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Rat Proestrus Uterine Fluid Contains a Large Molecular-Weight Protein Complex with Metalloendopeptidase Activity

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A neutral metalloendopeptidase has been identified in the secretion of the rat proestrous uterus. The native form of the enzyme secreted in the uterus is a high-mol-wt protein complex, which barely migrates in 3% native polyacrylamide gel electrophoresis. In 2% SDS, the enzyme resolves into 380-, 210-, and 110-kDa subunits. The 380- and 210-kDa proteins demonstrate peptidase activity in SDS-PAGE as detected by an enzyme overlay membrane impregnated with Suc-Ala-Ala-Phe-AFC. The uterine peptidase is substantially inhibited by EDTA, EGTA, 1,10 phenanthroline, thiorphan, DTT, and sodium dodecyl sulfate (SDS). The inhibition by 1,10 phenanthroline is reversed by Co^{2+}. The uterine enzyme has a pH optimum of 7.0, and readily hydrolyzes acetyl tetraalanine and Suc-Ala-Ala-Leu-pNA by releasing alanylalanine and Leu-pNA, respectively. Kinetic analysis of the peptidase using Suc-Ala-Ala-Leu-pNA as substrate yielded K_m and V_{max} values 0.9 m*M* and 4.3 μ*M*/min/3.5 μg protein, respectively. Negative staining of the purified uterine metalloprotease demonstrated spherical particles made up of several subunits. The earlier work had indicated that the uterine fluid metalloendopeptidase adhered to the head region of the ejaculated spermatozoa. It is very likely that this peptidase may be involved in assisting the fertilization process.

Key Words: Proestrus; uterine secretion; metalloendopeptidase.

Abbreviations: E64, *trans*-epoxysuccinyl-L-Leucylamido-(4 guanidino) butane; DTT, dithiothreitol; BAEE, benzoyl-arginine ethyl ester; Suc-Ala-Ala-Phe-AFC, succinyl alanyl alanyl phenylalanyl-7-amino-4-trifluoromethyl coumarin; Suc-Ala-Ala-Leu-pNA, succinyl-alanyl-alanyl-Leucyl-4-nitroanilide; Leu-pNA, leucyl-4-nitroanilide; Suc-Ala-Ala-Ala-AMC, succinyl-alanyl-alanyl-alanyl-7-amido-4 methyl coumarin; PMSF, phenylmethyl sulfonyl fluoride.

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Introduction

At proestrus, the rat uterus is engorged with watery fluid. At the time of mating, the fluid is retained in the uterus, and seminal components become admixed with it. This fluid is expelled following relaxation of the cervix during early estrus. It has been observed that the ovariectomized adult rat or immature rat treated with estradiol also accumulates fluid in the uterus. The exact role of this estradiol-dependent uterine secretion is not clearly understood. Ejaculated spermatozoa need to be capacitated during their transport through the female genital tract, and since the ejaculated spermatozoa are retained in the proestrous fluid several hours prior to the time of fertilization, we attempted to analyze the proestrous uterine fluid for the presence of factors that could participate in bringing about alterations in the sperm plasma membrane. We identified an endopeptidase in the proestrus secretion and in the uterine fluid of estrogenized immature rats, which hydrolyzed internal peptide bonds in peptides of different chain lengths *(1)*. Furthermore, the administration of progesterone along with estradiol negated the formation of the enzyme in the rat or mouse uterus *(2)*. The peptidase activity, which was inhibited by EDTA, was associated with a protein fraction that eluted with the void volume of Sephacryl S-200 and Sephacryl S-300 molecular sieve columns. Immunotracing of the endopeptidase using antisera to the S-200 fraction indicated intimate binding of the enzyme to the head region of ejaculated spermatozoa and was detected in the oviduct epithelium as well as in the luminal and glandular epithelia of the proestrus–estrus uterus *(3)*. In the above studies, we were unable to confirm the precise molecular size of the Sephacryl fraction, which demonstrated the peptidase activity. In the present investigation, we examine the molecular nature of the uterine fluid endopeptidase and show that the native enzyme secreted in the proestrous uterus is a large mol-wt protein that demonstrates metalloendopeptidase activity.

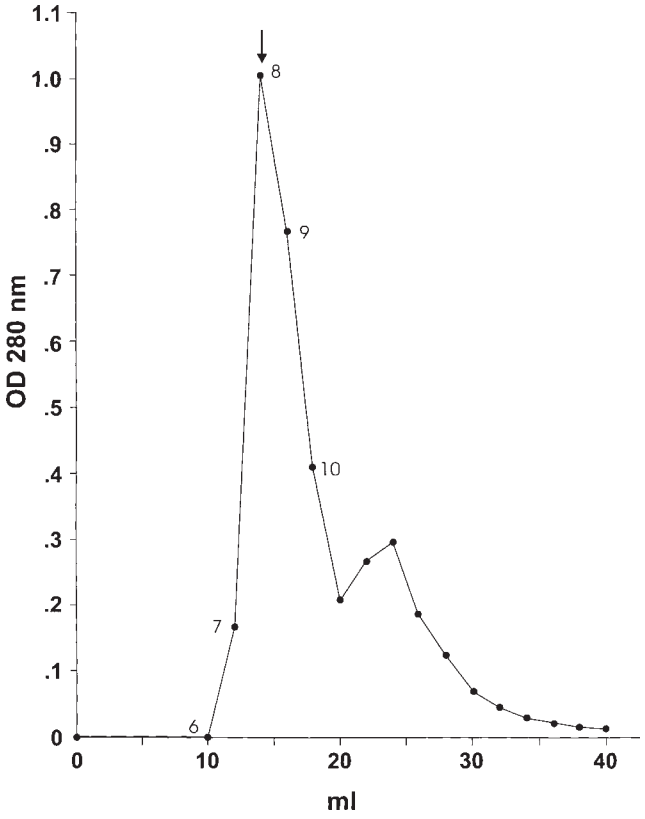

Fig. 1. Sephacryl S-300 HR gel filtration of proestrus fluid. The first protein peak that demonstrated peptidase activity was close to the void volume. The active fractions 7, 8, 9, and 10 were pooled for rechromatography. The arrow indicates protein peak demonstrating the enzyme activity.

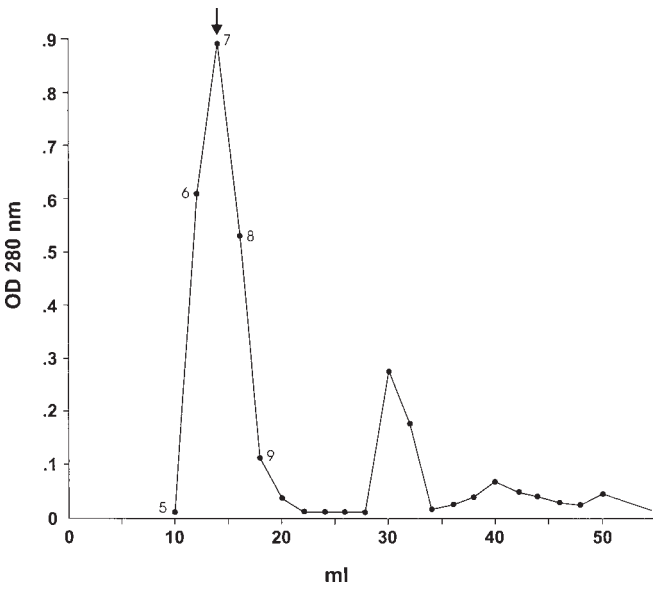

Fig. 2. Sephacryl S-300 HR rechromatography of fractions of the first protein peak of Fig. 1. Fractions 6, 7, 8, and 9 were pooled and concentrated for further purification. The arrow indicates the protein peak demonstrating enzyme activity. The second protein peak did not show any enzyme activity.

Results

Purification of Metalloendopeptidase

A Sephacryl S-300 HR (Pharmacia, Piscataway, NJ) molecular sieve column was used to isolate the uterine endopeptidase. Concentrated proestrus uterine fluid containing approx 3–4 mg of protein was layered on a Sephacryl S-300 HR column (1.6 × 27 cm) and 2-mL fractions were collected. The optical density of the eluted fractions at 280 nm is shown in Fig. 1. The major peak eluted close to the column void volume. Fractions 7, 8, 9, and 10 demonstrated significant peptidase activity toward the substrates Acetyl tetraalanine and Suc-Ala-Ala-Leu-pNA. The active fractions were pooled and concentrated using a Millipore filter (30-kDa mol-wt cutoff) and rechromatographed on the same Sephacryl S-300 HR column. The first protein peak (fractions 6, 7, 8, and 9) demonstrating peptidase activity (Fig. 2) were pooled and concentrated (S-300). The second protein peak in Fig. 2 did not possess any peptidase activity in the presence of acetyl tetraalanine ($AcAla_4$) or Suc-Ala-Ala-Leu-pNA.

The native PAGE of S-300 indicated the presence of at least three protein bands, which penetrated the separation gel, and a significant protein band, which remained at the origin of the 3% gel (Fig. 3). The peptidase activity of the protein bands in the gel, by enzyme overlay membrane, indicated that the enzyme activity was present only in the band that barely penetrated the 3% gel (Fig. 3).

SDS-PAGE of the crude uterine fluid, the S-300 fraction, and the electrolute (EL) was performed in 3–7.5% slab gel. The enzyme samples were preincubated in sample buffer containing 2% SDS, unreduced, for 1 h at room temperature. ISS staining of the SDS-PAGE of the EL yielded three protein bands, one of which was close to the beginning of 7.5% gel (Fig. 4, EL). The approximate molecular weights of these bands were 380, 210, and 110 kDa. EOM of the SDS-PAGE gel revealed enzyme activity was in 380- and 210-kDa mol-wt protein bands. The EOM of 380-kDa protein of EL demonstrated a very faint fluorescent band (Fig. 4). The SDS-PAGE of the crude uterine fluid, the S-300 fraction, and the EL indicated that the major enzyme activity as demonstrated by EOM was in 210-kDa protein. This peptide could be a product of the 380-kDa protein, which also showed distinct enzyme activity in the enzyme overlay membrane (Fig. 4). Neither the 110-kDa protein of the electrolute, the crude uterine fluid, nor the S-300 fraction demonstrated peptidase activity. It is possible that the 110-kDa protein is an impurity, or an inactive product, derived from the 210-kDa protein.

pH Optimum

The pH optimum of the uterine endopeptidase was 7.0 as determined by 0.1 *M* acetate buffers at pH 3.5, 4.3, 5.0, and 6.0 and 0.05 *M* Tris-HCl buffers at pH 7.0, 7.5, 8.0, and 9.0. The same pH optima were observed using the substrate $Ac\text{-}Ala_4$ and Suc-Ala-Ala-Leu-pNA.

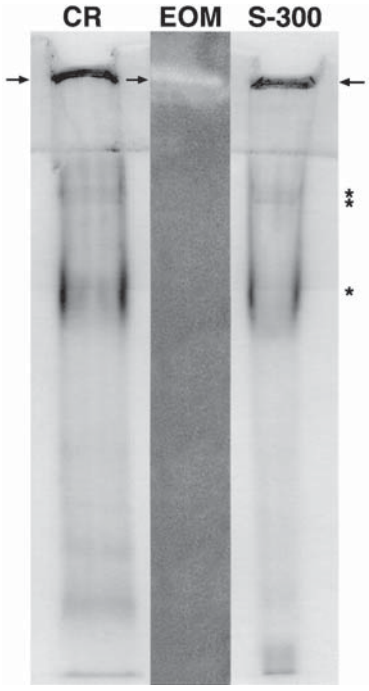

Fig. 3. Three to 5% native PAGE of CR-crude uterine fluid and S-300-Sephacryl fraction. CR and S-300 lanes are stained with ISS-blue, whereas the EOM lane is the enzyme overlay membrane incubated on the gel containing S-300 fraction. The arrow indicates the protein band in 3% gel demonstrating enzyme activity. The asterisks indicate the proteins in the S-300 fraction, which did not possess peptidase activity.

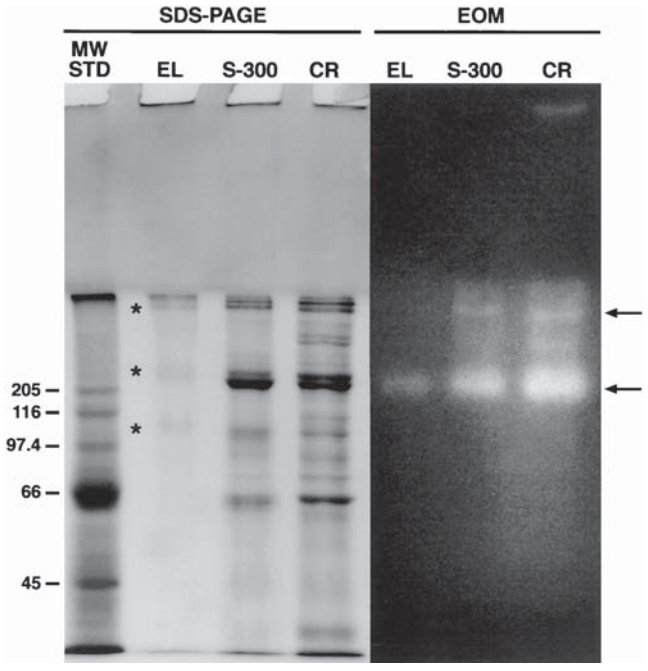

Fig. 4. Three to 7.5% SDS-PAGE and EOM of CR-crude uterine fluid, S-300-Sephacryl S-300 fraction, and EL—electrolute. Staining is done using ISS blue stain. Note 380- and 210-kDa proteins showing protease activity in EOM. EOM of 380-kDa protein of EL demonstrates very faint fluorescent band. MWSTD—molecular weight standards. The two arrows in EOM indicate 380- and 210-kDa proteins that demonstrate enzyme activity. The three asterisks in the SDS-PAGE of electrolute (EL) represent 380-, 210- and 110-kDa proteins.

Substrate Specificity

Several synthetic peptide substrates and large proteins were tested for peptidase activity (Table 1). No cleavage products were identified when large proteins, like, casein, gelatin, rat-tail collagen, fibronectin, immunoglubins-IgA, and IgG, were incubated with the enzyme for 4–6 h and analyzed by PAGE. Also, gelatin or casein was not hydrolyzed when incorporated in the PAGE (zymogram) by the uterine peptidase. Incubation of the uterine enzyme with azocoll or azocasein showed no chromogen release, indicating that collagen and casein were not substrates for the uterine enzyme. Of the synthetic peptides tested, only Ac-Ala_4 and Suc-Ala-Ala-Leu-pNA were suitable for the uterine peptidase assay. Incubation of the uterine peptidase with Ac-Ala_4 yielded Ala-Ala for up to 1 h. Suc-Ala-Ala-Phe-AMC, Suc-Ala-Ala-Phe-AFC, and Suc-Ala-Ala-Ala-AMC were hydrolyzed, but less efficiently as determined by the appearance of cleavage products at longer time intervals (2–4 h) by thin-layer chromatography. Our studies indicate that the uterine peptidase demonstrated substrate specificity and hydrolyzed specific peptide bonds of the synthetic peptides. Moreover, this substrate specificity was identical to that of the S-200 fraction previously described by us *(1)*. When Suc-Ala-Ala-Leu-pNA was used as substrate, Leu-pNA was the product of hydrolysis after 1 h of incubation. The addition of bacterial amino-peptidase to the incubation mixture yielded paranitroaniline, which was assayed colorimetrically. The use of Suc-Ala-Ala-Leu-pNA was preferred to study the uterine peptidase enzyme kinetics, because as little as 2–5 μg/mL of the enzyme was

Table 1
Substrates Used to Test the Peptidase Activity of Uterine Fluid Enzyme

Substrate	Cleavage Product
Ac-Ala-Ala-Ala-Ala	Ala-Ala active hydrolysis
Suc-Ala-Ala-Leu-pNA	Leu-pNA very active hydrolysis
Suc-Ala-Ala-Phe-AMC	Phe-AMC weak hydrolysis
Suc-Ala-Ala-Phe-AFC	Phe-AFC weak hydrolysis
Suc-Ala-Ala-Ala-AMC	Ala-AMC weak hydrolysis
CBZ-Gly-Pro-Leu-Ala-Pro	Nil
Gelatin	Nil
Casein	Nil
IgG (Rat)	Nil
IgA (Human)	Nil
BAEE	Nil
Azocasein	Nil
Azocoll	Nil
Polylysine	Nil
Collagen	Nil
Fibronectin	Nil

Table 2
Effect of Various Inhibitors on Uterine Fluid Peptidase Activity

Inhibitor	Concentration	% Inhibition	Substrate used
EDTA	5 m*M*	90	Ac-Ala_4
EGTA	5 m*M*	90	Ac-Ala_4
DTT	1 m*M*	93	Ac-Ala_4
1:10 Phenanthroline 5 m*M*	100	Ac-Ala_4	
1:10 Phenanthroline 5 m*M* + Cobalt chloride	5 m*M*	0	Ac-Ala_4
1:7 phenanthroline	5 m*M*	0	Ac-Ala_4
Mercuric chloride	0.4 m*M*	50	Ac-Ala_4
PMSF	1 m*M*	0	Ac-Ala_4
Soybean trypsin inhibitor	1 mg/mL	0	Ac-Ala_4
Benzamidine	200 μg/mL	0	Ac-Ala_4
Cystatin	50 μg/mL	0	Ac-Ala_4
Aprotinin	200 μg/mL	0	Ac-Ala_4
Antipain	500 μg/mL	0	Ac-Ala_4
Calpain inhibitor I or II	7 μg/mL	0	Ac-Ala_4
α2-macroglobulin	1 U/mL	0	Ac-Ala_4
Phosphoramidon	1 m*M*	0	Ac-Ala_4
E-64	2 mg/mL	0	Ac-Ala_4
Thiorphan	2 μg/mL	95	Ac-Ala_4 and Suc-Ala-Ala-Leu-BNA
Chymostatin	10 μg/mL	35	Ac-Ala_4 and Suc-Ala-Ala-Leu-pNA
SDS	0.1%	90	Ac-Ala_4 and Suc-Ala-Ala-Leu-pNA

adequate for the assay system, whereas 10 times more enzyme was needed for the assay with Ac-Ala_4 as substrate. The rate of hydrolysis of Suc-Ala-Ala-Leu-pNA was linear for the first 30 min with substrate concentration ranging from 0.1 to 1.0 m*M*.

Suc-Ala-Ala-Phe-AFC was used as substrate in the Enzyme Overlay Method (EOM) to detect uterine peptidase activity by PAGE. This peptide was readily available commercially and the fluorescent hydrolyzed products were easily detected by 1–4 h of incubation of the overlay membrane on the polyacrylamide gel.

Values of V_{max} and K_m were derived by the Lineweaver-Burk plot using Suc-Ala-Ala-Leu pNA as substrate. The average data points of three such independent assays are V_{max} = 4.3 μ*M*/min/3.5 μg protein and K_m = 0.9 m*M*.

Inhibition Studies

Uterine peptidase was significantly inhibited by chelating agents, like EDTA, EGTA, and 1,10 phenanthroline, but was not inhibited by 1,7 phenanthroline. It was also significantly inhibited by thiorphan, DTT, and sodium dodecyl sulfate (SDS) (Table 2). In order to study whether divalent cations were needed by the peptidase for optimal activity, the enzyme and the incubation medium were dialyzed against Chelex-100 (*see* Materials and Methods) for 24 h and tested for peptidase activity using Ac-Ala4 as substrate. The enzyme dialyzed against Chelex-100 did not lose any enzyme activity, which suggested that a metal ion may be incorporated within the peptidase, which is needed for its activity.

It is of interest to note that the activity of uterine enzyme was completely restored when Co^{2+} (equimolar concentration) was added to the 1,10 phenanthroline-inhibited incubation mixture. The uterine enzyme was not affected by serine-protease inhibitors, like PMSF, benzamidine, or soybean trypsin inhibitor. Also, the uterine enzyme differed from other known metalloendopeptidases by not being inhibited by phosphoramidon.

Electron Microscopy

Electron micrographs of negatively stained purified uterine peptidase (electrolute) indicated that the native enzyme may exist as 20- and 40-nm globular particles (Fig. 5). The staining pattern of these particles by uranyl acetate indicated that the spherical particles may be comprised of 4–6 subunits.

Discussion

In our earlier work *(1–6)*, we had identified an endopeptidase, which was inhibited by the chelating agents, in the proestrus fluid of rat. We had ascribed the peptidase activity of the Sephacryl fraction to the major protein band, which migrated in 7.5% native PAGE, but had ignored the protein band, which remained in the stacking gel. Later attempts to measure the peptidase activity in electrolutes of the protein band of native PAGE were unsuccessful. The attempt to identify the peptidase activity in the native PAGE zymograms using gelatin or casein as substrate also was unsuccessful. In the present study, we have been able to

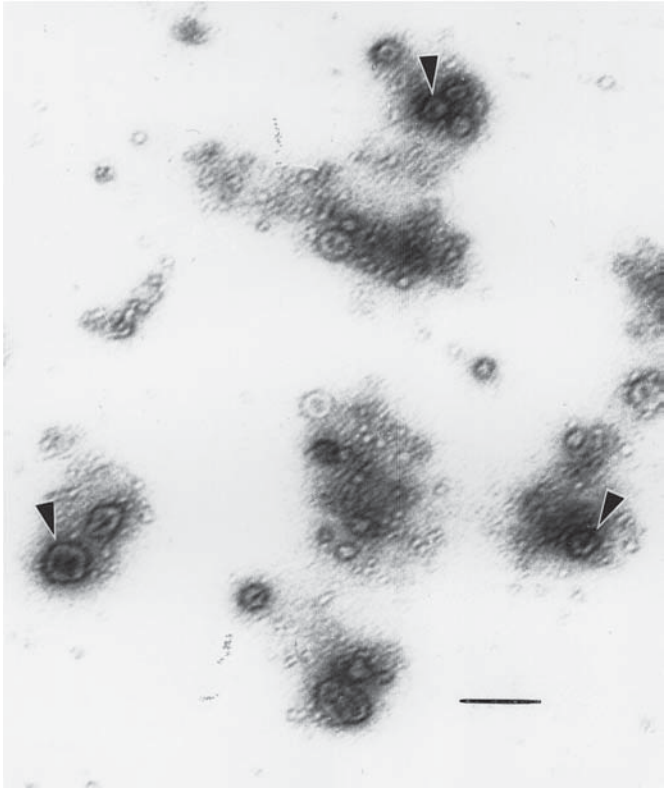

Fig. 5. Negative staining of the purified uterine peptidase (electrolute) by 2% uranyl acetate. Note globular form of protein particles. Some protein particles (arrowhead) demonstrate the presence of subunit structure accentuated by the negative stain. Bar represents 100 nm.

establish clearly the molecular nature of the uterine fluid peptidase using an enzyme overlay membrane impregnated with the substrate. All the peptidase activity was localized in a protein band that migrated only slightly in 3% native PAGE. The enzyme characteristics (substrate specificity, inhibition studies, pH optimum, detection by EOM) of the uterine fluid peptidase isolated by Sephacryl-300 HR column were identical to those of the enzyme fraction obtained by Sephacryl-200. Moreover, the Sephacryl-300 HR molecular sieve column was more effective in removing some small-mol-wt protein impurities than Sephacryl-S-200.

The uterine endopeptidase is a neutral endopeptidase, which is secreted in the proestrus uterus in the form of a large protein complex. The uterine enzyme is a metalloendopeptidase, since it is totally inhibited by chelating agents, like EDTA, EGTA, and 1,10 phenanthroline. Inhibition of 1,10 phenanthroline is completely reversed by Co^{2+}. Similar restoration of the enzyme activity by Co^{2+}, Zn^{2+}, or Mn^{2+} has been reported in metalloendopeptidases isolated from rat brain *(7)* and rat testis *(8)*. However, in the rat, the uterine metalloendopeptidase inhibition by 1,10 phenanthroline was reversed only by Co^{2+}, and not by Mn^{2+}, Ca^{2+}, or Zn^{2+}. Addition of Ca^{2+} ion to the incubation medium very slightly enhanced the peptidase activity, but the complete depletion of the cations in the assay medium using Chelex-100 (*see* Materials and Methods) did not negate the enzyme activity. Therefore, it seems that the metal ion needed for the enzyme activity may be an intrinsic component of the uterine metalloendopeptidase. More research is needed to identify possible metal components, which support the peptidase activity.

It has been suggested that the proteinases derived from the uterus or embryo may have a role in implantation *(9–11)*. Endopeptidases and arylamidases have been identified in uterine fluid of estrogen-treated rats *(12)*. An estrogen-induced hydrolytic enzyme, which was not inhibited by EDTA and DTT, has been identified in the mouse uterus *(13)*. Negishi and Koide *(14)* have characterized a 600-kDa estrus stage-specific proteinase by zymogram in the uterine fluid of rat, but it was not inhibited by SDS or 1,10 phenanthroline. Matrix metalloproteases identified in the epithelial and stromal cells of the uterus are believed to be involved in endometrial remodeling and blastocyst implantation *(11,15)*. These matrix metalloproteases participate in the breakdown of the protein components of extracellular matrix. Their enzyme characteristics, regarding substrate specificity and reaction to inhibitors differ widely from those of rat proestrus uterine fluid metalloendopeptidase. The latter did not hydrolyze substrates of the matrix metalloproteases, and its enzyme activity was not stimulated by *p*-aminophenyl mercuric acetate.

In the current study, the rat uterine fluid metalloendopeptidase hydrolyzed several synthetic peptides, but did not hydrolyze large proteins. The uterine fluid metalloendopeptidase lysed the zona pellucida of unfertilized, but not fertilized eggs *(4,16)*. It is likely, therefore, that the uterine metalloendopeptidase has a natural protein substrate in spermatozoa or in the zona pellucida, which we have not been able to identify.

The uterine enzyme was not inhibited by the inhibitors of serine proteases like PMSF. It was, however, significantly inhibited by thiorphan, a specific inhibitor of metalloprotease 24:11 *(17)*, but not by phosphoramidon, also a known inhibitor of metalloendopeptidases. The uterine metalloendopeptidase also differs from soluble metalloendopeptidase (24:15) isolated from brain *(7)* and testis *(8)*. The metalloendopeptidase 24:15 is stimulated in the presence of DTT, a potent inhibitor of the uterine enzyme. The uterine metalloendopeptidase seems to be quite different from other known metalloendopeptidases. Possible molecular homologies between the uterine metalloendopeptidase and other soluble and matrix metalloproteases should be investigated by N-terminal sequencing studies.

The electron microscopic studies of the native form of the uterine enzyme obtained from the stacking gel of native PAGE indicated that the enzyme may be released in the form of proteasome complex.

Metalloproteases have been implicated in several physiological roles, including myoblast fusion *(18)*, exocytosis

(19), implantation *(11,15,20)*, fertilization *(21)*, and the acrosome reaction *(22,23)*. We have in the current analysis identified and characterized a large-mol-wt protein complex with metalloendopeptidase activity in the proestrus fluid of the rat, which may be involved in fertilization and implantation processes.

Materials and Methods

Chemicals

Ac-Ala_4, EDTA, EGTA, 1,10 phenanthroline, 1,7 phenanthroline, thiorphan, phosphoramidon, E64, aprotinin, benzamidine, antipain, chymostatin, azocasein, and mol-wt standards were purchased from Sigma (St. Louis, MO). α_2-Macroglobulin, calpain-I and II, inhibitors, and cystatin were purchased from Boehringer Mannheim (Indianapolis, IN). Azocoll was purchased from Calbiochem (La Jolla, CA). Suc-Ala-Ala-Phe-AFC impregnated cellulose acetate membrane (enzyme overlay membrane) was purchased from Enzyme Systems Products (Livermore, CA). SDS (2-D electrophoresis grade) was purchased from Millipore (Bedford, MA). The substrate succinyl-alanyl-alanyl-leucyl-paranitroanilide (Suc-Ala-Ala-Leu-pNA) and the purified amino peptidase from *Streptomyces griseus* (SGAP) were generously donated by Shamaryahu Blumberg, Sackler Institute of Molecular Medicine, Tel Aviv University, Israel.

Collection of Proestrus Fluid

Daily vaginal smears of 50 adult female Wistar rats were recorded to determine the day of proestrus. The uterine luminal fluid was aspirated using a 1-mL syringe fitted with a 27-gage needle, on the day of proestrus between 8 PM and 10 PM. Approximately 0.15–0.2 mL of fluid was obtained/uterine horn. The pooled proestrus fluid was centrifuged at 1000*g* for 10 min at 4°C to sediment the cellular debris. The pooled proestrus fluid (15–18 mL) was later dialyzed against 20 m*M* Tris-saline buffer, pH 7.1 (TBS), for 24 h at 4°C. The retentate was concentrated fourfold by using Millipore ultrafree CL filters with 30-kDa mo-wt cutoff and stored at -20°C.

Chromatography

The concentrated proestrus fluid containing 3–5 mg protein was layered on a molecular sieve column (1.6 × 27 cm) of Sephacryl S-300 HR, which was equilibrated previously with 20 m*M* Tris-saline buffer, pH 7.1. The elution rate of the column was approx 1 mL/min. All the eluted samples (S-300) were assayed for the endopeptidase activity, protein estimation, and polyacrylamide gel electrophoresis (PAGE).

Protein Assay

Protein estimations were done according to the method of Bradford *(24)* using the Bio-Rad (Hercules, CA) reagent. Bovine serum albumin was used as standard protein.

Polyacrylamide Gel Electrophoresis (PAGE)

Native polyacrylamide slab gel electrophoresis (Native-PAGE) was performed on a miniprotean II electrophoresis apparatus (Bio-Rad) according the method described by Davis *(25)* for disk gel electrophoresis. The separation and stacking gels were 5 and 3%, respectively. SDS-PAGE was performed according to Laemmli *(26)* with separation and stacking gels of 7.5 and 3%, respectively. Protein was loaded on the gel (6–10 μg/lane) and the electrophoresis was run at 200 V until the dye in the sample buffer (bromophenol blue) reached the end of the gel. The protein sample was incubated with the sample buffer containing 2% SDS without any reducing agent for at least 1 h at room temperature. Staining of the native and SDS gels was done by using ISS blue stain (Integrated Separation Systems, Natick, MA). The molecular size of the protein bands was determined by the method described by Weber and Osborn *(27)*.

Enzyme Overlay Method (EOM)

Active proteolytic enzyme in the native or SDS gels was detected by overlaying the gel with a cellulose acetate membrane impregnated with substrate Suc-Ala-Ala-Phe-AFC, according to the method of Smith *(28)*. Before layering the samples, the gel was prerun for 10 min at 100 V. One sample set was run for ISS staining, and other set was run in the same gel for incubation with the enzyme overlay membrane. After incubation of the enzyme overlay membrane with the excised gel for 1 to 4 h, the membrane was photographed under a long-wavelength UV illuminator. Since SDS inhibits the peptidase, SDS-PAGE gels were preincubated first with 2.5% Triton X-100 for 1 h and later with TBS for 1 h, before overlaying the membrane.

Electrolution

The protein band in the native-PAGE corresponding to the fluorescent band in EOM was excised. The excised gel was cut into small pieces, and loaded into glass electrolution tubes, which contained Tris-glycine buffer, pH 8.6 without SDS. The electrolution was performed at 5°C for 18 hr in a Biorad electroluter model 422. The eluted protein was collected, dialyzed against TBS and concentrated for further study.

Enzyme Assay

The rate of generation of free amino groups by enzymatic action on *N*-acetyl tetraalanine was determined by the ninhydrin method *(29)* using L-alanine as standard. The complete system consisted of 5 m*M* Ac-Ala_4, enzyme fraction ranging from 30 to 150 μg/mL in 0.02 *M* Tris-saline buffer, pH 7.1. Of the ninhydrin-positive cleavage products, alanyl-alanine, was the only product during the first hour of the enzyme reaction as detected by thin-layer chromatography of the cleaved products. For inhibition studies, the appropriate concentration of enzyme inhibitor was added to the substrate mixture before addition of peptidase.

The hydrolyzed product was assayed after 30 min of incubation. In order to determine whether any metal ion was necessary for the optimal enzyme activity of the uterine fluid peptidase, an aliquot of S-300 fraction was dialyzed against 100–200 mesh Chelex-100 (Bio-Rad) for 24 h. The enzyme activity of the Chelex-treated uterine enzyme was compared with that of untreated S-300 fraction using Ac-Ala_4 as substrate. The TBS in this assay was also deprived of metal ions using Chelex-100.

The peptidase activity of the uterine fluid peptidase was also assayed using Suc-Ala-Ala-Leu-pNA and aminopeptidase from *S. griseus* (SGAP) by a two stage method *(30)*. The uterine enzyme liberated leucyl-paranitroanilide (Leu-pNA) from the substrate and the Leu-pNA was further cleaved by the bacterial amino peptidase, thus releasing the chromogenic product paranitroaniline, which was quantitated by noting its absorbance at 405 nm (*p*-nitroaniline $E_{405} = 10{,}600\ M^{-1}, cm^{-1}$) *(31)*. The assay was performed in 0.05 *M* Tris-HCl, pH 7.5/0.1 *M* NaCl at 37°C. The substrate (0.4 m*M*) was preincubated with the bacterial aminopeptidase (6 μg/mL) for at least an hour before the addition of uterine endopeptidase. The concentration of the uterine enzyme ranged from 2 to 15 μg/mL of the incubation mixture. The assay volume was 1 mL.

Electronmicroscopy

Negative staining of the purified uterine enzyme, which was obtained after electrolution of the enzyme-positive protein band of the native gel, was carried out on uncoated 400-mesh copper grids according to the method described by Malech and Albert *(32)*. The negative stain consisted of 2% aqueous uranyl acetate containing 25 μg/mL of bacitracin (Sigma, St. Louis, MO). Bacitracin was used to enhance "wetting" and film formation. Electron micrographs were taken at original magnifications of 5000–15,000 diameters at an accelerating voltage of 80 kV.

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