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Purification and some properties of bound cysteine proteinase inhibitor from cornified cells of newborn rat epidermis

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Cysteine proteinase inhibitor exists in two forms in terminally differentiated keratinocytes. One is readily soluble in 20 mM sodium phosphate buffer but the other is bound to the plasma membrane and is poorly soluble. The cysteine proteinase inhibitor (CPI) from the membrane was extracted from cornified epidermal layers of 2-day-old rats and its properties were compared with those of soluble CPI. This CPI (bound CPI) was solubilized in alkaline 8 M urea containing 2-mercaptoethanol from the residual tissue exhaustively treated with buffered 4 M urea. CPI was separated from keratin by ammonium sulfate precipitation and purified by means of papain affinity chromatography, ion exchange column chromatography and gel filtration. Bound CPI had an M_r value of about 16000, a pI value of 3.8 and was unstable at above 80 °C, while soluble CPI was of M_r 13000 and stable at above 80 °C. Both CPIs were stable at 4 °C in the range of 3.0–9.0. Bound CPI contained half cystine and the ratio of acidic-to-basic amino acids was 3.18. Bound CPI inhibited rat liver cathepsins B, H, and L but did not inhibit the activity of noncysteine proteinases. Papain activity was inhibited by bound CPI at three sites, noncompetitively, and the K_i value was calculated to be 0.11 nM.

Introduction

Cysteine proteinase inhibitors (CPIs) have been isolated from various tissue sources of numerous species. Although classification of group-specific inhibitors has not been totally established, CPIs have been subgrouped by their molecular weight, chemical structure and possible physiological functions. These groups are, in general, subdivided into low-molecular-weight (M_r 10000–14000) and high-molecular-weight (M_r 60000–160000) inhibitors. The low-molecular-weight CPIs were further grouped by their isoelectric point (pI), but each also exists in multiple isoelectric forms. Since the early study of Järvinen [1], CPIs from epidermal cells of rats [2,3] and humans [4,5] have been the subject of many investigations. The inhibitors in normal epidermis of rodents [1,4] and humans [4,5] possess acidic pI, while those in some skin diseases [6] or from oral mucosa [7] have a neutral pI. The mechanism of action of the inhibitors is noncompetitive and they have been shown to be synthesized in keratinocytes [2].

Immunohistochemical studies have demonstrated that much of the acidic inhibitor present in

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Abbreviations: Bz-Arg-βNA, N^{α}-benzoyl-DL-arginine-2-naphthylamide; Bz-Arg-OEt, N^{α}-benzoyl-DL-arginine ethyl ester; Bz-Tyr-OEt, N^{α}-benzoyl-L-tyrosine ethyl ester; CPI, cysteine proteinase inhibitor; PMSF, phenylmethylsulfonyl fluoride; PHMB, *p*-hydroxymercuribenzoate.

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keratinocytes, particularly in the proximal layers, is readily soluble but cysteine proteinase inhibitor, which is less soluble, appears to deposit by the plasma membrane of terminally differentiated cells [8]. In previous studies, we attempted to extract the less soluble inhibitor (bound inhibitor) and found that it is necessary to use an alkaline, 8 M urea solution containing mercaptoethanol [9]. In this study, we purified bound inhibitor and the chemical properties which were different from those of soluble CPI were comparatively characterized.

Materials and Methods

Materials. 2-day-old Sprague-Dawley strain rats were used. DEAE-cellulose (DE-52) was purchased from Whatman. Sepharose 4B, Sephadex G-100 and M_r marker proteins for electrophoresis were from Bio-Rad. Papain (2 × crystallized, type V), N^{α}-benzoyl-DL-arginine-2-naphthylamide (Bz-Arg-βNA), N^{α}-benzoyl-DL-arginine ethyl ester (Bz-Arg-OEt) and N^{α}-benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt) were from Sigma Chemical Co. *p*-Dimethylaminocinnamaldehyde was from Eastman Kodak Co. Trypsin, chymotrypsin, pepsin and cathepsin D were from Sigma Chemical Co., and E_{64} was kindly provided by Taisho Pharmaceutical Co., Japan.

Enzyme assays. A slightly modified method of Järvinen and Hopsu-Havu [10] was used for papain activity. Papain (1 μg protein) was activated in 1 ml of 0.1 M Tris-HCl (pH 7.5), 5 mM EDTA, 5 mM dithiothreitol at 37 °C for 5 min. The assay was initiated by adding 20 μl of 91 mM α-Bz-Arg-2-NA and the amount of β-naphthylamine released at 37 °C for 10 min was determined by colorimetric reaction with *p*-dimethylaminocinnamaldehyde. Rat liver cathepsins B and H were assayed by the same procedure at pH 6.0 and 6.8, respectively. 1 unit of enzyme was defined as the amount that hydrolyzed 1 μmol of substrate per min. Rat liver cathepsin L was assayed by a modification of the method of Starkey [11] with azocasein as the substrate. Spectrophotometrically, trypsin was measured at 255 nm with Bz-Arg-OEt [12], while chymotrypsin was measured at 250 nm with Bz-Tyr-OEt [13]. Cathepsin D was assayed with acid-denatured hemoglobin according to the method of Anson [14]. Pepsin was assayed with hemoglobin according to the method of Tang [15].

Inhibitor assay. The inhibitor was preincubated with cysteine proteinases (papain, cathepsin B, H or L) in activation steps as described above. Residual enzyme activity was compared with that in the reaction mixture without the inhibitor. Inhibitor activity to other proteinases was assayed after preincubation of the enzymes with inhibitor at 37 °C for 5 min.

Isolation of rat liver cathepsins. Perfused adult rat livers were homogenized with a Potter-Elvehjem homogenizer in 3 vols. of 0.25 M sucrose containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The lysosomal-light mitochondrial fraction prepared by the method of De Duve et al. [16] was lysed by freezing and thawing five times. After centrifugation at 105 000 × *g* for 60 min, cathepsins B, H and L were purified from the supernate by a modification of the method of Barret and Kirschke [17], first using ultrafiltration (Amicon, YM-5 membrane filter) and gel filtration on a Sephadex G-100 column (2.6 × 89 cm). Enzyme fractions, molecular weight range 20 000–30 000 (L-20), were pooled and separated by a CM Sephadex C-50 column equilibrated with 20 mM sodium acetate buffer, (pH 5.8), 5 mM EDTA and 5 mM 2-mercaptoethanol. Cathepsins B, H and L were eluted by a stepwise gradient of sodium chloride (60 mM, 200 mM and 500 mM, respectively). Cathepsin B was stored in 20% glycerol added 20 mM sodium phosphate buffer (pH 5.8) at −70 °C. Cathepsins H and L were stored in the same buffer containing 5 mM EDTA and 5 mM 2-mercaptoethanol.

Purification of epidermal CPI. The epidermis was separated from 2-day-old rat skin as described [2] and cornified cells mechanically scraped from the epidermis were maintained at −70 °C until use. Cornified cells (20 g, wet weight) were homogenized with a Ten-Broeck type glass-glass homogenizer in 10 vols. of 20 mM sodium (or stirred) phosphate buffer (pH 6.0), 0.28 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM *p*-hydroxymercuribenzoate (PHMB) (phosphate-buffered saline) and extracted at 4 °C overnight. The homogenate was centrifuged at 13 000 × *g* for 10 min. Soluble CPI was purified from the

supernate by ammonium sulfate fractionation (60–70% saturation), followed by DEAE-cellulose chromatography and Sephadex G-100 chromatography.

Precipitates of the phosphate-buffered saline extraction were washed twice in phosphate-buffered saline and subjected to sequential extraction to remove soluble CPI and other extractable proteins from the precipitates. It was extracted with 10 vols. of 50 mM sodium phosphate buffer (pH 7.0), 4 M urea, 1 mM PMSF and 1 mM PHMB, at room temperature overnight and centrifuged at $13000 \times g$ for 10 min. The final extraction was performed with 10 vols. of 20 mM Tris-HCl (pH 9.0), 8 M urea and 0.1 M 2-mercaptoethanol, 1 mM PMSF and 1 mM PHMB (buffer A) at 37°C overnight, and centrifuged at $13000 \times g$ for 10 min. Solid ammonium sulfate was added to the supernate (buffer A extract), which exhibited papain inhibitor activity, to give a 30% saturation, and centrifuged at $13000 \times g$ for 10 min. The supernate was dialyzed against 10 mM sodium phosphate buffer (pH 6.0, three times), concentrated on a YM-5 membrane filter, and applied to a CM-papain Sepharose column (bed volume 10 ml). According to the method of Järvinen [1], the inhibitor (bound CPI) was eluted with 20 mM Na_3PO_4 (pH 12.1) and dialyzed against 6 M urea in 10 mM Tris-HCl (pH 8.0). Purification of the bound CPI was initiated with chromatography on a DEAE-cellulose column (0.6×5 cm) equilibrated with the dialysis buffer. The inhibitor was eluted with 200 ml of a linear gradient of NaCl (0–250 mM) in 6 M urea in 20 mM Tris-HCl (pH 8.0). Fractions (50–70 mM NaCl) with the activity were pooled and dialyzed against 0.12 M NaCl in 20 mM sodium phosphate buffer (pH 7.2), concentrated to about 4 ml on a YM-5 membrane filter and applied to a Sephadex G-100 column (1.8×113 cm), which was equilibrated with the dialysis buffer. Flow rate was 12 ml/h; 5 ml fractions were collected.

Electrophoresis. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was performed with 12 and 15% slab gels by the method of Laemmli [18]. They were stained by the silver staining method of Oakley et al. [19] and Coomassie blue R-250 in 30% ethanol containing 10% acetic acid, respectively. Standard proteins (Bio-Rad, low M_r) were used to estimate molecular weight. Isoelectric focusing was performed according to the procedure in the manufacturer's (LKB) pamphlet using polyacrylamide gel with carrier ampholytes (2%, w/v) composed of Ampholines with pH ranges of 2.5–4.5, 4.0–6.0 and 6.0–8.0. After prerunning, bound CPI (50 mU) was loaded onto the alkaline pH side of the gel and the gel was run at 400 V for 6 h (Multiphore 2117, LKB). The pH of the focused gel was measured at 5-mm intervals by a microcombination pH electrode (SE-1600GC, Fujikagaku, Tokyo, Japan) at 2°C. The gel was sliced, 5-mm thick, homogenized and extracted at 4°C for 2 days in 500 μl of 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. After low-speed centrifugation of the gel suspension, the supernate (100 μl) was assayed for inhibitor activity.

pH stability of CPIs. To the inhibitor solutions(20 μl) which caused 50% inhibition of papain activity, 2 μl of 1 M buffer solutions of various pH values (acetate buffer, pH 3.5–6.0, sodium phosphate buffer, pH 6.0–7.5, and Tris-HCl, pH 7.0–9.0) were added and kept at 4°C. After 2 h, papain (1 μg) and activation buffer solution (500 μl) were added to give a 1-ml assay system and the remaining inhibitor activity was assayed as described above.

Heat stability of CPIs. Inhibitor in 10 mM Tris-HCl (pH 8.0, 20 μl), which caused 50% inhibition of papain, was heated at various temperatures for 5 min. After cooling on ice, papain and activation buffer were added and the inhibitory activity was assayed as described above.

Amino acid analysis. Amino acid composition was analyzed by the PICO-TAG method [20]. Protein solutions were desalted by chromatography on a Sephadex G-25 column using double distilled water as the eluent. Proteins, 100 pmol, were hydrolyzed in 6 N HCl at 110°C for 24 h in vacuo, derivatized with phenylisothiocyanate, and analyzed on a reverse phase PICO-TAG column (Waters Associates).

Preparation of CM-papain Sepharose. Papain, 100 mg, was activated with 5 mM dithiothreitol and 5 mM EDTA in 20 ml of 0.1 M Tris-HCl (pH 7.5) at room temperature for 10 min. It was treated with 20 mM iodoacetic acid and dialyzed against 0.5 M NaCl in 0.1 M $NaHCO_3$ (pH 8.3), and

coupled to pre-swollen CNBr-activated Sepharose 4B (2.9 g) at room temperature for 2 h. About 95% of the protein was coupled to the Sepharose 4B, as judged from protein estimation of the gel wash. The gel was treated with 0.1 M Tris-HCl (pH 8.0) at room temperature for 2 h and washed with 0.1 M $NaHCO_3$ (pH 8.3) and 0.15 M NaCl with 0.1 M sodium acetate buffer (pH 4.0) and 0.5 NaCl, and finally with 20 mM sodium phosphate buffer (pH 6.0).

Titration of the active site of papain and CPIs for papain. The active site of the papain solution was first titrated with E_{64}. 1 μg of activated papain was preincubated for 20 min with E_{64} (0–20 pmol). By assay for residual enzyme activity, the active site of 1 μg papain was found to be 9.0 pmol. Inhibition sites of CPIs for papain were then determined using the same papain solution. 1 μg of papain was preincubated for 5 min with CPI (0–15 pmol of soluble CPI or 0–10 pmol of bound CPI) at the activation step and enzyme activity was assayed. The binding ratio was calculated by extrapolation of the linearly inhibiting portion (at least 0–70% inhibition). The molar concentration of soluble and bound CPI was calculated assuming their M_r value to be 13000 and 16000, respectively.

Determination of K_i value and mechanism of inhibition. The K_i value and mechanism of inhibition were determined by the method of Henderson [21]. Inhibition of papain activity was measured under the condition that a non-linearity of the titration curve was obtained ($E_t/K_i = 13.6$). Total enzyme concentration (E_t) used was 1.5 nM and the total inhibitor concentration (I_t) was 0.8–1.45 nM.

Protein measurement. Protein was measured by the method of Lowry et al. [22] with bovine serum albumin as a standard.

Results

Extraction of bound CPI

The solubility of papain inhibitor activity from cornified cells in different extracts (Table I) was similar to that previously reported [9]. In a typical experiment, 87% (107000 mU/189 mg protein) of the inhibitor activity appeared in the first phosphate-buffered saline. Soluble CPI in the second and third phosphate-buffered salines was reduced to 4.6% (5600 mU/9.7 mg) and 0.4% (490 mU/2.2 mg protein) of the total activity, respectively. No activity was detected in 50 mM sodium phosphate buffer (pH 7.0), 4 M urea, although 67.5 mg protein was extracted. Buffer A extracted 6.9% of the total activity (8460 mU/720 mg protein).

Purification of bound CPI

Table II summarizes the yield of bound CPI at each purification step. Because 30% $(NH_4)_2SO_4$ precipitated 83% of total protein in buffer A extract, a 7-fold purification of the inhibitor was achieved. Furthermore, large amounts of proteins were precipitated, but not a majority of the activity during extensive dialysis; bound CPI was purified 44-fold. The bound CPI was attached to a CM-papain Sepharose column and eluted with 20 mM Na_3PO_4 (pH 12.1), but not with 3 M NaCl. This affinity of bound CPI was the same as that of acidic epidermal CPI reported by Järvinen [1]. By the next step with the DEAE-cellulose column, in the presence of 6 M urea, a 2100-fold purification

TABLE I

SOLUBILITY OF PAPAIN INHIBITOR ACTIVITIES FROM RAT CORNIFIED CELLS

Step	Protein (mg)	Activity [a] (mU)	Spec. act. (mU/mg)	Percent of total activity [b]
1st PBS	189	107000	566	87.6
2nd PBS	9.7	5600	577	4.6
3rd PBS	2.2	490	222	0.4
4 M urea (pH 7.0)	67.5	0	0	0
Buffer A	720	8460	11.8	6.9

[a] Enzyme activity of various fractions was assayed after dialysis of aliquots against cold water to remove PMSF and PHMB.
[b] Total activity denotes the sum at each step of inhibitory activity.

TABLE II

PURIFICATION OF BOUND CPI FROM 8 M UREA EXTRACT OF CORNIFIED CELLS AFTER REPEATED EXTRACTION OF SOLUBLE CPI

Step	Protein (mg)	Total activity [a] (mU)	Spec. act. (mU/mg)	Purification (fold)	Yield (1%)
Buffer A	4900	38000	7.7	1	100
30% $(NH_4)_2SO_4$ sup.	600	32000	53	7	85
Dialysis	70	23600	337	44	62
Papain Sepharose	7.4	15200	2070	270	40
DEAE-cellulose	0.46	7400	16000	2100	20
Sephadex G-100	0.31	7100	23000	3000	18.6
Papain Sepharose	0.096	4300	45000	5800	11.4

[a] 1 unit of inhibitor was defined as the amount that decreased papain activity by 1 unit.

was achieved. Recovery of the inhibitor was reduced by 90–95% in the absence of urea. This property contrasts bound CPI with soluble CPI which does not require urea during ion-exchange column chromatography. Bound CPI eluted at 50–70 mM NaCl was further chromatographed on a Sephadex G-100 column (Fig. 1). The approximate M_r value of bound-CPI was 16000 (Fig. 1, inset). Purification of about 6000-fold was achieved by rechromatography with CM-papain Sepharose. Specific activity of purified bound CPI was 45000 mU/mg, and was 2.6-fold higher than that of

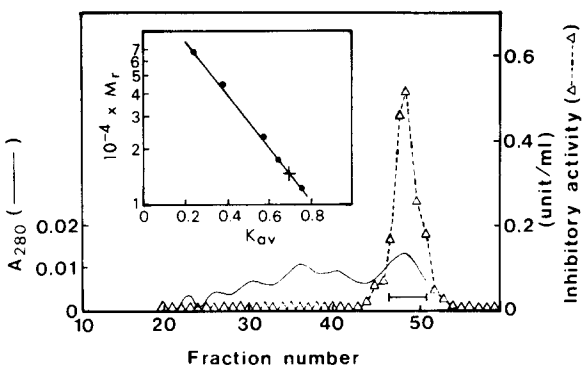

Fig. 1. Gel filtration of bound CPI. Fractions with inhibitory activity in DEAE-cellulose chromatography were pooled, dialyzed against equilibration buffer (20 mM sodium phosphate buffer, pH 7.2, 0.12 M NaCl), and applied to a Sephadex G-100 column (1.8×113 cm). Inset: molecular weight estimation of bound CPI. Elution volumes of M_r marker proteins were determined by a separate experiment. The M_r marker proteins used were bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), chymotrypsinogen A (M_r 25000), myoglobin (M_r 17800) and cytochrome *c* (M_r 12400). Flow rate, 12 ml/h; fractions of 5 ml were collected.

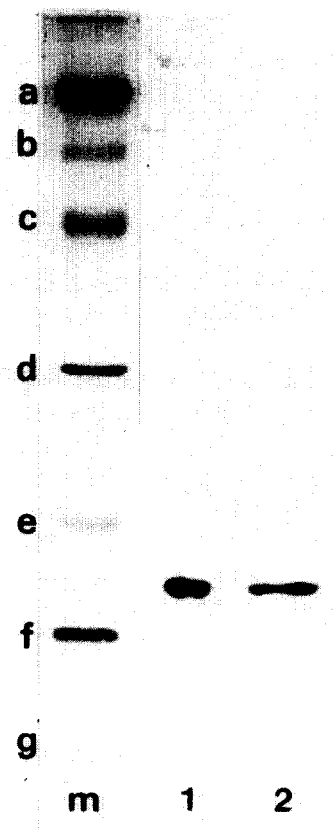

Fig. 2. SDS-polyacrylamide gel electrophoresis. Bound CPI (2.5 μg) was incubated with sample solution in the absence (lane 1) or presence (lane 2) of 2-mercaptoethanol. The gel was stained by Coomassie blue. Lane m, molecular weight markers: a, phosphorylase (M_r 92500); b, bovine serum albumin (M_r 66200); c, ovalbumin (M_r 45000); d, carbonic anhydrase (M_r 31000); e, soybean trypsin inhibitor (M_r 21500); f, lysozyme (M_r 14400); g, Bromophenol blue.

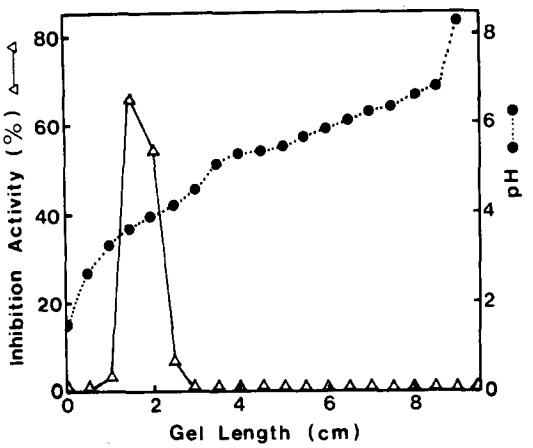

Fig. 3. Isoelectric focusing of bound CPI on polyacrylamide gel containing 2% Ampholine (pH 2.5–8.0). Inhibitor activity for papain extracted from 5 mm thick gels was assayed.

homogeneous soluble CPI (spec. act. 17100 mU/mg) purified by us according to the method of Ohtani et al. [4].

The purity of bound CPI was tested by SDS-polyacrylamide gel electrophoresis and the gel was stained by silver staining or Coomassie blue staining. Bound CPI showed a single protein band, M_r 15000–16000, in the presence and absence of 2-mercaptoethanol (Fig. 2). Molecular weight of the purified soluble CPI used in this study was comparatively determined with bound CPI; it was M_r 13000 as determined by both gel filtration and SDS-gel electrophoresis.

pI of bound CPI

The p*I* value determined by isoelectric focusing was 3.8 (Fig. 3). Recovery of inhibitor activity from the gel was 24%.

Amino acid analysis

The amino acid compositions of bound and soluble CPIs are compared in Table III. Half cystine is absent in soluble CPI, while it is 2.4 mol% in bound CPI. The lysine content was very low in bound CPI. The ratio of acidic-to-basic amino acids (A/B) of bound CPI was 2-fold higher than that of soluble CPI (1.57 and 3.18 in soluble CPI and bound CPI, respectively).

Stability

At temperatures above 80°C, bound CPI is unstable while soluble CPI is relatively stable (Fig. 4). After incubation at 90°C for 5 min, 40% of bound CPI and 70% of soluble CPI activity remained. Both CPIs were stable at 4°C in the pH range 3.0–9.0.

TABLE III

AMINO ACID COMPOSITION OF CPI

Amino acid	Mol%	
	soluble CPI	bound CPI
Asp	13.5	11.3
Glu	15.1	13.6
Ser	3.3	8.8
Gly	9.7	13.6
His	1.1	0.9
Arg	3.5	4.2
Thr	7.4	5.8
Ala	4.1	10.2
Pro	3.7	4.3
Tyr	3.1	2.6
Val	8.7	6.1
Met	2.2	1.1
Half-Cys	0.0	2.4
Ile	2.0	4.0
Leu	9.4	7.3
Phe	4.0	2.6
Lys	14.7	3.6

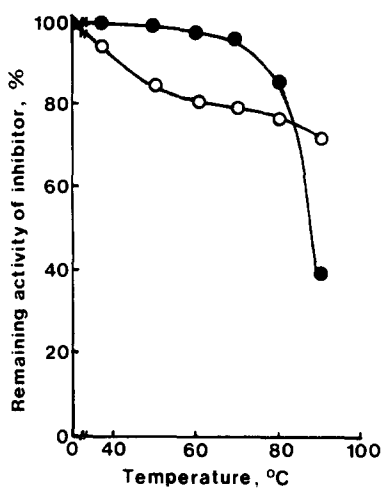

Fig. 4. Heat stability of CPIs. Inhibitor (in 20 µl of 10 mM Tris-HCl, pH 8.0) which causes 50% inhibition of papain (1 µg) was heated at various temperatures for 5 min. After cooling on ice, papain and activation buffer were added and the inhibitory activity was assayed (in a 1 ml system). Bound inhibitor lost its activity rapidly at temperatures above 80°C.

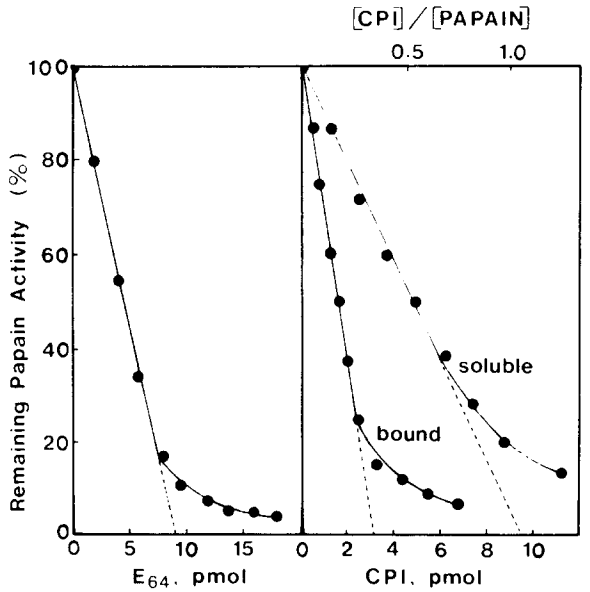

Fig. 5. Inhibitory sites of CPIs for papain. The active site of papain was measured by titration with E_{64} (left). 1 μg of papain contains 9 pmol of the active sites, indicating that 21% of the protein is active as papain, assuming the M_r value of the papain solution to be 23400. The papain solution was titrated with CPIs (right). Soluble CPI (17000 mU/mg) and bound CPI (45000 mU/mg) used were in the range of 0–15 pmol and 0–10 pmol, respectively. $E_t = 9$ nM. E_t/K_i in bound CPI = 82.

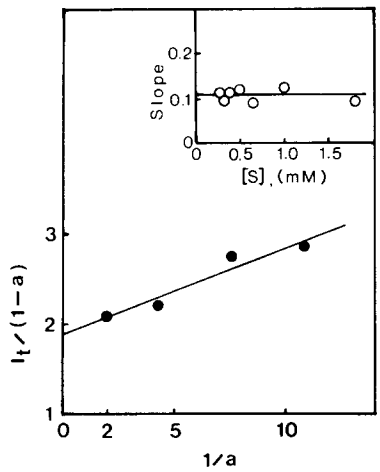

Fig. 6. Determination of the K_i value and mechanism of inhibition. Residual activity of papain was measured in the presence (0.9–1.64 nM) and absence of bound CPI at a fixed substrate concentration, 0.667 mM. The slope obtained with an Easson-Stedman plot was replotted (inset) against the substrate concentration (Bz-Arg-β-NA = 0.2–1.8 mM). The straight line parallel to the abscissa indicates noncompetitive inhibition and a K_i value of 0.11 nm. $E_t = 1.5$ nM; $E_t/K_i = 13.6$.

Inhibitory sites of CPIs for papain

As Fig. 5 shows, soluble and bound CPIs inhibited papain linearly up to 70–80% inhibition. Since inhibition was almost complete during the first 0.5 min of preincubation in both CPIs (data not shown), the deviation from the linearity at a higher inhibitor concentration may merely reflect reversible inhibition. By extrapolation of the linear portion, the binding ratio of papain to soluble and bound CPI was calculated to be 0.94 and 2.8, respectively. The nearest integer of the binding ratio 1 in soluble CPI indicates that the inhibitor contains one inhibitory site per molecule. While about three papain molecules bind to a bound CPI, suggesting three sites in the bound CPI.

TABLE IV

INHIBITION OF SOLUBLE AND BOUND CPI

Proteinase	Substrate	ID_{50} [a] (μg protein)	
		soluble CPI	bound CPI
Rat liver cathepsin B (0.18 μg)	Bz-Arg-βNA	4.6	0.131
Rat liver cathepsin H (0.08 μg)	Bz-Arg-βNA	0.88	0.034
Rat liver cathepsin L (0.09 μg)	azocasein	0.28	0.021
Papain (1 μg)	Bz-Arg-βNA	0.058	0.021
Rat liver cathepsin D (4 μg)	haemoglobin	> 50	> 50
Bovine pepsin (0.8 μg)	haemoglobin	> 50	> 50
Bovine trypsin (2 μg)	Bz-Arg-OEt [b]	> 50	> 20
Bovine chymotrypsin (2 μg)	Bz-Tyr-OEt	> 50	> 50

[a] Amount (μg protein) necessary for 50% inhibition of activity.
[b] OEt, ethoxyl.

Determination of K_i value and type of inhibition

Inhibition of papain activity by bound CPI was plotted (Fig. 6) in accordance with Eqn. 1 of Easson and Stedman [23]:

$$\frac{I_t}{1-a} = \frac{K_i}{a} + E_t \quad (1)$$

Where a is the fractional enzyme activity (V_i/V_0), and I_t and E_t the total inhibitor and enzyme concentration, respectively. A replot of the slope (Fig. 6, inset) against increasing substrate concentration demonstrated no dissociation of the enzyme-inhibitor complex (noncompetitive-type inhibition). The K_i value was found to be 0.11 nM.

Inhibition spectrum

Both bound and soluble CPI inhibited those cysteine proteinases tested but not cathepsin D, pepsin, trypsin and chymotrypsin (Table IV).

Discussion

CPI bound to epidermal cells was extracted and purified at about 6000-fold. The specific activity of bound CPI on papain hydrolysis of Bz-Arg-βNA was 3-times greater than that of CPI purified from the phosphate-buffered saline extract. Chemical properties of bound CPI simulated those of soluble CPI; both have similar molecular weights, belong to the same low-molecular-weight tissue inhibitor group and are stable at pH values ranging from 3.5 to 9. However, bound CPI differs from soluble CPI in its solubility from epidermis and heat stability. Bound CPI was not extracted in buffered 4 M urea which is suited to the extraction of histidine-rich protein from epidermis [24,25]. In addition, we found that bound CPI was not extractable in non-ionic detergent, NP-40 or Triton X-100, 2 M NaCl or 3 M KSCN (data not shown). Buffer A, which has been used for keratin extraction [26], is the only solution used so far to successfully extract bound CPI indicating that CPI exists in tissue as an insoluble component most probably by forming hydrogen bonding or S–S cross-linking and not by ionic or hydrophobic binding. However, bound CPI was easily separated from keratin and other proteins by $(NH)_2SO_4$ precipitation and followed by dialysis against 10 mM sodium phosphate buffer (pH 6.0). Most known tissue inhibitors are heat stable [27–32] as is soluble CPI, but bound CPI lost its activity at temperatures above 80 °C, as shown with serum inhibitors [1,33] and pig peripheral leucocytes [34]. In addition, bound CPI contains half cystine and has a more acidic pI than soluble CPI which does not contain half cystine.

Soluble CPI and liver inhibitor inhibit papain noncompetitively [3,35]. The methodology by which chicken egg white was originally shown to inhibit ficin uncompetitive [36] was found inappropriate by Anastasi et al. [37]. Using a low E_t/K_i ratio and a replot of the inhibitor reaction with enzyme, bound CPI was also found to inhibit papain noncompetitively. The affinity of the bound CPI ($K_i = 0.11$ nM) was comparable to cystatin-like inhibitor CPI-B ($K_i = 0.12$ nM) of human liver [32] and muscle inhibitor ($K_i = 0.1–1$ nM) of bovine [40], and was stronger than that of TPI(1) or TPI(2) ($K_i = 60$ nM and 457 nM, respectively) of rat epidermis [29] which correspond to soluble CPI.

A difference between bound CPI and various other CPIs was also found in relative inhibitor activity as regards rat liver cysteine proteinases. While soluble CPI [4], rat liver CPI [37] and bovine muscle CPI [38] inhibited cathepsin H more strongly than cathepsin B, a much smaller amount of bound CPI inhibited cathepsin B as well as cathepsins H and L. Furthermore, bound CPI has about three inhibitory sites, whereas soluble CPI has one. A low K_i value suggests that the inhibitor may have an unusual structure or repeated functional sequences in the molecule as is reported in kininogen [39] and in double-headed CPI-L [40].

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