Volume 303, number 2,3, 265–268 FEBS 11109 June 1992

The antibacterial peptide seminal plasmin alters permeability of the inner membrane of *E. coli*

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Received 17 March 1992

Seminal plasmin (SPLN) a 47-residue peptide, isolated from bovine seminal plasma, exhibits antibacterial activity against Gram-positive and Gram-negative bacteria. Although SPLN strongly inhibits the transcription of various natural and synthetic templates by *E. coli* RNA polymerase in vitro, it also associates with model membranes of phosphatidylcholine and phosphatidic acid. We have undertaken experiments to ascertain whether SPLN permeabilizes the bacterial inner membrane and thereby exerts its antibacterial activity, as in the case of recently isolated antibacterial peptides from mammalian sources. Our results show that SPLN affects the permeability properties of the bacterial inner membrane which is reflected by increased uptake of *ortho*-nitrophenylgalactoside (ONPG), which can normally be translocated only by protein transporters. SPLN has also been shown to act on the outer membrane, since divalent cations inhibit antibacterial activity.

Seminal plasmin; Antibacterial protein; Membrane permeability; Spheroplast

1. INTRODUCTION

Seminal plasmin (SPLN) a 47-residue peptide isolated from bovine seminal plasma [1], exhibits antibacterial activity against Gram-positive and Gram-negative bacteria [2,3] and inhibits the growth of yeast [4]. SPLN inhibits the in vitro transcription of various templates by *E. coli* RNA polymerase [4,5]. However, SPLN also associates with model membranes of phosphatidylcholine and phosphatidic acid [7], and binds to sperm plasma and acrosomal membranes [3]. Thus, SPLN clearly has a tendency to interact with membranes, although the sequence shows only one region which is more hydrophobic than rest of the peptide [8]. It is conceivable that the antibacterial activity of SPLN stems from its ability to change the permeability properties of the bacterial inner membrane, like some of the recently isolated antibacterial peptides [9–11] from mammalian sources. Hence, we have undertaken experiments to ascertain whether SPLN permeabilizes the bacterial inner membrane and thereby exerts its antibacterial activity.

2. EXPERIMENTAL

2.1. *Synthesis of SPLN*

The synthesis was carried out in a stepwise manner in the Applied Biosystems Model 431A peptide synthesizer using Fmoc chemistry. Single coupling was employed for the entire synthesis using the protocols supplied by the manufacturer. The resin was treated with trifluoroacetic acid, thioanisole, *meta*-cresol, and ethanedithiol to effect the removal of side chain protecting groups and separation of the peptide from the resin. The peptide was purified by HPLC on a HP 1090 instrument using a reverse phase Waters μBondapak C_{18} column. The purified peptide was characterized by amino acid analysis on a LKB 4151 Alpha plus analyzer and sequence analysis on an Applied Biosystems Model 470A sequencer. The antibacterial activity [12] and inhibition of *E. coli* RNA polymerase of synthetic and natural SPLN were compared and found to be identical. SPLN from bovine seminal fluid was isolated as described by Reddy and Bhargava [2].

Bacteriolytic activity of SPLN was monitored by following decrease in optical density at 600 nm (OD_{600}) of *E. coli* W160-37 cells, grown to logarithmic phase in minimal A medium [13] (10.5 g KH_2PO_4 4.5 g, K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.5 g sodium citrate, 0.1 mM $MgSO_4$, 0.1 g L-arginine and 1% glucose in 1 litre of water) in the presence of 5×10^{-4} M isopropylthiogalactoside (IPTG). The OD_{600} of the culture before adding SPLN was 0.2 to 0.3 ($2.0–3 \times 10^8$ cells/ml) at 37°C. Aliquots were withdrawn from bacterial cells incubated with and without SPLN at defined time periods and OD_{600} was measured. Bacteriolytic activity was also monitored by measuring the release of β-galactosidase from the cells in presence of SPLN. Aliquots of 150 μl were withdrawn at the same time intervals and diluted to 1 ml with assay buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$ and 0.05 M mercaptoethanol, pH 7.0) and spun down at 12,000 × g at 4°C. β-Galactosidase activity in the supernatant was measured using *ortho*-nitrophenylgalactoside (ONPG) [13]. In order to determine the permeability properties of the bacterial inner membrane in the presence of SPLN, the influx of ONPG into the sedimented cells from above was determined by incubating the cells in 1 ml of the assay buffer with ONPG at 37°C. The total enzyme activity of the sedimented cells in the absence of SPLN was measured after treating the cells with 0.1% sodium dodecyl sulphate/chloroform.

Antibacterial activity of SPLN in the absence and presence of divalent cations was determined by incubating logarithmically growing culture of *E. coli* diluted to an OD of 0.01 at 600 nm in the absence and presence of various concentrations of the appropriate cation at 37°C for 6 h and measuring the OD at 600 nm. The growth in the absence of SPLN under similar conditions was taken as control. The composition of the synthetic medium in which cells were grown was (g/l): NH_4Cl 0.5, $(NH_4)_2SO_4$ 0.5, KH_2PO_4 13.6, $MgSO_4 \cdot 7H_2O$ 0.02,

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$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ 0.0156, glucose 20, L-arginine hydrochloride 0.1 (2).

2.2. *Preparation of spheroplasts from* E. coli *cells*

Spheroplasts from *E. coli* W 160-37 cells were prepared essentially according to Ito et al. [14]. *E. coli* cells were grown in the above synthetic medium in the presence of 5×10^{-4} M IPTG, to an OD of 0.4 at 600 nm. The bacterial pellet from approximately 50 ml of the culture was washed with 0.05 M Tris–HCl (pH 8.1), suspended in 0.2 ml of 20% sucrose containing 0.03 M Tris–HCl (pH 8.1), and cells were converted into spheroplasts by incubation in an ice bath for 30 min with 1/10 volume of 1 mg/ml of lysozyme, freshly dissolved in 0.1 M EDTA (pH 7.0). Spheroplasts were collected by centrifugation at 15,000 × g for 15 min and resuspended in 10 ml of synthetic medium containing 20% sucrose. Aliquots of 1 ml of this preparation were used for lysis experiments.

The lytic activity of SPLN on the spheroplasts was determined by measuring the release of β-galactosidase after incubating aliquots of spheroplasts for 20 min with varying concentrations of SPLN at 37°C in 20% sucrose and determining the β-galactosidase activity [13] using ONPG in the supernatant and the pellets after centrifugation at 15,000 × *g* at 4°C. β-Galactosidase released as a result of treating the spheroplasts with water was taken as 100%. For determining the effect of divalent cations, the experiment was carried out in the presence of varying concentrations of the appropriate cation.

2.3. *Osmotic protection experiments*

Spheroplasts prepared from *E. coli* were suspended in 20% sucrose solution in minimal A medium and 30 mM of polyethylene glucol (PEG) of molecular mass 600, 1540, 3000 or 4000. Then, SPLN was added and the β-galactosidase activity was assayed after incubation for 20 min.

3. RESULTS

We have monitored the activity of the cytoplasmic enzyme β-galactosidase in *E. coli* in the presence of SPLN in order to ascertain the permeability status of the bacterial inner membrane. Fig. 1 shows the activity of β-galactosidase in the supernatant and in the cell pellet as a function of time and in the presence of 30 μM SPLN. In the pelletted cells there is considerable increase in β-galactosidase activity with increasing time in the presence of SPLN. However, very little β-galactosidase is released into the supernatant. It is well established that influx of ONPG through the bacterial inner membrane occurs through lac permease and, in the absece of the protein transporter, no influx of ONPG is possible. Enhanced activity of β-galactosidase in the presence of antibacterial agents would thus reflect the permeabilization of the bacterial membrane to ONPG [9]. Hence, SPLN clearly permeabilizes the bacterial inner membrane, providing additional pathways for ONPG influx. Also, as there is increased β-galactosidase activity as a function of time, it is unlikely that SPLN is internalized. If so, there could only be an initial increase in the permeability. Although the above exper-

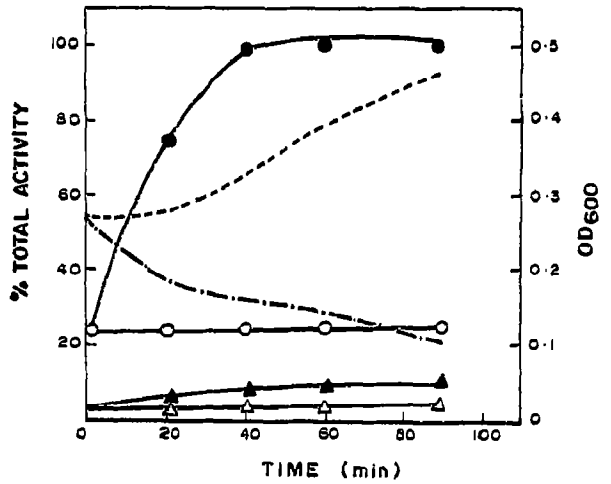

Fig. 1. Effect of SPLN on the influx of ONPG into *E. coli* cells. (○) Influx of ONPG into cell in the absence of SPLN; (●) influx into cells in the presence of 30 μM SPLN. β-Galactosidase activity in the supernatant in the absence of (△) and presence of (▲) of 30 μM seminal plasmin. OD_{600} of the cells as a function of time monitored in the absence (--) and presence (–) of SPLN is also shown. Total β-galactosidase activity was determined by treating the cells with 0.1% SDS/chloroform. The values on the ordinate are percentage of the total activity, normalized to OD_{600} of 1 and are taken as an indication of influx.

Table I

Effect of divalent cations on the inhibition of growth of *E. coli* W160-37 by seminal plasmin

Seminal plasmin (μM)	Cation	Concentration (mM)	A_{760} nm of the culture at 6 h	Inhibition of growth (%)	Relief of inhibition by the cation (%)
0	None	–	0.5	–	–
6	None	0.72	0.0	100	–
0	Zn^{2+}	0.72	0.53	0	–
6	Zn^{2+}	0.24	0.0	100	0
6	Zn^{2+}	0.48	0.12	76	24
6	Zn^{2+}	0.72	0.35	30	70
0	Ca^{2+}	0.72	0.57	0	–
6	Ca^{2+}	0.24	0.24	78	22
6	Ca^{2+}	0.48	0.42	27	73
0	Mn^{2+}	0.72	0.50	0	–
6	Mn^{2+}	0.24	0.13	74	26
6	Mn^{2+}	0.48	0.46	8	92

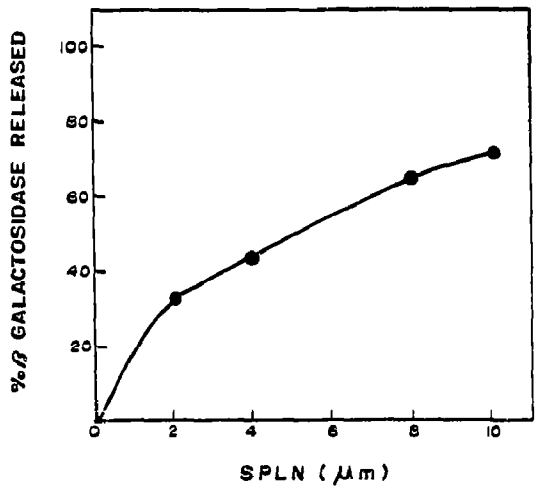

Fig. 2. Release of β-galactosidase from *E. coli* spheroplasts in the presence of SPLN.

iments were done with strain W160-37 similar results were obtained with other strains. The absence of β-galactosidase activity in the supernatant indicates that SPLN does not lyse bacterial cells in the time course of the experiments. During the course of the experiment there is a decrease in OD_{600} from 0.283 to 0.119. Although this decrease is 50%, the amount of β-galactosidase detected in the supernatant is not substantial. SPLN did not inhibit the activity of purified β-galactosidase, indicating that lack of activity in the supernatant is not due to inhibition of activity by the peptide.

It has been well established that divalent cations like Ca^{2+} stabilize the outer membrane structure [15–17]. Hence, we have examined the effect of these cations on the antibacterial activity of SPLN. The results are presented in Table I. The cations, Ca^{2+}, Mn^{2+} and Zn^{2+} clearly inhibit the antibacterial activity of SPLN, most probably by preventing its entry into bacterial cells and localization in the inner membrane. Thus, a stabilized outer membrane structure prevents the entry of SPLN and consequent localization in the inner membrane. Extensive studies on the permeabilization of red blood cell membranes by detergents and toxins have indicated that metal ions like Ca^{2+} and Zn^{2+} prevent colloid osmotic lysis, presumably by stabilizing the membrane. We examined whether these metal ions would also protect spheroplasts from osmotic lysis in the presence of SPLN. However, it was observed that even in millimolar range no protection against lysis was observed.

In order to get further insight into the nature of the pathway for ONPG in the inner membrane as a result of perturbation by SPLN, experiments were performed to determine the size of 'pores' in the membrane using osmoprotectants. However, for these experiments spheroplasts were used rather than intact cells as the outer membrane would form a barrier to molecules like PEG 4000 [8] which were used as osmoprotectants. Unlike with intact cells, β-galactosidase activity could be detected in the supernatant after spinning down the spheroplasts. Fig. 2 shows the % release of β-galactosidase as a function of SPLN concentration. It is evident that β-galactosidase is released even at very low amounts of SPLN, indicating that SPLN causes perturbation of the bacterial inner membrane even at very low concentrations. When the release of β-galactosidase in the presence of various osmoprotectants was determined, only 40% decrease in activity was observed even with PEG 4000 indicating the presence of lesions of ~40 Å. Thus lysis proceeds through a colloid osmotic mechanism similar to the lysis of red blood cells by various agents.

4. DISCUSSION

SPLN has antibacterial activity against a variety of organisms. Since SPLN inhibits the transcription of various natural and synthetic templates by *E. coli* RNA polymerase in vitro [4,5], it was presumed that its biological activity stemmed from this property. However, there has been no direct demonstration that SPLN enters bacteria cells in vivo. Our results show that SPLN has the ability to alter the permeability properties of the bacterial membrane as judged by increased ONPG influx as a function of time. Decrease in the efflux of β-galactosidase from spheroplasts in the presence of osmoprotectants indicates that a colloid osmotic type of lysis-mechanism operates. Since the influx of ONPG into *E. coli* cells increases with time at a fixed peptide concentration, it is unlikely that SPLN is internalized and exerts its activity on the RNA synthesis machinery. The inner membrane of bacteria contains the electron transport chain and enzymatic systems for energy generation. It is likely that association of SPLN results in formation of defects in the membrane resulting in extensive uncoupling of various pumps and other translocations. This causes a generalized permeability breakdown and subsequently cell death. Although autolysins are known to be induced due to perturbation of inner membrane [18], it is unlikely that induction of autolysins plays a major role in the antibacterial activity of SPLN as increased ONPG influx is also observed at pH 5, when autolysin induction does not take place [19].

The lipopolysaccharide layer in the bacterial outer membrane in Gram-negative bacteria forms a barrier to hydrophobic molecules or macromolecules like lysozyme. Also molecules with sizes >700 daltons cannot permeate through the outer membrane. Since SPLN would be cationic at pH 7.4, it can associate with the outer membrane like polymyxin and polylysine and perturb the outer membrane to an extent that it can gain entry to the inner membrane. Thus, the decrease in OD shown in Fig. 1 does not appear to signify cell lysis and probably reflects disruption of the outer membrane. Divalent cations are known to stabilize the outer mem-

brane structure by binding to lipopolysaccharide molecules and thus prevent entry of toxins [15–17]. Hence, it is likely that SPLN is prevented from reaching the inner membrane when the outer membrane is stabilized. It is thus unlikely that SPLN uses the pathway responsible for entry and localization of colicins in *E. coli* [20] or channels formed by porins.

SPLN does not have any sequence similarity with defensins [10] a class of antibacterial peptides isolated from neutrophils. Conformational studies have indicated that SPLN adopts an α-helical conformation particularly in hydrophobic environment [7] where high-resolution crystal structure of defensins has revealed a β-sheet conformation [21]. Thus SPLN and defensins do not share a common structural motif. However, our present work as well as studies on defensins [9] indicate that the antibacterial activity of these peptides stems from their ability to permeabilize the bacterial inner membrane. Hence, important pre-requisites for peptide antibacterial agent would be: (i) entry into the bacterial inner membrane by either making use of the colicin pathway or by perturbation of the outer membrane by virtue of being cationic; (ii) mild perturbation of bacterial membrane due to the presence of amphipathic structures which could be either helices of β-sheets.

Acknowledgements: We thank Imperial Cancer Research Fund Laboratories, London for providing time and chemicals on the Applied Biosystems 431A automated peptide synthesizer for the synthesis of SPLN. We also are grateful to Mr. V.M. Dhople for amino acid analysis. The inhibition of transcription of *E. coli* RNA polymerase by SPLN was kindly checked by Dr. D. Chatterji. We thank Dr. P.M. Bhargava for discussions.

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