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HUMAN CYSTATIN, A NEW PROTEIN INHIBITOR OF CYSTEINE PROTEINASES

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SUMMARY: A new low-molecular weight protein inhibitor of cysteine proteinases, human cystatin, was isolated from sera of patients with autoimmune diseases. It inhibits papain, human cathepsin H and cathepsin B. According to its partially determined amino-acid sequence, human cystatin is highly homologous to egg white cystatin, but only distantly related to stefin, the cytosolic protein inhibitor of cysteine proteinases isolated from human polymorphonuclear granulocytes. Very probably human cystatin is identical with human γ-trace, a microprotein of known sequence but hitherto unknown function.

Recent studies revealed the existence of two groups of protein inhibitors of cysteine proteinases of mammalian origin. One group of inhibitors has high molecular weights ranging from 60,000 to 175,000 and is present in plasma (reviewed in 1,2). The other group consists of low-molecular weight inhibitors with molecular masses of about 11,000 to 16,000. These inhibitors have been found in tissues (3-12) and, to a limited extent, in serum (1,13). Two inhibitors of the latter type were recently isolated from rat liver (14) and human polymorphonuclear granulocytes (15). Their complete primary structures have been determined (16-18). The two inhibitors are highly homologous to each other, but only distantly related to egg white cystatin (19).

Recently we found an increased level of low-molecular weight protein inhibiting papain in sera of patients suffering from autoimmune diseases. The present communication reports on isolation, properties and structure of a new protein inhibitor of cysteine proteinases from these sera.

MATERIALS AND METHODS

Materials. Human serum from patients with autoimmune diseases (Lupus erythematosus and glomerulonephritis) was prepared from fresh blood. Human liver cathepsin B and cathepsin H were prepared by the method developed for bovine spleen (20). Papain was from Sigma. Sephadex G-50, CNBr-activated Sepharose 4B, ampholines, and molecular weight standard proteins were from

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Pharmacia Fine Chemicals. CM-cellulose was purchased from Serva. S-carboxymethylated papain-Sepharose 4B was prepared as described previously (21). All other chemicals used were of analytical grade.

Enzyme and inhibitor assays. Enzyme activities of papain (EC 3.4.22.2.) and cathepsin B (EC 3.4.22.1.) on Benzoyl-DL-arginine-2-naphthylamide substrate and of cathepsin H (EC 3.4.22.16.) on L-Leucyl-2-naphthylamide substrate were determined according to Barrett (22). The inhibitory activity was determined under the conditions described for the corresponding enzyme assay. Prior to assays, a known amount of inhibitor was preincubated with the enzyme (3 µg) for 5 min at pH 7.0 and room temperature before adding the substrate. One inhibitor unit corresponds to the complete inhibition of 1 µg of papain.

Temperature stability. Inhibitor (3.7 µg) was incubated for 10 min at 80 °C and assayed for inhibition of papain as described before.

Purification of inhibitor. Serum (2400 ml) was concentrated by lyophilization to 720 ml. An aliquot of 120 ml was applied to a Sephadex G-50 column (6 x 110 cm) equilibrated and eluted with 0.01 M Tris buffer, pH 8.0, containing 0.5 M NaCl. This step was repeated and fractions inhibiting papain were collected. The last inhibitor peak (designated as Peak III Fig. 1) was concentrated using a Diaflo YM-2 membrane and applied to a S-carboxymethylated papain-Sepharose 4B column (3 x 15 cm) which had been previously washed with 0.01 M Tris/HCl buffer, pH 8.0, containing 1.0 M NaCl. After washing with this buffer, the fractions containing inhibitor were eluted with 0.01 M NaOH, pooled and dialyzed against 0.01 M phosphate buffer, pH 7.0. The dialysate was chromatographed on a column of CM-cellulose (3 x 15 cm) equilibrated with the same buffer. After elution with starting buffer, a linear gradient of NaCl (0-0.1 M) in a total volume of 400 ml was applied. Two inhibitor peaks were eluted and concentrated using a Diaflo YM-2 membrane.

Amino-acid analysis. Protein samples were hydrolysed in 6 N HCl at 105 °C for 24, 48, 120 h, and 24 h after performic acid oxidation. The reults from the amino-acid analyser (Kontron Liquimat II) were corrected for destruction and incomplete hydrolysis as indicated in the Table.

Amino-acid sequence analysis. The N-terminal amino-acid sequence of the inhibitor was determined by automated solid-phase Edman degradation in a non-commercial sequencer (23). Prior to degradation the protein was covalently attached to p-phenylene-diisothiocyanate activated aminopropyl glass (88 Å pore size) in water adjusted to pH 8 with solid sodium bicarbonate as described elsewhere (24). The released amino-acid phenylthiohydantoin derivatives were identified by quantitative high performance liquid chromatography operating on-line to the sequencer (23).

Other methods. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (25). Analytical gel electrofocusing was carried out on 1 mm thick 5 % polyacrylamide gel plates with ampholines of pH 2-10 following the manufacturer's instructions (Pharmacia Fine Chemicals). Protein was determined by the Lowry method (26).

RESULTS

From Fig. 1 it is evident that only a small fraction of the total inhibitory activity of serum on cysteine proteinases was found in the low-molecular weight fraction (Peak III). This fraction was subjected to affinity chromatography on a S-carboxymethylated papain-Sepharose 4B column. CM-cellulose chromatography as the last purification step resulted in two inhibitor peaks eluting with the starting buffer and with the NaCl gradient, respectively (not shown). Only the second peak was investigated. 0.29 mg of pure inhibitor was obtained from 2.4 l of serum.

As shown in Fig. 2, the final product was homogenous in SDS polyacrylamide gel electrophoresis, and its apparent molecular mass was determined to be

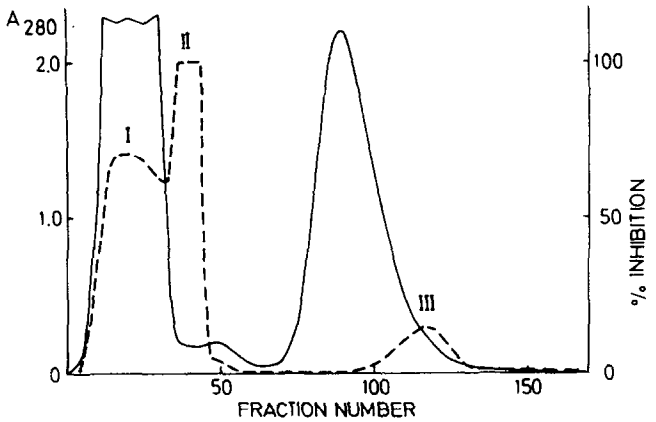

Fig. 1. Sephadex-G-50 chromatography of concentrated serum. Protein concentration (———) is indicated by the absorbance at 280 nm. Fractions of 12 ml were collected. For estimation of inhibitory activity on papain, 0.1 ml ofeach fraction was applied and the relative degree of inhibition is indicated (– – –).

approximately 15,000. Isoelectric focusing of the inhibitor resulted in a single band corresponding to an isoelectric point of approx. 8.0 (not shown).

The effect of the purified inhibitor on papain, human cathepsin B and human cathepsin H is shown in Fig. 3. The inhibitor inhibited all three

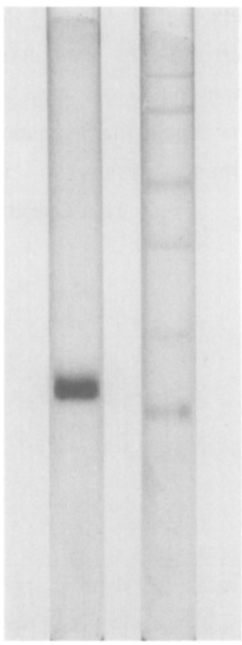

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified human cystatin. in a 10 % gel. The sample (4.5 µg) was preincubated for 30 min at 37 °C in 1 % SDS containing 1% 2-mercaptoethanol. Protein bands were stained with Coomassie Brilliant Blue G-250. Phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soyabean trypsin inhibitor and α-lactalbumin were used as molecular weight standards (right lane).

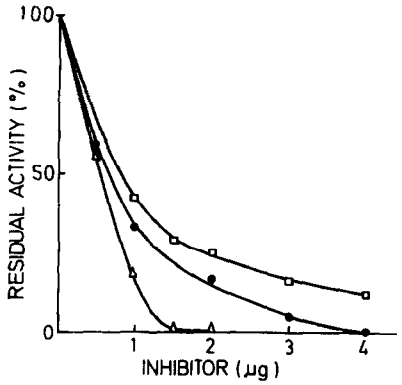

Fig. 3. Inhibitory effect of human cystatin on cysteine proteinases. Enzymes (3 μg each) were preincubated with increasing amounts of inhibitor and the remaining activities of papain (△—△), human cathepsin H (●—●) and human cathepsin B (□—□) were determined as described in Materials and Methods.

cysteine proteinases, but to a different extent. About one mol of inhibitor was needed for complete inhibition of one mol of papain and cathepsin H, whereas a several-fold excess of inhibitor did not completely inhibit cathepsin B. After incubation of the inhibitor for 10 min at 80 °C, no significant change of papain inhibition was observed, indicating that the protein inhibitor is thermostable.

N-terminal amino-acid sequencing of the inhibitor resulted in a homogenous sequence for the first 50 amino-acid residues. This sequence is highly homologous to the corresponding part of egg white cystatin, and probably distantly related to the corresponding sequence of stefin, the protein inhibitor of cysteine proteinases isolated from the cytosol of human polymorphonuclear granulocytes (Fig. 4). Computer search revealed that the

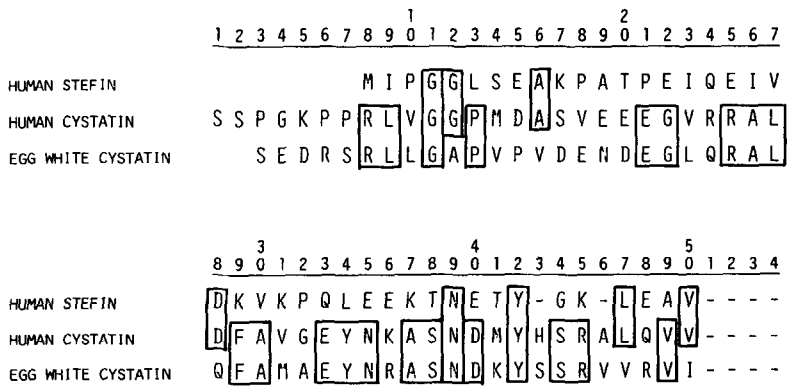

Fig. 4. Comparison of N-terminal amino-acid sequences of human cystatin (Ser-1 to Val-50) with the corresponding parts of human stefin (Met-1 to Val-41) and egg white cystatin (Ser-1 to Ile-48) using the alignment which gives the highest number of identical residues in this region. Common residues are shown in boxes.

determined N-terminal sequence of the new inhibitor is identical with the N-terminal sequence of human γ-trace, a protein of unknown function (27). Furthermore, the amino-acid compositions of the two proteins are identical within the usual limits of error. For comparison the amino-acid compositions of egg white cystatin and stefin are presented (Table).

DISCUSSION

In the present study we discovered a new low-molecular weight protein inhibitor of cysteine proteinases. According to its inhibition properties and its partially known amino-acid sequence this inhibitor is closely related to egg white cystatin (19,28) and should be named 'human cystatin'. Although the common properties of both cystatins are also similar to those of stefin (15-17) and the thiol proteinase inhibitor (TPI-B) from rat liver (14,18), the latter two inhibitors belong to a different family of cysteine proteinase inhibitors according to their primary structures. The cystatin and the stefin family, however, may be considered to be members of the same superfamily of low-molecular weight protein inhibitors of cysteine proteinases (W. M. et al., manuscript in preparation).

Table. Amino-acid composition of human cystatin, human γ-trace, egg white cystatin and human stefin.

	Human cystatin	Human γ-trace *	Egg white cystatin *	Human stefin *
Asp	12.02 (12)	12	10	11
Thr	7.39 (7)	7	5	7
Ser	8.60 (9)	9	12	2
Glu	12.35 (12)	12	16	15
Pro	7.68 (8)	8	5	5
Gly	8.31 (8)	8	5	8
Ala	10.00 (10)	10	7	5
Cys	3.86 (4)	4	4	0
Val	9.82 (10)	10	9	9
Met	2.94 (3)	3	2	2
Ile	1.99 (2)	2	6	4
Leu	8.01 (8)	8	10	8
Tyr	3.75 (4)	4	5	6
Phe	5.11 (5)	5	3	2
Lys	6.91 (7)	7	7	12
His	2.94 (3)	3	1	1
Arg	8.05 (8)	8	8	1
Trp	n.d. (0)	0	1	0

* as calculated from the amino-acid sequences (17,19,28).
The values for Thr and Ser were corrected for destruction by zero extrapolation of a quadratic regression line fitted to the yields obtained after 24, 48 and 120 h hydrolysis. Cys and Met were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation. The values after 120 h hydrolysis were selected for Val and Ile. The Trp content was estimated fron the A_{280}/A_{289} ratio.

Surprisingly, human cystatin seems to be identical with human γ-trace, a microprotein without known function which has been intensively studied. This protein has been isolated from the urine of patients with renal failure. Using immunological methods, γ-trace has been found in many tissues, but in very low concentrations in normal serum (27). These former observations provide strong evidence that human cystatin alias γ-trace is an intracellular inhibitor of cysteine proteinases.

Our preliminary results show that the concentration of human cystatin in normal serum is at least ten times lower than in the investigated sera of patients suffering from autoimmune diseases. One may speculate that the higher inhibitor level in the sera of patients is due to increased release of inhibitor from cells as a consequence of the autoimmune process.

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