ENZYME PURIFICATION BY SUBSTRATE ELUTION CHROMATOGRAPHY FROM PROCION DYE–POLYSACCHARIDE MATRICES

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Received 17 August 1976

1. Introduction

The most effective methods of enzyme purification are those that make use of interactions between enzymes and their specific ligands. In affinity chromatography, for example, the ligand is bound to an insoluble support and, depending on the specificity of the ligand-enzyme interaction, a single enzyme or group of enzymes can be bound to such an affinity polymer [1]. Disadvantages of such polymers are frequently their instability and high cost.

An alternative approach to enzyme purification is the elution of bound enzyme with a specific ligand [2]. In this case, proteins are bound non-specifically to a polymer and the binding is specifically overcome by complex formation of the enzyme with a ligand. In general cation exchange resins have been used as affinity polymers as these contain common ligands at which binding occurs, sometimes through groups located at or near the enzyme binding site. Upon elution with a specific substrate these binding sites are shielded against interaction with the ion exchanger as a result of complex formation between the enzyme and its substrate.

During characterisation studies on the enzyme Carboxypeptidase G, elaborated by *Pseudomonas* ATCC 25301 and which hydrolyses the amino-benzoylglutamic acid peptide bond in folic acid, methotrexate and *p*-aminobenzoyl glutamate, the enzyme was found to bind readily to Blue Dextran 2000, a high molecular weight dextran substituted with the mono-chlorotriazinyl dye Cibachron Blue F3GA.

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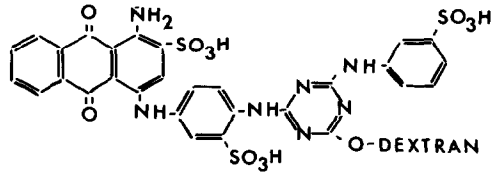
DEXTRAN BLUE
(Cibachron BLUE F3GA)



FOLIC ACID

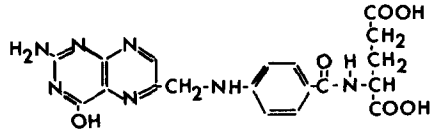


Fig.1.

A number of different dye polysaccharide conjugates were therefore synthesised by covalent attachment of the dyes to polysaccharide matrices and the affinity of the enzyme for these resins, its elution by substrate and the usefulness of these matrices in purification of the enzyme was determined.

2. Materials and methods

Procion dyes were a kind gift from ICI organics division, Leicester; Cibachron Blue F3GA was obtained from Ciba-Geigy (UK) Ltd., Manchester; Sepharose 4B, Sephadex G-100 and Blue Dextran 2000 from Pharmacia GB Ltd., London.

Dye–polysaccharide conjugates were prepared at ambient temperature (15–25°C) by the procedure of Dudman and Bishop [3]. To a vigorously-stirred suspension of Sepharose 4B (5 ml) or Sephadex G-100 (50 mg) in water (20 ml) was added a freshly-prepared solution of the dye (50 mg) in water (5 ml). After 5 min solid sodium chloride was added to a final concentration of 2% and after a further 30 min, sodium carbonate to a final concentration of 0.1% and the mixture stirred overnight to allow unreacted dye to be hydrolysed. The dyed resin was exhaustively washed on a glass sinter to remove unreacted dye, packed into small disposable columns (5 ml, Biorad Labs., Kent, UK) and equilibrated with 20 mM Tris–acetate buffer in the range pH 5.5 to pH 8.0 by constant washing.

In small scale studies, columns (5 ml) were loaded with aliquots of carboxypeptidase (50–200 units; 40 U/mg protein) equilibrated by dialysis against 20 mM Tris–acetate buffer at the appropriate pH value. The columns were then sequentially washed with 2 volumes of buffer, 2 volumes of buffer containing 2 mM *p*-aminobenzoyl glutamate and finally 2 volumes of buffer containing 1 M sodium chloride. The eluants at each stage were assayed for carboxypeptidase activity by a spectrophotometric assay using the folate analogue methotrexate as substrate [4].

The growth of *Pseudomonas* ATCC 25301, large scale purification and characterisation of the enzyme by conventional methods will be described elsewhere. To prepare an extract for large-scale affinity chromatography, bacterial paste (2 kg) was suspended in three volumes of 10 mM sodium acetate, pH 8.3 and the cells disrupted by two passages through a Manton-Gaulin homogeniser (APV, Crawley, UK) at 550 kg/cm^2. Cell debris was removed by centrifugation and the cell free supernatant adjusted to pH 5.5 by the addition of dilute acetic acid and the whole diluted 3-fold with deionised water. SP-Sephadex, A50 (100 g) swollen and equilibrated to the same pH and ionic strength was added and the suspension stirred overnight at 4°C. The resin was allowed to settle and, after decanting the supernatant, was transferred to a column (30 × 13 cm). The column (4 litres) was washed with two volumes of 10 mM sodium acetate buffer, pH 5.5 and carboxypeptidase activity was eluted with 25 mM Tris–acetate buffer, pH 9.0 and collected in 500 ml fractions.

The pooled extract (3.5 litres) was concentrated with an Amicon CH3 hollow fibre unit fitted with a HIP5 membrane to 1.5 litres, solid NaCl was added to a final concentration of 150 mM, the pH was rapidly adjusted to pH 5.5 by the addition of 3 N acetic acid and the precipitate of pteroic acid rapidly removed by centrifugation. The supernatant was adjusted to pH 7.0 with 1 N NaOH and, concentrated to 800 ml and dialysed against 20 mM Tris–acetate, pH 7.0. One half of the preparation (400 ml; 8400 units) was purified by conventional methods (table 2) while the other was purified by chromatography on a 320 ml (20 × 4.4 cm) Procion HE3B–Sephadex G-100 column, synthesised as described above.

Enzyme was adsorbed to the Procion column in 20 mM Tris–acetate, pH 7.0 and after washing the column with buffer to reduce the A_{280} the enzyme was eluted with the same buffer containing 2 mM *p*-aminobenzoyl glutamate, which was later removed by a double-dialysis against activated charcoal (5 g/litre).

Carboxypeptidase G was assayed using methotrexate as substrate [4] and units were defined as 1 μmol substrate hydrolysed per minute at 30°C. Samples were analysed throughout the purification for protein by the method of Lowry [5] and by SDS-polyacrylamide gel electrophoresis in 10% gels [6] (See fig.5).

3. Results and discussion

Studies with small columns of Sepharose 4B–dye conjugates demonstrated variation both in the capacity of the different dyes to bind enzyme and in the subsequent elution of the enzyme by the substrate *p*-aminobenzoyl glutamate (table 1). Folic acid, the more usual substrate for this enzyme was not used as the eluant since the enzymic product of the reaction, pteroic acid, is insoluble at pH 6.0. The procion red dye HE3B proved to have good adsorption capacity, specificity of elution and gave good enzyme recoveries.

Sephadex G-100 was substituted for Sepharose 4B as the insoluble support for ligand binding as the former exhibited a markedly higher substitution capacity for the dye. It was also found that the dye

Table 1
Substrate elution chromatography of carboxypeptidase G on Sepharose 4B–dye conjugates

Sepharose 4B-conjugate	Recovered activity %		
	20 mM Buffer	20 mM Buffer + 2 mM PABG	20 mM Buffer + 1 M NaCl
Procion Yellow MX-6G	1	0	75
Yellow MX-R	0	0	40
Orange MX-G	84	1	1
Blue H7GS	80	7.5	0
Blue MX-R	2	5	83
Blue MX-3G	1	1	48
Red H3B5	6	102	4
Red HE7B	0	54	43
Red HE3B	0	83	12
Red H3B	42	46	0
Cibachron Blue F3Ga	1	33	59
Sepharose 4B (control)	91	2.5	0

Carboxypeptidase G solution (1 ml) containing 40 units of activity (1 mg protein) was loaded on to 5 ml columns of each Sepharose 4B-dye conjugate, equilibrated in 20 mM Tris–acetate, pH 6.0 and at 15°C. Columns were washed with 10 ml of the same buffer followed by 10 ml buffer containing 2 mM *p*-aminobenzoyl glutamate (PABG) and finally 10 ml buffer containing 1 M NaCl. Column fractions (0.5 ml) were assayed for enzyme activity during each elution stage.

binding capacity of such matrices could be increased by using higher dye concentrations and lower reactant volumes during preparation of the dye–Sephadex conjugates. Stability of the matrices was excellent, no significant loss of enzyme binding capacity or leakage of the dye from the resin was detected in 6 months storage in buffer at +4°C. The matrices could be re-used repeatedly with no loss of capacity, provided that they were regenerated after use with 1 to 2 M NaCl.

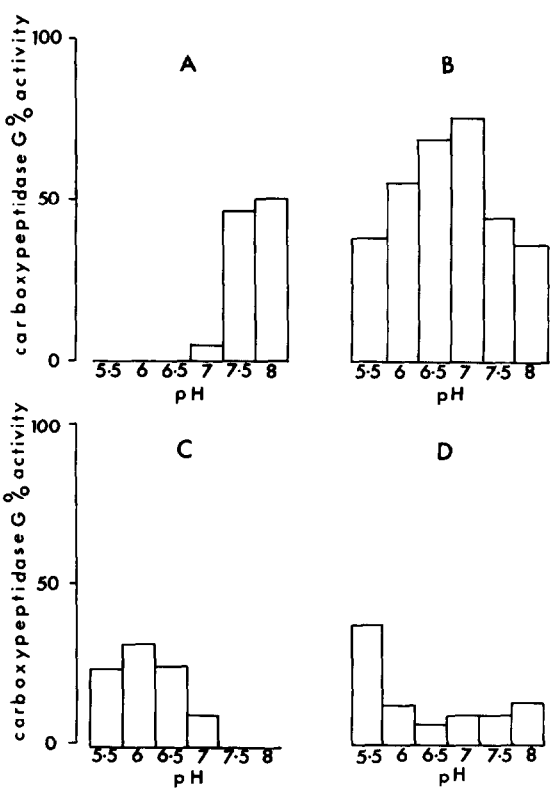


Fig. 2. Fate of carboxypeptidase G during binding and elution of Procion HE3B–Sephadex G-100 columns under different pH regimes. Aliquots (10 ml) of 20 mM Tris–acetate buffer ranging from pH 5.5 to pH 8.0 and containing 500 units of carboxypeptidase G activity (10 mg protein) were loaded on to 5 ml columns of Procion HE3B–Sephadex G-100 conjugate equilibrated in the corresponding buffer. Columns were washed with 10 ml of the same buffer (A) and then eluted, first with 10 ml buffer containing 2 mM *p*-aminobenzoyl glutamate (B) and finally with 10 ml buffer containing 1 M NaCl (C). Column fractions (0.5 ml) were assayed at each stage for enzyme activity (see Materials and methods) and enzyme lost on the columns was calculated (D).

The pH at which binding and elution were carried out had a marked effect on the recovery of the enzyme (fig.2). Below pH 7.0 there was strong binding of enzyme and elution became progressively more difficult. Above pH 7.0 much of the enzyme failed to bind to the column. The optimum conditions were at pH 6.5 and pH 7.0 and the latter was preferred for routine application as the enzyme eluted as a much sharper band.

The stronger binding of carboxypeptidase G at lower pH was emphasised in a second experiment, where enzyme was eluted with a NaCl-gradient from columns equilibrated in 20 mM Tris–acetate buffer at pH 6.0 and 7.0. At pH 6.0 enzyme activity peaked at 100–180 mM NaCl while at pH 7.0 elution was optimal at 50–100 mM NaCl.

The possibility of using soluble dye to elute enzyme was also investigated. Columns equilibrated at pH 7.0 were eluted with buffer containing 0.1% Procion HE3B dye (fig.3). The enzyme eluted as a sharp peak with the dye front, exhibiting true affinity chromatography with respect to the dye. Removal of

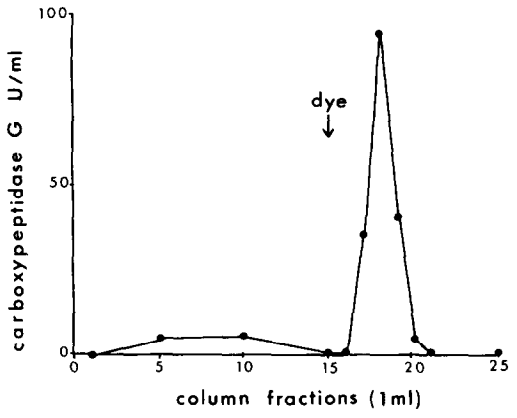


Fig.3. Elution of carboxypeptidase G from a Procion HE3B–Sephadex G-100 column using soluble Procion HE3B dye. A column (5 ml) of Procion HE3B–Sephadex G-100 equilibrated in 20 mM Tris–acetate buffer pH 7.0 was loaded with 200 units of carboxypeptidase G in the same buffer (5 ml). The column was washed with 10 ml buffer and then eluted with buffer containing 0.1% Procion HE3B dye. Column fractions (1 ml) were assayed for enzyme activity (see Materials and methods).

Table 2
Comparison of the purification of carboxypeptidase G by conventional and Procion–Sephadex techniques

Stage	Conventional method		Procion–Sephadex method	
	Total units ($U \times 10^{-3}$)	Specific activity (U/mg)	Total units ($U \times 10^{-3}$)	Specific activity (U/mg)
Cell Extract (from 1 kg cell paste)	10.2	0.2	10.2	0.2
SP–Sephadex (batch)	8.4	2.3	8.4	2.3
Procion–Sephadex	–	–	7.0	73
QAE–Sephadex	6.1	10.1	–	–
SP–Sephadex II (salt gradient)	5.2	36.7	–	–
SP–Sephadex III (pH gradient)	4.0	85.2	–	–
Sephadex G-150	3.2	154	5.4	151

All steps were carried out at +4°C including Procion–Sephadex elution. (See Materials and methods and fig.4 for details). The enzyme is homogeneous by both methods after the Sephadex G-150 column.

the dye from the enzyme was achieved by dialysis against activated charcoal, chromatography on QAE-Sephadex or running the enzyme-dye solution through Sephadex G-25 in 0.5 M NaCl at pH 9.0.

The usefulness of a Procion HE3B–Sephadex matrix in the large-scale purification of the enzyme was investigated. Carboxypeptidase G present in cell extracts of *Pseudomonas* ATCC 25301 was initially purified by batch adsorption and elution on SP–Sephadex. This step gave an 11-fold increase in specific activity and together with the subsequent acidification, served to remove pteroic acid, which inhibited binding of the enzyme to the Procion column. The binding capacity of the Procion column for the enzyme at this stage of enzyme purification (2.5 units/mg protein) was 20–70 U/ml matrix, i.e., approximately 0.33 mg enzyme protein per ml matrix, compared with 100–250 U/ml matrix for enzyme at stages of purification corresponding to specific activities of 10–70 U/mg protein (table 2).

The enzyme was eluted from the Procion column as a sharp peak with 2 mM *p*-aminobenzoyl glutamate in 20 mM Tris–acetate, pH 7.0 (fig.4). The exact point at which enzyme was eluted from the matrix was found to vary with the ratio of enzyme: matrix and the column flow rate. Slow flow rates allowed appreciable enzyme catalysed substrate hydrolysis, even at 4°C and excess matrix, i.e., more than the amount required to bind all the enzyme, resulted in the rebinding of enzyme lower down the column. This retarded the elution position from coincident with the applied substrate front to 1 to 2 column volumes behind the front. Following removal of *p*-aminobenzoyl glutamate, which interferes with protein determination, assay showed that carboxypeptidase G specific activity had been increased 30-fold to give approximately 50% homogeneous enzyme (table 2) and SDS-polyacrylamide gel electrophoresis showed only 2 major protein bands after the Procion column (fig.5). Subsequently

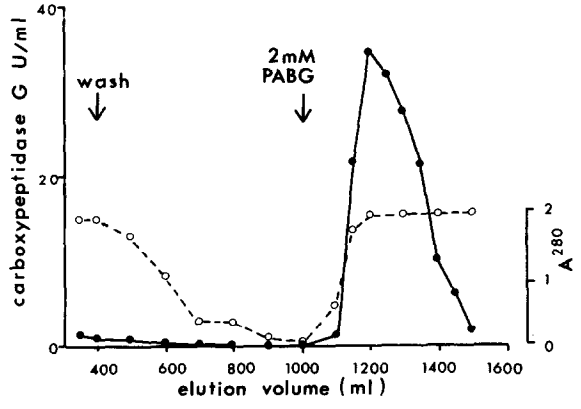


Fig.4. Elution of carboxypeptidase G from Procion Red HE3B–Sephadex G-100 with *p*-aminobenzoyl glutamate. 400 ml post SP–Sephadex pool (see Materials and methods) was loaded on to a 320 ml Procion Red HE3B–Sephadex G-100 column (20 X 4.4 cm) equilibrated with 20 mM Tris–acetate pH 7.0, at +4°C and 100 ml/h. The column was then washed with 600 ml 20 mM Tris–acetate, pH 7.0 followed by 600 ml 2 mM *p*-aminobenzoyl glutamate (PABG) in the same buffer. Flow rate was maintained at 100 ml/h and 10 ml fractions were collected.

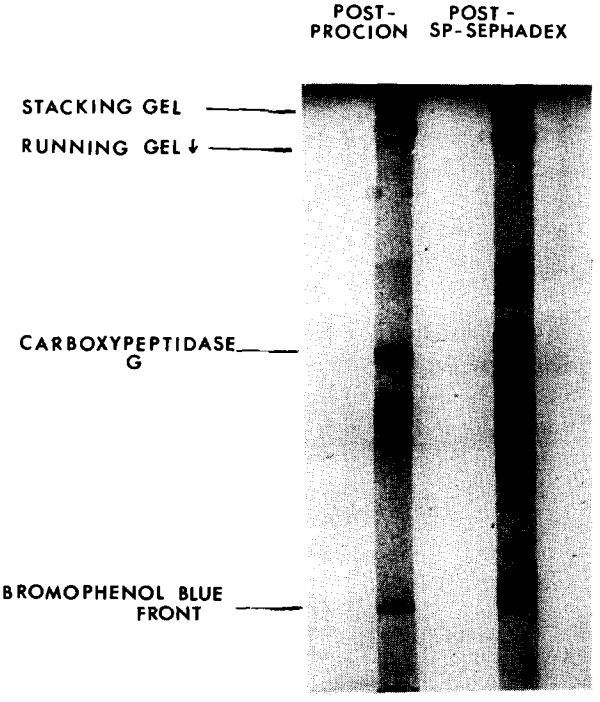


Fig.5. SDS-polyacrylamide gels of pre- and post-Procion HE3B–Sephadex G-100 column samples during large-scale purification of carboxypeptidase G. Samples (50 μl containing 1–2 mg/ml protein) of the post SP–Sephadex pool and the post Procion HE3B–Sephadex G-100 pool (see Materials and methods) were run on 10% SDS-polyacrylamide gels (6 mm × 10 cm) at 0.5 mA per gel for 16 h. Gels were stained for protein in 0.25% Coomassie Brilliant Blue R stain (Sigma Chemical Co) in 50% methanol/10% acetic acid (2 h) and then washed repeatedly in a 25% methanol/10% acetic acid mixture.

purification of the enzyme to homogeneity was achieved by gel filtration on Sephadex G-150 (table 2).

The resolving power of this technique as applied to carboxypeptidase G purification can be seen in table 2; the purification scheme using the Procion matrix contains fewer steps and overall gives a 50% higher recovery of enzyme. While it might be argued that the binding of carboxypeptidase G to Procion–Sephadex and SP–Sephadex is a similar process due to the presence of sulphonic acid-type residues in both resins, evidence that enzyme binding to the former is pseudo-affinity and to the latter is ionic may be inferred from the inability of *p*-aminobenzoyl glutamate to elute the enzyme from SP–Sephadex, and the fact that the Procion dye HE3B is a competitive inhibitor of methotrexate and *p*-aminobenzoyl glutamate hydrolysis. Comparison of the structure of the procion dyes with folic acid (see fig.1) suggests that strongly acidic and hydrophobic areas present in the molecule are important for enzyme binding. The correct orientation of such groupings is however critical, since relatively small differences in dye structure dramatically affects the binding of carboxypeptidase G to the particular Procion–Sephadex matrix.

Previous work has shown that other enzymes may be bound to Blue dextran and Blue Dextran–Sepharose, e.g., pyruvate kinase [7], phosphofructokinase [8] and more recently orotate phosphoribosyl transferase and orotidylate decarboxylase [9]. The structural similarities between nucleotide substrates and cofactors, and several Procion dyes can be demonstrated by the construction of molecular models and has recently been further indicated by the apparent affinity binding of the enzyme glycerokinase to a Procion–Sepharose column (Comer, Bruton and Atkinson, unpublished data). The usefulness of Procion and similar dyes in such systems is that they may be used in the chemically simple synthesis of inexpensive, stable and re-usable pseudo-affinity columns of wide application and that preparation of such Procion matrices does not require the use of cyanogen halides which are a potential hazard in other large-scale affinity–matrix syntheses.

Acknowledgement

The authors wish to thank the Cancer Research Campaign whose grant supported this work.

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