ISOLATION OF NONPROTAMINE PROTEINS FROM THE NUCLEI OF THE GONAD CELLS OF THE RUSSIAN STURGEON *Acipenser güldenstadti*

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

UDC 577.112.083

Eight nonprotamine proteins have extracted from the nuclei of the cells of the gonads of the Russian sturgeon with 0.35 M sodium chloride solution followed by fractionation of carboxymethyl-Sephadex G-25 and desalting on Bio-Gel P-2, and their amino acid compositions have been determined.

In recent years, the particular attention of research workers has been attracted to nonhistone chromosomal proteins playing an important role in the specific regulation of the transcription process [1]. The nonhistone proteins of the chromatin of somatic cells have been studied most intensively [2–5], and the proteins of the sex cells have been investigated to a smaller degree [6, 7].

In the chromatin from the gonads of the Russian sturgeon *Acipenser güldenstadti*, we have detected proteins differing from the protamines by a higher content of acidic amino acids, which have been called nonprotamine proteins [8]. The amino acid composition of a mixture of nonprotamine proteins and the hydrophobic/basic, neutral/basic, and acid/basic ratios have been determined for two components [8].

In the present paper we describe the development of a method for obtaining individual nonprotamine proteins in amounts suitable for further studies and give information on the determination on the amino acid compositions of eight proteins.

The nonprotamine proteins were previously [1] isolated from the chromatin, and their yield was fairly low. It is possible that part of the proteins was lost at the stage of purifying the chromatin. The extraction of native nuclei with a 0.14 M solution of NaCl, which is used for the purification of the chromatin, and subsequent analysis of the extract by gel filtration on Sephadex G-25 showed that in addition to the proteins of the nuclear sap (fraction A, Fig. 1a), a considerable amount of nonprotamine proteins passed into the solution (fractions B and C, Fig. 1a). Consequently, these proteins were subsequently obtained from whole nuclei by extraction with 0.35 M NaCl.

The nonprotamine proteins were separated from the other nuclear proteins by two methods: 1) gel filtration on Sephadex G-25. The proteins of the nuclear sap issued in the free volume of the column (Fraction A, Fig. 1b); the nonproteins were separated into two fractions: B and C (Fig. 1b); and 2) by Goodwin's method [9]. The proteins of the nuclear sap were removed from the extract with 2% TCA and the nonprotamine proteins were precipitated at pH 9 with acetone. For preparative purposes the precipitation method proved to more convenient, since the nonprotamine proteins obtained by gel filtrations had to be desalted again before fractionation, which was accompanied by losses of material. By Goodwin's material, from 1 kg of sturgeon milt it was possible to obtain about 2 g of a mixture of nonprotamine proteins.

The proteins obtained by gel filtration were analyzed by electrophoresis on paper in 0.1 N CH_3COOH, and eight components were detected. The nonprotamine proteins isolated by precipitation were subjected to electrophoresis in 10% polyacrylamide gel at pH 8 in the presence of sodium dodecyl sulfate; 10-12 components were identified with molecular weights of from 10,000 to 50,000 daltons.

The mixture of nonprotamine proteins was separated by ion-exchange chromatography on CM-Sephadex G-25 at two pH values — 8.8 and 8.0 (Fig. 2), using a gradient of NaCl

M. V. Lomonosov Moscow State University. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 507-512, July-August, 1982. Original article submitted October 1, 1981.

0009-3130/82/1804-0475$07.50

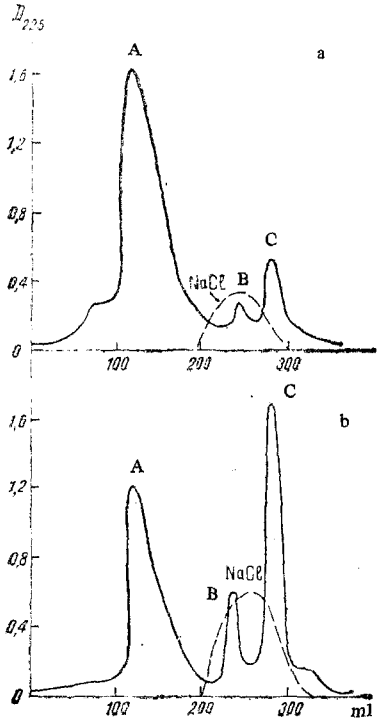

Fig. 1

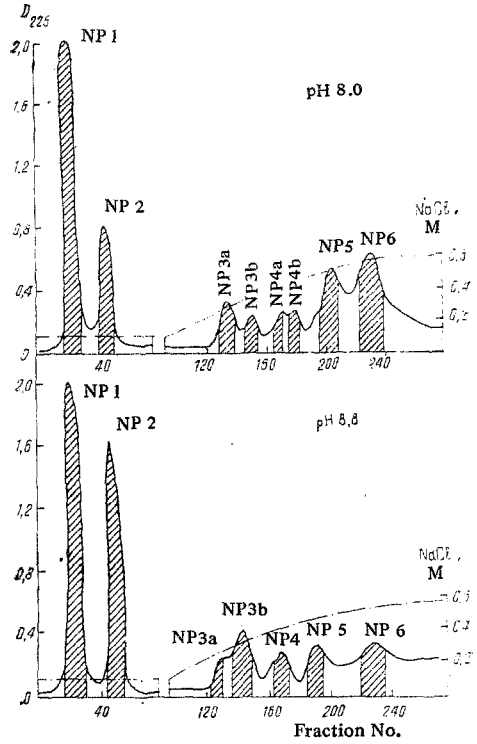

Fig. 2

Fig. 1. Chromatography of salt extracts from the nuclei of the cells of the sturgeon gonads on Sephadex G-25: a) 0.14 M NaCl extract; b) 0.35 M NaCl extract (2.5 × 100 cm columns; elution with 0.1 N CH_3COOH).

Fig. 2. Fractionation of the nonprotamine proteins on CM-Sephadex G-25: a) pH 8.0; b) pH 8.8 (2.5 × 100 cm column, 0.01 Na borate buffer).

concentrations of from 0.1 to 0.6 M. At pH 8.8 fractions NP 1 and NP 2 were obtained in high yield, and at pH 8.0 an increase in the yield of the components NP 5 and NP 6 was observed. This is apparently connected with differences in the basicities of the nonprotamine proteins. The more acidic components NP 1 and NP 2 dissolve better at high pH values, while the solubility of the less basic proteins NP 5 and NP 6 rises with a lowering of the pH. Thus, by using different pH values it is possible to obtain the required components in high yield.

The desalting of the nonprotamine proteins proved to be difficult. It was not possible to desalt them by gel filtration on Sephadexes G-10, G-25, and G-50. The elution volumes of the proteins and of the salt practically coincided, and in some cases the salt issued even before the protein (Fig. 3a, b). This is apparently due to the high affinity of the nonprotamine proteins for the matrix of the Sephadexes.

The best results were obtained by using Bio-Gel P-2 for desalting (Fig. 3c). By changing the amount of deposited proteins and the rate of elution, all the components isolated were desalted on Bio-Gel P-2. In some cases, moreover, an additional separation of the fractions took place (Fig. 4a).

The homogeneity of all the proteins was checked by gel filtration on Sephadexes G-50 and G-25. NP 2, NP 5, and NP 6 proved to be homogeneous.

Fraction NP 1 contained as impurities proteins of the nucleus that had not been precipitated completely by the 2% TCA. It was possible to remove these proteins by gel chromatography on Sephadex G-25 (Fig. 4b). The NP 3 fraction was separated into the components NP 3a, NP 3b, and NP 3c, and NP 4 into NP 4a and NP 4b. As the result of fractionation and subsequent purification, a total of eight components was isolated, four main ones: NP 1, NP 2, NP 5, and NP 6; and four minor ones: NP 3a, NP 3b, NP 4a, and NP 4b.

The amino acid compositions of the nonprotamine proteins are given in Table 1. For comparison, the same table gives the amino acid compositions of two nonhistone proteins from trout testes: HMG-T [6] and H 6 [7].

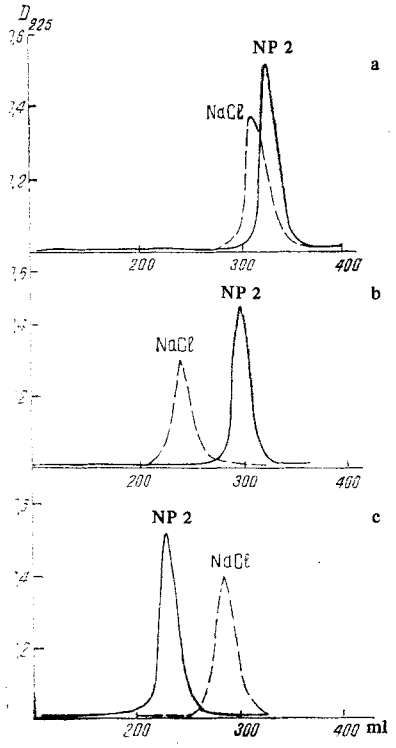

Fig. 3. Desalting of the NP 2 fraction: a) on Sephadex G-50 (2 × 150 cm column; elution with 0.15 N CH_3COOH); b) on Sephadex G-10 (2 × 150 cm column; 0.15 N CH_3COOH); c) on Bio-Gel P-2 (2.5 × 100 cm column; 0.15 N CH_3COOH).

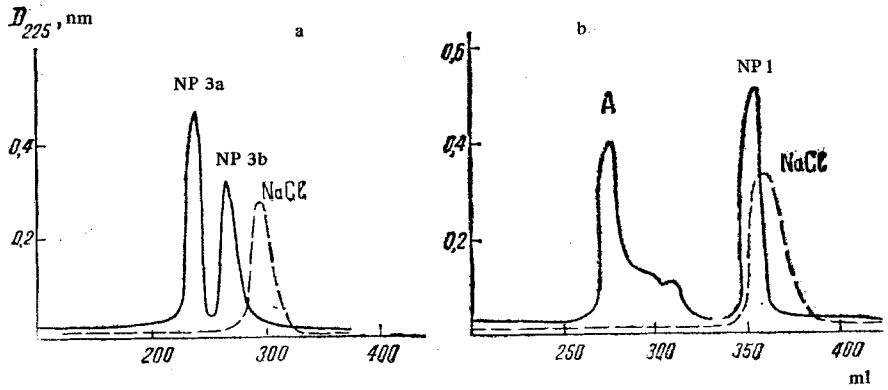

Fig. 4. Gel filtration of fraction NP 3a on Bio-Gel P-2 (a) and separation of the fraction NP 1 on Sephadex G-25 (b). Column 2.5 × 150 cm; elution with 0.15 N CH_3COOH.

The main components of the nonprotamine proteins can be separated into two groups differing by the amounts of acidic and basic amino acids that they contain. In NP 1 and NP 2 the total amount of acidic amino acids exceeds that of the basic ones; the acidic/basic amino acid is 1.2 for NP 1 and 2.0 for NP 2. The proteins NP 5 and NP 6, on the other hand, are distinguished by a high lysine content, which amounts to 20.5% in NP 5 and 21.8% in NP 6; the acidic/basic ratio for both these proteins is only 0.4.

Furthermore, these groups of proteins differ in their glycine and alanine contents. In NP 1, 17.3% of glycine was found, and in NP 2 there was 20.7%; NP 5 and 6 contained considerably less glycine but, in return, they had large amounts of alanine — 17.7% in NP 5 and 21.8% in NP 6.

TABLE 1. Amino Acid Compositions of the Nonprotamine Proteins from the Gonads of *Acipenser güldenstadti* and the Proteins HMG-T [6] and H 6 [7] from Trout Testes

Amino acid	HMG-T [6]	NP 1	NP 2	NP 3a	NP 3b	NP 4a	NP 4b	NP 5	NP 6	H 6
	mole %									
Lysine	15,39	13,2	6,1	6,9	12,1	5 7	3,6	20,5	23 9	23,1
Histidine	1,5	1 2	1 3	1 9	1 3	0,7	1,6	1 3	1 0	—
Arginine	5,26	4,1	1,5	11,2	8,6	3,5	2,7	3,3	2,2	4,2
Aspartic acid	11,45	8,6	6,2	6,2	7 7	6 2	6 4	4,8	4,8	6 7
Threonine	2,99	4,7	4 0	4 6	5 1	5,7	6,5	5,7	6,6	1,6
Serine	4,57	6,8	8,9	9,4	11,0	9,7	13,0	8 6	7,8	5,6
Glutamic acid	9,07	14,2	11,6	11 8	11,0	10,0	9,7	6,9	5,3	6,1
Proline	7,74	9,3	7,5	2,5	3,3	2,4	2,2	1,6	6,1	12 3
Glycine	17 31	17 3	20,7	15,1	15 4	12,3	14 4	4 2	5,1	7 4
Alanine	8,29	9 7	14 8	9,3	8,7	9 3	10,5	15,7	21,8	25,4
Valine	3,90	3,9	3,7	3 8	4 2	4,8	4,8	7,1	7,5	3,4
Methionine	2,04			0,6		1,1				
Isoleucine	1,63	2,0	1,9	2 0	1 4	2,3	1,9	—	—	—
Leucine	2,69	3,1	4,6	4 2	1,9	4,5	3,9	5,5	6,2	1,2
Tyrosine	2,28	0 6	4 4	6,8	2,3	13,1	11,5	1,3	0 7	—
Phenylalanine	3,35	1,0	2,8	3,7	2,8	9,2	7,2	1,5	1,5	—
Ratio of acidic/ basic amino acids	0,93	1,23	2 0	0,9	0 85	1,64	2 0	0,4	0 4	0,4

In a comparison of the amino acid compositions of the nonprotamine proteins from the sturgeon gonads and the proteins isolated from trout testes, attention is attracted by the similarity of the components NP 1 with HMG-T and NP 6 with H 6. This similarity is shown in the amounts of the acidic and basic amino acids and of glycine and alanine. Thus, for NP 1 and HMG-T the ratios of acidic to basic amino acids are of the same order: 1.23 and 0.93, respectively, and for NP 6 and H 6 they are the same — 0.4. In NP 1 and HMG-T, moreover, the amounts of glycine and valine are identical; NP 6 and H 6 contain almost the same amount of alanine.

There are no direct analogs among the nonhistone proteins of trout testes for the components NP 2 and NP 5 from sturgeon gonads. It is possible that such proteins have not yet been detected, or their presence is in fact characteristic for the sturgeon species.

The group of minor proteins we have designated NP 3 consists of at least three components having similar amino acid compositions which are in general outline characteristic for the other proteins of the nonhistone type.

The components NP 4a and NP 4b are present in very small amounts and differ from the other nonprotamine proteins by a higher content of aromatic amino acids.

EXPERIMENTAL

In the investigation we used carboxymethyl-Sephadex G-25 (Pharmacia), Bio-Gel P-2 (Bio-Rad), Filtrak FN3 chromatographic paper, and Standard proteins from Serva.

The initial material used was freshly frozen milt of Russian sturgeon caught in the lower basin of the Volga.

The isolation of the nuclei from the sturgeon gonads was performed by Ando's method [10]. All operations in the isolation of the nuclei and proteins were performed at 4°C.

The milt freed from fat (1000 g) was comminuted and homogenized in 3 liters of 0.14 M NaCl solution containing 0.015 M $MgCl_2$ and 0.5 mM benzenesulfonyl fluoride. The homogenate was passed through three layers of muslin and was centrifuged at 5000 rpm for 10 min. The precipitate of nuclei was washed three times with 0.14 M NaCl. The yield of moist nuclei was 450 g.

Extraction of the Nuclear Proteins. The moist nuclei (20 g) were homogenized in a blender with 50 ml of 0.14 M NaCl at 14,000 rpm for 5 min. The extract was centrifuged at 10,000 rpm for 15 min, clarified by filtration through a No. 4 glass filter, and chromatographed

on a 2 × 150 cm column of Sephadex G-25 (Fig. 1a). The residue of nuclei remaining after the treatment with 0.14 M NaCl was extracted similarly with 50 ml of 0.35 M NaCl, and the extract obtained was also studied by gel filtration on Sephadex G-25 (Fig. 1b).

Isolation of the Nonprotamine Proteins. A. The nuclei (100 g) were extracted three times with 200 ml of 0.35 M NaCl in a blender at 14,000 rpm for 5 min. The extract was separated from the solid matter by centrifugation at 10,000 rpm for 10 min and was clarified by filtration through a No. 4 glass filter. The residue from the evaporation of 200 ml of extract to dryness was dissolved in 5 ml of 0.1 N CH_3COOH, separated from the undissolved residue by centrifugation at 9000 rpm for 10 min, and applied to a column (6 × 100 cm) of Sephadex G-25. The proteins were eluted with 0.1 N CH_3COOH. The course of the separation was monitored spectrophotometrically at 225 nm. The elution curve had the same form as in an analytical experiment (Fig. 1b).

B. Goodwin's method [9]. The moist nuclei (250 g) were mixed in a homogenizer with 500 ml of 0.35 M NaCl at 8000 rpm for 15 min and the mixture was centrifuged (10,000 rpm, 10 min). The operation described was repeated three times. The supernatants were combined, and trichloroacetic acid was added to them to give a concentration of 2%. The resulting precipitate was separated out by centrifugation (5000 rpm, 15 min). To the solution were added ammonia to pH 9 and 4.5 liter of cooled acetone. The precipitate that deposited was separated off by centrifugation and it was washed with cold ethanol and with ether and was dried. This gave 1 g of the total preparation of nonprotamine proteins.

The fractionation of the nonprotamine proteins was carried out on carboxymethyl-Sephadex G-25 at two pH values, 8.0 and 8.8. A solution of 300 g of protein in 30 ml of 0.01 M sodium borate buffer (pH 8.0 or 8.8) containing 0.1 M NaCl was separated from undissolved material by centrifugation and was deposited on a column (2.5 × 100 cm) of Sephadex previously equilibrated with the initial buffer. The column was washed with 500 ml of borate buffer containing 0.1 M NaCl, and then an exponential gradient of NaCl concentrations of from 0.1 to 0.6 M was used. The volume of the mixer was 500 ml, and the rate of elution 24 ml/h. The course of fractionation was followed by the spectrophotometric examination of samples at 225 nm. Fractions corresponding to the chromatographic peaks were combined and subjected to further investigation.

The fractions were desalted by gel filtration on Bio-Gel P-2 in 0.15 N CH_3COOH. Column 2.5 × 100 cm, rate of elution 30 ml/h. Monitoring by spectrophotometry at 225 nm.

The homogeneity of the fractions was checked by gel chromatography on Sephadexes G-50 (column 2.0 × 150 cm, elution with water), and G-25 (column 2.5 × 150 cm, elution with 0.15 N CH_3COOH). The proteins in the eluates were determined from the absorption at 225 nm.

Paper electrophoresis was performed in 1 N CH_3COOH, first at 250 V for 1 h and then at 600 V for 1.5 h. The electrophoretograms were visualized with ninhydrin.

Electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate was done by the Weber–Osborn method [11]. The following were used as marker proteins: bovine albumin, ovalbumin, pepsin, β-lactoglobulin, and cytochrome c. The proteins were incubated in 0.02 M Na phosphate buffer, pH 7.0, containing 1% of SDS and 1% of mercaptoethanol (37°C, 16 h). A 10% polyacrylamide gel containing 0.2% of SDS was used. The time of electrophoresis was 7 h, the current strength 5 mA/tube, and the electrode buffer 0.1 M Na phosphate buffer mixture, pH 7.0, containing 0.1% of SDS. The proteins were stained with a 1% solution of Coomassie Blue in the isopropanol–10% acetic acid–water (25:10:65) system. The excess of dye was washed out with 10% acetic acid.

The amino acid compositions of the nonprotamine proteins were determined after hydrolysis with 6 N HCl (105°C, 24 h) on a Beckman amino acid analyzer.

SUMMARY

A preparative method has been developed for obtaining individual nonprotamine proteins, and their amino acid compositions have been determined.

LITERATURE CITED

1. N. V. El'tsina, Usp. Sovrem. Biol., 87, 3 (1979).
2. J. M. Walker, G. H. Goodwin, and E. W. Johns, Eur. J. Biochem., 62, 461 (1976).
3. J. H. Walker, B. M. Parker, and E. W. Johns, Int. J. Peptide Protein Res., 12, 269 (1978).
4. J. M. Walker, G. H. Goodwin, and E. W. Johns, FEBS Lett., 100, 394 (1979).

5. J. M. Walker, J. R. B. Hasting, and E. W. Johns, Eur. J. Biochem., 76, 461 (1977).
6. D. C. Weston, E. H. Peters, and G. H. Dixon, Eur. J. Biochem., 74, 53 (1977).
7. D. C. Watson, N. C. Wong, and G. H. Dixon, Eur. J. Biochem., 95, 193 (1979).
8. A. N. Bulanova, E. P. Yulikova, I. V. Kozhushkova, and A. B. Silaev, Khim. Prir. Soedin., 429 (1980).
9. G. H. Goodwin, R. H. Nicolas, and E. W. Johns, Biochem. Biophys. Acta, 405, 280 (1975).
10. S. Kawashima and T. Ando, J. Biochem. (Tokyo), 83, 1117 (1978).
11. K. Weber and M. Osborn, J. Biol. Chem., 244, 4406 (1979).