Biochem. J. (1980) **191**, 229–232
Printed in Great Britain

Affinity-chromatographic purification of human α_2-antiplasmin

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(*Received 17 March 1980/Accepted 5 June 1980*)

A new simple and efficient purification method for α_2-antiplasmin is described that is based on the interaction between α_2-antiplasmin and a fragment from elastase-digested plasminogen constituting the three *N*-terminal triple-loop structures in the plasmin A-chain (LBSI). After a single-step adsorption of the α_2-antiplasmin from plasminogen-depleted plasma to LBSI–Sepharose and elution with 6-aminohexanoic acid, an 80–90% pure preparation with a yield of 50–60% is obtained. The major impurity is fibrinogen, which can easily be removed by gel filtration, and, as a result, a homogeneous fully active α_2-antiplasmin preparation is obtained that has the same properties as previously described for α_2-antiplasmin. Evidence is put forward that a form of α_2-antiplasmin with less affinity for the lysine-binding sites in plasminogen may exist, even in unfractionated plasma.

The fibrinolytic process seems to be regulated at several levels, including plasminogen activation and plasmin inactivation (Wiman & Collen, 1978*a*). The latter part of the process is effected by α_2-antiplasmin, which is the most important plasmin inhibitor in plasma (Collen & Wiman, 1978). Since the discovery of α_2-antiplasmin (Moroi & Aoki, 1976; Müllertz & Clemmensen, 1976; Collen, 1976), several purification methods have been described. Most of these include affinity chromatography on insolubilized plasminogen as an important step (Moroi & Aoki, 1976; Wiman & Collen, 1977; Christensen & Clemmensen, 1978). The basis for this method is an interaction between the so-called lysine-binding sites in the plasmin A-chain and a complementary site in α_2-antiplasmin, as demonstrated by kinetic analysis (Wiman *et al.*, 1979). This interaction plays an important role in the rapid reaction between plasmin and α_2-antiplasmin and, physiologically, for the regulation of the fibrinolytic process (Wiman & Collen, 1978*a*,*b*; Wiman *et al.*, 1978, 1979).

In the present work we have used an insolubilized fragment from elastase-digested plasminogen, constituting the three *N*-terminal triple-loop structures (Sottrup-Jensen *et al.*, 1977) in the plasmin. A-chain (LBSI), for affinity-chromatographic purification of α_2-antiplasmin.

Abbreviation used: LBSI, fragment from elastase-digested plasminogen constituting the three *N*-terminal triple-loop structures.

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A preliminary account of this work was presented at the Seventh International Congress on Thrombosis and Haemostasis, held in London on 15–20 July 1979.

Materials and methods

Plasminogen and LBSI

Plasminogen was purified from plasma or Cohn fraction III by affinity chromatography on lysine–Sepharose (Deutsch & Mertz, 1970). The plasminogen preparations were dialysed against 0.1 M-NH_4HCO_3 and digested with elastase–Sepharose in the presence of 50 μM-aprotinin, as described previously (Wiman *et al.*, 1979). The digest was applied to a lysine–Sepharose column equilibrated with 0.1 M-NH_4HCO_3. The bound fragments were eluted by 0.1 M-NH_4HCO_3 containing 10 mM-6-aminohexanoic acid and subsequently chromatographed on Sephadex G-75 equilibrated with 0.1 M-NH_4HCO_3. Two major peaks were obtained; the first peak constitutes the three *N*-terminal triple-loop structures of the plasmin A-chain (LBSI) (Sottrup-Jensen *et al.*, 1977) and the second peak constitutes the fourth triple-loop structure (LBSII) in the plasmin A-chain. The freeze-dried LBSI fraction was dissolved in 0.1 M-sodium phosphate buffer, pH 8.0, and coupled to CNBr-activated Sepharose 4B (Axén *et al.*, 1967) at a ratio of about 5 mg of LBSI per ml of settled gel.

Activation of plasminogen to plasmin was performed with Sepharose-bound urokinase as described previously (Wiman & Wallén, 1973).

0306-3275/80/100229-04$01.50/1

Miscellaneous procedures

The procedures for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969), determination of α_2-antiplasmin activity (Wiman & Collen, 1978), electroimmunoassay (Laurell, 1966) by using a specific antiserum against α_2-antiplasmin (Wiman & Collen, 1977), amino-acid analysis (Wiman & Collen, 1977) and *N*-terminal amino-acid-sequence determination (Edman & Henschen, 1975) and crossed-immunoelectrophoresis (Clarke & Freeman, 1968) were essentially as described in the cited references.

Reagents

Aprotinin (Trasylol) was obtained from Bayer A.G. (Leverkusen, Germany) by courtesy of Dr. E. Philipp. Sepharose 4B was from Pharmacia (Uppsala, Sweden) and Ultrogel ACA 44 was from LKB (Stockholm, Sweden). Elastase was obtained from Sigma (St. Louis, MO, U.S.A.) and the plasmin substrate D-valyl-L-leucyl-L-lysine *p*-nitroanilide was a gift from Kabi Diagnostica (Mölndal, Sweden) by courtesy of Dr. P. Friberger.

Results

Purification of α_2-antiplasmin

Plasma was treated with lysine–Sepharose, in a batch procedure, as described previously (Wiman & Collen, 1977). About 1 litre of plasminogen-depleted plasma was stirred with 40–50g of LBSI–Sepharose at +5°C for 30–60min. After filtration on a Büchner funnel, the LBSI–Sepharose was washed with 0.04M-sodium phosphate buffer, pH7.0, and subsequently poured into a column. Elution was performed first with 0.04M-sodium phosphate buffer, pH7.0, containing 0.5M-NaCl and then repeated a second time with this buffer containing 10mM-6-aminohexanoic acid (Fig. 1). The material that was eluted with 6-aminohexanoic acid was dialysed against distilled water at +5°C. During this step a large portion of the protein, mainly consisting of fibrinogen, was precipitated and subsequently removed by centrifugation. The clear supernatant could be either concentrated by ultrafiltration and stored frozen or freeze-dried. This material is typically 80–90% pure α_2-antiplasmin, as judged from activity measurements, electroimmunoassay and protein determination. The yield of α_2-antiplasmin at this stage is typically 50–60% of that in the starting material. The 10–20% impurities remaining in the α_2-antiplasmin preparation are still mainly fibrinogen. If necessary this can easily be removed by gel filtration on Ultrogel ACA 44 in 0.04M-sodium phosphate buffer, pH7.0 (Fig. 2). The second peak, constituting the α_2-antiplasmin, was dialysed against distilled water and freeze-dried.

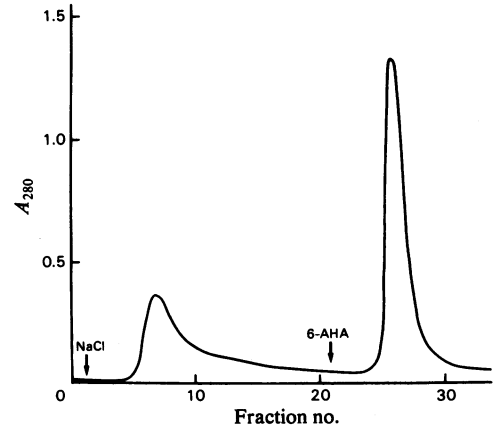

Fig. 1. *Affinity chromatography on LBSI–Sepharose* The α_2-antiplasmin was adsorbed on to LBSI–Sepharose (40ml of settled gel) in a batch procedure from about 1 litre of plasminogen-depleted plasma. The LBSI–Sepharose was washed on a Büchner funnel with 0.04M-sodium phosphate buffer, pH7.0, packed in a 5cm² × 8cm column and eluted first with the same phosphate buffer containing 0.5M-NaCl and subsequently with the phosphate buffer containing 10mM-6-aminohexanoic acid (6-AHA).

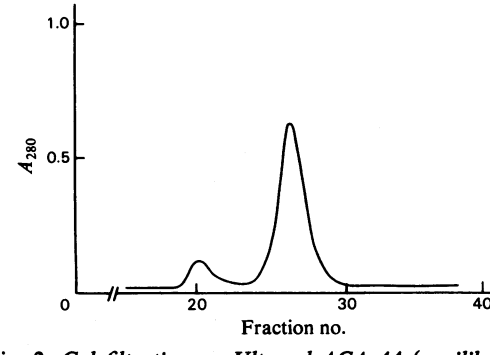

Fig. 2. *Gel filtration on Ultrogel ACA 44 (equilibrated with 0.04M-sodium phosphate buffer, pH7.0) of the 6-aminohexanoic acid eluate from the LBSI–Sepharose column*

Several batches of LBSI–Sepharose have so far been used over ten times and only a small gradual decrease in binding capacity has been noticed. However, some LBSI–Sepharose preparations do not seem to be more efficient in binding α_2-antiplasmin from plasminogen-depleted plasma than plasminogen–Sepharose. The reason for this has, at present, not been fully and systematically investigated, and is, therefore, not well understood. Nevertheless, these preparations of LBSI–Sepharose can still be used with a slightly modified version of

the method that gives very similar results. Thus the plasminogen-depleted plasma is first precipitated by the addition of an equal volume of 12% (w/v) poly(ethylene glycol) 6000 at +5°C. After stirring at +5°C for 30min and then centrifuged, the supernatant is treated with LBSI–Sepharose as described above.

Characterization of the purified α_2-antiplasmin

After the gel-filtration step the α_2-antiplasmin is homogeneous and fully active as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3), activity measurements and electroimmunoassay. Thus a solution of the purified α_2-antiplasmin preparation in 0.1M-sodium phosphate buffer, pH7.3, with a concentration of 91μg/ml (A_{280}, using $A^{1\%}_{1cm} = 6.70$; Wiman & Collen, 1977) contained 93±4 (S.D.) μg of α_2-antiplasmin activity and 90±5 (S.D.) μg of α_2-antiplasmin antigen per ml. The only detectable *N*-terminal amino acid is asparagine and the sequence is in agreement with previously published data (Wiman & Collen, 1979). The amino-acid composition is almost identical with that reported previously (Wiman & Collen, 1977), except for lysine, the content of which is found to be about 15% lower.

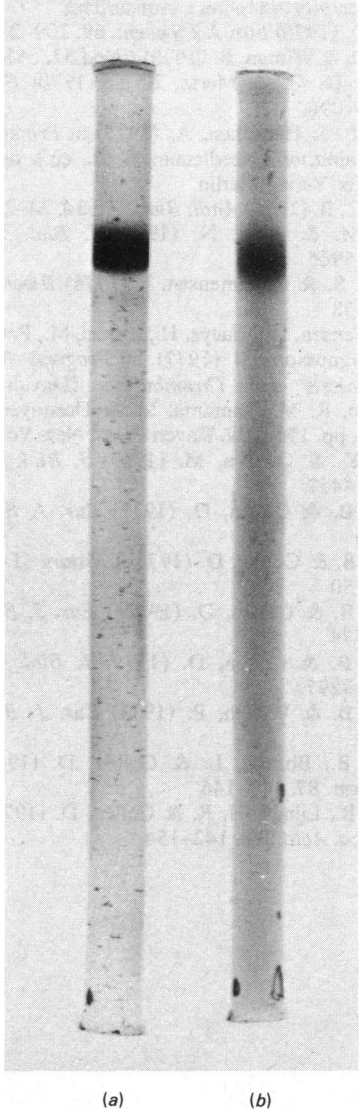

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified α_2-antiplasmin*
(*a*) Reduced sample (1% w/w dithioerythritol, 10min, 80°C); (*b*) non-reduced sample.

α_2-Antiplasmin with less affinity for LBSI–Sepharose

If plasminogen-depleted plasma is repeatedly treated with LBSI–Sepharose or if the adsorption is carried out by a column procedure, a portion of the α_2-antiplasmin antigen ranging between 25 and 40% is still not adsorbed, as demonstrated by electroimmunoassay. This form of α_2-antiplasmin still seems to be functional, since the major part of it forms a complex with plasmin. This is demonstrated in Fig. 4, which shows crossed immunoelectro-

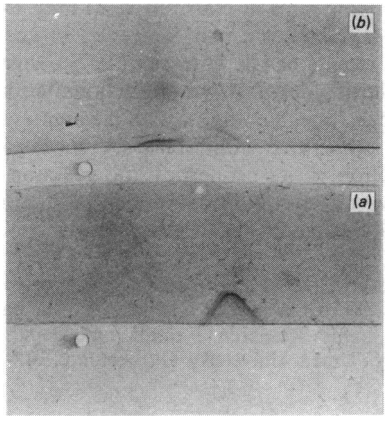

Fig. 4. *Crossed immunoelectrophoresis of* (*a*) *plasma depleted in plasminogen and adsorbed with LBSI–Sepharose (α_2-antiplasmin concentration about 0.3 μM) and* (*b*) *this plasma after addition of plasmin to a final concentration of 1 μM and incubation for 10min at room temperature*
A portion (5μl) of the samples was applied to the agarose gels and the first dimension run for 1.5h at a potential gradient at 40V/cm. The second dimension was run for 4h at a potential gradient of 40V/cm into a gel containing 5μl of antiserum against α_2-antiplasmin/ml.

phoresis of plasminogen-depleted plasma treated with LBSI–Sepharose after addition of plasmin to a final concentration of 1 μM.

Discussion

The new purification method for α_2-antiplasmin described in the present study has major advantages over previous methods in its simplicity and efficiency. Thus LBSI–Sepharose seems to have a higher capacity for adsorbing α_2-antiplasmin from plasminogen-depleted plasma than a molar equivalent of plasminogen–Sepharose. This is in agreement with the dissociation constants obtained for the interaction between α_2-antiplasmin and different plasminogen derived fragments (Wiman *et al.*, 1979). Another advantage is that LBSI does not contain a potential active site and cannot therefore bind α_2-antiplasmin irreversibly. Furthermore, no differences in physicochemical and stability properties of the α_2-antiplasmin prepared with the present method have been found when compared with our previous method (Wiman & Collen, 1977).

Christensen & Clemmensen (1978) and Clemmensen (1979) reported that a form of α_2-antiplasmin with no affinity for plasminogen–Sepharose existed in their preparations of α_2-antiplasmin partially purified by conventional techniques. The results obtained in the present study support this finding and further suggest that this form of α_2-antiplasmin with less affinity for the lysine-binding sites in plasmin(ogen) may exist even in unfractionated plasma. However, in accordance with the results of Clemmensen (1979), this form of α_2-antiplasmin seems to be a functional antiplasmin, since it can form a complex with plasmin even under highly competitive conditions. A more thorough investigation of its properties and reaction kinetics has to wait until a purification method for this form of α_2-antiplasmin has been achieved.

This study has been financially supported by the Swedish Medical Research Council (Project no. 05193) and the Lions Research Foundation, Department of Oncology, Umeå University (Project no. 148/79). The expert technical assistance of Ms. Åsa Almquist is gratefully acknowledged.

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