CHAPTER 4

DISCUSSIONS AND CONCLUSIONS

4.1 Discussions

Nile tilapia skin, the wastes from fillet processing industry, caused many seriously environmental problems. In this study, we tried to utilize and add value to these by-products since they still contained good amount of protein material. Bioactive peptides can be produced by enzymatic hydrolysis of fish gelatin (Vercruysse et al. 2005). These bioactive peptides are generally short peptides (2-20 amino acids) and have no functional activity exhibited in their native proteins. Upon certain proteolysis, their specific bioactive roles can be achieved and exert beneficial effects at target sites in the body after absorption (Chi et al. 2015; De Gobba et al. 2014). The bioactivities include the ACE inhibitory effect, antioxidative, immunomodulatory and antimicrobial activities (Raghavan and Kristinsson 2009; Thiansilakul et al. 2007; Zhang et al. 2008). The bioactivity of these peptides is affected by many different parameters, such as the source of protein, DH, peptide structure, amino acid composition, type of protease used and MW (Li et al. 2013; Memarpoor-Yazdi et al. 2013).

4.1.1 Production of Gelatin Hydrolysates and Their Degree of Hydrolysis

Gelatin was extracted from the Nile tilapia skin by hot water extraction. Our average production yield was 20.77 ± 0.80% wet weight basis and was higher than

that previously reported by Songchotikunpan et al. (2008) and Zhuang and Sun (2011), which were 18.1% and 12.9% gelatin yields, respectively. Other fish skins extraction for gelatin resulted in different yields, such as black tilapia (5.4%), red tilapia (7.8%) (Jamilah and Harvinder 2002), young Nile perch (12.3%) and adult Nile perch (16.0%) (Muyonga et al. 2004). The difference of gelatin yields between the Nile tilapia skin and other fish skins is noticed. The differences of composition, collagen content and the amount of soluble components in the skins take part in this variation. These factors depend on the species, the age of the fish and also the extraction method employed (Songchotikunpan et al. 2008).

To obtain the bioactive peptides, the gelatin was then separately hydrolyzed with bromelain, papain, trypsin, alcalase, flavourzyme and neutrase. During the first 30 min, the hydrolysis rate was high except for alcalase, and then the steady state of enzymatic reactions was achieved for each enzyme. After 4 hr of hydrolysis, the highest DH was discovered in the reactions with papain (59.55%), followed by flavourzyme (56.61%), bromelain (50.59%), trypsin (44.55%), alcalase (16.05%) and neutrase (15.71%), respectively. Our DH result of some enzymes is different from other reports. For the whole anchovy sprat (*Clupeonella engrauliformis*) study, flavourzyme, bromelain, papain and alcalase were used to hydrolyze these proteins. The DHs of each hydrolysate were 30.5 ± 2.1%, 43.05 ± 2.7%, 49.74 ± 2.6% and 55.8 ± 0.56%, respectively (Ovissipour et al. 2013). Lin and her colleagues (2014) hydrolyzed five low-value aquatic processing by-products, including Pacific mackerel, Spanish mackerel, hairtail, Japanese anchovy and horse mackerel, with alcalase. The obtained DHs ranged from 9.54 ± 0.47% to 10.71 ± 0.26%. In another Nile tilapia skin gelatin research, two steps

hydrolysis with multifect neutral followed by properase E gave the highest DH at 22.11% (Zhang et al. 2012). The difference of DH may arise from the different amino acid sequences of the substrates with the same applied enzyme, the different proteolytic activity of each enzyme on the same substrate, the ratio of enzyme to substrate and the hydrolysis time. The high DH means that hydrolysate should contain more of the low MW peptides than the low DH hydrolysate. If lower MW peptide is required, double digestion can be performed. The relationship between the peptide MW and their biological activities has been reported in several studies (Jeon et al. 1999; Vandanjon et al. 2009). Low MW fractions (<3000 Da) have been found as the most interesting bioactive peptides for nutritional and pharmaceutical applications (Saidi et al. 2014).

4.1.2 Bioactivities of Gelatin Hydrolysate with MW<10 kDa

Each hydrolysate exhibited great antioxidant activity assayed by various methods. For ABTS radical scavenging assay, flavourzyme, trypsin and bromelain hydrolysate from the Nile tilapia skin gelatin showed very high level of radical scavenging, while, neutrase and flavourzyme did not present good capacity for scavenging. In our study, within 10 min of reaction time, no activity was noticed from alcalase. The different level of ABTS activity was reported in other protein hydrolysates. For squid and tuna skin, the ABTS activity orderly decreased from alcalase, collagenase, trypsin and pepsin hydrolysates in both tuna and squid skin (Alemán et al. 2011a; Alemán et al. 2011b). The greatest ABTS radical scavenging capacity was observed in alcalase hydrolysate of whole anchovy sprat protein, while the lowest activity was noticed in flavourzyme hydrolysate (Ovissipour et al. 2013). This contradiction may result from the different starting

material and the DH in each study. Alcalase hydrolysate from tilapia skin gelatin in our study had DH of 16.05% compared to alcalase hydrolysate from squid (30.9%) and anchovy sprat (55.8%) (Alemán et al. 2011a; Ovissipour et al. 2013)

In linoleic acid model system, all hydrolysates from tilapia skin gelatin, except for alcalase and neutrase hydrolysate, could inhibit the autooxidation of lipid higher than 40%. Other studies reported that tilapia retorted skin gelatin hydrolysate could reduce the peroxidation rates of linoleic acid and had an inhibitory effect on lipid peroxidation (77.3%) similar to 10 ppm BHA (Yang et al. 2009). High level of inhibition was also found in purified tryptic hydrolysate of hoki skin gelatin, which was closed to inhibitory level of BHT (Mendis et al. 2005). The inhibition mechanism supposed to be the inhibition of chain propagation in lipid peroxidation cycle from the amino acids in bioactive peptides reacting with peroxyl radicals in the system. Some reported peptides that exhibited higher inhibition of lipid peroxidation consisted of specific hydrophobic amino acids, such as Gly, Leu, Phe and Pro, which were expected a higher interaction between peptide and fatty acids (Saiga et al. 2003).

FRAP and chelating activity determination involves with ferrous ion or iron (II) ion. The importance of iron ion is an ability to generate free radicals from Fenton reaction. Fenton reaction is the reaction between ferrous salts and peroxides that produce hydroxyl radicals. The attack of hydroxyl radicals can result in an initiation of lipid peroxidation in human body. Minimization of the Fe^{2+} concentration in the Fenton reaction provides protection against oxidative damage (Borah et al. 2011). All hydrolysates in this study showed the good reducing activity against ferrous ion complex. The similar results are noted in

other researches. The most reductive ability is noticed in neutrase hydrolysate followed by alcalase for *Arca subcrenata* (Song et al. 2008). In squid skin gelatin, the greatest reducing power observes in alcalase hydrolysate, whereas, trypsin hydrolysate of tuna skin gelatin presented the highest FRAP values (Alemán et al. 2011b). In contrast to our study, flavourzyme hydrolysate from whole anchovy sprat exhibited the lowest reductive capacity (Ovissipour et al. 2013).

For ferrous chelating, all tilapia skin gelatin hydrolysate could greatly trap the ferrous ion in the system higher than 75%, except papain hydrolysate (approximately 15%). Our chelation percentage was mostly higher than that previously reported in anchovy sprat protein by Ovissipour et al. (2013). The maximum chelating capacity for whole anchovy sprat hydrolysates was around 50%. Bromelain and papain hydrolysate were found to be the best ferrous ion chelator.

From our results and other studies, it was noticed that the levels of antioxidant activity of bioactive peptide ensue from the source of hydrolysate, the different enzymes activity and the DH. The difference of presented amino acid sequences in the each hydrolysate may respond to their distinct antioxidative capacity. However, it is not well understood about the bioactive mechanisms of such peptides and only a few studies have been made concerning the structure-activity relationship (Hernández-Ledesma et al. 2011; Schmelzer et al. 2007). Some studies have indicated which protease should be chosen to produce the desired fragment according to the effect required (Tavano 2013). Most researchers confirm that the antioxidative properties are a result of the different amino acids present in the peptides and no single antioxidant mechanism can represent the

overall antioxidant activity of the peptides. For instance, hydrophobic amino acid-rich peptides are expected to inhibit lipid peroxidation, acting as proton donors to the hydrophobic peroxyl radicals and as metal ion chelators. In this sense, peptides containing histidine in their sequences have been reported to act as metal ion chelators, perhaps because of their ring structure characteristic (Alemán et al. 2011b).

In the RAS system, ACE is a vital enzyme for regulating blood pressure. It converts angiotensin I into an active potent vasoconstrictor angiotensin II, resulting in the increase of blood pressure. Many studies reported the effect of protein hydrolysate in lowering blood pressure. Ichimaru et al. (2009) found out that hydrolysate from porcine skin collagen could effectively decrease blood pressure *in vivo*. In squid skin gelatin hydrolysate, alcalase hydrolysate was a great ACE inhibitor with IC_{50} of 0.34 mg/mL (Alemán et al. 2011b). Lin et al. (2012) also informed that pepsin hydrolysate of squid skin gelatin with MW lower than 2 kDa showed the best ACE inhibition capacity *in vitro* with IC_{50} of 0.33 mg/mL. Zhao and his colleagues (2009) hydrolyzed sea cucumber (*Acaudina molpadioidea*) body wall protein with bromelain and alcalase consecutively and reported that the hydrolysate with MW lower than 2 kDa exhibited high ACE inhibition. The evaluation of the ACE inhibitory activity of Nile tilapia gelatin hydrolysate was performed by Vo et al. (2011). Nile tilapia gelatin was separately hydrolyzed with alcalase, pronase E, pepsin and trypsin. Highest ACE inhibitory activity occurred in the alcalase hydrolysate, being higher than other specific (pepsin and trypsin) and nonspecific (pronase E) protease. In our study, all gelatin hydrolysates from Nile tilapia skin could greatly inhibit the ACE function.

Interestingly, our trypsin and alcalase hydrolysates could exhibit the similar level of inhibition against ACE.

Ngo et al. (2012) described that the common characteristic of ACE inhibitory peptides is the hydrophobicity of the N-terminus, which may conduce to the inhibitory activity. Tavano (2013) suggested that peptides possessing an ACE inhibitory effect are usually small fragments composing of 2 to 12 amino acids and often comprise of polar amino acid residues such as proline. Many studies indicate that C-terminal tripeptide residues are greatly influenced in competitive binding at the active site of ACE. In addition, the hydrophobic, or positively charged, amino acids at C-terminal are commonly found in the most effective ACE inhibitory peptides. It is suggested that the potent ACE inhibitor is the peptides that consist of hydrophobic amino acids at these positions. In this context, this helps explaining why anti-hypertensive peptide production is usually prepared by pepsin or trypsin hydrolysis. Pepsin favors the cleavage between hydrophobic residues, whereas, Arg- and Lys- cleavages generally occur with trypsin hydrolysis. However, the mechanisms involved in the ACE inhibitory effect should be further investigated.

Kizhakekuttu and Widlansky (2010) reported that hypertension could be exacerbated or may be induced with the emerging of excessive ROS. Thus, the natural diets that contained abundant of antioxidants could reduce blood pressure and cardiovascular risk. Nevertheless, the discontented results were noticed from the randomized trials and population studies using natural antioxidants and the reasons for these unexpected results were still not completely understood. Without sufficient knowledge about mechanisms of antioxidants on hypertension and the

specific target, any exact natural antioxidants for hypertension therapy still cannot be endorsed.

4.1.3 Partial Purification and Identification of Bioactive Peptide from Nile Tilapia Skin Gelatin

According to our results, trypsin and flavourzyme hydrolysates were suitable for purification to obtain low MW bioactive peptides. Both hydrolysates exhibited a great potent as antioxidant and antihypertensive agents. Crude hydrolysate of each enzyme was fractionated by gel filtration chromatography, which separates peptides in accordance to their MW. High MW peptides (more than 30 kDa) are eluted out from the column first because, with their large size, they cannot pass through the inside of Sephadex® G-50 beads, which have fractionation range from 1.5 to 30 kDa. Low MW peptides come out after that. For trypsin hydrolysate, TA, the high MW fraction, had lower in bioactivities compared to crude hydrolysate. The good antioxidant and antihypertensive activities were still exhibited in the low MW fractions, TB and TC. All flavourzyme fractions presented great ABTS value, except for FB fraction. Even though, all flavourzyme fractionates showed great antihypertensive activity, the highest inhibitory activity against ACE was observed in FB fraction. Many researchers also reported that the low MW peptide exhibited higher bioactivities than high MW peptide. Fan et al. (2012) evaluated the antioxidant properties of enzymatic hydrolysate of tilapia frame protein and found out that the peptide with MW lower than 1 kDa exhibited the highest antioxidant activity. Zhang et al. (2012) reported that the highest antioxidant activity was noticed in the low MW tilapia skin gelatin hydrolysate fractions with IC_{50} of 110.80 µg/mL. A low MW

tuna muscle hydrolysate using alcalase was prepared by Saidi et al. (2014). Alcalase hydrolysate was fractionated with ultrafiltration and nanofiltration membrane. The highest 2,2'-diphenyl-1-picrylhydrazyl (DPPH), as well as the hydroxyl radical scavenging activities were discovered in the nanofiltration permeate (MW<1 kDa). The retentate (MW 1-4 kDa) also exhibited the greatest superoxide radical and reducing power activities. The great antioxidative properties were also found in the low MW peptides from protein hydrolysate of Pacific hake (Cheung et al. 2012), croceine croaker muscle (Chi et al. 2015a) and bluefin leatherjacket heads (Chi et al. 2015b). Chalé et al. (2014) stated that the most active ACE inhibitory fraction was the peptide fraction from the *Mucuna pruriens* hydrolysed with pepsin-pancreatin that had MW lower than 1 kDa. Low MW (<2 kDa) of body wall protein from the sea cucumber showed more potent ACE inhibitory activity (Zhao et al. 2009). Jung and Kim (2006) revealed that low MW fraction exhibited more ACE inhibition than the high MW. In the study of sardine muscle hydrolysate, the increased ACE inhibition was particularly observed from the hydrolysate with more proteolysis (Matsui et al. 1993). Ngo et al. (2015) proposed that the skate gelatin hydrolyzed with alcalase and protease (MW below 1 kDa) exhibited the highest ACE inhibition. The highest inhibition of ACE was also noticed in the fraction below 5 kDa of *Chlorella ellipsoidea* hydrolysate (Ko et al. 2012). The similar results were also reported in other food protein researches, such as goat milk hydrolysate (Espejo-Carpio et al. 2013), yak milk casein hydrolysate (Mao et al. 2007), bovine casein hydrolysate (Jiang et al. 2010) and egg yolk protein (Eckert et al. 2014).

Consequently, the TB and FD fractions were further purified by ion exchange chromatography. Only one fraction (TB1) was obtained from TB, while, FD was fractionated into two fractions (FD1 and FD2) but still did not completely isolate from each other. This may come from the overall positive charge in those two peptides are resemble. Nevertheless, the bioactivities of all fractionates from ion exchange chromatography had reduced when compared with the starting fraction. Both bioactivities of TB1 were slightly reduced compared with those of original TB fraction. After FD fractionation, FD2 still exhibited good antioxidant activity compared to FD fraction, however, the great loss of antioxidative properties was observed in FD1. In additions, both FD1 and FD2 significantly lost their inhibitory activity of ACE. This effect may arise from the loss of synergistic peptides to cooperate with their antioxidant and antihypertensive functions (Raghavan and Kristinsson 2009).

Based on this finding, the purification with ion exchange chromatography may not be needed since the peptide fractions did not entirely separate and their bioactivities were deteriorated. Hence, TB and FD fractions were selected to be identified for their sequences by MALD-TOF/TOF MS/MS technique. Two spectra from TB fraction were correlated with the *Oreochromis niloticus* collagen peptides, which were identified for their sequences as GPEGPAGAR (peptide 1, MW 810.87 Da) and GETGPAGPAGAAGPAGPR (peptide 2, MW 1490.61 Da). The sequence of peptide 1 and peptide 2 were rich in Ala (A), Gly (G) and Pro (P), which are common abundant amino acids in fish skin. Interestingly, from their amino acid sequences, it was found that the hydrophobic terminal was presented in both of peptide 1 and peptide 2. As mentioned earlier that

hydrophobicity of N-terminus was the common characteristic of ACE inhibiting peptide, thus, it could be expected that peptide 1 and peptide 2 could greatly inhibit the activity of ACE. Ichimaru et al. (2009) examined the antihypertensive effect of active peptide from porcine skin collagen, which had sequence as Gly-Phe-Hyp-Gly-Pro. His team also evaluated the effect of Gly-Pro against ACE inhibitory activity since Gly-Pro sequence existed frequently in collagen. They found out that both peptides could decrease the blood pressure of spontaneously hypertensive rats. This may propose that GP sequence takes part in the inhibitory mechanism of peptide against ACE. No significant match for the spectra from FD fraction with any peptide in the database was presented. The reason why there was no significant matched peptide from FD fraction may arise from the activity of flavourzyme. Flavourzyme contains both exo- and endopeptidase (serine and aspartic) activities. Therefore, hydrolysis of Nile tilapia skin gelatin with flavourzyme can yield many hydrolyzed peptide sequences, some of which may be similar in size but had different sequences. Many previous researchers also reported the amino acid sequences of bioactive peptide from other sources that represented the different amino acid sequences from our study. Fan et al. (2012) hydrolyzed tilapia frame protein with trypsin and identified for amino acid sequence of antioxidant peptides. They reported that two antioxidant peptides from tilapia frame protein obtained by trypsin had amino acid sequences as DCGY (456.12 Da) and NYDEY (702.26 Da). Ngo et al. (2014) identified the peptides from alcalase skin gelatin of skate responsible to inhibition of ACE activity and their amino acid sequences were MVGSAPGVL (829 Da) and LGPLGHQ (720 Da). Cod skin gelatin was sequentially hydrolyzed with gastric

endopeptidases (pepsin, trypsin and α-chymotrypsin, respectively) and identified for the active peptide. It was found out that active peptide sequence was LLMLDNDLPP (1301 Da), which exhibited the potent non-competitive ACE inhibition and great antioxidant activities in protecting cellular macromolecules from reactive oxygen species and scavenging intracellular reactive oxygen species (Himaya et al. 2012). Zhao et al. (2009) studied the ACE inhibitory peptide from body wall protein of sea cucumber obtained from sequential hydrolysis with bromelain and alcalase. Peptide exhibiting great ACE inhibition was sequenced as MEGAQEAQGD (1035 Da). The ACE inhibitory activity of reported bioactive peptides was summarized in the Table 4.1 together with peptide 1 and peptide 2.

Table 4.1 The ACE inhibitory activity of bioactive peptides

Source	Sequence	ACE inhibitory activity
Sea cucumber (Zhao et al. 2009)	MEGAQEAQGD (1035 Da)	IC_{50} = 4.5 μM
Pacific cod (Himaya et al. 2012)	LLMLDNDLPP (1301 Da)	IC_{50} = 35.7 μM
Skate (Ngo et al. 2014)	LGPLGHO (720 Da)	IC_{50} = 4.22μM
	MVGSAPGVL (829 Da)	IC_{50} = 3.09 μM
Nile tilapia* (This study)	GPEGPAGAR (810.87 Da)	%inhibition = 37.36%
	GETGPAGPAGAAGPAGPR (1490.61 Da)	%inhibition = 40.52%

*the concentration of synthetic peptide of Nile tilapia was 1.0 mg/mL in this assay.

4.1.4 Molecular Interaction between Peptide 1 and Peptide 2 on the Active Site of ACE Determined by *in silico* Docking

Our identified peptides (peptide 1 and 2) were evaluated with *in silico* docking method for better understanding of the molecular interactions on the ACE

active site. Peptide 1 showed the best conformation for docking with the lowest binding affinity. This may be due to a larger surface area of the binding interaction and hence the higher number of hydrogen bonds occurred at the carbonyl, amino and hydroxyl groups of the ligand molecule. Peptide 2 also revealed a similar level of binding affinity compared to peptide 1, even though it had a longer chain and more surface area. However, some of the residues of peptide 2 appeared as sharp turns, which could render the overall complex less stable than that of peptide 1. Furthermore, when compared between the similar interactions among the ligands observed at the common enzyme residues, peptide 1 also has more interactions similar to those of the K-26 binding, even though it is considerably shorter than peptide 2. The binding conformation of peptide 1 in the active site canal also appeared more expanded than that found in the peptide 2 case, which usually indicated a more stabilized conformation with potentially fewer steric clashes occuring while the peptide threading through the enzyme. These characteristics suggested that peptide 1 may be slightly more superior to peptide 2 in term of fitting into the binding pocket of ACE, though having a similar level of binding affinity. Taken together, the docking study implied that the ACE-peptide 1 and ACE-peptide 2 complexes are more stable than the ACE-K-26 complex. Peptide 1 may be a better ligand for the ACE active site cleft than peptide 2, though not significantly. Nevertheless, the two synthetic peptides exhibited no significant difference in antihypertensive activity. In fact, peptide 2 inhibited the function of ACE slightly higher than that noticed from peptide 1. The key interactions of ACE-peptide 1 and ACE-K-26 complexes were compared and summarized in two-dimensional schematics below (Figure 4.1).

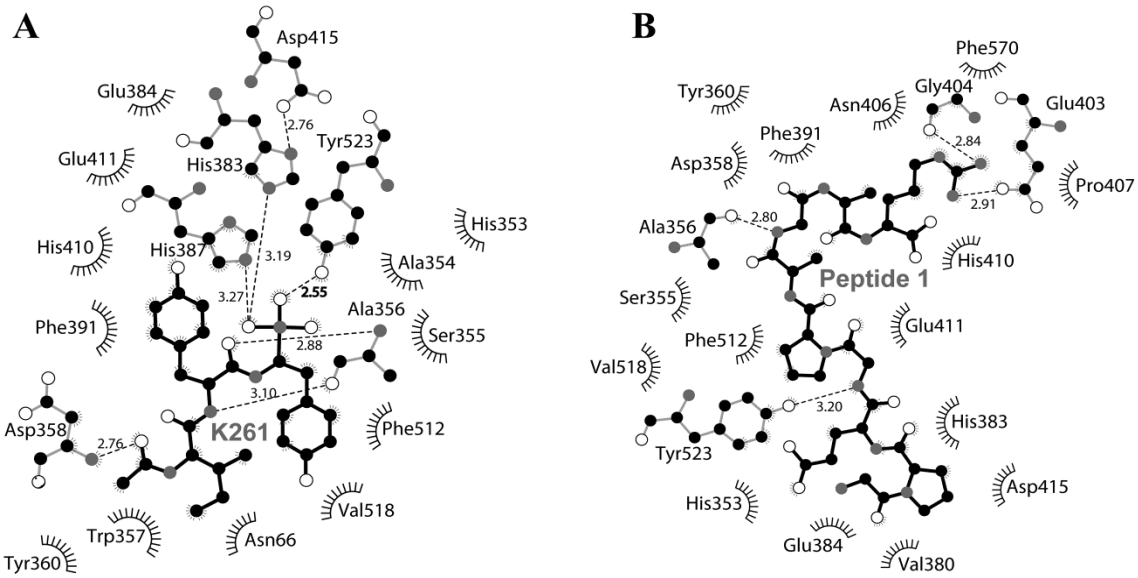

Figure 4.1 Two-dimensional schematics summarizing the key interaction between the ligands (black sticks) and the active site of ACE (gray sticks) analyzed by Ligplot (Wallace et al. 1995); (A) K-26 inhibitor and (B) peptide 1. Dashed lines indicate hydrogen bonding and 'eyelash' curves show unbonded hydrophobic interactions. Key amino acid residues of the enzyme for the binding are indicated. White atoms are oxygen and gray atoms are nitrogen. Distances of key interaction are indicated.

Compared with other reported peptide sequence, peptide 1 and peptide 2 contained both positive (R) and negative (E) charge residue in their sequence, which made them bind with Zn^{2+} molecule and Glu403 in the active site of ACE stronger than other peptide that composed with only negative or positive charge residue. Furthermore, the structure of both peptide 1 and peptide 2 was similar to the ACE inhibitors, of which the positive and negative charges exist along with the ring structure in their molecules (Scolnick 2001).

Interestingly, from the structure of peptide 1 and peptide 2, it would be predicted that these two peptides tolerate to pepsin and chymotrypsin hydrolysis in the digestive tract. Chymotrypsin preferentially cleaves at Phe (F), Tyr (Y) and Trp (W) in P1

position and the specificity of pepsin is Phe, Tyr, Trp and Leu (L) at P1 position (ExPASy 2015). These amino acids did not found in the sequence of peptide 1 or peptide 2, thus, peptide 1 and peptide 2 would still be 9 and 18 amino acids peptide, respectively, after absorption. However, further experiment would be performed to confirm that the digestive tract enzymes did not hydrolyzed peptide 1 and peptide 2 before absorption. ^{14}C labelled low molecular weight collagen hydrolysate was proved to be effectly absorbed into the blood after administered orally to Wistar rats (Watanabe-Kamiyama et al. 2010). From these findings, it could be implied that the bioactive peptides from trypsin hydrolysis of Nile tilapia skin were the potential antihypertensive and antioxidative agent from the natural source. However, for further development on commercial business, the cost for production of bioactive peptide must be concerned. The main problems for enzymatic hydrolysis are the cost of high purity enzymes are very expensive and using only crude enzyme may not achieve the required DH. To overcome these problems, double digestion or serial digestion with crude enzyme may be carried out to yield the low MW peptides. Another solution for this problem is recombinant DNA technique to produce the specific sequence bioactive peptide.

4.2 Conclusions

The Nile tilapia skin was extracted for gelatin using hot water extraction. The production yield of gelatin was higher than previously reported. Six proteases, including bromelain, papain, trypsin, alcalase, flavourzyme and neutrase, were applied to produce the gelatin hydrolysates. The gelatin hydrolysates were then examined for their bioactivities, both antioxidant and antihypertensive activities. The low MW fraction of tilapia skin gelatin hydrolyzed with trypsin and flavourzyme exhibited the best

antioxidant activities in all assays, including ABTS radical scavenging assay, FRAP assay, ferrous ion chelating assay and inhibition of lipid peroxidation. All low MW hydrolysates were found to be great antihypertensive agents.

Among these hydrolysates, trypsin and flavourzyme hydrolysates were selected for purification to obtain the purified bioactive peptide. Three fractionates (TA, TB and TC) were obtained from the trypsin hydrolysate, whereas, flavourzyme hydrolysate consisted of four peptide fractions (FA, FB, FC and FD). TB fraction exhibited high level of ABTS radical scavenging activity and inhibitory activity on ACE. In the flavourzyme fractions, the highest antioxidant and antihypertensive activities were noticed in FA and FB fraction, respectively. However, FB showed the lowest level of ABTS radical scavenging activity and, although, FA exhibited good antihypertensive property, its production yield was the lowest. FD fraction also presented great bioactivities in both. Further purification with ion exchange chromatography diminished the bioactivities from both fractions. Therefore, the FD fraction was subjected to identify for amino acid sequences along with TB fraction by MALDI-TOF/TOF. No peptide sequence from the FD fraction matched with any sequence in the database. Two peptide sequences were identified from TB fraction, which were GPEGPAGAR (MW 810.87 Da) and GETGPAGPAGAAGPAGPR (MW 1490.61 Da).

Docking analysis showed that both identified peptides from TB fraction could effectively bind with ACE and exhibited good inhibitory activities against ACE function. Thus, it can be concluded that gelatin hydrolysate from the Nile tilapia skin, especially the trypsin hydrolysate peptides, are novel candidates towards the development of the antihypertensive and antioxidant agent isolated from natural sources.