Comparison, characterization, and identification of proteases and protease inhibitors in epididymal fluids of domestic mammals. Matrix metalloproteinases are major fluid gelatinases

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Comparison, Characterization, and Identification of Proteases and Protease Inhibitors in Epididymal Fluids of Domestic Mammals. Matrix Metalloproteinases Are Major Fluid Gelatinases[1]

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ABSTRACT

The testicular and epididymal fluids of ram, boar, and stallion were analyzed by means of one-dimensional and two-dimensional gelatin gel zymography. Five main gelatinolytic bands were revealed in the ram and at least seven were observed in the boar and stallion. These proteolytic bands showed regionalized distribution throughout the organs. The two main proteolytic activities at around 54–66 kDa retrieved in all three species were inhibited by EDTA and phenanthroline, indicating that they were metallo-dependent enzymes. The activity of some of the low-molecular-weight gelatinases was also decreased by EDTA, whereas others were inhibited by serine protease inhibitors. One of the main proteases at 60–62 kDa from the caput fluid of the stallion and the ram was N-terminal sequenced; in both cases, high sequence homology was found with the N-terminal of the matrix-metalloproteinase-2 pro-form (pro-MMP-2). Antibodies against MMP-2, MMP-3, and MMP-9 gelatinases confirmed the regional distribution in the fluids of pre -, pro-, active, or degraded forms of these metalloproteases in all three species. We also observed the presence of acrosin in epididymal fluids, which was probably released by dead spermatozoa, but this enzyme did not explain all the serine protease activity. Moreover, the majority of this enzyme is bound to the protease inhibitor α_2-macroglobulin, which is present in the fluids of all three species. TIMP-2, a potent inhibitor of MMPs, was present in the fluid of the caput regions in the ram and boar, and in the caput and caudal fluids of the stallion. This study demonstrated that similar types of proteases and inhibitors are regionally distributed in the epididymal fluids of three domestic species, suggesting an identical role in the sperm maturation process, the plasticity of this organ, or both.

epididymis, gamete biology, male reproductive tact, sperm maturation

INTRODUCTION

Proteases and protease inhibitors play key roles in almost all physiological processes, including cell migration, cell signaling, and cell surface and tissue remodeling [1]. For example, a large number of proteases and protease inhibitors have been found in the testis, which undergoes constant remodeling due to spermatogenesis. Several of these proteases, such as procathepsin L, furin-type PC4, or neprylisins are involved in protein processing [2–4], whereas others such as the plasminogen activator and different matrix-metalloproteinases (MMPs) are involved in tissue remodeling, cell migration, or cell-cell interactions [5–8]. The presence of protease inhibitors with broad specificity such as α_2-macroglobulin and cystatin C [9, 10], protein C inhibitor [11], or more specific inhibitors such as the different tissue inhibitors of MMPs (TIMPs) [12] have also been documented in the testis. The equilibrium between proteases and their inhibitors appears to be critical for conserving the integrity of the blood-testis barrier and gamete development [11, 13]. The spermatozoa are also rich in different proteases, which are mainly localized within the acrosomal vesicle [14, 15]. The best known of these proteases is acrosin, which plays a role in sperm passage through the zona pellucida [16]. Keeping these sperm proteases in an inactive state is also critical to maintaining cell integrity to ensure reproductive function [11, 17].

In contrast to the proteases of the testis and the sperm cell, proteases involved in epididymal function have been given less attention. The epididymis links the testis to the vas deferens and it is during their sojourn in this organ that sperm acquire their fertilizing ability [18]. Epididymal sperm maturation involves large changes in specific membrane domains, mainly due to modifications in their protein composition. Some of these modifications result from very specific proteolytic processing leading to the disappearance or the redistribution of proteins among the different domains [18, 19]. Examples of these proteolytically transformed compounds are the different members of the ADAMs (i.e., a disintegrin and metalloprotease domain) family in rodents and monkeys (for reviews see [20–23]), the rat sperm CE9 antigen [24] and hyaluronidase (pH20/2B1) [25], the boar 135-kDa mannosidase [26], and the germinal form of angiotensin converting enzyme (ACE) in several domestic mammals [27].

In the present study we demonstrate that different MMPs and several gelatinolytic serine proteases are present in the testicular and epididymal fluids of three different domestic mammals (ram, boar, and stallion). We also demonstrate the regionalized presence of specific protease inhibitors that may control these activities in vivo. The role of these enzymes and inhibitors are discussed in view of the different functions of the epididymis.

MATERIALS AND METHODS

Chemicals

Low molecular weight standards for electrophoresis were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Specific protease inhibitors, EDTA, and all other reagents were of the best available grade (Sigma, Saint Quentin Fallavier, France; and Prolabo, Fontenay-sous-Bois, France).

Fluid and Sperm Collection

Epididymides and testes were obtained from adult Ile de France rams (eight animals), Large white boars (two animals) and selle Français stal-

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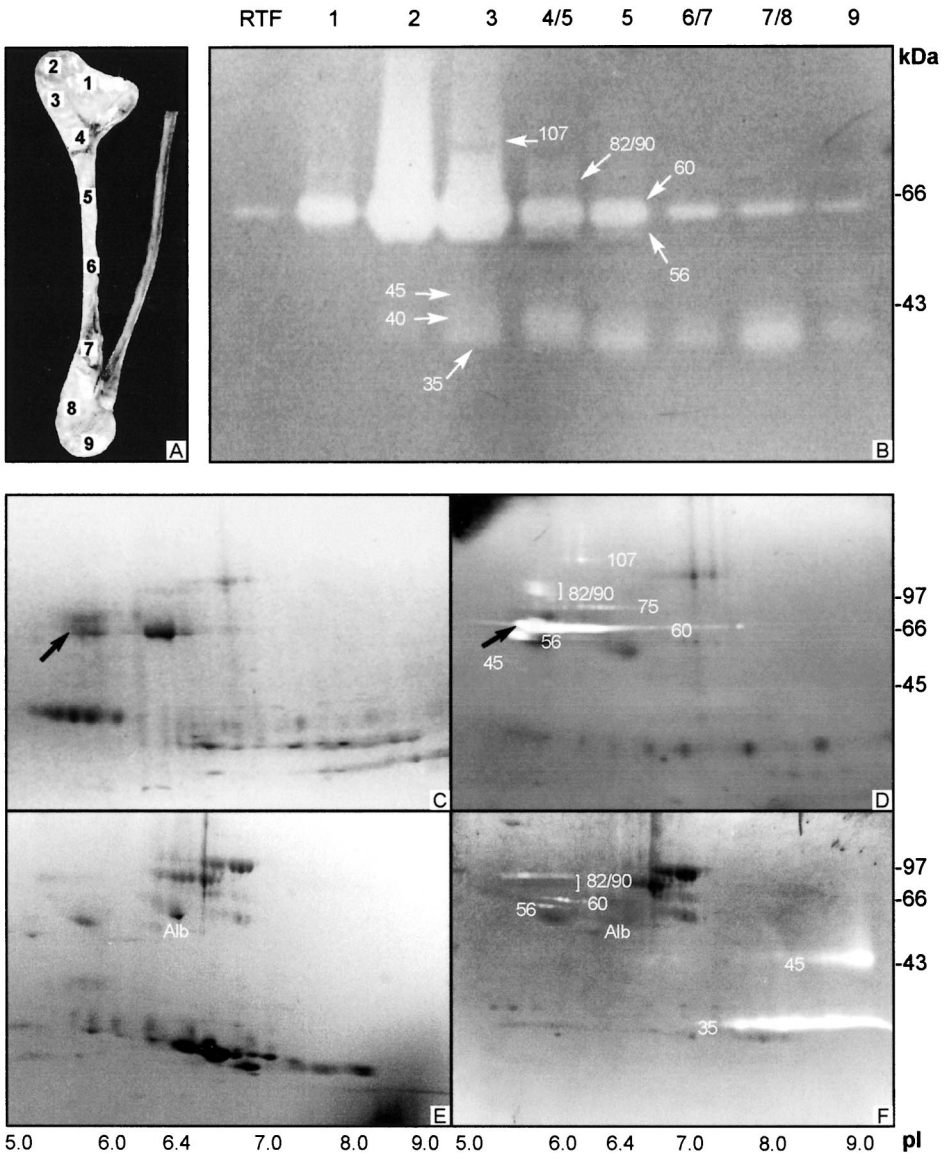

FIG. 1. Gelatinases in ram epididymal fluids. **A**) Ram epididymis showing the different zones where fluids were obtained (1–3, caput; 4–7, corpus; 8–9, cauda). **B**) One-dimensional gelatin zymogram of the rete testis fluid (RTF) and the epididymal fluids. Proteases appear as white bands on a black background. Ten micrograms of protein loaded per lane. **C** and **D**) Coomassie blue-stained two-dimensional nonreducing gel of the fluid from zones 2 (**C**) and 9 (**E**) and their equivalent gelatin zymogram (**D** and **F**, respectively). Separation of 0.3 mg of protein in **C**, 0.6 mg in **E**, 0.2 mg in **D**, and 0.4 mg in **E**.

lions (two animals) by surgical castration or from the local slaughterhouse. Testicular fluid was carefully collected by puncturing the rete testis. Fluids from the different epididymal zones (Figs. 1A, 2A, and 3A) were microperfused as previously described [28]. Spermatozoa were separated from the fluid by centrifugation (10 min at 15 000 × *g* at 4°C). The fluids were carefully removed and centrifuged again (10 min at 15 000 × *g*) and used directly or stored at −20°C.

Gel Electrophoresis

Protein isoelectrofocalization was performed as previously described [29] except that when indicated, samples were not treated with dithiothreitol to remain under nonreducing conditions. SDS 6%–16% polyacrylamide gel gradients were used for protein separation and β-mercaptoethanol was omitted from the sample buffer. The gels were stained with Coomassie blue. For zymography the gel solutions were supplemented with gelatin (0.15 mg/ml). After migration the zymograms were washed for 2 h in a water solution with 2.5% (w/v) Triton X-100 to allow in situ protein renaturation and incubated for 18 h at 37°C in the following solution: 50 mM Tris pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% (w/v) Tween 20 pH 7.5. Gelatinolysis was revealed by Coomassie blue staining (0.02% in methanol, acetic acid, and water, 3:1:6); The places where protease activity occurred appeared as white bands or areas on a blue background.

For the inhibition study, 6%–16% gelatin gels of fluid from zones 2 and 9 were run and the gel was cut into 2-cm-wide slices. Each slice was washed and incubated as above with one of the following inhibitors at the indicated concentration: EDTA, 1 mM; para-aminobenzamidine (PABA), 2 mM; aprotinin, 1 μg/ml; E64 (trans-epoxysuccinyl-L-amido(4-guanido)butane), 5 μg/ml; PMSF, 1 mM; AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride), 1 mM; bestatin, 5 μg/ml; leupeptin, 0.5 μg/ml; pepstatin, 1 μg/ml; phenanthroline, 1 mM; and antipain, 10 μg/ml. The zymogram slice was then developed and the inhibition was visualized by the absence or the decrease in intensity of the protease band as compared to a control lane obtained in the absence of inhibitor.

For N-terminal sequencing several two-dimensional gels and an equivalent zymogram were compared after migration. The Coomassie blue-stained spots of the normal gel, corresponding to the center of the area of protease activity, were carefully cut off. The pieces of gel were pooled and mashed with a pestle in a 2-ml microtube and supplemented with two volumes of reducing sample buffer (2×) before storage overnight at 4°C under constant shaking. The gel was removed by centrifugation (15 000 × *g*, for 10 min at 4°C), and the supernatant was boiled and loaded on 6%–16% SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride (PVDF), and the membrane was stained with Coomassie blue. The N-terminal of the visible bands were sequenced (Porton sequencer, Beckman Instruments, Fullerton, CA).

Western Blotting

Semi-dry transfer of proteins was performed over 2 h at 0.8 mA/cm^2 using Towbin buffer [30] for nitrocellulose or with cyclohexylamino-propanesulfonic acid (CAPS) pH 11 for PVDF. The Western blots were blocked with TBS 0.5% (w/v) Tween 20 and supplemented with 5% (w/v)

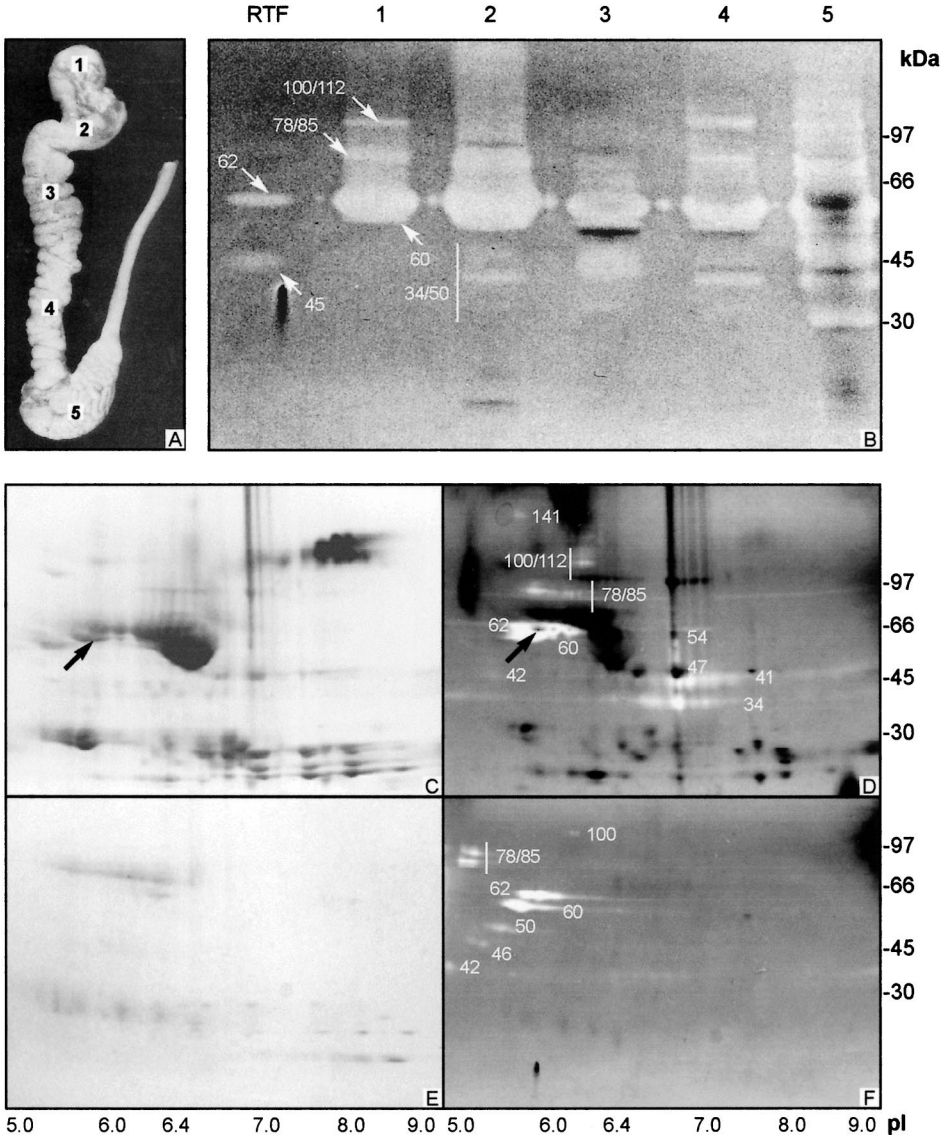

FIG. 2. Gelatinases in stallion epididymal fluids. **A**) Stallion epididymis showing the different zones where fluids were obtained (1–2, caput; 3–4, corpus; 5, cauda). **B**) One-dimensional gelatin zymogram of the rete testis fluid (RTF) and the epididymal fluids. About 15 μg protein loaded per lanes. **C** and **D**) Coomassie blue-stained two-dimensional nonreducing gel of the fluid from zones 2 (**C**) and 5 (**E**) and their equivalent gelatin zymogram (**D** and **F**, respectively). Separation of 0.35 mg of protein in **C**, 0.75 mg in **D**, 0.5 mg in **E**, and 1 mg in **F**.

lyophilized low-fat milk. The first antibodies were added at 1:1000 dilution for anti-MMPs, anti-α_2-macroglobulin, and TIMP2; and at 1:5000 for anti-acrosin. The blots were incubated under mild agitation overnight at 4°C or for 2 h at 37°C. The second antibody was a goat anti-rabbit antibody conjugated with peroxidase (dilution 1:5000; Sigma). The peroxidase was revealed with chemo-luminescent substrates (ECL$^+$, Amersham Pharmacia Biotech AB) and the images were recorded on film (X-Omat blue, NEN, Boston, MA).

Antibodies

Anti-MMPs. The antibodies used were commercially available rabbit polyclonal antibodies. The anti-MMP-2 were directed against synthetic peptides corresponding to the carboxyl terminal part of the of human enzyme; the hinge region (M4677; Sigma) or the hemopexin domain (M4552; Sigma). Both antibodies gave similar results although the polyclonal antibody against the hinge region was more reactive with the ram. The anti-MMP-9 (M5177; Sigma) and anti-MMP-3 (M4927; Sigma) were also directed against a synthetic peptide corresponding to the C-terminal hemopexin domain of human MMP-3 and MMP-9, respectively. Rabbit anti-α_2-macroglobulin polyclonal antibody (M-1893; Sigma) was raised against the human purified protein and anti-TIMP-2 (T8062; Sigma) was obtained against a synthetic peptide corresponding to the first loop of human TIMP-2.

Anti-acrosin. Acrosin isolation from ejaculated spermatozoa was adapted from the method described by Brown et al. [31]. The acid extract obtained from about 5×10^{10} cauda epididymal sperm was dialyzed against acidified deionized water, vacuum-dried, and dissolved in a mixture of 10% acetonitrile-0.1% trifluoroacetic acid (TFA) in deionized water. This extract was separated by reverse phase high-performance liquid chromatography (C18; 5 μm, 300 A, 3.9 × 150 mm; Waters, St. Quentin en Yveline, France) using a 10%–50% linear gradient (1 ml/min) with buffer A (deionized water, 0.1% TFA) and buffer B (10% deionized water, 90% acetonitrile, 0.1% TFA). Fractions showing the maximum protease activity at about 40 kDa on gelatin zymogram were further purified to homogeneity by gel filtration (Hiload 16/60, Superdex 200, Pharmacia). The identity of the purified protease was obtained by N-terminal amino acid sequencing. Two sequences were obtained at the same time: R-D-N-T-T-X-D-G-P-X-G-V-R-F-R, and I-I-G-G-Q-D-A-A-H-G-A-W-P-W-M. These sequences were identical to the recently published N-terminal sequences of the sheep acrosin light and heavy chains, respectively [32]. Positions 6 and 10 in the published light chain sequence corresponded to cystein residues, which could not be determined during the sequencing of our protein due to the Edman chemistry employed. The purified acrosin was then used to produce two polyclonal antibodies by a previously described procedure [27]. The final sera recognized only one main band at about 43 kDa on Western blotting of fresh SDS extracts from ram ejaculated or cauda epididymal sperm (not shown).

RESULTS

Presence of Gelatinases in Epididymal Fluids

Ram. One-dimensional gelatin zymogram of the fluids collected from the rete testis (RTF) at different levels of the ram epididymis (Fig. 1A) revealed several regionalized

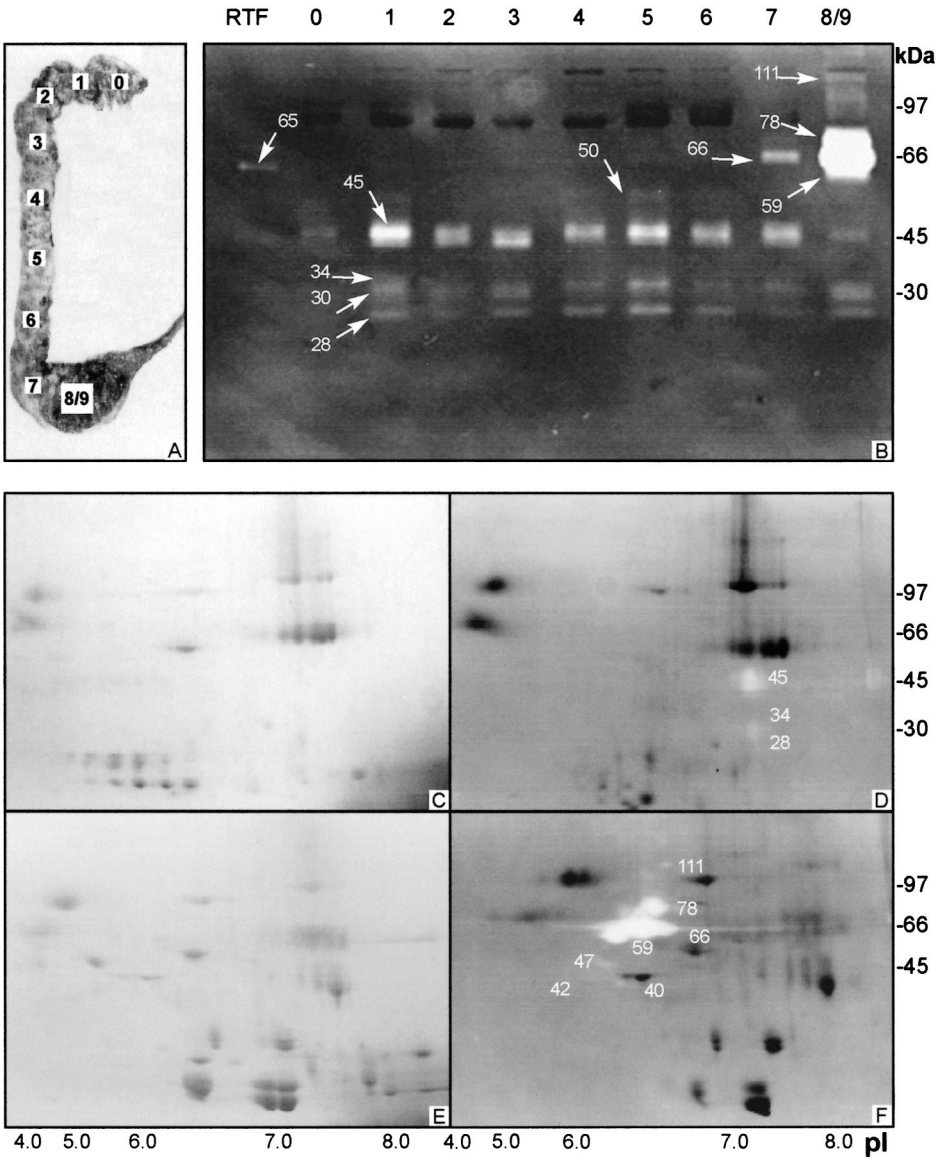

FIG. 3. Gelatinases in boar epididymal fluids. **A**) Boar epididymis showing the different zones where fluids were obtained (0–3, caput; 4–6, corpus; 7–9, cauda). **B**) One-dimensional gelatin zymogram of the rete testis fluid (RTF) and the epididymal fluids. Ten μg of protein loaded per lane. **C** and **D**) Coomassie blue-stained two-dimensional nonreducing gel of the fluid from zones 2 (**C**) and 8/9 (**E**) and their equivalent gelatin zymogram (**D** and **F**, respectively). Separation of 0.70 mg of protein (**C, D**, **E**, and **F**).

proteases with molecular weights ranging from 35 to 107 kDa (Fig. 1B and Table 1). The major protease activity was detected at about 60 kDa from the RTF to the caudal fluid and the quantity was maximal in zones 2 and 3. In these zones, the presence of proteases at 107 kDa and 82–90 kDa was only slightly visible due to the smear formed by the 60-kDa gelatinase. When the activity of this 60-kDa band decreased (zones 4–5 and 5), a 56-kDa gelatinase was observed. Proteases between 35 and 45 kDa appeared in the fluid of zone 2 and remained until the cauda epididymis. All these proteases varied in intensity and slightly in epididymal distribution between different animals (see also Fig. 5D).

Two-dimensional gel zymography of the fluid from zone 2 (Fig. 1D) confirmed the presence of the 107-kDa and 82- to 90-kDa proteases and also demonstrated that the 56-kDa protease was already present in this fluid. Other minor proteases such as the band at 75 kDa also became clearly visible. Comparison of this protease pattern with that obtained with the fluid of zone 9 (Fig. 1F) showed that the major gelatinolytic spots decreased in intensity and those of the 107-kDa and 75-kDa proteases disappeared. This also indicated that the 45-kDa acidic gelatinase detected in fluid from zone 2 may be different from the 45-kDa gelatinase present in zone 9 because they have very different isoelectric points (pIs; Table 1).

When the two-dimensional zymograms of fluids from zones 2 and 9 were compared to equivalent Coomassie blue-stained gels (Fig. 1, C and E), only the most acidic 60-kDa protease in fluid from zone 2 could be matched to a protein spot (see arrows in Fig. 1, C and D).

Stallion. The stallion rete testis and epididymidal fluids obtained from 5 different zones (Fig. 2A) were analyzed by one-dimensional zymography (Fig. 2B). Six to 10 major gelatinase bands with molecular weights ranging from 34 kDa to 112 kDa were detected (Table 1). In RTF, two gelatinases were present at 45 kDa and 62 kDa, and only the 62-kDa band remained from the rete testis to the cauda epididymidal fluid, with maximum activity in zone 2. Several other proteolytic bands appeared in the caput epididymidis; 3 major bands at 100–112, 78–85, and 60 kDa in zone 1 and several minor bands between 34 kDa and 50 kDa in zone 2. At least 10 different bands were visualized in the cauda (zone 5), suggesting the accumulation of proteases in this fluid and the appearance of new enzymes.

Two-dimensional zymography of fluids of zone 2 (caput)

TABLE 1. Presence and specificity of gelatinases from zones 2 and 9.

Ram				Stallion				Boar			
kDa	pI	Zone	Type*	kDa	pI	Zone	Type	kDa	pI	Zone	Type
				141	5.9	2	Metallo.				
				100–112	6.2	2	Metallo.	111	6.5	9	Metallo.
107	6.2	2	Metallo.	100	6.2	9	Metallo.	78	6.5	9	Metallo.
82/90	5.8	2 and 9	Metallo.	78–85	5.1–5.4	2 and 9	Metallo.	66	6–6.8	9	Metallo.
75	6–6.5	2	Metallo.	62	5.5–6.2	2 and 9	Metallo.	59	6.2–6.3	9	Metallo.
60	5.7–7.6	2 and 9	Metallo.	60	5.5–6.1	2 and 9	Metallo.	47	6–6.2	9	Metallo./Serine
56	5.6–5.9	2 and 9	Metallo.	54	7	2	Metallo.	45	7.5	2	Metallo./Serine
45	5.6	2	Metallo./Serine	50	5.6	9	Metallo.	42	6	9	ND
45	8.5–9	9	Metallo./Serine/E64	46	5.4	2	Metallo.	40	6.3	9	ND
40	ND	2 and 9	Metallo./Serine	47	7	9	Metallo.	34	7.5	2	ND
35	7.4–9	2 and 9	Serine/E64	42	5.1	2 and 9	Metallo.	28	7.5	2	ND
				41	7–7.2	2	Metallo.				
				34	7.1	2	Serine				

* Metallo., Inhibited by metalloprotease inhibitors; Serine, inhibited by serine protease inhibitors; E64, inhibited by cysteine protease inhibitor; ND, not determinated.

and zone 5 (cauda) (Fig. 2, D and F, respectively) confirmed the presence of these major gelatinases and revealed other less intense proteases such as the 141-kDa spot in zone 2. It also made it possible to demonstrate that the bands at 100–112 and 78–85 kDa were each formed by three closely migrating minor spots (100, 107, and 112 kDa; and 78, 81, and 85 kDa, respectively). The major 62-kDa gelatinase band present in zone 2 was separated into three spots, with the most acidic disappearing from the caudal fluid. The 42-kDa and 60-kDa proteases were also retrieved in the latter fluid but the 141-kDa and 100–112 kDa proteases had disappeared. The 78–85 kDa spots remained in the caudal fluid but apparently presented an acidic shift. The four gelatinases between 34 kDa and 54 kDa with pIs around 7–7.2 observed on the gel of zone 2 disappeared, but new spots with pIs between 5 and 6 appeared at 46 and 50 kDa. Only 7 gelatinases were revealed by two-dimensional zymography, whereas at least 10 protease bands were on the one-dimensional zymogram. The difference could be explained by the presence of acidic (pI <5) or basic (pI >9) proteases, which could not be resolved on two-dimensional zymography.

When the fluids of zones 2 and 9 were separated by two-dimensional gel and stained with Coomassie blue (Fig. 2, C and E), only 1 protein spot in the fluid from zone 2 matched a gelatinase spot (see arrows on Fig. 2, C and D).

Boar. Seven to 10 main different gelatinolytic bands with molecular weights ranging from 28 to 111 kDa were observed in the fluids collected from the rete testis and 9 different zones of the boar epididymis (Fig. 3, A and B; Table 1). A faint 65-kDa protease was restricted to the rete testis and zone 0 fluids. At least 4 gelatinolytic bands between 28 and 45 kDa appeared in zone 1, and were detected until zone 8/9. In this caudal fluid a large 66-kDa gelatinolytic band already detected in fluids from zone 7 increased sharply in intensity. Two other important 59- and 78-kDa bands were visualized only in this caudal fluid, as well as several higher molecular weight proteases (for example the 111-kDa band).

Two-dimensional zymography of fluids from zone 2 and zone 9 confirmed these results (Fig. 3, D and F, respectively; Table 1) and suggested that the gelatinases with molecular weights around 45 kDa detected in the fluid of zone 2 were different from the 40- to 47-kDa proteases present in caudal fluid. None of these proteases matched a major protein spot of the boar epididymal fluids (Fig. 3, C and E).

Specificity of Gelatinases from Epididymal Fluids

The types of gelatinase present in the fluids from zones 2 and 9 of all 3 species were determined on one-dimensional zymography by the action of specific inhibitors (see *Materials and Methods*). The main proteases for each species, situated between 56 and 66 kDa, and all gelatinases with higher molecular weights, were inhibited by metalloprotease inhibitors such as EDTA and phenanthroline. All the results are summarized in Table 1.

The minor gelatinases with molecular weights ranging between 40 and 45 kDa in the ram were partially inhibited by serine protease inhibitors (PABA, AEBSF, and antipain), whereas the 35-kDa band was entirely inhibited by E64 and serine protease inhibitors. EDTA, phenanthroline, and E64 also partially inhibited the 45-kDa gelatinase.

The major gelatinases in stallion epididymal fluids were entirely inhibited by EDTA and phenanthroline, whereas the band situated at 34 kDa was only partially inhibited by these inhibitors. The activity of this protease was also decreased by the presence of serine protease inhibitors.

The 45-kDa gelatinase detected in zone 2 in the boar was partially inhibited by the serine protease, metalloprotease inhibitors, and E64. In contrast, the gelatinase of the same molecular weight present in zone 9 was entirely inhibited by metalloprotease inhibitors. In these fluids all the gelatinases between 25 and 35 kDa were entirely inhibited by serine protease inhibitors.

Identification of One of the Major Gelatinases Present in Stallion and Ram Epididymal Fluids

In the stallion, we observed that a 62-kDa protein spot matched a gelatinase area in the fluid from zone 2 (arrows in Fig. 2, C and D). This spot was cut from Coomassie blue-stained gels and run on SDS-PAGE after extraction and reduction (see *Materials and Methods*). Two protein bands around 66 kDa were obtained after transfer on PVDF and were N-terminal sequenced. The sequence obtained for the heavier band was A-P-S-P-I-I, which perfectly fit the N-terminal of the pro-form of the human MMP2 (pro-MMP2/72 kDa gelatinase/gelatinase A/EC 3.4.24.24). The N-terminal sequence of the second band was A-V-V-F-D-P-K-P-A-L, and presented 90% homology with the α1B-glycoprotein (plasma protein XK).

The same protocol was applied to one spot corresponding to the 60-kDa gelatinase in the ram fluid from zone 2

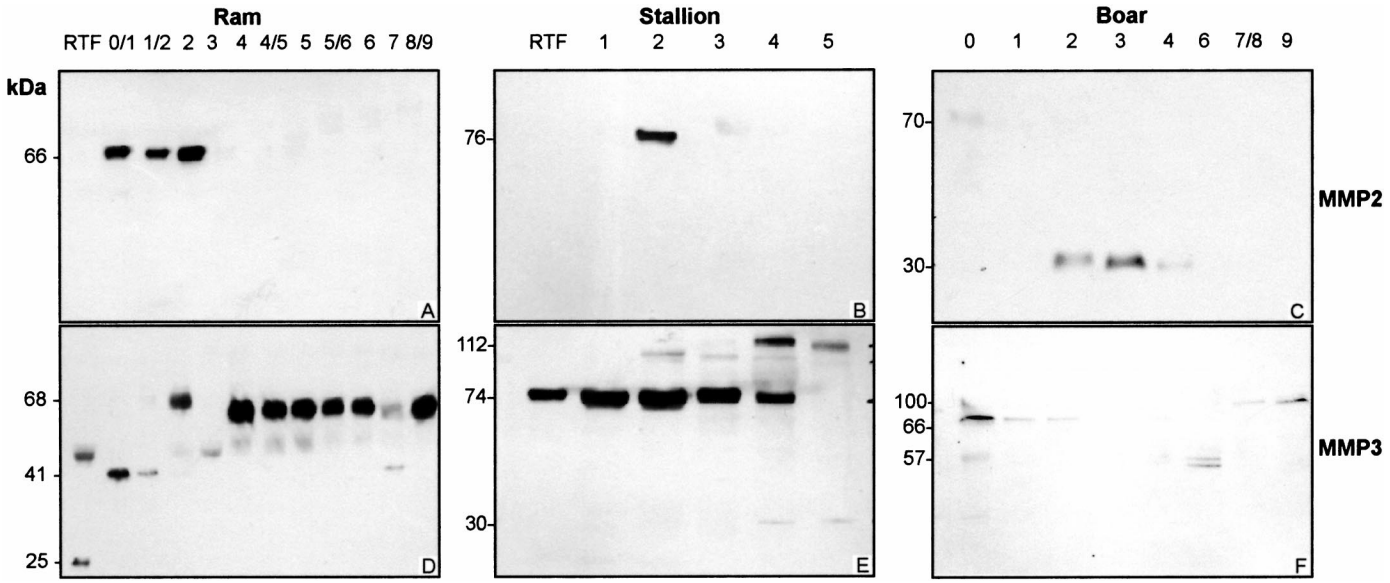

FIG. 4. Presence of MMP-2 and MMP-3 in epididymal fluids. Western blots of ram (**A** and **D**), stallion (**B** and **E**), and boar (**C** and **F**) epididymal fluids were probed with antibodies directed either against MMP-2 (**A**, **B**, and **C**) or MMP-3 (**D**, **E**, and **F**). About 50 μg protein were loaded per lane.

(see arrows in Fig. 1, C and D). One band was obtained after extraction and was sequenced. Its N-terminal was A-P-D-P-E-L-K-F-P-G, which also showed 70% homology with the N-terminal of the pro-form of MMP-2.

Immunodetection of MMPs in Epididymidal Fluids

Because one of the main gelatinases from zone 2 in the stallion and ram was identified as pro-MMP-2, and because the major gelatinases in epididymidal fluids from each mammal studied were also metalloproteases, we explored the presence of MMP2 by immunoblotting and also two other gelatinases of this family: MMP3 (stromelysin-1/EC 3.4.24.17) and MMP9 (gelatinase B/EC 3.4.24.35).

The Western blot probed with anti-MMP-2 antibodies detected one main immunoreactive 72-kDa band in the ram caput epididymal fluids (Fig. 4A and Table 2). Another less visible band was also observed at 56 kDa in the same zones with different animals (not shown). In the stallion, a 76-kDa band also restricted to the caput epididymis (zone 2) was immunodetected (Fig. 4B), confirming the result of the N-terminal sequencing. In the boar the antibodies revealed immunoreactive bands also restricted to the caput region: a faint band at 83 kDa from zones 0 to 2, and a more reactive 30-kDa band from zones 2 to 4 (Fig. 4C).

The anti-MMP-3 antibody reacted against different bands distributed throughout the epididymis in all three species (Fig. 4, D–F; Table 2). Five different bands were observed in the ram (Fig. 4D); the main reactive at 67 kDa was detected in the fluids from zones 4 to 9. A slightly higher molecular weight band at 68–70 kDa with increasing reactivity was observed from zones 0 to 2. A 41-kDa compound was detected in these caput fluids with decreasing

TABLE 2. Different forms of matrix metalloproteinases detected by Western blotting in testicular and epididymal fluids.

Theoretical detection	Ram	Stallion	Boar
MMP2			
72 kDa = pro-form (WB, Z)*	72 (0 → 2)†	76 (2)	83 (0 → 2)
66–68 kDa = active form (WB, Z)	ND	ND	ND
42 kDa = autolytic form (Z)	ND	ND	ND
31 kDa = domain C-term (WB)	ND	ND	30 (2 → 4)
?	55 (2, 3)	ND	ND
MMP3			
59–57 kDa = pro-form (WB, Z)	68 (0 → 2), 67 (4 → 9)	74 (T → 4)	74 (0 → 4)
45 kDa = active form (WB, Z)	45 (T, 2 → 9), 41 (0 → 2, 7)	ND	57 (0, 4 → 6)
28 kDa = autolytic form (Z)	ND	ND	ND
21 kDa = active form (Z)	ND	ND	ND
?	25 (T)	110 (4, 5), 100 (2 → 5), 30 (4, 5)	105 (7 → 9), 100 (7 → 9)
MMP9			
92–95 kDa = pro-form (WB, Z)	97 (9)	100 (5)	107 (T)
82–88 kDa = active form (WB, Z)	ND	ND	100 (0 → 2)
65 kDa = activation by cleavage C-term (Z)	ND	ND	ND
30 kDa = domain C-term (WB)	30 (9)	30 (5)	ND
?	70 (T → 2)	66 (2 → 5), 18 (5)	63 (T → 2), 45 (7), 15 (0 → 2)

* WB, Form detectable by Western blot; Z, form detectable by zymography.
† Molecular mass of the protein detected by Western blot and (in parenthesis) the epididymal zones where the protein was detected; T, rete testis fluid; ND, not detected; ?, unmatched form.

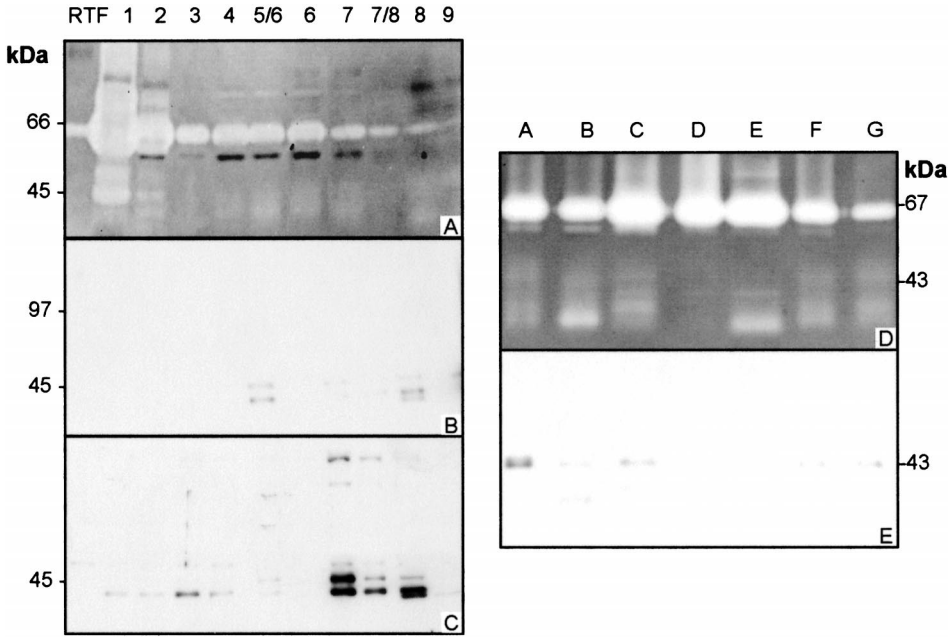

FIG. 5. Presence of acrosin in ram epididymal fluids. Fluids from the rete testis (RTF) and from the different epididymal zones were separated by one-dimensional gel zymography (**A**) or by SDS-PAGE under nonreducing (**B**) or reducing conditions (**C**) before transfer on nitrocellulose. The Western blots (**B** and **C**) were probed with the anti-acrosin antibody. The zone 2 fluid from seven different rams were separated for zymography (**D**) or blotted under non-reducing conditions and probed with the anti-acrosin antibody (**E**). In **A** and **D**, about 10 μg protein were loaded per lane, whereas in **B**, **C**, and **E**, 50 μg protein were loaded.

intensity, which was not visible in the corpus fluids, but reappeared in zone 7. In RTF a band at 25 kDa and one at 45 kDa (which was also slightly visible in fluids from zones 2 to 9) were detected. Four different bands were immunodetected in stallion and boar fluids (Fig. 4, E and F, respectively; Table 2). In the stallion, the main reactive band at 74 kDa was present from the RTF to the distal corpus region (zone 4), where a 110-kDa and a 30-kDa band became visible (zones 4–5). From the distal caput to the caudal region a band at about 100 kDa was also slightly reactive. In the boar, a 74-kDa band reacted from the RTF to zone 4, where a doublet of about 57 kDa, also detected in RTF, became visible and remained until zone 6. In the cauda they were replaced by 2 immunoreactive bands at about 100 kDa.

The MMP-9 antibody showed only low levels of reactivity in all three species and the results are summarized in Table 2. In some series of ram epididymidal fluids, this antibody detected a band at 70 kDa in the caput with decreasing intensity after zone 1, and bands at 97 and 30 kDa in caudal epididymal fluid. In the boar, two heavier molecular weight proteins (more than 100 kDa) and a protein at 63 kDa were observed from RTF to zone 2 and a 15-kDa band from zones 0 to 2. A band at 45 kDa was also detected in fluid from zone 7. A band at 66 kDa was detected in the stallion fluids from zones 2 to 5. In this last zone, 3 other bands were detected at 100, 30, and 18 kDa.

Presence of TIMP-2 Inhibitor in the Epididymal Fluids

TIMPs are specific inhibitors of MMPs, which are generally present at the same time as the matrix proteases. We therefore probed ram, boar, and stallion epididymal fluids with an anti-TIMP 2 antibody. Under nonreducing conditions, a band with a low level of intensity at about 97 kDa could be detected from the RTF to zones 2–3 in all species. In the ram RTF, the expected band at 21 kDa (the molecular weight of this TIMP) was also observed (not shown). In reducing conditions, the 21-kDa protein was visible in the ram RTF and appeared in boar RTF, whereas in the stallion, the 21-kDa band was detected only in the caudal epididymal fluid (not shown).

Immunodetection of Acrosin in Epididymal Fluids

Because some of the gelatinases around 40 kDa were serine proteases that were completely inhibited by PABA, a potent acrosin inhibitor [33], we made the assumption that this enzyme could be released by dead spermatozoa in the epididymal fluid. This was supported by our observation that the number of dead spermatozoa in the ram measured by the nuclear 4′,6′-diamidino-2-phenylidole staining [34] increased throughout epididymal transit from 7.3% ± 5.1% (mean ± SEM; n = 27) in the rete testis to 14.5% ± 4.7% (mean ± SEM; n = 20) in the caudal epididymidis.

Ram epididymal fluids were then analyzed both by zymography (Fig. 5A) and Western blotting using the polyclonal antibody obtained against the ovine sperm acrosin (Fig. 5, B and C). When the samples were not reduced before electrophoresis, this antibody recognized 3 bands at 40, 43, and 45 kDa in zones 5 to 9 (Fig. 5B). A high-molecular-weight band (>100 kDa) was detected from the RTF of zone 1 fluid in some series (not shown). Under reducing conditions (Fig. 5C), 3 bands at 40, 43, and 45 kDa were detected in almost all fluids with a large increase in caudal fluids. Reactive high molecular weight bands were also observed in these fluids. Several of the immunodetected bands of acrosin matched some of the gelatinase bands observed around 45 kDa in the fluids from different zones (Fig. 5, A and B). However, the presence at the same time of acrosin and gelatinolytic bands, as well as the number and intensity of these gelatinolytic bands, could vary considerably in the fluids of the same zone from different rams. For example, in 7 fluids from zone 2 (Fig. 5, D and E), gelatinase activities could be visualized even without detection of acrosin (see Fig. 5, lane E) and no relationship could be found between the level of activity and acrosin immunoreactivity.

When boar epididymal fluids were probed with the same anti-acrosin antibody under nonreducing conditions, a strong reaction was observed with a doublet of protein with a molecular weight of >110 kDa (Fig. 6A). Their reactivity showed a decreasing gradient of intensity from the caput to the cauda, where they almost disappeared. Bands around 45 kDa could also be detected in some fluids. These high

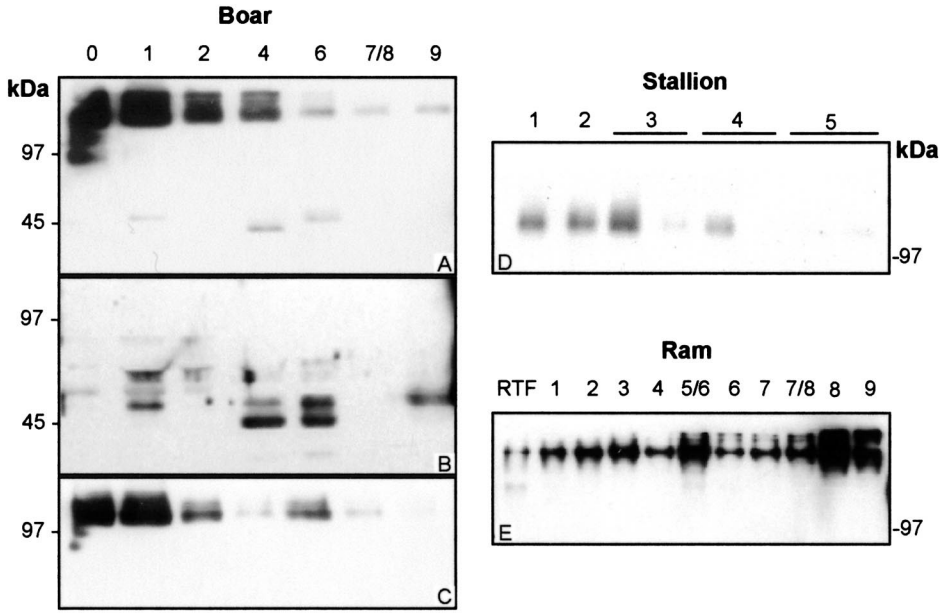

FIG. 6. Presence of acrosin and α_2-macroglobulin in epididymal fluids. Fluids from the rete testis (RTF) and from the different epididymal zones of the boar, stallion and ram were separated by SDS-PAGE under nonreducing (**A**, **C**, **D**, and **E**) or reducing conditions (**B**) before transfer on nitrocellulose. The Western blots were probed either with the anti-acrosin antibody (**A** and **B**) or the anti-α_2-macroglobulin antibody (**C**, **D**, and **E**). About 50 μg protein were loaded per lane.

molecular weight reactive bands were no longer visible under reducing conditions (Fig. 6B), but an increase in the reactivity of the bands around 45 kDa was noted.

In the stallion, a band with a molecular weight of >100 kDa was also detected in the fluid from the caput to zone 4 in nonreducing conditions, and a 47-kDa band was visible in the fluid of zone 3 (results not shown). In reduced gel conditions a less than 20-kDa band and a doublet around 50–55 kDa were detected in fluids of zone 2 and zone 4.

Presence of α_2-Macroglobulin Inhibitor in the Epididymal Fluids

In the boar, and to a lesser extent in the ram and stallion, anti-acrosin under nonreducing conditions reacted with high molecular weight proteins, which disappeared after reduction; this suggested that acrosin could be complexed with another protein and that this binding protein could be the α_2-macroglobulin inhibitor that has been previously reported in the epididymal fluids [35, 36]. The same boar epididymal fluids as in Figure 6A were then separated under nonreducing conditions, blotted, and probed with an anti-α_2-macroglobulin antibody (Fig. 6C). Two bands were immunoreactive with molecular weights >110 kDa, and both bands decreased in intensity from the caput to the caudal epididymis, where they became barely visible. When we compared the pattern of immunoreactivity between Figure 6, A and C, they were very similar. An identical pattern of immunoreaction was observed with the fluids from the stallion epididymidis (Fig. 6D). In the ram fluids (Fig. 6E), α_2-macroglobulin was present as in the boar and stallion, but with a different distribution. Two different bands were immunoreactive throughout the epididymis and their quantities even seemed to increase in the caudal fluid.

DISCUSSION

The use of monodimensional and bidimensional zymography allowed us to demonstrate the presence and the regionalized distribution of different types of gelatinase in the epididymal fluids of three domestic mammals (ram, boar, and stallion). Overall distribution of the main proteases was more similar in the ram and stallion than in the boar. For example, metalloprotease activity around 60 kDa was present in all epididymal fluids in the ram and stallion, but occurred mainly in the caudal epididymis in the boar. For all three species, all the gelatinases with molecular weights >54 kDa were metalloproteases. Each species also had low molecular weight gelatinases distributed throughout the epididymis. These minor gelatinases appeared sensitive to serine protease and also to metalloprotease inhibitors, and in some cases, to cystein protease inhibitor. The superposition on one-dimensional zymography of different gelatinases with similar molecular weights (as demonstrated by the two-dimensional zymography analysis) may explain some of the apparently mixed inhibition observed. Our results concerning the inhibition of gelatinases in the caput and caudal fluids and those concerning the distribution of these gelatinases obtained by two-dimensional zymography indicated that the most acidic proteases were in fact metalloproteases, whereas the basic ones were serine proteases. This also suggests that some of these acidic proteases could be degradation fragments of the higher molecular weight acidic metalloproteases.

One of the main metallogelatinases from the caput epididymis of the ram and stallion was identified as the proform of MMP2. The presence of this protein was further confirmed by using polyclonal anti-MMP-2 antibodies. These antibodies recognized protein bands in the caput fluid at 76 kDa in the stallion and ram. This corresponds to the molecular weight of the reduced pro-MMP-2 [1]. The difference in molecular weight of about 10–15 kDa that we observed between Western blotting and zymography could then be easily explained by the reducing and nonreducing gel conditions used. The second protein sequenced corresponded to the α1B-glycoprotein already described in the stallion epididymis [37], which is a serum protein with an unknown function that shows similarities with oprin, a serum metalloprotease inhibitor isolated from the opossum [38].

The antibodies against MMP3 and MMP9 indicated that these two metalloproteinases were also present in the fluids of all three species. MMP3 is present in all the fluids of the ram and the stallion. In the ram, degraded/active forms are observed in the caput, whereas the protein seems to be

in latent form from the corpus to the cauda. In the stallion, the immunoreactive protein, around 70 kDa, is restricted to the caput epididymidis. The molecular weight observed is higher than the 55–59 kDa expected for this protein but could result from the presence of greater (10–15 kDa) glycosylation than reported in other tissues [1, 39]. Alternatively, this protease could also form SDS-resistant complexes with TIMP inhibitors, but the reducing treatment for SDS-PAGE before blotting in our conditions should rule out this possibility.

Anti-MMP9 was the least reactive antibody. This was not due to lack of epitopic conservation but simply because there were low amounts of this protein in the fluids of each species. The 94- to 100-kDa pro-form was observed in the testicular and the caput fluids, whereas only degraded forms were visible in the caudal fluids.

It was difficult to ascertain the epididymal origin for each of the metalloproteinases. MMP2 and MMP9 have been shown to be synthesized in the testis mainly by Sertoli cells [5, 7, 8], and these proteins could be imported into the epididymis by the RTF. However, pro-MMP2 may be produced in the caput epididymidis in all three species because this enzyme was not detected or was in very low amounts in the rete testis and the proximal caput fluids. MMP9 was observed at both ends of the epididymis and therefore the caput protein may come from the testis, whereas the caudal MMP9 forms could be derived from a caudal neosynthesized pro-form. To our knowledge the presence of MMP3 has not been shown in the testis, but we observed pro- and active forms in the RTF and throughout the epididymis. Only degraded forms were observed in the caput epididymidis in the ram, whereas the pro-form was observed from the mid-caput to the cauda, suggesting the appearance of a new protein in these regions.

One of the main difficulties was also to match the gelatinolytic bands observed on zymography with the bands on Western blotting probed with anti-MMP antibodies. For example, except in the fluids from the caudal region, in the boar, no major 60- to 70-kDa gelatinases were found by zymography in the caput and corpus, whereas anti-MMPs indicated the presence of these proteases in these fluids. One explanation for this discrepancy is that zymography revealed gelatinase activities from the pro-, active, and degraded enzyme and also from inhibitor-protease complexes, whereas the antibodies were against the carboxy terminal part of these proteases, which can be released without affecting their activity. The second reason is the shift in molecular weight (which can be up to 10 kDa) between the reduced and nonreduced conditions used for the two techniques. We tried to extract several of the gelatinolytic spots from the two-dimensional zymograms and probed them by Western blotting with the different MMP antibodies, but we could not obtain an immunoreaction.

Some of the proteases present in the fluids were serine proteases and acrosin, which is a sperm cell protease, and seems to be one of these proteases. Acrosin is normally released from sperm at the time of the acrosomal reaction that occurs when the cell encounters the egg zona pellucida [14, 15]. However, when sperm cells die, they also release their acrosomal content. This protease is known to degrade gelatin and other extracellular matrix components [40], and the different acrosin forms observed between 40 and 55 kDa were due to the transformation of the pro-enzyme into activated/degraded forms [18, 31–33].

Other serine proteases such as trypsinogen [41] and the tissue and urokinase plasminogen activators [42] have been reported in the epididymal tissue, but their presence has not been demonstrated within the epididymal fluids. Members of the lysosomal proteases have also been described; cathepsins A, B, and D and procathepsin L have been immunolocalized within the epididymal epithelium with cell-specific and regional differences in their distribution, and three of these cathepsins (D, S, and procathepsin L) are secreted in the fluid [43–47]. These proteases, which belong to the papain family, should not be active under our assay conditions, although they may be some of the low molecular weight proteases inhibited by the general cysteine protease inhibitor E64.

We also found that protease inhibitors are present in the fluids of all three species and obtained evidence that they could complex the proteases (e.g., acrosin and the nonspecific inhibitor α_2-macroglobulin). This inhibitor could come from the testis where it is produced [9, 10], but it has been suggested that it is reabsorbed in the caput segment of the epididymis [35]. Effectively, the quantity of this inhibitor decreased considerably in the corpus fluid of the boar and stallion but not in the ram, suggesting that in this species it is only partially endocytosed or that it is produced in the lower part of the epididymis. TIMP-2, a more specific MMP inhibitor, was present in the caput epididymis. It certainly comes from the testis because its cDNA was not found in epididymis [48]. This inhibitor has a dual role: it allows activation of pro-MMP2 but it also inhibits MMPs by forming inactive complexes [49].

Several other protease inhibitors such as eppin [50]; protein C inhibitor [51]; HE4, an extracellular protease inhibitor [52]; and CRES, a cystatin-related inhibitor protein [53] have been described in the epididymal fluid or tissue. Whether some of these inhibitors could form complexes with MMPs or the other gelatinases present is not known. The presence of the inhibitor-protease complexes may explain some of the higher molecular weight bands detected by zymography and inhibited by metalloprotease inhibitors. Moreover, several different lipocalins with unknown functions are synthesized by the epithelium [54], and certain MMP9 isoforms could be associated with such molecules to form molecular weight complexes $>$100 kDa and even dimers of $>$200 kDa [55].

We did not analyze whether these proteases were active within the fluids. However, several results such as the fact that some of the proteases were degraded in the fluids suggested that they must be activated at some point during transit. Moreover, as set out in the *Introduction*, the sperm plasma membrane and also proteins within the fluid are submitted to proteolytic processing, implying that at least some proteases must be activated in situ. Serine protease activity has been suggested in fertilin processing, but this comes from in vitro experiments [20]. The types of protease involved in the other sperm membrane proteins processed during maturation are not known. Therefore, the role played by the gelatinases reported here in the proteolytic changes observed on the sperm membrane surface remains putative. The biological role of the different MMPs and all their physiological substrates have not been determined yet. These proteases are involved in many normal and disease processes involving the remodeling of the extracellular matrix, and some of these proteases also have ''sheddase'' type activity and thus can regulate the availability of bioactive molecules [1, 49, 56, 57]. In numerous mammals reproductive function is seasonal, and epididymal tissue is largely remodeled during the year in response to testicular activity. MMPs could then be involved in the plasticity of this

organ, because this phenomenon must include the intervention of a proteolytic-dependent reorganization of the epithelial cells and of their basement membrane. Moreover, a very tight blood-epididymal barrier exists, which is a continuity of the blood-testis barrier. The integrity of this barrier must be controlled by a very specific process involving equilibrium between proteases and protease inhibitors, as described for the blood-brain barrier [58]. It is interesting that none of the mice knocked out for MMPs reported to date show a male infertility phenotype, suggesting that these proteases could have redundant roles or that these enzymes have no critical function in sperm physiology, at least in rodents [49, 56].

One striking feature of our results is that large quantities of MMPs are present in the epididymal fluid. MMP2 is constitutively expressed in some tissues but at very low levels, whereas MMP3 and MMP9 are described as ''inducible'' genes that are under the control of different factors [1, 49, 56]. It is interesting that one of these factors is EMMPRIN or basigin, a member of the immunoglobulin family, which is also expressed on the spermatozoon membrane (reported as CE9 protein) and processed during epididymal maturation [59]. Further study of the relationship between a sperm cell derived protein and the active protein present in the epididymis may be important for understanding how the testis could control epididymal function and therefore sperm maturation.

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