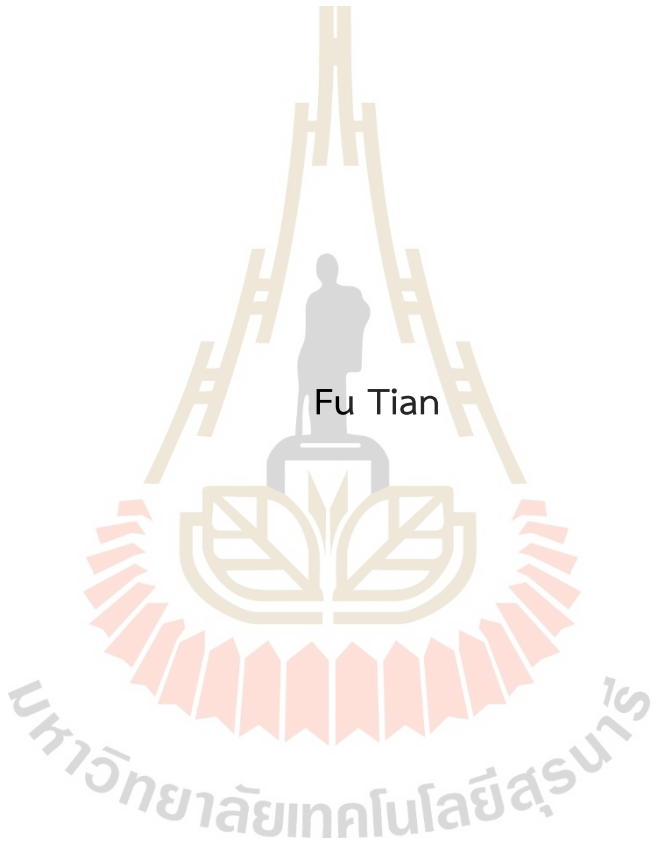
ISOLATION, IDENTIFICATION, AND MODE OF ACTION OF NOVEL ANTIBACTERIAL PEPTIDES FROM CHICKEN PLASMA HYDROLYSATES

Fu Tian



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การแยก การระบุชนิด และกลไกการออกฤทธิ์ของเพปไทด์ต้านแบคทีเรียจากโปรตีนไฮโดรไลเสทของพลาสมาเลือดไก่

นายฟู เทียน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ฟู เทียน : การแยก การระบุชนิด และกลไกการออกฤทธิ์ของเพปไทด์ต้านเชื้อแบคทีเรียจากโปรตีนไฮโดรไลเสทของพลาสมาเลือดไก่ (ISOLATION, IDENTIFICATION, AND MODE OF ACTION OF NOVEL ANTIBACTERIAL PEPTIDES FROM CHICKEN PLASMA HYDROLYSATES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.จิรวัฒน์ ยงสวัสดิกุล, 120 หน้า.

เพปไทด์ต้านแบคทีเรีย/การทำให้บริสุทธิ์/กลไกการออกฤทธิ์/การจำลองพลวัตเชิงโมเลกุล

เลือดไก่เป็นผลิตผลพลอยได้จากโรงเชือด ซึ่งมีการใช้ประโยชน์อย่างจำกัดและก่อให้เกิดปัญหาสิ่งแวดล้อม การนำเลือดไก่มาผ่านกระบวนการที่เหมาะสมจะเป็นประโยชน์อย่างยิ่งต่อการรักษาสิ่งแวดล้อมและมูลค่าทางเศรษฐกิจ วิกฤตการดื้อยาต้านแบคทีเรียหลายขนานที่ทวีความรุนแรงมากขึ้นและความเสี่ยงต่อสุขภาพจากสารกันบูดในอาหารนำไปสู่การหาแนวทางใหม่ในการควบคุมเชื้อแบคทีเรีย เพปไทด์ต้านจุลชีพ (Antimicrobial peptides, AMPs) ได้รับความสนใจอย่างต่อเนื่องเนื่องจากคุณสมบัติของเพปไทด์ที่มีเป้าหมายในการทำลายเยื่อหุ้มเซลล์และองค์ประกอบภายในเซลล์ เช่น ดีเอ็นเอ เอนไซม์ หรือโปรตีน ดังนั้นทำให้โอกาสที่เชื้อแบคทีเรียจะดื้อต่อเพปไทด์เกิดขึ้นน้อยมาก ดังนั้นวัตถุประสงค์ของงานวิจัยนี้คือแยกและระบุเพปไทด์ต้านจุลชีพจากไฮโดรไลเสทพลาสมาเลือดไก่

เพปไทด์ต้านแบคทีเรียชนิดใหม่ 2 ท่อนที่แยกได้จากไฮโดรไลเสทพลาสมาเลือดไก่จากการย่อยด้วยอัลคาเลส มีลำดับกรดอะมิโนคือ VSDH และ CCCPKAF มีฤทธิ์ต้านเชื้อ *Bacillus cereus* DMST 5040 ได้ดี โดยมีกลไกการออกฤทธิ์ที่หลากหลาย จากการวิเคราะห์การติดสีย้อมโพรพิเดียมไอโอไดด์พบว่าเพปไทด์ CCCPKAF มีฤทธิ์ทำลายเยื่อหุ้มเซลล์ และจากการทดสอบด้วยเทคนิคฟูเรียร์ทรานฟอร์มอินฟราเรดสเปกโทรสโกปีพบว่าเพปไทด์ VSDH มีผลต่อองค์ประกอบภายในเซลล์ ได้แก่ โปรตีนและกรดนิวคลีอิก เมื่อวิเคราะห์ด้วยเทคนิคการจำลองการจับกันระหว่างโมเลกุล (molecular docking) พบว่าเพปไทด์ VSDH มีความเจาะจงต่อเอนไซม์ที่เกี่ยวข้องกับการสังเคราะห์ดีเอ็นเอ นอกจากนี้ ยังพบว่าเพปไทด์ VSDH มีความสามารถในการจับโลหะ ส่งผลให้มีฤทธิ์ต้านเชื้อแบคทีเรีย

เพปไทด์ EADE ซึ่งเป็นเพปไทด์ประจุลบชนิดใหม่ที่แยกได้จากไฮโดรไลเสทพลาสมาเลือดไก่จากการย่อยด้วยเพปซินมีฤทธิ์ในการยับยั้งการเจริญของแบคทีเรีย *Staphylococcus aureus* ATCC 25923ที่ความเข้มข้น 0.5 มิลลิโมลาร์ เพปไทด์ EADE ยับยั้งแบคทีเรียโดยการจับกับแร่ธาตุที่จำเป็นต่อการเจริญของแบคทีเรีย โดยพบการเกิดสารประกอบเชิงซ้อนของ EADE กับ Ca^{2+} จากการวิเคราะห์โดยใช้เทคนิคนิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโทรสโกปี (NMR) และการจำลองพลวัตเชิงโมเลกุล

เพปไทด์ต้านจุลชีพ CP-1 (KPKVLLHA) ถูกแยกได้จากไฮโดรไลเสทพลาสมาเลือดไก่ที่ย่อยด้วยเพปซินด้วยเครื่องแฟลชโครมาโทกราฟฟี ด้วยคอลัมน์ C-18 พบว่าเพปไทด์ CP-1สามารถยับยั้งการเจริญของ *Salmonella Typhimurium* TISTR 292 ที่ความเข้มข้น 2 มิลลิโมลาร์ ผลการวิเคราะห์การติดสีย้อม โพรพิเดียมไอโอไดด์ด้วยกล้องคอนโฟคอน พบว่าเพปไทด์ CP-1ทำให้เยื่อหุ้มเซลล์ฉีกขาด และจากผลการวิเคราะห์โดยใช้เทคนิคการจำลองพลวัตเชิงโมเลกุลพบว่าเพปไทด์ CP-1มีเป้าหมายในการทำลายดีเอ็นเอด้วย นอกจากนี้ ยังพบการเสริมฤทธิ์กันของเพปไทด์ CP-1ที่ความเข้มข้น 1 มิลลิโมลาร์กับเพปไทด์ประจุลบ EADE ที่ความเข้มข้น 0.5 มิลลิโมลาร์ จากการทดสอบการวัดฟลูออเรสเซนต์ของสาร 1-*N*-ฟีนิลแนพธิลเอมีนพบว่า EADE เพิ่มการซึมผ่านของเยื่อหุ้มชั้นนอก ซึ่งอาจเกิดจาก EADE เข้าไปจับกับ Mg^{2+} หรือ Ca^{2+} ทำให้ CP-1 ซึมผ่านเยื่อหุ้มชั้นนอกไปที่เป้าหมายภายในเซลล์ได้ดียิ่งขึ้น ดังนั้นการใช้เพปไทด์ CP-1 ร่วมกับ EADE สามารถยับยั้งการเจริญของเชื้อ *S. Typhimurium* TISTR 292 ได้

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา Fu Tian

ปีการศึกษา 2564 ลายมือชื่ออาจารย์ที่ปรึกษา

FU TIAN : ISOLATION, IDENTIFICATION, AND MODE OF ACTION OF NOVEL ANTIBACTERIAL PEPTIDES FROM CHICKEN PLASMA HYDROLYSATES. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWATDIGUL, Ph.D., 120 PP.

ANTIBACTERIAL PEPTIDES/PURIFICTION/MECHANISMS/MOLECULAR DYNAMICS SIMULATION

Chicken blood is a byproduct from a slaughterhouse with limited utilization and causing environmental problems. Appropriate treatment of chicken blood is of great benefit to both environmental protection and economic value. The increasingly severe problem associated with multiple drug-resistant bacteria and the potential health risks from synthetic food preservatives have led to a demand for new strategies for microbial control. Antimicrobial peptides (AMPs) have recently attracted much attention as they act on multiple targets on membrane and intracellular components, such as DNA, enzymes, or protein. Thus, mutations conferring AMP resistance are less likely to occur. The objectives of this study were to isolate and identify AMPs from chicken blood plasma hydrolysates, and to elucidate their mechanisms of antibacterial action.

Two novel antibacterial peptides isolated from Alcalase-hydrolyzed chicken plasma hydrolysate were identified to be VSDH and CCCPKAF. They showed good antibacterial ability toward *Bacillus cereus* DMST 5040 with varied mechanisms. The CCCPKAF induced bacterial cell membrane disruption as evidenced by propidium iodide (PI) uptake. The VSDH targeted intracellular components, including proteins and nucleic acids as revealed by Synchrotron-based Fourier Transform Infrared (SR-FTIR). Molecular docking analysis revealed that VSDH showed good bonding affinity to various enzymes involved in DNA synthesis. Moreover, VSDH showed metal chelation ability, which could partly contribute to its antibacterial ability.

One novel anionic AMP, EADE, was also isolated from chicken blood plasma hydrolyzed by pepsin. EADE inhibited the growth of *Staphylococcus aureus* ATCC 25923 with minimal inhibitory concentration (MIC) of 0.5 mM. EADE inhibited growth by chelating trace elements that are required for bacterial growth. the results of NMR

and molecular dynamics (MD) simulation indicated that EADE formed stable complex with Ca^{2+}.

A novel cationic AMP, namely CP-1 (KPKVLLHA), was successfully isolated using C18-AQ flash chromatography from pepsin-hydrolyzed chicken plasma hydrolysate. CP-1 inhibited the growth of *Salmonella* Typhimurium TISTR 292 with MIC of 2 mM. Confocal laser scanning microscopy combined with PI showed cell membrane damage induced by CP-1. DNA was likely to be the second target of CP-1 as revealed by MD simulation. When combined with 0.5 mM EADE, MIC of CP-1 decreased to 1 mM, showing synergistic effect with anionic peptide. EADE possibly chelated Mg^{2+} or Ca^{2+}, which increased permeability of outer membrane as shown by 1-*N*-phenylnapthylamine (NPN). This ultimately allowed more influx of CP-1 to penetrate bacterial outer membrane to greater extent. Thus, CP-1 combined with EADE showed potential in controlling *S.* Typhimurium TISTR 292

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CHAPTER I
INTRODUCTION

1.1 Introduction

Due to overuse conventional antibiotics, several pathogenic microorganisms have become multi-drug resistant .Bacterial resistance is mainly caused by the mutation of one or more genes that are associated with detoxification of one or more toxins. Therefore, mutant bacteria may become resistant to conventional antibiotics, accompanied by an enhancement in their survival rates physically or genetically. (Sila et al., 2014). The novel antimicrobial agents need to be developed. Bioactive peptides with antibacterial activity could be natural potential candidates for substitution of conventional antibiotics (Ma et al., 2020).

Antimicrobial peptides (AMPs) are generally composed of 10-50 amino acids in length .Although no significant similarity in amino acid sequence has been established among different AMPs, some common characteristics do exist .These included two or more positively charged residues and presence of both hydrophilic and hydrophobic segments (Huan et al., 2020). AMPs have taken interest due to their unique antimicrobial mechanism against both Gram-positive and Gram-negative bacteria, and some multidrug-resistant strains .MIC value of the more effective AMPs is comparable to traditional antibiotics at 0.1-1.0 μg/ml (Beisswenger and Bals, 2005). Furthermore, these peptides possess broad spectrum and rapid microbial inactivation .Bacteria do not easily develop resistance to AMPs (Erdem Büyükkiraz and Kesmen, 2022). Most AMPs also exhibit antifungal, antiviral and anticancer properties (Zhang et al., 2021).

AMPs can find from several natural source such as microbial, insect, plant and animal (Huan et al., 2020). However, the content of natural AMPs in biological tissues is low, and the direct separation and extraction process is tedious, low yield and high cost. Enzymatic hydrolysis is another means to produce AMP, which is more efficient approach for production and separation.

Various enzymes Alcalase, pepsin, trypsin, papain, and flavourzyme (have been used to hydrolyze food proteins to obtain AMPs. Pu and Tang, (2017) reported an AMP, LVDHFPL, isolated from rice bran protein hydrolyzed by bromelain, showing potent antibacterial ability toward *Listeria monocytogenes*. Three AMPs generated from laba garlic protein hydrolysate by pepsin and trypsin, showed ability against *Escherichia coli* and *Sa .ureus* (Gao et al., 2019). Kong et al. (2020) isolated AMP inhibiting *E .coli* from Alcalase-hydrolyzed cottonseed .Among enzymes used, Alcalase was widely used to produce AMPs .Due to the wide range of amino acids recognized by Alcalase, the resulting protein hydrolysate contained small peptide .In addition, peptides contained hydrophobic characteristic, which is one of critical criteria (Tacias-Pascacio, 2020). Pepsin was widely used in the production of AMP from blood material, due to its commercial availability and specificity (Sanchez-Reinoso et al., 2021).

The mechanism of AMPs can divided into membrane disruption and intracellular reaction (Benfield and Henriques, 2020). Most AMPs reported thus far are cationic peptides .Positively charged peptides readily bound with negatively charged cell membranes via electrostatic interactions, and the lipid region of the membrane was penetrated and disorganized by hydrophobic residues of AMPs, causing destabilization of cell membrane (Brogden, 2005). For this reason, the number of postivie charges on peptides are used as an important criterion to screen potential AMPs .However, a few reports indicated that bacteria could introduce lysine into cell membrane through multiple peptide resistance factor (MprF) to reduce electronegative of cell membranem, resulting in reduction in activitis of cationic AMP. (Assoni et al., 2020). New features of AMPs showed be explored.

Anionic AMP (AAMP) was another group which was less studied .AAMPs are though to have evolved in response to bacterial resistance toward CAMPs .Normally, AAMPs need divalent metal ions for maximal activity, such as Dermcidin needed Zn^{2+} ions for pore formation and insertion into bacterial membrane (Becucci et al., 2014). In addition, an ovine pulmonary surfactant-associated anionic peptide)SAAP (used Zn^{2+} to form salt bridge with negative charged component of microbial membrane and crossed the membrane to complete action (Brogden et al., 1996). Unlike cationic AMPs, mechanisms of AAMPs remain elusive .Thus, study mechanism

of AAMP could increase its utilization and reduce the chance of emergence of resistant bacteria.

AMPs work synergitically with common antibiotics, which is another way to reduce the emergence of resistant bacteria and antibiotics utilization. AMPs promoted the adsorption of antibitoic by latering cell membrane permeability. This could reduce the dose of antibiotics and the risk of bacterial resistance (Yu et al., 2016). In addition, the synergistic effect between AMPs should also be explored. Jahangiri et al. (2021) reported that two CAMPs, P10 and Nisin showed synergistic effect agaisnt extensive durg-resistance *Acinetobacter baumannii*. However, the synergistic effect between AMP is only focus on cationic AAMPs. Thus far, synergistic effect between anionic and cationic AMPs is still not widely explored. This could shed light on more effective pathogen control strategey.

Chicken blood from a slaugherhouse could be a promising source for AMP production. It has been known that blood is a source of antimicrobial agents .Type of white blood cells known as granulocytes possess granules in their cytoplasm, which contain an array of antimicrobial molecules, such as defensins, cathelicidins, lysozyme, lactoferrin (Lehrer et al., 2005). Blood plasma is rich in protein, including albumin, globulin, and fibronectin .Albumin and globulin have been reported to possess antibacterial properties (Arzumanyan et al., 2019). Hydrolysis of blood plasma results in peptides exhibiting various bioactivities, including the antioxidant peptides isolated from duck plasma (Aiemratchanee et al., 2021). Discovering antimicrobial activity in chicken plasma hydrolysate would lead to increased value of chicken blood.

1.2 Research objectives

1.2.1 To produce AMPs derived from chicken plasma hydrolysate using Alcalase and pepsin.

1.2.2 To isolate, identify, and characterize AMPs generated from chicken plasma hydrolysate

1.2.3 To study mode of actions of peptides derived from chicken plasma hydrolysates.

1.2.4 To study synergistic effect of isolated CAMP and AAMP.

1.3 Research Hypotheses

Chicken blood plasma has potential as a protein source for production of AMPs .Alcalase and pepsin can be used to yield AMPs from chicken blood plasma . Moreover, some novel AMPs can be identified and show potent antibacterial ability toward various food pathogenic bacteria .In addition, these AMPs many exhibits different modes of action to inhibit bacterial growth .Different AMPs might work synergistically for controlling bacterial growth.

1.4 Scope of the study

Chicken blood plasma was hydrolyzed using two enzymes, Alcalase and pepsin, under the optimal conditions of 6 and 4 h, respectively .Fractionation of Alcalase-hydrolyzed sample was carried out using two chromatographic techniques, including size exclusion and reversed-phase chromatography .C18-AQ Flash chromatography was used for AMP separation of pepsin hydrolysate .Antibacterial ability of each fraction was tested and fraction exhibiting the highest activity was selected for peptide identification by LC-MS/MS .Peptides obtained from *de novo* peptides was systematically studied .Mode of action of synthetic peptides was investigated .In addition, synergistic effects between synthetic peptides were studied.

1.5 References

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CHAPTER II

LITERATURE REVIEWS

Antimicrobial resistant bacteria have emerged due to the overuse of conventional. The emergence of resistant bacteria which affect human health, also threaten agricultural livelihoods and global food security, has made it a major public health and economic issue in the world (Carratalá, Serna, Villaverde, Vázquez, and Ferrer-Miralles, 2020). Therefore, the development of new antimicrobial agents is urgent.

Antimicrobial peptides (AMPs) also known as peptide antibiotics which are a class of small molecule short peptides with broad-spectrum antimicrobial activity . AMPs are important barriers for the host's natural immune defense system against exogenous pathogens (Yeaman and Yount, 2003). AMPs have a different mechanism from traditional antibiotics to kill bacteria .Traditional antibiotics interrupt bacterial metabolism and cell growth by targeting specific receptors in bacteria .However, AMPs act on multiple targets on membrane and intracellular components such as DNA, enzyme, or protein (Brogden, 2005; Huang and Charron, 2017). Therefore, drug-resistance is unlikely to happen because there are multiple functional mechanisms and therapeutic targets. So far, over 3000 AMPs have been identified in the database. However only 7 AMPs are in clinical trial or approved by U.S .Food and Drug Administration)FDA .(They are colistin, gramicidin D, daptomycin, vancomycin, oritavancin, dalbavancin and telavancin (Krishnan, Choi, Jang, and Kim, 2020).

Protein is an important macronutrient as a source of essential amino acids and energy .In addition, basic nutrition of some food protein can provide extra health benefits through the release of bioactive peptides by enzymatic hydrolysis, fermentation, and gastrointestinal digestion .It is reported that protein hydrolysates deriving from several food proteins have wide biological activities, including immunomodulatory, anticancer, antihypertensive, antioxidant and anti-inflammatory . Thus, food protein might be another potential source for generate AMPs .The release of peptides upon hydrolysis by suitable protease could result in peptides

that function as AMPs .In addition, peptide preparation can also transform underutilized food protein and protein by-protein into valuable products, in which food companies are very interested. This review provides an insight about AMPs isolation from food protein .

2.1 Mode of actions

The antimicrobial mechanism of antimicrobial peptides can be divided into 2 types: membrane disruptive and non-membrane disruptive models

In membrane disrupting mechanism, the electrostatic interaction between cationic residues of the peptide and the anionic cell surface constitutes the first driver for AMP to approach cell membrane, and the hydrophobic interactions between the amphipathic domains of the peptides and the acyl chains of the lipid serves as the second driver (Brogden, 2005). Three main models namely the barrel-stave, toroidal and carpet-like model can be used to explain the membrane disrupting mechanism (Fig.2.1). The barrel-stave model AMPs can bind to the bacterial cell membrane and then insert into the hydrophobic area of the cell membrane to form a pore, leading to efflux of intracellular materials and then cell death .The AMP namely Alamethicin is a representative of the “barrel-stave ”model. The toroidal model is a state that peptides and lipid head groups are lined with lipid monolayer bending continuously from the top to the bottom as found in magainin 2. Carpet mode is an action of peptides accumulation on the outer leaflets of bacteria bilayer, perturb the surface tension leading to pore formation or make defect in the membrane .For example, LL-37 is representative of carpet model (Huan, Kong, Mou, and Yi, 2020). Overall, the membrane disruptive of AMPs is achieved by reaching the cell membrane and then aggregating to a threshold value and inserting into the membrane to form pores (barrel-stave or toroidal mode) or by disruptive the membrane into micelle structures (carpet model).

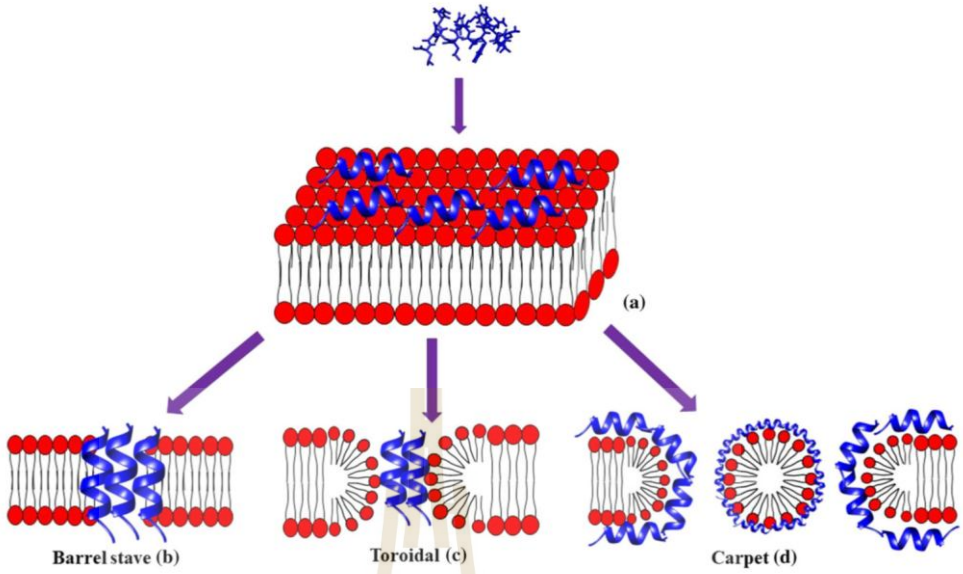

Fig. 2.1 Membrane broken mechanisms of AMPS (Manniello et al., 2021)

Nowadays, many evidence showed that mechanism of AMPs not only causes cell membrane damage, but also includes non-membrane disruptive mechanism (Fig. 2.2). AMPs can penetrate bacterial cell membrane without causing membrane disrupted, leading to bacterial death by interacting with intracellular such as, including DNA and proteins that involved in cellular division or protein synthesis (Manniello et al., 2021).

Although AMP-resistant bacteria have also emerged recently, it is difficult for bacteria to develop resistance to AMPs. First, AMPs mainly rely on interference with cell membranes and physical permeation to complete antimicrobial effects. It is difficult for bacteria to change their more conserved cell membrane structure to resistant AMPs .In addition, the AMPs also have multiple targets according to the characteristics of the bacteria, making it difficult for the bacteria to develop resistance in the short period of time (Lei et al., 2019)

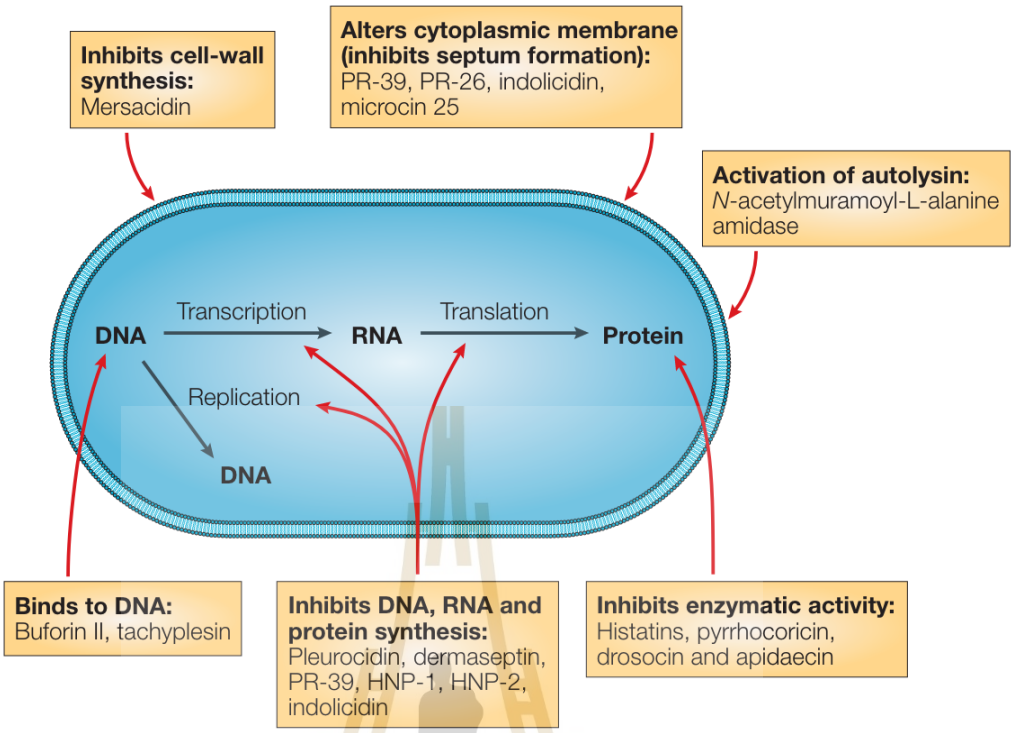

Fig. 2.2 Intracellular reaction of AMPs (Brogden, 2005).

2.2 Structural characteristics of AMPs

The strong antibacterial ability of AMPs is related to its structure. Many reports indicated that the specific amino acid residues, positive charge, hydrophobicity, and secondary structure of AMPs are important factors affecting their activity.

2.2.1 Charges of AMP

Although the composition and structure of AMPs from various source are different, they have some similarity in physicochemical properties. Most AMPs were relative small molecular weight, containing Lys, Arg or His which was positive charge(Arias, Piga, Hyndman, and Vogel, 2018). The positively charged amino acid residues allows AMPs to bind well to cell membrane, mainly due to the presence of negatively charged phospholipids in the membranes. This is prerequisite for the antimicrobial activity of AMPs (Lei et al., 2019). In addition, teichoic acid of Gram-positive bacteria and lipopolysaccharides of Gram-negative bacteria also contribute to the interaction with AMPs (Glattard, Salnikov, Aisenbrey, and Bechinger, 2016). Therefore, increasing the net positive charge of AMPs can improve the binding of the

AMPs to the bacterial membrane and increase the activity. However, excessive chares can disrupt the balance the structure and damage activity of the AMP (Mcphee and Hancock, 2005). Due to electrostatic action is necessary for binding of AMP to cell membrane, any factors that hiders the electrostatic adsorption of AMP would affect activity of AMPs. When free cations are present in the environment, they would compete with AMPs and bind to the bacterial surface, reducing the binding of AMPs to cell membrane, thus reducing the activity of the antimicrobial (Dong et al., 2012).

2.2.2 Hydrophobicity of AMPs

Hydrophobicity is another important factor that affects activity of AMPs. When AMPs are adsorbed on the cell membrane surface via electrostatic interaction, their hydrophobic parts are inserted into phospholipid bilayer and interact with the acyl chains, thus disrupting the cell membrane integrity (Schmidtchen, Pasupuleti, and Malmsten, 2014). However, once the threshold is exceeded, high hydrophobicity would promote self-polymerization in aqueous solution and reduce its penetration into the cell membrane, reducing its antibacterial activity of AMPs (Hädicke and Blume, 2016). In addition, hydrophobicity and cytotoxicity have been positively correlated (Wood et al., 2014). High hydrophobicity induces deeper insertion of AMP into hemoglobin, increasing its hemolytic properties.

2.2.3 Secondary structure of AMPs

Secondary structure of AMPs includes α-helical, β-sheet and random coil as detailed below, and some samples were showed in Table 2.1

2.2.3.1 α-Helical

According to the APD database (http://aps.unmc.edu/AP/main.pho). Natural α-Helical peptides represent the highest proportion of all identified AMPs. It is mainly derived from different species such as insects, fish, amphibians, mammals, and plants. Many studies have shown that α-helical peptides are converted into helical structures when it only contact membrane. This conversion leads to the spatial separation of hydrophilic and hydrophobic amino

acids, which are distributed on both sides of the helix axis to form an amphiphilic structure, which is important for membrane disruption (Uggerhøj et al., 2015).

Table 2.1 Representative AMPs with different secondary structures.

Categories	Special Representative	Sequence	Source	Reference
α-Helical	LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Human	(Dürr et al., 2006)
	Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	Bee	(Vlasak, Unger-Ullmann, Kreil, and Frischauf, 1983)
	Magainin-II	GIGKFLHSAGKFGKAFVGEIMNS	Frog	(Zasloff, 1987)
β-sheet	HNP-1	ACYCRIPACIAGGRRYGTCIYGGRKWAFCC	Human	(Liu and Schutte, 1998)
	Protegrin-1	RGGRLCYCRRRFCVCVGR	Pig	(Aumelas et al., 1996)
Random coil	Indolicidin	ILPWKWPWWPWRR	Cow	(Rozek, Friedrich, and Hancock, 2000)
	Tritrpticin	VRRFPWWWPFLRR	Pig	(Schibli, Nguyen, Kernaghan, Rekdal, and Vogel, 2006)
	PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	Pig	(Gudmundsson et al., 1995)

2.2.3.2 β-sheet

β-sheet is another major secondary structure of AMPs .β-Sheet of AMPs consist of 2-10 cysteine residues, forming 1-5 disulfide bonds to stabilize its conformation .For example, they become inactivated when Cys was replaced by acidic amino acids (Zhang et al., 2021). Unlike α-helical AMPs, which were a random coil structure in aqueous, β-sheet AMPs could be maintain a compact structure in aqueous solution (Ebenhan, Gheysens, Kruger, Zeevaart, and

Sathekge, 2014). Most β-sheet peptides play an important role in the immune system, as the not only protect against external pathogens, but also regulate the immune system.

2.2.3.3 Random coil

Most random coil AMPs were rich in Pro and Gly. Pro-rich AMPs were mostly found in mammals, and effective for Gram-negative bacteria, but not sensitive to Gram-positive bacteria. This may be due to the Gram-positive bacteria can secrete proteases to degrade Pro, thereby reducing the ability of Pro-rich AMPs. Gly-rich AMPs are mostly found in insects, and generally range in 8-30kDa (Zhang et al., 2021).

2.3 Other functions of AMPs

2.3.1 Antiviruses

Virus can infect all the organisms with cell and replicate themselves in the bodies. In addition to antibacterial activity, AMPs also had antiviral activity toward enveloped viruses. Such as, bovine AMP-13 can effectively inhibits the viral proliferation by disrupting viral protein synthesis and the viral gene expression in transmissible gastroenteritis virus (Liang et al., 2020). In addition, as one of the AMP found in human, LL-37 inhibits a variety of the enveloped virus, for example human immunodeficiency virus (HIV), influenza A virus (IAV), vaccinia virus (VV), HSV, dengue virus (DENV) and Zika virus (ZIKV) (Ahmed, Siman-Tov, Hall, Bhalla, and Narayanan, 2019). AMPs inhibit virus mainly by three ways: 1). Blocking the absorption and invasion of the virus to the host cell by interacting with specific virus receptor on the host cell membrane surface; 2). Destroying the virus envelope; 3).Altering the internal substance of the virus, inhibiting virus replication (Jung et al., 2019)

2.3.2 Anti fungus

More than 70,000 species of fungi have been identified .Among them, 399 species of fungi pose a great threat to human health (Victoria Castelli, Gabriel Derita, and Noelí López, 2017). There are many antifungal drugs available, but most of them target specific molecular receptors, easy to make fungi develop resistance and serious side effect on the body, especially on the liver (Rautenbach, Troskie,

and Vosloo, 2016). Therefore, the development of new antifungal drugs has become an another a hot issue for research. Many AMPs possess the function of antifungal. Madanchi et al. (2020) reported an AMP, namely AurH1, derived from aurein, which can inhibit *Candida albican*. Astacidin 1 isolated from hemocyanin of the freshwater crayfish *Pacifastacus leniusculus* was also showed good inhibition for *C.albican* (Choi and Lee, 2014). In addition, two chemically synthesized radish AMPs showed good inhibition against food spoilage yeast, such as *Zygosaccharomyces bailii* and *Z. rouxii* (Shwaiki, Arendt, and Lynch, 2020). Some mechanisms of antifungal AMPs have been proposed (1). Interfere with or inhibit the synthesis of fungal cell wall; (2) interact with fungal cell membrane and destroy membrane structure and function; (3) inhibit mitochondrial function and disrupt fungal respiration; (4) impact enzymes or nucleic or nucleic acids and other biological macromolecules of fungus and thereby impair their metabolic processes, such as induction of fungal autolysis (Dang et al., 2009).

2.3.3 Anticancer

AMPs are also effective on tumor cells. Due to the overexpression of various anionic substance)e.g., phosphatidylserne and O-glycosylated protein(, most tumor cell membranes surface has a negative charge, which provides the possibility for cationic AMPs to bind the surface of tumor cell membrane (Hoskin and Ramamoorthy, 2008; Mader and Hoskin, 2006). The AMPs exhibited anticancer properties by (1) recruiting immune cells (such as dendritic cells) to kill tumor cells, (2) causing tumor cells to necrotize or apoptosis, (3) inhibiting angiogenesis to eliminate tumor nutrition and prevent metastasis, and (4) interfere with the gene transcription and translation of tumor cells by activating certain regulatory functional proteins (Ma et al., 2020). Such as, LL-37, which inhibits tumor cells in colon cancer and gastric cancer. LL-37 induces apoptosis-inducing factor (AIF)/endonuclease G (Endo G) mediated apoptosis by activating the GPCR-p53-Bax/Bak/Bcl-2 signaling cascade in colon cancer cells (Piktel et al., 2016).

2.3.4 Antiparasites

Nowadays, parasitic disease are also one of the infectious diseases that have a great threat to the heath of livestock, poultry, and humans (Chalmers et al., 2020). As parasite resistance increases, thus dose the need for new therapies. Parasites are inhibited by AMP like cathelicidin and temporins-SHd (Abbassi et al., 2013).

Neshani et al. (2019) reported a marine synthetic AMP, namely Epi-1 can inhibit *Trichomonas vaginalis* by break its membrane. The mechanism of antiparasitic action of AMP is to act on the cell membrane surface of parasite, causing damage to the cell membrane and the outflow of intracellular ions and macromolecules, which eventually induces the death of the parasite (Mehta et al., 2014).

2.4 Sources of AMPs

2.4.1 Natural AMPs

Natural AMPs, also known as hot defense peptides, can be classified into three categories: microbial, plant and animal sources. The AMPs database (APD, https://aps.unmc.edu/) indicates that there are 3324 natural AMPs, 426 from microbial, 364 from plants, and 2446 from animals. Some of major natural AMPs are listed in Table 2.2

2.4.1.1 Microorganisms-derived AMPs

Microorganisms-derived AMPs are small molecular peptides, including cyclic peptides, glycopeptides and lipopeptides, which were secreted by microorganisms to protect themselves and kill or inhibit other bacteria and fungi (Huan et al., 2020). AMPs were secreted from bacteria, also known as bacteriocins, which are synthesized from the ribosomes of bacteria and are cationic antimicrobial peptides (Suganthi and Mohanasrinivasan, 2014). Bacteriocins can be classified into three types: Class I is lantibiotics which contain of 19-50 amino acids and undergo extensive post-translationally modifications to produce non-standard amino acids. Nisin is a most popular class I bacteriocin. Class II is small heat-stable, non-modified peptides, which molecular weight is less than 10kDa. Class III are large, heat-labile (> 10 kDa) bacteriocins, Helveticin M is one of the examples of class III bacteriocin produced by *Enterococcus faecium*, which disrupt the cell wall of Gram-positive bacteria and outer membrane of Gram-negative bacteria (Kumariya et al., 2019).

2.4.1.2 Insect-derived AMPs

Since the discovery of cecropin, more than 170 AMPs have been isolated and extracted from the insect (Toke, 2005). Insect-derived AMPs can be divided into 4 groups, 1). Cysteine-free AMPs, mainly representing cecropin, **α**-helical

Table 2.2 Some natural AMPs from different sources.

Origin	AMP	Structure	length	Charges	Source	target	Reference
Microbial	Nisin	Randomly coli	34	+4	*Lactococcus lactis*	G^+ bacteria	(Norouzi, Salimi, Halabian, and Fahimi, 2018)
	Gramicidin S	β-sheet	10	+2	*Bacillus brevis*	Broad spectrum	(Abraham et al., 2014)
	Plectasin	Helix and β-sheet	40	+1	Fungi	G^+ bacteria	(Mygind et al., 2005)
	Polymyxin b	Cyclic polypeptide	10	5	*Paenibacillus polymyxa*	G^- bacteria	(Polikanov, Aleksashin, Beckert, and Wilson, 2018)
	Thuricin CD	Heilx	30	-1	*Bacillus thuringiensis*	Clostridium difficile	(Rea et al., 2010)
Insect	Cecropin A	Helix	37	+7	Insect	Bacteria and Fungi	(Nguyen, Haney, and Vogel, 2011)
	Melittin	Helix	26	+7	Honebee	Broad spectrum	(Askari, Namaei, Ghazvini, and Hosseini, 2021)
	Pyrrhocoricin	β-sheet	20	+3	insect	*E.coli*	(Taniguchi et al., 2016)
	Crabrolin	Helix	13	+3	Hornet	G^+ and G^-	(Aschi et al., 2020)
	Ceratotoxin A	Helix	29	+6	Fly	G^+ and G^-	(Marchini, Giordano, Amons, Bernini, and Dallai, 1993)

Table 2.2 Some natural AMPs from different sources. (Continue)

Origin	AMP	Structure	length	Charges	Source	target	Reference
Animal	DCD-1	Helix	47	-2	Human	G^+ and G^-	(Steffen et al., 2006)
	Lactoferricin	Helix	49	+10	Human	Broad spectrum	(Chapple et al., 2004)
	Indolicidin	β-sheet	13	+13	bovine	G^+, G^- and fungi	(Rozek et al., 2000)
	AvBD2	β-sheet	36	+4	Chicken	G^+, G^-	(Lynn et al., 2004)(Lynn et al., 2004)
	SMAP-29	Helix	29	+9	Sheep	Bacteria, fungi	(Skerlavaj, Benincasa, Risso, Zanetti, and Gennaro, 1999)
	BMAP-27	Helix	26	+10	Bovine	Bacteria, cancer cell	(Yang et al., 2019)
	Chrysophsin-1	Helix	25	+6	Fish	G^+, G^-	(X. Wang, Zhu, Zhang, Yang, and Chen, 2012)
	PR-39	Rich in R and P	39	+11	pig	G^+, G^-	(Agerberth et al., 1996)
	Magainin II	Helix	23	+3	frog	Bacteria, fungi, protozoa	(Schäfer-Korting and Rolff, 2018)
	Buforin II	Helix	21	+6	Frog	Bacteria	(Cardoso et al., 2019)

Note: G+ = Gram-positive bacteria; G- = Gram-negative bacteria

structure, has strong activity against both Gram-positive and Gram-negative bacteria. 2). Cysteine-rich AMPs, such as the first insect defensins extract from *Sarcophaga peregrina*, which has strong activity against Gram-positive bacteria, but weak activity against negative bacteria (Matsuyama and Natori, 1988). 3). Proline-rich AMPs, mainly represented by apidaecins, which synthesized by the fat bodies of honeybees after infecting by pathogens, has activity for Gram-negative bacteria (Casteels, Ampe, Jacobs, Vaeck, and Tempst, 1989). 4). Glycine-rich AMPs, such as Attacins (Hultmark et al., 1983).

2.4.1.3 Plant-derived AMPs

AMPs have been isolated from various plants and plant organs such as stems, roots, seeds flowers and leaves. They exhibited effective bacterial activity against viruses, bacteria, fungi, parasites, and protozoa (Nawrot et al., 2014). The plant AMPs are mostly positively charged at physiological pH with molecular weights ranging from 2-10 kDa. The disulfide bonds in the plant AMPs which are composed of 4-12 cysteine residues stabilize their tertiary and quaternary structures and make the plant AMPs exceptionally stable to chemical, thermal, and enzymatic degradation. (Li, Hu, Jian, Xie, and Yang, 2021). Plant-derived AMPs can be divided into 8 major categories: thionis, plant defensins, hevein-like peptide, knottin-type peptide, α-hairpinin, lipid transfer protein, snakins, and cyclotide family (Li et al., 2021).

2.4.1.4 Animal-derived AMPs

a. Mammalian AMPs

In mammals, AMPs were found primarily within granules of neutrophils and in secretions from epithelial cells covering skin and mucosal surfaces (Hancock and Chapple, 1999). Cathelicidins and defensins are main families of mammalian AMPs. According to position of disulfide bonds, defensins can be divided into α-, β-, and θ-defensins (Reddy, Yedery, and Aranha, 2004; Skeate et al., 2020). Cathelicidins is another major mammalian AMPs is widely distributed in neutrophiles and released by protease hydrolysis for immunomodulatory activities, which found in human and other species. Such as LL-37, is usually detected in the skin of new born infants (Gschwandtner et al., 2014). Calthelicidins shared certain physicochemical

properties. Firstly, highly cationic with charge between +4 and +13. Secondly, cathelicidins in aqueous environments often have an unstructured structure, but when a membrane is present, they adopt an α-helical structure. Thirdly, some cathelicidin peptides are enriched in certain amino acids, such as tryptophan, or proline and arginine (porcine PR-39) (van Harten, van Woudenbergh, van Dijk, and Haagsman, 2018).

b. Chicken AMPs

The types and numbers of Chicken AMPs are relatively small. Most chicken AMPs are avian β-defensins (AvBD), also known as gallinacins. The AMPs from chicken are rich in arginine, lysine, and cysteine, and they share nine conserve residues, including the six cysteines that form the three-disulfide linkages, two glycines and one proline. Like the other β-defensins, chicken β-defensins also have broad-spectrum antimicrobial activity. When chicken was infected with Salmonella, the level of AvBD expression in the infected organs was increased (Michailidis, Avdi, and Argiriou, 2012). In addition, there defensins also showed active toward *E. coli*, *L. monocytogenes*, *S. typhimurium*, and *S. enteriditis* (Cheng et al., 2015; Yu, van der Linden, Sugiarto, and Anderson, 2010).

c. Amphibian-derived AMPs

Amphibian AMPs play an important role in protection of amphibians from pathogens (Rollins-Smith, 2009). Amphibian AMPs are mainly produced by frogs, and the most famous AMP from frogs is magainin; the skin secretions of frogs from genera *Xenopus*, *Silurana*, *Hymenochirus*, and *Pseudhymenochirus* under the Pipidae family are rich in AMPs (Conlon and Mechkarska, 2014; Mangoni and Casciaro, 2020). For amphibian-derived AMPs, although the sequence homology is low and the sequence variation is large, but their structures mostly belong to α-helical, and also have synergistic (Glattard et al., 2016).

2.4.2 Chemical modification AMPs

Due to several key drawbacks that limit the use of AMP as a clinical agent, such as high sensitivity to degradation by proteases and peptidases, toxicity when used systemically, and high production costs, (Breij et al., 2018; Mahlapuu et

al., 2016). therefore, there is a growing interest in designing new AMPs with enhanced antibacterial activity and fewer drawbacks based on naturally occurring AMP. (Han, Zhang, Lai, and Zhang, 2021). Higgs et al. (2007) reported that replacing Val with Arg at positions 33 and 39, or Ile and Thr at positions 12 and 38 were replaced with Arg. It was found that the increased inhibition activity was due to the increase in amount charge. Vasilchenko et al. (2017) reported that replacing all tryptophan residues on phenylalanine in D-form; the **α**-amino group in the main chain also modified by unsaturated fatty acid. Compared with indolicidin, this indolicidn-derived peptide more bactericidal, more resistant to proteinase K, and less toxic to mammalian cells. Most Natural AMPs have relatively long primary sequences, such as LL-37, well studied human cathelicaidin AMP, which has 37 amino acids (Duan et al., 2018). Long sequences are usually accompanied by high production costs, instability of proteases. Therefore, the minimally active or unwanted regions of a given AMP might first be identified and trimmed. Hao et al. (2009) reported that BF2-A, a derivative obtained by cut amino acid residues 5-21 at N-terminus of buforin 2, which has a 2-fold lower MIC than buforin 2. Additionally, the N-/C-terminal modifications may provide a simple but useful approach to strengthen the stability and effectiveness of AMP in vivo. Such as midation (C-terminal), acetylation (N-terminal), and methylation (N-terminal), which is generally applied to increase the resistance to peptidases and protease. (Brinckerhoff et al., 1999; de Breij et al., 2018; Rink et al., 2010). Although, Chemical modification can overcome the drawbacks of AMPs, but only small fraction peptides reached clinical trial phases (Table 2.3).

2.4.3 AMPs from protein hydrolysis

Hydrolysis protein with appropriate proteases was a method of AMPs. The enzymatic method is almost free of organic solvents and toxic chemical residues. Moreover, some protein lacking antimicrobial properties in their native conformation can be “activated ”by acquiring a different conformation or by enzymatic hydrolysis. After chymotrypsin hydrolysis of ovalbumin that is naturally without antimicrobial activity, the hydrolysate tends to have antimicrobial activity (Pellegrini, Hülsmeier, Hunziker, and Thomas, 2004). Bolscher et al. (2006) reported the production of antimicrobial peptides by enzymatic methods may be the most promising method for mass and low-cost production of AMPs.

Table 2.3 Clinical use and trail some AMPs.

Peptide name	Description	Target	Phase	Mechanism	length	charges	Reference
Nisin	lantibiotic	Gram-positive bacteria	approved	Depolarization of cell membrane	34	4	(Nayab et al., 2022)
Polymyxin B	Cyclic polypeptide	Gram-negative bacteria	approved	Membrane disruption	10	5	(Polikanov et al., 2018)
Daptomycin	lipopeptide	Gram-positive bacteria	approved	Membrane disruption	13	0	(Nayab et al., 2022)
Colistin	Cyclic polypeptide	Gram-negative bacteria	approved	Membrane disruption	10	5	(Couet et al., 2011)
Gramicidin	Polycyclic peptide	Gram-positive and negative bacteria	approved	Membrane disruption	16	0	(Gong et al., 2020)
D2A21	Synthetic peptide	Gram-positive and negative bacteria, fungi	III	Membrane disruption	23	9	(Chalekson, Neumeister, and Jaynes, 2002)
Pexiganan	Analog of magainin	Diabetic foot ulcers	III	Membrane disruption	22	9	(Rodríguez-Rojas et al., 2020)
Dusquetide	Analog of IDR-1	Oral mucositis	III	Immunomodulation	5	2	(Kudrimoti et al., 2016)

Table 2.3 Clinical use and trail some AMPs. (Continue)

Peptide name	Description	Target	Phase	Mechanism	length	charges	Reference
AB103	Peptide	Gram-negative	III	Immunomodulation	8	-1	NCT02469857
Surotomycin	Cyclic lipopeptide	C.difficile	III	Membrane disruption	13	-3	NCT01597505
OP-145	Derivative of LL-37	Middle ear infection	II	Membrane disruption	24	6	(Vila, Moreno-Morales, and Ballesté-Delpierre, 2020)
EA-230	oligopeptide	G- bacteria	II	Immunomodulation	4	0	(Hazam, Goyal, and Ramakrishnan, 2019)
LTX-109	Tripeptide	MRSA/G+	II	Membrane disruption	3	2	(Koo and Seo, 2019)
NP213	Cyclic peptide	Fungal infection	II	Membrane disruption	7	7	(Hara et al., 2008)
LL-37	Cathelicidin human	Bacteria, fungi,	II	Membrane disruption	37	6	(Overhage et al., 2008)
IDR-1	Bactenecin	MRSA	I	Membrane disruption	13	3	(J. Han, Jyoti, Song, and Jang, 2016)

2.5 Protein sources for AMPs

Food materials from plant and animals are potential sources for the isolation of AMPs. Isolation of peptide from food materials possess the benefits of natural availability, cost effectiveness and waste management. Some AMPs isolated from different source protein hydrolysates summary in Table 2.4

2.5.1 Dairy sources

Protein from milk is the primary source of bioactive peptides, which are released upon enzymatic hydrolysis during digestion or food processing (Théolier, Fliss, Jean, and Hammami, 2014). Milk protein (80 % casein and 20% whey) is a natural reservoir of bioactive peptides that exhibit an immune defense against server microbial infections and are used in formulation of potent drug with well-defined pharmaceutical effects (Khan, Pirzadeh, Förster, Shityakov, and Shariati, 2018). Studies have been proven that peptides are the potent inhibitor of pathogenic organisms.

2.5.1.1 Caseins

Casein is one of the most abundant sources of bioactive peptides. **K**-Casein is hydrolyzed by chymosin to product two peptides: (1) N-terminal **K**-casein *f* (1-105) and (2) hydrophilic and glycosylate C-terminal **K**-casein *f* (106-169) as also namely caseinomaropeptide (CMP) (Chen et al., 2021; Murakami, Lagarde, and Yuki, 1998). The glycosylated CMP and its derivative **K**-casein *f* (138-158) maybe responsible for the inhibition of adhesion of toxins and pathogens to the cell wall and protection against infection caused by *Streptococcus mutans*, *S. sanguis*, *Prophuromonas gingivlis* and *S. sobrinus*. In addition, trypsin digestion of **K**-casein resulted in hydrolysate inhibiting *S. aureus*, *E. coli* and *S. typhimurium* (Khan et al., 2018). Mudgil et al. (2022) reported that camel casein was hydrolyzed by Alcalase 2 h, the hydrolysate showed good activity toward *Candida albicans.*

2.5.1.2 Whey protein

Whey consists of major proteins including **α**-lactalbumin, glycomacorpeptide, **β**-lactoglobulin, protease peptone 3, immunoglobulins and serum albumin (Minj and Anand, 2020). Lactoferrin (Lf), is a glycoprotein, which consists of small fractions of milk protein. Arg and Try residues are rich in the Lf, that

Table 2.4 Antibacterial peptides derived from different source protein hydrolysates.

	Antimicrobial peptide	Protein source	Enzyme	Target bacteria	Reference
Milk	Isracidin *f* (1-23)	Bovine casein-α_{s1}	Chymosin	Gram positive and negative bacteria	(Hayes, Ross, Fitzgerald, Hill, and Stanton, 2006)
	Lactoferrin B *f* (18-36)	Lactoferrin	Pepsin and chymosin	Gram positive and negative bacteria	(Khan et al., 2018)
	Lactoferrin *f* (17-46)	Lactoferrin	Pepsin	*E.coli, L.monocytogenes,* viruses and fungi	
	Casecidins	Bovine casein-α_{s1}	Chymosin	Gram positive	(Haranahalli Nataraj, Naithani, Nagpal, and Behare, 2022)
	Casocidin-I *f* (150-188)	Bovine casein-α_{s2}	Trypsin	Gram-positive, negative, yeasts	
	Casocidin-I *f* (181-207)		Chymosin	Gram-positive, negative bacteria	
	Casocidin-I *f* (165-181)		Pepsin	Mainly Gram-positive bacteria	
	Kappacin *f* (106-169)	Bovine **K** casein	Pepsin	Mainly Gram-positive bacteria	(Mohanty et al., 2016)
	Kappacin *f* (42-49)		Chymosin	Gram-positive, negative	
	β-lactoglobulin *f* (15-20)	β-lactoglobulin	Trypsin	Gram-positive	(Pellegrini et al., 2001)(Pellegrini et al., 2001)
	β-lactoglobulin *f* (25-40)				
	β-lactoglobulin *f* (78-83)				

Table 2.4 Antibacterial peptides derived from different source protein hydrolysates. (Continue)

	VLSAADKGNVKAAWGKVGGHAAE VTLASHLPSDFTPAVSLDKFLANVSTVL	Bovine hemoglobin	Pepsin	*L. innocua*, *M. luteus*, *B. cereus*, *S. simulan*	(Daoud et al., 2005)
Meat	GFHI, DFHING, FHG, GLSDGEWQ	Bovine muscle	Thermolysin, proteinase K Alcalase	*L. monocytogenes, E. coli,* *S. typhimurium, B. cereus*， *P. aeruginosa and S. aureus*	(Jang, Jo, Kang, and Lee, 2008)
	Hydrolysates	Chicken liver	Alcalase	M. luteus	(Chakka, Elias, Jini, Sakhare, and Bhaskar, 2015)
		Porcine liver	Trypsin	*B. cereus, E. coli,* *L. monocytogenes, S. aureus*	(Verma, Chatli, Kumar, and Mehta, 2017)
		Sheep, deer, pig hemoglobin	Fungal protease	*E. coli, S. aureus and P. aeruginosa*	(Bah, Carne, McConnell, Mros, and Bekhit, 2016)

Table 2.4 Antibacterial peptides derived from different source protein hydrolysates. (Continue)

	YNHNF, WPTSFT, AVDRAV	Laba garlic	Pepsin and trypsin	*E. coli, S. aureus*	(Gao et al., 2019)
	HHRRFSLY, KFMPT, RRLFSDY	Cotton seed	Alcalase	*E. coli*	(Kong et al., 2020)(Kong et al., 2020)
Plant					
	LRRHASEGGHGPHW, EKLLGKQDKGVIIRA SSFSKGVQRAAF	Rice bran	Pepsin	*C.albicans, P. gingivalis*	(Taniguchi et al., 2017)(Taniguchi et al., 2017)
	Hydrolysates	Cowpea seed protein	Alcalase	Gram-positive and negavite bacteria	(Osman et al., 2021)
	CgPep 33	oyster	Alcalse/bromelin	Gram-positiv,Gram-negative, fungi	(Liu et al., 2008)
	GLPGPLGPAGPL	Mackerel	Protamex	*S. aureus*	(Ennaas et al., 2015)
marine					
	GLSRLFTALK	Anchovy cooking wastewater	Protamex	Gram-positive and Gram-negative	(Tang et al., 2015)
	Hydrolysate fraction	Half-fin acvhovy	pepsin	*E. coli*	(Song, Wei, Luo, and Wang, 2012)
		Shrimp head	alclase	*S. iniue, Y. rukeri*	(Rashidian et al., 2021)

facilitates interaction with cell (Farnaud and Evans, 2003). Bovine lactoferricin has 17-41 amino acid residues and is derived from the N-terminal of Lf, which generated by pepsin and exhibits antibacterial activity against Gram-positive and Gram-negative bacteria (Gruden and Poklar Ulrih, 2021). The bovine lactoferrin is hydrolyzed by trypsin, the peptide (< 5kDa) showed good inhibition of bacterial growth than the native lactoferrin (Lizzi et al. 2016). In addition, after trypsin digestion, four peptide fragment was generated from β-lactoglobulin, which inhibit gram-positive bacteria (Pellegrini, Dettling, Thomas, and Hunziker, 2001).

2.5.2 Meat and meat by-products

Meat is an important source of high-quality protein and play an important role in metabolism. Meat is becoming more interesting due to the economic and environmental benefits of using renewable animal by-products or by introducing innovative meat derived foods into human nutrition .AMPs can be generated from meat proteins by enzymatic hydrolysis. Several peptides with antimicrobial activity were isolated from blood by enzymatic hydrolysis. Antimicrobial activity of peptides derived from hemoglobin chain is, by far, the most studied. Such as Peptides TKAVEHLDDLPGALSELSDHKLRVDPVNFKLLSHSLL, LDDLPGALSE LSDLHAHKLR VDPVNFKLLSHSL, KLLSHSL and LLSHSL obtained from hydrolysis of bovine α-chain hemoglobin with pepsin, exhibited activity against *Kocuria luteus*, *Listeria innocua*, *E. coli* and *S. aureus* (Mora, Reig, and Toldrá, 2014). Kumari et al. (2022) used trypsin to hydrolysis the plasma of sheep, goat, and rabbit, all hydrolysates showed good activity against *E. coli*. Borrajo et al. (2020) reported flavoenzyme-hydrolyzed porcine liver showed antimicrobial activity toward Gram-positive and Gram-negative bacteria. In addition, chicken liver hydrolysates also showed moderate inhibition against *M.luteus*.

2.5.3 Marine

Marine organisms are a rich source of structurally diverse compounds with various biological activities, which adds great value to marine lives. (Boparai and Sharma, 2019). With marine species comprising approximately one half of the total global biodiversity, the sea provides a huge resource for novel compounds (Kim & Wijesekara, 2010). Ennaas et al. (2015) reported 4 antibacterial peptides (SIFIQRFTT,

RKSGDPLGR, AKPGDGAGSGPR, and GLPGPLGPAGPK) generated from by-product of Atlantic mackerel (*Scomber socmbrus*) showed activity against *S. aureus*. Bi et al. (2020) discovered an antibacterial peptide, namely Sm-A1 (GITDLRGMLKRLKKMK) from turbot (*Scophthalmus maximus*) that showed good activity toward both Gram-positive and Gram-negative bacteria. In addition, fraction of hydrolysate from shrimp (*Litopenaeus vannamei*) head wastes by Alcalase against *Sterptococcus iniae* (Rashidian, Abedian Kenari, and Nikkhah, 2021). Moreover, crustaceans, algae, and mollusks, such as oysters also show antimicrobial activity (Cunha and Pintado, 2022)

2.5.4 Egg

Egg, a functional food containing high biological value, has been studied for several bioactive peptides (Liu, Oey, Bremer, Carne, and Silcock, 2018). Tang et al. (2013) reported that AMPs obtained when ovalbumin was hydrolyzed by various enzymes (Flavourzyme, pepsin, trypsin and neutrase). AGLAPYKLKPIA that was obtained from the pepsin hydrolysate of ovotransferrin, inhibited both Gram-negative and Gram-positive bacteria (Ma et al., 2020). Al-Mohammadi et al. (2020) reported egg albumin was hydrolyzed by pepsin, resulting hydrolyzed egg albumin showed good antimicrobial ability. In addition, NTDGSTDYGILQINSR isolated from lysozyme hydrolysates by combination of papain and trypsin showed inhibitory effects on Gram-negative and Gram-positive bacteria (Memarpoor-Yazdi, Asoodeh, and Chamani, 2012). Although the egg white is the main line of defense against invading microorganism, several egg yolk components have also demonstrated antimicrobial activity, such as immunoglobulin (Ig)Y, egg yolk phosvitn (Mine and Kovacs-Nolan, 2005). Zambrowicz et al. (2012) reported that lecithin free egg yolk protein hydrolyzed by trypsin exhibited antimicrobial activity against *B. subtilis* and *B. cereus*. In addition, the combination hydrolysates of IgY and phosvitin effectively controlled enterotoxigenic *E. coli* (Gujral et al., 2017)

2.5.5 Plant

The antimicrobial activity of various plant-based peptide has been reported. Pu and Tang, (2017) reported that LVDHFPL, isolated from rice bran protein hydrolysate by bromelain, showed good antibacterial ability toward *L. monocytogenes*. Kong et al. (2020) reported three AMPs isolated from cotteenseed hydrolysate by Alcalase, which showed antibacterial activity toward *E. coli*. In

addition, Gao et al. (2019) reported AMPs against *E .coli* and *S. aureus*, generated from laba garlic protein hydrolysate by pepsin and trypsin.

2.6 Separation and purification of AMPs

Purification steps are necessary to screen AMPs in protein hydrolystae. Hydrolyzed protein exhibits different biological activities and physicochemical properties depending on their amino acid sequences, net charges, and molecular weights. Therefore, AMPs must be separated from protein hydrolysate based on their physicochemical properties to efficacy.

There have been different techniques used for the purification including gel filtration (GF), ion exchange chromatography (IE) and reversed phase high performance liquid chromatography (RP-HPLC). In GF, AMPs are fractionated based on their relative size. One of the most widely used column is Sephadex column. Besides, GF is also used to determine molecular weight (MW) of AMPs or remove low MW impurities (Sila et al., 2014). IE is widely used at the beginning of a purification scheme and is designed for the separation of ionizable compounds (Wang et al., 2015). IE is a separation technique, which depends on the interaction of the charge present on the surface of peptides and resin. It may be cationic or anionic exchange, depending on charges of peptides. Kong et al.(2020) used cationic exchange chromatography successfully separated AMP from cottonseed hydrolysates. RP-HPLC has been widely used for separation of AMPs with higher purify (Gao et al., 2019; Kong et al., 2020). In general, the higher hydrophobicity of AMPs may lead to enhanced membrane interference and selective cell damage (Nordahl et al., 2004),while a higher charge density may induce a strengthened electrostatic peptide-membrane interaction and reduce the toxicity to mammalian cells simultaneously (Kacprzyk et al., 2007)

2.7 Mechanism elucidation

2.7.1 Leakage of intracellular contents

By detecting the leakage of bacterial intracellular contents, the breakage of cell membrane can be quickly determined. These intracellular contents include

ions, ATP, DNA or RNA. Hou et al. (2019) used atomic absorption spectrometer to detect leakage of potassium ions, while ATP leakage was studied with an ATP kit, and based on the results of both methods, it was quickly determined that AMP influences *E. coli* cell membrane. In addition, DNA leakage was also used to determine cell membrane damage. When the bacterial membrane is damaged, DNA and RNA can be released from cell, which is easily detected at 260 mm. (Gao et al.,2019). Thus, these methods are useful to rapidly clarify the mechanism of action of AMP.

2.7.2 Membrane permeability assay

Membrane permeability test can be divided into inner membrane permeability and outer membrane permeability assay (Miao et al., 2016). Due to the lipopolysaccharide in the outer membrane of Gram-negative bacteria, some dyes cannot penetrate the intact outer membrane, but can easily across the damaged outer membrane and reach the cytoplasm (Vaara and Porro, 1996). The 1-*N*-phenyl-naphthylamine)NPN (was used to determine the permeability of the outer membranes. Fluorescence intensity is weak in aqueous solution but strong in hydrophobic environments (Wang et al., 2019).Thus, NPN can be used to detect the degree of damage to bacterial outer membrane by AMPs. For inner membrane permeability, it can be analyzed by β-galactosidase activity assay. Normally, β-galactosidase in the cell and released when the inner cell membrane is broken. When the added substrates *o*-nitrophenyl galactoside) ONPG (was cleaved by intracellular β-galactosidase, *o*-nitrophenol showed a significant absorbance at 405 nm, indicating that inner membrane permeabilization changes occur (Ma et al., 2020; Wang et al., 2019). In addition, propidium iodide (PI) also can be used to determined cell membrane permeability. PI is a membrane impermeable dye that stains nucleic acids in cell when cell membrane is destroyed. After stains with nucleic acid with higher fluorescent intensity. (Wang et al., 2019). Thus, the permeability of the cell membrane can be reflected by the intensity of the fluorescence signal.

2.7.3 Bacterial membrane depolarization assay

The bacterial membrane depolarization assay can be used to test the disruption of the cytoplasmic membrane. The membrane sensitive fluorescent dye used in this assay is diSC_3-5)3,3-'Dipropylthiadicarbocyanine iodide.(Since diSC_3-5 can

be taken up intracellularly by bacteria and concentrated on the cytoplasmic membrane, it leads to self-quenching of fluorescence .When the cell membrane is disrupted from polarization to depolarization, the $diSC_3$-5 is released into the extracellular medium or can be taken up into cytoplasm and form monomers with higher fluorescent intensity (Kim, Rajasekaran, and Shin, 2017)

2.7.4 Microscopy

2.7.4.1 Atomic force microscopy (AFM)

AFM is a sensitive technique that uses a cantilever at the tip to scan the sample surface and generate high spatial resolution. The force between the tip and the sample causes the cantilever to deflect, and the cantilever deflection is measured using a laser spot reflected onto a photodiode array to produce an image that reflects the change in surface height. (Swana, Nagarajan, and Camesano, 2021). AFM has been used to test change in the supported lipid membrane structure and thickness due to exposure to AMPs (Heath, Harrison, Strong, Evans, and Miller, 2018; Marín-Medina, Mescola, and Alessandrini, 2018). The structural transformation in lipid membranes exposed to AMP, namely protegrin-1 can be imaged using AFM and evidence of pore formation and worm-like micelles in the membrane can be seen (Lam et al. 2012). Theansungnoen et al. (2019) used AFM to image the effects of AMP (KT2) on *E. coli* and saw evidence of blisters or bubble on the surface. Furthermore, AFM can be used to assess the interaction between AMPs and DNA by observing their aggregation after incubation (Tang et al., 2009)

2.7.4.2 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) is a microscopy technique in which an image will be created with the electron beam transmitting through the sample. The sample is usually an ultra-thin slice less than 100 nm thick or a suspension on a grid. As the beam passes through the sample, the interaction of electrons with the sample will create an image. TEM has been frequently employed to study AMP effects on bacterial cells, since it offers unique insight into bacterial ultrastructure (Nicolas et al., 2019; Wang et al., 2019). TEM sample preparation requires embedded resin and ultrathin sections. Therefore, one limitation of TEM come into effect when using rod-shaped bacteria like *E. coli* or *B. cereus*, due to the random orientation of the bacteria in the resin, most of the cells are cross-sectional,

which makes it difficult to assess the AMP-induced phenotype. (Schäfer & Wenzel, 2020).This limitation was recently overcome by a flat embedding approach, resulting in mostly longitudinally sectioned cells (Wenzel et al., 2021)

2.7.4.3 Scanning electron microscope (SEM)

Unlike the Transmission Electron Microscope which uses transmitted electrons, the SEM uses emitted electrons. In the electron source the emitted electrons from a filament are collimated into a beam which is then focused on the sample surface through a set of lenses. The electrons interact with atoms in the sample, producing various signals that contain information about the surface topography. Thus, SEM provides information about the disruption of membrane integrity and change in cell morphology caused by AMPs rather than visualizing intracellular components. (Li et al., 2019). SEM has been successfully employed for antibiotic mode of action studies and is particularly interesting for AMPs which often cause cell surface defect. For example, Wang et al. (2019) using SEM to observed the *E. coli* treat with AMP, the pore formation on the cell surface.

2.7.5 Interaction with DNA

Some AMPs inhibit DNA synthesis as their antibacterial mechanism. The interaction of AMPs with DNA can be detected by gel electrophoresis, the electrophoretic migration of DNA bands is typically examined as a function of AMP concentration. When the rate of band migration is reduced or completely inhibited, this indicates the presence of interaction between the AMPs and DNA (Raheem and Straus, 2019). For example, the DNA binding activity of buforin II, an AMP isolated from Asian toad, was determined by agarose gel electrophoresis, leading to found that the mechanism of buforin II was affecting DNA, not membrane disruption (Park, Kim, and Kim, 1998). In addition, the interaction between bacterial DNA and peptides can also be detected by UV spectroscopy study. AMP can interact with DNA by binding to DNA base pairs via sliding of planar heteroaromatic groups. This combination usually leads to hypochromaticity on the UV-Vis absorption spectrum, and the more significant the hypochromaticity, the stronger the effect. When the double helix structure of DNA is damaged by AMP, it exhibited hyperchromicity, similarly, the more pronounced the hyperchromicity, the more severe the damage. (Zhang et al., 2021).

2.7.6 Synchrotron-based Fourier transform infrared (SR-FTIR)

Infrared spectroscopy has been used to characterize normal and cancerous tissues and cells since 1990 .It is one of the most used methods to study biological tissues and cells .Although the resolution of infrared microspectroscopy can reach diffraction limit, most current infrared microscopic spectrometers are equipped with traditional globar light source, whose heat source is only 1,000 ~ 1,500 K (Jamin et al., 1998), and whose brightness is weak, so when the test slit is reduced to less than 10 μm, its luminous flux is significantly reduced, seriously affecting the quality of infrared spectrum. Alternatively, synchrotron-based Fourier transform infrared) SR-FTIR (can provide a high signal-to-noise ratio) S/N (at ultraspatial resolutions to investigate molecular chemistry within the microstructures of samples (Wang et al., 2015). Synchrotron is a giant particle accelerator that turns electrons into extremely bright light, 100-1000 times brighter than that of a globar source (Wang et al., 2015).

All the main components of cells have conspicuous characteristic absorption peaks in the infrared .The main characteristic absorption of protein is the amide spectral band, Amide I)1600-1700 cm^{-1}, C=O stretch(; Amide II)1500-1600 cm^{-1}, C-N stretch(; Amide III)1200-1300 cm^{-1} .(In addition, the main characteristic absorption of lipid appears in 2959 cm^{-1})asymmetric C-H stretch of $-CH_3$(, 2922 cm^{-1})asymmetric C-H stretch of $-CH_2$ of fatty acids(; 2872 cm^{-1})symmetric C-H stretch of $-CH_3$ (2852 cm^{-1})symmetric C-H stretch of $-CH_2$ of fatty acids (and 1741cm^{-1})C=O stretch .(While the main characteristic absorption of nucleic acid appears in 1715 cm^{-1})C =O stretch of esters, RNA/DNA(; 1250-1220 cm^{-1})asymmetric P=O stretch of PO_2 (and 1085 cm^{-1} (Miller and Dumas, 2010).These targets can be chosen to monitor and analyze the cell changes caused by AMPs. Thus, SR-FTIR was widely used to study mode of action of antibiotic (Naksang et al., 2020; Thepbandit et al., 2021).

2.8 AMP from blood

Chicken blood from a slaughterhouse could be a promising source for producing antimicrobial peptides .Blood can be divided into two parts after centrifugation :plasma, the colorless part, and red blood cell, the red fraction .The

plasma contains high protein content, like thrombin, fibrinogen, immunoglobulin G or bovine serum-albumin (Bah, Bekhit, Carne, and McConnell, 2013). Red blood cell gives red color, representing 45 % of whole blood. Blood cell contains hemoglobin, a globular tetramer (64,500 Da) of four protoporphyrin groups, each including an iron atom in their center .This protein is broadly described as a rich source of bioactive peptides after enzymatic hydrolysis .As a byproduct, appropriate treatment of chicken blood is of great benefit to both environmental protection and economic development.

Hemoglobin is the main component of the erythrocytein function. In addition to its basic transporting oxygen, hemoglobin has been found to be a source of various biological peptides .Many antimicrobial peptides have been isolated from hemoglobin cleavage *in vivo* or from hemoglobin hydrolysis by chemical reagent, physical methods, or enzyme *in vitro*. Those antimicrobial peptides have multiple functions such as analgesic, antibiosis or promoter bacteria growth .Although some articles report that hemoglobin could promote bacteria growth, lots of research later suggest that some peptides also known as hemocidins are produced from chemical, physical or enzymolysis in *vitro* have antimicrobial activity (Beaubier et al., 2021; Hu et al., 2016; Karelin, Philippova, and Ivanov, 1995; Sanchez-Reinoso et al., 2021; Zhao, Piot, Gautier, and Cottenceau, 1996). Daoud et al. (2005) reported new peptides isolated from the hemoglobin hydrolysate treated by pepsin, corresponding to 107-136 fragment of the α chain of bovine hemoglobin .It exhibits antibacterial activity against *Micrococcus luteus* A270, *L. innocua*, *E. coli* and *S.* Enteritidis. AMPs isolated from chicken hemoglobin hydrolysates by papain exhibited rapid antimicrobial activity against 19 bacterial strains including 9 MRD bacterial strains (Hu et al., 2016). Bah et al. (2015) reported AMPs isolated from animal red blood cell hydrolysates deer, sheep, pig, and cattle (treated with fungal protease) FP400 and FPII were able to inhibit the growth of *E. coli*, *S. aureus* and *P. aeruginosa* .

Animal blood contains 60-70 %of plasma and 30-40 %of suspended red cells by weight. Composition and structure of human and animal plasma is exactly similar. Plasma protein is mainly composed of albumin, globulin, and fibrinogen, in which plasma albumin has the largest amount of 50 %. Globulin consists of 3 parts, α-, β -

and γ -globulin, accounting for about 23-27 .%Albumin is the earliest protein studied in plasma proteins and the molecule of albumin is a structure in which a peptide chain bends into a triple structure domain .About 48 %of the residues of albumin are arranged in α-helix and about 15 %are β-sheet .

Some bioactive peptides isolated from plasma such as angiotensin I-converting enzyme (ACE) inhibitory peptides isolate from porcine plasma (Aiemratchanee, Panyawechamontri, Phaophu, Reamtong, and Panbangred, 2021), antioxidant peptides isolated from duck plasma (Yang et al., 2020). However, few reports on antimicrobial activities of peptide produced from animal blood plasma hydrolysates. Thereby, plasma could be a potential source for production AMPs

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CHAPTER III

ISOLATION, IDENTIFICATION, AND MODE OF ACTION OF NOVEL ANTIBACTERIAL PEPTIDES FROM CHICKEN PLASMA HYDROLYSATES

3.1 Abstract

Two novel antibacterial peptides from chicken plasma hydrolysate were isolated and identified as VSDH and CCCPKAF. They showed effective antibacterial activity toward *Bacillus cereus* with varied modes of action. The peptide CCCPKAF caused cell membrane disintegration, as evidenced by propidium iodide (PI) uptake. In contrast, the peptide VSDH targeted intracellular molecules, including proteins and nucleic acids, as revealed by Synchrotron-based Fourier Transform Infrared (SR-FTIR). The secondary structure of intracellular proteins increased to a β-sheet structure concomitant with a decrease in the α-helix structure when exposed to 0.5 mM VSDH. Molecular docking analysis revealed that VSDH showed high binding affinity for the active sites of various enzymes involved in DNA synthesis. In addition, it showed good affinity for a chaperone protein (Dnak), resulting in misfolding of intracellular proteins. Nuclear magnetic resonance (NMR) and molecular dynamics simulations also indicated that VSDH chelated well with Mg^{2+}, which could partly contribute to its antibacterial activity.

Keywords: antibacterial peptides, SR-FTIR, molecular docking, NMR.

3.2 Introduction

The increasingly severe problems associated with multiple drug-resistant bacteria and the potential health risk from synthetic food preservative have led to a demand for new strategies for microbial control. Due to the single targets of traditional antibiotics, bacteria may develop tolerances through mutations and changes to antibiotic target site (von Wintersdorff et al., 2016). Antimicrobial peptides

(AMPs) have recently attracted much attention as they act on multiple targets on membrane and intracellular components, such as DNA, enzymes, or proteins (Brogden, 2005). Thus, mutations conferring AMP resistance are less likely to occur.

AMPs can be endogenously found in bacteria, insects, plants, and animals. Protein hydrolysate are another potential source of AMPs. The release of peptides upon hydrolysis by suitable protease(s) could result in peptides that function as AMPs. Pellegrini et al. (1999) reported that the hydrolysis of bovine α-lactalbumin by trypsin and chymotrypsin yielded three peptides with antibacterial activity toward Gram-positive bacteria, but the peptides were less effective against Gram-negative bacteria. The approach consisting of protein hydrolysis is more feasible than that of extracting endogenous AMPs.

Most AMPs reported thus far are cationic peptides containing 10-50 amino acid residues (Mahlapuu et al., 2016). Positively charged peptides readily bind with negatively charged cell membranes via electrostatic interactions, and the lipid region of the membrane is penetrated and disorganized by the hydrophobic residues of AMPs, causing destabilization of the cell membrane (Brogden, 2005). For this reason, the number of positive charges on peptides are used as an important indicator to screen potential AMPs. However, a few reports indicated that bacteria could introduce lysine into the cell membrane through MprF synthases to reduce the electronegativity of the cell membrane. Therefore, the probability of AMP binding to the cell membrane is reduced along with its antimicrobial activity (Nawrocki et al., 2014). The new features of cationic AMPs containing fewer positive charges or even anionic peptides should be explored.

The existing analytical tools for elucidating the mechanisms of AMPs include microscopy, flow cytometry and NMR (Li et al., 2021). The specific targets of AMPs cannot be clearly determined based on these techniques. Fourier transform infrared spectroscopy (FTIR) is an analytical technique that can detect the functional groups of compounds. The main components of bacterial cells have conspicuous characteristic absorption peaks in the infrared region (4000-400 cm^{-1}), such as peaks for lipids (3000-2800 cm^{-1}), proteins (1700-1500 cm^{-1}) and nucleic acids (1300-1100 cm^{-1}) (M. Wang et al., 2015). Traditional FTIR with a globar light source presents some limitations, particularly in terms of the diffraction effect of small areas of interest (Yu,

2004). Alternatively, synchrotron-based Fourier transform infrared (SR-FTIR) can provide a high signal-to-noise ratio (S/N) at ultraspatial resolutions to investigate molecular chemistry within the microstructures of samples. This technique has been widely used to investigate cellular changes in proteins, nucleic acids, and phospholipid components (Clède et al., 2013; Jamin et al., 2003). Although FT-IR can locate the cellular target components of AMPs, information on the specific target(s) responsible for inhibition is still missing. Molecular docking is another supporting tool that can reveal the plausible individual compounds that interact with AMPs ;details of the interactions can also be derived. The use of SR-FTIR in conjunction with molecular docking can provide useful insights into the potential cellular compounds that are targets of the AMPs of interest.

Chicken blood is a coproduct of slaughterhouses. Blood is a good source of nutrients, and it is considered a nonallergenic protein compared with soy and dairy proteins (Sorapukdee & Narunatsopanon, 2017). However, its consumption as food is limited in some cultures. Blood plasma is rich in proteins, including albumin, globulin, and fibronectin. Albumin and globulin have been reported to possess antibacterial properties (Arzumanyan et al., 2019). Discovering antimicrobial activity in chicken plasma hydrolysate would lead to the valorization of chicken blood.

The objective of this study was to investigate the antibacterial activities of peptides fractionated from chicken blood plasma hydrolysates. In addition, the mode of action of isolated peptides was systematically explored based on bacterial membrane integrity, cell damage and intracellular reactions. SR-FTIR and molecular docking were applied to gain more insights into the intracellular targets of antibacterial peptides. Finally, NMR and molecular dynamics simulations were applied to determine the metal chelating ability of the identified plasma AMP.

3.3 Materials and methods

3.3.1 Chicken plasma preparation

Fresh chicken blood was collected from a commercial slaughterhouse (Betagro, Lopburi, Thailand) with sodium citrate added to contain a final concentration of 4% to prevent clotting. Upon arrival, the samples were centrifuged at 2,000×g at 4℃ for 10 min to separate the plasma and blood cells. The plasma

fraction was dialyzed using a 3-kDa membrane against deionized water at 4°C for 18 h. The dialyzed sample was lyophilized, stored at -20°C, and used throughout the study.

3.3.2 Preparation of chicken plasma hydrolysates

The dialyzed plasma powder was dissolved in deionized water (100 mg/ml) and preincubated at 65.5°C for 10 min in a water bath. The pH of the plasma solution was adjusted to 9.6, and Alcalase 2.4 L (Novozymes Bagsvaerd, Denmark) was added at concentration of 4% (w/w) of the substrate. The pH was continuously adjusted to 9.6 using 1 M NaOH to maintain the optimal pH for the enzyme during the 6 h hydrolysis reaction. The reaction was terminated by heating the solution at 95°C for 10 min. Subsequently, the hydrolysates were rapidly cooled to 4°C in an ice bath, and the pH was adjusted to 7.0±0.1. Supernatants were collected by centrifuging at 10,000×g for 20 min and referred to as the chicken plasma hydrolysate (CPH) ,which was kept in a -80°C freezer throughout the study.

3.3.3 Isolation and identification of antibacterial peptides

One hundred microliters of the diluted CPH were loaded onto a Superdex peptide 10/300 GL column (GE Healthcare Bioscience Co., Uppsala, Sweden) equipped with fast protein liquid chromatography (FPLC; ÄKTA Pure 25, GE Healthcare Bioscience Co., Uppsala, Sweden). The mobile phase was DI water (A) and 30% ACN containing 0.1% TFA (v/v) (B). Stepwise elution at 0.8 ml/min was performed as follows: 20 ml, 2.5% B; 20 ml, 7.5% B; 20 ml, 12.5% B; 15 ml, 100%B , ml 35, .B %5.2 The peptides were detected at 214 nm and 280 nm. Each fraction was collected and lyophilized. Then, the inhibitory effects of these fractions on the growth of *Escherichia coli* and *Staphylococcus. aureus* was evaluated. The fraction with the strongest antibacterial inhibitory activity was further purified using a SOURCETM 5RPC ST 4.6/150 column (GE Healthcare Bioscience Co., Uppsala, Sweden). Peptides were eluted by mobile phase A (0.1% TFA in DI water, v/v) and mobile phase B (0.1% TFA in ACN, v/v). Stepwise elution at 0.2 ml/min was performed as follows: 5ml, 2% B; 5 ml, 10% B; 5 ml, 20% B; 5 ml, 30% B; 5 ml, 40% B; and 5 ml, 100% B and was adjusted to 2% B for 10 ml. The peptides were detected and tested as described above.

The fractionated peptide samples were injected into an Ultimate3000 Nano/Capillary LC System (Thermo Scientific, city, UK) coupled to a Hybrid quadrupole Q-Tof impact II™ (Bruker Daltonics, Bremen, Germany) equipped with a nanocaptive spray ion source. The peptides were enriched on a µ-Precolumn (300 µm i.d.×5 mm, C18 Pepmap 100; Thermo Scientific, Loughborough, UK) and separated with an Acclaim Pepmap RSLC C18 column (2 µm, 100 Å, nanoViper, 75 µm i.d.×15 cm; Thermo Scientific, Loughborough, UK). Mobile phases A and B containing 0.1% formic acid in water and 0. %1formic acid in 80% acetonitrile, respectively, were applied. A gradient of 5-55% solvent B was used at a constant flow rate 0.3 µl/min for 30 min. Electrospray ionization was performed using a Captive Spray at 1.6 kV. MS/MS spectra were obtained in positive-ion mode over the range (m/z) 150-1000 (Compass 1.9 software, Bruker Daltonics), and data were analyzed using PEAKS Studio 10.0 software (Waterloo, ON, Canada). Only peptides with "*de novo*" scores above 70% were selected for further analysis.

3.3.4 Antibacterial assay

The antibacterial activity of CPH, its fraction, and synthetic peptides were determined using a microbroth activity assay (Bah et al., 2015). Briefly, *S. aureus* ATCC 25923, *B. cereus* DMST 5040, *E. coli* ATCC 25992, *Salmonella* Typhimurium TISTR 292 were cultured at 37°C in trypticase soy broth (TSB) ,and pure colonies were obtained from trypticase soy agar (TSA). *Listeria* D 17303 *monocytogenes*were cultured at 37°C in TSB containing 0.6% yeast extract, and pure colonies were obtained from TSA containing 0.6% yeast extract. A single colony was diluted in Mueller Hinton Broth (MHB) broth to 1×10^5 CFU/ml. Fifty microliters of bacterial cultures were added to microtiter plate wells containing 50 µL of peptide samples and incubated at 37°C for 18 h. Turbidity was measured at 600 nm (Varioskan LUX Multimode Microplate Reader, Thermo Fisher Scientific, Waltham, MA, USA). Kanamycin at 20 ppm and DI water were prepared as positive and negative controls, respectively. The minimum inhibitory concentration (MIC) of peptides was determined. Briefly, twofold serial dilutions of each sample were incubated with each culture in a 96-well microplate at 37°C for 18 h. The lowest concentration of the peptide that showed growth inhibition as evaluated by the absorbance at 600 nm was determined to be the MIC.

3.3.5 DNA leakage

DNA leakage was analyzed by measuring the absorbance at 260 nm according to Gao et al. (2019) with some modifications. *B. cereus* in 10 mM PBS was adjusted to 1×10^6 CFU/ml. Then, 2×MIC of each synthetic peptide was added at a ratio of 1:1 (v/v) and incubated at 37°C. After time intervals of 0, 2, 4, and 6 h, the cell cultures were centrifuged at 3000 rpm, and the absorbance of the supernatant was measured at 260 nm using a μDropTM Plate A 2-3 (Thermo Scientific, USA) and a Varioskan LUX Multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). PBS at 10 mM was used as the negative control.

3.3.6 Scanning Electron Microscopy

The effect of synthetic peptides on morphology of *B.cereus* was investigated according to Wang et al. (2019) with some modification. Mid-logarithmic cells were treated with peptides at 1×MIC for 1 and 4 h. Subsequently, the cells were harvested and fixed with 2.5% glutaraldehyde at 4°C overnight and dehydrated with 20 % to 100 % acetone. The dried cells were coated with carbon. The specimens were observed at an accelerating voltage of 3 kV and at a magnification of 10,000 by SEM (ZWISS Gemini, Carl Zeiss, Germany).

3.3.7 Confocal laser scanning microscopy (CLSM) image analysis

B.cereus cells (3×10^8) in the mid-logarithmic phase were incubated with synthetic peptides at 1×MIC for 1 h. The cells were centrifuged at 1500×g for 5 min, washed 3 times with 10 mM PBS and resuspended with propidium iodide (PI) at a final concentration of 10 μg/mL in 10 mM PBS (pH 7.2). After incubation for 30 min at 4°C ,the unbound dye was removed by washing with excess PBS. Control cells were prepared in the absence of peptides. Then, 10 μL of the cell suspension was transferred onto a glass slide and observed using a Nikon A1Rsi CLSM at an excitation wavelength of 535 nm. Localization of the peptide targets followed Wang et al. (2019) with some modifications. *B. cereus* cells (3×10^8) were incubated with a FITC-labeled peptide at 1×MIC for 1 h. Then, the sample was centrifuged at 1,000×g for 10 min, washed 3 times with 10 mM PBS, and resuspended with PI (10 μg/ml) in PBS. After incubation for 30 min at 4°C, the unbound PI dye was removed by washing with excess PBS. The samples were smeared onto a glass slide and observed using a

Nikon A1Rsi CLSM at excitation wavelengths of 488 and 535 nm for FITC and PI, respectively.

3.3.8 Intracellular changes

3.3.8.1 SR-FITR Microspectroscopy

Bacterial cells were prepared following the method of Naksang et al. (2020) with some modifications. *B. cereus* cells were grown at 37°C in TSB with the addition of synthetic peptides at a final concentration of 1×MIC. The cells were taken at 0 and 4 h and centrifuged at 3000×g for 10 min. The cell pellets were washed 3 times with sterile 0.85% NaCl and DI water. The suspended culture was placed on a BaF_2 window, dried under vacuum for 2 h and stored in a desiccator to form a film before analysis. SR-FTIR microspectroscopy was carried out at the Beamline 4.1 instrument at the Synchrotron Light Research Institute (SLRI, Nakhon Ratchasima, Thailand). The FTIR spectra were obtained in a range of 800-3000 cm^{-1}. All spectra were smoothed, normalized, baseline corrected and calculated for the averages of the spectra using an OPUS 7.5 (Bruker, Ettlingen, Germany). Significant variations among data groups were identified by principal component analysis (PCA) in the spectral range of 3000–2800 and 1800–900 cm^{-1} using an Unscrambler 10.4 software (CAMO, Oslo, Norway). Data were processed by taking the second derivative using the Savitzky–Golay algorithm with seventeen points of smoothing to minimize the effects of variable baselines. The processed data were normalized with extended multiplicative signal correction (EMSC) to account for differences in sample thickness and correcting for scattering artifacts.

3.3.8.2 Gel retardation assay

The DNA extraction of *B.cereus* was carried out according to Neumann et al. (1992). The extracted DNA (700 ng) was mixed with various concentrations of synthetic peptides in 10 μL Tris-HCl buffer (10 mM, pH=8) and incubated at 37°C for 1 h. The ability of the peptides to bind with DNA was analyzed using agarose gel electrophoresis (1%). Gel retardation was visualized under UV illumination using a gel imaging system (Bio–Rad, Hercules, CA, USA).

3.3.8.3 Molecular docking studies

3.3.8.3.1 Construction of the homology model

Thymidylate kinase (TMK) ,thymidylate synthase (TS), dihydrofolate reductase (DHFR), and DNA gyrase subunits A and B were selected as targets for molecular docking because they are related to DNA synthesis. In addition, a chaperone protein (DnaK) was also selected because it is related to protein folding. Since the crystallographic structures of these proteins of B. cereus were not available, comparative modes were constructed using the I-TASSER server (Roy et al., 2010). The sequence for each protein was obtained from the UniProt database. The I-TASSER server generated five separate models from the FASTA input, and the top ranked model was selected based on the best available C-score. The C-score is the estimated global accuracy of the model, denoted as a value between [-5,2], with a score greater than -1.5 indicating a model of correct global topology (Yang and Zhang, 2015).

3.3.8.3.2 Docking

AutoDock Vina was used to predict binding conformations and affinities between the peptide (VSDH) and each enzyme. Docking simulations were performed according Trott & Olson, (2010). The structure of the VSDH was generated by PyMol 2.5. A grid box with dimensions of 50 Å × 50 Å × 50 Å was made to cover the entire binding pocket, including the active site with a specific grid spacing of 0.375 Å. The 3D structure picture file was prepared by PyMol 2.5. Vina scores were given as the predicted affinity of the VSDH to bind to each enzyme, which was calculated in kcal/mol.

3.3.8.4 Metal chelation

3.3.8.4.1 Effect metal ions on the growth of *B. cereus*

B. cereus was preincubated with VSDH (1×MIC) at 37°C for 18 h in MH broth. Turbidity was measured at 600 nm. Subsequently, 0.25 mM and 0.5 mM Mg^{2+} were added separately. The samples were further incubated for another 10 h, and turbidity was measured at 600 nm.

3.3.8.4.2 NMR

VSDH (20 mM) and $MgCl_2$ (20 mM) were mixed in DI water at 37°C for 30 min. The mixture was analyzed on a 500 MHz NMR spectrometer (Bruker AVANE III HD) with a CCP BBO 500 Cryoprobe at 25°C. Deuterated water was used as the solvent. ^{1}H and ^{13}C NMR spectra were collected at

frequencies of 500.363 and 125.816, respectively. The Bruker NMR software Topspin 3.5pl6 was used for data collection and data processing.

3.3.8.4.3 Molecular dynamics (MD) simulations

To verify the chelation mechanism, MD simulations were used. Briefly, the MD simulations were performed on a Yinfo Cloud Computing Platform (https//:cloud.yinfotek.com). The 3D structure of the peptide was generated by the platform tool and then used to manually construct the initial model of the peptide-metal complex. MD simulations were executed using an AmberTools20 package (Case et al.,2020) with the AMBER ff19SB (Tian et al., 2020) force filed. The system was solvated by a cubic water box using an OPC water model with a margin of 12 Å. Periodic boundary conditions (PBCs) were applied, and the net charge neutralized by Na^+ (or Cl^-). To remove improper atom contacts, the structure was first minimized by 2500 steps of steepest descent and 2500 steps of conjugate gradient, under a harmonic constraint of 10.0 kcal/(mol·Å2) on heavy atoms, then by 2500 steps of steepest descent and 2500 steps of conjugate gradient, under the same harmonic constraint on protein backbone atoms, and finally by 10000 steps of steepest descent and 10000 steps of conjugate gradient without constraint. After being fully minimized, the system was gradually heated to 300 K by a 20 ps NVT simulation. Subsequently, two-step equilibration phases were carried out: (1) a 200 ps NPT simulation with constraints on heavy atoms followed by (2) a 1000 ps NVT simulation without constraints. The temperature was maintained at 300 K using the Berendsen thermostat with a 1 ps coupling constant, and the pressure was maintained at 1 atm using a Monte Carlo barostat with a 1 ps relaxation time. Finally, the system was subjected to a 100 ns NVT simulation. The root-mean-square deviation (RMSD) was analyzed by the CPPTRAJ module

3.3.9 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software version 9, and statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test when more than 2 groups were compared. AT test was used when only 2 groups were compared.

3.4 Results and discussion

3.4.1 Effect of plasma hydrolysates on bacterial growth

CPH did not inhibit the growth of *E. coli* and *S. aureus*, which agreed with previous studies on plasma hydrolysates of cattle, sheep, deer, and pigs (Bah et al., 2015, 2016). After size-exclusion chromatography, 5 fractions were obtained, with peak B having the most effective antibacterial activity, with approximately 94% and 69% inhibition toward *S. aureus* and *E. coli*, respectively (Fig. 3.1 a, b). Peak D showed different trends, as it inhibited *S. aureus* but promoted the growth of *E. coli*. The plasma hydrolysates likely contained both nutrient peptides and antimicrobial peptides against *S. aureus* and *E. coli*. Based on these results, peak B was selected for further purification using an RPC column.

Four fractions were obtained after RPC (Fig.3.1 c). Only peak B-1 showed antibacterial ability toward *S. aureus* with 96.44% growth inhibition (Fig.3.1 d). Inhibition of *E. coli* was not found after RPC chromatography. Although multistep purification can result in higher purity, some active substances are lost (Tang et al., 2014). Based on these results, Peak B-1 was selected for peptide identification.

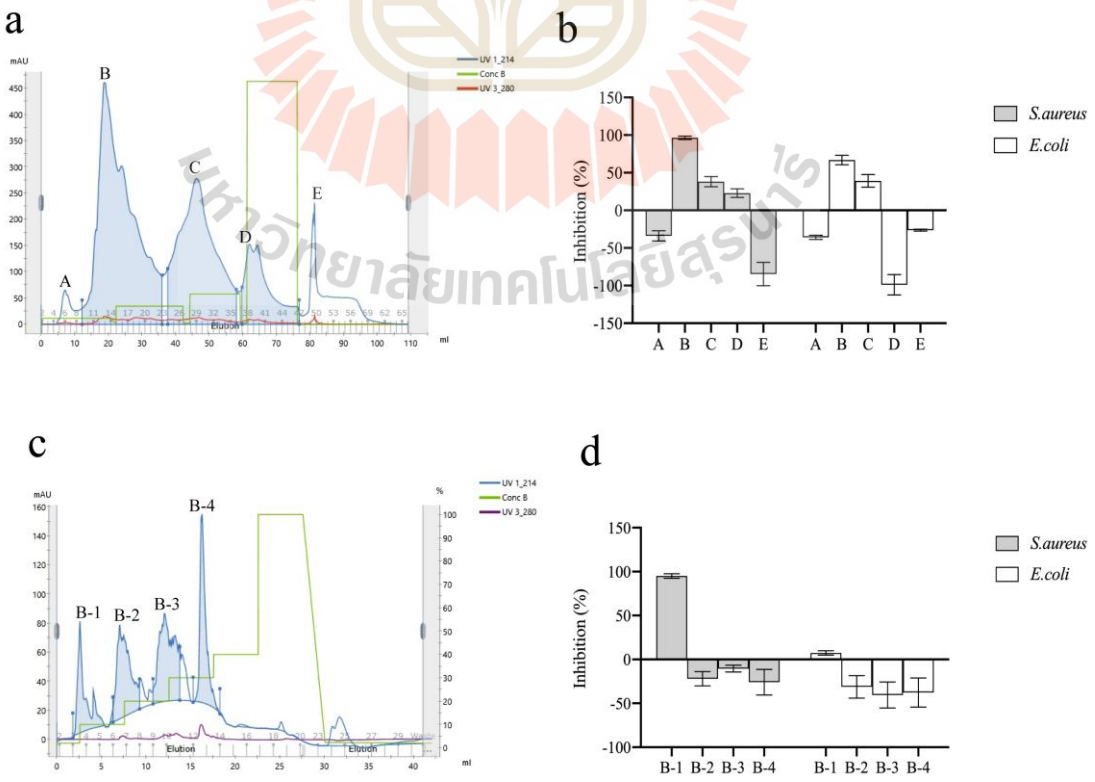


Fig. 3.1 a. Size exclusion chromatography of peptides from CPH; b. The antibacterial activities of different peptide fractions obtained by size-exclusion chromatography (4 mM *L*-Leu equivalents); c. RPC of peptides from Peak B; d. The antibacterial activities of different peptide fractions obtained by RPC (2 mM *L*-Leu equivalents). Data are presented as the mean±SD, n=3.

3.4.2 Identification of antibacterial peptides

A total of 7 peptides were identified in peak B-1, and their characteristics are summarized in Table 3.1. The MS/MS spectra of these peptides are displayed in Fig. 3.2. All identified peptides showed positively charged and hydrophobic residues, which have been reported to play role in antibacterial activity (Dashper et al., 2007). These peptides were considered novel peptides based on the antibacterial peptide database (http//:aps.unmc.edu). Among the seven peptides, VSDH and CCCPKAF showed good antibacterial activity (Fig.3.3). The MIC values of VSDH and CCCPKAF against *B. cereus* were 0.5 mM and 0.25 mM, respectively. In addition, MIC of VSDH against *S. aureus* was 0.5 mM.

Most of antibacterial peptides were amphiphilic and positively charged with net charges of +2 to +9. Peptides with a greater number of positive charges can promote better interaction with the cell membrane, resulting in membrane disruption (Brogden, 2005). The peptides (VSDH and CCCPKAF) identified in our study contained K and R, with net charges of 0 and +1, respectively, and showed good antibacterial ability against *B. cereus*. The smaller and more amphiphilic peptides can diffuse through the membrane to exert greater bacteriostatic effects (Wang et al., 2019). This might explain why the peptide VSDH with a relatively smaller mass exhibited good inhibitory activity. For the peptide CCCPKAF, the positive charge (+1) may contribute to the interaction with the negatively charged bacterial membrane. In addition, the cysteine (C) and proline (P) in CCCPKAF might contribute to its stronger antibacterial activity. An important role of the C residue is to form disulfide bonds to stabilize the β-hairpin or sheet structure (Bastos et al., 2018). In addition, the P residue is conducive to the formation of a linear structural conformation for AMPs and increases membrane permeability (Graf et al., 2017). Our peptides did not show antimicrobial activity against Gram-negative bacteria. AMPs specific for

Gram-negative bacteria must adsorb onto the lipopolysaccharide on the outer membranes and penetrate through the cytoplasmic membrane to induce membrane disintegration. Such peptides typically contain both positive charges and hydrophobic characteristics. Such peptides typically contain both positive charges and hydrophobic characteristics (Lei et al., 2019). Our results suggested that peptides with small molecular masses and fewer positively charged residues also exhibited antimicrobial properties and might possess different antimicrobial mechanisms from those of classic AMPs.

Table 3.1 Characteristics of antibacterial peptides of CPH identified by LC-MS/MS.

Peptide	MS (Da)	ALC (%)	Net charge	Parent protein
VSDH	457.17	75	0	Protein transport protein Sec31A
LLSR	488.30	84	1	SCO-spondin
VHVSSG	585.33	75	1	SCO-spondin
NATR	461.28	83	1	SCO-spondin
CCCPKAF	771.30	70	1	Immunoglobulin-like domain-containing receptor 2 isoform X1
HLKAA	539.27	75	2	Iron-dependent oxygenase
VQARSH	697.38	70	2	Cyclin-J isoform X1

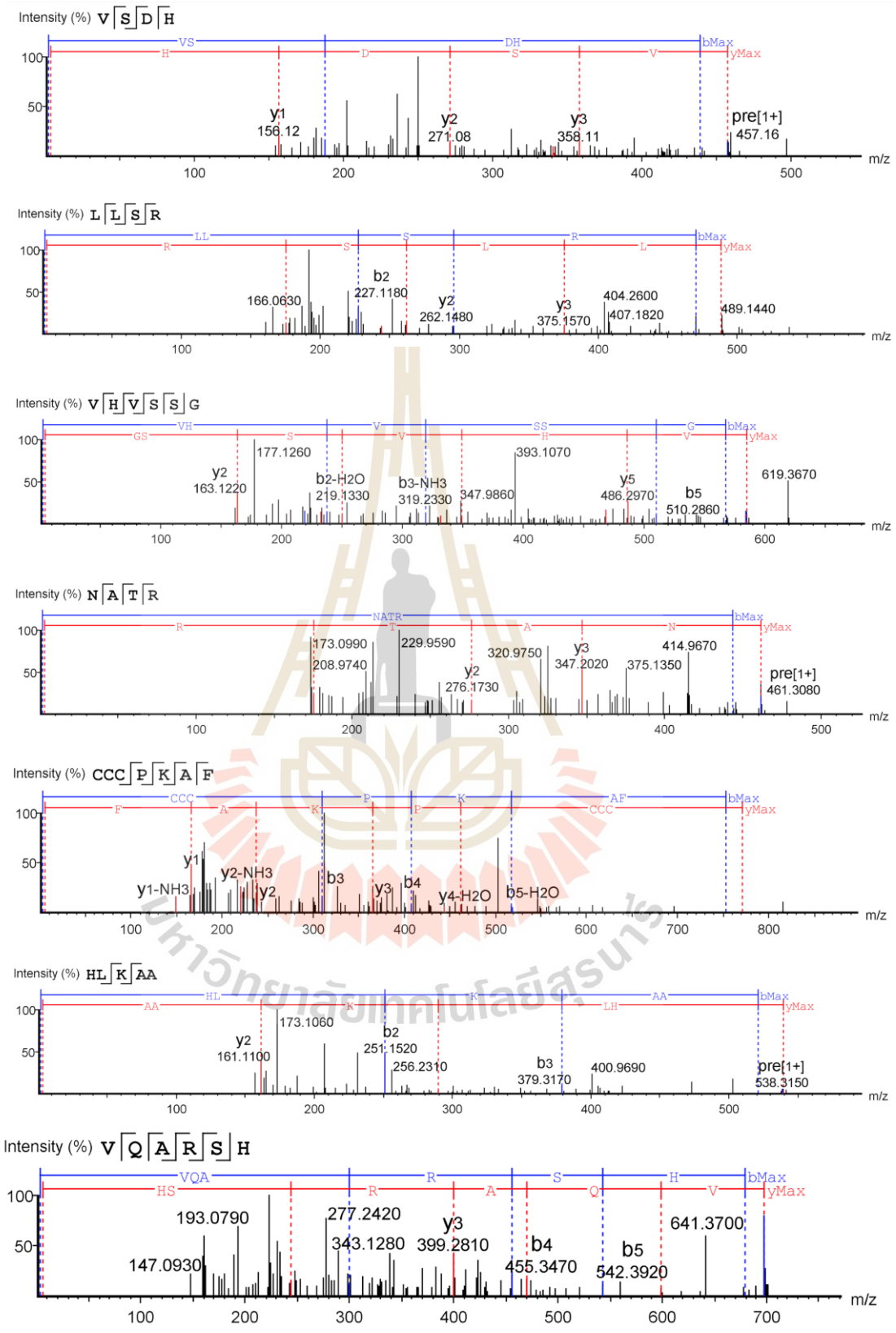


Fig. 3.2 MS/MS spectra of seven peptides in peak B-1 identified by LC–MS/MS.

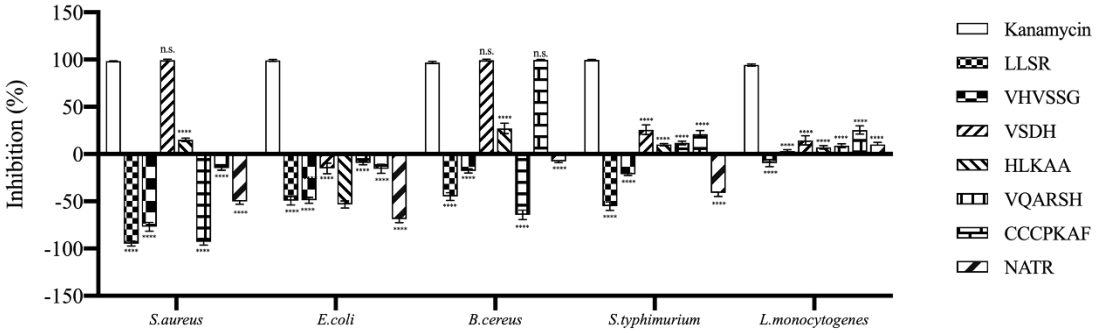


Fig 3.3 Antibacterial activities of various synthetic peptides derived from CPH at 2 mM. Data are presented as the mean±SD, n=3; ****p < 0.0001, n.s.: not significant (p > 0.05, compared with kanamycin)

3.4.3 Mechanisms of antibacterial activity

3.4.3.1 Leakage of nucleotides

When the bacterial membrane is damaged, DNA or RNA can be released from cells, which can be detected at 260 mm. The cells treated with CCCPKAF showed a drastic increase in absorbance during the first 2 h of exposure and remained stable afterward (Fig.3.4). However, VSDH did not induce any leakage of nucleotides. As a shorter peptide, VSDH was unlikely to cause cell membrane damage.

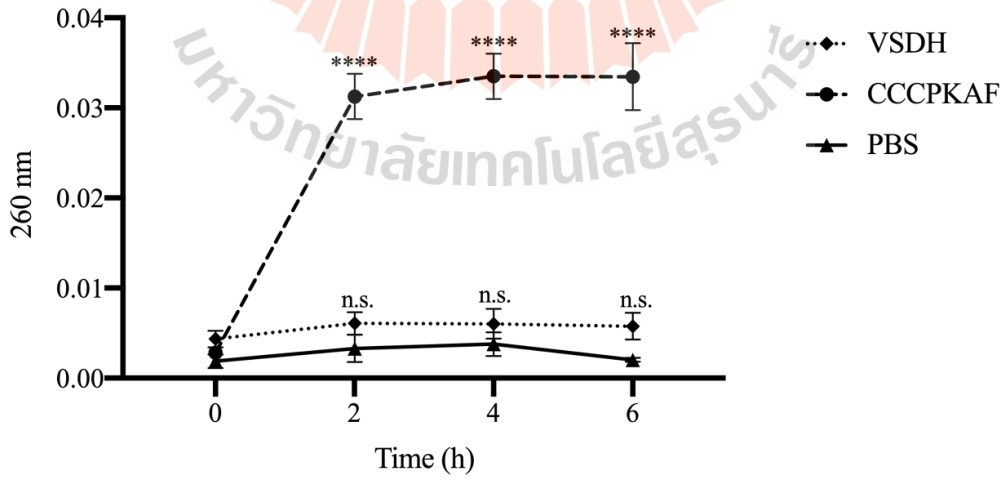


Fig.3.4 Effect of VSDH and CCCPKAF on DNA leakages from *B. cereus*. Data are presented as the mean±SD, n=3; ****p < 0.0001; n.s.: not significant at p > 0.05 compared with PBS.

3.4.3.2 Pore formation

In the control group (without peptide treatment), *B. cereus* showed typical cell morphological characteristics with regular rod shapes and smooth surfaces (Fig.3.5 a and b). After treatment with VSDH for 1 h and 4 h, deformations and wrinkles on the cellular surface were clearly observed (Fig.3.5 c and d). It should be noted that holes were not observed. These results suggested that VSDH penetrated the bacterial cell membranes without severely compromising their integrity. Miao et al. (2016) identified a dipeptide containing leucine and tyrosine connected with two phosphate molecules from kefir, which inhibited the growth of *E. coli* and *S. aureus* without causing severe cell membrane damage. When treated with CCCPKAF for 1 h, *B. cereus* cells exhibited significant alterations with noticeable holes and deformed cell structures (Fig.3.5 e). Prolonged exposure over 4 h resulted in cell collapse (Fig.3.5 f). These cell abnormalities indicated that CCCPKAF might have caused disintegration of the cell membranes, leading to cell death. Gao et al. (2019) also observed that incubation of *E. coli* and *S. aureus* with peptides isolated from laba garlic caused morphological changes. These results indicated that VSDH and CCCPKAF affected cell membranes in varied fashion.

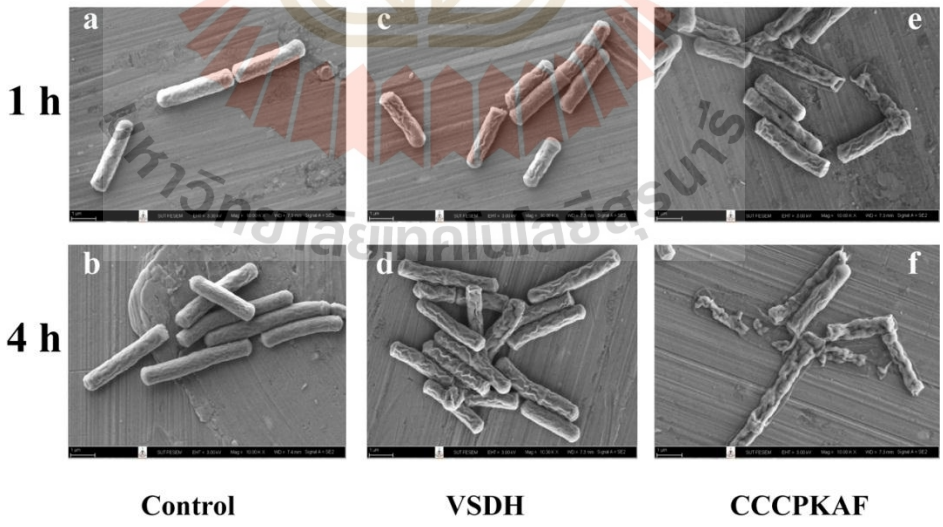
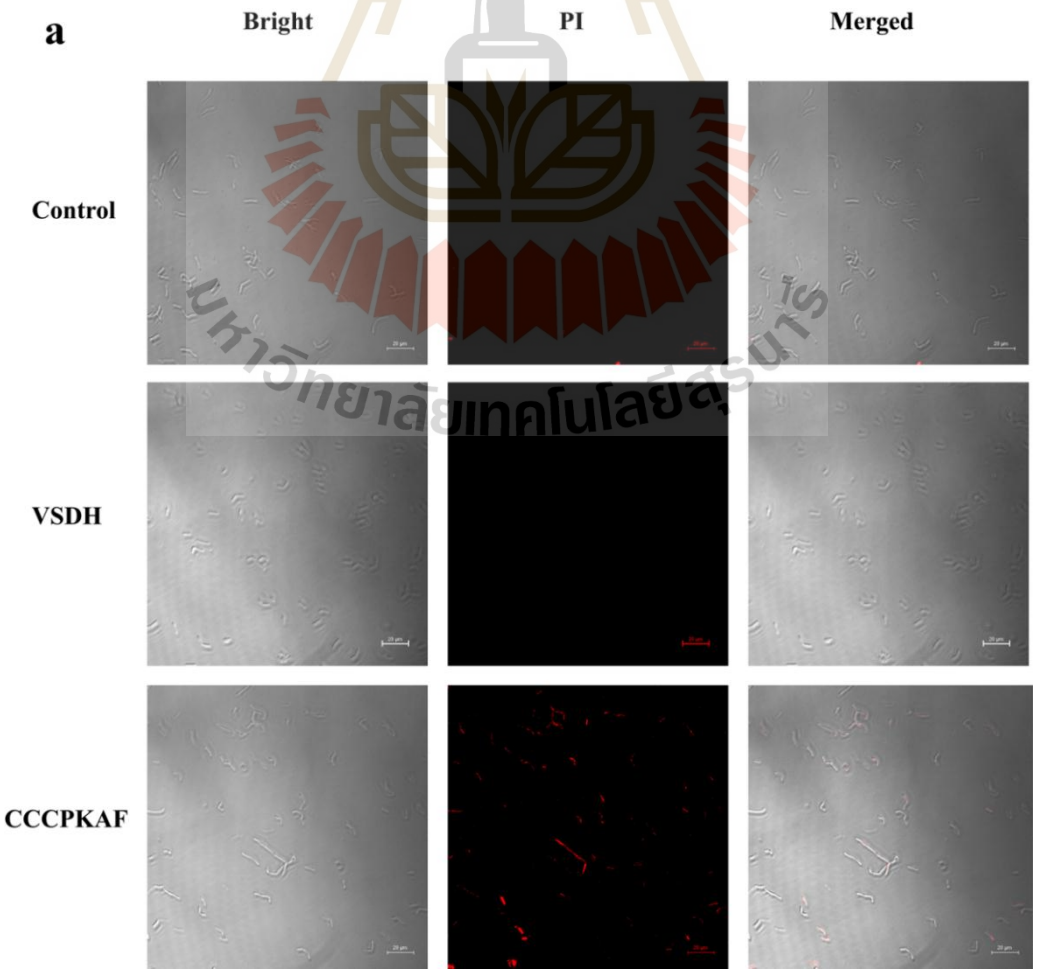


Fig. 3.5 Morphological changes of *B. cereus* treated with VSDH and CCCPKAF (1×MIC) observed by scanning electron microscopy: Control 1 and 4 h (a and b); VSDH 1 and 4 h (c and d); CCCPKAF 1 and 4 h (e and f).

3.4.3.3 Cell membrane disintegration

To investigate the membrane-perturbing activity of VSDH and CCCPKAF, CLSM was applied (Fig.3.6 a). PI is a membrane impermeable dye that stains nucleic acids in cells when the cell membrane is destroyed (Wang et al., 2019). The control (cells without peptide) displayed no appreciable fluorescent signal, indicating limited PI uptake. In contrast, cells emitted red fluorescence after treatment with CCCPKAF (1×MIC) for 1 h, confirming that CCCPKAF destroyed the integrity of the cell membrane. However, cells treated with VSDH for 1 h did not show an obvious red fluorescent signal, suggesting that the cell membrane integrity was intact after 1 h of exposure. This result indicated that VSDH penetrated cells and caused a bactericidal effect. The FITC-labeled VSDH also demonstrated that VSDH was localized in the cytoplasm without a distinct PI fluorescent signal (Fig.3.6 b). These results confirmed that VSDH was absorbed intracellularly without disturbing the integrity of the cell membranes.



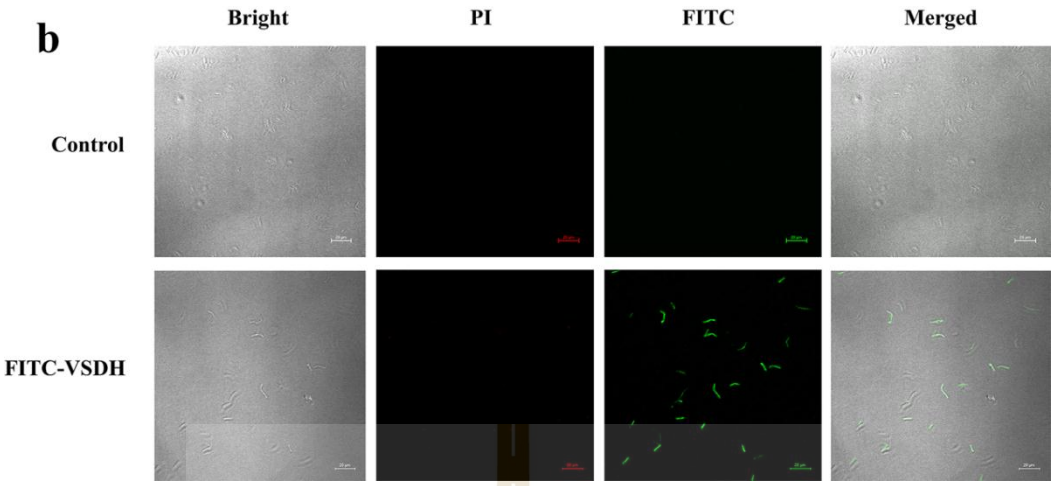


Fig. 3.6 CLSM images of *B. cereus* treated with chicken blood plasma peptides: a. VSDH and CCCPKAF; and b. FTIC-VSDH.

3.4.3.4 Interactions with intracellular components

The effect of VSDH on the intracellular components of *B. cereus* was further investigated using Synchrotron-based FTIR microspectroscopy (SR-FTIR). The FT-IR spectra for cells can be divided into 3 regions, namely, cellular fatty acids (3000-2800 cm^{-1}), the amide groups of proteins and peptides (1700-1500 cm^{-1}), and the phosphate group asymmetric stretching of nucleic acids (1350-1000 cm^{-1}) (Wang et al., 2015). After VSDH exposure for up to 4 h, changes in the intracellular components of *B.cereus* were evident (Fig.3.7 a). In Fig.3.7 b, peak shifts were noticed in the amide I (1654 cm^{-1} and 1630 cm^{-1}), α-helix (1654 cm^{-1}) and β-sheet (1630 cm^{-1}) structures (Barth & Zscherp, 2002). Among them, the peak at 1630 cm^{-1} was slightly higher after 4 h of treatment, indicating the unfolding of intracellular proteins to more β-sheet structures. A peak shift was also observed at 1240 cm^{-1} representing the phosphate group (P=O) asymmetric stretching of the phosphodiester bond of nucleic acids. These changes suggested that VSDH is likely an intracellular protein and a nucleic acid of *B. cereus*.

The PCA score plot of SR-FTIR demonstrated a clear separation between samples exposed to the peptide for 0 and 4 h (Fig.3.7 c). According to a loading plot (Fig.3.7 d), the separation was found to be associated with amide I (1674, 1650 and 1630 cm^{-1}) and nucleic acids (1240 cm^{-1}). The results from SR-FTIR

spectroscopy agreed with those of CLSM and SEM, which indicated that the VSDH passed through the membrane without significantly disturbing its integrity. VSDH mainly altered intracellular proteins and nucleotides, which might be one of the major causes of its antimicrobial activity.

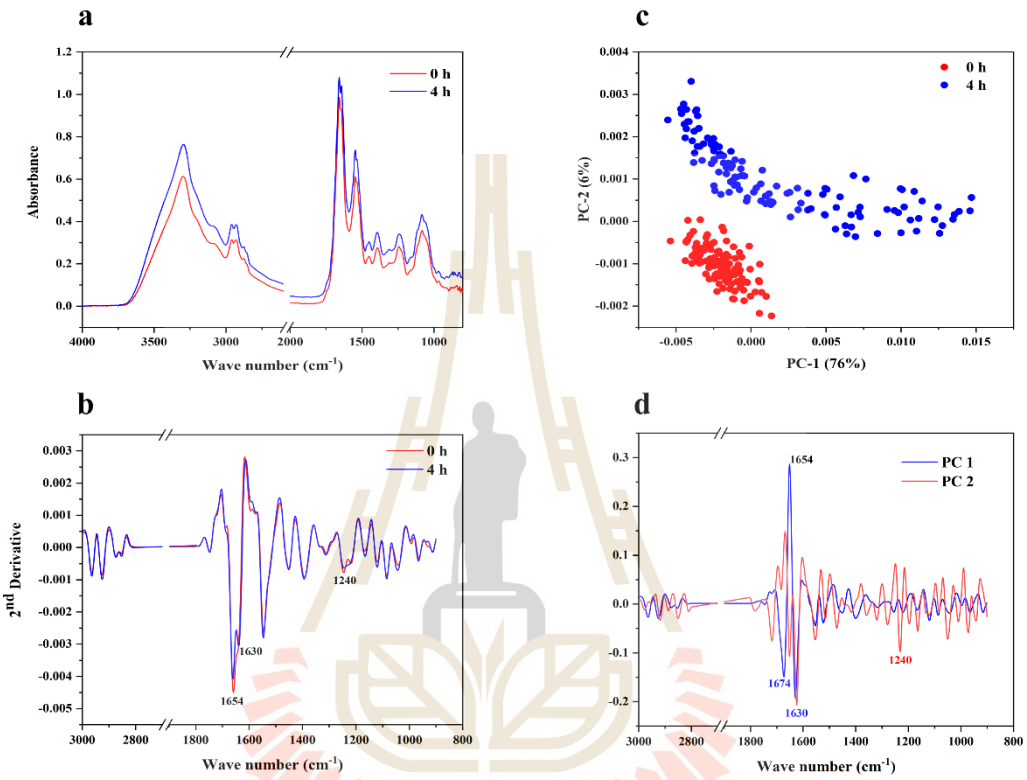


Fig. 3.7 SR-FTIR analysis of *B. cereus* treated with VSDH at 4 h. a. Representative average IR absorbance; b. Second derivatives of FT-IR spectra; c. PCA scores plot; d. their corresponding loadings plots.

3.4.3.5 DNA binding

Based on SR-FTIR, notable changes in nucleic acids were observed by VSDH treatment. Based on the in vitro tests, VSDH did not cause any changes in DNA migration at concentrations up to 4×MIC (data not shown), indicating a lack of peptide-DNA binding. The peptide VSDH contains a negatively charged amino acid (D), which hampers binding to negatively charged DNA. Thus, VSDH might interfere with DNA synthesis rather than exerting an effect through direct binding.

3.4.3.6 Molecular docking

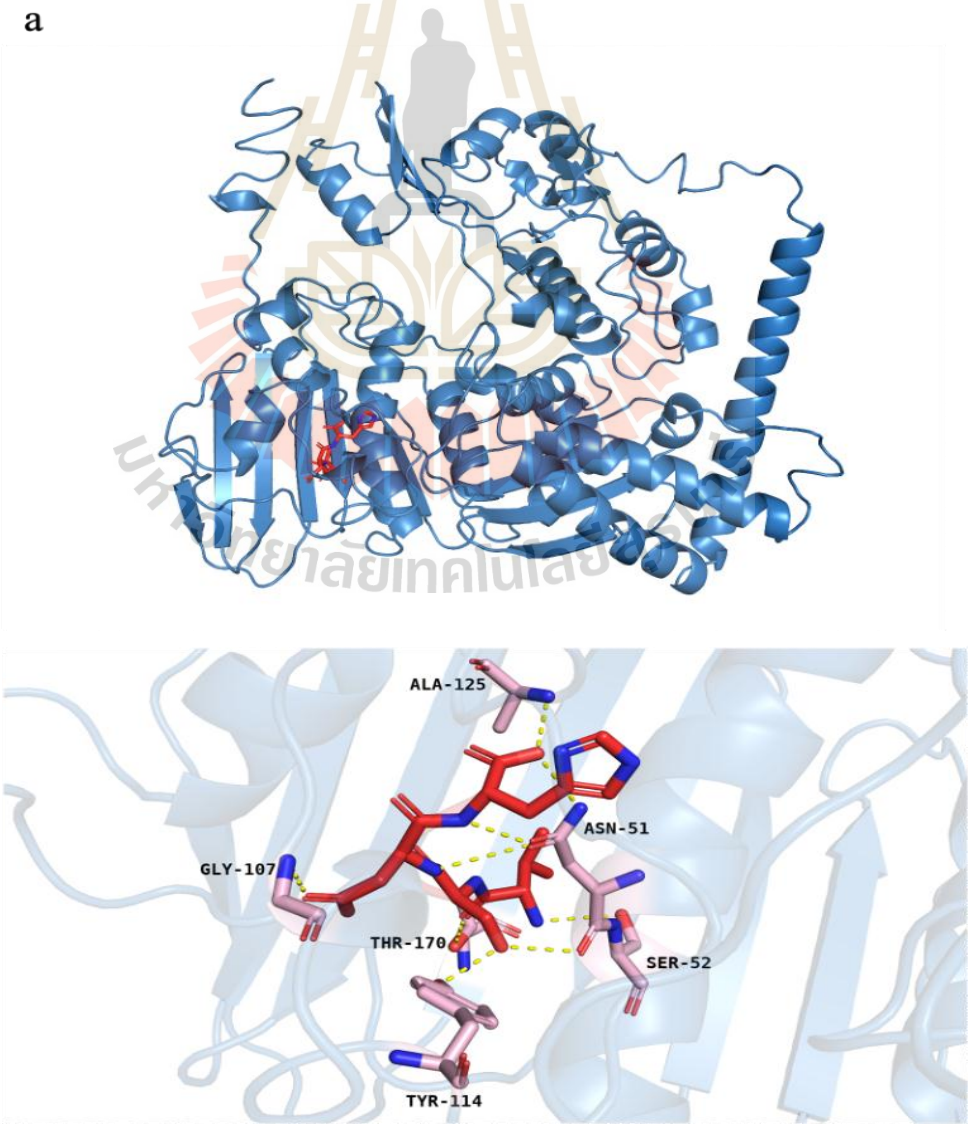
To gain more insights into how DNA interferes with VSDH, molecular docking was applied to elucidate interactions between the peptide and essential enzymes related to DNA synthesis, namely, thymidylare kinase (TMK), thymidylate synthase (TS), dihydrofolate reductase (DHFR), DNA gyrase subunit A and subunit B. Among the enzymes tested, the DNA gyrase subunit B showed the greatest peptide binding affinity of -8.4 kcal/mol (Table 3.2). The binding model suggested that binding between VSDH and the DNA gyrase subunit B occurred via hydrogen bonds involving Gly 107, Thr 170, Tyr 114, Ser 52, Asn 51 and Ala 125 in the binding pocket (Fig.3.8 a). DNA gyrase plays a key role in the process of DNA replication, transcription, and chromosome separation. DNA gyrase helps alleviate torsional tension by introducing negative supercoils to DNA molecules during DNA replication (Ashley et al., 2017). Thangaraj et al. (2018) also reported that quinoline peptides strongly interacted with DNA gyrase inhibiting the growth of *B. cereus*. In addition, DHFR is an enzyme catalyzing the reduction of dihydrofolate to tetrahydrofolate, thereby promoting thymidylate biosynthesis and improving DNA translation, RNA transcription and protein replication (He et al., 2020). Thus, it is vital for controlling cell proliferation. Based on molecular docking, VSDH showed good binding affinity to DHFR (Table 3.2), which would inhibit the enzyme activity and ultimately limit cell growth. DHFR inhibitors have been proven to be effective agents for treating bacterial infections, including those cause by Mycobacterium tuberculosis, *S. aureus*, and *E. coli* (He et al., 2020). Our results indicated that DNA gyrase and DHFR could be major targets for VSDH, interfering with DNA and protein synthesis, as evidenced by the SR-FTIR spectra.

Based on the SR-FTIR spectra, the alteration of intracellular proteins was evident after VSDH treatment. Kragol et al.(2001) reported that pyrrocoricin bound to the bacterial heat shock protein DnaK and affected chaperone-assisted protein folding, resulting in the growth inhibition of *E. coli*. Thus, the interaction between DnaK of *B. cereus* and VSDH was elucidated. The docking score for VSDH and DnaK was -9.2 kcal/mol, indicating good binding affinity. VSDH formed more hydrogen bonds with amino acid residues, including Asp 168, Asp 8, Asn 13, Thr 12, Thr 11, Gly 171 and Gly 312 (Fig.3.8 b). These results implied that VSDH also induced

protein misfolding, rendering changes in protein functions and ultimately inhibiting growth. It can be seen from the docking results that VSDH interfered with both DNA and protein synthesis in *B. cereus*.

Table 3.2 Docking energy of various enzymes related to DNA synthesis.

Receptor	Docking energy (kcal/mol)
DNA gyrase subunit B	-8.4
DHFR	-7.7
TS	-7.1
DNA gyrase subunit A	-6.7
TMK	-6.4



b

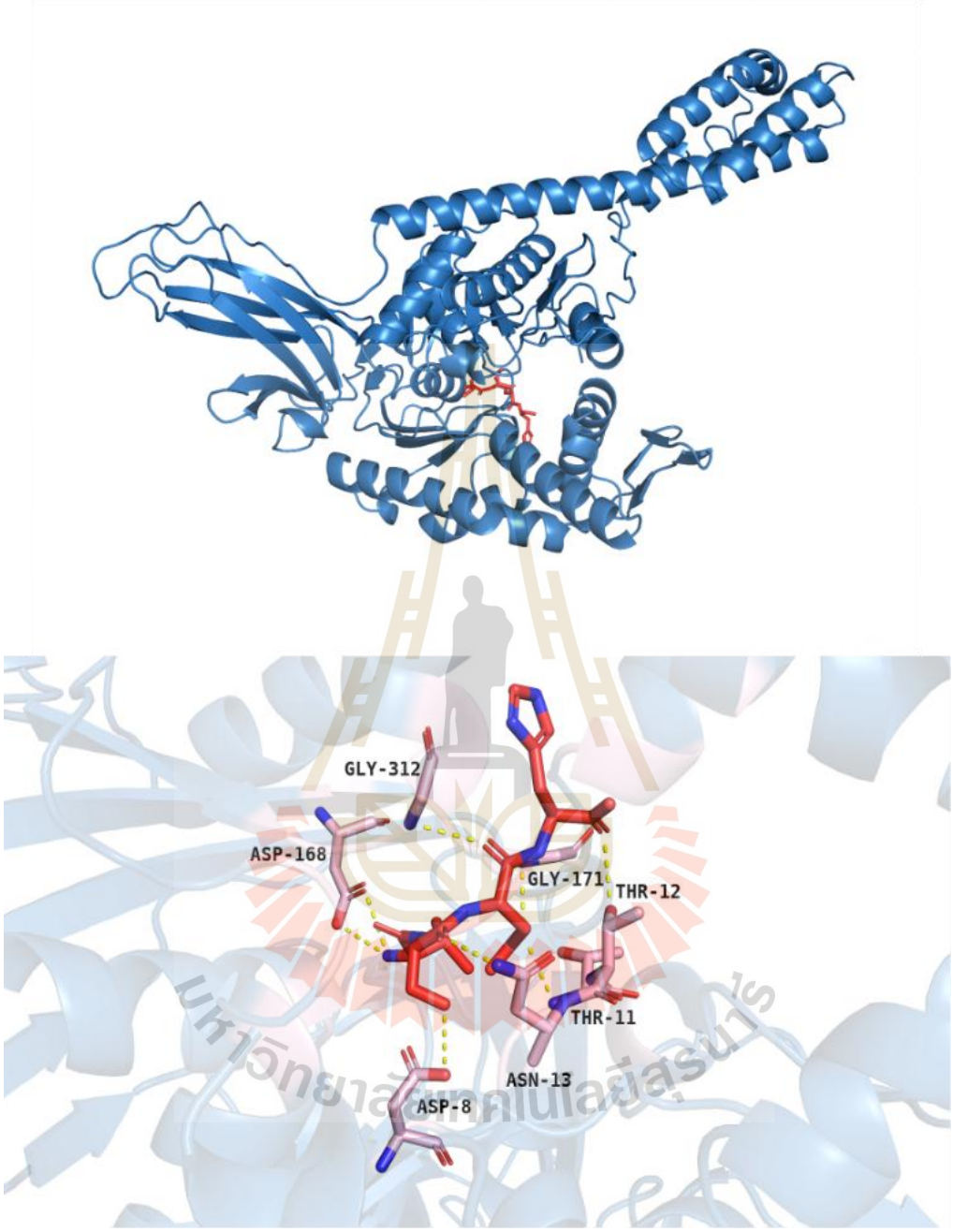
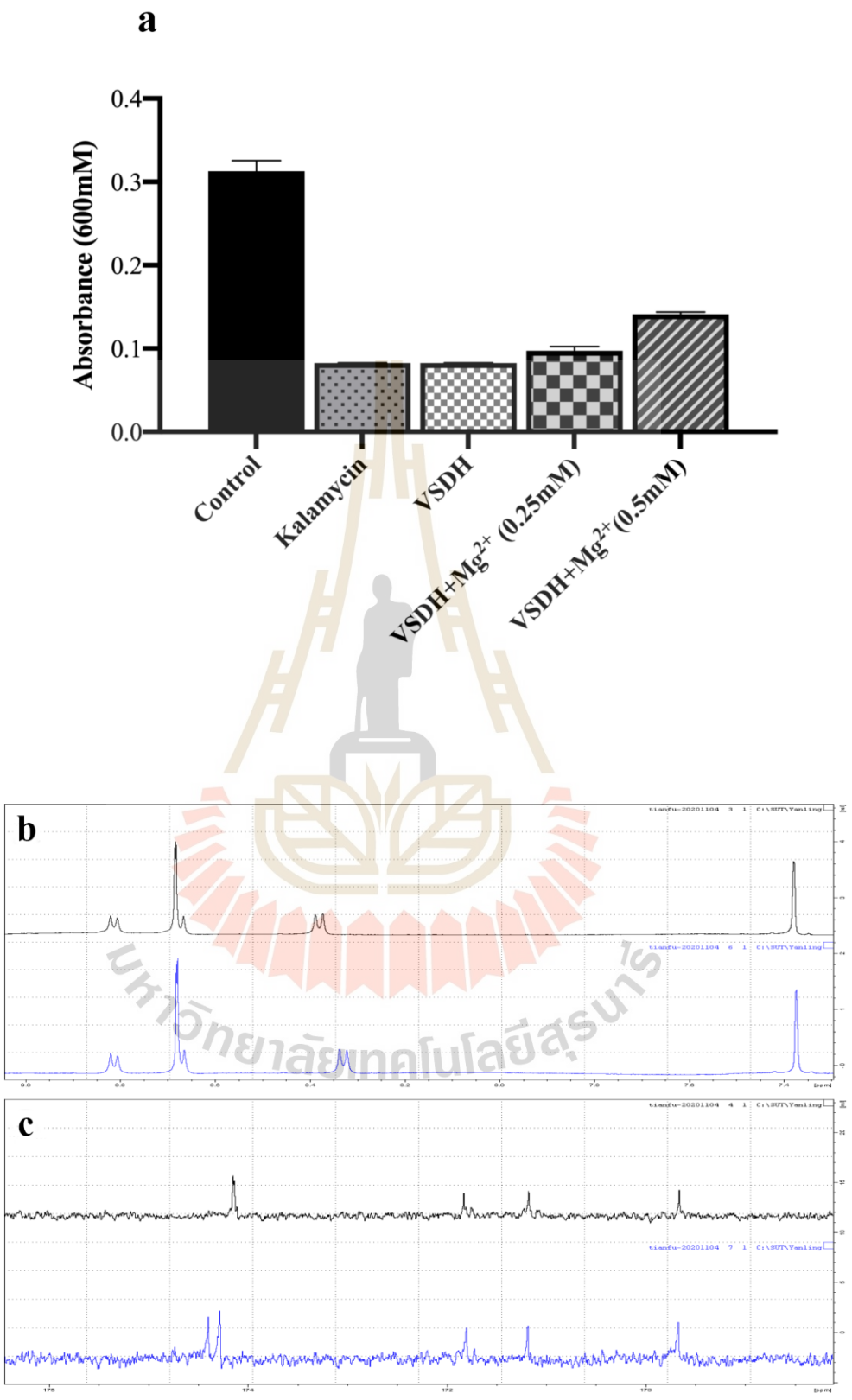


Fig. 3.8 Molecular docking of VSDH with enzymes related to DNA synthesis. a.DNA gyrase subunit B; b. DnaK. The backbone of the VSDH peptide is depicted in red lines; the active sites of both enzymes are depicted in pink lines; the yellow dots indicate hydrogen bonds.

3.4.3.7 Metal chelation

Histidine-rich AMPs can complex with metal ions via two nitrogen atoms of the imidazole ring (Brewer & Lajoie, 2000; Nishikawa & Ogawa, 2004). Corbin et al. (2008) reported that calprotectin, a protein found in human neutrophils, inhibits the growth of several bacteria and fungi by zinc and manganese chelation. Microplusin an antimicrobial peptide isolated from the Cattle Tick *Rhipicephalus Boophilus microplus*, inhibited *Micrococcus luteus* due to its copper II chelating ability. VSDH has a histidine residue at the C-terminus and contains a negatively charged amino acid (D). Moreover, the amino acid residues of DH resemble those of microplusin, which plays an important role in binding copper II (Silva et al., 2009). Magnesium (Mg^{2+}) is an essential trace element for protein synthesis that neutralizes the charge on ribosomal RNA to allow the proper folding of proteins. Insufficient intracellular Mg^{2+} ions lead to the disassembly of ribosomes (Pontes et al., 2016). Mg^{2+} is also vital for DNA synthesis as it is essential for DNA polymerase, catalyzing the incorporation of deoxynucleoside triphosphates into a growing DNA chain (Bermek et al., 2011). Thus, it was hypothesized that the Mg^{2+} chelating ability of VSDH might play some roles in its antibacterial ability. When *B. cereus* was cultured with VSDH for 18 h, Mg^{2+} (0.25 and 0.5 mM) was added to the medium, and incubation was prolonged for another 10 h. Growth appeared to recover at 0.5 mM Mg^{2+} addition (Fig.3.9 a). Chelation of Mg^{2+} induced the metal starvation of *B. cereus*, leading to its limited growth. Growth recovery upon Mg^{2+} addition confirmed the VSDH chelation ability. The ^{1}H NMR spectra also illustrated that theNH group showed a chemical shift at 8.32 ppm in the presence of Mg^{2+} (Fig.3.9 b). The ^{13}C NMR (Fig.3.9 c) spectra also revealed coordination between the C=O of Asp or the C-terminus and Mg^{2+}, as distinct peaks at approximately 174.2 ppm were observed. The possible chelation between VSDH and Mg^{2+} is illustrated in Fig. 3.9 d. The C-terminus of the two molecules VSDH and Asp-COOH were coordinated with Mg^{2+}. To verify the chelation mechanism, a peptide-metal complex was established, and a 20 ns molecular dynamics simulation was performed. The root mean square deviation (RMSD) (Fig. 3.9 e) during 20 ns implied high binding stability between VSDH and Mg^{2+}. Thus, these results demonstrated that VSDH chelated well with Mg^{2+}, which could be another vital mode of antimicrobial action.


a
Absorbance (600mM)
0.4
0.3
0.2
0.1
0.0
Control
Kalamycin
VSDH
VSDH+Mg2+ (0.25mM)
VSDH+Mg2+(0.5mM)
b
c

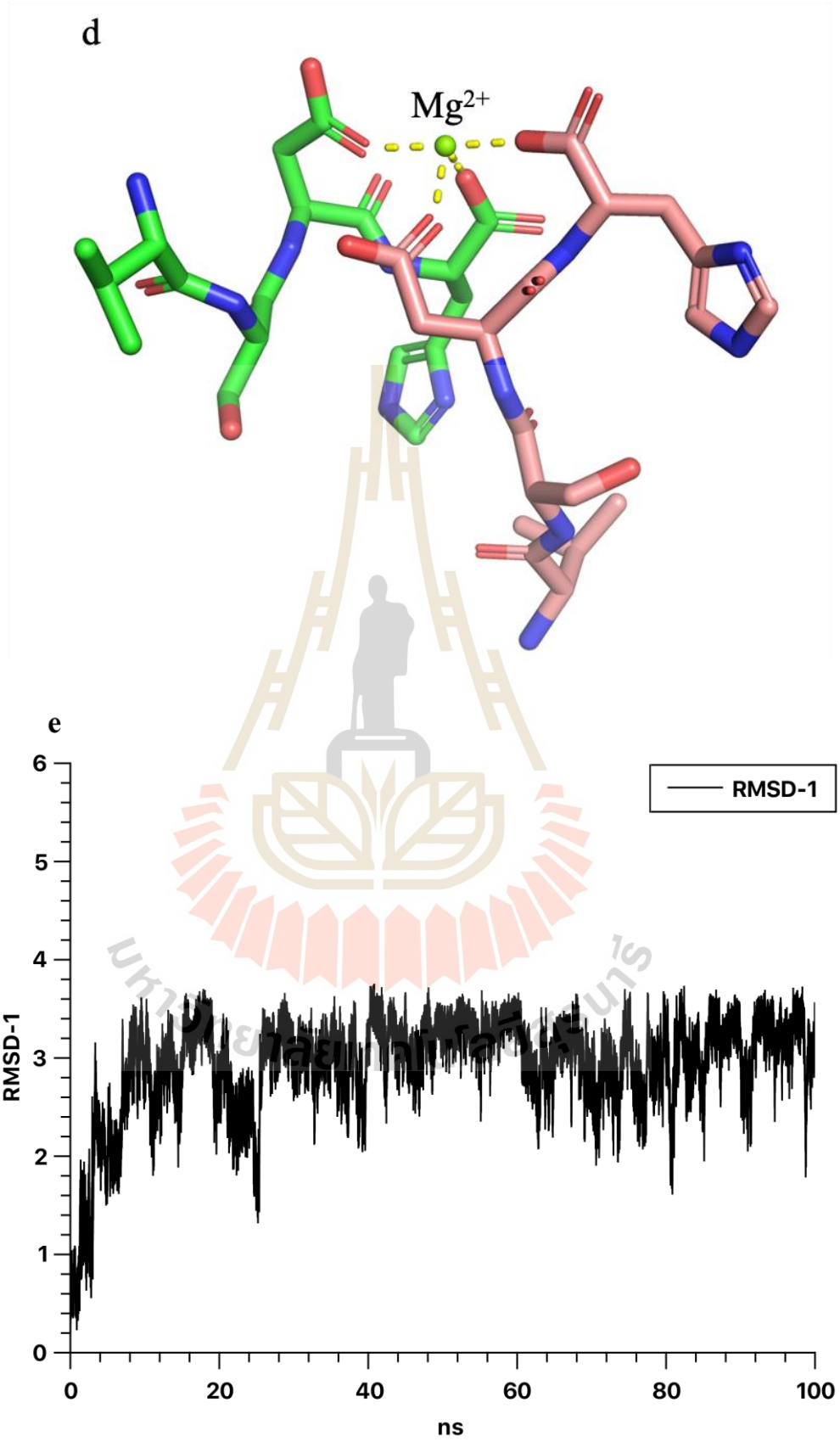

d
Mg2+
e
RMSD-1
RMSD-1
ns
0
1
2
3
4
5
6
0
20
40
60
80
100

Fig. 3.9 Metal chelating ability of VSDH during the growth of *B.cereus*. a. Cells were treated with 1×MIC for 18 h, and two concentrations of Mg^{2+} were added for an additional 10 h. Optical density was determined at 600 nm. Data are presented as the mean±SD, n=3; b. ^{1}H NMR spectra of the interaction between VSDH and Mg^{2+}; c. ^{13}C NMR spectra of the interaction between VSDH and Mg^{2+}; d. 3D structure of the VSDH-Mg^{2+} complex; e. Root mean square deviation (RMSD) plot for the VSDH-Mg^{2+} complex during 100 ns of molecular dynamics simulation.

3.5 Conclusions

The two novel antibacterial peptides, VSDH and CCCPKAF, isolated from chicken plasma hydrolysates displayed unique characteristics with different antibacterial modes of action toward *B. cereus*. CCCPKAF exhibited a lower positive charge and disturbed the integrity of the cell membrane, which is a typical characteristic of AMPs. In contrast, VSDH is a short neutral peptide showing multiple mechanisms. It passed through the cell membrane and reacted with various intracellular targets, including proteins and DNA. Molecular docking revealed that VSDH showed good binding affinity to the DNA gyrase subunit B and DnaK, resulting in the inhibition of DNA synthesis and in protein misfolding, respectively. VSDH also chelated Mg^{2+}, depriving an essential trace element for growth. These two peptides showed potential as emerging antimicrobial agents for food and pharmaceutical applications.

3.6 Acknowledgements

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CHAPTER IV

A novel short anionic antibacterial peptide, EADE, isolated from chicken plasma hydrolysates

4.1 Abstract

A novel anionic antibacterial peptide was successfully isolated by flash chromatography and identified to be EADE by LC-MS/MS. EADE inhibited the growth of *Staphylococus aureus* ATCC 25963 with minimal inhibitory concentration (MIC) of 0.5 mM. Propidium iodide (PI) uptake revealed that EADE did not disturb membrane integrity. Fluorescein isothiocyanate (FITC)-labeled EADE revealed EADE did not interact with cell. EADE exhibited bacteriostatic effect, which was fully reversed when culture medium was supplemented with 40μM Ca^{2+}. Other trace elements had no effect on survival improvement. Nuclear magnetic resonance (NMR) spectra revealed coordination of Ca^{2+} and carboxyl groups of E, D, E and C-terminus of the peptide. Molecular dynamics (MD) simulation indicated a Ca^{2+}-EADE conjugate was stable with tetrahedral coordination. Ca^{2+} sequestering ability of EADE appeared to be the main action for inhibiting *S. aureus* growth.

Keywords: anionic antibacterial peptide, metal chelation

4.2 Introduction

The rapid increase in antibiotic-resistant is due to the abuse and overuse of antibiotic. A large amount of studies have focused on the search for new antimicrobial agents to combat these serious threats (Wang et al., 2018). Antimicrobial peptides (AMPs) have gained attention as alternative antimicrobial agents due to its unique mechanisms. AMPs do not only attack bacterial membranes, but also penetrate the bacterial membrane and interfere with intracellular targets. Thus, multiple targets of AMPs make it difficult for resistant development (Zhu et al., 2022). In addition, AMPs also exhibit a broad spectrum against bacteria, fungi, and

yeast (Zasloff, 2002). Most antimicrobial peptides are cationic AMPs (CAMPs), containing 10-50 amino acid residues with a net charge between +2 and +11, which readily bind to negatively charged bacterial surface via electrostatic interaction (Mahlapuu et al., 2016). CAMPs should also contain 50 % hydrophobic amino acids, requiring for cell membrane disintegration (Brogden, 2005). Some CAMPs can traverse cell membrane and target intracellular constituents, such as DNA, enzymes, and proteins. However, some bacteria have evolved the strategy of incorporating positively charged polymers at cell surface, which reduces electronegativity of cell surface and lessen inhibition efficacy of CAMPs (Assoni et al., 2020). Thus, AMPs with varied modes of action should be sought.

Anionic AMPs (AAMPs) is another group which is less investigated. AAMPs have net charge ranging from -1 to -8 (Dennison et al., 2018). AAMPs are thought to have evolved in response to bacterial resistance toward CAMPs. Due to its anionic nature, AAMPs can thus function to target these bacteria, which can change their electronegativity. In addition, AAMPs also can utilized divalent metal ions, such as Zn^{2+}, to enhance its activity. CAMPs are ineffective in high salt condition. Therefore, AAMPs may be an alternative antimicrobial agent that can overcome limitation of CAMP (Paulmann et al., 2012). AAMPs are naturally present in insect, animals and human. Extraction of AAMPs from these sources for food application is not feasible due to high production costs and low efficiency. Hydrolysis protein with a specific protease is a better strategy with higher efficiency for AMPs production (Kong et al., 2020; Song et al., 2017; Tang et al., 2015), but reports of AAMPs by enzymatic hydrolysis is scarce.

EDTA has been reported to inhibit bacterial growth by depraving Mg^{2+}, Ca^{2+} and Fe^{2+}, which are essential trace elements for microbial growth (Ko et al., 2008). EDTA has been widely used as a preservative used in food. The consumers have good gained more interest in safter and more natural antibacterial agents in food. AAMPs that can interact with metal ions preventing their uptake by microorganisms could be an alternative to EDTA. Ovotransferrin (OVT) is an antimicrobial protein that relies on sequestering iron, an essential ion for bacterial growth (Bullen et al., 1978). Micropulsin (10,205 Da), an AAMP derived from tick, contains numerous His-rich regions, which strongly bind to metal ions. It showed bacteriostatic activity with Cu^{2+}

binding ability, limiting ion nutrients for *Micrococcus luteus* growth (Silva et al., 2009). AAMPs have potential to be developed as food preservatives. However, these AAMPs have relatively long primary sequence which sequences are usually accompanied by high production costs (Han et al., 2021). Thus, small molecules of AAMPs should receive more attention.

In this work, AAMP from chicken plasma hydrolysate was isolated and identified. Its mode of action was systematically investigated. Meanwhile, its metal chelating properties as related to its bacteriostatic were elucidated.

4.3 Materials and Methods

4.3.1 Chicken plasma preparation

Fresh chicken blood was collected from a commercial slaughterhouse (Betagro, Lopburi, Thailand) with sodium citrate added to contain a final concentration of 4% to prevent clotting. Upon arrival, the samples were centrifuged at 2,000×g at 4°C for 10 min and plasma was collected. Plasma was dialyzed in 3-kDa dialysis membrane in DI water at 4°C for 18 h. Subsequently, dialyzed samples were lyophilized and kept in a -20°C freezer.

4.3.2 Preparation of chicken plasma hydrolysate (CPH)

Plasma powder was dispersed in deionized water at concentration of 100mg/ml, and pH was adjusted to 2.5. Enzymatic hydrolysis was performed using porcine pepsin (Sigma-Aldrich, Chemie Gmbh, Steinhem, Germany) with an E/S ratio of 3/100 (w/w) at 37°C. After 4 h of hydrolysis, the reaction was stopped by heating at 95°C for 10 min. Then, the hydrolysates were rapidly cooled to 4°C in an ice bath. Finally, samples were centrifugated at 10,000 × g for 20 min and referred to as chicken plasma hydrolysate (CPH) which was kept in -80°C until use.

4.3.3 Purification and identification of antibacterial peptides

Two ml of diluted CPH was loaded onto a Puriflash C18-AQ 15 µm F0012 flash column (Interchim, Montlucon, France) equipped with Puriflash® 5.250 system (Interchim, Montlucon, France). The mobile phases were composed of 0.05 % (v/v) trifluoroacetic acid in water (A) and 0.05% trifluoroacetic acid in acetonitrile (B). The elution at 15 ml/min was performed using the following gradients: 2% B, 10 CV;

10% B, 10 CV; 20 % B, 10 CV; 20%-100% B, 5 CV; 100% B, 3 CV; 100%-2% B, 2 CV and 2%B, 5CV. The highest antibacterial activity fraction was selected for peptide identification. The highest antibacterial activity fraction was selected for peptide identification. Molecular mass and peptide sequence were analyzed on the Dionex UltiMate 3000 UHPLC+ focused Systems (Thermo Fisher Scientific Inc., Waltham, MA, U.S.) coupled with a micro TOF-QII mass spectrometer (Bruker Daltonics, Bremen, Germany). The AdvanceBio peptides plus (150 mm × 4.6 mm × 2.7 μm) (Agilent Technologies, Santa Clara, CA, USA) was used for peptide separation at a flow rate of 0.5 mL/min of mobile phase, using deionized water containing 0.1% formic acid as solvent A, and acetonitrile containing 0.1% formic acid as solvent B. The elution was performed as follows:0-5 min (2% B), 5-35 min (2-35% B), 35-40 min (35-95% B), 40-45 min (95% B), 45-47 min (95-2% B) and 47-55 min (2% B). The LC-QTOF data were collected and processed by Compass 1.3 and data were analyzed using the PEAKS Studio X pro software (Bioinformatic Solution Inc. Waterloo, Canada).

4.3.4 Determination of antibacterial activity

Antibacterial activity of fractions obtained from flash column chromatography and synthetic peptides were carried out according to Kong et al. (2020) with some modifications. Briefly, the single colony of *S. aureus* ATCC 25923, *Bacillus cereus* DMST 5040, *Escherichia coli* ATCC 25992 and *Salmonella* Typhimurium TISTR 292 were diluted to 1×10^5 CFU/mL with Mueller Hinton Broth (MHB), and 50 μL of diluted cell cultures were added to a 96-well microtiter plate. Fifty μL of each sample were sterilized by filtering through a 0.22 μm membrane and were added to each well and incubated at 37°C for 18 h. Positive and negative controls were also prepared using 20 ppm Kanamycin and DI water, respectively. Absorbance (OD_{600}) was determined using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA). The minimum inhibitory concentration (MIC) of each sample was determined. Briefly, 2-fold serial dilutions of each sample were incubated with each culture in a 96-well microtiter plate at 37°C for 18 h. Cell density was measured at 600 nm and the lowest concentration showing growth inhibition was determined as MIC.

4.3.5 Growth Kinetics

Growth kinetics of EADE was assessed according to Silva et al. (2009) with some modifications. Briefly, *S. aureus* were treated with EADE (4 × MIC) and incubated at 37°C for various time intervals of 0, 4, 8, 12 and 24 h. Enumeration was performed using a drop plate technique on PCA incubated at 37°C for 24 h. In addition, after 24 h of incubation, cells with EADE were collected and wash with sterile phosphate-buffered saline (PBS) by centrifugation and resuspended in MHB. Cell suspension was divided into two portions. One portion was incubated with EADE (4× MIC) at a ratio of 1:1 (v/v), and another portion was incubated with PBS at a ratio of 1:1 (v/v) and incubated for 24 h. Cell enumeration was determined by a drop plate technique.

4.3.6 Role of Zn^{2+} on antibacterial activity

The role of metal ions on antibacterial activity of the identified peptide, EADE, was Investigated according to Brogden et al. (1996) with slight modifications. EADE (0.25 and 0.5 mM) alone and EADE at 0.25 and 0.5 mM pre-incubated with Zn^{2+}(final concentration 20μM) for 1 h at 37°C, were incubated with bacterial cell (1×10^5 CFU/mL) at 37°C for 18 h, respectively. Then absorbance (OD_{600}) was determined using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA).

4.3.7 Confocal laser scanning microscopy image (CLSM)

The membrane integrity was determined using propidium iodide (PI) and observed by CLSM. Briefly, mid-logarithmic, *S. aureus* were treated with EADE at 1 × MIC for 1h, centrifuged at 2,000 × g for 5min, washed 3 times with 10 mM PBS, and PI was added to attain a final concentration of 10 μg/mL. After incubation for 30 min at 4°C in dark, cells were washed twice with PBS and re-suspended in 10 mM PBS. Then, 10 μL of suspension was transferred onto a glass slide and observed using a Nikon A1Rsi CLSM at excitation of 535 nm. Cells without peptide treatment were used as a control.

To further evaluate cellular localization of peptide were followed with slight modifications (Wang et al., 2019). *S aureus* (OD_{600}=1) were incubated with 1 × MIC FITC-labeled EADE at 37°C for 1 h. Subsequently, cells suspension was

centrifuged at 2,000 × g for 5 min, Cell pellets were washed three times and resuspended in 10 mM PBS. Samples were smeared on a glass slide and observed by Nikon A1 Rsi CLSM at excitation of 488 nm.

4.3.8 Scanning electron microscopy (SEM)

The morphological of the cell with and without EADE treatment were observed using a scanning electron microscope (ZEISS Gemini, Carl Zeiss, Germany). Briefly, mid-logarithmic *S. aureus* (OD_{600}=1) were treated with EADE at 1 × MIC at 37°C for 1 and 4 h. Subsequently, cells were harvested and fixed with 2.5 % glutaraldehyde at 4°C overnight and dehydrated by 20%-100% of acetone. Dried cells were coated with gold and observed at an accelerating voltage of 3kV and a magnification of 10,000

4.3.9 Metal chelation ability

4.3.9.1 Effect of metals ions on growth of *S. aureus*

Chelating ability of synthetic peptide was studied according to Silva et al. (2009) with some modifications. *S. aureus* (1×10^5 CFU/mL) incubated with 0.5 mM EADE in MHB supplemented with several trace elements, including 500 μM $MgCl_2$, 40 μM $CaCl_2$, 50 μM $FeCl_3$, 12.4 μM $ZnSO_4$, $CuCl_2$ and 2.5 μM $CoCl_2{\cdot}H_2O$. After 18 h of incubation at 37°C, the turbidity was measured at A_{600}. In another experiment, *S. aureus* were pre-incubated with 0.5 mM EADE for 18 h, then $CaCl_2$ was added (finally concentration 40 μM). Cells were incubated for another 18 h and turbidity was measured at A_{600}.

4.3.9.2 NMR spectroscopy

EADE (20 mM) and $CaCl_2$ (20 mM) were mixed at ratio of 1:1 (v/v) and incubated at 37°C for 30 min. Peptide was added Deuterated water at ratio of 4:1 (v/v) and then analyzed on a Bruker Avance III HD 500 MHz NMR spectrometer with a CCP BBO 500 Cryoprobe at 25°C. The ^{13}C NMR spectra were collected at 125.816. Burker NMR software Topspin 3.5p16 was used for collection and processing.

4.3.9.3 Molecular dynamics simulation

To further validate the chelation mechanism between EADE and Ca^{2+} or Mg^{2+}, molecular dynamic simulations were applied. The molecular dynamic simulation was performed in Yinfotek Cloud Computing Platform

(http://cloud.yinfoteck.com). The 3D structure of the EADE was generated using the platform's tools. Molecular dynamic simulation was executed using AMBER ff19SB force (Tian et al., 2020) and Amber Tools (Case et al., 2020). The system was solved using the OPC water model with a margin of 10 Å. Periodic boundary conditions were applied and $CaCl_2$ or $MgCl_2$ was used to neutralize net charge. The structure was minimized by 2500 steps of steepest descent and 2500 steps of conjugate gradient without constraint, subsequently, the system was gradually heated to 300 K by 20 ps NVT simulation. Finally, the system was subjected to a 30 ns NVT simulation.

4.3.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 9, and statistical significance was determined using one-way ANOVA and Tukey's multiple comparison test when more than 2 groups were compared. The paired t-test was used for comparison between 2 groups.

4.4 Results and discussion

4.4.1 Isolation and identification

AMPs were amphiphilic compounds and hydrophobic interactions of AMPs cell membrane were one of important features (Mahlapuu et al., 2016; Tang et al., 2008). Therefore, hydrophobicity of peptides can be used as a criterion for AMP isolation. After C18 AQ flash chromatographic separation, 7 peaks were obtained (Fig.4.1 a). Among them, peak 2 and peak 3 at 2 mM (*L*-leu equivalents) exhibited 95 % and 94 % inhibition toward, *S. aureus*, respectively. They also inhibited *S. typhimurium* at 90 and 94% at same concentration, respectively. Thus, peak 3 was selected for further studies.

Typically, AMPs contain about 50 % hydrophobic amino acid residues (Stark et al., 2002). Thus, they are likely eluted from the C18 column at higher ACN concentration. In this study, AMP fraction were eluted at 2% ACN, suggesting relatively high polarity nature of AMP derived from chicken plasma. An anionic peptide, EADE was identified from peak 3, which contained total net charge of -3 and total hydrophobic ratio of 25 % (Fig.4.1 b), corresponding to its early elution from C18-AQ column. The peptide EADE is considered as a novel peptide based on the antimicrobial peptide database (https://aps.unmc.edu). EADE showed good antibacterial

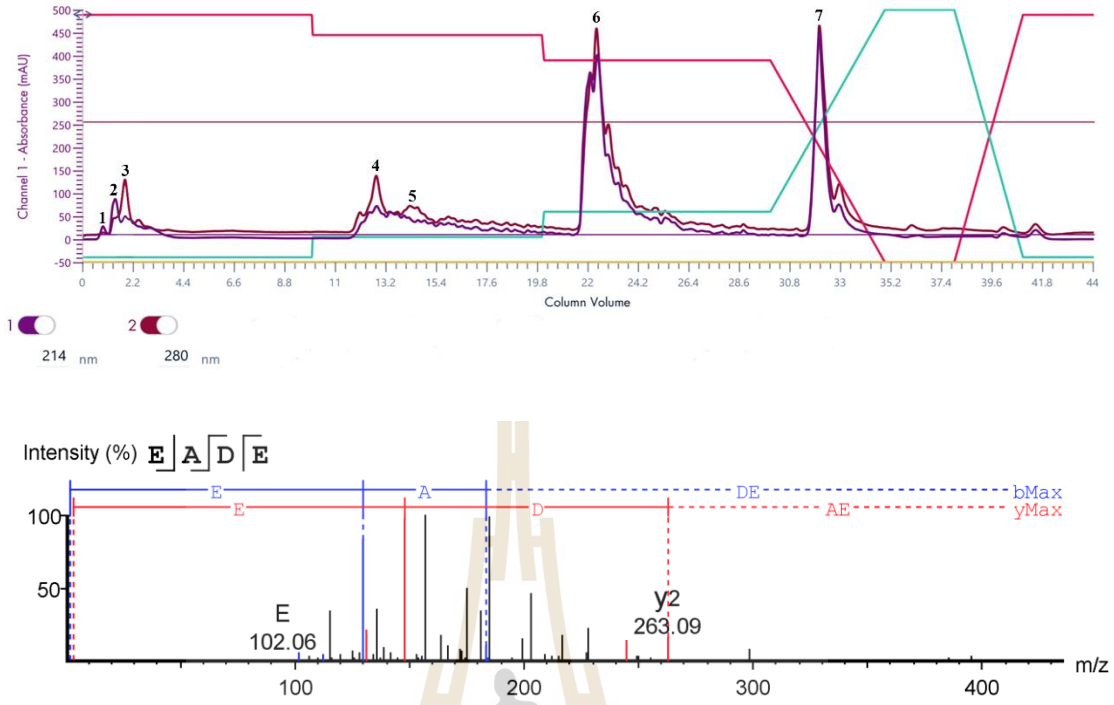

Fig. 4.1 a. C18-AQ flash chromatogram of peptides from CPH (red line= buffer A, green line=buffer B); b. MS/MS spectra of EADE.

activity toward to *S. aureus* with MIC of 0.5 mM. Tang et al., (2008) reported an AAMP isolated from Chinese traditional edible larvae of a housefly, VESWV which inhibited Gram-positive and gram-negative bacteria with MIC of 20-32 μg/mL and 18-20 μg/mL, respectively. Song et al., (2017) also found an AAMP from Maillard reaction products of half-fin anchovy hydrolysates/glucose, FFTQATDLLSR, which showed good activity toward *E. coli.* VESWV and FFTQATDLLSR shared a common characteristic with relatively high hydrophobicity of 60 % and 45 %, respectively and low negative charge (-1). Higher hydrophobicity allowed them to penetrate cell membrane and complete their antibacterial action. Brogden et al. (1996) reported that three AAMPs isolated from ovine pulmonary surfactant, namely, GDDDDDD, DDDDDDD and GADDDDD, have difficulties in binding to the cell surface due to their high electronegativity. Therefore, Zn ions are required to enhance their antibacterial activity. Zn ions can establish salt bridges between AAMPs and the negative components of the cell surface. Therefore, it promotes the binding of AAMPs to the bacterial surface and enhances its antibacterial activity (Almarwani et al., 2020). EADE

exhibited only 25 % hydrophobicity with strong negative charge of -3, which shared a similar net charge characteristic of GADDDDD. Therefore, EADE might require divalent metal ions to enhance its antibacterial ability.

4.4.2 Bacteriostatic effect of EADE

EADE at 2 mM (4 × MIC) was not lethal to *S. aureus*, but only suppressed its growth. In comparison with the control (Fig.4. 2), cells treated with EADE showed lower cell counts, approximately four orders of magnitude at 24 h. (Fig.4. 2 a). In addition, this effect was reserved after EADE was removed from medium (Fig. 4.2 b). therefore, these results indicated that EADE is bacteriostatic against *S. aureus.*

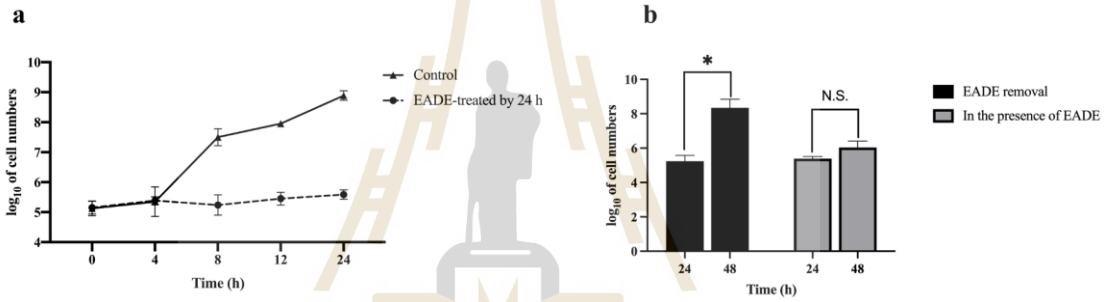

Fig. 4.2 Kinetics of *S. aureus* growth during treatment with EADE. a. Kinetics of *S. aureus* growth during 24 h; b. the bacteriostatic effect of EADE for *S. aureus*. N.S.: not significant ($p > 0.05$), *$p < 0.05$

4.4.3 Role of Zn^{2+} for antibacterial ability

Divalent metal ions, such as Zn^{2+}, have been reported to enhance antibacterial activity of AAMP (Łoboda et al., 2018). AAMPs used metal ions to form salt bridges with negatively charged constituents of microbial membranes, allowing interactions with cells and complete mode of action (Je**Ż**owska-Bojczuk and Stokowa-Sołtys, 2018). In addition, some AAMPs required metal ions as a structural cofactor. Dermicidin, which interacts with membrane phospholipids by forming ion channels, require the presence of Zn^{2+} to function properly. Dermicidin forms a hexametric channel by the trimerization of antiparallel peptide dimers. The dimers are stabilized by coordination to Zn^{2+} ions through residues Glu 5 and Asp 9 of one

peptide, and residues His 38 and Asp 42 from the second peptide (Łoboda et al., 2018). However, antibacterial activity of EADE was not affected by Zn^{2+} (Fig. 4.3), which could be related to various reasons. *S. aureus* can change the electronegativity by incorporating a positive charge, thus reducing the possibility of binding to AMP (Assoni et al., 2020), therefore, the addition of Zn might not facilitate interactions of EADE and bacterial cells. In addition, EADE contains Asp and Glu which are classified as "hard" base according to hard-soft acid-base theory (Łoboda et al., 2018). These carboxyl anions are unlikely to interact with Zn^{2+} to form hexametric channel due to Zn^{2+} are classified as borderline acid and preferred to borderline base.(Łoboda et al., 2018).

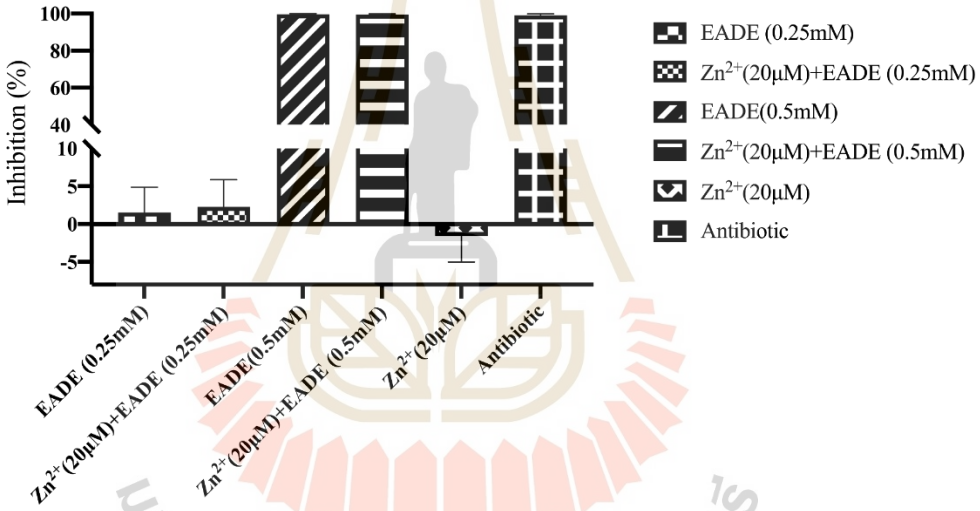

Fig. 4.3 Effect of 20 μM Zn^{2+} on antibacterial ability of EADE at 0.25 and 0.5 mM toward *S. aureus*. Data are mean±SD, n=3.

4.4.4 CLSM

A membrane impermeable dye, PI, is capable of staining nucleic acids in cells when membrane is disrupted. The control group (without EADE) showed a small fluorescent signal, which might be the results of natural cell death (Song et al., 2020). Cell treated with EADE for 1 h also did not show an obvious red fluorescent signal, suggesting that cell membrane integrity was intact after 1 h of exposure (Fig.4.4 a). Miao et al. (2016) reported a dipeptide containing leucine and tyrosine

connected with two phosphate molecules from kefir, inhibited the growth of *S. aureus* and *E. coli* by intracellular reaction without causing severe cell membrane damage. Thus, PI results suggest that bacteriostatic effect of EADE might not involve membrane disruption. To further determine the localization of EADE at the cellular level, the FITC-EADE was applied. After treatment with FITC-labeled EADE for 1 h, green fluorescent was not observed in cells (Fig.4.4 b), indicating that EADE was not absorbed.

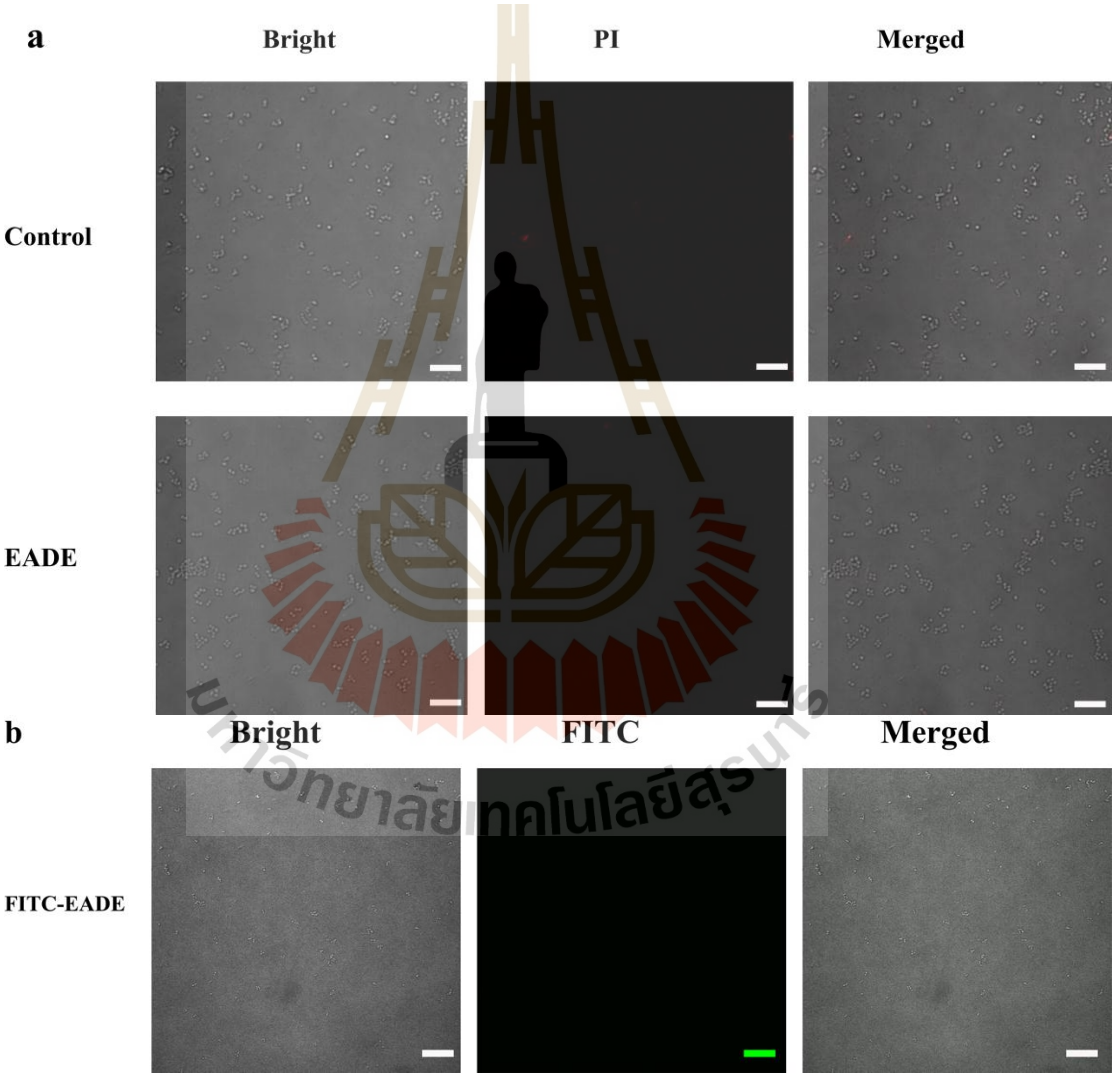

Fig. 4.4 a. CLSM images of *S.aureus* treated with EADE (1×MIC) and PI; b. *S. aureus* treated with FITC-EADE (1×MIC). Scale bar = 20 μm.

4.4.5 SEM

S.aureus without EADE served as control showed a smooth surface without any visible damage (Fig.4.5). Cell treated with EADE for 1 h also exhibited smooth surface. When exposure time was extended to 4 h, only a few cells appeared broken, but most cells remained intake. The SEM results indicated that EADE did not cause significant morphological changes and damage, confirming results of CLSM.

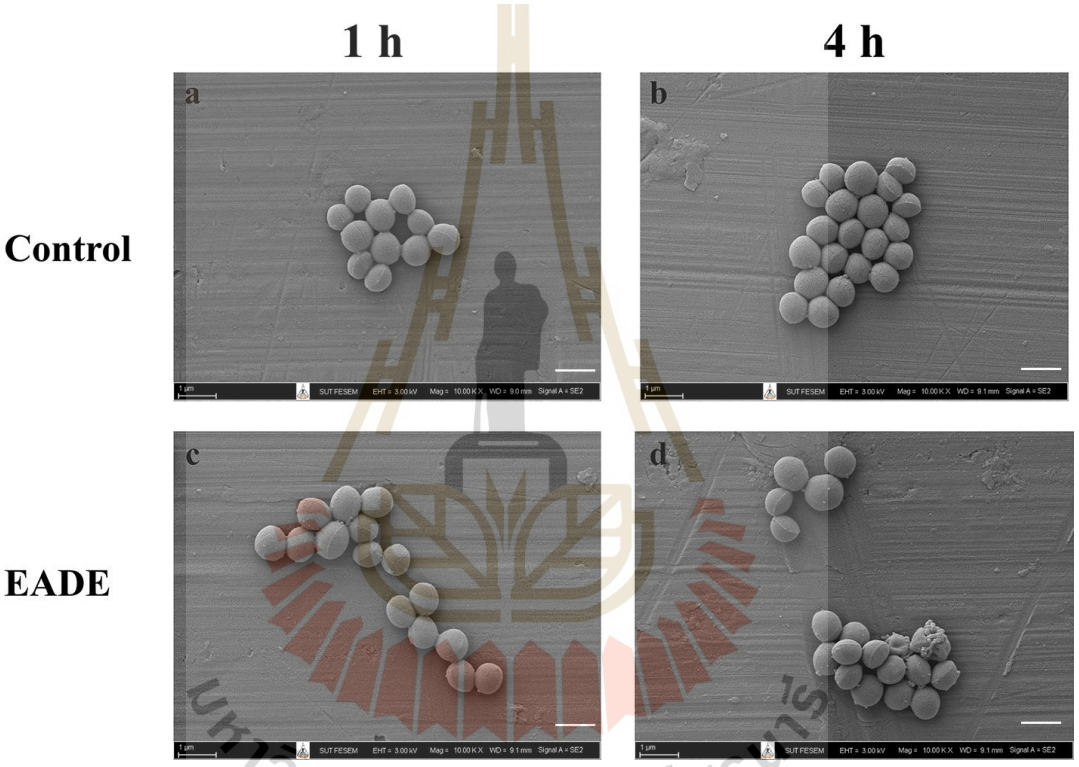

Fig. 4.5 Morphological changes of *S. aureus* treated with EADE (1 × MIC) for 1 and 4 h. scale bar = 1μm

4.4.6 Metal chelation

According to above results, EADE might not directly interact with cells to suppress its growth. One of possibilities of its bacteriostatic effect could be its metal chelating ability. When medium was supplement with various metal ions in the presence of EADE, an antimicrobial activity of EADE against *S. aureus* decreased when only Ca^{2+} was added (Fig.4.6 a). Moreover, Ca^{2+} restored bacterial growth when

added to cells pre-incubated with EADE for 18 h (Fig.4.6 b). Ca^{2+} is critical for many metabolic processes, including maintaining cell structure, membrane transport mechanisms (channels, primary and secondary transporters), chemotaxis, cell division and cell differentiation processes, such as sporulation and heterocyst formation (Dominguez, 2004; Smith, 1995). Herbaud, et al. (1998) reported that *B.subtilis* growth appeared to be intimately linked to the presence of Ca^{2+}, as normal growth was immediately restored at 1 mM Ca^{2+} addition to an almost no growing culture in EGTA containing LB medium. These results indicated that EADE may chelate Ca^{2+} and induce starvation. ^{13}C NMR spectrum also illustrated the binding of EADE and Ca^{2+} (Fig.4.7). The peak shift around 174-177 ppm revealed coordination between carboxylic acid of E, D, E or C-terminus of EADE complex. Amagai et al. (2018) reported that depletion of Ca^{2+} and Mg^{2+} significantly suppressed the *S. aureus* growth, but supplementation of Ca^{2+} or Mg^{2+} restored the *S. aureus*. However, in our study, only Ca^{2+} supplementation promoted *S. aureus* growth restoration. Thus, MD simulation was applied to study the chelation of Ca^{2+} or Mg^{2+} by EADE. After 30 ns MD simulation, the 3D structure of ion-EADE complex was studied. For Ca^{2+}-EADE complex (Fig.4.8 a), all four amino acids are involved in binding to Ca^{2+}, where the two carboxyl groups from E1 and C-terminus, the C=O of A2, and the water molecules forms a tetrahedral coordination. However, for Mg^{2+}-EADE complex, only D3 and E4 involved in binding to Mg^{2+}, and no tetrahedral coordination were present (Fig.4.8 c). The 3D structure indicated that EADE bound to Ca^{2+} with higher affinity than Mg^{2+}. The RMSD result of Ca^{2+}-EADE complex showed more profile than that of Mg^{2+}-EADE, suggesting better stability of complexation. It can be speculated that lesser extent of Mg^{2+}-EADE conjugation would lead to more available free Mg^{2+} for growth of *S. aureus.* In contrast, high affinity of EADE for Ca^{2+} resulted in bacterial starvation of Ca^{2+} and growth suppression.

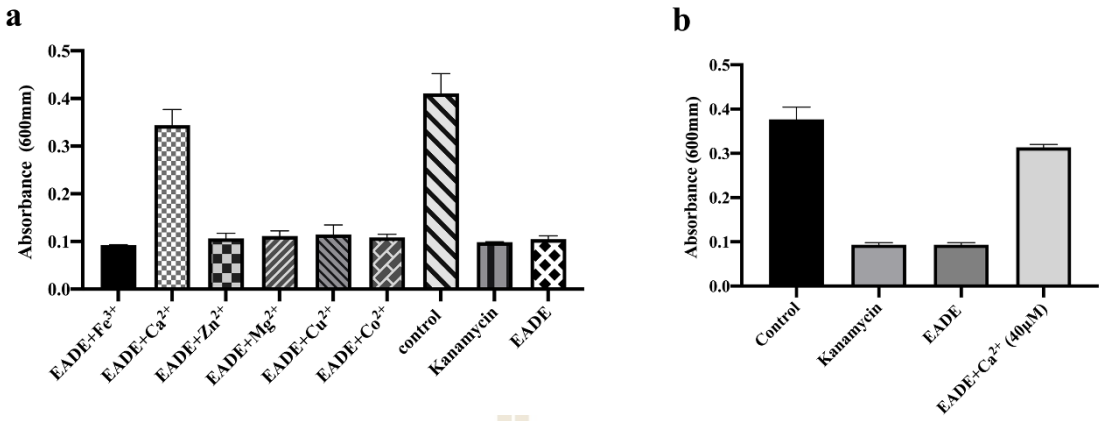

Fig. 4.6 a. Effect of various trace elements on antimicrobial activity of *S. aureus* in the presence of 0.5 mM EADE; b. Effect of Ca^{2+} supplementation on the growth of *S. aureus*, after 18 h pre-incubated with 0.5 mM EADE.

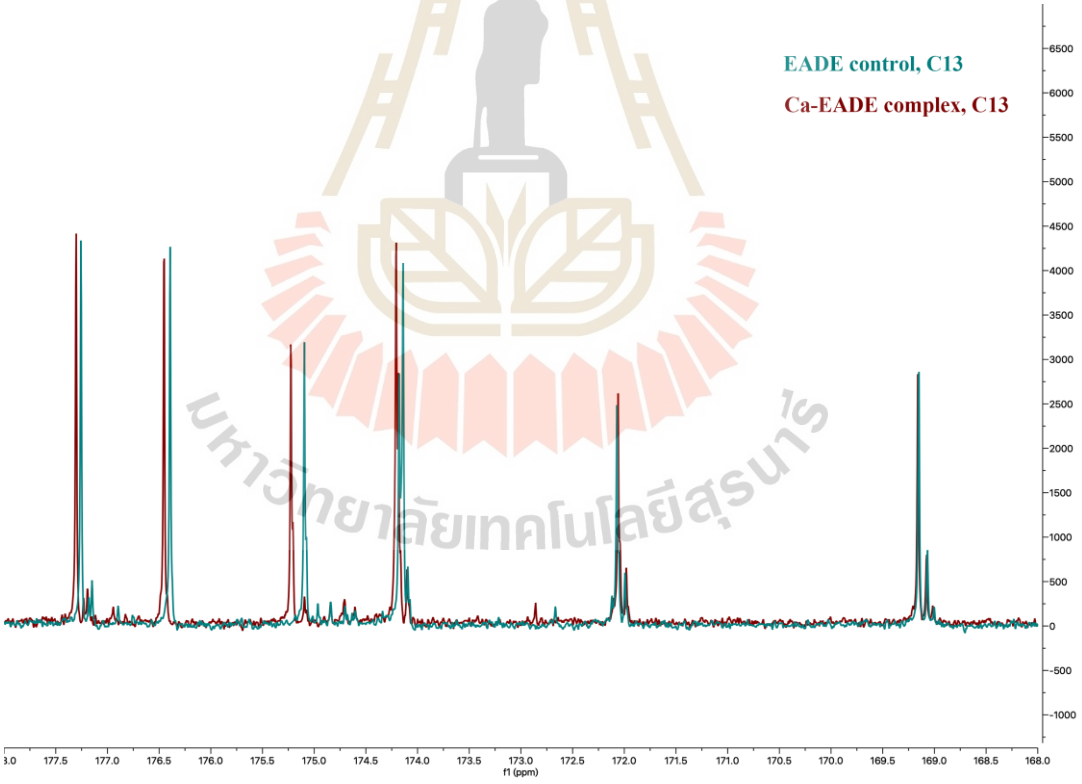

Fig.4.7 ^{13}C NMR of Ca^{2+}-EADE complex (20 mM:20mM).

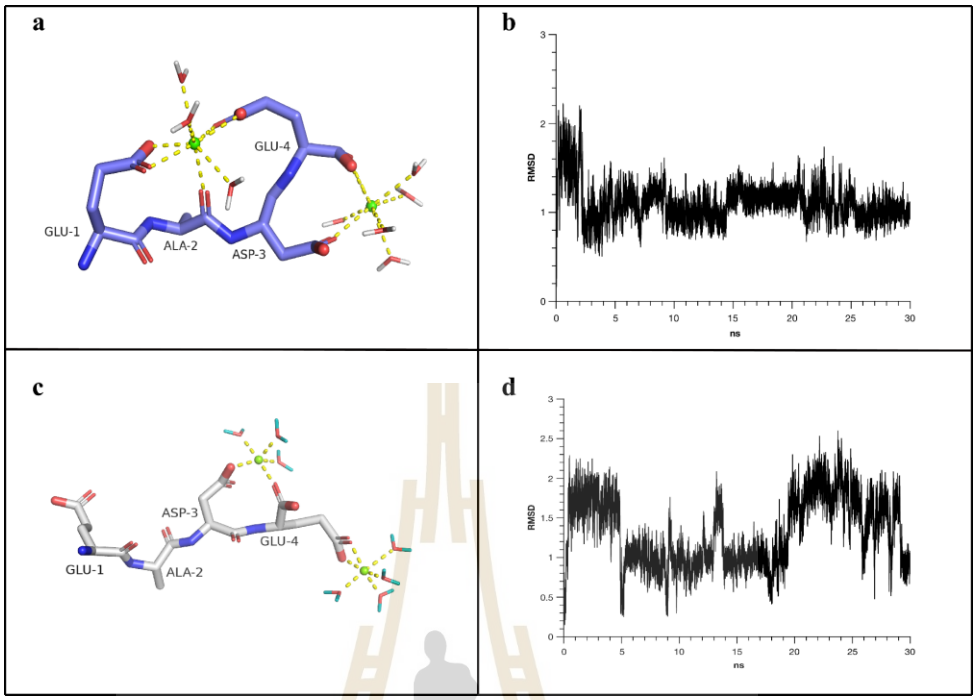

Fig. 4.8 a. 3D structure of Ca^{2+}-EADE complex, green ball=Ca, red and white sticks=H_2O; b. RSMD of Ca^{2+}-EADE complex; c. 3D structure of Mg^{2+}-EADE complex, light green ball=Mg, blue and red sticks=H_2O; d. RSMD of Mg-EADE complex

4.5 Conclusions

A novel anionic antibacterial peptide, EADE, was isolated from chicken plasma hydrolysate. The EADE is a bacteriostatic peptide, which inhibits the growth of bacteria by chelating calcium, EADE bound to Ca^{2+}, resulting in insufficient Ca^{2+} uptake and growth retardation. Thus, EADE could have potential as a natural antimicrobial agent to control growth of *S. aureus* in food application

4.6 References

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CHAPTER V

A CATIONIC ANTIBACTERIAL PEPTIDE ISOLATED FROM CHICKEN PLASMA HYDROLYSATES AND ITS SYNERGISTIC EFFECTY WITH THE ANIONIC ANTIBACTERIAL PEPTIDE

5.1 Abstract

A cationic peptide was successfully isolated by C18AQ flash chromatography and identified to be CP-1, KPKVLLHA. CP-1 inhibited the growth of *Salmonella* Typhimurium TISTR 292 with minimal inhibitory concentration (MIC) of 2 mM. Confocal laser scanning microscopy (CLSM) with propidium iodide (PI) showed that incubation of CP-1 with *S.* Typhimurium caused membrane damage, leading to the integrity of cell membrane and pores formation. Moreover, molecular dynamic simulation revealed interactions of CP-1 with DNA. The MIC value was reduced to 1 mM when combined with 0.5 mM. EADE, an anionic peptide isolated from chicken plasma. EADE possibly chelated Mg^{2+} or Ca^{2+} destabilizing and increasing the permeability of outer membrane stability as shown by 1-*N*-phenlnapthylamine (NPN) uptake assay, thus increasing the permeability of outer membrane to a greater extent. Thus, CP-1 combined with EADE showed potential application in controlling *S.* Typhimurium.

Keywords: antimicrobial peptide, chicken plasma, cationic, anionic, molecular dynamic simulation

5.2 Introduction

Due to the overuse and abuse of antibiotics, several antibiotic resistant bacteria have emerged. Bacteria develop antibiotic resistance by various mechanisms, including alternating the target of antibiotics, secreting enzyme to degrade antibiotics, changing membrane permeability to prevent antibiotics influx and developing membrane efflux pumps to exclude antibiotics (Si et al., 2021). Recently, antimicrobial peptide (AMPs) has attracted the attention as possible alternatives to

conventional antibiotics due to their unique mechanism. Most AMPs not only work by directly attacking bacterial membranes, but also can enter the cell and interfere with various intracellular targets (Brogden, 2005; Huang and Charron, 2017). Thus, multiple targets of AMPs result in lesser extent of resistance development (Zhu et al., 2022).

Food protein is considered as important source of AMPs after enzymatic hydrolysis (Song et al., 2020). AMPs can be obtained from different protein hydrolysates, including milk (Sah et al., 2018), eggs (Mohammadi Azad et al., 2017), fish (Tang et al., 2014), plant (Kong et al., 2020) and meat (Borrajo et al., 2019). Blood plasma rich in protein, like albumin, globulin, and fibronectin. Hydrolysis of blood plasma results in peptide showing various bioactivities including antioxidant peptides isolated from duck plasma (Yang et al., 2020), antihypertensive peptides generated from porcine blood plasma (Aiemratchanee et al., 2021). Antibacterial peptides from chicken plasma have not been reported.

When two or more AMPs are used in combination, they exert more efficacy than a single use of AMP application. The combination can reduce the risk of AMP resistance. Two AMPs, originally isolated from skin of frogs, called PGLa and magainin-2, showed synergistic effect against *E. coli* by creating a functionally more stable pores in the bacterial membrane (Westerhoff et al., 1995; Zerweck et al., 2017). AMPs also can combine with other antibacterial agents (McCloskey et al., 2014). AMPs might increase bacterial membrane permeability, providing increased access to the bacterial inner compartment for other agents (He et al., 2015). Polymyxin B binds to the lipid A portion of lipopolysaccharide (LPS) in Gram-negative bacteria and replaces Ca^{2+}, Mg^{2+} in the LPS layer. This process destroys the stability of LPS and increases the permeability of the outer membrane of the bacteria, to sensitizing bacteria to other antibiotics. According to previous studies, synergism mainly occurs between highly membrane-active AMPs, or between highly membrane-active AMPs and antibiotic with intracellular targets. However, the synergistic effect of AMPs like AAMPs with non-membrane disruption mechanism is less studied, like AAMPs is less studied. In our previous study, an AAMP, EADE, was found to only inhibit *S. aureus* by chelating Ca^{2+}. It also showed Mg^{2+} chelating ability. Therefore, whether AAMP can also increase the permeability of bacterial outer membrane and to sensitize bacteria to another CAMP should be evaluated.

Thus, the aim of this study was to isolate CAMP from chicken plasma and to study its mechanism. In addition, synergistic effect of EADE and CAMP was thoroughly investigated

5.3 Materials and Methods

5.3.1 Chicken plasma preparation

Fresh chicken blood was obtained from a slaughterhouse (Betagro, Lopburi, Thailand) and sodium citrate was added to final concentration of 4% to prevent clotting. Upon arrival, sample were centrifuged at 2,000×g for 10 min at 4°C to collect plasma. Plasma samples were dialyzed in 3-kDa dialysis membrane in DI water at 4°C for 18 h. subsequently, dialyzed samples were lyophilized and kept in a -20°C freezer.

5.3.2 Preparation of chicken plasma hydrolysates (CPH)

Plasma powder was dispersed in deionized water at concentration of 100 mg/ml, and pH was adjusted to 2.5. Enzymatic hydrolysis was performed using porcine pepsin (Sigma-Aldrich Chemie Gmbh, Steinhem, Germany) with an E/S ratio of 3/100 (w/w) at 37°C. After 4 h of hydrolysis, reaction was stopped by heating at 95°C and cooled to 4°C on ice bath. Finally, the sample was centrifuged at 10,000×g for 20 min and refereed to as chicken plasma hydrolysate (CPH) which was kept in a -80°C throughout the study.

5.3.3 Purification and identification of antibacterial peptides

Two mL of diluted CPH were loaded onto a Puriflash C18-AQ 15 µm F0012 flash column (Interchim, Montlucon, France). The mobile phases were composed 0.05% (v/v) trifluoroacetic acid in water (A) and 0.05% trifluoroacetic acid in acetonitrile (B). The elution at 15 ml/min was performed using the following gradients: 2% B, 10 CV; 10% B, 10 CV; 20% B, 10 CV; 20%-100% B, 5 CV; 100% B, 3 CV; 100%-2% B, 2 CV and 2% 5 CV. The highest antibacterial activity fraction was selected for peptide identification. Molecular mass and peptide sequence were analyzed by Dionex UltiMate 30000 UHPLC+ focused syntems (Thermo Fisher Scientific Inc., Waltham, MA, U.S.) coupled with a microTOF QII mass spectrometer (Bruker Daltonics, Bremen, Germany). The AdvanceBio peptides plus (150 mm×4.6

mm×2.7μm) (Agilent Technologies, Santa Clara, CA, USA) was used for peptide separation at a flow rate of 0.5 mL/min, using deionized water containing 0.1% formic acid as solvent A, and acetonitrile containing 0.1 % formic acid as solvent B. Elution was performed as follows: 0-5 min (2% B), 5-35 min (2-35% B), 35-40 min (35-95% B), 40-45 min (95% B), 45-47 min (95-2% B) and 47-55 min (2% B). The LC-QTOF data were collected and processed by compass 1.3 software and data were analyzed using the PEAKS Studio 10.0 software (Waterloo, ON, Canada).

5.3.4 Determination of antibacterial activity

Antibacterial activity of activity of fraction and synthetic peptide were carried out according to Fu et al. (2022). Briefly, the single colony of *Staphylococcus aureus* ATCC25923, *Bacillus cereus* DMST 5040, *Escherichia coli* ATCC 25992 and *Salmonella* Typhimurium TISTR 292 were diluted with Mueller Hinton Broth (MHB), and 50 μL of diluted cell cultures were added to a 96-well microtiter plate. Fifty μL of samples were sterilized by filtering through a 0.22 μm membrane and were added to each well and incubated at 37°C for 18 h. Positive and negative controls were also prepared using 20 mg/L Kanamycin and DI water, respectively. Absorbance (OD600) was determined using Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA). The minimum inhibitory concentration (MIC) of each sample was determined. Briefly, 2-fold serial dilutions of each sample were incubated with each culture in a 96-well microtiter plate at 37°C for 18 h. The lowest concentration showing growth inhibition as assessed by absorbance at 600 nm was determined as MIC.

5.3.5 Confocal laser scanning microscopy analysis (CLSM)

CLSM was evaluated according to method described by Wang et al. (2019) with slight modifications. Briefly, *S.* Typhimurium (OD=0.2) were incubated with synthetic peptide (1×MIC) at 37°C for 1h. The cells were centrifuged at 1500×g for 10 min, wash 2 times with 10 mM phosphate buffered saline (PBS) and resuspended with propidium iodide (PI) at a final concentration of 10μg/mL in 10 mM PBS (pH 7.2). After incubation for 30 min at 4°C, the unbound dye was removed by washing with excess PBS. Control cells were prepared in the absence of peptides. Then, 10 μL of the cell

suspension was transferred onto a glass slide and observed using a Nikon A1Rsi CLSM at an excitation wavelength of 535 nm.

5.3.6 Scanning Electron Microscopy (SEM)

The morphology of *S.* Typhimurium with and without CP-1 treatments were observed using a field emission scanning electron microscope (ZEISS Gemini, Carl Zeiss, Germany). Sample preparation was carried out by Wang et al.(2019) with slight modifications. Briefly, mid-logarithmic *S.* Typhimurium were treated with CP-1 at 1×MIC or the combined 0.5 mM CP-1 and 1 mM EADE at 37°C for 1 and 4 h. Subsequently, cells were harvested and fixed with 2.5% glutaraldehyde at 4°C overnight and dehydrated by 20-100% of acetone. Dried cells were coated with gold and observed at an accelerating voltage of 3 kV and a magnification of 10,000.

5.3.7 DNA binding ability by molecular simulation

The structure of peptide CP-1 was generated by tools of Yinfo Cloud Computing Platform (https://cloud.yinfotek.com). The crystal structure of DNA was obtained from protein data bank (PDB:1BNA). The binding conformations of CP-1 and DNA were predicated using a HDOCK web server based on a hybrid docking algorithm (http://hdock.phys.hust.edu.cn/) (Yan et al., 2017). The binding complex of peptide and DNA were further subjected to molecular dynamics (MD) simulations to investigate their bind stabilities. MD simulations was also performed on Yinfo Cloud Computing Platform. AMBER ff19SB and parmbsc 1 force fields were applied to CP-1 and DNA, respectively. MD simulations were performed using AMBER 20 software for 100 ns (Case et al., 2020).

5.3.8 Synergistic effect of AMPs

5.3.8.1 Outer membrane permeability

Briefly, single colony of *S.* Typhimurium TISTR 292 were diluted with MHB, and 50 μL of diluted cell cultures were added to the 96-well microtiter plate, then 50μL of the mixture of the anionic peptide, EADE, and the cationic peptide identified from this study, CP-1 at two ratio, (1:1 mM and 1:2 mM) were added to each well and incubated at 37°C for 18 h. Then, absorbance at 600 nm was determined.

In addition, the outer membrane permeability of *S* . Typhimurium was determined and carried out by Briers et al. (2011) with some modifications. Briefly, *S.*

Typhimurium were grown in Luria broth (LB) at 37°C for 6 h to attain OD_{600}=0.4. Cells cultures were centrifuged at 3,000×g, 10 min Cell pellets were resuspended in 5 mM HEPES buffer (pH 7.2). Subsequently, 100 μL of cell suspension was mixed with 50 μL of 40 mM NPN and 50 μL of 4 mM CP-1 or 50 μL of the mixture of CP-1 and EADE at ratio of 2:1 (4mM/2mM) in a sterile 96-well black plate. The fluorescence was recorded after incubation at room temperature for 1h at excitation and emission wavelength at 350 and 420 nm, respectively. The relative uptake factor was calculated using the following equation:

$$\text{1: Uptake factor peptides} = \frac{\text{Fluo (cells+peptides+NPN)} - \text{Fluo (cells+sample)}}{\text{Fluo (buffer+NPN)} - \text{Fulo (buffer)}}$$

$$\text{2: Uptake factor cells} = \frac{\text{Fluo (cells+NPN)} - \text{Fluo (cells)}}{\text{Fluo (buffer+NPN)} - \text{Fluo (buffer)}}$$

Relative uptake factor=Uptake factor peptides-Uptake factor cells

Note: Fluo=Fluorescence

5.3.8.2 Stability of heterodimer of CP-1 and EADE

The heterodimer of CP-1 and EADE was generated using a HDOCK web server (http://hdock.phys.hust.edu.cn/). Then, heterodimer of CP-1 and EADE were further subjected to molecular dynamics (MD) simulations to evaluate heterodimer stability. MD simulations were performed on a Yinfo Cloud Computing Platform (https://cloud.yinfotek.com). AMBER ff19SB force fields were applied. MD simulations were performed using AMBER 20 software 10 ns.

5.4 Results and discussion

5.4.1 Purification and dissociation

Crude protein hydrolysates were purified with Flash C18-AQ chromatograph, resulting in 7 fractions (Fig. 5.1). Antibacterial activity was detected in fraction 2 and 3. Fraction 3 was found to contain an anionic AMP identified to be EADE as reported previously (Tian et al. 2022). The fraction 2 was identified by LC-MS/MS to contain a cationic AMP, namely CP-1 composing of 8 amino acids residues,

KPKVLLHA (Fig.5.2) with molecular weight 905.13 Da. The CP-1 is considered a novel peptide based on the antibacterial peptide database (http://aps.unmc.edu). CP-1 inhibited *S.aureus, S. Typhimurium* and *E.coli* at 1 mM with approximately of 15%, 71% and 51%, respectively. MIC value of CP-1 against *S. Typhimurium* was 2 mM. Most cationic AMPs have similar features of at least 2 positively charged amino acids as well as higher hydrophobic proportion (≥50%). The positively charged amino acids promote the binding of the AMPs to the negatively charged components of the bacterial cell surface, and then the hydrophobic amino acids of AMP interact with the lipid bilayer, resulting in disruption of cell membrane. Examples of cationic AMPs included HHRRFSLY isolated from cottonseed hydrolysates which inhibit *E.coli* with the IC_{50} value of 0.26 mg/mL (Kong et al., 2020). GLSRSLFTALK isolated from protein hydrolysate of anchovy cooking wastewater, can inhibit 7 test bacteria, among which it was most sensitive to *S.aureus* ATCC25923 and *B.subtilis* ATCC 9372 with MIC value of 16 μg/mL (Tang et al., 2015). The antibacterial activity of CP-1 may be attributed to its positive charges (+3) and relatively high hydrophobicity (65%). For Gram-negative bacteria, AMP need to permeabilize or disrupt outer and cytoplasmic membranes (Li et al., 2017). The outer leaflet is primarily a coat of lipopolysaccharide (LPS). LPS molecules are decorated with a high number of negatively charged phosphate groups that are engaged in salt-bridges with divalent cations (e.g., Ca^{2+} and Mg^{2+}), which is main barrier for most antibiotics (Li et al., 2017). The cationic characteristic of CP-1 might displace divalent cations and destabilize LPS. This led to local disturbance in the outer membrane, which allows CP-1 to easily cross the outer membrane and reached the cell membrane to complete its killing action. Antimicrobial activity of CP-1 toward *S. aureus* was only 15%. It has been reported that *S. aureus* can incorporate positively charged polymers into the cell membrane, which reduces electronegativity of cell membrane, limiting interactions of cationic AMPs to bacterial cell (Assoni et al., 2020). This may be one of reasons why CP-1 was less effective against *S. aureus* than gram-negative *S. Typhimurium*.

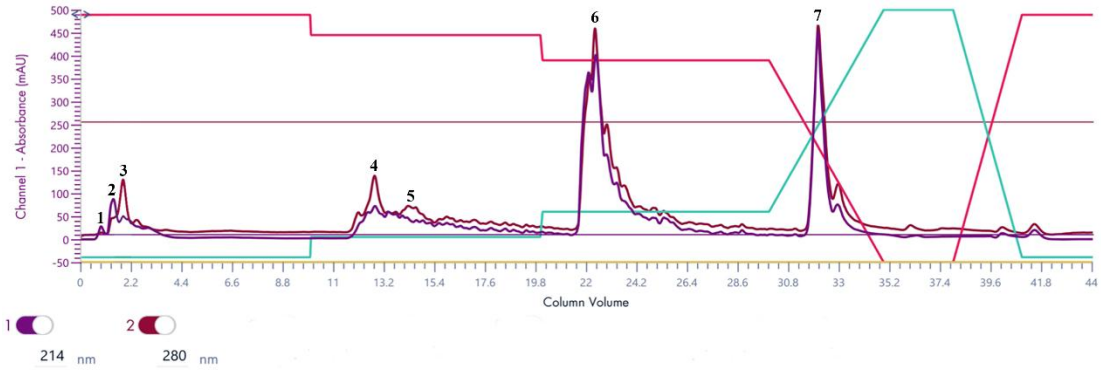

Fig. 5.1 C18-AQ flash chromatogram of peptides from chicken plasma hydrolysate.

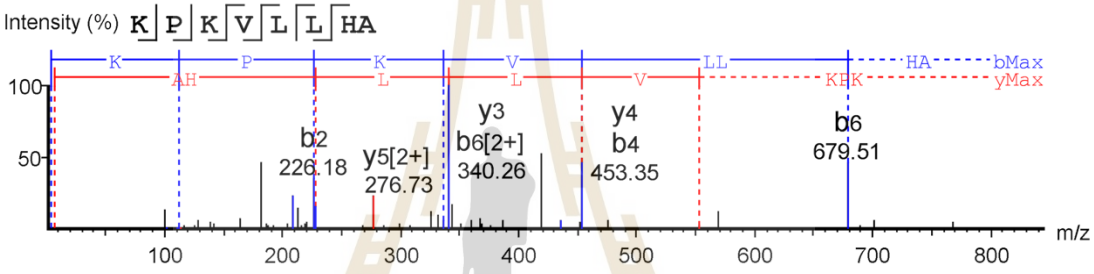

Fig. 5.2 MS/MS spectra of CP-1

5.4.2 Analysis of membrane integrity

Cell-impermeable dye PI emits fluorescence after binding to nucleic acids, indicating disintegration of cell membrane (Tang et al., 2014). There was no significant fluorescent signal observed in the control (without peptide), indicating full integrity of cell membranes (Fig. 5.3). A small portion of *S.* Typhimurium cells emitted red fluorescence after exposure to CP-1 for 1 h, suggesting disruption of cell membrane. AMP have been reported to increase membrane permeability. Exposure of peptide KDFPGRR for 30 min resulted in significant PI signal in *E.coli* (Song et al., 2020). Moreover, antibacterial peptide killed bacteria mainly by destroying cell membrane integrity, causing leakage of intracellular compounds and eventually cell death (Tang, Shi, Zhao, Hao, and Le, 2009). This study demonstrated that CP-1 destroyed cell membrane integrity of *S.* Typhimurium.

5.4.3 Scanning electron microscopy (SEM)

In the absence of CP-1, cell morphology of *S.* Typhimurium showed normal surface without noticeable damage (Fig. 5.4). After treatment with CP-1 for 1 h,

small pores were observed on cell surface and larger pore sizes were observed after 4 h exposure. It has been reported that AMPs interact directly with bacterial cell membrane, causing membrane permeability and rapid death (Li, Xiang, Zhang, Huang, and Su, 2012). Thus, SEM results provide morphological evidence that CP-1 can act on cell membrane, which is a typical characteristic of cationic antimicrobial peptide.

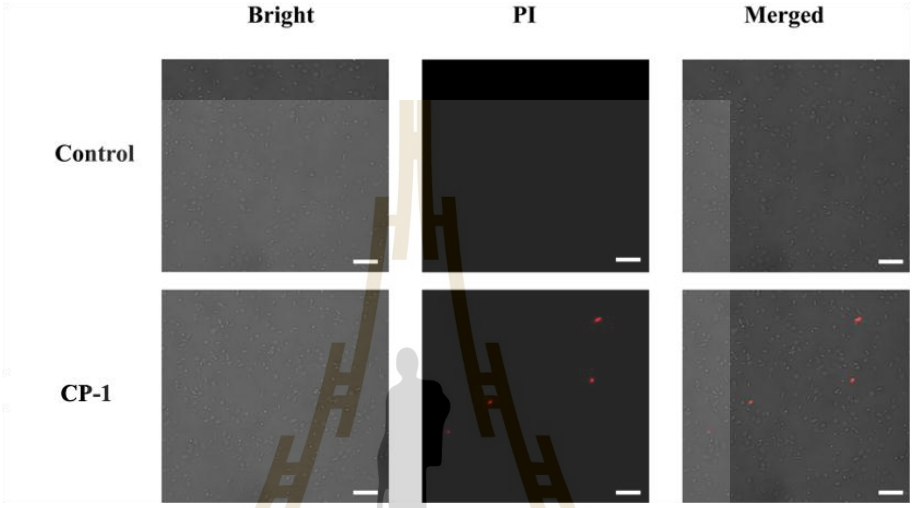

Fig. 5.3 CLSM images of *S.* Typhimurium treated with PI and CP-1 at 1×MIC. The scale bars=10 μm.

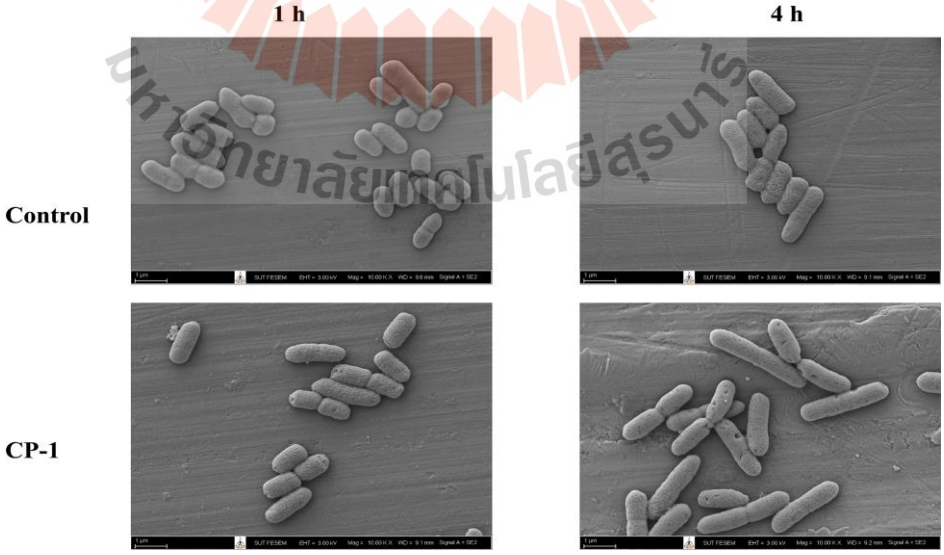

Fig. 5.4 SEM images of *S.* Typhimurium treated with CP-1 at 1×MIC for 1 and 4 h. (magnification: 10,000×). The scale bars=1 μm.

5.4.4 DNA-binding prediction by molecular simulation

In addition to inducing cell membrane disturbing, AMPs also can inhibit synthesis of intracellular targets such as DNA, enzymes, and protein. Peptide CM4 has been reported to permeate to cells and interacted with polyanionic DNA through its positive charges(Li et al., 2020). Thus, high positively charged CP-1 could result in high affinity with DNA. The MD simulation was used to predict the binding capacity of CP-1 and DNA. RMSD revealed that complex of CP-1 and DNA was stable. The stable binding conformation showed that CP-1 bound to DNA minor groove by forming intermolecular hydrogen bonds (Fig.5.5a). The N-terminal Lys of CP-1 contributed the most to the binding, forming hydrogen bond with the phospholipid backbone of DNA. In addition, Val 4 formed additional hydrogen bonds with phospholipid backbone of DNA. Based on these results, CP-1 might initially cause bacterial cell membrane damage, increasing permeability of cell membrane, and further interacting with intracellular targets, such as DNA.

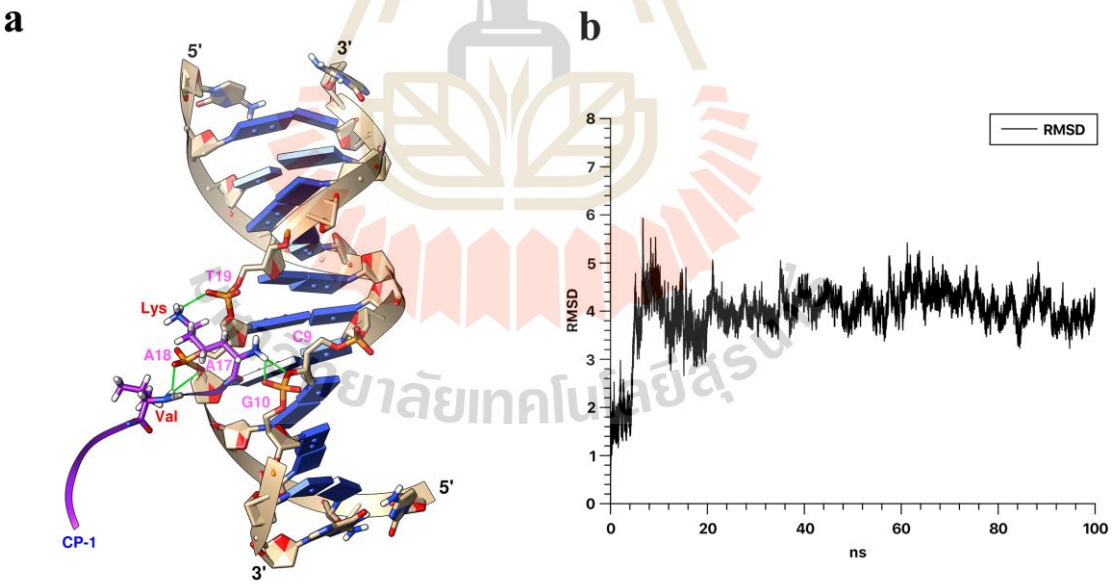

Fig. 5.5 a. Illustration of MD-equilibrated conformation between CP-1 and DNA; b. RMSD of binding complex between CP-1 and DNA.

5.4.5 Synergistic effect of CP-1 and EADE against *S.* Typhimurium

A combination of ethylenediaminetetraacetic acid (EDTA) and lysozyme is commonly used to disrupt Gram-negative bacteria. EDTA chelates Mg^{2+} and Ca^{2+}, destabilizing outer membrane of gram-negative bacteria. Subsequently, lysozyme enters periplasmic space and hydrolyzes 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in a peptidoglycan (Wooley and Blue, 1975). An anionic peptide, EADE, isolated from chicken plasma only inhibit *S. aureus* and exhibited potent chelating ability (Tian et al., 2022). It is plausible that EADE might be able to modify permeability of outer membrane and enhance antibacterial effect of CP-1. When 0.5 mM EADE was combined with 1 mM CP-1, the maximum antibacterial ability was attained (Fig.5.6 a). Permeability of outer membrane of *S.* Typhimurium treated with the combined peptides was greater than cell subjected to CP-1 alone (Fig.5.6 b). The SEM results also showed more, and larger pores were observed on the cell surface after treatment with the combined EADE and CP-1, , as comparing to CP-1 alone (Fig. 5.7). EADE was likely to change the permeability of outer membrane by chelating Mg^{2+} and Ca^{2+} on outer membrane, thus allowing CP-1 to cross the outer membrane and reach the cell membrane to complete its antibacterial action. Two AMPs namely Magainin2 and PGLa, isolated from skin of the African frog *Xenopus laevis*, were mixed at a 1:1 molar ratio, many heterodimers were found and membrane permeabilization were increased (Hara et al., 2001; Matsuzaki et al., 1998; Zerweck et al., 2017). Thus, synergy Magainin2 and PGLa seems to be due to the formation of heterodimers, which can insert into the cell membrane and change membrane permeabilization. (Ma et al., 2020). Synergism of EADE and CP-1 related to heterodimers formation could be another possibility. However, when MD was applied formation of heterodimer between these 2 peptides was unlikely (Fig.5. 8). With only 10 ns simulation, EADE and CP-1 were separated (Fig.5.8 a) and RMSD also revealed the unstable conjugation (Fig.5.8 b). Thus, it is more likely that the observed synergism was mainly attributed from chelation ability of EADE, which sequester Mg^{2+} and Ca^{2+} from outer membrane and disrupt outer membrane stability. This would eventually allow more intracellular accessibility of CP-1.

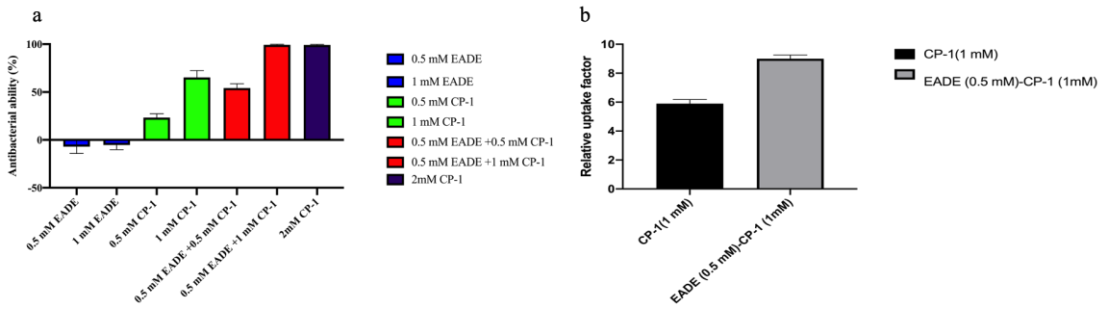

Fig. 5.6 a. Antibacterial ability toward *S.* Typhimurium as affected by different molar ratios of EADE and CP-1; b. Outer membrane permeability of *S.* Typhimurium treated by 1 mM CP-1 and EADE-CP-1 at 0.5:1 mM, respectively.

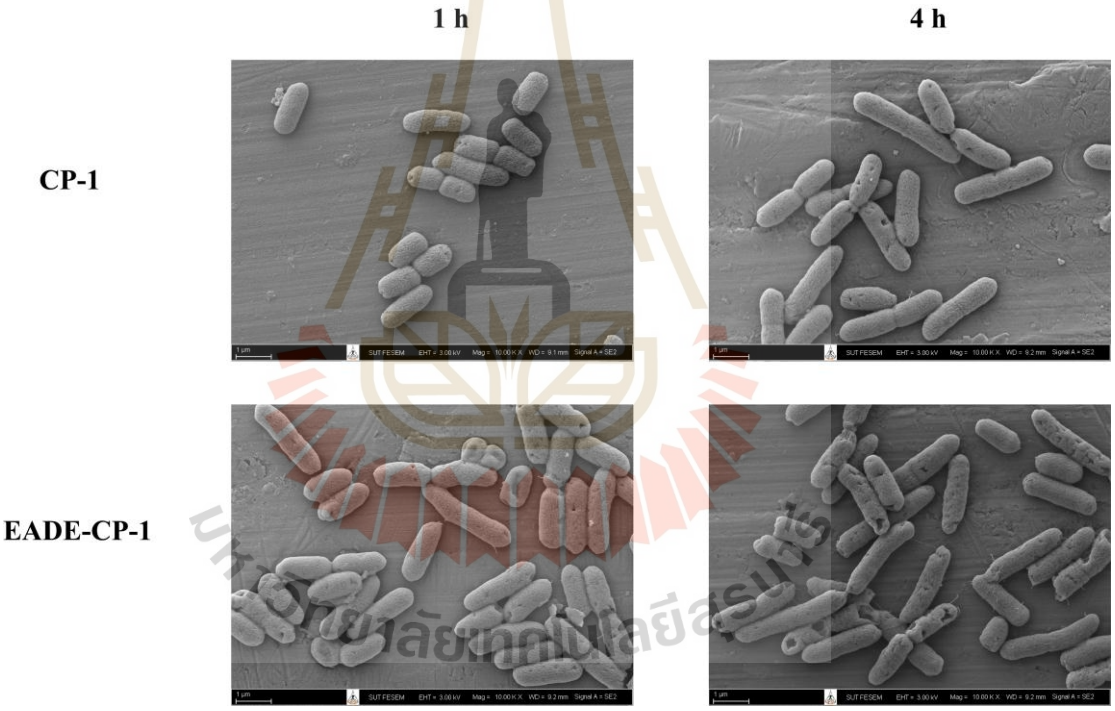

Fig. 5.7 SEM images showing morphological changes of *S.* Typhimurium subjected to 2 mM CP-1 and the combined EADE and CP-1 at 0.5:1 mM for 1 and 4 h. (magnification: 10,000×). The scale bars=1 μm

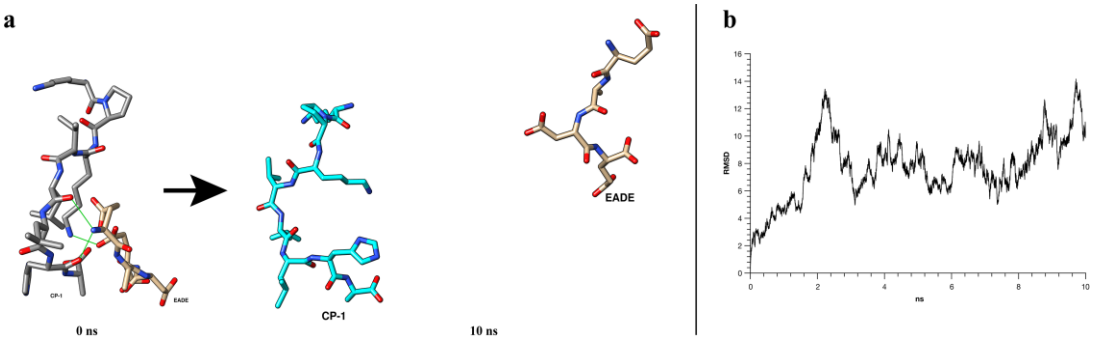

Fig. 5.8 a. Conformation of CP-1-EADE after 10 ns; b. RMSD of CP-1-EADE.

5.5 Conclusions

A cationic antibacterial peptide CP-1 isolated from chicken plasma hydrolysate showed good antibacterial activity towards *S.* Typhimurium with MIC value of 2 mM. CP-1 disturbed the integrity of the cell membrane. In addition, CP-1 showed good binding affinity to DNA, suggesting that DNA maybe one of intracellular targets for CP-1. Furthermore, an anionic peptide, EADE, was found to have synergistic effect with CP-1. The combined CP-1 and EADE could have potential in controlling Gram-negative bacteria, particularly, *S.* Typhimurium. Further studies on food system would be needed to verify the antibacterial activity of CP-1 and EADE in food matrix.

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CHAPTER VI

SUMMARY

Chicken blood plasma is a potential source for production of AMPs. Two novel AMPs, VSDH and CCCPKAF, were isolated from chicken plasma hydrolyzed by Alcalase. These two AMPs displayed unique characteristics with different modes of action toward *Bacillus cereus*. CCCPKAF exhibited lower positive charge and disturbed the integrity of the cell membrane, which is a typical characteristic of AMP. However, VSDH interacted with intracellular components, including DNA and protein synthesis as revealed by SR-FTIR and molecular docking. In addition, VSDH also chelated Mg^{2+}, depriving an essential trace element for growth.

Oher two novel AMPs, EADE and CP-1 (KPKVLLHA), were also isolated from pepsin-hydrolyzed chicken plasma. EADE was an anionic antibacterial peptide, which utilized metal chelation ability to inhibit *S. aureus.* EADE bound to Ca^{2+}, resulting in insufficient Ca^{2+} uptake and limited growth. CP-1 was a cationic AMP, showing antibacterial ability toward *Salmonella typhimurium*. CP-1 disturbed the integrity of cell membrane. In addition, DNA could be another target of CP-1 based on molecular simulation. Furthermore, EADE was found to have a synergistic effect on antibacterial activity with CP-1. When 0.5 mM of EADE was added, MIC of CP-1 was reduced by 50%. EADE changed permeability of outer membrane, allowing CP-1 to cross reach cell membrane to greater extent. Therefore, this research could enhance the utilization of chicken blood, and these peptides might be a promising source of natural antibacterial agents in food

BIOGRAPHY

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