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The molecular factors derived from potent bactericidal activity of reduced cryptdin-4

（還元型 cryptdin-4 の強力な殺菌活性の分子機構に関する研究）

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Abstract

Defensins, a major family of antimicrobial peptides (AMPs), are cysteine-rich AMPs with three disulphide bonds under normal oxidative conditions. For a long time, studies concerning defensins have focused on oxidised forms consisting of three disulphide bridges. However, it has been reported that reduced defensin exhibits bactericidal activity equal to or greater than that of its oxidised form. After that, it has been focused on the reduced defensins studies. α-Defensins secreted by small intestinal Paneth cells are known as cryptdins. Cryptdin-4 (Crp4) is the most widely studied cryptdin. Reduced Crp4 ($Crp4_{red}$) has been reported to exert potent bactericidal activity against enteric commensal and non-commensal bacteria, whereas oxidised Crp4 ($Crp4_{ox}$) is only active against non-commensal bacteria. An increase in reduced cryptdins is correlated with dysbiosis in a mouse model of Crohn's disease. This study suggests that alternative defensin activity is associated with disease mechanisms and progression. However, the precise bactericidal mechanism of both $Crp4_{ox}$ and $Crp4_{red}$ has yet to be fully elucidated, considering that $Crp4_{ox}$ permeabilises bacterial membranes, and that such permeabilisation is correlated with bactericidal activity. On the other hand, some studies have demonstrated that certain defensins do not disrupt cell membranes but exhibit other modes of action, defined as 'molecular targeting mechanisms', and have reported a fully new activity mechanism termed nanonets.

In this study, I aimed to elucidate the molecular factors that affect the potent bactericidal activity of $Crp4_{red}$. I tested free cysteine, structure, and hydrophobicity as molecular factors, and determined whether bacterial membrane interactions are critical for the mode of action of $Crp4_{red}$.

In Chapter 1, I describe the preparation of peptides to study the bactericidal activity of $Crp4_{red}$. Crp4 was co-expressed in *E. coli* to generate $Crp4_{ox}$, and $Crp4_{red}$ was obtained by completely reducing $Crp4_{ox}$. To study the function of the thiol group of cysteine residues, $Crp4_{red}$ was chemically modified with N-ethylmaleimide (NEM).

In Chapter 2, I sought to elucidate the bactericidal mechanism of $Crp4_{red}$ at the molecular level using the peptides described in the previous chapter; $Crp4_{ox}$, $Crp4_{red}$, NEM-Crp4, and 6C/S-Crp4, in which all Cys residues were substituted with Ser residues. The

bactericidal activities of these peptides against non-commensal and commensal bacteria were assessed. All peptides showed bactericidal activity against non-commensal bacteria. $Crp4_{red}$ and NEM-Crp4 exhibited bactericidal activity against one commensal, *Lactobacillus johnsonii* (*L. johnsonii*) whereas 6C/S-Crp4 was not effective against *L. johnsonii* but killed another commensal, *Bifidobacterium breve* (*B. breve*). On the other hand, $Crp4_{ox}$ did not exert any activity against either commensal bacteria. These results suggested that it is not important for the thiol group of cysteine residues in $Crp4_{red}$ to exhibit potent bactericidal activity. The secondary structure of the peptides showed no significant changes. Hydrophobicity was strongly correlated with bactericidal activity. These results suggested that the potent $Crp4_{red}$ bactericidal activity is derived from its hydrophobicity. Moreover, I attempted to determine whether bacterial membrane interactions are critical for the $Crp4_{red}$ mode of action. A liposome leakage assay using lipids extracted from *L. johnsonii* suggested a degree of correlation with bactericidal activity These results suggested that membrane interactions are crucial for potent $Crp4_{red}$ activity and that the potent bactericidal capacity of $Crp4_{red}$ is derived from its hydrophobicity and involves disruption of the bacterial membrane.

General Introduction

Antimicrobial peptides (AMPs) are small polypeptides that are produced by a vast number of organisms and play important roles in innate immunity to protect potential hosts from infectious microbes, exerting broad antimicrobial activity against gram-positive and gram-negative bacteria, yeast, fungi, and certain viruses (Brogden, 2005; Hancock & Lehrer, 1998; Michael E Selsted & Ouellette, 2005; Van Der Weerden et al., 2013; Wilson et al., 2013; Zasloff, 2002). AMPs have a net positive charge under physiological conditions and interact with negatively charged bacterial membranes. AMPs are classified into three major classes: cathelicidins, histatins, and defensins (Bahar & Ren, 2013; Michael E Selsted & Ouellette, 2005).

Defensins are a family of AMPs with common characteristics including a molecular weight of ~4.5 kDa, have three conserved disulphide bridges, and a triple-stranded β-sheet structure. Mammalian defensins are classified into α-, β-, and θ-defensins. α- and β-defensins are distinguished by the pattern of their three disulfide linkages, Cys^{I}-Cys^{VI}, Cys^{II}-Cys^{IV}, Cys^{III}-Cys^{V} for α-defensins, or Cys^{I}-Cys^{V}, Cys^{II}-Cys^{IV}, and Cys^{III}-Cys^{VI} for β-defensins. θ-defensins are structurally distinguished from α- and β-defensins, as they are backbone-cyclised (Ganz, 2003; Michael E Selsted & Ouellette, 2005). α-Defensins have conserved features, including an invariant disulphide bridge pattern of Cys^{I}-Cys^{VI}, Cys^{II}-Cys^{IV}, and Cys^{III}-Cys^{IV} (Chandrababu et al., 2009; Hadjicharalambous et al., 2008; Maemoto et al., 2004; M E Selsted & Harwig, 1989; Wanniarachchi et al., 2011), a canonical Arg-Glu salt bridge (Rajabi et al., 2008; Rosengren et al., 2006; Sato et al., 2012; Wu et al., 2005), a conserved Gly residue at position Cys^{III+8} (Xie et al., 2005) and high Arg levels relative to Lys (Schmidt et al., 2012). The tertiary structure of oxidised α-defensins consists of a triple-stranded β-sheet that is constrained by a disulphide bridge

(Jing et al., 2004; Maemoto et al., 2004). Although the role of reduced defensins has been overlooked over time, it has been reported that human β-defensin 1 (hBD-1) shows bactericidal activity equal to or greater than that of its oxidised form, demonstrating that reduced hBD1 and reduced human α-defensin 5 (HD-5) exist *in vivo* (Bjoern O Schroeder et al., 2011; C. Wang et al., 2016). The hBD1 reduced by thioredoxin in human colon and expression levels of thioredoxin are altered in inflammatory bowel disease patients, suggesting a correlation between reduced defensins and certain diseases (Jaeger et al., 2013; Bjoern O Schroeder et al., 2011).

α-Defensins secreted by small intestinal Paneth cells are known as cryptdins. Crp4 is the most studied cryptdin, including salt bridge mutants (Andersson et al., 2012; Rosengren et al., 2006), Arg to Lys substitutions (Schmidt et al., 2012), Arg charge-neutral substitutions (Tanabe et al., 2004), N-terminal mutants and Cys-null mutants (Satchell et al., 2003, Hadjicharalambous et al., 2008; Tanabe et al., 2004). Although these studies mainly involved oxidised Crp4, it has been reported that $Crp4_{red}$ shows potent bactericidal activity, with lower selectivity against enteric commensal bacteria, including *Bifidobacterium* and *Lactobacillus* sp., although $Crp4_{ox}$ does not display activity against these bacteria. However, $Crp4_{red}$ activity against non-commensal bacteria is equal to that of $Crp4_{ox}$ (Masuda et al., 2011). An increase in reduced cryptdins is correlated with dysbiosis in a murine model of Crohn's disease (Shimizu et al., 2020). This study suggests that alternative defensin activity is associated with disease mechanisms and progression. However, the precise bactericidal mechanisms of both $Crp4_{ox}$ and $Crp4_{red}$ have yet to be fully elucidated, although it has been reported that $Crp4_{ox}$ permeabilises bacterial membranes and such permeabilisation is correlated with bacterial killing activity (Figueredo et al., 2009; Masuda et al., 2011; Satchell et al., 2003).

A common antibacterial mechanism of AMPs, including defensin, involves interactions with negatively charged bacterial membranes which disrupt their integrity, although the mechanisms have not been fully elucidated for many of them (Zasloff 2002; Ganz 2003; Selsted and Ouellette 2005). However, a number of studies have demonstrated that certain AMPs, including human α-defensin, do not disrupt cell membranes, but rather exhibit other modes of action defined as 'molecular targeting mechanisms', in which AMPs interact with bacterial intercellular or membrane compounds (Breukink & de Kruijff, 2006; Brogden, 2005; Chang et al., 2012; de Leeuw et al., 2010; Lin et al., 2010). Fungal defensin, oyster defensin, and human β-defensin 3 (hBD-3) bind lipid II and inhibit cell wall biosynthesis (Goals et al., 2010; Sass et al., 2010; Schmitt et al., 2010). Oxidised hBD-1 enters the gram-negative bacterial periplasmic space via FepA and exerts its activity depending on DsbA and DsbB (Wendler et al., 2018). Reduced HD-5 strongly binds to lipopolysaccharide (LPS) and neutralises LPS (C. Wang et al., 2016). Moreover, a fully new mechanism of defensin activity has been reported, which oxidised human α-defensin 6 (HD-6), self-assembles to form extracellular nanonets, and prevents the translocation of bacteria across the epithelial barrier (Chu et al., 2012). Reduced HD-6 also forms a net-like structure to protect against invasive bacteria and differs from those of oxidised, reduced nanonets from exerting bactericidal activity (B. O. Schroeder et al., 2015). In contrast, only the reduced form of hBD-1 shows extracellular nanonet formation as well as bacterial membrane disruption (Raschig et al., 2017). These studies suggest that oxidised and reduced defensin antimicrobial mechanisms are different from each other. In addition, cysteines with free thiol groups, or cysteine residues alone, exhibit antimicrobial activity (Caldeira et al., 2013; Gouveia et al., 2012; Ohno et al., 2020; H. Wang et al., 2019; Yomogida et al., 1995). These studies suggest that free cysteine

residues may be involved in reduced defensin activity. Furthermore, some studies have suggested that structure and hydrophobicity might be related to reduced defensin activity (Klüver et al., 2005; Bjoern O Schroeder et al., 2011; Tai et al., 2014).

In this study, I aimed to test how molecular factors, free cysteine, structure, and hydrophobicity result in the potent bactericidal activity of $Crp4_{red}$, and to determine which kinds of bacterial membrane interactions are critical for the $Crp4_{red}$ mode of action. My data indicated that the potent activity of $Crp4_{red}$ is derived from its hydrophobicity and that molecular factors are crucial for bacterial membrane disruption.

In Chapter 1, I described the preparation of peptides to study bactericidal activity and critical molecular factors of $Crp4_{red}$. Chemical synthesed defensins have been widely used to study thier functions. However, synthesised defensins are expensive. My laboratory has reported a means to solve this bottleneck by using a recombinant *E. coli* expression system to enhance inclusion body formation to avoid toxicity and degradation and succeeded in producing $Crp4_{ox}$ (Tomisawa et al., 2013, 2015). To prepare $Crp4_{red}$, this procedure was modified. First, I attempted to add a reducing agent to the solubilisation step, but this approach failed. Finally, I succeeded in obtaining $Crp4_{red}$ to form reduced purified $Crp4_{ox}$. The function of cysteine is one of the essential factors mentioned above. To study the role of cysteine, specific residues can be substituted with other amino acids or unnatural amino acids (Chandrababu et al., 2009; Hadjicharalambous et al., 2008; Maemoto et al., 2004; Raschig et al., 2017; B. O. Schroeder et al., 2015; Wanniarachchi et al., 2011). However, the chemical synthesis approach used to produce mutants is costly, and recombinant peptide approaches take a long time. I attempted to produce chemically modified $Crp4_{red}$ to use the free thiol group, which is highly active and easy to alkylate (Crankshaw & Grant, 1996; Grant, 2017). I succeeded in obtaining NEM-Crp4 and

modified the cysteine residues with N-ethylmaleimide. This approach for obtaining a reduced defensin-inactivated free thiol group is easier and quicker than other methods and would accelerate reduced defensin studies.

In Chapter 2, I studied how molecular factors, free cysteine, structure, and hydrophobicity result in the potent bactericidal activity of $Crp4_{red}$. I assessed the bactericidal activities of $Crp4_{ox}$, $Crp4_{red}$, NEM-Crp4, and 6C/S-Crp4 by substituting Cys with Ser against non-commensal and commensal bacteria. All peptides showed bactericidal activity against non-commensal bacteria, whereas $Crp4_{red}$ and NEM-Crp4 exhibited bactericidal activity against commensal bacteria. Secondary structure was analysed using circular dichroism (CD) measurements, which indicated no significant differences. In contrast, the potent peptides exhibited high hydrophobicity which was strongly correlated with bactericidal activity. A liposome leakage assay for lipids extracted from commensal bacteria demonstrated a correlation with bactericidal activity, and the bactericidal activity of both $Crp4_{ox}$ and $Crp4_{red}$ was abolished by inhibition of electrostatic interactions. These findings suggested that the potent bactericidal activity of $Crp4_{red}$ is derived from its hydrophobicity, and that the bactericidal mechanism involves disruption of bacterial membranes. My findings provide a better understanding of the bactericidal mechanisms of $Crp4_{ox}$ and $Crp4_{red}$.

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Chapter1 :

Preparation of reduced cryptdin-4 and N-ethylmaleimide modification using recombinant oxidised cryptdin-4

1-1 Abstract

The α-defensins are small 3-4 kDa of cationic and amphiphilic peptides. They contribute to innate immunity to protect hosts by exerting antimicrobial activity. A large amount of peptide is essential to study its activity and structure. However, widely used chemical synthesis of α-defensin is high costly. Therefore, the recombinant technologies to produce α-defensin using *E. coli* are attractive because *E. coli* cultivation is inexpensive and grows fast. Unfortunately, α-defensins are toxic to *E. coli* and easy to be degraded due to its antibacterial activities and small molecular weights. Our laboratory previously has reported the production system of mouse α-defensin oxidized cryptdin-4 ($Crp4_{ox}$) using *E. coli* to enhance the inclusion body formation to avoid toxicity and degradation. I modified this technology to produce reduced cryptdin-4 ($Crp4_{red}$). Surprisingly, $Crp4_{red}$ was not acquired when reduced reagent was added solubilized inclusion body. I attempted to reduce purified $Crp4_{ox}$ and succeeded in obtaining $Crp4_{red}$. Moreover, cysteine residues of $Crp4_{red}$ were modified N-ethylmaleimide to produce NEM-Crp4. This approach is easy and quick method to produce $Crp4_{red}$ which is inactivated its free-thiol group. This production method would enhance the study of $Crp4_{red}$ and its chemical modification would open up the possibility of reduced defensin study.

1-2 Introduction

Antimicrobial peptides (AMPs) are produced a vast living organism and play an important role in innate immunity to protect host from infectious microbes (Brogden, 2005; Michael E Selsted & Ouellette, 2005; Zasloff, 2002). They are small cationic and amphiphilic peptides exhibiting broad antimicrobial activity against gram-positive and gram-negative bacteria, yeast, fungi and some viruses (Guaní-Guerra et al., 2010; Van Der Weerden et al., 2013; Wilson et al., 2013). AMPs are classified three major categories: cathelicidins, histatins and defensins (Bahar & Ren, 2013; Guaní-Guerra et al., 2010). The mammalian defensins are defined α- and β-defensins by their three disulphide bridge patterns. The α-defensins are small 3-4 kDa of cationic and amphiphilic peptides and have conserved features, including invariant disulphide bridge pattern of Cys^{I}-Cys^{VI}, Cys^{II}-Cys^{IV} and Cys^{III}-Cys^{IV} (Chandrababu et al., 2009; Hadjicharalambous et al., 2008; Maemoto et al., 2004; M E Selsted & Harwig, 1989; Wanniarachchi et al., 2011), a canonical Arg-Glu salt bridge (Rajabi et al., 2008; Rosengren et al., 2006; Sato et al., 2012; Wu et al., 2005), a conserved Gly residue at position Cys^{III+8} (Xie et al., 2005) and high Arg relative to Lys (Schmidt et al., 2012).

Cryptdin-4 (Crp4) is a mouse α-defensin secreted by small intestinal Paneth cells. Crp4 has the three disulphide bounds, conserved among in all α-defensins (Hadjicharalambous et al., 2008; Maemoto et al., 2004). The role of the bounds was believed to protect from protease degradation (Maemoto et al., 2004). However, it has been reported that $Crp4_{red}$ shows activity with lower selectivity against enteric commensal bacteria, including *Bifidobacterium* and *Lactobacillus* sp., although $Crp4_{ox}$ did not show activity against these bacteria. On the other hand, the activity against non-commensal bacteria of $Crp4_{red}$ was equal to that of $Crp4_{ox}$ (Masuda et al., 2011). Moreover, some human defensins shows

their reduced form exerting bactericidal activity equal to or greater than those of the oxidised form (B. O. Schroeder et al., 2015; Bjoern O Schroeder et al., 2011). In previous studies, Crp4 was prepared chemically synthesized or *Escherichia coli* (*E. coli*) expression system, 6-histidine-tagged linked to N-terminal of Crp4 (Hadjicharalambous et al., 2008; Jing et al., 2004; Maemoto et al., 2004; Masuda et al., 2011). However, the expression of α-defensins in *E. coli* has been difficult due to their toxicity to host and host-derived proteolytic degradation (Li, 2011). My laboratory has been reported to overcome this problem to coexpress aggregation-prone protein promoting an inclusion body formation (Tomisawa et al., 2015).

A large amount of reduced defensins is essential to study the structure, activity and mechanism of them. However, previous Crp4 expression system is the method to prepare $Crp4_{ox}$ and the chemical synthesis as reduced form is costly. I attempted to modify our previously reported method to produce large amount of $Crp4_{red}$.

To study reduced defensin, the cysteine function is one of essential factors. It has been reported cysteines with free thiol groups or cysteine residues alone exhibit antimicrobial activity (Caldeira et al., 2013; Gouveia et al., 2012; Ohno et al., 2020; Wang et al., 2019; Yomogida et al., 1995). The free cysteine residues in reduced human β-defensin 1 (hBD1) are critical for the unique mechanism of nanonets formation (Raschig et al., 2017). It has been widely used to study the role of cysteine the mutants substituted cysteine to other amino acids or unnatural amino acids (Chandrababu et al., 2009; Hadjicharalambous et al., 2008; Maemoto et al., 2004; Raschig et al., 2017; B. O. Schroeder et al., 2015; Wanniarachchi et al., 2011). However, chemical synthesis approach to produce some mutants is high costly and recombinant peptides approaches take a long time to construct a plasmid vector and need to study new production methods when a wild-type method is

not adopted to mutants. I attempted to produce chemically modified $Crp4_{red}$ to use the feature free-thiol group is highly active and easy to be alkylated (Crankshaw & Grant, 1996; Grant, 2017). This approach was fully succeeded in production of modified free-thiol group Crp4 easily and quickly.

These studies to produce reduced and Cys modified defensin would be more accelerated the reduced defensins studies.

1-3 Materials and Methods

Expression and purification of $Crp4_{ox}$

The pCOLADuet1 vector (Novagen), which contains two multiple cloning sites (MCS), was used to establish an expression system for Crp4. The *KSI* gene was cloned into the MCS1 site, and the *Crp4* gene was cloned into the MCS2 site. *Escherichia coli* BL21 (DE3) was transformed with the constructed pCOLA vectors and induced by isopropyl-β-D-thiogalactopyranoside, and bacterial cells were harvested after cultivation for 4 h at 37 °C. Cells were lysed by sonication, and insoluble pellet fractions containing inclusion bodies containing Crp4 and KSI were collected. The inclusion bodies were washed with 0.5 % Triton-X. The insoluble inclusion bodies were solubilised in solubilisation buffer (6 M urea, 20 mM Gly-NaOH, 3 mM ethylenediaminetetraacetic acid [EDTA], pH 9.0), and then incubated overnight at 37 °C to solubilise inclusion bodies and to refold Crp4. Cation exchange chromatography was performed to separate crude Crp4 from other proteins. Cellular debris was removed by centrifugation for 30 min at 4 °C. The refolded Crp4 was applied to an SP-Sepharose column (Cytiva) and eluted with a NaCl gradient. Eluate was dialysed against 0.1 % acetic acid overnight. The dialyzed sample was purified by reverse-phase high-pressure liquid chromatography (RP-HPLC) on an ODS column (Cosmosil $_5C_{18}$-AR-300; 10 × 250 mm; Nacalai Tesque). A linear gradient elution was performed using 20% to 40% acetonitrile in water containing 0.1% trifluoroacetic acid at a flow rate of 1 mL/min over 40 min. The purified recombinant Crp4 ($Crp4_{ox}$) was lyophilised and stored below −20 °C.

Reduction of $Crp4_{ox}$ to produce $Crp4_{red}$

The disulphide bonds of $Crp4_{ox}$ were reduced entirely to produce $Crp4_{red}$. Oxidised Crp4

was reduced by reducing buffer (6 M urea, 20 mM Gly-NaOH, 200 mM β-mercaptoethanol, pH 9.0) and incubated room temparture for 1 h. The reaction mixture was diluted 10-fold by 0.1% TFA containing buffer and purified by RP-HPLC under the same conditions as $Crp4_{ox}$. The purified $Crp4_{red}$ was lyophilised and stored at a temperature below −20 °C.

N-ethylmaleimide modification of $Crp4_{red}$ to produce NEM-Crp4

To prepare the Crp4 with thiol groups modified by N-ethylmaleimide (NEM), 1 ml of 200mM N-ethylmaleimide (NEM) in 200mM cirate buffer (pH 3.0) was added to 100 - 500 μg of $Crp4_{red}$. This mixture was incubated for 60 min at room temperature, and then injected for RP-HPLC under the same conditions as described above. The collected peptides were lyophilised and stored below −30 °C.

Characterization of peptides by MALDI-TOF-MS and AU-PAGE

The all peptides described above and 6C/S-Crp4 substituted all six Cys residues to Ser purchased from Sigma-Aldrich Japan were characterized , and disulphide bond formation was confirmed using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) and acid-urea polyacrylamide gel electrophoresis (AU-PAGE), in which the samples (1 μg) in 5% acetic acid were electrophoresed on a 12.5% acrylamide gel containing 5 % acetic and 5 M urea in 5% acetic acid running buffer at 150 V.

1-4 Results

Production of recombinant $Crp4_{ox}$, reduced it to prepare $Crp4_{red}$ and modified free-thiol group by N-ethylmaleimide

The primary structures of each peptide are shown in Fig. 1 and the production flowchart is described Fig. 2. $Crp4_{ox}$ was produced as recombinant peptide using aggregation-prone protein co-expression system previously reported (Tomisawa et al., 2015). This expression system has succeeded in the other AMPs, ABF-2 from *C. elegans* (Tomisawa et al., 2013). In this system, expression of target peptide is enhanced by coexpressed an aggregation-prone protein promoting an inclusion body formation, leading to avoid toxicity to host cells and degradation from host-derived protease. In previously reported system, interestingly, Crp4 was refolded at solubilization step although the solubilized buffer contained high concentration denaturant, 6 M Urea (Tomisawa et al., 2015). I attempted to modify this method to produce $Crp4_{red}$. To obtain reduce form, 200 mM β-mercaptoethanol was added as a reducing reagent to solubilisation buffer at solubilisation step (Fig. 2) and purified cation exchange chromatography and RP-HPLC. However, I did not obtain $Crp4_{red}$ at RP-HPLC purification (Fig. 3c). Next, I tried to use $Crp4_{ox}$ as a starting material. The three pairs of disulphide bonds were reduced by a reducing buffer described in materials and methods section and reduced peptide was directly applied to RP-HPLC purification. As shown in Fig. 3d and Table 1, $Crp4_{red}$ was obtained using purified $Crp4_{ox}$.

Modification of free-thiol group by N-ethylmaleimide to produce NEM-Crp4

Cysteine residues is related to reduced defensin activity (Raschig et al., 2017). To study the function of Cys residues, substitution to other amino acids or an unnatural amino acid

is widely used (Chandrababu et al., 2009; Hadjicharalambous et al., 2008; Maemoto et al., 2004; Raschig et al., 2017; B. O. Schroeder et al., 2015; Wanniarachchi et al., 2011). However, sometimes a substituted peptides does not obtain same method as the wild type. Actually, I could not obtain 6C/S-Crp4, all Cys residues substituted to Ser (data not shown). Chemical synthesis is an alternative method, but it is costly. Free-thiol group is known as active group and easy to be alkylated (Crankshaw & Grant, 1996; Grant, 2017). I attempted to produce chemically modified $Crp4_{red}$ to study the role of Cys residues of its bactericidal activity. The N-ethylmaleimide (NEM) was selected as a model acetylating reagent and the reaction scheme is described in Fig. 4. The Crp4red was reacted with NEM in 200mM citrate buffer (pH 3.0) and purified RP-HPLC. The NEM modification was confirmed the mass by MALDI-TOF-MS (Table 1).

Characterization of peptides by MALDI-TOF-MS and AU-PAGE

The produced peptides and purchased chemical synthesis 6C/S-Crp4 were evaluated using AU-PAGE and MALDI-TOF MS (Table 1 and Fig. 5). Results of AU-PAGE revealed that $Crp4_{ox}$ consisted of a triple-stranded β-sheet structure and showed a faster mobility than $Crp4_{red}$ and the other linear analogues, as previously reported (Maemoto et al., 2004; Masuda et al., 2011; Shimizu et al., 2020). $Crp4_{ox}$ showed a lower molecular weight than $Crp4_{red}$ through MALDI-TOF-MS analysis. These data indicated that $Crp4_{red}$ was completely reduced.

1-5 Discussion

Previously, my laboratory has reported efficient production system of $Crp4_{ox}$ (Tomisawa et al., 2015). In this system, Crp4 is coexpressed with aggregation-prone protein to promote an inclusion body formation, leading to avoid toxicity to host cells and degradation from host-derived protease. This approach was succeeded in another AMPs, ABF-2 from *C. elegans* (Tomisawa et al., 2013). Crp4 expressed in *E. coli* is refolded at solubilization step although the solubilized buffer contained high concentration denaturant. To obtain $Crp4_{red}$, I modified the solubilization step to add reducing agent. However, $Crp4_{red}$ peak was not observed at RP-HPLC purification. At cation exchange chromatography, the $Crp4_{red}$ peak was observed, and the fraction contained Crp4 by Tricine-SDS PAGE band (data not shown). I could not find the reason why $Crp4_{red}$ was lost but some reasons would be considered, such as misfolded or degraded residual host derived protease at dialysis step. Alternatively, I attempted to use $Crp4_{ox}$ as a starting material to produce $Crp4_{red}$. This approach was fully succeeded in obtaining $Crp4_{red}$. The reducing buffer composition is almost same solubilization buffer. The difference is that denaturant removal by dialysis or dilution. Dilution can reduce the denaturant concentration and changes to acidic pH more quickly than dialysis. This would lead avoiding misfold or using highly purified $Crp4_{ox}$ would prevent from degrading by host-derived protease.

The cysteine residues are critical for the nanonets formation of reduced hBD1 (Raschig et al., 2017). It has also been reported cysteines with free thiol groups or cysteine residues alone exhibit antimicrobial activity (Caldeira et al., 2013; Gouveia et al., 2012; Ohno et al., 2020; Wang et al., 2019; Yomogida et al., 1995). To study reduced defensin, the cysteine function is one of essential factors. The widely used approach is a substitution to

other amino acids or an unnatural amino acids (Chandrababu et al., 2009; Hadjicharalambous et al., 2008; Maemoto et al., 2004; Raschig et al., 2017; B. O. Schroeder et al., 2015; Wanniarachchi et al., 2011). However, chemical synthesis approach is costly and recombinant peptide approach takes a long time to construct a plasmid vector and sometimes be needed new production method when wild-type method is not adopted to substitutions. Free-thiol group is known as active group and easy to be alkylated (Crankshaw & Grant, 1996; Grant, 2017). I attempted to produce chemically modified $Crp4_{red}$ to study the role of Cys residues of its bactericidal activity. This approach could produce $Crp4_{red}$ modified and inactivated its thiol group easily and quickly. Moreover, the advantage of this approach is that there are many alkylating reagents which have various chemical features (Crankshaw & Grant, 1996; Grant, 2017; Kuznetsova et al., 2021; Pasquarello et al., 2004).

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Table 1. Calculated and observed m/z for matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF-MS) of the oxidised cryptdin-4 ($Crp4_{ox}$), reduced Crp4 ($Crp4_{red}$), Crp4 with cysteine (Cys) residues substituted with serine (Ser) peptide (6C/S-Crp4), and Crp4 with thiol groups modified by N-ethylmaleimide (NEM-Crp4).

Peptides	Calculated m/z	Observed m/z
$Crp4_{ox}$	3,755.5	3,756.1
$Crp4_{red}$	3,761.5	3,762.2
6C/S-Crp4	3,665.2	3,661.6
NEM-Crp4	4,512.1	4,510.9

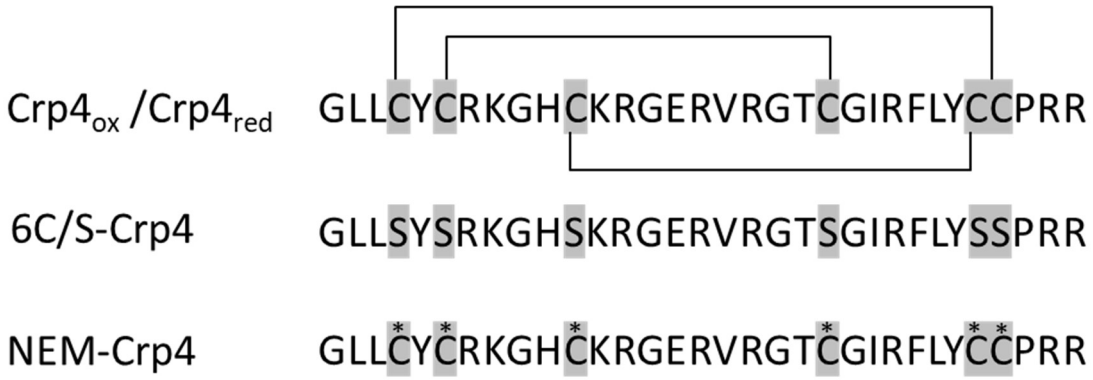

Fig. 1 The primary structures of cryptdin-4 (Crp4), Crp4 with cysteine (Cys) residues substituted with serine (Ser) peptide (6C/S-Crp4), and Crp4 with thiol groups modified by NEM (N-ethylmaleimide) (NEM-Crp4). Oxidised Crp4 ($Crp4_{ox}$) forms disulphide bonds between Cys^{I}-Cys^{VI}, Cys^{II}-Cys^{IV}, and Cys^{III}-Cys^{V}. The asterisks represent Cys residues modified by NEM

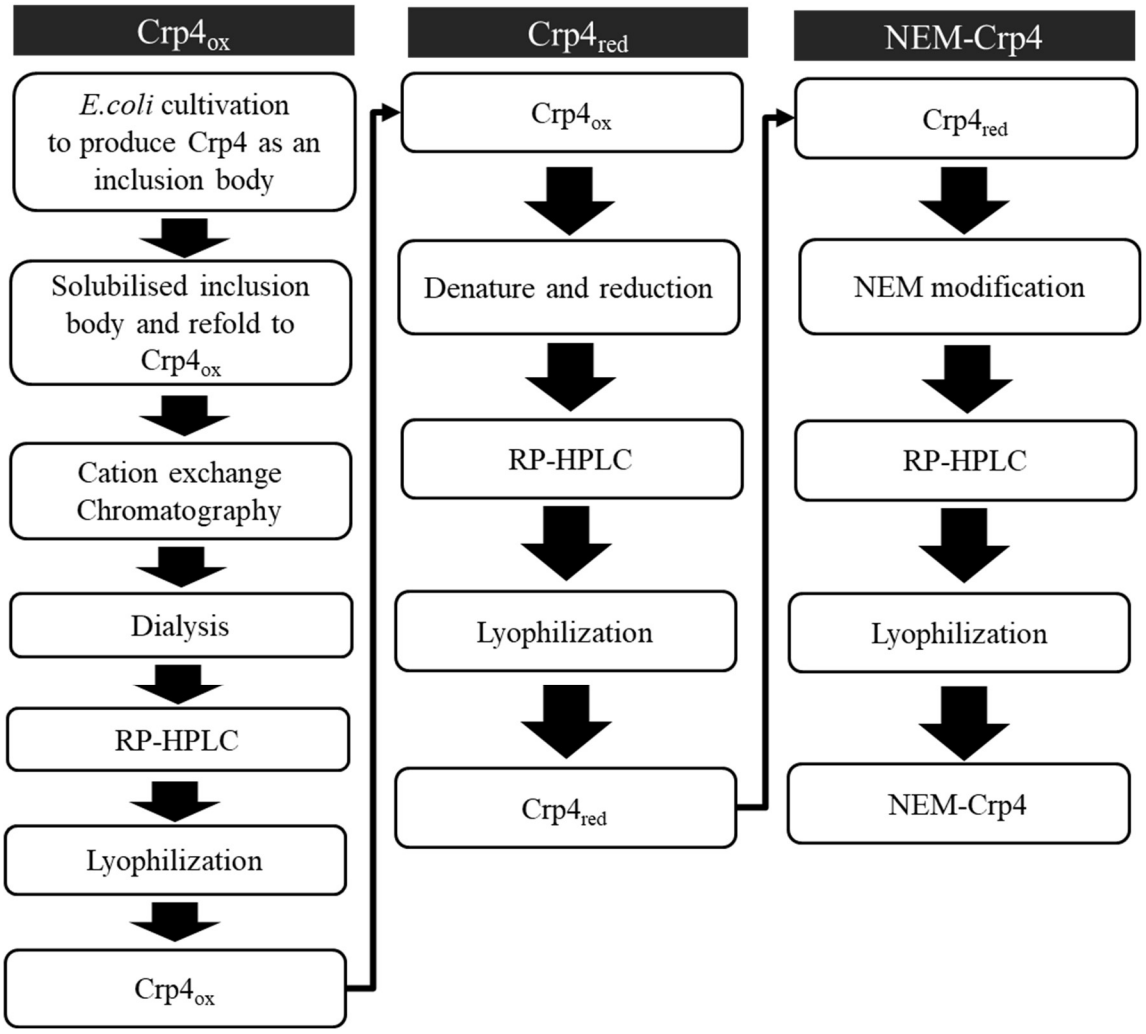

Fig. 2 The production flowchart from oxidized Crp4 (Crp4ox) through reduced (Crp4red) and NEM-Crp4.

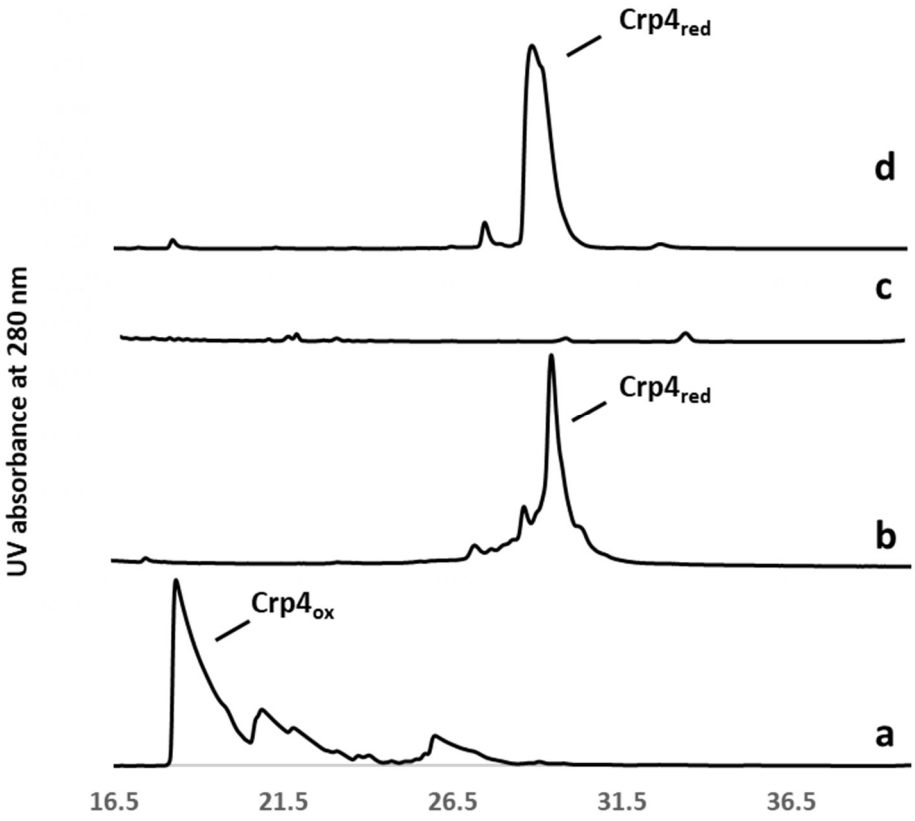

Fig. 3 Reversed-phase high-performance liquid chromatography (RP-HPLC) purification chromatogram of (a) oxidized Crp4 ($Crp4_{ox}$), (b) chemical synthesis Crp4 , (c) an inclusion body contained Crp4 solubilised in the presence of 200 mM β-mercaptoethanol and (d) purified $Crp4_{ox}$ reduced.

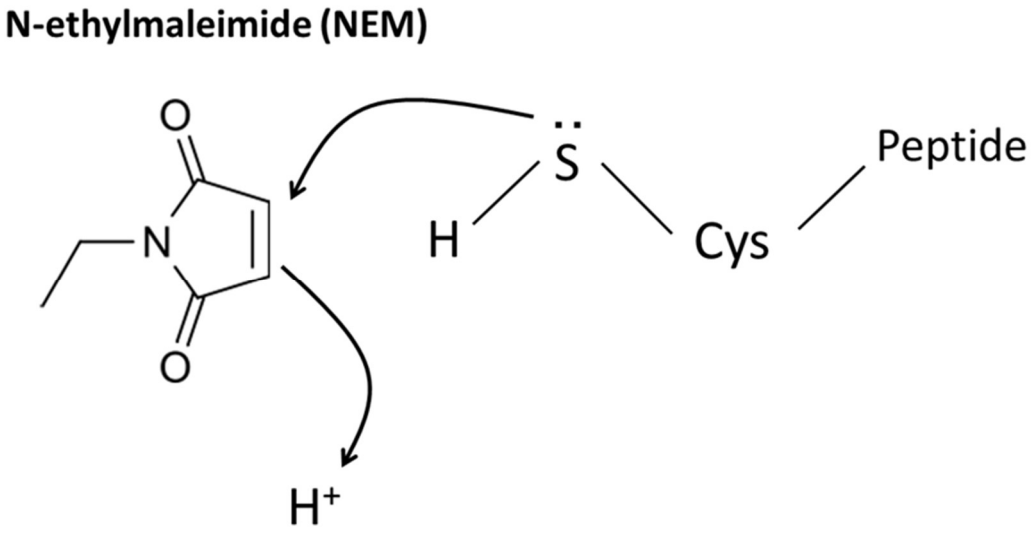

Fig. 4 The reaction scheme of N-ethylmaleimide (NEM) with thiol group of cysteine.

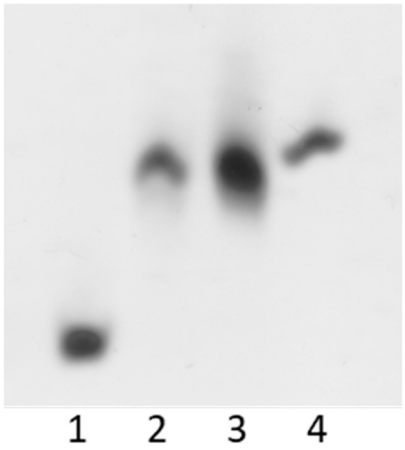

Fig. 5 acid-urea polyacrylamide gel electrophoresis (AU-PAGE) analysis of (1) $Crp4_{ox}$, (2) reduced Crp4 ($Crp4_{red}$), (3) 6C/S-Crp4, and (4) NEM-Crp4; 1.0 μg of each peptide was resolved by AU-PAGE.

Chapter2 :

Potent bactericidal activity of reduced cryptdin-4 derived from its hydrophobicity and mediated by bacterial membrane disruption

2-1 Abstract

Defensin is a cysteine-rich antimicrobial peptide with three disulphide bonds under normal oxidative conditions. Cryptdin-4 (Crp4) is a defensin secreted by Paneth cells in the small intestine of mice, and only reduced Crp4 ($Crp4_{red}$) shows activity against enteric commensal bacteria, although both oxidised Crp4 ($Crp4_{ox}$) and $Crp4_{red}$ can kill non-commensal bacteria. To investigate the molecular factors that affect the potent antimicrobial activity of $Crp4_{red}$, the bactericidal activities of $Crp4_{ox}$ and $Crp4_{red}$, Crp4 with all Cys residues substituted with Ser peptide (6C/S-Crp4), and Crp4 with all thiol groups modified by N-ethylmaleimide (NEM-Crp4) were assessed. All peptides showed bactericidal activity against non-commensal bacteria, whereas $Crp4_{red}$ and NEM-Crp4 showed bactericidal activity against commensal bacteria. These potent peptides exhibited high hydrophobicity, which was strongly correlated with membrane insertion. Intriguingly, $Crp4_{ox}$ formed electrostatic interactions with the membrane surface of bacteria, even without exerting bactericidal activity. Moreover, the bactericidal activity of both oxidised and reduced forms of Crp4 was abolished by inhibition of electrostatic interactions; this finding suggests that $Crp4_{red}$ targets bacterial membranes. Finally, a liposome leakage assay against lipids extracted from commensal bacteria demonstrated a correlation with bactericidal activity. These results suggest that the potent bactericidal activity of $Crp4_{red}$ is derived from its hydrophobicity, and the bactericidal mechanism involves disruption of the bacterial membrane. Findings from this study provide a better understanding of the bactericidal mechanism of both $Crp4_{ox}$ and $Crp4_{red}$.

2-2 Introduction

Defensins are cysteine-rich cationic antimicrobial peptides (AMPs) that act as effectors of innate immunity and protect the host from infectious microbes (Hancock and Lehrer 1998; Zasloff 2002; Ganz 2003; Selsted and Ouellette 2005; de Leeuw and Lu 2007). Human defensins are characterised as α-defensins or β-defensins based on the pattern of the three disulphide linkages (Selsted and Harwig 1989; Ganz 2003; Selsted and Ouellette 2005; Taylor et al. 2008). The tertiary structure of the oxidised form consists of a triple-stranded β-sheet that is constrained by three disulphide bonds (Jing et al. 2004; Maemoto et al. 2004; de Leeuw et al. 2010). Although the role of the reduced form of defensins has been overlooked for a long time, Schroder et al. demonstrated that human β-defensin 1 (hBD-1) is reduced by thioredoxin in human colon, and the reduced form shows bactericidal activity equal to or greater than that of the oxidised form (Schroeder et al. 2011; Jaeger et al. 2013). Moreover, altered expression levels of thioredoxin in inflammatory bowel disease patients have also been reported, suggesting a correlation between reduced defensins and some diseases (Schroeder et al. 2011; Jaeger et al. 2013).

Cryptdin-4 (Crp4) is a mouse α-defensin produced by small intestinal Paneth cells. Masuda et al. (2011) reported that only the reduced form of Crp4 ($Crp4_{red}$) showed activity with lower selectivity against enteric commensal bacteria, including *Bifidobacterium* and *Lactobacillus* sp., although oxidised Crp4 ($Crp4_{ox}$) did not show activity against these bacteria. On the other hand, the activity against non-commensal bacteria of $Crp4_{red}$ was equal to that of $Crp4_{ox}$ (Masuda et al. 2011). The increase in reduced cryptdins is correlated with dysbiosis in a Crohn's disease model mice (Shimizu et al. 2020). This study suggests that alternative defensin activities are related to disease mechanisms and progression. However, the precise bactericidal mechanism of both

$Crp4_{ox}$ and $Crp4_{red}$ has yet to be fully elucidated, although it has been reported that $Crp4_{ox}$ permeabilises bacterial membranes and its permeabilisation is correlated with bacterial killing activity (Satchell et al. 2003; Figueredo et al. 2009; Masuda et al. 2011).

Some studies have demonstrated that some AMPs do not disrupt the membrane but exhibit other modes of action defined as 'molecular targeting mechanisms', in which the AMPs interact with bacterial intercellular or membrane compounds (Brogden 2005; Breukink and de Kruijff 2006; de Leeuw et al. 2010; Lin et al. 2010; Sass et al. 2010; Schmitt et al. 2010; Schneider et al. 2010; Srinivas et al. 2010; Chang et al. 2012; Kandaswamy et al. 2013; Le et al. 2017; Wendler et al. 2018).

Moreover, Chu et al. (2012) demonstrated a fully new activity mechanism of defensin, which oxidised human α-defensin 6 (HD-6) self-assembles to form extracellular nanonets and prevents the translocation of bacteria across the epithelial barrier (Chu et al. 2012). Schroder et al. (2015) showed that reduced HD-6 not only forms a net-like structure to protect against invasive bacteria but also exerts bactericidal activity (Schroeder et al. 2015). In contrast, only the reduced form of hBD-1 showed extracellular nanonet formation as well as bacterial membrane disruption (Raschig et al. 2017). In addition, cysteines with free thiol groups or cysteine residues alone exhibit antimicrobial activity (Yomogida et al. 1995; Gouveia et al. 2012; Caldeira et al. 2013; Wang et al. 2019; Ohno et al. 2020). These studies suggest that free cysteines might be involved in reduced defensin activities. Furthermore, some studies have suggested that structures and hydrophobicity might be related to reduced defensin activities (Klüver et al. 2005; Schroeder et al. 2011; Tai et al. 2014).

In my study, I aimed to test how molecular factors, free cysteine, structure, and hydrophobicity cause the potent bactericidal activity of $Crp4_{red}$ and to determine which

kinds of bacterial membrane interactions are critical for the $Crp4_{red}$ mode of action. My data indicate that the potent activity of $Crp4_{red}$ is derived from its hydrophobicity and that its molecular factors are crucial for bacterial membrane disruption.

2-3 Materials and Methods

Expression, purification, and reduction of Crp4 and preparation of linear analogues

The peptides preparation methods are described in Chapter1. Briefly, Crp4 was coexpressed with aggregation-prone partner protein, KSI promoting an inclusion body using *Escherichia coli* BL21 (DE3). The harvested bacterial cells were lysed by sonication, and insoluble pellet fractions containing inclusion bodies containing Crp4 and KSI were collected. The inclusion bodies were washed with 0.5 % Triton-X and were solubilised in solubilisation buffer (6 M urea, 20 mM Gly-NaOH, 3 mM ethylenediaminetetraacetic acid [EDTA], pH 9.0), and then incubated overnight at 37 °C to solubilise inclusion bodies and to refold Crp4. Cation exchange chromatography was performed to separate crude Crp4 from other proteins. The refolded Crp4 was applied to cation exchange chromatography resin, SP-Sepharose column (Cytiva) and eluted with a NaCl gradient. Eluate was dialysed against 0.1 % acetic acid overnight. The dialysed sample was purified by reverse-phase high-pressure liquid chromatography (RP-HPLC) on an ODScolumn (Cosmosil $_5C_{18}$-AR-300; 10 × 250 mm; Nacalai Tesque). A linear gradient elution was performed using 20% to 40% acetonitrile in water containing 0.1% trifluoroacetic acid and the purified $Crp4_{ox}$ was lyophilized. The disulphide bonds of $Crp4_{ox}$ were reduced entirely to prepare $Crp4_{red}$ by 200 mM β-mercaptoethanol containing buffer and purified by RP-HPLC under the same conditions as $Crp4_{ox}$. The purified $Crp4_{red}$ with thiol groups were modified by N-ethylmaleimide (NEM) to prepare NEM-Crp4. $Crp4_{red}$ was reacted with 200 mM NEM in 200 mM citrate buffer (pH 3.0). This mixture was incubated for 60 min at room temperature, and then injected for RP-HPLC under the same conditions above. The collected peptides were lyophilized. The peptide with all six Cys residues of Crp4 substituted with Ser (6C/S-Crp4) was purchased from

Sigma-Aldrich Japan. The purity and hydrophobicity of all peptides was assessed using the aforementioned RP-HPLC conditions mentioned. The prepared peptides were characterised, and disulphide bond formation was confirmed using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) and acid-urea polyacrylamide gel electrophoresis (AU-PAGE). The brief methods were described in Chapter 1.

Bactericidal peptide assay

Lactobacillus johnsonii JCM 2012 (*L. johnsonii*) and *Bifidobacterium breve* JCM 1192 (*B. breve*) were used as models of commensal bacteria in the small intestine, while *Listeria monocytogenes* JCM 7671 (*L. monocytogenes*) and *Escherichia coli* ML35 ATCC 43827 (*E. coli* ML35) were used as non-commensal bacteria (Masuda et al. 2011). *L. johnsonii* was cultured in de Man, Rogosa, and Sharpe (MRS) broth (Oxoid). *B. breve* was cultured in reinforced clostridial medium (RCM) broth (Oxoid). Both commensal bacteria were cultured at 37 °C under anaerobic condition using the Anaero Pack system (Mitsubishi Gas Chemical). *L. monocytogenes* was grown in brain heart infusion (BHI) broth (Difco) and *E. coli* ML35 was cultured under aerobic condition in trypticase soy broth (TSB) (Difco) at 37 °C and 180 rpm. The exponential phase of bacterial growth was collected by centrifugation, washed, and resuspended in assay buffer containing 10 mM sodium phosphate buffer supplemented with 1 % medium. Bacterial suspensions were incubated with each peptide for 1 h at 37 °C using the Anaero Pack system for *L. johnsonii* and *B.breve*. Following incubation, the samples were diluted and plated on MRS, RCM, BHI or TSB agar plates. Surviving bacterial rates were determined relative to surviving colonies of peptide-unexposed control conditions after overnight incubation.

Western blotting to detect $Crp4_{ox}$ and $Crp4_{red}$ bound to bacteria

Western blotting was performed using the modified vacuum method (Tomisawa et al. 2013). Briefly, the bactericidal samples were used for western blotting. Treated bacteria were centrifuged and washed in 10 mM sodium phosphate buffer supplemented with 1 % medium. The washed bacterial pellets were resuspended in 1 × sodium dodecyl sulphate (SDS) sample buffer. After SDS-polyacrylamide gel electrophoresis (PAGE), the gel was blotted onto a polyvinylidene fluoride (PVDF) membrane at 1.5 mA/cm^2 for 40 min. The vacuum method was performed using a SNAP i.d. (Merck) system and blocking buffer (StabilGuard, SurModics), and then vacuumed. Next, anti-Crp4 antibody (Nakamura et al. 2013) was added and washed three times with phosphate-buffered saline with Tween 20 (PBS-T). Finally, Crp4 was detected using a secondary antibody (Goat Anti-Rat HRP Conjugate, Novus Biologicals) and ECL Prime western blotting detection reagent (Cytiva).

Lipid extraction from L. johnsonii

Lipids were extracted according to the modified Bligh and Dyer method (BLIGH and DYER 1959; Hugo et al. 2012). Briefly, early stationary-phase bacteria were collected by centrifugation and washed. Cell pellets were resuspended in chloroform-methanol-water (1:2:0.8) overnight at 4 °C, and then centrifuged. The supernatant was collected, and a second extraction was performed on the pellet. Chloroform-water (1:1) was added to each supernatant and centrifuged to separate the phases. The chloroform phase was collected and dried under a nitrogen stream.

Liposome preparation and calcein leak assay

Large unilamellar vesicles (LUVs) were prepared as previously described (Chongsiriwatana and Barron 2010). Briefly, residual chloroform of the extracted lipids was removed completely under vacuum for overnight. The dried lipids were hydrated in 10 mM Tris-HCl and 70 mM calcein (pH 7.4) for 1 h at 37 °C. The mixture was vortexed and subjected to three freeze-thaw cycles. The liposomes were then extruded 15 times through a Mini-Extruder (Avanti) using a 0.1 μm polycarbonate filter to produce LUVs. Untrapped calcein was removed by gel filtration using Sephadex G-50 DNA Grade (GE Healthcare), 10 mM Tris-HCl (pH 7.4), and 185 mM sucrose as the eluent buffer.

The calcein leak assay was determined fluorometrically in a 2 mL-plastic cell (F2000 Fluorescence Spectrophotometer, Hitachi). The leakage of calcein was monitored with excitation and emission wavelengths of 490 nm and 500–550 nm, respectively. Peptide-induced leakage was calculated using the following equation:

$$leak\ percentage\ (lsb\ \%) = \frac{(F - F_0)}{(F_{100} - F_0)} \times 100$$

where F is the fluorescence intensity induced by 10 μg/mL of peptides, F_0 is the fluorescence intensity of intact LUVs, and F_{100} is the fluorescence intensity after treatment with 0.1 % Triton X-100. Under the above conditions, 50 μL of calcein-loaded LUVs reproducibly induced approximately $F_{100} - F_0 = 3{,}000$.

CD measurement to analyze secondary structure

Circular dichroism (CD) spectra were collected in quartz cuvettes with a path length of 1 mm on a Jasco J-725 spectropolarimeter (Jasco). Spectral scans were performed from 250 to 195 nm, with a step resolution of 0.1 nm and a scanning speed of 50 nm min^{-1} at room temperature, a response time of 1.0 s, a bandwidth of 1.0 nm, and four-scan

averaging under a nitrogen atmosphere. Peptide samples (30 μM) were measured in 10 mM sodium phosphate buffer (pH 7.4), 40 % trifluoroethanol (TFE), and 10 mM SDS. The mean residue ellipticity values, [θ], were calculated using the following relation:

$$[\theta] = \frac{\theta_{observed}}{10 \times n \times C \times l}$$

where θ is the ellipticity measured in degrees, n is the number of amino acid residues, C is the peptide molar concentration, and l is the optical pass length of the cell in centimeters.

2-4 Results

Bactericidal peptide assay

The primary structures of assessed peptides are shown in Chapter 1 (Fig. 1 in Chapter 1). The prepared peptides evaluation data were shown in Chapter 1 (Table and Fig. 5 in Chapter 1). These data indicated that all peptide corrected mass and $Crp4_{red}$ was completely reduced. The bacteria to test bactericidal activity is shown in Table.

The colony counting assays using $Crp4_{ox}$ and $Crp4_{red}$ showed the same results as previously reported (Masuda et al. 2011). Two peptides exhibited bactericidal activity against the non-commensal bacteria, *L. monocytogenes* and *E. coli* ML35. $Crp4_{red}$ killed both the commensal bacteria *L. johnsonii* and *B. breve* (Fig. 1). However, $Crp4_{ox}$ did not show activity against *L. johnsonii* but showed weak activity against *B. breve* even at 10 μg/mL. It has been reported hBD-1 altered its activity under aerobic or anaerobic condition (Wendler et al. 2018). To determine the influence of culture environment, *L. johnsonii* was cultured under aerobic condition and exposed $Crp4_{ox}$ and $Crp4_{red}$. Unlikely hBD-1, both of Crp4 form did not alter bactericidal activity under aerobic or anaerobic condition (Fig. 1c, S1).

To investigate how molecular factors, free cysteine, structure, and hydrophobicity affect the potent bactericidal activity of $Crp4_{red}$, two linear analogues were prepared, and their activities were tested. The results showed that 6C/S-Crp4 and NEM-Crp4 killed both the non-commensal bacteria (Fig. 1a, b). The activities of NEM-Crp4 against both commensal bacteria were similar to that of $Crp4_{red}$. Moreover, 6C/S-Crp4 killed only *B. breve* at its highest concentration, 10 μg/mL, and its activity against *L. johnsonii* was similar to that of the oxidised form (Fig. 1c, d). These results suggest that the potent bactericidal activity of $Crp4_{red}$ is not simply derived from the thiol groups of its free

cysteine.

CD measurement to analyze secondary structure

The structure of each peptide was measured by CD in an aqueous buffer and in membrane mimetic conditions (organic modifier TFE and SDS micelles). The spectra of $Crp4_{ox}$, which did not show any significant changes, indicated that the structure of $Crp4_{ox}$ is stabilised by three disulphide bonds in any solution (Fig. 2a–c). In contrast, the spectra of $Crp4_{red}$ and linear analogues in aqueous environments were changed in organic solvents and SDS micelles. The $Crp4_{red}$ and linear analogues were typical of a random coil conformation in the aqueous solution, whereas in the presence of TFE, they showed a negative band at approximately 205 to 210 nm, indicating β-structure, and some α-helices were observed from the shoulder at about 220 nm (Liu et al. 2008; Scudiero et al. 2010; Bonucci et al. 2013). The CD spectra of $Crp4_{red}$ and NEM-Crp4 in SDS micelles were slightly different from those of TFE, but that of 6C/S-Crp4 was almost unchanged. These results suggest that the ease of structural change in the hydrophobic environment of the membrane may affect the activity of $Crp4_{red}$.

A previous study showed that the potent activity of reduced hBD-1 depends on hydrophobicity and free cysteine located at the C terminus (Schroeder et al. 2011). Based on this study, we measured the hydrophobicity of all the prepared peptides using RP-HPLC. The retention times of $Crp4_{red}$, NEM-Crp4, and 6C/S-Crp4 were longer than those of $Crp4_{ox}$, indicating that the hydrophobicity of these peptides was higher than that of $Crp4_{ox}$ (Fig. 3). These results indicate the possibility that the potent bactericidal activity of $Crp4_{red}$ is strongly related to hydrophobicity.

Interaction with peptides and bacterial membrane

Oxidised Crp4 and $Crp4_{red}$ bactericidal mode of action is considered to interact with the bacterial membrane and produce membrane permeabilisation (Satchell et al. 2003; Figueredo et al. 2009; Masuda et al. 2011). However, some defensins do not exert their activity by disrupting membranes, but interact with other target molecules such as cell wall precursor lipid II (de Leeuw et al. 2010; Sass et al. 2010). To investigate the direct interaction between $Crp4_{red}$ and the membrane, *L. johnsonii* lipids were extracted and calcein-entrapped liposomes without any putative target molecules were prepared. The data of dye leakage showed that $Crp4_{red}$ and NEM-Crp4 led to approximately 80% efflux, whereas $Crp4_{ox}$ and 6C/S-Crp4 produced limited leakage (Fig. 4). These results demonstrate a high correlation with bactericidal activity, suggesting that $Crp4_{red}$ activities are due to direct membrane disruption that results from their high hydrophobicity.

The bactericidal activity data by the addition of NaCl suggested that electrostatic interaction with the membrane is an essential first step in the interaction of both $Crp4_{red}$ and $Crp4_{ox}$ to exert activity on the non-commensal bacterium *L. monocytogenes* (Fig. 5a). The bactericidal activity of $Crp4_{red}$ against the commensal bacterium *L. johnsonii* was also abolished by NaCl. These results as well as those illustrated in Fig. 4 show that $Crp4_{ox}$, $Crp4_{red}$ and their linear analogues exhibit their activity through the common mode of action of defensins: electrostatic interaction with the bacterial membrane. Western blotting analysis showed that $Crp4_{red}$ was bound to non-commensal and commensal bacteria in a concentration-dependent manner (Fig. 5b, c). Notably, $Crp4_{ox}$ was also bound to both bacteria, even though there was no activity against *L. johnsonii.* These results also suggest that the higher membrane insertion and disruption ability of $Crp4_{red}$

with higher hydrophobicity is crucial after the electrostatic interaction with the surface of the bacterial membrane.

E. coli ML35 is the only gram-negative bacteria using this study and its membrane composed outer and inner membrane. To confirm outer membrane interaction, the NPN uptake assay was performed. The NPN is incorporated to membrane and fluoresces around hydrophobic environment when outer membrane is damaged (Fig. S2a). The dye incorporation to outer membrane was shown both of $Crp4_{ox}$ and $Crp4_{red}$, suggesting both forms attacked *E. coli* outer membrane (Fig. S2b). The bactericidal activity against *E. coli* ML35 was abolished in the presence of NaCl (Fig. S3a). Western blotting analysis showed that $Crp4_{ox}$ and $Crp4_{red}$ bound to bacteria and binding was inhibited by NaCl (Fig. S3b), suggesting electrostatic interaction with bacterial membrane firstly and follow outer membrane disruption. These results support that the membrane insertion and disruption is crucial for $Crp4_{red}$ bactericidal activity.

2-5 Discussion

Previously, it was reported that only reduced defensins showed potent bactericidal activity against enteric commensal bacteria (Masuda et al. 2011; Schroeder et al. 2011; Schroeder et al. 2015). A common antibacterial mechanism of AMPs, including defensin, interacts with negatively charged bacterial membranes and disrupts its integrity, although the mechanisms have not been fully elucidated in many of them (Zasloff 2002; Ganz 2003; Selsted and Ouellette 2005). For instance, some AMPs, including human α-defensin, interact with specific molecular components of bacteria component (Brogden 2005; Breukink and de Kruijff 2006; de Leeuw et al. 2010; Lin et al. 2010; Chang et al. 2012). Fungal defensin, oyster defensin, and hBD-3 were shown to bind lipid II and inhibit cell wall biosynthesis (Sass et al. 2010; Schmitt et al. 2010; Schneider et al. 2010). Wendler et al. (2018) showed that oxidised hBD-1 enters the Gram-negative bacterial periplasm space via FepA and exerts its activity depending on DsbA and DsbB (Wendler et al. 2018). In this study, however, we demonstrated that $Crp4_{red}$ exerts its activity through direct membrane interaction without any specific molecular target and that its potent activity is derived from its hydrophobicity.

To test how molecular factors cause the potent bactericidal activity of $Crp4_{red}$, I prepared two linear analogues with substituted or modified cysteine residues, 6C/S-Crp4 and NEM-Crp4. The activities of NEM-Crp4 and $Crp4_{red}$ against both commensal bacteria were equivalent. In addition, 6C/S-Crp4 killed *B. breve* but not *L. johnsonii* (Fig. 1c, d). These results suggest that the thiol group of free cysteine in $Crp4_{red}$ is not essential for its potent activity. Additionally, hBD-1 and HD-6 with all Cys residues substituted showed lower activity than those of wild types. Some studies have reported that Cys itself showed antimicrobial activity (Schroeder et al. 2011; Gouveia et al. 2012; Caldeira et al. 2013;

Schroeder et al. 2015; Raschig et al. 2017; Wang et al. 2019). The CD spectra of $Crp4_{red}$ and linear analogues showed secondary structure formation in TFE and SDS micelles (Fig. 2a–c), suggesting that they form a certain conformation in the bacterial membrane. The differences in the structure and ease of structural change in hydrophobic conditions might also be related to the activity of each peptide. I demonstrated that the two peptides $Crp4_{red}$ and NEM-Crp4 killed all non-commensal and commensal bacteria owing to a high hydrophobicity (Fig. 3), indicating that hydrophobicity is critical for the potent bactericidal activity of $Crp4_{red}$. The results that $Crp4_{ox}$ showed higher hydrophilicity is natural because hydrophobic residues are buried inside the molecule due to the formation of disulphide bonds and the globular structure of $Crp4_{ox}$. After formation of the tertiary structure of $Crp4_{ox}$, it had an amphipathic structure (Jing et al. 2004). This structure may also inhibit migration into the membrane of commensal bacteria. A previous study also demonstrated that C-terminal hydrophobicity is important for the potent bactericidal activity of reduced hBD-1 and antimicrobial activity of $Crp4_{ox}$ (Schroeder et al. 2011; Tai et al. 2014). Moreover, I demonstrated a strong correlation between bactericidal activity and membrane permeabilisation (Fig. 1c and Fig. 4), suggesting that the bactericidal mechanism of $Crp4_{red}$ is mainly the disruption of bacterial membrane integrity. Commensal bacteria showed resistance to $Crp4_{ox}$, though bound to the bacterial membrane surface (Fig. 5a, c), suggesting that the bactericidal activity of $Crp4_{ox}$ is dependent on bacterial membrane composition and its hydrophobicity. Previous studies indicated that HNP-1 prefers to interact with fungal model membranes compared with those of bacterial and mammalian (Gonçalves et al. 2012) and RegIIIα and showed leakage from phosphatidylcholine/phosphatidylserine (PC/PS) liposomes, whereas liposomes composed of total *E. coli* lipids were not induced (Mukherjee et al. 2014).

Moreover, maculatin 1.1, an α-helical AMP from Australian tree-frag species, changes secondary structure and membrane insertion, depending on the length of the hydrophobic core of lipids (Sani et al. 2012). The lipid composition of *Lactobacillus* sp. and *Bifidobacterium* sp. includes a higher proportion of long-chain fatty acids than that of *L. monocytogenes*; hence, the lipids in the former species are more hydrophobic than those in the latter species. Furthermore, *Lactobacillus* sp. has a high percentage of the long-chain C19:0 fatty acids (Veerkamp 1971; Mastronicolis et al. 2010; Gandhi and Shah 2016). Thus, the difference in membrane hydrophobicity between commensal and non-commensal bacteria may be one of the most important factors related to the selective antibacterial activity of $Crp4_{ox}$ and $Crp4_{red.}$

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Table. The bacteria list to test bactericidal assay

Non-Commensal/ Commensal	Bacteria	Culture condition	Gram stain
Non-Commensal	*Listeria monocytogenes* (*L. monocytogenes*)	Aerobic	Positive
	Escherichia coli ML35 (*E. coli* ML35)	Aerobic	Negative
Commensal	*Lactobacillus johnsonii* (*L. johnsonii*)	Anaerobic	Positive
	Bifidobacterium breve (*B. breve*)	Anaerobic	Positive

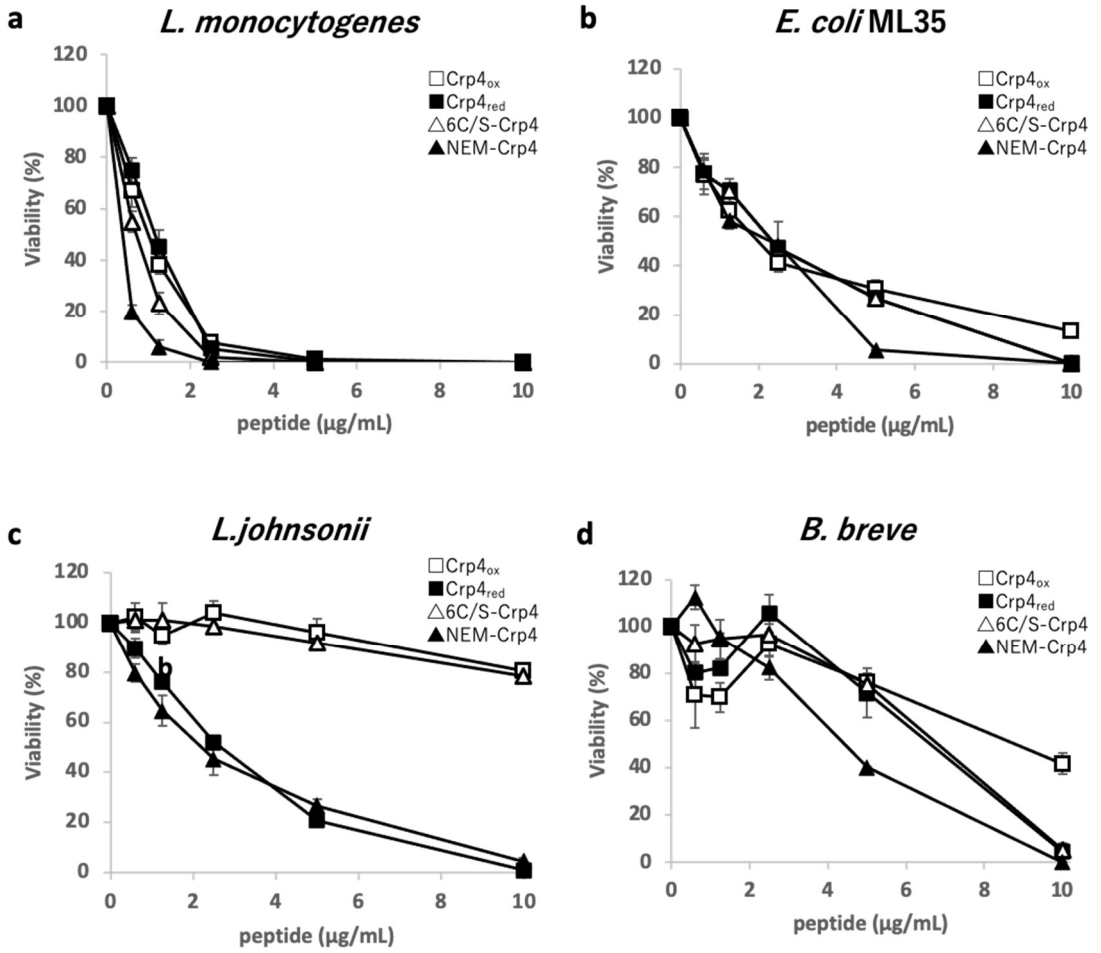

Fig. 1 Bactericidal activities of oxidised Crp4 ($Crp4_{ox}$) (□), reduced Crp4 ($Crp4_{red}$) (■), Crp4 with cysteine (Cys) residues substituted with serine (Ser) peptide (6C/S-Crp4) (△), and Crp4 with thiol groups modified by NEM (N-ethylmaleimide) (NEM-Crp4) (▲) against **(a)** *L. monocyogenes*, **(b)** *E. coli* ML35, **(c)** *L. johnsonii* and **(d)** *B. breve*. About 1×10^7 CFU/mL of bacteria were exposed to peptides at 0.6, 1.25, 2.5, 5, and 10 μg/mL. Data are presented as mean ± standard error of the mean (SEM). n = 6 for $Crp4_{ox}$ and $Crp4_{red}$ and n = 4 for 6C/S-Crp4 and NEM-Crp4 in **(a)** and **(c)**. n = 3 for all peptides in **(b)** and **(d)**

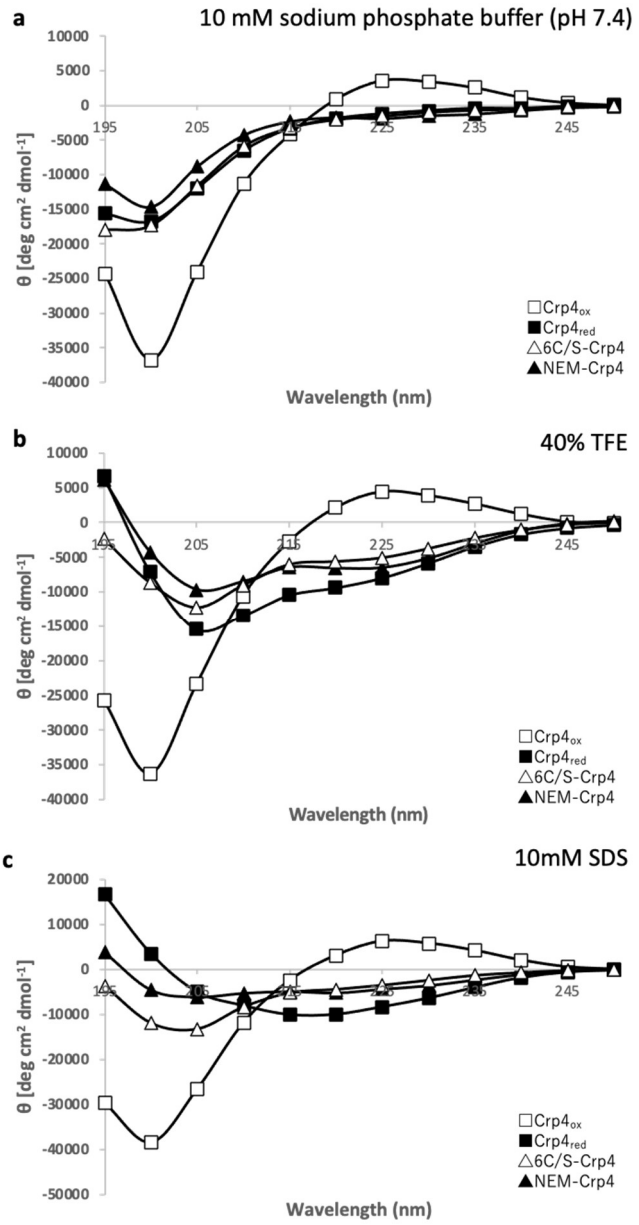

Fig. 2 Circular dichroism (CD) spectra measured from 250 to 195 nm at room temperature (25 °C). Each 30 μM of oxidised Crp4 ($Crp4_{ox}$) (□), reduced Crp4 ($Crp4_{red}$) (■), Crp4 with cysteine (Cys) residues substituted with serine (Ser) peptide (6C/S-Crp4) (△), and Crp4 with thiol groups modified by NEM (N-ethylmaleimide) (NEM-Crp4) (▲) was measured in **(a)** 10 mM sodium phosphate buffer (pH 7.4), **(b)** 40 % trifluoroethanol (TFE), and **(c)** 10 mM sodium dodecyl sulphate (SDS).

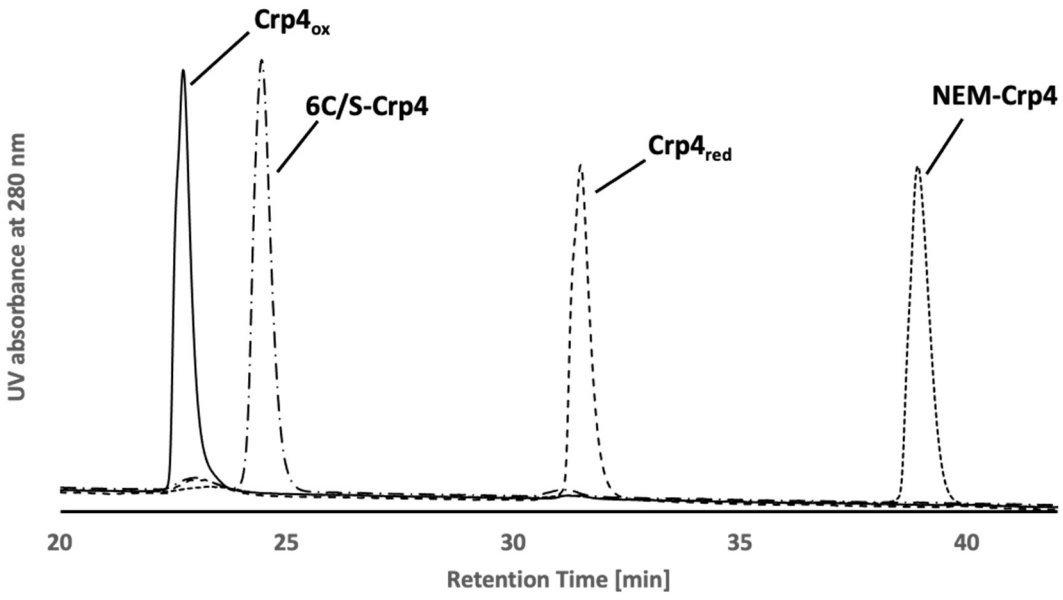

Fig. 3 Hydrophobicity of oxidised Crp4 ($Crp4_{ox}$), reduced Crp4 ($Crp4_{red}$), Crp4 with cysteine (Cys) residues substituted with serine (Ser) peptide (6C/S-Crp4), and Crp4 with thiol groups modified by NEM (N-ethylmaleimide) (NEM-Crp4) analysed using reversed-phase high-performance liquid chromatography (RP-HPLC). Approximately 10 μg of each peptide was injected, and early retention time indicated lower hydrophobicity.

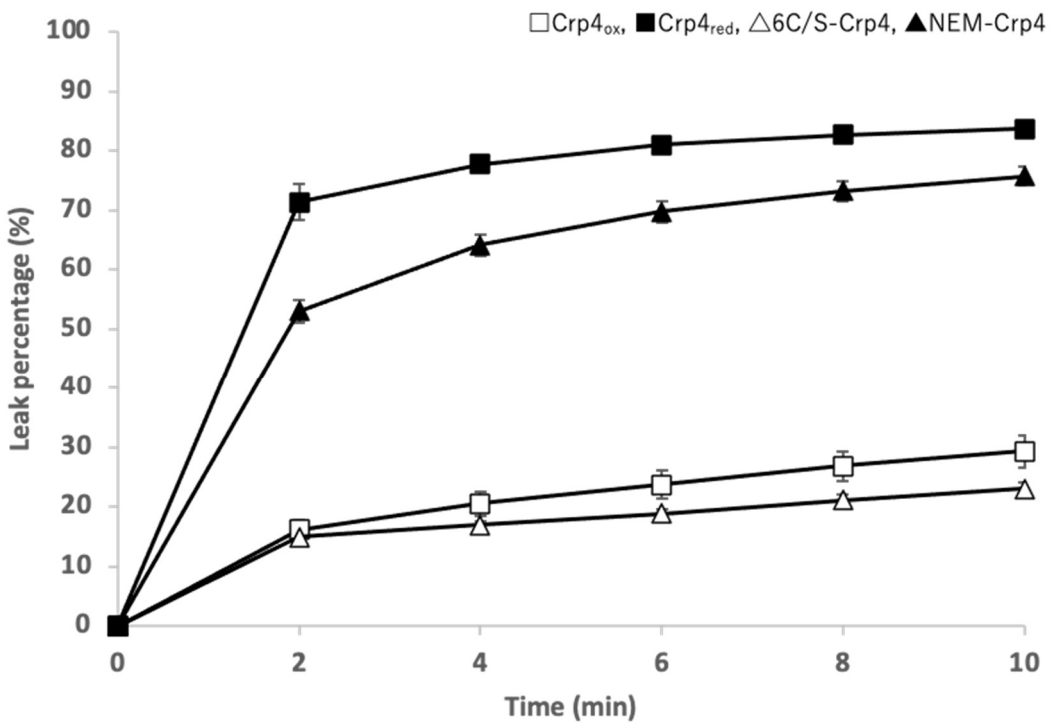

Fig. 4 The interaction of 10 µg/mL oxidised Crp4 ($Crp4_{ox}$) (□), reduced Crp4 ($Crp4_{red}$) (■), Crp4 with cysteine (Cys) residues substituted with serine (Ser) peptide (6C/S-Crp4) (△), and Crp4 with thiol groups modified by NEM (N-ethylmaleimide) (NEM-Crp4) (▲) with liposomes measured by calcein leakage from large unilamellar vesicles (LUVs), fluorometrically, which were prepared using *Lactobacillus. johnsonii* lipid extraction. Data are presented as mean ± standard error of the mean (SEM). n = 3 for all peptides

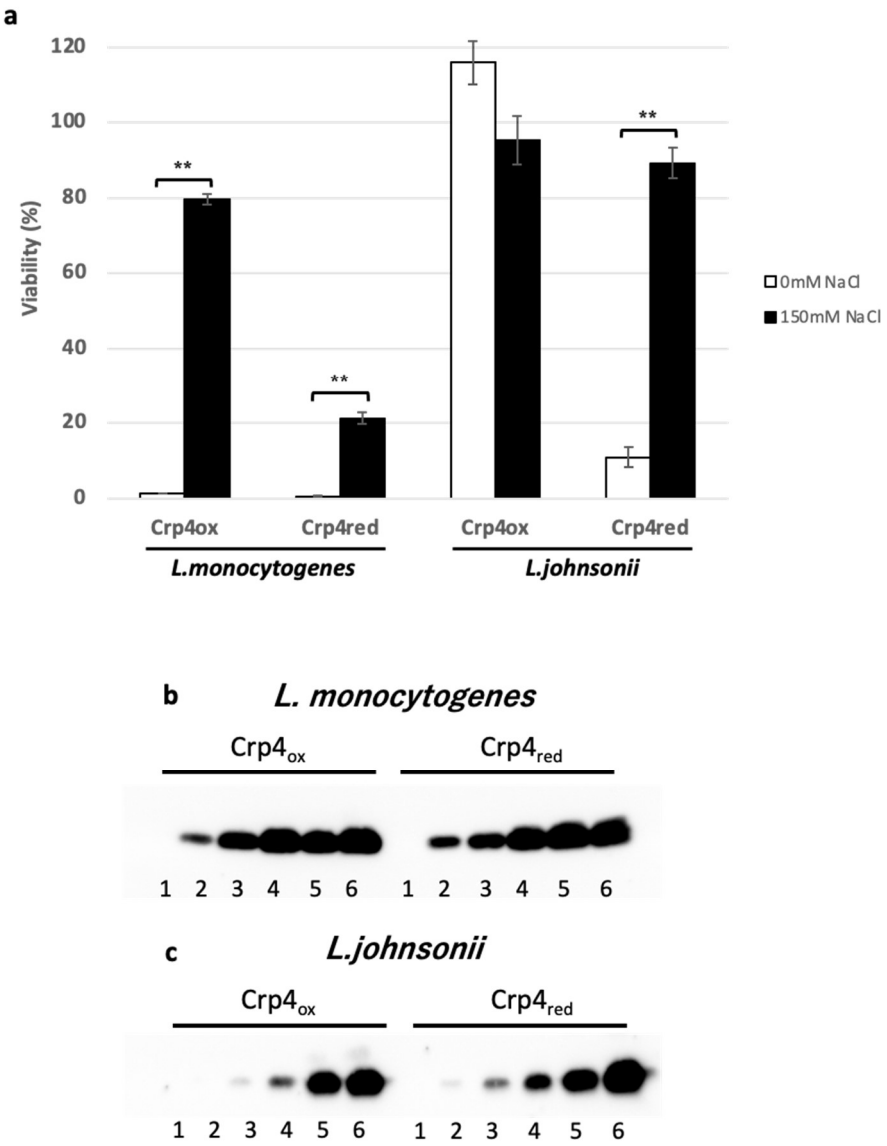

Fig. 5 (a) Bactericidal activity at 5 µg/mL in the presence or absence of 150 mM NaCl. The activities were assayed to determine influence of electrostatic interaction with peptides and bacterial membrane; oxidised Crp4 ($Crp4_{ox}$) and reduced Crp4 ($Crp4_{red}$) bound to **(b)** *L monocytogenes* and **(c)** *L. johnsonii* were confirmed using western blotting (1, 0 µg/mL; 2, 0.6 µg/mL; 3, 1.25 µg/mL; 4, 2.5 µg/mL; 5, 5 µg/mL; 6, 10 µg/mL). Data are presented as mean ± standard error of the mean (SEM) and were statistically evaluated using student's *t*-test with ** $P < 0.01$. n = 3 for all peptides

Supporting data

Materials and Methods

NPN uptake assay

The exponential phase of *E. coli* ML35 was collected by centrifugation, washed and resuspended in 10 mM HEPES, 20 mM glucose. The N-phenyl-1-naphthylamine (NPN) uptake assay was determined fluorometrically in a 2 mL-plastic cell (F2000 Fluorescence Spectrophotometer, Hitachi). The diluted bacterial suspension, NPN solution (10mM HEPES, 100 μM NPN) and peptides were mixed and the uptake percentage of NPN was measured with excitation and emission wavelengths of 355 nm and 380-430 nm, respectively after 5min incubation. Peptide-induced uptake percentage was calculated using the following equation:

$$leak\ percentage\ (lsb\ \%) = \frac{(F - F_0)}{(F_{100} - F_0)} \times 100$$

where F is the fluorescence intensity induced by 10 μg/mL of peptides, F_0 is the background fluorescence intensity, and F_{100} is the fluorescence intensity after treatment with 10 μM melittin.

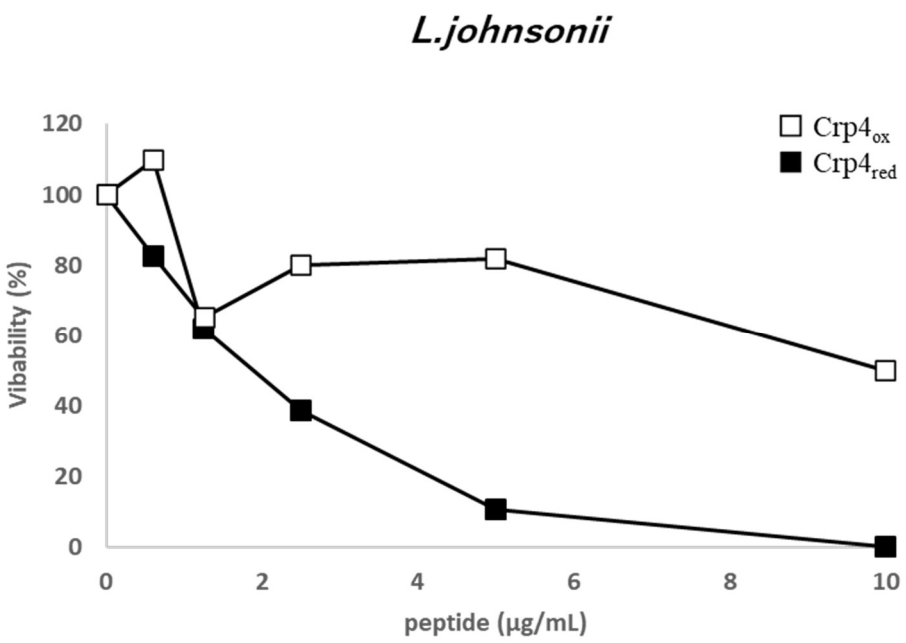

Fig. S1 Bactericidal activities of oxidised Crp4 ($Crp4_{ox}$) (□) and reduced Crp4 ($Crp4_{red}$) against *L. johnsonii* under aerobic condition. About 1×10^7 CFU/mL of bacteria were exposed to peptides at 0.6, 1.25, 2.5, 5, and 10 µg/mL.

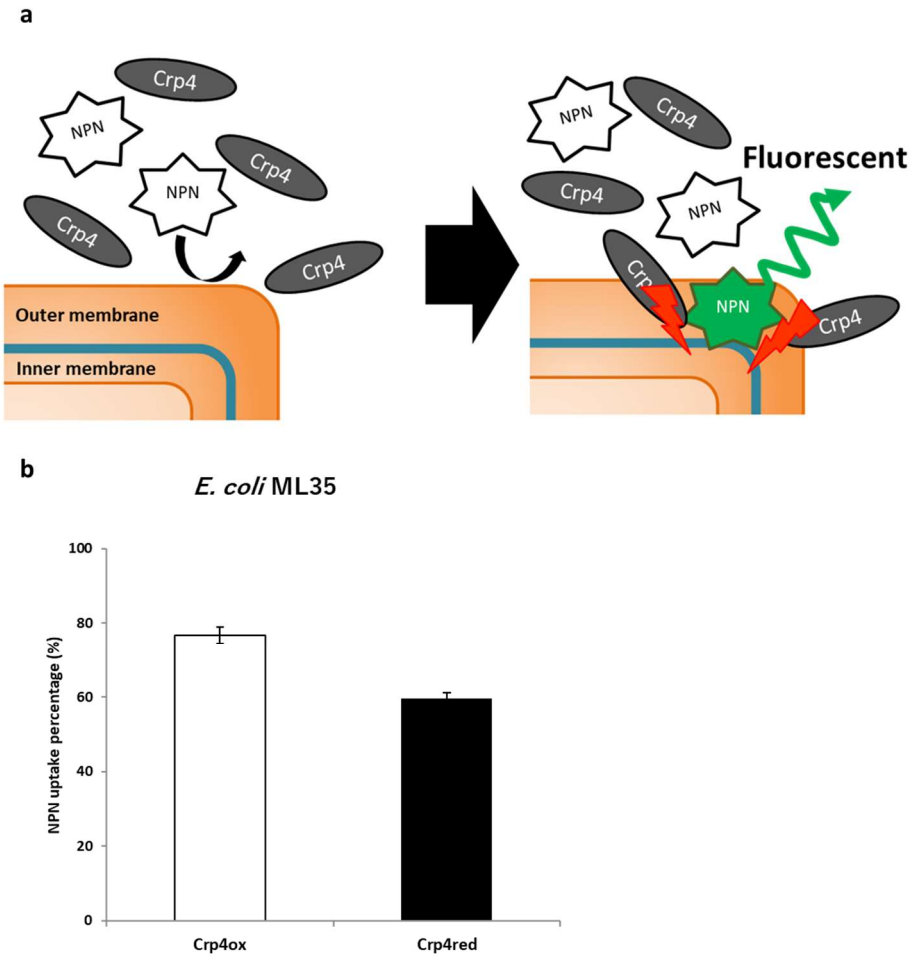

Fig. S2 (a) The illustration of NPN uptake assay. **(b)** The interaction of 10 µg/mL of oxidised Crp4 ($Crp4_{ox}$) (□) and reduced Crp4 ($Crp4_{red}$) (■) with *E. coli* ML35 outer membrane measured by NPN uptake assay. Data are presented as mean ± standard error of the mean (SEM). n = 3 for all peptides

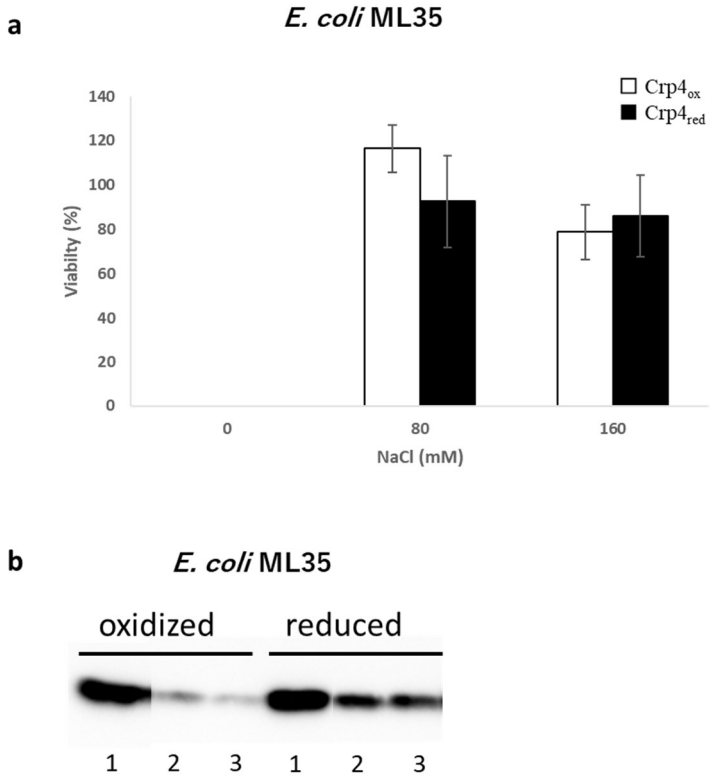

Fig. S3 (a) Bactericidal activity at 5 µg/mL in the presence or absence of 80 mM and 160 mM NaCl. The activities were assayed to determine influence of electrostatic interaction with peptides and bacterial membrane; oxidised Crp4 ($Crp4_{ox}$) and reduced Crp4 ($Crp4_{red}$) bound to **(b)** *E.coli* ML35 was confirmed using western blotting (1, 0 mM NaCl; 2, 80 mM NaCl; 3, 160 mM NaCl).

Conclusion

In this thesis, I revealed that the potent bactericidal activity of $Crp4_{red}$ is derived from its hydrophobicity and that its bactericidal mechanism involves the disruption of bacterial membranes.

In Chapter 1, I described a new production method for $Crp4_{red}$ which can be adopted for a large number of inexpensive peptide preparations. Recombinant purified $Crp4_{ox}$ expressed in *E. coli* was used as a starting material and was reduced to produce $Crp4_{red}$. Cysteine residues have been widely used as substitutes for other amino acids or unnatural amino acids to study the function of cysteine. However, conventional methods using chemically synthesised or recombinant peptides are costly and time-consuming. I have demonstrated an alternative approach to modify the thiol groups of cysteine residues. This method is easier and quicker than conventional methods and would accelerate reduced defensin studies.

In Chapter 2, I demonstrated that hydrophobicity is crucial for the potent bactericidal activity of $Crp4_{red}$ and disruption of the bacterial membrane. There was a strong correlation between bactericidal activity against commensal and non-commensal bacteria and peptide hydrophobicity. A liposome leakage assay for lipids extracted from commensal bacteria demonstrated a correlation with bactericidal activity, which was abolished by the inhibition of electrostatic interactions. These data suggested that the $Crp4_{red}$ bactericidal mechanism involves the disruption of bacterial membranes. My findings provide a better understanding of the bactericidal mechanisms involving $Crp4_{ox}$ and $Crp4_{red}$.

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