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# ISOLATION AND PARTIAL CHARACTERISATION OF A THIOL PROTEINASE INHIBITOR FROM HUMAN PLASMA

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## SUMMARY

A thiol proteinase inhibitor has been isolated from human plasma by ion exchange, salt-mediated hydrophobic and ion chelation chromatography. It was found to be electrophoretically heterogeneous (in both its native state and after isolation) giving a bimodal arc with an $\alpha_1$ and $\alpha_2$ peak in bidimensional immunoelectrophoresis. It was a good inhibitor of papain but only partially inhibited human kidney cathepsin B1 and did not inhibit the bacterial thiol proteinase, clostripain. Its mean protein concentration in adults sera was 42.5 $\pm$ 6.8 mg per dl.

## INTRODUCTION

There are seven well-characterised plasma proteinase inhibitors in man: antithrombin III, C1 inactivator, $\alpha_1$ chymotrypsin inhibitor, inter-$\alpha$-trypsin inhibitor, $\alpha_2$ macroglobulin, $\alpha_1$ trypsin inhibitor (1) and $\alpha_2$ plasmin inhibitor (2). Preliminary descriptions of two further inhibitors have been reported, $\beta_1$ collagenase inhibitor (3) and plasminogen activator inhibitor (4). Recently Sasaki et al. (5) have reported the presence in serum of an inhibitor ($\alpha_2$ thiol proteinase inhibitor) that appears to be specific for the thiol proteinases and which is immunologically distinct from $\alpha_2$ macroglobulin or any other inhibitor described above. However, they did not isolate the inhibitor and most of the characterisation was carried out on partially purified material. I wish to report the isolation and partial characterisation of a serum thiol proteinase inhibitor which is probably identical to that described by Sasaki and his group.

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## MATERIALS AND METHODS

### Materials.

Out-of-date citrated plasma, individual serum specimens from 120 adult blood donors aged between 19-40 years and 20 cord serum specimens from healthy full-term infants were all obtained from the Department of Haematology, University Hospital of Wales.

Sepharose 4B-L-phenylalanine was prepared as described by Doellgast and Fishman (6) and Sepharose 6B-bis carboxymethylamine by the method of Porath et al. (7). Cellulose DE52 was obtained from Whatman, Maidstone, Kent, Ultrogel AcA44 from LKB, Bromma, Sweden and all other chromatography media used from Pharmacia (UK) London.

Bovine chymotrypsin Type II, bovine trypsin Type III, clostripain and papain Type III were obtained from Sigma Chemicals, Poole, Dorset, and human kidney cathepsin B1 was a gift from Dr. M. Davies, KRUF Institute, Cardiff Royal Infirmary. Azocasein was prepared as described by Starkey (8).

Polyvalent antiserum to human serum and monospecific antisera to $\alpha_1$ chymotrypsin inhibitor, $\alpha_2$ macroblobulin, $\alpha_2$ plasmin inhibitor, thiol proteinase inhibitor and $\alpha_1$ trypsin inhibitor were raised in rabbits (9) from proteins isolated in this laboratory. Other monospecific antisera were obtained from Hoechst Pharmaceuticals, Hounslow, Middlesex.

### Analytical Methods.

Amino acids and hexosamines were determined on a Technicon AA1 Analyser after hydrolysis of the sample at 110°C for 20 h in either 3N mercaptoethanesulphonic acid (Pierce and Warriner, Chester, Cheshire) (10), or 6M HCl. Hexoses and fucose were determined by a procedure of Lee (11) and sialic acid determined by the method of Aminoff (12).

SDS polyacrylamide gel electrophoresis with 7.5% gels was carried in an LKB Multiphor 2117 Tank (13) and estimation of molecular weight was made with the aid of BDH molecular weight markers. Isoelectric focusing in polyacrylamide gels containing LKB Ampholines 4-6 was carried out in the LKB Multiphor 2117 (14).

Immunodiffusion, electroimmunoassay, 'fused rocket' electroimmunoassay and bidimensional immunoelectrophoresis were carried out as previously described (9, 15). Conditions for agarose electrophoresis were identical to that used to run the first dimension of the bidimensional immunoelectrophoresis.

### Assay of Proteinase Inhibition.

Papain inhibition was assayed by a procedure similar to that described by Sasaki et al. (5), except that a 1.2% azocasein solution replaced casein as the substrate. Cathepsin B1 activity and inhibition were measured at pH 6.0 with 6% azocasein as substrate. Inhibitions of clostripain, chymotrypsin and trypsin were measured by the same procedure except that the appropriate buffers for these enzymes were substituted for the buffer used in the papain assay and a 3% azocasein solution was used.

### Isolation of Thiol Proteinase Inhibitor.

All chromatography procedures were carried out at ambient temperature and the inhibitor located in fractions by 'fused rocket' immunoassay with monospecific antiserum.

The protein precipitated between 1.2 to 2.4 M $(NH_4)_2SO_4$ from a litre of plasma was taken up in about 200 ml of water and dialysed against 25 mM Tris Cl buffer containing 4.0 mM EDTA and 25 mM NaCl pH 7.8. After removal of the precipitate, the dialysed solution was loaded onto a column of Cellulose DE52 (3.2 x 45cm) previously equilibrated with the Tris-Cl-EDTA buffer containing 25 mM NaCl. After washing, the column was developed with a 2 litre gradient to a final buffer of 25 mM Tris Cl containing 4.0 mM EDTA and 500 mM NaCl

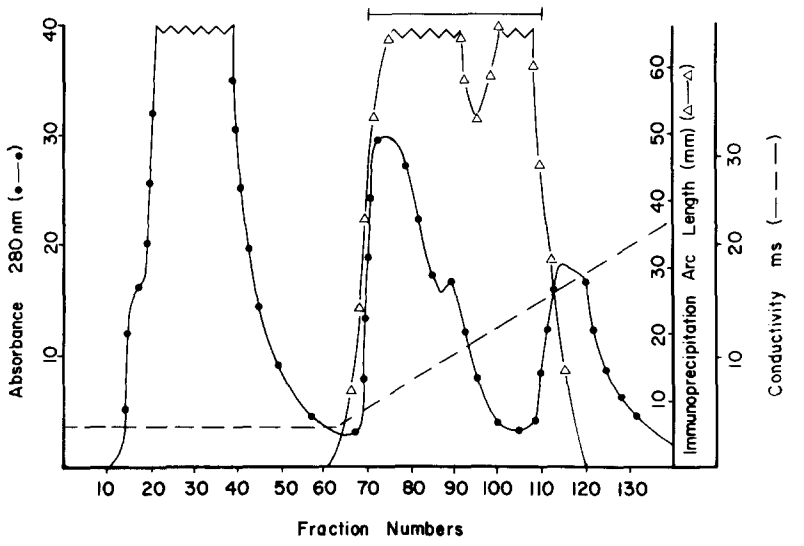


Fig. 1 DEAE cellulose chromatography of a plasma protein fraction that precipitated between 1.2 M to 2.4 M $(NH_4)_2SO_4$. Flow rate was 70ml per h and the fraction volume was 12ml. 1-----1 indicates fractions that were collected.

pH 7.8. The inhibitor, partially resolved into two components, was eluted between a $Na^+$ concentration of 35 mM to 150 mM and all fractions in this range were collected and bulked. (Fig. 1).

The pH of bulked fractions was adjusted to 6.5 with 1 M $H_3PO_4$ and $(NH_4)_2SO_4$ was added to 1.2 M. After standing overnight, the precipitate was removed and the supernatant was loaded onto a column of Sepharose-L-phenylalanine (2.2 x 60cm) in 100 mM potassium phosphate buffer pH 6.5, with 1.2 M $(NH_4)_2SO_4$. The column was washed with starting buffer until the blue ceruloplasmin fraction had been eluted and then developed with a 600 ml linear gradient to a final buffer of 50 mM potassium phosphate pH 6.8. The inhibitor was eluted as a single peak between 1.0 M to 0.6 M $(NH_4)_2SO_4$ (Fig. 2). The inhibitor fractions were bulked, made 2.5 M to $(NH_4)_2SO_4$ and after standing at $4^oC$ overnight, the precipitate was collected. It was taken up in 50 ml of distilled water and dialysed against 25 mM Tris Cl pH 8.2 containing 100 mM NaCl.

The solution was loaded onto a column of Sepharose-bis carboxymethylamine (2.2 x 40 cm); the upper 75% contained chelated copper (about 20 µM per ml). Prior to chromatography the column had been washed with 25 mM Tris Cl pH 8.1 containing 100 mM NaCl. It was found that the inhibitor was either unabsorbed or partially retarded; the retarded fraction represented 20% of the total inhibitor added to the column and contained serum proteins that were difficult to remove by other procedures. Thus only the unabsorbed inhibitor was collected (Fig. 3).

The pH of the collected fractions was adjusted to pH 6.5 with 1 M $H_3PO_4$ and K Cl added to 1.2 M and the sample was loaded onto a Phenyl-Sepharose Cl-4B column (1.5 x 15 cm) which had been equilibrated with 50 mM Tris $PO_4$ buffer pH 6.5 containing 1.2 M KCl. After washing until no further protein was eluted, the column was developed with a 400 ml gradient to a final buffer of 25 mM Tris Cl pH 8.2 containing 25% ethylene glycol. The inhibitor was eluted

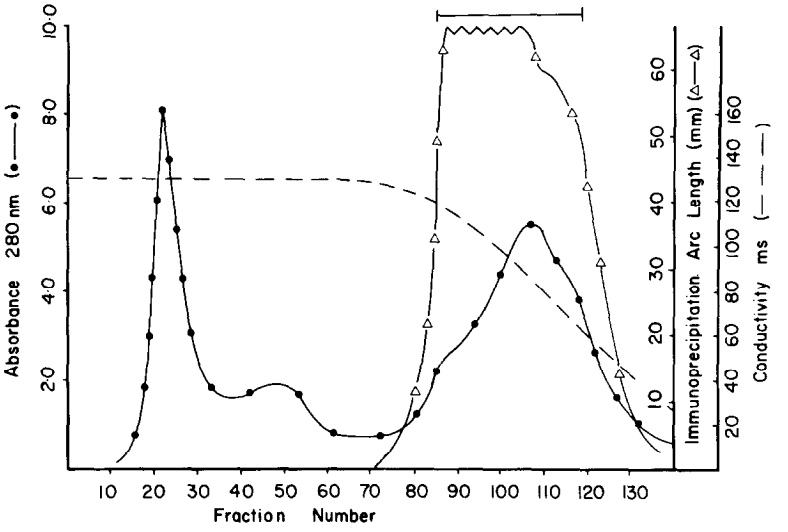


Fig. 2 Salt-mediated hydrophobic chromatography on Sepharose 4B-L phenylalanine of the thiol proteinase inhibitor fraction collected after ion exchange chromatography. Flow rate was 24ml per h and the fraction volume was 8.0 ml.
1-----1 indicates fractions collected.

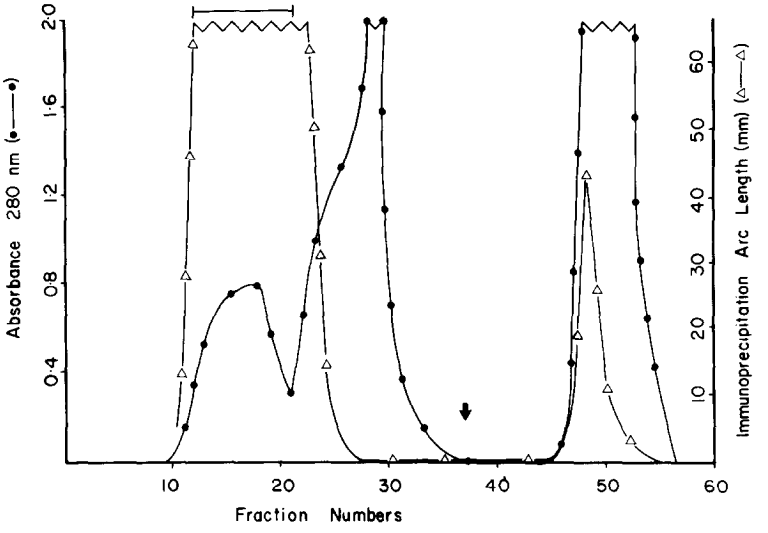


Fig. 3 Ion chelation chromatography on copper chelate of Sepharose 6B-bis-carboxymethylamine of inhibitor fraction isolated after chromatography on Sepharose 4B-L-phenylalanine. Flow rate was 24.2 ml per h and the fraction volume was 8.4 ml. Arrow indicates change from wash buffer (25 mM Tris Cl pH 8.1 with 100 mM NaCl) to 50 mM EDTA pH 7.1.
1-----1 indicates fractions collected.

TABLE 1

AMINO ACID AND CARBOHYDRATE COMPOSITION OF PLASMA THIOL PROTEINASE INHIBITOR

| | μg per mg Protein | μM per mM of Protein (2) |
|---|---|---|
| Aspartic Acid | 104.8 | 47.2 |
| Threonine | 68.4 | 34.4 |
| Serine | 53.6 | 30.6 |
| Glutamic Acid | 120.5 | 49.1 |
| Proline | 45.3 | 23.6 |
| Glycine | 24.8 | 19.8 |
| Alanine | 26.5 | 17.8 |
| Cystine (1) | 7.6 | 1.9 |
| Valine | 34.9 | 17.9 |
| Methionine | 4.8 | 1.9 |
| Isoleucine | 47.8 | 21.9 |
| Leucine | 55.8 | 25.5 |
| Tyrosine | 59.8 | 19.8 |
| Phenylalanine | 45.6 | 16.6 |
| Lysine | 66.9 | 27.4 |
| Histidine | 9.9 | 3.8 |
| Tryptophan | 17.3 | 5.1 |
| Arginine | 37.0 | 12.7 |
| Glucosamine | 13.6 | 4.1 |
| Galactosamine | 2.2 | 0.7 |
| Galactose | 51.7 | 15.5 |
| Mannose | 31.3 | 9.4 |
| Fucose | < 1.0 | - |
| Sialic Acid | 30.7 | 6.0 |

(1) Estimated after 20 h hydrolysis with 6M H Cl
(2) Assuming a molecular weight of 60,000

free of contaminants over most of the gradient. A stepwise elution instead of a gradient always resulted in either a poor yield or the presence of contaminants depending on the conditions used. The yield of the inhibitor from plasma, was between 20-25%.

## RESULTS AND DISCUSSION

The isolated thiol proteinase inhibitor was a glycoprotein containing about 10% carbohydrate and was also characterised by its high acicic amino acid content (Table 1). It had an $E_{280}^{1\%}$ of 5.9 and a pI of between 4.7 - 4.9 with 5 bands resolved. The protein had pronounced electrophoretic and molecular size heterogeneity. In agarose electrophoresis it separated into an $\alpha_1$ and an $\alpha_2$ band; the latter being the major component, (Fig. 4B) and this separation was reflected in the bimodal precipitation arc that developed with bidimensional electrophoresis (Fig. 4A). Two inhibitor peaks were resolved with estimated molecular weights of 90,000 and 170,000 when either plasma or

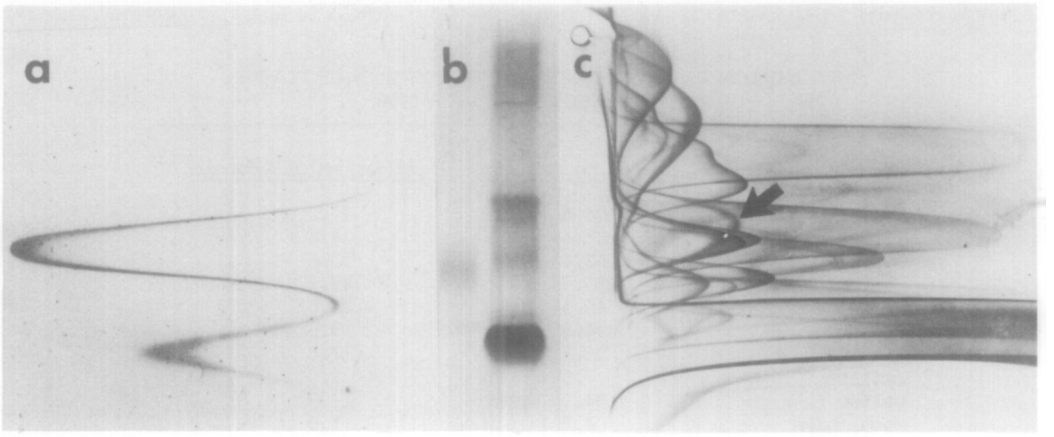


Fig. 4 Electrophoretograms of thiol proteinase inhibitor and whole plasma. A) Bidimensional electrophoresis of 4 µl of inhibitor solution (4 mg per ml) against 0.5 mls of antihuman serum. B) Agarose electrophoresis of inhibitor (4mg per ml) and plasma. C) Bi-dimensional electrophoresis of 4 µl of plasma (1 in 4 dilution) against 0.5 ml antihuman serum. Arrow indicates inhibitor arc in polyvalent pattern. Agarose electrophoretogram was fixed with 1.2% picric acid in 16% acetic acid. After drying, plates were stained with 0.2% coomassie blue in methanol: water; acetic acid (4: 6: 1 by volume).

purified inhibitor was separated by gel chromatography on Ultra-gel AcA44. Both components inhibited papain and appeared to be immunologically identical. The molecular weight was also estimated with SDS polyacrylamide electrophoresis. A single protein band was seen with an estimated molecular weight of 60,000 or 64,000 if pretreatment with 1% mercaptoethanol was omitted. No small peptide was seen in the SDS electrophoretogram of the reduced inhibitor and it seems likely that the difference in molecular weight was due to a conformation change caused by cleavage of an internal cystine bridge. The reason for the difference in molecular weight when measured by SDS polyacrylamide electrophoresis and gel chromatography is not known. In view of this pronounced heterogeneity which was not reported by Sasaki et al. (5), I suggest that the inhibitor should be termed $\alpha$ thiol proteinase inhibitor rather than $\alpha_2$ thiol proteinase inhibitor (5).

Papain was completely inhibited by the inhibitor but the activity of the cathepsin B1 preparation was only reduced by 50% by a two-molar excess of the

inhibitor (assuming a molecular weight of 60,000) and only 70% inhibition was achieved with a ten-molar excess. The enzyme was immunochemically pure and its activity was not inhibited by pepstatin (personal communication, M.Davies). Consequently this would suggest that it was a poor inhibitor of cathepsin B1 and raises questions concerning the inhibitor's physiological role. The inhibitor's major function may be to inhibit the neutral thiol proteinases that play some role in the inflammatory response (16). Alternatively, its inhibition specificity may be analagous to $\beta_1$ collagenase inhibitor which inhibits collagenase to varied degrees depending on the tissue source of the enzyme (3). There was no inhibition by the inhibitor of the bacterial thiol proteinase clostripain, or of bovine chymotrypsin and bovine trypsin.

The serum concentration in adults was 42.5 $\pm$ 6.8 mg/dl with no significant difference between the sexes and the cord serum concentration was 24.1 $\pm$ 3.5mg/dl. In view of its relatively high serum concentration (see also Fig. 4C), it is surprising that it has not been isolated before. The purified inhibitor did not crossreact with monospecific antiserum to antithrombin III, $\alpha_1$B glycoprotein, $\alpha_1$ chymotrypsin inhibitor, C1 inactivator, ceruloplasmin Gc globulin, haptoglobin, $\alpha_2$HS glycoprotein, inter-$\alpha$-trypsin inhibitor, $\alpha_2$ macroglobulin, $\alpha_2$ plasmin inhibitor, $\alpha_1$ trypsin inhibitor or $\alpha_2$ Znglycoprotein. Its electrophoretic heterogeneity resembled 3.8 S histidine-rich glycoprotein (17) but its properties were different. It had a different composition, pI and did not bind heparin.

## REFERENCES

1. Heimburger, N. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B., and Shaw, E. ed.) pp. 367-386, pub. Cold Spring Harbour Laboratories, New York.
2. Collen, D., and Wiman, B. (1978) Blood 51, 563-569.
3. Woolley, D.E., Roberts, D.R., and Evanson, J.M. (1976) Nature 261, 325-327.
4. Hedner, U. (1973) Thromb. Diath. Haemorrh. 30, 414-424.
5. Sasaki, M., Minakata, K., Yamamato, H., Niwa, M., Kato, T., and Ito, N. (1977) Biochem. Biophys. Res. Comm. 76, 917-924.
6. Doellgast, G.T., and Fishman, W.H. (1974) Biochem. J. 141, 103-112.
7. Porath, J., Carlsson, J., Olsson, I., and Belfrage, G. (1975) Nature 258, 598-599.

8. Starkey, P.M. (1977) in Proteinases in Mammalian Cells and Tissues (Barrett, A.J. ed.) pp. 82-83, Elsevier-North Holland Biomedical Press, Amsterdam.
9. Ryley, H.C., and Brogan, T.D. (1973) J.Clin. Path. 26, 852-856.
10. Penke, B., Ferenczi, R., and Kovacs, K. (1974) Anal. Biochem. 60, 45-50.
11. Lee, Y.C. (1972) in Methods of Enzymology (Ginsburg, V. ed.) vol. 28, pp. 63-70, Academic Press, New York and London.
12. Aminoff, D. (1961) Biochem. J. 81, 384-392.
13. Fehrstrom, H., and Moberg, U. (1977) L.K.B. Application Note 306.
14. Winters, A., Ek, K., and Andersson, U.B. (1977) L.K.B. Application Note 250.
15. Ryley, H.C. (1977) J. Chromat. 143, 411-414.
16. Hayashi, H. (1975) Int. Rev. Cytol. 40, 101-151.
17. Heimburger, N., Haupt, N., Kranz, T., and Baudner, S. (1972) Hoppe Seyler's Z Physiol. Chem. 353, 1137-1140.