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Rapid affinity chromatographic method for the isolation of human cathepsin H

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ABSTRACT

Cathepsin H was purified by a single-step affinity chromatographic method from crude human kidney extract. The affinity medium consisted of low-molecular-mass cysteine proteinase inhibitors from potato tubers (PCPIs) coupled to cyanogen bromide-activated Sepharose. The yield of the method is comparable to that of the classical methods. Isoelectric focusing and sodium dodecyl sulphate polyacrylamide electrophoresis showed high purity of the isolated cathepsin H. N-Terminal sequence analysis revealed that intact single-chain cathepsin H was obtained. Binding of the enzyme to the PCPI-Sepharose showed that a free SH group in the cysteine proteinase is not required for complex formation.

INTRODUCTION

Lysosomal cysteine proteinases cathepsins B (EC 3.4.22.1), H (EC 3.4.22.16), L (EC 3.4.22.15) and S (EC 3.4.22.–) play an important role in protein degradation processes [1]. On the bases of their primary structure they belong to the group of closely related proteins of the papain superfamily [2]. Since these enzymes have similar molecular mass and other biochemical properties, their separation by most of the classical isolation procedures is time-consuming. On the other hand, few methods involving affinity chromatography or covalent chromatography for the isolation of lysosomal cysteine proteinases have been described [3–6], but none of these methods was selective for cathepsin H. In the present work a single-step affinity chromatographic method for the isolation of cathepsin H from human kidney extract was developed. As an affinity medium the low-molecular-mass cysteine proteinase inhibitors from potato tubers (PCPIs) coupled to CNBr-activated Sepharose were used. These inhibitors were described recently [7,8] and may well be members of a new superfamily of inhibitors of cysteine proteinases, as judged by their primary structure and inhibitory properties. The kinetic data presented in this paper, together with previously published results [9], demonstrate sufficient selectivity in the inhibition of cysteine proteinases, which makes these inhibitors suitable candidates for the affinity chromatographic purification of lysosomal cysteine proteinases.

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EXPERIMENTAL

Materials

CNBr-activated Sepharose, 8–25% polyacrylamide gradient gel (Phast Gel Gradient), Pharmalyte (pH 3–10), broad calibration kit and low-molecular-mass calibration kit were supplied by Pharmacia-LKB (Uppsala, Sweden). Papain (twice crystallized, EC 3.4.22.2), Bz-Arg-NNap and Arg-NNap (Bz = benzoyl; NNap = 2-naphthylamide) were from Sigma (Munich, Germany). Z-Phe-Arg-NHMec (Z = benzyloxycarbo-

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nyl; NHMec = (4-methyl-7-coumaryl)amide) was purchased from Bachem (Bubendorf, Switzerland). L-3-Carboxy-2,3-*trans*-epoxypropionylleucylamido-4-guanidino butane (E-64) was from Peptide Research Foundation (Osaka, Japan). Trifluoroacetic acid was from Applied Biosystems (Forest City, CA, USA) and acetonitrile was from Rathburn (Walkerburn,UK). The Chromspher C_8 high-performance liquid chromatography (HPLC) column was from Chrompack (Frankfurt, Germany).

Electrophoresis was carried out on Phast System apparatus from Pharmacia-LKB. HPLC instrumentation used was from LDC/Milton Roy (Stone, UK). The 475A liquid-phase sequenator was from Applied Biosystems. Fluorescence was measured on a LS-3 fluorimeter from Perkin-Elmer (Beaconsfield, UK).

Highly purified PCPI with a p*I* of 8.3 (PCPI 8.3) was obtained as described [7]. Its active concentration was determined by titration with cathepsin L which was previously titrated with E-64 [10].

Human cathepsins B, H and L for kinetic measurements and antibody preparation were prepared as previously described [11,12].

Carboxymethylated papain was prepared by dissolving papain in 0.1 *M* phosphate buffer, pH 7.0, containing 2 m*M* dithioerithrol (DTE), 1 m*M* ethylenediaminetetraacetic acid disodium salt (EDTA) and 11 m*M* iodoacetic acid at room temperature. Undissolved material was filtered and discarded. The solution of the carboxymethylated papain was transferred by dialysis into 0.1 *M* phosphate buffer, pH 7.0, containing 0.5 *M* NaCl and 1 m*M* EDTA.

PCPI-Sepharose

Inhibitors of cysteine proteinases were partially purified from potato tubers of variety Pentland Squire as already described [7]. The low-molecular-mass inhibitory fractions eluted from Sephacryl S-200 were collected and used as ligands. More than 70% of these inhibitors were PCPI 8.3. A total of 30 mg of protein inhibitor was bound to 20 g (dry weight) of CNBr-activated Sepharose 4B, according to the procedure described by the manufacturer.

Crude kidney extract

The extract was prepared from human kidney as described [11]. The acidified and centrifuged homogenate was precipitated with ammonium sulphate to 20% saturation. The precipitate was discarded and the supernatant was again precipitated with ammonium sulphate to 70% saturation. The precipitate was suspended in 0.02 *M* phosphate buffer, pH 7.0, containing 0.5 *M* NaCl and 1 m*M* EDTA, and dialysed against the same buffer overnight at 4°C. The dialysate was centrifuged, divided into portions of 15 ml and stored at −20°C.

Affinity chromatography

A column (7 cm × 4 cm I.D.) containing 80 ml of PCPI-Sepharose was washed with 0.02 *M* phosphate buffer, pH 7.0, containing 0.5 *M* NaCl and 1 m*M* EDTA. A 15-ml volume of crude human kidney extract was slowly run into the gel. After application of the entire sample, washing was continued with 500 ml of the starting buffer at a flow-rate of 3.3 ml/min. Elution was performed with 0.02 *M* citrate buffer, pH 3.4, containing 1 m*M* EDTA, and finally with 0.01 *M* NaOH. Prior to elution 1.7 ml of 1 *M* ammonium acetate were added to each test tube in order to preserve enzymatic activity. Fractions eluted with the citrate buffer were concentrated and transferred by ultrafiltration on an Amicon YM-5 membrane into 0.02 *M* acetate buffer, pH 5.5, containing 1 m*M* EDTA. The affinity medium was stored at 4°C in 0.02 *M* phosphate buffer, pH 7.0, containing 1 *M* NaCl.

PAGE in the presence of SDS

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was carried out in a 8–25% polyacrylamide gradient gel on the Phast System apparatus as recommended by the manufacturer. Some samples were reduced with 5% β-mercaptoethanol at 100°C for 10 min prior to electrophoresis. Gels were stained with 0.1% Commassie Brilliant Blue G-250. Calibration was achieved using a low-molecular-mass calibration kit.

Analytical isoelectric focusing

Isoelectric focusing was carried out on Phast System apparatus as recommended by the manufacturer. Polyacrylamide plates with Pharmalyte carrier ampholines of pH 3–10 were used. A mixture of standard proteins was run parallel to the sample on the same gel.

Amino acid sequence determination

For the amino acid sequence determination samples were prepared by HPLC on the Chromspher C_8 column. Elution was started with buffer A which consisted of 1.00% (v/v) trifluoroacetic acid in water and was continued with a gradient of buffer B which contained 80% (v/v) acetonitrile in buffer A. The flow-rate was 1.0 ml/min and absorbance was monitored at 215 nm. The eluted peak was N-terminally sequenced using an Applied Biosystems 475A liquid-phase sequenator. Phenylthiohydantoin derivatives were identified on line using the attached 120A HPLC system [13].

Enzyme assays

Samples were tested for enzymic activity as described previously [10]. Cathepsin B and papain were tested with Bz-Arg-NNap, cathepsin H with Arg-NNap and cathepsin L with Z-Phe-Arg-NHMec as substrates. One unit (U) of enzyme activity hydrolyses 1 μmol of substrate per min.

Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed using immunoselective polyclonal antibodies against cathepins B, H and L. Antisera were raised according to Morton *et al.* [14] by injection of rabbits or sheep with human cathepsin B, cathepsin H or cathepsin L. Immunoselective antibodies were purified from rabbit and sheep antisera by the use of immunoadsorbent columns, containing human kidney cathepsin B, cathepsin H or cathepsin L bound to the CNBr-activated Sepharose 4B. Quantitative amounts of cathepsins B, H and L in different samples were determined using ELISAs as previously described [15,16].

Determination of protein

Proteins in the crude material were determined using the method of Lowry *et al.* [17]. The concentrations of proteins in the fractions and the concentration of the pure cathepsin H were determined by direct measurement of absorbance at 280 nm, assuming that the specific absorbance at 280 nm ($A^{1\%}$) for cathepsin H, which means absorbance of 1% cathepsin H solution, is 14 as for the bovine enzyme [18].

Determination of the inhibition constants K_i

Continuous fluorimetric assay was used to determine K_i values for the interactions between PCPI 8.3 and cathepsins B, H and L. To start the reaction, 0.01 ml of the enzyme at nanomolar concentration, preactivated in 4 mM DTE, was added to 1.99 ml of the mixture of substrate and inhibitor in the assay buffer. 0.1 M Phosphate buffer, containing 1.33 mM EDTA and 2 mM DTE, adjusted to pH 6.0 and 7.0, was used for cathepsins B and H, respectively. 0.4 M Sodium acetate buffer, containing 1.5 mM EDTA and 2 mM DTE, pH 5.5, was used for cathepsin L. The final concentrations of the substrates were 10 and 5 μM Z-Phe-Arg-NHMec for cathepsin B and L, respectively, and 10 μM Arg-NHMec for cathepsin H. The inhibitor concentration ranged from 3 to 500 nM active PCPI 8.3, depending on the enzyme. The spectrophotometric cuvette was thermostated at 25°C. The time dependence of fluorescence (exitation 370 nm, emission 460 nm) was monitored on a Perkin-Elmer LS-3 fluorimeter, connected to an IBM-XT computer running the program FLU [19]. When a steady state was reached, usually after 30 min, monitoring was stopped. Experimental data were fitted by nonlinear regresion analysis to the equation of Morrison [20]. K_i values were obtained from the relationship $K_i = k_{diss}/k_{ass}$. Each K_i value is the result of at least five experiments.

RESULTS AND DISCUSSION

The prepared PCPI-Sepharose was calculated to contain 16 nmol of protein ligand per ml of gel. The binding capacity of the affinity medium

for cathepsin H was 0.16 nmol/ml of gel. Hence only about 1% of bound inhibitor was interacting with enzyme molecules. This may have been partly due to steric hindrance by the gel matrix. The capacity of the affinity medium did not change substantially after eight applications.

In contrast to the inhibitors of the cystatin superfamily, the nature of binding of PCPIs to cysteine proteinases is not known. We observed that the binding and elution of cathepsin H from the affinity medium was not affected by the presence of 2 m*M* DTE. Furthermore, the same amounts of active and carboxymethylated papain bound to the PCPI-Sepharose column under the same conditions, thus indicating that the free SH-group of cysteine proteinases is not required for complex formation.

The binding and elution profile of the affinity chromatography is shown in Fig. 1. Unbound material showed activity towards Bz-Arg-NNap, Arg-NNap and Z-Phe-Arg-NHMec, thus demonstrating the presence of cathepsins B, H and L. The peak eluted with citrate buffer, pH 3.4, showed major activity towards Arg-NNap and minor activity towards Bz-Arg-NNap. This is characteristic of cathepsin H. Elution with 0.01 *M* NaOH resulted in a protein peak with a low content of all three activities. ELISAs of the same fractions confirmed the presence of all three cathepsins in the unbound material as well as in the alkali-eluted peak, whereas the peak eluted by citrate buffer contained only cathepsin H. To explain the behaviour of cathepsins B, H and L on PCPI-Sepharose, inhibition constants were determined using highly purified PCPI 8.3. The obtained K_i values were 130, 70 and 0.1 n*M* for cathepsins B, H and L, respectively. These values agree well with those previously determined for cathepsins B and L [9]. The general feature of the elution profile of the affinity chromatography agrees with the obtained K_i values. Under chosen conditions where cathepsin B is only slightly retarded, cathepsin H is weakly bound and can be eluted with a change to acidic conditions. The presence of cathepsin L in the unbound material cannot be adequately explained.

Table I summarises the results of the use of PCPI-Sepharose for cathepsin H isolation. A 350-μg amount of pure cathepsin H can be obtained from an ammonium sulphate extract in one experiment. The yield of about 10% is comparable with that of classical isolation methods [11,21]. Cathepsin H obtained by the affinity method showed *ca.* 30% lower specific activity

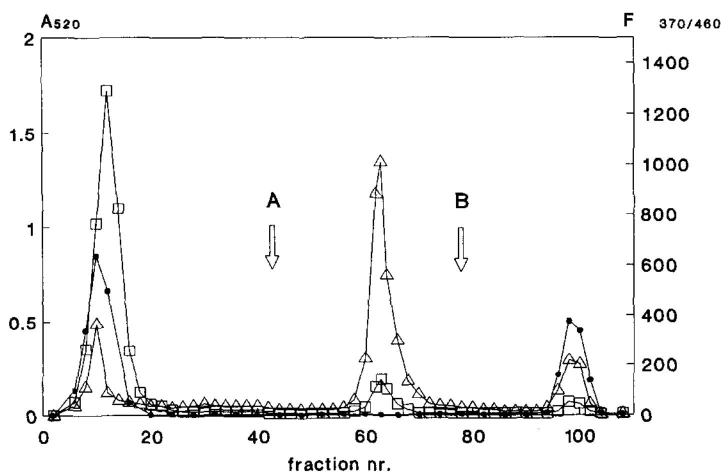

Fig. 1. Elution diagram of crude kidney extract on PCPI-Sepharose. Activities against Arg-NNap (△) and Bz-Arg-NNap (□) are expressed as absorbance at 520 nm, and activity against Z-Phe-Arg-NMec is expressed as *F*370/460 (●). Changes in elution conditions are marked by arrows: A = 0.02 *M* citrate buffer, pH 3.4; B = 0.01 *M* NaOH.

TABLE I

SUMMARY OF CATHEPSIN H PURIFICATION FROM HUMAN KIDNEY BY THE AFFINITY CHROMATOGRAPHY

The values originate from an experiment in which 15 ml of ammonium sulphate extract were applied to PCPI-Sepharose.

	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Acidic homogenate	1080	86	0.08	100	1
$(NH_4)_2SO_4$ extract	360	83	0.23	96	3
Cathepsin H	0.35	8.8	25.0	10	313

than cathepsin H obtained by the classical method [11]. This may be a consequence of the short exposure of cathepsin H to very low pH of 3.4.

The purity of cathepsin H eluted from the affinity medium was examined using electrophoresis. Isoelectric focusing revealed two main bands with p*I* 6.1 and 6.3 (Fig. 2). These values are in good agreement with results for human cathepsin H obtained by classical methods [11,21]. SDS-PAGE of the affinity chromatography-purified cathepsin H showed a single band at M_r 30 000 whether or not the sample was reduced (Fig. 3). SDS-PAGE of the classically isolated cathepsin H showed an identical pattern. N-Terminal sequence analysis of cathepsin H purified by the affinity method was performed after HPLC puri-

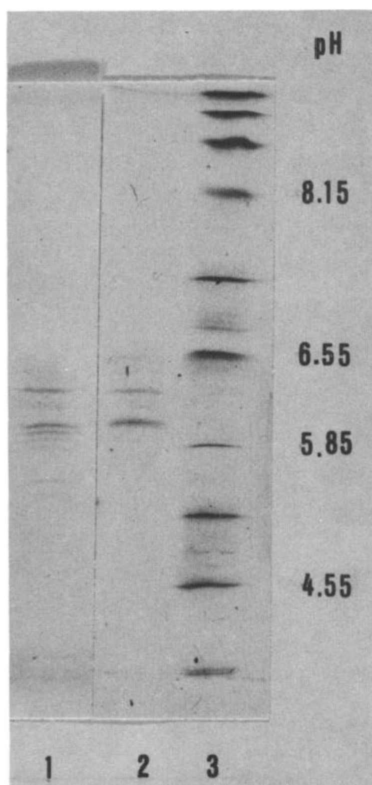

Fig. 2. Isoelectric focusing of cathepsin H. Electrophoresis was performed as described under Experimental. Lane 1: cathepsin H purified by the classical method; lane 2: cathepsin H purified by the affinity chromatographic method; lane 3: standard proteins.

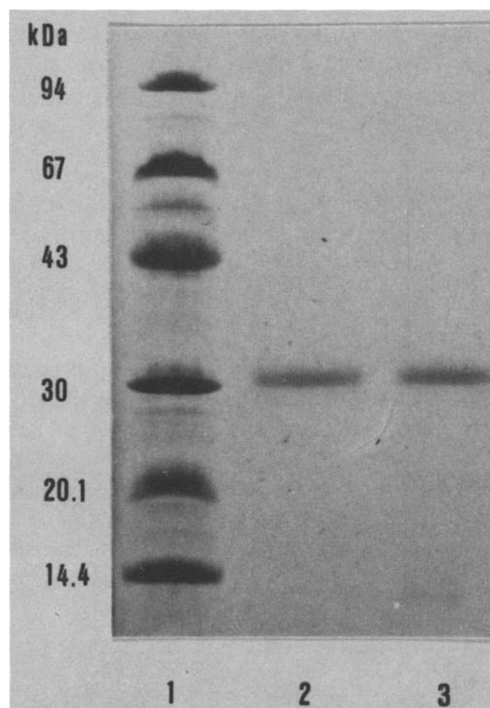

Fig. 3. SDS-PAGE of cathepsin H obtained using the affinity chromatographic method. Electrophoresis was performed on an 8–25% gradient gel as described under Experimental. Lane 1: standard proteins; lane 2: cathepsin H under non-reduced conditions; lane 3: cathepsin H reduced with β-mercaptoethanol for 10 min at 100°C prior to electrophoresis.

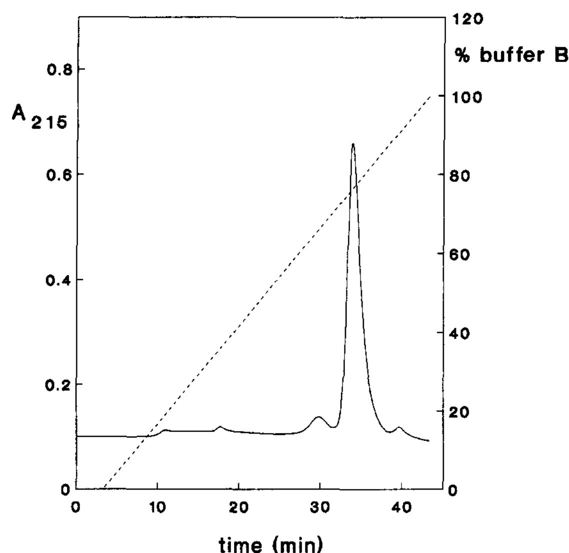

Fig. 4. HPLC diagram of purified cathepsin H. The solid line indicates protein concentration, and the dashed line the gradient of buffer B concentration as described under Experimental. Flow-rate was 1.0 ml/min.

fication. A major protein peak was eluted at about 62% of buffer B (Fig. 4). Sequence analysis of this protein revealed the presence of a mini-chain in an amount equivalent to that of the authentic cathepsin H molecule starting either with Tyr- or with Gly-Pro-Tyr-. No traces of the light chain were found. A similar result was observed for human cathepsin H obtained by the classical method, but in this case always some percentage of this enzyme was processed to the light and heavy chains [22]. Also, rat liver cathepsin H was isolated by a classical method as a 1:1 mixture of the single- and double-chain forms [23]. The correlation of different isolation procedures of cathepsin L and its occurrence in single- or double-chain forms was discussed by Pike and Dennison [24]. They concluded that the rapid isolation procedure results in single-chain cathepsin L. It is possible that this observation may be extended to cathepsin H.

Different affinity methods for cysteine proteinase isolation have been described. Blumberg *et al.* [25] introduced the -Gly-Gly-(OBzl)Tyr-Arg ligand for papain purification, but this method was not applicable to other cysteine proteinases [3]. The aminophenylmercuric ligand introduced for papain by Sluyterman and Wijdnes [26] was also applicable to cathepsin B [3] but the method was not selective. Covalent chromatography on activated thiol Sepharose 4B or thiopropyl-Sepharose 6B represented an advance in cysteine proteinase isolation [4,6,27,28]. A similar principle was used in covalent affinity chromatography on immobilized peptidylcystamine derivatives [5]. These methods are quite specific for cysteine proteinases but selectivity among cysteine proteinases is poor. A new form of affinity chromatography using Gly-Phe-Gly-semicarbazon as a ligand was introduced by Rich *et al.* [29]. The one-step method separated pure cathepsin B from crude human liver extract. The use of different semicarbazons as ligands was extended to ananain [30], histolysin [31] and chymopapain [32].

Our affinity method on PCPI-Sepharose demonstrated an inhibitor–enzyme interaction that was sufficiently selective to discriminate cathepsin H from other cysteine proteinases in the crude starting material. The single-step procedure described here yields 350 μg of pure human cathepsin H in a non-processed single-chain form. The yield of this method is comparable to that of the classical procedures, but the time and effort required are greatly reduced.

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