Identification of the region that plays an important role in determining antibacterial activity of bovine seminalplasmin

Narasimhaiah Sitaram*, Chilukuri Subbalakshmi, Viswanath Krishnakumari, Ramakrishnan Nagaraj

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

Received 30 August 1996; revised version received 21 November 1996

Abstract Seminalplasmin (SPLN) is a 47-residue protein isolated from bovine seminal plasma having potent antimicrobial activity against a broad spectrum of microorganisms. SPLN, also known as caltrin, acts as a calcium transport regulator in bovine sperms. Analysis of the sequence of SPLN reveals a 27-residue stretch with the sequence SLSRYAKLANRLANPKLLETFLSKWIG more hydrophobic than the rest of the protein. It is demonstrated that a synthetic peptide corresponding to this 27-residue segment has antimicrobial activity comparable to that of SPLN. It does not exhibit hemolytic activity at concentrations where antibacterial activity is observed. Since P27 can be conveniently obtained in large amounts by chemical synthesis, it could serve not only as a starting compound to obtain peptides with improved antibacterial activity but also to understand the role of SPLN in reproductive physiology.

Key words: Antimicrobial protein; Antimicrobial peptide; 27-residue peptide; Hemolytic activity; Secondary structure; Seminalplasmin/caltrin

1. Introduction

There has been considerable interest in determining the constituents of seminal plasma possessing antimicrobial activity from as early as 1949, following the observation that it had the ability to inhibit the growth of *S. aureus* [1,2]. The unequivocal demonstration of the presence of a small molecular weight, non-enzymatic protein in bovine seminal fluid came from the isolation and characterization of seminalplasmin (SPLN), a 47-residue peptide [3–5]. Antibacterial peptides have been shown to play an important role in the innate immunity of many animal species [6]. Antibacterial peptides also appear to be present in the reproductive tract. The gene for a putative 39-residue antibacterial peptide FALL-39 is expressed in human testis [7]. A 32-residue antibacterial peptide andropin has been characterized from ejaculatory duct of *Drosophila* [8]. The female accessory sex gland of the medfly *Ceratitis capitata* has been found to contain two 29-residue antibacterial peptides: ceratotoxins A and B [9]. Thus, the presence of antimicrobial peptides in the reproductive tract may serve an important function in offering protection against infection. SPLN exhibits antimicrobial activity against a variety of microorganisms [3,10]. The peptide also exhibited specificity in activity, i.e., it did not have hemolytic activity over a wide concentration range [11,12]. Interestingly, the peptide specifically lysed dividing cells in culture [11]. We have been interested in mechanisms by which SPLN exerts its activity. Several lines of evidence suggest that SPLN exerts its activity by permeabilizing the bacterial membranes [13] very much like the subsequently discovered endogenous antibacterial peptides from species right across the evolutionary scale like cecropins, defensins and magainins [6,14,15]. Since the interaction of SPLN with lipids appears to be important in the manifestation of its activity, the sequence of SPLN was analysed in order to delineate segments that would conceivably have a high potential to interact with lipids [12,16]. Two 13-residue stretches corresponding to 14–26 and 28–40 regions of SPLN, i.e., SLSRYAKLANRLA (SLS) and PKLLETFLSKWIG (SPF) (Fig. 1) having a good propensity to interact with lipids were identified [12]. We have investigated the activity of a 27-residue peptide, P27, with the sequence SLSRYAKLANRLANPKLLETFLSKWIG, encompassing both the 13-residue segments SLS and SPF. The results of our investigations are described in this paper.

2. Experimental procedures

2.1. Peptide preparation

Peptide P27 was synthesized by Fmoc chemistry on a Milligen Peptide Synthesizer and the peptide-bound resin was kindly provided by Dr. K.R. Kumaraswamy of Millipore (India) Pvt. Ltd. The peptide was cleaved from the resin with trifluoroacetic acid/thioanisole/meta-cresol/ethanedithiol (10:1:1:0.5, v/v). The crude peptide was purified by HPLC on a Bio-rad (Hi-Pore RP 304) C4, 250×4.6 mm column using the solvents A (H_2O containing 0.1% TFA) and B (CH_3CN containing 0.1% TFA). A linear gradient of 0–100% in 60 min at a flow rate of 1 ml/min was used. The desired peptide eluted at ~26.5 min. Composition of the peptide was checked by amino acid analysis on a LKB 4151 Alpha Plus Amino Acid Analyzer and was sequenced on an Applied Biosystems 473A Protein Sequencer.

2.2. Antimicrobial activity

The antimicrobial activities of the peptides were assayed by procedures described earlier under aerobic conditions [17]. Different concentrations of peptide were added to 1 ml of nutrient broth medium containing the inocula of test organism, in mid-logarithmic phase of growth, ~10^6CFU/ml. The nutrient broth contained 6 g of bacto nutrient broth (Difco) and 5 g of NaCl per liter. Microbial growth was determined by the increase in OD_{600} after incubation of the samples at 37°C or 30°C for 6–9 h. The lowest concentration that resulted in complete inhibition of growth was recorded as 100% minimal inhibitory concentration (MIC). The microorganisms were *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis*. The bactericidal effect of P27 against *E. coli* and *S. aureus* was determined by plating suitably diluted aliquots of the culture after exposure to different concentrations of the peptide for different specified time intervals on nutrient agar plates. The plates were incubated for 18 h at 37°C for colony counting.

Inner membrane permeability of *E. coli* W160.37 was monitored by measuring the β-galactosidase activity in the cells in stationary phase of growth after incubating the cells (in which the enzyme has been induced with 5×10^{-4} M IPTG), in the presence of different concentrations of the peptides using ONPG as the substrate [17]. Cells in-

*Corresponding authors: N. Sitaram/R. Nagaraj. Fax: 91-40-671195. E-mail: nraj@ccmb.globemail.com

PII S0014-5793(96)01406-8

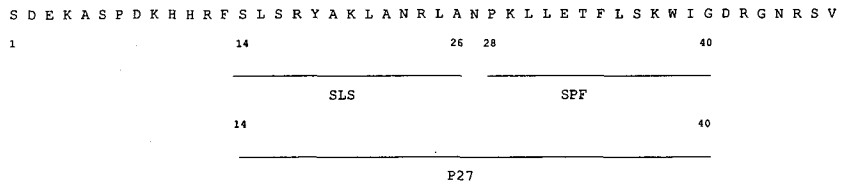

Fig. 1. Primary structure of bovine seminalplasmin and seminalplasmin-related peptides SLS, SPF and P27 corresponding to segments 14–26, 28–40 and 14–40 of seminalplasmin.

Table 1
Antimicrobial activity of SPLN-related peptides

Organism	MIC (μg/ml)			
	SPLN	SLS	SPF	P27
E. coli W160-37	20–30	60–70	50–60	10–20
Pseudomonas putida NCIM 2102	20	NA	NA	50–60
S. aureus PEP 4/3	< 50	NA	NA	40–50
B. subtilis	20–30	NA	100–120	10–20

NA: not active.

cubated in the absence of the peptide was taken as the control. OD measurements were made at 550 nm and 420 nm and ($A_{420}-1.75\times A_{550}$) was taken to measure the β-galactosidase activity.

2.3. Hemolytic assay

Erythrocytes from rat were isolated from heparinized blood by centrifugation and washed three times with phosphate-buffered saline (PBS, 5 mM phosphate buffer containing 0.15 M NaCl, pH 7.4) just before assays were performed. Aliquots of cell suspension ($\sim 10^7$/ml) in eppendorf tubes were incubated at 37°C with peptides in duplicates for 30 min with gentle mixing. The tubes were then centrifuged and the absorbance of the supernatants was measured at 540 nm. The lysis obtained with 1% Triton X-100 was considered 100%.

2.4. Circular dichroism studies

Spectra were recorded in a Jobin Yvon Mark V Spectropolarimeter. Peptides were dissolved at a concentration of 0.1 mM in Hepes buffer, pH 7.4, and trifluoroethanol. All spectra were recorded at 25°C and data are represented as mean residue ellipticities. Peptide concentration was quantitated by amino acid analysis. Spectra were deconvoluted by the convex constraint analysis (CCA) program kindly provided by Prof. G.D. Fasman [18].

3. Results and discussion

The antimicrobial spectrum of P27 is presented in Table 1. The peptide is more active than the other earlier described active shorter synthetic segments of SPLN, SPF [17] and SLS [19]. In fact, the potency against *E. coli* is comparable to that of SPLN. The peptide also exhibits activity against other microorganisms like SPLN with more or less comparable potency. Thus, the peptide spanning residues 14–40 of SPLN has antimicrobial activity comparable to that of SPLN, unlike the two 13-residue segments that exhibit moderate activity, preferentially against *E. coli*. In order to determine whether P27 acts as a bacteriostatic or bactericidal agent, the number of viable cells after incubating the *E. coli* and *S. aureus* in the presence of the peptide, in the case of *E. coli* and *S. aureus*, was determined. The results are presented in Table 2. At a peptide concentration of 15 μg/ml, *E. coli* culture is virtually sterile in a period of 2 h and in 1 h duration at a peptide concentration of 30 μg/ml. Similar results were also obtained with *S. aureus*. In order to determine whether P27 exerts its activity by permeabilizing the bacterial membrane, disruption of the *E. coli* inner membrane in the presence of the peptide was monitored and the data are presented in Fig. 2. It is evident that P27 effectively permeabilizes the *E. coli* inner membrane.

SPLN does not exhibit hemolytic activity even at high concentrations (150 μg/ml) whereas the 13-residue peptide SPF exhibits hemolytic activity at the same concentration range as its antimicrobial activity [17]. In the case of P27 whether the improved antibacterial activity was accompanied by an increase or decrease in hemolytic activity was then examined. Fig. 3 shows the hemolytic activity of P27. At its MICs for *E. coli*, *S. aureus* and *B. subtilis*, the hemolysis observed is practically zero. However, above 30 μg/ml hemolysis is observed with 100% lysis of erythrocytes at 180 μg/ml. The peptide segments outside the P27 sequence thus appear to have a role in determining the specificity of activity.

In order to examine whether the differences in the specificity of activity between P27 and SPLN can be attributed to the differences in the conformation of the two peptides, the structure of SPLN was examined in aqueous environment and TFE and the spectra are shown in Fig. 4A,B. In aqueous environment P27 is unordered, whereas in TFE it has a propensity to adopt α-helical structure. Deconvolution of the CD spectrum in TFE indicates a helical content of 65%. Fig. 4B

Table 2
Bactericidal effects of P27

Organism	Concentration of P27 (μg/ml)	Time of incubation (min)	% Viable cells
*E. coli**	15	0	100
		30	16
		60	3
		120	0
	30	0	100
		30	4
		60	0
		120	0
*S. aureus**	30	0	100
		30	0
		60	0

*5×10^6 CFU of the cells were incubated with different concentrations of the peptides in Difco nutrient broth (6 g/L) containing 5 g/L of NaCl.

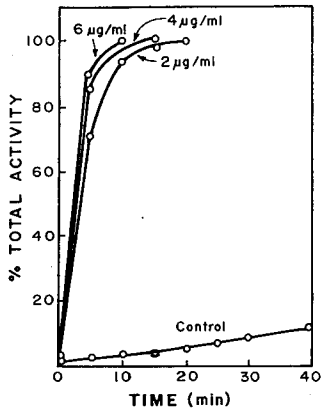

Fig. 2. Effect of P27 on the influx of ONPG into *E. coli* W 160-37. Cells were grown in nutrient broth containing 5×10^{-5} M IPTG overnight to a A_{600} of 1.0. Cells in the stationary phase of growth were diluted 100 times with phosphate buffer containing ONPG and incubated at 37°C with different concentrations of the peptides. The absorption at 420 nm and 550 nm were recorded at various time points. The value ($A_{420}-1.75\times A_{550}$) was taken to denote ONPG influx. Cells incubated in the absence of any peptide was taken as the control.

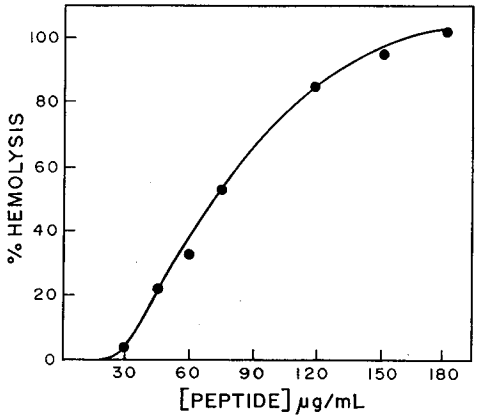

Fig. 3. Hemolysis of rat erythrocytes as a function of peptide concentration. Erythrocytes ($\sim10^7$/ml) were incubated in phosphate buffered (10 mM) isotonic saline containing various concentrations of P27 and SPF for 20 min.

indicates that SPLN is more ordered than P27 in aqueous environment. However, in TFE, the spectra of the two are considerably different. The lower values for SPLN and cross over at less than 200 nm would suggest a greater proportion of unordered structure. Deconvolution of the spectra indicate the presence of β-sheet and helical conformation in buffer with individual contribution of 25 and 4%, respectively, with a random contribution ~70%. In TFE, the extent of contribution of component corresponding to α-helical conformation increases to 30% with β-sheet structure contribution ~10%. The random contribution is ~50%. SPLN thus appears to have lower helical content than P27 with a small fraction existing β-sheet conformation.

There has been tremendous interest in structure–function correlation studies on endogenous antibacterial peptides not only with a view to understand their mechanism of action but also explore the possibility of using them as therapeutic agents. This has been necessary as resistance to clinically used antibacterial drugs are a serious problem in clinical medicine [20–23]. However, the lengths of the endogenous peptides like cecropins as well as the complex structures like in defensins have made it difficult to get short peptides derived from them with improved or even comparable activity. Several short peptides with good activity have indeed been synthesized but these are model peptides or hybrids of the toxin melittin and antimicrobial peptides [23,24]. In the case of SPLN, structure–function studies have lead to the identification of a peptide substantially shorter than the parent peptide having comparable antimicrobial activity. Though this 27-residue peptide does have antibacterial activity, it has hemolytic properties which SPLN does not have. It should be possible to remove this activity by suitable engineering as demonstrated for SPF [25]. Thus, P27 along with magainins and cecropins should be a good candidate for exploring the possibility of using peptides as antibacterial drugs.

SPLN has recently been shown to be a member of the *neuropeptide Y* gene family [26]. Apart from its antimicrobial activity SPLN, alternately known as caltrin [4,27,28], has several other properties including Ca^{2+} transport regulatory activity as well as modulation of zona pellucida-induced acrosome reaction in bovine sperms [29,30]. It has been proposed that, although SPLN inhibits spontaneous acrosomal exocytosis by inhibiting the Na^+/Ca^{2+} antiporter pathway, its presence on the sperm membrane is essential for bringing about the physiologically relevant zona pellucida agonist-induced acrosomal reaction in bovine sperms [29–31]. It would thus be of interest to examine whether P27 would exhibit activities

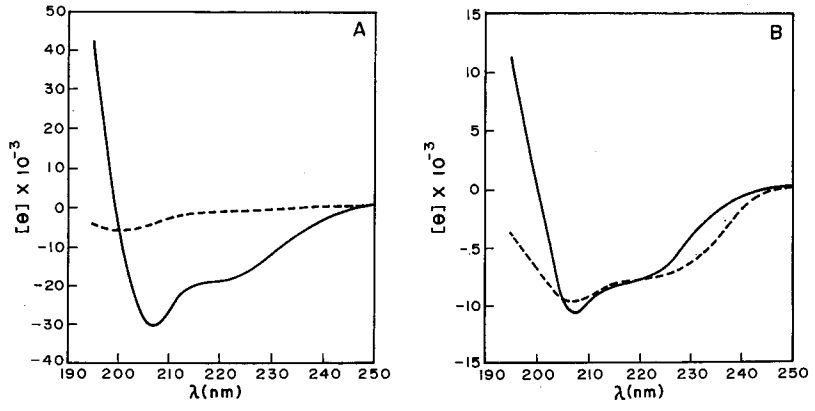

Fig. 4. CD spectra of P27 (0.1 mM) and SPLN (0.1 mM) at 25°C in Hepes buffer (broken line) (5 mM, pH 7.4) and TFE (solid line). (A) P27; (B), SPLN.

similar to that of caltrin on spermatozoa. Since P27 would be easier to obtain by chemical synthesis as compared to SPLN, the peptide would serve not only as a good candidate for peptide engineering to get antibacterial peptides with improved activity but also understand the role of SPLN in reproductive physiology.

Acknowledgements: Financial support from Department of Science and Technology, Government of India is gratefully acknowledged. We thank V.M. Dhople for amino acid analysis and M.V. Jagannadham for carrying out sequencing of P27.

References

[1] Rozansky, R., Gorevitch, J., Bazeninsky, A. and Eckerling, B. (1949) J. Lab. Clin. Med. 34, 1526–1529.
[2] Shivaji, S. (1984) Trends Biochem. Sci. 9, 104–107.
[3] Reddy, E.S.P. and Bhargava, P.M. (1979) Nature 279, 725–728.
[4] Sitaram, N., Krishna Kumari, V. and Bhargava, P.M. (1986) FEBS Lett. 201, 233–236.
[5] Sitaram, N. and Nagaraj, R. (1995) Bioessays 17, 417–422.
[6] Boman, H.G. (1995) Annu. Rev. Immun. 13, 61–92.
[7] Agerberth, B., Gunne, H., Odeberg, J., Kogner, P., Boman, H.G. and Gudmundsson, H.G. (1995) Proc. Natl. Acad. Sci. USA 92, 195–199.
[8] Samkovlis, C., Kylsten, P., Kimbrell, A., Engstorm, A. and Hultmark, D. (1991) EMBO J. 10, 417–422.
[9] Marchini, D., Giordano, P.C., Amons, R., Bemini, L.F. and Dallai, R. (1993) Insect Biochem. Molec. Biol. 23, 591–598.
[10] Shivaji, S., Scheit, K.H. and Bhargava, P.M. (1990) Proteins of Seminal Plasma. John Wiley, New York.
[11] Murti, T.R., Chandani, S.A., Khar, A., Reddy, G.L. and Bhargava, P.M. (1994) Biochim. Biophys. Acta 1221, 109–114.
[12] Sitaram, N. (1993) Structure-Function Correlations in Antibacterial and Cytolytic Peptides: Studies on Seminalplasmin, Seminalplasmin-derived and Related Synthetic Peptides. Ph.D. Thesis. Jawaharlal Nehru University, New Delhi.
[13] Sitaram, N., Krishna Kumari, V. and Nagaraj, R. (1992) FEBS Lett. 303, 265–268.
[14] Bevins, C.L. and Zasloff, M. (1990) Annu. Rev. Biochem. 59, 395–417.
[15] Lehrer, R.I., Lichtenstein, A.K. and Ganz, T. (1993) Annu. Rev. Immun. 11, 105–128.
[16] Sitaram, N. and Nagaraj, R. (1993) Biochemistry 32, 3124–3130.
[17] Sitaram, N. and Nagaraj, R. (1990) J. Biol. Chem. 265, 10438–10442.
[18] Perczel, A., Park, K. and Fasman, G.D. (1992) Anal. Biochem. 203, 83–93.
[19] Sitaram, N., Subbalakshmi, C. and Nagaraj, R. (1993) FEBS Lett. 328, 239–242.
[20] Silver, L.L. and Bostian, K.A. (1993) Antimic. Agents. Chemother. 37, 377–383.
[21] Chin, G.J. and Mark, J. (Ed.) (1994) Science 264, 359–393.
[22] Swartz, M.N. (1994) Proc. Natl. Acad. Sci. USA 91, 2420–2427.
[23] Blondelle, S.E. and Houghton, R.A. (1992) Biochemistry 31, 12688–12694.
[24] Andreu, D., Ubach, J., Boman, A., Wahlin, B., Wade, D., Merrifield, R.B. and Boman, H.G. (1992) FEBS Lett. 296, 190–194.
[25] Sitaram, N., Chandy, M., Pillai, R. and Nagaraj, R. (1992) Antimicrob. Agents. Chemother. 36, 2468–2472.
[26] Herzog, H., Hort, Y., Scheider, R. and Shine, T. (1995) Proc. Natl. Acad. Sci. USA 92, 594–598.
[27] Rufo, G.A., Singh, J.P., Babcock, D.F. and Lardy, H.A. (1982) J. Biol. Chem. 257, 4627–4632.
[28] Lewis, R.V., San Agustin, J., Kruggel, W. and Lardy, H.A. (1985) Proc. Natl. Acad. Sci. USA 82, 6490–6501.
[29] San Agustin, J.T., Hughes, P. and Lardy, H.A. (1987) FASEB. J. 1, 60–66.
[30] Clark, E.N., Corron, M.N. and Florman, H.N. (1993) J. Biol. Chem. 268, 5309–5316.
[31] San Agustin, J.T. and Lardy, H.A. (1989) J. Biol. Chem. 265, 6860–6867.