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THE USE OF IMMOBILIZED METHYLCHYMOTRYPSIN FOR THE PURIFICATION OF HUMAN AND SHEEP ALPHA-1-PROTEINASE INHIBITOR (a_1-PI)

JOANNA GRYBEL and TADEUSZ WILUSZ
Institute of Biochemistry and Molecular Biology, University of Wrocław, Tamka 2, 50-137 Wrocław, Poland

Abstract: α_1-proteinase inhibitor was isolated from albumin fractions of human and sheep plasma using a new method of purification using affinity chromatography on immobilized methylchymotrypsin in the presence of 5 M NaCl. The inhibitor was finally polished to homogenity either by chromatography on a Mono Q or a Sephacryl S-200 HR column. The presented method makes it possible to recover α_1-proteinase inhibitor which has been added to cow milk.

Key Words: α_1-Proteinase Inhibitor, Human Plasma, Sheep Plasma, Milk, Methylchymotrypsin, Affinity Chromatography, NaCl

INTRODUCTION

α_1-Proteinase inhibitor (α_1-PI), also known as α_1-antitrypsin (α_1-AT), is a dominant component of the α_1-fraction in human serum. It is the most important inhibitor of neutrophyl elastase, which, if uncontrolled, can cause emphysema in the lung as a result of extensive proteolysis of the structural proteins: collagen, elastin, and proteoglycan. α_1-Proteinase inhibitor inactivates elastase, protecting the lung from degradation. Human α_1-proteinase inhibitor is the best-characterized member of the serpin superfamily [1]. Serpins are irreversible suicide substrate-like inhibitors of serine proteinases, which have been found in vertebrates, and in insects, viruses and plants. In human serum, serpins constitute about 10% of the plasma proteins. They participate in a number of physiological

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Corresponding authors – Fax: (+48) 71-3752-608; E-mail: joanna@bf.uni.wroc.pl, wilusz@bf.uni.wroc.pl

Abbreviations used: α_1-PI - α_1-proteinase inhibitor; BApNA - *N*-α-benzoyl-DL-Arg-*p*-nitroanilide; Tris - Tris(hydroxymetyl)aminomethane; PMSF - phenylmethylsulfonyl fluoride; NPGB - *p*-Nitrophenyl *p*-guanidinobenzoate·HCl; CNBr - cyanogen bromide; TBS - Tris buffered saline.

processes, including blood coagulation, complement activation, fibrynolysis and inflammation, as well as in the control of cell mobility and chromatin folding, and the regulation of the nervous and endocrine systems [2]. They also have roles in angiogenesis, apoptosis, neoplasia and viral pathogenesis [for a review see 3]. For patients with α_1-proteinase inhibitor deficiency and emphysema, a replacement therapy (intravenous supplementation or inhalation) with purified inhibitor preparations is recommended by the American Thoracic Society [4]. The α_1-protease inhibitor is separated from human serum [4-11]; therefore, it is extremely expensive. Whatever procedure is used, simplifying the purification process of the inhibitor, whether from human serum or transgenic animals [12], may better satisfy the current demand for α_1-proteinase inhibitor. In this work, we report on the use of immobilized methylchymotrypsin in the presence of 5M NaCl for the purification of α_1-proteinase inhibitor from human serum.

MATERIALS AND METHODS

Human plasma was provided by Wrocław District Blood Bank. Sheep blood was collected from healthy animals in the Veterinary Department of Wrocław Agricultural University in a bottle containing sodium citrate in a final concentration of 3.8 g/l to prevent coagulation; the blood cells were separated by centrifugation at 2400 g for 20 min. at 22ºC. Bovine α-chymotrypsin (EC 3.4.21.1), BApNA, *N*-α-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, Tris, PMSF, 4-chloro-1-naphthol, rabbit polyclonal antibody against human α_1-protease inhibitor, Tween 20, bovine serum albumin and Coomassie Briliant Blue G-250 were from Sigma (St.Louis, MO, USA). NPGB was from ICN Biomedical (Costa Mesa, CA, USA). Methyl *p*-nitrobenzenesulfonate, CNBr and the reagents for SDS-PAGE were purchased from Fluka (Buchs, Switzerland). Sepharose 4B, Sephacryl S-200 HR, Mono Q column 5/5 and the molecular weight markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis were from Pharmacia LKB Biotechnology (Uppsala, Sweden). YM membrane filters were from Amicon (Danvers, MA, USA). Bovine trypsin (EC 3.4.21.4) was prepared according to Wilimowska-Pelc and Mejbaum-Katzenellenbogen [13]. Peroxidase-labelled goat antibodies anti-rabbit IgG were from Boehringer (Mannheim, Germany). Nitrocellulose transfer membrane was from Micron Separations Inc., (Westborough, MA, USA). All the other reagents were of the analytical grade.

Methylation of a-chymotrypsin

His 57 in bovine α-chymotrypsin was chemically modified to 3-methylhistidine according to Nakagawa and Bender [14].

Immobilization of methylchymotrypsin

Methylchymotrypsin (500 mg) was immobilized on CNBr activated Sepharose 4B (50 ml) according to March *et al.* [15].

Protein determination
Quantitation of protein levels was carried out as per the method of Bradford [19] with bovine albumin as a standard. The concentrations of trypsin and α-chymotrypsin were determined via spectrophotometric titration with NPGB [16].

Activity measurement
Enzyme activities were measured spectrophotometrically at 410 nm after 10 min. incubation at 37°C in 0.1 M Tris-HCl buffer, pH 8.0, with 20 mM $CaCl_2$, using BApNA for trypsin [17] and *N*-α-succinyl-Ala-Ala-Pro-*p*-nitroanilide for α-chymotrypsin [18] as substrates. One unit of the inhibitory activity was defined as the amount of protein required to reduce the activity of 2 μg of an enzyme to 50 % of the original value.

Electrophoresis and immunoblotting analysis
SDS-PAGE was performed according to Laemmli [20]. Gels were stained with 0.1 % Coomassie Brillant Blue R-250. For immunoblotting analysis, gels were electroblotted to a nitrocellulose tansfer membrane using 25 mM Tris/192 mM Glycine/20 % (v/v) methanol buffer, pH 8.3, according to Towbin *et al.* [21]. 0.03 % SDS was added to the transfer buffer. After the transfer (2 hr, 200 mA) the CN membrane was blocked overnight with 0.25 % gelatin in TBS, followed by three washes with TBS. The primary rabbit polyclonal antibody against α_1-proteinase inhibitor was diluted 1:1500 in TBS containing 0.5 % gelatin and the immunoreaction was run for 2 hours. The blot was then washed with TBS and incubated for 2 hours with peroxidase-labelled goat antibodies anti-rabbit IgG, diluted 1:6000 in TBS with 0.05 % Tween 20. The peroxidase reaction was carried out using 0.05 % 4-chloro-1-naphtol as a substrate in TBS containing 0.033 % H_2O_2.

N-terminal amino-acid sequence
N-terminal sequence analyses were performed on a gas-phase sequencer (Model 491, Perkin-Elmer/Applied Biosystems, Foster City, CA, USA). The PTH-derivatives were analyzed by online gradient HPLC on a SPHERI-5 PTH column (5m C_{18}; 220 x 2 mm i.d.; Perkin-Elmer) using a Model 140C Microgradient Delivery System equipped with a Model 785A Programmable Absorbance Detector (both from Perkin-Elmer/Applied Biosystems).

RESULTS

Purification of human a_1-proteinase inhibitor
All of the following steps were carried out at 5°C. 200 ml of human plasma was diluted with an equal volume of 10 mM Tris-HCl buffer, pH 8.0, containing 0.9 % NaCl. Saturated ammonium sulfate was added to achieve 0.45 saturation. After 3 hours, the pellet formed during centrifugation was discarded. From the supernatant, the albumin fractions were salted out by the addition of saturated

ammonium sulfate to the saturation of 0.8. After a further 3 hours, the formed precipitate was collected by centrifugation, dissolved in water and dialysed for 24 h against 0.05 M Tris-HCl buffer, pH 9.0.

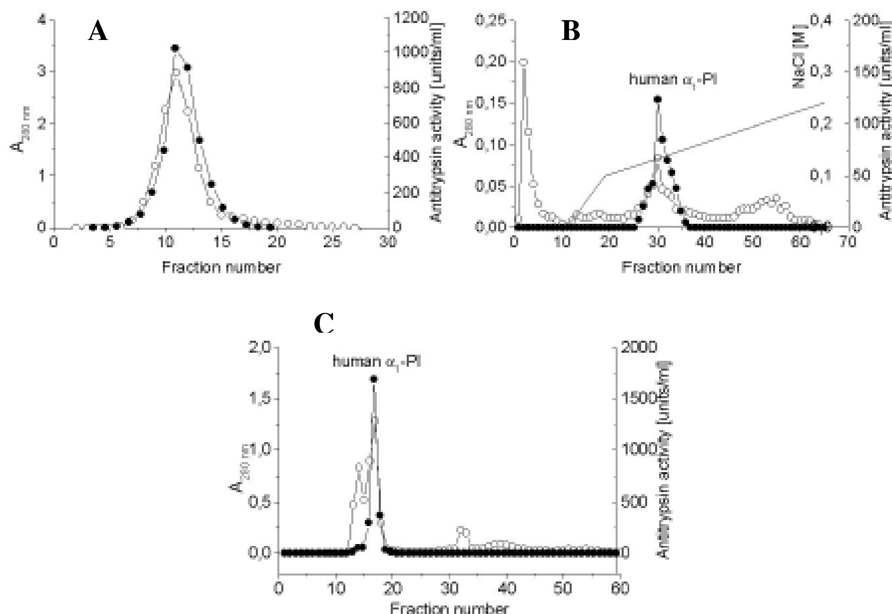

Fig. 1. Purification of human α_1-PI: **A** – affinity chromatography on immobilized methylchymotrypsin. A 100 ml sample containing 5 M NaCl at pH 9.0 was loaded on the methylchymotrypsin-Sepharose 4B column (29 x 75 mm), equlibrated with 0.05 M Tris-HCl pH 9.0, containing 5 M NaCl. The column was washed intensively with equilibrating buffer until the A_{280} value dropped below 0.02. The inhibitor was eluted with 0.05 M Tris-HCl buffer, pH 9.0. A 9 ml fraction was collected at a flow rate of 100 ml h^{-1} (the elution profile is shown in the picture). **B** – ion exchange chromatography. Partially purified α_1-PI was loaded on a Mono Q 5/5 column equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. Elution was performed by a gradient of NaCl in equilibration buffer. A 1 ml fraction was collected at a flow rate of 60 ml h^{-1}. **C** – size exclusion chromatography. After affinity chromatography the sample was loaded on a Sephacryl S-200 HR column (12 x 500 mm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. A 1.4 ml fraction was collected at a flow rate of 7 ml h^{-1}. —O—, absorbance at 280 nm; —●—, antitrypsin activity.

NaCl *in substantia* was added to the dialysed solution (albumin fraction) to a final concentration of 5 M, and applied to a methylchymotrypsin-Sepharose 4B column (29 x 75 mm), which had been equilibrated with 0.05 M Tris-HCl buffer, pH 9.0, containing 5 M NaCl. Unbound proteins were washed out with the starting buffer. The adsorbed α_1-protease inhibitor was then eluted with 0.05 M Tris-HCl buffer, pH 9.0 (Fig. 1A). The methylchymotrypsin-Sepharose 4B

was regenerated by washing with 0.01 M HCl, followed by a starting buffer. The fractions containing α_1-proteinase inhibitor were combined and dialysed for 24 h against the 0.05 M Tris-HCl buffer, pH 8.0, with several changes of the same buffer. After dialysis, the inhibitor was additionally purified in an FPLC system on a Mono Q 5/5 column equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. The inhibitor was eluted with a gradient of NaCl in the same buffer at flow rate of 60 ml h^{-1} (Fig. 1B). Alternatively α_1-proteinase inhibitor was concentrated on a YM 1 membrane and polished on a Sephacryl S-200 HR column (12 x 500 mm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0 (Fig. 1C). The fractions containing α_1-protease inhibitor were pooled and lyophilized. From 200 ml of human plasma, after Mono Q chromatography, 31.2 mg of α_1- protease inhibitor was obtained, whereas after Sephacryl S-200 HR as much as 33.8 mg of the inhibitor was gained. Polyacrylamide gel electrophoresis of the final preparation demonstrated a high degree of purity (Fig. 4A) and its identity as human α_1-proteinase inhibitor was confirmed by immunoelectrophoresis (Fig. 5A).

Purification of sheep a_1-proteinase inhibitor

Sheep plasma α_1-proteinase inhibitor was isolated following the procedure for the human plasma inhibitor described above. After chromatography on immobilized methylchymotrypsin in a 5 M concentration of NaCl (Fig. 2A), the inhibitor was further purified on a Mono Q column (Fig. 2B). From 200 ml of

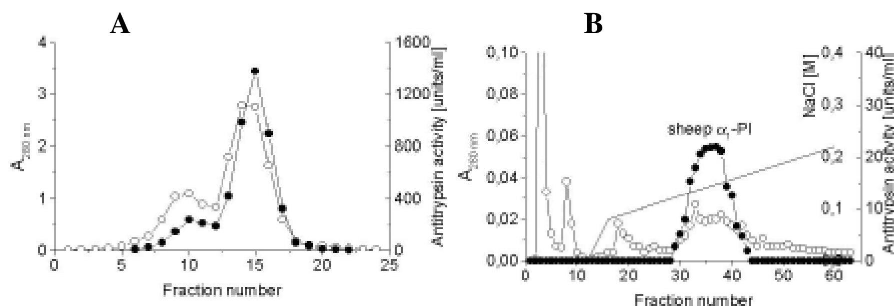

Fig. 2. Purification of sheep α_1-PI: **A** – affinity chromatography on immobilized methylchymotrypsin. A 100 ml sample containing 5 M NaCl at pH 9.0 was loaded on the methylchymotrypsin-Sepharose 4B column (29 x 75 mm), equlibrated with 0.05 M Tris-HCl pH 9.0, containing 5 M NaCl. The column was washed intensively with equilibrating buffer until the A_{280} value dropped below 0.02. The inhibitor was eluted with 0.05 M Tris-HCl buffer, pH 9.0. A 9 ml fraction was collected at a flow rate of 100 ml h^{-1} (the elution profile is shown in the picture). **B** – ion exchange chromatography. Partially purified α_1-PI was loaded on a Mono Q 5/5 column equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. The elution was performed by a gradient of NaCl in equilibration buffer. A 1 ml fraction was collected at a flow rate of 60 ml h^{-1}. —O—, absorbance at 280 nm; —●—, antitrypsin activity.

sheep plasma, as much as 23 mg of pure inhibitor (Fig. 4C) was obtained. The identity of the isolated protein with sheep plasma α_1-proteinase inhibitor [22] was confirmed by the identification of its N-terminal amino acid sequence: GVLQGHAVQE.

Recovery of human a_1-proteinase inhibitor from milk

9 mg of the previously purified human α_1-proteinase inhibitor dissolved in 3 ml of 0.15 M NaCl, was added to 100 ml of cow milk (3.2 % fat). The mixture was centrifuged at 3 500 x g for 30 minutes, and the fat from the top phase was removed. An equal volume of saturated ammonium sulfate was added to 90 ml of skim milk, and after 3 hours, this was centrifuged, and the proteins which had salted out were discarded. Albumin fractions from the supernatant were precipitated by increasing the ammonium sulphate to 0.8 saturation. The obtained precipitate was collected by centrifugation after 3 hours, dissolved in 5 ml of 0.02 M Tris-HCl buffer, pH 8.0, and dialysed overnight against the same buffer. NaCl was added to the clear dialysate to a final concentration of 5 M.

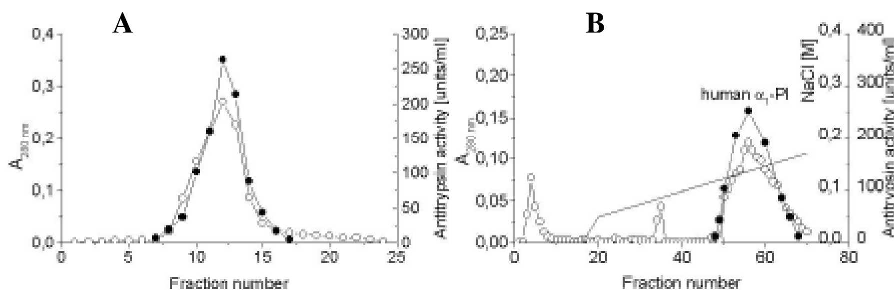

Fig. 3. Recovery of human α_1-PI from cow milk: **A** – affinity chromatography on immobilized methylchymotrypsin. A 20ml sample containing 5 M NaCl at pH 9.0 was loaded on the methylchymotrypsin-Sepharose 4B column (16 x 50 mm), equlibrated with 0.05 M Tris-HCl pH 9.0, containing 5 M NaCl. The column was washed intensively with equilibrating buffer until the A_{280} value dropped below 0.02. The inhibitor was eluted with 0.05 M Tris-HCl buffer, pH 9.0. A 9 ml fraction was collected at a flow rate of 100 ml h^{-1} (elution profile is shown in the picture). **B** – ion exchange chromatography. Partially purified α_1-PI was loaded on a Mono Q 5/5 column equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. The elution was performed by a gradient of NaCl in an equilibration buffer. 1 ml fractions were collected at a flow rate of 60 ml h^{-1}. —O—, absorbance at 280 nm; —●—, antitrypsin activity.

The inhibitor from the solution was isolated using immobilized methylchymotrypsin (Fig. 3A), followed by chromatography on a Mono Q column (Fig. 3B) in the conditions described above for human plasma α_1-proteinase inhibitor. Using this procedure, 3 mg out of 9 mg of α_1-proteinase inhibitor added to 100 ml of cow milk was recovered. The isolated inhibitor was

homogenous in SDS-PAGE (Fig. 4B). and its identity with human α_1-proteinase inhibitor was confirmed by immunoelectrophoresis (Fig. 5B).

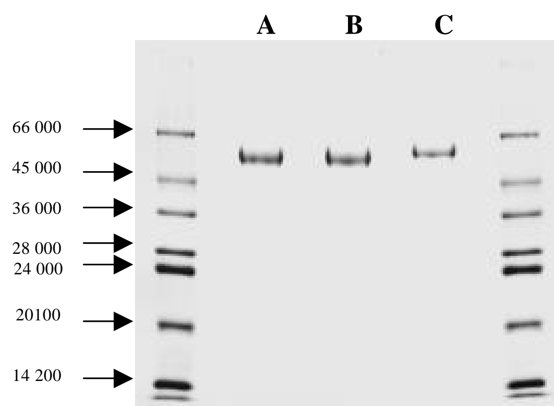

Fig. 4. SDS-PAGE of the purified α_1-proteinase inhibitor. Lane A, α_1-PI from human plasma after Mono Q chromatography – 1 µg; lane B, α_1-PI recovered from cow milk – 1 µg; lane C, α_1-PI from sheep plasma – 1 µg. The molecular weight markers are: bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36 kDa), bovine carbonic anhydrase (28 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin from bovine milk (14.2 kDa).

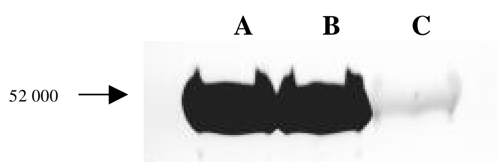

Fig. 5. Western blot of the purified α_1-proteinase inhibitor. Fractions of α_1-PI from human plasma after Mono Q chromatography (A – 3 µg); human α_1-PI recovered from cow milk (B – 3 µg); and α_1-PI from sheep plasma (C – 5 µg) were analyzed by SDS-PAGE, followed by electrophoretic transfer to nitrocellulose. The blot was subsequently probed using a polyconal antibody against α_1-proteinase inhibitor and a peroxidase-conjugated secondary antibody.

A summary of the purification procedure of α_1-protease inhibitor from human and sheep plasma, as well as its recovery from cow milk is shown in Tabs. 1, 2 and 3.

Tab. 1. Purification of human α_1-proteinase inhibitor from 200 ml of human plasma.

Purification step	Protein (mg)	Units	Specific activity (units/mg)	Purification fold	Yield (%)
Human plasma	14150.0	269506.0	19.0	1.0	100.0
Albumin fraction	7075.0	216952.0	30.7	1.6	80.5
Methylchymotrypsin	123.0	93518.6	760.0	40.0	34.7
Mono Q	31.2	47702.6	1528.9	80.5	17.7
Or Sephacryl S-200 HR	27.0	33990.3	1258.9	66.2	12.6

Tab. 2. Purification of sheep α_1-proteinase inhibitor from 200 ml of sheep plasma.

Purification step	Protein (mg)	Units	Specific activity (units/mg)	Purification fold	Yield (%)
Sheep plasma	10193.0	149840.0	14.4	1.0	100.0
Albumin fraction	5148.0	144895.3	28.1	1.9	96.7
Methylchymotrypsin	135.7	49464.9	372.1	25.8	33.7
Mono Q	23.0	23484.8	1044.0	72.5	16.0

Tab. 3. Recovery of 9 mg of human α_1-proteinase inhibitor added to 100 ml of cow milk.

Purification step	Protein (mg)	Units	Specific activity (units/mg)	Purification fold	Yield (%)
Cow milk with α_1-PI	16300.0	10758.0	0.7	1.0	100.0
Albumin fraction	2500.0	9350.0	3.7	5.7	92.0
Methylchymotrypsin	4.7	3975.3	845.8	1281.5	37.0
Mono Q	3.0	2800.0	933.3	1414.1	26.1

DISCUSSION

One of the most effective methods of proteinase inhibitor purification is an affinity chromatography on immobilized proteinases. However, this method is not without certain inconveniences. One of them is the hydrolysis of the reactive site peptide bond in some inhibitors. It takes place during the elution of proteins from the matrix in acidic conditions, and as a result, a mixture of virgin and modified forms of inhibitor molecules are produced [25]. In the event of the presence in a initial material of several isoinhibitors and/or inhibitors slightly differing in polipeptide chain length, the purification of the individual protein becomes a real problem. To avoid this, immobilized anhydrotrypsin and

anhydrochymotrypsin is used. These chemically-modified, catalytically-inactive enzymes, in which Ser 195 is converted to dehydroalanine, are still able to form reversible complexes with inhibitors, yielding upon dissociation non-modified inhibitors [26]. However, the procedure of the synthesis of these derivatives is time-consuming and inefficient.

The use of immobilized, fully active proteinases for the purification of α_1-PI, does not come into consideration at all, since it forms irreversible complexes with non-modified enzymes. That is why Drechsel *et al.* [10] used immobilized anhydrochymotrypsin for this purpose. However, the complex is so strong that a competitive inhibitor – chymostatin – is required to elute the α_1-PI. Chymostatin is rather expensive, which is why Drechsel's procedure is mainly usuable at the analytical scale.

In this paper, we present a new method of purification of α_1-PI from human and sheep serum. The crucial step of this procedure is an affinity chromatography on immobilized methylchymotrypsin in the presence of 5 M NaCl. Methylchymotrypsin is a catalytically-inactive derivative of α-chymotrypsin in which His 57 has been converted to 3-methylhistidine. The methylation procedure is simple and efficient [14]. Although deprived of catalytic activity, methylchymotrypsin maintains the ability to form complexes with substrates and inhibitors, especially in presence of 5 M NaCl. The high concentration of NaCl enables the adsorbtion of the inhibitor onto immobilized metylchymotrypsin, which can subsequently be eluted either with water or with any solution with a low salt concentration [23, 24].

α_1-PI only binds to immobilized methylchymotrypsin in presence of 5 M NaCl and can be released from the column upon decreasing the salt concentration, without the necessity of lowering pH. This is very important, since α_1-PI loses its activity in acidic conditions. Buffers of pH 6.0, 7.0, 8.0, and 9.0 were tested to find the optimal conditions of desorption for the α_1-protease inhibitor. It was established that pH 9.0 was the most effective for the elution of the inhibitor.

α_1-PI eluted from immobilized methylchymotrypsin was additionally polished on a Mono Q or alternatively on a Sephacryl S-200 HR column, yielding 31.2 mg and 27 mg of a homogenous protein, respectively, from 100 ml of human serum. Both techniques effectively remove contaminants from the inhibitor preparation. However, the chromatography on a Mono Q column is more convenient in use and gives a slightly higher yield.

When a human serum albumin fraction was applied onto the affinity column only 50 % of the α_1-PI was bound to immobilized methylchymotrypsin, even in an excess of an accessible ligand. After repetitive application of the unbound material onto the same column, again only about 50 % of the remaining activity was adsorbed. After the second chromatography, the α_1-PI was more contaminated; therefore, it required additional purification as on Mono Q and on Sephacryl S-200 HR columns. After the second chromatography, on

immobilized methylchymotrypsin we obtained an additional 16 mg of pure α_1-PI.

The presented method allows the isolation of the α_1-PI from the serum of other animal species, for example from sheep serum. The same procedure makes it possible to recover α_1-PI added to cow milk, and it might be useful for the purification of the recombinant inhibitor from the milk of a transgenic livestock.

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