INHIBITION OF FERTILIZATION *IN VIVO* BY PANCREATIC AND SEMINAL PLASMA TRYPSIN INHIBITORS

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Summary. Treatment of capacitated rabbit spermatozoa with pancreatic trypsin inhibitor or partially purified seminal plasma trypsin inhibitor and subsequent insemination of such spermatozoa into the oviducts of ovulated rabbits markedly inhibited fertilization. Washing the spermatozoa to remove excess inhibitor did not affect the antifertility action of pancreatic trypsin inhibitor. Seminal plasma trypsin inhibitor was purified by specific binding to a trypsin–maleic anhydride–ethylene copolymer and Sephadex G-25 and G-50 column chromatography. A 500-fold purification was obtained.

INTRODUCTION

A trypsin-like enzyme has been known to exist in mammalian spermatozoa for many years (Yamane, 1935a, b; Buruiana, 1956) and has recently been shown to be localized in the sperm acrosome (Stambaugh & Buckley, 1969; Zaneveld, Srivastava & Williams, 1969; Zaneveld & Williams, 1970). Although the enzyme is similar to trypsin and plasmin and is inhibited by trypsin inhibitors, it has some unique properties (Zaneveld, Polakoski & Williams in preparation) and we therefore propose the name ‘acrosin’ based on the nomenclature for proteolytic enzymes where the prefix refers to the source of the enzyme. Unfortunately the name ‘acrosomin’ has already been used to indicate a mucopolysaccharide in the acrosome (Clermont & Leblond, 1955). Acrosin appears to be used by spermatozoa to penetrate the zona pellucida of the ovum. Acrosomal extracts of epididymal and capacitated spermatozoa have high acrosin activity whereas activity cannot be detected in acrosomal extracts of ejaculated spermatozoa until after chromatography of the extracts (Zaneveld *et al.*, 1969). Treatment of epididymal spermatozoa with seminal plasma decreases acrosin activity and epididymal spermatozoa appear to acquire a trypsin inhibitor by contact with seminal plasma, the inhibitor being removed during capacitation or in acrosomal extracts by purification of acrosin (Zaneveld, Srivastava & Williams, 1970).

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Haendle, Fritz, Trautschold & Werle (1965) and Fritz, Schult, Hutzel, Wiedemann & Werle (1967) isolated a trypsin inhibitor from the seminal vesicles of mice using a resin to which trypsin was attached that specifically bound the inhibitor. This seminal vesicle trypsin inhibitor possessed many similarities to bovine pancreatic trypsin inhibitor, both having a molecular weight of approximately 6000 and a similar amino acid composition. Stambaugh, Brackett & Mastroianni (1969) showed that fertilization *in vitro* was inhibited by soybean trypsin inhibitor.

This report concerns the partial purification of rabbit seminal plasma trypsin inhibitor (SPTI) and the inhibition of fertilization *in vivo* by SPTI and pancreatic trypsin inhibitor.

MATERIALS AND METHODS

Isolation of seminal plasma trypsin inhibitor

Trypsin-maleic anhydride-ethylene copolymer was prepared according to the method of Levin, Pecht, Goldstein & Katchalski (1964). Four hundred mg of pure bovine pancreatic trypsin (Worthington Biochemical Company) combined with 1 g of maleic anhydride-ethylene copolymer (EMA31, Monsanto Company) to form the trypsin resin.

Semen was collected three times a week from forty New Zealand White rabbits. The spermatozoa were removed by centrifugation and the seminal plasma pooled and stored at -20°C . High molecular weight material, including decapacitation factor (DF), was removed from the seminal plasma by centrifuging at 105,000 *g* for 4 hr (Bedford & Chang, 1962). The supernatant solution was incubated at 37°C for 20 min with 1.4 g of lyophilized trypsin resin while shaking. The mixture was centrifuged at 6000 *g* for 15 min at 0°C and the supernatant solution removed. The precipitate was washed by centrifugation with 0.1 M-triethanolamine buffer, pH 8, containing 0.1 M-NaCl, until no protein could be detected in the supernate. The bound SPTI was removed from the trypsin-resin by suspension of the precipitate in 0.1 M-KCl-HCl buffer at pH 2 and incubated for 15 min at 37°C . The suspension was again centrifuged at 6000 *g* for 15 min at 0°C and the supernatant solution collected. This was repeated three times to release all the bound inhibitor. The trypsin resin was washed twice with glass-distilled water and lyophilized. The lyophilized copolymer is stable and can be used repeatedly.

The supernatant solutions were concentrated by flash-evaporation to 5 ml and desalted using a Sephadex G-25 column and elution with water at room temperature. The excluded fraction containing SPTI was lyophilized and used in the fertilization assays.

Seminal plasma trypsin inhibitor was further purified by addition of 20 mg lyophilized material to a Sephadex G-50 column and elution with 0.01 M-phosphate buffer, pH 7.0, at a rate of 10 drops/min.

Trypsin inhibitor assays were performed by incubating 0.1 ml of the inhibitor solution with 0.1 ml pancreatic trypsin (Worthington, twice crystallized), for 15 min at 25°C and assaying 0.1 ml of the mixture for trypsin activity by the method of Schwert & Takenake (1955) using benzoyl-arginine ethylester

(BAEE, Mann Res. Lab.) as substrate. Controls were similarly treated except that no trypsin inhibitor was present. Trypsin activity (1 mU) was defined as that amount of trypsin causing a rate of change of 0.001 optical density units/min. The purity of the SPTI-containing peaks was estimated by disc electrophoresis on polyacrylamide gel (Brewer & Ashworth, 1969).

Fertilization procedure in-vivo

Capacitated spermatozoa were obtained from mature New Zealand White rabbits by the method of Williams, Hamner, Weinman & Brackett (1964). Two hundred μg of pancreatic trypsin inhibitor (Worthington, crystallized) or 250 μg of SPTI were added to 10^5 spermatozoa and the suspension incubated for 15 min at 37°C . This amount of inhibitor is similar to that of low molecular weight DF (Williams, Robertson & Dukelow, 1969) usually employed in these

TEXT-FIG. 1. Disc electrophoresis of partially purified fractions containing seminal plasma trypsin inhibitor. Disc electrophoresis was performed at pH 8.3 (A and B) and 4.3 (C). (A) After desalting by Sephadex G-25; (B and C) After Sephadex G-50 chromatography.

assays. A larger amount of SPTI than pancreatic trypsin inhibitor (PTI) was used since the SPTI was less pure.

Human chorionic gonadotrophin (75 units, Spencer Mead, Inc.) was injected (i.v.) $12\frac{1}{2}$ hr before surgical insemination into mature, New Zealand White rabbits that had been kept in isolation for at least 18 days. One oviduct was used for the treated spermatozoa and the other for the control spermatozoa. A 0.25-ml syringe with a small Teflon needle was used to inseminate 0.05 ml (5×10^4 spermatozoa) of the sperm solution. The rabbits were killed 24 hr later, the ova flushed from the oviducts with Krebs-Ringer-phosphate and observed for cleavage. Overall egg recovery averages 85% in our laboratories.

RESULTS

Disc electrophoresis at pH 8.3 revealed the presence of one major and one minor component in the peak containing SPTI after Sephadex G-25 chromatography

(Text-fig. 1). The minor component was removed by further purification. Three components were obtained after Sephadex G-50 chromatography (Text-fig. 2). The major retarded component contained all the SPTI activity. This compo-

TEXT-FIG. 2. Elution profile of seminal plasma trypsin inhibitor (SPTI) subjected to Sephadex G-50 chromatography. Protein was eluted with 0.01 M-phosphate buffer, pH 7.0. Horizontal bar indicates pooled fractions containing SPTI.

TABLE 1

EFFECT OF MAMMALIAN TRYPSIN INHIBITORS ON FERTILIZATION IN VIVO

<i>Treatment of spermatozoa</i>	<i>No. of rabbits</i>	<i>Cleaved ova/ total no. of ova</i>	<i>% fertilization</i>
Pancreatic trypsin inhibitor (200 μ g/ 10^5 spermatozoa)	10	8/49	16.3
Control	10	28/37	75.7
Pancreatic trypsin inhibitor (200 μ g/ 10^5 spermatozoa)	7	2/22	9.1
Control	7	15/22	68.2
Seminal plasma trypsin inhibitor (250 μ g/ 10^5 spermatozoa)	5	1/14	7.1
Control	5	17/18	94.4

The mixture of trypsin and spermatozoa (0.05 ml, 5×10^5 spermatozoa) was inseminated into the oviducts of rabbits 12½ hr after administration of HCG, except in the second group of experiments in which the trypsin inhibitor was removed from the spermatozoa by centrifugation at 300 g for 5 min, discarding the supernatant solution and resuspending the spermatozoa in Krebs-Ringer-phosphate.

Controls were treated in the same way as the test samples, except that no trypsin inhibitor was added.

One μ g pancreatic trypsin inhibitor inhibits 3.6 mU pancreatic trypsin. One μ g seminal plasma trypsin inhibitor inhibits 2.4 mU pancreatic trypsin.

nent showed one band by disc electrophoresis at pH 8.3. At pH 4.3, two major adjacent bands and one faint band were obtained (Text-fig. 1). After Sephadex G-50 chromatography, SPTI had on a weight basis approximately a 500-fold

greater inhibitory effect on pancreatic trypsin than seminal plasma. One microgram of SPTI caused the inhibition of 10 mU pancreatic trypsin.

The motility of the capacitated spermatozoa was not affected by incubation with either PTI or SPTI if the preparations were salt-free. Both SPTI and PTI depressed fertilization (Table 1). SPTI caused 87.4% inhibition of fertility compared with the controls. The presence of trypsin inhibitor in the oviduct did not affect the fertility since approximately the same percentage inhibition of fertilization was obtained (59.4% versus 59.1%) if spermatozoa were inseminated with PTI or if the excess PTI was first removed by centrifugation and the spermatozoa resuspended in Krebs-Ringer-phosphate.

DISCUSSION

The results indicate that, besides DF, another enzyme inhibitor, SPTI, is present in the seminal plasma that can prevent fertilization. It is unlikely that the inhibition of fertilization by SPTI is caused by residual DF since all DF is removed from the seminal plasma by centrifugation at 105,000 *g* for 4 hr. Further, DF is not a trypsin inhibitor (Zaneveld & Williams, 1970) and will not bind to the trypsin-resin.

Fertilization can also be inhibited using PTI. Most likely SPTI and PTI act by inhibiting acrosin, a proteolytic enzyme present in the sperm acrosome so that penetration of the spermatozoa through the zona pellucida is no longer possible.

The trypsin inhibitor must pass through the outer acrosomal membrane to contact acrosin. It is therefore not surprising that high levels of soybean trypsin inhibitor were necessary to prevent fertilization *in vitro* (Stambaugh *et al.*, 1969). The molecular weight of soybean trypsin inhibitor is three to four times higher than most mammalian trypsin inhibitors and may penetrate the outer acrosomal membrane with difficulty.

Bedford & Chang (1962) found that the supernatant solution after centrifugation of rabbit seminal plasma at 105,000 *g* has little DF activity. Although SPTI is present in this supernatant solution, the amount of SPTI is probably too small to prevent fertilization. It is possible, however, that the small decreases in fertility (0 to 35%) caused by the supernatant solutions were due to the SPTI.

A high degree of purity of the seminal plasma trypsin inhibitor has been obtained. The faint band after electrophoresis at pH 4.3 is probably a contaminant but the heavy bands may both be caused by trypsin inhibitors of similar molecular weight since Fritz *et al.* (1967) obtained the same results when purifying a trypsin inhibitor from the seminal vesicles of mice. They showed the presence of two almost identical inhibitors possessing a molecular weight of approximately 6000. Since the SPTI is retarded by Sephadex G-50, its molecular weight might be in the same range. It appears quite likely that SPTI and the seminal vesicle trypsin inhibitor are the same or similar inhibitors.

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