Halocidin: a new antimicrobial peptide from hemocytes of the solitary tunicate, *Halocynthia aurantium*

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Abstract From hemocytes of the tunicate *Halocynthia aurantium* we purified a new antimicrobial peptide named halocidin. The native peptide had a mass of 3443 Da and comprised two different subunits containing 18 amino acid residues (WLNALLHHGLNCAKGVLA) and 15 residues (ALLHHGLNCAKGVLA), which were linked covalently by a single cystine disulfide bond. Two different monomers were separately synthesized and used to make three additional isoforms (15 residue homodimer, 18 residue homodimer, heterodimer). In antimicrobial assays performed with synthetic peptides of halocidin, it was confirmed that congeners of the 18 residue monomer were more active than those of the 15 residue monomer against methicillin-resistant *Staphylococcus aureus* and multidrug-resistant *Pseudomonas aeruginosa*. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Antimicrobial peptide; Tunicate; Hemocyte; Heterodimer; Antibiotic-resistant bacterium

1. Introduction

In recent years overuse of antibiotics has resulted in the rapid evolution of multiple drug-resistant bacteria, which have caused a variety of incurable infectious diseases. In the hope of inventing novel antimicrobial agents to control the increasing antibiotic-resistant bacteria, many cationic antimicrobial peptides have been isolated from diverse organisms over the last two decades [1–4]. It has been considered that the target of most antimicrobial peptides is the specific lipid bilayer of the bacterial membrane. This mechanism of action suggested that antimicrobial peptides might have an essential structure for attacking the bacterial membrane and retain their antimicrobial activities against resistant bacteria to conventional antibiotics, and also have minimal toxicity towards human cells [5]. Although much information has accumulated on the relationship between function and structure of antimicrobial peptides, search of natural peptides is still striving to identify a novel structure with omni-potency against a wide variety of pathogenic antibiotic-resistant bacteria.

Recently, we isolated an antimicrobial peptide, named 'di-cynthaurin', from the hemocytes of the tunicate, *Halocynthia aurantium* [6]. Di-cynthaurin contains an unpaired cysteine and forms a covalent homodimer, each monomer consisting of 30 amino acid residues. Here we report another antimicrobial peptide, 'halocidin', which is purified from the same source as di-cynthaurin. Halocidin also has a dimer structure, which is formed via a disulfide linkage between cysteines of two different sized monomers. This paper describes the purification and characterization of halocidin, and also contains data to allow comparison of the antimicrobial activities of halocidin and its congeners against two antibiotic-resistant bacteria with those of other previously known peptides.

2. Materials and methods

2.1. Purification of antimicrobial peptide from hemocytes of tunicates

Halocidin was isolated and purified from acid extracts of tunicate hemocytes that were prepared as described in previous works [6–8]. Briefly, living *H. aurantium* were purchased at a local sea market in Sockcho, South Korea. Their tunics were washed lightly with 70% ethanol, blotted dry and transected peribasally. The hemolymph was collected dripwise into a 50 ml test tube containing 150 mg of dry ethylenediaminetetraacetic acid. The collected material was pooled, passed through a sterile 74 μm mesh filter (Netwell, Corning Costar, Cambridge, MA, USA) and centrifuged at $300\times g$ for 10 min at 4°C. The hemocytes were resuspended in 30 ml of 0.34 M sucrose and then re-centrifuged as above. The recovered cells were resuspended in 10 ml of ice-cold 5% acetic acid, briefly sonicated and then 40 ml of 5% acetic acid was added. Thereafter the mixture was stirred overnight at 4°C and centrifuged at $20\,000\times g$ for 30 min at 4°C. The supernatant was used as a starting material to purify antimicrobial peptides.

A sample of supernatant containing at least 50 mg protein was loaded on a Sephadex G-50 gel filtration column equilibrated with 5% acetic acid. Each fraction from gel chromatography was tested for antibacterial activity in an ultrasensitive radial diffusion assay [9] and the active fractions were pooled and concentrated for further purification steps by preparative acid urea (AU)–polyacrylamide gel electrophoresis (PAGE) [10]. The AU–PAGE eluates were collected in 2 ml fractions and concentrated by vacuum centrifugation (Centra Evaporator, Bioneer, Korea). Every tenth fraction was electrophoresed on duplicate AU–PAGE gels. One gel was stained with Coomassie blue for monitoring the band and the other was used to identify antimicrobial activity of protein bands in a gel overlay assay [11]. Active fractions from preparative AU–PAGE were pooled and finally subjected to C18 reversed-phase (RP) HPLC column (Vydac 218TP54: The Separation Group, Hesperia, CA, USA). HPLC fractions were eluted in various linear gradients of acetonitrile containing 0.1% trifluoroacetic acid. For the first 10 min after loading sample, the column was washed with 5% acetonitrile at flow rate of 0.5 ml/min. Next the acetonitrile concentration was linearly increased by 1%/min for 60 min. Throughout the overall purification steps, we used a methicillin-resistant *Staphylococcus aureus* (MRSA-CCARM3001) in radial diffusion assays to confirm the antimicrobial activities of fractions.

2.2. Peptide characterization and synthesis

Lyophilized native halocidin was dissolved in 25 μl of 8 M urea and 25 μl of 0.4 M ammonium bicarbonate, pH 8.0. Next 5 μl of 45 mM dithiothreitol was added to the mixture and incubated at 50°C for

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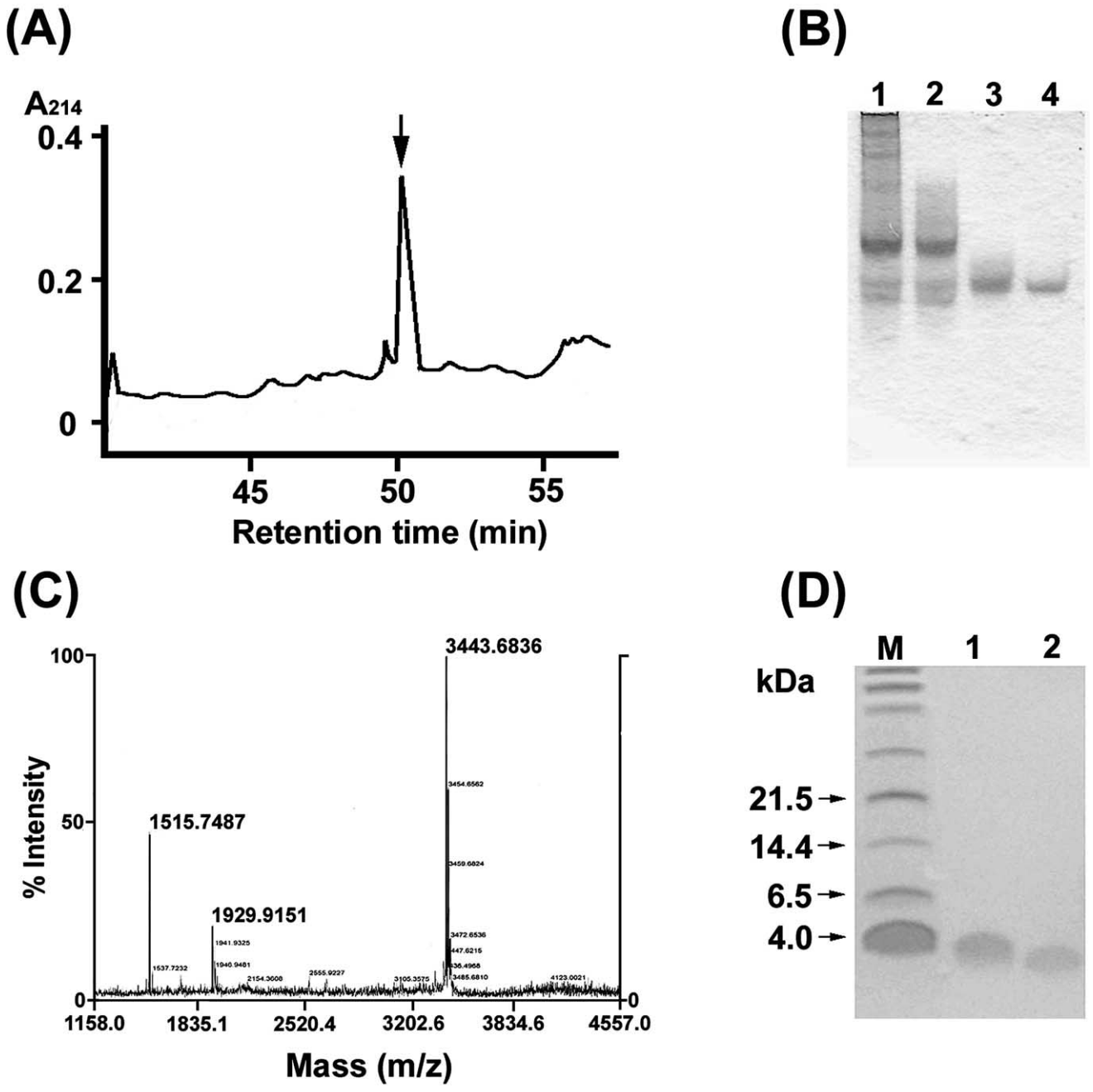

Fig. 1. Purification and MALDI mass spectrometric analysis of halocidin. A: C18 RP-HPLC as a final step to purify halocidin. Arrow indicates the peak including halocidin. B: AU–PAGE showing stages of purification. Lane 1, crude extracts of hemocytes; lane 2, active fractions from gel filtration; lane 3, active fractions from preparative AU–PAGE; lane 4, purified halocidin from RP-HPLC. C: MALDI mass analysis. The major peak appeared at *m/z* value of 3443.68 and two minor peaks at 1515.74 and 1929.91. D: Tricine SDS–PAGE gel with purified halocidin. Lane M, standard molecular weight markers; lane 1, natural (unreduced) halocidin; lane 2, halocidin reduced with dithiothreitol. PAGE gels were stained with Coomassie blue.

Table 1
Molecular masses (Da) of natural and synthetic peptides

	Peptide	Calculated mass	MALDI mass
Natural halocidin	15 monomer	1515.9	1515.74
	18 monomer	1929.4	1929.91
	heterodimer	3443.3	3443.68
Synthetic halocidin	15 monomer	1515.9	1515.70
	18 monomer	1929.4	1928.92
	15 homodimer	3029.8	3031.07
	18 homodimer	3856.8	3861.06
	heterodimer	3443.3	3445.04

15 min. To prepare vinylpyridine derivatives of halocidin monomers, 15 μl of vinylpyridine was added to the mixture that was cooled to room temperature and incubated in the dark for at least 30 min. Final reaction products were purified by RP-HPLC. Amino acid sequences of halocidin monomers were separately determined by gas-phase Edman degradation with a Procise 491 (Applied Biosystems, USA) instrument. The molecular mass of purified halocidin was measured by a matrix-assisted laser desorption ionization (MALDI) mass instrument (Voyager-DE STR, PerSeptive Biosystems, USA).

Two monomers (18 residue monomer and 15 residue monomer) of halocidin were separately synthesized by using the automated solid-phase peptide synthesizer (Pioneer, Applied Biosystems, USA) in the Korea Basic Science Institute and repurified by RP-HPLC to close to homogeneity. To prepare dimer forms, two synthetic peptides (1 mg/ml each) were mixed in 0.1 M ammonium bicarbonate and the mixture was left to stand open to the atmosphere for more than 72 h. Finally, synthetic halocidin congeners were purified by RP-HPLC, and each peak was identified by measuring its molecular mass by MALDI mass spectrometry.

2.3. Antimicrobial and hemolytic activity

Antimicrobial activities of peptides were measured in radial diffusion [9] and colony count assays [12]. For radial diffusion assay, stock peptides solutions were prepared in acidified distilled water (0.01% acetic acid) and 5 μl samples were introduced as a series of six serial two-fold dilutions. These ranged in concentration from 3.12 to 200 μg of peptide/ml and loaded into the wells (3 mm in diameter) in the underlay, in which washed mid-logarithmic phase bacteria were trapped. The underlay agars consisted of sterile citrate phosphate buffer (9 mM sodium phosphate, 1 mM sodium citrate, pH 7.4), 1% (w/v) type I (low electroendosmosis) agarose (A 6013; Sigma), and 3% tryptic soy broth (TSB: Difco, Detroit, MI, USA). After allowing 3 h for diffusion of peptides into underlay gels, a 10 ml nutrient-rich overlay gel containing 6% TSB and 1% agarose was poured. After the plates were incubated overnight to allow surviving bacteria to form colonies, the diameters of clearing zones, indicating antibacterial activity, were plotted against the $\log_{10}$ of the peptide concentrations. For colony count assay, peptides were mixed to final concentration of 5 μg/ml with mid-logarithmic phase MRSA (CCARM3001) or multidrug-resistant *Pseudomonas aeruginosa* (MDRPA-CCARM2002) in a sterile 10 mM sodium phosphate buffer (pH 7.4) containing 0.3 mg/ml of TSB powder. The mixtures, typically in a final volume of 100 μl, were incubated for the pre-determined times (5 min and 15 min) at 37°C in a shaking water bath and 20 μl aliquots were directly removed at each interval and plated on 1.5% Bacto-agar (Difco). The resulting colonies were counted after an overnight incubation. Both antimicrobial assays were repeated at least three times and mean values were used to make a graph. Magainin 1 and buforin 2 purchased from Sigma were used as standard peptides.

For hemolytic assay, 20 μl of peptides at pre-determined concentration was added to 180 μl of a 2.5% (V/V) suspension of human erythrocytes in phosphate-buffered saline (PBS). Melittin (Sigma), a hemolytic and α-helical peptide from bee venom, was used as positive control sample. The mixture was incubated for 30 min at 37°C and 600 μl of PBS was added to each tube. After centrifugation at $10\,000 \times g$ for 3 min, the supernatant was removed and the absorbance measured at 540 nm.

2.4. Other methods

A Model Mini-Protean 3 Cell (Bio-Rad) was used to perform Tricine SDS–PAGE [13] and AU–PAGE [14]. Peptide concentrations were measured with bicinchoninic acid (Sigma).

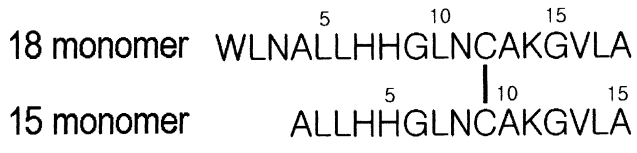

Fig. 2. Primary sequences of two subunits of halocidin. The sequences are shown in standard single letter code. Vertical bar represents a disulfide bond between cysteine residues of the 18 residue monomer and the 15 residue monomer.

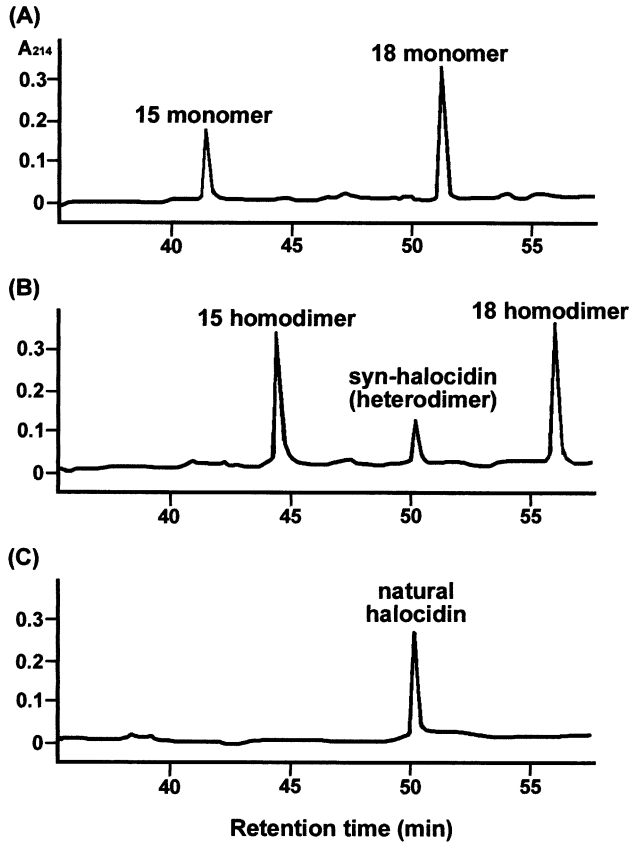

Fig. 3. RP-HPLC profiles with synthetic and natural halocidins. Each HPLC was performed in the same condition as in the purification of natural peptide. A: Two synthetic monomers, 15 monomer and 18 monomer, were eluted at 36.8% and 46.3% acetonitrile, respectively. B: Two homodimers, 15 homodimer and 18 homodimer, were eluted at 39.2% and 51.7% acetonitrile, respectively. The heterodimer, equivalent to natural halocidin, was eluted at the same concentration of acetonitrile as natural one. C: Natural halocidin was re-purified with RP-HPLC.

3. Results

3.1. Purification of halocidin

Halocidin was purified from acid extracts of *H. aurantium* hemocytes by a consecutive three-step procedure consisting of gel permeation chromatography, preparative AU–PAGE and RP-HPLC. Fig. 1A shows the RP-HPLC profile of active fractions eluted from the electrophoresis step that was especially powerful and afforded substantially pure halocidin (Fig. 1B). Halocidin was eluted at a retention time of 50.2 min corresponding to 45.2% acetonitrile.

3.2. Characteristics of halocidin

MALDI mass analysis of purified peptide showed three peaks with the predominant one having a mass of 3443.68 and the two less dominant ones having masses of 1929.91 and 1515.74 (Fig. 1C), suggesting halocidin has a dimer form as seen previously for di-cynthaurin [6]. This conclusion was confirmed by Tricine SDS–PAGE performed with non-reduced and reduced halocidin (Fig. 1D). Moreover, two peaks were detected in the profile of RP-HPLC used to repurify the reduced and modified peptides for amino acid sequencing (data not shown). Fig. 2 shows the primary structure

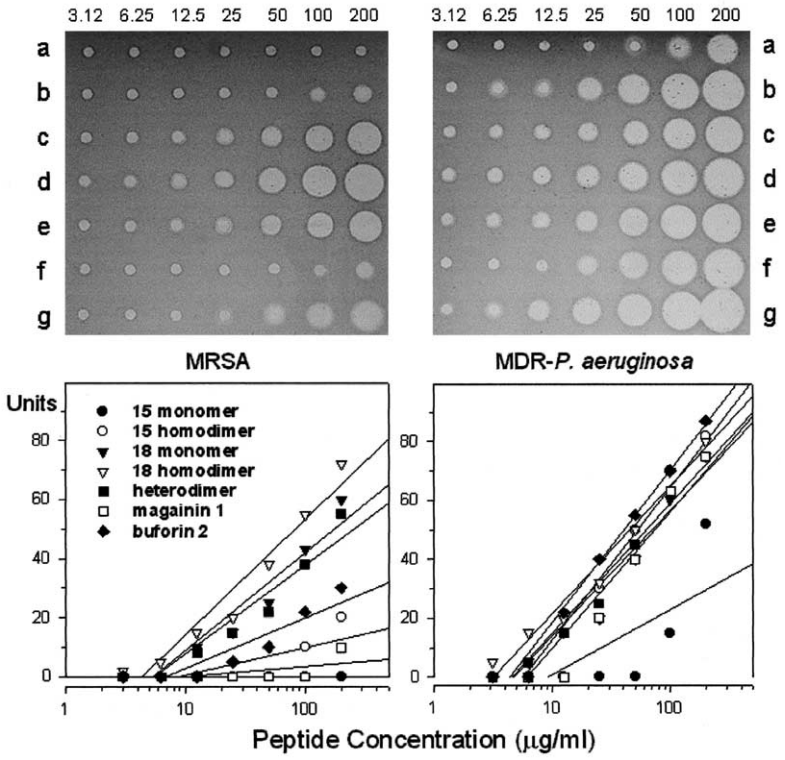

Fig. 4. Radial diffusion assays for antimicrobial activities of halocidin congeners against MRSA and MDRPA. Diameters of clearing zone have been expressed in units (1 mm = 10 units). The regression lines were computer generated, using a least-mean-squares program. Upper panels show photos of gels in radial diffusion assays. Numbers represent the concentration (μg/ml) of peptides loaded in each well. a: 15 monomer; b: 15 homodimer; c: 18 monomer; d: 18 homodimer; e: heterodimer; f: magainin 1; g: buforin 2. Antibiotic-resistant strains were obtained from the Culture Collection of Antibiotic Resistant Microbes (CCARM) at Seoul Women's University in Korea.

of halocidin. Our database search revealed that the amino acid sequence of halocidin is not homologous to that of any other known protein, indicating halocidin is a new peptide. Halocidin consists of two subunits that contain 18 and 15 residues, named 18 monomer and 15 monomer in this paper. Two monomers are linked by a single disulfide bond between the 12th Cys of the 18 monomer and the 9th Cys of the 15 monomer. In the case of many antimicrobial peptides including clavanins [7], styelins [8] and di-cynthaurin [6] from tunicates, they are C-terminally amidated. Therefore, we prepared synthetic peptides of the two monomers, each having an amidated C-terminal alanine. The measured masses of two syn-

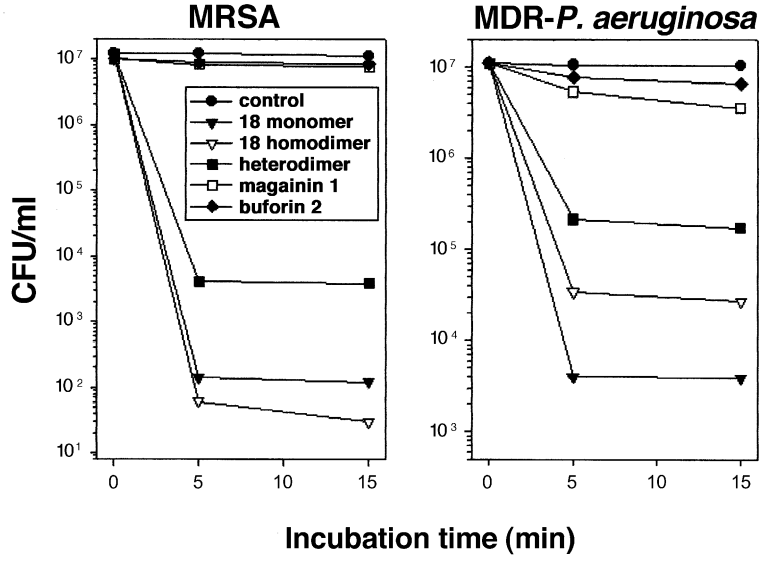

Fig. 5. Antimicrobial activities of peptides in colony count assay. Instead of peptides, an equivalent volume of 0.01% acetic acid to sample was added to each tube for control. Note 18 monomer and homodimer showed stronger activities than heterodimer after incubation for 5 min and 15 min, and standard peptides have little activity against both bacteria.

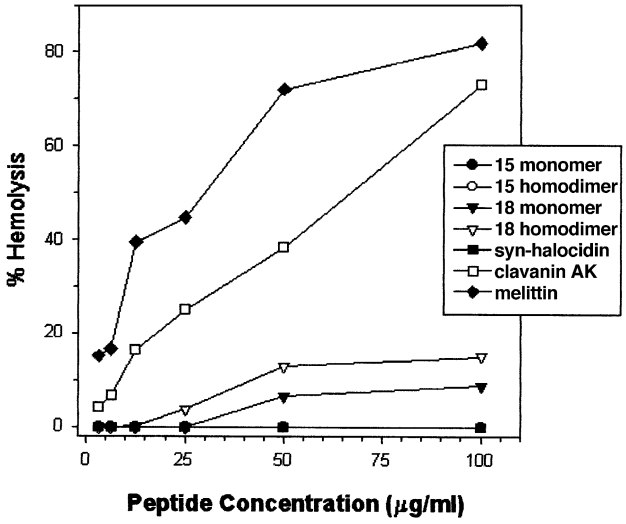

Fig. 6. Hemolytic activities for halocidin congeners. Triton-X100 1% was used for control of 100% hemolysis and 0.01% acetic acid for peptide-free control. Clavanin AK used as standard peptide is a clavanin A congener that has strong hemolytic activity [23]. Percent hemolysis was calculated with the following equation: Hemolysis (%) = (A_{540} of sample − A_{540} of peptide-free control)/(A_{540} of 100% control − A_{540} of peptide-free control) × 100.

thetic peptides almost agreed with those of their corresponding native forms and had the values calculated for the amidated forms (Table 1). Moreover the synthetic halocidin (heterodimer) consisting of two different monomers appeared at the same retention time (50.16 min: 45.16% acetonitrile) on RP-HPLC as the natural halocidin (Fig. 3).

3.3. Biological activities

We compared the abilities of synthetic halocidin congeners, which are 18 monomer, 15 monomer, 18 homodimer, 15 homodimer and heterodimer, with those of standard peptides, magainin 1 and buforin 2, to kill mid-logarithmic phase MRSA and MDRPA. While all peptides except the 15 monomer showed strong activities against MDRPA, only three peptides (18 monomer, 18 homodimer and heterodimer) apparently maintained their activities against MRSA in a radial diffusion assay (Fig. 4). In addition, our colony count assay revealed that 18 monomer, 18 homodimer and heterodimer reduced the number of viable MRSA by more than $3\log_{10}$–$5\log_{10}$ bacteria in 5 min, and viable MDRPA by more than $\log_{10}$–$3\log_{10}$. In contrast, other two standard peptides had no activity against both antibiotic-resistant bacteria even after 15 min incubation (Fig. 5). The hemolytic effects of halocidin congeners are shown in Fig. 6. Whereas 100 μg/ml of 18 monomer and 18 homodimer disrupted 8% and 16% of human erythrocytes, respectively, an equal concentration of other synthetic congeners of halocidin lacked hemolytic activity.

4. Discussion

In general, it has been reported that most antimicrobial peptides are positively charged and exert their activities by adapting to the anionic properties of bacterial cell envelopes. A number of studies showed that basic amino acids play a critical part in the mechanism of action of antimicrobial peptides as they attack bacterial cell membranes [15]. Each subunit of halocidin also has three basic amino acid residues (Fig. 2), which might contribute to the cationicity of halocidin. Compared with other cationic antimicrobial peptides, halocidin has relatively less positive net charge owing to its two histidines that have the smallest pK_a (about 6.5) of three cationic residues (Arg, Lys, His). Nevertheless halocidin retained a strong potency against MRSA and MDRPA in our two assay systems at pH 7.4. Moreover, it has been recently shown that many staphylococcal strains are resistant to the antimicrobial peptides [16,17], and established that the peptide-resistant bacteria neutralize the negatively charged groups of the cell wall by esterifying with the amino group of D-alanine, the latter reaction results in decreased interactions with cationic peptides [18]. Judging from the cationicity of halocidin and its activity against MRSA, we postulate therefore that halocidin may have an additional structural motif besides its cationicity, which is important for interaction with the bacterial cell wall.

Interestingly, halocidin has an intermolecular disulfide linkage, which is also confirmed in the structure of di-cynthaurin, an antimicrobial homodimeric peptide isolated from hemocytes of *H. aurantium* in our previous work [6]. Although halocidin is a heterodimeric peptide, unlike di-cynthaurin, its large subunit differs from the small one only by containing three additional N-terminal amino acid residues (Trp-Leu-Asn). Our antimicrobial assays revealed that 15 monomer and 15 homodimer were much less active than other halocidin congeners with an extra N-terminal part. What is the reason for the difference of activities between 15 monomer and 18 monomer congeners? We think one answer might be provided by a Trp residue at the N-terminus of the 18-mer. It is generally considered that the positioning of an aromatic residue at the N-terminal site is important for the antimicrobial activity of the peptide [19]. For instance, in the case of cecropins, well-known antimicrobial peptides from insects, it is confirmed that the exchange of Trp, the second amino acid of the N-terminal part, with a charged residue resulted in a prominent decrease of antibacterial activity [20]. On the other hand, our colony count assay revealed that halocidin congeners act much faster on bacteria than buforin 2 which requires introduction into the cell for its activity [21]. Taken together, although it is too early to determine the action mechanism of halocidin from our present data, we surmise that the main target of halocidin, like many other antimicrobial peptides including cecropins, might be the bacterial cell membrane [22].

Although we have not presented the antimicrobial spectra of halocidin congeners in this paper, we confirmed they retain strong activities against a wide variety of pathogenic bacteria. To design an ideal peptide antibiotic with a stronger activity against multidrug-resistant bacteria and with less hemolytic activity, we are now examining the biological activities of diverse synthetic peptides based on the halocidin structure.

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References

[1] Hancock, R.E. and Chapple, D.S. (1999) Antimicrob. Agents Chemother. 43, 1317–1323.
[2] Boman, H.G. (1995) Annu. Rev. Immunol. 13, 61–92.
[3] Broekaert, W.F., Terras, F.R., Cammue, B.P. and Osborn, R.W. (1995) Plant Physiol. 108, 1335–1358.

[4] Hancock, R.E. and Scott, M.G. (2000) Proc. Natl. Acad. Sci. USA 97, 8856–8861.
[5] Jacob, L. and Zasloff, M. (1994) Ciba Found. Symp. 186, 197–216.
[6] Lee, I.H., Lee, Y.S., Kim, C.H., Kim, C.R., Hong, T., Menzel, L., Boo, L.M., Pohl, J., Sherman, M.A., Waring, A. and Lehrer, R.I. (2001) Biochim. Biophys. Acta 1527, 141–148.
[7] Lee, I.H., Zhao, C., Cho, Y., Harwig, S.S., Cooper, E.L. and Lehrer, R.I. (1997) FEBS Lett. 400, 158–162.
[8] Lee, I.H., Cho, Y. and Lehrer, R.I. (1997) Comp. Biochem. Physiol. 118, 515–521.
[9] Steinberg, D.A. and Lehrer, R.I. (1997) Methods Mol. Biol. 78, 169–186.
[10] Harwig, S.S., Chen, N.P., Park, A.S. and Lehrer, R.I. (1993) Anal. Biochem. 208, 382–386.
[11] Lehrer, R.I., Rosenman, M., Harwig, S.S., Jackson, R. and Eisenhauer, P. (1991) J. Immunol. Methods 137, 167–173.
[12] Gilchrist, J.E., Donnelly, C.B., Peeler, J.T. and Campbell, J.E. (1977) J. Assoc. Off. Anal. Chem. 60, 807–812.
[13] Schagger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[14] Ganz, T., Selsted, M.E., Szklarek, D., Harwig, S.S., Daher, K., Bainton, D.F. and Lehrer, R.I. (1985) J. Clin. Invest. 76, 1427–1435.
[15] Hancock, R.E. and Diamond, G. (2000) Trends Microbiol. 8, 402–410.
[16] Peschel, A., Vuong, C., Otto, M. and Gotz, F. (2000) Antimicrob. Agents Chemother. 44, 2845–2847.
[17] Peschel, A., Otto, M., Jack, R.W., Kalbacher, H., Jung, G. and Gotz, F. (1999) J. Biol. Chem. 274, 8405–8410.
[18] Peschel, A. and Collins, L.V. (2001) Peptides 22, 1651–1659.
[19] Jacobs, R.E. and White, S.H. (1989) Biochemistry 28, 3421–3437.
[20] Steiner, H., Andreu, D. and Merrifield, R.B. (1988) Biochim. Biophys. Acta 939, 260–266.
[21] Park, C.B., Kim, H.S. and Kim, S.C. (1998) Biochem. Biophys. Res. Commun. 244, 253–257.
[22] Kagan, B.L., Selsted, M.E., Ganz, T. and Lehrer, R.I. (1990) Proc. Natl. Acad. Sci. USA 87, 210–214.
[23] Lee, I.H., Cho, Y. and Lehrer, R.I. (1997) Infect. Immun. 65, 2898–2903.