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Identification of a second membrane-active 13-residue peptide segment in the antimicrobial protein, bovine seminalplasmin

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Seminalplasmin (SPLN) is a 47-residue protein from bovine seminalplasma having broad-spectrum antibacterial activity. The protein has no hemolytic activity. SPLN interacts with lipid vesicles and its antibacterial activity appears to stem from its ability to permeabilize the bacterial plasma membrane. Analysis of SPLN's primary structure, with respect to its relative hydrophobicity and hydrophilicity, revealed a segment, PKLLETFLSKWIG, more hydrophobic than the rest of the protein. A synthetic peptide corresponding to this region had not only antibacterial activity but also hemolytic properties. Analysis of the SPLN sequence based on hydrophobic moment plots has revealed a second segment, SLSRYAKLANRLA, which could be membrane active. A synthetic peptide corresponding to this region shows only antibacterial activity with no hemolytic activity.

Antimicrobial protein; Antimicrobial peptide; 13-Residue peptide; Bovine seminalplasmin; Membrane activity; Hemolytic activity

1. INTRODUCTION

Peptides possessing antimicrobial activity play an important role in insect immunity [1], contribute to host defense mechanisms in higher mammals [2], and also prevent infection in frogs, particularly on the skin surface [3]. Although some of these peptides, like cecropins and defensins, have significant potency, as well as a broad antimicrobial spectrum, antiviral and antifungal properties [1,4,5], they are relatively long, composed of ~ 37 residues. Also, defensins adopt highly folded structures with three disulphide bridges [6]. Hence, synthesis of these peptides in large quantities, as would be needed for use as a drug, would be difficult and expensive, and consequently their use as therapeutic agents is likely to be limited. The biological activity of these peptides results from their ability to form ion channels through membranes [7,8]. It would thus be pertinent to identify and examine the membrane activity of short segments of these peptides, particularly those which adopt amphiphilic α-helical conformations, as they would help to reveal the minimum requirement for biological activity. Also, short peptides would be more amenable to synthesis, and consequently would be of use in therapeutic applications.

Seminalplasmin (SPLN) is a 47-residue protein from bovine seminalplasma (Fig. 1) that has broad-spectrum antibacterial activity with no hemolytic properties [9,10]. The protein appears to exert its activity by permeabilizing the bacterial plasma membrane [11]. It also interacts with lipid vesicles, and its biological activity can be rationalized in terms of lipid–peptide interactions [12]. With a view to delineate regions of the protein that might have features which would favour interaction with membranes, its primary structure was analyzed with respect to its relative hydrophobicity and hydrophilicity, based on the method of Kyte and Doolittle [13]. A region comprising 13 amino acids, PKLLETFLSKWIG (SPF), was identified to be more hydrophobic than the rest of the protein [14]. A synthetic peptide corresponding to this region had not only antimicrobial activity but also lysed erythrocytes. In this paper we report the identification of another 13-residue segment, SLSRYAKLANRLA (SLS), in SPLN, based on hydrophobic moment analysis, having only antibacterial activity without hemolytic properties.

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2. EXPERIMENTAL

Peptide synthesis was achieved on triethyleneglycoldimethylacrylate-crosslinked (1%) polystyrene resin using t-boc chemistry and protocols essentially as described earlier [15,16]. The peptide was purified by fast-performance liquid chromatography (FPLC) and characterized by amino acid analysis and sequencing on an Applied Biosystems 473A sequencer.

The antibacterial activity of the peptide was determined by incubating an inoculum of *E coli* W 160.37 (10^7 CFU/ml) in minimal A medium [17] for 6 h in the absence and presence of various concentrations of peptide. The value of A_{600} for control cultures, which contained no peptide, was taken as 100%.

Lipid vesicles with entrapped carboxyfluorescein (CF) were prepared as follows. A lipid film of dioleoylphosphatidyl choline (DOPC) (Avanti Polar Lipids) was dispersed in 5 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), pH 7.4, containing 50 mM

S D E K A S P D K H H R F S L S R Y A K L A N R L A N P K L L E T F L S K W I G D R G N R S V
1 10 20 30 40 47

Fig. 1. Primary structure of bovine seminalplasmin.

NaCl and 100 mM CF, and sonicated to clarity. Liposomes were separated from non-encapsulated CF by gel-filtration on Sephadex G-75 (elution buffer, 5 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA). The excitation and emission monochromators were set at 493 nm and 520 nm, respectively. Complete release of CF from lipid vesicles was obtained by addition of Triton X-100 (0.1% v/v) and the fluorescence value was taken as 100% release. Fluorescence was continuously monitored after addition of peptide to lipid vesicles.

3. RESULTS AND DISCUSSION

SPLN adopts an α-helical conformation, particularly in hydrophobic environment. Recent reports indicate that peptide fragments of a helical protein or peptide show marked conformational preference for helix and nascent helix [18–20]. Hence, we analyzed the sequence of SPLN with the hydrophobicity and hydrophobic moment plot according to the method of Eisenberg and Wesson for amphiphilic α-helices [21] with the use of an 11-residue window. Apart from the sequence of SPF, which falls in the area of surface-seeking peptides in the hydrophobic moment vs. hydrophobicity plot, another sequence, SLSRYAKLANRLA (SLS), was also found to fall in this region. This sequence has a hydrophobic moment of 0.68 and average hydrophobicity of −0.19. This sequence has also been postulated as the calmodulin-binding domain in SPLN, based on a similar analysis [22]. Hence, a peptide corresponding to this sequence was synthesized and its antimicrobial properties determined.

The inhibition of growth of *E. coli* in the presence of varying concentrations of peptide SLS is shown in Fig. 2. The data indicate a minimal inhibitory concentration of 60 μg/ml. At this concentration and above (up to 150 μg/ml) no lysis of erythrocytes was observed. The specific antibacterial activity of SLS can be rationalized in terms of differences in the interaction with the bacterial and red blood cell surfaces, as in the case of SPLN and SPF [12]. The presence of two arginine residues and one lysine residue would favour binding of SLS to the negatively charged lipopolysaccharide molecules located on the exterior of the outer membrane of *E. coli* [23] and disrupt the structure, facilitating entry into the cytoplasmic membrane. It is likely that SLS then permeabilizes the bacterial plasma membrane like SPLN and SPF. The peptide does not reach the lipid bilayer of the erythrocyte membrane, probably as a consequence of binding to sialic acid residues which project out of the membrane surface.

In order to confirm the membrane permeabilizing ability of SLS, we examined its ability to cause release of CF from model membranes. The fluorescence of CF

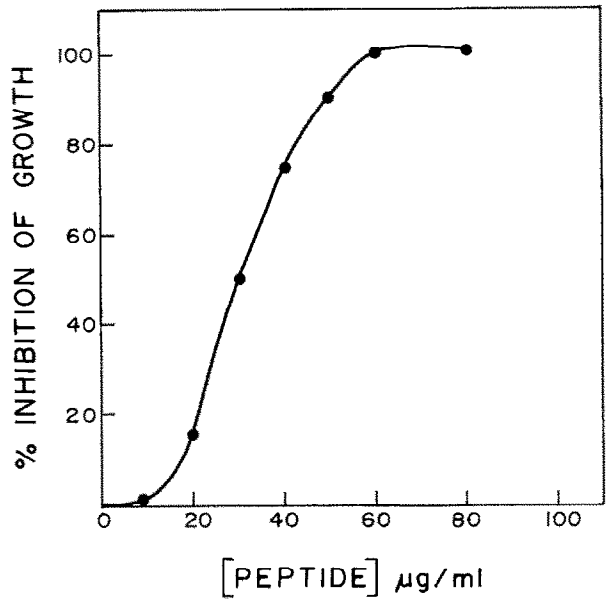

Fig. 2. Antimicrobial activity of SLS. *E. coli* W160-37 was incubated with varying concentrations of peptide in minimal A medium (1 ml) for 6 h. The absorbance at 600 nm was measured after 6 h and the value for control cultures which contained no peptides was taken as 100%.

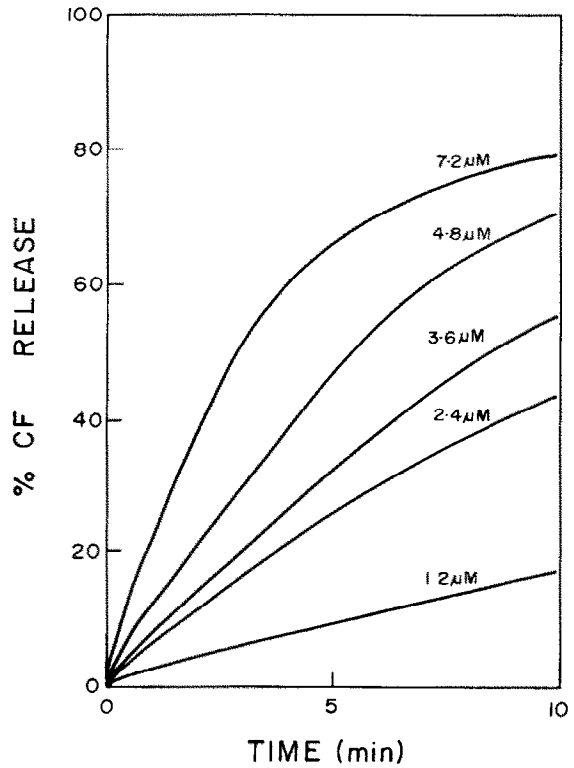

Fig. 3. Release of entrapped carboxyfluorescein (CF) from DOPC vesicles in the presence of varying concentrations of SLS. Lipid = 100 μM. The concentration of peptide is indicated on the traces.

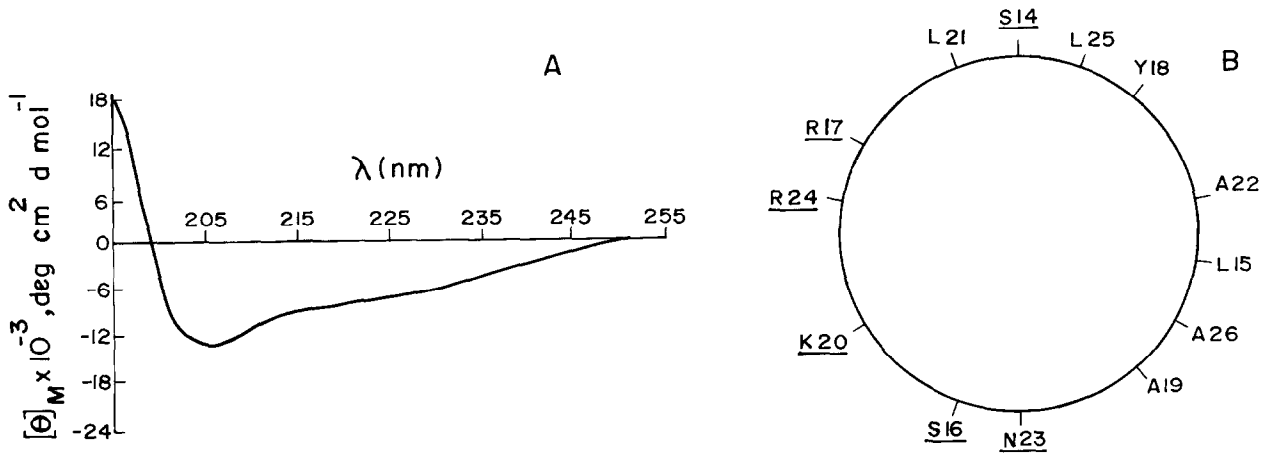

Fig. 4. Circular dichroism spectrum (CD) and helical wheel projection of SLS. (A) CD spectrum in micelles of SDS (30 mM), peptide concentration = 0.05 mg/ml. The spectrum was recorded in a Jobin Yvon dichrograph v spectropolarimeter at 25°C in cells of 1 mm path length. θ values are mean residue ellipticities. (B) Helical wheel projection.

is very weak when entrapped in lipid vesicles and enhanced considerably on dilution [24]. Hence increase of CF fluorescence is a measure of vesicle permeabilization. The CF release profiles from DOPC vesicles in the presence of varying concentrations of SLS as a function of time is shown in Fig. 3. CF release is clearly discernible. Similar results were obtained with lipid vesicles composed of phosphatidylethanolamine and phosphatidylglycerol which mimic the lipid composition of the bacterial inner membrane. It is evident that SLS, like SPLN and SPF, has the ability to form pores in model membranes.

In order to determine whether SLS has the propensity to adopt a helical structure, its conformation in an hydrophobic environment was examined by circular dichroism (CD) spectroscopy. The CD spectrum shown in Fig. 4A indicates a tendency of the peptide to adopt a helical conformation. The low helical content (~ 30%, estimated by the method of Taylor and Kaiser [25]) could arise due to the strong length dependence of the ellipticity values in peptides [26]. The axial projection of the helical conformation shown in Fig. 4B indicates the amphiphilic nature of the helix.

Thus both the 13-residue segments of SPLN predicted to be 'surface seeking' have antimicrobial activity. While SLS is not hemolytic, SPF has hemolytic activity. The peptides have the ability to form pores in lipid vesicles. The peptides span the region between residues 14 and 40 in SPLN, and our contention that regions 14–26 and 29–40 span the lipid bilayer and are responsible for forming channels is supported by the membrane activity of SLS and SPF. Regions 1–13 and 41–47 in SPLN have a net positive change and could play a role in effectively disrupting the lipopolysaccharide structure in the outer membrane of *E. coli*. In fact the minimal inhibitory concentration of SPLN (30 μg/ml) [14] is less than those of SLS and SPF (50 μg/ml).

Antimicrobial peptides, like ceropins and magainins, adopt predominantly helical conformations, and this structural feature is important for biological activity [27]. Based on hydrophobic moment and hydrophobicity analysis on a number of antimicrobial and hemolytic peptides, Eisenberg and Werson noted short peptide segments which would be strongly 'surface active' [21]. However, the membrane activity of these short peptides have not been examined. A similar analysis on SPLN reveals two segments that could be surface seeking. Our results indicate that 13-residue peptides corresponding to both the segments exhibit antimicrobial activity, indicating that the approach by Eisenberg and Werson is a useful method for identifying short membrane-active peptide segments.

Thus, peptide composed of only 13-residues can adopt helical structures which are amphiphilic and also exhibit antimicrobial activity. Identifying short bioactive segments in long peptide toxins and antibacterial peptides would help not only in rationalizing their biological activity in terms of structure but also help in generating short bioactive peptides that can be of possible therapeutic use.

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