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The Effects of Charge and Lipophilicity on the Antibacterial Activity of Undecapeptides derived from Bovine Lactoferricin

MORTEN B. STRØM[a], ØYSTEIN REKDAL[b] AND JOHN S. SVENDSEN[a]*

[a] Department of Chemistry, Faculty of Science, University of Tromsø, N-9037 Tromsø, Norway
[b] Institute of Medical Biology, Faculty of Medicine, University of Tromsø, N-9037 Tromsø, Norway

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Abstract: We have investigated the effects of charge and lipophilicity on the antibacterial activity of an undecapeptide (FKCRRWQWRMK) derived from the sequence of bovine lactoferricin. We prepared ten analogues that were modified by the incorporation of Ala, Tyr, Trp, Met and Arg residues, which are amino acids known to be important for the antibacterial activity of longer derivatives of lactoferricins. All undecapeptides contained the native Trp residues in positions 6 and 8, and the Arg residues in positions 5 and 9. Generally, the Gram-positive bacterium *Staphylococcus aureus* was more susceptible to these undecapeptides than the Gram-negative bacteria, and a higher antibacterial activity was observed against *Escherichia coli* than against *Pseudomonas aeruginosa*. The only exception was the peptide Undeca 9 (RRWYRWAWRMR-NH_2), which was almost equally active against all three test strains, displaying minimal inhibitory concentrations of 10 μg/ml (5.8 μM), 7.5 μg/ml (4.4 μM) and 5 μg/ml (2.9 μM) against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively. The peptides Undeca 6 (YRAWRWAWRWR-NH_2) and Undeca 7 (YRMWRWAWRWR-NH_2) were the two most active undecapeptides against *Staphylococcus aureus*, both displaying a minimal inhibitory concentration of 2.5 μg/ml (1.5 μM). The study showed that a level was reached in which undecapeptides having a net charge above +4 and containing three or four Trp residues all displayed a high antibacterial activity. All undecapeptides prepared were essentially non-haemolytic, but undecapeptides containing more than three Trp residues displayed 50% haemolysis of human red blood cells at concentrations above 400 μg/ml (>230 μM). Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antibacterial peptides; bovine lactoferricin; minimal inhibitory concentration; tryptophan; short peptides

INTRODUCTION

An increasing number of multiresistant bacteria, particularly in hospital environments, have resulted in an urgent need for new antibiotics. The pioneering works by the research group of Boman [1] who discovered the cecropins from the haemolymph of the giant silkmoth *Hyalophora cecropia*, of Selsted *et al.* [2–4] who characterized the rabbit and human defensins from neutrophils and of Zasloff [5] who discovered the magainins from the skin of the African clawed frog *Xenopus laevis*, have included the field of peptide chemistry in the development of prospective antibiotics. The isolation of peptides with antibacterial activity from a wide variety of other species suggests that antibacterial peptides constitute an important part of the innate immunity of animals, plants and insects [6].

*Correspondence to: J. S. Svendsen, Department of Chemistry, Faculty of Science, University of Tromsø, N-9037 Tromsø, Norway; e-mail: johns@chem.uit.no
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Abbreviations: ATCC American Type Culture Collection; EC_{50} concentration of peptide required for 50% haemolysis; LFB residues 17 to 31 of bovine lactoferrin; LFB 17–27 residues 17 to 27 of bovine lactoferrin; RBC human red blood cells.

A general feature of antibacterial peptides is a high content of positively charged residues, and the ability to adopt an amphipathic conformation upon interaction with the negatively charged phospholipids of the bacterial cell membrane. They are thought to function through increasing the permeability of the bacterial cell membrane by pore formation [7–9], although other targets, such as intracellular binding to DNA, are proposed as a mechanism of action by several authors [10–14]. The majority of these naturally occurring antibacterial peptides range in size from 11 to 50 amino acids and multiple disulphide bonds, as found in the defensins, often complicate their chemical synthesis [15,16].

Tomita *et al.* [17] and Bellamy *et al.* [18] discovered in 1991 that peptide fragments isolated after degradation of the mammalian protein lactoferrin by gastric pepsin displays a higher antibacterial activity than the native protein. These protein fragments were named lactoferricins and it was shown that bovine lactoferricin displays a higher antibacterial activity than human lactoferricin. Bovine lactoferricin is a 25 residue peptide encompassing residues 17 to 41 (LFB 17–41) of the mature bovine lactoferrin protein, and has a disulphide bond between the Cys residues in positions 19 and 36 [17,18]. However, shorter derivatives of bovine lactoferricin, which are devoid of the disulphide bond, also exhibit antibacterial activity. Kang *et al.* [19] have shown that an undecaderivative, encompassing amino acids 20 to 30 (LFB 20–30), retains much of the antibacterial activity of the larger bovine lactoferricin peptide.

We report here the antibacterial activity of a series of modified undecapeptides, which were based on our previous studies of a pentadecaderivative of bovine lactoferricin (LFB), encompassing residues 17 to 31 of the bovine lactoferrin protein [20,21]. The present undecapeptides were prepared with great diversities in both charge and lipophilicity by the incorporation of Ala, Tyr, Trp, Met and Arg residues, which are amino acids known to be important for the antibacterial activity of pentadecaderivatives of lactoferricins [21,22]. The peptides prepared were essentially non-haemolytic.

MATERIALS AND METHODS

Preparation of Peptides

All the peptides were synthesized by solid-phase methodology on a 9050 *Plus* PepSynthesizer (Milligen, Milford, MA, USA) using Fmoc-protection. Coupling with OPfp-esters was catalysed with HOBt (1.3 eq), whereas DIPEA (2.4 eq) was added when using *in situ* activation with HBTU (1 eq) in DMF. A 4-fold excess of amino acids was employed during every coupling step. The Fmoc-protected amino group was deprotected after each coupling step using 20% piperidine in DMF. The *C*-terminal amino acid was either pre-attached to a 4-hydroxymethylphenoxyacetic acid — polyethylene glycol — polystyrene resin (PerSeptive Biosystems, Hertford, UK), which ensured a free *C*-terminal carboxylic acid group after the final acidic treatment (see below), or coupled to an *in situ* Fmoc-deblocked 4-Fmoc-aminomethyl-(3,5-dimethoxy)phenoxyvaleric acid — polyethylene glycol — polystyrene resin (PerSeptive Biosystems, Hertford, UK), which ensured an amidated *C*-terminal end after the final acidic treatment. Cys was irreversibly protected with an Acm-group, whereas all other amino acids with reactive side chains were protected with acid labile protecting groups and deprotected during cleavage of the peptide from the solid support upon treatment with Reagent K for no more than 3 h [23]. The solid support was removed by filtration, the filtrate concentrated under reduced pressure, and the peptides precipitated from diethyl ether. The precipitates were washed several times with diethyl ether and dried under reduced pressure.

The peptides were purified on a RP-HPLC C18-column (Delta-Pak™ C18, 100 Å, 15 μm, 25 × 100 mm, Waters Corporation, Milford, MA, USA) using a mixture of water and acetonitrile (containing 0.1% TFA) as the mobile phase and employing UV-detection at 254 nm. All peptides were analysed for impurities on an analytical RP-HPLC C18-column (Delta-Pak™ C18, 100 Å, 5 μm, 3.9 × 150 mm, Waters Corporation) with a mixture of water and acetonitrile (containing 0.1% TFA) as the mobile phase. The purity of all peptides was found to be >98%. The integrity of the peptides was checked by positive ion electrospray ionization mass spectrometry on a VG Quattro quadrupole mass spectrometer (VG Instruments Inc., Altrincham, UK).

Antibacterial Activity

The bacterial test strains, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923, were grown in 2% Bacto Peptone water (Difco 1807-17-4) until exponential growth. A standard microdilution technique with an inoculum of 2×10^6 cfu per ml was

used. The minimal inhibitory concentration (MIC) of the peptides was determined in 2% Bacto Peptone water after incubation on 96-well polystyrene plates (NUNC A/S, prod. no. 167008, Roskilde, Denmark) overnight at 37 °C. The concentration series used was as follows: 200, 150, 100, 75, 50, 25, 20, 15, 10, 7.5, 5, 2.5, 1 and 0.5 μg/ml. Changes in the turbidity of the peptone-based growth-medium were detected at 540 nm by a Microwell System Reader 510 spectrophotometer (Organon Teknika, Boxtel, The Netherlands). The minimal bactericidal concentration (MBC) was determined by inoculating 10 μl of the overnight culture from each well on agar plates with PDM antibiotic sensitivity medium II (AB Biodisk, Solna, Sweden), and incubating for another night at 37 °C. All the peptides were tested in duplicates.

Haemolytic Activity

The haemolytic activity of the peptides was determined using human red blood cells (RBC) isolated from heparinized blood as described by Dathe *et al.* [24]. Briefly, the RBC were prepared from freshly collected human blood (4 ml) by centrifugation at 1500 rpm for 10 min at 4 °C. The cells were washed three times with phosphate buffered saline (pH 7.2–7.3) and diluted to 10% haematocrit. The RBC (1%) were incubated with peptide dissolved in phosphate buffered saline for 1 h at 37 °C. The concentration series used was as follows: 1000, 500, 100, 50, 10 and 1 μg/ml. The samples were centrifuged at 4000 rpm for 5 min before the absorbance of the supernatant was measured at 540 nm. Zero haemolysis (blank) and 100% haemolysis (control) were determined in phosphate buffered saline and 1% Triton X-100 (Sigma, St. Louis, MO, USA), respectively. The required concentration of peptide for 50% haemolysis (EC_{50}) was derived from the dose-response curve.

RESULTS AND DISCUSSION

Antibacterial Activity

Our previous results from an alanine-scan on the pentadecapeptide LFB suggest that the four *C*-terminal amino acid residues are indifferent residues, which can be excluded without severely affecting the antibacterial activity against *E. coli* [21]. As shown in Table 1, the observed antibacterial activity of the undecapeptide LFB 17–27 proved this assumption to be true. Furthermore, this peptide was also more active against *S. aureus* than LFB. A homologous undecapeptide has also been prepared by Kang *et al.* [19] and shown to possess antibacterial activity against *E. coli* and *B. subtilis* comparable to bovine lactoferricin. However, the undecapeptide reported by Kang *et al.* [19] lacks the three *N*-terminal Phe, Lys and Cys residues of LFB, but contains the Lys, Leu and Gly residues in positions 12, 13 and 14, respectively (see LFB numbering, Table 1).

The previous alanine-scan experiment on LFB also revealed that the Acm-protected Cys residue in position 3 and the Gln residue in position 7 (Table 1) are counterproductive residues, since a replacement of these residues with Ala increases the antibacterial activity against *E. coli* [21]. Similar alanine-modifications of the undecapeptide LFB 17–27 resulted in the peptide Undeca 1, which displayed the same activity as LFB 17–27 against *E. coli* and *S. aureus*, and an increased activity against *P. aeruginosa*. The alanine-modification was therefore preserved in most of the other undecapeptides prepared.

The incorporation of Tyr into the antibacterial undecapeptides was based on the results from a series of tyrosine-modified pentadecapeptides of a murine lactoferricin derivative recently investigated by us [22]. The tyrosine-modified murine pentadecapeptides showed increased affinities for SDS micelles, which correlated with an increased antibacterial activity, especially against *S. aureus* [22]. As shown for Undeca 2, Phe and Tyr served similar functions in the present undecapeptides since no change in antibacterial activity was observed when the *N*-terminal Phe residue was replaced by Tyr.

However, the results of Undeca 3 showed that the antibacterial activity could be increased against all test bacteria by the incorporation of a Trp residue, another aromatic amino acid. We have previously shown that the two Trp residues in positions 6 and 8 are the most important residues for the antibacterial activity of LFB [21]. Both Trp and Tyr residues have a preference for the water–phospholipid interface region when incorporated into biological membranes [25–27]. This interaction is thought to be stabilized by hydrogen bonding between the hydroxyl group of Tyr, or the imino group of Trp, and the polar parts of the membrane, as well as being affected by the dipole moment of the side chains. However, Yau *et al.* [28] have shown that indole analogues of Trp, which are

Table 1 Antibacterial and Haemolytic Activities of Undecapeptides Based on the Amino Acid Sequence of a Pentadecapeptide (LFB) of Bovine Lactoferricin

Name of peptide	Molecular weight[a]		Amino acid sequence (single letter code)[b]															MIC[c] *E. coli*	MIC[c] *P. aeruginosa*	MIC[c] *S. aureus*	Haemolysis[d] EC_{50}
	Obs.	(calc.)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15				
LFB	2064.8	(2064.5)	F	K	C	R	R	W	Q	W	R	M	K	K	L	G	A	50 (24)	Not tested	100 (48)	N.h.[e]
LFB 17–27	1695.2	(1695.8)	—	—	—	—	—	—	—	—	—	—	—					50 (30)	N.a.[f]	50 (30)	N.h.
Undeca 1	1535.0	(1535.4)	—	—	A	—	—	—	A	—	—	—	—					50 (33)	200 (130)	50 (33)	N.h.
Undeca 2	1551.9	(1550.8)	Y	—	A	—	—	—	A	—	—	—	—					50 (32)	200 (129)	50 (32)	N.h.
Undeca 3	1606.9	(1606.0)	Y	—	A	—	—	—	A	—	—	W	—					20 (12)	50 (31)	20 (12)	N.h.
Undeca 4	1636.9	(1636.0)	Y	—	A	W	—	—	A	—	—	W	—					20 (12)	75 (46)	10 (6.1)	540 (330)
Undeca 5	1635.9	(1635.4)	Y	—	A	W	—	—	A	—	—	W	K-NH_2					7.5 (4.6)	20 (12)	5 (3.1)	610 (373)
Undeca 6	1692.0	(1691.0)	Y	R	A	W	—	—	A	—	—	W	R-NH_2					10 (5.9)	20 (12)	2.5 (1.5)	570 (337)
Undeca 7	1752.1	(1751.0)	Y	R	M	W	—	—	A	—	—	W	R-NH_2					10 (5.7)	20 (11)	2.5 (1.4)	410 (234)
Undeca 8	1838.2	(1836.4)	Y	R	M	W	—	—	R	—	—	W	R-NH_2					7.5 (4.1)	20 (11)	5 (2.7)	530 (289)
Undeca 9	1722.0	(1721.0)	R	R	W	Y	—	—	A	—	—	M	R-NH_2					10 (5.8)	7.5 (4.4)	5 (2.9)	N.h.

[a] Observed and (calculated) molecular weight including the Acm-protecting group on cysteine. See the experimental section.
[b] The $-NH_2$ group denotes peptides with an amidated *C*-terminal carboxylic acid group.
[c] MIC: minimal inhibitory concentration in μg/ml and (μM).
[d] EC_{50}: concentration in μg/ml and (μM) required for 50% haemolysis.
[e] N.h.: no haemolytic activity within the concentration range tested, i.e. up to 1000 μg/ml (approx. 500 μM).
[f] N.a.: no antibacterial activity within the concentration range tested, i.e. up to 200 μg/ml (approx. 120 μM).

incapable of hydrogen bonding and have reduced dipole moments, also have a preference for the interface region of membranes. Similarly, we observed that the replacement of the *N*-terminal Phe residue with Tyr did not influence the antibacterial activity of Undeca 2. Therefore, the effect of the aromatic residues may be explained better by their rigid flat ring structure and by a possible π-electron interference with the cell membrane components [28]. Also the size of the aromatic residues is important for the activity of antibacterial peptides. Haug *et al.* [29] have recently demonstrated a strong correlation between the antibacterial activity of derivatives of LFB and the bulkiness of unnatural aromatic amino acids, which were substituted for the Trp residues in positions 6 and 8. Thus, compared with Tyr and Phe, the positive effect of Trp on the antibacterial activity of the present undecapeptides was due to its larger aromatic ring-size.

In the present study, the activity against *S. aureus* was further increased for Undeca 4, in which a fourth Trp residue was incorporated in replacement for the Arg residue in position 4 of Undeca 3. However, we observed no overall additive effect of incorporating more than three Trp residues since the activity of Undeca 4 against *P. aeruginosa* was reduced compared with Undeca 3. In fact, the results indicated that a reduction in the positive charge had a stronger negative effect than the expected positive effect of the incorporation of a fourth Trp residue.

Next to the incorporation of aromatic residues, the single most effective modification of the undecapeptides was the amidation of the *C*-terminal carboxylic acid group. This finding was in accordance with the investigations of Tomita *et al.* [30] on the active centre of bovine lactoferricin. Compared with the non-amidated peptide Undeca 4, its amidated derivative Undeca 5 displayed a marked increased antibacterial activity against all test bacteria. Amidation increases the net positive charge of the peptides by eliminating the negatively charged *C*-terminal carboxylate group, and thereby prevents electrostatic repulsions between the peptides and the negatively charged components of the bacterial surface. The *C*-terminal amide group may also protect the peptides against carboxypeptidases and thereby increase the lifetime of the peptides. In accordance with the investigations by Kang *et al.* [19], the importance of electrostatic interactions between the peptides and the negatively charged components of the bacterial cell membrane was also investigated by replacing the Lys residues in positions 2 and 11 with Arg. The side chain of Arg can interact both electrostatically and through hydrogen bond interactions with the negatively charged components of the bacterial surface, and thereby increase the interaction between the peptides and the bacterial surface. The resulting peptide, Undeca 6, displayed an increased antibacterial activity against *S. aureus* compared with Undeca 5. For their corresponding LFB derivative, Kang *et al.* [19] observed an increased antibacterial activity against both the Gram-positive bacterium *B. subtilis* and the Gram-negative bacterium *E. coli*. However, a similar increased affinity for the Gram-negative bacteria was not observed for the arginine-modified peptide Undeca 6, which may be a result of Undeca 5 already containing more than the minimum requirement of positively charged residues.

Somewhat surprisingly, the peptides Undeca 7 and Undeca 8 displayed similar antibacterial activities to the preceding peptides. In Undeca 7, the lipophilicity of the peptide was increased by replacing the Ala residue in position 3 of Undeca 6 with Met, whereas in Undeca 8 both the net charge and the lipophilicity were simultaneously increased by the replacements of the Ala residues in positions 3 and 7 of Undeca 6 with Met and Arg, respectively. Apparently, a limit had been reached after which further introduction of hydrophobic residues did not have any positive effect on the antibacterially activity of these undecapeptides. It should be commented that at the lowest peptide concentrations the error in the MIC determinations is thought to be one titre-step, and that small differences in the antibacterial activity of the most active undecapeptides may be hard to interpret. Considering this, the study revealed that the present undecapeptides that had a net positive charge above +4 and contained three or more Trp residues were all highly active and displayed small variations in their MIC values. This was also demonstrated for the last peptide of this series, Undeca 9, which in part was based on the sequence of a modified murine lactoferricin pentadecapeptide LFM R1,9 W8 recently prepared by us [22]. With its three Trp residues, Undeca 9 contained one Trp residue less than Undeca 8, but both peptides contained five Arg residues. Undeca 9 appeared to be the most active undecapeptide against *P. aeruginosa*, and it also displayed a high activity against the two other test strains. Noteworthily, Undeca 9 was also more antibacterialy active than LFM R1,9 W8, which for comparison displays MIC values against *E. coli, P. aeruginosa* and *S. aureus* of 20 μg/ml (10 μM), 200 μg/ml (98 μM)

and 75 μg/ml (37 μM), respectively ([22] and Strøm MB unpublished results).

Bactericidal Activity and Mechanisms of Resistance

The bactericidal activity of the peptides was evaluated by determining their minimal bactericidal concentration (MBC). The MBC value was the lowest peptide concentration that prevented bacterial colony formation when 10 μl of the overnight cultures were inoculated onto agar plates. For *E. coli* and *S. aureus* the MIC and MBC values were practically the same (MBC data are not shown). However, for *P. aeruginosa* the most active peptides displayed approximately 3-fold higher MBC values than MIC values, and implied that part of the bacterial population stayed alive at the MIC level of the peptides and formed colonies when the peptide concentration was lowered. Giacometti *et al.* [31] have reported that peptide-resistant mutants of *P. aeruginosa* can be selected after consecutive exposures of the cells to cecropin P1, magainin 2, indolicidin, nisin and ranalexin. The mechanism of resistance was not commented on by Giacometti *et al.* but could involve altered charge and lipid composition of the cell wall and cytoplasmic membrane, or other mechanisms such as increased peptidase activity, as was reported by Groisman [32] for *Salmonella sp.* However, it is noteworthy that the published genome sequence of *P. aeruginosa* has revealed the existence of an unusually high number of multidrug efflux systems, particularly of the so-called RND-family (resistance-nodulation-cell division) [33]. *P. aeruginosa* is predicted to have ten AcrB/Mex-type RND multidrug efflux systems, whereas *E. coli* has only four [33]. These efflux systems can pump drugs both from the periplasm and cytoplasm and give resistance against many drugs of clinical importance. Their substrate specificity is poorly understood, but a main requirement is that the substrates have a hydrophobic domain so that they can get access to the transporters via the cell membrane [34,35]. Conceivably, multidrug efflux pumps may also protect *P. aeruginosa* against hydrophobic antibacterial peptides and contribute to cell recovery once the peptide concentration is lowered. Further investigations to address this question are in progress at our laboratory.

Haemolytic Activity

Lysis of RBC was used as a coarse measurement of the toxicity of the peptides. In general, the haemolytic activity of the peptides was low (Table 1). The EC_{50} values were usually more than 20-fold higher than the MIC values against the least susceptible bacterial strain. It was noteworthy that the haemolytic activity was highly dependent on the number of Trp residues present in the peptides. Peptides with four Trp residues displayed a measurable, albeit low, haemolytic activity within the concentration range tested, while peptides with less than four Trp residues were non-haemolytic. An analogy exists to the tryptophan-rich antibacterial tridecapeptide indolicidin, which is strongly haemolytic [36,37]. Subbalakshmi *et al.* [38] showed that the haemolytic activity of indolicidin is almost completely abolished when all of its five Trp residues are replaced with Phe, or when four of them are replaced with Leu [39]. Thus, a close relationship between the amount of Trp residues and haemolytic activity of peptides may be a more general phenomenon. Hence, it would be of interest to investigate the haemolytic activity of indolicidin analogues containing only three Trp residues.

In conclusion, the results of our modified undecapeptides revealed the efficiency of a balanced mixture between cationic and aromatic residues. The most antibacterial active undecapeptides had a net positive charge above +4 and contained three or four Trp residues. The low toxicity makes these undecapeptides highly potent for therapeutical usage.

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