ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY ACTIVITY OF PROTEIN HYDROLYSATE FROM SEEDS OF THAI FRUITS

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ฤทธิ์ยับยั้งแอนจิโอเทนซิน I-คอนเวอร์ติงเอนไซม์ของโปรตีนไฮโดรไลเสตจากเมล็ดผลไม้ไทย

นายอรรถสิทธิ์ นุชประภา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY ACTIVITY OF PROTEIN HYDROLYSATE FROM SEEDS OF THAI FRUITS
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อรรถสิทธิ์ นุชประภา: ฤทธิ์ยับยั้งแอนจิโอเทนซิน I-คอนเวอร์ติงเอนไซม์ของโปรตีนไฮโดร-ไลเสตจากเมล็ดผลไม้ไทย อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. อมร เพชรสม, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร.อภิชาติ กาญจนทัต, 53 หน้า.

ระบบเรนิน-แอนจิโอเทนซินเป็นระบบหนึ่งในร่างกายที่มีผลเกี่ยวกับการควบคุมความดันโลหิต โดยเรนินจะส่งผลให้แอนจิโอแทนซิโนเจนปลดปล่อยแอนจิโอเทนซิน I และจะถูกเปลี่ยนไปเป็นแอนจิโอเทนซิน II ด้วยแอนจิโอเทนซินคอนเวอร์ติงเอนไซม์ หรือ ACE โดยเอนไซม์ชนิดนี้มีบทบาทต่อพยาธิสภาพของร่างกายในการควบคุมความดันโลหิตด้วยสองปฏิกิริยาที่เกี่ยวเนื่องกัน โดยจะไปเปลี่ยนแอนจิโอเทนซิน I ไปเป็นแอนจิโอเทนซิน II ซึ่งจะมีความสามารถสูงในการทำให้หลอดเลือดหดตัว และยังไปยับยั้งการทำงานของแบรดดีไคนินที่มีผลในการขยายของหลอดเลือด โปรตีนสกัดหยาบและโปรตีนไฮโดรไลเสตที่เตรียมโดยการนำไปบ่มกับเอนไซม์เพปซินและแพนครีเอตินจากเมล็ดผลไม้ไทย 4 ชนิด ได้แก่ เมล็ดมะละกอดิบ, เมล็ดมะละกอสุก, เมล็ดเงาะ, เมล็ดลำไย, และเมล็ดลิ้นจี่ได้ถูกนำมาทดสอบหาความสามารถในการยับยั้งกิจกรรมของเอนจิโอเทนซิน I-คอนเวอร์ติงเอนไซม์ในขั้นของหลอดทดลอง พบว่าโปรตีนไฮโดรไลเสตที่เตรียมจากเมล็ดลิ้นจี่มีความสามารถในการยับยั้งมากที่สุด โดยมีค่า IC_{50} ที่ 0.22±0.010 มิลลิกรัมโปรตีนต่อมิลลิลิตร เมื่อนำโปรตีนไฮโดรไลเสตของเมล็ดมะละกอดิบ, เมล็ดลำไย, และเมล็ดลิ้นจี่ไปทำการศึกษาจลนพลศาสตร์พบว่ามีการยับยั้งแบบไม่แข่งขัน และมีค่า K_i เท่ากับ 6.02, 2.82, และ5.62 มิลลิกรัมโปรตีนต่อมิลลิลิตร และยังพบว่าตัวยับยั้งทั้ง 3 ชนิดนี้ทำงานได้ดีในช่วงค่าความเป็นกรด-ด่างที่ 6 – 8 โดยที่จะทนต่ออุณหภูมิได้ตั้งแต่ -20 จนถึง 80 องศาเซลเซียส หลังจากนำโปรตีนไฮโดรไลเสตทั้ง 3 ชนิดไปทำบริสุทธิ์บางส่วนด้วยเทคนิคอัลตราฟิลเตรชันด้วยเยื่อกรองขนาด 10 กิโลดาลตันและ 5 กิโลดาลตัน พบว่าส่วนที่มีขนาดต่ำกว่า 5 กิโลดาลตันของเมล็ดลำไยมีความสามารถในการยับยั้ง ACE ได้ดีที่สุด โดยมีค่า IC_{50} อยู่ที่ 0.43±0.011 มิลลิกรัมโปรตีนต่อมิลลิลิตร จึงนำโปรตีนส่วนนี้ไปแยกเป็นเพปไทด์ด้วยเครื่องเอชพีแอลซีแบบรีเวิร์สเฟส พบว่าแยกได้เพปไทด์จำนวน 5 พีค จึงนำไปทำการวิเคราะห์หาลำดับของกรดอะมิโนด้วยเทคนิคแมสสเปกโตรเมตตรี พบว่าได้ลำดับกรดอะมิโน 9 สายและพบว่ามี 3 สายที่อาจจะมีผลในการยับยั้งกิจกรรมของ ACE ได้

สาขาวิชา: เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา: 2556 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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ATTHASITH NUCHPRAPHA: ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY ACTIVITY OF PROTEIN HYDROLYSATE FROM SEEDS OF THAI FRUITS. ADVISOR: ASSOC. PROF. AMORN PETSOM, Ph.D. CO-ADVISOR: ASST. PROF. APHICHART KARNCHANATAT, Ph.D., 53 pp.

Blood pressure regulation is partially dependent on the rennin-angiotensin system; renin acts on angiotensinogen to release angiotensin-I, which is further converted into the angiotensin II by the angiotensin I-converting enzyme (ACE). ACE plays a key physiological role in the regulation of blood pressure by virtue of two different reactions that it catalyzes: conversion of the inactive angiotensin I to the powerful vasoconstrictor angiotensin II, and inactivation of the vasodilator bradykinin. Crude extract and ammonium sulphate cut protein extracts, and their pepsin-pancreatin hydrolysates, from the seeds of 4 Thai fruits (i) *Carica papaya* L.; (papaya; unripe and ripe form), (ii) *Nephelium lappaceum* L. (rambutan) (iii) *Dimocarpus longan* Lour. subsp. (longan), and (iv) *Litchi chinensis* Sonn. (lychee) were screened for their in vitro angiotensin I- converting enzyme inhibitory (ACEI) activity. The highest activity of each fraction, protein hydrolysate of lychee seeds shows the highest potential of ACE inhibitors of IC_{50} value 0.22±0.010 mg protein/ml. The protein hydrolysate of unripe papaya seeds, longan seeds, and lychee seeds show uncompetitive and non-competitive inhibition with K_i values at 6.02, 2.82, and 5.62 mg protein/ml, with optimum pH in range of 6-8. After partial purified with ultrafiltration technique, UF-3 (below 5 kDa) of longan seeds show the highest inhibitory activity with IC_{50} values at 0.43±0.011 mg protein/ml. This fraction subjected to RP-HPLC, five peaks were separated, and subjected into LC/MS/MS for amino acids sequences analysis. The P1-F1, P3-F1, and P3-F4 are possibility the most inhibitory activity peptides.

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

Academic Year: 2013 Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

%	percentage
°C	degree Celsius
µg	microgram
µl	microliter
A	absorbance
BSA	Bovine serum albumin
CH_2CN	acetonitrile
cm	centimeter
Da	Dalton
g	gram
hr	hour
kDa	kilo Dalton
l	liter
LC/MS/MS	Liquid chromatography/Mass Spectrometry/Mass Spectrometry
M	molar
min	minute
ml	milliliter
mg	milligram
mM	milimolar
MW	molecular weight
NaCl	Sodium Chrloride

nm	nanometer
RP-HPLC	Reversed Phase-High Performance Liquid Chromatography
rpm	revolution per minute
TFA	trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
U	unit activity
v/v	volume by volume
w/v	weight by volume

CHAPTER I

INTRODUCTION

Hypertension, one of the most common worldwide diseases, is a chronic medical condition in which the resultant elevated blood pressure can damage the health. There are many associated risk factors, such as strokes, heart disease, chronic renal failure or aneurysm disease. There are many predisposition factors, such as a sedentary lifestyle, stress and visceral obesity, of hypertension, which are not restricted to the aged and elderly. The angiotensin I-converting enzyme (ACE, EC.3.4.15.1) plays a key physiological role in the control of blood pressure, in the Renin-Angiotensin System (RAS), which mediates control of the extracellular volume (i.e. that of the blood plasma, lymph and interstitial fluid) and arterial vasoconstriction. ACE catalyses the conversion of the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II and also degrades bradykinin, leading to the systematic dilation of the arteries and decrease in arterial blood pressure. Some of the ACE inhibitor (ACEI) peptides result in a decreased formation of angiotensin II and decreased blood pressure. For this reason, many studies have been directed towards the attempted synthesis of functional ACEIs without side-effects, such as captoprill or enalapril, which are currently used in the treatment of hypertensive patients. There is a strong trend towards developing natural ACE inhibitors (ACEI) for the treatment of hypertension.

Bioactive proteins and peptides have physiological properties and in recent times several studies have been done on identifying and optimizing the isolation of biopeptides from both plant and animal sources. These peptides are generated both *in vivo* and *in vitro* from the proteolytic hydrolysis of food proteins. Peptides with a wide range of regulatory effects have been discovered, including modulation of the immune defence, increased nutrient uptake, neuro-endocrine information transfer, antihypertensive, antithrombotic, antimicrobial, antigastric and opioid activity. These peptides have been

discovered in a diverse array of sources, including snake venom, spinach, whey proteins and mushrooms. However, the only legumes that have been investigated for biopeptides to the best of our knowledge are chickpeas, peas, cowpeas and soybeans; although it is assumed that since peanuts have a similar protein profile as these legumes then they will have similar biological activities too. Thus, researchers consider seeds as new major sources of bioactive protein hydrolysate and interesting in distinction from previous report. Previous reports describe small molecule organic compounds but a few reports about bioactive protein hydrolysate. This is the good reason to find new bioactive protein hydrolysate from Thai fruits seeds for medical, pharmaceutical, industrial applications or higher research.

CHAPTER II

LITERATURE REVIEWS

2.1 Hypertension

Hypertension is a worldwide epidemic problem, affecting about 20 % of world's adult population. It is the one of the major risk for the development of cardiovascular disease and it often called a silent killed because the persons with hypertension are asymptomatic for years (Gao *et al*., 2010). Hypertension is the most common serious chronic health problem because it carries a high risk factor for arteriosclerosis, stroke, myocardial infarction and end-stage renal disease (Je *et al*., 2005). Therefore, the decreasing of blood pressure to normal levels is crucial for preventing cardiovascular and renal disease. In the human body, blood pressure is regulated by renin-angiotensin system (RAS). RAS has been found to be a coordinated peptidic hormonal cascade for the control of cardiovascular, renal, and adrenal function governing fluid and electrolyte balance and arterial blood pressure (Carey and Siragy, 2003). Renin catalyzes the inactivated form of angiotensinogen to form angiotensin I, which further cleaved by angiotensin I-converting enzyme (ACE) to vasoconstrictor angiotensin II and damage bradykinin into an inactive metabolite (Ahn *et al*., 2012; Gao *et al*., 2010). (Figure 2.1)

2.2 Angiotensin-I converting enzyme (ACE)

Angiotensin-I converting enzyme (ACE) is a key enzyme in the regulation of blood pressure and electrolyte homeostasis. ACE belongs to the class of zinc proteases and located in the vascular endothelial lining of lungs. ACE acts as an exopeptidasse that cleaves dipeptides from the C-terminus of various oligopeptides. (Curtiss *et. al.* , 1978; Yang *et.al*., 1970). ACE is an important enzyme of the renin-angiotensin system, major

regulation of blood pressure in mammals (Tomatsu *et al*., 2013). A membrane-anchored dipeptide-liberating carboxypeptidase (peptidyldipeptide hydrolase, kinase II, EC 3.4.15.1) converts angiotensin I (a decapeptide; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) to the highly potent vasoconstrictor octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe + His-Leu), (Figure 2.2). The effects of angiotensin II including vasoconstriction, arterial constriction and blood pressure elevation are mediated by angiotensin type 1 receptors (AT1). Angiotensin I also bind to angiotensin type 2 receptor (AT2) which highly expressed in fortal mesenchymal tissues but poorly expressed in the adult. This enzyme also plays a key physiological role in the regulation of local levels of several endogenous bioactive peptides such as breaks down bradykinin, a vasodilator, further contributing to blood pressure elevation in the kinin-kallikrein system (Barbana and Boyce., 2010). The inhibition of ACE would be expected to prevent the formation of the hypertensive agent angiotensin II and to potentiate the hypotensive properties of bradykinin, leading to combined lowering of the blood pressure. Inhibitors of ACE are therefore widely used in therapy for hypertension, heart failure, myocardial infarction, and diabetic nephropathy.

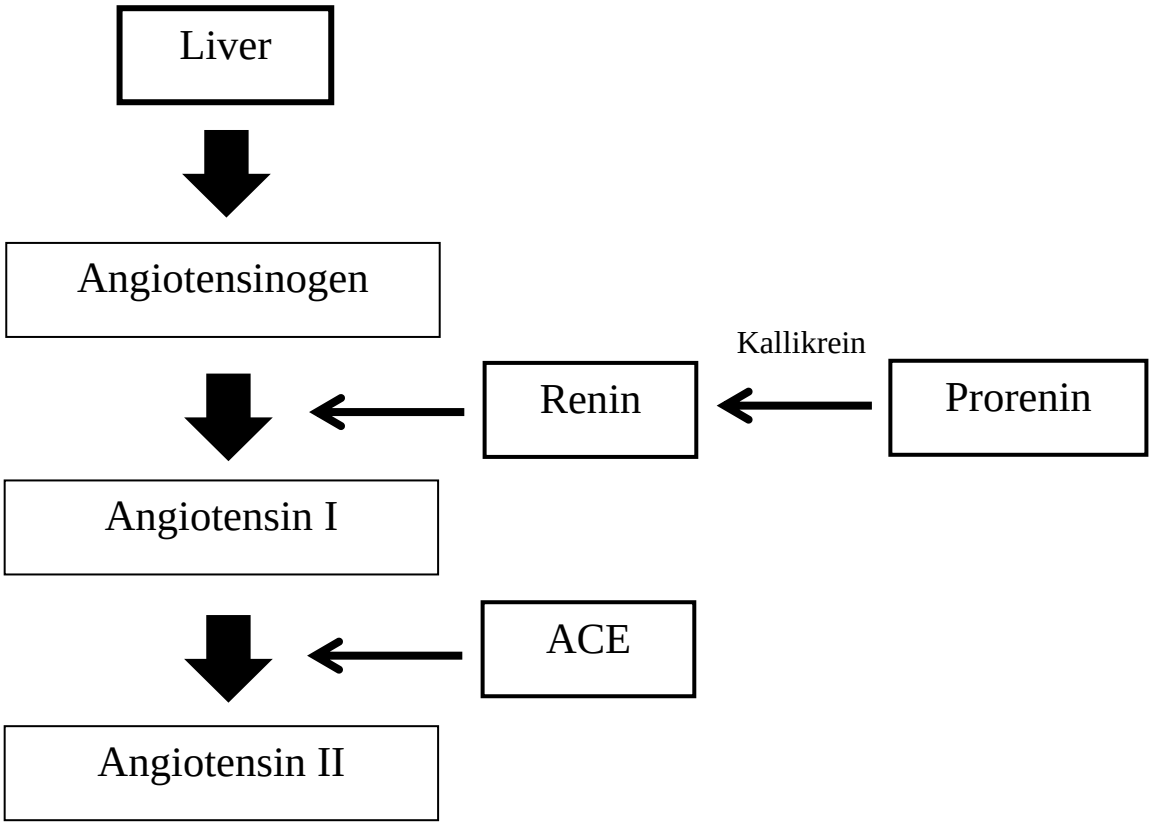


Figure 2.1 The renin-angiotensin system (RAS)

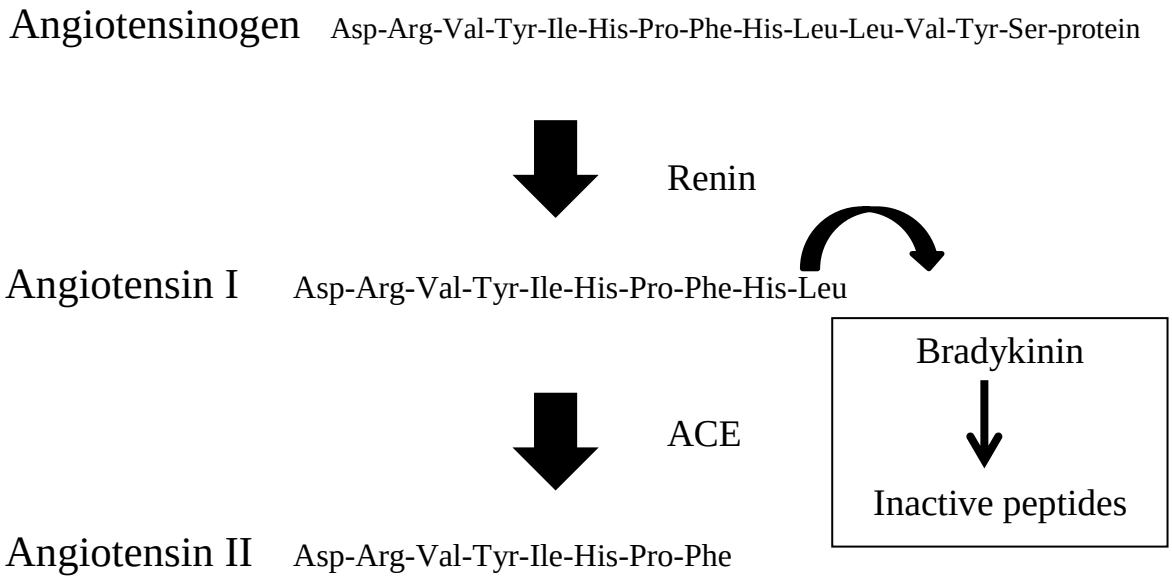
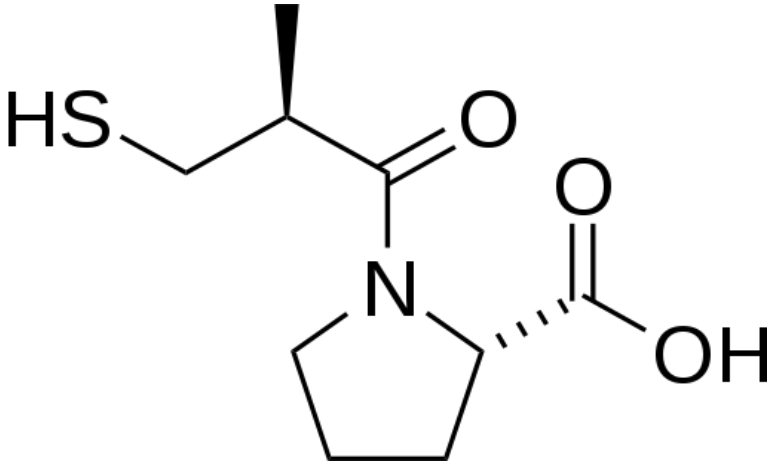


Figure 2.2 Angiotensin converting enzyme (ACE)

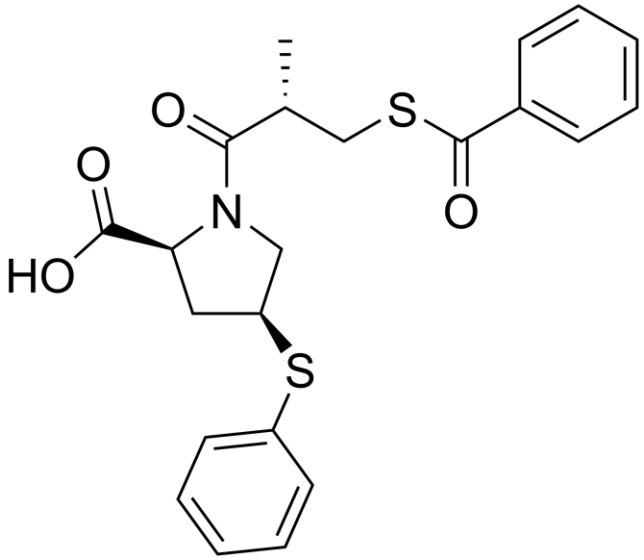
2.3 Angiotensin I-converting enzyme inhibitors (ACEI)

The inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension, the development of drugs to control high blood pressure, ACE inhibitor (ACEI) has become an important activity. The first anti-hypertensive effect *in vitro* was discovered in snake venom. Many studies have been attempted in the synthesis of ACEI such as captopril or D-3-mercapto-2-methylpropanoyl-L-proline is the first synthesis compound which an analog of Ala-Pro sequence, with sulfhydryl as a strong chelating group of zinc ion (Patchett *et al*., 1980). The ACEI can be divided into three group based on their molecular structure as sulfhydryl-containting agents such as captopril, zefenopril, fentiapril, and alacepril.

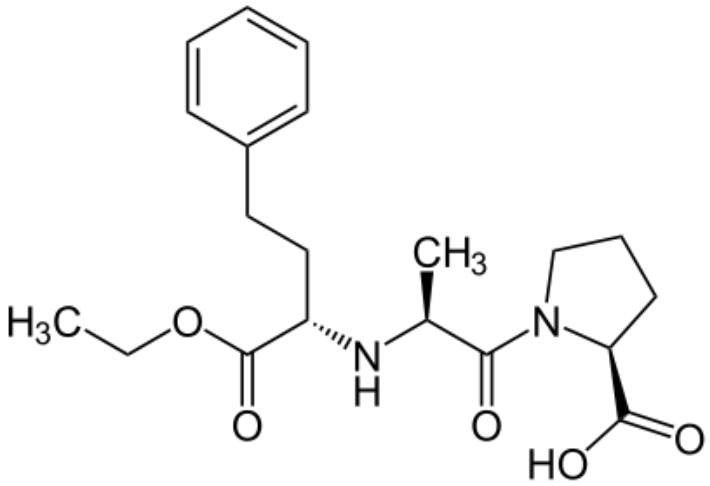
Decarboxylate-containing agents such as enalapril, remipril, quinapril, lisinopril, imidapril, perindopril, benazepril, and trandolapril. Fosinopril is the only phosphonate-containing agents in this group (Lawrie, 1991). (Figure 2.3) ACEI are current used in the treatment of hypertension and heart failure in humans (Ondetti *et al*., 1977). However, these synthetic drugs are believed to have certain side effects such as cough, taste disturbances, skin rashes or angioneurotic edema all of which might be intrinsically linked to synthetic ACEI (Kim & Wijesekara *et al*., 2010). Therefore, the research and development to find non-toxic and economical ACEI are necessary for the prevention and remedy of hypertension (Goretta *et al*., 2006).



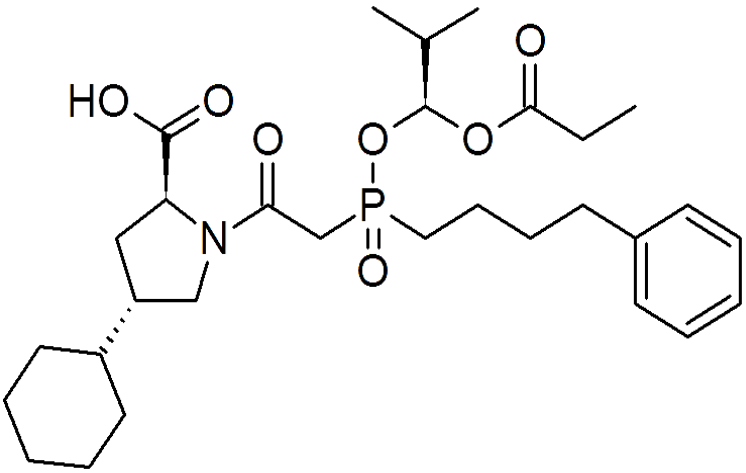
Captopril



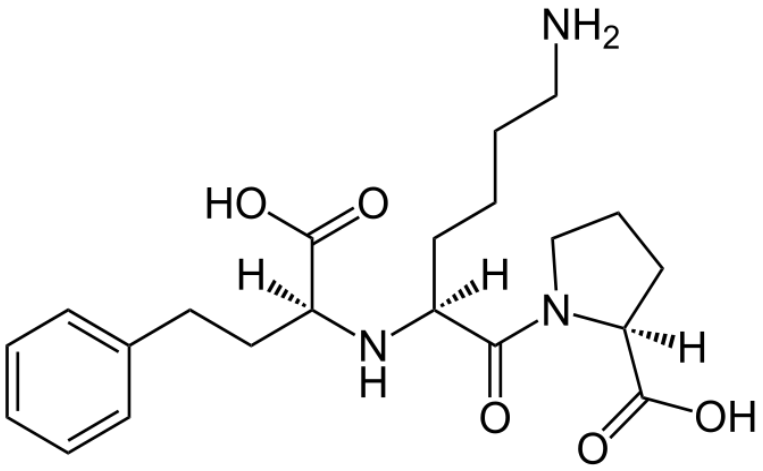
Zofenopril



Enalapril



Fosinopril



Lisinopril

Figure 2.3 Chemical structures of captopril, zofenopril, enalapril, fosinopril, and lisiopril.

ACE inhibitory peptides are also present in the amino acid sequences of several food proteins (Ariyoshi, 1993). The intrinsic bioactivities of the peptide encrypted in food proteins are latent until they are released ad activated by enzymatic hydrolysis, for example, during gastrointestinal digestion and food processing (Takano, 1998). Activated peptides are potential modulators of various regulatory processes in the living system. Therefore, food protein-derived inhibitors of ACE represent natural, physiologically active, food-grade components, which may provide health benefits beyond basic nutrition (Clare & Swaisgood, 2000). In particular, food protein-derived peptides may contribute to reducing the risk of developing cardiovascular disease through the consumption of ACE inhibitors as functional food ingredients (Meisel, 1993; FitzGerald & Meisel, 1999).

2.4 Source protein derives ACE inhibitory peptides

The procedures have generally been used in the identification and characterization of ACE-inhibitory peptides are (i) isolation from *in vitro* enzymatic hydrolysate (ii) *in vivo* gastrointestinal digestion , and (iii) chemical synthesis of peptides having identical or similar structures to those known to be bioactive. In some cases ACE-inhibitory peptides may be isolated from a food source without prior enzymatic processing, for example, from garlic (Suetsuma, 1998).

A widely variety of ACE inhibitory peptides have been identified and characterized from milk, animal (non-milk), plant, and miscellaneous protein sources. The ability of an ACE-inhibitory peptide is usually revealed as an IC_{50} value, which is equivalent to the concentration of peptide mediating a 50% inhibition of activity (Holmquist *et al*., 1979; Vermeirssen *et al*., 2002). In the majority of cases, the most frequently used analytical method to determine IC_{50} is based on the hydrolysis of hippuryl-histidine-leucine (HHL) (Crushman and Cheng, 1971). With the creation of new artificial substrates for ACE, alternative methods have been developed to quantify the IC_{50} of ACE inhibitory peptides (Elbl *et al*., 1994; Mehanna *et al*., 1999). Unfortunately, the use of various modifications of the method of Crushman and Cheng has made the comparison of IC_{50} value from different studies difficult because some reports do not detail the number of enzyme units used in the inhibition analyses or include and IC_{50} value for and ACE-inhibitory standard such as Captopril. (FitzGerald and Meisel, 2000)

As can be seen in Table 2.1, the majority of peptides are short-chain peptides with low molecular mass. This agreement with the crystallography studies, the active site of ACE cannot bind with the large peptide molecules (Natesh *et al*., 2003)

Table 2.1 Potent of ACE inhibitory peptides

Source	Enzyme	Amino acid sequence	IC_{50} (μM)	Reference
Garlic	No enzyme	FY NY NF	3.74 32.6 46.3	Suetsuna *et al*., 1998
Wheat	Alcalase	TF LY YL AF IY VF IVY VFPS TAPY TVPY TVVPG DIGYY DYVGN TYLGS GGVIPN APGAGVY	17.8 6.4 16.4 15.2 2.1 9.2 0.48 0.46 13.6 2 2.2 3.4 0.72 0.86 0.74 1.7	Matsui *et al*., 1999
Sunflower	Pepsin Pancratin	FVNPQAGS	6.9	Megias *et al*., 2006
Rapeseed	Gastrointestinal simulation	RIY VWIS	20 30	Marczak *et al*., 2003
Buckwheat	No enzyme	GPP	0.00625[a]	Ma *et al*., 2006
Mungbean	Alcalase	KDYRL VYPALR KLPAGYLF	26.5 82.5 13.4	Li *et al*., 2006

Table 2.1 (Continued)

Source	Enzyme	Amino acid sequence	IC_{50} (µM)	Reference
Seaweed pipefish	Papain Alcalase Neutrase Pronase Pepsin Trypsin	YFPHGP HWYYQA	0.62[a] 1.44[a]	Wijesekara *et al*., 2011
Hen egg white lysozyme	Pepsin Chymotrypsin Trypsin	MKA RGY VAW	25.7 61.9 2.86	Rao *et al*., 2012
Salmon byproduct	Alcalase Flavorzyme Neutrase Pepsin Protamex Trypsin	VWDPPKFD FEDWVPLSCF FNVPLWE	9.10 10.77 7.72	Ahn *et al*., 2012
Cornucopia mushroom	No enzyme	RLPSEFDLSAF-LRA RLSGQTIEVTS-EYLFRH	0.46[a] 1.14[a]	Jang *et al*., 2011
Wheat gliadin	Clarex Alcalase Esperase	PVILF	0.02[a]	Thewissen *et at*., 2011
Grass carp	Alcalase	VAP	0.00534[a]	Chen *et al*., 2011
Potato	Trypsin	GFR FK IMVAEAR GPCSR CFCTKPC MCESASSK	94.25 265.43 84.12 61.67 1.31 75.93	Huang *et al*., 2011

[a] IC_{50} values quoted are expressed as mg protein/ml.

2.5 Characterization of ACE inhibitory peptides

Inhibitors of ACE were developed for therapy of human hypertension without knowledge of the structure of human ACE, designed on the basis of an assumed mechanistic homology with carboxypeptidase A. Recently; the analysis of structure of ACE has shown the resembles zinc metalopeptidase. (Natash *et al*., 2003). The somatic form of ACE consists of two homologous domains (N- and C-domain) (Inagami, 1992), each of which contains an active site which catalyzes the hydrolysis of angiotensin I (Wei *et al*., 1992). ACE inhibitors may preferentially act on either ACE domain. However, the C-domain seems to be necessary for controlling blood pressure, suggesting that this domain is the dominant angiotensin-converting site. Although there is no known specific physiological substrate of the C-domain, the C-domain activity can be assessed specifically *in vitro* by use the synthetic substrate (HHL).

ACE prefer to have substrates or inhibitors that contain hydrophobic (aromatic or branched side chains) amino acid residues at the first of three C-terminal positions (Cheung, Wang, Ondetti, Sabo, & Crushman, 1980; Wu *et al*., 2006). Many naturally occurring peptidic inhibitors containing proline at C-terminus. This applies also for the highly active short-chain peptides. The majority of di- and tri-peptide inhibitors have a Tyr, Phe, Trp, or Pro residue at the C-terminal end, the Trp seems to be most effective in increasing in highly active inhibitors. Example, the result of Rao *et al*., the hen egg white lysozyme protein hydrolyzed by pepsin, α-chymotrypsin, and trypsin was purified to the tri-peptides, the peptide Val-Ala-Trp show the strongest ACE inhibitory activity with IC_{50} value of 2.86±0.08 µM (Rao *et al*., 2012). The sequence of the peptide has the structure-activity relationship described as above. Many similar tri-peptide sequences in other reports, such as Val-Ala-Pro (2 µM) (De Leo *et al*., 2009) and Ile-Met-Tyr (1.8 µM) (Matsui *et al*., 2002)

2.6 Purification and sequencing of ACE inhibitory peptides

ACE inhibitory peptides can be separated from the protein hydrolsate mixture by the various techniques of membrane-based reparation and chromatography techniques or various techniques in the research. For the example, the peptide Phe-Asn-Val-Pro-Leu-Tyr-Glu has been purified from salmon byproduct protein hydrolysate by alcalase hydrolysis. Ahn and his co-workers were loaded the protein hydrolysate to DEAE FF ion-exchange column. The active fraction was subjected to Sephadex G-25 gel filtration column. The active fraction was then subjected to reverse-phase HPLC on an ODS C_{18} column. The active peak which has the highest ACE inhibitory fraction was further purified by using the same column. Accurate molecular mass and amino acid sequence were determined by Hybrid Quadrupole-TOF LC/MS/MS mass spectrometer, sequence information was obtained by tandem MS analysis. (Ahn *et al*., 2012)

Moreover, ACE inhibitory peptide can be purified by another technique such as ultrafiltration by using ultrafiltration membrane bioreactor system with 30, 10 and 5 kDa of molecular weight cut-offs (MWCO) (Gao *et al*., 2010). Size-exclusion chromatography is based on their molecular size, also called gel filtration chromatography when operated with aqueous mobile phase or gel permeation chromatography when performed in organic mobile phases. Ion-exchange chromatography, capillary focusing and capillary electrophoresis were based on their charge properties.

Amino acid composition was studied by reversed-phase high performance liquid chromatography (RP-HPLC) system (Rozan *et al*., 2000), reversed phase columns are usually packed with bonding of octadecylsilyl coated silica. Organic solvents such as acetonitrile, methanol, and propanol were usually used as gradient elution mobile phase. Trifluoroacetic acid (TFA), is often added for improve the chromatographic peak shape into eluting solvents. The detection of amino acid was monitored at wavelength of UV visible with UV detector (Mohtar *et al*., 2012). To determination of the unknown

peptides, mass spectrometry chosen to determine the amino acid sequence and accurate molecular mass.

Two main techniques are electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) are adopted to determine the unknown peptides its call mass spectrometry techniques.

2.7 The Thai fruits seeds

Thai fruits are the unique identity and culture of Thailand for represents the fertility of the country. With the great physical geography and plentifully, the rich plant varities with good taste, nice quality of fruits which are beneficially. As mentioned above, the properties of the fruit Thailand that much more valuable, and in terms of healthy food. But the processing of fruits, canned, dried fruit or other products that made many wasted from fruits including seeds. The remaining seeds are mostly discarded without value or sold on the cheap to fuel production. From the previous study have been widely researches about the storage of nutrient in seed grains. The researcher possess that some kind of Thai fruits seeds such as papaya (*Carica papaya* L) longan (*Dimocarpus longan* Lour. subsp.), rambutan (*Nephelium lappaceum* L.), and lychee (*Litchi chinensis* Sonn.) might be the new source of bioactive protein hydrolysate. This study might be differently from that previously reported which is few research that reports a protein hydrolyzate containing bioactive in non-edible fruits seeds. It is extremely interesting to bring the research to find the active ingredient in protein hydrolysate, especially the new information to applies in the pharmaceutical industry or clinical research, and medicinal as an ingredient in the food industry, cosmetics industry, etc.

CHAPTER III

EXPERIMENTALS

3.1 Biological materials

The food processing waste, fresh 4 kinds of the Thai fruits seeds such as (i) *Carica papaya* L.; (papaya; unripe and ripe seeds), (ii) *Dimocarpus longan* Lour. subsp. (longan), (iii) *Litchi chinensis* Sonn. (lychee), and (iv) *Nephelium lappaceum* L. (rambutan) were obtained from canned fruit industry, Malee Sampran Public Co., Ltd. in Nakhon Pathom province and dried fruit industry, Kim Chua Group Co., Ltd. in Bangkok, Thailand. All the samples were quickly taken to the laboratory and kept in the dark and cold with 4 °C until used.

3.2 Chemical materials

Angiotensin Converting Enzyme ; ACE (E.C. 3.4.15.1) from rabbit lung, Bovine Serum Albumin (BSA), Hippuric acid as standard, Captopril as positive control, Hippuryl-L-Histidyl-L-Leucine (HHL) as substrate peptide, Other proteases, Pancreatin from porcine pancreas and Pepsin from porcine gastric mucosa were purchased from Sigma Chemicals Co. (USA). All other biochemicals and chemicals used in the investigation were of analytical grade.

3.3 Preparation of the crude extract from Thai fruits seeds

The preparation according to the method of Yodjun *et.al*., 2012, the 4 kinds of Thai fruits seeds were cleaned, pared, and removed the impurities and damaged seeds, then weight 1.5 kg (wet weight) to blended in 5 l of phosphate buffered saline (PBS; 20

mM phosphate buffer with 0.15 M NaCl pH 7.2) using a blender (Phillips, Indonesia) until homogenous texture and subsequently stirred overnight at 4 ^{o}C with an 4 fin propeller,using a low-speed agitator (IKA Labortechnik, Germany) at middle speed. The cheesecloth was used to filtration through double-layered to separate the suspension from the fluid and then the filtrate was clarified by centrifuged at 15,000 × g for 30 min at 4 ^{o}C, and the supernatant was harvested. Ammonium sulfate was slowly added with stirring to 80% saturation and subsequently stirred for overnight at 4 ^{o}C prior. The suspension was centrifuged at15,000 × g for 30 min at 4 ^{o}C to harvesting of the insoluble material (precipitate) as the crude extract. The crude extract was then dissolved in double-deionized water, dialyzed against excessive amounts of double-deionized water and lyophilized to yield as the crude protein.

3.4 Preparation of the protein hydrolysate

The crude proteins produced from the seeds of each fruit species were used as a substrate for production of the protein hydrolysate, by treatment with pepsin and pancreatin following the method of Magias *et. al*., 2006 with slightly modified. In brief, each crude protein was incubated with gastric enzyme pepsin until the final substrate/enzyme (v/w) concentration ratio was 20:1 and adjusted to pH 1.5 – 2.5 by 1M HCl. The hydrolysis was carried out for 180 min with shaking 180 rpm at 37 ^{o}C, and then inactivated the activity by adding 1 M NaOH to pH 7.0 - 8.0. The pancreatic enzyme, pancreatin was added to a 20:1 (v/w) substrate/enzyme ratio and shaken 180 rpm for 180 min at 37 ^{o}C. The hydrolysis (enzyme reaction) was stopped by heating at 80 ^{o}C for 20 min. Hydrolysates were clarified by centrifuged at 15,000 × g for 30 min at 4 ^{o}C for removed the insoluble. The supernatant was determanated the ACE inhibitory activity. The choice of these two proteases was to crudely mimic that in the human gastrointestinal tract.

3.5 ACE inhibitory activity assay

ACE inhibitory activity was measured according to the method of Je *et. al*., 2005 with slightly modified. 50 µl of crude proteins solution of 4 kinds of Thai fruits seeds was mixed with 50 µl of ACE (25 mU/ml) and pre-incubated at 37 ^{o}C for 10 min, after which time the mixture was re-incubated with 150 µl of substrate (10 mM HHL in PBS) for 30 min at 37 ^{o}C. Then, the reaction was stopped by adding 250 ml of 1M HCl. The hippuric acid was extracted with 500 µl of ethylacetate. After centrifugation at 15,000 × g and 4 ^{o}C for 15 min, 200 µl of the upper layer was transferred into another new test tube, and evaporated in a vacuum at room temperature. The hippuric acid was dissolved in 500 µl of double deionized water, measured the absorbance at 230 nm using an UV-spectrophotometer. A standard curve was constructed using a series of hippuric acid standards of known concentration to quantify the released hippuric acid in the assay mixture. Control use as non-inhibitor by using double deionized water instead. The inhibition potential can calculate by the equation below, and the concentration of ACE inhibitor required to inhibit 50% of the ACE activity under the above assay conditions was defined IC_{50}.

$$\% \text{ inhibition} = \frac{\text{A of contol} - \text{A of sample}}{\text{A of control} - \text{A of blank}} \times 100$$

3.6 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 (BSA) as the standard. The absorbance at 595 nm was monitored with a microplate reader.

3.7 Amino acid analysis

Whole seed of 4 kinds of freshThai fruits seeds were crushed with liquid nitrogen to fine powder. The measured methods according to Liu *et. al*., 1995 and Bosch *et. al*., 2006, the sample preparation conducted by weight sample into the test tube and added 5 ml of 6N HCl and place the reaction in heating block at 110 °C for 22 hrs. The internal standard was add into the hydrolysate and dilute with deionized water, mix the filtrate with AccQ-fluor derivatization buffer and AccQ-fluor reagent to derivatizing of the amino acid. Heat samples at 55 °C for 10 min in heating block. Five µl of samples were subjected to on a Hypersil Gold column C_{18} HPLC (4.6 x 150 mm, 3 µm, Waters Alliance) with 60% acetonitrile in sodium acetate buffer pH 4.90±0.05.

3.8 Inhibitory kinetics stud

To clarify the ACE inhibition pattern, the method of ACE inhibitory kinetics was used by according to Yodjun *et. al*., 2012. Different concentrations of substrate (1, 2, 3, 4, and 5 mM) and inhibitors (undiluted, diluted 2 folds, and 4 folds) were added to each reaction mixture and incubated with ACE at 37 °C. The inhibition kinetics of ACE in presence of protein hydrolysate was determined with Lineweaver-Burk plot.

3.9 pH resistance determination

To determine the pH resistance stability of protein hydrolysate, the method according to Rungsaeng *et. al*., 2013 by incubating the protein hydrolysates with broadly similar salinity levels by varies pH 2 -12 by buffers with ratio 1:4 (sample:buffer) at 37°C for 0, 30, 60, 90, and 120 min prior to assaying the ACE inhibitory activity. The buffers used in this experiment were 50 mM concentration are 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 (pH 8.0, 9.0 and 10.0) and glycine-NaOH (pH 10.0, 11.0 and 12.0). Control use as non-inhibitor by using double deionized water instead with the same ratio of buffers.

3.10 Temperature resistance determination

The thermostability of the protein hydrolysate were determined by method of Rungsaeng *et.al.*, 2013 with slightly modified, each 500 µl of protein hydrolysate was aliquot into 1.5 ml microfuge tubes and incubated at the designed temperatures as -20 °C (in ultra-low freezer), 0 °C (in freezer), 4 °C (in refrigerator), 10, 20 °C (in cooling chamber), 30, 40, 50, 60, 70, 80, and 90 °C (in water bath) for 0, 30, 60, 90, and 120 min. At every designed time take the sample vary quick to evaluate for ACE inhibitor activity assay.

3.11 Partial purification of the hydrolysate protein

The hydrolyzed protein was further applied to ultrafiltration (UF) according to method of Mohtar *et.al.*, 2012, the protein hydrolysate was fractionated into 3 parts (UF-1, UF-2, and UF-3) by the ultrafiltration membrane bioreactor system with 10 and 5 kDa molecular weight cut-offs (MWCO). UF-1 was not passed through the 10 kDa membrane. UF-2 was passed through the 10 kDa membrane but not passed through the 5 kDa membrane. UF-3 was passed through the 5 kDa. All of UF fractions were lyophilized in a freeze-drier for ACE inhibitory activity assay and protein content determination.

3.12 Isolation of ACEI peptides

After partial purified with ultrafiltration technique, UF-3 of protein hydrolsate of each species of Thai fruits seeds were fractionated by using reversed phase-hugh performance liquid chromatography (RP-HPLC, spectraSYSTEM, USA) on Shimpak C-

18 column (250 x 46 mm). The solvent system and conditions were according to Yodjun *et. al*., 2012. A linear gradient of acetonitrile from 0% to 70% containing 0.1% Trifluoroacetic acid (TFA) at flow rate 0.7 ml/min. 50 µl of fractionated sample (UF-3) were injected. The elution peaks were monitored at 280 nm and collected every minute, each single minutes of fraction was pooled and lyophilized.

3.13 Identification of ACEI peptides

The collected peaks of RP-HPLC fractions were re-suspended with 50% acetonitrile containing 0.1% formic acid and subjected to amaZon SL Ion Trap LC/MS/MS mass spectrometer (Bruker, MA, USA) coupled with ESI source. Instrumental control and data analysis were performed by using Bruker Daltonics trapControl version 7.0 and ESI campass 1.3 for amaZon DataAnalysis version 4.0. The spectra were recorded by the mass/charge (m/z) ranges of 200 – 1200 in both MS and MS/MS modes. The peptide sequencing module of the software calculations were used to process the MS/MS data and blast with Masco database.

3.14 Statistical analysis

All determinations, were done in triplicate, and the results are reported as the mean $\pm$ 1 standard error of the mean (SEM). Regression analyses and calculation of IC_{50} values was done using GraphPad Prism Version 6.00 for Windows (GraphPad Software Inc.).

CHAPTER IV

RESULT AND DISCUSSION

4.1 Screening of ACEI in seed samples

The potassium phosphate buffer extracted protein (crude extract), ammonium sulphate precipitated protein (crude protein) and the protein hydrolysates were determined for ACE inhibitory activity assay. Four Thai fruits seeds species from *Carica papaya* L.; (papaya; unripe and ripe seeds), *Nephelium lappaceum* L. (rambutan), *Dimocarpus longan* Lour. subsp. (longan), and *Litchi chinensis* Sonn. (lychee) were chosen the be observe in this experiment. The inhibition potential was reported as IC_{50} (the half maximal (50%) inhibition concentration (IC) of a substance) values which are calculated from regression equation derived from the percent inhibition versus protein concentration of sample (Table 2).

From Table 2, *L. chinensis* Sonn. (lychee) show the greatest ACE inhibitory activity in protein hydrolysate form with IC_{50} value at 0.22±0.010 mg protein / ml) follow by *D. longan* Lour. subsp. (longan) and *C. papaya* L. (in unripe form) with IC_{50} values at 0.74±0.006 and 1.04±0.002 mg protein / ml. The worst ACEI activities were *C. papaya* L. (in ripe form) and *N. lappaceum* L. (rambutan) with no detection of inhibition of the activity. Thus, the 3 kinds of fruit seeds with the IC_{50} measurable (unripe papaya seeds, longan seeds, and lychee seeds) were considered to characterization determination.

Table 4.1 The *in vitro* ACE inhibitory activity of crude extract, crude protein and protein hydrolysate of 4 kinds of Thai fruits seeds.

Plant seed species	IC_{50} values		
	crude extract (mg protein/ml)	**crude protein (mg protein/ml)**	**protein hydrolysate (mg protein/ml)**
C. papaya L. (papaya)			
Unriped	ND	1.43±0.012	1.04±0.002
Riped	ND	ND	ND
N. lappaceum L. (rambutan)	ND	ND	ND
D. longan Lour. subsp. (longan)	0.35±0.002	0.88±0.002	0.74±0.006
L. chinensis Sonn. (lychee)	ND	0.23±0.002	0.22±0.010

ND = Not detected

All data are shown as the average mean ± 1 SEM and are obtained from 3 replicated determinations.

4.2 Mechanism of the inhibition

The inhibition mode of ACEIs from Thai fruits seeds were evaluated by kinetic studies. Table 3, show the inhibition mode of the protein hydrolysate of each fruit seeds samples. From the table, that indicating 2 types of inhibition mode, the uncompetitive inhibition (unripe, papaya, and lychee seeds) which binding to the ACE-substrate complex not to free enzyme (Plamer, 2001), and the non-competitive inhibition (longan seeds) which binding with an enzyme molecule to produce dead-end complex by binding the different sites from the substrate (Ahn *et al*., 2012). These inhibition mode obtained by Lineweaver-Burk plots, the K_i values were determined are 6.02, 2.82, and 5.62 mg protein / ml, respectively. The K_m value, settle the ACE as the active enzyme and HHL as the substrate, was 0.04 mM with V_{max} was 7.0042 mM / min.

Most of the ACEIs that were derived from food protein hydrolysates belong to the competitive type such as natto (Akiko, Hiroshi, and Eiko., 1994) and fermented oyster sauce (Je *et al*., 2004). Cause by the competitive inhibitors are able to enter to the active center of ACE and interact with the active sites and prevent substrate to binding (Katayama *et al.*, 2008), Ruiz, Ramos and Recio., 2004), Rao *et al*., 2011). Some of isolated peptides show the non-competitive inhibition such as Pacific cod skin gelatin protein hydrolysate by using gastrointestinal enzymatic hydrolysis (Himaya *et al*., 2012) and uncompetitive inhibition in F7 of hen egg white lysozyme (HEWL) hydrolyzed by trypsin and papain with separated by RP-HPLC (Asoodeh *et al*., 2011).

Table 4.2 Inhibition modes with K_i of the 3 kinds of Thai fruits seeds protein hydrolysates

Plant seed species	**Inhibition mode**	K_i **(mg protein/ml)**
C. papaya L. (unripe papaya)	Uncompetitive	6.02
D. longan Lour. subsp. (longan)	Non-competitive	2.82
L. chinensis Sonn. (lychee)	Uncompetitive	5.62

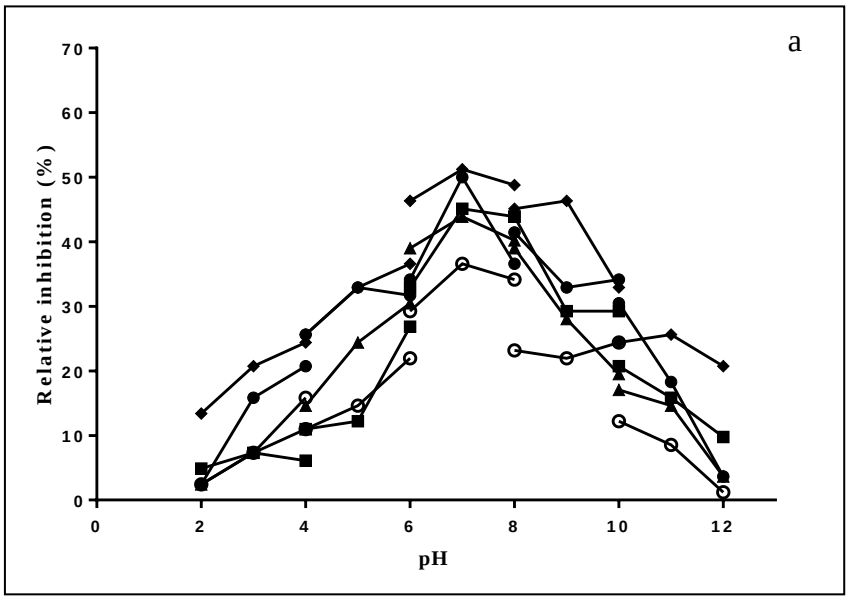
All data are shown as the average mean ± 1 SEM and are obtained from 3 replicated determinations.

4.3 pH resistance of the ACEIs

After incubated the protein hydrolysate with alter broad pH range of buffer from 2 – 12. Figure 4.1 showed the optimal ACEI activity unripe papaya seeds (fig. 4.1a) were at pH 6 – 8 in potassium phosphate buffer. The optimum pH of longan seeds (fig. 4.1b) was at pH 6 – 8, and the optimum pH of lychee seeds was at the range of 6 – 8 too (fig. 4.1c).

All of the samples had the excellent inhibition activity at 0 min after the incubation, the decreasing of activity affected from the extensive incubation time (30, 60, 90, and 120 min). This broad pH range makes the potentially excellent of enzyme for pharmaceutical industry and food derived proteins. At the other pH which had poor inhibition activity may suggested some of ion in buffer might slow down or block the ACEI activity at each pH values or the high and low excessively pH might destroyed or degraded ACEI activity peptides.

Yodjun et.al. (2012) reported the F75 of *Zingiber ottensii* rhizome showed the optimal ACEI activity at pH 4-5 and 8-11. Rungseang *et. al.* (2013) reported *Z. officinale* (post-DEAE cellulose unbound fraction) showed the optimal acetylcholinesterase inhibitory activity at pH 2-9 and 10-12.



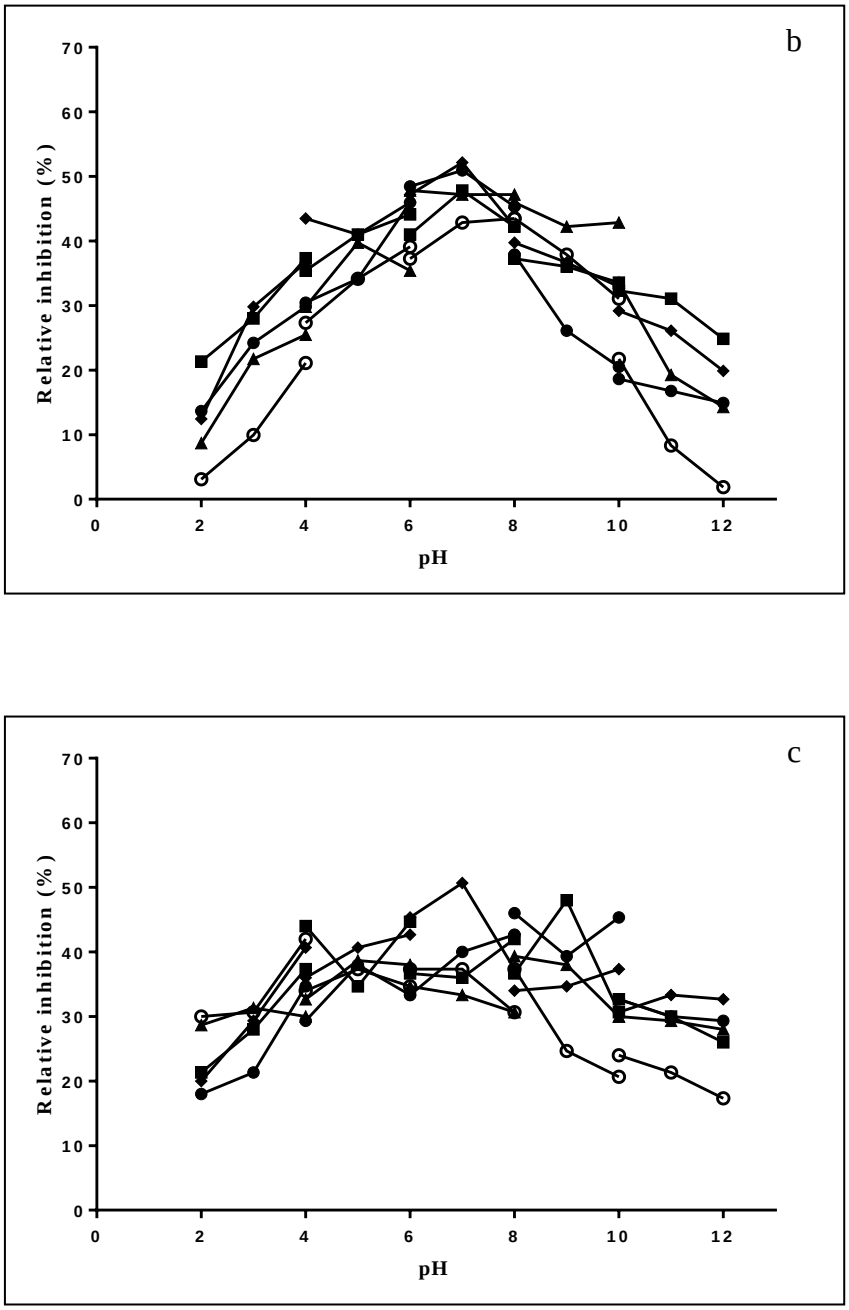
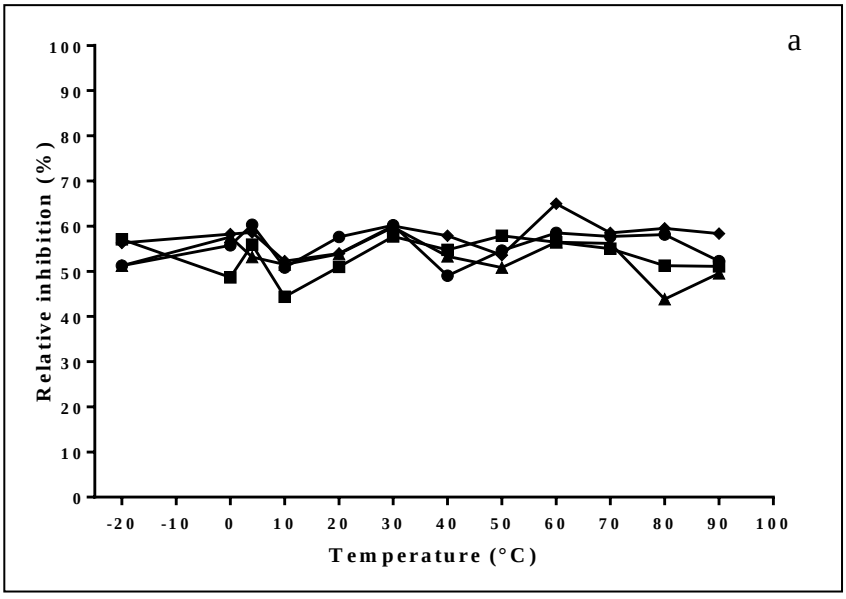


Figure 4.1 pH stability of ACEIs; a) unripe papaya seeds; b) longan seeds; and c) lychee seeds with various incubation times; 0 min (diamond); 30 min (close circle); 60 min (square); 90 min (triangle); and 120 min (open circle). All data are shown as the average mean ± 1 SEM and are obtained from 3 replicated determinations.

4.4 Temperature resistance of ACEI

The thermal stability of the ACEIs from various Thai fruits seeds protein hydrolysates are showed in Figure 4.2 (a – c). The relative inhibition activity of the ACEIs were wide in temperature range, most of ACEIs are giving the prominence relative percent inhibition are at -20 – 80 °C and decreased the inhibition ability at 90 °C with various incubated times (30, 60, 90, and 120 min). The higher temperature and longer incubation may cause the changing of the ability of ACEIs regions of the protein structure to binding enzyme.

From the previous studied, Yodjun *et.al*. (2012) reported the F75 of *Z. ottensii* rhizome showed the optimal ACEI activity at -20 - 60 °C. Rungseang *et. al.* (2013) reported *Z. officinale* (post-DEAE cellulose unbound fraction) showed the optimal acetylcholinesterase inhibitory activity at -20 – 60 °C.



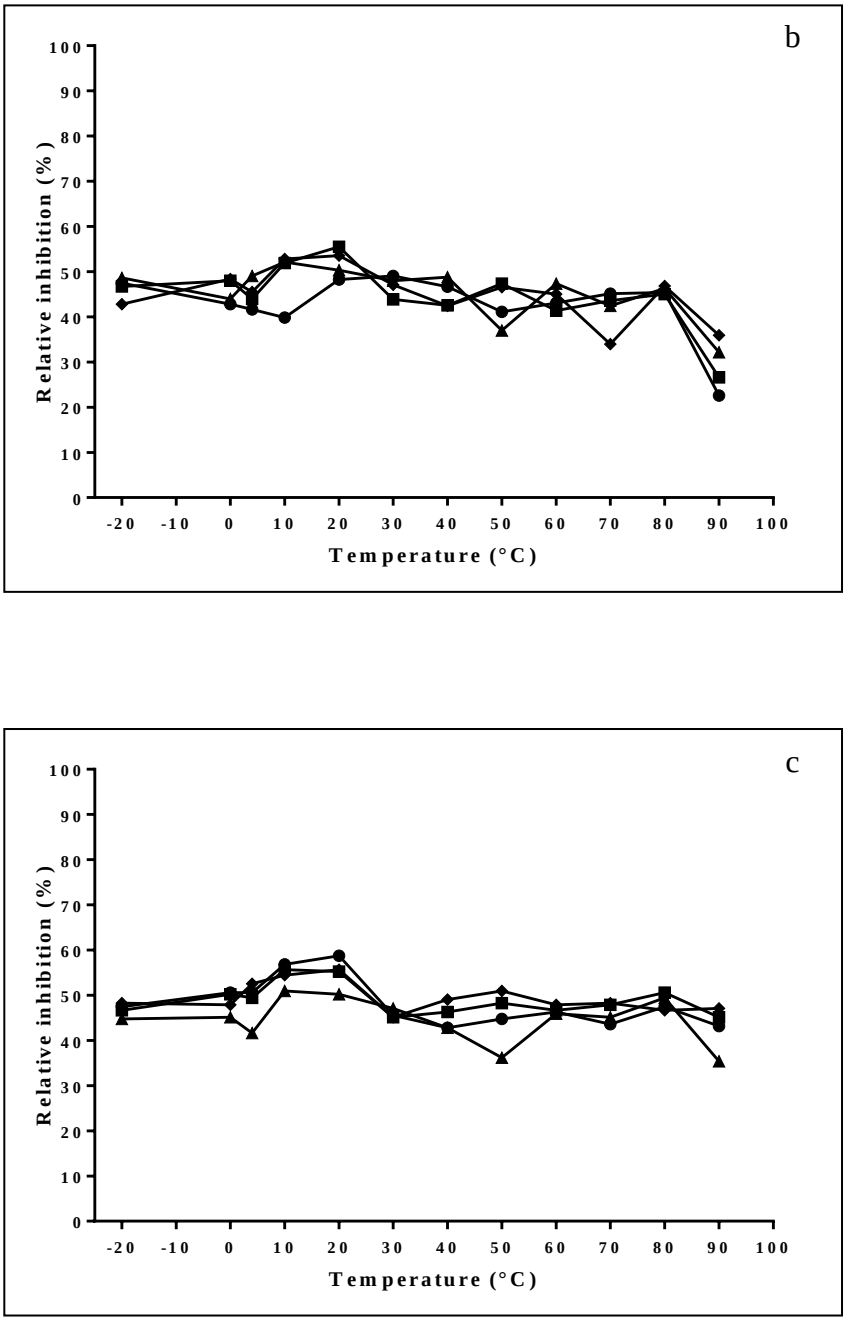


Figure 4.2 Thermostability of ACEIs; a) unripe papaya seeds; b) longan seeds; and c) lychee seeds. With varies time; 30 min (diamond); 60 min (close circle); 90 min (square); and 120 min (triangle). All data are shown as the average mean ± 1 SEM and are obtained from 3 replicated determinations.

4.5 Partial purification of the hydrolysate proteins

Protein hydrolysates were prepared from the crude protein of 4 kinds of Thai fruits seeds with pepsin and pancreatin were collected for further fractionation by ultrafiltration. Table 4.3 show the protein hydrolysates fractionated into 3 parts as UF-1 (>10 kDa), UF-2 (5-10 kDa), and UF-3 (<5 kDa) by ultrafiltration membrane bioreactor system. Table 4.3 also show the ACEIs activity of ultrafiltration fractions varied with the molecular mass distribution, the ACEIs activity were increased with decreasing of molecular weight cut-off. Thus, the low molecular weight of peptides indicated the higher inhibition activity than the high molecular weight. The UF-3 (< 5 kDa) of longan seeds protein hydrolysate showed the most potent of ACEI activity with IC_{50} value at 0.43±0.011 mg protein / ml. This fraction was further subjected into high performance liquid chromatography (HPLC).

Won-Ko *et al*. (2006) reported the yellowfine sole (*Limanda aspera*) frame protein hydrolysate were fractionated by ultrafiltration membrane bioreactor system into 3 ranges of MWCO as YFPH-I (30-10 kDa), YFPH-II (10-5 kDa), and YFPH-III (< 5 kDa) resulting the YFPH-III had the highest ACEI activity with an IC_{50} value of 0.883 mg protein / ml. Mohtar *et al*. (2012) reported from the winged bean (*Psophocarous tetragonolobus*) protein hydrolysate by four proteolytic enzymes as flavourzyme, alcalase, bromelain and papain was separated by ultrafiltration membrane bioreactor system with MWCO 10, 5 and 2 kDa and found that the 2 kDa had the highest ACEI activity with an IC_{50} value of 0.003 and 0.130 mg protein / ml.

Table 4.3 ACEIs activities of the protein hydrolysates fractionated by ultrafiltration.

Plant seed species	IC_{50} values		
	UF-1 (> 10 kDa) (mg protein/ml)	**UF-1 (10-5 kDa) (mg protein/ml)**	**UF-3 (< 5 kDa) (mg protein/ml)**
C papaya L. (unripe papaya)	19.77±0.011	4.68±0.007	ND
D. longan Lour. subsp. (longan)	9.25±0.017	1.95±0.006	0.43±0.011
L. chinensis Sonn. (lychee)	ND	1.47±0.005	ND

ND = Not detected

All data are shown as the average mean ± 1 SEM and are obtained from 3 replicated determinations.

4.6 Isolation of ACEI peptides

After partial purified with ultrafitration technique, UF-3 of longan seeds protein hydrolysate was the most ACEI activity potency. Thus, this fraction was further analysis by subjected to RP-HPLC on a Shimpak C_{18} column by using trifluoroacetic acid/acetonitrile solvent system to separation of the peptides. Figure 4.3 showed 5 peaks were eluted, the elution peaks was detected at 280 nm. The fractions were separately collected and named as P1 - P5. Each fractions were collected by subsequently retention time. P1 was collected at 3 - 5 min; P2 was collected at 5 - 7 min; P3 was collected at 7 - 8 min; P4 was collected at 8 - 10 min; and P5 was collected at 15 - 17 min, respectively. After purified, P1 - P5 were further analyzed by LC/MS/MS to identify the amino acid sequences.

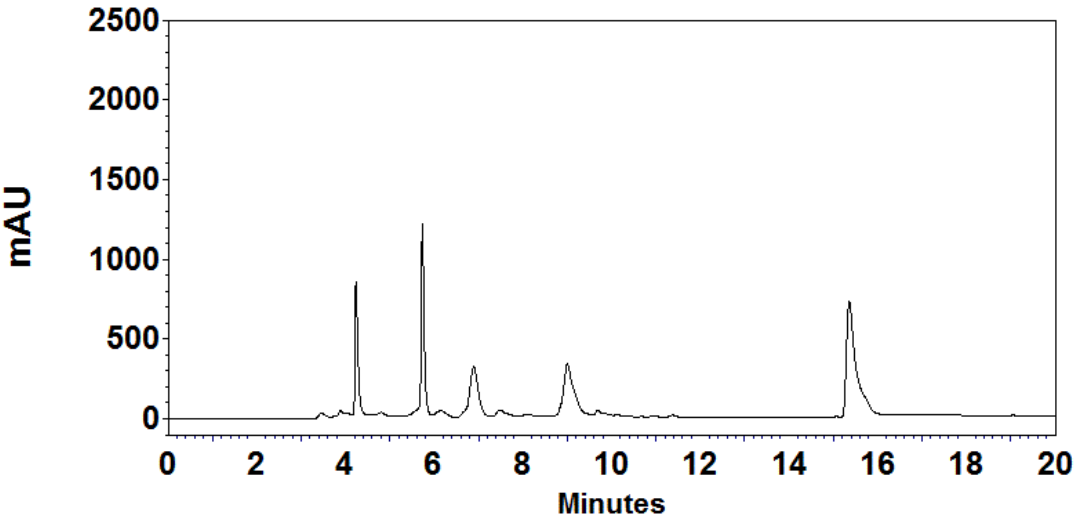


Figure 4.3 Preparative RP-HPLC profile of UF-3 longan seeds protein hydrolysate.

4.7 Identification of ACEI peptides

From RP-HPLC fractionated, five fractions were subjected to LC/MS/MS analysis for determination of the sequences of the peptides contained. The sequences of peptides identified are P1-F1 (Glu-Thr-Ser-Gly-Met-Lys-Pro-Thr-Glu-Leu) was related to Varicose-related protein in *Arabidopsis thaliana* and P1-F2 (His-Asp-Met-Arg-Ser-Cys-Cys-Val-Asp-Ile-Asp-His-Val-Ser-Leu-Tyr-Asn-Leu) was related to Pentatricopeptide repeat-containing protein At2g39620 in *A. thaliana*. The peptides sequence of P2 is P2-F1 (Leu-Val-Ser-Seer-Asp-Pro-Asp-Ile-Ser-Gln-Arg-Met-Phe) was related to enzyme Nicotianamine synthase in *Noccaea caerulescens*; the peptides sequences of P3 are P3-F1 (Ile-Ser-Ser-Met-Gly-Ile-Leu-Val-Cys-Leu) was related to enzyme vacuolar proton-pyrophosphatase in *Potamogeton distinctus*, P3-F2 (Thr-Asn-Gln-Val-Val-Ser-Glu-Met-Gly-Ile-Ala-Ala-Gly-Ala-Ala-Leu) was related to hypothetical protein OsI_04393 in *Oryza sativa* Indica Group, P3-F3 (Val-Arg-Ala-Met-Val-Ala-Glu-Cys-Leu) was related to hypothetical protein CARUB_v10000363mg in *Capsella rubella*, and P3-F4 (Ile-Ser-Tyr-Val-Val-Pro-Val-Tyr-Ile-Ala-Glu-Ile-Thr-Pro-Lys-Thr-Phe-Arg-Gly-Gly-Phe) was related to Beta integral membrane protein (gb|U43629) in *A. thaliana*. The peptides sequences of P4 are P4-F1 (Thr-Leu-Ala-Met-His-Tyr-Phe) was related to ferric reductase-like transmembrane component family protein in in *A. thaliana* and P4-F2 (Arg-Ser-Ile-Arg-Ile-Thr-Gly-Phe-Gly-Ser-Ser-Ser-Asp-Leu) was related to scarecrow transcription factor family protein in *A. lyrata* subsp. *lyrata*.

The previously has been reported that the ACEIs properties of peptides contain the positive charged amino acids (arginine/lysine) at C-terminal is important for ACE inhibition (Meisel, 1998). Moreover, it been reported about the positively charged amino acids in the middle position of tri-peptides had a stronger inhibition activity. For example, Ile-Arg-Tyr showed inhibitory activity five times stronger than Ile-Gln-Tyr (Majumder and Wu, 2010). From other studied, ACE might prefers to have substrate or inhibitors that contain tryptophan, tyrosine, phenylanine, proline, and a hydrophobic amino acid at the first three C-terminal position residues to contribute the inhibitory potency (Cheung *et al*., 1980; Wu *et al*., 2006)

From the results, the peptides sequences of UF-3 longan seeds protein hydrolysate were indicated the most of peptides contained hydrophobic amino acid at the C-terminal and positive charged amino acids in the middle of the peptides were P1-F1 (Glu-Thr-Ser-Gly-Met-Lys-Pro-Thr-Glu-Leu), P3-F1 (Ile-Ser-Ser-Met-Gly-Ile-Leu-Val-Cys-Leu), and P3-F4 (Ile-Ser-Tyr-Val-Val-Pro-Val-Tyr-Ile-Ala-Glu-Ile-Thr-Pro-Lys-Thr-Phe-Arg-Gly-Gly-Phe), these peptides were possibility the most inhibitory activity peptides. Thus, these peptides were decided to synthesis for further analysis.

4.8 Amino acid profile

The total amino acid contents of the crude protein of five kinds of Thai fruits seeds calculated on dry weight are shown in Table 4.4. The amino acid profile showed the amount of hydrophilic amino acids (unripe papaya seeds = 13.36, ripe papaya seeds = 12.87, rambutan seeds = 3.2, longan seeds = 2.63, and lychee seeds = 1.92 mg / 100mg protein) are higher than the hydrophobic amino acids (unripe papaya seeds = 8.48, ripe papaya seeds = 8.3, rambutan seeds = 2.69, longan seeds = 2.16, and lychee seeds = 1.75 mg / 100mg protein).

From the result, the high content of positive charge amino acids (arginine/lysine) is the two forms of papaya seeds, following by rambutan seeds, longan seeds, and lychee seeds, respectively. The high content of hydrophobic amino acids is two forms of papaya seeds, the moderate contents are rambutan seeds and longan seeds, with lychee seeds had moderate hydrophobic amino acids content.

Table 4.4 Amino acids content of four kinds of Thai fruit seeds

Amino acids	Contents (mg / 100 mg protein dry weight)				
	unripe papaya seeds	**ripe papaya seeds**	**rambutan seeds**	**longan seeds**	**lychee seeds**
Hydrophilic					
Aspartic acid	3.52	2.93	0.57	0.49	0.39
Serine	0.88	0.89	0.37	0.30	0.24
Glutamic acid	3.55	3.28	0.89	0.55	0.43
Histidine	0.36	0.28	0.13	0.11	0.09
Arginine	1.66	1.56	0.54	0.54	0.29
Threonine	0.92	1.02	0.32	0.29	0.24
Lysine	2.47	2.91	0.38	0.35	0.24
Total	13.36	12.87	3.2	2.63	1.92
Hydrophobic					
Glycine	0.50	0.41	0.63	0.44	0.26
Alanine	0.72	0.37	0.26	0.20	0.24
Proline	1.98	2.96	0.28	0.25	0.20
Tyrosine	2.29	2.38	0.18	0.14	0.09
Valine	0.74	0.53	0.39	0.29	0.26
Isoleucine	0.62	0.46	0.24	0.20	0.18
Leucine	0.92	0.68	0.42	0.36	0.30
Phenylalanine	0.71	0.51	0.29	0.28	0.22
Total	8.48	8.3	2.69	2.16	1.75

CHAPTER V

CONCLUSSION

ACE inhibitory peptides were produced from 4 kinds of the Thai fruits seeds, unripe and ripe papaya seeds, rambutan seeds, longan seeds, and lychee seeds. The crude proteins were hydrolysis by stimulation of human digestion by gastric enzyme as pepsin and pancreatin. The hydrolysates were partial purified by ultrafiltration technique with MWCO at 10 and 5 kDa to 3 size parts. After fractionated, UF-3 of longan seeds protein hydrolysate (below 5 kDa) had the highest ACE inhibitory activity potential (IC_{50} with 0.43±0.011 mg protein / ml). Thus, this fraction were subjected to RP-HPLC, the collected peak named P1-P5 were subjected to LC/MS/MS for identify the peptides sequences. Mass spectra showed 9 peptide sequences, but only P1-F1, P3-F1, and P3-F4 were possibility the most inhibitory activity peptides. Thus, these peptides were decided to synthesis for further analysis. This was the first study to show the production of antihypertensive peptides by enzymatic hydrolysis of protein from the seeds extract of Thai fruits. The result suggested that some Thai fruits could be a source of peptides that might be a potent source of ACEI bioactive compounds.

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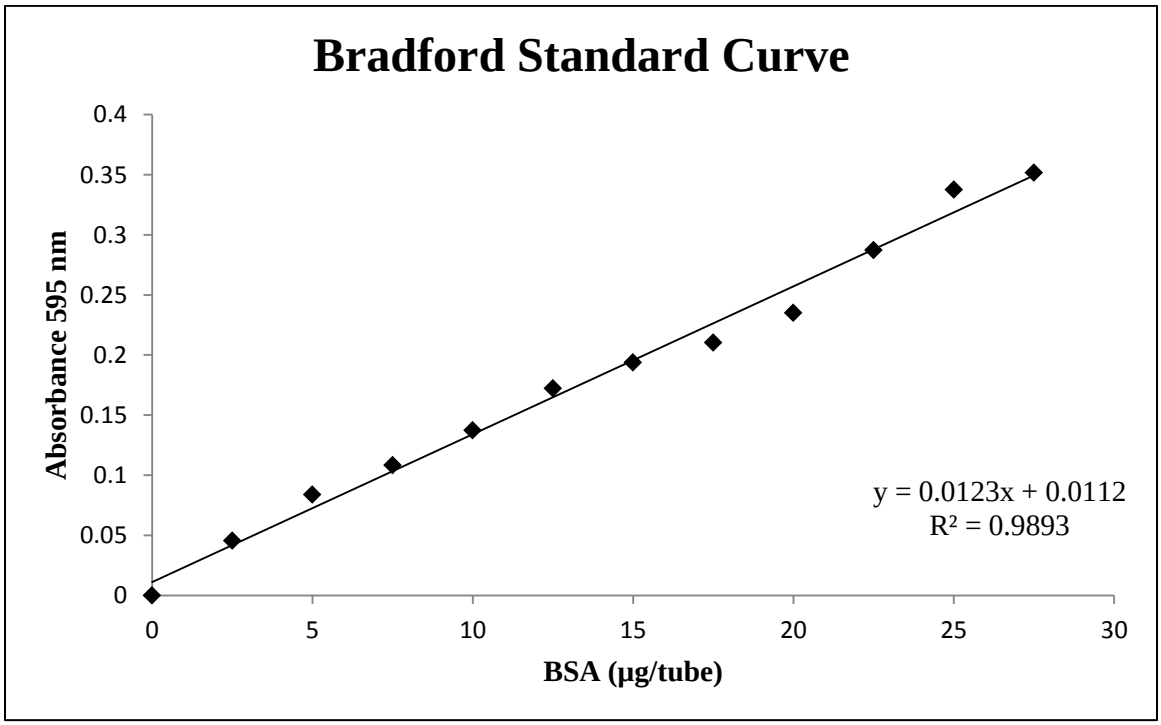
APPENDICES

APPENDIX A

Calibration curve for protein determination by Bradford method



APPENDIX B

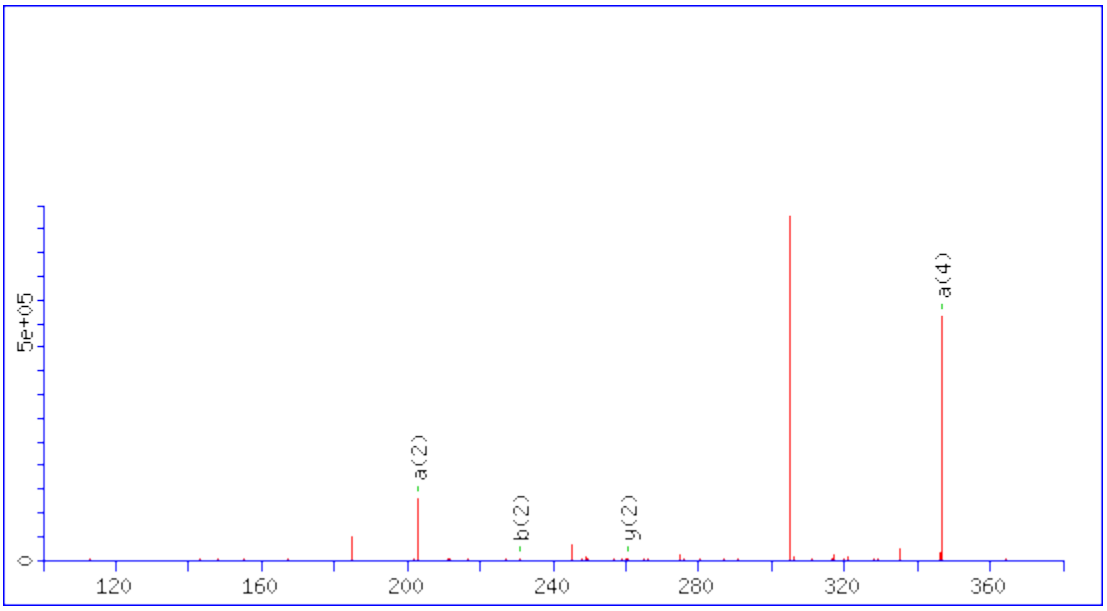
Amino acid abbreviations

Amino acid	Three-letter	One-letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
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

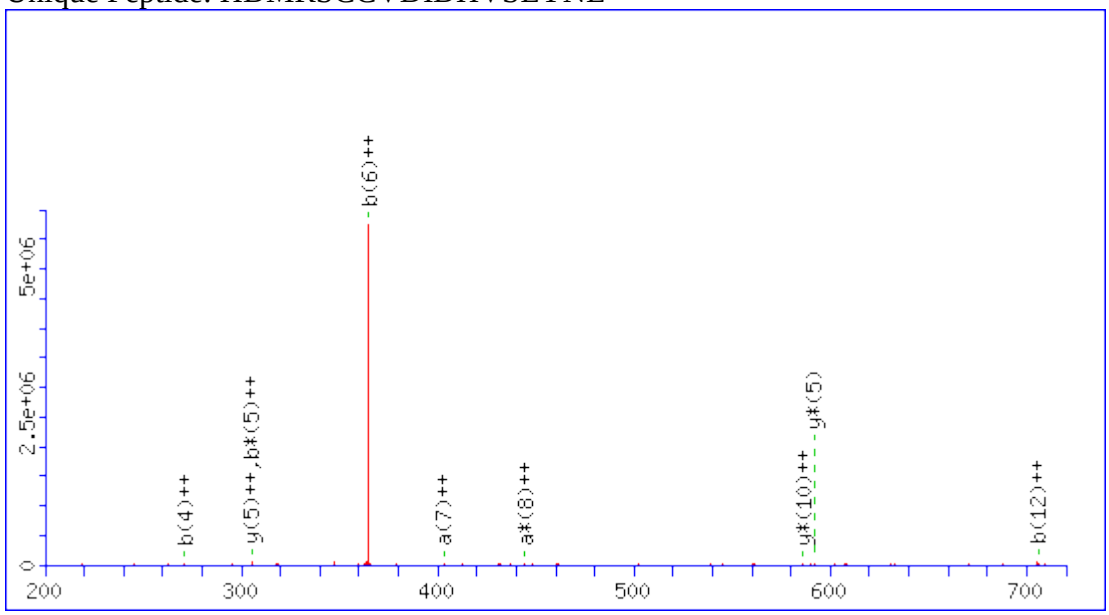
APPENDIX C

Molecular weight of 4 peaks from MS/MS spectrum of the UF-3 from longan seeds protein hydrolyste by RP-HPLC.

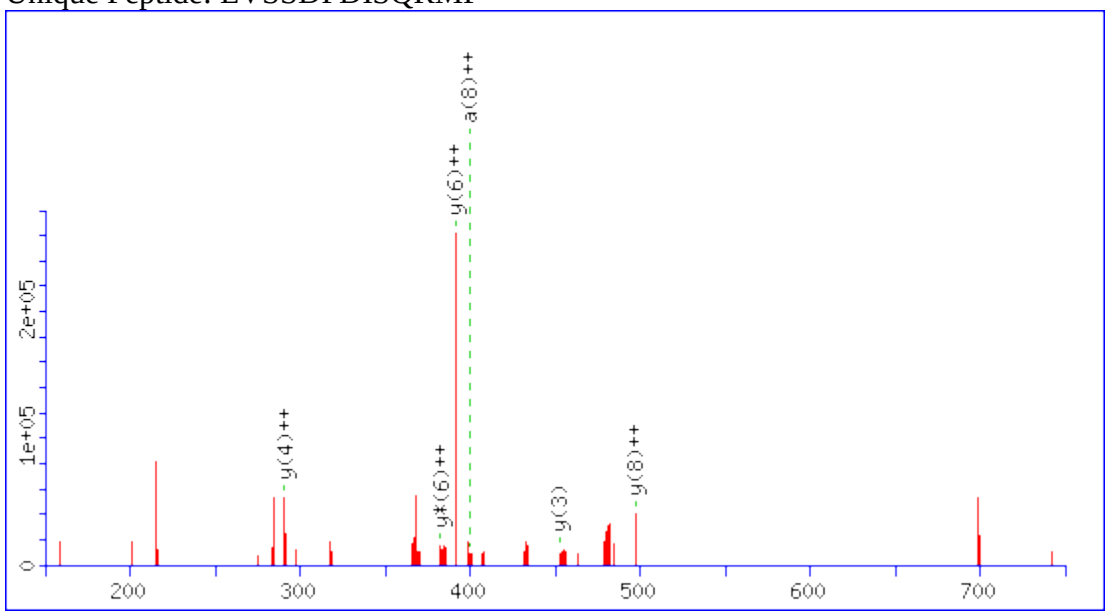
P1-F1
Observed: 365.0000
Mr(expt); 1091.9782
Mr(calc); 1091.5169
Unique Peptide: ETSGMKPTEL



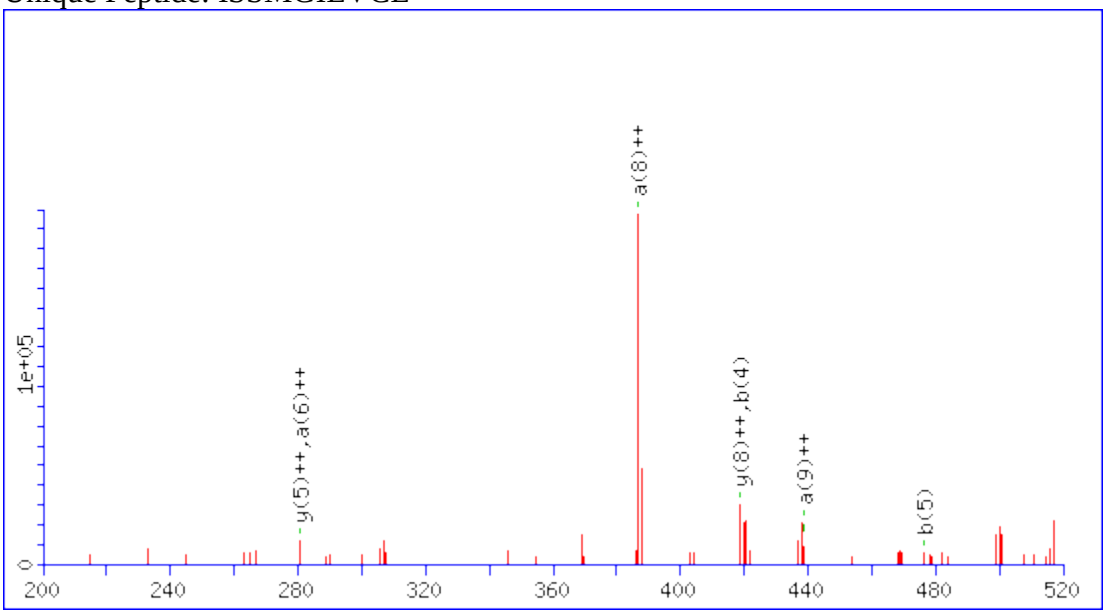
P1-F2
Observed: 707.0000
Mr(expt): 2117.9782
Mr(calc): 2118.9285
Unique Peptide: HDMRSCCVDIDHVSLYNL



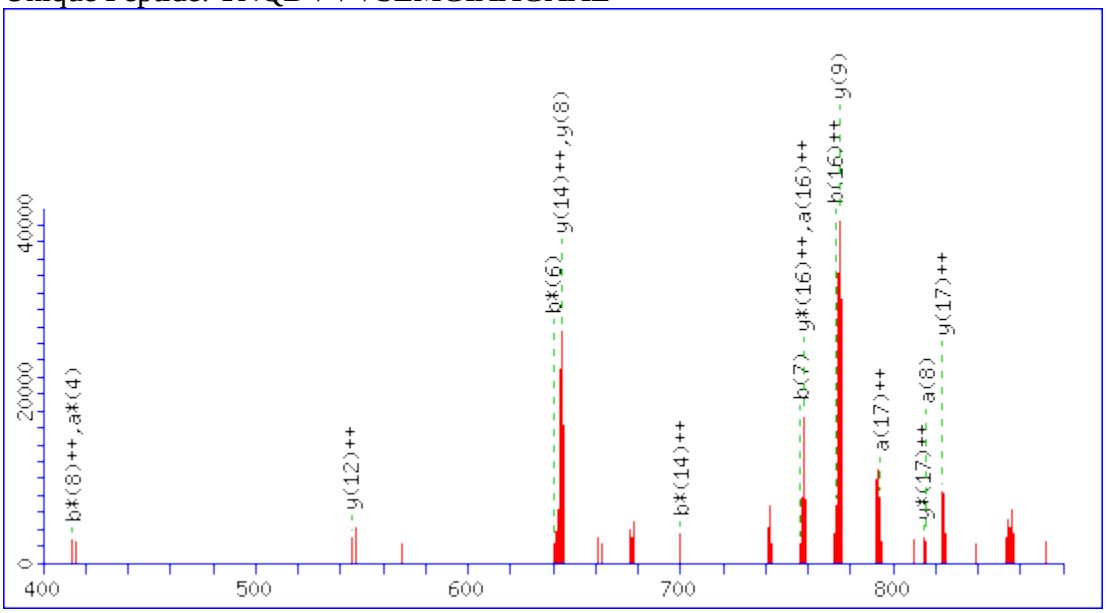
P2-F1
Observed: 498.7000
Mr(expt): 1493.0782
Mr(calc): 1493.7184
Unique Peptide: LVSSDPDISQRMF



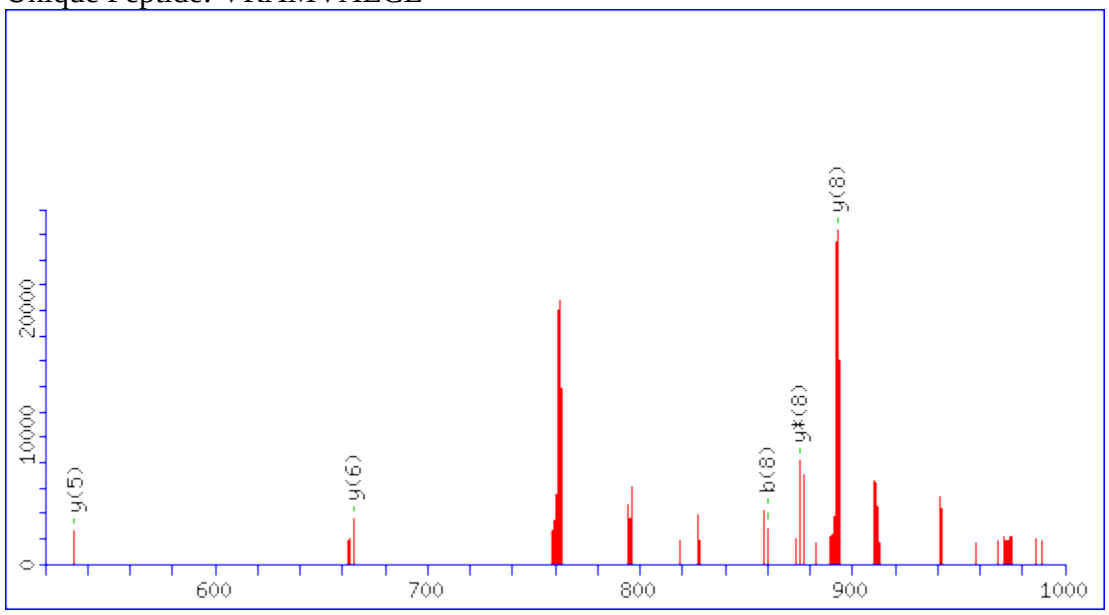
P3-F1
Observed: 518.1000
Mr(expt): 1034.1854
Mr(calc): 1034.5504
Unique Peptide: ISSMGILVCL



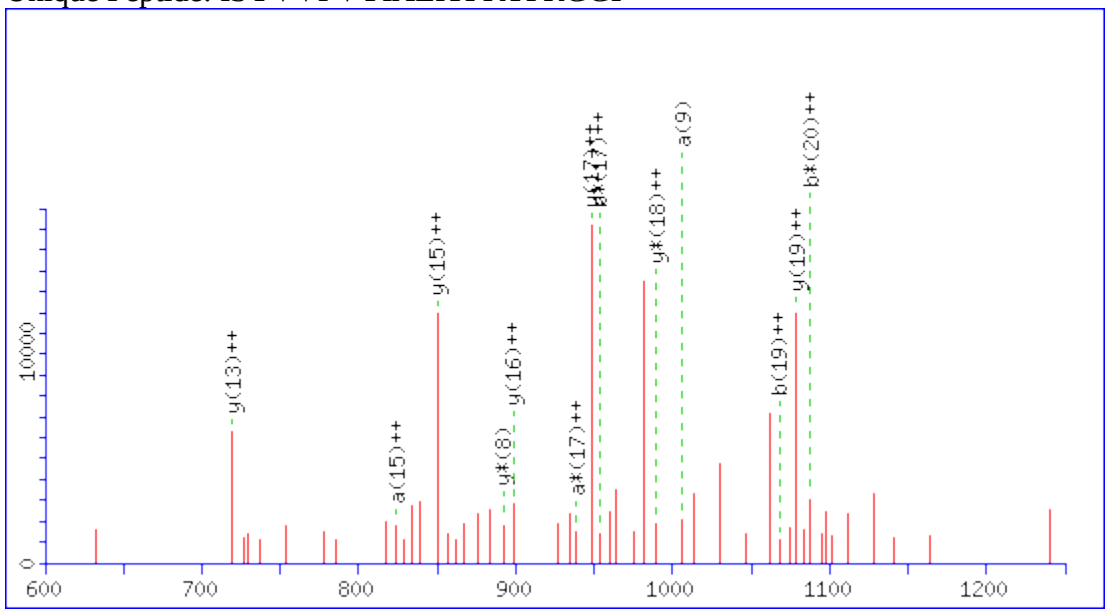
P3-F2
Observed: 874.0000
Mr(expt): 1745.9854
Mr(calc): 1744.8665
Unique Peptide: TNQDVVVSEMGIAAGAAL



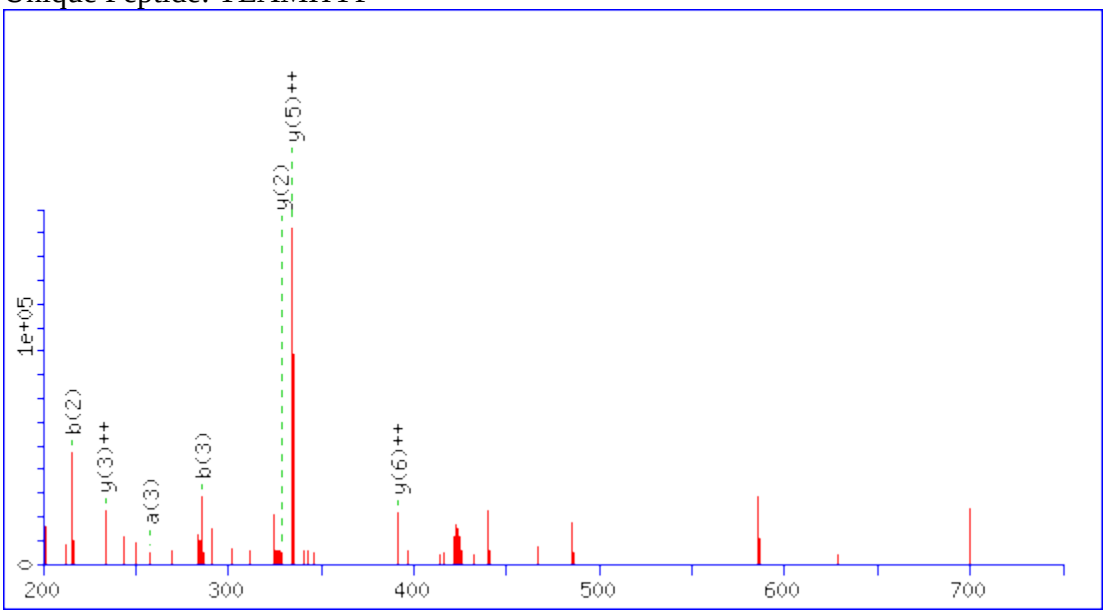
P3-F3
Observed: 992.0000
Mr(expt): 990.9927
Mr(calc): 990.4990
Unique Peptide: VRAMVAECL



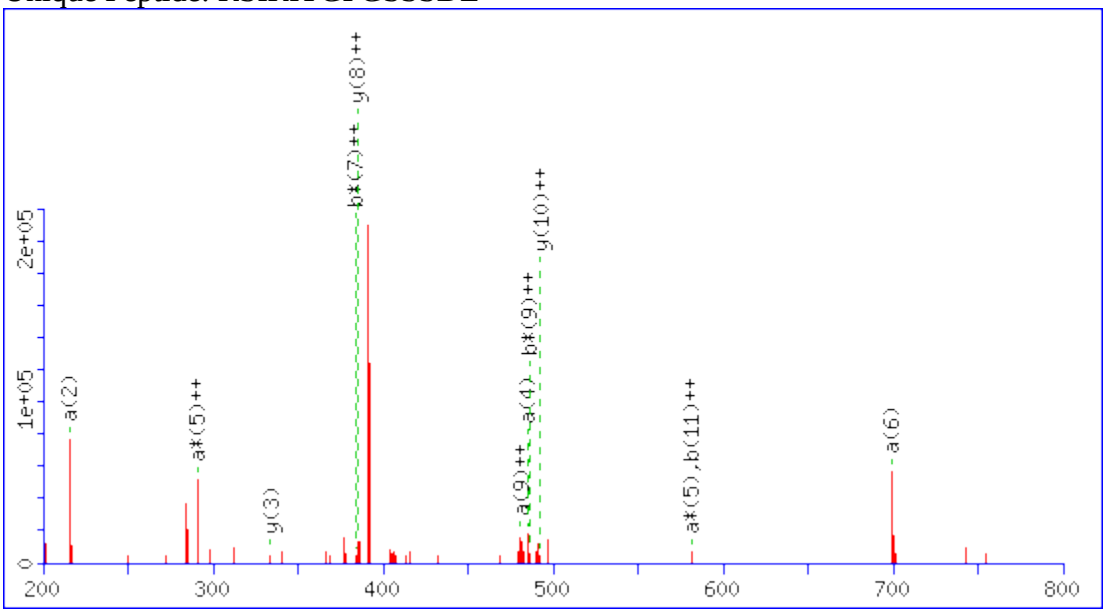
P3-F4
Observed: 1179.0000
Mr(expt): 2355.9854
Mr(calc): 2356.2831
Unique Peptide: ISYVVPVYIAEITPKTFRGGF



P4-F1
Observed: 442.0000
Mr(expt): 881.9854
Mr(calc): 881.4106
Unique Peptide: TLAMHYF



P4-F2
Observed: 499.0000
Mr(expt): 1493.9782
Mr(calc): 1494.7791
Unique Peptide: RSIRITGFGSSSDL



BIOGRAPHY

Mr. Atthasith Nuchprapha was born on 6 July 1988 in Suphanburi and he was raised in Angthong, Thailand. He graduated the secondary school from Angthong Pattamaroj Witthayakhom School and graduated with a Bachelor's Degree of Science in Microbiology from Department of Biology, Faculty of Science, Srinakharinwirot University since 2011

Academic presentations;

1. Nuchprapha, A., Karnchanatat, A. Angiotensin I-converting enzyme inhibitory activity of protein hydrolysate from seeds of Thai fruits, 13th FAOBMB International Congress of Biochemistry and Molecular Biology, 25-29 November 2012, Bangkok, Thailand.