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PROTAMINES

A STUDY OF THE AMINO-ACID SEQUENCE OF STURINE B

L. K. Evseenko, E. P. Yulikova, and A. B. Silaev

UDC 547.962.1.05.543.544.6

We have previously described the isolation of sturine B and the determination of its amino-acid composition and of the N- and C-terminal amino acids [1]. In the present paper we consider the hydrolysis of sturine B by trupsin, the fractionation of the mixture of peptides obtained, and the determination of their structure, and also the study of the N- and C-terminal sequences of sturine B with the aid of leucine aminopeptidase and carboxypeptidases A and B.

The investigations of the primary structure of a series of protamines have shown that in their molecules the neutral amino acids are arranged in pairs or even in triplets [2]. Thus is particularly characteristic for the hydroxy amino acids serine and threonine.

Sturine B contains the 18-19 arginine residues, one histidine residue and a fairly limited set of neutral amino acids: Ala_1, Gly_2, Ser_2, Pro_1, Glu_1. To determine their positions in the molecule of sturine B it appeared appropriate to use trypsin hydrolysis, in which the bonds formed by residues of neutral amino acids remain unchanged. The presence in the sturine B molecule of one proline residue, which considerably retards the hydrolysis of the bonds adjacent to it permitted us to hope to obtain fairly large fragments.

The combination of trypsin hydrolysis with the hydrolysis of sturine B by leucine aminopeptidase and carboxypeptidase made it possible to determine the bulk of the sequence of aminoacids in the protein.

In a preliminary experiment on the kinetics of the hydrolysis of sturine B by trypsin with the aid of the ninhydrin reaction, it was shown that the cleavage of the peptide bonds is practically complete after 6 h (Fig. 1).

To isolate the peptides formed, the 6-h hydrolyzate of the hydrochloride of sturine B was separated by ion-exchange chromatography on Amberlite CG-50 resin, using elution with Na borate buffer mixture by Ando's method [3], modified by ourselves. We lowered the pH of the buffer mixture to 7.4, since under the conditions of Ando's method (pH 8.0) the peptides are not completely absorbed on the resin; the elution was performed by increasing the NaCl concentration gradient-wise and not stepwise, which ensured a fairly good separation of the peptides.

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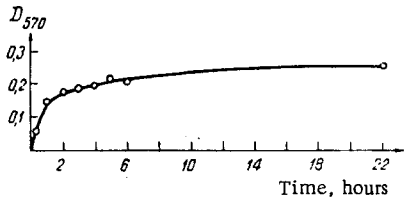

Fig. 1. Hydrolysis of sturine B by trypsin.

In this way, six chromatographic peaks were obtained which have been denoted by TI-TVI, respectively (Fig. 2). The fractions corresponding to TI-TVI were desalted on Amberlite SG-50 resin in the H^+ form equilibrated with 0.2 M Na acetate buffer mixture (pH 4.3). It must be mentioned that the sorption of the peptides on the resin took place completely only in a buffered column; otherwise the elution of the peptides began even in the free volume of the column.

The fractions were freeze-dried and their homogeneity was checked by gel filtration on Bip-Gel P-2 and by electrophoresis on paper in electrolytes 1 and 2. The peptides TII, TIV, and TV proved to be homogeneous. The fractions TI, TIII, and TVI consisted of at least two substances each. The TI and TIII fragments were separated by comparative electrophoresis on paper in system 2 into the peptides TI-1, TI-2, TIII-1 and TIII-2, respectively. Fraction TVI was separated by gel filtration on Bio-Gel P-2 into TVI-1 and TVI-2. The results of a determination of the amino-acid compositions of the peptides are given in Table 1.

On hydrolysis, short mainly di- and tripeptides were obtained, with the exception of the hexapeptide TIV. Consequently, to determine their amino-acid sequence it was sufficient to determine the N- and C-terminal amino acids. The N-terminal amino acid in the peptides TII, TIII, TV, TVI-1, and TVI-2 were determined by the dinitrophenyl method and in the peptides TI-1, TI-2, and TIV by the dansyl method; the C-terminal amino acids were determined by hydrolysis with carboxypeptidase B.

In all the peptides, the C-terminal position was occupied by arginine. This shows that the trypsin hydrolysis had taken place specifically.

From the hydrolyzate, two fractions consisting only of arginine – TIII-2 and TVI-2 – were isolated. We used the following method to determine whether they were free arginine or its peptides. Two aliquots of the substance were taken; one of them was hydrolyzed and the number of micromoles of arginine was determined on an amino-acid analyzer, and the other aliquot was dinitrophenylated, the completeness of dinitrophenylation being checked by paper electrophoresis in electrolyte 3, and it was then hydrolyzed and the amino-acid composition was determined. In this process, no arginine was found in the aqueous phase of the hydrolyzate of the fraction DNP-TIII-2, while DNP-TVI-2 still contained half the initial amount of arginine in the peptide. Consequently TIII-2 is free arginine and TVI-2 the dipeptide Arg-Arg; TIII-2 and TVI-2 also differ in their positions on the elution curve, TIII-2 issues after the peptides containing one arginine residue and TVI-2 after the peptides with two residues of basic amino acids.

The presence of the peptides Gly-Arg-Arg and Arg-Arg in the hydrolyzate shows that not all the bonds formed by arginine residues undergo cleavage. This can be explained by the assumption that in a block consisting of two to six arginine residues the splitting off of one arginine residue form the N- or the C-end is difficult because of its nearness to the free α-amino or α-carboxy group.

The peptides Ser-Ser-Arg and Pro-Glx-Arg were isolated in small amounts. Although there are only two serine residues in sturine B, it was found that they are adjacent to one another, as in other protamines. The peptide Pro-Glx-Arg could be formed only by the cleavage of an Arg-Pro bond. The cleavage of this bond is a fairly unusual phenomenon, but it has been observed in the trypsin hydrolysis of a number of protamines [4].

Of particular interest is the peptide TIV. Its amino-acid composition is Ser_2, Arg_2, Pro_1, Glu_1, its N-terminal amino acid serine and its C-terminal amino acid arginine. Since the peptides Ser-Ser-Arg and Pro-Glx-Arg were isolated from the hydrolyzate, it is obvious that they were formed from the peptide TIV, and its structure can be represented as Ser-Ser-Arg-Pro-Glx-Arg. The structure of the peptide was confirmed by the analysis of its sequence by the automatic Edman method.

To determine the C-terminal sequence of sturine B we used hydrolysis with carboxypeptidases A and B. It is known that carboxypeptidase B possesses not only the usual specificity with respect to basic substrates but also an activity similar to that of carboxypeptidase A [5]. Consequently, the hydrolysis of sturine B by

TABLE 1. Amino-Acid Composition and Structure of the Trypsin Peptides of Sturine B

Amino acid	Peptides								
	TI-1	TI-2	T	T-1	T-2	TI	T	TI-1	TI-2
His								0,146 (0,9)	
Arg	0,285 (1,0)	0,403 (1,0)	0,899 (1,0)	1,266 (1,0)	2,050	0,365 (2,2)	0,580 (2,1)	0,161 (1,0)	0,800
Ser	0,598 (2,0)					0,301 (1,8)			
Glu		0,396 (1,0)				0,169 (1,0)			
Pro		0,319 (0,8)				0,174 (1,0)			
Gly				1,277 (1,0)			0,278 (1,0)	0,162 (1,0)	
Ala			0,867 (1,0)						
N-Terminal amino acid	Ser	Pro	Ala	Gly	Arg	Ser	Gly	His	Arg
C-Terminal amino acid	Arg	Arg	Arg	Arg		Arg	Arg	Arg	Arg
Structure of the peptide	Ser-Ser-Arg	Pro-Glx-Arg	Ala-Arg	Gly-Arg	Arg	Ser-Ser-Arg--Pro-Glx-Arg	Gly-Arg_2	His-Gly-Arg	Arg-Arg

Note. The amino acids are given in micromoles (in residues).

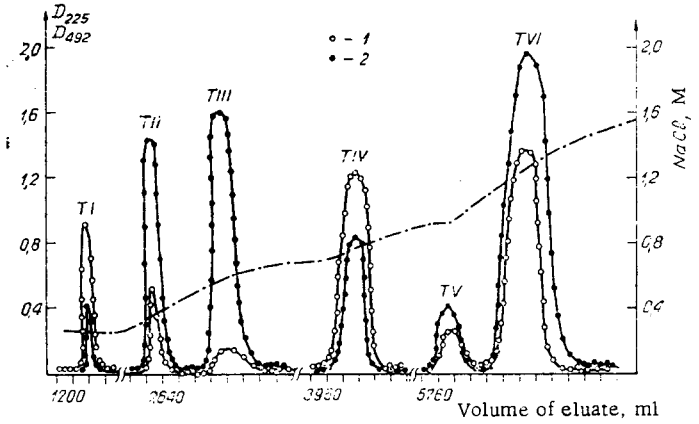

Fig. 2. Fractionation of the trypsin of peptides of sturine B on Amberlite CG-50 [1) D_{255}; 2) D_{492}].

carboxypeptidase B was performed in the presence of β-phenylpropionic acid – an inhibitor of carboxypeptidase A. Under these conditions, one μmole of protein yielded 3.7 μmole of arginine (Fig. 3a).

When the sturine was hydrolyzed by a mixture of carboxypeptidases A and B, in addition to arginine, only glycine was split off, 5.3 μmole of arginine being obtained per μmole of glycine (Fig. 3b). The apparent noncorrespondence of the amount of arginine split off in this experiment with the experiment with the inhibited carboxypeptidase B is explained by the fact that glycine is split off far more slowly than arginine and, moreover, an arginine block apparently follows glycine in the sturine B molecule. Thus, the C-terminal sequence of sturine B has the form ...Arg-Gly-Arg_4-OH. The N-terminal sequence of sturine B was investigated with the aid of leucine aminopeptidase. After hydrolysis for 13 h, from 1 μmole of protein 0.9 μmole of alanine, 5.0 μmole of arginine, and 1.7 μmole of serine had been split off (Fig. 3c). We have shown previously [1] that the N-terminal amino acid of sturine B is alanine. Since the molecule of the sturine contains only one alanine residue, the following N-terminal sequence can be written: H-Ala-Arg_{4-5}-Ser-Ser.... A comparison of this sequence with the structure of the peptide TIV made it possible to determine the position of the 11th and 12th amino-acid residues in the N-terminal segment of the sturine B molecule: H-Ala-Arg_{4-5}-Ser-Ser-Arg-Pro-Glx-Arg....

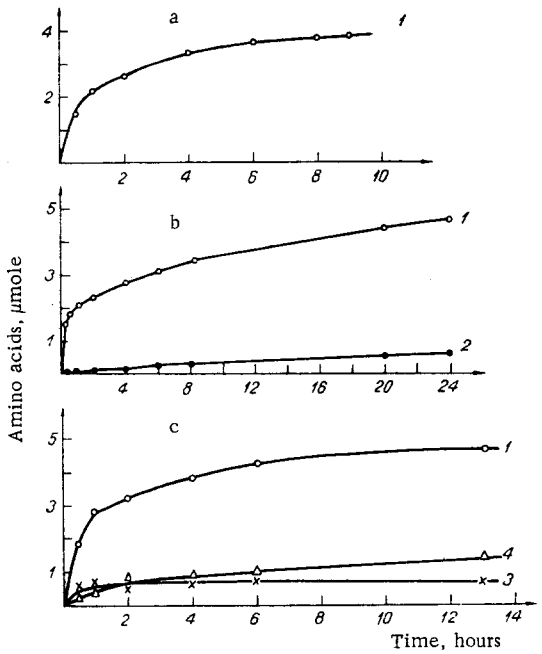

Fig. 3. Hydrolysis of sturine B by carboxypeptidase B (a), a mixture of carboxypeptidases A and B (a), and leucine aminopeptidase (c): 1) arginine; 2) glycine; 3) alanine; 4) serine.

Analysis of the results of trypsin hydrolysis together with those of a study of the N- and C-terminal sequences has enabled us to establish the following partial amino-acid sequence of sturine B: H-Ala-Arg_{4-5}-Ser-Ser-Arg-Pro-Glx-Arg_X-(His-Gly-Arg)-Arg_Y-Gly-Arg_4-OH.

EXPERIMENTAL

The sturine B was obtained as described previously [1].

The following enzymes were used in the investigation:

Trypsin (EC 3.4.4.4), type V "Reakhim"; the activity was determined by the hydrolysis of the p-nitrophenyl ester of benzoyl L-arginine [6];

Carboxypeptidase B (peptidyl-L-lysine hydrolase, EC 3.4.2.2), a product of the firm "Serva" from porcine pancreatic gland; before an experiment it was treated with diisopropyl phosphorofluoridate;

Carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.2.1), a product of the firm "Sigma," treated with diisopropylphosphorofluoridate. The activity of the carboxypeptidases was determined by the method proposed by L. A. Lublinskaya [7];

Leucine aminopeptidase (L-leucyl peptide hydrolase, EC 3.4.1.1), a product of the firm "Serva," from bovine crystalline lens was kindly given to us by V. M. Stepanov.

Electrophoresis was performed in Durrum instruments in the following electrolytes: 1) 1 N acetic acid; 2) pyridine–acetic acid–water, pH 5.6; 3) 85% formic acid–acetic acid–water (28 : 20 : 52).

Chromatography was performed in a thin layer of silica gel using the following systems: 1) chloroform–methanol–acetic acid (95 : 5 : 1); 2) butan-1-ol–25% ammonia (80 : 20); 3) acetone–isopropanol–25% ammonia (9 : 7 : 0.5); 4) acetone–isopropanol–25% ammonia (9 : 7 : 0.7); 5) chloroform–benzyl alcohol–ethyl acetate–acetic acid (5 : 4 : 5 : 0.2); 6) acetone–isopropanol–25% ammonia (9 : 7 : 2); 7) acetone–isopropanol–25% ammonia (9 : 7 : 3); 8) chloroform–benzyl alcohol–methanol–acetic acid (5 : 4 : 1 : 1).

Hydrolysis of Sturine B by Trypsin and Separation of the Mixture of Peptides Obtained. Sturine B (800 mg) was dissolved in 100 ml of 0.1 M triethylammonium carbonate buffer, pH 7.8 (37°C, 30 min). Then 20 mg of crystalline trypsin was added to the solution. The mixture was incubated at 37°C for 6 h. Hydrolysis was stopped by boiling the reaction mixture in the water bath for 10 min. The hydrolyzate was evaporated to dryness and dissolved in 20 ml of 0.2 M sodium borate buffer, pH 7.4.

The mixture of peptides so obtained was fractionated on Amberlite CG-50 cation-exchange resin (type II, Na^+ form) in 0.2 M Na borate buffer, pH 7.4, using a NaCl gradient (see Fig. 2). The column was 100 cm high and 2.5 cm wide, and the rate of elution was 36 ml/h. Samples with a volume of 6 ml were taken on an automatic fraction collector. The course of the fractionation was followed by the spectrophotometry of the sample at 225 nm with the aid of the Sakaguchi reagent [8].

Desalting of the Peptides. The peptides were desalted on a column (50×2.5 cm) containing Amberlite CG-50 resin in the H^+ form buffered with 0.2 M Na acetate buffer, pH 4.3. The combined eluates of the peptides were diluted three times with the buffer and passed through a column (50×3 cm) at the rate of 100 ml/h. The salts were eluted with 0.2 N acetic acid, and the peptides were eluted rapidly with 0.1 N hydrochloric acid in the cold (0°C). The sorption of the peptides on the resin and their elution was monitored by means of the Sakaguchi reagent. The acid eluates of the peptides were neutralized with Amberlite IRA-400 ion-exchange resin in the OH^- form and were freeze-dried.

Check on the Homogeneity of the Peptides. The homogeneity of the peptides was checked by electrophoresis in electrolytes 1 and 2 (500 V, 1.5 h) and by chromatography on Bio-Gel P-2. The peptides were passed through a column (150×1.5 cm) filled with Bio-Gel P-2 in 0.02 N HCl at the rate of 9 ml/h. The process was monitored by the spectrophotometry of the samples at 225 nm and by the Sakaguchi reaction.

The amino-acid compositions of the peptides were determined on a Hitachi KLA-2B amino-acid analyzer after hydrolysis with 6 N hydrochloric acid (105°C, 24 H).

Dinitrophenylation of the Peptides [9]. An aliquot of a peptide was dissolved in 1 ml of a 1% solution of triethylamine, and 0.2-0.3 ml of an ethanolic solution of fluorodinitrobenzene (FDNB, 20 mg/ml) was added. The mixture was kept at 37°C for 16 h. The excess of FDNB was extracted with ether. The peptide solution was evaporated to dryness and the residue was hydrolyzed with 6 N HCl (105°C, 16 h).

The DNP-amino acids were identified by thin-layer chromatography on silica gel; the ether-soluble derivatives in system 1 and the acid-soluble derivatives by double chromatography in system 2 with intermediate drying at 100°C.

The dansylation of the peptides was performed by Gray's method [10]. A peptide (0.05-0.1 μmole) was dissolved in 150 μl of 0.1 N $NaHCO_3$, and 150 μl of a solution of 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS chloride) in acetone (6 mg/ml) was added. The mixture was kept in the dark at 37°C for 1 h. The DNS-peptides were hydrolyzed with 6 N hydrochloric acid (105°C, 4 h). The acid was driven off by vacuum distillation, and the residue was dissolved in the minimum volume of acetone–1 N HCl (9:1); the DNS derivatives were identified by two-dimensional chromatography in a thin layer of silica gel G in systems 3 and 4 (first direction) and 5 (second direction), or 6 and 7 (first direction) and 8 (second direction).

Hydrolysis of Sturine B by Carboxypeptidase B. Sturin B (5 mg) was dissolved in 5 ml of 0.05 M tris-hydrochloride buffer, pH 8.05, containing 0.25 M NaCl and 0.01 M β-phenylpropionic acid, and 0.5 mg of carboxypeptidase B which had been treated with diisopropyl phosphorofluoridate was added. After predetermined times of incubation at 37°C, 0.8-ml samples were taken, acidified to pH 2.5, evaporated to dryness in vacuum, and analyzed on the Hitachi amino-acid analyzer.

Hydrolysis of Sturine B by a Mixture of Carboxypeptidases A and B. A suspension of carboxypeptidase A (0.1 ml, containing 100 μg of the enzyme) was diluted with 0.1 M NaCl solution (3 ml) and cooled to +4°C and, with stirring, 0.05 N NaOH was added dropwise until the enzyme had dissolved (pH 8.0-8.5).

The solution of carboxypeptidase A so obtained was added to a solution of sturine B in 0.05 M tris-hydrochloride buffer, pH 8.05 (enzyme-substrate ratio 1:100). Carboxypeptidase B was added to the same solution, again in an enzyme–substrate ratio of 1:100). The reaction mixture was kept at 37°C. Samples were taken and analyzed in the same way as in the experiment with carboxypeptidase B.

Hydrolysis of Sturine B by Leucine Aminopeptidase. Sturine B hydrochloride (3 mg) was dissolved in 6 ml of 0.05 M tris-HCl buffer, pH 8.05, containing 0.5 M sodium chloride and 0.005 M manganese chloride, and 0.9 ml of a solution of leucine aminopeptidase (2 mg/ml) was added (enzyme–substrate ratio 1:100). The mixture was incubated at 37°C, and 1-ml samples were acidified to pH 3, evaporated to dryness, and analyzed on the Hitachi amino-acid analyzer.

SUMMARY

The partial amino-acid sequence of sturine B has been determined on the basis of a study of the structure of the peptides from its trypsin hydrolysis and a determination of its N- and C-terminal sequence with the aid

of leucine aminopeptidase and carboxypeptidases A and B.

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SYNTHESIS OF A HEXAPEPTIDE CONTAINING VALINE, LEUCINE, AND GLUTAMIC ACID

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We have previously reported the synthesis and attractive properties of some optically active peptides containing residues of the amino acids glycine, alanine, leucine, proline, ornithine, lysine, and phenylalanine for blood-sucking mosquitoes [1, 2]. In the present paper we describe the preparation of the methyl ester of the hexapeptide N-benzyloxycarbonyl-(Cbz)-L-phenylalanyl-L-valyl-Oγ-methyl-L-glutamyl-L-leucyl-O-valyl-L-alanine and the intermediate di- and tripeptides with the aim of studying further the dependence of their properties on the amino-acid composition, the sequence and configuration of the amino acids, and the length of the peptide chain.

The hexapeptide (XII) was synthesized from two tripeptides by the carbodiimide method according to the Scheme shown. It must be noted that repeated reprecipitation was necessary to free the substance from dicyclohexylurea.

The dipeptides (VI) and (VIII) and the tripeptides (VII) were obtained by the mixed-anhydride method using isobutyl chloroformate. This method permitted the synthesis of homogeneous substances in good yield (65-98%) and of the glutamyl-containing dipeptide with a free carboxy group, which considerably facilitated the preparation of the desired hexapeptide.

The hydrazide (IX) was readily formed in methanolic solution and was crystallized under ether. The debenzyloxycarbonylation of the tripeptide (X) by hydrogenation over Pd black in methanolic solution in the presence of acetic acid took place without appreciable destruction of the peptide bonds. (See Scheme on next page.)

To confirm the amino-acid composition of the hexapeptide obtained, it was subjected to complete acid hydrolysis with 6 N hydrochloric acid at 105°C for 20 h. The hydrolyzate was investigated chromatographically and electrophoretically. On the chromatogram and on the electrophoretogram five ninhydrin-positive substanes were detected the mobilities of which coincided with those of alanine, valine, leucine, phenylalanine, and glutamic acid.

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