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Note

Purification of mannuronan C-5-epimerase by affinity chromatography on alginate–Sepharose

GUDMUND SKJÅK-BRÆK AND BJØRN LARSEN
Institute of Marine Biochemistry, University of Trondheim, N-7034 Trondheim (Norway)
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Affinity chromatography with substrate as the bound ligand is a powerful technique in the purification of enzymes, but successful application requires close simulation of the conditions that favour enzyme–substrate binding in free solution. Affinity chromatography of enzymes with polymeric substrates as ligands has been reported for lysozyme–chitin[1], protease–haemoglobin[2], DNA-polymerase–DNA[3], heparosan-*N*-sulphate D-glucosyluronic acid C-5-epimerase–heparan sulphate[4], and alginase–alginate[5]. In the last example, the alginate was attached covalently to a polyacrylamide matrix (hydrazine-activated Biogel P-20) *via* the reducing end-groups. We now report on the purification of mannuronan C-5-epimerase, which epimerises D-mannuronic into L-guluronic acid residues in the polymer chain, using a Sepharose 6B column containing alginate covalently linked to the hydroxyl groups.

The alginate used for coupling was isolated from *Laminaria digitata*[6], and from the soil bacterium *Azotobacter vinelandii*[7]. The composition of the alginate was determined by high-resolution, ^{1}H-n.m.r. spectroscopy[8,9]. The enzyme was isolated from liquid cultures of *Azotobacter vinelandii* by ammonium sulphate precipitation[10], and its activity was assayed on the basis of tritium release into water by using [5-^{3}H]alginate as substrate[11]. The [5-^{3}H]alginate, isolated from *Azotobacter vinelandii*, contained 95% of D-mannuronic acid and 5% of L-guluronic acid residues and had a specific activity of 6500 c.p.m./mg[11]. Carbohydrates and proteins were analysed by the phenol–sulphuric acid[12] and Folin–Ciocalteu methods[13], respectively.

Alginate was coupled to the Sepharose matrix as follows. Epoxy-activated Sepharose (Pharmacia, 8 g) was swollen and washed on a glass sinter (G-3) with distilled water (800 mL). The gel was then mixed with an equal volume of alginate solution containing bacterial alginate (ManA/GulA = 1.7; $\overline{\text{d.p.}}_n > 100$) (15 mg/mL) and alginate from *Laminaria digitata* (ManA/GulA = 9; $\overline{\text{d.p.}}_n$ 26.5) (10 mg/mL). The pH was adjusted to 12.4 with M NaOH and the coupling was effected by shaking for 16 h at 40°. Excess epoxy-groups were blocked by keeping the gel in M ethanolamine for 4 h at 20° and pH 9.0. The gel was washed with distilled water (400 mL)

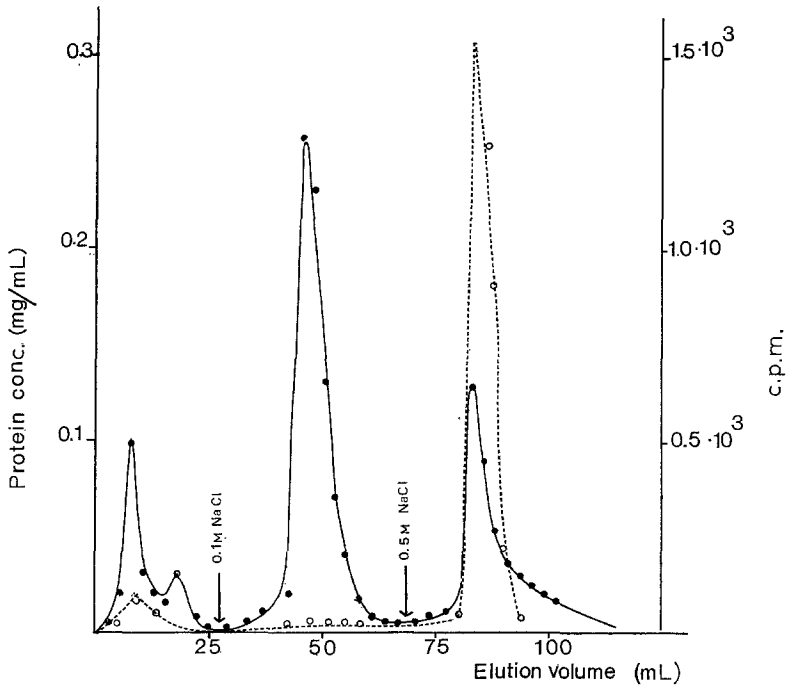

Fig. 1. Purification of mannuronan C-5-epimerase by affinity chromatography on alginate–Sepharose 6B. Crude enzyme (3 mL) isolated by ammonium sulphate precipitation (protein, 1.45 mg/mL) was applied to the column and eluted with 0.05M imidazole buffer (pH 6.8) containing 0.1 and 0.5M NaCl, respectively. The flow rate was 21 mL/h. The enzymic activity in each fraction (2.5 mL) was determined by mixing 0.2 mL of enzyme with 0.625 mg of [5-^{3}H]alginate (4000 c.p.m.) and 3.4mM $CaCl_2$ in a total volume of 1.75 mL. The reaction was stopped by adding 25 μL of aqueous NaCl (160 mg/mL) and 2 mL of ethanol. The supernatant after centrifugation (2.5 mL) was assayed for radioactivity in a liquid scintillation counter (Isocap 300); samples were counted for 20 min: —●—, protein concentration; --○--, enzyme activity.

TABLE I

PURIFICATION OF MANNURONAN C-5-EPIMERASE

Enzyme fraction	*Protein (mg/mL)*[a]	*Enzyme activity (c.p.m.)*[a]	*Specific activity (c.p.m./mg)*[a]	*Total protein in the peak (mg)*
Ammonium sulphate precipitate (50%)	1.45	1700	1170	4.35
Affinity chromatography				
Peak I	0.10	80	800	0.50
Peak II	0.26	00	—	2.38
Peak III	0.13	1520	11690	1.12

[a]Only the top fractions of each peak are given.

and then, alternately, with 0.1M acetate buffer (pH 4.5) and 0.1M borate buffer (pH 8.9), both containing 0.5M NaCl. The gel was packed into a column (16 × 100 mm), and equilibrated for 24 h with 0.05M imidazole buffer containing 0.34mM $CaCl_2$ and 0.5mM dithiothreitol.

The crude enzyme obtained by ammonium sulphate precipitation was dissolved in imidazole buffer and passed through a column of G-25 PD-10 (Pharmacia) to remove contaminants of low molecular weight and salt. The desalted solution was centrifuged at 10,000*g* for 15 min before it was applied to the column. The column was washed with two bed-volumes of the imidazole buffer, and the adsorbed proteins were eluted by stepwise increase of the ion strength of the buffer. The protein peaks (detected by absorption at 280 nm) were desalted by using a column of G-25 PD-10, and the enzymic activity and protein concentration were assayed.

The elution pattern in Fig. 1 has been reproduced more than 20 times with different batches of enzyme and different preparations of gel. The enzymic activity was always found in peak III (eluted with 0.5M NaCl). Occasionally, a low activity was also detected in peak I (Table I). The data given in Table I demonstrated a protein recovery of 80–90% and an approximately ten-fold purification of the enzyme. There appeared to be no unspecific binding to the column when all the epoxy groups were blocked by ethanolamine. Since the ligand (alginic acid) is charged at pH 6.8, the affinity gel might be expected to function as a cation exchanger, binding positively charged proteins unspecifically. However, experiments with various ion-exchange materials showed[14] that, at pH 6.8, the epimerase does not bind to such cation exchangers as CM-Sephadex or CM-Sepharose CL 6B, but binds strongly to such anion exchangers as DEAE-Sephadex A-25 and DEAE-Sepharose CL 6B.

On electrophoresis in polyacrylamide gradient gels, peak III from the affinity column gave a narrow band with a migration rate corresponding to a molecular weight of 120,000, and three weaker, but distinct, bands with higher molecular weights (~140,000, 350,000, and 420,000). After treatment with 2-mercaptoethanol, SDS-electrophoresis gave one strong band corresponding to a molecular weight of 120,000 and some faint bands with higher and lower mobility. Peak III had no main bands in common with the two other peaks.

Thus, it is concluded that the epimerase is retained on the column due to a biospecific adsorption, but that it will be contaminated during elution by other proteins retained due to an ion-exchange effect of the alginate. However, the method is very useful in allowing the isolation of a small number of the many proteins originally present in the enzyme preparation.

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