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Purification, subunit structure and inhibitor profile of cathepsin A

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ABSTRACT

Cathepsin A (EC 3.4.16.1), a lysosomal carboxypeptidase, has been purified 1374-fold from pig kidney. Purification steps included concanavalin A-Sepharose and phenyl-Sepharose chromatography and chromatofocusing. The specific activity (16.9 U/mg) of the purified enzyme was significantly higher than previously reported values. The enzyme preparation appeared homogeneous when analyzed by non-denaturing polyacrylamide gel electrophoresis and was free of detectable protease contamination. The molecular mass (M_r = 97 000), isoelectric point (5.0), and sensitivity to inhibitors were consistent with reported properties of cathepsin A. However, the previously reported three-peptide chain structure was not observed. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence or absence of 2-mercaptoethanol demonstrated that the enzyme is composed of two M_r 47 000 subunits, each of which dissociate in the presence of 2-mercaptoethanol into two polypeptide chains of 19 000 and 31 000.

INTRODUCTION

We previously have reported the presence of angiotensin carboxypeptidase (ACP) activity in a partially-purified preparation of renin from the human kidney [1]. This ACP sequentially removes the C-terminal leucine and histidine from angiotensin I (AI) to form the potent vasopressor hormone, angiotensin II (AII). The amino acid sequences of these and other peptides mentioned below are shown in Fig. 1.

Preliminary characterization [1] of the ACP re-

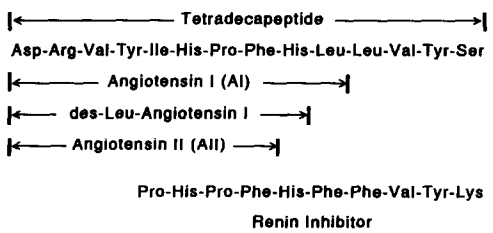

Fig. 1. Amino acid sequences and abbreviations of angiotensin-related peptides.

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vealed that it had a number of similarities to the lysosomal carboxypeptidase, cathepsin A (EC 3.4.16.1), including: (1) stimulation and stabilization by chloride ions and sucrose, (2) lack of requirement for sulfhydryl reagents for activity, (3) presence of an essential sulfhydryl group and (4) a similar molecular mass [2–4]. However, the ACP seemed to have higher activity on peptides with angiotensin-like amino acid sequences than on peptides with unrelated sequences. Besides its activity on AI and des-Leu-AI, the effect of ACP on AI was strongly inhibited by the synthetic tetradecapeptide renin substrate and by a decapeptide renin inhibitor, but it was only weakly inhibited or unaffected by unrelated peptides such as leucine enkephalin, substance P, bradykinin, Pro–Leu–Gly–amide and β-endorphin. AII, however, was neither a substrate nor an inhibitor [1].

These results did not substantiate the reported broad specificity for cathepsin A action on both N-blocked dipeptides [5–7] and natural peptides [7]. Matsuda [7] reported on the hydrolysis of adrenocorticotropin, AI, AII, bradykinin, oxytocin and substance P by cathepsin A. In particular, the hydrolysis of AII occurring nearly as rapidly as AI by cathepsin A is different from the hydrolytic properties of ACP. In addition, Longunov and Orekhovich [8] reported that bovine spleen cathepsin A cleaved Asn^1-Val^5-AII at the Tyr–Val bond, an endopeptidase cleavage. The work of Iodice and coworkers [9,10] demonstrated the carboxypeptidase nature of cathepsin A which suggests that the observation of endopeptidase activity in cathepsin A preparations is probably due to contamination by cathepsin D or other proteinases.

In a subsequent report [11], pig kidney ACP (AI as substrate) was shown by us to co-purify with cathepsin A (benzyloxycarbonyl-glutamyl-tyrosine (Z–Glu–Tyr) as substrate), and it was concluded that most, if not all, of the ACP activity of pig kidney is due to the action of cathepsin A. In light of these results, it was of interest to re-evaluate the substrate specificity of cathepsin A. The purpose of the present work was to prepare highly purified cathepsin A to be used to determine whether or not cathepsin A displays greater activity on peptides with angiotensin-like sequences compared to a variety of peptides with dissimilar sequences. We report here on the purification of cathepsin A from porcine kidney, its subunit structure, and its sensitivity to a variety of inhibitors.

MATERIALS AND METHODS

Materials

Benzyloxycarbonyl-glutamyl-tyrosine (Z–Glu–Tyr), benzyloxy-carbonylglutamic acid (Z-Glu), concanavalin A (Con A)-Sepharose, α-methylmannoside, fluorescein isothiocyanate (FITC)-casein, trypsin, cathepsin D, mersalyl acid 2-(N-morpholino)ethanesulfonic acid (MES), and 3-(N-morpholino)propanesulfonic acid (MOPS) were obtained from Sigma (St. Louis, MO, USA). DEAE-Sephadex, Sephadex G-200, phenyl-Sepharose, Polybuffer Exchanger 94 and Polybuffer 74 were supplied by Pharmacia (Piscataway, NJ, USA). Silver stain kits were from Bio-Rad (Richmond, CA, USA). Fresh pig kidneys were obtained locally from Fischer Packing (Louisville, KY, USA). Des-Leu-AI was custom synthesized by Bachem (Torrance, CA, USA) and all other peptides were purchased from Sigma.

Standard cathepsin A assay

Cathepsin A was assayed with Z–Glu–Tyr as the substrate, and the progress of the reaction was monitored by measurement of the Z–Glu formed by the HPLC method described previously [11]. The assay used 0.25 ml of 3 m*M* Z–Glu–Tyr in 0.1 *M* sodium acetate, 0.1 *M* NaCl, 0.1 *M* sucrose, 1 m*M* EDTA, pH 5.2, pre-warmed to 37°C. Enzyme was added, and the reaction was allowed to proceed for 5–15 min. The reaction was stopped by the addition of 0.75 ml of 250 m*M* phosphoric acid, and the Z–Glu was measured by HPLC. After addition of the phosphoric acid, the Z–Glu concentration was stable. A unit (U) of activity is defined as 1 μmol of product formed per minute. Specific activity is expressed as units per mg protein. Protein was measured by the method of Lowry *et al.* [12] with bovine serum albumin as the standard.

Purification

All of the purification procedures were carried out at 0–4°C. The buffer used for most steps in the purification procedure consisted of 0.1 *M* sodium acetate, 0.1 *M* NaCl, 0.1 *M* sucrose, 1 m*M* EDTA, pH 5.2, and will be referred to as buffer 1. Batches

of six to eight kidneys at a time were processed through the end of step 2. After 18–22 kidneys had been processed during one week, the preparations were pooled and further purified by Con A-Sepharose in step 3. These steps were repeated for two more weeks and then the three post-Con A-Sepharose preparations from a total of 66 kidneys were combined for further purification.

Step 1: tissue extraction

Sixty-six fresh pig kidneys weighing a total of 9.03 kg were obtained locally and processed either the same day or the following day. Whole kidneys were trimmed of most of the tubules and cut into small pieces. The pieces were homogenized in a blender at high speed for 30 s in two volumes of buffer 1. This crude extract was centrifuged at 1 000 *g* for 10 min. The supernatant was saved, and the pellet was re-homogenized in 1 volume of buffer 1. This second homogenate was centrifuged, and the supernatant was combined with the first supernate. The combined supernatants were filtered through five layers of cheesecloth.

Step 2: differential centrifugation

The filtrate was centrifuged at 16 000 *g* for 20 min. The pellet which comprises the mitochondrial/lysosomal fraction was suspended in two volumes of 10 m*M* sodium acetate, 20 m*M* NaCl, 1 m*M* EDTA, pH 5.2, frozen and thawed twice, and then centrifuged at 100 000 *g* for 60 min.

Step 3: ammonium sulfate fractionation

The protein in the 100 000 *g* supernatant was further purified and concentrated by collecting by centrifugation the protein precipitating between 25 and 60% saturation with ammonium sulfate. This pellet was dissolved in a small volume of buffer 1 and frozen until further processing was undertaken.

Step 4: Con A-Sepharose affinity chromatography

A column of Con A-Sepharose (18.6 × 2 cm; 58 ml) was equilibrated with buffer 1 (without EDTA). The enzyme from step 3 (approximately 300 U; 2.7 g) was applied to the column, and 16-ml fractions were collected. The column was washed with the same buffer supplemented with 0.5 *M* NaCl. The enzyme was eluted with 0.1 *M* sodium acetate, 0.5 *M* NaCl, 0.1 *M* sucrose, 1 m*M* EDTA, 0.5 *M* α-methylmannoside, pH 5.2. Fractions containing cathepsin A activity were pooled and concentrated by the addition of ammonium sulfate to 80% saturation. The pellet was resuspended in buffer 1 and frozen until further processed. This step was repeated two more times with the remainder of the enzyme from step 3.

Step 5: phenyl-Sepharose chromatography

A column (11.1 × 2.4 cm; 50 ml) of phenyl-Sepharose was equilibrated with buffer 1. The enzyme from step 4 (463 U, 800 mg) was applied to the column and 16-ml fractions were collected. After application of the sample, the column was washed with a low-ionic-strength buffer (buffer 2) consisting of 0.01 *M* sodium acetate, 0.02 *M* NaCl, 0.1 *M* sucrose, 1 m*M* EDTA, pH 5.2 until the A_{280} of the effluent was below 0.03. The enzyme was eluted with a linear gradient consisting of 250 ml of buffer 2 and 250 ml of 80% ethylene glycol in buffer 1. The active fractions were pooled, dialyzed briefly against buffer 1 to lower the ethylene glycol concentration, concentrated by ultrafiltration using an Amicon PM-30 membrane, and then dialyzed three times against five volumes of buffer 2.

Step 6: DEAE-Sephadex chromatography

A column (20 × 2.4 cm; 90.5 ml) of DEAE-Sephadex A-50 was equilibrated with buffer 2. The enzyme from step 5 (178 U, 46 mg) was applied to the column, and 200-ml fractions were collected. After application of the sample, the column was rinsed with three column volumes of buffer 2, and then a linear gradient was begun which consisted of 250 ml each of buffer 2 and buffer 2 with an additional 0.2 *M* NaCl added. Fractions 20–27 were pooled, and the protein was precipitated with 80% ammonium sulfate. The precipitated protein was collected by centrifugation and resuspended in a small volume of buffer 1.

Step 7: Sephadex G-200 chromatography

A column of Sephadex G-200 (63 × 2.6 cm; 334 ml) was equilibrated with buffer 1. One-half of the enzyme from step 6 (15 ml, 62 U, 5 mg) was applied, and 5-ml fractions were collected. This step was repeated using the remainder of the enzyme from step 6. Fractions containing cathepsin A activity (fractions 34–43) were pooled and concentrated by ultra-

filtration. This pooled and concentrated preparation was re-applied to the same Sephadex G-200 column to ensure complete removal of high-molecular-mass, inactive protein. Again, fractions 34–43 were pooled and concentrated by ultracentrifugation.

Step 8: Chromatofocusing

A column (19 × 1 cm; 14.9 ml) of Pharmacia Polybuffer Exchanger 94 was equilibrated with 0.025 *M* imidazole hydrochloride, 0.1 *M* sucrose and 1 m*M* EDTA, pH 6.8. The enzyme from step 7 was dialyzed briefly (about 4 h) against this starting buffer and applied to the column. The column was eluted with a 1:8 dilution of Pharmacia Polybuffer 74 containing 0.1 *M* sucrose and 1 m*M* EDTA, adjusted to pH 4.0 with HCl. Fractions of 1.8 ml were collected.

Electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out at 4–6°C in 7% polyacrylamide tube gels in the presence of 14 m*M* sodium acetate, 0.1 *M* sucrose and 20 m*M* NaCl at pH 4.0. Electrophoresis was performed in the direction of the cathode at 3 mA per gel tube for 1 and 3.5 h. Gels were stained with Coomassie Blue R250.

Discontinuous sodium dodecyl sulfate (SDS)-PAGE was performed by the method of Laemmli [13] using 0.7 mm 12% gels and the Bio-Rad Mini-Protean II Dual Slab Cell. In addition, the continuous SDS-PAGE system of Weber and Osborn [14] was performed using 7.5% polyacrylamide. Samples were prepared both with and without 2-mercaptoethanol (2-ME), heated at 95°C for 4 min, and run at 200 V (125 V for the Weber and Osborn method) until the bromphenol blue tracking dye reached the bottom of the slabs. Gels were stained either with Coomassie Blue or with silver.

Proteinase assays

The FITC–casein procedure described by Twining [15] was used. FITC–casein (Sigma Type III; 72 μg FITC/mg casein) was dissolved in 50 m*M* sodium phosphate, pH 7.0, at a concentration of 5 mg/ml. The reaction mixture contained 40 μl of enzyme, 80 μl FITC–casein, and 80 μl of the appropriate buffer. The assay buffer for trypsin was 100 m*M* sodium phosphate, 150 m*M* NaCl, pH 7.8. The buffer for cathepsin D was 250 m*M* sodium acetate, pH 4.5. The purified cathepsin A was assayed in the cathepsin D buffer and also in 250 m*M* sodium acetate, pH 5.2, containing 0.1 *M* NaCl and 0.1 *M* sucrose. The reaction was carried out in closed microfuge vials at 37°C for 1 h in the dark. The reaction was stopped by the addition of 480 μl of 5% trichloroacetic acid. The vials were allowed to stand at room temperature for 1 h in the dark and then centrifuged for 5 min in a Beckman microfuge. A 400-μl aliquot of the supernatant was added to 1.6 ml of 0.5 M Tris–HCl, pH 8.5. Fluorescence was determined with an excitation wavelength of 490 nm and an emission wavelength of 535 nm.

RESULTS

Cathepsin A was purified from pig kidney lysosomes by the sequential use of Con A-Sepharose chromatography, phenyl-Sepharose chromatography, DEAE-Sephadex chromatography, gel filtration Sephadex G-200 chromatography, and chromatofocusing.

The enzyme was bound tightly to Con A-Sepharose (Fig. 2) and required relatively high levels of

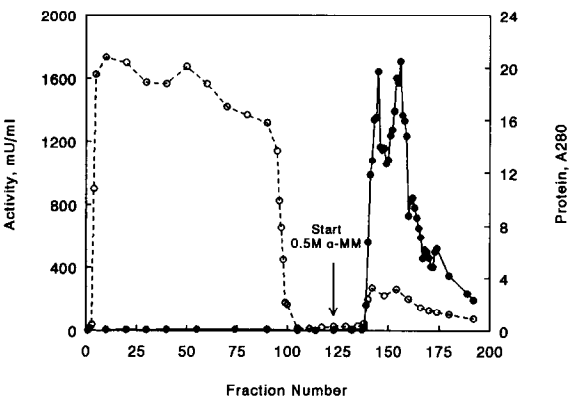

Fig. 2. Con A-Sepharose chromatography of cathepsin A. The 25–60% ammonium sulfate fraction of the lysosomal lysate was applied to a column (18.6 × 2 cm) of Con A-Sepharose equilibrated with buffer 1 (without EDTA). After application of the sample and rinsing with one column volume of buffer 1 (without EDTA) supplemented with 0.5 *M* NaCl, the enzyme was eluted with buffer 1 (with EDTA) containing 0.5 *M* NaCl and 0.5 *M* α-methylmannoside (α-MM). Fractions of 16 ml were collected. All fractions with cathepsin A activity were pooled. This is a representative pattern of the chromatography, which was performed three times. ● = Activity; ○ = A_{280}.

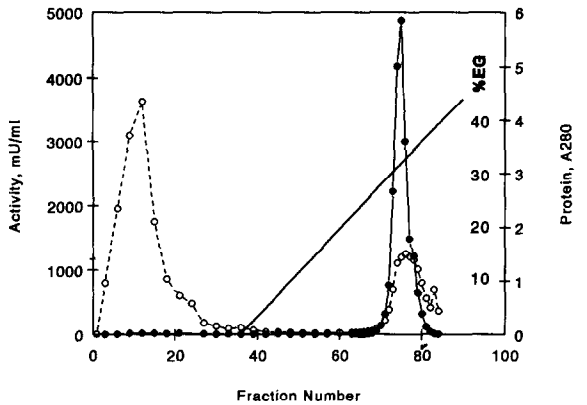

Fig. 3. Phenyl-Sepharose chromatography. The cathepsin A from Con A-Sepharose chromatography was applied to a column (11.1 × 2.4 cm) of phenyl-Sepharose equilibrated with buffer 1. After application of the sample, the column was rinsed with a low-ionic-strength buffer (0.01 *M* sodium acetate, 0.02 *M* NaCl, 0.1 *M* sucrose, 1 m*M* EDTA). The bound enzyme was eluted with a linear gradient from 0–80% ethylene glycol (EG). No additional activity and little additional protein eluted between 40 and 80% EG. Fractions of 16 ml were collected. ● = Activity; ○ = A_{280}.

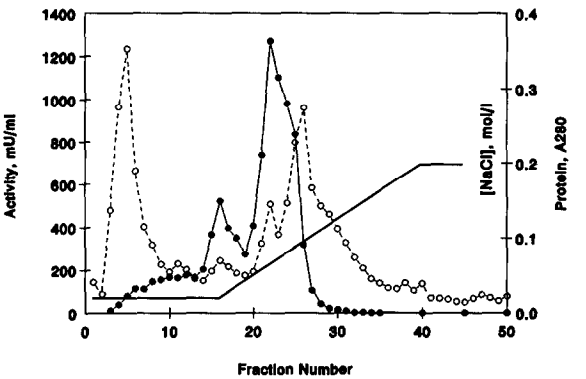

Fig. 4. DEAE-Sephadex chromatography. The cathepsin A recovered from the phenyl-Sepharose step, after concentration and dialysis against 0.01 *M* sodium acetate, 0.02 *M* NaCl, 0.1 *M* sucrose, 1 m*M* EDTA, pH 5.2, was applied to a column (20 × 2.4 cm) of DEAE-Sephadex equilibrated with the same buffer. The column was washed with three volumes of the same buffer and then a linear gradient from 0.02 to 0.2 *M* NaCl was begun. The major peak of activity eluted in fractions 20–27 and was pooled for further purification. Fractions of 20 ml were collected. ● = Activity; ○ = A_{280}.

α-methylmannoside to be eluted from the column. The multiple peaks of activity that are seen in Fig. 2 are partly due to the heterogeneity of the enzyme and partly due to stopping the flow during elution to allow the α-methylmannoside to displace the enzyme more completely. It is of interest to note that 0.1 *M* sucrose, which stabilized cathepsin A, did not interfere with its binding to Con A-Sepharose. This step effected a 5-fold purification with a 50% recovery.

Cathepsin A was further purified by hydrophobic interaction chromatography using phenyl-Sepharose (Fig. 3). The enzyme was bound tightly to phenyl-Sepharose and required about 30% ethylene glycol for elution. The specific activity was increased by a factor of 6.7 by this procedure, and the recovery was 38%.

Approximately 30% of the cathepsin A activity applied to the DEAE-Sephadex column did not bind under the conditions used (Fig. 4). High-molecular-mass forms of cathepsin A which do not bind to DEAE-Sephadex under these conditions had been reported previously [2,11,16–18]. Fractions 5–13 and fractions 14–19 were pooled separately, concentrated and applied to gel filtration chromatography (data not shown). The activity of both of these pools eluted with an apparent molecular mass of 97 000. These unbound forms of cath-

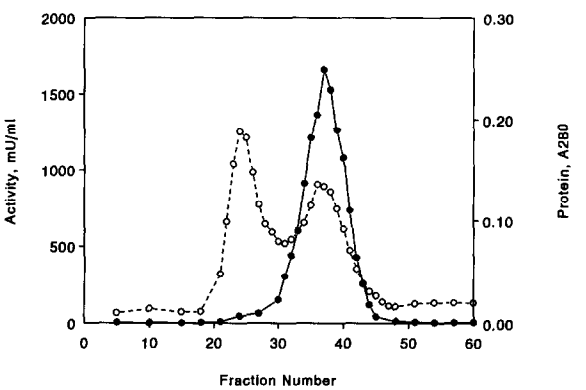

Fig. 5. First Sephadex G-200 chromatography. One-half of the enzyme recovered from the previous DEAE-Sephadex step was applied to a column (63 × 2.6 cm) of Sephadex G-200 equilibrated with buffer 1. The sample volume was 15 ml which was less than 5% of the column volume. Fractions of 5 ml each were collected. The major peak of activity, which eluted in fractions 34–43, was pooled. The peak of enzymatic activity in fraction 37 corresponds to a molecular mass of 97 000. This chromatography was repeated on the other half of the enzyme preparation. ● = Activity; ○ = A_{280}.

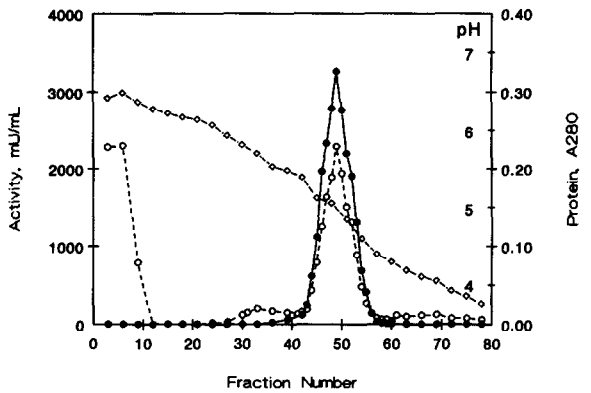

Fig. 6. Chromatofocusing of cathepsin A. A column (19 × 1 cm) of Polybuffer Exchanger 94 was equilibrated with 0.025 *M* imidiazole–HCl, 0.1 *M* sucrose, 1 m*M* EDTA, pH 6.8. The cathepsin A from the second Sephadex G-200 step was dialyzed briefly against the buffer and applied to the column. The pH gradient was generated by eluting the column with Polybuffer 74 (1:8 dilution) adjusted to pH 4.0. The fraction size was 1.8 ml. ● = Activity; ○ = A_{280}; ◇ = pH.

epsin A were not included in subsequent purification steps. The major enzyme form (60%) was bound to the DEAE-Sephadex column and subsequently eluted from it by the salt gradient. This step increased the specific activity by a factor of 3.2 and the recovery in this step was 73%.

After DEAE-Sephadex chromatography, the enzyme was further purified by Sephadex G-200 gel filtration chromatography (Fig. 5). A large amount of high-molecular-mass inactive protein was separated from the enzyme. The elution volume of this cathepsin A activity corresponded to a molecular mass of 97 000. The Sephadex G-200 chromatography was repeated (data not shown) to insure complete removal of the high-molecular-mass contaminant seen in Fig. 5. The combined purification of this step was 1.4-fold with a yield of 36%.

Only trace amounts of inactive protein were removed by chromatofocusing (Fig. 6), and the specific activity did not increase as a result of this step —the yield for this step was 82%. The isoelectric point (p*I*) of cathepsin A determined by this procedure was 5.0.

Using this purification procedure, summarized in Table I, the enzyme was purified nearly 1400-fold over the crude extract. The final specific activity was 16.9 U/mg, and the overall yield was 0.31%. This specific activity was 74% higher than the 9.7 U/mg measured for the cathepsin A purified by Kawamura *et al.* [4]. The composition of the buffer in our standard cathepsin A assay differed slightly from the assay buffer used in that study. Their buffer consisted of 50 m*M* acetate buffer (instead of 0.1 *M*), pH 5.2, 1.0 *M* sucrose (instead of 0.1 *M*) and 0.1 *M*

TABLE I

PURIFICATION SUMMARY

Step	Total activity (U)	Total protein (mg)	Yield (%)		Specific activity (U/mg)	Purification	
			Overall	Per step		Overall	Per step
Crude extract	11 800	959 000	(100)	–	0.0123	(1.0)	–
1000 *g* supernatant	10 200	580 000	86.4	86.4	0.0176	1.4	1.4
16 000 *g* pellet	4 500	170 000	38.1	44.1	0.0265	2.2	1.6
100 000 *g* supernatant	1 290	15 900	10.9	28.6	0.0811	6.6	3.0
$(NH_4)_2SO_4$ (25–60%)	916	8 035	7.8	71.6	0.114	9.3	1.4
Con A-Sepharose	463	800	3.9	50.0	0.579	47	5.1
Phenyl-Sepharose	178	46	1.5	38.5	3.87	315	6.7
DEAE-Sephadex	124	9.95	1.1	73.3	12.5	1016	3.2
Sephadex G-200	44.3	2.62	0.38	35.7	16.9	1374	1.4
Chromatofocusing	36.5	2.16	0.31	81.6	16.9	1374	1.0

KCl (instead of 0.1 *M* NaCl). In addition, their buffer did not include 1 m*M* EDTA. The Z–Glu–Tyr concentration was the same. In order to determine whether the increased specific activity was due to increased activity in our standard assay, the activity of the purified cathepsin A was compared using both conditions. Activity was 8% higher with our standard assay, therefore the increased specific activity is primarily due to increased purity compared to the preparation of Kawamura *et al.* [4]. Product formation was linear for at least one hour under both conditions.

As shown in Fig. 7, non-denaturing polyacrylamide tube gel electrophoresis (pH 4.0) of the purified enzyme showed a single protein band. Tubes 1 and 2 (Fig. 7) contained 10 μg of purified cathepsin A electrophoresed for 1 h and 3.5 h, respectively. There was no detectable fast moving contaminant in tube 1 and no detectable slow moving contaminant in tube 2. Tube 3 contained a mixture of bovine serum albumin (BSA), myoglobin and cytochrome *c* (0.5 μg each). There was good resolution of the proteins, and 0.5 μg were easily detected with the Coomassie Blue stain. If an acidic contaminant with an isoelectric point less than 4 were present, it would have a negative charge, migrate toward the anode, and therefore not enter the gel under these conditions. To check for such a contaminant, in a separate experiment, 10 μg of cathepsin A (tube 4) was electrophoresed under the same conditions except toward the anode for 3.5 h. No contamination by more acidic proteins was observed.

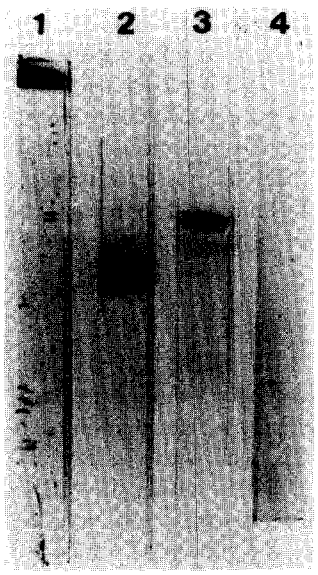

Fig. 7. Non-denaturing PAGE of purified cathepsin A. Electrophoresis was carried out at 4–6°C in 7% polyacrylamide tube gels in the presence of 14 m*M* sodium acetate, 20 m*M* NaCl, 0.1 *M* sucrose, pH 4.0. After application of the samples, electrophoresis was performed in the direction of the cathode at 3 mA per gel tube. Gels were stained with Coomassie Blue R250. Tubes: 1 = 10 μg cathepsin A run for 1 h; 2 = 10 μg cathepsin A run for 3.5 h; 3 = 0.5 μg each of BSA, cytochrome *c* and myoglobin run for 3.5 h; 4 = 10 μg cathepsin A run for 3.5 h in the direction of the anode.

To test for the possible presence of thiol-dependent carboxypeptidases, the effect on activity of the addition of 3 m*M* cysteine was determined. Cysteine had no effect on activity at pH 3.5 or 5.2, with Z–Glu–Tyr or AI as substrates. To test for the possible presence of proteinases, the activity of the purified cathepsin A on FITC–casein was compared with the activity of trypsin and cathepsin D (Fig. 8). Expressed as relative fluorescence units per h per μg of protein, the activities of trypsin at pH 7.8 and cathepsin D at pH 4.5 were 900 and 94, respectively. The activity of cathepsin A at pH 4.5 was 0.38, and its activity at pH 5.2 was 1.25. The maximum level of contamination with cathepsin D is estimated to be less than 0.4%, and it is probable that the low level of apparent endopeptidase activity by cathep-

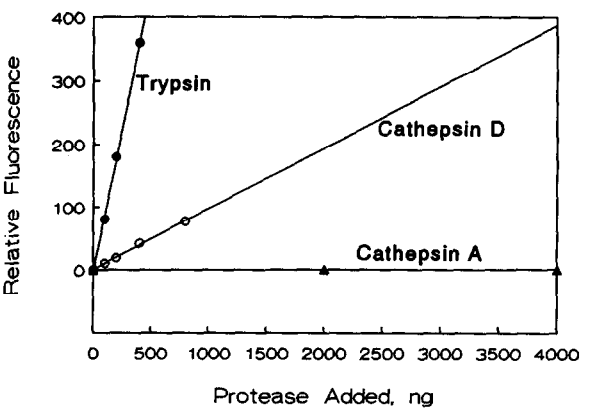

Fig. 8. FITC–Casein proteinase assay. The indicated amounts of trypsin, cathepsin D or the purified cathepsin A were incubated with FITC–casein at 37°C for 1 h. Trichloroacetic acid-soluble fluorescein was measured with an excitation wavelength of 490 nm and an emission wavelength of 525 nm. The small amount of activity by cathepsin A is equivalent to less than 0.4% contamination with cathepsin D.

sin A is actually due to its carboxypeptidase action releasing C-terminal FITC-labelled amino acids.

The effects of several inhibitors and potential activators were tested, and these results are summarized in Table II. This cathepsin A preparation was markedly inhibited by DFP (see Table II for abbreviations) which suggests that the enzyme has an activated serine in its active site. The chymotrypsin inhibitor, TPCK, had a moderate inhibitory effect, but the trypsin inhibitor, TLCK, did not. The inhibition by PCMBS, mersalyl acid (and possibly zinc) indicates the presence of an essential sulfhydryl moiety. The lack of inhibition by EDTA indicates that metal ions are not required for activity. These results are all consistent with previously reported properties of cathepsin A [4]. In addition, the lack of activation by divalent metal ions is evidence for the absence of metal ion-dependent carboxypeptidases in the preparation.

Discontinuous SDS-PAGE by the method of Laemmli [13] in the presence or absence of 2-ME (Fig. 9) demonstrated that the M_r 97 000 protein is composed of two apparently identical subunits of M_r 47 000 in size. Each of these subunits is composed of two disulfide-bonded peptide chains of M_r 31 000 and 19 000. Because these results did not agree (see Discussion) with those of Kawamura *et al.* [19] who used the continuous SDS-PAGE method of Weber and Osborn [14], a sample of cathepsin A was electrophoresed by this method with and without 2-ME. The results (not shown) were essentially the same as with the discontinuous method, although the apparent molecular masses were higher. The non-reduced subunit had an apparent M_r of 61 000, and the separated peptide chains were 35 000 and 24 000 in size.

TABLE II

EFFECTS OF INHIBITORS AND ACTIVATORS ON CATHEPSIN A ACTIVITY

Inhibition was tested with 68 μM AI as substrate at pH 5.8. Abbreviations: DFP = diisopropylfluorophosphate; TPCK = *p*-tosylphenylalanyl-chloromethylketone; TLCK = *p*-tosyl-lysyl-chloromethyl-ketone; PCMBS = *p*-chloromercuribenzene sulfonate.

Addition	Concentration (mol/l)	% of Control
None (Control)	–	(100)
DFP	$1 \cdot 10^{-4}$	22
TPCK	$1 \cdot 10^{-4}$	71
TLCK	$1 \cdot 10^{-4}$	96
PCMBS	$1 \cdot 10^{-4}$	36
Mersalyl acid	$1 \cdot 10^{-4}$	15
EDTA	$5 \cdot 10^{-3}$	98
$CaCl_2$	$5 \cdot 10^{-3}$	101
$MnCl_2$	$5 \cdot 10^{-3}$	100
$Zn(OAc)_2$	$5 \cdot 10^{-3}$	91

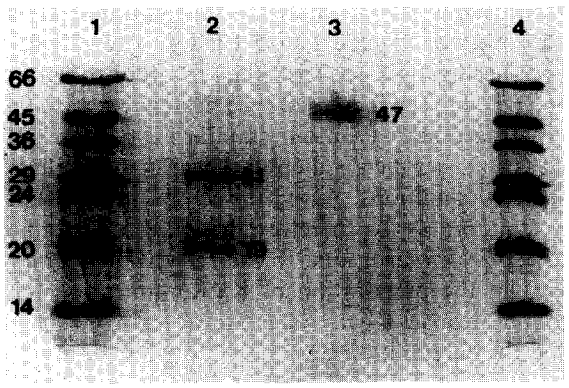

Fig. 9. Discontinuous SDS-PAGE of cathepsin A. SDS-PAGE was performed in 12% gels according to Laemmli [13] in the presence (lane 2) or absence (lane 3) of 2-mercaptoethanol. Cathepsin A samples were 2 μg. Lanes 1 and 4 molecular-mass ($\times 10^{-3}$) markers (BSA, 66 000; ovalbumin, 45 000; glyceraldehyde dehydrogenase, 36 000; carbonic anhydrase, 29 000; trypsinogen, 24 000; soybean trypsin inhibitor, 20 000; α-lactalbumin, 14 200). The gel was stained with Coomassie Blue R250.

DISCUSSION

Cathepsin A purified from pig kidney lysosomes by the methods described above has a molecular mass of 97 000. This agrees well with the 100 000 value reported [4] for pig kidney cathepsin A,S (S for small). The enzyme also was found to have the same isoelectric point, 5.0, and similar sensitivity to inhibitors. However, this enzyme, which was homogenous by non-denaturing PAGE, had a specific activity of 16.9 U/mg. The preparation by Kawamura *et al.* [4] had a specific activity of 9.7 U/mg. As mentioned in the results section, the assay conditions we used resulted in an 8% higher measured rate compared with the conditions used by Kawamura *et al.* [4] and accounts for only a small percentage of the increased specific activity of our preparation. Ap-

parently, our preparation is significantly more pure, although it is possible that the preparation of Kawamura *et al.* [4] was pure, but partially inactive.

Purification steps not previously used for cathepsin A include Con A-Sepharose chromatography, phenyl-Sepharose chromatography, and chromatofocusing. The strong binding to Con A-Sepharose and elution by α-methylmannoside provides evidence that the enzyme is a glycoprotein. This result was anticipated, since cathepsin A is a lysosomal enzyme [20–22]. This step provided a 7-fold purification. Preliminary experiments suggested that various gradients of α-methylglucoside and α-methylmannoside may be useful to separate various forms of cathepsin A. However, since some microheterogeneity may well exist in the carbohydrate portion of the enzyme, it was decided to avoid separation of enzyme forms at this point in the purification.

The enzyme was found to bind tightly to phenyl-Sepharose and required about 30% ethylene glycol for elution. After the subsequent ion-exchange and gel filtration chromatography steps, it was found that all three of the enzyme forms separated by the ion-exchange step had molecular masses of 97 000. None of the previously reported high-molecular-mass forms were observed [2,11,16–18]. Matsuda and Misaka [2] presented evidence that the high-molecular-mass forms of cathepsin A present in rat liver were aggregates of the low-molecular-mass (approximately 100 000) form and that hydrophobic bonds held the aggregates together. The most likely reason for the absence of the aggregates in the present preparation is their elimination in the phenyl-Sepharose step. It is not known whether this elimination was due to irreversible binding to the phenyl-Sepharose or to dissociation of the aggregates by the ethylene glycol.

The chomatofocusing step only removed a small amount of inactive protein and did not measurably increase the specific activity. The isoelectric point of 5.0 determined in this step is identical to that reported by Kawamura *et al.* [4] by isoelectric focusing.

During discontinuous SDS-PAGE (Fig. 9) in the absence of 2-ME, a sample of the enzyme migrated as a single protein with an M_r of 47 000. This result suggests that the 97 000 native enzyme is a homodimer. The 47 000 subunit completely dissociated into peptides of 31 000 and 19 000 in the presence of 2-ME. These results do not corroborate the subunit structure reported by Kawamura *et al.* [19]. Their SDS-PAGE gels, run only in the presence of 2-ME, show peptide chains of 55 000, 25 000 and 20 000, all having about the same staining intensity. These authors used the continuous SDS-PAGE method of Weber and Osborn [14]. Electrophoresis of our preparation with this method confirmed the subunit structure observed with discontinuous system (Fig. 9).

It is unlikely that the presence of their 55 000 peptide is due to incomplete reduction of disulfide bonds in their samples, since they showed that [^{32}P]-diisopropylfluorophosphate only labelled their 25 000 peptide. If their 55 000 peptide was composed of the two disulfide bonded chains (25 000 and 20 000 chains), it too should have been labelled. It is possible that their 55 000 peptide is a contaminant. As indicated above, the specific activity of our preparation was nearly twice that of theirs [4].

In summary, the purified preparation of pig kidney lysosomal cathepsin A described here had a higher specific activity than preparations previously described and was shown to be free of contaminating endopeptidases and other carboxypeptidases. The molecular mass, isoelectric point and sensitivity to inhibitors were consistent with the previously reported properties of cathepsin A. Although the subunit composition of our preparation of cathepsin A differed from that reported previously, our preparation was considerably more pure based on specific activity. The substrate specificity of this preparation of cathepsin A is currently under investigation.

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