

Preparation of High-Purity Urokinase Using Single-Step Hydrophobic Interaction Chromatography with p-Aminobenzamidine Ligand

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Abstract A novel process for urokinase purification was studied using p-aminobenzamidine as the ligand and sepharose 4B as the matrix. The adsorption, washing, and elution conditions were optimized by an unusual method. An adsorption buffer containing 2.5 M NaCl and 1% Tween 80 facilitated the adsorption of urokinase on the affinity media and prevented contaminants from binding to the p-aminobenzamidine affinity gel. It was found that 5% Tween 80 removed most of the contaminants from the affinity column. A 0.2 M glycine elution buffer containing 0.5 M NaCl (pH 3.0) was found to have a strong elution ability with a high recovery and purity of urokinase. A crude urokinase material of 231 IU/mg protein from human urine was purified to 124,300 IU/mg protein with a purification factor of 538 and yield of 86.7%. As a result, a high purity urokinase was obtained with only a single affinity chromatography step. The purification process was successfully scaled-up to a 2-l chromatography column. The resulting urokinase eluate could be directly lyophilized, thereby complying with Chinese pharmacopoeia (1995 version) standards.

Key words: Urokinase, purification, p-aminobenzamidine, Tween 80, glycine

Urokinase (EC.3.4.99.26), a serine protease, is a plasminogen activator that is widely used for thrombolysis. In general, a crude urokinase is purified to a high purity (more than 100,000 IU/mg protein) using a series of steps, including ion exchange, ultrafiltration concentration or ultrafiltration desalination, affinity chromatographies, gel chromatography, etc. Affinity chromatography is the most popular purification step, yet a single affinity chromatography step is unable

to obtain high purity urokinase from the crude material from human urine. As such, various ligands such as p-aminobenzamidine, α -N-benzenesulfonyl p-aminophenylamine, pGlu-Lye-Leu-Argal, benzidine, Dye, β -lactamine antibiotics, and monoclonal antibody [2, 8, 15, 17, 18, 19, 24], and various chromatography media such as Sepharose 4B, Sepharan HEMA, polystyrene-based medium, and macropore glass beads were used for the purification of urokinase [3, 6, 13, 21, 22]. Currently, p-aminobenzamidine and sepharose 4B are widely used as the ligand and matrix for urokinase purification, respectively. Usually, the affinity chromatography purification process is composed of the preparation of p-AB affinity media with cyanogen bromide activation, adsorption with phosphate buffer containing 0.4 M NaCl, washing with the above phosphate buffer, and elution with 0.1 M acetic acid buffer (pH 4.0) [8]. In the present study, a p-AB-Sepharose 4B affinity gel was prepared using four chemical reactions. The adsorption, washing, and elution conditions were investigated by systematic optimization, and the optimal conditions of adsorption, washing, and elution were found to be quite different from those in previous reports. Employing the above optimized conditions, crude urokinase material from human urine was purified to a high purity using just a single affinity chromatography.

MATERIALS AND METHODS

Materials and Apparatus

The Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden); the p-aminobenzamidine (p-AB) and Glu-Gly-Arg 7-amino-4-methylcoumarin (GGA-MCA) were from Sigma Chemicals Co. (St. Louis, U.S.A.). The N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimidehydrochloride (EDC) was obtained from Merck Co. (Darmstadt, Germany). The

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epichlorohydrin, succinic anhydride, and Tween 80 were of analytical grade. The bovine thrombin, plasminogen, fibrinogen, and urokinase reference standard were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The crude urokinase, prepared by a silica adsorption and salting out process from human urine, was obtained from the Shanghai Institute of Pharmaceutical Industry (Shanghai, China). The F 950 fluorospectrophotometer and 752 UV spectrophotometer were made by Shanghai No. 3 analytical instruments Factory (Shanghai, China). The chromatography column with a UV detector and fractionation collector was made by Pharmacia (Uppsala, Sweden).

Assay Methods

The epoxy groups in the epoxy activated gel were determined by the method of Sundberg and Porath [20]. The amino group was titrated by the method of Inman [9]. The carboxyl group and p-AB ligand in the wet gel were determined by the method of Hixson and Nishikawa [7]. The p-AB ligand density bound on the matrix was determined by measuring the absorbancy at 292 nm before and after the coupling reaction (p-AB exhibits a strong absorption at this wavelength with $E_{1\text{ cm}}^{1\%}=800$) [1]. The complete substitution of a free amino group in the aminated gel was checked by a ninhydrin test. One-tenth g of a succinylated gel was added to 1 ml of a 0.1% ninhydrin water solution and the mixture was boiled. The development of a purple color indicated the presence of an amino group. The urokinase activity was determined spectrofluorometrically by using GGA-MCA as the fluorogenic substrate, with the excitation at 380 nm and emission at 460 nm [12]. The activity of the final purified urokinase was compared with that produced by the bubbling method [14]. The protein concentration was determined by the absorbance at 280 nm (A_{280}). For the final purified urokinase, the protein concentration was determined by the A_{280} value with a coefficient of 0.74 ($E_{1\text{ cm}}^{1\%}=13.6$) [23].

Affinity Media Preparation

The affinity gel was prepared according to Cao *et al.* with modifications [4]. The reaction sequence is shown in

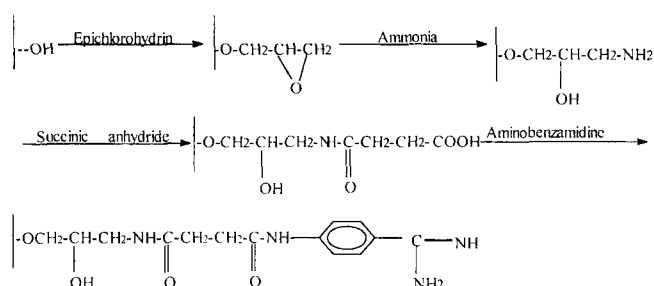


Fig. 1. Reaction sequence for the preparation of affinity gel.

Fig. 1. One hundred and fifty g of washed and suction-dried sepharose 4B beads was suspended in 250 ml of 0.4 M NaOH, then 60 ml of epichlorohydrin was added to the suspension. The suspension was incubated at 40°C for 2 h with shaking, transferred to a sintered glass filter, then the gel was washed extensively with water and suction-dried. The epoxy-activated sepharose 4B beads were suspended in 300 ml of a concentrated ammonia solution, incubated at 40°C for 3 h with shaking, then transferred to a sintered filter and the gel was successively washed with water, 1 M NaCl, then water. The aminated sepharose 4B beads were suspended in 200 ml of 0.1 M NaCl, then 50 g of succinic anhydride was added gradually while the pH was maintained at 6.0 by the addition of 5 M NaOH. The suspension was then allowed to stand for 5 h, filtered, and washed with water, 1 M NaCl, and water in turn. The succinylated sepharose 4B beads were suspended in 200 ml of water, then 1.5 g of p-AB and 2.0 g of EDC were added to the suspension. The suspension was maintained at room temperature for 12 h with shaking, while the pH value was adjusted to 4.2–4.6 with 1.0 M HCl. After the completion of the reaction, the resultant affinity gel was extensively washed with water, 1.0 M NaCl, and water, and stored in 0.02% sodium azide at 4°C.

Preparation of Crude Urokinase Solution

The solid crude urokinase material was mixed with 0.05 M Na_2HPO_4 (ratio of crude urokinase weight to buffer volume was 1:4), stirred for 2 h at 8°C, and then filtered under a vacuum. The filtrate was collected, then NaCl and Tween 80 were added so that the solution contained 2.5 M NaCl and 1% Tween 80. The pH of the solution was adjusted to 7.0 or otherwise specified pHs using 1 M NaOH or 1 M HCl.

Selection of Adsorption Conditions

One gram of a wet p-AB gel suction-dried was added to 10 ml of a crude urokinase solution (11,083 IU/ml) at pH 7.0, 7.2, 7.5, 7.8, and 8.0 (0.05 M phosphate and 2.5 M NaCl), with 0.5, 1.0, 1.5, 2.0, and 2.5 M NaCl (pH 7.0 and 0.05 M phosphate) and 0.1, 0.2, 0.5, and 1.0% Tween 80 (pH 7.0, 0.05 M phosphate and 2.5 M NaCl). All the samples were shaken for 8 hours at 8°C. The residual urokinase concentration was measured and the amount of adsorbed urokinase per gram of wet gel was calculated.

Selection of Washing Conditions

Eighty milliliters of crude urokinase (11,083 IU/ml) was mixed with 20 g of a wet p-AB gel (pH 7.0, 0.05 M phosphate, 2.5 M NaCl, and 1% Tween 80) and shaken for 8 h at 8°C. The loaded gel was filtered under a vacuum. The amount of urokinase bound to the gel was 24,100 IU/g wet gel. The wet gel, 1.0 g each, was then placed in test tubes. Ten milliliters of different washing solutions with

different concentrations were added to the test tubes. The tubes were then shaken for 8 h and the concentration of urokinase in the resulting washing solution was measured.

Selection of Elution Conditions

Eighty milliliters of a urokinase solution of 10,108 IU/ml (0.05 M phosphate, 2.5 M NaCl, and 1% Tween 80 at pH 7.0) was loaded on a column (1.520 cm) packed with 20 g of the wet gel (previously equilibrated by the same buffer) at a 0.2 ml/min flow rate. The loaded gel was washed with 50 ml of 5% Tween 80, and it was then taken out from the column and filtered under a vacuum. The activity of urokinase bound to the gel was 61,224 IU/g wet gel. One g each of the wet resin was placed in test tubes. Ten milliliters of different eluants with different concentrations and pHs were added to the test tubes. The tubes were then shaken for 8 h and the concentration of urokinase in the eluate was measured.

Breakthrough

A column (1.0×12 cm) was packed with 10 g of a wet p-AB gel and equilibrated with 0.05 M phosphate, 2.5 M NaCl, and 1% Tween 80 at pH 7.0. 11,083 IU/ml of urokinase (180 ml) containing the same buffer was then applied to the column at a flow rate of 0.2 ml/min. The effluent was collected and the urokinase concentration was measured.

Column Purification of Urokinase

One hundred milliliters of a crude urokinase solution of 11,083 IU/ml (100 ml) containing 0.05 M phosphate, 2.5 M NaCl, and 1% Tween 80 at pH 7.0 was applied to a column (1.0×12 cm) with 10 g of a wet p-AB gel (equilibrated by the same buffer) at a flow rate of 0.2 ml/min. The column was then washed with 50 ml of 5% Tween 80 and the loaded urokinase was eluted using 0.2 M glycine containing 0.5 M NaCl (pH 3.0). The concentrations of urokinase and protein in each stage were measured.

RESULTS AND DISCUSSION

Affinity Media Preparation

The affinity gel preparation was carried out by a reaction involving four steps. The epoxy, amino, carboxyl, and p-AB density was 88, 79, 79, and 44 $\mu\text{mol/g}$ wet gel, respectively. Thirty five $\mu\text{mol/g}$ of the wet gel carboxyl groups remained intact in the affinity gel. The p-AB directly reacted with the epoxy gel, yet the reaction activity was low and required a large excess of p-AB. Since direct coupling can lead to a spacer with an insufficient length, the present method used succinic anhydride to extend the spacer length, thereby forming a gel with a terminal carboxyl functionality. The coupling reactivity of p-AB

with the carboxyl gel was much higher than that with the epoxy-gel [10]. The ligand density of the affinity gel was controlled according to the quantities of p-AB and EDC added. Epichlorohydrin activation can bring about some degree of cross-linkage, which can increase the stability of the matrix without influencing the permeability [11]. The remaining epoxy groups are automatically hydrolyzed in a water environment, and have no influence on the later purification process. In addition, the residual amino functionality can enhance the nonspecific adsorption. Consequently, an excess amount of succinic anhydride was used to react with the aminated gel in the reaction step 2 (see Fig. 1), so that no free amino group would remain. This was confirmed by a negative ninhydrin test. The level of carboxyl groups in the succinylated gel was 79 $\mu\text{mol/g}$ wet gel, which also indicated the level of amino groups, thereby demonstrating that the conversion was complete. Parts of the carboxyl groups may have remained intact in the affinity gel; however, it is also believed that residual carboxyl groups can benefit the affinity chromatography of urokinase [16]. Most of the contaminant proteins had a low isoelectric point and were negatively charged at an adsorption pH of around 7.0, having been expelled from the carboxy-bearing matrix, whereas urokinase had a high isoelectric point (pI 8.8) and was positively charged, thereby favoring adsorption to the negatively charged matrix. As such, the carboxyl group density was controlled at a proper ratio. The preparation of the affinity gel was successfully scaled up to a two-liter gel volume with a good performance.

Optimization of Adsorption Conditions

The urokinase binding to the p-AB gel at pH 7.0–8.0 is shown in Fig. 2. The residual urokinase concentration and

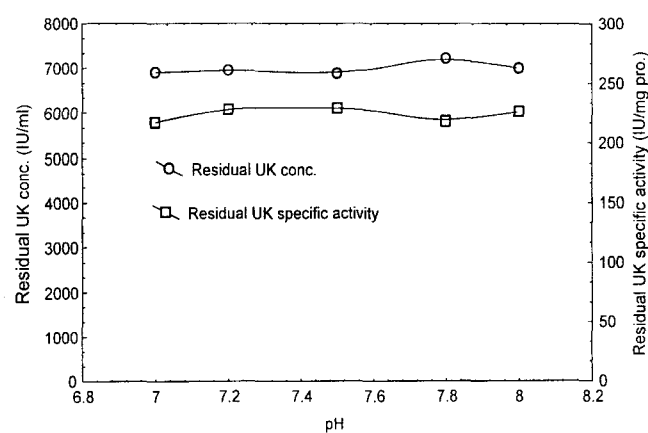


Fig. 2. Effect of phosphate pH on urokinase bound to p-AB gel. A batch adsorption test was carried out with different pHs. One gram of a wet p-AB gel suction-dried was added to 10 ml of crude urokinase solution (11,083 IU/ml), at pH 7.0, 7.2, 7.5, 7.8, and 8.0 (0.05 M phosphate and 2.5 M NaCl). All the samples were shaken for 8 h at 8°C. The residual urokinase concentration and specific activity were measured.

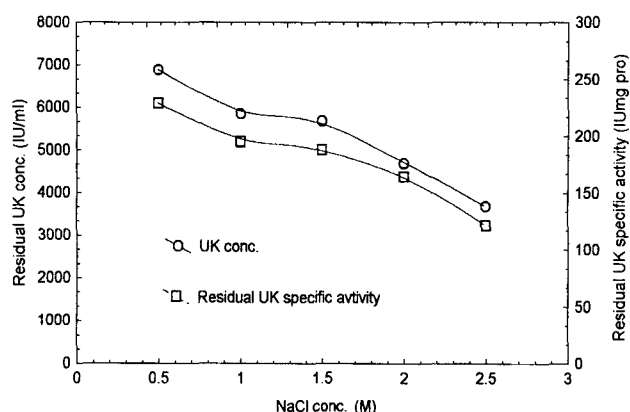


Fig. 3. Effect of NaCl concentration on urokinase bound on p-AB.

A batch adsorption test was carried out with different NaCl concentrations. One gram of a wet suction-dried p-AB gel was added to 10 ml of crude urokinase solution (11,083 IU/ml), at pH 7.0, 7.2, 7.5, 7.8, and 8.0 (0.05 M phosphate and 2.5 M NaCl). All the samples were shaken for 8 h at 8°C. The residual urokinase concentration and specific activity were measured.

specific activity were not so different, except for a slight change at pH 7.8. The residual concentrations were 7,233 IU/ml at pH 7.8 and around 6,900 IU/ml at other pH points. The specific activity of the residual urokinase slightly increased from 7.0 to 7.5 (217 and 229 IU/mg protein), and then decreased to around 218 IU/mg protein at pH 7.8. This means that the purity of the urokinase bound to the p-AB gel at pH 7.0 and 7.8 was higher than at other pH points. Therefore, the adsorption condition of pH 7.0 was found to be optimal, when considered both the maximal adsorption capacity and purity of the urokinase bound to the p-AB gel. In contrast, the NaCl concentration had an obvious influence on the urokinase bound to the p-AB gel (Fig. 3). Both the residual urokinase concentration and the specific activity decreased rapidly with an increase in the NaCl concentration. The residual concentration and specific activity at 2.5 M NaCl were 3,670 IU/ml and 121 IU/mg protein, respectively. The value of the residual concentration was almost one half of the value (6,892 IU/ml) at 0.5 M NaCl. The amount of urokinase bound to the p-AB increased by 80%. The residual urokinase specific activity was 121 IU/mg protein. The value was also almost one half of the value (229 IU/mg protein) at 0.5 M NaCl. This means that the purity of the urokinase bound to the p-AB gel increased with a high salt concentration. The p-AB ligand is a serine protease inhibitor with a benzene ring that has a hydrophobic structure and an amidine group. With a high salt concentration, the hydrophobic interaction between p-AB and urokinase is favorable. An amidine structure is also necessary to maintain the affinity between the ligand and the urokinase, because a hydrophobic interaction by itself can not obtain a high specificity. Since protein is precipitated due to salting out at a concentration

above 2.5 M NaCl, a NaCl concentration higher than 2.5 M is not recommended.

Tween 80 is a surfactant and it was expected that it would promote the hydrophobic interaction between the ligand and the target protein in hydrophobic chromatography. Sheng *et al.* used a 0.1 M phosphate buffer containing 0.1% Tween 80 as a washing step during urokinase purification using monoclonal antibodies as the ligand [17], yet no details were mentioned in their reports. In the current study, different concentrations of Tween 80 were added to the adsorption buffer and the effect on urokinase adsorption was investigated (see Fig. 4). The residual urokinase concentrations remained almost constant from 0 to 1% Tween 80, yet the residual urokinase specific activity decreased with an increase in the Tween 80 concentration. The specific activity was 98 IU/mg protein at 1% Tween 80, which was 22 IU/mg protein lower than the control. This shows that Tween 80 did inhibit contaminants from binding to the p-AB ligand, thereby benefiting the affinity between the urokinase and the ligand. It would appear that the surfactant Tween 80 promoted the binding between the hydrophobic benzene ring structure of the p-AB ligand and the hydrophobic domain of the urokinase. This phenomenon was similar to the binding of urokinase to p-AB at a high NaCl concentration, yet the exact reason for the phenomenon is still unclear.

Breakthrough Curve

The breakthrough curves of the crude urokinase solution are shown in Fig. 5. The concentration of urokinase increased when the effluent volume was between 100 and 160 ml and became constant above 160 ml. The curve was

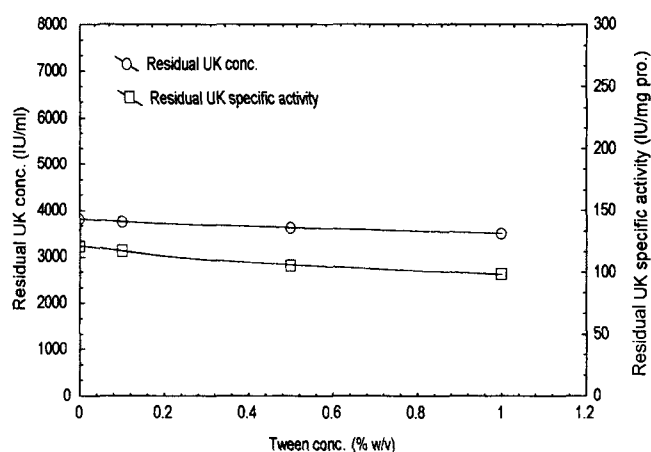


Fig. 4. Effect of Tween 80 concentration on the adsorption of urokinase to p-AB-sepharose 4B.

A batch adsorption test was carried out with different Tween 80 concentrations. One gram of a wet suction-dried p-AB gel was added to 10 ml of crude urokinase solution (11,083 IU/ml), at 0.5, 1.0, 1.5, 2.0, and 2.5 M NaCl (pH 7.0 and 0.05 M phosphate). All the samples were shaken for 8 h at 8°C. The residual urokinase concentration and specific activity were measured.

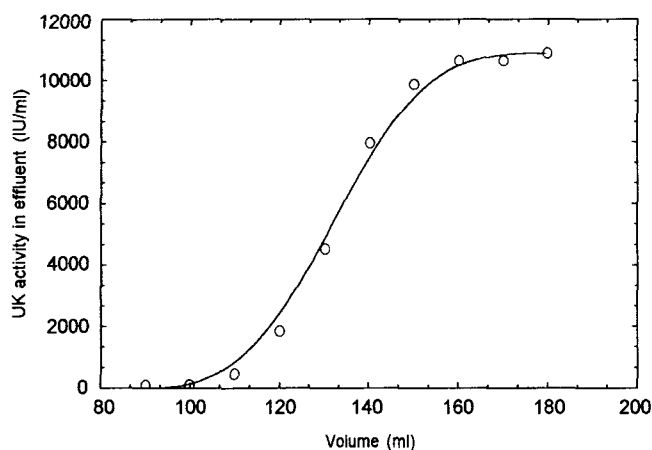


Fig. 5. Breakthrough of urokinase bound to p-AB affinity column.

A breakthrough curve was done with a packed p-AB gel column. The column (1.0×12 cm) was packed with 10 g of wet gel and equilibrated in 0.05 M phosphate, 2.5 M NaCl, and 1% Tween 80 at pH 7.0. Urokinase of 11,083 IU/ml (180 ml) in the same buffer was applied on the column at the flow rate of 0.2 ml/min. Maximal adsorption capacity was 142,700 IU/g wet gel.

a typical S shape. The maximum adsorption capacity was 142,700 IU/g wet gel. This value is useful when considering the amount of urokinase in the loading step, as 70–80% of the maximum adsorption capacity of urokinase was applied to the packed column. A higher percentage would result in a high product loss, while a lower percentage would result in low product purity due to the adsorption of contaminants.

Washing Conditions

Generally, the same buffer is used to wash the column after the feed is loaded. However, in the case of a feed that contains a high level of contaminants, the washing effect is not satisfactory. As such, a stronger washing buffer is required to improve the purity of the product. There are many kinds of contaminants in crude urokinase, and it is

important to remove as many of these contaminants in the washing step as possible. In the current study, many types of washing buffers were tested to identify a satisfactory washing step, and the results are shown in Table 1. Five % Tween 80 exhibited the lowest urokinase specific activity in the washing solution, although the residual urokinase concentration was a little high. This result was also similar to the case of Tween 80 under adsorption conditions (see Fig. 4). The results of Tween 80 containing NaCl were quite different. While the residual urokinase concentration increased, the specific activity also increased in the washing solution. This can result in product loss and a decrease in product purity. Therefore, 5% Tween 80 without NaCl was used for the washing step in all subsequent purification tests.

Optimization of Elution Conditions

The interaction between urokinase and p-AB is strong. A satisfactory eluant should easily elute the target protein with a high purity and high recovery. In previous literature, 0.1 M acetic acid (pH 4.0) has been used to elute urokinase from a p-AB gel [8], however, the recovery and purity of the product are not high. In the current study, various eluants were tested for the elution of urokinase. The selection of eluants was based on the change of pH, buffer type, salt concentration, and elution specificity. The results are shown in Table 2. One-tenth M Arginine (pH 9.0) exhibited a high recovery of 89.9%, yet the specific activity of the product was only 15,414 IU/mg protein. Arginine with an amidine structure was able to compete with a p-AB ligand for eluting urokinase, but its strong elution ability also eluted other contaminants. Two-tenth M glycine (pH 3.0) showed a recovery of 82.4% and specific activity of 39,422 IU/mg protein, which was better than the results with acetic acid and all the other eluants tested. As such, it was selected as a potential eluant. The further optimization of glycine as an eluant was carried out using different glycine concentrations, pHs, and NaCl concentrations. The results are shown in Figs. 6 to 8. The elution recovery

Table 1. Effect of Tween 80 concentration on washing out contaminants of urokinase bound to p-AB-sepharose 4B.

T ₈₀ conc. (%w/v)	UK conc. (IU/ml)	Protein conc. (mg/ml)	Specific activity (IU/mg)
0.1	1,083	4.0	271
0.5	1,214	5.6	214
1.0	1,258	6.4	196
5.0	1,279	11.0	116
0.1+(0.05M P+0.5 M NaCl, pH 7.0)	2,013	5.4	373
0.5+(0.05M P+0.5 M NaCl, pH 7.0)	2,233	8.0	279
1.0+(0.05M P+0.5 M NaCl, pH 7.0)	2,196	6.8	323
5.0+(0.05M P+0.5 M NaCl, pH 7.0)	2,238	11.4	196
0.05M P+0.5 M NaCl, pH 7.0	2,438	4.2	580
0.05M P+1.0 M NaCl, pH 7.0	2,241	4.6	487
0.05M P+2.5 M NaCl, pH 7.0	1,392	3.0	464

Table 2. Selection of eluants for urokinase bound to p-AB-sepharose 4B.

Eluants	UK conc. (IU/ml)	Pro. conc. (mg/ml)	Sp. activity (IU/mg)	Rec (%)
pH 9.0, 0.1 M Arg	5,503	0.357	15,414	89.9
pH 3.0, 0.2 M Gly	5,046	0.128	39,422	82.4
pH 9.0, 0.2 M Gly	3,380	0.188	28,644	55.2
pH 9.0, 0.05 M Tris	4,764	0.352	13,534	77.8
pH 3.0, 0.1 M HAC	3,835	0.122	31,434	62.6
pH 4.0, 0.1 M HAC	3,028	0.170	17,812	49.5
pH 4.0, 0.1 M Phosphate	4,032	0.160	25,200	70.0
pH 9.0, 0.1 M Carbonate	4,592	0.194	23,670	75.0

NaCl conc. 0.5 M, temperature 8°C.

UK: Urokinase; Pro: protein; Sp: specific; Rec: Recovery.

of urokinase decreased with an increase in the glycine concentration. Two-tenth M glycine exhibited the highest recovery of 82.5% and 1.0 M the lowest recovery of 59.2% (Fig. 6). Since a low glycine concentration is a highly hydrophilic milieu, the urokinase was easily eluted from its hydrophobic bond with ligand. However, a high glycine concentration creates a highly hydrophobic milieu, making it difficult to elute urokinase at a low pH. The elution results of urokinase at different pHs are shown in Fig. 7. A recovery of 83.0% was obtained at pH 3.0 and a satisfactory recovery was also achieved at pHs 2.8–3.2. Yet, a higher and lower pH decreased the elution recovery. Elution can change the affinity milieu for adsorption by pH, ion strength, buffer type, and other additives. In general, a change of pH is the most important way to elute an interesting protein. However, a lower pH can damage the activity of urokinase, while a higher pH can not produce a strong elution milieu. Consequently, the elution buffer

should contain a low concentration of salt to maintain protein activity. The elution recoveries of urokinase with different NaCl concentrations are shown in Fig. 8. The recovery decreased with an increase in the NaCl concentration. Although recoveries of 90.7 and 85.5% were obtained at 0.0 and 0.1 M NaCl, respectively, the specific activities of urokinase were only 12,702 and 23,466 IU/mg protein, respectively. In particular, when no NaCl was present in the elution buffer, the eluate became turbid due to the instability of the protein in a no-salt milieu [5, 16]. A satisfactory specific activity of 39,421 and recovery of 82.4 were obtained at 0.5 M NaCl, while a higher NaCl concentration resulted in a lower recovery and specific activity due to the highly hydrophobic milieu.

Column Purification

In the loading stage, the urokinase concentration in the effluent was very low. The loss of urokinase was less

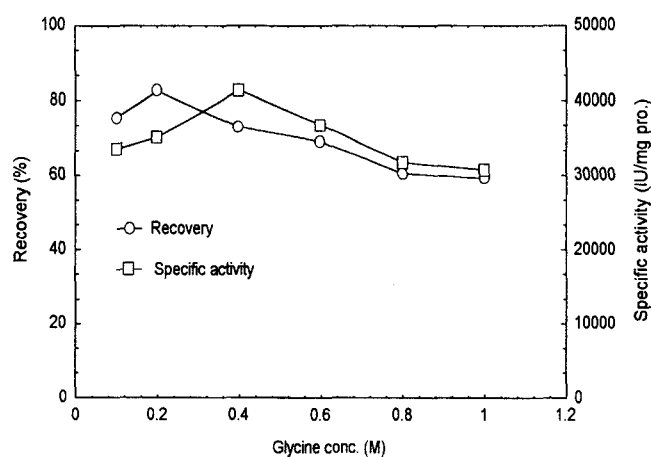


Fig. 6. Effect of glycine concentration on the elution of urokinase bound to p-AB-sepharose 4B.

A batch elution test was carried out with different glycine concentrations. Ten milliliters of glycine solution with different concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 M (pH 3.0, 0.5 M NaCl) were added to the tubes which contained 1 g of affinity gel bound to 61,224 IU urokinase. These tubes were shaken for 8 h at 8°C.

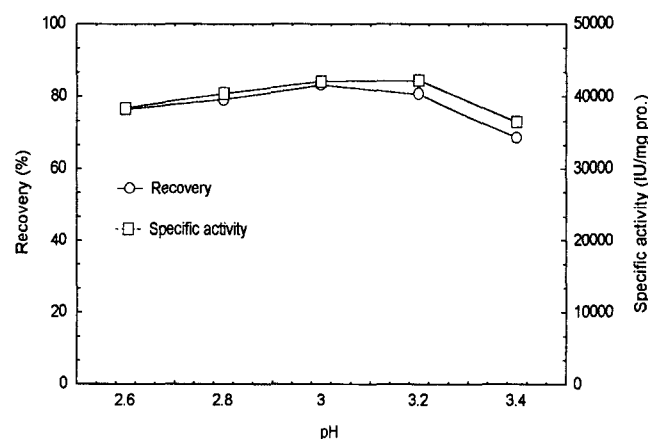


Fig. 7. Effect of glycine pH on the elution of urokinase bound to p-AB-sepharose 4B.

A batch elution test was carried out with different glycine pHs. Ten milliliters glycine solution with different pHs at 2.6, 2.8, 3.0, 3.2, and 3.4 (0.2 M glycine and 0.5 M NaCl) were added to tubes containing 1 g of affinity gel bound to urokinase of 61,224 IU. The tubes were shaken for 8 h at 8°C. The urokinase concentration and specific activity in the eluate were measured.

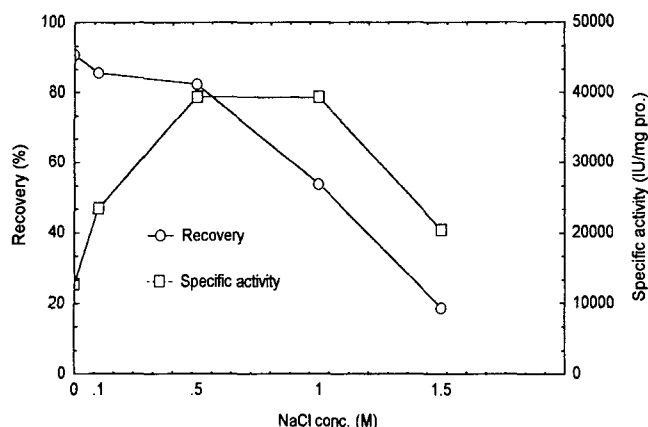


Fig. 8. Effect of NaCl concentration on the elution of urokinase bound to p-AB-sepharose 4B.

The batch elution test was carried out with different NaCl concentrations. Ten milliliters of 0.2 M glycine solution with different NaCl concentrations at 0, 0.1, 0.5, 1.0, and 1.5 M (pH 3.0) were added to the tubes containing 1 g of affinity gel bound to urokinase at 61,224 IU. The tubes were shaken for 8 h at 8°C.

than 3.0%. In the column purification, 5% Tween 80 and 0.1 M phosphate buffer containing 1.0 M NaCl (pH 4.0) were used in turn for washing the column. The loading, washing, and elution curves of the urokinase are shown in Fig. 9. The results of the column purification are shown in Table 3. Five % Tween 80 washed out a large amount of the contaminants and resulted in the lowest product loss (0.2%) in the washing step. This demonstrated that 5% Tween 80 was the best. The 0.1 M phosphate buffer containing 1.0 M NaCl (pH 4.1) was able to wash out small molecular weight urokinase [23] and the residual Tween 80 in the first washing step, thereby confirming a large molecular weight urokinase ratio of more than 90% [14]. In the elution stage, 0.2 M glycine containing 0.5 M NaCl (pH 3.0) exhibited the strongest elution ability. The recovery was 86.2% based on a material balance calculation in the elution step, and the specific activity was 124,300 IU/mg protein in a single purification step. When compared with previous reports, this is the highest purification factor with a single chromatography step. An HPLC analysis from Shanghai No. 1 Biochemical and Pharmaceutical Company (Shanghai, China) demonstrated that the purity of the product and ratio of high molecular weight urokinase

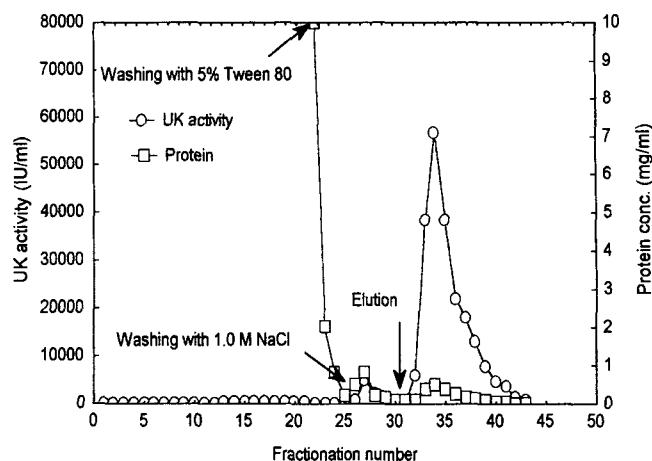


Fig. 9. Column purification curve of urokinase.

The column purification was done in a packed p-AB gel column (1.0×12 cm). One hundred milliliters of crude urokinase solution at 11,083 IU/ml containing 0.05 M phosphate buffer, 2.5 M NaCl, and 1% Tween 80 at pH 7.0 were applied onto a column (1.0×12 cm) with 10 g of a wet p-AB gel (equilibrated by the same buffer) at a flow rate of 0.2 ml/min. The column was washed with 50 ml of 5% Tween 80 and 0.1 M phosphate buffer containing 1.0 M NaCl (pH 4.1) in turn, then the loaded urokinase was eluted with 0.2 M glycine containing 0.5 M NaCl (pH 3.0).

to low molecular weight urokinase reached the standard of Chinese Pharmacopoeia (data not shown). Elution is one of the most important steps for affinity chromatography, as the recovery of eluted products is the key in determining the total product yield. As such, a good selectivity and high recovery are required in the elution stage, based on optimizing the elution conditions applied to the column purification. Holmberg *et al.* [8] previously purified urokinase under loading conditions of 0.1 M phosphate buffer containing 0.4 M NaCl (pH 7.0) and elution conditions of 0.1 M acetic acid containing 0.4 M NaCl (pH 4.0). A specific activity of 18,625 IU/mg urokinase was purified to 140,200 IU/mg with a purification factor of 7.5 with yield of 85–90% in a single chromatographic step. However, in the current study, a crude urokinase material with a specific activity of only 231 IU/mg protein was used, yet a high purity of urokinase was still obtained by a single affinity chromatography.

The experiment was scaled up to a two-liter affinity gel column (12×20 cm) and 100 million IU of urokinase was

Table 3. Results of column purification of urokinase.

Steps	Volume (ml)	UK conc. (IU/ml)	Pro conc. (mg/ml)	Recovery (%)	Sp activity (IU/mg pro)
Loading	120	10,108	44	–	231
Effluent	120	293	–	2.9	–
Washing 1 ^a	30	88	–	0.2	–
Washing 2 ^b	30	2,617	0.11	6.5	23,035
Elution	70	15,019	0.12	86.7	124,300

^a5% Tween 80; ^b0.1 M phosphate buffer containing 1.0 M NaCl (pH 7.0); UK: Urokinase; Pro: Protein; Sp: specific.

applied to the column. Similar product recovery and purity were obtained, yet the urokinase concentration in the eluate was as high as 110,000 IU/ml. As such, the eluate could be directly lyophilized with the required additives without a concentration step. The pyrogen of the solution was tested using the rabbit method, and the quality reached the Chinese Pharmacopoeia standard [13].

CONCLUSION

Using a single affinity chromatography step, crude urokinase was highly purified with a high recovery. When 5% Tween 80 was used to wash the column, most of the contaminants in the column were washed out. The successful purification process consisted of preparation of p-AB affinity media with four steps of chemical reactions, loading with high salt concentration, washing with high detergent concentration, and elution with glycine buffer. The process was also scaled-up to a two-liter affinity chromatography column.

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