AMINO ACID SEQUENCE OF BASIC ACROSIN INHIBITOR FROM BULL SEMINAL PLASMA

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The molecule of the inhibitor contains 57 amino acid residues whose sequence is the following: Pyr-Gly-Ala-Gln-Val-Asp-Cys-Ala-Glu-Phe-Lys-Asp-Pro-Lys-Val-Tyr-Cys-Thr-Arg-His-Ser-Asp -Pro-Gln-Cys-Gly-Ser-Asn-Gly-Glu-Thr-Tyr-Gly-Asn-Lys-Cys-Ala-Phe-Cys-Lys-Ala-Val-Met- -Lys-Ser-Gly-Gly-Lys-Ile-Asn-Leu-Lys-His-Arg-Gly-Cys-Lys. The N-terminal group of the inhibitor is pyrrolidone carboxylic acid. The sequential data were obtained by analyses of peptides isolated from tryptic and chymotryptic digests of the oxidized or carboxymethylated inhibitor. The molecular weight of the inhibitor is 6200.

The seminal plasma of mammals contains protease inhibitors. They are the natural antagonists of acrosin, an acrosomal protease, which plays a role in the penetration of the sperm through the zona pellucida of the ovum[1] and in other fertilization processes taking place in ovular cytoplasm[2]. The main function of all acrosin inhibitors present in the secretions and tissues of the male genital tract is to quickly block the acrosin activated prematurely. Protease inhibitors of seminal plasma are also important agents in the protective mechanism of the organism since they neutralize the action of granulocyte proteases[3] liberated to a higher degree during inflammatory processes. The presence of low molecular weight protease inhibitors has been demonstrated in seminal plasma of a number of mammals[4]. All these inhibitors are proteins of relatively low molecular weight and are highly stable in acid media. As yet the amino acid composition of inhibitors from seminal plasma of man[5], boar[6], guinea pig[6], and bull[7] has been determined. The structural data available so far are the complete covalent structure of the inhibitor from boar seminal plasma[8] and the partial amino acid sequence of the inhibitor from guinea pig[9]. Three low molecular weight inhibitors[10] were isolated from bull seminal plasma: two isoinhibitors of acidic character of molecular weight about 8700 and one basic inhibitor of molecular weight about 6800. Their molecular weights were determined by gel filtration[11].

This paper describes the elucidation of the complete amino acid sequence of the basic acrosin inhibitor from bull seminal plasma based on the analysis of the complete tryptic digest of the oxidized inhibitor, the limited tryptic digestion of the carboxymethylated and citraconylated inhibitor, and on the analysis of the chymotryptic digest of the oxidized inhibitor.

EXPERIMENTAL

Material

The acrosin inhibitor from bull seminal plasma was prepared as described in the preceding paper[7]. Trypsin, whose chymotryptic activity had been blocked by the reaction with chloro--[N-*p*-tosyl-L-phenylalanyl]methane[12], and chymotrypsin were from Worthington Biochemical Corp., Freehold, N. J., U.S.A. Thermolysin was a B-grade product of Calbiochem, Los Angeles, Cal., U.S.A. Subtilisin and iodoacetic acid were purchased from Koch and Light, Colnbrook, England. 2-Mercaptoethanol was from Fluka, Buchs, Switzerland and citraconic anhydride from Aldrich Europe, Beerse, Belgium. The reagents used for the thiazolinone degradation technique were commercial products purified as recommended by Edman and Henschen[13]. The remaining chemicals were commercial preparations of highest purity available. Sephadex G-25 (fine) and G-50 (superfine) were products of Pharmacia, Uppsala, Sweden. Silica gel coated aluminum sheets (Silufol), used for the identification of amino acid phenylthiohydantoins, were from Kavalier, Sázava, Czechoslovakia. Polyamide layer sheets, used for the identification of the dansyl derivatives of amino acids, were purchased from BDH Chemicals Ltd., Poole, England. Paper Whatman No 3MM paper for chromatography was a product of Whatman Biochemicals Ltd., Maidstone, England.

Methods

The cyanogen bromide cleavage of peptides was carried out according to Gross and Witkop[14]; the quantity of the reagent used was twice the weight of the peptide and the cleavage was allowed to proceed at 0°C for 48 h. Enzymic cleavage of 1% solution of the peptides by chymotrypsin, subtilisin, or thermolysin was carried out in 0·1M-NH_4HCO_3 at 37°C and a molar enzyme to substrate ratio of 1 : 40 for 4—6 h. The buffer used for thermolytic cleavage contained 1 mM-$CaCl_2$. The digestion was discontinued by lyophilization of the samples. Small peptides contained in the enzymic digests were purified on paper Whatman No 3MM by procedures S1—S3: descending electrophoresis[15] at pH 5·6 in the buffer water–pyridine–acetic acid (994 : 5 : 1, v/v) at a potential gradient of 30 V/cm (procedure S1), horizontal high voltage electrophoresis[16] at pH 1·9 in the system water–acetic acid–formic acid(16 : 3 : 1, v/v) at a potential gradient of 85 V/cm (procedure S2), and chromatography[17] in the system n-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12, v/v) (procedure S3). For the preparation of peptide maps a part of the enzymic digest (0·3 μmol) was applied to chromatographic paper and subjected to electrophoresis (procedure S1) in the first direction and to chromatography (procedure S3) in the second direction. The peptides were stained with 0·2% solution of ninhydrin in acetone and subsequently detected by the chlorination procedure and staining with the tolidine-iodide reagent[18]. The peptides were eluted by the buffer used for procedure S1. Amino acid analyses were carried out by the method of Spackman and coworkers[19] as modified by Benson and Patterson[20] in an amino acid analyzer of Czechoslovak make (Instrument Development Workshops, Cezchoslovak Academy of Sciences, Prague). The values given are not corrected. Half-cystine was determined as cysteic acid or carboxymethyl--cysteine. Homoserine was converted into homoserine lactone[21] before the analysis. The manual thiazoline degradation method[13] was carried out according to Blombäck and coworkers[22] with 0·2—1 μmol of peptide. Amino acid phenylthiohydantoins obtained by the conversion of the thiazolinones were identified by thin-layer chromatography on Silufol sheets in the following systems[23–25]: acetic acid-ethylene chloride (4 : 1), xylene–acetone (5 : 1), and chloroform–acetone (50 : 3). The phenylthiohydantoins were detected by iodine vapors and ammonia[23]. Alternatively, the peptides were degraded by the dansyl-Edman technique according to Gray and Smith[26]. For the determination of the N-terminal amino acid of large peptides by the dansyl

technique the procedure of Zanetta and coworkers[27] was used. The dansyl-amino acids were identified in all cases by thin-layer chromatography on polyamide layers[28] by the technique introduced by Hartley[29] and using additional solvent systems reported by Brosius[30]. The following systems were used: 1·5% formic acid, toluene–acetic acid (10 : 1), ethyl acetate–methanol–acetic acid (20 : 1 : 1), 50 mM-Na_3PO_4–ethanol (3 : 1), 1M-NH_4OH–ethanol (1 : 1), 0·15M-NH_4OH, and n-heptane–1-butanol–formic acid (10 : 10 : 1). Amino acid amides were determined by direct identification of amino acid phenylthiohydantoins and the results checked by determination of the electrophoretic[31] mobility of the peptides in the buffer water–pyridine–acetic acid (895 : 100 : 5, v/v) at pH 6·5.

Reduction and carboxymethylation of inhibitor. The protein (30 mg) was reduced by β-mercaptoethanol and the cysteine residues were carboxymethylated by the method of Crestfield and coworkers[32]. The S-carboxymethyl derivative of the protein was separated from the reaction products by gel filtration on a column (4·1 × 25 cm) of Sephadex G-25, equilibrated with 20mM-NH_4OH. The protein-containing fraction was lyophilized.

Citraconylation of the amino groups of the inhibitor. The protein (30 mg) was dissolved in 5 ml of 0·1M sodium pyrophosphate and treated with stirring with four portions (total volume 200 µl) of citraconic anhydride[33]. The reaction was carried out at 22°C and the pH was kept constant at 8·5 by titration with 4M-NaOH. At the end of the reaction the citraconylated inhibitor was separated from the reaction products on a column of Sephadex G-25 (4 × 25 cm) equilibrated with 0·15M-NH_4HCO_3.

Limited tryptic digestion. The carboxymethylated and citraconylated inhibitor (*c.* 30 mg) was digested by trypsin immediately after separation of reaction products by gel filtration in 0·15M-NH_4HCO_3. Trypsin (0·6 mg) was added to the solution of the modified protein and the mixture was incubated 1 h at 50°C. The digest was lyophilized, dissolved in 3 ml of water and again

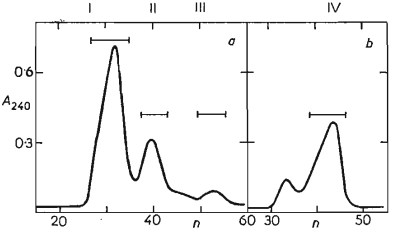

FIG. 1

Gel Filtration of Limited Tryptic Digest of S-Carboxymethylated and Decitraconylated Inhibitor

a First fractionation. Column of Sephadex G-25, *b* rechromatography of pooled fraction I. Column of Sephadex G-50. The columns (155 × 1·6 cm) were eluted by 50 mM-HCOOH. Fractions 4·3 ml/12 min, *n* number of fractions. The effluent was pooled as shown by horizontal bars.

lyophilized. To remove the citraconyl residues the lyophilized product was dissolved in 3 ml of 50% formic acid and set aside for 16 h at 23°C. The mixture of decitraconylated peptides was separated on a column of Sephadex G-25 under the conditions described in the legend to Fig. 1*a*. The mixture of large peptides contained in the first portion of the effluent was separated on a column of Sephadex G-50 under identical conditions. The course of the separation is shown in Fig. 1*b*.

Oxidation of inhibitor. The inhibitor (80 mg) was oxidized by performic acid[34] for 2·5 h at —7°C. After this period the action of performic acid was discontinued by stepwise addition of saturated sodium sulfite solution with cooling (temperature of reaction mixture +5°C). The removal of performic acid was checked by the reaction with a solution of tolidine and sodium iodide[18] which was negative. The solution of the oxidized inhibitor was freed of the reaction products by gel filtration on a column of Sephadex G-25 (4·1 × 25 cm) in 0·1M formic acid; the protein-containing fraction was lyophilized.

Complete tryptic digest of the oxidized inhibitor. The material (60 mg) was dissolved in 6 ml of 0·1M-NH_4HCO_3 and digested 2 h at 37°C with 0·75 mg of trypsin. Subsequently another 0·75 mg portion of trypsin was added and the digestion was continued for 2 additional hours. The digest was lyophilized, dissolved in 5 ml of water and again lyophilized. Individual peptides were isolated by electrophoretic and chromatographic procedures.

Chymotryptic digest of oxidized inhibitor. The protein (18 mg) was dissolved in 2 ml of 0·2M-NH_4HCO_3, 0·4 mg of chymotrypsin was added, and the digestion was allowed to proceed 6 h at 37°C. The digest was treated in the same manner as the tryptic digest.

Liberation of the blocked α-amino group of N-terminal tryptic peptide. The modified procedure developed by Blombäck and Doolittle[35] was used. Peptide T1 (0·5 μmol) was dissolved in 100 μl of 1M-NaOH and the solution was maintained 24 h at 23°C. After this period the reaction mixture was saturated with CO_2 to pH 5·6 and the peptide with the free amino group separated from the original peptide which did not react (*c.* 70%) by electrophoresis in system S1.

RESULTS AND DISCUSSION

In an earlier report[7] the amino acid composition of the basic inhibitor from bull seminal plasma was presented and evidence was adduced showing that the α-amino group of the inhibitor is blocked. The primary structure of the inhibitor was elucidated by analyses of peptides derived from its complete tryptic digest (T), limited tryptic digest (LT), and chymotryptic digest (C). The alignment of the sequential regions permitted us to propose the primary structure of the inhibitor shown in Fig. 2. A separate problem represented the elucidation of the N-terminal segment of the inhibitor which was found in the enzymic digest and identified in the form of peptides with a blocked α-amino group.

The sequential studies were based on the analysis of the complete tryptic digest of the oxidized inhibitor. The peptides contained in this digest were relatively well distributed all over the peptide map (Fig. 3) and were isolated by one-dimensional techniques in the systems described. The amino acid composition of these peptides is given in Table I and the results of their sequential analysis in Table II. One peptide only with blocked N-terminal group (T1) was isolated from the tryptic digest. This peptide

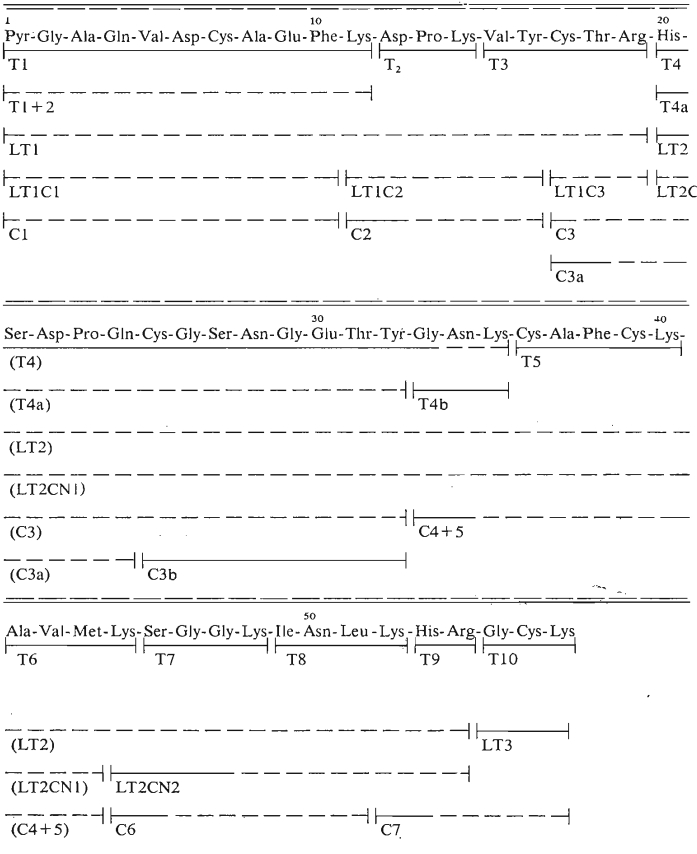

FIG. 2

Amino Acid Sequence of Basic Acrosin Inhibitor

The peptides are marked by horizontal bars, sequences determined in full by solid lines and sequences not determined by dashed lines. The symbols designating the peptides are explained in the legend to Table I and III.

was not stained with ninhydrin and could be detected by chlorination only. The N-terminal amino group of peptide T1 was reactivated by conversion using sodium hydroxide under the conditions sufficient for the transformation of pyrrolidone carboxylic acid to glutamic acid[35]. The amino acid sequence Glu-Gly-Ala- was established by the dansyl-Edman procedure. This result permits us to conclude that pyrrolidone carboxylic acid is the N-terminal residue of peptide T1. We may extrapolate at the same time that the N-terminal sequence of the inhibitor is Pyr-Gly--Ala. Acetyl or formyl groups which also could block the N-terminal α-amino

TABLE I

Amino Acid Composition and Yields of Peptides Isolated from Complete Tryptic (T) Digest of Oxidized Inhibitor and Fragments Arising from Additional Thermolytic (Th) or Subtilisin (S) Cleavage

The position designates serial numbers of amino acid residues in the inhibitor molecule of which the peptide consists.

Designation of peptide	Position	Yield %	Number of amino acid residues
T1	1—11	28	$Cys_{1.0}$, $Asp_{1.0}$, $Glu_{2.6}$, $Gly_{0.9}$, $Ala_{1.8}$, $Val_{0.9}$, $Phe_{1.0}$, $Lys_{1.0}$
T1Th1	1— 4	38	$Glu_{2.0}$, $Gly_{1.1}$, $Ala_{1.0}$
T1Th2	5— 9	55	$Cys_{0.8}$, $Asp_{0.8}$, $Glu_{1.0}$, $Ala_{0.8}$, $Val_{0.8}$
T1Th3	10—11	55	$Phe_{1.0}$, $Lys_{1.1}$
T1S1	1— 4	62	$Glu_{2.0}$, $Gly_{1.0}$, $Ala_{1.0}$
T1S2	5— 8	50	$Cys_{1.0}$, $Asp_{1.0}$, $Ala_{1.0}$, $Val_{1.2}$,
T1S3	9—11	38	$Glu_{1.0}$, $Phe_{1.0}$, $Lys_{1.0}$
T2	12—14	32	$Asp_{1.0}$, $Pro_{1.0}$, $Lys_{1.1}$
T1+2	1—14	10	$Cys_{1.0}$, $Asp_{2.0}$, $Glu_{2.6}$, $Pro_{0.8}$, $Gly_{0.9}$, $Ala_{1.8}$, $Val_{0.9}$, $Phe_{1.0}$, $Lys_{1.7}$
T3	15—19	45	$Cys_{1.2}$, $Thr_{1.1}$, $Val_{1.0}$, $Tyr_{0.7}$, $Arg_{0.9}$
T4	20—35	46	$Cys_{1.0}$, $Asp_{2.9}$, $Thr_{0.9}$, $Ser_{1.8}$, $Glu_{2.0}$, $Pro_{0.8}$, $Gly_{3.0}$, $Tyr_{0.8}$, $His_{0.9}$, $Lys_{1.0}$
T4a	20—32	13	$Cys_{1.0}$, $Asp_{2.0}$, $Thr_{0.9}$, $Ser_{1.8}$, $Glu_{2.0}$, $Pro_{0.8}$, $Gly_{2.1}$, $Tyr_{0.7}$, $His_{0.8}$
T4b	33—35	10	$Asp_{1.0}$, $Gly_{1.0}$, $Lys_{0.9}$
T5	36—40	38	$Cys_{2.0}$, $Ala_{1.0}$, $Phe_{0.9}$, $Lys_{1.0}$
T6	41—44	42	$Met_{1.0}$, $Ala_{1.0}$, $Val_{1.0}$, $Lys_{1.2}$
T7	45—48	46	$Ser_{0.9}$, $Gly_{1.9}$, $Lys_{1.0}$
T8	49—52	57	$Asp_{1.0}$, $Ile_{1.0}$, $Leu_{1.1}$, $Lys_{0.8}$
T9	53—54	59	$His_{1.0}$, $Arg_{1.0}$
T10	55—57	57	$Cys_{1.0}$, $Gly_{1.0}$, $Lys_{1.0}$

group are sufficiently stable under these conditions of cleavage. Peptide T1 was digested with subtilisin and thermolysin. The amino acid composition of peptides T1S1-T1S3 and T1Th1-T1Th3, resulting from this digestion, is also given in Table I. The N-terminal group of peptide T1S1 was set free under the conditions described above and the peptide was then sequenced. The distribution of amino acid amides in all peptides was also deduced from their net charge. The partial results obtained

TABLE II

Amino Acid Sequence and Net Charge of Peptides Isolated from the Complete Tryptic (T) and Chymotryptic (C) Digest of Oxidized Inhibitor, Including Data on Subtilisin (S) and Thermolysin (Th) Fragments of the Peptides

Designation of peptide	Net charge	Amino acid sequence
T1	−3	Pyr-Gly-Ala(Glx_2,Val,Asx_2,Cys,Ala,Phe,Lys)[a]
T1S1	−1	Pyr-Gly-Ala-Gln
T1S2	−2	Val-Asp-Cys-Ala
T1S3	0	Glu-Phe-Lys
T1Th1	−1	(Pyr,Gly,Ala,Gln)
T1Th2	−3	(Val,Asx,Cys,Ala,Glx)
T1Th3	+1	Phe-Lys
T2	0	Asp-Pro-Lys
T1+2	−3	(Pyr,Glx_2,Gly,Ala_2,Val,Asx_2,Cys,Phe,Pro,Lys_2)
T3	0	Val-Tyr-Cys-Thr-Arg
T4	−1	His-Ser-Asp-Pro-Gln-Cys-Gly-Ser-Asn-Gly-Glu-Thr-Tyr-Gly(Asx,Lys)
T4a	−2	His(Ser_2,Asx_2,Pro,Glx_2,Cys,Gly_2,Thr,Tyr)
T4b	+1	Gly-Asn-Lys
T5	−1	Cys-Ala-Phe-Cys-Lys
T6	+1	Ala-Val-Met-Lys
T7	+1	Ser-Gly-Gly-Lys
T8	+1	Ile-Asn-Leu-Lys
T9	+2	His-Arg
T10	0	Gly-Cys-Lys
C1	−4	(Pyr,Glx_2,Gly,Ala_2,Cys,Val,Asx,Phe)
C2	+1	Lys-Asp(Pro,Lys,Val,Tyr)
C3	−2	Cys(Asx_2,Thr_2,Ser_2,Glx_2,Pro,Gly_2,Cys,His,Arg,Tyr)
C3a	0	Cys-Thr(Asx,Ser,Pro,His,Arg,Glx)
C3b	−2	Cys-Gly-Ser-Asn-Gly-Glu-Thr-Tyr
C4+5	0	Gly-Asn(Ala_2,Cys_2,Val,Phe,Lys_2,Met)
C6	+2	Lys-Ser(Gly_2,Ile,Asx_2,Lys,Leu)
C7	n.d.[b]	Lys-His(Arg,Gly,Cys,Lys)

[a] Pyr stands for pyrrolidone carboxylic acid; [b] not determined.

by the analysis of the subtilisin and thermolysin digest of peptide T1, permit us to derive its amino acid sequence as follows:

T1	Pyr-Gly-Ala(Glx,Val,Asx,Cys,Ala,Glx,Phe,Lys)
T1S1	Pyr-Gly-Ala-Gln
T1S2	Val-Asp-Cys-Ala
T1S3	Glu-Phe-Lys
T1Th1	(Pyr,Gly,Ala,Gln)
T1Th2	(Val,Asx,Cys,Ala,Glx)
T1Th3	Phe-Lys
T1	Pyr-Gly-Ala-Gln-Val-Asp-Cys-Ala-Glu-Phe-Lys

All peptides (T1 – T10), which were expected to result from tryptic cleavage of the inhibitor with respect to its amino acid composition and to the specificity of trypsin, were isolated from the tryptic digest and sequentially characterized. An exception is peptide T1+2, in which the bond -Lys-Asp- (residues 11 and 12) was cleaved incompletely, and peptides T4a and T4b arising from the cleavage of the bond -Tyr-Gly- (residues 32 and 33). In view of the presence of peptide bonds hydrolyzed with difficulties the amino acid analysis of peptide T8 was checked with the 90h hydrolysate of the peptide. The complete amino acid sequence of all tryptic peptides was determined.

Additional information on the structure of the inhibitor molecule provided the analysis of the limited tryptic digest of the carboxymethylated and decitraconylated inhibitor. The fragments formed were isolated by gel filtration on Sephadex G-25 (Fig. 1*a*). Fraction I contains a mixture of fragment LT2 and uncleaved inhibitor. Fragment LT1 only is present in fraction II; fraction III contains fragment LT3. Fraction I was subjected to gel filtration on Sephadex G-50 as shown in Fig. 1*b* and afforded fragment LT2, contained in fraction IV. The data on the amino acid composition of these fragments and on peptides obtained by their additional degradation are given in Table III. In accordance with the content of two arginine residues in the inhibitor molecule the limited tryptic digest contained three fragments (LT1 to LT3) which account for the whole molecule of the inhibitor. Fragment LT1 is lacking a free α-amino group and represents the N-terminal 19-residue region of the inhibitor molecule. Fragment LT1 was subsequently digested with chymotrypsin to three peptides (LT1C1 – LT1C3) whose characteristics are shown in Fig. 4. The properties of the N-terminal peptide LT1C1 (Pyr,Gly,Ala,Glx,Val,Asx,Cys,Ala,Glx,Phe) are similar to those of peptide T1, except that peptide LT1C1 is by one lysine residue longer. Peptide LT1C2 represents the middle part of fragment LT1. Peptide LT1C3

TABLE III

Amino Acid Composition and Yields of Peptides Isolated from Limited Tryptic (LT) Digest of Carboxymethylated and Citraconylated Inhibitor, Including Data on Chymotryptic (C) of Cyanogen Bromide (CN) Fragments of the Peptides

The position designates serial number of amino acid residues in the inhibitor molecule of which the peptide consists.

Designation of peptide	Position	Yield %	Number of amino acid residues
LT1	1—19	50	$Asp_{2.1}$, $Thr_{0.7}$, $Glu_{2.7}$, $Pro_{1.0}$, $Gly_{1.3}$, $Ala_{1.8}$, $Val_{1.6}$, $Tyr_{0.7}$, $Phe_{0.9}$, $Cys_{1.8}$, $Lys_{2.1}$, $Arg_{0.8}$
LT1C1	1—10	23	$Asp_{1.0}$, $Cys_{1.2}$, $Glu_{2.8}$, $Gly_{1.0}$, $Ala_{2.0}$, $Val_{0.9}$, $Phe_{1.0}$
LT1C2	11—16	28	$Asp_{1.0}$, $Pro_{1.0}$, $Val_{1.0}$, $Tyr_{0.9}$, $Lys_{1.6}$
LT1C3	17—19	26	$Cys_{0.8}$, $Thr_{0.9}$, $Arg_{1.0}$
LT2	20—54	52	$Asp_{3.7}$, $Thr_{1.0}$, $Ser_{2.5}$, $Glu_{2.0}$, $Pro_{0.9}$, $Gly_{4.6}$, $Ala_{1.9}$, $Val_{1.1}$, $Met_{0.9}$, $Ile_{0.9}$, $Leu_{0.9}$, $Tyr_{0.9}$, $Phe_{1.0}$, $Cys_{3.1}$, $Lys_{5.2}$, $His_{1.9}$, $Arg_{1.1}$
LT2CN1	20—43	37	$Asp_{3.0}$, $Thr_{1.0}$, $Ser_{1.8}$, $Glu_{2.1}$, $Pro_{1.1}$, $Gly_{3.0}$, $Ala_{2.0}$, $Val_{1.0}$, $Tyr_{0.8}$, $Phe_{1.0}$, $Cys_{2.7}$, $Lys_{1.6}$, $His_{0.8}$, $Hse_{0.8}$ [a]
LT2CN2	44—54	27	$Asp_{1.1}$, $Ser_{1.0}$, $Gly_{2.1}$, $Ile_{0.9}$, $Leu_{1.1}$, $Lys_{2.9}$, $His_{0.9}$, $Arg_{0.9}$
LT3	55—57	69	$Gly_{1.1}$, $Cys_{0.9}$, $Lys_{1.0}$

[a] Hse stands for homoserine.

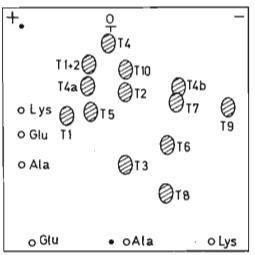

FIG. 3

Peptide Map of Tryptic Digest of Oxidized Inhibitor

First direction, electrophoresis at pH 5·6 (horizontally), second direction, descending electrophoresis (vertically). o origin, • reference mixture of amino acids (Lys,Glu,Ala).

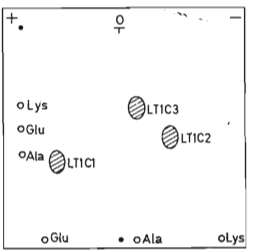

FIG. 4

Peptide Map of Chymotryptic Digest of Fragment LT1 Obtained by Limited Tryptin Digestion of the Inhibitor

See legend to Fig. 3 for details.

containing arginine provides information on the C-terminal region of fragment LT1. Fragment LT2 comprising the middle part of the inhibitor molecule (residues 20–54), is N-terminated by histidine and C-terminated by arginine. Fragment LT2 contains one methionine residue and was therefore cleaved by cyanogen bromide; peptides LT2CN1 and LT2CN2 were obtained by electrophoresis in system S1. The mobility of peptide LT2CN1 is m = −0·07 in terms of the mobility of aspartic

TABLE IV

Amino Acid Composition and Yields of Peptides Isolated from the Chymotryptic (C) Digest of the Oxidized Inhibitor

The position designates serial numbers of amino acid residues in the inhibitor molecule of which the peptide consist.

Designation of peptide	Position	Yield %	Number of amino acid residues
C1	1—10	30	$Asp_{1.0}$, $Cys_{1.1}$, $Glu_{2.7}$, $Gly_{1.0}$, $Ala_{1.9}$, $Val_{1.0}$, $Phe_{1.1}$
C2	11—16	39	$Lys_{2.0}$, $Asp_{1.1}$, $Pro_{1.0}$, $Val_{1.0}$, $Tyr_{0.9}$
C3	17—32	12	$His_{0.8}$, $Arg_{0.8}$, $Cys_{2.1}$, $Asp_{2.0}$, $Thr_{1.8}$, $Ser_{2.0}$, $Glu_{2.0}$, $Pro_{1.0}$, $Gly_{2.0}$, $Tyr_{0.8}$
C3a	17—24	21	$His_{1.0}$, $Arg_{1.0}$, $Cys_{1.0}$, $Asp_{1.0}$, $Thr_{0.9}$, $Ser_{1.0}$, $Glu_{1.0}$, $Tyr_{0.8}$
C3b	25—32	21	$Cys_{0.9}$, $Asp_{1.1}$, $Thr_{1.0}$, $Ser_{1.0}$, $Glu_{1.0}$, $Gly_{2.0}$, $Tyr_{0.8}$
C4+5	33—43	48	$Lys_{1.8}$, $Cys_{2.1}$, $Asp_{1.0}$, $Met_{0.8}$, $Gly_{1.0}$, $Ala_{2.0}$, $Val_{1.0}$, $Phe_{0.9}$
C6	44—51	34	$Lys_{2.0}$, $Asp_{1.0}$, $Ser_{0.9}$, $Gly_{2.0}$, $Ile_{1.0}$, $Leu_{1.1}$
C7	52—57	34	$Lys_{2.2}$, $His_{0.9}$, $Arg_{1.0}$, $Cys_{1.0}$, $Gly_{1.0}$

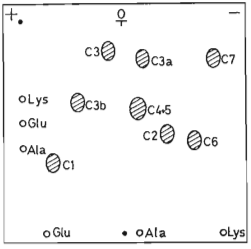

FIG. 5

Peptide Map of Chymotryptic Digest of Oxidized Inhibitor

See legend to Fig. 3 for details.

acid and the mobility of peptide LT2CN2, based on the mobility of lysine, is m = +1·12. The partial amino acid sequence of peptide LT2CN2, determined by the dansyl-Edman procedure, was Lys-Ser-Gly-Gly(Lys_2,Ile,Asx,Leu,His)Arg. The partial amino acid sequence of peptide LT2CN2 provides information on the order of tryptic peptides T7 and T8. One more fragment, LT3, Gly-Cys-Lys, containing residues 55–57 of the inhibitor, was isolated from the limited tryptic digest; this fragment does not contain arginine and represents the C-terminal region of the inhibitor.

The chymotryptic digest of the oxidized inhibitor was treated in the same manner as the tryptic digest. The peptide map of this digest and the designation of the peptides are shown in Fig. 5. Peptides C1–C7 were isolated whose amino acid composition is given in Table IV and partial amino acid sequences in Table II. Peptide C1, isolated from the chymotryptic digest, had its N-terminal α-amino group blocked; the amino acid composition and the electrophoretic properties of the peptide are identical with those of peptide LT1C1. All bonds regarded as sensitive to chymotrypsin were cleaved; additional cleavage of the bond at the carboxyl side of Gln (24) and Met (43) was also observed. By contrast, the bond between Phe (38) and Cys (39) was not cleaved. Peptides C3a and C8, whose sequences were partly characterized and which contain arginine residues, confirm the order of all three fragments (LT1–LT3) isolated from the limited tryptic digest of the inhibitor. The peptides isolated from the chymotryptic digest of the oxidized inhibitor afforded data permitting sequential regions, determined by the analysis of the tryptic digest, to be linked to one another unambiguously.

The derived primary structure of the acrosin inhibitor, consisting of 57 amino acid residues, is in perfect agreement with its amino acid analysis[7] and permits us to determine the exact molecular weight of the inhibitor. The original value of 6800 found by gel filtration should be corrected in view of the present data to 6200.

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REFERENCES

1. McRorie R. A., Williams W. L.: Ann. Rev. Biochem. *43*, 777 (1974).
2. Schiessler H., Arnold M., Ohlsson K., Fritz H.: Hoppe-Seyler's Z. Physiol. Chem. *357*, 1251 (1976).
3. Marushige Y., Marushige K.: Biochim. Biophys. Acta *519*, 1 (1978).
4. Haendle H., Fritz H., Trautschold I., Werle E.: Hoppe-Seyler's Z. Physiol. Chem. *343*, 185 (1965).
5. Schiessler H., Arnhold M., Fritz H. in the book: *Proteinase Inhibitors* (H. Fritz, H. Tschesche, L. J. Greene, E. Truscheit, Eds), 2nd Int. Res. Conf., Bayer Symp. V, p. 344. Springer Verlag Berlin, Heidelberg, New York 1974.

6. Fink E., Klein F., Hammer G., Müller-Bardorff G., Fritz H. in the book: *Proceedings of the International Research Conference on Proteinase Inhibitors* (H. Fritz, H. Tschesche, Eds), p. 225. Walter de Gruyter, Berlin, New York 1971.
7. Čechová D., Jonáková V., Sedláková E., Mach O.: Unpublished results.
8. Tschesche H., Kupfer S., Klauser R., Fink E., Fritz H. in the book: *Protides of the Biological Fluids* (H. Peeters, Ed.), 23rd Colloquium, p. 255. Pergamon Press, Oxford, New York 1976.
9. Fink E.: *Thesis*. Munich University, 1970. Cited from Tschesche H., Kupfer S., Klauser R., Fink E., Fritz H. in the book: *Protides of the Biological Fluids* (H. Peeters, Ed.), 23rd Colloquium, p. 255. Pergamon Press, Oxford, New York 1976.
10. Čechová D., Jonáková V., Sedláková E.: 12th FEBS Meeting, Dresden 1978, Abstr. No 2806.
11. Čechová D., Fritz H.: Hoppe-Seyler's Z. Physiol. Chem. *357*, 401 (1976).
12. Kostka V., Carpenter F. H.: J. Biol. Chem. *239*, 1799 (1964).
13. Edman P., Henschen A. in the book: *Protein Sequence Determination* (S. B. Needleman, Ed.), p. 232. Springer Verlag, Berlin, Heidelberg, New York 1975.
14. Gross E., Witkop B.: J. Biol. Chem. *237*, 1856 (1962).
15. Mikeš O.: This Journal *22*, 831 (1957).
16. Prusík Z., Štěpánek J.: J. Chromatogr. *87*, 73 (1973).
17. Walley S. G., Watson J.: Biochem. J. *55*, 328 (1953).
18. Reindel F., Hoppe W.: Ber. Deut. Chem. Ges. *87*, 1103 (1954).
19. Spackman D. H., Stein W. H., Morre S.: Anal. Chem. *30*, 1190 (1958).
20. Benson J. V. jr, Patterson J. A.: Anal. Chem. *37*, 1108 (1965).
21. Ambler R. P.: Biochem. J. *96*, 32p (1965).
22. Blombäck B., Blombäck M., Edman P., Hessel B.: Biochem. Biophys. Acta *115*, 371 (1966).
23. Grüner K.: Chem. Listy *64*, 1160 (1970).
24. Edman P., Sjöquist J. S.: Acta Chem. Scand. *10*, 1507 (1956).
25. Grüner K.: Personal communication.
26. Gray W. R., Smith J. F.: Anal. Biochem. *33*, 36 (1970).
27. Zanetta J. P., Vincendon G., Mandel P., Gombos G.: J. Chromatogr. *51*, 441 (1970).
28. Woods K. R., Wang K. T.: Biochim. Biophys. Acta *133*, 369 (1967).
29. Hartley B. S.: Biochem. J. *119*, 805 (1970).
30. Brosius J.: Biochemistry *17*, 501 (1978).
31. Offord R. E.: Nature (London) *211*, 591 (1966).
32. Crestfield A. M., Moore S., Stein W. H.: J. Biol. Chem. *238*, 622 (1963).
33. Dixon H. B. F., Perham R. N : Biochem J. *109*, 312 (1968).
34. Hirs C. H. W.: J. Biol. Chem. *219*, 611 (1956).
35. Blombäck B., Doolittle R. F.: Acta Chem. Scand. *17*, 1816 and 1819 (1963).

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