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Bombyx Cysteine Proteinase Inhibitor (BCPI) Homologous to Propeptide Regions of Cysteine Proteinases Is a Strong, Selective Inhibitor of Cathepsin L–like Cysteine Proteinases[1]

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***Bombyx* cysteine proteinase inhibitor (BCPI) is a novel cysteine proteinase inhibitor. The protein sequence is homologous to the proregions of certain cysteine proteinases. Here we report the mechanism of its inhibition of several cysteine proteinases. BCPI strongly inhibited *Bombyx* cysteine proteinase (BCP) activity with a K_i = 5.9 pM, and human cathepsin L with a K_i = 36 pM. The inhibition obeyed slow-binding kinetics. The inhibition of cathepsin H was much weaker (K_i = 82 nM), while inhibition of papain (K_i > 1 μM) and cathepsin B (K_i > 4 μM) was negligible. Following incubation with BCP, BCPI was first truncated at the C-terminal end, and then gradually degraded over time. The truncation mainly involved two C-terminal amino acid residues. Recombinant BCPI lacking the two C-terminal amino acid residues still retained substantial inhibitory activity. Our results indicate that BCPI is a stable and highly selective inhibitor of cathepsin L–like cysteine proteinases.**

Key words: *Bombyx*, cathepsin L, cysteine proteinase, inhibitor, propeptide.

Protein inhibitors of cysteine proteinases have been reported in various organisms, and cystatins and the cystatin superfamily have been studied extensively (*1–4*). In a previous study, we identified a novel cysteine proteinase inhibitor, *Bombyx* cysteine proteinase inhibitor (BCPI) (*5*). BCPI was found in the hemolymph of silkmoth *Bombyx mori* as a specific inhibitor of *Bombyx* cysteine proteinase (BCP) (*6*). BCP is a cathepsin L–like cysteine proteinase, belonging to a papain superfamily, and is also present in the hemolymph and accumulates in mature eggs (*7–9*). Two similar but distinct BCPIs have been isolated; BCPIα and BCPIβ. Both BCPIs are inhibitory for other cysteine proteinases such as papain, and cathepsins L and B, but do not inhibit trypsin or pepsin. Interestingly, the amino acid sequence of BCPI is significantly homologous to propeptide sequences of certain cysteine proteinases, such as baculovirus, plant, and protozoan cysteine proteinases (*5*). One protein analogous to BCPI has been reported (*10*). Denizot *et al.* (*10*) indicated that cytotoxic T-lymphocyte antigen (CTLA-2) is highly homologous to the proregion of mouse cathepsin L. They demonstrated the expression of CTLA-2 mRNA in activated T-cells and mast cells, but were not able to define its biological activities. The expression and purification of recombinant CTLA-2β has been reported, and the protein is a proteinase inhibitor capable of inhibiting cathepsin L–like cysteine proteinases (*11*). Based on these early findings, we proposed a new type of cysteine proteinase inhibitors, which resemble cysteine proteinase proregions but are distinct from cystatins (*5*). Several studies have revealed that the proregions of cysteine proteinases have inhibitory effects on the corresponding enzymes (*12–16*). Their most noticeable function is selective inhibition of the cognate enzyme. Fox and colleagues (*17*) were the first to show that the propeptide of cathepsin B selectively inhibited the mature form of cathepsin B with a K_i value in the nanomolar range (*17*). Similar results have been reported for other cysteine proteinases such as papain, and cathepsins L and S (*18–21*). Since BCPI and CTLA-2 are proteins homologous to cysteine proteinase proregions, it would be interesting to determine whether or not these inhibitors exhibit similar selectivity in their action. However, so far only these two proteins have been reported, and even less is known about the mechanism of the inhibition.

In the present study, we showed that BCPI selectively inhibits cathepsin L–like cysteine proteinases with K_i values in the picomolar range. The inhibition obeys a slow-binding kinetics. On incubation with BCP, BCPI was trun-

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Abbreviations: Arg-MCA, L-arginine 4-methylcoumarinyl-7-amide; BCP, *Bombyx* cysteine proteinase; BCPI, *Bombyx* cysteine proteinase inhibitor; CTLA-2, cytotoxic T-lymphocyte antigen-2; E-64, *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine; ESI/MS, electrospray-ionisation mass spectrometry; Z-Arg-Arg-MCA, benzyloxycarbonyl-L-arginyl-L-arginine 4-methylcoumarinyl-7-amide; Z-Phe-Arg-MCA, benzyloxycarbonyl-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide.

cated at the C-terminal, but retained its inhibitory activity.

EXPERIMENTAL PROCEDURES

Enzymes and Inhibitors—*N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-Agmatine (E-64), and enzyme substrates Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, and Arg-MCA were purchased from Peptide Institute (Osaka). BCP was purified and the activities were measured as described previously (*7, 22*). Cathepsin L (human liver), cathepsin B (human liver), and cathepsin H (human liver) were obtained from Calbiochem-Novabiochem (La Jolla, CA). Papain was purchased from Sigma (St. Louis, MO). The activities of these enzymes were measured according to the method of Barrett and Kirschke (*23*). The concentrations of active enzymes were determined by active-site titration using E-64 (*4*). All concentrations stated below are for the active forms of the enzymes. BCPI (BCPIβ) was purified according to the method described previously (*6*). Recombinant BCPIs were expressed in *Escherichia coli* and then purified (see below). Their purity was confirmed by SDS-PAGE, and only preparations giving single protein bands were employed. For inhibition of BCP, the reaction mixture (300 μl) contained 0.1 M sodium acetate, pH 5.5, 1 mM EDTA, 8 mM cysteine, 10 μM Z-Phe-Arg-MCA, 30 ng of activated BCP, and 10 μl of inhibitor. One unit of inhibitory activity was arbitrarily defined as the amount causing 50% inhibition of BCP activity under the conditions used. The protein concentrations of inhibitor proteins were determined using the predicted molar extinction coefficients at 280 nm (calculated from the amino acid sequences) (*24*), and by the method of Bradford using BSA as a standard (*25*). Since BCPI (BCPIβ) contains five tyrosine residues, the molar extinction coefficient was estimated to be 6,400 M^{-1} cm^{-1}.

Kinetic Measurements—The inhibition kinetics of BCP, cathepsins L and B, and papain were studied at 25°C under pseudo-first order conditions. The buffers used were 0.1 M sodium acetate buffer, pH 5.25 for BCP, and pH 5.5 for cathepsins L and B, and 0.1 M sodium phosphate buffer, pH 6.0, for papain. All buffers contained 1 mM EDTA and 8 mM cysteine. The substrates (5–20 μM) used were Z-Phe-Arg-MCA for BCP, cathepsin L and papain, and Z-Arg-Arg-MCA for cathepsin B. The reaction was initiated by the addition of 10 μl of enzyme (diluted with 0.1% Brij-35 and preactivated with 8 mM cysteine) to 990 μl of the buffer/substrate solution containing the inhibitor. The enzyme concentrations used were 7.2 pM for BCP, 1.7 pM for cathepsin L, 730 pM for cathepsin B, and 3.9 pM for papain. The progress curves were monitored continuously at excitation and emission wavelengths of 370 and 460 nm with a spectrofluorometer (model F2000, Hitachi). For inhibition of enzymes, nonlinearity in the initial stage of the reaction was observed, indicating the presence of a slow-binding inhibition process, as defined by the following equation:

$$[P] = v_s t + (v_i - v_s)[1-\exp(-k_{obs}t)]/k_{obs},$$

where P represents the product concentration, v_i and v_s the initial and steady-state velocities, respectively, and k_{obs} the apparent first-order rate constant for the inhibition (*26, 27*). The progress curves were computer-aided fitted by nonlinear regression analysis using the proFit 5.5 program (published by QuantumSoft, Zurich, Switzerland). The analysis was achieved with different inhibitor concentrations, and the individual parameters (v_i, v_s, and k_{obs}) were obtained for each progress curve. The k_{obs} values were fitted to the equation: $k_{obs} = k_{diss} + k_{ass}[I]/(1+ [S]/K_m)$, where k_{ass} and k_{diss} represent the association and dissociation rate constants, respectively, and K_m the Michaelis constant values for the corresponding enzymes (plotted as k_{ob} *versus* [I], the slope being $k_{ass}/(1 + [S]/K_m)$. Then, k_{ass} values were calculated. The k_{diss} values were obtained from individual measurements, $k_{diss} = k_{obs}v_s/v_i$, and K_i values were calculated from $K_i = k_{diss}/k_{ass}$.

The activity of cathepsin H in the presence of BCPI was measured under almost the same experimental conditions as described above, using 680 pM cathepsin H in 0.1 M potassium phosphate buffer, pH 6.8, containing Arg-MCA as the substrate. As inhibition of cathepsin H yielded linear plots of [P] *versus* time, the K_i value was determined from Dixon plots of the form 1/*V* *versus* [I] with two different substrate concentrations (10 and 20 μM) (*28*).

Treatment of BCPI with BCP and Preparation of Cleaved BCPI—BCPI was incubated at a final concentration of 0.3 mg/ml with BCP in a molar ratio of 50:1 at 37°C. The buffers used were the same as those used for the standard inhibition assay described above. As a control, 0.6 mM E-64 was added to the incubation mixture. After incubation, an aliquot of the mixture was taken and the reaction was stopped by adding 0.6 mM E-64. Then, samples were subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue R-250 or silver nitrate reagent (*6*). For preparation of a large amount of treated BCPI, 180 μg of BCPI was incubated with BCP for 2 h at 37°C. The incubated samples were purified by HPLC on a Aquapore RP-300, C8 column (Applied Biosystems, San Jose, CA). The chromatographic conditions were the same as described previously (*6*). HPLC gave one large peak at almost the same retention time as that of the intact BCPI, and fractions comprising the peak were collected. After confirming the purity and treatment by SDS-PAGE, the treated BCPI was first N-terminal sequenced with an automated protein sequencer (Model 473A; Applied Biosystems). For C-terminal sequencing, treated BCPI after HPLC was evaporated to dryness, dissolved in 50% acetonitrile containing 0.05% formic acid, and then subjected to electrospray-ionization mass spectrometry (ESI/MS) (SCIEX API-300; Perkin Elmer-Cetus, Foster City, CA). Circular dichroism (CD) spectra were obtained with a JASCO spectropolarimeter (J-600; Japan Spectroscopic). Near and far UV CD spectra were measured in cells with path lengths of 10 and 1 mm, respectively. All experiments were performed at room temperature (25°C) in 10 mM sodium acetate, pH 5.5.

Expression and Purification of Recombinant Proteins—Routine molecular cloning techniques were used (*29*). cDNA of BCPIβ was employed for expression of recombinant BCPIs (*5*). In order to express recombinant inhibitors, putative leader sequences were removed, and matured regions were expressed as fusion proteins with *E. coli* thioredoxins, allowing soluble expression of the recombinant proteins (*30*). Mutant BCPIs lacking C-terminal amino acid residues were expressed. They were rBCPI2D, rBCPI6D, and rBCPI10D, lacking 2, 6, and 10 amino acid residues, respectively. The primers used were designed based on the BCPIβ cDNA sequence, having additional restriction enzyme sites, *Nco*I sites for forward primers and *Sal*I sites for reverse primers. cDNA fragments were amplified from the

cDNA by means of PCR techniques. The amplified DNA fragments were digested with *Nco*I and *Sal*I, and then ligated into the *Nco*I and *Sal*I sites of pET32a plasmid vectors (Novagen, Madison, WI), joined to the 3′-end of the *E. coli* thioredoxin gene. The plasmid DNAs were used for transformation into *E. coli* XL1-Blue cells (Stratagene, La Jolla, CA), and were transferred to expression host strains *E. coli* BL21(DE3)pLys (Novagen). Expression was performed according to the method described previously (*31*). Between the thioredoxin and recombinant inhibitor protein, there lies a linker peptide. This linker peptide includes a His-Tag sequence (HisHisHisHisHisHis), which is used for purification of the fusion proteins, and the sequence of AspAspAspAspLys, which is the recognition sequence for protease enterokinase. Thus, the fusion proteins were first purified by affinity chromatography on His-Bind metal chelation resin, and then the recombinant inhibitor proteins were separated from the fusion proteins by cleavage with enterokinase. *E. coli* cell pellets were suspended and sonicated in 20 mM Tris-Cl, 0.5 M NaCl, 5 mM imidazole, 0.1% Triton X-100 containing 0.1 mM phenylmethylsulphonyl fluoride and 0.1 mg/ml lysozyme, and the resulting lysates were separated into soluble and insoluble fractions by centrifugation. The fusion proteins were expressed as soluble proteins, and then purified with the His-Bind resin (Novagen) according to the protocol supplied by the manufacturer. The recombinant inhibitor proteins were then cleaved away from the fusion proteins by digestion with enterokinase, and purified with an "Enterokinase cleavage capture kit" (Novagen). The homogeneity and molecular weights of the proteins were determined by SDS-PAGE.

RESULTS

Inhibition Kinetics of BCPI—BCPI caused typical slow-binding inhibition of BCP when activated BCP was added to a mixture of BCPI and Z-Phe-Arg-MCA (Fig. 1A). As BCP showed a broad pH activity curve around pH 5.0, the inhibition study was performed in the pH range of 4.0–6.0, slow-binding inhibition being exhibited (data not shown). With different BCPI concentrations, k_{obs}, v_i, and v_s values were obtained. A plot of k_{obs} *versus* [BCPI] remains linear in

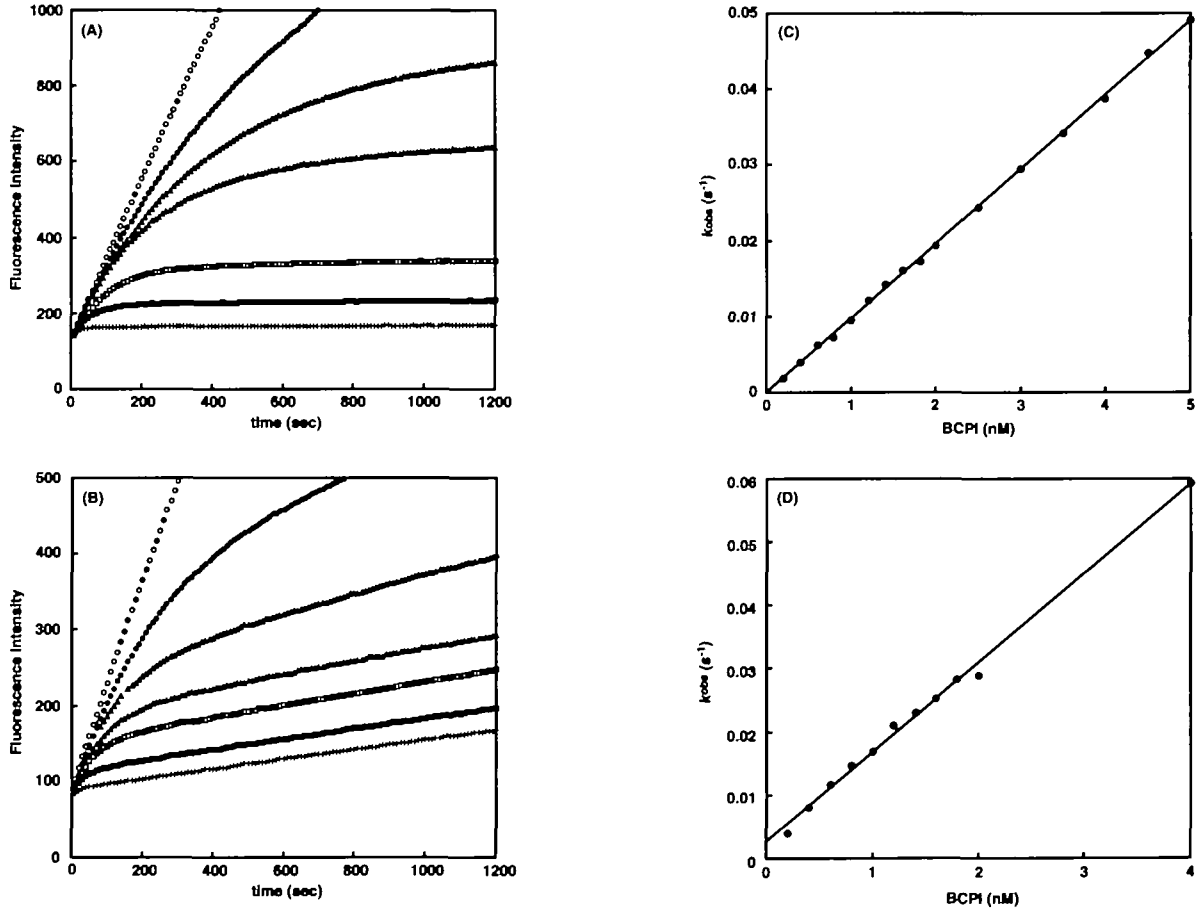

Fig. 1. **Slow-binding inhibition of BCP and cathepsin L by BCPI.** Measurements were performed at 25°C as described under "EXPERIMENTAL PROCEDURES." (A) Progress curves for the inhibition of 7.2 pM BCP by BCPI in the presence of 10 μM Z-Phe-Arg-MCA. The BCPI concentrations (nanomolar) were as follows: (open circles) 0; (closed circles) 0.20; (open triangles) 0.30; (closed triangles) 0.50; (open squares) 1.0; (closed squares) 2.0; (crosses) 5.0. (B) Progress curves for the inhibition of 1.7 pM cathepsin L by BCPI in the presence of 5 μM Z-Phe-Arg-MCA. The BCPI concentrations (nanomolar) were as follows: (open circles) 0; (closed circles) 0.20; (open triangles) 0.40; (closed triangles) 0.60; (open squares) 1.0; (closed squares) 2.0; (crosses) 4.0. Data points were plotted every 10 s. The progress curves were fitted by nonlinear regression analysis (see text), and the k_{obs} value was obtained for each BCPI concentration. Then, k_{obs} was plotted against BCPI concentration. (C) Plot of k_{obs} *versus* [BCPI] concentration for the inhibition of BCP. (D) Plot of k_{obs} *versus* [BCPI] concentration for the inhibition of cathepsin L.

the inhibitor concentration range of 0 to 5 nM, allowing us to calculate k_{ass} with the K_m value of BCP (12 μM) (Fig. 1C). k_{diss} was measured directly, and K_i was calculated from $K_i = k_{diss}/k_{ass}$. The K_i value was calculated to 5.9 pM, indicating that BCPI binds tightly to BCP. To confirm the accuracy of the K_i value, the K_i value was calculated from the relationship $v_i/v_s = 1 + [I] / K_{iapp}$, yielding K_i = 7.4 pM, which was in good agreement with the value calculated from k_{ass} and k_{diss}. As shown in Fig. 1 (B and D), BCPI also caused marked inhibition of human cathepsin L with a K_i = 36 pM and slow-binding kinetics. BCPI inhibited papain and cathepsin B through similar slow-binding inhibition mechanisms, but was much more weakly inhibitory for these enzymes. In contrast to the K_i values in the picomolar range for BCP and cathepsin L, K_i values in the micromolar range were calculated for papain (K_i > 1 μM) and cathepsin B (K_i > 4 μM). Under the experimental conditions used for the inhibition of cathepsin H, the progress curve for substrate hydrolysis appeared to be linear. Therefore, 1/*V versus* [BCPI] plots were obtained with two different substrate concentrations, and the plots yielded intercepting lines. A value of 82 nM was obtained for K_i. All K_i values obtained are summarized in Table I.

Truncation of BCPI by BCP at the C-Terminal End—Incubation of BCPI with BCP in the enzyme/inhibitor

TABLE I. **Inhibition constants of cysteine proteinases with BCPI.**

	K_i (nM)
BCP	0.0059 ± 0.0001[a]
Cathepsin L	0.0360 ± 0.016[a]
Cathepsin H	82.0 ± 0.5[b]
Papain	>1,000[a]
Cathepsin B	>4,000[a]

K_i values represent the means ± SEM for triplicate experiments. [a]Calculated from k_{obs}, v_i, and v_s. [b]Calculated from $1/v$ *vs.* [I] (see "EXPERIMENTAL PROCEDURES").

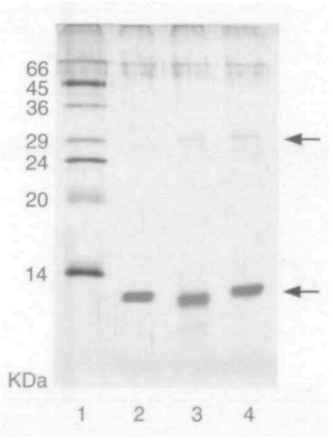

Fig. 2. **Cleavage of BCPI by BCP, as analyzed by SDS-PAGE.** BCPI was incubated with BCP in the molar enzyme to inhibitor ratio of 1:50 for 1 h at 37°C. Following incubation, samples were subjected to SDS-PAGE (17.5% polyacrylamide gel), and the gel was stained with silver nitrate reagent. Lane 1, molecular weight markers, with the sizes of standards shown on the left; lane 2, BCPI alone; lane 3, BCPI incubated with BCP; lane 4, BCPI incubated with BCP in the presence of E-64. The upper arrow indicates BCP and the lower one BCPI. See "EXPERIMENTAL PROCEDURES" for details.

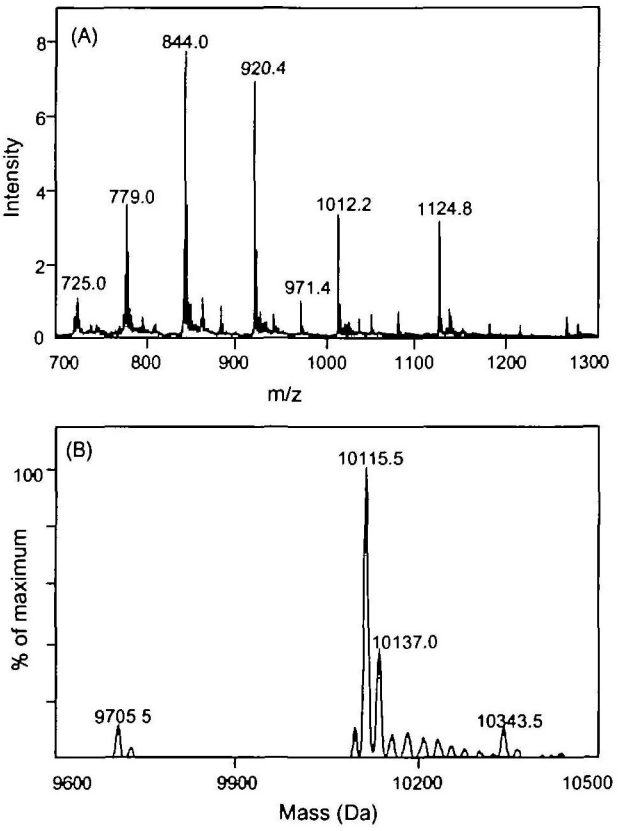

Fig. 3. **Mass spectrum of truncated BCPI.** BCPI was treated with BCP and then purified on a reversed-phase HPLC column. A sample was then injected into the mass spectrometer. (A) Observed ion-spray mass spectrum in the 700–1,300 m/z range. (B) Reconstructed mass spectrum obtained from the spectrum shown in (A) over the mass range of 9,600–10,500 Da.

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                1                                                    60
BCPI            ETDTPRHYDL NQAKELFEIF VKEHNREYKD DADRELHYQS FKKHLAEINQ LNEKNPYTT-
                                                  *              *   *
CTLA-2 alpha    -----AAPPP DPSLDNEWKE WKTKPAKAYN LNEKRHRRLV WEENKKKIEA HNADYEQGKT
                     ||| | |||||||||| |*| ||||| | ||||||| |||||||*|| |*|||| |||
CTLA-2 beta     -----AAPSP DPSLDNEWKE WKTTFAKAYS LDEERHRRLM WEENKKKIEA HNADYERGKT

               61                                                   120
BCPI            ---FGINKFA DYTPEEQQSR LGLRLPAKKT ..........  .......... ..........
                   * * *  * ****
CTLA-2 alpha    SFYMGLNQFS DLTPEEFKTN CYGNSLNRGE MAPDLPEYED LGKNSYLTPG RAQPE.....
                ||||*|*|*| *|****| || | | |  ||| ||||||||||| |||||||||| |||||
CTLA-2 beta     SFYMGLNQFS DLTPEEFRTN CCGSSVNCRGE MAPDLPEYED LGKNSYLTPG RAQPE.....
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Fig. 4. **Amino acid sequences of BCPI and CTLA-2.** Vertical lines indicate amino acid residues identical between CTLA-2α and CTLA-2β. Asterisks (*) indicate amino acid residues identical between BCPI and CTLA-2. Arrows indicate the sites of truncation of BCPI on treatment with BCP.

TABLE II. **Inhibition of BCP by recombinant BCPI.**

	Activity (units/μg)	K_i (nM)
BCPI	180 ± 17	0.0059 ± 0.0001
rBCPI	34.0 ± 5.7	0.110 ± 0.006
rBCPI2D	31.0 ± 4.7	0.360 ± 0.037
rBCPI6D	15.0 ± 2.1	2.40 ± 0.19
rBCPI10D	nsi	nsi

Inhibitory activities and K_i values are expressed as means ± SEM for triplicate experiments. K_i values were calculated from k_{obs}, v_i, and v_s (see "EXPERIMENTAL PROCEDURES"). nsi, no significant inhibition.

molar ratio of 1:50 at 37°C for 1 h resulted in a truncated BCPI, as revealed on SDS-PAGE (Fig. 2). Intact BCPI remained its full-length form for 20 min and then started to be gradually converted into a truncated form. The truncated form was stable for at least 4 h, but on longer (overnight) incubation this form was eventually degraded. Incubation of BCPI with human cathepsin L in the enzyme/inhibitor molar ratio of 1:100 at 37°C for 1 h resulted in similar truncation of the BCPI (data not shown). As a control, BCPI alone was incubated under the same buffer conditions, but there was no breakdown of the inhibitor even after overnight incubation. A cysteine proteinase inhibitor, E-64, completely blocked both the truncation and following breakdown of BCPI. For preparation of the truncated BCPI, a larger amount of BCPI was incubated with BCP, and then subjected to HPLC. The truncated BCPI still retained substantial inhibition activity toward BCP after HPLC preparation. In order to determine which terminal end of the BCPI was truncated, N- and C-terminal protein sequencing was performed. The N-terminal sequence was identical to that of the intact BCPI, indicating that the cleavage occurred at the C-terminus. For C-terminal sequencing, the truncated BCPI was subjected to ESI/MS. The spectrum obtained is shown in Fig. 3. The spectrum showed the presence of multiple-charged ions corresponding to a protein of a molecular mass of 10,114.05 ± 1.28 Da. This mass can be attributed to BCPI lacking two C-terminal amino acid residues (KT) (Fig. 4) with a calculated molecular mass of 10,113.2 Da. As a minor cleavage product (<10% of the total), the spectrum showed a protein of a molecular mass of 9,704.66 ± 0.78 Da, which can be attributed to BCPI lacking six C-terminal amino acid residues (LPAKKT) with a calculated molecular mass of 9,703.18 Da. As a control, intact BCPI was also subjected to ESI/MS, yielding an estimated molecular mass of 10,342.46 ± 1.01 Da, which clearly matched that of the intact BCPI (10,342.5 Da) (data not shown). This indicates that no post-translational modification of BCPI occurs.

Inhibitory Activities of Recombinant BCPIs—In order to clearly define the nature of the truncation at the C-terminal end, we prepared several recombinant BCPIs lacking C-terminal amino acid residues. The recombinant inhibitors differed from the wild-type protein by two additional amino acid residues (Ala-Met) at the N-terminal end. The recombinant BCPIs were expressed and purified, and then their inhibitory activity toward BCP was measured as described under "EXPERIMENTAL PROCEDURES." The recombinant inhibitors also caused slow-binding inhibition of BCP, and from the progress curves K_i values were calculated for each recombinant BCPI. All data obtained are shown in Table II. The inhibitory activity of the recombinant BCPI

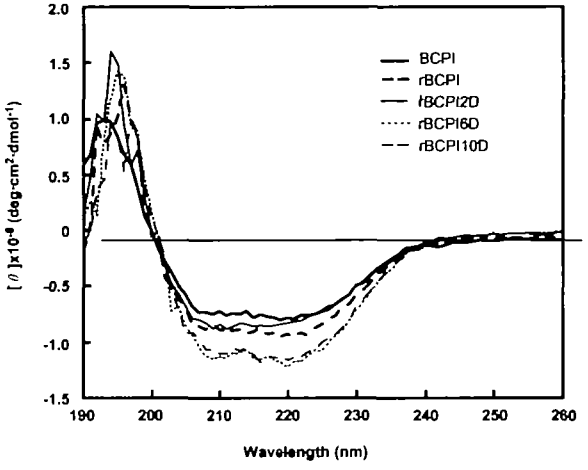

Fig. 5. **Far UV CD spectra of BCPI and recombinant BCPIs.** Spectra were measured at room temperature in 10 mM sodium acetate buffer, pH 5.5, at 10 μM protein concentration. BCPI, purified BCPI; rBCPI, recombinant BCPI; rBCPI2D, rBCPI lacking C-terminal two amino acids; rBCPI6D, rBCPI lacking the C-terminal six amino acids; rBCPI10D, rBCPI lacking the C-terminal ten amino acids.

containing the full-length amino acid sequence (rBCPI) was about 20% of that of native BCPI. The K_i value was about 20 times that of the native BCPI but was still in the nanomolar range of 0.110 nM. The inhibitory activity of the recombinant BCPI lacking the C-terminal 2 amino acid residues, which corresponds to the major truncated form, was also similar, although the K_i value was about three times that of the recombinant full-length BCPI. Further removal of C-terminal amino acid residues resulted in a loss of the inhibitory activity. Removal of 6 amino acid residues caused a significant decrease in the inhibitory activity (about half) and an increase in the K_i value to about 20 times that of the full-sized recombinant BCPI. The inhibitory activity of the recombinant BCPI lacking the C-terminal 10 amino acid residues was negligible. Far UV CD spectra of BCPI, the recombinant BCPI, and recombinant BCPIs lacking C-terminal amino acid residues are shown in Fig. 5. The spectrum of rBCPI is quite similar to that of BCPI, indicating that the secondary structure content of the recombinant BCPI is the same as that of the native BCPI. Moreover, the spectra remained quite similar upon deletion of the C-terminal. This indicates that truncation at the C-terminal end (two, six, and ten amino acid residues) does not cause a significant change in the secondary structure of BCPI. Near UV CD spectra of BCPI and rBCPI are devoid of a dichroic signal, indicating that BCPI is essentially devoid of a compact tertiary structure (data not shown).

DISCUSSION

BCPI is a strong inhibitor of BCP with a dissociation constant (K_i) of 5.9 pM. This value is comparable with dissociation constants obtained between cystatins and cysteine proteinases (*1–4*). Under the conditions used in the present experiments, inhibition by BCPI exhibited a slow-binding mechanism. Cystatins usually show slow-binding inhibition mechanisms when they inhibit cysteine proteinases. The propeptide of rat cathepsin B is a slow-binding inhibitor for

cathepsin B at pH 6.0 (*17*). In this case, the mechanism was pH-dependent; at pH 4.0, the propeptide behaved as a classical competitive inhibitor. Moreover, the K_i value increased markedly with a decrease in pH from 6.0 to 4.0. On the other hand, the inhibition mechanism of BCPI was not dependent on the pH in the range of 4.0–6.0. Further studies are necessary to determine whether the K_i values vary with changes in the pH. BCPI is a strong inhibitor not only for its corresponding enzyme, BCP, but also for human cathepsin L, with a K_i value of 36 pM. It would be interesting to compare these results with those for propeptide inhibitors. The propeptides of papain and papaya proteinase IV hardly inhibited cathepsins B; the K_i values were in the micromolar range, while they inhibited their cognate enzymes with nanomolar K_i values (*18*). In contrast, the propeptide of rat cathepsin B is a potent inhibitor of cathepsin B with a K_i value of 0.4 nM at pH 6.0, whereas the K_i value for the inhibition of papain is 5.6 μM (*17*). Cysteine proteinases belonging to the papain group can be divided into two subclasses: the cathepsin B class and the non-cathepsin B class, which includes papain, and cathepsins L, H, and S (*32*). It is therefore not surprising that the propeptide of cathepsin B does not show affinity for non-cathepsin B class enzymes such as papain, and *vice versa*. It is also reasonable that BCPI does not exhibit affinity for cathepsin B because BCPI is homologous to the proregions of cysteine proteinases belonging to the non-cathepsin B class (*5*). BCPI was, in fact, less inhibitory for cathepsin B (K_i > 4 μM). The cathepsin L propeptide is a much more selective inhibitor and is a potent inhibitor of cathepsin L with a K_i = 0.088 nM (*19*). It is noteworthy that not only cathepsin B but also papain are hardly inhibited by the propeptide (K_i > 1 μM). Based on these results, Carmona *et al.* (*19*) suggested that selective inhibition by the cathepsin L propeptide can be achieved within the non-cathepsin B class proteinases. Moreover, there may be weak selectivity even in inhibition within cathepsins (cathepsins L and S), because the K_i value for the inhibition of cathepsin S was much higher (K_i = 45 nM). The data obtained here show the possibility of similar selectivity for BCPI. BCPI was also a potent inhibitor of BCP and cathepsin L. It was a much weaker inhibitor of cathepsin H (K_i = 82 nM), and did not inhibit papain at up to 1 μM. Thus, it could be concluded that BCPI, like the cathepsin L propeptide, is a selective inhibitor of cathepsin L–like cysteine proteinases. BCPI is an inhibitor protein and is not a propeptide of BCP. This means that BCP is not a real cognate enzyme of BCPI. However, the characteristics of BCPI observed here strongly support the notion that BCPI is a functional propeptide originating from BCP.

It must be noted that contrary to our observations for BCPI, CTLA-2β was reported to be a non-selective inhibitor, inhibiting cathepsin L (K_i = 24 nM), papain (K_i = 25 nM), and cathepsin H (IC_{50} = 67 nM) (*11*). Two CTLA-2 proteins have been previously reported, CTLA-2α and CTLA-2β (*10*) (Fig. 4). The amino acid sequences of these two proteins are quite similar. We recently found that CTLA-2α may be a selective inhibitor of cathepsin L–like cysteine proteinases (Kurata *et al.*, in preparation). Although more precise kinetic studies are required, CTLA-2α, therefore, may be functionally different from CTLA-2β. This difference is interesting because there are also two similar types, BCPIα and BCPIβ, of BCPI.

BCPI was first truncated by BCP at the C-terminal end and then gradually degraded on prolonged incubation with the enzyme. The truncation mainly involves the two C-terminal amino acid residues. The cathepsin B propeptide has been reported to be cleaved by cysteine proteinases (*17*). The propeptide of cathepsin B was degraded into small peptides on 24 h incubation with cathepsin B or papain at pH 4.0. The propeptide of BCP was completely digested by BCP within 1 h under experimental conditions similar to those used here (Yamamoto *et al.*, unpublished data). No truncation has been observed for propeptide inhibitors. Cystatin C is known to be truncated by some endopeptidases (*33, 34*). Recombinant human cystatin C was truncated at the N-terminal end by cathepsin L. Truncation of cystatin C by 11 N-terminal amino acid residues resulted in a major loss of inhibitory activity. In contrast, using recombinant BCPIs, we showed in the present study that the truncated BCPI still retains sufficient inhibitory activity toward BCP. This suggests that the C-terminal end of BCPI may interact with the BCP active site and be cleaved by the enzyme, but the truncation does not strongly affect the affinity of the inhibitor for the enzyme. Data obtained on CD analysis also strongly support this suggestion.

Many efforts have been made to develop specific inhibitors that target individual proteinases, and the propeptides of cysteine proteinases have been specifically identified because of their high selectivity in inhibition (*21, 35, 36*). In particular, for parasite cysteine proteinases, such development is very important because these enzymes play a critical role in host invasion and in the following life cycle of the parasites. In this report, we showed that BCPI, a functional propeptide, is a stable, highly selective inhibitor. Studies involving recombinant BCPIs are in progress in our laboratories.

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