

## Development of Bioaffinity Chromatography for Uricase Purification

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### RINGKASAN

*Perencat urikas seperti urat, xanthin, oksonat dan sianurat dicuba sebagai ligand dalam penyediaan bahan biokeafinan dalam pengasingan kromatografi urikas. Hanya terbitan purin mempunyai kuasa penghalang, sementara terbitan s-triazin kehilangan kuasa penghalangnya terhadap urikas. Pengasingan kromatografi mengguna bahan biokeafinan memberi hasil yang lebih tinggi, dan penulenan yang lebih baik jika dibandingkan dengan teknik kromatografi biasa.*

### SUMMARY

*The uricase inhibitors urate, xanthine, oxonate and cyanurate were tried as ligands, for preparing bioaffinity support for chromatographic separation of uricase. Only derivatised purines showed inhibitory properties, whereas derivatised s-triazines seemed to lose their absorptive capacity. Chromatographic separation on a bioaffinity support produced higher yield and better purification of uricase than conventional chromatographic technique.*

### INTRODUCTION

Bioaffinity chromatography exploits the unique ability of individual proteins to bind ligands specifically and reversibly. Thus isolation of proteins by bioaffinity chromatography presents considerable advantages over conventional procedures for protein purification based on relatively small differences in physico-chemical properties between proteins in a mixture of proteins. Its use is now widespread and in principle this technique can be applied to purify enzymes, nucleic acids, hormones or hormone receptors (Cuatrecasas, 1972; Weetal, 1974).

Uricase (urate : oxygen oxidoreductase, EC 1.7.3.3) is an important enzyme for use in routine clinical analysis (Watts, 1974) and possibly for enzyme replacement therapy (Kissel *et al.* 1968). The production of highly purified uricase is very desirable for these purposes.

### MATERIALS AND METHODS

Uricase activity was assayed by measuring the amount of oxygen consumed in the enzyme-catalysed reaction, using the oxygen monitor

(Yellow Spring-Model 53) fitted to a Kipp and Zonen DB8 recorder. The reaction was carried out in 0.1M borate buffer pH 9.0 at 25°. The buffer (3.0 ml) and 10-100 $\mu$ l enzyme were equilibrated in the reaction chamber and the reaction initiated by addition of 100 $\mu$ l urate solution.

Urate solution was prepared by dissolving 50mg uric acid (BDH) and 40mg lithium carbonate in (BDH) in warm water and making up to 100ml.

One unit of uricase activity is equivalent to one  $\mu$ mole of oxygen consumed min<sup>-1</sup>.

#### *Preparation of uricase extract*

Uricase extract was prepared from porcine liver by the following procedure.

- a) Homogenation in alkaline buffer
  - b) Heat treatment
  - c) n-Butanol separation
  - d) Ammonium sulphate precipitation
- Details of the procedure will be presented in a paper (in preparation) or may be referred to Salleh (1978).

*Ion-exchange chromatography*

DEAE-cellulose (DE-52) was prepared for use according to the instruction of the manufacturer (Whatman). The ion-exchanger was packed in a glass column (1 × 12 cm) to a height of 10 cm and equilibrated with 10mM borate buffer at the pH under investigation. Trials were carried out at pH 8.5, 9.0 and 9.5.

The enzyme extract was initially dialysed in the equilibrating buffer for 16h. The dialysed sample (5 ml) was applied to the column and washed with the equilibrating buffer at 0.5ml. min<sup>-1</sup>. The elution was carried out using a linear salt gradient (0-0.25M) of ammonium sulphate or sodium chloride.

Protein was monitored at 280nm.

*Bioaffinity chromatography*

Sepharose 4B (Pharmacia) was activated with 1,4-butanediol diglycidyl ether (Aldrich) by the method developed by Sunberg and Porath (1974) and oxirane group determination was carried out as set out by the same workers.

Uric acid (BDH), xanthine (Sigma), potassium oxonate (Aldrich) and cyanuric acid (Aldrich) were tested as ligands. Each substance (1g) was dissolved in distilled water and adjusted to pH 12.0 with 1M NaOH, and made up to 100ml. Activated Sepharose was incubated with the solution of potential ligand for 20h at 25°. The Sepharose-ligand was washed with 0.1M NaOH and distilled water. The amount of ligand coupled to the gel was determined by the Kjeldahl method (Salleh, 1978).

To eliminate uncoupled oxirane groups on the Sepharose the gel was reincubated in 1M n-ethanolamine for a further 12h.

The chromatographic column was prepared by packing 2g suction-dried Sepharose-ligand in a glass column (1 × 7cm) and the gel equilibrated with 0.1M borate containing 1mM EDTA pH 9.0.

Uricase extract was dialysed in the equilibrating buffer for 16h prior to application to the column.

Different flow rates were tested to achieve adsorption. Uricase adsorbed onto the affinity support was eluted by 25mm oxonate in 0.1M borate buffer pH 9.0. The enzyme was separated from the inhibitor by gel filtration through a column of Sephadex G-25 (Pharmacia).

**RESULTS**

The elution profile shown in Fig. 1 represent typical results obtained by chromatographic separation on a column of DEAE-cellulose. At pH 8.5, 95% of uricase activity in the input sample (9 units) were adsorped onto a 7ml DEAE-cellulose bed, but only 10 to 15% of the activity were finally eluted. About 5-fold purification was achieved in the uricase recovered. Variation of pH in adsorption and elution processes did not significantly alter the elution profile. Using either ammonium sulphate or sodium chloride in the elution did not show any significant variation. A similar elution profile was obtained when ammonium sulphate or sodium chloride gradients were used.

In the activation of Sepharose, 28-30 mole of oxirane group g<sup>-1</sup> of suction dried gel was obtained. Table 1 summarises the results obtained when different ligand was incubated with the activated Sepharose.

TABLE 1

Amount of ligands attached to activated Sepharose 4B. Each value is the average of three preparations.

Concentration of coupling solution	$\mu$ moles ligand g <sup>-1</sup> . gel
Uric acid	
1g/100ml (0.059M)	14.35
Xanthine	
1g/100ml (0.066M)	19.1
Cyanuric acid	
1g/100ml (0.078M)	13.8
Oxanic acid	
1g/100ml (0.064M)	5.2

Fig. 2 shows the capacity of Sepharose-urate and Sepharose-xanthine as adsorbents for uricase. Although there was more xanthine bound per g gel (Table 1) the capacity of the Sepharose-xanthine was only about 65% that of Sepharose-urate.

No adsorbtion was achieved when uricase extract was passed through Sepharose-oxonate and Sepharose-cyanurate supports, even at very low flow rate. No adsorbtion was achieved by the batch method.

Fig. 3 shows the adsorption and elution profile of uricase on the Sepharose-urate support.

BIOAFFINITY CHROMATOGRAPHY FOR URICASE PURIFICATION

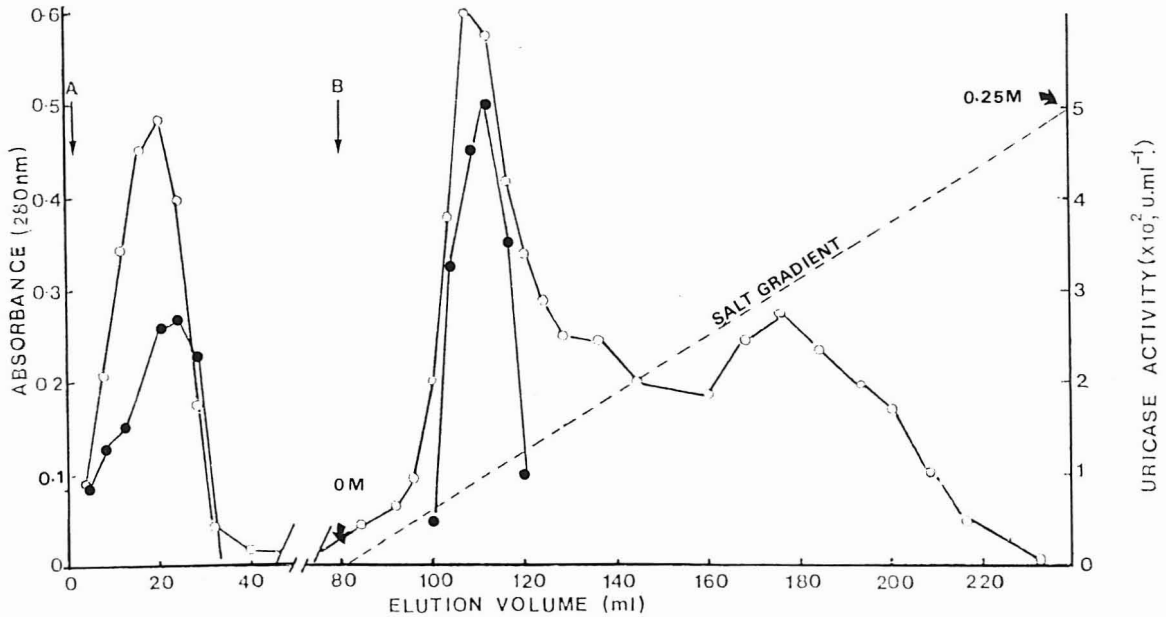


Fig. 1. Elution profile of uricase separation on DEAE - cellulose. (●) indicates uricase activity (○) indicates protein concentration and (---) represents salt gradient. The sample was applied at point A and the salt gradient at point B.

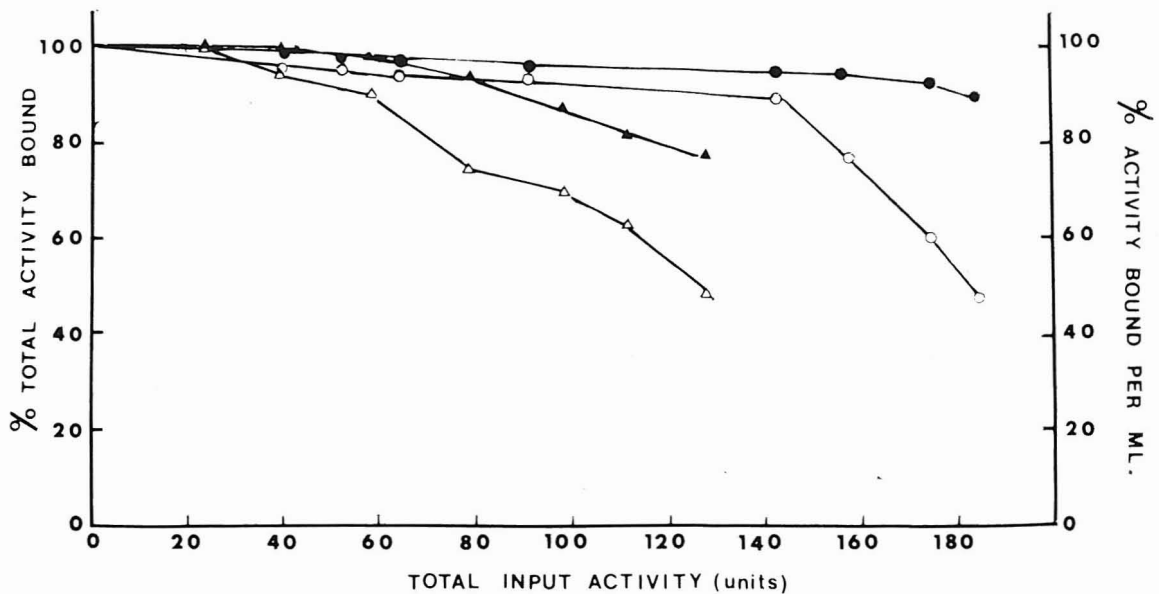


Fig. 2. The capacity of Sepharose ligand for bioaffinity binding of uricase. The percentage of total activity adsorbed onto Sepharose-urate (●) and Sepharose-xanthine (○) and the percentage of activity bound per ml of input sample onto Sepharose-urate (▲) and Sepharose-xanthine (△) are illustrated.

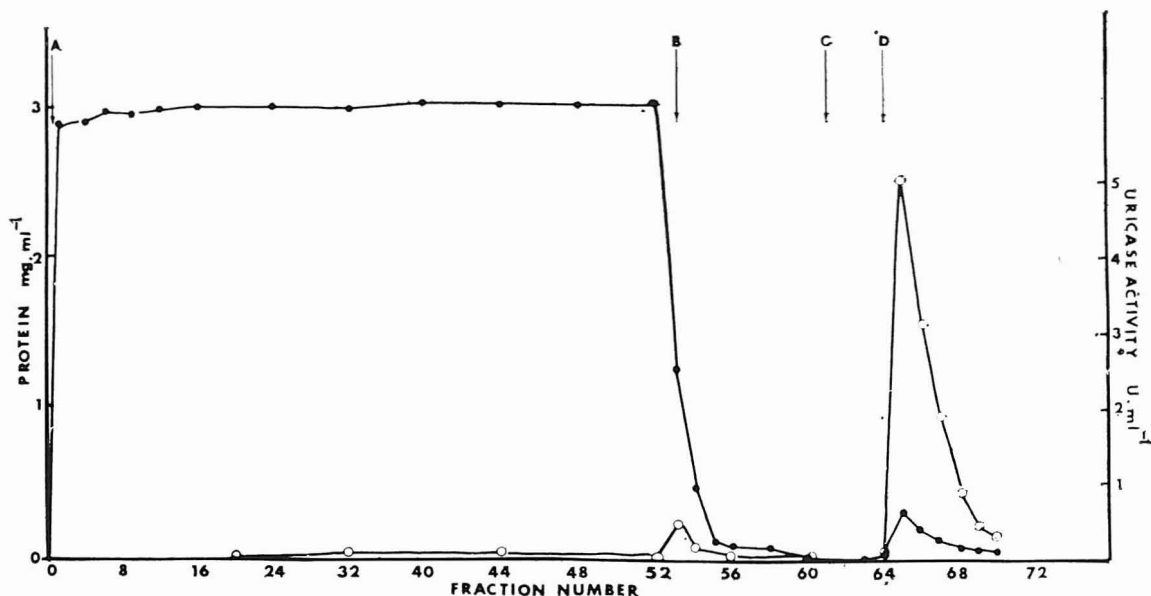


Fig. 3. Adsorption and elution profile of uricase on Sepharose-urate. (○) denotes uricase activity, and (●) denotes protein concentration. Sample was applied at point A, buffer containing 0.5M NaCl at point B, equilibrating buffer at point C and eluant was applied at point D. 8 ml fractions were collected. A total of 117 units of uricase was applied and 85 units were recovered in the eluates.

About 70% of total input activity of enzyme was recovered. This is very much higher than the recovery from ion-exchange chromatography. About 80 fold purification was achieved using either Sepharose-urate or Sepharose-xanthine support.

## DISCUSSION

The behaviour of uricase on DEAE-cellulose may be explained by the high affinity between opposite charges of the enzyme and the ion-exchanger, indicating that the uricase molecules are polyanionic under the conditions studied. Truscoe (1967) showed that uricase was irreversibly inhibited by cationic detergents, comprising quaternary ammonium salts, due to the formation of complexes. In another work, long chain alkyl groups seemed to be involved in the inhibitory effect (Truscoe, 1968). Nevertheless, the formation of these complexes may contribute to the inability to elute uricase from the ion-exchanger.

Fig. 4 shows the possible structures of the Sepharose-ligand complexes. The affinity for uricase of Sepharose-urate and Sepharose-

xanthine showed that the derivatisation process did not eliminate their bioaffinity characteristic. It is rather surprising that derivatised oxonate and cyanurate showed no affinity at all for uricase. Fridovich (1965) showed that oxonic acid ( $k_i - 1 \times 10^{-5}M$ ) and cyanuric acid ( $k_i - 3 \times 10^{-5}M$ ) were competitive inhibitors of uricase. Other s-triazines were also shown to be inhibitory to a lesser degree. The s-triazine ring which is similar in part to the purine ring was considered to be the essential configuration for the inhibitory properties. However, our experiments showed that derivatised s-triazines actually lose their inhibitory property. This may be due to curling up of the long linkage arm between support and ligand, making the latter inaccessible to the enzyme. Otherwise, the attachment of a long methylene chain to the s-triazine ring has completely altered its enzyme specificity.

When this work begun no procedure for the purification of uricase by affinity chromatography had been published. However, a procedure in which 8-aminoxanthine was used as a ligand, with comparable purification achieved, was published by Watanabe and Suga (1978 a, b).

# BIOAFFINITY CHROMATOGRAPHY FOR URICASE PURIFICATION

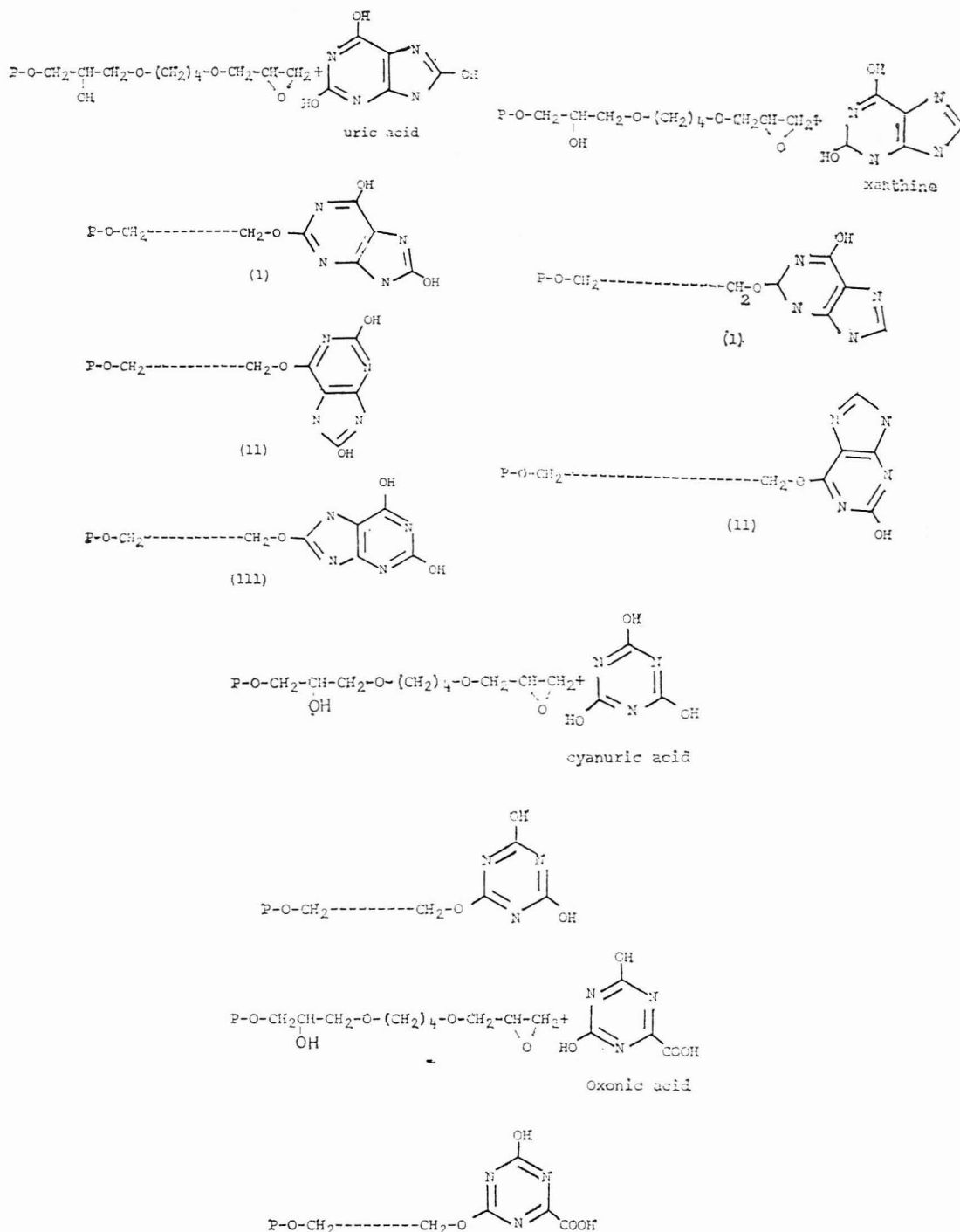


Fig. 4. Possible structures of Sepharose-ligand supports.

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