Components of human split ejaculates II. Enzymes and proteinase inhibitors

P. F. Tauber,* L. J. D. Zaneveld,† D. Propping‡ and
G. F. B. Schumacher

*Laboratory for Reproductive Biochemistry and Immunology, Department of Obstetrics and Gynecology,
University of Chicago, Chicago, Illinois 60637, U.S.A.*

Summary. Lysozyme, α -amylase, neutral proteinase and plasminogen activator were most concentrated in the initial portion of the ejaculate that consists mostly of Cowper's gland and prostate gland fluids as well as spermatozoa. The concentration of the high molecular weight proteinase inhibitors, α_1 -antitrypsin and α_{1X} -antichymotrypsin, was essentially unaltered throughout the ejaculate fractions, although their absolute amounts showed an increase towards the final fraction. By contrast, the total inhibitory activity towards pancreatic trypsin was highest both in concentration and amount in the last fraction, thus indicating that the seminal vesicles are its primary source. Plasminogen, prothrombin, Factor XIII, and the proteinase inhibitors antithrombin III, α_2 -macroglobulin, inter- α -trypsin inhibitor and C₁s-inactivator could not be detected immunochemically in whole ejaculates, and indicates the dissimilarity between the coagulation/liquefaction processes of semen and blood.

Introduction

Knowledge of the quantity and distribution of the seminal constituents throughout the ejaculate is essential to determine the mechanisms involved in the human seminal coagulation–liquefaction process (Tauber, Propping, Zaneveld & Schumacher, 1973; Zaneveld, Schumacher, Tauber & Propping, 1974; Tauber, Zaneveld, Propping & Schumacher, 1975). Tauber *et al.* (1975) showed that the immunoglobulins IgG and IgA, albumin and transferrin, are at higher concentrations in the first portion of the split ejaculate which contains most of the spermatozoa and secretions from the prostate and Cowper's gland. Lactoferrin distribution, however, is similar to that of fructose and is therefore mainly a product of the seminal vesicles. The present paper is a report of the various lytic enzymes and enzyme-related components in the same series of split ejaculates.

Materials and Methods

Details of the collection, preparation and storage of the split ejaculates were described by Tauber *et al.* (1975). Sperm count and fructose concentrations were used as control parameters to ensure proper collection. Lysozyme, α -amylase and plasminogen activator were determined by the micro-radial diffusion methods of Schumacher & Schill (1972) and Schill & Schumacher (1972) with a minor modification in the plasminogen activator test. A human urokinase preparation (Leo Pharmaceutical, Copenhagen; obtained through Polysciences Inc., Warrington, Pennsylvania) was used as

* Present address: University of Essen, Department of Obstetrics and Gynecology, Hufelandstr. 55, 4300 Essen, West Germany.

† Present address: Department of Physiology, College of Medicine, University of Illinois at the Medical Center, 901 South Wolcott Street, Chicago, Illinois 60680, U.S.A.

‡ Present address: Hospital for Women, Harvard Medical School, 221 Longwood Avenue, Boston, Massachusetts 02115, U.S.A.

standard and contained in the stock solution 2200 PlougU/ml in 0.85% NaCl at -20°C . Saline dilutions varied from 2.2 to 2200 PlougU/ml (0.7 PlougU of urokinase activity = 1 CTA unit = release of 46.2×10^{-3} μM -methanol from α -acetyl-L-lysine methyl ester).

Neutral proteinase activity of semen was assayed using a modified gelatin plate method (Zaneveld, Schumacher & Travis, 1973). Kodak projector slide plates were exposed, developed, fixed, extensively washed and dried. They were cut to fit LKB frames (Gelman Instruments, Ann Arbor, Michigan) used for immunodiffusion tests and overlaid with 10.0 ml melted agar (1%) in 0.1 M-sodium diethylbarbiturate buffer, pH 7.8, containing 0.1 M-NaCl. Wells of 1.5 mm diameter were punched in the agar and filled with standard solutions and samples. Bovine pancreatic trypsin (Worthington Biochemical Corporation; 0.63–40 $\mu\text{g}/\text{ml}$) in 0.1 M-borate buffer, pH 8.0, containing 0.05 M- CaCl_2 , was used as standard. The agar layers were removed after 16 hr at 37°C in a moist chamber and the diameter of the lysis zones measured. The proteolytic activity of the samples was expressed in equivalents of μg bovine pancreatic trypsin/ml.

The total amount of trypsin inhibitory activity was tested by mixing 10 μl of the seminal test sample with 25 μl bovine pancreatic trypsin (100 $\mu\text{g}/\text{ml}$ in the borate buffer mentioned above). After incubation for 5 min at room temperature, 3.0 ml benzoyl arginine ethyl ester (BAEE; 0.2 mg/ml: Sigma Chemical Company, St. Louis, Missouri) was added (Schwert & Takenaka, 1955). The change in optical density at 253 nm was recorded over 5 min. As a control, the same amount of trypsin was incubated with buffer. One mi.u. of inhibitor was defined as the amount that prevented hydrolysis of 1 μmol BAEE. Background esterolytic activity was measured by adding 10 μl of each split ejaculate sample to the BAEE and recording the activity. Appropriate adjustments were made in the final figures.

The concentrations of α_1 -antitrypsin and $\alpha_{1\text{X}}$ -antichymotrypsin were determined by radial immunodiffusion (Schumacher, 1970) using monospecific antisera and a standardized and stabilized reference serum (Behring Diagnostics, Somerville, N.J.). Plasminogen, prothrombin, Factor XIII, antithrombin III, α_2 -macroglobulin, inter- α -trypsin inhibitor and C_1s -inactivator were determined by Ouchterlony double immunodiffusion using Hyland plates, Type D (Hyland Laboratory, Travenol Division, Costa Mesa, Ca.) and monospecific antisera (Behring Diagnostics). Pooled normal human serum and/or plasma served as control. The mathematical and statistical evaluations were performed as described previously (Tauber *et al.*, 1975).

Results

The mean results are shown in Table 1; interested readers may contact the authors for the detailed figures.

Enzymes

Lysozyme. Although the highest concentration was usually found in the initial fraction of the ejaculate, the mean amount of lysozyme increased towards Fraction III. Due to the larger volumes of Fractions II and III, the differences were not statistically significant.

α -Amylase. The highest concentrations were found in Fraction I (Fraction I versus Fraction II, $P = 0.01$; Fraction I versus Fraction III, $P = 0.025$), as were the mean amounts.

Neutral proteinase. Mean concentrations and mean amounts showed a distinct decrease in distribution from the initial to the final fraction of the ejaculate. The differences between the concentrations of Fractions I and II as well as Fractions I and III were statistically significant ($P < 0.0005$). All donors exhibited the same pattern of decrease. No neutral proteinase activity could be found in the final portion of one donor and only 1/7 samples of Fraction II from this donor contained proteolytic activity, in contrast to 4/5 samples in his Fraction I.

Plasminogen activator. This decreased in concentration and amount from the first to the last fraction as did the neutral proteinase. The differences between Fractions I and II ($P = 0.01$) and I and III ($P = 0.0005$) were statistically significant.

Table 1. The mean concentrations (per ml seminal plasma) and amounts (per total fraction volume) of various enzymes and inhibitors in the split ejaculates obtained from 10 men

Enzymes/inhibitors	Split ejaculates						Whole ejaculates	
	Fraction I			Fraction II			Fraction III	
	Conc.	Amount	%	Conc.	Amount	%	Conc.	Amount
Lysozyme (μg chicken EW equiv.)	80	50	28	60	60	33	70	180
α -Amylase (μg bovine pancreatic amylase equiv.)	0.52	0.39	36	0.35	0.33	30	0.36	1.09
Neutral proteinase (μg bovine pancreatic trypsin equiv.)	2.44	1.88	61	0.76	0.70	23	0.47	3.08
Plasminogen activator (PlougU urokinase equiv.)	23.05	14.91	37	14.34	13.49	33	9.27	40.61
Total trypsin inhibitor (mi.u.)	3777	2688	23	4635	4297	37	4992	11586
α_1 -Antitrypsin (μg)	80	57	25	90	85	37	90	230
$\alpha_{1\text{r}}$ -Antichymotrypsin (μg)	40	30	25	40	40	33	40	120

Plasminogen, prothrombin and Factor XIII. These enzymes were not detectable in 10 normal seminal plasma samples by means of the Ouchterlony double immunodiffusion.

Proteinase inhibitors

Total trypsin inhibitory activity. The concentration and amount of total inhibitory activity increased from Fraction I to Fraction III, although statistical differences were not present. The distribution varied considerably between the donors, but without individual extremes.

α_1 -Antitrypsin. The mean concentrations and amounts of α_1 -antitrypsin increased slightly from Fraction I to Fraction III, although the differences were not statistically significant.

α_{1X} -Antichymotrypsin. The levels of α_{1X} -antichymotrypsin were approximately one-half those of α_1 -antitrypsin. The mean concentrations of the three Fractions were identical, but the mean amounts increased towards the last fraction.

Antithrombin III, α_2 -macroglobulin, inter- α -trypsin inhibitor and C_{1s} -inactivator. None of these enzyme inhibitors was detectable in 12 whole seminal plasma samples by means of Ouchterlony double immunodiffusion.

Discussion

The previously reported sperm counts and fructose levels in the three portions of the split ejaculates (Tauber *et al.*, 1975) and the values found in the present study are shown in Text-fig. 1. The patterns are characteristically related to the origin of a component. Text-figure 1 shows that the neutral proteinase and the plasminogen activator are mainly derived from the prostate or Cowper's gland, or that these enzymes are associated with spermatozoa and epididymal fluid. By contrast, the total trypsin inhibitor pattern is more similar to that of fructose. Both α_1 -antitrypsin and α_{1X} -antichymotrypsin are almost equally distributed among the three portions, indicating contribution by both the prostate gland and seminal vesicles.

The presence of lysozyme in human seminal plasma has been demonstrated previously (Hirschhäuser & Kionke, 1971; Schill & Schumacher, 1973). Lysozyme values in whole human semen specimens vary considerably, although no statistical differences can be found between semen samples of different quality in regard to sperm count and motility (Schumacher, 1974). The mean concentration of lysozyme in this study confirms previous results (Schill & Schumacher, 1973). Values 3–10 times higher were found by Hirschhäuser & Eliasson (1972), probably due to different lysozyme preparations used as reference standard. The mean lysozyme curve in Text-fig. 1 is close to the theoretical line of equal distribution, i.e. the accessory glands contribute equally, although the mean concentration is slightly higher in the first fraction. This is in agreement with the observations of Hirschhäuser & Eliasson (1972) and Schumacher (1974).

The amount of diffusible lysozyme was assessed in the genital organs of a 31-year-old man removed at autopsy. Small tissue cylinders were punched from the luminal portions of the tract and the amount of lysozyme released/mg wet weight of tissue was determined as described by Schumacher & Pearl (1969). Remarkably high levels of lysozyme were present in epididymal tissue (0.036 μ g). The prostate also contained high levels (0.029 μ g in the middle lobe and 0.032 μ g in the lateral lobe), in contrast to the testis (0.016 μ g), the ampulla (0.022 μ g) and the vas deferens (0.022 μ g). The seminal vesicles contained 0.026 μ g lysozyme, explaining why the distribution curve of this enzyme (Text-fig. 1) does not favour the first portion of the ejaculate more extensively.

α -Amylase levels in normal human semen samples show wide variations and do not differ considerably from those in 'abnormal' specimens (Moon & Bunge, 1968). As in our results, these authors also found the highest concentration of α -amylase in the first portion of the ejaculate and the computed mean concentration in the whole ejaculate is in agreement with the results of Schill & Schumacher (1973).

The neutral proteinase activity of human semen is probably due to a single enzyme that has been called 'chymotrypsin-like enzyme' although it is dissimilar to chymotrypsin in almost all of its properties except for its BTEE hydrolysing activity. The enzyme has been partly purified and charac-

Text-fig. 1. Distribution profile of enzymatic components in human ejaculates. The curves reflect the concentration changes of the components during the ejaculation process. Curves below the dotted line represent contributions that originate mainly from the seminal vesicles, and curves above the line from other parts of the male genital tract. The two vertical lines indicate the average accumulated volumes of split ejaculate Fractions I and II, respectively. For further details see text.

terized by Lundquist, Thorsteinsson & Buus (1955), Syner & Moghissi (1972) and Fritz, Arnhold, Förg-Brey, Zaneveld & Schumacher (1972), and reintroduction of the name 'seminin', first proposed for the proteolytic enzyme in semen by Oettle (1950), has been suggested (Zaneveld *et al.*, 1974). The distribution pattern of neutral proteinase in split ejaculates is almost identical to that of spermatozoa. More than 60% of the total amount of ejaculate is present in the first portion, which represents approximately 25% of the volume of the whole ejaculate. Fraction III contains 45% of the total volume and contributes only 16% to the total amount of neutral proteinase activity, indicating that very little neutral proteinase originates from the seminal vesicles.

The plasminogen activator is an ubiquitous part of the fibrinolytic system and is also present in seminal fluid (Harvey, 1949; VonKaulla & Shettles, 1954; Rasmussen & Albrechtsen, 1960). In Text-fig. 1, the plasminogen activator is highest in the first portion of the ejaculate and its main source may therefore be the prostate. No correlation is found, however, between the amount of prostatic fluid and the concentration of activator in semen (Harvey 1949; Ying, Day, Whitmore & Tagnon, 1956); histochemical data (Kester, 1971) also seem to support this latter observation, indicating that other male accessory glands may be the source of seminal plasminogen activator.

Two plasminogen activators (SPA 1: mol. wt 68,000; SPA 2: mol. wt 72,000) have been purified and characterized from human seminal plasma and are immunochemically related to urokinase (Propping, Tauber, Zaneveld & Schumacher, 1974). The distribution pattern of plasminogen activator in the ejaculate is very similar to that of the neutral proteinase (Text-fig. 1), but the mol. wt of the latter is 30,000 (Syner & Moghissi, 1972) and the two enzymes can be distinguished by Sephadex G-75 gel filtration (Propping *et al.*, 1974).

The presence of low molecular weight proteinase inhibitors and α_1 -antitrypsin in human seminal plasma has been previously established (Händle, Fritz, Trautschold & Werle, 1965; Schumacher, 1970; Fink, Jaumann, Fritz, Ingrisich & Werle, 1971), yet our results show the presence of another serum proteinase inhibitor, α_{1X} -antichymotrypsin, although in rather low concentrations. The total trypsin inhibitory activity of the combined fractions, representing both the low and high molecular weight inhibitors, correlates with the data of Fink *et al.* (1973). This similarity was confirmed by measuring the inhibitory effect of 10 seminal fluid pools towards bovine pancreatic trypsin as described in the 'Materials and Methods'. The mean $\pm$ S.D. trypsin inhibitory activity was 4716 ± 1310 mi.u./ml (range 2964–6864 mi.u./ml), and was very close to the combined value from the amounts of each split ejaculate, thus illustrating the accuracy of the test system.

The physiological role of the high molecular weight inhibitors is not established, but at least one of the low molecular weight inhibitors may be involved in the capacitation and fertilization process through its inhibition of sperm acrosin (Zaneveld, Robertson, Kessler & Williams, 1971; Zaneveld, Dragoje & Schumacher, 1972). None of these inhibitors, with the possible exception of α_{1X} -antichymotrypsin, inhibits neutral proteinase of human seminal plasma (Fritz *et al.*, 1972). The serum inhibitors are rather evenly distributed throughout the ejaculate (Text-fig. 1), whereas the total trypsin inhibitory activity has a fructose-like distribution. Thus, the main source of the low molecular weight inhibitors in man is the seminal vesicles, as in laboratory animals (Händle *et al.*, 1965).

The involvement of several seminal enzymes in the liquefaction of the human seminal coagulum has been suggested (Bunge & Sherman, 1954; Ying *et al.*, 1956; Rasmussen & Albrechtsen, 1960; Syner & Moghissi, 1972; Hirschhäuser & Eliasson, 1972). The first portion of the ejaculate enhances the liquefaction of the coagulated third portion which normally lyses quite slowly, indicating that the liquefying factor(s) are primarily in the first fraction (Tauber *et al.*, 1973). This applies to most of the enzymes investigated, particularly the plasminogen activator and neutral proteinase. It is of interest that Fraction III of one donor did not possess neutral proteinase activity and required an extremely long time to liquefy (2 hr). Addition of Fraction I from a normal donor to this coagulum resulted in rapid liquefaction. The particular component(s) involved in seminal liquefaction cannot yet be identified but the absence of plasminogen, prothrombin, fibrinogen and Factor XIII from seminal plasma indicates a dissimilarity between the seminal coagulation-liquefaction system and that of blood coagulation and fibrinolysis.

We thank Mrs P. Crawford, Mr B. Dragoje and Mr B. Jemison for their excellent technical assistance. This research was supported by the Ford Foundation Grant No. 690-0108, USPHS Grant No. NIH-HD-06315, and a Biomedical Center for Population Research Grant No. NIH-HD-07110.

References

- BUNGE, R.G. & SHERMAN, J.K. (1954) Liquefaction of human semen by alpha-amylase. *Fert. Steril.* **5**, 353–356.
- FINK, E., JAUMANN, E., FRITZ, H., INGRISCH, H. & WERLE, E. (1971) Protease Inhibitoren im menschlichen Spermaplasma. Isolierung durch Affinitätschromatographie und Hemmverhalten. *Hoppe Seyler's Z. Physiol. Chem.* **352**, 1591–1594.
- FINK, E., FRITZ, H., JAUMANN, E., SCHIESSLER, H., FÖRG-BREY, B. & WERLE, E. (1973) Protein proteinase inhibitors in male sex glands and their secretions. *Prot. Biol. Fluids* **20**, 425–431.
- FRITZ, H., ARNHOLD, M., FÖRG-BREY, B., ZANEVELD, L.J.D. & SCHUMACHER, G.F.B. (1972) Verhalten der chymotrypsinähnlichen Proteinase aus Human-sperma gegenüber Protein-Proteinase-Inhibitoren. *Hoppe Seyler's Z. Physiol. Chem.* **353**, 1651–1653.
- HÄNDLE, H., FRITZ, H., TRAUTSCHOLD, I. & WERLE, E. (1965) Über einen hormonabhängigen Inhibitor für proteolytische Enzyme in männlichen akzessorischen Geschlechtsdrüsen und im Sperma. *Hoppe Seyler's Z. Physiol. Chem.* **343**, 185–188.
- HARVEY, C. (1949) Fibrinolysin in human semen. *Stud. Fert.* **1**, 11–17.
- HIRSCHHÄUSER, C. & ELIASSON, R. (1972) Origin and possible function of muramidase (lysozyme) in human seminal plasma. *Life Sci.* **11**, 149–154.
- HIRSCHHÄUSER, C. & KIONKE, M. (1971) Zur Biochemie des fibrinolytischen Systems im Spermaplasma. *Fortschr. Androl.* **2**, 118–122.

- KESTER, R.C. (1971) The distribution of the plasminogen activator in the male genital tract. *J. clin. Path.* **24**, 726-731.
- LUNDQUIST, F., THORSTEINSSON, T. & BUUS, O. (1955) Purification and properties of some enzymes in human seminal plasma. *Biochem. J.* **59**, 69-79.
- MOON, K.H. & BUNGE, R.G. (1968) Observations on the biochemistry of human semen. *Fert. Steril.* **19**, 977-981.
- OETTL, A.G. (1950) Fibrinolytic factors in human semen. *Stud. Fert.* **2**, 71.
- PROPPING, D., TAUBER, P.F., ZANEVELD, L.J.D. & SCHUMACHER, G.F.B. (1974) Purification and characterization of two plasminogen activators from human seminal plasma. *Fedn Proc. Fedn Am. Socs exp. Biol.* **33**, 289.
- RASMUSSEN, J. & ALBRECHTSEN, O.K. (1960) Fibrinolytic activity in human seminal plasma. *Fert. Steril.* **11**, 264-277.
- SCHILL, W.B. & SCHUMACHER, G.F.B. (1972) Radial diffusion in gel for microdetermination of enzymes. I. Muramidase, alpha-amylase, DNase I, RNase A, acid phosphatase, and alkaline phosphatase. *Analyt. Biochem.* **46**, 502-533.
- SCHILL, W.B. & SCHUMACHER, G.F.B. (1973) Micro radial diffusion in gel. Methods for the quantitative assessment of soluble proteins in genital secretions. In *The Biology of the Cervix*, pp. 173-200. Eds R. J. Blandau & K. S. Moghissi. University of Chicago Press.
- SCHUMACHER, G.F.B. (1970) Alpha-antitrypsin in genital secretions. *J. Reprod. Med.* **5**, 13-19.
- SCHUMACHER, G.F.B. (1974) Lysozyme in human genital secretions. In *Lysozyme*, pp. 427-447. Eds E. F. Osseimann, R. E. Canfield & S. Beychok. Academic Press, New York.
- SCHUMACHER, G.F.B. & PEARL, M.J. (1969) Muramidase (lysozyme) in cervical secretions. *Prot. Biol. Fluids* **16**, 525-534.
- SCHUMACHER, G.F.B. & SCHILL, W.B. (1972) Radial diffusion in gel for microdetermination of enzymes. II. Plasminogen activator, elastase, and nonspecific proteases. *Analyt. Biochem.* **48**, 9-26.
- SCHWERT, G.W. & TAKENAKA, Y. (1955) A spectrophotometric determination of trypsin and chymotrypsin. *Biochim. biophys. Acta* **16**, 570-575.
- SYNER, F.N. & MOGHISSI, K.S. (1972) Purification and properties of a human seminal proteinase. *Biochem. J.* **126**, 1135-1140.
- TAUBER, P.F., PROPPING, D., ZANEVELD, L.J.D. & SCHUMACHER, G.F.B. (1973) Biochemical studies on the lysis of human split ejaculates. *Biol. Reprod.* **9**, 62.
- TAUBER, P.F., ZANEVELD, L.J.D., PROPPING, D. & SCHUMACHER, G.F.B. (1975) Components of human split ejaculates. I. Spermatozoa, fructose, immunoglobulins, albumin, transferrin, lactoferrin and other plasma proteins. *J. Reprod. Fert.* **43**, 249-267.
- VONKAULLA, K.N. & SHETTLES, L.B. (1954) Beitrag zur Kenntnis des proteolytischen Fermentsystems im menschlichen Spermaplasma, mucus cervicalis, Tubarschleimhaut und Liquor folliculi. *Klin. Wschr.* **32**, 468-472.
- YING, S.H., DAY, E., WHITMORE, W.F. & TAGNON, H.J. (1956) Fibrinolytic activity in human prostatic fluid and semen. *Fert. Steril.* **7**, 80-87.
- ZANEVELD, L.J.D., ROBERTSON, R.T., KESSLER, M. & WILLIAMS, W.L. (1971) Inhibition of fertilization *in vivo* by pancreatic and seminal plasma trypsin inhibitors. *J. Reprod. Fert.* **25**, 387-392.
- ZANEVELD, L.J.D., DRAGOJE, B.M. & SCHUMACHER, G.F.B. (1972) Acrosomal proteinase and proteinase inhibitor of human spermatozoa. *Science, N.Y.* **177**, 702-703.
- ZANEVELD, L.J.D., SCHUMACHER, G.F.B. & TRAVIS, J. (1973) Human sperm acrosomal proteinase: antibody inhibition and immunologic dissimilarity to human pancreatic trypsin. *Fert. Steril.* **24**, 479-484.
- ZANEVELD, L.J.D., SCHUMACHER, G.F.B., TAUBER, P.F. & PROPPING, D. (1974) Proteinase inhibitors and proteinases of human semen. In *Proteinase Inhibitors*, pp. 136-146. Eds H. Fritz, H. Tschesche, L. J. Greene & E. Truscheit. Springer-Verlag, New York.

Received 6 May 1975