A Mode of Action of Alginate Lyase from *Turbo cornutus* on Sodium Alginate[†]

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Alginate lyase from *Turbo cornutus* acted on sodium alginate and resulted in a rapid decrease in viscosity and a gradual increase in reducing power of the substrate solution. The result of gel filtration of the reaction products indicated that oligouronides were liberated as the final product. The fact that the enzyme attacked preferentially on the mannuronate-rich moieties of alginate molecule was demonstrated by an analysis with paper electrophoresis of the reaction products after acid treatment, which separated the oligouronides from the polyuronides.

It has been demonstrated that alginic acid is a co-polymer of D-mannuronic acid and L-guluronic acid.¹⁾ Atkins *et al.*²⁾ have revealed that D-mannuronic acid residues are β -linked in the CI conformation and L-guluronic acid residues are α -linked in the IC conformation.

Alginate-degrading enzymes from different sources have been studied by many investigators. Nakada and Sweeny³⁾ isolated two types of the enzymes from abalone hepatopancreas, which were designated as alginase I and II. The former appeared to be specific for D-mannuronic acid and the latter for L-guluronic acid. Nisizawa *et al.*⁴⁾ reported two enzyme components from marine mollusc, which showed a high activity toward mannuronides. The enzymes from bacterial origin, pseudomonad, specific for guluronides have also been described by Davidson *et al.*⁵⁾ and Min *et al.*⁶⁾ More recently, Boyd and Turvey⁷⁾ isolated guluronate lyase from *Klebsiella aerogenes*.

In the previous paper, Muramatsu *et al.*⁸⁾ purified alginate lyase [EC 4.2.2.3] from the mid-gut gland of *Turbo cornutus* and disclosed the enzymic properties. The present study deals with a mode of degradation of alginate by this enzyme.

MATERIALS AND METHODS

Materials. Sodium alginate and propylene glycol alginate were obtained from Wako Pure Chemical Industries Ltd. and Tokyo Kasei Industries Co., respectively, and used without further purification. Sephadex G-15 was a product of Pharmacia. The purified alginate lyase was prepared as described previously⁸⁾ except that the lyophilization was replaced by ultrafiltration on a Diaflo membrane UM 10.

Assay of lyase activity and viscosity measurement. Alginate lyase activity and viscosity of the reaction mixtures were conducted according to the methods described in the previous paper.⁸⁾

Measurement of reducing power. Reducing power in the reaction mixture was determined according to the method of Somogyi⁹⁾ and expressed as equivalent of mannose.

Acid hydrolysis of sodium alginate. The procedure described by Haug and Larsen¹⁰⁾ was applied on intact or enzymatically degraded alginate.

Paper electrophoresis. Paper electrophoresis as described by Haug and Larsen¹¹⁾ was used for qualitative analysis and separation of the uronides produced by enzymic degradation of alginate. In a run, two paper strips (Toyo filter paper No. 51A, 3.5×40 cm) were used in a Toyo paper electrophoretic apparatus PE2 at a voltage of 17~20/cm for 2 hr at room temperature. The buffer used was 10 mM borax solution containing 5 mM calcium chloride. Location of uronides was determined by dipping the paper in a 2.5% aniline trichloroacetate-ethylacetate solution.¹²⁾

Enzymic degradation of alginate and propylene glycol alginate. The reaction mixture which contained 2 ml

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of 0.2% sodium alginate (0.26% of propylene glycol alginate) in 50 mM Tris-HCl buffer, pH 8.0 and 0.1 ml of the enzyme was incubated at 30°C for 120 min. For prolonged incubation (22 and 44 hr), another reaction mixture having the same composition was incubated in the presence of toluene. At 22 hr, an equal amount of the fresh enzyme was added to 1 ml of the residual mixture, and the reaction was allowed to proceed for another period of 22 hr. The reaction was terminated by heating the mixture in a boiling water bath for 3 min. An aliquot of 0.5 ml of the mixture was loaded onto a column of Sephadex G-15 and eluted with 50 mM phosphate buffer, pH 7.0, at a flow rate of 8 ml per hour. The elution position of mono-uronide from the column was obtained by eluting separately either mannose or glucuronic acid as standard in the same condition.

Determination of uronides, unsaturated uronides and mannose. The uronides and unsaturated uronides, which were eluted from the column, were determined by measuring absorbance at 530 nm in the carbazole reaction as modified by Bitter and Muir¹³⁾ and by absorbance at 235 nm,⁸⁾ respectively. Mannose as standard from the column was detected by phenol-sulfuric method.¹⁴⁾

Preparation of enzymatically degraded alginate for paper electrophoresis. Sodium alginate (100 mg) was dissolved in 5 ml of 10 mM phosphate buffer, pH 8.3. Insoluble materials were removed by centrifugation. To the supernatant, 2 ml of the enzyme solution (1.3 units in the same buffer) were added and the reaction mixture was incubated in the presence of toluene at 30°C for 22 hr. An equal amount of the enzyme was added at each time interval of 22 hr. The reaction was terminated by heating the mixture in a boiling water bath for 3 min, and the mixture was cooled in tap water. By dropwise addition of dilute sulfuric acid, the pH of the reaction mixture was brought to 1.5. The resulting precipitate was separated by centrifugation, washed with dilute sulfuric acid (pH 1.5), followed by ethanol, and dried *in vacuo* (acid insoluble). A part of this dried material was used for the gel filtration which was performed as described above at a flow rate of 18 ml per hour. Another part was hydrolyzed with sulfuric acid and analysed by paper electrophoresis as described above. The supernatant separated from the precipitate was neutralized with solid calcium carbonate and centrifuged. The "gypsum" was rinsed with a small amount of water and centrifuged. The combined clear supernatant was evaporated *in vacuo*. The dried material was dissolved in water, and a small amount of insoluble material was removed by centrifugation (acid soluble). A portion of this solution was chromatographed on Sephadex G-15. Another portion was dried *in vacuo* and then hydrolyzed with sulfuric acid,

as described above, to examine uronic acid by paper electrophoresis.

RESULTS

Changes in viscosity and reducing power

Degradation of alginate by the enzyme is illustrated in Fig. 1. The initial viscosity decreased by seventy percent within 5 min after initiation of the reaction. After 30 min, further decrease in the viscosity was observed. The reducing end groups increased gradually with increased incubation time up to about 2.5 hr. A degree of 9% of maximum reducing power was attained at this stage and 15% at 22 hr. These observations indicate that the enzyme acts on alginate at random, and the resulting oligouronides and short chain polyuronides are not easily attacked by the enzyme.

FIG. 1. Degradation of Alginate by the Enzyme as Reflected by Changes in Viscosity and Reducing Power.

(A) The reaction mixture contained 10 ml of 0.2% alginate in 50 mM Tris-HCl buffer, pH 8.0 and 0.2 ml of the enzyme. (B) The reaction mixture (20.2 ml) had the same composition as in (A). An aliquot of 2 ml was withdrawn at each time interval and tested for reducing power.

Gel filtration of reaction products

The reaction products obtained at different reaction stages were loaded on top of a column

FIG. 2. Gel Filtration of the Reaction Products on a Sephadex G-15 Column (1.5 × 87 cm).

The reaction mixture was incubated for 120 min (O—O), 22 hr (O—O) and 44 hr (●—●). Elution was performed with 50 mM phosphate buffer, pH 7.0, at a flow rate of 8 ml/hr. The abbreviation *Ve* represents the elution volume of the mannose as standard. x—x; intact alginate solution.

FIG. 3. Gel Filtration of Propylene Glycol Alginate Incubated with the Enzyme on a Sephadex G-15 Column (1.5 × 87 cm).

The reaction mixture was incubated for 44 hr (●—●). Elution was performed as described in Fig. 2. The abbreviation *Ve* is the same as in Fig. 2. x—x; intact propylene glycol alginate solution.

of Sephadex G-15 and eluted as shown in Fig. 2. Alginate, which was not treated with the enzyme, gave a viscous solution and showed a relatively broad peak. Upon digestion with the enzyme, this peak turned narrow and a couple of small peaks appeared on prolonged incubation, indicating production of the oliguronides. Any compounds corresponding to monouronide were not observed on the chromatograms. With propylene glycol alginate, as shown in Fig. 3, only a trace

amount of oliguronides was detected.

Paper electrophoresis of acid hydrolysate of intact and enzymatically degraded alginate

A mixture of the acid hydrolysate of intact alginate and glucuronic acid as a standard was applied on the paper strip and the electrophoresis was carried out as described in the text (Fig. 4A). From the electrophoretic pattern which showed three bands, the slowest moving band could be assigned to guluronic acid, the fastest moving one to glucuronic acid, and the middle band to mannuronic acid.¹⁰⁾ The relative mobilities of guluronic acid and glucuronic acid to that of mannuronic acid were 0.88 and 1.2, respectively. These values of guluronic acid and mannuronic acid were very close to the values calculated from the pattern reported by Haug and Larsen,¹¹⁾ while the value of glucuronic acid was slightly lower. The acid hydrolysate of the "acid insoluble" fraction obtained from the enzymic digestion showed two bands which corresponded to mannuronic acid and guluronic acid (Fig. 4B). Each concentration of these uronides on the paper was not analyzed in the present investigation. The "acid soluble"

FIG. 4. Paper Electrophoretic Patterns of Acid Hydrolysates of Intact and Enzymatically Degraded Alginates.

The paper electrophoresis was carried out at a voltage of 17~20/cm for 2 hr at room temperature in 10 mM borax solution containing 5 mM calcium chloride. The color was developed by dipping the paper in aniline trichloroacetate-ethylacetate solution.

(A), intact alginate; (B), "acid insoluble" fraction; (C), "acid soluble" fraction.

FIG. 5. Gel Filtration of Enzymatically Degraded Alginate after Acid Treatment (pH 1.5) on a Sephadex G-15 Column (1.5×98 cm).

Each fraction was loaded on a separate column and eluted with the same buffer as in Fig. 2 at a flow rate of 18 ml/hr. The abbreviation Ve represents the elution volume of glucuronic acid as standard. (A), "acid soluble" fraction; (B), "acid insoluble" fraction.

fraction gave a band which was in good agreement with the mobility of mannuronic acid (Fig. 4C), and the additional band of glucuronic acid added as standard.

That acid treatment was effective in separating the oligouronides from the short chain polyuronide of the reaction products was also demonstrated by gel filtration of the products on Sephadex G-15 (Fig. 5). The "acid insoluble" fraction was eluted only at the void volume (around 68 ml) of the column, showing lack of oligouronide, while the "acid soluble" fraction showed multiple peaks, including the fairly large oligouronides. The peaks seemed to be mainly composed of mannuronic acid, as judged by the paper electrophoretic pattern.

DISCUSSION

A mode of action of alginate lyases from various sources has been proposed. It is assumed that the enzymes from bacterial origin (pseudomonad,^{5,6} *Klebsiella aerogenes*⁷) acted preferentially on guluronosyl linkages and the enzymes from molluscs^{4,16} on mannuronosyl linkages. Two types of the enzymes which were specific for each uronide were isolated from abalone by Nakada and Sweeny.³ On the alginate lyase from *Turbo cornutus*, the authors examined the degradation mode toward alginate and the specificity for the linkages.

From the results shown in Fig. 1, it is obvious

that the enzyme cleaved the glycosidic linkages in an endo-type manner by the eliminase action,⁸ resulting in a rapid fall in viscosity and a slow increase in reducing power. In the initial stage of the reaction, the oligouronides were not liberated in a detectable amount. The oligouronides became barely observable only after prolonged incubation. On the basis of this fact, it seems probable that the longer chain polyuronides are preferentially bound by the enzyme rather than the shorter chains, suggesting a broad distribution of the sub-sites for the substrate binding on the enzyme molecule. Requirement of the free carboxyl groups of alginate for the enzyme action is shown in Fig. 3. Gel filtration of the reaction products of propylene glycol alginate incubated with the enzyme for 44 hr indicated only the breakthrough peak at the void volume of the column, accompanying a trace amount of the oligouronides. As reported in the previous paper,⁸ propylene glycol alginate is slightly degraded by the enzyme. This is most likely due to the incomplete esterification of the carboxyl groups of alginate.

The chemical compositions of intact and enzymatically degraded alginate were investigated. Mannuronic and guluronic acids were detected in the hydrolysates of intact alginate by paper electrophoresis. This is in good agreement with the data reported by Fischer and Dörfel¹⁶ and Haug and Larsen.¹¹ Separation of the oligouronides from the

polyuronides of digests was achieved by lowering the pH of the reaction mixture to about 1.5, as indicated in Fig. 5. This procedure facilitated chemical analysis of the products. Two fractions, "acid insoluble" and "acid soluble," were analyzed separately to identify the species of uronic acids present. As judged by the electrophoretic pattern, the "acid insoluble" fraction was composed of the two species, containing a relatively large amount of guluronic acid as compared to mannuronic acid, although this analysis was not quantitatively carried out. The "acid soluble" fraction showed a main single band, which corresponded to mannuronic acid. However, possibility of coexistence with a small amount of guluronic acid could not be excluded. From the patterns shown in Figs. 4 and 5, it is evident, in any event, that the oligouronides liberated from alginate by treatment with the enzyme are mostly composed of mannuronic acid, and that the mannuronate-rich linkages are more favorably attacked by the enzyme in an endo-type fashion.

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REFERENCES

- 1) E. Percival and R. H. McDowell, "Chemistry and Enzymology of Marine Algal Polysaccharides," Academic Press Inc., New York, N. Y., 1967, p. 99.
- 2) E. O. T. Atkins, W. Mackie and E. D. Smolko, *Nature*, **225**, 626 (1970).
- 3) H. I. Nakada and P. C. Sweeny, *J. Biol. Chem.*, **242**, 845 (1967).
- 4) K. Nishizawa, S. Fujibayashi and Y. Kashiwabara, *J. Biochem.*, **64**, 25 (1968).
- 5) I. W. Davidson, I. W. Sutherland and C. J. Lawson, *Biochem. J.*, **159**, 707 (1976).
- 6) K. H. Min, S. F. Sasaki, Y. Kashiwabara, H. Suzuki and K. Nisizawa, *J. Biochem.*, **81**, 539 (1977).
- 7) J. Boyd and J. R. Turvey, *Carbohydr. Res.*, **57**, 163 (1977).
- 8) T. Muramatsu, S. Hirose and M. Katayose, *Agric. Biol. Chem.*, **41**, 1939 (1977).
- 9) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
- 10) A. Haug and B. Larsen, *Acta Chem. Scand.*, **16**, 1908 (1962).
- 11) A. Haug and B. Larsen, *ibid.*, **15**, 1395 (1961).
- 12) M. Gee and R. M. McCready, *Anal. Chem.*, **29**, 257 (1957).
- 13) T. Bitter and H. M. Muir, *Anal. Biochem.*, **4**, 330 (1962).
- 14) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Robers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
- 15) L. A. Elyakova and V. V. Favorov, *Biochim. Biophys. Acta*, **358**, 341 (1974).
- 16) F. G. Fischer and H. Dörfel, *Hoppe-Seyler's Z. physiol. Chem.*, **302**, 186 (1955).