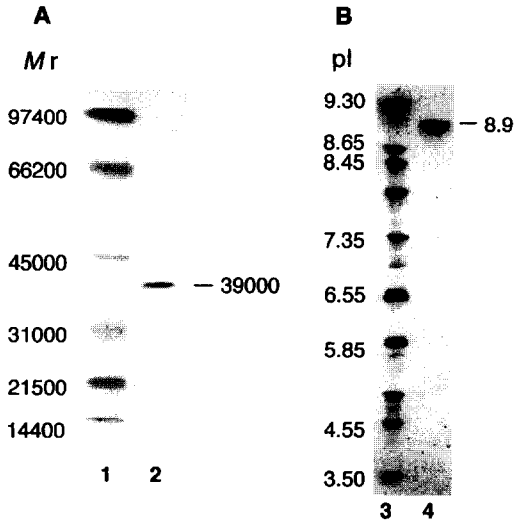

Fig. 1. SDS–PAGE (A) and IEF (B) of purified alginate lyase from *E. cloacae* M-1. The protein was stained with Coomassie brilliant blue R-250. Lane 1, molecular mass marker proteins (SDS–PAGE standard low, Bio-Rad); lanes 2 and 4, purified intracellular alginate lyase of *E. cloacae* M-1; lane 3, pI marker proteins (pI calibration kit 3-10, Pharmacia).

composition turned out to have an effect on activity and stability of the enzyme, and the enzyme indicated the highest activity in Tris–HCl buffer. However, the enzyme was unstable when the enzyme was stored in 50 mM Tris–HCl buffer.

The sequence of the 20 amino acids at the N-terminal site of the purified enzyme was A–V–P–A–P–G–D–K–F–E–L–S–G–W–S–L–S–V–P–V–, and this sequence was completely identical with that of *K. pneumoniae* [24].

The properties of the purified enzyme were quite similar to those of the extracellular alginate lyase from *E. cloacae* [7]. However, the intracellular enzyme was slightly more stable than the extracellular enzyme, and the optimum temperature of the intracellular enzyme (40 °C) was higher than that of the extracellular enzyme (30 °C). Moreover, the intracellular enzyme activity was obtained about 300 times higher than the extracellular enzyme activity from 8 L of culture broth. Hence, we used the intracellular enzyme for further studies on the substrate specificity and mode of action of the alginate lyase.

Substrate specificity of the purified enzyme.—The enzyme predominantly attacked poly(GulA) and poly(ManA/GulA), and hardly attacked poly(ManA). Especially, the enzyme rapidly degraded poly(GulA), and produced oligouronic acids with a wide range of dp. Oligouronic acids having a dp > 5 were further degraded, and unsaturated triuronic acid was accumulated as the major end-product [Fig. 2, poly(GulA)]. These results suggest that the intracellular alginate lyase was a kind of polyguluronate lyase [(1 → 4)-α-L-guluronan lyase, EC 4.2.2.11], and the specificity of the enzyme was similar to those of several other polyguluronate lyases, which also produced unsaturated triuronic acids as the major end-product from poly(GulA) [2,7,23,25,26].

Mode of action of the enzyme toward oligoguluronic acids.—The action pattern of the purified polyguluronate lyase was examined using oligo(GulA)s (dp 2–9) as substrates. The reaction mixture (100 μL) containing each oligo(GulA) (final concn. 2 mM), and 0.02 units of the alginate lyase

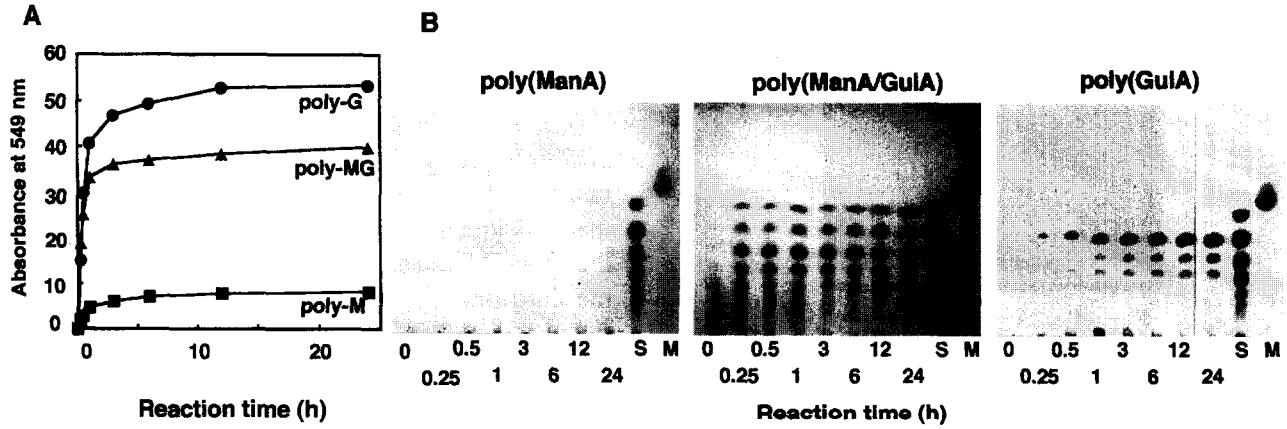

Fig. 2. Course of enzyme reaction on poly(ManA), poly(ManA/GulA), and poly(GulA) analyzed by the TBA method (A) and by TLC (B). The sugars on plates were detected with H_2SO_4. S, unsaturated diuronic acid to unsaturated heptauronic acid from top to bottom; M, mannuronic acid lactone.

was incubated for 6 h at 30 °C, and the products in the mixture were analyzed by FACE.

Fig. 3 shows a FACE analysis of the reaction products. The enzyme did not degrade oligo(GulA)s having a dp < 4 (data not shown) as effective as the polyguluronate lyase from *K. aerogenus* [23]. On the other hand, our enzyme degraded oligo(GulA)s having a dp 5–9, and gave several kinds of oligouronic acids. Penta(GulA) was degraded to di- and triuronic acids (lane 1 in Fig. 3), and hexa(GulA) was degraded to di- and tetrauronic acids or triuronic acids (lane 2 in Fig. 3). Furthermore, the enzyme rapidly degraded hepta-, octa-, and nona(GulA)s and gave di- and triuronic acids as end products (lanes 3–5 in Fig. 3). These results suggest that the reaction rate increased depending on the dp of substrate as reported for polymannuronate lyase from *Turbo cornutus* [12].

Next, the enzyme reaction was investigated to clarify the cleavage site of the oligo(GulA)s. Fig. 4 shows a TLC of the enzymatic degradation products of penta- and hexa(GulA)s. The enzyme produced unsaturated triuronic acid from penta(GulA) (lane 1 in Fig. 4), and unsaturated tri- and tetrauronic acids from hexa(GulA) (lane 2 in Fig. 4). As polyguluronate lyase cleaves α-(1 → 4)-gulosyluronic acid linkages and produces oligouronic acids having unsaturated uronic acid residues at the non-reducing end, unsaturated triuronic acid and unsaturated tri- and tetrauronic acids were generated at the reducing end side of penta(GulA) and hexa(GulA), respectively. On the other hand, reduced penta- and

Fig. 3. FACE analysis of degradation products of oligo(GulA) by *E. cloacae* M-1 polyguluronate lyase. The reaction was carried out for 6 h with 0.02 units of the enzyme in 100 μL reaction mixture containing 2 mM oligo(GulA). Lane S, authentic GulA to nona(GulA) from bottom to top (each 2 nmol per lane); lanes 1–5, enzymatic degradation products of penta(GulA) to nona(GulA).

Fig. 4. TLC of degradation products of penta(GulA) and hexa(GulA) by *E. cloacae* M-1 polyguluronate lyase. Unsaturated sugars were detected with the TBA reagent. Lane S, authentic unsaturated di(GulA) to unsaturated tetra(GulA) from top to bottom; lane 1, reaction products of penta(GulA); lane 2, reaction products of hexa(GulA).

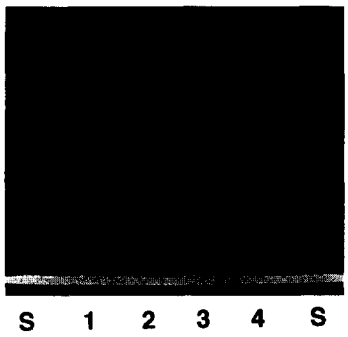

Fig. 5. FACE analysis of degradation products of reduced penta(GulA) and reduced hexa(GulA). Lane S, authentic GulA to nona(GulA) from bottom to top; lane 1, reduced penta(GulA); lane 2, degradation products of reduced penta(GulA); lane 3, reduced hexa(GulA); lane 4, degradation products of reduced hexa(GulA).

hexa(GulA)s were also subjected to enzymatic degradation under the same conditions described above. Fig. 5 shows a FACE analysis of the reaction products with the purified alginate lyase. As is evident from Fig. 5, diuronic acid was generated from reduced penta(GulA) (lane 2 in Fig. 5), and di- and triuronic acids were generated from reduced hexa(GulA) (lane 4 in Fig. 5). It is clear that these oligouronic acids were generated from the non-reducing end side of the substrates, because ANTS reacts with the reducing end of oligouronic acids. Thus, these results suggest that the purified polyguluronate lyase cleaved the second glycosidic linkage from the non-reducing end of penta(GulA). In addition, the enzyme cleaved the second or the third glycosidic linkage from the non-reducing end of hexa(GulA) with a preference for the second linkage. A linear response was obtained between the intensity and quantity of oligouronic acids in FACE analysis [15], and both di- and tetrauronic acids appeared to have almost the same band intensity as that of triuronic acid in FACE analysis (lane 2 in Fig. 3). As 1 mol of hexa(GulA) afforded 2 mol of triuronic acid by enzymatic degradation, the bond cleavage frequency of the second linkage was two times higher than that of the third linkage. In the case of reduced hexa(GulA), the purified enzyme cleaved the second and the third glycosidic linkage from the non-reducing end at the same level (lane 4 in Fig. 5). This phenomenon suggests that the structural change of the reducing end of hexa(GulA) changed the bond cleavage frequency.

To analyze the initial products of the enzymatic degradation of oligo(GulA) having high dp, both parent and reduced hepta-, octa-, and nona(GulA)s were incubated with the dilute enzyme solution. The reaction mixture (100 μL) containing each oligo(GulA) (final concn. 2 mM) and 0.002 units of the alginate lyase was incubated for 15 min at 30 °C, and the products in the mixture were analyzed. FACE analysis of the initial products of the enzymatic degradation of each oligo(GulA) are shown in Fig. 6. Di- and pentauronic acids were produced from hepta(GulA) (lane 2 in Fig. 6A), and only diuronic acid was produced from reduced hepta(GulA) (lane 8 in Fig. 6B). Thus, these results indicate that the enzyme cleaved the second glycosidic linkage from the non-reducing end of hepta(GulA). By comparing the cleavage pattern of Fig. 6A and 6B, it can be deduced that the enzyme cleaved the second or the

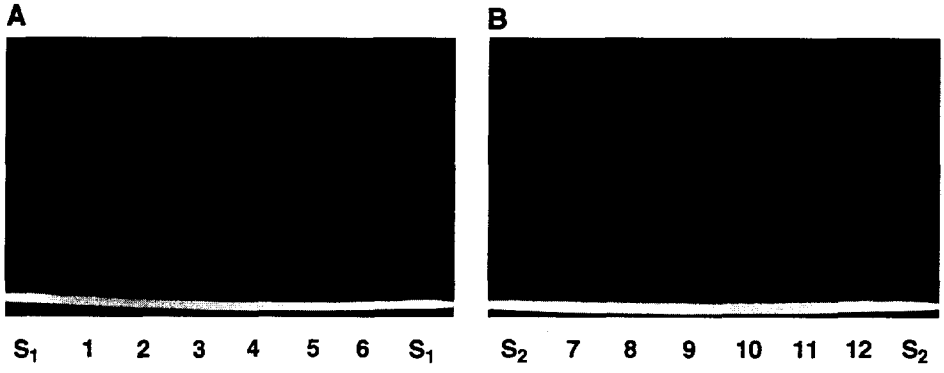

Fig. 6. FACE analysis of degradation products of oligo(GulA) with high dp by *E. cloacae* M-1 polyguluronate lyase. The reaction was carried out for 15 min with 0.002 units of the enzyme in 100 μL reaction mixture containing 2 mM substrate. (A) degradation products of parent hepta(GulA), octa(GulA), and nona(GulA); (B) degradation products of reduced hepta(GulA), octa(GulA), and nona(GulA); lane S_1, acid hydrolysate of poly(GulA); lane S_2, authentic GulA to nona(GulA) from bottom to top; lane 1, hepta(GulA), lane 2, degradation products of hepta(GulA); lane 3, octa(GulA); lane 4, degradation products of octa(GulA); lane 5, nona(GulA); lane 6, degradation products of nona(GulA); lane 7, reduced hepta(GulA), lane 8, degradation products of reduced hepta(GulA); lane 9, reduced octa(GulA); lane 10, degradation products of reduced octa(GulA); lane 11, reduced nona(GulA); lane 12, degradation products of reduced nona(GulA).

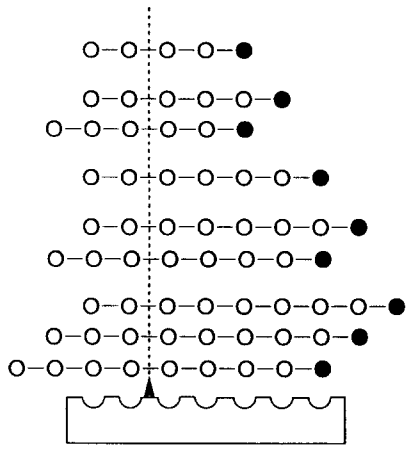

Fig. 7. Possible binding and degradation mechanisms of oligo(GulA) by polyguluronate lyase from *E. cloacae* M-1. An arrowhead indicates the position of the catalytic site. ○, α-(1 → 4)-linked L-guluronic acid residue; ●, reducing end of L-guluronic acid residue.

third glycosidic linkage of octa(GulA). In the case of nona(GulA), several oligouronic acids (dp 2–7) were produced from parent nona(GulA), and di- and triuronic acids were produced from reduced nona(GulA). However, the amount of tetrauronic acid from reduced nona(GulA) was much lower than that from parent nona(GulA). This also may be due to the structural change of the reducing end of nona(GulA). The enzyme, therefore, cleaved the second, the third, or the fourth glycosidic linkage from the non-reducing end of nona(GulA).

Earlier, Thoma et al. presented the subsite profile of the active site of *Bacillus subtilis* α-amylase by the analysis of product patterns [27]. We used the same concept for the interpretation of the degradation reaction of the polyguluronate lyase acting on oligo(GulA). Possible binding and degradation mechanisms of oligo(GulA) by polyguluronate lyase from *E. cloacae* M-1 are summarized in Fig. 7. The enzyme did not degrade oligo(GulA)s with a dp ≤ 4, and degraded penta(GulA) to dimer and unsaturated trimer. However, the result of five subsites can not explain the cleavage pattern of hexa(GulA) and higher oligo(GulA)s. For instance, hexaguluronic acid was cleaved at two positions, the second and the third glycosidic linkage from the non-reducing end, and hepta(GulA) had a single preferential site of cleavage by the enzyme. Additionally, the degradation rate of hepta(GulA) by this enzyme remarkably increased, compared with that of hexa(GulA). So, the number of subsites was estimated to be seven for this enzyme. The catalytic site of the enzyme is located between the second and the third subsites from the non-reducing end.

Some investigators have reported that the presence of a terminal unsaturated uronic acid residue remarkably affected the degradation rate of polygalacturonate lyase [28,29]. So, further studies are in progress to elucidate the mode of action of this enzyme toward a series of unsaturated oligo(GulA)s.

Acknowledgements

We are thankful to Dr. T. Ishii, National Forestry and Forest Products Research Institute, for the FABMS analysis of oligouronic acids. We thank Dr. T. Ozawa of the Institute of Applied Biochemistry, University of Tsukuba, for the circular dichroism analysis of alginate. This work was supported, in part, by Grants-in-Aid (06760070) from the Ministry of Education, Science and Culture, Japan.

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