Simultaneous isolation of human kidney cathepsins B, H, L and C and their characterisation

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Abstract

A procedure for the simultaneous isolation of four cysteine proteinases, cathepsins B, H, L and C, from human kidney is described. The method includes concentration of the acidified homogenate by ammonium sulphate precipitation. The resuspended and dialysed precipitate was chromatographed on DEAE-cellulose DE-32, to allow separation of cathepsins H and C from cathepsins B and L. The main isoform of cathepsin H was separated from cathepsin C by cation-exchange chromatography on CM-Sephadex C-50. These two enzymes were further purified by covalent chromatography on thiopropyl Sepharose and gel permeation on Sephacryl S-200. The last step allowed separation of cathepsin C and the minor isoform of cathepsin H. Purification of the other two enzymes, cathepsins B and L, was carried out on thiol Sepharose, followed by chromatography on CM-Sepharose C-50. In this step, pure cathepsin L was obtained, while two isoforms of cathepsin B had to be finally purified on Sephacryl S-200 columns. The purity of each enzyme was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis, isoelectric focusing on polyacrylamide gels and N-terminal sequencing. The activities of the purified cathepsins B, H and L were determined in terms of k_{cat}/K_M for three substrates, Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA. The method produced 25 mg of cathepsin B, 6.5 mg of cathepsin H, 1.5 mg of cathepsin L and 3.8 mg of cathepsin C from 3.5 kg of human kidney.

Keywords: Cathepsins; Enzymes

1. Introduction

Mammalian tissues contain a number of cysteine proteinases, including cathepsins B (EC 3.4.22.1), H (EC 3.4.22.16), L (EC 3.4.22.15), S (EC 3.4.22.27) and C (EC 3.4.14.1), located in lysosomes [1–3]. They play important roles in intracellular protein degradation and turnover [4], bone resorption [5], emphysema [6], prohormone activation [7–9], cancer

metastasis [10], muscular dystrophy [11] and rheumatoid arthritis [12]. As their amino acid sequences show strong similarity to that of papain, they are considered part of the papain superfamily of cysteine proteinases [13–15]. All of them are synthesized as larger precursors, which are subsequently processed to mature single-chain or double-chain enzymes [16–19]. On the basis of their activities, they can be divided into cathepsin B, which acts predominantly as a carboxypeptidase [20,21], cathepsins L and S, which work exclusively as endopeptidases [21,22], cathepsin H, which acts preferentially

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as an aminopeptidase [23], and cathepsin C, which is classified as dipeptidylaminopeptidase I [24].

These enzymes have been isolated from a large number of sources using different methods. One of them is simultaneous isolation of cathepsins B, H, and L [25], which produces only small amounts of these enzymes. In contrast, our aim was to develop a procedure which would render possible the isolation of pure and active cathepsins from human tissue in the same procedure and in sufficient quantities for further investigation of their roles in normal and pathological states of the human organism.

2. Experimental

2.1. Materials

Sephacryl S-200 superfine, CM-Sephadex C-50, activated thiol Sepharose 4B, thiopropyl Sepharose 6B, 8–25% polyacrylamide gradient gels, 7.5% polyacrylamide homogeneous gels, Pharmalyte (pH 3–10), broad calibration kit and low molecular-mass calibration kit were supplied by Pharmacia-LKB (Uppsala, Sweden). DEAE-cellulose DE-32 was from Whatman (Maidstone, UK). Bz-D,L-Arg-NA (Bz = benzoyl; NA = 2-naphthylamide), Arg-NA, Gly-Phe-MNA (MNA = 4-methoxy-2-naphthyl-amide) were from Sigma (Munich, Germany). Z-Phe-Arg-MCA [Z = benzyloxycarbonyl; MCA = (4-methyl-7-coumaryl)amide], Z-Arg-Arg-MCA and Arg-MCA were purchased from Bachem (Buben-dorf, Switzerland). L-*trans*-Epoxysuccinylleucyl-amido(3-methyl)butane (Ep-475) was from Peptide Research Foundation (Osaka, Japan). Bio-Rad Pro-tein Assay Dye Reagent was from Bio-Rad (Munich, Germany). Spectra/Por 1 dialysis membrane was from Spectrum (Los Angeles, CA, USA). The Chromspher C_8 high-performance liquid chromatog-raphy (HPLC) column was from Chrompack (Frank-furt, Germany).

Electrophoresis was performed on PhastSystem Apparatus from Pharmacia-LKB. The HPLC instru-mentation used was from LDC/Milton Roy (Stone, UK). The 475A liquid-phase sequencer was from Applied Biosystems. Fluorescence was measured on a LS-3 fluorimeter from Perkin-Elmer (Beaconsfield, UK).

Stefin A from human leukocytes was prepared in our laboratory as described [26]. Its activity was 100% inhibitory as determined by titration of papain, which was pre-titrated with Ep-475.

2.2. Purification of enzymes

A 3500-g quantity of frozen human kidneys was partially thawed, minced and homogenized at a ratio of 1:1.5 with 0.5% NaCl and 1 mM EDTA. The homogenate was acidified to pH 4.2 with 2 M HCl, as reported [27]. The acidified extract was left overnight at 4°C and centrifuged at 4200 g for 20 min in a Sorvall centrifuge. The supernatant was concentrated with ammonium sulphate. The proteins which precipitated between 20 and 70% ammonium sulphate saturation were collected by centrifugation, resuspended in 5 mM EDTA, dialysed against 0.02 M phosphate buffer, pH 6.0, containing 1 mM EDTA, and applied to a column of DEAE-cellulose DE-32 (20 × 3 cm), equilibrated with the same buffer. Fractions of about 10 ml, eluted at flow-rate of 33 ml/h with 0.25 M NaCl, were later used for purification of cathepsins B and L, while unbound material was used for purification of cathepsins H and C. Fractions containing cathepsins H and C were concentrated in an Amicon ultrafilter with a YM-10 membrane, dialysed against 0.02 M sodium acetate buffer, pH 5.2, containing 1 mM EDTA, and applied to a column of CM-Sephadex C-50 (35 × 2.5 cm), equilibrated with the same buffer. After elution of the unbound material, a gradient of NaCl concen-tration increasing to 0.4 M was applied. Two over-lapping peaks of activity towards Arg-NA and a broad peak of activity towards Z-Phe-Arg-MCA were eluted at a flow-rate of 13.8 ml/h. The first peak active towards Arg-NA, containing the main form of cathepsin H, was reduced with 20 mM cysteine and 1 mM dithioeritrol (DTE) in 0.1 mM phosphate buffer, pH 7.0, for 30 min at room temperature and afterwards separated from low M_r thiols on a Sephadex G-25 column (20 × 2 cm), equilibrated with 0.1 M sodium acetate buffer, pH 4.0, containing 0.3 M NaCl and 1 mM EDTA. Proteins were eluted at a flow-rate of 33 ml/h in fractions of 8 ml and mixed in a beaker with 50 g of wet weight thiopropyl Sepharose 6B, equilibrated with the same buffer (pH 4.0) for 1 h at room

temperature as described [28]. After the elution of unbound material, bound proteins were eluted batch-wise with 0.1 M phosphate buffer, pH 6.8, containing 0.3 M NaCl and 1 mM EDTA. The main form of cathepsin H was finally purified on a column of Sephacryl S-200 (120 × 2 cm), equilibrated with 0.1 M sodium acetate buffer, pH 5.5, containing 0.3 M NaCl and 1 mM EDTA, at a flow-rate of 13.8 ml/h and with a fraction size of 6 ml. The second peak of activity towards Arg-NA, together with a broad peak of activity towards Z-Phe-Arg-MCA from a CM-Sephadex C-50 column, was reduced, desalted and further purified on thiopropyl Sepharose 6B and Sephacryl S-200 columns in the same way as the main form of cathepsin H. The peak active towards Arg-NA from the Sephacryl S-200 column corresponded to the minor form of cathepsin H (cathepsin H$_1$); the first peak active towards Z-Phe-Arg-MCA corresponded to cathepsin C; the second Z-Phe-Arg-MCA active peak was further purified on DEAE cellulose DE-32 (14 × 1.5 cm), equilibrated with 0.02 M phosphate buffer, pH 7.5, containing 1 mM EDTA. After a gradient application of 0–0.6 M NaCl, a small amount of Z-Phe-Arg-MCA and Z-Arg-Arg-MCA activity was obtained.

Material eluted with 0.25 M NaCl from the first DEAE cellulose column, containing cathepsins B and L, was reduced with 5 mM DTE for 30 min at room temperature, dialysed against 0.1 M phosphate buffer, pH 6.0, containing 0.3 M NaCl and 1 mM EDTA, and mixed with 200 g of wet weight thiol Sepharose 4B, equilibrated with the same buffer at 4°C overnight. The suspension was then poured into a column and after the elution of unbound material, 20 mM cysteine was added to the starting buffer to elute covalently bound material at a flow-rate of 33 ml/h, similar to the method described in Ref. [29]. Fractions eluted with 20 mM cysteine were concentrated, dialysed against 0.02 M sodium acetate buffer, pH 5.2, containing 1 mM EDTA, and applied to a CM-Sephadex C-50 (35 × 2.5 cm I.D.) column, equilibrated with the same buffer. Unbound material was discarded and a 0–0.3 M NaCl gradient elution was applied, followed by elution with 0.3 M NaCl and 0.6 M NaCl in the same buffer at a flow-rate of 25 ml/h and with a fraction size of 8 ml. By the gradient elution, two isoforms of cathepsin B were obtained. They were separately purified on the

Sephacryl S-200 columns (120 × 2 cm I.D.) at a flow-rate of 13.8 ml/h and with a fraction size of 6 ml. Cathepsin L was obtained by elution of the CM-Sephadex C-50 column with 0.6 M NaCl.

All purified enzymes were stored at −20°C in 0.02 M sodium acetate buffer, pH 5.2, containing 1 mM EDTA.

2.3. Protein determination

Protein concentrations were determined using the Bio-Rad Protein Assay [30]. The standard curve was obtained using bovine serum albumin. Protein concentrations, in eluted fractions and in purified samples, were determined by the absorbance measurements at 280 nm, assuming that A_{280} (1%) for cathepsin B is 18, and for cathepsins H, L and C is 14, as determined for bovine cathepsins B and H, respectively [31].

2.4. Enzyme assays

During the isolation procedure, cathepsins B and H were assayed using 4 mM Bz-D,L-Arg-NA and Arg-NA, respectively, as described [30]. The same method was used for cathepsin C determination with 50 μM Gly-Phe-MNA as the substrate. Cathepsin L was assayed using 5 μM Z-Phe-Arg-MCA [21]. This substrate was also cleaved by cathepsins B and C. As assay buffers, we used 0.1 M phosphate, containing 5 mM cysteine and 1.5 mM EDTA, pH 6.0 and 7.0, for cathepsins B and H, respectively. A 0.4 M sodium acetate buffer, containing 5 mM DTE, 20 mM NaCl and 1.5 mM EDTA, pH 5.5, was used in the determination of cathepsins L and C.

Active site titrations of enzymes were carried out essentially as described [21]. Enzymes were diluted in the same buffers as mentioned above except for cathepsin C, where 0.1 M Tris–HCl buffer, pH 7.0, containing 5 mM DTE and 1.5 mM EDTA, was used. Cathepsin L (0.07 μM) and 0.1 μM cathepsin B (all final concentrations) were titrated with Ep-475; 0.05 μM cathepsin H and 0.03 μM cathepsin C were titrated with 100% active stefin A. Residual activities were determined as stated above. For cathepsin C, Gly-Phe-MNA was used as the substrate.

2.5. Determination of k_{cat}/K_M

The substrates used were Z-Phe-Arg-MCA, Z-Arg-Arg-MCA and Arg-MCA. Each substrate was used at a final concentration far below its K_M value for the particular enzyme. This enabled us to determine the catalytic constant, k_{cat}/K_M, by use of Eq. 1:

$$\nu = (k_{cat}/K_M)[E_o][S] \qquad (1)$$

where ν is the initial velocity, k_{cat} the catalytic rate constant, K_M the Michaelis constant, E_o the total concentration of the enzyme and S the substrate concentration. The buffers were the same as those used in the enzyme assays. Each enzyme was activated in the buffer solution in a fluorimetric cuvette thermostated at 37°C. After 5 min, substrate was added in a negligible volume. The release of product was monitored continuously on a Perkin-Elmer LS-3 fluorimeter, connected to an IBM-XT computer running the Flusys software [33]. The fluorimeter was calibrated with 7-MCA as the standard at excitation and emission wavelengths of 370 nm and 460 nm, respectively.

2.6. PAGE in the presence of SDS

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was carried out in an 8–25% polyacrylamide gradient gel, or in a 7.5% polyacryamide homogeneous gel, on the PhastSystem apparatus, as recommended by the manufacturer. Where stated, samples were reduced with 5% 2-mercaptoethanol at 100°C for 5 min prior to electrophoresis. Gels were calibrated using a low-molecular-mass calibration kit. All gels were stained with 0.2% Coomassie Brilliant Blue G-250.

2.7. Analytical isoelectric focusing

Isoelectric focusing was carried out on polyacrylamide plates with Pharmalyte carrier ampholines in the pH range of 3–10, using the PhastSystem apparatus, as recommended by the manufacturer. A mixture of standard proteins was

run parallel to the samples. Gels were stained with Coomassie Brilliant Blue G-250.

2.8. N-terminal sequencing

Prior to N-terminal sequencing, samples were dialysed in 10% (v/v) acetic acid in water, or desalinated by HPLC on a Chromspher C_8 column. Automated sequence analysis was performed on an Applied Biosystems 475 liquid sequencer connected on-line to a 120 A phenylthiohydantoin-amino acid analyser from the same manufacturer.

3. Results and discussion

The flow diagram of the isolation procedure is shown in Scheme 1. The entire purification procedure was carried out at 4°C using simple chromatographic methods. Kidneys were chosen as the most appropriate organ for the isolation of these enzymes on the basis of our previous work with cathepsins H and L. This choice is in agreement with the distribution of cathepsin H in rat tissues [34]. Furthermore, the amount of cathepsin B isolated by
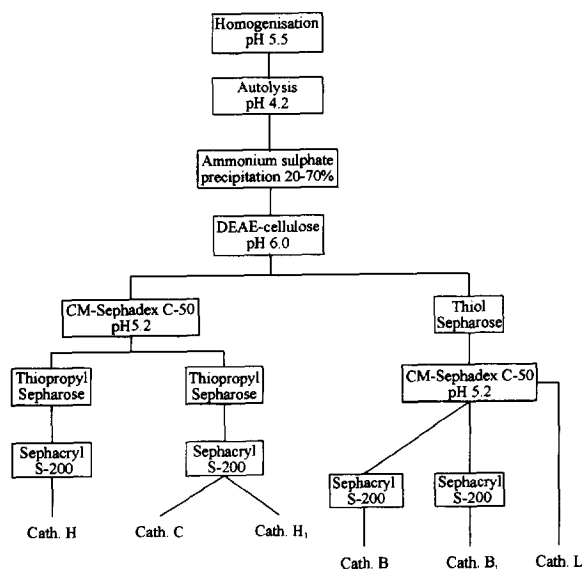
Scheme. 1. Flow diagram of the simultaneous procedure for the isolation of cathepsins B, H, L and C from human kidney.

the same method from human liver or kidney was similar [35]. The human kidney extract was prepared as is generally recommended for cysteine proteinase isolation [27,29,36–40] except for autolysis, which was performed at lower temperatures (4°C) overnight, since we found it more convenient for extraction of cathepsins B and H. Concentration of the extracted proteins was achieved by ammonium sulphate precipitation rather than with acetone, since ammonium sulphate precipitation is more appropriate for larger volumes. By the first chromatographic step on DEAE cellulose at pH 6.0, we managed to separate cathepsins H and C from cathepsins B and L. This step was crucial in cathepsin H isolation, since later binding of cathepsin H to thiopropyl Sepharose was more efficient in the absence of cathepsin B [27]. Prior to binding to thiopropyl Sepharose, cathepsins H and C were separated on CM-Sephadex C-50 at pH 5.2, using a NaCl con-

centration gradient (Fig. 1). Fractions containing the main cathepsin H activity were eluted at 0.1–0.14 M NaCl and activated, desalted and bound to thiopropyl Sepharose as suggested [28]. The unbound material did not contain any Arg-NA activity, and the release of cathepsin H from thiopropyl Sepharose was achieved by soaking the Sepharose in 20 mM cysteine in phosphate buffer, pH 6.8, at 4°C overnight. The final cathepsin H purification was carried out on a high-resolution Sephacryl S-200 column (Fig. 2). The fractions from the CM-Sephadex C-50 column, containing a minor part of Arg-NA activity and a broad peak of Z-Phe-Arg-MCA activity (Fig. 1), were purified in the same way as cathepsin H, using covalent chromatography and gel filtration (Fig. 3). The high-molecular-mass peak of Z-Phe-Arg-MCA activity from the Sephacryl S-200 column represented purified cathepsin C, and the Arg-NA activity peak corresponded to a minor isoform of cathepsin H (cathepsin H_1). The second Z-Phe-Arg-MCA activity peak in the 30 000 M_r range was further purified on DEAE-cellulose at pH 7.5 and characterised on the basis of enzymatic activities as a
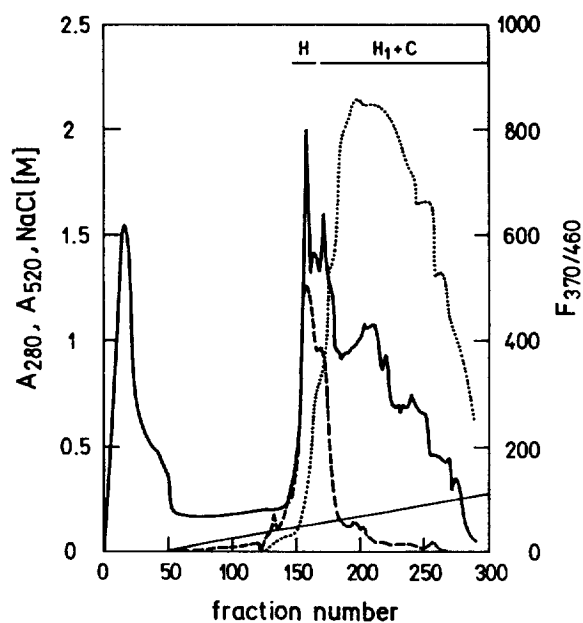

Fig. 1. Cation-exchange chromatography on CM-Sephadex C-50, equilibrated with 0.02 M sodium acetate buffer, containing 1 mM EDTA, of unbound fractions from DEAE-cellulose. The column was eluted with a NaCl gradient (thin line). Proteins were monitored at A_{280} (solid line), activity towards Arg-NA is expressed as A_{520} (dashed line) and towards Z-Phe-Arg-MCA as $F_{370/460}$ (dotted line). The fractions combined for further purification are indicated by horizontal bars.
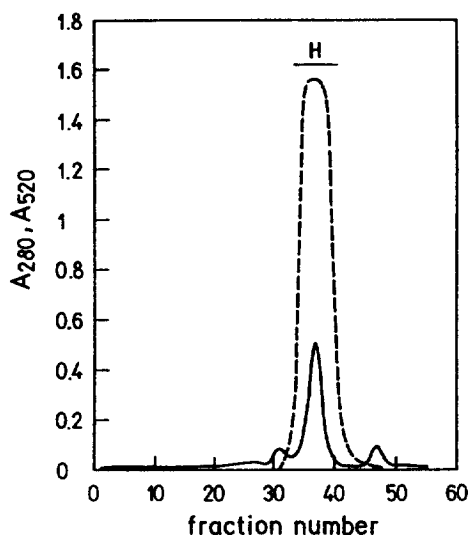
Fig. 2. Final purification of cathepsin H by gel permeation on Sephacryl S-200. Proteins were measured as A_{280} (solid line) and activity towards Arg-NA is expressed as A_{520} (dashed line). The fractions containing purified cathepsin H are indicated by horizontal bars.
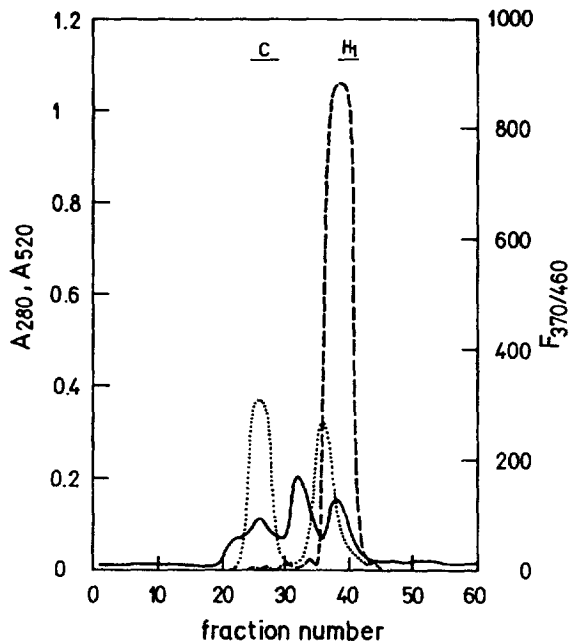
Fig. 3. Final purification of cathepsin C and cathepsin H_1 by gel permeation on Sephacryl S-200. Protein was measured as A_{280} (solid line). Activity towards Arg-NA is expressed as A_{520} (dashed line), and activity towards Z-Phe-Arg-MCA as $F_{370/460}$ (dotted line). The fractions containing pure cathepsin C and cathepsin H_1, respectively, are indicated by horizontal bars.

Fig. 4. Cation-exchange chromatography on CM-Sephadex C-50, equilibrated with 0.02 M sodium acetate buffer, containing 1 mM EDTA, of fractions eluted with 0.25 M NaCl from DEAE-cellulose. The column was eluted with a NaCl gradient, 0.3 M NaCl and 0.6 M NaCl as indicated (thin line). Proteins were measured as A_{280} (solid line), activity towards Bz-D,L-Arg-NA is expressed as A_{520} (dashed line) and activity towards Z-Phe-Arg-MCA as $F_{370/460}$ (dotted line). The fractions combined for further purification of cathepsins B and B_1 and fractions containing pure cathepsin L are indicated by horizontal bars.

minor amount of cathepsin B, which was not further investigated.

Cathepsin B and L activities, obtained by the first step from DEAE-cellulose (Scheme 1), were activated and extensively dialysed to remove DTE prior to binding to thiol Sepharose. Unbound material from thiol Sepharose contained some Bz-D,L-Arg-NA and Z-Phe-Arg-MCA activity, but was discarded. Elution of bound material was performed with 20 mM cysteine overnight. Purification was continued on a CM-Sphadex C-50 column, where two isoforms of cathepsin B and cathepsin L were separated (Fig. 4). Cathepsin L appeared to be pure after this step, while both isoforms of cathepsin B had to be separately purified on Sephacryl S-200 columns. A typical profile of the separation of the main isoform of cathepsin B, designated as cathepsin B, is shown in Fig. 5. A similar profile appeared when the minor isoform of cathepsin B, named cathepsin B_1 was purified on a Sephacryl S-200 column.
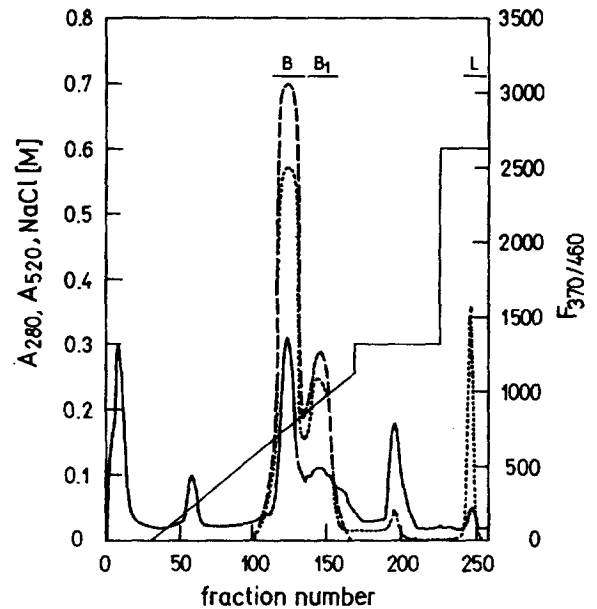
In a typical experiment, the amounts of purified cathepsins obtained from 3.5 kg of human kidney were as follows: B, 25 mg; B_1, 2.1 mg; H, 6.5 mg; H_1, 0.4 mg; L, 1.5 mg and C, 3.8 mg. The relatively high amount of starting material was used with the aim of obtaining greater amounts of purified enzymes in a relatively short time, although this resulted in somewhat lower yields than when the separation is run using a small amount of the starting material [25]. The amounts of enzymes obtained are similar to those obtained using our previously described method [27] or the method of Moin et al. [41], but lower than in other methods [37–40,42]. The reason may be that these methods were optimised for a single enzyme. The amount of purified cathepsin H_1 is low, but the other half of it remained unpurified, as is evident from Fig. 3. The yield of cathepsin C was virtually the same as that obtained
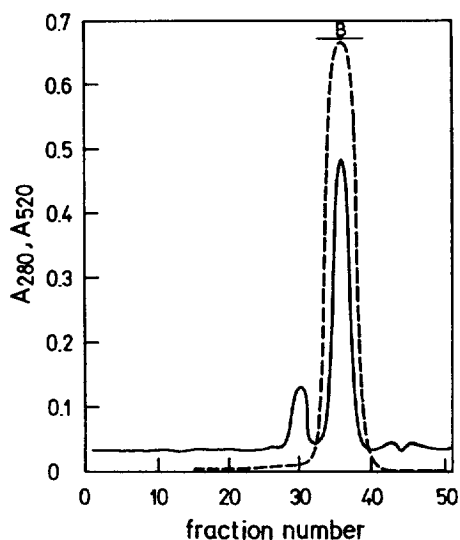
Fig. 5. Final purification of cathepsin B by gel permeation on Sephacryl S-200. Proteins were measured as A_{280} (solid line) and activity towards Bz-D,L-Arg-NA is expressed as A_{520} (dashed line). The combined fractions containing pure cathepsin B are indicated by horizontal bars.

detected in our SDS–PAGE. The minor isoform, cathepsin B_1, showed two bands with M_rs of 28 000 and 24 000 under reductive conditions (Fig. 6a), representing a mixture of single chain and double chain forms of cathepsin B, respectively [13]. Human liver cathepsin B, purified by the affinity method on the semicarbazone of Gly-Phe-glycinal linked to Sepharose 4B [42], was almost completely cleaved into heavy and light chains, while cathepsin B from different human tumour tissues obtained by Moin et al. [41], using basically the same method as Rich et al. [42] also showed considerable amounts of single chain cathepsin B. However, no information is available at present about the isolation of pure single chain cathepsin B from human tissue. We did not detect a doublet on SDS–PAGE representing glyco-sylated and non-glycosylated heavy chains of cathep-
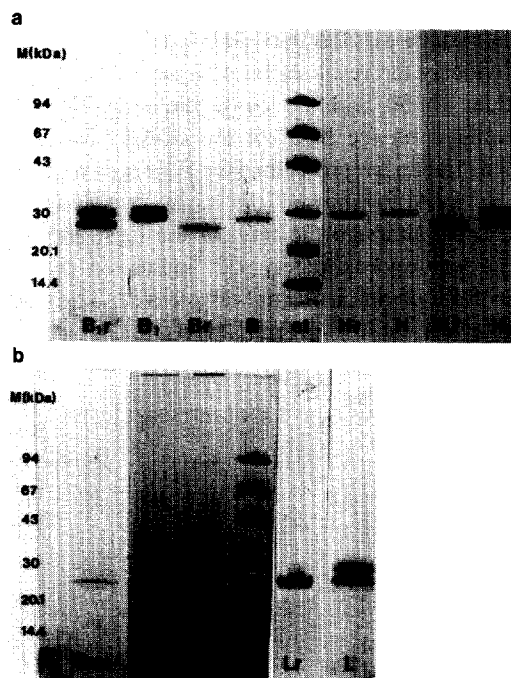
for cathepsin C isolated from human spleen [44], but somewhat lower than that isolated from human kidney by Dolenc et al. [44], who completed the isolation procedure without freezing the samples. The amount of isolated cathepsin L was rather low if compared to previous procedures [39,40,45]. It remains to be determined whether the binding of cathepsin L to thiol Sepharose would be more successful in the absence of cathepsin B, which could be removed by CM-cellulose chromatography prior to covalent chromatography.

3 1. Cathepsin B characterisation

SDS–PAGE, under non-reducing conditions for both isoforms of cathepsin B, showed a band with a M_r of 28 000. If samples were reduced prior to SDS–PAGE, the main isoform of cathepsin B showed only a single band with a M_r of 24 000, representing the heavy chain, while the light chain with a M_r of about 5000, which is linked to the heavy chain by disulphide bonds, could not be

Fig. 6. SDS–polyacrylamide gel electrophoresis of purified enzymes. (a) Cathepsin B, B; cathepsin B_1, B_1; cathepsin H, H; cathepsin H_1, H_1; standard proteins, st. (b) Cathepsin C, C; cathepsin L, L. When the sample was reduced prior to electrophoresis, the letter "r" is added to the symbol. Electrophoresis of cathepsin C reduced in the presence of 5 M urea is designated as Cr(u).

sin B, as was detected for cathepsin B from human liver [41] and human kidney [46].

The purity of our preparation of cathepsin B was also tested by isoelectric focusing. The main cathepsin B isoform, completely cleaved into heavy and light chains, showed a single band with a pI of 5.3, while the cathepsin B$_1$ isoform, consisting of a mixture of single chain and double chain enzyme, showed two bands with pIs of 5.3 and 5.5, respectively (Fig. 7a). The results can be compared with isoelectric focusing of six isozymes of human liver cathepsin B with pIs ranging from 4.5 to 5.5 [36].

N-Terminal sequencing of the main cathepsin B isoform revealed two N-terminal sequences (VSVE- and LPAS-) belonging to the heavy and light chains of cathepsin B, respectively, in an approximate molar ratio of 1:1, showing that the sample was completely cleaved into two polypeptide chains. This is consistent with previous results of the complete amino acid sequence of human cathepsin B [14]. It should be mentioned that the two-chain form of cathepsin B is obviously the result of limited proteolysis between residues 47–50, with the loss of the dipeptide, as was determined on the basis of the cDNA sequence of cloned human prepro-cathepsin B [47]. The cathepsin B$_1$ isoform showed the same two N-terminal sequences, although the ratio of the light and heavy chains was approximately 1:2, indicating that only about 50% of this isoform was cleaved. The finding
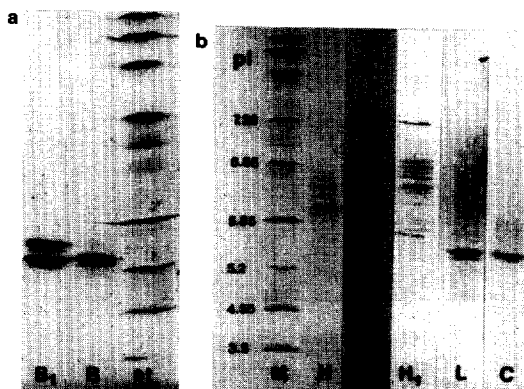
supports our decision to name the two forms of cathepsin B as isozymes, since differences in their polypeptide chains is minimal.

For further biochemical characterisation of cathepsin B, the activities of the isozymes towards different synthetic substrates have been determined. The k_{cat}/K_M was chosen as a measure of specificity of the enzyme for the particular substrate. The exact concentration of the enzyme was determined by active site titration. Both isoforms of cathepsin B were determined to be 75–85% active. Their k_{cat}/K_M values for Z-Phe-Arg-MCA and Z-Arg-Arg-MCA are summarized in Table 1. No significant difference in activity between the isoforms is apparent. It can be concluded that the cleavage of the enzyme into light and heavy chains does not alter its enzymatic activity towards these substrates. Similar results were obtained for different forms of porcine liver cathepsin B for some protein and synthetic substrates, but surprisingly, the cleaved cathepsin B showed much higher aldolase inactivating activity than did the uncleaved form [48]. Our determinations of k_{cat}/K_M for Z-Phe-Arg-MCA are in good agreement with those of Barrett [49] for human cathepsin B, Wada and Tanabe [50] for chicken cathepsin B, and Takahashi et al. [51] for the porcine enzyme. On the other hand, the discrepancies with some other k_{cat}/K_M determinations [21,25,52] are obvious, which may result from the different methods used or because of incompletely purified enzyme. However, it can be concluded that substrates with Phe on the P$_2$ position (as defined by Schechter and Berger [53]) are much more susceptible to cleavage with cathepsin B than those with Arg on the P$_2$ position, if in both cases P$_1$ is occupied ([21,41,54,55]).

Fig. 7. Isoelectric focusing of purified enzymes. (a) Cathepsin B$_1$, B$_j$; standard proteins, st. (b) Cathepsin H isolated in two purification runs (H), cathepsin H$_1$, H$_j$; cathepsin L, L; cathepsin C, C.

Table 1
k_{cat}/K_M values for cathepsins B, H and L

Substrate	k_{cat}/K_M (s^{-1} M^{-1})		
	Z-Phe-Arg-MCA	Z-Arg-Arg-MCA	Arg-MCA
Cathepsin B	359·10^3	129·10^3	N.D.[a]
Cathepsin B$_1$	307·10^3	140·10^3	N.D.
Cathepsin H	1.6·10^3	0.2·10^3	39·10^3
Cathepsin H$_1$	1.9·10^3	0.3·10^3	48·10^3
Cathepsin L	2760·10^3	26·10^3	N.D.

[a] N.D.=not determined.

3.2. Cathepsin H characterisation

The main form of cathepsin H, reduced with 2-mercaptoethanol or unreduced, showed a single band with a M_r of 30 000 (Fig. 6a), as reported previously [27,38,56]. Cathepsin H_1 showed two bands with M_rs of 30 000 and 26 000, when run unreduced in SDS–PAGE, while a doublet with a M_r of around 26 000 appeared when the sample was reduced with 5% of 2-mercaptoethanol, prior to electrophoresis (Fig. 6a). It can be concluded that the majority of cathepsin H was isolated as uncleaved enzyme, while cathepsin H_1 represents the completely cleaved enzyme, consisting of heavy and light chains which are linked by disulphide bonds. Two bands with M_rs of 30 000 and 25 000 were also observed by Dalet-Fumeron et al. [25] for reduced human liver cathepsin H on SDS–PAGE.

In isoelectric focusing, the two forms of cathepsin H behaved differently (Fig. 7b). The main form from most preparations exhibited two main bands with p*I*s of 6.1 and 6.3, or in some preparations a single band with a p*I* of 6.1. Similar values were observed also by other authors [25,27,38,56]. Cathepsin H_1 showed several bands with considerably higher p*I* values of 6.3–6.8. This heterogeneity in p*I* values may be the consequence of differences in N-terminal extensions of the heavy chain, the extent of cleavage into two-chain enzyme, oligosaccharide structure or phosphorylation.

N-Terminal sequencing was performed only with the main cathepsin H form, showing two main bands on isoelectric focusing. The results were in agreement with those already reported [15]. Four N-terminal sequences were obtained. Two of these, approximately equimolar, represented single chain cathepsin H starting with YPPS- or with GPYP-, which is also the beginning of the heavy chain. The light chain and mini chain of the proregion, starting with GIPY- and EPQ-, respectively, were also detected.

Active site titration with stefin A revealed that the main form of cathepsin H was 70–80% active, while cathepsin H_1 showed about 40% activity. The enzymatic properties of both forms were tested on three substrates (Table 1). Both forms exhibited very similar k_{cat}/K_M values for each of the tested sub-

strates. The value for Arg-MCA in our determination is a little higher than in Ref. [21], but within the same order of magnitude. The discrepancy can be attributed to the less accurate determination of the active enzyme concentration by titration of the enzyme with E-64 compared to that obtained with the tight binding inhibitor, stefin A [27]. The determinations of Dalet-Fumeron et al. [25] cannot be compared with ours, since their ratio of activities towards the N-terminally blocked substrate Z-Phe-Arg-MCA and free Arg-MCA was near 1:1, while our determination showed a ratio of about 1:25 for the same substrates. Rothe and Dodt [23] observed a 25-fold decrease in k_{cat}/K_M for N-terminally blocked Bz-Arg-MCA compared to Arg-MCA. Obviously, cathepsin H exhibits a preference for N-terminally free substrates, even though they are small molecules.

3.3. Cathepsin L characterisation

The isolated cathepsin L showed two bands with M_rs of 30 000 and 25 000 in SDS–PAGE, when run unreduced. If reduced prior to electrophoresis, a single band with a M_r of 25 000 was obtained (Fig. 6b), indicating that mature cathepsin L is cleaved into heavy and light chains connected only by S–S bonds [39,40].

Isoelectric focusing of purified cathepsin L resulted in two bands close together with p*I*s of about 5.4 (Fig. 5.4). The existence of several multiple isoeletric forms of human liver cathepsin L has been reported [25,39]. On the basis of our experience working with bovine cathepsin B from lymph nodes and spleen [31,37], and from the work of other authors dealing with porcine cathepsin B from liver and kidney [51], the conclusion can be drawn that a different organ from the same species can be the reason for the differences in the isoelectric focusing pattern of an enzyme.

N-Terminal sequencing of our cathepsin L sample revealed an equimolar ratio of two sequences starting with APRS- and NNKY- representing heavy and light chains, respectively, as already reported [15,57].

Active site titration of the isolated cathepsin L with Ep-475 indicated that 40–50% of the enzyme is

active. Its activity towards two different substrates was determined in terms of k_{cat}/K_M (Table 1). The activity towards Z-Phe-Arg-MCA was 100 times greater than towards Z-Arg-Arg-MCA, as had also been observed for human liver cathepsin L [39]. The k_{cat}/K_M value of 2550×10^{-3} M^{-1} s^{-1} for Z-Phe-Arg-MCA, is in good agreement with some determinations [21,25], and is the same order of magnitude as in others [39,52].

3.4. Cathepsin C characterisation

The high molecular mass of cathepsin C is evident from the elution profile obtained from an Sephacryl S-200 column (Fig. 3). A more accurate molecular mass of cathepsin C was determined by SDS–PAGE in 7.5% polyacrylamide homogeneous gels and in 8–25% polyacrylamide gradient gels. In a 7.5% gel, a band with a M_r of 200 000 appeared when cathepsin C was run unreduced. With prior reduction, a band with a M_r of 55 000 was obtained (not shown). In 8–25% gels, unreduced cathepsin C could not penetrate into the gel, while the reduced sample showed two bands with M_rs of 24 000 and 29 000, respectively and a weak band with a M_r of about 55 000 (Fig. 6b). The oligomeric character of the 200 kDa cathepsin C was established many years ago [58], but its quaternary structure is still a matter of discussion. Ishidoh et al. [17] and Muno et al. [19], studying the rat enzyme, showed that procathepsin C has an unusually long proregion, and that the mature enzyme consists of heavy chains with a molecular mass of 25 kDa and C-terminally located light chains with a molecular mass of 7.8 kDa, which are homologous to the heavy and light chains of proteinases of the papain superfamily. Dolenc et al. [44], using polyacrylamide gel electrophoresis, obtained a M_r of around 50 000 for the human cathepsin C subunit, which, after reduction, dissociated into heavy and light chains of mature cathepsin C with M_rs of 22 000 and 6000, respectively. Our results are quite consistent with this, although it seems that our cathepsin C, when run in an 8–25% gel, was not completely reduced, so we were able to detect three bands with M_rs of 55 000, 24 000 and 29 000 representing a 50 kDa subunit of cathepsin C, heavy chain and unseparated heavy and light chains, re-

spectively. If the sample was reduced in the presence of 5 M urea, only one band with a M_r of 24 000 was observed (Fig. 6b). Other authors [43], working with human spleen cathepsin C reduced with 2-mercaptoethanol, detected only one band with a M_r of 24 000 in SDS–PAGE.

Isoelectric focusing of the cathepsin C which we have isolated showed a single band with a pI of 5.4 (Fig. 7b), which is identical to that of the human spleen enzyme [43]. The value is lower than that determined by electrophoretic titration of human kidney cathepsin C [44]. The difference could arise from different methods used for pI determination or from different isolation procedures.

N-Terminal sequencing of unreduced cathepsin C revealed three sequences starting with DTPAXC-, LPQSW- and DPFNP-, indicating N-termini of proregion, heavy and light chains of cathepsin C, respectively, in accordance with the observations of Dolenc et al. [44]. If cathepsin C was dialysed against 5 mM DTE through a membrane with a 6–8 kDa cut-off point prior to N-terminal analysis, only the sequence of heavy chain was obtained, indicating that the light chain and the peptide of proregion with DTPAXC-N-terminal sequence are connected to the heavy chain only by disulphide bonds and that their M_rs are lower than 8000.

During the isolation procedure, the activity of cathepsin C was followed with Z-Phe-Arg-MCA in the presence of 20 mM NaCl and reducing agent. Its reactivity towards this substrate was reported by Liao and Lenny [59], although cathepsin C is designated as an enzyme capable of sequentially removing dipeptides from the N-termini of suitable substrates, such as Gly-Phe-NA [43,44,60,61].

The low sensitivity of cathepsin C to Ep-475 was observed. A 50 molar excess of the inhibitor was needed for about 60% inhibition of the enzyme, as similarly reported for some other epoxysuccinyl peptide inhibitors [44,60]. Cathepsin C was inhibited rapidly with stefin A. A titration curve with this inhibitor could be obtained, when residual activity was tested with Gly-Phe-MNA as substrate.

In conclusion, a procedure for the simultaneous isolation of four human cysteine proteinases cathepsins B, H, L and C of the papain superfamily has been developed. The method is suitable for the

isolation of large amounts of these enzymes in a single run using simple chromatographic methods. Also, two isozymes of cathepsins B and H, respectively, were obtained. The characterisation of these enzymes convinced us that the method provides pure and highly active cathepsins suitable for further biochemical and physiological investigations. Unfortunately, the method did not enable us to obtain human cathepsin S, despite its predicted existence in human liver [62].

Acknowledgments

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