# DISULFIDE BONDS OF TRYPSIN INHIBITOR FROM COW COLOSTRUM

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One of the disulfide bonds of trypsin inhibitor from cow colostrum was reduced by sodium borohydride and the free SH-groups were carboxymethylated. The modified inhibitor was specifically cleaved by trypsin. Gel filtration of the digest on a column of Sephadex G-50 fine afforded two disulfide peptides in which disulfide bonds between half-cystine residues Nos 8—58 and Nos 33—54 were determined. The third bond, 17—41, was determined by the identification of the carboxymethylated cysteines.

The primary structure of trypsin inhibitor from cow colostrum has been known[1] for some time. The inhibitor molecule consists of one polypeptide chain of 67 amino acids and contains three disulfide bonds.The molecule is resistant in native state to trypsin and chymotrypsin. It is nonspecifically cleaved by pepsin[2] and by the peptidase from *Aspergillus flavus*[3]. We have not been able to obtain the disulfide peptides in pure form from these digests. We met with the same difficulties when we tried to cleave the molecule by partial acid hydrolysis[3]. We have decided therefore to disrupt the three-dimensional structure of the inhibitor molecule by the reduction of one disulfide bond and to carboxymethylate the arising free SH-groups, as described for the basic pancreatic trypsin inhibitor[4]. The modified inhibitor was susceptible to tryptic cleavage. The two specific tryptic disulfide peptides were isolated, the two carboxymethyl-cysteine residues were identified, and the three disulfide bonds of the trypsin inhibitor from cow colostrum were thus determined.

## EXPERIMENTAL

### Material

Cow colostrum obtained on the first day after the delivery was provided by the Collective Farm, district Nymburk, Bohemia. Sepharose 4 B and all types of Sephadex used were products of Pharmacia (Uppsala, Sweden). 5,5′-Dithio-bis(2-nitrobenzoic acid) was purchased from Sigma,

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U.S.A. Sodium borohydride was from Merck, Germany. Trypsin was a 2-times crystallized, salt-free preparation of Worthington Biochemical Corp., U.S.A.

## Methods

*Isolation of inhibitor.* The inhibitor was isolated by the method described earlier[5] except that the inhibitor, which had been precipitated from the solution in 2·5% trichloroacetic acid by ammonium sulfate, was desalted and attached to a column of trypsin Sepharose. The conditions of preparation of the trypsin-Sepharose column and of its operation were analogous to those reported by Kassell and Marcyniszyn[6] for the isolation of the inhibitor from bovine organs. After displacement and desalting, the inhibitor (400 mg) was placed on a column of CM-Sephadex (2·6 × 27 cm) under the conditions described in a preceding paper[5]. The column was equilibrated with 0·05M potassium formate adjusted to pH 3·5 by formic acid. The inhibitor was eluted by a linear gradient of increasing ionic strength (0 to 0·2M-KCl). The volume of both the mixing device and of the reservoir was 1·5 l. Fractions 15 ml in volume were collected at 30 min intervals. The pooled fractions corresponding to the middle portion of the peak and showing antitryptic activity were used in subsequent work.

*The determination of antitryptic activity and the quantitative amino-acid analyses* were described in detail elsewhere[1,5]. The samples for analysis were hydrolyzed 20 h at 112°C.

*The identification of the trypsin inhibitor-trypsin complex* was carried out by the measurement of inhibitory activity after the release of the inhibitor from the complex by 2·5% trichloroacetic acid[7].

*Reduction by sodium borohydride.* The procedure of Light and Sinha[8], as used by Kress and Laskowski[4] for the reduction of one disulfide bond in the basic pancreatic trypsin inhibitor, was followed. Trypsin inhibitor from cow colostrum (45 mg) was dissolved in 0·02% solution of EDTA in water (25 ml) at 2°C. The pH of the solution was adjusted to 8·8 by 0·2M-NaOH and the volume was made up to 3 ml by EDTA. The solution was kept under a nitrogen barrier. Cold 0·2M solution (3 ml) of sodium borohydride in water was then added. The nitrogen barrier was restored and kept at 2°C, 60 min in a water bath. The reaction was discontinued by acidification of the mixture to pH 2·7 by 2M hydrochloric acid. The mixture was then kept in ice until treated further (2 h). An aliquot was withdrawn for the determination of free sulfhydryl groups.

*Determination of free sulfhydryl groups.* The samples for the determination of free sulfhydryl groups by the Ellman method[9,10] were withdrawn from the reaction mixture and pipetted into cold water (final volume 2 ml); the pH was immediately adjusted to 3·0 by 2M hydrochloric acid. They were kept in ice until treated further. A 1 ml sample (0·05—0·10 μmol of protein) was used for the determination of the free SH-groups. To this sample 0·5 ml of water was added, followed immediately by 1 ml of 1M-Tris buffer and 1M phosphate buffer at pH 8·1, and by 0·5 ml of 0·002M 5,5′-dithio-bis(2-nitrobenzoic acid) solution in 0·01M sodium phosphate at pH 8·1. The reaction was allowed to proceed 30 min in a dark room. The absorbance at 412 nm was then measured. The content of free sulfhydryl groups was read off a calibration curve for 0·01—0·5 μmol of cysteine.

*Carboxymethylation of partly reduced inhibitor.* Iodoacetic acid (160 mg) in 1 ml of 1M-NaOH was added to the reduced inhibitor under a nitrogen barrier. The pH of the reaction mixture was maintained at 8·2 by the addition of 2M-NaOH, 30 min, under a nitrogen barrier. The reaction was discontinued by acidification to pH 3·4 by 4M hydrochloric acid. The excess of the reagent was removed on a column of Sephadex G-25 (1·2 . 35 cm), equilibrated with 0·02M ammonium carbonate. The protein-containing fractions were pooled (volume 12 ml). An aliquot

(0·5 ml) was taken to dryness and used for amino-acid analysis. The rest was subjected to tryptic cleavage.

*Cleavage of carboxymethylated inhibitor by trypsin.* Trypsin (10 mg) dissolved in 0·5 ml of 0·001M hydrochloric acid was added to the solution (11·5 ml) of the partly reduced and carbomethylated inhibitor in 0·02M ammonium carbonate. The cleavage was allowed to proceed 4 h at 37°C. The lyophilized tryptic digest was dissolved in 2 ml of 0·02M ammonium carbonate and subjected to gel filtration on a column of Sephadex G-50 fine (2·9 . 71 cm), equilibrated with 0·02M ammonium carbonate. The absorbance of the fractions (6 ml/15 min) was measured at 280 and 230 nm. Fractions 2 though 4 were rechromatographed on a column of Sephadex G-50 fine (2·5 . 58 cm) under identical conditions.

*Reduction of disulfide groups and aminoethylation of cysteine residues of the peptide from fraction* 4. The peptide (2 mg) was dissolved in 1 ml of 0·02M ammonium carbonate and 2 drops of mercaptoethanol were added. After 30 min of reaction at room temperature, 2 drops of ethylene imine were added and the mixture was set aside for 1 h. The excess of the reagent was removed on a column of Sephadex G-10 (1·5 . 10 cm), equilibrated with 0·02M ammonium carbonate.

*Cleavage of aminoethylated peptide by trypsin.* The total quantity (2 mg) of the peptide, dissolved in 0·02M ammonium carbonate was digested by trypsin (40 μg in 50 μl of water), 2 h at 37°C. The digest was dried in a desiccator *in vacuo* (oil pump).

*The methods of isolation and detection of small peptides.* The Pauly reaction, oxidation, high voltage electrophoresis in buffers at pH 1·9 and 5·6, as well as the descending chromatography in the system 1-butanol–acetic acid–pyridine-water (15 : 10 : 3 : 12) were described in an earlier paper[1]. The ninhydrin staining was carried out by 0·1% ninhydrin. Where spots were to be eluted and the eluates subjected to amino-acid analysis, 0·01% ninhydrin was used for the detection. The analytical values of the N-terminal amino acid were then up to 50% lower.

## RESULTS AND DISCUSSION

### *Isolation of Inhibitor*

The method of isolation was modified compared to the procedure described earlier[5] We have found that there are several forms of the inhibitor, isoinhibitors, in the colostrum. These isoinhibitors differ only little in amino-acid composition. These isoinhibitors were not fractionated before this study and a mixture of several isoinhibitors was therefore used to start with. Although we have obtained evidence of amino-acid replacements in our study, this phenomenon did not affect the determination of the disulfide bonds.

### *Reduction of Inhibitor, Carboxymethylation, and Tryptic Cleavage*

As obvious from the course of the reduction in 0·1M sodium borohydride (Fig. 1), one disulfide bond is reduced quickly and the reaction then stops. The same result was obtained in 0·2M borohydride. The partly reduced inhibitor was carboxymethylated since it showed high antitryptic activity and was resistant to trypsin. The carboxymethylated inhibitor was not active. The residual activity of the sample (5%)

TABLE I

Amino Acid Analyses of Peptides F 3, OX 1 and OX 2

The number of residues per mol is given. The values were obtained on 20-h hydrolysates and are not corrected. Half-cystine was determined as cysteic acid in the oxidized sample.

| Amino acid | F 3 | OX 1 | OX 2 | Amino acid | F 3 | OX 1 | OX 2 |
|---|---|---|---|---|---|---|---|
| Lysine | 1·02 | 0·77 | — | Glutamic Acid | 5·78 | 3·05 | 3·00 |
| Arginine | 0·98 | — | 1·00 | Proline | 4·80 | 1·93 | 2·70 |
| Half-Cystine | 2·05 | 0·81 | 0·88 | Alanine | 1·02 | — | 0·95 |
| Aspartic Acid | 2·10 | 1·00 | 1·10 | Isoleucine | 0·79 | 0·77[a] | — |
| Threonine | 1·95 | 0·95 | 0·72 | Leucine | 2·00 | — | 2·00 |
| Serine | 0·89 | 0·80 | — | Phenylalanine | 0·81 | — | 0·47[a] |

[a] The values of the N-terminal amino acid are decreased by its partial reaction with ninhydrin.

TABLE II

Amino-Acid Analyses of Peptides F 4 and AE 1 through AE 4

The number of residues per mol is given. The values were obtained on 20-h hydrolysates and are not corrected. Half-cystine was determined as cysteic acid in the oxidized sample.

| Amino acid | F 4 | AE 1 | AE 2 | AE 3 | AE 4 |
|---|---|---|---|---|---|
| Arginine | 0·98 | — | — | — | 1·00 |
| Half-cystine | 1·95 | — | — | — | — |
| Aspartic Acid | 5·84 | 2·00 | — | 4·00 | — |
| Threonine | 3·40 | 1·08 | — | 2·60 | — |
| Serine | 1·84 | 1·78 | — | 0·80 | — |
| Glutamic Acid | 4·10 | — | — | 3·50 | — |
| Proline | 1·05 | — | — | 0·85 | — |
| Glycine | 3·20 | — | — | 3·00 | — |
| Alanine | 0·82 | — | 0·70[a] | — | — |
| Methionine | 0·85 | — | — | — | — |
| Leucine | 1·05 | — | — | — | 0·80[a] |
| Tyrosine | 2·70 | 1·47[a] | — | 0·70 | — |
| Phenylalanine | 2·78 | 0·90 | — | 1·60 | — |
| Carboxymethyl-Cysteine | 1·09 | — | — | 0·98 | — |
| Aminoethyl-Cysteine | — | — | 1·00 | 0·75 | — |

[a] The values of the N-terminal amino acid are decreased by its partial reaction with ninhydrin.

obviously can be ascribed to the remaining intact inhibitor. We had to use a larger quantity of trypsin than usual to cleave the carboxymethylated inhibitor because a portion was bound in complex with the intact inhibitor.

*Fractionation of Tryptic Digest*

The tryptic digest was subjected to gel filtration on Sephadex G-50 fine (Fig. 2). Fractions corresponding to individual peaks were pooled in 9 fractions which will be designated in the order of their emergence from the column. Fractions 2,3 and 4 were rechromatographed. A comparison of peptide maps of oxidized and unoxidized samples of all fractions with the peptide map of the tryptic digest of the oxidized inhibitor provided information on the distribution of the peptides from the inhibitor in the individual fractions. Fractions 5,7, 8 and 9 did not contain peptides from the inhibitor and were discarded. These fractions probably contain small peptides from

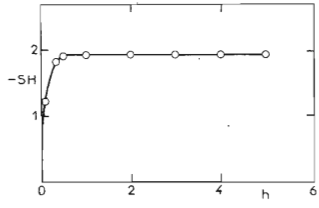


FIG. 1

Rate of Reduction of Trypsin Inhibitor from Cow Colostrum in 0·1M Sodium Borohydride

h Time of reduction in hours, —SH sulfhydryl groups released (mol per mol of inhibitor).

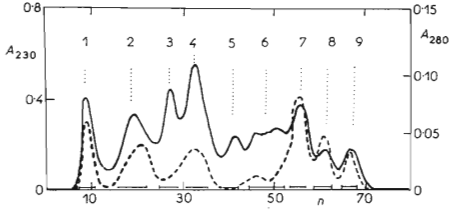


FIG. 2

Gel Filtration of Tryptic Digest of Partly Reduced and Carboxymethylated Inhibitor on Sephadex G-50

The column (2·9 . 71 cm) was eluted by 0·02M ammonium carbonate. Fractions (n) 6 ml/15 min. Full line, absorbance at 230 nm, broken line, absorbance at 280 nm. 1—9, pooled fractions.

autolyzed trypsin whose large quantities were used for the digestion. The complex of trypsin with the intact inhibitor was found in fraction 1. The quantitative amino-acid analysis of rechromatographed fraction 2 showed a high content of histidine and valine in the sample; these amino acids, however, do not exist in the molecule of the trypsin inhibitor from cow colostrum[1]. They originate probably also from the trypsin autolysate. Therefore we did not investigate this fraction either and only fractions 3,4 and 5 were studied.

*Fraction 3*

A comparison of the peptide maps of the oxidized and the unoxidized sample of fraction 3 showed the presence of disulfide peptide F 3 which yielded two peptides after oxidation. They were subjected to amino-acid analysis (Table I). The remaining portion of the peptide was dried and oxidized by performic acid. The oxidized peptides obtained were resolved on paper using high-voltage electrophoresis at pH 1·9 in the first direction and descending chromatography in the second direction (Fig. 3). The dilute ninhydrin solution was used for the detection. The peptides were eluted, precipitated, and subjected to quantitative amino-acid analysis (Table I). Since the primary structure of the trypsin inhibitor from cow colostrum is known[1], the composition and distribution of tryptic peptides in the molecule is also known. We were able therefore to identify the peptides obtained from their amino-acid analyses.

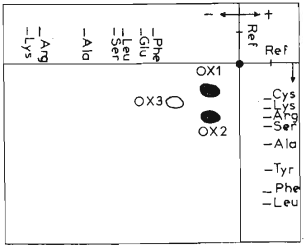


FIG. 3

Peptide Map of Oxidized Disulfide Peptide F 3

Horizontally high-voltage electrophoresis at pH 1·9, vertically, descending chromatography. Ref. reference amino acids. OX1 to OX 3, oxidized peptides.

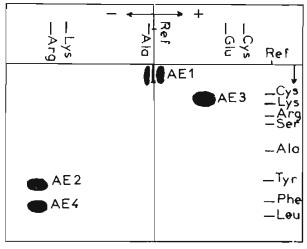


FIG. 4

Peptide Map of Tryptic Digest of Aminoethylated Peptide F 4

Horizontally, high-voltage electrophoresis at pH 5·6, vertically, descending chromatography. Ref. reference amino acids. AE 1 trough AE 4, peptides obtained.

The analysis of peptide OX 1 corresponds to the peptide derived from the C-terminus of the molecule, *i.e.*

$$\overset{57}{\text{Ile}}\text{-Cys-Glu-Pro-Pro-Gln-Gln-Thr-Asp-Lys-}\overset{67}{\text{Ser}}.$$

The analysis of peptide OX 2 corresponds to the N-terminal peptide

$$\overset{1}{\text{Phe}}\text{-Gln-Thr-Pro-Pro-Asp-Leu-Cys-Gln-Leu-Pro-Gln-Ala-}\overset{14}{\text{Arg}}.$$

The amino-acid composition of the acidic and neutral amino acids of peptide OX 3 shows one threonine residue less. The content of the basic amino acids was not determined. This peptide was obtained in a yield which was only 20% of the yield of peptide OX 2. It obviously represents the N-terminal peptide of another isoinhibitor. The investigation of the disulfide peptide from fraction 3 showed the disulfide bond between the half-cystine residue at positions 8 and 58.

*Fraction 4*

Only one peptide, F 4, was observed on the peptide maps of the oxidized and unoxidized sample of fraction 4. The amino-acid analysis (Table II), however, indicates the content of two half-cysteines. We have therefore concluded that the disulfide bond is present in one single peptide. From its amino-acid composition (Table II), peptide F 4 contains residues at positions 24 through 56. The peptide contains 2 half-cystines and 1 carboxymethyl-cysteine. It was necessary to determine their order. The peptide was cleaved by trypsin at the aminoethyl-cysteine residues. The digest was resolved by a combination of high-voltage electrophoresis at pH 5·6 and descending chromatography (Fig. 4). The peptide map was stained by 0·01% ninhydrin, the spots were cut out, the peptides eluted and analyzed (Table II). Peptide AE 1 was obtained in two spots which gave a positive test with the Pauly reagent. From its amino-acid composition it was identified as -Tyr-Phe-Tyr-Asx-Ser-$\overset{24}{}$-Thr-Ser-Asn$\overset{31}{}$. The fact that this peptide gave two spots whose eluates showed identical amino acid composition is most likely the result of the difference in the content of the sugar moiety bound probably to an aspartyl residue. The peptide does not contain AECys, and results from nonspecific cleavage at the asparagine residue. It is followed by peptide AE 2 -Ala-AECys$\overset{32}{}$. Peptide AE 3, also Pauly positive, contains carboxymethyl-cysteine in addition to aminoethyl-cysteine. Since it is a tryptic peptide, we assume that it is C-terminated by aminoethyl-cysteine. We identified this peptide as $\overset{33}{}$Glu-Pro-Phe-Thr-Tyr-Gly-Gly-CMCys$\overset{34}{}$-Gln-Gly-Asn-Asn-Asx-Asn-Phe-Glu-Thr-Thr-Glu-Met-AECys$\overset{41}{}$. Peptide AE 4 -Leu-Arg- is at the N-

terminus of the whole peptide. The investigation of fraction 4 showed a disulfide bond between the half-cystines at positions 33 and 54 and a carboxymethyl-cysteine residue at position 41.

*Fraction 6*

The major peptide on the map of fraction 6 showed both mobility at pH 1·9 and $R_F$ on descending chromatography the same as peptide -Gly-Pro-Cys-Lys (15, 17, 18) from the tryptic digest of oxidized or S-sulfonated inhibitor[1]. We focused our attention on the purification of this peptide. Fraction 6 was applied as a 20 cm long line to paper

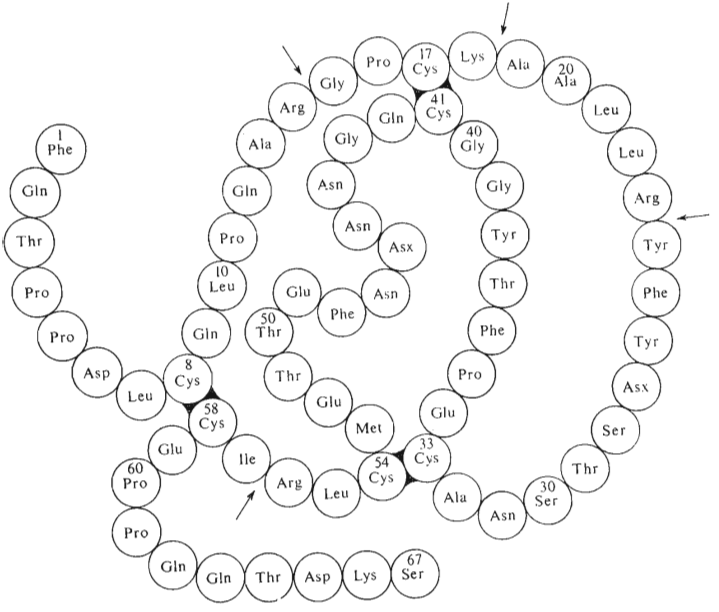


FIG. 5

Location of Disulfide Bonds in Molecule of Trypsin Inhibitor from Cow Colostrum

The arrows designate sites of tryptic cleavage of the partly reduced and carboxymethylated inhibitor.

and separated by high-voltage electrophoresis at pH 1·9. The band containing the peptide was cut out, sawn to a sheet of paper and subjected to descending chromatography. The peptide was eluted and its amino-acid composition determined (Gly 1·00; Pro 0·98; CMCys 0·90; Lys 1·04). The peptide was identified as -Gly-Pro-CMCys-Lys-$^{15}$ $^{17}$ $^{18}$ The location of the second carboxymethyl-cysteine residue in fraction 4 was determined. The third disulfide bond links together half-cystines at positions 17 and 41.

In this study we determined the positions of all disulfide bonds in the molecule of the trypsin inhibitor from cow colostrum (Fig. 5). With respect to their order in the molecule, they link half-cystines 1–6, 2–4 and 3–5. The same positions of disulfide bonds were found in the pancreatic trypsin inhibitor whose primary structure is more than to one third identical with the structure of the trypsin inhibitor from cow colostrum[11]. The identity of the disulfide bonds indicates also an identity in the three-dimensional structure of the two inhibitors. This is also evidenced by the fact that the same disulfide bond (2–4) in the colostrum trypsin inhibitor is preferentially reduced, as has been reported for the basic pancreatic trypsin inhibitor[4,12]. An explanation why exactly this bond is preferentially cleaved offers the three-dimensional structure of the pancreatic inhibitor[13]. Bond 2–4 is located on the surface of the molecule and thus exposed to the surrounding medium. The same is obviously true for the trypsin inhibitor from cow colostrum.

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