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Cystatin, a protein inhibitor of cysteine proteinases

Improved purification from egg white, characterization, and detection in chicken serum

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The protein from chicken egg white that inhibits cysteine proteinases, and has been named 'cystatin', was purified by ovomucin precipitation, affinity chromatography on carboxymethylpapain–Sepharose and chromatofocusing. The final purification step separated two major forms of the protein (pI 6.5 and 5.6), with a total recovery of about 20% from egg white. By use of affinity chromatography and immunodiffusion it was shown that the inhibitor is also present at low concentrations in the serum of male and female chickens. Tryptic peptide maps of the separated forms 1 and 2 of egg-white cystatin were closely similar, and each form had the *N*-terminal sequence Ser-Glx-Asx. The two forms showed complete immunological identity, and neither contained carbohydrate. K_i values for the inhibition of cysteine proteinases were as follows: papain ($<1\times10^{-11}$ M), cathepsin B (8×10^{-10} M), cathepsin H (about 2×10^{-8} M) and cathepsin L (about 3×10^{-12} M). Some other cysteine proteinases, and several non-cysteine proteinases, were found not to be significantly inhibited by cystatin. The inhibition of the exopeptidase dipeptidyl peptidase I by cystatin was confirmed and the K_i found to be 2×10^{-10} M. Inhibitor complexes with active cysteine proteinases and the inactive derivatives formed by treatment with iodoacetate, E-64 [L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane] and benzyloxycarbonylphenylalanylalanyldiazomethane were demonstrated by isoelectric focusing and cation-exchange chromatography. The complexes dissociated in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (with or without reduction) with no sign of fragmentation of the inhibitor. Cystatin was found not to contain a free thiol group, and there was no indication that disulphide exchange plays any part in the mechanism of inhibition.

In contrast with the vast amount of information that has been obtained on the protein inhibitors of serine proteinases (Laskowski & Kato, 1980), very little is known of inhibitors of the cysteine proteinases. Cysteine proteinases are abundant in the body, however, and are responsible for much of the intracellular proteolysis (Kirschke *et al.*, 1980), so the means by which their activities are controlled are of great interest.

A low-M_r protein that inhibits ficin, papain, cathepsin B and dipeptidyl peptidase I (cathepsin C) has previously been isolated in small quantities from chicken egg white (Fossum & Whitaker, 1968; Sen & Whitaker, 1973; Keilová & Tomášek, 1974). This protein, for which we have proposed the name 'cystatin' (Barrett, 1981), is of particular interest for several reasons. It forms complexes with cysteine proteinases even after their catalytic sites have been inactivated by bulky active-site-directed reagents; this characteristic distinguishes cystatin from any

Abbreviations used: the abbreviations used for amino acid derivatives and *N*-terminal groups are based on the standard conventions [*Biochem. J.* (1972) **126**, 773–780]. The *C*-terminal groups are: CH_2Cl, chloromethane; NMec, 7-(4-methyl)coumarylamide; NNap, 2-naphthylamide; $NPhNO_2$, nitroanilide; $OPhNO_2$, *p*-nitrophenyl ester; CHN_2, diazomethane. Other abbreviations are: Tos, tosyl; Cm-papain, *S*-carboxymethylpapain; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane; f.p.l.c., fast protein liquid chromatography (Pharmacia system); h.p.l.c., high-pressure liquid chromatography (Du Pont system); SDS, sodium dodecyl sulphate.

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other proteinase inhibitor previously studied. However, the egg-white inhibitor has similarities to a group of proteins found in mammalian skin (Järvinen, 1976, 1978), squamous-cell carcinomata (Rinne, 1980), rat liver (Kominami *et al.*, 1981), and other tissues. This raises the possibility that there is a homologous family of 'cystatins' that might well be of widespread biological importance. As a first step in the investigation of this possibility we have developed a new method for the isolation of egg-white cystatin, studied its properties, and shown that it exists in chicken serum as well as egg white.

Experimental

Materials

Brij 35 and iodoacetic acid were obtained from BDH Chemicals, *p*-chloromercuribenzoic acid from Aldrich Chemical Co., and papain (twice-crystallized), avidin (affinity-purified), ficin, clostripain, L-cysteine (free base) and dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride from Sigma (London) Chemical Co. Carbowax 20m, a brand of poly(ethylene glycol), was from Raymond A. Lamb, North Acton, London NW10 6JL, U.K. Z-Phe-Arg-NMec (hydrochloride), Arg-NMec, Z-Arg-Arg-NNap and Z-Gly-Phe-NNap were purchased from Bachem Feinchemikalien AG, CH-4416 Bubendorf, Switzerland. Z-Arg-Arg-NMec was from Universal Biologicals, Cambridge CB5 8BA, U.K. Azocasein was prepared as described by Barrett & Kirschke (1981). E-64 was the gift of Dr. K. Hanada, Taisho Pharmaceutical Co., Saitama, Japan. Z-Phe-Ala-CHN_2 was the gift of Dr. E. Shaw, Brookhaven National Laboratory, Upton, NY, U.S.A. Ampholines were products of LKB Instruments or Pharmacia. Sepharose 4B, Polybuffer exchanger 94 and Polybuffer 74 were from Pharmacia. Phenyl isothiocyanate was obtained from Pierce and Warriner (U.K.) Ltd., Chester, Cheshire CH1 4EF, U.K. The polyamide t.l.c. sheets were from BDH Chemicals.

Cathepsins B and H from human liver were prepared as described by Schwartz & Barrett (1980); the cathepsin B was then further purified by chromatofocusing. Rat liver cathepsin L was the gift of Dr. H. Kirschke (Physiologisch-Chemisches Institute, Halle, German Democratic Republic), dipeptidyl peptidase I from bovine spleen the gift of Dr. J. K. McDonald (Department of Biochemistry, Medical University of South Carolina, Charleston, SC, U.S.A.), the streptococcal proteinase the gift of Dr. S. Elliott (Department of Pathology, University of Cambridge, Cambridge, U.K.), and porcine pancreatic elastase the gift of Dr. J. Travis (Department of Biochemistry, University of Georgia, Athens, GA, U.S.A.). For some experiments, papain was prepared by the method of Baines & Brocklehurst (1979), and the fully active enzyme was isolated essentially as described by Stuchbury *et al.* (1975). Papaya peptidase was obtained from papaya (*Carica papaya*) latex by a method based on that of Robinson (1975). Bromelain (stem) was the crystalline suspension from the Boehringer Corporation (London) Ltd. Trypsin (Sigma type XII) was treated with Tos-Phe-CH_2Cl to inactivate chymotrypsin.

Chicken eggs were purchased from local food stores, and were of unknown variety. Chicken serum was a gift of Dr. I. R. Dickson of this laboratory.

Methods

Preparation of Cm-papain–Sepharose. Cm-papain–Sepharose for affinity chromatography was prepared as follows. Papain (500 mg) was activated with 2 mM-cysteine and 1 mM-disodium EDTA in 75 ml of 0.1 M-sodium phosphate buffer, pH 6.0, for 10 min at 20°C, and allowed to react with 10 mM (final concn.)-iodoacetic acid. The solution was dialysed against 0.1 M-$NaHCO_3$, and stirred overnight with 500 g (wet weight) of Sepharose 4B that had been activated with CNBr (50 mg/g) as described by Axén & Ernback (1971) in 500 ml of 0.1 M-$NaHCO_3$/NaOH, pH 9.0. The gel was then treated with 0.1 M-glycine for 1 h and washed at pH 3 and pH 11. The amount of protein coupled to the gel was determined by the method of Lowry *et al.* (1951) to be 0.6 mg/g of hydrated gel.

Electrophoresis. Polyacrylamide-gel electrophoresis was done in the discontinuous 2-amino-2-methylpropane-1,3-diol (Ammediol)/glycine/HCl system described by Bury (1981), in slab gels containing 12.5% (w/v) total acrylamide. Samples containing cysteine proteinases were treated with iodoacetate before they were electrophoresed in the presence of SDS under reducing conditions (Barrett *et al.*, 1979).

Analytical isoelectric focusing. Isoelectric focusing was done in slab gels prepared on GelBond film (Bioproducts, Rockland, ME 04841, U.S.A.) from 1% (w/v) LKB agarose EF, 10% (w/v) sorbitol and 1% (w/v) Ampholines. The Ampholines comprised a mixture (2:1, v/v) of pH 8–10.5 and pH 3–10 ranges. The Pharmacia apparatus type FBE 3000 was used essentially as described by the manufacturers. The wicks were soaked in 0.5 M-NaOH and 0.5 M-acetic acid respectively, and samples were applied to the gel on slips of 3MM filter paper midway between the anode and cathode. The run was for 3 h, and gels were fixed in trichloroacetic acid solution (10%, w/v, for 1 h, and 1% overnight) before being washed for 30 min in water, dried in warm air, and stained with Coomassie Brilliant Blue R, as described for immunodiffusion plates by Barrett (1974).

Enzyme assays. Dipeptidyl peptidase I was assayed with Gly-Phe-NNap (McDonald *et al.*, 1969). Clostripain and streptococcal proteinase were assayed with Z-Arg-NMec and Z-Lys-OPhNO$_2$ respectively, as described by Barrett *et al.* (1982). Cathepsin B was assayed with Z-Arg-Arg-NNap or Z-Phe-Arg-NMec, cathepsin L with Z-Phe-Arg-NMec and cathepsin H with Arg-NMec, all as described by Barrett & Kirschke (1981).

Active-site titration of papain solutions was by stoichiometric inactivation with E-64 (Barrett *et al.*, 1982).

Ficin and stem bromelain were assayed with azocasein (1.5%, w/v) in 0.10M-sodium phosphate buffer, pH 6.0, containing 2mM-cysteine (final concn.), at 50°C for 30min. The reaction, in 1.0ml total volume, was stopped by addition of 5ml of 3% (w/v) trichloroacetic acid. Activity was determined as ΔA_{366} in the filtrate.

Trypsin, chymotrypsin, pancreatic elastase, cathepsin G and thermolysin were also assayed with azocasein, except that the buffer was 0.10M-Tris/HCl, pH 8.1, containing 20mM-CaCl$_2$ (final concn.).

Quantification of cystatin by inhibition of papain. Two procedures were used, one with Bz-Arg-NPhNO$_2$ as substrate, which requires rather large amounts of cystatin and has been described (Barrett, 1981), and a micro-scale method with Z-Phe-Arg-NMec as substrate. The details of the latter method were as follows.

The stock assay buffer was 200mM-K$_2$HPO$_4$/200mM-KH$_2$PO$_4$/4mM-EDTA/4mM-dithiothreitol, pH 6.8. Samples (10, 20, 30 ... 90μl) of cystatin solution in 0.1% Brij 35 were mixed with 50μl of stock assay buffer, and made up to 140μl with 0.1% Brij 35. Brij 35, a non-ionic detergent, was included in many of the buffer solutions because it decreases non-specific adsorption of many proteins to surfaces. Papain (20μl of an 8nM solution standardized by titration as mentioned above) was added, and the mixtures incubated for 10min at 40°C. Stock assay buffer (4.0ml) and 0.1% Brij 35 (8.8ml) were mixed, and 0.59ml added to each tube. After a few minutes for re-equilibration to 40°C, 0.25ml of 20μM-Z-Phe-Arg-NMec substrate solution was added, and the assays were completed in the usual way (Barrett & Kirschke, 1981). Results in the linear part of the dose–response curve (up to about 50% inhibition) were extrapolated to give the molar concentration of cystatin (on the assumption of a 1:1 binding ratio).

Ultrafiltration. Solutions of cystatin were transferred from one buffer to another and concentrated by ultrafiltration under pressure of nitrogen, in an Amicon cell fitted with a PM-10 membrane.

Purification of cystatin

The inhibitor was purified in three main steps: precipitation of ovomucin, affinity chromatography and chromatofocusing. Preparations were usually made from 72 chicken eggs.

Removal of ovomucin. The egg whites were separated from the yolks and blended with an equal volume of 0.25% NaCl by use of a mixer/homogenizer (Silverson Machines Ltd., London S.E.1, U.K.). The pH was adjusted to 6.5 with 5M-sodium formate buffer, pH 3.0, and the precipitate was removed by centrifugation (30min at 2100*g*) and discarded (Matsushima, 1958).

Affinity chromatography. The supernatant was stirred overnight with 500g (damp wt.) of Cm-papain–Sepharose which had been equilibrated with 50mM-phosphate buffer, pH 6.5, containing 0.5M-NaCl and 0.1% Brij 35. The adsorbent was collected by centrifugation (30min at 340*g*), resuspended in buffer as above, and washed by further cycles of centrifugation and resuspension in buffer until the A_{280} approached zero. The Cm-papain–Sepharose was packed into a column (50mm diameter), and washed again with the pH 6.5 buffer, but with 10% (v/v) glycerol in place of the Brij 35. After passage of 2 bed-volumes of the pH 6.5 buffer, the bound protein was eluted with 50mM-K$_3$PO$_4$, pH 11.5, in 0.5M-NaCl and 10% glycerol (without detergent). Fractions representing the single peak of protein detected by A_{280} in the effluent were tested for inhibitory activity in the Bz-Arg-NPhNO$_2$ assay. The active fractions were combined and the pH adjusted to 7.4 with 5M-sodium formate buffer, pH 3.0. The protein solution was transferred into 25mM-imidazole/HCl, pH 7.4, and concentrated by ultrafiltration.

Chromatofocusing. The sample was run on a column (15cm × 1cm) of Polybuffer exchanger 94 that had been equilibrated with 25mM-imidazole/HCl, pH 7.4. Polybuffer 74 (diluted 8-fold and adjusted to pH 4.0 with 1M-HCl) was used as eluent at a flow rate of 10ml/h. The protein from fractions inhibitory for papain was run in electrophoresis, and pools representing the separate forms of the inhibitor were made.

Determination of $A_{280}^{0.1\%}$. After chromatofocusing, the two separate forms of cystatin were precipitated from 80%-satd. (NH$_4$)$_2$SO$_4$ to remove Polybuffer; the precipitates were collected by centrifugation and washed twice with 80%-satd. (NH$_4$)$_2$SO$_4$. The proteins were transferred into water by ultrafiltration, A_{280} was measured, and the protein content was determined by the Lowry *et al.* (1951) method. The solutions were then freeze-dried to constant weight, and the weights of the proteins determined.

Determination of hexosamine. The method of Blix (1948) was used with hydrolysates prepared in 6M-HCl during 4h at 105°C. Samples were also run in a Locarte amino acid analyser, in comparison with standards of glucosamine and galactosamine.

Peptide mapping. Cystatin, 3mg in 4ml of

6 M-guanidinium chloride, was reduced with dithiothreitol and carboxymethylated with iodoacetic acid by a standard method and freeze-dried. The protein was then dissolved in 1 ml of 1% NH_4HCO_3. Trypsin (1%, w/w) was added, and the reaction was allowed to proceed for 1 h at 37°C. The same amount of trypsin was added again, and, after reaction for a further 1 h, the solution was made 5% (v/v) with respect to formic acid and freeze-dried. The peptide mixture corresponding to 20 μg of protein was run in h.p.l.c. on a Zorbax ODS column in a gradient from water to 80% acetonitrile, all containing 0.1% trifluoroacetic acid (Bennett *et al.*, 1981), and the effluent was monitored at 220 nm.

Determination of free thiol groups. The method of Ellman (1959) was used, but in some experiments the protein was first subjected to mild reduction with 2 mM-cysteine in 50 mM-potassium phosphate buffer, pH 6.8, for 15 min at 40°C. The cysteine was removed by running the sample on a column of Sephadex G-25 in 25 mM-sodium acetate buffer, pH 5.0.

Identification of N-terminal amino acids. The *N*-terminal sequence of the two forms of cystatin was determined by the dansyl–Edman method (Gray, 1972*a*,*b*). Toluene was substituted for benzene in the solvent system of Woods & Wang (1967) for the resolution of dansyl-amino acids in polyamide t.l.c. (Croft, 1972).

Immunological methods. Rabbits were immunized by intramuscular injection of cystatin (form 1 or 2) with Freund's complete adjuvant (0.8 mg followed by a further 0.5 mg 2 weeks later).

Double immunodiffusion was in 1% agarose in 20 mM-phosphate buffer, pH 7.2, containing 0.15 M-NaCl, and was allowed to proceed for 24 h. Plates were washed overnight in 1% (w/v) NaCl, dried, and stained with Coomassie Brilliant Blue R, as described by Barrett (1974).

Radial-immunodiffusion assays were done essentially as described by Mancini *et al.* (1965). Plates were 1% agarose as above, but contained 8% (v/v) antiserum to the purified inhibitor, and were standardized with a solution of the purified cystatin. Diffusion was allowed to proceed for 48 h.

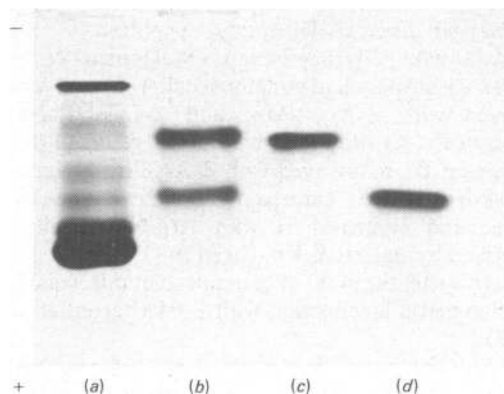

Fig. 1. *Polyacrylamide-gel electrophoresis at various stages in the purification of cystatin from egg white*
The samples were taken after: (*a*) mixing egg white with NaCl; (*b*) Cm-papain–Sepharose chromatography; (*c*) chromatofocusing (cystatin form 1); and (*d*) chromatofocusing (cystatin form 2).

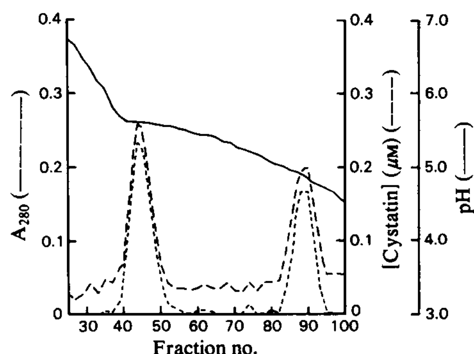

Fig. 2. *Separation of the two forms of cystatin by chromatofocusing*
Distribution of cystatin (– – – –) and protein (————) in effluent fractions from a column of Polybuffer exchanger 94. Cystatin was quantified in the Bz-DL-Arg-$NPhNO_2$ assay as described under 'Methods'. The pH of the effluent (——) is shown.

Results

Purification of cystatin

After the initial precipitation of ovomucin, affinity chromatography gave a product consisting mainly of two proteins differing in mobility in non-SDS polyacrylamide-gel electrophoresis (Fig. 1). Chromatofocusing separated the two proteins in peaks of similar specific inhibitory activity (Fig. 2).

The two proteins gave reactions of complete immunological identity when run in immunodiffusion against antisera raised against each of them individually; they also gave reactions of complete identity with the antigen in untreated egg white (Fig. 3). The components were therefore considered to be forms of a single protein, cystatin, and were referred to as 'form 1' and 'form 2', in order of elution from the chromatofocusing column. In fact, further minor forms could be resolved with the higher resolution of chromatofocusing on the Mono-P column of a Pharmacia f.p.l.c. system. From 72 eggs we typically obtained about 35 mg of cystatin in the two separated forms, a recovery of about 20% (Table 1).

Table 1. *Purification of cystatin*

The results are from a typical preparation with 72 eggs. The values for protein in the first two stages were determined by the method of Lowry *et al.* (1951), and in the later stages by A_{280} ($A^{0.1\%}_{280,1\,cm} = 0.87$). Cystatin was quantified by radial immunodiffusion, the reference standard being the final purified product, which was shown independently to be homogeneous by electrophoresis. Percentage purity was calculated as (mg of cystatin/mg of protein) × 100.

Stage	Volume (ml)	Protein (mg)	Cystatin (mg)	Purity (%)	Enrichment (fold)	Yield (%)
Egg white plus NaCl	4600	393600	193	0.05	1	100
Cm-papain–Sepharose	102	82.9	67.5	81.4	1628	34.9
Chromatofocusing						
Form 1	47.3	24.7	24.7	100	2000	12.8
Form 2	35.5	12.8	12.8	100	2000	6.6

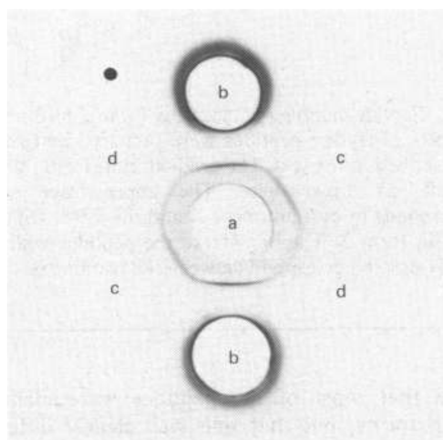

Fig. 3. *Immunological identity of the two forms of cystatin*

Rabbit anti-(cystatin form 1) serum (a) was allowed to diffuse against (b) whole egg white (40 μl) and forms 1 (c) and 2 (d) of cystatin separated by chromatofocusing (1 μg each). The plate was developed and stained as described under 'Methods'.

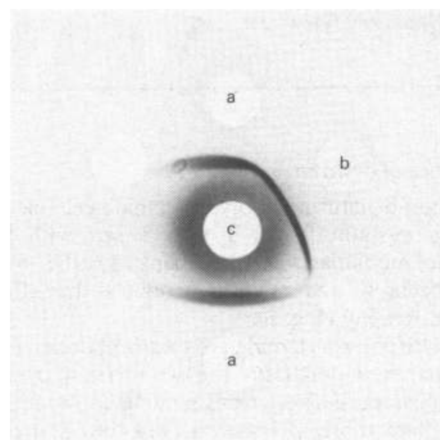

Fig. 4. *Identification of cystatin in chicken serum*

Serum samples run on Cm-papain–Sepharose as described in the text (a) were allowed to diffuse against rabbit anti-(cystatin form 1) serum (c), alongside pure cystatin (form 1) (b).

However, for many experiments the mixed forms (of which about 70 mg was obtained) were satisfactory.

We found freezing or freeze-drying of purified cystatin in water to lead to loss of activity, as others have found previously (Keilová & Tomášek, 1974), but solutions in 20% (v/v) glycerol were stored at −20°C, and solutions in 50 mM-NH_4HCO_3, pH 7.5, were freeze-dried, with full retention of activity. Cystatin was very stable at alkaline pH; for example, no loss in activity was detectable after storage at pH 11 and at 4°C overnight.

Detection of cystatin in serum of male and female chickens

When chicken serum was examined directly by immunodiffusion or immunoelectrophoresis against anti-cystatin serum, no precipitin lines were detected. To provide a more sensitive test, serum (15 ml) was passed through a column (4 cm³) of Cm-papain–Sepharose, and the column was washed with 40 ml of phosphate buffer, pH 6.5, before elution with phosphate buffer, pH 11.5, as usual. Fractions (0.5 ml) were collected and tested by double immunodiffusion for the presence of cystatin. The stained plate showed faint precipitin lines for three fractions, which were combined and concentrated 5-fold by dialysis against Carbowax. In immunodiffusion, a sharp precipitin line was then detected, which showed complete identity with the inhibitor purified from egg white (Fig. 4). The concentration was measured by radial immunodiffusion and found to be equivalent to 1 μg of cystatin/ml of serum (which may be compared with 80 μl in egg white). Similar results were obtained whether the serum was from male or female chickens.

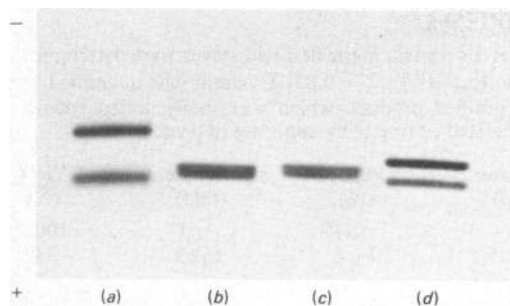

Fig. 5. *SDS/polyacrylamide-gel electrophoresis with reduction*
The samples were (*a*) avidin and cytochrome *c*, (*b*) cystatin form 1, (*c*) cystatin form 2 and (*d*) myoglobin and lysozyme.

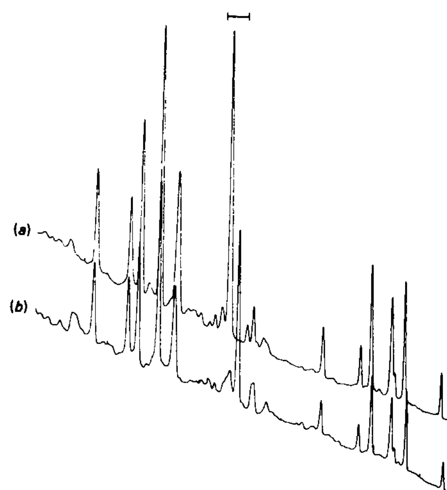

Fig. 6. *Peptide mapping of cystatins 1 and 2 by h.p.l.c.*
Samples of tryptic peptides were prepared and run as described in the text. The gradient runs from right to left, as drawn here. The upper trace (*a*) corresponds to cystatin form 1, and the lower (*b*) to cystatin form 2. The bar marks the peptides which clearly do not correspond between the two forms.

Properties of cystatin

In non-denaturing polyacrylamide-gel electrophoresis cystatin forms 1 and 2 ran with very different mobilities, corresponding to the major components of the mixture produced by affinity chromatography (Fig. 1).

In SDS/polyacrylamide-gel electrophoresis (with reduction) the mobilities of the two forms of cystatin were indistinguishable, corresponding to M_r slightly greater than those of lysozyme (14400) and cytochrome *c* (12400), and less than myoglobin (17600) and the avidin subunit (17000) (M_r values being those reported by Dunker & Rueckert, 1969), and thus consistent with M_r about 15000 (Fig. 5). It seems, however, that the mobility of cystatin in the SDS/polyacrylamide-gel system is anomalous, since the determination of the amino acid sequence of the protein has recently shown the M_r to be 12500 (C. Schwabe, A. Anastasi & A. J. Barrett, unpublished work).

Cystatin forms 1 and 2 had pI values (determined by analytical isoelectric focusing) of 6.5 and 5.6 respectively.

Colour yield in the assay of Lowry *et al.* (1951), and $A_{280,1cm}^{0.1\%}$, were determined by use of solutions of known dry-weight concentration. The Lowry colour yield was 0.72 that of bovine serum albumin. The $A_{280}^{0.1\%}$ was 0.87 for both forms, which agreed well with the value of 0.888 reported by Fossum & Whitaker (1968) for a less highly purified preparation.

The *N*-terminal sequence of both forms of cystatin was Ser-Glx-Asx. No hexosamine was detected in cystatin hydrolysates either by the method of Blix (1948), or in the amino acid analyser, and we conclude that cystatin is not a glycoprotein.

Peptide mapping by h.p.l.c. of the tryptic peptides of forms 1 and 2 of cystatin (see under 'Methods') showed that most of the peptides were identical between forms, but that one was clearly different (Fig. 6). This peptide has been isolated, and further work on it is needed (C. Schwabe, A. Anastasi & A. J. Barrett, unpublished work).

Cystatin as a proteinase inhibitor

Assay of cystatin by inhibition of papain. Despite the very high affinity of cystatin for papain (see below) and high rate constant for association (M. J. H. Nicklin & A. J. Barrett, unpublished work), care was necessary to ensure that the interaction approached stoichiometry when the very low concentration of papain appropriate to the fluorimetric assay was used. With the preincubation of enzyme and inhibitor in a small volume (see under 'Methods'), the percentage inhibition in a dose–response curve increased linearly at least to 50%, and this allowed reproducible quantification.

On the basis of a 1:1 stoichiometry of reaction, and the $A_{280}^{1\%}$ and M_r values for cystatin given above, it was found that preparations ranged from 50 to nearly 100% activity. The most active preparations showed essentially complete incorporation of protein into complexes with papain, when examined by analytical isoelectric focusing (Fig. 7), or with papaya peptidase in f.p.l.c. on a column of Mono-S at pH 5.0.

The f.p.l.c. system at pH 5.0 clearly showed the formation of complexes between cystatin and

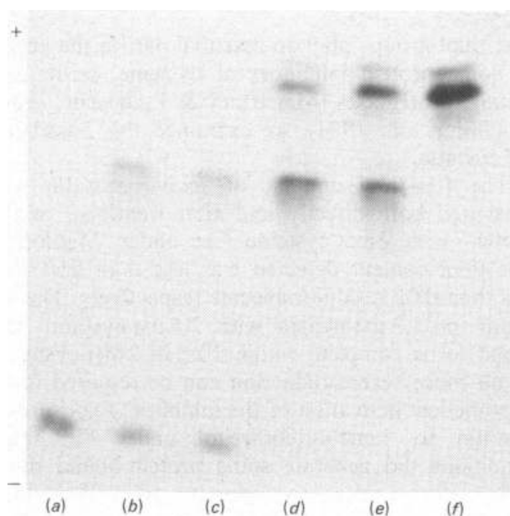

Fig. 7. *Demonstration of papain–cystatin complexes in isoelectric focusing*

Samples run on the flat-bed analytical isoelectric-focusing gel were mixtures of cystatin solution (1.06mg/ml) and papain (100μM in 2mM-dithiothreitol). Samples (*a*)–(*e*) contained 2μl of cystatin with 0, 2.5, 3.3, 8.0 and 15μl of papain respectively. Sample (*f*) was 20μl of water with 15μl of papain.

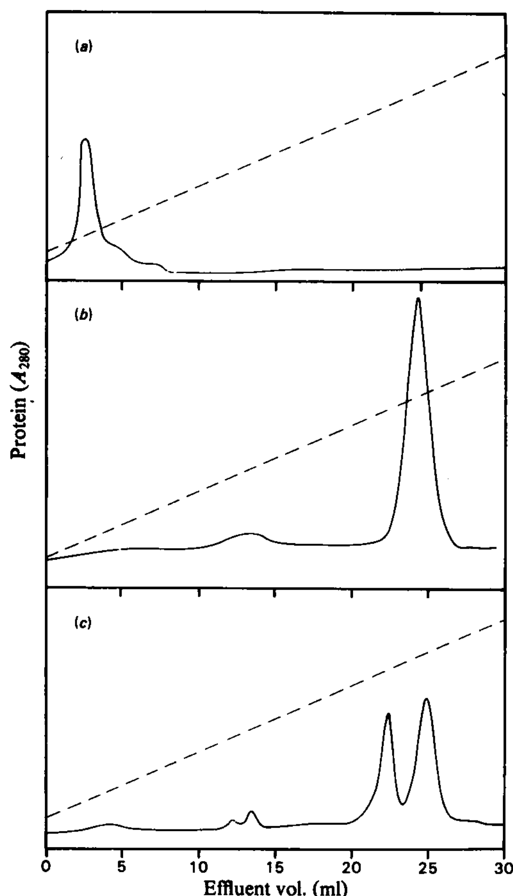

Fig. 8. *Demonstration of papaya-peptidase–cystatin complexes in f.p.l.c.*

The samples were run on the Mono-S column of the f.p.l.c. apparatus in a sodium acetate buffer, pH 5.0, the gradient (----) increasing from 50mM to 800mM over 37ml. Samples were (*a*) cystatin (0.3mg), (*b*) iodoacetate-inactivated papaya peptidase (1.0mg) and (*c*) the mixture of the two. The shift of the cystatin to a new peak near that of papaya peptidase in (*c*) is attributable to complex-formation. ——, A_{280}.

carboxymethylated papaya peptidase (Fig. 8). A sodium malonate buffer system, pH 5.5, allowed separation of cystatin form 2 from papain, and complex-formation was demonstrated with papain that had been allowed to react with E-64 or Z-Phe-Ala-CHN_2, both very bulky active-site-directed blocking groups.

Specificity of inhibition. Cystatin was tested for the capacity to inhibit a variety of cysteine, serine and metallo-proteinases.

In addition to papain, some, but not all, other cysteine proteinases were inhibited. Ficin and stem bromelain (each 5μg of commercial enzyme in the 1 ml assay mixture; see under 'Methods') were assayed in the presence of 2μg or 100μg of cystatin (mixed forms). It was found that the ficin was 43% inhibited by 2μg of cystatin, whereas the bromelain was less than 10% inhibited by 100μg of cystatin. Neither clostripain (0.05μg) nor streptococcal proteinase (15μg) showed significant inhibition by 100μg of cystatin. Human cathepsins B, H and L were strongly inhibited by cystatin (see below).

Four serine proteinases, bovine trypsin, bovine α-chymotrypsin, porcine elastase and human leucocyte cathepsin G (each 5μg), were tested for susceptibility to cystatin in assays with azocasein as substrate. Cystatin (200μg) caused not more than 10% inhibition of any of them.

A metalloproteinase, thermolysin (0.5μg), was assayed under the same conditions as the serine proteinases; 100μg of cystatin gave no detectable inhibition.

Dipeptidyl peptidase I was inhibited by cystatin, as reported by Keilová & Tomášek (1975). The apparent K_i was 3×10^{-10}M under the conditions of the standard assay (with 200μM-substrate), but the inhibition was found to be of mixed competitive and non-competitive type (M. J. H. Nicklin, unpublished work).

Inhibition constants for papain and cathepsins B, H and L. Cystatin proved to be a very tight-binding inhibitor of papain. We were unable to detect any

dissociation of complexes at pH 6.8, and the conditions were such that dissociation would have been detected had K_i been greater than 10^{-11} M.

Cathepsin B (2 nM, 40 μl) was activated with 40 μl of 100 mM-dithiothreitol in buffer for 30s, and diluted with 3.84 ml of sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA. Z-Arg-Arg-NNap in water (40 μl) was added to give a concentration of 100 μM or 200 μM (a set of measurements was made at each concentration). The mixtures were incubated at 30°C, and the liberation of 2-naphthylamine was recorded fluorimetrically ($\lambda_{excitation}$ 340 nm, $\lambda_{emission}$ 414 nm) until a steady initial rate could be measured. Then 40 μl of cystatin solution was added to give concentrations up to 2 nM. The rate of hydrolysis was allowed to relax to the steady state (during about 60 min), and the results were replotted by the method of Dixon (1953) to give a K_i of 8×10^{-10} M.

Cathepsin H was assayed with Arg-NMec at pH 6.8, in the presence of a series of concentrations of cystatin, and the results calculated by the method of Henderson (1972) to give a K_i of approx. 2×10^{-8} M.

The K_i of cathepsin L was very low and the enzyme was unstable in dilute solution, so that K_i could only be estimated at all because of an unusually high rate constant of association. In principle, the experiments were as for cathepsin B, but the conditions were as follows: the final cathepsin L concentration (by titration) was 5 pM, the activator was 200 mM-cysteine for 30 s, the range of inhibitor concentrations (final) giving consistent data was 48–72 pM, and the temperature was 25°C. A high (20 μM) concentration of the substrate Z-Phe-Arg-NMec was used, despite the fact the rate of inhibition by cystatin was decreased, because it stabilized the enzyme. 7-Amino-4-methylcoumarin release was monitored continuously ($\lambda_{excitation}$ 360 nm, $\lambda_{emission}$ 460 nm). Data were replotted as initial rate/steady-state inhibited rate against inhibitor concentration. K_i (apparent) was approx. 1×10^{-11} at this substrate concentration, three times the K_m (Barrett & Kirschke, 1981). The mechanism was not investigated, but if it had been purely competitive, the true K_i would have been about 3×10^{-12} M.

Sen & Whitaker (1973) reported that the inhibition of ficin by cystatin is non-competitive and the K_i is much greater than the values we have obtained for papain, cathepsin B and cathepsin L. Their conclusions are invalidated, however, by the fact that the experiments were done with E_t (total enzyme concn.) much greater than K_i, whereas E_t should be less than one-tenth K_i for the type of replot used [as discussed by Henderson (1972)].

Exclusion of any role for a thiol group in the mechanism of inhibition. Since it has been proposed that thiol groups play an essential part in the action of some protein inhibitors of cysteine, serine and metallo-proteinases (Macartney & Tschesche, 1980; Kopitar *et al.*, 1981), we examined this possibility for cystatin.

The free-thiol content of native cystatin was measured both directly and after treatment of the protein with 2 mM-cysteine (see under 'Methods'). The thiol content detected was less than 0.01 and less than 0.02 residue/molecule respectively. The reaction of 1.3 μM-papain with 2.5 μM-cystatin was found to be complete within 10 s, in 2 mM-cysteine, so no more severe reduction can be required for a hypothetical activation of the inhibitor. Exposure of cystatin to 1 mM-dithiothreitol under the same conditions did generate some protein-bound thiol, however, equivalent to reduction of a disulphide bridge in one third of the molecules.

Cystatin was also treated with 50 mM-iodoacetate in 50 mM-sodium phosphate buffer, pH 6.8, at 40°C for 15 min, either directly or after treatment with cysteine as above. The excess iodoacetate was removed by gel chromatography and the inhibitor samples tested for their capacity to inhibit papain; the activities were indistinguishable by titration from that of control untreated inhibitor.

These findings, together with the great stability of cystatin to alkali, which would not be expected of a thiol-dependent protein, lead us to conclude that cystatin does not inhibit by any mechanism involving a thiol group on the inhibitor molecule. The molecule contains four cysteine residues, which apparently form two disulphide bridges (C. Schwabe, A. Anastasi & A. J. Barrett, unpublished work).

SDS/polyacrylamide-gel electrophoresis of cystatin–papain complexes. Preparations of the complex between papain and cystatin form 1 were treated with iodoacetate, and electrophoresed both with and without reduction. When papain had not been in excess, only bands corresponding to free papain and free inhibitor were observed. There was no evidence of the formation of SDS-stable complexes or of the cleavage of the polypeptide chain of the inhibitor, which occur with some inhibitors of serine proteinases (Laskowski & Kato, 1980).

Discussion

Affinity chromatography of cystatin on Cm-papain–Sepharose provided almost a one-step purification procedure for the mixed forms. Use of the ligand enzyme in its carboxymethylated form exploited the affinity of the inhibitor for the modified enzyme, and provided an affinity medium much simpler to use than active-papain–Sepharose would have been. It was convenient that cystatin proved to

be stable at alkaline pH. Chromatofocusing proved to be an excellent method for separation of the forms of the inhibitor.

Each of the two major forms of cystatin is a single polypeptide chain, and they have identical *N*-terminal sequences and give closely similar peptide maps. The difference in charge is apparently due to one or a few amino acid substitutions in one region of the molecule. We did not detect any significant amount of hexosamine in cystatin, and therefore agree with the conclusion of Sen & Whitaker (1973) that it is not a glycoprotein. Fossum & Whitaker (1968) found the egg-white inhibitor to be active against ficin and papain, but not bromelain (another plant cysteine proteinase), and not trypsin, chymotrypsin or subtilisin (serine proteinases) or the metalloproteinases of *Pseudomonas aeruginosa*, *Ps. vulgaris*, and some *Bacillus* species. Keilová & Tomášek (1974) showed inhibition of cathepsin B and dipeptidyl peptidase I (cathepsin C), but not cathepsins D and E (both aspartic proteinases). We have confirmed the inhibition of papain, ficin and cathepsin B, and the lack of inhibition of bromelain, trypsin and chymotrypsin. Cathepsins H and L and papaya peptidase also were inhibited, but another cysteine proteinase, clostripain, and other serine proteinases, pancreatic elastase and cathepsin G, were not. Since the protein inhibits a broad range of cysteine proteinases, and not serine, metallo- or aspartic proteinases, the name 'cystatin' seems appropriate.

The mechanism of inhibition by cystatin has yet to be established. We see no indication that the inhibitor has an available thiol group, so there is nothing to suggest a disulphide-exchange mechanism of inhibition such as has been proposed for an inhibitor of similar M_r from bovine spleen (Kopitar *et al.*, 1981). The lack of cleavage of cystatin in complexes, as judged by SDS/polyacrylamide-gel electrophoresis, does not exclude the possibility of a covalent interaction by the 'standard mechanism' put forward by Laskowski & Kato (1980), but obviously does nothing to support it.

Complexes were formed with proteinases in which the catalytic cysteine was alkylated, even by very bulky substituents. This extends the previous reports for the mercuri derivatives of papain, ficin and cathepsin B, and carboxymethylated ficin (Sen & Whitaker, 1973; Keilová & Tomášek, 1974). These findings raise the possibility that, in the complex, the surfaces of proteinase and cystatin molecules are not closely associated in the region of Cys-25 (in the papain numbering).

The occurrence of cystatin in serum of male and female chickens indicates that the protein is not synthesized exclusively as a component of the egg. This would be consistent with the possibility that it is related to the inhibitors with similar properties that exist in the tissues of various mammals and which were mentioned in the introduction.

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