AMINO ACID SEQUENCES OF STURIN A AND OF NUDIVENTRIN A

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Nudiventrin and sturin have been fractionated and it has been shown that each of them consists of two components, A and B, their ratio in sturin being 10 : 1. The amino acid sequences of these proteins have been determined and it has been shown that they have the same primary structure.

Nudiventrin A and sturin A are protamines forming constituents of the nucleoprotein complexes of the gonads of the sturgeons Acipenser nudiventris and A. sturio, respectively. It appeared of interest to compare the amino acid sequence of the protamines isolated from fish of one and the same family. The results of this investigation are given in the present paper.

Sturins A and B were separated as described previously [1] with a slight change in the conditions of fractionation, using a stepwise gradient of NaCl (1.55 and 1.62 M). Under these conditions, sturin A issued from the column at a lower concentration of sodium chloride than previously [1] (Fig. 1).

Like the majority of protamines [2], nudiventrin is heterogeneous. When an attempt was made to separate it under the conditions for the fractionation of the total sturin, two fractions were obtained, each of which was enriched with one of the components. Obviously, incomplete separation of component A from component B took place under these conditions. To obtain pure component A we used a stepwise-exponential concentration of sodium chloride (1.4 M → 1.6 M; 1.6 M) (Fig. 1). It was found that the amount of component A in nudiventrin was ten times greater than the amount in B, while in sturin this ratio is 2 : 1.

In the desalting of the protein solutions, for better sorption on the ion-exchange resin dilution was carried out with sodium acetate buffer.

The rechromatography of the desalted sturin A and nudiventrin A on CM-Sephadex C-25, their gel filtration on Bio-Gel P-10, the presence of single N-terminal amino acids (dinitrophenylation and dansylation methods) and individual C-terminal amino acids (CPB [carboxypeptidase B] and the dansylation method), and also a determination of the N-terminal sequences of these proteins by the automatic Edman method [3, 4] permitted the conclusion that the proteins isolated were homogeneous.

The amino acid compositions and N- and C-terminal amino acids of the proteins investigated are given below.

Amino acid	Nudiventrin A	Sturin A
Lysine	6,3 (6)	5,4 (5—6)
Histidine	3,2 (3)	3,1 (3)
Arginine	14,0 (14)	12,82 (13)
Threonine	0,9 (1)	0,95 (1)
Serine	1,91 (2)	1,94 (2)
Glycine	1,1 (1)	1,0 (1)
Alanine	2,0 (2)	2,02 (2)
Leucine	1,0 (1)	1,0 (1)
N-amino acid	Ala	Ala
C-terminal amino acid	Lys	Lys

Thus, the protamines studied differ by their lysine and arginine contents. To establish their primary structures, the proteins were subjected to hydrolysis with thermolysin, which does not clear the bonds formed by residues of basic amino acids, and the peptides obtained were analyzed; in addition, the N-terminal sequences of nudiventrin A and sturin A were studied by the automatic Edman method and the C-terminal sequence with CPB [3, 4].

The separation of the peptides of thermolysin hydrolyzates of the proteins investigated gave twelve fractions in each case (Th1-Th12) (Fig. 2). The elution curves of the peptides obtained in the hydrolysis of nudiven-

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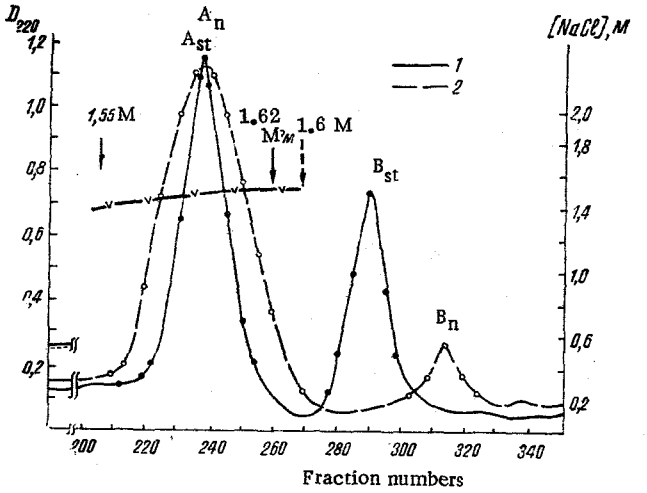


Fig. 1. Fractionation of sturin (1) (170 mg) and of nudiventrin (2) (170 mg) on CM-Sephadex C-25 (column 2.5 × 50 cm) in 0.05 M sodium acetate buffer (pH 5.7); fraction volume 3 ml, rate of elution 30 ml/h.

trin A and sturin A coincided completely. It is likely that the action of thermolysin on the molecules of these proteins formed the same fragments.

The fractions corresponding to the chromatographic peaks Th8-Th12 contained arginine (Sakagushi reaction) [5]. They are desalted by Ando's method [6]. Fractions Th3-Th7 were desalted by Blok's method [7].

Solutions of the peptides were tested for homogeneity by paper electrophoresis, and also from the finding of single N- and C-terminal acids (hydrolysis with CPB and CPA [carboxypeptidases B and A]).

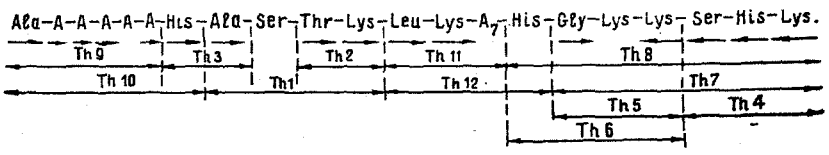
We found that the high arginine contents in the proteins studied and in the peptides Th9, Th10, Th11, and Th12 (Table 1 and 2) led to an increased error of analysis. Therefore, to calculate the amino acid compositions of these proteins and peptides we introduced a correction factor for arginine. Its value was determined from a calibration curve using standard solutions of arginine with the following concentrations (μM): 0.15, 0.2, 0.5, 0.7, and 1. It was established that Th9 and Th10 each contained 5 arginine residues, Th11 and Th12, contained seven and nudiventrin A and sturin A each contained 12 arginine residues.

The amino acid sequences of Th2, Th3, Th4, Th5, Th9, and Th10 (Tables 1 and 2) unambiguously followed from their amino acid compositions and the analysis of the N- and C-terminal amino acids of these peptides.

The structure of peptide Th1 (Tables 1 and 2) was established on the basis of the structures of Th2, which is part of Th1 and is formed by the cleavage of the ···-Ser ↓ Thr··· bond to give N-terminal Thr, there being a single Thr residue in the molecule of each of the proteins investigated.

To reconstruct the C-terminal part of nudiventrin A (sturin A) we carried out the hydrolysis of Th7 with CPB. According to the results obtained, the proteins studied had the following sequence of amino acids in the C-terminal section: ···Lys-Ser-His-Lys.

The hydrolysis of the protamines with thermolysin formed several overlapping peptides: Th10 and Th3, at His; Th1 and Th3, at Ala; and Th12 and Th8 at His. The results obtained in the investigation of the amino acid sequences of these proteins by the automatic Edman method [3, 4] and of the peptides of thermolysin hydrolysates of nudiventrin A and sturin A permit us to state that components A of the protamines studied have the same primary structure, which coincides with the primary structure of stellin A [8] and can be represented as follows



→) sequence determined by means of a sequenator; ←) by hydrolysis with CPB; A) arginine; Th) peptides from thermolysin hydrolysates of sturin A and nudiventrin A.

TABLE 1. Amino Acid Compositions and Structures of the Thermolysin Peptides of Sturin A

Symbol of the peptide	Amino acid										Sequence of the peptide
	Lys	His	Arg	Ser	Gly	Ala	Leu	Thr	N-terminal	C-terminal	
Th 1	1,04 (1)			0,92 (1)		1 (1)		0,82 (1)	Ala	Lys	Ala-(Ser-Thr)-Lys
Th 2	1 (1)							0,8 (1)	Thr		Thr-Lys
Th 3		0,85 (1)				1 (1)			His		His-Ala
Th 4	1 (1)	1 (1)		0,9 (1)					Ser	Lys	Ser-His-Lys
Th 5	2,1 (2)				1 (1)				Gly		Gly-Lys_2
Th 6	1,9 (2)	0,9 (1)			1 (1)				His	Lys	His-(Gly-Lys)-Lys
Th 7	3,2 (3)	1 (1)		0,94 (1)	1 (1)				Gly	Lys	Gly-Lys-Lys-Ser-His-Lys
Th 8	3,2 (3)	2 (2)		1 (1)	1 (1)				His	Lys	His-(Gly-Lys_2-Ser-His-)-Lys
Th 9			5 (5)			1 (1)			Ala		Ala-Arg_5
Th 10		0,95 (1)	5 (5)			1 (1)			Ala	His	Ala-Arg_5-His
Th 11	1 (1)		6,9 (7)				1 (1)		Leu	Arg	Leu-(Lys-Arg_6)-Arg
Th 12	1 (1)	1 (1)	7 (7)				1 (1)		Leu	His	Leu-(Lys-Arg_6)-Arg-Lys

TABLE 2. Amino Acid Compositions and Structures of the Thermolysin Peptides of Nudiventrin A

Symbol of the peptide	Amino acid										Sequence of the peptide
	Lys	His	Arg	Ser	Gly	Ala	Leu	Thr	N-terminal	C-terminal	
Th 1	1 (1)			0,93 (1)		1 (1)		0,91 (1)	Ala	Lys	Ala-(Ser-Thr)-Lys
Th 2	1 (1)							0,81 (1)	Thr		Thr-Lys
Th 3		0,94 (1)				1 (1)			His		His-Ala
Th 4	1 (1)	0,8 (1)		1 (1)					Ser	Lys	Ser-His-Lys
Th 5	1,85 (2)				1 (1)				Gly		Gly-Lys_2
Th 6	1,96 (2)	1 (1)			1 (1)				His	Lys	His-(Gly-Lys)-Lys
Th 7	3,1 (3)	0,9 (1)		0,9 (1)	1 (1)				Gly	Lys	Gly-Lys-Lys-Ser-His-Lys
Th 8	2,8 (3)	2,1 (2)		0,95 (1)	1 (1)				His	Lys	His-(Gly-Lys_2-Ser-His)-Lys
Th 9			5 (5)			1 (1)			Ala		Ala-Arg_5
Th 10		0,9 (1)	4,8 (5)			1 (1)			Ala	His	Ala-Arg_5-His
Th 11	1 (1)		7 (7)				1 (1)		Leu	Arg	Leu-(Lys-Arg_6)-Arg
Th 12	1 (1)	1 (1)	7 (7)				1 (1)		Leu	His	Leu-(Lys-Arg_6)-Arg-His

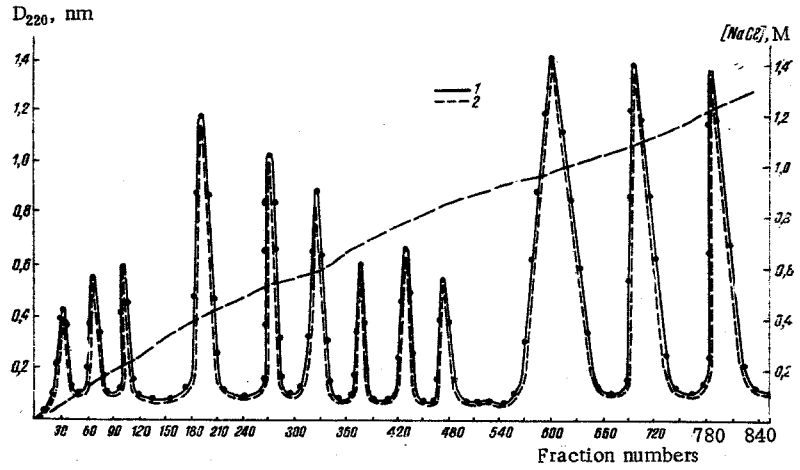


Fig. 2. Fractionation of the thermolysin peptides of sturin A (1) (200 mg) and of nudiventrin A (2) (200 mg) on C-25 CM-Sephadex (column 2.5 × 50 cm) in sodium acetate buffer (pH 5.7). Mixer 0.3 liter; fraction volume 3 ml; rate of elution 30 ml/h.

Thus, the chromatins of three species of sturgeon differ only by the ratio of the protamines present in them: in the chromatin of Acipenser sturia the amount of component A is twice that of component B, in A. stellatus it is three times greater [9], and in A. nudiventris it is ten times greater.

EXPERIMENTAL

Nudiventrin and sturin were isolated by Ando's method [6] from the milt of A. nudiventris and A. sturio, caught in the mouth of the R. Kura, respectively. The following reagents were used in the work: thermolysin (E.C. 3.4.4), carboxypeptidase A (E.C. 3.4.2.2.1), carboxypeptidase B (E.C. 3.4.2.2), and dansyl chlorine (GFR).

Paper electrophoresis was carried out in a Durrum instrument in 1 N acetic acid.

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

In all the experiments on fractionation, gel filtration, and desalting, the course of elution was followed from the absorption of samples at 220 nm.

The amino acid compositions of sturin A, nudiventrin A, and the peptides were determined on a Hitachi model KLA-3B amino acid analyzer after they had been hydrolyzed in 5.7 N hydrochloric acid under standard conditions.

The fractionation of the proteins and peptides (Figs. 1 and 2) was performed on C-25 CM-Sephadex in 0.05 N sodium acetate buffer, pH 5.7 (buffer A). The total sturin (or nudiventrin) was dissolved in buffer A (12 ml) and deposited on the column. Elution was performed with a stepwise gradient of NaCl. The fractions corresponding to component A (or B) of sturin (or nudiventrin) were combined and desalted.

A mixture of the peptides of sturin A (or nudiventrin A) was dissolved in 0.05 N sodium acetate buffer, pH 5.7 (15 ml) and was deposited on a column. Elution was performed with a stepwise-exponential concentration gradient of NaCl.

Desalting of the Proteins and Peptides. Solutions of proteins were diluted with buffer A to a 0.5 M concentration of sodium chloride and were deposited at the rate of 18 ml/h on a column filled with Amberlite CG-50 in the H^+ form equilibrated with buffer A. The salts were eluted with a 0.5 M solution of acetic acid. Cold ethanol was added to the eluate (1 : 3). The sturin A (or nudiventrin A) separated out in the form of a white precipitate, which was washed with cold ethanol to pH 5 and was reprecipitated.

From 170 mg of total sturin we isolated 50 mg of sturin A and 25 mg of sturin B, and from 170 mg of total nudiventrin we isolated 70 mg of nudiventrin A and 7 mg of nudiventrin B.

Peptides Th3-Th7 [7] were desalted in a column (0.9 × 6 cm) filled with Dowex 50 × 5. A solution of a peptide in 80% ethanol was deposited on the column at the rate of 15 ml/h. The column was eluted with 15 ml of 80% ammonia solution in 80% ethanol, 15 ml of 80% ethanol, 15 ml of water, 15 ml of a 4 N solution of ammonia, and water.

Solutions of peptide Th8-Th12 were diluted three- to sixfold with water acidified to pH 3-4, and deposited on a column (2 × 3 cm) filled with Amberlite CG-50 (H^+ form) equilibrated with 0.01 N sodium acetate buffer, pH 5.7, at the rate of 20 ml/h. The salts were eluted with a 0.5 N solution of acetic acid and with water to pH 5. The peptides were eluted with 0.1 N hydrochloric acid. The eluates of the peptides were treated with Amberlite IRA-400 in the OH^- form to pH 5-6.

The gel filtration of sturin A (or nudiventrin A) was carried out on a column (1.5 × 130 cm) filled with Bio-Gel P-10 equilibrated with a 0.01 N solution of hydrochloric acid. A solution of 15 mg of the protein in 0.01 N hydrochloric acid was deposited on the column at the rate of 15 ml/h. Elution was effected with a 0.01 N solution of hydrochloric acid.

The N-terminal amino acids in sturin A, nudiventrin A, and the peptides were determined by Gray's method [11]. The dansyl(amino acid)s were identified by micro thin-layer chromatography [12] in systems 1 and 2 (direction I) and 3 (direction II).

C-Terminal Amino Acids. To aliquots of the peptides (Th1, Th4, Th6, Th8, and TH11) and the proteins was added triethylammonium carbonate buffer, pH 8.0, until the final concentration was 0.5 M, and then CPB (E : S = 1 : 400) and a solution of benzylsulfonyl fluoride to a final concentration of $5 \cdot 10^{-3}$ M. After predetermined intervals of time (0, 5, 15, 45, 90, 120, and 180 min), samples were taken from the reaction mixture and were acidified with 0.5 M acetic acid to pH 3-4. The amino acids split out were analyzed in an amino acid analyzer. In some cases they were determined by the dansylation method followed by the TLC of the dansyl derivatives of the amino acids.

Thermolysin Hydrolysis. A solution of 200 mg of sturin A (or nudiventrin A) in 0.02 M tris-hydrochloride buffer, pH 8.05, at a concentration of protein in the reaction mixture of 1 mole/ml was treated with thermolysin (E : S = 1 : 100) and incubation was carried out at 37°C for 4.5 h. Hydrolysis was stopped by the addition of 0.5 N acetic acid to pH 3. The mixture was evaporated in a rotary evaporator.

SUMMARY

Nudiventrin and sturin have been fractionated and it has been shown that each of these consists of two components, A and B, their ratio in sturin being 2 : 1 and in nudiventrin 10 : 1.

The amino acid sequences of these proteins have been determined and it has been shown that they have the same primary structure.

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