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Characterization of protamines from four avian species

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No data are available on the protamines of birds, with the exception of galline. We have characterized the protamines from four species of birds belonging to four different orders. All of them have very similar properties. They have been purified by carboxymethylcellulose chromatography and analyzed with respect to amino acid composition and electrophoretic behaviour. They are very arginine-rich proteins (63.4–67.3%) but do not contain lysine. Serine (12.0–18.2%), tyrosine (5.8–9.0%) and glycine (4.5–7.1%), along with arginine, make up the bulk of the amino acid residues in these molecules. The electrophoretic mobility of bird protamines in acetic acid-urea-polyacrylamide gels is intermediate between that of somatic histones and salmine. The molecular size, estimated from amino acid analysis and electrophoretic migration, is 65 ± 5 amino acid residues.

Chromatin; Protamine; Nuclear protein; (Sperm)

1. INTRODUCTION

The sperm proteins are highly basic molecules, very variable in animal evolution, that are found in the sperm nuclei of most animal species [1]. In vertebrates, the composition and sequence of fish and mammalian protamines are well known [2,3]. In birds, only the primary structure of fowl protamine is known [4] and in the classes Amphibia and Reptilia, only approximate results on sperm protein composition in a few species have been published [5,6]. Among the known vertebrate protamines, arginine is the main and sometimes only basic residue, being organized largely in 4–7 unit groupings along the molecule [2–4]. The electrostatic interaction between arginine residues and anionic groups of DNA is the main cause of condensation of sperm nuclei. This interaction could possibly be regulated in vivo through reversible post-transcriptional modifications of some of the other amino acid residues obtained in the protamine molecule, such as enzymatic phosphorylation-dephosphorylation of serines [7] and, additionally in mammals, the making and breaking of cystine bridges [8]. The reversible character of the arginine-DNA interaction is in conformity with the decondensation of sperm nuclei during fertilization [9] and justifies the presence of amino acids capable of modification. From this point of view, the fowl protamine is particularly interesting among the vertebrates due to its considerable content of serine residues (11 serines compared to 2–4 in fish and mammals). Mollusc protamines also have a very large amount of serine, as reviewed by Subirana [10]. Here, we have carried out the purification of 4 bird protamines from different orders and have analysed their amino acid composition, molecular size and electrophoretic behaviour.

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2. MATERIALS AND METHODS

2.1. *Isolation of nuclei from spermatozoa and advanced testicular spermatids*

To obtain protamine-containing nuclei, we used testis and deferent ductus from birds more than 1

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year old. The following amounts of tissues were used: 40 g from the duck *Anas platyrhynchos* (order Anseriformes); 15 g from the quail *Coturnix coturnix* (order Galliformes); 8 g from the pigeon *Columba livia* (order Columbiformes) and the parakeet *Melopsittacus undulatus* (order Psittaciformes). Each tissue was homogenized separately, in 2 M sucrose/0.1% Triton X-100 containing 50 mM benzamidine chloride and 10 mM citric acid (final pH 2.3) as proteolytic inhibitors. After centrifugation at 58000 × *g* for 2 h, the nuclear sediment was homogenized in 10 mM Tris, pH 8.0/20 mM EDTA and nuclei were collected again by centrifugation (6000 × *g* for 10 min). The new sediment was resuspended in 10 mM Tris, pH 8.0, sonicated for 6 min and centrifuged. The last sediment contained nuclei from spermatozoa and advanced testicular spermatids [11]. All operations were performed at 4°C.

2.2. *Protamine purification and analysis*

Nuclei were homogenized in 0.25 M HCl. The extracts were precipitated with 6 vols cold acetone and thoroughly washed with acetone/0.1 N HCl (6:1, v/v) and finally with acetone. The proteins were dried and dissolved in 0.05 N acetate buffer, pH 6/0.2 M NaCl containing 50 mM benzamidine chloride, then loaded onto a 7 × 1 cm carboxymethylcellulose column (Whatman CM 52). The column was washed first with 0.05 N acetate buffer, pH 6.0/0.1 M NaCl to remove benzamidine chloride. This was followed by a wash with 0.05 N acetate buffer, pH 6.0/0.8 N NaCl to discharge somatic histones. Finally, a 0.8–1.6 M NaCl gradient was applied to elute the protamines. The collected fractions were read at 225 nm, precipitated with 25% trichloroacetic acid and washed with acetone. Duck protamine obtained in this manner was purified further by electroelution using preparative acrylamide gels [12]. For electrophoresis we utilized the acetic acid/urea method of Panyim and Chalkley [13], since protamines precipitate in SDS solutions. Amino acid analyses were performed as in [14].

3. RESULTS

3.1. *Protamine purification*

The 0.25 N HCl extract from spermatozoa and spermatidal nuclei in all 4 species examined contains histones, protamines and some other minority proteins. Somatic histones are eluted from the CM-cellulose column upon washing with 0.05 N acetate/0.8 M NaCl buffer, while protamines from quail, pigeon and parakeet are eluted practically without any contamination at ionic strengths within the range 1.4–1.6 M NaCl. Duck protamine, however, shows heterogeneous behaviour on the column. It can be seen in fig.1A that part of this protamine is eluted at 0.9 M NaCl, together with some minor bands, while the rest of

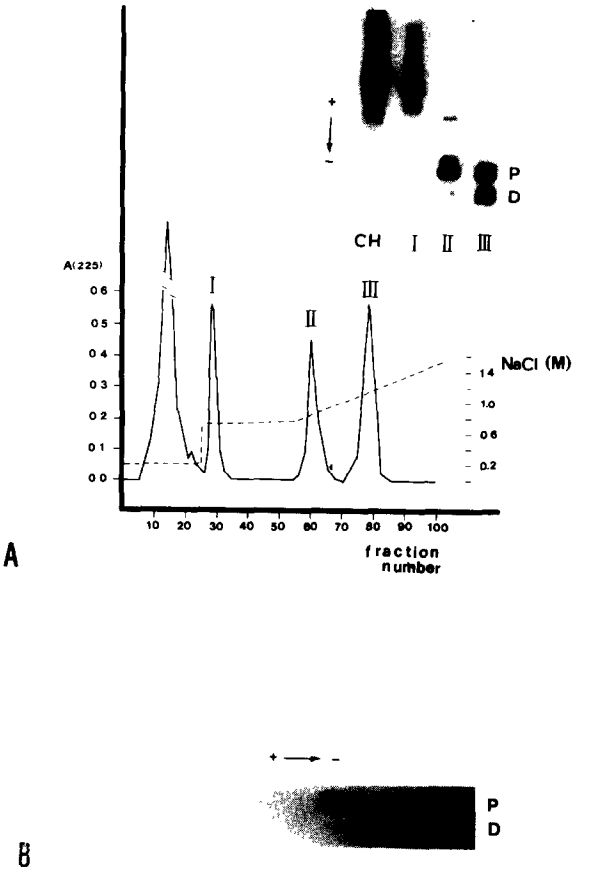

Fig.1. (A) Heterogeneous behaviour of duck protamine upon CM-cellulose chromatography. The first peak from the column (not numbered) is due to benzamidine chloride. P, protamine; D, peptide derived from partial degradation of P; CH, core histone standard. (Insert) Acetic acid-urea-polyacrylamide gel electrophoretogram of fractions I–III. (B) Electrophoretogram of duck protamine (P) and degradation peptide (D) after electroelution from a preparative gel.

the protamine is eluted at 1.2 M NaCl, together with a peptide having higher electrophoretic mobility. The amount of this peptide is directly related to the process of obtaining nuclei, being highest when no proteolytic inhibitors are used. We suspect, therefore, that it is a degradation product of protamine, similar to those mentioned by Nakano et al. [4] in their chicken protamine studies. Although the protamine and its degradation product elute together by column chromatography, they are well separated by preparative electrodialysis (fig.1B).

3.2. *Electrophoretic mobilities and amino acid analysis*

In fig.2 we observe that the electrophoretic mobilities of bird protamines from the 4 orders are similar. Chicken protamine, salmine and somatic histones are used as electrophoretic markers in this experiment. The quail preparation (fig.2e) also shows a lighter band moving more slowly than protamine. Table 1 indicates the amino acid composition of bird protamines. The estimated molecular size as a function of amino acid analysis and electrophoretic behaviour gives a value of 65 ± 5 amino acids. Although the overall compositions are similar in each bird, glutamic acid and leucine are found only in the parakeet protamine. Pigeon protamine (fig.2d) has the simplest amino acid composition.

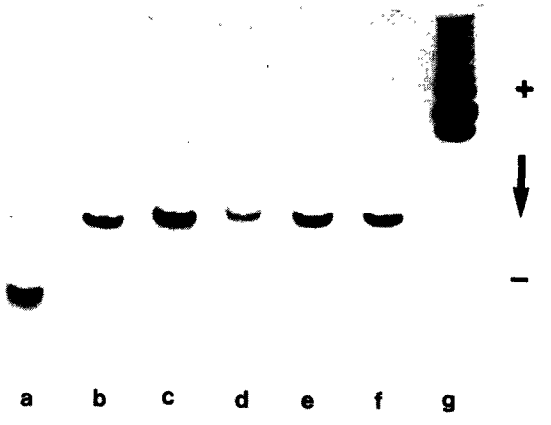

Fig.2. Acetic acid-urea polyacrylamide gel electrophoretogram of purified bird protamines. Salmine standard (a), parakeet protamine (b), duck protamine (c), pigeon protamine (d), quail protamine (e), chicken protamine standard (f), and core histone standard (g).

Table 1

Amino acid composition (in mol%) of protamines from quail, pigeon, duck and parakeet compared to chicken protamine

	Rooster[a]	Quail	Pigeon	Duck	Parakeet
Arg	58.5	67.3	63.4	63.4	65.7
Thr	1.6	1.4	1.6	1.2	–
Ser	17.2	12.0	18.2	14.0	13.5
Glu	–	–	trace	–	4.4
Pro	3.5	–	–	2.5	–
Gly	8.6	6.5	7.1	6.5	4.5
Ala	3.2	1.9	1.6	1.5	–
Val	1.7	1.6	–	1.9	2.0
Leu	–	–	–	–	2.1
Tyr	6.2	8.1	8.1	9.0	5.8
X[b]	–	–	–	–	2.1

[a] From Nakano et al. [4]

[b] X elutes at pH 6.4 just before histidine. It has not yet been identified

4. DISCUSSION

Protamines and sperm basic proteins have a general function, namely the reversible condensation of nuclei. In addition, certain types of sperm basic proteins display particular functions, for example displacement of proteins preexisting in nuclei, such as histones or intermediate proteins. In the case of the avian species examined, the results show that 90–96% of bird protamines are formed only by 4 types of amino acid residues: arginine, serine, tyrosine and glycine. Arginine (58.5–67.3%) and serine (12.0–18.2%) are the most important quantitatively, while tyrosine and glycine are present in the ranges 5.8–9.0% and 4.5–8.6%, respectively (we include chicken protamine in these values). Also, arginine and serine are the two amino acid residues almost always found in protamines or sperm basic proteins from all species examined. It is obvious that they relate to the universal function of sperm basic proteins due to their great capacity of interaction: in the case of the guanidino group of arginine by interaction with DNA and in the case of serine by

phosphorylation of the hydroxyl group, possibly regulated by extracellular stimuli [9,15]. Tyrosine function is not so clear, although it is a frequent component of sperm basic proteins [10]. In the five bird protamines examined thus far there are 4–6 residues per molecule. These could play a role in the placement and interaction of protamine with DNA by hydrogen bonding with the base pairs [16]. The relatively high glycine concentration in bird protamines is also a characteristic which makes them different from the known protamines of bony fish and mammals. Other interpretations regarding the presence of particular amino acid residues can be made. However, it is particularly interesting to note the increase in cooperativity obtained in the interaction of protamine compared with polyarginine [17]. It is also striking to note the overall similarity amongst the bird protamines we have studied. Other zoological classes show a much more extensive variation in size and composition of sperm basic proteins [10]. This might be related to the very uniform method of reproduction within this class of animals, namely internal fertilization and egg laying.

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