クルマエビの頭部廃棄物に見出された抗菌およびAC
E阻害ペプチドに関する研究

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Master's Thesis

STUDY ON CHARACTERIZATION AND STRUCTURE OF ANTIBACTERIAL AND ACE INHIBITORY PEPTIDES DERIVED FROM THE HEAD WASTES OF KURUMA SHRIMP, *Marsupenaeus japonicus*

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修士学位論文内容要旨
Abstract

専　攻 Major	食機能保全科学	氏　名 Name	Zhou Jie
論文題目 Title			Study on characterization and structure of antibacterial and ACE inhibitory peptides derived from the head wastes of kuruma shrimp, *Marsupenaeus japonicus* (クルマエビの頭部廃棄物に見出された抗菌および ACE 阻害ペプチドに関する研究)

　　Over the past few decades, extensive attention regarding on the preparation, characterization and application of protein hydrolysates and natural peptides has been aroused owing to their diverse promising bio-functions. The shrimp heads, as a main by-product of industry processing, are rich in high quality protein, which is a desired ingredient to prepare bioactive peptides with considerable pharmacological potential. However, to our present knowledge, no studies have reported the biological function of protein hydrolysate from kuruma shrimp by-product. Therefore, this study aimed to investigate the antibacterial and Angiotensin I-converting enzyme (ACE) inhibitory activities of protein hydrolysate from kuruma shrimp head, as well as to purify and identify the potential peptide sequence of protein hydrolysate.

　　In chapter 2, in order to screen out suitable proteases for enzymatic hydrolysis, the kuruma shrimp head protein was independently hydrolyzed by three common commercial proteases (pepsin, trypsin and papain). As a result, papain hydrolysate exhibited the strongest antibacterial and ACE inhibitory activities and was selected for subsequent research. Protein/peptide profile of papain hydrolysate analyzed by SDS-PAGE and HPLC-SEC showed a rich composition of small peptides ranging in size below 5 kDa.

　　In chapter 3, in order to elucidate the target components that exhibited corresponding biological activity, kuruma shrimp head papain hydrolysate (KSHPH) were purified using two stages RP-HPLC. After first stage of RP-HPLC, the fraction KSHPH-F_9, which showed the strongest antibacterial activity with the MIC values range from 4.17 to 16.67 mg/ml and the highest ACE inhibitory activities with the IC_{50} value of 0.97 ± 0.04 mg/ml, was selected for further purification. Then, after second stage of RP-HPLC, the molecular mass and amino acid sequence of the peptide fraction (KSHPH-$F_{9\text{-I}}$) with strongest antibacterial activity and the peptide fraction (KSHPH-$F_{9\text{-III}}$) with highest ACE inhibitory activity were measured by LC-MS and LC-MS/MS. The MIC values of KSHPH-$F_{9\text{-I}}$ was 0.67 to 3.33 mg/ml and was noted to show the higher rates of sensitivity to Gram-positive strains than Gram-negative strains. The IC_{50} value of KSHPH-$F_{9\text{-III}}$ which was purified 42.22-fold from KSHPH using two stage of RP-HPLC was 0.045 ± 0.005 mg/ml. The resulting peptide sequence of KSHPH-$F_{9\text{-I}}$ and KSHPH-$F_{9\text{-III}}$ were identified as Val-Thr-Val-Pro and Ala-Arg-Leu/Ile, respectively.

　　In chapter 4, in order to clarify the structural characterization of antibacterial peptide, the 3D structure predication and physicochemical properties analysis of peptide VTVP were carried out. It was found that the peptide VTVP was a desirable hydrophobic peptide with a well-defined random coil conformation. Using a Lineweaver-Burk plots, the ACE inhibitory peptide, ARL/I, indicated a competitive inhibition characteristic and with the inhibitor constant value (Ki) of 36.19 ± 0.28 μg/ml. The inhibitory mechanism of peptide inhibitor and ACE were elucidated by molecular docking simulation. The results of docking revealed that hydrogen bonds were the main interaction force responsible for binding between the peptide inhibitor and the active sites of ACE. Finally, the results of hemolytic activity assay suggested that all purified peptides showed no toxic effect on rabbit red blood cells even if used at high concentration.

　　In conclusion, the study illuminated the potential biological activities of papain hydrolysate from kuruma shrimp head, and successfully identified the antibacterial peptide VTVP and ACE inhibitory peptide ARL/I. It is anticipated that these results can provide new promising possibility and opportunity for practical application in food and pharmaceutical industries.

Content

Abbreviation of Amino Acids

Table of amino acids and their abbreviation, which used to describe the deduced amino acid sequence in this paper.

Amino Acid	Abbreviation (3 Letter)	Abbreviation (1 Letter)
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Abbreviations

KSHH	Kuruma shrimp head hydrolysate
KSHPH	Kuruma shrimp head papain hydrolysate
ACE	Angiotensin I-converting enzyme
RAS	Renin-angiotensin system
KKS	Kallikrein-kinin system
FAO	Food and agriculture organization
PVR	Peripheral vascular resistance
BP	Blood pressure
PCR	Polymerase chain reaction
CFU	Colony-forming unit
HHL	Hippuryl-$_L$-Histidyl-$_L$-Leucine
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
HPLC	High performance liquid chromatography
RP-HPLC	Reversed-phase high performance liquid chromatography
IC$_{50}$	Inhibitor concentration that inhibits 50% of ACE activity
MIC	Minimum inhibitory concentration
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
UPLC	Ultra performance liquid chromatography
ESI	Electrospray ionization
3D	3 Dimensions
GRAVY	Grand average of hydropathicity

Chapter 1 General introduction

Crustaceans species, such as shrimp, crab, lobster, etc., are cultured widely throughout the world. Shrimp, an important member of the crustacean family, which has constituted to a major part of crustacean consumption in recent years (Mao et al., 2017). In particular, the shrimp production in Asia has rising steadily these years, and accounted for over 80% of the worldwide total yield (FAO, 2018). However, due to the export demand and people's consumer preferences, most of shrimp is usually processed as meat, remained head, shell and tail portions as by-products (Knorr, 1991). In general, the shrimp heads account for 30%–50% of whole shrimp weight, which varies among species and processing methods (Sachindra et al., 2005; Sila et al., 2012). Thus, the accumulation of biowastes without reasonable utilization have led to lots of problems, including the squander of biological resources and the troubles of waste disposal, as well as environmental pollution. Shrimp head waste is rich in high quality protein (50% to 65%, dry weight basis) (Mizani et al., 2005), which is a excellent route to process the marine bioactive peptides with considerable pharmacological potential.

1.1 Production of marine bioactive peptides

Over the years, several studies have reported that the bioactive peptides from marine organism sources, these peptides exhibited various biological functions including antibacterial activity (Sila et al., 2014b), antihypertensive activity (Nii et al., 2008), antioxidant activity (Wang et al., 2013), immunomodulatory activity (Hou et al., 2012), anticancer activity (Doyen et al., 2011), anti-inflammatory activity (Ahn et al., 2015), anticoagulant activity (Cheng et al., 2018), and other biological activities (Fig. 1-1). These bioactive peptides, usually contain 2–20 amino acid residues in length, are encrypted within the sequence of parent protein and can be released during the hydrolysis and/or food processing (Harnedy & FitzGerald, 2012). Biological function of peptides are mainly based on their amino acid composition and sequence (Harnedy

& FitzGerald, 2012). Protein hydrolysates are composed of peptides, oligopeptides and free amino acids that are produced via protein hydrolysis in varying degrees (Clare & Swaisgood, 2000).

Basically, marine bioactive peptides are produced from various protein sources by three methods: (1) chemical hydrolysis, peptide bonds of protein are cleaved by either acid or alkali solutions; (2) Microbial fermentation, degradation of proteins through the fermentation of particular proteolytic microorganisms; (3) enzymatic hydrolysis, hydrolysis of protein substances using exogenous proteolytic enzymes in vitro (Nasri, 2017). In addition, several other approaches also used to produce highly efficiency bioactive peptides with known structure, including chemical synthesis, recombinant DNA technology, and enzymatic synthesis (Nasri, 2017).

The schematic representation of general protein hydrolysis process is illustrated in Fig. 1-2. Compared to other treatment, the obvious advantages conferred by using enzymatic hydrolysis method include: (1) the high substrate and reaction specificity of biocatalysis process; (2) mild reaction conditions and easy to control the process to obtain reproducible bioactive protein hydrolysate; (3) the product is natural status and suitable for subsequent purification steps; (4) bioactive peptides produced by enzymatic digestion do not involve organic solvents or toxic chemicals, which can be an excellent alternatives for food and pharmaceutical industries (Kim & Wijesekara, 2010; Nasri, 2017; Simpson et al., 1998).

1.2 Biological activities of marine bioactive peptides

Marine bioactive peptides have widely been isolated and identified from marine organisms as well as marine fish processing by-products (Harnedy & FitzGerald, 2012). These peptides contribute a large potential application values to functional foods, nutraceuticals, and pharmaceuticals industries as the promising ingredients (Kim & Wijesekara, 2010). The overall composition of marine bioactive peptides is similar, but the peptides possessed different structural characteristics owing to their

different biological activities. During the past few decades, the antibacterial peptides and angiotensin I-converting enzyme (ACE) inhibitory peptides are ranked among the most widely studied bioactive peptides (Jemil et al., 2017).

1.2.1 Antibacterial activity

Since the first natural antibiotic, penicillin, has been discovered by Alexander Fleming in 1929, many new classes of antibiotics have been exploited and applied to improve the quality of human lifespan by successful treatment variety of infections caused by bacteria over the followed few decades (Cézard et al., 2011). Stating from the 80s, the number of newfound antibiotic chemical structures declined dramatically, however, a rapidly increase of antibiotic-resistant bacteria due to the widespread use and abuse of traditional antibiotic (Cézard et al., 2011; Wald et al., 2016). Therefore, it is urgent to seek for possible alternatives to traditional antibiotic chemotherapy. Antibacterial peptides, small molecules composed of less than fifty amino acid residues, are essential components of the innate immune system and produced by nearly all living organisms in a defense strategy to resist invading microorganisms (Cézard et al., 2011). Accordingly, the research and analysis of antibacterial peptides applying to alternative antibacterial strategies have increased with each passing days. Since the diverse composition and arrangement of amino acids of antibacterial peptides, the structure and function of them are not entirely the same. However, antibacterial peptides have several physicochemical properties, which are crucial for its activity. Firstly, while some potent peptides with no charge or negative charge have been reported (Bougherra et al., 2017; Sila et al., 2014a), most antibacterial peptides have a net positive charge. The antibacterial peptides with positive charge can interact with the negative charge microbial outer membrane by electrostatic force due to the presence of phosphate groups within lipopolysaccharide (LPS) for Gram negative bacteria and with lipoteichoic acids for Gram positive bacteria, respectively (Jenssen et al., 2006). Certainly, the presence of excessive charges can inhibit the activity through

interfering with the structure (Tossi et al., 2000). Another essential characteristic of antibacterial peptides is their hydrophobicity, which controls the peptide to penetrate the lipid bilayers and induce cell lysis (Aoki & Ueda, 2013). However, the over increased hydrophobicity may leads to diminished antibacterial activity and increased cytotoxicity (Pasupuleti et al., 2012). Most of antibacterial peptides contain 50% hydrophobic residues in their sequence (Kobbi et al., 2015). In addition, the majority of antibacterial peptides show amphipathicity, which means peptides both have hydrophilic (binds phospholipids) and hydrophobic (binds lipids) areas (Ahmed &Hammami, 2019). The membrane permeabilization mechanism depends on the amphiphilic structure of antibacterial peptides. Generally, the secondary structure of antibacterial peptides can be categorized into four major classes: α-helix, β-sheet, loop and extended peptides (Ahmed & Hammami, 2019). The regular secondary structure of α-helix is more common in antibacterial peptides. It was noteworthy that the activity of antibacterial peptide is not lie on a particular property, rather the properties of antibacterial peptides, such as charge, hydrophobicity, amphipathicity, and second structure, are interrelated. Therefore, various approaches such as modification, novel design, and model prediction can be used to improve the physiological activity of antibacterial peptides (Kang et al., 2017).

In general, the mechanism of action of antibacterial peptides can be divided into two main groups: 1) membrane disruptive mode, which caused pore formation and membrane permeabilization, and 2) non-membrane disruptive mode, which act on the intracellular targets without change the membrane integrity (Powers & Hancock, 2003). In both group, the association between antibacterial peptides and the bacterial cell membrane occurs through electrostatic attraction. This attraction promotes the ultimate interaction of antibacterial peptides with the lipid bilayers. During this process, the peptides can gradually aggregate onto the surface of the membrane. Two different kinds of states ("S state" and "I state") formed depending on several parameters, including the nature of the antibacterial peptides, the membrane or the peptide concentration (Huang, 2000). Originally, the peptides tend to be adsorbed to the

bilayers with an orientation parallel at a low peptide-to-lipid ratio. The S state can induce a thinning/stretching of the membrane, while does not cause the membrane damage immediately. As the peptide-to-lipid ratio increases and then reaches a threshold, the orientation of antibacterial peptides change to the perpendicular to the bilayers, which means that the "I state" forming and peptides begin to insert into the membrane, eventually resulting in pore formation (Cézard et al., 2011; Huang, 2000). As illuminated in Fig. 1-3(A-C), three kinds of membrane disruptive modes have been discovered, including the disruption of the lipid bilayer, the membrane is deformed and bent during the insertion process (toroidal pore model), or the membrane is neither deformed nor bent during the insertion process (barrel-stave model), and the thinning of the lipid bilayer followed by dissolution of the membrane (carpet model) (Ahmed & Hammami, 2019; Cézard et al., 2011; Kang et al., 2017). However, in some cases, the killing of microorganisms may occur with very little to even no membrane disruption (Cézard et al., 2011). As for non membrane disruptive mode (Fig. 1-3), the cell can uptake the antibacterial peptides via direct penetration or endocystosis (Cudic & Otvos Jr, 2002; Kang et al., 2017). Once translocated in the cytoplasm, the peptides are able to act through many ways: bind to the DNA, RNA or protein and inhibit the synthesis processes; inhibition of cell-well synthesis; inhibition of enzymatic activity; inhibition of septum formation and/or activation of autolysin (Cézard et al., 2011; Scocchi et al., 2016).

An increasing number of antibacterial peptides have been reported in recent years, which naturally existed in the haemolymph of the many marine invertebrates or released from protein substrate upon enzymatic hydrolysis process. Some examples of antibacterial peptides derived from marine invertebrates are summarized in Table 1-1. In 1996, a proline-rich 6.5 kDa antibacterial peptide has been isolated from the haemocytes of the shore crab, *Carcinus maenas* (Schnapp et al., 1996). Three years later, a cysteine-rich 11.5 kDa polypeptide has been reported from the granular haemocytes of same crab species to show antibacterial activity against Gram-positive and salt tolerant bacteria (Relf et al., 1999). In addition, an approximately 3.7 kDa,

basic, antibacterial peptide (designated callinectin) was purified from the hemocytes of the blue crab, *Callinectes sapidus* (Khoo et al., 1999). A family of antimicrobial peptides, known as penaeidins 1 to 3, derived from the hemolymph of shrimps *Penaeus vannamei*, inhibited the growth of fungi and bacteria with a predominant activity against Gram-positive bacteria (Destoumieux et al., 1997). After that, some putative homologues of the 11.5 kDa antibacterial peptides and the penaeidins have been characterized in other shrimp species, including *Litopenaeus vannamei* and *Litopenaeus setiferus* (Bartlett et al., 2002; Cuthbertson et al., 2002). Furthermore, an antibacterial peptide (astacidin 1) with 16 amino acid residues was found in plasma of the crayfish, *Pacifastacus leniusculus* (Lee et al., 2003). Moreover, several peptide fractions have been isolated from the protein hydrolysate of marine organisms, including the Atlantic rock crab *Cancer irroratus* (Beaulieu et al., 2013), the sponge crab *Dromia dehaani* (Anbuchezian et al., 2018), the oyster *Crassostrea gigas* (Liu et al., 2008), the blue mussel *Mytilus edulis* (Dong et al., 2012), and so on.

Up to now, a total of 2,661 antibacterial peptides have been found from different living organisms (aps.unmc.edu/AP/database/antiB.php). However, there is no clear indication regarding the current use of these peptides as antibiotics. So, these antibacterial peptides may have a potential effect to replace the antibiotic existed and applied to the pharmaceutical as well as food nutrition industries.

1.2.2 Antihypertensive activity

High blood pressure, or hypertension, which is defined as systolic blood pressure above 130 mm Hg and/or diastolic blood pressure above 80 mm Hg (Egan, 2018). Hypertension is an important risk factor for cardiovascular disease (CVD), affecting approximately 29% of adult population worldwide which is predicted to increase to 2.5 billion in the year 2024 (Barman & Baishya, 2019). ACE is a dipeptide hydrolase that play a crucial physiological role in regulating blood pressure in vivo. As shown in Figure 1-4, the renin-angiotensin system (RAS) and kallikrein-kinin system (KKS)

constitute the important hormonal regulation system for blood pressure. Renin is an enzyme secreted from the kidneys which hydrolyses plasma angiotensinogen releasing the angiotensin-I, a decapeptide, and subsequently the inactive angiotensin-I converted to the potent vasoconstrictor angiotensin-II through cleaving the C-terminal His-Leu dipeptide by ACE action, resulting in peripheral vascular resistant and blood pressure elevation (Barbana & Boye, 2011). Furthermore, ACE also converted the bradykinin to inactive products, inhibited the prostagladin synthesis, which further promote the increase of blood pressure (Barbana & Boye, 2011). Therefore, inhibition of ACE is a pivotal factor to lowering the blood pressure and treatment of the symptom of hypertension.

The first known ACE inhibitors are originally isolated from the venom of *Bothrops jararaca*, and many synthetic ACE inhibitors are used to prevent and treat the hypertension until now, such as captopril, enalapril, lisinopril and ramipril (Ondetti et al., 1971; Patchett et al., 1980). Although these synthetic drugs show a significant effect in treating hypertension and related diseases, some adverse side effects, such as cough, allergic, reactions, taste disturbance, and skin rashes, have been caused inevitably (Jao et al., 2012). Thus, searching for a kind of ACE inhibitors without undesirable side effects has become a focal subject of public interest. Over the last few decades, various ACE inhibitory peptides derived from marine invertebrate sources have been reported extensively, including shrimp (Hai-Lun et al., 2006; Wang et al., 2008b; Nii et al., 2008), clam (Tsai et al., 2008; Tsai et al., 2006), oyster (Wang et al., 2008a; Shiozaki et al., 2010), sea cucumber (Zhao et al., 2009), and cuttlefish (Balti at al., 2010)(Table 1-2). These findings suggest that the marine proteins may be a good source to produce ACE inhibitory peptides. As compared with synthetic drugs, marine-derived ACE inhibitory peptides can be used as effective alternatives for application of pharmaceutical and health industry because of the increasing interest for safety and economical cost of drugs.

The inhibition mode of ACE inhibitory peptides is a significant index for determining their inhibitory activity, which can be evaluated using Lineweaver-Burk

plots. The binding mode of inhibitors and ACE is reversible inhibition, including three different kinds of mode: 1) competitive inhibition, 2) noncompetitive inhibition, and 3) uncompetitive inhibition (Jao et al., 2012). Generally, most of ACE inhibitory peptides have been reported to act as competitive inhibitors; however, several noncompetitive and uncompetitive ACE inhibitory peptides have also been observed from various protein sources (Jao et al., 2012). In competitive inhibition mode, the inhibitors can bind to the active site to block it or to inhibitor binding site that is remote from the active site so as to alter the enzyme conformation such that the substrate cannot approach the active site of ACE (Hong et al., 2008). In this mode, the K_m (Michaelis-Menten constant) value of reaction increased with the rise of inhibitor concentration while the V_{max} (maximum velocity) value of enzyme reaction remain unchanged. The noncompetitive inhibition mode shows that both the inhibitor and the substrate can be bound to the ACE at any given point of time. Already formed enzyme-substrate-inhibitor complex cannot release a product but can only be converted back into the enzyme-substrate complex or the enzyme-inhibitor complex (Si et al., 2009). In this mode, the K_m value of reaction remain unchanged with the rise of inhibitor concentration while the V_{max} value of enzyme reaction decreased over time. In the uncompetitive inhibition system, the inhibitor can bind only to the enzyme-substrate complex and decrease the maximum enzyme activity, so that it takes longer for the substrate or product to leave the active site (Jao et al., 2012). Both K_m value and V_{max} value of enzyme reaction decrease as the reaction proceeds.

Actually, the ACE inhibitory activity of peptides in vitro does not always correlate with their antihypertensive effect in vivo (Jao et al., 2012). Thus, the vitro-simulated gastrointestinal digestion is a simple and effective way to simulate the effect in vivo. Theoretically, there are two ways to describe the antihypertensive effect of peptides in vivo after oral administration. First, the peptides maintain their intact structure and second, they are hydrolyzed into active products, after the action of digestive enzymes, absorbed in the intestine and eventually play their antihypertensive role (Jao et al., 2012). Therefore, the truth antihypertensive effect of ACE inhibitory

peptides need to be proved by in-vitro simulated gastrointestinal digestion, animal and clinical studies.

1.2.3 Other biological activities

The possible roles of marine-derived bioactive peptides have been demonstrated to possess antioxidant activity by scavenging free radicals and reactive oxygen species to prevent oxidative damage and anticoagulant activity by prolongation both the thrombin time and activated partial thromboplastin time (Cheng et al., 2018; Wang et al., 2013). In addition, the metal binding capacity of peptides, including zinc-binding peptide and calcium-binding peptide have been reported recently (Chen et al., 2013; Jung & Kim, 2007). The zinc-binding peptide derived from oyster protein hydrolysate has provided a reference for the development of oyster protein-based zinc supplements to improve the zinc deficiency in human body (Chen et al., 2013). The calcium-binding peptide isolated from pepsinolytic hydrolysates of hoki (*Johnius belengerii*) frame can be recommended to oriental people with lactose indigestion or intolerance and calcium dietary supplements as alternative to milk and dairy products (Jung & Kim, 2007). Naturally occurring bioactive peptides derived from marine organism have shown a wide range of application that worth further study to discover and explore more potential value.

1.3 Research objectives

Kuruma shrimp, *Marsupenaeus japonicus*, is one of the economic important species of shrimp culture. Since it has characteristic brownish red bands and is named 'kuruma' for its wheel-like curled shape (Fig. 1-5). As shown in Fig. 1-6, the kuruma shrimp mainly distributed in Japan, Australia, and many Southeast Asian countries (Rosenberry, 2003). From the statistical information of FAO, in 2017, the global production of kuruma shrimp was about 55,143 tons (www.fao.org/fishery/statistics/ zh). A large amount of high quality protein, which stored in the shrimp by-product, is

provided a sufficient raw materials for research. Although several bioactive peptides have been isolated and characterized from various marine organism sources, only a few peptides have been reported from crustaceans. Especially, to our present knowledge, no studies have analysed the antibacterial and ACE inhibitory activities of protein hydrolysate from kuruma shrimp by-product in vitro and in vivo.

To sum up, the overall objective of this research is to investigate the antibacterial and ACE inhibitory activities of head protein hydrolysate from kuruma shrimp.

The specific objectives of this research are:

1) To prepare and analyze the antibacterial and ACE inhibitory activities of head protein hydrolysate from kuruma shrimp.

2) To purify and identify the potential antibacterial and ACE inhibitory peptides released from head wastes of kuruma shrimp by treatment with papain.

3) To illuminate the relationship between peptide structure and corresponding activity.
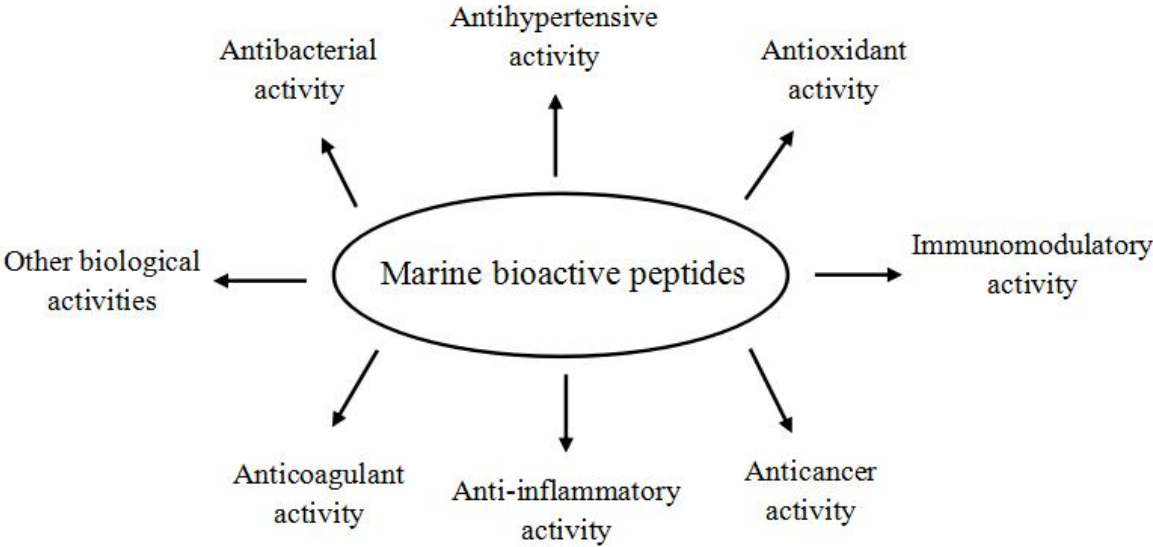
1.4 Figures and Tables

Fig. 1-1 Biological properties of marine bioactive peptides relevant to the promotion of human health and disease prevention.
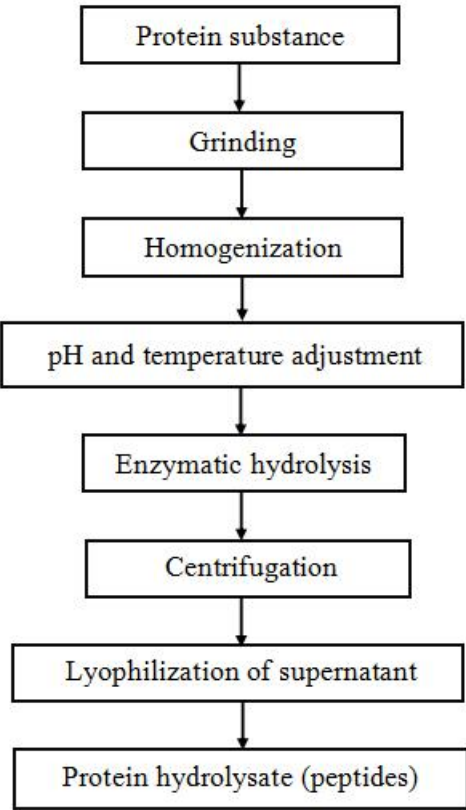
Fig. 1-2 Flow chart showing various steps of protein hydrolysate production.
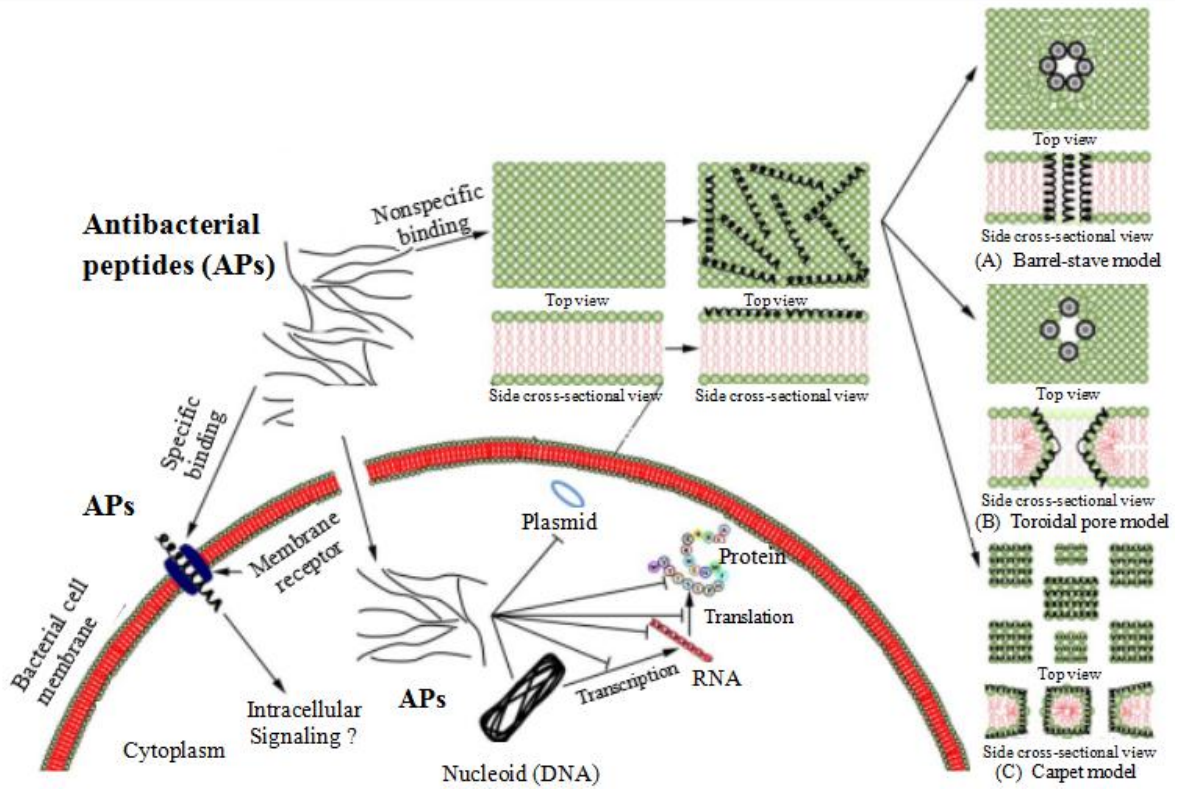
Fig. 1-3 Overview of mechanism of action of antibacterial peptides. (Ref.:Ahmed & Hammami, 2019)

Fig. 1-4 Schematic diagram of the renin-angiotensin system and kallikrein-kinin system. PVR: Peripheral vascular resistance; BP: Blood pressure.

Fig. 1-5 The morphology of kuruma shrimp.

Fig. 1-6 Geographical distribution of kuruma shrimp. The area of identified kuruma shrimp is shown by red color (Source: FAO FishStat, www.fao. org/fishery/species/25 84/en).

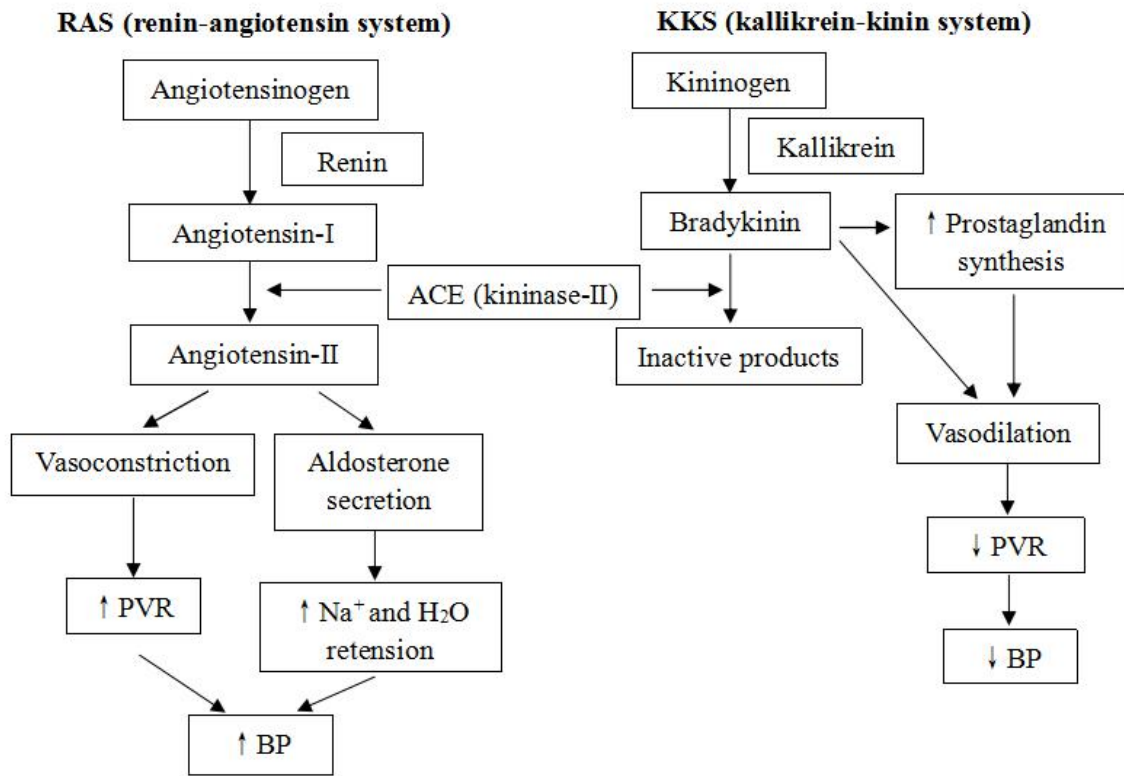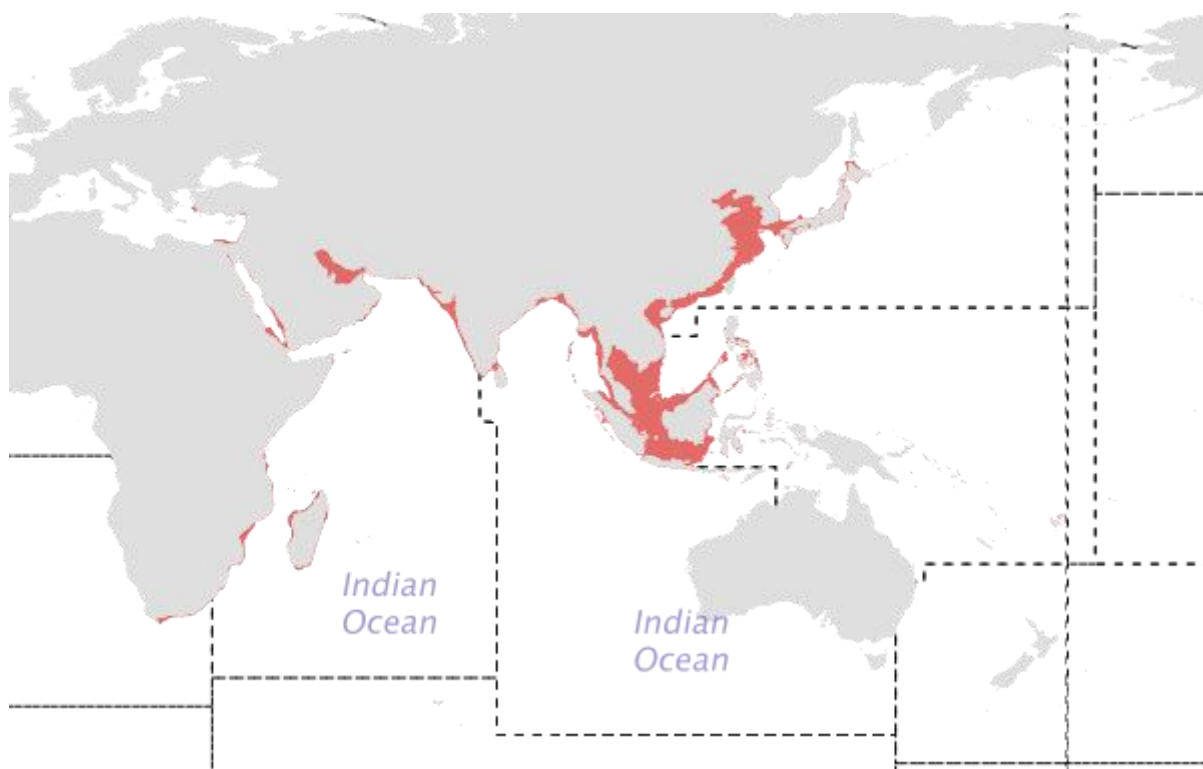Table 1-1 Antibacterial peptides derived from marine invertebrates

Group	Species	Peptide	Reference
Crab	*Carcinus maenas*	6.5 kDa	Schnapp et al., 1996
		Crustin Cm1 (11.5 kDa)	Relf et al., 1999
	Callinectes sapidus	Callinectin (3.7 kDa)	Khoo et al., 1999
	Cancer irroratus	200 to 750 Da	Beaulieu et al., 2013
	Dromia dehaani	Dromidin (513 Da)	Anbuchezian et al., 2018
Shrimp	*Penaeus vannamei*	Penaeidins 1 to 3	Destoumieux et al., 1997
	Litopenaeus vannamei	Crustin Lv1–6	Bartlett et al., 2002
	Litopenaeus setiferus	Crustin Ls1–3	
		Penaeidin 4	Cuthbertson et al., 2002
Crayfish	*Pacifastacus leniusculus*	Astacidin 1	Lee et al., 2003
Oyster	*Crassostrea gigas*	*CgPep33*	Liu et al., 2008
Mussel	*Mytilus edulis*	MAMP fraction	Dong et al., 2012

Table 1-2 ACE inhibitory peptides derived from marine invertebrates

Group	Species	Peptide	Reference
Shrimp	*Acetes chinensis*	Phe-Cys-Val-Leu-Arg-Pro, Ile-Phe-Val-Pro-Ala-Phe, Lys-Pro-Pro-Glu-Thr-Val	Hai-Lun et al., 2006
		Asp-Pro,Gly-Thr-Gly, Ser-Thr	Wang et al., 2008b
	Plesionika izumiae	Val-Trp-Tyr-His-Thr, Val-Trp	Nii et al., 2008
Clam	*Meretrix lusoria*	Tyr-Asn	Tsai et al., 2008
	Corbicula fluminea	Val-Lys-Lys, Val-Lys-Pro	Tsai et al., 2006
Oyster	*Crassostrea talienwhanensis Crosse*	Val-Val-Tyr-Pro-Trp-Tyr-Glu-Arg-Phe	Wang et al., 2008a
	Crassostrea gigas	Asp-Leu-Thr-Asp-Tyr	Shiozaki et al., 2010
Sea cucumber	*Acaudina molpadioidea*	Met-Glu-Gly-Ala-Gln-Glu-Ala-Gln-Gly-Asp	Zhao et al., 2009
Cuttlefish	*Sepia officinalis*	Val-Tyr-Ala-Pro, Val-Ile-Ile-Phe, Met-Ala-Trp	Balti at al., 2010

Chapter 2 Analysis of antibacterial and angiotensin I-converting enzyme (ACE) inhibitory activities from kuruma shrimp head hydrolysate (KSHH)

2.1 Introduction

Several studies indicated that shrimp head protein hydrolysate is rich in a variety of amino acids, peptides and protein, which have potential for recovery and utilization as additives or alternatives in food nutrition and pharmaceutical industries (Ruttanapornvareesakul et al., 2006). As we know, the desirable effect of enzymatic hydrolysis is due to it avoids the extremes of chemical and physical treatments and thus minimizes undesirable reactions (Clemente, 2000). A considerable amount of effects have been devoted to develop protein hydrolysate from shrimp by-product by enzymatic hydrolysis. However, the functional properties of shrimp head protein hydrolysate and their subsequent application are poorly reported.

In this chapter, the antibacterial and ACE inhibitory activities of head protein hydrolysate derived from kuruma shrimp treated with several proteolytic enzymes were investigated respectively. Then, the molecular weight distribution of protein hydrolysate was determined by SDS-polyacrylamide gel electrophoresis and size exclusion chromatography.

2.2 Materials and Methods

2.2.1 Materials

Kuruma shrimp specimens were collected in living status from a local aquafarm in Amakusa city, Kumamoto prefecture, Japan. The shrimp heads were separated and used for hydrolysate preparation.

Pepsin and papain were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Trypsin and ACE from rabbit lung were obtained from Sigma-Aldrich

Co. (Tokyo, Japan).

2.2.2 Shrimp species identification

DNA was extracted from shrimp specimen using QuickGene DNA tissue kit S (FUJIFILM Co. Ltd, Tokyo, Japan) according to the manufacturer's instructions. Specifically, 1.5 ml microcentrifuge tube containing about 15~20 mg of chopped tissue with 180 µl of Tissue Lysis Buffer (MDT), and 20 µl of Proteinase K (EDT) were mixed and incubated at 55 °C for 60 min. After incubation, the supernatant was moved to a new sterilized tube by centrifugation at 10,000 rpm for 3 min. 180 µl of Lysis Buffer (LDT) was added, vortexed 15 s and followed by a quick spin down. After incubated at 70 °C for 10 min, a quick spin down was performed again. 240 µl of 100% (v/v) ethanol was added and mixed thoroughly. The lysate was then transferred to a cartridge of the automatic nucleic-acid isolation system QuickGene-810 and the "DNA tissue mode" was selected with a major modification in the elution time to maximum. A final volume of 200 µl was collected and the samples of genomic DNA were ready to be used immediately or stored at −20 °C until use.

The absorbance (A) of the DNA extracts were measured using the BioSpec-nano UV-VIS Spectrophotometer (SHIMADZU, Kyoto, Japan). The A260/A280 ratio of pure DNA extracts were ranged from 1.8 to 2.0.

To amplify a partial region of the mitochondrial 16S rRNA gene, PCR was carried out using one pair of primers 16SarL and 16SbrH (Palumbi et al., 2002). The specific amplified fragment region of mtDNA and the primer sequence were shown in the Fig. 2-1, and Table 2-1. PCR was performed in 25 µl total volume that containing 2.0 µl of 10×ExTaq buffer, 1µl of each primer (10 µM), 0.2 µl of TaKaRa Ex Taq DNA polymerase (5 U/µl), 2.0 µl of dNTP Mix (2.5 mM), and 2 µl of extracted template DNA (20-30 ng), and was made up to the reaction volumn with sterile water. The PCR was conducted in a Program Temp Control System PC-701 (ASTEC Co. Ltd., Fukuoka, Japan) under the following condition: (i) 30 s at 98 °C, (ii) 30 cycles of 10 s

at 98 °C, 30 s at 53 °C, 1 min at 72 °C, and (iii) 4 min at 72 °C. The length and purity of the PCR products were checked on 1.5% (wt/vol) agarose gel electrophoresis. The target band was observed using a luminescent image analyzer LAS-4000 mini (FUJIFILM Co. Ltd., Tokyo, Japan).

Before the Labeling, 5 µl of the PCR product was purified using 2 µl of ten times diluted ExoSAP-IT® Express PCR product Cleanup (Affymetrix Inc, Santa Clara, CA). The mixture was incubated at 37 °C for 60 min, and followed by heating at 85 °C for 15 min to inactivate the enzyme.

Sequencing of the purified PCR products has preformed using the BigDye Terminater v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA,USA). Each 10 µl reaction liquid containing 3.5 µl of the previously purified PCR products, 1 µl of primer (10 µM), 1 µl of BigDye Terminater v3.1, 1.5 µl of 5× sequencing buffer, and was made up to 10 µl with sterilized water. Then, PCR reactions were submitted to 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. After labeling reaction, the unreacted fluorescent terminators were removed by the ethanol precipitation method.

The obtained PCR products (dry) mixed with 15 µl of Hi-Di Formamide (Applied Byosystems, Foster City, CA,USA) were incubated at 95 °C for 3 min with stir continuously, and then cooled by ice for 2 min. The sequenced products were run on an ABI PRISM®3130 Genetic Analyzer (Applied Biosystems, Foster City, CA,USA) and analyzed using DNA sequencing analysis software SeqEd Version1.0.3 (PerkinElmer). The sequence similarity was searched in the NCBI database by using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.3 Preparation of KSHH

Heads from kuruma shrimp were used as raw material to produce the protein hydrolysate. Fresh shrimp head was ground in an electric food processor at 4 °C. The minced shrimp head was then homogenized in a blender (ULTRA-TURRAX T25, IKA,

Germany) with distilled water added till the final substrate concentration to 1:2 (W:V). After 5 min, the pH of the mixture was adjusted to the optimal value for each enzyme using 5 mol/l HCl or 5 mol/l NaOH. The hydrolysis reaction was started by the addition of enzyme (enzyme/substrate, 10,000 U/g). During the hydrolysis, the pH of the mixture was maintained at the desired value by adding the 5 mol/l NaOH continuously. After 4 hours of hydrolysis, the hydrolysate was heated at 95 °C for 10 min to inactivate the enzymes, followed by adjusted the pH of the hydrolysate to 7.0. Then, the protein hydrolysate was centrifuged at 12,000 × g for 20 min at 4 °C. Next, the supernatant was recovered and freeze-dried and then stored at 4 °C until subsequent use. Hydrolysis conditions for the preparation of KSHH are summarized in Table 2-2.

2.2.4 Determination of peptide content

The peptide content of mixture before and after enzymatic hydrolysis was measured by the OPA method with some modification (Church et al., 1983). The sample solution (20 mg/ml) was filtered by 0.2 μm membrane and the filtrate passed through ultrafiltration membrane with molecular weight (MW) cut-off of 5 kDa (EMD Millipore Corporation, Billerica, MA, USA). The infiltrate was defined as small peptides (MW < 5 kDa). Fifty milliliters of fresh reagent was prepared by mixing 25 ml of 100 mM borax, 2.5 ml of 20% (W/W) sodium dodecyl sulfate (SDS), 40 mg o-phthaldialdehyde (OPA) solution (dissolved in 1 mL methanol) and 44 mg dithiothreitol (DTT) and then adjusted the volume to 50 ml with deionized water. Ten microliter of this infiltrate was mixed with 1 ml reagent. The reaction mixture was incubated for 2 min at room temperature, and the absorbance at 340 nm was measured with UV-1800 spectrophotometer (SHIMADZU, kyoto, Japan). The peptide content was quantified using serine as standard.

2.2.5 Antibacterial activity assay

Bacterial species tested in this study were as follow: *Escherichia coli* Y1090, *Staphylococcus aureus* NBRC 102135, *Micrococcus luteus* NBRC 3066, *Shewanella putrefaciens* IAM 1509, and *Listonella anguillarum* NBRC 13266. The antibacterial activity of KSHH was assessed by paper disc diffusion assay on Luria-Bertani (LB) agar plates (Indu et al., 2006).

The strains were inoculated into 5 ml of sterile LB and incubated 15 hours at 37 °C for *S. aureus*, *E. coli* and 35 °C for other bacterial strains. After CFU counting, the bacterial concentration was adjusted to 1.0×10^7 CFU/ml with sterile saline solution. After a short vortex homogenization, 100 microlitres of bacteria solution were added to every plate to make it evenly coated. Filter paper discs of 8 mm diameter have been pre-sterilized by a high-pressue steam sterilizer (BS-325, TOMY Co., Ltd., Tokyo, Japan) for 20 min at 121 °C. Using an ethanol dipped and flamed forceps, these discs were aseptically placed over nutrient agar plates seeded with the respective test microorganisms. Then, samples were added to these discs. The plates were incubated 24 hours at 37 °C for *S. aureus*, *E. coli* and 35 °C for other bacterial strains. Antibacterial activity was measured as the diameter of the clear zone of inhibition compared to a positive control, tetracycline, and a negative control, sterile saline solution in plates. All assays were performed in triplicate.

2.2.6 ACE inhibitory activity assay

The ACE inhibitory activity was measured as described by the method of Cushman & Cheng (1971) with some detailed modification. This assay was performed by using Hippuryl-L-histidyl-L-leucine (HHL) as substrate and UV spectrophotometry to detect the production of hippuric acid. A sample solution (80 µl) with 200 µl of HHL solution (2.5 mM) was pre-incubated at 37 °C for 5 min. ACE (20 µl, 0.2 U/ml) was added to start the reaction. After 60 min of incubation at 37 °C, the enzymatic reaction was stopped by adding 100 µl of 1 M HCl. The hippuric acid formed was

extracted with 600 µl of ethyl acetate from which 400 µl was evaporated. The residue was dissolved in 1 ml distilled water and its absorbance was measured at 228 nm (UV-1800 spectrophotometer, SHIMADZU, kyoto, Japan). The inhibition activity was calculated using the following equation: ACE inhibition activity (%) = [1 − (S − Sb) / (C − Cb)] × 100; where C, Cb, S and Sb represent the absorbance of control (100% activity), blank inhibitor (HHL alone), sample (inhibitor peptide) and blank sample (peptide alone). The IC_{50} value (the concentration of inhibitor that is able to inhibit 50% of the ACE activity) was calculated by plotting the % ACE inhibition against the different concentrations of peptide.

2.2.7 Determination of molecular weight distribution

According to the results of antibacterial activity and ACE inhibitory activity of KSHH, it was shown that the papain hydrolysate possessed the highest antibacterial activity and the strongest ACE inhibitory activity among three hydrolysates, and the KSHH hydrolyzed by papain have been named as KSHPH. To illuminate the peptide molecular weight distribution of KSHPH, the test of SDS-polyacrylamide gel electrophoresis and gel filtration chromatography have been done in this section.

2.2.7.1 SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15% handmade gel to obtain the protein profile of KSHPH. The electrophoresis was carried out using an AE-7300 (Atto, Japan) compact PAGE system. 1× Running buffer (500 ml MilliQ water containing Tris Base 1.5 g, glycine 7.2 g, SDS 5 g) was filled into the electrophoresis container. Before SDS-PAGE, sample solution was mixed with the sample buffer [0.125 M Tris-HCl buffer (pH 6.8) containing 4% SDS and 10% β-mercaptoethanol] in 3:1 (v/v). All solutions were heated at 100 °C for 3 min and then filled into the 15% acrylamide gel. After being run, the gel was stained with a Rapid Coomassie Brilliant Blue R-250 (Kanto Chemical Co.,

Inc. Tokyo, Japan) and washed with a destaining solution (25 ml methanol, 35 ml acetic acid, and 440 ml MilliQ water). The Precision Plus Protein Standards (Bio-Rad Laboratiries, Hercules, CA, USA) were used as molecular markers.

2.2.7.2 Size exclusion chromatography

The molecular weight distribution of KSHPH was determined by size exclusion chromatography (SEC-HPLC) using a HPLC system (LC-20A, SHIMADZU Corporation, Kyoto, Japan) equipped with a multiwavelength detector (MD-2010 plus, JASCO Corporation, Tokyo, Japan) and a Jasco LC Net II /ADC Chromatography Data solutions and the software ChromNAV version 1.07.01 (JASCO Corporation, Tokyo, Japan). The Superdex 75 10/300 GL column (1 × 30 cm, GE Healthcare Biosciences, Buckinghamshire, UK) was used in this test. KSHPH (1 g) was suspended in 15 ml of MilliQ water, filtered through 0.2 μm membrane filter and then separated onto the column, eluted at a flow rate of 0.4 ml/min and monitored at 280 nm. The HPLC system was calibrated with four molecular weight markers: Albumin (66,463 Da), Carbonic Anhydrase (30,000 Da), Cytochrome c (12,384 Da), and Aprotinin (6,512 Da) (Sigma-Aldrich, St.Louis, MO, USA).

2.3 Results

2.3.1 Shrimp species identification

The shrimp species identification assay has been conducted firstly to ensure the species accuracy of raw material. The developed primer pair successfully amplified the desired DNA fragment from the test sample. Amplicon of about 500 bp DNA fragment with primer pair designed based on mitochondrial 16S rRNA sequence was obtained (Fig. 2-2). In addition, a partial 16S rRNA sequence of 491 bp was amplified using the primers (Fig. 2-3). Blast analysis revealed that this 449 bp except both primers' region had a 100% similarity with the partial sequence of 16S rRNA gene from

Marsupenaeus japonicus.

2.3.2 Antibacterial activity

To screen the suitable enzyme for proteolysis to generate antibacterial peptides, kuruma shrimp heads were independently hydrolyzed for 4 h with pepsin, trypsin and papain at their own optimum conditions. Three kinds of hydrolysates were tested against two bacteria strains: *E. coli* Y1090 and *S. aureus* NBRC 102135. As shown in Fig. 2-4 and Table 2-3, trypsin and papain hydrolysates showed antibacterial activity against *S. aureus*, while not any antibacterial activity was detected in pepsin hydrolysate. The size dimension of inhibition zone was related to the dry weight of KSHH. It was found that papain hydrolysate possessed the strongest antibacterial activity against *S. aureus*. Based on the results, papain hydrolysate was chosen for subsequent antibacterial peptide purification and named as KSHPH. Then, three kinds of bacteria strains (*M. luteus*, *S. putrefacien*, and *L. anguillarum*) were added to test the antibacterial activity of KSHPH.

Fig. 2-5 and Table 2-4 represent the antibacterial activity of KSHPH against totally five bacteria strains. 40 mg of KSHPH (dry weight) showed obvious inhibition zone against *S. aureus*, *M. luteus* , and *L. anguillarum*, whereas no inhibitory activity against *E. coli* and *S. putrefaciens*. Among all the strains, KSHPH was found to be more effective against *S. aureus* and *L. anguillarum*. The results of bacteria sensitive test indicated that KSHPH has a low antibacterial activity when compared with tetracycline (positive control), so the subsequent purification experiments of KSHPH was crucial. At the same time, these five kinds of bacteria strains were selected for the antibacterial activity test in the following study.

2.3.3 ACE inhibitory activity

To produce ACE inhibitory peptides, the head wastes of kuruma shrimp were separately hydrolyzed using several commercial protease. The peptide content of

KSHH was calculated by the calibration curve (Fig. 2-6). Then, three types of protease hydrolysate were compared on the peptide content and ACE IC_{50} value (Table 2-5). The peptide content and IC_{50} value of shrimp head wastes without protease treatment were 2.33 and 8.41 mg/ml, respectively. After treatment with protease, peptide content increased from 8.69 to 9.73 mg/ml. On the other hand, the IC_{50} value of hydrolysate decreased from 1.90 to 2.52 mg/ml. From these results, it was preliminarily speculated that the enzyme hydrolysis of shrimp head wastes probably released lots of small molecular peptides, which significantly enhanced the ACE inhibitory activity of protease hydrolysate. Among these protease hydrolysate, the strongest IC_{50} value was exhibited in papain hydrolysate at 1.90 mg/ml. Therefore, the KSHPH was selected for next purification, which is same with the antibacterial activity assay.

2.3.4 Molecular weight distribution

The extent of protein degradation by papain was estimated by SDS-PAGE analysis and size exclusion chromatography method. The molecular weight of shrimp head wastes before and after hydrolysis have been estimated by comparing them with the standard marker proteins with molecular weight between 250~10 kDa. The electrophoretic pattern of shrimp head wastes showed that there were so many high molecular weight protein in sample before papain cleavage (Fig. 2-7, lane 2). By contrast, after papain treatment, the bands of all high molecular weight proteins were completely disappeared. The protein molecular weight of KSHPH was almost below 25 kDa and most of them are below 10 kDa (Fig. 2-7, lane 1). The result means that papain was efficient to degrade the high molecular weight proteins of the substrate mixture to low molecular weight peptides.

In addition, the molecular weight distribution was also analyzed by size exclusion chromatography on Superdex 75 10/300 GL column. During gel filtration, the substances elute from the column in order of decreasing molecular weight. Larger proteins and peptides do not enter the pores of the column (stationary phase) as readily,

but pass through the fluid volume of the column faster than smaller proteins and peptides. The linear relationship between molecular weight and retention time was established by the four molecular weight standards (Fig. 2-8). Thus, the real molecular weight distribution of KSHPH was calculated according to the calibration curve (Fig. 2-8B). As shown in Fig. 2-9A, the KSHPH were separated into five fractions. The molecular weight of peak 1 was from 5,000 to 40,000 Da, peak 2 was from 2,000 to 5,000 Da, peak 3 was from 500 to 2,000 Da, and peak 4 and 5 were less than 500 Da (Fig. 2-9B). It appeared that the low molecular weight peptide ranges of 0–500 Da were the most detected with an abundance of 36.80% in comparison with other fractions.

2.4 Discussion

Marine organisms are rich in high-quality protein, which is a good source to produce bioactive peptides. In most instances, several bioactive peptides are inactive within the sequence of the parent protein and can be released through proteolytic processing, it shows that the hydrolysis of peptide bonds is important in liberating the potent peptides (Udenigwe & Aluko, 2012). Basically, there are three method can be used to prepared the bioactive protein hydrolysate: chemical hydrolysis process, enzymatic hydrolysis and microbial fermentation (Kim & Wijesekara, 2010; Nasri, 2017). Compared to other methods, enzymatic hydrolysis is more suitable for food and pharmaceutical production, due to the milder conditions, easy to control, and it avoids the use of organic solvents or toxic chemicals treatment that ensure the safety and cleanliness of the end products (Kim & Wijesekara, 2010; Nasri, 2017). Plenty of parameters influenced the biological activities of protein hydrolysate, including the protease specificity, the protein substrate and the hydrolysis conditions (Balti et al., 2010; Jemil et al., 2017). Thus, three commonly used commercial enzymes (pepsin, trypsin and papain) were selected to produce bioactive peptides with different chain lengths and amino acid sequences. The kuruma shrimp head proteins were cleaved at

different cutting sites by proteases, producing protein hydrolysates with varying antibacterial and ACE inhibitory activities, due to having different peptide compositions. The higher efficiency exhibited in papain hydrolysate according to the results of antibacterial activity and ACE inhibitory activity (Fig. 2-4, Tables 2-3 and 2-5). The result of ACE inhibitory assay was in accordance with Zarei et al (2015) who reported that papain hydrolysate of palm kernel cake exhibited the highest ACE inhibitory activity than other protease hydrolysate. Papain is definitely suitable for producing biological protein hydrolysate. Abdel-Hamid et al. (2016) have found two main antibacterial peptides from camel whey hydrolyzed by papain. Kim et al. (2011) have isolated and characterized a novel dipeptide with ACE inhibitory activity from papain hydrolysate of jellyfish, *Nemopilema nomurai*. Papain has broad specificity for catalyzing the hydrolysis of peptide bond that release peptides with amino acid, such as leucine or glycine. In addition, papain is particularly suited for cutting amino acid bearing a large hydrophobic side chain at the P2 position (Martínez-Cruz et al., 2014; Zarei et al., 2015). The IC_{50} values of all hydrolysates are present in Table 2-5. Compared with the previous reported values for marine protein hydrolysate ranging from 0.17 to 501.7 mg/ml, the IC_{50} values of hydrolysate from shrimp, shark meat, mackerel bone are lower than others (He et al., 2007). Thus, it was found that the KSHPH had a moderate ACE inhibitory activity.

The result of SDS-PAGE was basically consistent with the result of SEC we determined. It highlighted that the KSHPH had a high content in peptides with a molecular weight less than 5 kDa, and existed a considerable number of oligopeptides. This mass range indicated that the KSHPH had an appropriate degree of hydrolysis, which is benefit for generating bioactive peptides such as antibacterial and ACE inhibitory peptides. The SDS-PAGE patterns of shrimp head wastes mixture before and after hydrolysis were typical to those previously reported for protein hydrolysate of bovine casein (Bougherra et al., 2017), *penaeus kerathurus* (Limam et al., 2008), flounder fish muscle (Ko et al., 2016), camel whey (Abdel-Hamid et al., 2016) and so on. Meanwhile, Doyen et al., (2012) also found that the low molecular weight peptide

ranges of 300-700 Da showed the abundance of 80.2% in the initial snow crab by-products hydrolysate.

In conclusion, the KSHPH was selected for further purification steps due to its stronger antibacterial and ACE inhibitory activities. Meanwhile, these results provide a new idea for the recycling and utilization of shrimp by-products. However, the protein hydrolysate (KSHPH) is a mixed solution with no clear indication of specific peptides that associated with antibacterial and ACE inhibitory activities. In next chapter, 1) active fractions were purified by using two stage of RP-HPLC, 2) molecular mass and amino acid sequence of active peptide fractions were determined by LC-MS and LC-MS/MS.
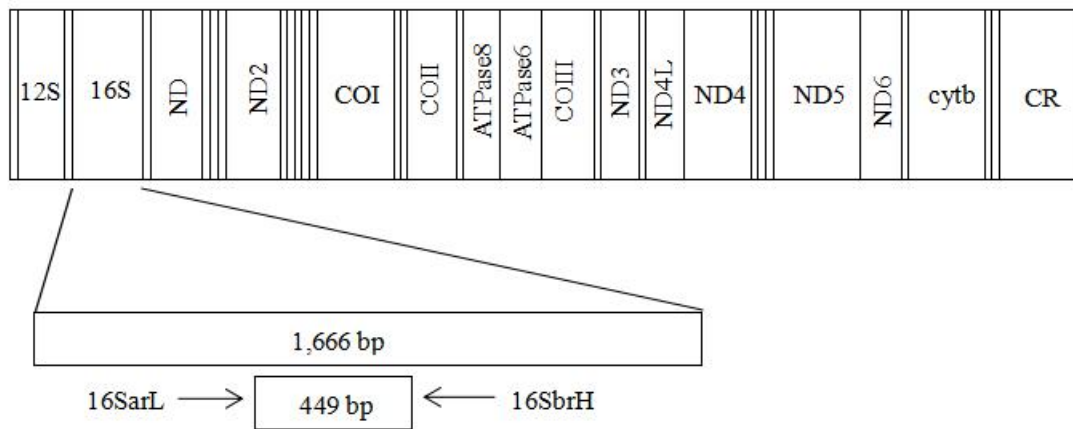
2.5 Figure and Tables

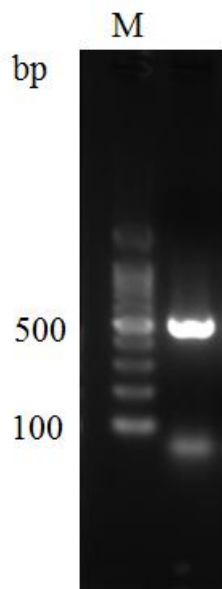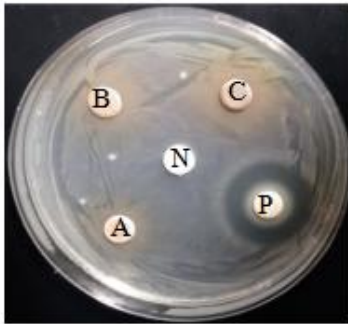Fig. 2-1 Typical structure of mtDNA and the sequencing regions for species identification.

Fig. 2-2 1.2% Agarose gel electropherogram of mitochondrial 16S rRNA PCR amplification products from the shrimp species. Line M: Marker.
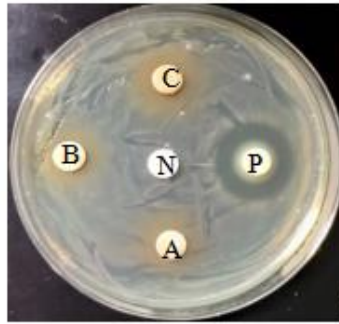
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Sample                              1 CTGCCCACTG ATTTGTTTTA AAGGGCCGCG GTATATTGAC CGTGCGAAGG     50
Marsupenaeus japonicus (AY742271.1) 5 .......... .......... .......... .......... ..........     54
Marsupenaeus japonicus (AF279820.1) 4 .......... .......... .......... .......... ..........     53
Marsupenaeus japonicus (AP006346.1) 13302 .......... .......... .......... .......... .......... 13253

Sample                              51 TAGCATAATC ATTAGTCTTT TAATTGGAGG CTTGTATGAA TGGTTGGACA    100
Marsupenaeus japonicus (AY742271.1) 55 .......... .......... .......... .......... ..........    104
Marsupenaeus japonicus (AF279820.1) 54 .......... .......... .......... .......... ..........    103
Marsupenaeus japonicus (AP006346.1) 13252 .......... .......... .......... .......... .......... 13203

Sample                              101 AAAAGTAAGC TGTCTCGATT ATAATAATTG AACTTAACTT TTAAGTGAAA    150
Marsupenaeus japonicus (AY742271.1) 105 .......... .......... .......... .......... ..........    154
Marsupenaeus japonicus (AF279820.1) 104 .......... .......... .......... .......... ..........    153
Marsupenaeus japonicus (AP006346.1) 13202 .......... .......... .......... .......... .......... 13153

Sample                              151 AGGCTTAAAT GTTTCAGGGG GACGATAAGA CCCTATAAAG CTTGACAATA    200
Marsupenaeus japonicus (AY742271.1) 155 .......... .......... .......... .......... ..........    204
Marsupenaeus japonicus (AF279820.1) 154 .......... .......... .......... .......... ..........    203
Marsupenaeus japonicus (AP006346.1) 13152 .......... .......... .......... .......... .......... 13103

Sample                              201 ACTTCGTTAT ATTATAAATT GTTAGTATAA CTTGATTTTA ACGGGGGTTT    250
Marsupenaeus japonicus (AY742271.1) 205 .......... .......... .......... .......... ..........    254
Marsupenaeus japonicus (AF279820.1) 204 .......... .......... .......... .......... ..........    253
Marsupenaeus japonicus (AP006346.1) 13102 .......... .......... .......... .......... .......... 13053

Sample                              251 GTTTCGTTGG GGCGACGGGA ATATAATAAA TAACTGTTCT TTTAAATATA    300
Marsupenaeus japonicus (AY742271.1) 255 .......... .......... .......... .......... ..........    304
Marsupenaeus japonicus (AF279820.1) 254 .......... .......... .......... .......... ..........    303
Marsupenaeus japonicus (AP006346.1) 13052 .......... .......... .......... .......... .......... 13003

Sample                              301 ATTACAAAAA TGTTTGGTAA ATAATTGATC CTCTATTAGA GATTAAAAGA    350
Marsupenaeus japonicus (AY742271.1) 305 .......... .......... .......... .......... ..........    354
Marsupenaeus japonicus (AF279820.1) 304 .......... .......... .......... .......... ..........    353
Marsupenaeus japonicus (AP006346.1) 13002 .......... .......... .......... .......... .......... 12953

Sample                              351 TTAAGTTACT TTAGGGATAA CAGCGTAATC TTCTTTGAGA GTCCACATCG    400
Marsupenaeus japonicus (AY742271.1) 355 .......... .......... .......... .......... ..........    404
Marsupenaeus japonicus (AF279820.1) 354 .......... .......... .......... .......... ..........    403
Marsupenaeus japonicus (AP006346.1) 12952 .......... .......... .......... .......... .......... 12903

Sample                              401 ACAAGAAGGT TTGCGACCTC GATGTTGAAT TAAGGTATCC TTATAATGC     449
Marsupenaeus japonicus (AY742271.1) 405 .......... .......... .......... .......... .........     453
Marsupenaeus japonicus (AF279820.1) 404 .......... .......... .......... .......... .........     452
Marsupenaeus japonicus (AP006346.1) 12902 .......... .......... .......... .......... ......... 12854
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Fig. 2-3 Comparison of partial nucleotide sequence of 16s rRNA region between sample and shrimp species. The identical residues are shown by dot.
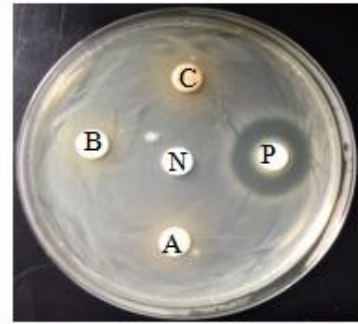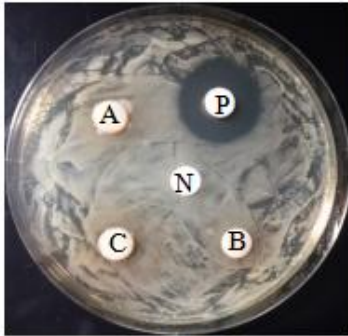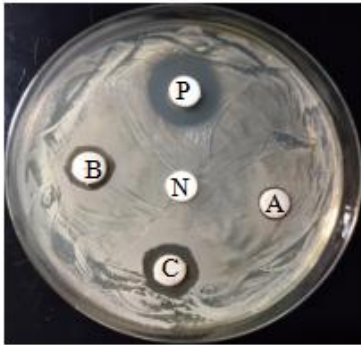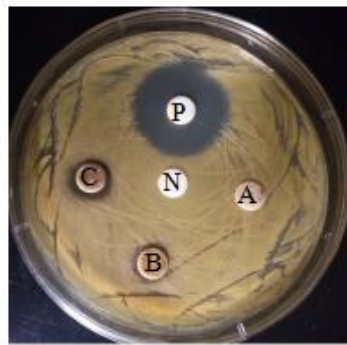
Fig. 2-4 Antibacterial activity of KSHH against *E. coli* ans *S. aureus*. KSHH (dry weight): A, 20 mg; B, 30 mg; C, 40 mg. P, tetracycline: *E.coli* Y 1090, 375 μg; *S. aureus* NBRC 102135, 10 μg. N, sterile saline solution.
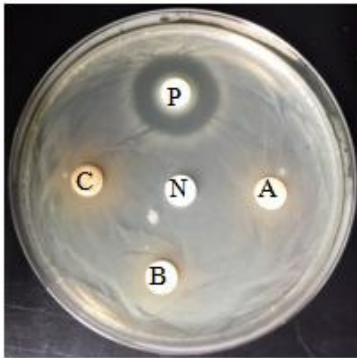
Gram-positive

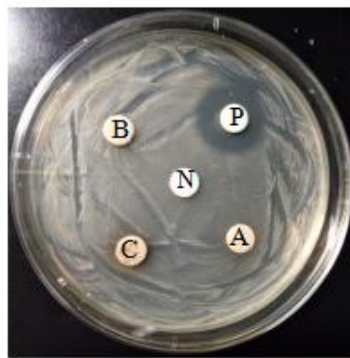S. aureus NBRC 102135   M. luteus NBRC 3066
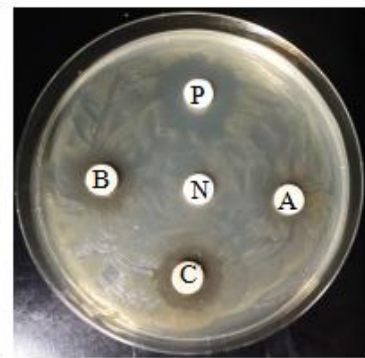
Gram-negative

E. coli Y 1090   S. putrefaciens IAM 1509   L. anguillarum NBRC 13266

Fig. 2-5 Antibacterial activity of KSHPH. KSHPH (dry weight): A, 20 mg; B, 30 mg; C, 40 mg. P, tetracycline: *S. aureus* NBRC 102135, 10 µg; *M. luteus* NBRC 3066, 10 µg; *E. coli* Y 1090, 375 µg; *S. putrefaciens* IAM 1509, 17.5 µg; *L. anguillarum* NBRC 13266, 7.5 µg. N, sterile saline solution.
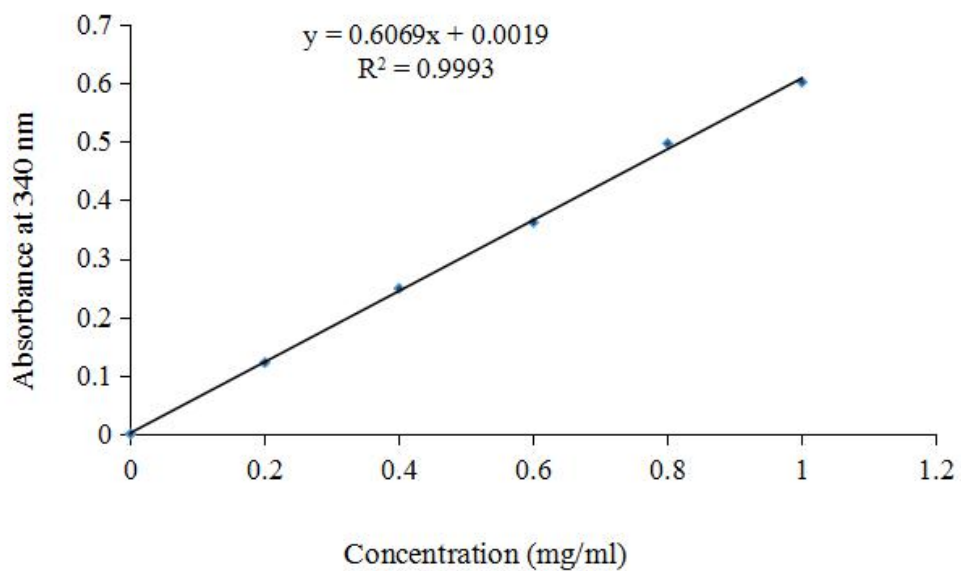
Fig. 2-6 Calibration curve for determination of peptide content.
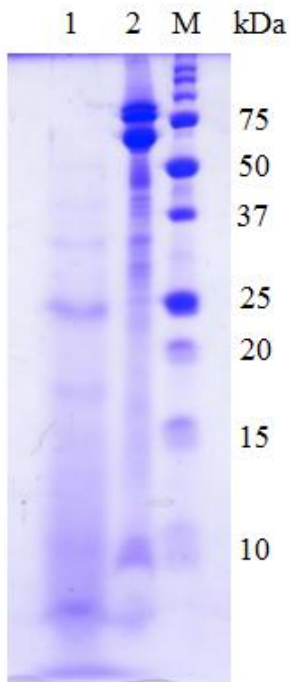
Fig. 2-7 Electrophoresis profile on 15% SDS-PAGE of KSHPH. M, protein marker; lane 1, KSHPH; lane 2, shrimp head wastes without protease treatment.
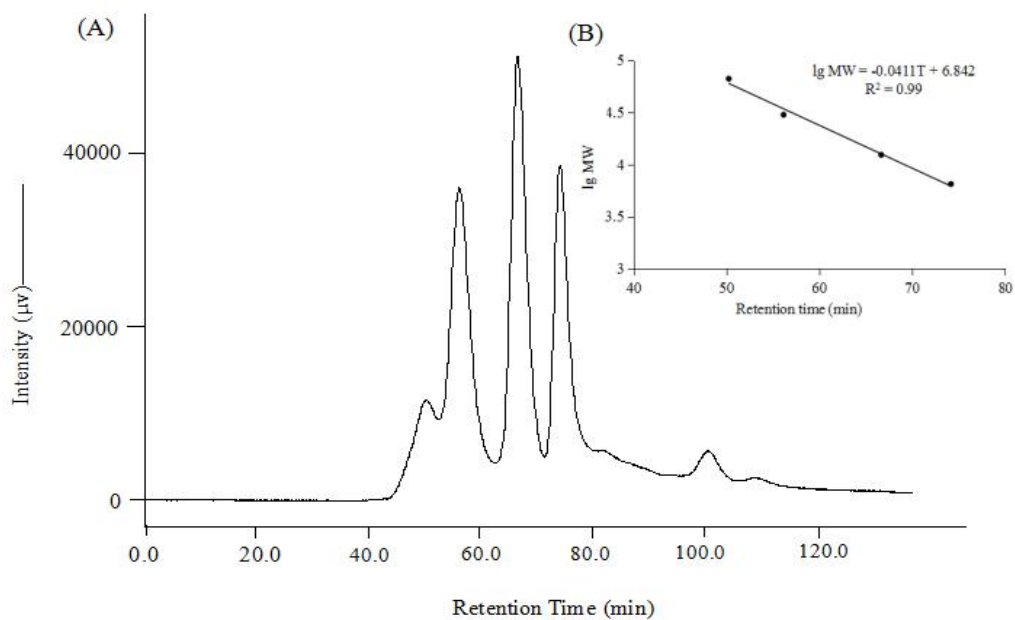
Fig. 2-8 Size exclusion chromatogram on Superdex 75 10/300 GL column of four molecular weight markers (A) and the calibration curve (B).
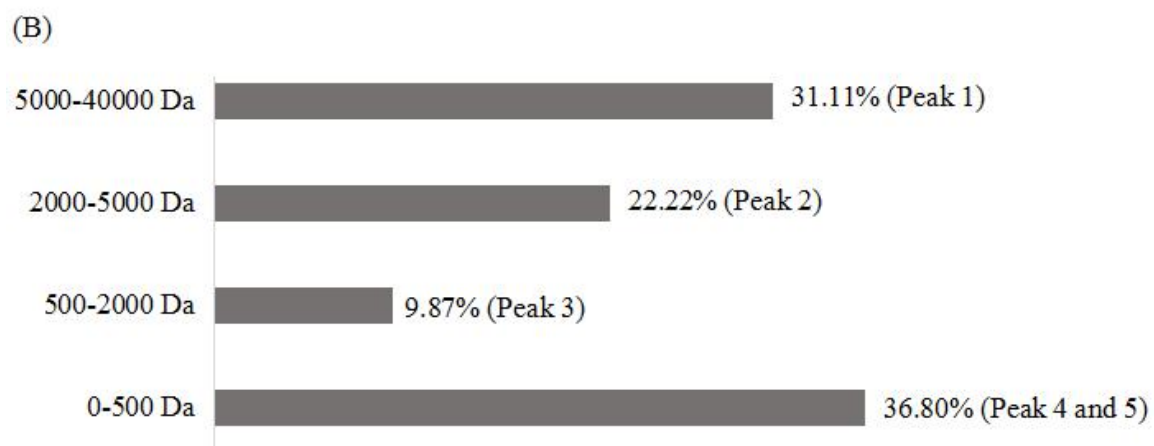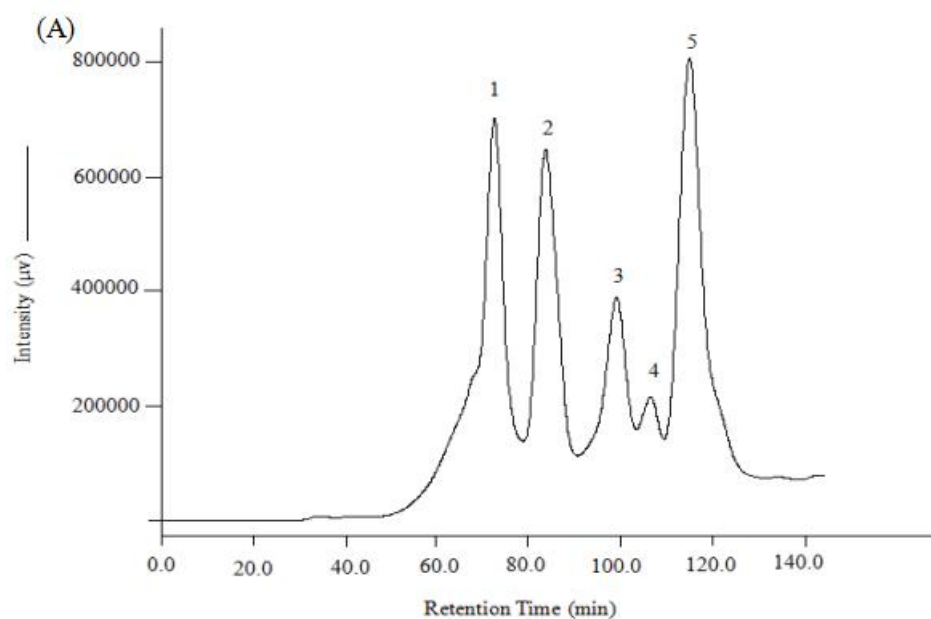
Fig. 2-9 Size exclusion chromatogram on Superdex 75 10/300 GL column of KSHPH (A) and the molecular weight distribution of KSHPH calculated by calibration curve (B).

Table 2-1 Primers used for PCR amplification of special identification assay

Primers	Sequence
16SarL	5'-CGCCTGTTTATCAAAAACAT-3'
16SbrH	5'-CCGGTCTGAACTCAGATCACGT-3'

Table 2-2 Optimum conditions of enzymatic hydrolysis for various enzymes

Enzyme	pH	Temperature (°C)
Pepsin	2.2	37
Trypsin	8.0	37
Papain	7.0	50

Table 2-3 Antibacterial activity of KSHH against *E.coli* ans *S.aureus*

Bacterial species	Zone of inhibition (mm)								
	Pepsin hydrolysate			Trypsin hydrolysate			Papain hydrolysate		
	A	B	C	A	B	C	A	B	C
E. coli Y 1090	—	—	—	—	—	—	—	—	—
S. aureus NBRC 102135	—	—	—	—	+	++	+	++	+++

KSHH (dry weight): A, 20 mg; B, 30 mg; C, 40 mg.

Inhibition zones: ++++: >20 mm; +++: 20~16 mm; ++: 15~11 mm; +: <11 mm, and －: no activity.

Table 2-4 Antibacterial activity of KSHPH

Bacterial species	Zone of inhibition (mm)		
	A	B	C
S. aureus NBRC 102135	+	++	+++
M. luteus NBRC 3066	—	+	++
E. coli Y1090	—	—	—
S. putrefaciens IAM 1509	—	—	—
L. anguillarum NBRC 13266	+	++	+++

KSHPH (dry weight): A, 20 mg; B, 30 mg; C, 40 mg.

Inhibition zones: ++++: >20 mm; +++: 20~16 mm; ++: 15~11 mm; +: <11 mm, and －: no activity.

Table 2-5 Effect of proteases on peptide content and ACE IC_{50} of KSHH

Sample	Peptide content (mg/ml)	IC_{50} (mg/ml)
Shrimp head wastes without protease treatment	2.33 ± 0.04	8.41 ± 0.18
Pepsin hydrolysate	8.97 ± 0.07	2.39 ± 0.06
Trypsin hydrolysate	8.69 ± 0.03	2.52 ± 0.00
Papain hydrolysate	9.73 ± 0.05	1.90 ± 0.03

Data are presented as mean $\pm$ SD (n=3).

Chapter 3 Purification and identification of antibacterial and ACE inhibitory peptides from kuruma shrimp head papain hydrolysate (KSHPH)

3.1 Introduction

Peptides with different biological activities (antibacterial, antihypertensive, antioxidant, antitumor, etc) have been present in many species, especially, the marine organisms (Cheung et al., 2015). After protein hydrolysate preparation, the most essential step to obtain the bioactive peptides was the purification. Up to now, there are various purification strategies to separate bioactive peptides from protein hydrolysate, including the traditional methods and some novel methods. Eleven antibacterial peptides have been isolated from barbel muscle protein hydrolysate by gel filtration and reverse-phase high performance liquid chromatography (RP-HPLC) (Sila et al., 2014b). It has been also reported that the magnetic liposome extraction combined with the RP-HPLC could successfully purify a novel antibacterial peptide from the hydrolysate of highland barley (Pei et al., 2018a). Zhao et al. (2009) have reported that an ACE inhibitory peptide was isolated from the *Acaudina molpadioidea* hydrolysate, using the chromatographic methods including gel filtration, ion-exchange chromatography and RP-HPLC. In addition, Salampessy et al. (2017) have isolated five main ACE inhibitory peptides from leatherjacket protein hydrolysates using ultrafiltration and followed by RP-HPLC. RP-HPLC is widely used in the purification step. Various bioactive peptides have been reported from protein hydrolysate using two stages of RP-HPLC, such as izumi shrimp (Nii et al., 2008), hen egg white lysozyme (Rao et al., 2012), ostrich egg white ovalbumin (Khueychai et al., 2018), and bovine α_{S1}-casein (McCann et al., 2006).

In this chapter, the obtained KSHPH was purified by two stages of RP-HPLC. Then, the molecular weight and amino acid sequence of peptides in the most active fractions were determined by LC-MS and LC-MS/MS.

3.2 Materials and Methods

3.2.1 Purification of KSHPH

HPLC separation was performed on a LC-20A HPLC system (SHIMADZU Corporation, Kyoto, Japan) equipped with a MID-2010 plus multiwavelength detector (JASCO Corporation, Tokyo, Japan) and a Jasco LC Net II /ADC Chromatography Data solutions and the software ChromNAV version 1.07.01 (JASCO Corporation, Tokyo, Japan).

The freeze-dried powder of KSHPH was suspended in distilled water at a concentration of 100 mg/ml, filtered through 0.20 µm filters, and then separated by RP-HPLC on a TSKgel ODS-80TM column (250 × 4.6 mm, Tosoh Corp., Tokyo, Japan). Sample was injected at a volume of 20 µl. The column was pre-equilibrated with eluent A (water containing 0.1% trifluoroacetic acid (TFA)) for 10 min, then peptides were eluted with a linear gradient of eluent B (acetonitrile containing 0.1% TFA) at a flow rate of 1.0 ml/min. On-line UV absorbance scans were performed at 220 nm. Major fractions were collected, lyophilized and determined for their antibacterial and ACE inhibitory activity. The fractions, which showed strongest antibacterial activity and highest ACE inhibitory activity, were re-dissolved at 10 mg/ml and further separated by second stage RP-HPLC on a Mightysil RP-18 column (150-4.6, 5µm, Kanto Chemical Co., Inc, Tokyo, Japan). The elution was conducted at a flow rate of 0.5 ml/min using a linear gradient of acetonitrile containing 0.1% TFA. The elution peaks were detected at a wavelength of 220 nm. The fractions with highest antibacterial and ACE inhibitory activities were collected, pooled and lyophilized, respectively. The purity of the active fractions were analyzed by using same analytical column in the second step RP-HPLC. Then, the pure fractions exhibiting the strongest antibacterial and ACE inhibitory activities were followed by identification of molecular mass and amino acid sequence.

3.2.2 Antibacterial assay

3.2.2.1 Antibacterial activity assay

Bacterial species tested in this study are as follow: *Escherichia coli* Y1090, *Staphylococcus aureus* NBRC 102135, *Micrococcus luteus* NBRC 3066, *Shewanella putrefaciens* IAM 1509, and *Listonella anguillarum* NBRC 13266. The antibacterial activity of the separated fractions was assessed by paper disc diffusion assay described in 2.2.5 of Chapter 2.

3.2.2.2 Minimum inhibitory concentration determination

Minimum inhibitory concentration (MIC) assays were performed using the method of Bougherra et al. (2017) with some modification. The most active fraction of RP-HPLC was dissolved at a definite concentration (80 mg/ml for first step of RP-HPLC and 8 mg/ml for second step of RP-HPLC, respectively) in distilled water and then 2-fold serial dilutions were prepared in a 96-well microplates. The test mixture of each well comprised 50 µl of LB medium, 50 µl of peptide test solution and 100 µl of bacterial suspension (1×10^6 CFU/ml). The inhibition of bacterial growth was monitored by measuring absorbance at 620 nm on a Multiskan FC microplates reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) after 18 h of incubation at 37 °C. Absorbance of well corresponding to decreasing concentrations of the peptide fraction was compared to that of well of a negative control consisting of LB medium and a positive control, tetracycline. Experiments were performed in triplicate.

The MIC was defined as the lowest concentration of peptide fraction that caused no visible increase of absorbance at 620 nm after incubation at 37 °C for 18 h without shaking.

3.2.3 ACE inhibitory activity assay

The ACE inhibitory activity and IC$_{50}$ value of active fractions were determined by

using a UV spectrophotometer (UV-1800, SHIMADZU, Kyoto, Japan) followed with the description in 2.2.6.

3.2.4 Peptide sequence identification

The LC-MS and LC-MS/MS analysis were performed using a Waters ACQUITY UPLC system coupled to a TQ detector (Waters, Milford, MA, USA). The purified peptides (0.5 mg/ml) were dissolved in 80% acetonitrile containing 0.1% (v/v) formic acid, and then ten microliters of the sample filtered through a 0.2 μm filter membrane was automatically injected to the UPLC system, equipped with an TSK gel Amide-80 column (3 μm, 2.0 mm ID×15 cm; TOSOH, Japan). The eluent A was MilliQ water, and the eluent B was acetonitrile. The flow rate of elution was 0.2 ml/min and the gradient consisted of 90% B for 5 min, followed by a linear decrease to 50% B in 20 min. The UPLC eluent was directly injected into the TQ detector which was equipped with an electrospray ionization (ESI) sourse.

MS spectra were recorded in the positive mode using the full scan method firstly from 100 to 2000 m/z. The molecular weight of peptides were detected by a charged $[M+H]^+$ state analysis in the mass spectrum. The capillary voltage was 3.0 kV and the sample cone voltage was 30 V for detection. The source temperature was 120 °C and the desolvation temperature was 350 °C. The desolvation and cone gas flow rates were set to 600 and 50 L/hr, respectively. All mass spectra were acquired in centroid mode. The peptide sequence was measured from the collision-induced dissociation (CID) spectrum of the protonated analyse $[M+H]^+$ by tandem MS experiments. The daughters mode was used for operation with the collision energy ramp from 60 to 80 V. The amino acid sequence of peptides were performed using MassLynx software version 4.1 and confirmed by manual validation.

3.3 Results

3.3.1 Purification of antibacterial peptide

Enzymatic hydrolysis of kuruma shrimp head wastes generates a complex mixture of active and inactive peptides with different molecular weight and amino acid sequence. To purified the antibacterial peptides, KSHPH was separated by two stage of RP-HPLC. After separated by first stage of RP-HPLC, KSHPH was fractionated into eleven major fractions. The elution profile is shown in Fig. 3-1. Fractions associated with each peak have been collected separately, concentrated by lyophilization and then evaluated for antibacterial activity. As shown in Fig. 3-2 and Table 3-1, six peptide fractions (F_1; F_2; F_3; F_5; F_8 and F_9) exhibited antibacterial activity against the tested bacterial species, but only F_9 fraction showed antibacterial activity against all the tested strains. Meanwhile, the F_9 fraction showed the highest level of activity among the active fractions and was named KSHPH-F_9. For the five bacterial strains, *S. aureus*, *M. luteus* and *L. anguillarum* were noted to show the higher rates of sensitivity to the KSHPH-F_9, respectively.

The KSHPH-F_9 peptide fraction showed antibacterial activity against Gram-positive and Gram-negative bacteria. The MIC was defined as the lowest peptide concentration that completed inhibited the strain growth after 18 h of incubation. The KSHPH-F_9 was found to inhibit the growth of *S. aureus*, *M. luteus*, *S. putrefaciens*, *L. anguillarum* and *E. coli* and the MIC values are shown in Table 3-2. The peptide fraction displayed significantly higher antibacterial activity against *L. anguillarum*, *S. aureus* and *M. luteus* with MIC values equal to 4.17 mg/ml, 5.00 mg/ml, and 5.00 mg/ml, respectively. In contrast, the lowest inhibition was noticed against *E. coli* with MIC values of 16.67 mg/ml. The results showed that the KSHPH-F_9 displayed the wide range of antibacterial activity with MIC values ranging between 4.17 mg/ml to 16.67 mg/ml.

Further purification of KSHPH-F_9 was conducted with second stage of RP-HPLC

to give for five major fractions (F$_{9\text{-}I}$ –F$_{9\text{-}V}$) (Fig. 3-3). Each fraction was collected and subsequently assayed for the antibacterial activity against *S. aureus*, *M. luteus*, *S. putrefaciens*, and *E. coli*. As illustrated in Fig. 3-4 and Table 3-3, the antibacterial activity was observed only in two fractions (F$_{9\text{-}I}$ and F$_{9\text{-}II}$) and was absent in other fractions. Among them, F$_{9\text{-}I}$ fraction showed the strongest antibacterial activity against all the tested strains and has been called KSHPH-F$_{9\text{-}I}$. For the four bacterial strains, same with the previous results, *S. aureus*, and *M. luteus* were noted to show the highest rates of sensitivity to the KSHPH-F$_{9\text{-}I}$.

Meanwhile, the antibacterial efficacy of KSHPH-F$_{9\text{-}I}$ was expressed as MIC and the results are shown in Table 3-4. The KSHPH-F$_{9\text{-}I}$ was found to inhibit the growth of *S. aureus*, *M. luteus*, *S. putrefaciens* and *E. coli* with different degree of inhibition. The peptide fraction displayed significantly higher antibacterial activity against *S. aureus* and *M. luteus* with MIC values equal to 0.67 mg/ml, and 0.83 mg/ml, respectively. In contrast, the KSHPH-F$_{9\text{-}I}$ showed lower sensitivity against *S. putrefaciens* and *E. coli* with the MIC values of 1.00 mg/ml and 3.33 mg/ml, respectively. Thus, after the second stage of RP-HPLC purification, the MIC values of KSHPH-F$_{9\text{-}I}$ was about 5–7 times higher than KSHPH-F$_9$.

3.3.2 Purification of ACE inhibitory peptide

The kuruma shrimp head hydrolysate, obtained by treatment with papain, which displayed the highest ACE inhibitory activity, was separated on first stage of RP-HPLC using TSKgel ODS-80TM column. As reported in Fig. 3-1, there are eleven major absorbance peaks (F$_1$–F$_{11}$) were mentioned at 220 nm. As shown in Fig. 3-5, ACE inhibitory activity at 8 mg/ml was widely observed in all fractions, and the F$_9$ fraction exhibited the highest inhibitory activity (87.42%) with an IC$_{50}$ value of 0.97 ± 0.04 mg/ml. Thus, the KSHPH-F$_9$ has both the strongest antibacterial activity and highest ACE inhibitory activity among all the fractions. Moreover, the F$_9$ fraction was eluted at 100% acetonitrile, which indicates that the potential peptides have a high

hydrophobicity in their amino acid sequence. The KSHPH-F_9 was then further isolation using Mightysil RP-18 column and fractionated into five major fractions (F_{9-I} –F_{9-V}) (Fig. 3-3). Among those fractions, F_{9-III} exhibited the strongest ACE inhibitory activity at 0.5 mg/ml (an IC_{50} value of 0.045 ± 0.005 mg/ml) and named as KSHPH-F_{9-III} (Fig. 3-6). Concrete results obtained during purification steps are summarized in Table 3-5. The KSHPH-F_{9-III} was purified 42.22-fold from KSHPH using two stages of RP-HPLC purification.

3.3.3 Peptide sequence identification

Peptides in fraction KSHPH-F_{9-I} and fraction KSHPH-F_{9-III} were determined by ESI-MS for molecular mass and ESI-MS/MS for amino acid sequence. According to the result of peak purity determination (Fig. 3-7), the purity of KSHPH-F_{9-I} and KSHPH-F_{9-III} was more than 98% (data not shown). The low impurity content of fractions indicated that these purified fractions were suitable for LC-MS identification.

The MS and MS/MS spectrum charts of target fraction were shown in Figs. 3-8 and 3-9, respectively. The accurate relative molecular mass of the KSHPH-F_{9-I}, deduced from the m/z value of $[M+H]^+$ by subtraction of one mass unit for the attached proton, is 414.47 Da (Fig. 3-8A). Similarly, It can be calculated that the precise relative molecular mass of KSHPH-F_{9-III} is 358.33 Da (Fig. 3-9A).

According to the results of molecular mass and tandem MS spectra shown in Fig. 3-8B, the amino acid sequence of peptide (KSHPH-F_{9-I}) identified is Val-Thr-Val-Pro. Based on the results of Table 3-4, the peptide Val-Thr-Val-Pro exhibited antibacterial activity, with the MIC values between 1.62 mM and 8.03 mM (data was obtained from the molecular mass of VTVP and the Table 3-4) against the test bacteria. For the selected microorganisms, the antibacterial activity of peptide Val-Thr-Val-Pro was more pronounced against Gram-positive bacteria than Gram-negative bacteria. Likewise, peptide sequence of KSHPH-F_{9-III} was identified as Ala-Arg-Leu/Ile (Fig. 3-9B). This peptide fraction exhibited the highest ACE inhibitory activity with the IC_{50}

value of 125.58 µM (data was obtained from the molecular mass of ARL/I and the Table 3-5). Since the Leu and Ile share exactly the same molecular weight, the precise amino acid sequence of KSHPH-F$_{9\text{-III}}$ can not be determined by MS/MS spectrum alone. In order to clarify the peptide sequence of KSHPH-F$_{9\text{-III}}$, further study is needed.

3.4 Discussion

To purify the active antibacterial and ACE inhibitory peptides, KSHPH has been fractionated sequentially using two stage of RP-HPLC. Finally, the fraction KSHPH-F$_{9\text{-I}}$ with strongest antibacterial activity and the fraction KSHPH-F$_{9\text{-III}}$ with highest ACE inhibitory activity were isolated from KSHPH, and then the peptide sequences of these fractions were identified using ESI-MS/MS.

Fraction KSHPH-F$_{9\text{-I}}$ was identified that composed of one pure peptide with the molecular mass of 414.47 Da, corresponding to the peptide sequence of Val-Thr-Val-Pro (VTVP). The antibacterial peptide sequence VTVP has not been reported in previous study or related websites. The antibacterial activity of peptides largely dependent on several factors, including the molecular mass, structure diversity (Shai, 2002), hydrophobicity (Kustanovich et al., 2002) and specific-amino acid composition, such as proline, histidine, arginine, cysteines and glycine (Andreu & Rivas, 1998). The antibacterial peptide (VTVP) in present study was a tetrapeptide and characterized by a hydrophobic ratio of 50%. Up to now, numbers of antibacterial peptides have been determined to be short peptides (2–20 amino acids), of which nearly 50% are hydrophobic residues (Abdel-Hamid et al., 2016; Kobbi et al., 2015). It was reported that two major peptides (414.05 and 456.06 Da mass), with high antibacterial activity, were detected in purified fraction of papain hydrolysed camel whey (Abdel-Hamid et al., 2016). A new antibacterial peptide, Dromidin, resulted in the value 513.0 Da was isolated and identified from the hemolymph of the crab *Dromia dehaani* (Anbuchezian et al., 2018). In addition, several short peptides were also identified from various

origins, including the green juice alfalfa (Kobbi et al., 2015), the barbel muscle (Sila et al., 2014b), the whey protein (Théolier et al., 2013) and so on. Furthermore, it has been reported that the hydrophobicity of antibacterial peptides showed important effect to enable peptide to penetrate bacterial cells and induce membrane lysis, and the overly increase ratio of hydrophobicity of antibacterial peptide sequence correlates with its low selectivity and toxicity toward mammalian cells (Aoki & Ueda, 2013; Kang et al., 2017). The hydrophobicity of antibacterial peptides is a modulator of cell membrane activity and excessively strong hydrophobicity would hold the peptide back to transport to the target microorganism (Li et al., 2013). The short peptide VTVP exhibited the widest range of antibacterial activity with MIC values ranging between 1.62 and 8.03 mM. These results were in agreement with the previous findings reported by Bougherra et al. (2017) and Kobbi et al. (2015), which showed that the active peptide is a short peptide and does not have the classical characteristic of the antibacterial peptides. Generally, the classical antibacterial peptides have a net positive charge that enable them to absorb by electrostatic interaction on the negatively charged bacterial membranes (Kang et al., 2017). In addition, the antibacterial peptides show various degree of antibacterial activity based on their secondary structure, with the α–helix, β–sheet or a mixture of both are often more active (Brogden, 2005). Certainly, some peptides without aforementioned secondary structures have also strong antibacterial activity. However, the peptide VTVP has no net charge, and it's too short to present a ordered secondary structure (random coil structure), which may account for the moderate antibacterial activity of it. The antibacterial peptides with a random coil structure can be structured in aqueous solution when they are in contact with biological membrane (Powers & Hancock, 2003). However, the mechanism of action of antibacterial peptide needs further study.

The amino acid sequence of KSHPH-F$_{9-III}$ was identified as Ala-Arg-Leu/Ile (ARL/I), with molecular mass of 358.33 Da. The peptide sequence ARL/I was partial similar to some ACE inhibitory peptide sequences, which have been reported in other protein origins. The identified ACE inhibitory peptide Ala-Phe-Leu (IC$_{50}$ of 65.2 μM)

has been previously separated from the enzymatic hydrolysate of marine shrimp, *Acetes chinensis* (Hai-Lun et al., 2006), Ala-Phe-Leu (IC$_{50}$ of 63.8 µM) and Ala-Glu-Leu (IC$_{50}$ of 57.1 µM) from two microalgae, *Chlorella vulgaris* and *Spirulina platensis* (Suetsuna & Chen, 2001), Ala-Ser-Leu (IC$_{50}$ of 102.15 µM) from the silkworm pupa protein (Wu et al., 2015), Ile-Arg (IC$_{50}$ of 306.4 µM) from marine sponge, *stylotella aurantium* (Ko et al., 2017) and the Ala-Arg (IC$_{50}$ of 570.78 µM) from trevally, *Pseudocaranx sp* (Salampessy et al., 2015). However, the ACE inhibitory tri-peptide, Ala-Arg-Leu/Ile, with the IC$_{50}$ value of 125.58 µM, has not been previously isolated from other sources. In general, the ACE inhibitory activity of peptides is associated with the chain length and the amino acid sequence of peptides. The ACE inhibitory peptides usually contain 2 to 12 amino acids, and a lower molecular weight may contribute to higher ACE inhibitory activity of purified peptides than high molecular weight (Yamamoto et al., 1994; Ko et al., 2012; Wu et al., 2015). In addition, the peptides are more likely to be potential therapeutic substitutes for treatment of hypertension, since the short-chain peptides, especially di- and tri-peptides, are more easily absorbed in the gastrointestinal tract (Matthews & Adibi, 1976). The peptide Ala-Arg-Leu/Ile had a hydrophobic ratio of 66.67%. The hydrophobicity is another pivotal factor to promote the ACE inhibitory activity of peptides. The highly hydrophilic property could made the peptide inaccessible to the active site of ACE, since the hydrophilic-hydrophobic balance is a vital factor in biologically active moleculars (Kohmura et al., 1989). Wu et al. (2006a) studied the quantitative structure-activity relationships of ACE inhibitory peptides composing of 168 dipeptides and 140 tripeptides based on a database. The results indicated that the most favorable tripeptides residues, were the aromatic amino acids in the carboxyl terminus, while the positively charged amino acids were preferred for the middle position, and the hydrophobic amino acids were preferred for the amino terminus (Wu et al., 2006a). In the present study, the peptide ARL/I did have the Arg residue, a positively charged amino acid located in the middle position, and the Ala residues, a hydrophobic amino acid located in the amino terminus. The carboxyl terminal amino

acid of peptide ARL/I is Leucine or Isoleucine, which is a hydrophobic amino acid. Actually, the hydrophobic amino acid residues at the amino or carboxyl terminus were also essential to form the ACE inhibitory peptides (Asoodeh et al., 2016; Gómez-Ruiz et al., 2007). Hence, the peptide Ala-Arg-Leu/Ile, has a hydrophobic amino acid (Leu/Ile) at the carboxy terminal, which may also contribute to the ACE inhibitory activity of peptide, significantly. Numbers of ACE inhibitory peptides have been reported with Leu/Ile at their carboxyl terminus, such as Leu-Leu (Pan et al., 2012), Leu-Gly-Ile (Li et al., 2002), Val-Tyr-Ile (Matsufuji et al., 1995), Cys-Val-Leu, Phe-Cys-Val-Leu (Hai-Lun et al., 2006). Certainly, further study on the relationship between the structure and function of ACE inhibitory peptide would be needed to analysis and understand the effects of ACE inhibitory peptides more deeply.

Conclusively, the novel antibacterial peptide (VTVP) and the novel ACE-inhibitory peptide (ARL/I) were purified from the KSHPH using two stage of RP-HPLC. These peptides may have potential uses in food or health-related applications. Therefore, the information on their structural and hemolytic properties are necessary. In next chapter, 1) to predict and analysis the relationship between peptide structure and activity, the 3D model of KSHPH-F_{9-I} and molecular docking simulation of KSHPH-F_{9-III} were studied, and then to confirm the drug safety, 2) the hemolytic activity of all peptides was investigated.
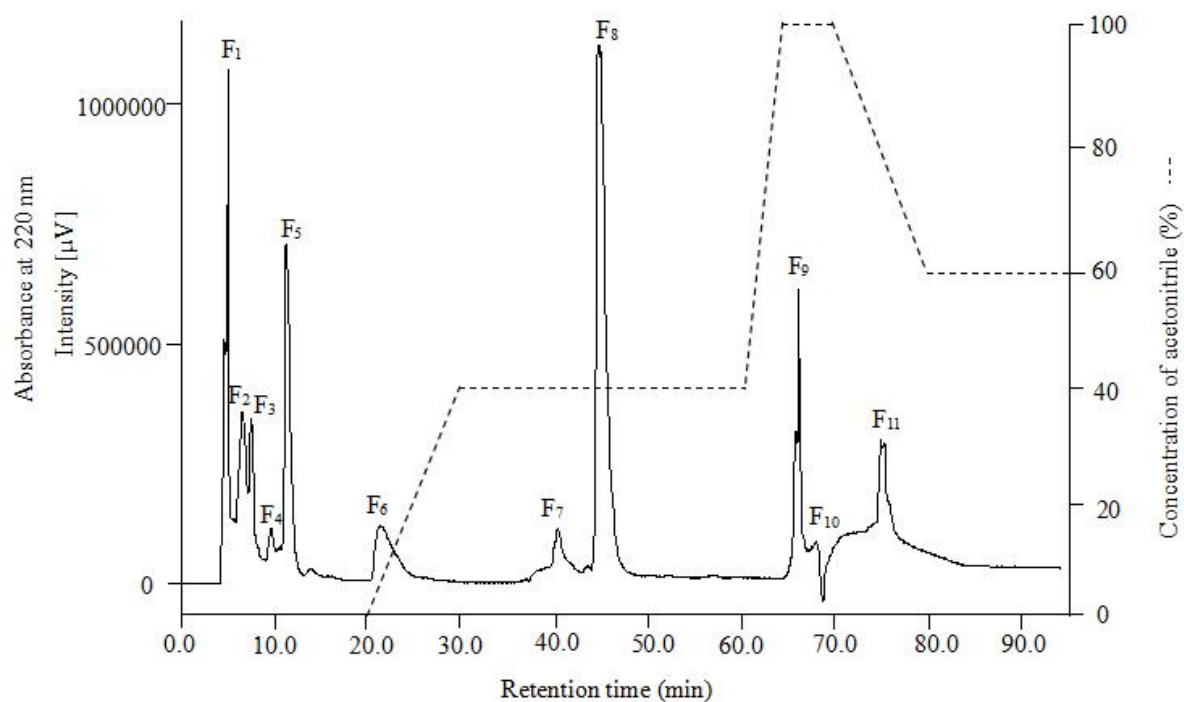
3.5 Figures and Tables

Fig. 3-1 RP-HPLC profile of KSHPH obtained by treatment with papain.

Column: TSKgel ODS - 80TM (φ46×250 mm)

Mobile phase: A: 0.1% TFA in distilled water (v/v)

B: 0.1% TFA in acetonitrile (v/v)

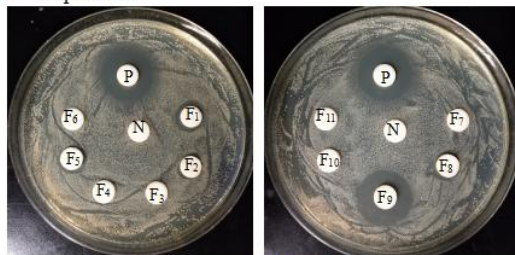Gradient: 0~20 min, 100% A; 20~30 min, 100% A→60% A/40% B; 30~60 min, 60% A/40% B; 60~65 min, 60% A/40% B→100% B; 65~70 min, 100% B; 70~80 min, 100% B→40% A/60% B.
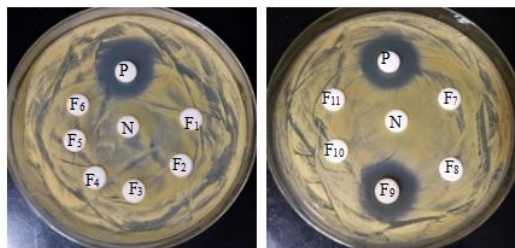
Injection: 20 μl
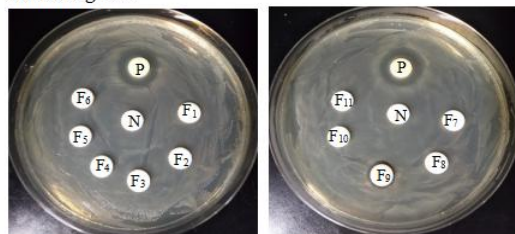
Flow rate: 1.0 ml/min

Detection: Absorbance at 220 nm

Fig. 3-2 Antibacterial activity of peptide frctions collected from first stage of RP-HPLC against *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Shewanella putrefaciens*, and *Listonella anguillarum*. $F_1 \sim F_{11}$:16 mg of dry weight of fractions. P, tetracycline: *S.aureus* NBRC 102135, 10 μg; *M.luteus* NBRC 3066, 10 μg; *E.coli* Y 1090, 375 μg; *S. putrefaciens* IAM 1509, 17.5 μg; *L. anguillarum* NBRC 13266, 7.5 μg. N, sterile saline solution.

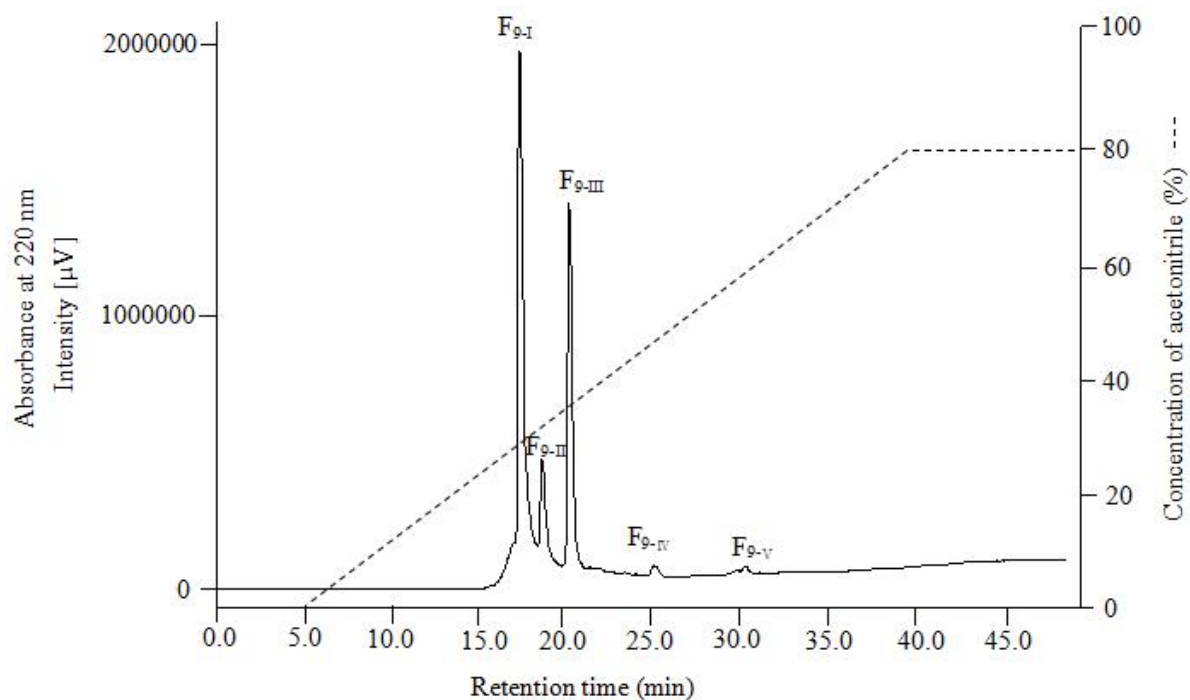Fig. 3-3 RP-HPLC profile of KSHPH-F$_9$ recovered from TSKgel ODS - 80TM column.

       Column: Mightysil RP-18 150-4.6, 5 μm

       Mobile phase: A: 0.1% TFA in distilled water (v/v)

                   B: 0.1% TFA in acetonitrile (v/v)
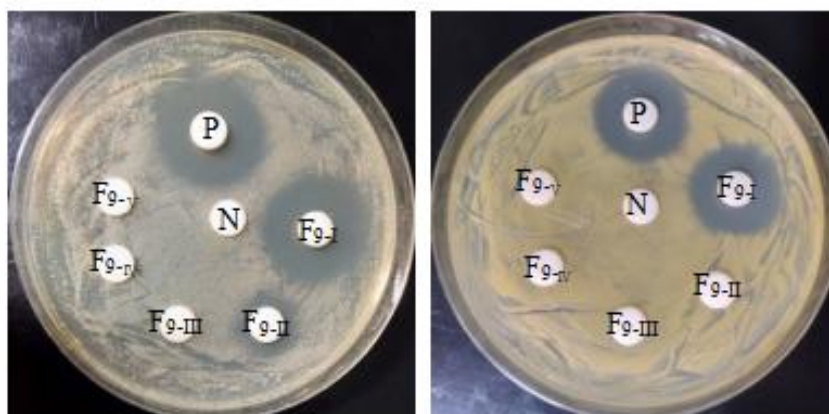
       Gradient: 0~5 min, 100% A; 5~40 min, 100% A→20% A/80% B; 40~50 min, 20% A/80% B.

       Injection: 20 μl

       Flow rate: 0.5 ml/min

       Detection: Absorbance at 220 nm

Gram-positive

S. aureus NBRC 102135   *M. luteus* NBRC 3066

Gram-negative
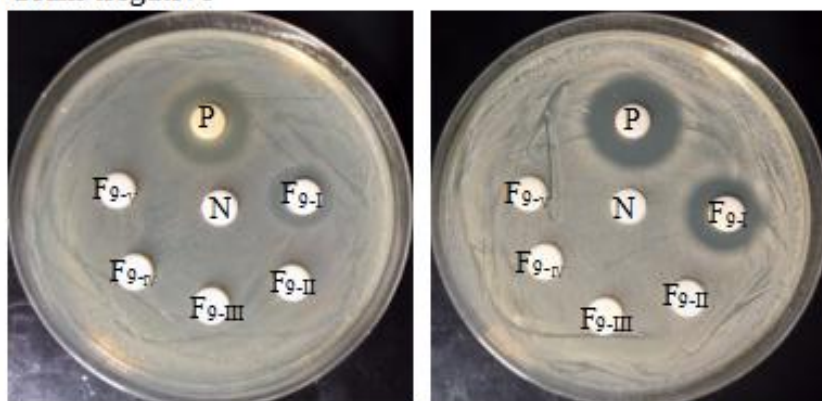
E. coli Y 1090   *S. putrefaciens* IAM 1509

Fig. 3-4 Antibacterial activity of peptide fractions collected from second stage of RP-HPLC against *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, and *Shewanella putrefaciens*. $F_{9-I} - F_{9-V}$: 2.4 mg of dry weight of fractions. P, tetracycline: *S. aureus* NBRC 102135, 10 μg; *M. luteus* NBRC 3066, 10 μg; *E. coli* Y 1090, 375 μg; *S. putrefaciens* IAM 1509, 17.5 μg. N, sterile saline solution.
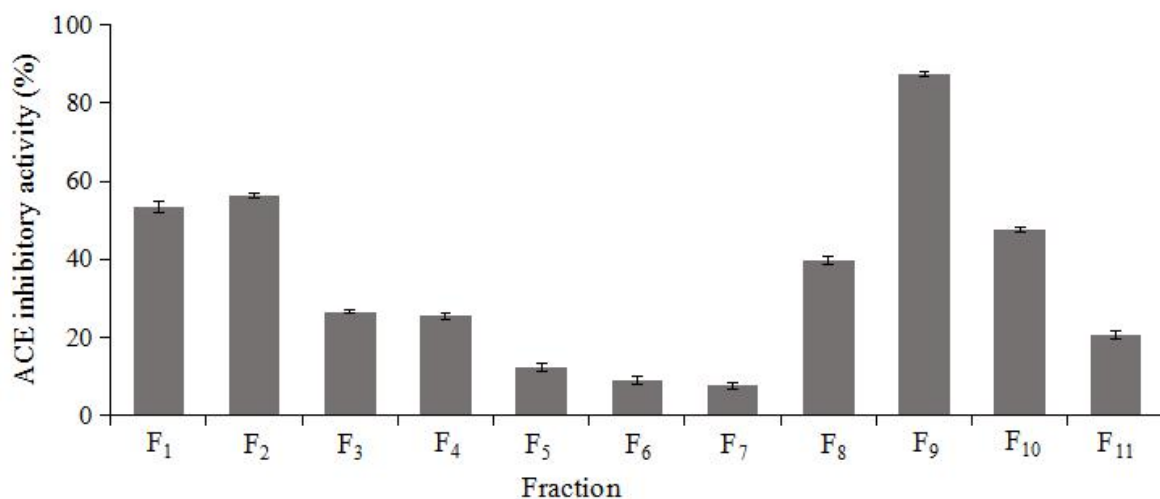
Fig. 3-5 ACE inhibitory activity of peptide frctions collected from first stage of RP-HPLC. ACE inhibitory activity of each fraction ($F_1 - F_{11}$) was at the test concentration of 8 mg/ml.
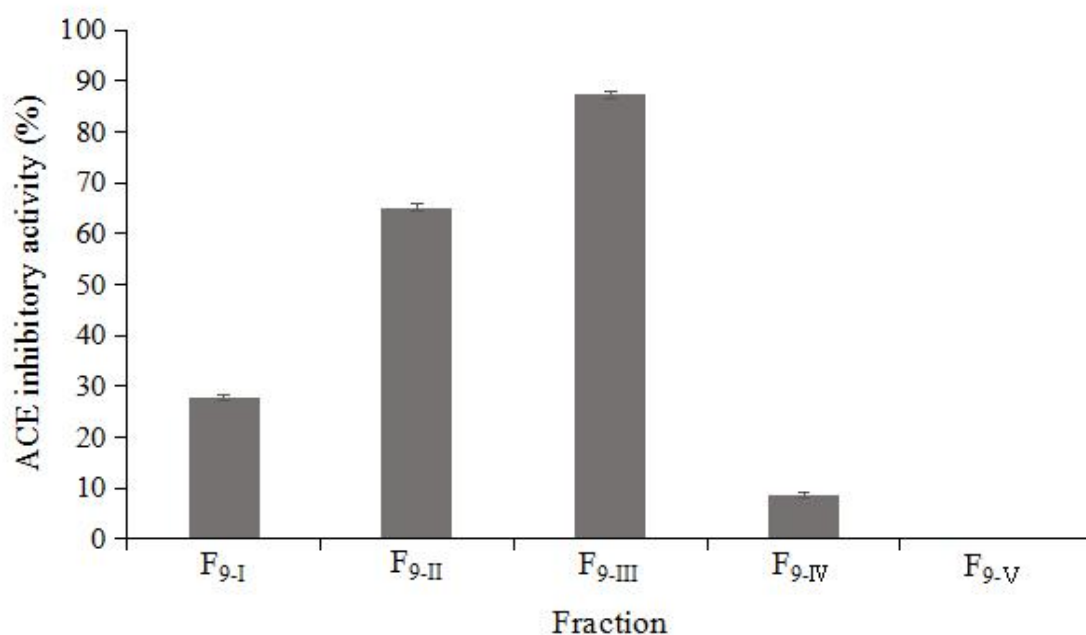
Fig. 3-6 ACE inhibitory activity of peptide fractions collected from second stage of RP-HPLC. ACE inhibitory activity of each fraction ($F_{9-I} - F_{9-V}$) was at the test concentration of 0.5 mg/ml.
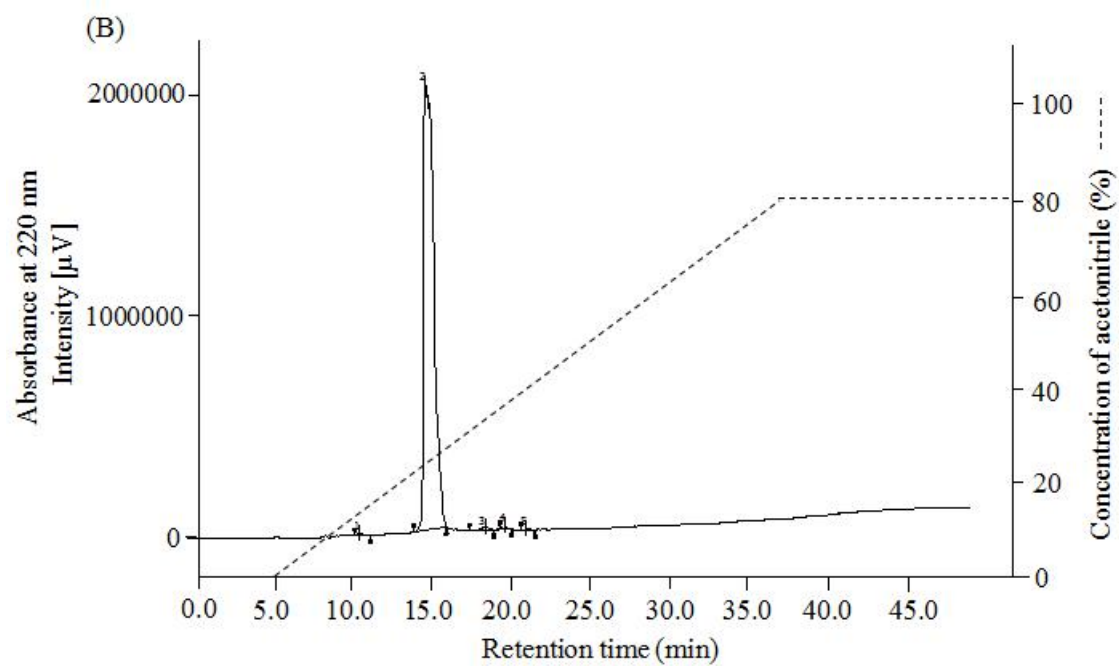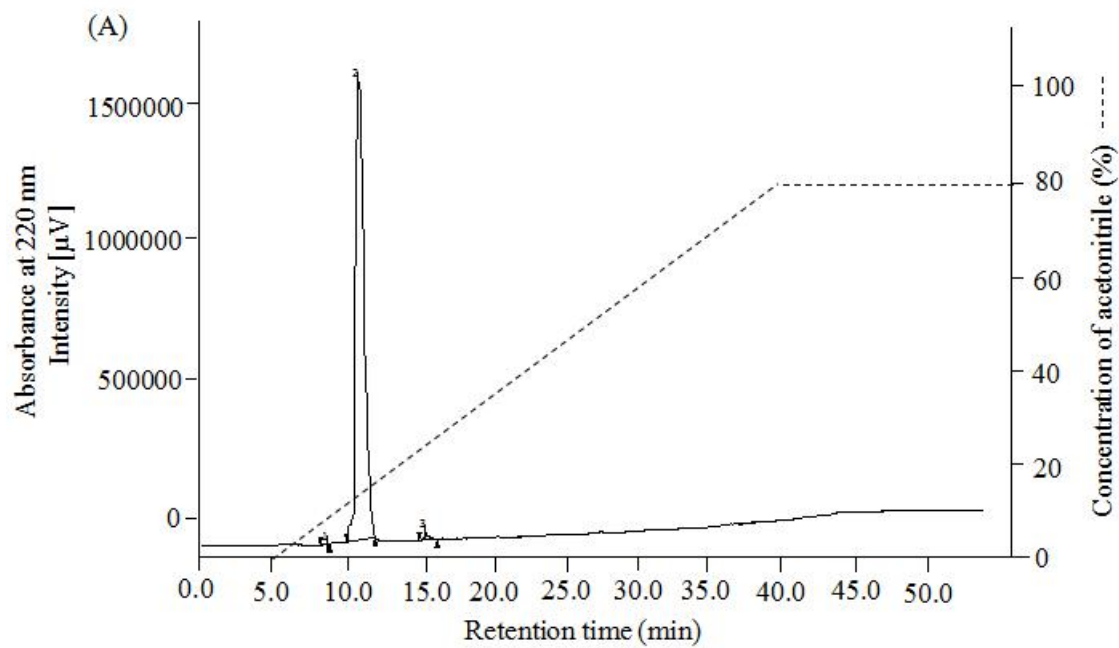
Fig. 3-7 RP-HPLC profile of KSHPH-F$_{9\text{-I}}$ (A) and KSHPH-F$_{9\text{-III}}$ (B) on Mightysil RP-18 column.
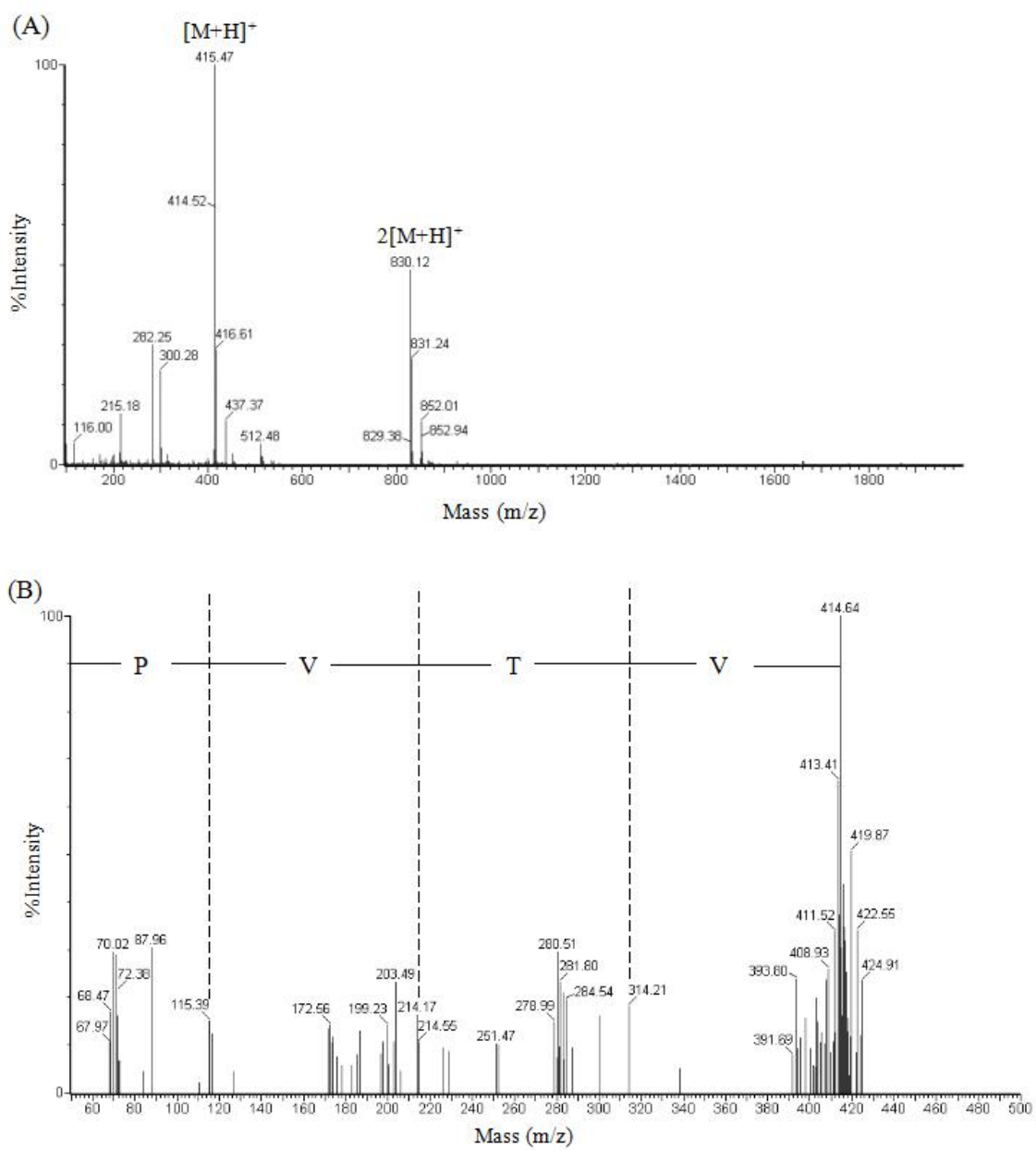
Fig. 3-8 Identification of the molecular mass and amino acid sequence of the KSHPH-F$_{9-I}$. (A) ESI-MS spectrum. (B) ESI-MS/MS spectrum of m/z 415.47.
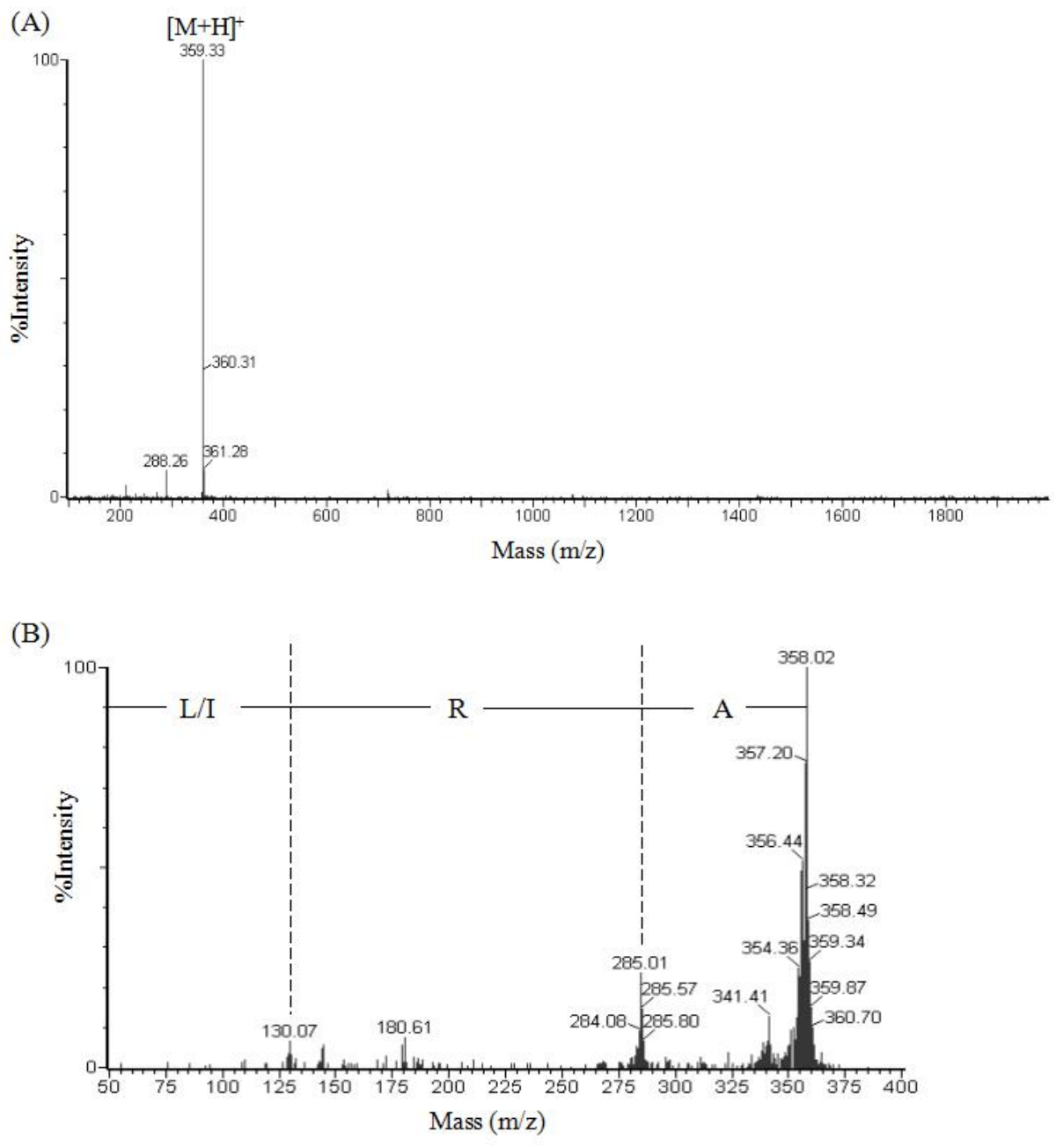
Fig. 3-9 Identification of the molecular mass and amino acid sequence of the KSHPH-F$_{9-III}$. (A) ESI-MS spectrum. (B) ESI-MS/MS spectrum of peak m/z 359.33.

Table 3-1 Antibacterial activity of peptide fractions collected from KSHPH purified by first stage of RP-HPLC

Bacterial species	RP-HPLC fractions										
	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}	F_{11}
S. aureus NBRC 102135	++	+	−	−	++	−	−	+	++++	−	−
M. luteus NBRC 3066	−	−	−	−	−	−	−	−	++++	−	−
E.coli Y1090	−	−	−	−	−	−	−	−	+	−	−
S. putrefaciens IAM 1509	+	−	−	−	−	−	−	−	++	−	−
L. anguillarum NBRC 13266	+++	++	++	−	++	−	−	+	++++	−	−

The sample of each fraction was add 16 mg of dry weight on paper disc respectively.

Inhibition zones: ++++: >20 mm; +++: 20~16 mm; ++: 15~11 mm; +: <11mm, and −: No activity.

Table 3-2 MIC values of RP-HPLC fraction (KSHPH-F$_9$) against bacteria strains

	Bacterial species	MIC values (mg/ml)	
		KSHPH-F$_9$	Tetracycline
Gram (＋)	*S. aureus* NBRC 102135	5.00	0.0017
	M. luteus NBRC 3066	5.00	0.0017
Gram (－)	*E. coli* Y1090	16.67	0.3333
	S. putrefaciens IAM 1509	6.67	0.0025
	L. anguillarum NBRC 13266	4.17	0.0013

Initial concentration of KSHPH-F$_9$ (mg/ml): *S.aureus* NBRC 102135, *M.luteus* NBRC 3066, *E.coli* Y 1090, *S. putrefaciens* IAM 1509, *L. anguillarum* NBRC 13266, 80 mg/ml.

Initial concentration of tetracycline (mg/ml): *S.aureus* NBRC 102135, *M.luteus* NBRC 3066, *S. putrefaciens* IAM 1509, *L. anguillarum* NBRC 13266, 0.32 mg/ml; *E.coli* Y 1090, 1.6 mg/ml.

Table 3-3 Antibacterial activity of peptide fractions collected from KSHPH-F$_9$ purified by second stage of RP-HPLC

Bacterial species	RP-HPLC fractions				
	F$_{9\text{-I}}$	F$_{9\text{-II}}$	F$_{9\text{-III}}$	F$_{9\text{-IV}}$	F$_{9\text{-V}}$
S. aureus NBRC 102135	++++	++	—	—	—
M. luteus NBRC 3066	++++	—	—	—	—
E. coli Y1090	++	—	—	—	—
S. putrefaciens IAM 1509	+++	—	—	—	—

The sample of each fraction was add 2.4 mg of dry weight on paper disc respectively.

Inhibition zones: ++++: >20 mm; +++: 20~16 mm; ++: 15~11 mm; +: <11mm, and —: No activity.

Table 3-4 MIC values of RP-HPLC fraction (KSHPH-F$_{9\text{-I}}$) against bacteria strains

	Bacterial species	MIC values (mg/ml)	
		KSHPH-F$_{9\text{-I}}$	Tetracycline
Gram (+)	*S. aureus* NBRC 102135	0.67	0.0017
	M. luteus NBRC 3066	0.83	0.0017
Gram (−)	*E. coli* Y1090	3.33	0.3333
	S. putrefaciens IAM 1509	1.00	0.0025

Initial concentration of KSHPH-F$_{9\text{-I}}$ (mg/ml): *S. aureus* NBRC 102135, *M. luteus* NBRC 3066, *E. coli* Y 1090, *S. putrefaciens* IAM 1509, 8 mg/ml.

Initial concentration of tetracycline (mg/ml): *S. aureus* NBRC 102135, *M. luteus* NBRC 3066, *S. putrefaciens* IAM 1509, 0.32 mg/ml; *E. coli* Y 1090, 1.6 mg/ml.

Table 3-5 Purification of ACE inhibitory peptide of shrimp head hydrolysate from *M. japonicus*.

Purification step	IC$_{50}$ value (mg/ml)[a,b]	Purification fold
KSHPH	1.90 ± 0.03	1.00
KSHPH-F$_9$	0.97 ± 0.04	1.96
KSHPH-F$_{9\text{-III}}$	0.045 ± 0.005	42.22

[a] IC$_{50}$ values was defined as the concentration of inhibitor required to inhibit 50% of ACE activity.

[b] Values are presented as mean ± SD (n=3).

Chapter 4 Structural and hemolytic properties of antibacterial and ACE inhibitory peptides

4.1 Introduction

The emergence of multidrug-resistant bacteria currently represents one of the tough challenge of human health and the medical community. Thus, there is urgent need to develop novel antibacterial agents with different modes of action than the conventionally used antibiotics, which target specific proteins, eukaryotic peptides act on bacterial membranes and other generalized targets, such as DNA and RNA (Da Costa et al., 2015). Traditionally, the search for novel antibacterial peptides involved the preparation, purification and identification of active peptides from various natural sources. The specificity and bioactivity of antibacterial peptides largely depend on the amino acid sequence and structure, as well as some physicochemical properties, such as net charge, the degree of helicity, hydrophobicity and amphipathicity (Ahmed & Hammami, 2019). Therefore, the structural simulation and analysis of physicochemical properties of antibacterial peptides are critical for effective explanation of the structure-activity relationship of peptides.

The human body contains two isoforms of ACE: testis ACE (tACE) and somatic ACE (sACE). Both of them are transcribed from the same gene in a tissue-specific manner. sACE consists of two homologous domains (N and C domain). There is an evidence showing that N domain has no effect on blood pressure, whereas C domain is the dominant angiotensin-converting site (Fan et al., 2019; Natesh et al., 2003). tACE has only one domain that identical to the C domain of sACE, except for a unique 36-residue sequence at its N-terminus (Fan et al., 2019). The ACE inhibitor can get into the inside of ACE and form a stable complex with ACE, preventing other substrates contact with the active site of ACE. In ACE, each homologous domain has a zinc-binding motif HEXXH (active site), which form a ligand with two histidines (His383 and His387), a downstream glutamate (Glu411), and an acetate ion (from the

crystallization medium) (Natesh et al., 2003). The ACE inhibitor can interact with the ACE by binding peptide residues to ACE active sites, through hydrogen bonds and hydrophobic interactions; besides, making a direct interaction with zinc binding ligand (Fan et al., 2019; Pina & Roque, 2009). Peptide size and sequence are two key points that determine the potency, mode of action, and bioavailability of ACE inhibitory peptides. Generally, compared with large peptides, short peptides show higher inhibitory effect, since the binding channel of ACE is too narrow to accomodate the large ones (Natesh et al., 2003). Although the ACE inhibitory effect of purified peptides has been demonstrated in previous study, the exact mode of interaction and the molecular mechanisms between ACE inhibitory peptides and ACE structure remains unclear.

In this chapter, the study was aimed to illuminate: 1) structural characterization of antibacterial peptide by software analysis and 3D model prediction, 2) ACE inhibition pattern and interaction mode of ACE inhibitory peptide to ACE by using molecular docking simulation, and 3) hemolytic activity of active peptides.

4.2 Materials and Methods

4.2.1 Structural characterization of antibacterial peptide

The physicochemical properties of peptides including theoretical mass, net charge, GRAVY (Grand average of hydropathicity) and extinction coefficient were calculated using a peptide property calculator (http://www.novoprolabs.com/tools/calc_peptide_ property). The structural characteristic of peptide was deduced by a mathematical model of the Network Protein Sequence analysis Internet server of the Pole Bio-informatique Lyonnais (http://pbil.ibcp.fr). Meanwhile, The minimum energy 3D structures of identified peptides were modeled by the Hyperchem 8.0 software. The steepest descent algorithm was used to minimize the energy of peptide.

4.2.2 Determination of ACE inhibition pattern

To clarify the ACE inhibition pattern of purified peptide (KSHPH-F$_{9\text{-III}}$), the assay was conducted with various concentrations of HHL substrate (4, 2, 1, and 0.5 mM) in the absence and presence of different concentrations of the peptide inhibitor (40 and 80 µg/ml) according to the method of Barbana & Boye (2011). Lineweaver-Burk plots of 1/absorbance versus 1/HHL were used to determine the type of enzyme inhibition. Additionally, the inhibition constant Ki of ACE inhibitory peptide was investigated by Dixon plot.

4.2.3 Molecular docking simulation

To elucidate the inhibitory mechanism of peptide to ACE, the molecular docking of ARL/I with ACE was studied. The crystal structure of ACE used in this research was downloaded from the RCSB PDB Protein Data Bank (http://www.rcsb.org) with the code 1O86.pdb (ACE-lisinopril complex), which represents the human tACE in complex with lipinopril at 2 Å resolution. The 3D structure of purified ACE inhibitory peptide was constructed using Hyperchem 8.0 software. The structure was energy minimized using steepest descent algorithm.

The molecular docking was conducted using flexible docking tool of AutoDock 4.2 (TSRI, USA). Before docking, all water molecules and the inhibitor lisinopril were removed, whereas the zinc and chloride atoms were retained in the active site. Then, the polar hydrogens and atom type were added to the ACE model. The docking runs were carried out with a radius of 4 Å, with coordinates x: 40.484, y: 33.632, and z: 47.188. The Lamarckian genetic algorithm (LGA) was used to search the optimal binding sites during docking simulation. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 25,000,000 energy evaluations. The population size was set to 150. Lisinopril was docking as a reference for active sites. The best ranked docking pose of purified peptide in the active site of ACE was obtained according to the scores and binding-energy values.

4.2.4 Hemolytic activity assay

The hemolytic activity of the purified peptide fractions was determined using rabbit red blood cells (Nippon Bio-Supp.center, Tokyo, Japan), as described by Khueychai et al. (2018) and Tan et al. (2013) with some modifications. 5 ml blood cells were centrifuged for 5 min at 2,000 × g to isolate the erythrocytes, washed three times in an isotonic phosphate buffered saline (PBS) solution (pH 7.4) until the buffer turned clear and resuspended in PBS to the initial blood volume. The cell suspensions of 50 µl (final 2% erythrocytes) were mixed with various concentrations of the peptide fraction of 50 µl, and then to give a final volume of 200 µl added with PBS solution. The mixtures were then incubated at 37 ℃ for 30 min. After incubation, the mixtures were centrifuged at 2,000 × g for 5 min, and the supernatant was transferred to a new 96-well plate. Release of hemoglobin was monitored by measurement of absorbance at 540 nm using a Multiskan FC microplates reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). PBS solution and 1% (v/v) Triton X-100 were used as negative and positive controls, respectively. The percentage of hemolysis was calculated as follows: Hemolysis % = (At − Anc) / (Apc − Anc) × 100. Where At, Anc, and Apc represent the absorbance of test, absorbance of negative control and absorbance of positive control, respectively.

The hemolytic properties of materials can be divided into three different categories according to the ASTM standard F 756-00 (2004). The hemolytic activity with a hemolysis percentage over 5%, slight hemolytic activity with a hemolysis ratio between 5 and 2%, and no hemolytic activity with the hemolysis index less than 2%.

4.3 Results

4.3.1 Structural characterization of antibacterial peptide

The physicochemical properties and theoretical structure of peptide VTVP were shown in Fig. 4-1. The theoretical mass of VTVP was 414.49 g/mol, which was

consistent with the molecular mass determined by LC-MS. The peptide VTVP has no net charge in neutral solution. The value of GRAVY was 1.53 > 0, indicating it is a hydrophobic peptide, which possess quite a few hydrophobic residues in sequence. The antibacterial peptide present a random coil structure. As for the 3D model, The theoretical minimum energy state for "VTVP" is Energy = 7.6983 kcal/mol; Gradient = 0.0994.

4.3.2 ACE inhibition pattern of ACE inhibitory peptide

The ACE inhibition pattern of purified peptide Ala-Arg-Leu/Ile explained the characteristics of peptide binding to ACE and inhibit the enzyme activity. As shown in Fig. 4-2A, the line of varying concentrations of purified peptide with intersecting at common intercept on y-axis indicated that the V_{max} (maximum velocity) value of enzyme reaction remain unchanged. However, the K_m (Michaelis-Menten constant) values of reaction increased with the rise of inhibitor concentration. These results indicate that the ARL/I peptide was competitive inhibitor, and the inhibitor constant Ki value was 36.19 ± 0.28 μg/ml (Fig. 4-2B). The competitive inhibitor suggested that the inhibitor can enter the interior of ACE and interact with the ACE active sites and prevent substrate binding.

4.3.3 Molecular mechanism between the ACE inhibitory peptide and ACE

The purified peptide sequence (ARL/I) was used to examine its effect on ACE inhibitory activity by molecular docking analysis and further results obtained from ACE inhibition assay in vitro. The chemical structural formulas of purified peptides were generated using the software Hyperchem 8.0, with the theoretical minimum energy state for "ARL" is Energy = 9.3343 kcal/mol, Gradient = 0.0988; "ARI" is Energy = 10.2867 kcal/mol, Gradient = 0.0994.

The docking study of the tripeptide ARL at the ACE catalytic site in the presence of Zn(II) has shown the best returned pose in Fig. 4-3 with the binding energy value of

–9.22 kJ/mol. As exhibited in Fig. 4-3A and B, the best peptide docking site of ARL was located in the deep narrow channel of the ACE active site. The best pose of enzyme-inhibitor complex was stabilized by hydrogen bonds, and hydrophobic, hydrophilic and electrostatic interactions (Pan et al., 2011; Wu et al., 2015). The hydrogen bonds interaction force was particularly important for docking complex among these forces (Wu et al., 2015). Details of peptide ARL interaction with ACE residues after docking are shown in Fig. 4-3C. The values of the hydrogen bond parameters of the best pose (ARL) are exhibited in Table 4-1. The carbonyl group of the C-terminal leucine residue established hydrogen bonds with the Lys511, the amide group of Gln281, and the –OH from Tyr520. The carbonyl group of the first peptide bond between alanine and arginine established hydrogen bond interaction with the residue Tyr523 and His383, and the amine group of the first peptide bond established hydrogen bond interaction with the residue Glu384. An additional hydrogen bond was observed in the carbonyl group of the second peptide bond between arginine and leucine residues and the aromatic residue His513. The amine group of the N-terminal alanine residue interacts with the residues Ala354 and Glu384 via hydrogen bonding. In addition, the Zinc (II) ion is also an critical component in ACE catalysis and is partly responsible for the binding strength between ACE and their inhibitors (Pan et al., 2011; Pina & Roque, 2009). In the original ACE configuration, the Zinc ion tetra-coordinated with the three residues (His383, His387 and Glu411) and CH3COOXT (Fig. 4-4). The specific values of bond length are shown in Table 4-2. After molecular docking of ARL, the carbonyl group of the first peptide bond between alanine and arginine residues that replaced the CH3COOXT (Fig. 4-3D). The three residues (His383, His387 and Glu411) and their atoms coordinated with the zinc ion basicly made no changes after ARL docking (Table 4-2).

The best pose for docking simulation of ARI is shown in Figs. 4-5 A and B, with the binding energy value of –9.34 kJ/mol. The small molecular ligand (ARI) successfully enters the deep narrow channel of ACE after docking. Details of peptide ARI interaction with ACE residues after docking are shown in Fig. 4-5C. The

hydrogen bond interactions of enzyme-inhibitor complex of ARI were partly similar to the complex of ARL (Table 4-1), besides, the amine group of the first peptide bond between alanine and arginine established hydrogen bonds with the residues Ala354 and the Glu384. Moreover, an additional hydrogen bond was regarded in the intermediate arginine residue and the residue Glu162. The ARI pose obtained also showed the ability to coordinate with the zinc ion through the carbonyl group of the first peptide bond between alanine and arginine residues (Fig. 4-5D). Together with the three residues (His383, His387 and Glu411) coordinated with zinc ion, the ARI around the zinc ion formed a distorted tetrahedral geometry (Table 4-2).

According to the above results, the binding of peptide ARL/I in the active site of ACE indicated a competitive inhibition characteristic, which is highly agreement with the results of ACE inhibition pattern obtained from the Lineweaver-burk plot.

4.3.4 Hemolytic activity

Since the hemolytic activity of active peptides greatly limits their application, the purified peptide fractions were tested for hemolytic activity. Each peptide was assayed at various concentrations, the highest concentration of samples was corresponding to five times the highest MIC values of antibacterial peptide and the IC_{50} value of ACE inhibitory peptide. When KSHPH-F_{9-I} was incubated with rabbit erythrocytes at concentrations of 1.25-15 mg/ml, all the hemolysis percentages were below 2%, it means no hemolysis was observed (Fig. 4-6). Similarly, the hemolytic effect on KSHPH-F_{9-III} at concentrations of 0.05-0.25 mg/ml (Fig. 4-7). Therefore, these results reveal that these peptides would be no-toxic even if used at high concentrations.

4.4 Discussion

The GRAVY value is defined as the sum of hydrophathy values of all amino acids divided by the protein length, which can be used to measure the hydrophobicity of a specific peptide or protein. The GRAVY value of peptide VTVP was more than zero,

and the hydrophobicity rate was 50%, the results indicated that this peptide shows a reasonable hydrophobic structure. Antibacterial peptides usually have one or more hydrophobic centers, and the mechanism of action of peptide is related to its hydrophobic nature (Pei et al., 2018a). The software analysis indicated that the secondary structure of purified peptide (VTVP) is mainly the random coil conformation. Actually, the random coil is a desirable confirmation that can protect the peptides against adverse conditions in actual environment (Pei et al., 2018b). Numbers of antibacterial peptides with random coil structure in aqueous solution have been reported (Bu et al., 2011; Pei et al., 2018b). The majority of antibacterial peptides are amphipathic having both hydrophilic (binds phospholipids) and hydrophobic (binds lipids) domains, which make a great contribution to peptide binding and inserting to the bacterial membrane and ultimately leads to the bacterial lysis and death (Ahmed & Hammami, 2019). The peptide VTVP was too short to form a typical amphiphilic structure like α-helical peptide, however, the alternating hydrophobic and hydrophilic amino acid sequence of VTVP may also generate a kind of amphiphilic structure that plays a significant role when peptide contact to the cell membrane of bacteria. The alternating tetrapeptide sequence has been reported for the preparation of nano-materials (Cui et al., 2009).

The ACE inhibition pattern of purified peptide elucidates how it binds to ACE and inhibit its activity. The peptides ARL and ARI were competitive inhibitor, which indicated that the inhibitor peptide can competitively bind to the catalytic site of ACE and prevent other substrate binding. Actually, competitive ACE inhibitor peptides were the most widely reported in the world. For example, the peptide YV (Khueychai et al., 2018), YN (Tsai et al., 2008), ASL (Wu et al., 2015), MEVFVP (Ko et al., 2016), GVGSPY (Sasaki et al., 2019), were identified to be competitive inhibitors. Furthermore, some commercial synthetic drugs, such as lisinopril and captopril, also have a competitive property (Lopez & Terrell, 2019; Vidt et al., 1982).

Despite the ACE inhibitory peptides as an alternative for control of hypertension have been extensively studied, the precise molecular mechanisms between active

peptides and ACE site are not fully understood. Therefore, the docking simulation between the purified peptide (ARL/I) and ACE was analyzed using flexible docking tool of AutoDock 4.2 software. Based on ACE's catalytic mechanism and relevant references, three main active site pockets (S1, S1' and S2') of ACE were identified (Fan et al., 2019; Pina & Roque, 2009). S1 pocket includes residues Ala354, Glu384 and Tyr523, while residues Gln281, His353, Lys511, His513 and Tyr520 correspond to the S2' pocket and S1' pocket only contains Glu162 residue (Pina & Roque, 2009). The docking results revealed that peptide ARL established hydrogen bonds with the S1 pocket (Ala354, Glu384 and Tyr523) and S2' pocket (Gln281, Lys511, His513 and Tyr520) of ACE. The peptide ARI established hydrogen bonds with all aforementioned active site residues of ARL, especially, the hydrogen bond with the residue Glu162 is also present in the docking of ARI at the S1' pocket of ACE. The result of docking simulation supports the theory that these three active site pockets of ACE possess different affinity for different amino acid on the substrate. Mostly aromatic amino acids and proline show affinity for S1 pocket also Ala, Val and Leu. Ile is more favorable for S1' active pocket. Pro and Leu in the substrate sequence are most favorable for the S2' pocket with regard to the affinity exerted on the enzyme (Jung et al., 2006). The zinc ion at the ACE active site plays a crucial role for ACE activity. A complete inactivation of the enzyme was reported in studies where mutations of metal-coordinating residues in human sACE were created, providing evidence for the significance of zinc ion (Kim et al., 2003). After molecular docking, it was found that the original group CH3COOXT was substitute by peptide inhibitor, and the initial value of bond length of zinc ion with its surrounding atoms made some changes, and some atoms of peptide (ARL/I) were nearly closed to the zinc ion (Table 4-2). The amine groups of peptides ARL, ARI were closed to the zinc ion and coordinating with zinc ion. Therefore, the ACE lost its activity. These results are to some extent in concordance with previous studies based on the molecular recognition between bioactive peptides and ACE (pan et al., 2011; Pina & Roque, 2009).

The hemolytic ability is one of the important indexes to evaluate the safety of

active peptide. Even at the high concentration, the hemolysis levels of purified peptide are almost close to the basal levels. Generally, the bioactive peptides exhibiting no/low hemolytic activity that regard as having potential to apply in food or health-related industries (Pei et al., 2018b).

In conclusion, the 3D model and physicochemical properties analysis of antibacterial peptide (VTVP) were benefit for further understood the relationship between peptide structure and antibacterial activity. The ACE inhibitory peptide (ARL/I) has been demonstrated to have competitive inhibitory properties. Through analyzing the molecular interaction between ACE and active, the molecular docking simulation further elucidated the relationship between ACE inhibitory activity of peptides and ACE active sites. All active peptides purified in this research had no hemolytic effect. These research results are conducive to the practical application and subsequent development of active peptides.

4.5 Figures and Tables
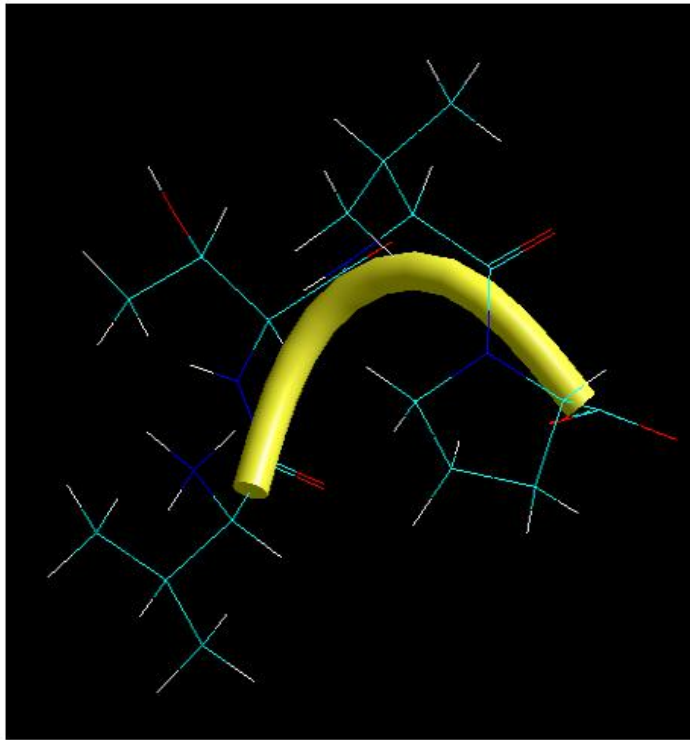
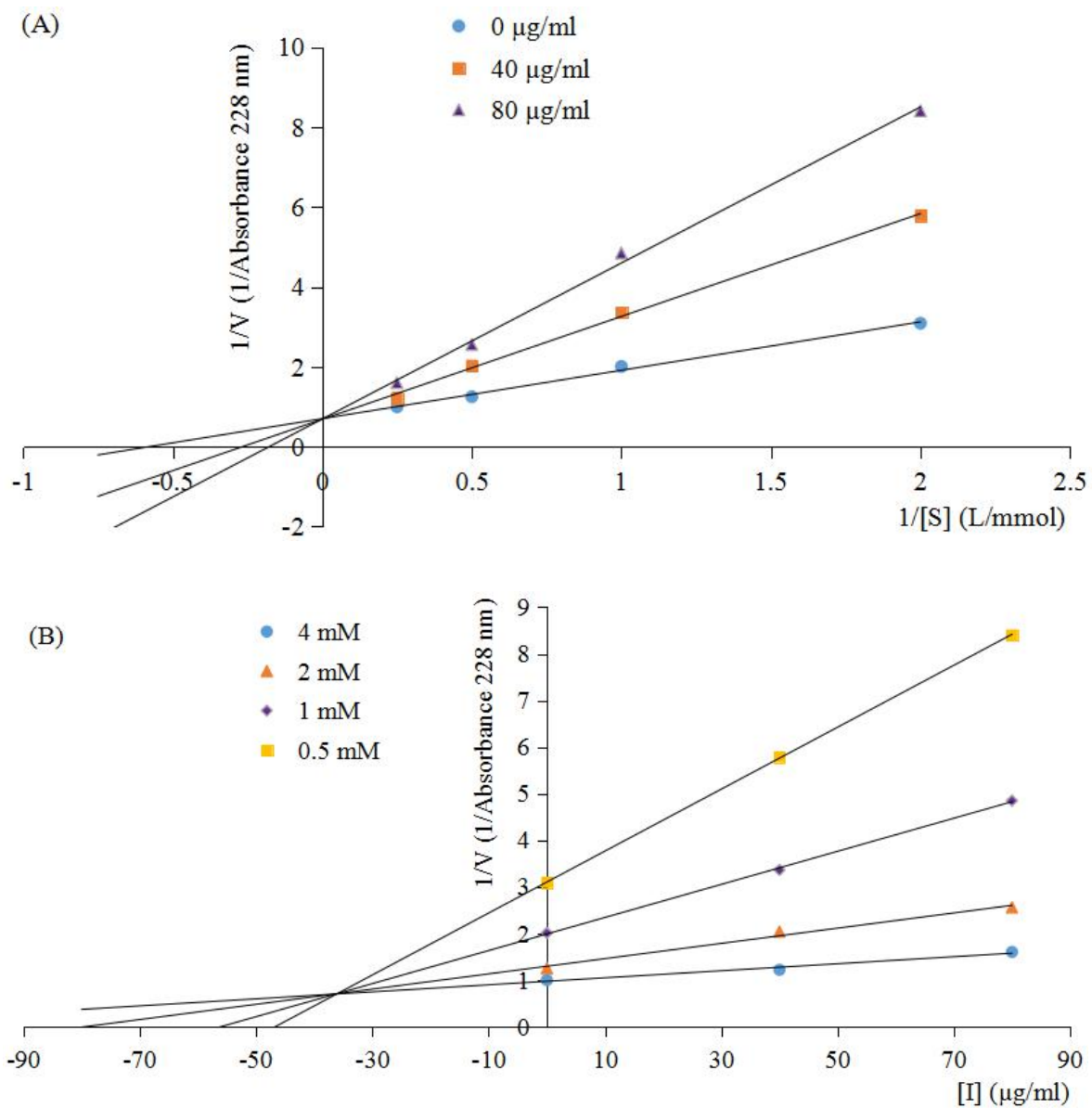Fig. 4-1 Structural characterization information and the theoretical 3D structure of antibacterial peptide "VTVP".

Fig. 4-2 Lineweaver-Burk plot of ACE inhibition of purified peptide (KSHPH-F$_{9\text{-III}}$) at concentrations of 40 and 80 μg/ml (A); the Dixon plot was used to calculate the inhibition constant Ki of peptide (B).

Fig. 4-3 Molecular docking between ACE and ARL. (A) General overview of docking pose of ARL (blue stick mode) at the ACE site. (B) Local overview of docking pose of ARL at the ACE site. (C) The interaction between peptide ARL and ACE. The hydrogen bonds are shown in yellow dashed lines and zinc coordination bonds in oranges dashed lines. (D) ARL zinc coordination at the ACE active site.

Fig. 4-4 Details of the zinc ion tetra-coordinated with the ACE residues before molecular docking.
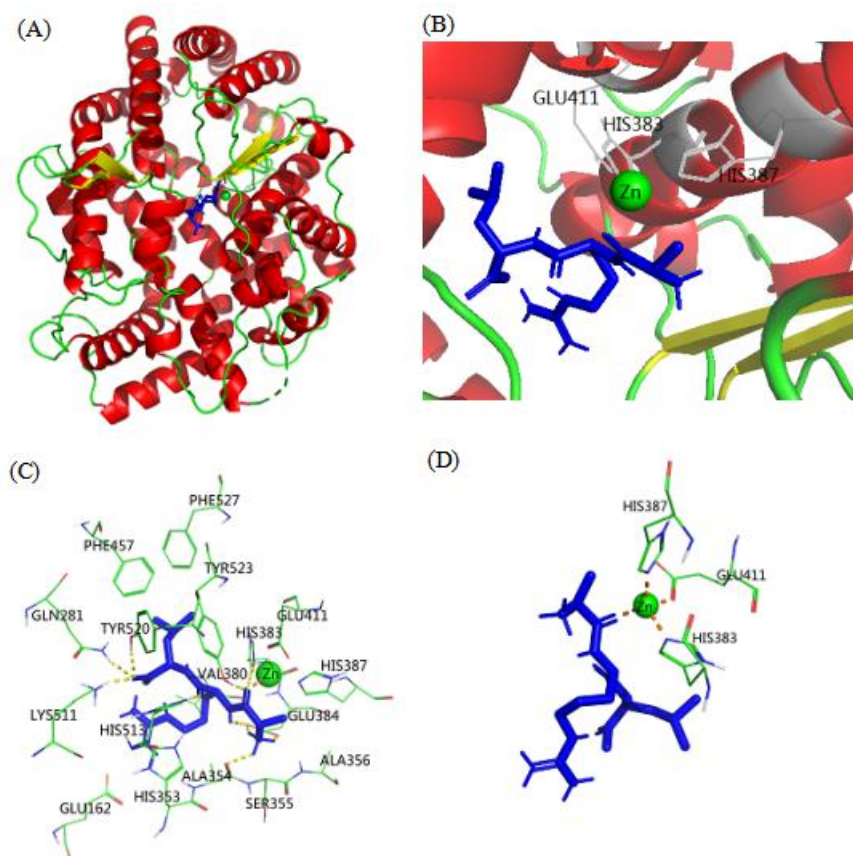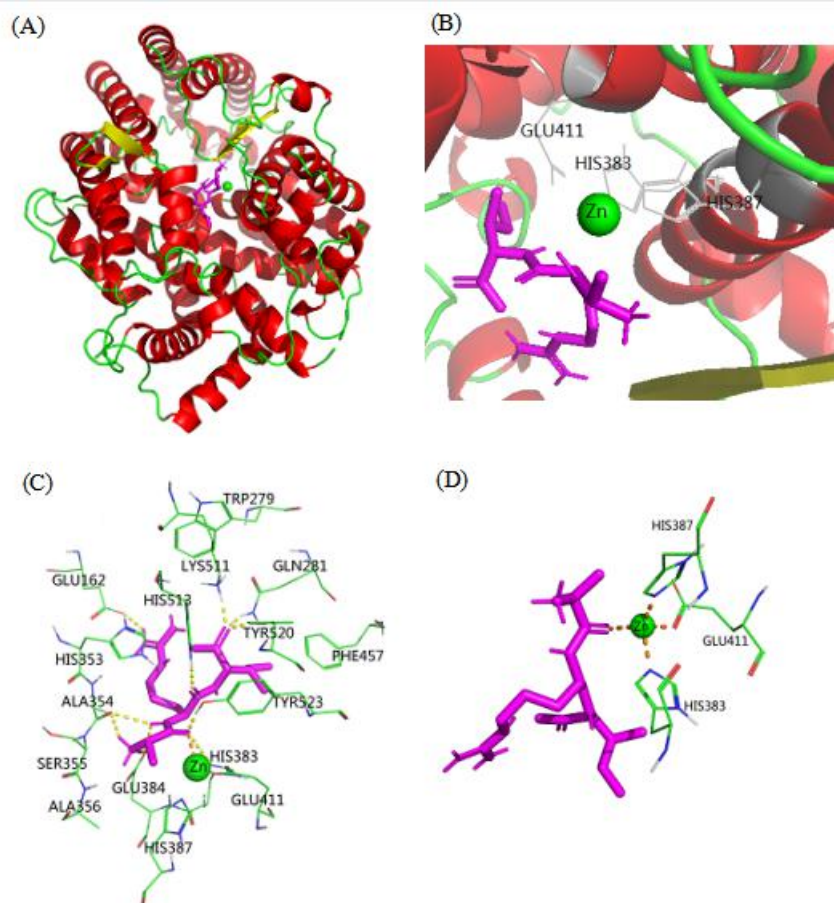
Fig. 4-5 Molecular docking between ACE and ARI. (A) General overview of docking pose of ARI (magenta stick mode) at the ACE site. (B) Local overview of docking pose of ARI at the ACE site. (C) The interaction between peptide ARI and ACE. The hydrogen bonds are shown in yellow dashed lines and zinc coordination bonds in oranges dashed lines. (D) ARI zinc coordination at the ACE active site.
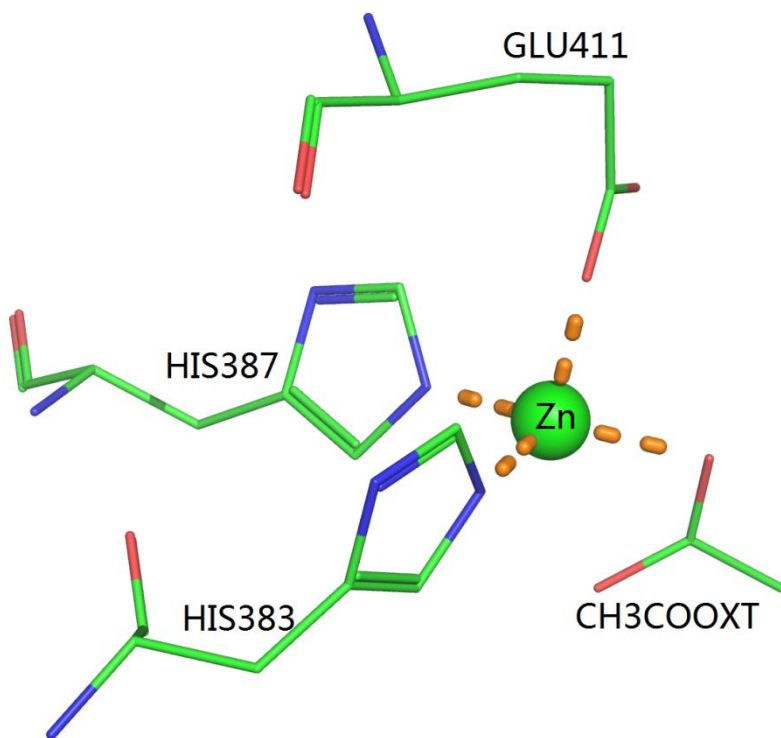
Fig. 4-6 Hemolytic activity of KSHPH-F$_{9\text{-I}}$ against rabbit red blood cells.
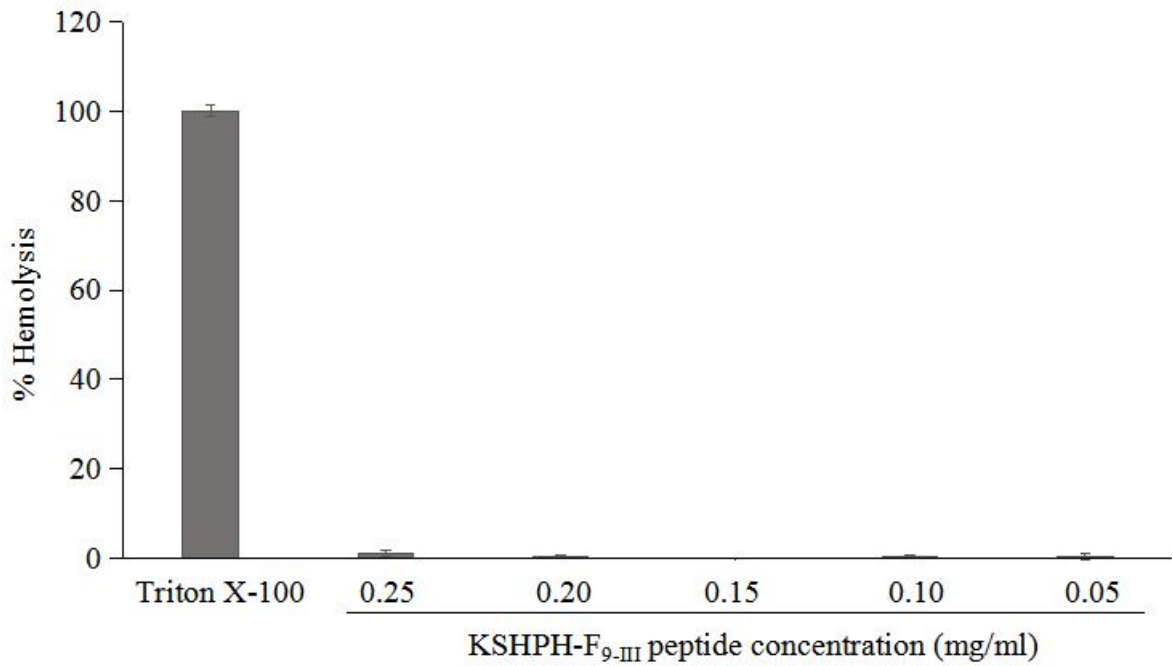
Fig. 4-7 Hemolytic activity of KSHPH-F$_{9\text{-III}}$ against rabbit red blood cells.

Table 4-1 Hydrogen bonds observed between best bioactive peptide poses obtained from docking results and ACE

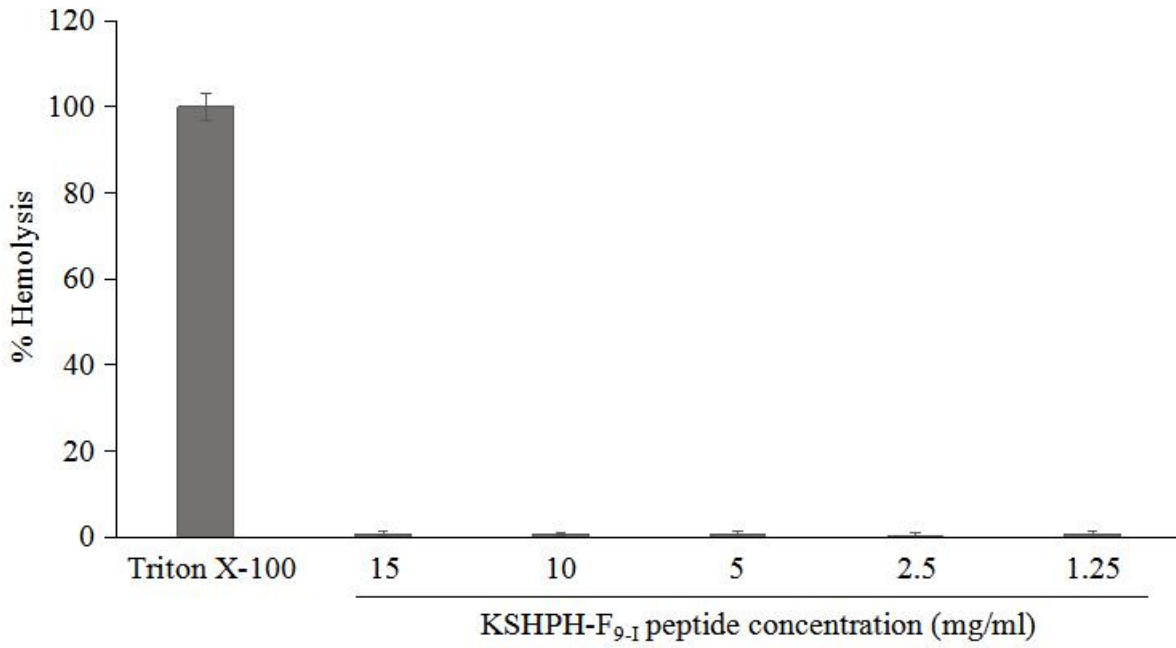|	Hydrogen bond obtained from molecular docking			
	ARL	Distance (Å)	ARI	Distance (Å)
Glu162: O(E2)	×		√	2.17
Gln281: N(E2)	√	2.21	√	1.93
Ala354: O	√	2.13	√	2.22
Ala354: O			√	2.59
His383: N(E2)	√	3.35	√	3.35
Glu384: O(E2)	√	1.78	√	1.90
Glu384: O(E2)	√	2.08	√	2.27
Glu384: O(E2)	√	2.18		
Lys511: N(Z)	√	1.69	√	1.70
His513: N(E2)	√	2.20	√	2.22
Tyr520: O(H)	√	2.14	√	1.92
Tyr523: O(H)	√	1.81	√	1.71

In XXX123: Y(00), XXX represents the abbreviation of amino acid molecule, 123 represents the serial number of this amino acid molecule on ACE, Y represents the atom involved into the interaction and 00 represents the serial number of this atom on this amino acid molecule.

√, existence of hydrogen bond interactions; ×, nonexistence of hydrogen bond interactions.

Table 4-2 Atoms from ACE residues and bioactive peptides (poses obtained from docking results) involved in the interaction with Zn^{2+} before and after docking

	Residues involved in interaction with Zn^{2+}					
	Initial ACE	Distance (Å)	ARL	Distance (Å)	ARI	Distance (Å)
His383: N(E2)	√	2.02	√	2.04	√	2.04
His387: N(E2)	√	2.06	√	2.07	√	2.07
Glu411: O(E1)	√	1.97	√	2.00	√	2.00
CH3COOXT	√	2.06	×	–	×	–
ARL: O(4)	×	–	√	2.08	×	–
ARI: O(4)	×	–	×	–	√	2.20

In XXX123: Y(00), XXX represents the abbreviation of amino acid molecule, 123 represents the serial number of this amino acid molecule on ACE, Y represents the atom involved into the interaction and 00 represents the serial number of this atom on this amino acid molecule.

√, existence of hydrogen bond interactions; ×, nonexistence of hydrogen bond interactions.

Chapter 5 Summary of main finding and discussion

6.1 Summary

1) Kuruma shrimp is a kind of high economic value shrimp species thriving in Japan. Kuruma shrimp head protein was independently hydrolyzed by three commonly used proteases to prepare protein hydrolysates. Among these investigated, papain hydrolysate exhibited the strongest antibacterial and ACE inhibitory activities. Protein/ peptide profile analyzed by SDS-PAGE and HPLC-SEC showed a rich composition of small peptides ranging in size below 5 kDa in papain hydrolysate.

2) Kuruma shrimp head papain hydrolysate (KSHPH) was purified by using two stages of RP-HPLC. Fractionation of protein hydrolysate using RP-HPLC revealed a direct relationship between hydrophobicity and antibacterial/ACE inhibitory activity. After purification, two peptide fractions were selected out, including the fraction KSHPH-F_{9-I}, which showed the strongest antibacterial activity with the MIC values range from 0.67 to 3.33 mg/ml against different tested microorganisms, and the fraction KSHPH-F_{9-III}, which exhibited the highest ACE inhibitory activity with the IC_{50} value of 0.045 ± 0.005 mg/ml. The peptide sequences of KSHPH-F_{9-I} and KSHPH-F_{9-III} fractions were identified Val-Thr-Val-Pro and Ala-Arg-Leu/Ile by LC-MS/MS.

3) The 3D structure predication and physicochemical properties analysis suggested that the antibacterial peptide VTVP was a desirable hydrophobic peptide with a well-defined random coil conformation. The Lineweaver-Burk plots indicated a competitive inhibition pattern of purified peptide ARL/I with the inhibitor constant value (Ki) of 36.19 ± 0.28 μg/ml. The interaction mode and molecular mechanism of peptide inhibitor and ACE were elucidated by molecular docking simulation. The results of docking revealed that hydrogen bonds were the mainly interaction force responsible for binding between ARL and ACE active sites (S1 and S2' pockets), and between ARI and ACE active sites (S1, S1' and S2' pockets). In addition, the peptide

ARL/I can form the coordination bonds with zinc ion that may make ACE deactivation. All purified peptides showed no toxic effect on rabbit red blood cells.

6.2 Discussion

Recently, extensive attention regarding on the preparation, characterization and application of protein hydrolysates and natural peptides has been aroused owing to their diverse promising bio-functions. Protein hydrolysates are preferentially produced by enzymatic hydrolysis of different protein sources by adding suitable proteinases under controlled condition, followed by downstream purification to isolate target biological peptides from a complex mixture solution (Nasri, 2017). Therefore, the downstream purification step is crucial for producing potent and desire peptides. RP-HPLC is regarded as the most frequently used and efficient method to separate the reaction mixture components to obtain the active peptides. In this study, novel antibacterial peptide VTVP and ACE inhibitory peptide ARL/I have been found from the papain hydrolysate of kuruma shrimp head wastes.

VTVP was identified as antibacterial peptide with moderate antibacterial activity against Gram-positive (*S. aureus* and *M. luteus*) and Gram-negative (*E. coli* and *S. putrefaciens*) bacteria. These bacteria are common pathogen indicators that can be used to evaluate the antibacterial spectrum and antibacterial efficacy of peptides. The peptide VTVP was noted to show the higher rates of sensitivity to Gram-positive strains than Gram-negative strains, but the mode of action remains unknown. Peptide VTVP is a tetrapeptide with desirable hydrophobic ratio (50%). The sequence identified in the present study has never been reported to be antibacterial, VTVP is thus a new antibacterial sequence. The purified antibacterial peptide (VTVP) has a low molecular mass, different from the conventional antibacterial peptides that have a relatively high molecular mass. However, previous studies implied that some antibacterial peptides with low molecular mass also showed antibacterial activity that can't be ignored. Actually, the small size of oligopeptides render them better

application in food and medical development as their bioavailability is high and they are amenable to chemical modifications to enhance potency (He et al., 2014). In recent years, series of short chain chemical modified peptides were designed and synthesized. Three oligopeptides have been isolated using a combinatorial yeast overlay method, as well as their chemical derivatives (N–terminal acetylation, C–terminal amidation and hydroxyl phosphorylation) were synthesized to compare the antibacterial potency (He et al., 2014). Some unique groups, including *p*-Hydroxy cinnamic acid (pHCA), acetic anhydride (Ac), cinnamic acid (CIN), and 3-(4-hydroxyphenyl) propionic acid (HPPA) were used to modify the N–terminal of tetrapeptides to increase their antibacterial activity (Bisht et al., 2007). Even to enhance the antibacterial efficacy of active peptides by cyclization, which is regard as a systematical improvement both in the pharmacodynamics and pharmacokinetic properties (Chakraborty et al., 2015).

ARL/I was identified as ACE inhibitory peptide with competitive inhibition characteristic. This result suggested that the peptide inhibitor may play an ACE inhibitory role through followed two ways: 1)binding to the active site of ACE to block substrate binding again, 2) binding to the inhibitor-binding site that is remote from the active site of ACE so as to alter the enzyme conformation such that the substrate cannot approach the active site. Thus, the accuracy molecular mechanism of interaction between ACE and peptide inhibitor has been predicted by using molecular docking simulation. Peptide size and hydrophobicity of sequence are two critical indexes in determining the affinity with ACE active site. Since the ACE was a complex polymer protein with lots of α-helical structures, the binding channel of ACE is too narrow to accommodate the large peptides (Natesh et al., 2003). Three milk protein derived peptides IPA, FP and GKP could enter the ACE channel, and binding to the ACE catalytic site with hydrogen bonds, hydrophobic, and electrostatic interactions, as well as coordinate with zinc ion (Pan et al., 2011). Similarly, a short peptide YSK also can forming hydrogen bonds with the active pockets of human ACE (Wang et al., 2017). By contrast, the large peptide ALPMHIR with the lower ACE inhibitory efficiency since it was hard to access the binding channel and just located outside the

active site of ACE (Pina & Roque, 2009). In addition, the hydrophobicty of peptide is also make a great contribution to ACE inhibitory activity. The main catalytic sites of ACE show obvious hydrophobicity, which makes the C domain of ACE form a integral hydrophobic environment (Wu et al., 2006b). Usually, the ACE inhibitory peptides prefer competitive mode that contain a hydrophobic amino aicd at the N/C-terminal (Khueychai et al., 2018). Hence, I can confirm that the peptide sequence ARL/I show potent ACE inhibitory activity. The molecular docking simulation revealed that the detailed action mode of ARL/I. The interaction binding between ARL/I and ACE was mainly stabilized by hydrogen bonds, as well as forming a cooridinate bond with Zn^{2+}.

Based on the above results and discussion, antibacterial peptide VTVP and ACE inhibitory peptide ARL/I have been purified from the papain hydrolysate of kuruma shrimp head wastes by using two stages of RP-HPLC. The successful isolation of these active peptides proved that the protein hydrolysate of shrimp head wastes had a variety of biological activity, which provided a new possibility for subsequent research and application.

6.3 Prospect for future study

Further research should be carried out based on the current findings in present study, e.g., to further investigate other biological activities of protein hydrolysate of kuruma shrimp head, to analysis and determine the mode and mechanism action of antibacterial peptide VTVP, to explore the antihypertensive effect and gastrointestinal digestion and absorption ability of ACE inhibitory peptide ARL/I. It is anticipated that further studies could provide new promising opportunity for developing potential supplementary and therapeutic agents for food and pharmaceutical industries.

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