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BIOSYNTHESIS OF ALGINATE: PURIFICATION AND CHARACTERISATION OF MANNURONAN C-5-EPIMERASE FROM *Azotobacter vinelandii*

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ABSTRACT

A mannuronan C-5-epimerase from the soil bacterium *Azotobacter vinelandii* has been highly purified and characterised. The enzyme is a single polypeptide chain with a mobility in gradient and SDS electrophoresis corresponding to a mol. wt. of 122,000; it has a pI of 4, highest activity at pH 7.0, and an absolute requirement for Ca^{2+}. The K_m has been estimated for two different calcium concentrations, using [5-^{3}H]alginate as substrate. A rapid purification procedure based on fast protein liquid chromatography on MonoQ (HO^-) resin is described.

INTRODUCTION

An extracellular mannuronan C-5-epimerase can be isolated from liquid cultures of *Azotobacter vinelandii*. This enzyme is active during the biosynthesis of alginate by converting D-mannuronic acid (M) residues into L-guluronic acid (G) in the polymer chain[1–3]. The enzyme can epimerise both bacterial and algal alginate having a wide range in monomer composition and sequence of units. "Poly(mannuronic acid)" (*i.e.*, F_M >0.96), prepared from *Ascophyllum nodosum*, can be epimerised to ~70% G by the bacterial enzyme. When alginates with a higher content of guluronic acid were used[4] as substrate, the G content in the epimerised polymer reached 90%. The enzyme activity is accompanied by an exchange of H-5 with water, but this reaction does not involve[5] such redox co-enzymes as NAD^+ or $NADP^+$. Calcium ions play an essential part in the epimerisation process. Apart from stimulating the reaction, the concentration of Ca^{2+} also influences the epimerisation pattern. Epimerisation at low concentrations (0.86mM) favours the introduction of neighbouring G-units, thus creating G-blocks, whereas high concentrations of Ca^{2+} favour introduction of single G-units, thus resulting in a polymer with a more alternating structure[6].

In previous papers[7,8], we described a new assay for enzyme activity and a purification procedure based on affinity chromatography. We now report further purification and some properties of the highly purified enzyme.

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EXPERIMENTAL

Alginate preparations. — The alginate used as a ligand for the preparation of the alginate–Sepharose was isolated from *Azotobacter vinelandii.* As determined by ^{1}H-n.m.r. spectroscopy, this alginate had a guluronic acid content (F_G) of 0.37 and d.p. >100, and contained 12% of OAc. The C-5-tritiated alginate used in the enzyme assay was isolated from the same bacterium and fractionated as described previously[7]. This procedure gave an alginate with F_M 0.95 and a specific activity of 10,000 c.p.m./mg. The alginate used in the n.m.r. assay was isolated from the intracellular medulla of *Ascophyllum nodosum* receptacles and further fractionated, after mild hydrolysis with acid, by Ca^{2+} precipitation to obtain an alginate with a high content of mannuronic acid (F_M >0.95). The C-5-epimerase activity was assayed as the amount of tritium released into water when [5-^{3}H]alginate was incubated with the enzyme.

The incubation mixture consisted of [5-^{3}H]alginate (0.25 mL, 2.5 mg/mL; F_M 0.95; specific activity, 10,000 c.p.m./mg), collidine buffer (1 mL, pH 7.0), and calcium chloride (added to 3.4mM), and the volume was adjusted to 1.65 mL. The mixture was inoculated with enzyme solution (0.2 mL, ~1 mg of protein per mL) and stored for 2 h at 30°. The reaction was stopped by adding sodium chloride to 0.2% followed by ethanol (2 mL). After centrifugation, the radioactivity of the supernatant solution was determined by using a scintillation counter. The background in control samples containing substrate, but no enzyme, was 2–3% of the total activity added. In the kinetic studies, control samples were used for each concentration of substrate.

Enzyme assay based on ^{1}H-n.m.r. spectroscopy[9]. — "Poly(mannuronic acid)" (7.5 mg), isolated from *Ascophyllum nodosum*, was dissolved in collidine buffer (6.0 mL, pH 6.8), calcium chloride was added to 3.4mM, and the volume was adjusted to 9.6 mL with distilled water. The mixture was incubated with enzyme solution (0.4 mL) for 16 h at 30°. The reaction was stopped by transferring the sample to a dialysis bag, and the alginate was precipiated by adding 0.01M HCl dropwise to pH 3.5. The alginate was dialysed for 24 h against 0.05M HCl to remove Ca^{2+}, dissolved by neutralisation, and then dialysed exhaustively against distilled water. After freeze-drying, the alginate was dissolved in D_2O (0.4 mL) and the ^{1}H-n.m.r. spectrum was recorded[9] with a 100-MHz Jeol FX-100 spectrometer.

Purification of the enzyme. — The enzyme was isolated from liquid cultures of *Azotobacter vinelandii* by precipitation with ammonium sulphate. The precipitate at 50% saturation was dissolved in 0.05M imidazole/HCl buffer (pH 6.8) containing 0.34mM $CaCl_2$ and 0.5mM dithiothreitol (DTT), and desalted on a prepacked column (PD-10) of Sephadex G-25 (Pharmacia) equilibrated with the same buffer. This "crude" preparation was eluted from a column of Sephadex A-25 by a stepwise increase in ionic strength. The epimerase was eluted with 0.4M NaCl, and this fraction was desalted and then applied to a column (10 × 120 mm) of alginate–Sepharose. Proteins bound by non-specific interactions were eluted with 0.1M

NaCl. The epimerase was eluted as one sharp peak with 0.5M NaCl. The fraction containing the purified enzyme was desalted on a column of Sephadex G-25 equilibrated with 0.05M imidazole buffer (pH 6.8) and kept frozen at −30° or freeze-dried. The column of alginate–Sepharose was prepared by coupling[8] bacterial alginate to epoxyactivated Sepharose.

Gel filtration was carried out on Sephacryl-300 SF gel. A solution of crude enzyme (40 mg) in Tris/HCl buffer (0.05M, pH 7.0) containing 0.34mM $CaCl_2$, 0.5mM DTT, and 0.1M Na_2SO_4 was applied to a column (12 × 700 mm) of Sephacryl-300 SF and eluted with the same Tris/HCl buffer at 15 mL/h. Activity was analysed using [5-^{3}H]alginate as substrate. The active proteins were also incubated with "poly(mannuronic acid)" and the conversion was analysed by n.m.r. spectroscopy. The molecular weights of the proteins eluted were estimated on the basis of K_{av} values for globular protein standards.

Gel electrophoresis was carried out in polyacrylamide gradient gels (PAG 4/30, Pharmacia) in 0.01M Tris/0.08M boric acid buffer containing 2.5mM EDTA, and run for 16 h at 150 V. The gels were fixed for 30 min in aqueous 10% sulfosalicylic acid and stained in 0.1% Coomassie Brilliant Blue R-250 in aqueous 25% methanol and 10% acetic acid. SDS electrophoresis was carried out in the same gradient gel after the protein samples has been treated with 2-mercaptoethanol and SDS. The buffer was 0.04M Tris/sodium acetate containing 25mM EDTA and 2% of SDS. Standard proteins were used as molecular weight markers, and the molecular weight was calculated as described[10].

Fast protein liquid chromatography. — Crude and affinity-purified enzyme were chromatographied on a MonoQ anion-exchanger using an FPLC™ system (Pharmacia). All solutions were filtered through a 0.22-μm Millipore filter, and the protein samples were centrifuged at 10,000*g* for 1 h to remove particles and aggregates. Samples of 500 μL were applied to the column and eluted with 0.05M imidazole/HCl buffer (pH 7.0) containing 0.34mM $CaCl_2$ and 0.5mM DTT. The fractions were desalted on a prepacked column (PD-10) of Sephadex G-25, and the activity was assayed by the [^{3}H]alginate method. The active fractions were also incubated with "poly(mannuronic acid)" and the conversion was measured by n.m.r. spectroscopy.

RESULTS AND DISCUSSION

The purification of mannuronan C-5-epimerase, based on a two-step process involving ion-exchange and affinity chromatography, gave a highly purified enzyme. Affinity chromatography on alginate–Sepharose is an effective and rapid method of purification. However, since the ligand (alginate) is ionised at pH 6.8, the column might also be expected to function by cation exchange, binding positively charged proteins non-specifically. Since the epimerase also binds to anion exchangers at pH 6.8, chromatography on A-25 Sephadex was used prior to the affinity step in order to remove the positively charged proteins. By eluting the

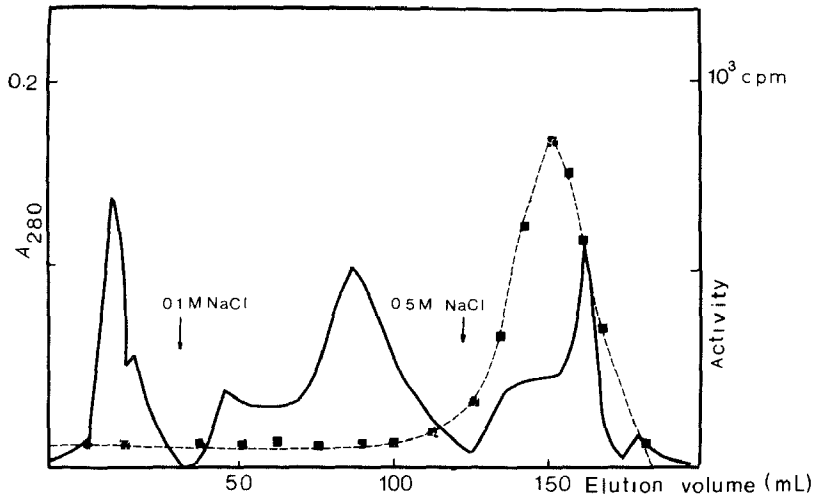

Fig. 1. Purification of mannuronan C-5-epimerase on A-25 Sephadex: enzyme activity assayed by ^{3}H-release, ---■---; A_{280}, ———.

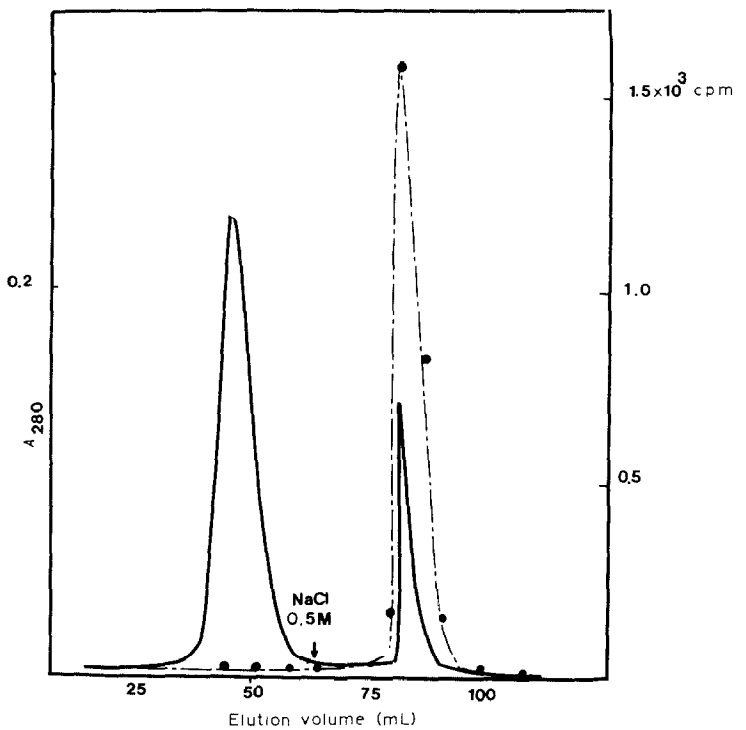

Fig. 2. Purification of mannuronan C-5-epimerase on alginate–Sepharose. enzyme activity assayed by ^{3}H-release, ---●---; A_{280}, ———.

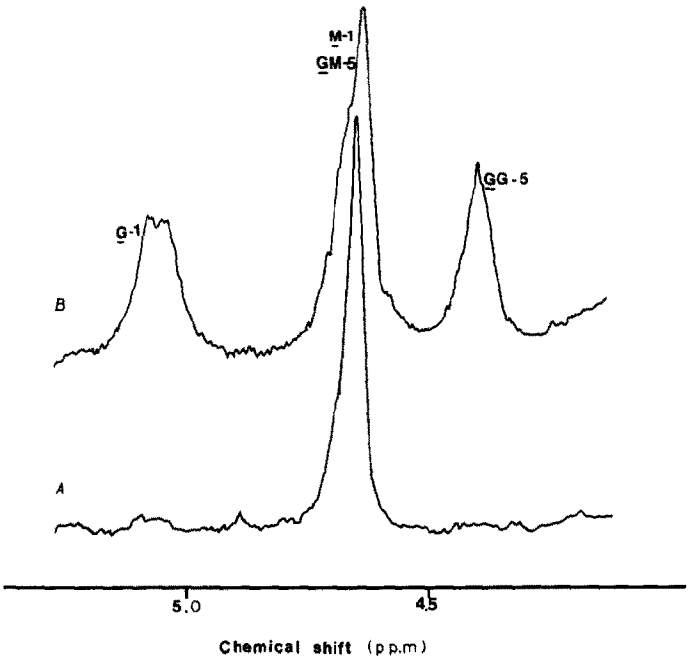

Fig. 3. A section from a 99.6-MHz F.t. ^{1}H-n.m.r. spectrum (solutions in D_2O) of alginate from *A. nodosum*: *A*, before epimerisation; *B*, after epimerisation with highly purified epimerase.

TABLE I

THE COMPOSITION AND DIAD FREQUENCIES OF EPIMERISED "POLY(MANNURONIC ACID)" ISOLATED FROM *Ascophyllum nodosum*

Sample	F_G	F_M	F_{GG}	F_{GM}	F_{MM}
"Poly-M" epimerised with highly purified enzyme	0.42	0.58	0.32	0.10	0.48
"Poly-M" epimerised with f.p.l.c.-purified enzyme	0.32	0.68	0.23	0.09	0.59

column with a stepwise increase in ionic strength, in the buffer, the epimerase was separated from the cationic, as well as from some of the contaminating anionic, proteins (Fig. 1). This procedure also removed an alginate lyase occasionally present in the crude preparation. In the subsequent purification step on alginate–Sepharose, only proteins with biospecificity towards the ligand (alginate) should be retained on the column. A typical clution pattern from the affinity column is shown in Fig. 2. The activity of the highly purified enzyme was confirmed by the n.m.r. spectroscopic assay. The results are shown in Fig. 3, and demonstrate a very efficient conversion by the purified enzyme of a "poly(mannuronic acid)" into a polymer containing 42% of guluronic acid (Table I). Analytical isoelectric focusing

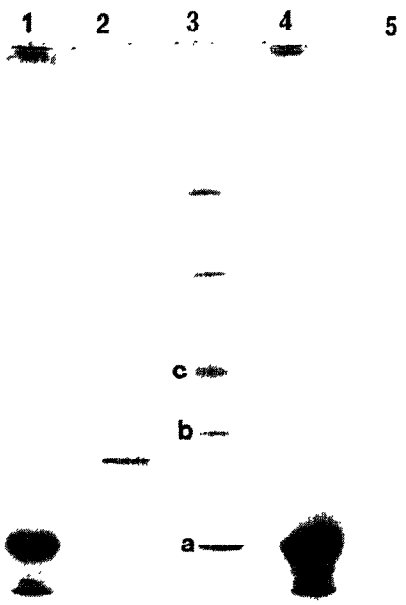

Fig. 4. Gradient gel electrophoresis (PAG 4/30) of the hghly purified enzyme: 1 and 4, crude enzyme after ammonium sulphate precipitation; 2 and 5, purified enzyme; 3, standards [a, BSA (mol. wt. 67,000); b, LDH (140,000); c, catalase (230,000)].

in polyacrylamide gel showed that most of the proteins in the crude preparation had isoelectric points below 4, with some very strong bands around 3.2–3.5, and only a few in the area 7–9. The purified enzyme gave one strong band corresponding to pI 4.5 and two weak, very narrow bands with pI 3.5, thus demonstrating the efficiency of the purification system.

If, during the coupling process, bacterial alginate was replaced by an algal alginate having approximately the same monomer composition and d.p., the epimerase did not bind to the affinity column under the same conditions, but was only retarded. Chemically, the most conspicuous difference between these two ligands is the *O*-actyl groups present in the bacterial alginate[11]. Since these groups are likely to be removed during the coupling process, they cannot be expected to influence the binding of epimerase. At present, no explanation can be offered for the difference between the two types of alginate with respect to epimerase binding.

When subjected to gel electrophoresis in polyacrylamide gradient gels, the purified enzyme gave one distinct, narrow band with a mobility corresponding to a mol. wt. of 122,000. Some very faint bands, corresponding to a mol. wt. of ~360,000 and 440,000, could also be seen (Fig. 4). That the 122,000-dalton protein is responsible for the epimerase activity was indicated by gel-filtration experiments. When a crude enzyme preparation was chromatographed on Sephacryl 300, the active proteins were eluted with a K_{av} slightly lower than that of aldolase (mol. wt. 158,000). Since the activity peak is broad, it cannot be ruled out that proteins with

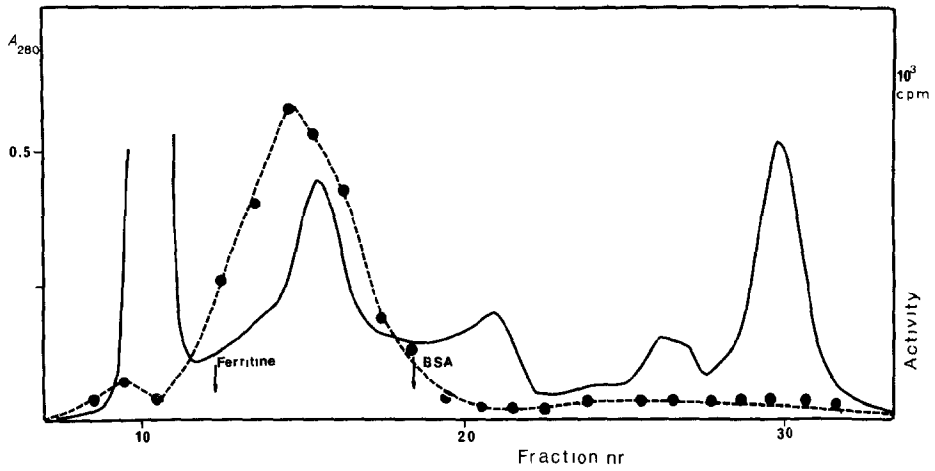

Fig. 5. Gel filtration of a crude preparation of mannuronan C-5-epimerase on Sephacryl 300 SF: enzyme activity assayed by ^{3}H-release, ---●---; A_{280}, ———.

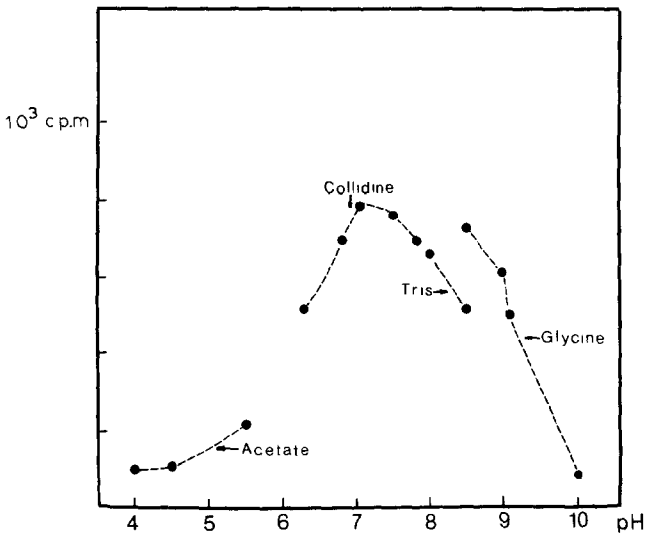

Fig. 6. Enzyme activity as a function of pH, assayed by ^{3}H-release in the buffers 0.05M HOAc/NaOH (pH 4.0–5.6), 0.05M collidine/HCl (pH 6.3–6.9), 0.05M Tris/HCl (pH 8.0–8.6), and 0.05M glycine/NaOH (pH 8.6–10.0).

higher mol. wt. have epimerase activity (Fig. 5). When the purified enzyme was reduced with 2-mercaptoethanol and subjected to SDS electrophoresis in polyacrylamide gradient gels, it gave a strong band with mobility corresponding to a mol. wt. 120,000, which suggests that the epimerase consists of a single polypeptide chain.

If the crude enzyme preparation is free of alginate lyase activity, the A-25

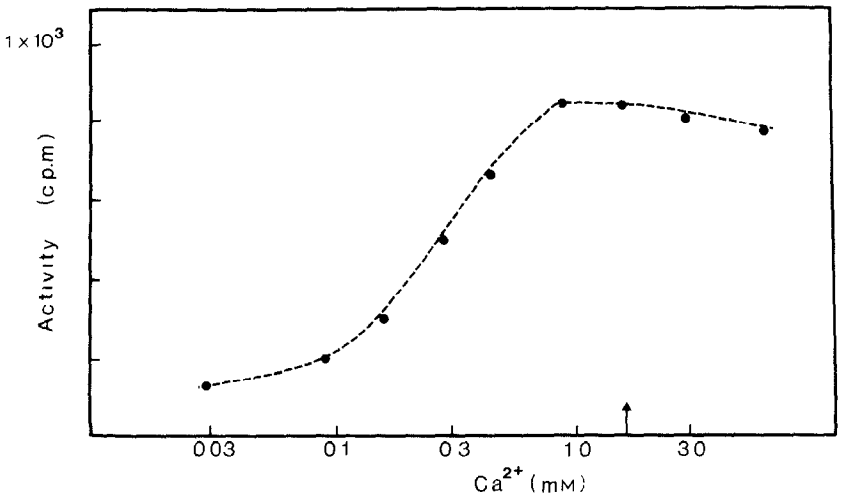

Fig. 7. Enzyme activity as a function of Ca^{2+}, assayed by ^3H-release. Equimolar concentration of calcium ions is indicated by →.

anion-exchanger can be replaced by a cation exchanger that will not retain the epimerase, which can then be applied directly to the alginate–Sepharose-column without desalting. Consequently, this procedure is less time-consuming and causes less dilution. The affinity column can be used for months, and alginate lyase-contaminated crude preparations applied directly on the column do not seem to reduce the binding capacity or resolution.

The correlation between the enzyme activity and pH is given in Fig. 6. The purified enzyme was active over a broad range of pH, and the highest activity was obtained with collidine/HCl buffer (pH 7.0) in accordance with the results obtained with the crude enzyme[2].

The influence of Ca^{2+} on the enzyme activity is shown in Fig. 7. The enzyme has an absolute requirement for Ca^{2+}, but no activity could be detected at <0.01mM. The enzyme activity is stimulated over a wide range of concentrations of Ca^{2+} with a marked increase between 0.2 and 1.0mM, where the activity was maximal. With the crude enzyme preparation, a slightly higher concentration of Ca^{2+} was needed to obtain the same activity. This is to be expected if contaminating proteins compete with the substrate and the epimerase for Ca^{2+}. Isoelectric focusing experiments showed that the major proportion of these proteins have pI values below 4.5.

The K_m value has been determined for the purified enzyme. Since the concentration of Ca^{2+} influences both reaction rate and epimerisation pattern, the kinetic experiments were performed for low (0.68mM) and high (8.6mM) concentrations of Ca^{2+}. The [5-^{3}H]alginate was used as substrate and the samples were incubated for 30 min. The K_m value was determined by the Lineweaver–Burk procedure and the results are given in Fig. 8. For hydrated sodium mannuronate

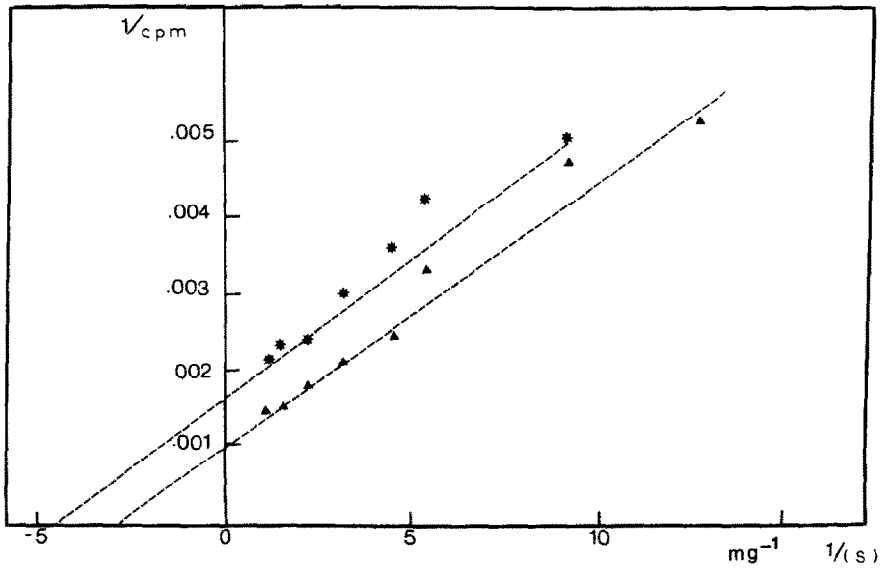

Fig. 8. Lineweaver–Burk plot for the purified mannuronan C-5-epimerase: 0.68 (---★---) and 8.6mM calcium chloride (---▲---).

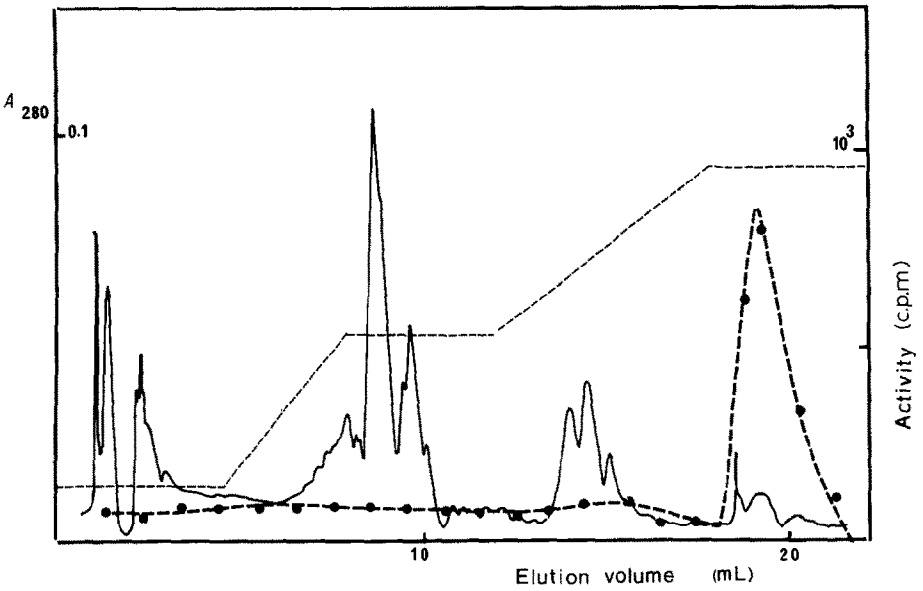

Fig. 9. Elution pattern of a crude mannuronan C-5-epimerase preparation on MonoQ (F.p.l.c.): gradient 0.1→0.6M sodium chloride, ------; enzyme activity assayed by ^{3}H-release, ---●---; A_{280}, ———.

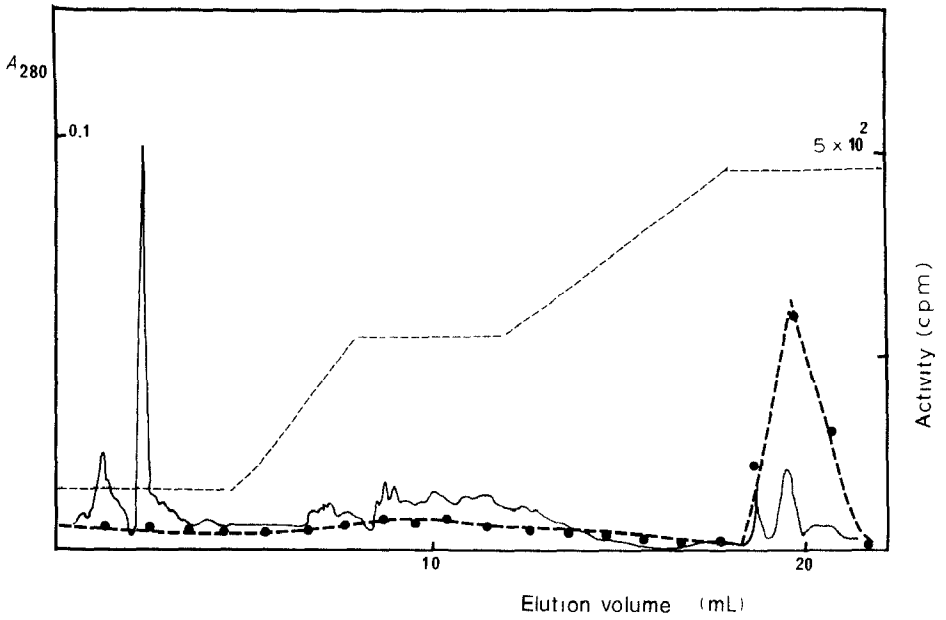

Fig. 10. Elution pattern of an affinity-chromatographed epimerase on MonoQ (F.p.l c.) gradient 0.1→0.6M sodium chloride, ------; enzyme activity assayed by ^{3}H-release, ---●---; A_{280}, ———

(mol. wt. 216), the K_m was 0.90×10^{-3} M and 0.62×10^{-3} M for high and low [Ca^{2+}], respectively. These values are similar to those reported[12] for a mannuronan C-5-epimerase isolated from the brown seaweed *Ishige okamurai*. The observed difference in the K_m values suggests that Ca^{2+} has an influence on the affinity of the epimerase for alginate. It is not known whether this is due to a conformational change of the enzyme or the substrate. The Lineweaver–Burk plots (Fig. 8) might suggest a nonlinear relationship. A more thorough discussion of this anomaly, based on an n.m.r. spectroscopic study of the epimerisation process, will be published elsewhere.

A rapid separation procedure based on fast protein liquid chromatography on a monobead anion-exchange (MonoQ™) is demonstrated in Figs. 9 and 10. This technique is useful for analytical and semi-preparative work. Location of activity, as assayed using [^{3}H]alginate, was confirmed by incubating the fraction with "poly M", and the conversion was analysed by n.m.r. spectroscopy. The results in Table I show that the ammonium sulphate-precipitated enzyme purified on the MonoQ™ column converts "poly(mannuronic acid)" into a polymer containing 32% of guluronic acid.

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REFERENCES

1 B. LARSEN AND A. HAUG, *Carbohydr. Res.*, 17 (1971) 287–296.
2 A. HAUG AND B. LARSEN, *Carbohydr. Res.*, 17 (1971) 297–308.
3 B. LARSEN, *Proc. Int. Seaweed Symp., XIth, Goethenburg*, 1981, pp. 7–34.
4 G. SKJÅK-BRÆK, in G. O. PHILLIPS (Ed.), *Application of Hydrocolloids in Food: Progress in Food and Nutrition Science*, Pergamon, Oxford, 1983, pp. 523–528.
5 B. LARSEN AND H. GRASDALEN, *Carbohydr. Res.*, 92 (1981) 163–167.
6 R. OFSTAD AND B. LARSEN, *Proc. Int. Seaweed Symp., XIth*, Goethenburg, 1981, pp. 485–494.
7 G. SKJÅK-BRÆK AND B. LARSEN, *Carbohydr. Res.*, 103 (1982) 133–136.
8 G. SKJÅK-BRÆK AND B. LARSEN, *Carbohydr. Res.*, 103 (1982) 137–140.
9 H. GRASDALEN AND B. LARSEN, *Carbohydr. Res.*, 68 (1979) 23–31.
10 M. N. NEVILLE, *J. Biol. Chem.*, 246 (1971) 6228–6234.
11 I. W. DAVIDSON, I. W. SUTHERLAND, AND C. J. LAWSON, *J. Gen. Microbiol.*, 98 (1977) 603–606.
12 M. ISHIKAWA AND K. N. NISIZAWA, *Bull. Jpn. Soc. Sci. Fish.*, 47 (1981) 137–140.