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ACTION OF TRYPSIN ON A NUCLEOPROTEIN FROM STURGEON GONADS

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A nucleoprotein has been isolated from the gonads of the Caspian sturgeon and its composition has been determined. It has been shown that it contains 55% of DNA, 2% of RNA, 36% of protamines, and about 7% of nonprotamine proteins of nonbasic nature. The nucleoprotein has been hydrolyzed with trypsin, and the amino acid compositions of some hydrolysis products have been studied. On the basis of the results obtained, the hypothesis has been put forward of a possible linkage of the DNA with the basic proteins. It has been shown that protamines react with the DNA through the basic amino acid residues located at various regions of their molecules.

One of the approaches to the study of the structure of the chromatin of the somatic cells is the use of proteolytic enzymes, especially trypsin [1].

Trypsin obviously causes the cleavage of the peptide bond mainly at those carboxyl groups of arginine and lysine that are not included in interaction with DNA. This action disturbs the structure of the nucleoprotein complex, a weakening of the bond of certain sections of the protein molecules with the DNA takes place, and this is accompanied by the migration of individual peptide fragments from the complex. Analysis of these fragments, and also of the peptides remaining bound with the DNA on trypsin hydrolysis enables us to determine which of the proteins present in a given type of chromatin interact more strongly with the DNA.

We have used this method to investigate the chromatin from sturgeon gonads.

We have previously studied the primary structure of the proteins of basic nature — protamines — present in this complex [2, 3]. The general characteristics of other components of it have been obtained in the course of the present investigation.

By using trypsin hydrolysis to study the nucleoproteins from sturgeon gonads we hoped to obtain certain information on the nature of the interaction of the components of chromatin and possible sites of contact of the proteins with the DNA.

The nucleoprotein was isolated from sturgeon gonads by the method of Kardykov et al. [4]. At all stages of the isolation process, to prevent proteolysis we added benzenesulfonyl fluoride. In this method of isolation, the bulk of the somatic chromatin passes into solution [4]. We have noted that the nucleoprotein from sturgeon sperm obtained under these conditions proved to be mainly insoluble. Subsequently, all operations were performed with this insoluble fraction of the DNA.

The amounts of DNA and RNA in the DNP, determined by the methods of Spirin [5] and Davidson [6], amounted to 55% and 2%, respectively.

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The nucleotide composition of the isolated chromatin after its hydrolysis with perchloric acid was determined on a Varian analyzer (mol. %): adenine, 28.3; thymine, 27.7; guanine, 22.3; cytosine, 20.1; 5-methylcytosine, 1.65.

The amount of protein components in the DNP was determined in the following way. Hydrochloric acid completely extracted the protamines (sturin A and sturin B) from a dry sample of the nucleoprotein. The extract was evaporated, hydrolyzed, and analyzed on the amino acid analyzer. The ratio of components A and B was calculated from the results of analysis on the basis of the amounts of amino acids present only in the sturin A molecule (leucine) or only in the sturine B molecule (proline). The amount of leucine was 0.35 μmole and of proline 0.16 μmole; i.e., the ratio of components A and B was 2:1.

Taking into account the molecular weights of these protamines* and their ratio in the nucleoprotein, we calculated that the amount of sturine in the complex was ∿36% of the total weight of the DNP.

The chromatin residue was treated with a solution of perchloric acid until the DNA and RNA had been eliminated completely [1], the amount of them in the DNP being ∿57%. After these extractions, substances of protein nature remained in the residue (∿7% of the weight of the nucleoprotein). To determine their overall amino acid composition, an analysis was carried out on the chromatin residue from which hydrochloric and perchloric acids had completely eliminated the protamines, the DNA, and the RNA [1]. From the results of analyses, they contained a considerable amount of glycine, alanine, serine, acidic amino acids, and also lysine, threonine, leucine, and other amino acids and a small amount of arginine. In the protamines, arginine makes up ∿70% of the weight of their molecules. By analogy with the nonhistone proteins of the somatic chromatin, we have called these proteins nonprotamine proteins.

The nucleoprotein isolated from sturgeon sperm was hydrolyzed with trypsin (E:S = 1:10). As a result of the reaction, the chromatin swelled with the formation of a viscous gel, which was separated by centrifuging into a precipitate P and a soluble fraction S.

Ninhydrin-positive substances were detected in the soluble fraction of the reaction mixture by electrophoresis. Apparently, under the action of trypsin on the chromatin, peptide fragments of the proteins pass into solution.

In a control experiment, ninhydrin-positive substances were absent.

We assumed that the peptides more strongly bound to the DNA should remain in the precipitate. For their isolation, the precipitate was treated with acid (P_{se}) [1].

To determine the nature of the peptides passing into solution and those remaining in the precipitate, we determined the total amino acid compositions of S and P_{se}. In addition, the solution of the hydrochloric extract of the precipitate was subjected to fractionation on carboxymethyl-Sephadex G-25.

It was found that the action of trypsin on sturgeon chromatin led to an increase in the amount of hydrophobic, neutral, and acidic amino acids and a simultaneous fall in the amount of basic amino acids in S as compared with P_{se}. Consequently, the ratio of hydrophobic, neutral, and acidic amino acids to basic amino acids increases in S, in contrast to P_{se}. A similar feature has been observed in the tryptic hydrolysis of somatic chromatin [7].

	P_{se}	S
Hydrophobic*/lysine + arginine	0.15	0.5
Neutral†/lysine + arginine	0.54	1.2
Acidic‡/lysine + arginine	0.19	0.53

*Including valine, leucine, and isoleucine.
†Including threonine, serine, proline, glycine, and alanine.
‡Including aspartic and glutamic acids.

Analysis of the fractions obtained by the chromatography of S and P_{se} on carboxymethyl-Sephadex G-25 showed that among the protein fragments migrating into the solution on trypsin

*Molecular weight of sturin A 3560, of sturin B 3594 (calculated from the results of amino acid analysis).

TABLE 1. Amino Acid Compositions of the Fractions of P_{se}

Amino acid	Amino acid compositions of the fractions, μmole (residues)				
	T_3	T_4	T_5	T_6	T_8
Lysine	0,4 (1)		0,84 (2)		
Histidine	0,33 (0.8)		0.46 (1)		
Arginine		0,54 (2)		0,96 (2)	1,3 (3)
Serine	0,4 (1)	0,5 (2)	0,45 (1)		
Glutamic acid		0,3 (1)			
Proline		0,3 (1)			
Glycine					0,46
Alanine				0,5	
N-terminal amino acid	Serine	Serine	Lysine	Alanine	Glycine

hydrolysis two substances were present in the largest amount, which contained a considerable amount of acidic and neutral amino acids (mol. %: aspartic acid, 15, 8.5; glutamic acid, 18.5, 12.7; serine, 7, 10.4; glycine, 9.5, 10.3; alanine, 7.6, 13) and a small amount of arginine and lysine (2, 6 and 4, 8 mol.%). They probably arose from the nonprotamine proteins present in the sturgeon chromatin. Arginine was absent from the other fractions of S.

The amino acid compositions and N-terminal amino acids of some peptides of the hydrochloric acid extract are shown in Table 1. It must be mentioned that in P_{se} we found a peptide (T_9) consisting of several arginine residues, which was shown by amino acid analysis and by paper electrophoresis using arginine as standard.

The peptides given in Table 1 can be considered as fragments of sturins A and B derived from various parts of their molecules:

Sturin A

Ala-A-A-A-A-A-His-Ala-Ser-Thr-Lys-Leu-Lys-A-A-A-A-A-A-A-His-Gly-Lys-Lys-Ser-
-His-Lys

Sturin B

Ala-A-A-A-A-A-Ser-Ser-A-Pro-Gln-A-A-A-A-A-A-A-His-Gly-A-A-A-A-Gly-A-A

On the basis of the facts given on the amino acid compositions of the peptides, it is possible to put forward the hypothesis that the protamines in the nucleoprotein complex are strongly bound to the DNA and retain this bond even under the action of trypsin on the chromatin.

The role of the nonprotamine proteins in the formation of the nucleoprotein is still not clear. The study of the structure of these proteins and of their possible bonds with the DNA will be continued.

EXPERIMENTAL

The initial material consisted of ripe milt of the Caspian sturgeon collected in the period of the autumn spawning in September, 1977, in the mouth of the Volga (town of Astrakhan'). The milt was frozen at −70°C.

The trypsin (E.C. 3.4.4.4) used in the investigation was a Serva (GFR) product with an activity of 40 units/mg.

Electrophoresis was performed in a Durrum apparatus on Filtrak FN-3 and Whatman 3MM (United Kingdom) papers in a 1 N solution of acetic acid. The arginine and the peptides were determined colorimetrically by Sakaguchi's method [8].

Chromatography was performed in a thin layer of silica gel [9] using the following solvent systems [10]: 1) acetone–isopropanol–25% ammonia solution (9:7:0.5); 2) acetone–isopropanol–25% ammonia solution (9:7:0.7); and 3) chloroform–benzyl alcohol–ethyl acetate–glacial acetic acid (6:4:5:0.2).

Hydrolysis of the Peptides and Proteins. A weighed sample of peptide or protein was treated with a tenfold excess of 5.7 N hydrochloric acid and the mixture was frozen and, then,

after the air had been pumped out, it was heated at 105°C for 24 h. The excess of acid was eliminated by three evaporations with water.

The amino acid compositions of the proteins and peptides were determined on a Hitachi KLA-3B amino acid analyzer.

The chromatin was isolated mainly by the method of V. A. Kadykov et al. [4]. To suppress enzymatic activity, an inhibitor (benzenesulfonyl fluoride) was added to a final concentration of $5 \cdot 10^{-4}$ M. The resulting solution of chromatin in deionized water was clarified at 18,000 rpm for 1 h in a High Speed centrifuge. The supernatant was carefully poured off. The precipitate was stored at +4°C.

The sample of DNP was washed with ethanol, with a mixture of ethanol and ether (1:1), and with ether and was dried at 105°C for 1 h.

The DNA and RNA were isolated from the DNP by the method of Davidson and Smellie [6]. To 5 mg of dry DNP was added 0.25 ml of 0.3 N caustic potash and the mixture was kept at 37°C for 18 h. Then 57% perchloric acid was added to the hydrolyzate to give pH 1. The precipitate that deposited was separated off by centrifuging and was washed with water. The amounts of RNA and DNA were calculated by Spirin's method [5]. The precipitate was washed with ethanol and was dried at 100°C for 1 h. Then the dry precipitate was treated with 5 ml of 0.5 N perchloric acid and was boiled in the water bath for 20 min. The amounts of DNA and DNP were calculated by Spirin's method [5].

It was found that 5 mg of DNP contained 2.75 mg of DNA and 0.1 mg of RNA.

The nucleotide composition was determined on a Varian analyzer. A weighed sample of DNP was previously treated with 0.075 ml of 57% perchloric acid at 105°C for 75 min, after which the reaction mixture was diluted with water to 1 ml.

The amounts of proteins and DNP were calculated from the amino acid analysis of the hydrochloric acid extract of the chromatin. The dry DNP (5.1 mg) was carefully ground in a mortar with a 0.25 N solution of hydrochloric acid, and the mixture was centrifuged at 8000 rpm for 10 min. The completeness of extraction was checked spectrophotometrically. The combined extracts were evaporated, hydrolyzed, and analyzed on the amino acid analyzer.

The DNA and RNA were extracted from the chromatin residue with 0.5 N perchloric acid [1] on the boiling water bath until the absorption at 270 nm was nil. It was found that 5.1 mg of dry DNP contained 1.82 mg of protamines, which corresponds to ~36% of the weight of the chromatin, while 7% of the weight of the DNP was due to nonprotamine proteins.

Hydrolysis of Sturgeon Chromatin with Trypsin. The crude DNP (1.86 g) was incubated in 30 ml of 0.05 N triethylammonium carbonate buffer, pH 8, at 37°C for 1 h, and trypsin was added to a final concentration of 0.15 mg/ml. In a control experiment, the chromatin was kept in the same buffer at 37°C without the addition of trypsin. Hydrolysis was stopped after 3 h by the addition of inhibitor (benzenesulfonyl chloride) to a final concentration of $5 \cdot 10^{-3}$ M. Aliquots of the reaction mixture and of the mixture from the control experiment were analyzed by paper electrophoresis. The reaction mixture was centrifuged at 8000 rpm for 20 min. The solution and the precipitate were analyzed separately.

Fractionation of a Solution of the Trypsin Hydrolysate of the Chromatin. An aliquot of the solution was evaporated, and the residue was dissolved in 5 ml of 0.05 M sodium acetate buffer, pH 5.7, and deposited on a column (1.5 × 50 cm) filled with carboxymethyl-Sephadex G-25 equilibrated with the same buffer. The peptides were eluted by using an exponential sodium chloride gradient. The fractions were checked for the presence of arginine by the Sakaguchi reaction.

The basic peptides from the precipitate were extracted with 0.25 N hydrochloric acid solution (10-ml portions) at 4°C to zero absorption at 220 nm [1]. The combined acid extracts were neutralized with the resin Amberlite IRA-400 (OH^- form) and were freeze-dried.

The mixture of basic peptides was fractionated on carboxymethyl-Sephadex G-25 in 0.05 M sodium acetate buffer, pH 5.7, using an NaCl gradient. Column 2.5 × 50 cm; rate of elution 30 ml/h. Samples with a volume of 3 ml were collected in an automatic fraction collector. The course of fractionation was followed by the spectrophotometric examination of the samples at 220 nm. The fractions obtained were designated T-1-T-11.

Desalting of the Basic Peptides. To each fraction of P_{Se} was added 0.05 M sodium acetate buffer, pH 5.7, to give a concentration of the salt in the solution of 0.3-0.4 M, and it was deposited on a column (2 × 10 cm) filled with Amberlite G-50 resin in the H^+ form equilibrated with the same buffer. The salts were eluted with 0.25 N acetic acid. The peptides were eluted in the cold with 0.1 N hydrochloric acid. The process was followed by means of the spectrophotometry of samples at 220 nm. The eluates were neutralized with Amberlite IRA-400 resin in the OH^- form to pH 5.0.

The dansylation of the peptides was performed by Gray's method [11]. The dansylated amino acids were identified by micro thin-layer chromatography [10] in systems 1 and 2 (direction I) and 3 (direction II).

SUMMARY

The composition of the nucleoprotein complex from sturgeon gonads has been established, and its hydrolysis with trypsin has been effected. It has been shown that the protamines intreact strongly with the DNA by the basic amino acid residues located in various regions of their molecules.

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