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A HEAT STABLE LOW MOLECULAR WEIGHT INHIBITOR OF LYSOSOMAL CYSTEINE PROTEINASES IN HUMAN SERUM

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SUMMARY : A new proteinase inhibitor of lysosomal cysteine proteinases has been isolated from human serum. In spite of its low concentration, kinetics studies indicate that it may be of physiological relevance given the low Ki values found for its interaction with human cathepsin B and cathepsin H.

Human serum contains a specific inhibitor for cysteine proteinases which may appear under different molecular forms (1-4).

This protein strongly inhibits papain and ficin (1-3), but exhibits a rather weak inhibiting capacity on lysosomal cysteine proteinases such as cathepsin B, H or L (2, 3, 5-7). Though the lack of precise kinetics data does not permit any conclusion to be drawn as yet, the question of its biological significance may however be raised.

This paper gives evidence for the presence in human serum of a low Mr heat stable component which inhibits liver cathepsins B and H in such a way that, given the low Ki values found for its interaction with these enzymes, it could readily be of physiological relevance.

Whether or not this inhibitor corresponds to a tissue inhibitor of cysteine proteinases still remains questionable but the first known properties of this compound seem to make it specific.

MATERIAL AND METHODS

Normal human serum was prepared from the blood of a pool of healthy donors.

Ultrogel AcA34 was obtained from Industrie Biologique Française , DEAE Sephacel and Sephadex G 50 fine from Pharmacia France, Bz-Arg-NNap from Sigma, Z-Arg-Arg-NNap and Arg-NNap from Bachem, Bubendorf, Switzerland, Fast

ABBREVIATIONS USED : αTPI,α thiol proteinase inhibitor ; Bz, α-N-benzoyl ; Z , α-N- benzyloxycarbonyl ; N-Nap, 2 naphtylamide ; E_{64} , L-trans-epoxysuccinylleucylamido (4-guanidino) butane.

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garnet GBC fluoroborate from Serva, Heidelberg, FRG, and E_{64} from the peptide Research Foundation, Osaka, Japan.

Human liver cathepsin B was prepared as previously described for rat cathepsin B (8).

Preparation of human cathepsin H has been detailed elsewhere (7). Brie fly, the three first steps were common with those of the cathepsin B isolation procedure (8). The fourth step of ion exchange chromatography was carried out as described by Schwartz and Barrett (9) except DEAE Sephadex was used instead of DEAE cellulose (DE 52). Cathepsin H passed straight through the column and was then submitted to two successive gel filtration on Sephadex G 75 superfine. Both proteinases were stored at - 70°C in 0.1 M acetate buffer (pH 4.6) 2 mM EDTA, 1 mM dithioerythrol, 0.1 M NaCl until use.

PURIFICATION PROCEDURE : 35 ml of fresh human serum were applied to a column (5 x 50 cm) of Ultrogel AcA 34 equilibrated in 10 mM Tris HCl buffer (pH 7.4) 150 mM NaCl. Elution was performed at a flow rate of 25 ml/hour. The last UV absorbing peak eluted after the major protein peaks (fig. 1 a) was pooled, concentrated to about 6 ml (Amicon UM 05 membrane), dialyzed against 10 mM Tris HCl buffer (pH 8.7) using a PD 10 Pharmacia column and applied to a column (1.6 x 15 cm) of DEAE Sephacel equilibrated in the same buffer.

Fractions collected after washing with this buffer were assayed for their cathepsin H inhibiting capacity as described under "enzyme assays". The first cathepsin H inhibiting peak was concentrated to 5-6 ml and then passed through a column (3.2 x 88 cm) of Sephadex G 50 equilibrated in 10 mM Tris HCl buffer (pH 8.7) 150 mM NaCl, 0.1 % Brij 35. Eluted fractions were scarcely detectable in UV spectrophotometry and were therefore solely assayed for their cathepsin H inhibiting capacity (fig. 1 c), then they were pooled, concentrated to 5 ml and stored at - 70°C until use.

The second minor cathepsin H inhibiting peak eluted slightly later from the DEAE Sephacel column and representing about 10 % of the total inhibiting capacity was not taken into account.

Evaluation of the inhibitor Mr was made after calibration of the Sephadex G 50 column with low Mr markers (human serum albumin Mr = 68,000, chymotrypsinogen A Mr = 25,000 , ribonuclease Mr = 13,700 , aprotinin Mr = 7,000).

ENZYME ASSAYS

Active site titrations of cathepsin B and cathepsin H were carried out essentially as described by Barrettusing the epoxide inhibitor E_{64} (10) and Z-Arg-Arg-NNap and Arg-NNap as substrates respectively.

Cathepsin B was thus found to be 44 % active and cathepsin H 35 %.

Titration of the inhibitor by cathepsin B and cathepsin H: Cathepsin B (0.23 µM) was incubated for 10 min. at 40°C with increasing amounts of inhibitor in 0.1 M sodium-phosphate buffer (pH 6.0), 1.33 mM EDTA, 3 mM cysteine, 0.1 % Brij 35 (final volume 360 µl) before adding 10 µl of 22 mM Bz-Arg-NNap to start the reaction. After 10 min. the liberated 2 naphtylamine was assayed colorimetrically at 520 nm with Fast garnet GBC fluoroborate as described by Barrett(11). The same procedure was used for cathepsin H 0.086 µM in 0.04 M Sodium Phosphate buffer (pH 6.8), 1.33 mM EDTA, 3 mM cysteine, 0.1 % Brij 35 and using Arg-NNap (0.5 mM) as a substrate.

Ki determination: Ki values were determined for both enzymes using the EASSON-STEDMAN plot (12). This was made possible by lowering enzyme concentration in the assays so that a non linearity of the titration curve was obtained (12). Very sensitive substrates have to be used to carry out these experiments where the enzyme concentration is frequently in the nanomolar range. For this reason, Z-Arg-Arg-NNap was used instead fo Bz-Arg-NNap for cathepsin B assays whereas Arg-NNap was found suitable for all assays with cathepsin H.

The latter was assayed at 5.8×10^{-9} M with increasing amounts of inhibitor (1.7 to 12.5×10^{-9} M) following the same procedure as before and

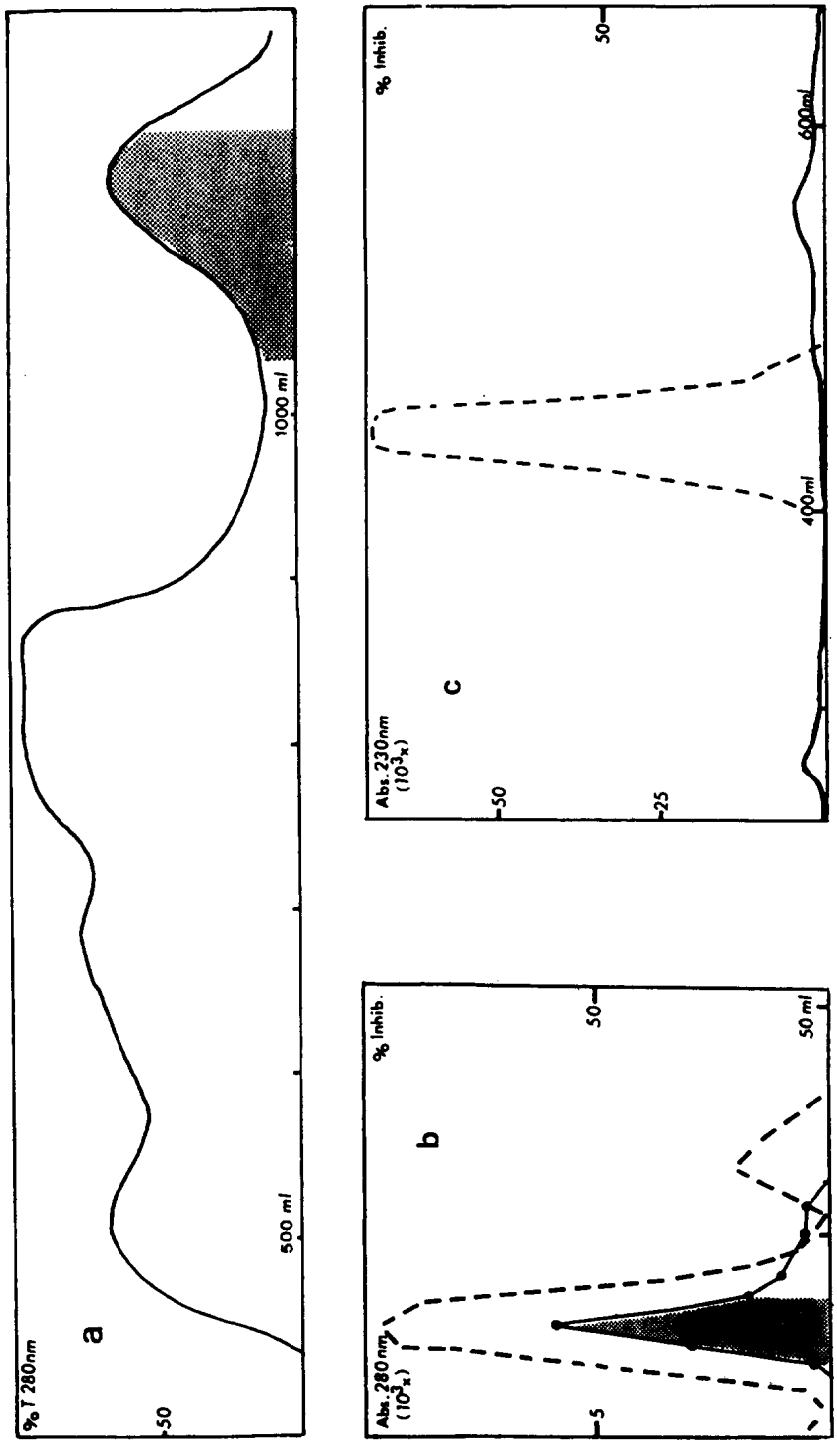

Fig. 1 : Purification of the low Mr lysosomal proteinase inhibitor from human serum. a) Chromatography of fresh human serum on Ultrogel AcA 34. Fractions corresponding to the shaded area were treated as indicated under "Purification procedure" before being applied to a DEAE Sephacel column. The dotted line of (b) indicates cathepsin H inhibition. The shaded area of (b) was finally chromatographed on Sephadex G 50 (c). Cathepsin H inhibiting fractions (dotted line) were concentrated for use in kinetics measurements.

cathepsin B was used at 1.58×10^{-8} M with inhibitor concentrations varying from 1.36 to 17×10^{-8} M.

Temperature stability. Inhibitor (4.5 µM) was incubated for 10 min at 90°C then assayed for its cathepsin H inhibitory activity as described before.

RESULTS AND DISCUSSION

Though lysosomal cysteine proteinases are largely involved in the protein turnover and may thus be classed among the most active proteinases in the body (11), little is known about their "in vivo" regulation. The physiological relevance of proteinase inhibitors mainly depends on their affinity and their rate of interaction with proteinases which are related to the kinetics constants of the equilibrium reaction as well as to the inhibitor concentration. The kinetics properties of this new inhibitor have been therefore investigated before its physicochemical characterization was completed in order to first appraise its physiological function.

The decrease in cathepsin H activity upon addition of increasing concentrations of inhibitor to a constant amount (0.086 µM) of enzyme gives a straight line up to almost zero activity (fig. 2). A complete association between both partners has been therefore completed under these conditions which permit accurate titration of the inhibitor (4.5 µM) and allow us to suppose a very low Ki value : since the binding depends mainly on the $\frac{Eo}{Ki}$ ratio , conditions for obtaining this result are fulfilled when $\frac{EO}{Ki} > 100$ provided the reaction is reversible (14). From these data and considering a 1:1 stoichiometry, the plasma concentration of the inhibitor may be estimated next to 10^{-6} M.

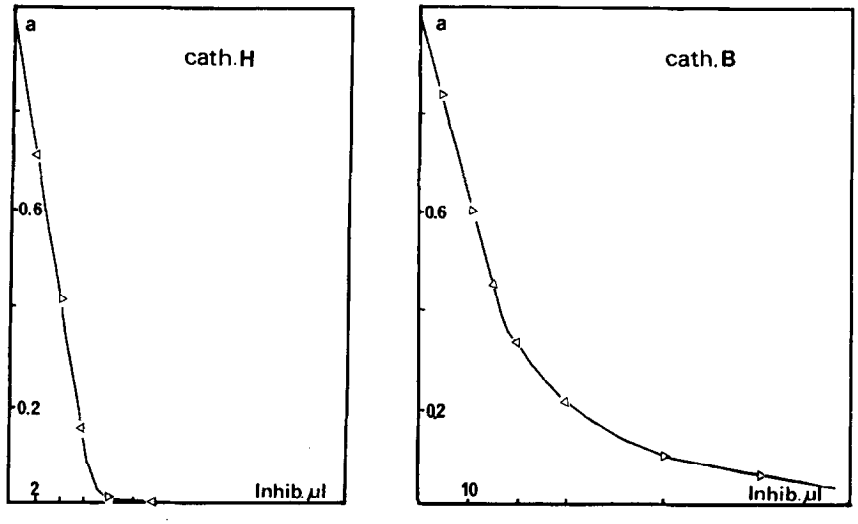

Fig. 2 : Residual fractional activity ($a = \frac{E}{Eo}$) of cathepsin H (0.086 µM) and cathepsin B (0.23 µM) on Arg-NNap and Bz-Arg-NNap respectively upon addition of increasing volumes of inhibitor. Molar concentration of active inhibitor may be calculated from the curve on the left which is linear up to almost zero activity.

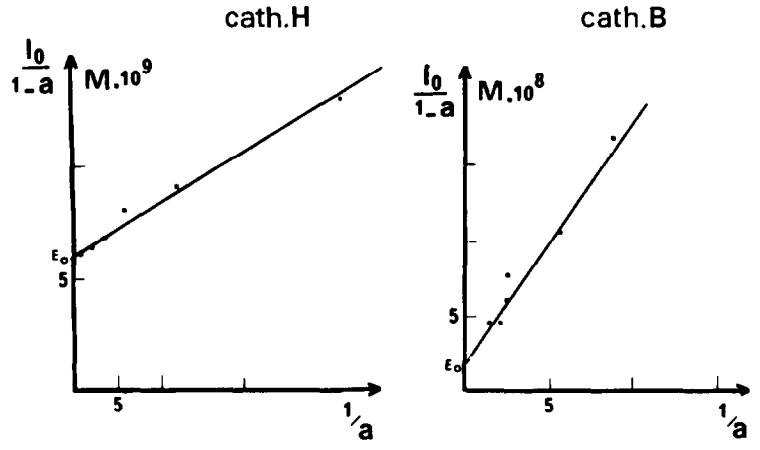

Fig. 3 : Determination of Ki in accordance with equation [1]. The residual fractional activity $a = \frac{E}{E_0}$ of cathepsin H (5.8×10^{-9} M) and cathepsin B (1.58×10^{-8} M) on Arg-NNap and Z-Arg-Arg-NNap respectively was first calculated for increasing-concentrations of inhibitor as described under " Material and Methods".The slope of the straight lines obtained corresponds to Ki or Ki app.

When cathepsin B is assayed under almost similar conditions (Eo = 0.23 μM) the last portion of the curve is not linear (fig. 2). Therefore, the equivalence point cannot be precisely determined under these conditions. This result however gives evidence for a reversibility of the reaction since it has been found not to be time dependent (15).Moreover it presupposes a weaker inhibition of cathepsin B by the serum inhibitor as compared to cathepsin H. This was confirmed by the calculation of Ki values using the Easson and Stedman plot. When working at enzyme concentrations low enough to be in the same order of magnitude than that of Ki a reversibility of the proteinase-inhibitor association was observed, which permits the calculation of Ki for both proteinases (16) using the following equation :

$$\frac{Io}{1 - a} = \frac{Ki}{a} + Eo \qquad [1]$$

Where a is the fractional enzyme activity, Io and Eo the initial inhibitor and enzyme concentration respectively. When plotting $\frac{Io}{1-a}$ vs $\frac{1}{a}$ the slope of the straight line obtained corresponds to Ki (fig. 3), provided no dissociation of the enzyme inhibitor complex occurs upon substrate addition.

This is simply verified by working at two widely different substrate concentrations.

No significant substrate dependent dissociation was found working with cathepsin H on Arg-NNap but this phenomenon was observed with cathepsin B

reacting with Z-Arg-Arg-NNap. In this case, the slope corresponds to Ki (app) which is related to Ki as follows (16):

$$Ki\ (app) = Ki\ (1 + \frac{S}{Km}) \qquad [2]$$

Using a Km value of 0.19 mM (17) Ki may be determined according to equation [2].

The reaction between human liver cathepsin B and the low Mr serum inhibitor was characterized by $Ki = 1.6 \times 10^{-8}$ M whereas $Ki = 2.2 \times 10^{-10}$M was found for the reaction between this inhibitor and human liver cathepsin H.

After incubation of the inhibitor for 10 min. at 90°C no significant change of activity towards cathepsin B or H was recorded demonstrating the heat stability of the molecule. On the other hand a molecular weight of 10,500 ± 1,000 was estimated from the G 50 gel filtration analysis.

Both of these properties as well as the strong inhibition exhibited by this serum inhibitor towards cathepsin H raise the question of a possible relationship with tissue cysteine proteinase-inhibitors.

As recently described, (18, 19) potent inhibitors of cathepsin H have been purified from rat lung and rat liver cytosol. This common feature appears all the more significant as no potential inhibitor of cathepsin H but iodoacetic or iodoacetamide had been previously demonstrated to give a total inhibition of this enzyme (9). However attempts to demonstrate the presence in serum of low Mr cysteine proteinase inhibitors remained unsuccessful(20).

Given its strong cathepsin B and H inhibiting properties the inhibitor described in the present study differs significantly from the αTPI of high Mr previously described in human serum which preferentially inhibits heterologous cysteine proteinases such as papain. Recently, Iwata et al., (21) have demonstrated two varieties of cysteine proteinase-inhibitors in rat serum which are probably related to those found in human given their enzyme inhibitory properties.

The low Ki values found for the interaction between the human low Mr cysteine proteinase-inhibitor and human cathepsins B and H demonstrate the potential physiological activity of the inhibitor in serum in spite of its rather low concentration since Ki values are several order of magnitude lower than its expected concentration. However a more probable extraplasmatic or intracellular function has to be considered given its apparent restricted specificity for cysteine proteinases. Whether or not this inhibitor is present outside the circulation in sufficient amount to have a physiological efficiency remains to be elucidated but its low Mr would support this hypothesis all the more than plasma proteinase inhibitors with higher Mr have been previously located in the extravascular space or even inside the cell (22).

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