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Synthesis and Biological Characterization of Indolicidin Analogues

Yong-beom Lim, Jae-chul Pyun and Jong-sang Park*

Department of Chemistry, College of Natural Sciences,
Seoul National University, Seoul 151-742, Korea
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Abstract : Indolicidin has been known to have a broad spectrum of antimicrobial activities against Gram negative and positive bacteria. Its eight analogues were chemically synthesized. The analogue design was based on the analysis of sequence to elucidate the role of some residues in the antibacterial mechanism of indolicidin. Bactericidal activities were assayed against *Escherichia coli* and *Proteus vulgaris*, and the membrane perturbing abilities of the peptides were assayed using a dye containing liposome. Among the eight analogues, [Gly^4,Gly^6]-Indo, [Ile^6,Ile^8]-Indo, [Lys^{12}]-Indo and [Thr^2,Tyr^9]-Indo showed enhanced antibacterial activities. These results suggest that proline and cationic residues are important in the bactericidal activity of indolicidin. We tried to describe the antimicrobial mechanism of indolicidin with these results.
Key words : antimicrobial peptide, arsenazo III, critical concentration, indolicidin, liposome

Cytoplasmic granules of polymorphonuclear leukocytes (neutrophils) contain various antimicrobial peptides such as PR-39 (Agerberth *et al.*, 1991, Storich *et al.*, 1993), FALL-39 (Agerberth *et al.*, 1995), cathelin (Ritonja *et al.*, 1989), Bac5 (Frank *et al.*, 1990), Bac7 (Frank *et al.*, 1990), CAP18 (Larrick *et al.*, 1991), eNAP-2 (Couto *et al.*, 1993), PMAP-23 (Zanetti *et al.*, 1994), PMAP-36 (Storich *et al.*, 1994) and indolicidin (Selsted *et al.*, 1992). All these peptides possess a highly conserved region of about 130 amino acids in the preproprotein (Strukelj *et al.*, 1995). This preproregion includes the propeptide of about 100 residues highly similar to cathelin, a protein isolated from pig leukocytes and reported to be a cystein protease inhibitor (Kopitar *et al.*, 1989), but it was retracted later (Lenarcic *et al.*, 1993). A number of antibacterial peptides (peptide antibiotics) are important in innate immunity because they can function without either high specificity or memory (Boman *et al.*, 1995). The innate immune system permits the host to curb and delay microbial growth shortly after an infection.

Indolicidin is an antimicrobial tridecapeptide isolated from the cytoplasmic granules of bovine neutrophils (Selsted *et al.*, 1992). It contains a high proportion of a few kinds of amino acids (5 Trp, 3 Pro, 2 Arg) and can be classified as a linear peptide without Cys and with a high proportion of Trp and Pro residues (Boman *et al.*, 1995). The peptides containing high contents of Pro and Arg are PR-39 (Agerberth *et al.*, 1991, Storich *et al.*, 1993), Bac5 (Frank *et al.*, 1990), Bac7 (Frank *et al.*, 1990), Apidaecin (Casteel *et al.*, 1989), Drosocin (Bulet *et al.*, 1993) and indolicidin. PR-39 stopped DNA synthesis within a few minutes and induced a limited degradation of proteins within the same time (Boman *et al.*, 1993). It was suggested that Bac7 may manifest its activity by a bacteriostatic rather than a bacteriolytic mechanism (Tani *et al.*, 1995). It has been suggested that indolicidin might partition into bilayer membranes to avoid aqueous exposure of its hydrophobic domains (Ahmad *et al.*, 1995). It was observed that indolicidin is strongly cytotoxic to rat and human T lymphocytes, and suggested to be a local regulator inhibiting clonal expansion of T lymphocytes during ongoing immune responses (Schluesener *et al.*, 1993).

In this study, we have synthesized indolicidin and its eight analogues. Residues that have high proportions in indolicidin such Trp, Pro and Arg were substituted. The bactericidal activities of the peptides were investigated, and the membrane perturbing abilities of indolicidin and its analogues were assayed using a dye containing phosphatidylcholin (PC) liposome. Some analogues showed enhanced activity. Comparisons between these experiments were used to explain the antimicrobial activity of indolicidin.

*To whom correspondence should be addressed.
Tel : 82-2-880-6660, Fax : 82-2-889-1568
e-mail : pfjspark@plaza.snu.ac.kr

Materials and Methods

Materials

N-α-Fmoc amino acids and Rink amide MBHA resin were obtained from Anaspec (San Jose, USA). Fmoc-L-tyrosine(o-t-butyl), Fmoc-L-arginine(pbf), Fmoc-L-lysine(boc), Fmoc-L-threonine(o-t-butyl). Wang resin and L-α-Phosphatidylcholine (from Egg Yolk) were obtained from Sigma (St. Louis, USA). Muller Hinton medium was purchased from Difco (Detroit, USA). Arsenazo III was obtained from Aldrich (Milwaukee, USA).

Syntheses of indolicidin and the analogues

The peptide was synthesized by solid phase peptide synthesis (SPPS) using N-α-Fmoc-amino acids. HPLC was performed on Gilson model 712 (Villiers-le-Bel, France) with detection by UV absorption at 280 nm. The peptides were eluted using H_2O with 0.1% TFA (trifluoroacetic acid) and acetonitrile with 0.1% TFA. Analytical C18 column was purchased from Vydac (Hesperia, USA). Semi-preparative C18 column (Bondapak™ C18) was from Waters (Milford, USA). The major peak in HPLC was collected and used for assays. Their molecular weights were determined by electrospray ionization-mass spectroscopy (ESI-MASS) (Table. 1).

Antimicrobial susceptibility test

The antimicrobial activities of the peptides were tested both against *Escherichia coli* ATCC 47000 and *Proteus vulgaris* NRRL B-123. The bacteria were grown in TSB (tryptic soy broth) at 37℃ overnight. The bacteria with 1×10^5 CFU were spreaded in Muller Hinton agar plates and discs containing serially diluted peptides were seeded. The plates were incubated for 24 h at 37℃ and zones of inhibition were measured. Critical concentrations were calculated from inhibition zone diameter as described (Barry *et al.*, 1981).

Table 1. Sequence of indolicidin analogues and their molecular weights[a]

Name	Sequence	M.W.
indolicidin	H-ILPWKWPWWPWRR-NH_2	1906.9
indo-OH	H-ILPWKWPWWPWRR-**OH**	1908.1
[Ile^6,Ile^8]-Indo	H-ILPWK **I** P **I** WPWRR-NH_2	1759.7
[Gly^4,Gly^6]-Indo	H-ILP**G** K**G**PWWPWRR-NH_2	1648.3
[Gly^{13}]-Indo	H-ILPWKWPWWPWR**G**-NH_2	1806.7
[Gly^{12}]-Indo	H-ILPWKWPWWPW**G**R-NH_2	1806.5
[Lys^{12}]-Indo	H-ILPWKWPWWPW**K**R-NH_2	1878.9
[Ala^7]-Indo	H-ILPWKW**A**WWPWRR-NH_2	1879.8
[Thr^2,Tyr^9]-Indo	H-I**T**PWKWPW**Y**PWRR-NH_2	1871.2

[a]M.W (molecular weight) was determined by ESI-MASS.

The leakage test

Liposomes containing metallochromic dye arsenazo III (AIII) were prepared as described previously (Weissmann *et al.*, 1976). Briefly, AIII is a metallochromic dye which undergoes a striking change of color from red to blue due to the change of a absorption maximum from 560 nm to 605 and 660 nm upon complexing the calcium. The molar absorptivity of its difference spectrum (with and without Ca^{2+}) is in the order of 10^4 (Budesinsky *et al.*, 1969). Egg yolk PC (lecithin) in chloroform was dried under a stream of nitrogen and lyophilized *in vacuo* overnight. A solution containing 3mM AIII, 5mM Hepes (pH 7.4) was added to the lyophilized lipid and vortexed vigorously to make multilamella vesicles (MLVs). The MLVs were sonicated in 60 min in a bath-type sonicator to make small unilamella vesicles (SUVs). To enlarge encapsulation efficiencies, repeated freezing and thawing (10 cycles) were performed. The dye containing liposome was separated from free dye by Sephadex G-50 column (Pharmacia); elution was carried out with 0.145 M NaCl, 0.145 M KCl, 5 mM Hepes (pH 7.4). The fractions eluted first were collected. The concentration of lipid was determined by Stewart assay (Stewart *et al.*, 1980).

Serially diluted peptides and $CaCl_2$ (10 mM final concentration) were added to the dye containing liposomes (38 μg/ml). The liposomes were incubated for 30 min at 37℃. Absorbance changes were measured at 660 nm.

Results and Discussion

Indolicidin has a high proportion of tryptophans, prolines and arginines. It is very short and its sequence is unique. It belongs to the cathelin family of antibiotics (Levy *et al.*, 1993). Antimicrobial mechanism of such peptides is not well characterized yet. It was suggested by fluorescence emission spectra that tryptophan residues of indolicidin partition from buffer solution into the comparatively less polar environment of the lipid bilayer (Ahmad *et al.*, 1995). It was proposed that PR-39 induced a selective degradation of some protein components required for completion of an ongoing round of DNA replication (Boman *et al.*, 1993). Bac7 may manifest its activity by a bacteriostatic rather than a bacteriolytic mechanism (Tani *et al.*, 1995). D-form of apidaecin was found to be totally inactive on all bacteria tested suggesting that its target is protein (Casteel *et al.*, 1994).

The antibacterial mechanism of indolicidin is not revealed yet. Some of relatively well characterized peptide antibiotics are Cecropin A (Christensen *et al.*, 1988), Magainins (Duclohier *et al.*, 1989), etc. They

are known to form ion channels in planar lipid membranes. To some extent, the antimicrobial mechanism of indolicidin is likely due to the partitioning of indolicidin into membrane lipid bilayer.

Analogue design

Analogues were designed using the following rationale:

1) High contents of hydrophobic amino acid tryptophan may be important in the partitioning of indolicidin into bacterial membrane.

2) Arginine, prevalent in many peptide antibiotics (Boman *et al.*, 1995), cationic in physiological pH, is likely to be important among the amino acids constituting indolicidin. Many peptide antibiotics are cationic. The suggested mechanism by which they cross the outer membrane to gain access to the inner membrane is by compromising with the outer membrane barrier by binding the lipopolysaccharide layer and disrupting the membrane structure (Saberwal *et al.*, 1994).

3) As there are three proline residues in indolicidin, it may have rigid structure. Replacement of proline residues would induce a large conformational change.

Influence of the replacement of tryptophan residues

The proportion of tryptophan residues in indolicidin is about 40%. So, tryptophan residues may play an important role among the amino acids in indolicidin. As tryptophan is the most hydrophobic amino acids with hydrophilicity value -3.4. (Hopp *et al*, 1981), it may promote partitioning of indolicidin into the inner hydrophobic region of lipid bilayer. As shown in Table 1, tryptophan residues at the site of 4th, 6th and 8th residues of indolicidin were replaced with less hydrophobic amino acids like isoleucine (hydrophilicity value; -1.8) and glycine (hydrophilicity value; 0.0). Critical concentration values in Fig. 1 and Fig. 2 shows that [Ile^6, Ile^8]-Indo and [Gly^4, Gly^6]-Indo have enhanced bacterial killing activities than the wild type of indolicidin. The calculated critical concentration value tends to be somewhat lower than the MIC value (Barry *et al.*, 1981). We initially thought that activity would be reduced in this case. This indicates that the high contents of tryp-

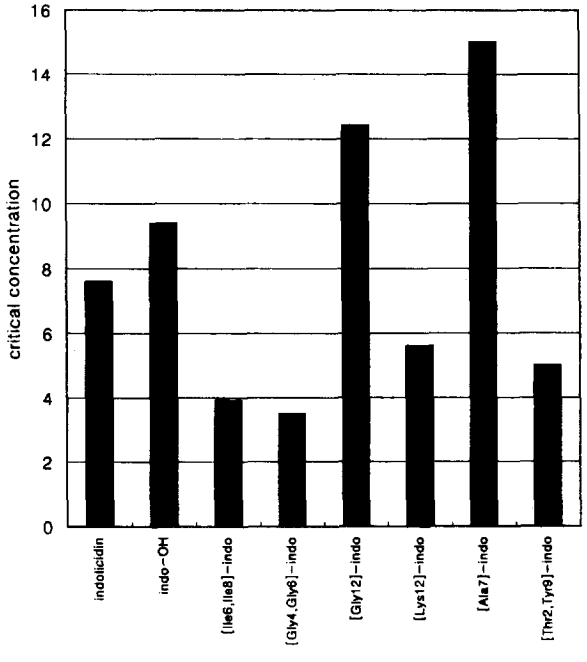

Fig. 1. Critical concentrations of indolicidin and its analogues against *E. coli*. Critical concentrations (μg/ml) were determined from inhibition zone diameter as described (Barry *et al.*, 1981).

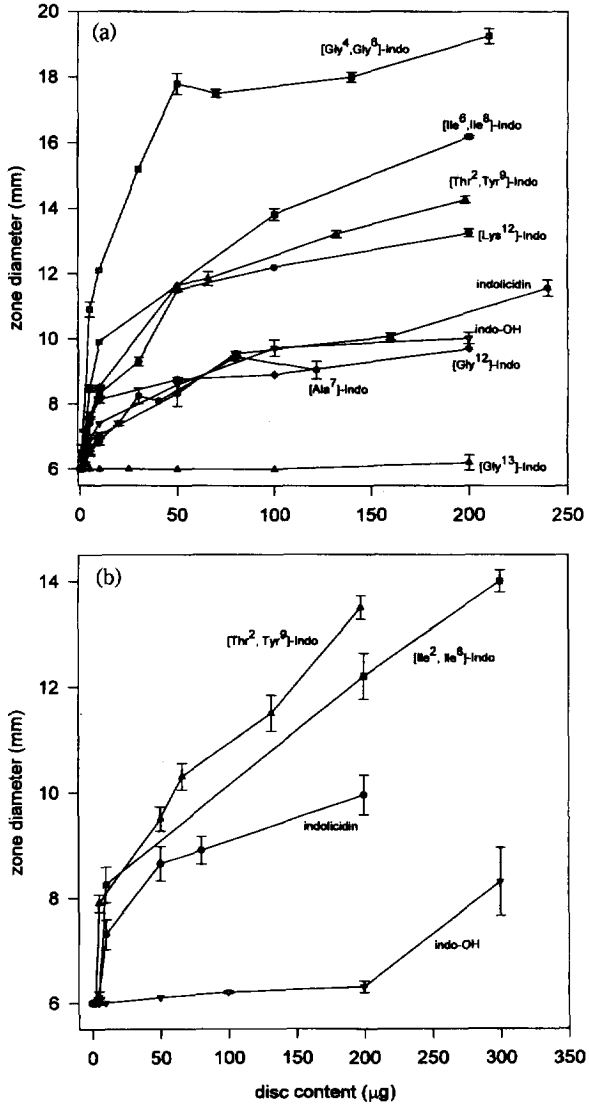

Fig. 2. (a) Antimicrobial susceptibility test for *E. coli*. (b) for *P. vulgaris*. Plates were seeded with discs containing serially diluted peptides and after incubation for 24 h at 37°C, inhibition zone diameter was measured. These data are the mean values of three replicate experiments.

tophans in indolicidin can tolerate some replacements by a moderately hydrophobic amino acids, isoleucine and glycine. But, they showed lower model membrane perturbing abilities than indolicidin as shown in Fig. 3. Although this results do not reflect the actual process occurred in the real cytoplasmic membrane of bacterium, we can imagine that membrane perturbing activity of indolicidin may not be crucial in its antimicrobial mechanism.

Influence of the replacement of cationic residues

Indolicidin has two arginine residues in its carboxyterminal. Each arginine was substituted by glycine respectively in [Gly^{12}]-Indo and [Gly^{13}]-Indo. As shown in Fig. 1 and Fig. 2, there was a drastic reduction of activity in [Gly^{13}]-Indo. Arg^{12} substitution by Gly also had negative effect on its activity. This suggest that positive charge in indolicidin is important in some kind of interaction with lipopolysaccharide in bacterial outer membrane or with other components in or near membrane. In [Lys^{12}]-Indo, activity was even enhanced. Arg and Lys are similar in structure, charge and their basic nature, thus Arg deletion may have been compensated with Lys.

Influence of the replacement of proline

Pro^7 substitution in the middle of the indolicidin by Ala lowered its activity much in [Ala^7]-Indo (Fig. 1 and 2). There is a possibility that indolicidin may self-associate at the higher concentrations (Ahmad *et al.*, 1995). As there are three prolines in indolicidin, structural requirement may be important in self-association or in other kind of interaction.

Other substitutions

Indolicidin has amidated carboxy terminal. When terminal amide was replaced to terminal acid in indo-OH, bacterial killing activity against *Escherichia coli* and membrane perturbing ability were not changed much as compared with indolicidin (Fig.1 and 2). This result implies that amidation in indolicidin is not so important in its antimicrobial activity.

Many antimicrobial peptides exert their activity by forming amphiphilic α-helical structure. [Thr^2, Tyr^9]-Indo was designed to enhance amphiphilicity if indolicidin forms amphiphilic α-helix. Bactericidal activity was enhanced (Fig. 1 and 2).

Comparison of activities against Gram negative and positive bacteria

Indolicidin and its analogues were slightly more active towards Gram negative bactera, *E. coli* than to Gram positive bacteria, *P. vulgaris* (Fig. 2). Not all the peptides assayed for *E. coli* were used for *P. vulgaris*. [Ile^6, Ile^8]-Indo and [Thr^2, Tyr^9]-Indo which showed enhanced activity against *E. coli* were also more active to *P. vulgaris* than indolicidin. This described the fact that the antimocrobial mechanism of indolicidin may be similar in both kinds of bacteria.

Antimicrobial mechanism of indolicidin.

The leakage of dye from model membranes of phos-

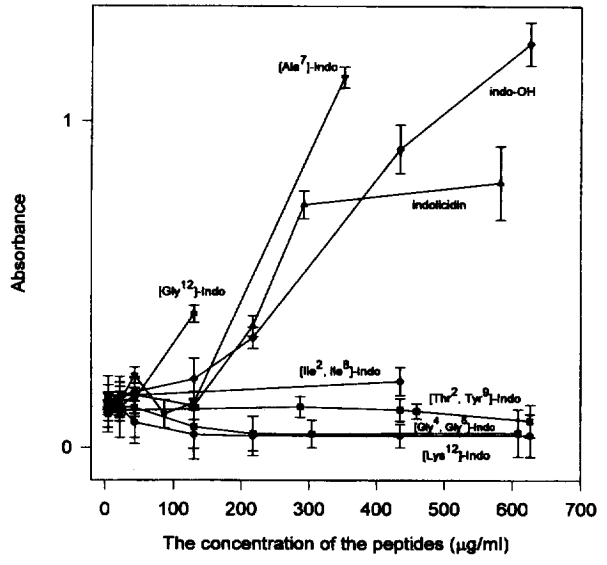

Fig. 3. (a) Leakage of dye (AIII) from phosphatidylcholine liposomes (38 mg/ml) by indolicidin and its analogues. Liposomes were incubated for 30 min at 37°C with the peptides. Absorbance was measured at 660 nm. These data are the mean values of three replicate experiments.

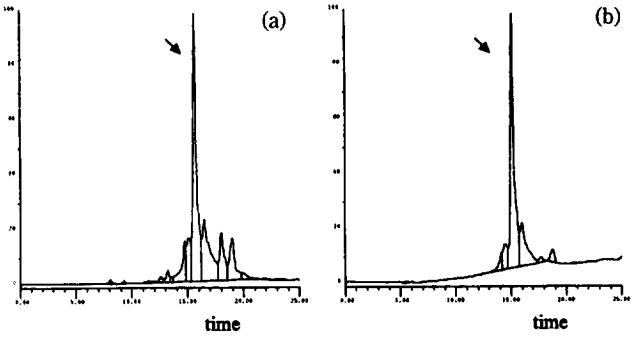

Fig. 4. When indolicidin and indo-OH were mixed to liposome, aggregates were formed. The aggregates were centrifuged and seperated by the immiscible mixture of water and chloroform. Quantification of lipid contents by Stewart assay confirmed that there was lipid in the chloroform layer. (a) HPLC profile of crude indo-OH. (b) HPLC profile of water layer when indo-OH was added to liposomes and the aggregates were separated by water and chloroform mixture. Solvent A; H_2O (0.1% TFA) , Solvent B; acetonitrile (0.1% TFA). Linear gradient of acetonitrile from 20% to 75%. Liposomes were made in 20 mM Hepes, pH 7.4.

phatidylcholine was investigated (Fig. 3). When indolicidin and indo-OH were added to L[AIII] or L, aggregates were formed. The aggregates were centrifuged and separated by the immiscible mixture of chloroform and water, and confirmed to be a peptide and lipid (Fig. 4). This suggests that indolicidin interacts with model membrane strongly. But, whether this phenomenon would occur in the actual bacterial membrane is unclear.

Positively charged arginine and proline were important in the antimicrobial activity. But some deletions in tryptophan were not crucial. [Ile^6, Ile^8]-Indo, [Gly^4, Gly^6]-Indo, [Lys^{12}]-Indo and [Thr^2, Tyr^9]-Indo that showed enhanced activities than indolicidin itself in antimicrobial susceptibility test were nearly inactive in membrane perturbing abilities. This finding is intriguing. Indolicidin may be membrane active possibly by the high contents of tryptophans and positively charged residues, but it is probable that some other bactericidal mechanisms may exist.

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References

Agerberth, B., Lee, J. Y., Berman, T., Carlquist, M., Boman, H. G. and Mutt, V. (1991) *European J. Biochem.* **202**, 849.

Agerberth, B., Gunne, H., Odeberg, J., Kogner, P., Boman, H. G. and Gudmundsson, G. H. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 195.

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

Barry, A.L. (1981) in *Antibiotics in Laboratory Medicine* (Lorian, V., ed.), p. 11, Williams & Wilkins, Baltimore, London

Boman, H.G., Agerberth, B. and Boman, A. (1993) *Infect. Immun.* **61**, 2978.

Boman, H. G. (1995) *Annu. Rev. Immunol.* **13**, 61.

Budesinsky, B. (1969) in Chelates in Analtical Chemistry (Flashka, H. A., Barnard, A. J., eds.), vol. 2, pp. 1-91, Dekker, New York.

Bulet, P., Dimarcq, J. L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Vandorsselaer, A. and Hoffmann, J. A. (1993) *J. Biol. Chem.* **268**, 14893.

Casteels, P., Ampe, C., Jacobs, F., Vaeck, M. and Tempst, P. (1989) *EMBO J.* **8**, 2387.

Casteels, P. and Tempst, P. (1994) *Biochem. Biophys. Res. Commun.* **189**, 339.

Couto, M. A., Harwig, S. S. L. and Lehrer, R. I. (1993) *Infect. Immun.* **61**. 2991.

Christensen, B., Fink, J., Merrifield, R. B. and Mauzerall, D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5072.

Duclohier, H., Spach, G. and Molle, G. (1989) *Biophys. J.* **56**, 1017.

Frank, R. W., Gennaro, R., Schneider, K., Przybylski, M. and Romeo, D. (1990) *J. Biol. Chem.* **265**, 18871.

Hopp, T. P. and Woods, K. R. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3824.

Kopitar, M., Ritonja, A., Popovic, T., Gabrijelcic, D., Krizj, I and Turk, V. (1989) *Biol. Chem. Hoppe-Seyler* **370**, 1145.

Larrick, J. W., Morgan, J. G., Palings, I., Hirata, M. and Yen, M. H. (1991) *Biochem. Biophys. Res. Commun.* **179**, 170.

Lenarcic, B., Ritonja, A., Dolenc, I., Stoka, V., Berbic, S., Pungercar, J., Strukelj, B and Turk, V. (1993) *FEBS Lett.* **336**, 289.

Levy, O., Weiss, J., Zarember, K., Ooi, C. E. and Elsbach, P. (1993) *J. Biol. Chem.* **268**, 6058.

Ritonja, A., Kopitar, M., Jerala, R. and Turk, V. (1989) *FEBS Lett.* **255**, 211.

Saberwal, G. and Nagaraj, R. (1994) *Biochim. Biophys. Acta.* **1197**, 109.

Schluesener, H. J., Radermacher, S., Melms, A. and Jung, S. (1993) *J. Neuroimmunol.* **47**, 199.

Selsted, M.E., Novotny, M. J., Morris, W. J., Tang, Y. Q., Smith, W. and Cullor, J. S. (1992) *J. Biol. Chem.* **267**, 4292.

Stewart, J.C.M. (1980) *Anal. Biochem.* **104**, 10.

Storich, P. and Zanetti, M. (1993) *Biochem. Biophys. Res. Commun.* **196**, 1058.

Storich, P., Scocchi, M., Tossi, A., Gennaro, R. and Zanetti, M. (1994) *FEBS Lett.* **337**, 303.

Strukelj, B., Pungercar, J., Kopitar, G., Renko, M., Lenarcic, B., Berbic, S. and Turk, V. (1995) *Biol. Chem. Hoppe-Seyler.* **376**, 507.

Tani, A., Lee, S., Oishi, O., Aoyagi, H. and Ohno, M. (1995) *J. Biochem.* **117**, 560.

Weissmann, G., Collins, T., Evers, A. and Dunham, P. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 510.

Zanetti, M., Storici, P., Tossi, A., Scocchi, M. and Gennaro, R. (1994) *J. Biol. Chem.* **269**, 7855.