THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 18, Issue of June 25, pp. 10438–10442, 1990
Printed in U.S.A.

A Synthetic 13-Residue Peptide Corresponding to the Hydrophobic Region of Bovine Seminalplasmin Has Antibacterial Activity and Also Causes Lysis of Red Blood Cells*

(Received for publication, December 15, 1989)

Narasimhaiah Sitaram and Ramakrishnan Nagaraj‡

From the Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

Seminalplasmin (SPLN), a 47-residue peptide present in bovine seminal plasma, is one of the few proteins isolated from mammalian sources having potent antibacterial activity. SPLN also interacts with sperm acrosomal and plasma membranes. On the basis of analysis of the primary structure of SPLN with respect to its relative hydrophobicity and hydrophilicity, a region comprising of 13-amino acids, Pro-Lys-Leu-Leu-Glu-Thr-Phe-Leu-Ser-Lys-Trp-Ile-Gly, has been delineated. It is demonstrated that a synthetic peptide corresponding to this 13-residue region inhibits growth of *Escherichia coli* like SPLN and also has the ability to lyse red blood cells.

A large number of peptides comprising approximately 15–40 residues, isolated from diverse sources such as insect venom (1, 2) and insect hemolymph (3) have potent antibacterial activity. Many of these peptides also exhibit lytic activity against eukaryotic cells (1, 4, 5). The biological activity of these peptides stem from their ability to spontaneously partition into the lipid bilayer of membranes and bring about reorganization in the arrangement of fatty acid acyl chains, thereby altering the physical properties of the membrane (1, 2, 6). Extensive structure-function studies indicate that almost all such peptides tend to adopt α-helical structure in hydrophobic environment (2, 6). The amino acid sequence of these peptides are such that the helix is amphiphilic in nature. In addition, these peptides have charged residues either flanking the amphiphilic region or present in the helical sequence. Their presence has been shown to be important for membrane-destabilizing activity (2, 6).

Seminalplasmin (SPLN),[1] a 47-residue peptide, present in bovine seminal plasma (7), is one of the few proteins isolated from mammalian sources, having potent antibacterial activity. Seminalplasmin also increases the fluidity of sperm acrosomal membrane and sperm plasma membrane (8) and causes lysis of dividing eukaryotic cells.[2] In this paper, we have analyzed the primary structure of SPLN (9) with respect to its relative hydrophobicity and hydrophilicity. We have delineated a region comprising 13 amino acids, which is considerably more hydrophobic than the rest of the protein. We demonstrate that this hydrophobic 13-residue peptide inhibits growth of *Escherichia coli* like SPLN but also has the ability to lyse red blood cells which SPLN does not.

MATERIALS AND METHODS

Amino acids, *O*-benzyl serine, γ-benzyl glutamate, and PEG 600 were from Sigma. PEG 1540, PEG 3000, and PEG 4000 were from Indian sources and Merrifield resin was from Vega Biotechnologies Inc. Side chain-protected Lys and Trp derivatives, *i.e.* Lys(2ClZ) and Trp(HCO) and α-NH_2-protected Boc-amino acids were prepared by established procedures. Solvents and reagents were from Spectrochem, Bombay, India.

Peptide Synthesis—The peptide corresponding to the sequence H_2N-Pro-Lys-Leu-Leu-Glu-Thr—Phe-Leu-Ser-Lys-Trp-Ile-Gly-COOH (SPF) was synthesized by solid-phase methods, manually. The peptide chain was assembled on Merrifield resin (1% cross-linked). The coupling of the first amino acid to the resin and the subsequent steps were carried out essentially as described in earlier reports (10–12). The Boc group was employed for the protection of α-amino groups. The side chain protecting groups were 2 ClZ for Lys, OBzl for Glu, Bzl for Ser and Thr, and formyl for Trp. Cleavage of the peptide from the solid-support was achieved by treatment with trifluoroacetic acid:thioanisole:metacresol:ethanedithiol in the ratio (10:1:1:1, v/v) at room temperature for 12 h. After removal of the trifluoroacetic acid, the residue was triturated with ether to yield the peptide. After extensive washing with ether, the peptide was purified by HPLC using a Beckman Ultrapore RPMC 5-μm (4.6 × 75 mm) C-8 column. The purified peptide was further characterized by amino acid analysis on an LKB 4151 Alpha Plus amino acid analyzer and sequencing on an Applied Biosystems 470A protein sequencer connected to an on-line phenylthiohydantoin analyzer (model 120A).

Stock solution of the peptide was prepared in methanol and the concentration determined by quantitative amino acid analysis.

Antibacterial Activity—Logarithmically growing cultures of *E. coli* W160-37 (initial A_{760} = 0.01) were incubated with various concentrations of SPF- and HPLC-purified seminal plasmin (9) in different aliquots. After 6 h, the A_{760} of the culture was determined. An A_{760} value of 0.5–0.6 was observed for the control culture for which no peptide was added and this value was taken as 100 for the purpose of calculation of percentage inhibition.

The bacteriolytic activity of SPF on *E. coli* was determined by measuring the release of β-galactosidase (13) and alkaline phosphatase (14). *E. coli* W160-37 cells grown to log phase in minimal A medium (13) containing 0.4% (w/v) lactose, were incubated with the peptide at 37 °C. Aliquots of 150 μl withdrawn at different times diluted to 1 ml of 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 was determined in the cell-free supernatant using ONPG (13). In order to determine the permeability properties of the bacterial inner membrane in the presence of SPF, the influx of ONPG into the sedimented cells from above, was determined by incubating the cells in 1 ml of buffer with ONPG at 37 °C. The total enzyme activity of the sedimented cells in the absence of SPF was measured after treating the cells with 0.1% SDS/chloroform. Similar experiments were also performed with *E. coli* CSH 57, a multi-auxotrophic strain (lac y) grown

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed.

[1] The abbreviations used are: SPLN, seminalplasmin; SPF, H_2N-Pro-Lys-Leu-Leu-Glu-Thr — Phe-Leu-Ser-Lys-Trp-Ile-Gly - COOH; Boc, *t*-butyloxycarbonyl; Bzl, benzyl ether; 2ClZ, 2-chlorobenzyloxycarbonyl; IPTG, isopropylthiogalactoside; OBzl, benzyl ester; ONPG, *ortho*-nitrophenylgalactoside, PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.

[2] P. M. Bhargava, personal communication.

on minimal A medium supplemented with all necessary growth requirements and 5×10^{-4} isopropylthiogalactoside (13).

For experiments on the release of alkaline phosphatase, *E. coli* C-90 strain was used and the enzyme assayed only in the supernatant.

Lysis of Erythrocytes—Rat erythrocytes were isolated and the buffy coat removed by centrifugation of freshly collected blood and washing three times with isotonic saline. They were incubated at 37 °C in 10 mM phosphate-buffered saline PBS with different concentrations of SPF for different time periods, centrifuged, and absorbance in the supernatant was measured at 540 nm. The absorbance obtained by treatment of erythrocytes with 1% Triton X-100 was taken as 100%.

Osmotic Protection—Erythrocytes were suspended in 0.135 M NaCl, 5 mM phosphate buffer (pH 7.4) in 30 mM solution of one of the following substances. D-Mannitol, sucrose, raffinose, PEG of molecular weights 600, 1540, 3000, or 4000 (15). Then, peptide was added and hemolysis determined as above after incubation for 30 min at 37 °C. The molecular diameters of the substances used were taken as mannitol, 7 Å; sucrose, 9 Å; raffinose, 11 Å; PEG 600, 16 Å; PEG 1540, 24 Å; PEG 3000, 30 Å; and PEG 4000, 38 Å (16).

RESULTS

The hydropathic profile of SPLN was examined by the method of Kyte and Doolittle (17) and the profile observed is shown in Fig. 1. A hydrophobic segment from residues 28 to 40 is clearly discernible. The peptide Pro-Lys-Leu-Leu-Glu-Thr-Phe-Leu-Ser-Lys-Trp-Ile-Gly (SPF) corresponding to this region was synthesized by solid-phase methods and assayed for antibacterial and cell lytic activity.

Antibacterial Activity—The percentage inhibition of the growth of logarithimically growing cultures of *E. coli* W160-37 on incubation with SPF and seminalplasmin at different concentrations is summarized in Table I. The minimal inhibitory concentration of SPF is 50 μg/ml and, in the case of seminalplasmin, ~30 μg/ml. Antibacterial activity of SPF can conceivably arise due to its ability to lyse bacteria or alter the permeability properties of the bacterial inner membrane resulting in depolarization. Therefore, the bacteriolytic activity of SPF and its effect on the permeability properties of the bacterial inner membrane were investigated. Incubation of *E. coli* W160-37, CSH 57 (lac y), and C-90 cells with varying concentrations of SPF for different intervals of time and analysis of the cell-free supernatant did not reveal the presence of β-galactosidase (a cytoplasmic enzyme) and alkaline phosphatase (a periplasmic enzyme). Therefore, SPF does not have the ability to lyse bacteria. The activity of β-galactosidase in sedimented *E. coli* W 160-37 cells as a function of time and peptide concentration is shown in Fig. 2. At a peptide concentration of 100 μg/ml, there is a gradual increase in the activity of β-galactosidase until about 30 min, followed by a rapid rise in activity, approaching the value of the enzyme activity when the inner membrane of *E. coli* is permeabilized by chloroform and detergents. At a peptide concentration of 200 μg/ml, a very rapid increase in enzyme activity is observed even at 30 min and the maximum activity is reached in 60 min. The enhanced activity of β-galctosidase in sedimented *E. coli* cells in presence of SPF as compared to the control suggests the presence of an additional pathway for the influx of ONPG through the bacterial inner membrane in addition to the transporter protein lac permease. In order to confirm that SPF permeabilizes the inner membrane of *E. coli*, thereby providing an additional pathway for influx of ONPG, experiments were done in a strain which lacks lac permease. Fig. 3 shows the activity of β-galactosidase in sedimented *E. coli* CSH 57 (lac y) in which β-galactosidase has been induced by isopropylthiogalactoside in presence of SPF. Considerable β-galactosidase activity is detected confirming the ability of the peptide SPF to permeabilize the inner membrane of *E. coli*.

Interaction of SPF with Red Blood Cells—Fig. 4 shows the hemolysis of erythrocytes as a function of the concentration of the peptide. Up to 6 μM, very little lysis is observed. Beyond 6 μM, considerable lysis is observed and 100% lysis is observed at 30 μM. The time course of lysis of erythrocytes by the peptide is shown in Fig. 5. It is evident that hemolysis occurs gradually and is not a rapid process. In order to determine whether the lysis was due to a colloid-osmotic process and

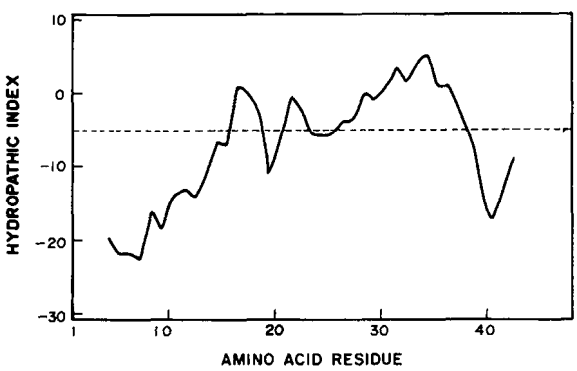

FIG. 1. **Hydropathic index plot of SPLN sequence.** The SPLN sequence was analyzed by the method of Kyte and Doolittle (17) averaging over a range of 7 amino acids. Hydrophobic domains are *above the dotted line* and hydrophilic domains are *below the dotted line*.

TABLE I

Antimicrobial activity of SPF and seminalplasmin

Compound	Concentration	Inhibition
	μg/ml	%
SPF	10	0
	20	25
	30	60
	40	90
	50	100
Seminalplasmin	10	15
	20	95
	30	100

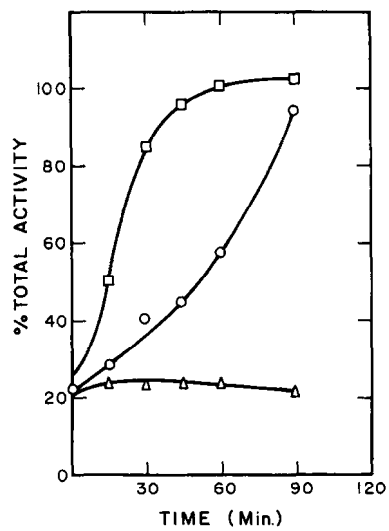

FIG. 2. **Effect of SPF on the influx of ONPG into *E. coli* W160-37.** △, control (no SPF); ○, 100 μg/ml SPF; and □, 200 μg/ml SPF. The cells were grown in minimal A medium containing 0.4% (w/v) lactose to a A_{600} of 0.6 and incubated with SPF at 37 °C for the stated period. The cells were centrifuged and the influx of ONPG through the inner membrane of the cells was estimated by the activity of β-galactosidase in the cytoplasm. Total β-galactosidase was determined by treating the cells with 0.1% SDS/chloroform and this activity was taken as 100%. The values in the *y* axis are percentages of the total activity and are taken as an indicator of the influx of ONPG.

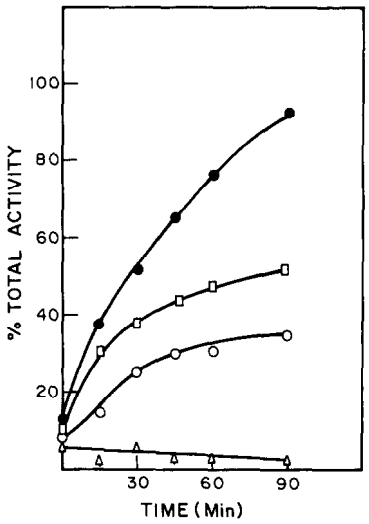

FIG. 3. **Effect of SPF on the influx of ONPG into *E. coli* CSH 47 (lac y).** △, control (no SPF); ○, 100 μg/ml SPF; □, 200 μg/ml SPF; ●, 400 μg/ml SPF. The cells were grown in minimal A medium with 5×10^{-4} M isopropylthiogalactoside to an A_{600} of 0.6 and incubated with SPF at 37 °C for the stated period. The cells were centrifuged and the influx of ONPG through the inner membrane of the cells was estimated by the activity of β-galactosidase in the cytoplasm. Total activity of β-galactosidase was determined as described in the legend for Fig. 2. The values in the *y* axis are percentages of the total activity and are taken as an indicator of the influx of ONPG.

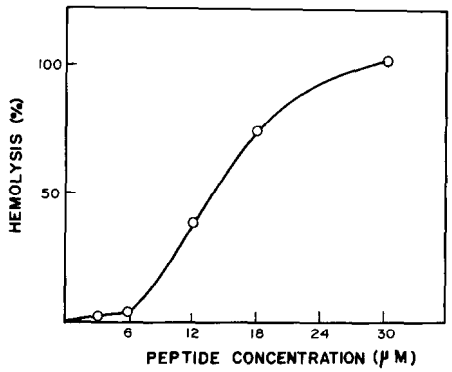

FIG. 4. **Hemolysis of rat erythrocytes as a function of SPF concentration.** Erythrocytes (0.5%, v/v) were incubated in phosphate-buffered (10 mM) isotonic saline containing various concentrations of SPF for 20 min.

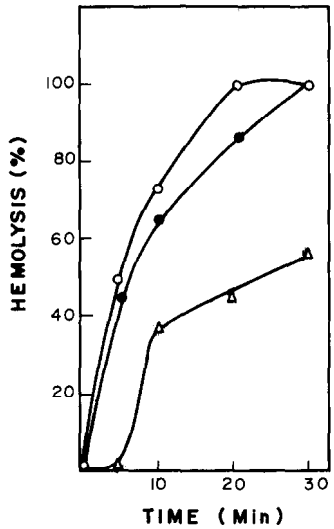

FIG. 5. **Hemolysis of rat erythrocytes by SPF as a function of time.** △, 12 μM; ●, 18 μM; ○, 24 μM. Experimental conditions were identical to that of Fig. 4 except that hemolysis was determined at different times of incubation with different concentrations of SPF.

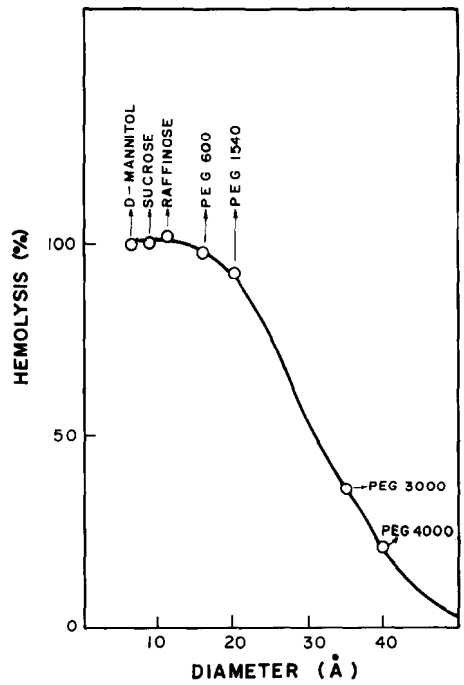

FIG. 6. **SPF-induced hemolysis in the presence of various osmoprotectants.** The erythrocytes (0.5%, v/v) were suspended in 0.135 M NaCl, 5 mM phosphate buffer (pH 7.4), and 30 mM protectant. Subsequently, SPF (final concentration 30 μM) was added and hemolysis was determined after incubation for 30 min at 37 °C.

also to determine the size of the membrane lesion, the hemolysis by the red blood cells was studied in the presence of peptides and various osmoprotectants. The lysis data in the presence of osmoprotectants is presented in Fig. 6. Protection against lysis is observed to some extent in the presence of PEG 3000 and to a large extent in the presence of PEG 4000 indicating that the lesions produced by the peptide are 36–40 Å in diameter. The osmotic protection at various concentrations of the peptide was investigated next. The data shown in Fig. 7 indicates that even at a peptide concentration of 8 μM where ~40% lysis is observed in the absence of any osmoprotectant, lysis is prevented only by PEG 3000 and 4000. The perturbation of the red blood cell membrane did not, however, result in release of membrane fragments into the supernatant as no phospholipid was detected by lipid estimation assays, in the lysis experiments. The membrane-perturbing ability of SPF against *E. coli* and erythrocytes was completely abolished on pre-incubation of peptide with trypsin, showing that shorter fragments of SPF generated by trypsin do not possess membrane-perturbing ability. No lysis of erythrocytes was observed in the presence of seminalplasmin.

In order to gain insight into the structural basis of the membrane-perturbing ability of SPF, the circular dichroism spectrum of the peptide was examined in trifluoroethanol and micelles of SDS (Fig. 8). Two minima, approximately 205 and 220 nm, and a cross-over, ~200 nm, were observed. While percentage of α-helix, β-structure, or random structure present in SPF cannot be estimated due to its small size, the spectrum is characteristic of peptides in predominantly α-helical conformation (18). The helical-wheel projection (19) of the peptide (Fig. 9) clearly indicates the amphiphilic nature of the peptide. Estimation of the hydrophobic moment yielded a value of 0.41.

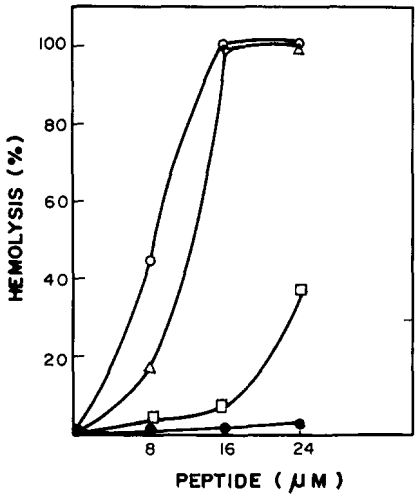

FIG. 7. **Protection of hemolysis by osmoprotectants at various concentrations of SPF.** ○, PEG 600; △, PEG 1540; □, PEG 3000; ●, PEG 4000. Assay conditions were same as in Fig. 6 except that the concentration of SPF was varied.

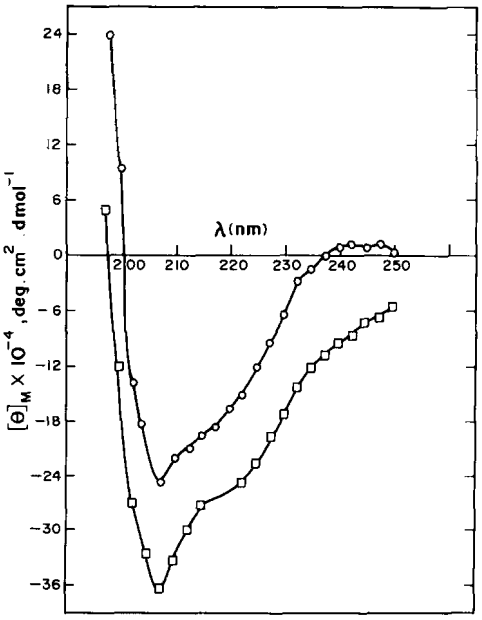

FIG. 8. **CD spectra of SPF in TFE (□) and 30 mM SDS (○).** The concentration of the peptide was 100 μg/ml. Spectra were recorded on a Jasco J-20 spectropolarimeter in a 1-mm cell at 25 °C. $[\Theta]_M$ values are expressed as molar ellipticities.

DISCUSSION

The peptide corresponding to the most hydrophobic region of seminalplasmin, Pro-Lys-Leu-Leu-Glu-Thr-Phe-Leu-Ser-Lys-Trp-Ile-Gly, has antibacterial and cell lytic activity. Since SPF does not lyse *E. coli* its antibacterial activity appears to stem from its ability to insert into the inner membrane of *E. coli* and render it permeable to substrates like ONPG which cannot cross the membrane barrier without the presence of a protein transporter. The breakdown in the permeability barrier and the consequential depolarization of the inner membrane results in the inhibition of the growth of *E. coli* but not of their lysis. SPF, however, lysed erythrocytes. The gradual release of hemoglobin during lysis as well as the lysis protection experiments indicate that a colloid-osmotic process is involved in hemolysis. Although SPF is comprised of only 13 amino acid residues, circular dichroism studies indicate that SPF adopts an α-helical structure particularly in hydrophobic environment. Edmundson wheel projection indicates that the

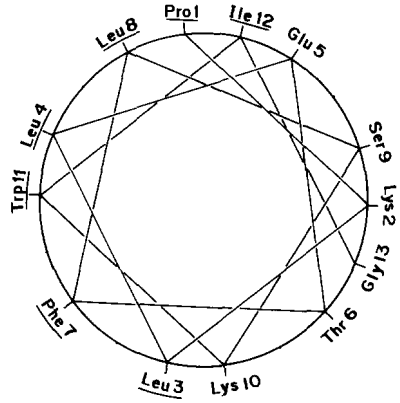

FIG. 9. **Projection of the sequence of SPF as an α-helical wheel according to Schiffer and Edmundson (19).** Hydrophobic residues are *underlined.*

helix is amphiphilic in nature with a polar face comprising of Glu, Ser, Lys, Gly, and Thr residues and an apolar face comprising Ile, Pro, Leu, Trp, and Phe residues. A hydrophobic moment (20) of ~0.41 is obtained for SPF which is comparable to many of the recently reported membrane-active peptides (21, 22). Thus SPF is one of the very few 13-residue peptides having antibacterial and hemolytic activity. It thus appears that the length of the peptide chain and thereby the length of α-helix is not a crucial factor for membrane activity. Results reported in this paper as well as studies on the 13-residue peptides crabrolin (21) and bombolitin (21) and recently reported model peptides (22) indicates that juxtaposition of the side chains in the helix (that is, the extent of amphilicity) is an important factor for membrane activity.

The gradual release of hemoglobin and the lysis protection experiments also argue against a mechanism of lysis involving solubilization of the membrane by a detergent-like action of the peptide. Lysis protection experiments indicate pores of 40 Å. Unlike in the case of hemolysis induced by gramicidins and melittin (15) the size of the lesion seems independent of the peptide concentration. It therefore seems possible that SPF aggregates in the membrane phase to form hydrophilic channels, in spite of its short length. A 13-residue NH_2-terminal fragment of alamethicin has been shown to be active in ion transport and uncoupling oxidative phosphorylation in mitochondria (23–25).

The peptide SPF corresponds to the single hydrophobic domain in seminal plasmin. The membrane-perturbing ability of SPF was examined with a view to evaluate the importance of this region in the antibacterial and eukaryotic cell lytic activity of SPLN (26). The peptide has bacteriostatic activity but does not lyse bacterial cells, whereas SPLN has both bacteriostatic and bacteriolytic activity. SPF exhibits lytic activity against erythrocytes and dividing cells, whereas SPLN specifically lyses only dividing cells. However preliminary observations indicate that SPF, like SPLN, increases the fluidity of sperm plasma membrane. While regions other than the sequence corresponding to SPF clearly modulate the antibacterial and selective eukaryotic cell lytic activity of SPLN, we propose that the sequence corresponding to SPF is primarily responsible for the membrane-perturbing ability of SPLN. Evaluation of the biological activity of analogs of SPLN without the SPF sequence or with variants of this sequence would help in confirming our hypothesis. Studies directed toward this end are currently in progress.

Acknowledgments—We thank Dr. P. M. Bhargava for comments and discussion. We are grateful to V. M. Dhople for amino acid

analysis and Krishna Kumari for help in purification of SPF by HPLC.

REFERENCES

1. Bernheimer, A. W., and Rudy, B. (1986) *Biochim. Biophys. Acta* **864,** 123–141
2. DeGrado, W. F. (1988) *Adv. Protein Chem.* **39,** 51–124
3. Steiner, H., Multmark, D., Engstrom, A., Bennich, H., and Boman, H. G. (1981) *Nature* **292,** 246–248
4. Thompson, S. A., Tachibana, K., Nakanishi, K., and Kubota, I. (1986) *Science* **233,** 341–343
5. Lazarovia, P., Primor, N., and Loew, L. M. (1986) *J. Biol. Chem.* (1986) **261,** 16704–16713
6. Kaiser, E. T., and Kezdy, F. J. (1987) *Annu. Rev. Biophys. Bioeng.* **16,** 561–81
7. Reddy, E. S. P., and Bhargava, P. M. (1979) *Nature* **279,** 725–728
8. Shivaji, S., Scheit, K. H., and Bhargava, P. M. (1989) *Proteins of Seminalplasma* pp. 331–356, John Wiley & Sons, New York
9. Sitaram, N., Krishna Kumari, V., and Bhargava, P. M. (1986) *FEBS Lett.* **201,** 233–236
10. Laxma Reddy, G., Bikshapathy, E., and Nagaraj, R. (1985) *Tetrahedron Lett.* **26,** 4257–4260
11. Laxma Reddy, G., and Nagaraj, R. (1986) *Proc. Indian Acad. Sci. (Chem. Sci.)* **97,** 71–75
12. Laxma Reddy, G., and Nagaraj, R. (1989) *J. Biol. Chem.* **264,** 16591–16597
13. Miller, J. H. (1972) *Experiments in Molecular Genetics,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Garen, A., and Levinthal, C. (1960) *Biochim. Biophys. Acta* **38,** 470–483
15. Katsu, T., Ninomiya, C., Kuroko, M., Kobayashi, H., Hirota, T., and Fujita, Y. (1988) *Biochim. Biophys. Acta* **939,** 57–63
16. Scherrer, R., and Gerhardt, P. (1971) *J. Bacteriol.* **107,** 718–735
17. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132
18. Woody, R. W. (1982) *The Peptides* (Udenfriend, S., and Meienhofer, J., eds) Vol. 7, pp. 16–114, Academic Press, New York
19. Schiffer, M., and Edmundson, A. B. (1967) *Biophys. J.* **7,** 121–135
20. Eisenberg, D., Weiss, R. M., and Terwilliger, T. C. (1982) *Nature* **299,** 371–374
21. Argiolas, A., and Pisano, J. J. (1985) *J. Biol. Chem.* **260,** 1437–1444
22. Suenaga, M., Lee, S., Park, N. G., Aoyagi, H., Kato, T., Umeda, A., and Amako, K. (1989) *Biochim. Biophys. Acta* **981,** 143–150
23. Nagaraj, R., Mathew, M. K., and Balaram, P. (1980) *FEBS Lett.* **121,** 365–368
24. Mathew, M. K., Nagaraj, R., and Balaram, P. (1981) *Biochem. Biophys. Res. Commun.* **98,** 548–555
25. Mathew, M. K., Nagaraj, R., and Balaram, P. (1982) *J. Biol. Chem.* **257,** 2170–2176
26. Chitnis, S. N., Prasad, K. S. N., and Bhargava, P. M. (1987) *J. Gen. Microbiol.* **133,** 1265–1271