DISULFIDE BONDS OF BASIC ACROSIN INHIBITOR (BUSI II) FROM BULL SEMINAL PLASMA

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The thermolysin digest of the basic acrosin inhibitor (molecular weight 6 200) was resolved on a column of Sephadex G-15 and subsequently by electrophoretic and chromatographic techniques. Cysteine peptides, which link together half-cystine residues 7 and 39, 17 and 36, and 25 and 57 by disulfide bonds, were isolated. The structure of the molecule of the basic acrosin inhibitor corresponds to structures of inhibitors of the Kazal type. The amino acid sequence of a few residues in the basic acrosin inhibitor has been revised.

According to the present state of knowledge the inhibitors of serine proteinases can be divided into several groups with respect to their molecular weights, structural homologies, difference in the active centers, and with respect to the number and positions of half-cystine residues and the way in which the latter are linked by disulfide bonds[1].

Two acrosin inhibitors, the acidic inhibitor BUSI I and the basic inhibitor BUSI II, have been isolated from bull seminal plasma; these inhibitors differ in isoelectric points, size, amino acid composition, and inhibitory power[2]. The main function of the acrosin inhibitors present in the secretions and tissues of the male genital tract is a rapid blocking of prematurely activated acrosin. The basic acrosin inhibitor from bull seminal plasma is a low molecular weight protein of 6 200 whose isoelectric point lies at 10·5 and which is stable at pH 3. Inhibitor BUSI II in native state inhibits acrosin, trypsin, and chymotrypsin. It is inactive against kallikrein[3]. The molecule of BUSI II consists of 57 amino acid residues including 6 half-cystines[4] in positions 7, 17, 25, 36, 39, and 57. The basic acrosin inhibitor shows structural homology with porcine pancreatic trypsin inhibitor[5] which belongs to the class of proteinase inhibitors of Kazal type[6].

This paper completes the information on the structure of the molecule of BUSI II and deals with the determination of the positions of the disulfide bonds which are a criterion necessary for the classification of the inhibitors.

EXPERIMENTAL

Material and Methods

The basic acrosin inhibitor from bull seminal plasma was prepared as described in the preceding communication[2]. The enzymes used in this study and the preparation of the digest have been described elsewhere[7]. The material for amino acid analysis was hydrolyzed in 6 mol l^{-1} HCl at 110°C *in vacuo*. The analyses were carried out in Durrum D-500 amino acid analyzer. Cystine was determined as cysteic acid after oxidation of the peptides in performic acid[8]. The N-terminal amino acid sequences of the peptides were assayed by the double coupling method of Chang[9] using 4-N,N-dimethylaminoazobenzene 4′-isothiocyanate. The reagents used for the stepwise degradation were obtained from Pierce Eurochemie (Rotterdam, Holland), 4-Dimethylamino-azobenzene isothiocyanate (DABITC) was from Fluka (Buchs, Switzerland). The methods used for the separation of peptide mixtures, such as chromatography and electrophoresis on paper and cellulose thin layers, and the reagents for the detection of peptides, including the technical materials, have been described elsewhere[7].

Preparation and Treatment of Thermolysin Digest of Acrosin Inhibitor

The inhibitor (5·2 μmol; 32·4 mg) was dissolved in 3 ml of 0·2 mol l^{-1} pyridine adjusted to pH 6·5 by acetic acid and containing 2 mmol l^{-1} $CaCl_2$. Thermolysin (2·7 mg) was added and hydrolysis of the inhibitor was allowed to proceed 48 h at 37°C. The digest was lyophilized and resolved on a column of Sephadex G-15. The experimental data and the elution profile are shown in Fig. 1. Aliquots (50 μl) were removed from the individual fractions and applied to Whatman No 3MM chromatography paper; the chromatogram was developed in the system 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12, v/v). The cystine peptides were stained with a methanolic solution[10] of sodium nitroprusside and sodium cyanide. Selected fractions were pooled and marked A, B, and C. These fractions were subsequently resolved by paper electrophoresis or chromatography. The disulfide peptides were cleaved by oxidation in performic acid[11]; the oxidation of peptides containing histidine or tyrosine was carried out under milder conditions[12]. The pairs of cysteic acid peptides resulting from the oxidation of the individual cystine peptides were purified by thin-layer techniques on cellulose.

RESULTS AND DISCUSSION

The disulfide bonds of the basic acrosin inhibitor were determined by the analysis of peptides from the thermolysin digest of the native inhibitor. On the basis of the knowledge of the specificity of the enzyme it was reasonable to assume that the peptide chain of the inhibitor would be cleaved also between half-cystines 36 and 39 and that the digest would contain all cystine peptides necessary for the determination of the three disulfide bonds. Thermolysin moreover contains no half-cystine residues in its molecule and therefore peptides which might be derived from the autolysis of the enzyme cannot negatively affect the determination of cystine. It was shown in preliminary experiments that a part of the inhibitor remains uncleaved after 8 to 16 h of hydrolysis. The digestion with thermolysin was therefore prolonged to 48 h. The separation of the digest by gel filtration is illustrated by Fig. 1 which also shows

the pooling of fractions containing identical disulfide peptides. The amino acid composition of the peptides isolated from these fractions is given in Table I, together with the composition of peptides formed by their oxidation in performic acid.

Fraction A contained a smaller quantity of longer fragments of the inhibitor which were characterized only partly. The amino acid composition of fragment FA indicates that it represents a part of the inhibitor molecule in which one disulfide bridge only was cleaved. This fragment probably involves the part of the peptide chain between residues 16 and 31 linked through a disulfide bond to the region between residues 33 and 37 and through another disulfide bond also to the fragment containing residues 55 to 57. The system of numbering of the amino acid residues in the inhibitor molecule is shown in Fig. 2. Fraction A also contains the peptides which were formed by the autolysis of thermolysin; the latter contains three tryptophan residues which contribute to the relatively high absorbance value of this fraction.

Two cystine peptides, designated D1 and D3 were isolated from fraction B. When the N-terminal sequence of peptide 1 was examined two amino acid residues, valine and alanine, were found in the first degradation step and aspartic acid and phenylalanine in the second. This finding together with other data, including the amino acid analysis of peptide D1 and of its fragments D1ox1 and D1ox2 formed

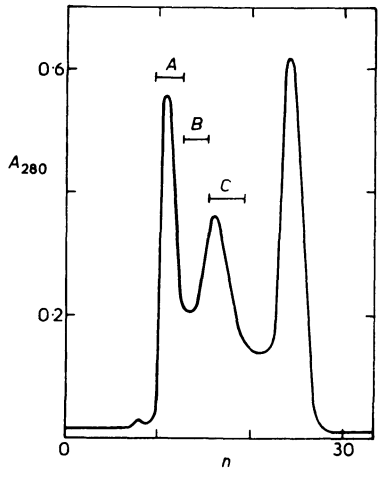
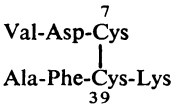


FIG. 1

Gel filtration of thermolysin digest of basic acrosin inhibitor (32·4 mg) on column of Sephadex G-15. The column (1·6 × 52 cm) was equilibrated with 0·01 mol l^{-1} NH_4HCO_3. Fractions 3·5 ml/10 min, *n* fraction number. The absorbance was measured at 280 nm. The effluents were pooled as shown by horizontal bars. *A*, *B*, and *C*, fractions containing cystine peptides

by oxidative cleavage, and the amino acid sequence of these fragments indicate that cystine peptide D1 consists of two chains.

Peptide D1:



The disulfide bond between half-cystines 7 and 39 holds together two parts of the inhibitor molecule involving residues 5 to 7 and 37 to 40. Cystine peptide D3 was also isolated from fraction B. A characteristic feature of its amino acid composition

TABLE I

Amino acid composition of peptides FA, Th1, cystine peptides (*D*), and products obtained by their oxidation (Dox). The Table shows the number of residues per mol of peptide. The values were not corrected for destruction or incomplete release. Half-cystine was determined as cysteic acid in oxidized samples

Designation of peptide	Residues	Amino acid analysis
FA	16—37 and 55—57	Ala 0·8, Asp 3·0, Arg 0·8, Cys 2·7, Glu 2·1, Gly 4·0, His 1·0, Lys 2·2, Pro 0·8, Ser 1·8, Thr 1·2
Th1	18—20	Arg 1·0, Glu 0·9, Thr 0·8
D1	5—7 and 37—40	Ala 0·9, Asp 1·1, Cys 2·2, Lys 1·0, Phe 1·0, Val 1·0
D1ox1	5—7	Asp 1·0, Cys 1·1, Val 1·0
D1ox2	37—40	Ala 0·9, Cys 1·1, Lys 1·0, Phe 1·0
D2	16—17 and 33—36	Asp 1·0, Cys 1·6, Gly 1·1, Lys 1·0, Tyr 0·7
D2ox1	16—17	Cys 1·0, Tyr 0·7
D2ox2	33—36	Asp 0·9, Cys 1·0, Gly 1·1, Lys 1·0
D3	21—25 and 55—57	Asp 1·3, Cys 1·7, Gly 1·0, His 0·8, Lys 0·7, Pro 1·0, Ser 1·0
D3ox1	21—25	Asp 1·0, Cys 1·1, His 0·9, Pro 1·0, Ser 0·9
D3ox2	55—57	Cys 1·1, Gly 1·0, Lys 1·0

is that it contains one of the two histidine residues present in the inhibitor molecule. When the N-terminal amino acid sequence of peptide D3 was examined, serine and glycine were found in the first degradation step, and asparagine and lysine in the second. After oxidation of peptide D3 fragments D3ox1, Ser-Asn-Pro-His-Cys, and D3ox2, Gly-Lys-Cys, were isolated. These data permit the structure of the parent disulfide peptide D3 to be determined.

Peptide D3:

$$\begin{array}{l} \overset{25}{\text{Ser-Asn-Pro-His-Cys}} \\ \qquad\qquad\qquad\quad | \\ \qquad\qquad\quad \underset{57}{\text{Gly-Lys-Cys}} \end{array}$$

Peptide D3 represents two regions of the inhibitor molecule linked together by a disulfide bond between half-cystines 25 and 57. Tripeptide Th1, Thr-Arg-Glu (residues 18–20) was isolated from fraction B in addition to the cystine peptides. This peptide, together with the knowledge of disulfide peptide D3 leads us to revise the order of residues 20–24 and the amino acid sequence Gly-Lys-Cys (55–57) incorrectly determined in earlier work[4]. The sequence Gly-Lys-Cys is in accordance with the data on the amino acid sequence of the acrosin inhibitor obtained by NMR spectra measurement[13].

Cystine peptide D2 was isolated from fraction C. The N-terminal amino acids of this peptide are tyrosine and glycine. After peptide D2 had been oxidized and the products resolved, dipeptide D20X1 Tyr-Cys and peptide D20X2, Gly-Asn-Lys-Cys, were isolated. These partial data permit the structure of disulfide peptide D2 to be derived as follows:

Peptide D2:

$$\begin{array}{l} \overset{17}{\text{Tyr-Cys}} \\ \qquad\quad | \\ \underset{36}{\text{Gly-Asn-Lys-Cys}} \end{array}$$

Disulfide peptide D2 determines the link between the two remaining half-cystine residues 17 and 36 thus permitting the complete covalent structure of the basic acrosin inhibitor to be derived as shown in Fig. 2.

Information necessary for a comparison of the three-dimensional structures of molecules of inhibitors of the Kunitz[14] and Kazal type has been obtained by X-ray diffraction analysis. The two inhibitor types differ in the spatial orientation of disulfide bonds and in the manner in which the individual half-cystine residues are linked one to another. The half-cystine residues are marked in the text which follows by Roman numerals according to the order of their occurrence in the primary structure of the inhibitors. The disulfide bond which links together half-cystines *II*

and *IV* in bovine basic trypsin inhibitor[15] (Kunitz type) is localized on the surface of the molecule. The remaining disulfide bonds are oriented to the center of the molecule. A characteristic feature of inhibitor molecules of the Kunitz type[14] are disulfide bonds[16] between half-cystines *I* and *VI*, *II* and *IV*, and *III* and *V*. The three-dimensional structure of Japanese quail ovomucoid[17] (3rd domain), which may serve as a model of inhibitors of the Kazal type, has no disulfide bond on the surface of the molecule. Inhibitors of this type[6] have disulfide bonds[18] linking together half-cystines *I* and *V*, *II* and *IV*, and *III* and *VI*.

It has been shown in this paper that the disulfide bonds of the basic acrosin inhibitor link together the half-cystine residues as follows: *I*–*V*, *II*–*IV*, and *II*–*VI*. Hence, according to the disulfide bonds the inhibitor can be classified as an inhibitor of the Kazal type. This conclusion is also evidenced by the finding that the basic acrosin

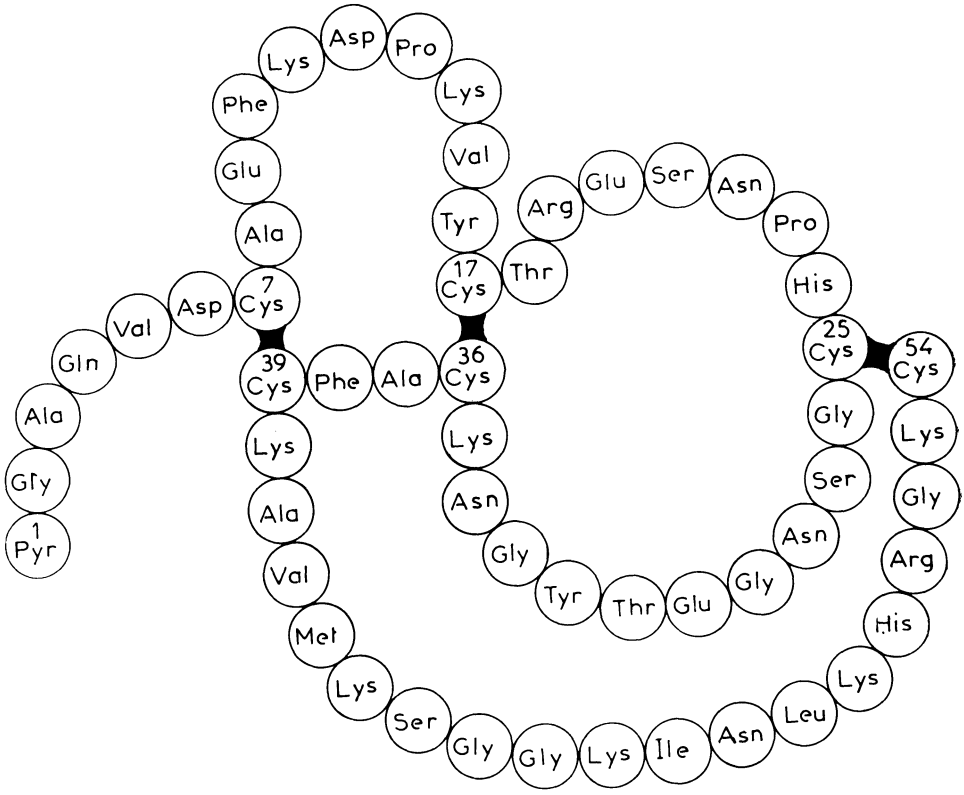


FIG. 2
Covalent structure of basic acrosin inhibitor from bull seminal plasma

inhibitor cannot undergo a partial reduction of its disulfide bonds by sodium borohydride (unpublished observations) which proceeds smoothly with the basic pancreatic trypsin inhibitor[19] and the cow colostrum inhibitor[20]. It may be therefore assumed that all the disulfide bonds of the basic acrosin inhibitor will also be oriented in its three-dimensional structure to the center of the molecule, as shown by X-ray diffraction analysis[17] of inhibitors of the Kazal type.

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