FEBS Letters 395 (1996) 48–52 FEBS 17602

Requirements for antibacterial and hemolytic activities in the bovine neutrophil derived 13-residue peptide indolicidin

C. Subbalakshmi, V. Krishnakumari, R. Nagaraj*, N. Sitaram

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

Received 30 June 1996; revised version received 25 August 1996

Abstract **The antimicrobial and hemolytic activities of the 13-residue peptide indolicidin (ILPWKWPWWPWRR-NH_2), present in bovine neutrophils, and its analogs have been determined with a view to gaining insight into the structural roles of tryptophan and proline. Peptides where proline was replaced by alanine and tryptophan by phenylalanine showed antibacterial activities comparable to that of indolicidin. The peptides do not exhibit a strong propensity to occur in either helical or β-sheet conformation. The peptides also do not appear to exert their activity by permeabilizing the bacterial plasma membrane unlike other endogenous antibacterial peptides. The presence of tryptophan appears to be essential for hemolytic activity as the phenylalanine analog does not exhibit any hemolytic activity.**

Key words: Indolicidin; Antimicrobial activity; Hemolytic activity; Membrane permeabilization

1. Introduction

Indolicidin is a 13-residue peptide amide, isolated and characterized from the cytoplasmic granules of bovine neutrophils, having potent antibacterial activity [1]. The primary structure of the peptide ILPWKWPWWPWRR-NH_2 (IL), is characterized by 5 tryptophan residues, which is the highest mole percentage of any known protein sequence and is unique among known endogenous antibacterial peptides [2–5]. In addition, the tryptophan residues are interspersed with prolines. Although it is presumed to act by disrupting membranes [6], its mechanism of action remains to be established unequivocally. Considerable attention has been focused in recent years on structure-activity relationships in short (i.e. 15–20 residues) antibacterial peptides [7–12] as they have the potential to be used as therapeutic agents in treating bacterial infections due to the serious problem of resistance to several of the currently used antibiotics [13–15]. It has been demonstrated that by judicious positioning of hydrophobic and cationic amino acids, it is possible to generate peptides with specific antibacterial activity [9,10,12]. Indolicidin, though possessing potent antibacterial activity, is highly cytotoxic, rendering it unsuitable as a potential therapeutic molecule. In an effort to gain insight into the structural roles of tryptophan and proline residues in indolicidin and explore the possibility of modulating its activity, we have compared its biological properties with peptides wherein the 3 prolines have been replaced by alanines, i.e. ILAWKWAWWAWRR-NH_2 (ILA) and 5 tryptophans replaced by phenylalanines, i.e. ILPFKFPFFPFRR-NH_2 (ILF). We have observed that ILA has biological activities comparable to that of IL whereas ILF exhibits only potent antimicrobial activity without any hemolytic activity.

2. Materials and methods

2.1. Synthesis of peptides

Indolicidin (IL) and its two analogs ILA and ILF were synthesized by the solid-phase method on a semiautomated synthesizer (LKB Biolynx 4175) using Novasyn KR resin (Novabiochem, UK) by fluorenylmethoxycarbonyl (Fmoc) chemistry [16]. All amino acids were added as Fmoc-hydroxybenzotriazole active esters. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/thioanisole/phenol/water/ethanedithiol (16.5:1:1:1:0.5 v/v) for 5 h at room temperature. The peptides were checked for purity on HPLC using a reverse-phase column (Bio Rad C4 Hi-Pore RP 304, 250×4.6 mm), using a solvent system of 0.1% aqueous TFA and acetonitrile. All the peptides were > 90% pure. However, HPLC purified peptides were used for all studies. The peptides were characterized by amino acid and sequence analyses on a LKB 4151 Alpha plus analyser and 473A Applied Biosystems gas phase sequencer, respectively.

2.2. Antimicrobial activity

The antimicrobial activities of the peptides were determined in liquid medium by a modified method described by Lehrer et al. [17], by growing the microorganisms to late logarithmic phase in nutrient broth (Bacto Difco nutrient broth in the case of bacteria and YEPD in the case of yeast) and diluting it to an OD_{600} of 0.1 ($\sim 10^7$ CFU/ml) in 10 mM sodium phosphate buffer (pH 7.4). Aliquots of 900 μl of the culture so diluted were incubated with different concentrations of the peptides for 1 h at the end of which 100 ml of 10×nutrient broth was added to each of the tubes and further incubated for a period of 6–9 h. All incubations were carried out at 37°C. Microbial growth was determined by measuring the increase in OD_{600}. The lowest concentration of the peptide that resulted in complete inhibition of growth was recorded as its minimal inhibitory concentration (MIC). The microorganisms used were *Escherichia coli* (W 160.37) *Staphylococcus aureus*, *Saccharomyces cerevisiae* and *Candida utilis*. Peptide concentrations in stock solutions were determined by amino acid analysis.

2.3. Outer membrane permeability

Outer membrane permeability was assessed by *N*-phenyl-1-*N*-naphthylamine (NPN, Sigma) uptake assay [18]. *E. coli* W 160-37 cells were grown to late logarithmic phase in bactonutrient broth (Difco) and the cells obtained were washed twice with 5 mM HEPES buffer (pH 7.4). A 1 ml aliquot of the cells so prepared and adjusted to an OD_{600} of 0.5 in the same buffer containing 10 μM of NPN was taken for each experiment. The excitation monochromator was set at 350 nm and the emission at 420 nm was continuously monitored after addition of the peptide from an aqueous stock solution. The experiment was carried out at 25°C and was continuously stirred.

2.4. Inner membrane permeability

Inner membrane (IM) permeability in the presence of the peptides was assessed by the *O*-nitrophenyl-3-D-galactoside (ONPG) influx method as described earlier by Lehrer et al [19]. Briefly, *E. coli* W 160-37 cells were grown to late logarithmic phase in bacto nutrient broth (Difco) in the presence of 5×10^{-4} M isopropyl thiogalactoside (IPTG) for inducing the cytoplasmic enzyme β-galactosidase. This was diluted to an OD_{600} of 0.03 with 10 mM sodium phosphate buffer (pH 7.4) containing 1.67 mM ONPG. Aliquots of this were incubated with

*Corresponding author (and N. Sitaram). Fax: (91) (40) 671195.

0014-5793/96/$12.00 © 1996 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(96)00996-9

Table 1
Antimicrobial activity of indolicidin and its analogs

Peptides	MIC (μg/ml) against			
	E. coli (W 160 37)	*S. aureus* (ATCC 8530)	*S. cerevisiae* (PEP 4/3)	*C. utilis* (CBS 4511)
IL	20–25	2–4	20–25	20–25
ILA	20–25	2–4	10–15	10–15
ILF	25–30	2–4	20–25	20–25

different concentrations of peptides at 37°C and the influx of ONPG into the cells was monitored by OD measurements at 420 and 550 nm at fixed time intervals. ($A_{420}-1.7\times A_{550}$) reflects ONPG influx into the cells and was used as an indicator of the permeability status of the IM.

2.5. Hemolytic activity

Hemolytic activities of the peptides on rat erythrocytes were determined as described earlier [20]. Briefly, erythrocytes were isolated from heparinized rat blood by centrifugation after washing three times with phosphate-buffered saline (PBS, 5 mM phosphate buffer containing 0.15 M NaCl pH 7.4). Aliquots of $\sim 10^7$/ml cells were incubated with different concentrations of the peptides for 30 min at 37°C with gentle mixing. The tubes were then centrifuged and the absorbance of the supernatants were measured at 540 nm. The lysis obtained with 1% Triton X-100 was taken as 100%.

PBS containing a 30 mM concentration of one of the following osmoprotectants, D-mannitol, sucrose, raffinose, PEG of molecular weights 600, 1000 and 3000, in the above assay was used to determine the protection provided by them against hemolysis by the peptides. The molecular diameters (Å) of the substances used were taken as mannitol, 7; sucrose, 9; raffinose, 11; PEG 600, 16; PEG 1000, 20 and PEG 3000, 30 [20].

2.6. Light scattering

Aggregation of IL, ILA and ILF as a function of concentration in aqueous solution was monitored by 90° angle scattering on a spectrofluorimeter by fixing both the excitation and emission monochromators at 600 nm.

All fluorescence experiments were performed on a Hitachi 650-10S spectrofluorimeter.

2.7. CD studies

CD spectra of the peptides were recorded in 5 mM HEPES buffer pH 7.4 and TFE in a Jobin Yvon Dichrograph V Spectropolarimeter calibrated with d_{10}-camphorsulfonic acid, in a cell of 1 mm path length, at 25°C. CD band intensities are represented as mean residue ellipticity. The spectra in TFE were independent of peptide concentration in the range 0.05–0.2 mM.

3. Results

The antimicrobial activity of indolicidin and its analogs are summarized in Table 1. The analogs as well as the parent compound exhibit activity against *E. coli*, *S. aureus*, *S. cerevisiae* and *C. utilis* with comparable potency. Thus, the changes effected in indolicidin, i.e. P→A and W→F, do not appear to have any effect on the antimicrobial properties.

Since a large number of endogenous antibacterial peptides exert their activity by disrupting the outer membrane and subsequently permeabilizing the inner membrane in Gram-negative microorganisms [2–4], we examined the effect of indolicidin and its analogs on the integrity of outer and inner membranes of *E. coli*. The data presented in Fig. 1 indicate that all the three peptides disrupt the OM very effectively. The ability of the peptides to permeabilize the *E. coli* inner membrane was assessed by monitoring the influx of ONPG, a chromogenic substrate for β-galactosidase, a cytoplasmic enzyme. The assay indicated that indolicidin and its analogs do

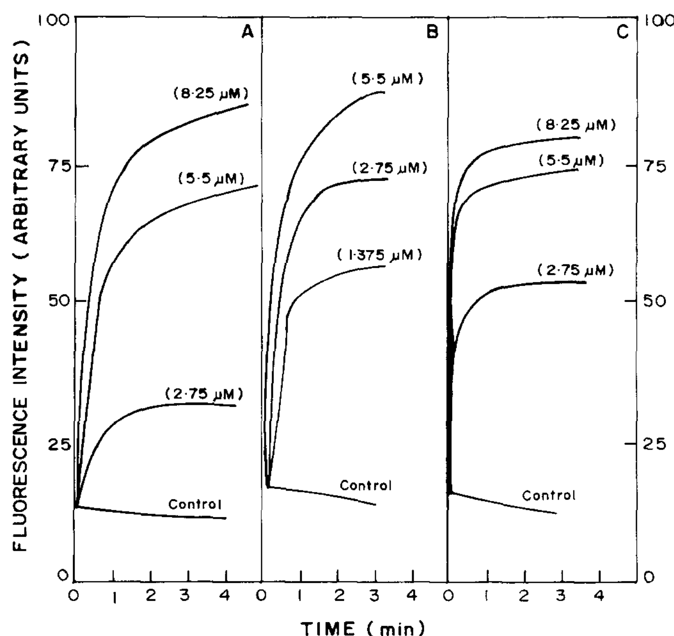

Fig. 1. Effect of indolicidin (IL) and analogs on the outer membrane permeability of *E. coli* measured as increase in the fluorescence intensity of *N*-phenyl-1-naphthylamine (NPN). Cells grown to late logarithmic phase and adjusted to an A_{600} of 0.5 were suspended in 0.5 mM HEPES buffer (pH 7.4) containing 10 mM NPN. Varying concentrations of peptides were added from an aqueous stock solution and the increase in fluorescence was continuously recorded. (A), IL; (B), ILA and (C), ILF.

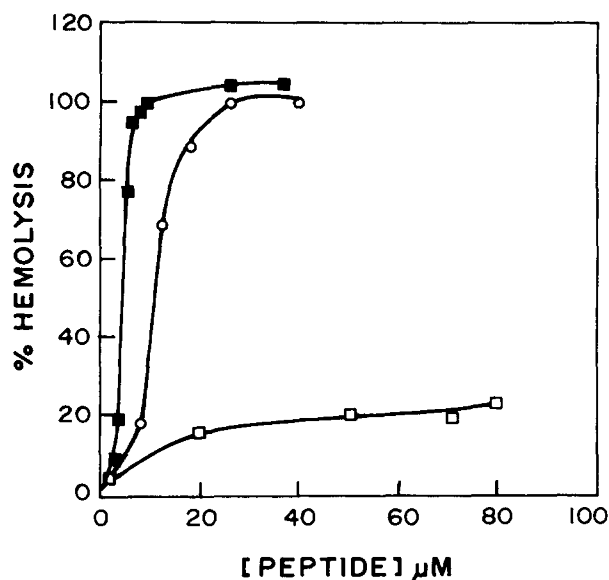

Fig. 2. Hemolysis of rat erythrocytes as a function of peptide concentration. Erythrocytes (0.5% v/v) were incubated in phosphate-buffered (5 mM) saline containing varying concentrations of the peptides for 20 min. (○) IL; (■) ILA; (□) ILF.

not permeabilize the inner membrane up to a concentration of 150 μg/ml which is 6-fold excess of their MICs, unlike defensins and other antibacterial peptides [19–21]. The inability of the peptides to permeabilize the bacterial inner membrane was further confirmed by monitoring the respiration of *E. coli* in a Gilson 5/6 oxygraph fitted with a Clark-type oxygen electrode. No change in respiration was observed immediately on addition of peptide unlike in the case of peptides which exert their activity by membrane permeabilization.

Indolicidin has the ability to lyse erythrocytes [6]. Hence, the effect of the P→A and W→F changes on hemolytic activities was evaluated with the help of ILA and ILF. The data is presented in Fig. 2. While P→A change results in increased activity over indolicidin, the W→F change abolishes hemolytic activity. In order to determine whether lysis was due to a colloid-osmotic process and also to determine the size of the membrane lesion, the hemolysis of red blood cells was studied in the presence of peptides and various osmoprotectants. The data is presented in Fig. 3. Mannitol, sucrose and raffinose offer either no protection or partial protection, whereas PEG 1000 offers partial protection and 3000 offers almost complete protection to hemolysis. The size of the lesions produced appears to be ~30 Å in the case of IL and ILA.

Indolicidin has been shown to aggregate in aqueous medium. The effect of P→A and W→F change on aggregation was examined. The data shown in Fig. 4 indicates that ILA (P→A change) aggregates to a greater extent than indolicidin whereas ILF (W→F) does not. The aggregation properties correlate with the ability of the peptide to cause hemolysis. Thus, strong aggregating tendency and the overall hydrophobicity due to W, presumably, as the hydrophobic index of F is less than that of W [22,23], appear to lead to potent hemolytic activity.

The conformations of IL, ILA and ILF were examined in aqueous buffer and trifluoroethanol (TFE). In buffer, the spectra were characteristic of peptides in unordered conformation. The spectra in TFE are shown in Fig. 5. The spectrum of IL indicates that a substantial population of the molecules is in random conformation even in TFE, presumably due to the presence of 3 P residues. The spectrum of ILA shows a shoulder at ~205 nm and a minimum at ~221 nm with a cross-over at ~197 nm. Clearly, a greater proportion of molecules is in ordered conformation as compared to IL. Deconvolution of the spectrum by the method of convex constraint analysis [24,25] indicates random and distorted helical structure. The spectrum of ILF indicates that a substantial fraction of the molecules populates unordered conformation. However, for all the 3 peptides, unambiguous interpretation of the CD spectra is difficult as the contribution from the substantial number of aromatic residues in the peptides could distort the spectra.

4. Discussion

Indolicidin a 13-residue peptide, is a part of the repertoire of endogenous antibacterial peptides present in bovine neutrophils [1]. The presence of 5 tryptophans and 3 prolines in its sequence is unique among known endogenous antibacterial peptides. In an effort to gain an insight into the mechanism of action of indolicidin and the functional roles of P and W, we have investigated how indolicidin exerts its antibacterial and hemolytic activities and evaluated the biological activities of the analogs. Now, proline is an effective α-helix and β-sheet breaker. The presence of 3 prolines would not favour ordered

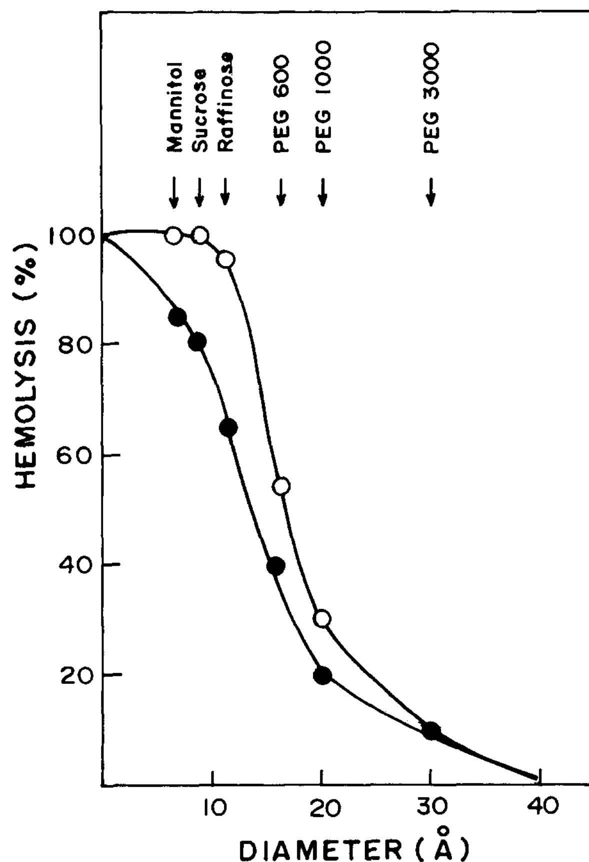

Fig. 3. Hemolysis induced by peptides in the presence of various osmoprotectants. Erythrocytes (0.5% v/v) were suspended in 0.135 mM NaCl, 5 mM phosphate buffer (pH 7.4) and 30 mM protectants. Subsequently, peptides were added and hemolysis was determined after incubation for 30 min at 37°C. (○) IL; (●) ILA.

structure in indolicidin. In order to evaluate the structural role of prolines, they were replaced by an α-helix former, alanine. The tryptophans were replaced by the aromatic amino acid phenylalanine in order to determine whether the tryptophans were essential for activity.

Indolicidin shows broad spectrum activity and for this, P and W do not appear to be essential, as ILA and ILF show almost identical activity profiles. All the peptides studied, being highly cationic, appear to enter Gram-negative bacteria by disrupting the outer membrane (OM) structure. However, the site of action does not appear to be the bacterial plasma membrane like other endogenous neutrophil peptides such as defensins [19]. However, regarding hemolytic activity, P→A change results in increased hemolytic activity and W→F change results in a drastic reduction in activity. Correlation of activity with structure in the case of IL, ILA and ILF by CD spectroscopy is hampered as the aromatic residues may distort the spectra in the region of interest, i.e. 195–250 nm. However, qualitatively it appears that the peptides do not have a strong propensity for α-helical conformation. In IL and ILF where 3 prolines are present, a substantial proportion of the molecules are in unordered conformation. Convex constraint analysis indicates a distorted helical structure for ILA with a substantial proportion of molecules populating random structures.

In conclusion, the 13-residue tryptophan-rich peptide from bovine neutrophils does not exert its antibacterial activity by permeabilizing the bacterial plasma membrane, unlike other peptides of neutrophil origin like defensins. The proline and tryptophan residues do not appear to be essential for antimicrobial activity. The preponderance of aromatic residues and the overall cationic nature might favor interaction with nucleic acids and it is conceivable that IL exerts its activity by interacting with nucleic acids and preventing either replication or transcription. However, for hemolytic activity, Trp appears to be essential. Our investigations have also led to the identification of an analog of indolicidin, ILPFKFPFFPFRR-NH_2, possessing only broad-spectrum antimicrobial activity, but devoid of hemolytic activity and thus could be a potential therapeutic agent.

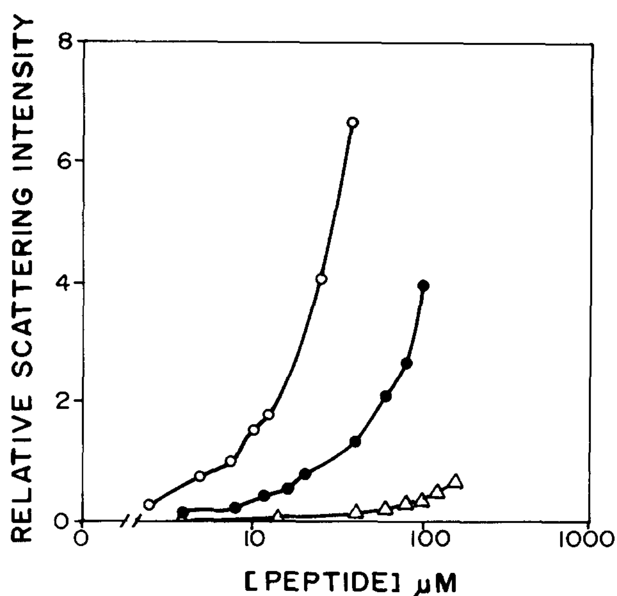

Fig. 4. Light scattering (90° angle) of aqueous solutions of peptides. Peptides were in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl. The amount of light scattered at 90° was measured using a spectrofluorimeter with excitation and emission monochromators set at 600 nm. (●) IL; (○) ILA, (△) ILF.

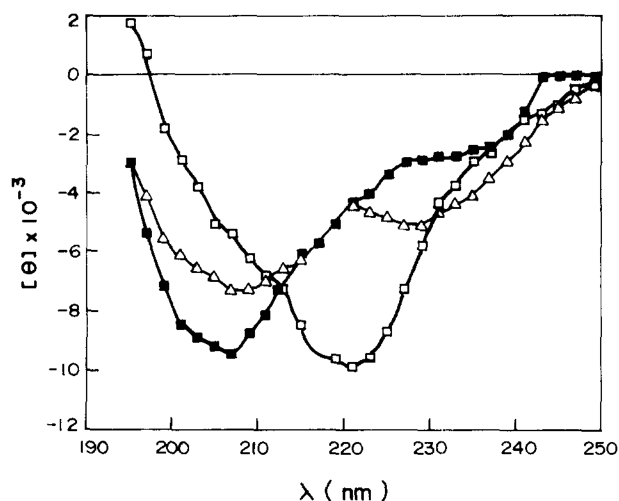

Fig. 5. Circular dichroism spectra of peptides in trifluoroethanol. Peptide concentration was 0.1 mM. (■) IL; (□) ILA; (△) ILF.

Acknowledgements: We thank V.M. Dhople for amino acid analysis and M.V. Jagannadham for checking the sequences of the peptides.This research was supported in part by the Department of Science and Technology, Government of India.

References

[1] Selsted, M.E., Novotny, M.J., Morris, W.L., Tang, Y.Q., Smith, W. and Cullor, J.S. (1992) J. Biol. Chem. 267, 4292–4295.

[2] Lehrer, R.I., Lichtenstein, A.K. and Ganz, T. (1993) Annu. Rev. Immunol. 11, 105–128.

[3] Saberwal, G. and Nagaraj, R. (1994) Biochim. Biophys. Acta 1197, 109–132.

[4] Boman, H.G. (1995) Annu. Rev. Immunol. 13, 61–62.

[5] Blondelle, S.E. and Houghten, R.A. (1992) Biochemistry 31, 12688–12694.

[6] Ahmad, I., Perkins, W.R., Lupan, D.M., Selsted, M.E. and Janoff, A.S. (1995) Biochim. Biophys. Acta 1237, 109–114.

[7] Andreu, D., Ubach, J., Boman, A., Wahlin, B., Wade, D., Merrifield, R.B. and Boman, H.G. (1992) FEBS Lett. 295, 190–194.

[8] Wade, D., Andreu, D., Mitchell, S.A., Silveira, A.M.V., Boman, A., Boman, H.G. and Merrifield, R.B. (1992) Int. J. Pept. Protein Res. 40, 429–436.

[9] Sitaram, N., Chandy, M., Pillai, R. and Nagaraj, R. (1992) Antimicrob. Agents Chemother. 36, 2468–2472.

[10] Sitaram, N., Subbalakshmi, C. and Nagaraj, R. (1995) Int. J. Pept. Protein Res. 46, 166–173.

[11] Merrifield, R.B., Juvvadi, P., Andreu, D., Ubach, J., Boman, A. and Boman, H.G. (1995) Proc. Natl. Acad. Sci. USA 92, 3449–3453.

[12] Thennarasu, S. and Nagaraj, R. (1995) Int. J. Pept. Protein Res. 46, 480–486.

[13] Neu, H.C. (1992) Science 257, 1064–1073.

[14] Silver, L.L. and Bostian, K.A. (1993) Antimicrob. Agents Chemother. 37,377–383.

[15] Swartz, M.N. (1994) Science 91, 2420–2427.

[16] Atherton, E. and Sheppard, R.C. (1989) Solid Phase Peptide Synthesis: A Practical Approach, IRL, Oxford.

[17] Lehrer, R.I., Rosenman, M., Harwig, S.S.L., Jackson, R. and Eisenhauer, P. (1991) J. Immunol. Methods 137, 167–173.

[18] Loh, B., Grant, C. and Hancock, R.E.W. (1984) Antimicrob. Agents. Chemother. 26, 546–551.

[19] Lehrer, R.I., Barton, A., Daher, K.A., Harwig, S.S.L., Ganz, T. and Selsted, M.E. (1989) J. Clin. Invest. 84, 553–561.

[20] Sitaram, N. and Nagaraj, R. (1990) J. Biol. Chem. 265, 10438–10442.
[21] Sitaram, N., Krishnakumari, V. and Nagaraj, R. (1992) FEBS Lett. 303, 265–268.
[22] Janin, J. (1979) Nature 277, 491–493.
[23] Faucheri, J.L. (1985) Trends Biochem. Sci. 268
[24] Perczel, A., Hollosi, M., Tusnady, G. and Fasman, G.D. (1991) Protein Eng. 4, 669–679.
[25] Perczel, A., Park, K. and Fasman, G.D. (1992) Anal. Biochem. 203, 83–93.