Isolation and partial characterization of rat sperm tail fibrous sheath proteins and comparison with rabbit and human spermatozoa using a polyclonal antiserum

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Rat sperm tail fibrous sheath was isolated using mechanical and chemical dissection methods from spermatozoa collected from the cauda epididymis. The procedures used to isolate the fibrous sheath were monitored by phase-contrast microscopy and purity was verified by electron microscopy. SDS-PAGE of isolated total fibrous sheath revealed at least 17 bands when stained with Coomassie brilliant blue and 20 bands with silver stain. The most intensely staining proteins, using both staining methods, were a double band at 80–87 kDa, and a band at 28.5 kDa, whereas with silver staining, bands at 66.2 kDa and 32.7 kDa were also intensely stained. Electroelution following SDS-PAGE was used to isolate 11 of these proteins (116.4, 87.5, 80.9, 66.2, 57.2, 49.7, 46.8, 37.3, 32.7, 28.5 and 15.5 kDa). Amino acid analysis revealed that these proteins were abundant in aspartic and glutamic acid, glycine, serine and leucine, while histidine and phenylalanine were of low abundance. The content of cystine varied widely from 9.4% to 1.4%. The amino termini of the 80.9 kDa, 32.7 kDa, 28.5 kDa and 15.5 kDa proteins were blocked. Immunofluorescence microscopy demonstrated that a polyclonal antiserum to isolated rat fibrous sheath was localized to the principal piece of the rat, rabbit and human spermatozoa, but in the rabbit it also labelled the equatorial region of the head. Western blotting detected all protein bands in isolated fibrous sheath and a similar range of proteins in the spermatozoa of rat and rabbit. Human sperm proteins of 116 kDa and 80 kDa were immunoreactive in common with other species, and there was only weak crossreactivity with the other proteins. These data suggest the presence of common epitopes in the proteins of all three species.

Introduction

The movement of mammalian spermatozoa depends on their flagellae, which consist of several cytoskeletal components including the axoneme, the outer dense fibres and the fibrous sheath. The fibrous sheath is a component of the principal piece, which begins at the termination of the mid-piece mitochondrial sheath and extends for most of the remaining length of the flagella. The fibrous sheath is composed of two cytoskeletal elements, longitudinal columns and numerous transverse ribs, which surround the outer dense fibres composed of a central medulla and a thin limiting cortex (Fawcett, 1970, 1975; Sapsford *et al.*, 1970). The longitudinal columns, which run in positions equivalent to 3 and 8 of the outer dense fibre system of the mid-piece, are assembled during the early part of spermiogenesis in rats, whereas the transverse ribs arise during the second half of spermiogenesis, and both proceed from distal to proximal in the developing flagellum (Irons and

Clermont, 1982; Oko and Clermont 1989; Clermont *et al.*, 1990).

There have been several studies using antibodies that recognize epitopes on proteins present in mouse fibrous sheath (Sakai *et al.*, 1986), human fibrous sheath (Jassim *et al.*, 1990, 1991; Jassim, 1991) and rat fibrous sheath (Oko, 1988), and a monoclonal antibody that recognizes a 67 kDa antigen in rat, mouse and hamster spermatozoa (Fenderson *et al.*, 1988). It has been reported that two monoclonal antibodies, S69 and S70, recognize the principal piece of the human sperm flagellum (Beecher *et al.*, 1993).

The fibrous sheath is an insoluble keratin-like structure with extensive disulfide linkages (Bedford and Calvin, 1974; O'Brien and Bellve, 1980) and, judged by its morphological, biochemical, physical and immunological features, it is proposed that it is a modified form of the intermediate filament proteins (Eddy, 1988; Eddy *et al.*, 1992; Jassim *et al.*, 1992). The major proteins that compose the rat fibrous sheath are of molecular masses of 75–80, 27.5 and 14.4 kDa (Olson *et al.*, 1976; Oko, 1988) and are highly phosphorylated owing to their high content of serine (Brito *et al.*, 1989). Comparative studies of the fibrous

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sheath and outer dense fibres by immunochemistry (Oko, 1988) and peptide mapping (Brito *et al.*, 1989) suggest that the 14.4 kDa polypeptide of the fibrous sheath is common to the outer dense fibres, whereas the two larger proteins, 80 kDa and 24 kDa, are unrelated to the outer dense fibre proteins. Brito *et al.* (1989) reported the isolation and amino acid composition of three of the fibrous sheath proteins in rats.

The present study extends our knowledge of the composition of the fibrous sheath by the isolation of additional proteins and by providing the amino acid composition of 11 of its protein components. In addition, the sperm proteins among different species (human, rabbit and rat) were compared using a polyclonal antibody raised against isolated rat fibrous sheath.

Materials and Methods

Experimental animals

Spermatozoa were obtained from Sprague–Dawley rats (Central Animal House, Monash University), 90–110 days old, killed by CO₂ anaesthesia. These investigations were approved by Monash University Standing Committee on Ethics in Animal Experimentation and conform to the NHMRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes. Rabbit spermatozoa were collected from adult New Zealand white rabbits by artificial vagina and human spermatozoa were provided by the Andrology Department at Monash Medical Centre.

Preparation of rat sperm tails

The rat cauda epididymides were dissected and spermatozoa released by mincing with razor blades in several drops of 0.05 mol sodium phosphate buffer l⁻¹, pH 7.5, 0.15 mol NaCl l⁻¹, containing 0.2 mmol phenylmethylsulfonyl fluoride l⁻¹ (PBS–PMSF). The sperm tails were isolated from the heads using the protocol developed by Calvin (1976). The spermatozoa were filtered through 180 µm nylon mesh and washed twice in PBS–PMSF at 400 g for 10 min. Isolated spermatozoa were decapitated by sonication twice (Sonifier model B-30, Branson Sonic Power Co., Shelton, CT) at 30% output for 30 s at 4°C and, after washing with PBS–PMSF, the decapitated spermatozoa were centrifuged through a sucrose step gradient (75, 70, 65% w/v) at 100 000 g for 80 min (Beckman SW 28 rotor). The sperm tails, removed from the 65–70% interface, were suspended in PBS–PMSF, and centrifuged through a sucrose step gradient 65% and 75% (w/v) at 100 000 g for 60 min to increase the purity of sperm tails. Isolated sperm tails from the 65–75% sucrose interface were washed and centrifuged at 10 000 g for 10 min (Sorvall SS34 rotor) with PBS–PMSF, and subsequently used for the isolation of the fibrous sheath. Each procedure was verified by phase-contrast microscopy.

Isolation of fibrous sheath

The procedure used to isolate the fibrous sheath from the sperm tails was a modification of the method of Olson *et al.*

(1976). The isolated sperm tails were suspended for 15 min in 50 mmol Tris–HCl l⁻¹, pH 9.0, 2% (v/v) Triton X-100 and 5 mmol DTT l⁻¹ with gentle shaking at 4°C; the supernatant was discarded and the procedure repeated. After washing with 50 mmol Tris–HCl l⁻¹, pH 9.0, containing 0.2 mmol PMSF l⁻¹, the sperm tails were resuspended in 25 mmol Tris–HCl l⁻¹, pH 8.0, 4.5 mol urea l⁻¹, 25 mmol DTT l⁻¹ and shaken for 5 h at 4°C. The resultant insoluble fraction was suspended in 25 mmol Tris–HCl l⁻¹, pH 8.0, containing 25% (w/v) sucrose and centrifuged through a sucrose step gradient comprising 25, 50, 75% (w/v) at 100 000 g for 50 min. The final insoluble fraction was removed from the 50–75% sucrose interface and washed with 25 mmol Tris–HCl l⁻¹ at 50 000 g for 20 min. Each step of the procedure was monitored by phase-contrast and electron microscopy.

Preparation of sperm proteins

Rat, rabbit and human spermatozoa were solubilized in 4.5 mol urea l⁻¹, 2% (w/v) SDS, 25 mmol DTT l⁻¹ by shaking at room temperature for 5 h. The insoluble gel-like material was removed by centrifugation at 10 000 g for 20 min. The protein content was estimated using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA), which is similar to that described by Lowry *et al.* (1951), but is more tolerant of detergents.

Electron microscopy

Samples from each step of the isolation procedure were fixed in 2.5% (v/v) glutaraldehyde in 0.1 mol sodium cacodylate l⁻¹ buffer, pH 7.4, overnight at 4°C, and postfixed in 1% (v/v) osmium tetroxide. After dehydration in alcohol, samples were embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and examined on a Jeol II electron microscope.

SDS-PAGE

The isolated fibrous sheath was solubilized in 25 mmol Tris–HCl l⁻¹, pH 8, 1% (w/v) SDS, 2 mmol DTT l⁻¹ for 90 min with shaking at room temperature. The insoluble fraction, composed mainly of a few remaining sperm heads and remnants of the outer dense fibre cortex, were removed by centrifugation. The apparent molecular size of proteins was determined using 12.5% (w/v) SDS-polyacrylamide gels (Laemmli, 1970) under reducing conditions; 40–50 µg of protein per lane was used for Coomassie brilliant blue staining and 1 µg of protein for silver staining with mini gels (Wray *et al.*, 1981). The molecular size was calibrated using molecular weight standards (Bio-Rad) and densitometry was performed using a LKB 2202 Ultrascan Densitometer (Bromma).

Antibody preparation

The isolated rat fibrous sheath was solubilized and the insoluble material removed (as described in the previous section) and emulsified 1:1 in Freund's complete adjuvant (Commonwealth Serum Laboratories, Melbourne). New

Zealand white rabbits were injected s.c. into multiple sites around the neck and shoulders with 1 ml of emulsion containing 75 µg fibrous sheath. The rabbits were boosted with 25 µg of fibrous sheath in Freund's incomplete adjuvant at intervals of 2 weeks. Serum was collected after each boost and tested using western blotting.

Western blotting

Approximately 10 µg of total sperm protein from rat, rabbit and human together with the purified rat fibrous sheath was run on 12.5% (w/v) SDS-PAGE and then transferred onto nitrocellulose membrane (BA 85, Schleicher and Schuell, Dassel) in a 20 mmol Tris l⁻¹, 150 mmol glycine l⁻¹ buffer containing 20% (v/v) methanol at 60 V overnight (Towbin *et al.*, 1979). The transfer buffer was then changed to 25 mmol phosphate buffer l⁻¹, pH 6.5, 20% methanol (v/v), and transferred at 30 V for a further 5 h. The excess protein-binding sites on the membrane were blocked with 0.05 mol phosphate l⁻¹ buffer, pH 7.5, 0.15 mol NaCl l⁻¹ (PBS), containing 1% (w/v) non-fat milk powder for 1 h. The membrane was probed overnight at room temperature with a rabbit polyclonal antiserum (JMR 2) raised against whole purified rat fibrous sheath or pre-immune serum diluted 1:1000 with PBS containing 1% (v/v) Tween 20 (Sigma, St Louis, MO). The membrane was then incubated for 3 h with a biotinylated sheep antirabbit immunoglobulin antiserum (Silenus Laboratories, Melbourne), diluted 1:3000 in PBS-Tween. This was followed by incubation for 1 h with streptavidin-horse radish peroxidase (Silenus) diluted 1:8000 in PBS-Tween. The blot was developed with 10 mg 3,3'-diaminobenzidine (DAB, Sigma) diluted in 15 ml of 20 mmol Tris-HCl l⁻¹, pH 7.5, 0.15 mol NaCl l⁻¹.

Isolation of fibrous sheath protein components

Individual proteins were isolated with a high degree of purity by cutting out the bands lightly stained with Coomassie brilliant blue from a 12.5% reduced SDS-PAGE gel and performing electroelution (Hunkapiller *et al.*, 1983) to recover the proteins from the gel. The purity of the bands was determined by SDS-PAGE and silver staining. Protein recovery was determined by the Bio-Rad DC protein assay and by amino acid analysis. The samples were stored at -20°C.

Amino acid analysis

Samples for amino acid analysis were precipitated with 8 volumes of 100% cold methanol (-20°C); the precipitate was resuspended in 20 ml of water and precipitated for a second time with cold methanol. The samples were then lyophilized and hydrolysed in a vacuum in constantly boiling 6 mol HCl l⁻¹ containing 0.2% (w/v) phenol for 22 h at 110°C. Samples were then analysed using the Millipore Picotag method (Millipore, Bedford, MA; Cohen and Strydom, 1988). The losses incurred by the hydrolysis, as measured in control proteins, were minimal (Stanton *et al.*, 1992) except for tyrosine (26%). Methionine and cystine (including cysteine) content was estimated by oxidizing the samples to methionine sulphone and cysteic acid, respectively, using performic acid. Samples

were analysed at least twice in separate hydrolysates and the results averaged. The average coefficient of variation between runs was less than 2%.

Amino-terminal sequencing

Proteins were sequenced for five cycles and amino acids identified by an online Applied Biosystems 470A gas phase sequencer (Applied Biosystems Inc, Foster, CA). Isolated abundant proteins (80.9 kDa; 28.5 kDa) were applied directly or proteins from solubilized fibrous sheath (80.9 kDa, 28.5 kDa, 32.7 kDa and 15.5 kDa) were transferred to PVDF membrane (Immobilon-PSQ, Millipore Corp, Bedford, MA).

Immunofluorescence microscopy

Rat cauda epididymal spermatozoa and ejaculated rabbit and human spermatozoa were washed in PBS-PMSF, and then spotted on glass slides precoated with poly-L-lysine and fixed with 2% (v/v) formaldehyde for 30 min at 4°C, and then processed for indirect immunofluorescence as described by Almahbobi and Hall (1993). In brief, the attached spermatozoa were extracted with 1% (v/v) Triton X-100 in 10 mmol Pipes l⁻¹, pH 6.8, 0.1 mol NaCl l⁻¹, 3 mmol MgCl₂ l⁻¹, 20% (w/v) sucrose, for 5 min at 4°C. Nonspecific binding and autofluorescence were reduced by treating the spermatozoa with 1.5% (w/v) glycine for 20 min and 1% (w/v) sodium borohydride for 30 min. The spermatozoa were then incubated with the anti-fibrous sheath antiserum (JMR 2), diluted 1:100 for 60 min, and then with sheep anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate (Silenus, Melbourne; 1:50) for 30 min. Nonspecific staining was determined by replacing the primary antiserum with preimmune rabbit serum or with PBS. Autofluorescence and aldehyde-induced fluorescence were tested by omitting the second antibody before and after fixation. Photography and printing were performed at the same settings.

Results

Isolation of fibrous sheath

Extraction of isolated cauda epididymal spermatozoa in Triton X-100 and DTT completely solubilized the plasma membrane and mitochondrial sheath. The outer dense fibres of the middle piece were exposed, while the fibrous sheath of the principal piece remained intact. The microtubules of the axoneme were disrupted, as shown in Fig. 1a. After extraction with urea and DTT, the disrupted axoneme and most of the outer dense fibres were solubilized; however, some remnants of the outer dense fibre cortex remained in the isolated fibrous sheath preparation (Fig. 1b). The yield from each experiment (12 rats) was 2.9 ± 0.4 mg of fibrous sheath protein, as estimated by amino acid analysis.

SDS-PAGE

SDS-PAGE of isolated fibrous sheath proteins revealed at least 17 bands stained by Coomassie brilliant blue (Fig. 2a)

Fig. 1. Electron micrographs showing the effect of the chemical dissection used in the isolation of fibrous sheath. (a) Cross-sections of the middle and the principal piece of the flagella after treatment with Triton X-100 and DTT. The plasma membrane and mitochondrial sheath were solubilized exposing the outer dense fibres (ODF), the fibrous sheath (FS), and the axoneme (AXN). (b) Cross-sections after further treatment with urea and DTT. The axoneme and most of the outer dense fibres were solubilized. Remnants of outer dense fibres, mostly the cortex portion (ODF) remained. Note that the fibrous sheath (FS) remained intact. Scale bars represent 200 nm.

and 20 different major and minor proteins by silver staining (Fig. 2b). The most intensely stained proteins were the double band at 80–87 kDa, and the 28.5 kDa band using both staining methods, whereas the 66.2 kDa and 32.7 kDa bands were apparently abundant only using silver staining. In contrast, the apparent abundance of the 15.5 kDa protein was greater with Coomassie brilliant blue staining than with silver staining. The densitometry profile of total fibrous sheath protein in a silver stained gel indicates that the 80–87 kDa band is composed of at least three protein species. The relative quantities of the ten most abundant proteins, as calculated from densitometry (Fig. 3) of a silver stained gel, are shown in Table 1. The 80–87 kDa proteins account for almost half the protein composition and, together with the 28.5 kDa and 32.7 kDa proteins, make up 88% of the rat fibrous sheath protein components.

Isolation of fibrous sheath protein components

Electroelution was followed by SDS-PAGE, and ten of the fibrous sheath proteins were isolated (Fig. 2c). The 15.5 kDa protein was also isolated; however, due to its low yield and poor silver staining property, this protein did not show sufficient intensity to be photographed. The yield of these highly purified proteins is shown in Table 1. The total yield of the combined proteins was $62.34 \pm 1.97 \mu\text{g}$ for the 12 rats, which represents 2.1% of the total protein in the isolated fibrous sheath. This yield was low considering that the purification involved only a two-step procedure. Other means of purification such as reverse phase, ion exchange and gel filtration chromatography were unsuccessful in separating the individual proteins. The yields of each protein were disproportionate to their abundance in the original material (Table 1). The 28.5 kDa protein had the highest yield of recovered protein; however, this was only 2.9% of the total amount of the 28.5 kDa protein present. The most abundant proteins (87.5–80 kDa) had a yield of only 0.5% of the available protein. Other proteins had high yield, such as the 116 kDa protein; the 37.3 kDa protein, which was almost undetectable, yielded 4.12 μg .

Amino acid analysis

Amino acid composition of whole fibrous sheath is shown in Table 2, and is characterized by high values for aspartic acid (representing aspartic acid and asparagine) and glutamic acid (representing glutamic acid and glutamine). Serine, leucine and cystine (representing cysteine and cystine) were abundant and there was a low content of histidine, phenylalanine and isoleucine.

The amino acid compositions of the 11 purified proteins (Table 2) were also characteristically high in aspartic and glutamic acid, although the 15.5 kDa protein contained less aspartic acid (5.8%) and the 32.7 kDa protein contained the least glutamic acid (6.8%). Although the amino acid compositions of all 11 proteins were similar, there were, in most proteins, some differences that were above the 2% reproducibility. This finding suggests that most, if not all, of the proteins are unique rather than fragments of different sizes from a parent protein. The content of cystine varied widely between the different molecular mass species, from 9.4% in the 46.8 kDa to 1.4% in the 80.9 kDa and 49.7 kDa species. The loss of tyrosine on hydrolysis was high when estimated in standard proteins in our system (Stanton *et al.*, 1992); however, three of the isolated proteins, 49.7 kDa, 37.3 kDa and 32.7 kDa, had a high content of tyrosine, compared with the other proteins. Attempts to sequence the 80 kDa, 32.7 kDa, 28.5 kDa and 15.5 kDa proteins were unsuccessful, presumably owing to blocking of the amino terminus.

Sperm proteins

SDS-PAGE of solubilized proteins from human, rabbit and rat spermatozoa are compared with that of isolated rat fibrous sheath (Fig. 4). Protein bands of similar staining intensity were seen corresponding to 116 kDa, 80–87 kDa, 57.2 kDa

Fig. 2. SDS-PAGE using 12.5% gels of rat total fibrous sheath proteins (FS) stained with (a) Coomassie brilliant blue and (b) silver stain. These are compared with the isolated individual proteins (c) which were also silver stained. The apparent molecular size of each isolated protein is given in kDa. STD: standards.

Fig. 3. Densitometry profile of silver stained SDS-PAGE of the isolated total rat fibrous sheath, showing two leading shoulders on the peak in the 80–90 kDa region, suggesting at least three proteins that have a similar molecular mass. The positions of the molecular mass standards (kDa) are marked with arrows.

32.7 kDa, 28.5 kDa and 15.5 kDa, although some species-specific bands of different molecular masses were present. Western blot analysis of isolated rat fibrous sheath with immune serum raised against purified rat fibrous sheath showed

Table 1. The abundance and yields of the isolated rat fibrous sheath proteins from the cauda epididymides of 12 rats

Protein band (kDa)	Total protein (%)	Yield (μ g)	Yield (%)
116.4	2.0	5.14 ± 0.55	8.8
87.5	nd ^a	1.57 ± 0.40	
80.0	48.6	5.87 ± 1.49	0.5
66.2	2.7	4.39 ± 0.05	5.6
57.2	2.9	6.32 ± 2.40	7.5
49.6	0.8	3.38 ± 1.11	14.4
46.8	2.4	1.61 ± 0.31	2.3
37.3	nd ^b	4.12 ± 2.04	
32.7	10.6	3.49 ± 0.66	1.1
28.5	28.7	24.09 ± 12.55	2.9
15.5	1.3	2.35 ± 0.07	6.2
Total	100	62.34 ± 1.97	2.1

^aDetected as part of the 80–87 band.

^bUnresolved by densitometry.

crossreactivity with fibrous sheath proteins, corresponding to their apparent abundance on SDS-PAGE gels. The 116 kDa and 80 kDa polypeptides of the rat, rabbit and human sperm proteins were strongly immunoreactive. The rat and rabbit sperm proteins had similar immunoreactive proteins of 80–87 kDa, 57.2 kDa, 32.7 kDa and 28.5 kDa; however, there was no immunoreactivity or weak immunoreactivity with the rest of the human sperm proteins. The preimmune serum did not crossreact with any bands.

Table 2. Amino acid composition of the rat fibrous sheath protein (FS) and the isolated protein components identified by apparent molecular mass (kDa)

	FS	Apparent molecular mass (kDa)										
		116.4	87.5	80.9	66.2	57.2	49.7	46.8	37.3	32.7	28.5	15.5
Asp	10.1	11.1	11.8	15.3	13.6	10.1	13.9	11.5	8.8	8.0	11.1	5.8
Glx	12.3	11.7	10.9	12.6	10.9	9.0	11.2	10.5	9.3	6.8	11.6	9.0
Ser	8.8	7.6	7.4	7.7	5.6	5.6	5.9	6.6	5.5	6.0	5.6	7.3
Gly	5.8	8.8	8.9	7.8	7.8	9.2	8.8	11.4	8.3	9.6	6.0	11.3
His	1.5	1.4	1.3	3.3	1.6	3.2	1.1	1.1	1.3	1.2	1.3	1.2
Arg	4.7	3.7	4.2	3.1	3.9	3.7	3.3	3.1	3.2	4.0	4.7	7.0
Thr	3.8	4.9	4.0	3.1	3.7	4.3	3.4	3.4	3.2	4.6	3.6	6.0
Ala	6.1	9.3	8.2	8.9	7.1	8.3	7.4	4.7	8.2	9.1	6.0	10.3
Pro	5.9	5.8	2.3	4.5	7.3	6.1	4.6	4.1	6.0	4.9	4.5	6.5
Tyr	2.0	0.5	0.6	0.9	4.6	1.1	11.1	4.1	8.4	7.1	1.4	0.6
Val	4.8	6.3	5.7	5.2	6.9	7.0	4.7	5.5	6.9	6.7	3.3	6.0
Met	4.3	2.2	3.2	3.0	3.3	5.7	2.8	3.0	5.9	3.5	3.5	1.2
Cys	8.8	4.3	7.5	1.4	3.4	1.6	1.4	9.4	3.3	4.3	8.4	4.8
Ile	3.2	4.4	5.0	4.1	4.8	6.9	4.8	5.5	5.1	5.4	4.6	4.9
Leu	8.5	8.7	8.9	8.7	6.8	8.8	7.4	7.2	8.1	7.8	10.6	9.4
Phe	2.2	3.7	1.8	2.1	2.6	3.0	2.2	2.9	2.5	3.3	6.1	1.6
Lys	7.1	5.8	8.3	8.5	6.1	6.5	5.9	5.9	6.1	7.6	7.7	7.0

Fig. 4. (a) SDS-PAGE of solubilized (1) rat, (2) rabbit, and (3) human spermatozoa together with (4) isolated total fibrous sheath stained with Coomassie brilliant blue. (b) Western blot of (1) human, (2) rabbit and (3) rat spermatozoa, and (4) isolated rat fibrous sheath, probed with a polyclonal antibody against rat fibrous sheath. STD: standards.

Immunofluorescence microscopy

Positive staining with the antiserum was confined to the principal piece of rat spermatozoa (Fig. 5b) since, as monitored by phase-contrast microscopy (Fig. 5a), the head showed no sign of staining. All the controls showed complete negative staining throughout the whole structure of the spermatozoa (Fig. 5d). The fluorescent signal seen within the middle piece was due to neither crossreactivity of the antibody nor non-specific binding, since these signals were always detected in this region without the addition of the second antibody, indicating an autofluorescent property. Specific staining was

Fig. 5. Immunofluorescence localization of rat fibrous sheath proteins. Phase-contrast (a, c) and fluorescence (b, d) micrographs of the same fields of rat spermatozoa probed with an anti-rat fibrous sheath antiserum raised in rabbits (a, b), or preimmune rabbit serum (c, d). Positive staining is shown mainly in the principal piece of the spermatozoa (large arrows). Small arrows indicate the middle piece where the autofluorescence is clear in (b) and (d). The principal piece shows no staining in control (d) and no crossreactivity is seen in the sperm head (arrowheads). Scale bar represents 7 μ m.

also seen exclusively in the principal piece of human and rabbit spermatozoa (Figs 6b and d, respectively). The antiserum also showed crossreactivity in the equatorial segment of rabbit sperm heads. All controls displayed the same phenomenon of autofluorescence in the middle piece (Fig. 6e; data for rabbit spermatozoa not shown) as did the rat spermatozoa.

Fig. 6. Immunofluorescence localization of rabbit and human fibrous sheath proteins. Phase-contrast (a, c) and fluorescence (b, d, e) micrographs of the same fields of rabbit (a, b) and human (c, d, e) spermatozoa probed with a rabbit anti-rat fibrous sheath antiserum. No fluorescein isothiocyanate-conjugated second antibody was used in panel (e). In both species, immunostaining is seen only in the principal piece (large arrows), while the middle piece shows only an autofluorescent background (small arrows). Specific staining is also seen in the equatorial region of the rabbit sperm head (arrowheads). Scale bar represents 7 μ m.

Discussion

This study demonstrates that it is possible to isolate a large number of the individual proteins that compose the fibrous sheath of the rat sperm tail. Other studies have isolated the fibrous sheath and separated its individual components by gel electrophoresis (Olson *et al.*, 1976; Oko, 1988) but only Brito *et al.* (1989) have been able to isolate sufficient quantities of three of these components to obtain amino acid analysis. Our data confirm these findings and extend the number of components that can be isolated using two biochemical procedures combined with several physicochemical separation techniques. Although the losses during the electroelution step are large, the process does enable isolation of sufficient quantities for amino acid analysis and attempts at amino-terminal sequencing. The latter technique has not yielded any sequences to date due to amino-terminal blocking of the 80 kDa, 32.7 kDa, 28.5 kDa and 15.5 kDa proteins. Attempts to use gel filtration, ion exchange or reverse phase HPLC were unsuccessful in purifying these proteins.

Chemical dissection of spermatozoa using SDS, Triton X-100, urea and DTT has been used to characterize various sperm structures by their selective solubilization properties (Millette *et al.*, 1973; Olson *et al.*, 1976; Calvin, 1976; Vera *et al.*, 1984; Oko, 1988; Jassim *et al.*, 1992). In the study reported here, a slight modification of the method developed by Olson *et al.* (1976) was used. The majority of the sperm tail components was solubilized by treatment with urea and DTT, followed by extraction with Triton X-100 and DTT. Although

this treatment left the fibrous sheath intact, remnants of the outer dense fibre cortex remained. This suggests that there is a complex three-dimensional structural organization of the outer dense fibres, with a different protein composition in the cortex from that in the medulla. Contamination by the outer dense fibre in our fibrous sheath preparation was minimized by solubilizing the fibrous sheath in 2% SDS and DTT, which allowed the removal of the outer dense fibre cortex by centrifugation.

The amino acid composition of the 80 kDa and 24 kDa proteins reported by Brito *et al.* (1989) are similar to the composition of the 80 kDa and 28.5 kDa proteins in the study reported here. However, the 15.5 kDa protein in this study differs significantly from the 12 kDa protein of Brito *et al.* (1989), particularly in serine, alanine, tyrosine, valine and isoleucine content. These differences may relate to contamination of the fibrous sheath preparation by the cortex of the outer dense fibre using the preparative scheme outlined. In the study reported here, a further step was introduced to remove this contamination, whereas no comment was made regarding such material in the study by Brito *et al.* (1989). In addition, data on the amino acid composition of a further six proteins isolated from the fibrous sheath are presented here. It is clear that amino acid sequences are necessary to identify the genes encoding these proteins as a prelude to determining their role in the formation of the fibrous sheath. However, in view of the fact that the amino termini of a number of these proteins were blocked, the amino acid composition provides a method of characterizing some of these proteins.

It has been suggested that the proteins of the fibrous sheath and the outer dense fibres may be similar in view of their features under electron microscopy and the observation that antibodies to fibrous sheath proteins crossreacted with those in the outer dense fibres in the study by Oko (1988). Our antibody to whole fibrous sheath did not crossreact with the outer dense fibre proteins on western blots (data not shown) and, furthermore, the immunofluorescence localization of the epitopes recognized by the fibrous sheath antiserum were confined to the principal piece of rat, rabbit and human spermatozoa and did not localize to the region of the mid-piece. Again, these differences may relate to the purity of the fibrous sheath preparations used to immunize rabbits. The fact that the 116 kDa and 80 kDa proteins of all species crossreact with the antiserum to the rat fibrous sheath raises the possibility that these proteins share common antigenic determinants. Furthermore, additional fibrous sheath proteins common to the rat and rabbit are detected by the antiserum. The equatorial segment of the rabbit sperm head also crossreacted strongly with the antiserum and the reasons for this are unclear. In many species, the equatorial segment exhibits properties that distinguish it from other regions of the head, but it is surprising that a flagella protein or part thereof is represented in this region.

The availability of the antiserum offers the possibility of screening λ gt11 expression libraries with a view to cloning the genes encoding for the proteins that appear to be amino-terminally blocked. Antibodies to fibrous sheath proteins have been described in a number of studies raising the prospect of their use to screen libraries (Sakai *et al.*, 1986; Fenderson *et al.*, 1988; Jassim, 1991; Jassim *et al.*, 1991; Beecher *et al.*, 1993). Alternatively, approaches such as those outlined by Morales *et al.* (1994), who obtained an internal amino acid sequence of the 27 kDa outer dense fibre protein, may be applicable to the fibrous sheath proteins. The demonstration that it is possible to isolate sufficient quantities of a number of these proteins for amino acid analysis should provide the basis for further studies of their structure.

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