Purification and Properties of a Human Seminal Proteinase

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1. A method is described for the purification of a proteinase, present in human seminal plasma and previously shown to accelerate migration of spermatozoa through cervical mucus *in vitro*. A 25-fold purification was achieved in three steps, consisting of ammonium sulphate fractionation, chromatography on CM-cellulose and gel filtration. 2. The enzyme displays some properties similar to chymotrypsin: pH optimum 7.5–8.0; substrate preference of casein, haemoglobin and benzoyltyrosine ethyl ester but not benzoylarginine ethyl ester; mol.wt. 33000. However, it is unaffected by 1 mM-di-isopropyl phosphofluoridate or 1 mM metal cations, and in this respect differs from chymotrypsin. 3. The properties of the enzyme strongly resemble those of the 'chymotrypsin-like' enzyme discovered in seminal plasma by Lundquist *et al.* (1955). 4. The use of dimethyl-casein permitted the performance of enzyme assays at substrate concentrations five times higher (up to 50 mg/ml) than could be achieved with ordinary casein (10 mg/ml).

The presence of proteinase (Huggins & Neal, 1942; Lundquist, 1952; Lundquist *et al.*, 1955) and peptidase (Lundquist *et al.*, 1955) activity in human seminal plasma has been known for some time. Elucidation of their function, however, remains incomplete. As reviewed by Mann (1964), one suggested role is their action as components of the fibrinolytic system responsible for the liquefaction of ejaculated semen. Evidence that at least two proteolytic enzymes, a proteinase and a fibrinolysin, may be involved in this process has been reported (Lundquist *et al.*, 1955). More recent studies suggest that these enzymes may also be important in sperm transport. Moghissi & Syner (1970a) examined the effect of a proteinase isolated from human seminal plasma on the migration of spermatozoa through human cervical mucus *in vitro*. In a test system consisting of measured amounts of fresh ejaculate and cervical mucus, addition of purified enzyme to the mucus produced an acceleration in penetration of spermatozoa through the cervical secretion. Further, concomitant changes in the rheological properties of the mucus after the addition of proteinase suggested that some degree of hydrolysis of the mucin had occurred (Moghissi & Syner, 1970b).

In the present paper, a method for the purification of the seminal proteinase that influences spermatozoal migration *in vitro* is described, as are measurements of pH optimum, substrate specificity, molecular size and effects of activators and inhibitors.

Experimental

Materials

CM-Sephadex (CM-50) was obtained from Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A. Chymotrypsin was purchased from Calbiochem, Los Angeles, Calif., U.S.A. Tris, trypsin inhibitors and α -naphthyl acetate were supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. Bio-Gel materials were obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Di-isopropyl phosphofluoridate was supplied by Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Casein, glutarylphenylalanine β -naphthylamide, benzoylarginine β -naphthylamide, benzoylarginine ethyl ester, benzoyltyrosine ethyl ester and amino acid derivatives of β -naphthylamide were purchased from Mann Research Laboratories, New York, N.Y., U.S.A. Methyl-Cellosolve was obtained from Pierce Chemical Co., Rockford, Ill., U.S.A.

Pooled human semen was provided in frozen form from fertility clinics. Fresh ejaculates were obtained from donors.

Analytical methods

Protein. This was determined by the method of Lowry *et al.* (1951).

Proteinase. Assays were performed in 1 ml of reaction mixture containing enzyme and casein (5 mg)

or other protein substrates in 0.05M-tris-HCl, pH7.5. Incubations were carried out at 40°C for 1 h and reactions stopped by addition of 1 ml of cold 10% (w/v) trichloroacetic acid. Enzyme controls were incubated in the absence of casein and treated with casein immediately before deproteinization. Colour development in the trichloroacetic acid filtrates (0.5ml) was accomplished with the Folin phenol reagent (Lowry *et al.*, 1951) and read against a tyrosine standard (0.08 μ mol) at 650nm after 40 min. Proteinase activity was expressed as μ mol of acid-soluble tyrosine released/h under these conditions of assay.

Peptidase and esterase. These were assayed fluorimetrically (Greenberg, 1962) with alanine β -naphthylamide and α -naphthyl acetate respectively as substrates. The final concentration of substrate in the reaction mixture was 0.2mM, in a volume of 3.5 ml. Linear reaction rates were followed at 30°C with a fluorimeter coupled to a linear-log recorder.

Purification methods

All preparative procedures were carried out at 0–5°C unless otherwise indicated.

Pooled human semen (100ml) was centrifuged at 600g for 10 min to sediment spermatozoa. After an additional centrifugation at 20000g for 20 min the clarified plasma was dialysed against 10 litres of water. This procedure accomplished the removal of 15–20% of the Lowry-reactive material without a measurable loss in proteolytic activity.

Ammonium sulphate fractionation. To 50ml of dialysed seminal plasma was added 243mg of $(\text{NH}_4)_2\text{SO}_4$ /ml to give 40% saturation. After 1 h the proteins that precipitated were removed by centrifugation for 10 min at 20000g. The supernatant was adjusted to 50% saturation by addition of 97mg of $(\text{NH}_4)_2\text{SO}_4$ /ml of this solution.

Additional fractions were obtained by increasing the $(\text{NH}_4)_2\text{SO}_4$ saturation by 10% increments up to 80% saturation. The proteins that sedimented by this procedure were dissolved in 10ml of water and dialysed overnight against 6 litres of water. The dialysed fractions were centrifuged at 20000g for 10 min to remove insoluble material and the supernatants assayed for enzyme activity and protein.

Chromatography on Sephadex CM-50. For batch preparation of the proteinase 3g of Sephadex CM-50 was allowed to swell in water and washed according to the manufacturer's directions. After removal of the wash by suction, the slurry was washed twice with 800ml of starting buffer (0.04M-sodium phosphate, pH6.1) to remove 'fines' and allowed to equilibrate in 800ml of this buffer overnight. Ammonium sulphate fraction (10ml), previously dialysed against starting buffer and containing approx. 40mg of protein/ml, was applied to a column bed

(5cm $\times$ 6.5cm) of Sephadex CM-50 and eluted with starting buffer identical in composition with that used for washing and equilibrating the resin.

Gel filtration. Columns of Bio-Gel P-100 or P-200 were used for purification and also to obtain an estimate of molecular size of the proteinase. Preparation and calibration of the columns was performed essentially by the procedure of Leach & O'Shea (1965), except that in the present study polyacrylamide (Bio-Gel) was the separating medium and 0.05M-tris-HCl-0.05M-NaCl, pH7.5, was the developing buffer. After the column had been poured, the flow rate was allowed to equilibrate for a few days until it stabilized at 20ml/h.

Results

Yields

Ammonium sulphate fractionation. Most of the proteinase activity was precipitated between 40 and 70% ammonium sulphate saturation. Below 40%, and above 70%, saturation insignificant amounts of proteinase precipitated.

Chromatography on CM-Sephadex. Peptidase activity passed through the CM-Sephadex without adsorption, but the proteinase remained on the column and was eluted with 0.05M-tris-HCl-0.05M-NaCl buffer, pH7.5 (Fig. 1). Esterolytic activity, which is often associated with proteolytic enzymes, was resolved into two components, the major one being in the proteinase fraction. Experience with several columns of Sephadex CM-50 demonstrated that this esterase peak always coincided with the proteinase peak. Consequently, it was found expedient for routine preparations to localize the proteinase fraction by monitoring the column eluates with α -naphthyl acetate as substrate. However, in later purification stages by gel filtration, this assay procedure could not be used since proteinase (casein-hydrolysing enzyme) became separated from esterase (naphthyl acetate-hydrolysing enzyme) activity. The yields of proteinase for several batch preparations ranged from 35 to 46%, with a 2.5-fold increase in specific activity. The proteinase fraction (CF_2) was dialysed against several changes of water and concentrated by freeze-drying.

Gel filtration. Freeze-dried fraction CF_2 was reconstituted with 2.5ml of 0.05M-tris-HCl-0.05M-NaCl, pH7.5, and applied to a column (2cm $\times$ 50cm) of Bio-Gel P-200. Elution with developing buffer produced two proteinase fractions (Fig. 2). The first was a minor one (G-1) and had a low specific activity. For three columns the yield of this fraction varied from 10% to only 5% of the total casein-hydrolysing activity eluted. Results obtained from a fourth column demonstrated its complete absence. Consequently, further characterization of this minor component

Fig. 1. Chromatography of 10ml of ammonium sulphate fraction (40-70% saturation) on Sephadex CM-50 (5 cm $\times$ 6.5 cm)

A total of 410mg of protein was applied and 10ml fractions were collected. For details see the text. (a) Peptidase; (b) proteinase; (c) esterase; (d) protein. The arrows indicate where buffer was changed from 0.04M-sodium phosphate, pH 6.1, to 0.05M-tris-HCl-0.05M-NaCl, pH 7.5.

was not pursued in the present study. The major proteinase fraction (G-2) had an elution volume of 118 ml and a relative elution volume (elution volume/void volume) of 1.90. The esterase activity in human seminal plasma is polymorphic and showed two peaks of activity, neither of which coincided with the proteinase peak. The separation of proteinase from esterase activity, though not complete, was sufficient to demonstrate that the two activities reside in separate molecules. In this respect, the seminal proteinase differs from chymotrypsin and trypsin, which can utilize a variety of esters as substrate, including α -naphthyl acetate. Fraction G-2 (tubes 57-63) was dialysed against water and freeze-dried for use in the spermatozoa-migration experiments (Moghissi & Syner, 1970a). For characterization work, freeze-dried fraction G-2 was dissolved in 2 ml of water and

Fig. 2. Gel filtration of the proteinase on Bio-Gel P-200

The sample applied consisted of the fraction CF₂ (Fig. 1), dialysed, concentrated by freeze-drying and dissolved in 2.5 ml of buffer. The column was 2 cm $\times$ 50 cm. For details see the text. (a) Protein; (b) proteinase; (c) esterase.

used in the studies outlined below. The yield and specific activity of the proteinase after each purification step are shown in Table 1.

Properties of proteinase fraction G-2

pH optimum. The pH-dependent properties of the proteinase with casein as substrate consisted of a moderately sharp zone of maximum activity between pH 7.5 and 8.0 and no activity below pH 6.0 or above pH 10.0. On this basis the enzyme can be classified as a neutral proteinase.

Substrate saturation and specificity. In the standard assay for proteinase activity the final concentration of casein in the reaction mixture is 5 mg/ml. However, to obtain zero-order reaction conditions with the seminal proteinase much higher casein concentrations were necessary. Because of solubility problems with ordinary heat-denatured casein at concentrations above 10 mg/ml, *NN*-dimethyl-casein, prepared by the method of Lin *et al.* (1969), was used. With this derivative, maximum activity for the seminal protease was obtained at a final substrate concentration

Table 1. *Purification of proteinase from 50 ml of dialysed seminal plasma*

Procedure	For details see the text.				
	Vol. (ml)	Protein (mg/ml)	Total activity ($\mu\text{mol/h}$)	Specific activity ($\mu\text{mol/h per mg of protein}$)	Yield (% of starting material)
Seminal plasma	50	15.0	25.5	0.034	100
$(\text{NH}_4)_2\text{SO}_4$ fraction precipitated between 40 and 70% saturation	10	41.3	20.7	0.050	81
Sephadex CM-50	2.5	15.3	7.90	0.257	31
Bio-Gel P-200 fraction G-2	2.0	2.8	4.90	0.860	19

Fig. 3. *Comparison of the activities of chymotrypsin and fraction G-2 proteinase*

Dimethyl-casein was used as substrate. ▲, Fraction G-2 proteinase (100 $\mu\text{g/ml}$); ●, chymotrypsin (1 $\mu\text{g/ml}$).

of 20 mg/ml (Fig. 3) whereas the corresponding value for chymotrypsin was only 5 mg/ml.

Other substances were also examined as substrates for the seminal proteinase (Table 2). At a concentration of 5 mg/ml human haemoglobin was one-third as active as casein. Other human proteins tested at the same concentration and conditions of assay included γ -globulin, albumin and transferrin, and all were inactive. Several synthetic substrates were examined but none showed appreciable activity with the seminal proteinase. At a concentration of 0.2 mM, benzoylarginine ethyl ester and benzoylarginine naphthylamide (trypsin substrates) and benzoyltyrosine ethyl ester and glutarylphenylalanine naphthylamide (chymotrypsin substrates) showed no activity with dilute enzyme solutions that normally displayed significant activity with casein. By increasing the

amount of enzyme tenfold it was possible to demonstrate activity with benzoyltyrosine ethyl ester but not with benzoylarginine ethyl ester or the amide substrates (glutarylphenylalanine naphthylamide and benzoylarginine naphthylamide). Peptidase substrates tested at a concentration of 0.2 mM included the β -naphthylamides of alanine, leucine, phenylalanine, tyrosine and methionine, and all were inactive.

Effect of cations. Before the effect of metal cations was tested, the seminal enzyme was dialysed sequentially against 0.01 M-EDTA and water. For the assay, the cations Ca^{2+} , Fe^{3+} , Mg^{2+} and Mn^{2+} were incubated with the enzyme for 30 min before addition of casein. At a final concentration of 1 mM the cations had no effect on proteinase activity.

Inhibitors. Di-isopropyl phosphofluoridate and ϵ -aminohexanoic acid (plasmin inhibitor) at concentrations up to 1 mM did not inhibit the proteinase. The soya-bean trypsin inhibitors, types II-0 and IS (Sigma Chemical Co., St. Louis, Mo., U.S.A.), were also without effect at a final concentration of 1 mg/ml.

Molecular size. A column (2 cm $\times$ 92 cm) of Bio-Gel P-100 was prepared in 0.05 M-tris-HCl, pH 7.5, and calibrated with known proteins (Fig. 4). The elution volume for the seminal proteinase was 154 ml and corresponded to a molecular weight of 33000.

Discussion

The properties of the seminal proteinase described in this paper are very similar to those of the 'chymotrypsin-like' enzyme originally demonstrated in human seminal plasma by Lundquist *et al.* (1955). The activity of both enzymes is unaffected by di-isopropyl phosphofluoridate or metal cations and the pH for maximum activity is the same (7.5–8.0). The two proteinases also hydrolyse haemoglobin and tyrosine esters in addition to casein. In view of these similarities, it is suggested that the seminal proteinase is identical with the 'chymotrypsin-like' enzyme discovered by Lundquist *et al.* (1955).

The use of dimethyl-casein proved to be advantageous since it allowed determinations of proteinase

Table 2. Activity of seminal proteinase towards various substrates

The activity of the protein substrates is expressed as described in the text. The amount of enzyme used with the synthetic substrates was ten times that used with the protein substrates. Activity with benzoyltyrosine ethyl ester was determined by the method of Hummel (1959). Activity with benzoylarginine ethyl ester was determined by the procedure of Bergmeyer (1965). For the types of amino acid naphthylamides used, see the text.

Substrate	Final substrate concn.	Activity
Casein	5 mg/ml	0.045 μ mol/h
Haemoglobin	5 mg/ml	0.017 μ mol/h
Albumin	5 mg/ml	0
Transferrin	5 mg/ml	0
γ -Globulin	5 mg/ml	0
Benzoyltyrosine ethyl ester	0.2 mM	0.01 μ mol/min
Benzoylarginine ethyl ester	0.2 mM	0
α -Naphthylamides	0.2 mM	0

Fig. 4. Molecular weight of seminal proteinase

A calibration curve of known proteins on a column (2 cm $\times$ 92 cm) of Bio-Gel P-100 is shown. in, Insulin, mol.wt. 6000; cc, cytochrome c, mol.wt. 12000; ct, chymotrypsin, mol.wt. 24000; alb, albumin, mol.wt. 66000. The arrow indicates the elution position of the fraction G-2 proteinase. Void volume (V_0) = 105 ml; elution volume (V) = 154 ml for the fraction G-2 proteinase.

activity to be made at substrate concentrations considerably higher (up to 50 mg/ml) than those obtainable with untreated casein (10 mg/ml). Although the lower amount is sufficient to provide conditions of substrate saturation for pancreatic chymotrypsin, a concentration of 20 mg/ml is needed for the seminal enzyme. This disparity in casein requirement, and lack of effect of di-isopropyl phosphorfluoridate and

metal ions on activity, constitute the major points of difference between the seminal enzyme and pancreatic chymotrypsin.

The purification procedure employed in the present study had low efficiency. The yield of product amounted to 19% of the starting material with a 25-fold increase in specific activity. The greatest loss of enzyme activity occurred during the chromatography on Sephadex CM-50, where only 38% of the proteinase applied to the column was recovered. The enzyme is relatively stable and is not seriously affected by freezing but loses all activity at 100°C for 10 min.

Compared with chymotrypsin the seminal proteinase preparation demonstrated low activity towards casein (Fig. 3): 96.0 μ mol/h per mg of protein and 0.86 μ mol/h per mg of protein respectively. This low activity is due, partly at least, to the impurity of the seminal proteinase. When analysed by polyacrylamide electrophoresis at pH 8.6 (Davis, 1964) the fraction G-2 demonstrates three prominent, anodic bands, which migrate close together and have different staining intensities with Amido Schwarz.

It is possible that the function of the seminal protease is concerned with thinning of the cervical mucus as well as the digestion of seminal-plasma proteins. Proteolytic enzymes, such as trypsin and chymotrypsin, have been found to hydrolyse human as well as bovine cervical mucus and to produce physical and chemical changes (Neuhaus & Moghissi, 1962; Syner & Moghissi, 1971). The highly viscous property of the mucus is due mainly to a mucin consisting of 25% protein and 75% carbohydrate with a molecular weight of 4×10^6 (Gibbons, 1959). Presumably, the accelerated migration of spermatozoa *in vitro* through cervical mucus treated with seminal proteinase was due to the enzyme's hydrolytic action on the mucin. Spermatozoa also contain chymotrypsin-like activity, which is probably derived from the seminal

plasma and becomes adsorbed to the spermatozoa after ejaculation (Stambaugh & Buckley, 1970). It is possible that the seminal chymotrypsin-like enzyme produces some hydrolytic action on cervical mucus *in vivo* in association with the spermatozoal cell.

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