*Journal of Chromatography*, 278 (1983) 53—61
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1822

# THE USE OF THIOL—DISULPHIDE EXCHANGE CHROMATOGRAPHY FOR THE AUTOMATED ISOLATION OF $\alpha_1$-ANTITRYPSIN AND OTHER PLASMA PROTEINS WITH REACTIVE THIOL GROUPS

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(First received March 28th, 1983; revised manuscript received June 15th, 1983)

## SUMMARY

A method has been developed for the rapid isolation of $\alpha_1$-antitrypsin and other thiol proteins from plasma by an automated chromatography system. The thiol-proteins are initially bound to matrix-linked activated thiol-compounds by an SH—SS interchange reaction. The mixed disulphides are then reduced in two steps and subfractionated by passage through Blue-Sepharose and AH-Sepharose columns. The rate of the interchange reactions varies with the microenvironment of the reacting thiols. $\alpha_1$-Antitrypsin is recovered with 95% purity in 60% yield within two days from 1 l of plasma.

## INTRODUCTION

Current methods for the isolation of $\alpha_1$-antitrypsin from plasma are usually designed to recover below 100 mg in each cycle and most procedures include ion-exchange chromatography [1—4]. Fractions containing the bulk (80—90%) of $\alpha_1$-antitrypsin are selected after each chromatographic step from the elution diagrams. Since this entails partial exclusion of the most basic and acid fractions at each chromatography step, the final product will contain $\alpha_1$-antitrypsin with a microheterogeneity that differs from that of native plasma [5]. We describe here a method principally based on thiol—disulphide interchange reactions [6] for semiautomatic isolation of $\alpha_1$-antitrypsin with retained microheterogeneity in high yield and in weekly gram quantities. The fractionation procedure is monitored by a programmable controller.

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## MATERIALS AND METHODS

L-Cysteine·HCl and 2-mercaptoethanol were purchased from BDH (Poole, U.K.) and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), dithiothreitol from Sigma (St. Louis, MO, U.S.A.). AH-Sepharose 4B, Blue-Sepharose CL-6B, cyanogen bromide (CNBr)-activated Sepharose 4B, heparin-Sepharose CL-6B, thiopropyl-Sepharose 6B, thiol-Sepharose 4B and dextran sulphate (sodium salt) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and polyethyleneglycol M 4000 from Kebo (Stockholm, Sweden).

Antisera to all major plasma proteins were available at the laboratory.

Agarose gel electrophoresis, immunoelectrophoresis and electroimmunoassay were used for screening purposes and for quantitation of specific proteins.

### *κ-Chain-Sepharose*

κ-Chains isolated from urine of myeloma patients were activated to κ-thionitrobenzoate (κ-TNB) [5]. κ-Chains (5 g) were dissolved in 75 ml Tris—HCl (1 *M*, pH 8) with $Na_2$EDTA (0.1 *M*) and saturated with DTNB. The solution was kept at room temperature for 4 days to obtain mainly monomeric κ-TNB. The amount of the mixed disulphide κ-TNB required for Sepharose-coupling was passed through 8—10 volumes Sephadex G 75, equilibrated with 0.1 *M* $NaHCO_3$ containing 0.5 *M* sodium chloride, to separate κ-TNB from TNB and DTNB. κ-Chains (1 g) with TNB-blocked COOH-terminal cysteinyls were conjugated with 20 g CNBr-activated Sepharose 4B as recommended by the manufacturer. About 40 μmol κ-chains (23,000 dalton) were bound per 100 ml Sepharose.

### *Plasma preparations*

Plasma was available from the hospital blood bank. Low (LD) and very low density (VLD) lipoproteins were removed from plasma, either by passage through a heparin-Sepharose column (2 ml plasma per ml heparin-Sepharose) [7] which allowed recovery of a series of proteins, or by precipitation at 4°C with 20 ml dextran sulphate (10%) and 100 ml (1 *M*) calcium chloride per l plasma [8]. Plasma was mildly reduced by adding 2-mercaptoethanol to a concentration of 0.02 *M*, to cleave mixed disulphides and interprotein SS-bridges. All low-molecular thiol compounds were removed by dialysis overnight with running tap-water. Polyethyleneglycol was added to a concentration of 4% (w/v) at 4°C, and the plasma was centrifuged 1 h later. Salts were added to the supernatant in a final concentration of 0.05 *M* Tris, 0.2 *M* sodium chloride, 5 m*M* $Na_2$EDTA and 3 m*M* $NaN_3$, and the pH was adjusted to 8.1—8.0 with 6 *M* hydrochloric acid under vigorous stirring. This diluted plasma was stored frozen until the fractionation was started. The plasma albumin content had decreased from 45 to about 30 g/l.

### *Solutions for chromatography*

Buffer 1: 0.05 *M* Tris buffer, pH 8.1, containing 0.2 *M* sodium chloride, 5 m*M* $Na_2$EDTA and 3 m*M* $NaN_3$. Buffer 2: 0.05 *M* sodium phosphate buffer, pH 5.5, containing 0.5 *M* sodium chloride. Reducing solution 1: 1 mg DTNB and 0.25 mg dithiothreitol per ml buffer 1. Reducing solution 2: buffer 1 with

20 m$M$ 2-mercaptoethanol. DTNB solution of $\kappa$-chain reactivation: 0.5 mg DTNB per ml buffer 1. Buffer 1 with additional 1.3 $M$ sodium chloride was used as a regeneration solution for ridding Blue-Sepharose and AH-Sepharose of proteins.

## EXPERIMENTAL

### *The flow system for the fractionation of thiol-proteins*

The principle design is shown in Fig. 1 and Table I. Plasma rid of VLD- and LD-lipoproteins is reduced, dialyzed, and pumped through a $\kappa$-chain Sepharose column with DTNB-activated thiol groups. $\alpha_1$-Antitrypsin and some other plasma proteins are retained in the column by SH—SS exchange reactions. The retained (SS-linked) proteins are later eluted by a two-step reduction. Albumin is removed by passing the primary eluate through a Blue-Sepharose column. The eluate is pumped on through an AH-Sepharose column which retains pre-albumin and other minor contaminants, while $\alpha_1$-antitrypsin and a mixed Ig-fraction pass through to be recovered as eluate 1. These proteins are separated by ammonium sulphate fractionation. Other thiol-proteins are recovered in eluates II, III and IV.

All solution inlets are furnished with filters. Transmission tubes (0.075 mm), glass T connectors and nylon nipples of Technicon design are used throughout as links.

Arrows in Fig. 1 indicate direction of flow. The Sepharose columns are

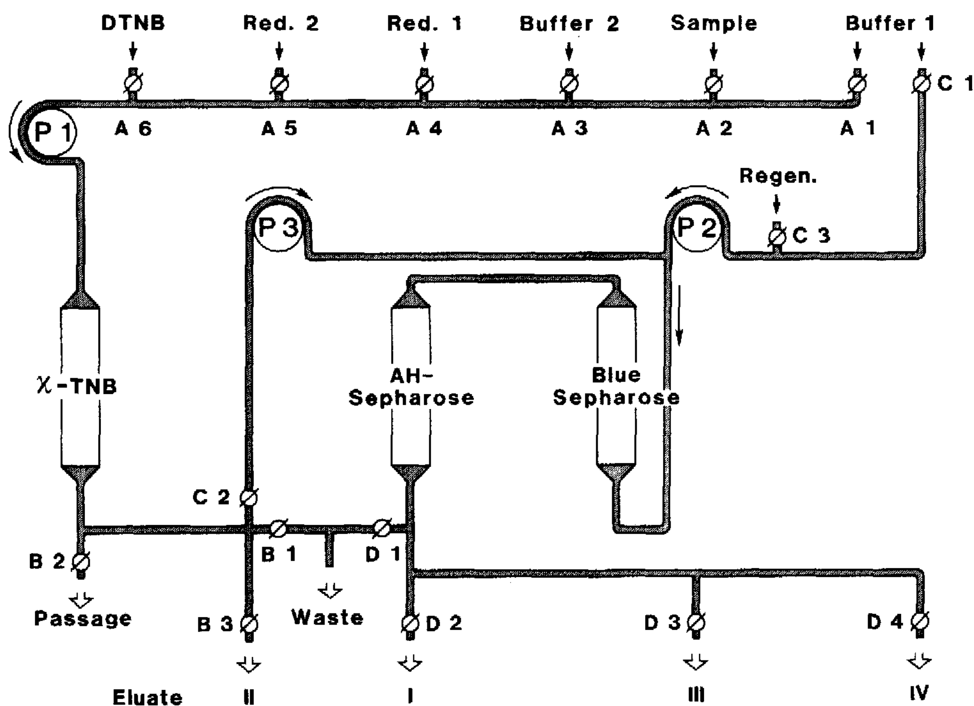



Fig. 1. Scheme of flow system for retention elution and subfractionation of thiol-proteins. A, B, C and D indicate valves and P pumps. The activation time and order of pumps and valves are given in Table I. For details see text.

TABLE I

PROGRAMME FOR FLOW SYSTEM GIVEN IN FIG. 1

Standard activation programme with a pump flow-rate for 1 and 3 of 180 ml/h, and 110 ml/h for P2; 450 ml $\kappa$-Sepharose, 150 ml Blue-Sepharose and 170 ml AH-Sepharose columns suitable for fractionation of 500 ml de-lipidated, mildly reduced plasma (final volume 800 ml). Time is given in minutes.

| Step | Time | Valve | P1 | Valve | Valve | P2 | P3 | Valve |
|---|---|---|---|---|---|---|---|---|
| 1 | 230 | A1 | + | B1 | | | | |
| 2 | 120 | A2 | + | B1 | | | | |
| 3 | 155 | A2 | + | B2 | | | | |
| 4 | 120 | A1 | + | B2 | | | | |
| 5 | 115 | A3 | + | B1 | | | | |
| 6 | 230 | A1 | + | B1 | | | | |
| 7 | 55 | A4 | + | B1 | C1 | + | | D1 |
| 8 | 65 | A1 | + | B1 | C1 | + | | D1 |
| 9 | 70 | A5 | + | | C2 | | + | D1 |
| 10 | 40 | A1 | + | B1 | C1 | + | | D1 |
| 11 | 120 | A1 | + | B3 | C3 | + | | D2 |
| 12 | 125 | A6 | + | B1 | C1 | + | | D3 |
| 13 | 85 | A6 | + | B1 | C1 | + | | D4 |

loaded from the bottom upwards, their inlets being provided with 10-ml bubble traps with a clamped tube as top outlet. The inlet and outlet of each column is connected to a mechanically adjustable double-channel four-port valve (SRV-4, Pharmacia), which permits the channel system to be flushed by cleansing solutions that bypass the columns. These details have been excluded from Fig. 1.

The three chromatography tubes (300 mm × 50 mm I.D.) are furnished with adaptors at top and bottom (Pharmacia A 50). The 16 valves were of type: Skinner valve B10 (6 W, 24/50 V) (New Britain, CT, U.S.A.). The heat generated in the activated valves may raise the temperature of the valve body to some 55°C, which may cause accumulation of a thin ring of protein precipitate inside the rim of the valve membrane. Since this precipitate may finally disturb the valve function after 25—50 h, valves of latch type are therefore preferred at positions A2 and B2, through which the bulk of heat labile plasma protein passes.

Three LKB multiplex peristaltic pumps are used. Pumps 1 and 3 are calibrated to the same flow-rate (180 ml/h) and pump 2 slower (110 ml/h). The pump tubes are exchanged prophylactically every third month, no flow-rate adjustment being needed in between. A Mitsubishi programmable controller, Melsec-K, activates pumps and valves in accordance with an easily exchangeable programme from a Melsec-K7PUE programming unit. The controller is equipped with two KYOI units (each a 16-point element I/O unit).

The columns are emptied and repacked every second month, assuming four complete cycles to be run each week. The $\kappa$-chain-Sepharose and the Blue-Sepharose have been used for two years without apparent loss in exchange capacity, while the AH-Sepharose had to be replaced within three months (= 12—15 regeneration cycles). Increasing amount of prealbumin in eluate 1 indicates that it is time for renewal of this ion exchanger.

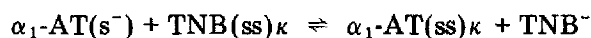
*Practical comments*

De-aeration of the solutions is unnecessary if bubble traps are mounted at the column inlets. The reducing solutions 1 and 2 are prepared daily and retain sufficient reducing capacity overnight without having to be flushed with nitrogen. No disturbing build-up of pressure has been observed in the tube system during repetitive cycles, when the flow-rate was kept at 3 ml/min and the LD lipoproteins are carefully removed from the plasma. The Sepharose packing stabilizes after a few cycles, and some millimetres of free fluid layer are observed above the filters of the inlet adaptors. These need not be adjusted. Smooth flow through the columns is indicated if an even yellow zone passes when DTNB is pumped through.

The outlet from the AH-Sepharose was connected to a fraction collector, and the samples were analysed by electroimmunoassay to find the optimal period for activation of valve D2. Identical daily volumes in the receptacles of the four eluate outlets indicate correct flow and valve functions.

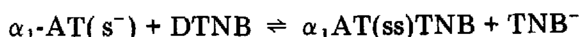
*Development of the method for fractionation of thiol proteins*

The SH—SS interchange reaction, on the passage of mildly reduced plasma at pH 7—8, was used to compare the efficiency of DTNB-activated thiopropyl Sepharose (-O-$CH_2$-CHOH-$CH_2$-SH), glutathione Sepharose (-GluCysGly), and $\kappa$-chain Sepharose (Glu-Cys) [6] in retaining both albumin and $\alpha_1$-antitrypsin. Both proteins have one reactive thiol group, but the ratio of the plasma concentrations of mercaptoalbumin and $\alpha_1$-antitrypsin is above 15 to 1. The results showed that the interchange reaction with the activated thiopropyl group was roughly proportional to the albumin:$\alpha_1$-antitrypsin ratio in the passing solution, while activated glutathione bound to Sepharose retained $\alpha_1$-antitrypsin 30 times faster than it did albumin, and activated $\kappa$-chains enriched $\alpha_1$-antitrypsin 150 times faster than they did albumin, at a flow-rate of the protein solution through the column of 3 ml/min. Since the amount of albumin retained is increased by recycling the sample, or by retarding the flow, the solution was passed through only once to keep the albumin retention at a minimum. We have shown previously [6] that SS-linked plasma proteins vary slightly in their sensitivity to reduction; thus, further enrichment of $\alpha_1$-antitrypsin to albumin could be achieved by a two-step reduction (elution) programme. The coupling reaction used in the column



$$\alpha_1\text{-AT(s}^-) + \text{TNB(ss)}\kappa \rightleftharpoons \alpha_1\text{-AT(ss)}\kappa + \text{TNB}^-$$

was forced to the left by excess $TNB^-$ in reducing solution 1. Any released $\alpha_1$-AT($s^-$) formed a soluble mixed disulphide with DTNB in reducing solution 1 according to



$$\alpha_1\text{-AT(s}^-) + \text{DTNB} \rightleftharpoons \alpha_1\text{AT(ss)TNB} + \text{TNB}^-$$

The mixed disulphide was eluted in front of and overlapping $TNB^-$. In the second elution step, 0.02 *M* 2-mercaptoethanol released all remaining $\kappa$-linked plasma proteins. This eluate (II) was harvested without further purification

through valve B3, while the solution with the proteins first released was transferred to a Blue-Sepharose column, the size of which was chosen to efficiently remove albumin. The third column was loaded with AH-Sepharose, which had been found to link prealbumin stronger, compared with $\alpha_1$-antitrypsin, than other anionic exchangers. The proteins remaining in the solution after its passage through the AH-Sepharose column, were harvested through valve D2 (eluate I) and consisted mainly of $\alpha_1$-antitrypsin and immunoglobulins. The latter are quantitatively precipitated by 1.97 *M* ammonium sulphate.

The AH-Sepharose and Blue-Sepharose were regenerated by the passage of a strong salt solution which released proteins that were collected in the consecutive eluates III and IV — eluate III containing proteins from the AH-Sepharose with some admixture of proteins from the Blue-Sepharose column. The bulk of the latter are recovered in eluate IV.

Proteins in all four eluates were precipitated with ammonium sulphate in two steps (1.97 *M* and 3.08 *M*) with intervening centrifugation.

## RESULTS

After repeated activation and reduction of the $\kappa$-chain Sepharose with DTNB and 2-mercaptoethanol, 210 $\mu$mol $TNB^-$ per l Sepharose was recovered on reduction. During each loading with plasma the total amount of thiol proteins linked per l $\kappa$-Sepharose was about 25 $\mu$mol. The average recovery of plasma proteins in ten experiments is given in Table II. The plasma used in these experiments had been de-lipidated by the passage of heparin-Sepharose. The proteins reported in the tables are those which were obtained in mg quantities when loading a 450-ml $\kappa$-chain column with 300 ml plasma.

TABLE II

MAJOR PROTEINS RECOVERED FROM EACH SH—SS CYCLE WITH ACTIVATED $\kappa$-CHAINS

Values are mg protein recovered from 300 ml plasma in eluate.

| Proteins* | Eluate I | | Eluate II | | Eluate III | | Eluate IV | |
|---|---|---|---|---|---|---|---|---|
| | a | b | a | b | a | b | a | b |
| $\alpha_1$-Antitrypsin | 4 | 235 | 0.3 | 0.4 | 3 | 24 | — | — |
| Albumin | 0.2 | 1 | 13 | 87 | 8 | 79 | 1 | 44 |
| Prealbumin | — | 1 | (1) | 6 | 3 | 14 | trace | — |
| $\alpha_2$-Macroglobulin | 18 | — | 37 | — | 72 | — | 2 | — |
| IgA | 12 | — | 50 | — | 32 | — | 1 | — |
| IgM | 20 | — | 41 | — | 16 | — | 0.5 | — |
| IgG | 10 | — | 12 | — | 3 | — | — | — |
| C3 | 0.3 | — | 0.8 | — | 4 | — | 0.05 | — |
| C4 | 0.08 | — | 0.4 | — | 3 | — | 0.1 | — |
| Hc-globulin | 0.08 | 0.02 | 0.3 | — | 0.3 | 0.05 | 0.05 | 0.01 |
| HDL | (0.9)** | | (3) | | (11) | | (1) | |

*Protein content of each eluate (I—IV) is given after precipitation with 1.97 *M* and 3.08 *M* ammonium sulphate as a and b, respectively.

**Values between brackets indicate atypical immunoreactivity.

The following proteins were searched for but not found: orosomucoid, antichymotrypsin, inter-α-trypsin inhibitor, ceruloplasmin, antithrombin III, $\alpha_2$-antiplasmin, Cl-esterase inhibitor, HS-globulin, prothrombin, plasminogen, pseudocholinesterase, fibronectin, haemopexin, thyroxin-binding globulin, sex hormone-binding globulin, P-component, CRP, $\alpha_2$-microglobulin, β-microglobulin, C4-binding globulin. The following average percentages were obtained for the recovery of each protein, when calculated from the protein load: $\alpha_1$-antitrypsin (66%), prealbumin (23%), IgM (32%), $\alpha_2$-macroglobulin (17%), IgA (16%), transcortin (10%), HC- or $\alpha_1$-microglobulin (9%), C4 (4%), albumin (2%), C3 (2%), HDL (2%), haptoglobins (1%), IgG (0.8%), transferrin (0.3%), Gc-globulin (trace), LDL (trace).

The recovery given for IgM in Table II may be too high, since we did not check the molecular size, and since the fractions were not strongly reduced before immunochemical estimation. The recovery values for C3, C4 and HDL are also approximate, because the electroimmunoassay analyses indicated atypical immunologic reactivity with partial blockage of antigenic determinants. Generally, the agarose gel electrophoretic patterns of the final four fractions agreed well with the concentrations of the various protein species identified in them. However, a major $\alpha_1$-component of low solubility in eluate II, and the major $\alpha_2$-fraction of low solubility in eluate IV have not yet been identified.

The proteins in eluate II are cleaved off from the κ-chains together with $TNB^-$ by 2-mercaptoethanol, and eluted with excess 2-mercaptoethanol, mainly as free thiol proteins, while the proteins in eluates I, III and IV are recovered as mixtures of mixed disulphides with DTNB, or as SS-linked mixed proteins. This may explain why slightly atypical electrophoretic mobility and broad electrophoretic zones are obtained. The more anodal position of prealbumin and albumin in eluate III than in eluate II is due to the negative charge added by linked $TNB^-$ (not shown).

The various proteins are converted within 24 h to mixed Cys-disulphides by the addition of 0.1 *M* freshly prepared cysteine·HCl under pH adjustment to 7. This reaction is accelerated by the addition of activated carbon which adsorbs $TNB^-$. The proteins are stored as stabile mixed disulphides with cysteine in 0.1 *M* glycine of pH 6—7, 0.05 *M* $Na_2EDTA$ 0.15 *M* sodium chloride and 3 m*M* $NaN_3$. The purity of $\alpha_1$-antitrypsin in eluate Ib and of mercaptoalbumin in IVb is 95% or higher. The microheterogeneity of the final $\alpha_1$-antitrypsin fraction agreed on isoelectric focusing with that of $\alpha_1$-antitrypsin in the original plasma (not shown).

## DISCUSSION

Covalent chromatography of thiol proteins, based on 4,4-dithiodipyridine activated glutathione linked to Sepharose, was introduced by Brocklehurst et al. [9]. The rate of the SH—SS interchange reaction is primarily determined by the p*K* of the protein thiols, which must be borne in mind when designing experiments. These p*K* values are not easy to estimate, as they vary with the micro-environment of the thiol on the protein surface [10]. Our early finding of κ-chains, linked by SS-bonds to plasma proteins, suggested that κ-chains

reacted faster with $\alpha_1$-antitrypsin than prealbumin, and prealbumin than albumin, when the plasma concentration of the various proteins was considered [11]. Electrostatic influence from the micro-environment of the reactive thiols seems to effect the SH—SS interchange rate strongly, judging by the sequences of the three proteins which we now know. The reaction rates at neutral pH with $\kappa$-chains, of the thiols of $\alpha_1$-antitrypsin (232) (—His—Cys—Lts—) [12], of prealbumin (10) (—Lys—Cys—Pro—), and of albumin (34) (—Gln—Cys—Pro), decrease in corresponding order. The adjacent Glu to Cys in $\kappa$-chains seems to facilitate the interchange reaction of proteins with Cys and adjacent basic residues in the sequence. The same series of reaction rates has been observed for $\kappa$- and $\lambda$-chains (C-terminals: —Glu—Cys—Ser) and the three proteins, but the rate is much slower for $\lambda$-chains, suggesting that the ultimate seryl induces some steric hindrance.

It may be considered sophisticated to utilize terminal $\kappa$-chain thiols as SH—SS exchangers, but these small proteins are available in large amounts in the urine of many myeloma patients, and are easily recovered in any clinical laboratory. While their large size limits the usefulness of $\kappa$-chains as SH—SS exchangers, their fast reaction with $\alpha_1$-antitrypsin makes them preferred for the isolation of $\alpha_1$-antitrypsin with native microheterogeneity — at least until more efficient, but similarly selective, SH—SS exchangers have been synthesized. A peptide of the four to six C-terminal amino acids of $\kappa$-chains may be a more efficient exchanger than the complete $\kappa$-chain. Substitutions around the two reacting cysteinyls affect the SH—SS exchange rate, and an optimal electrostatic effect may exist for each specific protein; thus, it would be advantageous to have a range of thiol peptides to chose between for use in this type of chromatography.

The protein recoveries presented in Table II refer to proteins with reactive thiols in pooled and stored human plasma; thus, complementary studies have to be performed on freshly collected, individual plasma before any conclusions may be drawn about the physiological state of the proteins. The large amount of $\alpha_2$-macroglobulin, C3 and C4 obtained, may have been caused by thiols released on cleavage of their labile thiol esters after blood sampling. Though plasma IgA is well known for formation of SS-linked complexes in plasma, IgM is not, so the large amount recovered was unexpected. Further studies of the effect of the mild reduction (0.02 *M* 2-mercaptoethanol) on the various native IgM species are necessary before any valid conclusions may be drawn about the physiological implications of our findings.

## ACKNOWLEDGEMENTS

This work has been supported by grants from the Swedish Medical Research Council (Project No. B83-03X-00581-19C) and First of May Flower Annual Campaign for Children's Health.

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