PROTAMINES

FRACTIONATION OF STURINE FROM Acipenser güldenstädti ON CARBOXYMETHYL-SEPHADEX C-25

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Protamines are a special group of nuclear proteins. They are distinguished by a high content of residues of basic amino acids, mainly arginine, and by the absence of aromatic amino-acid residues. Another feature of the protamines is their heterogeneity. Some of the protamines suppress the growth of various pathogenic microbes and cause an inhibition of the growth of individual types of tumors [1-4]. Of this group of proteins, those that have been studied in most detail are salmine, clupeine, and iridine, the primary structure of which is known [5, 6]. The least-studied protamines are those of the true sturgeons. The amino-acid compositions and terminal amino acids of two of them – stelline [7] and total sturine [8, 9] – have been determined.

Together with workers of the Central Order of Lenin Institute for the Further Training of Doctors, we have isolated protamines from the gonads of the true sturgeons of the Caspian Sea (common sturgeon, Acipenser sturio L., the Caspian sturgeon, A. stellatus Pallas, and "ship," A. nudiventris, Lov.) [10]. A preliminary study of their chemical compositions has shown that they are all heterogeneous and contain arginine, lysine, histidine, glycine, alanine, proline, serine, threonine, leucine, and glutamic acid.

Sturine, isolated from the gonads of Acipenser güldenstädti (sturgeon) and stelline from Acipenser stellatus (Caspian sturgeon) cause 60-80% inhibition of the growth of solid and ascitic tumors in mice. The protamine from the gonads of Acipenser nudiventris ("ship") is less active.

To obtain individual protons, we used several methods of fractionation: gel filtration on Sephadex G-25 and G-50 and on Bio-Gels P-4, P-6, and P-10, chromatography on CM-cellulose and carboxymethyl-Sephadex C-25 at various pH values and with a gradient of NaCl concentrations. The most effective separation was observed on CM-Sephadex.

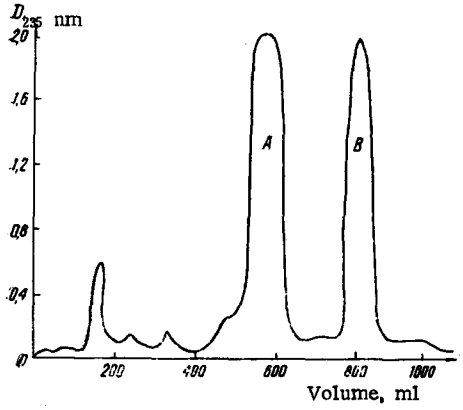

Fig. 1. Separation of sturine on carboxymethyl-Sephadex C-25.

In the present paper we describe in detail the fractionation of sturine on CM-Sephadex C-25 and the results of a study of the amino-acid composition of the proteins isolated. Sturine sulfate, obtained from the nucleoprotamine by Ando's method [11] was fractionated.

In a preliminary experiment we determined the conditions for the adsorption of protamine at various pH values of buffer solutions. The best results were obtained in 0.02 M phosphate buffer mixture with pH 7. At higher pH values, the protein was not adsorbed completely on the support, and at pH values below 7, conversely, it was bound to the Sephadex too firmly.

The protein was eluted from the CM-Sephadex with 0.02 M phosphate buffer mixture (pH 7) containing sodium chloride in

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TABLE 1. Amino-Acid Composition of the Sturines from Acipenser güldenstädti

Amino acids	Sturine				
	initial	A		B	
		μmole/mg	No. of residues	μmole/mg	No. of residues
Lysine	0,61	0,65	5,61	—	—
Histidine	0,39	0,39	3,38	0,19	1,04
Arginine	2,51	1,79	15,46	3,98	22,2
Threonine	0,12	0,11	0,96	—	—
Serine	0,31	0,25	2,19	0,32	1,79
Glutamic acid	0,07	—	—	0,18	1,00
Proline	0,06	—	—	0,18	1,00
Glycine	0,23	0,15	1,28	0,37	2,05
Alanine	0,29	2,26	2,22	0,17	0,95
Leucine	0,10	0,12	1,0	—	—

various concentrations. Elution began when the concentration of sodium chloride in the eluting buffer was not less than 1 M. For the preliminary separation of the heterogeneous mixture of sturines, we performed the adsorption of the total protein on CM-Sephadex from 0.02 M buffer mixture (pH 7), and for elution we used the same buffer mixture with a gradient of sodium chloride concentrations from 1 to 1.9 M. Under such fractionation conditions, two components of sturine were isolated, which we have called sturines A and B (Fig. 1). Sturine A differs considerably from sturine B (Table 1). Sturine B contains about 75% of arginine and a small group of neutral amino acids. These include serine, proline, glycine, and alanine and, of the amino acids rarely found in protamines, histidine and glutamic acid.

The composition of sturine A includes threonine, serine, glycine, alanine, and leucine. In addition to arginine, sturine A contains histidine and lysine. These amino acids have also been found in other protamines isolated from the roe of the true sturgeons – in the total sturine from Acipenser sturio [8] and in the fractions of stelline obtained from CM-cellulose under various conditions of elution [7, 12]. The presence of lysine and histidine is probably characteristic for individual representatives of this type of protamines.

The homogeneity of sturine B was confirmed by means of a series of methods supplementing one another: chromatography on carboxymethyl-Sephadex (rechromatography), gel filtration on Bio-Gel P-10, paper electrophoresis, and disc electrophoresis in polyacrylamide gel.

The elution curves from CM-Sephadex and from Bio-Gel P-10 show a single symmetrical peak. On paper electrophoresis in 30% acetic acid and in formic acid – acetic acid – water (28 : 20 : 52) only one spot, revealed by means of the Sakagush reagent was found. On disc electrophoresis of solution of the protein with a concentration of 30-60 γ in 15% polyacrylamide gel (pH 4.0) with subsequent measurement of the density of coloration in an ERJ-10 microdensitometer (Italy), symmetrical peaks were found which showed that they contained 86-88% protein. The results presented show the fairly high homogeneity of sturine B.

After rechromatography on CM-Sephadex, sturine B had the following composition: Arg_{22}, His_1, Ser_2, Glx_1, Pro_1, Gly_2, Ala_1. The N-terminal acid was determined by Sanger's method [13]. The C-terminal acid was determined with the aid of carboxypeptidase B. The N-terminal position in sturine B is occupied by alanine and in the C-terminal section there is a protein consisting of four arginine residues (3.95 μmole of arginine per μmole of protein).

EXPERIMENTAL

As the starting material we used the fresh roe of sturgeon trapped in the mouth of the R. Kura on April 17, 1968. The roe was frozen with dry ice.

The nucleoprotamines were isolated by Ando's method [11] at 0-4°C. The comminuted roe was stirred with a threefold volume of Ringer solution (0.65% of NaCl, 0.014% of KCl, 0.012% of $CaCl_2$, 0.012% of $NaHCO_3$, 0.001% of NaH_2PO_4) and was passed through close-woven fabric. The suspension were centrifuged at 10,000 rpm, and the precipitate was washed with Ringer solution containing 0.2% of sodium citrate, and with ethanol, acetone, and ether, and was dried in the air. From 4350 g of frozen roe 680 g of nucleoprotamines was obtained.

Sturine was isolated from the nucleoprotamine by Ando's method [11] at 0-4°C. The nucleoprotamine (10 g) was stirred with 600 ml of a 2 M solution of sodium chloride, and the viscous solution formed was treated with 100 ml of a saturated solution of copper sulfate. The precipitate was separated off by centrifuging and was washed with cold water. To the combined filtrate was added an 8 M solution of sodium picrate and the mixture was left overnight in the refrigerator. Then the precipitate was separated by centrifuging and was carefully washed with sodium picrate solution and cold water. The picrate was dissolved in 67% (by weight) aqueous acetone, and 2 N sulfuric acid to a pH of 3 and a threefold volume of ethanol were added. The protamine sulfate was filtered off and purified by four reprecipitations from aqueous solution with ethanol. This gave 1.25 g of sturine sulfate.

Fractionation of Sturine Sulfate on Carboxymethyl-Sephadex C-25. The protein (350 mg) was dissolved in 10 ml of 0.02 M phosphate buffer mixture (pH 7) containing 0.5 M sodium chloride and was transferred to a column (50 × 2 cm) of carboxymethyl-Sephadex C-25 (capacity 4.5±0.5 meq/g, 40-120 nm, Pharmacia) equilibrated with the same buffer mixture. Gradient elution was performed by increasing the concentration of sodium chloride in the solution from 1 to 1.9 M; the rate of elution was 16 ml/h. Fractions with a volume of 4 ml were collected by means of a ZPK_{206}-B automatic collector (Poland). Fractionation was monitored by spectrophotometry at 225 nm on an SF-4 instrument. The fractions were combined and desalted on Amberlite IRS-50 ion-exchange resin in the H^+ form at 0-4°C by Ando's method [11]. The salts were washed first with 0.3 N acetic acid and then with water.

The protamine was eluted with 0.10 N hydrochloric acid at 4°C. The eluates were neutralized with Amberlite IRA-400 ion-exchange resin in the OH^- form to pH 5.0. The solutions were freeze-dried, giving 80 mg of sturine A and 50 mg of sturine B.

The rechromatography of sturine B was performed on a column with dimensions of 1 × 40 cm as described above.

Chromatography of Sturine B on Bio-Gel P-10. Sturine B (47 mg) was dissolved in 5 ml of 0.2 N acetic acid and transferred to a column (3.5 × 50 cm) of Bio-Gel P-10 equilibrated with 0.2 N acetic acid. The protamine was eluted with the same acid at the rate of 20 ml/min.

The disc electrophoresis of sturine B was performed as described by Debabov and Rebentish [14] in 15% polyacrylamide gel (30-60 γ of sturine B per 0.6 × 10-cm tube, using a glycine buffer, pH 4.0, with a time of electrophoresis of 3 h at a current strength of 5 mA per tube). The electrophoregrams were stained with a 0.5% solution Coomassie Blue, the excess of dyestuff was washed out with 7% acetic acid, and the bands were densitometered in transmitted light on an ERJ-10 instrument.

The complete hydrolysis of the sturines was performed with triple-distilled 5.7 N hydrochloric acid at 105-109°C for 24 h.

The amino-acid composition of the sturine was determined with a Hitachi KLA-3B automatic amino-acid analyzer.

The dinitrophenylation of sturine B was performed by Sanger's method [13] (37°C, 48 h). The amount of the N-terminal amino acid was established by two-dimensional chromatography on silica gel G in systems 1) chloroform–methanol–acetic acid (95 : 5 : 1) and 2) butan-1-ol–concentrated ammonia (80 : 20).

The hydrolysis of sturine B was performed with Serva carboxypeptidase B (E.C. 3.4.2.2, peptidyl-L-lysine hydrolase) from porcine pancreatic gland treated with diisopropyl phosphorofluoridate. The sturine B (5 mg) was incubated in 3.8 ml of 2 M tris hydrochloride buffer, pH 8.1, with carboxypeptidase B (ratio of enzyme to substrate 1 : 1000) at 37°C for 16-h; the hydrolyzate was acidified and evaporated to dryness, and the residue was analyzed on the amino-acid analyzer.

Members of the Institute of Physiology, Academy of Sciences of the Azerbaidzhan SSR, Prof. M. A. Mekhtiev and Student Scientific Worker E. G. Gauzer assisted in the collection of the material for investigation, and workers of the Department of Chromatography, Interfaculty Laboratory of Bioorganic Chemistry of Moscow State University L. A. Baratova and L. P. Belyanova performed the analyses.

SUMMARY

1. A convenient method of fractionating the mixture of sturines from the ripe gonads of Acipenser güldenstädti on carboxymethyl-Sephadex C-25 has been proposed.

2. The amino-acid composition of two sturines – sturine A and sturine B – and the terminal amino acids in sturine B have been determined.

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