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Primary structure of rabbit sperm protamine, the first protamine of its type with an aberrant N-terminal

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Rabbit protamine was extracted from *S*-(pyridylethylated) sperm cell nuclei with hydrochloric acid and then isolated by reversed-phase HPLC. The primary structure was determined by amino acid sequence analysis of the total protein and of fragments obtained by digestion with endoproteinase Lys-C and thermolysin. The protamine contains 49 amino acid residues and is clearly homologous with mammalian type 1 protamines, 47% of the positions being invariant. Surprisingly, rabbit protamine possesses an N-terminal valine residue, whereas all mammalian and several non-mammalian protamine sequences of this type start with alanine, the N-terminal region being remarkably conserved during evolution.

Protamine; Sperm histone; DNA-binding protein; Amino acid sequence; HPLC; (Rabbit sperm cell)

1. INTRODUCTION

In many animal species the chromatin organization changes dramatically during the haploid phase of spermatogenesis [1,2]. The characteristic nucleosome structure of the somatic cell nuclei vanishes and instead a highly condensed and inert type of chromatin is formed. In parallel, histones and non-histones are gradually substituted by the small, very basic protamines. Much detailed information is available about the structural organization of somatic chromatin and histone-DNA interaction (cf. [3,4]). In contrast, the understanding of sperm chromatin structure and protamine-DNA interaction is still fragmentary.

Most mammalian species seem to contain a single type of protamine in their sperm cells, but in man [5–8] and mouse [9] two main types of protamines (types 1 and 2) have been found. These two types show no convincing structural homology. However, human and mouse type 1 protamines are homologous with the single (or main) protamines of boar [10], ram [11], bull [12] and stallion [13,14]. It could be expected that proteins forming the corresponding, compact complexes with DNA show a high degree of mutual resemblance and are evolutionary conserved, and indeed the six protamine sequences contain similar, highly basic arginine clusters, which bind to the acidic DNA. Furthermore, certain half-cystine residues, involved in disulfide stabilisation of sperm chromatin, as well as the N-terminal hexapeptide sequence Ala-Arg-Tyr-Arg-Cys-Cys- have so far appeared as invariant [14]. However, in the present communication the first type 1 protamine with an aberrant N-terminal is reported, the rabbit sperm protamine.

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2. EXPERIMENTAL

2.1. *Preparation of spermatozoa*

Epididymal tissue was prepared from New Zealand rabbits, minced with scissors and swirled with phosphate-buffered saline. The suspended sperm cells were filtered through a 100 μm nylon gauze, and washed with phosphate-buffered saline.

2.2. *Isolation of protamine*

Rabbit protamine was purified in a similar way as other protamines [7,14]. Spermatozoa from 3 rabbits (7.7×10^7 cells)

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were successively extracted with 10 ml of 0.5 M acetic acid for 12 h, 5 ml of 8 M guanidine hydrochloride, 0.1 M acetate, pH 4, for 1.5 h using sonification, and water, nuclear material being recovered by centrifugation between extractions. The nuclear pellet was then suspended in 5 ml of 0.1 M Tris chloride, pH 7.5, with 250 μl mercaptoethanol for 1 h, subsequently 375 μl 4-vinylpyridine (Fluka) were added. After 1.5 h, *S*-(pyridylethylated) protamine material was extracted from the pelleted nuclei with 5 ml of 0.5 M hydrochloric acid for 1 h. The extract was directly applied to HPLC.

2.3. *HPLC procedure*

Reversed-phase high-performance liquid chromatography (HPLC) was performed with a Milton Roy chromatography unit, a 25 × 0.46 cm Nucleosil C-18 column (Macherey-Nagel; 10 μm particles, 300 Å pores), and various gradients between 0.1% (v/v) trifluoroacetic acid in water and in acetonitrile at a flow of 1 ml/min and room temperature.

2.4. *Gel electrophoresis*

Vertical slab gels (0.1 × 8.5 × 10 cm) containing 15% acrylamide, 2.5 M urea and 0.9 M acetic acid as electrode solution were used [14].

2.5. *Proteolytic digestion*

Isolated *S*-(pyridylethylated) protamine (20 nmol) was incubated with 20 μg endoproteinase Lys-C (Boehringer, Mannheim; 3 U/mg) in 600 μl of 0.1 M Tris chloride, pH 8.6, for 2 h at 37°C, acidified and subjected to HPLC. The C-terminal fragment (6 nmol) isolated by HPLC from the previous cleavage was further digested with 1.3 μg thermolysin (Calbiochem; 9390 U/mg) in 100 μl of 0.1 M ammonium hydrogencarbonate, pH 7.8, 1 mM calcium chloride for 0.5 h at 37°C, acidified and applied to HPLC.

2.6. *Amino acid and sequence analysis*

Compositions were determined after hydrolysis in 0.2 ml of 6 M hydrochloric acid for 24 h at 110°C in a Biotronik amino acid analyser under conditions where *S*-(pyridylethyl)cysteine (Fluka) and *N*-(methyl)alanine (Serva) separate from other amino acids [15]. For sequence analysis, the Edman method was carried out in a prototype spinning-cup sequenator [16]. Phenylthiohydantoin derivatives were identified in an isocratic HPLC system which also separates the derivatives of *S*-(pyridylethyl)cysteine and *N*-(methyl)alanine [15].

3. RESULTS

Rabbit protamine was isolated from epididymal spermatozoa by a similar procedure as used for human and stallion protamines [7,14]. The protamine-DNA complex of the nuclei was mercaptolysed and *S*-alkylated in situ and then dissociated by hydrochloric acid, whereafter the *S*-(pyridylethylated) protamine material could be subjected to reversed-phase HPLC (fig.1). Two, incompletely resolved components appeared at an acetonitrile concentration typical for mammalian protamines, i.e. about 20%. The components showed highly similar, protamine-like electrophoretic mobilities (fig.2). Also the amino acid compositions (table 1) were virtually identical and typical for protamines, i.e. very rich in arginine and half-cystine, but devoid of alanine, which otherwise is part of the characteristic N-terminus of mammalian type 1 protamines [5,7–14]. The composition is similar to those published earlier [17,18]. Subjecting the HPLC components to short N-terminal analyses identical sequences were found: Val-Arg-Tyr-Arg-Cys-Cys-Arg-Ser-Gln-Ser-Arg-. Through lack of an explanation for the heterogeneity no difference was made between the components in subsequent analyses.

The highly unexpected finding of a valine residue in the first position of a protamine sequence prompted a more thorough examination.

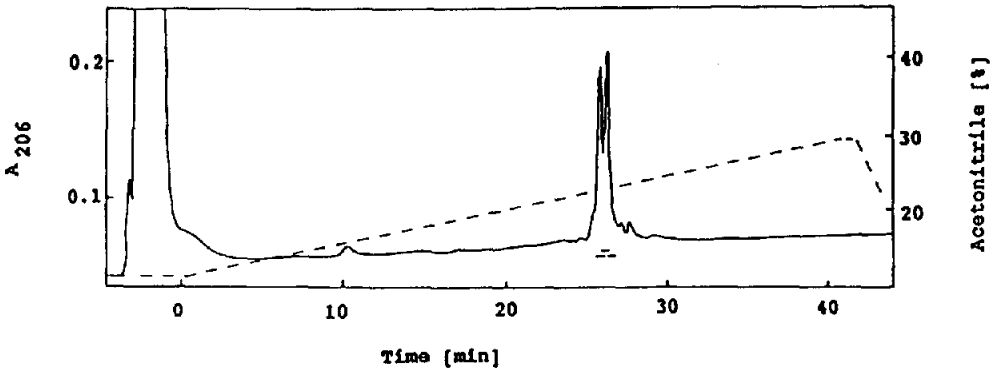

Fig.1. Isolation of *S*-(pyridylethylated) rabbit protamine by reversed-phase HPLC on a Nucleosil C-18 column and elution with a linear gradient from 10 to 30% acetonitrile in 0.1% trifluoroacetic acid in 40 min. Horizontal bars indicate components analysed.

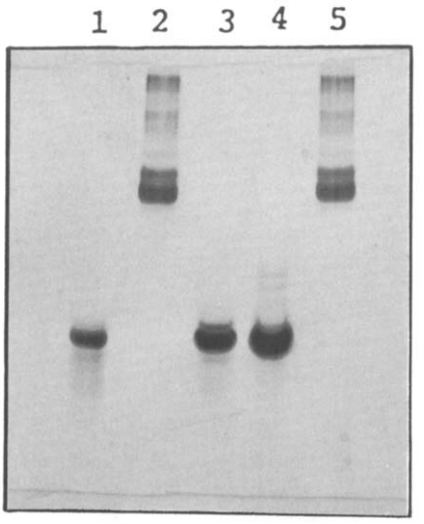

Fig.2. Acetic acid/urea gel electrophoresis of *S*-(pyridylethylated) rabbit protamine; (1) first component, (3) intermediate part and (4) second component in fig.1; (2) and (5) calf thymus histone (Sigma).

For this purpose the phenylthiohydantoin derivative from the first sequenator cycle was cochromatographed with a standard mixture of phenylthiohydantoin derivatives (fig.3). The derivative of the N-terminal residue coeluted perfectly with the valine derivative. It was then considered that what appeared as a valine residue might in fact be a posttranslationally modified alanine residue, e.g. an *N*-(methylated) alanine, which repeatedly has been observed in nucleic acid-associated proteins [19]. However, the presence of *N*-(methyl)alanine in rabbit protamine could be ruled out, as its phenylthiohydantoin derivative elutes in a different position (between those of proline and tryptophan) than that of valine and free *N*-(methyl)alanine appears on amino acid analysis in a position (between serine and homoserine) where no peak was detected in rabbit protamine hydrolysates. Furthermore, only one additional valine residue was detected in the rest of the sequence (see below), two residues being expected from the composition (table 1).

Table 1

Amino acid composition of rabbit protamine components (fig.1) and fragments from endoproteinase Lys-C (K) and thermolysin (T) digestion (fig.4) as mol residue/mol peptide; (a) uncorrected values from amino acid analysis, (b) values from sequence

Amino acid	First pool	Last pool		K_a		K_b		T_a		T_b	
	(a)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
D/N	–	–	–	–	–	–	–	–	–	–	–
T	1.5	1.8	2	1.7	2	–	–	1.0	1	–	–
S	2.9	2.7	3	–	–	3.0	3	–	–	–	–
E/Q	2.1	2.0	2	–	–	2.2	2	–	–	–	–
P	–	–	–	–	–	–	–	–	–	–	–
G	–	–	–	–	–	–	–	–	–	–	–
A	–	–	–	–	–	–	–	–	–	–	–
V	1.9	1.8	2	–	–	1.9	2	–	–	–	–
M	–	–	–	–	–	–	–	–	–	–	–
I	–	–	–	–	–	–	–	–	–	–	–
L	1.1	1.0	1	0.9	1	–	–	–	–	1.0	1
Y	2.8	2.7	3	1.5	2	1.0	1	–	–	0.9	1
F	–	–	–	–	–	–	–	–	–	–	–
K	1.2	1.0	1	–	–	1.2	1	–	–	–	–
H	–	–	–	–	–	–	–	–	–	–	–
R	26.2	25.9	26	5.4	5	20.9	21	2.2	2	3.0	3
W*	–	–	–	–	–	–	–	–	–	–	–
C**	8.1	9.1	9	2.9	3	4.4	6	1.5	2	0.8	1
Total			49		13		36		5		6

* Not determined

** Determined as *S*-(pyridylethyl) cysteine

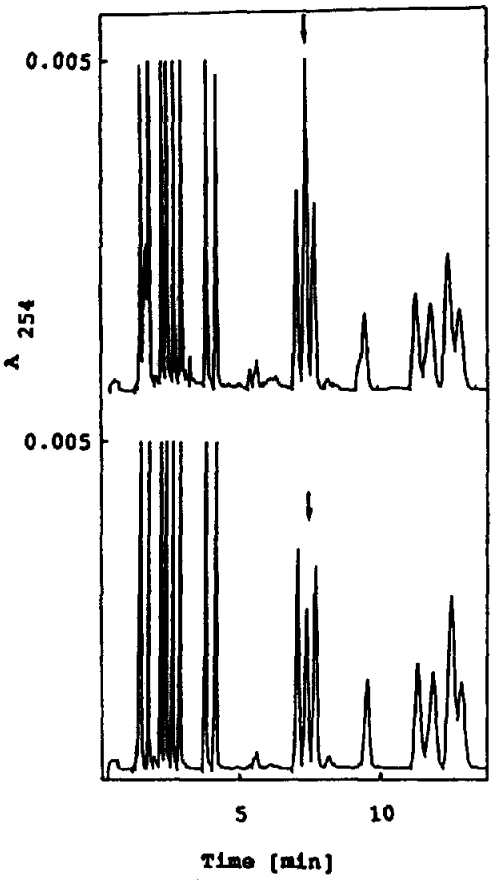

Fig.3. Identification of rabbit protamine N-terminal amino acid by isocratic reversed-phase HPLC; standard mixture of phenylthiohydantoin amino acids in the presence (upper panel) and absence (lower panel) of N-terminal residue; (↓) position of valine derivative.

To complete the sequence determination 7 nmol of protamine were subjected to an extended sequenator run. It was now possible to identify all 49 residues of the sequence (fig.4). The results were confirmed by analysing fragments obtained by cleaving the protamine at the single lysine residue with endoproteinase Lys-C. The fragments were separated by HPLC (fig.5) and their compositions (table 1) and sequences (fig.4) determined. The C-terminal fragment was further digested with thermolysin, the major subfragments were isolated by HPLC (fig.5) and again analysed for compositions (table 1) and sequences (fig.4). All data were mutually consistent.

4. DISCUSSION

The complete amino acid sequence of rabbit sperm protamine has been elucidated by protein chemical methods. It shows strong homology with other mammalian type 1 protamines, 47% of the positions being invariant (fig.6). The alignment demonstrates that strongly basic arginine clusters, which presumably bind to the acidic DNA, tyrosines, which may act as DNA intercalators, and most half-cystines, which disulfide-crosslink sperm chromatin, all are evolutionarily conserved. The presence of valine instead of alanine at the N-terminus is a surprise, as the constancy of N-terminal alanine in all previously sequenced mammalian type 1 protamines [5,7–14] seemed to indicate its functional importance. In fact, many non-mammalian arginine-rich protamines also possess an N-terminal alanine, the N-terminal sequences being reminiscent of the mammalian counterparts, i.e. Ala-Arg-Tyr-Arg- in galline from rooster [20], Ala-Arg-Ser-Arg- in scylliorhinine Z3 from dog-fish [21] and Ala-Arg-Arg-Arg- in clupeines YI and Z from herring [22], and sturine B and stelline A from sturgeons [23]. All other protamines of this type have proline as the N-terminal residue. It may seem noteworthy that the precursors of mouse [24] and human [25]

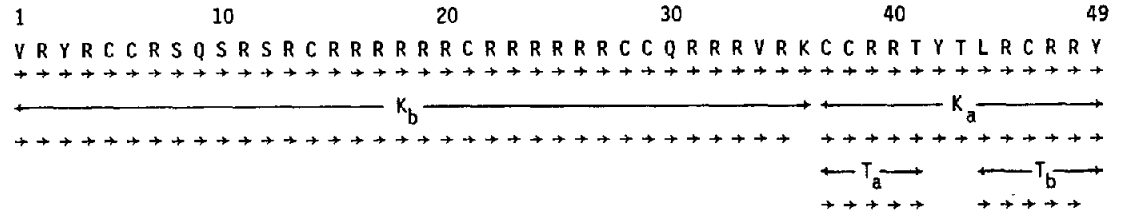

Fig.4. Amino acid sequence of rabbit protamine. (←→) Denotes length of peptide, (→) amino acid residue identified, (K) an endoproteinase Lys-C digestion fragment, (T) a thermolysinolytic fragment.

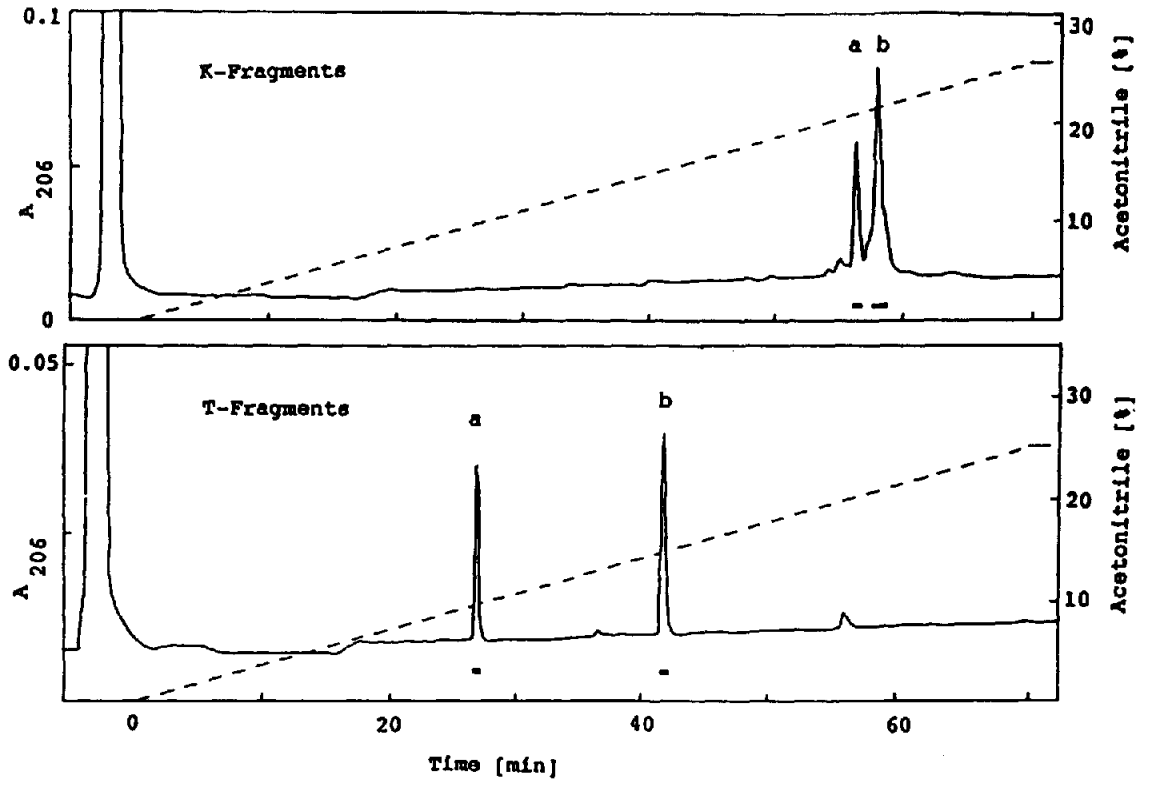

Fig.5. Fractionation of digests of *S*-(pyridylethylated) rabbit protamine by reversed-phase HPLC on a Nucleosil C-18 column and elution with linear gradients from 0 to 25% acetonitrile in 0.1% trifluoroacetic acid in 75 min; endoproteinase Lys-C digest (K) and thermolysin digest (T). Horizontal bars indicate components analysed.

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Rabbit     V r Y r C C r S Q S r S r C - r r r r r r C r r r r r r C C Q r r r - V r K C C r - r - T Y T - L r C r r Y -
Mouse 1    A r Y r C C r S K S r S r C - r r r r r r C r r r r r r C C r r r r r r - - C C r r r r S Y T - I r C K K Y -
Rat        A r Y r C C r S K S r S r C - r r r r r r C r r r r r r C C r r r r r r - - C C r r r r S Y T - F r C K r Y -
Human 1    A r Y r C C r S Q S r S r Y Y r Q r Q r - S r r r r r r S C Q T r r r A M r C C r P r - - Y r - P r C r r H -
Boar       A r Y r C C r S H S r S r C - r P r r r r C r r r r r r C C P r r r r r A V C C r - r - - Y T V I r C r r C -
Stallion   A r Y r C C r S Q S Q S r C r r r r r r r C r r r r r r S V r Q r r - - - V C C r - r - - Y T V L r C r r r r
Bull       A r Y r C C L T H S G S r C r r r r r r r C r r r r r r r F G r r r r r r - V C C r - r - - Y T V I r C T r Q -
Ram        A r Y r C C L T H S r S r C r r r r r r r C r r r r r r r F G r r r r r r - V C C r - r - - Y T V V r C T r Q -
Goat       A r Y r C C L T H S r S r C r r r r r r r C r r r r r r r F G r r r r r r - V C C r - r - - Y T V V r C T r Q -

Invariant  r Y r C C     S  S r    r  r  r    r r r r r r       r r       C C r  r    Y      r C
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Fig.6. Alignment of mammalian protamine type 1 sequences from rabbit (this work), mouse [9], human [5,7,8], boar [10], stallion [3,14], bull [12], ram [11], rat and goat (our unpublished work). For clarity R has been indicated by r. Gaps (–) have been introduced to maximize homology.

type 2 protamines have the identical N-terminus as rabbit type 1 protamine, i.e. Val-Arg-Tyr-Arg-. However, no type 2 protamine could be detected in rabbit epididymal spermatozoa.

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