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High-performance liquid chromatographic method for the simultaneous purification of cathepsins B, H and L from human liver

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ABSTRACT

A high-performance liquid chromatographic procedure for the isolation of the three cysteine proteinases, namely cathepsins B, H and L, is described. The method is based on the following four steps. (1) A classical AcA 44 gel permeation separation with a 30–70% ammonium sulphate fraction from the human liver homogenate is used to remove the non-enzymic high-molecular-mass components. (2) Preparative cation-exchange chromatography on a CM-SW TSK column can separate the three proteinases. (3) An anion-exchange step on a semi-preparative DEAE-SW TSK column for the cathepsin H fraction is used to remove a small amount of cathepsins B and L activities. (4) The three separated enzymes are purified on an analytical TSK gel 2000 SW column. The purity of each enzyme is assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis and electrofocusing on polyacrylamide gels. To check the activities of the purified proteinases, the kinetic constants [Michaelis constant (K_M) and catalytic constant (K_{cat})] and the ratio K_{cat}/K_M against the fluorigenic substrates Arg-NH-Mec, Z-Arg-Arg-NH-Mec and Z-Phe-Arg-NH-Mec after active-site titration using E-64, were determined. Z-Phe-Phe-CNH_2 was also used as a specific inhibitor of cathepsin L. This method requires only 6 g of human liver, and gives a high yield of the three lysosomal cysteine-proteinases: thus, about 150 μg of cathepsin B and 50 μg each of cathepsins L and H are obtained in a single run.

INTRODUCTION

Lysosomal cysteine proteinases are the most active enzymes involved in the intracellular protein breakdown [1]. Cathepsins B (CB, EC 3.4.22.1), H (CH, EC 3.4.22.16) and L (CL, EC 3.4.22.15) belong to this group of cellular proteinases and are members of the papain superfamily [2]. They have been isolated from different species (human, ox, sheep, pig, rat, chicken) and tissues (liver, kidney, spleen) [1–7], and their complete amino acid sequences have been reported by several groups [7–12]. However, the previously reported isolation methods necessitated many steps, the yields were low, the simultaneous isolation of the three proteinases was not possible, and large amounts of starting material were required [1–9]. When small organs or pieces of tissue are available, purification remains difficult. For example, cathepsin B-like proteinases have been character-

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ized in malignant tissues and cells, but studies at the molecular level are impossible without isolation [13–15].

This paper describes a high-performance liquid chromatographic (HPLC) procedure for the simultaneous isolation of these three cathepsins from human liver in high yield. A four-step purification scheme is proposed with only a few grams of tissue, permitting analysis of a large number of small samples for routine studies. The physico-chemical and kinetic properties of the purified proteinases were closely related to those reported previously.

EXPERIMENTAL

Materials

Frozen human liver was used as the starting material for the purification procedure. Ampholines (pH range 3.5–10.0), standard proteins and prestained standard proteins for immunoblotting were purchased from Pharmacia (Saint Quentin en Yvelines, France), Serva (Sant Germain en Laye, France) and Bethesda Research Laboratory (BRL) (Herblay, France), respectively. Triton-X 100 was from Sigma (St. Louis, MO, USA). AcA 44 gel was supplied by the Industrie Biologique Française (Gennevilliers, France) This gel was packed in a glass column (75.0 cm × 3.0 cm I.D.). The following TSK HPLC columns from Tosoh (Tokyo, Japan) were used: a cation-exchanger CM (carboxymethyl) 3SW (15.0 cm × 2.15 cm I.D.), an anion-exchanger DEAE (diethylaminoethyl) 5PW (7.5 cm × 0.75 cm I.D.) and a gel permeation 2000SW (30 cm × 0.75 cm I.D.). The HPLC apparatus "System Gold" was from Beckman (Gagny, France). Z-Phe-Arg-NH-Mec, Z-Arg-Arg-NH-Mec, Arg-NH-Mec, Z-Phe-Phe-CHN_2 and NH_2-Mec[a] were purchased from Nova-Biochem (Laufelfingen, Switzerland), and E-64 (L-3-carboxy-2.3-*trans*-epoxypropionylleucylamido-4-guanidino butane) was from the Protein Research Foundation (Osaka, Japan). Fluorescence measurements were carried out an a Kontron SFM 25 spectrofluorimeter at 347 nm (excitation) and 440 nm (emission) using NH_2-Mec for calibration.

All other reagents were of analytical grade.

Purification

Frozen liver (100 g) was thawed, rinsed with the extraction solution (0.15 *M* NaCl pH 3.8, 1 m*M* ethylenediaminetetraacetic acid, disodium salt, EDTA) and homogenized in 200 ml of extraction solution containing 0.2% Triton-X100 in a Waring blender. The pH was then acidified to 3.8 with 1 *M* HCl, and the enzymes were extracted by stirring overnight at 4°C. The insoluble material was removed by centrifugation (15 min, 3000 *g*), and a 30–70% $(NH_4)_2SO_4$ protein fraction was precipitated from the supernatant. After centrifugation (15 min of 3000 *g*),

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[a] Z = Benzyloxycarbonyl; NH-Mec = 4-methyl-7-coumarylamide; CHN_2 = diazomethane.

the pellet was suspended and extensively dialyzed against 0.15 *M* acetate buffer (pH 4.6) containing 0.1 *M* NaCl and 1 m*M* EDTA. The equilibrated fraction (33 ml) was centrifuged as described above and then frozen in 2-ml aliquots.

A 2-ml fraction, corresponding to 6 g of starting tissue, was run through an Ultrogel AcA 44 column in the intitial buffer at 4°C and 15 ml/h. The cysteine proteinase activity in the eluate was checked against Z-Phe-Arg-NH-Mec: 0.1 m*M* substrate was incubated in 500 μl of 0.1 *M* phosphate (pH 6.0) 1 m*M* dithioerythritol DTE and 2 m*M* EDTA (activation buffer) after addition of 50 μl of elution sample. The reaction was stopped after 15 min at 37°C, and the fluorescence measured as previously reported [1,3]. Pooled active fractions were concentrated and dialysed against 0.1 *M* acetate buffer (pH 5.3). Cation-exchange chromatography on a CM 3SW TSK column, equilibrated in the same buffer, was carried out. The sample (2 ml) was injected and, after elution of the breakthrough peak, a three-step NaCl gradient was developed at 2.0 ml/min: 0.00–0.15 *M* NaCl during 150 min; 0.15–0.40 *M* NaCl during 60 min; 0.40–1.00 *M* NaCl during 10 min. A final washing step (1.0 *M* to 0.0 *M* NaCl during 10 min) was then performed. Cathepsin H was checked using Arg-NH-Mec with the pH 6.8 activation buffer, cathepsins B and L were checked using Z-Phe-Arg-NH-Mec and Z-Arg-Arg-NH-Mec with the pH 6.0 activation buffer, as reported above. The three proteinase activities were pooled and concentrated separately. In some cases, the cathepsin B activity was pooled in two different fractions on the basis of two different peaks of activity. Cathepsins B and L were frozen, and cathepsin H was further purified by anion-exchange chromatography on a DEAE-5PW column equilibrated with 20 m*M* phosphate buffer (pH 6.0). The sample (1 ml) was applied and after elution of the breakthrough peak, a linear NaCl gradient (0–0.5 *M* NaCl, during 30 min) was applied. The column was then reequilibrated with the starting buffer. Cathepsin H, B and L activities were determined as described above. Cathepsin H fractions were concentrated, and the small amounts of separated cathepsins B and L activities were discarded. The purification of the three cysteine proteinases was achieved by gel permeation on a 2000 SW TSK column equilibrated with 0.15 *M* acetate buffer (pH 4.6) containing 0.1 *M* NaCl and 1 m*M* EDTA. After sample application (20 μl), a 1.0 ml/min flow-rate was used for elution. Enzymic activities were determined as described above, and active fractions were concentrated. As previously reported, the protein concentrations of the purified enzymes were determined at 280 nm [3,16] and the purified proteinases were stored at −20°C for futher studies.

Polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed as reported by Laemmli [17] in 15% slab gels. Samples were incubated with a mixture of 1% SDS, 20% glycerol and 2% DTE before boiling for 5 min.

Isoelectrofocusing in polyacrylamide gels was carried out as described previ-

ously [17] using an ampholine pH gradient of 3.5–10.0 and a gel containing 7% acrylamide, 0.2% bisacrylamide and 20% sucrose.

For both methods, staining was performed by using Coomassie violet R 150. Immunoblotting was performed by using sheep polyclonal antibodies directed against human liver cathepsin B, as reported before [13].

Chromatographic pH titration profiles

These experiments were carried out by using the TSK-CM-3SW column. Both the proteinase sample [CB and CL, 40 μg (500 μl); CH 20 μg (200 μl)] and the column were equilibrated with 0.1 *M* acetate buffer (pH 5.3). Each proteinase sample was injected separately into the column. After a total column elution volume, a pH gradient was applied by using 0.1 *M* acetate buffer (pH 6.0) at a flow-rate of 2 ml/min during 60 min. The true gradient was drawn by pH measurements into the eluted fractions (2 ml/tube). Each enzyme was located using the fluorigenic substrates as described before. For cathepsin H a reverse pH titration profile experiment was carried out, starting in 0.1 *M* acetate buffer (pH 6.0) for both the column and the sample. In this case, the cathepsin H activity was located in the total column elution volume, so a pH gradient was not useful.

Characterization of cysteine proteinases activities

Concentrations of active enzymes were determined by titration with E-64 as described previously [1,5,18]. Catalytic constants (K_{cat}) were estimated using stopped assays as follows: small amounts of proteinases ($3 \cdot 10^{-10}$ *M*) were diluted in 100 μl of activation buffer (pH 6.0 for CB and CL, pH 6.8 for CH). After 10 min of preincubation, the substrate was added and incubation at 37°C was carried out. Six different incubation times (0, 5, 10, 15, 30 and 60 min) and three different fluorigenic substrates (Z-Phe-Arg-NH-Mec, Z-Arg-Arg-NH-Mec and Arg-NH-Mec) were used. The substrate concentrations used were $9 \cdot 10^{-4}$ *M* for CB and CH and $9 \cdot 10^{-5}$ *M* for CL. The reaction was stopped and the fluorescence measured as reported previously [1,3,17]. Alternatively, intitial rate assays were performed as already described [13]. Quantitation of freed NH_2-Mec was carried out by using a calibration curve drawn with an NH_2-Mec solution, and results were plotted as a function of time.

Six different substrate concentrations were used for the determination of the Michaelis constant (K_M). The concentration ranges were 0.9–$9 \cdot 10^{-4}$ *M* and 0.9–$9 \cdot 10^{-6}$ *M* for CB, CH and CL, respectively. Nanomolar enzyme concentrations were used, and other experimental conditions were unchanged. Results were analysed by the Lineweaver–Burk method [19]. As previously reported [3,13], characterization was achieved using Z-Phe-Phe-CNH_2 in the concentration ranges $4 \cdot 10^{-8}$ *M* to $4 \cdot 10^{-6}$ *M* and $4 \cdot 10^{-5}$ *M* to $4 \cdot 10^{-4}$ *M* for cathepsin L and cathepsin B, respectively.

RESULTS

Purification

Gel permeation is very useful for the removal of high-molecular-mass proteins: this step gives a clean and enriched cysteine proteinase fraction suitable for subsequent HPLC purification.

Cation-exchange chromatography can separate the three cysteine proteinases (Fig. 1): using its aminopeptidase activity against Arg-NH-Mec, cathepsin H was located in the first peak. Cathepsin B, which cleaves both endopeptidase sub-

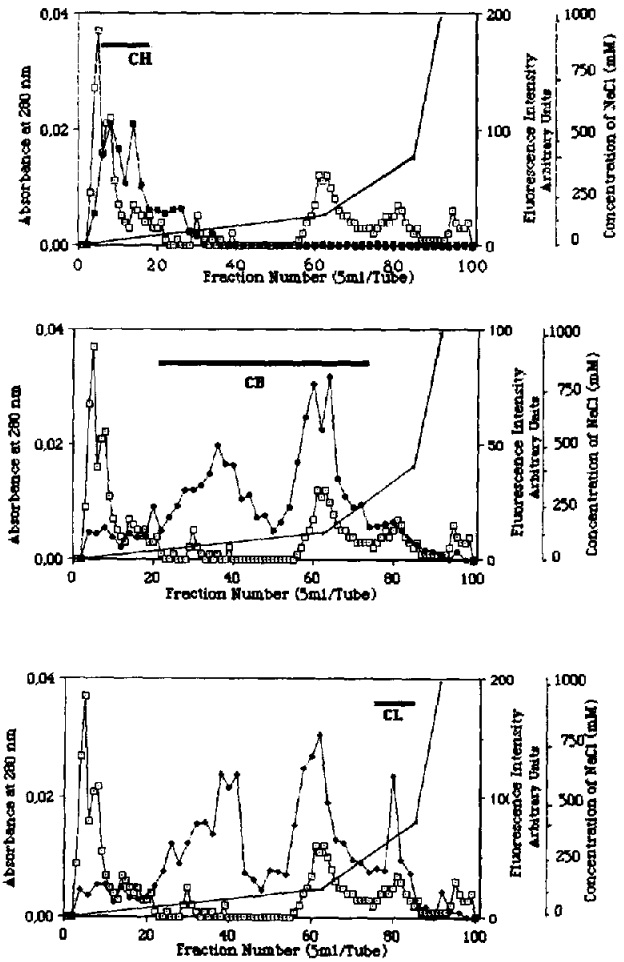

Fig. 1. Cation-exchange chromatography of the cysteine proteinase fraction. The sample (2 ml) was dialysed overnight against 1 l of 100 m*M* sodium acetate buffer (pH 5.3). Then it was injected into a CM 3SW HPLC column equilibrated with the same buffer. Elution was carried out with a three-step NaCl gradient (—). Fractions were monitored at 280 nm for proteins (□), and assayed for cathepsin B activity (●), cathepsin H activity (■) and cathepsins L and B activities (◆). The three separated proteinases were pooled as indicated by horizontal bars.

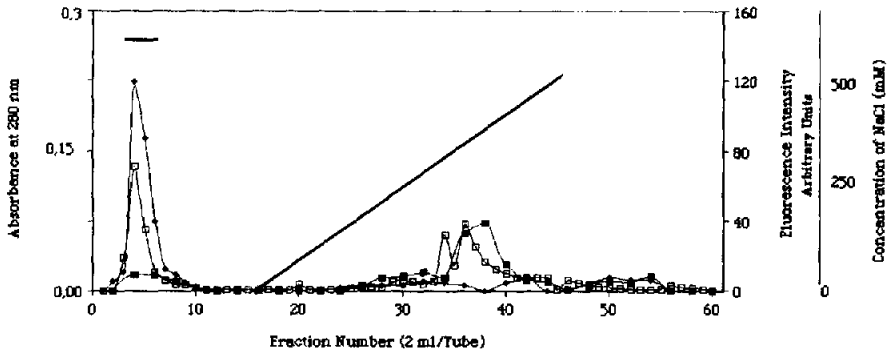

Fig. 2. Anion-exchange chromatography of the cathepsin H fraction. The sample (1 ml) was dialysed overnight against 1 l of 20 m*M* phosphate buffer (pH 6.0) and injected into a DEAE 5 PW column equilibrated with the same buffer. The column was eluted with a linear NaCl gradient (–). Proteins in the fractions were monitored at 280 nm (□), and assayed for cathepsin H activity (◆) and cathepsins L and B activities (■). Cathepsin H fractions were pooled as indicated by the horizontal bar.

strates, *i.e.* Z-Phe-Arg-NH-Mec and Z-Arg-Arg-NH-Mec, gave a broader peak that eluted between 100 and 200 m*M* NaCl. Cathepsin L was located by its single endopeptidase activity against Z-Phe-Arg-NH-Mec: this proteinase is firmly bound to the resin and gives a single sharp peak around 400 m*M* NaCl. The cathepsin B activity separated from this step into two different peaks was pooled. In some cases, both peaks were pooled separately for subsequent studies.

In order to remove small amounts of cathepsin B, the cathepsin H fraction was subjected to anion-exchange chromatography: cathepsin H eluted in the breakthrough peak, and contaminant cathepsin B and L activities bound to the resin could be separated (Fig. 2).

The purification of the three separated proteinases was achieved by gel permeation chromatography (Fig. 3). In both the whole homogenate and the ammonium sulphate fraction, cysteine proteinase activities were difficult to quantitate: the high protein concentration, acting as a competing substrate, led to an underestimate of enzymic activities. Consequently, the yield was expressed as the purified active enzyme per gram of solid tissue. After five different purifications, the mean concentration of each enzyme was estimated (Table I).

Characterization of purified proteinases

SDS-PAGE with a reducing agent gave bands at both 30 000 and 25 000 for the three proteinases. At the bottom of the gel, a light chain could be seen corresponding to these enzymes (Fig. 4a). On the basis of previous electrophoretic studies [3,5], and from a knowledge of the sequence of the three cathepsins, *i.e.* the presence of both heavy and light chains in disulphide-linked and single-chain form [4,7–9] the following explanation can be proposed: the 30 000 band represents the single-chain form and the 25 000 band the heavy chain of the two-chain form. The light component located at the bottom of the gel could be the light chain.

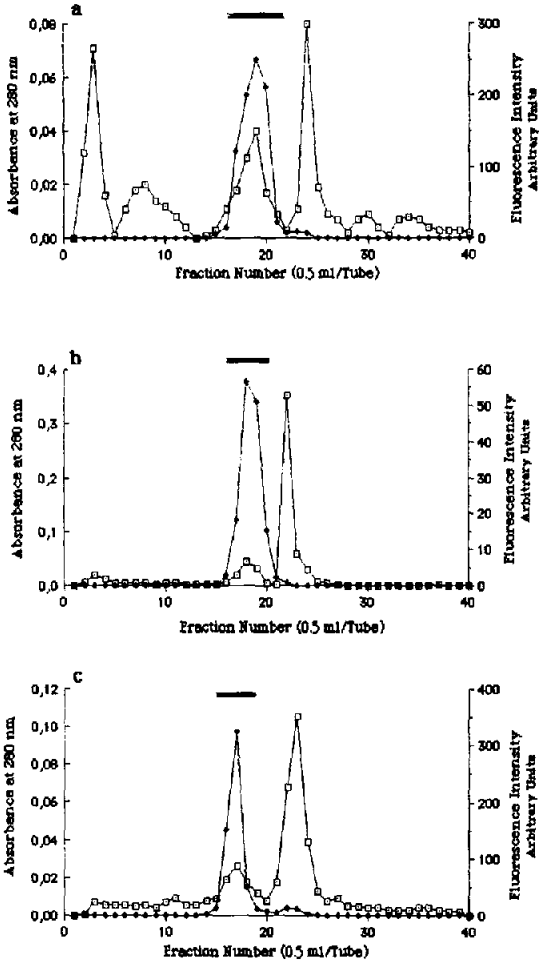

Fig. 3. HPLC gel permeation of the separated cysteine proteinases. The sample (20 μl) was injected into a TSK gel 2000 SW column and eluted with 150 m*M* sodium acetate buffer (pH 4.6) containing 100 m*M* NaCl and 1 m*M* EDTA. Proteins in the fractions were monitored at 280 nm (_□), and assayed for cathepsin B activity (a) cathepsin H activity (b) and cathepsin L activity (c).

The immunoblotting study of cathepsin B gave similar results. Nevertheless, the light component was not seen, probably owing to the loss of the light component during the electrophoretic transfer, whose speed is inversely proportional to the molecular mass. Alternatively, the cathepsin B antibodies could be directed only against the major component, *i.e.* the heavy chain (Fig. 4b). When cathepsin B was separated into two different fractions by cation exchange, the results were similar: SDS-PAGE and immunoblotting showed both the 30 000 and 25 000 forms. Consequently, these results are not shown.

Following gel electrofocusing, pH isoelectric values of 5.5, 5.7 and 6.2 were found for cathepsins B, L and H, respectively (Fig. 5). To explain the discre-

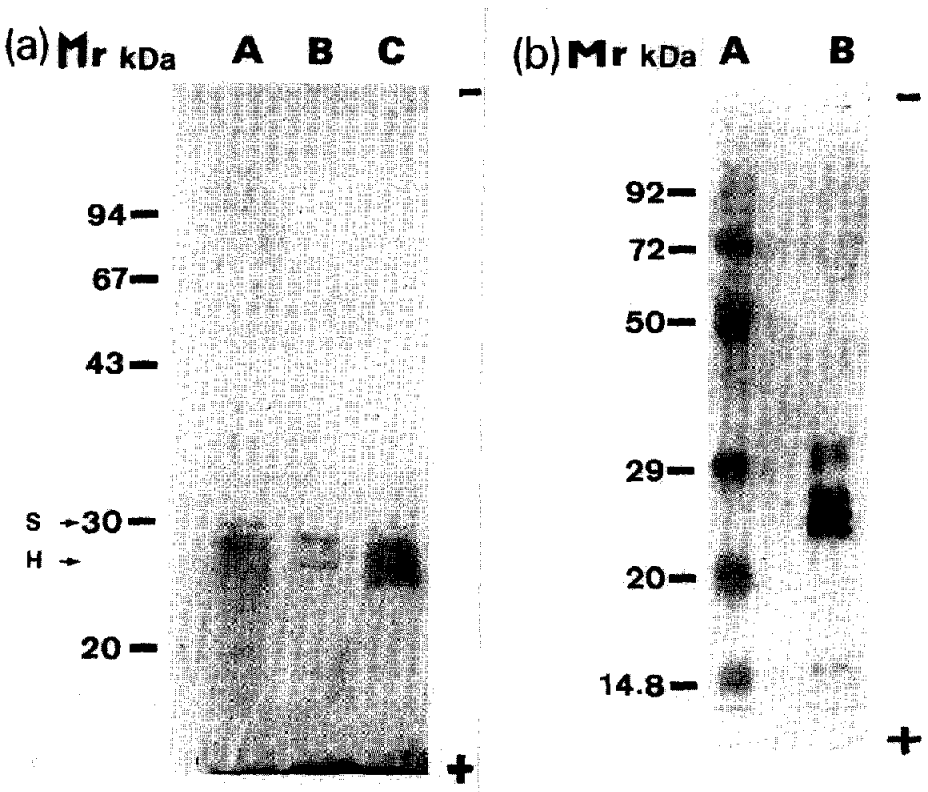

Fig. 4. (a) SDS-PAGE of purified human liver cysteine proteinases under reducing conditions. A 15-μg sample of purified enzymes was run on 15% slab gels. Proteins were stained by Coomassie violet R 150 after trichloroacetic acid fixation. (A) Cathepsin L; (B) cathepsin H; (C) cathepsin B; (S) single chain form; (H) heavy chain. The following standards were used: soya-bean trypsin inhibitor (20 000), carbonic anhydrase (30 000), ovalbumin (43 000), bovine serum albumin (67 000), phosphorylase B (94 000). (b) Immunoblotting of purified human liver cathepsin B. A 15-μg sample of purified enzyme was submitted to SDS-PAGE on a 15% slab gel and subsequently transferred to a nitrocellulose sheet for 1 h. The sheet was then washed, and bound immunoglobulins were detected with rabbit anti-sheep immunoglobulin G–peroxidase conjugate (0.5 μg/ml) using 4-chloro-1-naphthol. Prestained BRL standards were used after correction of their molecular mass with the same standards as in Fig. 6.

TABLE I

AMOUNTS OF CYSTEINE PROTEINASES SIMULTANEOUSLY PURIFIED FROM HUMAN LIVER, AND COMPARISON WITH PREVIOUS PUBLISHED RESULTS

Amount of liver, 1 g.

Compound	Amount (mean ± S.D., n = 5) (μg)	Value from ref. 20 (μg)	Value from ref. 21 (μg)	Value from ref. 23 (μg)
CB	25.7 ± 15.0			16.5
CH	1.10 ± 6.0	2.0		
CL	9.3 ± 4.0		0.5	

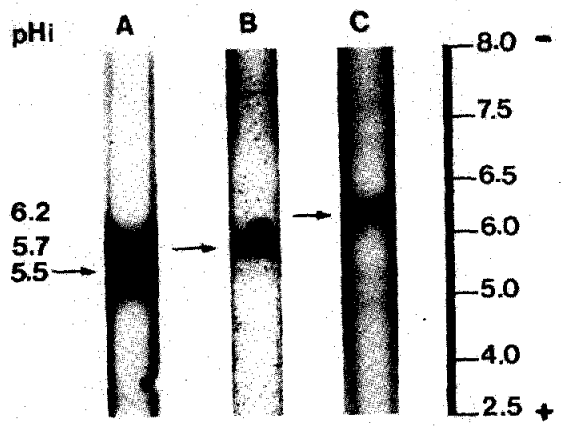

Fig. 5. Analytical gel electrofocusing of purified human liver cysteine proteinases. A 15-μg sample of purified enzymes was run on a 3.5–10 pH gradient overnight and stained as described in Fig. 6. The pH gradient was measured on three other gels cut in 5-mm strips, which were immersed for two days at 4°C in 1 ml of distilled water. The pH scale (mean ± S.D., $n = 4$) is drawn on the right (A) cathepsin B; (B) cathepsin L; (C) cathepsin H.

pancies between the cation-exchange chromatography and the pH isoelectric values found for the three proteinases, the chromatographic pH titration profile was drawn between 5.3 and 6.0 (Fig. 6). Cathepsin H eluted in the starting buffer, *i.e.* pH 5.3, cathepsin B was located at pH 5.43, and cathepsin L gave five peaks at pH 5.40 (minor), 5.45, 5.54, 5.80 and 5.90. When the titration profile was obtained at pH 6.0, cathepsin H also eluted in the starting buffer. These results indicate that the net charge of CH between 5.3 and 6.0 is near zero. Anion-exchange chromatography of CH at pH 6.0 gave similar results and corresponds to the same charge.

Active-site titration by E64 gave concentrations of active enzymes of between 80% and 100%, when they were calculated from the total purified protein concentration in the sample.

Kinetic properties of the three proteinases are summarized in Table II: both cathepsin B and L were endopeptidases The highest K_{cat}/K_M ratio was obtained against Z-Arg-Arg-NH-Mec for cathepsin B. Cathepsin L gave the best K_{cat}/K_M ratio against Z-Phe-Arg-NH-Mec. Cathepsin H acted as an aminopeptidase and as an endopeptidase against Arg-NH-Mec and Z-Arg-Arg-NH-Mec, respectively. Cathepsin B activity was partially inhibited by $4 \cdot 10^{-4}$ M of Z-Phe-Phe-CNH_2. In contrast, complete inhibition of cathepsin L activity was observed at the concentration of $3 \cdot 10^{-7}$ M.

DISCUSSION

To date, HPLC has been used only as the final step in the purification of the cysteine proteinases [20–22]. Consequently, simultaneous isolation of cathepsins

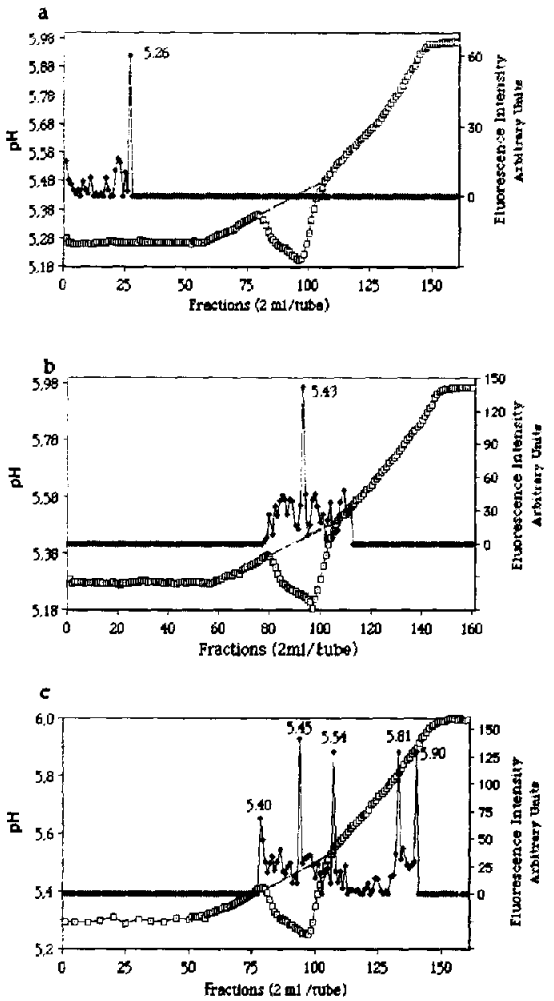

Fig. 6. Chromatographic pH titration profiles of the three purified cysteine proteinases on the TSK-CM-3SW column. The enzymes, equilibrated in 0.1 *M* acetate buffer (pH 5.30), were run separately on the column equilibrated in the same buffer. After a total column elution volume, the pH gradient was started by using 100 m*M* acetate buffer (pH 6.0). The true pH values (□) were monitored during the experiment. The pH gradient depletion due to the buffering power of eluted proteins was corrected (broken line) for the calculation. Enzymic activities were located as described previously by using the three fluorigenic substrates. Cathepsin L activity, assayed with Z-Arg-Arg-NH-Mec, was not significant: this result is not shown.

B, H and L was impossible and the yields were low. For example, 700 μg of purified cathepsin L was obtained from 1.4 kg of human liver [21] and 8 mg of cathepsin H from 4 kg of human kidney [20]. A better result was obtained by affinity chromatographic purification of human liver cathepsin B (10 mg of purified enzyme from 600 g of starting material) [23]. Our results shown in Table I,

compared with those discussed above, show that our method is the most efficient. For the first time, simultaneous purification of the three cysteine proteinases from the same tissue sample was possible, and the highest yields could be obtained by this three-step HPLC method. Alternatively, cloning and expression of cysteine proteinase genes would be useful for production of these enzymes. However, cathepsin B expression in *Escherichia coli* is unsuccessful [24]. Correct cloning and processing of these proteins probably requires a mammalian cell expression system. To date, cysteine proteinases are available only by tissue extraction and purification.

Cathepsins B, H and L were found as single-chain or two-chain enzymes. These findings are in agreement with numerous reports concerning the structural properties of these proteinases [3,4,20–23]. Nevertheless, the ratio between the single-chain and two-chain forms seems to be linked to the purification method, as reported recently for cathepsin L [25]. On the other hand, different cathepsin B isoforms were seen after the HPLC cation-exchange step [6,26], and no significant differences were found in the properties of these isoforms. It is tempting to think that single-chain and disulphide-linked heavy and light chains of cathepsin B could be distinguished at this level. However, this separation seems to be a consequence of minor structural modifications not detectable by the physico-chemical and enzymic methods reported here (data not shown). In this work, pH isoelectric values were similar to those reported before [17,18,20,21]. These values are related to the chromatographic titration profiles found for cathepsins B and L. However, cathepsin H exhibits a chromatographic titration profile different from that expected from pH isoelectric values. Similar differences were previously reported by other groups [1,20].

The kinetic data summarized in Table II illustrate the differences between the active sites of cathepsins B and L: in accordance with Schechter and Berger's subsite definition [27], cathepsin B hydrolyses a peptide bond with two polar residues in P1 and P2. In contrast, maximal hydrolysis was obtained with cathepsin L when a hydrophobic side-chain was present in the P2 position.

Cathepsin L exhibits a higher affinity for synthetic substrates than cathepsin B, probably owing to the high digestion potential of cathepsin L against protein substrates, as recently reported for basement membranes [28]. Sensitivity to Z-Phe-Phe-CNH_2 is higher for cathepsin L than for cathepsin B, probably owing to a hydrophobic binding pocket in cathepsin L. Cathepsin H was found as an endoaminopeptidase as previously reported [1]. Active- site titration of the three proteinases by the epoxide E-64 was also observed. Purified cysteine proteinases were obtained with maximal activity (80–100% of the protein concentration), better than those described previously [3,4,17,18,20–23]. However, a mixture of single-chain and two-chain enzymes was used in this kinetic study. As shown previously [22], both single and two-chain cathepsin B bind to α_2-macroglobulin as endopeptidases. Thus the enzymic properties of both molecular forms seem to be similar. Nevertheless, further investigations are needed in order to separate

TABLE II

KINETIC PARAMETERS OF CATHEPSINS B, H AND L

Values were measured as described in Experimental. N.D. = not determined.

Substrate	Cathepsin B			Cathepsin H			Cathepsin L		
	K_{cat} (s^{-1})	K_M (mM)	K_{cat}/K_M (10^3 s^{-1} M^{-1})	K_{cat} (s^{-1})	K_M (mM)	K_{cat}/K_M (10^3 s^{-1} M^{-1})	K_{cat} (s^{-1})	K_M (mM)	K_{cat}/K_M (10^3 s^{-1} M^{-1})
Z-Phe-Arg-NH-Mec	49	0.39	125	0	N.D.	N.D.	17	0.0066	2561
Z-Arg-Arg-NH-Mec	685	0.28	2446	0.59	0.90	0.655	6	0.0330	182
Arg-NH-Mec	0	N.D.	N.D.	0.64	0.70	0.914	0	N.D.	N.D.

single- and two-chain cysteine proteinases. These separate species would be useful in a study of the catalytic properties of the cysteine proteinases.

In conclusion, a simple HPLC method applicable to small tissue samples is described here for the purification of the cysteine proteinases cathepsins B, H and L. The physico-chemical and catalytic properties of these purified enzymes are similar to those obtained by using more sophisticated methods. In future, this new HPLC application will enable us to study cysteine proteinases in a purified state.

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