CHAPTER 1

INTRODUCTION

Aquatic capture fisheries provide more than 50% of the total world fish production. Of this, over 70% has been utilized as processed products (Kim and Mendis 2006). The fish industry is a major economic source worldwide. For many countries, especially in developing countries, fish protein is a major source of nutrients (Chalamaiah et al. 2012). It composes of many functional nutrients, such as polysaccharides, polyunsaturated fatty acids, minerals, vitamins, enzymes and bioactive peptides (Pomponi 1999).

1.1 Historical Background and Significance of Problems

The Nile tilapia, *Oreochromis niloticus*, is one of the popular species in freshwater aquaculture. It is predicted that tilapia will become the most important aquaculture crop in this century. Hence, the demand of tilapia in all forms is increasing steadily in the global market (FAO 2013). The main tilapia producers in Asia are China, the Philippines, Thailand and Taiwan (El-Sayed 2006). However, the more tilapia processing industries increase, the more by-products are produced. Only 40% of the fish is produced for human consumption with more than 60% of by-products generated from the fish processing industry are wastes, which include skin, head, fins and bones (Dekkers et al. 2011). These large quantities of wastes would create serious pollution and disposal problems. Selling the wastes as low market-value products, such as animal

feed, fish meat and fertilizer, is a good solution to these problems. However, these by-products still consist of a good amount of protein-riched materials (Hsu 2010). Particularly, fish skin is a rich source of collagen and gelatin. Gelatin is a high molecular weight polypeptide mixture derived from collagen after being heated to 45 °C or higher, and is widely used in food and cosmetic industries (Cheng et al. 2012; Gómez-Guillén et al. 2011).

Food proteins not only provide amino acids for the growth and health maintenance of humans, but they also serve as a precursor for physiologically active peptides. Many health promoting peptides have also been identified from food protein hydrolysates. These bioactive peptides are generally short peptides (2-20 amino acids) and their native proteins often have no functional activity. 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). To regain the essential nutrients and bioactive components from fish wastes, several techniques have been applied, such as solvent extraction, enzymatic hydrolysis or microbial fermentation (Najafian and Babji 2012; Vercruysse et al. 2005). Among these three methods, enzymatic hydrolysis of fish gelatin is more suitable, since it results in a production of bioactive peptides without organic solvents or toxic chemical residuals (Vercruysse et al. 2005). Many studies have reported that gelatin hydrolysate produced from the fish skin exhibits great antioxidant activity (Gómez-Guillén et al. 2011; Jongjareonrak et al. 2008; Mendis et al. 2005; Yang et al. 2008; Zhang et al. 2012; Zhuang and Sun 2011) and antihypertensive activity (Byun and Kim 2001; Kittiphattanabawon et al. 2013; Vo et al. 2011;).

In this study, gelatin hydrolysates were firstly prepared from enzymatic hydrolysis of Nile tilapia skin gelatin. Each gelatin hydrolysate was quatified for their antioxidant and antihypertensive activity. Two hydrolysates with the highest bioactivites were chosen for purification and identification for their bioactive peptides sequences. The molecular docking of identified peptides will provide the information of molecular interactions between the peptides and angiotensin-I-converting enzyme (ACE) for better understanding.

1.2 Nile Tilapia (*Oreochromis niloticus*)

The Nile tilapia (*Oreochromis niloticus*), or local name 'Pla Nin', is a freshwater fish belonging to the Cichlidae family, Order *Perciformes* and Class *Actinopterygii* (Figure 1.1). They originated from Africa but have been introduced worldwide. Tilapia is one of the most popular aquaculture fish due to its good attributes, including their fast growth rate, wide range of environmental condition tolerance, stress and disease resistance, the ability to reproduce in captivity, short generation time, and low trophic levels of feeding (El-Sayed 2006). Nile tilapia is a deep-bodied fish with cycloid scales and regular dark vertical stripes throughout the depth of its silver body (Figure 1.1). In the breeding season, its skin often turns red (Picker and Griffiths 2011). It can grow to a maximum length of 62 cm and a weight of 3.65 kg. The average size is around 20 cm (Bwanika et al. 2004). The food sources of Nile tilapia are not only some aquatic plants, including phytoplankton and periphyton, but also invertebrates, benthic fauna, detritus, bacterial films and even other fishes and fish eggs (FAO 2015a). It can live longer than 10 years (GISD 2015). The limiting growth factors are food availability and water temperature (Kapetsky and Nath 1997). Their optimal growth is achieved at 31-36 °C

and declines with decreasing temperature. In aquaculture ponds, Nile tilapia can reach sexual maturity at the age of 5-6 months (FAO 2015a).

Figure 1.1 Image of the Nile tilapia, *Oreochromis niloticus* (Source: nar.er.usgs.gov)

1.3 Bioactive Peptides

Food proteins are abundant source for the production of biological active peptides. As a whole parental protein, these peptides do not exhibit any activity. Solvent extraction, enzymatic hydrolysis and microbial fermentation are the methods to liberate the bioactive peptides from food proteins. Among these three methods, enzymatic hydrolysis is a more preferable method with no organic solvents or toxic chemicals left in the products (Kim and Wijesekara 2010). Furthermore, recombinant DNA technique and peptide synthesis are an alternative production of the bioactive peptide apart from those three conventional methods as summarized in Figure 1.2. Generally, bioactive peptides are short peptides (usually 3-20 amino acid residues) and their amino acid compositions and sequences play an important role in their activities. The specific activities can be achieved with certain proteolysis (Chi et al. 2015; De Gobba et al. 2014). Various biological functions of bioactive peptides (Figure 1.3), such as antihypertension, immunomodulatory, antithrombotic, antioxidant, antimicrobial and

anticancer activities, are dependent on the amino acid sequence of the peptides. The structure of peptide and other properties, including hydrophobicity, charge or microelement binding, are important for the multifunction of their activities. Moreover, the bioactivity of these peptides are also influenced from many parameters, such as protein source, degree of hydrolysis (DH), type of protease used and their molecular weight (MW) (Li et al. 2013; Memarpoor-Yazdi et al. 2013). Several studies reported revalance between the MW of peptides and their biological activities (Jeon et al. 1999; Vandanjon et al. 2009). For nutritional and pharmaceutical applications, the most interesting bioactive peptides are the low MW fractions (<3000 Da) (Saidi et al. 2014). Bioactive peptides derived from marine organisms exhibited many physiological functions, including antihypertensive, antioxidant, anticoagulant and antimicrobial activities (Kim and Wijesekara 2010).

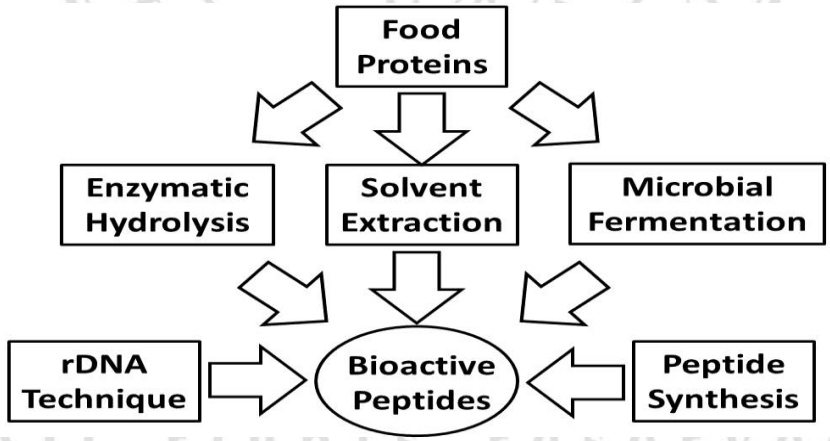

Figure 1.2 Methods for production of bioactive peptides

(Adapted from: Danquah and Agyei 2012)

The most used enzymes for production of bioactive peptides are pepsin, chymotrypsin and particularly trypsin. Nevertheless, other enzymes, such as alcalase and thermolysin, have also been widely studied. The different liberated fragments

resulted from the digestions of distinct enzymes generate a wide range of actions peptides (Tavano 2013). The mechanisms of bioactive peptides are not totally understood and the structure-activity relationship has been discussed in only a few studies. Some studies implied that protease can be used to acquire the desired peptide fragment with required effect. The first ACE inhibitory peptides have been reported by Oshima et al. (1979). The ACE inhibitory peptides are usually small fragments (containing 2-20 amino acids). Tripeptide residues are responsible in competitive binding to the active site of ACE. Hydrophobic (aromatic or branched side chains) amino acids at the C-terminal positions or positively charged by lysine (Lys) and arginine (Arg) at the C-terminal residue are commonly noticed in the most effective ACE inhibitory peptides. That is why a successful antihypertensive peptide production uses pepsin (with favored hydrolysis between hydrophobic residues) and trypsin (specific cleavage after Arg- and Lys-) for enzymatic hydrolysis. Moreover, some research works have reported that the inhibitory activity of peptides may depend on the unique amino acid composition and on the hydrophobicity of the C-terminus of the peptides. The peptide composed of proline (Pro) or hydroxyproline (Hyp) residue at the C-terminal region exhibits great ACE inhibition property. Bacterial and fungal proteases are then used for hydrolysis since Pro resists other digestive proteases. Normally, the last three hydrophobic amino acids of the C-terminal are also found in ACE inhibitors (Espejo-Carpio et al. 2013; Korhonen and Pihlanto 2006; Li et al. 2004; Zhang et al. 2013).

The antioxidant activity of bioactive peptides relies on the DH and also the enzyme employed during the hydrolysis step. The exact mechanism is not clearly explained, nevertheless, some studies reported that some aromatic amino acids and

histidine are vital for the antioxidant activity. The presence of hydrophobic amino acids in the bioactive peptides also relate to their antioxidant potency. The capability of antioxidant peptides is associated with the ability to interrupt the radical chain reaction during lipid oxidation and also to chelate pro-oxidant metal ions, which depends on the presence of certain amino acid residues in the peptides, such as tyrosine, histidine, methionine and tryptophan. Hydrophobic amino acids are abundant in gelatin peptides, helping in their emulsifying ability. Hence, bioactive peptides generated from marine gelatin are expected to exhibit greater antioxidant effects among other antioxidant peptide sequences (Kim and Wijesekara 2010; Mendis et al. 2005; Tavano 2013).

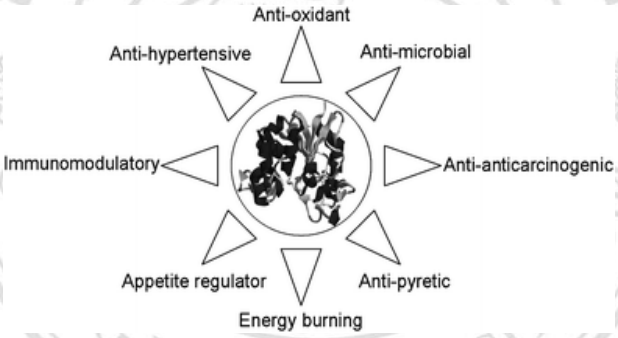

Figure 1.3 Health beneficial bioactivities of bioactive peptides (Nagpal et al. 2011)

1.3.1 Enzymes used in this study

As aforementioned, enzymatic hydrolysis of food protein is the most suitable method to produce the bioactive peptides. Many proteases can be used for hydrolysis of food protein, depending on what amino acid sequences they require or what bioactivities are of interest. In this study, six proteases are applied to Nile tilapia skin gelatin to produce different amino acid sequences of bioactive peptides. The specificity of each enzyme is summarized in Table 1.1.

Table 1.1 Specificity of the enzyme used in this study (BIOZYM 2015; FAO 2015b; Sigma-Aldrich 2015; Worthington 2015)

Enzyme	Source	Specificity
Bromelain (EC 3.4.22.32)	Pineapple stem	C-terminal of Lys, Ala, Tyr or Gly
Papain (EC 3.4.22.2)	Papaya latex	Broad specificity (C-terminal of basic amino acid, Leu or Gly, except the next is Val)
Trypsin (EC 3.4.21.4)	Pancreas	C-terminal of Lys or Arg except next amino acid is Pro
Alcalase (EC 3.4.21.62)	*Bacillus licheniformis*	Broad specificity (preferable C-terminal of a large uncharged residue)
Flavourzyme (EC 3.4.11.1)	*Aspergillus oryzae*	C-terminal of Ser or Asp N-terminal of amino acids (exopeptidase)
Neutrase (EC 3.4.24.28)	*Bacillus amyloliquifaciens*	N-terminal of non-polar amino acid

1.4 Hypertension

High blood pressure, or also called hypertension, is a serious condition and is one of the most occurring health problems in the world. One of the important independent risk factors for cardiovascular diseases is associated with hypertension. Hypertension also associates with myocardial infraction, coronary heart disease, stroke, kidney failure, heart failure and vascular dementia. It was estimated that one third of the world adult population is suffered from this condition (Sharp et al. 2011; Zhou et al. 2013).

The main vital system that controls the blood pressure is the renin-angiotensin system (RAS), the pathway of which the main regulators are renin and ACE. In 1956, ACE, a zinc dependent dipeptidyl corboxypeptidase (EC 3.4.15.1), was firstly isolated from horse blood as a hypertension-converting enzyme (Kim and Wijesekara 2010). In RAS system (Figure 1.4), an active renin is converted from prorenin by a trypsin-like enzyme. Angiotensinogen is cleaved by renin to form angiotensin I (ANG-I). Subsequently, ACE converts ANG-I into potent vasoconstrictor angiotensin II (ANG-II). ACE can also hydrolyze the vasodilator bradykinin, a nonapeptide involved in lowering blood pressure, into an inactive metabolite, resulting in the up-regulation of blood pressure. The ANG-II action that involved with aldosterone release and sodium concentration increase contributes to blood pressure augmentation. While, degraded bradykinin reduces the ability of the blood vessels to relax after the contraction (Fernández-Musoles et al. 2013; He et al. 2013).

ACE not only catalyses the hydrolysis of bradykinin, but also degrades many neuropeptides that may interact with the cardiovascular system, including enkephalins, neurotensin and substance P (Kim and Wijesekara 2010). The endothelium of somatic tissues and testis are the source of the ACE production, a pivotal component in RAS. The difference of ACE from endothelium and ACE from testis lies in their numbers of active domain sites. Two active domain sites are presented in the endothelium ACE, both domains are great targets for ACE inhibitors, while ACE from testis, a shorter-specific form, has only one active site (Donoghue et al. 2000). Hence, one of the effective therapeutic methods for the treatment of hypertension is the inhibition of ACE activity. By inhibiting activity of ACE, the ANG-II generation is reduced and,

consequently, bradykinin is less hydrolyzed, which conduces to a down-regulation of the blood pressure.

The first natural ACE inhibitory peptides were from snake venom, which lead to the development of many synthetic ACE inhibitor drugs such as captopril, enalapril and lisinopril. These drugs are also used in heart failure treatment in humans. However, it was hypothesized that the synthetic ACE inhibitors are the cause of some certain side effects, such as cough, taste disturbance, skin rash or angioneurotic edema. Therefore, searching for natural effective ACE inhibitors is gaining more interest. ACE inhibitory peptides are alternative ACE inhibitors, which are extensively studied in recent years (Espejo-Carpio et al. 2013; Kim and Wijesekara 2010). The target for ACE inhibitor is the active site binding pocket of the ACE, at which ACE inhibitor coordinates with zinc ion leading to inhibition of ACE activity (Akif et al 2010a; Akif et al 2010b).

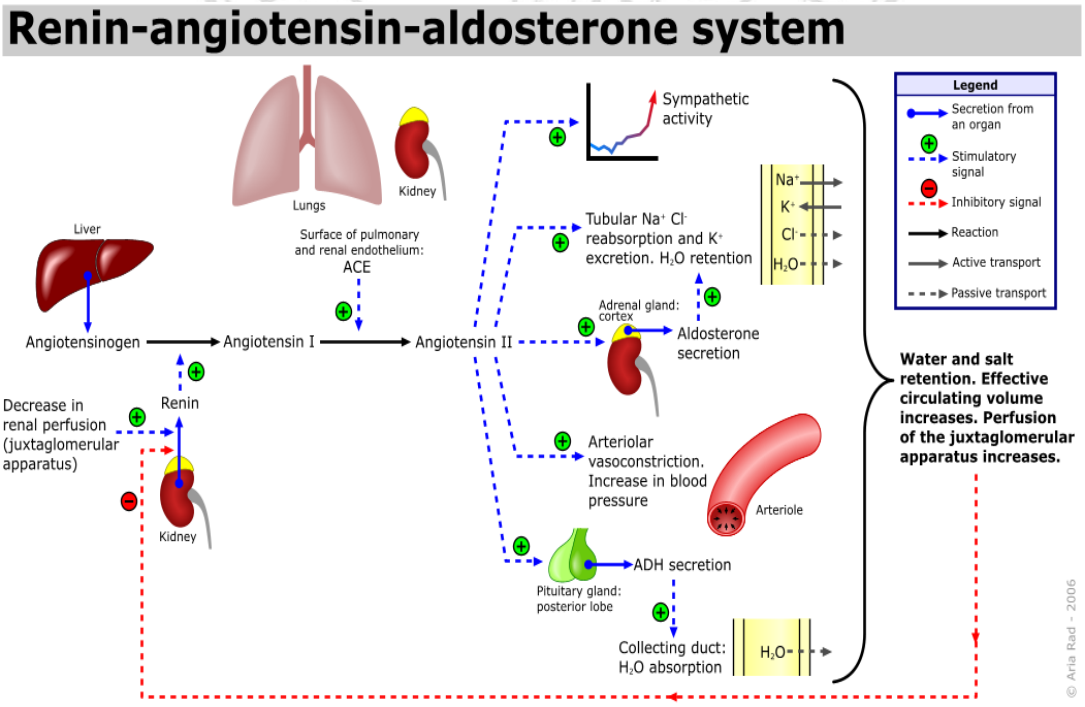

Figure 1.4 The renin-angiotensin system (RAS)

(Source: https://en.wikipedia.org/wiki/Angiotensin-converting_enzyme)

1.5 Antioxidants and Free Radicals

Any substance at comparatively low concentration that can significantly inhibit or delay the oxidation of the substrate is called antioxidant, which includes non-enzymatic compounds (like antioxidant vitamins, trace elements, coenzymes and cofactors) as well as enzymes (such as superoxide dismutase and glutathione peroxidase) (Sies 1993). The principle of the antioxidant defensive system is divided into 3 major systems based on their functions, which are (1) prevention, the first line of the defense against reactive oxygen species by preventing their formation, (2) interception, suppressing chain initiation and/or breaking chain propagation reactions, and (3) repair, repairing the damage once it has occurred.

Reactive oxygen species (ROS), reactive nitrogen species (RNS) and free radicals are normally generated within aerobic organisms, especially humans and other vertebrates, during cellular respiration (Table 1.2). The oxidation of fats and oils can occur during the processing or storage step. Additionally, air pollutants, oxidants in tobacco and UV radition are the other sources to generate the oxidants (Sarmadi and Ismail 2010). With the presence of unpaired electrons, this makes the free radicals unstable and very active to attract the electron from other substances or molecules in the body, resulting in cell or tissue damage (Chalamaiah et al. 2012). In general, the ROS can cause damage in proteins, mutation in DNA and oxidation of membrane phospholipids (Lee et al. 2004). Therefore, the ROS and free radicals play a crucial role as a promoter in several diseases such as hypertension, cancer, inflammation, neurodegenerative disorders, diabetes, aging problems, Parkinson's disease and Alzheimer disease (Bougatef et al. 2009; Ngo et al. 2010).

Under normal circumstances, ROS can be removed by antioxidant defense systems. However, in some conditions, the endogenous defense system fails to protect the body against ROS, resulting in oxidative stress. Oxidative stress is a condition that the elimination of highly reactive molecules (ROS and RNS) is inadequate or lower than the generation. Hence, the demand of synthetic and natural antioxidants increases. Though the synthetic antioxidants are efficient, they may also cause some toxic and hazardous effects. Therefore, the interest of natural antioxidants from food resources is growing especially for their potential health benefits with no or little side effects (Sarmadi and Ismail 2010).

Table 1.2 Intercellular oxidants generation pathways (Klaunig and Kamendulis 2004)

1. Generation of reactive oxygen species via reduction of molecular oxygen

   $O_2 + e^- \longrightarrow O_2^{-\bullet}$ (superoxide anion)

   $O_2^{-\bullet} + H_2O \longrightarrow HO_2^{\bullet}$ (hydroperoxyl radical)

   $HO_2^{\bullet} + e^- + H \longrightarrow H_2O_2$ (hydrogen peroxide)

   $H_2O_2 + e^- \longrightarrow OH^- + OH^{\bullet}$ (hydroxyl radical)

2. Production of reactive nitrogen species

   L-Arginine + $O_2 \longrightarrow NO^{\bullet}$ (nitric oxide) + L-Citrulline

   $O_2^{-\bullet} + NO^{\bullet} \longrightarrow ONOO^-$ (peroxynitrite)

   $ONOO^- + CO_2 \longrightarrow ONOOCO_2^-$ (nitrosoperoxy carbonate)

   $ONOOCO_2^- \longrightarrow NO_2^{\bullet}$ (nitrogen dioxide) + $CO_3^{-\bullet}$ (carbonate anion radical)

3. Fenton reaction

   $H_2O_2 + Fe^{2+} \longrightarrow OH^- + OH^{\bullet} + Fe^{3+}$

1.5.1 Measuring the Antioxidant Activities

Depending on the mechanism involved, the antioxidant capacity assays can be generally classified into two groups: hydrogen atom transfer (HAT) reaction and single electron transfer (SET) reaction-based methods (Figure 1.5 and Table 1.3). The ability to quench free radicals by donating hydrogen atom to form stable compounds is the principle of HAT-based methods. The measurement of antioxidant activity is relied on the kinetic competition, which are more related to the radical chain-breaking antioxidant capacity. HAT assays are quite fast, normally can be done within minutes or seconds, and not affected by the solvent or pH. However, reducing agents and metals can lead to abnormally high reactivity, making HAT assays more complex. The major antioxidants in HAT assays are metal chelators (e.g., EDTA, preventive) and chain-breaking antioxidants (e.g., BHT, sacrificial) acting as hydrogen atom donors (Huang et al. 2005; Prior et al. 2005). The potential of antioxidants to reduce other molecules, together with metals, carbonyls and radicals, by transferring one electron, is the basis of the SET-based methods. Various assays are available to measure the reducing capacity of antioxidant according to ET-based mechanism. The assays are carried out in acidic (ferric ion reducing antioxidant power, FRAP), neutral (trolox equivalent antioxidant capacity, TEAC) or basic (total phenols assay by Folin-Ciocalteu reagent) conditions. In comparison with HAT methods, SET-based methods are relatively slow, pH dependent and need more time to complete the reactions. The potential of deprotonation and ionization of the reactive functional groups involve the relative reactivity. Reactivity is generally measured

in relative to the percentage decrease in product rather than its kinetics or total capacity (Thitilertdecha 2010).

According to the HAT and SET mechanisms, the dominant antioxidant activity depends on the medium and the type of antioxidant so that, with different radicals or oxidant sources, the antioxidants may respond diversely. For example, an antioxidant that possesses strong ion chelation activity may not exhibit any activity in other assays. Therefore, it is clear that the "total antioxidant activity" of a particular sample should not be measured by only one method. Both lipophilic and hydrophilic capacity should be reflected when assessing the "total antioxidant activity". In addition, for physiological activity, hydrogen atom transfer (radical quenching) and electron transport (radical reduction) have to be distinguished (Prior et al., 2005).

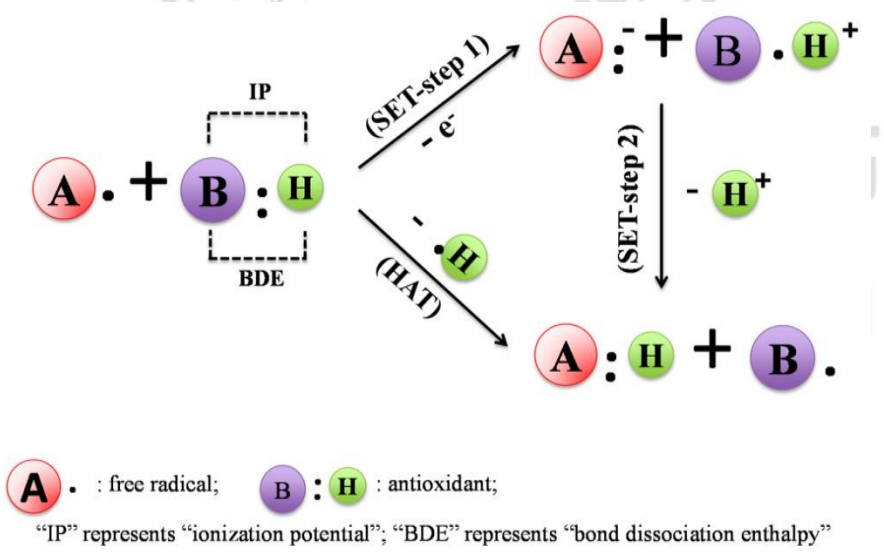

Figure 1.5 Reaction of antioxidant with free radical (Liang and Kitts 2014)

Table 1.3 *In vitro* antioxidant activity assays

Assays involving HAT reactions	
$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet}$	ORAC (Oxygen radical absorbance capacity)
$ROO^{\bullet} + LH \longrightarrow ROOH + L^{\bullet}$	TRAP (total radical trapping antioxidant parameter)
	Croton or β-carotene bleaching assay
	IOU (inhibited oxygen uptake)
	Inhibition of lipid oxidation
	Chemiluminescence (CL)
	TOSC (total oxidant scavenging capacity)
Assays by SET reaction:	
$M(n) + e \text{ (from AH)} \longrightarrow$	FRAP (ferric ion reducing antioxidant power)
$AH^{\bullet+} + M(n-1)$	Copper(II) reduction capacity
Assays utilizing both HAT and SET mechanism	
	TEAC (Trolox equivalent antioxidant capacity)
	DPPH (diphenyl-1-picrylhydrazyl)
	Total phenols assay by Folin-Ciocalteu reagent

1) Trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC assay is based on the ability of antioxidants to scavenge the long-life radical anion $ABTS^{+\bullet}$. Peroxyl radicals or other oxidants oxidize the ABTS to its radical, $ABTS^{+\bullet}$, which is intensely colored (Figure 1.6). The ability of tested compounds to decrease the color reacting directly with the $ABTS^{+\bullet}$ radical is measured as the antioxidant capacity and expressed as relative equivalent to Trolox. This method is simple to operate,

quickly reacts with antioxidants, and functions at a wide pH range, which can be applied to the study of pH effects on antioxidant mechanisms. For these reasons, TEAC method becomes a popular method for studying antioxidant capacity. TEAC reactions can be automated and adapted to microplates, flow injection and stopped flow. However, thermodynamically, a compound that has a redox potential lower than ABTS (0.68 V) can reduce $ABTS^{+\bullet}$, too. Many phenolic compounds have lower redox potential than that. For slow reaction, interpreting in short duration (4-6 min) may result in lower TEAC values since the reaction does not reach its endpoint (Prior et al. 2005).

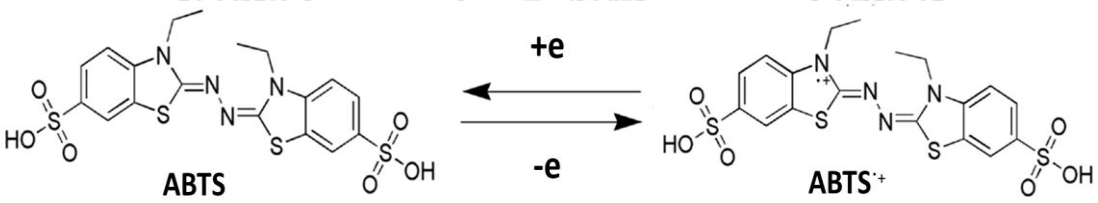

Figure 1.6 Reaction of ABTS (Christopher et al. 2014)

2) Ferric ion Reducing Antioxidant Power (FRAP) assay

FRAP assay is originally developed to measure the reducing power in plasma. This method is based on the reducing power of oxidants to reduce ferric to ferrous ion by electron donation. The extent of conjugation in polyphenols and degree of hydroxylation is associated with the reducing power. Low pH condition of FRAP assay helps to maintain iron solubility. FRAP method is easy, speedy, inexpensive and requires less specialized equipment, which can be performed using automated, semiautomatic or manual methods. However, the ferric ion used in FRAP assay is not relevant

to antioxidant activity mechanistically and physiologically. Besides, FRAP cannot detect compounds that act by radical quenching (HAT) and is limited to measure thiol antioxidants, such as glutathione (Thitilertdecha 2010).

3) Inhibition of lipid peroxidation

Hydroxyl radicals are the products from the reaction between peroxides and ferrous ion (from iron complex), which is termed as Fenton reaction. Produced hydroxyl radicals can react with fatty acids or fatty acyl side chain, resulting in stimulation and initiation of lipid peroxidation. To prevent the damage from Fenton reaction, chelating Fe^{2+} into an inert stabilized redox state reduces the concentration of Fe^{2+} involved in Fenton reaction, leading to prevention of the oxidative damage from Fenton reaction and subsequent initiation of lipid peroxidation chain reaction (Chandra Mohan et al. 2012; Winterbourn 1995). An unstable species, hydroperoxides, generated from linoleic acid oxidation plays a key role in the further autoxidation of lipids and then decomposes into many oxidized products (Figure 1.7). To investigate the production of hydroperoxides from the oxidation of linoleic acid, thiocyanate system is used to provide the blood-red color of ferric thiocyanate, of which ferric ion is resulted from the reaction between ferrous ion and hydroperoxide radicals. The presence of antioxidants delays the lipid oxidation causing the lower rate of peroxide formation. In food-based system, lipid oxidation is the radical source for occurring oxidative reactions. A limitation of this method is the low stability of hydroperoxides and extensive oxidation of lipids, which can occur without any build-up of hydroperoxides. Therefore, the significant

inhibition of transient hydroperoxides by antioxidants may cause false negative detection by this procedure. Hence, to confirm that hydroperoxides are built up in the test conditions chosen for the substrate, the control samples are required (Thitilertdecha 2010).

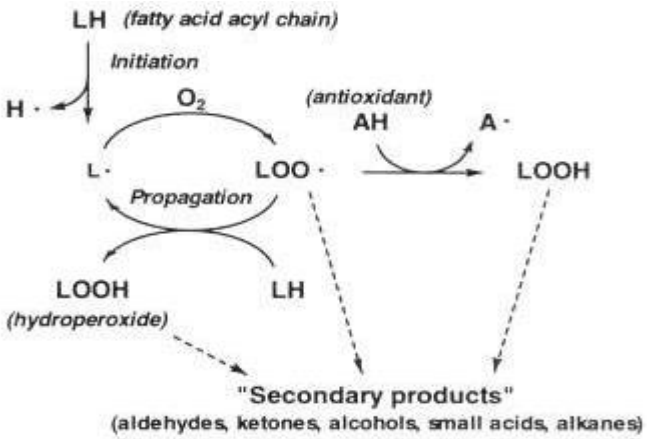

Figure 1.7 Mechanism of lipid autoxidation
(Source: http://www.fao.org/docrep/v7180e/v7180e06.htm)

1.6 Mass Spectrometry (MS)

MS is an analytical technique for the determination of elemental composition of a sample or molecule. This technique consists of two major steps: (1) ionizing chemical compounds to generate charged molecules or molecule fragments; and (2) measuring the mass-to-charge (*m/z*) ratio of the resulted charged particles. Normally, MS is the technique used to identify the physical sample composition by generating a mass spectrum representing the masses of the sample components. Mass spectrum is obtained from a mass spectrometer measurement. Basically, mass spectrometer can be divided into three parts: an ion source, which converts sample molecules in gas phase into ions; a mass analyzer, which sorts the ions according to their masses by applying electromagnetic fields; and a detector system that measures and calculates the

abundance of each presenting ion. Several ionization techniques are applied for the ion source, including electron ionization, chemical ionization, plasma desorption, matrix-assisted laser desorption/ionization (MALDI), thermospray, atmospheric pressure ionization (API), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). For ion separation, many analyzers have been developed such as quadrupole, ion trap, time of flight (TOF), TOF/TOF, fourier transform ion cyclotron (FT-ICR) and hybrid analyzers (quadrupole-TOF). The common used detectors are photographic plate, faraday cup, electron multipliers and electro-optical ion detectors. Apart from these three parts, a computer is another important tool for the MS analysis. The basic functions of computer are controlling the MS operation, processing the obtained signals and interpreting of the data (De Hoffmann and Stroobant 2007; Westermeier and Naven 2002). The typical procedures within the mass spectrometer are (1) sample ions production, (2) different ions masses separation, (3) ions detection of each mass produced, and (4) data collection for generating the mass spectrum (Figure 1.8).

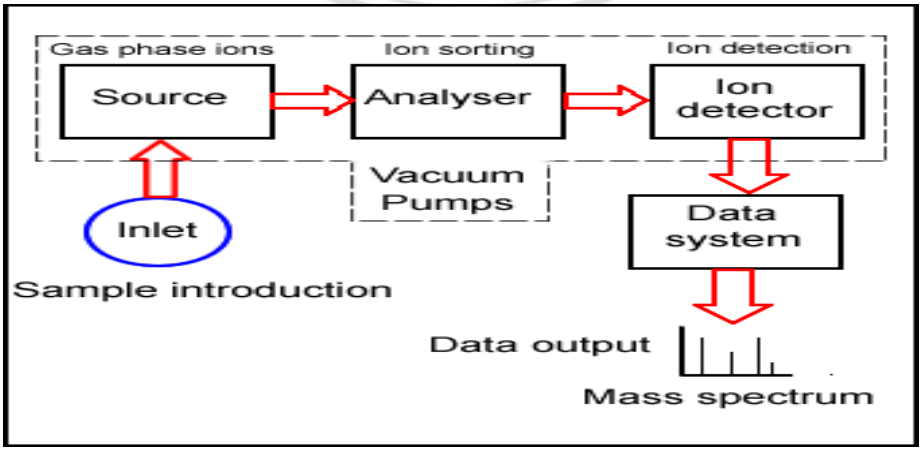

Figure 1.8 Schematic of mass spectrometry diagram
(Source: www.hull.ac.uk/chemistry/masspec3/principles%20of%20ms.html)

Nowadays, for protein identification from the biological extracted samples, MS is becoming the potential tool (Dunham et al. 2012). Before applying with the MS, the protein mixtures are digested into peptides via chemical or enzymatic methods. Charged particles in the gas phase are generated from the peptides and followed by *m/z* ratios separation. The protein identification generally utilizes two detection modes; MS and MS/MS (or tandem mass spectrometry) mode. The actual masses of peptide residues are measured, which indicate the composition of digested proteins in the MS mode, resulting in peptide mass fingerprinting (PMF) for database search. In the MS/MS mode, the actual peptide masses are determined before being fragmented and measured the masses again, resulting in peptide fragment fingerprint (PFF) spectrum, which is usually used for peptide sequence determination (Westermeier and Naven 2002). The schematic diagram for protein identification is displayed in Figure 1.9.

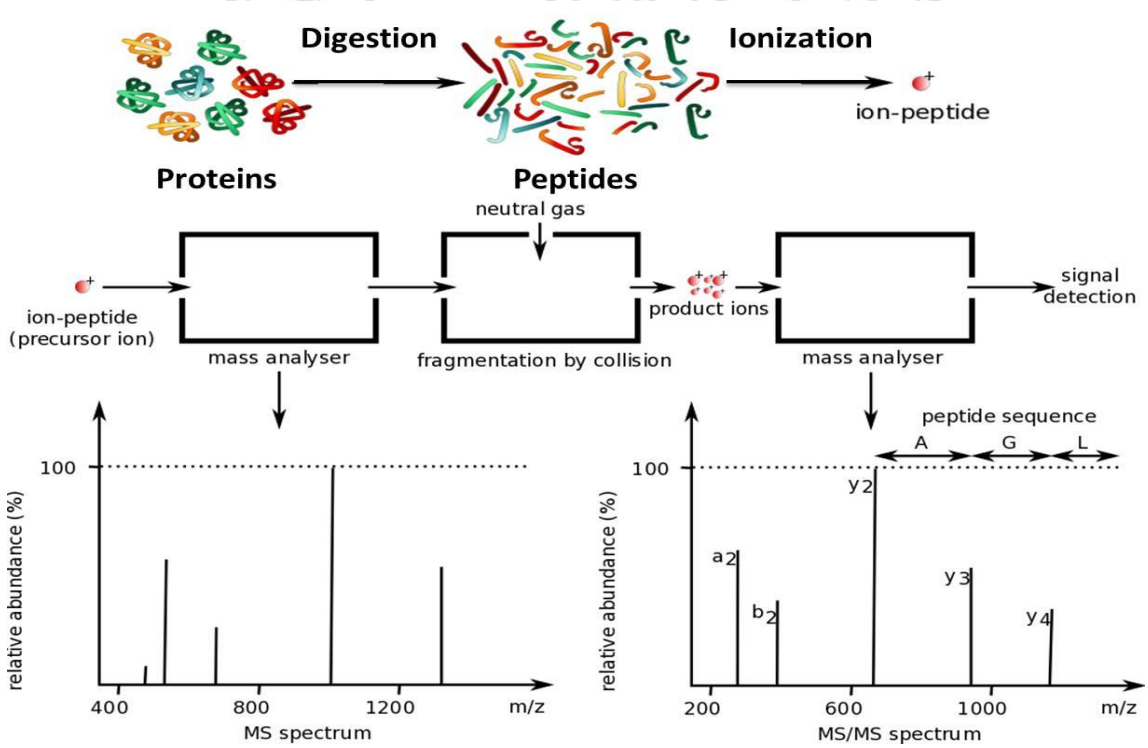

Figure 1.9 Schematic diagram of protein identification

(Modified from Source: https://en.wikipedia.org/wiki/Protein_mass_spectrometry)

Depending on the appropriation and objective of application, the types of instruments are considered for MS analysis. For peptide sequencing, two methods are mainly applied to identify the sequence, which are MALDI-TOF/TOF-MS and LC-MS/MS.

1.6.1 MALDI-TOF/TOF-MS

TOF/TOF analyzer is often coupled with MALDI ion source and uses MS mode for protein identification (Medzihradszky et al. 2000). The mixture, containing mixed peptides with matrix that strongly absorbs at the laser wavelength, is dried out and peptide molecules become incorporated into the crystal lattice of the matrix. The laser beam is focused onto the surface of the matrix, causing desorption and ionization of the matrix and the sample molecules to generate the matrix and analyte ions with positive or negative charges under vacuum condition (Figure 1.10).

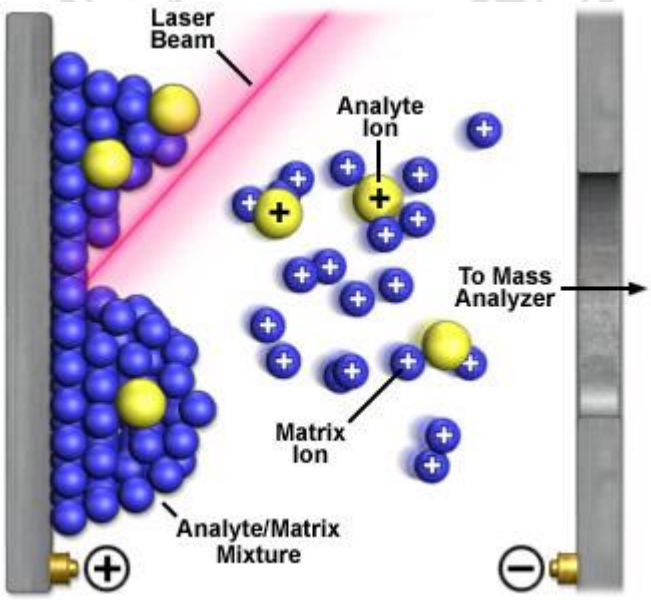

Figure 1.10 Schematic diagram of MALDI

(Source: https://nationalmaglab.org/images/users/icr/techniques/ionization-maldi.jpg)

The gas-phase ions are accelerated by an electrostatic field and extracted into the TOF/TOF analyzer (Figure 1.11). Each ion mass is measured according to the time of flight of ion in TOF flight tube. This instrument uses a short linear TOF and a reflectron TOF as the first and the second analyzers, respectively. Two TOF analyzers are separated by ion deflection gate and collision cell. After released from the collision cell, the precursor ions and its fragments are reaccelerated, resulting in a reduction of kinetic energy spread of the chosen precursor ions and its fragment ions, which allows the one-step detection of ion spectra (De Hoffmann and Stroobant 2007).

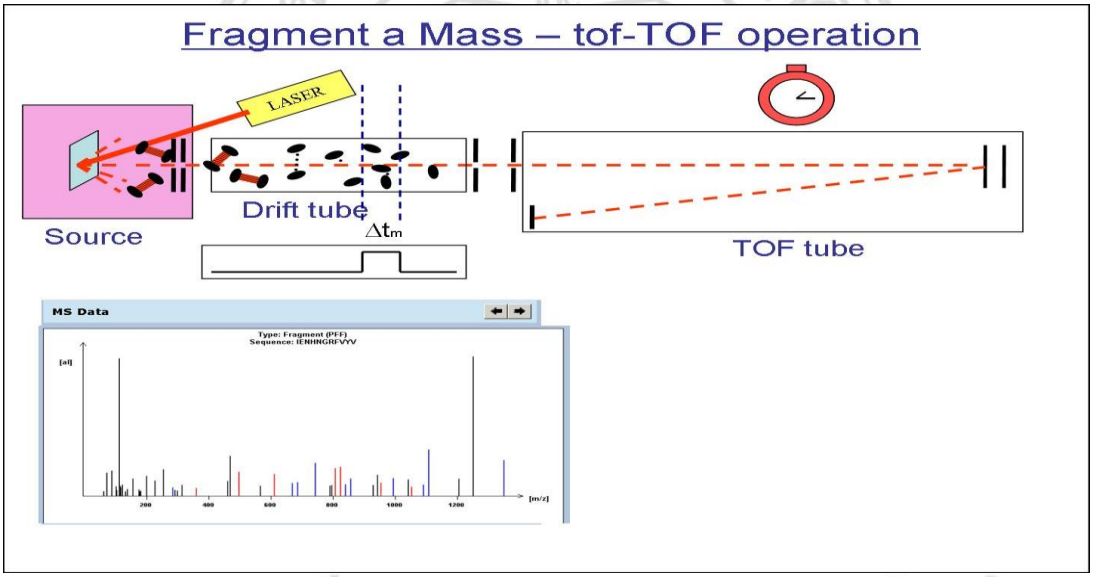

Figure 1.11 Schematic diagram of TOF/TOF mass analyzer
(Source: http://www.mpipz.mpg.de/44542/MALDI-TOF-TOF_MS_MS)

1.6.2 LC-MS/MS

LC-MS/MS is the high throughput technique commonly used for proteomic analysis (Figure 1.12). Briefly, the sample solution is introduced into the ion source for ionization and fragmentation after being eluted from the LC column. The ions with a particular *m/z* ratio are selected as precursor ions for dissociation process for the propose of characterizing the precursor ion in the first mass

analyzer. The precursor ions, or parent ions, are decomposed into product ions, termed as 'daughter ions', in the collision cell by a collisionally activated dissociation (CAD) process. An array of product ions produced by the decomposition of a particular precursor provides a product ion spectrum (Pitija 2009).

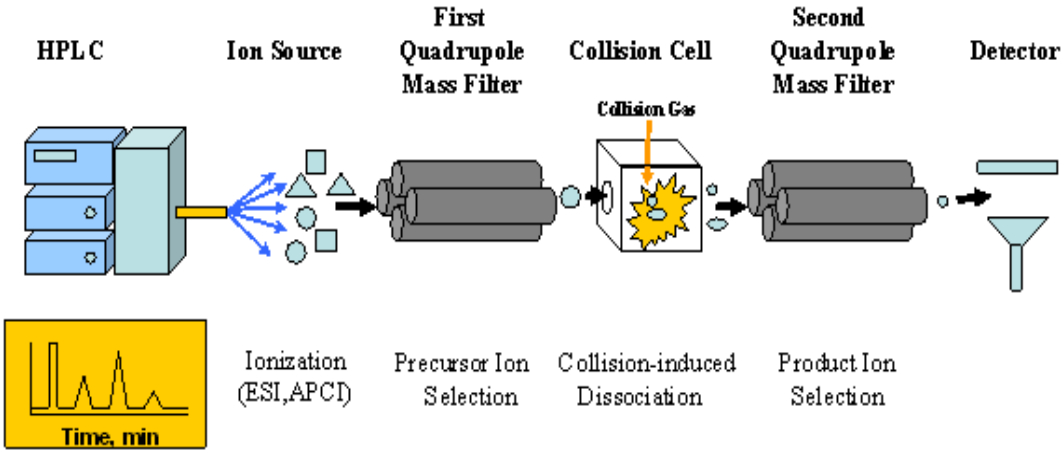

Figure 1.12 Basic principles of LC-MS/MS

(Source: http://www.toray-research.co.jp/kinougenri/biology/bio_004.html)

1.7 Molecular Docking

The emerging of Molecular Docking field was driven by the needs of structural molecular biology and structure-based drug discovery. The growth of powerful and available computers and the easy access to the database of small molecules and proteins are the great factors to make Molecular Docking became more facilitated. The goal of an automated Molecular Docking software is to predict and understand the molecular interaction, including binding modes, posture and binding affinity prediction. Normally, Molecular Docking is performed between a ligand, which is a small molecule, and a target macromolecule, commonly known as 'ligand-protein' docking. However, the

protein-protein docking is becoming more interesting. A scoring function is used in Molecular Docking program in general, which can be seen as an attempt to approximate the standard chemical potentials of the system (Tateing 2012).

Molecular docking is the method to evaluate feasible binding geometries (or often called binding poses or modes) of a putative ligand with a protein target in the three-dimensional view. The ligand positioning relative to the receptor and their conformational states are presented in the binding poses. The correct estimation of free binding energy is an essential consideration of any ligand-receptor interaction since a large number of small molecules can be rapidly and accurately docked into the binding site of receptors via a docking method. Any docking must accomplish these three basic tasks: (1) the binding-site characterization; (2) ligand positioning onto the binding site; and (3) the evaluation of interaction strength for that particular ligand-receptor complex. Three categories of docking procedures are classified depending on the approximation level: (1) rigid body docking (both protein and ligand are treated as rigid bodies); (2) semiflexible docking (only the ligand is considered flexible); and (3) fully flexible docking (both protein and ligand are treated as flexible molecules).

Proteins are highly flexible molecules that exist in multiple conformational states with low-energy barriers separating them. Protein motions can be divided into three categories: small-scale fast motions; large-scale slow domain motions; and renaturation upon the ligand binding. Flexibility of a protein is especially important in the docking process while using low-resolution protein models arising from comparative modeling approaches. On the other hand, ligand flexibility is relatively easier to handle computationally and has now become standard in docking routines. The multiple conformations of the ligands are stored in the database. The incremental construction

method is commonly used to divide the ligand into fragments and incrementally build within the receptor binding site. Exhaustive computational techniques for examining conformational space (molecular dynamics, simulated annealing and Monte Carlo) can be used on conditions that the pace of the search algorithm does not penalize the virtual screening process (Nokthai 2010).

Generally, three protocols are widely employed in Molecular Docking approach, which are GOLD, AutoDock and LigandFit method (Tateing 2012). In this research, AutoDock Vina method was performed to predict the interaction between the bioactive peptides and the ACE. In an AutoDock method, a united-atom model is used for the ligand and receptor presented only polar hydrogens. Partial atomic charges are assigned to the ligand. To correct the scoring functions, the ligand must be assigned for Gasteiger partial charges, since the scoring functions would use Gasteiger charges on the ligand for calibration. The optimized variants of simulated annealing are used in the AutoDock method. Simulated annealing may be regarded as having both local and global search aspects, which utilizes a generic algorithm for global searching and performs the energy minimization with a local search method (Tateing 2012). AutoDock Vina is the improved version developed from AutoDock. AutoDock Vina provides more accurate binding mode predictions, is faster and thus significantly shortens its running time. Vina's user interface designs also contribute to its ease of use as the docking molecule structures and the specification of the search space including the binding site are all that are required (Trott and Olson, 2010).

1.8 Scopes and Aims of This Research

This research focused on utilization and salvation of the Nile tilapia skins, the by-products from fillet processing, to become more valuable products. This also included a determination of their biological activities of the Nile tilapia skin gelatin hydrolysates, which were prepared by various protease enzymes (bromelain, papain, alcalase, trypsin, flavourzyme and neutrase). Our work also extended to the purification and identification of the bioactive peptides derived from the hydrolysates that exhibited great bioactivities. The molecular interactions between identified peptides and ACE were investigated biochemically and computationally to gain better insights on their potencies.