Limited Proteolysis of the Bovine Pancreatic Secretory Trypsin Inhibitor at Acid pH*

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SUMMARY

The bovine pancreatic secretory trypsin inhibitor (Kazal's inhibitor) undergoes a limited and specific proteolysis by catalytic amounts of trypsin in the presence of calcium ions at acidic pH. The pH optimum for both the initial rate of reaction and the extent of reaction is pH 2.7. Incubation of inhibitor with 0.7 mole % trypsin at pH 2.7 for 3 hours at 25°C results in the modification of 25% of the inhibitor molecules. We have shown, by isolation and characterization of peptide fragments, that a single arginyl-isoleucyl peptide bond (residues 18 and 19) is hydrolyzed. This modified inhibitor is active, but is inactive when arginine 18 is removed by carboxypeptidase B. The features of the reaction thus far examined are similar to those described by Ozawa and Laskowski (J. Biol. Chem., 241, 3955 (1966)) for the soybean trypsin inhibitor-trypsin system. On this basis the arginine residue at position 18 is identified as the reactive site of the inhibitor.

Finkenstadt and Laskowski (1) and Ozawa and Laskowski (2) have demonstrated limited proteolytic cleavage of the soybean trypsin inhibitor and chicken ovomucoid trypsin inhibitor by catalytic amounts of trypsin at pH 3.75. A single bond is hydrolyzed: arginyl-isoleucyl (residues 64 and 65) in the soybean trypsin inhibitor, and arginyl-alanyl in cluck ovomucoid. The sensitive peptide bond has been denoted the "reactive site" (2). Both modified inhibitors are active, although the rate of reaction of modified STI with trypsin is slower than that of unmodified inhibitor. When the newly exposed COOH-terminal arginine residue of either modified inhibitor is removed by treatment with carboxypeptidase B, inhibitor activity is lost. The resynthesis of the peptide bond (3) and the enzymatic replacement of the arginyl residue by a lysyl residue at position 64 in STI has been presented as evidence for the existence of an equilibrium among trypsin, intact inhibitor, trypsin-inhibitor complex, and modified inhibitor (4). Feinstein and Feeney (5) have demonstrated that one form of catalytically inactive trypsin, L-l-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, does form a complex with the chicken ovomucoid inhibitor (but not with soybean trypsin inhibitor) as determined by a "competitive enzyme assay" and electrophoresis. In order to examine the role of the newly formed amino group of trypsin-modified STI and chicken ovomucoid (i.e. isoleucine and alanine, respectively), Haynes and Feeney (6) allowed the modified inhibitors to react with trinitrobenzenesulfonic acid. STI was rapidly and almost completely inactivated, whereas chicken ovomucoid was relatively unaffected. It would therefore appear that there are at least two modes of interaction between trypsin and polypeptide trypsin inhibitor.

In a study of the interaction of pancreatic trypsin inhibitors with trypsin, we have found that partial proteolysis occurs when the bovine pancreatic secretory trypsin inhibitor is incubated with catalytic amounts of trypsin in the presence of calcium ions at pH 2.7. The trypsin-modified inhibitor is active, but loses its activity on incubation with carboxypeptidase B. In this report we describe some of the conditions affecting the partial proteolysis reaction and show that a single bond, arginyl-isoleucyl (residues 18 and 19), is cleaved. While our studies were in progress, Tschesche reported that when pancreatic secretory inhibitors are incubated with stoichiometric amounts of trypsin at pH 7.8, specific cleavage occurs at a lysine bond in the porcine inhibitor and at an arginine bond in the bovine inhibitor (7).

At the present time, it is not clear whether the acid pH partial proteolysis reaction actually occurs at neutral pH and, if so, whether it is a necessary intermediate on the path of reactions which lead to inactive trypsin when trypsin interacts with naturally occurring polypeptide inhibitors.

EXPERIMENTAL PROCEDURE

Materials

Bovine pancreatic secretory trypsin inhibitor was purified by equilibrium chromatography on DEAE-cellulose at pH 9.0 (8) in 0.028 M Tris-hydrochloride (9), from fractions supplied by...
Dear P. J. Burek of Eli Lilly and Company, Indianapolis, Indiana.

This material, isolated as a by-product of insulin preparation (10), is identical in specific activity, amino acid composition, and chromatographic behavior with the inhibitor isolated in our laboratory directly from pancreatic juice. Basic pancreatic trypsin inhibitor (Kunitz's inhibitor), Lot 3185-L1g, was the gift of Dr. P. J. Melle of Armour Pharmaceutical Company, Kankakee, Illinois, and Novo porcine trypsin was purchased from Enzyme Development Company, New York. p-Toluenesulfonyl-L-arginine methyl ester and trans-aconitic acid were purchased from Worthington. Porcine trypsin (Lot K258256) was a gift from Dr. P. J. Melle of Armour Pharmaceutical Company, Kankakee, Illinois, and Novo porcine trypsin was purchased from Enzyme Development Company, New York. p-Toluenesulfonyl-L-arginine methyl and trans-aconitic acid were purchased from Calbiochem. Sephadex G-25, G-50, and G-75 were purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. All other chemicals were reagent grade.

** Gel Filtration**

Columns (0.9 x 400 cm) of Sephadex G-25 and Sephadex G-50 were prepared from gels first swollen in 50% acetic acid and then equilibrated in pyridine-acetate buffer, pH 3.1 (0.2 M in pyridine) (11). A layer, 2 to 3 mm, of glass beads was placed above the layered gel disc in each column. Two columns, 0.9 x 200 cm, were connected in series by polyethylene tubing above the sintered glass disc in each column. Two columns, 0.9 x 400 cm, were purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. All other chemicals were reagent grade.

**Amino Acid Analysis**

Samples of 0.1 to 0.2 pmole were hydrolyzed in an evacuated, sealed tube with 1 ml of twice distilled, constant boiling HCl. The tubes were held at 110° for 22 hours. Amino acid analysis of the hydrolysates was performed according to the method of Spackman, Stein, and Moore (14) with a Beckman-Spinco model 120 analyzer. Inhibitor and peptide concentrations were determined by amino acid analysis after 22-hour acid hydrolysis.

**Edman Degradation**

A subtractive procedure was carried out at the level of 0.1 pmole per aliquot by the method of Grey (15) as modified by Elzings, Lai, and Hirs (16). The peptides were subjected to three cycles of the Edman reaction but were extracted with butyl acetate only once, i.e. immediately before hydrolysis.

**Trypsin and Inhibitor Assays**

Trypsin activity and inhibition were determined by the pH-stat procedure with the use of p-toluenesulfonfyl-L-arginine methyl ester as described previously (8). One unit of trypsin activity corresponds to the hydrolysis of p-toluenesulfonyl-L-arginine methyl ester at the rate of 1 pmole per min. The molarity of active trypsin was calculated on the basis of a specific activity of 410 units per mg and a molecular weight of 23,800. One unit of inhibitor activity is the amount that inhibits 1 unit of trypsin activity. The standard deviation of replicate inhibitor assays was 1.1%. Inhibitor values are the average of duplicate assays, with a 95% confidence interval of 3.2%.

**Modification of Inhibitor and Its Analytical Determination**

Inhibitor was incubated with catalytic amounts of trypsin at 25° in 0.04 M CaCl₂ at several pH values between 1.83 and 7.75. Sodium trans-aconitate, 0.018 M, was used to buffer the solutions except at pH 1.83 and pH 7.75, where 0.018 M HCl and 0.08 M sodium borate, respectively, were used. Unless otherwise specified the inhibitor concentration was ~0.13 pmole per ml and that of bovine trypsin was 0.036 mg per ml. The molar ratio of active trypsin to inhibitor was 1:150. Volumes of up to 2 ml were incubated in glass stopped, 2 ml volumetric tubes. pH measurements were made on 250-μl aliquots in similar tubes with a Corning pH meter, model 12, and combination electrode No. 4385-L-15 (Arthur H. Thomas Company, Philadelphia).

In order to determine the extent of modification, the trypsin incubation mixture was treated with carboxypeptidase B and the loss of inhibitor activity was determined. The pH of 100 μl of the trypsin incubation mixture was made alkaline by the addition of 200 μl of 0.1 M sodium borate buffer. The composition of the borate buffer was chosen to give a final pH of 7.65 to 7.85. Carboxypeptidase B, 34 μg in 10 μl of 0.1 M NaCl, was added and the mixture was incubated at 25° for 24 hours. The sensitivity of inhibitor to inactivation by carboxypeptidase B has been used as an index of the extent of modification by trypsin.

**Identification of Extent and Site of Peptide Bond Cleavage**

**Modification of Inhibitor—Bovine PST1 (1.3 μmole) was treated with 0.85 mg of bovine trypsin (245 units per mg) in 10 ml of 0.018 M trans-aconitic acid containing 5 X 10⁻⁴ M CaCl₂, pH 2.7, at 25° for 3 hours. The molar ratio of active trypsin to inhibitor was 1:150. An aliquot was removed for treatment with carboxypeptidase B in order to determine the extent of modification (as determined by inactivation after carboxypeptidase B treatment). The pH was then corrected with ammonia to pH 7.72 and carboxypeptidase B (Lot COBC 7A), 7.875 Azso units in 0.97 ml, was added. The reaction was stopped after 25 hours at 25° by lyophilization.

**Separation of Peptides and Determination of Free Amino Acids**

The trypsin- and carboxypeptidase B-treated bovine PST1 reaction mixture was applied to a column, 0.9 x 140 cm, of Sephadex G-75 previously equilibrated with 0.01 M ammonium acetate, pH 7.9. The mixture of modified and intact inhibitor (Fig. 1, Peak I) was lyophilized and filtered through a column, 0.9 x 20 cm, of Dowex 1 equilibrated in 0.1 M acetic acid before oxidation with performic acid by the method of Hirs (17). The oxidized peptides were lyophilized twice to remove reagents and then subjected to gel filtration on a Sephadex G-25 column, 0.9 x 400 cm, at pH 3.1 (Fig. 2). The small fragment (Fig. 2, Peak II) was also filtered through a Sephadex G-25 column, 0.9 x 400 cm, pH 3.1 (cf. Fig. 3).

The low molecular weight fraction (Fig. 1, Peak III) was pooled and exhaustively lyophilized. An aliquot was hydrolyzed

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1 L. J. Greene and J. S. Giordano, Jr., data to be published.

2 Carboxypeptidase B was dialyzed against 0.1 M NaCl-0.01 M sodium borate, pH 8.3, at 4° for 22 hours in order to reduce the level of free amino acids.
for 22 hours, and another portion was dissolved directly in 0.2 M sodium citrate, pH 2.2. The amino acid content of both samples was determined on the amino acid analyzer.

RESULTS

Site of Peptide Bond Cleavage at pH 2.7

The reaction mixture contained bovine PSTI which had been incubated with 0.7 mole % trypsin at pH 2.7 for 3 hours followed by treatment with carboxypeptidase B at pH 7.72 for 25 hours. Gel filtration on Sephadex G-75 at pH 7.9 (cf. Fig. 1) separated the mixture of modified and intact inhibitor, Peak II, from carboxypeptidase B and trypsin-inhibitor complex (0.7 mole % of total bovine PSTI), Peak I, and from low molecular weight materials, Peak III. The high ratio of absorbance at 570 nm (ninhydrin reaction after alkaline hydrolysis) to absorbance at 280 nm of Peak II is a reflection of the low concentration of aromatic amino acids in bovine PSTI. The separation of bovine PSTI from carboxypeptidase B and trypsin is also indicated by the shape of the elution profile and the results of amino acid analysis (cf. Table I, Column 2). The absorbance at 280 nm of Peak III is due to aconitate.

Arginine Release—Peak III (Fig. 1) contained 0.24 and 0.09 eq of free arginine and lysine, respectively, per mole of bovine PSTI applied to the column. The net amount of basic amino acids released was 0.19 and 0.01 eq of arginine and lysine, respectively, per mole of bovine PSTI when corrected for the contribution of carboxypeptidase B and trypsin incubated under similar conditions. The amount of free arginine recovered, 0.19 eq, is in relatively close agreement with a 25% decrease in inhibitor activity observed after carboxypeptidase B treatment, and with the amount of peptide fragments recovered (cf. Table III, below).

Isolation and Characterization of Peptides—The mixture of modified and intact bovine PSTI (Fig. 1, Peak II) after performic acid oxidation. The column, 0.9 × 400 cm, was equilibrated with pyridine-acetate buffer, pH 3.1. Peptides were located by ninhydrin analysis after alkaline hydrolysis. The fractions indicated by the bars were combined. The amino acid compositions of the peptides are presented in Table I, Columns 3, 4, and 5.

Fig. 1. Gel filtration on Sephadex G-75 of reaction mixture containing bovine pancreatic secretory trypsin inhibitor, trypsin, and carboxypeptidase B. The column, 0.9 × 140 cm, was equilibrated with 0.01 m ammonium acetate, pH 7.9. Peptides were located by ninhydrin analysis after alkaline hydrolysis (○), and by ultraviolet absorbance at 280 nm (■). The fractions indicated by the bars were combined.

Fig. 2. Gel filtration on Sephadex G-50 of a mixture of intact and modified bovine PSTI (Fig. 1, Peak II) after performic acid oxidation. The column, 0.9 × 400 cm, was equilibrated with pyridine-acetate buffer, pH 3.1. Peptides were located by ninhydrin analysis after alkaline hydrolysis. The fractions indicated by the bars were combined. The amino acid compositions of the peptides are presented in Table I, Columns 3, 4, and 5.

Fig. 4. The observed lysine values are not reliable because of incomplete removal of the ε-phenylthiocarbamyl group on hydrolysis with 6 N HCl at 110° C for 22 hours. Tyrosine is destroyed by the conditions used; however, there is no interference with Edman degradation or internal peptide cleavage. Since this study was...
The values for cysteic acid are included in Table II, but they have been neglected in the interpretation of the results from Edman degradation. The values were high for the starting material (i.e., Cycle 0) and more variable than those for most other amino acids. The 6 cysteic acid residues in the oxidized polypeptide do not occupy any of the positions within residues 1 through 3 or 18 through 21.6

**Peptide I (Fig. 9)**—This peptide has been identified as the oxidized single polypeptide chain derived from intact bovine PSTI on the basis of (a) amino acid analysis (compare Column 3 with Column 1, Table I), (b) Sephadex G-50 elution position, and (c) identification of amino-terminal amino acids. The data presented in Table II indicate that the first three amino-terminal residues are Asx-Ile-Leu. We have assigned the sequence Ile-Tyr-Asx to the amino terminus of the peptide on the basis of the isolation, characterization, and similar behavior during Edman degradation of the hexapeptide, Ile-Tyr-Asx-Pro-Val-aminoethylcysteine from trypsin digests of reduced, aminoethylated bovine PSTI.2

**Peptide II (Fig. 2)**—This peptide contains 38 amino acid residues per molecule and corresponds to the COOH-terminal region of the molecule (cf. Table I, Column 4). During the subtractive Edman degradation, approximately 1 residue of isoleucine was lost in the first cycle; there was no change of composition after the second cycle, and aspartic acid decreased by 1 residue in the third cycle. The efficiency of the third step indicates that degradation had, in fact, occurred during the second cycle. Therefore, either lysine or tyrosine occupies that position.4 We have assigned the sequence Ile-Tyr-Asx to the amino terminus of the peptide on the basis of the isolation, characterization, and similar behavior during Edman degradation of the hexapeptide, Ile-Tyr-Asx-Pro-Val-aminoethylcysteine from trypsin digests of reduced, aminoethylated bovine PSTI.2

**Peptide III (Fig. 9)** This peptide contains 17 residues; its amino acid composition is presented in Table I, Column 5. When the peptide was subjected to gel filtration on a Sephadex G-25 column, 0.9 × 400 cm, it was recovered as a single peak serine, respectively, per molecule (cf. Tables I and II). These amino acids plus glycine have also been observed as variable contaminants in unrelated preparations examined in our laboratory when the work was in progress. They have been neglected in assigning the amino acid composition to each fragment, i.e., values in parentheses. Our studies of the amino acid sequence of bovine PSTI indicate that the 1 alanine and 2 serine residues present in bovine PSTI occupy position 7 and positions 32 and 53, respectively (L. J. Greene and D. C. Bartelt, data to be published).
and there was no change in amino acid composition (compare Columns 5 and 6, Table I). The amino-terminal sequence determined by subtractive Edman degradation, Asx-Ile-Leu, indicates that the peptide is derived from the amino-terminal end of the molecule.

Recovery of Peptides and Free Arginine—The recovery of peptides and amino acids has been calculated by dividing the amount recovered by the total amount of bovine PSTI (both intact and modified) applied to the column. The results are summarized in Table III. The fragments (small, large, and free arginine), which account for all of the amino acids present in native inhibitor (cf. Table I), were recovered in essentially identical yields. The data summarized in Table III indicate that a single arginine-peptide bond, residues 18 and 19, was hydrolyzed in approximately 25% of the bovine PSTI molecules. This quantitative recovery of the fragments, and essentially quantitative recovery of free arginine, as well as the failure of the ninhydrin reaction to show the presence of other peptides or free amino acids (cf. Figs. 2 and 3), may be taken as strong evidence for the conclusion that the partial proteolysis reaction is extremely selective.

The data also indicate that the same proportion of the molecules modified by cleavage at arginine residue 18 are also sensitive to inactivation by carboxypeptidase B. The selective cleavage is shown diagrammatically in Fig. 4. We have also examined the products obtained when bovine PSTI is modified by trypsin but not exposed to carboxypeptidase B. As expected, the fragments corresponding to residues 1 through 18 as well as 19 through 56 and 1 through 56 were isolated; the yields were comparable to those reported in Table III.

**TABLE II**

<table>
<thead>
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<th>Peptide and No. of cycles</th>
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<th>Ile</th>
<th>Leu</th>
<th>Cys</th>
<th>Asx</th>
<th>Tyr</th>
<th>Ser</th>
<th>Recovery**</th>
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<tr>
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<td></td>
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<tr>
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<td>3.92</td>
<td>0.19</td>
<td>1.81</td>
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<td>Peptide III (Fig. 3)</td>
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* The amount of peptide relative to starting material.

** This residue has been identified as tyrosine (cf. "Results").

**TABLE III**

<table>
<thead>
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<th>Recovery**</th>
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<td>Small fragment—residues 1 through 17 (Fig. 2, Peak III, and Fig. 3)</td>
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<td>Large fragment—residues 19 through 56 (Fig. 2, Peak II)</td>
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<tr>
<td>Arginine—residue 18 (Fig. 1, Peak III)</td>
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<td>Intact bovine PSTI—residues 1 through 56 (Fig. 2, Peak I)</td>
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<td>Inhibitor activity</td>
</tr>
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</table>

* Expressed as moles of peptide recovered per mole of bovine PSTI (modified + intact) applied to the column. This method of calculation permits direct comparison of arginine release with peptide fragments.

** This value may be considered a lower limit because a restricted region of the peak was combined for analysis (compare Fig. 2, Peak II, and Fig. 1, Peak III).

* Determined on an aliquot of the reaction mixture after incubation with trypsin. The value was obtained by dividing the amount of inhibitor activity remaining after carboxypeptidase B treatment by the amount of inhibitor activity present before treatment.
Determination of Extent of Inhibitor Modification

The sensitivity of inhibitor to inactivation by carboxypeptidase B could be used as a quantitative measure of the proteolysis reaction because intact inhibitor retains activity during incubation with carboxypeptidase B. In a series of control experiments we have found that the activity of inhibitor exposed to catalytic amounts of trypsin over the range, pH 1.8 to 7.6, is constant and undiminished (relative to inhibitor in the absence of trypsin) unless the material is later incubated with carboxypeptidase B. Both modified and unmodified inhibitor react with trypsin completely in less than 2 min under the conditions of the inhibitor assay. The conditions used for carboxypeptidase B incubation, per se, do not influence the observed value. For example, there is no change in the extent of inactivation when modified inhibitor plus 0.7 mole % trypsin, after 3 hours of incubation at pH 2.7, are incubated at pH 7.4 for up to 48 hours before treatment with carboxypeptidase B. The extent of inactivation is also independent of a 5-fold range of carboxypeptidase B concentration and duration of incubation with carboxypeptidase B. The conditions used for carboxypeptidase treatment (see "Experimental Procedure") were selected to permit its use as an indicator system. However, the fact that inactivation is equivalent to the hydrolysis of a single peptide bond has been shown for only one set of conditions: pH 2.7, 0.7 mole % trypsin, and 3 hours. Studies are in progress to characterize the product (or products).

Fig. 4. Schematic representation of the partial proteolysis of bovine pancreatic secretory trypsin inhibitor by catalytic amounts of trypsin at acid pH.

Fig. 5. Modification of bovine pancreatic secretory trypsin inhibitor by bovine trypsin at pH 3.22 and 25°C. The extent of modification was determined by measuring the amount of inhibitor activity destroyed by carboxypeptidase B (CpB). The relative concentration of trypsin to bovine PSTI was 0.7 mole %.

Fig. 6. Extent of modification of bovine PSTI by 0.7 mole % bovine trypsin as a function of pH. •, 5 hours of incubation; ○, 16 hours of incubation. The extent of modification was determined as indicated in the legend to Fig. 5. CpB, carboxypeptidase B.

Fig. 7. Initial rate of modification of bovine pancreatic secretory trypsin inhibitor by 0.7 mole % bovine trypsin as a function of pH. The extent of modification was determined as indicated in the legend to Fig. 5. CpB, carboxypeptidase B.
when the partial proteolysis reaction is carried out at other pH values.

**Dependence of Extent and Rate of Modification on pH**—The time course of the reaction of bovine PSTI with 0.7 mole % bovine trypsin at pH 3.22 is illustrated in Fig. 5. The reaction reaches an apparent plateau after about 5 hours of incubation; however, the modification does continue, although at a diminished rate, for up to 30 hours. Although the phenomenon probably reflects an asymptotic approach to an equilibrium of the kind described for STI, we prefer to use the term “plateau” in this report because we have not yet examined the question of reversibility. The modification of bovine PSTI by porcine trypsin at pH 3.2 proceeds at a much faster rate than observed for bovine trypsin. However, the extent of modification is the same for both species of trypsin. When incubation is carried out at other pH values, characteristic plateau values are reached. At pH 2.7 the plateau value appears to be independent of a 10-fold range of trypsin concentration, i.e., 0.7 to 7.0 mole %.

The effects of pH on the extent and the initial rate of the modification reaction are illustrated in Figs. 6 and 7, respectively. The pH profiles for both parameters are essentially superimposable. There is a sharp maximum at pH 2.7. This is strikingly different from the usual alkaline pH optimum for the action of trypsin on protein, peptide, and ester substrates. In the pH range near neutrality there is no evidence of modification after 5 hours of incubation, and, if it existed at all after 16 hours, it is of the order of 2%. The pH profile for both extent and initial rate of modification resemble a titration curve for at least two ionizable groups with $K_a$ values of about 2.4 and 3.4, respectively. They suggest but by no means establish that the first carboxyl group must be ionized and the second un-ionized for modification to occur.

**Behavior of Basic Pancreatic Trypsin Inhibitor**—Similar attempts were made to modify the basic pancreatic trypsin inhibitor with trypsin and subsequently to inactivate it with carboxypeptidase B. No modification, as determined by inactivation or the release of basic amino acids, was observed within the pH range, 2.7 to 3.5, at 25° and 35°, with either bovine or porcine trypsin at the 0.7 mole % level.

**DISCUSSION**

These experiments show that the bovine pancreatic secretory trypsin inhibitor undergoes a proteolysis reaction similar to that described by Ozawa and Laskowski (2) for soybean trypsin inhibitor and ovomucoid trypsin inhibitor (2, 5). The features of the reaction common to the three types of inhibitors are (a) proteolysis by catalytic amounts of trypsin at acid pH, (b) hydrolysis of a specific arginine bond, and (c) inactivation of the modified inhibitor when the arginine residue is removed by carboxypeptidase B. Those features of the reaction concerned with equilibrium and reversibility are currently under investigation.

We have shown, by isolation and characterization of peptide fragments, that a single arginyl-isoleucyl bond is hydrolyzed at positions 18 and 19. Removal by carboxypeptidase B of the COOH-terminal arginine residue thus formed leads to inactivation of the inhibitor. On this basis, the arginine residue at position 18 is identified as the reactive site of bovine PSTI. The amino acid sequence in the vicinity of arginine 18 is

- Gly-Cys-Arg---His-Tyr---

The presence of proline in position 17 explains why large amounts of carboxypeptidase B (7 mole %) were required to release arginine at position 18. In addition to cysteine at position 16 a second cysteine residue is present in position 9. Therefore, there are two sites for the small fragment to be attached by disulfide bridges to the remainder of the inhibitor after cleavage of the arginyl-isoleucyl bond. This is consistent with the suggestion of Ozawa and Laskowski (2) that the reactive site of many or all trypsin inhibitors is contained within a disulfide loop. This point will be further clarified when the positions of the disulfide bridges of bovine PSTI are determined.

Tschesche has reported that porcine and bovine pancreatic trypsin inhibitors are hydrolyzed at lysine and arginine residues, respectively, upon interaction with trypsin (7). After incubation of porcine inhibitor with 0.9 eq (90 mole %) of trypsin at pH 7.8 for 4 hours, he separated trypsin from the mixture of intact and modified inhibitors by gel filtration at pH 2.0. Intact inhibitor and two modified forms were identified by electrophoretic mobility in a system calibrated with intact and modified inhibitor (Lys-X bond broken). Our experiments differ significantly from those of Tschesche in the pH of the reaction and the stoichiometry of the reactants. A comparison of our results with those of Tschesche must await further study of the reaction at pH 7.8; in particular, the identification of the cleaved peptide bond.

Our studies extend the phenomenon originally described by Laskowski to the bovine pancreatic secretory trypsin inhibitor. Our experiments describe a partial proteolysis reaction which occurs maximally at pH 2.7. The results neither support nor contradict the suggestion that a partial proteolysis reaction is a necessary prerequisite for the inhibition of trypsin at neutral pH.

**Acknowledgments**—It is with pleasure that we acknowledge the assistance of D. C. Bartelt, J. S. Giordano, Jr., and R. Shapanka in the preparation of the peptide fragments and the determination of amino acid sequences. We express our appreciation to Dr. P. J. Burck and E. L. Grimm, Biochemical Research, Eli Lilly and Company, for fractions containing bovine secretory trypsin inhibitor, and to Dr. E. Sachs, Laboratoire
Choay, Paris, France, for samples of basic pancreatic trypsin inhibitor.

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