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A novel matrix for high performance affinity chromatography and its application in the purification of antithrombin III

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Abstract

Viscose fiber, a regenerated cellulose, was evaluated for using as a novel matrix for high performance affinity chromatography. With a one-step activation with epichlorohydrin, heparin can be readily covalently attached to the matrix. This heparin–viscose fiber material was used for purifying antithrombin III (AT III) from human plasma. The purity of the AT III from this one-step purification is 93% as measured by SDS-PAGE and the protein recovery yield is about 90%. This column is highly specific as described by the dissociation constant of the complex of immobilized heparin and AT III, which was 2.83×10^{-5} mol/L. And more important, this viscose fiber material demonstrated its excellent mechanical property that allows the flow rate to reach up to 900 cm/h or more.

Keywords: Viscose fiber; High performance affinity chromatography; Heparin; Antithrombin III

1. Introduction

Affinity chromatography is one of the most powerful techniques in selective purification and isolation of a great number of compounds [1]. This technique has the purification power to eliminate steps, increase yields and improve process economics [2]. High performance affinity chromatography (HPAC) was introduced by Ohlson et al. [3], who combined two chromatographic techniques of high performance liquid chromatography and affinity chromatography. Due to its specificity, rapidity and high performance, HPAC has gained more and more attention and is being used increasingly for large-scale bimolecular purifications [4,5]. Success of HPAC depends on many factors. The type of matrix is one of the most important factors. The physical and chemical properties of matrix constitute dominant effects on the chromatographic performance. The availability of cost-effective and efficient support material is also critical important for advancements in bioprocess technology [6].

The matrix used for HPAC can be roughly divided into two groups, namely, inorganic and organic media. Generally, the inorganic polymers, such as silica, have good mechanical stability and can be easily derived to introduce functional groups [7], but suffer from poor chemical stability and high non-specific adsorption caused by its residual silanol groups [8]. The synthetic organic polymers are suitable from a chemical stability point of view, but some of these possess low biocompatibility due to their hydrophobic character, for example, polystyrene [1].

Being an easily available natural polymer, cellulose has played an important role in affinity chromatography [9,10]. Compared with the synthetic organic polymers, cellulose and its derivatives have hydrophilic surfaces and are biocompatible [11,12]. Various cellulose media derived from regenerated and non-regenerated cellulose are commercially available. However, the high degree of endogenous crystallinity in natural cellulose makes it less suitable for affinity chromatography. The crystalline regions possess

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much less accessible hydroxyl groups than amorphous regions and may exhibit non-specific adsorption as well as low derivatization ability. The regenerated cellulose does not have such crystalline regions and possess more accessible hydroxyl groups that reduce the effect of non-specific adsorption and enhance reactivity in chemical reactions. Covalent crosslinks in regenerated cellulose also improve its rigidity and mechanical stability.

In the present work, viscose fiber, a regenerated cellulose, was studied as a novel and potential matrix for HPAC. To investigate its affinity chromatographic behaviors, heparin was used as a model ligand. The affinity chromatographic behaviors of heparin–viscose fibers were evaluated. The application of heparin–viscose fibers in isolating antithrombin III from human plasma was also investigated.

2. Experimental

2.1. Materials and equipment

The viscose fiber was provided by Baoding Swan Chemical Fiber Group Corporation (Hebei, China). Antithrombin III (AT III) was a gift from Hualan Bioengineering Company Ltd. (Henan, China). Heparin sodium salt (140 U/mg) was purchased from Jiangsu Changzhou Biochemistry Institute (Jiangsu, China).

Epichlorohydrin was from Jinda Fine Chemical Factory (Tianjin, China). Protein markers were purchased from Shanghai Biochemistry Institute of CAS (Shanghai, China). Water used to prepare aqueous buffers was triple distilled. The other chemical reagents used in the study were of analytical grade. The buffers and solutions were filtered through 0.22 μm filters prior to use.

The HPLC apparatus consists of a DIONEX AGP-1 gradient system (Dionex Corporation, USA) with a Kratos Spectroflow 757 variable-wavelength detector (ABI, USA). The output from the detector was connected to a WDL-95 HPLC Workstation (Dalian Institute of Chemical Physics, Dalian, China).

Scanning electron micrography (SEM) analysis was carried out on a KYKY-2800 scanning electron microscope (KYKY Apparatus Factory, Chinese Academy of Sciences, Beijing, China). Circular dichroism spectra were measured with a Jasco J-810 spectropolarimeter (Jasco, Japan). UV absorbance spectra were determined on a Techcomp 8500 UV–vis spectrometer (Tianmei Corporation, Shanghai, China).

2.2. Preparation of heparin–viscose fiber and control packing media

Briefly, fibrous viscose (2.4 g) was suspended in a solution consisting of 20 mL of 2.4 mol/L NaOH and 20 mL of epichlorohydrin. The reaction mixture was incubated at 50 °C for 3 h. The unreacted epichlorohydrin was removed by extensive washing with distilled water [13]. The content of epoxy groups on the surface of epichlorohvdrin–viscose fibers was measured by Keen's method [14].

Heparin sodium salt (373 mg) dissolved in 10 mL of 0.04 mol/L HCl was stirred at 100 °C for 30min. After cooling to room temperature, partially hydrolyzed heparin was recovered by precipitation with four volumes of ethanol.

The mixture of epichlorohydrin modified viscose fibers (2.0 g) and partially hydrolyzed heparin (250 mg) in 20 mL of 0.5 mol/L Na_2CO_3 (pH 11) was incubated at 50 °C for 72 h with shaking. The obtained heparin–viscose fibers were thoroughly washed with water to remove unreacted heparin. The residual epoxy groups on the heparin–viscose fibers were blocked by reacting with 20 mL of 1.0 mol/L ethanolamine (pH 9.0) for 8 h.

The packing material for the control column was prepared using the same procedures, which included a surface activation step with epichlorohydrin, a coupling step without the addition of heparin and an end-capping step with ethanolamine as those used in the affinity column preparation.

2.3. High performance affinity chromatography of antithrombin III on heparin immobilized viscose fibers

The resultant heparin–viscose fibers and the control packing media were suspended in ethanol and were slurry packed into stainless steel columns (70 mm × 5.0 mm i.d.) at 100 kg/cm^2, respectively.

Without additional mention, all chromatographic experiments were performed with step-wise elution mode at room temperature. A 30 mg/mL AT III solution was prepared with triple distilled water. The loading buffer was 0.01 mol/L sodium phosphate (pH 4.5) containing 0.1 mol/L NaCl. The elution buffer was 0.01 mol/L sodium phosphate (pH 7.4) containing 0.5 mol/L NaCl. The flow rate was 300 cm/h. A 5 μL aliquot of AT III was injected on the column. After washing with loading buffer for 5 min, the bound AT III was eluted from column with elution buffer and detected at 220 nm.

2.4. Standard curve of antithrombin III

The standard curve was prepared using pure AT III as the standard. The different concentrations of pure AT III were prepared as 0.024, 0.048, 0.096, 0.12 mg/mL. The absorbance was detected at 220 nm. The correlation coefficient (r) of the standard curve was equal to 0.997 ($n = 4$). From this curve, the recovery of AT III from affinity column could be determined.

2.5. Circular dichroism determination

Circular dichroism measurements were performed at 25 °C on a Jasco series 810 spectropolarimeter. Quartz made cylindrical cell with a path length of 1 cm was utilized to obtain spectral data in the 200–260 nm region. Two samples were prepared for evaluating the effect of the loading and elution conditions on the structure of the protein. Sample "a" was prepared by loading pure AT III onto the heparin column

at pH 4.5 and eluting at pH 7.4. Sample "b" was prepared by loading pure AT III and eluting at pH 7.4.

2.6. Purification of antithrombin III from human plasma with heparin-viscose column

The starting material for the purification of AT III was normal human plasma, which were screened to confirm the absence of hepatitis B surface antigen and antibody to human immunodeficiency virus. Human plasma was filtered through a 0.22 μm membrane filter to remove cryoprecipitate. Vitamin K-dependent coagulation factors were removed by adsorption on diethylaminoethyl Sephadex A-50, according to the method described by Hoffmann [15]. Then 5 mL of the treated plasma was loaded continuously onto the pre-equilibrated heparin-viscose column. The flow rate was 300 cm/h. Unbound specific compounds were washed thoroughly with 0.01 mol/L sodium phosphate (pH 4.5) containing 0.1 mol/L NaCl and 0.5 mol/L NaCl, successively. The bound AT III was eluted from column with 0.01 mol/L sodium phosphate (pH 7.4) containing 0.5 mol/L NaCl at a flow rate of 900 cm/h. The elution was monitored at 280 nm.

2.7. Characterization of purified antithrombin III

The purity of the eluted fractions from heparin-viscose column was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels using a DYY-8C electrophoresis system (LiuYi Apparatus Factory, Beijing, China) with a DYCZ-24D electrophoresis cell (gel size: 8.2 cm × 8.2 cm). The separated proteins and protein markers were stained with Blue Coomassie R-250. The following proteins were used as markers: rabbit phosphorylase b, M_r 97400; bovine serum albumin, M_r, 66200; rabbit actin, M_r 43000; bovine carbonic anhydrase, M_r 31000 and trypsin inhibitor, M_r 20100.

The gels were scanned using a GIS-2010 densitometer (Tanon, Shanghai, China) for quantification of protein bands.

3. Results and discussion

3.1. Characterization of viscose fibers

The viscose fiber is a kind of regenerated cellulose fiber and widely used in industries of weaving, knitting and threads making. It is commercially available and inexpensive. In our experiment, the viscose fiber with a diameter of 30 μm was cut into short fibers with an average length of about one hundred micrometers. Morphology analysis by scanning electron microscope (Fig. 1) showed that the viscose fibers looked like cylinders with characteristically concavo–convex surface and there seems to be no pores on the surface. The concavo–convex surface provides more specific surface area, which enables high ligand density and high binding capacity. The non-porous nature of viscose fiber makes it possess high mechanical strength. Another unique character of this viscose fiber is that it is soft in organic solvent but rigid in aqueous solution. After suspended in ethanol, it can be easily slurry-packed into a stainless steel column at 100 kg/cm^2. When replacing ethanol with an aqueous solution, the fibers become rigid, which leads to a lower backpressure, better performance and longer lifetime. The investigation of the relationship between flow rate and column backpressure confirmed that viscose fibers expressed good mechanical stability in aqueous solution. As shown in Fig. 2, the column backpressure increased linearly with increasing flow rate during the test range from 60 to 1500 cm/h. When the flow rate was up to 1500 cm/h. the backpressure of the column was about 14 MPa and there was no evidence that the fibers were compressed to cause fouling. Due to its excellent flow characteristics under pressure, the viscose fiber would be a promising support for fast affinity purification.

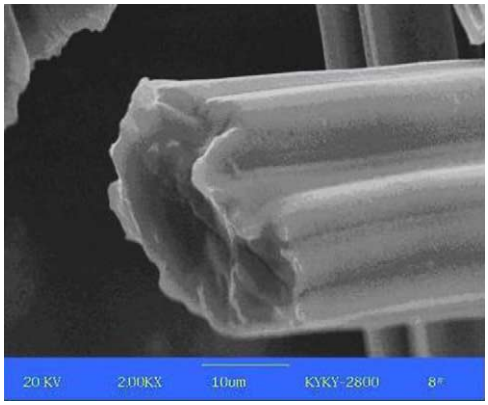

Fig. 1. Scanning electron micrograph of the morphology of the viscose fibers. Magnification: 2000×.

3.2. Immobilization of heparin on viscose fibers

Heparin is known for its anticoagulant activity and interacts with AT III specifically. Heparin-immobilized media has been widely used as an affinity adsorbent for the separation and purification of plasma components in blood-coagulation systems [16,17]. Because viscose is lack of active group, the attachment of heparin is made in two steps: activation and

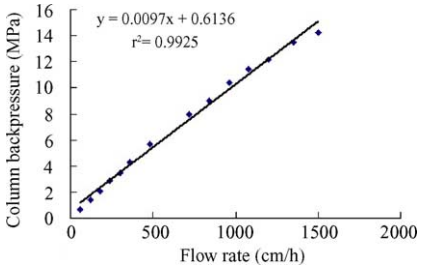

Fig. 2. Relationship between flow rate and backpressure of the control column. Column: stainless steel column (70 mm × 5.0 mm i.d.) packed with control packing media; mobile phase: water.

Table 1
Optimization of the activation of viscose by epichlorohydrin using different NaOH concentrations

Concentration of NaOH (mol/L)	Amount of epoxy groups on viscose (mmol/g fibers)
1.0	0.28
1.5	0.53
2.4	0.65
3.0	0.72

coupling. The viscose fibers were first activated with a bifunctional reagent, epichlorohydrin in an alkaline condition. Then heparin was attached to the viscose fibers through a reaction between its amino groups and the epoxy groups on the surface of epichlorohydrin–viscose. Epichlorohydrin also acted as the spacer between viscose and heparin. which increased the flexibility of the ligand and allowed an effective binding between heparin and its specific protein.

In order to generate a reasonable amount of epoxy groups on the surface of epichlorohydrin–viscose fibers, the concentration of NaOH in the activation step was optimized. As shown in Table 1. the amount of epoxy groups increased along with increasing the concentration of NaOH. However, considering that an extremely alkaline condition might have negative effect on the chemical stability of viscose fibers, a NaOH concentration of 2.4 mol/L was used in the activation reaction according to Guo's work [12], The concentration of epoxy groups on the obtained epichlorohydrin–viscose fibers was 0.65 mmol/g fibers.

Before coupling, heparin was reacted with diluted HC1. which made more amino groups of heparin exposed by partially hydrolyzing the *N*-sulfate from heparin. The partially hydrolyzed heparin can be readily reacted with the epoxy groups of epichlorohydrin–viscose fibers. The maximum binding capacity to AT III on the heparin media made of this coupling method has been revealed in our previous work [16]. After coupling reaction, the excess of epoxy groups remained on the heparin–viscose fibers was deactivated by ethanolamine. which would eliminate the non-specific adsorption of proteins. No epoxy groups were detected after ethanolamine end-capping step.

3.3. Optimization of high performance affinity chromatography conditions

In order to maximize binding capacity, several parameters, including pH, ionic strength and flow rate of mobile phase, were optimized. The pH values showed significant influence on the effective adsorption of AT III on the heparin-immobilized viscose fibers. Maximum adsorption capacity was achieved at pH 4.5. The binding capacities decreased greatly at other pH values. When the pH value was higher than 7, almost no adsorption occurred. So a 0.01 mol/L sodium phosphate solution with pH 4.5 was chosen as the loading buffer and a 0.01 mol/L sodium phosphate solution with pH 7.4 was chosen as the elution buffer.

Usually, the suitable pH value of loading buffer for protein purification was chosen as 7.4 in order to mimic the physiological buffer condition. But in this experiment, the optimized pH of loading buffer decreased to 4.5. Compared with other researches, the coupling method of heparin and matrix in this study is quite different from them. The reason for the pH requirement at 4.5 for binding instead of 7.4 was probably due to the pretreatment of heparin with hydrogen chloride at 100 °C. This probably caused partial hydrolysis of heparin and loss of some negatively charged sulfate groups, which will decrease the binding to the positive charges on AT III. Because pI value of AT III is about 5.0, a lower pH will make the protein more positively charged, which in turn to strengthen the interactions between heparin and AT III. When the buffer pH is 7.4, AT III is more negatively charged so as to hardly interact with immobilized heparin.

Since there was almost no AT III binding to heparin affinity column at pH 7.4, the elution buffer was chosen as 0.01 mol/L sodium phosphate buffer containing 0.5 mol/L NaCl at pH 7.4. The recovery of AT III from heparin–viscose fiber column was 90% at this condition, which was determined spectrophotometrically by standard curve method.

The concentration of NaCl in elution buffer was generally used about 1.0 mol/L or more in AT III purification reported by Peterson and Blackburn [18], as well as Funahashi [19]. In their cases, the pH values of loading and elution buffers were the same as about 7, so high concentration of NaCl was needed to elute the AT III. In this study, the pH values of loading and elution buffers were quite different as 4.5 and 7.4, respectively. According to the study of buffer pH, almost no adsorption of AT III on heparin affinity column was observed at 0.01 mol/L phosphate buffer (pH 7.4), so the concentration of NaCl in the elution buffer was used as 0.5 mol/L. Moreover, due to the high protein recovery at this condition, the relatively low salt concentration used to elute the AT III from the column is reasonable.

The dynamic binding capacity of the media was investigated at different flow rate. As shown in Fig. 3, when ionic strength of loading buffer was used as 0.01 mol/L sodium phosphate (pH 4.5), the influence of flow rate on binding capacity could be neglectable. When the 0.01 mol/L sodium phosphate (pH 4.5) containing 0.1 mol/L NaCl was used as loading buffer, the binding capacity significantly decreased with increasing flow rates. Therefore, when loading buffer with relatively high ionic strength was used to avoid non-specific adsorption, the flow rate of loading step must be maintained at 300 cm/h or lower in order to gain the maximum adsorption. Despite the necessity of maintaining relatively low flow rate at loading step, fast purification could still be achieved at equilibrating and elution steps by using flow rate of 900 cm/h or more. The binding capacity was determined by overloading the column with AT III. The adsorbed AT III was eluted and measured spectrophotometrically as 1.62 mg/g fibers.

With the optimized conditions, anithrombin III exhibited apparently specific binding activity to heparin–viscose fibers,

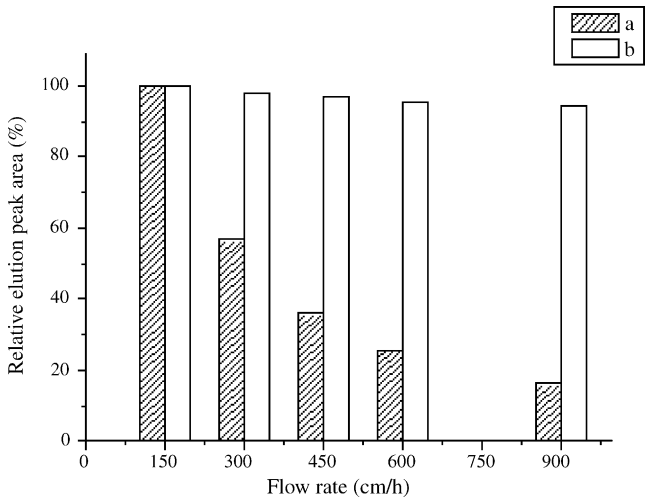

Fig. 3. Effect of flow rate in the sample-loading step on the dynamic binding capacity of AT III on the heparin–viscose fibers column, (a) loading buffer, 0.01 mol/L sodium phosphate (pH 4.5) containing 0.1 mol/L NaCl; (b) loading buffer, 0.01 mol/L sodium phosphate (pH 4.5). The column was pre-equilibrated with loading buffer. Then 150 μg (5 μL) of AT III was injected on the column. After washing with loading buffer for 5 min, the bound AT III was eluted with 0.01 mol/L sodium phosphate (pH 7.4) containing 0.5 mol/L NaCl. The detection wavelength was 220 nm.

while no retention of AT III was observed on the control column at the same condition (as shown in Fig. 4).

To address the concern that the AT III binding to the column at pH 4.5, circular dichroism (CD) measurement for the sample with and without experiencing a pH 4.5 loading condition were carried out. CD is a standard method to evaluate the secondary structural characteristics of protein [20].

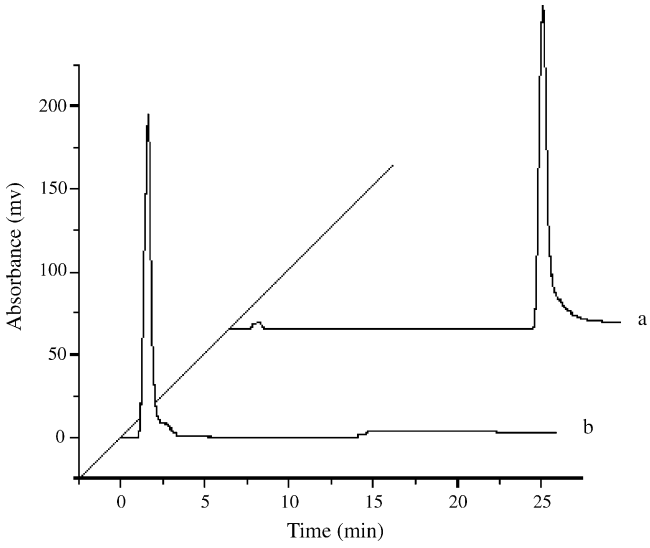

Fig. 4. Chromatographic behaviors of antithrombin III on the heparin–viscose fibers column (a) and the control column (b). The columns were pre-equilibrated with loading buffer. Then 150 μg (5 μL) of AT III was injected on the columns. After washing with loading buffer for 5 min, the bound AT III was eluted with 0.01 mol/L sodium phosphate (pH 7.4) containing 0.5 mol/L NaCl. The flow rate was 300 cm/h and the wavelength was 220 nm.

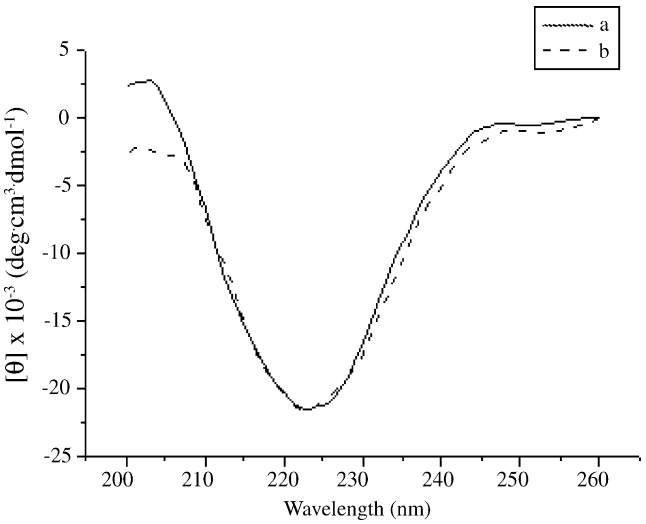

Fig. 5. Circular dichroism spectra of AT III with (a) and without (b) experiencing pH4.5 binding step. Wavelength region was 200–260 nm. Operating temperature was 25 °C.

As shown in the Fig. 5, the CD spectra of AT III with and without experiencing pH4.5 binding step superimposed each other well, suggesting that they have very similar secondary structure characteristics. The structural similarities between AT III with and without experiencing pH 4.5 were also verified by identical second derivatives of the UV absorbance spectra (data not shown).

3.4. Determination of the dissociation constant of the complex of immobilized heparin and antithrombin III

The interaction between heparin–viscose fibers and AT III was evaluated by analytical HPAC [21,22]. The AT III solutions containing different concentrations were passed through the heparin-viscose column until plateaus of maximum absorbance occurred, respectively (shown in Fig. 6). At this time, the eluate had the same concentration as the initial applied AT III solution. The procedure was monitored at 280 nm. The variation of elution volume $\overline{V}$ was plotted according to the equation [23]:

$$\frac{1}{\overline{V} - V_0} = \frac{K_d}{M_T} + \frac{1}{M_T}[P]_0$$

Where $\overline{V}$ and V_0 are the elution volume at which the affinity matrix is half-saturated and the void volume of the column, respectively (L), M_T is the total amount of immobilized heparin (mol), $[P]_0$ is the initial concentration of AT III solution (mol/L) and K_d is the dissociation constant of the complex of immobilized heparin and AT III (mol/L). From the plot of $1/(\overline{V} - V_0)$ versus $[P]_0$ shown in Fig. 6, K_d can be calculated by the ratio of intercept/slope. $K_d = 2.83 \times 10^{-5}$ mol/L, which is similar to the result obtained in our previous work [16]. The apparent dissociation constant obtained is within the range of 10^{-4}–10^{-8} mol/L, which is suitable for affinity applications [24].

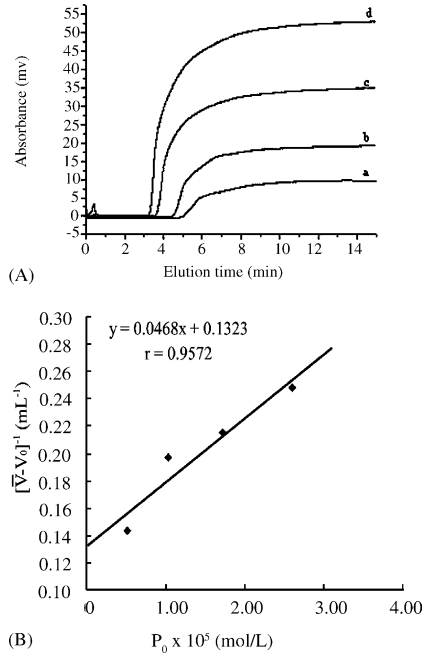

Fig. 6. Frontal chromatography of AT III on the column packed with heparin–viscose fibers (A). The concentrations of AT III were as follows: (a) 0.52×10^{-5} mol/L; (b) 1.03×10^{-5} mol/L; (c) 1.72×10^{-5} mol/L and (d) 2.59×10^{-5} mol/L. The flow rate was 300 cm/h and the loading buffer was 0.01 mol/L sodium phosphate (pH 4.5) containing 0.1 mol/L NaCl, wavelength: 280 nm. Plot of $1/(\overline{V} - V_0)$ vs. $[P]_0$ (B). The linear regression equation and correlation coefficient were shown.

3.5. Purification of antithrombin III from human plasma

AT III from human plasma was purified on the heparin-immobilized viscose column. After removal of cryoprecipitate and vitamin K-dependent coagulation factors, the plasma was directly loaded on the affinity column. After loading, the column was washed with 0.01 mol/L sodium phosphate (pH 4.5) containing 0.1 mol/L NaCl and 0.5 mol/L NaCl until the unbound compounds were completely eluted. The flow rate was 300 cm/h at sample loading and washing steps. The AT III fraction was eluted from column by changing mobile phase to 0.01 mol/L sodium phosphate (pH 7.4) containing 0.5 mol/L NaCl at a flow-rate of 900 cm/h (Fig. 7).

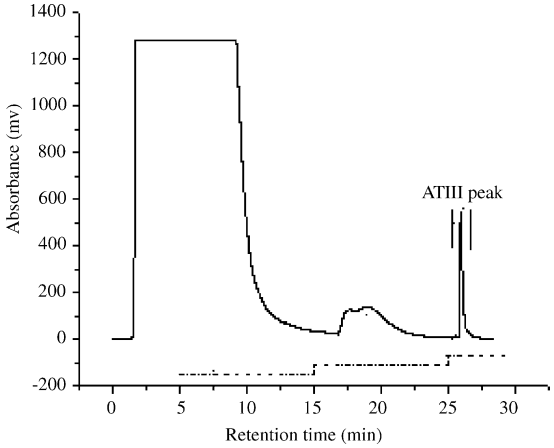

Fig. 7. Chromatogram of human plasma on heparin-viscose column. The column was equilibrated with 0.01 mol/L sodium phosphate–0.1 mol/L NaCl, pH 4.5, and then 5 mL of human plasma was loaded. Eluents, 5–15 min, 0.01 mol/L sodium phosphate–0.1 mol/L NaCl, pH 4.5, 15–25 min, 0.01 mol/L sodium phosphate–0.5 mol/L NaCl, pH 4.5 (flow rate, 300 cm/h), 25–30 min, 0.01 mol/L sodium phosphate–0.5 mol/L NaCl, pH 7.4 (flow rate, 900 cm/h); wavelength, 280 nm. The column effluents were collected between the two vertical lines.

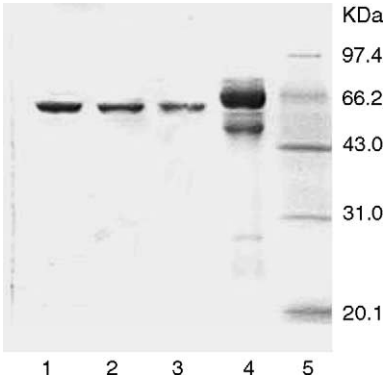

Fig. 8. SDS-PAGE analysis of fractions from human plasma separated on heparin-viscose affinity column. Lanes: 1 and 2, eluted fractions from different chromatographic runs; 3, standard AT III; 4, human plasma; 5, molecular mass markers.

The AT III fraction was collected between the two vertical lines indicated in Fig. 7 and was analyzed by SDS-PAGE. As the electrophoretogram in Fig. 8 shown, the purified AT III exhibited one major band, which was consistent with the standard AT III band (M_r, 57000 measured by MALDI-TOF-MS). Densitometric scanning of the gel indicated that the purity of the eluted AT III was 93%.

4. Conclusion

Viscose fibers have been demonstrated to be suitable as a novel matrix of HPAC. The excellent recovery and purity of the isolated AT III from human plasma in this one step HPAC suggest that this material is suitable for the isolation of biological materials. The mechanical property of this material makes it fit for fast protein isolation. Furthermore, the low cost of viscose fiber makes it very attractive and great potential for large-scale industrial applications.

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