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SPERM BASIC NUCLEAR PROTEINS IN THE BIVALVE MOLLUSC *MESODESMA DONACIUM*: CHARACTERIZATION AND COMPARISON WITH HISTONE-LIKE AND PROTAMINE-LIKE PROTEINS OF OTHER MOLLUSCS

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Abstract—1. The sperm nuclei of the marine bivalve mollusc *Mesodesma donacium* contain four somatic-type histones (H2A, H2B, H3 and H4), one sperm histone H1-like and a sperm-specific protein, named by us M1.

2. The histone H1-like is soluble in 5% perchloric acid and rich in lysine (32.8%).

3. The sperm-specific protein (M1) presents a high content of lysine (27.9%) and a low content of arginine (5.5%) in comparison to the related proteins of the other bivalves. It has a clear trypsin-resistant core.

4. We compared our results with previous data on bivalve sperm protein.

INTRODUCTION

The phylum Mollusca has a great number of different species (Storer *et al.*, 1979) in which the protein composition in the sperm nuclei shows a considerable variability of the sperm-specific proteins within and between closely related taxonomic groups (Bloch, 1969; Kasinsky, 1989). This seems to support suggestions about their evolutionary relationship (Subirana *et al.*, 1973). Until now, the organization of the sperm nuclei from some groups of molluscs has remained unknown, as have the features and content of their sperm proteins. Recently, however, several authors have analyzed the basic nuclear proteins in sperm of archeogastropods, patellogastropods, mesogastropods and species of polyplacophora (Daban *et al.*, 1990; Daban *et al.*, 1991; Daban *et al.*, 1991). On the other hand, several studies have been carried out on the sperm basic proteins in the class Bivalvia (Zalensky and Zalenskaya, 1980; Olivares *et al.*, 1986a,b; Ausió, 1986; Ausió *et al.*, 1987) which show some differences in size and structure, but all of them share an almost similar amino acid composition. Ausió and Van Holde (1988a) have found, associated with the four core histones in mature sperm of *Spisula solidissima*, a spermatic histone H1 with a trypsin-resistant core, molecular weight, solubility in 5% perchloric acid and amino acid composition similar to other histones of the H1 family. Similar observations have been carried out by Olivares *et al.* (1986a), in the sperm protein Pt2 of *Protothaca thaca* and by Giancotti *et al.* (1983) in the razor clam *Ensis minor*. The role played by these proteins in the structure and function of the nucleoprotein complex is under analysis (Ausió and Van Holde, 1987; Olivares *et al.*, 1987; Olivares and Ruiz, 1991).

With the aim of increasing knowledge of the chromosomal protein composition, we have accomplished the characterization of these proteins in *Mesodesma donacium* as well as the comparison between the main sperm-specific protein studied here with other previously analyzed species within the class Bivalvia.

MATERIAL AND METHODS

Biological material

Specimens of *M. donacium* were collected off the Chilean coast, in the VII Region and immediately processed. Mature male gonads of *M. donacium* were shaken mildly in a beaker with filtered sea water at 0–4°C and living spermatozoa were released spontaneously.

Sperm nuclei purification and preparation of basic nuclear proteins

All operations, except for chromatographical purification and trypsin digestion, were performed at 4°C as described by Olivares *et al.* (1986a). A sperm-containing suspension was treated as described by Ausió and Subirana (1982) for obtaining purified nuclei. Protein extracts were obtained as in Olivares *et al.* (1986a). Phenylmethylsulfonyl fluoride (PMSF) (0.1 mM) was present as proteolytic inhibitor throughout the procedure.

Protein purification

The basic nuclear proteins were extracted with 0.25 N HCl as described elsewhere (Olivares *et al.*, 1986a). Further fractionation of the acid-soluble fraction was achieved by partial fractionation with 5% perchloric acid and/or chromatography. The 5% perchloric extraction was performed as follows: the crude HCl extracts were suspended

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in 10 vol of 5% perchloric acid, vortexed immediately and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was precipitated with acetone/HCl at −20°C and finally was vacuum-dried (Ausió, 1988). Ion-exchange chromatography was performed with CM-C25 Sephadex in 50 mM sodium acetate buffer at pH 6.7. The histones and sperm-specific proteins were eluted from the column in the presence of 1 and 2 M NaCl, respectively.

Gel electrophoresis

Proteins were analyzed by acetic acid–urea 15% polyacrylamide (Panyim and Chalkely, 1969) and SDS-18% polyacrylamide gel electrophoresis in first and second dimension as described Thomas and Kornberg (1978). The gels were stained with 0.1% Amido Black 10B in 7% acetic acid.

Amino acid analysis

The amino acid analyses were carried out by previously described methods.

Trypsin digestion

Trypsin digestion of the sperm-specific protein (M1) was carried out as described by Olivares *et al.* (1986a).

RESULTS

Figure 1A shows an acetic acid–urea 15% polyacrylamide gel of total basic proteins from *Mesodesma donacium* sperm compared to *Protothaca thaca* sperm proteins. From this figure it is evident that there is coexistence between histones and one major fraction of low electrophoretic mobility. We have shown that the four histones H2A, H2B, H3 and H4 are present in the mature sperm of the surf clam *P. thaca* together with an additional band which, in the system, runs very close to H3 histone and which is extractable in 5% perchloric acid (Olivares *et al.*, 1986a). Besides, a sperm-specific protein, named by us M1 (lane 1) is found. In the case of *M. donacium* sperm, the situation is very similar to *P. thaca*. However, the major fraction (M1) runs faster than protein (Pt 1) and the first histone fraction migrates slightly above the histone H1 of *P. thaca* (lane 2). When we study these proteins in SDS-18% polyacrylamide gel (Fig. 1B) we become aware of the total histones from *P. thaca* sperm as indicated above (lane 1). These proteins are compared with those proceeding from sperm of *M. donacium* (lane 2). The electrophoretic migration is totally similar. With the aim of obtaining a purified fraction H1 from sperm of *M. donacium*, we extracted the total proteins with 5% perchloric acid as described in Materials and Methods. The result can be observed in lane 3.

The bidimensional electrophoretic analysis (Fig. 2) corroborates the fact that protein M1 is completely absent from SDS gel, as a consequence of low solubility in this detergent (Olivares *et al.*, 1986a; Ausió, 1986). The other proteins are very well resolved. Thus, the main spot (in amount) runs in the region of H1 standard (not shown) and the other four spots apparently migrate to the core region such as in the first dimension. These results are in agreement with the assumption that these proteins really correspond to histone proteins.

In order to purify the fraction M1, we have performed chromatography on carboxymethyl–C25 Sephadex as shown in Fig. 3. Protein M1 was obtained in a pure form (peak 2) whereas the histone proteins co-eluted together in peak 1. Probably, the duplicity of peak 2 is due to the presence of two oligomeric forms of the protein.

Amino acid composition of the sperm-specific protein isolated as described above is shown in Tables 1 and 2. As can be observed in Table 1, the protein M1 exhibits some common compositional features of the sperm-specific components, such as 20–30% lysine, 20–25% serine and 10% alanine. However, protein M1 has a low content of arginine (5.5%) in comparison to the related proteins; Pt1 of *P. thaca* (Olivares *et al.*, 1986a), EM6 of *E. minor* (Giancotti *et al.*, 1983) and PLI of *M. nasuta* (Ausió, 1988b). Therefore, we think that this protein might not be assigned as a protamine-like component because of its low content of arginine. High amounts of this residue are characteristic of the species with protamines interacting with

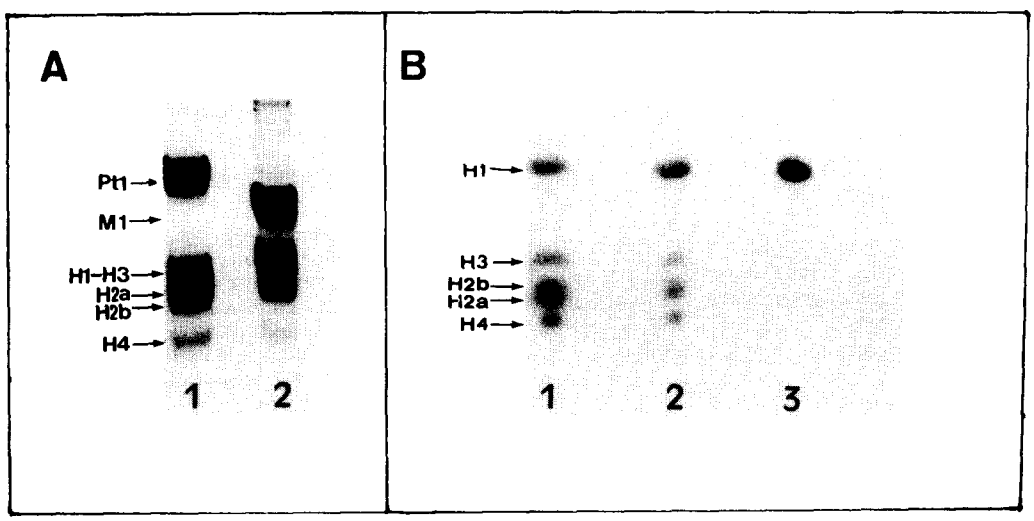

Fig. 1. Electrophoretic mobility of basic nuclear proteins from sperm of *Mesodesma donacium* in polyacrylamide gel containing acetic acid–urea (A) and (B) SDS. Lane 1, *Protothaca thaca*, lane 2, *Mesodesma donacium* and lane 3, histone H1-like from *Mesodesma donacium*. Origin is at top of gel.

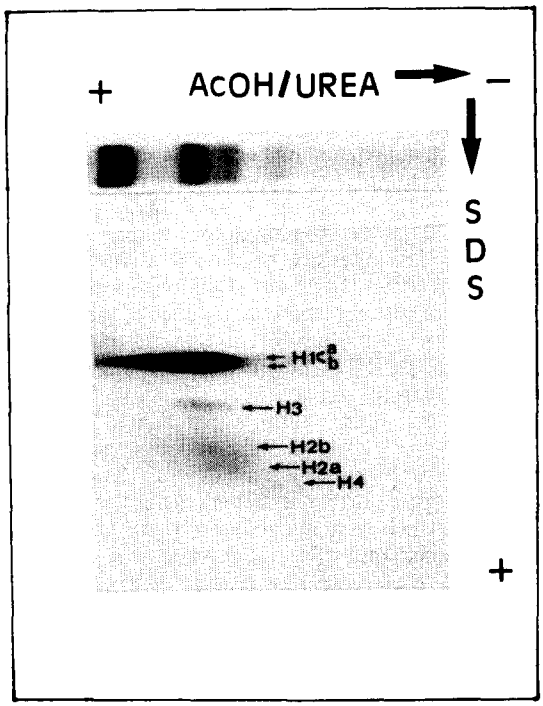

Fig. 2. Electrophoresis of basic nuclear proteins from sperm of *Mesodesma donacium*. First dimension is 15% acrylamide–acetic acid–urea and second dimension is 18% acrylamide–0.1% SDS.

Table 1. Amino acid composition (mol%) of the sperm-specific protein M1 of *Mesodesma donacium* in comparison to the protamine-like and other histones of the H1 family

	Pt1	PL-SP	EM6	PLI	H1(ct)	H5	M1
K	32.7	24.8	24.9	21.8	26.8	23.6	27.9
H	1.0		4.0	2.3		1.9	trace
R	27.9	23.1	21.7	26.9	1.8	12.4	5.5
D	0.7	0.6	2.4	0.8	2.5	1.7	3.4
T	1.2	4.3	2.0	4.0	5.6	3.2	4.0
S	22.1	21.7	18.9	20.2	5.6	11.9	21.4
E	0.5	0.6	1.8	0.8	3.7	4.3	5.3
P	1.7	2.4	trace	1.8	9.2	4.7	2.7
G	3.2	3.0	2.6	2.2	7.2	5.3	8.8
A	5.3	14.2	12.7	11.3	24.3	16.3	13.4
C	n.d.			0.7			
V	1.2	2.3	2.3	2.4	5.4	4.2	2.3
M	0.1	0.4	0.9	0.2		0.4	
I	0.7	0.5	1.2	1.4	1.5	3.2	1.7
L	1.0	1.7	2.9	2.1	4.5	4.7	3.6
Y	0.3	0.3	0.6	0.5	0.9	1.2	
F	0.3	0.3	1.0	0.7	0.9	0.6	
W		0.3					

Pt1, protamine-like protein from *P. thaca* sperm (Olivares *et al.*, 1986a).
PL-SP, protamine-like protein from *S. solidissima* sperm (Ausió and Subirana, 1982).
EM6, protamine-like protein from *E. minor* Sperm (Giancotti *et al.*, 1983).
PLI, histone-like protein from *M. nasuta* sperm (Ausió, 1988).
H1-CT, histone H1 from calf thymus (Mayes and Jhons, 1982).
H5, histone H5 from chicken erythrocyte (Mayes and Jhons, 1982).

DNA in their sperms. On the other hand, the high content of lysine, as well as the relative percentage of the other amino acids, represents features to bring this protein close in composition to that of the histone H1 family. However, this protein M1 lacks aromatic residues in comparison to calf thymus histone H1 and chicken erythrocyte histone H5 (Table 1). In all cases, the Pt2 protein from *P. thaca*, a sperm-specific histone H1, also is lacking aromatic residues (Table 2). Table 2 shows the amino acid analysis composition of this protein in comparison to

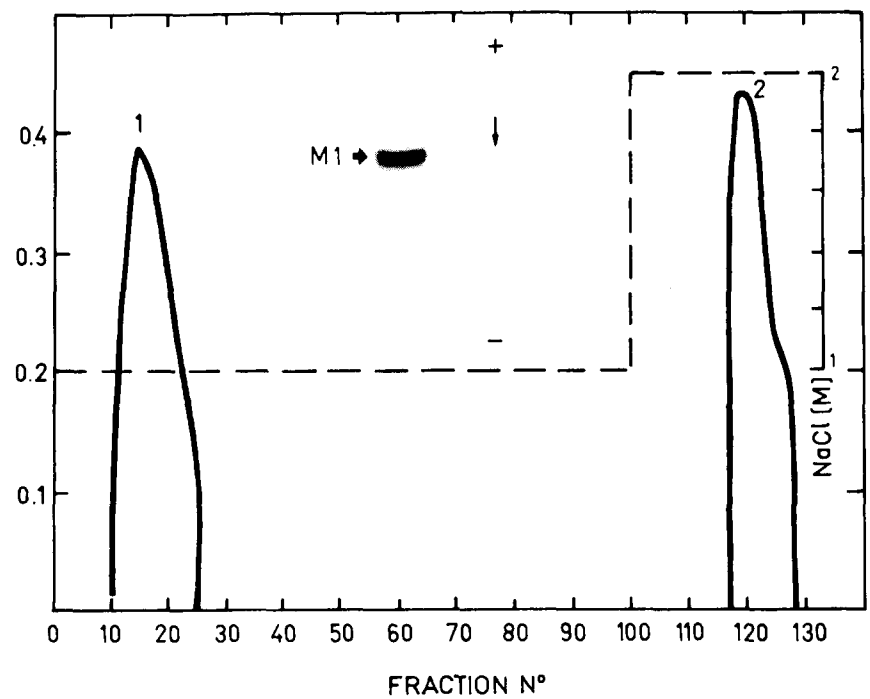

Fig. 3. Ion-exchange chromatography on CM–C25-Sephadex of nuclear proteins extracted from *Mesodesma donacium* sperm. Peak 1, histone proteins; peak 2, protein M1. (---) Discontinue gradient saline. The inset shows the purity of the M1 fraction, which is achieved with this technique.

Table 2. Amino acid composition (mol%) of the sperm histone H1 variants (H1a and H1b) of *Mesodesma donacium* in comparison to other representative members of the histone H1 family

	Pt2	H1-SSP	H1-PSP	H1-CT	H5-CE	H1-CE	M1* (H1a-H1b)
K	36.3	30.3	29.5	26.8	23.6	30.0	32.8
H	0.7	0.5	1.0		1.9		2.4
R	6.1	1.7	11.0	1.8	12.4	2.4	2.3
T	5.0	5.8	1.9	5.6	3.2	3.7	5.8
S	7.1	5.0	6.0	5.6	11.9	6.3	6.5
E	2.1	2.8	2.3	3.7	4.3	5.0	3.4
P	7.6	9.2	7.3	9.2	4.7	9.5	6.3
G	4.8	4.8	4.2	7.2	5.3	5.8	5.2
A	22.5	25.5	24.9	24.3	16.3	25.7	24.0
C		1.1–1.2					
V	1.0	3.7	3.7	5.4	4.2	4.1	2.7
M		0.5	1.8		0.4		
I	1.4	2.2	1.0	1.5	3.2	0.6	2.5
L	2.3	3.0	2.2	4.5	4.7	3.8	2.9
Y		1.2	0.9	0.9	1.2	0.3	
F		0.6	0.4	0.9	0.6	0.3	0.8

Pt2, histone H1 from *P. thaca* sperm (Olivares *et al.*, 1986a).
H1-SSP, histone-like protein from *S. solidissima* sperm (Ausió, 1986).
H1-PSP, histone H1 from *P. angulosus* (Strickland *et al.*, 1976).
H1-CT, histone H1 from calf thymus (Mayes and Jhons, 1982).
H1-CE, histone H5 from chicken erythrocyte (Mayes and Jhons, 1982).
H1-CE, histone from chicken erythrocyte (Tsai and Hnilica, 1975).

other proteins of the histone H1 family. Thus, we appreciate that the protein H1 from *M. donacium* has a high content of lysine (32.8%) and alanine (24%) which is characteristic of the histone H1 from somatic or sperm sources. On the other hand, this protein is extractable in 5% perchloric acid and it runs very close to histone H3 in acetic acid–urea gels (Fig. 1, lane 2).

A feature of histones is that they possess a folded trypsin-resistant domain. Protein M1 was digested with trypsin as outlined in Materials and Methods. Figure 4 shows the time course of digestion as visualized on acetic acid–urea gels. Protein M1 gives a limit product fragment as is typically found in all the members of the H1 family. It is important to note here the special behavior that protein M1 presents. In fact, this protein clearly presents an aggregate at the top of the gel, at 0 min.

This situation has been also observed in the whole sperm protein of this species (Fig. 1, lane 2). When digestion is in progress this aggregate disappears and a trypsin-resistant core appears. However, we have observed the presence of a band that appears at 90 min of digestion running above the limit product fragment. The intensity of this band increases to reach its maximum at 2 hr of digestion. We think that this band might correspond to a dimer of the trypsin-resistant core of protein M1. In fact, this protein has a strong tendency to polymerize in the absence of reductor reagent. As a similar situation has been observed by other authors in related proteins (Barbero *et al.*, 1980; Ausió and Van Holde, 1988; Ausió, 1988) this result is probably in relation to the existence of one or two cysteine residues in the core fragment which were not reduced in the presence of β-mercaptoethanol. The latter result has been described by Ausió and Van Holde (1988) and Ausió (1988). It is also important to note that the samples were treated under denturing conditions (the core protein is unfolded) and the oxidation of the protein is highly enhanced.

In all cases, the cysteine residue was not detected in our analysis (Table 1) but it is known to be easily destroyed during the acid hydrolysis conditions employed in the amino acid analysis.

DISCUSSION

The analyses of the basic nuclear proteins associated with DNA in sperms of the bivalve molluscs have shown the coexistence of a complete set of histones of the somatic type, together with histone and protamine variants (Subirana *et al.*, 1973; Zalensky and Zalenskaya, 1980; Uschewa *et al.*, 1985; Ausió, 1986; Olivares *et al.*, 1986a,b; Subirana and Colom, 1987). In the species analyzed here

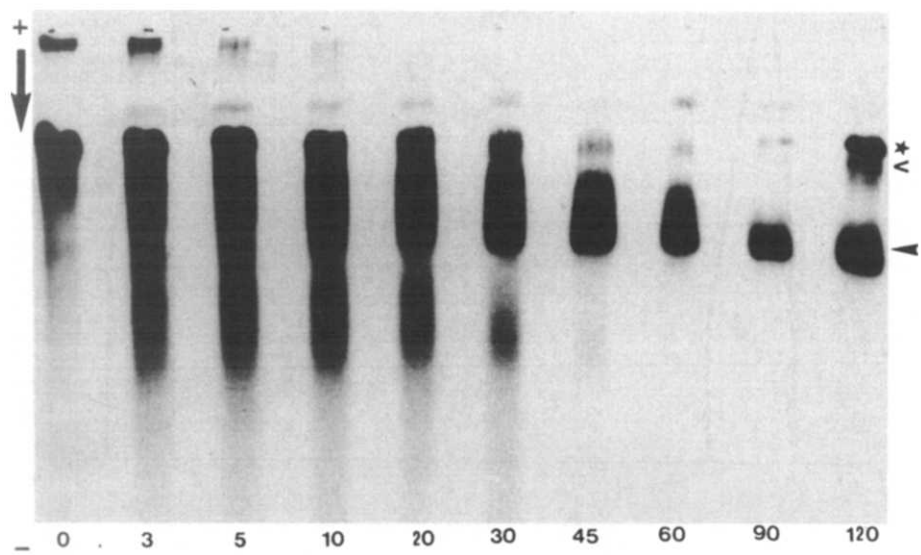

Fig. 4. Time course of digestion of protein M1 by trypsin. The arrow indicates the limit-product fragment; (★) points out the possible dimer generated in absence of β-mercaptoethanol by the trypsin-resistant core; (<) shows the fraction of whole protein M1 undigested yet. Origin is at top of gel.

(*Mesodesma donacium*), we have observed a complete set of histones. Thus, the core histones present a very similar relative electrophoretic mobility to those proceeding from sperm of *Protothaca thaca*, both in urea–acetic acid gel (Fig. 1A), but completely similar in SDS gel (Fig. 1B). The two-dimensional gel electrophoresis of ripe sperm is clearly indicative of the presence of all histones H2A, H2B, H3, H4 and a sperm-specific histone H1 which is seen as two electrophoretic spots. These spots correspond to two sperm histone variants (H1a and H1b) (Fig. 2). It is important to note that the relative mobility of sperm-specific histone H1 and (H1a and H1b) of *M. donacium* bears a strong resemblance to the histone H5 from chicken erythrocytes when they are run together in acetic acid–urea gel (not shown). The comparison has validity because histone H5 is a final differentiation-specific variant from red blood cells in several higher taxonomic groups. However, the amino acid composition of sperm-specific histone H1 (H1a and H1b) is more similar to typical somatic histone H1 than H5 (Table 2). Therefore, the higher mobility shown by histone H1 (H1a and H1b) of *M. donacium* can be originated by a smaller molecular weight than its counterpart H1.

A similar protein (Pt2) has been described in the surf clam *Protothaca thaca* (Olivares *et al.*, 1986a) and in the razor clam *Ensis minor* (Giancotti *et al.*, 1983).

Besides the histone complement, the sperm of *M. donacium* contains one sperm-specific protein (M1). This protein has particular features such as (1) its amino acid composition is reminiscent of the components of family H1 (rich in lysine residues) and (2) it contains a trypsin-resistant core. The presence of proteins with special characteristics associated with DNA in the sperm of bivalves seems to be a common occurrence within the taxonomic group. A peculiarity of this protein is its capacity to aggregate easily as whole protein, as well as when it is in the form of a trypsin-resistant core (Fig. 4). Probably, this is in direct relation to the presence of cysteine residues in this region. This situation has been observed in related proteins from this taxonomic group (Ausió, 1988; Ausió and Van Holde, 1988b).

In summary, we think that the protein M1 might be assigned within the category of histone H1-like. In all cases, this protein is unusual, because all the sperm proteins described in other bivalves have a high level of lysine and also arginine.

However, the composition of protein M1 might be related to the histone H5 (Table 2) which is induced in the H1 family.

The coexistence between unusual histone variants with somatic types of histones in the genetic material prompts several questions: How does the interaction between both types of proteins and with DNA take place? Which is the chromatin structure in these sperm nuclei? What is the role, if any, played by this protein in the early stage of development following fertilization? The answers to these questions are beginning to be found.

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