Binding and Cleavage by Trypsin and Chymotrypsin at the Reactive Sites of Proteinase Inhibitors from Brazilian Pink Beans (*Phaseolus vulgaris*, Variety Rosinha G2)

Chao Wu and John R. Whitaker*

Department of Food Science and Technology, University of California, Davis, California 95616

Binding and cleavage of selected trypsin/chymotrypsin inhibitors from Brazilian pink beans (BPB) were studied. Trypsin binding to BPB inhibitors B(B) and B(C) was tightest at pH 7–9; looser complexes were formed at pHs below 7 and above 9. Inhibitor B(B) bound 2 trypsin molecules/mol in the absence of chymotrypsin; with both enzymes present only chymotrypsin–inhibitor–trypsin (1:1:1) complex was observed. Three cleavage derivatives of B(B), Bt_1, Bt_2, and Bc (t, trypsin; c, chymotrypsin) were isolated. Bt_1 and Bc had full activity against trypsin but reduced activity against chymotrypsin. Bt_2 had reduced activity against both enzymes. Chymotrypsin resynthesized the chymotrypsin reactive site in Bt_2 at pH 7.0; trypsin did not resynthesize either site. The results conclusively showed that trypsin preferentially cleaved the chymotrypsin-binding site, as confirmed by use of carboxypeptidases A and B.

INTRODUCTION

Naturally occurring proteinase inhibitors are found in a wide variety of plant and animal tissues. Proteinase inhibitors in animal tissues play critical roles in the regulation of numerous physiological processes including intracellular protein catabolism, processing of precursor proteins, blood coagulation, immunological processes, and other self-defense mechanisms. The physiological functions of plant proteinase inhibitors, on the other hand, are still not clear. Three functions have been proposed for plant proteinase inhibitors, i.e., as regulatory agents in controlling endogenous proteinases, as storage proteins, or as protective agents directed against insect or microbial proteinases (Ryan, 1973; Liener and Kakade, 1980).

The chemistry and biochemistry of proteinase inhibitors have been studied in detail not only because of their biological importance but also because they provide excellent models for specific interactions between macromolecules (Means et al., 1974). The recognition and binding between antigen and antibody, DNA and DNA binding protein, and complement and its receptor and interactions between enzyme subunits are other examples of interactions between macromolecules having great biological importance and therefore under active investigation. Nevertheless, studies on interactions between proteinases and their proteinaceous inhibitors, because of the ease in following the interaction by measuring enzyme activity left, still contribute most to our understanding of macromolecular interactions.

Most proteinase inhibitors from *Phaseolus vulgaris* that have been well studied belong to the Bowman–Birk family (Sgarbieri and Whitaker, 1982; Garcia-Olmedo et al., 1987). They are all double headed and have similar structural features to the soybean Bowman–Birk inhibitor. The inhibitors have various specificities. Most of the inhibitors from *P. vulgaris* are double headed against trypsin and chymotrypsin, although some are against trypsin and elastase (Wilson and Laskowski, 1975) or two molecules of trypsin (Chu and Chi, 1965; Wagner and Riehm, 1967).

We have purified to homogeneity three inhibitors from Brazilian pink beans (*P. vulgaris* var. Rosinha G2) (Whitaker and Sgarbieri, 1981) and four inhibitors from red kidney beans (*P. vulgaris* var. Linden) (Wu and Whitaker, 1990). All, except one [R(B1)], are double headed against trypsin and chymotrypsin (Wu and Whitaker, 1990; Wu and Whitaker, 1991). These inhibitors have very similar properties and homologous sequence and structure (Wu and Whitaker, 1991). In this paper, we describe our further studies on Brazilian pink bean inhibitors with respect to characteristics of binding to trypsin and chymotrypsin and the interaction mechanism.

MATERIALS AND METHODS

Materials. Three inhibitors from Brazilian pink beans (*P. vulgaris* var. Rosinha G2) were purified according to the methods of Whitaker and Sgarbieri (1981) and were designated B(A), B(B), and B(C). These inhibitors are very similar in terms of their properties, sequences and structures (Wu and Whitaker, 1991); therefore, further detailed studies on binding to trypsin and chymotrypsin focused mainly on one or two of the inhibitors.

Bovine pancreas trypsin (type III), carboxypeptidase A–PMSF, carboxypeptidase B–DFP, N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BANA), *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA), *p*-nitrophenyl-*p'*-guanidinobenzoate hydrochloride, *p*-nitrophenyl acetate, acrylamide, sodium dodecyl sulfate (SDS), and 5,5'-dithiobis(2-nitrobenzoic acid) were from Sigma Chemical Co. α-Chymotrypsin was from Worthington Biochemical Corp. *N,N'*-Methylenebis(acrylamide) and ammonium persulfate were from Bio-Rad Laboratories. All the chemicals were of analytical grade. Deionized water was used in all the experiments.

Methods. *Trypsin and α-Chymotrypsin Assays.* Trypsin and chymotrypsin activities were determined spectrometrically by measuring the rate of absorbance increase at 410 nm using N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPA) and *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA) as substrates for trypsin and chymotrypsin, respectively (Erlanger et al., 1961, 1966). Substrate was added after enzyme and inhibitor had been incubated at 30 °C for selected periods of time (usually 10 min).

Inhibition Constant Determination. The dissociation constants of trypsin–inhibitor complexes (K_i) in this study were determined according to the method of Bieth (1974). BANA was used as substrate because of its high stability at different pHs.

Gel Electrophoresis of Enzyme–Inhibitor Complexes. Electrophoresis of enzyme–inhibitor complexes was carried out on polyacrylamide gels with continuous buffer systems. The inhibitors were mixed with trypsin or chymotrypsin in various molar ratios in 0.02 M Tris buffer and allowed to stand for 10 min. An aliquot of the solution containing 10–20 μg of total protein was

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loaded onto a 12% gel. For anodic PAGE the electrophoresis buffer was 0.1 M Tris/glycine buffer (pH 9.5). For cathodic PAGE 0.07 M β-alanine/acetate buffer (pH 4.5) was used for electrophoresis. Electrophoresis was carried out in the Mighty small slab gel (7 × 8 cm, Hoeffer Scientific Instruments) with a gel thickness of 0.75 mm. Bromophenol blue and methyl green were included as tracking dyes in samples for anodic and cathodic electrophoresis, respectively. Constant current of 12 mA was employed, and the electrophoresis took 30–50 min. After electrophoresis, gels were fixed in 10% sulfosalicylic acid for 20 min and stained with 0.1% Coomassie Blue R-250 in 25% methanol and 10% acetic acid for 1–2 h. Gels were then destained in 7% acetic acid solution until the background was clear.

Gel Filtration Chromatography of Enzyme–Inhibitor Complexes. Enzyme–inhibitor complexes were also chromatographed by gel filtration on a FPLC system. A Superose 12 HR10/30 column was equilibrated with 0.22 M Tris buffer containing 0.25 M NaCl (pH 8.10). Inhibitor B(B) and enzymes were mixed in various ratios in the above buffer and allowed to stand at room temperature for 10 min. An aliquot (100 μL) containing 20–30 μg of total protein was loaded to the column. The same buffer was used for the elution. The inhibitor and each enzyme were also chromatographed on the same column individually to determine their elution volumes. Absorbance at 214 nm was monitored. Flow rate was 0.5 mL/min.

Limited Proteolysis. To determine the best conditions for controlled cleavage of inhibitors by proteinases at the reactive sites, the inhibitors were incubated with catalytic amounts of trypsin or chymotrypsin (molar ratio of enzyme to inhibitor of 1:50) at different pHs (2.3–5.0) at room temperature (23 °C) for various periods of time. At the end of an incubation period, an aliquot of the solution was transferred into a buffer at pH 6.8 containing 30% glycerol and stored at −20 °C before electrophoresis analysis. The extent of reaction was followed by measuring the relative amounts of cleavage products at different times by gel electrophoresis (10 μg of each sample was loaded); differently cleaved products were quantitated by using a laser scanning densitometer after the gels were stained. Controls were also made with inhibitors incubated under the same conditions but without enzyme addition. No change in total activity and electrophoresis patterns was observed for controls at the end of incubation. In a preparative procedure, inhibitors were dissolved (2 mg/mL) in 0.04 M glycine buffer (pH 2.5), and trypsin or chymotrypsin (40 μg/mL) was added. The solution was incubated at room temperature for 4 days, and the reaction was stopped by adjusting the pH to 7.0 and storing the solution at −20 °C. Under such conditions, nearly all of the inhibitor was cleaved.

Isolation of Cleaved Inhibitors. Electrophoresis under native conditions was used to separate cleaved inhibitors from the original (intact) inhibitors and to recover them. After controlled cleavage, 1–2 mg of the mixture of intact and cleaved inhibitors was dissolved in about 0.4 mL of sample buffer and loaded onto a cast polyacrylamide slab gel. The buffer system was the same as in the normal native gel electrophoresis system for analytical purpose (Davis, 1964) except there was only one big sample well and the gel was thicker (1.5 mm). After electrophoresis, the gel was immersed in 10% trichloroacetic acid solution. After less than 10 min, the white precipitated inhibitor in the gel could be seen. The bands were then cut out, and the inhibitors were electroeluted according to the method of Hunkapiller (1983).

When there was essentially a single product after the controlled cleavage, ion-exchange chromatography with a DEAE-Sephacel column (1.5 × 10 cm) was used to separate the cleaved inhibitor from the enzyme. The column was equilibrated with 0.02 M ammonium acetate (pH 6.0). The cleaved inhibitor was eluted with a linear gradient of 0.02–0.5 M ammonium acetate buffer (pH 6.0).

Treatment with Carboxypeptidases A and B. Inhibitors, after limited proteolysis and isolation, were dissolved in 0.1 M ammonium bicarbonate buffer (pH 8.20). Carboxypeptidase A–PMSF or carboxypeptidase B–DFP (4% of inhibitor weight) was added, and the solution was incubated at room temperature. At different times, aliquots were taken from each sample and assayed for antitrypsin and antichymotrypsin activity by using the same inhibitor without carboxypeptidase treatment as control.

Treatment of Reactive Site Cleaved Inhibitor with Trypsin and Chymotrypsin at Neutral pH. Inhibitor with both reactive sites cleaved (Bt_2) was dissolved in 0.1 M NH_4HCO_3 buffer, pH adjusted to 7.0 with acetic acid; equal or excess molar amount of trypsin or chymotrypsin was added. The solution was allowed to stand at room temperature for 1 h before 100% trichloroacetic acid (w/v) was added to make a final TCA concentration of 5%. The samples were then centrifuged to remove the precipitate (denatured enzyme), and the supernatant was adjusted to pH 7.0 by adding 2 M Tris (free base). The neutralized supernatant was dialyzed against water and lyophilized.

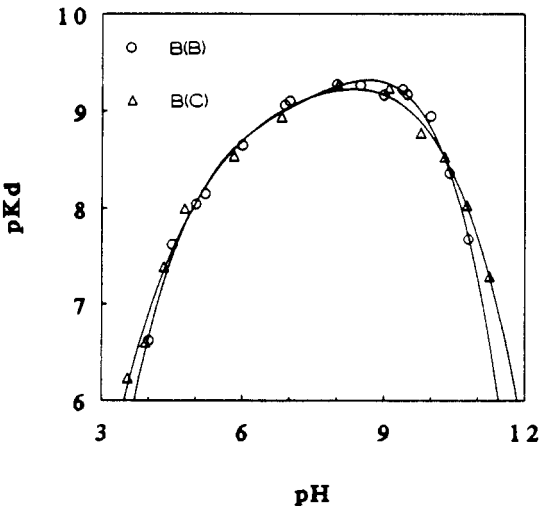

Figure 1. Effect of pH on K_d of inhibitors B(B) and B(C) for trypsin. The buffer contained 0.1 M each of acetate, MES, Tris, and glycine. BAPA was used as substrate. K_ds were determined at 30 °C according to Bieth's (1974) method.

RESULTS

Effect of pH on K_d of Enzyme–Inhibitor Complexes. The effect of pH on dissociation constants of trypsin–inhibitor complexes of B(B) and B(C) was studied. The results are shown in Figure 1. K_d values of both inhibitors as a function of pH were similar. Plots of pK_d vs pH showed nonsymmetric curves. The binding between trypsin and inhibitors was very tight over a very broad pH range (K_d less than 10^{-8} M). The optimal binding for both inhibitors was at pH 8–9. The binding became looser as the pH increased to above 10 or decreased to below 5.

Gel Electrophoresis of Enzyme–Inhibitor Complexes. The enzyme–inhibitor complexes were studied by gel electrophoresis using a continuous buffer system. Figure 2 shows the cathodic and anodic electrophoresis patterns of complexes of BPB inhibitors with trypsin and chymotrypsin. The electrophoresis for both buffer systems took 30–50 min; therefore, complexes with half-lives in the same order of magnitude should be visible as bands in the gel. The number of complexes observed on the gels generally matched what one would predict on the basis of the results from stoichiometry studies (Wu and Whitaker, 1991) except inhibitor B(B), which formed two complexes with trypsin (Figure 2a, lanes 4–6) and one complex with chymotrypsin (Figure 2b, lanes 2–4).

The two complexes of inhibitor B(B) with trypsin seen on the gel were the complex of one trypsin–one inhibitor and the complex of two trypsin–one inhibitor. From the fact that the faster migrating band (C1) dominated when the inhibitor was in excess (Figure 2a, lane 6) whereas the slower migrating band (C2) dominated when trypsin was in excess (Figure 2a, lane 4), one knows that C1 was the complex of one trypsin–one inhibitor and C2 was the complex of two trypsin–one inhibitor. A minor band above the major trypsin–inhibitor complex bands for inhibitor B(A) can be observed when trypsin was in excess (Figure

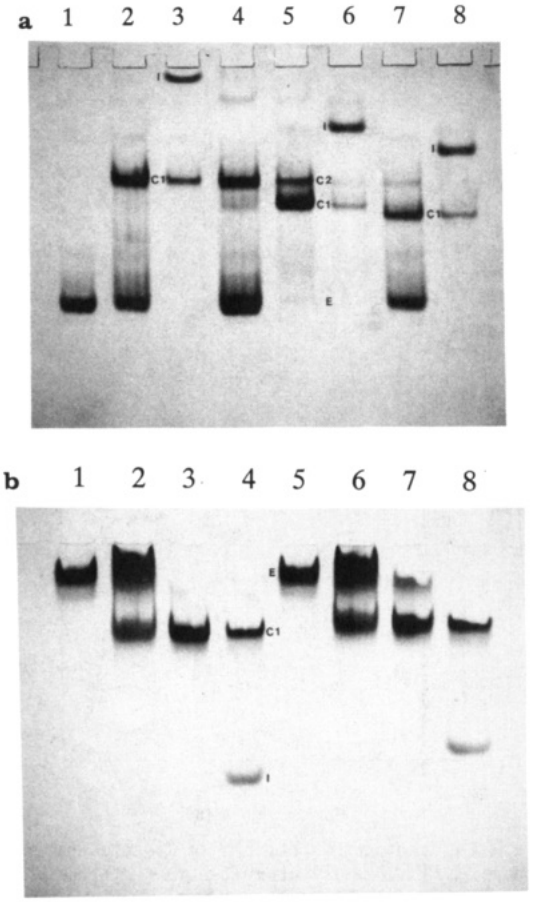

Figure 2. Electrophoresis patterns of complexes of BPB inhibitors with trypsin and chymotrypsin on polyacrylamide gel. Inhibitors were mixed with trypsin in different ratios and incubated for 10 min before they were loaded to the gel. Labels of the bands: E, trypsin or chymotrypsin; I, inhibitor; C1, E–I complex with 1:1 ratio; C2, E–I complex with 2:1 ratio. (a) Cathodic electrophoresis of inhibitors with trypsin. Sample description:

lane	sample	molar ratio (inhibitor:trypsin)
1	trypsin	
2	B(C):trypsin	1.0:2.0
3	B(C):trypsin	3.0:1.0
4	B(B):trypsin	1.0:4.0
5	B(B):trypsin	1.0:1.5
6	B(B):trypsin	3.0:1.0
7	B(A):trypsin	1.0:2.0
8	B(A):trypsin	3.0:1.0

(b) Anodic electrophoresis of inhibitors with chymotrypsin. Sample description:

lane	sample	molar ratio (inhibitor:chymotrypsin)
1	chymotrypsin	
2	B(B):chymotrypsin	1.0:3.0
3	B(B):chymotrypsin	1.0:1.0
4	B(B):chymotrypsin	3.0:1.0
5	chymotrypsin	
6	B(A):chymotrypsin	1.0:3.0
7	B(A):chymotrypsin	1.0:1.0
8	B(A):chymotrypsin	3.0:1.0

2a, lane 7). This indicated that complexes of inhibitor B(A) with trypsin can also bind an additional trypsin molecule, although the second binding is weak. The observed extra binding site for trypsin is most probably due to the nonspecific binding of trypsin to the chymotrypsin site of the inhibitor.

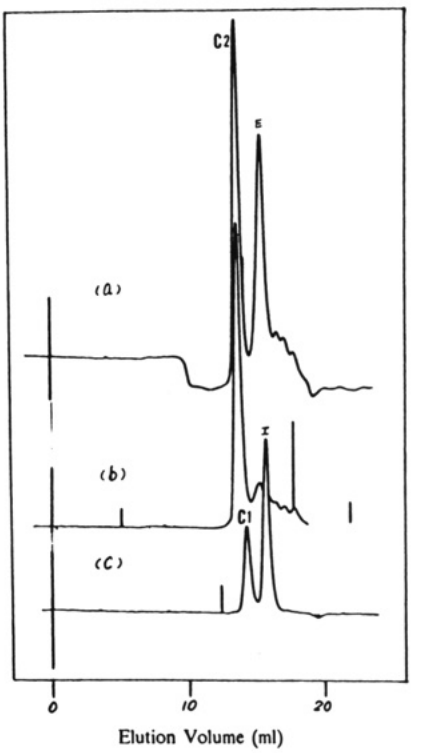

Figure 3. Chromatography of inhibitor–trypsin complexes by FPLC gel filtration column (Superose 12 HR10/30). Inhibitor and enzyme were mixed at different ratios in 20 mM Tris buffer (pH 8.10) and incubated at room temperature for 10 min before they were loaded onto the column. The elution buffer was 20 mM Tris (pH 8.10) containing 0.25 M NaCl. Flow rate was 0.5 mL/min. (a) B(B):trypsin = 1:3; (b) B(B):trypsin = 1:1.5; (c) B(B):trypsin = 3:1. Peaks of the E–I complexes are labeled C1 and C2 according to the number of trypsin molecules in complex. E, trypsin; I, inhibitor.

Gel Filtration of Enzyme–Inhibitor Complexes. Figures 3–5 show the chromatography of E–I complexes by gel filtration. Two different complexes of B(B) with trypsin were observed. The smaller complex (C1) appeared when inhibitor was in excess (Figure 3c), whereas the complex C2 became larger when trypsin was in excess (Figure 3a,b); therefore, the smaller complex should have one trypsin and one inhibitor, and the larger complex should have two trypsins and one inhibitor. Since trypsin does not form dimers under the conditions of the experiment as shown by gel filtration chromatography of trypsin, the two trypsin molecules in the larger complex C2 should both bind to the inhibitor. Only one complex (C1) could be observed when B(B) was mixed with chymotrypsin, even with 3× excess of chymotrypsin (Figure 4).

When an excess of both trypsin and chymotrypsin was incubated with the inhibitor, a new complex (C3) with a size bigger than any of the trypsin–inhibitor complexes and chymotrypsin–inhibitor complex(es) was observed (Figure 5a). This should be the three-way complex between inhibitor, trypsin, and chymotrypsin; this observed peak (C3) supported previous data that the binding to trypsin and chymotrypsin was independent (Wu and Whitaker, 1991).

The gel filtration results indicated that there were at least two binding sites for trypsin and one site for chymotrypsin on B(B). From binding stoichiometry studies, it was shown that inhibitor B(B) binds to one trypsin molecule and one chymotrypsin molecule independently. Similar to the result in gel electrophoresis of enzyme–inhibitor complexes shown above, the apparent extra binding site for trypsin may be due to the binding of trypsin

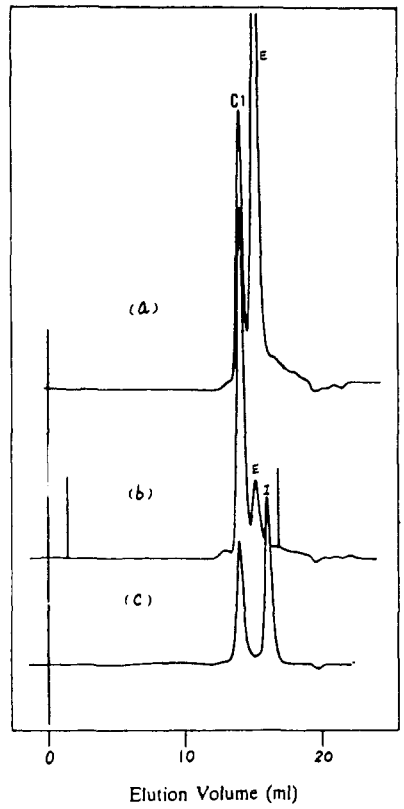

Figure 4. Chromatography of inhibitor–chymotrypsin complexes by FPLC gel filtration column (Superose 12 HR10/30). Experimental conditions were the same as in Figure 3. (a) B(B):chymotrypsin = 1:3; (b) B(B):chymotrypsin = 1:1.5; (c) B(B):chymotrypsin = 3:1. Peaks of the E–I complexes are labeled C1 since only one chymotrypsin molecule was bound to the inhibitor. E, chymotrypsin; I, inhibitor.

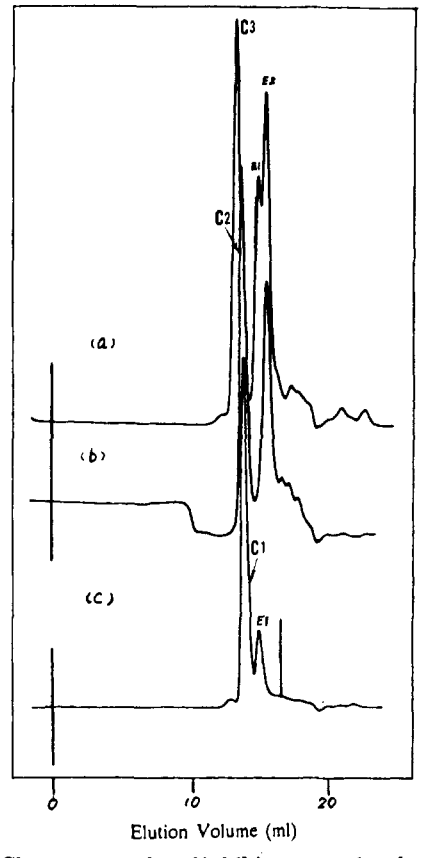

Figure 5. Chromatography of inhibitor–trypsin–chymotrypsin complexes by FPLC gel filtration column (Superose 12 HR10/30). Experimental conditions were the same as in Figure 3. (a) B(B):chymotrypsin:trypsin = 1:2.5:2.5; (b) B(B):trypsin = 1:3 (same as Figure 3a); (c) B(B):chymotrypsin = 1:1.5 (same as Figure 4b). Peaks of the E–I complexes are labeled C1 (one chymotrypsin–one inhibitor), C2 (two trypsin–one inhibitor), or C3 (trypsin–inhibitor–chymotrypsin). E1, chymotrypsin; E2, trypsin.

to the chymotrypsin binding site. Assuming no conformational change after complexation and the second-order rate constant for the complexation to be 10^6 M^{-1} s^{-1}, the half-life of enzyme–inhibitor complexes would be 11.6 min with K_d of 10^{-7} M. Any complexes with K_d greater than that probably would not be found by gel electrophoresis or gel filtration chromatography under the conditions for the above experiments because 30–50 min was needed for either of the experiments. Therefore, should the observed extra trypsin binding site be due to the nonspecific binding to the chymotrypsin site, the binding is still quite tight.

Activity assay of different complexes separated by gel filtration chromatography gave further information on the nature of these complexes (Figure 6). As shown in Figure 6a, the complex (C1) formed with trypsin when inhibitor was in excess still has chymotrypsin inhibitory activity; however, its trypsin inhibitory activity, although significant, is much lower than its chymotrypsin inhibitory activity, indicating that in this complex the trypsin site is taken but the chymotrypsin site is vacant. The binding of complex to another trypsin appeared to be nonstoichiometric under these conditions. The second peak off the column is that of B(B) with full trypsin and chymotrypsin inhibitory activities. With excess of trypsin, the complex was larger and had no trypsin binding ability (Figure 6b). In fact, the second trypsin appeared to be very loosely bound and became partially dissociated from the complex during activity assay. The complex still bound chymotrypsin as shown by its chymotrypsin inhibitory activity; whether the chymotrypsin site was initially vacant, however, cannot be deduced from this result since the chymotrypsin binding could occur with concomitant displacement of the second trypsin should it be at the chymotrypsin site.

Figure 6c clearly shows that the complex with chymotrypsin can still bind trypsin as shown by its inhibitory activity against trypsin. The complex can no longer inhibit chymotrypsin. Binding to chymotrypsin appeared to be looser than to trypsin; chymotrypsin was partially released from the complex in assay solution. The activity of the released chymotrypsin from the complex was measured and indicated by the negative peak of inhibitory activity against chymotrypsin.

The complex of trypsin–inhibitor–chymotrypsin can be seen in Figure 6d. The complex is neither active against trypsin nor against chymotrypsin. The binding to trypsin is apparently tighter than to chymotrypsin; no residual trypsin activity could be detected in the ternary complex between inhibitor, trypsin, and chymotrypsin, whereas some chymotrypsin apparently became free and gave substantial activity. This, however, could be mainly due to longer assay time for chymotrypsin than for trypsin.

Controlled Cleavage at the Reactive Site. Limited proteolysis of inhibitor B(B) was studied as a model to find the best condition for cleavage at the reactive sites with trypsin. Electrophoresis of inhibitors treated with trypsin at different pHs and for different times was carried out. Figure 7a shows the cleavage of B(B) by trypsin at pH 2.3. Quantitation of the extent of cleavage was done by measuring the intensity of the bands of cleaved inhibitor on the electrophoresis gel with a scanning laser densitometer. Reaction rates at different pHs are shown in Figure 7b. At higher pH, the extent of the cleavage reaction was

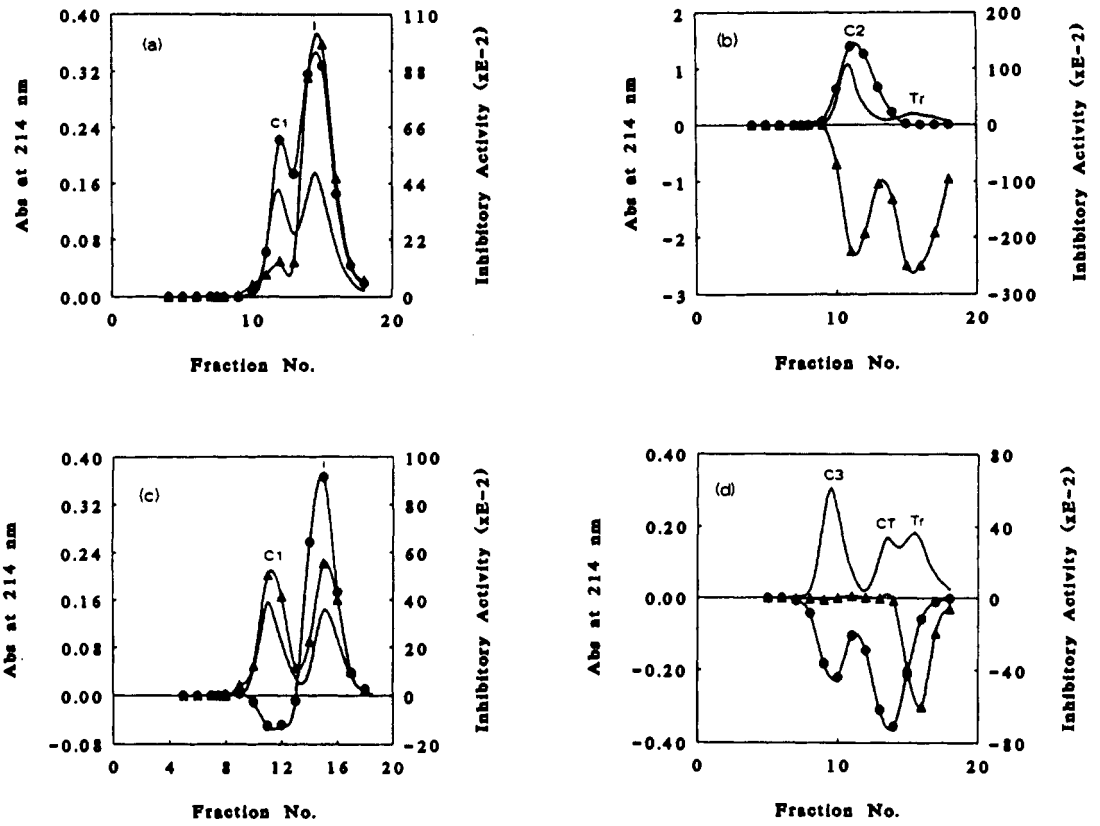

Figure 6. Chromatography of inhibitor–trypsin–chymotrypsin complexes by FPLC gel filtration column (Superose 12 HR10/30). Experimental conditions were the same as in Figure 3 except larger amounts of samples were loaded and 0.4-mL fractions were collected. Collection of fractions started when 10 mL was eluted after loading of the sample. Inhibitory activities against trypsin (▲) and chymotrypsin (●) were assayed separately; (—) protein absorbance at 214 nm. Inhibitory activity against trypsin was measured in the presence of chymotrypsin and vice versa. Inhibitory activities were expressed as micrograms of trypsin or chymotrypsin inhibited by 40 μL of sample. Note the negative inhibitory activities against trypsin and chymotrypsin reflected the presence of enzyme activity of trypsin and chymotrypsin, respectively. (a) 100 μg of B(B), B(B):trypsin = 3:1; (b) 60 μg of B(B), B(B):trypsin = 1:3; (c) 60 μg of B(B), B(B):chymotrypsin = 1:3; (d) 40 μg of B(B), B(B):chymotrypsin:trypsin = 1:2:2.

too small in our experimental time frame (result not included); fitting of the data for kinetics would be meaningless. At pH 3.5 and lower, substantial amounts of the inhibitor were cleaved after 100 h. The reaction apparently followed first-order kinetics with respect to inhibitor concentration (Figure 7b).

The rate of hydrolysis was higher at lower pH within the pH range we tested. At pH 2.3 and 3.0 after 4 days of incubation at room temperature, a majority of the B(B) was converted to the modified form. Substantial second-bond cleavage could be observed after 48 h at pH 2.3 (Figure 7a). The specific bond hydrolysis was much slower at higher pH. The rate constants at pH 2.3 appeared to decrease as the reaction progressed. At pH 2.3, essentially one bond of the inhibitor was cleaved after 48 h; the slope of the semilogarithmic plot decreased significantly after that time (Figure 7b). Since inhibitor B(B) binds two trypsins, the results may indicate that one site is more difficult to cleave than the other one when subjected to trypsin at low pH and thus the kinetics of cleavage is biphasic. More data points would be needed to verify this.

Figure 8 shows the PAGE patterns of the inhibitors after limited proteolysis using conditions chosen on the basis of the above experiments. The cleaved inhibitors were isolated by DEAE-Sephacel ion-exchange chromatography or preparative gel electrophoresis. The number of peptide bonds cleaved in each inhibitor can be deduced from the relative positions on the gel. Thus, Bt_1 had one bond cleaved, Bt_2 had two bonds cleaved, and Bc had only one bond cleaved. The relative inhibitory activities of these preparations against trypsin and chymotrypsin are listed in Table I. The inhibitor cleaved by chymotrypsin (Bc) and the inhibitor cleaved once by trypsin (Bt_1) are similar in activities. They retained full activity against trypsin but only partial activity against chymotrypsin. The inhibitor with two bonds cleaved (Bt_2) was less active against both trypsin and chymotrypsin than the intact inhibitor B(B). The decreased inhibitory activities after cleavage at the reactive sites can be explained by slower binding kinetics or simply looser binding due to possible conformational change at the reactive sites.

Treatment with Carboxypeptidases. The inhibitors cleaved by limited proteolysis were treated with carboxypeptidases to confirm that the cleavages were at the reactive sites and to know which site was cleaved. The change of inhibitory activity against trypsin and chymotrypsin after carboxypeptidase treatment is shown in Figure 9. As a control experiment, buffer instead of carboxypeptidases was added to the cleaved inhibitors and incubated for the same periods of time. No loss of inhibitory activities in the controls was found. When intact inhibitor B(B) was incubated with carboxypeptidases under the same conditions, no loss of inhibitory activities was found, indicating either the C terminus of the intact inhibitor is buried inside the molecule and not subject to carboxypeptidase cleavage or any hydrolysis at the C-terminal end does not affect the inhibitory activities.

The loss of inhibitory activities of the cleaved inhibitors after carboxypeptidase treatments (Figure 9) confirmed that the cleavages were at the reactive sites. It also demonstrated that the C-terminal residues at the reactive

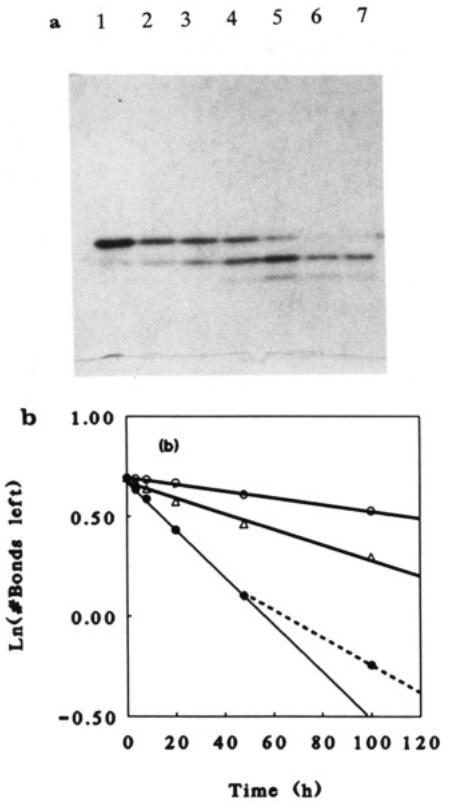

Figure 7. Cleavage of inhibitor B(B) by trypsin at different pHs. (a) Electrophoresis patterns of inhibitors cleaved at pH 2.3 for different periods of time. (Lane 1) Inhibitor B(B); (lanes 2–7, B(B) cleaved for 3.5, 8.0, 20, 48, 100, and 100 h, respectively. (b) Reaction rates at different pHs. Calculation was based on two reactive sites per mole (thus two bonds per mole were subjected to the cleavage; see text). Number of bonds cleaved per mole was calculated by summation of 1 × percent inhibitor with one bond cleaved and 2 × percent inhibitor with two bonds cleaved. (●) pH 2.3; (△) pH 3.0; (○) pH 3.5.

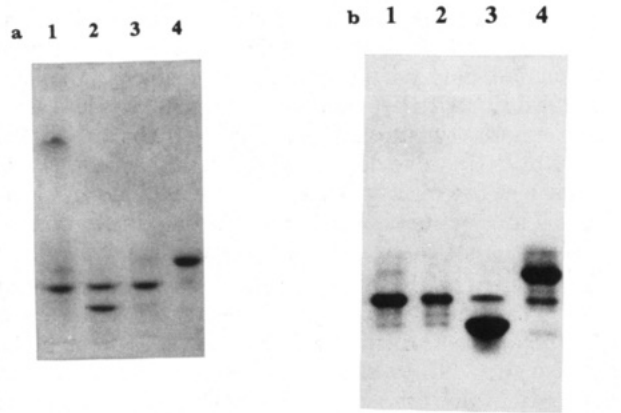

Figure 8. PAGE patterns of inhibitor B(B) after cleavage with trypsin or chymotrypsin (a) and isolated modified inhibitors (b). Cleaved inhibitors were isolated by ion-exchange chromatography or preparative electrophoresis. (a) (Lane 1) B(B) modified by chymotrypsin for 100 h at pH 2.5; (lane 2) B(B) treated with trypsin for 100 h at pH 2.5; (lane 3) B(B) treated with trypsin for 100 h at pH 3.0; (lane 4) B(B). (b) (Lane 1) Bc; (lane 2) Bt_1; (lane 3) Bt_2; (lane 4) B(B).

sites were important to the inhibitory activities; removing these residues resulted in major loss of inhibitory activity.

The determination of the site cleaved in each inhibitor on the basis of this result (Figure 9) was a surprise. Inhibitor B(B) with one bond cleaved by trypsin (Bt_1) and by chymotrypsin (Bc) both showed loss of inhibitory activity against chymotrypsin after incubation with CPA and no loss of activity against trypsin (Figure 9a,c),

Table I. Relative Activities of Different Inhibitor Preparations

inhibitor	% inhibitory activity[a] against trypsin	% inhibitory activity[a] against chymotrypsin
Bt_1	95.5	34.5
Bt_2	42.4	33.7
Bc	94.1	32.7
Bt_2–Tr[b]	37.5	27.8
Bt_2–CT[c]	39.8	78.6

[a] Specific activity (micrograms of trypsin inhibited per microgram of inhibitor) relative to that of intact inhibitor B(B). [b] Bt_2 after complexation with equal molar amount of trypsin followed by rapid acid dissociation. Complexation with 2× molar excess of trypsin gave the same result. [c] Bt_2 after complexation with equal molar amount of chymotrypsin followed by rapid acid dissociation.

indicating that in both Bt_1 and Bc the chymotrypsin site was cleaved but the trypsin site was intact. The inhibitor with two bonds cleaved (Bt_2) by trypsin had both the trypsin and chymotrypsin sites cleaved since the trypsin inhibitory activity was lost upon treatment with carboxypeptidase B and the chymotrypsin inhibitory activity was lost with carboxypeptidase A (Figure 9b). These results establish that trypsin still interacts with the inhibitor at the chymotrypsin site even at low pH. The fact that the chymotrypsin site was cleaved by trypsin preferentially to the trypsin site suggests that the interaction of trypsin with the chymotrypsin site favors the cleavage of the reactive site peptide bond, perhaps because of the looser complex formed.

Resynthesis of the Reactive Site. Bt_2 was allowed to complex with equal molar or excess of trypsin or chymotrypsin for 1 h at room temperature and then isolated by rapid dissociation with acid. Figure 10a shows the PAGE patterns of the inhibitor after such treatment and parts b and c of Figure 10 show their activity change after treatments with carboxypeptidases A and B. The activities of these inhibitors treated with equal or excess molar of trypsin or chymotrypsin are also listed in Table I.

After complexation with chymotrypsin and rapid dissociation, one of the cleaved bonds appeared to be mostly resynthesized on the basis of the PAGE pattern (Figure 10a, lane 3), whereas complexation with trypsin followed by rapid dissociation did not change the migration pattern of the inhibitor in PAGE (Figure 10a, lane 4). Complexation with trypsin followed by rapid acid dissociation only slightly increased the migration toward the anode. The results on the activity change after treatments with carboxypeptidases A and B show that after complexation with chymotrypsin and rapid dissociation, the chymotrypsin site was no longer subject to CPA digestion (Figure 10b), confirming that the intact chymotrypsin site was regenerated. The small loss of chymotrypsin inhibitory activity in this sample is believed to be due to incomplete regeneration of the peptide bond at the reactive site. Electrophoresis pattern of this sample (Figure 10a, lane 3) shows that a minor portion of the inhibitor still has both trypsin and chymotrypsin inhibitory sites cleaved. Complexation with trypsin and rapid dissociation as performed under the conditions in these experiments did not regenerate the intact trypsin site as evidenced by similar loss of trypsin inhibitory activity after treatment with CPB before and after the complexation with trypsin (Figure 10c). The slightly increased migration distance on PAGE may suggest loss of a small oligopeptide such as Ser-Gly-His-Arg from the N terminus (Wu and Whitaker, 1991) during treatment with trypsin.

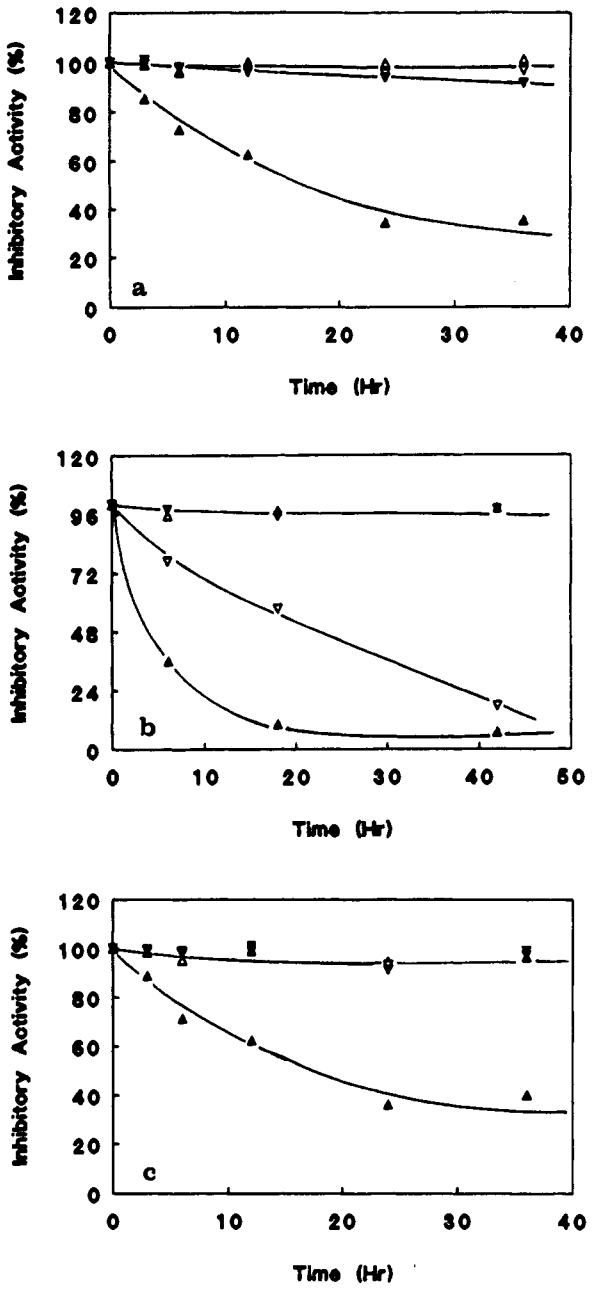

Figure 9. Effect of carboxypeptidase A and carboxypeptidase B treatment on tryptic and chymotryptic inhibitory activity of Bt_1 (a), Bt_2 (b), and Bc (c). Activity against trypsin was measured in the presence of chymotrypsin and vice versa. (△) Trypsin inhibitory activity left after treatment with carboxypeptidase A; (▲) chymotrypsin inhibitory activity left after treatment with carboxypeptidase A; (▽) trypsin inhibitory activity left after treatment with carboxypeptidase B; (▼) chymotrypsin inhibitory activity left after treatment with carboxypeptidase B.

DISCUSSION

The existence of separate and independent reactive sites for trypsin and chymotrypsin is a general characteristic (besides high cystine and low molecular weights) of Bowman–Birk type inhibitors (Sgarbieri and Whitaker, 1982; Birk, 1985; Norioka et al., 1988). However, not all Bowman–Birk inhibitors bind 2 mol of enzyme at their reactive sites independently. For instance, inhibitor II from azuki bean (Yoshikawa et al., 1977; Yoshikawa and Ogura, 1978) cannot complex simultaneously with trypsin and chymotrypsin, although it has two separate reactive sites. Tur-Sinai et al. (1972) observed a similar phenomenon with the peanut inhibitor; the complex with trypsin lacked antichymotrypsin activity, and the complex with chymotrypsin had no antitrypsin activity.

Inhibitors in this study have separate and independent reactive sites, one against trypsin, one against chymotrypsin. Independence of the reactive sites is clearly shown by the ability of the complex with one enzyme to inhibit the other enzyme. Inhibitor B(B) can bind two molecules of trypsin in the absence of chymotrypsin on the basis of the results of gel electrophoresis and gel filtration chromatography of enzyme–inhibitor complexes. However, since the complex with chymotrypsin could only bind one trypsin as shown by the result from its gel filtration chromatography, this complex indicates that the second trypsin molecule should bind to the chymotrypsin reactive site.

Most protein inhibitors of serine proteinases act by a standard inhibition mechanism (Laskowski and Kato, 1980). The inhibitors bind to enzymes in the manner of a good substrate, but very tightly, and are cleaved very slowly, if at all, at the reactive site (Read and James, 1986). Inhibitors obeying the standard mechanism have very low K_d, indicating tight enzyme–inhibitor complexes, and low K_{hyd} (equilibrium constant for hydrolysis of the reactive site peptide bond) (Finkenstadt et al., 1974; Laskowski and Kato, 1980; Read and James, 1986; Kato et al., 1987). Therefore, cleavage at the reactive sites is usually not feasible at neutral pH.

Reactive site cleaved inhibitors can be prepared by incubating the inhibitor with catalytic quantities of enzyme for a long time at low pH as was done in this study. Similar splitting of a single bond at the reactive site upon exposure to catalytic quantities of enzyme has been found in most protein trypsin inhibitors (Laskowski and Sealock, 1970). There has been no report, however, that trypsin will also cleave the chymotrypsin reactive site. Nevertheless, such cleavage observed in our study is consistent with the result that trypsin binds to the chymotrypsin site.

A low pH is required for trypsin (or chymotrypsin) to split the peptide bond at the reactive sites, presumably because of the loose complexes at low pH. Perhaps the loose binding permits a small percentage of the trypsin or chymotrypsin to be "free" at the reactive site, but never "free" for long enough to escape from the inhibitor. Such "freedom" provides an opportunity for water molecules to go inside the complex at the reactive site for the hydrolysis of the bond. At pH below 4, K_d is at least 2 orders of magnitude larger than at neutral pH (Figure 1), consistent with the assumption that loose binding is a requisite for peptide bond cleavage at the reactive site. Laskowski (1970) showed that K_{hyd} of soybean trypsin inhibitor by trypsin was minimal at neutral pH but increased greatly as pH was adjusted below 4 or above 9. There has not been much study of the kinetics of reactive site cleavage at different pHs. Assuming the effect of pH on K_{hyd} was mainly on the rate constant of cleavage, the effect of pH on the rate of cleavage would be similar to the pH effect on K_d, consistent again with the observation that the rate of cleavage was higher at lower pH.

Although there has not been a report in the literature that trypsin can cleave the chymotrypsin site, it should not be conceived as totally unexpected. A precedent for this is the study of Omichi et al. (1980), which showed that the reactive site of a subtilisin inhibitor, which binds subtilisin very tightly but binds α-chymotrypsin much more loosely, can be cleaved easily by α-chymotrypsin. Also, the reactive site has usually been named after the first enzyme against which inhibitors were found. Therefore, Weder has suggested the term "cognate enzyme" instead

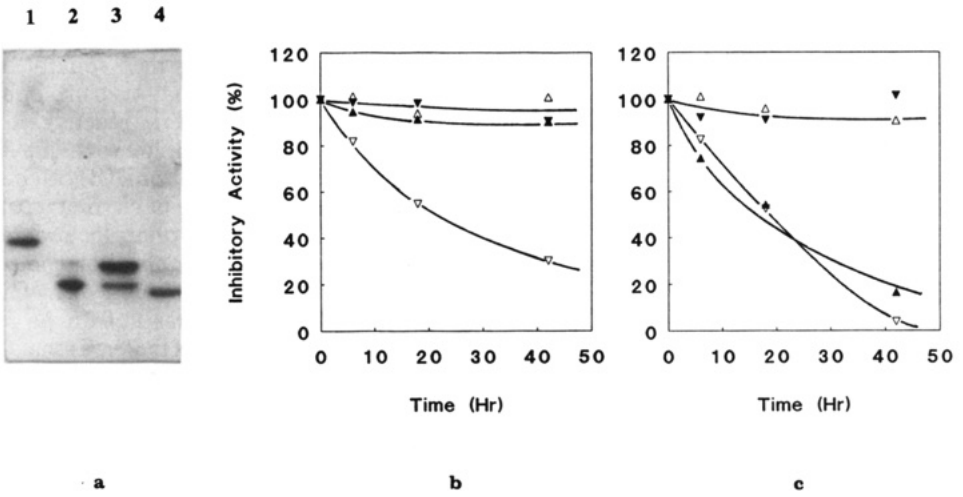

Figure 10. Resynthesis of the reactive sites. (a) PAGE patterns of the modified inhibitors after complexation with enzyme and rapid dissociation. (Lane 1) B(B); (lane 2) Bt_2; (lane 3) Bt_2 after complexation with chymotrypsin and rapid dissociation; (lane 4) Bt_2 after complexation with trypsin and rapid dissociation. (b) Activity change of sample in lane 3 of (a) after treatment with carboxypeptidases. (c) Activity change of sample in lane 4 of (a) after treatment with carboxypeptidases. (△) Trypsin inhibitory activity left after treatment with carboxypeptidase A; (▲) chymotrypsin inhibitory activity left after treatment with carboxypeptidase A; (▽) trypsin inhibitory activity left after treatment with carboxypeptidase B; (▼) chymotrypsin inhibitory activity left after treatment with carboxypeptidase B.

of "target enzyme" for the enzymes inhibited (personal communication).

Our observation that the chymotrypsin site is cleaved first by trypsin was a surprise to us. At neutral to acid pH, binding of trypsin is preferentially to the trypsin site rather than to the chymotrypsin site as evidenced by the electrophoresis and chromatography of complexes with trypsin. Since binding of trypsin to the chymotrypsin site is estimated to be at least 2 orders of magnitude looser than binding to the trypsin site, the preferential cleavage of chymotrypsin site at pH 2.3 supports the assumption that looser binding favors the cleavage. At even lower pH at which the interaction of trypsin with the chymotrypsin site becomes too weak for cleavage, preferential cleavage at the trypsin site by trypsin might be possible.

That the first cleavage by trypsin is at the chymotrypsin site was also confirmed by the experiment of resynthesizing the cleaved peptide bond by treating with chymotrypsin. Hixon and Laskowski (1970) first showed that by kinetically controlled dissociation of the enzyme–inhibitor complex only virgin inhibitor was regenerated. In this study, treatment with trypsin did not resynthesize the peptide bond at the chymotrypsin reactive site; in fact, even the peptide bond at the trypsin reactive site was not regenerated.

Failure to find resynthesis of the reactive site(s) by trypsin could be because, at acid pH, the dissociation to modified inhibitor is faster than that to intact inhibitor. The dissociation of the E–I complex by acid has been studied with other proteinase inhibitors. It was found that the products of so-called kinetically controlled dissociation were predominantly native inhibitors, and this was thought to be a general phenomenon (Laskowski and Sealock, 1970; Laskowski and Kato, 1980). However, Tonomura et al. (1985) showed that acidification of a complex between subtilisin and subtilisin inhibitor SSI to pH 2.5 produced predominantly reactive site cleaved SSI with a pentapeptide also cleaved from the N-terminal end. The ratio of the inhibitor with reactive site intact to the modified inhibitor increased as the complex was acidified to lower pH. The trypsin reactive site of inhibitor B(B) may be similar to SSI. Faster dissociation to inhibitor with intact reactive site than to modified inhibitor would take place only at lower pH. The incomplete regeneration of the chymotrypsin reactive site peptide bond (Figure 10a, lane 3) might have reflected the relative rates of the two competing processes (dissociation to give intact reactive site and to give cleaved reactive site).

Another explanation for the failure to resynthesize the trypsin reactive site peptide bond is that, although the dissociation to intact inhibitor was faster, not enough time was allowed for the complex to reach equilibrium so the inhibitor was mainly in the modified form before acid dissociation. More work is needed to determine the reasons why trypsin was unable to re-form the peptide bond at the reactive site(s) of Bt_2 under the conditions used.

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