ฤทธิ์ยับยั้งแอนจิโอเทนซินI-คอนเวอร์ติงเอนไซม์ของโปรตีนและเพปไทด์ที่บริสุทธิ์จากเหง้าของพืชวงศ์ขิง

นางสาวมณีรัตน์ ยอดจันทร์

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ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY ACTIVITY OF PURIFIED PROTEINS AND PEPTIDES FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS

Miss Maneerat Yodjun

A Thesis Submitted in Partial Fulfillment of the Requirements

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By	Miss Maneerat Yodjun
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Polkit Sangvanich, Ph.D.
Thesis Co-advisor	Aphichart Karnchanatat, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……………………………………………….. Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr. rer. nat.)

THESIS COMMITTEE

……………………………………………….. Chairman

(Associate Professor Sirirat Rengpipat, Ph.D.)

……………………………………………….. Thesis Advisor

(Associate Professor Polkit Sangvanich, Ph.D.)

……………………………………………….. Thesis Co-advisor

(Aphichart Karnchanatat, Ph.D.)

……………………………………………….. Examiner

(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

……………………………………………….. Examiner

(Associate Professor Nongnuj Muangsin, Ph.D.)

……………………………………………….. External Examiner

(Prapas Khorphueng, Ph.D.)

มณีรัตน์ ยอดจันทร์: ฤทธิ์ยับยั้งแอนจิโอเทนซินI-คอนเวอร์ติงเอนไซม์ของโปรตีนและเพปไทด์ที่บริสุทธิ์จากเหง้าของพืชวงศ์ขิง อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. พลกฤษณ์ แสงวณิช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร. อภิชาติ กาญจนทัต, 59 หน้า

แอนจิโอเทนซินI-คอนเวอร์ติง เอนไซม์ มีความสำคัญเกี่ยวข้องข้องกับภาวะความดันโลหิตสูงซึ่งมีฤทธิ์ทำให้เกิดการเปลี่ยน แอนจิโอเทนซิน I ไปเป็นแอนจิโอเทนซิน II ซึ่งมีผลต่อการลดความดันโลหิตได้ ในงานวิจัยนี้โปรตีนของพืชวงศ์ขิงที่บริสุทธิ์และโปรตีนที่ถูกย่อยด้วยเปปซิน และนำมาตรวจสอบฤทธิ์การยับยั้งแอนจิโอเทนซิน I-คอนเวอร์ติง เอนไซม์ โปรตีนสกัดหยาบจากเหง้าของพืชวงศ์ขิง 15 ชนิด นำมาตรวจสอบฤทธิ์การยับยั้งของแอนจิโอเทนซินI-คอนเวอร์ติงเอนไซม์ โดยการหาฤทธิ์การยับยั้งของโปรตีนซึ่งเป็นการศึกษาในขั้นหลอดทดลอง โปรตีนสกัดหยาบจากเหง้าของไพลดำมีฤทธิ์การยับยั้งของแอนจิโอเทนซินI-คอนเวอร์ติงเอนไซม์สูงสุดมีค่าเท่ากับ7.30×10^{-7} มิลลิกรัมโปรตีนต่อมิลลิลิตรจากนั้นนำโปรตีนมาทำให้บริสุทธิ์โดยเทคนิคโครมาโทกราฟีแบบแลกเปลี่ยนไอออนด้วยคอลัมน์ SP sepharose โดยชะแบบเป็นลำดับขั้น สามารถแยกโปรตีนสกัดหยาบได้เป็น 5 ส่วน คือ unbound F25 F50 F75 และ F100 ตามลำดับ โดยส่วน F75 มีปริมาณโปรตีนสูงที่สุด เมื่อใช้เทคนิคพอลิอะคริลาไมด์เจลอิเล็กโตรฟอเรซิสแบบเสียสภาพ พบว่าส่วนของ F75 มีโปรตีนบริสุทธิ์อย่างน้อย 1 ชนิดที่มีขนาด 20.7 กิโลดาลตัน เมื่อวิเคราะห์ลำดับกรดอะมิโนภายในโมเลกุลโดยการตัดด้วยทริปซิน แล้ววิเคราะห์ชิ้นส่วนที่ถูกย่อยด้วยเครื่อง LC-MS/MS พบว่าลำดับอะมิโนของไพลดำมีความคล้ายคลึงกับกลุ่มไคติเนส ทำการศึกษาฤทธิ์ของสารยับยั้งแอนจิโอเทนซินI-คอนเวอร์ติงเอนไซม์ของ F75 มีเสถียรภาพที่อุณหภูมิ -20 จนถึง 60 องศาเซลเซียส เป็นเวลา 30 นาทีและที่ค่าความเป็นกรด-ด่าง ซึ่งมีค่าลดลงในช่วงพีเอช 6 และเพิ่มขึ้นสูงช่วง 8-12 และนำโปรตีนมาศึกษาทางจลนพลศาสตร์พบว่ามีค่า K_i เท่ากับ 9.1×10^{-5} มิลลิกรัมโปรตีนต่อมิลลิลิตร ในส่วนของเพปไทด์พบว่าโปรตีนจากเหง้าของไพลเหลืองที่ทำการย่อยด้วยเปปซินที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 30 นาที สามารถหาฤทธิ์ของสารยับยั้งเอนไซม์แอนจิโอเทนซินI-คอนเวอร์ติงเอนไซม์ ได้ค่า IC_{50} เท่ากับ 0.38±0.012 มิลลิกรัมโปรตีนต่อมิลลิลิตร หลังจากนั้นนำโปรตีนจากเหง้าของไพลเหลืองที่ทำการย่อยด้วยเปปซินมาแยกเพปไทด์โดย เครื่องเอชพีแอลซีแบบรีเวอร์เฟส พบว่าได้เพปไทด์มีลักษณะเป็นพีคเดียว และนำมาตรวจสอบฤทธิ์ของสารยับยั้งแอนจิโอเทนซินI-คอนเวอร์ติงเอนไซม์ ได้ค่า IC_{50} เท่ากับ 0.011±0.012 มิลลิกรัมโปรตีนต่อมิลลิลิตร เมื่อศึกษาทางจลศาสตร์พบว่าเพปไทด์มีค่า K_i เท่ากับ 1.25×10^{-6} มิลลิกรัมโปรตีนต่อมิลลิลิตรและเพปไทด์ของสารยับยั้งแอนจิโอเทนซินI-คอนเวอร์ติงเอนไซม์ มีการยับยั้งแบบแข่งขัน โดยลำดับกรดอะมิโนของเพปไทด์ไพลเหลืองที่พบคือ โปรลีน-อะลานีน-กลูตามิก-ไกลซีน-ฮิสติดีน-เซอรีน ซึ่งมีความคล้ายคลึงกับลำดับกรดอะมิโนของโปรตีนไมโทคอนเดรียจากมันฝรั่ง

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต..
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MANEERAT YODJUN: ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY ACTIVITY OF PURIFIED PROTEINS AND PEPTIDES FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., CO-ADVISOR: APHICHART KARNCHANATAT, Ph.D., 59 pp.

Angiotensin I- converting enzyme (ACE) plays a major role in the regulation of blood pressure by virtue of two different reactions that it catalyzes: conversion of the inactive decapeptide angiotensin I to a powerful vasoconstrictor and salt-retaining octapeptide angiotensin II, and inactivation of the vasodilator and natriuretic nonapeptide, bradykinin. Blood pressure-lowering activities of pure protein and pepsin hydrolysates of protein of Zingiberaceae plants were assayed in vitro by inhibition of the angiotensin I-converting enzyme. The crude protein of 15 plants in Zingiberaceae family were screened for their ACE inhibitory by in vitro ACE inhibitory activity protein was isolated from Zingiberaceae plants. Crude protein of *Z. ottensi. rhizome* was high activity, having IC_{50} of 7.30×10^{-7} mg protein/ml. Rhizomes proteins were isolated and then purified by stepwise eluted SP sepharose chromatography. Five unbound fractions obtained from purification step were called F25, F50, F75, and F100. The highest protein content was found in the F75 fraction. Results form native and reducing SDS-PAGE indicated that the F75 was single protein gave an estimated size of about 20.7 kDa. The tryptic fragments of the ACEI were sequenced using LC-MS/MS analysis, it resulted suggested that its amino acid sequence is similar to chitinase. The effect ACEI activity of F75 was largely stable at temperature between -20 and 60 °C (at a 30 min exposure). The pH inhibition effect of fraction F75 was negated at pH 6 and steeply reduced at pH 8 – 12. This protein exhibited a strong ACE inhibitory activity which K_i was of 9.1×10^{-5} mg protein/ml. For the analysis of peptides from *Z. cassumunar* which derived from pepsin hydrolysates at 37°C for 30 min possessed ACE inhibition at IC_{50} of 0.38±0.012 mg/ml, after fractionation by RP-HPLC was ascribed to a single peptide with IC_{50} for ACE inhibitory at 0.011±0.012 mg/ml. The peptide was a potent competitive inhibitor of ACE with a K_i of 1.25×10^{-6} mg protein/ml. The sequence of the peptide from *Z. cassumunar* was found to be Pro-Ala-Glu-Gly-His-Ser, which is similar to the mitochondrial protein sequence from *Solanum tuberosum* L.

Field of Study : Biotechnology   Student's Signature ....

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LIST OF ABBREVIATIONS

%	percentage
^{O}C	degree celsion
µg	microgram
µl	microliter
A	Absorbance
BSA	Bovine serum albumin
cm	centimeter
Da	Dalton
EDTA	Ethyllenediamine tetraacetic acid
g	gram
hr	hour
kDa	kilodalton
l	liter
LC-MS-MS	Liquid Chromatography-Mass Spectrometry-Mass Spectrometry
M	Molar
mA	Miliampere
mg	Miligram
min	minute
ml	milliliter
mM	milimolar
MW	Molecular weight
NaCl	Sodium Chloride
PAGE	Polyacrylamind gel electrophoresis
rpm	revolution per minute

RT	Room temperture
SDS	Sodium dodecyl sulfate
TEMED	N,N,N’,N’-tetramethyl ethylenediamine
Tris	Tris(hydroxymethyl)amiomethane
U	Unit activity
V	Volt
V/V	Volumn by volumn
W/V	Weight/volumn

CHAPTER l

INTRODUCTION

Hypertension is a chronic medical condition in which the blood pressure that damages health one of the most common worldwide disease. There are many risk factors for stroke, heart disease, chronic renal failure or aneurysm disease (Guyton *et al.*, 2006). There are many factors such as sedentary lifestyle, stress, visceral obesity of hypertension, which no longer relegated to the aged and elderly (Egan et al., 2004). The angiotensin I-converting enzyme (ACE, EC.3.4.15.1) play a key physiological role in the control of blood pressure, in the Renin-Angiotensin System (RAS) (Ganten D. et al., 1984), which mediates extracellular volume (i.e. that of the blood plasma, lymph and interstilial fluid) and arterial vasoconstriction. ACE catalyes the conversion of decapeptide angiotensin I to the potent vasoconstrictor angiotensin II and degrades bradykinin, leading to systematic dialation of the arteries and decrease in arterial blood pressure (Kostis *et al.*, 1987). Some of these angiotensin I-converting enzyme inhibitory peptides result in the decreased formation of angiotensin II and decrease blood pressure. For this reason, many studies have been directed toward the attempted synthesis of ACE inhibitors, such as captoprill or alacepril, which are currently used in the treatment of hypertensive patients, they are entirely without side-effects. (Brown *et al.*, 1998) So the trend has been toward developing natural ACE inhibitors for the treatment of hypertension.

ACE inhibitory protein and peptide from frequently consumed foods are attracting considerable interest because they are more natural and safer when compared with ACE inhibitory drugs. ACE inhibitory proteins and peptide have been

isolated from various animal and plant food sources. Recently, the plant food sources, soy bean and related products have been the most widely stuied. Sunflower (*Helianthus annuus* L.) (Megias C. et al., 2004) and potato (*Solanum tuberosum*) (Pihlanto A. et al., 2008). The values of sunflower and potato could be increased if processed to contain ACE inhibitory protein and peptide.

Zingiberaceae is a family of flowering plants consisting of aromatic perennial herbs with creeping horizontal or tuberous rhizomes (Larsen, 1980). It has more than 1300 species, has a antropical distribution found in the tropics of Africa, Asia and the Americas, with its greatest diversity in Southeast Asia. Many species are important medical plants, spices or ornametal plants. Although new bioactive peptides are discovered and characterized year-by-year, novel bioactive peptides (in particular from herbal origins) are still needed because of their attractive identities, such as in oral administration of “natural” medicines. The objective of this study is to investigate the angiotensin I-converting enzyme inhibitory activity of peptides from Zingiberaceae rhizomes.

CHAPTER ll
LITERATURE REVIEWS

2.1 Hypertension

Hypertension is now a major problem threatening people health in the world. It is a risk factor for developing cardiovascular diseases (arteriosclerosis, stroke and myocardial infraction) and end-stage renal disease, and is often called a "silent killer" because persons with hypertension are often asymptomatic for years (Je et al., 2005). Hypertension (defined as a blood pressure ≥140/90 mmHg) is an extremely common comorbid condition in diabetes, affecting ~20–60% of patients with diabetes, depending on obesity, ethnicity, and age. In type 2 diabetes, hypertension is often present as part of the metabolic syndrome of insulin resistance also including central obesity and dyslipidemia. In type 1 diabetes, hypertension may reflect the onset of diabetic nephropathy. Hypertension substantially increases the risk of both macrovascular and microvascular complications, including stroke, coronary artery disease, and peripheral vascular disease, retinopathy, nephropathy, and possibly neuropathy. In recent years, adequate data from well-designed randomized clinical trials have demonstrated the effectiveness of aggressive treatment of hypertension in reducing both types of diabetes complications (*Arauz-Pacheco C et al., 2002*). Terapy versus placebo in reducing outcomes including cardiovascular events and microvascular complications of retinopathy and progression of nephropathy. These studies used different drug classes, including angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), diuretics, and β-blockers, as the initial step in therapy. All of these agents were superior to placebo; however, it must be noted that many patients required three or more drugs to achieve the specified target levels of blood pressure control. Overall there is strong evidence that

pharmacologic therapy of hypertension in patients with diabetes is effective in producing substantial decreases in cardiovascular and microvascular diseases *(Bakris GL et al., 2000).* Since the past two decades, the renin-angiotensin system (RAS) has been found to be a coordinated peptide hormonal cascade for the control of cardiovascular, renal and adrenal functions governing fluid and electrolyte balance and arterial blood pressure (Carey and Siragy, 2003).

2.2 The renin-angiotensin-aldosterone system (RAAS)

Renin is an acid proteinase containing ~350 amino acids. It is generated from the inactive precursor prorenin, by the action of kallikrein (EC 3.4.21.34) (Ondetti, M. A. and Cushman, D. W. ,1982). The main source of renin is the juxtaglomerular cells of the kidney; however, renin has also been isolated from the submaxillary gland and from amniotic fluid. Several factors influence the release of renin, including renal perfusion pressure, salt depletion, and stimulation of ß2-receptors by aldosterone (Deszi, L., 2000). Renin is responsible for liberation of angiotensin I from ATN (Inagami, T., 1992). Inhibition of renin activity may be achieved as a result of angiotensin (Ang) II production and numerous pharmacological agents. The concentration of ATN in plasma is generally never high enough to saturate renin; therefore changes in the concentration of ATN may influence the rate of Ang II production (Inagami, T. , 1992). The RAS is 1 of the major regulators of BP, electrolyte balance, renal, neuronal, and endocrine functions associated with cardiovascular control in the body. RASs specific to the brain (Philips, M. I., 1987), placenta (Poisner, A. M. , 1998), bone marrow (Haznedaroglu, I. C. and Öztürk, M. A., 2003), and pancreas (Leung, P. S., 2003) have been identified. As can be seen in (Figure 1), RAS begins with the inactive precursor angiotensinogen (ATN). ATN is a glycopeptide with a molecular weight of ~60 kDa (Inagami, T., 1992). ATN is

distributed in numerous tissues in addition to plasma and cerebrospinal fluid. ATN is the only known precursor of angiotensin I as well as the only known substrate for renin (EC 3.4.23.15)

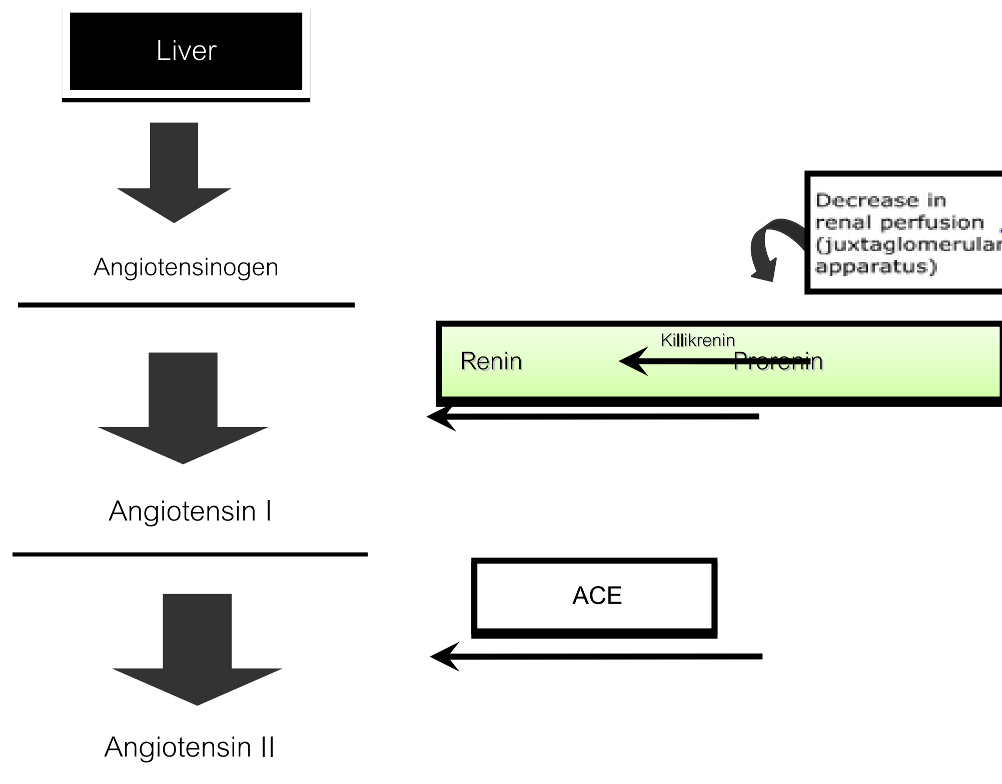


Figure 1. The renin-angiotensin system.

2.3 Characteristics of ACE

ACE was originally isolated in 1956 as a “hypertension-converting enzyme” (Skeggs et al., 1956). It plays an important role in the resin-angiotensin system (RAS) which regulates blood pressure and fluid homeostasis in human. Angiotensin-I-converting enzyme (ACE; EC 3.4.15.1) is a dipeptidyl carboxypeptidase that elevates blood pressure by producing the vasoconstrictor angiotensin II and degrading the vasodilator bradykinin (Campbell, 1987). ACE plays an important role in the regulation of blood pressure as well as fluid and salt balance in mammals. It is a

dipeptidylcarboxypeptidase which converts the inactive ecapeptide, angiotensin I, into a potent vasoconstrictor, the octapeptide angiotensin II. Moreover, ACE inactivates bradykinin, a vasodilatory peptide. Hence, ACE raises blood pressure (Neutel, J. M. et al., 1999). The main effector molecule of the RAS, angiotensin II, is produced through an enzymatic cascade consisting of renin, an aspartic protease that first cleaves angiotensinogen to form the decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu,, and ACE that then further cleaves angiotensin I into the octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe | His-Leu) by removing the C-terminal dipeptide His-Leu (Lavoie and Sigmund, 2003). The resulting angiotensin II is a potent vasoconstrictor, stimulates the release of aldosterone and antidiuretic hormone or vasopressin, and increases the retention of sodium and water and the regeneration of rennin. These effects directly act in concert to raise blood pressure. A nonapeptide derivative of angiotensin I, des-Asp1-angiotensin I which prevents infarction- and non-infarction-related cardiac injuries and disorders, can be cleaved the dipeptide His-Leu by ACE to produce Angiotensin III (Asp | Arg-Val-Tyr-Ile-His-Pro-Phe) (Murray and FitzGerald, 2007) which has 40% of the vasoconstriction activity of Angiotensin II (Figure 2). In addition, ACE also termed kininase II, inactivates the vasodilators bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, 1-9) and kallidin (Lys-bradykinin) in kallikreinkinin system by cleaving the C-terminal dipeptide Phe-Arg. ACE eventually cleaves further its primary metabolite bradykinin (1-7) into the shorter fragment bradykinin (1-5) (Sivieri *et al*., 2007) (Figure 2).

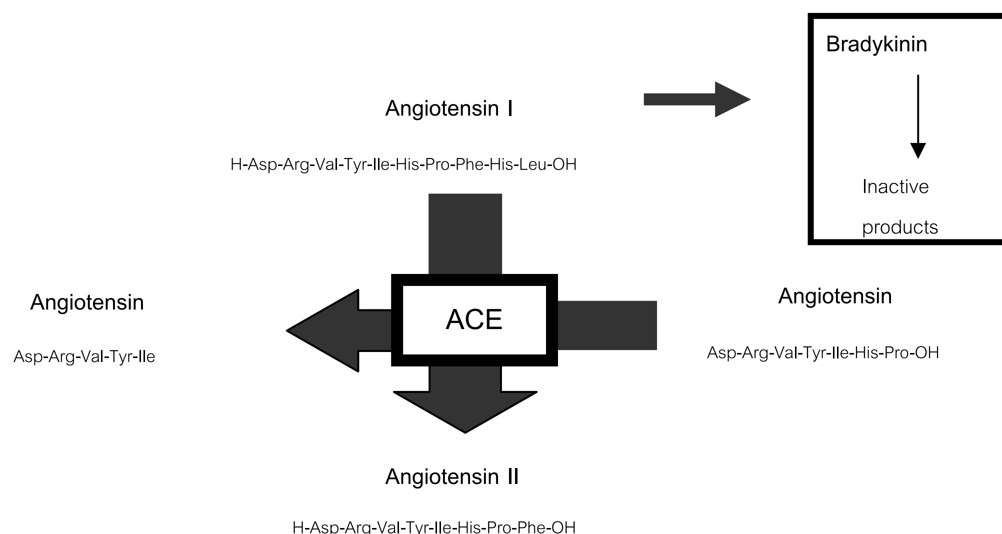


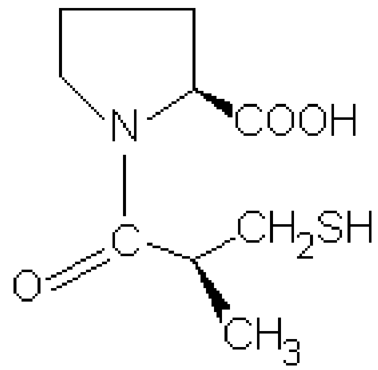
Figure 2. Angiotensin Converting Enzyme(ACE)

There are a number of methods used to quantify ACE activity. These include using hippuryl-L-histidyl-L-leucine, which can be quantified spectrophotometrically (Cushman, D. W. and Cheung, H. S., 1971) or by reversed-phase high performance chromatography (Mehanna, A. S. and Dowling, M., 1999). Additionally, ACE activity may be quantified using 2-furanacryloyl-L-phenylalanyl-L-glycyl-L-glycine (Holmquist, B., Bünning, P. and Riordan, J. F., 1979). Fluorometric analysis of ACE activity is also possible using the fluorophore-labeled tripeptide dansyltriglycine (Elbl, G. and Wagner, H., 1994). The potency of an ACE inhibitor is usually expressed as an IC_{50} (concentration of material mediating 50% inhibition of ACE activity) value, which is equivalent to the concentration of inhibitor mediating 50% inhibition of activity.

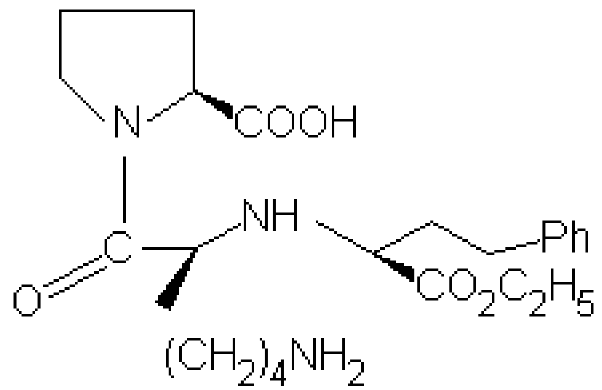
2.4 ACE inhibitors

Angiotensin-converting enzyme (ACE) inhibitors are some of the most commonly prescribed medications for hypertension. Indeed, they were cited in a recent survey of primary care supervisors in Australia (Heran BS et al., 2008) as the treatment most often recommended by guidelines and favored over other antihypertensive drugs as first-line agents. This enthusiasm for ACE inhibitors is somewhat inconsistent with current recommendations, (Charles P et al., 2009) which prefer thiazide diuretics as first-line medication for uncomplicated cases of hypertension. ACE inhibitors are seen as more appropriate for first-line use when other high-risk conditions are present, such as diabetes. Still, given clinicians' favorable experience with ACE inhibitors and the increasing prevalence of type 2 diabetes in the population, it is clear that ACE inhibitors will maintain an important role in the treatment of hypertension. The first such compound, captopril or D-3-mercapto-2-methylpropanoyl-L-proline, is an analog of Ala-Pro sequence, with sulfhydryl as a strong chelating group of zinc ion. Its adverse effects, that were the same as caused by mercapto-containging penicillamine, prompted the design of non-sulfhydryl ACE inhibitors (Patchett *et al*., 1980). ACE inhibitors can be classified as Sulfhydryl containing ACE inhibitors structurally related to captopril (eg, Fentiapril, Pivalopril, zefenopril, alacepril) (figure 3.); Dicarboxyl-containing ACE inhibitors structurally related to enalapril (eg, lisinopril, benazepril, quinapril, moexipril, ramipril, spirapril, perindopril, spirapril, pentopril, cilazapril); phosphorous containing ACE inhibitors structurally related to fosinopril (Lawrie, R, 1991). .

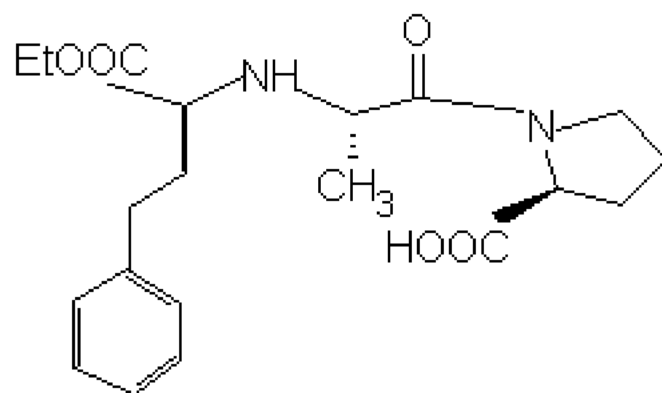
Captopril



Lisinopril



Enalapril



Fusinopril

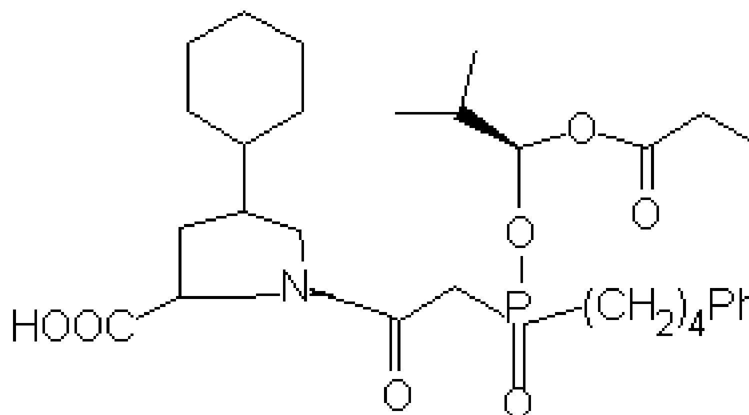


Figure 3. Chemical structure of captopril, lisinopril, enalapril, and fosinopril.

Several adverse side effects such as hypotension, increased potassium levels, reduced renal function, cough, angioedema, skin rashes, and fetal abnormalities have been associated with synthetic ACE inhibitory drugs (Ames, R. P., 1983). A common side effect of ACE inhibitors is a dry cough appearing in 5-20% of patients and may result in the discontinuation of treatment. Another serious problem is angioedema which affects 0.1-0.5% of patients and can be life-threatening. The two side effects have generally been attributed to the altered concentrations of bradykinin (Acharya *et al.*, 2003). Natural inhibitors of ACE have been identified within the primary sequences of a range of food proteins (Ariyoshi, Y., 1993).

2.5 Plant food sources derived inhibitors of ACE

ACE inhibitory peptides can be produced during the manufacture of a range of dairy products. (Meisel et al., 1997) demonstrated that secondary proteolysis during cheese ripening leads to the production of ACE inhibitory peptides. In a previous study, high ACE inhibitory activity in a tryptic digest of pea protein isolate was found,

suggesting that the pea may be an alternative source of ACE inhibitory peptides (Vermeirssen et al., 2002). The ACE inhibitor from buckwheat (Fagopyrum esculentum Moench) was identified to be a tripeptide, Gly-Pro-Pro, having IC50 value of 6.25 lg protein/ml. It is seen that, of the individual caseins, f(25–27) from αs1-casein is a potent in vitro inhibitor of ACE having an IC50 of 2.0 µmol/L and that f(208–216) of bovine serum albumin has an IC50 of 3.0 µM/L. While the structure activity relationship for food-derived ACE inhibitors has not been established, it appears that binding to ACE is strongly influenced by the C-terminal tripeptide sequence. Many substrates and competitive inhibitors of ACE contain hydrophobic amino acids in this region. A number of potent food protein derived ACE inhibitors contain proline at the C-terminus. Furthermore, several ACE inhibitors contain lysine or arginine as the C-terminal residue. It has been postulated that the positive charge associated with the side-chain groups of these amino acids contributes to ACE inhibitory potency (Meisel, H., 1993). The naturally occurring peptides with ACE inhibitory activity were first obtained from snake venom (Ondetti et al., 1971). These ACE inhibitors contained 5–13 amino acid residues per molecule, and most of them had a C-terminal sequence of Ala-Pro or Pro-Pro. (Oshima, Shimabukuro, & Nagasawa., 1979) reported ACE inhibitory peptides produced from food proteins by digestive protease. ACE inhibitory peptides can be produced by solvent extraction, enzyme hydrolysis, and microbial fermentation of food proteins. For pea protein, the highest ACE activity is reached early in the simulated stomach phase using pepsin treatment and the level is maintained during the simulated small intestine phase using trypsin-chymotrypsin treatment (Vermeirssen et al., 2004). In other several studies, the plant protein hydyolysates generated during pepsin digestion had greater ACE inhibitory activities than those after subsequent digestion with pancreatin, which

suggests that pepsin-produced inhibitory peptides are subsequently hydrolyzed during pancreatic hydrolysis (Yang *et al*., 2003; Megías *et al*., 2004; Yang *et al*., 2004).

Table 1. Potent ACE inhibitory peptides derived from plant foods.

Source[a]	Parent protein	Enzyme	Amino acid sequence	IC_{50}(μM)	Reference
Soybean	Whole protein	Alcalase	DLP	4.8	Wu and Ding (2001)
			DG	12.3	
		Pepsin	YLAGNQ	14	Chan *et al.* (2003)
			FFL	37	
			VMDKPQR	39	
			IYLL	42	
		Fermentation	HHL	2.2	Shin *et al.* (2001)
			WL	29.9	Kuba *et al.* (2003)
			IFL	44.8	
	Protein isolate	Protease D3	NEGPLV	21	Kodera and Nio *et al.* (2006)
			PNNKPFQ	33	
			YVVFK	44	
	Glycine	Protease P	VPIVP	1.69	Gouda *et al.* (2006)
Mung bean	Protein isolate	Alcalase	KDYRL	26.5	Li *et al.* (2006)
			KLPAGTLF	13.4	
Sunflower	Protein isolate	Pepsin-pancreatin	FVNPQAGS	6.9	Megias et al. (2006)
Rice	Protein isolate	Alcalase	TQVY	18.2	Li *et al.* (2007)
Corn	Gluten	Alcalase	AY	14.2	Yang *et al.* (2006)
Broccoli	Water extract	No enzyme	YPK	10.5	Lee *et al.* (2006)
Mushroom	Water extract	No enzyme	GEP	40	Lee *et al.* (2004)
Garlic	Water extract	No enzyme	FY	3.74	Suetsuna *et al.* (1998)
			NY	32.6	
			NF	46.3	

Table 1. (Continued)

Source[a]	Parent protein	Enzyme	Amino acid sequence	IC_{50}(µM)	Reference
Buckwheat	Whole protein	Pepsin-chymotrysin	VK	13	Li *et al.* (2003)
			FY	25	
			YQY	4	
			PSY	16	
			LGI	29	
			ITF	49	
			INSQ	36	
	Water extract	No enzyme	GPP	6.25	Ma *et al.* (2006)
Whet	Germ protein	Alcalase	TF	17.8	Matsui et al. (1999)
			LY	6.4	
			YL	16.4	
			AF	15.2	
			IY	2.1	
			VF	9.2	
			IVY	0.48	
			VFPS	0.46	
			TAPY	13.6	
			TVPY	2	
			TVVPG	2.2	
			DIGYY	3.4	
			DYVGN	0.72	
			TYLGS	0.86	
			GGVIPN	0.74	
			APGAGVY	1.7	

[a] the content in the blank position is the same as that in the last row of the same column; b IC50 values quoted are expressed as µg/ml; cIC50 values quoted are expressed as µg; d the *in vivo* assay has been conducted.

2.6 characterization of ACE inhibitory peptides

ACE inhibitory peptides are generally short sequences, which is in agreement with the results of (Natesh et al., 2003) who showed that the active site of ACE cannot accommodate large peptide molecules. The C-terminal tripeptide strongly influences the binding of substrate or inhibitor to ACE. ACE appears to have a preference to a substrate or a competitive inhibitor containing hydrophobic (aromatic or branched

side-chains) amino acids in the C-terminal tripeptide. C-terminal lysine, leucine, isoleucine, valine may also contribute significantly to increasing ACE inhibitory activity of peptides (Murray and FitzGerald, 2007). It is suggested that arginine and phenylalanine residues in RSFCA are essential for a specific interaction with ACE and ACE inhibition (Kumada *et al*., 2007). It is well established that in vitro incubation of milk proteins with gastrointestinal proteinase preparations enriched in pepsin, trypsin, and chymotrypsin activities results in the release of ACE inhibitory peptides. ACE inhibitors have been developed to prevent angiotensin II production in cardiovascular diseases and utilized in clinical applications since the discovery of ACE inhibitor in snake venom (Ferreira et al., 1970). In this study, the peptides were produced by hydrolysis of cottonseed protein with different protease, including alcalase, flavourzyme, trypsin, neutrase, papain and pepsin. The most potent ACE inhibitory activity hydroly- sate was obtained by papain hydrolysis of CPH. Further, the papain-hydroylsate was separated with ultrafiltration into 4 parts (UF-I, UF-II, UF-III, UF-IV) (Dandan Gao et al,. 2010). ACE appears to require the L-configuration of amino acids at position three from the C-terminal (Murray and FitzGerald, 2007). That peptide conformation, i.e. the structure adopted in a specific environment, is also expected to contribute to ACE inhibitory potency. The *C*-terminal tripeptide residues may interact with subsites at the active site of ACE (Ondetti and Cushman, 1982). ACE prefers to have substrates or competitive inhibitors that contain hydrophobic amino acid residues such as proline, phenylalanine, and tyrosine at three positions from the *C*-terminal (Cheung *et al*., 1980). Most of the naturally occurring peptide inhibitors contain proline at their *C*-terminal.

2.7 *In vitro* activity of plant food – derived ACE inhibitory peptides.

The ACE inhibitory activity was assayed by modification of the method of (Cushman and Cheung., 1971). In all cases, the enzyme is presented with peptide substrate, the hydrolysis of which is measured by detecting the formation of products. The Angiotensin I converting enzyme, Hippuryl-Histidyl-Leucine (Hip-His-Leu), was used as a substrate. Captopril was purchased from the Sigma Chemical Co. (St. Louis, USA). The release of two products hippuric acid (HA) and L-His-L-Leu (HL) from the substrate hippuryl-Lhistydyl-L-Leucine (Hip-His-Leu, HHL) hydrolysis by ACE is directly related to ACE activity. Instead of measuring the absorbance of extracted HA at 228 nm as in the original method, an aliquot of product mixture may be directly injected to HPLC system 38 to quantify the release of HA (Wu and Ding, 2002). The inhibition mode of ACE-catalyzed hydrolysis of HHL is determined by Lineweaver-Burk plots. Competitive ACE inhibitory peptides are most frequently reported and have been identified from mushroom extracts (Choi *et al*., 2001), chickpea (Pedroche *et al*., 2002) and soy (Wu and Ding, 2002) protein hydrolysates.

2.8 Purification and sequence of plant food – derived ACE inhibitory peptides

ACE inhibitory peptides can be separated from a hydrolysate mixture by various kinds of membrane-based separation and chromatography techniques. The peptide Gly-Pro-Leu was purified from Alaskan pollack skin gelatin hydrolysates according to a previously reported method from our laboratory (Byun and Kim, 2001). In 2001 Noh and Song previously reported that membrane filtration with a 1000 Da molecular weight cut-off could be a useful processing method for the purification of ACE inhibitors, because most potent ACE inhibitory substances have molecular

weights below or around 1000 Da (Ariyosh., 1993). Buckwheat protein extract was filtered using an YM-10 membrane. An ACE inhibitor was purified using consecutive chromatographic methods including: ion-exchange chromatography, gel filtration chromatography, and reverse-phase high performance liquid chromatography (Min-Suk Ma et al., 2006). Enzymatic hydrolysate of fermented soybean products can be treated using a mixture of water, acetonitrile and trifluoroacetic acid to extract active peptides (Gibbs *et al*., 2004). Other chromatography techniques include ion-exchange chromatography (IEC), capillary electrophoresis (CE), capillary isoelectric focusing (CIEF), and size-exclusion chromatography (SEC). IEC, CE and CIEF separate peptides based on their charge properties, while SEC is a separation method based on molecular size. SEC is also named gel filtration chromatography when operated in an aqueous mobile phase or gel permeation chromatography when performed in organic mobile phases (Wang and Gonzalez de Mejia, 2005; Shahid and Zhong, 2008).

Analytical reversed-phase high performance liquid chromatography (RP-HPLC) was carried out using a Waters HPLC system. Frequently, reversed phase columns are packed with a chemically bonded octadecylsilyl coated silica; such columns are referred to as C-18 and are very non-polar. Other popular bonded columns have dodecylsilyl, octasilyl, or phenylsilyl packings. Gradient elution is usually practiced with gradually increased organic solvent (acetonitrile, methanol, propanol) concentration. The result is that the more polar components of peptide mixture elute first. Trifouroacetic acid (TFA), is often added to the eluting solvents to improve the chromatographic peak shape. The ACE inhibitory peptide of douchi qu pure-cultured were fractionated into four major peaks by gel filtration chromatography on Sephadex G-25 (Jian-Hua Zhang et al., 2006). After elution with 0.1% TFA at a flow rate of 1 ml/min for 3 min, 20 ll of samples, which had filtered

through 0.45 lm filters, were injected into the HPLC. A linear gradient of 0– 60% acetonitrile in 0.1% TFA was applied over 60 min at the same flow rate. The elution was monitored at 220 nm with a UV detector (Suetsuna,1998; Yust et al., 2003).

For unknown peptides, mass spectrometry methods are adopted to determine molecular mass and amino acid sequence. Electrospray ionization (ESI) and matrixassisted laser desorption/ionization (MALDI) are two main techniques for measuring molecular mass. ACE inhibitory dipeptide was isolated, and its molecular mass and amino acid sequence were determined as 238.2 Da and Gly-Tyr, respectively, by LC-ESI/MS. The results of this study suggest that silk fibroin byproducts have the possibility to become an effective source for ACE inhibitory peptides (F. Zhou et al., 2010). The amino acid composition of the purified peptide was determined after hydrolysis in vacuo with 5.8 M HCl at 110 °C for 24 h. The sequence of the peptide was found to be NH2-Val-Leu-Ileu-Val-Pro-COOH and corresponded to Val397-Pro401 of the glycinin subunit G2 (Swiss Prot: P04405) of soybean (*G. max*). MALDI-TOF of the peptide indicated the presence of a single peptide of *m*/*z* 578.9. The amino terminal sequence of the peptide was determined by Edman degradation on an automated gas-phase sequencer (K.G. Mallikarjungouda., 2006).

2.9 Bioactivity Protein and peptide

The term bioactivity refers to food components that can affect biological processes or substances. They therefore have an impact on body function or condition and, ultimately, general health. Extensive research is underway to investigate the potential of bioactive components in food for improving health. It is now common practice to hydrolyse protein for specific applications, such as hypoallergenic

products, infant formula and clinical nutrition (Clemente A., 2000). Recently, research studies have shown that the most complete protein source is whey protein. Whey contains all the essential amino acids in the proper ratios and is considered a complete protein. As a matter of fact, whey protein is superior to and has a higher biological value than eggs, milk, meat or fish. It is also superior to vegetable-based proteins such as soy, potato, rice, wheat, and beans. The biological value is a measurement of how well the body can use the protein for growth and maintenance (Bucci L, 2002). Proteins may also be hydrolysed to produce biologically active peptides, which can be added to foods as part of a complete hydrolysate or as partly purified peptides. The biological activities associated with whey peptides include cholesterol-reducing activity, antibacterial activity (Recio I and Visser S., 1999), antithrombotic activity, opioid-like activity , antioxidant activity and antihypertensive activity . The term “bioactive protein (or peptide)” can also mean intact molecule or fragments enzymatically digested from whole protein molecule (Wang and Mejia, 2005). Casein (a major protein component in milk) digested fragments had many biological activities such as antimutagenic, antibacterial, immunomodulator, and enhance mineral uptake activities (Pihlanto and Korhonen, 2000) while digested soy protein fragments exhibit many activities in the same manner such as protease, trypsin inhibitor, anticancer, hypotensive, ACE inhibitor activities (Wang and Mejia, 2005), blood cholesterol and total lipid reduction (Pihlanto and Korhonen, 2003). It is well established that the inhibition of ACE by peptides results in a decrease in blood pressure (Clare DA, Swaisgood HE., 2000). Numerous potential ACE inhibitory peptides deduced from in vitro activity measurements have been reported (Kim SK et al., 2001).

2.10 The herb: Zingeberaceae family

Zingiberaceae, or the Ginger family, is a family of flowering plants consisting of aromatic perennial herbs with creeping horizontal or tuberous rhizomes, comprising ca. 52 genera and more than 1300 speciesIt is important natural resources that provide many useful products for food, spices, medicines, dyes, perfume and aesthetics to man.

Zingiberaceae is divided into 4 tribes (Sirirugsa P. , 1999)

- HEDYCHIEAE
- ZINGIBEREAE
- ALPINEAE
- GLOBBEAE

Zingiberaceae of Thailand

		Total no. of sp.	**No.of sp. in Thailand**
HEDYCHIEAE	Boesenbergia	45	14
	Caulokaempferia	10	5
	Cautleya	5	1
	Curcuma	80	50
	Curcumorpha	1	1
	Haniffia	2	2
	Hedychium	60	20
	Kaempferia	50	15
	Scaphochlamys	25	2
	Stahlianthus	7	1
ZINGIBEREAE	Zingiber	90	35
ALPINIEAE	Alpinia	250	20
	Amomum	125	20
	Elettariopsis	30	3
	Etlingera	60	4-5
	Geostachys	16	3
	Hornstedtia	35	1-2
	Pomereschia	2	1
GLOBBEAE	Gagnepainia	3	3
	Globba	70	40

The Thai Zingiberaceae is to complete the medicinal revision of the family and to be published in the rhizome of Thailand. Most medical applications are reported to be achieved by using its rhizome, but the properties of the plant reported

in different documents were appeared to depend on local knowledge. Asides from its medicinal properties, this Southeast Asia native ginger can also be used for ornamental purposes and is even used as a spice and fresh food (Ravindran P. and Babu K., 2005). The objective of this study was to investigate a new protein with α-glucosidase inhibitory activity from the rhizomes of Zingiber ottensii The objective of this study was to investigate a new protein with α-glucosidase inhibitory activity from the rhizomes of Zingiber ottensii. The amino acid sequence of an internal fragment of this purified Z. ottensii rhizomal protein had a similarity to the sequence from the plant cysteine proteinase family (Tiengburanatam N., 2009). In addition, it is linked to poultice in postnatal treatment and as an appetizer (Ravindran P. and Babu K., 2005). In 2008 the composition of the essential oil of *Zingiber cassumunar* Roxb. from Bangladesh was examined by gas chromatography mass spectroscopy (GC-MS). Sixty-four components were identified in leaf oil and 32 components were identified in the rhizome oil, accounting for 94.60% and 98.56% of the total yields, respectively (Md. Nazrul Islam Bhuiyan., 2008).

CHAPTER III

EXPERIMENTAL

3.1 Materials and Chemicals

The fresh rhizomes of Zingiberaceae plants were periodically purchased (October 2008-June 2009) from Chatuchak park market in Bangkok, Thailand.

Acetic acid (Merck Ag Darmstadt, Germany)

Acetonitrile

Acetylthiocholine iodide (Fluka, Germany)

Acetylcholinesterase from electric eel (Sigma,U.S.A)

Acrylamind (Plusone Pharmacia Biotech, Sweden)

Angiotensin Converting Enzyme from rabbit lung (Sigma,U.S.A)

Bis-acrylamide (Promega, USA)

Bovine serum albumin (Sigma, USA)

Bromophenol Blue (USB, USA)

Captopril (Fluka, China)

Coomassie Brilliant Blue G-250 (USB, USA)

Di- Potassiumhydrogen phosphate (Merck Ag Darmstadt, Germany)

5,5'-Dithio-bio(2-nitrobenzoic acid) (Sigma,U.S.A)

Eserine (Sigma,U.S.A)

Ethylenediaminetetraacetic acid, EDTA (Sigma, USA)

Ethanol (Merck Ag Darmstadt, Germany)

Ethyl acetate (Ajax Finechem, New Zealand)

Hippuric acid 98% (Sigma,U.S.A)

Hippuryl-Histidyl-Leucine acetate salt (Sigma,U.S.A)

Hydrochloric acid (J.T. Baker, USA)

Pepsin from porcine gastric mucosa power (Sigma, Germany)

Potassium dihydrogen phosphate (Merck Ag Darmstadt, Germany)

Sodium azide (Merck Ag Darmstadt, Germany)

Sodium chloride (Merck Ag Darmstadt, Germany)

Sodium hydroxide (Merck Ag Darmstadt, Germany)

Standard Molecular Weight Marker (Sigma, U.S.A)

Tetramethylethylenediamine, TEMED (Plusone Pharmacia Biotech, Sweden)
Tris (USB, U.S.A)
Trifluoroacetic acid (Fluka, Germany)

3.2 Equipment

Autopipette (Pipetman, Gilson, France)
Dialysis bag (Snake Skin Dialysis Tubing, Pierce, U.S.A)
Electrophoresis unit (Hoefer mini VE, Amersham Pharmacia Biotech, Sweden)
Freeze dryer (Labconco, U.S.A)
High Speed Refrigerated Centrifuge (Kubota 6500, Japan)formance Liquid
High Performance Liquid Chromatography (Spectra system/spectra series, Fortune Scientific)
Hot plate stirrer (HL instrument, Thailand)
Laminar Flow (Safety Lab, Asian Chemical and Engineering Co., Ltd., Thailand)
LC/MS/MS mass spectrometry
Microcentrifuge (Tomy MTX-150)
Orbital Shaker (OS-10 Biosan, Latvia)
pH meter (Mettler Toledo, U.S.A)
Pipette tips (Bioline, U.S.A)
Spectrophotometer (Synergy HT Biotek, USA)
Speed vacuum centrifuge (Heto-Holten, Denmark)
Ultrasonic (leaner D200, D.S.C.,)
Vortex mixer (Vortex-Genie2, Scientific Industries, U.S.A)
Water Bath (NTT-1200 Tokyo kikakikai, Japan)
384-well microtter plate (greiner, USA)

3.3 Chemical and biological materials

Fresh rhizomes of Zingiberaceae plants were purchased from Chatuchak park market in Bangkok, Thailand. Angiotensin Converting Enzyme (E.C. 3.4.15.1; ACE) from rabbit lung, Hippuric acid, Hippuryl-L-Histidyl-L-Leucine (HHL), and Pepsin (E.C. 3.4.23.1) from porcine gastric mucosa and were purchased from Sigma Chemicals Co. (USA). All other biochemicals and chemicals used in the investigation were of analytical grade.

3.4 Preparation of the Zingiberaceae rhizomes extract

Rhizomes of Zingiberaceae plants (1.5 kg wet weight) were peeled, cut into small pieces and then homogenized in 5 L of PBS (0.15 M NaCl/20 mM Phosphate buffer, pH 7.2) using a blender and then left with stirring overnight at 4°C by an agitator. The suspension was then clarified by filtration through a double-layered cheesecloth followed by centrifugation at 15,000×g for 30 min. The clarified supernatant was then harvested and ammonium sulfate added, with stirring, to 80% saturation and then left with stirring overnight at 4°C. The precipitate was collected from the suspension by centrifugation at 15,000×g for 30 min with discarding of the supernatant. The pelleted material was then dissolved in PBS, dialyzed against excess water and then freeze dried.

3.5 Purification of Protein from the Rhizomes of *Z. ottensii*

The protein solution of *Z. ottensi.*. was applied to SP sepharose (Amersham Pharmacia Biotech, UK) cation exchange column from the method described by Tipthara (2007). The crude protein solution was re-dissolved in 10 ml of deionized water and each of about 5 ml was loaded into a 5 ml loop of automatic liquid chromatography system (AKTA prime, Amersham bioscience, Sweden) connected to 15 cm length SP-Sepharose fast flow column (Amersham Biosciences, 17-0729-10, Sweden) and microcomputer. After equilibration with buffer A (20mM Tris-HCl pH 7.2). The bound fraction was eluted with a stepwise gradient formed by the addition of 25, 50, 75, and 100% buffer B (20 mM Tris-HCl pH 7.2 with 1 M NaCl); flow rate = 2 ml/min; 10 ml per fraction collected; proteinataeous peaks were monitored at 280nm. Individal fractions were dried using speed vacuum and their assay of ACE inhibitory activity.

3.6 Hydrolysis of protein from Zingiberaceae rhizomes by pepsin

The proteins from Zingiberaceae rhizomes were dissovlved in PBS (1 mg/ml) and digest by pepsin (500 U/ml in 0.1 M HCl) 500 µl. Incubation various time for 0, 30, 60, 90, 120, and 180 min at 37°C, pepsin digestion was adjusted with KOH to pH 7.2 and boiled for 15 min. Further digestion was carried out centrifuge 15,000×g at 4°C for 30 min (Arihara K. et al., 2001). The supernatant was measured of ACE inhibitory activity.

3.7 ACE inhibitory activity assay

ACE inhibitory activity was measured according to the methods of Je *et al.*, 2008. Crude proteins of Zingiberaceae solution (50 µl) with 50 µl of ACE (25 munits/ml) was pre-incubated at 37°C for 10 min, After which the mixture was re-incubated with 150 µl of substrate (10 mM HHL in PBS) for 30 min at 37°C, the reaction was stopped by adding 250 µl of 1M HCl. The hippuric acid was extracted with 500 µl of ethylacetate. After centrifugation 15,000×g at 4°C for 15 min, 200 µl of the upper layer was transferred into a test tube, and evaporated in a vaccum at room temperature. The hippuric acid was dissolved in 500 µl of distilled water, measure absorbance at 250 nm using an UV-spectrophotometer.

3.8 Isolation and of ACE inhibitory peptide.

The supernatant containing a suite of peptides was injected further fractionated by RP-HPLC on a C-18 Shimpak column (250×46 mm) using of 0.1% Trifluoroacetic acid (TFA) and 70% acetonenitrite (CH_3CN) in water containing 0.05% TFA at flow rate of 0.7 ml/min traversing from (0-60% in 60 min). The peptides were detected at 230 nm. Individal fractions were dried using speed vacuum and their assay of ACE inhibitory activity.

3.9 Determination of the protein content

The protein concentration was determined following the standard Bradford assay (Bradford, 1976), with dilutions of a known concentration of bovine serum

albumin as the standard. The absorbance at 595 nm was monitored with a microplate reader

3.10 ACE inhibitory Kinetics

Various concentrations of the substrate HHL were incubated with ACE in the presence of the purified peptide at 37 ^{o}C . The hippuric acid released was determined as described previously. A Calibration curve for standard hippuric acid was constructed from the hippuric acid released. The type of inhibition was determined from a Lineweaver-Burk plot.

3.10.1 pH resistance determination

To determine the pH resistance of purified protein, the procedure were carried out as follows. Each 200 µl of fraction F75 solvated and diluted in 20 mM Phosphate buffer, pH 7.2 was separately transferred into a 1.5 ml eppendorf tube and dried using a SpeedVac Concentrators. The protein was then re-solvated in 200 µl of 20 mM phosphate buffer, pH 7.2 set to the desired pH and incubated at 37°C for 30 min prior to assaying for ACE inhibitory activity. The pH buffers used in this experiment were all at 50 mM concentration and were composed of; glycine-HCl (pH 2.0, 3.0 and 4.0), sodium acetate (pH 4.0, 5.0 and 6.0), potassium phosphate (pH 6.0, 7.0 and 8.0), Tris-HCl (8.0, 9.0 and 10.0) and glycine-NaOH (10.0, 11.0 and 12.0). All buffers and the non-buffered pH solutions were adjusted to the final pH using 1 N NaOH or 1 M HCl, as appropriate. The obtained ODs were calculated for percentage of inhibition using ACE inhibitory activity assay.

3.10.2 Temperature resistance determination

Each 200 µl of purified protein (fraction F75) in 20 mM Tris-HCl buffer pH 7.2 was aliquoted into a 1.5 ml eppendorf tube in triplicate and incubated at the temperature previously designed as; freezer (<4°C), cooling bath (10°C), laboratory room (20°C), water bath (30, 45, 50, 60, 70, 80 and 90°C), for 1 h. After centrifugation at 10,000 x g for 15 min at 4°C to pull the liquid down to the bottom of the tube these samples were evaluated for ACE inhibitory activity assay.

3.11 Reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis

For determining of proteins during the purification step and monitoring for molecular size distribution pattern of obtained protein, native and reducing SDS-PAGE was performed follwing the procedure of Bollag (Bollag et al., 1996) and Lamelli (Lamelli 1970). A 15% (w/v) acrylamide separating gel and a 5% (w/v) acrylamide stacking gel were prepared using Biorad descent electrophoresis set. Samples were mixed with sample buffer by sample: sample buffer = 4:1 (v:v). After the gel set well, two cassettes were loaded into the chamber and running buffer was poured in. The electric current was set at 40 mA (double panels) and 280 V. Until marker front line reached the cassette edges, the system was stop and the gels were taken to stain with staining solution (0.1% w/v coomassie brilliant blue R250 in 20% ethanol in deionized water) for overnight. Then, the gels were washed with destain solution (20% v/v acetic acid and 20% v/v methanol in deionized water) and the solution would be always changed until blue color absence from gel textures. Relative molecular weights were achieved by comparison with coresolved sample bands from molecular markers (Low molecular weight SDS maker, 1704461, Amersham bioscience, Sweden). Native PAGE and SDS-PAGE were performed in the same procedure (as described above) except that the native PAGE contained no 2-mercaptoethanol and SDS in the sample buffer, gels and running buffers and no needed to boil the samples. Equipment used in all these analysis was power supply (Amersham, model EPS 301, Pharmacia Biotech, UK) and Vertical Electrophoresis Chamber set (Hoefer model miniVE, Pharmacia Biotech, UK). Tricine reducing SDS-PAGE was carried out as the same SDS-PAGE procedure accepted that tricine were used instead of glycine (at the equal weight) and the percentage of acrylamide in both separating and stacking gel were half reduced.

3.12 Identification of ACE inhibitory peptide

The molecular weight of the purified ACE inhibitory was determined using liguid chromatography-mass spectrometry (LC-MS) with MALDI-TOF. ACE inhibitory sequence was identified though the Edman degradation using the liquid phase peptide sequenator.

The sample preparation process followed the published method of Tiptara et al. (2008). Each band in the electrophoretic gel was excised, cut into small pieces (ca. 1 mm3) and washed with 100 µl deionized water. The gel pieces were destained by adding 200 µl of a 2:1 (v/v) ratio of acetonitrile: 25 mM NH4HCO3 for 15 min, and this step was performed several times until the gel pieces were completely destained. The supernatant was removed and gels were then dehydrated by adding 200 µl acetonitrile for 15 min prior to drying in a vacuum centrifuge. Then 50 µl of a 10 mM DTT solution in 100 mM NH_4HCO_3 was added, and the proteins were reduced for 1 h at 56°C. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and gels were incubated for 45 min at room temperature in the dark. The solution was then removed, the gel pieces were dehydrated in acetonitrile and the solvent evaporated off before adding 10 µl of a trypsin solution (proteomics grade, Sigma) (10 ng/µl in 50 mM NH_4HCO_3). After allowing the gel plug to swell for 15 min at 4oC, 30 µL of 50 mM $NH_4HCO_3$was added and the digestion allowed to proceed at 37°C overnight. The supernatant was then harvested following centrifugation at 10000 x g for 1 min. The remaining peptides in the gel were extracted with a solution of 50% (v/v) acetronitrile containing 5% (v/v) formic acid for 10 min with shaking, and subsequently pooled with the supernatant and taken to dryness.

Locations of the sequence fragments obtained from trypsinized tandem MS on matched intact peptide molecule were deduced by comparisons among all three dimension images of trimmed sequence which the intact peptide was trimmed from the given sequence. The three dimension images were created from intact target protein with the maximum matching percentage to the F75 fragments using 3Djigsaw online software version 2.0 (http://bmm.cancerresearchuk.org/~3djigsaw/). Data was rendered by using RasWin Molecular Graphics Window version 2.6 (freeware, available at http://www.umass.edu/microbio/rasmol/getras.htm). The three dimension images of the molecules were set as cartoons style and exported as JPEG images and the matched locations were labeled using Microsoft Paing version 5.1 (build 2600.xpsp_sp2_rtm.040803-2158).

3.13. Statistical analysis

All determinations, except for ACEI activity, were done in triplicate, and the results are reported as the mean + 1 standard error of the mean (SEM). Regression analyses and calculation of IC_{50} values was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc.).

CHAPTER lV

RESULT AND DISCUSSION

A. The study of ACE inhibitory activity of protein of Zingiberaceae plants

4.1 Screening for ACEI in plant samples

In this study we screened the ammonium sulphate cut fractions from the rhizomes of 15 Zingiberaceae plant species for ACEI. The IC_{50} values were calculated from the regression equation obtained from evaluation of different concentrations of each test extract (Table 4.1). Of the six crude proteins screened, all five were positive for ACEI activity with good inhibitory activity (low IC_{50} values) being observed for *Boesenbergia pandurata*, *Curcuma aromatica*, *Curcuma zedoaria*, and *Zingiber ottensii Zingiber* (Table 4.1). From the result *Z. ottensii* showed strong ACE inhibitory in the crude protein , which most potent ACE inhibitory activity (IC_{50} $7.30\times10^{-7} \pm 0.01$ mg/ml). According to these results several candidates have been selected for further studies.

Table 4.1 The *in vitro* ACEI activity in the ammonium sulphate cut fractions of 15 Thai species from within the Zingiberaceae family[a].

Scientific name	IC_{50} value (mg/ml)[b]
Alpinia galanga (Linn.) Swartz.	ND
Boesenbergia pandurata Roxb.	$2.43\times10^{-5} \pm 0.02$
Curcuma aeruginosa Roxb.	ND
Curcuma amarissima Roscoe.	ND
Curcuma aromatica.	$6.97\times10^{-5} \pm 0.01$
Curcuma longa Linn.	ND
Curcuma sp. (Kan-ta-ma-la)	ND
Curcuma xanthorrhiza Roxb.	ND
Curcuma zedoaria (Berg) Roscoe.	$7.63\times10^{-5} \pm 0.02$
Hedychium coronarium.	ND
Kaempferia galanga Linn.	ND
Zingiber cassumunar	$2.10\times10^{-5} \pm 0.01$
Zingiber officinale Roscoe.	ND
Zingiber ottensii Valeton.	$7.30\times10^{-7} \pm 0.01$
Zingiber zerumbet Smith.	ND

[a]Data are shown as the mean $\pm$ 1 SEM and are derived from 3 replicate enrichments
[b]crude protein represent the crude homogenate and ammonium sulphate cut fraction, respectively.
ND = Not detected

4.2. Purification of ACEI with ion exchange chromatography

The ACEI activity from *Z. ottensii* rhizomes was enriched to apparent homogeneity using a two-step procedure. The crude rhizome homogenate was first precipitated with 80% saturation ammonium sulfate and the precipitate harvested by

centrifugation, dialyzed with distilled water at 4 ºC and dried by lyophilization. Secondly, the ammonium sulphate cut fraction was subjected to SP-sepharose column chromatography in 20 mM phosphate buffer (pH 7.2) and eluted in the same buffer with a 0, 0.25, 0.5, 0.75 and 1 M NaCl stepwise gradient. The fractions containing proteins that eluted from the SP-sepharose column were screened for ACEI activity (Figure 1). Five distinct protein peaks were isolated, being the unbound fraction, and the bound proteins that were then eluted at 0.25 (F25), 0.5 (F50), 0.75 (F75), and 1.00 (F100) M NaCl. Most of the protein appeared to be unbound with decreasing amounts with increasing adhesion to the column (increasing salt levels to elute it) such that the F75 peak was very small (Figure 4.1). However, ACEI activities were only detected in the F75 fraction, which accounted for just over 90% of the total recovered protein (Figure 4.2 and Table 4.1). Thus, the F75 ACEI fraction was selected for further characterization.

Table 4.2 The protein yield and the ACEI in each enriched fraction[a].

Fraction	ACEI activity	
	IC_{50} (mg/ml)	**Maximal inhibition (%)**
Fraction unbound	0.0035±0.02	66.7±0.02
Fraction F25	0.0012±0.02	79.9±0.02
Fraction F50	ND	ND
Fraction F75	0.00063±0.01	85.0±0.03
Fraction F100	ND	ND

[a]Data are shown as the mean ± 1 SEM and are derived from 3 replicate enrichments
[b]Crude protein represent the crude homogenate and ammonium sulphate cut fraction, respectively.
ND = Not detected

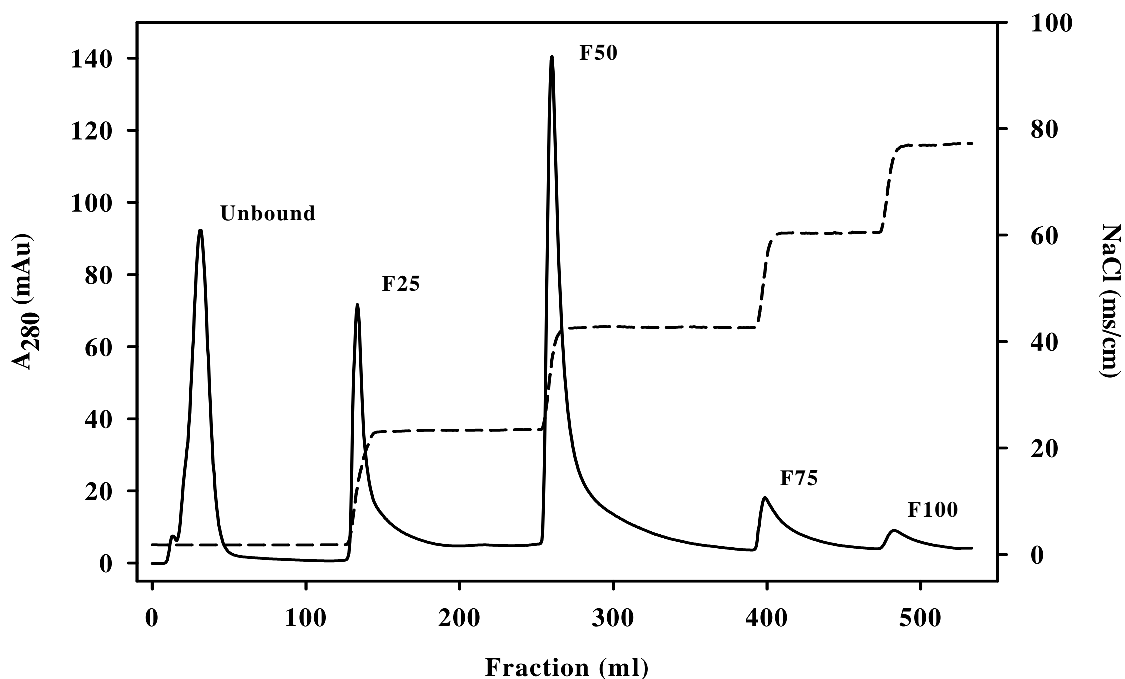


Figure 4.1 SP-sepharose chromatogram of the ammonium sulphate cut fraction of *Z. ottensii* rhizome proteins (50 mg) with stepwise NaCl elution (0.00, 0.25, 0.50, 0.75 and 1.00 M).

4.3. Molecular weight determination by reducing SDS-PAGE

The protein fractions with ACEI activity from each enrichment stage were analyzed for purity and protein pattern by reducing SDS-PAGE resolution (Figure 4.2). The implication that the enriched post-SP-sepharose-F75 fraction was a relatively homogenous protein preparation was supported by the presence of a single band after reducing SDS-PAGE analysis, and gave an estimated size of about 20.7 kDa (Figure 4.2). That an apparent high level of purity was attained by just a single step chromatography purification is of relevance since this is easier, has a lower time and cost of purification cost, and should avoid the significant yield losses seen with multiple processing steps (Demir *et al.*, 2008; Wang and Ng, 2002; Ye *et al.*, 2001; Rameshwaram and Nadimpalli, 2008).

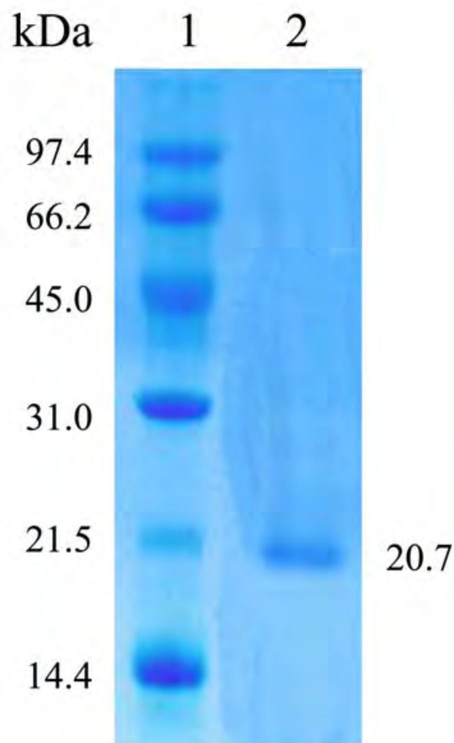


Figure 4.2 Reducing SDS-PAGE analysis of the enriched post-SP-sepharose-F75 fraction. Lane 1, molecular weight standards; Lane 2, post-SP-sepharose-F75 fraction.

4.4. Characterization of the ACEI activity

Current research into ACEIs is driven by their potential applications in medical research. In this context, the determination of the physicochemical parameters characterizing the stability of the inhibitors is essential to select effective and stable inhibitors under a large variety of environmental conditions. Moreover, the knowledge of their structural features is fundamental to understanding the inhibitor-enzyme interactions and allows novel approaches in the use of synthetic or modified inhibitors for drug design.

4.4.1 Temperature resistance determination

The thermal stability profile of the enriched ACEI (post-SP-sepharose-F75 fraction) from *Z. ottensii* is shown in Figure 4.3. The relative activity of this ACEI was stable over a relatively wide temperature range (-20 - 60 ^{o}C at a 30 min exposure) with more than 80% relative activity being retained at 80 ^{o}C for 30 min. One possible reason was the higher temperature and longer time incubation range caused a change

in the ACEI protein structure at regions that are involved in binding to ACE. A similar thermal stability has been observed for the proteolytic α-glucosidase inhibitor from the rhizomes of *Z. ottensii* with a high degree of stability over 0 - 65 °C that then decreased at higher temperatures (Tiengburanatam *et al.*, 2010).

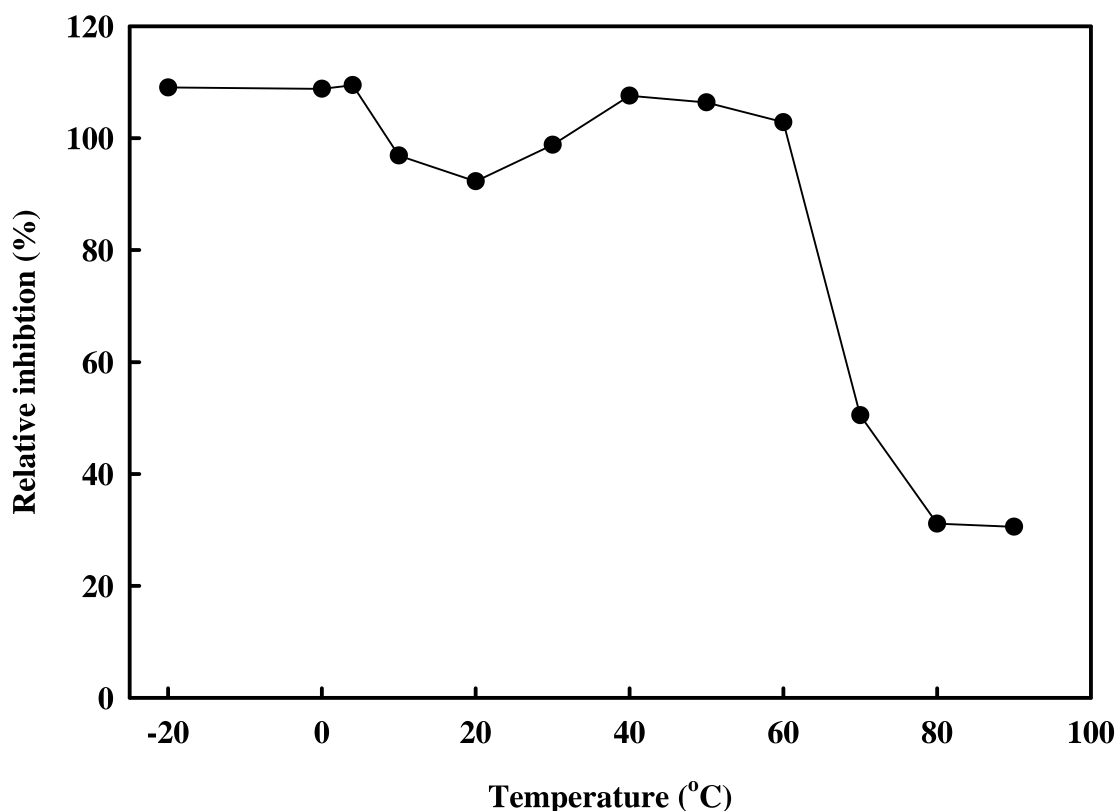


Figure 4.3 Thermostability of the enriched ACEI from *Z. ottensii* rhizomes (post-SP-sepharose-F75 fraction). The assay was performed in 20 mM phosphate buffer pH 7.2 at various temperatures for 30 min. Data are shown as the mean ± 1 SEM and are derived from triplicate experiments.

4.4.2. pH resistance of the ACEI activity

The residual ACEI activity, as a relative % inhibition, as a function of the pH was largely unaffected giving a broad pH optimum. This makes it a potentially excellent enzyme for the food and pharmaceutical industry. Changing the F75 protein preincubation buffer pH and salts revealed that the inhibition effect of fraction F75 was negated at pH 6 - 8 and steeply reduced at pH 8 - 12 (Figure 4.4). However, some

buffer-dependent affects were seen, especially at pH 6 (Figure 4.4) where a very low ACEI activity was seen in sodium acetate but not in potassium phosphate. Thus, some inhibitor-ion interactions might block or slow down the ACEI activity at such pH values. These are potential pitfalls in all, including this ACEI activity, enzyme assays and also in potential biotechnological applications where changing buffers is difficult or expensive (except, perhaps, for immobilized enzymes).

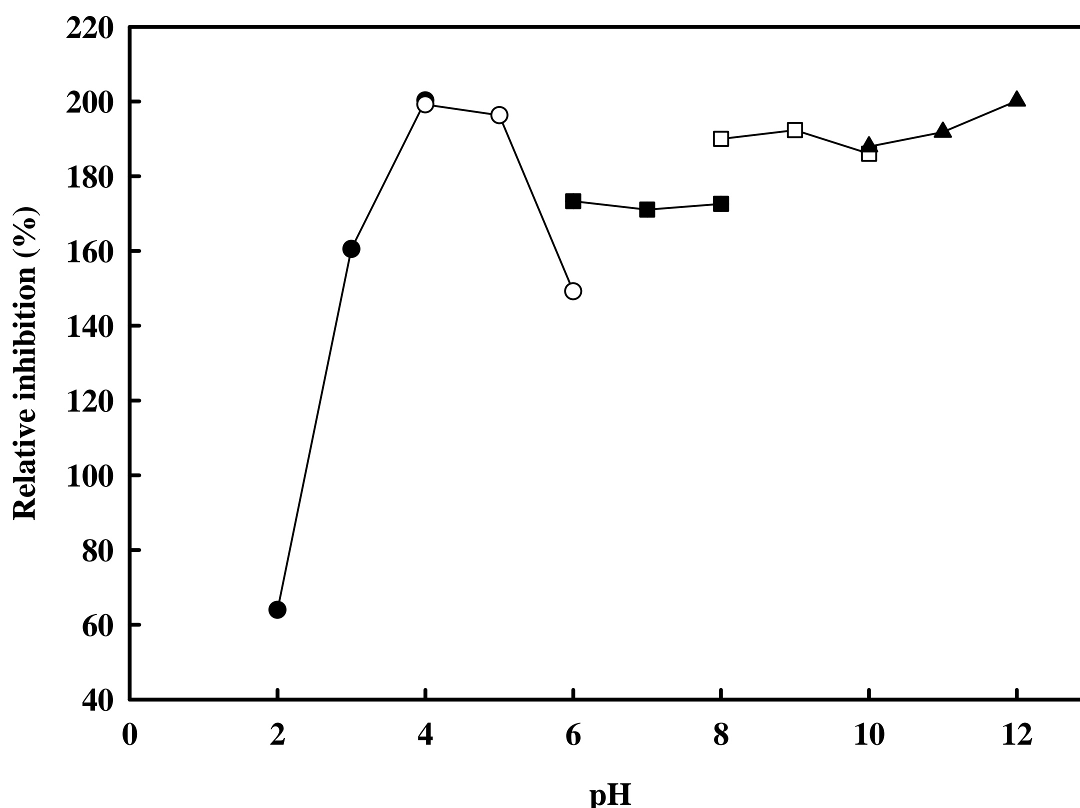


Figure 4.4 pH stability of enriched ACEI from *Z. ottensii* rhizomes (post-SP-sepharose-F75 fraction). Pretreatment was with (closed circle) 20 mM glycine-HCl (pH 2-4), (open circle) 20 mM sodium acetate (pH 4-6), (open square) 20 mM potassium phosphate (pH 6-8), (closed square) 20 mM Tris-HCl (pH 8-10), and (triangle) 20 mM glycine-NaOH (pH 10-12). Data are shown as the mean ±1 SD and are derived from three repeats.

4.4.3. Mechanism of inhibition

The inhibition mode of the ACEI from ginger was analyzed by double reciprocal (Lineweaver-Burk) plots (Figure 4.5). The K_m value, with hippuryl-L-

histidyl-L-leucine as the substrate and ACE as the active enzyme, was 0.0043 mg with a V_{max} of 188.68 µM / min. When the ACEI was added to the enzyme mixture at various concentrations, the kinetics demonstrated a competitive inhibition mechanism with a K_i value of 9.1×10^{-5} mg protein / ml. Kinetic determinations suggested that these compounds inhibit the enzyme activity by competing with the substrate for the active site

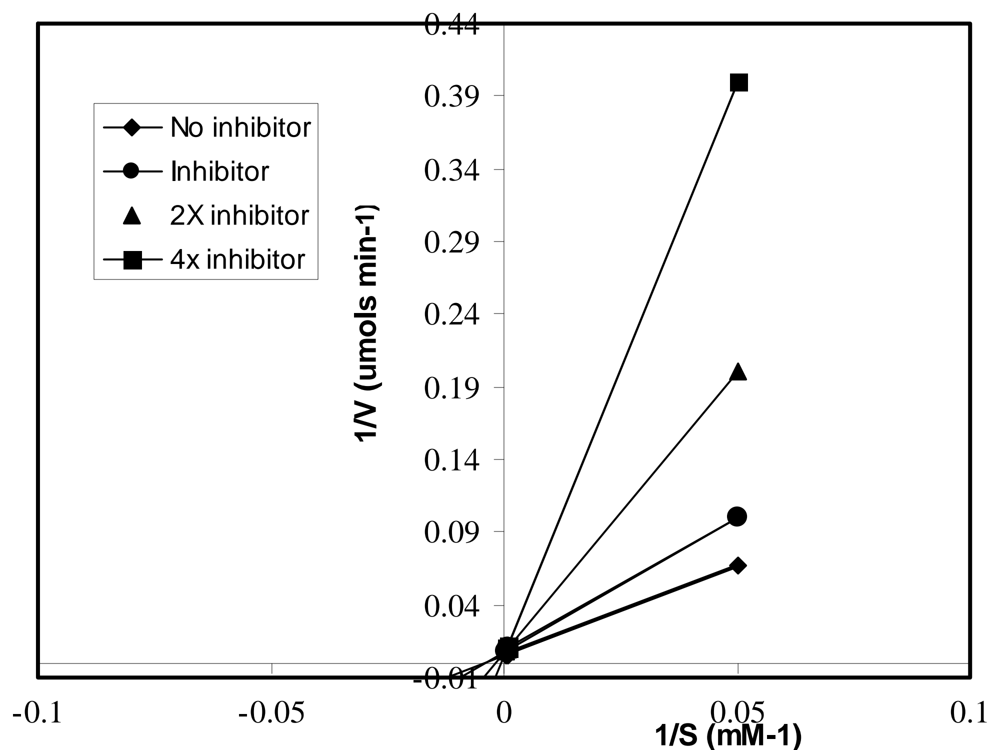


Figure 4.5 Lineweaver-Burk plots derived from the inhibition of ACE by the ACEI from *Z. ottensii* rhizomes. ACEI was treated with each stated concentration of hippuryl-L-histidyl-L-leucine (0.04-0.4 mM)in the presence of the ACEI .

4.5. Potential ACEI protein identification

Amino acid sequences of the tryptic peptide fragments were deduced by LC-MS/MS analysis. Five sequences (GPLKLSYNYNYGPQK, GNQAVFNR, HLFQQDGELVDLNMMR, YGGYNYGAPGK, and TNAENEVTLK) were gained from software analysis (*De novo* deducing). All fragments were aligned to those homologs available in the NCBI GenBank and UniProt databases. Only one sequence obtained, GPLKLSYNYNYGPQK, was also BLASTp searched against the GenBank and UniProt nr database alone, revealing 100% amino acid sequence similarity to that

of part of chitinase (Figure 4.6). As a result, the query sequence, GPLKLSYNYNYGPQK as shown in Figure 4.7.

Accession number	Organism		Sequence	
	Zingiber ottensii (F75)		GPLKLSYNYNYGPQK	
Q688M5	*Oryza sativa* (Chitinase 9 precursor)	199	GPIQLSYNYNYGP	211
Q6SPQ7	*Bambusa oldhamii*(Chitinase)	200	GPIQLSYNYNYGP	212
Q41795	*Zea mays* (Class I acidic chitinase)	135	GPIQLSYNYNYGP	147
Q7X9F4	*Galega orientalis* (Class Ib chitinase 2)	203	GPIQLTYNYNYGP	215
P85084	*Carica papaya* (Endochitinase)	115	GPLQLSWNYNYGP	127
Q7M1Q9	*Phytolacca Americana* (Chitinase A)	108	GPLQLSWNYNYGP	120
Q9ZWS3	*Nicotiana tabacum* (Chitinase 134)	136	GPIQISYNYNYGP	148
Q9SQL3	*Poa pratensis* (Chitinase)	191	GPIQISYNYNYGP	123
Q9FS45	*Vitis vinifera* (Chitinase precursor)	197	GPIQISYNYNYGP	209
Q6RH76	*Capsicum annuum* (Chitinase class I)	98	GPIQISYNYNYGP	110

Figure 4.6 Amino acid sequence from the tryptic fragments of the ACEI from *Z. ottensii* rhizomes (post-SP-sepharose-F75 fraction). Comparisons are made with other chitinase from the chitinase family that showed the highest sequence identity in BLASTp searches of the NCBI and SwissProt databases. Accession codes (UniProt/GenBank) are shown.

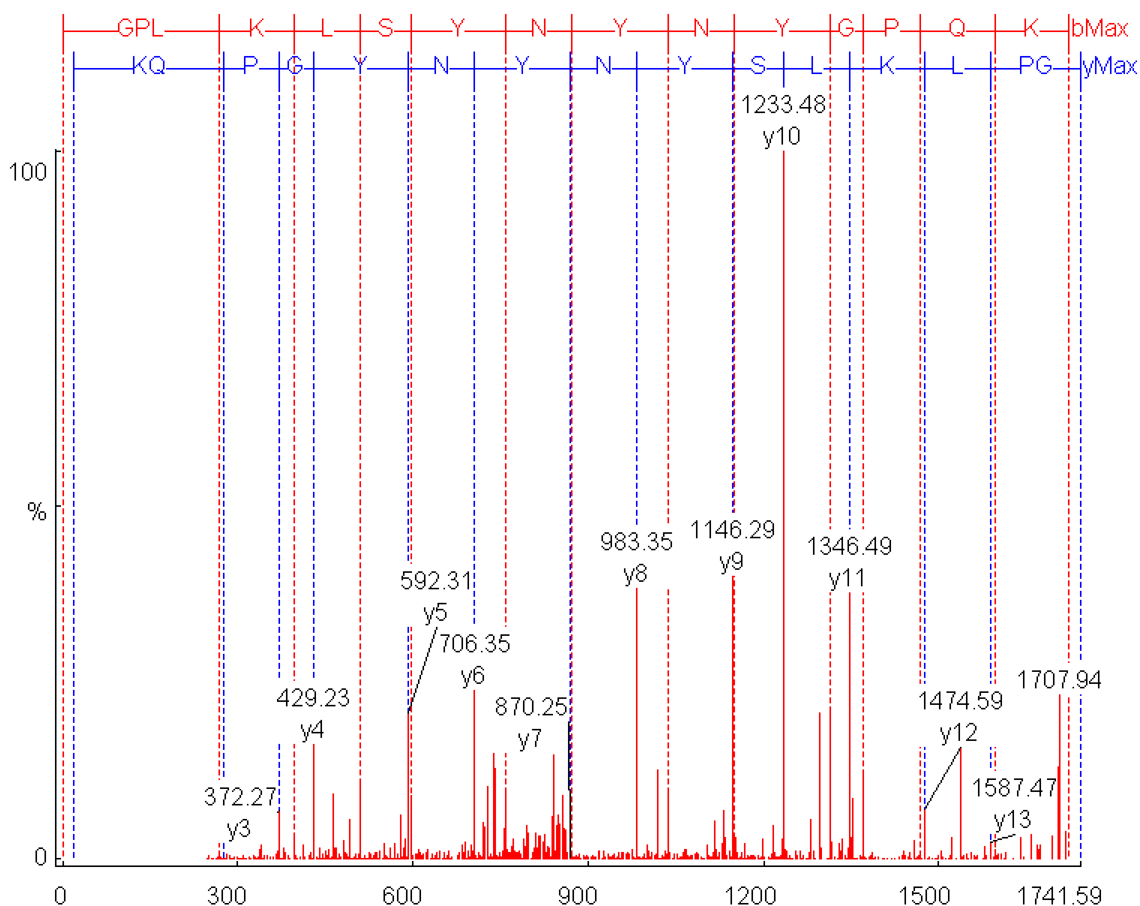


Figure 4.7 LC/MS/MS spectra of the tryptic digest of the F75 from the sequence GPLKLSYNYNYGPQK.

B. The study of ACE inhibitory activity of peptide derived from crude protein of Zingiberaceae plants

4.6 Zingiberaceae plants protein hydrolysate containing ACE inhibitor peptides

The proteins extracted from Zingiberaceae plants were digested with pepsin. The inhibitory activity of the protein hydrolydastes from *Z. cassumunar* was digested at 37 ^{o}C for 30 min, which most potent ACE inhibitory activity (IC_{50} 0.38 ± 0.012 µg/ml) The RP-HPLC separation of the pepsin digests from *Z. cassumunar* on a C-18 column using the TFA/CH_3CN solvent system. The chromatographic profile (Figure 4.8) indicated the presence of peptides peak and fractionation each 10 min were assay for ACE inhibitory activity. The fraction 3 exhibited the highest ACE inhibitory activity at 30-40 min with IC_{50} value 0.011 ± 0.012 µg/ml, which a single peak.

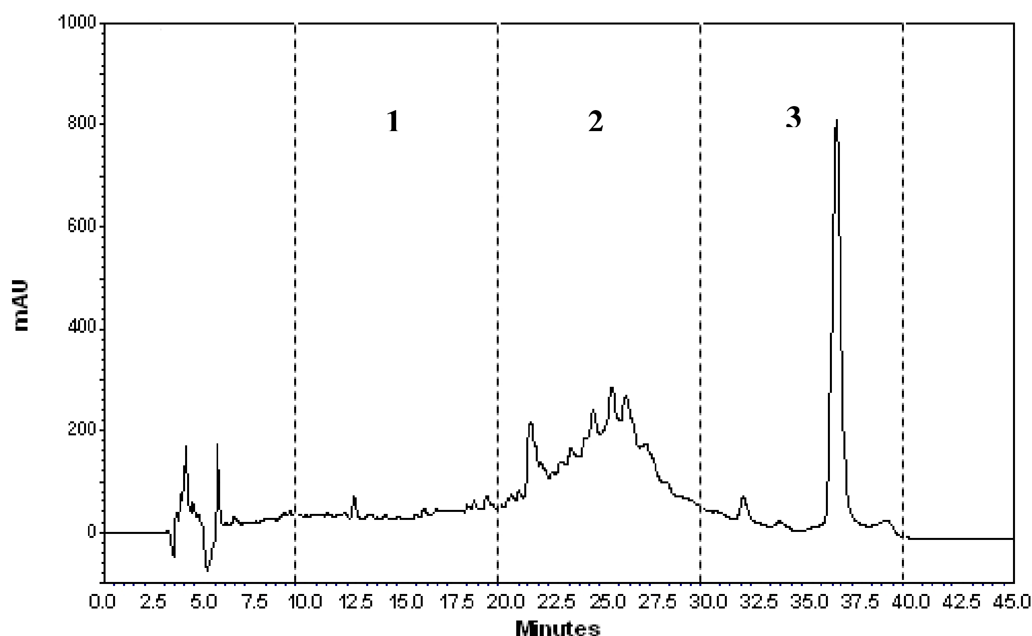


Figure 4.8 RP-HPLC profile of protein hydrolysdates with pepsin from *Z. cassumunar*.

The ACE inhibitory peptides derived from *Z. cassumuna* were identified by MALDI-TOF mass spectrometer. Peptide fraction 3 (Figure 4.9) is reported as the most potent ACE inhibitory fraction, which indicated the presence of single peptide of m/z. The molecular weight of ACE inhibitory peptide of Z. *cassumuna* was identified using MALDI-TOF mass spectrometer, which is m/z of 522.23. Consequently, it molecular weight was determined to be 521.23 Da. The amino acid sequence of the peptide was determined by Edman degradation. The sequence of the peptide was found to be Pro-Ala-Glu-Gly-His-Ser. The sequence amino acid peptide isolated in this study supported the importance of Gly-His-Ser at the carboxyl terminal. Emmemann *et al*. (1994) also reported the Rieske iron-sulfur protein from potato mitochondria. However, the amino acid sequence of their peptides and their parents were unique making comparisons difficult. Mallikarjun (2006) also reported the ACE inhibitory peptide glycinin, the 11S Globulin of soybean *(Glycine max)*. It was identified its amino acid sequence which is Val-Leu-Ile-Val-Pro using Edman degradation and peptide; m/z of 578.9. Consequently, it is the same order of the molecular weight if not lower than the report from Mallikarjun (2006).

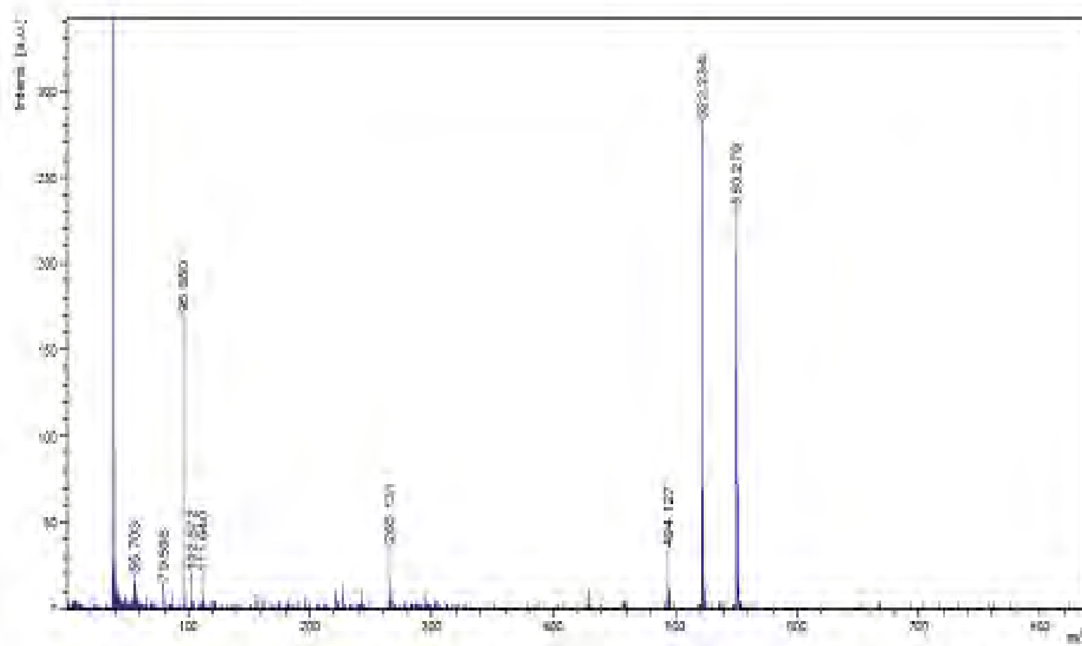


Figure 4.9 MALDI-TOF spectrum of the peptide at 30-40 min by RP-HPLC from *Z. cassumunar*.

4.7 Kinetic parameters of peptide with ACEI

The ACE inhibitory was studied to elucidate the mechanism of action the peptide from RP-HPLC to determine the hippuric acid released was used to determine the initial velocity of the ACE. Lineweaver-Burk plots were used to estimate the modes of ACE inhibition by the *Z. cassumunar* peptide. The effect of inhibitior concentration on the varying substrate concentration indicated that peptide was also a competitive inhibitor. The inhibition calculated constant K_i is 1.25×10^{-6} mg protein/ml (Figure 4.10).

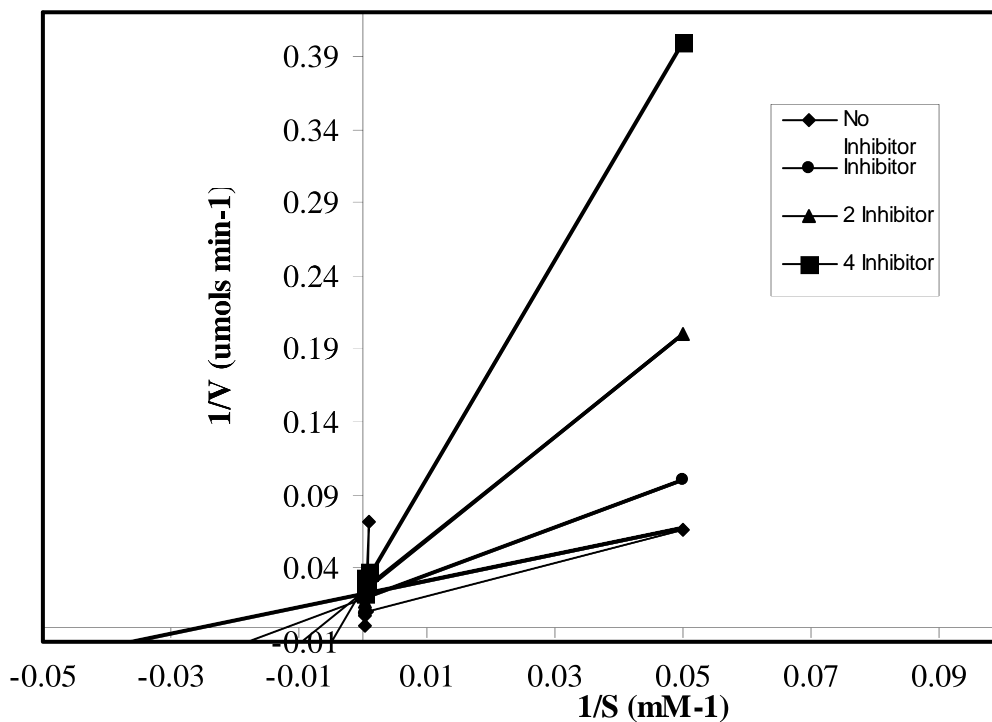


Figure 4.10 Lineweaver-Burk plots derived from the inhibition of ACE by the ACEI peptide from *Z. cassumunar* rhizomes. ACE was treated with each stated concentration of hippuryl-L-histidyl-L-leucine (0.04-0.4 mM) in the presence of the ACEI peptide.

CHAPTER V

CONCLUSION

Z. *ottensii* showed percent ACE inhibitory activity; IC_{50} of the crude protein was 7.30×10^{-7} ± 0.01mg/ml.. The rhizomes of Z. *ottensii* was purified, using a single-step moderate cation exchange chromatography. The protein exhibited a strong ACE inhibitory activity which K_i was 9.1×10^{-5} mg protein / ml.. The ACE inhibitory peptides could be derived from Z. *cassumunar* and results showed that a simple pattern of peptide, is a single peptide was purified with ACE inhibitory activity. The in vitro study is a good starting point of ACE inhibitory proteins and further research would be performed for the in vivo anti-hypertentive activities.

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APPENDICES

APPENDICE A

Zingiberaceae plants

Scientific name	Thai name
Alpinia galanga (L.) Swartz	ข่า
Boesenbergia pandurata (Roxb.)	กระชายดำ
Curcuma aeruginosa Roxb.	ว่านมหาเมฆ
C. amarissima Roscoe	ขมิ้นดำ
C. aromatic	ว่านนางคำ
C. longa L.	ขมิ้นชัน
Curcuma sp.	คัณฑมาลา
C. comosa	ว่านชักมดลูก
C. zedoaria (Berg) Roscoe	ขมิ้นอ้อย
Hedychium coronarium Roem.	ว่านมหาหงส์
Kaempferia galanga L.	เปราะหอม
Zingiber cassumunar	ไพลเหลือง
Z. officinale Roscoe	ขิง
Z. ottensii Valeton.	ไพลดำ
Z. zerumbet (L.) Smith	กระทือ

APPENDICE B

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solutions

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

10% SDS (w/v)

Sodium dodecyl sulfate (SDS) 10 g

50% Glycerol (w/v)

100% Glycerol 50 ml

Added 50 ml of distilled water

1% Bromophenol blue (w/v)

Bromophenol blue 100 mg

Brought to 10 ml with distilled water and stirred until dissolved.

Filtration will remove aggregated dye.

2. Working solution

Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)

Acrylamide 29.2 g

N,N,-methylene-bis-acrylamide 0.8 g

Adjust volume to 100 ml with distilled water

Solution B (1.5 M Tris-HCl pH 8.8, 0.4% SDS)

2 M Tris-HCl (pH 8.8)	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (0.5 M Tris-HCl pH 6.8, 0.4% SDS)

1 M Tris-HCl (pH 6.8)	50 ml
10% SDS	4 ml
Distilled water	46 ml

10% Ammonium persulfate

Ammonium persulfate	0.5 g
Distilled water	5 ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS)

Tris (hydroxymethyl)-aminomethane	3 g
Glycine	14.4 g
SDS	1 g

Dissolved in distilled water to 1 litre without pH adjustment
(final pH should be 8.3)

5x sample buffer
(60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 14.4 mM 2-mercaptoethanol)

1 M Tris-HCl (pH 6.8)	0.6 ml
Glycerol	5 ml
10% SDS	2 ml
1% Bromophenol blue	1 ml
2-mercaptoethanol	0.5 ml
Distilled water	0.9 ml

3. SDS-PAGE

15% Separating gel

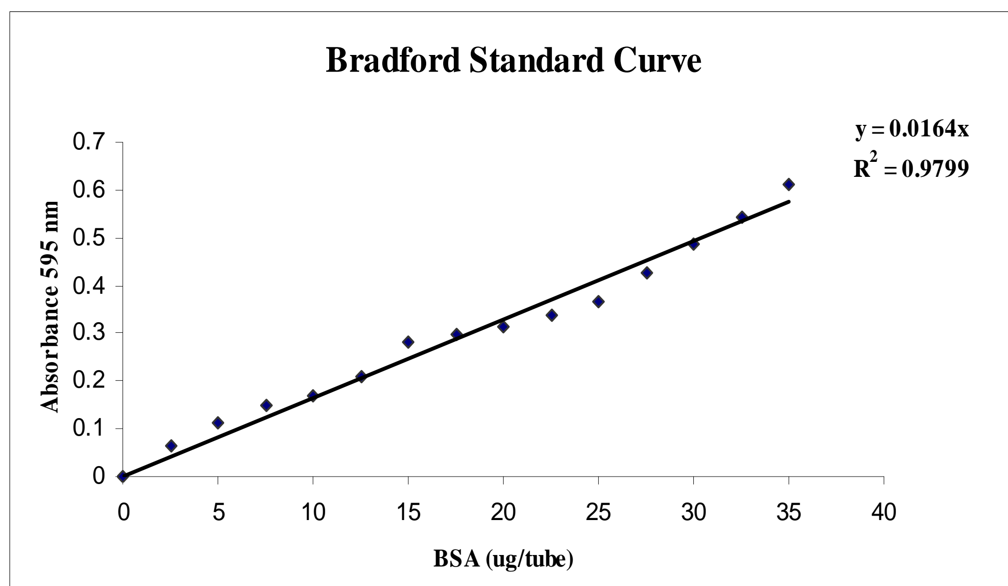
Solution A	10.0	ml
Solution B	5.0	ml
Distilled water	5.0	ml
10% Ammonium persulfate	100	µl
TEMED	10	µl

5.0% Stacking gel

Solution A	0.67	ml
Solution B	1.0	ml
Distilled water	2.3	ml
10% Ammonium persulfate	30	µl
TEMED	5.0	µl

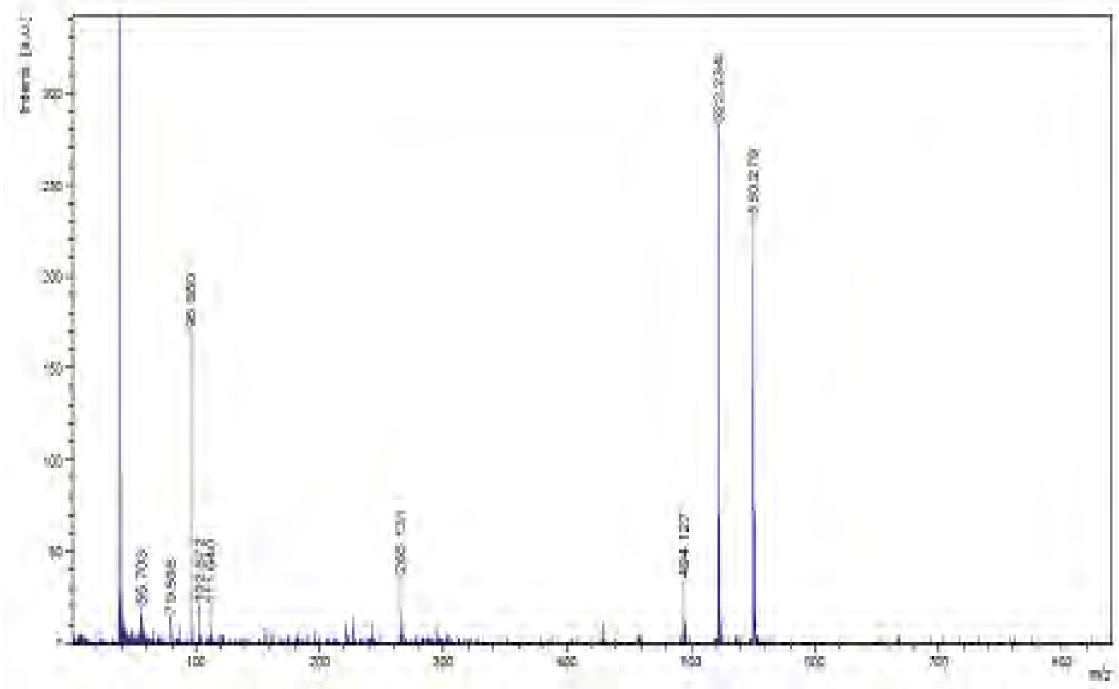
APPENDICE C

Calibration curve for protein determination by Bradford method



APPENDICE D

Molecular weight from MALDI-TOF spectrum of the peptide at 30-40 min by RP-HPLC from Z. cassumunar.



APPENDICE E

Amino acid abbreviations

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

BIOGRAPHY

Miss Maneerat Yodjun was born on November 20, 1985 in Phichit, Thailand. She graduated with a Bachelor Degree of Science from Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2008. She had been studies for a Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2009.

Academic presentation;

1.) Yodjun, M., Sangvanich, P., and Karnchanatat, Angiotensin I-converting Enzyme Inhibitory Activity from the Peptides of the Rhizomes of Zingiberaceae Plants, The 12th Graduate Research Conference Khon Kaen University 2011, 28 January 2011, Khon Kaen, Thailand.