AN ENDOPEPTIDASE IN THE UTERINE SECRETION OF THE PROESTROUS RAT AND ITS RELATION TO A SPERM DECAPITATING FACTOR

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Summary. - A proteolytic enzyme has been isolated from the luminal fluid of the uterus of proestrous rats and from the uterine fluid of immature female rats treated with estradiol. When tested with synthetic oligopeptides as substrates, the enzyme was found to differ in specificity from the uterine dipeptidase (Smith, 1948; Albers et al., 1961) and aminopeptidase (Schmidt et al., 1967) previously reported. It was found to act as an endopeptidase hydrolyzing peptides of different chain lengths such as Leu↓Ala, Leu↓Ala↓Ala, Ac-Ala-Ala↓Ala↓Ala and the S-peptide of bovine pancreatic ribonuclease (20 amino acid residues; Richards, 1958) which is hydrolyzed into at least five fragments. The enzyme requires Ca^{++} (optimum 2-4 mM) and has a pH optimum at pH 7.0. The purified enzyme moved as a single band in acrylamide-gel electrophoresis. On the basis of its characteristics it cannot be identified with any one of the listed hydrolytic enzymes (Barman, 1969) of mammalian origin.

The luminal fluid of the uterus of proestrous rats was also shown to contain a non-dialyzable factor which on incubation with epididymal spermatozoa of rat or mice brings about separation of the head from the midpiece-tail segment; it does not decapitate epididymal spermatozoa of the bull, ram or guinea-pig (Fig. 1). Sperm decapitation is inhibited by the secretions of the coagulating gland or the seminal vesicles. The decapitating activity from uterine fluid remained associated with the peptidase activity described above throughout the fractionating procedure adopted; furthermore, the pH optimum, requirement for Ca^{++} and behaviour towards various inhibitors are the same for both activities.

Isolation. - The uterus of the rat is distended with watery fluid on the day of proestrus. This fluid was aspirated by syringe, centrifuged at 1000 x g

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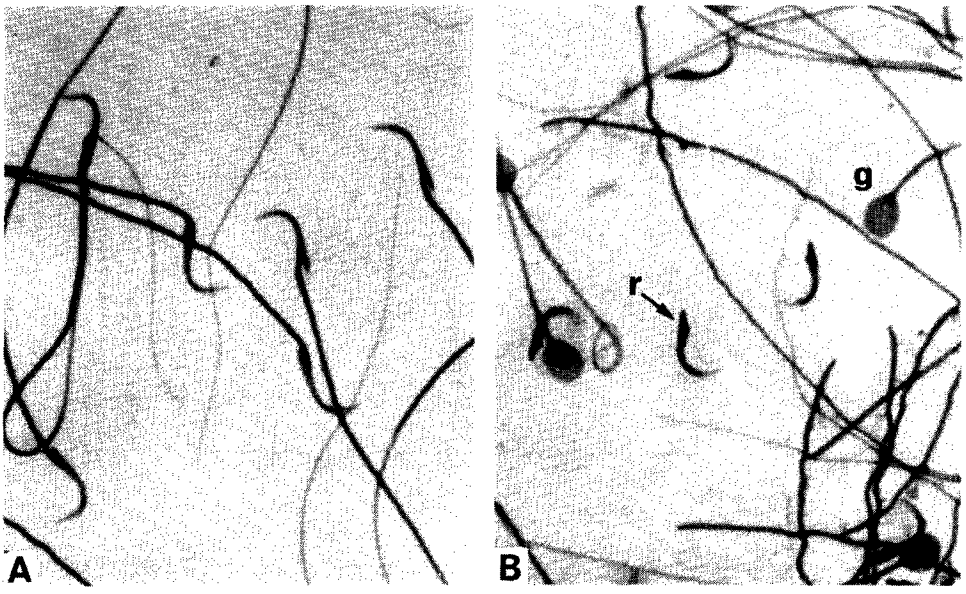

Figure 1. Smears of epididymal spermatozoa incubated for 3 h at 38°C in a medium composed of 0.05 ml saline, 0.01 ml 0.04 M $CaCl_2$ and 0.01 ml 0.01M Na-pyrophosphate, at a final concentration of 6 x 10^6 spermatozoa/ml. A, rat sperm incubated without decapitating factor; B, mixture of rat and guinea-pig sperm incubated with decapitating factor (0.13 ml of Sephadex fraction S_{3-4}); r, detached head of rat spermatozoon; g, intact guinea-pig spermatozoa.

for 10 min at 2°C to sediment cellular debris, and dialyzed against distilled water for 48 hrs at 3°C. The retentate was concentrated by ultrafiltration to 4 ml, yielding a protein concentration of approximately 3-4 mg/ml, and layered on a Sephadex G150 column (1.8 x 112 cm) previously equilibrated with 0.05M Tris-HCl buffer pH 7.1 at 3°C. Elution was carried out with the same buffer, collecting 3.5 ml fractions, and each fraction was assayed for (i) proteolytic activity, using N-acetyl tetra-L-alanine as the substrate (v. i) and (ii) rat epididymal sperm-decapitating activity. The first five fractions following the void volume exhibited both activities. These fractions were analyzed by acrylamide-gel electrophoresis. The eluate fractions active in both assays showed a common electrophoretic component; two of the active fractions (S_{3-4}) showed only this single band (Fig. 2) and sedimented as a symmetrical peak of 7.2 S, suggesting a molecular weight unusually high for an endopeptidase. This major peak was accompanied by a small

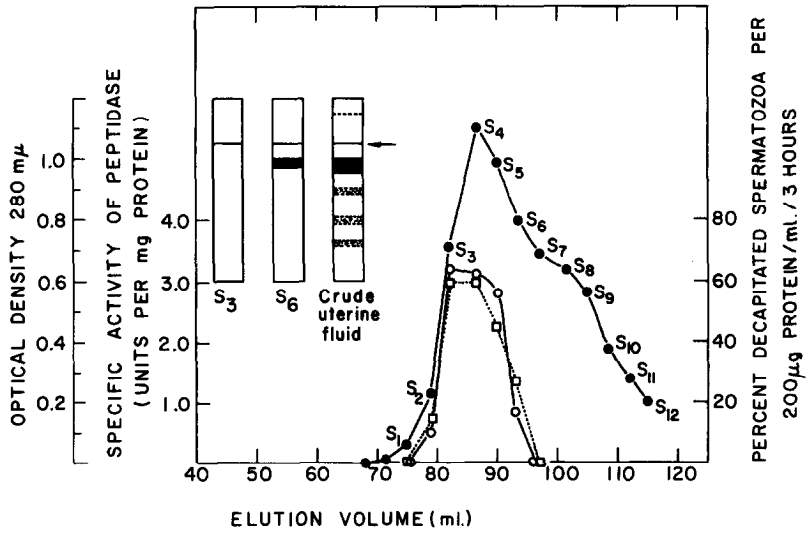

Figure 2. Sequential sephadex G150 chromatography and acrylamide-gel electrophoresis of uterine fluid proteins. ●—● optical density; ○—○ specific activity of peptidase; □—□ specific activity of sperm decapitating factor. The incubation mixture for assessing peptidase activity contained 0.1 ml 0.1 M Ac-tetra-L-alanine, 0.02 ml 0.04M $CaCl_2$, 0.07 ml 0.1 M Tris buffer pH 7.3, 0.05 ml eluate fraction and 0.01 ml 0.01 M Na-pyrophosphate. The extent of cleavage of the substrate was determined by ninhydrin colour development after incubation at 38°C for 30 min. One enzyme unit was that amount of enzyme which generated one micromole of amine per min. For sperm decapitating activity, epididymal spermatozoa were incubated for 3 hr with 0.13 ml of each eluate fraction under the conditions described in legend to Fig. 1. Activity was expressed as percentage of spermatozoa decapitated per unit weight of uterine protein. Inset represents electrophoretic pattern on acrylamide-gel of eluate fractions S_3 and S_6, and of crude uterine fluid proteins. Arrow points at component common to all fractions active in the two assays.

amount (<10%) of a heavier component (s = 13S).

Substrate specificity of the peptidase from uterine fluid. - The substrate specificity of the purified enzyme (fraction S_{3-4}, protein concentration 300 μg/ml by the method of Lowry, et al., 1951) was tested with synthetic oligopeptides of different amino acid composition and chain lengths, and with peptides substituted at their terminal amino-groups. Dipeptides with a C-terminal alanine such as Ala-Ala, Leu-Ala, and Tyr-Ala were readily hydrolyzed. Slow hydrolysis was observed with Ser-Ala and none with Gly-Ala or Val-Ala. No hydrolysis of dipeptides was observed when alanine was the N-terminal acid, e.g. with Ala-Leu,

Ala-Phe, Ala-Gly, Ala-Asp, Ala-Glu and Ala-Pro. Oligopeptides of longer chain length were also hydrolyzed: Leu-Ala-Ala, Ala-Ala-Phe-Arg, Ala-Ala-Ala-Ala. The octapeptide (1-8) and the S-peptide (1-20) of RNase were both cleaved into at least five different products.

The enzyme acted as an endopeptidase, since Ac-Ala-Ala-Ala-Ala was found to be initially cleaved into Ac-Ala-Ala and Ala-Ala; Ala-Ala-Ala-Ala into Ala-Ala; Z-Ala-Ala-Leu into Z-Ala and Ala-Leu; and since it yielded peptide fragments, rather than free amino acids, from the octapeptide or the S-peptide of pancreatic ribonuclease. Substrates of carboxypeptidase A, such as Z-Gly-Gly-Leu, of carboxypeptidase B or trypsin, such as penta-lysine, and of chymotrypsin such as N-acetyl-tyrosine ethyl ester, were not hydrolyzed.

Assay of peptidase activity and kinetic analysis. - The rate of generation of free amino groups by action of the enzyme on N-acetyl tetra-L-alanine was determined by the ninhydrin method (Cocking and Yemm, 1954), with alanine as the standard. The complete system consisted of acetyl tetra-L-alanine (5 mM), $CaCl_2$ (3.2 mM), enzyme fraction S_{3-4} (300 μg protein/ml, 0.1 - 0.6 ml per one ml of assay solution) and sodium pyrophosphate (4×10^{-4} M) in 0.05 M Tris-HCl buffer pH 7.1. Of the ninhydrin-positive cleavage products, Ala-Ala and alanine, the former was the principal product during the first two hours of the enzyme reaction, as shown by paper electrophoresis and paper chromatography. The rate of hydrolysis was linear for the first 60 mins of incubation and was proportional to enzyme concentration up to 300 μg protein/ml. The Michaelis constant $K_m = 2.9 \times 10^{-3}$ M and the maximal velocity $V_{max} = 1.6 \times 10^{-5}$ mole per liter per minute were obtained from Lineweaver-Burk plots using initial rates at an enzyme concentration of 45 μg protein per ml. The plots were linear in the substrate concentration range investigated (10^{-3} to 10^{-2} M for acetyl tetra-L-alanine).

Activators and inhibitors of proteolytic and sperm-decapitating activities. - The proteolytic enzyme and the sperm decapitating factor were activated by Ca^{++} ions; the optimal concentration of $CaCl_2$ for both activities was in the range 2 - 4 mM. Sperm decapitation was completely inhibited by 0.1 M NaF, 0.06 M Na-citrate, 5×10^{-3} EDTA, 0.05 M Na-pyrophosphate, 0.03% Na-dodecyl sulphate. Hydrolysis of acetyl tetra-L-alanine was inhibited to the extent of 87% by 6×10^{-3} M EDTA, 47% by 0.01 M pyrophosphate, 32% by 0.12 M NaF or 0.06 M Na-citrate, 78% by 0.03% Na-dodecyl sulphate. The proteolytic and sperm-decapitating activities were not inhibited by 10^{-3} M diisopropyl fluorophosphate.

It is pertinent that chymotrypsin, trypsin and pronase at 25 μg/ml concentration decapitated 100% of epididymal spermatozoa during two hours of incubation at 38°C. These observations lend support to the hypothesis that the proteolytic activity of uterine fluid is responsible for the sperm breakage effect.

Ejaculated spermatozoa, i.e. spermatozoa that have been exposed to the secretions of the male accessory glands, remain intact in the uterine lumen for at least 12 hours after mating, but are susceptible to decapitation by uterine fluid after washing with saline. Addition of coagulating gland fluid (1:10) or seminal vesicle secretion (1:100) protected epididymal or washed ejaculated spermatozoa against the decapitating factor of proestrous uterine fluid in vitro.

Discussion. - Rabbit and human uterine extracts have been shown to contain peptidases which hydrolyze glycine-containing di- and tri-peptides, but endopeptidase activity was not found (Smith, 1948). Furthermore, an aminopeptidase was shown to be present in human endometrial tissue during the secretory phase of the cycle (Schmidt et al., 1969); this enzyme was also shown to be present in rat uterus (Schmidt et al., 1967). Albers et al. (1961) found peptidase activity in oestrous fluid of rat and rabbit, and an increase of this activity in the uterine tissue was observed during the proestrous and oestrous phase of the cycle. The dipeptide Ala-Gly used by these authors as substrate is not cleaved by our enzyme preparation. The present work then establishes the presence in the uterine fluid of proestrous rats of an endopeptidase not previously described. This protease is closely associated, and may be identical, with a factor enhancing epididymal sperm fragility in vitro, an effect mimicked by several other proteolytic enzymes.

In vitro penetration of rat ova by sperm was first achieved by pretreating the ova with chymotrypsin (Toyoda and Chang, 1968). It thus seems possible that the proteolytic enzyme of uterine fluid may play a role in the initiation of the fertilization process. Whether this enzyme is also involved in sperm capacitation (Noyes, 1953) remains to be examined.

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