Bacteriolytic Activity of Seminalplasmin

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Seminalplasmin, an antimicrobial protein from bovine seminal plasma, lysed both Gram-positive and Gram-negative bacteria but not *Candida albicans*. The lytic activity was not lysozyme-like and was not affected by inhibitors of RNA or protein synthesis or by azide; it was strongly inhibited by divalent cations like Ca^{2+} , Mn^{2+} and Mg^{2+} at millimolar concentrations. Maximum lysis of *Escherichia coli* was obtained at 37 °C; heat treatment of *E. coli* drastically reduced its susceptibility to lysis by seminalplasmin. *E. coli* cells in the stationary phase of growth were lysed much less than those in the exponential phase, and those grown in an enriched medium were lysed much more than those grown in a minimal medium. It appears that the lytic activity of seminalplasmin is due to the activation of an autolysin.

INTRODUCTION

Seminalplasmin is an antimicrobial protein isolated from bovine seminal plasma (Reddy & Bhargava, 1979). It inhibits the growth of many Gram-positive and Gram-negative bacteria and yeasts by entering the cells and inhibiting RNA synthesis (Reddy & Bhargava, 1979; Bhargava, 1981*a, b*; Shivaji, 1984; Rao & Bhargava, 1985; Scheit & Bhargava, 1985; Scheit *et al.*, 1985). *In vitro*, seminalplasmin binds to RNA polymerases and reverse transcriptases and inhibits their activity (Scheit *et al.*, 1979; Rao *et al.*, 1983; Reddy *et al.*, 1983; Scheit & Zimmer, 1984). We now report that concentrations of seminalplasmin higher than those required for the inhibition of RNA synthesis lyse *Escherichia coli* cells. The lysis may be the result of the activation of an autolysin.

METHODS

Micro-organisms, media and reagents. *E. coli* strain C90 (Bachmann, 1972), which is inducible for β -galactosidase, *Bacillus subtilis* NCIM 2162, and *Candida albicans* NCIM 3100, obtained from the National Collection of Industrial Micro-organisms (NCIM), Poona, India, were used. *E. coli* and *B. subtilis* were grown at 37 °C in glucose-minimal A medium (Miller, 1972) with 0.1 mM-MgSO₄, unless stated otherwise. *C. albicans* was grown at 30 °C in a yeast extract/glucose medium (1%, w/v, yeast extract/3%, w/v, glucose). Dried cells of *Micrococcus luteus* and protease type XI were obtained from Sigma. Seminalplasmin and antiseminalplasmin were prepared from bovine seminal plasma as described earlier (Reddy & Bhargava, 1979; Rao & Bhargava, 1985).

Lytic activity of seminalplasmin. This was measured by following the decrease in optical density at 600 nm (OD₆₀₀) of microbial cells, grown to exponential phase except where indicated, initially suspended at an OD₆₀₀ of 0.15–0.3 (1.5–3.0 × 10⁸ cells ml⁻¹) in either minimal medium containing 0.1 mM-Mg²⁺ (which medium was used unless otherwise mentioned), or Tris/HCl buffer (10 mM; pH 7.4) containing 0.1 M-NaCl, and incubated with the desired amount of seminalplasmin at 37 °C (unless otherwise stated) for the desired period. Seminalplasmin dissolved in 20–40 µl distilled water was added to 1 ml of the cell suspension; addition of this amount of water alone had no lytic effect. In some cases in which the medium used for studying lysis was different from that used for growth, the cells were sedimented by centrifugation and resuspended in the desired medium. This treatment did not result in any cell lysis.

The kinetics of lysis by seminalplasmin was studied either by periodic measurements of OD₆₀₀, or by recording continuously the decrease in OD₆₀₀ of cells incubated with seminalplasmin in a cuvette maintained at 37 °C.

RNA synthesis. This was studied either by pulse labelling or by continuous labelling. For pulse labelling, *E. coli*

Fig. 1

Fig. 2

Fig. 1. Kinetics of the lysis of viable *E. coli* (A and B) and *B. subtilis* (C) cells by seminalplasmin ($200 \mu\text{g ml}^{-1}$). *E. coli* cells were grown in LB medium (A) or minimal medium (B), and *B. subtilis* cells in minimal medium. The cells were sedimented in a microcentrifuge for 15 min and resuspended in minimal medium, and lysis was followed by continuously recording the OD_{600} .

Fig. 2. Kinetics of the release of β -galactosidase from *E. coli* cells treated with seminalplasmin ($200 \mu\text{g ml}^{-1}$). The cells were grown in minimal medium containing 0.4% (w/v) lactose and incubated with seminalplasmin at 37°C . Samples of 0.1 ml taken at different times were diluted to 1 ml with cold minimal medium and spun down at $120000 g$ for 15 min at 4°C . β -Galactosidase activity was determined in the cell-free supernatant as described in Methods. The OD_{600} of the culture incubated with seminalplasmin was 0.25 at 0 min and 0.06 at 60 min.

cells were suspended at an OD_{600} of 0.3 in 1 ml minimal medium containing 0.1 mM-Mg^{2+} , and incubated at 37°C . At various times, a 0.1 ml sample of the culture was pulse-labelled with [^3H]uridine [$20 \mu\text{Ci}$ (740 kBq); sp. act. 14 Ci mmol^{-1} ($518 \text{ GBq mmol}^{-1}$)] for 20 s at 37°C . Cold 10% (w/v) trichloroacetic acid (TCA, 1 ml) was added followed by bovine serum albumin (0.5 mg), and the TCA-insoluble material was collected on Whatman no. 1 filter paper discs, washed with cold 5% TCA, dried and counted in a toluene-based scintillation fluid. For continuous labelling, *E. coli* cells (OD_{600} 0.3) were incubated with [^3H]uridine [$200 \mu\text{Ci}$ (7.4 MBq) ml^{-1} ; sp. act. 14 Ci mmol^{-1}] at 37°C in minimal medium containing 0.1 mM-Mg^{2+} . At various times, 0.1 ml samples of the culture were processed as above.

β -Galactosidase activity. This was measured in the cell supernatant using *o*-nitrophenyl- β -galactoside as the substrate (Miller, 1972); the total enzyme activity in the culture was measured after toluenization of the cells.

RESULTS

Cells of *E. coli* growing exponentially were lysed by seminalplasmin (Fig. 1). In a large number of experiments, *E. coli* ($1.5\text{--}3.0 \times 10^8 \text{ cells ml}^{-1}$) treated with $200\text{--}300 \mu\text{g}$ seminalplasmin ml^{-1} showed a 25–90% decrease in OD_{600} in 1–2 h. The time course of lysis by seminalplasmin was similar to that of autolysis induced by agents such as EDTA in showing an initial exponential phase followed by a plateau (Leduc & Van Heijenoort, 1980; Leduc *et al.*, 1982). Lysis of *E. coli* was also demonstrated by measurement of the release of β -galactosidase from cells in which the enzyme has been induced by growth in lactose-minimal A medium (Fig. 2).

E. coli cells grown in an enriched medium (LB; Miller, 1972) were significantly more susceptible to lysis than cells grown in minimal medium (Fig. 1). (Assays for lysis were done only in minimal medium, irrespective of the medium in which the cells had been grown, since cells suspended in the enriched medium were not lysed at all by seminalplasmin, presumably due to degradation or complexing of seminalplasmin by the constituents of the medium.) Dense cell suspensions ($10^9\text{--}10^{10} \text{ cells ml}^{-1}$) were also lysed by seminalplasmin, provided that higher

Fig. 3

Fig. 3. Effect of pH on the lysis of *E. coli* cells by seminalplasmin ($200 \mu\text{g ml}^{-1}$). Cells were grown in minimal medium containing 10 mM-citrate adjusted to pH 5.0 or 7.5, sedimented in a microcentrifuge for 10 min and resuspended in the above medium at pH 5.0 or 7.5, and lysis was followed by continuous recording of OD_{600} . —, Cells grown and lysed at pH 7.5; ---, cells grown at pH 7.5 and lysed at pH 5.0; ····, cells grown and lysed at pH 5.0; - · - ·, cells grown at pH 5.0 and lysed at pH 7.5.

Fig. 4

Fig. 4. Comparison of the kinetics of lysis (a) and of the inhibition of RNA synthesis (b) by seminalplasmin in exponentially growing *E. coli* cells. ○, $100 \mu\text{g seminalplasmin ml}^{-1}$; ●, $200 \mu\text{g seminalplasmin ml}^{-1}$. RNA synthesis was studied by pulse labelling as described in Methods. Lysis was followed by measuring the OD_{600} at various time intervals.

concentrations (about $1\text{--}2 \text{ mg ml}^{-1}$) of the protein were used. *B. subtilis* cells grown in minimal medium were also lysed by seminalplasmin, the kinetics of lysis being similar to that observed in the case of *E. coli* (Fig. 1). Cells of *C. albicans* (which is susceptible to growth inhibition by seminalplasmin; Scheit & Bhargava, 1985) or dried cells of *M. luteus* were not lysed even by high concentrations ($400 \mu\text{g ml}^{-1}$) of seminalplasmin.

E. coli cells grown at pH 5.0 were much less susceptible to lysis by seminalplasmin, at pH 5.0 or pH 7.5, than were those grown at pH 7.5 (Fig. 3). The lytic activity of seminalplasmin was much less pronounced at pH 5.0 than at pH 7.5 for cells grown at either pH (Fig. 3). In this regard, seminalplasmin-induced lysis is similar to penicillin-induced lysis (Goodell *et al.*, 1976).

Seminalplasmin inhibits RNA synthesis in *E. coli* and other micro-organisms (Reddy & Bhargava, 1979; Bhargava, 1981*a, b*; Shivaji, 1984; Scheit & Bhargava, 1985; Scheit *et al.*, 1985). To determine whether the inhibition of RNA synthesis by seminalplasmin is a consequence of the lysis of cells, we looked at the time course of both processes, at two seminalplasmin concentrations (Fig. 4); at the lower concentration ($100 \mu\text{g ml}^{-1}$), at which seminalplasmin dramatically inhibited growth of and RNA synthesis in *E. coli*, no significant lysis was observed. Further, *E. coli* cells in the stationary phase of growth seemed to be far less susceptible to lysis by seminalplasmin than exponentially growing cells (Fig. 5); the rate of RNA

Fig. 5. Lytic effect of seminalplasmin ($200 \mu\text{g ml}^{-1}$) on *E. coli* at various stages of growth. A sample of the growing culture was diluted with minimal medium to OD_{600} 0.20 before the addition of seminalplasmin, and lysis was followed by measurement of the decrease in OD_{600} after incubation for 1 h. ●, Growth; ○, lysis.

synthesis in stationary phase cells, on the other hand, was sensitive to inhibition by seminalplasmin (inhibition $\approx 45\%$ with $100 \mu\text{g ml}^{-1}$ and $\approx 70\%$ with $200 \mu\text{g ml}^{-1}$ at 12 min, beyond which no further RNA synthesis took place in the presence of seminalplasmin; not shown).

The lysis by seminalplasmin of *E. coli* cells (OD_{600} 0.2) killed by heating for 15 min at 80°C was 16% of that observed with cells that were not heat-treated. However, seminalplasmin lysed *E. coli* cells that had been killed by a 2 h treatment with rifampicin ($50 \mu\text{g ml}^{-1}$), with the same efficiency as it lysed exponentially growing viable cells. The optimum temperature for the lysis of *E. coli* cells by seminalplasmin was 37°C ; lysis at 0°C was 30.3% and at 55°C , 8.2% of that at 37°C .

Seminalplasmin inhibits RNA synthesis in various micro-organisms, and subsequently kills them (Reddy & Bhargava, 1979; Scheit & Bhargava, 1985; Scheit *et al.*, 1985). Antiseminalplasmin, another protein isolated from bovine seminal fluid, reverses the inhibition of growth and of RNA synthesis by seminalplasmin by preventing the latter's entry into the cells (Rao & Bhargava, 1985). Antiseminalplasmin ($200 \mu\text{g ml}^{-1}$) inhibited the lytic activity of seminalplasmin even if added during the course of lysis (Fig. 6). We have recently shown that calcium ions, too, reverse the inhibitory effects of seminalplasmin (N. Sitaram, K. H. Scheit and P. M. Bhargava, unpublished); Ca^{2+} and other divalent cations also inhibited the lytic activity of seminalplasmin (Fig. 7).

The protein synthesis inhibitor chloramphenicol ($200 \mu\text{g ml}^{-1}$), and the respiratory inhibitor azide (1 mg ml^{-1}), had no effect on the lysis of *E. coli* cells by seminalplasmin even when the cells were preincubated with the above agents for 30 min before being treated with seminalplasmin; lysis (measured after 1 h) was, in fact, stimulated by about 50% in both cases. Under the same conditions, the non-specific protease type XI (Sigma; $500 \mu\text{g ml}^{-1}$) totally abolished the lytic activity of seminalplasmin. In a medium of high osmolarity (0.5 M-sucrose or mannitol in minimal medium), the lysis of *E. coli* by seminalplasmin after 1 h was 60% more than that in the normal medium; no protoplasts or ghosts were formed even in the high-osmolarity medium.

DISCUSSION

We demonstrate here that seminalplasmin possesses bacteriolytic activity. Since this protein has an M_r of about 6000 (Theil & Scheit, 1983), it can be calculated from the data given above that for lysis to occur, about 10^8 molecules of seminalplasmin per *E. coli* cell need to be present in the incubation medium. As dried *M. luteus* cells, which act as an excellent substrate for

Fig. 6

Fig. 6. Effect of antiseminalplasmin on the lysis of *E. coli* cells by seminalplasmin ($200 \mu\text{g ml}^{-1}$). Cells grown in minimal medium were treated with seminalplasmin and lysis was followed by continuous recording of OD_{600} . Antiseminalplasmin was added at the times stated after the addition of seminalplasmin: (a) no antiseminalplasmin added; (b) antiseminalplasmin ($200 \mu\text{g ml}^{-1}$) added at 0 min; (c) antiseminalplasmin ($400 \mu\text{g ml}^{-1}$) added at 0 min; (d) antiseminalplasmin ($200 \mu\text{g ml}^{-1}$) added at 5 min.

Fig. 7

Fig. 7. Effect of divalent cations on the lysis of *E. coli* cells by seminalplasmin ($200 \mu\text{g ml}^{-1}$) in Tris/HCl buffer containing 0.1 M-NaCl . Lysis was followed by measurement of the decrease in OD_{600} over 1 h. In the control (no added cation), there was a 60% reduction in OD_{600} over the initial value of 0.2.

lysozyme, were not lysed, it is clear that the lytic activity of seminalplasmin is not like that of lysozyme.

Seminalplasmin is known to inhibit RNA synthesis in bacteria and yeasts. We show here that higher concentrations of seminalplasmin are required for lysis than for the inhibition of RNA synthesis. Further, seminalplasmin inhibits RNA synthesis in, but does not lyse, cells in the stationary phase of growth. It would therefore appear that the two effects of seminalplasmin – that is, the inhibition of RNA synthesis that eventually leads to death of the cell without lysis, and the lytic effect – are independent of each other.

The observation that lysis begins almost immediately after the addition of seminalplasmin would suggest that the lytic activity of seminalplasmin involves the cell wall. Agents such as β -lysin that lyse bacteria by affecting the cell membrane lead to a decrease in the optical density of the culture only after several hours even though some of the cellular constituents leak out at an

early stage (Matheson & Donaldson, 1968; Donaldson & Tew, 1977). Apart from colicin M (Schaller *et al.*, 1982), seminalplasmin is the only protein known to lyse a Gram-negative bacterium like *E. coli* in the absence of agents such as EDTA. It also lyses the Gram-positive organism *B. subtilis*.

Active uptake of seminalplasmin does not seem to be required for lysis of *E. coli*, as the lysis was not inhibited by azide. Nor did protein synthesis appear to be necessary, since chloramphenicol or rifampicin-treated cells were lysed as rapidly as (or more rapidly than) untreated cells. In this respect, lysis by seminalplasmin resembles EDTA-induced lysis and differs from lysis by agents such as penicillin (Leduc *et al.*, 1982). Existing evidence strongly suggests that seminalplasmin kills micro-organisms by entering the cell and inhibiting RNA synthesis (Reddy & Bhargava, 1979; Scheit & Bhargava, 1985; Scheit *et al.*, 1985). Inhibition of seminalplasmin-induced lysis of *E. coli* by antiseminalplasmin and Ca^{2+} , which appear to prevent the entry of seminalplasmin into the cells (Rao & Bhargava, 1985; N. Sitaram, K. H. Scheit & P. M. Bhargava, unpublished), also suggests that the permeation of seminalplasmin, at least through the outer membrane, is required for lysis.

With regard to the mechanism of the lysis of *E. coli* by seminalplasmin there are two possibilities: seminalplasmin may lyse the cells directly as, for example, lysozyme does, or it may lead, directly or indirectly, to the activation of an autolysin. The first possibility would seem unlikely as seminalplasmin had no effect on heat-treated *E. coli* or dried *M. luteus* cells. The latter possibility is supported by the following observations: (a) as in the case of autolysis induced by various methods (Leduc & Van Heijenoort, 1980; Leduc *et al.*, 1982; Lubitz *et al.*, 1984), lysis by seminalplasmin of cells grown in rich medium was greater than that of cells grown in minimal medium, and lysis of cells in the stationary phase was less than that of exponentially growing cells; and (b) like penicillin-induced lysis, seminalplasmin-induced lysis was much less pronounced when cells were grown at pH 5.0, or lysis was carried out at pH 5.0, than at higher pH. We are at present in the process of isolating seminalplasmin-resistant mutants, which may allow us to test this possibility. If seminalplasmin does indeed activate an autolysin, the observation that cells preincubated with rifampicin for 2 h were lysed by seminalplasmin would suggest that the autolysin has a long half-life.

Seminalplasmin, unlike EDTA, does not bind to divalent cations such as Ca^{2+} (N. Sitaram, K. H. Scheit & P. M. Bhargava, unpublished); its use may, therefore, be preferable to that of lysozyme for lysis of *E. coli* under certain conditions because the latter requires EDTA for its action.

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