Isolation, characterization and possible mode of action of antiseminalplasmin, a new protein that inhibits the antimicrobial activity of seminalplasmin

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The isolation from bovine seminal plasma and purification of a new protein called 'antiseminalplasmin', which reverses the inhibition of the growth of, and RNA synthesis in, *Escherichia coli* by seminalplasmin (another protein of bovine seminal plasma), is described. Antiseminalplasmin, a weakly acidic protein, has a minimum M_r of about 39000 and appears to consist of three acidic peptide chains that move close to each other on electrophoresis on cellulose acetate strips or on sodium dodecyl sulphate/18%-(w/v)-polyacrylamide gels. Antiseminalplasmin has a tendency to oligomerize at slightly alkaline pH values; it does not bind to seminalplasmin or to DNA, and does not reverse the inhibition by seminalplasmin of transcription *in vitro* by purified *E. coli* RNA polymerase. It appears that antiseminalplasmin may act by binding to the cell surface and preventing the entry of seminalplasmin into the cells. By itself, antiseminalplasmin has no effect on the growth of *E. coli*.

Bovine seminal plasma has been shown to contain an antimicrobial protein, seminalplasmin, which acts, among other micro-organisms, on *Escherichia coli* (Reddy & Bhargava, 1979; Bhargava, 1981; Rao *et al.*, 1983; Theil & Scheit, 1983*a,b*). Here we describe the isolation, purification and some properties of another protein from bovine seminal plasma, in the presence of which seminalplasmin can no longer inhibit the growth of *E. coli*, and which we have named 'antiseminalplasmin'.

Previous work (Reddy & Bhargava, 1979; Scheit *et al.*, 1979) suggested that seminalplasmin kills *E. coli* by entering the cell and specifically inhibiting the synthesis of rRNA. Antiseminalplasmin reverses this inhibition; it appears to act by preventing the entry of seminalplasmin into the cell.

Materials and methods

Materials

Bovine semen was obtained and seminal plasma prepared from it as described previously (Walton, 1945; Reddy & Bhargava, 1979; Reddy *et al.*, 1979). Cellulose acetate strips (size 36cm × 5cm)

for electrophoresis were obtained from Oxoid Division of Oxo Ltd., London E.C.4., U.K. ³H-labelled *E. coli* RNA was isolated as described by Reddy *et al.* (1979); it was denatured by heating at 60°C for 5 min in 0.05M-Tris/HCl buffer, pH 7.6, containing 0.02M-MgCl₂ and 0.35M-KCl, and then chilled rapidly in a solid-CO₂/acetone bath. Seminalplasmin was prepared as described previously (Reddy & Bhargava, 1979).

Isolation of antiseminalplasmin

Seminal plasma (100–150 ml) was dialysed at 4°C against two 4-litre changes each of 6mM-Tris/HCl buffer, pH 7.4, for 18 h, and chromatographed on a column (6cm × 70cm) of DEAE-Sephadex A-25, prepared in, and equilibrated with, the above buffer. Fractions (10 ml) were collected at a flow rate of approx. 1 ml/min. The adsorbed protein was eluted by a gradient (1000 ml) of 0–2M-NaCl in the above-mentioned buffer; two major peaks were obtained [Fig. 1*a(i)*]. The pooled fractions comprising the first peak (DP1) were dialysed for 48 h at 4°C against distilled water and freeze-dried. DP1 (6 g) was dissolved in 400 ml of 0.05M-Tris/HCl buffer, pH 8.3, containing 0.05M-KCl and 0.1 mM-EDTA, and further fractionated on a column (6cm × 70cm) of phosphocellulose P-11 prepared in, and equilibrated with, the above-mentioned buffer. The fractions representing non-adsorbed protein material [peak 1 in Fig. 1*a(ii)*]

Abbreviations used: RNAase, ribonuclease; SDS, sodium dodecyl sulphate.

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were pooled, dialysed overnight at 4°C against distilled water and freeze-dried. About 400 mg of the freeze-dried non-adsorbed protein material from the phosphocellulose column was dissolved in 0.06M-Tris/HCl buffer, pH 7.4, containing 0.5M-NaCl and chromatographed on a column (2.5cm × 150cm) of Bio-Gel P-100 equilibrated with the above buffer, at a flow rate of 40ml/h. Three peaks were obtained [Fig. 1a(iii)]. The second peak (peak 2) had the antiseminalplasmin activity; it was dialysed overnight at 4°C against distilled water and freeze-dried.

The freeze-dried protein fraction (50mg) from Bio-Gel P-100 was dissolved in 5ml of 0.02M-acetate buffer, pH 5.2, and chromatographed on an RNAase affinity column (5ml bed volume) of agarose-5'-(*p*-aminophenylphospho)uridine 2'(3')-phosphate equilibrated with 0.02M-acetate buffer, pH 5.2. The adsorbed protein was eluted with dilute acetic acid (Wilchek & Gorecki, 1969) to allow re-use of the column, and the pooled non-adsorbed fraction, representing over 95% of the protein loaded on to the column and containing the antiseminalplasmin activity, was dialysed against distilled water and freeze-dried. This preparation, called 'antiseminalplasmin preparation 1', was assayed for proteolytic activity with seminalplasmin as the substrate, as described below. If it was found to be free of proteolytic activity, it was used as such; if not, a portion (40mg) was dissolved in 6ml of 0.1M-acetate buffer, pH 3.5, containing 1M-NaCl, and chromatographed [Fig. 1a(iv)] on a haemoglobin-agarose column (5ml bed volume) equilibrated with 0.1M-acetate buffer, pH 5, containing 1M-NaCl. The non-adsorbed protein fractions (representing over 75% of the protein loaded on to the column) were pooled and the adsorbed protein was eluted with 0.1M-Tris/HCl buffer, pH 8.6, containing 1M-NaCl, to allow re-use of the column. The non-adsorbed fraction was dialysed against distilled water and freeze-dried. This protein fraction is referred to as 'antiseminalplasmin preparation 2'. Pure antiseminalplasmin as well as preparations containing this protein at various stages of purification (Fig. 1) was stable for more than a year at -20°C.

Electrophoresis, ultracentrifugation, isoelectric focusing and chromatography

Non-SDS/polyacrylamide-gel electrophoresis was carried out on cylindrical gels in two ways. In method 1, 10%-(w/v)-polyacrylamide gels were used and the run carried out in 0.05M-Tris/glycine buffer, pH 8.4, at 5mA/gel for 3h. In method 2, 15% polyacrylamide gels were used and the run carried out in a β -alanine/acetic acid buffer, pH 4.5, in the cold at 6–8mA/tube for 3–4h (Reisfield *et al.*, 1962); in both the methods the gels were fixed in 12.5% (w/v) trichloroacetic acid for 15 min and stained with Coomassie Blue R-250 (McKnight, 1977). SDS/polyacrylamide-gel electrophoresis on 10% polyacrylamide cylindrical gels was carried out in the absence or in the presence of 2-mercaptoethanol as stated previously (Reddy & Bhargava, 1979). SDS/polyacrylamide-gel electrophoresis on 18%-polyacrylamide slab gels was performed as described by Laemmli (1970).

Electrophoresis on cellulose acetate strips was performed in 0.5M-borate buffer, pH 8.3, containing 6M-urea, 10mM-2-mercaptoethanol and 1mM-EDTA, for 2h at 800V per strip, with 100 μ g of the protein; the strips were stained with 0.1% Amido Black (Schaffner & Weissman, 1973).

Sedimentation-velocity runs were made in 6mM-Tris/HCl buffer, pH 7.4, at 59780rev./min in a Beckman analytical ultracentrifuge at 28°C, with a protein solution of $A_{1\%}^{1\%} = 5$ or 10, in 6mM-Tris/HCl buffer, pH 7.4, or in 6mM-Tris/HCl buffer, pH 7.4, containing 1% SDS; the run time was 85min in the former and 90min in the latter case. Archibald and synthetic-boundary runs were made in the above Tris/HCl buffer at 8000rev./min, a protein solution of $A_{1\%}^{1\%} = 10$ being used.

Isoelectric focusing on polyacrylamide gels (0.4cm × 10cm) in the presence of urea was done as described by O'Farrell (1975), with 100 μ g of the protein and a pH 3.5–10 gradient.

Isoelectric-focusing runs on a column (LKB; 110ml) were performed as described previously (Mandel & Marmur, 1968), by using a pH 3.5–10 gradient with the pH 5–8 range expanded.

Fig. 1. Isolation and purification of antiseminalplasmin

(a) (i) Chromatography of 100–150ml of dialysed bovine seminal plasma on DEAE-Sephadex A-25; (ii) chromatography of 6000 $A_{1\%}^{1\%}$ units of the first peak [DP1; within the broken lines in (i)] from the DEAE-Sephadex column, on phosphocellulose (the unadsorbed fraction was pooled and is not shown; the gradient was applied at the origin on the x-axis); (iii) chromatography of 400 $A_{1\%}^{1\%}$ units of the non-adsorbed peak (peak 1) from the phosphocellulose column on Bio-Gel P-100; (iv) chromatography, on haemoglobin-agarose, of 50 $A_{1\%}^{1\%}$ units of the fraction of peak 2 from the Bio-Gel column that was non-adsorbed on a RNAase affinity column. (b) Polyacrylamide-gel electrophoresis of antiseminalplasmin preparation 2: (i), 100 μ g on a 10% gel, with Tris/glycine buffer, pH 8.3, in the absence of SDS; (ii) 100 μ g on a 15% gel, with β -alanine/acetic acid buffer, pH 4.8, in the absence of SDS; (iii), 100 μ g on 10% cylindrical gels in the presence of SDS with mercaptoethanol (3) and without mercaptoethanol (4), with cytochrome *c* (two different concentrations) as marker (1 and 2). (c) Purification scheme.

The h.p.l.c. run was made on a Kontron (Zurich, Switzerland) instrument with a Nucleosil C-18 column and a 0–80% gradient of solvent B [acetonitrile/0.1 M-sodium phosphate, pH 2.1 (4:1, v/v)] in solvent A (0.1 M-sodium phosphate, pH 2.1); the gradient time was 90 min.

For chromatography on concanavalin A-Sepharose, antiserinalplasmin (50 µg) was run on a column (30 ml bed volume) equilibrated with 0.05 M-Tris/HCl buffer, pH 7.3, containing 0.05 M-NaCl, 1 mM-MnCl₂ and 1 mM-CaCl₂, at a flow rate of 60 drops/10 min at 4°C. The adsorbed protein was eluted with 0.1 M-sodium borate buffer, pH 10.

Amino acid analyses

The amino acid analyses were performed in the usual way on a Beckman automatic and computerized amino acid analyser, after oxidation of the protein with performic acid (Hirs, 1956) and hydrolysis with 6 M-HCl for 20 h under vacuum at 110°C; it was shown that the protein was completely hydrolysed during this period. Tryptophan was not determined.

Assay of antiserinalplasmin

Antiserinalplasmin activity during purification was monitored by measuring the relief of inhibition of growth of *E. coli* by seminalplasmin (Reddy & Bhargava, 1979). A 6 ml portion of an exponentially growing culture of *E. coli* W160-37 ($A_{580}^{1\%} = 0.01$, equivalent to approx. 1×10^7 cells/ml) was incubated with the stated concentration of seminalplasmin and the antiserinalplasmin preparation to be tested. After 6 h, the $A_{580}^{1\%}$ of the culture was measured. The $A_{580}^{1\%}$ (~0.5–0.7) of the culture to which no seminalplasmin was added was taken as 100 for the purpose of calculation of the percentage inhibition; samples to which either only seminalplasmin or only antiserinalplasmin was added were run as controls. Antiserinalplasmin never showed any inhibition of the growth of *E. coli*. Trypsin (final concn. 2 µg/ml) was added at 37°C in 0.01 ml of 0.1 M-phosphate buffer, pH 7.6, to the culture when the extracellular seminalplasmin or antiserinalplasmin, as the case may be, in the incubation medium, was intended to be destroyed; trypsin by itself had no effect on the growth of *E. coli*. All additions were carried out at 37°C. In one set of experiments designed to study the nature of association of antiserinalplasmin with the cells, extracellular antiserinalplasmin was removed by decantation after pelleting of the cells by centrifugation after incubation of the culture with antiserinalplasmin (or seminalplasmin) for 60 min at 37°C; the cells were then resuspended in the original volume of fresh medium containing antiserinalplasmin or seminalplasmin as required. For heat-denaturation studies, anti-

seminalplasmin in 6 mM-Tris/HCl buffer, pH 7.4, was heated for 10 min at 90°C in a boiling-water bath; the protein was not precipitated by this treatment.

Effect of antiserinalplasmin on the inhibition of RNA synthesis by seminalplasmin in pulse-labelling experiments

A 6 ml portion of an exponentially growing culture of *E. coli* ($A_{580}^{1\%} = 0.2$ –0.3) was incubated with seminalplasmin (150 µg/ml). To another 6 ml portion of the same culture incubated with seminalplasmin as described above, antiserinalplasmin (300 µg/ml) was added at the specified time. At the desired time after incubation with seminalplasmin or with seminalplasmin and antiserinalplasmin, a 0.5 ml portion of the culture was pulse-labelled for 20 s with [³H]uridine (0.32 µg, 11.8×10^6 c.p.m.) added in 0.2 ml of the growth medium, and the RNA synthesis measured as described previously (Reddy & Bhargava, 1979). Antiserinalplasmin by itself had no effect on the pulse-labelling of RNA.

Effect of antiserinalplasmin on the inhibition of RNA synthesis by seminalplasmin in continuous labelling experiments

Portions (11 ml) of an exponentially growing culture of *E. coli* ($A_{580}^{1\%} = 0.3$) were incubated for various periods at 37°C in the presence of [³H]uridine [7.83 µg (3.5×10^6 c.p.m.)/ml], with or without seminalplasmin (150 µg/ml), or with seminalplasmin (150 µg/ml) and antiserinalplasmin (300 µg/ml). At each time point, a 0.5 ml portion from each of the incubated cultures was precipitated with 5 ml of ice-cold 5% (w/v) trichloroacetic acid (Reddy & Bhargava, 1979). The radioactivity (x) in the precipitate was taken as a measure of the labelling of the total nucleic acid (RNA and DNA). Another 0.5 ml portion from the culture was treated with an equal volume of 1 M-KOH for 18 h at 37°C, neutralized and then made 5% with respect to trichloroacetic acid; the radioactivity (y) in this precipitate gave the extent of labelling of DNA. $x - y$ was taken as a measure of the labelling of RNA.

Binding of antiserinalplasmin and DNA

Antiserinalplasmin (200 µg) and seminalplasmin (80 µg) were incubated at 37°C for 30 min in 6 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl. The mixture was then electrophoresed on a non-SDS-containing gel by method 2 (described above). Antiserinalplasmin and seminalplasmin were run separately as controls.

[³H]Poly(dA·dT) (8750 c.p.m.; 2.4 nmol of dA·dT) diluted with unlabelled poly(dA·dT) (0.5 $A_{260}^{1\%}$ unit) was incubated in 1 ml of Tris/HCl buffer, pH 7.9, containing 0.05 M-NaCl and 10 mM-

MgCl₂, with 80 µg of antiseminalplasmin for 30 min at 0°C, and filtered through a nitrocellulose Millipore membrane filter (0.45 µm pore size) that had previously been soaked for 1 h in the above buffer. The filter was washed slowly with 5 ml of the ice-cold buffer, dried and counted for radioactivity. A control without antiseminalplasmin was run concurrently.

Determination of protein and sugar

The absorption coefficient of antiseminalplasmin was determined with bovine serum albumin as a standard, and using Lowry's method of protein determination. Routinely, an $A_{280}^{1\%}$ of 1 was taken to be equivalent to 1 mg of protein/ml in the case of antiseminalplasmin. The total reducing sugars in antiseminalplasmin were determined by the phenol/H₂SO₄ method (Dubois *et al.*, 1956), with galactose as the standard.

Measurement of radioactivity

¹⁴C- and ³H-containing samples on Whatman paper filters were counted for radioactivity in a toluene-based scintillation mixture; to the ³H-containing filters, 100 µl of water was first added, followed by 1 ml of NCS tissue solubilizer (The Radiochemical Centre, Amersham, Bucks., U.K.) for 1 h, before the addition of the scintillation mixture. Aqueous samples containing ¹⁴C or ³H were counted for radioactivity in Bray's scintillation mixture.

Measurement of RNAase activity

The nuclease activity was determined with native and heat-denatured ³H-labelled *E. coli* RNA (1.2 $A_{260}^{1\%}$; 1×10^5 c.p.m.) or [¹⁴C]poly(rU) (5×10^4 c.p.m.) as the substrate in 0.1 ml (final volume) as described previously (Reddy *et al.*, 1979).

Measurement of DNAase activity

The hydrolysis of [³H]poly(dA·dT) (8750 c.p.m.; 2.4 µmol of dA·dT) by the antiseminalplasmin preparation (220 µg/ml) was carried out at 37°C for 10–60 min, in 0.1 ml (final volume) of the same buffer, and the extent of hydrolysis determined as in the case of RNAase assay.

Determination of proteolytic activity on seminalplasmin

The assay mixture contained, in a total volume of 20 µl: 5 µl of seminalplasmin (50 µg), 5 µl of antiseminalplasmin (100 µg) and 10 µl of 6 mM-Tris/HCl buffer, pH 7.4; it was incubated at 37°C for 30 min and the reaction was stopped by the addition of 20 µl of SDS-containing sample incubation buffer (Laemmli, 1970), and heating for 2 min in a boiling-water bath. The extent of proteolysis

was determined by SDS/polyacrylamide-gel electrophoresis on an 18%-polyacrylamide slab gel on which the two proteins were well separated, followed by staining with Coomassie Blue R-250 (McKnight, 1977) and quantitative densitometry.

Effect of antiseminalplasmin on the inhibition by seminalplasmin of transcription by E. coli RNA polymerase

The final reaction mixture contained, in 0.1 ml of 0.04 M-Tris/HCl buffer, pH 7.8 (Smagowicz & Scheit, 1977): [¹⁴C]ATP (1.5×10^5 c.p.m.; 1.5 mM) *E. coli* RNA polymerase holoenzyme (3 µg; sp. activity 16000 units/mg, assayed with phage-T₇ DNA as the template; 95% pure as judged by SDS/polyacrylamide-gel electrophoresis), poly(dT) (0.86 $A_{260}^{1\%}$ unit) and seminalplasmin and antiseminalplasmin at the concentrations stated in Table 4. The reaction mixture was incubated at 37°C for 30 min without [¹⁴C]ATP, and the reaction started by the addition of [¹⁴C]ATP. Aliquots (20 µl) were withdrawn at 10 min, and the incorporation of [¹⁴C]ATP into acid-insoluble material determined as described previously (Smagowicz & Scheit, 1977).

Results and discussion

Isolation and purification of antiseminalplasmin

Antiseminalplasmin was obtained from dialysed bovine seminal plasma by sequential chromatography on columns of DEAE-Sephadex, phosphocellulose P-11, Bio-Gel P-100, a RNAase affinity column and a haemoglobin-agarose affinity column, as shown schematically in Fig. 1(c); typical elution profiles from the first, second, third and fifth columns are given in Figs. 1(ai), 1(aii), 1(aiii) and 1(aiv) respectively. Antiseminalplasmin-containing fractions represented 18–25%, 9–12%, 0.8–1.4% and 0.6–1.4% of the total seminal plasma proteins, at the DEAE-Sephadex, phosphocellulose, Bio-Gel and haemoglobin-agarose stages; bovine seminal plasma contains 30–80 mg of protein/ml. The material eluted from the RNAase affinity column, called 'antiseminalplasmin preparation 1' (Fig. 1) was, in most cases, contaminated, to an extent of up to 30% with two proteinases (one acidic, with a pH optimum of 3.0, which is inactive at pH 7, and the other neutral, with a pH optimum of 7.4). These proteinases, which were recently purified to homogeneity (V. N. Rao & P. M. Bhargava, unpublished work), were removed quantitatively on passage of antiseminalplasmin preparation 1 through a haemoglobin-agarose column; this proteinase-free preparation is called 'antiseminalplasmin preparation 2' (Fig. 1). In several experiments, however, antiseminalplasmin preparation 1 showed no proteinase activity. All experiments re-

ported here were done either with proteinase-free antiserinalplasmin preparation 1 or with antiserinalplasmin preparation 2. The properties of, and the results obtained with, the two preparations, were identical, except that when contamination with the acidic proteinase before the haemoglobin-agarose run was high, antiserinalplasmin preparation 1 contained traces (less than 1%) of hydrolytic products derived from the haemoglobin-agarose column, as a result of action of this proteinase on the column material. Even though the column was run at pH 5, the material was loaded at pH 3.5; haemoglobin is cleaved at pH 3.5 by the acidic proteinase mentioned above. (Neither of the proteinases is active on antiserinalplasmin under the conditions used, though seminalplasmin is one of the best substrates for both the proteinases.) The hydrolytic products mentioned above could be revealed by polyacrylamide-gel electrophoresis on 18%-polyacrylamide slab gels. That they were derived from the column material was shown by polyacrylamide-gel electrophoresis of the proteinase by itself, after passage through the haemoglobin-agarose column.

Antiserinalplasmin gave a single band on non-SDS/polyacrylamide-gel electrophoresis at pH 4.8; on SDS/polyacrylamide-gel electrophoresis on 15%-polyacrylamide gels in the absence (apparent M_r 38 500) or in the presence (apparent M_r 14 600) of mercaptoethanol (Fig. 1b) and on isoelectric-focusing gels (Fig. 2). It gave a single symmetrical peak in sedimentation runs in the analytical ultracentrifuge, in the absence (4.4S) or in the presence (1.1S) of SDS. The M_r obtained by Archibald and synthetic-boundary runs was about 36 000. It also appeared homogeneous in h.p.l.c. runs. Antiserinalplasmin, however, gave three close, but clearly separated, bands on electrophoresis on cellulose acetate in the presence of urea and mercaptoethanol (Fig. 3), and two (sometimes three) bands on electrophoresis on 10%-polyacryl-

amide slab gels in the presence of SDS and mercaptoethanol (Fig. 4; the bands above the two main bands representing the subunits of antiserinalplasmin were shown to be derived from the haemoglobin-agarose column as mentioned above). These observations indicate that antiserinalplasmin probably consists of three non-identical peptide chains, each of M_r between 12 000 and 15 000.

Antiserinalplasmin, on SDS/polyacrylamide-gel electrophoresis at pH 8.3, gave one major and a series of minor bands that corresponded to integral multiples of the major band in M_r [Fig. 1b(i)], in which two of these bands can be seen; at closer to neutral pH, up to ten sharp bands could be observed. These observations suggest pH-dependent oligomerization of the protein.

The amino acid composition of antiserinalplasmin (preparation 2) was (in mol/100 mol of total amino acids): Lys_{5.4}His_{2.2}Arg_{3.7}Asx_{12.6}Thr_{7.3}Ser_{7.1}Glx_{10.7}Pro_{8.5}Gly_{6.8}Ala_{5.1}Cys_{2.5}Val_{4.0}Met_{2.0}Ile_{4.8}Leu_{8.0}Tyr_{5.1}Phe_{4.2}. Isoelectric-focusing runs on a column gave a pI between 6 and 7. Antiserinalplasmin was shown to be a glycoprotein containing 12% galactose equivalents of carbohydrate moieties. It bound strongly to concanavalin A-Sepharose and could be eluted, only in an inactive state, by 0.1M-borate buffer, pH 10. It had no deoxyribonuclease activity, and its absorption coefficient (1% solution in 6mM-Tris/HCl buffer, pH 7.4) at 280nm was 9.8.

Nucleolytic activity of antiserinalplasmin

All antiserinalplasmin preparations (1 or 2) isolated so far have contained traces of a ribonucleo-

Fig. 2. Isoelectric focusing of antiserinalplasmin preparation 2 on a polyacrylamide gel

Fig. 3. Electrophoresis of antiserinalplasmin preparation 1 on cellulose acetate strips

Fig. 4. SDS/polyacrylamide-gel electrophoresis of antiserinalplasmin preparation 2 on 18% polyacrylamide gels

lytic activity specific for pyrimidines, even after repeated passage through the RNAase affinity column (Table 1). This activity was always less than 1% of the activity of RNAase A (EC 3.1.27.5) on a weight-by-weight basis; the specific activity appeared to be constant from preparation to preparation. The band(s) corresponding to antiseminalplasmin obtained on non-SDS/polyacrylamide-gel electrophoresis also showed RNAase activity; the known RNAases of bovine seminal plasma are basic and would move in the other direction (that is, away from the gel) under the conditions used for polyacrylamide-gel electrophoresis. These observations suggest that the low level of RNAase activity observed in the purified antiseminalplasmin preparations may be an integral property of this protein, although, of course, the possibility that it represents a contaminant that we were not able to remove cannot be ruled out.

Antiseminalplasmin did not show any DNAase activity towards poly(dA·dT).

Biological activity of antiseminalplasmin and its mechanism of action

Seminalplasmin strongly inhibits the growth of *E. coli* and transcription *in vitro* by *E. coli* RNA polymerase (EC 2.7.7.6) (Reddy & Bhargava, 1979; Scheit *et al.*, 1979). When antiseminalplasmin was added to a culture of *E. coli* along with seminalplasmin, no inhibition of the growth of *E. coli* was observed. Table 2 gives the results of representative experiments demonstrating the above activity of antiseminalplasmin at various stages of purification. This activity of antiseminalplasmin was lost completely on heating at 90°C for 10 min.

For 50–100% reversal of the inhibition of growth of *E. coli* by seminalplasmin the amount of antiseminalplasmin required, on a w/w basis, was two

Table 1. Ribonuclease activity, on native and heat-denatured ³H-labelled *E. coli* RNA and on [¹⁴C]poly(rU), of antiseminalplasmin preparation 2

The untreated sample had 18620, 18600 and 7625 c.p.m. in the case of native RNA, heat-denatured RNA and poly(rU) respectively, in the acid-insoluble fraction; the percentages given are of these values.

Protein preparation		Radioactivity rendered acid-soluble (%)		
Description	Concn. (µg/ml)	Poly(rU)	Native RNA	Heat-denatured RNA
Antiseminalplasmin	200	–	10	47
preparation 2	120	10	–	–
Ribonuclease A	20	–	79	100
	1	38	–	–

Table 2. Reversal, by antiseminalplasmin at various stages of its purification, of the inhibition of growth of *E. coli* by seminalplasmin

Seminalplasmin, when used alone at each of the concentrations mentioned, gave virtually 100% inhibition of growth. The absorbance of the control culture (without seminalplasmin or antiseminalplasmin) was between 0.50 and 0.64.

Antiseminalplasmin preparation	[Seminalplasmin] (µg/ml)	[Antiseminalplasmin] (µg/ml)	Seminalplasmin/antiseminalplasmin ratio (w/w)	Inhibition of growth of <i>E. coli</i> (%)
DEAE-Sephadex fraction DP1	25	800	1:32	50
Phosphocellulose peak 1	25	250	1:8	50
Antiseminalplasmin preparation 1 (from Bio-Gel)	25	50	1:2	50
Antiseminalplasmin preparation 2 (from haemoglobin-agarose)				
Batch 1	25	50	1:2	50
Batch 2	25	80	1:3.8	0
Batch 3	25	125	1:5	0
Batch 4	25	150	1:6	0
Batch 5	37	154	1:4.2	0
Batch 6	40	90	1:2.3	0

to five times the amount of seminalplasmin used. Since the above antiseminalplasmin/seminalplasmin ratio suggested the requirement for a molar ratio of the two proteins for the activity of antiseminalplasmin, it seemed possible that antiseminalplasmin acts by binding to seminalplasmin. However, non-SDS/polyacrylamide-gel electrophoresis (Fig. 5) of an incubated mixture of the two proteins did not suggest very much binding. Polyacrylamide-gel electrophoresis was performed at a pH of 4.8, where seminalplasmin moves close to the dye front but antiseminalplasmin hardly moves (in the experiment of Figs. 5b and 5c, it had not entered the gel). In the event of formation of a stable complex between the two proteins, as was previously observed between seminalplasmin and RNA polymerase (Scheit *et al.*, 1979) or avian-myeloblastosis-virus reverse transcriptase (EC 2.7.7.7) (Reddy *et al.*, 1983), one would expect a retardation of the movement of seminalplasmin on the gel; no such retardation was observed. Antiseminalplasmin did not exhibit any proteolytic activity towards seminalplasmin; the two proteins were quantitatively separated from an incubated mixture by SDS/polyacrylamide-gel electrophoresis on a gradient gel. Other commonly used substrates of proteolytic enzymes, too, were not hydrolysed by antiseminalplasmin.

Seminalplasmin has been shown to act by inhibiting, in whole *E. coli* cells, the synthesis of

rRNA. Antiseminalplasmin, when added along with seminalplasmin, reversed the inhibition of RNA synthesis in *E. coli* in a continuous-labelling experiment (Fig. 6). In pulse-labelling experiments, antiseminalplasmin, when added 12.5 min after seminalplasmin, at which time the inhibition of the labelling of RNA by seminalplasmin had reached a plateau (Reddy & Bhargava, 1979), completely reversed the inhibition (Fig. 7a). However, when antiseminalplasmin was added 30 min after the addition of seminalplasmin, no such reversal was observed (Fig. 7b).

Addition of antiseminalplasmin up to 20 min (or trypsin up to 10 min; see Reddy & Bhargava, 1979) after the addition of seminalplasmin still resulted in reversal of the inhibition of the growth of *E. coli*, whereas addition of antiseminalplasmin (or trypsin) at 30 min (or later) after the addition of seminalplasmin had no effect on growth inhibition by seminalplasmin (Table 3). Thus the reversal by antiseminalplasmin of the inhibition of growth and of RNA synthesis in *E. coli* by seminalplasmin seemed to go hand in hand.

Fig. 5. Binding of antiseminalplasmin to seminalplasmin as revealed by non-SDS/polyacrylamide-gel electrophoresis. Seminalplasmin, antiseminalplasmin preparation 2, or a mixture of the two proteins, was incubated and electrophoresed. (a) antiseminalplasmin (200 µg); (b) Seminalplasmin (80 µg); (c) seminalplasmin (80 µg) plus antiseminalplasmin (200 µg).

Fig. 6. Effect of antiseminalplasmin on inhibition of RNA synthesis in whole *E. coli* cells by seminalplasmin. Three separate portions of an exponentially growing culture of *E. coli* were incubated at 37°C, one without seminalplasmin (●), one with seminalplasmin (■, 150 µg/ml), and one with seminalplasmin (150 µg/ml) and antiseminalplasmin preparation 2 (300 µg/ml) together (▲). [^3H]Uridine was added at zero time to all the three cultures and its incorporation into RNA measured in samples taken at the stated times.

Fig. 7. Effect of antiseminalplasmin, added at 12.5 min or 30 min after the addition of seminalplasmin, on the pulse-labelling of RNA in an exponentially growing culture of *E. coli*

Seminalplasmin (150 $\mu\text{g}/\text{ml}$) was added to two portions of the culture of *E. coli* at zero time. At various times, sub-portions from one of the portions were pulse-labelled (for 20 s) with $[^3\text{H}]$ uridine and the radioactivity incorporated into RNA determined. To the second portion, antiseminalplasmin preparation 2 (300 $\mu\text{g}/\text{ml}$) was added at 12.5 min (a) or 30 min (b) after the addition of seminalplasmin, and sub-portions pulse-labelled as above; arrows indicate the time of addition of antiseminalplasmin. The control, run in triplicate, received neither seminalplasmin nor antiseminalplasmin, but was pulse-labelled as described above. Δ , Control (without seminalplasmin or antiseminalplasmin); $\blacksquare$, seminalplasmin; $\bullet$, seminalplasmin + anti-seminalplasmin.

It has previously been reported (Reddy & Bhargava, 1979) that, for seminalplasmin to irreversibly inhibit the growth of *E. coli*, the cells need to be incubated with the protein for about 30 min. It has also been shown (N. S. Rao, K. S. N. Prasad & P. M. Bhargava, unpublished work) that seminalplasmin, at the concentrations used in the present study, although preventing the growth of the culture if left in contact with it for 30 min, killed the cells only after several hours, the exact period de-

pending on the initial concentrations of the cells and of seminalplasmin. The observations reported here would therefore suggest that once seminalplasmin had inhibited the growth of the culture, antiseminalplasmin could not reverse this inhibition.

Seminalplasmin acts on several organisms (Gram-positive and Gram-negative bacteria, as well as yeasts, such as *E. coli*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Klebsiella*, *Streptococcus faecalis*, *Salmonella typhimurium*, staphylococci, *Candida albicans* and *Cryptococcus neoformans*), by inhibiting the synthesis of RNA, preferentially of rRNA (Reddy & Bhargava, 1979; K. H. Scheit, M. Zimmer, N. S. Rao, K. S. N. Prasad & P. M. Bhargava, unpublished work). In at least *E. coli*, it does not have any effect on protein or DNA synthesis in the whole cell and is therefore unlikely to act by binding to the cell membrane and preventing the entry of precursors and nutrient. Further, if seminalplasmin were to act at the cell membrane, it would probably do so by binding to a receptor. Such receptors (e.g. for the colicins) are known to be species-specific; seminalplasmin, on the other hand, acts on a variety of microbial species. It is therefore likely that seminalplasmin acts by entering the cell, and that once enough seminalplasmin has entered the cells to inhibit the growth of the culture irreversibly, a process that perhaps takes about 30 min under the conditions used here (Reddy & Bhargava, 1979; see also the results with trypsin in Expt. 2 of Table 3), antiseminalplasmin is unable to reverse this inhibition. This suggests that antiseminalplasmin may act by binding, strongly and irreversibly, to the cell membrane and preventing the entry of seminalplasmin. This view is supported by the results of an experiment in which a culture of *E. coli* was incubated for 60 min with antiseminalplasmin and the protein was removed by centrifugation and decantation; the sedimented cells freed of the extracellular antiseminalplasmin were no longer susceptible to seminalplasmin (Table 3, Expt. 3).

Antiseminalplasmin did not reverse the inhibition of transcription of poly(dT) by *E. coli* RNA polymerase in an 'in vitro' system (Table 4). Further, antiseminalplasmin did not show any measurable binding to DNA. Therefore antiseminalplasmin is unlikely to act by preventing the inhibition of transcription by seminalplasmin within the cell, by binding either to the RNA polymerase or to DNA. In fact, if antiseminalplasmin were to act by penetrating the cell, the ribonuclease activity associated with the antiseminalplasmin preparation used should prove toxic to the cell by destroying the transcripts, as was found to be the case *in vitro* for all pyrimidine-containing transcripts. Antiseminalplasmin (preparation 1 or 2)

Table 3. *Effect of antiseminalplasmin preparation 2, added at different times before or after the addition of seminalplasmin, on the inhibition of growth of E. coli by seminalplasmin*

The growth-inhibition assays were carried out as described under 'Assay of antiseminalplasmin'. The $A_{550}^{1\%}$ of the culture at zero time was 0.01 (10^7 cells/ml). The growth in the control (without seminalplasmin, antiseminalplasmin or trypsin) after 6 h corresponded to an $A_{550}^{1\%}$ of 0.63 in Expt. 1, 0.53 in Expt. 2, and 0.65 in Expt. 3. Trypsin, where used, was added at the stated time after the addition of seminalplasmin to destroy the latter. In Expt. 3, the culture, after incubation for 60 min with the zero-time additions mentioned in the Table, was centrifuged and the sedimented cells resuspended in fresh medium containing no seminalplasmin or antiseminalplasmin; all the 60 min additions were done to this resuspended culture, which was then further incubated for 6 h.

Expt. no.	Time (in min) of the addition of :			Final concn. ($\mu\text{g/ml}$)			Inhibition of the growth at the end of the assay (%)
	Seminal-plasmin	Antiseminal-plasmin	Trypsin	Seminal-plasmin	Antiseminal-plasmin	Trypsin	
1	0	—	—	20	—	—	100
	0	0	—	20	20	—	25
	—	0	—	—	20	—	0
	0	—	0	20	—	1	7
	—	—	0	—	—	1	0
	0	60	—	20	20	—	100
	0	—	60	20	—	1	100
2	0	—	—	30	—	—	100
	0	0	—	30	90	—	0
	0	5	—	30	90	—	0
	0	10	—	30	90	—	0
	0	15	—	30	90	—	0
	0	20	—	30	90	—	0
	0	30	—	30	90	—	100
	0	60	—	30	90	—	100
	10	0	—	30	90	—	0
	30	0	—	30	90	—	0
	0	—	0	30	—	2	0
	0	—	10	30	—	2	0
	0	—	30	30	—	2	100
	0	—	—	30	—	—	100
3	60	—	—	40	—	—	100
	60	60	—	40	150	—	0
	0	60	—	40	150	—	100
	60	0	—	40	150	—	0
	—	60	—	0	150	—	0

Table 4. *Effect of antiseminalplasmin preparation 2 on the inhibition of transcription in vitro of poly(dT) by seminalplasmin, using purified E. coli RNA polymerase*

The transcription was carried out for 10 min, with [^{14}C]ATP as the substrate, and measured by paper-strip chromatography.

Final concn. ($\mu\text{g/ml}$)		Radioactivity in RNA	
Seminalplasmin	Antiseminal-plasmin	(c.p.m./20 μl)	(% of control)
0	0	2966	100
100	0	1160	39
0	200	3000	101
100	200	1200	40

was found to be completely non-toxic to *E. coli* and had no effect whatsoever on its growth, even at concentrations as high as 400 $\mu\text{g/ml}$.

It therefore appears likely that antiseminal-

plasmin acts by binding to the cell surface and preventing the entry of seminalplasmin, although other possibilities, such as an association of antiseminalplasmin in whole cells with another protein

such as to make antiseminalplasmin capable of inhibiting transcription and masking its RNAase activity, cannot be ruled out.

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