Fractionation by high-performance liquid chromatography and characterization of the highly related protamines from the sperm of the marine worm *Platynereis dumerilii*

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The protamines from the emitted sperm nuclei of the marine annelid *Platynereis dumerilii* have been purified from the whole basic protein by gel filtration and ion-exchange chromatography and further fractionated by reverse-phase high-performance liquid chromatography. Four main fractions were obtained and characterized by their electrophoretic mobilities and amino acid compositions. All these protamines are characterized by high arginine and serine contents and by the presence of equivalent amounts of asparagine, threonine and valine. Each of these fractions is heterogeneous. After re-fractionation the amino acid composition and the N-terminal sequence of the samples indicate that each protein corresponds to a variant of the same family of highly related protamines.

Protamine (Sperm) HPLC

1. INTRODUCTION

During spermatogenesis, important biological and morphological changes take place in the cellular and nuclear organization. In a number of animal species, spermatogenesis is characterized by the replacement of the somatic type histones by a set of small arginine-rich proteins: the protamines. However, histones are maintained in chromatins of spermatozoa in some vertebrates [1–3] as well as in many invertebrates such as echinoderms [4,5], crustaceans [6] and molluscs in which histones are detected either alone [7] or in association with protamines [8–10].

In most of the mammals (bull, boar, ram and stallion) as well as in the rooster, amino acid compositions and sequence data indicate that each species contains a single homogeneous protamine [11–14]. This contrasts with the situation observed in human [15] and fish [16] protamines in which heterogeneity of the protamine fraction results either from the presence of different proteins or from the existence of a set of variants with the same structural basis. In most of the teleost fishes [16] and in the squid [8], protamines occur as a mixture of closely related molecules. The second situation is illustrated by the protamines of the dogfish [17]: recent studies have shown that sperm nuclei in this species contain four different basic proteins. One is a typical arginine-rich protamine, whereas the three other components are cysteine-rich protamines similar to that found in mammals.

Little information is available concerning the homogeneity or heterogeneity of invertebrate protamines. The only known amino acid composition concerns whole protamines. In bivalve molluscs, the presence of a specific arginine-, lysine-rich protamine-like protein is generally observed [18–20] with almost equal amounts of both basic amino acids. Protamines from the sperm of gastropods [8,21], cephalopods [8] and annelids [10,22,23] have the same major characteristics: the

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two basic amino acids arginine (46%) and lysine (11%) are present and high amounts of serine (17%) and alanine (9%) are observed. These four amino acids represent approx. 84% of all the amino acids. Cysteine, aromatic amino acids and methionine are absent.

In the purified nuclei of the emitted sperm of the annelid *Platynereis dumerilii*, a rambling polychaete, the histones are maintained in the chromatin. The presence of additional specific H1 histones [24] and the coexistence of a complex family of protamines can give us information on the respective role of these proteins in the mechanism of condensation of the DNA which occurs in the nucleus of the male gamete during spermatogenesis. The prerequisite for these studies is the determination of the structure of these proteins. The work reported here deals with the isolation and characterization of the highly related protamines from the emitted spermatozoa of the annelid. Their amino acid compositions and their amino-terminal sequences reveal that each of them corresponds to a variant of the same protein which is representative of the invertebrate protamine-type.

2. MATERIALS AND METHODS

Marine annelids were fished at the 'Station Biologique d'Arcachon' (France) at the first and last quarters of the moon at the beginning of July [25]. Male worms were placed in a bucket and the emission of sperm was obtained by thermal shock in the presence of a few females [26].

The purification of the sperm nuclei and the extraction of basic proteins have been described [10].

2.1. *Isolation of protamines*

Protamines were obtained either as in [10] after chromatography of the P10 protamine extract on carboxymethylcellulose, or directly obtained after chromatography of the 5% $HClO_4$ insoluble basic proteins on a CM-cellulose (Whatman CM 52) column (2 × 37 cm) equilibrated with 0.2 M NaCl in 0.02 M ammonium acetate/acetic acid, pH 5.0. The proteins were dissolved in the same buffer and applied to the column. The elution was performed with a linear gradient from 0.2 to 1.7 M NaCl in acetate buffer. Proteins were dialysed against 0.01 M HCl (Spectrapor membrane tubing, M_r cut off: 3500) and dried under vacuum.

2.2. *Protamine fractionation by high-performance liquid chromatography*

The fractionation of the protamine sample was performed by reverse-phase high-performance liquid chromatography using an LKB 2 150/2 152 apparatus with a fixed wavelength ultraviolet monitor. The protein sample (2 mg) dissolved in 0.2 ml of 0.08% trifluoroacetic acid (TFA) was loaded onto a Lichrosorb RP 18 (5 μm) (0.4 × 25 cm) equipped with a Lichrosorb RP 18 (7 μm) guard column (0.4 × 3 cm), equilibrated with 0.08% TFA. Proteins were eluted with a complex gradient (see fig.2A,B for details) of 0–50% acetonitrile in 0.08% TFA for 75 or 110 min. All runs were carried out at room temperature at a constant flow rate of 0.8 ml/min. The solvents were degassed for 10 min by sonication and for 1 min under vacuum with stirring prior to use.

2.3. *Analytical gel electrophoresis*

Histone and protamine preparations were controlled by polyacrylamide gel electrophoresis as in [27].

Samples dissolved in 8 M urea, 0.9 M acetic acid and 0.5 M β-mercaptoethanol were run in slab gels (14 × 16 × 0.075 cm) containing 15% acrylamide and 2.5 M urea.

2.4. *Amino acid analysis*

Protamine samples were hydrolysed in vacuo at 110°C in 6 M HCl for 24 and 72 h. One drop of 1% phenol was added to each sample to prevent excessive degradation of tyrosine. Amino acid analyses were performed on a Beckman 119 CL amino acid analyser.

2.5. *Amino-terminal sequence determination*

The amino-terminal sequences of the protamines were determined by manual Edman degradation performed as in [28]. Phenylthiohydantoin derivatives of amino acids were identified by high-performance liquid chromatography on a column of C 18 micro-Bondapack as in [29] using a Waters Associates apparatus.

3. RESULTS AND DISCUSSION

The basic proteins extracted from the emitted sperm nuclei of the annelid *Platynereis dumerilii* consist of a complex mixture of histones and protamines (fig.1, lane 2). The protamine fraction can be separated from the 'core' histones by chromatography of the 5% $HClO_4$-insoluble basic proteins (fig.1, lane 3) on carboxymethyl-cellulose as shown in [10]. It appears highly heterogeneous by polyacrylamide gel electrophoresis and is constituted of slow moving components (fig.1, lanes 4 and 5). A part of the protamine fraction (P1) comigrates with H2A histone and the other part migrates more slowly than the *Platynereis* H1 histone and comigrates with calf thymus H1 histone.

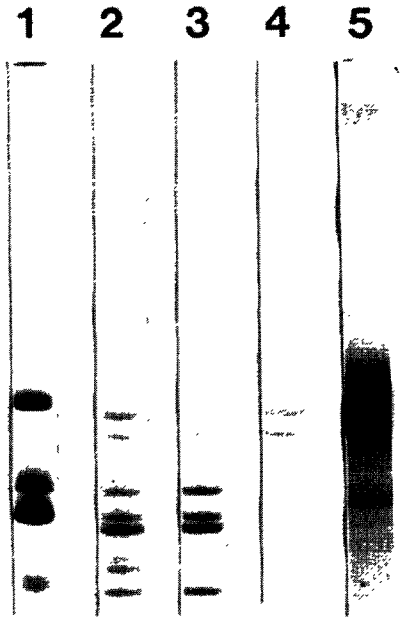


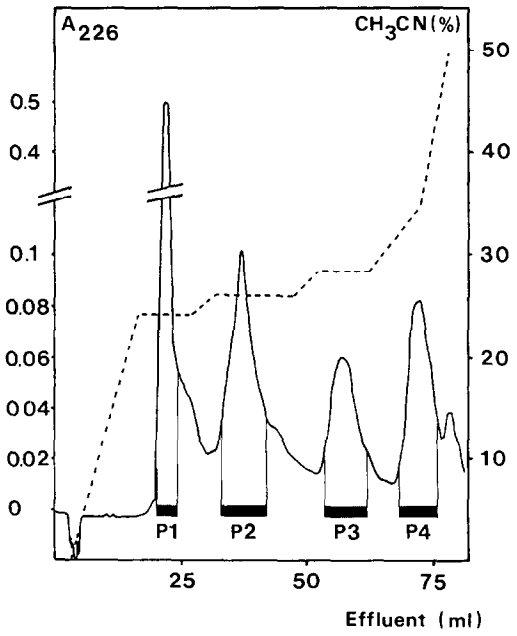
Fig.1. Analytical polyacrylamide gel electrophoresis of whole histone from calf thymus (slot 1), whole basic proteins from the annelid sperm (slot 2), 5% $HClO_4$-insoluble proteins (slot 3), and protamine fraction obtained after CM-cellulose ion-exchange chromatography (slot 4) with overloading sample (slot 5). Electrophoresis was on 0.9 M acetic acid, 2.5 M urea and 15% acrylamide slab gels. Gels were stained overnight with amido black in methanol/acetic acid/water (200:70:730, v/v).

The same electrophoretic heterogeneity is observed with protamine sample extracted from one worm.

Four fractions (P1–P4) were separated by reverse-phase high-performance liquid chromatography (fig.2A). They were eluted at 24, 26, 28 and 35% acetonitrile, respectively, and separately pooled as shown. The first three fractions appear heterogeneous by gel electrophoresis (fig.2B). The amino acid composition of the unfractionated protamine sample and of the four fractions are presented in table 1. As three or four analyses were performed on each of the protamine samples, the observed differences are highly significant. These proteins are devoid of cysteine, tyrosine and histidine. They are mainly characterized by a high content of arginine (42–46% of the total number of residues) and of serine and alanine. Both basic amino acids (arginine and lysine) are present in the four fractions. These four amino acids represent 80–84% of the total number of residues. These major characteristics are generally found in invertebrate protamines, especially in protamines from gastropods [8–21] and annelids [22,23].

The progressive increase of the amount of hydrophobic residues from P1 to P4 and the parallel increase of the size of the molecule could explain the increasing retention time in reverse-phase high-performance liquid chromatography. Threonine is found at the amino terminal end and arginine is found at the carboxy terminal end of the four protamine fractions. The same number of valine (1) (which was used to calculate the number of residues), aspartic acid (2) and threonine (2) residues is observed in all protamines. Likewise, the amount of glutamic acid, proline and lysine is the same for P2, P3 and P4, and the number of leucine is the same for P1 and P2 and for P3 and P4, respectively. All these data strongly suggest that P1, P2, P3 and P4 are four closely related families of protamine molecules. The confirmation of the size of these proteins by SDS-polyacrylamide gel electrophoresis was rendered impossible because all four fractions are insoluble in SDS. However, amino acid analyses indicate that their molecular mass must be between 6 and 8 kDa.

The amino acid sequence at the terminus of P1 is Thr-Ala-Lys (and Arg)-Arg-Asn-Ala (and Ser)-. Two different phenylthiohydantoin derivatives



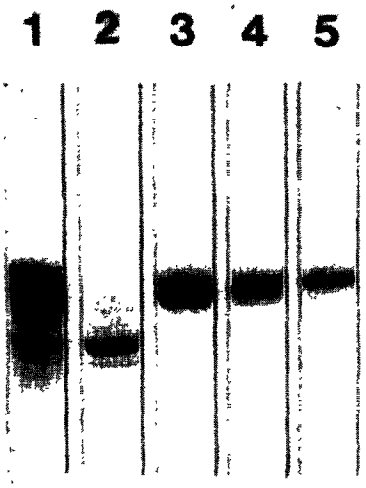


Fig.2. (A) Separation of the 4 protamine fractions by high-performance liquid chromatography. 2 mg protein obtained after CM-cellulose chromatography (slot 4 in fig.1) were dissolved in 0.2 ml of 0.08% TFA, kept overnight at 20°C, centrifuged (10000 × *g* for 1 min) and loaded onto a Lichrosorb RP 18 (5 μm, 0.4 × 25 cm) column equilibrated with 0.08% TFA. The proteins were eluted with a gradient of 0% acetonitrile (dotted line) for 2 min, from 0 to 24% for 13 min, 24% for 10 min, from 24 to 26% for 5 min, 26% for 15 min, from 26 to 28% for 5 min, 28% for 10 min, from 28 to 35% for 10 min and from 35 to 50% for 5 min. Fractions of 1 ml were collected at a constant flow rate of 0.8 ml/min. The elution of proteins was monitored at 226 nm. (B) Polyacrylamide gel electrophoresis of protamine fractions from the sperm of the annelid. Slot 1, unfractionated protamine; slot 2–5, protamine P1–P4 of A, respectively. See legend to fig.1.

were found after the second and the fifth cycles and were relevant to the high degree of heterogeneity already observed by gel electrophoresis, inside the P1 protamine fraction.

For P2 and P3, the N-terminal sequence is Thr-Ala-Lys-Lys (and Arg)-Asn-Ser-: two different phenylthiohydantoin derivatives were found after the third cycle. P4 was characterized by the following N-terminal sequence: Thr-Ala-Lys-Arg-Asn-Ser without replacement of amino acids. A great similarity in N-terminal sequences is found in the four protamines. It is in all cases:

Thr-Ala-Lys-Arg-Asn-Ser
or or or
Arg Lys Ala

Such degrees of heterogeneity were also observed when a longer gradient was used for the fractionation of the whole protamine sample by high-performance liquid chromatography (fig.3A). In this way, we have collected eight fractions for P1, three for P2 and P3 and P4 appears to be homogeneous (electrophoretic control in fig.3B).

The differences observed in the amino acid compositions of each protamine fraction P1–P4 appear to be highly significant and are in part confirmed by the partial primary structure we have now determined: presence of one proline and one glutamic acid in the P2, P3 and P4 fractions – absence of these two amino acids in P1; presence of two residues of leucine in P3 and P4 and only one in P1 and P2. These differences determine the classification in four families of protamine. Within these protamine families, the variants exhibit differences in the amount of the amino acids already highly represented (Arg, Ser and Ala). Inside each family, the variants show no difference as far as the characteristics which place them in this family are concerned. For example, the eight variants of P1 possess two residues of glycine, one leucine but neither glutamic acid nor proline.

Table 1

Amino acid composition of the four protamine fractions from the sperm of *Platynereis dumerilii*

	Unfractionated protamine (%)	P1		P2		P3		P4	
		%	MR	%	MR	%	MR	%	MR
Asp	3.5	3.81	1.94 (2)	3.33	1.90 (2)	3.45	2.18 (2)	3.02	2.10 (2)
Thr	3.6	4.01	2.05 (2)	3.18	1.82 (2)	3.13	1.98 (2)	3.24	2.25 (2)
Ser	25.8	20.97	10.70 (11)	20.48	11.70 (12)	20.30	12.85 (13)	20.00	13.89 (14)
Glu	1.8	–	–	0.98	0.56 (1)	1.21	0.77 (1)	0.81	0.56 (1)
Pro	1.7	–	–	1.09	0.62 (1)	1.24	0.78 (1)	1.30	0.90 (1)
Gly	4.8	3.90	1.97 (2)	4.77	2.73 (3)	4.97	3.15 (3)	5.12	3.56 (3–4)
Ala	11.3	11.98	6.11 (6)	11.60	6.63 (7)	12.41	7.85 (8)	11.66	8.10 (8)
Val	2.2	1.96	1.00 (1)	1.75	1.00 (1)	1.58	1.00 (1)	1.44	1.00 (1)
Ile	0.9	0.43	0.22 –	0.47	0.27 –	0.65	0.41 –	0.56	0.39 –
Leu	3.0	1.72	0.88 (1)	2.39	1.37 (1)	2.82	1.78 (2)	2.45	1.70 (2)
Phe	0.8	–	–	–	– –	0.59	0.37 –	0.58	0.40 –
Lys	7.5	4.75	2.42 (2)	6.36	3.63 (4)	5.84	3.70 (4)	6.17	4.28 (4)
Arg	39.2	46.46	23.10 (24)	43.59	24.91 (25)	41.92	26.47 (27)	43.65	30.31 (30)
TMR	–	–	(51)	–	(59)	–	(64)	–	(68–69)
N-terminal			Thr		Thr		Thr		Thr
C-terminal			Arg		Arg		Arg		Arg

3 or 4 analyses were performed for each of the 4 protamine fractions. Results are expressed in molecules per cent molecules of amino acid. Values for threonine and serine are zero-time extrapolations. The experimental precision is ± 0.05%. MR, molar ratio of each amino acid to valine. The experimental precision is ± 0.2%. Values in parentheses are approximative molar ratios to valine. TMR, approximative total molar ratio of all the amino acids to valine. Traces of methionine (0.6%), tyrosine (0.5%) and histidine (0.3%) are present in the unfractionated protamine and absent in purified fractions

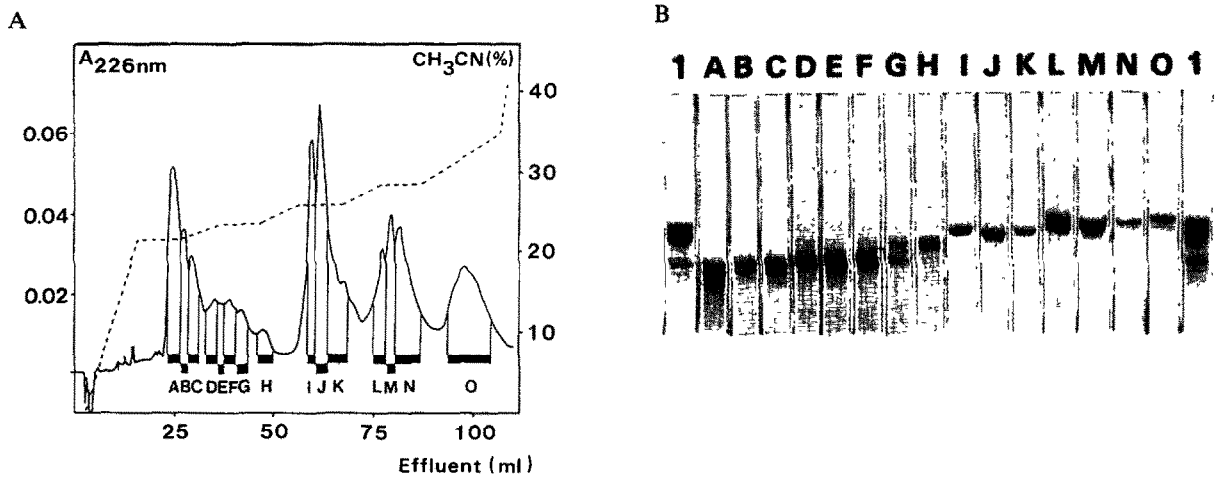


Fig.3. (A) Fractionation of the protamine family by high-performance liquid chromatography. See legend to fig.2A. The proteins were eluted with a linear gradient of 0% acetonitrile (dotted line) for 2 min, from 0 to 21% for 13 min, 21% for 10 min, from 21 to 23% for 10 min, 23% for 10 min, from 23 to 26% for 10 min, 26% for 10 min, from 26 to 28% for 10 min, 28% for 10 min, from 28 to 35% for 20 min and from 35 to 50% for 5 min. (B) Polyacrylamide gel electrophoresis of protamine fractions collected after high-pressure liquid chromatography. Slot 1, unfractionated protamine; slot A–O, fractions A–O in A, respectively. See legend to fig.1.

The apparent heterogeneity of these proteins on polyacrylamide gel electrophoresis can be attributed to the presence of structural variants. However, digestion of the protamines with alkaline phosphatase does not modify the electrophoretic mobility of this sample (gels not shown). Moreover no phosphoamino acids (threonine and serine) were identified after 2 h hydrolyses of the protamine sample with 6 M HCl.

The respective primary structure of the variants contained in the protamine fractions P1, P2, P3 and P4 and the variations in the positions of amino acids specific to each variant are presently under investigation and their determination will give us information about the complexity of the basic protein equipment of the condensed chromatin.

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