

Biochemical Studies of Metalloendoprotease Activity in the Spermatozoa of Three Mammalian Species

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Ejaculated porcine and human spermatozoa, hamster spermatozoa from the cauda epididymidis, isolated hamster sperm heads and hamster cytoplasmic droplets contained activity that hydrolyzed the metalloendoprotease substrate ABZ-Ala-Gly-Leu-Ala-NBA (AAGLAN). Hamster sperm heads were isolated by treating spermatozoa with proteinase K and removing sperm tails with Dowex-50W beads. Hamster sperm activity was characterized using spermatozoa from which cytoplasmic droplets were removed by sonication and centrifugation. Porcine sperm preparations were essentially free of cytoplasmic droplets, while human sperm preparations retained somewhat more droplet material. Activity from all of these sources was inhibited by the metalloendoprotease inhibitors phosphoramidon, 1,10-phenanthroline, CBZ-D-Phe and CBZ-L-Phe but was not competitively inhibited by the metalloendoprotease substrate CBZ-Ser-Leu-amide. The AAGLAN hydrolyzing activity found in intact spermatozoa of all three species had a pH optimum of 6.2, while the optimum of the hamster sperm cytoplasmic droplet activity was 7.0. In addition, hamster sperm preparations were inhibited by ZnCl₂ and dithiothreitol, but were not affected by toluene, benzamidine or chymostatin. The AAGLAN hydrolyzing activity of hamster sperm preparations was reduced, but not eliminated, by dialysis. It is concluded that spermatozoa from all three species, hamster sperm heads and hamster cytoplasmic droplets contain metalloendoprotease activity. Furthermore, metalloendoprotease activity found in hamster cytoplasmic droplets is different from that found in spermatozoa.

Key words: spermatozoa, metalloendoprotease, cytoplasmic droplets, human, porcine, hamster.

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Fertilization includes two sperm membrane fusion events (Yanagimachi, 1981): the fusion of the spermatozoon and the egg plasma membrane and the acrosome reaction, which is the fusion of the sperm outer acrosomal membrane with the overlying sperm plasma membrane. In mammalian spermatozoa, the latter fusion is followed by the fenestration of the fused membranes, resulting in the release of acrosomal contents that may have a role in egg penetration by the spermatozoa (Yanagimachi, 1981).

Protease activity of golden hamster spermatozoa has been implicated in both of these fusion events *in vitro*. Dravland et al (1984) reported that synthetic trypsin inhibitors inhibit the membrane fusion events of the hamster sperm acrosome reaction, and Dravland and Meizel (1982) have reported that such inhibitors also decrease fusion of previously acrosome-reacted hamster spermatozoa with zona-free hamster eggs. Metalloendoprotease activity has been implicated recently in a number of fusion events. Specific metalloendoprotease inhibitors were shown to inhibit myoblast fusion (Couch and Strittmatter, 1983), synaptic transmission at mammalian neuromuscular junctions (Baxter et al, 1983), neurotransmitter release from the retina of *Xenopus laevis* (Frederick et al, 1984) and exocytosis in mast cells and chromaffin cells (Mundy and Strittmatter, 1985). In several of those studies, the presence of metalloendoprotease activity was detected using the fluorescent substrate ABZ-Ala-Gly-Leu-Ala-NBA (AAGLAN).

Metalloendoprotease activity has also been shown to exist in the male mammalian reproductive tract. Koren et al, (1975) found metalloendoprotease activity in rat testis and have suggested a role in the release of spermatozoa from the copulatory plug. Bovine epididymal and seminal fluids both contain metalloendoprotease activity, most being found in the cytoplasmic droplets lost during sperm maturation. A small amount of activity was demonstrated in pure bovine sperm preparations (Lessley and Garner, 1983). McRorie et al (1976) reported the presence of an enzyme, acrolysin, resembling the metalloendoprotease thermolysin in rabbit sperm acrosomal extracts and suggested that it has a role in the conversion of the acrosomal proenzyme proacrosin to its active form, acrosin. There is also evidence of metalloendoprotease involvement in the acrosome reaction. An inhibitor of metalloendoproteases, 1,10 phenanthroline, and a dipeptide metalloendoprotease substrate, CBZ-Gly-Phe-NH₂, were shown to inhibit the acrosome reaction of the sea urchin, *Strongylocentrotus purpuratus* (Farach et al, 1985). Preliminary studies by Thomas et al (1985) have shown that the metalloendoprotease substrate CBZ-Ser-Leu-NH₂ but not the control dipeptide derivative CBZ-Gly-Gly-NH₂ inhibits the human sperm acrosome reaction.

In this paper, we report the presence of metalloendoprotease activity in golden hamster, porcine and human spermatozoa, partially characterize the activity and demonstrate that it is present in the hamster sperm head.

Materials and Methods

Materials

The following were purchased: carbobenzoxy (CBZ) dipeptide derivatives from Vega (Tucson, AZ); phosphoramidon, ClCH₂CO(N-OH)Phe-Ala-Ala-NH₂, and 2-aminobenzoyl-Ala-Gly-Leu-Ala-4-nitrobenzylamide (AAGLAN) from Enzyme Systems Products (Livermore, CA); 1, 10-phenanthroline, Percoll, proteinase K (from *Tritirachium album* type XI), MES (2-[N-morpholino]ethane sulfonic acid), chymostatin, benzamidine-HCl and Dowex 50W beads from Sigma (St. Louis, MO), HEPES from Research Organics Inc. (Cleveland, OH), and glass beads (0.25-0.3 mm, B. Braun Melsuger) and Spectropor No 4 dialysis tubing (molecular weight cut-off 12000-14000) from VWR Scientific (San Francisco, CA). Other organic and inorganic chemicals were reagent grade purchased from Mallinckrodt (St. Louis, MO). All media were made with water purified in a Barnstead, Nanopure water purification system (Boston, MA). Golden hamsters (11 to 20 weeks old) were purchased from Simonsen's Labs, Inc. (Gilroy, CA). Human ejaculates were obtained by masturbation from healthy donors and porcine ejaculates were

obtained from sexually mature boars (Duroc, Hampshire and crossbred) using the gloved hand technique and the sperm-rich fractions were filtered through mira cloth (Calbiochem).

Preparations from Whole Spermatozoa

Hamster spermatozoa were removed from the cauda epididymidis of healthy golden hamsters (Meizel et al, 1980) and suspended in 1.0 ml of a phosphate buffered saline (PBS) medium, which consisted of 138 mM NaCl, 8.54 mM KCl, 6.34 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.93 mM CaCl₂, 0.492 mM MgCl₂ and 1.0 mM benzamidine, pH 7.3. This suspension was then layered over a Percoll gradient that consisted of one ml each of 95%, 47.5% and 35% Percoll layers in 5 mM HEPES, 191 mM NaCl, pH 7.4. After centrifugation at 800 × g for 20 minutes (all centrifugation steps were performed at room temperature unless otherwise specified), the highly motile spermatozoa in the 95/47.5 interface were washed twice by resuspension in PBS medium and centrifugation for 5 min at 800 × g. The pellet was then resuspended in 4 volumes of homogenization buffer (1 mM benzamidine, 50 mM HEPES, pH 8.0) and homogenized with 20 strokes in a Wheaton homogenizer followed by three 30-watt bursts for 10 sec each with a sonifier (Heat Systems). The sonicated preparation was centrifuged for 30 sec at 9,000 × g in a Beckman microfuge to remove nuclear and flagellar material and the supernatant was used in enzyme assays.

Porcine spermatozoa were prepared similarly except that the Percoll gradient consisted of one ml each of 72, 63, 45 and 36% Percoll in 5 mM HEPES, 191 mM NaCl, pH 7.4 and centrifugation was at 300 × g for 20 min. The resulting pellet was then washed and homogenized as described above for hamster spermatozoa.

Human semen ejaculates were allowed to liquefy for 30 min, layered over a two-step gradient consisting of 0.5 ml each of 95% and 47.5% Percoll in 50 mM Hepes, 191 mM NaCl, pH 7.4, and centrifuged at 500 × g for 20 min. The resulting pellet was washed twice at 500 × g for 10 min, resuspended in homogenization buffer and sonicated with three 20-watt bursts for 10 sec each. The sonicated preparation was then centrifuged as described above for hamster spermatozoa to remove nuclear and flagellar material.

Cytoplasmic Droplet-free Hamster Sperm Preparation

Hamster spermatozoa were centrifuged through a Percoll gradient, then washed twice as described above, and the resulting pellet was diluted to a concentration of 9.0 × 10⁶ spermatozoa/ml with 13% sucrose in PBS medium. To disrupt cytoplasmic droplets that were still attached, the spermatozoa were subjected to sonication (10-12 watts) for 1.5 min. After centrifuging the sonicated preparation at 800 × g for 5 min, the pellet was resuspended in the same medium and centrifuged again in the same manner. The pellet was then homogenized, sonicated and centrifuged as described above for whole sperm preparations.

Preparation of Pure Hamster Sperm Heads

Hamster spermatozoa obtained from the cauda epididymidis were washed twice (800 × g, 5 min) in 10 ml of PBS

medium and the remaining pellet was resuspended to a concentration of 9×10^6 spermatozoa/ml in the same medium. After a 10-min. incubation at 37 C in a volume of 1.0 ml, 0.1 mg/ml proteinase K was added and the suspension was allowed to incubate for 10 more minutes. This was followed by vortexing for 30 sec. Tails and whole spermatozoa were removed by adding Dowex 50W beads that had been prewashed 4 times in PBS medium (without benzamidine) to neutralize the pH. Dowex 50W beads (0.5 gms) were added to 10 ml of the sperm preparation. The beads were allowed to settle, and then the supernatant was removed and added to fresh beads. This step was repeated three times. The final supernatant was centrifuged ($800 \times g$, 5 min) and the pellet was spun through a Percoll gradient as described above for the preparation of highly motile hamster spermatozoa. The pellet and the 95%/47.5% interface, which contained heads, were pooled, washed twice as described above for whole hamster sperm preparations, sonicated in 4 volumes of homogenization buffer and centrifuged as described above for whole human sperm preparations.

Purification of Hamster Sperm Cytoplasmic Droplets

After centrifugation of hamster spermatozoa on a Percoll gradient (described above), the interface between 35% Percoll and the remaining PBS medium above it was enriched in cytoplasmic droplets (detected by phase contrast microscopy at $500 \times$ magnification). This droplet layer was resuspended in 10 ml PBS medium and centrifuged for 20 min at $800 \times g$. To remove spermatozoa, the resulting pellet was resuspended in PBS medium and eluted through a funnel containing glass beads (prepared as described by Llanos et al, 1982). This elution was repeated and the eluent was centrifuged at $800 \times g$ for 20 min. The resulting pellet was resuspended in PBS medium. Cytoplasmic droplets were collected by centrifugation at $800 \times g$ (20 min), resuspended in 4 volumes of homogenization buffer and sonicated (two 20-watt bursts of 10 sec each). The sonicated preparation was centrifuged for 30 sec at $9000 \times g$ and the supernatant was used in assays.

Dialysis of Cytoplasmic Droplet-free Hamster Sperm Preparations

Spectrapor dialysis tubing was prepared as described by Llanos et al (1982). Sperm preparations were dialyzed against 1000 volumes of 50 mM HEPES, pH 8.0, overnight at 4 C with one change of solution. Metalloendoprotease activity from these preparations was compared with preparations that were not dialysed but left at 4 C for an equal amount of time.

Metalloendoprotease Assay

The fluorescence of the 2-amino benzoyl group in AAGLAN is quenched, but increased fluorescence occurs when AAGLAN is proteolytically cleaved (Kam et al, 1979). Samples to be assayed (from 1-12 mg protein, 10-80 μ l) were added to 50 mM HEPES, 25 mM MES, 1mM benzamidine, 10 mM CaCl_2 , pH 6.2 (total volume of 180 μ l). The assay was begun with the addition of 20 μ l of 4 mM AAGLAN in 10% dimethylformamide and was run

for 2 to 10 h at 37 C. At selected times, incubations were concluded by the addition of 1 ml of water followed by centrifugation for 4 min at $9000 \times g$. The fluorescence of the supernatant was read in a spectrofluorometer at excitation and emission wavelengths of 340 nm and 415 nm, respectively. The relative fluorescence was compared with that produced by complete hydrolysis of AAGLAN in the same medium by 4 ng thermolysin. Each assay was done in duplicate.

In studies involving the effect of various additions on metalloendoprotease activity, 5 μ l of test solution were substituted for an equal volume of enzyme assay buffer. CBZ derivatives (1 mM) and $\text{ClCH}_2\text{CO}(\text{N-OH})\text{Phe-Ala-Ala-NH}_2$ were dissolved in 100% DMSO, 1,10 phenanthroline (80 mM) was dissolved in 10% ethanol and NP-40 (0.05 mg/ml), dithiothreitol (0.1-1.0 mM), EDTA (1.0 mM), phosphoramidon (80 mM), CaCl_2 (1.0-10 mM), ZnCl_2 (0.1-1.0 mM) and NaCl (10-26 mM) were dissolved in homogenization buffer. Controls contained the same solvents.

For pH dependence studies, 50 μ l of the homogenization medium and 100 μ l of 50 mM HEPES, 50 mM MES, 1 mM benzamidine of an appropriate pH were added in order to obtain the final desired pH. The conductivity of the buffers used in the pH dependence studies was standardized (5 mmho) by adding appropriate levels of NaCl. Conductivity was monitored using a conductivity meter (Radiometer, Copenhagen). In ion studies, CaCl_2 was omitted from the assay medium.

Electron Microscopy

After the last wash, the cytoplasmic droplet-free hamster sperm pellet was resuspended in 80 μ l of 0.2 M cacodylate, 2% sucrose, pH 7.4, and 100 μ l fixative was added (2.5% glutaraldehyde in the resuspension buffer). The mixture was allowed to stand for 1 to 2 h and then centrifuged for 30 sec at $9000 \times g$. The resulting supernatant was removed without disturbing the pellet and replaced with 200 μ l cacodylate buffer. The pellet was then kept refrigerated for up to a week. The sperm pellet was resuspended in 7% agar at 44 C for 1 h. The agar was cooled to room temperature and rinsed in buffer and then water before being stained *en bloc* with 2% aqueous uranyl acetate for 10 min. Samples were dehydrated in a series of graded acetate solutions and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were stained in 4% uranyl acetate (5-7 min) and Reynolds' (Reynolds, 1963) lead citrate (5 min). Sections were viewed in a Phillips EM-500 electron microscope.

Results

Metalloendoprotease Activity in Spermatozoa

Sperm preparations of the three species examined contained AAGLAN hydrolyzing activity (Table 1). Enzyme assays, using the cytoplasmic droplet-free hamster sperm preparations, were shown to be concentration dependent (data not shown) and essentially linear with respect to time at a wide range of pH levels (Figure 1). It should be noted that activity was

TABLE 1. AAGLAN Hydrolyzing Activity of Pig, Human, and Hamster Spermatozoa and Hamster Cytoplasmic Droplets*

	Specific Activity (pmoles AAGLAN/ hr/mg protein)	Activity/Particle† (pmoles AAGLAN/ hr/10 ⁷ particles)
Hamster spermatozoa	154 ± 18.0‡	12.4 ± 1.10‡
Cytoplasmic droplet-free hamster spermatozoa	303 ± 59.0§	4.84 ± 2.40§
Hamster sperm heads	220 ± 240‡	2.20 ± 1.20
Hamster cytoplasmic droplets	146 ± 110	0.96 ± 0.12
Porcine spermatozoa	83.0¶	0.96 ± 0.12‡
Human spermatozoa	146 ± 21.0‡	1.56 ± 0.68

*All preparations were frozen once prior to assays.

†Particles are intact hamster, human, or porcine spermatozoa, hamster sperm heads, or hamster sperm cytoplasmic droplets.

‡Values are the mean ± SD for three preparations.

§Values are the mean ± SD for four preparations.

||Values are the mean ± SD for five preparations.

¶Value is the average of two values.

probably not due to bacterial contamination during prolonged incubation because addition of toluene (Table 3) to the incubation medium (to reduce bacterial growth) did not reduce metalloendoprotease activity. This hydrolysis was not due to sperm acrosin since isolated porcine acrosin did not hydrolyze the substrate (data not shown).

Localization of Metalloendoprotease Activity in Spermatozoa

Activity was found in both hamster spermatozoa and free cytoplasmic droplets (residual cytoplasm lost during epididymal maturation as membrane vesicles, Figure 2A). Since many hamster spermatozoa from the cauda epididymidis retained attached cytoplasmic droplets (30-60%), cytoplasmic droplet-free sperm preparations also were made. These preparations contained no intact cytoplasmic droplets as determined by phase contrast microscopy (Figure 2B) or by transmission electron microscopic analysis. It is unlikely that cytoplasmic droplet membrane fragment contamination contributed to the metalloendoprotease activity in these purified preparations because when isolated cytoplasmic droplets were subjected to the same treatment as cytoplasmic droplet-free hamster spermatozoa (sonication, 10-12 watts for 1.5 min; centrifugation twice for 5 min at 800 × g, homogenization and sonication of the pellet, and centrifugation of the sonicated pellet, 9000 × g for 30 seconds), no activity was found in the pellet. In por-

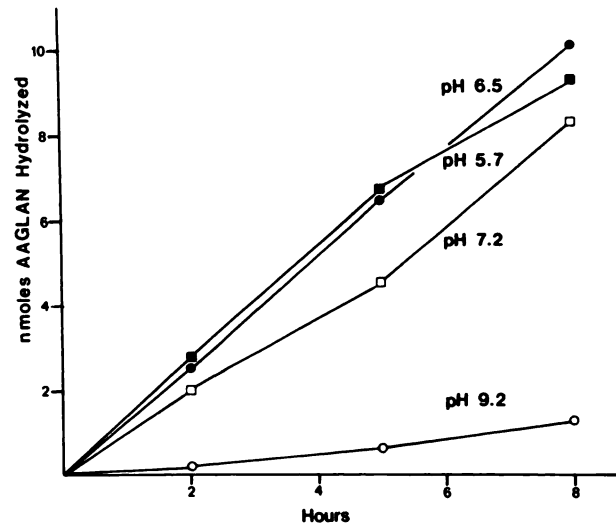


Fig. 1. A typical time course of metalloendoprotease assays at four different pH values using cytoplasmic droplet-free hamster sperm preparations. Each point represents the average of duplicates.

cine, whole hamster and human sperm preparations, some spermatozoa still retained cytoplasmic droplets attached to the midpiece (1-2 droplets/100 porcine spermatozoa, 30-50/100 hamster whole spermatozoa and 6-12/100 human spermatozoa).

To determine whether the AAGLAN hydrolyzing activity was in the hamster sperm head, sperm heads that were from 5 to 20% acrosome-intact (by phase contrast microscopy) were isolated (Figure 2C). There were always less than 1% free cytoplasmic droplets and never more than 2% tail contamination. Millette et al (1973) had previously used trypsin to cleave mouse and rat sperm heads from tails, and Eksittikul and Chulavatnatol (1980) utilized various anion exchange beads in the isolation of rat sperm tails. These procedures were modified in the present study, using proteinase K and Dowex 50W cation exchange beads during the separation of hamster sperm heads and tails. Proteinase K itself has AAGLAN hydrolyzing activity, but this activity was not inhibited by either 1,10-phenanthroline or phosphoramidon (data not shown). The amount of proteinase K activity left in the hamster sperm head preparations could be completely inhibited by chymostatin, a chymotrypsin inhibitor which also partially inhibits hamster sperm MEP activity (17%). Therefore, in the sperm head assays, chymostatin (17 μM) was added to the assay medium and a correction was made for the slight inhibition of sperm enzyme activity by chymostatin. The AAGLAN

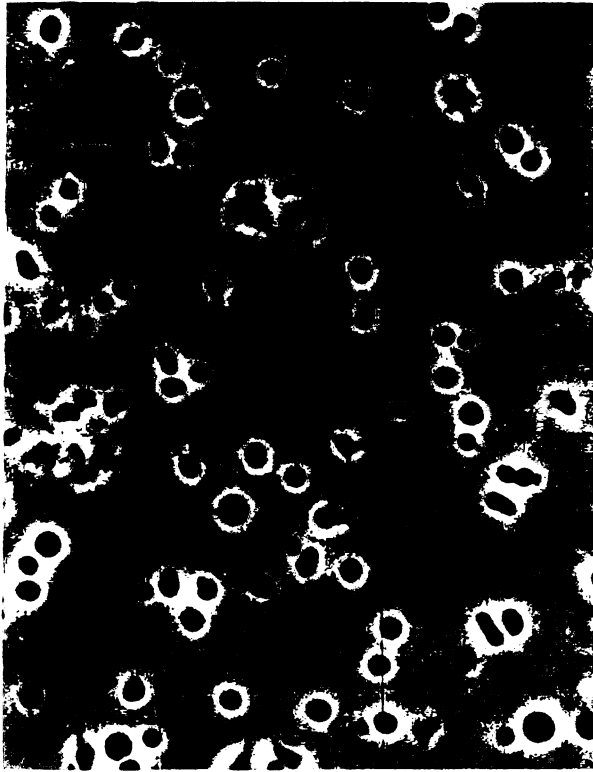


Fig. 2A, B. Phase contrast microscopy (500 X) of hamster spermatozoa free of cytoplasmic droplets, sperm heads and cytoplasmic droplets. A) Hamster sperm cytoplasmic droplets isolated by Percoll gradient centrifugation, followed by elution from glass bead columns, B) cytoplasmic droplet-free hamster spermatozoa prepared by sonicating intact spermatozoa.



hydrolyzing activity in the hamster sperm head preparations showed inhibition by 1,10-phenanthroline and phosphoramidon similar to that in cytoplasmic droplet-free hamster sperm preparations (Table 2).

Transmission Microscopic Analysis of Cytoplasmic Droplet-free Hamster Sperm Preparations

Transmission electron microscopy revealed that about one third (by subjective examination) of those spermatozoa that appeared acrosome-intact by phase contrast microscopy were also acrosomal matrix-intact (Figure 3A), and the rest appeared to have outer acrosomal membranes that were disrupted and no acrosomal matrix (Figure 3B). The plasma membrane was absent entirely from around the head and the principal piece of the tail in all spermatozoa and in all but a few cases from the tail midpiece.

Characterization of Sperm

Metalloendoprotease Activity

Metalloendoproteases are commonly characterized

Fig. 2C. Phase contrast microscopy (500 X) of hamster sperm heads from intact spermatozoa that were treated with proteinase K followed by tail removal by binding to Dowex-50W beads.

TABLE 2. Effect of Inhibitors on AAGLAN Hydrolysis by Porcine, Human, and Hamster Spermatozoa and Hamster Cytoplasmic Droplet Preparations

		Percent of Control*				
		Human Spermatozoa	Porcine Spermatozoa	Hamster Spermatozoa†	Hamster Sperm Heads	Cytoplasmic Droplets
Reversible inhibitors						
Phosphoramidon	(80 μ M)	15 \pm 2.0	—	29 \pm 9.3	26 \pm 14	44 \pm 7.4
CBZ-D-Phe	(1 mM)	ND‡	ND	53 \pm 7.0	ND	ND
CBZ-L-Phe	(1 mM)	41 \pm 13	—	48 \pm 2.2	ND	13 \pm 2.0
Irreversible inhibitor						
ClCH ₂ CO(NOH)Phe-Ala-Ala-NH ₂	(1 mM)	48 \pm 16	76.5§	103 \pm 7.5	ND	ND
Dipeptide derivatives						
CBZ-Ser-Leu Amide	(1 mM)	125 \pm 13	93 \pm 4.0	114 \pm 2.0	ND	ND
CBZ-Gly-Gly Amide	(1 mM)	110 \pm 24	90 \pm 14	111 \pm 9.7	ND	ND
Chelating agents						
1,10-Phenanthroline	(1.1 mM)	0 \pm 0	3 \pm 5.2	0.33 \pm 0.58	0.67 \pm 1.1	2.7 \pm 4.6
EDTA	(1 mM)	ND	ND	—	ND	ND

*Values are the mean \pm SD for three preparations. Uninhibited activities ranged from 0.31 to 0.60 nmoles AAGLAN hydrolyzed/hr in metalloendoprotease assays of cytoplasmic droplet preparations, 0.15–1.50 nmoles AAGLAN hydrolyzed/h for hamster sperm preparations, 0.14–0.23 nmoles AAGLAN hydrolyzed/h for porcine sperm preparations, 0.08–0.40 nmoles AAGLAN hydrolyzed/h for human sperm preparations, and 0.07–0.17 nmoles AAGLAN hydrolyzed/h for hamster sperm head preparations.

†Cytoplasmic droplet-free hamster spermatozoa.

‡ND = not determined.

§Value is the average for two preparations.

by their ability to be inhibited by metal chelators and phosphoramidon (Bond and Beynon, 1985). Although chelating agents are also inhibitors of other proteases, phosphoramidon is a specific inhibitor of metalloendoproteases (Suda et al, 1973; Kitagishi and Hiromi, 1984). Both 1,10-phenanthroline and phosphoramidon were effective inhibitors against human, porcine, and hamster sperm preparations (Table 2). Metalloendoproteases are active against substrates in which the amide group is contributed by uncharged aromatic or aliphatic amino acids such as phenylalanine or leucine (Moriyama, 1974; Chlebowski and Coleman, 1976). Neither the metalloendoprotease substrate carbobenzoxy-Ser-Leu-amide, nor the dipeptide derivative carbobenzoxy-Gly-Gly-amide, which is not a substrate, however, inhibited enzyme activity against AAGLAN in any of the samples tested. It is important to note that AAGLAN hydrolysis by thermolysin also was not inhibited by carbobenzoxy-Ser-Leu-amide under our assay conditions. The irreversible metalloendoprotease inhibitor, ClCH₂CO(N-OH)Phe-Ala-Ala-NH₂ (see Nishino and Powers, 1980 for studies of similar inhibitors), was effective against human and porcine sperm enzyme preparations but not hamster sperm preparations (Table 2). The trypsin inhibitor benzamidine had no

TABLE 3. Effect of Various Reagents on AAGLAN Hydrolysis by Cytoplasmic Droplet-free Hamster Sperm Preparations

		Percent of Control Hydrolysis*
Cations		
CaCl ₂	(1.0 mM)	141 \pm 18.8
CaCl ₂	(10 mM)	193 \pm 23
NaCl	(10 mM)	155 \pm 31
NaCl	(26 mM)†	204 \pm 2.0
ZnCl ₂	(0.1 mM)	51 \pm 6.0
ZnCl ₂	(1.0 mM)	1.3 \pm 2.3
Detergent		
NP-40	(0.05 mg/ml)	241 \pm 31
Sulphydryl reducing agent		
Dithiothreitol	(0.1 mM)	88 \pm 6.2
Dithiothreitol	(1.0 mM)	16.3 \pm 9.6
Toluene‡		
96 \pm 13		
Serine protease inhibitors		
Benzamidine	(1.0 mM)	96 \pm 5.1
Chymostatin	(17 μ M)	83 \pm 4.5

*Values are the means \pm SD of three preparations. Activities ranged from 0.22 to 1.5 nmoles AAGLAN hydrolyzed/h.

†NaCl (26 mM) is the same ionic strength as 10 mM CaCl₂.

‡One drop of toluene was added to the surface of the incubation medium.

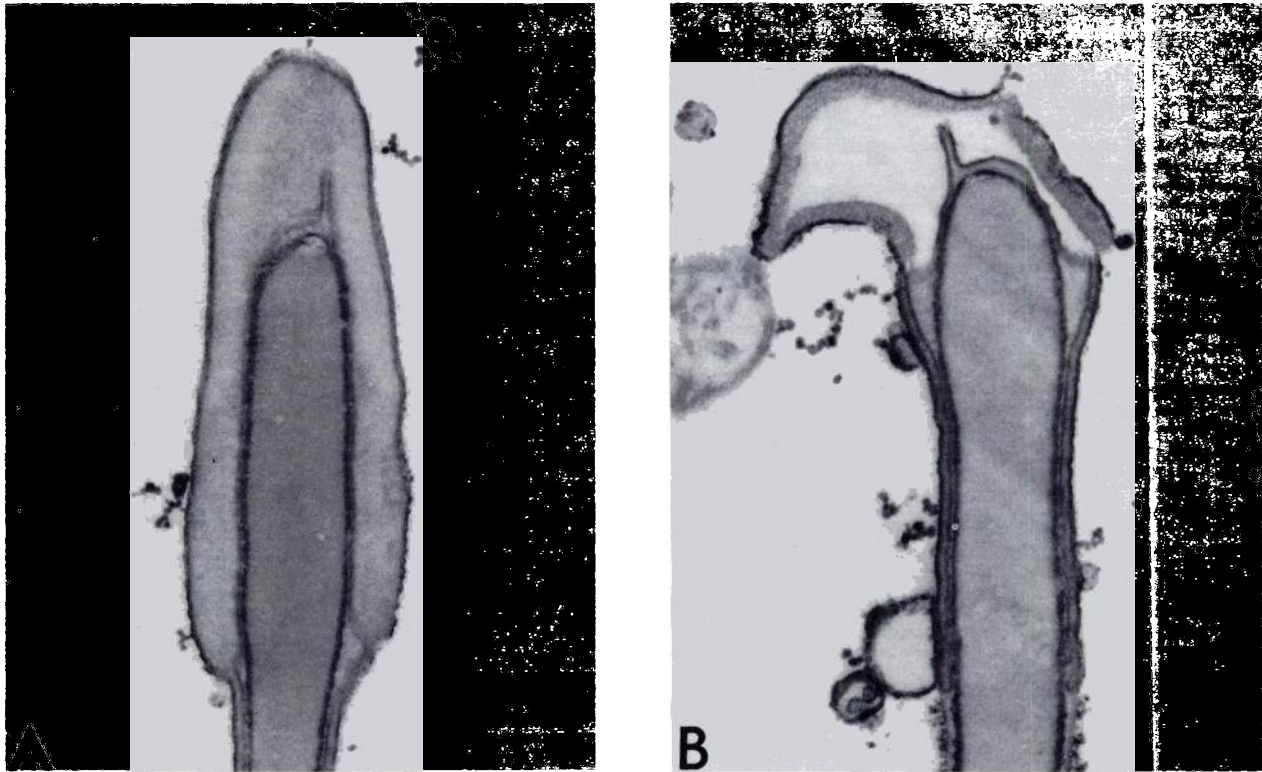


Fig. 3. Transmission electron micrographs of cytoplasmic droplet-free spermatozoa. a) micrograph showing intact outer acrosomal membrane (arrow) and acrosomal matrix, b) micrograph showing disrupted outer acrosomal membrane and absence of acrosomal matrix. $\times 45,000$.

effect on hamster sperm metalloendoprotease activity (Table 3).

The pH dependence curves indicate that the enzyme activity in the spermatozoa of all three species has an optimal pH of about 6.0 (Figures 4-6). Figure 1

shows that the activity is linear over a wide pH range. The maximal pH of AAGLAN hydrolyzing activity in cytoplasmic droplets, on the other hand, is closer to 7.0 (Figure 4). Although 10 mM CaCl_2 doubled cytoplasmic droplet-free hamster sperm activity, this

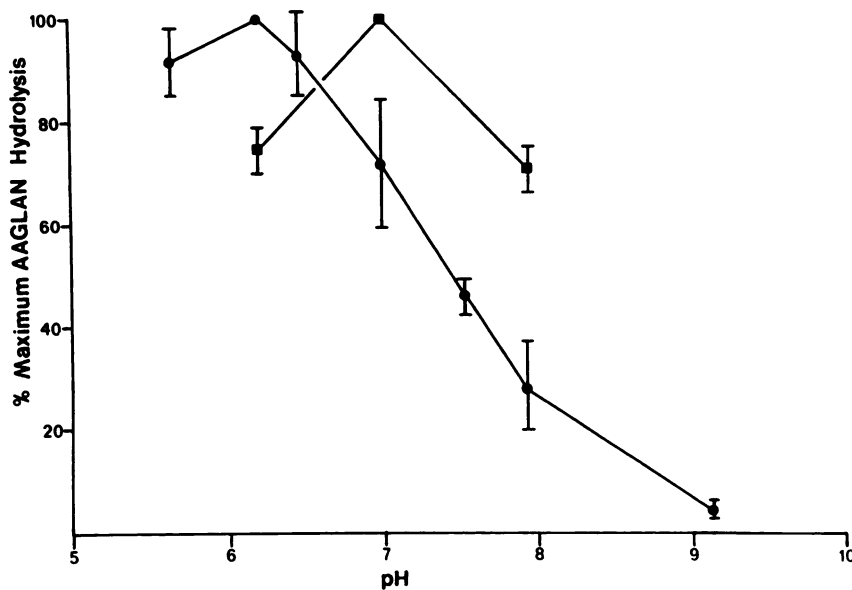
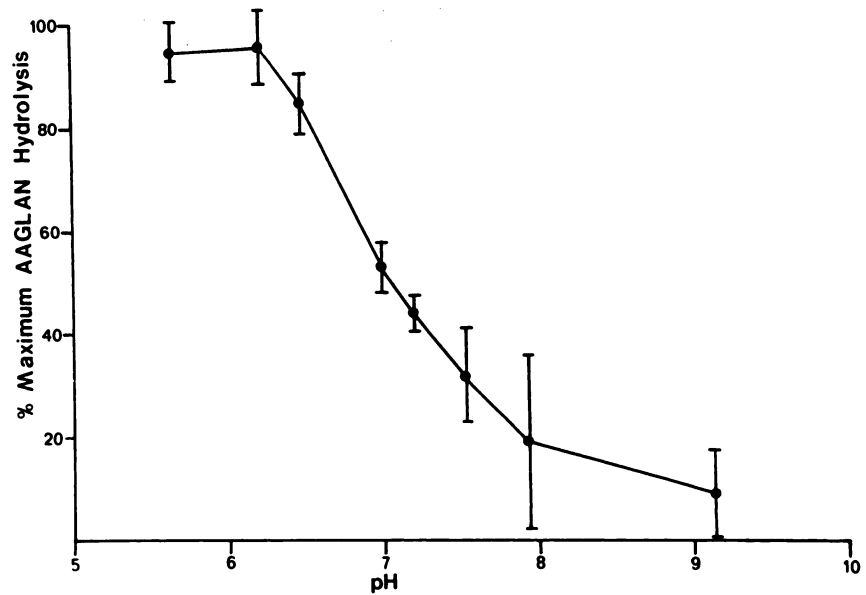


Fig. 4. A pH profile of AAGLAN-hydrolyzing activities in cytoplasmic droplet-free hamster spermatozoa (●) and in cytoplasmic droplets (■). Activities ranged from 0.2-1.7 nmoles AAGLAN hydrolyzed/h for cytoplasmic droplets and 0.25-1.6 nmoles AAGLAN hydrolyzed/h for cytoplasmic droplet-free hamster sperm preparations. Points represent the mean \pm SD for three preparations.

Fig. 5. A pH profile of AAGLAN-hydrolyzing activity in porcine spermatozoa. Activities ranged from 0.3 to 0.5 nmoles AAGLAN hydrolyzed/h at eight pH values. Points represent the mean \pm SD for three preparations.



phenomenon was most likely due to an increase in ionic strength, since adding NaCl of the same conductivity (26 mM) had a similar effect (Table 3).

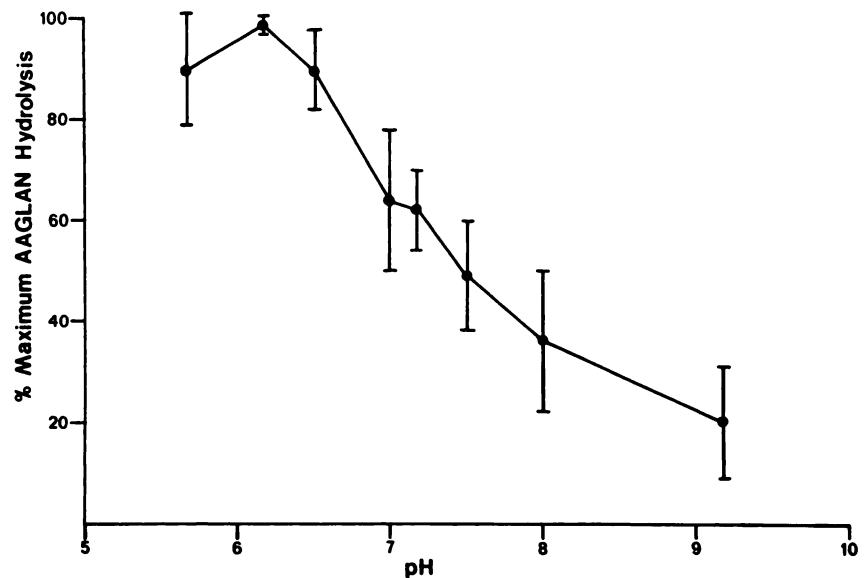
The removal of ions by dialysis did not eliminate the activity of cytoplasmic droplet-free hamster sperm preparations. These preparations retained $46\% \pm 26$ (mean \pm SD, $n = 3$) of their AAGLAN hydrolyzing activity.

Discussion

In this study, the fluorescent protease substrate AAGLAN was used to demonstrate the presence of metalloendoprotease activity in hamster, human and

porcine spermatozoa and hamster sperm heads. AAGLAN, developed by Nishino and Powers (1980) as a convenient and sensitive assay method for metalloendoproteases, is hydrolyzed by the metalloendoprotease thermolysin and the serine protease subtilisin BPN, but not by bovine chymotrypsin A (Nishino and Powers, 1980). Porcine acrosin, a serine protease found in the acrosome of porcine spermatozoa, did not hydrolyze the substrate. In the present studies, however, human, porcine and hamster spermatozoa and hamster sperm head preparations did exhibit AAGLAN hydrolyzing activity. In addition, activity in these preparations was inhibited by 1,10-

Fig. 6. A pH profile of AAGLAN-hydrolyzing activity in human spermatozoa. Activities ranged from 0.3 to 0.6 nmoles AAGLAN hydrolyzed/h at eight pH values. Points represent the mean \pm SD for three preparations.



phenanthroline, a strong chelating agent that removes zinc from metalloproteins (Vallee et al, 1960) and the metalloendoprotease inhibitor phosphoramidon. To classify a protease as a metalloendoprotease, it is important to demonstrate that the metal is bound tightly enough so that dialysis does not remove it (Bond and Beynon, 1985). Indeed, in the present studies, substantial activity remained in cytoplasmic droplet-free hamster sperm preparations after dialysis. In metal-activated enzymes, which are also inhibited by metal chelators, metals would be eliminated by dialysis because of their loose association with the protease (Bond and Benyon, 1985). Although activity was decreased by dialysis, it is likely that much of this loss was due to denaturation or degradation of metalloendoprotease activity during dialysis, rather than to loss of metal-activated enzyme activity. This conclusion is based on the high percentage of inhibition of the undialyzed preparation by the specific metalloendoprotease inhibitor phosphoramidon (>70%, Table 2). The present work provides very strong evidence for the existence of sperm metalloendoprotease activity: the inhibition by phosphoramidon, 1,10-phenanthroline and CBZ-D (and L)-Phe; the lack of inhibition by benzamidine and chymostatin and the presence of appreciable activity after dialysis. Final proof, however, awaits the testing of inhibitors of all of the other classes of protease on the purified enzyme. In this paper, we have classified this activity as endoprotease activity because the substrate, AAGLAN, is hydrolyzed internally. Before it can be determined if this protease activity is acting as an endoprotease *in vivo*, the natural substrate must be determined.

It is unlikely that this activity was due to contamination by other semen constituents since spermatozoa were centrifuged through a Percoll gradient with seminal plasma, epididymal fluid and free, non-sperm particulate material remaining above the Percoll gradient. In addition, spermatozoa were washed twice with large volumes of wash medium after the gradient step. It is possible that some of the activity was due to enzyme absorbed by the sperm head membrane, but such activity would have to be tightly bound and could well have a function in fertilization.

Although we did detect AAGLAN hydrolyzing activity in hamster sperm preparations shown to be free of retained or unretained cytoplasmic droplets, such activity was also found in hamster sperm cytoplasmic droplets. The pH optimum of the droplet activity was 7.0, rather than the 6.2 optimum of the sperm enzyme, but the droplet activity and the sperm

activity were both inhibited by 1,10-phenanthroline, phosphoramidon and the metalloendoprotease reversible inhibitor CBZ-L-Phe. Even though free cytoplasmic droplets were removed from all sperm preparations by Percoll gradient centrifugation, some of the activity assayed in porcine and human sperm preparations could have been due to cytoplasmic droplets retained by the sperm midpieces since 1 to 2% of porcine spermatozoa and 6 to 12% of human spermatozoa still had a droplet attached at the midpiece. Human spermatozoa also can retain some droplet remnants around the sperm neck (Pedersen and Fawcett, 1976), but we did not attempt to count the number of such spermatozoa.

There have been several other reports of metalloendoprotease activity in mammalian spermatozoa. Koren and Milkovic (1973) found a collagenase-like peptidase in ejaculated human spermatozoa and epididymal bovine and rat spermatozoa, but metalloendoprotease inhibitors and chelating agents were not used to verify the nature of the enzyme. McRorie et al, (1976) reported the presence of a metalloendoprotease, acrolysin, in rabbit sperm acrosomes. They used Triton X-100 together with Hyamine 2389 to isolate acrosomal membranes, a method that also has been shown to extract enzymes from sites other than the acrosome (Stambaugh and Smith, 1973). Acrolysin was inhibited by EDTA, 1,10-phenanthroline, and the specific metalloendoprotease inhibitor phosphoramidon (Suda et al, 1973; Kitagishi and Hiromi, 1984). Lessley and Garner (1983, 1984) found metalloendoprotease activity in bovine ejaculated spermatozoa that were free of cytoplasmic droplets and in bovine epididymal spermatozoa. They used the Pz-peptide, 4-phenylazobenzoyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg, as the substrate and, based on the specific bonds cleaved, detected two enzymes: a soluble sperm peptidase (Pz-peptidase B) and a particulate-bound sperm peptidase (Pz-peptidase A). Both activities were strongly inhibited by 1,10-phenanthroline, but Pz-peptidase A was not inhibited by phosphoramidon, while Pz-peptidase B was strongly inhibited. The pH optima of these enzymes were also different (pH 5.9 for Pz-peptidase A and 6.9 for Pz-peptidase B). Pz-peptidase B was found mainly in spermatozoa while Pz-peptidase A also was found in cytoplasmic droplets and, to a small extent, in particulate-free seminal plasma. Pz-peptidase B, a soluble enzyme, bears some resemblance to the metalloendoprotease activity found in the hamster spermatozoa. Both activities were inhibited by 1,10-phenanthroline and phosphoramidon and, to a small extent, by chymos-

tatin and 0.1 mM dithiothreitol. Zinc inhibited the hamster sperm enzyme, but not the bovine enzyme, and the pH optima were different. It must be kept in mind, however, when comparing the present results with those of Lessley and Garner, that very different assay conditions were used. Berruti and McRorie (1985) found both a soluble and a particulate-bound Pz-peptidase in cytoplasmic droplet-free ejaculated boar sperm preparations, but did not find Pz-peptidase activity in particle-free boar seminal plasma.

Two preliminary studies have suggested that metalloendoproteases have a role in fertilization. Farach et al., (1985) inhibited sea urchin (*S. purpuratus*) fertilization with 1,10-phenanthroline and the metalloendoprotease substrate CBZ-Gly-Phe-NH₂. Thomas et al., (1985) were able to inhibit the human sperm acrosome reaction with the metalloendoprotease substrate CBZ-Ser-Leu-NH₂, but not with the dipeptide derivative CBZ-Gly-Gly-NH₂, which is not a substrate for that protease. Metalloendoprotease substrates have also been shown to inhibit events involving membrane fusion in several somatic cells (Mundy and Strittmatter, 1985).

Although, as mentioned above, metalloendoprotease activity has been reported to be present in the spermatozoa of several mammalian species, it had never been shown conclusively to exist in the sperm head where it presumably should be located for a role in the fusion events of fertilization. In this report, we demonstrated for the first time that metalloendoprotease activity is present in the sperm head. The activity in isolated sperm heads was inhibited by 1,10-phenanthroline and phosphoramidon. Hamster sperm tails were not isolated, and so we have not eliminated the possibility that they also contain metalloendoprotease activity. Isolated hamster sperm heads contained only 18% and 45%, respectively, of the total activity of intact and cytoplasmic droplet-free spermatozoa (Table 1). However, in view of the fact that so many isolated sperm heads lost the periacrosomal plasma membrane, head cytosol, outer acrosomal membrane and acrosomal contents, these percentages of retained activity are not insignificant. The higher specific activity and lower total activity of the cytoplasmic droplet-free sperm preparations compared with the intact hamster sperm preparations (Table 1) were probably due to the complete loss of the plasma membrane and cytoplasmic droplets and the partial loss of the acrosomal membrane in the former. Since substantial activity remained in that plasma membrane-free preparation, at least some of the activity in the spermatozoa is not associated with

the head plasma membrane.

Although we did demonstrate the presence of metalloendoprotease activity in the spermatozoa of three mammals, CBZ-Ser-Leu-amide, a metalloendoprotease substrate that inhibited the human sperm acrosome reaction (Thomas et al, 1985), did not inhibit the activity in any of these sperm preparations. This substrate was also ineffective against thermolysin hydrolysis of AAGLAN in the present study. The lack of inhibition could have been due to early hydrolysis of the CB2-Ser-Leu-amide during the prolonged incubation time required for this assay. Inhibition may have required an even higher CB2-Ser-Leu-amide concentration than that used in this study. It is also possible that the putative metalloendoprotease involved in the human sperm acrosome reaction and the activities described in the present paper are not identical. The former could have been masked by the latter in these assays or else some other as yet unidentified protease with a similar substrate specificity, but which is not a metalloendoprotease, may have been inhibited in the acrosome reaction studies. For this reason, future work will involve further studies of metalloendoproteases and other proteases found in human spermatozoa.

Note-in-Proof

After the submission of this manuscript, it was reported that PZ-peptidases A and B are present in intact human spermatozoa in addition to other human semen components (Lessley BA, Garner DL. Identification and distribution of PZ-peptidases A and B in human semen. *J Androl* 1985; 6:372-378).

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