PEPTIDES INHIBITING ANGIOTENSIN I-CONVERTING ENZYME DERIVED FROM EDIBLE MUSHROOMS

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ABSTRACT

Hypertension is one of the major health problems worldwide. Bioactive peptides that inhibit angiotensin I-converting enzyme (ACE) in the blood pressure regulation system (renin-angiotensin system) can contribute to the prevention and treatment of hypertension. Mushroom species studied are high in protein content, which makes them potentially a good source of antihypertensive peptides. Among the nine edible mushrooms evaluated, protein extracts from *Pleurotus cystidiosus* (E1Pc and E5Pc) and *Agaricus bisporus* (E1Ab and E3Ab) exhibited high levels of ACE inhibitory activity. The protein extracts were fractionated by reverse-phase high performance liquid chromatography (RPHPLC) into six fractions each. Fraction 3 from E5Pc (E5PcF3) and fraction 6 from E3Ab (E3AbF6) with the highest ACE inhibitory activity were selected for proteomic analysis. SDS-PAGE analysis showed E5PcF3 consisted mainly of low molecular weight proteins while E3AbF6 contained a variety of high to low molecular weight proteins. There were 22 protein clusters detected by SELDI-TOF-MS analysis with five common peaks found in E5PcF3 and E3AbF6, which had *m/z* values in the range of 3940 to 11413. Further purification by size exclusion chromatography (SEC) yielded seven fractions. SEC fraction 1 of E5PcF3 (E5PcF3C1) as well as SEC fraction 1 and 4 of E3AbF6 (E3AbF6C1 and E3AbF6C4) showed the highest percentages of ACE inhibitory activity. Nine peptides with potential ACE inhibitory activity were selected from the list of peptides obtained by LC/MS/MS analysis. Two peptides were from E5PcF3C1 (AHEPVK, GPSMR) while five peptides (KVAGPK, FALPC, RIGLF, EGEPR, APSAK) and three peptides (AHEPVK, GVQGPM, PSSNK) were selected from E3AbF6C1 and E3AbF6C4, respectively. The nine peptides were chemically synthesized and tested for their IC_{50} values and their stability against gastrointestinal enzymes (pepsin, trypsin and chymotrypsin). The lowest IC_{50} value was exhibited by AHEPVK, where 50% of ACE activity was reduced at a concentration of 62.6 μM. This

was followed by RIGLF and PSSNK, where their IC_{50} values were 115.9 and 129.3 μM, respectively. Interestingly, the ACE inhibitory activity of the peptides was stable at the acidic and basic pH of stomach and intestine. Their biological activity was also retained after gastrointestinal digestion. BIOPEP analysis predicted AHEPVK was stable throughout the digestion process. The remaining eight peptides could be further hydrolyzed by the digestive enzymes to produce fragments of peptides, 2-4 amino acids in length with enhanced ACE inhibitory activity. The inhibition pattern of the three most potent peptides (AHEPVK, RIGLF and PSSNK) was determined by Lineweaver Burk plot. AHEPVK and RIGLF exhibited competitive inhibition pattern against the ACE while PSSNK showed noncompetitive inhibition pattern. Therefore, the peptides tested in the current study, particularly AHEPVK, RIGLF and PSSNK could be potential ACE inhibitors from mushroom. The widely available mushroom source and rare food allergy cases makes the peptides a potential source to be applied as ingredient in functional foods, dietary supplements or produced as pharmaceutical antihypertensive drugs.

ABSTRAK

Hipertensi merupakan salah satu masalah kesihatan utama di seluruh dunia. Peptida bioaktif yang merencat enzim angiotensin-I converting enzyme (ACE) dalam sistem pengawalaturan tekanan darah (sistem renin-angiotensin) boleh menyumbang kepada pencegahan dan rawatan hipertensi. Cendawan yang dikaji mempunyai kandungan protein yang tinggi. Ini menjadikannya sumber yang baik untuk peptida antihipertensi. Antara sembilan cendawan yang dikaji, ekstrak protein daripada *Pleurotus cystidiosus* (E1Pc dan E5Pc) dan *Agaricus bisporus* (E1Ab dan E3Ab) mempunyai aktiviti antihipertensi yang tinggi. Setiap ekstrak protein tersebut difraksi kepada enam fraksi dengan menggunakan 'Reverse Phase High Performance Liquid Chromatography' (RPHPLC). Fraksi 3 daripada E5Pc (E5PcF3) dan fraksi 6 daripada E3Ab (E3AbF6) dengan aktiviti antihipertensi tertinggi telah dipilih untuk analisis proteomik. Analisis SDS-PAGE menunjukkan E5PcF3 terdiri terutamanya daripada protein dengan berat molekul rendah manakala E3AbF6 mengandungi protein dengan pelbagai berat molekul tinggi dan rendah. Terdapat 22 kelompok protein dikesan oleh analisis SELDI-TOF-MS dengan lima puncak yang sama ditemui dalam E5PcF3 dan E3AbF6, yang mempunyai nilai *m/z* dalam lingkungan 3940-11413. Penulenan selanjutnya dengan menggunakan 'Size Exclusion Chromatography' (SEC) menghasilkan tujuh fraksi. Fraksi SEC 1 daripada E5PcF3 (E5PcF3C1) serta fraksi SEC 1 dan 4 daripada E3AbF6 (E3AbF6C1 dan E3AbF6C4) menunjukkan peratusan aktiviti antihipertensi tertinggi. Analisis LC/MS/MS daripada tiga fraksi SEC tersebut telah mengenalpasti sembilan urutan peptida yang berpotensi dalam merencat aktiviti ACE. Dua peptida dari E5PcF3C1 (AHEPVK, GPSMR) manakala lima peptida (KVAGPK, FALPC, RIGLF, EGEPR, APSAK) dan tiga peptida (AHEPVK, GVQGPM, PSSNK) telah dipilih, masing-masing dari E3AbF6C1 dan E3AbF6C4. Sembilan peptida tersebut telah disintesis secara kimia

dan diuji nilai IC_{50} dan kestabilan terhadap enzim pencernaan (pepsin, tripsin dan kimotripsin). Nilai IC_{50} terendah ditunjukkan oleh AHEPVK, di mana 50% aktiviti ACE telah disekat pada kepekatan 62.6 μM. Ini diikuti oleh RIGLF dan PSSNK, dengan nilai IC_{50} masing-masing ialah 115.9 dan 129.3 μM. Semua peptida menunjukkan aktiviti perencatan enzim ACE yang stabil pada pH berasid dan beralkali dalam perut dan usus. Aktiviti biologi mereka juga dikekalkan selepas pencernaan. Analisis BIOPEP menunjukkan AHEPVK adalah stabil sepanjang proses pencernaan. Baki lapan peptida berkemungkinan dihidrolisiskan oleh enzim pencernaan untuk menghasilkan serpihan peptida, 2-4 amino asid panjang yang mempunyai aktiviti perencatan enzim ACE yang meningkat. Corak perencatan tiga peptida yang paling potent (AHEPVK, RIGLF dan PSSNK) ditentukan oleh plot Lineweaver Burk. AHEPVK dan RIGLF menunjukkan corak perencatan kompetitif manakala PSSNK menunjukkan corak perencatan tak kompetitif. Oleh itu, peptida yang diuji, terutamanya AHEPVK, RIGLF dan PSSNK adalah perencat enzim ACE yang berpotensi dari cendawan. Sumber cendawan yang senang didapati dan jarang menyebabkan kes alahan makanan membuatkan peptida tersebut sumber yang berpotensi untuk digunakan sebagai ramuan dalam diet tambahan atau dihasilkan sebagai ubat antihipertensi.

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	Definition
%	Percentage
ACE	Angiotensin I-Converting Enzyme
a.k.a.	Also known as
ANOVA	Analysis of variance
ca.	approximately
Da	Dalton
dH_2O	Distilled water
EDM	Expression Difference Mapping
e.g.	for example
et al.	and others
etc.	et cetera
g	gram
GABA	γ-aminobutyric acid
H	Hour
HCl	Hydrochloric acid
IC_{50}	Concentration of an ACE inhibitor required to inhibit 50% of ACE activity
i.e.	that is (to say)
L	Litre
LC/MS/MS	Liquid Chromatography Mass Spectrometry
mAU	milli Absorbance Unit
mg	milligram
µg	microgram

min	minute
ml	millilitre
µl	microlitre
ND	Not detected
Ret.	Retention time
RPHPLC	Reverse Phase High Performance Liquid Chromatography
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SELDI-TOF-MS	Surface-Enhanced Laser Desorption Ionisation Time-of-Flight Mass Spectrometry
TFA	Trifluroacetic acid
V	Volt

1.0 INTRODUCTION

Hypertension or high blood pressure is defined as persistent elevation of systolic blood pressure greater than 140 mmHg or diastolic blood pressure greater than 90 mmHg. Hypertension affects about 30% of the adult population in most countries and has become one of the leading public health problems worldwide (Lawes *et al.*, 2008). It is one of the risk factors for cardiovascular diseases, such as stroke, heart failure, cardiac arrhythmia and arteriosclerosis (Yunus *et al.*, 2004). It is estimated that approximately 7.6 million of premature deaths were attributed to high blood pressure worldwide (Lawes *et al.*, 2008). The number of adults with hypertension is predicted to increase to a total of 1.56 billion in the year 2025 (Kearney *et al.*, 2005).

The most common blood pressure regulation mechanism has been associated with the renin-angiotensin system. Renin is a protease synthesized by the kidney. It converts angiotensinogen from the liver to angiotensin I, which is a biologically inactive decapeptide. Later, angiotensin I is converted to an active octapeptide vasoconstrictor, angiotensin II, by the action of angiotensin I-converting enzyme (ACE). This reaction will cause the contraction of blood vessels and thereby leads to hypertension. Another blood pressure regulation pathway involves a peptide called bradykinin. It causes blood vessels to enlarge and therefore lowering the blood pressure (Lam *et al.*, 2009). ACE inhibitors block the conversion of angiotensin I to angiotensin II and inhibit the degradation of bradykinin and thus lowers the blood pressure (Sweitzer, 2003; Tom *et al.*, 2002). Therefore, ACE inhibitors have become an important therapeutic approach in the development of antihypertensive drugs.

Many attempts have been made in the production of artificial synthetic ACE inhibitors. Currently, synthetic ACE inhibitor drugs such as captopril, enalapril and lisinopril are used for hypertension therapy in clinical practice. However, synthetic drugs may cause undesirable side effects such as cough, taste disturbances, skin rashes

and angioedema in hypertensive patients, who usually require life-long medication (Brown & Vaughan, 1998; Murray & Crowther, 1998). Since the first discovery of ACE inhibitory peptide from snake venom (Borgheresi *et al.*, 1996), many studies were carried out to find ACE inhibitory peptides from natural sources such as high protein food. Previous studies have reported on the successful purification of ACE inhibitory peptides from foods such as tuna (Lee *et al.*, 2010), buckwheat (Ma *et al.*, 2006), milk (Nakamura *et al.*, 1995) and potato (Pihlanto *et al.*, 2008).

Mushrooms have received increasing attention in recent years because of its nutrition with health-stimulating properties and medicinal effects. Beside nutritional value, the unique colour, taste and aroma of the mushrooms which can sometimes stimulate one's appetite are also the reasons that attract their consumption by humans (Chang, 2008). It is not easy to separate edible and medicinal mushrooms because many of the common edible species that are beneficial in the prevention and treatment of various human diseases and several medically related mushrooms that are also eaten. The most reported medicinal mushroom with blood pressure lowering effect is *Ganoderma lucidum* (Guillamón *et al.*, 2010). Previous studies have also reported on the antihypertensive effect of some edible mushrooms. *Lentinula edodes*, *Flammulina velutipes* and *Pleurotus* species such as *P. cornucopiae*, *P. nebrodensis* and *P. sajor-caju* have been reported to possess hypotensive activity (Hagiwara *et al.*, 2005; Kim *et al.*, 2002; Miyazawa *et al.*, 2008; Tam *et al.*, 1986; Wasser, 2005).

Mushrooms are rich in protein content. The amount of crude protein in mushrooms is ranked below most animal meats but above most other foods, such as milk, vegetables and fruits (Chang & Miles, 2004). The high protein content of mushrooms makes them a potential source of antihypertensive peptide. Besides, the mushroom industry is becoming popular in Malaysia. Hence, a large amount of mushrooms can be obtained from the mushroom farms in Malaysia for protein

extraction. Previous studies have shown successful purification of ACE inhibitory peptides from fruiting bodies of edible mushrooms, *Tricholoma giganteum* (Lee *et al.*, 2004), *Grifola frondosa* (Choi *et al.*, 2001), *Pholiota adiposa* (Koo *et al.*, 2006) and *P. cornucopiae* (Jang *et al.*, 2011).

Thus, based on the reasons mentioned above, the objectives of this study are

1. To evaluate the antihypertensive activity of crude water extracts and proteins from fruiting bodies of nine selected species of edible mushrooms.
2. To carry out bioassay-guided purification of angiotensin I-converting enzyme (ACE) inhibitory peptide from the selected active protein fractions.
3. To test the effect of gastrointestinal digestion on the ACE inhibitory activity of the selected peptides and to determine their ACE inhibition pattern.

2.0 LITERATURE REVIEW

2.1 Hypertension

Blood pressure is the force created by the heart as it pushes blood into the arteries through the circulatory system. It is recorded in two numbers. A normal blood pressure has a reading below 120/80 mm/Hg. The "120" is the systolic blood pressure representing the pressure while the heart contracts to pump blood to the body. The "80" is the diastolic blood pressure representing the pressure when the heart relaxes between beats. A person diagnosed with high blood pressure or hypertension usually has blood pressure reading above 140/90 mm/Hg (Lewis, 2009). It occurs when the arteries become narrowed because of blood clots or cholesterol and plaque build-up in the arteries. Thus, the heart has to pump harder to force the blood to pass through them (Valeo, 2013).

There are two categories of hypertension, primary and secondary hypertension. Primary hypertension, which is also called essential hypertension, is the most common type of hypertension, affecting 95% of hypertensive patients. The cause of primary hypertension is unknown. It is usually linked to genetics, race, unhealthy diet, lack of exercise and obesity (Carretero & Oparil, 2000). Thus, the diagnosis of primary hypertension is made after excluding known causes that comprise secondary hypertension. Secondary hypertension is less common compared to primary hypertension. It affects only 5% of hypertensive patients (Mayet & Coats, 1998). It can be caused by conditions that affect the kidneys, arteries, heart or endocrine system and can be a side effect of many medications. It can also occur during pregnancy (Chiong *et al.*, 2008).

Hypertension has been on the rise worldwide. According to World Health Organization (WHO), it is estimated that nearly one billion people globally have high

blood pressure (Chockalingam *et al.,* 2006). Kearney *et al.* (2005) predicted that the number of adults with hypertension would increase to approximately 1.56 billion in the year 2025. In Malaysia, four out of ten adults suffer from hypertension (Yunus *et al.*, 2004). Hypertension is one of the major risk factors for cardiovascular diseases, such as atherosclerosis, myocardial infarction, stroke and renal failure (Whitworth, 2003). Cardiovascular disease has been the principal cause of death in government hospitals in Malaysia. It accounted for about 23-26% of deaths from 1994 to 2001 (Zambahari, 2004). This high rate is most probably due to the low awareness, low rates of treatment and poor control rates of hypertension among the Malaysians (Lim & Morad, 2004).

Hypertension is a 'silent killer' because it usually causes no symptoms until the people who had it measure their blood pressure (Moser, 2007). Some of the symptoms may develop in people with markedly elevated blood pressure, which includes headache, dizziness, blurred vision, nausea and chest pain. People often do not seek medical care until they have symptoms arising from the organ damage caused by long-term high blood pressure. Examples of organ damage commonly seen in chronic high blood pressure are heart attack, heart failure, stroke, kidney failure, vision loss and leg pain when walking. Thus, besides measuring blood pressure, doctors will also inspect the eyes of hypertensive patients for thickening or narrowing blood vessels, examination of the heart for unusual sounds or beats, examination of the enlargement of kidneys and swelling in the neck (goiter) which is caused by overactive or underactive thyroid, a condition that can elevate blood pressure (Moser, 1992).

There are different ways to treat hypertension, which involves lifestyle modification alone or with pharmacological treatment. Lifestyle changes, such as losing weight, exercise, limit alcohol intake, reduce sodium intake, stop smoking and taking a well balanced diet rich in fresh fruits and vegetables are able to control the blood pressure level and prevent hypertension (Barbosa-Filho *et al.*, 2006). Meta-analysis

carried out by Neter and coworkers showed that weight loss makes an important contribution to the treatment of hypertension, especially in subjects taking antihypertensive medication (Neter *et al.*, 2003). Based on the findings by Geleijnse *et al.* (2003) and Bray *et al.* (2004), a great reduction in blood pressure level was observed among adults with high potassium and reduced sodium intake. A control trial on 2234 participants have shown a dose-response relationship between the mean percentage of alcohol reduction and the mean blood pressure reduction (Xin *et al.*, 2001). In addition, The National Heart, Lung and Blood Institute (NHLBI), U.S. has developed a diet plan known as DASH diet (Dietary Approaches to Stop Hypertension) to prevent and control hypertension. The diet is rich in fruits, vegetables, whole grains and low-fat dairy foods. The diet plan has been proven to significantly lower the blood pressure (Svetkey *et al.*, 1999). Beside blood pressure lowering effect, a cohort study among middle-aged women during 24 years of follow-up showed association of DASH diet with a lower risk of coronary heart disease and stroke (Fung *et al.*, 2008). Lifestyle changes may effectively lower the blood pressure, however it requires patience and motivation.

Previous study has reported on the successful reduction of blood pressure and its associated symptoms by massage therapy (Hernandez-Reif *et al.*, 2000). Other treatments such as acupuncture (Weil *et al.*, 2007), Qigong (Cheung *et al.*, 2005), Tai Chi (Tsai *et al.*, 2003) and yoga (Cohen *et al.*, 2011) have also been reported to show hypotensive effect.

Beside lifestyle changes and drug medication, botanical medicines can also help to normalize blood pressure. Yarnell and Abascal (2001) have reported on the effectiveness of rauwolfia, garlic and hibiscus to reduce the blood pressure reading. Oral administration of extracts from fruiting bodies of reishi has been proven to show hypotensive effect in spontaneously hypertensive rats (Kumakura *et al.*, 2008). Non-herbal supplements such as fish oil (Knapp & FitzGerald, 1989), co-enzyme Q10

(Hodgson *et al.*, 2002), green algae (Merchant *et al.*, 2002), vitamin C (Duffy *et al.*, 1999) and combination of vitamins and minerals (Farvid *et al.*, 2004) had also shown successful blood pressure lowering effect. Ernst (2005) had summarized the clinical trial reports on the treatment of hypertension by lifestyle modification, herbal medicines and non-herbal supplements. Therefore, people have to be alerted about the importance of consuming dietary supplements from natural products to maintain their health and prevent diseases; "prevention is better than cure".

2.2 Antihypertensive drugs

There are many classes of antihypertensive drugs, which lower blood pressure by different means. The most widely used are diuretics, beta-blockers, alpha-blockers, $alpha_2$-agonists, calcium channel blockers, angiotensin II receptor antagonists and ACE inhibitors (Barbosa-Filho *et al.*, 2006).

Diuretics help the kidneys to eliminate excess salt and water from the body's tissues and blood, thereby, cause vasodilatory effect and reduce the blood pressure (Ernst & Mann, 2011). It is recommended for patients with mild to moderate hypertension. Three main classes of diuretics are thiazide-diuretics, loop diuretics and potassium sparing diuretics (Shah *et al.*, 2004). The increase of serum uric acid concentration induced by diuretics can trigger a gouty attack. Besides formation of gout, other side effects of diuretics include interstitial nephritis, ototoxicity, sun sensitivity and urinary frequency (Ernst & Mann, 2011).

Alpha-blockers and beta-blockers are antihypertensive drugs that reduce the blood pressure by blocking the action of the sympathetic nervous system and reduce the force of heart muscle's contractions. Alpha-blockers and beta-blockers are not recommended to be used as first-line therapy for hypertension. They can be considered as second-line

or third-line agents when drugs from other classes are not sufficient to achieve target blood pressure (Bangalore & Messerli, 2008; Rossitto *et al.*, 2010). Consumption of beta-blockers have undesirable adverse effects, such as drowsiness, sleep disturbance, visual hallucinations, depression, blurring of vision and reducing the physical performance of body (Bangalore & Messerli, 2008; Bengtsson, 1984). Side effects of alpha-blockers include dizziness, fatigue and somnolence (Rossitto *et al.*, 2010). Furthermore, National Institute for Health and Clinical Excellence in the UK has downgraded the role of beta-blockers due to their risk of provoking type 2 diabetes (NICE, 2006).

Alpha$_2$-agonist can help to treat hypertension by stimulating the brain to open peripheral arteries and ease the blood flow. They are usually administered in combination with a diuretic. Clonidine, a type of alpha$_2$-agonist has been used as an antihypertensive agent since the late sixties. Administration of clonidine may cause side effects such as drowsiness, dry mouth, increase of blood glucose concentration and impotence (Basker *et al.*, 2009).

Another type of antihypertensive drug, calcium channel blockers or also known as calcium antagonists work by blocking the entry of calcium into muscle cells in artery walls and thus reduce the arterial pressure and decrease the blood pressure (Hernández-Hernández *et al.*, 2002). Opie and Schall (2002) had reported that calcium channel blockers gave a higher risk of myocardial infarction. Besides myocardial infarction, consumption of calcium channel blockers had also been associated to the higher risk of mortality and cancer (Cheng & Behar, 1997).

ACE inhibitors regulate the blood pressure through the renin-angiotensin system and the kallikrein-kinin system (Murphey *et al.*, 2003). Its mechanism of action will be discussed in detail in the next part of the thesis (Section 2.3).

The different classes of antihypertensive drugs mentioned above have been shown to effectively lower blood pressure in hypertensive patients. The frequency of antihypertensive agents used differs according to countries. For example, calcium channel blocker is preferred in Japan whereas beta-blockers and diuretic are the most widely prescribed antihypertensive drugs in the U.S. and Europe (Higashi *et al.*, 2000; Muratani *et al.*, 1996). National Institute for Health and Clinical Excellence in the UK has recommended ACE inhibitor as the first choice for the treatment of hypertension (NICE, 2006). According to Forni *et al.* (2010), the choice of the antihypertensive drug prescribed should depend on its side-effect profile. Some antihypertensive drugs are better than others in specific groups of patients. For example, ACE inhibitors are preferred to be prescribed to treat hypertension in patients with diabetes (Opie & Schall, 2002). Some studies have reported on the benefits of combination therapy compared to monotherapy. The treatment involving the combination of two or more antihypertensive agents requires less time to achieve target blood pressure and better tolerability compared to higher dose monotherapy (Frank, 2008). Example of combination therapy include ACE inhibitor and calcium antagonists (Forni *et al.*, 2010).

2.3 ACE inhibitors

Blood pressure is controlled by a number of different interacting biochemical pathways. It can be increased or decreased depending on the pathway that predominates at any given time (Hong *et al.*, 2008). The most common blood pressure control has been associated with the renin-angiotensin system. Renin is a protease synthesized by the kidney. It converts the angiotensinogen from the liver to angiotensin I, which is a biologically inactive decapeptide. Later, angiotensin I is converted to an active octapeptide vasoconstrictor, angiotensin II, by the action of ACE. This reaction will cause the contraction of blood vessels and thereby raising the blood pressure (Kumar *et*

al., 2010). Another blood pressure pathway involves a peptide called bradykinin. It can cause the enlargement of blood vessels and thereby lowering the blood pressure. In this pathway, ACE has a promoting effect in the degradation and inactivation of bradykinin (Lam *et al.*, 2009; Pina & Roque, 2008). ACE inhibitors have the effect to suppress the conversion of angiotensin I to angiotensin II and inhibit the degradation of bradykinin and further lowering the blood pressure (Kumar *et al.*, 2010; Sweitzer, 2003). Thus, ACE inhibitors have become an important therapeutic approach in the development of antihypertensive drugs.

Besides being used as a drug for patients with hypertension, ACE inhibitor also plays a role in the regulation of other types of diseases. According to O'Keefe *et al.* (2001), ACE inhibitor is effective in the treatment of patients with atherosclerosis. There is also an improvement in diagnosis when ACE inhibitors were supplied to patients who have diabetes but with normal blood pressure. It reduces the new-onset of type 2 diabetes through unknown mechanisms (O'Keefe *et al.*, 2001). The Heart Outcomes Prevention Evaluation Study Investigators (HOPE) reported that ramipril, a type of ACE inhibitor, is effective in the reduction of death rate, myocardial infarction, stroke revascularization, cardiac arrest, heart failure and complications related to diabetes (HOPE, 2000). Another type of ACE inhibitor, captopril has also been tested to be effective in the protection against deterioration in renal function in insulin-dependent diabetic nephropathy (Lewis *et al.*, 1993).

There is a correlation between cardiovascular diseases, diabetes and hypertension. Cardiovascular diseases are the major causes of mortality in persons with diabetes and hypertension (Sowers *et al.*, 2001). Pharmacologic therapy that interrupts the renin-angiotensin system may reduce cardiovascular and renal diseases in diabetic patient with hypertension (Sowers & Haffner, 2002). This was supported by a report showing ACE inhibitor therapy reduced the probability of hypertensive patients to develop type

2 diabetes (Sowers *et al.*, 2001). In contrast, hypertensive patients who were taking another type of antihypertensive medication, beta-blockers had a higher risk of getting diabetes (Gress *et al.*, 2000). Therefore, ACE inhibitors are prescribed as the first line treatment for hypertensive patients with diabetes (Vivian & Rubinstein, 2002).

Basically, ACE inhibitors can be divided into three groups based on their reactive moiety that binds with ACE. The first group consists of captopril and zofenopril which binds with ACE by a sulfhydryl (SH) linkage. *In vitro* studies suggest that the presence of SH may confer properties other than ACE inhibition to these drugs. For instance captopril and zofenopril has exhibited cardioprotective and free radical scavenging abilities in animal studies (Liu *et al.*, 1992). The second group of ACE inhibitor contains a phosphinyl (PO_2) as its reactive moiety. Fosinopril is the only ACE inhibitor that was categorized in this group (Brown & Vaughan, 1998). The largest group of ACE inhibitor contain a carboxyl (CO_2). It consists of enalapril, ramipril, quinapril, perindopril, lisinopril, imidapril and trandolapril (Buttar, 1997).

Various synthetic ACE inhibitors such as captopril, ramipril, enalapril, cilazapril, pentopril, trandolapril, perindopril, lisinopril, alacepril and imidaprilat may effectively block the conversion of angiotensin I to angiotensin II or inhibit the breakdown of bradykinin. However, these pharmaceutical products may have side effects such as cough, taste disturbances, angioedema, skin rashes and fetal anomalies in pregnant woman (Brown & Vaughan, 1998). The incidence of cough has been reported in patients receiving captopril, enalapril, lisinopril and ramipril (Salami & Katibi, 2005). This side effect was higher during captopril and enalapril treatment (Yesil *et al.*, 1994). The presence of SH group in captopril has been reported to confer toxicity such as taste disturbance, skin rashes and proteinuria (Reid, 1997). Administration of benazepril has been proven to develop hyperkalemia whereas captopril, lisinopril and enalapril may cause the incidence of angioedema (Ahuja *et al.*, 2000; Jason, 1992; Kharasch, 1992).

Thus, this has raised the awareness among the scientists to search for ACE inhibitors from natural sources to replace the chemically synthesized drugs and to reduce the cost of current drug therapy.

The ACE inhibitory activity can be quantified with methods that include colorimetric, fluorometric, radiochemical and chromatographic method (Murray *et al.*, 2004). In general, most of the ACE inhibitory assays rely on the principle of determining hippuric acid formation from hippuryl-histidyl-leucine (HHL) resulted from the removal of the C-terminal dipeptide histidylleucine by the action of ACE. The hippuric acid formed is extracted with ethyl acetate and measured at 228 nm by a spectrophotometer (Cushman & Cheung, 1971). However, the unhydrolyzed HHL or the presence of other compounds in the sample extracts were also easily extracted into the ethyl acetate and absorbed at 228 nm. Thus, this may cause the overestimation of the ACE activity. To overcome these problems, Lam *et al.* (2007) had introduced a colorimetric method using a new synthetic substrate, 3-hydroxybutyrylglycyl-glycine (3HB-GGG) for measuring ACE inhibiting activity. The substrate, 3HB-GGG is cleaved into amino acids (Gly and Gly-Gly) and 3-hydroxybutyric (3HB) by the actions of ACE (Lam *et al.*, 2007). The ACE inhibitory activity is measured spectrophotometrically according to the amount of cleaved 3-hydroxybutyric (3HB) from 3HB-GGG (Lam *et al.*, 2009). This assay kit is available commercially and known as ACE Kit-WST (Dojindo Laboratories, Kumamoto, Japan). The ACE inhibitory activity in the current study was tested using the ACE-kit WST.

2.4 Edible mushrooms and antihypertensive effect

Mushrooms are defined as macrofungi with distinctive and visible fruiting bodies which can be either epigeous or hypogeous (Chang, 2008). Edible mushrooms are used

extensively in culinary especially among the Chinese, European and Japanese (Chang, 2008). Beside nutritional value, the unique colour, taste and aroma of mushrooms are the reasons that attract their consumption by humans (Guillamón *et al.*, 2010). Mushrooms contain large amount of protein, carbohydrates, fibre and low in fat (Chang & Miles, 2004). Besides, mushrooms can be a good source of vitamins B_1, B_2, B_{12}, C and D as well as mineral elements, such as calcium, potassium, magnesium, phosphorus, copper, iron and selenium. (Chang, 2008; Chang & Buswell, 1996). Thus, they are a good diet for people with hypertension (Moura *et al.*, 2007).

It is not easy to differentiate edible and medicinal mushrooms because many of the common edible species are beneficial in the prevention and treatment of various human diseases and several medically related mushrooms are also eaten (Guillamón *et al.*, 2010). Among 14,000-15,000 species of mushrooms in the world, about 1800 species of mushrooms have been estimated to contain potential medicinal properties (Chang, 2008). Edible mushrooms which are widely cultivated by the local mushroom growers and sold in the supermarkets in Malaysia are oyster mushrooms (*Pleurotus* spp.), shiitake [*Lentinula edodes* (Berk.) Pegler], jelly mushroom (*Auricularia* spp.) and paddy straw mushroom [*Volvariella volvacea* (Bull.) Singer] (Chang & Lee, 2004). Besides the locally cultivated mushroom, some imported mushrooms are also found in the supermarkets in Malaysia. Examples are Portabella, white button mushroom [*Agaricus bisporus* (J.E. Lange) Imbach] and shimeji (*Hypsizygus* sp.). Others are edible mushrooms collected in the wild e.g. *Schizophyllum commune* Fr. and *Termitomyces* spp. which are sometimes available at wet markets. Truffles (*Tuber* spp.), maitake [*Grifola frondosa* (Dicks.) Gray] and monkey's head [*Hericium erinaceus* (Bull.) Pers.] are normally found in speciality shops or in selected restaurants (Lee *et al.*, 2009b).

Many mushrooms have been reported to possess antihypertensive effect. The most reported medicinal mushroom with blood pressure lowering effect is *Ganoderma lucidum* (Curtis) P. Karst. (Guillamón *et al.*, 2010; Paterson, 2006). Mycelium extract of *G. lucidum* has exhibited blood pressure lowering effect in animal studies (Lee & Rhee, 1990). Ten triterpenes isolated from *G. lucidum* have been shown to have ACE inhibitory activity (Morigiwa *et al.*, 1986). Besides *G. lucidum*, some common edible mushrooms have also been reported to show hypotensive effect, namely maitake and oyster mushroom (Agrawal *et al.*, 2010; Talpur *et al.*, 2002).

Some studies suggested that hypotension can be induced by the mushroom extracts as an impact resulting from the primary effect. For instance, suppression of sympathetic nervous system was reported to be involved in hypotension (Sato *et al.*, 1987). According to an *in vivo* study carried out by Lee and Rhee (1990), extract of *G. lucidum* showed a decreased in the systolic and diastolic blood pressure as well as inhibition of renal efferent sympathetic nerve activity in the animals. However, it did not decrease the heart rate. Therefore, it was suggested that the hypotensive action of *G. lucidum* was due to its suppression effect on the sympathetic nerve activity (Lee & Rhee, 1990). Another example, seaweeds were reported to exhibit marked antihyperlipidemic activity (Ren *et al.*, 1994a). Enhanced excretion of cholesterol into faeces that resulted from consumption of seaweed has been suggested as an important factor for reducing blood pressure (Ren *et al.*, 1994b). Mushrooms, such as *L. edodes* and *H. erinaceus* have been reported as potent hypolipidemic agents (Yang *et al.*, 2003a; Yoon *et al.*, 2011). Thus, the reduction of cholesterol due to the consumption of mushrooms can help to reduce blood pressure in hypertensive patients.

Mushrooms can be a good source of antihypertensive drugs. Compared to most pharmaceuticals, the biologically active compounds that are extracted from mushrooms have extraordinarily low toxicity, even at high doses (Chang & Miles, 2004). The

antihypertensive effects reported in the previous studies on the nine edible mushroom species tested in the current study are as follows:

2.4.1 *Agaricus bisporus*

Agaricus bisporus is commonly known as button mushroom. It grows naturally in grasslands, fields and meadows in North America and Europe. It is the most cultivated mushroom globally and the most consumed mushroom in Western countries (Jeong *et al.*, 2010). It is cultivated in more than 100 countries, which produced about 2 million metric tons of fresh weight in 1997 (Chang & Miles, 2004). Its fruiting body is either white or brown colour according to variety.

In hypercholesterolemic rats, consumption of *A. bisporus* had decreased their plasma total cholesterol, low density lipoprotein, hepatic cholesterol and triglyceride concentration (Jeong *et al.*, 2010). This is in line with the study conducted by Chen *et al.* (2012c), which reported that the fruiting body of *A. bisporus* having high amount of lovastatin, a compound with cholesterol lowering effect. Lovastatin has been proven to reduce the risk of coronary heart disease (Furberg *et al.*, 1994). Beside lovastatin, *A. bisporus* also contains γ-aminobutyric acid (GABA) which has been demonstrated to have hypotensive effect in *in vivo* studies (Tanaka *et al.*, 2009).

2.4.2 *Flammulina velutipes*

Flammulina velutipes (Curtis) Singer is commonly known as enoki in Japan or golden needle mushroom (*Jin zhen gu*) in China. It is sometimes called winter mushroom. It grows naturally on the stumps of the Chinese Hackberry tree, called enoki in Japanese and also on some other trees such as mulberry and persimmon trees. China, Japan, Korea and Taiwan are the major producers of *F. velutipes* with worldwide production of

284,000 metric ton in 1997 (Chang & Miles, 2004). There is a difference in appearance between wild and cultivated *F. velutipes*. Cultivated *F. velutipes* have white colour fruiting bodies with long and thin stem while their wild types usually have a dark brown colour with shorter and thicker stem. Supermarkets usually sell cultivated form of *F. velutipes*.

Flammulina velutipes has been reported to contain fibrinolytic enzyme which exhibits a remarkable degree of fibrinolytic activity (Park *et al.*, 2007). Fibrin is formed from fibrinogen by the action of protease thrombin and it is then polymerized to form clot over a wound site (Falvo *et al.*, 2010). Fibrinolysis is an important process in the body that keeps fibrin blood clots from growing. Fibrin clot formation and fibrinolysis are normally well balanced in the body system (Cesarman-Maus & Hajjar, 2005). However, factors such as dysfunction or disease of the liver could lead to impaired fibrinolysis, which would subsequently cause the occurrence of cardiovascular diseases (Tabak *et al.*, 2009). Clinical studies have demonstrated a positive correlation in the increased level of plasma fibrinogen and prevalence of hypertension (Poli *et al.*, 2000; Shankar *et al.*, 2006). This could be due to the relation of fibrinogen to cause the increase in blood viscosity (Letcher *et al.*, 1981), hyperinsulinemia (Vâlek *et al.*, 1995) and inflammation (Engstrom *et al.*, 2002) which are among the factors involved in the elevation of blood pressure. Therefore, *F. velutipes* could exhibit its blood pressure lowering effect through fibrinolytic activity. Besides fibrinolytic enzyme, high amount of GABA and lovastatin have been detected in the fruiting body of *F. velutipes* (Chen *et al.*, 2012c). GABA and lovastatin is a potent hypotensive and cholesterol lowering compound, respectively (Furberg *et al.*, 1994; Tanaka *et al.*, 2009).

2.4.3 *Hericium erinaceus*

Hericium erinaceus is commonly known as *Hou tou gu* or Monkey's head in China due to its fruiting body that looks like the head of a baby monkey. It is known as Yamabushitake in Japan and sometimes referred to as the bear's head or lion's mane mushroom. Its fruiting body is white in colour but tends to be yellowish. Initially, it was available only in the wild where it grows on old and dead wood in the deep forest but now it is being grown on substrates in plastic bag cultures. The largest producer of *H. erinaceus* is China with the annual production of approximately 2800 metric ton in 1998 (Chang & Miles, 2004).

Hericium erinaceus was found to have the ability to reduce serum triglyceride and total cholesterol levels as well as decrease the body weight and fat weight (Hiwatashi *et al.*, 2010; Wang *et al.*, 2005). Besides, it also showed to have hypolipidemic effects in *in vivo* study (Yang *et al.*, 2003a). Thereby, it is an ideal food for hypertension and cardiovascular disease patients.

2.4.4 *Lentinula edodes*

Lentinula edodes is commonly known as shiitake. It is a Japanese name derived from the mushroom associated tree, *shii* tree (*Castanaopsis cuspidate* Schottly) and *take*, Japanese word for mushroom (Wasser, 2005). It is the second most cultivated mushroom worldwide, which produce 1,564,000 metric ton in 1997 (Chang & Miles, 2004). Initially, it is grown mainly in Asian countries but now it has spread to the United States, Australia, Canada, Brazil and a few European countries (Chang & Miles, 2004).

Lentinula edodes has been reported to contain therapeutic properties for treating cardiovascular disease where it can lower cholesterol level in plasma and remove lipids

in liver (Rai *et al.*, 2005). Kabir *et al.* (1987) had reported on the blood pressure lowering effect in spontaneously hypertensive rats fed with shiitake. A compound known as eritadenine was found in *L. edodes* which is a hypocholesterolemic agent (Yoon *et al.*, 2011). Eritadenine can accelerate the excretion of ingested cholesterol and its metabolic decomposition (Sugiyama *et al.*, 1995). Besides, *L. edodes* was also found to contain high amount of ergothioneine (Chen *et al.*, 2012c). Dietary consumption of ergothioneine has been associated with reduced risk of cardiovascular disease (Martin, 2010). A high amount of vitamin D has been detected in *L. edodes* that were grown under exposure to ultraviolet lights (Lee *et al.*, 2009a; Mattila *et al.*, 2002a). Several clinical studies have shown association between vitamin D and hypertension. Hypertensive patients who received ultraviolet B (UVB) exposure 3 times weekly for 6 weeks have shown significant reduction in systolic and diastolic blood pressure (Krause *et al.*, 1998). Elderly women who received dietary supplement of vitamin D showed significant reduction of blood pressure after 8 weeks (Pfeifer *et al.*, 2001). Interestingly, the blood pressure lowering effect of vitamin D has been associated to the renin-angiotensin system (Li, 2003). Vitamin D was reported to increase the productions of intracellular calcium which is a suppressor to the renin activity (Burgess *et al.*, 1990). Furthermore, vitamin D induces relaxation of vascular smooth muscle cells which would improve dilation of arterial blood vessels and subsequently decreases blood pressure (Tarcin *et al.*, 2009).

2.4.5 *Pleurotus* species

Pleurotus spp. is commonly known as oyster mushroom. The word *Pleurotus* derives from Greek which means ‘side ear’. About 875.6 thousand metric ton of *Pleurotus* spp were produced in 1997 (Chang & Miles, 2004). Various species of this wood-rotting fungus are found globally. It is cultivated in many countries in Europe and Asia. One of

the reasons for its popularity is its ability to grow on a wide range of substrates due to its characteristic that secrete a wide range of enzymes, which can degrade polysaccharides, such as lignin, cellulose and hemicelluloses found in the biomass of forest and agricultural crop residues. This mushroom fruits at a relatively high temperature. Thus, it can be grown in tropical and subtropical areas, as well as in temperate regions during the summer season (Chang & Miles, 2004). There are five *Pleurotus* spp cultivated in Malaysia, which includes *P. citrinopileatus* Singer (yellow oyster mushroom), *P. cystidiosus* O.K. Mill. (abalone mushroom), *P. flabellatus* (Berk and Br.) Sacc (pink oyster mushroom), *P. florida* (Mont.) Singer (white oyster mushroom) and *P. pulmonarius* (Fr.) Quđ (a.k.a. *P. sajor-caju*) (grey oyster mushroom).

A significant reduction in blood pressure was observed in hypertensive patients after the consumption of oyster mushroom (Agrawal *et al.*, 2010). *In vivo* studies have also shown *Pleurotus* spp contain effective lipid lowering properties (Alam *et al.*, 2011; Schneider *et al.*, 2011), cholesterol lowering effect (Rai *et al.*, 2005) and antihypertensive effects (Miyazawa *et al.*, 2008). The results found in the *in vivo* studies were in line with the results of the bioactive compounds found in *Pleurotus* spp. D-mannitol, a phytochemical from *Pleurotus* spp was reported to exhibit hypotensive effect (Hagiwara *et al.*, 2005). Fruiting body of *Pleurotus* spp. also have high amount of ergothioneine (Chen *et al.*, 2012c). Dietary consumption of ergothioneine has been associated with reduced risk of cardiovascular disease (Martin, 2010). Similarly, a cholesterol lowering compound, lovastatin was also detected in large amount in the fruiting bodies of *Pleurotus* spp (Chen *et al.*, 2012c).

2.5 Mushrooms proteins and ACE inhibitory peptides

According to Chang and Miles (2004), the protein content of cultivated mushrooms ranges from 1.75-5.9% of their fresh weight. This is in line with the report by Manzi *et*

al. (1999), who reported the protein content of *P. ostreatus*, *P. eryngii*, *P. pulmunarius* and *L. edodes* in the range of 1.26 to 5.75 g/100 g of fresh weight (Manzi *et al.*, 1999). On a dry weight basis, the protein content in mushrooms may vary from as little as 4-8% for species of *Auricularia* to as high as 43% for *Volvariella volvacea* (Chang, 1999). The protein content in mushroom is about 2-12 times of the amount found in vegetables and fruits. Milk has been reported to have 2.9-3.3% protein. On the other hand, the protein content of various meat is as follows: pork (9-16%), beef (12-20%), chicken (18-20%), fish (18-20%) (Chang, 1999). Thus, it can be concluded that the amount of crude protein in mushrooms rank below animal meats but well above most other foods, such as vegetables, fruits and milk.

The role of proteins as physiologically active components in the diet is being increasingly recognized (Iwaniak & Dziuba, 2009). A previous study had reported on the inverse relationship of dietary protein with blood pressure (Stamler *et al.*, 1996). This could be due to the bioactive peptides encrypted within the sequence of proteins. Bioactive peptides have been defined as specific fragment that have a positive impact on the body systems upon administration, namely the cardiovascular, digestive, immune and nervous systems (Kitts & Weiler, 2003). Bioactive peptides are inactive within the sequence of the parent protein and can be released in three ways, which are enzyme hydrolysis by digestive enzyme, hydrolysis by proteolytic microorganisms and through food processing (Korhonen & Pihlanto, 2006). Some natural or synthesized peptides which act on the renin-angiotensin system have the ability to prevent and treat hypertension through inhibition of angiotensin I, angiotensin converting enzyme (ACE) and Ang II type 1 receptor (AT1) (Hong *et al.*, 2008; Korhonen & Pihlanto, 2003; Korhonen & Pihlanto, 2006). Since the first discovery of ACE inhibitory peptide in snake venom, there has been an increased interest in finding ACE inhibitory peptides from food proteins.

Many food proteins or artificial synthetic products are a good source of these inhibitory peptides (Korhonen & Pihlanto, 2003). Examples of the successful purification of ACE inhibitors from food proteins are shown in Table 2.1. Most of the peptides listed in Table 2.1 consist of short amino acid sequences and low molecular masses. This is in line with the report by Natesh *et al.* (2003), which stated low molecular weight peptides have added advantage as ACE inhibitor because large peptide molecules are restricted from fitting into the active site of ACE.

Although ACE inhibitory peptides from food proteins may have lower ACE inhibitory activity *in vitro* compared to the ACE inhibitory drugs, they do not have the harmful side effects and also lower the cost of healthcare (Hong *et al.*, 2008). Besides *in vitro* studies, several tests in spontaneously hypertensive rats also suggest a significant suppression of the development of hypertension with a diet rich in ACE inhibitory peptides (Lee *et al.*, 2010; Wang *et al.*, 2008). Thus, ACE inhibitory peptides are not only effective for treatment of hypertension, it can also be used as functional food for prevention of hypertension. There are several ACE inhibitory peptides from bonita and sardine which have been commercialized in Japan and Canada. Examples of the product names are Valtyron®, PeptAce, Vasotensin® and Levenorm® (Harnedy & FitzGerald, 2012). These products are claimed to be suitable for consumption as functional food by individuals with mild hypertension. Additionally, these products were reported to be free of side effects (EFSA Panel on Dietetic Products Nutrition and Allergies, 2010a; EFSA Panel on Dietetic Products Nutrition and Allergies, 2010b). Therefore, this has increased the interest among researchers in finding ACE inhibitory peptides in other commonly consumed foods.

Previous studies have showed successful purification of ACE inhibitory peptides from fruiting bodies of edible mushrooms, *Grifola frondosa* (Choi *et al.*, 2001), *Tricholoma giganteum* (Lee *et al.*, 2004), *Pholiota adiposa* (Koo *et al.*, 2006) and

Pleurotus cornucopiae (Jang *et al.*, 2011). The comparison of their peptide sequences, molecular masses, IC_{50} values and kinetic mode of inhibition are illustrated in Table 2.2. Peptides purified from *G. frondosa*, *T. giganteum* and *P. adiposa* have short amino acid sequences, which was in the range of 3-6 amino acids in length whereas ACE inhibitory peptides from *P. cornucopiae* consist of 14-17 amino acids in length. The IC_{50} values for the ACE inhibitory activity were in the range of 3.2-539.9 μM.

Table 2.1: ACE inhibitory peptides derived from food protein.

Food source	Peptide sequence	Molecular mass (Da)	[b] IC_{50} (μM)	Reference
Bonita fish	LKPNM	601.7	2.4	Fujita and Yoshikawa (1999)
Broccoli	YPK	[a]NM	23.7	Lee *et al.* (2006)
Buckwheat	GPP	270.56	23.1	Ma *et al.* (2006)
Clam	VKP	NM	3.7	Tsai *et al.* (2006)
	VKK	NM	1045	
Cuttlefish	VYAP	448.2	6.1	Balti *et al.* (2010)
	VIIF	491.1	8.7	
	MAW	407.2	16.32	
Egg	RVPSL	571.6	20	Liu *et al.* (2010a)
Garlic	SY	269	66.3	Suetsuna (1998)
	SF	253	130.2	
Milk	PYVRYL	809.9	2.4	Quiros *et al.* (2005)
Mung bean	KDYRL	693.8	26.5	Li *et al.* (2006)
	VTPALR	655.8	82.4	
Oyster	DLYDY	687.7	143	Shiozaki *et al.* (2010)

Table 2.1 (Continued)

Rice	TQVY	NM	18.2	Li *et al.* (2007)
Salmon	AP	186.18	0.322	Gu *et al.* (2011)
	VR	273.30	1.214	
Sea squirt	AHIII	565.7	37.1	Ko *et al.* (2011)
Soy meal	DLP	NM	4.8	Wu and Ding (2002)
	DG	NM	12.3	
Soybean	LVQGS	495.70	43.7	Rho *et al.* (2009)
Spinach	MRWRD	762.8	2.1	Yang *et al.* (2003b)
	MRW	473.6	0.6	
	LRIPVA	667.8	0.38	
Sweet	GTEKC	NM	275.8	Huang *et al.* (2008)
potato	VKAGE	NM	141.56	
	KIEL	NM	849.7	
	SNIP	NM	228.3	
	VRL	NM	208.6	
	RF	NM	392.2	
	VVGAK	472.5	1.14	Huang *et al.* (2011a)
	GPCSR	518.5	61.67	Huang *et al.* (2011b)
Wakame	AIYK	494	213	Suetsuna and Nakano (2000)
	YKYY	636	64.2	
	KFYG	514	90.5	
	YNKL	537	21	

[a] NM=Not mentioned

[b] IC_{50} denotes the concentration of an ACE inhibitor required to inhibit 50% of ACE activity.

Table 2.2: ACE inhibitory peptides derived from edible mushrooms.

Mushroom sp	Peptide sequence	Molecular mass (Da)	[a] IC_{50} (μM)	Inhibition pattern	Reference
Grifola frondosa	VIEKYP	747.8	129.7	Competitive	Choi *et al.* (2001)
Tricholoma giganteum	GQP	301	3.2	Competitive	Lee *et al.* (2004)
Pholiota adiposa	GQGGP	414	254	Competitive	Koo *et al.* (2006)
Pleurotus cornucopiae	RLPSEFDLSAFLRA	1622.85	277.3	Competitive	Jang *et al.* (2011)
	RLSGQTIEVTSEYLFRH	2937.26	539.9	Noncompetitive	

[a] IC_{50} denotes the concentration of an ACE inhibitor required to inhibit 50% of ACE activity.

2.6 Purification of ACE inhibitory peptide

2.6.1 Extraction of proteins from mushroom

Proteins can be recovered from inside (intracellular proteins) and outside (extracellular proteins) of the cell. Most of the proteins studied in the early days were isolated from extracellular fluids because it was easy to obtain and extracellular proteins are more stable (Scopes, 1994). Intracellular proteins were obtained by disrupting the cells and the proteins will be released into the aqueous extract. Various cell disruption methods have been developed which include physical, chemical, enzymatic and mechanical

methods (Middelberg, 1995). Table 2.3 showed the principles of cell disruption techniques commonly used in the laboratory.

Plant cells are difficult to disrupt because they have cellulosic cell walls. Mushrooms also contain cell walls which are made of chitin (Vetter, 2007). Hence, they require vigorous mechanical techniques to break up the cell wall. Previous studies had reported on the higher concentration of proteins obtained from fungi using sonicator, bead milling and homogenization (Klimek-Ochab *et al.*, 2011; Taskova *et al.*, 2006). In the current study, homogenization using a waring blender was used to extract proteins from mushroom.

Table 2.3: Principles of cell disruption methods.

Cell disruption methods		Principle
Physical	Osmotic shock	This method involved suspension of cells in hypotonic solution which would cause the rupture of cells and release its cellular content (Klimek-Ochab *et al.*, 2011).
	Freeze-thaw	This technique involves freezing of cells in freezer followed by thawing at room temperature. Multiple cycles of freezing-thawing are necessary for efficient lysis of cells (Ward & Swiatek, 2009).
Chemical	Detergent	Suspension of cells in solution containing detergent to solubilise the cell membrane. Commonly used detergents include Triton-X and Tween series. However, proteins could be denatured in the presence of detergents and an additional purification polishing

Table 2.3 (Continued)

Chemical		step has to be carried out to remove the detergent from the product. Hence, this method is avoided where possible (Klimek-Ochab *et al.*, 2011).
	Solvents	Suspension of cells in organic solvents such as acetone and toluene which acts on the cell membrane by solubilising its phospholipids and release the integral proteins and subcellular components (Dey *et al.*, 1997). Limitation of using solvents is similar to detergent i.e. they have to be removed from samples after the cell disruption process (Scopes, 1994).
Enzymatic		Suspension of cells in solution containing enzymes that digest the cell wall. The enzymes commonly used include lysozyme, zymolase, cellulase, pectinase, etc. The disadvantage of enzymatic method is the instability of enzyme which will subsequently cause the method to be irreproducible (Taskova *et al.*, 2006).
Mechanical	Bead mill	Sample is placed in a vessel containing small beads. Cell disruption of sample is due to the grinding action of the rolling beads and the impact resulting from the cascading beads (Taskova *et al.*, 2006).
	Homogenizer	Homogenizing of samples in a blender will break up large cells and shears apart smaller ones (Scopes, 1994).

Table 2.3 (Continued)

Mechanical	Sonicator	Sample is subjected to high frequency ultrasound (above 16kHz). Tiny bubbles formed by the high frequency vibration will release mechanical energy in the form of shock waves equivalent to several thousand atmosphere of pressure (Scopes, 1994).
	French press	The device consists of a cylinder fitted with a plunger. Sample placed in the cylinder is pressurized using the plunger (Scopes, 1994).

2.6.2 Protein separation by ammonium sulphate

The distribution of hydrophilic and hydrophobic residues at the surface of the protein molecule will determine its solubility in various solutions. Hence, different types of proteins in a solution can be separated by causing some of the proteins to precipitate by altering some properties in the solution, such as by changing the ionic strength, pH and by adding miscible organic solvents, inert solutes or polymers (Scopes, 1994). Precipitation by organic solvents such as alcohol or acetone is not commonly used because of its tendency to denature protein at room temperature. Precipitation with polyethylene glycol (PEG) is also not commonly used due to the difficulty to remove it from the protein solutions (Ahmed, 2005). Salting out of proteins by ammonium sulphate precipitation is usually used because ammonium sulphate has high solubility even at lower temperatures which is important as proteins have to be maintained in cold condition. Addition of salt to the solution will increase the surface tension of water and subsequently increase the hydrophobic interaction between protein and water (Wingfield, 2001). Proteins in the solution tend to fold to decrease their surface area in an attempt to minimize contact with the solution. This will cause the aggregation and

precipitation of proteins. The precipitate can be collected by centrifugation. Proteins with larger amount of hydrophobic surface precipitate at lower salt concentrations. Table 2.4 showed the amount of ammonium sulphate required to precipitate proteins at a certain salt saturation.

Table 2.4: Amount of ammonium sulphate (in grams) required per litre of solution from initial percentage saturation to target percentage saturation at 4^{o}C.

		Target % saturation									
		10	20	30	40	50	60	70	80	90	100
		Amount of ammonium sulphate (g) required per litre of solution									
Initial % saturation	0	56	114	176	243	313	390	472	561	662	767
	10		57	118	183	251	326	406	494	592	694
	20			59	123	189	262	340	424	520	619
	30				62	127	198	273	356	449	546
	40					63	132	205	285	375	469
	50						66	137	214	302	392
	60							69	143	227	314
	70								72	153	237
	80									77	157
	90										79

(Source: http://www.encorbio.com/protocols/AM-SO4.htm)

2.6.3 Pooling of protein fractions by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a useful method to separate he components of a protein mixture. It can also be used to estimate the molecular weight of proteins. In the SDS-PAGE analysis, proteins were denatured by boiling. SDS, an anionic detergent which was added to the samples would bind to the proteins and further denature secondary and non disulfide linked tertiary structures of the proteins (Scopes, 1994). A negative charge was applied to each protein in proportion to its mass. The proteins were then loaded onto a gel consisting of acrylamide and bisacrylamide. An electric field is applied across the gel causing the negatively charged proteins to migrate across the gel towards the positive electrode. Each protein will move differently through the gel matrix. Small proteins will migrate easily through the pores in the gel. Therefore they are able to travel farther down the gel. Larger proteins will have more difficulties to move in the gel matrix. Therefore, they will remain closer to the point of origin (Manz *et al.*, 2004; Westermeier, 2011). After electrophoresis, the gel is stained either with Coomassie Brilliant Blue or silver staining to allow visualization of the separated proteins. In the current study, proteins precipitated by ammonium sulphate were visualised by SDS-PAGE to determine the similarity of protein profiles in the salt fractions. Fractions with similar protein profiles were pooled in the same group.

2.6.4 Protein separation by column chromatography

Column chromatography is the most common method used for protein separation. A solution containing protein mixtures is flowed through a column which is packed with stationary phase material. There are a wide range of stationary phase materials, which include silica, dextran or cellulose (Janson, 2011). Different proteins interact differently with the stationary phase material. Therefore, they are separated based on their degree

of adsorption to the column material where each protein will require different time to pass through the column. Usually protein purification protocol involves with one or more chromatographic steps. The chromatographic steps used in the current study were reverse phase chromatography (RPHPLC) and size exclusion chromatography (SEC).

The column material used in the RPHPLC is usually silica based. Proteins are eluted according to their hydrophobicity by a gradient of increasing concentrations of mobile phase, i.e. organic solvent containing an ionic modifier. The most commonly used mobile phase is acetonitrile containing trifluoroacetic acid (TFA). More hydrophobic protein will be eluted later from the column (Aguilar, 2004).

In SEC, proteins are separated according to size and shape. The SEC column consists of beads with different pore sizes. The separation depends on the ability of the proteins to enter the channels in the porous beads. Small proteins have more potential channels that they can access but larger proteins are excluded from the channels and will pass quickly between the beads. Therefore, proteins are separated in order of decreasing molecular weight, with the largest protein eluting from the column first (Stanton, 2004).

2.7 Mass spectrometry analysis of ACE inhibitory peptides

Biological activities of proteins are related to the amino acid composition, sequence and size of peptides. Mass spectrometry (MS) has been established as the primary method for protein identification from biological origin. It is an analytical technique that measures the mass to charge ratio of charged particles. MS instruments consist of three modules; an ion source, a mass analyzer and a detector (Manz *et al.*, 2004).

An ion source is an electro-magnetic device that is used to ionize the sample to be analyzed. The ionization methods commonly used in proteomics are Matrix Assisted

Laser Desorption Ionization (MALDI), Surface-Enhanced Laser Desorption Ionization (SELDI) and Electrospray Ionization (ESI). MALDI uses a solid plate where a UV active matrix is spotted on the plate together with the protein sample. The laser hits the spot on the crystallized matrix and transfers energy from the matrix molecule to the sample which will then vaporizes the sample and sending a plume of ions into the MALDI source. These plumes of ions are collected and sent out simultaneously to a mass analyzer. The principle of SELDI is similar to MALDI. It involves the ionization of samples that have been co-crystallized with a matrix on a target surface. However, unlike MALDI which has a single target surface, SELDI has different ProteinChip chromatographic surfaces that are uniquely designed to retain proteins from complex mixtures according to their specific properties, such as hydrophobic, anionic, cationic or metal ions. These ProteinChips array are commercially known as H50, Q10, CM10 and IMAC30 arrays, respectively. Proteins were left to interact with the spot surface by letting it to incubate for a period of time. Proteins and contaminants that do not bind to the spot surface are then removed by washing followed by the addition of energy-absorbing matrix to the spot. Samples are ionized by a pulsed-UV laser beam and their molecular masses were measured by mass analyzer (Seibert *et al.*, 2004; Vorderwülbecke *et al.*, 2005). In ESI, initially protein samples were injected through a charged capillary. This will allow charged particles to be formed at the capillary tips. Evaporation of the particles increases the charge of the particles and explodes into smaller droplets. Repeated evaporation and fission will lead to the formation of small charged droplets. These ions will then travel into the analyzer to be scanned one mass at a time (Aebersold & Mann, 2003; Manz *et al.*, 2004). ESI coupled with liquid chromatography (LC) is usually used for the detection of peptides from a complex mixture. Prior separation by LC enables the detection of peptides with similar masses by distinct resolution. According to Zhang *et al.* (2013), attempting to electrospray a

mixture of analytes simultaneously without prior separation would result in the ionization of only the most hydrophobic molecules. A more sensitive application, nanoLC-ESI/MS/MS has been developed and widely used in proteome analysis. The separation of peptides by LC at a flow rate of nanolitre per minute followed by direct electrospray into the mass spectrometer has been proven to improve the sensitivity of peptide separations and subsequently increased the amount of proteins detected, especially the low abundant proteins (Wilm & Mann, 2001).

The mass analyzer is the component that separates the ions created from the ion sources by their mass-to-charge ratios. Usually MALDI and SELDI are attached to a Time of Flight (TOF) mass analyzer. Ions generated in the MALDI and SELDI source are held for a brief time and pulsed into the TOF mass analyzer at the same time. Ions with lower mass and higher velocity will hit the detector first. On the other hand, quadrupole mass analyzer is widely used together with ESI. It acts as a mass-selective filter. Only the ions in a certain range of mass to charge ratio are passed through the system at a time (Trauger *et al.*, 2002; Vorderwülbecke *et al.*, 2005).

The detector records the charged or current produced when an ion hits a surface. The signals from these charged or currents will produce a mass spectrum. For each mass spectrum, software is used to determine the peptide masses by matching it with the protein database (Wiśniewski, 2008).

Prior to MS analysis, protein was digested with a proteolytic enzyme, usually trypsin that will cleave the protein at specific sites to create a complex peptide mixture. The peptides are subjected to mass spectrometric analysis. The masses of these peptides provide a peptide mass fingerprinting which can be used in database searches to identify the protein of interest. This protein profiling technique is known as ‘bottom-up’ approach and has been widely used in proteomic research (Aebersold & Mann, 2003).

NanoLC-ESI/MS/MS has been used by previous studies for protein identification of ACE inhibitory peptide after purification by reversed phase and size exclusion chromatography (Balti *et al.*, 2010; Ghassem *et al.*, 2011; Zhao *et al.*, 2009). Thus, in the current study, this method has been selected to study the protein profiling of ACE inhibitors in mushrooms.

According to Vermeirssen et al., (2004), the bioactivity of protein or peptide is inversely correlated to chain length. Previous studies had reported on the higher ACE inhibitory activity exhibited by lower molecular weight peptides. Guang and Phillips (2009) had summarized a list of potent low molecular weight ACE inhibitory peptides derived from plant foods. SELDI-TOF-MS has been reported as the most effective method in profiling low molecular weight proteins and peptides (<20 kDa), providing a complementary visualization technique to 2D-PAGE (Issaq *et al.*, 2003). Thus, SELDI-TOF-MS has been used for the protein profiling analysis of low molecular weight proteins in the current study. Hydrophobic ACE inhibitor has been reported to be more effective than hydrophilic ACE inhibitor (Moskowitz, 2002; Moskowitz & Johnson, 2004). Therefore, ProteinChip Array H50 with hydrophobic surface was used in the SELDI-TOF-MS analysis in the current study.

2.8 Structure-activity relationship of ACE inhibitory peptides

There are two isoforms of human ACE, somatic (sACE) and testicular (tACE) forms. Somatic ACE and tACE is composed of 1277 and 701 amino acid, respectively (Pina & Roque, 2008). Somatic ACE is expressed in most tissues and it has two homologous catalytic domains (N- and C-domains). Testicular ACE exhibits only one domain that is identical to the C-domain of sACE (Pina & Roque, 2008). The N-domain is involved in the control of hematopoietic stem cell proliferation. The C-domain is the dominant site involved in blood pressure regulation and it consists of three subsites, S', S1' and S2'

(Phelan & Kerins, 2011). ACE hydrolyses peptides by the removal of a dipeptide from the C-terminus, as in the cleavage of His-Leu from angiotensin-I to convert it to the vasoconstrictor angiotensin-II (Coates, 2003). Accordingly, the C-terminal of the inhibitory peptides is important for the inhibitory effect of the ACE enzyme.

Some authors have proposed structural requirements for potent ACE inhibitory peptide. Curtis *et al.* (2002) reported that peptides with potent ACE inhibitory activity usually consist of 2-5 amino acids in length and contain one or a combination of an aromatic amino acid, a branched-chain amino acid and an imino proline. There were also reports which suggested that potent ACE inhibitors contain hydrophobic amino acid residues such as proline, phenylalanine, tryptophan and tyrosine at the C-terminal whereas the N-terminal usually consists of branched aliphatic amino acid such as valine, isoleucine and arginine (Byun & Kim, 2002; Cheung *et al.*, 1980).

More recent studies have involved the utilisation of quantitative structure activity relationship (QSAR) model. This computational method is a useful tool to search information relating chemical structure of peptides to biological activities. It has been applied for predicting potential ACE inhibitor by peptide structures. According to the QSAR model established by Pripp and co-workers, increased side chain hydrophobicity in the C-terminal and decreased side chain size of the penultimate amino acid enhanced ACE inhibitory potential of peptides up to six amino acids in length (Pripp *et al.*, 2004; Pripp *et al.*, 2005). QSAR model reported by Wu *et al.* (2006a) showed that amino acid with bulky side chain and hydrophobic side chain is preferred for dipeptides. For tripeptides, aromatic amino acids were preferred for the C-terminal, while positively charged amino acids were preferred for the middle position and hydrophobic amino acids were preferred for the N-terminal (Wu *et al.*, 2006a). For tetrapeptides, tyrosine and cysteine are preferred for the first position from the C-terminus, histidine, tryptophan and methionine for the second position with isoleucine, leucine, valine and

methionine for the third position and tryptophan for the fourth position (Wu *et al.*, 2006b). He *et al.* (2012) had built a QSAR model with an artificial neural network. Their finding shows that protein with high content of hydrophobic amino acid, especially aromatic amino acids is suitable to produce ACE inhibitory peptides.

Small molecular weight peptides have been reported to be more effective in ACE inhibitory activity. Several studies had shown the optimum molecular weight of ACE inhibitory peptides are less than 3 kDa (He *et al.*, 2006; Jiang *et al.*, 2010; Miguel & Aleixandre, 2006). This is due to the capability of small peptides to pass the gastrointestinal tract, easily absorbed and reach the peripheral target sites (Quiros *et al.*, 2005). In addition, large peptide molecules are restricted from fitting into the active site of ACE (Natesh *et al.*, 2003).

2.9 Effect of gastrointestinal digestion on the ACE inhibitory peptides

Proteins or peptides delivered by the oral route have to be able to withstand the strong pH and maintain their biological activities throughout the digestion processes in the gastrointestinal tracts before they reach their target site inside the body. The most important sites for the digestion of proteins and peptides are the stomach and the small intestine. Gastric juice in the stomach contains mainly hydrochloric acid (HCl) and protease enzyme, pepsin. The approximate pH of the gastric juice is about pH 2.0. The low pH of the gastric juice will denature the ingested proteins, making them easier to be digested enzymatically. Besides, the acidic condition in the stomach is required for activation of pepsin. Pepsin prefers to cleave proteins at N-terminal of aromatic amino acid, such as phenylalanine, tryptophan and tyrosine (Fruton *et al.*, 1961).

Although the chemical digestion of protein is initiated by pepsin in the stomach, most of the digestion and absorption processes of protein take place in the small intestine (Mahato *et al.*, 2003). It is a tube-like structure consisting of three major

regions, duodenum, jejunum and ileum (McClements & Li, 2010). Proteolytic enzymes in the small intestine include trypsin and chymotrypsin. They are secreted by the pancreas. In contrast to pepsin, the pancreatic enzymes function optimally at moderate alkaline pH, approximately pH 8.0. Trypsin preferentially cleaves peptide bonds on the C-terminal side of lysine and arginine side chains (Olsen *et al.*, 2004). Chymotrypsin is less specific than trypsin. They cleave proteins at C-terminal of a variety of amino acid, mostly those of aromatic or large hydrophobic amino acid residues, such as phenylalanine, tyrosine and tryptophan (Okada *et al.*, 2008).

Some peptides with potent *in vitro* ACE inhibiting activity failed to show antihypertensive activity after oral administration due to the possible hydrolysis of these peptides by gastrointestinal enzymes (Fujita *et al.*, 2000). However some peptides showed higher ACE inhibitory activity after gastrointestinal digestion. They are known as pro-drug type inhibitor (Li *et al.*, 2004). These peptides are expected to show long lasting antihypertensive activity after oral administration (Vercruysse *et al.*, 2010). Preliminary experiment by gastrointestinal enzyme incubation *in vitro* provides an easy method to evaluate the fate of these peptides after oral administration.

2.10 Inhibition pattern of inhibitors against ACE enzyme

Theoretically, the inhibition pattern of enzyme inhibitor can be separated into three groups: competitive, noncompetitive and uncompetitive. In the competitive inhibition, both the substrate (S) and inhibitor (I) compete for the same active site of enzyme (E). Only the ES complex leads to product formation. In the noncompetitive inhibition, the inhibitor tends to bind to the enzyme at a site other than the active site while in the uncompetitive inhibition, the inhibitor binds only to the ES complex. The ESI complex formed in the noncompetitive and uncompetitive inhibition mode will cause the inactivation of enzyme, hence it is unable to produce product (Copeland, 2005;

Jao *et al.*, 2012). The inhibition mode of ACE inhibitory peptides can be evaluated by performing Lineweaver-Burk plots of 1/absorbance change per minute (1/V) versus 1/substrate concentration (1/[S]). ACE inhibition was measured by varying the concentration of substrate in the absence and presence of different concentrations of inhibitor. The example of graphs obtained for competitive, noncompetitive and uncompetitive inhibition mode is shown in Figure 2.1. Competitive inhibitors have the same y-intercept as uninhibited enzyme but different x-intercepts. Noncompetitive inhibition produces plots with the same x-intercepts as uninhibited enzyme but different y-intercepts whereas uncompetitive inhibition produces different intercepts on both the y- and x-axes (Chang, 2005).

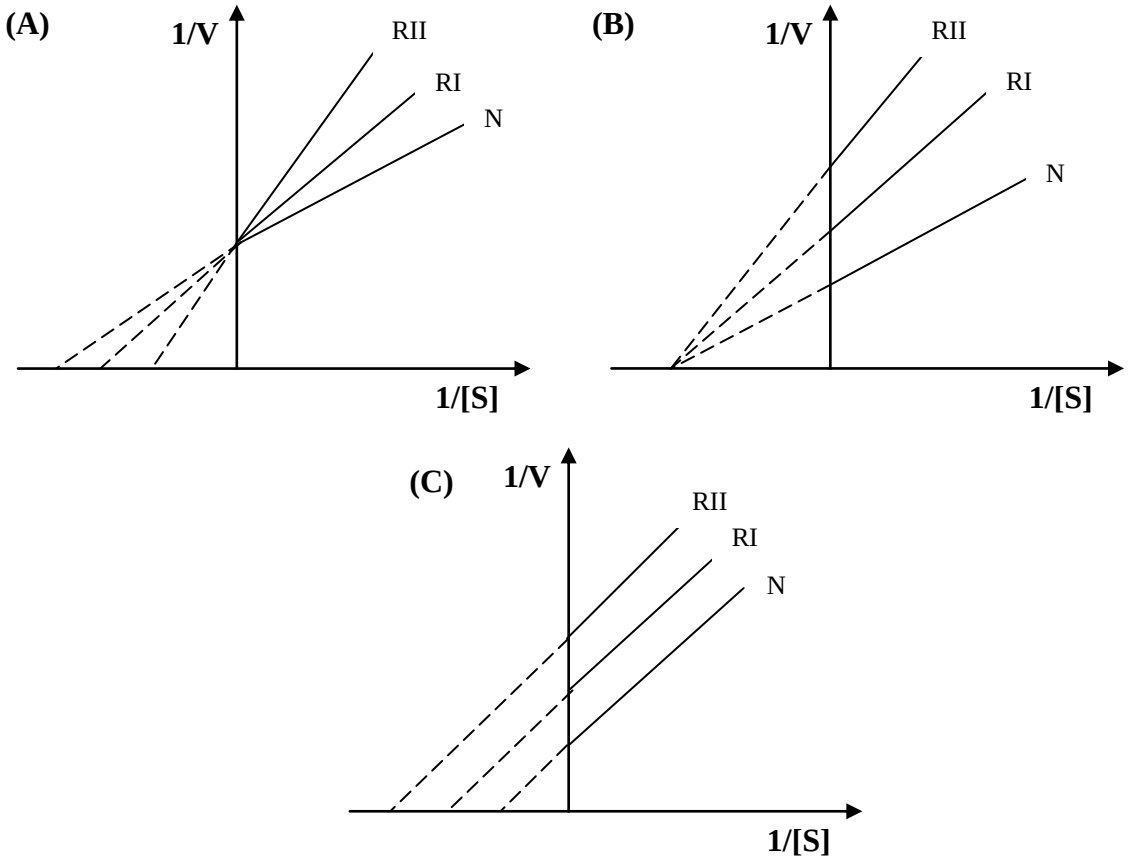

Figure 2.1: Lineweaver Burk plot of (A) competitive, (B) noncompetitive and (C) uncompetitive inhibition.

N: No inhibitor; RI: With inhibitor at concentration I; RII: With inhibitor at concentration II

3.0 MATERIALS AND METHODS

3.1 Fruiting body of edible mushrooms

The nine selected species of fresh mushroom fruiting bodies: *Agaricus bisporus* (button mushroom), *Flammulina velutipes* (golden needle) and *Lentinula edodes* (shiitake) were purchased from the local grocery store, Aeon Co. (M) Bhd. while *Hericium erinaceus* (monkey's head mushroom), *Pleurotus citrinopileatus* (yellow oyster mushroom), *P. cystidiosus* (abalone mushroom), *P. flabellatus* (pink oyster mushroom), *P. florida* (white oyster mushroom) and *P. pulmonarius* (grey oyster mushroom) were purchased from a local mushroom farm, Ganofarm Sdn Bhd. The fruiting bodies of the nine edible mushrooms tested are shown in Figures 3.1.

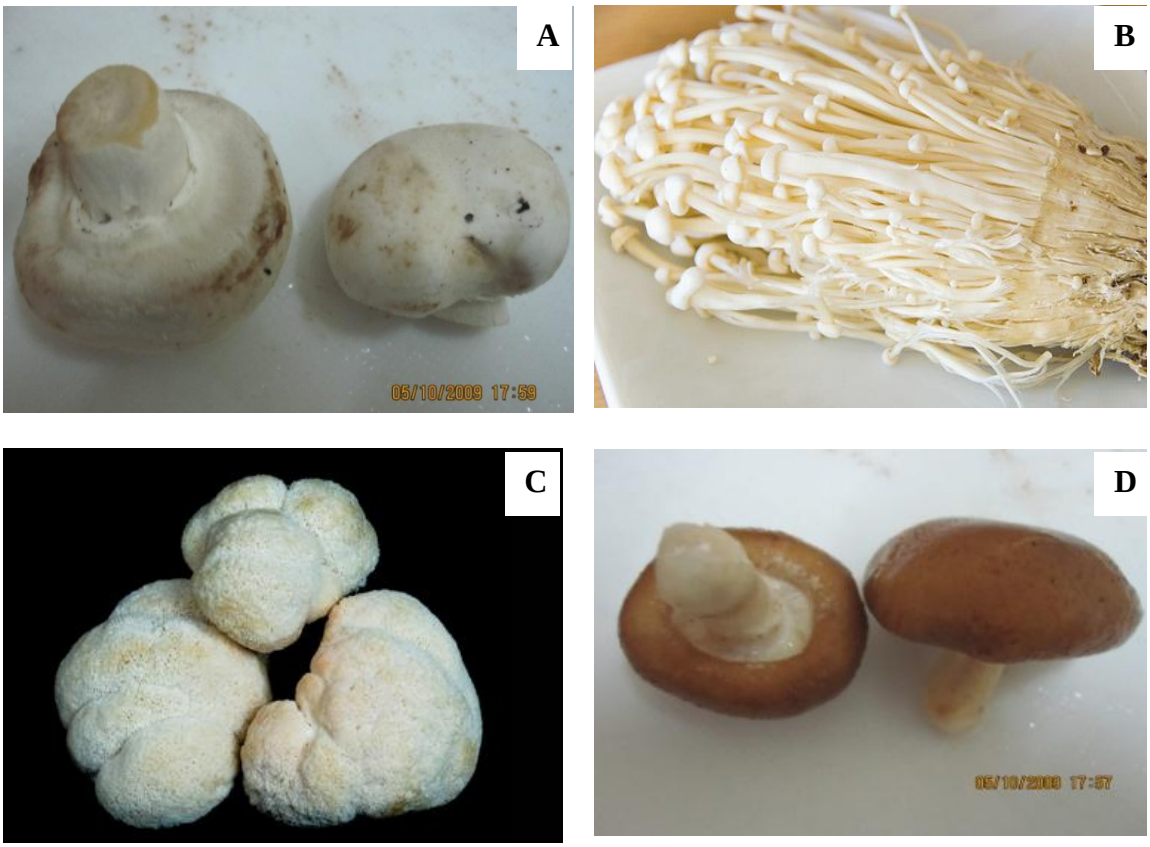

Figure 3.1: Fruiting bodies of edible mushrooms studied.

A: *Agaricus bisporus*; B: *Flammulina velutipes*; C: *Hericium erinaceus*; D: *Lentinula edodes;*

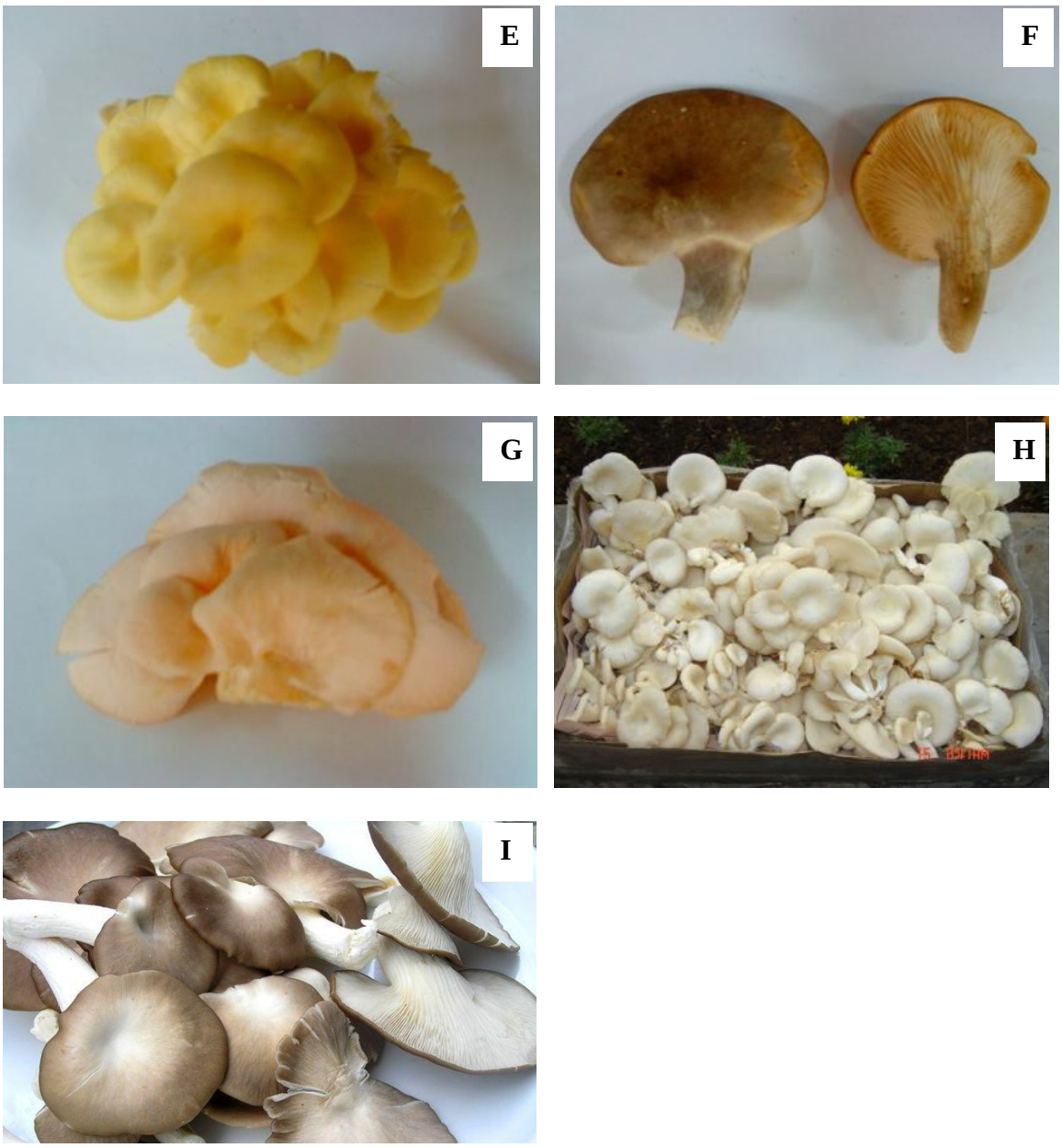

Figure 3.1 (Continued).

E: *Pleurotus citrinopileatus*; F: *Pleurotus cystidiosus*; G: *Pleurotus flabellatus*; H: *Pleurotus florida*; I: *Pleurotus pulmonarius*

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3.2 Protein extraction from fruiting bodies of edible mushrooms

Fruiting bodies of the nine edible mushrooms were cleaned and sliced. Each mushroom species was blended with distilled water at a ratio of 1:2 (w/v). The mixture was filtered and centrifuged to remove unwanted debris. Proteins were precipitated out from the

mushroom extracts using ammonium sulphate precipitation method. The whole process of protein precipitation was carried out with continuous stirring on a magnetic stirrer. The beaker containing 100 ml water extract of mushroom was kept chilled by placing it in an ice bucket. First, 5.6 g of ammonium sulphate (Merck, Darmstadt, Germany) was weight out and added slowly to the water extract. After the last bit of salt has dissolved, the solution was left to stir for another 30 min to allow complete equilibration between dissolved and aggregated proteins. The solution was then centrifuged at 10,000 rpm (ca. 8400 x *g*) for 15 min at 4^{o}C. The pellet was dissolved in distilled water and kept for further analysis. The supernatant was used for precipitation of proteins at 20% salt saturation by the addition of 5.7 g ammonium sulphate followed by the same steps mentioned above. The concentration of ammonium sulphate was increased stepwise until it reached 100% salt saturation. The amount of ammonium sulphate required for each targeted percentage of salt saturation was illustrated in Table 2.4 (page 28). The protein extracts obtained from 10-100% salt saturation were then dialysed using snakeskin dialysis tubing with a cut-off of 3,500 Da (Thermo Scientific, Rockford, IL, USA) to remove the salt from the sample. The protein extracts were dialyzed in a cold room (4 ℃) against distilled water under continuous stirring with a magnetic stirrer for 48 hours. Water was changed four times throughout the process. Dialyzed protein extracts were freeze-dried and stored at -20 ^{o}C for further analysis.

3.3 Estimation of protein content in the crude water extracts and protein extracts of edible mushrooms

Protein content was estimated using Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). It is a colorimetric method measured with a microplate reader. First, the reagent A and reagent B provided in the assay kit was mixed at a ratio of 1:50 to produce working reagent. Twenty five microlitre of each

sample was then mixed with 200 µl of the working reagent in a microplate well by shaking the plate on a plate shaker for 30 seconds. The plate was then covered and incubated at 37 ^{o}C for 30 minutes. After the plate was cooled to room temperature, the absorbance of the colour formed was measured with a Sunrise™ ELISA microplate reader (Tecan, Grödig, Austria) at 562 nm. Protein content of the samples was estimated by comparing their absorbance values against the standard curve of bovine serum albumin (BSA). The volume used for the preparation of each diluted BSA standards from the initial stock solution of 2000 µg/ml was shown in Table 3.1. A batch consisting of the nine concentrations of diluted BSA standards was prepared and tested alongside the protein samples each time the assay was performed.

Table 3.1: Volume (µl) used for the preparation of diluted BSA standards from the initial stock 2000 µg/ml.

Vial	dH_2O (µl)	BSA (µl)	Final BSA concentration (µg/ml)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B	750
E	325	325 of vial C	500
F	325	325 of vial E	250
G	325	325 of vial F	125
H	400	100 of vial G	25
I	400	0	0 (Blank)

3.4 SDS-PAGE analysis of protein extracts from edible mushrooms

SDS-PAGE was used as a parameter to pool the protein extracts precipitated from different percentages of salt saturation. It was also used to visualize the molecular weights of the proteins in RPHPLC fractions exhibiting the highest ACE inhibitory activity. The method was carried out in a vertical slab gel apparatus according to the modified method of Laemmli (1970). The gel consists of separating gel (16% polyacrylamide) and stacking gel (4% polyacrylamide) was prepared by mixing the solutions according to the protocol given in Appendix A (I). Protein sample was mixed with SDS-PAGE sample buffer at a ratio of 3:1 (v/v) and boiled for 5 minutes. The mixture and broad range SDS-PAGE standard markers (Bio-Rad Laboratories, Hercules, USA) were then loaded into the wells of the gel. Electrophoresis was conducted at a constant current of 60 V to move the proteins through the stacking gel followed by 100 V to resolve the proteins in the separating gel.

The protein bands were then stained by silver staining according to the method by Heukeshoven and Dernick (1988). Protocols for the preparation of solutions for silver staining were illustrated in Appendix A (II). A hundred millilitre of solution was used per gel. All washing and incubation steps were carried out with constant gentle shaking on a shaker. Following electrophoresis, the gel was immersed immediately in the fixing solution for at least 30 min. Fixation will restrict protein movement from the gel matrix and remove interfering ions and detergent from the gel. Fixing solution was then replaced with sensitizing solution to increase the sensitivity and contrast of the staining. After incubated for 30 min, the gel was washed three times with distilled water, 5 min each time. This was followed by incubation of the gel in silver solution for 20 min. The gel was then rinsed with distilled water twice, 1 min each time to remove the excess of unbound silver ions. After that, the gel was left to incubate in the developing solution until the bands become visible. The colour development was stopped by replacing the

developing solution with stopping solution and left shaken for 10 min. The gel was then washed three times in distilled water for 10 min each time and kept in preserving solution.

3.5 Estimation of ACE inhibitory activity

Antihypertensive activity of crude water extracts and protein fractions were tested using ACE inhibitory assay kit (ACE kit-WST, Dojindo Laboratories, Kumamoto, Japan). The assay kit was supplied with substrate buffer, enzyme working solution and indicator working solution. The crude mushroom extracts and the protein fractions were tested at 10 mg/ml and 10 µg/ml protein, respectively. The RPHPLC and SEC fractions were tested at 1 µg/ml. First, 20 µl of sample solution was added into the 96-well plate followed by 20 µl of substrate buffer. There were two blanks required for the assay, blank 1 (positive control without ACE inhibition) and blank 2 (reagent blank). Deionized water was added in the blank 1 and blank 2 wells instead of sample solution. Then, 20 µl of enzyme working solution were added into sample wells and blank 1. Deionized water was added in blank 2 well instead of enzyme working solution. The amount of each solution for sample, blank 1 and blank 2 were summarized in Table 3.2.

Table 3.2 Amount of each solution (µl) added in the well for sample, blank 1 and blank 2 in the ACE inhibitory assay.

Solutions (µl)	Sample	Blank 1	Blank 2
Sample solution	20	-	-
Deionized water	-	20	40
Substrate buffer	20	20	20
Enzyme working solution	20	20	-
Indicator working solution	200	200	200

The plate was then covered and incubated in waterbath at 37 ^{o}C. After 60 minutes of incubation, 200 µl of indicator working solution was added into each well. The plate was incubated at room temperature for 10 minutes. The absorbances of the samples were measured with a Sunrise^{TM} ELISA microplate reader (Tecan, Grödig, Austria) at 450 nm. The ACE inhibitory activity of the samples tested was calculated using the following equation:

$$\text{ACE inhibitory activity (\%)} = [(A_{\text{blank 1}} - A_{\text{sample}}) / (A_{\text{blank 1}} - A_{\text{blank 2}})] \text{ X } 100$$

where A_{sample} is the absorbance of the solution containing ACE and the sample, $A_{\text{blank 1}}$ is the absorbance of positive control (without ACE inhibition) and $A_{\text{blank 2}}$ is the absorbance of reagent blank.

The concentration of the ACE inhibitor required to inhibit 50% of the ACE activity under the above assay condition was defined as IC_{50}.

3.6 Purification of selected active protein extracts from *P. cystidiosus* and *A. bisporus* by RPHPLC

Purification of four selected protein extracts (E1Pc, E5Pc, E1Ab and E3Ab) with active ACE inhibitory activity was carried out using an HPLC system equipped with a SCL-10AVP system controller, LC-10ATVP solvent delivery unit, SPD-M10AVP UV-Vis diode array detector and DGU-12A degasser (Shimadzu, Kyoto, Japan). The column used in the current study was a Chromolith® SemiPrep RP-18 column (100-10 mm, Merck, Darmstadt, Germany). First, the protein extracts were filtered through a whatman syringe filter (13 mm x 0.45 µm) before being injected into the column. The crude proteins were eluted with an increasing gradient of HPLC grade acetonitrile (Merck, Darmstadt, Germany) containing 0.05% TFA (Sigma-Aldrich, St. Louis, MO, USA), from 0% to 50% in 10 min at a flow rate of 3.5 ml/min. The UV absorbance of the eluent was monitored at 220 nm. All the protein extracts were fractionated according

to the peaks obtained. The fractions were then freeze-dried and their percentages of ACE inhibitory activity were determined at a concentration of 1 µg/ml protein.

3.7 Proteomic analysis of RPHPLC fractions with active ACE inhibitory activity

RPHPLC fractions from *P. cystidiosus* (E5PcF3) and *A. bisporus* (E3AbF6) were analysed using SDS-PAGE and SELDI-TOF-MS. SDS-PAGE was performed based on the method described in Section 3.4 (page 42) while SELDI-TOF-MS analysis was carried out using hydrophobic H50 ProteinChip® arrays (Bio-Rad Laboratories, Hercules, CA, USA). The arrays were first equilibrated with binding buffer, which consisted of 0.5% TFA in 50% acetonitrile (v/v). Then, 5 µl samples containing 0.5 µg of protein were spotted on the arrays. After the arrays were air-dried, 2 µl of CHCA in 0.5% TFA in 50% acetonitrile (v/v) was added onto the arrays and they were left to air-dry. The analyses were carried out on the Bio-Rad ProteinChip SELDI system PCS4000 (Bio-Rad Laboratories, Hercules, CA, USA). Data collection was carried out in positive ion mode using the following acquisition settings: mass range of 0 to 20 kDa, focus mass of 10 kDa. Laser energies used for the shot sequences were based on the following settings: warming shots 1000 nJ and data shots 900 nJ. Spectra were calibrated using an external calibration against a mixture of standards consisting of somatostatin (1637.9 Da), arg-insulin (5969.0 Da) and cytochrome c (12263.3 Da).

Expression Difference Map (EDM) analysis on the SELDI-TOF-MS spectra was performed using the ProteinChip Data Manager 3.5 (Bio-Rad Laboratories, Hercules, CA, USA). Peaks with mass to charge ratio (*m/z*) between 2000 and 30000 were analysed. Mean peak intensities from four replicate samples were used to determine the *p*-value. Peak clusters were done using second-pass peak selection with S/N > 2, within

0.1% of the mass window and allowing estimated peaks to be included; $p<0.01$ was considered statistically significant.

3.8 Purification of active RPHPLC fractions by SEC

RPHPLC fractions from *P. cystidiosus* (E5PcF3) and *A. bisporus* (E3AbF6) were further purified by SEC using Biosep SEC-S2000 column (300 x 7.8 mm, Phenomenex, Torrance, CA, USA). Analysis was performed on a HPLC system equipped with SCL-10AVP system controller, LC-10ATVP solvent delivery unit, SPD-M10AVP UV-Vis diode array detector and DGU-12A degasser (Shimadzu, Kyoto, Japan). The mobile phase consists of 45% acetonitrile containing 0.1% TFA. The flow rate was 1.0 ml/min and the effluent was monitored at 214 nm. The RPHPLC fractions were fractionated according to the peaks obtained. The fractions were then freeze-dried and their percentages of ACE inhibitory activity were determined according to the method described in Section 3.5 (page 43).

3.9 Identification of peptide sequence by Liquid Chromatography Mass Spectrometry (LC/MS/MS)

Identification of the peptides present in E5PcF3C1, E3AbF6C1 and E3AbF6C4 were carried out by LC/MS/MS. The analysis was conducted by Proteomics International, Perth, Australia. Basically, the SEC fractions were digested with trypsin and the peptides extracted were analysed by electrospray ionisation mass spectrometry using the Ultimate 3000 nano HPLC system (Dionex, Sunnyvale, CA, USA) coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA). Peptides were loaded onto a C18 PepMap100, 3 μm (LC Packings) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). Protein identification was carried

out using Mascot sequence matching software (Matrix Science) with Ludwig NR database.

3.10 Peptide synthesis

Based on the LC/MS/MS data, nine potential ACE inhibitory peptides were selected from E5PcF3C1, E3AbF6C1 and E3AbF6C4. The peptide sequences selected were AHEPVK, GPSMR, KVAGPK, FALPC, RIGLF, EGEPR, APSAK, GVQGPM and PSSNK. They were chemically synthesized by Peptron, Inc., Republic of Korea. The purity of the synthesized peptides were >98% measured by RPHPLC and MS analysis.

3.11 Effect of simulated gastrointestinal digestion on the ACE inhibitory activity of selected peptides

Simulated gastrointestinal digestion of the nine synthesized peptides was carried out *in vitro* by the method of Liu et al., (2010b). The peptide solution (0.1 mg/ml, 0.5 ml) was incubated with 0.5 ml of a 0.05% pepsin solution (0.1 M HCl at pH 2.0) for 2.5 h at 37^{o}C. In successive pepsin-pancreatin digestion test, the peptide solution was adjusted to pH 8.0 after the pepsin digestion. Then, 0.5 ml of pancreatin solution, i.e. potassium phosphate buffer (0.1 M, pH 8.0) containing 0.025% (w/v) chymotrypsin and 0.025% (w/v) trypsin were added into the solution. The mixture was incubated for another 2.5 h at 37 ^{o}C. Control consisting of peptide solution incubated in buffer solutions only, i.e. HCl and potassium phosphate buffer, were carried out alongside the experiment. Two negative controls were also carried out together with the experiment. The first negative control comprised only the buffer solutions, i.e. without peptide solution and gastrointestinal enzymes. The second negative control consists of buffer solutions with gastrointestinal enzymes but void of peptide solution. Protocols for preparation of HCl

and potassium phosphate buffer were illustrated in Appendix A (III). After the enzymatic treatment, the samples were boiled for 10 min to stop the digestion and then centrifuged at 10,000 rpm (ca. 8400 x *g*) for 10 min. The supernatants were freeze-dried and used for measurement of ACE inhibitory activity. The stability of the purified peptides against gastrointestinal enzymes were analysed by injecting the samples into the SEC column. Prediction of proteolytic fragment released after digestion was carried out based on the BIOPEP database (www.uwm.edu.pl/biochemia).

3.12 Determination of ACE inhibition pattern

The inhibition pattern of the most potent peptides, AHEPVK, RIGLF and PSSNK on the ACE activity were determined spectrophotometrically using HHL (Sigma-Aldrich, St. Louis, MO, USA) as substrate. Basically, 20 µl of ACE solution (0.1 UN/ml; Sigma-Aldrich, St. Louis, MO, USA) and 50 µl of peptides were incubated with 200 µl of various HHL concentrations (0.63, 1.25, 2.50 and 5.00 mM). The enzymatic reaction was terminated by the addition of 250 µl of 1.0 M HCl. The liberated hippuric acid was extracted with ethyl acetate and evaporated under vacuum condition. The hippuric acid residue was re-dissolved in 1.0 ml of distilled water and the absorbance was determined at 228 nm using a spectrophotometer (SmartSpec™ Plus Spectrophotometer, Bio-Rad Laboratories, Hercules, USA). The kinetics of ACE inhibition in the presence and absence of each peptide (0.00, 0.05 and 0.50 mg/ml) was determined by Lineweaver-Burk plots.

4.0 RESULTS AND DISCUSSION

4.1 Protein content and ACE inhibitory activity of crude water extracts from edible mushrooms

4.1.1 Protein content of crude water extracts

Mushrooms were reported to have high protein content, which rank above most fruits and vegetables (Chang & Miles, 2004). The protein content in the crude water extracts of the nine mushroom species tested in the current study was estimated by BCA protein assay kit. Referring to Figure 4.1, the protein content was in the range of 0.73 to 2.70 g protein/100 g fresh weight. Crude water extracts of *L. edodes* and *P. citrinopileatus* had the highest protein content. This was followed by *P. flabellatus* and *P. cystidiosus* with the protein content of 2.47 and 2.07 g protein/100g fresh weight, respectively. The protein content of *A. bisporus* and *F. velutipes* were 1.70 and 1.53 g protein/100 g fresh weight, respectively. Crude water extracts of *H. erinaceus* contain 0.96 g protein/100 g fresh weight. The most commonly cultivated *Pleurotus* species in Malaysia, *P. pulmonarius* and *P. florida* contain 0.75 and 0.73 g protein/100 g fresh weight, respectively. They had the lowest protein content compared to the other mushroom species tested in the current study.

Agaricus bisporus in the current study had a similar protein content compared to samples of *A. bisporus* from Italy (1.63 g/ 100 g fresh weight) and Northeast Portugal (1.23 g/ 100 g fresh weight) (Manzi *et al.*, 2001; Reis *et al.*, 2012). Mattila *et al.* (2002b) has reported slightly higher protein content in *A. bisporus*, which was 2.09 g/100 g fresh weight. *Lentinula edodes* also showed similar protein content compared to samples of *L. edodes* from Turkey, which had a protein concentration in the range of 2.07 to 2.94 g/100 g fresh weight (Cağlarirmak, 2007). However, lower protein content for *L. edodes* was reported in samples from Italy (1.53 g/100 g fresh weight) and Northeast Portugal

(0.89 g/ 100 g fresh weight) (Manzi *et al.*, 2001; Reis *et al.*, 2012). *Pleurotus pulmonarius* in the current study has a lower protein content compared to *P. sajor-caju* as determined by Cağlarirmak (2007) and Bonatti *et al.* (2004), who reported 1.76 g and 1.54 g/ 100 g fresh weight, respectively. Substrates used for mushroom cultivation, temperature and humidity during growth as well as the age of the harvested mushroom have been reported to influence the chemical composition and nutritional value of mushrooms (Chang & Miles, 2004; Dundar *et al.*, 2008). Hence, different pre- and post-harvest conditions may cause variations in the protein content of mushrooms.

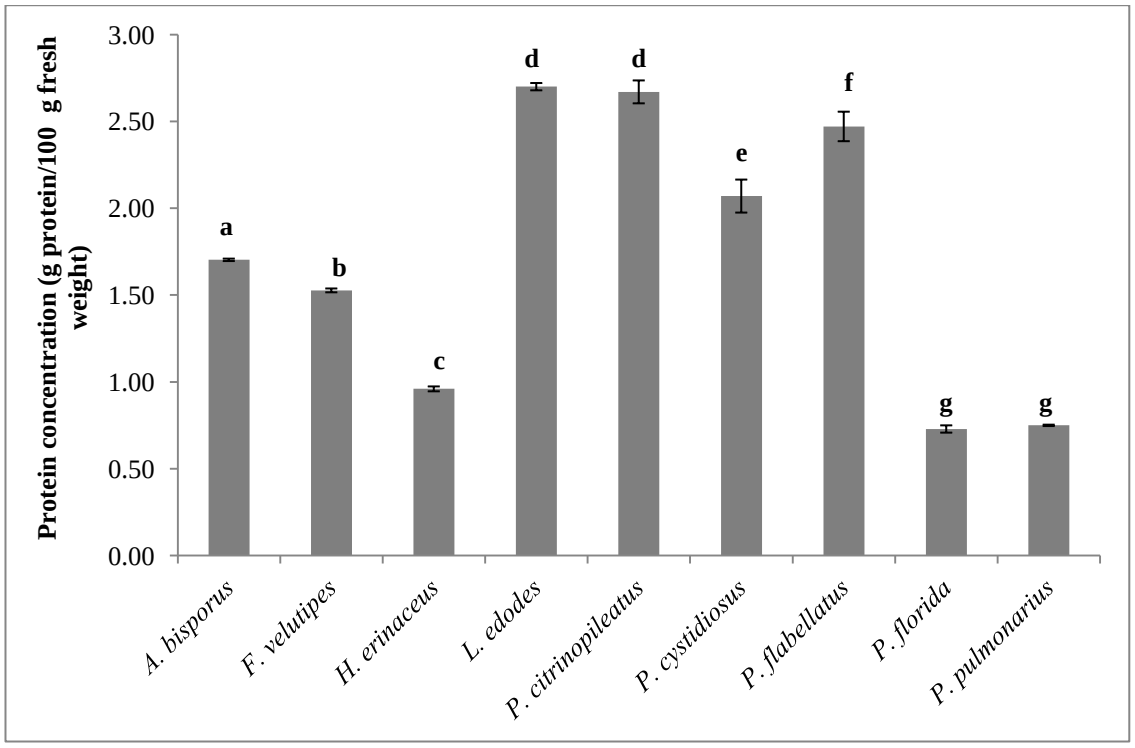


Figure 4.1: Protein concentration (g protein/100 g fresh weight) of crude water extracts from nine selected species of edible mushrooms.

All values were expressed as mean ± standard deviation (n=3). Means with different lower case alphabet letters are significantly different ($p<0.05$).

4.1.2 ACE inhibitory activity of crude water extracts

The crude water extracts from the nine edible mushroom species were tested for their ACE inhibitory activity at a concentration of 10 mg/ml. This concentration was selected as it was the effective concentration at which all the mushroom species tested could exert their bioactivity. Referring to the result obtained in Figure 4.2, all the nine mushroom species tested inhibited at least 70% of the ACE activity. The highest ACE inhibitory activity was exhibited by *F. velutipes* and *L. edodes* where both the crude water extracts showed 95.5% of ACE inhibition. This is in line with the report by Chen *et al.* (2012c), which showed *F. velutipes* and *L. edodes* has high amount of GABA, or also known as γ-aminobutyric acid. It is a secondary metabolite present in many vegetables and fruits. It has been proven to have blood pressure lowering effect in animal studies (Hayakawa *et al.*, 2004; Shizuka *et al.*, 2004). Based on the study by Zhi and Omori (2001), GABA has ACE inhibiting ability. This is supported by the report on the positive correlation between GABA content and ACE inhibitory activity in cheese (Wang *et al.*, 2010). Besides, Watanabe *et al.* (2002), suggested that peptides and GABA in *L. edodes* played a role in the inhibition of ACE activity. (Chen *et al.*, 2012c). In the current study, crude water extract of *A. bisporus* exhibited 87.2% of ACE inhibitory activity. Interestingly, high GABA content was also reported in this mushroom.

Crude water extract of *H. erinaceus* at concentration of 10 mg/ml showed 90.1% of ACE inhibitory activity. There were not many reported studies on the ACE inhibitory activity of *H. erinaceus*. Based on the report by Abdullah *et al.* (2012), hot water extract of *H. erinacues* inhibited 50% of the ACE activity at a concentration of 580 mg/ml. Compared to the current study, the ACE inhibitory activity of the crude water extract of *H. erinaceus* which did not undergo boiling process exhibited higher ACE inhibitory activity. This may suggest that cold water extraction is a better technique to extract

ACE inhibitors compared to hot water extraction. This is in line with the report by Choi *et al.* (2001), which showed most of the mushrooms tested, especially *G. frondosa* has higher ACE inhibitory activity in cold water extracts compared to hot water extracts.

Among the *Pleurotus* spp. tested in the current study, *P. florida* and *P. pulmonarius* exhibited the highest ACE inhibitory activity with 87.2% and 85.3% of the ACE activity inhibited, respectively. The remaining three *Pleurotus* spp., namely *P. citrinopileatus*, *P. cystidiosus* and *P. flabellatus* showed the percentages of ACE inhibitory activity in the range of 71.9-81.4%. Aqueous extract from *P. sajor-caju* (a.k.a. *P. pulmonarius*) has been reported to have hypotensive effect by the regulation of the renin-angiotensin system (Chang & Miles, 2004). Previous studies have reported on the identification of D-mannitol as one of the main phytochemicals with ACE inhibitory effect in *P. cornucopiae* and *P. citrinopileatus* (Hagiwara *et al.*, 2005; Suzuki *et al.*, 2006).

The ACE inhibitory activity of mushrooms reported in the current study was higher compared to previous reports. At a concentration of 25 mg/ml, water extracts of *A. bisporus* and *P. sajor-caju* were reported to inhibit 27.3% and 38.7% of ACE, respectively, while different strains of *F. velutipes* inhibited ACE activity in the range of 13.7-32.9% (Lee *et al.*, 2004). Kim and co-workers (2002) also reported a lower ACE inhibitory activity for *L. edodes* and *F. velutipes*, where 50% of ACE activity was inhibited at concentrations of 18.4 to 39.3 and 7.4 to 22.6 mg/ml, respectively. The conditions of extraction of ACE inhibitor from mushrooms have been reported to influence the level of ACE inhibitory activity. The optimum extraction conditions for *Tricholoma giganteum* and *Pholiota* sp. were at 30^{o}C for 3 hours and 1 hour, respectively (Lee *et al.*, 2004). *Grifola frondosa* required a longer extraction time, i.e., 12 hours (Choi *et al.*, 2001). Apparently, the higher ACE inhibitory activity obtained in the current study compared to previous studies shows that the extraction technique

employed in the current study was sufficient to extract the ACE inhibitors from mushrooms.

According to the study by Barbosa-Filho *et al.* (2006), natural compounds showing active ACE inhibition include proteins, flavonoids, alkaloids, sesquiterpenoids and terpenes. Among these, protein was the most reported compound showing ACE inhibitory activity. Therefore, further analysis was carried out to study the ACE inhibitory activity of proteins in the mushrooms tested in the current study.

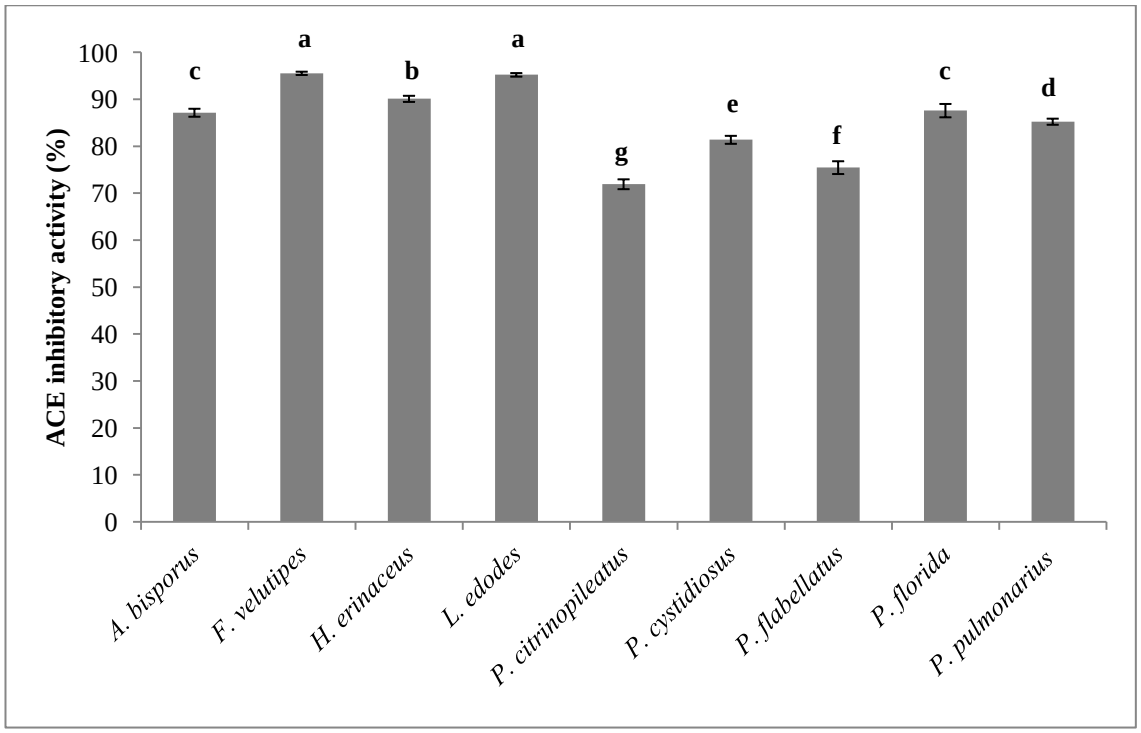


Figure 4.2: Percentages of ACE inhibitory activity of crude water extracts from nine selected species of edible mushrooms.

All values were expressed as mean ± standard deviation (n=3). Means with different lower case alphabet letters are significantly different ($p<0.05$).

ACE inhibitory activity was tested at 10 mg/ml.

In the current study, there was no correlation between the protein content and the ACE inhibitory activity (R^2 = 0.1442). This is supported by a report by Apostolidis *et al.* (2006) which also showed no correlation between the two parameters. This may indicate that other compounds in the crude water extract could have interfered or competed with proteins to bind to the active site of ACE. Therefore, protein purification is important to eliminate other compounds that might hinder the true effect of these proteins.

In addition, the disassociation between the ACE inhibitory activity and protein content may also show that the type of protein rather than the protein content that could have affected the ACE inhibitory activity. Some studies have focused on the purification of peptides from protein precursor with known ACE inhibitory activity. Examples of these proteins include casein (Jiang *et al.*, 2010; Minervini *et al.*, 2003), collagen (Saiga *et al.*, 2008; Sato *et al.*, 2012), elastin (Sato *et al.*, 2012), troponin (Katayama *et al.*, 2004), actin (Yokoyama *et al.*, 1992), myosin (Katayama *et al.*, 2007; Terashima *et al.*, 2010) and ovalbumin (Miguel *et al.*, 2004). Currently, there is limited study reported on the proteomic analysis of proteins present in mushrooms, in particular edible mushrooms. Two studies have reported on the proteomics of edible mushrooms, *P. tuber-regium* Fr. (Chen *et al.*, 2012b), *H. erinaceus* and *Sparassis crispa* (Wulfen) Fr. (Horie *et al.*, 2008). However, these studies were focused on the identification of proteins present at different stages of mushroom life cycle instead of their biological activity. Referring to the review by Erjavec *et al.* (2012), the author and co-workers have listed a table on the biological activities of bioactive proteins and protein extracts from mushrooms. The bioactivities listed include antifungal, antibacterial, antiviral, antiparasitic, antitumour and immunomodulatory. Antihypertensive or ACE inhibitory activity was not included in the list. Even though there were reports on the ACE inhibitory peptides purified from edible mushrooms, such as *G. frondosa* (Choi *et al.*,

2001), *T. giganteum* (Lee *et al.*, 2004) and *P. cornucopiae* (Jang *et al.*, 2011), the protein precursors for the bioactive peptides were not determined. In view of these limitations, the purification of ACE inhibitory peptides in the current study was not targeted on certain protein in mushrooms. The protein mixtures in the mushrooms tested were fractionated by bioassay-guided purification methods to determine the potent ACE inhibitory peptides.

4.2 Comparison of protein contents in the protein extracts precipitated from edible mushrooms by ammonium sulphate

Proteins were precipitated from the water extracts of the nine edible mushrooms by different percentages of salt saturation ranging from 10% to 100% (S10 to S100). Figure 4.3 shows a comparison of the protein concentrations in the protein extracts of each mushroom species tested. Most mushroom species in the current study had the highest concentration of protein precipitated at S50 to S70, except for *A. bisporus* (S40) and *P. pulmonarius* (S90). Theoretically, proteins with large numbers of hydrophobic residues should precipitate out first. Hence, mushrooms tested in the current study contain high amounts of amphipathic proteins. According to He *et al.* (2012), proteins that contain abundant hydrophobic amino acids are a good source to produce ACE inhibitory peptide. This is because peptide with potent ACE inhibitory activity usually contain hydrophobic amino acid residues at the C-terminal and branched aliphatic amino acid at the N-terminal (Curtis *et al.*, 2002). In addition, Wang *et al.* (2011a) and He *et al.* (2012) have reported that apricot almond meal and wheat protein isolates which contained 50.08% and 42.84% hydrophobic amino acids, respectively are good sources of potent ACE inhibitory peptides. Therefore, the high amount of proteins precipitated at S50 to S70 in the nine mushroom species tested in the current study shows they can be a good source of ACE inhibitory peptides.

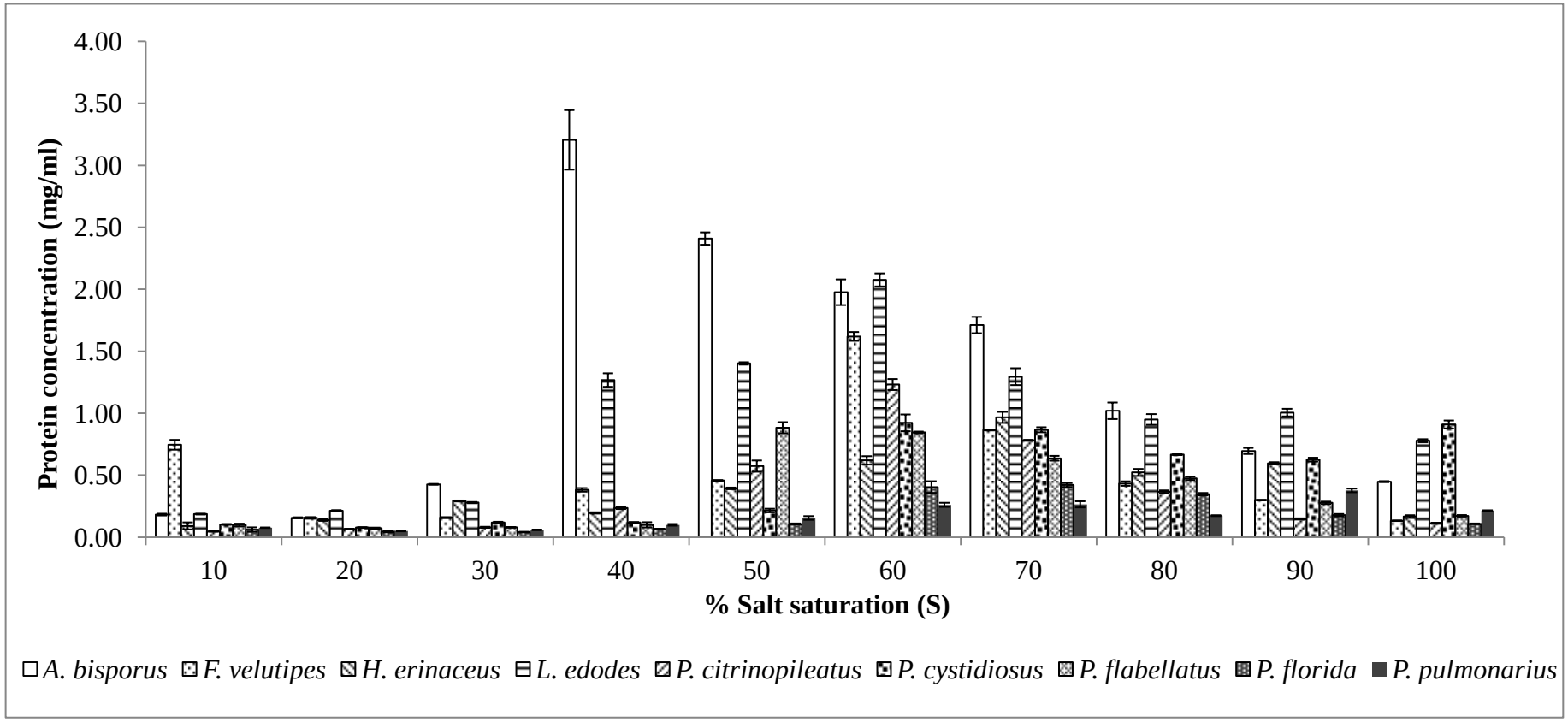


Figure 4.3: Protein concentration of protein extracts precipitated at ten percentages of salt saturation (S10-S100) from nine selected species of edible mushrooms.

All values were expressed as mean ± standard deviation (n=3).

4.3 SDS-PAGE analysis of protein extracts precipitated from edible mushrooms by ammonium sulphate

The ten ammonium sulphate precipitated protein extracts from the nine mushroom species were subjected to SDS-PAGE analysis. Figures of the gels were illustrated in Appendix B. Protein extracts with similar SDS-PAGE protein band profiles were pooled in the same group as shown in Table 4.1. Protein extracts from *H. erinaceus* and *P. citrinopileatus* were pooled into four groups while *A. bisporus*, *P. flabellatus*, *P. florida* and *P. pulmonarius* were pooled into five groups. *Flammulina velutipes*, *L. edodes* and *P. cystidiosus* were divided into six groups.

Table 4.1: Pooling of protein extracts precipitated at 10-100% salt saturation (S10-S100).

Mushroom species	*Pooled protein extracts (E)					
	1	2	3	4	5	6
A. bisporus	S10-20	S30	S40	S50-60	S70-100	-
F. velutipes	S10-30	S40	S50-60	S70	S80	S90-100
H. erinaceus	S10-30	S40-50	S60-70	S80-100	-	-
L. edodes	S10-20	S30-40	S50	S60	S70-80	S90-100
P. citrinopileatus	S10-40	S50	S60-70	S80-100	-	-
P. cystidiosus	S10-20	S30-50	S60	S70-80	S90	S100
P. flabellatus	S10-40	S50	S60	S70-80	S90-100	-
P. florida	S10-40	S50	S60-70	S80	S90-100	-
P. pulmonarius	S10-20	S30-50	S60	S70	S80-100	-

* Pooling of protein extracts were based on the similarity of protein bands observed on SDS-PAGE profile.

4.4 Comparison of ACE inhibitory activity in pooled protein extracts from edible mushrooms

The use of ammonium sulphate precipitation method for purifying protein has the advantages of high protein recovery and maintaining proteins in their native state (Jiménez *et al.*, 2012). There were a few previously reported studies which have utilized ammonium sulphate as their first step in the bioassay-guided method for purification of bioactive proteins from plants. Sheih *et al.* (2009) have purified ACE inhibitory peptides from algae at 20% and 80% salt saturation. Arulpandi and Sangeetha (2012) have purified protein with antibacterial activity at 40-80% salt saturation. Alpha-amylase inhibitor have been extracted from tuber at 80% salt saturation (McEwan *et al.*, 2010). Referring to Table 4.2, the ability of the pooled protein extracts to exhibit ACE inhibitory activity at a concentration as low as 10 µg/ml shows that ammonium sulphate precipitation method has successfully recovered the proteins from the mushroom species without adversely affecting their ACE inhibitory activity.

As reported in Figure 4.2 (page 53), crude water extracts from all the nine mushroom species showed a comparably high ACE inhibitory activity. However, referring to Table 4.2, protein extracts from the nine mushroom species showed variations of ACE inhibitory activity. Some protein extracts such as E1 to E4 of *H. erinaceus* have exhibited low ACE inhibitory activity. It is possible to suggest that non-protein ACE inhibitors could play a major role in these mushrooms. Isolation and purification of non-protein ACE inhibitors from several mushrooms have been reported. Kiyoto *et al.* (2008) identified L-pipecolic acid from water extract of edible mushroom, *Sarcodon aspratus* (Berk.) as ACE inhibitor with moderate activity (IC_{50} = 23.7 mg/ml). A sugar alcohol, D-mannitol isolated from *P. cornucopiae* has been identified as the active compound that inhibited the ACE activity at IC_{50} value of 3.0 mg/ml (Hagiwara *et al.*, 2005).

Based on the percentage of salt saturation, proteins in E1 have the most hydrophobic residue, and proteins with less hydrophobicity were precipitated in the latter protein extracts. Proteins with high content of hydrophobic amino acid was reported to have more potential to produce high ACE inhibitory activity (He *et al.*, 2012). Referring to the result illustrated in Table 4.2, there was no correlation between the ACE inhibitory activity and hydrophobicity of the protein extracts. The percentage of ACE inhibitory activity does not decrease proportionally with the reduction of hydrophobicity of the protein extracts. For example, theoretically E2 of *A. bisporus* is more hydrophobic than E3. However, E3 has exhibited higher ACE inhibitory activity than E2 with 71.4% and 8.3% of ACE inhibited, respectively. This shows that apart from hydrophobicity, the presence of other characteristics could also have contributions in the ACE inhibition ability of proteins. Pripp and Ardo (2007) have found a positive correlation between increased ACE inhibition and bitterness of peptides. Besides, several authors have reported on the relationship of the structure and activity of ACE inhibitory peptides (Guang & Phillips, 2012; Pripp *et al.*, 2004; Wu *et al.*, 2006b). Juan and Cho (2005) have constructed a three dimensional structure-activity relationship model to analyse the binding affinity of ACE inhibitor to the active site of ACE.

The ammonium sulphate precipitation method has fractionated the proteins in the crude water extracts according to the hydrophobic interaction between protein and water after the addition of salt (Wingfield, 2001). According to the study reported by Gomez-Ruiz *et al.* (2002), there could be a synergistic effect among peptides in cheese to exhibit potent ACE inhibitory activity. Likewise, this synergistic effect was also proposed in the studies on production of ACE inhibitory peptides from fermented milk and tilapia (Gobbetti *et al.*, 2000; Raghavan & Kristinsson, 2009). Therefore, in the current study, the protein extracts with lower ACE inhibitory activity, such as E1 to E4 of *H. erinaceus* may not indicate that the proteins present were not potent ACE

inhibitors. There could be a synergistic effect among the proteins or peptides when present together in the crude water extracts in inhibiting the ACE activity, where when separated, the activity can no longer be observed (Gomez-Ruiz *et al.*, 2002; Raghavan & Kristinsson, 2009). However, the aim of the current study is to identify potent ACE inhibitory peptides which act individually. Therefore, only protein extracts with potentially high ACE inhibitory activity were selected for further analysis.

Among the protein extracts, E1 from *P. cystidiosus* (E1Pc) showed the highest ACE inhibitory activity, blocking 96.2% of ACE activity in the inhibition assay at 10 µg/ml. This was followed by E5 of *P. cystidiosus* (E5Pc) with 86.7% ACE inhibition. E1 and E3 of *A. bisporus* (E1Ab and E3Ab) also showed significantly higher ACE inhibitory activity compared to the other protein extracts with 78.4% and 71.4% of ACE enzyme inhibited, respectively. Referring to Table 4.1 (page 57), proteins in E1Pc and E5Pc were precipitated at 10-20% and 90% salt saturation, respectively. Proteins in E1Ab and E3Ab were precipitated at 10-20% and 40%, respectively. Based on the percentages of salt saturation, these protein extracts particularly E1Pc and E1Ab most probably have higher hydrophobicity. Therefore, they can be a good source of ACE inhibitory peptides as indicated by He *et al.* (2012). Besides high content of hydrophobic amino acid, potent ACE inhibitors have preference for certain amino acid at their C- and N-termini. For example, proline, phenylalanine, tryptophan and tyrosine were preferred at C-terminal whereas valine, isoleucin and arginine were preferred as N-terminal residues (Cheung *et al.*, 1980). Therefore, the four protein extracts, i.e. E1Pc, E5Pc, E1Ab and E3Ab with the most potent ACE inhibitory activity were selected for further purification by RPHPLC to further characterize the ACE inhibitory activity of the proteins.

Table 4.2: Percentages of ACE inhibitory activity of the pooled protein extracts from the nine selected species of edible mushrooms.

Mushroom species	Pooled protein extracts (E)					
	1	2	3	4	5	6
A. bisporus (Ab)	**78.4 ± 4.4** ^{s}	8.3 ± 3.2 ^{abcd}	**71.4 ± 0.9** ^{r}	39.3 ± 1.3 ^{p}	8.1 ± 2.8 ^{abcd}	-
F. velutipes	18.3 ± 3.5 ^{fghi}	15.8 ± 0.8 ^{efg}	12.6 ± 2.2 ^{cdef}	13.7 ± 3.2 ^{def}	16.4 ± 3.7 ^{efgh}	19.8 ± 0.5 ^{ghij}
H. erinaceus	5.4 ± 3.4 ^{a}	5.9 ± 2.9 ^{a}	6.3 ± 1.8 ^{ab}	6.9 ± 4.6 ^{abc}	-	-
L. edodes	11.7 ± 2.1 ^{bcde}	16.6 ± 6.3 ^{efgh}	24.9 ± 2.8 ^{jkl}	5.7 ± 2.9 ^{a}	3.3 ± 1.9 ^{a}	4.6 ± 2.6 ^{a}
P. citrinopileatus	41.1 ± 4.4 ^{p}	20.9 ± 1.6 ^{ghij}	15.2 ± 4.9 ^{efg}	20.2 ± 1.5 ^{ghij}	-	-
P. cystidiosus (Pc)	**96.2 ± 0.6** ^{u}	47.5 ± 2.7 ^{q}	31.6 ± 6.1 ^{mno}	31.5 ± 2.3 ^{mno}	**86.7 ± 2.8** ^{t}	48.3 ± 2.6 ^{q}
P. flabellatus	28.4 ± 3.1 ^{klm}	36.5 ± 4.1 ^{op}	21.8 ± 3.9 ^{hij}	19.9 ± 3.0 ^{ghij}	22.0 ± 3.4 ^{hij}	-
P. florida	25.5 ± 2.3 ^{jkl}	24.2 ± 8.5 ^{jk}	17.0 ± 3.1 ^{efgh}	22.9 ± 4.7 ^{ijk}	4.5 ± 4.2 ^{a}	-
P. pulmonarius	41.2 ± 7.3 ^{p}	30.6 ± 0.8 ^{lmn}	35.9 ± 2.2 ^{nop}	32.2 ± 4.2 ^{mno}	5.5 ± 1.7 ^{a}	-

All values are expressed as mean ± standard deviation (n=3). Means with different lower case alphabet letters are significantly different ($p<0.05$).

ACE inhibitory activity was tested at 10 µg/ml protein. Protein extracts highlighted in bold were selected for further purification.

4.5 Partial purification of ACE inhibitors from selected pooled protein extracts of *P. cystidiosus* and *A. bisporus* by RPHPLC

As discussed in Section 4.4, proteins with potent ACE inhibitory activity usually contain abundant hydrophobic amino acid (He *et al.*, 2012). Therefore, the four protein extracts with the most potent ACE inhibitory activity from *P. cystidiosus* (E1Pc, E5Pc) and A. *bisporus* (E1Ab and E3Ab) were further fractionated based on hydrophobicity by RPHPLC. The RPHPLC chromatograms obtained are shown in Figure 4.4. Both protein extracts from *P. cystidiosus* had different peak patterns. However, both protein extracts from *A. bisporus* showed very similar peak patterns. This could be due to the percentages of salt saturation for E1Ab (S10 to S20) and E3Ab (S40) were quite close. Thus, some proteins with similar characteristics could be precipitated within these salt saturation solutions.

Six RPHPLC fractions, i.e. F1 to F6 were collected from each protein extract. They were freeze-dried and evaluated for ACE-inhibitory activity. Referring to Table 4.3, RPHPLC fractions collected from E1Pc and E1Ab had higher recovery rate percentages compared to E5Pc and E3Ab, respectively. Percentages of proteins recovered from RPHPLC fractions of E1Pc and E5Pc were ranging from 6.16-21.78% and 4.95-17.48%, respectively. Whilst percentages of proteins recovered from RPHPLC fractions of E1Ab and E3Ab ranged from 6.69-19.14% and 5.35-17.18%, respectively. RPHPLC utilized hydrophobic stationary phase. Proteins with high net hydrophobicity have more tendencies to participate in hydrophobic interactions with the stationary phase of RPHPLC (Scopes, 1994). Based on the percentages of salt saturation, proteins in E1Pc and E1Ab were more hydrophobic than E5Pc and E3Ab. Therefore, the higher hydrophobicity characteristic of the proteins in E1Pc and E1Ab has more affinity to bind to the stationary phase of RPHPLC and subsequently caused the higher recovery in the two protein extracts.

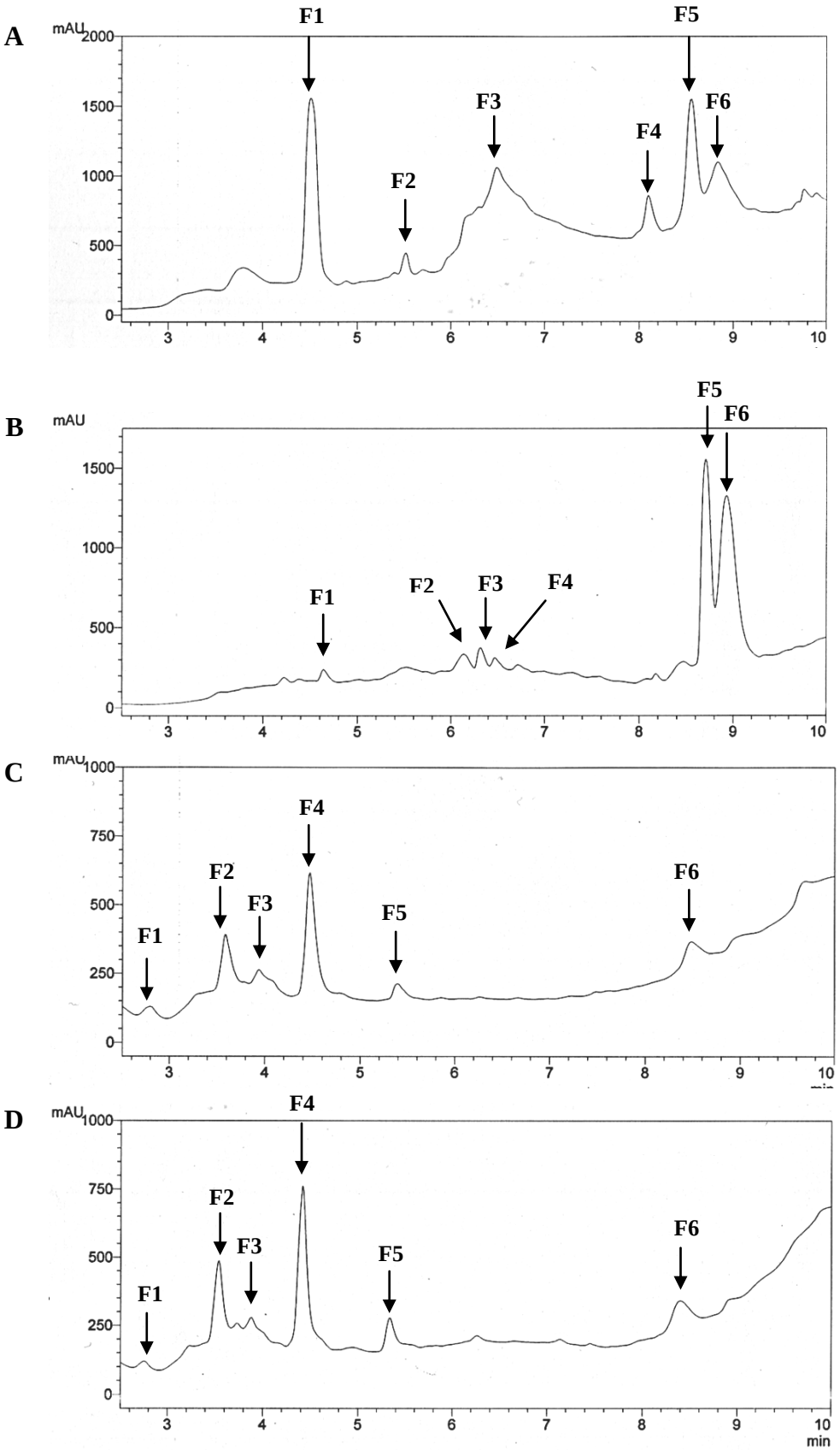


Figure 4.4: RPHPLC chromatograms of protein extracts from (A) E1Pc, (B) E5Pc, (C) E1Ab and (D) E3Ab.

Fractions collected were labelled as F1 to F6.

Table 4.3: Percentages of protein recovery yield and percentages of ACE inhibitory activity of RPHPLC fractions from selected protein extracts.

RPHPLC fractions	Protein extracts							
	E1Pc		E5Pc		E1Ab		E3Ab	
	% Recovery	*% inhibition	% Recovery	*% inhibition	% Recovery	*% inhibition	% Recovery	*% inhibition
F1	15.75	3.3 ± 0.6 ^{a}	5.53	19.2 ± 4.5 ^{de}	9.62	9.2 ± 3.8 ^{bc}	6.06	7.7 ± 2.2 ^{abc}
F2	6.16	7.7 ± 5.0 ^{ab}	4.95	28.4 ± 5.9 ^{fg}	12.00	7.4 ± 2.9 ^{abc}	10.16	12.9 ± 2.3 ^{cd}
F3	21.78	10.6 ± 3.2 ^{abc}	5.36	**30.8 ± 5.6 ^{g}**	7.22	1.2 ± 2.7 ^{a}	6.90	14.4 ± 7.6 ^{cd}
F4	9.74	25.9 ± 2.9 ^{efg}	8.33	21.4 ± 4.5 ^{def}	19.14	4.4 ± 2.1 ^{ab}	17.18	12.2 ± 5.7 ^{cd}
F5	15.06	21.9 ± 5.4 ^{def}	14.46	19.1 ± 4.7 ^{de}	6.69	3.8 ± 4.7 ^{ab}	5.35	16.8 ± 2.7 ^{d}
F6	13.24	14.7 ± 6.8 ^{bcd}	17.48	17.4 ± 4.1 ^{cd}	7.84	19.3 ± 6.8 ^{de}	6.15	**25.7 ± 4.2 ^{e}**
Total	81.73	-	56.11	-	62.51	-	51.80	-

*All values are expressed as mean ± standard deviation (n=3). Means with different lower case alphabet letters within the same mushroom species are significantly different ($p<0.05$).

ACE inhibitory activity of RPHPLC fractions was tested at 1 µg/ml protein. RPHPLC fractions highlighted in bold were selected for further analysis.

The total percentages of proteins collected in the RPHPLC fractions of E1Pc and E5Pc were 81.73% and 56.11%, respectively. On the other hand, a total of 62.51% and 51.80% of proteins were collected by RPHPLC fractions of E1Ab and E3Ab, respectively. This shows that not all the proteins were recovered from the four protein extracts. Previous work has shown that proteins denature and lose their bioactivity in 50% aqueous-acetonitrile solution (Scopes, 1994). In view of this complication, proteins were eluted from the RPHPLC column to a maximum of 50% acetonitrile. Proteins eluted by higher than 50% acetonitrile were eliminated from analysis.

Despite of this drawback, acetonitrile was still utilized as the mobile phase in the current study because of its high optical transparency in the detection wavelengths used for protein and peptide analysis, which is slightly above 200 nm (Aguilar, 2004). In addition, the lower viscosity and lower chemical reactivity of acetonitrile makes it a good choice for liquid chromatography (Gekko *et al.*, 1998). Compared to alcohols such as methanol and 2-propanol, mobile phases containing acetonitrile was reported to exert greater retention (Aguilar, 2004).

Most of the previously reported studies on the purification of ACE inhibitory peptides have used RPHPLC profile with elution of acetonitrile up to not more than 50%. This could be that the authors were aware of the bioactivity loss of proteins at 50% aqueous-acetonitrile solution. There were studies that utilized the same RPHPLC profile used in the current study, i.e. elution of proteins using a linear gradient of acetonitrile at 0-50%. This include studies on the purification of ACE inhibitory peptides from tuna (Qian *et al.*, 2007), porcine (Muguruma *et al.*, 2009), cuttlefish (Balti *et al.*, 2010) egg white (Asoodeh *et al.*, 2012) and mushroom *G. frondosa* (Choi *et al.*, 2001). Some studies have eluted the proteins at a lower percentage of acetonitrile. For example ACE inhibitory peptides were purified from fermented soybean (Kuba *et al.*, 2003), freshwater clam (Tsai *et al.*, 2006) and corn gluten (Yang *et al.*, 2007) at a linear

gradient of 0-40% acetonitrile. ACE inhibitory peptides from broccoli and buckwheat were eluted at a gradient of 5-20% acetonitrile (Lee *et al.*, 2006; Ma *et al.*, 2006). Even though some studies on purification of ACE inhibitory peptides have eluted proteins to a percentage of acetonitrile higher than 50%, the fraction that exhibited the highest ACE inhibitory activity were proteins eluted at lower than 50% acetonitrile. For example, ACE inhibitory peptide was purified from grass carp at linear gradient of acetonitrile from 5-60% for 60 min. The fraction exhibited the highest ACE inhibitory activity was eluted at a retention time of approximately 40 min (Chen *et al.*, 2012a). Likewise, ACE inhibitory peptide from salmon was eluted from 0-70% acetonitrile for 33 min. The most active fraction was eluted at a retention time of approximately 21 min (Ahn *et al.*, 2012). Thus, the four protein extracts in the current study was further purified by RPHPLC to a maximum of 50% acetonitrile.

ACE inhibitory activity of the protein fractions collected from RPHPLC are shown in Table 4.3 (page 64). The RPHPLC fractions were tested at a lower concentration compared to protein extracts, i.e. 1 μg/ml. Approximately 3.3-25.9% of ACE was inhibited by the RPHPLC fractions from E1Pc, while the ACE inhibitory activity of the RPHPLC fractions from E5Pc was in the range of 17.4-30.8%. RPHPLC fractions from E1Ab and E3Ab inhibited ACE activity in the range of 1.2-19.3% and 7.7-25.7%, respectively. Based on the percentages of ammonium sulphate used, proteins in E1Pc and E1Ab have higher hydrophobicity compared to proteins in E5Pc and E3Ab. Referring to Table 4.3, mostly RPHPLC fractions collected from E5Pc and E3Ab has higher ACE inhibitory activity compared to RPHPLC fractions collected from E1Pc and E1Ab, respectively. This shows that proteins with intermediate hydrophobicity in E5Pc and E3Ab have higher ACE inhibitory activity. According to He *et al.* (2012), proteins that contain abundant hydrophobic amino acid can be a good source of ACE inhibitory peptides. For example, potent ACE inhibitory peptides have been purified from apricot

almond meal and defatted wheat germ protein which contain 50.08% and 42.54% of hydrophobic amino acids, respectively (He *et al.*, 2012; Wang *et al.*, 2011a). However hydrophobicity is not the only characteristic for ACE inhibitor. Apart from abundant hydrophobic amino acids, the presence of some amino acids with other characteristic can be an added advantage for the proteins to be a good source of ACE inhibitory peptides. Some studies have predicted the preference of amino acid with certain characteristic for each position in potent ACE inhibitory peptide. For example, a tripeptide with potential ACE inhibitory activity was predicted to have preference for aromatic amino acids at the carboxyl terminus, a positively charged amino acids for the middle position and hydrophobic amino acids for the amino terminus (Wu *et al.*, 2006a). Therefore, this may explain the higher ACE inhibitory activity in E5Pc and E3Ab which contain proteins with intermediate hydrophobicity compared to E1Pc and E1Ab, respectively.

Referring to Table 4.3, the highest ACE inhibitory activity in *P. cystidiosus* was exhibited by F3 of E5Pc whereas in *A. bisporus*, it was exhibited by F6 of E3Ab. The percentage of proteins recovered from E5PcF3 was 5.36% which was the second lowest amount obtained among the RPHPLC fractions of E5Pc. On the other hand, 6.15% of proteins were recovered from E3AbF6. It was the third lowest amount of protein obtained among the RPHPLC fractions of E3Ab. This may suggest that the active ACE inhibitory proteins in *P. cystidiosus* and *A. bisporus* were present in low abundance. According to Ning *et al.* (2008), usually the low abundance proteins that plays important roles in biological process. Therefore, the low recovery percentage with the highest ACE inhibitory activity exhibited by E5PcF3 and E3AbF6 shows that these RPHPLC fractions consist of low abundance proteins which could be potential source of ACE inhibitory peptides.

In the previous studies, different ACE inhibitory peptides had been purified from different species of edible mushrooms, namely *G. frondosa* (Choi *et al.*, 2001), *T. giganteum* (Lee *et al.*, 2004) and *P. cornucopiae* (Jang *et al.*, 2011). This shows that different proteins in the mushroom species were responsible for the ACE inhibitory activity. Hence, the RPHPLC fraction with the highest ACE inhibitory activity from *P. cystidiosus* (E5PcF3) and *A. bisporus* (E3AbF6) were selected for proteomic analysis by SDS-PAGE and SELDI-TOF-MS to compare their protein characteristics.

4.6 Proteomic analysis of selected RPHPLC fractions with active ACE inhibitory activity

Proteomic analysis of the two most active RPHPLC fractions from *P. cystidiosus* (E5PcF3) and *A. bisporus* (E3AbF6) were carried out by SDS-PAGE and SELDI-TOF-MS. Referring to Figure 4.5, SDS-PAGE of the two RPHPLC fractions revealed the majority of proteins present in E5PcF3 and E3AbF6 were of low molecular weight, i.e. with molecular masses less than 30 kDa. Some reports from previous studies on food-derived proteins with ACE inhibitory abilities also feature relatively low molecular weight. For example, buckwheat proteins with molecular masses less than 10 kDa has exhibited higher anti-ACE activity than its high molecular weight fractions (Ma *et al.*, 2006). In the current study, E5PcF3 and E3AbF6 have protein bands with molecular masses of 7 and 12 kDa as well as 5, 8 and 13 kDa, respectively. Proteins with molecular masses of 3 to 10 kDa have been reported to exhibit moderate ACE inhibitory activity (Chiang *et al.*, 2006; Jiang *et al.*, 2010). Therefore, it is possible to suggest that these low molecular weight proteins could be responsible for triggering the ACE inhibitory activity in E5PcF3 and E3AbF6.

There were two protein bands common to E5PcF3 and E3AbF6 of molecular masses 16 and 18 kDa. A distinct band at 17 kDa was detected in E5PcF3 which was not visualised in E3AbF6. On the other hand, E3AbF6 contained eight high molecular weight protein bands which are not detected in E5PcF3. According to Natesh *et al.* (2003), large peptide molecules are restricted from fitting into the active site of ACE. Therefore the ACE inhibitory activity in E3AbF6 might not be attributable to these high molecular weight proteins.

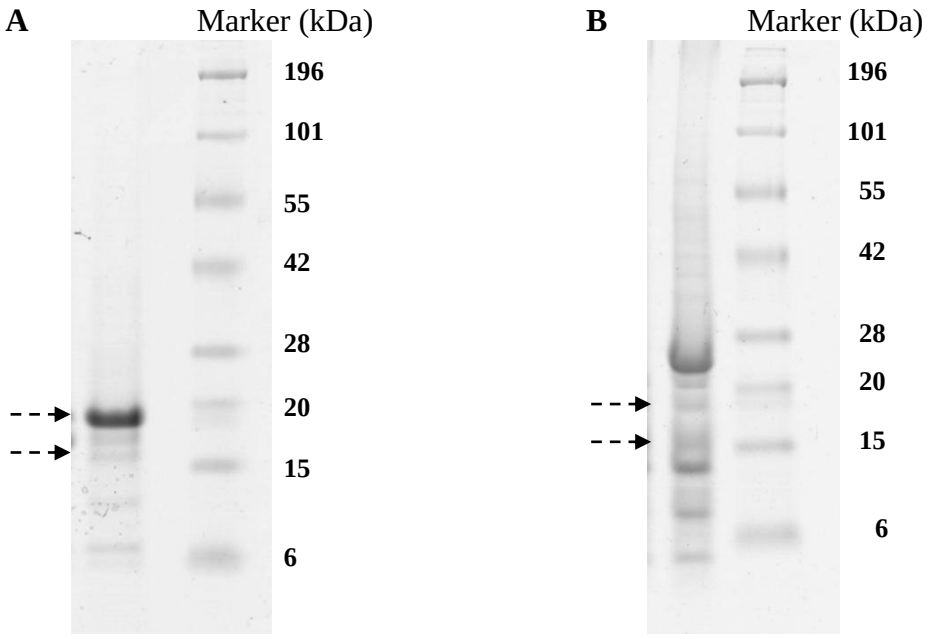


Figure 4.5: SDS-PAGE analysis of (A) E5PcF3 and (B) E3AbF6.

- - → : Proteins with similar molecular weight detected in E5PcF3 and E3AbF6.

SELDI-TOF-MS has been reported to be the most effective method for profiling low molecular weight proteins and peptides (<30 kDa), providing a complementary visualisation technique to SDS-PAGE data (Issaq *et al.*, 2003). SELDI-TOF-MS spectra of E5PcF3 and E3AbF6 are shown in Figure 4.6. There were 22 protein clusters identified in the EDM analysis of the two RPHPLC fractions as shown in Table 4.4.

SELDI peaks lower than *m/z* 3500 were detected with high intensity in the SELDI-TOF-MS spectra of E3AbF6. These peaks were not detected in E5PcF3. In the current study, proteins lower than 3,500 Da were expected to be removed during dialysis of the protein extracts by snakeskin dialysis tubing (3,500 Da cut-off). As reported previously, intact protein structures may become unstable in 50% aqueous-acetonitrile mixtures (Gekko *et al.*, 1998). Based on the location of E3AbF6 in the RPHPLC chromatogram (Figure 4.4D, page 63), the fraction was collected from the gradient in a water-acetonitrile mixture close to 50% (water: acetonitrile). Therefore, the detection of proteins lower than 3,500 Da in the SELDI spectra of E3AbF6 could represent broken-down or fragmented proteins from the unstable proteins in the aqueous-acetonitrile mixture.

There were five common peaks detected in E5PcF3 and E3AbF6 (*m/z* values 3940, 4254, 5496, 8300 and 11413). The high intensity of these common peaks shows they could play a role in the high levels of ACE inhibitory activity exhibited by the two RPHPLC fractions. Besides the five common peaks, there were seven extra peaks with *m/z* values of 5291, 7876 to 8244 and 8516 to 8629 detected only in E5PcF3, while E3AbF6 contained three peaks with *m/z* values of 5906.9, 6022.5 and 8291.8 that were not detected in E5PcF3. Interestingly the *m/z* values of these proteins were in the range of 3000 to 10000. Therefore, this range of proteins could be the potential anti-ACE agent which played a role in the higher ACE inhibitory activity exhibited in E5PcF3 and E3AbF6.

Based on the proteomic analysis of E5PcF3 and E3AbF6 discussed above, the RPHPLC fractions still consist of a mixture of proteins. Therefore, the protein mixtures in E5PcF3 and E3AbF6 were further fractionated by SEC column according to molecular size to further reduce the complexity of proteins for detection of ACE inhibitory proteins.

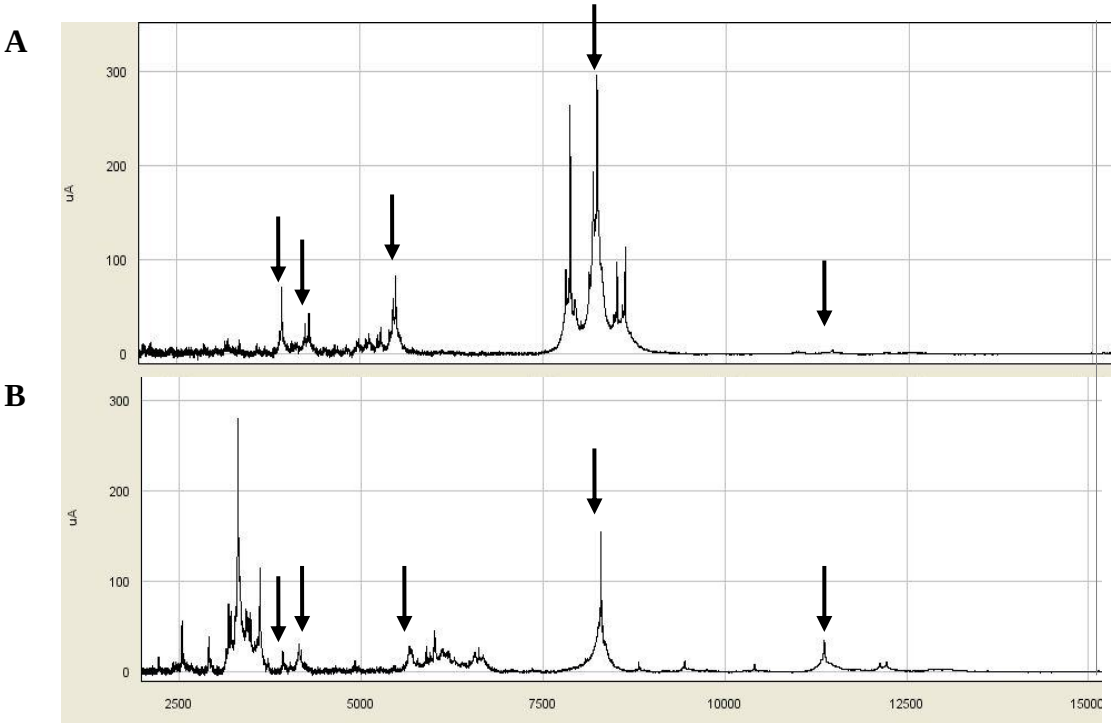


Figure 4.6: SELDI-TOF-MS spectra of (A) E5PcF3 and (B) E3AbF6.

⟶ : Proteins with similar molecular weight detected in E5PcF3 and E3AbF6.

Table 4.4: EDM analysis of peak intensities observed in SELDI-TOF-MS spectra of E5PcF3 and E3AbF6.

[a]Protein cluster *m/z*	[b]*p*-value	[c]Peak intensities (μA)	
		E5PcF3	E3AbF6
2552.0 ± 0.9	0.021	ND	59.9 ± 5.7
2919.2 ± 0.9	0.021	ND	39.4 ± 4.3
3189.0 ± 1.3	0.021	ND	70.9 ± 11.4
3223.2 ± 1.1	0.021	ND	60.1 ± 11.9
3317.7 ± 0.9	0.021	ND	295.7 ± 7.7
3621.2 ± 1.0	0.021	ND	102.0 ± 19.2
3940.6 ± 2.8	0.021	66.9 ± 2.8	22.4 ± 2.3
4254.8 ± 1.0	0.021	42.1 ± 2.6	30.6 ± 3.0

Table 4.4 (Continued)

5291.2 ± 0.5	0.021	25.9 ± 0.5	ND
5496.6 ± 0.4	0.021	85.0 ± 5.4	35.2 ± 4.0
5906.9 ± 2.3	0.021	ND	30.1 ± 2.0
6022.5 ± 0.8	0.021	ND	42.5 ± 7.4
7876.5 ± 0.6	0.021	226.2 ± 39.4	ND
8193.5 ± 0.6	0.021	189.2 ± 5.4	ND
8223.6 ± 1.1	0.021	141.5 ± 4.9	ND
8244.2 ± 0.6	0.021	284.9 ± 13.5	ND
8291.8 ± 2.3	0.040	ND	76.5 ± 3.9
8305.8 ± 1.4	0.021	88.5 ± 2.5	155.3 ± 14.7
8328.2 ± 22.1	0.021	80.3 ± 2.6	50.1 ± 4.5
8516.6 ± 0.6	0.021	80.3 ± 13.9	ND
8629.5 ± 0.7	0.021	95.2 ± 14.9	ND
11413.5 ± 2.7	0.021	5.0 ± 0.3	39.6 ± 3.4

[a]Proteins with similar molecular mass were clustered during EDM analysis. The *m/z* value for each protein cluster was presented as mean ± standard deviation (n=4).

[b]Values with $p<0.05$ indicate the protein peaks were statistically significant in the two samples based on peak intensity.

[c]Peak intensities are presented as mean ± standard deviation (n=4).

ND: Peaks were not detected. Intensities of ND peaks are only estimated values for clustering purposes during EDM analysis. Thus their intensities are not shown.

Protein clusters highlighted in bold are common proteins detected in E5PcF3 and E3AbF6.

4.7 Further purification of selected RPHPLC fractions with active ACE inhibitory activity by SEC

Clinical trials have suggested that protein consumption, especially from plant sources could reduce blood pressure (Elliott *et al.*, 2006). Low molecular weight compounds possess advantages of high absorbability, high permeability and long term stability (Arai *et al.*, 2013). Based on the report by Natesh *et al.* (2003), large protein molecules are restricted from fitting into the active site of ACE. Previous studies have reported that low molecular weight fractions of filtrates from ultrafiltration exhibited higher anti-ACE activity. For example, protein filtrate from tilapia with molecular weight cut off lower than 30 kDa showed higher percentages of ACE inhibitory activity (Raghavan & Kristinsson, 2009). In addition, potent ACE inhibitory proteins purified from water extracts of edible mushrooms such as *P. cornucopiae*, *P. adiposa* and *T. giganteum* were exhibited by filtrate of 5000 molecular weight cut-off (Jang *et al.*, 2011; Koo *et al.*, 2006; Lee *et al.*, 2004). Therefore, this has intrigued us to further purify the proteins with lower molecular weight. In the current study, the mobile phase used for SEC, i.e 45% acetonitrile containing 0.1% TFA was an optimised method for the separation of low molecular weight proteins (< 20 kDa) provided by the manufacturer (McGinley & Knudsen, 2010).

The SEC chromatograms of E5PcF3 and E3AbF6 are shown in Figure 4.7. There were seven fractions collected from each sample and labelled as C1 to C7. Referring to Table 4.5, higher recovery rate percentages was collected from SEC fractions of E5PcF3 compared to E3AbF6. The total percentages of proteins recovered in E5PcF3 and E3AbF6 were 83.4% and 21.0%, respectively. As mentioned earlier, the SEC profiling has been optimized for the separation of low molecular weight proteins. The high percentage of protein recovered in E5PcF3 may suggest that low molecular weight proteins dominate in E5PcF3. This is in accordance with the result of SDS-PAGE in

Figure 4.5 (page 69), which showed only protein bands with molecular masses lower than 30 kDa were visualized in E5PcF3. On the other hand, SDS-PAGE analysis shows that E3AbF6 consist of both high and low molecular weight proteins. The lower protein recovery in SEC fractionation of E3AbF6 may presume that the extract contained higher amount of high molecular weight proteins.

The ACE inhibitory activity of SEC fractions was tested at 1 μg/ml. Referring to Table 4.5, most of the SEC fractions collected from E5PcF3 exhibited higher percentages of ACE inhibitory activity compared to E3AbF6. The ACE inhibitory activity exhibited by SEC fractions of E5PcF3 was in the range of 5.6-27.4% whereas 0.9-18.7% of ACE activity was blocked by SEC fractions of E3AbF6. Among the SEC fractions, E5PcF3C1, E3AbF6C1 and E3AbF6C4 exhibited the highest ACE inhibitory activity. Based on the position of the three peaks in the SEC chromatogram shown in Figure 4.7, the molecular masses of peaks C1 and C4 were estimated to be lower than 20 and 10 kDa, respectively. Previous studies have shown successful purification of potent ACE inhibitory peptides from food proteins in this range of molecular masses. Protein fraction from sea cucumber below 20 kDa exhibited the highest percentage of ACE inhibitory activity (Forghani *et al.*, 2012) whilst protein fraction from tuna with molecular masses of 5-10 kDa exhibited high percentage of anti-ACE activity (Lee *et al.*, 2010). In addition, ACE inhibitory peptide from *G. frondosa* was purified from filtrate with molecular weight cut off 10,000 (Ohtsuru *et al.*, 2000). Therefore, the three SEC fractions, i.e. E5PcF3C1, E3AbF6C1 and E3AbF6C4 could be a potential source of potent ACE inhibitory peptides. These three SEC fractions were selected for further analysis by LC/MS/MS.

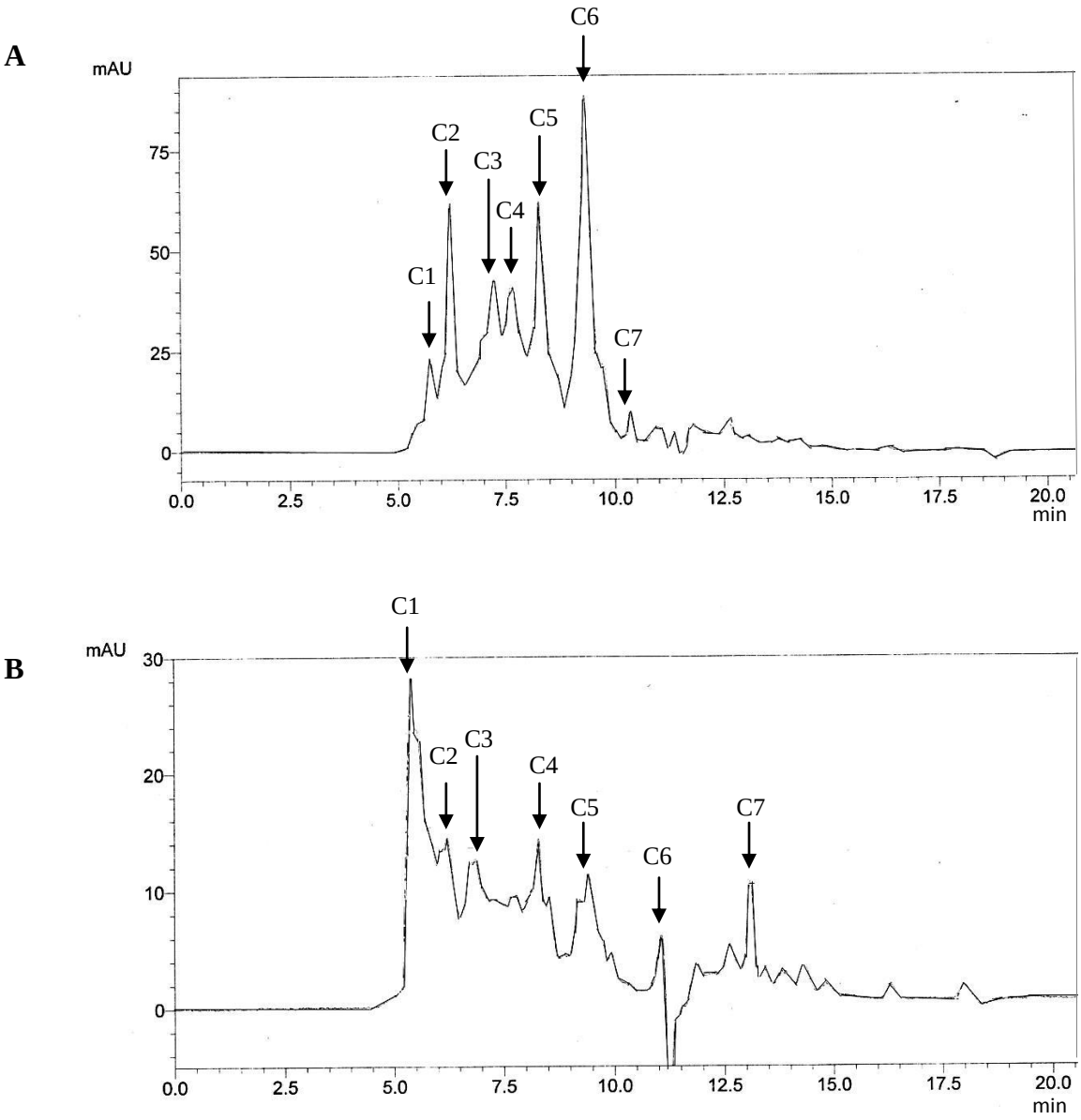


Figure 4.7: SEC chromatograms of (A) E5PcF3 and (B) E3AbF6.

Fractions collected were labelled as C1 to C7.

Table 4.5: Percentages of protein recovery yield and percentages of ACE inhibitory activity of the SEC fractions.

SEC fractions	RPHPLC fractions			
	E5PcF3		E3AbF6	
	% Recovery	*% inhibition	% Recovery	*% inhibition
C1	3.6	**27.4 ± 2.7 [a]**	4.5	**18.7 ± 3.2 [e]**
C2	3.9	5.6 ± 2.2 [bc]	2.9	5.8 ± 2.3 [bc]
C3	24.6	7.5 ± 0.8 [cd]	5.1	0.9 ± 0.6 [f]
C4	12.8	7.9 ± 4.7 [cd]	4.0	**16.1 ± 0.5 [e]**
C5	9.6	5.9 ± 2.2 [bc]	1.7	6.4 ± 1.5 [bcd]
C6	12.3	8.7 ± 2.5 [cd]	0.7	6.6 ± 2.5 [bcd]
C7	16.6	10.1 ± 0.52 [d]	2.1	3.4 ± 1.8 [bf]
Total	83.4	-	21.0	-

All values are expressed as mean ± standard deviation (n=3).

*ACE inhibitory activity of SEC fractions was tested at 1 µg/ml protein. Means with different lower case alphabet letters are significantly different ($p<0.05$).

SEC fractions highlighted in bold were selected for further analysis.

4.8 LC/MS/MS analysis of potent ACE inhibitor from SEC fractions

The amino acid sequence of peptides in E5PcF3C1, E3AbF6C1 and E3AbF6C4 were determined by LC/MS/MS. The list of all the peptide sequences including the score, protein name, molecular masses of the proteins and species of origins were illustrated in Appendix C. Most of the proteins identified in the three SEC fractions were hypothetical proteins, i.e. proteins predicted from amino acid sequences only and the protein functions were unknown. All the three SEC fractions analyzed in the current study contain ribosomal protein. It is one of the common proteins found in the cell wall

of mushrooms (Chen *et al.*, 2012b). It plays a role in the biogenesis of ribosome for different stages of translation process and involved in RNA folding, protein assembly, rRNA processing, stabilization of the subunit structure and interacts with protein folding factors at the exit tunnel of the ribosome (Ferreira-Cerca *et al.*, 2005). Interestingly, peptides from ribosomal protein in rapeseed have been reported as potent ACE inhibitor (Marczak *et al.*, 2003). Therefore, the ribosomal proteins in E5PcF3C1, E3AbF6C1 and E3AbF6C4 could be a potential source of ACE inhibitory peptide.

Actin is also a protein commonly found in the mushroom cell wall (Chen *et al.*, 2012b). In fungi, it is a cytoskeleton protein involved in the initiation of cell wall formation and functions in the fundamental processes of cell polarity, cytokinesis and endocytosis (Aghamohammadzadeh & Ayscough, 2010). ACE inhibitory peptides have been identified in actin from animal sources such as chicken and fish (Fujita *et al.*, 2000; Yokoyama *et al.*, 1992). Likewise, peptide isolated from actin of plant, i.e. rapeseed has also exhibited potent ACE inhibitory activity. This may suggest actin in E5PcF3C1 could have contributions in the ACE inhibitory activity exhibited by the particular SEC fraction.

Another common protein in mushroom, guanosine triphosphate (GTP)-binding protein has been detected in E5PcF3C1. It is a protein associated with the biosynthesis of capsular polysaccharides, i.e. β-glucans and plays a major role in the regulation of cell wall morphogenesis (Kang & Cabib, 1986). Glutathione S-transferase found in E3AbF6C1 is a protein associated with detoxification processes and participate in the cellular response to oxidative stress (Thuillier *et al.*, 2011). Currently, there is no report on the association of ACE inhibitory peptides with GTP-binding protein or glutathione S-transferase.

Due to the detection of mostly hypothetical proteins with unknown function in the three SEC fractions, the amino acid sequence of peptides was taken into consideration in the selection of peptides with potential ACE inhibitory activity. The physicochemical properties of amino acids have been reported to influence the biological activity of peptides as ACE inhibitor. For example, hydrophobicity of amino acids has been indicated to have the greatest influence on ACE inhibitory activity. In other words, amino acid with greater hydrophobicity shows higher ACE inhibitory activity (He *et al.*, 2012). According to Pripp and co-workers, hydrophobicity of C-terminal enhanced the ACE inhibitory activity of potential peptides up to six amino acids in length (Pripp *et al.*, 2004; Pripp *et al.*, 2005). Likewise, Curtis *et al.* (2002) indicated bioactive peptides are typically 2-5 amino acids in length. Besides hydrophobicity of C-terminal, Wu *et al.* (2006a) has suggested branched aliphatic amino acid for the N-terminal of potential ACE inhibitory peptides. Therefore, in the current study, potential ACE inhibitors were selected from the peptide list based on the structural requirement of potential ACE inhibitory peptides mentioned above, i.e. less than six amino acids and consists of hydrophobic and branched aliphatic amino acid at the C- and N-termini, respectively. The physicochemical properties of the twenty amino acids, which include hydrophobicity, charge and chemical structure were listed in Appendix D.

Total of ten peptide sequences have been selected as potential ACE inhibitory peptides. Two peptides were selected from E5PcF3C1. There were five and three peptides chosen from E3AbF6C1 and E3AbF6C4, respectively. The peptide sequences and molecular masses were listed in Table 4.6. LC/MS/MS spectra of the peptides were illustrated in Appendix E. There is a similar peptide sequence, i.e. AHEPVK detected in E5PcF3C1 and E3AbF6C4. It could be a common peptide found in *P. cystidiosus* and *A. bisporus*. The subsequent analyses were carried out based on nine peptide sequences.

The nine selected ACE inhibitory peptides contain five to six amino acids in length. This is in accordance with the maximum length of bioactive peptides as mentioned in previous reports (Curtis *et al.*, 2002; Pripp *et al.*, 2004).

Table 4.6: List of peptide sequences, molecular masses and IC_{50} values of the selected potential ACE inhibitors.

SEC fraction	Peptide (P)	Peptide sequence	Observed mass	Calculated mass	*IC_{50} (μM)
E5PcF3C1	1	AHEPVK	679.51	679.37	62.8 ± 5.8 [a]
	2	GPSMR	547.73	546.26	277.5 ± 9.9 [b]
E3AbF6C1	3	KVAGPK	599.39	598.38	304.9 ± 7.1 [b]
	4	FALPC	549.29	549.26	690.9 ± 5.3 [c]
	5	RIGLF	605.35	604.37	115.9 ± 10.4 [a]
	6	EGEPR	587.80	586.27	728.2 ± 6.6 [c]
	7	APSAK	474.25	472.26	325.7 ± 6.5 [b]
E3AbF6C4	1	AHEPVK	679.51	679.37	62.8 ± 5.8 [a]
	8	GVQGPM	604.37	603.27	736.7 ± 4.8 [c]
	9	PSSNK	531.24	531.27	129.3 ± 16.6 [a]

*IC_{50} values were expressed as mean ± standard deviation (n=3). Means with different lower case alphabet letters are significantly different ($p<0.05$).

As indicated in the report by Wu *et al.* (2006a), the N-terminal of potential ACE inhibitory peptides usually consist of aliphatic amino acids. This type of amino acids have side chains that contain only hydrogen and carbon atoms, which include alanine, glycine, isoleucine, leucine, valine and proline (Betts & Russell, 2003). As shown in

Table 4.6, the first two positions of the N-terminal for the nine peptides have either one or both position with aliphatic amino acids whereas for the C-terminal, greater hydrophobicity of amino acids may increase the ACE inhibitory activity of the particular peptide (He *et al.*, 2012), Amino acid with hydrophobic characteristic include alanine, isoleucine, leucine, methionine, proline, tryptophan and valine. Referring to the peptide sequences shown in Table 4.6, the first two positions of the C-terminal contain either one or both residues with hydrophobic amino acids.

The nine selected peptides were chemically synthesized to determine the IC_{50} values of ACE inhibition. The extrapolated graphs for the determination of IC_{50} values for the nine synthetic peptides were illustrated in Appendix F. Referring to Table 4.6, the nine synthetic peptides showed variations of IC_{50} values, ranging from 62.8-736.7 μM with the lowest IC_{50} value exhibited by P1. Interestingly, this peptide was detected in both E5PcF3C1 and E3AbF6C4. It could be a common peptide in *P. cystidiosus* and *A. bisporus*. The second potent ACE inhibitory activity was exhibited by P5 with IC_{50} value of 115.9 μM. This was followed by P9 with IC_{50} value of 129.3 μM. The IC_{50} values of these three peptides were not significantly different ($p<0.05$). Peptides P2, P3 and P7 have also exhibited insignificant differences ($p<0.05$) with IC_{50} values in the range of 277.5-325.7 μM. The remaining three peptides, P4, P6 and P8 have blocked 50% of the ACE activity at a higher concentration, ranging from 690.9-736.7 μM.

Previous studies have reported on the successful purification of ACE inhibitory peptides from edible mushrooms. Among them, peptides from *G. frondosa* and *P. adiposa* have the same length of amino acids with the peptides in the current study, i.e. 5-6 amino acids. The IC_{50} value of peptides VIEKYP and GQGGP from *G. frondosa* and *P. adiposa* were 129.7 and 254 μM, respectively (Choi *et al.*, 2001; Koo *et al.*, 2006). Apparently, the three most active peptides tested in the current study, namely P1, P5 and P9 has comparable activity with peptide from *G. frondosa* and more potent

activity than peptide from *P. adiposa*. There were also pentapeptides and hexapeptides derived from other food sources that exhibited comparable ACE inhibitory activity with the three most potent peptides in the current study. Peptides LVQGS and VTPALR from soybean and mung bean blocked 50% of the ACE activity at a concentration of 43.7 and 82.4 μM, respectively (Li *et al.*, 2006; Rho *et al.*, 2009). Pentapeptides, GPCSR and VKAGE from sweet potato exhibited IC_{50} values of 61.67 and 141.56 μM, respectively (Huang *et al.*, 2008; Huang *et al.*, 2011b). There were also pentapeptides showing higher ACE inhibitory activity compared to the peptides in the current study. For example LKPNM, a peptide from bonita fish which has been commercialized as supplement in Canada and Japan. It has the ability to block 50% of the ACE activity at a concentration of 2.4 μM (Fujita & Yoshikawa, 1999). Previous studies have also reported on the purification of shorter ACE inhibitory peptides. i.e. 2-4 amino acids. Some of these peptides have shown higher ACE inhibitory activity than the pentapeptides and hexapeptides in the current study. However, there were also peptides that exhibited lower ACE inhibitory activity. For example, tripeptides from clam, VKP and VKK have IC_{50} values of 3.7 and 1045 μM, respectively. Nine peptides with 2-4 amino acids purified from sweet potato exhibited IC_{50} values of 208.6-849.7 μM (Huang *et al.*, 2008). There were also studies reported on the isolation of longer peptide sequences with ACE inhibitory activity. For example, two peptides have been extracted from *P. cornucopiae* with 14 and 17 amino acids in length. These peptides exhibited IC_{50} values of 277.3 and 539.9 μM, respectively (Jang *et al.*, 2011). However, shorter peptides are preferred due to the advantages of being capable of passing the gastrointestinal tract, easily absorbed and reach the peripheral target sites (Quiros *et al.*, 2005). Therefore, the nine peptides with five to six amino acids tested in the current study could be potential ACE inhibitors and warrant further study.

The amino acid sequences of the nine peptides tested in the current study were compared with the potential ACE inhibitory peptides reported in previous studies. C-terminal has been reported to have strong influence on the ACE inhibitory activity (Cheung *et al.*, 1980; Wijesekara & Kim, 2010). This is because binding of inhibitor to the ACE enzyme takes place predominantly via the C-terminal residues (Jao *et al.*, 2012). Therefore, the comparison of amino acid sequences reported in the current study was mostly focused on the C-terminal residue. Pentapeptide from sea squirt, AHIII (Ko *et al.*, 2011) has a similar amino acid sequence with P1, AHEPVK. The first two amino acids at N-terminal were alanine and histidine. Besides P1, there were three peptides in the current study, P3 (KVAGPK), P7 (APSAK) and P9 (PSSNK) which have lysine at the C-terminal. Lysine is an aliphatic amino acid. There were reports demonstrated that potential ACE inhibitory peptides posses lysine at their C-terminal. Examples include VVGAK, YPK, AIYK and VKK from sweet potato, broccoli, wakame and clam, respectively (Huang *et al.*, 2011a; Lee *et al.*, 2006; Suetsuna & Nakano, 2000; Tsai *et al.*, 2006). Pentapeptide ACE inhibitor from sweet potato, GPCSR (Huang *et al.*, 2011b) has a similar peptide sequence with P2, GPSMR. Besides P2, another peptide that has arginine at the C-terminal is P6 (EGEPR). Previous reports on ACE inhibitory peptides that contain this aliphatic amino acid at the C-terminal include VR and VTPALR from salmon and mung bean, respectively (Gu *et al.*, 2011; Li *et al.*, 2006). P5 (RIGLF) which has the second lowest IC_{50} value contains phenylalanine at the C-terminal. Peptide with aromatic amino acid at the C-terminal has been reported to exhibit strong ACE inhibitory effect (Cheung *et al.*, 1980). Previously reported ACE inhibitory peptides with phenylalanine at the C-terminal include VIIF and YYAPF from cuttlefish and wine, respectively (Balti *et al.*, 2010; Takayanagi & Yokotsuka, 1999). Cysteine has been reported to have modulatory effect on the renin-angiotensin system (Vasdev *et al.*, 2009). In the current study, P4 (FALPC) contains cysteine at its C-terminal. A

potential ACE inhibitory peptide, GTEKC with cysteine at the C-terminal has been isolated from sweet potato (Huang *et al.*, 2008). LKPNM, a peptide from bonita fish has been commercialized as supplement in Canada and Japan (Harnedy & FitzGerald, 2012). It has strong hydrophobic amino acid, methionine at the C-terminal. Interestingly, peptide P8 (GVQGPM) in the current study also contains methionine at the C-terminal. In addition, glycine has been reported as one of the major constituent of ACE inhibitory peptides (Byun & Kim, 2001; Wu *et al.*, 2013). In the current study, there were five peptides, P2, P3, P5, P6 and P8 containing glycine either at the N-terminal or middle position. Therefore, the similarity of amino acid residues of the nine peptides in the current study with previously reported ACE inhibitors shows the nine peptides from *P. cystidiousus* and *A. biposrus* could be potential ACE inhibitors. Although the IC_{50} value of the nine peptides were higher compared to some previously reported ACE inhibitory peptides from other food sources, the three most active peptides, namely P1, P5 and P9 have better activity compared to previously reported edible mushrooms (Choi *et al.*, 2001; Jang *et al.*, 2011; Koo *et al.*, 2006). Besides, mushrooms are easy to cultivate. *Pleurotus* spp. and *A. bisporus* are among the most cultivated mushrooms worldwide (Sanchez, 2010). Hence, a sufficient supply of these mushrooms species could be obtained for extraction of potential ACE inhibitory peptides. Additionally, mushroom allergy from eating is very rare (Koivikko & Savolainen, 1988). Therefore, these could be the added advantages for the production of mushroom as pharmaceutical drugs.

Table 4.7 summarizes the percentages of ACE inhibitory activity of fractions obtained from each purification steps. Water extracts of *P. cystidiosus* and *A. bisporus* have showed 81.4% and 87.2% of ACE inhibition when tested at 10 mg/ml. The protein extracts were tested at a lower concentration than the water extract, i.e. 10 µg/ml and the ACE inhibitory activity was maintained at 86.7% and 71.4% for *P. cystidiosus* and

Table 4.7: Summary of the purification steps of ACE inhibitors from *P. cystidiosus* and *A. bisporus*.

Purification steps (concentration)	*P. cystidiosus*		*A. bisporus*	
	ACE inhibition (%)	*Purification fold	ACE inhibition (%)	*Purification fold
Water extract (10 mg/ml)	81.4	-	87.2	-
Pooled protein extract (10 µg/ml)	86.7	-	71.4	-
RPHPLC (1 µg/ml)	30.8	1.0	25.7	1.0
SEC (1 µg/ml)	27.4 (C1)	0.9	18.7 (C1)	0.7
			16.1 (C4)	0.6
Synthesised peptides (1 µg/ml)	27.5 (P1)	0.9	27.5 (P1)	1.1
	23.9 (P2)	0.8	15.0 (P3)	0.6
			22.0 (P4)	0.9
			27.6 (P5)	1.1
			26.4 (P6)	1.0
			21.6 (P7)	0.8
			32.7 (P8)	1.3
			22.9 (P9)	0.9

*Purification fold was determined by dividing the percentage of ACE inhibition from each purification step with the percentage of ACE inhibition from RPHPLC.

A. bisporus, respectively. The stable ACE inhibitory activity in spite of the reduction of concentration by 1000 fold showed proteins in the two mushroom species tested in the current study played a major role in ACE inhibition. This is in accordance with the report by Barbosa-Filho *et al.* (2006) that showed protein as the most reported compound showing active ACE inhibitory activity.

The proteins were then fractionated by RPHPLC according to hydrophobicity and the concentration used to test their ACE inhibitory activity was reduced to 1 µg/ml. The most active RPHPLC fraction from *P. cystidiosus* and *A. bisporus* had inhibited 30.8% and 25.7% of ACE activity, respectively. Proteins in these RPHPLC fractions were then further fractionated based on their molecular size by SEC. The most active SEC fraction from *P. cystidiosus* inhibited 27.4% of the ACE activity at 1 µg/ml. Two SEC fractions from *A. bisporus* have exhibited the highest ACE inhibitory activity, i.e. 18.7% and 16.1%. The RPHPLC and SEC fractions possessed bioactivities even at a reduced 10 fold concentration than the concentration of protein extract indicated fractionation of proteins has reduced the complexity of protein mixture hence, the potential protein is able to better express its ACE inhibitory activity. The enhancement of ACE inhibitory activity by fractionation has been reported by Ning *et al.* (2008). Based on the purification fold, a slight reduction in the ACE inhibitory activity of the two SEC fractions compared to RPHPLC fraction in *A. bisporus* may indicate a possibility of synergistic activity among the proteins in RPHPLC fraction of *A. bisporus*, Synergistic activity has been proposed for peptides in cheese, tilapia, pea and whey (Gomez-Ruiz *et al.*, 2002; Raghavan & Kristinsson, 2009; Vermeirssen *et al.*, 2005). Fractionation of RPHPLC fraction by SEC could have prevented the respective proteins from exhibiting their activity.

Referring to Table 4.7, two peptides have been selected from *P. cystidiosus* whilst eight peptides were chosen from *A. bisporus*, The ACE inhibitory activity of the two

peptides from *P. cystidiosus* have shown similar percentages of inhibition with their SEC fraction. Tripeptides from broccoli and buckwheat have also been reported to exhibit similar activity with their native proteins in HPLC fraction (Lee *et al.*, 2006; Ma *et al.*, 2006). Therefore, the similar ACE inhibitory activity of the P1 and P2 compared to SEC fraction in the current study showed that the two peptides may have major contribution to the ACE inhibitory activity in SEC fraction of *P. cystidiosus*. The eight peptides from *A. bisporus* have exhibited a range of percentages of ACE inhibitory activity, i.e. 15.0-32.7%. Based on the purification fold, some of the peptides exhibited better ACE inhibitory activity compared to their protein precursors in the SEC fractions. Increased ACE inhibitory peptides compared to their native proteins have been reported in studies from food sources, such as cuttlefish and fish (Balti *et al.*, 2010; Ghassem *et al.*, 2011).

The stability of peptides in terms of their bioactivity after oral administration is of high concern in the protein pharmaceutical industry (Mahato *et al.*, 2003). Therefore, the nine chemically synthesized peptides in the current study were further analyzed for their ACE inhibitory activity after gastrointestinal digestion. Preliminary experiment by gastrointestinal enzyme incubation *in vitro* provides an easy method to evaluate the fate of these peptides after oral administration.

4.9 Effect of simulated gastrointestinal digestion on the selected peptides

4.9.1 ACE inhibitory activity of peptides after gastrointestinal digestion

Simulated gastrointestinal digestion of the nine synthetic peptides was carried out by *in vitro* assay. Studies have shown that most proteins were emptied from the stomach in two hours after ingestion (Chen *et al.*, 1962; Gupta *et al.*, 1958). Besides, at 40^{o}C most proteins are completely digested by enzymes within a 2 to 3 hours period (Hill &

Schmidt, 1962). Prolong hydrolysis time has been reported to cause excessive digestion and consequently produced inactive peptides (Zhu *et al.*, 2010). Peptides hydrolysed for 2 to 3 hours exhibited the highest ACE inhibitory activity (Jiang *et al.*, 2007; Kumar *et al.*, 2013). Hence, the peptides in the current study were incubated in the digestive enzyme solutions for 2.5 h. There were studies that utilized the same length of incubation time used in the current study. This include studies on the purification of ACE inhibitory peptides from whey (Bamdad *et al.*, 2009), cotton leafworm (Vercruysse *et al.*, 2009) and insect cells (Staljanssens *et al.*, 2011).

Control consists of percentages of ACE inhibitory activity of the peptides incubated in buffer only, without digestive enzymes. Referring to the results obtained in Table 4.8, the peptides in control group exhibited variations in the percentages of ACE inhibitory activity. The lowest ACE inhibitory activity was exhibited by P6 at 24.5%. This was followed by P4 and P8, where 53.3% and 53.8% of ACE was inhibited, respectively. P1 and P5 had the highest ACE inhibitory activity. Both of the peptides had inhibited 80.3% of the ACE activity.

A negative control was carried out alongside the experiment which consists of the ACE inhibitory activity of the buffer without peptide. The result obtained showed the buffer, which contains HCl and potassium phosphate buffer also exhibited a slight ACE inhibitory activity by blocking 31.5% of the ACE activity. According to Saboury and Moosavi-Movahedi (1995), the state of surrounding solvent such as pH, ionic strength, presence of hydrogen bond breaking solutes and amphiphilic compounds could disrupt the protein structure which lead to the loss of its biochemical activity. In the actual human body, the ACE enzymes work in physiological conditions which are optimum for the ACE enzyme. In the current study, ionic strength and pH of the buffers could have slightly affected the structure of the ACE and hence inhibit its activity.

Therefore, future work can be carried out by *in vivo* to test the actual ACE inhibitory activity of the peptides studied.

Table 4.8: ACE inhibitory activity of the nine selected peptides and negative control after simulated gastrointestinal digestion.

Peptide (P)	ACE inhibitory activity (%)		
	Control	Pep	Pep + T + C
1	80.3 ± 7.7 [a]	95.4 ± 0.2 [a]	95.9 ± 0.3 [a]
2	67.1 ± 4.5 [b]	92.2 ± 2.3 [ab]	96.1 ± 0.5 [a]
3	70.0 ± 4.2 [b]	93.8 ± 0.8 [ab]	95.8 ± 0.3 [a]
4	53.3 ± 6.7 [c]	90.9 ± 6.4 [b]	95.3 ± 0.5 [a]
5	80.3 ± 4.3 [a]	94.3 ± 1.7 [ab]	95.9 ± 0.3 [a]
6	24.5 ± 6.5 [d]	94.3 ± 1.7 [ab]	95.8 ± 0.3 [a]
7	68.7 ± 1.4 [b]	92.9 ± 2.1 [ab]	95.2 ± 0.3 [a]
8	53.8 ± 4.2 [c]	93.5 ± 1.4 [ab]	95.9 ± 0.9 [a]
9	63.3 ± 8.0 [b]	93.2 ± 0.8 [ab]	91.5 ± 4.7 [b]
Negative control	31.5 ± 0.1 [d]	63.9 ± 3.0 [c]	84.7 ± 2.0 [c]

Control: Peptide incubated with buffer only; Pep: Peptide incubated with pepsin;

Pep + T + C: Peptide incubated with pepsin for 2.5 hours followed by trypsin and chymotrypsin.

Negative controls were carried out without the presence of peptide.

ACE inhibitory activity was expressed as mean ± standard deviation (n=3). Means with different lower case alphabet letters within a column are significantly different ($p<0.05$).

Referring to Table 4.8, all the peptides except P6 have significantly higher ACE inhibitory activity than the negative control. This shows the bioactivity of peptides was not affected by the acidic and basic pH of buffer which could mimic the pH in the stomach and intestine. ACE inhibitory peptides which were pH stable have been isolated from protein red deer plasma, algae waste and oyster (Liu *et al.*, 2010b; Sheih *et al.*, 2009; Wang *et al.*, 2008).

The effect of gastrointestinal digestion on the ACE inhibitory activity of the peptides was assessed by two experiments. Evaluation of the ACE inhibitory activity of peptides digested with pepsin mimics the bioactivity of these peptides when it reached the stomach whilst the ACE inhibitory activity of peptides digested with pepsin for 2.5 hours followed by trypsin and chymotrypsin mimics the bioavailability of these peptides when it reached the intestine. As shown in Table 4.8, all the nine peptides had enhanced ACE inhibitory activity after digested by pepsin. The ACE inhibitory activity was further increased after digestion by trypsin and chymotrypsin. The ACE inhibitory activity of digestive enzymes without peptide was determined and served as negative control. The result obtained showed the negative controls also exhibited ACE inhibitory activity. Buffer containing pepsin but without peptide has inhibited 63.9% of ACE activity whilst buffer containing pepsin, trypsin and chymotrypsin but without peptide inhibited 84.7% of ACE activity. As mentioned in the previous paragraph, the buffer condition could have affected the molecular structure of some ACE that lead to the loss of its bioactivity. Additionally, the pH and ionic strength of the buffers could also cause some of the digestive enzymes, i.e. pepsin, trypsin and chymotrypsin to breakdown and release amino acid fragments which could have ACE inhibition activity. Currently, there were no studies reported on the possible ACE inhibition effect of the digestive enzymes. However, based on the report by Tang *et al.* (1973), the amino acid sequence for one of the digestive enzymes, i.e. pepsin contain high amount of hydrophobic amino

acid, which could have ACE inhibitory activity. Referring to Table 4.8, even though the negative controls also exert ACE inhibitory activity, the percentages exhibited by the negative controls were still statistically lower than the percentages obtained by all the nine peptides. This may indicate the peptides could still retain their biological activity after gastrointestinal digestion. Future work could be done by *in vivo* study to prove the actual ACE inhibitory activity of the peptides after gastrointestinal digestion.

There were studies reported on the production of pro-drug type ACE inhibitory peptides. These peptides are converted to true inhibitors by ACE or gastrointestinal proteases and claimed to have long lasting antihypertensive activity after oral administration (Fujita *et al.*, 2000; Zhao *et al.*, 2009). Pro-drug type ACE inhibitory peptide, LKPNM from fish protein which has been commercialized as supplement was reported to exhibit higher ACE inhibitory activity after oral administration. It was suggested to be due to the hydrolysis of LKPNM to produce LKP, which has eight-fold higher ACE inhibitory activity than LKPNM (Fujita & Yoshikawa, 1999). Likewise, ACE inhibitory activity of peptides isolated from fermented milk and sea cucumber has intensified inhibitory activity after incubation with gastrointestinal proteases (Hernández-Ledesma *et al.*, 2004; Zhao *et al.*, 2009). Terashima and co-workers (2011) have reported on the improved ACE inhibitory activity of the peptide derived from chicken meat by shortening the peptide length from 10 amino acids to two amino acids. Therefore, it is possible to suggest that the nine peptides tested in the current study could be further hydrolysed by gastrointestinal proteases to produce di- or tripeptides with higher ACE inhibitory activity. Hence, the nine peptides were further analyzed for the possible released of fragments during gastrointestinal digestion.

4.9.2 Bioinformatic-aided prediction of peptide released during gastrointestinal digestion

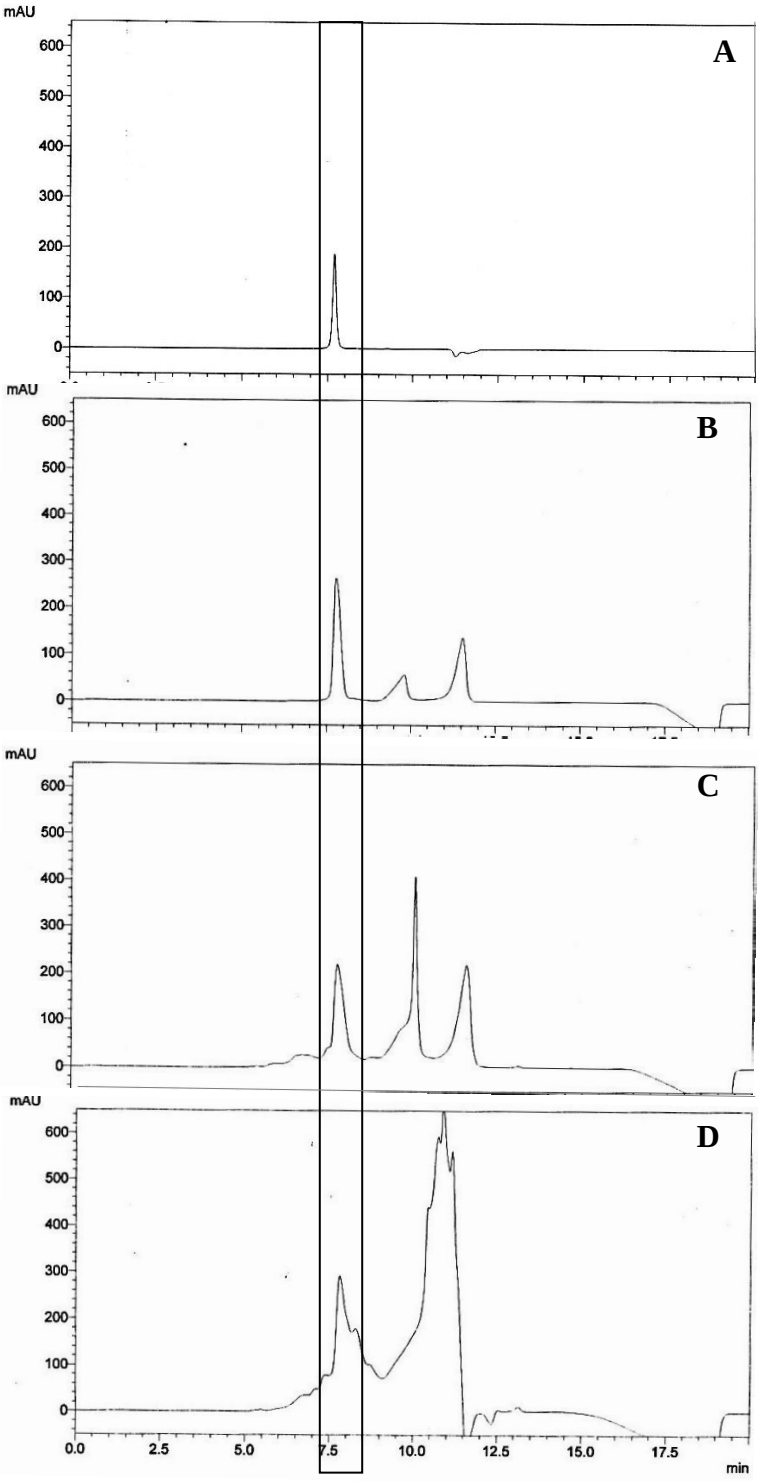
There are a number of online programs in peptide science designed to provide information such as physicochemical properties of amino acid residues, predictions of the peptide structure and proteolytic cleavage sites (Minkiewicz *et al.*, 2008; Minkiewicz *et al.*, 2009). BIOPEP database (www.uwm.edu.pl/biochemia) is one of the online programs that can serve as a tool to predict the possible proteolysis products from a protein or peptide sequence and define the possible biological activity of the proteolysis fragments (Iwaniak & Dziuba, 2011). All the nine peptide sequences had been analysed by BIOPEP. The predicted fragments released during pepsin and pepsin-pancreatin digestion were shown in the panel C and D of Figures 4.8-4.16. Referring to the result obtained, all the peptides tested, except P4 and P5, were predicted to be stable in the stomach before they reach the small intestine.

The stability of the peptides after simulated gastrointestinal digestion was analyzed by injecting the samples into SEC column. The chromatograms obtained were illustrated in Figures 4.8-4.16. The chromatograms of negative controls which consist of buffers only without gastrointestinal enzymes and peptide were shown in Appendix G (I) and G (II). There were two buffer peaks observed in each solution. Peaks at retention time of 9.49 and 11.45 min were observed in HCl whilst peaks at retention time of 9.77 and 11.31 min were observed in potassium phosphate buffer. These may explain the extra two peaks obtained in all the chromatograms in panel B of Figures 4.8-4.16, which consist of peptide incubated with buffer only.

The second negative control consists of buffer solutions with gastrointestinal enzymes but void of peptide solution. The chromatograms obtained were illustrated in Appendix G (III) and G (IV). It is important to note that some of the peptides in the

current study have peaks eluted at similar retention time with the gastrointestinal enzymes. As shown in Figures 4.8A (page 93) and 4.10A (page 96), peptides P1 and P3 were eluted at 7.80 min. Based on the chromatograms in Appendix G (III) and G (IV), the pepsin and pepsin-pancreatin enzymes also have a peak at 7.80 min with peak height 16 and 22, respectively. The negative controls were injected into the SEC column at 5-fold lower concentration than the *in vitro* peptide digestion assay. Therefore, the pepsin and pepsin-pancreatin enzyme peak could be eluted at a height of approximately 80 and 110, respectively in the chromatograms in panel C and D of Figures 4.8 and 4.10. On the other hand, peptides P2 (page 95), P5 (page 100), P6 (page 101), P7 (page 102) and P9 (page 105) were eluted at 8.30 min. Based on the SEC chromatograms in Appendix G (III) and G (IV), peak height of pepsin and pepsin-pancreatin enzyme at 8.30 min were 15 and 43, respectively. As mentioned previously, the pepsin and pepsin-pancreatin enzymes were injected at 5-fold lower concentration than the peptide digestion assay. Therefore, these gastrointestinal enzymes could have a peak height of approximately 75 and 215, respectively in the chromatograms in panel C and D of Figures 4.9, 4.12, 4.13, 4.14 and 4.16.

Referring to Table 4.8 (page 88), P1 incubated in buffer without digestive enzymes exhibited a high percentage of ACE inhibitory activity, i.e. 80.3%. According to Wang *et al.* (2011b), the preferential parameters for hexapeptides with potent ACE inhibitory activity are hydrophobic and stereo properties. In addition, hydrophobic ACE inhibitor has been reported to show higher efficiency than hydrophilic ACE inhibitor (He *et al.*, 2012; Moskowitz & Johnson, 2004; Sun *et al.*, 2011). Based on the amino acid sequence, P1 has high amount of hydrophobic amino acid. This may explain the high ACE inhibitory activity of P1. According to the BIOPEP analysis, P1 was not hydrolysed by the three digestive enzymes. It remained stable throughout the digestion process. As shown in Figure 4.8C and D, the peptide peak at 7.80 min has a peak height



Ret.	7.80
Height	195
Area	2901

Ret.	7.80
Height	263
Area	4216

Ret.	7.80
Height	321 (Enzyme height = 80)
Area	13284

*Fragment: AHEPVK

Ret.	7.80
Height	325 (Enzyme height = 110)
Area	9179

*Fragment: AHEPVK

Figure 4.8: SEC chromatograms of P1 with retention time (min), peak height (mAU), peak area (mAU) and predicted fragments released after digestion.

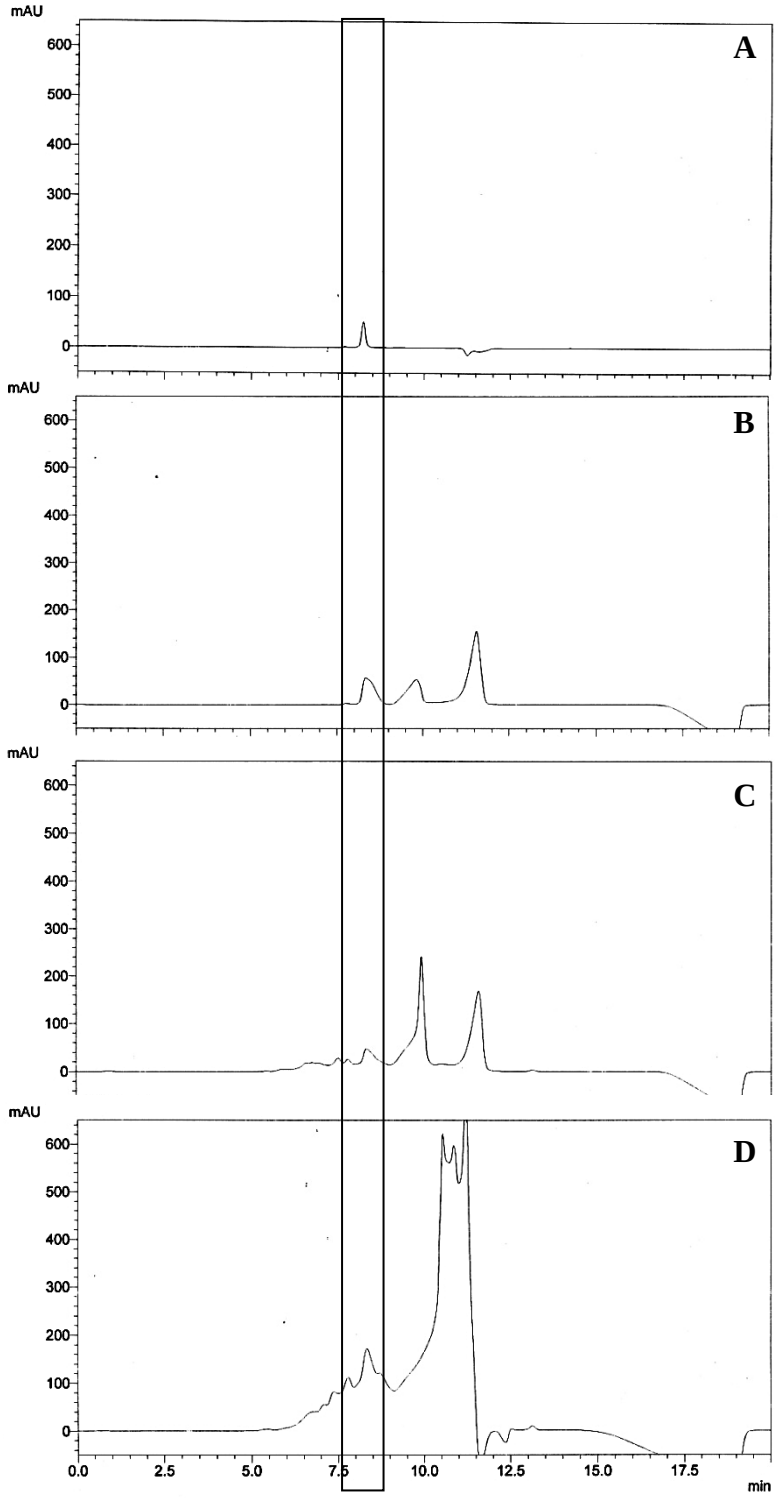
(A) Pure peptide (B) Peptide incubated with buffer only, (C) Peptide incubated with pepsin and (D) Peptide incubated with pepsin followed by trypsin and chymotrypsin.

*Prediction of proteolytic fragment released was carried out based on the BIOPEP database (www.uwm.edu.pl/biochemia).

of 321 and 325, respectively. Deduction of peak height for digestive enzymes, pepsin and pepsin-pancreatin enzyme, i.e. 80 and 110, respectively shows the peptide peak were retained at approximately the same height with the peptide incubated in buffer only (Figure 4.8B). This may verify the result in BIOPEP analysis that AHEPVK was not digested by the digestive enzymes.

According to BIOPEP analysis, P2 has been predicted to remain stable after pepsin digestion. However, referring to Figure 4.9C, the peptide peak at 8.33 min has a height lower than the peak of enzyme pepsin, i.e. 75. This suggests that P2 could have been hydrolyzed in the simulated pepsin digestion. Referring to Table 4.8 (page 88), P2 still managed to exhibit ACE inhibitory activity after pepsin digestion. Future work could be carried out by *in vivo* study to prove the actual ACE inhibitory activity of P2 after gastrointestinal digestion. Besides, 3D structural docking assay could be carried out to reveal the clear mechanism of ACE inhibition by P2. BIOPEP analysis revealed P2 would be hydrolyzed by pepsin-pancreatin enzymes to release dipeptide sequences, GP and SM from its precursor. GP has been previously reported to exhibit ACE inhibitory activity with IC_{50} value of 252.6 μM (Byun & Kim, 2002). There were no previous reports on the ACE inhibitory activity of SM. However, dipeptide ACE inhibitors with serine at the N-terminus have been isolated from garlic. These dipeptides, namely SY and SF have inhibited 50% of ACE activity at concentrations of 66.3 and 130.2 μM, respectively (Suetsuna, 1998). Based on the peak height detected at 8.33 min in Figure 4.9D, i.e. 214 it could be the peak of enzyme pepsin-pancreatin. The detection of only the enzyme peak may confirm the hydrolysis of P2 to release the potent ACE inhibitors, GP and SM.

BIOPEP analysis shows P3 would remain stable during pepsin digestion. Referring to Figure 4.10C, the peak eluted at 7.79 min has higher height than the enzyme peak. It may contain the unhydrolyzed P3. The peptide was predicted to release



Ret.	8.23
Height	50
Area	437

Ret.	8.25
Height	57
Area	1459

Ret.	8.33
Height	47 (Enzyme height = 75)
Area	1661

*Fragment: GPSMR

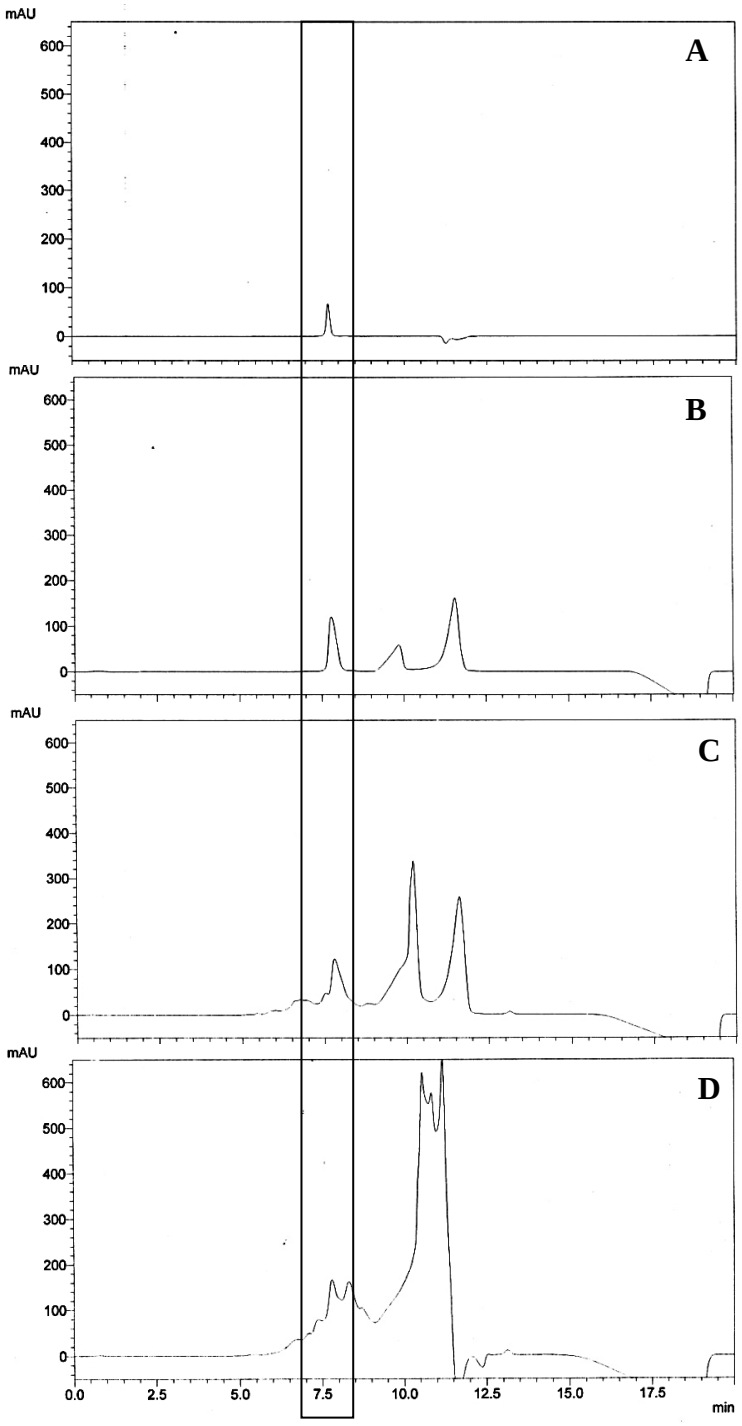
Ret.	8.33
Height	214 (Enzyme height = 215)
Area	7084

*Fragment: GP, SM, R

Figure 4.9: SEC chromatograms of P2 with retention time (min), peak height (mAU), peak area (mAU) and predicted fragments released after digestion.

(A) Pure peptide (B) Peptide incubated with buffer only, (C) Peptide incubated with pepsin and (D) Peptide incubated with pepsin followed by trypsin and chymotrypsin.

*Prediction of proteolytic fragment released was carried out based on the BIOPEP database (www.uwm.edu.pl/biochemia).



Ret.	7.78
Height	68
Area	626

Ret.	7.78
Height	138
Area	4203

Ret.	7.79
Height	148 (Enzyme height = 80)
Area	5164

*Fragment: KVAGPK

Ret.	7.80
Height	172 (Enzyme height = 110)
Area	5858

*Fragment : K, VAGP, K

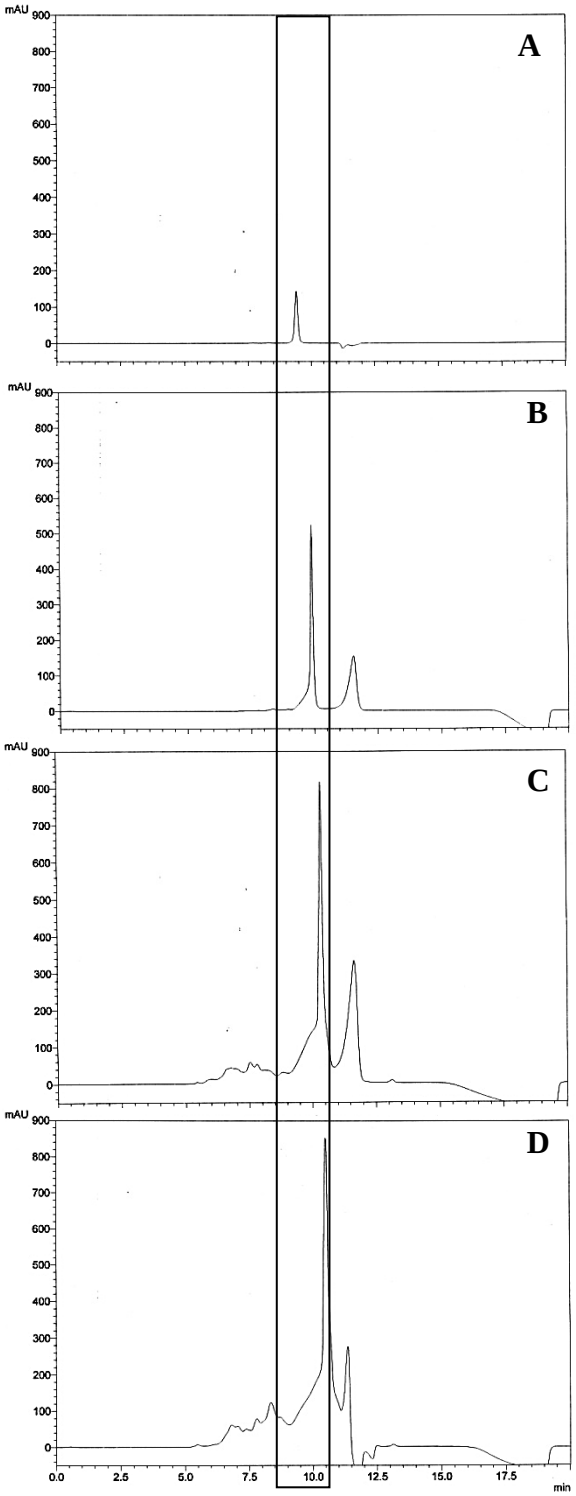
Figure 4.10: SEC chromatograms of P3 with retention time (min), peak height (mAU), peak area (mAU) and predicted fragments released after digestion.

(A) Pure peptide (B) Peptide incubated with buffer only, (C) Peptide incubated with pepsin and (D) Peptide incubated with pepsin followed by trypsin and chymotrypsin.

*Prediction of proteolytic fragment released was carried out based on the BIOPEP database (www.uwm.edu.pl/biochemia).

VAGP from its precursor after digested by pepsin-pancreatin enzymes. The tetrapeptide released contain strong hydrophobic aliphatic amino acid, valine at the N-terminus and imino acid, proline at the C-terminus. This is in accordance with the requirement of peptides with strong ACE inhibitory activity (Cheung *et al.*, 1980; Guang & Phillips, 2012). Furthermore, a tetrapeptide with similar peptide sequence, i.e. VYAP has been isolated from cuttlefish. It was reported to exhibit IC_{50} value of 6.1 μM (Balti *et al.*, 2010). Tripeptide, VKP containing amino acid valine and proline at the N-and C-termini has also exhibited high ACE inhibitory activity with IC_{50} value of 3.7 μM (Tsai *et al.*, 2006). In Figure 4.10D, the peak eluted at 7.80 min has a height of 172, which is slightly higher than the enzyme peak, 110. In SEC, protein or peptides are separated according to size (Stanton, 2004). The release of lysine from the N- and C-termini of P3 may not have much effect on its molecular weight. Therefore, VAGP could have been eluted at the similar retention time as KVAGPK at 7.80 min.

BIOPEP analysis of P4 shows dipeptides, AL and PC would be released from the precursor after pepsin digestion. Only the dipeptide AL remained after pepsin-pancreatin digestion. Currently, there were no reported studies on the ACE inhibitory activity of dipeptide AL. Based on the amino acid sequence, the dipeptide consists of hydrophobic amino acids, alanine and leucine. The release of this hydrophobic peptide may have contributed to the higher ACE inhibitory activity of P4 after digestion (Vermeirssen *et al.*, 2005). Referring to the SEC chromatogram in Figure 4.11A, the peptide was eluted at a retention time of approximately 9.41 min. As illustrated in Appendix G (I) and G (II), HCl and potassium phosphate buffer have a peak at 9.49 and 9.77 min, respectively. Therefore, the peak eluted at approximately 9.61 min in Figure 4.11B could consist of buffers peak together with peptide P4. The combination of buffer and peptide peaks makes it difficult to observe the stability of the peptide by SEC. Theoretically, smaller peptides would be eluted from the SEC column at a later time



Ret.	9.41
Height	150
Area	2302

Ret.	9.61
Height	559
Area	8020

Ret.	10.36
Height	863
Area	2174

*Fragment: F, AL, PC

Ret.	10.52
Height	902
Area	30999

*Fragment: F, AL, P, C

Figure 4.11: SEC chromatograms of P4 with retention time (min), peak height (mAU), peak area (mAU) and predicted fragments released after digestion.

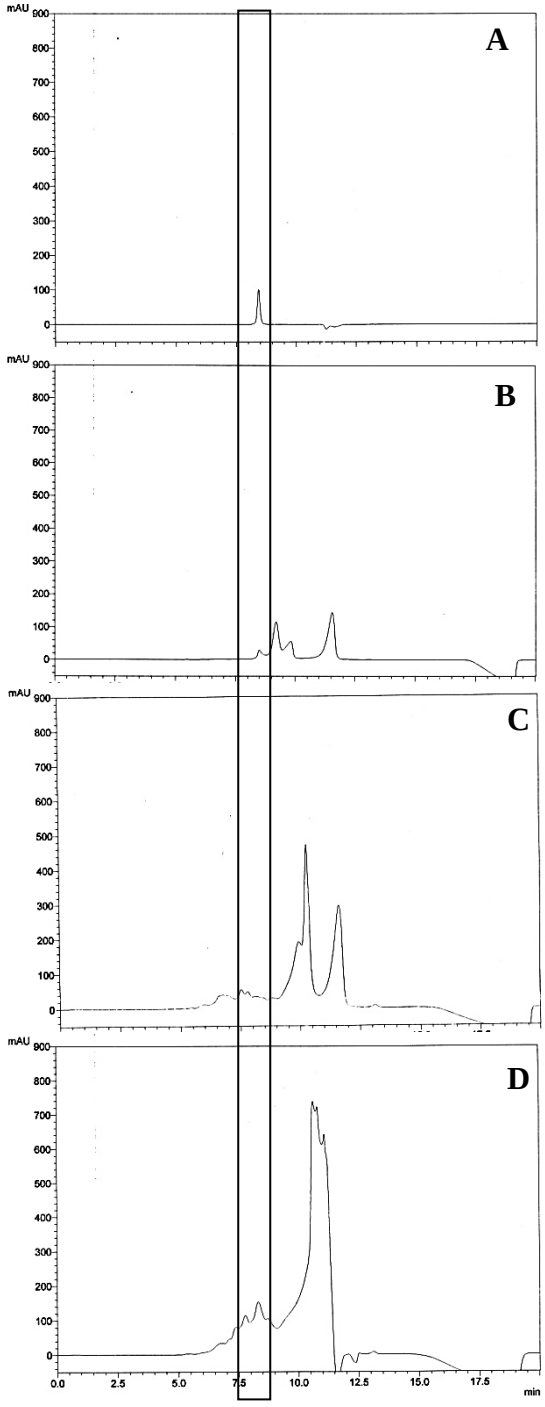
(A) Pure peptide (B) Peptide incubated with buffer only, (C) Peptide incubated with pepsin and (D) Peptide incubated with pepsin followed by trypsin and chymotrypsin.

*Prediction of proteolytic fragment released was carried out based on the BIOPEP database (www.uwm.edu.pl/biochemia).

(Stanton, 2004). The peak eluted at 10 to 11 min in Figure 4.11D was higher compared to the chromatograms obtained by other peptides after pepsin-pancreatin digestion. Therefore, this may confirm the hydrolysis of P4 to smaller fragments by gastrointestinal enzymes.

Based on the result in Table 4.8 (page 88), P5 has exhibited the same high percentage of ACE inhibitory activity as P1 when incubated in buffer without digestive enzymes. However, unlike P1 which is stable throughout the digestion process, P5 was predicted to be further hydrolyzed in the digestive tract. It would release tetrapeptide, RIGL after pepsin digestion and further hydrolyzed to release tripeptide, IGL by pancreatin enzymes (Figure 4.12). Although there were no previous reports on the ACE inhibitory activity of IGL, its amino acid sequence fit the criteria of a potent ACE inhibitor. It has branched aliphatic amino acid, isoleucine at the N-terminus and strong hydrophobic amino acid, leucine at the C-terminus (Wu *et al.*, 2006a). In addition, glycine has been reported as the most favourable amino acid in potent ACE inhibitor of tripeptides (Wang *et al.*, 2011b). According to the SEC chromatograms of P5 in Figure 4.12, peptide digested with pepsin and pepsin-pancreatin enzymes has eluted the peptide peak with approximately the same height with enzyme. This shows peak P5 has disappeared after digestion which may indicate the peptide has undergone further hydrolysis as predicted by the BIOPEP analysis.

Peptides P6 and P7 were predicted to remain stable during pepsin digestion. However, they were predicted to release dipeptide sequences, GE and AP, respectively after pepsin-pancreatin digestion. These dipeptide sequences has been reported to block 50% of the ACE activity at a concentration of 5.4 and 230 μM, respectively (Cheung *et al.*, 1980). Besides dipeptide AP, P7 was also predicted to release tripeptide, SAK after pepsin-pancreatin digestion. The SEC chromatograms in Figure 4.13C and 4.14C showed peak eluted at 8.30 min have a height of 124 and 167, respectively. They were



Ret.	8.43
Height	101
Area	880

Ret.	8.50
Height	30
Area	522

Ret.	8.44
Height	62 (Enzyme height = 75)
Area	771

*Fragment: RIGL, F

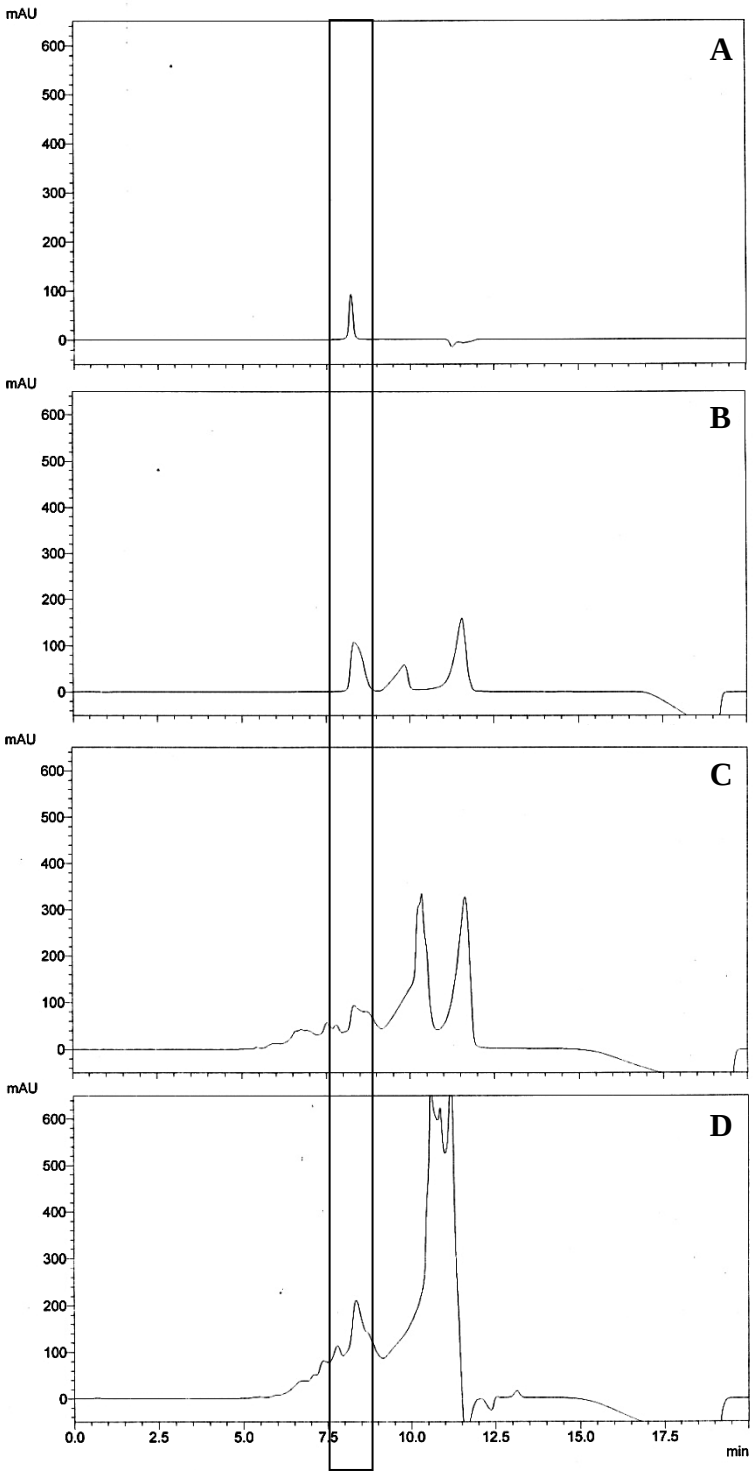
Ret.	8.42
Height	188 (Enzyme height = 215)
Area	6189

*Fragment: R, IGL, F

Figure 4.12: SEC chromatograms of P5 with retention time (min), peak height (mAU), peak area (mAU) and predicted fragments released after digestion.

(A) Pure peptide (B) Peptide incubated with buffer only, (C) Peptide incubated with pepsin and (D) Peptide incubated with pepsin followed by trypsin and chymotrypsin.

*Prediction of proteolytic fragment released was carried out based on the BIOPEP database (www.uwm.edu.pl/biochemia).



Ret.	8.26
Height	94
Area	1007

Ret.	8.30
Height	107
Area	2738

Ret.	8.33
Height	124 (Enzyme height = 75)
Area	3907

*Fragment: EGEPR

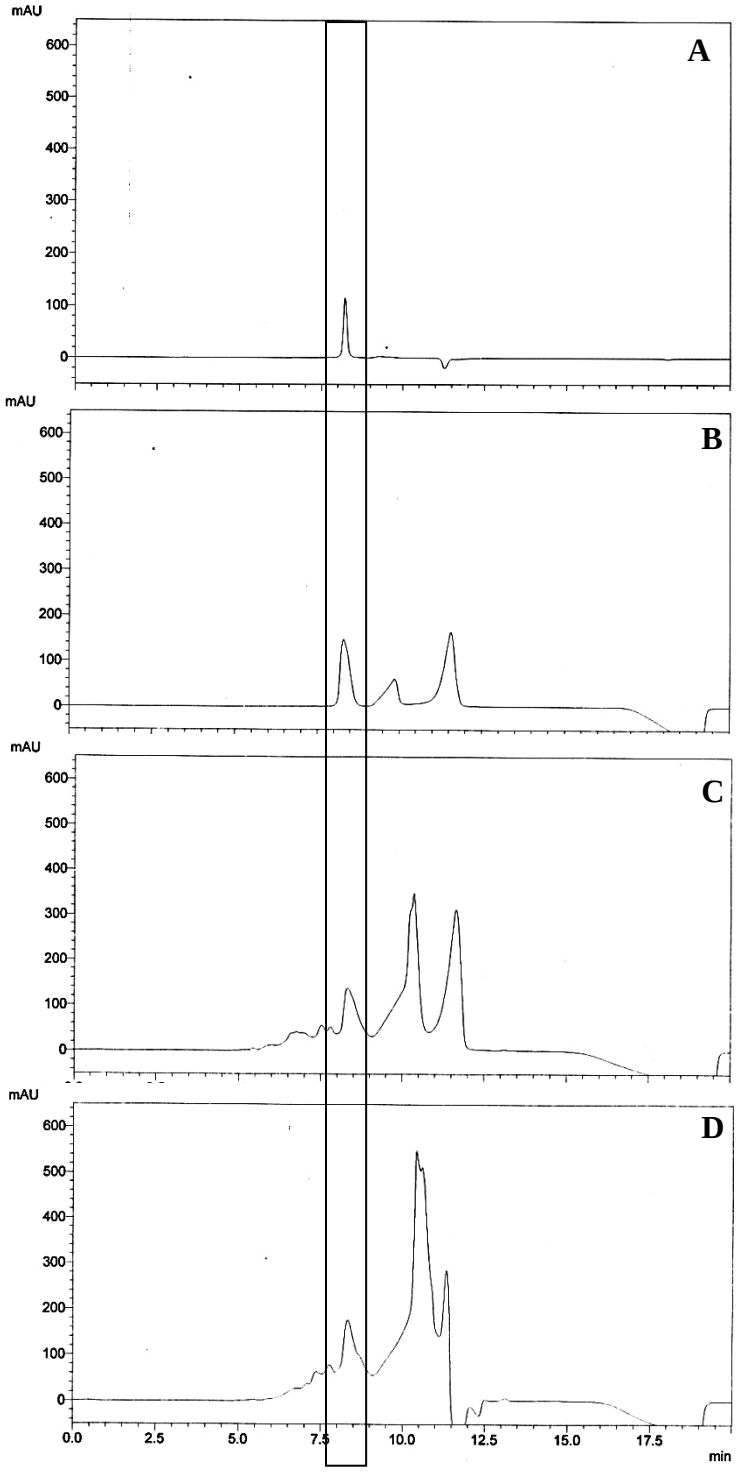
Ret.	8.35
Height	220 (Enzyme height = 215)
Area	12381

*Fragment: E, GE, P, R

Figure 4.13: SEC chromatograms of P6 with retention time (min), peak height (mAU), peak area (mAU) and predicted fragments released after digestion.

(A) Pure peptide (B) Peptide incubated with buffer only, (C) Peptide incubated with pepsin and (D) Peptide incubated with pepsin followed by trypsin and chymotrypsin.

*Prediction of proteolytic fragment released was carried out based on the BIOPEP database (www.uwm.edu.pl/biochemia).



Ret.	8.25
Height	97
Area	2080

Ret.	8.29
Height	146
Area	2994

Ret.	8.31
Height	167 (Enzyme height = 75)
Area	6957

*Fragment: APSAK

Ret.	8.32
Height	216 (Enzyme height = 215)
Area	9970

*Fragment: AP, SAK

Figure 4.14: SEC chromatograms of P7 with retention time (min), peak height (mAU), peak area (mAU) and predicted fragments released after digestion.

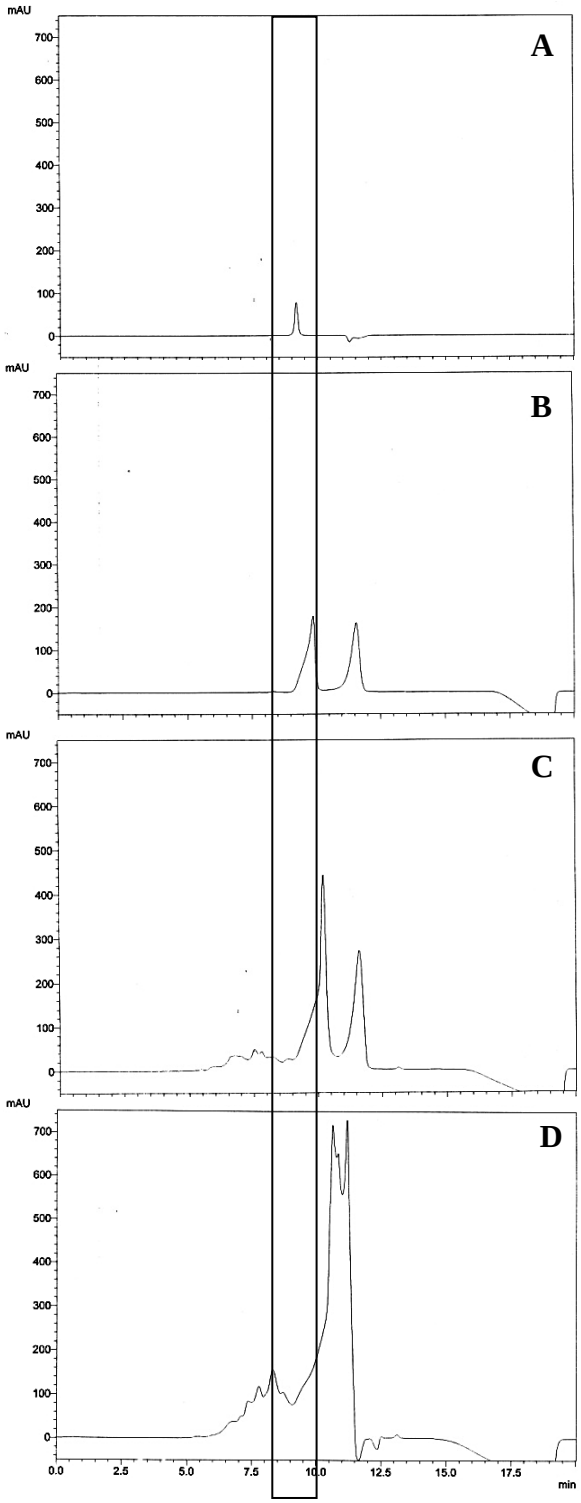
(A) Pure peptide (B) Peptide incubated with buffer only, (C) Peptide incubated with pepsin and (D) Peptide incubated with pepsin followed by trypsin and chymotrypsin.

*Prediction of proteolytic fragment released was carried out based on the BIOPEP database (www.uwm.edu.pl/biochemia).

higher than the estimated enzyme peak, i.e. 75. This may suggest the peptides were still available and not hydrolyzed after incubation with the enzyme pepsin. In Figures 4.13D and 4.14D, the peaks were eluted at almost the same peak height with the digestive enzyme, i.e. 215, which indicate the peptides were not detected in the SEC chromatograms. Therefore, this may confirm the hydrolysis of the peptides by pepsin-pancreatin enzymes as predicted by the BIOPEP analysis.

Peptide P8 was predicted to remain stable after pepsin digestion. It will release dipeptide, GP and tripeptide, GVQ after pepsin-pancreatin digestion. Dipeptide, GP has been previously reported as potent ACE inhibitor with IC_{50} value of 252.6 μM (Byun & Kim, 2002). Based on the SEC chromatogram of peptide P8 in Figure 4.15A, the peptide was eluted at a retention time of approximately 9.38 min. As illustrated in Appendix G (I) and G (II), HCl and potassium phosphate buffer have a peak at 9.49 and 9.77 min, respectively. Therefore, the peak eluted at approximately 9.89 min in Figure 4.15B could consist of buffers peak together with peptide P8. The combination of buffer and peptide peaks makes it difficult to observe the stability of the peptide by SEC. Theoretically, smaller peptides would be eluted from the SEC column at a later time (Stanton, 2004). The higher peak eluted at 10 to 11 min in Figure 4.15D compared to the chromatograms obtained by other peptides after pepsin-pancreatin digestion may confirm the hydrolysis of P8 to smaller fragments by gastrointestinal enzymes.

According to the BIOPEP analysis, P9 was expected to remain stable during pepsin digestion. It will release tetrapeptide, SSNK from its precursor after being digested by pepsin-pancreatin enzymes. It has been claimed that the most preferential characteristic of tetrapeptides with ACE inhibitory activity are stereo and electrical amino acid such as lysine (Wang *et al.*, 2011b). Peptides with amino acid serine at the N-terminal have been isolated from garlic. These dipeptides, namely SY and SF have



Ret.	9.38
Height	84
Area	1616

Ret.	9.89
Height	208
Area	6950

Ret.	10.24
Height	486
Area	17539

*Fragment: GVQGPM

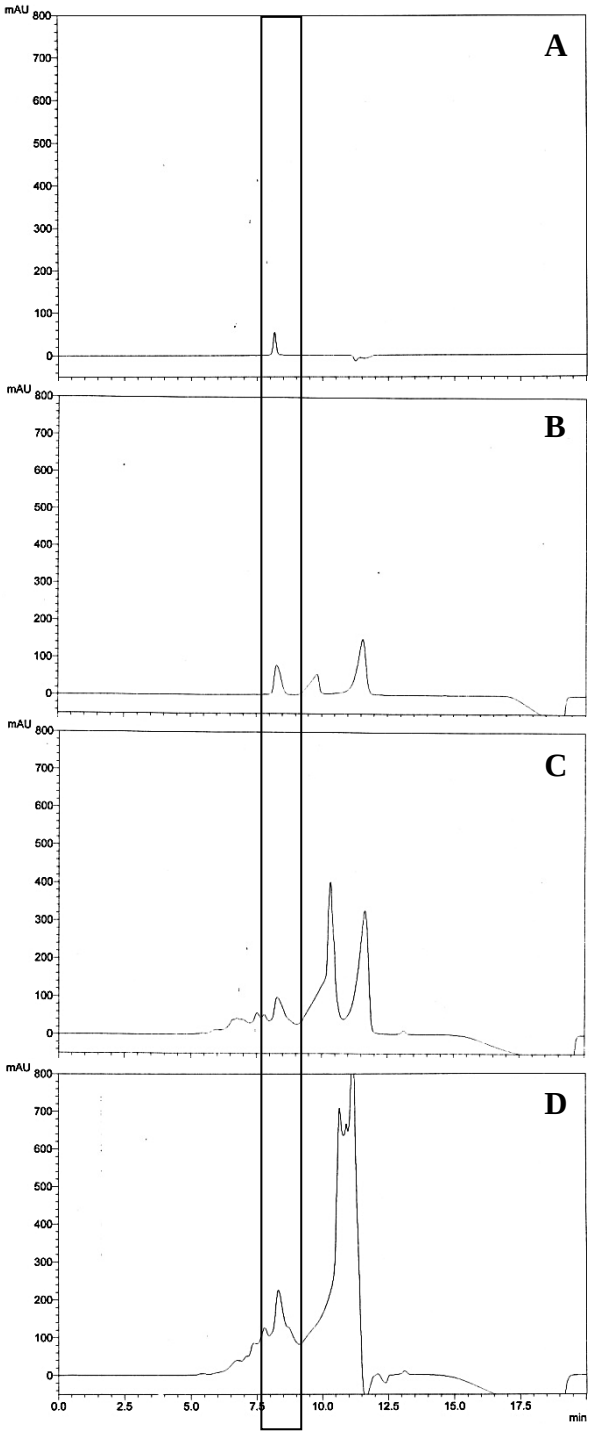
Ret.	10.64
Height	763
Area	29637

*Fragment: GVQ, GP, M

Figure 4.15: SEC chromatograms of P8 with retention time (min), peak height (mAU), peak area (mAU) and predicted fragments released after digestion.

(A) Pure peptide (B) Peptide incubated with buffer only, (C) Peptide incubated with pepsin and (D) Peptide incubated with pepsin followed by trypsin and chymotrypsin.

*Prediction of proteolytic fragment released was carried out based on the BIOPEP database (www.uwm.edu.pl/biochemia).



Ret.	8.20
Height	54
Area	459

Ret.	8.28
Height	79
Area	1445

Ret.	8.29
Height	126 (Enzyme height = 75)
Area	5419

*Fragment: PSSNK

Ret.	8.32
Height	253 (Enzyme height = 215)
Area	11766

*Fragment: P, SSNK

Figure 4.16: SEC chromatograms of P9 with retention time (min), peak height (mAU), peak area (mAU) and predicted fragments released after digestion.

(A) Pure peptide (B) Peptide incubated with buffer only, (C) Peptide incubated with pepsin and (D) Peptide incubated with pepsin followed by trypsin and chymotrypsin.

*Prediction of proteolytic fragment released was carried out based on the BIOPEP database (www.uwm.edu.pl/biochemia).

exhibited potent ACE inhibitory activity with IC_{50} values 66.3 and 130.2 μM, respectively (Suetsuna, 1998). Therefore, the SSNK which contain serine and lysine at its N- and C-termini, respectively may warrant further analysis of this peptide as potential ACE inhibitor. Referring to Figure 4.16C, the peak eluted at 8.29 min has a higher height than the enzyme peak. It may contain the unhydrolyzed P9. In Figure 4.16D, the peak eluted at 8.32 min has a height of 253, which is slightly higher than the enzyme peak. The release of imino acid, proline from P9 after pepsin-pancreatin digestion might not have much effect on its molecular weight. SEC separates protein or peptide based on molecular weight (Stanton, 2004). Therefore, SSNK could have been eluted at almost the same retention time as PSSNK at 8.3 min.

The bioavailability of ACE inhibitory peptides after administration is of high concern. The peptides have to reach the blood stream in an active form in order to act as ACE inhibitors. Injection has been recognised as an effective way of delivering peptide drugs to the body (Brown, 2005). However, patient compliance with drug administration regimens by this parenteral route is generally poor due to the pain and discomfort associated with injections. Besides, patients may encounter possible infections caused by inappropriate use or reuse of needles. Hence, oral route remains the most acceptable way of delivering drugs (Shaji & Patole, 2008).

Several studies have been carried out to investigate the pharmacokinetic behaviour of ACE inhibitory peptides. Oral administration of tripeptides IPP and VPP have exerted bioavailability of approximately 0.1% in pigs (Pijl *et al.*, 2008). In humans, picomolar concentration was detected in plasma following consumption of yoghurt enriched with these two tripeptides (Foltz *et al.*, 2007). Furthermore, ACE inhibitory peptide from sardine hydrolysate which has been commercialized by the product name Valtyron® is claimed to demonstrate efficient blood pressure control by daily intake of as low as 0.6 g/serving. There are 31 peptide sequences identified in this product.

Among the peptides, dipeptide VY has been determined as the main ACE inhibitor. A single dose of 0.6 g of the sardine peptide product contains 0.6-0.96 mg VY (EFSA Panel on Dietetic Products Nutrition and Allergies, 2010b). An absolute bioavailability of 0.014% was detected in plasma after oral administration of 12 mg of VY (EFSA Panel on Dietetic Products Nutrition and Allergies, 2010b). According to Matsui *et al.* (2004), ACE inhibitory peptides preferably to accumulate in organs, such as liver, kidneys, heart and lungs rather than in the plasma. This is in accordance with the report by Miguel *et al.* (2007), in which the ACE activity in lung, kidney and aorta is greatly inhibited compared to plasma ACE in SHR after treatment with egg white hydrolysate. This may suggest that these organs could be a target site associated with the antihypertensive action of ACE inhibitory peptides. In addition to ACE inhibitory activity, some studies have demonstrated the existence of peptides that exert their blood pressure lowering effects through vascular relaxation (Sipola *et al.*, 2002). Based on the *in vivo* studies, these peptides exhibited their antihypertensive action either by direct or indirect action on vascular smooth muscle (Kouno *et al.*, 2005; Maes *et al.*, 2004).

According to Foltz *et al.* (2007), the oral bioavailability of peptides increases with decreasing chain length. Peptides less than 5 amino acids in length have shown higher oral bioavailability (Craik, 2006). Several studies have demonstrated significant decrease in systolic blood pressure by oral administration of peptides with five to six amino acids in length. After oral administration of pentapeptide WVPSV and VVYPW at a dose of 10 mg/kg, the systolic blood pressure of spontaneously hypertensive rats (SHR) decreased by 22.5 and 9.6 mmHg, respectively (Ren *et al.*, 2011). In another study, hexapeptide IPPVPP and VPPIPP lowered the systolic blood pressure by 21 and 17.4 mmHg, respectively in SHR at a dose of 1.5 mg/kg (Ding *et al.*, 2013). This may suggest that the nine peptides in the current study which consisted of five to six amino acids in length could have high oral bioavailability as an antihypertensive drug.

The main challenge of drug delivery through oral administration has been associated with membrane permeability of the peptides in the gastrointestinal tract (Shaji & Patole, 2008). The intestinal epithelium selectively controls the entry of nutrients and drugs to the systemic circulation. Therefore, it is important to understand the mechanism of absorption of substances at the intestinal membrane for designing peptides with improve delivery across the barrier and subsequently increase its bioavailability to reach the target site. Possible routes of drug transport across the membrane include passive diffusion, facilitated transport and carrier-mediated transport system (Mahato *et al.,* 2003). Some researchers have carried out investigations to predict the intestinal permeability of peptides in the digestive system which involve either *in silico, in vitro, in situ, ex vivo* or *in vivo* method (Antunes *et al.,* 2013). According to Webb (1990), small peptides, mostly di- and tri-peptides have better permeability through the intestinal membrane. This is in accordance with the report by Mahato *et al.* (2003) which shows transporters in the membrane are designed to transport 2 to 3 amino acid peptides into the cells. Tetrapeptides would be hydrolyzed primarily by the membrane peptidases prior to absorption. This may presume that the peptides in the current study which have been predicted to undergo further hydrolysis in the digestive system to release fragments of 2 to 4 amino acids in length may have better absorbability across the intestinal membrane and thereby have high probability to reach its target site.

In the current study, eight peptides were predicted to undergo further hydrolysis and released fragments consisting of 2 to 4 amino acids which fit the structural requirement of potential ACE inhibitor. Peptide P1 was predicted to remain stable throughout the digestion process. BIOPEP database only served as a tool to predict the possible proteolysis products from the precursor. Future work can be carried out by *in*

vivo study to prove the actual ACE inhibitory activity of the peptides after administration and to investigate the bioavailability of the peptides after digestion.

4.10 Determination of ACE inhibition pattern

ACE inhibition pattern explains how the peptide binds to ACE and inhibit its activity. Based on the IC_{50} values, three synthetic peptides, namely P1, P5 and P9 which have the most potent ACE inhibitory activity were selected to determine their inhibition pattern against the ACE. According to the Lineweaver-Burk plot in Figures 4.17A and 4.17B, P1 and P5 showed competitive inhibition pattern against the ACE. This indicates that P1 and P5 bind to the active site of ACE to block it from binding to the substrate. ACE has been reported to prefer peptide with competitive inhibition pattern (Chel-Guerrero *et al.*, 2012; Hong *et al.*, 2008). Besides, ACE has higher affinity to bind with peptides containing hydrophobic amino acid especially with aromatic side chain at the three positions of the C-terminal (Li *et al.*, 2004). Referring to the amino acid sequence of P1 and P5 at Table 4.6 (page 79), the three positions of the C-terminal consist of hydrophobic amino acids. Therefore, this may explain the competitive inhibition pattern exhibited by these peptides.

The inhibition pattern exhibited by P1 and P5 are similar with the ACE inhibitory peptides purified from edible mushrooms, *G. frondosa*, *P. adiposa*, *T. giganteum* and peptide F2-1A of *P. cornucopiae* (Choi *et al.*, 2001; Jang *et al.*, 2011; Koo *et al.*, 2006; Lee *et al.*, 2004). Besides edible mushrooms, there were reports on competitive ACE inhibitors from food-derived peptides, such as milk, oyster sauce, cheese and freshwater clam (Gobbetti *et al.*, 2000; Je *et al.*, 2005; Ruiz *et al.*, 2004; Tsai *et al.*, 2006). In addition, the first orally administered ACE inhibitory antihypertensive drug, captopril also inhibited the ACE in a competitive pattern (Bhuyan & Mugesh, 2011). Therefore,

these shows P1 and P5 are potential ACE inhibitors which can be developed as nutraceuticals.

Referring to Figure 4.17C, P9 exhibited noncompetitive inhibition pattern against the ACE enzyme. This shows that P9 tends to bind together with the substrate to the enzyme at any given time. The enzyme-substrate-inhibitor complex formed cannot produce product and can only be converted back to the enzyme-substrate complex or enzyme-inhibitor complex (Jao *et al.*, 2012). Noncompetitive inhibition pattern has been reported in ACE inhibitory peptide derived from edible mushroom, *P. cornucopiae* (Jang *et al.*, 2011). This inhibition mechanism is also observed in food proteins such as fermented soybean (Kuba *et al.*, 2003), oyster (Shiozaki *et al.*, 2010; Wang *et al.*, 2008) and egg (Memarpoor-Yazdi *et al.*, 2012).

Structure-activity relationship studies on ACE inhibitory peptides revealed positively charged C-terminal, such as arginine and lysine have low affinity with the ACE active site (Li *et al.*, 2004). However, these peptides have exhibited potential ACE inhibiting activity. Examples of ACE inhibitory peptides with positive charge amino acid at their C-terminus include AKK from sardine muscle (Matsufuji *et al.*, 1994) and MKR from hen egg white lysozyme (Rao *et al.*, 2012). These peptides were suggested to inhibit the ACE by binding to allosteric site that is different from the catalytic site of the enzyme (Li *et al.*, 2004). Interestingly, P9 contains a positively charged amino acid, lysine at its C-teminus that may explain its noncompetitive inhibition pattern against the ACE.

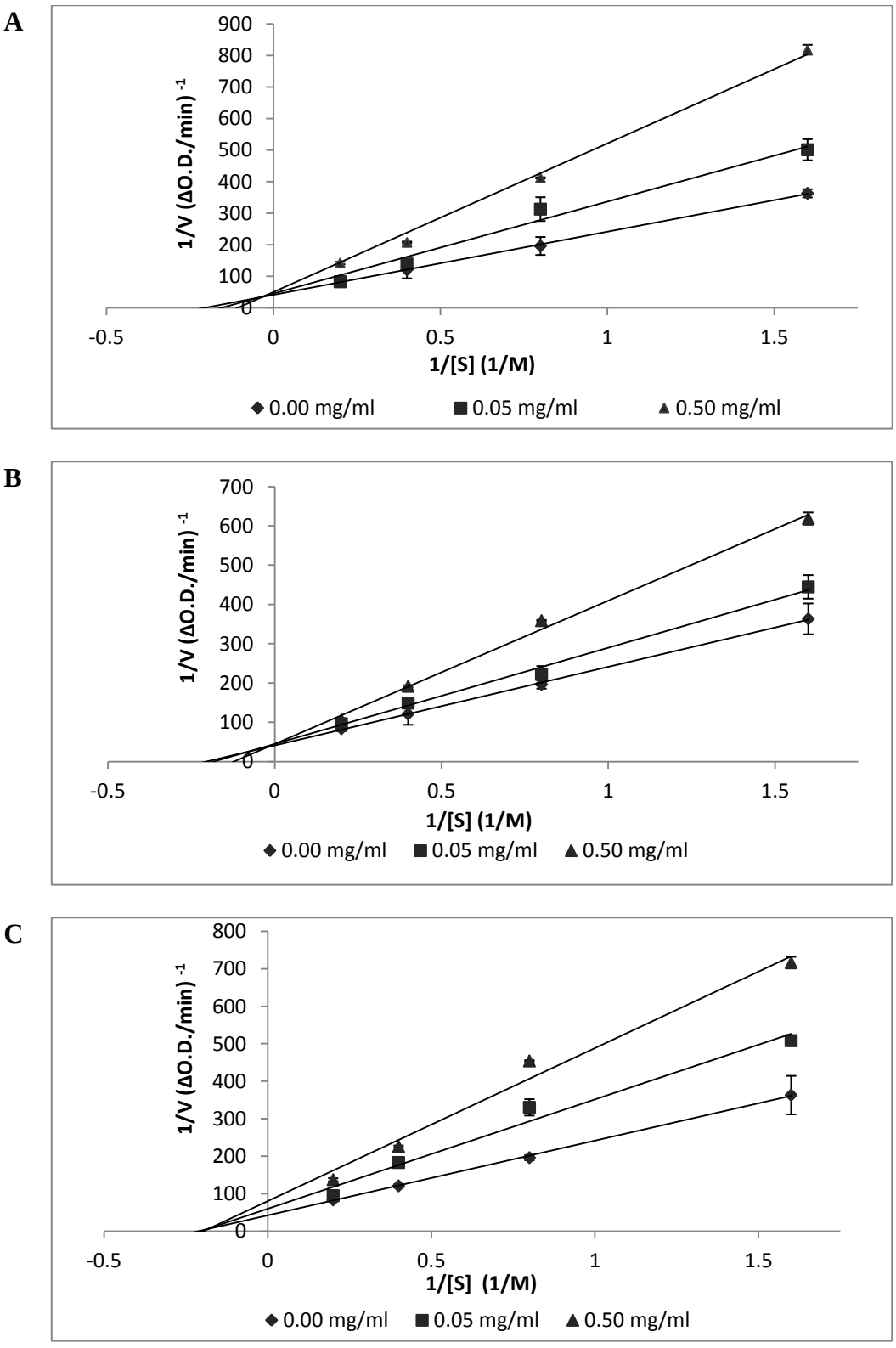


Figure 4.17: Lineweaver-Burk plot of ACE inhibitory activity of (A) P1, (B) P5 and (C) P9.

ACE inhibitory activity was determined in the presence (0.05 mg/ml and 0.5 mg/ml) or absence of peptide (control).

5.0 CONCLUSION

Edible mushrooms are rich in protein and are potential source of bioactive peptides. Compared to previously reported edible mushrooms, the higher inhibition of ACE activity by water extracts of nine mushroom species tested in the current study viz. *A. bisporus, F. velutipes, H. erinaceus, L. edodes, P. citrinopileatus, P. cystidiosus, P. flabellatus, P. florida* and *P. pulmonarius* revealed cold water extraction is suitable to extract ACE inhibitors. Most of the mushrooms studied have the highest concentration of protein precipitated by ammonium sulphate at S50 to S70 indicating that the mushrooms contain high amounts of amphipathic proteins. These mushrooms could be a good source of ACE inhibitory peptides as previous studies have reported proteins containing approximately 50% hydrophobic amino acid as good ACE inhibitors.

For each mushroom species, the protein fractions were pooled according to the similarity of protein bands observed by SDS-PAGE. The ACE inhibitory activity of the pooled protein extracts were tested at a concentration of 10 µg/ml. The highest ACE inhibitory activity was exhibited by E1Pc and E5Pc from *P. cystidiosus*, inhibiting 96.2% and 86.7% of ACE activity, respectively. This was followed by E1Ab and E3Ab from *A. bisporus* that inhibits 78.4% and 71.4% of ACE activity, respectively. Protein extracts from the other mushroom species that exhibited lower ACE inhibitory activity may indicate synergistic effect among the proteins or peptides.

Fractionation based on hydrophobicity by RPHPLC of these four protein extracts (E1Pc, E5Pc, E1Ab and E3Ab) yielded six peaks each (F1-F6). Percentages of proteins recovered from E1Pc and E5Pc fractions ranged from 6.16-21.78% and 4.95-17.48%, respectively while fractions from E1Ab and E3Ab ranged from 6.69-19.14% and 5.35-17.18%, respectively. The ACE inhibitory activity of the RPHPLC fractions was tested at a concentration of 1 µg/ml. The highest ACE inhibitory activity was exhibited by E5PcF3 with 30.8% of ACE inhibited. Among the RPHPLC fractions of *A.*

bisporus, the highest ACE inhibitory activity was exhibited by E3AbF6 with 25.7% of ACE inhibited. Proteomic analysis of these two RPHPLC fractions revealed the majority of proteins present in E5PcF3 and E3AbF6 were of low molecular weight, i.e. with molecular masses less than 30 kDa. The detection of SELDI peaks with high intensity at *m/z* ranging from 3-10 kDa shows this range of proteins could be the potential anti-ACE agent which has played a role in the higher ACE inhibitory activity exhibited in E5PcF3 and E3AbF6.

E5PcF3 and E3AbF6 were further fractionated by SEC column according to molecular size to further reduce the complexity of proteins and seven peaks were collected each (C1-C7). The total percentages of proteins recovered in E5PcF3 and E3AbF6 were 83.4% and 21.0%, respectively. The ACE inhibitory activity of the SEC fractions was tested at a concentration of 1 μg/ml. In E5PcF3, the highest ACE inhibitory activity was exhibited by fraction C1 with 27.4% of ACE inhibition. In E3AbF6, the most potent ACE inhibitory activity was exhibited by fractions C1 and C4 with 18.7% and 16.1% of ACE inhibited, respectively. Based on the position of the three peaks in the SEC chromatogram, the molecular masses of peaks C1 and C4 were estimated to be lower than 20 and 10 kDa, respectively. These three SEC fractions have been further analyzed by LC/MS/MS.

Nine potential ACE inhibitors, AHEPVK, GPSMR, KVAGPK, FALPC, RIGLF, EGEPR, APSAK, GVQGPM and PSSNK were selected from the peptide list of LC/MS/MS analysis. Selection were based on the structural requirement of potential ACE inhibitory peptides, i.e. less than six amino acids and consists of hydrophobic and branched aliphatic amino acid at the C- and N-termini, respectively. These nine peptides were chemically synthesized and their ACE inhibitory activity was determined by IC_{50} values. The result obtained showed the IC_{50} values varied from 62.8-736.7 μM with the three most potent ACE inhibitory activity exhibited by P1 (AHEPVK), P5 (RIGLF) and

P9 (PSSNK). Although the IC_{50} value of these three most potent peptides were higher compared to some previously reported ACE inhibitory peptides from other food sources, these three peptides have better activity compared to previously reported edible mushrooms, *G. frondosa*, *P. adiposa* and *P. cornucopiae*.

The effect of gastrointestinal digestion on the ACE inhibitory activity of the nine synthetic peptides was determined by *in vitro* study. The result obtained showed the nine peptides were pH stable and they were able to maintain their ACE inhibitory activity even after gastrointestinal digestion. BIOPEP analysis of the nine peptides showed they could be broken down by digestive enzymes to 2-4 amino acids in length which could exhibit better ACE inhibitory activity. This may suggest the peptides may have increased ACE inhibitory activity after oral administration. Future work could be done by *in vivo* study to reveal the clear mechanism of ACE inhibition by the peptides.

Based on the IC_{50} values, three most active peptides were selected for the determination of ACE inhibition pattern. P1 (AHEPVK) and P5 (RIGLF) have exhibited competitive inhibition pattern whereas P9 (PSSNK) showed noncompetitive inhibition pattern. The inhibition pattern exhibited by P1 and P5 was similar with the ACE inhibitory peptides purified from edible mushrooms, *G. frondosa*, *P. adiposa*, *T. giganteum* and peptide F2-1A of *P. cornucopiae*. Noncompetitive inhibition pattern has been reported in ACE inhibitory peptide derived from peptide F2-1B of *P. cornucopiae*.

In conclusion, the nine peptides tested in the current study, in particular P1, P5 and P9 could be potential ACE inhibitors. Although captopril and some previously reported food derived peptides have stronger ACE inhibition activity than the peptides in the current study, the nine peptides were derived from mushrooms which could be easily obtained and have very low probability of causing food allergy. These peptides could be applied as ingredient in functional foods, dietary supplements or pharmaceuticals as an antihypertensive agent.

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APPENDICES

Appendix A: Protocols for solutions

I. Solutions for SDS-PAGE

Solution A: 30% acrylamide: bisacrylamide solution (30.8% T: 2.7% bis)

Acrylamide	30.0 g
N, N'-methylenebisacrylamide	0.8 g

The solution was made up to 100 ml by the addition of distilled water and deionised by stirring for 1 hour or overnight with a small quantity of Amberlite IRN-150L. The solution was filtered and stored in a dark bottle at 4^{o}C. It can be kept for less than 3 months.

Solution B: 4X running gel buffer (1.5 M Tris-HCl, pH 8.8)

Tris base	18.17 g

The Tris base was dissolved with distilled water. After adjusting the pH to 8.8 using concentrated HCl, the solution was made up to 100 ml with distilled water. It was kept at 4^{o}C.

Solution C: 10% (w/v) SDS

SDS	10.0 g

The SDS was dissolved with 100 ml of distilled water. The solution can be kept at room temperature.

Solution D: Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

Tris base 6.06 g

The Tris base was dissolved with distilled water. After adjusting the pH to 6.8 using concentrated HCl, the solution was made up to 100 ml with distilled water. It was kept at 4^{o}C.

10% (w/v) ammonium persulphate (APS)

APS 0.05 g

The APS was dissolved in 0.5 ml of distilled water. The solution was prepared fresh prior to gel casting.

Volume of solutions required for the preparation of 16% separating gel and 4% stacking gel.

Solutions	16% Separating gel Volume (ml)	4% Stacking gel Volume (ml)
A	10.68	0.65
B	5.00	-
C	0.20	0.05
D	-	1.25
dH_2O	4.02	3.05
10% APS	0.10	0.025
TEMED	0.0066	0.005

The volume of solutions stated above was for the preparation of two gels.

10% APS and TEMED were added immediately to the solutions prior to casting.

Tank buffer : 25 mM Tris, 198 mM glycine, 0.1% (w/v) SDS, pH 8.3

Tris base	3.03 g
Glycine	14.4 g
SDS	1.0

The solution was made up to 1 L with distilled water and stored at room temperature.

SDS-PAGE sample buffer: 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT, 62.5 mM Tris-HCl, pH 6.8

Bromophenol blue	0.02 g
Glycerol	2.0 ml
SDS	0.4 g
Dithiothreitol (DTT)	0.2 g
Solution D	2.5 ml

The solution was made up to 20 ml with distilled water and kept at -20^{o}C in aliquots.

II. Solutions for silver staining

Fixing solution: 40% (v/v) ethanol, 10% acetic acid

Ethanol	40.0 ml
Acetic acid glacial	10.0 ml

The solution was made up to 100 ml with distilled water

Sensitizing solution: 30% (v/v) ethanol, 0.13% (v/v) glutaryldehyde, 0.5 M sodium acetate, 8 mM sodium thiosulphate

Ethanol	30.0 ml
50% (w/v) glutaryldehyde	0.5 ml
Sodium acetate trihydrate	6.8 g
Sodium thiosulphate	0.2 g

The solution was made up to 100 ml with distilled water. Glutaryldehyde was added into the solution immediately before use.

Silver solution: 0.25% (w/v) silver nitrate, 0.5% (v/v) formaldehyde

Silver nitrate	0.25 g
37% (v/v) formaldehyde	0.50 ml

The solution was made up to 100 ml with distilled water. Formaldehyde was added immediately into the solution before use.

Developing solution: 0.24 M sodium carbonate, 0.04% (v/v) formaldehyde

Sodium carbonate	2.5 g
37% (v/v) formaldehyde	40.0 µl
5% (w/v) sodium thiosulphate	2.8 µl

The solution was made up to 100 ml with distilled water. Formaldehyde was added immediately into the solution before use.

Stopping solution: 40 mM ethylenediaminetetraacetic acid

Ethylenediaminetetraacetic acid	1.46 g

The solution was made up to 100 ml with distilled water.

Preserving solution: 30% (v/v) ethanol, 4% (v/v) glycerol

Ethanol	30.0 ml
87% (v/v) glycerol	4.6 ml

The solution was made up to 100 ml with distilled water.

III. Solutions for simulated gastrointestinal digestion test

HCl: (0.1 M, pH 2.0)

HCl	8.17 ml

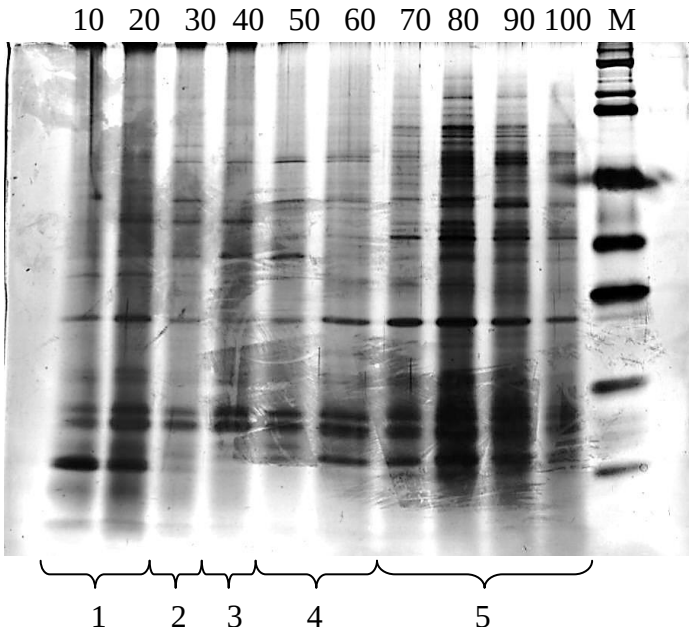
The HCl was dissolved in 500 ml of distilled water. After adjusting the pH to 2.0, the solution was made up to 1L by the addition of distilled water.

Potassium phosphate buffer: (0.1 M, pH 8.0)

Potassium phosphate monobasic	0.52 g
Potassium phosphate dibasic	16.73 g

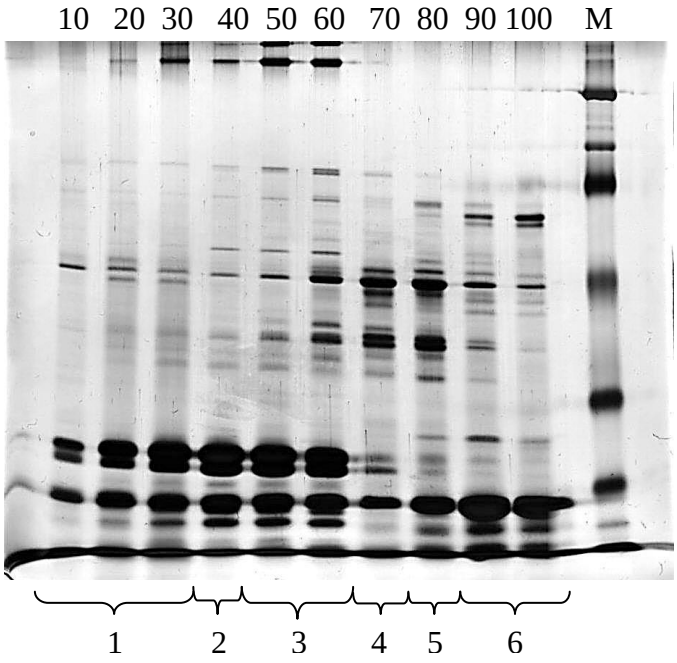
Potassium phosphate monobasic and potassium phosphate dibasic were dissolved in distilled water. After adjusting the pH to 8.0, the solution was made up to 1L.

Appendix B: SDS-PAGE for pooling



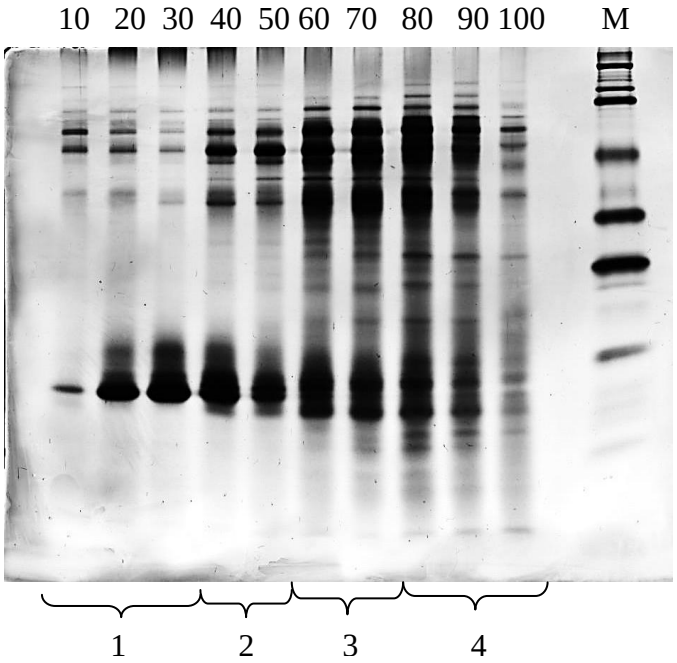
I. SDS-PAGE analysis of protein fractions from *Agaricus bisporus* precipitated at ten percentages of salt saturation.

*Protein fractions were pooled into five groups.



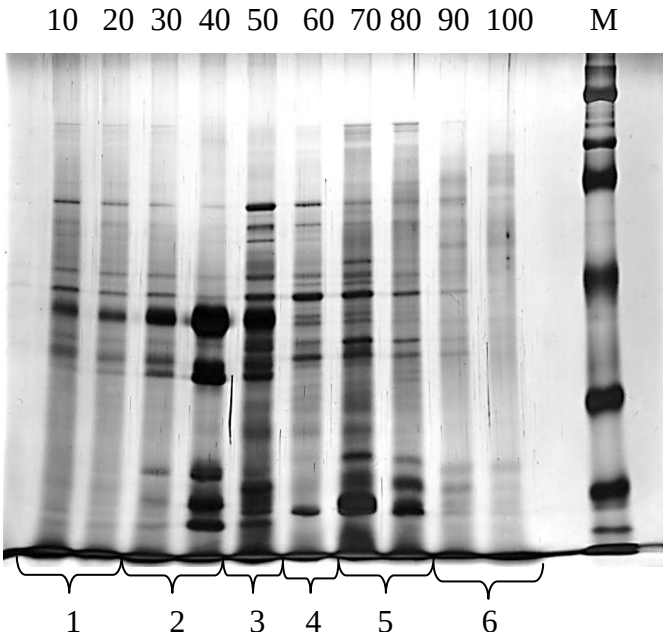
II. SDS-PAGE analysis of protein fractions from *Flammulina velutipes* precipitated at ten percentages of salt saturation.

*Protein fractions were pooled into six groups.



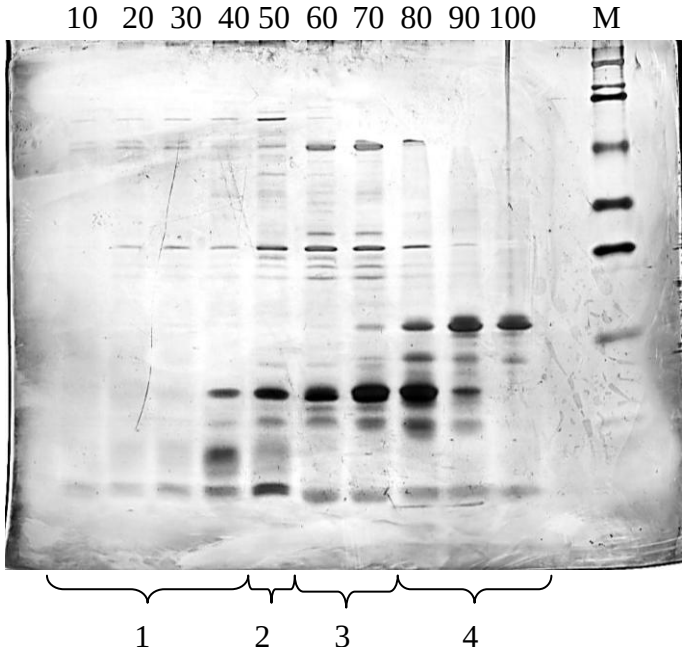
III. SDS-PAGE analysis of protein fractions from *Hericium erinaceus* precipitated at ten percentages of salt saturation.

*Protein fractions were pooled into four groups.



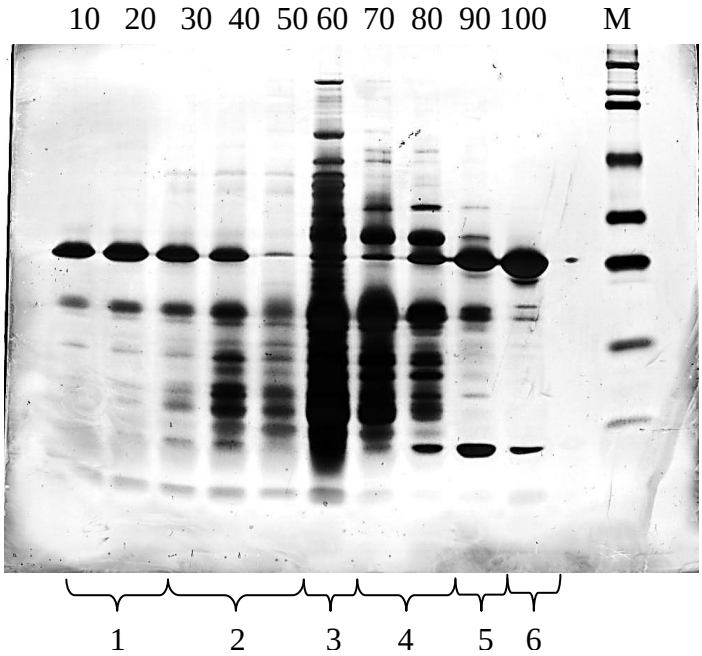
IV. SDS-PAGE analysis of protein fractions from *Lentinula edodes* precipitated at ten percentages of salt saturation.

*Protein fractions were pooled into six groups.



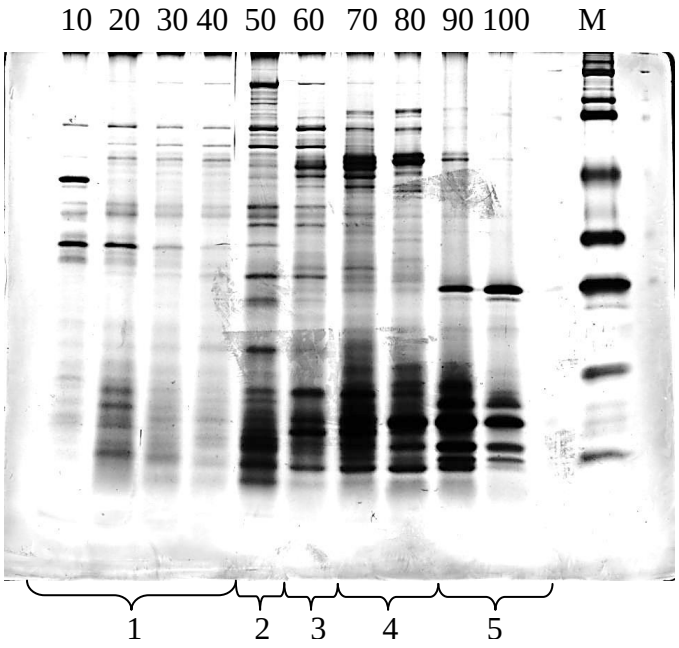
V. SDS-PAGE analysis of protein fractions from *Pleurotus citrinopileatus* precipitated at ten percentages of salt saturation.

*Protein fractions were pooled into four groups.



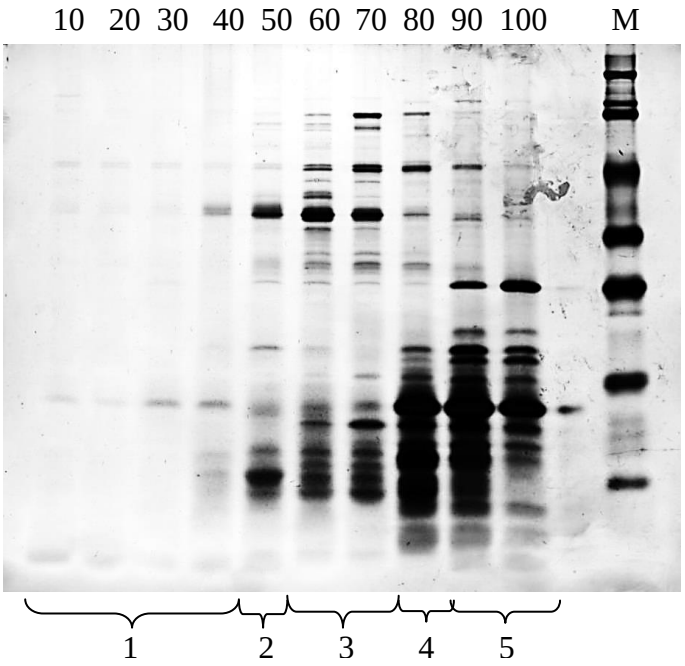
VI. SDS-PAGE analysis of protein fractions from *Pleurotus cystidiosus* precipitated at ten percentages of salt saturation.

*Protein fractions were pooled into six groups.



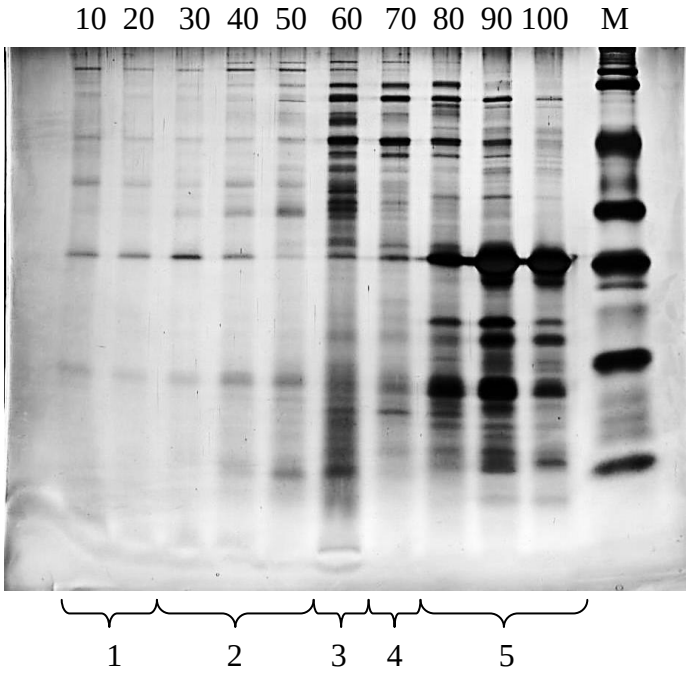
VII. SDS-PAGE analysis of protein fractions from *Pleurotus flabellatus* precipitated at ten percentages of salt saturation.

*Protein fractions were pooled into five groups.



VIII. SDS-PAGE analysis of protein fractions from *Pleurotus florida* precipitated at ten percentages of salt saturation.

*Protein fractions were pooled into five groups.



IX. SDS-PAGE analysis of protein fractions from *Pleurotus pulmonarius* precipitated at ten percentages of salt saturation.

*Protein fractions were pooled into five groups.

Appendix C: LC/MS/MS Mascot result (score, peptide sequence, protein name, protein molecular mass and origin).

I. Mascot result of E5PcF3C1

Score	Peptide sequence	Protein name	Mr (Da)	Origin
37	MPASSLLDSLNR	Phytanoyl-CoA dioxygenase	44319	*Colletotrichum graminicola*
32	NPGALPAPTLSRAK	AGL283Wp	39310	*Ashbya gossypii*
32	QQKPLVVSSPMPYR + [O]	Hypothetical protein	37599	*Ajellomyces capsulata*
31	VAPEEHPVLLTEAPINPK	Actin	41605	*Lipomyces starkeyi*
28	LLAEGKIQVHKPK	Hypothetical protein	37041	*Nectria haematococca*
28	NATYANNLASLNK	Palmitoyl-protein thioesterase	35152	*Coprinopsis cinerea*
25	YYPGEVCLRTTDVAIINK	Hypothetical protein	32641	*Batrachochytrium dendrobatidis*
25	LMLTDLGIVPGKK	Hypothetical protein	38234	*Puccinia graminis f. sp. tritici*
25	LNPSPLIGGPVPGGR	Hypothetical protein	14016	*Puccinia graminis f. sp. tritici*

Mascot result of E5PcF3C1 (Continued)

24	MTSSSPTAKK + [O]	Hypothetical protein	35599	*Pyrenophora teres f. teres*
24	IDTVLNAIEAEQAEFR	Peroxisomal membrane protein (Pex14p)	38420	*Saccharomyces cerevisiae*
24	YLIAGATGGLGAEVLNYFVK	Hypothetical protein	34383	*Aspergillus terreus*
24	AVMYPNSRIFR + [O]	Hypothetical protein	32710	*Aspergillus niger*
24	AGSEAIIMKSYR +[O]	Hypothetical protein	37932	*Batrachochytrium dendrobatidis*
24	RLCGLCGPR	Hypothetical protein	24966	*Paracoccidioides brasiliensis*
23	TMKPVETVLK +[O]	Putative GTP-binding protein	21194	*Leucocoprinus cf. zamurensis*
23	MDSSITAMTSWGMAR + [O]	Hypothetical protein	37895	*Chaetomium globosum*
23	EGEEEGAAGPVK	Transcriptional activator	37401	*Arthroderma benhamiae*
22	FQHCISQLDK	Hypothetical protein	18914	*Myceliophthora thermophila*
22	QSLENSMEFPK	Hypothetical protein	27371	*Phaeosphaeria nodorum*

Mascot result of E5PcF3C1 (Continued)

22	MDPGMVWLFYAYR + [O]	Hypothetical protein	33262	*Hypocrea jecorina*
22	KPEESTSFQSR	Hypothetical protein	25957	*Ajellomyces capsulata*
22	AIEADPVTTK	Hypothetical protein	40215	*Mycosphaerella graminicola*
21	TNGGSNKEGGDCAVPLK	Hypothetical protein	20474	*Arthroderma benhamiae*
21	KCGIFCTDGEQAK	Hypothetical protein	29586	*Sordaria macrospora*
21	KSADQVPIGLIPR	DNA replication licensing factor MCM7	16758	*Tephromela atra var. calcarea*
21	RSLVAPPNTQGIAAR	Hypothetical protein	34395	*Arthroderma otae*
21	QLFQLRDLFK	Hypothetical protein	35745	*Cordyceps militaris*
21	AGGINAQRPLR	Hypothetical protein	21883	*Puccinia graminis f. sp. tritici*
21	MMQPESVAR + [O]	Hypothetical protein	24774	*Moniliophthora perniciosa*
21	ETVAGEPVR	Hypothetical protein	15206	*Ajellomyces capsulata*
20	ALNGHVTVISVEYR	Hypothetical protein	23389	*Aspergillus terreus*

Mascot result of E5PcF3C1 (Continued)

20	TMPVDIPK + [O]	Hypothetical protein	33219	*Yarrowia lipolytica*
20	RPVAGMVR + [O]	Hypothetical protein	24169	*Verticillium albo-atrum*
20	HFYSSLHTMVR + [O]	Hypothetical protein	32404	*Mycosphaerella graminicola*
20	FASWDIFFADER	6-phosphogluconolactonase	28266	*Arthroderma gypseum*
20	RCGHGALFAALAK	Hypothetical protein	34511	*Moniliophthora perniciosa*
19	FAQLAMK [O]	Hypothetical protein	31191	*Spathaspora passalidarum*
19	SIYEEAARR	Methyltransferase type 11	29475	*Grosmannia clavigera*
19	QQGSQAGNQWNEGAR	Hypothetical protein	29198	*Moniliophthora perniciosa*
18	MSTVPNFK + [O]	Hypothetical protein	34274	*Postia placenta*
17	AVIVKNFDEGTK	60S ribosomal protein	15700	*Batrachochytrium dendrobatidis*
17	KPATATTSK	Hypothetical protein	21593	*Pyrenophora teres f. teres*
16	AMEQTLLTSAMK+ [O]	Hypothetical protein	22803	*Nectria haematococca*

Mascot result of E5PcF3C1 (Continued)

16	ETEVYAVDFNPSK	Hypothetical protein	35034	*Encephalitozoon intestinalis*
15	WHAWVAK	Hypothetical protein	34243	*Arthrobotrys oligospora*
14	IVSPGMGKPPPPMLLQSLK+ [O]	Hypothetical protein	29834	*Verticillium albo-atrum*
14	QDSFKEFIK	Hypothetical protein	16573	*Puccinia graminis f. sp. tritici*
14	FEGIMGKK+ [O]	Hypothetical protein	34242	*Ajellomyces dermatitidis*
12	AHEPVK	Hypothetical protein	15610	*Pyrenophora teres f. teres*
12	ADDFR	FUN81 protein	23426	*Arthroderma otae*
10	YTLSC	Hypothetical protein	38494	*Arthrobotrys oligospora*
10	GMGGPR	RAS small monomeric GTPase	31661	*Cordyceps militaris*
7	FGGNI	Hypothetical protein	36569	*Moniliophthora perniciosa*
7	EAFSLFSQKMDGEK+ [O]	Hypothetical protein	21281	*Sclerotinia sclerotiorum*
7	KPAAP	Hypothetical protein	24545	*Schizophyllum commune*

Mascot result of E5PcF3C1 (Continued)

7	DPAIK	NADP-dependent leukotriene B4 12-hydroxydehydrogenase	27822	*Paracoccidioides brasiliensis*
7	LVESG	Hypothetical protein	19524	*Ajellomyces capsulatus*
6	YLSHA	Hypothetical protein	22569	*Schizophyllum commune*
4	GPSMR	Hypothetical protein	22519	*Paracoccidioides brasiliensis*

II. Mascot result of E3AbF6C1

Score	Peptide sequence	Protein name	Mr (Da)	Origin
30	EPESSSPDVK	Hypothetical protein	20199	*Colletotrichum graminicola*
29	SLLESEDEGCAR	Hypothetical protein	22091	*Ajellomyces capsulata*
27	KDISSGGLNLNFVK	Hypothetical protein	28662	*Candida tenuis*
25	MSDSTPTPSGLAAK + [O]	Hypothetical protein	31587	*Aspergillus clavatus*
24	QRDDSPDAVR	Hypothetical protein	21299	*Arthroderma gypseum*

Mascot result of E3AbF6C1 (Continued)

22	TPPQGSHGCTHK	Hypothetical protein	32448	*Botryotinia fuckeliana*
22	AAIDLQLEFKATEK	Hypothetical protein	22680	*Botryotinia fuckeliana*
22	IEEMYVVMNTPLGLFGER + [O]	Hypothetical protein	31698	*Botryotinia fuckeliana*
19	LLVGMQHRTPIK+ [O]	Hypothetical protein	15998	*Puccinia graminis f. sp. tritici*
19	KYHSIEATGGDVNK	Whole genome shotgun sequence assembly, scaffold_1, strain Mel28	17044	*Tuber melanosporum*
19	DAWMGSSGYPMSFTR+ [O]	Hypothetical protein	19549	*Nectria haematococca*
18	GSNDSPSLPALATDSK	Hypothetical protein	28489	*Neurospora tetrasperma*
18	CGPPPSADR	Hypothetical protein	20744	Verticillium albo-atrum
17	QLYCEGILDAFGSK	Hypothetical protein	26294	*Phaeosphaeria nodorum*
17	GHPHGGIVFSDLK	Oxidoreductase, short-chain dehydrogenase/reductase family	32445	*Neosartorya fumigata*
17	DMPGQDCR	Hypothetical protein	31447	*Ajellomyces capsulata*
16	TYVVPEGLAEFK	50S ribosomal protein YmL27	16080	*Arthroderma gypseum*

Mascot result of E3AbF6C1 (Continued)

16	QPPQSDPK	Hypothetical protein	29760	*Vanderwaltozyma polyspora*
15	AHAPR	Hypothetical protein	21658	*Schizophyllum commune*
13	GPVVK	Hypothetical protein	20145	*Paracoccidioides brasiliensis*
12	RMGEDGTPAK + [O]	Hypothetical protein	28755	*Pyrenophora teres f. teres*
12	KNPPPER	Hypothetical protein	16972	*Sclerotinia sclerotiorum*
12	LNPQG	DNA repair protein	21460	*Arthroderma otae*
10	GPVTLEMR	Hypothetical protein	24366	*Podospora anserina*
9	RIGLF	Hypothetical protein	21335	*Sclerotinia sclerotiorum*
9	DFAGK	Hypothetical protein	27560	*Schizophyllum commune*
9	GPVVQG	OB-fold nucleic acid binding domain-containing protein	27408	*Verticillium albo-atrum*
9	VAPAN	Hypothetical protein	25500	*Arthrobotrys oligospora*
8	GGPHR	Hypothetical protein	27447	*Arthroderma benhamiae*
8	FALPC	Hypothetical protein	34031	*Arthrobotrys oligospora*
8	KPLGG	Hypothetical protein	27214	*Neurospora crassa*

Mascot result of E3AbF6C1 (Continued)

7	GFHGG	Hypothetical protein	22199	*Chaetomium globosum*
7	LPSPA	Hypothetical protein	16332	*Penicillium chrysogenum*
6	APSAK	Hypothetical protein	16273	*Schizophyllum commune*
6	YSAGK	Metallo-beta-lactamase superfamily protein	25992	*Paracoccidioides brasiliensis*
6	FGSGN	BZIP transcription factor	14497	*Magnaporthe oryzae*
6	GCFGA	Hypothetical protein	20067	*Melampsora larici-populina*
5	KVAGPK	Hypothetical protein	28222	*Ustilago maydis*
5	FPTSK	Hypothetical protein	14585	*Puccinia graminis f. sp. tritici*
5	QAFTT	Hypothetical protein	17335	*Serpula lacrymans var. lacrymans*
5	EGEPR	Threonine synthase	17893	*Pichia angusta*
5	YDCTA	Hypothetical protein	15944	*Aspergillus terreus*
4	FGAAA	Hypothetical protein	26129	*Aspergillus terreus*
3	APGPP	Acetylcholinesterase	24265	*Verticillium albo-atrum*
2	AGGAY	Hypothetical protein	19524	*Sporisorium reilianum*

Mascot result of E3AbF6C1 (Continued)

1	TGGPR	Hypothetical protein	15137	*Puccinia graminis f. sp. tritici*
0	MGGFG+ [O]	Strain CBS138 chromosome E complete sequence	32989	*Candida glabrata*
0	AYITL	Ribosomal protein	23260	*Scheffersomyces stipitis*
0	FSAAS	Hypothetical protein	17539	*Moniliophthora perniciosa*

III. Mascot result of E3AbF6C4

Score	Peptide sequence	Protein name	Mr (Da)	Origin
32	DPILGGAQNK	Hypothetical protein	15263	*Botryotinia fuckeliana*
25	GDQLMWFK + [O]	Hypothetical protein	14051	*Melampsora larici-populina*
24	RPKGLLGGPSAR	Hypothetical protein	15151	*Arthroderma benhamiae*
24	AARPQPGK	Hypothetical protein	13327	*Puccinia graminis f. sp. tritici*
24	AILRGQPR	Hypothetical protein	11502	*Colletotrichum graminicola*
21	QHWRPCEIHGK	Hypothetical protein	13479	*Neurospora tetrasperma*

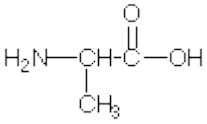
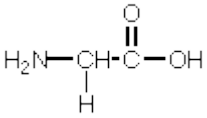
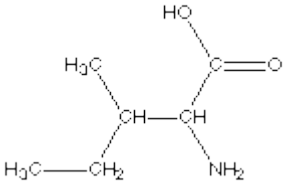
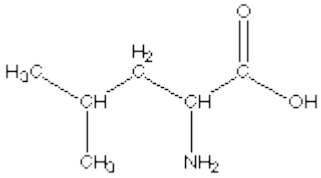
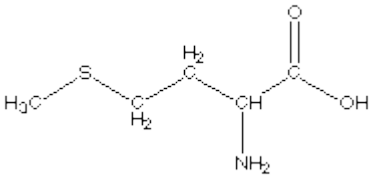
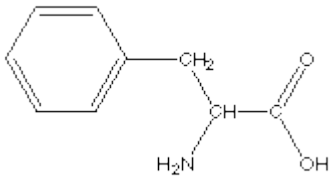
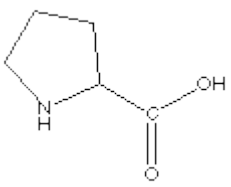
Mascot result of E3AbF6C4 (Continued)

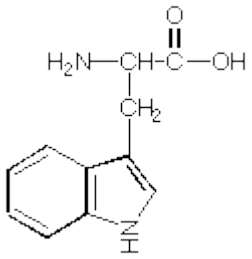
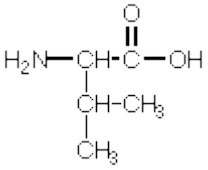
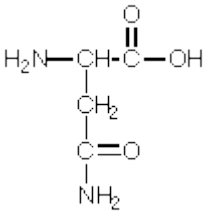
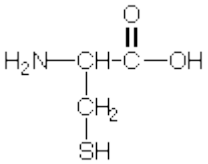
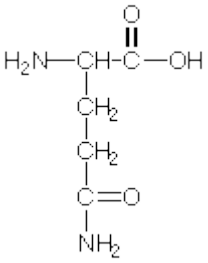
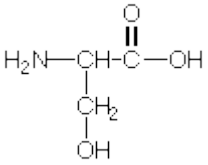
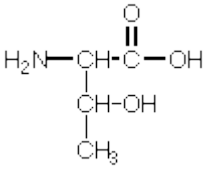
21	GVQGPM + [O]	Hypothetical protein	19893	*Melampsora larici-populina*
21	DVLAGPVSVARVRPR	Hypothetical protein	7587	*Aspergillus niger*
21	ECDPYISK	Hypothetical protein	20465	*Pichia pastoris*
21	AFLEELK	Hypothetical protein	17753	*Schizosaccharomyces japonicus*
20	QPRPSANK	Hypothetical protein	16815	*Magnaporthe oryzae*
20	YSILASWI	Hypothetical protein	14227	*Ajellomyces dermatitidis*
19	RAPQADLAK	Hypothetical protein	18674	*Puccinia graminis f. sp. tritici*
18	FQRGNSLSAVTSK	Hypothetical protein	11707	*Cordycep militaris*
18	VAAGAMLASPKSL + [O]	Hypothetical protein	7919	*Botryotinia fuckeliana*
18	GPNGSMFK	Hypothetical protein	16052	*Postia placenta*
18	DFAFGATR	Hypothetical protein	14961	*Ajellomyces dermatitidis*
15	MDPRMLLEMYMSMAGAR	Hypothetical protein	20135	*Puccinia graminis f. sp. tritici*
15	HVFNSCKK	Hypothetical protein	11324	*Leptosphaeria maculans*
11	DIPAC	Hypothetical protein	16181	*Neosartorya fumigata*

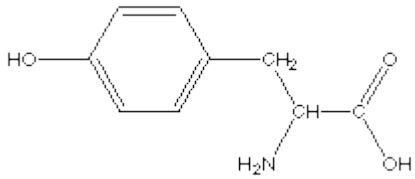
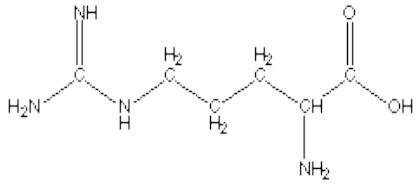
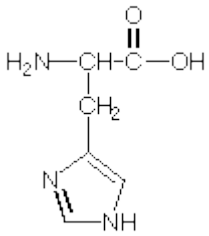
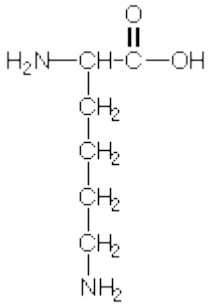
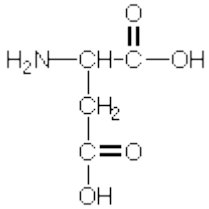
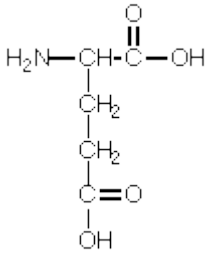
Mascot result of E3AbF6C4 (Continued)

13	AHEPVK	Hypothetical protein	15610	*Pyrenophora teres f. teres*
9	ALPPL	Hypothetical protein	14011	*Arthroderma otae*
8	PSSNK	Hypothetical protein	15080	*Botryotinia fuckeliana*
7	DTYGG	Hypothetical protein	11396	*Ajellomyces capsulata*
7	ENVLFG	MRP-like ABC transporter	13589	*Botryotinia fuckeliana*
5	DGPID	Hypothetical protein	11627	*Pyrenophora tritici-repentis*
3	IPMAK + [O]	Hypothetical protein	14259	*Puccinia graminis f. sp. tritici*
3	PLGPK	Hypothetical protein	16453	*Ajellomyces dermatitidis*
3	KPLNG	Hypothetical protein	17557	*Paracoccidioides brasiliensis*
2	AGGAY	Hypothetical protein	19524	*Sporisorium reilianum*
1	GSAPK	60S ribosomal protein	17831	*Ajellomyces dermatitidis*

Appendix D: Symbol, properties and structure of amino acids

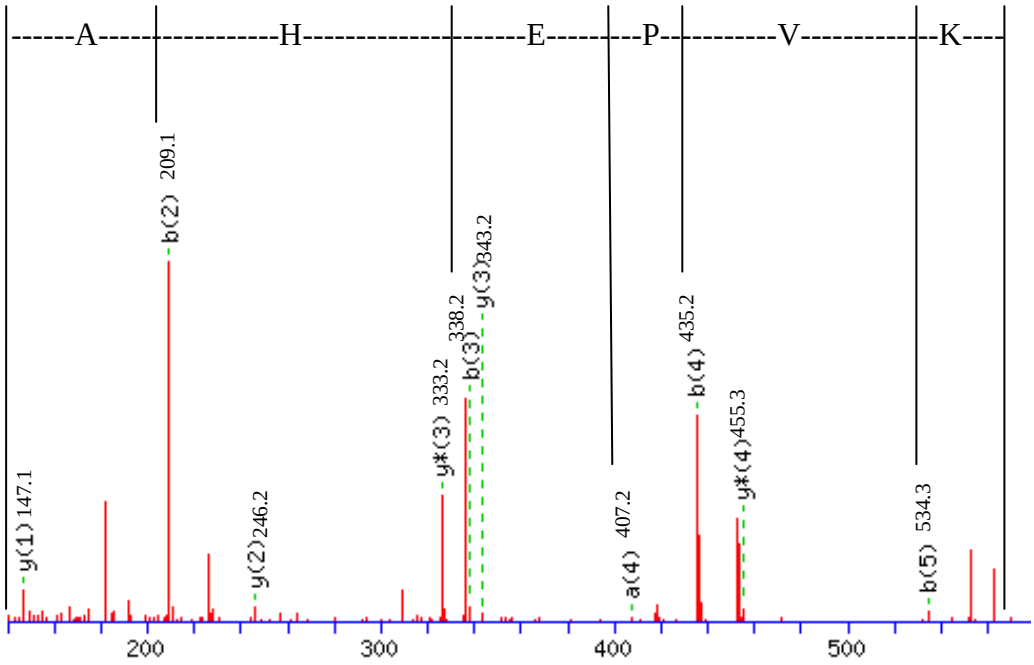
Amino acid	Symbol		Properties	Structure
	1-Letter	3-Letter		
Alanine	A	Ala	Hydrophobic Neutral	
Glycine	G	Gly		
Isoleucine	I	Ile		
Leucine	L	Leu		
Methionine	M	Met		
Phenylalanine	F	Phe		
Proline	P	Pro		

Tryptophan	W	Trp	Hydrophobic Neutral	
Valine	V	Val		
Asparagine	N	Asn	Hydrophilic Neutral	
Cysteine	C	Cys		
Glutamine	Q	Gln		
Serine	S	Ser		
Threonine	T	Thr		

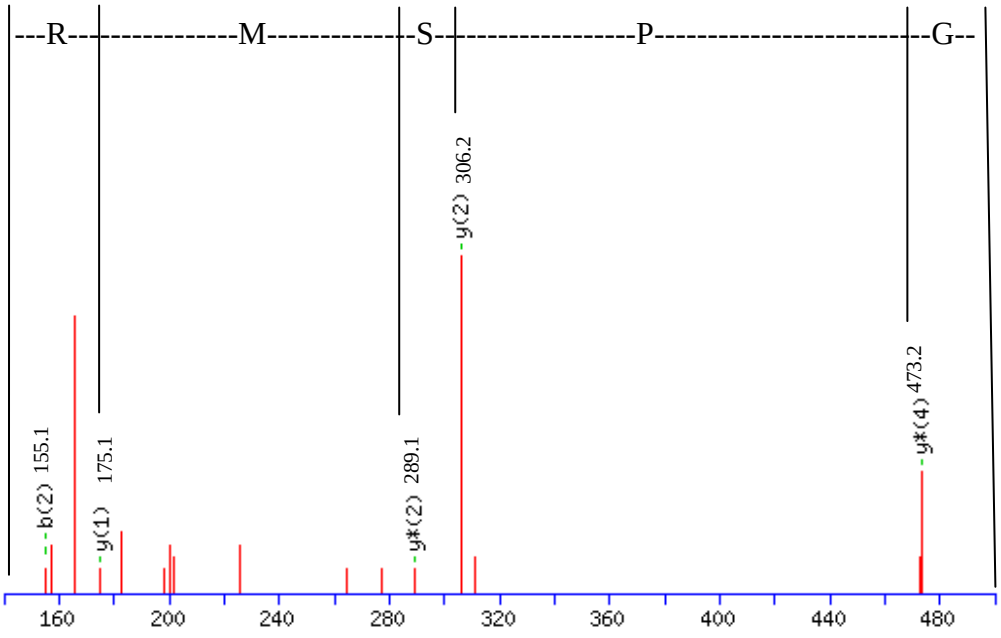
Tyrosine	Y	Tyr	Hydrophilic Neutral	
Arginine	R	Arg	Hydrophilic Charged (+)	
Histidine	H	His		
Lysine	K	Lys		
Aspartate	D	Asp	Hydrophilic Charged (-)	
Glutamate	E	Glu		

(Source: http://biomedapps.curtin.edu.au/biochem/tutorials/AAs/AA.html)

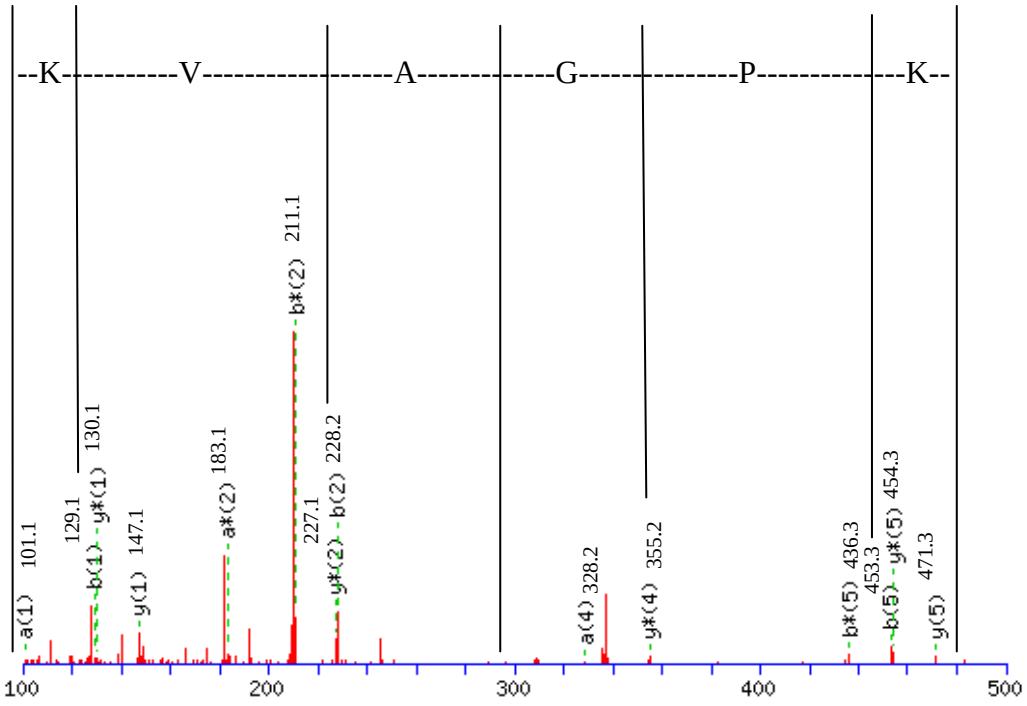
Appendix E: LC/MS/MS spectra of selected peptides



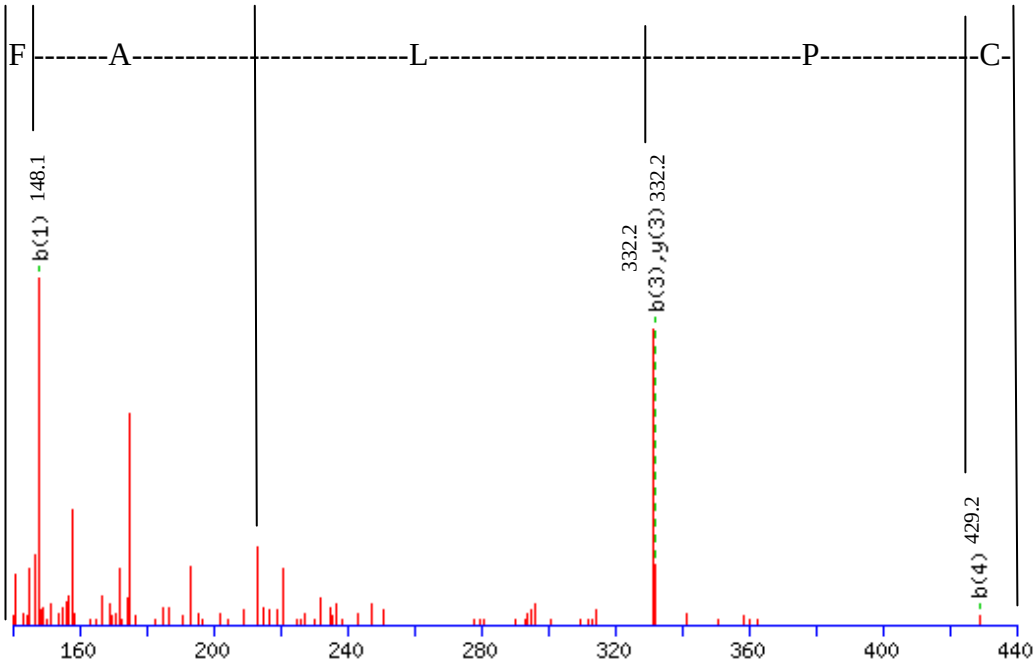
I. LC/MS/MS spectra of peptide 1 (AHEPVK)



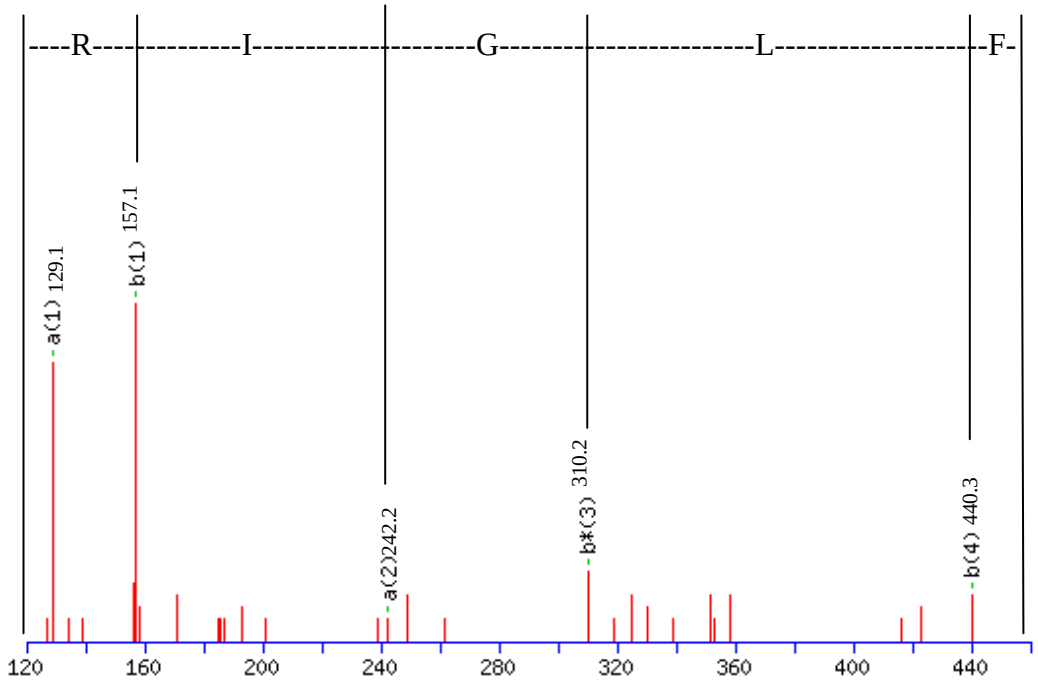
II. LC/MS/MS spectra of peptide 2 (GPSMR)



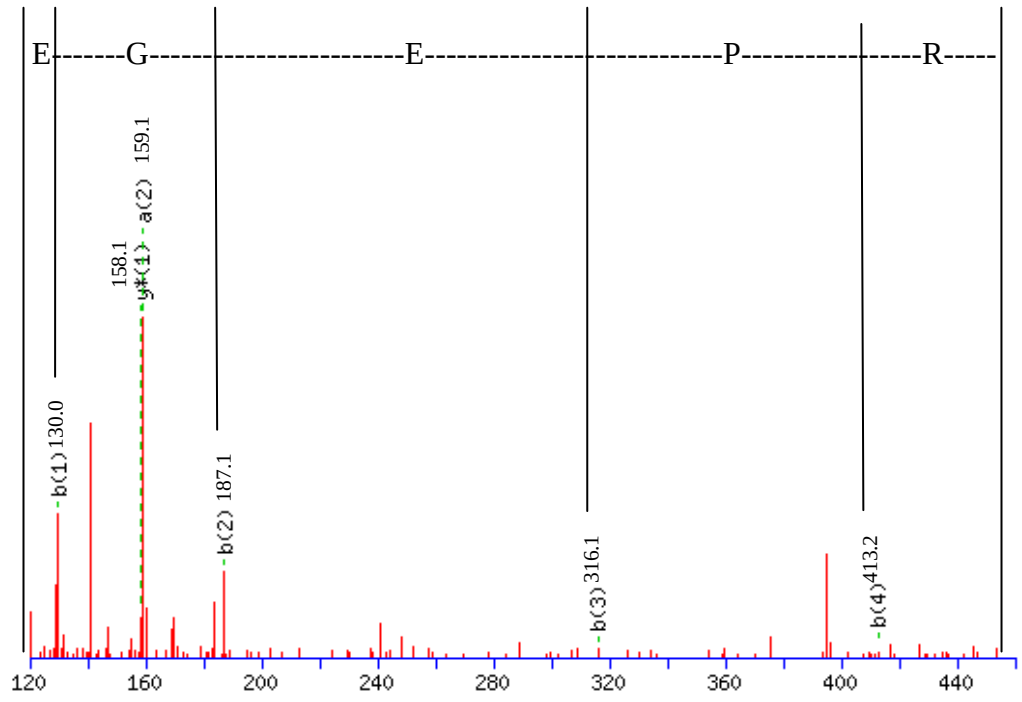
III. LC/MS/MS spectra of peptide 3 (KVAGPK)



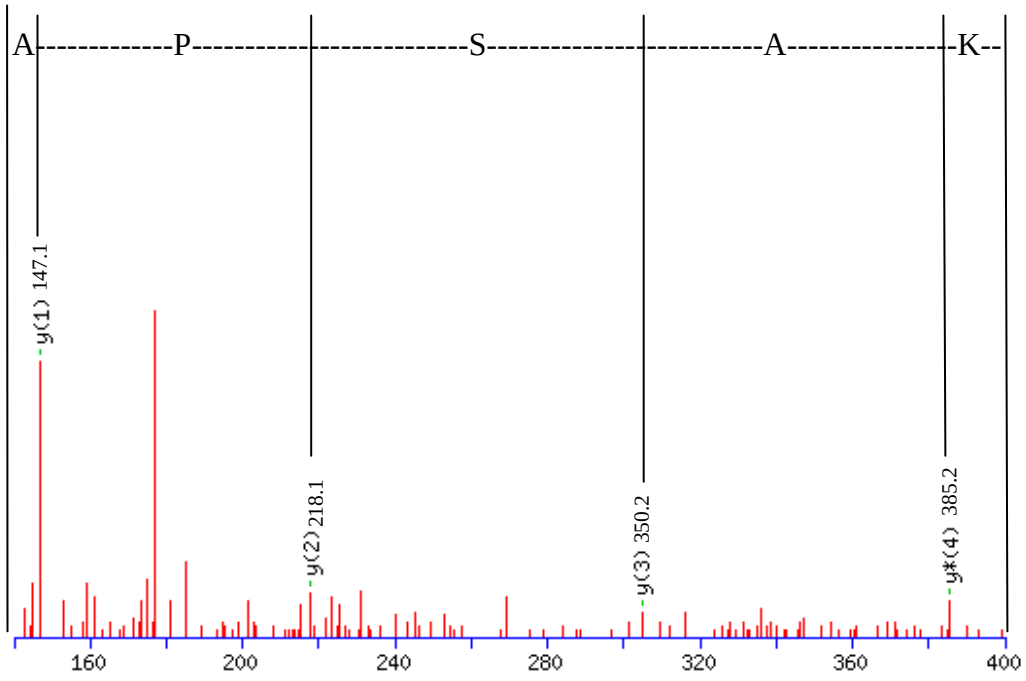
IV. LC/MS/MS spectra of peptide 4 (FALPC)



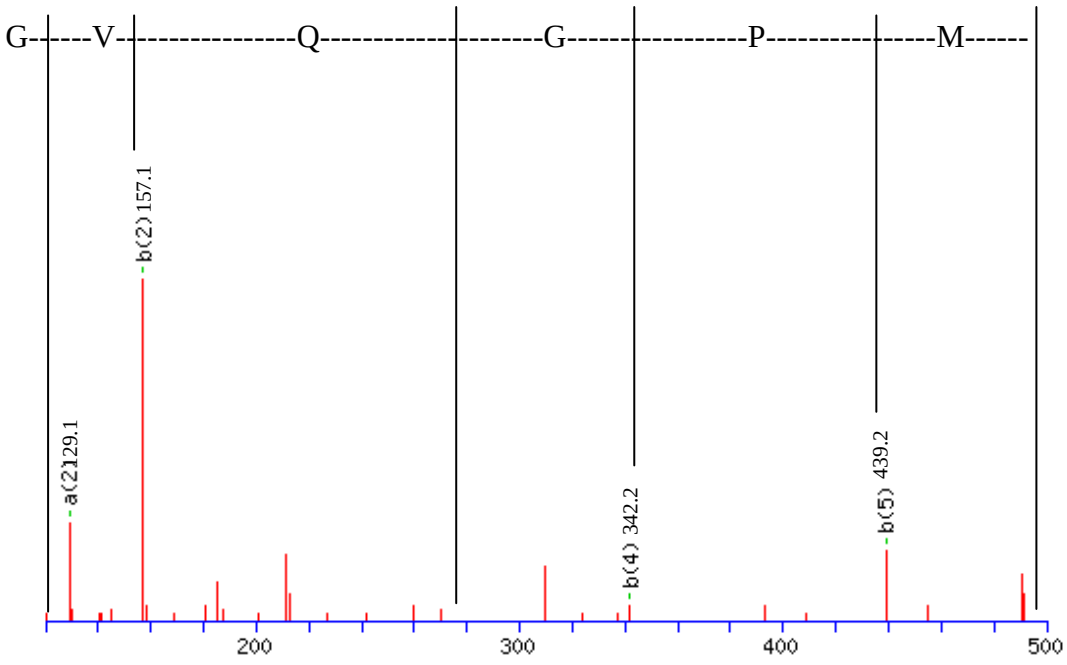
V. LC/MS/MS spectra of peptide 5 (RIGLF)



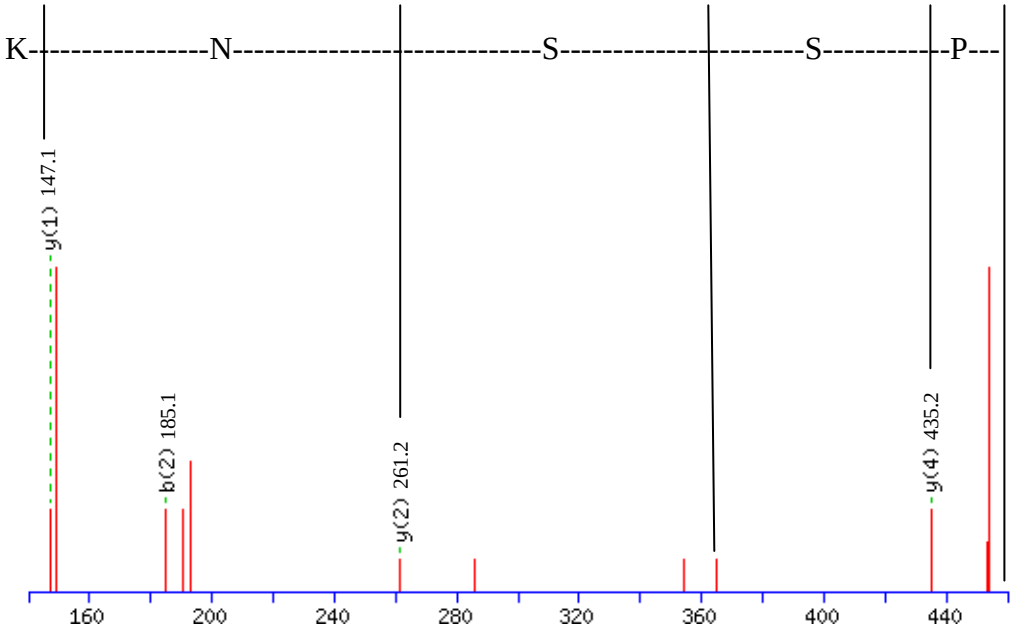
VI. LC/MS/MS spectra of peptide 6 (EGEPR)



VII. LC/MS/MS spectra of peptide 7 (APSAK)

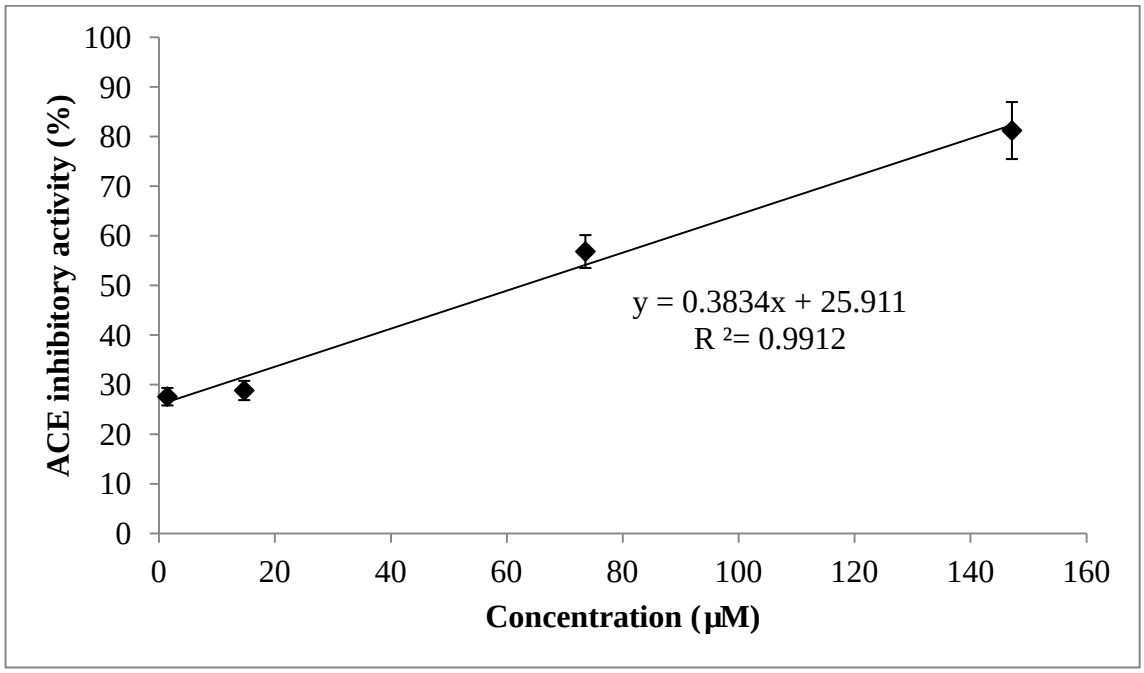


VIII. LC/MS/MS spectra of peptide 8 (GVQGPM)

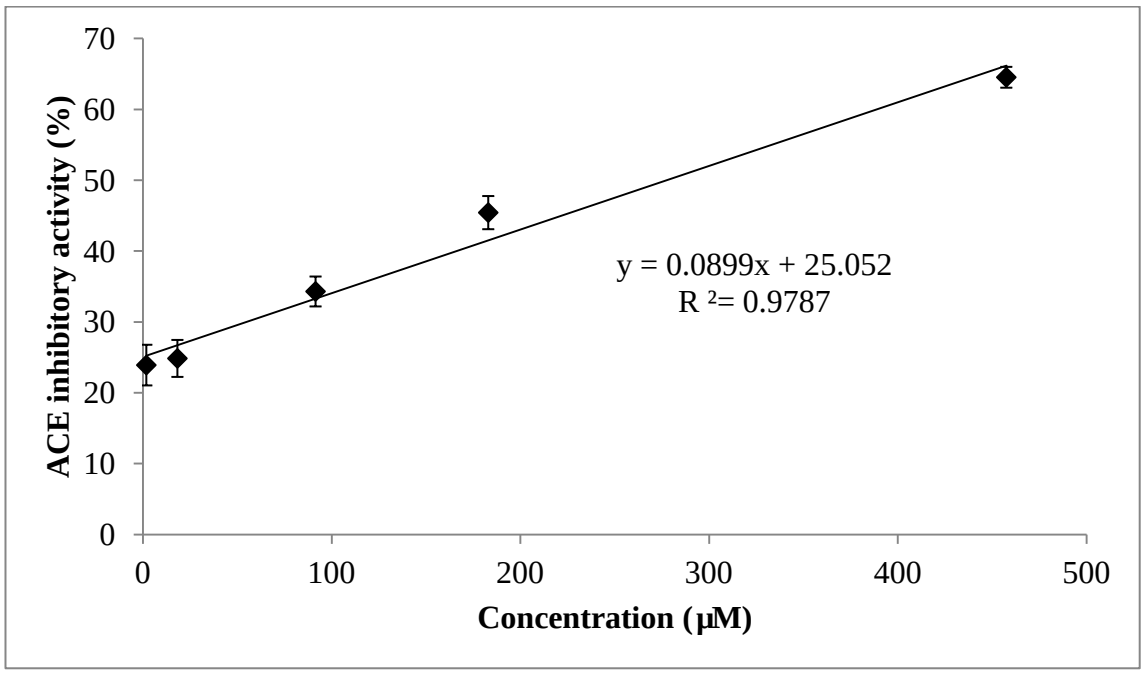


IX. LC/MS/MS spectra of peptide 9 (PSSNK)

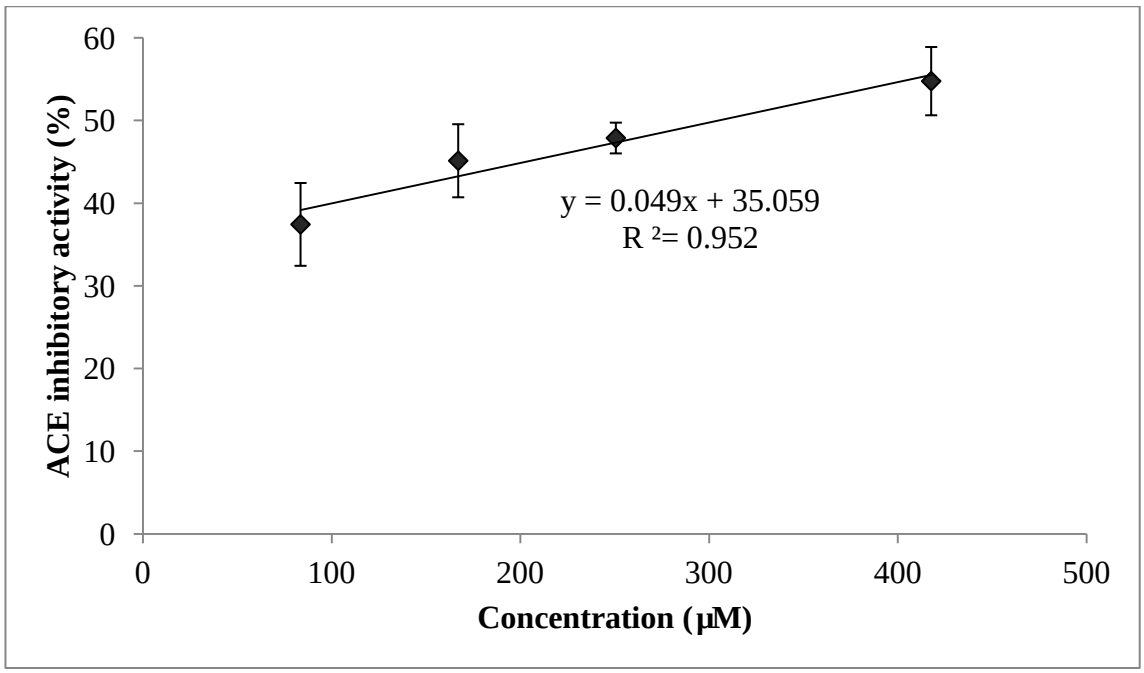
Appendix F: Extrapolation of graph for the determination of IC_{50} value



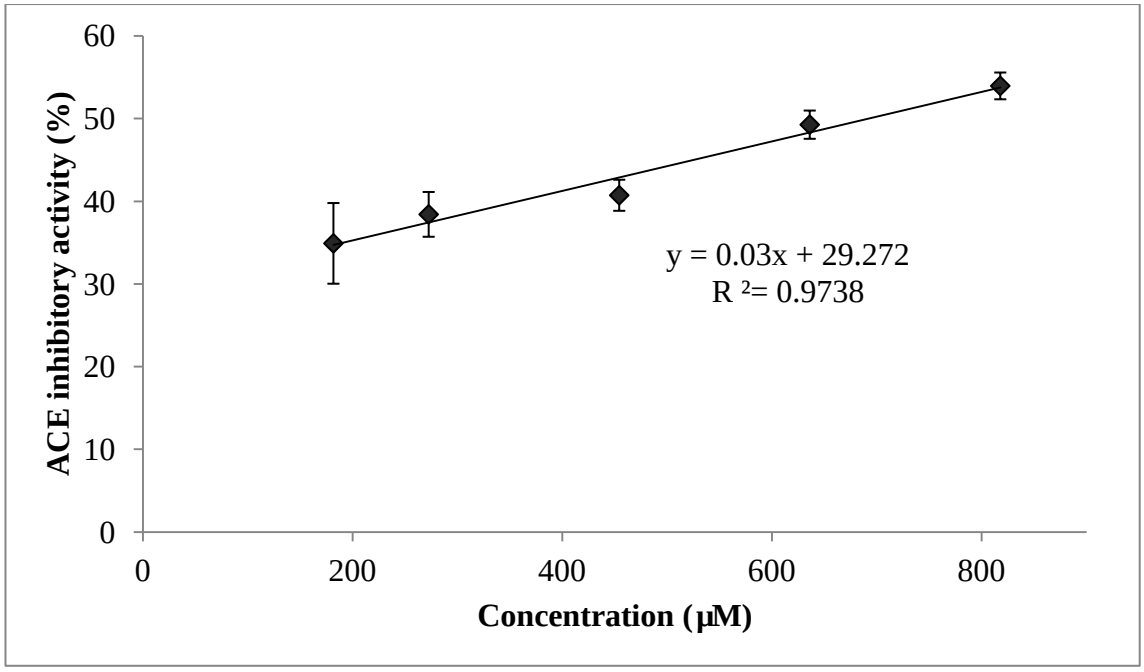
I. Graph for the determination of IC_{50} value of peptide 1 (AHEPVK).



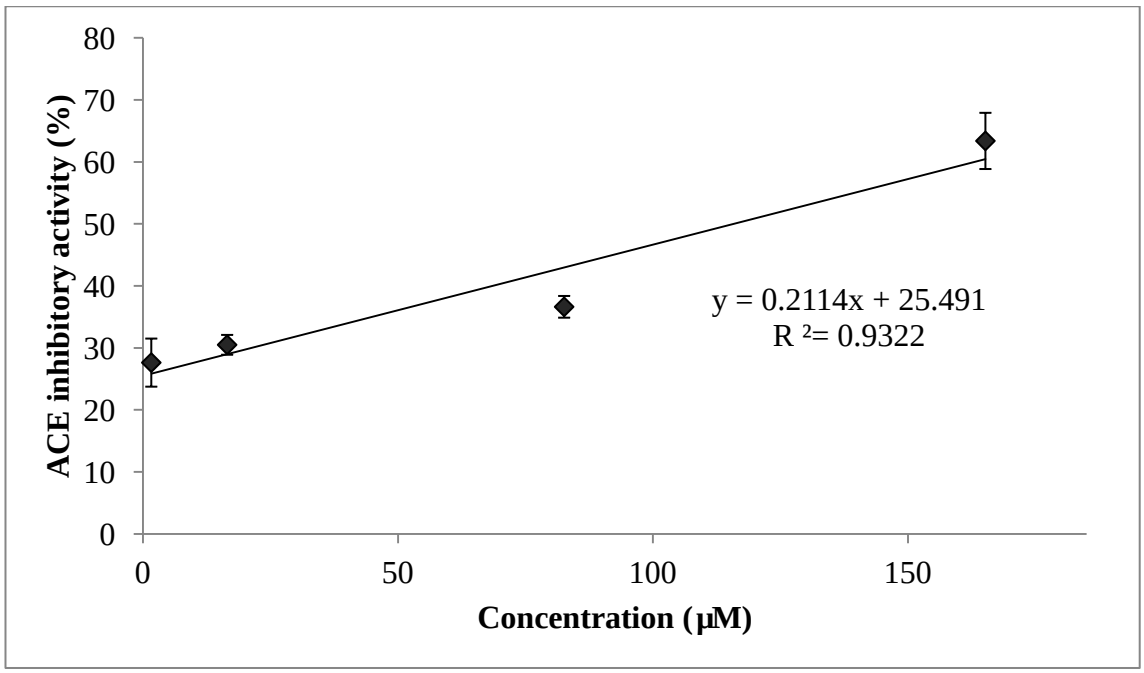
II. Graph for the determination of IC_{50} value of peptide 2 (GPSMR).



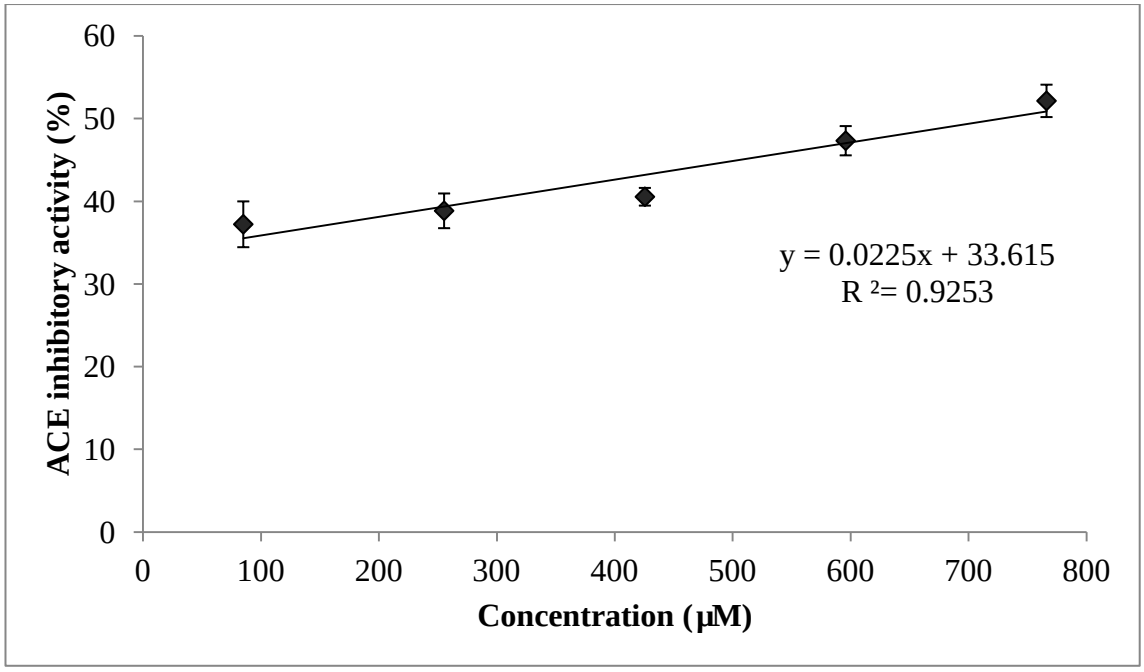
III. Graph for the determination of IC_{50} value of peptide 3 (KVAGPK).



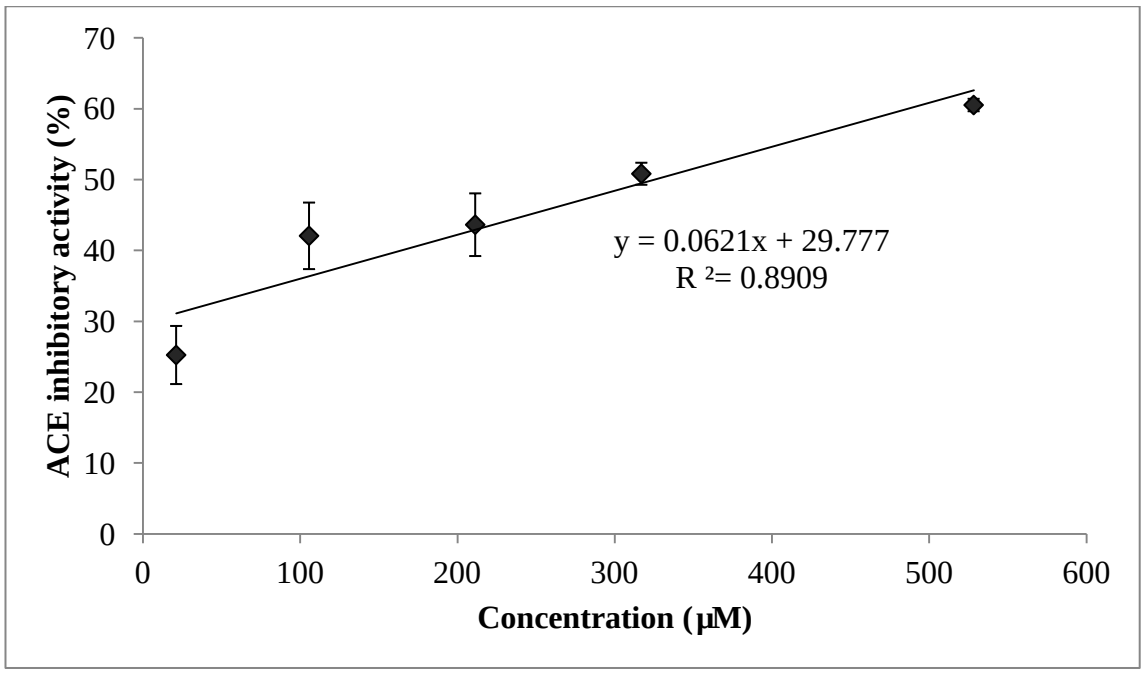
IV. Graph for the determination of IC_{50} value of peptide 4 (FALPC).



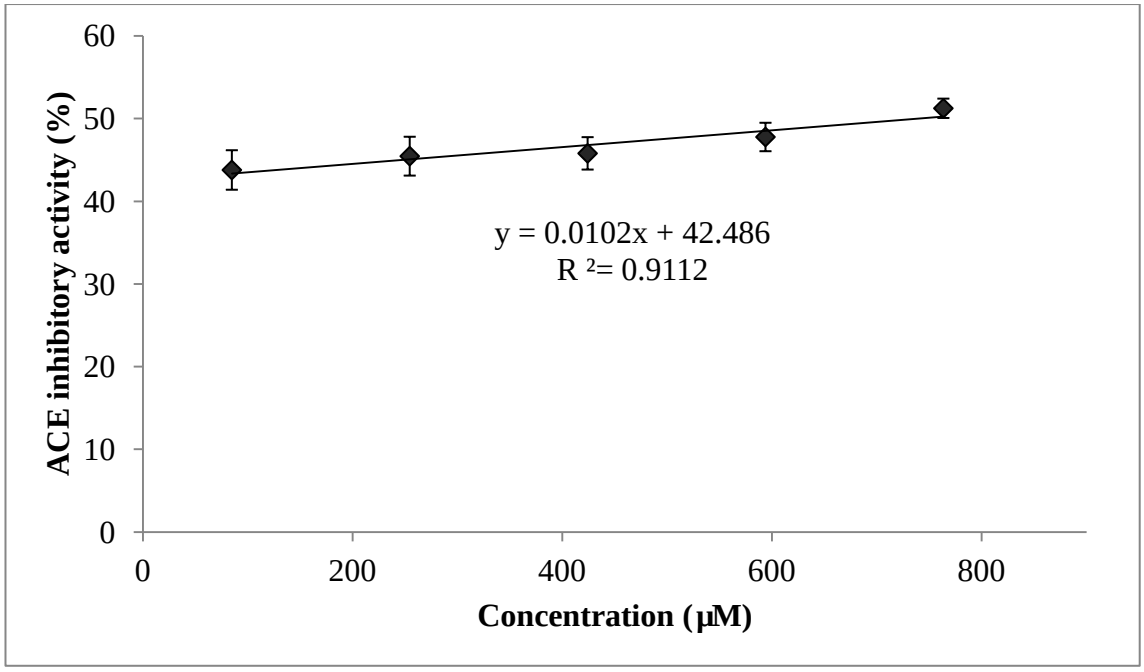
V. Graph for the determination of IC_{50} value of peptide 5 (RIGLF).



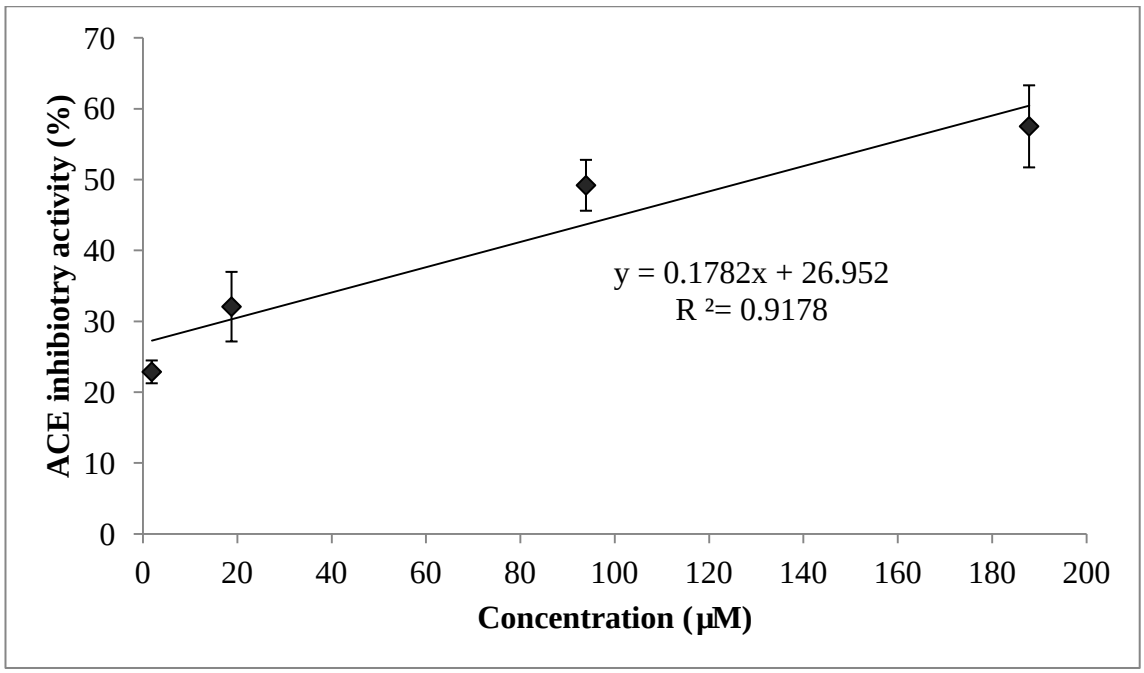
VI. Graph for the determination of IC_{50} value of peptide 6 (EGEPR).



VII. Graph for the determination of IC_{50} value of peptide 7 (APSAK).

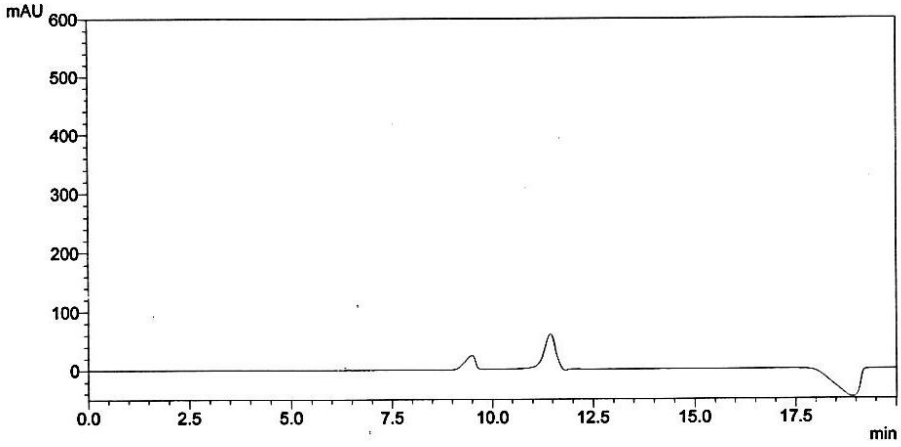


VIII. Graph for the determination of IC_{50} value of peptide 8 (GVQGPM).



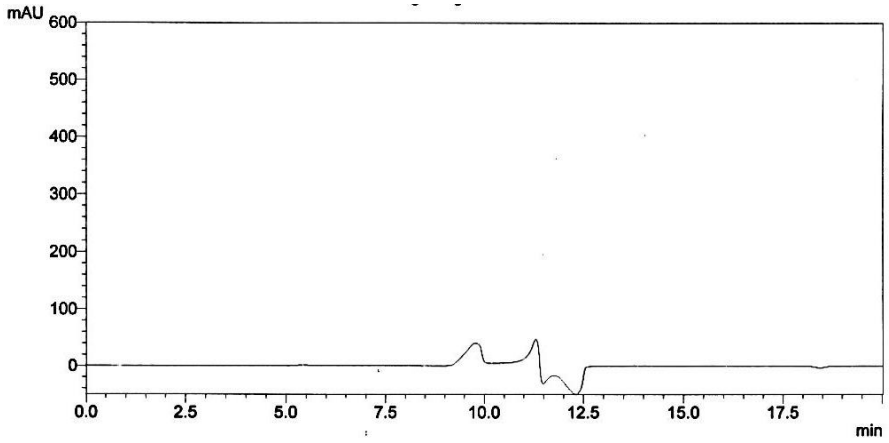
IX. Graph for the determination of IC_{50} value of peptide 9 (PSSNK).

Appendix G: SEC chromatograms of negative controls



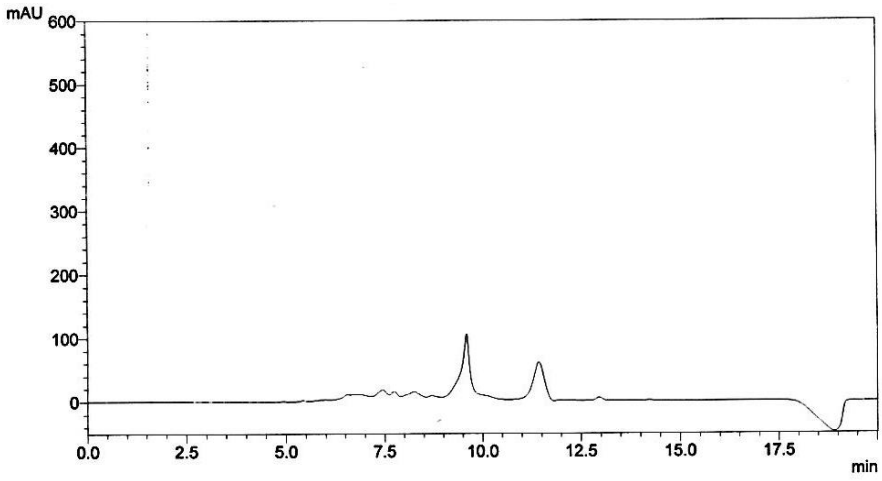
Peak No.	Ret.	Height	Area
1	9.49	24	470
2	11.45	61	1356

I. SEC chromatogram of HCl with retention time (min), peak height (mAU) and peak area (mAU).



Peak No.	Ret.	Height	Area
1	9.77	50	1710
2	11.31	76	2769

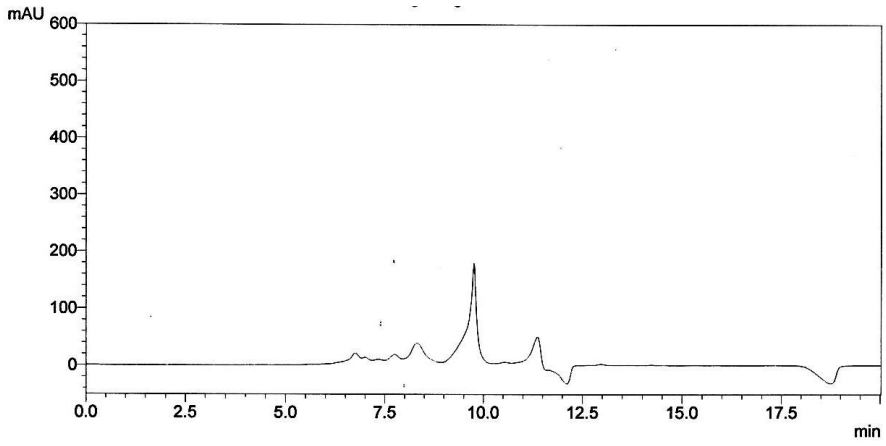
II. SEC chromatogram of potassium phosphate buffer with retention time (min), peak height (mAU) and peak area (mAU).



Peak No.	Ret.	Height	Area
1	7.45	18	359
2	7.76	16	206
3	8.26	15	438
4	9.60	106	2198
5	11.44	62	1321

III. SEC chromatogram of pepsin in HCl with retention time (min), peak height (mAU) and peak area (mAU).

* The concentration used for injection into the SEC column was 5-fold lower than the concentration used in the peptide digestion assay.



Peak No.	Ret.	Height	Area
1	7.32	13	221
2	7.75	22	474
3	8.31	43	1361
4	9.76	185	3630
5	11.37	58	1243

IV. SEC chromatogram of pepsin, trypsin and chymotrypsin in HCl and potassium phosphate buffer with retention time (min), peak height (mAU) and peak area (mAU).

* The concentration used for injection into the SEC column was 5-fold lower than the concentration used in the peptide digestion assay.

Appendix H: List of publications

I. Proceedings

Lau, C.C., Abdullah, N., Shuib, A.S. & Aminudin N. (2009, September). *Angiotensin I-converting enzyme inhibitor from freeze-dried and fresh Pleurotus sajor-caju*. Poster presented at The 5th International Medicinal Mushroom Conference, Nantong, China.

Lau, C.C., Abdullah, N. & Shuib, A.S. (2011, October). *Characterization of antihypertensive peptides from Pleurotus cystidiosus O.K. Miller (abalone mushroom)*. Paper presented at The 7th International Conference on Mushroom Biology and Mushroom Products, Arcachon, France.

Lau, C.C., Abdullah, N. & Shuib, A.S. (2012, November). *Bioactive peptides from edible mushrooms with antihypertensive activity*. Paper presented in conjunction with Ph.D visit from Wageningen University, The Netherlands to University of Malaya.

Lau, C.C., Abdullah, N. & Shuib, A.S. (2013, January). *Identification of ACE inhibitory peptides from Pleurotus cystidiosus O.K. Miller and Agaricus bisporus (J.E. Lange) Imbach by LC-MS/MS*. Paper presented at Proteomics Symposium, Faculty of Medicine, University of Malaya.

II. Article in academic journals

Lau, C.C., Abdullah, N., Shuib, A.S. & Aminudin, N. (2012). Proteomic analysis of antihypertensive proteins in edible mushrooms. *Journal of Agricultural and Food Chemistry*. *60*, 12341-12348.

Lau, C.C., Abdullah, N., Aminudin, N. & Shuib, A.S. (2013). Effect of freeze-drying process on the property of angiotensin I-converting enzyme inhibitory peptides in grey oyster mushrooms. *Drying Technology: An International Journal*. *31*(13-14), 1693-1700.

Lau, C.C., Abdullah, N., Shuib, A.S. & Aminudin, N. (2013). Novel angiotensin I-converting enzyme inhibitory peptides derived from edible mushroom *Agaricus bisporus* (J.E. Lange) Imbach identified by LC-MS/MS. *Food Chemistry*. Article in press.

Lau, C.C., Abdullah, N. & Shuib, A.S. (2013). Novel angiotensin I-converting enzyme inhibitory peptides derived from an edible mushroom, *Pleurotus cystidiosus* O.K. Miller identified by LC-MS/MS. *BMC Complementary and Alternative Medicine*. (Article under review after corrections).

P-2-5: ANGIOTENSIN-I-CONVERTING ENZYME INHIBITOR FROM FREEZE-DRIED AND FRESH *PLEUROTUS SAJOR-CAJU*

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Hypertension is one of the risk factors for cardiovascular diseases, such as stroke, heart failure, cardiac arrhythmia and arteriosclerosis. It can be prevented and treated by the inhibition of angiotensin-I- converting enzyme (ACE) in the cardiovascular system. The earliest discovery of ACE inhibitor was from snake venom. Subsequently, many attempts have been made in the production of artificial synthetic ACE inhibitors. However, synthetic drugs are believed to have certain side effects such as cough, taste disturbances and skin rashes. Therefore, interest in identifying ACE inhibitors from food sources has increased. Many ACE inhibitory peptides have been discovered, among which some are derived from food-proteins, such as milk, buckwheat, potato and tuna. Mushroom is rich in protein content which ranks below most animal meats but well above most other foods, for instance vegetables and fruits. Previous studies have showed successful purification of ACE inhibitors from fruiting bodies of edible mushroom, *Tricholoma giganteum* and *Grifola frondosa*. *Pleurotus sajor-caju* is the most common edible mushroom known worldwide. The majority of mushroom growers in Malaysia prefer to grow *P. sajor-caju* compared to the other types of mushrooms because they are local favourites, easy to cultivate, have high yield potential and high nutritional value. Therefore, it can be easily obtained in the market to produce into pharmaceutical product. Freeze-drying (lyophilisation) is one of the most common ways of preserving protein. But sometimes bioactivity can be lost during freeze-drying. Thus, the purpose of this study is to compare, isolate and identify the ACE inhibitory substances from fruiting bodies of fresh *P. sajor-caju* (FPSC) and freeze-dried *P. sajor-caju* (DPSC). Water extracts of FPSC and DPSC were prepared by homogenizing the FPSC and DPSC in distilled water at a ratio of 1:2 and 1:10, respectively. The mixtures were then filtered to get the protein extracts of FPSC and DPSC. Proteins were purified from the FPSC and DPSC using ammonium sulphate precipitation method from 10% to 100% salt saturation (S10 to S100). This was followed by dialysis to remove the salt and unwanted low-molecular-weight solute from the sample. The protein content was measured using bis-cinchonic acid (BCA) protein assay kit. Protein fractions from DPSC contained higher protein concentration (144-993 µg/mL protein) compared to FPSC (51-377 µg/mL protein). The highest protein content for DPSC and FPSC was obtained at S70 and S90, respectively. The ACE inhibitory activity of the protein fractions were assayed by *in vitro* using hippuryl-histidyl-leucine (HHL) as substrate. At a concentration of 25 µg/mL protein, protein fraction at S70 of DPSC exhibited the highest inhibition of the ACE enzyme by 86.08%, followed by S60 (77.22%) and S30 (37.97%). However, among FPSC, only protein fractions at S30, S50, S60 and S100 showed ACE inhibitory activity ranging from 2.13%-27.66%. The active protein fractions will be further purified by SDS gel electrophoresis to determine the molecular weight and sequence of the ACE inhibitory protein.

Abstracts of oral presentations

3-22- Characterization of antihypertensive peptides from *Pleurotus cystidiosus* O.K. Miller (Abalone mushroom)

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Hypertension is one of the risk factors for cardiovascular diseases, such as stroke, heart failure, cardiac arrhythmia and arteriosclerosis [1]. Hypertension has been on the rise in Malaysia over the past 10 years [2]. It can be prevented and treated by the inhibition of angiotensin-I- converting enzyme (ACE) in the cardiovascular system [3]. Many ACE inhibitory peptides have been discovered, among which some are derived from food-proteins, such as milk, buckwheat, potato and tuna [4]. Previous studies have also showed successful purification of ACE inhibitors from fruiting bodies of edible mushroom, *Tricholoma giganteum* and *Grifola frondosa* [3, 5]. Excess production of reactive oxygen species (ROS) will contribute to hypertension. Antioxidant administration may help to prevent and treat hypertension [6]. *Pleurotus cystidiosus* has been proven to show strong antioxidant effect [7]. Therefore, it can be a good source of an antihypertensive drug. Thus, the objective of the current study is to purify the ACE inhibitory peptide from the fruit body of *P. cystidiosus*.

The antihypertensive activity in the current study was tested based on the percentage of ACE inhibited in the renin-angiotensin system. Crude water extract of *P. cystidiosus* fruit body showed high antihypertensive activity where 81.39% of ACE was inhibited at a concentration of 10 mg/ml. Proteins were precipitated from the crude water extract using ammonium sulphate precipitation method from 10% to 100% salt saturation (S10-S100). The crude proteins were grouped into 6 fractions according to the protein bands observed by SDS PAGE. Protein content of the 6 fractions were measured using Bis-cinchonic acid (BCA) protein assay kit, and was in the range of 182.73 to 930.00 µg/ml. The ACE inhibitory activity of the protein fractions were tested at a concentration of 10 µg/ml. The highest antihypertensive activity was obtained by fraction 1 (S10-S20) where 96.22% of ACE was inhibited. This was followed by fraction 5 (S90) where 86.73% of ACE was inhibited. The remaining protein fractions showed less than 50% ACE inhibitory activity. Thus, fraction 1 and fraction 5 were selected to do further purification by high performance liquid chromatography (HPLC). The proteins were eluted at an increasing gradient of acetonitrile in 0.05% TFA from 0 to 50% in 20 min. The protein fractions were sub-fractionated according to the HPLC peak obtained. Fraction 1 was divided into 7 sub-fractions (F1a-F1g) while fraction 5 was divided into 8 sub-fractions (F5a-F5h). The ACE inhibitory activities of the HPLC fractions were tested at 1 µg/ml. For fraction 1, F1a, F1c and F1e were among the best ACE inhibitors, while for fraction 5, the best ACE inhibitors were F5a, F5b and F5g. The HPLC fractions were loaded into the SDS PAGE to check the purity of the samples. Further work will be carried out to identify the protein sequence from *P. cystidiosus* with high ACE inhibitory activity.

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Oral 1

BIOACTIVE PEPTIDES FROM EDIBLE MUSHROOMS WITH ANTIHYPERTENSIVE ACTIVITY

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Hypertension is one of the major health problems worldwide. Bioactive peptides that inhibit Angiotensin I-Converting Enzyme (ACE) in the blood pressure regulation system (renin-angiotensin system) can contribute to the prevention and treatment of hypertension. Mushrooms are high in protein content, which makes them a potentially good source of antihypertensive peptides. Among the nine edible mushrooms tested, protein extracts from *Pleurotus cystidiosus* had high levels of antihypertensive activity. The protein extracts were further purified by reverse-phase high performance liquid chromatography (RP-HPLC) and size exclusion chromatography (SEC). LC-MS/MS analysis of the active protein had identified two peptide sequences (AHEPVK and GPSMR) with potential ACE inhibitory activity. The IC_{50} values of the synthesized peptides were 48.45 and 114.92 µg/ml, respectively. Interestingly, the two peptides had enhanced antihypertensive activity after gastrointestinal digestion. Although the ACE inhibition effect of the two peptides are lower compared to captopril (antihypertensive drug having side effects), the peptides are derived from a food source that can be consumed daily.

IDENTIFICATION OF ACE INHIBITORY PEPTIDES FROM *PLEUROTUS CYSTIDIOSUS* O.K. MILLER AND *AGARICUS BISPORUS* (J.E. LANGE) IMBACH BY LC-MS/MS

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Hypertension is one of the major health problems worldwide. Bioactive peptides that inhibit Angiotensin I-Converting Enzyme (ACE) in the blood pressure regulation system (renin-angiotensin system) can contribute to the prevention and treatment of hypertension. Mushrooms are high in protein content, which makes them a potentially good source of antihypertensive peptides. Protein extracts from *Pleurotus cystidiosus* and *Agaricus bisporus* having high levels of antihypertensive activity were further purified by reverse-phase high performance liquid chromatography and size exclusion chromatography. LC-MS/MS analysis of the active protein had identified nine peptide sequences with potential ACE inhibitory activity. These peptides have common characteristics of potent ACE inhibitory peptides, which usually consist of less than six amino acids in length, high content of hydrophobic amino acid with aromatic and branched aliphatic amino acid at the C and N-termini, respectively. The IC_{50} values of the synthesized peptides were in the range of 42.69 to 435.86 µg/ml. The nine peptides exhibited enhanced antihypertensive activity after gastrointestinal digestion. These peptides are from food source hence should have no side effects.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Proteomic Analysis of Antihypertensive Proteins in Edible Mushrooms

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ABSTRACT: Mushrooms are high in protein content, which makes them potentially a good source of antihypertensive peptides. Among the mushrooms tested, protein extracts from *Pleurotus cystidiosus* (E1Pc and E5Pc) and *Agaricus bisporus* (E1Ab and E3Ab) had high levels of antihypertensive activity. The protein extracts were fractionated by reverse-phase high-performance liquid chromatography (RPHPLC) into six fractions. Fraction 3 from E5Pc (E5PcF3) and fraction 6 from E3Ab (E3AbF6) had the highest antihypertensive activities. SDS-PAGE analysis showed E5PcF3 consisted mainly of low molecular weight proteins, whereas E3AbF6 contained a variety of high to low molecular weight proteins. There were 22 protein clusters detected by SELDI-TOF-MS analysis with five common peaks found in E5PcF3 and E3AbF6, which had m/z values in the range of 3940−11413. This study suggests that the antihypertensive activity in the two mushroom species could be due to proteins with molecular masses ranging from 3 to 10 kDa.

KEYWORDS: *Agaricus bisporus, Pleurotus cystidiosus, ACE inhibitory peptides, SELDI-TOF-MS*

INTRODUCTION

Hypertension or high blood pressure has been on the rise worldwide. According to the World Health Organization (WHO), nearly 1 billion people globally have high blood pressure, and it is predicted to increase to a total of 1.56 billion in the year 2025.[1] Hypertension is one of the major risk factors for cardiovascular diseases such as stroke, coronary heart disease, and peripheral artery disease.[2]

Angiotensin converting enzyme (ACE) is a dipeptidyl carboxypeptidase of the renin−angiotensin system that plays a key role in blood pressure homeostasis and the salt balance of mammals. ACE is involved in the conversion of angiotensin I to angiotensin II. This reaction ultimately causes the contraction of blood vessels and thereby leads to hypertension. Today, specific synthetic inhibitors of ACE are used to treat hypertension, congestive heart failure, and myocardial infarction.[3] However, these inhibitors can induce powerful side effects, such as angioedema.[4]

Nutritional factors play an important role in the prevention and treatment of hypertension. In addition to pharmacological treatment, a moderate lowering of blood pressure can be obtained by a nutritional approach. Many ACE inhibitory peptides have been detected in food proteins such as milk,[5] buckwheat,[6] potato,[7] tuna,[8] oyster,[9] broccoli,[10] cheese,[11] and chickpea.[12] Furthermore, naturally occurring ACE inhibitory proteins have been proven to be effective as in vivo antihypertensive agents.[13] Proteins with a high content of hydrophobic amino acids are good sources of ACE inhibitory peptides because high hydrophilicity will make the proteins inaccessible to the active site of ACE.[14,15] Structural analysis of tripeptides with high ACE inhibitory activity revealed they usually contain amino acid residues with aromatic and hydrophobic side chains at the carboxyl and amino termini, respectively, whereas positively charged amino acids are preferred for the middle position.[16] These peptides have been found to be less active than the synthetic ACE inhibitors; however, they might play an important role as antihypertensive agents as they can be part of the daily diet in the form of functional foods and are perceived as natural and safe by consumers.[17]

Mushrooms are favorite dishes in Oriental and Western countries. Besides their nutritional value, the unique color, taste, and aroma of mushrooms are the reasons that attract their consumption by humans.[18] Mushrooms are high in protein content; the amount of protein in mushrooms is below that in most animal meats but above that in most other foods, such as wheat and milk.[19] Previous studies have shown successful purification of ACE inhibitory peptides from the fruiting bodies of edible mushrooms, such as *Tricholoma giganteum*,[20] *Grifola frondosa*,[21] and *Pleurotus cornucopiae*.[22] Hence, mushrooms can be a good source of bioactive peptides. Currently, there are no studies available profiling antihypertensive peptides in edible mushrooms, and proteomics is an excellent tool to do this. Therefore, the objective of the current study was to compare the antihypertensive activity of proteins in edible mushrooms and carry out a proteomic analysis of selected potent antihypertensive proteins.

MATERIALS AND METHODS

Materials. Sporocarps (or fruiting bodies) of edible mushrooms *Agaricus bisporus* (button mushroom), *Flammulina velutipes* (golden needle), and *Lentinus edodes* (shiitake) were purchased from a local supermarket, whereas *Hericium erinaceus* (monkey's head mushroom), *Pleurotus citrinopileatus* (yellow oyster mushroom), *Pleurotus cystidiosus* (abalone mushroom), *Pleurotus flabellatus* (pink oyster mushroom), *Pleurotus florida* (white oyster mushroom), and *Pleurotus sajor-caju* (gray oyster mushroom) were purchased from a local mushroom farm, Ganofarm Sdn Bhd, Malaysia. α-Cyano-4-hydroxycinnamic acid (CHCA) (used for SELDI-TOF-MS analysis) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals, such as ammonium sulfate,

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Effect of Freeze-Drying Process on the Property of Angiotensin I-Converting Enzyme Inhibitory Peptides in Grey Oyster Mushrooms

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The effect of freeze-drying on the properties of angiotensin I-converting enzyme (ACE) peptide inhibitors in mushrooms was investigated. At least a two-fold higher amount of protein was extracted from freeze-dried compared to fresh fruiting bodies. Proteomic analysis showed freeze-drying has preserved a wide range of proteins in mushroom, including low abundance proteins which could be potential ACE inhibitors. The ACE inhibitory activity of proteins was not lost by freeze-drying. Therefore, freeze-drying could be applied for industrial-scale drying of mushroom fruiting bodies for the extraction of ACE inhibitory peptides from food for the production of functional foods and nutraceuticals.

Keywords Bioactive peptides; Edible mushroom; Hypertension; SDS-PAGE; SELDI-TOF-MS

INTRODUCTION

Angiotensin I-converting enzyme (ACE) inhibitors are the most commonly available and widely prescribed class of antihypertensive drugs.[1] ACE catalyzes the conversion of angiotensin I to angiotensin II, a vasoconstrictive octapeptide. It also has a promoting effect in the degradation and inactivation of bradykinin, a vasodilator.[2] Therefore, ACE inhibitors regulate the blood pressure by suppressing the conversion of angiotensin I to angiotensin II and inhibit the degradation of bradykinin. ACE inhibitors have also been used as a drug therapy for heart attack and diabetes.[3] Synthetic ACE inhibitor drugs (for example, captopril) have been reported to cause undesirable side-effects such as cough, taste disturbance, skin rashes, and angioedema.[4] Several ACE inhibitory peptides have been successfully purified from food proteins and commercialized in Japan and Canada under the product names of Vasotensin®, Levenorm®, Peptide ACE 3000, Lapis Support, Valtyron®, Stabilium® 200, and Protizen®.[5] These are peptides derived from bonita and sardine. These food-derived peptides were reported to be free of side-effects.[6,7] Therefore, this has increased interest from researchers in finding ACE inhibitory peptides in other commonly consumed foods.

Mushrooms are known to be rich in protein content.[8] They are used as a meat substitute and as a protein source in vegan diets.[9] Previous studies have shown successful purification of ACE inhibitory peptides from the fruiting bodies of edible mushrooms such as *Grifola frondosa*,[10] *Pleurotus cornucopiae*,[11] and *Tricholoma giganteum*.[12] *Pleurotus* spp., commonly known as the oyster mushroom, is among the most popular cultivated edible mushrooms worldwide due to its medicinal properties, vigorous growth, and undemanding cultivation conditions.[13] Among the *Pleurotus* spp., *P. pulmonarius* is the most commonly cultivated in Malaysia. Aqueous extract from *P. sajor-caju* (a.k.a. *P. pulmonarius*) has been reported to have a hypotensive effect by the regulation of the renin-angiotensin system.[14] Therefore, it may be considered as a good source of ACE inhibitory peptides.

Purification of bioactive proteins from perishable foods has to undergo stages of processing which may cause various changes in physical, chemical, and biological characteristics.[15] The delicate nature of most proteins with poor long-term stability causes denaturation or loss of bioactivity after processing. This has been one of the major problems in developing proteins as a therapeutic product.[16] Freeze-drying is the most widely used processing method for protein- or peptide-based drugs.[17] It can improve the storage stability of proteins.[15] However, contradicting reports also exist. According to Tang and Pikal,[18] the low temperature of freeze-drying does not guarantee protein stability because some proteins may experience cold denaturation

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