The Structure of the Bovine Pancreatic Secretory Trypsin Inhibitor—Kazal's Inhibitor

III. DETERMINATION OF THE DISULFIDE BONDS AND PROTEOLYSIS BY THERMOLYSIN

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SUMMARY

The three disulfide bonds of bovine pancreatic secretory trypsin inhibitor were assigned on the basis of the structures of cystine peptides isolated from thermolysin hydrolysates of the native inhibitor prepared at pH 6.5. The peptides were isolated by chromatographic procedures with Sephadex G-75, Sephadex G-25, and Dowex 50-X2. Five major cystine peptides were oxidized with performic acid and the cysteic acid fragments were separated by chromatography on Dowex 50-X2 and by high voltage paper electrophoresis. The cysteic acid peptides were located in the amino acid sequence of the inhibitor (Greene, L. J., and Bartelt, D. C., J. Biol. Chem., 244, 2646 (1969)) on the basis of amino acid composition and end group determination. Cysteine peptides corresponding to disulfide bonds I-V, II-IV, and III-VI were isolated in 55, 44, and 91% yield, respectively.

Intermediates in the degradation of the inhibitor by thermolysin having either one or two peptide bonds hydrolyzed per molecule were isolated. The sites of hydrolysis were identified as the peptide bonds Ile-Leu (residues 2 to 3) and Glu-Val (residues 12 to 13).
acid hydrolysis. Cysteic acid peptides suitable for structural studies were prepared by performic acid oxidation of cystine peptides by the procedure of Hirn (23). The values reported for cysteic acid have not been corrected for incomplete oxidation in either case.

Edman Degradation—The procedures used for subtractive Edman degradation have been described in Bartelt and Greene (24).

Detection of Cystine Peptides—Column effluents were not monitored for cystine peptides by specific volumetric procedures. The effluent was combined on the basis of ninhydrin color after alkaline hydrolysis. Duplicate aliquots of these pools were submitted to acid hydrolysis with and without prior performic acid oxidation by the method of Moore (22).

Bovine PSTI—These experiments were performed on two samples of inhibitor prepared from fractions of bovine pancreas by procedures developed for the isolation of inhibitor from bovine pancreatic juice (14). The results documented here were obtained on the same sample of inhibitor as was used for the amino acid sequence determination of the inhibitor (3, 16). The second sample was carried through the Dowex 50-X2 fractionation step (cf. Fig. 1) with essentially the same results with respect to chromatographic elution patterns and amino acid composition of peptides.

Thermolysin Hydrolysis—In order to minimize the disulfide interchange reaction, the hydrolysis was conducted at pH 6.5, 37°C, and cysteine peptides were isolated under acidic conditions (25-28).

Thermolysin (10.4 mg of protein) was suspended in 0.02 M calcium acetate at 0°C and solubilized by the dropwise addition of 0.2 N NaOH until the pH reached 11.5 (29). The pH of the solution was immediately adjusted to pH 8.2 with 0.2 N acetic acid. The protein concentration was adjusted to 1.04 mg per ml by dilution with 0.1 M MES buffer (2-(N-morpholino)ethanesulfonic acid), pH 6.5, containing 0.002 M calcium chloride. The activity of thermolysin solubilized under these conditions (determined by a spectrophotometric procedure with FAGLA 6.5, 0.002 M MES buffer, pH 6.5, 0.002 M calcium chloride at 0°C for 48 hours) was comparable to that of thermolysin prepared at pH 8.5.

Five micromoles of bovine PSTI (32.5 mg) and 0.2 mg of thermolysin were incubated in 10 ml of 0.1 M MES buffer, pH 6.5, 0.002 M calcium chloride at 0°C for 48 hours. The reaction was stopped by adjusting the solution to pH 3.0 with glacial acetic acid and the hydrolysate was submitted to gel filtration on Sephadex G-75. Cystine peptides were isolated by the procedures summarized in Fig. 1.

High Voltage Electrophoresis—Peptides were subjected to electrophoresis (Electrophorator D, Gilson Medical Electronics) on Whatman 3MM paper at pH 6.5 (25 ml of pyridine, 1 ml of acetic acid, and 225 ml of H2O) and at pH 3.5 (1 ml of pyridine, 10 ml of acetic acid, and 180 ml of H2O). Guide strips were developed with ninhydrin (0.5% in acetone). Acidic peptides were eluted from the paper with 50% aqueous pyridine and neutral peptides with 0.1 M ammonium hydroxide.

The products of performic acid oxidation of Peptide I-I (0.2 µmole) were separated by electrophoresis at pH 6.5 for 30 min at 52 volts per cm. The oxidation products of Peptide II-3 (0.2 µmole) were prepared by electrophoresis of Peptide II-3 at pH 6.5 for 40 min at 73 volts per cm.

Peptides present in Fraction IV (Fig. 3) were separated into components IV-1, IV-2, and IV-3 by electrophoresis at pH 6.5 for 30 min at 52 volts per cm. Fraction IV-3 was resubmitted to electrophoresis at pH 3.5 for 150 min at 52 volts per cm to prepare Fractions IV-3-A, IV-3-B, and IV-3-C. Fraction V (Fig. 3) was purified by electrophoresis at pH 3.5 for 150 min at 52 volts per cm to prepare Fractions V-1, V-2, V-3, and V-4.

Calculation of Recovery of Peptides—The recoveries of peptides are based on the results of amino acid analysis. They were corrected for material consumed for detection and amino acid analysis but not for chromatographic losses. The yield of cystine peptides has been calculated on the basis of the extensively hydrolyzed inhibitor (Fraction 2, Fig. 2) which represents 75% of the inhibitor treated with thermolysin for 48 hours. Similarly, the data for sites of and the extent of thermolysin hydrolysis of bovine PSTI summarized in Fig. 8 are based on peptides isolated from Fraction 2.

RESULTS

The hydrolysis of bovine PSTI by thermolysin leads to the formation of two classes of products which are separable by gel filtration on Sephadex G-75 (Fig. 2). Fraction I contains a mixture of partially hydrolyzed inhibitors, with one or two peptide bonds cleaved per molecule. Fraction 2 is a mixture of small peptides resulting from the extensive hydrolysis of bovine PSTI by thermolysin. The amino acid compositions of both fractions were essentially the same as the starting material (cf. Table I).
FIG. 2. Gel filtration on Sephadex G-75 of the products of thermolysin hydrolysis of 5 μmoles of bovine PST1 (cf. "Experimental Procedure"). The column, 1.8 × 100 cm, was equilibrated and developed with 0.2 M pyridine acetate buffer, pH 3.1, at 23°C. The effluent was collected in 4 ml fractions. Peptides were located by ninhydrin analysis after alkaline hydrolysis. The fractions indicated by the bars combined.

**TABLE I**

Amino acid composition of bovine pancreatic secretory trypsin inhibitor and products formed from inhibitor by action of thermolysin (Fig. 2)

| Amino acid   | Inhibitor | Partially hydrolyzed inhibitor Fraction 1 | Extensively hydrolyzed inhibitor Fraction 2 |
|--------------|-----------|------------------------------------------|--------------------------------------------|
| Lysine       | 3.12 (3)  | 3.20                                     | 3.20                                       |
| Arginine     | 3.03 (3)  | 3.11                                     | 2.91                                       |
| Cysteic acid | 6.11 (6)  | 5.76                                     | 6.36                                       |
| Aspartic acid| 6.96 (7)  | 6.23*                                    | 7.00                                       |
| Threonine    | 3.87 (4)  | 3.77                                     | 3.84                                       |
| Serine       | 2.00 (2)  | 2.00                                     | 2.07                                       |
| Glutamic acid| 7.14 (7)  | 6.96                                     | 7.00                                       |
| Proline      | 4.27 (4)  | 3.96                                     | 4.27                                       |
| Glycine      | 5.12 (5)  | 5.03                                     | 5.26                                       |
| Alanine      | 1.17 (1)  | 1.19                                     | 1.14                                       |
| Valine       | 4.02 (4)  | 3.93                                     | 4.18                                       |
| Methionine   | 0.84 (1)  | 0.77                                     | 0.77                                       |
| Isoleucine   | 2.97 (3)  | 2.83*                                    | 3.12                                       |
| Leucine      | 3.84 (4)  | 3.85                                     | 3.57                                       |
| Tyrosine     | 1.95 (2)  | 1.82                                     | 1.97                                       |

Yield*        | 25%       | 75%                                      |

* Determined after performic acid oxidation.

The assignment of the disulfide bonds based on the isolation of cystine peptides in Fraction 2 will be presented first, followed by the characterization of partially hydrolyzed inhibitor present in Fraction 1.

Assignment of Disulfide Bonds

Isolation of Cystine Peptides from Fraction 2 (Fig. 2)—The amino acid content of Fraction 2 accounted for all of the amino acids in bovine PST1 and was recovered in 75% yield relative to the amount of inhibitor incubated with thermolysin. After one cycle of subtractive Edman degradation the amino acid content of Fraction 2 decreased by approximately 12 to 14 residues per equivalent of inhibitor, showing that the mixture contained many small peptides with an average size of 4 to 5 residues per molecule.

The flow diagram given in Fig. 1 summarizes the procedures used for the isolation of cystine peptides from Fraction 2. The system of nomenclature used specifies the route of purification by identifying the elution diagram peaks from which the purified peptides have been prepared (cf. Fig. 1). Cystine peptides were effectively separated from smaller peptides when Fraction 2 was submitted to gel filtration on Sephadex G-25 as shown in Fig. 3. The effluent corresponding to the filled bars I and II contained 88% of the cystine peptides applied to the column. I and II were further fractionated on Dowex 50-X2 (Fig. 4). The filled bars indicate elute containing cystine Peptides I-1, I-3, I-4, and I-7 derived from Pool I (Fig. 4, top) and Peptides II-3, II-8, and II-9 derived from Pool II (Fig. 4, bottom). Peaks I-7 and II-9 contained less than 3% of the cystine present in Fraction 2. This material was not sufficiently homogeneous nor present in high enough quantities to be further characterized.

Peptides I-1, I-3, I-4, I-3, and II-8 were oxidized with performic acid and the resulting cystine peptides were isolated either by chromatography on Dowex 50-X2 or by high voltage...
Fig. 4. Chromatography on Dowex 50-X2 of Fractions I (top) and II (bottom) from the Sephadex G-25 filtration (Fig. 3). The columns, 0.9 X 60 cm, were equilibrated with 0.2 M pyridine acetate buffer, pH 3.1, and developed with a linear gradient (250 ml each) of starting buffer and 2.0 M pyridine acetate buffer, pH 5.0. Each sample contained a mixture of peptides derived from 1.2 pmoles of extensively digested inhibitor. The columns were located by ninydrin reaction after alkaline hydrolysis. The fractions indicated by the bars were combined. The solid bars indicate effluent containing cystine peptides. •••••• absorbance at 570 nm (ninhydrin reaction); ——, effluent pH.

paper electrophoresis. The identification of the peptides is based on the amino acid composition, determination of amino terminal residue by subtractive Edman degradation, and is interpreted in terms of the sequence of the inhibitor. No further information was required for the unique assignment of these peptides within the amino acid sequence of bovine PSTI. The 6 half-cystine residues of the inhibitor have been assigned Roman numerals starting from the amino-terminal portion of the molecule.

Disulfide Bond I-V—Cystine Peptide II-8 was coeluted with the tetrapeptide Leu-Gly-Arg-Glu from the Dowex 50-X2 column (Fig. 4, bottom). However, this contaminating peptide was effectively separated on Dowex 50 from cysteic acid peptides after performic acid oxidation of the mixture (Fig. 5, bottom). The elution position of the tetrapeptide was unchanged because it was not susceptible to performic acid oxidation. The yield of Peptide II-8 was 55%. The amino acid compositions and results of subtractive Edman degradation are given in Table II. These data show that a disulfide bond links residues 9 and 38.

Disulfide Bond II-IV—Peptide I-4 was isolated in 44% yield after chromatography on Dowex 50-X2 (Fig. 4, top). Two cysteic acid peptides, I-4-A and I-4-B, were prepared by performic acid oxidation of I-4 by chromatography on Dowex 50-X2 (Fig. 5, top). The analytical data for these peptides are given in Table III. The results show that a disulfide bond connects residues 16 and 35.

A mixture of related peptides, I-3, was also isolated in 19% yield (Fig. 4, top). I-3 had exactly the same amino acid composition as Peptide I-4. The cysteic acid fragments had also the same amino acid composition as I-4-A and I-4-B but had different elution positions from the Dowex 50-X2 column. These peptides probably represent a mixture of products result.
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from deamidation, imide or β-peptide bond formation involving residues 14 to 15 (Asn-Gly) and residues 33 to 34 (Asn-Glu) (cf. References 31, 32).

Disulfide Bond III-VI—Two cystine peptides, I-1 and II-3, corresponding to bond III-VI were released by the action of thermolysin on bovine PSTI. The peptides were homogeneous after chromatography on Dowex 50 X-2 (Fig. 4) and were isolated in 31 and 60% yield, respectively. After oxidation of the cystine peptides with performic acid the cysteic acid peptides were purified by high voltage paper electrophoresis. The amino acid composition and results of subtractive Edman degradation are given in Table IV. The data show that a disulfide bond connects residues 24 and 56 in the inhibitor.

A two-dimensional schematic diagram of the structure of bovine PSTI showing the arrangement of the disulfide bonds and the sequence of the amino acids is given in Fig. 6. The amino acid residues given in the mottled circles indicate the cystine peptides characterized in this study. The yields of these peptides, based on the amount of extensively hydrolyzed inhibitor (Fraction 2, Fig. 2) are: I-V, 55%; II-IV, 44%; and III-VI, 91%.

Table III

Disulfide bond II-IV of bovine PSTI

| Amino acid | Peptide I-4 | Peptide I-4-A | Peptide I-4-B |
|------------|-------------|---------------|---------------|
|            | I-4 residues 13 to 18 | I-4-A residues 13 to 18 | I-4-B residues 13 to 18 |
| Cysteic acid | 1.99 (2) | 1.05 | 1.10 | 1.09 |
| Aspartic acid | 1.84 (2) | 0.95 | 1.01 | 0.99 |
| Serine | 0.65 (1) | 0.60 | 0.22 |
| Glycine | 1.10 (1) | 1.23 | 1.14 |
| Glutamic acid | 1.14 (1) | 0.98 | 0.90 |
| Valine | 0.93 (1) | 0.94 | 0.32 |
| Arginine | 0.97 (1) | 0.93 | 0.92 |
| Edman recovery | 89% | 101% |

Table IV

Disulfide bond III-VI of bovine PSTI

| Peptide | Rf | Amino acid |
|---------|----|------------|
| I-1     | 0.62 | Cys, Ser, Gly, Val, Asp, Thr |
| I-1-A   | 0.88 | 0.97 | 1.04 | 1.11 |
| Edman degradation (90%) | 1.05 | 0.33 | 0.83 | 1.11 |
| I-1-B   | 0.80 | 1.11 | 2.11 | 0.86 | 1.13 | 0.83 |
| Edman degradation (98%) | 1.00 | 1.65 | 0.12 | 1.02 | 0.79 |
| II-3    | 0.62 | 0.98 | 0.43 | 0.89 | 1.12 |
| Edman degradation (99%) | 0.98 | 1.00 | 1.02 |
| II-3-B  | 0.75 | 0.87 | 1.13 | 0.20 |
Characterization of Partially Hydrolyzed Inhibitor

The amino acid composition of Fraction 1 (Fig. 2) corresponded to that of bovine PSTI less ~0.7 eq of aspartic acid and isoleucine because of the loss of the amino-terminal dipeptide Asn-Ile (cf. Table I). The sites of cleavage in this mixture of peptides were determined by oxidation with performic acid followed by separation of the fragments by gel filtration on a 200-cm column of Sephadex G-50 (Fig. 7, left panel). The low molecular weight material, Peak R, was a mixture of peptides corresponding to residues 1 to 12 and 3 to 12 in the molar ratio 1:2 (cf. Table V). The peptides in Fraction A were partially resolved by a second passage through a column of Sephadex G-50 with an effective length of 400 cm (cf. Fig. 7, right panel). The data given in Table V show that A-1 corresponds to residues 3 to 56 plus a small amount of peptide corresponding to residues 1 to 56, and that A-2 contains residues 13 to 56. These assignments were confirmed by the results of subtractive Edman degradation where the amino-terminal residues of A-1 and A-2 were shown to be primarily leucine and valine. On the basis of the amounts of each peptide recovered, the following composition can be given for the mixture of partially hydrolyzed inhibitors: 20% corresponds to a cleavage only at residues 12 to 13; 35% is inhibitor with only the bond at residues 2 to 3 hydrolyzed; 40% of the molecules have both bonds, residues 2 to 3 and 12 to 13 cleaved; and approximately 5% is intact inhibitor. On the basis of the relative simplicity of the elution diagrams and the analytical data we conclude: (a) the partially hydrolyzed inhibitor is a mixture of peptides with bonds cleaved at residues 2 to 3 (Ile-Leu) and residues 12 to 13 (Glu-Val); and (b) no other peptide bonds are cleaved in the early stages of hydrolysis.

Fraction 1 was resubmitted to hydrolysis with thermolysin in order to determine whether the partially hydrolyzed inhibitors were intermediates in the production of extensively hydrolyzed inhibitor. On the basis of the Sephadex G-75 elution diagram, amino acid composition and results of one cycle of subtractive Edman degradation, the extensively hydrolyzed material, obtained in 50% yield, corresponded to Fraction 2 of the first hydrolysis.

These results show that Fraction 1 is an intermediate in the production of Fraction 2 and indicate that the initial event in the hydrolysis of intact bovine PSTI is the cleavage of two bonds at residues 2 to 3 and 12 to 13, followed by the slow cleavage of one or more bonds leading to the extensive degradation of the molecule.

Characterization of Noncystine Peptides

Peptides devoid of cystine were present in every fraction of the Sephadex G-25 effluent (Fig. 3). Most of these peptides in Fractions I and II were obtained in homogeneous form during the purification of cysteine peptides (Fig. 4) and cysteic acid peptides (Fig. 5, bottom). Fraction III contained a tetrapeptide and the peptides in Fractions IV and V were isolated by high voltage paper electrophoresis. These peptides and their yields are given in Table VI. The peptides marked with asterisks had integral molar ratios of the constituent amino acids. The others, although somewhat contaminated, could be identified on the basis of the sequence of bovine PSTI. The free amino acids

The number of each peptide recovered, the following composition can be given for the mixture of partially hydrolyzed inhibitors: 20% corresponds to a cleavage only at residues 12 to 13; 35% is inhibitor with only the bond at residues 2 to 3 hydrolyzed; 40% of the molecules have both bonds, residues 2 to 3 and 12 to 13 cleaved; and approximately 5% is intact inhibitor. On the basis of the relative simplicity of the elution diagrams and the analytical data we conclude: (a) the partially hydrolyzed inhibitor is a mixture of peptides with bonds cleaved at residues 2 to 3 (Ile-Leu) and residues 12 to 13 (Glu-Val); and (b) no other peptide bonds are cleaved in the early stages of hydrolysis.

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### Table V

| Peptide | A-1, residues 1 to 56 and 3 to 56 | A-2, residues 13 to 56 | B, residues 12 and 3 to 12 |
|---------|---------------------------------|-----------------------|--------------------------|
| Lysine  | 2.99 (3)                        | 2.13 (2)              | 1.01 (1)                 |
| Arginine| 2.98 (3)                        | 2.22 (2)              | 1.04 (1)                 |
| Cysteic acid | 6.08 (6)               | 4.91 (5)              | 1.06 (1)                 |
| Aspartic acid | 6.08 (6-7)           | 5.26 (5)              | 1.23 (1-2)               |
| Methionine sulfone | 1.03 (1)       | 0.89 (4)              | 1.01 (1)                 |
| Threonine | 3.74 (4)                      | 2.83 (3)              | 1.01 (1)                 |
| Serine  | 2.05 (2)                        | 2.05 (2)              | 1.01 (1)                 |
| Glutamic acid | 6.83 (7)             | 5.37 (6)              | 2.22 (2)                 |
| Proline | 4.16 (4)                        | 4.37 (4)              | 1.01 (1)                 |
| Glycine | 5.16 (5)                        | 5.50 (4)              | 1.11 (1)                 |
| Alanine | 1.16 (1)                        | 0.60 (0)              | 0.99 (1)                 |
| Valine  | 4.06 (4)                        | 3.94 (4)              | 0.99 (1)                 |
| Isoleucine | 2.29 (2-3)          | 1.84 (2)              | 0.98 (1)                 |
| Leucine | 3.60 (4)                        | 2.92 (3)              | 0.96 (1)                 |
| Tyrosine| 1.48 (2)                        | 1.46 (2)              | 1.01 (1)                 |

* This peptide appears to be contaminated with material containing proline, glycine, and alanine.
TABLE VI
Nonscissile peptides formed from inhibitor by action of thermolysin (Fraction 2, Fig. 2)

Peptides were identified on the basis of amino acid composition in terms of the amino acid sequence of the inhibitor (3). The asterisk indicates peptides having integral molar ratios of constituent amino acids. The remaining peptides, although present in mixtures were identifiable because of differences in concentrations. The fraction number indicates the method of preparation of the peptide and the numbers in parentheses give the electrophoretic mobility relative to glycine except for Peptides I-VI which are related to aspartic acid. The yields of peptides are based on the amount of extensively hydrolyzed inhibitor Fraction 2, Fig. 2. The yields of free amino acids are reported as percentage of 1 residue per molecule inhibitor.

| Residue No. | Fraction (Rp) | Peptide | Yield |
|-------------|---------------|---------|-------|
| 1-2         | V-3(1.82)     | *Asn-Ile | 32%   |
| 1-3         | IV-3-B(1.52)  | Asn-Tle-Leu | 3%    |
| 3-6         | II-7          | *Leu-Gly-Arg-Glu | 66%   |
| 10-12       | IV-2(0.30)    | *Thr-Asn-Glu | 02%   |
| 19-22       | III           | *Ile-Tyr-Asn-Pro | 46%   |
| 19-22       | IV-2-A(1.24)  | *Ile-Tyr-Asn-Pro | 14%   |
| 20-23       | IV-1(0.53)    | *Thr-Asp-Gly | 38%   |
| 29-30       | IV-3-B(1.53)  | Val-Thr | 19%   |
| 29-31       | IV-3-A(1.24)  | Val-Thr-Tyr | 21%   |
| 31-33       | V-2(1.32)     | Tyr-Ser-Asn | 6%    |
| 32-33       | V-4(1.51)     | Ser-Asn | 2%    |
| 39-47       | II-5          | Met-Glu Asn-Lys-Glu | 30%   |
| 40-47       | I-5, II-5     | Glu-Asn-Lys-Glu-Arg-Glu-Thr-Pro | 9% |
| 48-49       | IV-3-C(1.80)  | *Val-Leu | 26%   |
| 50-52       | II 6          | Ile-Glu-Lys | 67%   |
| V-1         | Valine | 19%   |
| V-1         | Isoleucine | 7%    |
| V-1         | Leucine | 14%   |

listed in the table were determined directly on the amino acid analyzer on a sample which had not been hydrolyzed.

*Sites of Thermolysin Hydrolysis*

The peptide bonds cleaved by thermolysin at pH 6.5, 37°, for 48 hours are given in Fig. 8. The extent of cleavage is based on extensively hydrolyzed inhibitor (Fraction 2). Those numbers must be considered minimum values because they were calculated on the basis of the yield of recovered and identifiable peptides.

**Discussion**

The most striking feature of these results is that thermolysin, acting on bovine PST1, produced a small number of cystine peptides in high yield: 55, 44, and 91% for disulfide bridges I-V, II-IV, and III-VI, respectively. The use of column chromatographic procedures was advantageous not only for the preparation of the cystine peptides but also for the recognition and isolation of partially hydrolyzed inhibitor as well as the identification and characterization of nonscissile peptides. The cysteic acid peptides were located in the amino acid sequence of bovine PST1 on the basis of amino acid composition and end group determination. All of the analytical data obtained in this study were consistent with the reported sequence of the inhibitor (3).

Furthermore, we have not found evidence for heterogeneity of disulfide bonds either in homogeneous peptides or mixtures of peptides. We estimate that we would have detected 10% of disulfide heterogeneity, if present. Evidence for amide heterogeneity was found for asparagine, residues 14 and 33 (Peptide I-3) but this may have been the result of the acidic conditions used for gel filtration and Dowex 50 chromatography (31).

The sites of peptide bond cleavage and the extent of hydrolysis by thermolysin acting on bovine PST1 are summarized in Fig. 8. Peptide bonds involving the amino group of leucine, isoleucine, valine, alanine, serine, threonine, and methionine were readily hydrolyzed whereas tryosine, glutamic acid, and glycine were cleaved less rapidly. It is noteworthy that the peptide bond Pro-Val was hydrolyzed to the extent of 91% and 53% for residues 22 to 23 and 47 to 48, respectively. The hydrolysis of sequence Cys-Leu-Leu-Cys (residues 35 to 38) was crucial for the determination of the disulfide bonds. Thermolysin hydrolyzed the Cys-Leu and Leu-Leu bonds to the extent of at least 68 and 55%, respectively. In preliminary experiments we attempted to digest intact inhibitor with pepsin at pH 2.2 followed by trypsin at pH 6.5. Only one disulfide bond, II-III-VI, could be identified, presumably because of the lack of hydrolysis of Cys-Leu-Leu-Cys sequence by pepsin and the difficulty of separating closely related peptide resulting from incomplete hydrolysis (27). The specificity of thermolysin hydrolysis of intact bovine PST1 at pH 6.5 is similar to that reported by other investigators who used thermolysin at pH 8 to 8.5 on cytochrome c (33), azurin (34), Kunitz inhibitor (35), and peptides (35-38). The first application of the use of thermolysin for the determination of disulfide bonds was reported by Jentsch for bovine insulin (39). An important difference in the experimental procedure is that we conducted the thermolysin hydrolysis at pH 6.5 to reduce disulfide interchange (25-28). Jentsch (39) digested insulin at pH 8.0 but did not present data on disulfide interchange.

The hydrolysis of intact bovine PSTI by thermolysin leads to the formation of high molecular weight intermediates having only one or two peptide bonds cleaved per molecule and a mixture of small peptides. Presumably the initial cleavages take place at exposed or partially unfolded species and hydrolysis at the third or subsequent bonds destabilizes the structure, leading
to exposure of more bonds to proteolytic activity, finally resulting in the formation of extensively degraded peptides (cf. Reference 40). On this basis, residues 2 to 3 and 12 to 13 of bovine PSTI are exposed and accessible to the action of thermolysin (cf. Fig. 6). Similar limited proteolysis reactions occur when ribonuclease is treated with subtilisin or elastase leading to the formation of ribonuclease S (41) and ribonuclease E (42, 43). We are attempting to separate the modified forms of bovine PSTI present in Fraction 1 for further chemical, physical, and inhibitor studies.

In the absence of crystallographic information, an analysis of the significance of the two-dimensional structure given in Fig. 6 must necessarily be incomplete. However, certain features deserve comment. The carboxyl-terminal cysteine residue VI, is linked to the cysteine I at position 24. The other two disulfide bonds, I-V and II-IV, form a ring containing 12 amino acid residues. The disposition of the disulfide bonds appears to give the molecule a compact structure which may be responsible in part for its stability at acid pH and elevated temperature (13). The reactive site (44) of the molecule, Arg-Ile (residues 18 to 19), is located within a disulfide loop of 9 amino acid residues between cysteines II and III (45). After hydrolysis of the Arg-Ile bond, both fragments remain attached by two other disulfide bonds, I-V and II-IV. This structure supports the Laskowski (44) that reactive site of inhibitors is located between cystine residues.

The 6 half-cystine residues of cow, pig (24, 46) and sheep (47) pancreatic secretory trypsin inhibitors occupy the same positions in the amino acid sequences of these polypeptides. On this basis it would be expected that the disposition of the disulfide bonds of pig and sheep PSTI will be the same as found for bovine PSTI.

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At the International Research Conference on Proteinase Inhibitors (Munich, November, 1970), Tschesche reported that porcine PSTI contains a III-VI disulfide bond. At the same meeting we presented a preliminary report of our results documenting the disposition of the three disulfide bonds of bovine PSTI. In the symposium volume, the paper of Tschesche et al. (43) contained data for the remaining disulfide bonds of the porcine inhibitor. They are identical with those in bovine PSTI.
The Structure of the Bovine Pancreatic Secretory Trypsin Inhibitor—Kazal's Inhibitor: III. DETERMINATION OF THE DISULFIDE BONDS AND PROTEOLYSIS BY THERMOLYSIN
Odette Guy, Roslyn Shapanka and Lewis J. Greene

J. Biol. Chem. 1971, 246:7740-7747.

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