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Structure–biological activity relationships of 11-residue highly basic peptide segment of bovine lactoferrin

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The antimicrobial peptide, lactoferricin, is generated upon the gastric pepsin cleavage of lactoferrin and has many basic and hydrophobic amino acid residues essential for its biological activity. To investigate the structure–antimicrobial activity relationships, the basic amino acid-rich region of bovine lactoferricin (BLFC), RRWQWRMKKLG, was selected. Using chemically synthesized BLFC and its substituted peptides, the antimicrobial activities of the peptides were tested by determining the minimal inhibitory concentration (MIC) of *Escherichia coli* and *Bacillus subtilis* and the disruption of the outer cell membrane of *E. coli*, and the peptide's toxicities were assayed by hemolysis. The short peptide (B3) composed of only 11 residues had similar antimicrobial activities while losing most of the hemolytic activities as compared with the 25 residue-long ones (B1 and B2). The short peptides (B3, B5 and B7) with double arginines at the *N*-termini had more potent antimicrobial activity than those (B4 and B6) with lysine. However, no antimicrobial and hemolytic activities were found in B8, in which all basic amino acids were substituted with glutamic acid, and in B9, in which all hydrophobic amino acids were substituted with alanine. The circular dichroism (CD) spectra of the short peptides in 30 mM SDS were correlated with their antimicrobial activities. These results suggested that the 11-residue peptide of BLFC is involved in the interaction with bacterial phospholipid membranes and plays an important role in antimicrobial activity with little or no hemolytic activity. © Munksgaard 1996.

Key words: antimicrobial activity; bovine lactoferrin; CD spectra; hemolysis; lactoferricin

Lactoferrin, found in most exocrine secretions of mammals, is an iron-binding glycoprotein and has broad-spectrum antimicrobial activities. It is considered to be a major protecting component from bacteria at the mucosal surfaces and in colostrum and milk, thereby protecting infants from infectious diseases (1–3). Clinical studies indicate that only about 1–6% of ingested lactoferrin is excreted intact in the feces of breast-fed infants (4). Apparently, most ingested lactoferrin is degraded to low molecular weight peptides during passage through the gastrointestinal tract (5), and one of enzymatic digests of lactoferrin is considered to possess bioactive properties that may have physiological significance in nursing infants.

A pepsin hydrolysate of bovine lactoferrin has more potent antimicrobial activity than undigested lactoferrin (6) and is named bovine lactoferricin (BLFC). BLFC is composed of 25 amino acid residues with the sequence FKCRRWQWRMKKLGAPSITCVRRAF, derived from the *N*-terminal loop region of bovine lactoferrin (7). A distinct structural feature of the identified domain is the relatively high proportion and asymmetric clustering of basic amino acids. Eight of the 25 residues in BLFC are basic residues, and among them 6 basic residues are clustered at the *N*-terminus with α-helical propensity. The antimicrobial activity of BLFC seems to be correlated with this region.

In this paper we have focused a segment comprising 11 residues, RRWQWRMKKLG, which is considerably more basic and hydrophobic than the rest of BLFC and has amphipathic α-helical propensity. To investigate the peptide structure–biological activity relationships, BLFC, the short peptide and their analogues were synthesized. Their antimicrobial activities against *E. coli* and *B. subtilis* were assayed and the outer membrane disruption of *E. coli* was determined by monitoring the released β-lactamase. Their hemolytic activity was also determined and their CD spectra were measured in order to evaluate their secondary structures in various conditions.

EXPERIMENTAL PROCEDURES

Peptide synthesis and purification. Peptide syntheses were performed by the solid phase method (8) using

Fmoc as the N^{α}-amino protecting group. All the peptides were purified by HPLC on a reversed-phase C_{18}-column. The sequence data of the synthetic peptides used in this study are listed in Table 1.

Determination of minimal inhibitory concentration (MIC). *Escherichia coli* 0111 and *Bacillus subtilis* were used to investigate the antimicrobial activities of BLFC and its derivatives. *E. coli* was chosen as a model of Gram-negative bacteria and *B. subtilis* as Gram-positive bacteria. The bacteria were grown in Luria–Bertani medium (LB, 10 g of bactotryptone, 5 g of yeast extract and 10 g of NaCl per litre) at 37 °C overnight and diluted in a basal medium of 1% bactopeptone (Difco) to 1:200 [i.e. a final bacterial suspension containing $(2–8) \times 10^6$ colony forming units (CFU)/mL]. The peptides dissolved in 1% bacto-peptone were added to the 100 μL of bacterial suspension at concentrations varying from 25 or 30 μg/mL in the case of *B. subtilis* and 90 or 120 μg/mL in the case of *E. coli*, for serial two-fold dilutions in triplicate, and then incubated overnight at 37 °C in a shaking incubator. The MIC was defined as the lowest concentration of the peptide at which there was no change in all three series in absorbance, between time 0 and 18 h at 620 nm.

Bacterial growth kinetics in the presence of the peptides. The cultures which were grown in LB medium overnight were diluted in 1% bacto-peptone to a final cell density of 0.3 absorbance at 600 nm. *E. coli* and *B. subtilis* were treated with 100 and 50 μg/mL of the peptides, respectively, and incubated at 37 °C. The bacterial growth was determined by absorbance at 600 nm.

Determination of outer membrane disruption of E. coli. The disruption of the outer membrane was monitored by measuring the β-lactamase activity from *E. coli* 0111 harboring pUC19 plasmid. The bacteria were grown to log phase in LB medium containing 100 μg/mL of ampicillin, harvested, and washed with phosphate-buffered saline (PBS; 35 mM phosphate buffer and 0.15 M NaCl, pH 7.0). The cells were resuspended in PBS to reach a final cell density of 1 absorbance unit at 600 nm, and then incubated with various concentrations of B3 at 37 °C. β-Lactamase activity in the cell-free supernatant was determined by ampicillin decomposition at different times (9). The total enzyme activity of the cell was measured with the lysed cell, and this activity was taken as 100%.

Hemolytic assays. The hemolytic activities of the peptides were determined using human red blood cells (RBCs). The RBCs were isolated from heparinized blood by centrifugation and washed twice with PBS just prior to the assay. Four hundred μg/mL of the peptide in PBS was mixed with an equal volume of a 1% solution (v/v) of RBCs suspended in PBS. Following 1 h incubation at 37 °C, the samples were centrifuged at $1000 \times g$ for 5 min. The supernatant was separated, and its absorbance was measured at 414 nm. Blank hemolysis and 100% hemolysis controls were determined using centrifugates of RBCs suspended in PBS and 1% Triton X-100, respectively.

Circular dichroism. Circular dichroism (CD) spectra were recorded on a Jasco J720 spectropolarimeter. The spectra were measured at 25 °C using a 1 mm pathlength cell. The concentration of peptides was 0.1 mg/mL in solutions of 0, 50% (v/v) NMR grade trifluoroethanol (TFE) and 30 mM sodium dodecyl sulfate (SDS) containing 10 mM sodium phosphate, pH 7.0. The mean residue ellipticity (MRE) is given in deg cm^2 $dmol^{-1}$.

RESULTS

The purities of the synthetic peptides were above 95% as measured by analytical RP-HPLC. The antimicrobial and hemolytic activities of the peptides and their secondary structures were studied using these peptides.

TABLE 1

Amino acid sequences of synthetic peptides used in this study

Peptides	Sequences																								Remarks
B1 (BLFC)	F	K	C	R	R	W	Q	W	R	M	K	K	L	G	A	P	S	I	T	C	V	R	R	A	F	Native BLFC
B2	–[a]	–	S	–	–	–	–	–	–	–	–	–	–	–	–	–		–	–	S	–	–	–	–	–	S-substituted BLFC
B3				–	–	–	–	–	–	–	–	–	–													Core region of BLFC
B4				K	K	–	–	–	K	–	–	–	–													3R→K
B5				–	–	–	–	–	–	–	R	R	–													2K→R
B6				K	K	–	–	–	–	–	–	–	–													2R→K
B7				–	–	–	–	R	W	–	–	–	–													WR→RW
B8				E	E	–	–	–	E	–	E	E	–													5 basic aas→E
B9				–	–	A	A	A	–	A	–	–	A													5 hydrophobic aas→A

[a] Each dashed line (–) indicates the same amino acid in BLFC.

Antimicrobial and hemolytic activities of the peptides

The antimicrobial activities of the peptides were determined by measuring MICs and growth kinetics. The MICs of the long peptides, BLFC (B1) and B2, composed of 25 amino acid residues, were 6.3 and 7.5 μg/mL for *B. subtilis* and 22.5 and 30 μg/mL for *E. coli,* respectively (Table 2). These results indicated that the disulfide bond of BLFC might not be critical for antimicrobial activity, and agreed with the result of Bellamy *et al.* (7) using the synthetic analog containing covalently blocked (acetoamidomethyl-) cysteine residue. Since the loop structure of BLFC is not essential for antimicrobial activity, we focused the short basic rich sequence (B3) with an amphipathic α-helical propensity at the *N*-terminus of BLFC (Fig. 1). B3 was mainly composed of basic and hydrophobic residues. This unique property of B3 was thought to be useful for analyzing the structure–activity relationships.

B3 and its substituted peptides (B4–B7) also had similar antimicrobial activity compared to the long peptides (B1 and B2). The MIC ranges of the peptides were 6.3–15 μg/mL for *B. subtilis* and 11.3–30 μg/mL for *E. coli* (Table 2). Among the peptides, B5 with arginine substituted for all basic amino acids of B3 showed the most effective antimicrobial activity against both *B. subtilis* and *E. coli.* The short peptides (B3, B5 and B7) with double arginine residues at their *N*-termini showed 1.5- to 2-fold activity compared to those (B4 and B6) with double lysine residues (Table 2). The results indicated that arginine may be more effective than lysine as basic amino acids on antimicrobial activities. B7, originating from B3, which adopted a classically amphipathic α-helical structure (Fig. 1), showed similar antimicrobial activity against both *E. coli* and *B. subtilis* compared to its native form (B3) (Table 2). However, B8, with glutamic acid substituted for basic amino acid residues, and B9, with alanine substituted for all hydrophobic residues, displayed little or no antimicrobial activity, indicating that the basic and hydrophobic residues are indeed essential for antimicrobial activity.

TABLE 2

MIC and hemolytic activity of BLFC-derived peptides

Peptides	MIC (μg/mL)		% Hemolysis
	B. subtilis	*E. coli*	(200 μg/mL, 1 h)
B1	6.3	22.5	10
B2	7.5	30	4.6
B3	7.5	22.5	1.8
B4	15	30	0.74
B5	6.3	11.3	1.5
B6	15	30	1
B7	7.5	22.5	0.4
B8	>30	>120	0
B9	>30	>120	0

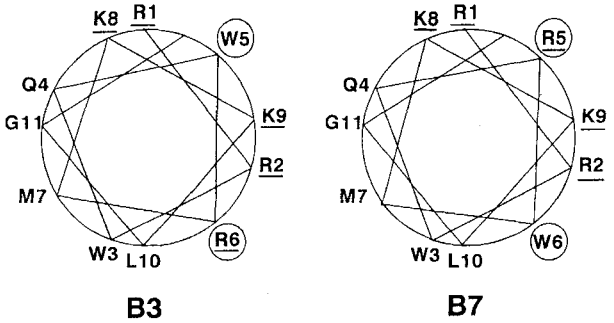

FIGURE 1

Helical wheel representation of B3 and its substituted peptide (WR→RW), B7. The switched amino acids are circled and the basic residues are underlined.

Many antimicrobial peptides have been known to lyse the RBC membrane as well as bacterial cell membrane. We have investigated the hemolytic activity of peptides whether they are appropriate for possible therapeutic uses. Although the short peptides (B3–B7) composed of 11-residues had similar antimicrobial activity with the long peptides (B1 and B2), the peptides showed much less hemolytic activity (Table 2). The hemolyses of RBCs treated with the short peptides ranged between 0.4 and 1.8%, whereas the hemolyses treated with B1 and B2 were 10 and 4.6%, respectively. Among these peptides, B7, which was fitted to an amphipathic α-helical structure, showed the lowest hemolytic values. The peptides (B8 and B9) with no antimicrobial activity also showed no hemolytic activity (Table 2).

The killing kinetics of the short peptides with basic residues (B3–B6) at high bacterial concentrations was significantly dependent on their *N*-terminal basic residues (Figs. 2 and 3). The peptides (B3 and B5) with double arginine at their *N*-termini inhibited *B. subtilis* growth more efficiently than those (B4 and B6) with double lysine (Fig. 2). When *B. subtilis* was treated with 50 μg/mL of the peptides, initial absorbances at 600 nm of the cultures were increased from 0.3 to 0.43 (B3 and B5) and 0.38 (B4 and B6) (Fig. 2). These phenomena might be due to the bacterial aggregation by the addition of peptides. B3 and B5 effectively inhibited *B. subtilis* growth up to 500 min. However, B4 and B6 inhibited the bacterial growth until 100 and 200 min, respectively, and then the cells were regrown (Fig. 2). Growth inhibitions of *E. coli* by peptide treatment (Fig. 3) were less efficient than that of *B. subtilis*, and B3 and B5 had more effective killing kinetics than B4 and B6. These results may be related to the outer membrane of *E. coli.*

Since BLFC has been known to disrupt the outer membrane of Gram-negative bacteria by releasing lipopolysaccharide (LPS) (10), disruption of the outer membrane of *E. coli* was monitored by measuring β-lactamase activity harboring pUC19 plasmid,

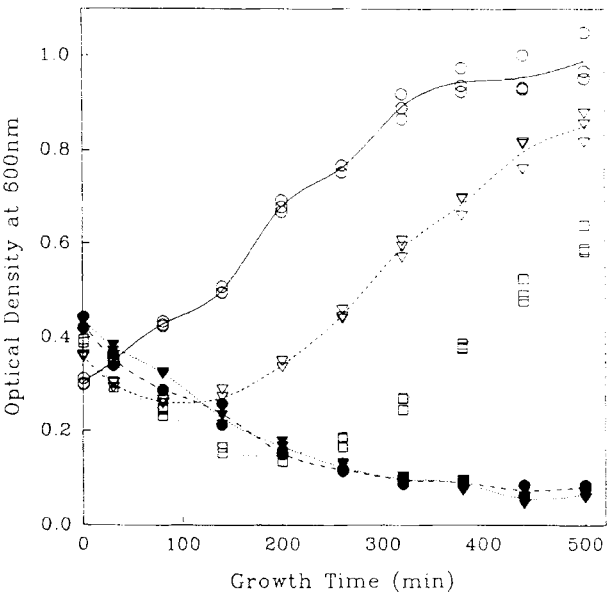

FIGURE 2

The antimicrobial effect of the substituted peptides of B3 against *B. subtilis*. Bacteria were grown to $Abs_{600}=0.3$ and then peptides B3 (●), B4 (▽), B5 (▼) and B6 (□) were added to a final concentration of 50 μg/mL. The control group is indicated by the symbol ○.

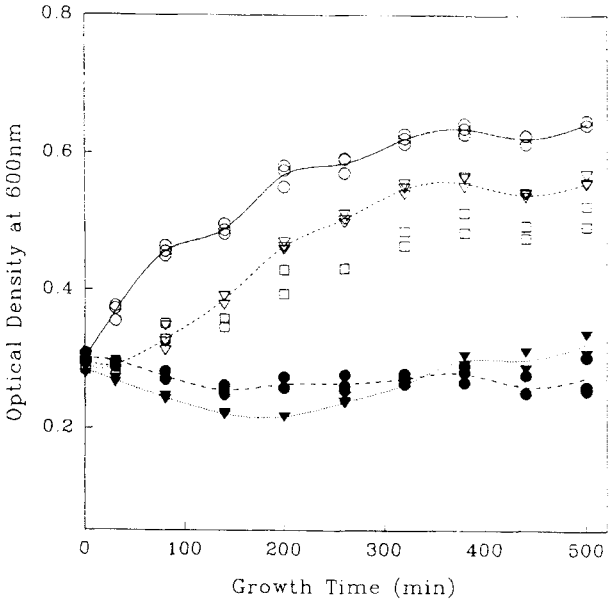

FIGURE 3

Antimicrobial effect of the BLFC-derived peptides against *E. coli*. Bacteria were grown to $Abs_{600}=0.3$ and peptides B3 (●), B4 (▽), B5 (▼) and B6 (□) were added to a final concentration of 100 μg/mL. The control group is indicated by symbol ○.

since β-lactamase is a typical periplasmic enzyme (9). As shown in Fig. 4, B3 induced the disruption of the outer membrane in dose-dependent and time-dependent manners, indicating that B3 could have disrupted the outer membrane by the release of LPS.

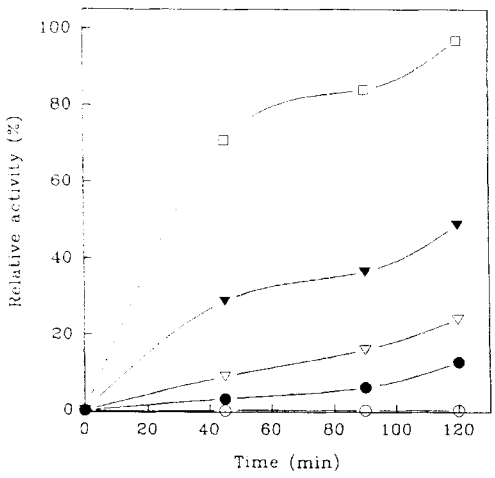

FIGURE 4

Effect of B3 peptide on the disruption of outer membrane. The bacteria were grown to log phase, harvested and suspended in PBS to reach a final cell concentration of $Abs_{600}=1$ and incubated with the following concentration (in mg/mL) of B3 peptide : 0 (○), 0.2 (●), 0.5 (▽), 1.0 (▼) and 2.0 (□). Aliquots were withdrawn at different time intervals, and the disruption of the outer membrane was determined as the relative activity, in which percentages are compared to the total activity of β-lactamase.

Relationships between antimicrobial activity and the secondary structures of the peptides

To investigate whether the antimicrobial activity of the peptides is related to their secondary structures, CD analyses were performed. As illustrated in Fig. 5, all the CD spectra of the peptides in aqueous solution showed random conformations with intensive negative bands below 200 nm regardless of the differences of their antimicrobial activity. The peptides in 50% TFE adopted the α-helical structure. It is known that TFE induces an α-helical conformation in many peptides because this conformation with the maximal hydrogen bonded backbone is energetically favorable (11). Whereas 25-residue peptides (B1 and B2) in 50% TFE had strong negative bands at 207 nm and a negative shoulder around 220 nm, 11-residue peptides (B3–B9) had weak negative bands around 205 nm and negative shoulders around 218 nm. The low helical tendency of the short peptides could be due to the strong length dependence of the ellipticity values in the peptides. However, no relationships between the secondary structures in 50% TFE and antimicrobial activity were found. Although B8 with glutamic acid substituted for all basic amino acids did not show antimicrobial and hemolytic activity (Table 2), its CD spectrum was similar to that of the native form, B3.

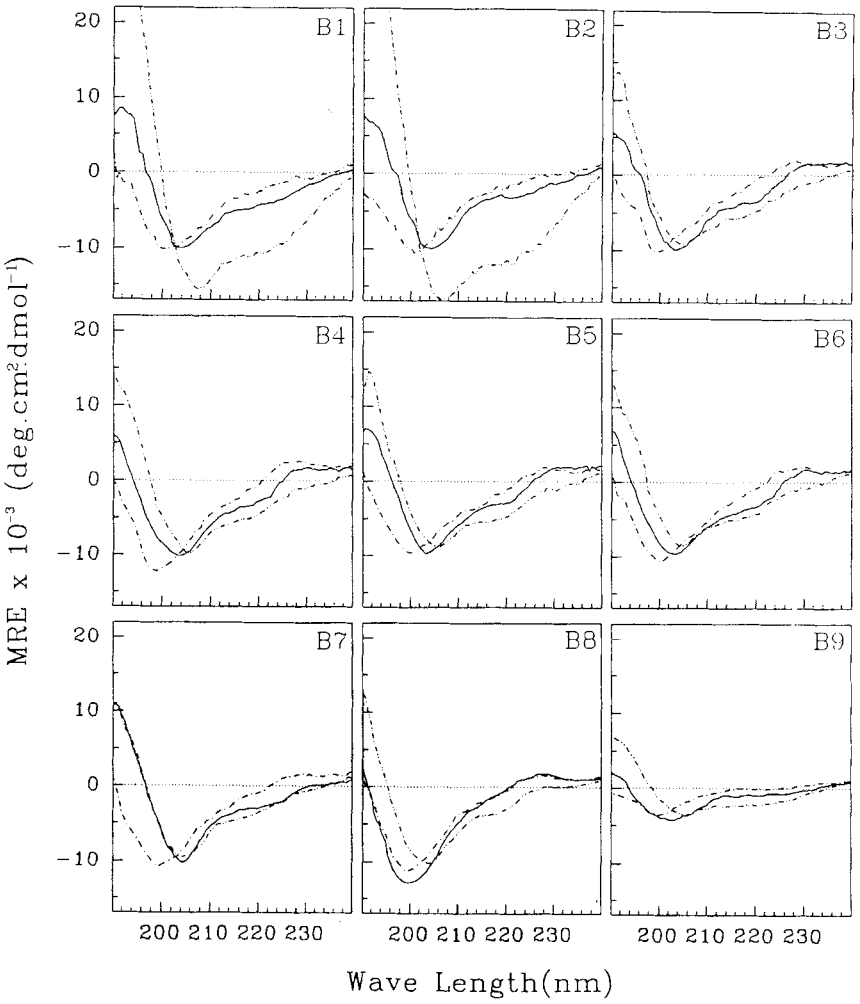

FIGURE 5

The CD spectra of various peptides in aqueous solution (—·—·—), 50% TFE (—··—··—) and 30 mM SDS (—) containing 10 mM sodium phosphate buffer. MRE is mean residue ellipticity.

In 30 mM SDS, which mimics a condition of an acidic lipid membrane, the CD spectra of the peptides appeared in different patterns. The conformations of the long peptides (B1 and B2) in 30 mM SDS were more ordered than those in aqueous solution, but much less ordered those in 50% TFE (Fig. 5). Moreover, the CD spectra of long peptides in 30 mM SDS were similar to those of the short peptides (B3–B7). These results suggested that only small portions of the long peptides interact with the SDS molecule. The CD spectra of the short peptides (B3–B7) had blue shifted bands around 203–204 nm and slightly increased ellipticity values at 220 nm in comparison with those in 50% TFE, except B7, which is designed to fit to an amphipathic α-helical structure (Fig. 1). The CD spectrum of B7 in 30 mM SDS was similar to that in 50% TFE (Fig. 5). However, overall CD spectra of the peptides (B3–B7) dissolved in 30 mM SDS showed similar patterns in 50% TFE, indicating that most portions of the short peptides were involved in interaction with the SDS molecule. The acidic-substituted peptide (B8) and the alanine substituted peptide (B9) in SDS showed similar CD spectra to ones in aqueous solution. These results suggested that the interaction of peptides with SDS mimicking the lipid membrane may be correlated with their antimicrobial activity.

DISCUSSION

This report describes the use of the short peptides composed of 11 amino acid residues derived from bovine lactoferrin as an antimicrobial peptide. It has been recently discovered that a more effective antimicrobial peptide, BLFC, is generated on the gastric pepsin cleavage of lactoferrin (6). Both bovine lactoferrin and lactoferricin are known to have higher antimicrobial activity than human lactoferrin and

lactoferricin, respectively (7). Among the sequences of BLFC, RRWQWRMKKLG, which adopted an amphipathic α-helical structure (Fig. 1), was thought to play an important role in the antimicrobial activity of BLFC. The antimicrobial activity of the short peptide (B3) with the above sequence agreed with our assumption (Table 2). The corresponding regions in human and murine lactoferrins are less basic and less hydrophobic (7), and these structural properties are related to the lower antimicrobial activity. In our study, the corresponding peptide with the sequence KSFQWQRNMRKVRG, derived from human lactoferrin, showed low antimicrobial activity (data not shown).

The lower MIC values of the peptides on *B. subtilis* as compared with *E. coli* (Table 2) suggested that BLFC and the short peptides seemed to interact directly with the cell membrane of *B. subtilis*, and then kill it by forming ion channels. In growth kinetics, *B. subtilis* was rapidly killed by the short peptides (Fig. 2). The re-growth of *B. subtilis* in the cultures treated with peptides (B4 and B6) may be attributed to the lower antimicrobial activity and/or higher degradation rate by proteolytic enzymes secreted by *B. subtilis*. The outer membrane of *E. coli* seemed to act as a barrier prohibiting the direct access of the peptides to its cell membrane. Thus a larger amount of the peptides may be needed to kill *E. coli* than *B. subtilis* (Table 2), and the growth kinetics of *E. coli* showed the inefficient killing rate through the incubation of the peptides (Fig. 3). These results suggested that the cell membranes of both *B. subtilis* and *E. coli* may be targets of the peptides for killing the cell, and the outer membrane of *E. coli* may act as a barrier for the peptides. To evaluate the killing mechanism of *E. coli* by the peptides, further studies are needed.

It seemed that B3 acted directly on the outer cell membrane of non-growing *E. coli* in buffer (PBS), although a high concentration (2 mg/mL) of B3 was required for full disruption of the large number of *E. coli* (1 absorbance unit at 600 nm) (Fig. 4). This result may be due to the high bacterial concentration and non-growing state of *E. coli*. The lipid membrane of growing cells is generally thought to be looser than that of non-growing cells, and antimicrobial peptides are more effective in a growing condition than in a non-growing condition. Evidence to support this explanation has been reported. Attacins, a family of the large antimicrobial proteins from insects, kill only growing bacteria (12), and PR-39, a arginine and proline rich peptide, kills growing *E. coli* considerably faster than non-growing *E. coli* (13). However, it was also reported that there was no difference between growing and non-growing *E. coli* killed by cecropin P1 (13).

In the substitution study, the potent antimicrobial activities of B3 and B5 with double arginine residues that at their *N*-termini may be related to their bulky guanidinium group. The peptides with two arginine residues at their *N*-terminus could disrupt the bacterial cell membrane more effectively than those with the relatively small lysine residue in the 11 residue peptides (Table 2, Figs. 2 and 3). However, this side-group effect cannot be applied to all antimicrobial peptides. The antimicrobial activity of the model peptide with a sequence, KLKLLLLLKLK-NH_2, significantly decreased when all lysines were substituted with arginine (14). This discrepancy may be related to the differences of their sequences and/or hydrophobic residues.

The amphipathic basic peptides (B1–B7) adopted α-helical structures in both TFE and SDS, indicating that hydrophobic conditions are required for maintaining the α-helical structure. However, their α-helical propensities were quite different according to the peptide length. The α-helical propensities of the long peptides (B1 and B2) in SDS were considerably weaker than those in TFE (Fig. 5), indicating that partial regions of B1 and B2 were involved in interaction with SDS, whereas most regions of the short peptides (B3–B7) were involved in interaction with SDS. The results suggested that the sequence composed of 11 residues of BLFC may be important for interaction with a bacterial cell membrane. The basic and hydrophobic residues were critical for antimicrobial activity and bacterial membrane binding. The acidic substituted peptide (B8) and alanine substituted peptide (B9) showed little or no antimicrobial activity and interaction with SDS. It is generally known that cationic amphipathic α-helical structures are related to antimicrobial activity by forming ion channels through membrane bilayers (15–17). Many antimicrobial peptides, such as cecropins (18) and magainins (19), are included in this family. Although a length of ca. 20 residues is necessary to provide an α-helix capable of spanning the lipid bilayer (15), the cationic amphipathic α-helical peptides composed of 8–12 residues were found to form ion channels (17), and agreed well with our results.

We have investigated the hemolytic activity of peptides as to whether they are appropriate for possible therapeutic agents. In this study, the short peptides had less hemolytic activity than the long peptides, maintaining similar antimicrobial activity (Table 2). Recent studies (20) suggested that the short peptides (> 14-mer) had a much lower hemolytic activity while keeping their antimicrobial activity. Thus making a short peptide seems to be beneficial for the development of peptide antibiotics with low toxicity.

In conclusion, the 11-residue peptide derived from BLFC showed similar antimicrobial activity to BLFC, but had lower hemolytic activity. The double arginine residues at the *N*-terminus of the short peptide were more important for the effective antimi-

crobial activity than lysine residues. Most regions of the short peptide were involved in interaction with SDS, indicating that the corresponding sequence is critical to bacterial membrane interaction. The data of CD spectra of the peptides were correlated with their antimicrobial activity.

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REFERENCES

1. Brock, J.H. (1980) *Arch. Dis. Child* **55**, 417–421
2. Bullen, J.J. (1981) *Rev. Infect. Dis.* **3**, 1127–1138
3. Reiter, B. (1983) *Int. J. Tissue React.* **5**, 87–96
4. Davidson, L.A. & Lonnerdal, B. (1987) *Acta Pediatr. Scand.* **76**, 733–740
5. Spik, G., Brunet, B., Mazuvier-Dehaire, C. & Montrenil, J. (1982) *Acta Pediatr. Scand.* **71**, 979–985
6. Tomita, M., Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H. & Kawase, K. (1991) *J. Dairy Sci.* **74**, 4137–4142
7. Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. & Tomita, M. (1992) *Biochim. Biophys. Acta* **1121**, 130–136
8. Barany, G. & Merrifield, R.B. (1980) in *The Peptides. Analysis, Synthesis, Biology* (Gross, E. & Meienhofer, J. Eds.) Vol. 2, pp. 1–284, Academic Press, New York
9. Liu, J. & Walsh C.T. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4028–4032
10. Ellison, R.T., Giehl, T.J., Tomita, M. & Yamauchi, K. (1993) *Infect. Immun.* **61**, 719–728
11. Steiner, H. (1982) *FEBS Lett.* **137**, 283–287
12. Hultmark, D., Engstrom, A., Adersson, K., Steiner, H., Bennich, H. & Boman, H.G. (1983) *EMBO J.* **2**, 571–576
13. Boman, H.G., Agerberth, B. & Boman, A. (1993) *Infect. Immun.* **61**, 2978–2984
14. Alvarez-Bravo, J., Kurata, S. & Natori, S. (1994) *Biochem. J.* **302**, 535–538
15. Lear, J.D., Wasserman, Z.R. & Degardo, W.F. (1988) *Science* **240**, 1177–1181
16. Agawa, Y., Lee, S., Ono, S., Aoyogi, H., Ohno, M., Taniguchi, T., Anzai, K. & Kirino, Y. (1991) *J. Biol. Chem.* **266**, 20218–20222
17. Anzai, K., Hamasuna, M., Kadono, H., Lee, S., Aoyagi, H. and Kirino, Y. (1991) *Biochim. Biophys. Acta.* **1064**, 256–266
18. Boman, H.G. & Hultmark, D. (1987) *Annu. Rev. Microbiol.* **41**, 103–128
19. Bevins, C.L. & Zasloff, M. (1990) *Annu. Rev. Biochem.* **59**, 395–414
20. Blondelle, S.E. & Houghten, R.A. (1992) *Biochemistry* **31**, 12688–12694

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