Final Report

Design, synthesis and characterization of antimicrobial and antibiofilm peptides derived from *Crocodylus siamensis* hemoglobin with high bacterial cell selectivity

Assist. Prof. Dr. Nisachon Jangpromma

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Assist. Prof. Dr. Nisachon Jangpromma

Department of Integrated Science, Forensic Science Program, Faculty of Science, Khon Kaen University

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Abstract (บทคัดย่อ)

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Investigator : Assist. Prof. Dr. Nisachon Jangpromma

E-mail Address : nisaja@kku.ac.th

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บทคัดย่อ

จากการศึกษาฤทธิ์ทางชีวภาพของฮีโมโกลบินจากเลือดจระเข้สยาม (*Crocodylus siamensis*) พบเปปไทด์ที่มีฤทธิ์ทางชีวภาพที่หลากหลาย คือ เปปไทด์ QL17 (QAIIHNEKVQAHGKKVL) ซึ่งเป็นเปปไทด์ที่มีความสามารถในการต้านเชื้อจุลชีพ ลดการอักเสบ และลดการเกิดออกซิเดชัน อย่างไรก็ตามเพื่อเพิ่มประสิทธิภาพของเปปไทด์ให้มีความจำเพาะของฤทธิ์ทางชีวภาพมากยิ่งขึ้น งานวิจัยชิ้นนี้จึงได้ทำการออกแบบเปปไทด์ชนิดใหม่โดยใช้เปปไทด์ดังกล่าวเป็นแม่แบบ การออกแบบทำโดยการเพิ่มประจุบวกให้กับเปปไทด์ด้วยกรดอะมิโน lysine (K) และ arginine (R) ในขณะที่ความเป็นไฮโดรโฟรบิกของเปปไทด์นั้นจะเพิ่มด้วยการเติมกรดอะมิโน tryptophan (W) โดยจะใช้แผนภาพจาก helical wheel projection ในการทำนายโครงสร้างของเปปไทด์ จากผลการออกแบบเปปไทด์ทำให้ได้เปปไทด์เส้นใหม่จำนวน 5 เส้น ได้แก่ IL15NJ1 (IKHWEKVWKHGKKVL), IL15NJ2 (IKHWKKVWKHGKKVL), IL15NJ3 (IKHWKKVWKHAKKVL), IL15NJ4 (IKHWKKVWKHWKKKL) และ IL15NJ5 (IRHWRRVWRHWRRRL) ซึ่งมีประจุบวก +4, +6, +6, +7, +7 ตามลำดับ และมีค่าไฮโดรโฟรบิกเฉลี่ยในช่วง 40-46% เมื่อทำการทดสอบฤทธิ์ต้านแบคทีเรีย พบว่าเปปไทด์ทั้ง 5 เส้น มีความสามารถในการต้านแบคทีเรียเพิ่มขึ้น และมีความเป็นพิษต่ำทั้งต่อเซลล์เม็ดเลือดแดงของมนุษย์ (human red blood cell), เซลล์เม็ดเลือดขาวมนุษย์ (human peripheral blood mononuclear cell; PBMC), เซลล์เคอราติโนไซต์จากมนุษย์ (HaCaT), เซลล์ไฟโบรบลาสต์จากมนุษย์ (human fibroblast cell) และเซลล์แมคโครฟาจของหนู (RAW 264.7) นอกจากนี้เมื่อทำการศึกษาความจำเพาะของเปปไทด์ต่อแบคทีเรีย พบว่าเปปไทด์ IL15NJ3 แสดงความจำเพาะสูงสุดในการทำลายเชื้อ

แบคทีเรียโดยแสดงค่า selectivity index ถึง 500 เมื่อเทียบกับเปปไทด์แม่แบบ และจากการศึกษาโดยใช้เทคนิค flow cytometry สามารถยืนยันได้ว่าเปปไทด์ IL15NJ3 มีความจำเพาะในการทำลายเชื้อแบคทีเรียมากกว่าเซลล์เพาะเลี้ยงของมนุษย์ ผลจากการศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดแสดงให้เห็นว่าเปปไทด์ IL15NJ3 มีกลไกในการเข้าทำลายแบคทีเรียผ่านทางเมมเบรน นอกจากนี้เปปไทด์ IL15NJ3 ยังแสดงกิจกรรมต้านการสร้างไบโอฟิล์มของแบคทีเรีย *Pseudomonas aeruginosa* ได้ เมื่อทำการศึกษาการแสดงออกของโปรตีนจากเชื้อแบคทีเรียที่สร้างไบโอฟิล์มเมื่อได้รับเปปไทด์ IL15NJ3 โดยใช้เทคนิคโปรติโอมิกส์ พบว่าการแสดงออกของโปรตีนในกลุ่มที่ทำหน้าที่ในการเคลื่อนที่และยึดเกาะกับพื้นผิว เช่น flagellin protein และ type 1 fimbrial protein มีการแสดงออกที่ลดลง รวมถึงการแสดงออกที่ลดลงของโปรตีนที่เกี่ยวข้องในระบบ quorum sensing เช่น TrbL protein และ phenazine biosynthesis protein เมื่อศึกษาฤทธิ์ทางชีวภาพอื่นๆ พบว่าฮีโมโกลบินจากเลือดจระเข้สยามและฮีโมโกลบินไฮโดรไลเสต (CHHs) ที่ได้จากการย่อยด้วยเอนไซม์ทริปซินและปาเปน ที่ความเข้มข้น 125-500 ไมโครกรัมต่อมิลลิลิตร แสดงฤทธิ์ต้านการอักเสบ ด้วยการลดปริมาณไนตริกออกไซด์ (NO) ที่ถูกสร้างขึ้นจากการกระตุ้นด้วย LPS ในเซลล์แมคโครฟาจ (RAW 264.7) โดยพบว่า CHH ที่ได้จากการย่อยด้วยปาเปนเป็นเวลา 2 ชั่วโมง แสดงฤทธิ์การต้านอักเสบได้ดีที่สุด สำหรับเปปไทด์สังเคราะห์ IL15NJ3 ที่ความเข้มข้น 100 และ 200 ไมโครกรัมต่อมิลลิลิตร แสดงกิจกรรมต้านการอักเสบได้อย่างมีประสิทธิภาพ และยังพบกิจกรรมต้านการเกิดออกซิเดชันของ Hb, CHHs และเปปไทด์สังเคราะห์ทุกเส้น ซึ่งจากผลการวิจัยทั้งหมดแสดงให้เห็นว่าเปปไทด์ IL15NJ3 มีศักยภาพอย่างมากในการแสดงฤทธิ์ทางชีวภาพที่หลากหลาย ทั้งฤทธิ์ต้านอักเสบ ฤทธิ์ต้านออกซิเดชัน ฤทธิ์ต้านเชื้อแบคทีเรียทั้งแกรมบวกและแกรมลบ โดยเฉพาะอย่างยิ่งความจำเพาะต่อการต้านเชื้อแบคทีเรียกลุ่มที่สร้างไบโอฟิล์ม ดังนั้นองค์ความรู้ที่ได้จากงานวิจัยนี้น่าจะเป็นประโยชน์ในแง่ของการพัฒนาใช้เปปไทด์สำหรับเป็นสารปฏิชีวนะทางเลือกใหม่ในการรักษาโรคติดเชื้อในอนาคต

คำสำคัญ : เปปไทด์ต้านจุลชีพ, ยับยั้งไบโอฟิล์ม, ดื้อยาปฏิชีวนะ, การเลือกเซลล์, จระเข้

Abstract

In a previous study, we reported a peptide derived from *Crocodylus siamensis* hemoglobin hydrolysate, named QL17 (QAIIHNEKVQAHGKKVL); that exhibited antimicrobial, anti-inflammation and antioxidant. To improve the antimicrobial activity of this peptide, it was used as the template to design novel effective peptides. The helical wheel diagram was used to monitor and evaluate amphipathic after the position change and substitution of certain amino acids. Lysine (K) and arginine (R) were appropriately selected to extend the hydrophilicity, whereas hydrophobic residues such as leucine (L), isoleucine (I) or tryptophan (W) were used to increase the hydrophobicity. As appropriate, the results of novel peptides design were given 5 peptides; named as IL15NJ1 (IKHWEKVWKHGKKVL), IL15NJ2 (IKHWKKVWKHGKKVL), IL15NJ3 (IKHWKKVWKHAKKVL), IL15NJ4 (IKHWKKVWKHWKKKL) and IL15NJ5 (IRHWRRVWRHWRRRL). There was net charge +4, +6, +6, +7 and +7, respectively which containing hydrophobicity about 40-46%. The results found that all novel designed peptides have potent antimicrobial efficacy than template and less cytotoxic to macrophage cell, human red blood cell, human white blood cell and human skin cell (i.e. human keratinocyte (HaCaT) and normal human dermal fibroblasts (NHDF). The IL15NJ3 displayed great antimicrobial against both Gram-positive and Gram-negative bacterial. The cell selective of IL15NJ3, as selectivity index, indicating the value was increased up to 500-fold compared to QL17 parental peptide. In agreement with the results of cell-selective, the flow cytometry results revealed that IL15NJ3 exhibited antibacterial efficacy over than mammalian cell membranes leading to maximize antimicrobial activity while minimizing to toxicity. The scanning electron microscope showed that IL15NJ3 interacted with outer membrane components, activated a structural modification damaging cell membranes, which lead to death of bacteria cell. Interestingly, IL15NJ3 could inhibit *Pseudomonas aeruginosa* biofilm and successfully disrupted biofilm degradation. Moreover, the protein expression response from *P. aeruginosa* biofilm formation when treated with IL15NJ3 was then investigated using proteomic approach. The result demonstrated that the expression of proteins in the group that acted in the movement and adhesion to the surface, such as flagellin protein and type 1 fimbrial protein was reduced

in their expression level. In addition, the expression of proteins involved in the quorum sensing system, such as TrbL protein and phenazine biosynthesis protein, also decreased. Considering other biological effects, our result found that the intact hemoglobin (Hb) and *Crocodylus siamensis* hemoglobin hydrolysates (CHHs) derived from trypsin and papain digestion at concentrations of 125-500 μg/ml show a decrease in nitric oxide production in a dose-dependent manner. The strongest anti-inflammatory activity was found for CHH derived from 2-h papain hydrolysis. For IL15NJ3 peptide, the results exhibited the reduction of NO production by LPS-stimulated RAW264.7 cell at the concentration of 100 and 200 μg/ml. Finally, Hb, CHHs and all of designed peptides were suppressed free radical which showed antioxidant activity. All observation herein revealed that IL15NJ3 peptide could be promising to develop broad-ranging medical peptides with the ability to treat and prevent against drug-resistant bacteria that produce biofilms via the suppression of bacteria endotoxins and scavenge radical.

Keywords : Antimicrobial peptide, Antibiofilm, Antibiotic resistance, Cell selectivity, Crocodile

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CHAPTER I
INTRODUCTION

1.1 Introduction to the research problem and its significance

Antimicrobial resistance is a complex global public health challenge, and no single or simple strategy will suffice to fully contain the emergence and spread of infectious organisms that become resistant to the available antimicrobial drugs. The development of antimicrobial resistance is a natural phenomenon in microorganisms, and is accelerated by the selective pressure exerted by use and misuse of antimicrobial agents in humans and animals. The current lack of new antimicrobials on the horizon to replace those that become ineffective brings added urgency to the need to protect the efficacy of existing drugs. Similarly in the case of bacteria in biofilm also show up to 1000 times greater tolerance to antibiotics and biocides (Ceri et al. 1999). One of those bacterial resistance is bacterial biofilms that are communities of microorganisms living at an interphase (solid-liquid or liquid-air), where they attach to each other through the extracellular polymeric substance (EPS) also known as the biofilm matrix. The EPS, which consists of extracellular DNA, proteins, lipids and polysaccharides, offers structural stability and protection (Flemming and Wingender, 2010). Bacterial infections account for a significant proportion of the global infectious disease burden, and morbidity and mortality rates caused by infectious microbial agents pose serious public health concerns. This is exacerbated by increasing resistance to antibiotics which is significant in an era where the development of new, synthetic antibacterial drugs lags the emergence of antimicrobial resistance (Mbah et al. 2012). It is therefore paramount to broaden the search for new antimicrobial substances, including the exploitation of novel sources where possible (Song et al. 2012).

The Antimicrobial peptides (AMPs) are one of among agents that have been interested, isolated and characterized from a wide range of animal, plant and bacterial species and are known to play important roles in the host defence system and innate immunity of all species (Zasloff, 1992; Andreu et al. 1998; Zasloff, 2002 and Hancock et al. 2006). AMPs are effective at low micromolar concentrations against a broad range of microorganisms, including in many cases those resistant to traditional antibiotics (Hurdle et al. 2011). AMPs are defined as short (10–50 amino acids) peptides possessing an overall positive charge (in general, +2 to +9) and a large percentage (≥

30%) of hydrophobic amino acids (Zhang et al. 2016). Unlike conventional antibiotics that kill or inhibit the growth of microorganisms by targeting various biosynthetic processes in growing bacteria such as proteins synthesis, RNA synthesis, DNA synthesis, bacterial cell wall synthesis (i.e. the synthesis of peptidoglycan or folic acid) or enzyme inhibitors. AMPs can destabilize and compromise the physical integrity of the bacterial membrane and therefore are unlikely to evoke bacterial resistance (Ahmad et al., 2012; Zhang et al. 2016). In this context, AMPs appear as an alternative weapon against infectious antibiotic-resistant bacteria. However, therapeutic applications of these AMPs have been hindered by their cytotoxicity in mammalian cells or ability to lyse eukaryotic cells (Porto et al., 2012; Zhang et al. 2016). To resolve this obstacle, numerous structure-function studies of both natural and synthetic antimicrobial peptides have been employed to improve AMPs activity against the interested pathogen and reduce toxicity at the therapeutic dose. It has therefore become necessary to broaden the search for new antimicrobial substances including the exploitation of novel, natural sources where possible (Song et al., 2012). One such growing area of research has begun to focus on protein hydrolysates of animal origin (Arroume et al., 2006; Arroume et al., 2008; Adje et al., 2011; Osman et al., 2016). Among natural agents, crocodile bloods are considered to be a rich source of bioactive substances that suitable for therapeutic applications. One of crocodile blood major components is hemoglobin which carries oxygen throughout the body. It had been long associated with a broad spectrum of biological activity, for instance including antimicrobial (Srihongthong et al., 2012; Pakdeesuwan et al., 2017), antioxidant (Jandaruang et al., 2012; Srihongthong et al., 2012; Phosri et al., 2014; Maijaroen et al., 2016; Pakdeesuwan et al., 2017; Phosri et al., 2017) and anti-inflammatory activity (Phosri et al., 2014; Jangpromma et al., 2017; Phosri et al., 2017). Recent studied of Lueangsakulthai et al (unpublished work, with permission) reported that a peptic hemoglobin hydrolysate from *Crocodylus siamensis* has also been shown to possess antibacterial activity represented the MIC_{50} values against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* at concentrations of 20, 20, 20 and 10 mg/ml (w/v), respectively. Antibacterial peptide QAIIHNEKVQAHGKKVL (QL17) from *C. siamensis* hemoglobin hydrolysate with 41% hydrophobicity and +2 net charges was identified. However, natural peptide had short half-life in mammalian reticulocytes (*in vitro*) when estimated by ProtParam tool (http://web.expasy.org/protparam). As the future designing of novel AMPs will need to minimize the cytotoxicity in mammalian

cells and enhance antibacterial activity for use as antibiotic drugs (Maccari et al. 2013). Toward this goal, design and synthesized peptide should be urgently and extensively investigated. Rational design of AMPs is a modern approached to antibiotic development which can classify into three major classes including physicochemical, template-based and *de novo* methods. Two methods use a previously known AMP as the basis for designing studies. While physicochemical approaches generate several analogues with different physicochemical properties, the template-based methods search for size reduction, adding selectivity and/or killer activity to known sequences. Furthermore, *de novo* methods that generate AMP without a template sequence, using only frequencies or patterns (Porto et al. 2012).

The activity of many AMPs depends principally on membrane permeabilization or lysis, and several mechanisms of action have been proposed, ranging from the formation of highly organized transmembrane pores, to that of the more dynamic supramolecular peptide/lipid complexes leading to toroidal pores, or to membrane damage via a relatively disorganized, detergent-like disaggregation due to hydrophobic and/or electrostatic interactions with the membrane surface (Zelezetsky and Tossi 2006). One important class of membrane-interacting AMPs assumes an amphipathic, α-helical conformation that permits insertion of a well defined hydrophobic sector into the lipid bilayer (Tossi et al. 2000; Zelezetsky and Tossi 2006). They represent one of the most successful designs in nature, being produced by all types of organisms from bacteria to mammals (Zelezetsky and Tossi 2006). Generally, AMPs possessed an overall positive charge and a large percentage of hydrophobic amino acids which permit the peptides to fold into amphipathic conformations upon contact with cell membranes, and the positively charged polar face help the molecules bind to the biomembrane via electrostatic interaction with the negatively charged head groups of phospholipids. Then, the nonpolar face of the peptides allows insertion into the membrane through hydrophobic interactions, causing increased permeability and loss of barrier function of bacterial cells (Brogden, 2005; Zhang et al. 2016). As a rule of thumb, the cationicity of a peptide especially lysine (K) or arginine (R) was used to increases in direct proportion with inclusion of polar amino acids. Moreover, incorporation of hydrophobic residues such as alanine (A), leucine (L), isoleucine (I), phenylalanine (F) or tryptophan (W) to stabilizes the peptide structure and plays an important role in the membrane interaction by anchoring into the membrane interfacial region (Zelezetsky and Tossi 2006;

Thankappan et al. 2013). Consequently, based on the properties of amino acids and the QL17 peptide template, several series of peptides were designed and synthesized by using standard Fmoc solid phase of GL biochem (Shianghai, PR China). Each peptide was purified to ≥ 95% purity by using reverse-phase high-performance liquid chromatography (RP-HPLC). The molecular weight of each peptide was confirmed using electrospray ionisation mass spectrometry (ESI-MS). The *in vitro* antimicrobial and antibiofilm activity of the peptide against human pathogenic strains with different concentration and time intervals were performed. Moreover, the hemolytic property and the cytotoxicity to normal cells line were also determined to confirm the non-toxicity of peptide.

1.2 Objectives

The present research was aim to design, synthesis and characterize antibacterial peptide using natural peptides template from Siamese crocodile (*C. siamensis*) in order to improve their half-life, enhance performance of antibacterial activity and potent antibiofilm activity with cell selectivity, non-toxic against normal cell lines as well as no hemolytic activity.

1.3 Scope of research

The QL17, hemoglobin hydrolysate from *C. siamensis* (QAIIHNEKVQAHGKKVL) was used as the template to design several series of peptides in order to improve their half-life, enhance performance of antibacterial activity and potent antibiofilm activity with cell selectivity, non-toxic against normal cell lines as well as no hemolytic activity. The peptide was synthesized then was characterized their secondary structure in solution (water) and membrane-mimicking environments (1 mM liposome, 50 mM SDS, 20 mM Tris–HCl and 50% TFE) by using Circular dichroism (CD). The antimicrobial activity against Gram-positive and Gram-negative bacteria was determined by broth dilution and disc diffusion assay. The hemolytic property against human erythrocytes and the cytotoxicity against human peripheral blood mono nuclear cell (PBMC), Macrophage RAW 264.7 cells, normal human keratinocytes (HaCaT) and normal human dermal fibroblast (NHDF) were explored in order to screening of the non-toxic concentration rage of peptides. The selectivity index (SI) and flow cytometry was proposed to evaluate the selectivity of peptides. The potential membrane

destruction mechanisms of the peptides were investigated using flow cytometry and microscopic techniques. The antibiofilm of *P. aeruginosa* was studied in stage of inhibition of bacterial initial attachment, inhibition of biofilm formation and degradation. Moreover its mechanism was investigated to determine the effects of peptides on bacterial cells.

1.4 The anticipated outcomes

4.1 Novel synthetic peptide have potent antimicrobial activity and cell selective.

4.2 Mode of action of novel antimicrobial peptide is acquired.

4.3 Ability of novel peptide against biofilm, inflammatory and oxidant are acquired.

CHAPTER II
LITERATURE REVIEW

For several decades antimicrobial resistance (AMR) has been a growing threat to the effective treatment of an increasing range of infections caused by bacteria, parasites, viruses and fungi. AMR results in reduced efficacy of antibacterial, antiparasitic, antiviral and antifungal drugs, making the treatment of patients difficult, costly, or even impossible. The impact on particularly vulnerable patients is most obvious, resulting in prolonged illness and increased mortality. The magnitude of the problem worldwide and the impact of AMR on human health, and on costs for the health-care sector and the wider societal impact, are still largely unknown. Some estimates of the economic effects of AMR have been attempted, and the findings are disturbing. For example, the yearly cost to the US health system alone has been estimated at US $21 to $34 billion dollars, accompanied by more than 8 million additional days in hospital. AMR is a complex global public health challenge, and no single or simple strategy will suffice to fully contain the emergence and spread of infectious organisms that become resistant to the available antimicrobial drugs (WHO report). Some of the problematic drug-resistant pathogens encountered today include *Staphylococcus aureus***,** *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Bacillus subtilis*.

Escherichia coli

Escherichia coli is a gram-negative rod-shaped that normally inhabits the intestines of humans and other mammals. Most strains of *E. coli* are harmless, but actually some stains are important pathogenic causing serious illness, such as hemorrhagic colitis and hemolytic uremic syndrome (Wang et al., 2015). *E. coli* can be divided into four classifications base on their specific virulence factors and phenotypic traits (Jafari et al., 2012). Frist, enteropathogenic *E. coli* (EPEC), is a special kind of *E. coli* that lets it attach to intestinal cells, causes acute intestinal infections in infants in the developing world. Infection typically spreads through contaminated food and water and leads to severe, watery diarrhea (Chen and Frankel, 2004). Second, enterotoxigenic *E. coli* (ETEC) can produced enterotoxins, which may be heat labile and/or heat stable, and colonization factors which allow the organisms to readily colonize the small intestine and thus cause diarrhea (Qadri et al., 2005). Vero toxin-producing/Shiga toxin-

producing *E. coli* (VTEC/STEC) which include its well-known subgroup enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC). These produce Shiga toxin or Shiga-like toxin (verotoxin), which is the essential virulence factor, which can cause diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, and death (Smith et al., 2014). Finally, diffusely adherent *E. coli* (DAEC), which is the bacteria uniformly, cover the entire cell surface. DAEC is a cause of acute diarrhea (Isabel et al., 2002).

Klebsiella pneumonia

Klebsiella pneumonia is a gram-negative opportunistic pathogen, which causes hospital-acquired urinary tract infections, pneumonia, septicemias, and soft tissue infections. The principal pathogenic reservoirs for transmission of Klebsiella are the gastrointestinal tract and the hands of hospital personnel. Because of their ability to spread rapidly in the hospital environment, these bacteria tend to cause nosocomial outbreaks. (Podschun and Ullmann, 1998).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an efficient biofilm-forming gram-negative, rod-shaped opportunistic pathogenic bacterium responsible for both acute and chronic infections. It is commonly found in soil, water and man-made environments, however, this bacterium has also been known to cause serious disease in animals and humans often times with generalized inflammation of the lungs, urinary tract and kidneys (Cotton et al., 2009; Das et al., 2017). A long-standing problem in patient care is the ability of *P. aerugionsa*, to form biofilms on implanted and indwelling devices. These localized infections can be difficult to detect with routine clinical microbiology and very frequently fail to resolve with aggressive antibiotic therapy (Mulcahy et al., 2014). Moreover, biofilms of *P. aeruginosa* can cause chronic opportunistic infections, which are a serious problem for medical care in industrialized societies, especially for immunocompromised patients and the elderly. They often cannot be treated effectively with traditional antibiotic therapy. Biofilms seem to protect these bacteria from adverse environmental factors. Several hypotheses have been formed to explain the reduced susceptibility of biofilms to antimicrobials (Mulcahy et al., 2014). It has been observed that biofilm-forming bacterial strains develop antibiotic resistance very fast. Thus, the treatment of microbial biofilm with conventional antibiotics becomes troublesome.

Therefore, the uses of new antibacterial and anti-biofilm compounds have drawn more attention in order to prevent microbial growth and biofilm formation (Das et al., 2017).

Staphylococcus aureus

Staphylococcus aureus is gram-positive bacterium that naturally presents on human skin and find ubiquitously in the environment, which can cause numerous illnesses, from minor skin infections to life-threatening diseases, such as abscesses, pneumonia, meningitis, endocarditis, and septicemia (Chang et al., 2013). Moreover, *S. aureus* is one of the most commonly isolated foodborne pathogen and it is considered as a major cause of foodborne illnesses worldwide. *S. aureus* had capable to produce a numerous of toxins including *Staphylococcal enterotoxins*, toxic shock syndrome toxin-1 (TSST-1) and leukocidin, which cause damage to biological membranes by interacting directly with the host, leading to the cell death (Rubab et al., 2018).

Staphylococcus epidermidis

Staphylococcus epidermidis is a gram-positive bacterium, commensal bacterium that colonizes the skin and mucous membranes of mammals and is the most prevalent staphylococcal species found in humans (Fey and Olson, 2010). S. *epidermidis* is prominent role in the hospital-acquired and foreign body infections, such as breast prostheses, peritoneal dialysis catheters, and vascular grafts (Demirer et al., 2001).

Bacillus subtilis

Bacillus subtilis is a gram-positive, spore-forming bacterium, that normally found on soil and capable grow in diverse environments including the gastrointestinal tracts of animals. *B. subtilis* has also known to related genera troublesome to food producers on account of their resistant endospores (Earl et al., 2008). *B. subtilis* has been implicated in foodborne illness with vomiting as the commonest symptom, but accompanying diarrhoea was frequently reported too (Logan, 2011).

One of serious antimicrobial resistance (AMR) is bacterial biofilms that are communities of microorganisms living at an interphase (solid-liquid or liquid-air), where they attach to each other through the extracellular polymeric substance (EPS) also known as the biofilm matrix. The EPS, which consists of extracellular DNA, proteins, lipids and polysaccharides, offers structural stability and protection (Flemming and Wingender, 2010). One of the most important EPS is alginate that controlled by the *algACD* operon, is a linear polymer has been shown to increase biofilm attachment to

solid surfaces. Of high importance to alginate productions are the *algD* genes that encode the guanosine diphosphate (GDP) manose dehydrogenase enzymes, which rate-limiting those catalyses the production of GDP-mannuronic acid from GDP-mannose of *P. aeruginosa* (Cotton et al., 2009; Laverty et al., 2014). Then microcolonies expand into a mature biofilm. Also during this stage, EPS that serve as an adhesive matrix and trap nutrients from the environment continue to be produced. In *P. aeruginosa* biofilm model, produced the outer membrane porin from *OprF* gene, is a general outer membrane porin that acts as transport of extracellular products into the cell (nutrient and oxygen) (Fito-Boncompte et al., 2011). Furthermore, the increase in cell density also leads to the activation of quorum-sensing systems. Quorum sensing (QS) or cell-to cell signaling controls the expression of extracellular virulence factors. The most defined quorum sensing pathways in *Pseudomonas aeruginosa* are the *las* and *rhl* systems. The *las* system comprises of the transcriptional activator protein LasR and the autoinducer synthase enzyme LasI, coded for by *lasR* and *lasI*, respectively (Laverty et al., 2014; Rasamiravaka et al., 2015). Biofilms constitute a protected mode of growth that allows survival in a hostile environment. The structures that form in biofilms contain channels in which nutrients can circulate (DeBeer et al., 1994), and cells in different regions of a biofilm exhibit different patterns of gene expression (Davies et al., 1993). The complexity of biofilm structure and metabolism has led to the analogy of biofilms to tissues of higher organisms (Costerton et al., 1995). These sessile biofilm communities can give rise to nonsessile individuals, planktonic bacteria that can rapidly multiply and disperse. Biofilms develop preferentially on inert surfaces, or on dead tissue, and occur commonly on medical devices and fragments of dead tissue such as sequestra of dead bone (Lambe et al., 1991); they can also form on living tissues, as in the case of endocarditis. Biofilms grow slowly, in one or more locations, and biofilm infections are often slow to produce overt symptoms (Ward et al., 1992). Sessile bacterial cells can release antigens and stimulate the production of antibodies, but the antibodies are not effective in killing bacteria within biofilms and may cause immune complex damage to surrounding tissues (Cochrane et al., 1988). Even in individuals with excellent cellular and humoral immune reactions, biofilm infections are rarely resolved by the host defense mechanisms (Khoury et al., 1992). However antibiotic therapy typically reverses the symptoms caused by planktonic cells released from the biofilm, but fails to kill the biofilm (Marrie et al., 1982). For this reason biofilm infections typically show recurring symptoms, after cycles of antibiotic therapy, until

the sessile population is surgically removed from the body. Planktonic bacterial cells are released from biofilms, and evidence supports the notion that there is a natural pattern of programmed detachment (Costerton et al., 1995). Moreover bacterial biofilms are inherently resistant to antimicrobial agents. One mechanism of biofilm resistance to antimicrobial agents is the failure of an agent to penetrate the full depth of the biofilm. Polymeric substances like those that make up the matrix of a biofilm are known to retard the diffusion of antibiotics (Cheema et al., 1986), and solutes in general diffuse at slower rates within biofilms than they do in water (Stewart, 1998). Antibiotics have been shown to penetrate biofilms readily in some cases and poorly in others, depending on the particular agent and biofilm (Hoyle et al., 1992). A second hypothesis to explain reduced biofilm susceptibility to antibiotics posits that at least some of the cells in a biofilm experience nutrient limitation and therefore exist in a slow-growing or starved state (Brown et al., 1988). A third mechanism of reduced biofilm susceptibility, which is more speculative than the preceding hypotheses, is that at least some of the cells in a biofilm adopt a distinct and protected biofilm phenotype. This phenotype is not a response to nutrient limitation; it is a biologically programmed response to growth on a surface.

In this light, it is not surprising that an impressive number of chronic bacterial infections involve bacterial biofilms, which are not easily eradicated by conventional antibiotic therapy. Thus, there is an urgent need to develop new therapies against this important human pathogen. New approaches, such as dispersal agents and microbial interference, are currently being developed for treating biofilm-associated infections (Taylor et al., 2011; Shah et al., 2013; Nguyen et al., 2015). As microorganisms are developing resistance to all the major antibiotics, there is an increased demand for the development of alternative methods for resisting microbial infections (Babu et al., 2016; Chatterjee et al., 2016). In this daunting scenario, host defense (antimicrobial) peptides (HDPs) have emerged as a promising alternative to traditional antibiotics for the treatment of persistent infections caused by biofilms (Jorge et al., 2012; de la Fuente-Núñez et al., 2016). HDPs were originally termed antimicrobial peptides (AMPs) that constitute the major component of the innate immune system of most living organisms, including mammals, insects, bacteria and fungi. In conferring protection to the organism from microbial attack, these molecules exhibit multiple mechanisms of action and, consequently, a low potential to select for resistance in bacteria (Fjell et al., 2012; de la Fuente-Núñez et al., 2016). The AMPs may have a

broad spectrum of antibacterial and antifungal activities. Moreover, in some cases, antiviral, antiparasitic and antitumor activities have also been observed (Nijnik and Hancock, 2009; Porto et al., 2012). These peptides are generally small in size ranging from 12 to at least 50 amino acids in length and are cationic due to the presence of excess lysine or arginine amino acid residues (Porto et al., 2012; de la Fuente-Núñez and Hancock, 2015). AMPs also contain a high proportion (usually>50%) of hydrophobic residues, which allows them to interact with, and often translocate across bacterial and host membranes, thus enabling their antimicrobial, immunomodulatory and antibiofilm activities (de la Fuente-Núñez and Hancock, 2015). Such physical characteristics allow HDPs to interact with membranes and translocate into negatively charged bacterial and host cells, thus enabling their diverse biological properties. These include their ability to directly kill planktonic microorganisms through their well-studied antimicrobial activity, modulation of the immune system to control infections by means of their immunomodulatory properties, and their antibiofilm activity that enables them to inhibit and disperse biofilms (Figure 2.1) (de la Fuente-Núñez et al., 2016).

The AMPs are molecules of great relevance to the pharmaceutical, biotechnology and food industries. The structural diversity and chemical nature displayed by these molecules is a condition that has led researchers to consider them as natural antibiotics, an innovative alternative to conventional antibiotics as a new class of drugs to prevent and treat systemic and topical infections (Gordon et al., 2005; Porto et al. 2012). Due to these facts, some AMPs are already utilized with clinical and commercial purposes, including ambicin (nisin), polymixin B and gramicidin S (Bradshaw, 2003; Porto et al. 2012). However restrictions on the use of AMPs for therapeutic use have been limited by their cytotoxicity in mammalian cells or ability to lyse eukaryotic cells (Porto et al., 2012; Zhang et al. 2016). To overcome this obstacle, numerous structure-function studies of both natural and synthetic antimicrobial peptides have been employed to improve AMPs activity against the interested pathogen and reduce toxicity at the therapeutic dose. It has therefore become necessary to broaden the search for new antimicrobial substances including the exploitation of novel, natural sources where possible (Song et al., 2012). One such growing area of research has begun to focus on protein hydrolysates of animal origin, such as the use of goat whey protein hydrolyzed by treatment with alcalase, and which shows broad spectrum antibacterial activity (Osman et al., 2016). Similarly, a peptic hemoglobin hydrolysate

from bovine hemoglobin has also been shown to possess antibacterial activity (Adje et al., 2011; Arroume et al., 2006; Arroume et al., 2008; Daoud et al., 2005; Froidevaux et al., 2001). In this context, crocodile blood represents one of the natural origins that considered a rich and valuable source for bioactive compounds. Scientific research covering potential applications for crocodile blood is predominantly focused on the utilization as treatment or supplement for human health. Notable examples are the application as alternative antibiotics and artificial blood products (Shim-Prydon and Camacho-Barreto, 2007). Moreover, traditional Chinese medicine uses crocodile blood and other components, such as oil, bile and gall bladder to treat a broad variety of ailments in patients, ranging from bronchitis, coughing, allergy, skin problems, high blood pressure and cancer (Shim-Prydon and Camacho-Barreto, 2007). With regard to our previous studies about blood of the Siamese crocodile (*Crocodylus siamensis*), we found potent antimicrobial property in all investigated components, including serum (Preecharram et al., 2008), plasma (Preecharram et al., 2010; Kommanee et al., 2012), hemoglobin (cHb) (Srihongthong et al., 2012) and white blood cell extract (cWBC) (Pata et al., 2011). In addition, these blood components were shown to exhibit promising anti-cancer, anti-inflammatory, antioxidant and wound healing activity (Phosri etal., 2014; Theansungnoen et al., 2014; Patathananone et al., 2015; Jangpromma et al., 2016; Pakdeesuwan et al., 2016; Phosri et al., 2017). With respect to cHb, our previous studies by Lueangsakulthai et al (unpublished work, with permission) demonstrated that hemoglobin hydrolysate from *C. siamensis* blood (CHH), hydrolysed for 8 h with pepsin has been shown to possess antibacterial activity represented the MIC_{50} values against *E. coli*, *S. aureus*, *K. pneumoniae* and *P. aeruginosa* at concentrations of 20, 20, 20 and 10 mg/ml (w/v), respectively. The active peptide from 8h-CHH was determined as QAIIHNEKVQAHGKKVL name QL17 with 41% hydrophobicity and +2 net. However natural peptide had short half-life in mammalian reticulocytes (*in vitro*) when estimated by ProtParam tool (http://web.expasy.org/protparam). As the future design of novel AMPs will need to minimize the cytotoxicity in mammalian cells and enhance antibacterial activity for use as antibiotic drugs (Maccari et al, 2013). Toward this goal, design and synthesized peptide should be urgently and extensively investigated. Consequently, the series of peptides have been design and synthesis either by modification of QL17 natural templates combine with physicochemical method and have been optimized for improved cell selectivity and enhanced antimicrobial and potent antibiofilm activity.

The first rational design methods were based on the most commonly proposed AMP mechanism of action, which is membrane disruption. This process is first mediated by electrostatic interactions among positive charged residues and negatively charged lipid heads, and then by insertions of hydrophobic residues into the membrane (Porto et al., 2012). Cationic α-helical amino acids represent an important class of naturally occurring AMPs, well scrutinized and characterized biologically (Thankappan et al. 2013). Using this approach, Dathe et al. (1997) (in Porto et al., 2012) developed several magainin 2 analogues and an 18 residue model peptide with KLA repetitions, modulating their activity by changing only hydrophobicity, hydrophobic moment and the angle of positively charged face helix. Moreover other features were conserved, such as helix propensity and total charge. This showed that when the hydrophobicity and hydrophobic moment increase, the antimicrobial and hemolytic activities from those peptides also increase (Dathe et al., 1997 in Porto et al., 2012). On the other hand, very low hydrophobicity abolishes the antimicrobial activity of those peptides, which can be compensated by increasing the hydrophobic moment. Therefore, while increasing those parameters the peptide becomes unspecific, a selective peptide may be reached with moderated hydrophobicity, increasing the hydrophobic moment and keeping the angle of charged face small (Porto et al., 2012).

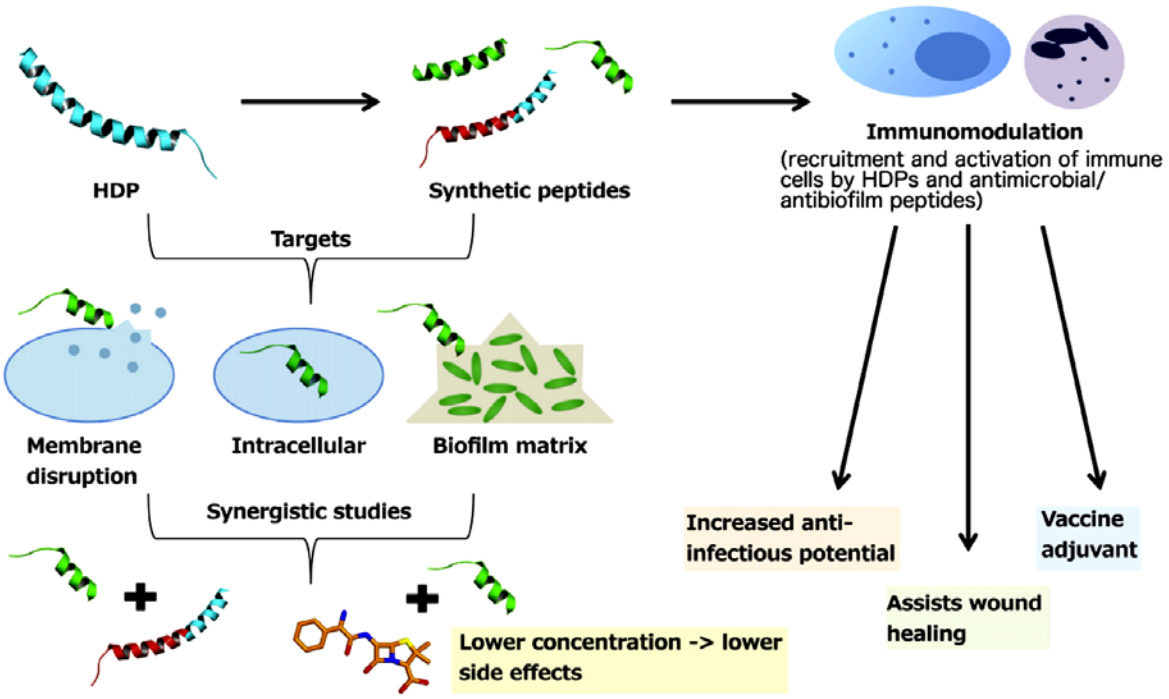


Figure 2.1 Potential biotechnological uses of HDPs and their synthetic analogs. HDPs and their derivatives can act both by direct killing of biofilms (alone or in combination with conventional antibiotics), being able to cause damage to the membrane of the targets cells, as well as by interfering with the homeostasis of the intracellular environment; and by immunomodulation, where the peptides possess the ability to recruit and activate cells from the immune system, facilitating bacterial clearance and increasing wound healing (de la Fuente-Núñez et al., 2016).

CHAPTER III
RESEARCH METHODOLOGY

3.1 Design of antimicrobial peptides

Antibacterial peptide QAIIHNEKVQAHGKKVL (QL17) from *Crocodylus siamensis* hemoglobin hydrolysate with 41% hydrophobicity and +2 net charges was selected as a template for design several series of novel peptide with cell selective and enhanced their performance against resistant bacteria and potent antibiofilm activity. The cationicity of a peptide especially lysine (K) or arginine (R) was used to extend positively charge whereas the hydrophobic residues such as alanine (A), leucine (L), isoleucine (I), phenylalanine (F) or tryptophan (W) to selected to invariable of peptide structure. We therefore designed new peptide to have the charges at around 4-7 with 40-46% of hydrophobicity. The 2-D structures of QL17 and its derivatives were constructed and the helical wheel projection was constructed online using the Emboss :: Pepwheel: http://www.tcdb.org/progs/?tool=pepwheel.

3.2 Solid-phase peptides synthesis

Fmoc solid phase methodology (GL Biochem, Shanghai, China) was obtained to synthesize all peptides. Then, the purification of all peptides was estimated using reversed phase high-performance liquid chromatography. ≥95% purity was choosing. The resulted peptides were confirmed molecular mass by electrospray ionization-mass spectrometry.

3.3 Circular dichroism (CD) spectrum of the peptides

Peptides (0.1 mg/ml) in PBS were dissolved to a suspension of phosphate buffered saline solution, and trifluoroethanol (TFE). CD spectra was recorded at a scanning speed of 20 nm/min in wavelength from 176 to 260 nm using circular dichroism spectroscopy. Data are displayed as mean residue elasticities.

3.4 Antimicrobial activity

3.4.1 Microbial growth conditions

The bacterial applied were *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 27736, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis*

ATCC 6633, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228. All bacterial was grown in Nutrient broth to mid-log phase at 37 °C.

3.4.2 MIC and MBC measurements

The broth microdilution assay was utilized to evaluate antimicrobial efficacy of all peptide. The experiment was performed by follow previous study (Zhang et al. 2016). In sort, the bacteria suspension at concentration $1x10^6$ CFU/ml (in a 50 µl final volume) was loaded to each well together with 50 µl of serially diluted peptide in sterile 96-well plate. Incubation was 18-22 h at 37 °C, the value of OD at 600 nm in each well was measured for bacterial growth with microplate reader. The inhibition was estimated as following formula; %inhibition = [(OD_{600} of control - OD_{600} of sample) / (OD_{600} of control - OD_{600} of blank)] × 100. The minimum concentration that inhibited more than 90% of bacterial growth was exposed as the minimal inhibitory concentration (MIC). The spread plate was also tested.

The spread plate assay was performed using nutrient agar. After broth microdilution method, 100 µl of the co-culture medium in each well was pipetted and spread on nutrient agar plate. Plates were maintained at 37 °C for 24 h. The colony growth around the plate was counted to analytical antimicrobial activity. The minimal bactericidal concentration (MBC) is lowest concentration that not has growth of bacterial on agar plate.

To insist bactericidal efficacy of peptide, resazurin test was used to determine MBC of peptide according to Elahikh et al. (2016). In sort, 0.01% w/v resazurin dyes (at 50 µl) was loaded per each well and maintained again a 37 °C for 18 h. Blue coloration is representing that peptides can completely kill the bacteria, while wells with pink coloration represent that bacterial can growth in this condition. The minimum concentration of the wells with blue coloration was considered to be MBC value.

3.5 Toxicity and cytotoxicity

3.5.1 The peptides ability toward red blood cell hemolysis

The ability of peptides toward red blood cell hemolysis was evaluated with hemolysis assay. Fresh human erythrocytes were carefully mixed with PBS (1:3) and centrifuge at 853 rpm × g for 2 min. Cell pellet was diluted to 2% in PBS. 100 µl of 2% hRBCs was incubated together with 10 µl of two-fold serial dilutions of peptides (final concentrations ranging from 12.5-100 µg/ml). The sample was maintained for 1 h at 37

°C. After treatments, plate was centrifuged. The supernatant was taken to determine absorbance at 415 nm using a microplate reader. All result hemolysis was compared with 1% Triton X-100; it is cause 100% hemolysis. The hemolysis was estimated by using the formulation as following; % hemolytic activity = (A_{415} of test sample / A_{415} of positive control) × 100. 1% Triton X-100 was applied as positive control and the negative control as PBS buffer.

3.5.2 Preparation of peripheral blood mononuclear cells (PBMCs)

Whole blood sample from healthy humans was obtained from Blood Transfusion Center Faculty of Medicine Khon Kaen University (ethical approval number HE601010). PBMCs were isolated using Ficoll medium (GE Healthcare, Uppsala, Sweden). Whole blood was mixed together with PBS (1:1) and gently overlay on Ficoll medium. Further, mixture solution was centrifuged for 40 min at 20 °C. PBMCs layer were collected and transferred to sterile tube and washed with PBS. Then, centrifuge at 200 × g was used to separate layer of PBMCs for 15 min at 20 °C. After that, cell pellet was suspended in media (RPMI 1640) and kept at 37 °C to further use.

3.5.3 The peptides ability toward cytotoxicity

The human peripheral blood mononuclear cells (PBMC), normal human keratinocytes (HaCaT), normal human dermal fibroblast (NHDF) and macrophage RAW 264.7 cells were used elucidated the toxicity of peptide by a standard MTT assay according to the method of (Phosri et al., 2014). Cells were seeded into 96-well plate. Plate was maintained at 37 °C for overnight. Afterward, serial diluted peptides were added to the cell culture. After incubated on 5 % (v/v) CO_2 incubator for 24 h, medium culture was removed and MTT solution (in a 0.05 mg/ml final concentration) was loaded. The plate was maintained for 30 mins. Thereafter, medium was removed and DMSO was loaded per well. The absorbance at 570 nm was determined on a microplate reader. Cell viability was estimated following equation; % viability = (A_{570} of test sample / A_{570} of untreated control) x 100.

3.6 Selectivity index

Selectivity index (SI) is a normal parameter used to quantify selectivity of AMPs. SI is defined as the ratio between the concentrations due to 50% lysis of red blood cell

(HC_{50}) or 50% cell death of normal cell death (IC_{50}) and the minimum bactericidal concentration (MBC) following formula; SI = HC_{50} or IC_{50} / MIC.

3.7 Time-killing kinetics measurement

An overnight growth of *E. coli* ATCC25922 and *S. epidermidis* ATCC12228 were diluted into nutrient broth to $1x10^6$ CFU/ml. The time-killing kinetics of peptide was determined as described previously by Yaraksa et al. (2014). 50 µl of diluted bacterial suspension were incubated together with 50 µl of the peptide solution (final concentration of 1xMIC). And the mixtures were incubated at 37 °C. Aliquots of 10 µl treatment solutions were removed at regular time intervals (0, 15, 30, 60, 180, 360, 540, 720, 900, and 1080 mins), diluted and cultured on nutrient agar plates for 24 h. Then, bacterial cells were counted in colony-forming units (CFU). Results were assayed using plotting a graph of the log CFU/ml against incubation time.

3.8 Cell selectivity

Fibroblast (NHDF), *E. coli* and *S. epidermidis* cells were selected to determined cell selectivity of FITC-labelled peptide using flow cytometry technique. Briefly, $2x10^5$ cells/ml of NHDF cells were seeded into 12-well plate and incubated for overnight. After incubation, cells were co-incubated with 0.5xMIC, 1xMIC and 2xMIC of FITC-labelled peptide, which growing *E. coli* and *S. epidermidis* at log-phase. After 1 h later, NHDF cells were harvested by trypsinization. Then, all cells were centrifuged at 12,000 rpm for 10 min. Cell pellets were washed with PBS buffer and re-centrifuged. Cells were dissolved with Annexin binding buffer. Cell selective was measured using flow cytometer.

3.9 Scanning Electron Microscopy (SEM) technique

A modified SEM method was utilized to determine the effect of peptide on the bacterial membrane. In sort, bacterial were treated with peptide at concentration of 0.5xMIC, 1xMIC and 2xMIC. The treatments were incubated for 1 h at 37 °C. After that, the cell pellets were gently pipetted on 0.2 µm polycarbonate membrane for 10 min. Then, 2.5% (v/v) glutaraldehyde was added for fixation of cells for 1h. Bacterial cells were dehydrated in a series of ethanol containing 30%, 50%, 70%, 90% and 100% for

15 min at each concentration. Dry bacterial specimen was placed on a stub with carbon tape. Finally, stub was overlay using gold palladium and photographed by SEM.

3.10 Antibiofilm activity

The activity of peptide inhibits *P. aeruginosa* performed biofilm in 24 h was studied according to previously described method with some modification (Mishra et al., 2017). Briefly, cultures of *P. aeruginosa* were diluted to $1x10^6$ CFU/ml in LB medium and cultured at 37 °C for 24 h. Then, 100 µl of peptide solution was pipetted to well and again maintained at 37 °C for 24 h. Planktonic bacterial cells were gently to remove using pipette out and rinsed with PBS to clear non-attach cells. After that, 1.2% glutaraldehyde was pipetted to each well, maintained for 30 min and removed solution. Adherent cell was stained by 0.41% crystal violet dye for 2 min and rinsed with PBS. Then, 95% ethanol was added to each well for dissolve crystal violet in cells. The absorbance at 595 nm was estimated by using microplate reader. The results were reported compare to untreated control. The initial attachment inhibition of peptide was calculated following formula; %initial attachment inhibition = 100 –[(A595 of test sample / A595 of untreated control) x 100].

3.11 Proteome analysis in *P. aerogenosa* biofilms

P. aerogenosa wide-type strain ATCC27853 was performed to biofilm formation expression analysis. After *P. aerogenosa* was treated with or without of peptide at 1xMIC for 24 h, the cells were harvested by centrifugation. The extraction of protein from cells pellet was eventually brought out by 0.5% SDS in 50mM Tris-HCl pH 7.0 and sonication at 80%, 3sec using sonicator. The supernatant solution was precipitated in cold-buffer containing 12.5%TCA in acetone for 16 h, washed protein pellet with cold-acetone. Protein concentration was measured using Lowry method (Lowry, 1951). Trypsin was then added (ratio 1:20), and the protein was incubated at 37 °C for 12 h. Peptide was separated by UPLC, C18-Reverse phase chromatography and the mass of peptide was measured using Mass Spectrometer (n=3) version SynaptHDMS (Waters, U.K.). Quantitative data was analyzed using DecyderMS 2.0 program (Johansson et al., 2006; Thorsell et al., 2007). The protein was identified by Mascot program (Perkins et al., 1999) base on Homo sapiens database (Swissport, Dec, 18). The expression of protein was showed in Heatmap using Multi Experiment Viewer (Mev) software version 4.6.1

(Howe et al., 2010). The function of protein expressed was classified by PseudoCAP-based functional classes (http://www.pseudomonas.com) (Winsor et al., 2016)

3.12 NO production via LPS-induced RAW264.7 cells

NO production was determined by measuring the accumulation of the nitrite level in culture medium. The RAW264.7 cells at the density of 2×10^4 cells/well were stimulated with LPS (100 ng/mL) with or without peptide (final concentration ranging from 0-200 µg/ml) for 24 h. Then, 100 ml of culture mediums from each condition was incubated at room temperature with the equal volume of Griess reagent (1 % sulfanilamide in 5% phosphoric acid and 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride) for 10 min. The absorbance at 540 nm was measured using a microplate reader. The cell was treated with LPS only as a positive control while without LPS and samples as an untreated group. All samples were compared with LPS group. The NO production was calculated following equation;

%NO production = [(A540 of test sample / A540 of LPS) x 100]

3.13 Antioxidant activity

For 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay, double distilled water (50 µl) was added to a 96-well plate. Next, CHHs (50 µl) and 0.0004 M DPPH (50 µl) were added and mixed for 5 min. The reaction was kept in dark for 25 min and absorbance was measured at 490 nm using DPPH with double distilled water as a blank. All samples were analyzed in triplicate. Radical scavenging activity was determined as the percentage antioxidant inhibition (%AI) value that was calculated based on the followingformula;

$$\% \text{ Antioxidant inhibition} = [(Abs_{control} - Abs_{test})/Abs_{control}] \times 100$$

The antioxidant activity using ABTS (free radical cation) reagent [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) with potassium persulphate] was modified from Phosri et al. (2014). The ABTS stock was diluted in distilled water, to an absorbance of 0.70 ± 0.02 at 734 nm. 10 µl of peptide was added to 100 µl of diluted ABTS solution at room temperature for 6 min. The absorbance value was determined at 734 nm using microplate reader. The ABTS scavenging activity of the samples was estimated by using the formulation as following;

$$\% \text{ ABTS scavenging} = [(A734_{control} - A734_{test\ sample}) / A734_{control}] \times 100.$$

CHAPTER IV
RESULTS

4.1 Peptide design, characterization and synthesis

Antibacterial peptide QAIIHNEKVQAHGKKVL (QL17) from *Crocodylus siamensis* hemoglobin hydrolysate with 41% hydrophobicity and +2 net charges was selected as a template for design several series of novel peptide. Removal of glutamine (Q) and alanine (A) amino acid at the N-terminal of QL17 was performed resulting in new peptide namely IL15. The series of IL15 peptide analogues were continually designed based on effect of positively charge and hydrophobic property. The Ile2 and Ala9 of IL15 was replaced with Lys in order to enhance the positively charge, whereas Asn4 and Gln8 was replaced with Trp which is a hydrophobic amino acid, to increase the hydrophobicity property. This peptide that was named IL15NJ1 contained positive net charge +4 and 40 % hydrophobicity. The Glu5 of IL15NJ1 was further substituted with Lys that called IL15NJ2. The IL15NJ2 contained +6 total net charges and also 40 % hydrophobicity. Therefrom, Gly11 and Val14 of IL15NJ2 were replaced with Trp and Lys respectively and called IL15NJ4 which the positively charge was increased to +7 and the hydrophobicity was still maintained at 40%. The increasing of positively charge was designed in hydrophilic face of peptide. Furthermore, we design novel peptide based on effect of hydrophobicity. Tryptophan (W), which is a hydrophobic amino acid, was selected to increase the hydrophobic property. The Gly11 of IL15NJ2 was substituted with Ala, named IL15NJ3 results in increase in hydrophobicity from 40% to 46%. The literatures have been described that arginine display cytotoxicity on red blood cell higher than lysine. Thus, in order to study the effect of positively charged residue on the cytotoxicity and antimicrobial activity of peptide, the comparison of arginine and lysine was constructed. The Lys2, 5, 6, 9, 12, 13, and 14 residues of IL15NJ4 were substituted with Arg to generate new derivative named IL15NJ5. It has total net charge +7 and 40% hydrophobicity correspond to the positively charged of IL15NJ4. The sequences and physicochemical properties of designed peptides were shown in Table 4.1. The peptides design in above were generated for preserve the amphipathic α-helix by replacing the hydrophobic residues on one segment and the cationic residues on the other segment as displayed in helical wheel data (Figure 4.1).

The predictions of secondary structure of all peptides were displayed as Figure 4.2. The QL17 and IL15 peptide were contained C-terminal random coil, α-helix and N-terminal random coil. IL15NJ1 and IL15NJ2 comprised of C-terminal random coil and α-helix. Moreover, IL15NJ3, IL15NJ4 and IL15NJ5 had shown only α-helix.

The HPLC profile in Figure 4.3A-4.9A indicated that all of synthetic peptides showed high purify. The purity of the synthetic peptides was all more than 95%. Mass spectrometry result was showed in Figure 4.3B-4.9B. We found that molecular weight between the experimental and calculated by ProtParam tool online showed no different. Suggest that peptides were generated to the required particular.

Table 4.1 The sequences and physicochemical properties of designed peptides.

Peptide	Sequence	Calculated mass MW[a]	Observed mass MW[b]	% Hydrophobicity	Net charge	Half-life in mammalian reticulocytes
QL17	QAIIHNEKVQAHGKKVL	1913.25	1913.27	41%	+2	0.8 hours
IL15	IIHNEKVQAHGKKVL	1714.04	1714.02	40%	+2	20 hours
IL15NJ1	IKHWEKVWKHGKKVL	1916.35	1916.31	40%	+4	20 hours
IL15NJ2	IKHWKKVWKHGKKVL	1915.40	1915.37	40%	+6	20 hours
IL15NJ3	IKHWKKVWKHAKKVL	1929.43	1929.40	46%	+6	20 hours
IL15NJ4	IKHWKKVWKHWKKKL	2073.61	2073.57	40%	+7	20 hours
IL15NJ5	IRHWRRVWRHWRRRL	2269.70	2269.66	40%	+7	20 hours

[a]Molecular weight (MW) was calculated using ProtParam tool online at website: (http://web.expasy.org/protparam).

[b]Molecular weight as estimated by mass spectroscopy

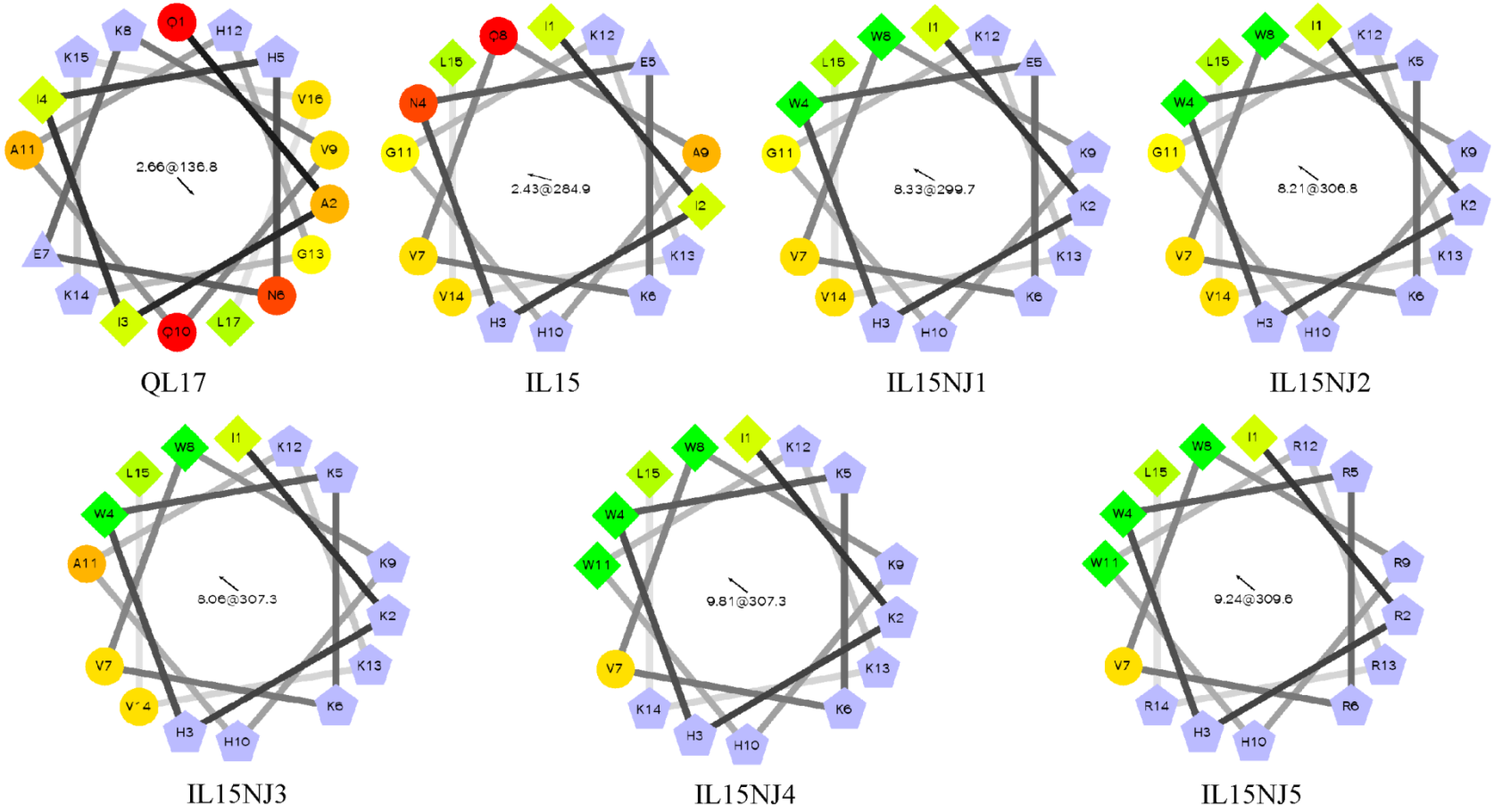


Figure 4.1 Helical wheel projection diagrams of the QL17 peptide and its derivative peptides. By default, the output presents the polar/basic residues in red, polar/acid residues in blue, polar/uncharged residues in green and nonpolar residues in yellow.

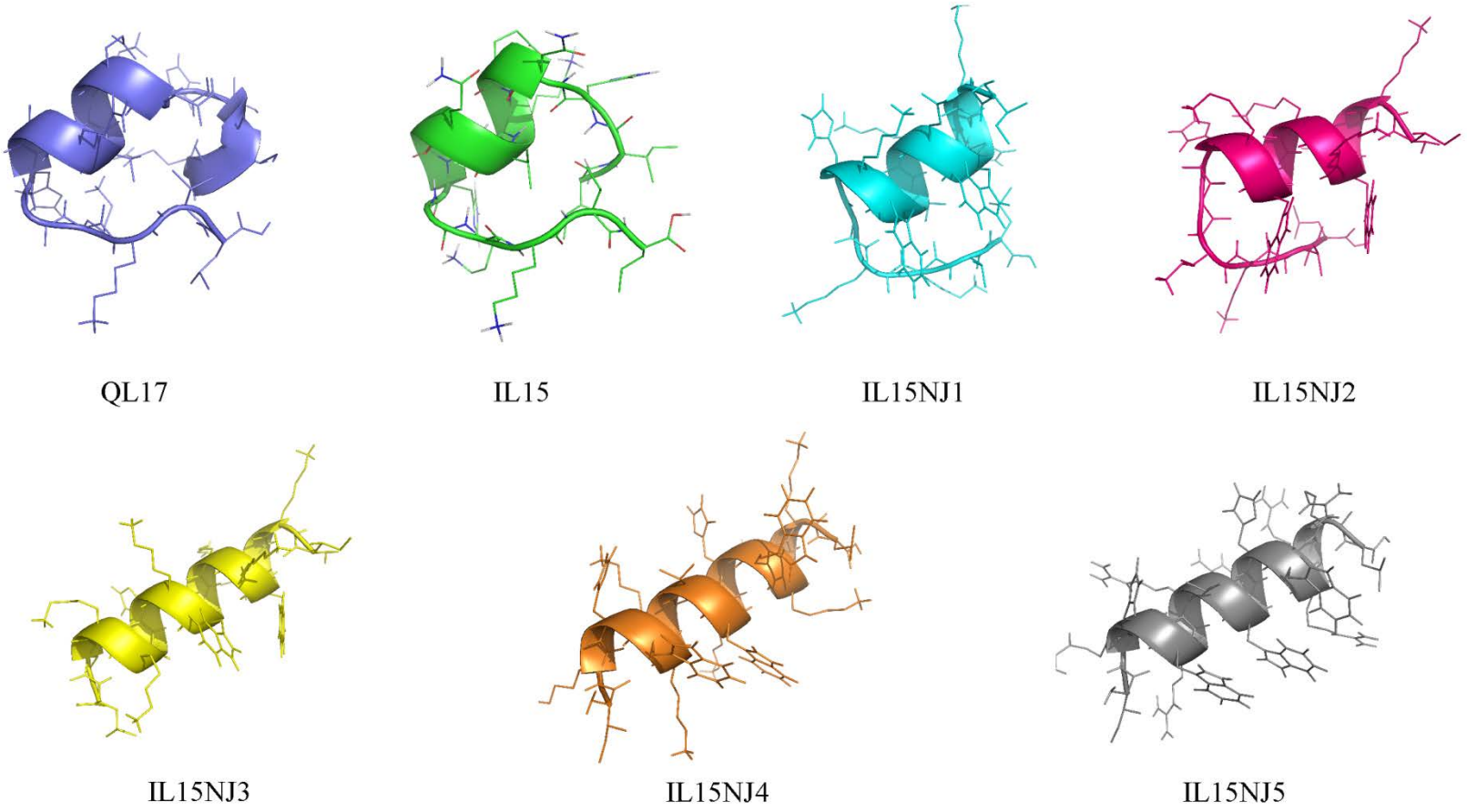
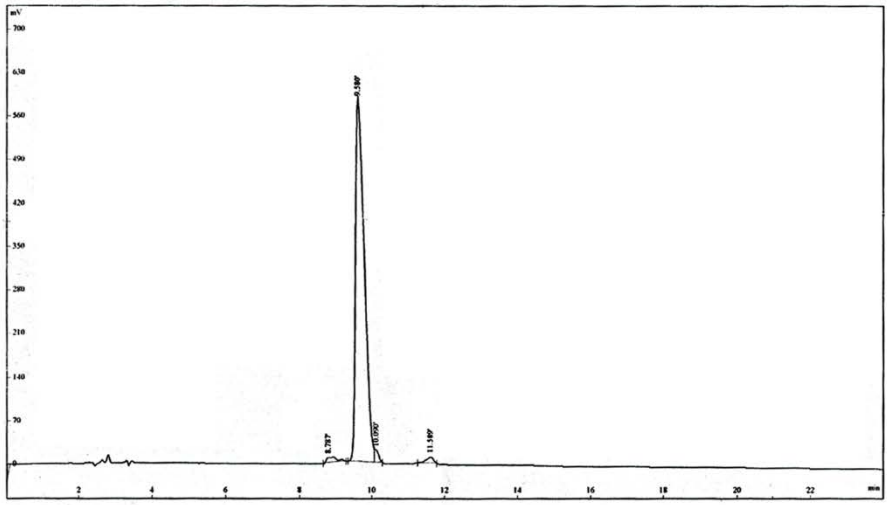


Figure 4.2 The secondary structure predicted of the QL17 peptide and its derivative peptides. All peptide structure was performed by PEP-FOLD servers.

A



Rank	Time	Conc.	Area	Height
1	8.787	1.874	194475	9458
2	9.580	95.47	9908173	589797
3	10.090	1.226	127287	20297
4	11.589	1.423	147725	10137
Total		100	10377660	629689

B

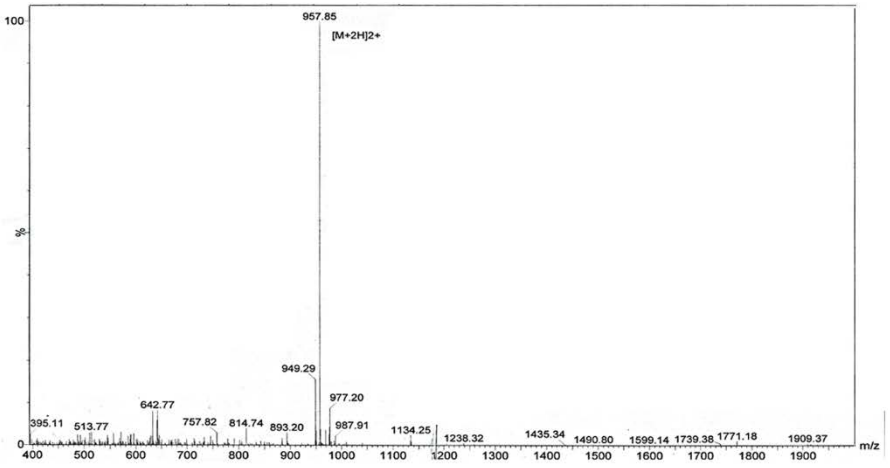
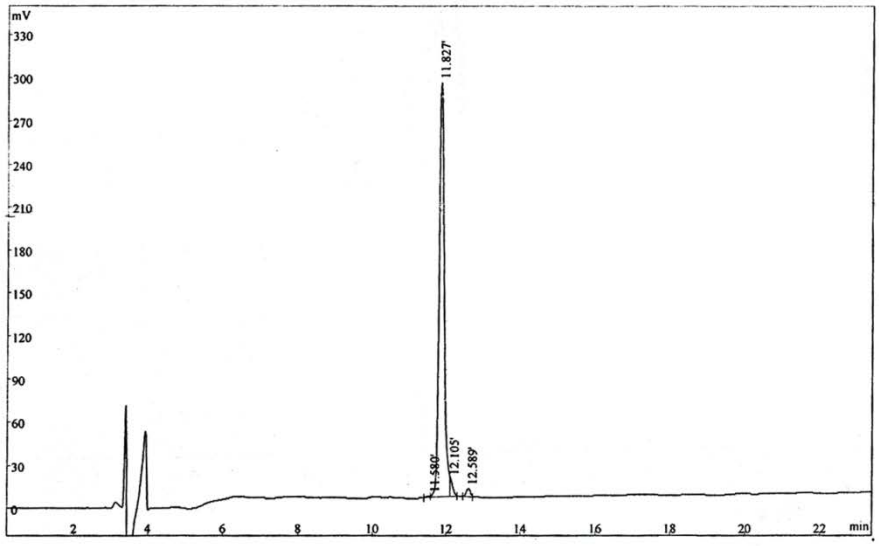


Figure 4.3 HPLC profile (A) and mass data (B) of QL17.

A



Peak No.	Ret Time	Height	Area	Conc.
1	11.580	851	4991	0.1921
2	11.827	288688	2512011	96.65
3	12.105	11731	46128	1.775
4	12.589	5505	35836	1.379
Total:				**100.0000**

B

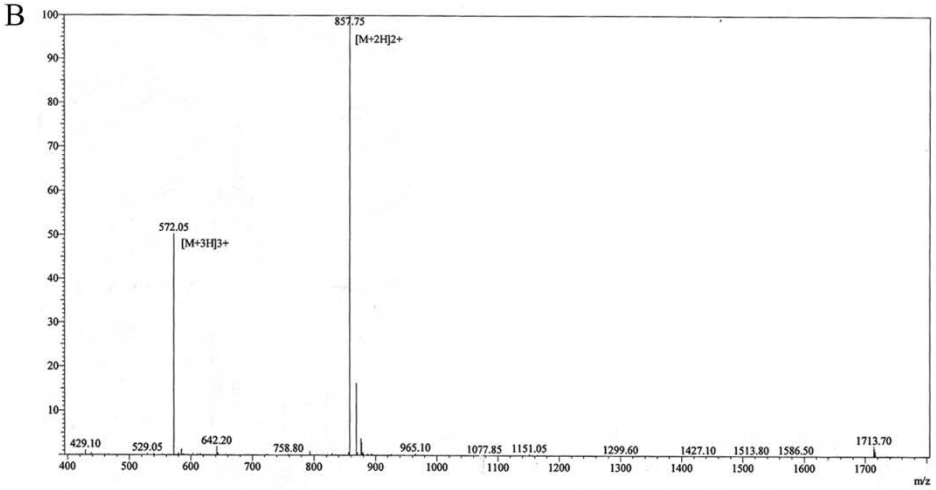
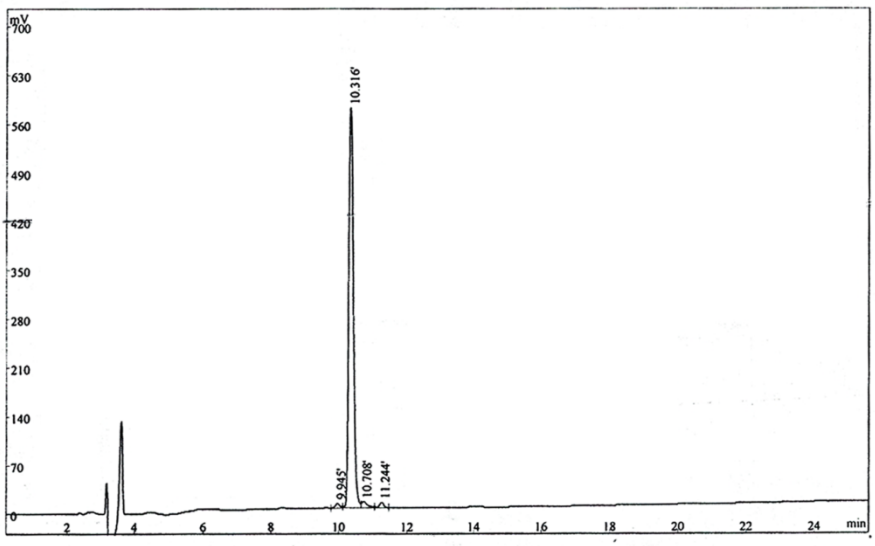


Figure 4.4 HPLC profile (A) and mass data (B) of IL15.

A



Peak No.	Ret Time	Height	Area	Conc.
1	9.945	6283	49418	0.9715
2	10.316	573482	4874123	95.82
3	10.708	9015	104281	2.05
4	11.244	7425	58984	1.16
Total:				**100.0000**

B

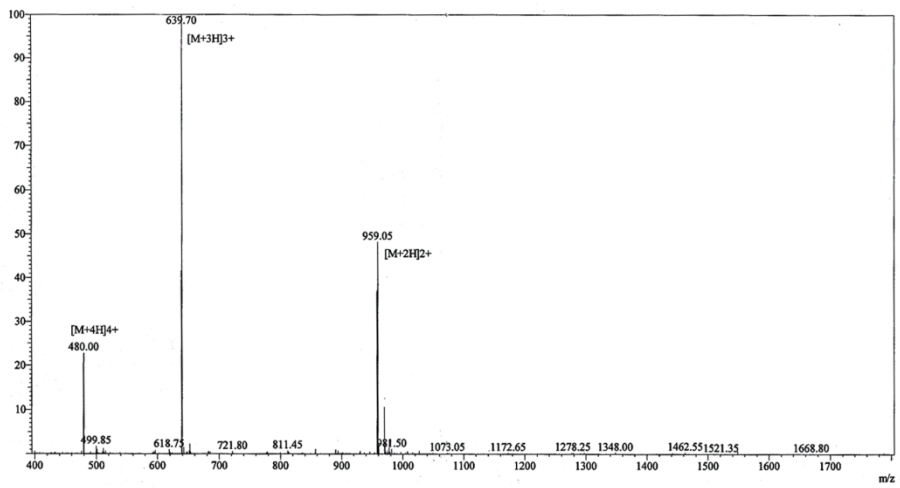
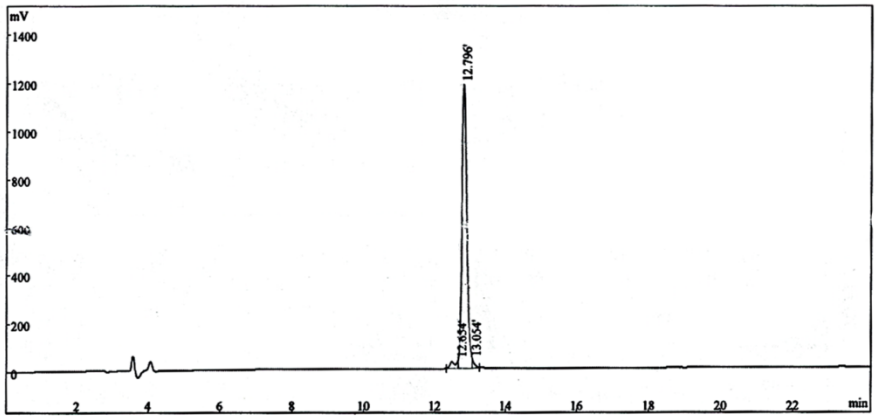


Figure 4.5 HPLC profile (A) and mass data (B) of IL15NJ1.

A



Rank	Time	Conc.	Area	Height
1	12.654	3.016	325371	28486
2	12.796	95.93	10348250	1173876
3	13.054	1.057	114061	31334
Total		100	10787682	1233696

B

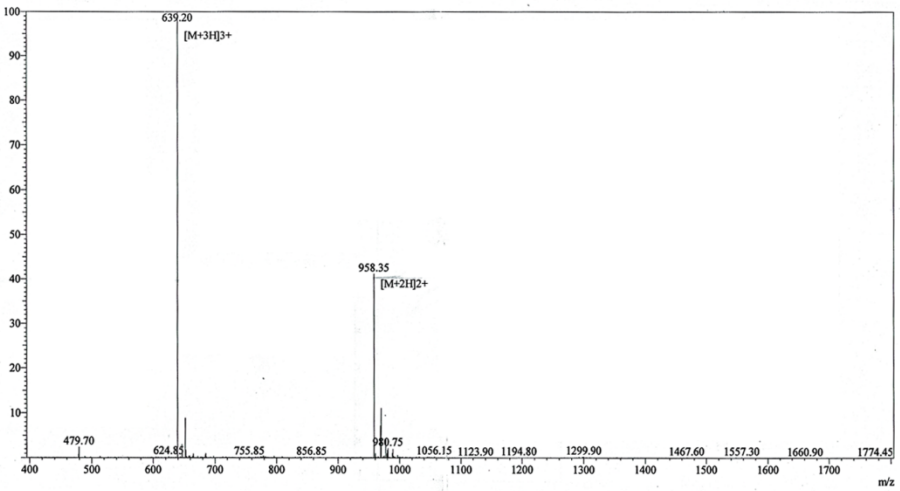
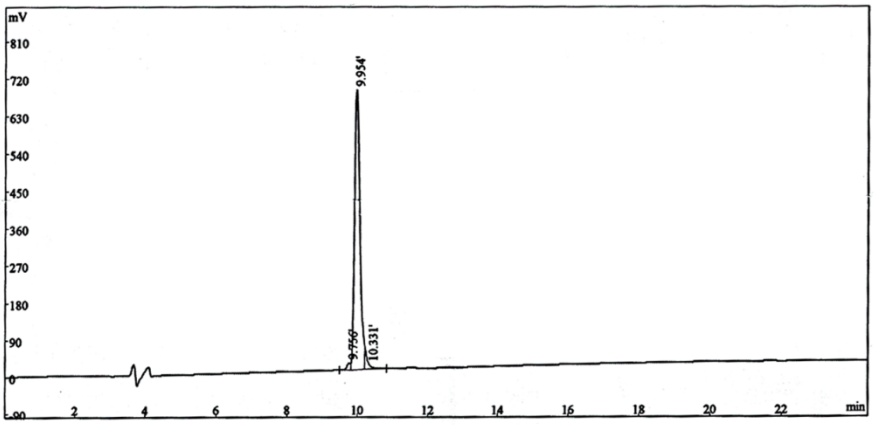


Figure 4.6 HPLC profile (A) and mass data (B) of IL15NJ2.

A



Rank	Time	Name	Conc.	Area	Height
1	9.756		1.469	101703	14645
2	9.954		95.36	6600904	672983
3	10.331		3.167	219204	8105
Total			100	6921811	695733

B

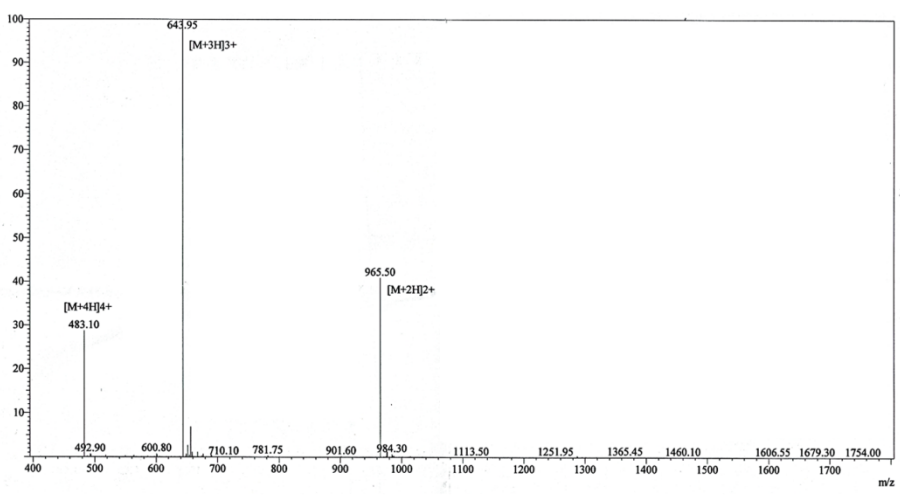
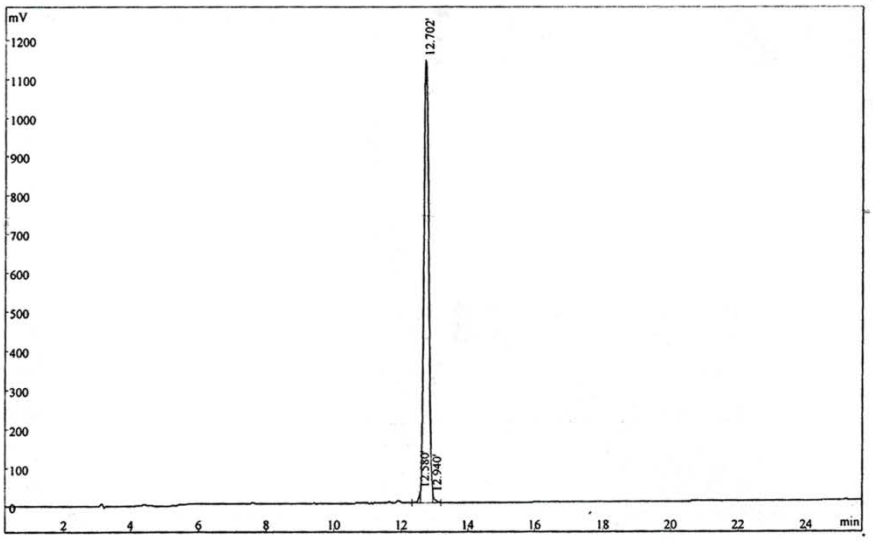


Figure 4.7 HPLC profile (A) and mass data (B) of IL15NJ3.

A



Peak No.	Ret Time	Height	Area	Conc.
1	12.580	27799	88055	0.8292
2	12.702	1139726	10476532	98.66
3	12.940	17799	54646	0.5146
Total:				**100.0000**

B

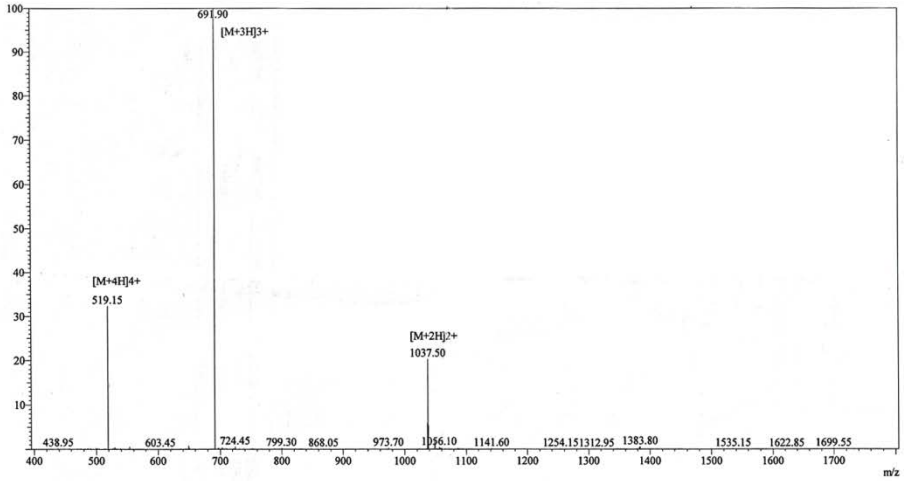
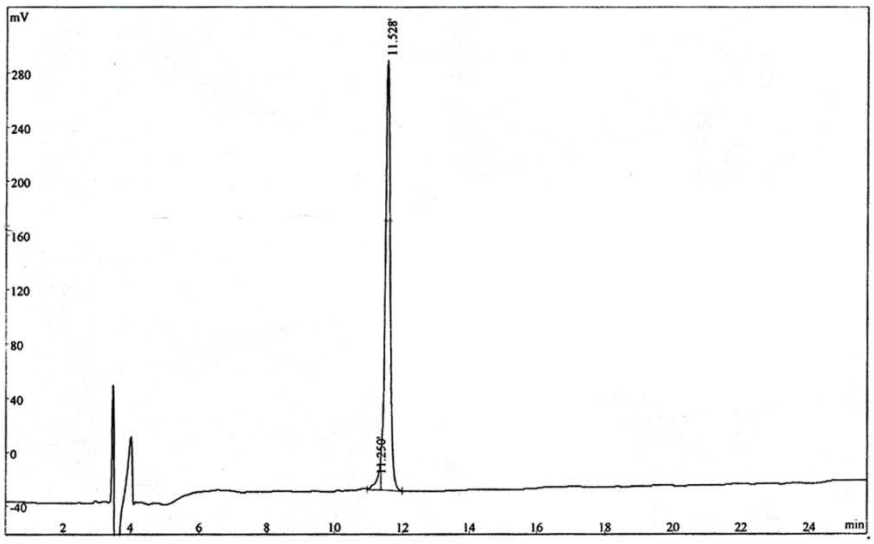


Figure 4.8 HPLC profile (A) and mass data (B) of IL15NJ4.

A



Peak No.	Ret Time	Height	Area	Conc.
1	11.250	8299	141433	4.653
2	11.528	317623	2897907	95.35
Total:				**100.0000**

B

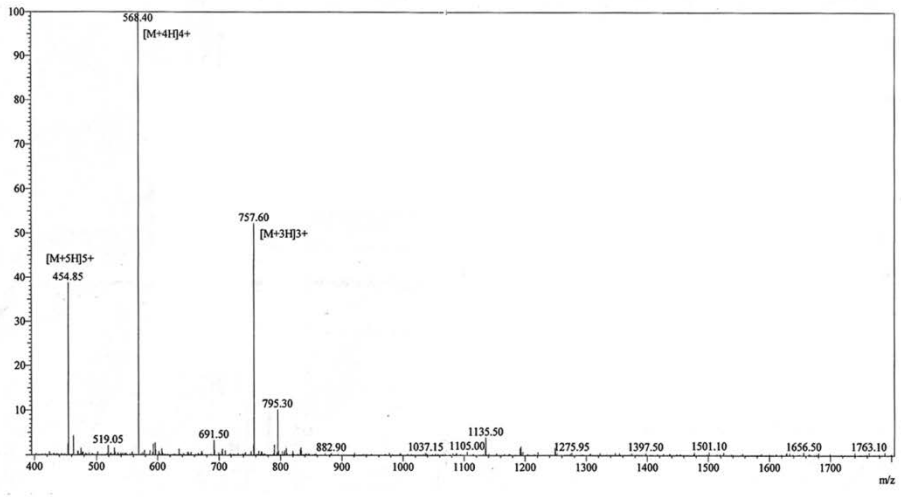


Figure 4.9 HPLC profile (A) and mass data (B) of IL15NJ5.

4.2 Secondary structure of the peptides by CD spectroscopy

The secondary structure of all peptides was evaluated in various environments such included PBS (aqueous environment) and 50% trifluoroethanol (TFE, mimicking the hydrophobic environment of the microbial membrane) by CD spectroscopy. Figure 4.10 displayed spectrums of peptide for 1 mg/ml of PBS and 50% TFE. In the exposure of water, all peptide displays random coil conformation as evaluated by deep and shallow minimums at 200 and 228 nm, respectively. In contrast, under mimicked-membrane (50% TFE) environment, the peptides were exhibited α-helical structure, as demonstrated by the behavior of two double negative bands at 208 and 222 nm. Substitution the number of positive residues slightly increased the helical, noticeable from the lower mean residue ellipticity values at 222 nm. In the presence of high hydrophobicity, IL15NJ3 exhibited strong α-helical characteristic.

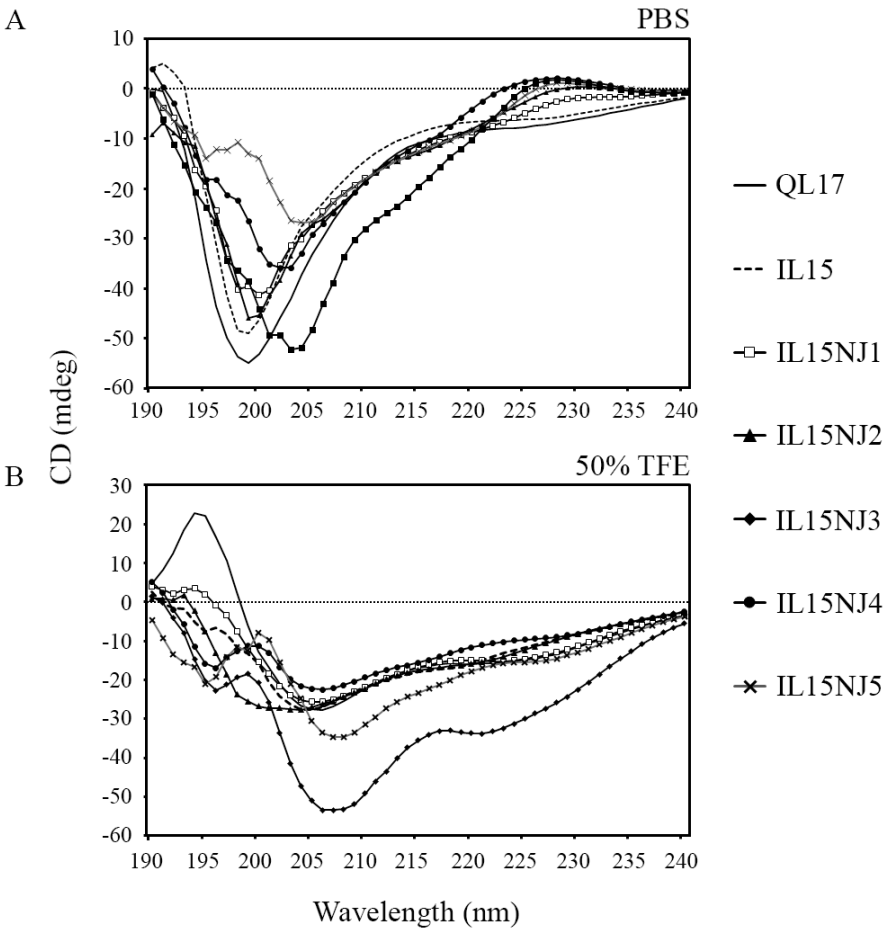


Figure 4.10 The CD spectrum of all peptides in any environment, (A) PBS and (B) 50% TFE.

4.3 Activities of peptides against microbial

The antimicrobial activity of all peptides and rational antibiotic were evaluated *in vitro* against difference microbial strains are given in Table 4.2. The results obviously showed that synthetic QL17, IL15 and IL15NJ1 have poor antimicrobial activity, with MIC > 200 µg/ml. Similarly, the result of IL15NJ2 also exhibited low antimicrobial activity against *K. pneumonia, P. aeruginosa, S. aureus* and *S. epidermidis* (MIC > 200 µg/ml), except for *E. coli* and *B. subtilis* stain that showed the MIC values at 100 and µg/ml, respectively. Conversely, the antibacterial activity of IL15NJ3, IL15NJ4 and IL15NJ5 considerably against all of tested bacterial strains were higher than those of QL17, IL15, IL15NJ1 and IL15NJ2. The MIC values were found to range between 4-12 for IL15NJ3, 4-12 for IL15NJ4 and 8-75 for IL15N5, respectively. Whereas, the MBC values were found to range between 6-25 for IL15NJ3, 6-50 for IL15NJ4 and 12-100 for IL15N5, respectively. Among all the peptides, IL15NJ3 exhibited the most potent AMPs followed by IL15NJ4 and IL15NJ5.

Table 4.2 MICs and MBCs of peptides against several bacterial strains.

Microorganism	QL17		IL15		IL15NJ1		IL15NJ2		IL15NJ3		IL15NJ4		IL15NJ5	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
E. coli	[a]>200	>200	>200	>200	>200	>200	100	150	4	6	4	6	15	25
K. pneumoniae	>200	>200	>200	>200	>200	>200	>200	>200	10	25	14	50	75	100
P. aeruginosa	>200	>200	>200	>200	>200	>200	>200	>200	10	18	10	14	35	50
S. aureus	>200	>200	>200	>200	>200	>200	>200	>200	12	25	12	25	25	50
B. subtilis	>200	>200	>200	>200	>200	>200	50	60	8	12	6	10	8	12
S. epidermidis	>200	>200	>200	>200	>200	>200	>200	>200	9	12	9	12	12	15

[a] > mean there is percentage inhibition less than 90

4.4 Toxicity of peptides

4.4.1 Effect of peptides on lysis of human erythrocytes

In order to determine the toxicity effects of parent and novel designed peptides, hemolytic activity assay was measured against human erythrocytes. The percentage of hemolysis was determined by considering the absorbance value of human erythrocytes treated with 1% Triton-X100 to represent 100% hemolysis and phosphate buffer saline (PBS) treated group was used as a negative control. As a result, all of tested peptides at the concentration ranging from 1.56 to 200 µg/ml exhibited insignificant lower hemoglobin release than that of Triton-X100 (<10%) (Figure 4.11A). At the concentration of 250-2000 µg/ml, the hemolysis of QL17, IL15, IL15NJ1, IL15NJ2, IL15NJ3 and IL15NJ4 on human erythrocyte was about 15% (Figure 4.11B). The slightly increased in hemolytic rate was found in IL15NJ3 which increasing hydrophobicity to 46%. Considering to the peptide substitution with K and R, the toxicity to human erythrocytes in K (IL15NJ4) was lower than R (IL15NJ5). At the concentration of 2000 µg/ml, K (IL15NJ4) was exhibited 20% hemolysis, while the toxicity to human erythrocytes at 60% was observed in R (IL15NJ5). The hemolytic concentration 50% (HC_{50}) value that defined as the lowest peptide concentration caused 50% hemolysis for IL15NJ5 was 896 µg/ml (Table 4.3). However, the hemolytic activity results suggested that all of peptides at the concentration at MIC and MBC had no or slightly toxic towards red blood cells.

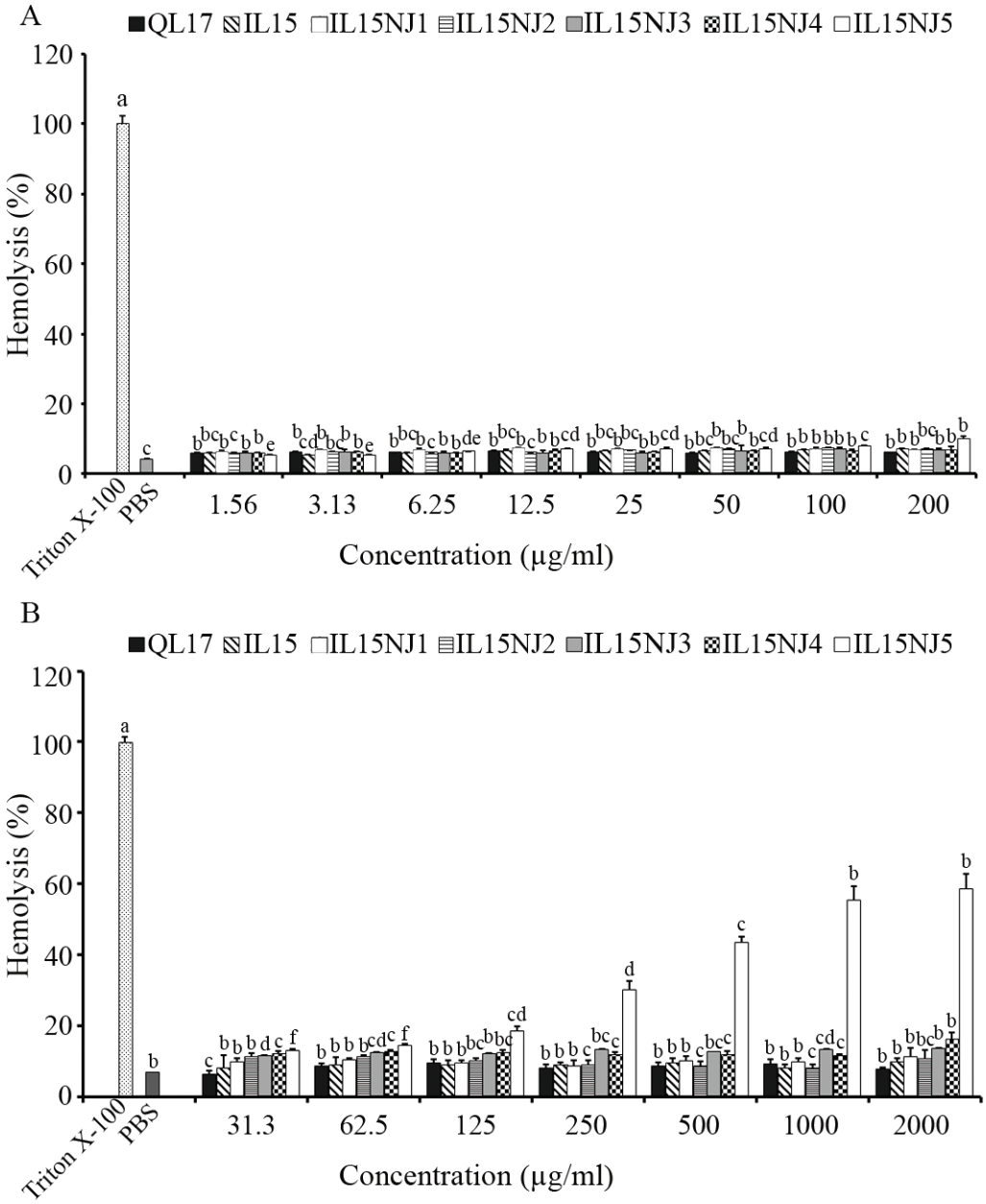


Figure 4.11 Hemolytic activity of peptides against human erythrocytes. (A) human erythrocytes incubated with 1.56-200 μg/ml of peptides. (B) human erythrocytes incubated with 31.3-2000 μg/ml of peptides. Each value is expressed as the mean ± SD. Different letters (a-f) on the top of each bar indicated statistically significant differences ($p < 0.05$).

Table 4.3 The concentration of peptide that induced human red blood cell hemolysis to 50%.

Peptide	Hemolysis concentration$_{50}$ (μg/ml)
QL17	>2000
IL15	>2000
IL15NJ1	>2000
IL15NJ2	>2000
IL15NJ3	>2000
IL15NJ4	>2000
IL15NJ5	896

4.4.2 Cytotoxicity observation on normal cell

The cytotoxicity effect of QL17, IL15, IL15NJ1, IL15NJ2, IL15NJ3, IL15NJ4 and IL15NJ5 were evaluated toward normal cell lines using MTT assay. The concentration corresponding to 50% cell death (IC_{50}) was determined from a plot of peptide absorbance versus peptide concentration. IC_{50} was the peptide concentration corresponding to one-half-maximum absorbance was illustrated in Table 4.4. At the concentration of 200 μg/ml, the highest percentage of cell viability for all mammalian cell lines was observed in IL15NJ1 and IL15NJ2 (>80%), while IL15NJ3, IL15NJ4 and IL15NJ5 showed cells viability less than 80% (Figure 4.12-4.13, A-D). The IL15NJ5 has the highest toxicity toward mammalian cells (i.e. 113 μg/ml for PBMC, 251 μg/ml for RAW 264.7 and 200 μg/ml for HaCaT). The IC_{50} values of IL15NJ4 and IL15NJ3 for RAW 264.7 were 142 μg/ml and 198 μg/ml, respectively (Figure 12-13). The increasing of toxicity could be observed when positively net charge of peptide enhanced to +7 (IL15NJ4 and IL15NJ5).

Table 4.4 The concentration corresponding to 50% cell death toward normal cell lines.

Peptides	IC_{50} (µg/ml)			
	PBMC	**RAW 264.7**	**HaCaT**	**NHDF**
QL17	>200	>200	>200	>200
IL15	>200	>200	>200	>200
IL15NJ1	>200	>200	>200	>200
IL15NJ2	>200	>200	>200	>200
IL15NJ3	>200	198	>200	>200
IL15NJ4	>200	142	>200	>200
IL15NJ5	113	251	200	>200

4.5 Selectivity index

The selectivity index (SI) of the peptides was calculated as the ratio of the 50% hemolysis (HC_{50}) or 50% cytotoxic in normal cell line (IC_{50}) concentration of peptide to MIC of the testing antimicrobial peptides. The high SI index was indicated the greater selectivity of peptides toward bacterial cell membranes over the mammalian cell membranes. Considering HC_{50} values, the highest tested concentration of 2000 µg/ml were used for QL17, IL15, IL15NJ1, IL15NJ2, IL15NJ3 and IL15NJ4 SI calculation, while the concentration of 896 µg/ml (HC_{50}) was used for IL15NJ5 SI calculation. Even for normal cells, the SI was calculated by using the highest tested concentration of 200 µg/ml, except for IL15NJ3 and IL15NJ4 treated RAW 264.7 cell and IL15NJ5 treated PBMC, RAW 264.7 and HaCaT cells. The calculated SI values are shown in Table 4.5 and Table 4.6. The obtained results suggested that IL15NJ3 exhibited the highest cell selectivity than those of QL17, IL15, IL15NJ1, IL15NJ2, IL15NJ4 and IL15NJ5 (Table 4.5 and Table 4.6). Therefore, IL15NJ3 was considered for used in the further experiments.

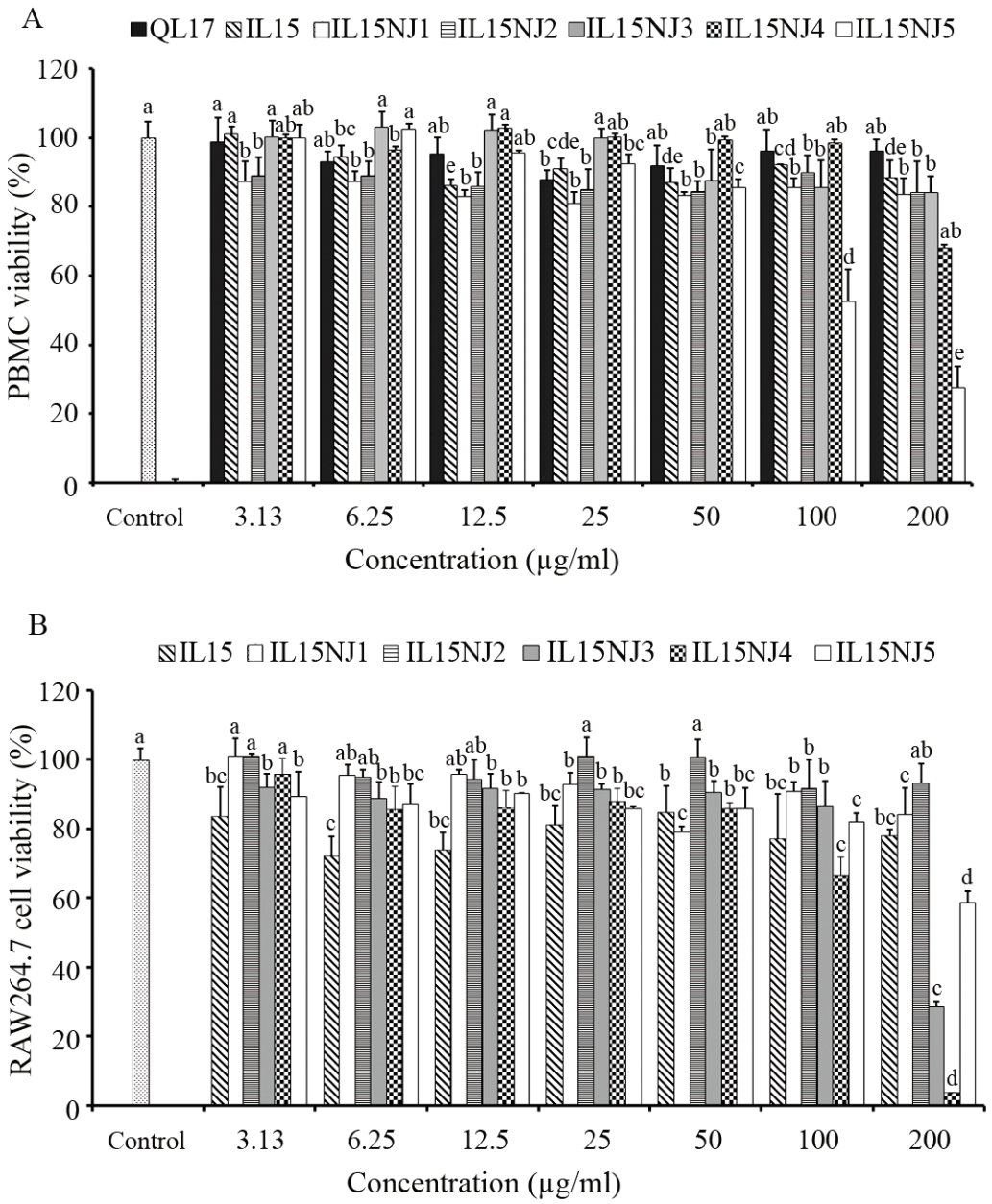


Figure 4.12 Cytotoxicity of peptides determined using MTT assay against PBMC (A) and RAW 264.7 cell (B) following exposed at a final concentration of peptides from 3.13-200 µg/ml. Each value is expressed as the mean ± SD. Different letters (a-e) on the top of each bar indicated statistically significant differences ($p < 0.05$).

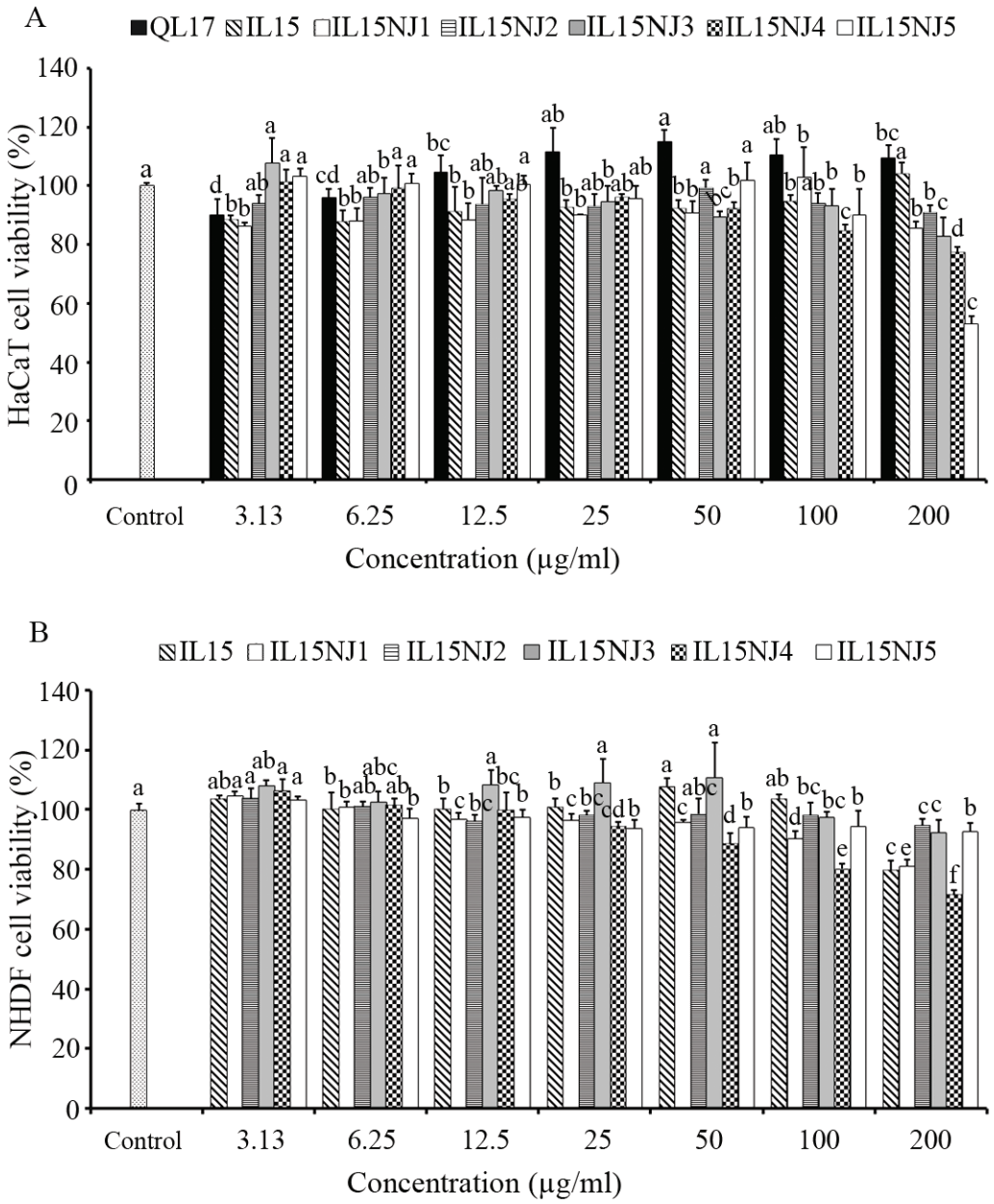


Figure 4.13 Cytotoxicity of peptides determined using MTT assay against HaCaT cell (A) and NHDF cell (B) following exposed at a final concentration of peptides from 3.13-200 µg/ml. Each value is expressed as the mean ± SD. Different letters (a-f) on the top of each bar indicated statistically significant differences ($p < 0.05$).

Table 4.5 Selectivity index of peptide against Gram-negative bacteria.

Peptide	Selectivity index														
	E. coli					***K. pneumonia***					***P. aeruginosa***				
	RBC	**PBMC**	**RAW 264.7**	**HaCaT**	**NHDF**	**RBC**	**PBMC**	**RAW 264.7**	**HaCaT**	**NHDF**	**RBC**	**PBMC**	**RAW 264.7**	**HaCaT**	**NHDF**
QL17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL15NJ1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL15NJ2	20	2	2	2	2	-	-	-	-	-	-	-	-	-	-
IL15NJ3	500	50	50	50	50	200	20	20	20	20	200	20	20	20	20
IL15NJ4	500	50	36	50	50	143	14	10	14	14	200	20	14	20	20
IL15NJ5	60	8	17	13	13	12	2	3	3	3	26	3	7	6	6

Table 4.6 Selectivity index of peptide against Gram-positive bacteria.

Peptide	Selectivity index														
	S. aureus					***B. subtilis***					***S. epidermidis***				
	RBC	**PBMC**	**RAW 264.7**	**HaCaT**	**NHDF**	**RBC**	**PBMC**	**RAW 264.7**	**HaCaT**	**NHDF**	**RBC**	**PBMC**	**RAW 264.7**	**HaCaT**	**NHDF**
QL17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL15NJ1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL15NJ2	-	-	-	-	-	40	4	4	4	4	-	-	-	-	-
IL15NJ3	167	17	17	17	17	250	25	25	25	25	222	22	22	22	22
IL15NJ4	167	17	12	17	17	333	33	24	33	33	222	22	16	22	22
IL15NJ5	36	5	10	8	8	112	14	31	25	25	75	9	21	17	17

4.6 Time kinetics of killing bacteria

The growth kinetic rate of *E. coli* and *S. epidermidis* after exposure with IL15NJ3 at MIC concentration was evaluated base on time killing kinetics assay. The results showed that IL15NJ3 were able to inhibit the growth kinetic rate of *E. coli* and *S. epidermidis* within 60 min and 180 min, repectively (Figure 4.14 A and B). The colony number of each peptide treated conditions was lower than untreated controls. The peptide displayed more quickly against Gram-negative bacteria than Gram-positive bacterial. Moreover, the time-kill kinetic results exhibited the complete killing of tested *E. coli* and *S. epidermidis* in 180 min and 360 min, respectively.

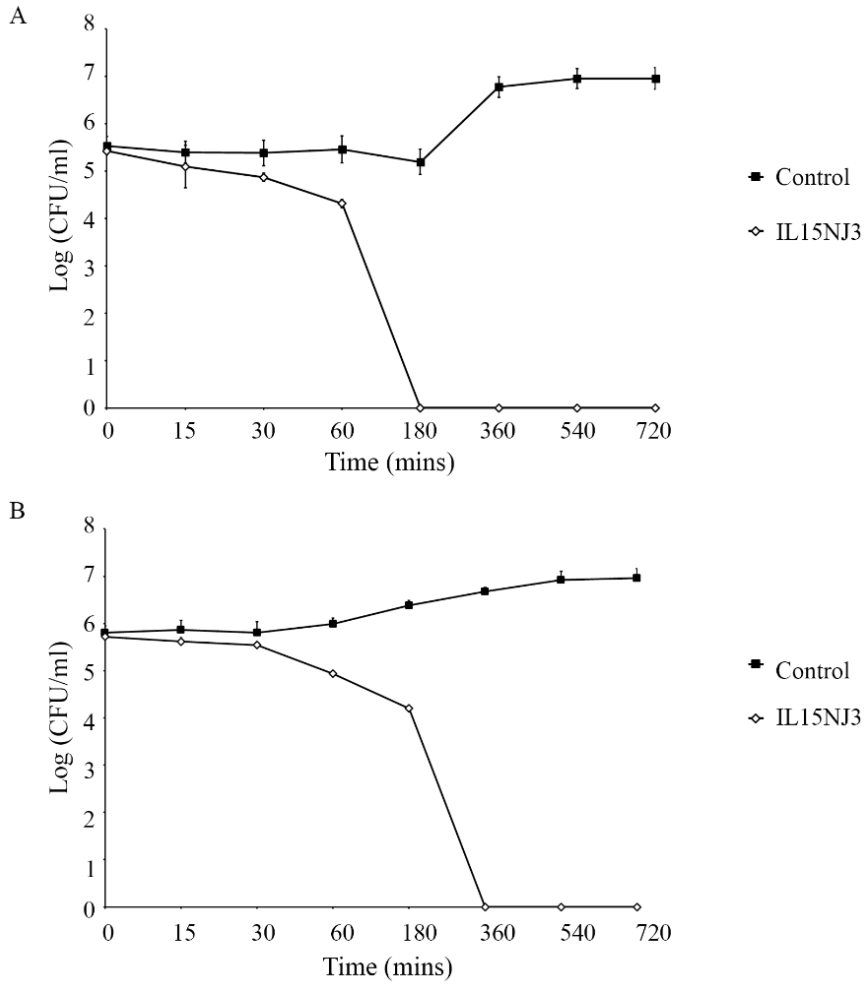


Figure 4.14 Killing kinetic efficient of IL15NJ3. (A) time-kill study of *E. coli* and (B) time-kill study of *S. epidermidis* treated with IL15NJ3 at 1×MIC concentration for 0, 15, 30, 60, 180, 360, 540, 720 min.

4.7 Peptide selectivity using flow cytometry technique

Cell selectivity of IL15NJ3 peptide was evaluated using flow cytometry technique. Flow cytometry results demonstrated fluorescence signal of peptide-labeled FITC was only 0.4%, 0.1% and 0% for the absence peptide-labeled FITC treated NHDF, *E. coli* and *S. epidermidis* groups, respectively, indicating viable cell membranes (Figure 4.15 and 4.16). Whereas, the 78.7%, 95.9% and 98.4% of FITC signal was observed in 0.5 MIC, 1×MIC and 2×MIC peptide-labeled FITC treated *E. coli* Gram-negative groups, respectively. The peptide-labeled FITC treated *E. coli* groups exhibited fluorescence signal higher than NHDF cell groups that were 1.3%, 3.9% and 4.9%, respectively (Figure 4.15). The similar results were obtained in the *S. epidermidis* Gram-positive groups, as 52.3%, 68.3% and 72.1% of FITC signal was observed in 0.5 MIC, 1×MIC and 2×MIC peptide-labeled FITC treated *S. epidermidis* while in the NHDF cells exhibited FITC fluorescence about 0.8%, 2.8% and 14.5% for 0.5 MIC, 1×MIC and 2×MIC peptide-labeled FITC treatment, respectively (Figure 4.16). The enhance of intensity in FITC positive with a dose-dependent manner indicated that IL15NJ3 peptide was capable to damage the *E. coli* and *S. epidermis* cells. Additionally, IL15NJ3 peptide showed high selective toxicity against bacterial cells over mammalian cells.

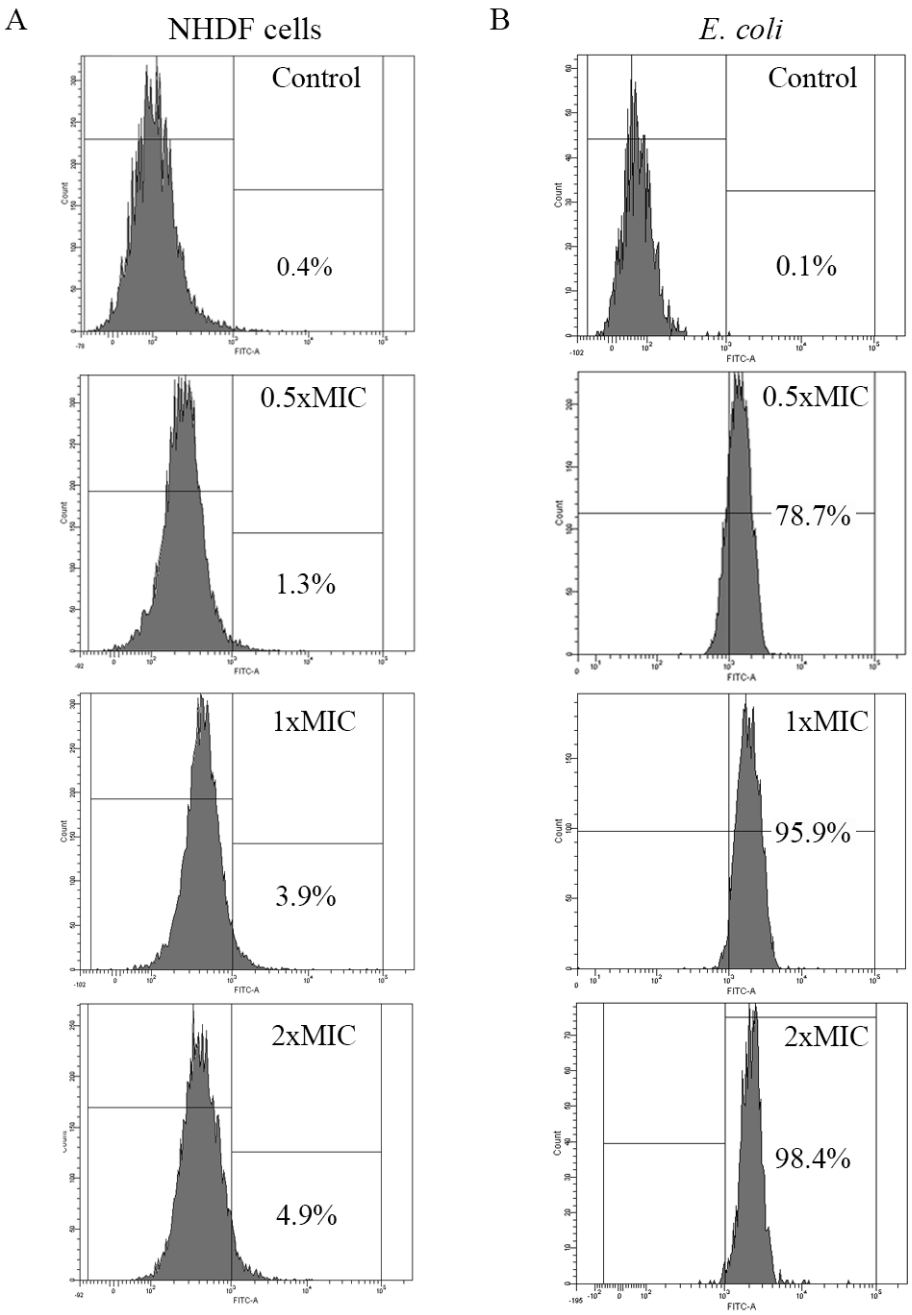


Figure 4.15 The cell selective of FITC-labelled IL15NJ3 to NHDF cells and *E. coli*, as determined by flow cytometry technique. The control group in both mammalian and bacterial cells was processed without peptide-labeled FITC. The treatment groups were co-incubated with 0.5 MIC, 1×MIC and 2×MIC of peptide-labeled FITC. (A) peptide-labeled FITC treated NHDF cells and (B) peptide-labeled FITC treated *E. coli* Gram-negative bacteria.

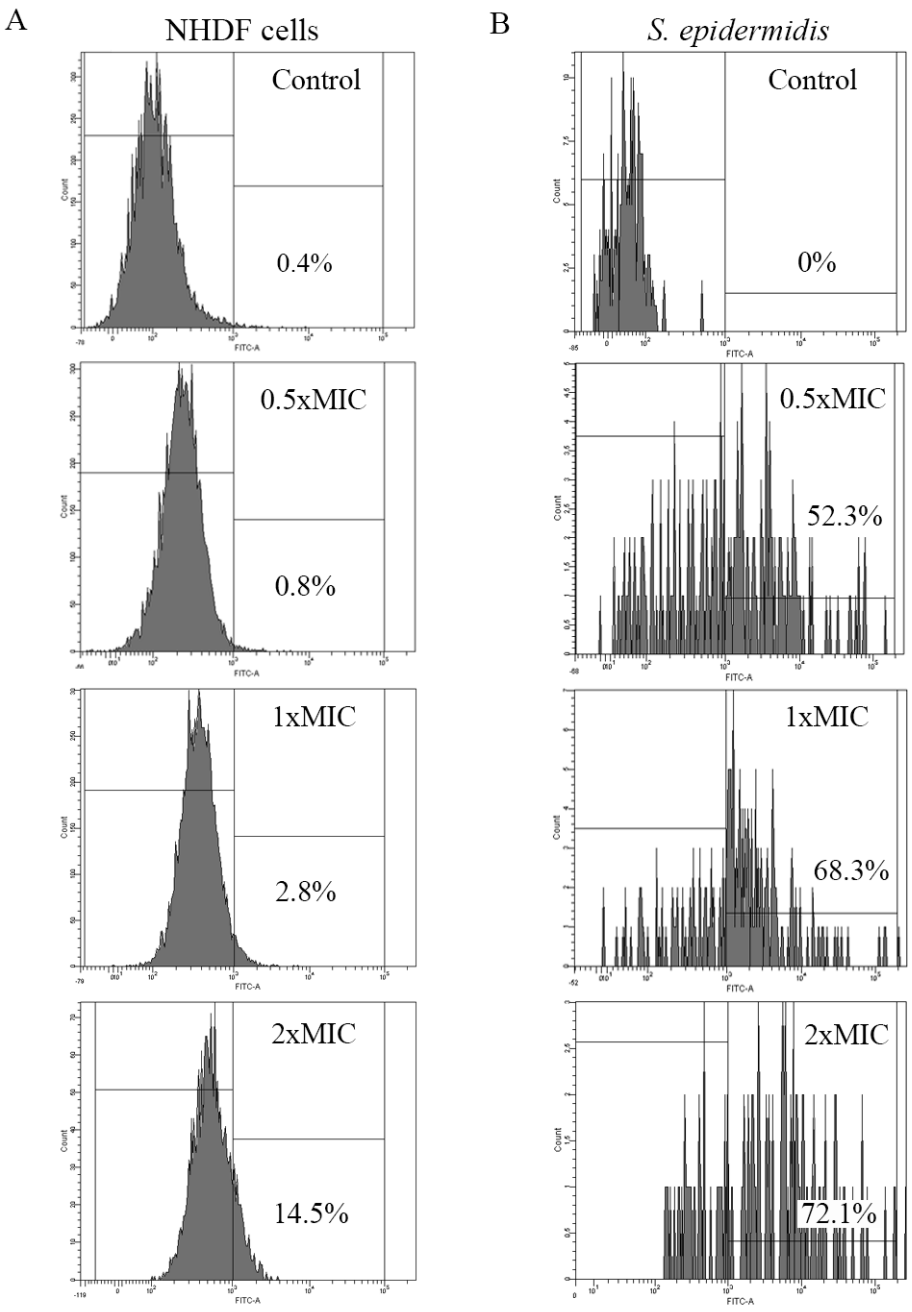


Figure 4.16 The cell selective of FITC-labelled IL15NJ3 to NHDF cells and *S. epidermidis*, as determined by flow cytometry technique. The control group in both mammalian and bacterial cells was processed without peptide-labeled FITC. The treatment groups were co-incubated with 0.5 MIC, 1×MIC and 2×MIC of peptide-labeled FITC. (A) peptide-labeled FITC treated NHDF cells and (B) peptide-labeled FITC treated *S. epidermidis* Gram-positive bacteria.

4.8 Ability of peptide on bacterial membrane disruption

The morphology change of *E. coli* and *S. epidermidis* were observed base on scanning electron microscopy (SEM) method. The result in Figure 4.17A demonstrated that *E. coli* cells were incubated without IL15NJ3 (untreated control) displayed smooth membrane surface and represented rod-shaped morphology. While, the exposure of peptide to *E. coli* for 1 h showed the bacterial cell membrane destruction in dose-dependent manner (Figure 4.17B-D). In the treatment of 0.5×MIC, the external membrane of the *E. coli* cells was altered to rough and exhibited cell clump (Figure 4.17B). After 1×MIC treatment, the surface membranes of *E. coli* were exhibited dramatic shrinking and became ruptured of the cell wall (Figure 4.17C). For a highest of tested concentration (2×MIC), the cell walls of bacteria were showed seriously degraded and collapsed, and absolutely damaged (Figure 4.17D).

For *S. epidermidis*, SEM micrographs showed that the bacterial cells were intact coccal morphology with a smooth surface membrane (Figure 4.17E). However, significant destroys and lysis of bacterial cells membrane were found after treatment with the various concentrations of peptide (0.5×MIC, 1×MIC and 2×MIC) to *S. epidermidis* cells for 1 h (Figure 4.17F-H). After treatment with peptide at 0.5×MIC, the *S. epidermidis* cells membrane were exhibited slight damage (Figure 4.17F). For up to 1×MIC of peptide treatment, the shrinking of bacterial cell membrane was found after 1 h (Figure 4.17G). IL15NJ3 at 2×MIC was clearly exhibited complete loss of integrity of *S. epidermidis* cell membrane and cell wall resulting in the released of intracellular content into an outer area (Figure 4.17H). This data suggested that IL15NJ3 provide bacteriostatic or bactericidal via interrupt membrane.

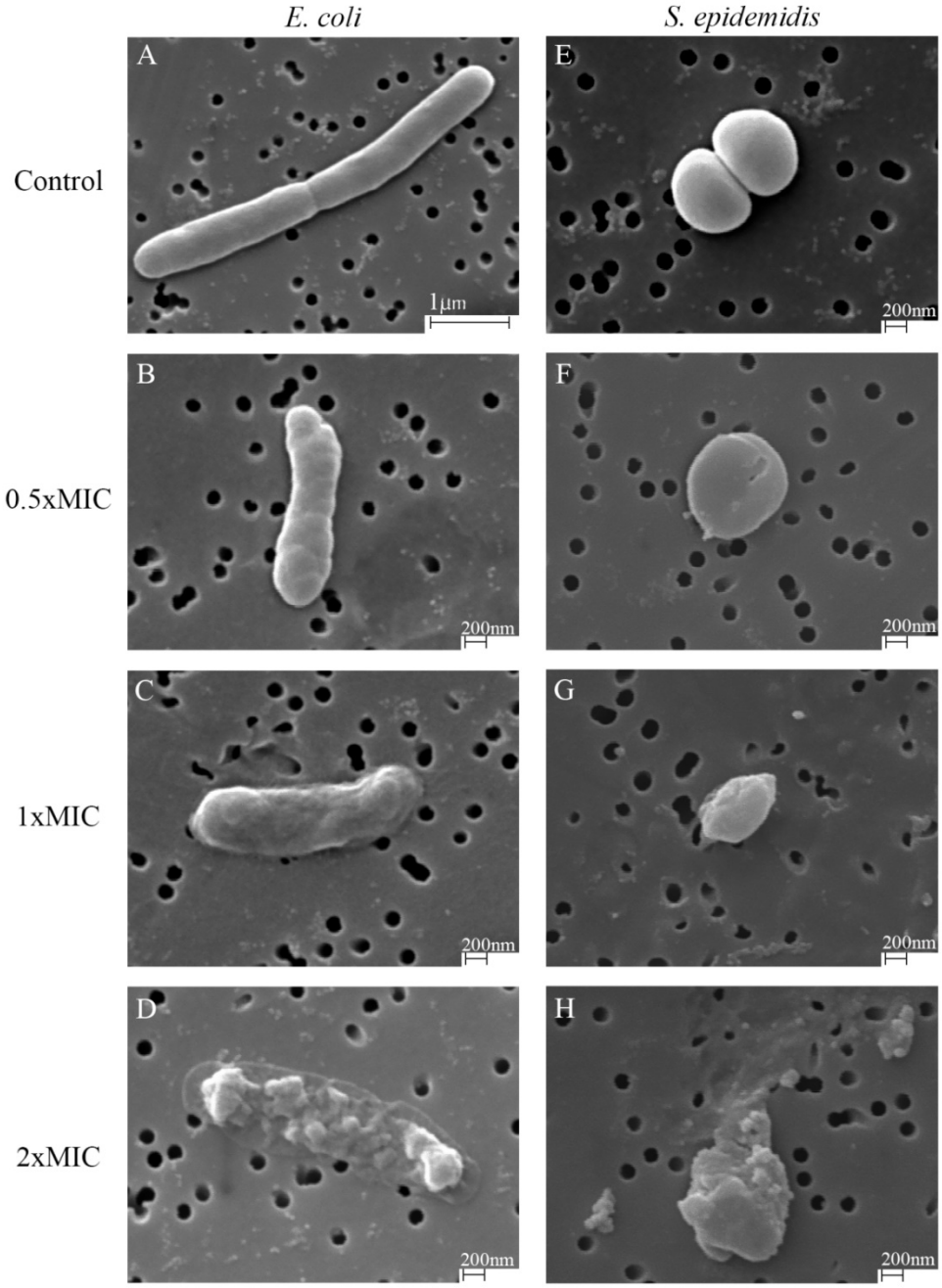


Figure 4.17 The SEM images of *E. coli* and *S. epidermidis* after exposure to IL15NJ3 at 0.5×MIC, 1×MIC and 2×MIC concentration for 1 h. (A) *E. coli* without IL15NJ3, (B) *E. coli* with 0.5×MIC IL15NJ3, (C) *E. coli* with 1×MIC IL15NJ3, (D) *E. coli* with 2×MIC IL15NJ3, (E) *S. epidermidis* without IL15NJ3, (F) *S. epidermidis* with 0.5×MIC IL15NJ3, (G) *S. epidermidis* with 1×MIC IL15NJ3, and (H) *S. epidermidis* with 2×MIC IL15NJ3.

4.9 Antibiofilm activity

The ability of IL15NJ3 to inhibit preformed biofilm was consequently determined. As displayed in Figure 4.18, the significantly reduced the preformed biofilm of *P. aerogenosa* were observed at 1×MIC, 2×MIC and 4×MIC of peptide. Preformed biofilm is considered as the final stage for biofilm development. In this stage bacterial in biofilms must detachment and spread cells into the environment to colonize new space. Hence, it can conclude that IL15NJ3 excellently inhibit biofilm development in *P. aeruginosa* biofilm.

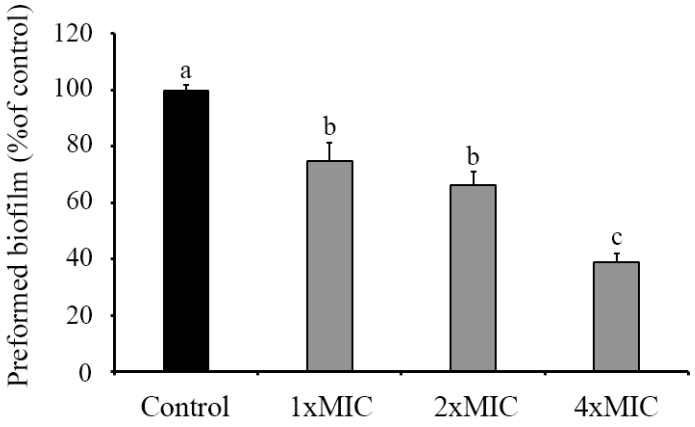


Figure 4.18 Anti-biofilm results of the IL15NJ3 peptide. Each value is expressed as the mean ± SD. Different letters (a-c) on the top of each bar indicated statistically significant differences ($p < 0.05$).

4.10 Proteomics analysis in *P. aerogenosa* biofilms

The expression levels of protein in *P. aerogenosa* biofilms with or without peptide were displayed in the Heatmap diagram (Figure 4.19). This shows the 200 different quantity of protein from *P. aerogenosa* when compared between control and IL15NJ3 peptide treated conditions. Of these proteins identified, 190 proteins were exhibited in both conditions, 6 proteins were found only in untreated control *P. aerogenosa* biofilms, whereas 4 proteins were found only in IL15NJ3 treated *P. aerogenosa* biofilms (Figure 4.20). The type of proteins that found only in each condition was illustrated in Table 4.7. Then, all of 200 proteins were classified for their function using PseudoCAP category. As demonstrated in Figure 4.21, the 55.5% of proteins were identified as unknown function, whereas 44.5% of protein cloud identified to be known function. Depend on PseudoCAP, the 44.5% of the known

proteins functional cloud divided into 21 function classes as demonstrated in Table 4.8. The example of protein fictional class was the amino acid biosynthesis and metabolism class. The 4.4% of known function protein was found in this class. Remarkably main of the proteins of this class were expressed at equal levels between the two conditions. The protein class of carbon compound catabolism had 1 down-regulated protein (allantoicase), 1 equal expression of protein (phosphogluconate dehydratase) and 2 up-regulated proteins (aliphatic amidase and glyceraldehyde-3-phosphate dehydrogenase). Three proteins of central intermediary metabolism class were up-regulated in two conditions that were metallothionein, alkaline phosphatase and glutathione-dependent formaldehyde dehydrogenase. Moreover, only one protein (DNA primase) was up-regulated in the DNA replication, recombination, modification and repair class when exposed to peptide.

The largest single structure of a bacterium is the bacterial membrane. The homeostasis of this structure is maintained by upholding the integrity of the cell that plays an important role in the survival of bacteria in the diverse environments. Therefore, the proteins that associated with the structure of bacteria were mainly observed. The results in Table 9 also demonstrated the 5.9% of protein class of fatty acid and phospholipid metabolism that showed 1 down-regulated protein (alpha/beta hydrolase), 2 up-regulation proteins (cardiolipin synthase, partial and acyl-CoA dehydrogenase) and 1 equal expression protein in both conditions (acyltransferase). The class of transcriptional regulators was slightly expressed at high levels. The highest percentages of proteins (19.1%) were found in the translation, post-translational modification, degradation class.

The flagellin in motility and attachment class and type 1 fimbrial protein in cell adhesion class were found down-regulated in their expression level in IL15NJ3 peptide treated *P. aeruginosa* biofilm. In addition, TrbL protein (plasmid) and phenazine biosynthesis protein in protein secretion system and secreted factors (toxins, enzymes, alginate) class were down-regulated. By PseudoCAP, these proteins have been previously document involved with quorum sensing pathway in *P. aeruginosa* biofilm formation (www.pseudomonas.com).

Table 4.7 The listed of protein expression found in *P. aeruginosa* biofilm that with or without IL15NJ3.

ID	Name
Control group	
gi|1524107607	LysR family transcriptional regulator
gi|1148843158	phenazine biosynthesis protein, partial
gi|1205073142	SulP family inorganic anion transporter, partial
gi|1244535903	5-oxo-L-prolinase
gi|685986031	periplasmic-like protein
gi|685977940	alpha/beta hydrolase
Treated group	
gi|1543310493	DUF262 domain-containing protein
gi|1159854540	zinc ribbon domain-containing protein
gi|1546849704	amino acid permease
gi|959949557	hypothetical protein

Figure 4.19 The heatmap diagram of *P. aeruginosa* biofilm formation with or without IL15NJ3.

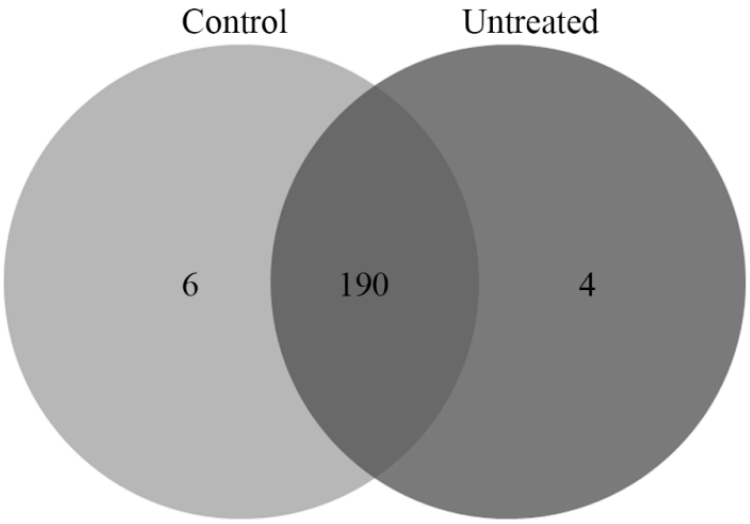


Figure 4.20 The Venn-diagram display the quantity of shared and exclusive protein between control and treated condition from *P. aeruginosa* biofilm.

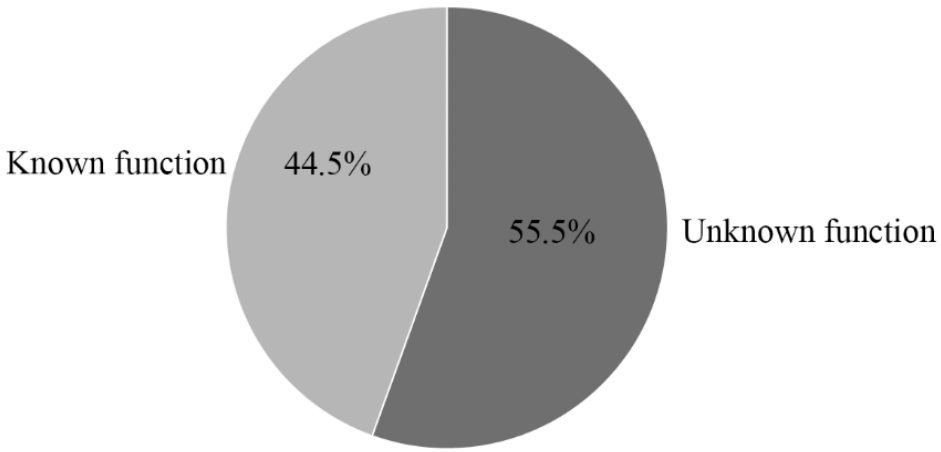


Figure 4.21 The percentage of group of protein functional classification.

Table 4.8 PseudoCAP functional classification of the protein in *P. aeruginosa* biofilm formation.

No.	Functional Class	Protein expression (No.)				%protein in functional class
		Down	Up	Equal	Total	
1	Adaptation, protection	-	-	1. mo+B161lecular chaperone DnaK 2. universal stress protein, partial 3. cold-shock protein	3	4.4
2	Amino acid biosynthesis and metabolism	-	1. arginine deiminase	1. argininosuccinate lyase 2. 4-hydroxyphenylpyruvate dioxygenase	3	4.4
3	Antibiotic resistance and susceptibility	-	-	1. antibiotic biosynthesis monooxygenase	1	1.5
4	Carbon compound catabolism	1. allantoicase	1. aliphatic amidase 2. glyceraldehyde-3-phosphate dehydrogenase	1. phosphogluconate dehydratase	4	5.9
5	Cell adhesion	1. type 1 fimbrial protein	-	-	1	1.5
6	Central intermediary metabolism	-	1. metallothionein 2. alkaline phosphatase 3. glutathione-dependent formaldehyde dehydrogenase	-	3	4.4

Table 4.8 PseudoCAP functional classification of the protein in *P. aeruginosa*-biofilm formation. (Cont.)

No.	Functional Class	Protein expression (No.)				%protein in functional class
		Down	Up	Equal	Total	
7	Chaperones & heat shock proteins	0	0	1. chaperonin GroEL	1	1.5
8	Chemotaxis	1. chemotaxis protein CheA	0	1. methyl-accepting chemotaxis protein	2	2.9
9	DNA replication, recombination, modification and repair	0	1. DNA primase	1. HU family DNA-binding protein	2	2.9
10	Energy metabolism	0	1. succinate dehydrogenase iron-sulfur subunit 2. succinate dehydrogenase flavoprotein subunit 3. F0F1 ATP synthase subunit beta	1. electron transfer flavoprotein subunit alpha/FixB family protein 2. cytochrome c	5	8.8
11	Fatty acid and phospholipid metabolism	1. alpha/beta hydrolase	1. cardiolipin synthase, partial 2. acyl-CoA dehydrogenase	1. acyltransferase	4	5.9

Table 4.8 PseudoCAP functional classification of the protein in *P. aeruginosa*-biofilm formation. (Cont.)

No.	Functional Class	Protein expression (No.)				%protein in functional class
		Down	Up	Equal	Total	
12	Motility & Attachment	1. flagellin	0	1. diguanylate cyclase	2	2.9
13	Nucleotide biosynthesis and metabolism	0	1. ribonucleotide-diphosphate reductase subunit beta	0	1	1.5
14	Protein secretion system	1. TrbL protein (plasmid)	0	0	1	1.5
15	Putative enzymes	0	1. dihydrolipoyllysine-residue acetyltransferase, partial 2. DNA helicase	1. EAL domain-containing Protein 2. UDP-N-acetylglucosamine diphosphorylase/ glucosamine-1-phosphate N-acetyltransferase, partial 3. GTP-binding protein, partial	5	8.8
16	Related to phage, transposon, or plasmid	1. conjugal transfer protein TraG	1. conjugal transfer protein 2. transposase	0	3	4.4

Table 4.8 PseudoCAP functional classification of the protein in *P. aeruginosa*-biofilm formation. (Cont.)

No.	Functional Class	Protein expression (No.)				%protein in functional class
		Down	Up	Equal	Total	
17	Secreted Factors (toxins, enzymes, alginate)	1. phenazine biosynthesis protein, partial	1. non-ribosomal peptide synthetase	1. triacylglycerol lipase	3	4.4
18	Transcription, RNA processing and degradation	1. DNA-directed RNA polymerase subunit beta′	1. DNA-directed RNA polymerase subunit beta	0	2	2.9
19	Transcriptional regulators	1. LysR family transcriptional regulator	1. AraC family transcriptional regulator	0	2	2.9
20	Translation, post-translational modification, degradation	1. elongation factor G 2. 30S ribosomal protein S14 3. D-tyrosyl-tRNA(Tyr) deacylase 4. ribonuclease R 5. cAMP-activated global transcriptional regulator CRP	1. elongation factor Tu, partial	1. peptidase 2. 30S ribosomal protein S1 3. 50S ribosomal protein L11 4. elongation factor Tu	13	19.1

Table 4.8 PseudoCAP functional classification of the protein in *P. aeruginosa*-biofilm formation. (Cont.)

No.	Functional Class	Protein expression (No.)				%protein in functional class
		Down	Up	Equal	Total	
20	Translation, post-translational modification, degradation	6. sigma-54-dependent Fis family transcriptional regulator 7. 50S ribosomal protein L1 8. RHS repeat protein, partial			13	19.1
21	Transport of small molecules	1. MFS transporter	1. amino acid permease	1. ABC transporter substrate-binding protein 2. polyamine ABC transporter substrate-binding protein	4	5.9

4.11 Anti-inflammatory activity

The anti-inflammatory activity of intact hemoglobin (Hb) and *Crocodylus siamensis* hemoglobin hydrolysates (CHHs) from trypsin and papain digestion at different incubation times (2, 4, 6 and 8 h) were evaluated on the NO production against macrophage RAW 264.7 cells. After induction of inflammation in macrophage RAW 264.7 cells by LPS addition for 24 h, the percentage of nitric oxide production was defined as 100%. Figure 4.22, intact Hb and CHHs at concentrations of 125-500 μg/ml show a decrease in nitric oxide production in a dose-dependent manner. CHH derived from papain hydrolysis by 2-h hydrolysis (2h-CHHp), 4h-CHHp, 6h-CHHp and 8h-CHHp at a concentration of 500 μg/ml show nitric oxide production at 46.86, 66.26, 52.27 and 52.94%, respectively. CHH derived from trypsin hydrolysis by 8-h hydrolysis (8h-CHHt) at a concentration of 500 μg/ml show nitric oxide production at 76.50%. This result shows that CHHs derived from papain hydrolysis had high efficacy to reduce nitric oxide production than CHHs derived from trypsin hydrolysis

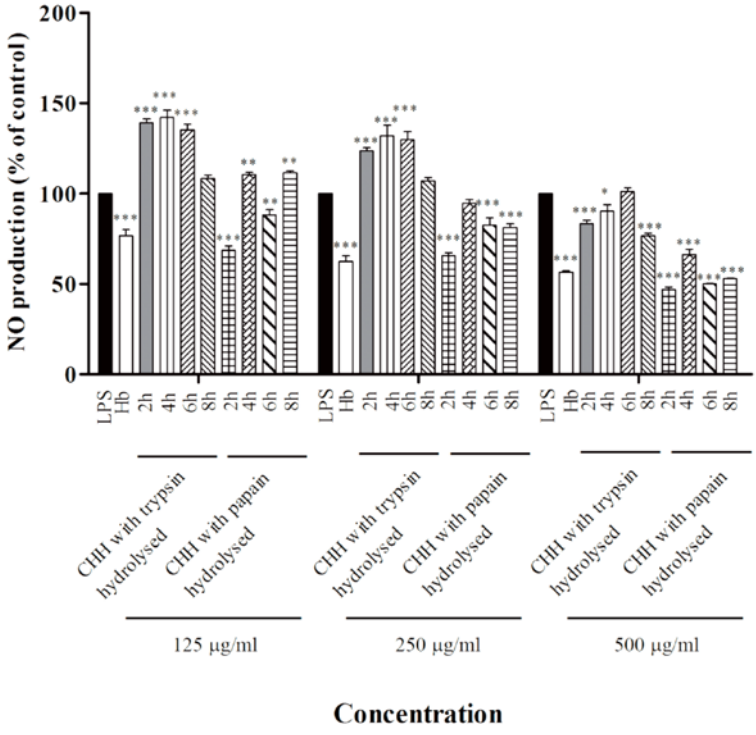


Figure 4.22 The effect of CHHs derived from trypsin and papain digestion on NO production in LPS-activated macrophage RAW 264.7 cells. Each bar displays the mean ± SEM of four demonstrations. (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$) probability levels compared with LPS treatment alone.

The effect of IL15NJ3 peptide on anti-inflammatory activity was constructed. The RAW264.7 macrophage cells were co-incubated with lipopolysaccharide (LPS) for 24 h. Then, the nitric oxide (NO) production was measured as nitrite concentration in the culture medium by Griess reagent assay. NO production was significantly decreased in the presence of IL15NJ3 at the concentration ranging from 100-200 µg/ml. At 100 and 200 µg/ml of IL15NJ3, the percentages of NO production were 19 and 54%, respectively (Figure 4.23). Our data indicated that IL15NJ3 shown anti-inflammatory activity though inhibition of NO production.

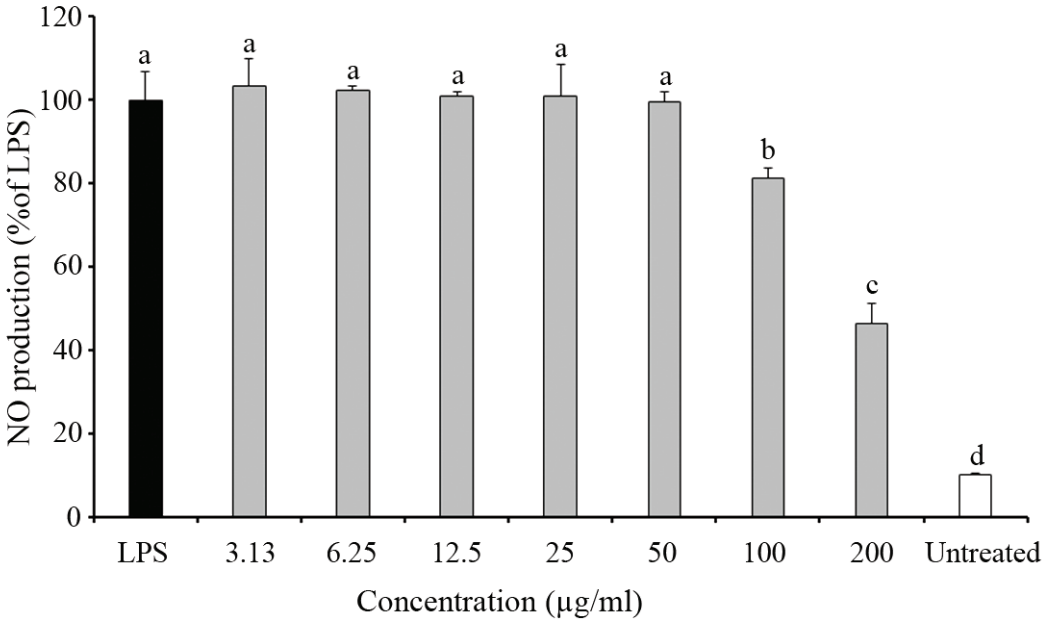


Figure 4.23 The effect of IL15NJ3 on NO production by LPS-stimulated macrophages. Each value is expressed as the mean ± SD. Different letters (a-d) on the top of each bar indicated statistically significant differences ($p < 0.05$).

4.12 Antioxidant activity

DPPH method was to determine the antioxidant activity of *Crocodylus siamensis* hemoglobin hydrolysates (CHHs) obtained from trypsin and papain digestion at different incubation times (2, 4, 6 and 8 h). DPPH scavenging activity of CHH derived from trypsin was similar to intact hemoglobin (Hb). CHH derived from papain hydrolysis by 8-h hydrolysis (8h-CHHp) showed the highest DPPH

scavenging activity at 56.86% antioxidant inhibition with IC50 value of 31 µg/ml (Figure 4.24)

ABTS was used to evaluate antioxidant activity of all synthetic peptides. As shown in Figure 4.25, all designed peptide exhibited significantly increase ABTS scavenging as dose dependent manner. Indeed, the design peptide indicated excellent antioxidant efficacy when compared to their parent peptide (QL17 and IL15) at all experiment concentration (3.13 - 200 ug/ml). Regarding to the highest concentration (200 ug/ml), the ABTS-scavenging efficacy of QL17 and IL15 were calculated to be 11% and 12%, whereas the ABTS suppression of design peptide (i.e. IL15NJ1, IL15NJ2, IL15NJ3, IL15NJ4 and IL15NJ5) exhibited the significant suppression percentage about 61%, 63%, 66%, 66% and 52%, respective. These finding suggested that all design peptide were inhibition free radical greater than parent peptide.

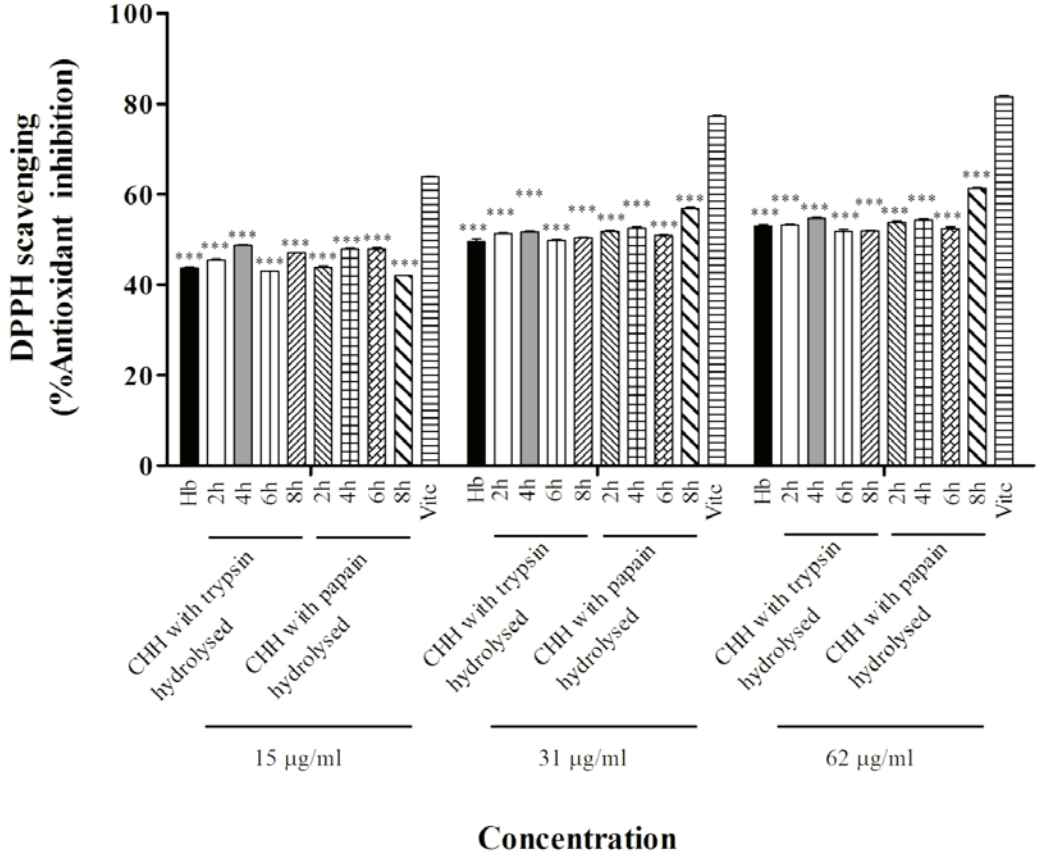


Figure 4.24 Antioxidant effect of CHHs derived from trypsin and papain digestion in DPPH radical scavengingassay. Each bar displays the mean ± SEM of three demonstrations. (*** $P < 0.001$) probability levels compared with vitamin C.

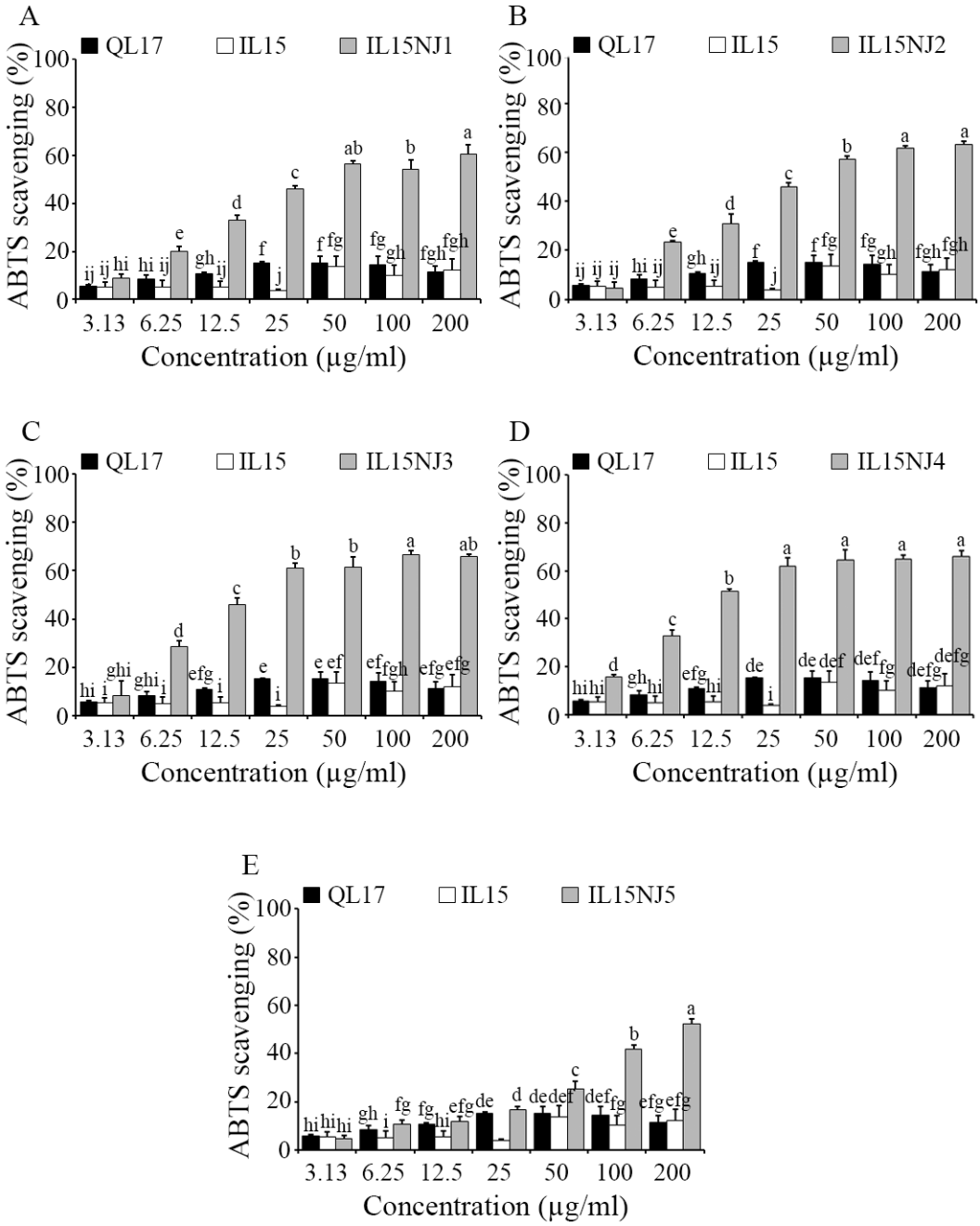


Figure 4.25 ABTS radical scavenging activity of IL15NJ1 (A), IL15NJ2 (B), IL15NJ3 (C), IL15NJ4 (D) and IL15NJ5 (E) were compared with both QL17 and IL15. Each value is expressed as the mean ± SD. Different letters (a-j) on the top of each bar indicated statistically significant differences ($p < 0.05$) compared all treatment.

CHAPTER V
DISCUSSION

Antimicrobial peptides (AMPs) are alternative routes to manage resistant bacteria. They are the crucial polypeptides in host defense, and stand as a significant weapon in the innate immune system to potentially kill a broad range of Gram-negative and Gram-positive bacteria, viruses, and parasites (Reinhardt and Neundorf, 2016; Zhang et al., 2016; Bahar and Ren, 2013). Previously, there was a report that QL17 with the multifunction of antimicrobial activity, anti-inflammatory activity, and antioxidant activity. However, these multifunctional peptides were found to relatively low antimicrobial peptide and had short half-life in mammalian reticulocytes (in vitro) when estimated by ProtParam tool. In addition, as the application use consideration, peptide will need to minimize the cytotoxicity in mammalian cells with the effective antimicrobial activity. Therefore, in order to achieve the potent AMPs with very low cytotoxicity, the design of novel AMPs follow by the combination of template base design and physiochemical properties method was performed using QLl7 as template. Based on the evidence that the complete amphipathic peptide exhibited the excellent bactericidal activity and cell specificity (Zhang et al., 2016; Khara et al., 2017), the peptide was designed by replacing the hydrophobic amino acid tryptophan (Trp) on hydrophobic part and the cationic amino acid lysine (Lys) or arginine (Arg) on the hydrophilic part. Trp is a hydrophobic amino acid with an aromatic side chain that was purposed to help AMP for adhering to the bacterial membrane and to interrupt membranes by affecting the interfacial area of the lipid bilayers (Han et al., 2016). For lysine, lysine provided the positive charge for interacting with the negative charge of the exterior of microbial lipid membranes, resulting in the interruption of bacteria membranes (Zhang et al., 2016). These cationic amino acids can also be considered to interact with negatively charged exterior of microbial lipid membranes (Reinhardt and Neundorf, 2016). As their positive charge enhancement, peptides were observed to display higher binding with bacteria membranes and antimicrobial efficacy.

In this research, the novel design peptides were observed the key physiochemical properties using the online tool. The positively net charge of design peptide was found in the ranging from +2 to +7, and the percentage of hydrophobicity was observed in the ranging from 40%-46%. Generally, the excellent antimicrobial peptide shares some

common feature such include exhibit positive net charge ranging from +2 to +9, large hydrophobicity as ≥ 30% and short (<50 residues) (Hancock and Sahl, 2006). Our peptides were found to have such properties in the range of the good antimicrobial peptide, suggesting the high possibility of these peptide to exhibit the well antimicrobial activity.

The evaluation of the secondary conformations of peptide under the membrane-mimicking environment using CD spectroscopy was performed, the CD result exhibited that all peptide was observed to form random coil conformation in aqueous solution, while, in the membrane-mimicking environment (50% TFE), all peptide was observed to adopt to be α -helical structure. This result agreed with the previous reports that AMPs were formed to the α-helical structure in the membrane environment which significantly increased the partition of peptide to the membrane and corresponded to antimicrobial efficacy (Lyu et al., 2016; Beevers and Dixon, 2015; Chen et al., 2010).

After the investigation of the key physiochemical properties, antibacterial activity was further investigated. The collected result indicated design peptide with the increase of positive net charge amino acid residues exhibited the increase of antibacterial against Gram-negative and Gram-positive bacteria. When compare the design peptides with the same hydrophobicity, the increase of positive net charge was found to increase antimicrobial activity, which agreed with the previous report that the increase of the positive net charge in peptide was found to increase antibacterial activity, resulting from the positive charge of peptide facilitate the peptide to interaction with negative charge bacterial membrane through electrostatic interaction (Zhang et al., 2016; Han et al., 2016). Whereas, when compare the design peptides with the same positive net charge, the increase of hydrophobicity was found to increase antimicrobial activity. Moreover, we also found that the increase of both positive charge and hydrophobicity, IL15NJ3, displayed the highest potent antimicrobial efficacy from among all peptides. These evident suggested that positive net charge and hydrophobicity of peptide play a key role in antibacterial activity. In comparison between Lys and Arg in the modification of the peptides, the result indicated that IL15NJ4 containing Lys showed antimicrobial property better than IL15NJ5 containing Arg. This might be the result from the smaller size of the amino acid side chain may lead to the deep insertion of the peptide into the membrane (Zhang et al., 2016).

For investigation the possibility of the design peptides in the application use, the toxicity of the peptides against mammalian cells was evaluated by measuring their

erythrocyte lysis effect and normal mammalian cell line viability. The significant difference of the hemolytic efficiency and cytotoxicity with the increase of the positive net charge from +4 to +6 was not found. Whereas, the highest positive net charge (IL15NJ4 (+7)) was observed to slightly toxic to RBC, PBMC, HaCaT and NHDF cell and the high toxicity against RAW264.7 cells was found at only the highest tested concentration. Furthermore, the result also showed that increase of the total of positive residues Lys could enhanced both microbial and hemolysis. This result agreed with the previous report that the increase of positive net charge of peptide to +7 could enhance antimicrobial activity, while the remarkable increasing of undesirable hemolysis was occurred (Jiang et al., 2008). These results indicated that there is the threshold of the increase of positive net of antimicrobial peptide for elevation of antimicrobial activity. For hydrophobicity of peptides, IL15NJ3 (+46 hydrophobic) exhibited the higher selective index (SI) than IL15NJ2 (+40 hydrophobic), suggesting the hydrophobicity of the peptides could increase the cell selectivity of peptides. Furthermore, substitution of Arg to Lys was found to increase more hemolytic efficiency and cytotoxicity. It is possible that larger R-side chain of Arg residues than Lys might be cause the high interfere with mammalian cell (Zhang et al., 2016). The SI was popularly utilized to attribute the representation of the cell selectivity of antimicrobial therapeutic agent. The higher SI rate demonstrated the excellent antimicrobial specificity. The SI of IL15NJ4 toward Gram-negative and Gram-positive bacteria was increased approximately 6 and 25-fold, respectively, as compared to IL15NJ2 (Table 7-8). When Compared IL15NJ3 with IL15NJ4, the SI of IL15NJ4 toward Gram-negative and Gram-positive bacteria was observed to slightly lower. These findings suggested that increase of positive net charged residues and hydrophobicity in suitable place will enhance the SI of peptide (Khara et al., 2017; Matsuzaki, 2009). Previously, Yin et al. (2012) exhibited that the cell selective of AMPs do not involve only one factor but rather require a good balance among (i) peptide helicity, (ii) optimum hydrophobicity of the core segment, (iii) positive charges and their distribution, (iv) dimerization and/or oligomerization ability in the membrane, and (v) minimization of aggregation.

Based on the great SI and antibacterial activity, IL15NJ3 was selected to investigate the effect for killing the Gram-positive and Gram-negative bacteria in stationary-phase of development. In 1xMIC, IL15NJ3 was found to mostly kill the *E. coli* (Gram-negative) within 60 min, while this peptide was observed to mostly kill the *S. epidermidis* within 180 min, suggesting that the effectiveness of peptide to kill

bacteria is based on the components of bacterial membrane. Although, the gram-positive bacteria lack the outer membrane, however, gram-positive bacteria have the peptidoglycan thicker than gram-positive bacteria (Malanovic and Lohner, 2016). Thus, the thick peptidoglycan might prevent the damage peptide better than the thin peptidoglycan, resulting in the higher antimicrobial activity toward Gram-negative bacteria when compare to gram positive bacteria.

To confirm cell specificity, IL15NJ3 was selected for the selective study. After the holding IL15NJ3-labelled FITC together with bacteria cells or mammalian cell line at the same time, FITC fluorescence intensity was increased in *E. coli* and *S. epidermidis* in dose-dependent manner. In contrast, the incubation with mammalian cells line, FITC fluorescence intensity was not increased when increase concentration of peptide, demonstrating that IL15NJ3 showed the selection with microbial higher than mammalian cell membranes, leading to maximize of antimicrobial activity with the minimize of toxicity.

To understand the killing mechanism of peptide toward bacterial, SEM was carried out to investigate morphology change. The result showed that the treatment of bacterial cell with IL15NJ3 resulted in the dramatic shrinking or blebbing, collapsed, and the absolute damaged on the cell surface. The previous studies have found that the general α-helical antimicrobial peptides showed antimicrobial efficiency by interrupt membrane integrity (Khara et al., 2017; Zhang et al., 2016; Reinhardt and Neundorf, 2016). The first process of AMPs killing bacteria was the electrostatic interaction between peptides with negative net charge components of phospholipids in the outer membrane (Zhang et al., 2016). After that, the peptides can subsequently create the membrane leaking, leading to both intrinsic and extrinsic metabolites free diffusing and bacteria death (Khara et al., 2017).

Most frequently infections in humans are caused by bacterial biofilms, which *P. aeruginosa* has the potentiality to transform from free living (planktonic) to biofilm phenotypic process of living. *P. aeruginosa* biofilms occur in a kind of clinical settings in cystic fibrosis lung, urinary catheters, and contact lenses (Waite et al., 2005). The bacterial in the community of biofilm was coated with polysaccharides, DNA, and/or proteins (Flemming and Wingender, 2010). The ultimate dramatic appearance of biofilm-related infections is that they are extremely resistant to conventional antibiotics against planktonic cells (de la Fuente-Nunez, 2015). Therefore, the exposure of antimicrobial agents that combat both planktonic microorganisms and biofilms is a key

for public health. The present study found that IL15NJ3 inhibited the initial attachment and biofilm formation process of *P. aeruginosa*. The ability of AMPs to inhibit bacterial biofilm might be the result from the inhibition of biofilm directly against planktonic bacteria, resulting in the suppressing of biofilm formation by reducing of the population of bacterial cells. Previously, LL-37 peptides, cationic and helical antimicrobial peptide, can affect the proteins expression that inhibits biofilm formation (Overhage et al., 2008). Thus, it is possible that IL15NJ3 might also inhibit bacterial biofilm by affecting the expression of protein involves with biofilm formation.

Therefore, in order to investigate the effect of peptide toward the expression of protein involves with biofilm formation and understands more inhibitory mechanism of peptide against *P. aeruginosa* biofilm, proteomic analysis was used. The expression levels of protein in *P. aerogenosa* biofilms with or without peptide were displayed in the heatmap diagram. This result showed the 200 different quantity of protein from *P. aerogenosa* when compared between control and treatment. All protein expression was separated by PseudoCAP category. The result found that 55.5% of protein cloud unknown function where 44.5% of protein cloud identified to known function. Depend on PseudoCAP examination, the main of the known proteins was found to categorize in 21 functional classes of protein. Interestingly, when considering the known function proteins, the results indicated IL15NJ3 could suppress protein expression of TrbL (plasmid) protein which is important in quorum sensing (QS) pathway (Winsor et al., 2016). Quorum sensing is the important process for *P. aeruginosa* virulence property, motility and biofilm formation, which is essential for cell communication for controlling population density. *P. aeruginosa* has two major QS systems, composing with las and rhl, which drive the production (throughout synthases LasI and RhlI) and the perception (by the transcription factors LasR and RhlR) of the autoinducer signaling molecules that involved with the formation of bioflim (Jimenez et al., 2012). Previous research exhibited that the suppressing of *P. aeruginosa* quorum sensing (QS) system interrupted biofilm formation and virulence effect (Rasamiravaka et al., 2015). In addition, TrbL protein (plasmid) was also important for phenazine biosynthesis. The phenazine is influences to the swarming motility and the surface to volume ratio of mature biofilms (Ramos et al., 2010). Furthermore, IL15NJ3 was observed to down regulate the type 1 fimbrial protein and flagellin. These proteins are the key protein of *P. aeruginosa* for cell motility, adhesion, and attachment (Koczan et al., 2011; Merritt

et al., 2007). Therefore, it is possible that this peptide could inhibited *P. aeruginosa*-biofilm formation though reduced adhesion and motility of planktonic cell.

Host innate immune responses during sepsis were found to involve with the presence of gram-negative bacterial outer membrane components. Lipopolysaccharide (LPS) is one of bacteria membrane components which is produced by gram-negative bacteria and could elevate the inflammation process by stimulation the production of NO. Thus, the inhibition of inflammation process causing from sepsis by interfering the LPS-stimulated inflammation is interested. This research, IL15NJ3 was found to potentially suppress NO levels in LPS-stimulated RAW264.7 cell, suggesting the potent ability of peptide to prevention of inflammation. Previously, AMPs were report to neutralize the endotoxin-induced NO and inflammatory mediator production by LPS via the direct bind to LPS or prevent the binding of LPS with LPS-binding protein (LBP) (Scott and Hancock, 2000; Scott et al., 2007). As a result, it is possible that IL15NJ3 might prevent the LPS-stimulated inflammation by using its positive segment to interact with phosphate groups of LPS via electrostatic interactions and hydrophobic residues to from hydrophobic interaction with non-polar region of LPS, resulting in LPS cannot bind to LBP and macrophages were not activated the inflammation process.

After the infection with bacteria, outer bacterial membrane component, including LPS (Gram-negative) and LTA (Gram-positive), can activated immune system of host cell to produce free radical and caused the oxidative stress in the biological system. Oxidative stress is the causes of numerous diseases, comprising with rheumatoid arthritis, chronic asthma, multiple sclerosis, diabetes, cancer, cardiovascular problems and other degenerative disorders (Cai et al., 2014). Therefore, the suppression of oxidative stress via the scavenging of free radical is the interesting way to prevent the effect of oxidative stress. This research, all of designed peptides were found to exhibit ABTS radical scavenging activity. The amino acid composition and sequence plays a crucial role in antioxidant activity of peptide. The higher content of hydrophobic amino acids in peptides was found to show the greater scavenge radical (Zou et al., 2016). The peptides containing His and Trp residues could exhibit the property of chelates capacity (Phosri et al., 2014; Zou et al., 2016). The positively charge residues (Arg and Lys) is believed to evince a significant part in ion chelation activity (Memarpoor-Yazdi et al., 2012; Zou et al., 2016). As the result, the antioxidant activity of designed peptides might result from amino acid residues such as Trp, Arg, and Lys.

REFERENCES

Adje, E.Y., Balti, R., Kouach, M., Dhulster, P., Guillochon, D., Arroume, N.N. 2011. Obtaining antimicrobial peptides by controlled peptic hydrolysis of bovine hemoglobin. Int J Biol Macromol. 49, 143-153.

Ahmad, A., Ahmad, E., Rabbani, G., Haque, S., Arshad, M., Khan R.H., 2012. Identification and design of antimicrobial peptides for therapeutic applications. Current Protein and Peptide Science. 13, 211-223.

Andreu, D., Rivas, L., 1998. Animal antimicrobial peptides: an overview. Biopolymers. 47, 415-433.

Arroume, N.N., Delval, V.D., Adje, E.Y., Traisnel, J., Krier, F., Mary, P., Kouach, M., Briand, G., Guillochon, D. 2008. Bovine hemoglobin: An attractive source of antibacterial peptides. Peptides 29, 969-977.

Arroume, N.N., Delval, V.D., Miloudi, K., Daoud, R., Krier, F., Kouach, M., Briand, G., Guillochon, D. 2006. Isolation and characterization of four antibacterial peptides from bovine hemoglobin. Peptides. 27, 2082-2089.

Babu, S.S., Mathew, S., Kalarikkal, N., Thomas, S., Radhakrishnan, E.K. 2016. Antimicrobial, antibiofilm, and microbial barrier properties of poly (e-caprolactone)/cloisite 30B thin films. 3 Biotech. 6, 249, doi: 10.1007/s13205-016-0559-7.

Bahar, A.A., Ren, D. 2013. Antimicrobial Peptides. Pharmaceuticals. 6, 1543-1575.

Beevers. A.J., Dixon, A.M. 2010. Helical membrane peptides to modulate cell function. Chem Soc Rev. 39, 2146-2157.

Brogden, K.A., 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol. 3, 238-250.

Brown, M.R., Allison, D.G., Gilbert, P., 1988. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? J. Antimicrob. Chemother. 22, 777–780.

Cai, Y., Luo, Q., Sun, M., Corke, H. 2014. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 74, 2157-2184.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D., and Buret, A., 1999. The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. Journal of Clinical Microbiology. 6, 1771-1776.

Chang, Y., Yang, C., Sun, R., Cheng, Y., Kao W., Yang, P. 2013. Rapid single cell detection of *Staphylococcus aureus* by aptamer-conjugated gold nanoparticles. Scientific Reports. 3, DOI: 10.1038/srep01863.

Chatterjee, M., Anju, C., Biswas, L., Kumar, V.A., Mohan, C.G., Biswas, R., 2016. Antibiotic resistance in Pseudomonas aeruginosa and alternative therapeutic options. Int J Med Microbiol. 306, 48-58.

Cheema, M.S., Rassing, J.E., Marriott, C., 1986. The diffusion characteristics of antibiotics in mucus glycoprotein. J. Pharm. Pharmacol. 38, 53.

Chen, H.D., Frankel, G., 2004. Enteropathogenic *Escherichia coli*: unravelling pathogenesis. FEMS Micro. Bio. Rev. 29, 83-98.

Cochrane, D.M.G., Brown, M.R.W., Anwar, H., Weller, P.H., Lams, K., Costertons, J.W., 1988. Antibody response to *Pseudomonas aeruginosa* surface protein antigens in a rat model of chronic lung infection. J. Med. Microbiol. 27, 255-261.

Costerton, J.W., Lewandowski, Z., 1995. Microbial biofilm. Annu. Rev. Microbiol. 49, 711.

Cotton, L.A., Graham, R.J., Lee, R.J., 2009. The Role of Alginate in *P. aeruginosa* PAO1 Biofilm Structural Resistance to Gentamicin and Ciprofloxacin. J. Exp. Microbiol Immunol. 13, 58-62.

Daoud, R., Dubois, V., Dodita, L. B., Arroume, N. N., Krier, F., Chihib, N. E., Mary, P., Kouach, M., Briand, G., Guillochon, D., 2005. New antibacterial peptide derived from bovine hemoglobin. Peptides. 26, 713-719.

Das, A., Das, M.C., Sandhu, P., Das, N., Tribedi, P., De, U.C., Akhter, Y., Bhattacharjee, S., 2017. Antibiofilm activity of *Parkia javanica* against *Pseudomonas aeruginosa*: a study with fruit extract. RSC Advances. 7, 5497-5513.

Dathe, M., Wieprecht, T., Nikolenko, H., Hande,l L., Maloy, W.L., MacDonald, D.L., Beyermann, M., Bienert, M., Hydrophobicity, hydrophobic moment and angle subtended by charged residues modulate antibacterial and haemolytic activity of amphipathic helical peptides. FEBS Lett. 403, 208-212.

Davies, G.D., Chakrabarty, M.A., Geesey, G.G., 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 59, 1181-1186.

de la Fuente-Núñez, C., and Hancock, R.E., 2015. Using anti-biofilm peptides to treat antibiotic-resistant bacterial infections. Postdoc J. 3, 1-8.

de la Fuente-Núñez, C., Cardoso, M.H., de Souza Cândido ,E., Franco, O.L., Hancock, R.E., 2016. Synthetic antibiofilm peptides. Biochim Biophys Acta. 1858, 1061-1069.

DeBeer, D., Stoodley, P., Lewandowski, Z., 1994. Effects of biofilm structures on oxygen distribution and mass transport. Biotech. Bioeng. 43, 1131-1138.

Demirer, S., GecËim, I.E., Aydõnuraz, K., lu, H.A., Yerdel, M.A., Kuterdem, E., 2001. Affinity of *Staphylococcus epidermidis* to Various Prosthetic Graft Materials. J Surg Res. 99, 70-74.

Earl, A.M., Losick, R., Kolter, R., 2008. Ecology and genomics of *Bacillus subtilis*. Trends Microbiol. 16, 269.

Fey, P.D., Olson, M.E., 2010. Current concepts in biofilm formation of *Staphylococcus epidermidis*. Future Microbiol. 5, 917-933.

Fito-Boncompte, L., Chapalain, A., Bouffartigues, E., Chaker, H., Lesouhaitier, O., Gicquel, G., Bazire, A., Madi, A., Connil, N., Véron, W., Taupin, L., Toussaint, B., Cornelis, P., Wei, Q., Shioya, K., Déziel, E., Feuilloley, M.G.J., Orange, N., Dufour, A., Chevalier S. 2011. Full virulence of *Pseudomonas aeruginosa* requires OprF. Infect Immun. 79, 1176-1186.

Fjell, C.D., Hiss, J.A., Hancock, R.E., Schneider, G., 2012. Designing antimicrobial peptides: form follows function. , Nat. Rev. Drug Discov. 11, 37-51.

Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nature Reviews Microbiology. 9, 623– 633.

Froidevaux, R., Kriera, F., Arroume, N. N., Marko, D. V., Kosciarz, E., Ruckebusch, C., Dhulster P. Guillochon D., 2001. Antibacterial activity of a pepsin-derived bovine hemoglobin fragment. FEBS Letters. 491, 159-163.

Global strategy for containment of antimicrobial resistance. Geneva, World Health Organization, 2001. (http://www.who.int/drugresistance/WHO_Global_ Strategy_English.pdf, accessed 8 July 2017).

Gordon, Y.J., Romanowski, E.G., McDermott, A.M. 2005. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. Curr Eye Res. 30, 505-515.

Han, H.M., Gopal, R., Park, Y. 2016. Design and membrane-disruption mechanism of charge-enriched AMPs exhibiting cell selectivity, high-salt resistance, and anti-biofilm properties. Amino Acids. 48, 505-522.

Hancock, R.E.W., Sahl, H.G., 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol. 24, 1551–1557.

Howe, E., Holton, K., Nair, S., Schlauch, A., Sinha, R. 2010. MeV: Multi Experiment Viewer. In Biomedical Informatics for Cancer Research. Quackenbush J. 267-277.

Hoyle, B.D., Alcantara, J., Costerton, J.W., 1992. *Pseudomonas aeruginosa* biofilm as a diffusion barrier to Piperacillin. Antimicrob. Agents Chemother. 36, 2054-2056.

Hurdle, J.G., O'Neill, A.J., Chopra, I. & Lee, R.E., 2011. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nature Reviews Microbiology 9, 62-z75.

Isabel C.A. Scaletsky, I.C.A., Fabbricotti, S.H., Carvalho, R.L.B., Nunes, C.R., Maranhão, H.S., Morais, M.B. Fagundes-Neto U., 2002. Diffusely Adherent *Escherichia coli* as a Cause of Acute Diarrhea in Young Children in Northeast Brazil: a Case-Control Study. J Clin Microbiol. 40, 645-648.

Jafari, A., MM Aslani, M.M., Bouzari, S. 2012. *Escherichia coli*: a brief review of diarrheagenic pathotypes and their role in diarrheal diseases in Iran. Iran J Microbiol. 4, 102-117.

Jangpromma, N., Preecharram, S., Srilert, T., Maijaroen, S., Mahakunakorn, P., Nualkaew, N., Daduang, S., Klaynongsruang, S., 2016. *In Vitro* and *in Vivo* Wound Healing Properties of Plasma and Serum from Crocodylus siamensis Blood. Journal of Microbiology and biotechnology. 26, 1140-1147.

Jangpromma, N., Poolperm, N., Pornsri, K., Anwised, P., Kabbua, T., Phosri, S., Daduang, S., Klaynongsruang, S. 2017. Proteomics profiling and inflammatory factor gene expression in LPS-stimulated RAW 264.7 cells treated with *Crocodylus siamensis* hemoglobin. Chiang Mai J. Sci. 44(3), 800-815.

Jiang, Z., Vasil, A.I., Hale, J.D., Hancock, R.E., Vasil, M.L., Hodges, R.S. 2008. Effects of net charge and the number of positively charged residues on the biological activity of amphipathic alpha-helical cationic antimicrobial peptides. Biopolymers. 90, 369-383.

Johansson, C., Samskog, J., Sundstrom, L., Wadensten, H.., Bjorkesten, L., Flensburg, J. 2006. Differential expression analysis of Escherichia coli proteins using a novel software for relative quantitation of LC-MS/MS data. Proteomics. 6, 4475-4485.

Jorge, P., Lourenço, A., Pereira, M.O., 2012. New trends in peptide-based anti-biofilm strategies:a review of recent achievements and bioinformatic approaches, Biofouling. 28, 1033-1061.

Khara, J.S., Obuobi, S., Wang, Y., Hamilton, M.S., Robertson, B.D., Newton, S.M. 2017. Disruption of drug-resistant biofilms using *de* novo designed short α-helical antimicrobial peptides with idealized facial amphiphilicity. Acta Biomater. 57, 103-114.

Khoury, A.E., Lam, K., Ellis, B., Costerton, J.W., 1992. Prevention and control of bacterial infections associated with medical devices. Am. Soc. Artif. Intern. Organs J. 38, 174-178.

Kommanee, J., Preecharram, S., Daduang, S., Temsiripong, Y., Dhiravisit, A., Yamada, Y., Thammasirirak, S., 2012. Antibacterial activity of plasma from crocodile (*Crocodylus siamensis*) against pathogenic bacteria. Ann Clin Microbiol Antimicrob. 11:22.

Lambe, D.W., Ferguson, K.P., Mayberry-Carson, K.J., Tober-Meyer, B., Costerton, J. W., 1991. Foreign-body-associated experimental osteomyelitis induced with Bacteroides fragilis and Staphylococcus epidermidis in rabbits. Clin. Orthop. 266, 285–294.

Laverty, G., Gorman, S.P., Gilmore, B.F. 2014. Biomolecular Mechanisms of *Pseudomonas aeruginosa* and *Escherichia coli* Biofilm Formation. Pathogens. 3, 596-632.

Logan, N.A., 2011. *Bacillus* and relatives in foodborne illness. J Appl Microbiol. 112, 417-429.

Lomize, M.A., Pogozheva, I.D., Joo, H., Mosberg, H.I., Lomize, A.L. 2012. OPM database and PPM web server: resources for positioning of proteins in membranes. Nucleic Acids Res. 40(Database issue), D370-6.

Lowry, O.H., Rosbrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J Biol Chem. 193, 265-275.

Lyu, Y., Yang, Y., Lyu, X., Dong, N., Shan, A. 2016. Antimicrobial activity, improved cell selectivity and mode of action of short PMAP-36-derived peptides against bacteria and *Candida.* Sci Rep. 6. doi: 10.1038/srep27258.

Maccari, G., Di Luca, M., Nifosí, R., Cardarelli, F., Signore, G., Boccardi, C., Bifone, A., 2013. Antimicrobial peptides design by evolutionary multiobjective optimization. PLoS Comput Biol. 9, e1003212. doi: 10.1371/journal.pcbi.1003212.

Maijaroen, S., Anwised, P., Klaynongsruang, S., Daduang, S., Boonmee, A. 2016. Comparison of recombinant a-hemoglobin from Crocodylus siamensis expressed in different cloning vectors and their biological properties. Protein Expression and Purification. 118, 55e63

Malanovic, N., Lohner, K. 2016. Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. Biochim Biophys Acta. 1858, 936-946.

Marrie, T.J., Nelligan, J., Costerton, J.W., 1982. A scanning and transmission electron microscopic study of an infected endocardial pacemaker lead. Circulation 66, 1339-1341.

Matsuzaki, K., 2009. Control of cell selectivity of antimicrobial peptides. Biochimica et Biophysica Acta. 1788, 1687-1692

Mbah, J.A., Ngemenya, M.N., Abawah, A.L., Babiaka, S.B., Nubed, L.N., Nyongbela, K.D., Lemuh, N.D. and Efange, S.M.N., 2012. Bioassay-guided discovery of antibacterial agents: *in vitro* screening of Peperomia vulcanica, Peperomia fernandopoioana and Scleria striatinux. Ann Clin Microbiol Antimicrob. 11, 10.

Memarpoor-Yazdi, M., Asoodehbc, A., Chamani, J. 2012. A novel antioxidant and antimicrobial peptide from hen egg white lysozyme hydrolysates. J Funct Foods. 4, 278-286.

Merritt, P.M., Danhorn, T., Fuqua, C. 2007. Motility and Chemotaxis in *Agrobacterium tumefaciens* Surface Attachment and Biofilm Formation. J Bacteriol. 189, 8005-8014.

Mishra, B., Lushnikova, T., Golla, R.M., Wang, X., Wang, G. 2017. Design and surface immobilization of short anti-biofilm peptides. Acta Biomater. 49, 316-328.

Mulcahy, L.R., Isabella, V.M., Lewis, K., 2013. *Pseudomonas aeruginosa* biofilms in disease. Microb Ecol. 68(1), 1-12. doi: 10.1007/s00248-013-0297-x.

Nguyen, T.K., Duong, H.T.T., Selvanayagam, R., Boyer, C., Barraud, N., 2015. Iron oxide nanoparticle-mediated hyperthermia stimulates dispersal in bacterial biofilms and enhances antibiotic efficacy. Sci. Rep. 5, 18385.

Osman, A., Goda, H. A., Hamid, M.A., Badran, S.M., Otte, J., 2016. Antibacterial peptides generated by alcalase hydrolysis of goat whey. LWT - Food Sci Technol. 65, 480-486.

Overhage, J., Campisano, A., Bains, M., Ellen C.W. Torfs, E.C.W., Rehm, B.H.A., Hancock, R.E.W., 2008. Human Host Defense Peptide LL-37 Prevents Bacterial Biofilm Formation. Infect Immun. 76, 4176-4182.

Pakdeesuwan, A., Araki, T., Daduang, S., Payoungkiattikun, W., Jangpromma, N., Klaynongsruang, S., 2017. *In vivo* wound healing activity of Crocodile (*Crocodylus siamensis*) hemoglobin and evaluation of antibacterial and antioxidant properties of hemoglobin and hemoglobin hydrolysate. J. Microbiol. Biotechnol. 27, 26-35.

Pata, S., Yaraksa, N., Daduang, S., Temsiripong, Y., Svasti, J., Araki, T., Thammasirirak, S., 2011. Characterization of the novel antibacterial peptide Leucrocin from crocodile (*Crocodylus siamensis*) white blood cell extracts. Dev Comp Immunol. 35, 545-553.

Patathananone, S., Thammasirirak, S., Daduang, J., Chung, J.G., Temsiripong, Y., Daduang, S., 2016. Bioactive compounds from crocodile (*Crocodylus siamensis*) white blood cells Induced apoptotic cell death in HeLa cells. Environ Toxicol. 31, 986-997.

Perkins, D.N., Pappin, D.J.C., Creasy, D.M., Cottrell, J.S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis. 20, 3551-3567.

Phosri, S., Jangpromma, N., Patramanon, R., Kongyingyoes, B., Mahakunakorn, P., Klaynongsruang, S., 2017. Effects of hemoglobin and whole blood of *Crocodylus siamensis* against hydrogen peroxide-induced oxidative damage in human lung fibroblast (MRC-5) and inflammation in mice. Inflammation. 40, 205-220**.**

Phosri, S., Mahakunakorn, P., Lueangsakulthai, J., Jangpromma, N., Swatsitang, P., Daduang, S., Dhiravisit, A., Thammasirirak, S., 2014. An investigation of antioxidant and anti-inflammatory activities from blood components of crocodile (*Crocodylus siamensis*). Protein J. 33, 484-492.

Podschun, R., Ullmann, U., 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. 11, 589-603.

Porto, W.F., Silva, O.N., Franco, O.L., William, F.P., Osmar, N.S., Octávio L.F., 2012. Prediction and Rational Design of Antimicrobial Peptides, Protein Structure, Dr. Eshel Faraggi (Ed.), InTech, DOI: 10.5772/38023. Available from: https://www.intechopen.com/books/protein-structure/ prediction-and-rational-design-of-antimicrobial-peptides.

Preecharram, S., Daduang, S., Bunyatratchata, W., Araki, T., Thammasirirak, S., 2008. Antibacterial activity from Siamese crocodile (*Crocodylus siamensis*) serum. Afr J Biotechnol. 7, 3121-3128.

Preecharram, S., Jearranaiprepame, P., Daduang, S., Temsiripong, Y., Somdee, T., Fukamizo, T., Svasti, J., Araki, T., Thammasirirak, S., 2010. Isolation and characterisation of crocosin, an antibacterial compound from crocodile (*Crocodylus siamensis*) plasma. Anim Sci. 81, 393-401.

Qadri, F., Svennerholm, A., Faruque, A.S.G., Sack, R.B., 2005. Enterotoxigenic *Escherichia coli* in Developing Countries: Epidemiology, Microbiology, Clinical Features, Treatment, and Prevention. Clin Microbiol Rev. 18, 465-483.

Ramos, I., Dietrich, L.E., Price-Whelan, A., Newman, D.K. 2010. Phenazines affect biofilm formation by *Pseudomonas aeruginosa* in similar ways at various scales. Res Microbiol. 161, 187-191.

Rasamiravaka, T., Labtani, Q., Duez, P., Jaziri, M.E. 2015. The Formation of Biofilms by *Pseudomonas aeruginosa*: A Review of the Natural and Synthetic Compounds Interfering with Control Mechanisms. Biomed Res. Int. doi: 10.1155/2015/759348.

Reinhardt, A., Neundorf, I. 2016. Design and application of antimicrobial peptide conjugates. Int J Mol Sci. 17: 701.

Rubab, M., Shahbaz, H.M., Olaimat, A.N., Oh, D. 2018. Biosensors for rapid and sensitive detection of *Staphylococcus aureus* in food. Biosensors and Bioelectronics. 105, 49-57.

Scott, M.G., Hancock, R.E. 2000. Cationic antimicrobial peptides and their multifunctional role in the immune system. Crit Rev Immunol. 20, 407-431.

Scott. M.G., Dullaghan, E., Mookherjee, N., Glavas, N., Waldbrook, M., Thompson, A. 2007. An anti-infective peptide that selectively modulates the innate immune response. Nat Biotechnol. 25, 5-472.

Shah, S.R., Tatara, A.M., D'Souza, R.N., Mikos, A.G., Kasper, F.K., 2013. Evolving strategies for preventing biofilm on implantable materials. Mater. Today. 16, 177-182.

Shim-Prydon, G., Camacho-Barreto, H., 2007. New animal products. New uses and markets for by-products and the co-products of crocodile, emu, goat, kangaroo and rabbit, A report for the Rural Industries Research and Development Corporation. RIRDC publication no 06/117, RIRDC project no DAQ 320A. pp. 65.

Smith, J.L., Fratamico, P.M., Gunther IV, N.W., 2014. Chapter Three - Shiga Toxin-Producing *Escherichia coli*. Adv Appl Microbiol. 86, 145-197.

Song, R., Wei, R.B., Luo, H.Y. and Wang, D.F., 2012. Isolation and Characterization of an Antibacterial Peptide Fraction from the Pepsin Hydrolysate of Half-Fin Anchovy (*Setipinna taty*). Molecules 17, 2980–2991.

Srihongthong, S., Pakdeesuwan, A., Daduang, S., Araki, T., Dhiravisit, A., Thammasirirak, S., 2012. Complete amino acid sequence of globin chains and biological activity of fragmented crocodile hemoglobin (*Crocodylus siamensis*). Protein J. 31, 466-476.

Stewart, P.S., 1998. A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. Biotechnol Bioeng. 59, 261-272.

Taylor, E., Webster, T.J., 2011. Reducing infections through nanotechnology and nanoparticles. Int J Nanomedicine. 6, 1463-1473.

Thankappan, B., Jeyarajan, S., Hiroaki, S., Anbarasu, K., Natarajaseenivasan, K., Fujii, N., 2013. Antimicrobial and antibiofilm activity of designed and synthesized antimicrobial peptide, KABT-AMP. Appl Biochem Biotechnol. 170, 1184-1193.

Theansungnoen, T., Yaraksa, N., Daduang, S., Dhiravisit, A., Thammasirirak, S., 2014. Purification and characterization of antioxidant peptides from leukocyte extract of *Crocodylus siamensis*. Protein J. 33, 24-31.

Thévenet, P., Shen, Y., Maupetit, J., Guyon, F., Derreumaux, P., Tufféry, P. 2012. PEP-FOLD: an updated de novo structure prediction server for both linear and disulfide bonded cyclic peptides. Nucleic Acids Res. 40, W288-W293.

Thorsell, A., Portelius, E., Blennow, K., Westman, B.A. 2007. Evaluation of sample fractionation using microscale liquid-phase isoelectric focusing on mass spectrometric identification and quantitation of proteins in a SILAC experiment. Rapid Commun Mass Spectrom. 21, 771-778.

Tossi, A., Sandri, L., Giangaspero, A., 2000. Amphipathic, alpha-helical antimicrobial peptides. Biopolymers. 55, 4-30.

Waite, R.D., Papakonstantinopoulou, A., Littler, E., Curtis, M.A. 2005. Transcriptome analysis of *Pseudomonas aeruginosa* growth: comparison of gene expression in planktonic cultures and developing and mature biofilms. J Bacteriol. 187, 6571-6576.

Wang, C., Chang, T., Yang, H., Cui, M., 2015. Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella Enteritidis*, *Escherichia coli* and *Listeria monocytogenes*. Food cont. 47, 231-236.

Ward, H.K., Olson, E.M., Lam, K., Costerton, W.J., 1992. Mechanism of persistent infection associated with peritoneal implant. J. Med. Microbiol. 36, 406-413.

Winsor, G.L., Griffiths, E.J., Lo, R., Dhillon, B.K., Shay, J.A., Brinkman, F.S. 2016. Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. Nucleic Acids Res. doi: 10.1093/nar/gkv1227 (Database issue).

Yaraksa, N., Anunthawan, T., Theansungnoen, T., Daduang, S., Araki, T., Dhiravisit, A. 2014. Design and synthesis of cationic antibacterial peptide based on Leucrocin I sequence, antibacterial peptide from crocodile (*Crocodylus siamensis*) white blood cell extracts. J Antibiot (Tokyo). 67, 205-212.

Yin, L.M., Edwards M.A., Li, J., Yip, C.M., Deber, C.M. 2012. Roles of hydrophobicity and charge distribution of cationic antimicrobial peptides in peptide-membrane interactions. J Biol Chem, 287, 7738-7745.

Zasloff, M., 1992. Antibiotic peptides as mediators of innate immunity. Curr Opin Immunol. 4, 3-7.

Zasloff, M., 2002. Antimicrobial peptides of multicellular organisms. Nature. 415, 389-395.

Zelezetsky, I., Tossi, A., 2006. Alpha-helical antimicrobial peptides-using a sequence template to guide structure-activity relationship studies. Biochim Biophys Acta. 1758, 1436-1449.

Zhang, S.K., Song, J.W., Gong, F., Li, S.B., Chang, H.Y., Xie, H.M., Gao, H.W., Tan, Y.X., Ji, S.P., 2016. Design of an α-helical antimicrobial peptide with improved cell-selective and potent anti-biofilm activity. Scientific reports. 6, 27394.

Zou, T.B., He, T.P., Li, H.B., Tang, H.W., Xia, E.Q. 2016. The Structure-Activity Relationship of the Antioxidant Peptides from Natural Proteins. Molecules. 21, 72.

OUTPUT

Publications in academic journal

Jiraporn Lueangsakulthai, Nicholas Michael, Theeranan Temsiripong, Watcharee Khunkitti, Sompong Klaynongsruang, **Nisachon Jangpromma**. (2019). Antioxidant and anti-inflammatory activities of the siamese crocodile (*Crocodylus siamensis*) hemoglobin hydrolysate derived from trypsin and papain hydrolysis. Chiang Mai Journal of Science. 46(5): 915-929.

Sirinthip Sosiangdi, Anupong Tankrathok, Sittiruk Roytrakul, Sakda Daduang, Sompong Klaynongsruang, **Nisachon Jangpromma**. Design, synthesis and characterization of antimicrobial and antibiofilm peptides derived from *Crocodylus siamensis* hemoglobin with high bacterial cell selectivity. Manuscript preparation for the protein journal (impact factor 1.029).

Academic conference

Nisachon Jangpromma, Boonpob Nowichai, Anupong Joompang, Jiraporn Lueangsakulthai, Sakda Daduang, Sompong Klaynongsruang (2018). A potent antioxidant and anti-inflammatory properties of modified peptide from *Crocodylus siamensis* hemoglobin hydrolysate. The 6th Biochemistry and Molecular Biology International Conference (6th BMB): Networking in Biosciences for Creativity and Innovation. June 20-22, 2018. Rayong Resort, Rayong, Thailand.

Sirinthip Sosiangdi, Anupong Tankrathok, Sompong Klaynongsruang, **Nisachon Jangpromma** (2018). Potential antibacterial activity of designed *Crocodylus siamensis* hemoglobin-based peptides. The 6th Biochemistry and Molecular Biology International Conference (6th BMB): Networking in Biosciences for Creativity and Innovation. June 20-22, 2018. Rayong Resort, Rayong, Thailand.

Nisachon Jangpromma, Sirinthip Sosiangdi, Boonpob Nowichai, Anupong Tankrathok, Sakda Daduang, Sompong Klaynongsruang (2018). Antibacterial and anti-inflammatory activity of designed and synthesized a α-helical peptide from *Crocodylus siamensis* hemoglobin hydrolysate. The 13th International Symposium of the Protein Society of Thailand. 7-9 August 2018. The Convention Center, Chulabhorn Research Institute during, 2018. Bangkok, Thailand.

Nisachon Jangpromma, Santi Phosri, Boonpob Nowichai, Anupong Joompang, Sompong Klaynongsruang (2018). Antioxidant activity of cationic amphipathic peptide modified from IL15 peptide from Siamese crocodile hemoglobin hydrolysate. The 44th Congress on Science and Technology of Thailand (STT44) Science and Technology in the Disruptive Era. 29-30 October 2018. Bangkok International Trade and Exhibition Center (BITEC), Thailand, 2018. Bangkok, Thailand.

Sirinthip Sosiangdi, Sompong Klaynongsruang, **Nisachon Jangpromma** (2018). Antimicrobial effect of novel synthetic peptide designed from *Crocodylus siamensis* hemoglobin hydrolysate on *Escherichia coli*. The 44th Congress on Science and Technology of Thailand (STT44) Science and Technology in the Disruptive Era. 29-30 October 2018. Bangkok International Trade and Exhibition Center (BITEC), Thailand, 2018. Bangkok, Thailand.

APPENDIX 1

Jiraporn Lueangsakulthai, Nicholas Michael, Theeranan Temsiripong, Watcharee Khunkitti, Sompong Klaynongsruang, **Nisachon Jangpromma**. (2019). Antioxidant and anti-inflammatory activities of the siamese crocodile (*Crocodylus siamensis*) hemoglobin hydrolysate derived from trypsin and papain hydrolysis. Chiang Mai Journal of Science. 46(5): 915-929.

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Contributed Paper

Antioxidant and Anti-inflammatory Activities of the Siamese Crocodile (*Crocodylus siamensis*) Hemoglobin Hydrolysate Derived from Trypsin and Papain Hydrolysis

Jiraporn Lueangsakulthai [a,b], Nicholas Michael [c], Theeranan Temsiripong [d], Watcharee Khunkitti [e], Sompong Klaynongsruang [b,f], Nisachon Jangpromma* [b,g]

[a] College of Public Health and Human Sciences, School of Biological and Population Health Sciences, Oregon State University, Oregon, 97331, United States.
[b] Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Thailand.
[c] Department of Chemistry, The University of Reading, Reading, RG6 6UR, United Kingdom.
[d] Srirachamoda Co., Ltd. 383 Moo 4, Nongkham, Sriracha, Chonburi 20230, Thailand.
[e] Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand.
[f] Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand.
[g] Department of Integrated Science, Forensic Science Program, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand.
*Author for correspondence; e-mail: nisaja@kku.ac.th

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Abstract

Crocodylus siamensis hemoglobin hydrolysates (CHHs) were obtained by trypsin and papain digestion at different incubation times (2, 4, 6 and 8 h) at 37 °C and subjected to antioxidant and anti-inflammatory activity assessment. DPPH scavenging activity of CHH derived from trypsin was similar to intact Hb. CHH derived from papain hydrolysis by 8-h hydrolysis (8h-CHHp) showed the highest DPPH scavenging activity at 56.86% antioxidant inhibition with IC_{50} value of 31 μg/ml. CHH derived from papain hydrolysis by 2-h hydrolysis (2h-CHHp) showed the highest reducing power activity at 0.99 mM Trolox equivalent and 4h-CHHt showed reducing activity at 0.16 mM Trolox equivalent (at concentration of 500 μg/ml). The linoleic peroxidation activity of CHH derived from papain hydrolysis by 6-h hydrolysis (6h-CHHp) and CHH derived from trypsin hydrolysis by 8-h hydrolysis (8h-CHHt) was increased in a dose-dependent manner with IC_{50} value of 5 μg/ml. The strongest anti-inflammatory activity was found for 2h-CHHp, which displayed a high efficacy in decreasing NO production of macrophage RAW 264.7 cells (46.86%) with no toxicity and significantly reduced pro-inflammatory cytokines interleukin-6 (IL-6) production to about 25.73 pg/ml. Taken collectively, the results of this work demonstrate that CHHs derived from papain hydrolysis possesses antioxidant and anti-inflammatory activities, which provides support for the application against inflammation and oxidative stress-related disorders.

Keywords: hemoglobin hydrolysate, papain hydrolysis, trypsin hydrolysis, anti-inflammatory, antioxidant

1. INTRODUCTION

Free radicals are created as a consequence of adenosine triphosphate (ATP) production by the mitochondria. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS). At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, they generate oxidative stress, a deleterious process that can damage all cell structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA) [1-2]. Oxidative stress plays a major part in the development of chronic diseases in humans such as cancer, arthritis, aging, autoimmune disorders, diabetes, infection, cardiovascular, inflammation and neurodegenerative diseases [3]. Within the human body has various mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced, or externally supplied through foods and/or supplements. Endogenous and exogenous antioxidants act as "free radical scavengers" by preventing and repairing damages that caused by ROS and RNS [4]. Inflammation is the immune system's physiological response to injury or infection [5]. Acute inflammation is a part of the body defense response, chronic inflammation is thought to be lead to numerous diseases for example cancer, diabetes, cardiovascular, pulmonary, and neurological diseases [5-6]. However, high expression of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 have been exhibited to have a role in oxidative stress-induced inflammation [7-8]. The imbalance between reactive oxygen species and endogenous antioxidant defense mechanisms leads to an unhealthy circle which may affect cellular components and trigger various diseases including inflammation [9].

Protein hydrolysate have begun to attract a lot of attention because of the growing belief that protein hydrolysate should possess health-promoting qualities. Recent biochemical research has shown that the protein hydrolysate not only furnishes amino acids but also provides bioactive peptides after digestion [10-11]. Consequently, bioactive peptides produced from both animal and plant sources are now being intensively investigated and have been reported to have antioxidant [12-13] and anti-inflammatory activity [14-16].

Crocodylus siamensis, commonly called Siamese crocodile, is a small freshwater crocodilian populating parts of Southeast Asia. Recently, several components of *C. siamensis* blood, i.e. plasma, serum, white blood cells and hemoglobin have been reported to possess a broad spectrum of biological properties, mainly attributed to the abundance of a number of biologically active peptides and proteins. Among these, hemoglobin constitutes the most abundant component and has been shown to exhibit antioxidant [17-22], antimicrobial [17-18, 22] and anti-inflammatory activity [19, 23-25].

Therefore, this study is aimed at investigating the antioxidant and anti-inflammatory of *C. siamensis* hemoglobin hydrolysates (CHHs) derived from trypsin and papain digestion. 2,2-diphenyl-1-picrylhydrazyl (DPPH), linoleic peroxidation and ferric reducing power assays were conducted to determine the antioxidant activity whereas 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nitric oxide (NO) (of macrophage RAW 264.7 cells), IL-10 and IL-6 assays were used to investigate the anti-inflammatory activity. The amino acid composition was analyzed to determine the amino acid composition of protein hydrolysate.

2. MATERIALS AND METHODS

2.1 Crocodile Hemoglobin Preparation

Crocodile (*C. siamensis*) blood was purchased from Sriracha Moda Farm., Ltd., Chon Buri, Thailand. The animal ethic approval record number is ACUC-KKU-52/60 (reviewed and approved by the animal ethics committee of

Khon Kaen University). Crocodile blood samples were withdrawn from the supravertibral branch of the internal jugular vein of crocodiles aged between 1-3 years. Blood was collected and transferred to 15-ml sterile tubes containing 0.08 g of EDTA. Blood samples were stored at 4 °C overnight to allow blood cells to settle. Red blood cells (bottom layer) were collected in sterile tubes. Isolated red blood cells were washed three times with phosphate buffer saline (PBS), pH 7.0, and centrifuged at 3,000 × *g* for 5 min at 4 °C. Ice-cold distilled water of five-fold volume was added to the RBC pellet, followed by vigorous mixing and allowing the mixture to settle for 10 min. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the supernatant was collected for lyophilization and then stored at -70 °C.

2.2 Trypsin and Papain Hydrolysis

Enzymatic hydrolysis was performed according to the method of Yu *et al.* [26]. Shortly, the hemoglobin solution was digested by trypsin / papain with a ratio of enzyme to substrate of 1:100 (w/w) at 37 °C for 2, 4, 6 and 8 h and boiled at 95 °C for 10 min to quench the reaction by inactivating the enzyme. The hydrolysis condition was performed at pH 7.5 (adjusted with 1 M HCl), followed by removal of insoluble components by centrifugation at 7,168 × *g* for 20 min. The supernatant was collected and adjusted to pH 7.0 by addition of 1 M HCl or 1 M NaOH. Finally, the supernatants were lyophilized and stored at -20 °C.

2.3 Degree of Hydrolysis

The degree of hydrolysis was determined by following the method of Benjakul *et al.* [27]. Briefly, 125 µl of CHHs (1 mg/ml) were added to 2.0 ml of 0.21 M sodium phosphate buffer, pH 8.2, followed by addition of 1 ml of 0.01% TNBS solution. The mixture was incubated in a water bath at 50 °C for 30 min in the dark, then 2 ml of 0.1 M sodium sulfite was added to stop the reaction. The mixture was then allowed to cool for 15 min. The absorbance was measured at 420 nm and the α-amino acid content expressed in terms of L-leucine. The percentage of the degree hydrolysis was calculated using the formula:

$$DH = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

where L_0 determines the amount of α-amino acid expressed in the sample, L_t corresponds to the amount of α-amino acid released at time t and L_{max} determines the maximum amount of α-amino acid after hydrolysis by 5 M HCl at 100 °C for 24 h.

2.4 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

Double distilled water (50 µl) was added to a 96-well plate. Next, CHHs (50 µl) and 0.0004 M DPPH (50 µl) were added and mixed for 5 min. The reaction was kept in dark for 25 min and absorbance was measured at 490 nm using DPPH with double distilled water as a blank. All samples were analyzed in triplicate. Radical scavenging activity was determined as the percentage antioxidant inhibition (%AI) value that was calculated based on the following formula;

$$\% \text{ Antioxidant inhibition} = [(Abs_{cont} - Abs_{test})/Abs_{cont}] \times 100$$

2.5 Ferric Reducing Power Assay

Ferric reducing power assay was modified from the method of Girgih *et al.* [28]. CHHs (100 µl) and positive control (glutathione) (100 µl) were added. Then 250 µl of 0.2 M phosphate buffer pH 6.6 and 150 µl of double distilled water were added. Potassium hexacyanoferrate 1% (w/v) 250 µl was added, vortexed and incubated at 50 °C, for 20 min. The reaction was stopped by adding 250 µl of 10% TCA and incubated for 10 min before centrifugation at 800 × *g* for

10 min. Reactions were then performed in a 96-well plate. The reaction contains sample (30 µl), double distilled water (160 µl) and 0.1% (w/v) ferric chloride (10 µl). The reaction was mixed and incubated at room temperature for 10 min. The absorbance was measured at 700 nm and all samples were analyzed in triplicate. The activities were measured as the equivalent to trolox value and were calculated based on the equation of the standard curve of the positive control.

2.6 Linoleic Peroxidation Assay

This assay was performed following the method of Ledesma *et al.* [29] with modifications. CHHs (5-500 µg/ml) (50 µl) were mixed with 50 µl of linoleic acid and 0.07 M ABAP (10 µl). The solution was mixed for 10 min. Acetic acid 20% (v/v) 150 µl was added and incubated at 70 °C, for 1h and reactions were performed in a 96-well plate. The reaction contains sample (20 µl), 75% ethanol (160 µl), 15% Ammonium thiocyanate (10 µl) and 10 mM ferrous chloride (10 µl). The reaction was mixed and incubated at room temperature for 3 min. The absorbance was measured at 500 nm. All samples were analyzed in triplicate. The activity was determined as the percentage antioxidant inhibition (%AI) value that was calculated using the following formula;

$$\% \text{ AI (antioxidant inhibition)} = [Abs_{cont} - Abs_{test})/Abs_{cont}] \times 100$$

2.7 Measurement of Nitric Oxide and Cell Viability

This assay was conducted according to the method of Lueangsakulthai *et al.* [25]. RAW 264.7 cells (1×10^5 cells/ml) were cultured on a 96-well plate overnight. CHHs (125-500 µg/ml) were co-incubated with LPS (100 ng/ml) and the resulting solution incubated with RAW 264.7 cells. Another incubation was set up between LPS and RAW 264.7 cells at 37 °C in a 5% CO_2 humidified atmosphere for 24 h. After incubation for 24 h (NO assay), 100 µl of culture medium from each CHHs were mixed with 100 µl of Griess reagent and incubated at 25 °C for 10 min. The absorbance was measured at 540 nm using a microplate reader (BioRad, Model 680, USA). Nitric oxide (NO) production was calculated as percentage of control. After incubation for 24 h (Cell viability assay), the medium was discarded. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ml) was added to RAW 264.7 cells and incubated at 37 °C in a 5% CO_2 humidified atmosphere for 30 min before the medium was discarded. DMSO was added and the reaction mixture incubated at 25 °C for 30 min. The absorbance was measured at 570 nm and the cell viability evaluated by comparing the absorbance with that of the control for each sample. All samples were analyzed in quadruplicate.

2.8 Measurement of IL-10 and IL-6

Aliquots of culture medium employed in the NO assay were further used for determination of IL-10 and IL-6 expression using the ELISA kit and following the instructions in the manufacturer's manual (R&D, Minneapolis, MN, USA).

2.9 Amino Acid Composition Analysis

Amino acids were determined by using ion-exchange chromatography. Their contents were identified using the amino acid analyzer (Biochrom 30+, Cambridge, UK) with post-column ninhydrin derivation and spectrophotometric detection. One hundred microliters of the prepared mixture was automatically injected into an amino acid analyzer.

2.10 Statistical Analysis

Statistical analysis was performed using ANOVA and followed by Dunnett's test (Prism 5.0, GraphPad Inc., San Diego, CA, USA). Data are presented as mean ± SEM. A value of $P <$

0.05 was accepted to be significant (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$).

3. RESULTS AND DISCUSSION

3.1 Degree of Hydrolysis (DH)

To obtain active protein fragments, crocodile hemoglobin was hydrolyzed by trypsin and papain digestion at different reaction times 2, 4, 6 and 8 h. Trypsin prefers to hydrolyze at Arg and Lys N-terminal position, while papain prefers to hydrolyze at hydrophobic side chain amino acid residues [30]. Trypsin is produced in the intestine and is one of the main digestive enzymes in the digestive system of humans and many other animals. Trypsin was used in order to mimic the intestinal digestion. Papain is one of the enzymes that used in commercial to produce active peptides. Degree of hydrolysis (DH) is defined as the proportion of cleaved peptide bonds in a protein hydrolysate. The TNBS method is based on the reaction of primary amino groups with trinitro-benzene-sulfonic acid (TNBS) reagent [31]. The extent of hemoglobin hydrolysate using trypsin digestion was evaluated by degree of hydrolysis (DH) which was 3.64%, 4.31%, 6.65% and 9.61% for 2 h, 4 h, 6 h and 8 h of incubation, respectively. The extent of hemoglobin hydrolysate using papain digestion was 5.75%, 8.05%, 11.27% and 18.94% for 2 h, 4 h, 6 h and 8 h of incubation, respectively (Figure 1). Results indicated that cleavage of peptide bonds, free amino acid and small peptides were higher content in longer enzymatic hydrolysis. Degree of hydrolysis was increased when incubation time rose. Trypsin has specific hydrolyzed position resulting in a slight increase of DH when compared with papain hydrolysis. Several protein hydrolysates that were hydrolyzed by trypsin and papain exhibited antioxidant [32-34] and anti-inflammatory activity [35]. Moreover, the radical scavenging activities of the hydrolysates were positively correlated with the DH (%) [36].

3.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

DPPH method is widely used for antioxidants screening because of its ability to scavenge oxidants compound [37]. In this assay, the different concentrations of CHHs (15-62 μg/ml) showed antioxidant activity. CHH derived from papain hydrolysis has higher antioxidant activity than CHH derived from trypsin hydrolysis, CHH derived from papain hydrolysis by 8-h hydrolysis (8h-CHHp) at the concentration of 15, 31 (IC_{50} value) and 62 μg/ml showed

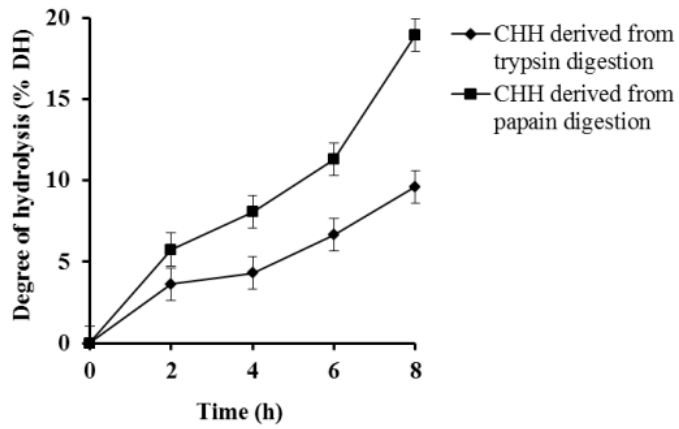

Figure 1. Degree of hydrolysis (%DH) of CHH after trypsin and papain digestion for 2, 4, 6 and 8 h. Hemoglobin hydrolyzed enzymatically displayed a direct correlation between the rate of hydrolysis (DH) and the time of incubation (h).

42.03, 56.86 and 61.28% antioxidant inhibition (AI), respectively. CHH derived from trypsin hydrolysis by 4-h hydrolysis (4h-CHHt) at the concentration of 15, 31 (IC_{50} value) and 62 µg/ml showed 48.73, 51.68 and 54.66% antioxidant inhibition (AI), respectively (Figure 2). CHH derived from papain hydrolysis had better scavenging effect when compared with that of CHH derived from trypsin hydrolysis. This result suggests that the increased degree of hydrolysis of CHH derived from papain hydrolysis could enhance the hydrolyzation in small protonated peptides resulting in an increase in DPPH scavenging activity. Scavenging of DPPH radical by a proton donating substance changes color from violet to yellow, which is detectable at absorbance of 490 nm [38]. Moreover, CHH derived from papain hydrolysis showed the ability to quench the DPPH radicals similar to the papain hydrolyzed from *Camellia oleifera* seed cake protein hydrolysate [32].

3.3 Ferric Reducing Power Assay

Ferric reducing power assay is the method that exhibited an ability to reduce ferric cyanide complex $[Fe^{3+}(CN^{-})_6]$ into ferrous cyanide complex $[Fe^{2+}(CN^{-})_6]$ by donating electrons. The results show that CHH derived from trypsin hydrolysis by 4-h hydrolysis (4h-CHHt), CHH derived from trypsin hydrolysis by 6-h hydrolysis (6h-CHHt), CHH derived from papain hydrolysis by 2-h hydrolysis (2h-CHHp) and CHH derived from papain hydrolysis by 8-h hydrolysis (8h-CHHp) at the concentration (500 µg/ml) displayed significant ferric ion reducing power. 2h-CHHp displayed the highest reduction activity equivalent to Trolox of about 0.99 mM, while intact Hb, 4h-CHHt, 6h-CHHt and 8h-CHHp displayed reduction equivalents of 0.82, 0.16, 0.05 and 0.81 mM Trolox, respectively (Figure 3). Moreover, 500 µg/ml glutathione (positive control) effected significant ferric ion reduction equivalent to Trolox at 18.60 mM (data not shown). An absorbance increase can be correlated to the reducing ability of antioxidant. The compound with antioxidant reacts with potassium ferricyanide to form potassium ferrocyanide and this reacts with ferric trichloride, yielding ferric ferrocyanide, a blue coloured complex, with a maximum absorbance at 700 nm. It has been reported that protein hydrolysates containing Ile, His, Tyr, Pro and Lys residues with high reducing power show antioxidant activity as well as a great ability to donate electrons to form stable compounds and thereby interrupt the free radical chain reactions [39-40].

3.4 Linoleic Peroxidation Assay

Lipid peroxidation is considered to be a free radical process which occurs from unsaturated lipid oxidation process. Lipid peroxidation can damage double layer cell membranes leading to numerous products such as hydrocarbon compounds, ketone and aldehyde (especially malondialdehyde, MDA) [41]. The results of the linoleic peroxidation assay reveal that the different concentrations of CHHs (5-500 µg/ml) effected significant inhibition of linoleic peroxidation in a dose-dependent manner when compared with Trolox. CHH derived from trypsin hydrolysis by 8-h hydrolysis (8h-CHHt) at the concentration of 5 (IC_{50} value), 50 and 500 µg/ml showed 76.34, 96.99 and 97.85% linoleic peroxidation inhibition, respectively. Meanwhile CHH derived from papain hydrolysis by 6-h hydrolysis (6h-CHHp) at the concentration of 5 (IC_{50} value), 50 and 500 µg/ml showed 91.95, 100 and 103% linoleic peroxidation inhibition, respectively (Figure 4). Moreover, Trolox (5, 50 and 500 µg/ml) showed 90.18, 92.73 and 93.99% of linoleic peroxidation inhibition and intact Hb (5, 50 and 500 µg/ml) showed 88.12, 88.99 and 91.55% inhibition. The results indicated that CHH derived from papain hydrolysis had significantly better linoleic peroxidation inhibition when compared with that of CHH derived from trypsin hydrolysis.

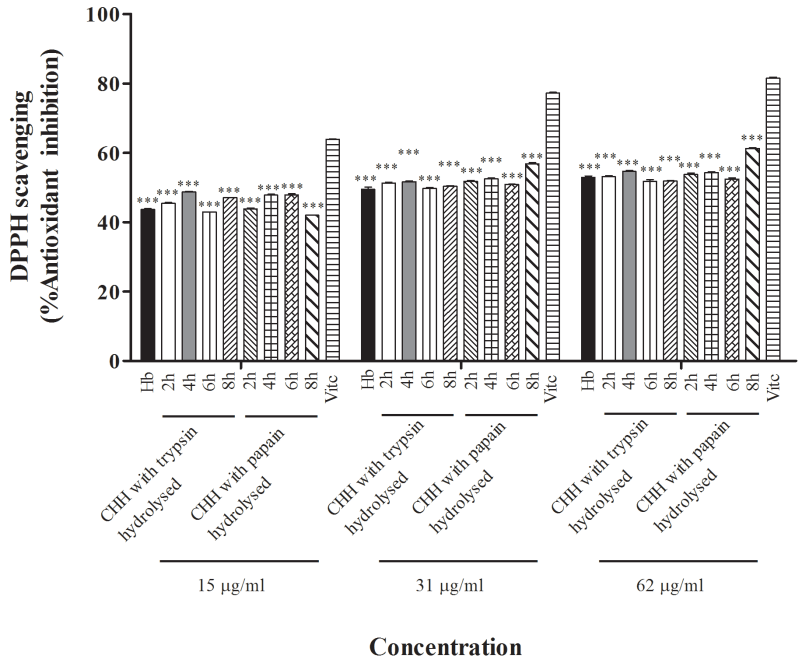

Figure 2. Antioxidant effect of CHHs derived from trypsin and papain digestion in DPPH radical scavengingassay. Each bar displays the mean ± SEM of three demonstrations. (*** $P <$ 0.001) probability levels compared with vit C.

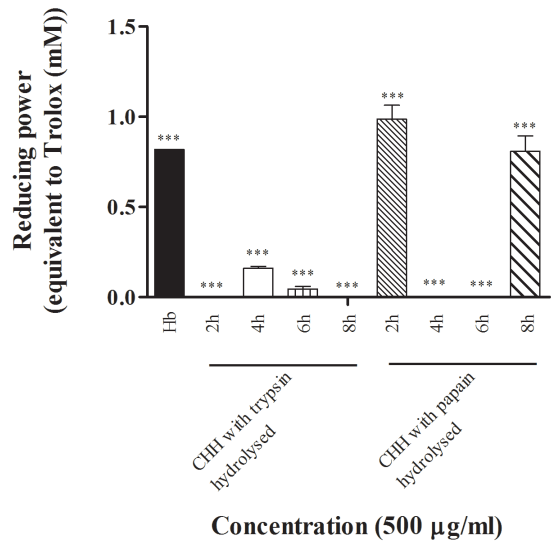

Figure 3. Reducing power of CHHs derived from trypsin and papain digestion at a concentration of 500 µg/ml expressed in Trolox equivalents. Each bar displays the mean ± SEM of three demonstrations. (*** $P <$ 0.001) probability levels compared with glutathione.

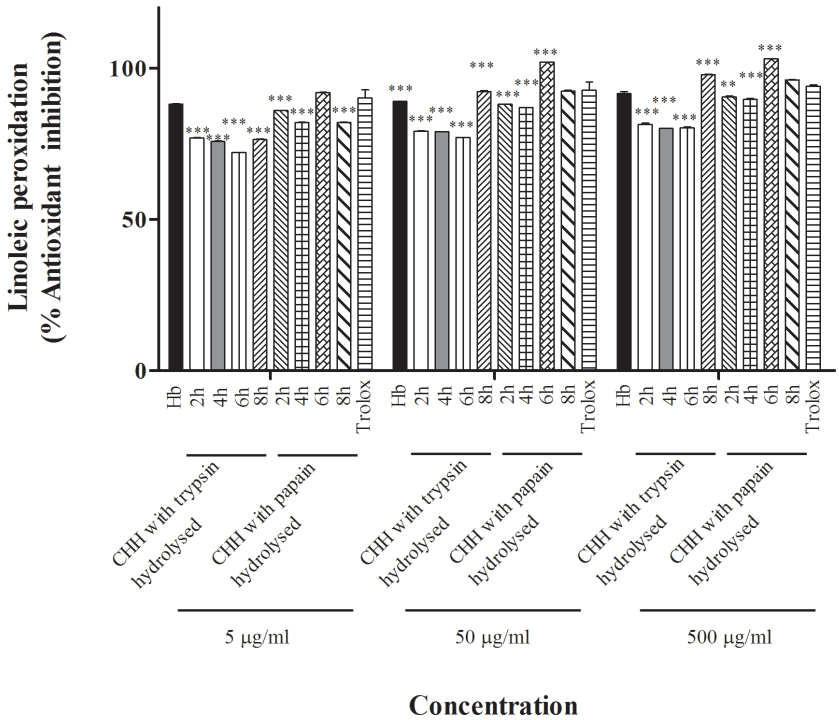

Figure 4. Linoleic peroxidation activity of CHHs derived from trypsin and papain digestion at a concentration of 5-500 µg/ml. Each bar displays the mean ± SEM of three demonstrations. (** $P < 0.01$ and *** $P < 0.001$) probability levels compared with Trolox.

The inhibitory effect of CHHp was similar to the hydrolysate of hoki skin gelatin where amino acid composition is rich in Gly, Pro, Glu, and Ala residues [42].

3.5 Measurement of Nitric Oxide, Cell Viability and Inflammatory Cytokines (IL-10 and IL-6) Production

The anti-inflammatory activity of intact Hb and CHHs were evaluated on the NO production, cell viability and inflammatory cytokines production against macrophage RAW 264.7 cells. The capability of inhibiting either the activity or production of nitric oxide (NO) can reduce the detrimental effects of inflammation. After induction of inflammation in macrophage RAW 264.7 cells by LPS addition for 24 h, the percentage of nitric oxide production was defined as 100%. Figure 5a, intact Hb and CHHs at concentrations of 125-500 µg/ml show a decrease in nitric oxide production in a dose-dependent manner. CHH derived from papain hydrolysis by 2-h hydrolysis (2h-CHHp), 4h-CHHp, 6h-CHHp and 8h-CHHp at a concentration of 500 µg/ml show nitric oxide production at 46.86, 66.26, 52.27 and 52.94%, respectively. CHH derived from trypsin hydrolysis by 8-h hydrolysis (8h-CHHt) at a concentration of 500 µg/ml show nitric oxide production at 76.50%. In order to evaluate cytotoxic effects of CHHs against macrophage RAW 264.7 cells, the viability of RAW 264.7 cells treated with defined concentrations of CHHs were examined (Figure 5b). The results indicate that all CHHs derived from trypsin hydrolysis and papain hydrolysis had no observable effect on cell viability. This result shows that CHHs derived from papain hydrolysis had high efficacy

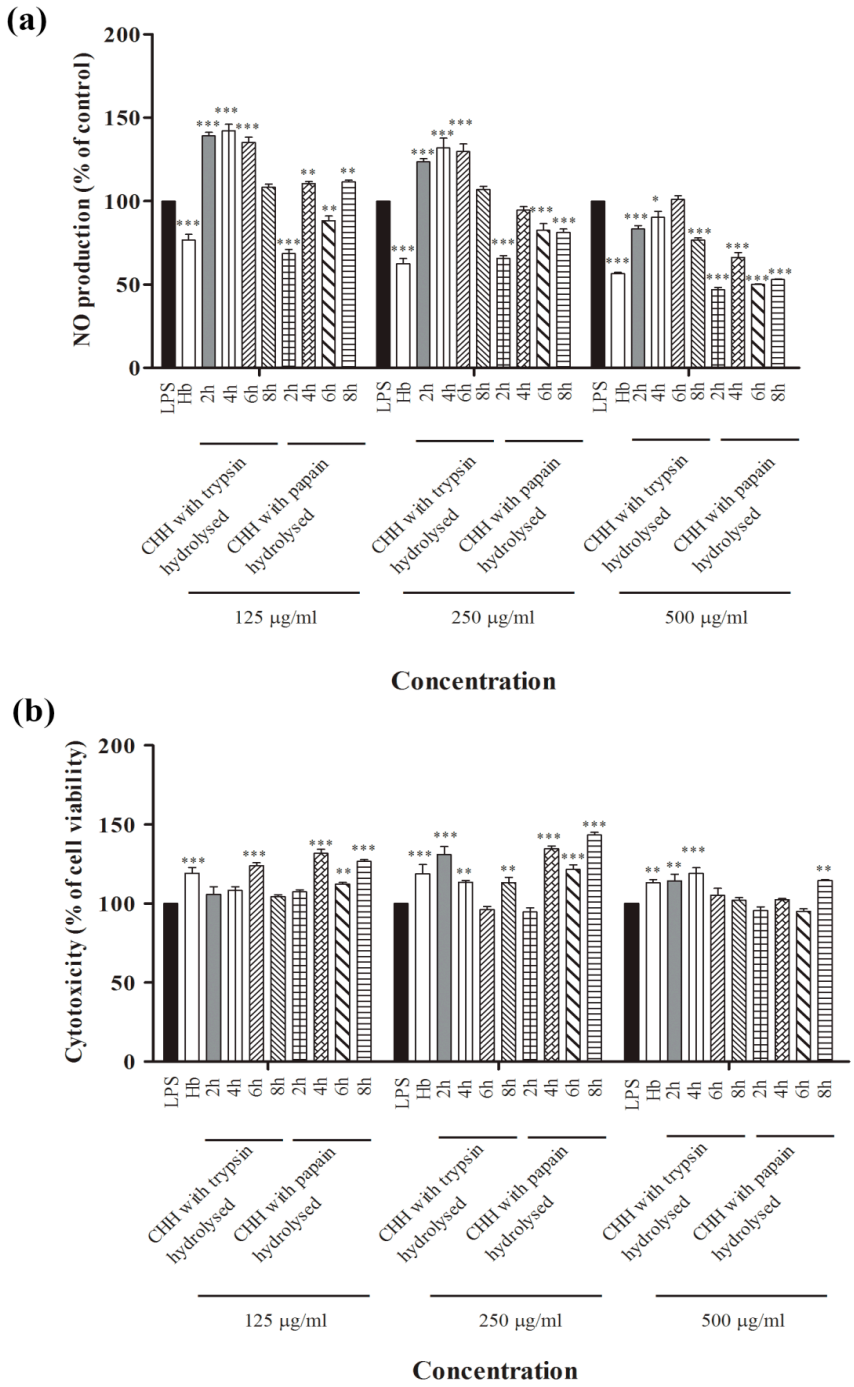

Figure 5. (a) The effect of CHHs derived from trypsin and papain digestion on NO production in LPS-activated macrophage RAW 264.7 cells and (b) the cytotoxicity (cell viability) of CHHs derived from trypsin and papain digestion on macrophage RAW 264.7 cells determined by the MTT assay. Each bar displays the mean ± SEM of four demonstrations. (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$) probability levels compared with LPS treatment alone.

to reduce nitric oxide production than CHHs derived from trypsin hydrolysis. The report of O'Sullivan *et al.* [35] showed that bovine lung hydrolysates prepared using papain exhibited anti-inflammatory activity by decreasing IL-6, IL-1β, and NO production in RAW264.7 cells and IL-2 production in Jurkat T cells. However, the decrease was likely due to cytotoxicity of this hydrolysate toward these cell lines. With excellent agreement with Phosri *et al.* [24], Jangpromma *et al.* [23] reported that crocodile Hb provides anti-inflammatory activity via the suppression of nitric oxide synthase (NOS), which inhibits the NO production and decreases inducible nitric oxide synthase (iNOS). The reports of Lueangsakulthai *et al.* [25] revealed that hemoglobin hydrolysate with pepsin digestion showed anti-inflammatory activity with decreasing pro-inflammatory cytokines and cytokine mediator production such as NO, IL-6, IL-1β and PGE_2. Similarly, the current results indicated that 2h-CHHp showed the highest activity to inhibit IL-6 production (25.73 pg/ml) compared to LPS. 4h-CHHp, 6h-CHHp and 8h-CHHp showed the activity to inhibit IL-6 production about 28.36, 26.60 and 29.84 pg/ml, respectively (Figure 6a). Notably, 4h-CHHt, 6h-CHHt and 6h-CHHp showed significantly ability to reduce IL-10 production to about 90.41, 91.52 and 87.07 pg/ml compared to LPS (Figure 6b). 2h-CHHp showed non-significantly ability to reduce anti-inflammatory cytokines IL-10 (109.30 pg/ml). The results conclude that CHH derived from papain hydrolysis has better efficacy than trypsin hydrolysis, CHH derived from papain hydrolysis by 2-h hydrolysis (2h-CHHp) was found to exhibit anti-inflammatory effects without toxic towards macrophage RAW 264.7 cells and possessed ability to decrease pro-inflammatory cytokine IL-6 production. The IL-6 is mediator for the production of inflammatory biomarkers, which consequently facilitates the progression of inflammation. IL-10 is an anti-inflammatory cytokine, which facilitates the progression of anti-inflammation. IL-6 also induces the expression of multiple factors with anti-inflammatory properties, including IL-1R antagonist, soluble TNF receptors, IL-10, acute phase reactants, glucocorticoids, protease inhibitors (such as tissue inhibitor of metalloproteinase-1), and suppressors of cytokine signaling $(SOCS)^3$ proteins [43]. IL-6 was decreased resulting in the decreasing of IL-10 production. Thus, the decreasing of IL-6 cytokines could retard or alleviate inflammation [44]. The collected results in this work indicate that the anti-inflammatory activity of CHH might be related to an interaction with the JAK/STAT pathway by their ability to decrease pro-inflammatory cytokine [45].

3.6 Amino Acid Composition Analysis

The amino acid composition is a characteristic feature of this protein. CHH derived from trypsin hydrolysis by 4-h hydrolysis (4h-CHHt) and CHH derived from papain hydrolysis by 2-h hydrolysis (2h-CHHp) both exhibited higher activity than other hydrolysates and the latter showed better antioxidant and anti-inflammatory activity. Both 4h-CHHt and 2h-CHHp were evaluated for their amino acid composition. 4h-CHHt contains 10 amino acids, including Thr, Gly, Cys, Val, Ile, Leu, Tyr, Phe, His and Lys at 9.74, 48.91, 15.14, 174.45, 61.11, 179.96, 32.76, 67.21, 137.30 and 101.24 μmol/l, respectively. 2h-CHHp contains 15 amino acids, including Thr, Glu, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, His, Orn and Lys at 32.96, 66.94, 400.44, 276.62, 594.30, 24.93, 294.25, 28.36, 77.07, 563.54, 143.83, 246.75, 259.23, 38.97 and 103.06 μmol/l, respectively (Table 1). The papain hydrolysis showed higher antioxidant and anti-inflammatory activity than trypsin hydrolysis, the amino acid composition results revealed that papain hydrolysis has more amino acid residues that contain a higher quantity and variety of amino

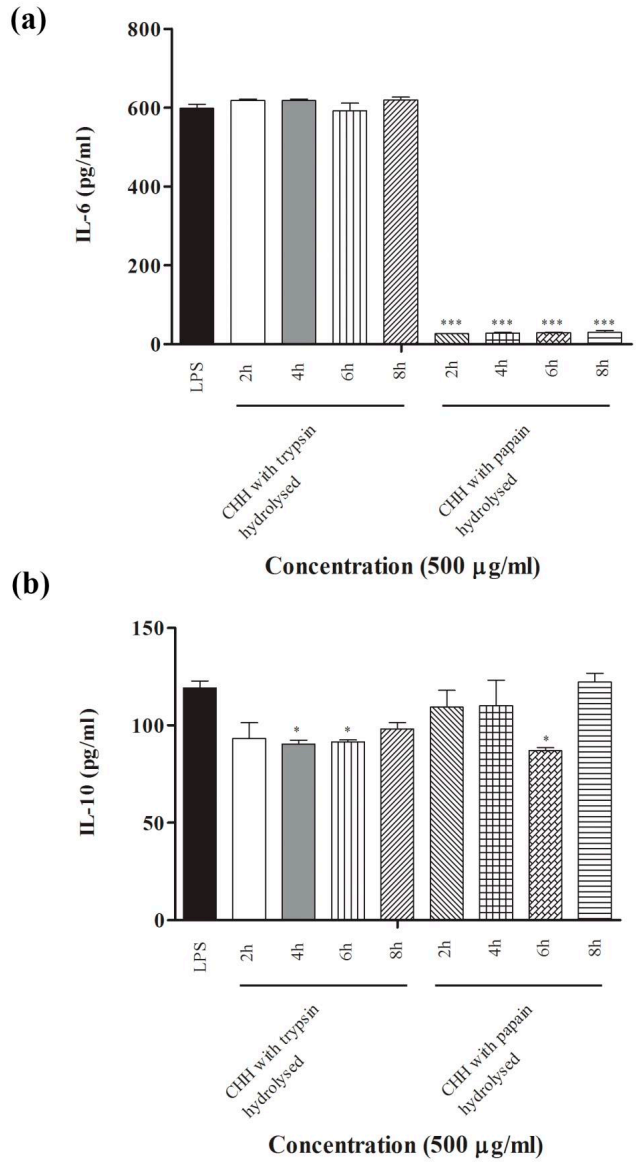

Figure 6. The effect of CHHs derived from trypsin and papain digestion at a concentration of 500 µg/ml on LPS-stimulated (a) IL-6 and (b) IL-10 productions. Each bar represents the mean ± SEM of three demonstrations. (* $P < 0.05$ and *** $P < 0.001$) probability levels compared with LPS treatment alone.

acids than trypsin hydrolysis. Several studies showed that antioxidant protein hydrolysates hydrolyzed by trypsin and papain are enriched in Pro, Ala, Leu, Tyr, Phe, Cys, Gly, His and Val residues [46-51] which are major constituent amino acids in CHH papain hydrolysis. The antioxidant and anti-inflammatory activity of protein derived from papain hydrolysis results from elements of antioxidant amino acid residues and thiol groups that are presented in their molecule. In a previous study, Qian *et al.* [39] reported that Ile, His, Tyr, Pro and

Table 1. Amino acid composition of CHH derived from trypsin hydrolysis by 4-h hydrolysis (4h-CHHt) and CHH derived from papain hydrolysis by 2-h hydrolysis (2h-CHHp).

Amino acid (μmol/l)	Sample	
	4h-CHHt	2h-CHHp
L-Aspartic (Asp)	-	-
L-Threonine (Thr)	9.74	32.96
L-Serine (Ser)	-	-
L-Glutamic (Glu)	-	66.94
L-Proline (Pro)	-	400.44
L-Glycine (Gly)	48.91	276.62
L-Alanine (Ala)	-	594.30
L-Cystine (Cys)	15.14	24.93
L-Valine (Val)	174.45	294.25
L-Methionine (Met)	-	28.36
L-Isoleucine (Ile)	61.11	77.07
L-Leucine (Leu)	179.96	563.54
L-Tyrosine (Tyr)	32.76	143.83
L-Phenylalanine (Phe)	62.71	246.75
L-Histidine (His)	137.30	259.23
L-Ornitine (Orn)	-	38.97
L-Lysine (Lys)	101.24	103.06
L-Arginine (Arg)	-	-

- Defines non-detection.

Lys are assumed to contribute to the reducing power of protein hydrolysates. In addition, peptides containing His and Tyr residues have been documented to exhibit protective effects against lipid peroxidation [52]. Hydrophobic amino acid side chains (e.g. Leu, Phe, Val and Ile) as well as positively charged amino acids (Lys, Arg and His) were documented to have a major influence on the anti-inflammatory activity of peptides [53-58].

4. Conclusion

Hemoglobin hydrolysate was derived from trypsin and papain hydrolysis of *C. siamensis* hemoglobin. CHH derived from papain hydrolysis is not only able to attenuate radical secretions of DPPH, ferric reducing and linoleic peroxide, but is also able to suppress LPS-induced nitric oxide production and pro-inflammatory cytokine IL-6 secretions in murine macrophages. These findings clearly indicate that CHH derived from papain hydrolysis possesses antioxidant and anti-inflammatory activities, which provide support for the application of hemoglobin hydrolysate against inflammation and oxidative stress-related disorders, however, further studies are required to identify active peptide sequences.

ACKNOWLEDGEMENTS

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REFERENCES

[1] Young I.S. and Woodside J.V., *Clin. Pathol.*, 2001; **54**: 176-186. DOI 10.1136/jcp.54.3.176.

[2] Halliwell B. and Gutteridge J.M.C., *Free Radicals in Biology and Medicine*, 4th Edn., Clarendon, Oxford, 2007.

[3] Pham-huy L.A., He H. and Pham-huy C., *Int. J. Biomed. Sci.*, 2008; **4**: 89-96.

[4] Valko M., Rhodes C.J., Moncol J., Izakovic M. and Mazur M., *Chemico-Biol. Int.*, 2006; **160**: 1-40. DOI: 10.1016/j.cbi.2005.12.009.

[5] Aggarwal B.B., Shishodia S., Sandur S.K., Pandey M.K. and Sethi G., *Biochem. Pharmacol.*, 2006; **72**: 1605-1621. DOI 10.1016/j.bcp.2006.06.029.

[6] Khansari N., Shakiba Y. and Mahmoudi M., *Recent Pat Inflamm Allergy Drug Discov.*, 2009; **3**: 73-80. DOI 10.2174/187221309787158371.

[7] Federico A., Morgillo F., Tuccillo C., Ciardiello F. and Loguercio C., *Int. J. Cancer*, 2007; **121**: 2381-2386. DOI 10.1002/ijc.23192.

[8] Hussain S.P. and Harris C.C., *Int. J. Cancer*, 2007; **121**: 2373-2380. DOI 10.1002/ijc.23173.

[9] Ravipati A.S., Zhang L., Koyyalamudi S.R., Jeong S.C., Reddy N., Bartlett J., Smith P.T., Shanmugam K., Münch G., Wu M.J., Satyanarayanan M. and Vysetti B., *BMC Complement. Altern. Med.*, 2012; **12**. DOI 10.1186/1472-6882-12-173.

[10] Clare D.A. and Swaisgood H.E., *J. Dairy Sci.*, 2000; **83**: 1187-1195. DOI 10.3168/jds.S0022-0302(00)74983-6.

[11] Ofori J.A. and Hsieh Y.H.P., *In food additive Prof. Yehia El-Samragy* (ed). The use of blood and derived products as food additives, InTech; 2012. p. 229-256.

[12] Fukada Y., Mizutani S., Nomura S., Hara W., Matsui R., Nagai K., Murakami Y., Washio N., Ikemoto N. and Terashima M., *J. Food Sci. Technol.*, 2016; **53**: 2476-2481. DOI 10.1007/s13197-016-2233-9.

[13] Shazly A.B., He Z., El-Aziz M.A., Zeng M., Zhang S., Qin F. and Chen J., *Food Chem.*, 2017; **232**: 753-762. DOI 10.1016/j.foodchem.2017.04.071.

[14] Mukhopadhya A., Noronha N., Bahar B., Ryan M.T., Murray BA., Kelly PM., O'Loughlin I.B., O'Doherty J.V. and Sweeney T., *Food. Sci. Nutr.*, 2014; **2**: 712-723. DOI 10.1002/fsn3.153.

[15] Ahn C.B, Cho Y.S. and Je J.Y., *Food Chem.*,2015; **168**: 151-156. DOI 10.1016/j.foodchem.2014.05.112.

[16] Joshi I., Sudhakar S. and Nazeer R.A., *Appl. Biochem. Biotechnol.*, 2016; **180**: 1128-1140. DOI 10.1007/s12010-016-2156-y.

[17] Jandaruang J., Siritapetawee J., Thumanu K., Songsiriritthigul C., Krittanai C., Daduang S., Dhiravisit A. and Thammasirirak S., *Protein J.*, 2012; **31**: 43-50. DOI 10.1007/s10930-011-9372-7.

[18] Srihongthong S., Pakdeesuwan A., Daduang S., Araki T., Dhiravisit A. and Thammasirirak S., *Protein J.*, 2012; **31**: 466-476. DOI 10.1007/s10930-012-9424-7.

[19] Phosri S., Mahakunakorn P., Lueangsakulthai J., Jangpromma N., Swatsitang P., Daduang

S., Dhiravisit A. and Thammasirirak S., *Protein J*., 2014; **33**: 484-492. DOI 10.1007/s10930-014-9581-y.

[20] Maijaroen S., Anwised P., Klaynongsruang S., Daduang S. and Boonmee A., *Protein Expres. Purif*., 2016; **118**: 55-63. DOI 10.1016/j.pep.2015.09.028.

[21] Pakdeesuwan A., Araki T., Daduang S., Payoungkiattikun W., Jangpromma N. and Klaynongsruang S., *J. Microbiol. Biotechnol*., 2017; **27**: 26-35. DOI 10.4014/jmb.1603.03046.

[22] Lueangsakulthai J., Jangpromma N., Temsiripong T., McKendrick J.E., Khunkitti W., Maddocks S.E. and Klaynongsruang S., *J. Appl. Microbiol*., 2017; **123**: 819-831. DOI 10.1111/jam.13539.

[23] Jangpromma N., Poolperm N., Pornsri K., Anwised P., Kabbua T., Phosri S., Daduang S. and Klaynongsruang S., *Chiang Mai J. Sci*., 2017; **44**: 800-815.

[24] Phosri S., Jangpromma N., Patramanon R., Kongyingyoes B., Mahakunakorn P. and Klaynongsruang S., *Inflammation*, 2017; **40**: 205-220. DOI 10.1007/s10753-016-0471-7.

[25] Lueangsakulthai J., Phosri S., Theansungnoen T., Jangpromma N., Temsiripong T., Mckendrick J.E., Khunkitti W. and Klaynongsruang S., *Biotechnol. Appl. Biochem*., 2018; **65**: 455-466. DOI 10.1002/bab.1628.

[26] Yu Y., Hu J., Miyaguchi Y., Bai X., Du Y. and Lin B., *Peptides*, 2006; **27**: 2950-2956. DOI 10.1016/j.peptides.2006.05.025.

[27] Benjakul S. and Morrissey M., *J. Agric. Food Chem*., 1997; **45**: 3423-3430. DOI 10.1021/jf970294g.

[28] Girgih A.T., Udenigwe C.C. and Aluko R.E., *J. Am. Oil Chem. Soc*., 2011; **88**: 381-389. DOI 10.1007/s11746-010-1686-7.

[29] Ledesma B.H., Hsieh C.C. and Delumen B.O., *Biochem. Biophys. Res. Commun*., 2009; **390**: 803-808. DOI 10.1016/j.bbrc.2009.10.053.

[30] Tavano O.L., *J. Mol. Catal. B Enzym*., 2013; **90**: 1-11. DOI 10.1016/j.molcatb.2013.01.011.

[31] Adler-Nissen J., *J. Agric. Food Chem*., 1979: **6**: 1256-1262. DOI 10.1021/jf60226a042.

[32] Li X., Deng J., Shen S., Li T., Yuan M., Yang R. and Ding C., *J. Food Sci. Technol*., 2015; **52**: 5681-5690. DOI 10.1007/s13197-014-1693-z.

[33] Shu G., Zhang Q., Chen H., Wan H. and Li H., *Acta Univ. Cibiniensis, Ser. E: Food Technol*., 2015; **19**: 65-74. DOI 10.1515/aucft-2015-0015.

[34] Afify A.E.M.M.R., El Baroty G.S., El Baz F.K., El Baky H.H. and Murad A.S., *J. Genet. Eng. Biotechnol,* 2018; **16**: 399-408. DOI 10.1016/j.jgeb.2018.01.002.

[35] O' Sullivan S.M., Lafarga T., Hayes M. and O' Brien N.M., *J. Food Biochem*., 2017; **41**: e12406. DOI 10.1111/jfbc.12406.

[36] Chi C.F., Hu F.Y., Wang B., Li Z.R. and Luo H.Y., *Mar. Drugs*, 2015; **13**: 2580-2601. DOI 10.3390/md13052580.

[37] Shukla S. and Mehta A., *Chiang Mai J. Sci*., 2017; **44**: 929-938.

[38] Yun-hui C., Zhang W. and Shi-ying X., *J. Cent. South Univ. T*., 2006; **13**: 160-165.

[39] Qian Z., Jung W., Byun H. and Kim S., *Bioresour. Technol*., 2008; **99**: 3365-3371. DOI 10.1016/j.biortech.2007.04.005.

[40] Han Y., Byun S.H., Park J.H. and Kim S.B., *Int. J. Food Sci. Technol*., 2015; **50**: 1996-2003. DOI 10.1111/ijfs.12890.

[41] Halliwell B., *Proc. Nutr. Soc*., 1987; **46**: 13-26. DOI 10.1079/PNS19870004.

[42] Mendis E., Rajapakse N. and Kim S.K., *J.*

Agric. Food Chem., 2005; **53**: 581-587. DOI 10.1021/jf048877v.

[43] Ahmed S.T. and Ivashkiv L.B., *J. Immunol.,* 2000; **165**: 5227-5237. DOI 10.4049/jimmunol.165.9.5227.

[44] Mao X.Y., Cheng X., Wang X. and Wu S.J., *Food Chem.*, 2011; **126**: 484-490. DOI 10.1016/j.foodchem.2010.11.025

[45] Yoshimura A., Nishinakamura H., Matsumura Y. and Hanada T., *Arthritis Res. Ther.*, 2005; **7**: 100-110. DOI 10.1186/ar1741.

[46] Jun S., Park P., Jung W. and Kim S., *Eur. Food Res. Technol.*, 2004; **219**: 20-26. DOI 10.1007/s00217-004-0882-9.

[47] Je J.Y., Qian Z., Byun H. and Kim S., *Process Biochem.*, 2007; **42**: 840-846. DOI 10.1016/j.procbio.2007.02.006.

[48] Kim S.Y., Je J.Y. and Kim S.K., *J. Nutr. Biochem.*, 2007; **18**: 31-38. DOI 10.1016/j.jnutbio.2006.02.006.

[49] Ren J., Zhao M., Shi J., Wang J., Jiang Y., Cui C., Kakuda Y. and Xue S.J., *Food Chem.*, 2008; **108**: 727-736. DOI 10.1016/j.foodchem.2007.11.010.

[50] Ao J. and Li B., *Food Sci. Technol. Int.*, 2012; **18**: 425-434. DOI 10.1177/1082013211428219.

[51] Feng P., Ding H., Lin H. and Chen W., *Sci. Rep.*, 2017; **7**: 7449. DOI 10.1038/s41598-017-08115-6.

[52] Moure A., Dominguez H. and Parajo J.C., *Process Biochem.*, 2006; **41**: 447-456. DOI 10.1016/j.procbio.2005.07.014.

[53] Vogel H.J., Schibli D.J., Jing W., Lohmeier-vogel E.K., Epand R.F. and Epand R.M., *Biochem. Cell Biol.*, 2002; **80**: 49-63. DOI 10.1139/o01-213.

[54] Nan Y.H., Park K.H., Jeon Y.J., Park Y.K., Park I.S., Hahm K.S. and Shin S.Y., *Protein Peptide Lett.*, 2007; **14**: 1003-1007. DOI 10.2174/092986607782541042.

[55] Kovacs-Nolan J., Zhang H., Ibuki M., Nakamori T., Yoshiura K. and Turner P.V., *Biochim. Biophys. Acta*, 2012; **1820**: 1753-1763. DOI 10.1016/j.bbagen.2012.07.007.

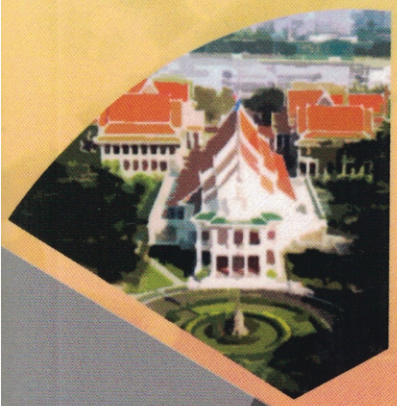
[56] Chatterton D.E., Nguyen D.N., Bering S.B. and Sangild P.T., *Int. J. Biochem. Cell Biol.*, 2013; **45**: 1730-1747. DOI 10.1016/j.biocel.2013.04.028.

[57] Majumder K., Chakrabarti S., Davidge S.T. and Wu J.J., *J. Agric. Food Chem.*, 2013; **61**: 2120-2129. DOI 10.1021/jf3046076.

[58] Kwak S.J., Kim C.S., Choi M.S., Park T., Sung M.K., Yun J.W., Yoo H., Mine Y. and Yu R., *J. Med. Food*, 2016; **19**: 678-685. DOI 10.1089/jmf.2016.3685.

APPENDIX 2

Nisachon Jangpromma, Boonpob Nowichai, Anupong Joompang, Jiraporn Lueangsakulthai, Sakda Daduang, Sompong Klaynongsruang (2018). A potent antioxidant and anti-inflammatory properties of modified peptide from *Crocodylus siamensis* hemoglobin hydrolysate. The 6th Biochemistry and Molecular Biology International Conference (6th BMB): Networking in Biosciences for Creativity and Innovation. June 20-22, 2018. Rayong Resort, Rayong, Thailand.



The 6th International Conference on Biochemistry and Molecular Biology

"NETWORKING IN MOLECULAR BIOSCIENCES TOWARDS CREATIVITY AND INNOVATION"

June 20-22, 2018

Rayong Resort, Rayong, Thailand

PROGRAM AND ABSTRACTS

Organized by

- Biochemistry and Molecular Biology Section of the Science Society of Thailand under the Patronage of His Majesty the King
- Department of Biochemistry, Faculty of Science, Chulalongkorn University

LIST OF ORAL AND POSTER PRESENTERS

S1-P-26

A POTENT ANTIOXIDANT AND ANTI-INFLAMMATORY PROPERTIES OF MODIFIED PEPTIDE FROM *Crocodylus siamensis* HEMOGLOBIN HYDROLYSATE

Nisachon Jangpromma[1,2,*], Boonpob Nowichai[2,3], Anupong Joompang[2,3], Jiraporn Lueangsakulthai[2,3], Sakda Daduang[4], Sompong Klaynongsruang[2,3]

[1] *Office of the Dean (Forensic Science Program), Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*

[2] *Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*

[3] *Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*

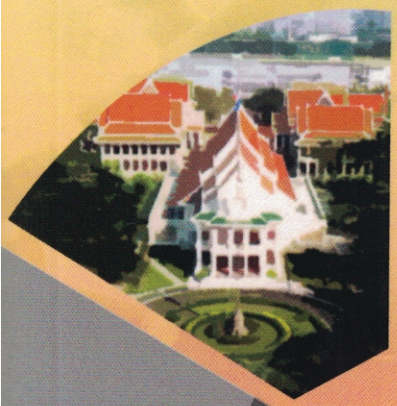
[4] *Division of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand*

*E-mail: nisaja@kku.ac.th

Recently, several peptides have been reported for their biological functions and promised for the clinical therapy. This due to the peptide eases modifications and rapid synthesis. Consequently, this work was demonstrated the potent antioxidant and anti-inflammatory of IL-R (IRHWRRVWRHWRRRL), the modified peptide design by using IL15 (IIHNEKVQAHGKKVL) derived from *Crocodylus siamensis* hemoglobin peptic hydrolysates as a template. Antioxidant activity was measured according to the DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals scavenging method. The results revealed that tryptophan (W) substitution of IL-R emerged out as the higher antioxidant than IL15 with a concentration dependent manner. At the concentration of 50, 100 and 200 μg/ml, IL15 showed inhibition percentages of 16.7%, 17.3% and 22.5%, while IL-R showed inhibition percentages of 25.1%, 43.7% and 50.8%, respectively. Moreover, IL-R displayed anti-inflammatory property with a high efficacy in decreasing NO production of lipopolysaccharide (LPS) stimulated macrophage RAW 264.7 cells. The dose dependent suppression of NO approximately 13.3%, 53.6% and 77.7% were observed when co-treatment with the defined concentrations of IL-R. Additionally, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay guaranteed that IL-R at a concentration of 50-200 μg/ml had no cytotoxic effects on RAW 264.7 cells. It is therefore concluded that IL-R peptide possesses a great potential to scavenge free radical and inhibit NO production in macrophage cells.

APPENDIX 3

Sirinthip Sosiangdi, Anupong Tankrathok, Sompong Klaynongsruang, **Nisachon Jangpromma** (2018). Potential antibacterial activity of designed *Crocodylus siamensis* hemoglobin-based peptides. The 6th Biochemistry and Molecular Biology International Conference (6th BMB): Networking in Biosciences for Creativity and Innovation. June 20-22, 2018. Rayong Resort, Rayong, Thailand.



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LIST OF ORAL AND POSTER PRESENTERS

S4-P-11 POTENTIAL ANTIBACTERIAL ACTIVITY OF DESIGNED *Crocodylus siamensis* HEMOGLOBIN-BASED PEPTIDES

Sirinthip Sosiangdi[1,2], Anupong Tankrathok[3], Sompong Klaynongsruang[1,2], Nisachon Jangpromma[2,4,*]

[1] *Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*
[2] *Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*
[3] *Department of Biotechnology, Faculty of Agro-Industrial Technology, Kalasin University, Kalasin 46000, Thailand*
[4] *Office of the Dean, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*

*E-mail: nisaja@kku.ac.th

In a previous study, we reported an antibacterial peptide derived from *Crocodylus siamensis* hemoglobin hydrolysate, named QL17 (QAIIHNEKVQ AHGKKVL); however this peptide has a narrow spectrum of activity. To improve the antimicrobial activity of the peptide, it was used as the template to design novel effective peptides. The helical wheel diagram was used to monitor and evaluate hydrophobicity and hydrophilicity after the positional change and substitution of certain amino acids. Lysine (K) and arginine (R) were appropriately selected to extend the hydrophilicity, whereas hydrophobic residues such as leucine (L), isoleucine (I) or tryptophan (W) were used to increase the hydrophobicity. As appropriate, two novel peptides were synthesized and named as IL-K (IKHWKKVWKHWKKKL) and IL-R (IRHWRRVWRHWRRRL), which had the same hydrophobicity and net charge at 40% and +7, respectively. Evaluation of the antimicrobial activity by broth microdilution assay revealed that IL-K had a slightly higher inhibition activity than IL-R at concentrations of 12.5, 25, 50 and 100 µg/ml. Both peptides had approximately 2-fold percentage inhibition higher than penicillin and as the QL17 parental peptide against *Klebsiella pneumoniae* and *Staphylococcus aureus*. Our findings suggest that the novel designed peptides are promising to be new antibacterial agents for further development and for use as antibiotics.

The 6th International Conference on Biochemistry and Molecular Biology

Potential antibacterial activity of designed *Crocodylus siamensis* hemoglobin-based peptides

Sirinthip Sosiangdi[1,2], Anupong Tankrathok[3], Sompong Klaynongsruang[1,2], Nisachon Jangpromma[2,4,*]

[1]Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[2]Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[3]Department of Biotechnology, Faculty of Agro-Industrial Technology, Kalasin University, Kalasin 46000, Thailand
[4]Office of the Dean, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
*E-mail: nisaja@kku.ac.th

Abstract

In a previous study, we reported an antibacterial peptide derived from *Crocodylus siamensis* hemoglobin hydrolysate, named QL17 (QAIIHNEKVQAHGKKVL); however this peptide has a narrow spectrum of activity. To improve the antimicrobial activity of the peptide, it was used as the template to design novel effective peptides. The helical wheel diagram was used to monitor and evaluate hydrophobicity and hydrophilicity after the positional change and substitution of certain amino acids. Lysine (K) and arginine (R) were appropriately selected to extend the hydrophilicity, whereas hydrophobic residues such as leucine (L), isoleucine (I) or tryptophan (W) were used to increase the hydrophobicity. As appropriate, two novel peptides were synthesized and named as IL-K (IKHWKKVWKHWKKKL) and IL-R (IRHWRRVWRHWRRRL), which had the same hydrophobicity and net charge at 40% and +7, respectively. Evaluation of the antimicrobial activity by broth microdilution assay revealed that IL-K had a slightly higher inhibition activity than IL-R at concentrations of 12.5, 25, 50 and 100 µg/ml. Both peptides had approximately 2-fold percentage inhibition higher than penicillin and as the QL17 parental peptide against *Klebsiella pneumoniae* and *Staphylococcus aureus*. Our findings suggest that the novel designed peptides are promising to be new antibacterial agents for further development and for use as antibiotics.

Introduction

Over the last decade, drug resistance to bacteria has caused serious problems in bacterial infectious disease therapeutics. Thereby, this has become an issue of interest to the World Health Organization (WHO) to find solutions to the problem of drugs being powerless or having limited use. Drug resistance makes the treatment of patients difficult, requiring increased healthcare costs and even unfortunately making it impossible to eradicate certain pathogen bacteria. Accordingly, it is urgently necessary to improve or develop new medical drugs to demolish those resistant bacteria. To overcome this obstacle, antimicrobial peptides (AMPs) are alternative routes to manage resistant bacteria. They are crucial polypeptides in host defense, and stand as a significant weapon in the innate immune system to the potent killing of a broad range of Gram-negative and Gram-positive bacteria, viruses and parasites at low concentrations[1,2,3]. Unlike traditional antibiotic drugs interrupting bacterial growth by various specific intracellular targets, including inhibiting synthesis of DNA, RNA, protein, or cell wall and enzyme inhibitors, on the contrary most AMPs target membranolytic mechanisms

via barrel-stave, carpet and toroidal-pore models to directly disrupt bacterial membrane permeability[4]. In addition to other bacterial killing mechanisms, some AMPs can diffuse inside the cell and eventually inhibit particular intracellular processes[3,5,6]. AMPs generally consist of a short length of 10-50 amino acids along with positive net charges such as +2 to +9. These characteristics help them to react with phospholipid groups of negatively charged bacterial membrane components via electrostatic forces[2]. Moreover, important AMP features enhancing bacterial attack are amphipathicity, in which they should comprise segments of hydrophilic and hydrophobic parts. Such features contribute to AMPs accelerated approach to the bacterial cell membrane and then insert themselves through the cell wall to adhere to phospholipids in the lipid bilayer. When several AMPs cross the bacterial membrane and associate with each other, they can subsequently create membrane leaking, leading to both intrinsic and extrinsic metabolites freely diffusing and bacteria death[4,6].

Our previous studies have reported that a *Crocodylus siamensis* hemoglobin hydrolysate-derived antimicrobial peptide, QL17, displayed MIC_{50} values killing Gram-negative strains *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and Gram-positive strain *Staphylococcus aureus* at concentrations ranging from 10 to 20 mg/ml (w/v). The QL17 peptide containing 17 amino acid residues has 41% hydrophobicity and +2 net charges. The peptide killed bacterial cells through iron dysregulation and an oxidative stress mechanism[7]. However, this peptide has relatively low antibacterial activity.

The improvement of natural peptides to increase the bactericidal rate has been presented by various methods. The first method is *de novo* design that chooses patterns or frequencies of amino acids to create new AMPs without a previously known sequence. But it is restricted to establishing an AMP similar database[8]. The second method is physicochemical, based on structural and chemical properties of amino acid composition[8]. The last method is a template-based design performing by substitution or deletion, particularly in amino acids. It can create novel AMPs with enhanced antimicrobial effect and cell selectivity[2]. Actually, this method needs some information about the physicochemical properties to develop novel AMPs[8]. In the present study, physicochemical and template-based methods were selected to design novel peptides to improve antimicrobial activity.

In this study, QL17 (QAIIHNEKVQAHGKKVL) was used as a peptide template for designing novel peptides with improvement of antimicrobial activity. The QL17 template was substituted with positively charged amino acid residue on a polar segment. The antimicrobial efficiency against Gram-negative and Gram-positive bacteria of peptides was subsequently measured.

Methodology

Peptides design

Antibacterial peptide QAIIHNEKVQAHGKKVL (QL17) from *C. siamensis* hemoglobin hydrolysate with 41% hydrophobicity and +2 net charges was selected as a template for a novel peptide design. The cationicity of a peptide, especially lysine (K) or arginine (R), was used to increase polarity, whereas the hydrophobic residues such as leucine (L), isoleucine (I) or tryptophan (W) were selected to stabilize the peptide structure. The charges of new peptides were designed at around +7 with 40-46% hydrophobicity. The helical wheel projections of QL17 and its analogues were constructed online using the NetWheels: Peptides Helical Wheel and Net projections maker: http://lbqp.unb.br/NetWheels/.

Peptide synthesis

The parent peptide and designed peptides were synthesized using Fmoc solid phase methodology (GL Biochem, Shanghai, China). Peptides were purified by reversed phase high-

performance liquid chromatography. The purity of peptides was greater than or equal to 95%. Then, resulting peptides had their molecular masses confirmed by electrospray ionization-mass spectrometry.

Antimicrobial activity assay

The bacteria used were *Klebsiella pneumoniae* ATCC 27736 and *Staphylococcus aureus* ATCC 25923. Antimicrobial activity of all peptides was determined using the broth microdilution assay. The method was performed according to a previous study[2]. Briefly, bacterial cells were grown to mid-log phase in nutrient broth at 37 °C, and the bacteria were diluted to 10^6 CFU/ml. 50 µl of bacterial suspension was added to each well, together with 50 µl of two-fold serially diluted peptide in a 96-well plate. After incubation at 37 °C for 18-22 h, the value of OD at 600 nm in each well was observed for bacterial growth with a microplate reader (Biochrom, Cambridge, UK). The percentage of inhibition was calculated according to the following formula: % inhibition = $[(OD_{600\ nm,\ control} - OD_{600\ nm,\ sample}) / (OD_{600\ nm,\ control} - OD_{600\ nm,\ blank})] \times 100$. All % inhibition tests were performed in triplicate.

Statistical analysis

Statistical values of all experimental results were calculated using ANOVA, followed by Duncan's test using statistix 8.0. These data are presented as mean ± SD. The *p* value of < 0.05 was considered as being significant.

Results and Discussion

Peptide design and characterization

In this study, the antimicrobial peptide QL17 was used as a template. Novel peptides, namely IL-K and IL-R, were designed by substituting Lys (K) or Arg (R) residues in hydrophilic segments, and Trp (W) residues in hydrophobic segments of parental peptide. However, the replacement of some amino acid residues (Asn, Gln and Gly) in the parent peptide is less essential for membrane-penetrated activity of the peptide[9,10]. Hence, we replaced negatively charged parts and some amino acids with selected amino acids Lys or Arg to increase the hydrophilic area, while Trp was chosen to increase the hydrophobic area. The implementation of our study was to design peptides with preserved facial amphipathic helical parts with an increase of the cationic charge and with a large percentage (≥ 30%) of hydrophobic amino acids[2]. The hydrophilic residues of Lys and Arg were selected to study toxicity, because previous reports have demonstrated that Arg shows toxicity over Lys even when both of them show the same polarity[2]. On the other hand, the selection of Trp was made because the aromatic side chain of Trp can form hydrogen bonds with the phospholipid bilayer of a bacterial cell membrane interface, whereby the peptide is able to permeate deeply and lyse the cell membrane[9,4].

Table 1 shows the sequences and physicochemical properties of the parent peptide and novel peptides. The molecular weights of QL17, IL-K and IL-R were 1913.25, 2073.61 and 2269.70 Da, respectively. Furthermore, the net positive charges on IL-K and IL-R were +7, while, the charge of QL17 was +2. Cationicity is the one crucial features of the antimicrobial efficacy of peptides. Besides, it is involved in interaction formation between cationic peptides and negative charges on the bacterial cell wall, which contain phosphate-head groups of the lipid membrane[11]. On the other hand, the mammalian cell membrane had zwitterionic phospholipids such as phosphatidylcholine, phosphatidylserine and cholesterol that interrupt the attachment of peptides into the membrane[10]. Another feature of AMPs is their hydrophobicity property. Both parent peptide (QL17) and novel peptides (IL-K and IL-R) have a high value of hydrophobicity at 41%, 40% and 40%, respectively. The hydrophobicity of peptides help them to penetrate into the cell membrane, leading to cell membrane pore formation and the occurrence of cell death[9]. However, bulky hydrophobic residues resulted in

strong affinity for mammalian cell membranes and lower cell selectivity[4]. Hence, designed peptides with altered hydrophobic amino acid residues must be considered. The helical wheel projections present the amino acid sequences and properties (Figure 1).

Table 1. Sequences and physicochemical properties of the parent peptide and novel peptides.

Peptide	Sequence	MW (Da)[a]	% Hp[b]	Net charge
QL17	QAIIHNEKVQAHGKKVL	1913.25	41.18%	+2
IL-K	IKHWKKVWKHWKKKL	2073.61	40%	+7
IL-R	IRHWRRVWRHWRRRL	2269.70	40%	+7

[a]Molecular weight (MW) was calculated using an online tool at website https://web.expasy.org/compute_pi/

[b]Percentage of hydrophobicity (% Hp) was calculated using an online tool at website https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php

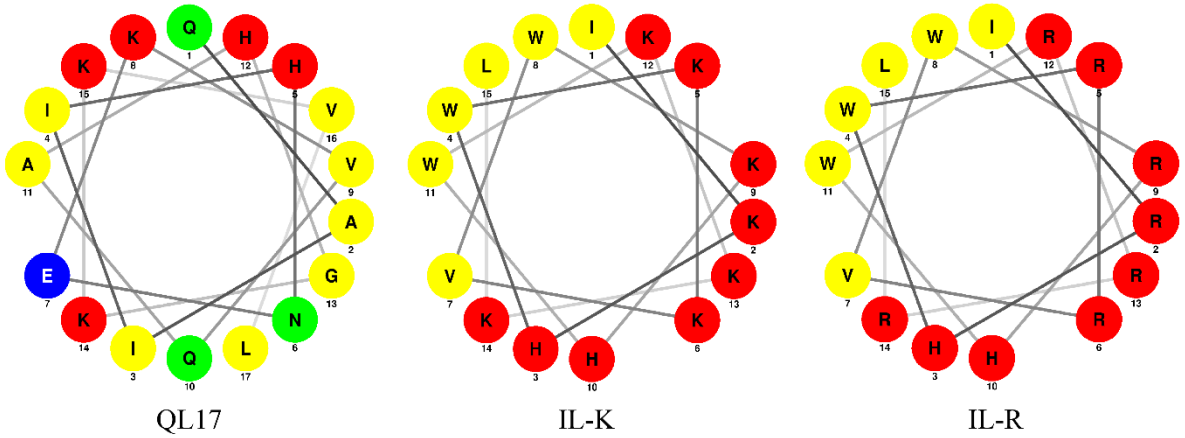

Figure 1. Helical wheel diagrams of the QL17 peptide and its derivative peptides. By default the output presents the polar/basic residues in red, polar/acid residues in blue, polar/uncharged residues in green, and nonpolar residues in yellow.

Antimicrobial activity

The antimicrobial activities of peptides and a conventional antibiotic, penicillin, were evaluated against Gram-negative (*K. pneumoniae*) and Gram-positive (*S. aureus*) bacteria. IL-K and IL-R exhibited higher antimicrobial activity against both Gram-negative and Gram-positive bacteria than penicillin and QL17 at concentrations ranging between 12.5-100 µg/ml (Figure 2). Noticeably, IL-K and IL-R displayed higher antimicrobial activity against *S. aureus* than *K. pneumoniae* at the same concentrations (ranging from 12.5-100 µg/ml). The A Gram-negative bacterium has an outer membrane to protect and reduce the damage induced by a peptide. In contrast, Gram-positive bacteria lack the outer membrane, thus peptides can directly damage cell walls and activate the loss of membrane permeability[4]. The treatment of IL-K on *K. pneumoniae* and *S. aureus* in concentrations of 100 µg/ml displayed a 3-fold and 1-fold percentage inhibition, respectively, compared to parental QL17 and penicillin. Likewise, IL-R showed a 2-fold and 1-fold inhibition against *K. pneumonia*, compared with parental QL17 and penicillin, respectively. In addition, IL-R exhibited increased killing efficiency on *S. aureus* by 3-fold and 2-fold compared with QL17 and penicillin, respectively. The results indicate that IL-K showed significantly better antimicrobial property than IL-R. Decrease in the size of the amino acid side chain may lead to the deep insertion of the peptide into the membrane. The results demonstrate that the positive charge of the peptide plays a major role in antimicrobial efficiency. These investigations are supported by the premise that the addition of positively

charged amino acid residues is efficacious for the initial electrostatic interactions between peptides and negatively charged lipid heads[10,1].

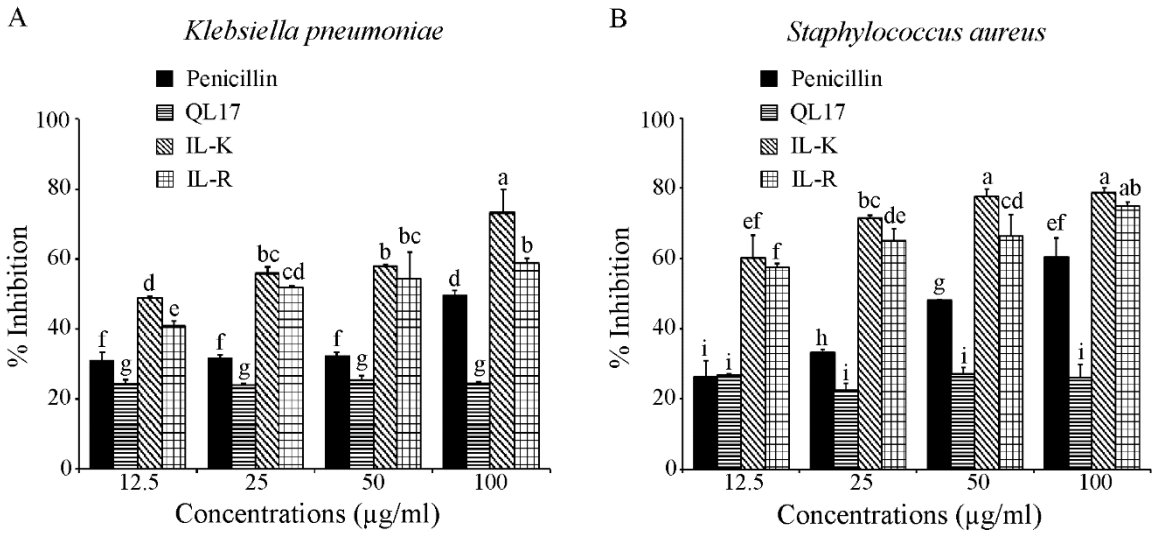

Figure 2. The inhibition percentage of *K. pneumoniae* (A) and *S. aureus* (B) treated with different concentrations of the parent peptide and novel peptides. The letters at the top of each bar indicate significant difference between treatments (p<0.05).

Conclusion

Two novel template-based designed peptides, namely IL-K and IL-R, which were designed by increasing the positive net charges, exhibited an increase in antibacterial property against Gram-negative (*K. pneumoniae* ATCC 27736) and Gram-positive (*S. aureus* ATCC 25923) bacteria when compared to that of a template peptide, QL17. Substitution with Lys residue in the hydrophilic segment of the parent peptide could obtain higher antimicrobial activity than the substitution of Arg. From the results, it is suggested that the size of the peptide affects the bacteria killing mechanism. In addition, a smaller peptide might easily access and insert itself into the membrane, resulting in bacterial cell damage. Hence, the novel designed peptides could be used as an alternative choice for therapeutic agents in the future.

References

1. Reinhardt A, Neundorf I. Int J Mol Sci. 2016;17:701.
2. Zhang S, Song J, Gong F, Li S, Chang H, Xie H, Gao H, Tan Y, Ji S. Sci Rep. 2016;6:273-294.
3. Bahar AA, Ren D. Pharmac. 2013;6:1543-1575.
4. Khara JS, Obuobi S, Wang Y, Hamilton MS, Robertson BD, Newton SM, Yang YY, Langford PR, Ee PLR. Acta Biomater. 2017;57:103–114.
5. Kim Y, Kim N, Lee J, Jang J, Park Y, Park S, Jang M. Biochem Biophys Res Commun. 2015;463:322-328.
6. Intorasoot S. Bull Chiang Mai Assoc Med Sci. 2013;46:1-19.
7. Lueangsakulthai J, Jangpromma N, Temsiripong T, McKendrick JE, Khunkitti W, Maddocks SE, Klaynongsruang S. Appl Microbiol. 2017;123:819-831.
8. Porto WF, Silva ON, Franco OL, William FP, Osmar NS, Octávio LF, Protein Structure. 2012;377-396. doi: 10.5772/38023.
9. Bi X, Wang C, Ma L, Sun Y, Shang D. J Appl Microbiol. 2013;115:663-672.
10. Liu B, Huang H, Yang Z, Liu B, Gou S, Zhong C, Han X, Zhang Y, Nia J, Wang R. Peptides. 2017;88:115–125.

11. Jindal HM, Le CF, Mohd Yusof MY, Velayuthan RD, Lee VS, Zain SM, Isa DM, Sekaran SD. PLoS ONE. 2015;10:e0128532. doi:10.1371/journal.pone.0128532.
12. Han HM, Gopal R, Park Y. Amino Acids. 2016;48:505-522.

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APPENDIX 4

Nisachon Jangpromma, Sirinthip Sosiangdi, Boonpob Nowichai, Anupong Tankrathok, Sakda Daduang, Sompong Klaynongsruang (2018). Antibacterial and anti-inflammatory activity of designed and synthesized a α-helical peptide from *Crocodylus siamensis* hemoglobin hydrolysate. The 13th International Symposium of the Protein Society of Thailand. 7-9 August 2018. The Convention Center, Chulabhorn Research Institute during, 2018. Bangkok, Thailand.

ABSTRACTS AND PROCEEDINGS

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Antibacterial and anti-inflammatory activity of designed and synthesized a α-helical peptide from *Crocodylus siamensis* hemoglobin hydrolysate

Nisachon Jangpromma[1,2,*], Sirinthip Sosiangdi[2,3], Boonpob Nowichai[2,3], Anupong Tankrathok[4], Sakda Daduang[5], and Sompong Klaynongsruang[2,3]

[1] Department of Integrated Science, Forensic Science Program, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

[2] Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

[3] Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

[4] Department of Biotechnology, Faculty of Agro-Industrial Technology, Kalasin University, Kalasin 46000, Thailand

[5] Division of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand

***Email:** nisaja@kku.ac.th

ABSTRACT

The natural antimicrobial peptide, IL15 (IIHNEKVQAHGKKVL) was previously purified from pepsin digestion of *Crocodylus siamensis* hemoglobin. This peptide was used as the template to design a novel peptide in order to enhance performance of its antibacterial activity. The α-helical arginine (R) rich (+7 net charge) peptide, called IL-R (IRHWRRVWRHWRRRL) was designed from IL15 template and synthesized. The antimicrobial activity of the synthesized peptides was quantitatively determined by reduction in number of *Staphylococcus epidermidis* ATCC 12228 corresponding to their optical density at 600 nm. The minimum inhibitory concentration (MIC) of IL-R was 12 μg/ml. While, the template, IL15 peptide showed MIC at >200 μg/ml. Moreover, IL-R elicited a significant decrease in the amount of nitric oxide (NO) production of lipopolysaccharide (LPS) stimulated macrophage RAW 264.7 cells. The half maximal inhibitory concentration (IC_{50}) value of IL-R was found to be 103 μg/ml. Using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, the obtained data guaranteed that IL-R at a concentration of 25-200 μg/ml had no cytotoxic effects on RAW 264.7 cells. And the designed peptide also was nontoxic effects to human red blood cells.

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APPENDIX 5

Nisachon Jangpromma, Santi Phosri, Boonpob Nowichai, Anupong Joompang, Sompong Klaynongsruang (2018). Antioxidant activity of cationic amphipathic peptide modified from IL15 peptide from Siamese crocodile hemoglobin hydrolysate. The 44th Congress on Science and Technology of Thailand (STT44) Science and Technology in the Disruptive Era. 29-30 October 2018. Bangkok International Trade and Exhibition Center (BITEC), Thailand, 2018. Bangkok, Thailand.

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ABSTRACTS BOOK

B1_005_PA: ANTIOXIDANT ACTIVITY OF CATIONIC AMPHIPATHIC PEPTIDE MODIFIED FROM IL15 PEPTIDE FROM SIAMESE CROCODILE HEMOGLOBIN HYDROLYSATE

Nisachon Jangpromma[1,2,*], Santi Phosri[3], Boonpob Nowichai[2,4], Anupong Joompang[2,4] and Sompong Klaynongsruang[2,4]

[1]Department of Integrated Science, Forensic Science Program, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

[2]Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

[3]Office of Education, Faculty of Engineering, Burapha University, Chonburi 20131, Thailand

[4]Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

*email: nisaja@kku.ac.th

Abstract: Known antimicrobial and antioxidant peptide IL15 (IIHNEKVQAHGKKVL) from *Crocodylus siamensis* hemoglobin hydrolysate was used as a template for the design of new cationic amphipathic peptide IL-K (IKHWKKVWKHWKKKL). Antioxidant activity was measured according to the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radicals scavenging method. The results indicated that IL-K exhibited higher antioxidant than IL15. The average percentage of radicals scavenging by 64% was observed in IL-K, while the average efficiency in radicals scavenging of IL15 was 16%. To determine the antioxidant activity of both peptides on HaCaT human keratinocytes cell, hydrogen peroxide (H_2O_2) was selected as the oxidant. The 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide solution (MTT) assay demonstrated that the oxidative damage induced by H_2O_2 to HaCaT cells led to a significant reduction of cell growth (21% cell viability). A protective effect against this damage was observed upon co-culture of the cells with IL15 and also IL-K. The HaCaT cell viability of IL15 was determined as 29% while IL-K showed cell viability as 35%, respectively. It is therefore concluded that IL-K that modified from Siamese crocodile hemoglobin possesses a great potential to scavenge free radical in cells.

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APPENDIX 6

Sirinthip Sosiangdi, Sompong Klaynongsruang, **Nisachon Jangpromma** (2018). Antimicrobial effect of novel synthetic peptide designed from *Crocodylus siamensis* hemoglobin hydrolysate on *Escherichia coli*. The 44th Congress on Science and Technology of Thailand (STT44) Science and Technology in the Disruptive Era. 29-30 October 2018. Bangkok International Trade and Exhibition Center (BITEC), Thailand, 2018. Bangkok, Thailand.

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ABSTRACTS BOOK

B2_002_PA: ANTIMICROBIAL EFFECT OF NOVEL SYNTHETIC PEPTIDE DESIGNED FROM *Crocodylus siamensis* HEMOGLOBIN HYDROLYSATE ON *Escherichia coli*

Sirinthip Sosiangdi,[1,2] Sompong Klaynongsruang,[1,2] Nisachon Jangpromma[2,3,*]
[1]Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[2]Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[3]Department of Integrated Science, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
*e-mail: nisaja@kku.ac.th

Abstract: The hydrolyzed *Crocodylus siamensis* hemoglobin peptide namely IL15 (IIHNEKVQAHGKKVL) was previously described for their antibacterial activity. However, this peptide had short half-life in mammalian reticulocytes (*in vitro*) when estimated by ProtParam tool. Accordingly, the present research was aimed to design novel antimicrobial peptide by substitution of lysine (K) residue in IL15 to extend the cationic net charge from +2 to +6. Whereas tryptophan (W) residue was chosen to increase the percentage of hydrophobicity from 40% to 46%. The antimicrobial activity was evaluated against Gram-negative bacterium using broth microdilution assay and plate count methods. IL15-modified exhibited strong antimicrobial activity against *Escherichia coli* with minimum inhibitory concentration (MIC, that percentage inhibition more than 90) value of 4 µg/ml and minimum bactericidal concentration (MBC, that killed bacteria) value of 6 µg/ml. Furthermore, the kinetic killing analysis results showed that IL15-modified could destroy *E. coli* within one hour at MIC value. Mode of action of this peptide was further elucidated using scanning electron microscope (SEM) technique. The results revealed that the bacterial cell membrane is dramatic destruction as a dose-dependent manner. This study is expected that the IL15- modified peptide can develop as the next generation of antibiotics and might solve antimicrobial resistant problem in the future.