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RESEARCH NOTES

AFFINITY CHROMATOGRAPHY PURIFICATION OF UROKINASE WITH EPICHLOROHYDRIN ACTIVATED AGAROSE MATRIX*

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1 INTRODUCTION

In literature, most matrices of affinity chromatography for urokinase (EC 3.4.99.26) purification were prepared by cyanogen bromide activation[1－4]. However, these adsorbents usually suffered from the drawback of leakage of the ligand, particularly in alkaline medium, because of the instability of the isourea linkage between the ligand and the spacer or agarose. Moreover, the positively charged imido group of the *N*-substituted isourea derivative and the hydrophobicity of the spacers might promote nonspecific adsorption. On the contrary, the adsorbents prepared by the method of epoxy-activated agarose are stable under alkaline medium and exhibit lower nonspecific adsorption, since the ether or alkylamine bond formed between ligand and agarose is stable and the spacer introduced is hydrophilic[5－7]. In this study, an epichlorohydrin activated matrix was used for the purification of urokinase. The preparation strategy was: agarose activated by epichlorohydrin forming epoxy-activated gel, the gel suspended in concentrated ammonia solution converting into aminated gel, and the latter reacted with succinic anhydride forming carboxyl gel. Finally, the carboxyl gel was coupled with *p*-aminobenzamidine (*p*-AB) in the presence of *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimidehydrochloride (EDC) to form the affinity gel (See Fig.1).

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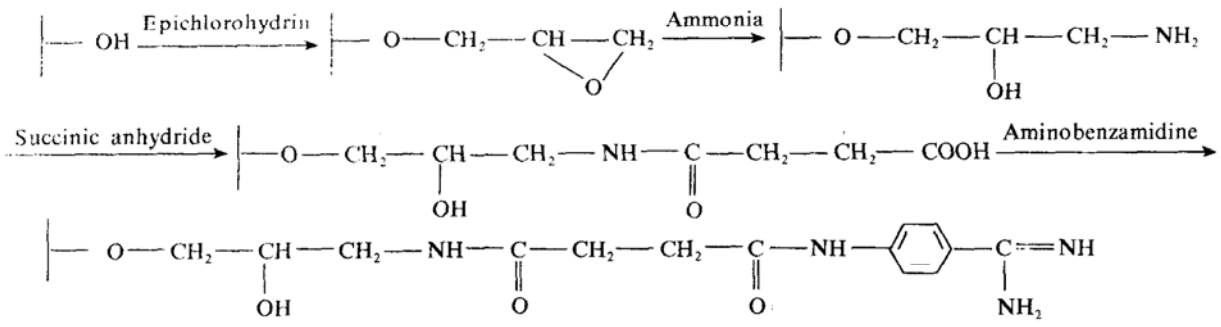

Figure 1 Reaction sequence for the preparation of affinity gel

2 MATERIALS AND METHOD

2.1 Materials

Sepharose 4B was purchased from Pharmacia; *p*-AB was from Sigma Co.; EDC was obtained from Merck Co. Epichlorohydrin and succinic anhydride were of analytical grade; Bovine thrombin, plasminogen, fibrinogen and urokinase reference standard were from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing. Crude urokinase was obtained from Shanghai Institute of Pharmaceutical Industry.

2.2 Affinity media preparation

Three variants of media A, B, C of varying ligand densities were prepared as follows: Three 12 g portions of washed and suction-dried agarose beads were each suspended in 15 ml of water and mixed with 6.5 ml of 2 mol · L^{-1} NaOH, then 1.0 ml, 1.2 ml and 1.5 ml of epichlorohydrin, respectively were added to each individual suspension. The suspensions were incubated at 40 ℃ for 2h with shaking, then transferred to a sintered glass filter and the gels were washed extensively with water and suction-fried. The next two steps of amination and succinylation were identical for the three variants.

The epoxy-activated agarose beads were suspended in 15 ml of concentrated ammonia solution, incubated at 40 ℃ for 3 h with shaking, then transferred to a sintered filter and the gel was in turn washed with water, 1 mol · L^{-1} NaCl and water.

The aminated agarose beads were suspended in 10 ml of 0.1 mol · L^{-1} NaCl. And 1.5g of succinic anhydride was added gradually with pH value maintained at 6 by adding solid NaOH. Then the suspension was allowed to stand for 5 h, filtered and washed with water, 1 mol · L^{-1} NaCl and water.

The three variants of succinylated agarose beads were each suspended in 10 ml of water, then 70,100 and 150 mg of *p*-AB and 100,150 and 200 mg of EDC were added to each suspension respectively. The suspensions were maintained at room temperature for 12 h with shaking and pH value was adjusted at 4.5−6.0 with HCl. After the completion of the reaction, the resultant affinity gels were extensively washed with water, 1 mol · L^{-1} NaCl and water and stored in 0.02% sodium azide at 4 ℃ .

2.3 Affinity chromatography

Affinity gel was packed in a 1×15cm chromatographic column, equilibrated with 0.05 mol · L^{-1} phosphate buffer, pH 7.2, containing 0.5 mol · L^{-1} NaCl at 4−10 ℃. A 20 ml of crude urokinase solution of 30,000 IU · ml^{-1} concentration was applied to the

column at a flow rate of 0.2 ml • min^{-1}. The solution pH value and electric conductivity were pre-adjusted to be the same as those of the equilibrating buffer. The column was then washed with the buffer. Elution was performed with 0.1 mol • L^{-1} acetate buffer, pH 4.0, containing 0.5 mol • L^{-1} NaCl at a flow rate of 0.2ml • min^{-1} with the absorbance A_{280nm} recorded. The protein and enzyme activity of the eluate were determined.

2.4 Binding kinetics

An 8.0g of suction-dried affinity gel was equilibrated with 0.05mol • L^{-1} phosphate buffer, pH 7.2, containing 0.5 mol • L^{-1} NaCl, and filtered. A 12 ml of crude urokinase in the same buffer solution of 30000 IU • ml^{-1} concentration was mixed with the affinity gel and stirred at 8 – 10 ℃. Samples were taken at 0, 5, 20, 40, 60, 90 and 120 min intervals, and centrifuged; the supernatant enzyme activity was determined.

2.5 Assay methods

Epoxy groups in epoxy-activated gel were determined by the method of Sundberg and Porath[8]. Carboxyl group and ligand in the wet gel were determined by the method of Hixson and Nishikawa[9]. The complete substitution of free amino group in aminated gel was checked by ninhydrin test[10]. 0.1g of succinylated gel was added to 1 ml of 0.1% ninhydrin water solution, and the mixture was boiled. Purple color developed was the positive sign of the presence of amino group. Urokinase activity was determined by the bubbling method[11]. Protein was determined by the absorbance at 280 nm (A_{280}).

3 RESULTS AND DISCUSSION

Three variants of epoxy-activated gels with different epoxy group densities were prepared using different quantities of epichlorohydrin. This might bring about some degree of cross-linkage. The cross-linkage could increase the stability of the matrix with little influence on the permeability[5].

p-AB could directly coupled with the epoxy gel, but the reaction activity was low[6], demanding an excess amount of *p*-AB. Moreover, direct coupling might lead to a spacer with insufficient length. The present method used succinic anhydride to extend the spacer length, forming a gel with terminal carboxyl functionality. The coupling reactivity of *p*-AB with carboxyl gel was much higher than that with epoxy-gel[6]. The ligand density of the affinity gel was controlled by the quantities of *p*-AB and EDC added. Some carboxyl groups might have remained intact in the affinity gel. It was believed that residual carboxyl groups might benefit the affinity chromatography of the urokinase[2]. The active group densities of prepared and intermediate gel were shown in Table 1.

The elution curves from matrices A, B, C were similar, and hence only that from matrix A was shown in Fig. 2. The overall results of chromatographic separation were summarized in Table 2.

The density of *p*-AB on affinity gel had great influence on the adsorption and elution of urokinase. The results shown in Table 2 suggested that a low-ligand-density gel (matrix A) might not have the urokinase completely bound in solution, thereby resulting in a low recovery. On the other hand, a high-ligand-density gel (matrix

Table 1 Densities of active groups in various gels during different steps of preparation [μ mol·(g wet gel)$^{-1}$]

Medium	Epoxy①	Carboxyl②	Ligand③	Residual carboxyl
A	47	45	22	23
B	70	67	39	28
C	81	78	54	24

① in epoxyl-activated gel;
② in succinylated gel;
③ in finished gel.

Table 2 Results of affinity chromatography purification of urokinase in media of different ligand densities

Medium	Recovery, %	Specific activity, IU·A_{280}^{-1}	Purification factor
A	55	72000	144
B	89	68000	136
C	92	54000	108

C) might bind much more other proteins due to low urokinase-specificity of *p*-AB, thereby resulting in a low purification factor. As a compromise, matrix B with a ligand density of ca. 40 μmol·(g wet gel)$^{-1}$ appeared to be the most satisfactory one.

The batch binding curves of urokinase on matrices A, B, C were shown in Fig.3. For matrix A urokinase needed a longer time, about 1h, to attain adsorption equilibrium. The residual concentration of urokinase in the solution was 17000 IU·ml^{-1}. For both matrices B and C adsorption equilibrium was reached in about 20 min. The residual concentration of urokinase were as low as 2900 IU·ml^{-1} and 1800 IU·ml^{-1} respectively. The urokinase concentration in the solution decreased rapidly by 40% in the first 5 min. The quantity and rate of adsorption of urokinase on affinity gel increased rapidly with increases in ligand density to an extent of about 40μmol·g^{-1}, beyond which no significant increases were observed.

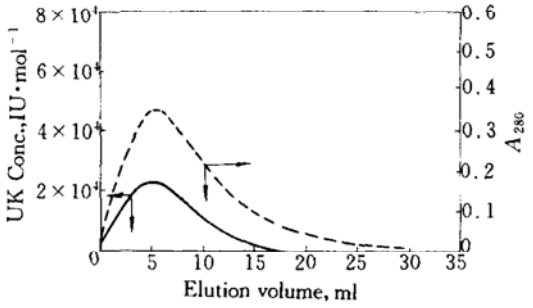

Figure 2 Elution curve from matrix A

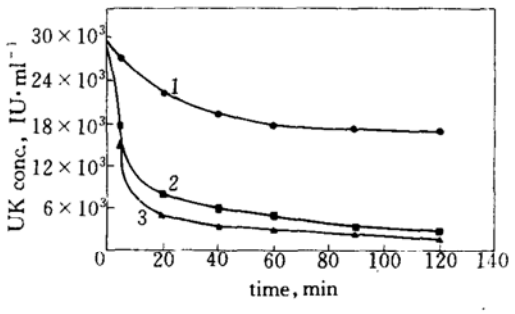

Figure 3 Binding kinetics of urokinase on matrix A, B, C
1—Matrix A; 2—Matrix B; 3—Matrix C

In this study, the specific activity of the urokinase used was as low as 500 IU·A_{280}^{-1}. The purity of urokinase could not meet the demand through a single chromatography

treatment. To avoid this shortcoming repeated chromatography or pre-purification with other methods for the crude urokinase would be needed.

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