Biochem J. (1980) **191**, 487–497
Printed in Great Britain

Human cathepsin H

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(*Received 31 March 1980/Accepted 25 June 1980*)

Cathepsin H was purified from human liver by a method involving autolysis and acetone fractionation, and chromatography on DEAE-cellulose, Ultrogel AcA 54, hydroxyapatite and concanavalin A–Sepharose. The procedure allowed for the simultaneous isolation of cathepsin B and cathepsin D. Cathepsin H was shown to consist of a single polypeptide chain of 28000 mol.wt., and affinity for concanavalin A–Sepharose indicated that it was a glycoprotein. The enzyme existed in multiple isoelectric forms, the two major forms having pI values of 6.0 and 6.4; it hydrolysed azocasein (pH optimum 5.5), benzoylarginine 2-naphthylamide (Bz-Arg-NNap), leucyl 2-naphthylamide (Leu-NNap), arginine 4-methyl-7-coumarylamide (Arg-NMec) and arginine 2-naphthylamide (Arg-NNap), (pH optimum 6.8). Arg-NNap and Arg-NMec, unlike Bz-Arg-NNap, were not hydrolysed by human cathepsin B. Cathepsin H was similar to cathepsin B in being irreversibly inactivated by exposure to alkaline pH. Sensitivity to chemical inhibitors was generally similar to that of other cysteine proteinases, but the enzyme was unaffected by 1 μM-leupeptin, which gave essentially complete inhibition of the other lysosomal cysteine proteinases, cathepsins B and L.

The proteinase activity associated with mammalian lysosomes has been largely attributed to cathepsin B and cathepsin D, a cysteine proteinase and an aspartic proteinase respectively, in the terminology of Barrett (1980*a*). More recently, two additional cysteine proteinases, with molecular weights similar to that of cathepsin B (25000), were isolated from lysosomes of rat liver, and called cathepsin H and cathepsin L (Kirschke *et al.*, 1976*a*). Cathepsin H was distinct in hyrolysing both blocked and unblocked amino acid naphthylamides.

The purpose of the present paper is to report the purification of cathepsin H from human liver and the properties of the purified enzyme. The purification procedure allows for the simultaneous isolation of both cathepsin B and cathepsin D from the same tissue sample. The properties of human cathepsin H are discussed in conjunction with reports of similar activities from other mammalian sources, and it is suggested that these represent forms of the same enzyme in different species.

Abbreviations used: Bz, α-*N*-benzoyl; Z, α-*N*-benzyloxycarbonyl; NNap, 2-naphthylamide; NNapOMe, 2-(4-methoxy)naphthylamide; $NPhNO_2$, 4-nitroanilide; NMec, 4-methyl-7-coumarylamide; Tos, tosyl; $-CH_2Cl$, -chloromethane; $-CHN_2$, -diazomethane; SDS, sodium dodecyl sulphate.

Materials and general methods

Arg-NNap·HCl, Leu-NNap·HCl, α-methyl-D-mannoside (grade III), cytochrome *c* (horse heart, type IIa), carbonic anhydrase (bovine erythrocyte), albumin (bovine serum, crystallized), phosphorylase *a* (rabbit muscle, twice-crystallized), ovalbumin (crystallized, grade VI), α-chymotrypsinogen *a* (bovine pancreas, six-times-crystallized, type II), transferrin (human), puromycin dihydrochloride, soya-bean trypsin inhibitor (type II-S), phenylmethanesulphonyl fluoride, iodoacetamide, Tos-Phe-CH_2Cl, and Coomassie Brilliant Blue were purchased from Sigma (London) Chemical Co. Ltd., Kingston upon Thames, Surrey KT2 7BH, U.K. Oxidized B-chain of bovine insulin was from Schwarz/Mann, Orangeburg, NY, U.S.A. Bz-DL-Arg-NNap·HCl, Arg-NNapOMe·HCl, Bz-DL-Arg-$NPhNO_2$·HCl, Arg-NMec and Tos-Lys-CH_2Cl were purchased from Bachem, CH-4416 Bubendorf, Switzerland. Iodoacetic acid (specially purified for biochemical work), Brij 35 (polyoxyethylene dodecyl ether), and hydroxyapatite suspension were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. *N*-Ethylmaleimide (Gold Label) and 4-aminophenylmercuric acetate were from Aldrich Chemical Co. Ltd., Gillingham, Dorset SP8 4BR, U.K. The microbial inhibitors, pepstatin A, leupeptin and

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chymostatin B (Umezawa & Aoyagi, 1977), were supplied by the Peptide Research Foundation, 476 Ina, Minoh-shi, Osaka 562, Japan. Ampholines and Ultrogel AcA 54 were purchased from LKB Instruments Ltd., South Croydon, Surrey CR2 9PX, U.K., and concanavalin A–Sepharose was from Pharmacia (G.B.) Ltd., London W5 5SS, U.K.

DEAE-cellulose (DE-52) was supplied by Reeve Angel Scientific Ltd., London EC4V 6AY, U.K. Hyamine 1622 [benzethonium chloride, *NN*-dimethyl-*N*-(2-{2-[4-(1,1,3,3-tetramethylbutyl)-phenoxy]ethoxy}ethyl)benzenemethanaminium chloride] was a product of Lennig Chemicals Ltd., Croydon CR9 3NB, Surrey, U.K.

Arquad 2C-50 [a 50% (w/v) solution of crude didodecylammonium chloride also containing analogues of longer and shorter chain length, in aq. 50% (v/v) propan-2-ol] was obtained from Akzo Chemie U.K. Ltd., London SW18 2LS, U.K., and is also available from Armak Co., P.O. Box 1805, Chicago, IL 60690, U.S.A. When required for use, the Arquad 2C-50 was diluted with 4 vol. of water and warmed until the mixture became clear; this was used while still clear at less than 45°C.

Bovine haemoglobin was prepared as described by Barrett (1970). Azocasein was prepared by treating casein with diazotized sodium sulphanilate, the method being otherwise as described by Charney & Tomarelli (1947); the material had an $A^{1\%}_{1cm}$ at 366nm of 24.0. α_2-Macroglobulin was purified from human plasma by the method of Barrett *et al.* (1979).

Organomercurial Sepharose was prepared by coupling 4-aminophenylmercuric acetate to Sepharose 4B as described by Barrett (1973), except that CNBr activation of the Sepharose was done in 5M-phosphate buffer as recommended by Porath *et al.* (1973). The gel was washed also with 1% glycine just before the pretreatment with cysteine. The capacity of the newly prepared adsorbent was determined, as described by Barrett (1973), to be 2.9μmol/g wet weight.

Sodium tetrathionate ($Na_2S_4O_6$) was prepared in our laboratory by Dr. David Sunter as described by Liu & Inglis (1972), and recrystallized twice from water/ethanol Z-Arg-Arg-NNap·2HCl was supplied by Dr. C. G. Knight of this laboratory, having been synthesized by an original method (Knight, 1980). Immunoglobin G was purified from sheep serum by Dr. M. E. Davies of this laboratory, as described previously (Davies *et al.*, 1978).

Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 were kindly given by Dr. E. N. Shaw, Brookhaven National Laboratory, Upton, NY, U.S.A. Leu-CH_2Cl was kindly given by Dr. H. Kirschke, Physiologisch-Chemisches Institut der Martin-Luther Universität Halle-Wittenberg, DDR-402 Halle (Saale), German Democratic Republic. Aprotinin (as Trasylol) was the gift of Dr. E. Philipp, Bayer A. G., D-5600 Wuppertal 7, Federal Republic of Germany.

Samples of post-mortem human liver, free from disease, were kindly contributed by Dr. P. Stovin, Papworth Hospital, Cambridge, U.K.; they were stored at −20°C until required for use.

Assays of cathepsin H and cathepsin B

The method used with the naphthylamide substrates was that described by Barrett (1972, 1976) for cathepsin B, in which 2-naphthylamine is determined colorimetrically after diazo-coupling with Fast Garnet GBC (diazotized *o*-amino-azotoluene). For cathepsin H the buffer composition was modified to 37.5mM-K_2HPO_4/37.5mM-KH_2PO_4, to give pH 6.8 instead of 6.0; the cysteine concentration was raised from 2 to 3mM and that of EDTA was maintained at 1mM. Routine assays were with 1mM-Arg-NNap for cathepsin H and 0.2mM-Z-Arg-Arg-NNap for cathepsin B. All assays were for 10min at 40°C after 5min pre-incubation without substrate. One unit of activity released 1μmol of 2-naphthylamine/min.

Assay of proteolytic activity

Cathepsin D was assayed with 2% (w/v) haemoglobin at pH 3.5 (Barrett, 1970). The proteolytic activities of cathepsins B and H were measured with azocasein as substrate. The enzyme (0.125ml) was preincubated with 0.25ml of incubation buffer for 5min at 40°C, and then 0.125ml of 6% (w/v) azocasein was added and the mixture incubated for 10min. The incubation buffer was 0.15M-potassium phosphate or sodium acetate buffer of appropriate pH, with 2mM-EDTA, and 4mM- or 6mM-cysteine for cathepsin B or cathepsin H respectively. The assay was stopped by addition of 2.5ml of 3% (w/v) trichloroacetic acid. The mixture was filtered and the A_{366} of the soluble reaction products measured. Blanks were prepared by the addition of the enzyme sample only after the reaction had been stopped with trichloroacetic acid. One unit of activity represents the hydrolysis of 1μg of azocasein/min at 40°C.

Determination of protein

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and, during the course of enzyme purifications, by measurement of A_{280}, for which $A^{1\%}_{1cm}$ was assumed to be 10.0.

SDS/polyacrylamide-gel electrophoresis

This was done as described by Barrett *et al.* (1979), by using a modification (Bury & Barrett,

1980) of the 2-amino-2-methylpropan-1,3-diol (Ammediol)/glycine/HCl system of Wykoff *et al.* (1977). Separating gels of 12.5% total acrylamide concentration (2.6% of this as methylenebisacrylamide) were used.

Analytical isoelectric focusing

The method was that of Barrett (1970), and 1% (w/v) carrier ampholines of pH range 3.5–10 were used. Gels were stained for cathepsin H activity by the method described by Barrett (1973) for cathepsin B, except that the incubation buffer was modified as described above for the quantitative assay, and Arg-NNapOMe was used as substrate. Some gels were bisected longitudinally, and one half used to detect enzymic activity, and the other half cut into 5mm segments, which were eluted overnight in 0.5ml of water for the measurement of pH.

Molecular-weight determination by gel chromatography

A column of Ultrogel AcA 54 (87cm × 1.6cm; 174cm^3), equilibrated with 20mM-KH_2PO_4/Na_2HPO_4 buffer, pH6.0, containing 0.20M-NaCl and 1mM-disodium EDTA, was used at a flow rate of 4ml/h per cm^2. A sample of purified cathepsin H (0.1 unit) was applied in 3.5ml of the column buffer, which also contained 2% (w/v) sucrose and 16mg of each of the column-calibration proteins: ovalbumin (45000 mol.wt.), α-chymotrypsinogen *a* (25000) and cytochrome *c* (13000).

Determination of kinetic parameters

Reaction rates were measured in triplicate for at least five substrate concentrations and analysed by the method of Wilkinson (1961) to determine K_m and V, and then $k_{cat.}$ (i.e. V/e; the molarity of enzyme, e, being calculated from protein concentration and the mol.wt. of 28000). The range of s/K_m was at least 0.2–2.0 for each of the substrates tested.

In inhibition experiments, values for K_i (app.) were determined from dose–response curves (v_o/v_i versus i) as described by Laidler (1958), *i* being the molar concentration of inhibitor.

Testing of potential inhibitors

This was done essentially as described by Barrett (1973) for human cathepsin B. A stock solution of purified cathepsin H (67milliunits/ml) was adjusted to 1mM-EDTA and pre-activated with 3mM-cysteine for 5min at 22°C. This was then diluted 1:3 (v/v) with 0.01% bovine serum albumin solution through which O_2-free N_2 had been bubbled for 30min. Portions (0.5ml) of the diluted enzyme solution were each mixed with 1.5ml of 0.1M-potassium phosphate buffer, pH6.8, containing a potential inhibitor, and incubated for 10min at 22°C. Mixtures were then preincubated for 5min at 40°C and assayed for 10min with Arg-NNap as described above. Reagent blanks were also run for each potential inhibitor. The percentage inhibition was determined by comparing the activities with those measured for positive controls that contained no inhibitor in the pH6.8 buffer. Of the compounds used, only Arquad 2C-50 interfered with the colour yield in the reaction of 2-naphthylamine with Fast Garnet GBC under the conditions used. In this case an additional set of positive controls was included in which the appropriate concentration of Arquad 2C-50 was introduced immediately before the Fast Garnet GBC coupling reagent was added.

Specific methods and results

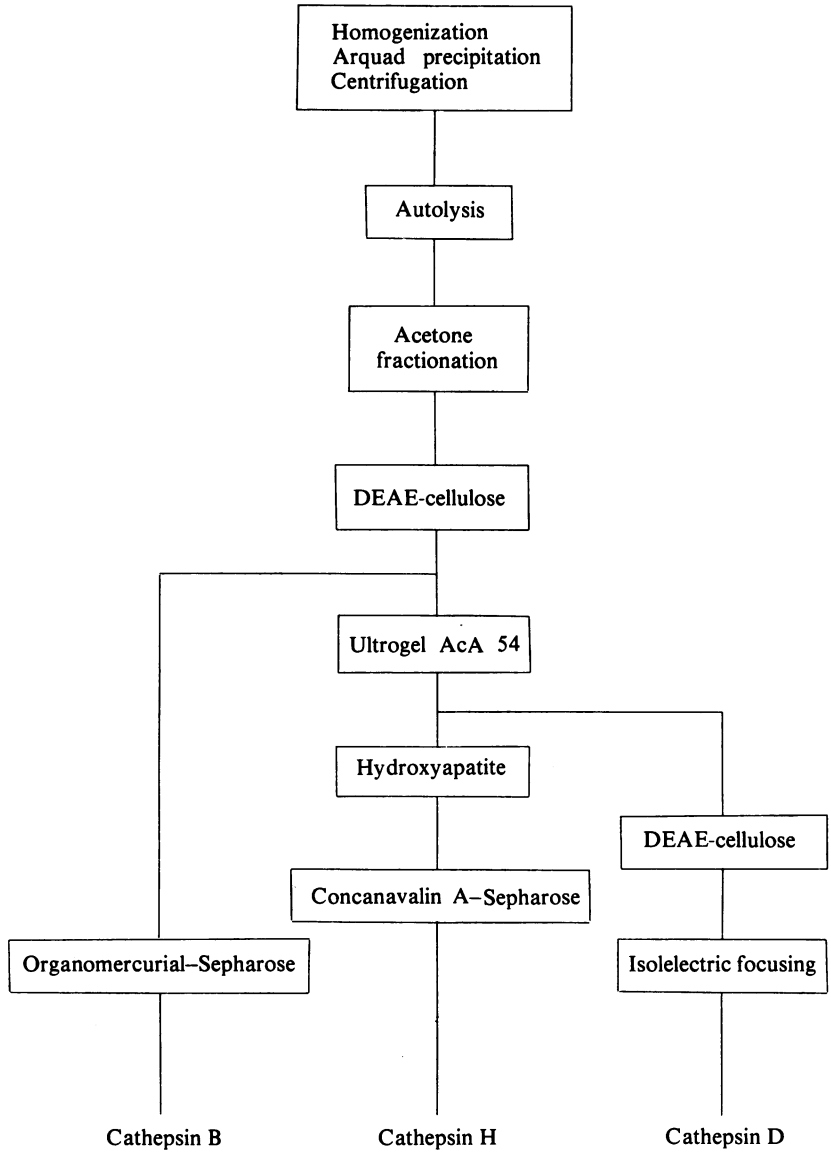
Purification of cathepsin H

The initial stages of the purification were similar to those used previously for the isolation of human cathepsins B and D (Barrett, 1973). Cathepsin H was separated from cathepsin B and cathepsin D in subsequent stages, and finally isolated in highly purified form. The purification procedure is summarized in the form of a flow diagram in Scheme 1. All operations, unless specified otherwise, were performed at 4°C.

Extraction, autolysis and acetone fractionation

Human liver, having been thawed at 4°C overnight, was trimmed of fat and connective tissue and cut into small pieces (about 1cm^3). Four 250g portions were each dispersed in 500ml of a solution of 1% (w/v) NaCl, 2% (v/v) butan-1-ol, and 10mM-disodium EDTA, at 4°C, by use of an MSE Atomix blender for 2.5min. The homogenate was treated with Arquad 2C-50 and centrifuged to remove particulate matter, subjected to autolysis overnight at pH4.5 and 40°C, and then fractionated with acetone, all as described by Barrett (1973). The 47–64% acetone precipitate was collected by centrifugation at 1500***g*** for 15min at −5°C in the MSE Mistral 6L centrifuge. This was redissolved in 5mM-disodium EDTA to a total volume of 50ml and dialysed overnight against 5 litres of water at 4°C.

In these initial stages of purification there was a reproducible increase in total activity of cathepsin H (Table 1), just as was found previously for cathepsin B (Barrett, 1973). This was attributed to the presence in the homogenate of one or more inhibitors that were eliminated by autolysis and acetone fractionation. The low apparent recovery of cathepsin H activity in the Arquad extract was explained by additional inhibition of the enzyme by the cationic detergent (see Table 2), and also interference with the diazo coupling of reaction product in the assay (see the Materials and general methods section).



Scheme 1. *Flow diagram summarizing the procedure for purification of cathepsin H from human liver*
Experimental details are given in the text and quantitative results are presented in Table 1.

Table 1. *Purification of human cathepsin H*
The yields of protein and enzymic activity are from 1 kg of human liver. The values for protein in the first three stages were determined by the method of Lowry *et al.* (1951).

	Protein (g)	Activity (units)	Specific activity (units/ml per A_{280} unit)	Yield (%)
Homogenate	183	95.6	0.0005	(100)
Arquad supernatant	72	22.4	0.0003	23
Autolysed extract	41	93.7	0.0023	98
Acetone (47–64%) fraction	1.37	98.6	0.073	103
DEAE-cellulose	0.70	67.0	0.096	70
Ultrogel AcA 54	0.033	40.2	1.23	42
Hydroxyapatite	0.012	24.4	2.01	26
Concanavalin A–Sepharose	0.006	17.8	2.90	19

DEAE-cellulose chromatography

The dialysed product of acetone fractionation (about 150ml) was adjusted to pH6.0 by dropwise addition of 2M-Tris/HCl buffer, pH9.0, and applied, at a flow rate of 10ml/h per cm^2, to a column (14cm x 2.6cm, 70cm^3) of DEAE-cellulose, equilibrated with 20mM-sodium phosphate buffer, pH6.0, containing 1% butan-1-ol. The ion-exchanger was then washed with the 20mM-buffer (about 1 bed volume) until the A_{280} fell to the starting value. Assays of individual column fractions indicated that cathepsin H (with cathepsin D) was not adsorbed under these conditions, and all fractions comprising the A_{280} peak were combined.

The DEAE-cellulose column provided a rapid and efficient method for removing nearly half of the contaminating protein (Table 1) and separating cathepsin H from cathepsin B, which was adsorbed to the column. A little cathepsin B sometimes contaminated the cathepsin H pool, but was removed during subsequent hydroxyapatite chromatography (see below). Cathepsin B was obtained in good yield and free from activity towards Arg-NNap by stepwise elution with 0.2M-NaCl in the 20mM-sodium phosphate buffer, and retained for further purification.

Gel chromatography on Ultrogel AcA 54

The non-adsorbed pool from DEAE-cellulose was concentrated to 7–8ml under pressure of N_2 by use of an Amicon Diaflo apparatus fitted with a Sartorious 12-136 membrane, and then made up to 9ml by adding 0.9ml of a 10-fold stock solution of the column buffer (see below) and water. The concentrated sample was run on a column (92.6cm x 2.5cm, 455cm^3) of Ultrogel AcA 54 in 20mM-KH_2PO_4/Na_2HPO_4 buffer, pH6.0, containing 0.20M-NaCl, 1mM-disodium EDTA and 0.1% Brij 35. The column was run at 4ml/h per cm^2, and a typical elution profile is shown in Fig. 1. Cathepsin H was eluted after the major protein peaks, typically with a 10–15-fold increase in specific activity (Table 1). The separation, based on molecular size, allowed cathepsin H (28000 mol.wt.) to be completely resolved from cathepsin D (45000 mol.wt.), which was retained as a by-product for further purification.

Hydroxyapatite chromatography

The pool of cathepsin H obtained by gel filtration was dialysed overnight against 2 litres of 20mM-KH_2PO_4/Na_2HPO_4 buffer, pH6.0, and then applied to a column (21cm x 1.1cm, 20cm^3) of hydroxyapatite equilibrated with the same buffer. The column was eluted with a gradient (400ml) increasing to 150mM-KH_2PO_4/Na_2HPO_4, pH6.0, at a flow rate of 7ml/h per cm^2. Cathepsin H was eluted within a portion of the gradient corresponding to 60–85mM-phosphate (Fig. 2). Analysis of the enzyme pool by SDS/polyacylamide-gel electrophoresis revealed two major polypeptide bands, one of which corresponded to cathepsin H (Fig. 3).

Chromatography on concanavalin A–Sepharose

The pool of activity from hydroxyapatite was adjusted to 0.2M-NaCl, 1mM-$CaCl_2$, 1mM-$MnCl_2$

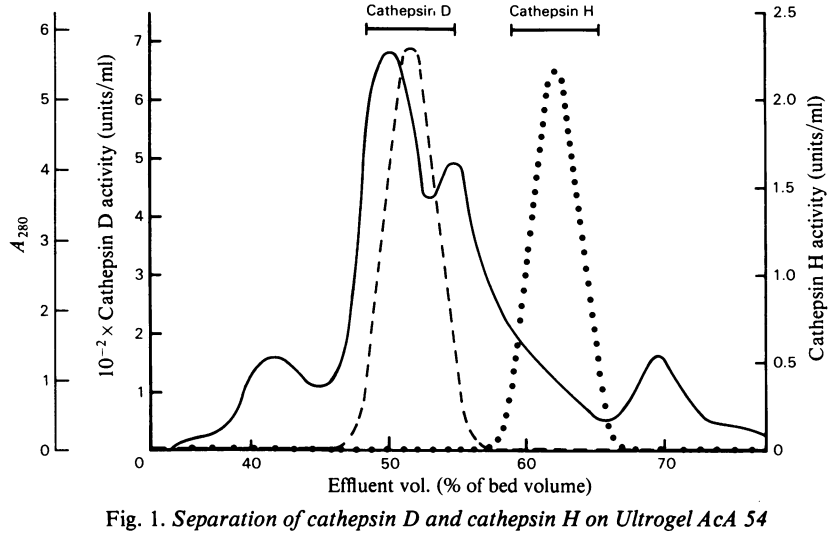


Fig. 1. *Separation of cathepsin D and cathepsin H on Ultrogel AcA 54*

Distribution of cathepsin D (----) and cathepsin H (····) activities, and protein measured as A_{280} (——), in effluent from a column of Ultrogel AcA 54. The fractions combined for further purification are indicated by the horizontal bars.

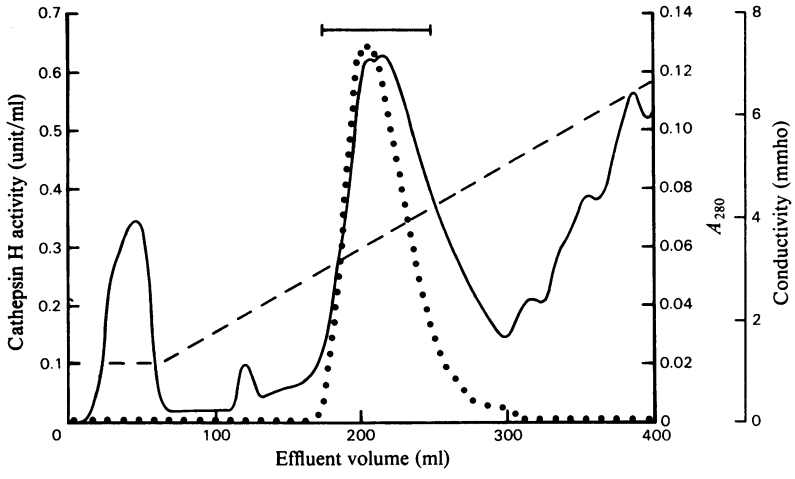


Fig. 2. *Chromatography of partially purified cathepsin H on hydroxyapatite*
Distribution of cathepsin H activity (····) and protein measured as A_{280} (——) in effluent from a column of hydroxyapatite, eluted with a gradient of 20–150 mM-KH_2PO_4/Na_2HPO_4 as described in the text. The conductivity (– – – –) of the effluent is shown. The fractions combined for further purification are indicated by the horizontal bar.

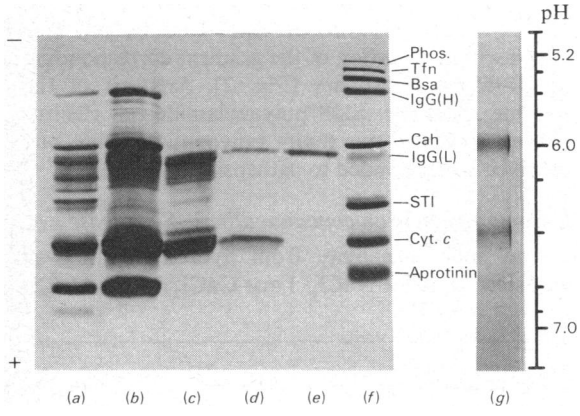


Fig. 3. *SDS/polyacrylamide-gel electrophoresis at various stages in the purification of cathepsin H from human liver, and isoelectric focusing of the pure enzyme*
The samples, run after reduction with 2-mercaptoethanol, were taken after (*a*) acetone fractionation, (*b*) DEAE-cellulose chromatography, (*c*) Ultrogel AcA 54 chromatography. (*d*) hydroxyapatite chromatography, and (*e*) concanavalin A–Sepharose chromatography. A mixture of standard proteins was also run, after reduction, in lane (*f*); the bands correspond to phosphorylase *a* (100000 mol.wt.) (phos.), transferrin (78000) (Tfn), bovine serum albumin (68000) (Bsa), immunoglobulin G heavy chain (50000) [IgG(H)], carbonic anhydrase (29000) (Cah), immunoglobulin G light chain (25000) [IgG(L)], soya-bean trypsin inhibitor (21000) (STI), cytochrome *c* (12750) (Cyt. *c*) and aprotinin (6500). The isoelectric-focusing gel (*g*) was stained for enzymic activity with Arg-NNapOMe as substrate, and the pH values determined as described in the Methods section.

and 0.1% Brij 35, and run at 6 ml/h per cm^2 on a column (6.4 cm × 1 cm, 5 cm^3) of concanavalin A–Sepharose, equilibrated with 20 mM-KH_2PO_4/Na_2HPO_4 buffer, pH 6.0, containing 0.2 M-NaCl, 1 mM-$CaCl_2$, 1 mM-$MnCl_2$ and 0.1% Brij 35. The column was washed with 3–4 bed volumes of starting buffer, and the enzymic activity was eluted as a broad peak with 50 mM-α-methyl D-mannoside in the same buffer (Fig. 4). Analysis of column fractions by SDS/polyacrylamide-gel electrophoresis revealed that the major inactive protein contained in the sample was not adsorbed to the column under starting conditions, and thus was efficiently separated from cathepsin H (Fig. 3). Occasionally samples from hydroxyapatite contained minor components in addition to the two referred to here; for these it was necessary to elute the concanavalin A–Sepharose column with a gradient (20 bed volumes) of 0–100 mM α-methyl D-mannoside. In either case, the fractions containing activity were combined to form the pool of purified enzyme used for all subsequent studies.

Storage

The purified enzyme was stored at 4°C or −20°C in 50–100 mM-sodium acetate buffer, pH 5.5, containing 1 mM-disodium EDTA, 0.1% Brij 35 and 5 mM-sodium tetrathionate. Under these conditions, there was 90–100% retention of activity after 1 month, but recoveries fell as low as 50% if the pH were as high as 6.0, or the tetrathionate were omitted.

Purification of cathepsin B and cathepsin D

The pool of cathepsin B eluted stepwise from

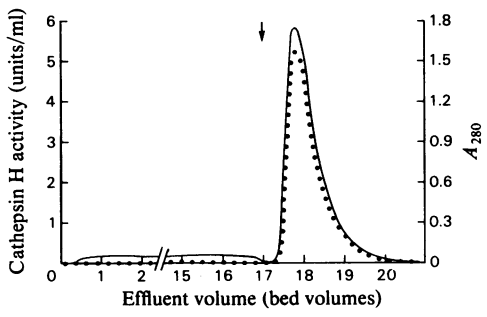


Fig. 4. *Chromatography of partially purified cathepsin H on concanavalin A–Sepharose*
Distribution of cathepsin H activity (····) and protein measured as A_{280} (————) in effluent from a column of concanavalin A–Sepharose. After the non-adsorbed protein had been eluted under starting conditions, cathepsin H was displaced by 50mM-α-methyl D-mannoside (indicated by the arrow).

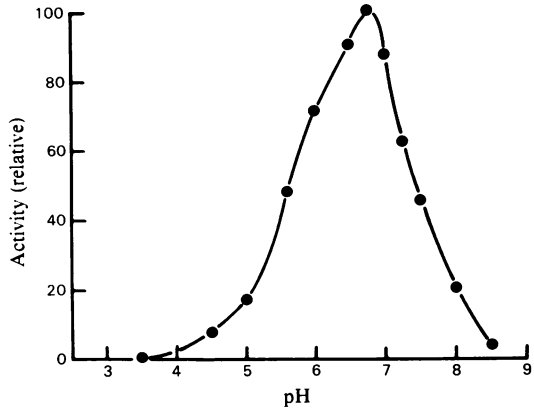


Fig. 5. *pH-dependence of cathepsin H activity*
Activity was determined against 1mM-Arg-NMec, with sodium formate, sodium acetate, sodium phosphate, and Tris/HCl buffers, as described in the text.

DEAE-cellulose was further purified by affinity chromatography on a column (9.4cm × 2.6cm, 50cm³) of organomercurial–Sepharose, equilibrated with 50mM-sodium acetate buffer, pH5.5, containing 0.2M-NaCl, 1mM-disodium EDTA and 1% butan-1-ol. The enzyme pool was first mixed with 0.25 vol. of a 5-fold concentrated solution of the column buffer, and then applied to the column at a flow rate of 20ml/h per cm². The column was eluted with the buffer until the A_{280} fell close to the starting value, and then the enzymic activity was eluted as a sharp peak with either 10mM-2-mercaptoethanol or 10mM-sodium tetrathionate in the column buffer. Specific activities of the purified product were within the range previously determined for pure human cathepsin B (Barrett, 1973).

Cathepsin D, having been separated from cathepsin H on Ultrogel AcA 54, was further purified on DEAE-cellulose and the major isoenzymes isolated by preparative isoelectric focusing as described by Barrett (1970).

Some properties of purified cathepsin H

Specific activity and absorption coefficient. The activity of purified human cathepsin H with Arg-NNap as substrate was 2.9units/ml per A_{280} unit. When protein was determined by the method of Lowry *et al.* (1951), the $A^{1\%}_{280,\,1cm}$ was found to be 12.2, and the specific activity was therefore 3.5units/mg.

Stability to pH and temperature. The effect of pH on the enzyme was demonstrated as follows. Cathepsin H (20munits in 25μl of 1.2mM-sodium phosphate buffer, pH6.0) was mixed with 25μl of an appropriate 0.10M-buffer and left for 60min at 22°C, and then assayed at pH6.8. Sodium formate (pH3.5–4.5), sodium acetate (pH4.5–5.6), sodium phosphate (pH5.6–7.5) and Tris/HCl (pH7.5–8.5) buffers were used. It was found that the enzymic activity was not affected by the exposure to acid pH, but was unstable at pH values above 7.0. Only 20 and 10% of the initial activity remained after exposure to pH7.5 and 8.5 respectively.

The heat stability of pure human cathepsin H was tested by incubating the enzyme, at 0.027 and 0.27mg/ml, in 20mM-sodium phosphate buffer, pH6.5, for 30min at temperatures in the range 30–60°C. The samples were assayed as usual at 40°C. At both concentrations the enzyme lost 10–15% of its activity during exposure to 50°C and more than 80% at 60°C.

pH-dependence of activity. When human cathepsin H was assayed with Arg-NNap (1mM, in the series of buffers used to determine pH stability), the pH optimum was found to be 6.8 (Fig. 5). A sharper decline in activity was measured at alkaline pH than at acid pH, and this was attributed to irreversible inactivation of the enzyme above neutral pH (see above).

The enzyme was also assayed under the same conditions with the protein substrate azocasein. Optimal activity (± 20%) was measured in the range pH5–6, and the activity at pH6.8 was 50% of the maximum.

Substrate specificity. Human cathepsin H hydrolysed the naphthylamide substrates Arg-NNap and Leu-NNap, and also the *N*-substituted derivative Bz-DL-Arg-NNap (Table 2), thus exhibiting activities characteristic of both an aminopeptidase and an endopeptidase. The specific activities of cathepsin H and cathepsin B on various naphthylamide sub-

strates are compared in Table 2. Little or no hydrolysis of Bz-DL-Arg-NPhNO$_2$ was detected, but this compound inhibited the hydrolysis of Arg-NNap with an apparent K_i of 3.2×10^{-4}M. Kinetic parameters were measured as described in the Methods section, and values obtained for the hydrolysis of Arg-NNap and Bz-Arg-NNap by human cathepsin H are given in Table 3. The value of $k_{cat.}/K_m$ obtained for Bz-Arg-NNap is close to that reported by Knight (1980) for human cathepsin B.

The kinetics of hydrolysis of Arg-NMec by human cathepsin H were also studied, the reaction being monitored fluorimetrically (Barrett, 1980*b*) (Table 3). The fluorimetric substrate had similar kinetic properties to the naphthyl amide, but provides an assay that is 75-fold more sensitive and avoids the use of the potentially carcinogenic naphthylamide substrate. It has been suggested that Arg-NMec be used in routine assays for cathepsin H, and Z-Phe-Arg-NMec for cathepsin B (Barrett, 1980*b*).

The proteolytic activity of human cathepsin H was assessed by using azocasein as substrate (see the Methods section) and the specific activity was found to be 15.6 units/mg at optimal pH (5.5). For comparison, the specific activity of human cathepsin B was measured at its optimal pH (6.0) and found to be 269 units/mg.

Behaviour on organomercurial–Sepharose. Organomercurial–Sepharose, which had been shown to be a very useful affinity adsorbent for human cathepsin B (Barrett, 1973), was unsuitable for use in the purification of cathepsin H. About 75% of the cathepsin H in the Ultrogel AcA 54 pool (see Table 1) was not adsorbed, even if first exposed to reducing conditions. In contrast, all of the cathepsin B that had been eluted stepwise from DEAE-cellulose (see above) was adsorbed. It is not clear why cathepsin H has a low affinity for the organomercurial adsorbent, but one possibility is that binding of the aminophenylmercury ligand to the essential thiol group is sterically hindered; it should be noted that the ligand was coupled directly to the Sepharose without a 'spacer'.

Molecular weight. The molecular weight of cathepsin H was determined by gel chromatography on a calibrated column of Ultrogel AcA 54 as described in the Methods section. The elution volume of cathepsin H activity corresponded to a mol.wt. of 28000.

Purified cathepsin H was run in polyacrylamide slab gels in the presence of SDS. A single polypeptide band was detected with a mobility corresponding to a mol.wt. of 28000 (Fig. 3). The same result was obtained whether the sample was first reduced by exposure to 2-mercaptoethanol or run un-reduced after treatment with iodoacetate.

Gel electrophoresis of the native enzyme. Purified cathepsin H was subjected to disc electrophoresis under acid conditions (Reisfeld *et al.*, 1962), and one protein band of low mobility was identified. This showed activity against Arg-NNapOMe, a substrate for cathepsin H (see the Materials and general methods section).

Isoelectric focusing. Purified cathepsin H was run in isoelectric-focusing gels, which were then stained for enzymic activity as described in the Methods section. Two major forms of the enzyme were identified with pI values of 6.0 and 6.4 (Fig. 3).

Determination of the total cathepsin H activity in gels homogenized after focusing revealed that only 20% of the activity was recovered, but consistency in results obtained with many samples suggested that the method gave a reliable analysis of the multiple forms of cathepsin H. Very similar findings were reported for cathepsin B by Barrett (1973).

Inhibitors. Potential inhibitors were tested as described in the Methods section, and the results are presented in Table 4.

Table 2. *Specific activities of human cathepsins B and H on some naphthylamide substrates*

Activities were measured at pH 6.8 for cathepsin H and at pH 6.0 for cathepsin B as described in the Methods section (1 unit releases 1 μmol of 2-naphthylamine/min at 40°C). Protein was measured by the method of Lowry *et al.* (1951).

	Specific activity (units/mg of protein)	
Substrate	Cathepsin H	Cathepsin B
Arg-NNap	3.54	0.00
Leu-NNap	1.95	0.00
Bz-DL-Arg-NNap	1.73	5.4
Z-Arg-Arg-NNap	0.15	154.3

Table 3. *Kinetic constants of some substrates of cathepsin H*

The values given were measured as described in the Methods section. The values for Bz-Arg-NNap were calculated for the L-isomer from data obtained with the racemate.

Substrate	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($s^{-1} \cdot M^{-1}$)
Arg-NNap	1.81	0.097	1.87×10^4
Bz-Arg-NNap	1.82	0.637	2.86×10^3
Arg-NMec	2.535	0.150	1.69×10^4

Table 4. *Effect of potential inhibitors on human cathepsin H*

The enzyme (8.3 munits in 2.0 ml) was preincubated with the compound at pH 6.8 for 10 min at 22°C and then for an additional 5 min at 40°C, before the 10 min incubation with Arg-NNap. The values are means of at least two determinations. See the Materials and general methods section for details.

Compound	Final concn. (mM)	Inhibition (%)
Iodoacetic acid	1.0	99
Iodoacetamide	1.0	99
N-Ethylmaleimide	1.0	69
4-Aminophenylmercuric acetate	1.0	93
Phenylmethanesulphonyl fluoride	1.0	3
Tos-Phe-CH_2Cl	0.01	79
Tos-Lys-CH_2Cl	0.01	60
Leu-CH_2Cl	0.001	85
Chymostatin B	0.016	47
Leupeptin	0.021	29
	0.001	2
Pepstatin	0.014	0
Z-Phe-Phe-CHN_2	0.01	31
Z-PHe-Ala-CHN_2	0.01	11
Soya-bean trypsin inhibitor	0.1	23
Hyamine 1622	1.0	52
Arquad 2C-50	1% (v/v)	26
Puromycin	1.0	36

Powerful inhibition of human cathepsin H by iodoacetic acid, iodoacetamide and 4-aminophenylmercuric acetate confirmed its classification as a cysteine proteinase.

Phenylmethanesulphonyl fluoride did not significantly alter the activity of human cathepsin H. This compound has, however, been found to inhibit cathepsin B (Barrett, 1973) and rat cathepsin H (Kirschke *et al.*, 1977*a*), but not rat cathepsin L (Kirschke *et al.*, 1977*b*).

The inhibition of cathepsin H by Tos-Phe-CH_2Cl and Tos-Lys-CH_2Cl is consistent with the high reactivity of the cysteine residue at the active site of the enzyme. Cathepsin B (Barrett, 1973) and cathepsin L (Kirschke *et al.*, 1977*b*) are likewise inhibited. The chloromethane derivative of L-leucine was found to be especially potent as an inhibitor of human cathepsin H; this has also been shown for the rat enzyme (Kirschke *et al.*, 1976*a*).

Cathepsin H was inhibited by the microbial peptide aldehydes chymostatin and leupeptin, but much less strongly than were human cathepsins B and L (Knight, 1980; Schwartz, W. N. & Barrett, A. J., unpublished). Thus cathepsin H was essentially unaffected by a leupeptin concentration (1 μM) that caused complete inhibition of cathepsins B and L. The K_i of leupeptin for human cathepsin H was determined to be 6.9×10^{-6} M as compared with 1.4×10^{-8} M for cathepsin B (Knight, 1980). Pepstatin, a potent inhibitor of aspartic proteinases, had no effect on cathepsin H.

Peptidyldiazomethanes that have been found to be irreversible inhibitors of cathepsin B (Watanabe *et al.*, 1979) were tested with cathepsin H. Under our incubation conditions, Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 produced 11–31% inactivation. In contrast, Z-Phe-Ala-CHN_2 produced complete inactivation of human cathepsin B at this concentration. Kirschke *et al.* (1980) failed to obtain significant inhibition of rat cathepsin H with either inhibitor at 10^{-4} M, under what appear to be the same conditions.

The inhibition of human cathepsin H by 0.1 mM soya-bean trypsin inhibitor was probably non-specific. This concentration of the inhibitor is about 20-fold that commonly used for specific inhibition of serine proteinases.

Puromycin inhibited human cathepsin H, but only weakly compared with its effect on non-lysosomal leucine naphthylamidase activities reported in rat brain (Marks *et al.*, 1968) and in human muscle (Bury & Pennington, 1973). The cationic detergent Hyamine 1622 is also an inhibitor of these naphthylamidases, and its effect on cathepsin H was likewise relatively weak. Arquad 2C-50, which was made use of in the purification procedure (see above), is another cationic detergent that was shown to be a weak inhibitor of cathepsin H.

Interaction with α_2-macroglobulin. α_2-Macroglobulin has the unique property of binding endopeptidases with retention of their activity against low-molecular-weight substrates (Barrett *et al.*, 1979). The binding of human cathepsin H to α_2-macroglobulin was demonstrated as follows. Cathepsin H (3 nmol) was incubated with 15 nmol of human α_2-macroglobulin for 30 min at 37°C in

2.8ml of sodium acetate buffer, pH 5.5, containing 3mM-cysteine and 1mM-disodium EDTA. The mixture was then run on Ultrogel AcA 54, and column fractions assayed with Arg-NNap.

It was found that more than 90% of the enzymic activity was eluted with α_2-macroglobulin (725000 mol.wt.) in the void volume, instead of at the elution volume characteristic of cathepsin H. Electrophoresis of the α_2-macroglobulin–cathepsin H complex as described by Barrett *et al.* (1979) showed that the α_2-macroglobulin had undergone the 'slow'-to-'fast' transition characteristic of the reaction of α_2-macroglobulin with endopeptidases. Likewise, the characteristic cleavage of the quarter subunit was detected by SDS/polyacrylamide-gel electrophoresis.

Discussion

It is now clear that human liver contains at least three cysteine proteinases, cathepsin B, cathepsin H and cathepsin L (Barrett, 1980*c*). All three enzymes have mol.wts. in the range 25000–30000, and cathepsin L is readily distinguished from the other two by the fact that it has little or no activity on Bz-Arg-NNap, or most other synthetic substrates, although it is a powerful proteinase.

Like human cathepsin B (Barrett, 1973), cathepsin H hydrolysed Bz-Arg-NNap and was inactivated by exposure to alkaline pH. It differed from cathepsin B, however, in being active on unblocked amino acid naphthylamides (e.g. Arg-NNap) as well as the blocked compounds. It was this characteristic which prompted Kirschke *et al.* (1977*a*) to describe rat liver cathepsin H as an endoaminopeptidase.

The activities of cathepsins B and H are also clearly distinguished by their susceptibilities to the microbial peptide aldehyde inhibitor leupeptin. Thus human cathepsin H is unaffected by 1μM-leupeptin, which gives essentially complete inhibition of cathepsin B (and cathepsin L). As proteins, human cathepsins B and H are easily separated by use of DEAE-cellulose or concanavalin A–Sepharose under the conditions described in the Results section. Human cathepsin L has a much higher affinity for CM (carboxymethyl)-cellulose at pH 5.0 than the other two enzymes (W. N. Schwartz, unpublished work).

Being hydrolysed by both cathepsin B and cathepsin H, Bz-Arg-2-NNap is not an ideal test substrate for either enzyme. In contrast, specific assays for cathepsin B can be made with Z-Arg-Arg-2-NNap (McDonald & Ellis, 1975; Knight, 1980) or Z-Phe-Arg-NMec (Barrett, 1980*b*). Specific assays for cathepsin H can be made with Arg-NNap or Arg-NMec (Barrett, 1980*b*). These substrates are susceptible to other aminopeptidases and amino acid naphthylamidases, but most such enzymes have little activity at pH 6.8 in the presence of cysteine, EDTA and phosphate, and at low Cl^- concentrations (McDonald & Schwabe, 1977), i.e. under the conditions of the cathepsin H assay. As a further control, assays can be made with the inclusion of 0.1mM-puromycin, which inhibits the aminopeptidases, but not cathepsin H. Even with the specific substrates, however, accurate determination of the enzymes in crude tissue extracts is made difficult by the presence of endogenous inhibitors of the cysteine proteinases.

Cathepsin H was first isolated from rat liver by Kirschke *et al.* (1976*b*). We have found human cathepsin H to show many of the properties reported for the rat enzyme (Kirschke *et al.*, 1977*a*, 1980); these include molecular weight, affinity for concanavalin A–Sepharose, substrate specificity, sensitivity to leupeptin and instability to alkaline pH. There are species differences, however, in isoelectric point and stability to heating at 60°C. The enzymes also differ (by less than one pH unit) in their pH optimum.

Two enzymes, described as 'α-*N*-benzoyl-arginine 2-naphthylamide hydrolases' have been isolated from rat skin and rabbit lung respectively (Järvinen & Hopsu-Havu, 1975; Singh & Kalnitsky, 1978). The properties of these enzymes strongly suggest that they may also represent forms of cathepsin H. Thus they are cysteine proteinases of mol.wt. 27000–29000, which hydrolyse unblocked as well as blocked amino acid naphthylamide substrates. Nevertheless, the rabbit lung enzyme differs from human cathepsin H in its alkali-stability (Singh & Kalnitsky, 1980) and collagenolytic activity (Singh *et al.*, 1978). Kirschke *et al.* (1980) have shown that neither rat nor human cathepsin H has detectable collagenolytic activity.

Other cathepsin H-like enzymes have been detected by Davidson & Poole (1975), Hardy & Pennington (1979), Husain (1976) and W. N. Schwartz (unpublished work) in preparations of rat liver, rat skeletal muscle, calf liver and human skeletal muscle respectively. Lysosomal activity against leucine naphthylamide has been detected both histochemically and as a contaminant in preparations of cathepsin B (Sylvén, 1968; Sylvén & Snellman, 1974). This activity probably corresponds to that of cathepsin H, and has previously been ascribed to a lysosomal aminopeptidase (McDonald & Schwabe, 1977).

In view of the widespread distribution of activity with the characteristics of cathepsin H, it seems likely that this enzyme will be found to occur generally in the lysosomes of mammalian cells.

We thank the Muscular Dystrophy Association of America for provision of a fellowship to W. N. S.

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