Cathepsin B from human renal cortex

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(Received 22 February 1982/Accepted 30 March 1982)

Cysteine-proteinase activity was observed in homogenates of human-cadaver renal cortex. This activity co-purified with renin enzymic activity until separation by aminohexyl-Sepharose–pepstatin affinity chromatography. The cysteine proteinase was purified 1780-fold after the following successive chromatographic procedures: Sephadex G-75, DEAE-cellulose DE-52, and an organomercurial affinity resin. The proteinase activity was dependent upon activation by thiol-containing compounds such as dithiothreitol, as well as by EDTA, and was inhibited by the thiol-group-specific alkylating reagents iodoacetic acid and *N*-ethylmaleimide. DE-52 cellulose chromatography resolved the cysteine proteinase into two components. On the basis of molecular size (26000 daltons), activity as a function of pH, stability as a function of pH, substrate specificity and thermal lability, the major component (95%) has been identified as cathepsin B. The DE-52 cellulose elution pattern of the minor component (5%) is suggestive of cathepsin H [Schwartz & Barrett (1980) *Biochem. J.* **191**, 487–497]. Enzymic activity was determined with synthetic substrates, in particular α -*N*-benzoyl-DL-arginine 2-naphthylamide (Bz-Arg-NNap), thus precluding the detection of cathepsin L [Kirschke, Langner, Wiederanders, Ansorge, Bohley & Broghammer (1976) *Acta Biol. Med. Germ.* **35**, 285–299]. Inhibition by dimethyl sulphoxide was observed in the determination of $K_m = 7.0 \pm 0.4$ mM for the substrate Bz-Arg-NNap, and care must therefore be taken in the preparation of substrate solutions.

As the presence and importance of control patterns in biological systems have been recognized, more attention has focused upon tissue proteinases. Selective patterns of protein degradation are responsible for the control of protein turnover and thereby exert a regulatory function upon enzyme levels in response to metabolic processes (Segal, 1976; Otto, 1971). Post-translational events that are mediated through selective protein cleavages include the removal of signal peptides, activation of inactive proenzymes, and release of hormones from inactive precursors (Campbell & Blobel, 1976; Steiner *et al.*, 1975; Graf & Kenessey, 1976; Quinn & Judah, 1978).

The identification of higher-molecular-weight less-active forms of renin in both kidney and plasma,

amid speculation regarding the mechanisms of activation and inactivation of pro-renin and renin respectively (Inagami *et al.*, 1977), directed our attention to the investigation of cysteine proteinases in renal tissue.

During the purification of renin from human-cadaver renal cortex, cysteine-proteinase activity was observed to co-purify with renin enzymic activity until its separation from renin by affinity chromatography on aminohexyl-Sepharose–pepstatin. Renin was absorbed to the pepstatin–resin and cysteine-proteinase activity was recovered in the effluent. Subsequent purification of the cysteine proteinase included $(\text{NH}_4)_2\text{SO}_4$ fractionation of the effluent and the three successive chromatographic procedures: Sephadex G-75 gel filtration, DEAE-cellulose ion-exchange chromatography and organomercurial affinity chromatography (described below).

The proteolytic activity herein described was dependent upon activation by thiol-group-containing compounds such as dithiothreitol and was inhibited by thiol-specific reagents such as *N*-ethyl-

Abbreviations used: SDS, sodium dodecyl sulphate; Bz-Arg-NNap, α -*N*-benzoyl-DL-arginine 2-naphthylamide; Z-Arg-Arg-NNap, benzyloxycarbonyl-L-arginyl-L-arginine 2-naphthylamide; Z-Gly-Gly-Arg-NNap, benzyloxycarbonylglycylglycyl-L-arginine 2-naphthylamide; ArgN, argininamide; Me₂SO, dimethyl sulphoxide.

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maleimide and iodoacetic acid. Upon chromatography on DE-52 cellulose, the proteinase activity was resolved into two components. On the basis of molecular size, activity as a function of pH, stability as a function of pH, substrate specificity and thermal lability, the major component was identified as cathepsin B. The elution pattern of the minor component is suggestive of cathepsin H (Schwartz & Barrett, 1980; Towatari & Katanuma, 1978; Hardy & Pennington, 1979).

A specific function for this renal cysteine proteinase *in vivo* has yet to be identified. Although the proteinase activity herein described can generate renin enzymic activity from both renin-suppressed and anephric human plasma substrate, this action can be reproduced by a variety of serine and aspartic proteinases and thus is presumed to be non-specific (Slater *et al.*, 1979; Slater & Haber, 1979).

Some results were presented in part at the International Congress of Biochemistry, held in Toronto, Canada, in 1979.

Experimental

Materials

The following materials were obtained from Sigma: Bz-Arg-NNap, Arg-NNap·HCl, Arg-Arg-Nap·3HCl, Bz-Gly-Gly-Arg-NNap·HCl, dithiothreitol, *N*-ethylmaleimide, leupeptin, Brij 35, mersalyl (*O*-{[3-(hydroxymercuri)-2-methoxypropyl]-carbamoyl}phenoxyacetic acid), Tris, NADH, L-cysteine, 5,5-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, and α -glycerophosphate dehydrogenase with triose isomerase. Chromatographic materials were obtained as follows: Sephadex G-75 and Sephadex G-100 from Pharmacia; AcA 44 resin from LKB; Affi-gel no. 501 from Bio-Rad; DE-52 and CM-52 celluloses from Whatman.

Materials for electrophoresis were obtained as follows: acrylamide, bisacrylamide, ammonium persulphate and electrophoretically pure SDS from Bio-Rad. *NNN'*-Tetramethylethylenediamine was purchased from Eastman Kodak.

Methods

The enzyme was isolated from a homogenate of kidney cortex obtained by dissection from human cadaver kidneys free of intrinsic renal disease and obtained within 24 h of death and with permission of next of kin. The thiol-activated proteinase co-purified with human renin until aminohexyl-Sepharose-pepstatin affinity chromatography. These steps were described previously (Slater & Strout, 1981). All chromatographic procedures described below were performed at 4°C.

Enzyme assays. Cysteine-proteinase activity was measured with substrate Bz-Arg-NNap as described

by Barrett (1972, 1976). One unit of activity was defined as release of 1 μ mol of product/min. The concentration of Me_2SO in the standard assay procedure was 2.5% (v/v).

Aldolase activity was determined by the method of Blostein & Rutter (1963).

Sephadex G-75 chromatography. After aminohexyl-Sepharose-pepstatin affinity chromatography, the cysteine-proteinase activity recovered in the non-bound protein fraction was precipitated by $(\text{NH}_4)_2\text{SO}_4$ at 65% saturation. The precipitate, dissolved in 50 mM-sodium acetate, pH 5.5, containing 1 mM-EDTA and 200 mM-NaCl, was applied to a column (95 cm $\times$ 4 cm) of Sephadex G-75. Procedural details are described in the legend of Fig. 1 (below).

DE-52 cellulose chromatography: A DE-52 cellulose column (30 cm $\times$ 0.9 cm) was equilibrated with 1 mM-EDTA in 5 mM-potassium phosphate, pH 6.5. The protein was eluted with a linear gradient established with 250 ml of equilibration buffer in the mixing chamber and 250 ml of equilibration buffer containing 400 mM-NaCl in the reservoir. The flow rate was maintained at 30 ml/h while applying the protein and then decreased to 20 ml/h during the elution period. Fractions (2.2 ml) were collected.

Organomercurial affinity chromatography. A column (17 cm $\times$ 1.5 cm) of Affi-gel no. 501 was prepared as described by Barrett (1973). Before the protein was applied, the column was washed and equilibrated with 50 mM-sodium acetate, pH 5.5, containing 1 mM-EDTA and 200 mM-NaCl.

After DE-52 cellulose chromatography each active peak was processed separately. The active material was dialysed overnight against the organomercury-affinity-column equilibration buffer before applying it to the column. The column was eluted with the same buffer until the non-bound protein was recovered. Bound protein was eluted with equilibration buffer containing 10 mM-cysteine. Fractions (3 ml) were collected at a flow rate of 30 ml/h. After dialysis to remove cysteine, the active enzyme was stored at -20°C .

Gel electrophoresis. Slab-gel electrophoresis was performed in a Bio-Rad model no. 220 apparatus. SDS/polyacrylamide-gel electrophoresis was performed in slab and disc gels as described by Laemmli (1970).

All other procedures are described in the Results section.

Results

The proteinase activity was purified in parallel with renin activity as outlined in Scheme 1. The thiol-activated Bz-Arg-NNap-hydrolysing activity recovered in the non-bound protein fraction was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to

Scheme 1. Flow diagram of purification procedure

65% saturation. This step produced an 18.7-fold purification with a 21% overall recovery of cysteine-proteinase activity. The precipitate was further purified by molecular-sieve chromatography on a Sephadex G-75 column as illustrated in Fig. 1. The same column was calibrated with standard proteins for molecular-weight determination, and the molecular weight of the cysteine-proteinase active material determined by this procedure was $26\,000 \pm 1000$.

When DE-52 cellulose chromatography was performed as described under 'Methods', two enzymically active peaks were resolved. A representative chromatogram is presented in Fig. 2. The specific activity of the first peak, designated 'DE-I' ranged from 0.15 to 0.25 unit/ A_{280} unit. Although the specific activity of the second peak, designated 'DE-II', varied from 2.0 to 3.0 units in a series of chromatographic runs, the specific activity was essentially constant across the peak for each

Fig. 1. *Sephadex G-75 chromatography*

The Sephadex G-75 column (95 cm $\times$ 4.0 cm) was equilibrated and eluted with 50 mM-sodium acetate, pH 5.5, containing 200 mM-NaCl and 1 mM-EDTA. A 33 ml portion of protein solution (30 A_{280} units/ml) was applied to the column. Fractions (5.5 ml) were collected at a flow rate of 100 ml/h. The column was monitored for protein by A_{280} and for enzymic activity by Bz-Arg-NNap hydrolysis.

particular run. About 95% of the activity was associated with the more active DE-II material. DE-I was a minor component representing less than 5% of the total activity.

Separation of cathepsin B with an organomercurial affinity column has been described (Barrett, 1973). In our work the commercial resin Affi-gel no.

Fig. 2. *DE-52 cellulose chromatography*

The DE-52 cellulose column, 30 cm $\times$ 0.9 cm, was equilibrated with 5 mM-potassium phosphate, pH 6.5, containing 1 mM-EDTA. 43.4 A_{280} units of protein were applied to the column. The protein was eluted with a linear gradient established with 250 ml of 5 mM-potassium phosphate buffer, pH 6.5, containing 1 mM-EDTA and 400 mM-NaCl in the reservoir. Fractions (2.2 ml) were collected at a flow rate of 20 ml/h.

501 from Bio-Rad was used and applied as described under 'Methods'. Attempts to employ this resin were made at every stage, starting with the $(\text{NH}_4)_2\text{SO}_4$ precipitate of the non-pepstatin-bound material. Before the DE-52 cellulose separation, use of the organomercurial column was unsuccessful. The procedure was characterized by erratic binding patterns, which were attributed to competing cysteine residues of protein contaminants.

Each of the two active proteinase components, obtained after DE-52 cellulose chromatography, was chromatographed separately on the Affi-gel no. 501 column. The bound protein was eluted with the equilibration buffer containing 10 mM-cysteine. A typical separation is illustrated in Fig. 3.

A summary of purification is presented in Table 1. Separation with the organomercury affinity resin provided the purest material obtained in the course of the present study. The active peak after affinity chromatography from DE-I is designated 'Pro-A'; the active peak after affinity chromatography obtained from DE-II is designated 'Pro-B'. The low yield and instability of Pro-A precluded its further characterization. Hence the protein in DE-II and Pro-B was the material subjected to further characterization in the present study.

Throughout the purification procedure it was not possible to obtain a preparation that would consis-

Fig. 3. Organomercurial Affi-gel 501 chromatography. The Affi-gel no. 501 column (17 cm $\times$ 1.5 cm) was prepared as described in the text. A 28 A_{280} unit portion of post-DE-52 cellulose peak-II material dialysed overnight against the equilibration buffer was applied to the column. The column was eluted with equilibration buffer until all non-bound protein was recovered. Bound protein was eluted with the same buffer containing 10 mM-cysteine. Fractions (3 ml) were collected at a flow rate of 30 ml/h.

tently reveal a single band on polyacrylamide-disc-gel electrophoresis. Thus far we have been unable to determine whether this observation is related to an autolytic process, isoenzymes or a co-purifying impurity. An additional probe employed to establish the state of purity was SDS/polyacrylamide-gel electrophoresis; electrophoresis of 5 μ g of the Pro-B material revealed one sharp major band, with some trailing and one minor trace

band (Fig. 4). Molecular weights determined for the protein bands were 25 800 for the major band and 34 200 for the trace band.

Properties of the cysteine proteinase

pH optimum. Bz-Arg-NNap hydrolase activity as a function of pH was evaluated from pH 4.0 to 7.5. The pH optimum was found to be pH 6.2–6.4.

Stability as a function of pH. Enzyme stability as a function of pH was investigated from pH 4.0 to 7.5 at 22°C. Enzyme stability was constant from pH 5.0 to 7.0. From pH 7.0 to pH 7.5 enzyme stability decreases sharply and the enzyme was completely inactive at pH 8.0. From pH 5.0 to pH 4.0 enzyme stability was less affected; 90% of the activity was observed at pH 4.0.

Thermal lability. Thermal sensitivity was evaluated at pH 6.0. For a period of 80 min the enzyme was equally stable at 4 and 22°C. At 37°C a slow but finite decrease in activity was observed, and at 58°C over 90% of the activity was lost within 10 min.

Substrate specificity. To assess the substrate specificity of the enzyme, the hydrolysis of a series of arginine 2-naphthylamide derivatives was investigated. The order of reactivity was Z-Arg-Arg-NNap > Bz-Arg-NNap > Arg-Arg-NNap > Z-Gly-Gly-Arg-NNap > ArgN (see Table 2). The K_m for the enzyme with Bz-Arg-NNap as substrate was 7.0 ± 0.4 mM (on the basis of two determinations), a value similar to that previously reported (Barrett, 1977; Knight, 1980).

Effect of dimethyl sulphoxide. In the process of determining K_m , a decrease in activity with an increase in Me₂SO concentration was observed. The presence of 7.5% (v/v) Me₂SO in the preparation of the standard curve did not affect the absorption; thus attention was focused upon enzyme inactivation. The concentration of Me₂SO in the standard assay was 2.5% (v/v). At 5% Me₂SO the enzyme activity was decreased by 14.5%, at 7% Me₂SO the activity decrease was 40%, and at 10% Me₂SO the decrease was 46.6%. It was clear from these data that enzyme activity was extremely sensitive to the concentration of Me₂SO. Since Me₂SO was necessary to dissolve the substrate, all activity measurements were made while maintaining the Me₂SO concentration at 2.5% (v/v).

Inactivation of aldolase. Inactivation of aldolase by the cysteine proteinase was also observed. Aldolase was incubated in the presence of the cysteine proteinase at 37°C. Aldolase was assayed by the method described by Blostein & Rutter (1963). Over 70% of the activity was lost in the first 30 min.

Inhibition. The effect of a series of known cysteine-proteinase inhibitors upon the cysteine proteinase activity was determined. The enzyme was

Table 1. *Purification of human renal-cortex cysteine proteinase*

The yields of protein and activity are from 3 kg of human-cadaver kidney cortex. The values for protein recorded through the pepstatin affinity column were determined by the method of Lowry *et al.* (1951).

	Total protein (g)	Total activity (units)	Specific activity (units/g or A_{280})	Purification (fold)	Recovery (%)
Homogenate supernatant	92.4	314	0.0037		
DEAE-cellulose batch elution	24.3	339	0.014	3.7	108
(NH ₄) ₂ SO ₄ (65% satd.)	10.8	228	0.024	6.4	92
Pepstatin non-bound	4.2	100	0.024	6.4	32
	(A_{280} units)				
(NH ₄) ₂ SO ₄ (0–65% satd.)	960	67	0.07	18.7	21
Sephadex G-75	57.4	77.5	1.35	365	24.7
DE-52 cellulose					
Peak DE-I		4.4	0.25		1.4
Peak DE-II	32.0	86.3	2.71	732	27.5
Affi-gel no. 501					
DE-I peak			1.00	270	
DE-II peak	13.7				
(a) Immediate assay		90.4	6.6	1784	
(b) Assayed after dialysis		64.4	4.7	1270	20.5

Table 2. *Substrate specificity*

Assays were performed by using the standard procedure. The reactions were initiated by the addition of 50 μ l of substrate in Me₂SO. The final substrate concentration in the assay was 4.5 mM, except for [Z-Arg-Arg-NNap], which was 2.25 mM.

Substrate	Specific activity (μ mol $\cdot$ min ⁻¹ $\cdot$ A_{280} ⁻¹)	Relative activity (Bz-Arg-NNap $\equiv$ 1.0)
1. Z-Arg-Arg-NNap $\cdot$ HCl	43.8	10.3
2. Bz-Arg-NNap $\cdot$ HCl	4.25	1
3. Arg-Arg-NNap $\cdot$ 3HCl	2.55	0.60
4. Z-Gly-Gly-Arg-NNap	1.08	0.25
5. Arg-NNap $\cdot$ HCl	0.0	0.0

incubated with the selected inhibitor for 5 min at 37°C before initiating the reaction with substrate. Under these conditions the enzyme was inhibited 100% by 10 μ M-iodoacetic acid, 75% by 1 μ M-leupeptin, and 18% in the presence of 10 μ M-phenylmethanesulphonyl fluoride. Soya-bean trypsin inhibitor did not inhibit at 0.1 mM. An inactivation of 50% was observed after a 30 min incubation with 4 mM-*N*-ethylmaleimide.

Discussion

Three distinct tissue cysteine proteinases of lysosomal origin, cathepsins B, H and L, have been described (Barrett, 1973; Schwartz & Barrett, 1980; Kirschke *et al.*, 1976). Of these, cathepsin B isolated from human liver has been the best characterized. In the present study, two cysteine-proteinase activities have been detected in human renal cortex.

The Bz-Arg-NNap hydrolase activity of both proteinases required EDTA and the presence of a thiol compound for activation. The major compo-

nent, studied in greater detail, was inhibited by Hg²⁺, *N*-ethylmaleimide, iodoacetic acid and leupeptin, a spectrum of inhibition consistent with cathepsin B (Barrett, 1977; Evans & Etherington, 1978; MacGregor *et al.*, 1979). Other properties consistent with cathepsin B were the pH optimum of the Bz-Arg-NNap assay (Barrett, 1973; Singh & Kalnitsky, 1978; Szego *et al.*, 1976; Towatari *et al.*, 1979), alkaline denaturation (Barrett, 1977; Warwas & Dobryszczyka, 1976; Towatari *et al.*, 1979; MacGregor *et al.*, 1979), thermal lability (Towatari *et al.*, 1978; Tallan *et al.*, 1952) and inactivation of aldolase (Bond & Barrett, 1980; Towatari *et al.*, 1979; Singh & Kalnitsky, 1978).

The order of substrate specificity determined with arginine 2-naphthylamide analogues was: Z-Arg-Arg-NNap > Bz-Arg-NNap > Arg-Arg-NNap > Z-Gly-Gly-Arg-NNap > ArgN. This pattern indicates a preference for a blocked α -amino group and two successive basic residues on the C-terminus of the scissile amide bond. This spectrum of substrate

Fig. 4. SDS/polyacrylamide-gel electrophoresis. Tracks 1 and 4, protein standards (Bio-Rad): phosphorylase *b* (M_r 100000), bovine serum albumin (M_r 66000), ovalbumin (M_r 44000), carbonic anhydrase (M_r 34000), soya-bean trypsin inhibitor (M_r 22000) and lysozyme (M_r 14000). Tracks 2 and 3, cysteine proteinase, Pro-B ($5\mu\text{g}$). To $100\mu\text{l}$ of sample an equal volume of 10% (final concn., w/v) SDS containing 1.25% (v/v) mercaptoethanol and 12.5% (v/v) glycerol was added. After placing the samples in a boiling-water bath for 5 min, 20 or $40\mu\text{l}$ was applied to an 11% (w/v)-polyacrylamide gel containing SDS at pH 8.8.

specificity is similar to that reported for human liver cathepsin B (Barrett, 1973; Schwartz & Barrett, 1980).

The molecular weight of the major band by molecular-sieve chromatography was $26\,000 \pm 1000$. The molecular weight by SDS/polyacrylamide-gel electrophoresis was 25800 for the major band and 34200 for the trace band. The molecular weight is

consistent with values reported for cathepsin B isolated from other sources (Barrett, 1973; Keilova, 1971; Distelmaier *et al.*, 1972; Evans & Etherington, 1978; MacGregor *et al.*, 1979; Takahashi *et al.*, 1979).

After DE-52-cellulose ion-exchange chromatography, two thiol-activated proteinase activities were identified. In the standard assay, DE-I was 0.1–0.2-fold as active as DE-II. The low yield of DE-I limited further characterization of this material. All the evidence accrued at this point supports our initial proposal (Gounaris & Slater, 1979) that the major component of renal proteinase activity herein described is cathepsin B. The chromatographic elution pattern on DE-52 cellulose of the minor component is suggestive of cathepsin H; however, the possibility that DE-I is a partially active degradation product of DE-II cannot be excluded. In the present study enzymic activity was determined with synthetic substrates. Consequently the presence of cathepsin L was not detected (Kirschke *et al.*, 1976).

As yet there is no evidence to support a unique physiological function for renal cathepsin B. The enzyme does catalyse the production of active renin-suppressed and anephric human plasma (Slater *et al.*, 1979). Further study, however, has not substantiated a previous report suggesting that the conversion of the high-molecular-weight form of renin into a low-molecular-weight form may be mediated by a cysteine proteinase (Murakami *et al.*, 1980). Thus the role of renal cathepsin B remains unidentified at this time. Possible functions range from selective protein degradation, which contributes to control of specific protein levels, to unique protein processing and activation phenomena.

Since this manuscript was originally submitted, the activation of prorenin by kidney cathepsin B isoenzymes isolated from pig kidney has been reported (Takahashi *et al.*, 1982). Although this information does support a specific role for cathepsin B in kidney tissue, definitive evidence has not been obtained.

We thank Dr. A. J. Barrett for generously providing Fast Garnet, Dr. A. J. Barrett, Dr. W. D. Carlson and Dr. E. Haber for general advice, Mr. H. Vincent Strout, Jr., Ms. Elena Bardensteyn and Mr. Scott Arnold for technical assistance, and Ms. J. A. Tavano and Ms. P. M. McBride for manuscript preparation. This work was supported by the National Heart, Lung and Blood Institute (grant no. HL-21247 to E. E. S.) and by a gift from R. J. Reynolds Industries. E. E. S. is an Established Investigator of the American Heart Association.

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