โปรตีนที่มีฤทธิ์ทางชีวภาพจากเหง้าไพลดำ *Zingiber ottensii* Valeton

นายณัฐชัย เที่ยงบูรณธรรม

BIOACTIVE PROTEIN FROM RHIZOMES OF *Zingiber ottensii* Valeton

Mr. Nathachai Tiengburanatam

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
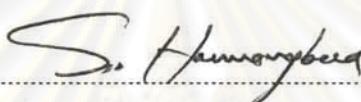**Faculty of Science**
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Thesis Title   BIOACTIVE PROTEIN FROM RHIZOMES OF *Zingiber ottensii* Valeton

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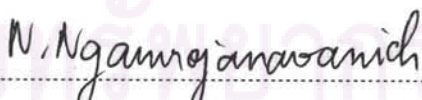
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ฉัฐชัย เที่ยงบูรณธรรม: โปรตีนที่มีฤทธิ์ทางชีวภาพจากเหง้าไพลดำ *Zingiber ottensii* Valeton (BIOACTIVE PROTEIN FROM RHIZOMES OF *Zingiber ottensii* Valeton) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ดร. อภิชาติ กาญจนทัต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร. พลกฤษณ์ แสงวณิช, 78 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อค้นหาโปรตีนที่มีฤทธิ์ทางชีวภาพจากเหง้าไพลดำ และศึกษาลักษณะสมบัติของโปรตีนที่แยกได้ โดยทำการสกัดโปรตีนจากเหง้าไพลดำด้วยสารละลายบัฟเฟอร์ Tris-HCl 20 มิลลิโมลาร์ ที่ค่าความเป็นกรดด่าง 7.2 จากนั้นนำโปรตีนมาทำให้บริสุทธิ์โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัวที่ 80 เปอร์เซนต์ ทำโปรตีนให้บริสุทธิ์โดยเทคนิคโครมาโทกราฟีแบบแลกเปลี่ยนอิออนด้วยคอลัมน์ SP sepharose โดยชะแบบเป็นลำดับขั้นด้วยสารละลายบัฟเฟอร์ Tris-HCl 20 มิลลิโมลาร์ ที่มีโซเดียมคลอไรด์ 1 โมลาร์ ที่ค่าความเป็นกรดด่าง 7.2 สามารถแยกโปรตีนสกัดหยาบได้เป็น 5 ส่วน คือ unbound F25 F50 F75 และ F100 ตามลำดับ โดยส่วน F50 มีปริมาณโปรตีนสูงที่สุด เมื่อใช้เทคนิคพอลิอะคริลาไมด์เจลอิเล็กโตรฟอเรซิสแบบไม่เสียสภาพ และเสียสภาพ พบว่าส่วนของ F50 เป็นโปรตีนบริสุทธิ์ที่มีอย่างน้อยสามหน่วยย่อยขนาด 32.5 15.2 และ 13.8 ตามลำดับ ผลการย้อมสีแบบ Periodic staining พบว่าเป็นไกลโคโปรตีน และผลทดสอบหาปริมาณคาร์โบไฮเดรตด้วยวิธี phenol-sulfuric พบว่ามีสัดส่วนเป็นคาร์โบไฮเดรตถึง 26.30 ± 1.01 เปอร์เซ็นต์โดยน้ำหนัก เมื่อวิเคราะห์ลำดับกรดอะมิโนภายในโปรตีนชนิดนี้พบว่า ลำดับของกรดอะมิโนที่ได้มีความคล้ายกับซิสเตอีน โปรติเอส ผลการทดสอบฤทธิ์ทางชีวภาพพบว่า โปรตีนชนิดนี้มีกิจกรรมของการยับยั้งแอลฟา-กลูโคซิเดสสูง เมื่อศึกษาจลนพลศาสตร์ของกิจกรรมยับยั้งแอลฟา-กลูโคซิเดส จะได้เปอร์เซ็นต์การยับยั้งสูงสุดที่ 77.5 เปอร์เซ็นต์ ค่า IC_{50} เท่ากับ 30.15 ไมโครกรัมต่อมิลลิลิตร และค่า K_i เท่ากับ 140 ไมโครโมล ส่วนค่า K_m เท่ากับ 2.35 ไมโครโมล และ V_{max} เท่ากับ 0.11 มิลลิโมลต่อนาที กิจกรรมของยับยั้งแอลฟา-กลูโคซิเดสของโปรตีนชนิดนี้ มีเสถียรภาพของกิจกรรมของการยับยั้งแอลฟา-กลูโคซิเดสของโปรตีนชนิดนี้ที่ค่าความเป็นกรด-ด่างเท่ากับ 2 จนถึง 10 และที่อุณหภูมิ 4 จนถึง 35 องศาเซลเซียส และสูงสุดที่ 65 องศาเซลเซียส โปรตีนชนิดนี้สามารถยับยั้งการเจริญเติบโตของเชื้อราโรคพืช *Collectotrichum cassiicola*, *Exserohilum turcicum* และ *Fusarium oxysporum* ได้ที่ความเข้มข้น 23.6 ถึง 47.2 ไมโครกรัม แต่ไม่สามารถยับยั้งการเจริญเติบโตของเชื้อจุลินทรีย์สี่ชนิด คือ *Bacillus subtilis*, *Candida albican*, *Escherichia coli* และ *Staphylococcus aureus* นอกจากนี้ยังพบว่าโปรตีนชนิดนี้ สามารถยับยั้งการเจริญของเซลล์มะเร็งตับชนิด HEP-G2 (IC_{50} = 1.130 ไมโครกรัม) และเซลล์มะเร็งลำไส้ชนิด SW620 (IC_{50} = 5.370 ไมโครกรัม) จากผลการศึกษาแสดงให้เห็นว่า สามารถแยกและศึกษาฤทธิ์ทางชีวภาพของไกลโคโปรตีนชนิดหนึ่งจากเหง้าไพลดำได้เป็นผลสำเร็จ

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต _____
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4973882123: MAJOR BIOTECHNOLOGY
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NATHACHAI TIENGBURANATAM: BIOACTIVE PROTEIN FROM RHIZOMES OF *Zingiber ottensii* Valeton THESIS ADVISOR: APHICHART KARNCHANATAT, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., 78 pp.

The objectives of this study were to find out bioactive protein from *Zingiber ottensii* rhizome and study properties of purified protein. Protein from the rhizome was isolated using 20 mM Tris-HCl buffer, salting out with 80% $(NH_4)_2SO_4$, and purified by stepwise eluted (1 M NaCl) SP sepharose chromatography. Five fractions obtained from purification step were unbound, F25, F50, F75, and F100 which the highest protein content was found in the F50 fraction. Results form native and reducing SDS-PAGE indicated that the F50 was single protein contained at least three subunits with sizes 32.5, 15.2, and 13.8 kDa. Periodic staining and phenol-sulfuric assay showed that the F50 was glycoprotein and contained 26.30±1.01% (by weight) carbohydrate moiety, respectively. By trypsinized and LC-MS/MS analysis, it was found that amino acid sequence of F50 was the most resemble to cysteine protease. Bioassay results indicated that the F50 contained α-glucosidase inhibitory activity which the maximum inhibiton, IC_{50}, and *Ki* were 100%, 30.15 μg/ml, and 140 μmol, respectively. The activity was stable at pH range 2-10 and at temperature up to 65°C. Furthermore, the F50 could inhibit growth of three plant pathogenic fungi, *Fusarium oxysporum*, *Exserohilum turicicum*, *Colectrotrichum cassiicola*, at a concentration of 23.6 to 42.7 μg, but no any effect on test bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis*). The F50 could also inhibit hepatoma cancer (HEP-G2; IC_{50} = 1.130 μg) and colon cancer (SW620; IC_{50} = 5.370 μg) proliferation. This could be concluded that a glycoprotein from *Z. ottensii* was successfully purified and its bioactivities were also characterized.

Field of Study: Biotechnology   Student's Signature: *Nathachai*

Academic Year: 2009   Advisor's Signature: *Aphichart Karnchanatat*

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LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
μg	microgram
μl	microlitre
A	Absorbance
BLAST	Basic local alignment search tool
BSA	bovine serum albumin
cm	centimeter
Da	Dalton
EDTA	Ethylenediamine tetraacetic acid
ESI/MS/MS	Electrospray ionisation/Mass spectrometry/Mass spectrometry;
g	gram
hr	hour
IC_{50}	The half maximal inhibitory
kDa	kilodaton
l	litre
LC/MS/MS	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
M	molar
mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
N	normal
nm	nanometer
NaCl	Sodium chloride
PAGE	polyacrylamide gel electrophoresis
PDA	Potato dextrose agar

rpm	revolution per minute
SDS	sodium dodecyl sulfate
TBS	0.15 M NaCl / 20 mM Tris-HCl buffer, pH 7.2
TEMED	*N,N,N',N'*-tetramethyl ethylenediamine
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
U	Unit activity
V	Volt
V/V	volume by volume
W/V	weight by volume
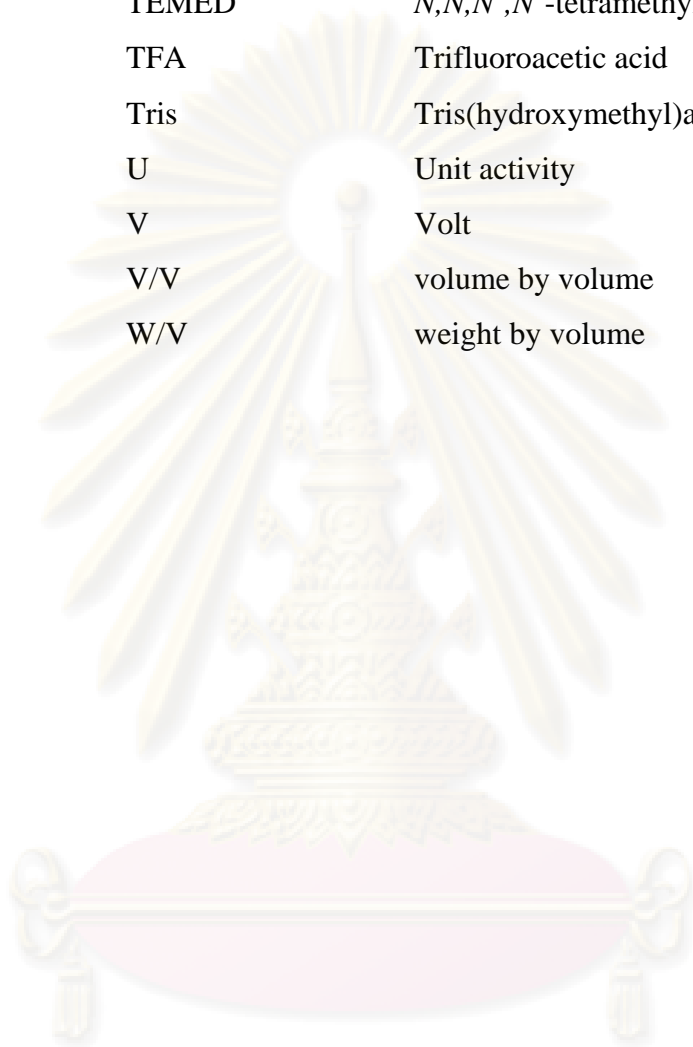
CHAPTER l

INTRODUCTION

People nowadays suffer from newly discovered and already existed diseases according to currently used drugs appear to be insufficiently responded. Some formerly effective drugs are no longer useful since target pathogens raise their resistances to the drugs. An explicit example is that streptomycin and *p*-amiosalicylic acid which was favorably used to treat *Mycobacterium tuberculosis* were recently found to be ineffective (Johnson, 2009). Next, benzyl penicillin (penicillin G), early time antibiotic prominently used in fighting with general pathogens, was become very little effect to most pathogens found in human digestive system (betterhealth, 2010). Finally, it was found that ampicillin now could not affect to some mutant strains of *Klebsiella aerogenes* anymore (Hamiltion-Miller, 1965). Some drugs are about to abolish because of unacceptable side-effects such as Phenformin, a biguanide derivative used for type 2 diabetes treating, was already banned due to highly lactic acidosis side-effect incidents it caused (Ching, *et al*., 2008). Many diseases and pathogens today are still short in efficiently treated drugs while many of susceptible pathogens are continuously developing their resistant abilities to the existed drugs. Thus, demands for novel active substances are always increase along the time and the phenomenon is always true even in the future. Besides lacking of satisfactory efficient drugs, increasing of healthcare demands are also a crucial factor making researches for alternative drugs with further or better activities interested in various areas. In addition to fully synthesized drugs, many high potential bioactive substances were already discovered from natural sources so far. Among these substances, one interested but still very few concerned was proteins or peptides. Owing to protein possesses an important identity in activity diversity because of its huge molecular variations in form of amino acid series and numbers together with manufacturing machines (eukaryotic and prokaryotic cells) available, it was thus interested by modern research topics in particular searching for proper templates for producing desired peptides biotechnologically and investigate for their activities. Thus, peptide sequences and their certain activities are valuable target found out from various organisms or specimens from natural origins including animals, plants, and

microorganisms. For the natural source point of view, Thai herbs are well accepted from worldwide people due to their outstanding pharmacological properties. However, many herbs are still lacking of scientific studies enough to be used as effective drugs in medical application. One of them is *Zingiber ottensii* Valeton, an annual plant belong to Zingiberaceae family. In Thailand, rhizome of this plant was used to cure bruises and gastrointestinal ulcers while it was use to treat convulsion and lumbago in Malaysia (Boukouvalas J. and Wang J., 2008). Although some volatile oils had been already studied, but there was no bioactive protein from this plant reported until now. Consequently, the aims of this study were to purify bioactive protein from the rhizomes of *Z. ottensii* and to assay them for bioactivity including antifungal, antimicrobial, cytotoxic, alpha-glucosidase inhibition activities, and protease activities.

CHAPTER ll

LITERATURE REVIEWS

2.1 Protein diversity

It is well known that protein is a kind of biomolecule (or can be called macromolecule) with the most diverse functions or activities. In addition to simple functions such as being structural components (for examples; collagen, microfilament, and microtubule), enzymes, hormones, receptors, transporters, immune species, storage proteins, and moving organelles (for examples; flagella, cilia, and spindle fibers), the protein can also involve in cell to cell or cell to environment signal transduction elements (for instances; integrins, fibronectin, decorin, protein and kinase C) (Voet, 2007). The heart of protein function is amino acid sequence which can be called "peptide". The diversity of protein functions come from difference of peptide sequences, both in amino acid type ordered and chain length. Some protein templates can be shuffled within their own sequences and become new difference sequence outcomes while some templates can be shuffled or trimmed of some sequences after transcription, so called "splicing". Moreover, nascent synthesized peptides are possibly managed by many post-translation modification procedures presented in different kinds of cells that make different in overall structures of final proteins. These mechanisms all cause changes in molecular structures and properties of proteins towards different three dimension compatibilities suitable for various functions (Elliott and Elliott, 2009). Owing to the proteins contain vastly varieties of forms and functions which most of changes can be manipulated in practical ways, they thus were interested by modern researches due to more alternative modification choices of both production processes and final products.

2.2 Bioactive protein

It can be summarized from the contexts mentioned in various documents that the "bioactive protein" or "biological active protein" is protein (or peptide) which can make changes in metabolisms or behaviors of organisms by organelle, cell, tissue, organ, or even whole organism levels (Pihlanto and Korhonen, 2003; Wang and Mejia, 2005; Silva *et al.*, 2006; Kokkoli et al., 2006; Aneiros and Garateix, 2004;

Ohtani *et al*., 1999). Such changes can be either advantageous or disadvantageous ways to individual organism or cell depended on its physiological and biochemical statuses. Milk immunoglobulins (Igs) can help calves in protecting of bacterial invasion but cause adverse effects in milk fermenting process as they also inhibit good microorganism growth. Some lectins can increase T-cell proliferation by their mitogenic activities but cause red blood cell agglutinated in the same time (Pihlanto and Korhonen, 2003). 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, 2003; Iwaniak and Minkiewicz, 2007) 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). Interestedly, it was found that some single fragmented peptide could contain more than one activity in the same time. A peptide fragment isolated from *Capparis spinosa*, a kind of water melon, possessed amino acid sequence resemble to a part of imidazole-glycerol phosphate synthase enzyme. It could inhibit proliferation of liver cancer cell (HepG2), intestinal cancer cell (HT29), breast cancer cell (MCF-7) whereas it could also inhibit the reverse transcriptase enzyme from HIV-1 virus in vitro and also inhibit *Valsa mali* fungi growth (Lam and Ng, 2008). Iwasaki *et al*., (2007) claimed about diasteromeric nanomer peptides derived from beetle defensin that it could not only inhibit various bacterial growths such as *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*, but could also inhibit mouse myeloma (P3-X63-Ag8.653) growth *in vitro* by $IC_{50} =$ 35 μM. It might be one of high potential peptide applied in antibiotic synergistic treatment and anticancer chemotherapy.

Bioactive protein can be found in most organisms including animals, plants and microorganisms. For the point of protein contents, all animals are mostly protein rich sources with completely amino acid type compositions while microorganisms are lesser but still flourished enough to be used as protein manufacturing places. Plants contain very least amount of protein contents and some proteins are lacking of certain amino acids but nearly all are found to be novels and having peculiar properties

(Wang and Mejia, 2005). Anyways, extraction from natural sources is not the favorite ways in preparation of bioactive proteins for any applications except researches which small amounts of the protein may be enough to use for investigation and cost per unit is minor concerned. The appropriate way for mass production is producing in microorganisms that the benefits practically gained are lower cost, short time spent, and more protein amount obtained with more convenience handling. There are some synthesized bioactive peptides commercially available now. For examples lactate dehydrogenase, antibodies, and glycerol dehydrogenase (Sigma-Aldrich, 2010). Most of peptide usages were found in research works and the lesser were also found in medical area for therapeutic purposes such as hirudin (a peptide from leech contained anti-blood clotting activity) was used to treat patients with blood coagulation system disorder or varicose vain (Eriksson, 1997), bradykinin (a peptide early found in human serum) was used as blood vessel dilation and anti-hypotension drugs (Hashimoto *et al*., 1973), and exenatide (glucagon-like peptide previously found in Gila monster lizard) was used to stimulate insulin production in Type 2 diabetes patients (Tripathy, 2008). Besides therapeutic properties, bioactive proteins are also frequently concerned in food researches areas as functional food topics in form of "bifunctional or thrifuntional peptides" which benefits at least dietary protein source, sensory improvement, and physiological improvement properties. This can be a strong potential way improving nutritional value for food industries. It was found that many proteins from foodstuffs contained some biological active properties which could be used to improve human health. The activities usually mentioned in functional food are anticancer, antioxidant, immunomodulator, ACE-inhibitor, cardiovascular curing and protecting, antibacterial and antifungal activities. Moreover, some were found that their pure form contained certain properties which could be used as therapeutic drugs in specified diseases such as Casoplatelin, an anti-trombin peptide found in enzymatically digested milk which could inhibit blood clotting and depositing, could be used in curing patients with thrombosis, stroke, or varicose vein (Iwaniak and Minkiewicz, 2007).

There are many bioactive peptides with various activities reported recently. Although those activities of peptides are very diverse, but identifying of all activities in single peptide is impossible for any individual laboratory. Thus, cogitated group of better feasible activities may be selected for identification. In this study,

hemagglutinating, α-glucosidase inhibitory, antimicrobial, and cytotoxic activities were chosen. Furthermore, protease activity was lastly found from amino acid sequence identification and bioimformatic alignment through internet network. Then, the activity was eventually confirmed and the protease becomes one of the activities of purified protein.

2.3 Hemagglutinating activity by lectins

Lectins are a group of protein that can bind to carbohydrate (which can be in form of sugar, oligosaccharide, or polysaccharide) specifically. Binding of the lectins is differed from those enzymes, anti-lectin antibodies, and other carbohydrate specific binding protein on that they will never change any bound-carbohydrate properties, not convert such carbohydrate to other substances, not come form immune origin, and being reversible binding. In addition to carbohydrate binding specifically, the lectins can cause cells agglutinated and glycoprotein or carbohydrate precipitated. That is why the lectins are sometimes called "agglutinin" (Sharon, and Lis, 2007; Peumans and Damme, 1995). Since most lectins have two or more carbohydrate binding sites in their molecules, which can make cross-linkages between cells or carbohydrate containing molecules and form solid network. However, there are also some certain lectins that presented in momovalent binding site, and thus can not agglutinate cells or precipitate carbohydrate.

Some lectins contain more than one type of acting site or one activity in single molecules so that they can bind to carbohydrate and can exhibit other behaviors such as enzymatic activity (which make this lectin called "lectzyme"), mitogenic activity, and transportation activity in the same time (Sharon, and Lis, 2007). From these phenomena, the lectins can be classified into three types according to their acting sites as "merolecins" (the lectins with only single carbohydrate binding domain, usually small single peptides), "hololectins" (the lectins with two resemble carbohydrate binding domains), "Chimerolectins" (the lectins contains both carbohydrate binding domain and other well-defined biological active domains which act dependently of previous domain) (Peumans and Damme, 1995). Beside this classification, the lectins can also be classified by their ligand specificities in two manners. The first is that by sizes of binding ligand which the lectins can be divided into two group; the lectins that specifically binding to monosaccharides as well as oligosaccharides and the

lectins that specifically binding to only oligosaccharides (Sharon, and Lis, 2007). The second classification manner is relatively old style that was set up during little details of lectin's information known. Thus, they were separated by their legand specificity only in sugar types such as mannose or glucose specific lectins, galactose specific lectins, and sialic acid specific lectins. However, they were recently found that most lectins tended to recognize certain three dimension structure than monosaccharide specificity. Thus, this classification style may not up to date because many of lectins formally grouped in one class are now no longer suitable for such class. Anyways, it may be familiar to some authors and may also found in some present documents.

Although the lectins have been found in human, animal, plant, and microorganisms, but it looks like that plant lectins were the most investigated for details (Sharon, and Lis, 2007; Chandra *et al*., 2006). Most lectins are present in seed cotyledons of the plant (but also found in any other parts such as roots, stems, rhizomes, and leaves in lesser amounts). In such tissues, most lectins are located within cytoplasm or protein bodies inside the cells (Moreira *et al*., 1991). In general, the lectins with the same ligand specificity contain different binding abilities mainly depended on their sources meant different genetic material that produce different lectins with different in three dimension structures. For instances, Tipthara *et al*., (2007) successfully purified a mannose specific lectin with strong rabbit hemagglutinating activity (0.017 µg of minimum amount that hemagglutination presented) of from *Curcuma Zedoaria*, Thipthara *et al*., (2008) also purified many lectins with weak activity (0.140 to 0.190 mg minimum amount that hemagglutination presented), Wong *et al*., (2008) purified mannose/glucose specific lectin with extremely strong activity (83.063 ng minimum amount that hemagglutination presented) from *Castanopsis chinensis*. Most of plant lectins become a set of important tools for glycobiology achievements. They are also applied in detection, isolation, and characterization of glycoconjugated substances mainly in glycoprotein, proteoglycan, and modified polysaccharides (Sharon, and Lis, 2007). The lectins are also advantages in immunology, histochemistry, pathology, and physiology areas. One familiar instance which the lectin usage is clearly seen is ABO blood type identification using blood group specific lectin such as concanavalin A, a lectin derived from jack bean seed (*Canavalia ensiformis*) that can specifically bind to non-

reducin α-terminal mannose. This blood groups determination is based on presence or absence of specific glycoprotein on red blood cells that the lectins can bind and make red blood cells agglutination (Moreira *et al*., 1991) by forming network with red blood cells and then can not be collected as button like form in the U shape bottom well. From this incident, a method widely used for lectin screenings or characterizations mainly involved cells agglutination, especially red blood cells from various animals (Sharon, and Lis, 2007). The lectins also have other roles in mammals. There was evidences indicated that the lectins played the important roles in cell differentiation, cell movement and phagocytosis, cell to cell and cells to matrix substances communication, cell organization in tissues, and embryo morphogenesis (Moreira et al., 1991).

On the other hand, consuming of lectins also may cause adverse results in some cases. Several lectins such as concanavalin A and wheat germ agglutinin (WGA) are toxic to mammalian cells, but relatively low compared with other toxic substances such as approximately 1000 times lower than ricin (an toxic albumin from Caster bean). It is believed that production and accumulation of toxic lectins in some plants are a kind of defending mechanisms which plants develop for protecting them form certain plant eating organisms such as insects and mammals (Sharon, and Lis, 2007; Peumans and Damme, 1995) and plant pathogens. Aside from defense mechanisms, the lectins also have their essential roles in plant-microorganism symbiosis, cell differentiation, pollen recognition, cell wall elongation, and as a reserved protein (Moreira *et al*., 1991). Interestingly, some plant lectins were found to be well react with viral surface glycoprotein and were hoped to use in controlling many diseases originated from viruses which current methods are still inadequate controllable efficiencies. Balzarini, J. *et al*., (2004) isolated two mannose-specific lectins from *Galanthus nivalis* (snowdrop) (GNA) and *Hippeastrum* sp. hybrid (Amaryllis) (HHA) and found that they contained *in vitro* anti-HIV virus activities ranged from 0.12 to 1.2 μg/ml for GNA and from 0.18 to 0.70 μg/ml for HHA depended on tested viral nature.

2.4 Important of α-glucosidase and its inhibitors

The α-glucosidases (EC 3.2.1.20) (Park H., et al., 2008; Gao H and Kawabata J, 2005) (EC 3.2.1.10, EC 3.2.1.48, and EC 3.2.1.106) (Zhu Y., *et al*., 2008) or maltases (Taylor J., 1994) are a group of enzymes that catalyze the cleavage of the α-glycosidic linkage of carbohydrate molecules from the non-reducing end and release free glucose from their substrates. They function particularly well with disaccharide and oligosaccharide substrates (Taylor J., 1994; Melo E., *et al*., 2006). In the case of humans, the enzyme is located around the brush-border cells of the intestine and plays a fundamental role in releasing glucose from dietary carbohydrates, thereby allowing efficient absorption. However, this can lead to an undesired increase in the blood sugar levels for diabetes type II patients and people who are in obesity that need lower dietary glucoses for keeping their weight loss or at least constant.

In addition to insulin treating which can reduce blood sugar but not the proper way to lower glucose accumulation, there are many alternative ways to reduce blood glucose. For examples; begin with reduce each meal consumption, nutritional management, treated with drugs that reduce carbohydrate digested enzyme releasing, and treating with the drugs that being the enzyme inhibitors (Silverberg, 2008). Treating with enzyme inhibitor is one of physiological safer way in case of healthy individual without hypoglycemia symptom and without treating with any other blood sugar reducing drugs (Brewer, D., 2006). Brewer D. (2006) suggested that α-glucosidase inhibitor was the safer method. It gave adequate blood sugar reducing result with fewer metabolic side effects and low incident of hypoglycemia compared with former sulfonylureas and insulin. Furthermore, the α-glucosidase inhibitor, a relatively recent interested topic, can not only decrease the postprandial blood glucose levels, but also prevent or cure some chronic diseases such as, cancer, viral infections and hepatitis (Park H., *et al*., 2008). This inhibitor was found to be difference forms presented in various plant origins. Moreover, many of inhibitors from plant were reported as having other benefits more than blood sugar reducing. For instance, castanospermine, an alkaloid from the black bean or Moreton Bay chestnut tree, has already been characterized as an α-glucosidase inhibitor that can effectively prevent mice from succumbing to Dengue virus infection and also to allow infected mice to

recover more quickly (Whitby K., *et al.*, 2005). The effect appeared to depend on the ability of castanospermine to block the viral α-glucosidase enzyme activity.

Apart from medical or healthcare benefits, α-glucosidase inhibitors are also involved in modern biotechnological studies to improve beer (Taylor J., 1994), sake (Iwata H., *et al.*, 2002), and other brewing industries, as certain glucose/total sugar ratios of the ferment are necessary for good product qualities. Since the inhibitors mentioned in those three documents were high activities, they were proven as worked even in crude extract form of wheat germ. One more advantage of this inhibitor group is that some of them contain a transglycosylation activity which may be used in specific oligosaccharide synthesis (Mala S., *et al.*, 2001).

Many of α-glucosidase inhibitors have been discovered, studied, and approved so far. The three most familiar inhibitors currently used in medicine are acarbose (pseudotetrasaccharide), miglitol (1-deoxynojirimycin) (Ladas S., *et al.*, 1992), and N-butyl-1-deoxynojirimycin (Melo E., *et al.*, 2006). However, to date almost all reported inhibitors are sugar or oligosaccharide derivatives (Evan S., *et al.*, 1985); and other small molecules, such as flavonoid analogues (Gao H. and Kawabata J., 2005), β-lactam, which already contains antibiotic activity at the same time (Lee D., 2000), the isoflavone genistein from soy bean (Lee D. and Lee S., 2001) and alkaloid (Whitby K., *et al.*, 2005). Moreover, there were uncharacterized inhibitors in form of the unknown substances from plant extracts, such as the methanol extract from the Devil tree, *Alstonia scholaris* (Jonganurakkun N., *et al.*, 2007), the water extract of the *Morus alba* leaf (Yogisha S. and Raveesha K., 2009), *n*-butanol extract of *Chaenomeles sinensis* fruit (Sancheti S., *et al.*, 2009), and the ethanol extracts of *Entada rheedii* and *Archidendron jiringa* seed coats, *Albizia lebbeck* and *Albizia lebbeckoides* bark and *Parkia speciosa* pericarp (Tunsaringkarn T., *et al.*, 2008) reported as high potential inhibitor in some documents. Most sources were found to contain from a moderate to a very high inhibition activity. Nevertheless, reports about proteinatious substances with α-glucosidase inhibitory activity are very few so far, especially from herbs. Further more, many approved inhibitors currently used in Type II diabetes treating (for examples: acabose, voglubose, and miglitol) are all synthetic drugs and their prominent adverse side-effects were also reported in many documents. The side-effects usually mentioned in literatures were flatulence, diarrhea, abdominal

pain, and also some other gastrointestinal symptoms (Brewer, D., 2006). Thus, this is mainly reason for nowadays scientists for seeking new inhibitors in novel sources. One popular source mostly concerned is herb, the most vast varieties and valuable source of substances.

Thailand has a high diversity of plants and possesses a rich biodiversity of herbs, many of which have widely accepted therapeutic properties. Since enzyme inhibition is one of the natural life mechanisms, it is therefore possible, if not likely, that this favorable activity will be present in many kinds of plants. On the other hand, although the existing inhibitors give good glycaemic control, there are still many serious side effects reported. For example, the acarbose application was found to contribute a high risk from adverse gastrointestinal effects (Van de Laar F., *et al*., 2005), while pseumatosis cystoides of intestine was linked to steroid-induced diabetic patient treated with vogibose (Tsujimoto T., *et al*., 2008). For these reasons, the discovery of new inhibitors of α-glucosidase may be beneficial, if not necessary, as they will be expected to become alternative weapons in the struggle against various healthcare problems, as well as being potentially highly valuable research and industrial tools in future.

2.5 Peptides with antimicrobial activity

There are at least three reasons for the need in finding out new alternative antimicrobial substances from natural sources. The first reason is that people nowadays concern about toxic of synthetic substances including daily contact chemicals or even drugs used in medical or healthcare purposes (Hafidh, *et al*., 2009). Any synthetic drugs were avoided in order to keep physiological cleans as belief. Thus, natural substances were used increasingly instead as well as any substances used for antimicrobial purposes. The second reason is that new alternative drugs are human hope for better fighting with existed diseases and pathogens. They may replace currently used drugs in points of more efficiency, more abundant, lower side-effect or safer or even lower production cost. It is fact that most alive organisms should have some mechanisms or substances fight with all time contacting pathogens so that they can be survived in nature. Although a plenty of antibiotics were discovered after first time Fleming's declaration, but they were still relatively low amounts compared with overall real natural antimicrobial substances. This mean the natural sources still

flourish with novel antimicrobial substances waiting for discovered. Additional small aspect may be raised here. The natural substances are usually good leading compound sources for mostly synthetic drug from the long past due to their diversities are far from human imagination. New chemical structures are always found in natural resources as higher frequency than artificial deducing structures. The final reason is that the mechanism used to synthesize natural substances are available and they are usually can be imitated in small, medium, and even large scale production with present biotechnological knowledge which looks easier than newly designed plants.

Regardless of small molecule such as phenolic compounds (Maddox *et al*., 2010), flavonoids (Tewtrakul, 2003), and alkaloids (Okwu and Igara, 2009) that were reported by various works as potential antimicrobial substances; the proteins with the same activity were also used in many fields, especially medical and industry. Some of antimicrobial peptides are mentioned below. Defensin is a large group of conserved antibacterial peptides found in many types of organisms such as insect, plant, small animal, and even human.

Plectacin, one of potentially antibacterial peptide belonged to defensin group, composted of highly cystein content in peptide chain. The plectacin was isolated from a saprophyte fungus, *Pseudoplectania nigrella*. It could inhibit the growth of many bacterial (in particular, *Streptococcus pneumonia* included mostly currently drug resistant strains), fungi, and virus. Thus this peptide was hoped to replace synthetic antibiotics which were nowadays no longer effective for many resistance strains and scientists were trying to use its nucleotide sequence as template to produce in large scale quantity. The plectacin could be easily purified and were now available in antimicrobial market. Low toxic was found in mice by various tests (Mygind *et al*., 2005).

Hemocidins, a peptide derived from hemoglobin or myoglobin digestion that release the fragment formally hided inside the protein molecule, was proven that it could specifically inhibit *Escherichia coli* with high efficiency (Mygind *et al*., 2005). Staphostatin group peptides were one of high potential antibacterial agents produced from certain stains of *Staphylococcus aureus* which was also pathogen in the same time. It could inhibit cystein protease and serine protease enzyme which were the tool the *Staphylococcus aureus* itself used as host invasion and disease development. Other highly potential peptides with antibacterial activity are bacteriocins from many

bacteria, magainin from frog, mellitin from bee, LL-37 from human, tachyplesin from horseshoe crab, and indolicidin from bovine were well studied and grouped into antimicrobial class (Mygind *et al*., 2005). In addition, there are many peptides approved by US FDA for using with foodstuffs, for examples, lactoferrin and bacteriocins. The lactoferrin (or some document may spell as "lactopherin") is a peptide fragment found in milk, saliva, tear, and many tissues including the peptide secreted from neutrophil. Its former name was lactotransferrin and belonged to transferin protein family. Thus, it can act more than antimicrobial activity. This peptide can not bind to Fe^{2+} only, but it can transfer this mineral also. The lactoferrin can inhibit broad types of bacteria, fungi, protozoa, including inhibit HIV virus from host cell invasion (*in vitro*). (Hancock and Chapple, 1999; and Adlerova, 2008). While bacteriocins are groups of peptides produced from various bacteria (mostly from lactic acid bacteria) and can relative specifically inhibit other bacteria. The peptides were proven as safe, no toxic effect to eukaryotic organisms, and can be destroyed by digestive proteases. The antimicrobial activities included broad type of bacteria and fungi (both pathogenic and food spoilage microorganisms). The function mode of these peptides is bacteriocides. The important members of this group are nicin and warnerin which were extremely studied. These two peptides were used in many foods industrial and were also included in some quality assurance programs (Galvez *et al*., 2007).

2.6 Cytotoxic activity

Form the contexts claimed by various documents, cytotoxic substances mean any species that can harm to monitored cells. In general, the cells are eventually died. In some cases, the cytotoxicity refers to ability to cause cell or organism death. The word "cytotoxic substances" thus have wide ranges of scope. It can refer to native human cells or abnormal cells such as tumor or malignant cells. It can also refer to any kind of cell's death such as necrosis and apoptosis. However, the word "cytotoxic" or "cytotoxicity" meant in medical reports were usually get into mainly two denotations; the certain substance which contains ability to kill cancer or tumor cells, the certain substance which can kill certain animals or certain animal cells (including human) (Chang, 1983; Mizutani and Yoshida, 1990; Clark *et al*., 1976; Kaewdoungdee, 2006; Galvez *et al*., 2003). In immunology, cytotoxicity can be

meant both the substance causing target cell death and ability of certain T-lymphocytes in killing target cells. Such target cells can be normal cell which infected by pathogens or normal cell which has been transformed to be abnormal one. The latter can also be called "cell-mediate cytotoxicity" (Mizutani and Yoshida, 1990). The cytotoxicity in bioactive protein point of view, thus can be mean the ability of bioactive protein to kill undesired cells such as tumor, benign, malignant, genetically changed and pathogenic infected cells in particular cancer cells.

To determine whether certain bioactive protein contains cytotoxicity to undesired cells or not, it was frequently tested with various cell lines involved. The cell lines included BT474, CHAGO, HEP-G2, KATO-3, CH-liver, and SW620. The BT474 or in ATCC name HTB-20 is epithelial cell line originated from human mammary gland. It was isolated by Lasfargues E. and Coutinho W.G. in 1979 from a solid, invasive ductal carcinoma of the breast. The abnormality bore with hypertetraploid range and the chromosome designated N11, N13, and N22 are absence. Some other chromosome also exhibit over and under represented manners. It has ability to adhere to container surface, forms normally compact multi-layer colonies, and rarely becomes confluent (Berkeley Lab, 2010). The CHAGO, human lung cancer cell line derived from a bronchogenic carcinoma synthesize and secrete the alpha subunit of glycoprotein hormone into culture medium (Wong and Biswas, 1985). The HEP-G2, derived from human hepatoblastoma. This cell line was proven for free from known hepatotropic viral agents. It can express wide variety of liver-specific metabolic functions. Two interested liver metabolic which the HEP-G2 can produce are cholesterol and triglyceride metabolism facilities including primary bile acids. Confluent Hep G2 monolayers express normal low-density lipoprotein (LDL) receptors and chylomicrons, very low-density lipoproteins (VLDL), LDL, and high-density lipoproteins (Javitt, 1990). The KATO-3, human gastric carcinoma signet ring cell lines, obtained from Sekiguchi's stomach (depositor). Its identity is spherical shape cell with adherent ability and being monolayer upon long term cultivation. This cell line contain blood type B; Rh^+ antigen and other well characterized identities were its stemline chromosome number is hypotetraploid with the 2S component occurring at 6.2%. Nine markers were common to most S metaphases, four markers were less frequent (CLS, 2010). The SW620 is human colon originated cancer cell isolated from human abdominal mass (blood group A, Rh^+). This cell line was

initiated by A. Leibovitz, *et al*., from a lymph node in the same manner as was the primary adenocarcinoma from which SW480 was derived the previous year. The cell shape is small spherical and bipolar cells with lacking of microvilli. The cells synthesize only small quantities of carcinoembryonic antigen (CEA), and are highly tumorigenic in nude mice. This cell line can produce keratin. There is a G to A mutation in codon 273 of the p53 gene resulting in an Arg to His substitution. The line is proven to express c-myc, K-ras, H-ras, N-ras, Myb, sis and fos oncogenes (ATCC, 2010).

2.7 Protease definition and importance

Proteolytic enzyme is a kind of enzyme belonged to hydrolase class. It has ability to cleavage proteins or peptides at peptide bond with certain specificity. This group of enzyme plays the important role in protein metabolism, especially protein turn over, recycling, and tissue rearrangement (Salas, 2008). Moreover, they also involved in large amount of vital biological processes such as signal transductions, defense mechanisms, growth developments, cell proliferations, immune actions, blood coagulation, cell mobilization, and even apoptosis (programmed cell death) (Moffitt, 2007; Lopez-Otín, 2008). Beside this, the proteolytic enzymes also exhibit two important roles in industrial and medical areas. The beneficial usages of the protease in industrial including food processing such as meat tenderization (Demir *et al*., 2008; Fahmy, 2004) and cheese production (Akuzawa, 1987), detergent enhancement such as protenatious stain removing (Najafi *et al*., 2005), pharmaceutical, leather production to remove soft proteins or tissue from animal skins (Demir *et al*., 2008), agroindustries, and waste water treatment such as meat industry waste degradation (Najafi *et al*., 2005). For medical advantages, there were many applications reported. The examples are plasmin which is human serum protease involved in fibrinolysis and used as clot resolving, cathepsin family which is a set of protease involved in various diseases (etc., cancer promotion and prevention, heart disease development, and certain parasitic infections) was intensely studied for finding proper inhibitors or regulatory ways in order to overcome such diseases (Pihlanto and Korhonen, 2003; Lutgens *et al*., 2007).

There are many terms involved in proteolytic activities that cause people confuse in usages. However, Barrett and McDonald (1986) had defined these terms in

their letters well.   By their obviously definitions, "Protease" is an enzyme that degrades protein by hydrolysis of peptide bond which can be divided in to two sub groups, proteinase and peptidase. The "proteinase" is the enzyme that specifically degrades intact protein molecules while the "peptidase" is the enzyme that only acts to the peptide fragments. On the other hand, there were additional two terms also used concurrently, exopeptidase and endopeptidase. The "exopeptidase" refers to proteolytic enzyme that cleavages terminal residue of intact proteins or intact peptides even they are with or without blocked residues while the "endopeptidase" cleavages only inner residues. In some cases, proteolytic enzymes can act to intact protein molecule only to terminal residues while the same enzyme can better cleavage inner parts when the substrates are in denature or loosing form. These enzymes are also called "endopeptidases".

The proteases, also a group of enzymes, have been classified into 6 mechanistic classes by the International Union of Biochemistry as cysteine protease, serine protease, aspartic protease, metaloprotease, threonine protease, and unknown type protease which composts of the enzymes that can not grouped into any of five previous categories or the functional amino acid in active site were unknown (Dubey *et al.*, 2007). The seventh new class has been recently reported as glutamic acid protease (Demir *et al.*, 2008). Each class has a characteristic by certain functional amino acid located within active site. Moreover, they are different in amino acid sequence towards three dimension structures and hence different in catalytic mechanisms. Nevertheless, their active sites usually need nucleophillic species to correctly act with specific substrate configurations so that each enzyme type contain nucleophillic amino acid or metal ion (usually zinc) in their active sites as designated by the group's names. The cystiene protease contains six main families; papain, calpains, clostripains, streptococcal cysteine proteases, viral cysteineproteases and most recently established, caspases (or apopains) (Dubey *et al.*, 2007).

Many practical used proteases tend to be microorganism origins because of they are easier to grow and harvest for enzymes (Najafi *et al.*, 2005). However, proteases from plant are also interested in term of novel amino acid sequences that can be used as template for protease synthesis in microorganism by genetic engineering. Furthermore, many protease isolations, identifications, and characterizations from plant were reported so far. Many of them were also appeared to

be used in some industrial applications. Phaseolain, a carboxypeptidase present in French bean leaves extract was well characterized as contained two subunit with one was proteinase C, having optimum pH at 3.0-4.0, and their amino acid composition were known (Carey, W.F., 1971). Salas, C.E. (2008) reviewed that a plant cysteine protease group which mostly bore Caricaceae plant defensive functions in unripe fruit faced with physical stresses. These enzymes worked resemble to human serine proteases and had much potential to be used as drug involved in blood clotting and would healing including immunomodulation, digestion improvement, and neoplastic alterations (Salas, 2008).

Several methods can be used in protease activity determination. In former procedure, intensity of protease activity could be measured. The method involving incubated sample with soluble casein digestion, intact casein precipitation with trichloroacetic acid, removing precipitated undigested casein, and quantitatively measures amount of remained protein in supernatant solution (Akuzawa, 1987). In recently, new improved method with more sensitivity and more comfortable was developed as chromogenic derivatives of certain protein were synthesized. Proteolysis activity determination using azocasein instead of ordinary casein was one of such chromogenic approach. The azocasein is synthetic non-specific protease substrate which after the molecule was cleaved by protease it will release azo dye group that can be read at 340 or 440 nm (Iversen, 1995). The overall procedure is like previous casein method except determination of soluble protein amount in final solution. The azocasein method uses simple spectrophotometry techniques which more comfortable and sensitive than previous method.

2.8 The herb: *Zingiber ottensii* **Valeton**

Taxonomic status of the plant, *Zingiber ottensii* Valeton

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Liliopsida

Order: Zingiberales

Family: Zingiberaceae

Genus: Zingiber

Specie: *Zingiber ottensii* Valeton

(A)   **(B)**   **(C)**
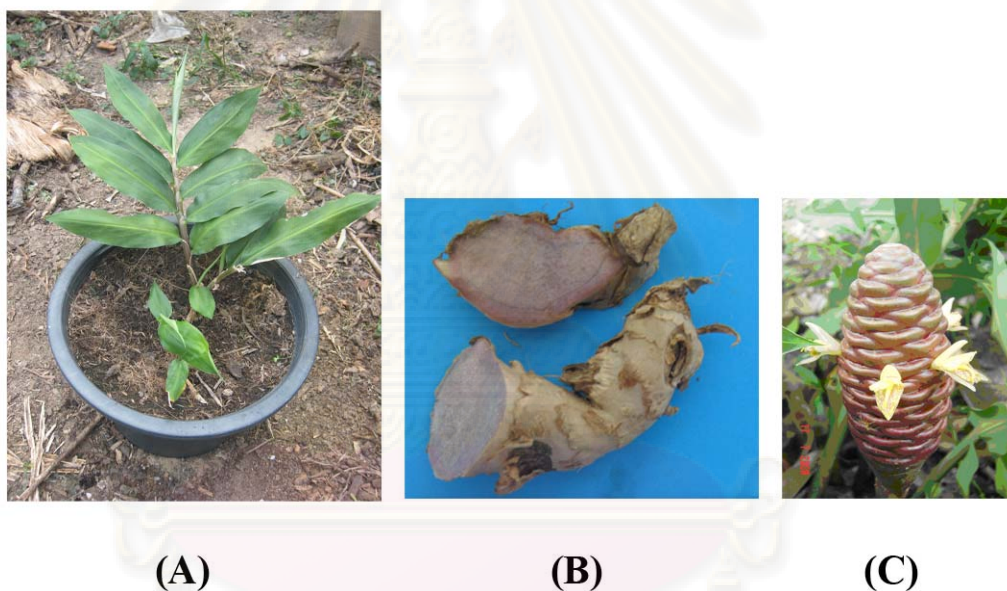
Figure 2.1 (A) Physical of *Z. ottensii*. (B) *Z. ottensii* rhizomes. (C) Flowering of *Z. ottensii* shows pink mottled and yellow labellum.

In 1918, this monocot plant was first reported by Valeton, T. as specimen found in Bogor town of Java, Indonesia (Valeton, 1918).   The *Z. ottensii* called (phonetically) "Plai-Dam" in Thai, is a medicinal plant that belongs to the Zingiberaceae family. Three characters distinguished this plant from other Zingiberaceae members are dark-purple rhizome texture, pale yellow Labellum, and pink mottled (Sirirugsa, 1998) (Figure 2.1). Distributions of this plant were reported to be spread widely and abundant in Southeast Asia while Sirirugsa, P (1998) referred

Malaysia and Sumatra as mostly found location. By isozyme comparison, it was found that the genetic of this plant is very closely related to *Z. zerumbet* and *Z. montanum*. Since *Z. ottensii* has reddish stem which is attractive, some were domestically cultivated for ornamental purposes. Some local people used it as appetizer (Ravindran and Babu 2005). 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. This may suggest potential cultivar (genetic) differences or environmental influences in the chemical composition, but this remains unknown. Nevertheless, these medicinal usages have included as a traditional sedative lotion remedy for convulsion (Akiyama K, *et al*., 2006) and lumbago treatment in Malaysia (Boukouvalas J. and Wang J., 2008; Sirirugsa, 1998). 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). In addition, it is linked to poultice in postnatal treatment and as an appetizer (Ravindran P. and Babu K., 2005). In Thailand, *Z. ottensii* has been traditional used to cure external bruises and gastrointestinal ulcers. Chemical constituents of the plant were also reported in rhizome. Three sesquiterpenes (humulene, humulene epoxide, and zerumbone) and diterpene were found in rhizome (Ravindran P. and Babu K., 2005). In addition, Malek *et al*., (2005) were successfully identified 28 components in its rhizome. The most abundant essential oil was zerumbone which constituted 37% of the total oil while other main constituents were terpinen-4-ol (16.8%), (α-humulene (10.9%) and sabinene (7.2%). Akiyama *et al*., (2006) reported four additional well identified terpenoids of 16 known compounds extracted from the rhizome and all molecular structures had been successfully discovered. Such four terpenoids were 1,10,10-trimethylbicyclo[7,4,0]tridecane-3,6-dione (1), (E)-14-hydroxy-15-norlabda-8(17),12-dien-16-al (2), (E)-labda-8(17),12,14-trien-15(16)-olide (3), (E)-14,-15,16-trinorlabda-8(17),11-dien-13-oic acid (4), and rel-(3R,5S)-3,5-dihydroxy-1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)heptane. Although some reports have addressed the chemical constituents of *Z. ottensii*, and in particular the essential oils, there still are no any report about bioactive protein from this plant.

CHAPTER III

EXPERIMENTAL

3.1 Materials

The fresh rhizomes of *Z. ottensii* were periodically purchased (October 2008-June 2009) from Chatuchak park market in Bangkok, Thailand. A voucher specimen (BKF No. 60689) was deposited at The Forest Herbarium (BKF), Royal Forest Department, Bangkok, Thailand. Three plant pathogenic fungal strains used in antifungal bioassay [*Collectotrichum cassicola* (DOAC 1196), *Exserohilum turcicum* (DOAC 0549), and *Fusarium oxysporum* (DOAC 1258)] were obtained from the Division of Plant Disease and Microbiology, Department of Agriculture, Bangkok, Thailand. Four bacteria strains (*Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus*) and six human tumor cell lines [BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric), CH-liver (liver), and SW620 (colon)] were obtained from The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand and maintained in nearby laboratory. Animal bloods (Goat, Goose, Mouse, Rat, and Sheep) were purchased from National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom, Thailand. Human blood specimen (with blood group A, B, AB, and O) were kindly donated from Thai Red Cross Society, Bangkok, Thailand. Ammonium sulfate, acrylamide, bis-acrylamide, hydrochloric acid, mercaptoethanol, TEMED (Tetramethylethylenediamine) and Tris (hydroxymethyl) aminomethane (MERCK) were purchased from the Merck group, Germany. Ammonium persulfate, Coomassie Blue G-250, glacial acetic acid, methanol, sodium chloride, sodium hydroxide and sodium dodecylsulfate (BDH) were purchased from VWR International, USA. The α-glucosidase from *Saccharomyces cereviseae* was purchased from Sigma-Aldrich Co. Ltd, USA. All other chemicals were analytical grade.

3.2 Extraction of Protein from the Rhizomes of *Z. ottensii*

Protein was isolated from the rhizomes using the procedure described by Tiptara *et al*., (2008) and Samarkina *et al*., (2009) with some modifications that 20 mM Tris-HCl (pH 7.2) buffer was used instead of phosphate buffer and no EDTA

included in the system. The procedure began with the rhizomes were peeled, minced into small cubes (about 5 x 5 x 5 mm), and soaked in 20 mM Tris-HCl buffer pH 7.2, containing 0.15 M NaCl in order to avoid as much as oxidation by air exposure. With a prepared rhizome to buffer (20 mM Tris-HCl, pH 7.2) ratio of about 1:4 (v/v), the small cubes were homogenized using a blender (Philips, HR 2061, Indonesia) until no remaining small pieces could be seen. The homogenate was then stirred overnight (IKa, RW 20N, Labotechnik Inc., Japan) at 4^oC. After centrifugation at 15,000 x g, 4^oC for 15 min, the supernatant was collected and transferred into a new container (5 L plastic tub), and further agitated at 4^oC overnight. Anhydrous ammonium sulfate was gradually added to make an 80% saturated solution and left for six hours to let the precipitation complete. The suspension was then harvested by centrifugation at 15,000 x g, 4^oC for 15 min, and the pellet (after resolvation in 20 ml of deionized water) was then dialyzed against five liters of deionized water, under 4^oC temperature, using dialysis tubing with 3500 Dalton MW cut-off (SnakeSkin, Thermo scientific Co., Ltd., USA). The water was changed three times during the two hours dialysis period. The obtained crude protein was finally freeze-dried and the powder was kept at -20 ^{o}C until use.

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

The protein purification process was slightly modified from the method described by Tipthara (2007), in that Tris-HCl buffer pH 7.2 and a SP sepharose (Amersham Pharmacia Biotech, UK) column were used instead. The procedure began with 100 mg of crude protein powder 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 the 20mM Tris-HCl pH 7.2 buffer, the sample was injected into the column. Conditions used for this cation-exchange chromatography purification were: buffer A = 20 mM Tris-HCl pH 7.2; buffer B = 20 mM Tris-HCl pH 7.2 with 1 M NaCl; stepwise elution was used at 25, 50, 75, and 100% buffer B; flow rate = 2 ml/min; 10 ml per fraction collected; proteinataeous peaks were monitored at 280nm. Data was analyzed and interpreted by Prime view version 1.00 (Amersham biosciences). After the process, all fractions in the same peak were pooled and directed to 3 times dialyzed at 4^oC for overnight

against 5 liters of deionized water. Finally, dialyzed liquid was freeze-dry and the powder was kept at -20°C refrigerator until used.

3.4 Protein amount determination

Protein contents were determined by Bradford's procedure as described by Bollag (1996). Bovine serum albumin (BSA) was used as standard, and a standard curve derived from the average of three determinations of a 5-30 µg/ml BSA concentration series was created for every determination. The working procedure began with 50 µl of samples which were two-fold diluted with deionized water in a 96 well flat-bottom plate. The series of standard BSA solutions were then placed into the appropriate wells. After 50 µl of Bradford's reagent was added to each analyzed well, the plate was shaken with round orbit plate shaker (Biosan, OS-10, Latvia) at 190 rpm for 1 min and then left for 15 minutes. Finally, the plate was read at 595 nm using an ELISA plate reader (Biotek Synergy HT, Biotek instrument, USA). The OD of the obtained samples was used to calculate the protein concentration using the linear equation computed from the standard curve. During the column chromatographic separations, the elution profiles of proteins were determined by measuring the absorbance at 280 nm.

3.5 Native and reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis

For determining of purity and monitoring for molecular size distribution pattern of obtained protein, native and reducing SDS-PAGE were carried out as procedures described by Bollag *et al*., (1996) and 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.

3.6 Glycoprotein determination

In gel periodic acid-Schiff's staining technique was modified from the method described in Trivedi *et al*., (1983). After electrophoresis, the gel was carefully taken into 1% periodic acid in 5% acetic acid (submerge under liquid level) and the container was shaken for 5 min. Then, the gel was rinsed twice with distilled water at the same volume and replaced with Schiff's reagent (prepared 1 day before used). After incubated in dark room temperature for overnight, the gel was 3 times washed with distilled water and soaked in 5% sodium metabisulfite in 5% acetic acid solution for 20 min. Finally, pink-purple color was slowly developed when the gel was repeatedly washed in distilled water until obvious intensity appears.

3.7 Carbohydrate content determination

The phenol-sulfuric technique was slightly modified from the procedure had been mentioned (Demir et al., 2008; Knutson, 1997) with scaling up and used glucose as standard instead. The F50 protein was serially diluted and a 500 µl of each dilution was transferred into 15 ml glass tubes. Next, a 500 µl of 4% phenol solution was added. After thoroughly mixing, the tubes were then left at room temperature for 5 min. Then, 4 ml of conc H_2SO_4 was added in each tube immediately and the tube was carefully mixed using vortex mixer (Vortex-2 Genie, Scientific industries Co., Ltd, USA). Each 100 µl of the samples were transferred to each well of microtitre plate

and read at 492 nm. Obtained data was calculated for sugar content (glucose equivalent) using standard curve developed from glucose by the same procedure.

3.8 α-glucosidase inhibition assay

The assay method was modified from that reported previously (Park *et al.*, 2008; Iwata *et al.*, 2002) using α-glucosidase from baker's yeast (Sigma, G5003, Sigma-Aldrich Co., Ltd., USA) as the working enzyme and *p*-nitrophenyl-α-D-glucopyranoside (PNPG) (Sigma, N1377, Sigma-Aldrich Co., Ltd., USA) as the substrate. The amount of the enzyme and substrate used in the assay was optimized for every new lot of enzyme and substrate purchased, and selected so as to give approximately 0.8 OD (after the reaction was stopped) within 30 min incubation at 37°C. Briefly, 40 μl of the serially diluted sample was transferred to each well of a 96 well microtiter plate. Then, 40 μl of enzyme (0.05 U per well, in 50 mM sodium acetate buffer, pH 5.5) was added in each well and the plate was shaken (190 rpm) on an orbital shaker (Biosan, model OS-10, Latvia) for 1 minute to mix the reagents. After incubation at 37°C for 30 minutes, 40 μl of the substrate (0.005 mM per well, in 50 mM sodium acetate buffer, pH 5.5) was added to each well. The plate was shaken again for two min and then incubated at 37°C for 30 minutes. The reaction was then stopped by addition of 40 ml of 1 M sodium carbonate solution to each well, shaken to mix and then the plate was read at 400 nm with an ELISA plate reader (Biotek© Instrument Inc, USA). Controls had 40 μl buffer added instead of samples, while blanks had 40 μl buffer added instead of the enzyme. The % inhibition was calculated using the following equation;

$$\% \text{ Inhibition} = \frac{(OD_{control}-OD_{blank})-(OD_{sample}- OD_{blank})}{(OD_{control}- OD_{blank})} \times 100$$

3.8.1 IC_{50} Determination

The IC50 determination was performed as per the α-glucosidase inhibition assay described in 3.7, but the amount of inhibitor was varied from 1.0-100.0 μg/ml, the upper limit representing the limit of solubility of the protein. The partial purified protein fraction used in this determination was freshly diluted from lyophilized power.

3.8.2 Determination of kinetic parameters

The procedure began with each 500 µl from the three concentrations (0, 1.0 and 2.0 µg/ml) of fraction F50 which solvated and diluted in 50 mM acetate buffer pH 5.5, was added into separate 15 ml glass test tubes containing 500 µl of 0.1 U/ml α-glucosidase in the same pH buffer. Then, all tubes were mixed thoroughly with vortex mixer and taken to incubate in a 37°C water bath. Immediately after 0.05 mM PNPG substrate in 50 mM acetate buffer pH 5.5 was added and mixed thoroughly, 50 µl of mixture was begun to sampling and transferred into 96 wells microtiter plate containing 50 µl of 1 M sodium carbonate solution in each well. The samples were taken at 3 min interval until the mixture finish. Then the plate was shaken in orbital shaker at 190 rpm for 3 min and taken to read at 400 nm by ELISA plate reader. Obtained ODs were converted to α-glucosidase products towards substrate changing amount per time. The Km and Vmax values of the enzyme including the inhibitor's Ki value were eventually determined from corresponding Lineweaver-Burk plots using SigmaPlot for window version 11.0 (Systat software, Inc, USA).

3.8.3 pH resistance determination

To determine the pH resistance of purified protein, the procedure were carried out as follows. Each 200 µl of fraction F50 (50 µg/ml) solvated and diluted in deionized water 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 the appropriate buffer or deionized water set to the desired pH and incubated at 37°C for 1 h prior to assaying for α-glucosidase inhibitory activity as described in 3.7. 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 the equation described in 3.8

3.8.4 Temperature resistance determination

Each 200 µl of purified protein (fraction F50) (50 µg/ml) protein 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 ($<4^{o}$C), cooling bath (15°C), laboratory room (25°C), water bath (35, 45, 55, 65, 75, 85 and 95°C), for 1 h. After centrifugation at 15,000 x g for 15 min at 4°C to pull the liquid down to the bottom of the tube these samples were evaluated for α-glucosidase inhibitory activity as procedure described in 3.7.

3.9 Hemagglutination assay

The procedure of hemagglutination assay was followed to the method described by Tipthara (2007) and Tipthara (2008). Animal bloods were centrifuged at low speed (1500 rpm) for 10 min and supernatant was discarded. The red cell pellets were washed 3 times with 20 mM Tris-HCl pH 7.2 buffer supplemented with 0.9% NaCl or until clean supernatant appeared. Each 50 μl of tested sample was transferred into a well of 96 well microtiter plate. Serial dilution was carried out in 20 mM Tris-HCl pH 7.2 buffer supplemented with 0.9% NaCl. Then, each 50 μl of 2% red blood cell in 20 mM Tris-HCl pH 7.2 buffer supplemented with 0.9% NaCl was added in to each sample well. The plate was shaken using orbital shaker at 190 rpm for 1 min. After that, the plate was covered and incubated at room temperature until the agglutination sign was seen in the positive control well (20 μg/ml hemagglutinin in the same buffer).

3.10 Anti-bacterial activity assay

The liquid dilution test procedure for antibacterial (proliferation and/or viability) activity was modified from Kim *et al*., (2005) Test bacteria were grown in YM broth (10 g/l peptone, 5g/l yeast extract, pH 7.0), and about 6 hours before the assay, each bacterial culture was adjusted to a final cell concentration of approximately 5×10^{3} cfu/ml in sterile 2X YM medium. The purified protein test sample was serially diluted in triplicate wells of a sterile 96-well plate with sterile deionized water, leaving 50 μl of each final dilution remaining in each well. Next, 50 μl of the prepared test bacterial dilutions were then added to the diluted purified protein in each well, mixed thoroughly, and the plate incubated at 37°C for 6-8 hours until a visible turbid solution was present in the control set of wells (no purified protein added to the bacterial suspension, just the same volume of sterile deionized water only). The bacterial growth in all wells was then measured as a function of the

increased absorbance (optical density) at 620 nm, and related to that of the controls (100% growth and 0% inhibition).

In addition, antibacterial activity, again both bactericidal and bacteristatic without discrimination between them, was also assayed via the standard agar diffusion test procedure, with slight modification from Wang *et al*., (2005). Five ml of warm (47°C) YM agar (0.7% (w/v)) with the appropriate bacterial dilution freshly added and mixed was quickly poured on sterile Petri-dishes containing ~10 ml of set cool YM agar (1.5% (w/v) agar) and left until the top agar set. Then, sterile dry paper discs were placed in the plate at about 1 cm distance from the edge and 10 μl of each purified protein and 25 μM chloramphenicol (positive control) dilutions were added into each disc. Each plate was then were incubated at 37°C for 12 hours to allow the bacteria to grow and the diameter of the clear zone of inhibition around each disc was measured.

3.11 Anti-fungal activity assay

The antifungal activity, in terms of colony growth inhibition, was screened by a similar method to that reported by Wang and Ng (2002). In brief, each of the target fungi species were cultured on 90 x 15 mm Petri dishes containing 10 ml potato dextrose agar (PDA). Before well developed mycelial colonies were present three sterile filter paper discs (6.25 mm diameter and 1 mm thick) were placed on each plate 1 cm away from the mycelial colony edge, 120 degrees from each other, with one disc for the control blank (10 μl of sterile distilled water) and two for appropriate dilutions of the test sample (purified protein) dissolved in sterile distilled water (also at 10 μl per disc) and filtered through 0.45 μm pore size cellulose acetate membrane filter (Satorius stedim, 1106-47-N, Germany). After addition of 10 μl of the respective solution to the respective disc on the culture plate, they were incubated at 25°C until the mycelial growth reached the edge of the control (distilled water) disc. The crescents of inhibition around the sample discs were then measured and used as a marker of the relative inhibitory power of the protein against test fungi. Each assay was performed with 5 such replicate plates per dilution.

3.12 Cytotoxicity assay for human malignant cell lines

The bioassay for the *in vitro* antiproliferative activity (including cytotoxicity without discrimination of the two activities) towards five human malignant cell lines, BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon), was performed in routine tissue culture. Cells were maintained in complete media, comprised of RPMI-1640 supplemented with 10 % (v/v) FCS and 2.0 mM L-glutamine at 37^oC under a 5% (v/v) CO_2 atmosphere. Cells were trypsinized, aspirated and washed before being seeding at a final density of 5 x 10^3 cells/µl in 200 µl of complete media per well in a 96 well plate and cultured for 24 h. After that, serial dilutions of the purified protein were added (0-35 µg/ml final concentration in a total volume of 200 µl complete media) into each well, mixed and the cultures incubated for 72 h. Next, 10 µl of MTT (3-[5, 5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was added to each well and incubated for a further 4 h before the media was carefully aspirated off and the adhered cells gently washed with RPMI-1640 (w/o FCS and other supplements) to remove all remaining media prior to adding 150 µl DMSO per well and leaving for 30 min. The cell remnants and solution were then aspirated to ensure all the cells were lysed and the crystals dissolved, and the absorbance at 540 nm was measured using a microtiter reader. Each assay was performed with triplicate wells with 10 µg/l doxorubicin and CH-Liver cell line w/o purified protein as the positive and negative controls, respectively.

3.13 Internal amino acid sequence determination by LC/MS/MS

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 mm^3) 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 NH_4HCO_3 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^oC. 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 4^oC, 30 µL of 50 mM NH_4HCO_3 was added and the digestion allowed to proceed at 37^oC 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.

The likely amino acid sequence of each internal fragment of the trypsinized was analyzed by LC/MS/MS mass spectrometry. The extracted tryptic peptides were then subjected to LC-nano ESI/MS/MS. All collected LC/MS/MS data were processed and submitted to a MASCOT search of the NCBI database. The following criteria were used in the Mascot search: trypsin cleavage specificity with up to three missed cleavage sites, cysteine carbamidomethyl fixed modification, methionine oxidation variable modifications, ± 0.2 Da peptide tolerance and MS/MS tolerance, and ESI-TRAP fragmentation scoring.

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 F50 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).

3.14 Protease activity determination

The determination of protease activity in solution was modified from that previously reported (Sigma, 1998; Iversen, 1995). Briefly, 500 µl of each fraction was transferred to a 1.5 ml eppendorf tube and 500 µl of a 1% (w/v) azocasein (Sigma-Aldrich, A2765, USA) solution in 20 mM Tris-HCl (pH 8.0) was added, thoroughly mixed, and then incubated at 37^oC for 30 min. Next, 500 µl of a 5% (w/v)

trichloroacetic acid solution was added, thoroughly mixed and left for 30 min at 37°C before clarification of the precipitate by centrifugation at 15,000 x g (Tomy MTX-150, Japan) for 15 min. After that, 100 μl aliquots of supernatant were transferred into the wells of a microtitre plate, 50 μl of 10% (w/v) NaOH solution added, mixed and the absorbance was read at 340 nm with an ELISA plate reader.

3.15 Proximate analysis of *Z. ottensii* rhizome texture

Proximate analysis was mainly followed the method mentioned by Jaafar *et al*., (2009) with some modifications. The detail of each procedure was as described below.

3.15.1 Moisture and ash content determination

Each 5 g of minced sample was loaded in known weight porcelain crucibles and dried in hot air oven at 105°C. The crucible was weighted after dried sample look could be seen until stable weight observed. The weight from this step was recorded and used to calculate for moisture content. The crucible was then further heated at 600°C for 3 hours using electrical furnace (Thermolyne model 30400, USA) and allowed it to cool down overnight. The crucible was eventually weight again and the weight in this step was used to calculate for ash content.

3.15.2 Protein content determination by Kjeldahl method

A 2 g of grinded sample was loaded into 500 ml Kjeldahl flask and taken to mini Kjeldahl set (Gerhardt, UK) installed in fume hood. A 200 ml of Conc H$_2$SO$_4$ and 1 g of mixed catalyst were added and the flask was refluxed for 6 hours. After that, the flask was distilled for 15 min and the effluence was catch by boric acid solution with phenolphthalein indicator. The liquid then was tritrated against 0.1 N H$_2$SO$_4$. The amount of nitrogen was eventually converted to protein content with 6.25 multiply factor.

3.15.3 Lipid content determination by Sohxlet method

A 10 g of grinded sample was loaded into 25 x 100 mm thimble and placed into Sohxlet glass set. Petroleum ether was filled in round bottom flask and connected to the glass set. The Sohxlet extraction was performed at middle level

heater for overnight. Next, petroleum extract was transferred to rotary evaporator (Buchi, japan) to remove as much solvent as possible. The remaining liquid was weighted and the weight of round bottom flask was subtracted out.

3.15.4 Carbohydrate and energy content calculation

The nutritional carbohydrate content in the texture of *Z. ottensii* rhizome was calculated by following formula;

% Carbohydrate = 100-[% moisture - % ash - % protein - % lipid]

The energy of was calculated by converting the amount of protein carbohydrate and lipid into amount of calories by these factors, each gram of carbohydrate or protein = 4 kcal and each gram of lipid = 9 kcal.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Protein isolation from *Z. ottensii* rhizome

The extraction ratio, a 1:4 (v/v) ratio of minced rhizome: 20 mM Tris-HCl buffer pH 7.2, was found to be suitable as the blended residual solid phase was approximately one third of the total volume under the liquid phase (data not shown). A lower buffer ratio might allow the residue to pack too fast and the mixture would become more viscous and be harder to mix. In turn, a higher buffer ratio would decrease the extracted protein concentration. The rhizome contains a large proportion of coarse starch that settled down very quickly, as seen in the bottom of container, after the mixture was poured out. Thus, the residue quickly stuck when ever it was left for any time.

The average protein yield obtained from the extractions of the rhizome batch reported here was 1.31±0.20 g/kg fresh rhizome weight, but it was observed that the amount of protein (per kg rhizome mass) obtained varied amongst the different rhizome batches. The weight could be converted to percentage as 0.13% fresh weight or 7.93% dry weight (estimated from the rhizome texture with average 83.50% moisture content). Masuzawa *et al*., (1977) had reported the protein content in rhizomes of *Miscanthus sacchariflorus* which ranged from 2.9 to 4.0% dry weight and young rhizome exhibited higher amount of protein than the old one. In contrast, Lipke *et al*., (1964) had reported 0.05 and 0.08% fresh weight of protein content in Tanweed bud and rhizome, respectively. Our total protein content was relatively high compared with those two reported methods, (Masuzawa *et al*., 1977; Lipke *et al*., 1964). It could be suggested that this single step purification was effective enough in protein preparation at least for the *Z. ottensii* rhizome. On the other hand, it might be indicated that differences of protein contents were not affected from extraction methods only, but also other factors; for examples age, plant parts, and genetic nature. The *Z. ottensii* rhizomes used in this study was selected for identical size, thickness, flawless and appearance. However, all were obtained from natural sources and so were not controlled or standardized for agrochemical, geochemical and climatic variations in the environment, age and cultivar genetics. Certainly, the rhizomes were

noted to vary in color (purple) shades and numbers of bud or inner cavities, which may indicate differences in their ages and constituents. Whether these also reflect differences in the protein content in general, or the specific protein of this report is unknown.

4.2 Protein purification

Preliminary testing found that solubility of the crude protein was 6.7-7.2 mg/ml in Tris-HCl buffer pH 7.2. At the saturation point, the solution became slightly cloudy. However, the residue which was not occurred in this point would be appeared at more than 15 mg/ml. This might because there was more than single protein in its composition and the saturated condition might come from one of them while the others remained unsaturated. Thus, all samples loaded into the chromatographic separation column were from the same extraction lot to ensure sample homogeneity, and at a 5 mg/ml concentration to ensure that no precipitation occurred in the separating column.

It was known that different proteins require different separation techniques in successive isolation and purification stages, for example, alocasin was isolated from giant taro (*Alocasia macrorrhiza*) rhizomes using 10 mM Tris-HCl pH 7.4 buffer without a $(NH_4)_2SO_4$ salting out step, but the crude protein was only obtained at a low yield of 384 mg/kg rhizome (compared with this study which gained 1.31 ± 0.20 g/kg rhizome), albeit after many chromatographic purification steps (Wang H., 2003). The protein purification step in this study used chromatography with SP-sepharose gel separation, a moderate cation-exchanger, to separate each protein in the crude extract using a 0 to 1 M NaCl gradient elution. The eluted pattern appeared to be a broad clump of bounded content with long tail eluting from about 0.27 M NaCl upwards (data not shown). The chromatogram indicated that gradient elution was not a suitable purifying condition for this protein mixture. This might because different proteins contained in this mixture possessed so small different in positive charges to be separated by gradient, little-by-little changing in solution's ionic strength. In alternative point of view, this might indicated that the extracted proteins have nearly similar positive charge density and that there was no clear partition (separation). It is possible that at the working pH condition (Tris-HCl buffer pH 7.2), the protein mixture, which can act as a buffer, had enough time to compensate gradually for the

increase in the ionic strength from the gradient elution and retain the previous net negative charge. Thus, a stepwise elution was next performed, which resulted in much clearer and delineated peaks (Figure 4.1).
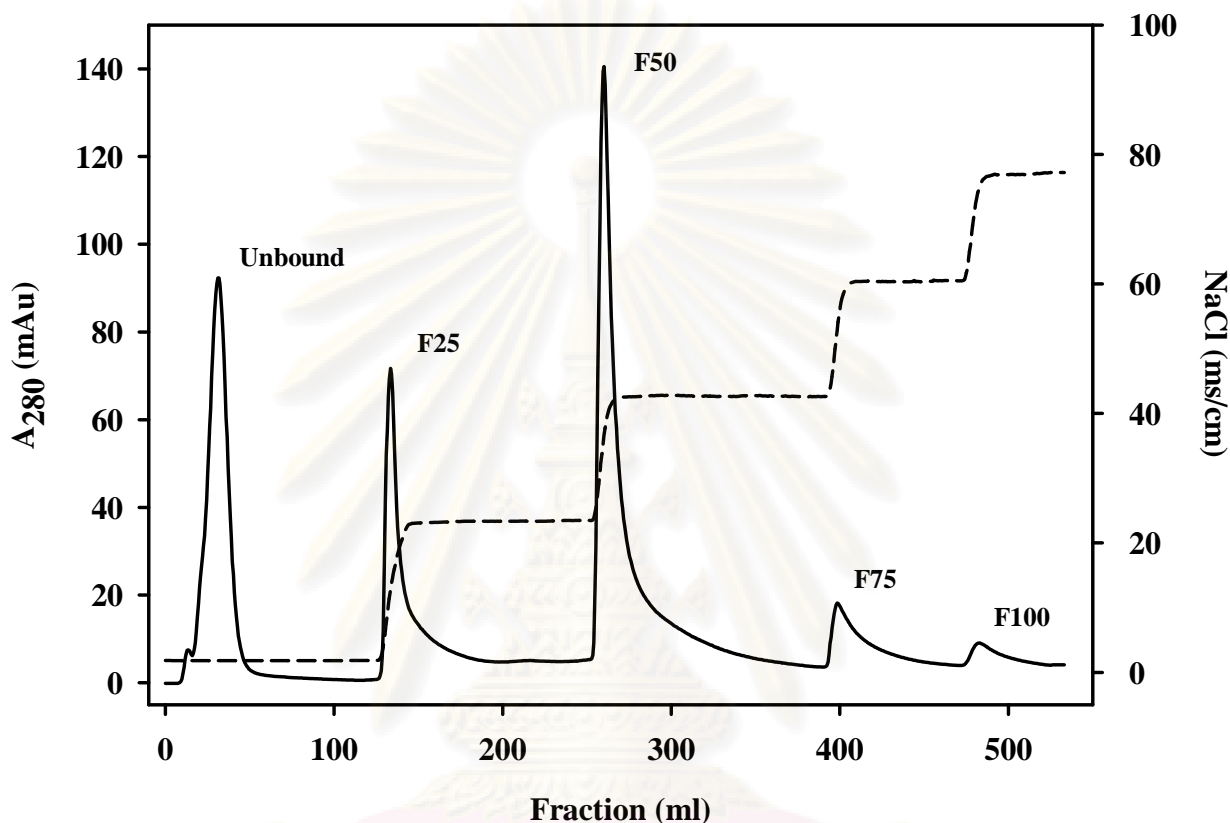
Figure 4.1 SP-sepharose chromatogram of the crude *Z. ottensii* rhizome protein extract (50 mg) with stepwise NaCl elution (0.25, 0.50, 0.75 and 1.00 M).

From the stepwise elution, five distinct peaks were isolated, unbound, and F25, F50, F75 and F100 from the bound fractions named according to the percentage of buffer B used in their elution. It was found that the peak of fraction F50 was the highest, followed by unbound and F25 respectively. Quantification of the protein content in the five fractions revealed the highest protein content was found in F50 (Table 4.1), in accord with it having the largest peak in the SP sepharose chromatogram. This could be the effects of salting out process (80% saturated $(NH_4)_2SO_4$) which allowed this protein dominance and ion-exchange chromatography condition used in the study which suitable for ionic strength of the F50 molecule.

Since the F50 was purified by SP sepharose, a strong cation exchanger with -CH_2-SO_3- functional group (Anonymous, 1999), the molecular charge should be moderate positive because of approximately middle salt concentration eluting. However, fractions F25 and U were ordered next in terms of protein yield (Table 4.1), in contrast to the chromatogram result (Figure 4.2). This can be explained by two non-mutually exclusive assumptions. Firstly, the unbound peak may not all be protein, but may also be elevated by the presence of other small molecules which absorb light at 280 nm. Secondly, the protein in fraction Unbound may contain less UV absorbing residues (tryptophan, phenylalanine and histidine) than that in fraction F25. Both fraction F75 and F100 contained too low an amount of protein to be of use in any further investigation. So, they were neglected. Note, however, that from 50 mg of loaded protein, only 29 mg (58%) were recovered in the unbound and four bound peaks (Table 4.1), meaning that some 42% of the loaded protein was lost, perhaps still bound to the column.

The F50 fraction was chosen in further analysis because of satisfied purity (Figure 4.2 A) and highest amount (Figure 4.1), meanwhile, F25 fraction contained more than one protein and could not be successfully separated so far. Many benefits achieved from single step chromatography purification; easier and more comfortable, lower time spent lower purification cost, and able to avoid more loss beyond many processing steps as seen in some literatures (Demir *et al*., 2008; Wang and Ng, 2002; Ye *et al*., 2001; Rameshwaram and Nadimpalli, 2008).

4.3 Purity checking by native and reducing SDS-PAGE

The protein from each step of purification was analyzed for purity and protein pattern by native-PAGE (Figure 4.2 A), where after SP Sepharose affinity chromatograpy a single protein from F50 band on the native-PAGE gel was observed, indicating that the purified protein obtained from the SP sepharose column should be a relatively pure protein.Reducing SDS-PAGE analysis revealed three bands, at about 32.5, 15.2, and 13.8 kDa were present in the F50 fraction (Figure 4.2B). If fraction F50 contains a single protein, then it must consist of at least three subunits. Since the highest intensity bands were seen in the lower molecular weight, it is possible that the F50 fraction protein might consist of one large and at least two small subunits.
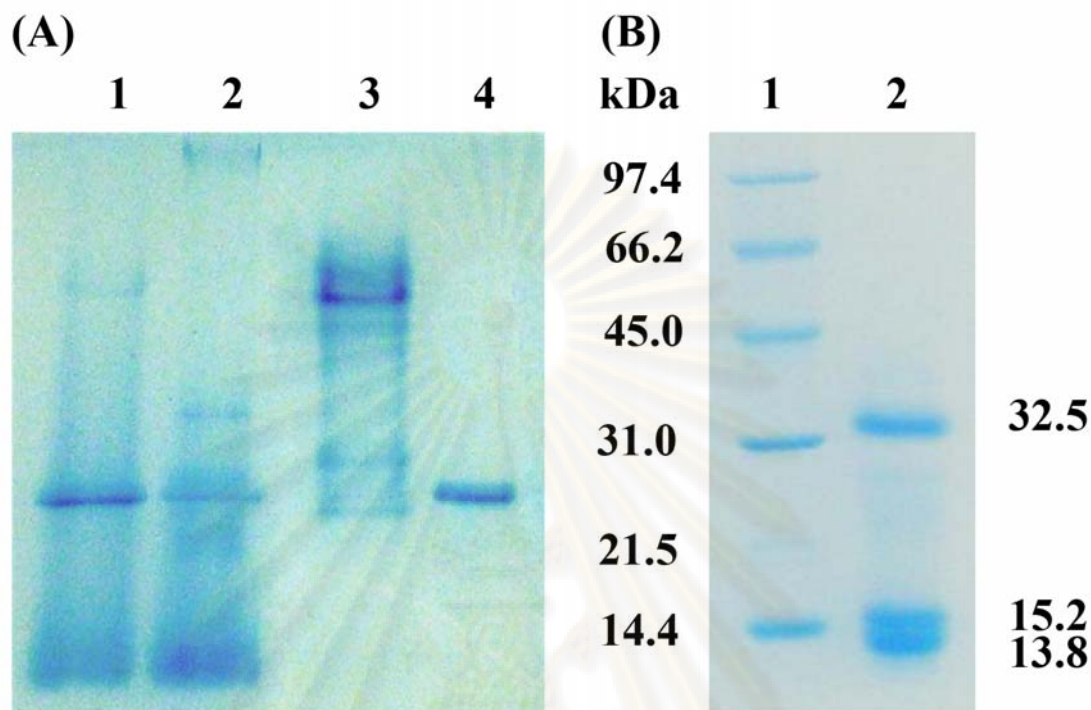
Figure 4.2. (A) Coomassie blue stained native-PAGE of the *Z. ottensii* rhizome protein from each step of purification where lanes 1-4 show 20 µg of total protein from (1) the crude extract (homogenate), (2) the 80% ammonium sulphate precipitated and kept fraction (3) the F25 bound SP sepahrose fraction discarded and (4) the F50 bound fraction kept. (B) Reducing SDS-PAGE analysis of the *Z. ottensii* rhizome protein purification. Lane 1, molecular weight standards; Lane 2, fraction F50.

4.4 Carbohydrate content determination

From phenol-sulfuric determination and periodic acid Schiff's staining results, the F50 molecule was glycoprotein containing 26.30±1.01% of carbohydrate content (glucose equivalent weight compared to 100% protein weight). The amounts of carbohydrate content achieved by phenol-sulfuric acid assay for unbound fraction (0%), F25 (363.29±1.00%), and F50 (26.30±1.01%) were consistent to the results from native and SDS-PAGE and periodic acid-Schiff's determination which the carbohydrate content in F25 was highest and no carbohydrate (also protein) found in

unbound fraction. It could be deduced that unbound fraction, the highest peak (Figure 4.1), was possibly small molecule (s) with neutral or negative charge and having the least carbohydrate content. Moreover, the F25 fraction might contain proteoglycan substances because of nearly 3.5 times carbohydrate content of protein part existed and no additional band present in periodic-acid Schiff's staining gel which could be pointed out as non-proteinateous polysaccharide (Figure 4.3).

Figure 4.3 Periodic acid-Schiff's staining of protein separated by reducing SDS-PAGE form (1) the 80% ammonium sulphate precipitated and kept fraction (2) the unbound fraction (3) F25 bound SP sepahrose fraction discarded and (4) the F50 bound fraction.

4.5 α-glucosidase activity

Since the crude extract had already been preliminary characterized and found to have α-glucosidase inhibition activity (97.5% inhibition using 50 mg crude protein/U α-glucosidase) (Table 4.1), the Unbound, F25 and F50 fractions were then evaluated for any α-glucosidase inhibitory activities. It was found that among these fractions the F50 contain highest α-glucosidase inhibitory activity (Table 4.1). Then, the F50 was further characterized for inhibitory kinetics such as percentage of maximum inhibition, IC_{50}, and inhibition constant (Ki). From the inhibition data

obtained using different concentrations of fraction F50, the data was processed and the best fit curve was derived using the commercial Sigmaplot version 11 software (Figure 4.4). The maximum inhibition and IC_{50} were calculated as 100% and 30.15 μg/ml, respectively. Cuminaldehyde, a small non-protein molecule isolated from *Cuminum cyminum* L. seeds, was reported to have an inhibition activity against Sprague-Dawley rat α-glucosidase with an IC_{50} of 0.5 mg/ml, which is 1.8 times lower than that of acarbose (Lee, 2005).

Table 4.1 The amount of protein in each purified fraction, after loading 50 mg total crude protein extract onto the column, and the α-glucosidase inhibitory activity.

Fraction	Protein yield (mg)	α-glucosidase inhibitory activity Maximal inhibition (%)
Crude extract	50.00	97.50±10.17
Fraction Unbound	1.55±0.60	36.74± 0.18
Fraction F25	4.20±3.00	28.54± 4.22
Fraction F50	21.35±8.00	77.50± 4.27
Fraction F75	1.15±0.80	[a]Nd
Fraction F100	0.75±0.50	[a]Nd

[a]Nd = Not determined
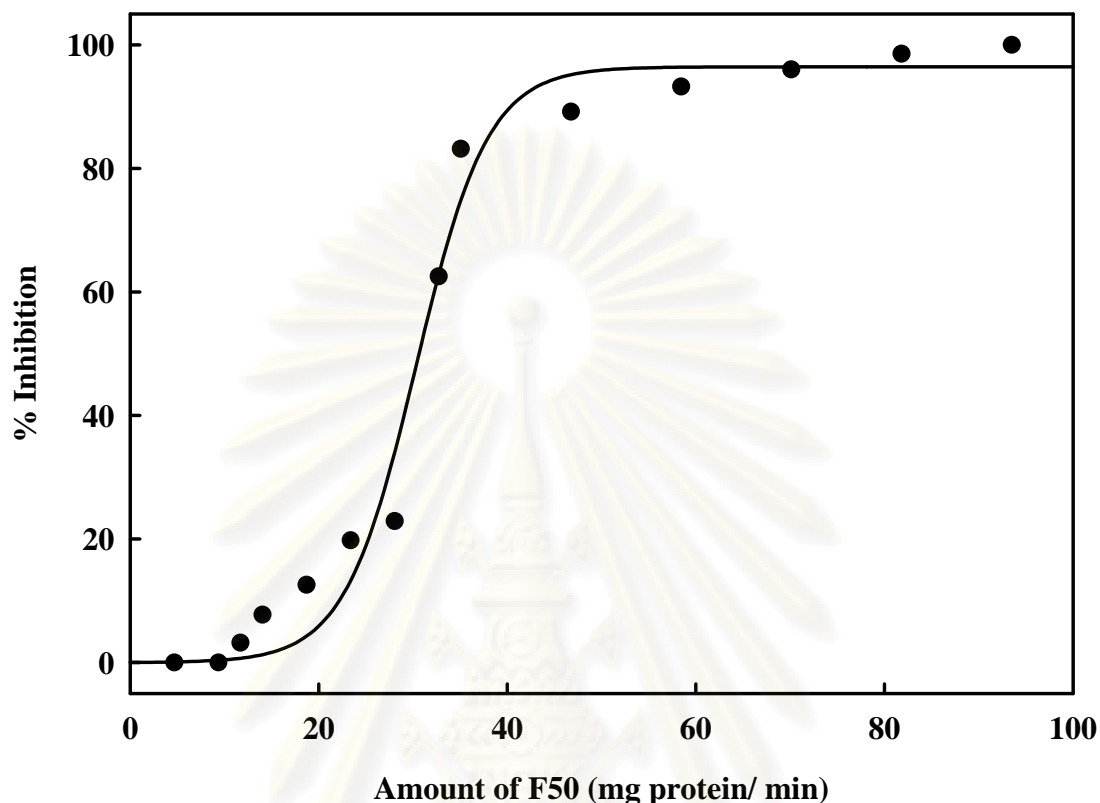
Figure 4.4 Dose-dependent inhibition of α-glucosidase by the *Z. ottensii* rhizome fraction F50 protein.

Although, different α-glucosidase sources might result in different relative Acarbose inhibitory activities, for targeting human α-glucosidase the enzyme from baker's yeast was used as a preliminary testing tool. Anyway, it could be approximately deduced that the fraction F50 had a 16.1-fold and 8.9-fold greater inhibitory activity than cuminaldehyde and acarbose, respectively. Furthermore, the crude protein preparation from *Z. ottensii* reported here revealed a 97.5% maximum inhibition activity compared to the 82.07% reported for the crude protein (after 90% saturated $(NH_4)_2SO_4$ salting out) from the flower extract of *Sesbania grandiflora* (Boonmee, 2007). Moreover, after Superdex G200 and DEAE cellulose purification of the α-glucosidase inhibitory activity of reduced the maximal observed inhibition level to 74.0%, which concurs with our result here that after SP Sepharose

purification the F50 fraction revealed a 77.5% maximum α-glucosidase inhibitory activity compared to ~97% in the crude *Z. ottensii* rhizome extract. However, it could be seen that the inhibitory protein from *Z. ottensii* rhizomes was apparently slightly stronger than that from *S. grandiflora* flowers. In addition, the IC_{50} of F50 (30.15 μg/ml) was somewhat similar, given the differences in assays, to the IC_{50} obtained from the *Morus alba* leaf extract (28.1 μg/ml) (Yogisha and Raveesha, 2009). From the data obtained using fraction F50, the calculated Ki was 140 μmol while the Km and Vmax were 2.35 μmol and 0.11 mM/min, respectively (Figure 4.5). In addition F50 was characterized as a reversible competitive inhibitor where its linear curve intercepted the no-inhibit α-glucosidase curve.
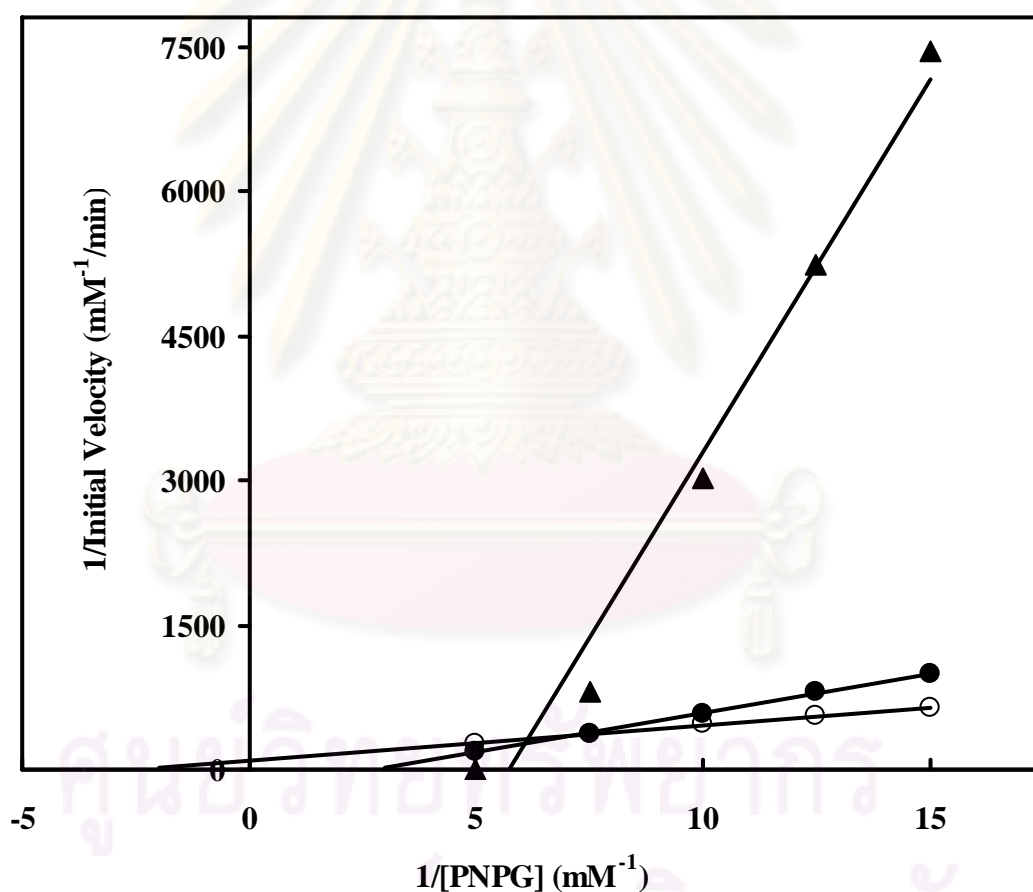
Figure 4.5 Lineweaver-Burk plot of α-glucosidase kinetics with and without different concentrations of the *Z. ottensii* rhizome fraction F50 protein. (open circle) 0 μg/ml, (closed circle) 1 μg/ml, and (triangle) 2 μg/ml. Data are shown as the mean ±1 SD and are derived from three repeats.

No significant change in the inhibition activity of fraction F50 was seen when pre-treated within the temperature range of 4-35°C, while at 45°C and, especially, 55°C, the observed inhibition activity was slightly decreased (Figure 4.6). One possible reason was that the higher temperature range caused a change in the structure of fraction F50 (or even α-glucosidase enzyme) reducing its ability to bind to the enzyme active site. Thus, it can be deduced that the F50 fraction inhibitor is likely to be affected in the 45-55°C temperature range. The inhibitory activity was maximal at pre-incubation at 65°C and decreased markedly thereafter at higher temperatures. The maximal inhibition at 65°C may, therefore, be regarded as the optimum temperature for this system. The baker's yeast enzyme used in this system had a poor activity at 65°C, in common with other *S. cereviceae* derived α-glucosidases which are reported to have a maximal optima at close 30°C (Ahmed *et al*., 2007), preventing co-incubations of enzyme and inhibitor together. However, that the F50 α-glucosidase inhibitor could be used at a rather high temperature means that α-glucosidases from other sources that are more thermotolerant should be applied in future studies. The decrease in the inhibitory effect at temperatures over 65°C could obviously be explained by degradation or denaturation of the F50 away from the active conformational state.
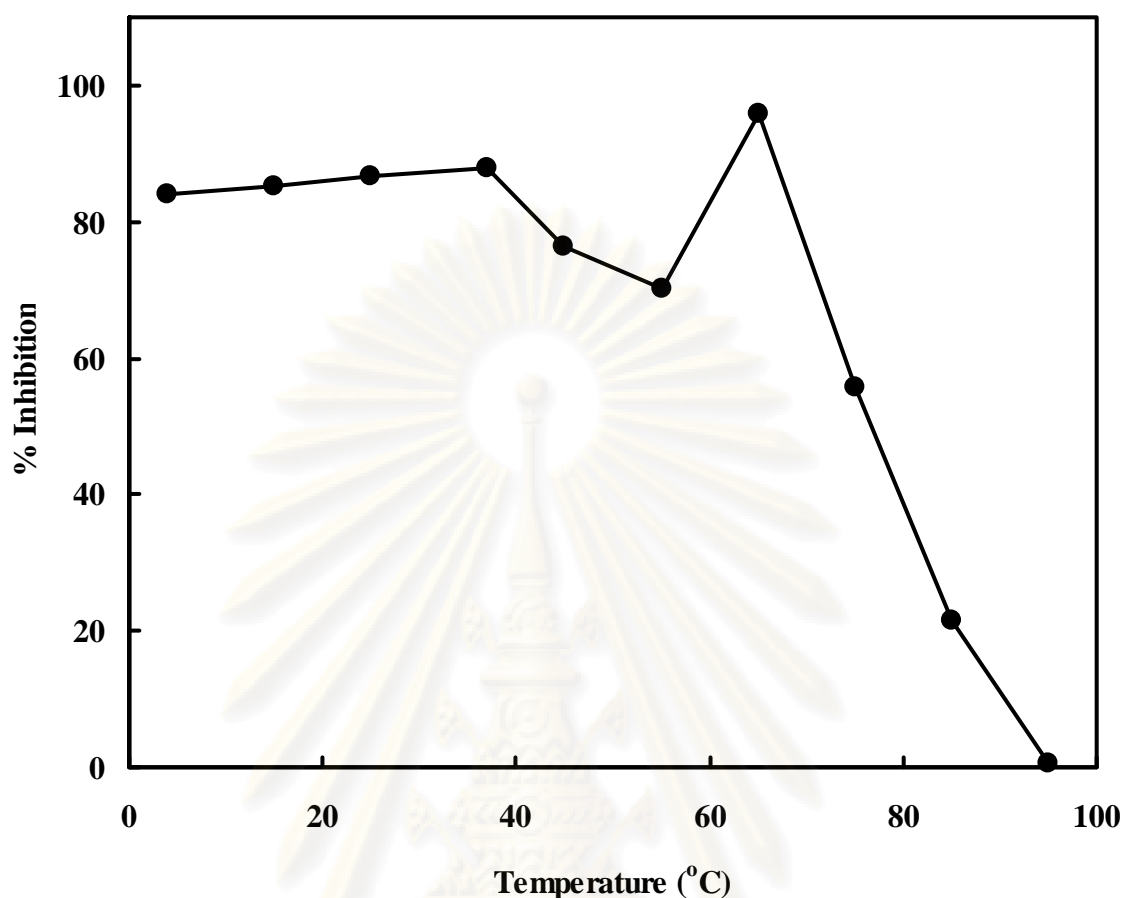
Figure 4.6 Effect of preincubation of fraction F50 at different temperatures for the subsequent α-glucosidase inhibitory activity. Data are shown as the mean ±1 SD and are derived from three repeats.

Changing the F50 protein preincubation buffer pH and salts revealed that the inhibition effect of fraction F50 was negated at pH 7.0 and steeply reduced at pH 10.0 (Figure 4.7). The optimal pH for the *S. cereviceae* α-glucosidase used was 6.8 (supplier's information sheet). In agreement with this, the optimum pH for baker's yeast (*S. cereviceae*) α-glucosidase has been reported to be about pH 6.0 and 5.8-7.0, respectively (Ahmed, 2007, and Shevchenko, 2001). Thus, at pH at 7.0, the concentration of F50 used (50 μg/ml) might be too limited to overcome the strongest activities of the α-glucosidase enzyme. A higher amount of the F50 inhibitor would be needed in this case. However, the optimal pH of the F50 inhibitor may lie within the acidic range (pH 2.0-6.0) since high inhibition percentages (more than 90%) were

obtained in this range. In which case, pH 7 is simply a suboptimal pH for the F50 inhibitor. Weaker inhibition activities (69.3-82.4%) were noted again as the pH increased to 8.0-9.0 before dropping off at pH 10 above the pI of fraction F50. The apparently high activity at pH 11.0 or 12.0 seen with buffer (Figure 4.7), but not without (Figure 4.8), should be ignored as an artifact. Although clear differential ion effects were seen (compare at pH 4, glycine acid vs. acetate; at pH 8, phosphate vs. Tris; and at pH 10, Tris vs. glycine base), this is more complicated as, in contrast to the preincubation of F50 at different temperatures, the preincubation at different pH values will also directly affect the α-glucosidase assay by changing the assay pH, since the volume and buffer capacity of the preincubation and assay mixture are equal on mixing, or 1:2 after substrate addition.
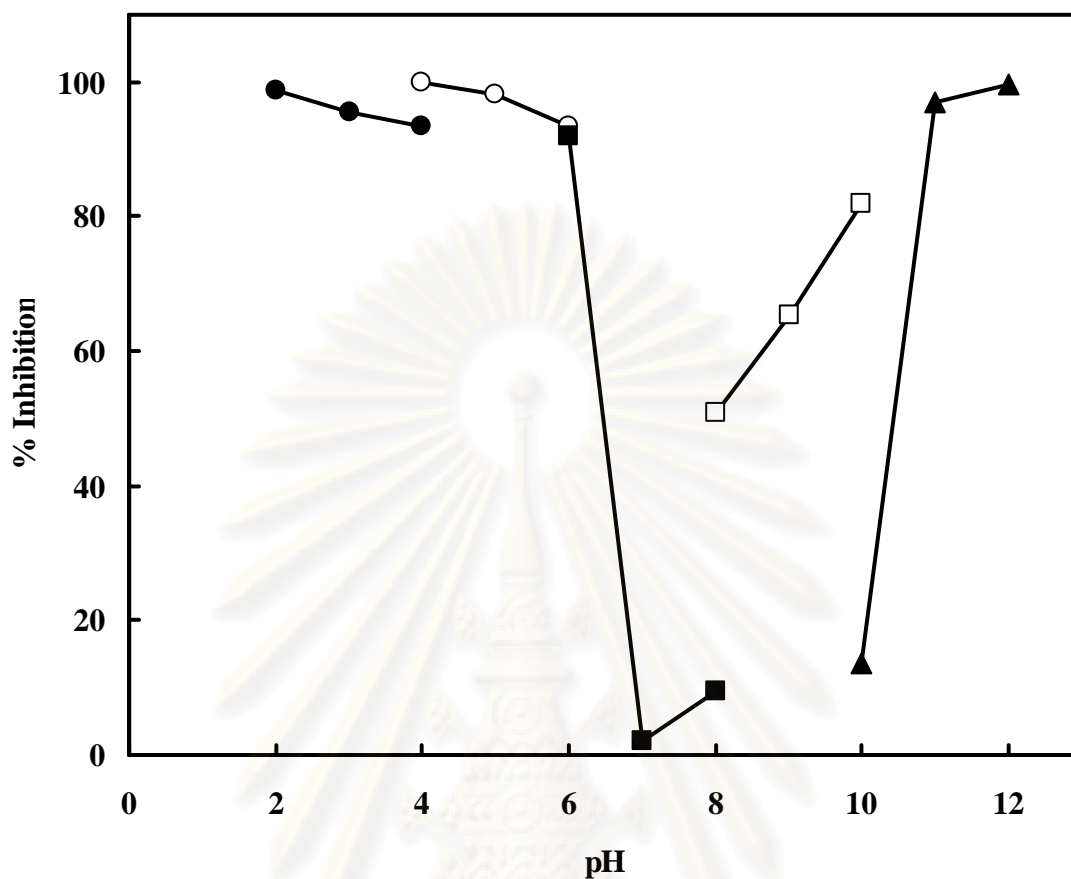
Figure 4.7 Effect of preincubation of fraction F50 in different pH and buffer solutions on the subsequent α-glucosidase inhibitory activity. Pretreatment was with (closed circle) 20 mM glycine-HCl (pH 2-4), (open circle) 20 mM sodium acetate (pH 4-6), (open square) 20 mM potassium phosphate (pH 6-8), (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.

To evaluate the potential role of differential ion effects from the different buffers, and limit the above changes to the assay pH, the pH tolerance assay was repeated only using a non-buffer system for the F50 protein pre-incubation (distilled water with pH set by HCl or NaOH addition only). Under these conditions, no significant decrease in the observed level of α-glucosidase inhibition was seen within a pH range from 2.0-10.0 (Figure 4.8). However, the activity sharply declined at pH values above 10. By comparison between Figures 4.7 and 4.8, three points could be

inferred. The first point was that there were ion effects evident with different buffers at the same pH as discussed above. The second was that pH values over 10.0 were not assayable, which was in agreement with the observation that α-glucosidase activity rapidly declined as the pH increased above 7.0 (Ahmed, 2007). Finally, there were declinations in inhibition activities at pH 7.0 and 10.0 (Figure 4.7) was the result from buffering ions. Thus, some inhibitor-ion interactions might block or slow down the inhibitor activity at such pH values. These were some possibly pitfalls in α-glucosidase inhibition assay which researchers must keep in mind in dealing with the system.
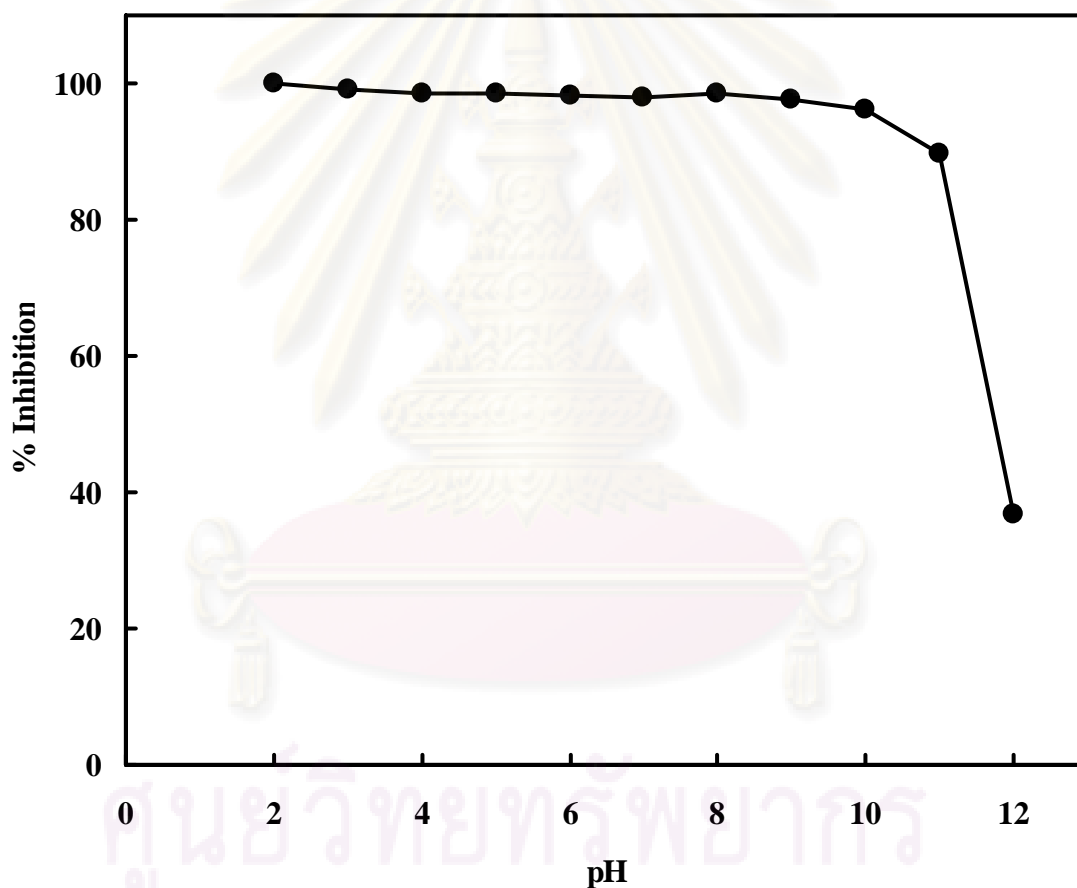
Figure 4.8 Effect preincubation of fraction F50 in different pH (buffer absence) solutions for the subsequent α-glucosidase inhibitory activity. Data are shown as the mean ±1 SD and are derived from three repeats.

4.6 Hemagglutination activity

Hemagglutination was performed in all protein preparation steps and all purified fractions which firstly used rabbit red blood cells as determination tool. The results were shown in table 4.2. It can be seen from the Table 4.2 that the crude extract, salting out, and F25 protein contained hemagglutination activities at the titre 2^5, 2^7-2^8, and up to 2^4, respectively. Since the salting out step (solid powder) gained more protein contents than crude extracted protein (solution), the salting out protein thus exhibited more hemagglutinating activity than crude extracted protein. The F25 fraction exhibited lower and fluctuating activities which were formally no reliable conclusion. However, it was just able to explain after the F50 protein had been identified as protease and the protease activity had been detected (will be described later).

Table 4.2 Hemagglutination activity of protein obtained from each purification step.

Fraction	Agglutination (titer)
Crude extract	2^5
Salting out	2^7-2^8
Fraction Unbound	-
Fraction F25	0-2^4
Fraction F50	-
Fraction F75	-
Fraction F100	-

Six different animal bloods (goose, guinea pig, rabbit, rat, mouse, and sheep) were determined the most suitable blood for F50. The hemagglutinating results were shown in table 4.3. It was found that the rat blood was the most suitable as it gave highest agglutinating titre at 2^9. The rabbit blood which was formally used in hemagglutination screening gave lower titre (2^4). The guinea pig and sheep bloods gave lowest agglutination (2^2) while no agglutination presented in the goose blood. Surprisingly, the mouse red blood cell was lysed (as confirmed by microscopy observation) by the cloudy red solution became clearly dark red in this experiment.

The mouse red blood cell was also test with 0.01% phytohemagglutinin and the lysed phenomenon was least. These indicated that it was one or more unknown molecular property of F50 that could disrupt the mouse red blood cell membrane. It was not complement system because all soluble proteins in the blood plasmas were already washed out. One possible reason was that the membrane of mouse red blood cell contains some glycoproteins which could be recognized by lectin from F50 and thus precipitate at a location and was removed out while the protease part of F50 worked (the F50 also contain protease activity which will be described later). Thus, in this case, the F50 could possibly be "lectzyme", the multi-binding site lectin with other activity than carbohydrate binding (Sharon, and Lis, 2007).

Table 4.3 Hemagglutinating activity of the F50 from the rhizomes of *Z. ottensii* against animal erythrocytes.

Erythrocyte source	Agglutination (titer)
Mouse	lysed
Rat	2^9
Guinea pig	2^2
Goose	0
Sheep	2^2
Rabbit	2^4

Human blood types (A, B, AB, O) were also examined for hemagglutination assay with F50 and no agglutination was found in all human blood types. The sugar inhibition assay appeared that only four sugars (fructose, *N*-acetyl glucosamine, glucose, and methyl-D-glucopyranoside) gave positive result by 2^2-2^4 titers. However, the activities were relatively low (no obvious specificity found), uncertain and unstable. Almost all activities would loss with in 24 hours after crude protein powder re-dissolved. This might because of the protease activity of F50 itself. Heamagglutination with additional minerals mostly gave no enhancement results except calcium chloride and ferric chloride which appeared slightly more agglutination dilution (about 1 to 2 titers but unstable). It suggested that calcium ion

and ferric ion could specifically affect the binding of this lectin while other divalent cation such as manganese ion not affected the binding.

4.7 Anti-bacterial activity against normal flora

The F50 was also tested for anti bacterial activities against four normal flora (*B. subtilis*, *S. aureus*, *P. aeruginosa*, and *E. coli*) by both liquid dilution test and agar diffusion test (or can be called Kirby-Bauer disk-diffusion method). It was found that no inhibition (clear zone) presented around discs filled with F50 at all given concentration of all bacterial cultures in agar diffusion test. Furthermore, the liquid diffusion test also gave the same direction result. There was no clear solution presented in any well after the broth cultures were incubated beyond 16 hours (the standard method requires 24 to 48 hours). These suggested that the F50 contained no antibacterial activities against (at least) four given bacterial strains. It could be possibly that the bacteria could grow faster than the F50 protease activity to destroy their extracelllular enzymes.

4.8 Anti-fungal activity against plant pathogenic fungi

The purified F50, when applied at a dose of either 23.6 µg or 47.2 µg/0.3 cm^2 disc showed antifungal activity against the three tested phytopathogenic fungal species, *E. turicicum*, *F. oxysporum* and *C. cassiicola*, but the level of inhibition attained was significantly higher at 47.22 µg/0.3 cm^2 disc on all three isolates (Figure 4.9). Antifungal peptides reported to date usually exhibit multiple bioactivities but have only a relatively weak antifungal activity, compared to the other activities. For instance, the lectin purified from kidney beans showed an antifungal efficiency against *F. oxysproum*, *Rhizoctonia solani* and *Coprinus comatus* that is somewhat similar to that seen here for purified protein on *E. turicicum*, but the kidney bean lectin could also inhibit HIV-1 reverse transcriptase and displayed a strong α-glucosidase enzymic activity (Ye *et al*., 2001). In addition, the cyclophilin-like antifungal protein (CLAP) from chickpea seeds was found to contain antifungal activity against *Mycosphaerella arachidicola* at a fairly similar level to that seen here for purified F50 against *C. cassiicola*, but CLAP could also inhibit HIV-1 reverse transcriptase up to 97.8±1.84% at 227.7 µM concentration (Ye *et al*., 2002). Thus, the

antifungal efficiency of F50, which corresponded to other reported peptides, is regarded as a mild to moderate growth inhibitor, depending upon the fungal strain.
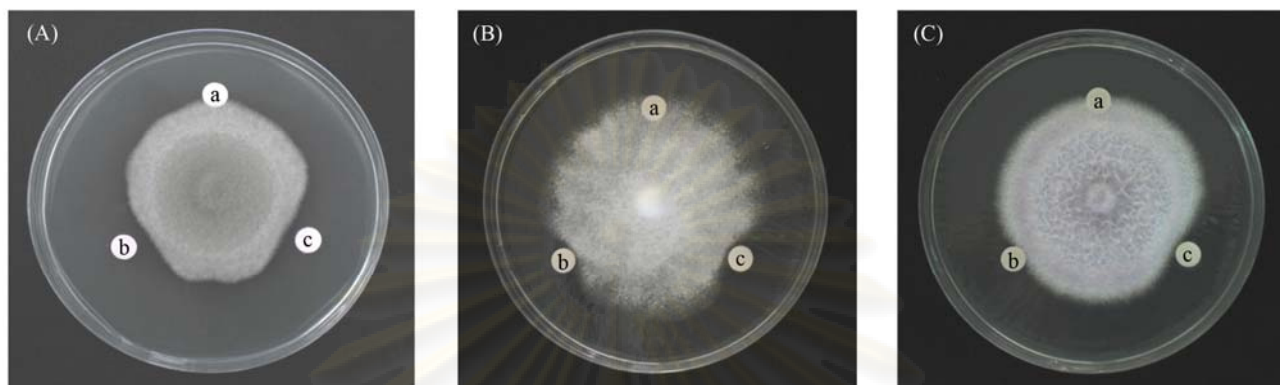
Figure 4.9 Disc diffusion test showing the antifungal (colony growth inhibition) effect of the purified protein against (A) *C. cassiicola*, (B) *E. turicicum* and (C) *F. oxysporum*. Each plate shows (a) the negative control (10 μl of 20 mM Tris-HCl buffer pH 7.4), (b) 23.6 μg F50/0.3 cm^2 disc and (c) 47.2 μg F50/0.3 cm^2 disc.

4.9 Cytotoxicity against cancer cells

The anti-proliferative or cytotoxic effect of F50 highest efficiency against HEP-G2 (hepatoma cancer) with an IC$_{50}$ value of 1.134 μg/ml down to the lowest for SW620 (colon cancer) with an IC$_{50}$ of 5.370 μg/ml. However, the dose-dependent effect (inhibition of proliferation and or cytotoxicity) was different between the two cell lines (Figure 4). For the SW620 cell line, although a larger IC$_{50}$ was evident, a greater degree of inhibition (just under 75%) over a narrower dose range was obtained than that seen with the HEPG2 cell line which displayed a 4.7 fold lower IC$_{50}$ value but a lower maximal inhibition level (~50%) spread over a larger dose range. This could suggest different mechanisms, be that receptors, with different Ka values, or differences in the number and duration of receptor crosslinking or in internalization pathways etc.
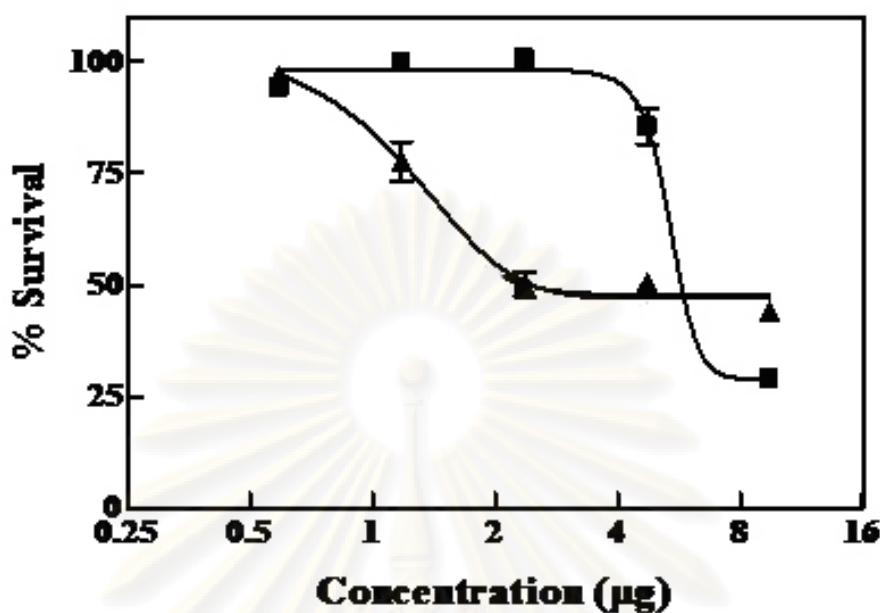
Figure 4.10 *In vitro* inhibition of cell cytotoxicity activity of F50 protein against the (■) colon cancer (SW620) and (▲) hepatoma cancer (HEP-G2) cell lines in tissue culture. Each data point represents the mean ± 1 SD of triplicate determinations.

4.10 Protein identification

Amino acid sequences of the tryptic peptide fragments obtained from the three different potential subunits of purified protein were deduced by LC-MS/MS analysis. Five sequences (AVANQPVSVTMDAAGR, NRNH, GCEGG, WPYR and NWGESGYIR) were gained from software analysis (*De novo* deducing) (Figure 4.11). All fragments were aligned to those homologs available in the NCBI GenBank and UniProt databases. The longest two sequences, AVANQPVSVTMDAAGR and NWGESGYIR, were also BLASTp searched against the GenBank and UniProt nr database alone, revealing 100% amino acid sequence similarity to that in zingipain-2, zingipain-1, cysteine protease GP2a, and cysteine protease GP2b (all from *Zingiber officinale* rhizome) for the first peptide, and 100% similarity to Actinidin Act2a protein from kiwi fruit (*Actinidia deliciosa*) for NWGESGYIR. Indeed, these two peptide fragment sequences were used to design degenerate oligomer nucleotide primers for further RT-PCR amplification based studies to develop probes and clone

the gene transcripts for recombinant protein expression (data not shown). The remaining three shorter fragments, NRNH, GCEGG and WPYR, did not exactly match any part of the available cysteine protease sequences but they are too short to look to look for reliable specific mismatched sites. Thus, they may represent polymorphic regions within the protein, or be derived from a different subunit/ protein. Nevertheless, from the data derived from the two larger peptide sequences, purified F50 is likely to be a member of the cysteine protease family (Figure 4.11), although whether it is comprised of one, two or three subunits and their role, if so, is yet to be established. Regardless, the notion of purified protein being a cysteine glycoprotease opens up the ability to evaluate the reasons for the other observed results, such as the negative antibacterial and positive antifungal activity. As a result, the query sequence, AVANQPVSVTMDAAGR and, NWGESGYIR as shown in (Figure 4.12, and 4.13 respectively.

Accession number	Organism		Sequence	
	Zingiber ottensii (F50)		AVANQPVSVTMDAAGR NRNH GCEGG WPYR NWGESGYIR	
P82473	*Zingiber officinale* (Cysteine proteinase GP-I)	126	AVANQPVSVTMDAAGRDFQLYRNGIFTGSCNISANHYRTVGGRETENDK-DYWTVKNSWGKNWGESGYIR	194
Q5ILG7	*Zingiber officinale* (Cysteine protease gp2a)	267	AAANQPISVGIDASGRNFQLYHSGIFTGSCNTSLNHGVTVVGYGTENGN-DYWIVKNSWGENWGNSGYIL	335
Q5ILG5	*Zingiber officinale* (Cysteine protease gp3a)	267	AAANQPISVGIDASGRNFQLYHSGIFTGSCNTSLNHGVTVVGYGTENGN-DYWIVKNSWGENWGNSGYIL	335
Q40675	*Oryza sativa* (Cysteine protease precursor)	268	AVANQPVAVAVEASGQDFQFYSEGVFTGECGTDLDHGVAAVGYGITRDGTKYWIVKNSWGEDWGERGYIR	337
P84346	*Jacaratia mexicana* (Mexicain)	214	AIANQPVSVVTDSRGRGFQFYKGGIYEGPCGTNTDHAVTAVGYGKT-----YLLLKNSWGPNWGEKGYIR	188
O22500	*Zea mays* (Cysteine proteinase Mir3)	258	AVANQPVSVAIBAAGTAFQLYSSGIFTGSCGTRLDHGVTAVGYGTENGK-DYWIVKNSWGSSWGESGYVR	326
Q6F6A7	*Daucus carota* (Cysteine protease)	218	AVAHQPVSVAIBAGGRAFQLYESGIFNGLCGTDLDHGVVAVGYGTEDGK-DYWIVRNSWGPNWGESGYIR	286
Q6F6A9	*Daucus carota* (Cysteine protease)	275	AVAHQPVSVAIBAGGRAFQLYESGVFTGQCGTELDHGVVAVGYGSENGK-DYWIVRNSWGPDWGESGYIR	343
Q9SLY9	*Zea mays* (Cysteine protease)	258	AVANQPVSVAIBAAGTQFQLYSSGIFTGSCGTALDHGVTAVGYGTENGK-DYWIVKNSWGSSWGESGYVR	326
Q6Y1E4	*Trifolium repens* (Cysteine protease 12)	251	AVANQPISVAIDASGSDFQFYKSGVFTGSCGTELDHGVTAVGYGISNDGTKYWLVKNSWGTDWGEFGYIR	320

Figure 4.11 Amino acid sequence from the tryptic fragments of the purified *Z. ottensii* rhizome fraction F50 protein. Comparisons are made with other cysteine proteinases from the cysteine proteinase family that showed the highest sequence homology in BLASTP searches of the NCBI and SwissProt databases.
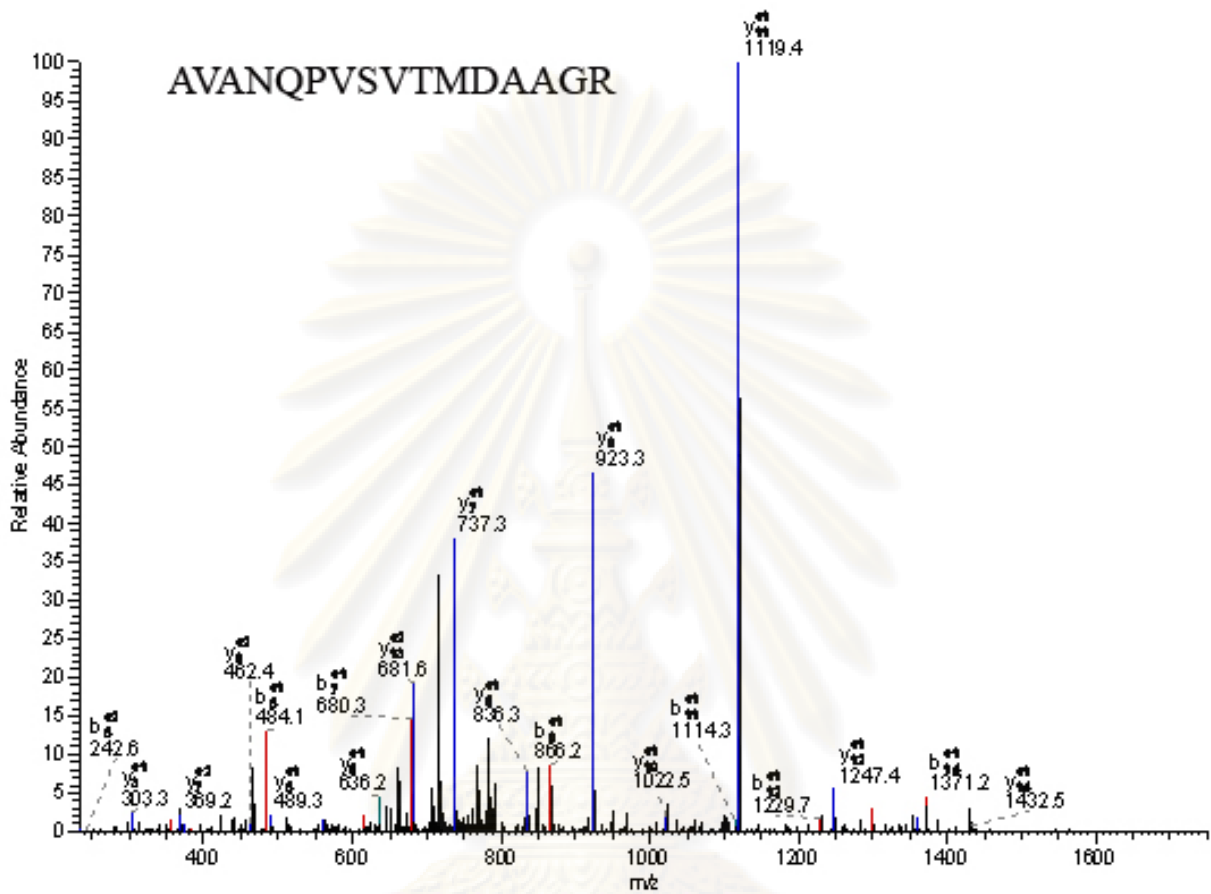
Figure 4.12 LC/MS/MS spectra of the tryptic digest of the F50 from the sequence AVANQPVSVTMDAAGR.
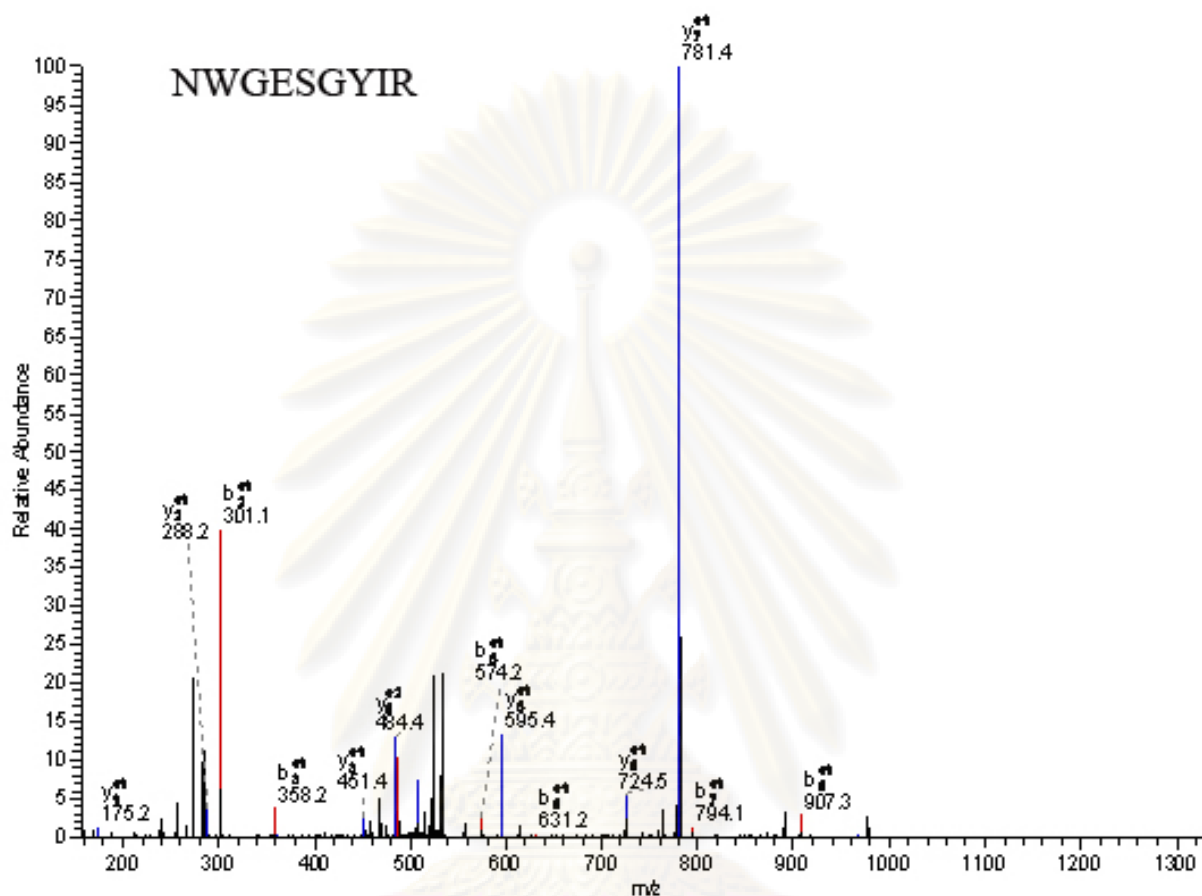
Figure 4.13 LC/MS/MS spectra of the tryptic digest of the F50 from the sequence NWGESGYIR.

4.11 3D-labeling of intact protein matched with obtained fragments

    The locations of fragments mentioned in 4.10 were defined on intact peptide sequence maximum matched, in this case, zingipain. Two zingipain sequences (Zingipain-I and Zingipain-II) were reported (Choi, K.H., and Laursen, R.A., 2000). Those two sequences were rendered in three dimension images and molecular arrangements were the same. However, this comparison was possibly for structure only; dealt with molecular charges or other parameters need other suitable methods. The sites of each fragment were showed in figure 4.14.

Figure 4.14 Three dimension structure of Zingipain illustrated the location of two main matched fragments inside (white wedge, the "AVANQPVSVTMDAAGR" sequence; red wedge, the "NWGESGYIR" fragment).

Figure 4.14 was one most possible model simulated from whole Zingipain-I sequence had been reported. It was indicated that both fragments were laid nearly on outside or surface of the molecules, one (pointed by white wedge) was possibly helix and the other (pointed by red wedge) was looped conformation. Indeed, the whole Zingipain-I sequence was also aligned to the other known peptide sequences and estimated for possible domain inside. Result shown that only the catalytic domain presented in the sequence was C1 hydrolase which located on from the 41st residue up to about 60th residue (but the sequence here was too short to be use for model creation). These two fragments (residues ranged from 126 to 137 and from 185 to 194) thus were not located in such catalytic side. However, with the work of Choi, K.H., and Laursen, R.A., (2000), the whole Zingipain-I and II sequence had been

already obtained and the catalytic domain was already estimated. It should be cover the 27th (cysteine) and 161st (histidine) residues which were cysteine-histidine essentially catalytic active residue character. Thus, our two fragments (residue from 126-137 and from 185-194) were regarded as part of catalytic site. One the other hand, it could be deduced form the figure 4.14 that the F50 protein might have similar structure, if so, many helical domain indicated the resistibility of molecule against temperature and other stresses. Analyzing data from RasWin software indicated seven helixes, 18 strands, and 25 turns (loops) presents. In addition, there were possibly 127 hydrogen bonds inside the molecule. The evident could be supported by our result shown that the F50 could resist upto $60^{o}C$ temperature (Figure 4.6) and up to pH 10 (Figure 4.8).

4.12 Protease activity determination

Further assay was set to confirm the protease activity according to information obtained from the tandem MS analysis. It was found that degradation levels of azocasein were matched with the eluted protein pattern (figure 4.15). The F50 fraction contained highest protease activity and the lesser was found in the F25 fraction. This result indicated the F50 fraction was actually contained protease activities in different levels. This is one evident confirming that the sequence identified information could also be used as guiding for screening direction. The finding of F50's protease activity made the former suspicions clear. It might explain how hemagglutination activity lost so quickly and might also play the role in α-glucosidase inhibitory activity which was still waiting for proving. It was also a bit of reason for answering why the F50 was the dominant protein and why single purification was enough.
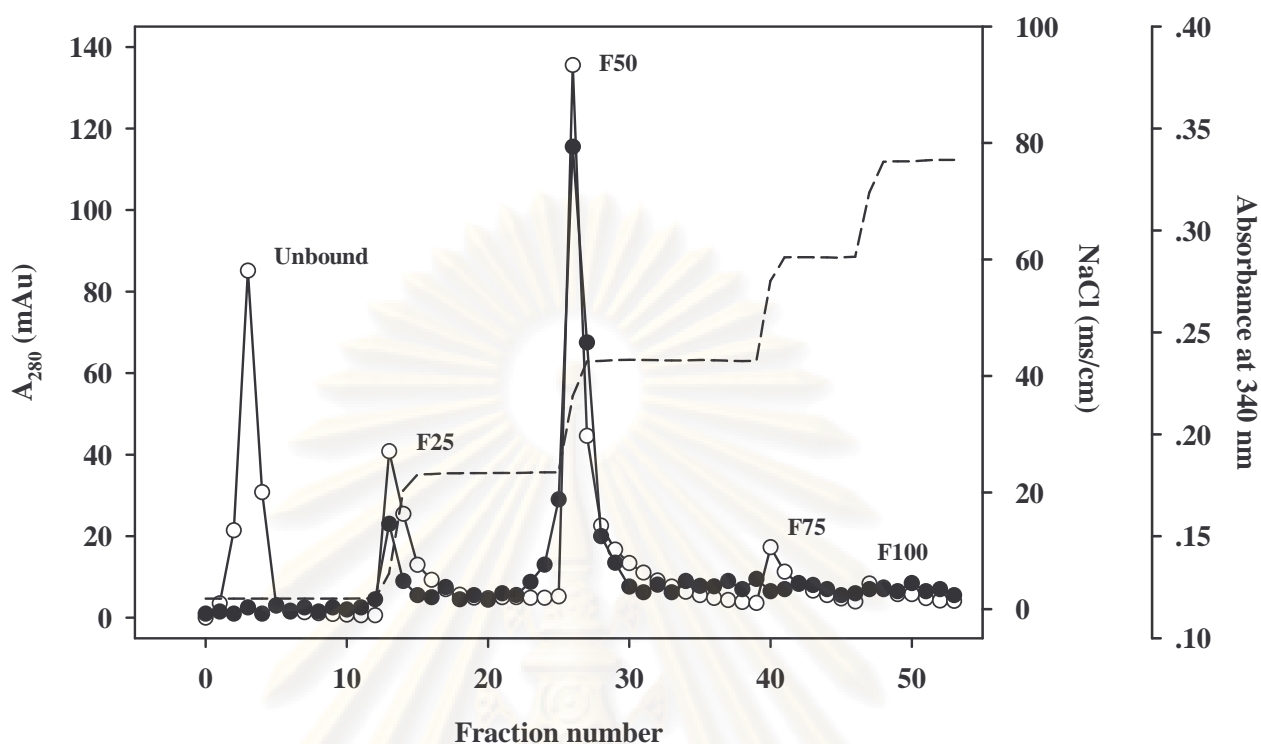
Figure 4.15 SP-sepharose chromatogram of the crude *Z. ottensii* rhizome protein extract (50 mg) with stepwise NaCl elution (0.00, 0.25, 0.50, 0.75 and 1.00 M). Fractions were assayed for (○) absorbance at 280 nm and (●) protease activity. Profile shown is representative of 3 independent trials.

4.12 Proximate analysis

Since the F50 contain α-glucosidase inhibitory activity, the *Z. ottensii* rhizome was expected to be promoted as new foodstuff's ingredients, spice, or supplement. Thus, the nutritional compositions of the rhizome which was currently unknown should be found out, in particular protein content, in order to achieve the information about proper recommended amount per meal or per day for the customer in applying the rhizome to healthcare purposes.

Table 4.4 Nutritional values of *Z. ottensii* obtained from proximate analysis

Criteria	Percent of dry weight
Total dry mass	100.00
Organic matter	88.92 ± 0.74
Fat	16.11± 0.06
Protein	6.31± 0.03
Mineral (Ash)	11.08± 0.12
Carbohydrate	66.50

Proximate analysis was carried out in fresh rhizome and the result was calculated in dry weight basis (table 4.4). The moisture of *Z. ottensii* rhizome were 83.67±0.16 which was lower than ginger (93.5-96.5%) (Anonymous, 1992). Although genetic different could be the major factor for this different as it caused variation in the rhizome structures, but it was also possibly depended on post-harvest time which allows the specimen loss their moisture. From the table 4.6, the protein content obtained from kjeldahl analysis method was 6.31±0.03% which was much higher than protein extracted from the rhizome in this study (1.31±0.20 g/kg rhizome or about 0.13%). This was not only originated from species differences but also from different analytical methods used.

In case of the Kjeldahl, all nitrogenous substances including nucleic acid, nitrate, alkaloid, etc can also increase the outcome nitrogen value toward protein amount after converted with the factor 6.25. Moreover, the Kjeldahl method was not detect soluble protein only, but it could also digest the insoluble protein out to the mixture solution which led to increase the nitrogen value out come. Thus, protein determined by the Kjeldahl method was usually higher than staining spectroscopy method. By comparison between *Z. ottensii* and ginger protein determined by the same Kjeldahl method, the protein content in *Z. ottensii* (about 6.3%) was lower than half of ginger protein (14.28% dry weight) (Anonymous, 1992). The origin of difference could be genetic, age, cultivated climates, etc. However, the proximate protein content could be used to estimate the protein ratio. In this case, from the IC_{50}

30.15 μg/ml (or 30.15 mg/l) of α-glucosidase inhibitory activity, it meant 30 mg of F50 could reduce half of glucose digestion from a liter of meal volume (contained about 70% carbohydrate by weight). Thus, (from ~6% protein) around 0.5 g of dry rhizome per meal should be able to reduce dietary glucose releasing. For various evidences previously mentioned, the *Z. ottensii* rhizome thus had much potential in blood-glucose caring meal replacing any other synthetic drugs, but the assumption was needed to be proven in future in term of *in situ* efficiency, health safety, and other necessary aspects that ensure people to use this herb in daily lives or as therapeutic purposes, for example anti-diabetic drug.
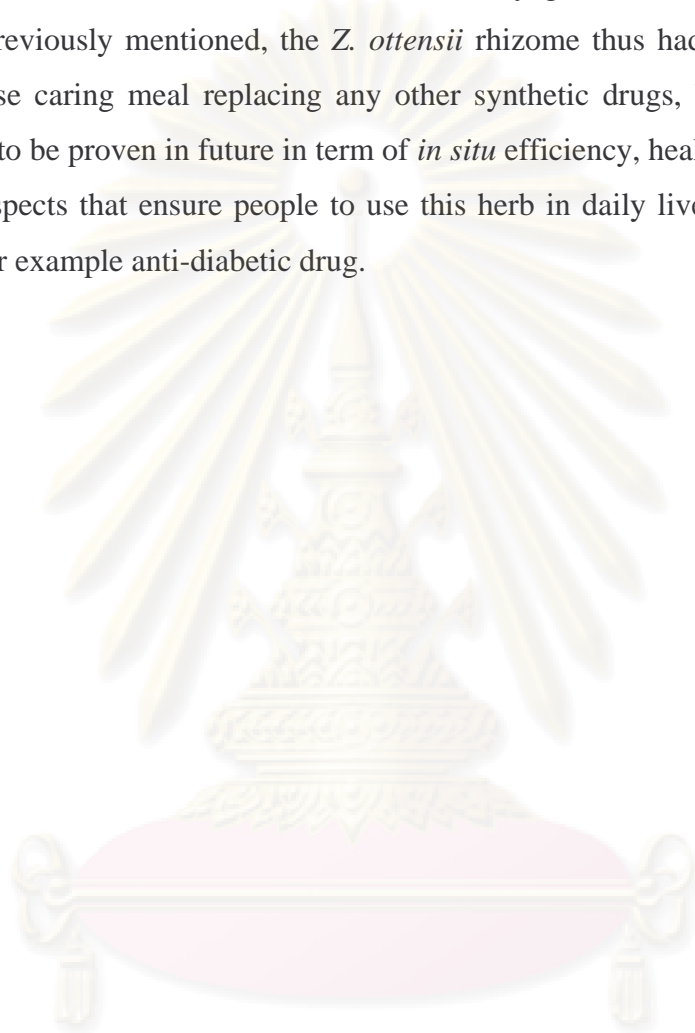
CHAPTER V

CONCLUSION

It could be concluded that a bioactive protein was successfully purified from the rhizomes of *Z. ottensii*, named F50, using a single-step moderate cation exchange chromatography. The F50 was found to be glycoprotein and to contain at least three components of 32.5, 15.2 and 13.8 kDa. The protein exhibited a strong α-glucosidase inhibition activity which the maximum % inhibition, IC_{50}, and Ki were 100%, 30.15 μg/ml, and 140 μmol, respectively. The activity was stable at the temperature up to $65^{\circ}C$ and pH ranges 2 to 10. Moreover, the F50 also contained mild to moderate antifungal (against *C. cassiicola* > *E. turicicum* > *F. oxysporum*) and anti-cancer (HEP-G2 and SW620) but no antibacterial activities (*in vitro*). This study can be a guideline for other researchers to deal with the protein in some other plant resources. Further research is required in conducting to characterize this protein in more details, such as its inhibitory mechanism, or molecular active site, interaction with other inhibitors, side effects, other targeted molecules and related therapeutic possibilities. As an alternative approach, the evaluation into the potential for using this protein as a food additive for healthcare purposes could be pursued.

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APPENDICES

APPENDIX A

MEDIA

The media were prepared by sterilization in the autoclave at 121 $^\circ$C for 15 minutes.

1. Potato dextrose agar (PDA)

Potato, peeled and diced	200	g
Glucose	20.0	g
Agar	15.0	g
Distilled water	1,000	ml

Boil 200 g of peels, dried potato for 1 hr in 1000 ml. of distilled water. Filter, and make up the filtrate to one liter. Add the glucose and agar and dissolve by streaming.

2. NA (Nutrient Agar)

Peptone	5.0	g
Beef extract	3.0	g
Agar	20.0	g
Distilled water	1,000	ml

3. NB (Nutrient Broth)

Peptone	5.0	g
Beef extract	3.0	g
Distilled water	1,000	ml

4. YMA (Yeast Malt Agar)

Yeast extract	3.0	g
Malt extract	3.0	g
Glucose	10.0	g
Peptone	5.0	g
Agar	20	g
Distilled water	1,000	ml

5. YMB (Yeast Malt Broth)

Yeast extract	3.0	g
Malt extract	3.0	g

Glucose	10.0	g
Peptone	5.0	g
Distilled water	1,000	ml

APPENDIX 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

APPENDIX C

Calibration curve for protein determination by Bradford method

$Y = 0.0155X$

$R^2 = 0.9881$

APPENDIX D

Calibration curve for carbohydrate content by Dubois method

$Y = 0.003X$
$R^2 = 0.9916$
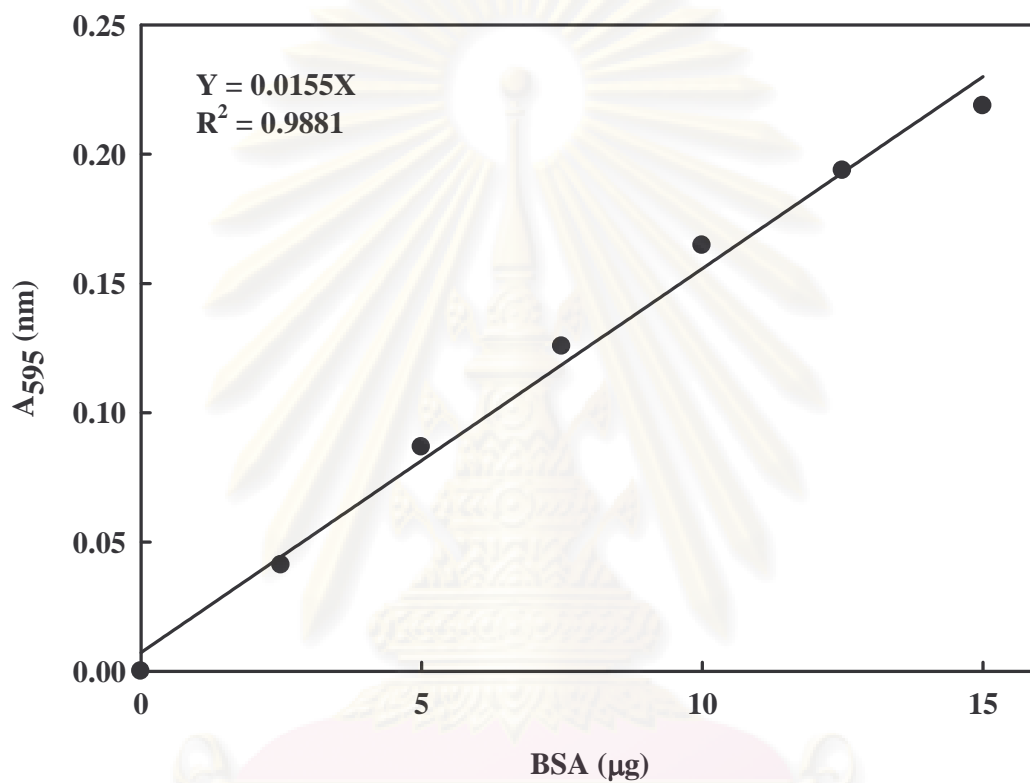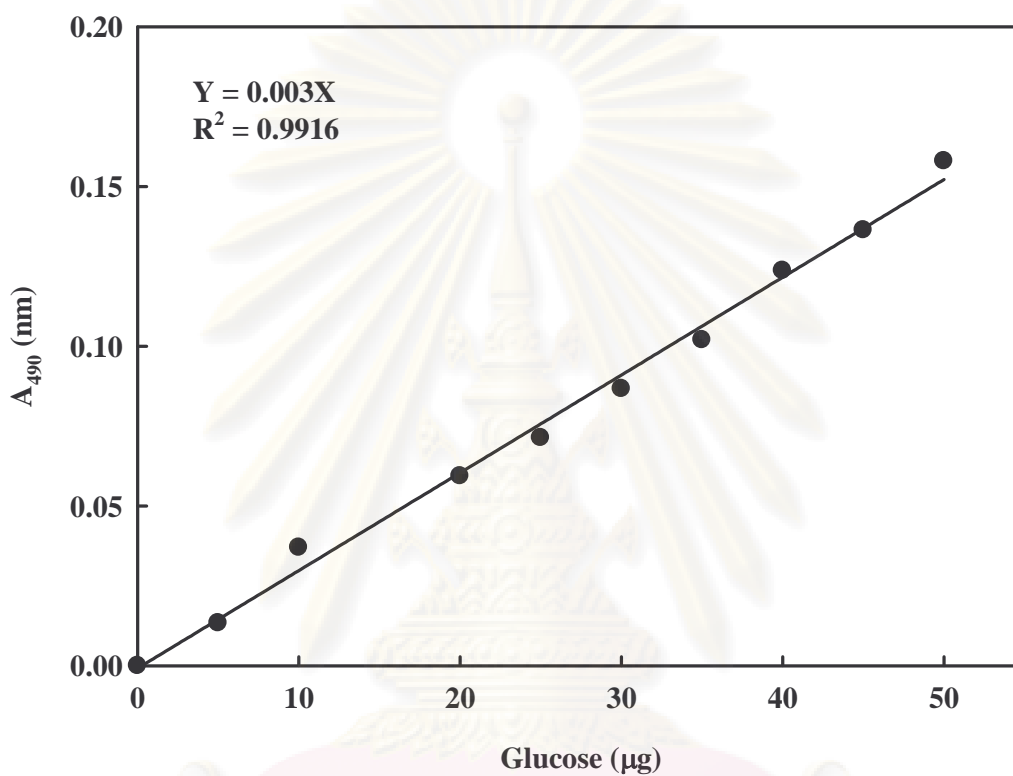
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จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX 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

APPENDIX F

Amino acid sequence of Zingipain-I

```
  1 dvlpdsidwr ekgavvpvkn qggcgscwaf daiaavegin qivtgdlisl seqqlvdcst
 61 rnhgceggwp yrafqyiinn gginseehyp ytgtngtcdt kenahvvsid syrnvpsnde
121 kslqkavanq pvsvtmdaag rdfqlyrngi ftgscnisan hyrtvggret endkdywtvk
181 nswgknwges gyirvernia essgkcgiai spsypikexx x
```

Amino acid sequence of Zingipain-II

```
  1 ddlpdsidwr engavvpvkn qggcgscwaf stvaavegin qivtgdlisl seqqlvdctt
 61 anhgcrggwm npafqfivnn gginseetyp yrgqdgicns tvnapvvsid syenvpshne
121 qslqkavanq pvsvtmdaag rdfqlyrsgi ftgscnisan haltvvgygt endkdfwivk
181 nswgknwges gyiraernie npdgkcgitr fasypvkkgt n
```

BIOGRAPHY

Mr. Nathachai Tiengburanatam was born on 11 December 1967 in Bangkok. He was graduated in 1989 with B.Sc. degree from Chiangmai University and further got Master in Biochemistry at the same academy in 1998. After graduated, he got the first job as senior technician of Thai acrylic fiber Co.,Ltd., Saraburi for 8 months. Next, he changed to be sale representative of various science and medical instruments and supplies during 1989 to 1992 such as Olic (Thailand) Co.,Ltd, Northern chemical and glassware Inc., and Kosin medical supply Co., Ltd. He started the family's business as administrative partner during 1992 to 1993 at Boonure Supermaket, Lampoon. Next, he changed to be research assistance of "Aflatoxin test kit development project" during 1994-1995 at department of biochemistry, faculty of medicine, Chiangmai University and admitted as student over there. After finished M.Sc. degree in 1998, he became an assistance of "Dengue vaccine development project" during 1998-1999 at department of microbiology, faculty of medicine, Chiangmai University. Next, he changed to be a researcher of Biogas advisory unit of the university for about 2 years. Finally, he became an instructor of Rajamangala University of technology from 2001 up to now. The organization named Lampang Agricultural Research and Training Center (LARTC) located at 202 M11 Lampang-Ngav Rd, Pichi subdistrict, Muang district, Lampang, Thailand. He was supported by the original affiliation to further study at Chulalongkorn university for Ph.D. from 2006 to 2009.

Academic presentation;

1) Tiengburanatam, N., Sangvanich, P., and Karnchanatat, A. A Novel α-Glucosidase Inhibitor Protein from the Rhizomes of *Zingiber ottensii* Valeton, The 3rd Technology and Innovation for Sustainable Development Conference (TISD2010), 4-6 March 2010, Nong Khai, Thailand.

2) Tiengburanatam, N., Sangvanich, P., Boonmee, A and Karnchanatat, A. A Novel α-Glucosidase Inhibitor Protein from the Rhizomes of *Zingiber ottensii* Valeton. (2010). *Applied Biochemistry and Biotechnology*, DOI 10.1007/s12010-010-8971-7.