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Journal of Chromatography A, 944 (2002) 203–210

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Recovery of antithrombin III from milk by expanded bed chromatography

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Abstract

In the present work, a new method of purification for antithrombin was developed using an expanded bed chromatography technique. Milk fat was removed by centrifugation and caseins were precipitated selectively by addition of zinc chloride. Crude skim milk was then directly fed to an expanded bed column containing the ion-exchange matrix. The use of a cation-exchanger (P-11) resulted in 100% adsorption and 13% recovery whereas the use of an anion-exchanger (DE-52) resulted in 100% adsorption and 84% recovery and up to five-fold purification of antithrombin. The buffer, 25 m*M* Tris–HCl pH 8.0; the eluting agent, 2 *M* $(NH_4)_2SO_4$; and 100% expansion of settled bed were determined to be the optimum conditions for the purification of antithrombin by ion-exchange expanded bed chromatography. A comparison of column diameters revealed that the elution yields increase by two-fold while the column diameter increases from 1 to 2.5 cm. However, antithrombin III was concentrated to a higher degree by using the column with an internal diameter of 1 cm.

Keywords: Milk; Expanded bed chromatography; Antithrombin; Glycoproteins; Proteins; Enzyme inhibitors

1. Introduction

Antithrombin III (ATIII) is an M_r 58 000 plasma glycoprotein which consists of a single polypeptide chain containing 432 amino acid residues. It is synthesised in the vascular endothelial cells and in the liver. ATIII is a serine protease inhibitor and is essential for the regulation of blood coagulation, i.e. major regulatory protein of haemostasis and thrombosis. It has found widespread clinical use in the treatment of venous thrombosis and pulmonary embolism. The plasma concentration of ATIII in normal individuals is approximately 150 mg/l [1,2].

Numerous procedures using various chromatographic techniques have been devised for the purification and separation of therapeutic proteins including antithrombin [3–9]. Hoffman [3] developed a large scale method for the preparation of a highly purified ATIII concentrate from human blood plasma based on the use of heparin affinity chromatography. The final product had an activity of 6.48 IU ATIII per mg protein. The purification of antithrombin and other proteinase inhibitors have similarly been accomplished by the adsorption of antithrombin onto heparin covalently attached to a solid matrix such as Sepharose, Ultrogel or AminoFractogel as the first step. Additional purification is then obtained by precipitation with poly(ethylene glycol) (PEG) or ammonium sulphate and by the application of ion-exchange techniques on Mono Q by fast protein liquid chromatography (FPLC) [4,5]. The recovery of antithrombin after affinity and ion-exchange chromatography steps were 30% and 20%, respectively

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0021-9673/02/$ – see front matter
PII: S0021-9673(01)01102-5

[5]. Dawidowicz et al. have developed an alternative ATIII analysis by affinity chromatography using heparin chemically bonded to a dextran layer and immobilised on controlled pore glass (CPG) [6]. Furthermore, membrane ion-exchanger (strongly basic Q-type ion-exchanger) and affinity membranes (heparin coupled to amine modified/epoxy-activated membranes by reductive amination) are reported to be versatile tools for the concentration and purification of recombinant ATIII with a purity of 75% [7]. The purification of recombinant ATIII from CHO cells was achieved by using a three-step procedure consisting of ion-exchange chromatography (strongly basic Sartoband membrane ion-exchanger), affinity chromatography (heparin coupled to CPG) and gel filtration [8,9]. For monitoring of rhATII during cell culture processes and subsequent purification steps, reversed-phase high-performance liquid chromatography (HPLC) was employed using a C_4 column [8]. Hydrophobic interaction chromatography (using a TSK Phenyl 5PW column) was also utilised for the quantification as well as for the separation of native human antithrombin and a partially denatured form of AT [10].

The expression of complex therapeutic proteins in the milk of transgenic dairy animals has been a challenge over mammalian cell culture systems. Milk is a safe and renewable source of commercially important proteins. Human diagnostic or pharmaceutically active, therapeutic proteins (e.g. protein C, α_1-antitrypsin, salmon calcitonin) have been so far purified from milk of transgenic livestock using established technologies of the dairy and pharmaceutical industries [11–15]. In the present work, we describe the application of expanded bed chromatography technology as an alternative downstream process for the purification of antithrombin from milk. The suitability of ion-exchange adsorbents was first investigated for the separation of antithrombin from the whey phase. The target protein is desired to adsorb onto the column and the caseins and the bulk of whey proteins are expected to pass through the ion-exchange bed. The partially purified antithrombin was recovered by a suitable eluting agent from the column. The effects of column dimensions, elution mode and buffer type on the adsorption and elution performance of the expanded bed column were therefore studied at various operating conditions.

2. Experimental

2.1. Protein

Antithrombin III (Atenativ 500 IU) was kindly provided by Pharmacia Medical Products (Istanbul, Turkey).

2.2. Ion-exchange adsorbents

The ion-exchangers used as adsorbents, P-11 and DE-52, were purchased from Whatman (Maidstone, UK). They were prepared as described previously [16].

2.3. Buffers

The compositions of mobile phases used in columns are as follows:

Phosphate buffer: 50 m*M* K_2HPO_4 and 50 m*M* KH_2PO_4, pH adjusted to 7.0 with KH_2PO_4.

Sodium acetate buffer: 50 m*M* $C_2H_3NaO_2.3H_2O$, pH adjusted to 5.5 with acetic acid.

Tris buffer: 25 m*M* $NH_2C(CH_2OH)_3$, pH adjusted to 8.0 with 5 *M* HCl.

20 m*M* $NH_2C(CH_2OH)_3$, pH adjusted to 7.0 with 5 *M* HCl.

L-Histidine buffer: 20 m*M* L-histidine, 0.5 *M* EDTA, and β-mercaptoethanol, pH adjusted to 6.0 with 5 *M* HCl.

All chemicals, except acetic acid (Aksin, Istanbul, Turkey) were purchased from Merck (Darmstadt, Germany).

2.4. Preparation of skim milk

The milk (Mis Milk, Istanbul, Turkey) was diluted with 0.2 *M* EDTA (pH 7.0) at a ratio of 1:1 to break up and solubilise the caseins and fat of the milk. The mixture was centrifuged at 3000 *g* and 4°C for 30 min by using a Sorvall RC 28S centrifuge (DuPont, CT, USA). Fat phase (top) and precipitates (pellet) were removed from the whey phase. The supernatant (skim milk) was stored at −90°C for several months.

2.5. Precipitation of α- and β-caseins of milk by 4 mM Zn^{2+}

Stored skim milk was thawed at 4°C in the

refrigerator. A volume of 0.2 ml of ATIII (Atenativ 500 IU) at a concentration of 1000 mg/l was added to the skim milk; then 2 ml of buffer, [i.e. 50 m*M* Tris–HCl buffer (pH 8.0) or 40 m*M* Tris–HCl buffer (pH 7.0), or 40 m*M* L-histidine buffer (pH 6.0), or 100 m*M* phosphate buffer (pH 7.0)], 0.032 ml 0.5 *M* $ZnCl_2$ and 1.768 ml water were also added making up to 4 ml solution. It was shaken for 30 min at room temperature. The mixture was then centrifuged at 4500–5200 *g* for 30 min in an Eppendorf centrifuge (Hamburg, Germany). α- and β-caseins are collected as paste and discarded. The supernatant containing ATIII was then fed to the expanded bed chromatography column.

2.6. Expanded bed chromatography experiments

The expanded bed chromatography columns (1.0×20 cm, 1.5×15 cm, and 2.5×10 cm) and adaptors (I.D. 1, 1.5, and 2.5 cm) were purchased from Bio-Rad (Hercules, CA, USA). The adaptor that was used to minimise the volume of liquid above the expanded bed had a variable working length of 1–14 cm. The Model EP-1 Econo Pump is a two-channel, bi-directional, variable speed peristaltic pump from Bio-Rad. The columns packed with the relevant ion-exchange adsorbents were fluidised and equilibrated with buffer solution to give a stable expanded bed height prior to adsorption studies. The crude skim milk containing antithrombin placed in a reservoir was pumped by a peristaltic pump upwards through the column. The column was then washed with the buffer to remove the unbound components and monitored spectrophotometrically. The bound target protein, antithrombin, was recovered using the isocratic elution method. In isocratic elution, the elution mode was downward, using 0.25 or 0.5 *M* NaCl or 2 *M* $(NH_4)_2SO_4$ in a relevant buffer solution as the eluting agents. The presence of the antithrombin was determined in the feedstock, flowthrough, wash and eluate.

2.7. Antithrombin assay

The antithrombin concentration was determined by the radial immunodiffusion (RID) method and confirmed by high-performance hydrophobic interaction chromatography measurements as well as by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. RID plates were purchased from the Binding site (Birmingham, UK), and ATIII levels were measured using the procedure as described by the manufacturer.

ATIII was also quantified by a HPLC system with a Phenyl 5PW column from Waters (Milford, MA, USA) at room temperature. A 25 m*M* Tris–HCl buffer, pH 8.0 (buffer A) and 2 *M* $(NH_4)_2SO_4$ in 25 m*M* Tris–HCl buffer (buffer B) was used for elution of ATIII by a linear gradient [10]. The concentration of ATIII in skim milk was determined by integration of the peak area.

The purity of the ATIII was assessed by SDS–PAGE under denaturing conditions. A 12% separating gel and 5% stacking gel containing acrylamide and TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) were used to run the injected proteins which were mixed with sample buffer [1 ml 0.5 *M* Tris–HCl pH 6.8 buffer, 0.8 ml glycerol, 1.6 ml 10% (w/v) SDS, 0.4 ml 2-β-mercaptoethanol and 0.2 ml 0.05% (w/v) Bromophenol Blue] at 1:1 ratio in the electrophoresis tank whose reservoirs were previously filled with running buffer (3 g Tris base, 14.4 g glycine and 1 g SDS to 1 l).

3. Results and discussion

This paper presents the first results for the direct extraction of antithrombin from milk by expanded bed chromatography — without any affinity purification. Milk is a multiphasic fluid composed of a fat emulsion, a micellular casein dispersion, a colloidal suspension of lipoproteins, and a solution of proteins, mineral salts, vitamins, organic acids, and minor components. Total protein in cow milk is 3.5%, casein is 2.8%, whey protein is 0.7% and fat is 3.7% [15]. Any purification of human therapeutic proteins expressed in transgenic dairy animal milk will require a ‘‘clean-up’’ of milk early in the process [13]. In the present study, milk was handled as explained in the Experimental section and the selective removal of caseins was achieved by the addition of $ZnCl_2$ as will be discussed below. Skimming is reported to remove >85% of milk lipids [13]. The EDTA-treated skim milk (whey phase) was then fed to the expanded bed column.

The skim milk loading was always kept below the breakthrough point to avoid any loss of yield of the

target protein, antithrombin, which is very expensive. The operational flow-rates were between 40 and 70 cm/h depending on the column diameter. There was no loss of antithrombin during the flowthrough (100% adsorption) and wash stages. Elution of antithrombin was performed in down-flow packed bed mode. The adsorbed antithrombin was eluted as a sharp peak. A 2–5-fold enriched antithrombin fraction was obtained by 8–10-fold bed volumes of buffer solution. The experimental data indicate that antithrombin is adsorbed mostly on the surface of the ion-exchanger and less into the interior of the cellulosic ion-exchange matrix which results in a slightly asymmetric elution curve with a slight tailing.

In the present work, one cycle of purification process including milk handling (30 min)+casein precipitation (60 min)+column equilibration (40 min)+loading (10 min)+wash (10 min)+elution (50–60 min) took 3.5 h.

3.1. Determination of Zn^{2+} concentration for the precipitation of α- and β-caseins in milk

The interaction of Zn^{2+} ions which form coordinate covalent bonds with caseins are previously used for the selective precipitation of porcine α- and β-caseins [12]. Caseins are major milk proteins and thus the predominant impurities in whey. α-Lactalbumin, a soluble whey protein, is also reported to bind zinc and calcium [15]. Therefore, the optimum concentration of Zn^{2+} for the precipitation of α- and β-caseins in EDTA-treated milk was determined using samples containing 1000 mg/l of ATIII and 2–10 m*M* Zn^{2+} in skim milk. Table 1 summarises the solubility of milk proteins as measured by A_{280} in the presence of different concentrations of this cation. The optimum concentration of Zn^{2+} to recover ATIII and to precipitate α- and β-caseins was found to be 4 m*M* for all buffers used. The SDS–PAGE analysis of the samples (Fig. 1) also confirms that α- and β-caseins (M_r 31 000–42 000) below ATIII (M_r 58 000) band are no more visible due to their removal from the milk.

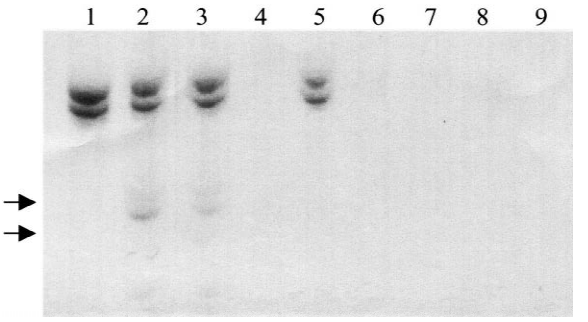

Fig. 1. SDS–PAGE analysis after $ZnCl_2$ precipitation using 25 m*M* Tris–HCl buffer, pH 8.0 [Lanes: (1) Atenativ; (2) 0 m*M* Zn^{2+}; (3) 2 m*M* Zn^{2+}; (4) space; (5) 4 m*M* Zn^{2+}; (6) 6 m*M* Zn^{2+}; (7) 8 m*M* Zn^{2+}; (8) 10 m*M* Zn^{2+}; (9) space].

3.2. Choice of ion-exchange matrix

In the development of a purification process for antithrombin, the first step is the choice of the most appropriate ion-exchange adsorbent. Two different ion-exchange matrices, i.e. cation-exchanger P-11, and anion-exchanger DE-52 were used in this study (Table 2).

The cation-exchanger P-11 was tested using 50 m*M* sodium acetate buffer at pH 5.5 in a 1 cm I.D. column; the settled and expanded bed heights were 3 and 10 cm, respectively. The skim milk containing 1.905 mg antithrombin was loaded onto the column, of which 100% was adsorbed and 12.66% was eluted. These results indicated that P-11 with its orthophosphate functional group and ammonium

Table 1
Precipitation with $ZnCl_2$: solubility of milk proteins measured by A_{280}

Concentration of Zn^{+2} (m*M*)	20 m*M* Tris–HCl pH 7.0	25 m*M* Tris–HCl pH 8.0	20 m*M* L-histidine pH 6.0
2	1.0871	0.7751	0.8553
4	0.1424	0.1342	0.1179
6	0.1375	0.0442	0.1271
8	0.1310	0.0449	0.1490
10	0.1338	0.0592	0.1010

Table 2
Comparison of different ion-exchange adsorbents

ATIII fed (mg)	Ion-exchanger	Eluting agent	ATIII in feed (mg/l)	ATIII eluted fraction (mg/l)	Elution yield (%)	Purification factor
1.630	DE-52	2 *M* $(NH_4)_2SO_4$	326	634	79.09	1.95
1.905	P-11	0.25 *M* NaCl	381	42.3	12.66	0.11

Adsorption yield, 100%; settled bed height, 3.0 cm; expanded bed height 10 cm.

counter ion is not suitable for antithrombin purification.

The anion-exchanger DE-52 was similarly tested in a 20 cm×1 cm I.D. column using 25 m*M* Tris–HCl buffer (pH 8.0). The experimental conditions were kept the same as in the cation-exchange column. The anion-exchange column yielded 80% recovery of antithrombin.

The chromatograms obtained with P-11 and DE-52 adsorbents are compared in Fig. 2. The type of ion-exchanger used affected the peak position and shape of the elution curves. The major whey proteins have acidic iso-electric points [13] and therefore ion-exchange chromatography with a basic adsorbent and buffer pH are better for the purification of ATIII.

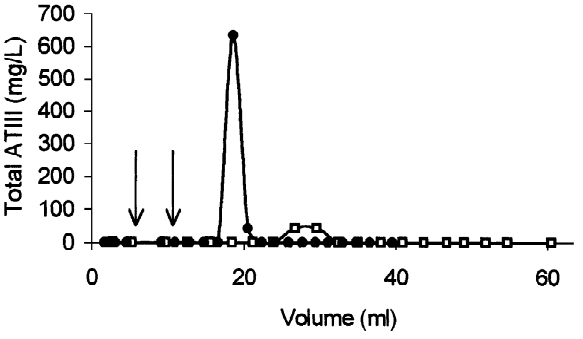

Fig. 2. Comparison of expanded bed chromatography experiments using different ion-exchange adsorbents. □, P-11 cation-exchanger; ●, DE-52 anion-exchanger. (Arrows indicate the start of wash and elution stages).

3.3. Comparison of buffers and eluting agents

The most important factor in the choice of buffer solution depends on the pH and ionic strength to be used. ATIII is soluble in the serum of Atenativ between pH 6.5 and 7.5. A set of experiments was performed in DE-52 anion-exchange column (20×1 cm) using buffers at different pH, i.e., 25 m*M* Tris–HCl, pH 8.0; 20 m*M* Tris–HCl, pH 7.0; 50 m*M* phosphate, pH 7.0, and 20 m*M* L-histidine, pH 6.0 (Table 3).

Two buffers, namely 25 m*M* Tris–HCl, pH 8.0, and 20 m*M* L-histidine, pH 6.0, resulted in 84% recovery and a two-fold purification of antithrombin. On the other hand, 50 m*M* phosphate buffer (pH 7.0) resulted in 43.3% recovery of antithrombin during the washing stage, and yielded no antithrombin during the elution stage.

In order to determine the best eluting agent for the recovery of ATIII, a set of experiments (Table 4) were performed with two eluting agents at different molarities, i.e., 0.25 *M*–0.5 *M* NaCl, 1 *M*–2 *M*

Table 3
Comparison of buffer systems

Buffer	ATIII in feed (mg/l)	ATIII fed (mg)	Eluting agent	Adsorption yield (%)	Elution yield (%)	ATIII in eluted fraction (mg/l)	Purification factor
25 m*M* Tris– HCl, pH 8.0	183	0.915	0.25 *M* NaCl	100	83.98	526	2.87
20 m*M* Tris– HCl, pH 7.0	381	1.905	0.25 *M* NaCl	100	71.20	381	1.00
50 m*M* phosphate, pH 7.0	439	2.195	0.25 *M* NaCl	56.67	0	326	0.48
20 m*M* L-histidine, pH 6.0	265	1.325	0.25 *M* NaCl	100	84.18	500	1.90

Settled bed height, 3.1 cm; expanded bed height, 10 cm.

Table 4
Comparison of eluting agents

ATIII fed (mg)	Eluting agent	ATIII in feed (mg/l)	ATIII in eluted fraction (mg/l)	Purification factor
0.915	0.25 *M* NaCl	183	526	2.87
0.915	0.5 *M* NaCl	183	305	1.67
0.915	1.0 *M* $(NH_4)_2$ SO_4	183	174	0.95
0.640	2.0 *M* $(NH_4)_2$ SO_4	128	645	5.00

Adsorption yield, 100%.

$(NH_4)_2SO_4$. Tris–HCl buffer (25 m*M*, pH 8.0) was used in DE-52 anion-exchange column (20×1 cm) which had 3 cm settled bed and 10 cm expanded bed heights, respectively.

The use of 0.25 *M* NaCl and 2 *M* $(NH_4)_2SO_4$ as eluting agents resulted in approximately three- and five-fold purification of ATIII. So 2 *M* $(NH_4)_2SO_4$ was determined as the convenient eluting agent for the best recovery of ATIII.

3.4. Effect of initial concentration of ATIII

The effects of initial concentrations of ATIII on the adsorption and elution performance were investigated in DE-52 anion-exchange column (20 cm×1 cm). The experimental conditions were as follows: 3 cm settled bed height, 10 cm expanded bed height, 25 m*M* Tris–HCl buffer, pH 8.0, and 2 *M* $(NH_4)_2SO_4$ as the eluting agent. The experimental results summarised in Table 5 indicate that as the initial concentration of ATIII in milk increases, although the adsorption yield is 100%, the elution yield and purification factor decrease. An extremely high value of elution yield was obtained with 128 mg/l initial antithrombin in milk, possibly due to the presence of the additive albumin, which was used for the stabilisation of ATIII. Replacement of albumin with sucrose as the stabiliser is reported to give better results in the purification of factor VIII/von Willebrand factor [17].

Table 5
Effect of initial concentration of ATIII

ATIII fed (mg)	ATIII in feed (mg/l)	ATIII in eluted fraction (mg/l)	Elution yield (%)	Purification factor
1.790	358	451	77.88	1.26
1.685	337	634	58.30	1.88
1.630	326	634	79.09	1.95
0.640	128	645	120.85	5.00

Adsorption yield, 100%.

3.5. Effect of settled bed height and expanded bed height

The adsorption of antithrombin in the skim milk was carried out at different volumes of ion-exchanger and at different degrees of bed expansion in 1 cm internal diameter and 20 cm height column using 25 m*M* Tris–HCl buffer, pH 8.0. Experimental conditions and results are summarised in Table 6.

The elution yields were found to be similar in columns with 6 cm settled bed heights (18.85 cm^3 DE-52). Recovery of 36.4% and 1.45-fold purification was obtained by 100% bed expansion. In experiments with 3 cm settled bed heights (9.43 cm^3 DE-52), on the other hand, the elution yields fluctuated although the purification factors were nearly same. The target protein, antithrombin, appeared earlier during elution in the experiment with 100 percent expansion (3 cm SBH– 6 cm EBH) resulting in 64% recovery. Recovery of 78% and 1.26-fold purification was obtained by 233% bed expansion (Table 6). It should here be noted that the initial concentration of ATIII at 3.5-fold (233%) bed expansion is slightly lower than those used in other experiments. Nevertheless, these results are consistent with those reported in literature [18], i.e., the effective expanded bed operation is achieved when

Table 6
Effect of expanded bed height

ATIII in feed (mg/l)	ATIII fed (mg)	Settled bed height (cm)	Expanded bed height (cm)	ATIII in eluted fraction (mg/l)	Elution yield (%)	Purification factor
539	5.390	6	20	677	35.72	1.26
539	5.390	6	15	706	28.87	1.31
539	5.390	6	12	782	36.40	1.45
358	1.790	3	10	451	77.88	1.26
500	2.500	3	7.5	648	49.45	1.30
500	2.500	3	6	648	64.06	1.30

25 m*M* Tris–HCl, pH 8.0; adsorption yield, 100%.

the volume of expanded bed is between two to three times of the packed bed material.

3.6. Column geometry

The objective of these experiments is to find the best column geometry for antithrombin purification. The anion-exchange columns of 20 cm×1 cm diameter, 10 cm×2.5 cm diameter and 15 cm×1.5 cm diameter were compared using the same volume of DE-52 adsorbents (18.85 cm^3) and 25 m*M* Tris–HCl buffer. The feedstock (skim milk) loaded onto the columns contained similar amounts of antithrombin (500–539 mg/l). The bed expansion was kept constant at 100%. Adsorption, washing and elution stages are shown in Fig. 3.

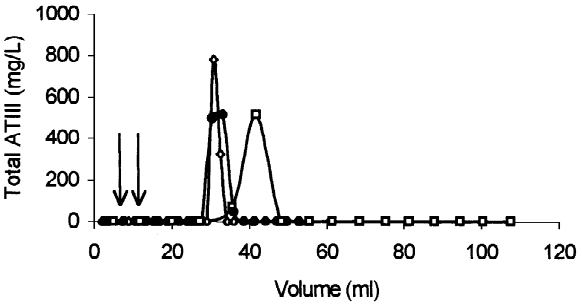

Fig. 3. Comparison of different column geometries in expanded bed chromatography. ◊, 20 cm×1 cm column; ●, 15 cm×1.5 cm column and □, 10 cm×2.5 cm column.

The adsorption yields are 100% in all these experiments. The elution yields increase by two-fold while the column diameter increases from 1 to 2.5 cm. However, ATIII is concentrated best in the smallest diameter column of 1 cm. The above mentioned experiments reveal that up to five-fold purification can be obtained in 1 cm internal diameter column by using optimum operating conditions (Table 7).

Table 7
Effect of column geometry

ATIII fed (mg)	Column (cm×cm)	Settled bed height (cm)	Expanded bed height (cm)	ATIII in eluted fraction (mg/l)	Elution yield (%)	Purification factor
5.390	20×1	6.0	12.0	782	36.40	1.45
5.000	15×1.5	2.7	5.4	513	58.60	1.03
5.000	10×2.5	1.0	2.0	513	73.80	1.03

4. Conclusion

Proteinase inhibitors like ATIII, α-antitrypsin and protein C are plasma proteins, and they are the third largest group in the plasma after albumin and the immunoglobulins [5]. Among them, the transgenic human antithrombin is the first therapeutic product derived from transgenic goats that has entered human clinical trials. The commercial potential of transgenic animal bioreactors for the production of high value human therapeutic proteins has made these processes very attractive. However, the high protein concentrations and the reactive protease cascades in dairy milk necessitates the development of new and effective purification technologies for these transgenic proteins rather than simply using the standard protein purification procedures.

Incorporation of a chromatographic technique early in the sequence of purification steps will lead to higher product yields and a less expensive purification process. The present study shows that expanded bed chromatography with ion-exchange adsorbent in combination with a priori zinc precipitation can yield a good resolution with the ease of processing. The purification process of ATIII from milk by expanded bed chromatography resulted in concentrated and partially purified antithrombin ready for the next purification step. In order to achieve antithrombin at a required purity, subsequent chromatographic techniques such as affinity, hydrophobic interaction, reversed-phase or membrane processing can be further applied. Nevertheless, the recovery of antithrombin on elution from the expanded bed compares well with other results shown in literature for a less specific method, i.e. ion-exchange chromatography. Ion-exchange adsorbents are preferred for their relative cheapness. Expanded bed chromatography, thus, offers an economic and short preliminary processing eliminating the need for intermittent solid removal steps. Other purification techniques like affinity or immunoaffinity chromatography used at initial stages for high value proteins are very expensive and cause the cost of downstream processing of the protein at commercial scale to be extremely high and consequently its manufacture to be infeasible. The ion-exchange expanded bed chromatography allows the application of these expensive specialty resins at later stages of purification in smaller amounts and thus leads to the reduction in cost.

Acknowledgements

The authors gratefully acknowledge the financial support provided by the Bogaziçi University Research Fund and State Planning Office through the projects 01A502 and 98K120880; also Pharmacia–Turkey for providing antithrombin (Atenativ).

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