โปรตีนที่มีฤทธิ์ยับยั้งอะเซทิลโคลีนเอสเทอเรสจากเหง้าของพืชวงศ์ขิง

นางสาวปอลิน รุ่งแสง

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ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF PROTEINS FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS

Miss Porlin Rungsaeng

A Thesis Submitted in Partial Fulfillment of the Requirements

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ปอลิน รุ่งแสง: โปรตีนที่มีฤทธิ์ยับยั้งอะเซทิลโคลีนเอสเทอเรสจากเหง้าของพืชวงศ์ขิง (ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF PROTEINS FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. อภิชาติ กาญจนทัต, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.พลกฤษณ์ แสงวณิช , 64 หน้า.

สารที่มีฤทธิ์ยับยั้งอะเซทิลโคลีนเอสเทอเรสมักถูกใช้เป็นยาที่ใช้ในการรักษาอาการของผู้ป่วยโรคอัลไซเมอร์ งานวิจัยนี้มีความสนใจนำเหง้าของพืชวงศ์ขิง 15 ชนิดมาทำการศึกษาหาโปรตีนที่มีกิจกรรมการยับยั้งอะเซทิลโคลีนเอสเทอเรส ส่วนสกัดหยาบและส่วนโปรตีนหยาบที่ทำการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตพบว่าขิงมีกิจกรรมการยับยั้งการทำงานของอะเซทิลโคลีน-เอสเทอเรสดีที่สุด ซึ่งจากการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตอิ่มตัวที่ 80 เปอร์เซ็นต์ และทำให้บริสุทธิ์โดยโครมาโทกราฟีแบบแลกเปลี่ยนประจุที่มี ดีอีเออี เซลลูโลสเป็นตัวค้ำจุน พบว่าส่วนของโปรตีนที่ไม่ติดกับคอลัมน์ มีกิจกรรมการยับยั้งการทำงานของอะเซทิลโคลีนเอสเทอเรสที่ดี เมื่อใช้เทคนิคพอลิอะคริลาไมด์เจลอิเล็กโตรฟอเรซิสแบบไม่เสียสภาพและเสียสภาพ พบว่าโปรตีนที่ไม่ติดกับคอลัมน์ มีโปรตีนบริสุทธิ์อย่างน้อย 1 ชนิดที่มีขนาด 33.5 กิโลดาลตัน เมื่อวิเคราะห์ลำดับกรดอะมิโนภายในโมเลกุลโดยการตัดด้วยทริปซิน แล้ววิเคราะห์ชิ้นส่วนที่ถูกย่อยด้วยเครื่อง LC-MS/MS พบเพปไทด์ 2 สายที่มี 16 กรดอะมิโนที่มีความคล้าย 100 เปอร์เซ็นต์กับซินจิเพน หนึ่ง (ซิเต-อีน โปรติเอสจากขิง) จากการทำไซโมกราฟฟี พบว่าแถบที่มีกิจกรรมการยับยั้ง อะเซทิลโคลีนเอส-เทอเรสนั้นมีกิจกรรมของโปรติเอสด้วย สารยับยั้งอะเซทิลโคลีนเอสเทอเรสมีเสถียรภาพที่อุณหภูมิ -20 จนถึง 60 องศาเซลเซียส เป็นเวลานาน 120 นาทีและที่ค่าความเป็นกรด-ด่างเท่ากับ 2 จนถึง 12 นอกจากนั้นสารยับยั้งอะเซทิลโคลีนเอสเทอเรสยังสามารถถูกกระตุ้นได้ด้วย Mn^{2+} และ Cu^{2+} ที่ 1 - 10 มิลลิโมลาร์ ถูกทำให้มีกิจกรรมการยับยั้งอะเซทิลโคลีนเอสเทอเรสลดลงในระดับกลางได้ด้วย Ca^{2+}, Fe^{2+}, Mg^{2+} และ Zn^{2+} ที่ 1-5 มิลลิโมลาร์ และถูกยับยั้งกิจกรรมการยับยั้งอะเซทิลโคลีนเอสเทอเรสที่ 10 มิลลิโมลาร์ นอกจากนั้น Hg^{2+} และ EDTA จะยับยั้งกิจกรรมการยับยั้งกิจกรรมอะเซทิลโคลีนเอส-เทอเรส สำหรับการวิเคราะห์จลนพลศาสตร์ของกิจกรรมยับยั้งอะเซทิลโคลีนเอสเทอเรส พบว่ามีการยับยั้งแบบไม่แข่งขันชนิด Non-competivive ที่มีค่า Ki เท่ากับ 9.31 มิลลิกรัมต่อมิลลิลิตร Vmax เท่ากับ 3.41 มิลลิโมลาร์ต่อนาที และ Km เท่ากับ 130.6 มิลลิโมลาร์

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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PORLIN RUNGSAENG: ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF PROTEINS FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS. ADVISOR: APHICHART KARNCHANATAT, Ph.D., CO-ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., 64 pp.

Acetylcholinesterase inhibitors (AChEIs) have been used as drugs for the symptomatic treatment of Alzheimer"s disease (AD). In order to search for new AChEIs, 15 Zingiberaceae plants were tested for AChEI activity in rhizome extracts. The crude homogenate and ammonium sulphate cut fraction of *Zingiber officinale* contained a significant AChEI activity. Eighty % saturation $(NH_4)_2SO_4$ precipitation and DEAE-cellulose ion-exchange chromatography (unbound fraction) enriched the protein to a single band on non-denaturing and reducing SDS-PAGE (ca 33.5 kDa), but in-gel-tryptic digestion with LC-MS/MS resolution revealed two heterogenous peptides, a 16 amino acid fragment with 100% similarity to Zingipain-1, a cysteine protease from *Zingiber officinale*, suggesting the preparation was heterogeneous. Gelatin-degrading zymography showed that the AChEI containing band also contained protease activity. The AChEI activity was largely stable between -20 – 60 °C (at least over 120 mins), and over a broad pH range (2 - 12). The AChEI activity was stimulated strongly by Mn^{2+} and Cu^{2+} at 1 - 10 mM, and weakly by Ca^{2+}, Fe^{2+}, Mg^{2+} and Zn^{2+} at 1 – 5 mM but inhibited at 10 mM. In contrast, Hg^{2+} and EDTA were very and moderately strongly inhibitory. AChEI exhibited non-competitive inhibition of AChE for the hydrolysis of acetylthiocholine iodide with a K_i value of 9.31 mg/ml, a V_{max} of 3.41 mM/min and a K_m of 130.6 mM.

Field of Study : Biotechnology Student"s Signature .....
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LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
μg	microgram
μL	microlitre
A	absorbance
Ach	acetylcholine
AChE	acetylcholinesterase
AChEIs	acetylcholinesterase inhibitors
AD	Alzheimer‟s disease
ATCI	acetylthiocholine iodide
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CNS	central nervous system
cm	centimeter
Da	dalton
DTNB	5,5‟-dithiobis (2-nitrobenzoic acid)
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
K_m	Michaelis-Menten constant
L	litre
LC/MS/MS	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
M	molar
mA	milliampere
mg	milligram
min	minute

mL	millilitre
mM	millimolar
MW	molecular weight
N	normal
nm	nanometer
NaCl	sodium chloride
PAGE	polyacrylamide gel electrophoresis
rpm	revolution per minute
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TEMED	*N,N,N',N'*-tetramethyl ethylenediamine
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
U	Unit activity
V	volt
V_{max}	maximum velocity
V/V	volume by volume
W/V	weight by volume

CHAPTER 1

INTRODUCTION

Aging is a universal natural biological process that takes place in all organisms, leading to progressive and deleterious changes in the body. It is now widely accepted that aging is a multifarious event resulting from the collective effects of genetic variation, environmental risk factors, nutritional factors and life style (Harman, 1998). With the inferences of these factors, the bodies of multicellular organisms, including humans, undergo progressive deterioration of physical functions, loss of homeostasis and an increased susceptibility to diseases with the aging process. Aging has a great impact on the brain functions, with a tendency in old age to have decreased memory, including recognition memory functions (James *et al*., 2008), short term recall (Gazzaley *et al*., 2007; Gilchrist *et al*., 2008;), and long term memories as well as the speed of processing (Park *et al*., 2002). Although aging is always associated with a decline in physiological functions, it is not necessarily associated with diseases (Anton *et al*., 2005). The aging process can be divided into two broad types, normal and pathological. In this context, normal aging is the result of the natural maturational process with gradual changes in cognitive functions. Such cognitive changes may be inevitable, with humans eventually experiencing deterioration in their memory even if they are not diagnosed with dementia (Christensen, 2001). On the other hand, pathological aging is always associated with non-normal factors, such as diseases and brain trauma (Reese *et al*., 2000). It is believed that the causes of aging-associated brain diseases are linked with several factors, such as lifestyle, cardiovascular diseases and genetic variation.

Neurodegenerative disease is generic term applied to a variety of conditions arising from the chronic breakdown and deterioration of neurons, particularly those of the central nervous system (CNS). In addition, these neurons many accumulate aggregated proteins which cause dysfunction. Alzheimer‟s disease (AD) is an aging-associated progressive neurodegenerative disease and is the major cause of dementia. It leads to cognitive impairment and behavioral changes (Nussbaum and Ellis, 2003). Owing to the global aging problem in the human population, there is no doubt that AD will become a major health concern. The clinical symptoms of AD, such as

memory impairment and language difficulties, usually become noticeable many years after the onset of the underlying pathological changes. Thus the effectiveness of current treatment is limited, partly because it is initiated when clinical symptoms become significant, which is typically after severe neuronal damage has already taken place. There is an urgent need to develop potent neuroprotective agents for the prevention as well as the treatment of AD. Indeed, the treatment of AD is an increasing and significant proportion of the medical aid costs in many countries (Evans, 1990). The certain cause of AD is still unknown, but the most plausible etiology of AD is the dysfunction and loss of cholinergic neurons in the brain, particularly in the region involved in learning and memory, as described in the cholinergic deficit hypothesis (Perry, 1986). This hypothesis claims that the cause of AD is from decreasing levels of the cholinergic neurotransmitter, acetylcholine (ACh). Therefore, the best-developed approach used to treatment AD, at the present, is the use of acetylcholinesterase inhibitors (AChEIs) to elevate the level of ACh and enhance the function of remaining ACh receptors in the brain.

AChEIs increase the availability of the neurotransmitter ACh by inhibiting its degradation enzyme, AChE. Clinical studies have shown that these AChEIs can provide modest improvement in the cognitive and global measures of relevance to dementia (Almkvist *et al*., 2004; Persson *et al*., 2009; Rosler *et al*., 1999; Winblad *et al*., 2001). Four commercial AChEIs approved by the US Food and Drug Administration (US FDA) and presently used in AD therapy are tacrine (Arrieta, 1998; Qizibash *et al*., 1998), donepezil (Jones, 2003), rivastigmine (Polinsky, 1998) and galanthamine (Jones, 2003). Although all commercial AChEIs are effective in AD treatment, they have undesirable side effects and are very expensive. Therefore, the search for new AChEIs is still ongoing. Many pharmaceutical industries have invested effort and capital to evaluate natural potential sources of drugs for the prevention and treatment of AD. In traditional folklore medicine, many plants have been used as a nerve tonic and to improve cognitive function. Therefore, such folklore based medicinal plants that are reputed as a nerve tonic and used for memory improvement are very attractive candidates as potential sources of new drugs against and to prevent AD.

The Zingiberaceae is a well-known plant family in Southeast Asia and many of its species are used in traditional folklore medicine for the effective treatment of several diseases. They are perennial herbs that are widely cultivated in Thailand and

the tropical regions of Asia, and have been commonly used as medicinal plants and spices in Thailand. The rhizomes of these plants possess diverse biological activities, including anti-microbial (Yamada *et al*., 1992; Hiserodt *et al*., 1998), anti-ulcer (Al-Yahya *et al*., 1990; Matsuda *et al*., 2003), anti-inflammatory (Araujo and Leon, 2001), anti-oxidant (Selvam *et al*., 1995), cytotoxic and anti-tumor (Itokawa *et al*., 1987; Murakami *et al*., 1995, 2000; Pal *et al*., 2001), vasorelaxant (Othman *et al*., 2002), anti-spasmodic (Ammon and Wahl, 1991), anti-hepatotoxic (Hikino *et al*., 1985) and anti-depressant activities (Noro *et al*., 1983; Yu *et al*., 2002). Although there have been many reports concerning the active chemical constituents of these plants including the rhizomes, and some biological activities of these species, these have largely been reported on the non-proteinaceous compounds. Indeed, only a few reports have focused on the bioactive peptides or proteins from these plants.

Proteases play an important role in regulating the biological processes in plants, such as stress responses, recognition of pathogens, induction of effective defense responses, mobilization of storage proteins during germination and the initiation of cell death or senescence (Schaller, 2004). Plant proteases also exhibit broad substrate specificity and are active over a wide pH and temperature range, and in the presence of organic compounds as well as other additives. Therefore, plant proteases may turn out to be an efficient choice in the pharmaceutical, medicinal, food and biotechnological industries (Kaneda and Tominaga, 1975).

The objective of this study was to investigate *in vitro* AChEI activities of proteins from Zingiberaceae rhizomes. Here, using azocasein digestion as the bioactivity readout for protease activity to guide purification, we report on the enrichment of a protease from Zingiberaceae rhizomes, and its likely identity, as deduced by tryptic peptide sequencing and homology searches.

CHAPTER ll

LITERATURE REVIEWS

2.1 Alzheimer's disease

2.1.1 History and definition

The clinical entity known as Alzheimer‟s disease (AD) clearly existed long before 1907, when German physician Alois Alzheimer described the clinical course and changes in the brain of a 55-year-old woman dying after a 4-year history of progressive dementia. In his report of Auguste, he demonstrated neurofibrillary tangles (NFTs) using the newly developed Bielschowsky silver impregnation method and observed cortical "miliary foci" or senile plaques (SPs) (Alzheimer, 1907), described 15 years later by Blocq and Marinesco. Sections of the brain of Auguste have been recovered in Munich, and micrographs from these historic slides confirmed the presence of typical histopathological changes of AD in the neocortex. In the second case, Johann F., described by Alzheimer in 1911 (Alzheimer, 1911), only plaques and no tangles were found, corresponding to the "plaque only" type of AD, and DNA extracted from this material revealed an ApoE 3/3 genotype (Bick, 1999).

AD is a chronic neurological disorder characterized by memory impairment, cognitive dysfunction, behavioral disturbances, and deficits in activities of daily living. (Adams *et al*., 1984; Aisen and Davis, 1997 and Jann, 1998). AD is originated from abnormalities of nervous system which cause dementia symptoms to the patient. Hallmark abnormalities are deposits of the protein fragment beta-amyloid (plaques) and twisted strands of the protein tau (tangles). In AD, as in other types of dementia, increasing numbers of nerve cells deteriorate and die. A healthy adult brain has 100 billion nerve cells, or neurons, with long branching extensions connected at 100 trillion points. At these connections, called synapses, information flows in tiny chemical pulses released by one neuron and taken up by the receiving cell. Different strengths and patterns of signals move constantly through the brain‟s circuits, creating the cellular basis of memories, thoughts and skills. In AD, information transfer at the synapses begins to fail, the number of synapses declines and eventually cells die. Brains with advanced Alzheimer‟s show dramatic shrinkage from cell loss and widespread debris from dead and dying neurons.

2.1.2 A cause of Alzheimer's disease

Although the causes of AD are not yet known, most experts agree that Alzheimer‟s, like other common chronic conditions, probably develops as a result of multiple factors rather than a single cause

2.1.2.1 Age factor

The greatest risk factor for AD is advancing age, but Alzheimer‟s is not a normal part of aging. Most Americans with AD are aged 65 or older, although individuals younger than age 65 can also develop the disease. When Alzheimer‟s or another dementia is recognized in a person under age 65, these conditions are referred to as "younger-onset" or "early-onset" Alzheimer‟s or "younger-onset" or "early-onset" dementia. (Francis, *et al*., 1999 and Terry, and Buccafuscos, 2003)

2.1.2.2 Genetic factor

A small percentage of AD cases, probably less than 1 percent, are caused by rare genetic variations found in a small number of families worldwide. These variations involve chromosome 21 on the gene for the amyloid precursor protein, chromosome 14 on the gene for the presenilin 1 protein and chromosome 1 on the gene for presenilin 2. In these inherited forms of Alzheimer‟s, the disease tends to develop before age 65, sometimes in individuals as young as 30. A genetic factor in late-onset AD (AD developing at age 65 or older) is apolipoprotein E-e4 (ApoE-e4). ApoE-e4 is one of three common forms of the ApoE gene, which provides the blueprint for a protein that carries cholesterol in the bloodstream. Everyone inherits one form of the ApoE gene from each of his or her parents. Those who inherit one ApoE-e4 gene have increased risk of developing AD. Those who inherit two ApoE-e4 genes have an even higher risk. However, inheriting one or two copies of the gene does not guarantee that the individual will develop Alzheimer‟s.

2.1.2.3 Acetylcholine factors:

The oldest hypothesis is the "cholinergic hypothesis". (Francis, *et al*., 1999) It states that Alzheimer's begins as a deficiency in the production of acetylcholine. Acetylcholine is a neurotransmitter inhibited by acetylcholinesterase (AChE), considered to play a role in the pathology of AD (Hebert *et al*., 1995). AChE are present in the brain and are detected among neurofibrillary tangles and neuritic plaques (Beard *et al*., 1995). Despite the unknown etiology of AD, elevation of acetylcholine amount through AChE enzyme inhibition has been accepted as the most

effective treatment strategy against AD (Arnold and Kumar, 1993). Therefore, AChE inhibitors have become the remarkable alternatives in treatment of AD.

2.1.2.4 Other factors

Other factors, then, are involved with nerve destruction in many of these patients. There's no lifestyle factor that's been conclusively shown to reduce your risk of Alzheimer's disease. However, some evidence suggests that the same factors that put you at risk of heart disease may also increase the chance that you'll develop Alzheimer's. Examples include: Smoking, High blood pressure, High cholesterol, Poorly controlled diabetes. These risk factors are also linked to vascular dementia, a type of cognitive decline caused by damaged blood vessels in the brain. Many people with cognitive decline have brain changes characteristic of both Alzheimer‟s disease and vascular dementia. Some researchers think that each condition helps fuel the damage caused by the other. (Kivipelto, *et al*., 2005; Pendlebury, and Rothwell, 2009; Raji, *et al*., 2009; Solomon, *et al*., 2009; Tsivgoulis, *et al*., 2009; Whitmer, *et al*., 2008 and Wu, *et al*., 2008)

2.2 Acetylcholinesterase (AChE) Inhibitory

2.2.1 AChE and Important of AChE Inhibitory

The acetylcholinesterase enzyme (AChE) is an ubiquitous class of serine hydrolases that hydrolyze choline esters with various efficiency. In vertebrates, two forms of acetylcholinesterase enzyme (ChE) encoded by two distinct genes occur, AChE and butyrylcholinesterase (BuChE) (Massoulie *et al*., 1993). The main function of AChE is the rapid hydrolysis of neurotransmitter acetylcholine (ACh) at cholinergic synapses (Taylor and Radic, 1994). AChE is present in nervous tissue, muscles (Brimijoin, 1983), plasma and blood cells (Heller and Hanahan, 1972 and Szelenyi *et al*., 1982) and displays a complex molecular polymorphism of quaternary structure.

The acetylcholinesterase enzyme (AChE) inhibitors are the standard of therapy for treatment of patients with Alzheimer disease (AD) and are the only class of drugs approved by the Food and Drug Administration (FDA) for treatment of this condition. Recent attempts to treat AD have been focused on enhancing cholinergic function, using either cholinergic receptor agonists (Cutler and Sramek, 1995) or, most commonly, using AChE inhibitors (Giacobini, 1996). AChE Inhibitory in AD therapy should boost endogenous levels of AChE in the brain and thereby enhance cholinergic neurotransmission. Several AchE inhibitory (Doody, 1999; Enz *et al*.,

1993; Sugimoto *et al*., 2000 and Polinsky, 1998;) of diverse chemical structure and mechanism of action are used for treatment of AD. These compounds are structurally diverse, possess unique patterns of specificities for various forms of ChE. They use distinct mechanisms of enzyme inhibition, present unique adverse event profiles, and offer relatively similar mean gains in cognitive abilities to patients with AD in controlled clinical trials. AChE inhibitors are the most effective approach to treat the cognitive symptoms of AD and other possible therapeutic applications in the treatment of Parkinson‟s disease, senile dementia, and myasthenia gravis, among others. (Anonymous, 2000; Brenner, 2000 and Rahman and Choudhary, 2001). Treatments with AchE Inhibitory have generally been beneficial in meliorating a global cognitive dysfunction and, more specifically, are most effective in improving attention (Parnetti *et al*., 1997).

AChE inhibitors such as tacrine, donepezil and rivastigmine are commonly used synthetic drugs for the treatment of Alzheimer‟s disease; however, these drugs are limited in use due to their adverse side-effects and are effective only against the mild type of AD (Schneider, 2001). Hence, recent efforts have focused on plant phytochemicals as natural sources of effective acetylcholinesterase inhibitors with little or no side effects which could be use as dietary intervention in the management of this disease (Conforti *et al*., 2007 and Orhan *et al*., 2004;).

2.2.2 The Mechanisms

The systematic biochemical investigation of the brains of patients with Alzheimer‟s disease began in the late 1960s and early 1970s. The hope was that a clearly defined neurochemical abnormality would be identified, providing the basis for the development of rational therapeutic interventions analogous to levodopa treatment of Parkinson‟s disease. Support for this perspective came in the mid-1970s with reports of substantial neocortical deficits in the enzyme responsible for the synthesis of acetylcholine (ACh), choline acetyltransferase (ChAT). (Bowen, *et al*., 1976; Davies, and Maloney, 1976; and Perry, *et al*., 1977) Subsequent discoveries of reduced choline uptake (Rylett *et al*., 1983), ACh release (Nilsson *et al*., 1986) and loss of cholinergic perikarya from the nucleus basalis of Meynert (Whitehouse *et al*., 1982) confirmed a substantial pre-synaptic cholinergic deficit. These studies, together with the emerging role of ACh in learning and memory (Drachman and Leavitt, 1974), led to the “cholinergic hypothesis of Alzheimers disease” (Figure 2.1 and 2.2). The action in Alzheimer's disease 1) reduced cortical cholinergic innervation; 2)

reduced corticocortical glutamatergic neurotransmission due to neuron or synapse loss; 3) reduced coupling of muscarinic M1 receptors (mAChR) to second messenger system?; 4) shift of tau to the hyperphosphoryalted state precursor of neurofibrillary tangles; 5) reduced secretion of soluble amyloid precursor protein (sAPP); 6) increased production of β-amyloid protein; 7) decreased glutamate (Glu) production. (It is hypothesised that these changes give rise to the clinical symptoms of Alzheimer‟s disease and contribute to the spread of pathology.) (Francis *et al*., 1993; Nitsch *et al*., 1996; Palmer and Gershon, 1990)

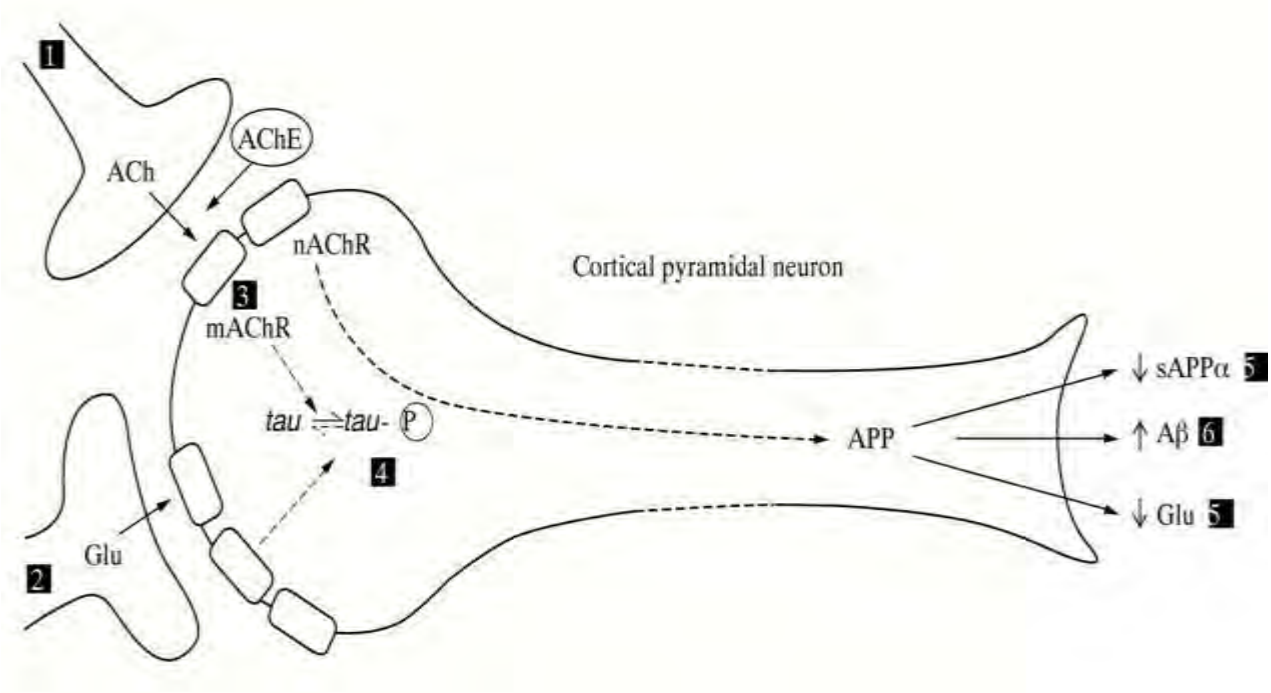

Figure 2.1. A proposed neurochemical changes in Alzheimer‟s disease

AChE inhibitors increase cholinergic activity by inhibiting the intrasynaptic activity of acetylcholinesterase, the enzyme responsible for the elimination of acetylcholine in the synapse. By inhibiting this enzyme, the intrasynaptic residence of available acetylcholine is increased and the likelihood of postsynaptic stimulation is enhanced. The action of AChE inhibitors 1) AChE inhibitors reduce the breakdown of endogenously released ACh, resulting in greater activation of postsynaptic ACh receptors; hypothesised consequences; 2) reduced phosphorylation of tau; 3) secretion of amyloid precursor protein (sAPP) returned towards normal; 4) reduced ß-amyloid

production; 5) glutamatergic neurotransmission returns towards normal, possibly due to activation of muscarinic and nicotinic receptors.

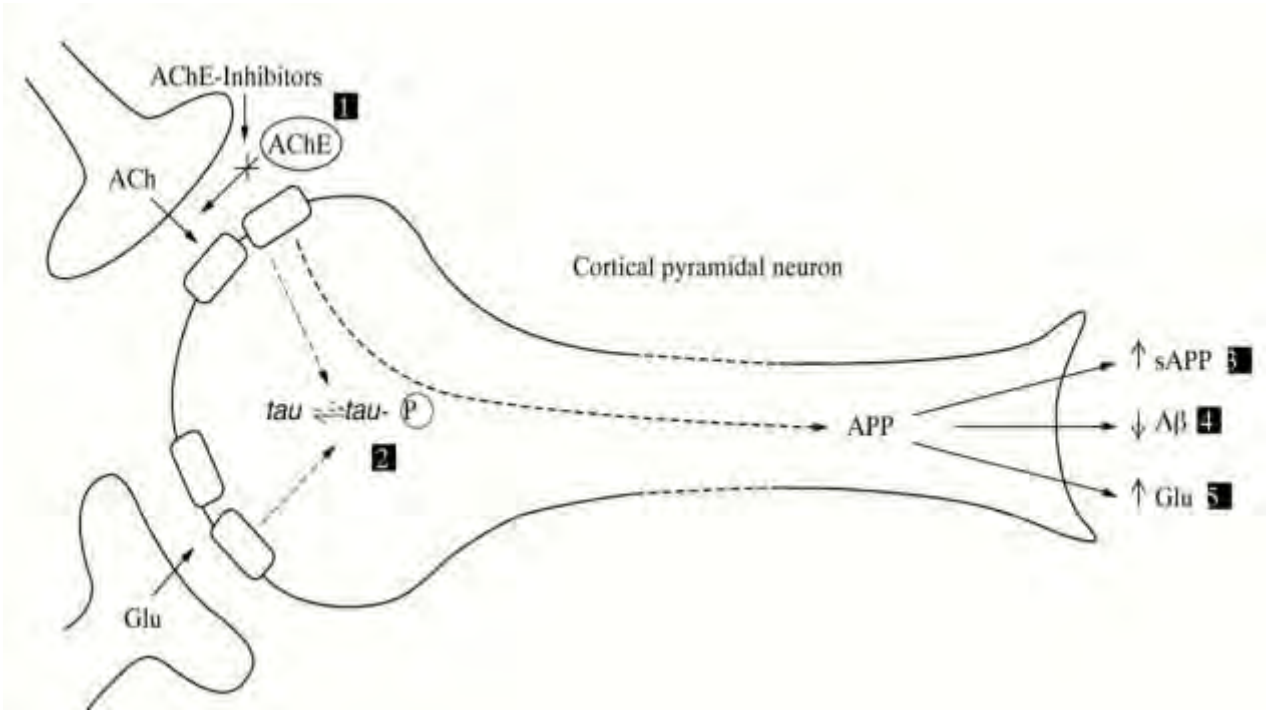

Figure 2.2 Rectification of neurotransmission with cholinesterase inhibitors

2.2.3 Distribution of AChE Inhibitors

2.2.3.1 AChE Inhibitor from marine sponge

3-Alkylpyridinium polymer type compounds (Figure. 2.3) isolated from the water extract of the marine sponge *Reniera sarai* collected from the North Adriatic Sea had a potent acetylcholinesterase inhibitory activity. These compounds inhibited the acetylcholinesterase enzyme of recombinant insect, electric eel and human erythrocyte origins and butyrylcholinesterase of horse sera origin at the IC_{50} values of 0.06 μM, 0.08 μM, 0.57 μM and 0.14 μM, respectively. (Bunc *et al*., 2002 ; Malovrh *et al*., 1999 ; Sepcic *et al*., 1997 and Sepcic *et al*., 1999)

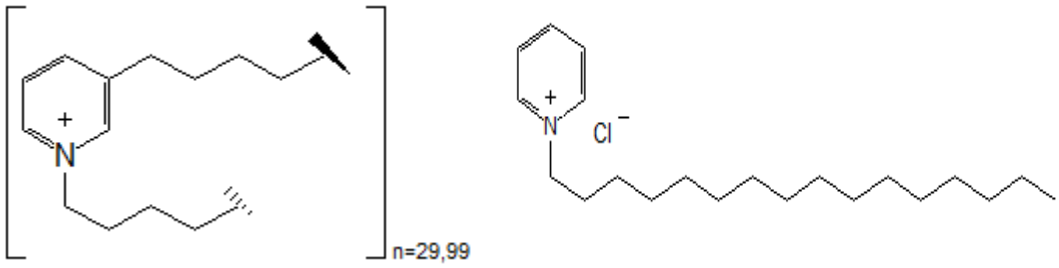

Figure. 2.3. Structures of some acetylcholinesterase inhibitors obtained from *Reniera sarai*

2.2.3.2 AChE Inhibitor from microorganism

Vizoltricine (Figure 2.4) isolated from the microorganism *Fusarium tricinctum*, is a potent acetylcholinesterase inhibitor (IC_{50}= 4.0 x 10^{-4} mM). In addition, its N-methyl derivative was found to have four times greater inhibition than vizoltricine itself (IC_{50}= 7.0 x 10^{-5} mM) (Solfrizzo M., and Visconti A., 1994; Visconti A., and Solfrizzo M., 1994)

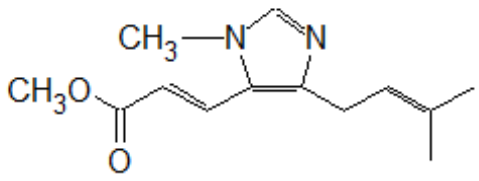

Figure 2.4. Structures of some acetylcholinesterase inhibitors obtained from *Fusarium tricinctum*

2.2.3.3 AChE Inhibitors from plant

A variety of plants has been reported to show AChE inhibitory activity and so may be relevant to the treatment of neurodegenerative disorders such as AD. *Bacopa monniera* and *Ginkgo biloba* are well-known cognitive enhancers in Indian and Chinese traditional medicine systems. Standardized extracts of *Bacopa monniera* and *G. biloba* both showed a dose-dependent inhibitory effect on AChE activity (Das *et al*., 2002). Eighty percent methanolic extract of *Myricaria elegans* Royle was found to have significant AChE inhibitory activity (Ahmad *et al*., 2003).

Methanolic extracts of seven herbs *Acorus calamus*, *Acorus gramineus*, *Bupleurm facaltum*, *Dioscorea batatas*, *Epimedium koreanum*, *Poria cocos* and *Zizyphi jujuba*, used in traditional Korean medicine for improvement of memory and cognition in old age have been tested for cholinesterase inhibitory properties and significant inhibition of the enzyme was shown by extracts from *Acorus calamus* and *E. koreanum* (Oh *et al*., 2004). Ingkaninan *et al*., 2000, 2003) screened the methanolic extracts of 32 plants used in Thai traditional rejuvenating and neurotonic remedies, for inhibitory activity on AChE and found that the extracts from roots of *Stephania suberosa* and *Tabernaemontana divaricata* showed significant inhibitory activity.

The chloroform:methanol (1:1) extracts of a number of the plant species namely *Corydalis solida* (L.) *Swartz subsp*. solida and *Glaucium corniculatum* (L.) J. H. Rudolph (Papaveraceae), *Rhododendron ponticum* L. subsp. *ponticum* and *Rhododendron luteum* Sweet. (Ericaceae), *Buxus sempervirens* L. (Buxaceae), *Vicia faba* L. (Fabaceae), *Robinia pseudoacacia* L. (Caeselpiniaceae), *Tribulus terrestris* L. and *Zygophyllum fabago* L. (Zygophyllaceae), *Lycopodium clavatum* L. (Lycopodiaceae), *Fumaria vaillantii* Lois., *Fumaria capreolata* L., *Fumaria kralikii* Jordan, *Fumaria asepala* Boiss., *Fumaria densiflora* DC., *Fumaria flabellata* L., *Fumaria petteri* Reichb. subsp. *thuretii* (Boiss.) Pugsley, *Fumaria macrocarpa* Boiss. ex Hausskn., *Fumaria cilicica* Hauskkn., Fumaria parviflora Lam. and *Fumaria judaica* Boiss. (Fumariaceae) were screened for their anti-cholinesterase activity (Orhan et al., 2004). The extracts of *Rhododendron ponticum*, *Rhododendron luteum*, *Corydalis solida*, *Glaucium corniculatum*, and *Buxus sempervirens* showed remarkable inhibitory activity above 50% inhibition rate at 1 mg/ml.

Amongst plants that have been investigated for dementia therapy, *Salvia* is one of the most numerous genera within the family Lamiaceae and grows in many parts of the world. It causes inhibition of AChE as well as nicotinic activity (Perry *et al*., 2000, 2001).

2.2.3.4 AChE Inhibitor from animal

The Fasciculin II was extracted from the venom of the green mamba, *Dendroaspis angusticeps.* Fasciculin II, a potential inhibitor of acetylcholinesterase (AChE), was tested on two types of Aplysia cholinergic recepters: H type, opening Cl^- channels; and D type, opening cationic channels. Evoked postsynaptic inhibitory responses and responses to ionophoretic application of acetylcholine (ACh) or

carbachol onto H-type receptors were potentiated in the presence of fasciculin II at 10-9 M, whereas the same concentration of this drug was without effect on the evoked postsynaptic excitatory responses or on the application of ACh or carbachol on D-type receptors. They concluded that Fasciculin II was a good inhibitor of acetylcholinesterase on neoru-neuronal preparations *in vivo*. (Fossier, *et al*., 1986).

2.2.4 Biological activities of AChE Inhibitors

2.4.4.1 Assays for AChE Inhibitory activity

Several methods for screening of AChE inhibitory activity from natural resources has been reported based on Ellman‟s reactions (Ellman *et al*., 1961). Moreover, Spectrophotometric determination thin-layer chromatography method (Ingkaninan *et al*., 2000; Marston *et al*., 2002) and micro-plate assay (Brlihlmann *et al*., 2004 and Ingkaninan *et al*., 2000) have been reported to be useful. HPLC method for detection of AChE inhibition on immobilized AChE column (Andrisano *et al*., 2001) and HPLC with on-line coupled UV–MS–biochemical detection for AChE inhibitory activity (Ingkaninan et al., 2000) have also been reported.

2.2.4.2 Phytoconstituents having acetylcholinesterase inhibitory activity

Work on new bioactive compounds from medicinal plants has led to the isolation and structure elucidation of a number of exciting new pharmacophores. A structures of these compounds are shown in Figure 2.5. *Physostigma venenosum* was used traditionally in Africa as a ritual poison, claimed to determine the guilt or innocence of person accused of a crime. Treatment with the indole alkaloid physostigmine, an AChE inhibitor isolated from *P. venenosum*, has improved cognitive function in several in vivo studies. Physostigmine, a short-acting reversible AChE inhibitor, is also reported to have shown significant cognitive benefits in both normal and AD patients, but clinical use may be limited by its short half-life, which would require multiple daily dosing (Da-Yuan *et al*., 1996; Mukherjee, 2001).

Chemical structure of physostigmine has provided a template for the development of rivastigmine, an AChE inhibitor that is licensed for use in the UK for the symptomatic treatment of mild-to-moderately severe AD. Rivastigmine is reported to inhibit AChE in the cortex and hippocampus, brain areas involved in cognition. Thus, it is apparent that plant-derived alkaloid AChE inhibitors may be important for the development of more appropriate drug candidates for the treatment of AD.

Galanthus nivalis was used traditionally in Bulgaria and Turkey for neurological conditions. Galantamine is an Amaryllidaceae alkaloid obtained from *Galanthus nivalis* L. Galantamine is reported to be more selective for AChE than butyrylcholinesterase, and provides complete oral bioavailability. It is licensed in Europe for AD treatment, was well tolerated and significantly improved cognitive function when administered to AD patients, in multi-center randomized controlled trials (Lopez *et al*., 2002). Initially derived from extracts of snowdrop and daffodil bulbs, this phenanthrene alkaloid is now synthetically produced. It is a reversible competitive AChE inhibitor that also allosterically modulates nicotinic receptors (this effect is probably independent of its cholinesterase inhibition). It has an elimination half-life of about 6 h. Metabolism produces four compounds, one of which is more active as a cholinesterase inhibitor than galantamine itself. Over 2000 patients have been involved in double-blind placebo-controlled trials of galantamine where positive effects on cognitive symptoms have been associated with significant benefits in activities of daily living (Da-Yuan *et al*., 1996). Other Amaryllidaceae alkaloids such as assoanine, epinorgalantamine, oxoassoanine, sanguinine, 11-hydroxygalantamine have also been reported to possess AChE activity (Lopez *et al*., 2002).

The lycopodium alkaloid huperzine A related to the quinolizidines, is a potent, yet reversible, inhibitor of AChE and is used in China for treating patients with myasthenia gravis and AD. The source of huperzine A is *Huperzia serrata*, a moss that has been used for treating contusions, strains, hematuria and swelling in Chinese folk medicine (Wang and Tang., 1998). It improved memory retention processes in cognitively impaired aged and adult rats (Raves *et al*., 1997). In a multicenter, double blind trial, huperzine A significantly improved memory and behavior in AD patients, and was reported to be more selective for AChE than butyrylcholinesterase and less toxic than the synthetic AChE inhibitors donepezil and tacrine. It may also have potential in the attenuation of memory deficits and neuronal damage that occurs after ischemia, so may therefore is beneficial in the treatment of cerebrovascular-type dementia (Raves *et al*., 1997).

Numerous essential oils and their monoterpene constituents have been investigated for their effects on AChE, and have shown weak inhibitory activity. For example, the essential oils from *Melissa officinalis* and *Rosmarinus officinalis* have been reported to inhibit erythrocyte AChE in vitro (Howes *et al*., 2003a, b). Other monoterpenes that are reported to inhibit AChE include geraniol, 3-carene, α-

caryophyllene and limonene. The structural diversity of the active anticholinesterase terpenoids complicates the prediction of potential structure–activity relationships. One feature associated with AChE inhibition is a hydrophobic ligand. The hydrophobic active site of AChE is reported to be susceptible to hydrophobic interactions. Monoterpenes consist of a hydrocarbon skeleton, which may contribute to their anti-cholinesterase activity. Monoterpenes may be cyclic (e.g. 1, 8-cineole and apinene) or acyclic (e.g. geraniol and linalool), a feature that may also influence anti cholinesterase activity. Further investigations may determine if a cyclic component or particular functional group favors AChE inhibition. Considering the relatively weak anti-cholinesterase activity of terpenoids reported to date, it is unlikely that they may be used therapeutically for cognitive disorders. However, analogues of active terpenoid compounds may be developed to enhance efficacy. More recently, the stilbene oligomer viniferin from Caragana chamlague, has also been identified as reversible and non-competitive inhibitor of AChE (Da-Yuan *et al*., 1996). Structure–activity relationship suggested that the nitrogen substituents at C-3 and/or C-20 of steroidal skeleton and the hydrophobic properties of the pregnane skeleton are the key structural features contributed to the inhibitory potency of pregnane-type steroidal alkaloids against AChE (Khalid *et al*., 2004).

Bioassay-guided fractionation of the methanolic extract resulted in the isolation of three furanocoumarins, isoimperatorin, imperatorin and oxypeucedanin as active principles from the methanolic extract of the roots of Angelica dahurica, which inhibited AChE activity in a dose-dependent manner (Kim *et al*., 2002). In a bioassay-guided search for AChE inhibitors four isoquinoline alkaloids, corynoxidine, protopine, palmatine and berberine have been isolated from the methanolic extract of the aerial parts of *Corydalis speciosa* (Kim *et al*., 2004). Bioassay-directed phytochemical investigations on a number of medicinal plants of Pakistan and Iran have led to the isolation of AChE inhibitors such as buxamine B, N, N-dimethyl buxapapine, sarsalignone and vaganine (Rahman and Choudhary, 2001). Indole alkaloids coronaridine, voacangine, voacangine hydroxyindolenine and rupicoline isolated from the chloroform extract of stalk of *Tabernaemontana australis* showed anti-cholinesterasic activity at the same concentration as the reference compounds physostigmine and galantamine, by thin-layer chromatography assay using the

modified Ellman‟s method (Andrade et al., 2005). Ursolic acid obtained from *Origanum majorana* has also been reported to possess AChE inhibitory activity (Chung et al., 2001).

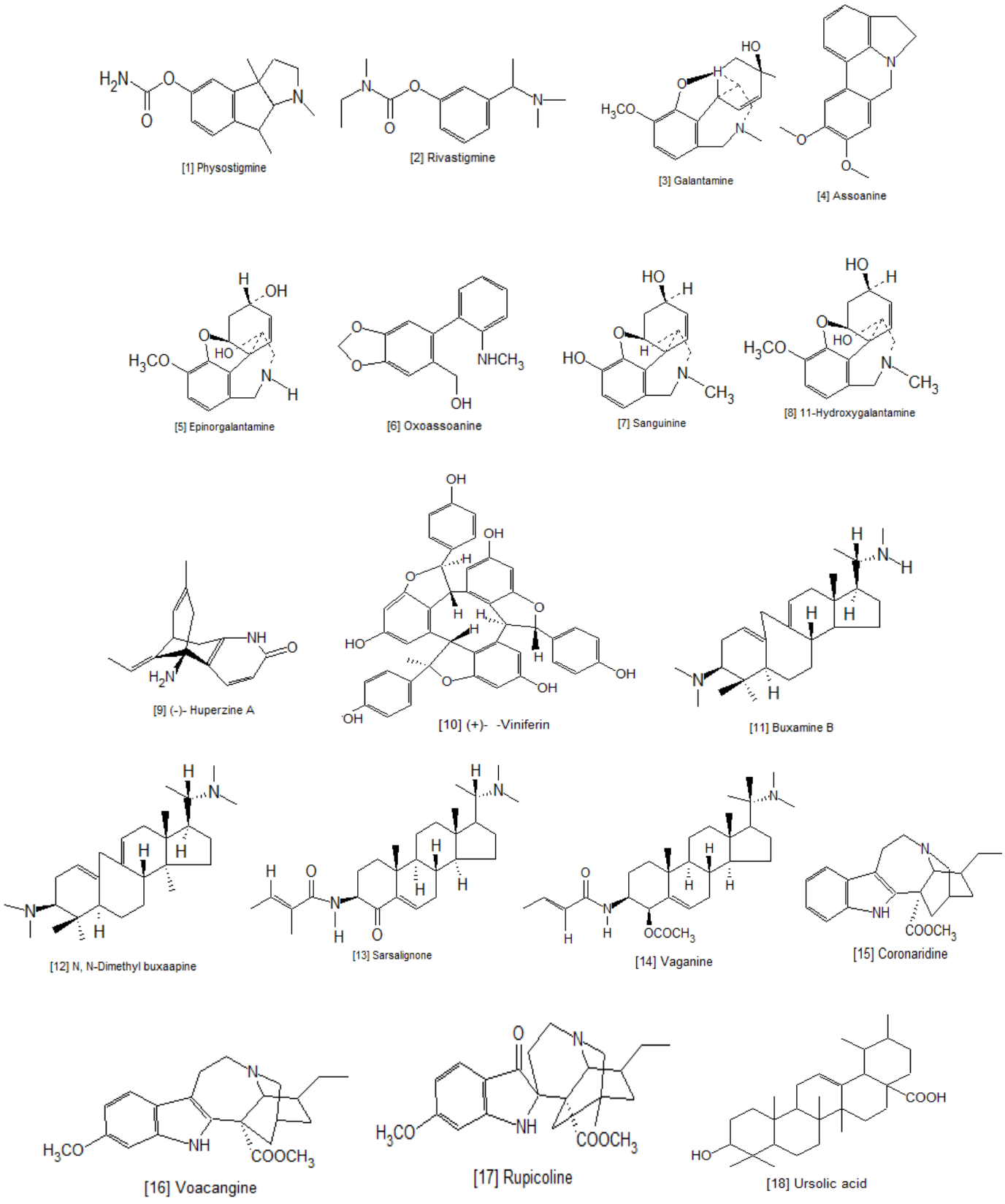

Figure 2.5. Structures of some acetylcholinesterase inhibitors obtained from plants.

2.3 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) (Lopez-Otín, 2008 and Moffitt, 2007). 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 and 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 (Lutgens *et al*., 2007 and Pihlanto and Korhonen, 2003).

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, 1971). Salas (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.

2.4 Family of Zingiberaceae

The Zingiberaceae is the largest family of the order Zingiberales, with approximately 53 genera and over 1,300 species. Species of the Zingiberaceae are the ground plants of the tropical forests. They mostly grow in damp and humid shady places. They are also found infrequently in secondary forest. Some species can fully expose to the sun, and grow on high elevation. An important characteristic is the presence of essential oils in tissues, strong aromatic and medicinal properties. Zingiberaceae plants including terpenes, alcohols, ketones, flavonoids, carotenoids and phytoestrogens. (Habsah et al., 2000; Mau et al., 2003; Suhai 2006) The Zingiberaceae have been reported for their biological activities in antifungal, antioxidant, insecticidal, and anti-inflammatory activities. Plants in this family are abundant in Asia including Thailand and there is still lacking of report concerned with acetylcholinesterase inhibitory activity in these herbs, thus it is interested to search among these herbs for the activity in order to apply in pharmacological area in future.

The 13 Zingiberaceae species were screened for antimicrobial and antioxidant activities. The antimicrobial activity of most of the extracts was antibacterial with only the methanol extract of Costus discolor showing very potent antifungal activity against only Aspergillus ochraceos and all the extracts showed strong antioxidant activity comparable with or higher that of a-tocopherol. (Habsah *et al*., 2000).

The water extract of *Zingiber officinale* showed 6-Gingerol, and was mostly found in the rhizome in concentrations of 130-7,138 ppm. The major compound in Curcuma is curcumin, which has concentrations as high as 38,000 ppm in species

(Suhai., 2006). Report of the pharmacological activities of curcumin including anti-fungal, anti-oxidant, insecticidal anti-microbial effects, anti-inflammatory activities and anti-cancer. (Kuttan *et al*., 1985; Negi *et al*., 1999; Sidhu *et al*., 1998; Sirat, 1994; Sirat *et al*., 1996 and Sirat and Liamen 1995)

The juice of *Zingiber officinale* administered at a dose of 4 ml/kg p.o. daily for 6 weeks significantly prevented hyperglycaemia and hypoinsulinaemia in streptozotocin (STZ)-induced type I diabetic rats. It also produced a significant increase in insulin levels and a decrease in fasting glucose levels in diabetic rats. In an oral glucose tolerance test, the extract was found to decrease the glucose level and to increase the insulin level significantly in STZ-diabetic rats and suggested that hypoglycemic activity of the juice of *Zingiber officinale* in type I diabetic rats possibly involved 5-HT receptors (Akhani *et al*., 2004).

In 2009, Kheeree *et al* reported antifungal and antiproliferative activities of Lectin from the rhizomes of *Curcuma amarissima* Roscoe. A lectin was purified from the rhizomes of Curcuma amarissima Roscoe by aqueous extraction, fractionation with 80% saturated ammonium sulfate, and a combination of affinity and gel chromatography. The molecular mass of the purified lectin was 32.4 kDa. The lectin showed no significant specificity in its ability to hemagglutinate erythrocytes from human blood groups (A, B, AB, and O), but for other animals, it only agglutinated rabbit and rat, and not mouse, guinea pig, goose, and sheep erythrocytes. The lectin was stable at temperatures below 40°C, but the hemagglutinating activity halved when it was heated to 45–85°C and was completely lost at 95°C. The hemagglutinating activity was more stable at 80°C than at 70°C and was rapidly inactivated at 90°C. It showed a maximum hemagglutination activity within the pH range of 8.0–11.0. The amino acid sequence of an internal fragment of this purified lectin showed sequence similarity to other members of the leucoagglutinating phytohemagglutinin precursor family, whilst the complete lectin inhibited the in vitro growth of three plant pathogenic fungi, *Fusarium oxysporum, Exserohilum turicicum*, and *Colectrotrichum cassiicola*, at a concentration of 17.5 to 35 µg, and showed in vitro cytotoxicity against the BT474 breast cancer cell line with an IC_{50} of approximately 21.2 µg. (Kheeree *et al*., 2009).

In 2010, Tiengburanatam *et al* reported a new protein with α-glucosidase inhibitory activity from the rhizomes of *Zingiber ottensii*. Valeton. This protein was found to have three likely sub-unit types, 32.5, 15.2, and 13.8 kDa. The kinetics of the

inhibition of α-glucosidase from Saccharomyces cerevisiae by standard enzymatic methods indicated the maximum percent inhibition; IC_{50} and K_i of this protein were 77.5%, 30.15 µg/ml, and 140 µmol, while the Km and Vmax were 2.35 µmol and 0.11 mM/min, respectively. The inhibitory action was pH-independent within the pH range 2–10, but was potentially affected by buffer salts, and was relatively temperaturestable between 4–35 °C, with a maximum activity at 65 °C. The amino acid sequence of an internal fragment of this purified *Z. ottensii* rhizomal protein had a similarity to the sequence from the plant cysteine proteinase family. (Tiengburanatam *et al*., 2010).

In 2010, Oboh *et al* reported inhibitory effects of water extractable phytochemicals of red and white ginger on acetylcholinesterase activities in rat brain (in vitro). The results revealed that both extracts inhibited acetylcholinesterase (AChE) in a dose-dependent manner (in the range of 0–6.76 mg ml^{-1}). The IC_{50} values of acetylcholinesterase inhibitory activity of red and white ginger were 3.03 and 2.86 mg/ml respectively. Therefore, some possible mechanism by the ginger extracts exerts anti-Alzheimer properties could be through the inhibition of acetylcholinesterase activities.

CHAPTER III

EXPERIMENTAL

3.1 Material and methods

3.1.1 Plant materials

The fresh rhizomes of 15 plants in Zingiberaceae family were periodically (April 2010 - May 2010) purchased from Chatuchak park market in Bangkok, Thailand.

3.1.2 Chemical and biological materials

AChE type V-S from electric eel (658 U/mg of solid), acetylthiocholine iodide (ATCI), azocasein, bovine serum albumin (BSA), DEAE-cellulose, 5,5‟-dithiobis (2-nitrobenzoic acid) (DTNB), trichloroacetic and trypsin (1,040 U/mg of solid) were purchased from Sigma-Aldrich (USA). The reagents used in polyacrylamide gel electrophoresis (PAGE) were obtained from Plusone Pharmacia Biotech (Sweden), except the low molecular weight calibration kit, used as standard molecular weight marker proteins, which was purchased from Amersham Pharmacia Biotech (UK). All other biochemical reagents and general chemicals used in the investigation were of analytical grade.

3.1.3 Preparation of the Zingiberaceae rhizomes extract

The rhizomes (1.5 kg wet weight) of the 15 selected plant species from within the Zingiberaceae family were peeled, cut into small pieces (~ 10 × 10 × 10 mm) and then homogenized in 5 L of PBS (20 mM phosphate buffer (pH 7.2) containing 0.15 M NaCl) using a blender and then left with stirring overnight at 4 ^{o}C. The suspension was then clarified by filtration through double-layered cheesecloth followed by centrifugation at 15,000 × g for 30 min. The clarified supernatant („crude homogenate‟) was harvested and ammonium sulfate added, with stirring, to 80% saturation and then left with stirring overnight at 4 ^{o}C. The precipitate was collected from the suspension by centrifugation at 15,000 × g for 30 min with discarding of the supernatant. The pelleted materials were then dissolved in PBS, dialyzed (3,500 MWCO) against 3 changes of 5 L of water at 4 ^{o}C and then freeze dried. This is referred to as the “ammonium sulphate cut fraction”.

3.1.4 Acetylcholinesterase inhibition assay

The AChEI activity was measured *in vitro* by a modification of Ellman's method (Ellman *et al*., 1961). The assay contained 125 µl of 3 mM DTNB, 50 µl of 50 mM Tris-HCl buffer (pH 8) with 0.1% (w/v) BSA, the plant extract at different concentrations in 50 mM Tris-HCl buffer (pH 8), 25 µl of 0.22 U/ml AChE and 25 µl of 15 mM ATCI. The reaction was mixed in a micro-well plate and incubated for 1 hr at 37 ^{o}C. The enzymatic reaction of AChE hydrolyses the acetyl group of ATCI to yield thiocholine that can then react with DTNB to form 5-thionitrobenzoate, a colored anion that absorbs UV light at 415 nm. The percent inhibition was calculated using the formula: [(control absorbance - sample absorbance) / control absorbance] ×100. The concentrations of the tested samples that inhibited the hydrolysis of substrate (ATCI) by 50% (IC_{50}) were determined by linear regression analysis between the inhibition percentages against the extract concentrations.

3.1.5 Protease activity assay

The determination of protease activity in solution was modified from that previously reported (Tiengburanatam *et al*., 2010) Briefly, 500 µl of each sample was transferred to a 1.5 ml eppendorf tube and 500 µl of a 1% (w/v) azocasein solution in 100 mM sodium acetate buffer (pH 4.5) was added, thoroughly mixed, and then incubated at 37 ^{o}C 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 ^{o}C before clarification of the precipitate by centrifugation at 15,000 × g 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 a spectrophotometer. The protease activity of sample was calculated with reference to a trypsin standard curve in terms of protease units. Increasing the absorbance by one unit was considered to be one unit of activity (Chandrashekar and Gujar, 2003).

3.1.6 Ion exchange chromatography

The ammonium sulphate cut fraction was resolvated in 20 mM phosphate buffer (pH 7.2) and loaded (10 ml at 25 mg/ml total protein) into a DEAE-cellulose column (1.6 cm i.d. x 15 cm length) pre-equilibrated in at least five column-volumes of 20 mM phosphate buffer (pH 7.2), and then eluted from the column using the same buffer with a flow rate of 1.0 ml / min, but with a stepwise gradient of 0, 0.25, 0.50, 0.75 and 1.0 M NaCl (step gradient increases). Each collected fraction (10 ml) was

screened for protein content (section 3.1.7) and AChEI and protease activities (sections 3.1.4 and 3.1.5, respectively). The fractions containing such activity were combined and dialyzed and freeze-dried as in section 3.1.3, and kept at -20 ^{o}C until use. Each fraction is known as the „post-DEAE-cellulose AChEI X fraction", where x stands for unbound, F25, F50, F75 or F100 for the unbound and bound fractions eluted at 0.25, 0.5, 0.75 and 1.0 M NaCl, respectively.

3.1.7 Protein content determination

For evaluation of protein levels in the DEAE-cellulose column chromatography, the elution peak profiles of the proteins were determined by measuring the absorbance at 280 nm. For all other samples the protein contents were determined by Bradford"s procedure (Bradford, 1976), using BSA as the standard with four different concentrations (5, 10, 15 and 20 µg/ml) to construct the calibration curve. For each serial 2-fold dilution of the sample in deionized water, 50 µl aliquots were transferred into each of three wells of a microtiter plate and 50 µl of Bradford"s reagent added to each well, shaken for 5 min and then left for 10 min before reading the absorbance at 595 nm with an ELISA plate reader. The obtained absorbance was converted to protein concentration using the linear equation computed from the standard curve.

3.1.8 Determination of the protein pattern by native-PAGE

The protein from each step of the purification was analyzed for its native protein pattern according to the method of Bollag *et al*. (1996), using a 10% and 5% (w/v) acrylamide separating and stacking gel, respectively. Tris-glycine buffer pH 8.3 was used as the electrode buffer, and gels were run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. After electrophoresis, the resolved proteins in the gel were visualized by Coomassie blue R-250 staining (0.1% (w/v) Coomassie blue R-250 in 10% (v/v) acetic acid and 45% (v/v) methanol) and several changes of destaining solution (10% (v/v) acetic acid and 45% (v/v) methanol) until the background was clear.

3.1.9 Zymography of gelatin-containing native-PAGE

To test for the presence of protease activity by zymography (Ambili and Sudhakaran, 1998) the sample was applied to a 10% (w/v) acrylamide separating gel containing 1% (w/v) gelatin. After electrophoresis, the gel was washed in 2.5% (w/v) Triton X-100 solution for 30 min at room temperature followed by incubation in

reaction buffer (20 mM phosphate buffer (pH 7.2), 1% (v/v) Triton X-100 and 25 mM $CaCl_2$) at 37 ^oC for overnight. The gels were developed using Coomassie blue R-250 solution (section 3.1.8) and the protease activity appeared as white band against a blue background.

3.1.10 Sodium dodecyl sulphate polyacrylamide gel electrophoresis and size estimation

Discontinuous reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared with 0.1% (w/v) SDS in 12.5% and 5% (w/v) acrylamide separating and stacking gels, respectively, with Tris-glycine buffer (pH 8.3) containing 0.1% (w/v) SDS as the electrode buffer, according to the procedure of Laemmli (1970). Samples to be analyzed were treated with reducing sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. Molecular weight standards were co-resolved in the gel alongside the samples to determine the subunit molecular weight of the purified protein(s). After electrophoresis, the proteins in the gel were visualized by standard Coomassie blue R-250 (section 3.1.8).

3.1.11 Effect of temperature on the AChEI activity

The effect of temperature on the AChEI activity was determined by incubating AChEI samples in 20 mM phosphate buffer (pH 7.2) at various temperatures (30 – 90 °C at 10 ^oC intervals) for 30, 60, 90 or 120 min, cooling to 4 °C and then assaying the residual AChEI activity as described in section 3.1.4 The activities attained were compared with the control which was set as 100% and are reported as the relative activities compared to that of the control. In all cases, AChE was used and at least three replicates were done for each assay.

3.1.12 The pH-dependence of the AChEI activity

Incubating the AChEI in buffers of broadly similar salinity levels but varying in pH from 2 - 12 was used to assess the pH stability and the pH optima of the AChEI. The buffers used (all 20 mM) were glycine-HCl (pH 2 - 4), sodium acetate (pH 4 - 6), potassium phosphate (pH 6 - 8), Tris-HCl (pH 8 - 10) and glycine-NaOH (pH 10 - 12). The purified AChEI was mixed in each of the different buffer-pH compositions, and left for 30, 60, 90 or 120 min at room temperature prior to assaying for AChEI activity (section 3.1.4). The activities attained were compared relative to that of the

control (set as 100%) and so are reported as the % relative activity. In all cases, AChE was used and at least three replicates were done for each assay.

3.1.13 Effect of metal ions on the AChEI activity

The effect of different divalent metal cations and the chelating agent EDTA, on the AChEI activity was evaluated. The enriched (post-DEAE-cellulose unbound) AChEI fraction (1 mg / ml) was incubated for 30 min with one of the divalent cation salts of Ca^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} (all as chlorides), Cu^{2+} or Zn^{2+} (as sulphates) or EDTA at 1, 5 or 10 mM with continuous shaking. After that, the AChEI activity was determined as described (section 3.1.4) using at least three replicates for each assay.

3.1.14 Estimation of kinetic parameters

The rate of hydrolysis of ACTI by AChE over an ATCI concentration range of 0.04 - 0.4 mM ($ATCI^{-1}$ = 2.5 - 25 mM^{-1}) in the presence or absence of various concentrations of the enriched (post-DEAE-cellulose unbound) AChEI fraction was measured. Then, the data from enzyme assays was subjected to double-reciprocal **(**Lineweaver-Burk) plot analysis to determine the Michaelis-Menten constant (K_m), maximum velocity (V_{max}) and inhibition mode of the enriched post-DEAE-cellulose unbound AChEI fraction .

3.1.15 Internal amino acid sequencing by liquid chromatography / mass spectrometry / mass spectrometry (LC/MS/MS)

3.1.15.1. *In situ* (in gel) trypsinization

The sample preparation process followed the published method of Mortz *et al*. (1994). The single band from the SDS-PAGE resolution of the post-DEAE-cellulose unbound AChEI fraction was excised from the gel, 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×rpm for 1 min. The remaining peptides in the gel were extracted with a solution of 50% (v/v) acetonitrile containing 5% (v/v) formic acid for 10 min with shaking, and subsequently pooled with the previous supernatants and taken to dryness.

3.1.15.2. LC-MS/MS and peptide blasting

The likely amino acid sequence of each internal fragment of the trypsinized protein was analyzed by LC/MS/MS. 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 (http://www.matrixscience.com) search of the NCBI database (http://blast.ncbi.nlm.nih.gov). 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 (Mortz *et al*., 1994).

3.1.16 Statistical analysis

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

CHAPTER IV

RESULT AND DISCUSSION

AD is the most common form of dementia among the elderly and manifests as a decreased level of ACh in the brain areas that are related to memory and learning (Lahiri *et al*., 2002). Based on the cholinergic hypothesis that memory impairments in patients suffering from AD result from a defect in the cholinergic system, an important approach to treat this disease is to enhance the ACh level in the brain by inhibition of the ACh degrading AChE enzyme. Indeed, AChEIs have been widely used in the treatment of AD and the only effective treatments currently available for AD are aimed at the cholinergic system using anti-ChE compounds. Nature is an unlimited resource for providing chemicals and biological compounds which are unique and complex insofar as their chemical synthesis seems impossible. The AChEI activity of some plants has been established already (Mukherjee *et al*., 2007).

4.1. Screening for AChEI activity in plant samples

In this study we screened the crude homogenates and ammonium sulphate cut fractions from the rhizomes of 15 Zingiberaceae plant species for protease activity using the azocasein assay (section 3.1.5) and further screened the extracts of all 15 Zingiberaceae plant species for AChEI activity using a modification of the method of Ellman *et al*. (1961). The IC_{50} values were calculated from the regression equation obtained from evaluation of different concentrations of each test extract (Table 4.1). Of the six crude homogenates screened, all six were positive for AChEI activity with good inhibitory activity (low IC_{50} values) being observed for *Boesenbergia pandurata*, *Hedychium coranarium* and *Zingiber officinale* (Table 4.1). Screening of the ammonium sulphate cut fraction of five of the fifteen species revealed all to be positive for AChEI activity, and three of these were different plant species to those screened as crude homogenates making a total of 9 / 9 positive species. Of note is that of the two species screened as both a crude homogenate and an ammonium sulphate cut fraction (*Z. officinale* and *Curcuma amarissima*), the IC_{50} value actually increased after ammonium sulphate fractionation. The crude homogenate and ammonium sulphate cut fraction of *Z. officinale* (ginger) showed relatively high AChEI activities,

with IC_{50} values of 22.4 ± 52 and 137.3 ± 55 μg / ml, respectively, and so was selected for further enrichment of the AChEI activity and evaluation.

Protease activity was found in the extracts of 14 / 15 of the plant species tested from the Zingiberaceae family, as evaluated using a modification of the method of Tiengburanatam *et al*., (2010), with the 15th species, *B. pandurata*, being negative (Table 4.1). The level of protease activity varied some 66.1-fold in the crude homogenate between the 14 positive tested plant species, ranging from very low in *C. zedoaria* to very high in *Alpinia galanga*, with most falling within the 20 - 120 U / mg protein range. However, in all bar two cases (*C. zeodaria* and *K. galanga* which remained the same and increased 1.91-fold, respectively), the protease specific activity was significantly lower (1.32- to 11.3-fold, with most at 2.9- to 3.7-fold) after 80% saturated ammonium sulphate precipitation, although whether this reflects loss in the non-precipitated fraction or else a loss of activity due to proteolysis or other forms of denaturation of a loss of essential cofactors is unknown. Regardless, although *Z. officinale* showed only a moderate and a slight protease activity in the crude homogenate and ammonium sulphate cut fraction (76.9 ± 0.04 and 21.6 ± 0.1 U / mg protein, respectively), it revealed a strong AChEI activity and so, as already stated above, was selected for further analysis, given this research is focused upon proteases that show potent AChEI activities.

Table 4.1 The protease activity and *in vitro* AChEI activity in the rhizome extracts of 15 Thai species from within the Zingiberaceae family[a].

Scientific name	IC_{50} value (μg/ml)		Protease activity (U/mg protein)	
	crude extract[b]	crude protein[c]	crude extract[b]	crude protein[c]
Alpinia galanga (Linn.) Swartz.	ND	ND	640.6 ± 0.394	56.6 ± 0.131
Boesenbergia pandurata Roxb.	24.3 ± 37	ND	ND	ND
Curcuma aeruginosa Roxb.	ND	ND	212.8 ± 0.086	120.2 ± 0.035
Curcuma amarissima Roscoe.	348.4 ± 100	466.1 ± 58	34.5 ± 0.047	10.8 ± 0.092
Curcuma aromatica.	ND	ND	21.6 ± 0.035	9.74 ± 0.252
Curcuma longa Linn.	67.2 ± 78	ND	66.8 ± 0.049	9.71 ± 0.020
Curcuma sp.	ND	ND	20.6 ± 0.015	15.6 ± 0.035
Curcuma xanthorrhiza Roxb.	ND	704.3 ± 41	33.3 ± 0.122	8.84 ± 0.081
Curcuma zedoaria (Berg) Roscoe.	ND	393.9 ± 19	9.69 ± 0.074	9.99 ± 0.022
Hedychium coronarium.	25.7 ± 63	ND	59.6 ± 0.060	11.3 ± 0.105
Kaempferia galanga Linn.	112.9 ± 75	ND	24.2 ± 0.020	46.3 ± 0.081
Zingiber cassumunar	ND	ND	42.0 ± 0.105	14.4 ± 0.262
Zingiber officinale Roscoe.	22.4 ± 52	137.3 ± 55	76.9 ± 0.043	21.6 ± 0.110
Zingiber ottensii Valeton.	ND	265.7 ± 40	113.4 ± 0.097	33.9 ± 0.240
Zingiber zerumbet Smith.	ND	ND	70.7 ± 0.157	19.0 ± 0.092

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

4.2. Purification of AChEI with ion exchange chromatography

The AChEI activity from ginger rhizomes was enriched to apparent homogeneity using a two-step procedure. The crude rhizome homogenate was first precipitated with 80% saturation ammonium sulfate and the precipitate harvested by centrifugation, dialyzed with distilled water at 4ºC and dried by lyophilization. Secondly, the ammonium sulphate cut fraction was subjected to DEAE-cellulose

anion exchange column chromatography in 20 mM phosphate buffer (pH 7.2) and eluted in the same buffer with a 0, 0.25, 0.5, 0.75 and 1 M NaCl stepwise gradient. The fractions containing proteins that eluted from the DEAE-cellulose column were screened for protease and AChEI activity (Figure 4.1). Four distinct protein peaks were isolated, being the unbound fraction, and the bound proteins that were then eluted at 0.25 (F25), 0.5 (F50) and 0.75 (F75) M NaCl. Most of the protein appeared to be unbound with decreasing amounts with increasing adhesion to the column (increasing salt levels to elute it) such that the F75 peak was very small (Figure 4.1). However, both protease and AChEI activities were only detected in the unbound fraction, which accounted for just over 90% of the total recovered protein (Figure 4.1 and Table 4.2). Thus, the post-DEAE-cellulose unbound AChEI fraction was selected for further characterization.

Table 4.2 The protein yield and the AChEI and protease activities in each enriched fraction[a].

Fraction	AChEI activity		Protease activity
	Protein yield (μg)	Maximal inhibition (%)	(U/mg protein)
Crude extract[b]	151.1 ± 53	92.4 ± 0.017	76.9 ± 0.043
Crude protein[c]	798.5 ± 124	76.7 ± 0.068	21.6 ± 0.110
Fraction unbound	72.3 ± 30	88.4 ± 0.023	786.3 ± 0.454
Fraction F25	30.5 ± 230	ND	ND
Fraction F50	24.1 ± 190	ND	ND
Fraction F75	ND	ND	ND

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

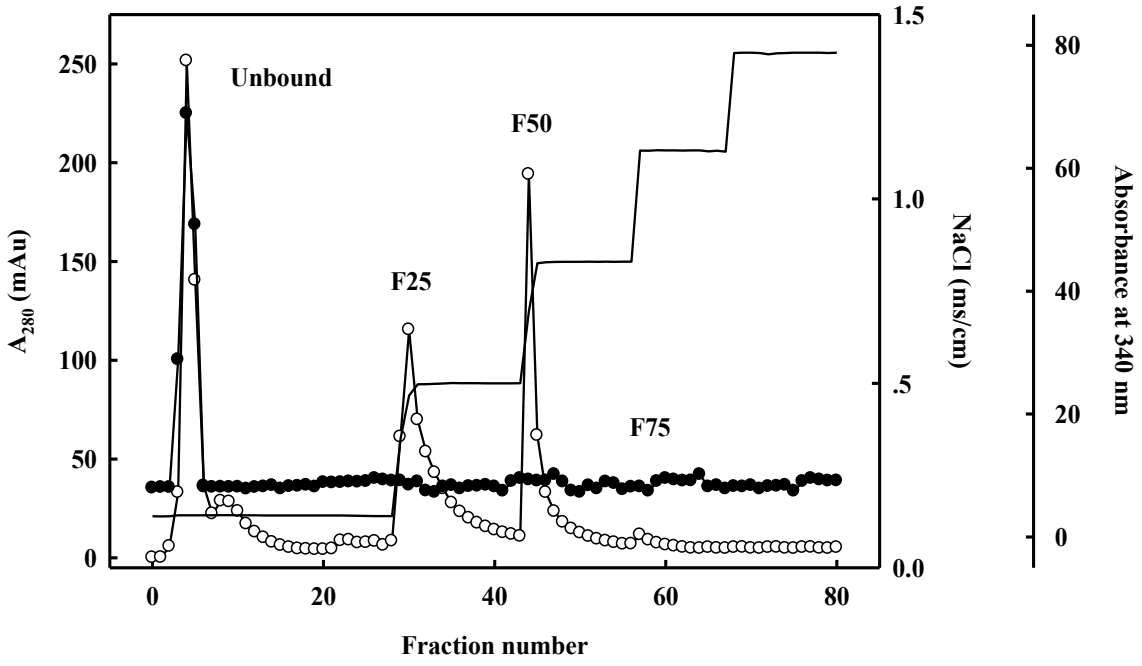

Figure 4.1 DEAE-cellulose chromatogram of the ammonium sulphate cut fraction of *Z. officinale* rhizome proteins (50 mg) with stepwise NaCl elution (0.00, 0.25, 0.50, 0.75 and 1.00 M). Fractions were assayed for (○) absorbance at 280 nm and (●) protease activity. Profile shown is representative of three independent trials.

4.3. Purity checking by native, zymography and reducing SDS-PAGE

The protein fractions with AChEI activity from each enrichment stage were analyzed for purity and protein pattern by native-PAGE and reducing SDS-PAGE resolution (Figure 4.2). Native PAGE resolution revealed an apparent single band for the post-DEAE-cellulose unbound fraction, which also coincided in migration pattern with the apparent protease activity in the gelatin zymography (Figure 4.2A). The implication that the enriched post-DEAE-cellulose unbound fraction was a relatively homogenous protein preparation was supported by the presence of a single band after reducing SDS-PAGE analysis, and gave an estimated size of about 35.5 kDa (Figure 4.2B). This size is in good agreement with the previously published biochemical characteristics for zingipain with a reported size of about 34.8 kDa (Adulyatham and Owusa-Apenten, 2005). That an apparent high level of purity was attained by just a single step chromatographic purification which is of relevance since this is easier, has a lower time and cost of purification, and should avoid the significant yield losses

seen with multiple processing steps (Demir *et al*., 2008; Rameshwaram and Nadimpalli, 2008; Wang and Ng, 2002 and Ye *et al*., 2001;).

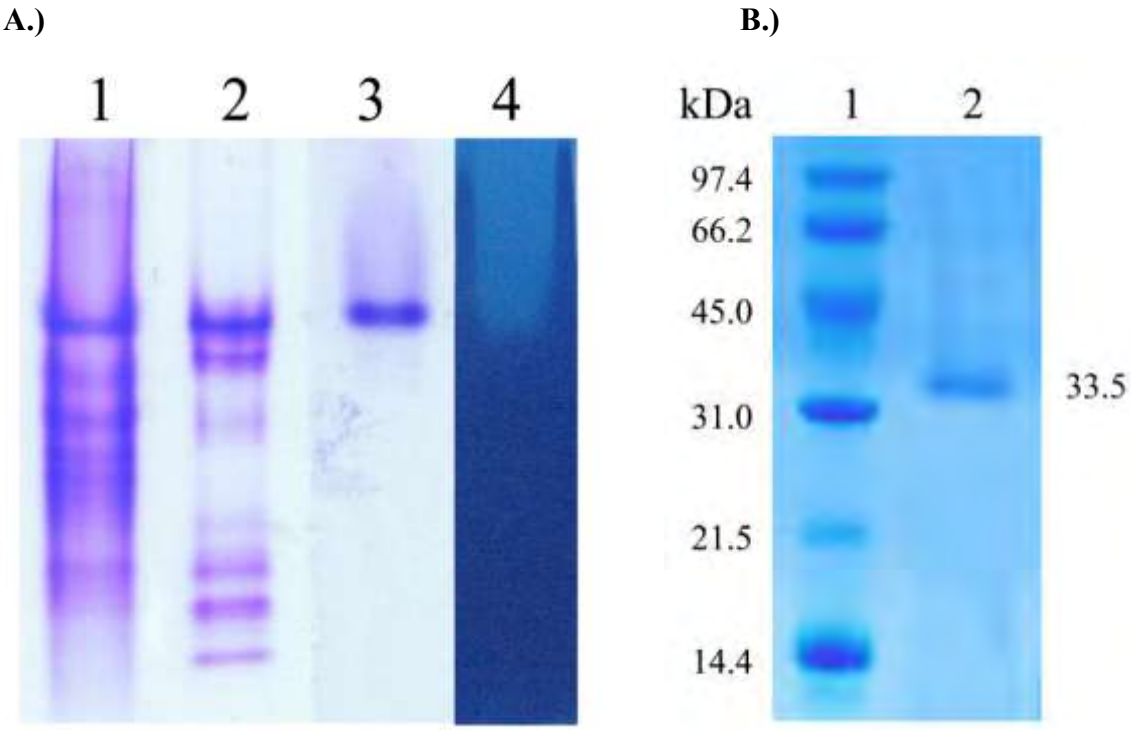

Figure 4.2 (A) Coomassie blue stained non-denaturing PAGE of the *Z. officinale* rhizome protein from each step of enrichment. Lanes 1 - 4 show 20 µg of total protein from (1) the crude homogenate, (2) the 80% saturation ammonium sulphate cut fraction and (3) the post-DEAE-cellulose unbound fraction and (4) gelatin-protease staining of the post-DEAE-cellulose unbound fraction. (B) Reducing SDS-PAGE analysis of the enriched post-DEAE-cellulose unbound fraction. Lane 1, molecular weight standards; Lane 2, post-DEAE-cellulose unbound fraction. The native and denaturing gels and protease zymographs shown are representative of three independent trials.

4.4. Characterization of the AChEI activity

Current research into AChEIs is driven by their potential applications in medical research. In this context, the determination of the physicochemical parameters characterizing the stability of the inhibitors is essential to select effective and stable inhibitors under a large variety of environmental conditions. Moreover, the

knowledge of their structural features is fundamental to understanding the inhibitor-enzyme interactions and allow novel approaches in the use of synthetic or modified inhibitors for drug design.

4.4.1 Temperature resistance determination

The thermal stability profile of the enriched AChEI (post-DEAE-cellulose unbound fraction) from ginger is shown in Figure 4.3 The relative activity of this AChEI was stable over a relatively wide temperature range (-20 – 90 ^{o}C at a 30 min exposure) with more than 60% relative activity being retained at 90 ^{o}C for 30 min. Except for at 90 ^{o}C, increasing the incubation time from 30 to 60 min only slightly decreased the resulting AChEI activity at each temperature, but further increases in the preincubation (exposure) time to 90 or 120 min decreased the AChEI activity further. However, the trend of a relatively thermostable activity up to 60 ^{o}C remained. Between -20 to 60 ºC the AChEI preparation had an activity over the range of 100 to 40%, respectively. However, no activity was evident after 120, 90 and 60 mins at 70, 80 and 90 ºC, respectively. One possible reason was the higher temperature and longer time incubation range caused a change in the AChEI protein structure at regions that are involved in binding to AChE. A similar thermal stability has been observed for the proteolytic α-glucosidase inhibitor from the rhizomes of *Z. ottensii* with a high degree of stability over 0 – 65 °C that then decreased at higher temperatures (Tiengburanatam *et al*., 2010).

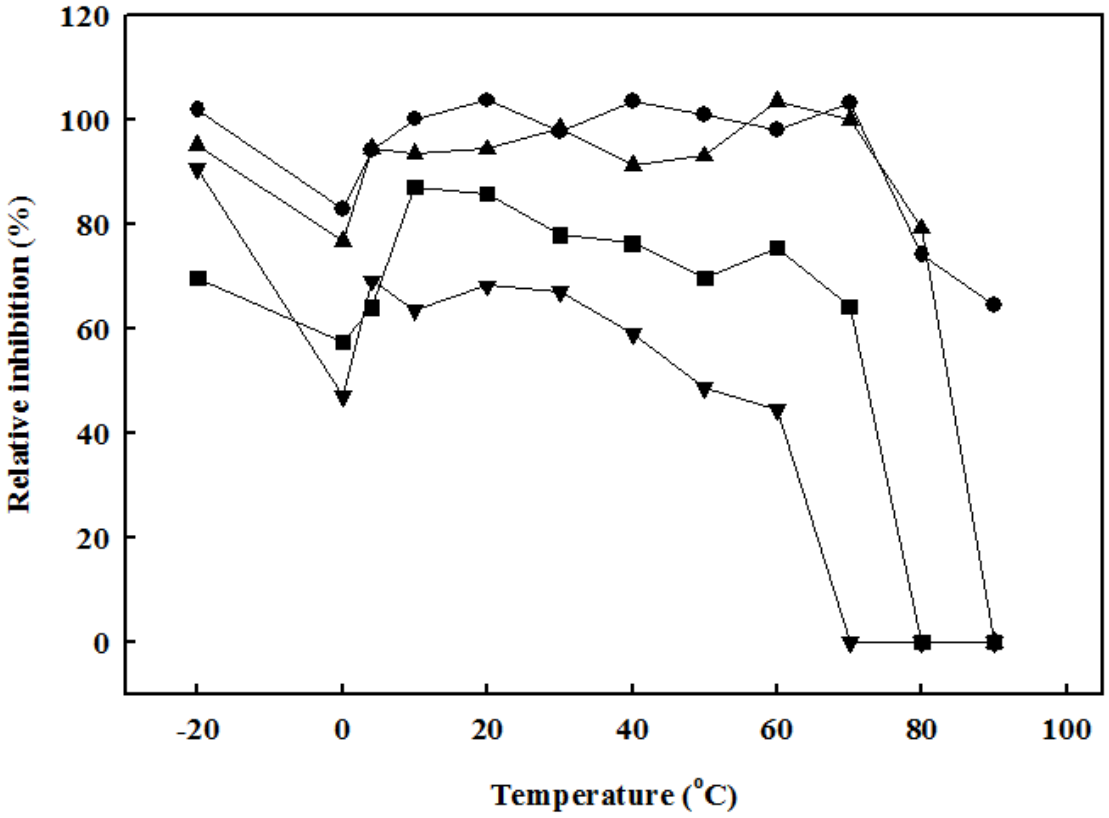

Figure 4.3 Thermostability of the enriched AChEI from *Z. officinale* rhizomes (post-DEAE-cellulose unbound fraction). The assay was performed in 20 mM phosphate buffer pH 7.2 at various temperatures for (●) 30, (▲) 60, (■) 90 and 120 (▼) min. Data are shown as the mean ± 1 SEM and are derived from triplicate experiments.

4.4.2. pH resistance of the AChEI activity

The residual AChEI activity, as a relative % inhibition, as a function of the pH was largely unaffected giving a broad pH optimum. This makes it a potentially excellent enzyme for the food and pharmaceutical industry. However, some buffer-dependent affects were seen, especially at pH 10.0 (Figure 4.4) where a very low AChEI activity was seen in Tris-HCl but not in Glycine-NaOH. Thus, some inhibitor-ion interactions might block or slow down the AChEI activity at such pH values. These are potential pitfalls in all, including this AChEI activity, enzyme assays and also in potential biotechnological applications where changing buffers is difficult or expensive (except, perhaps, for immobilized enzymes).

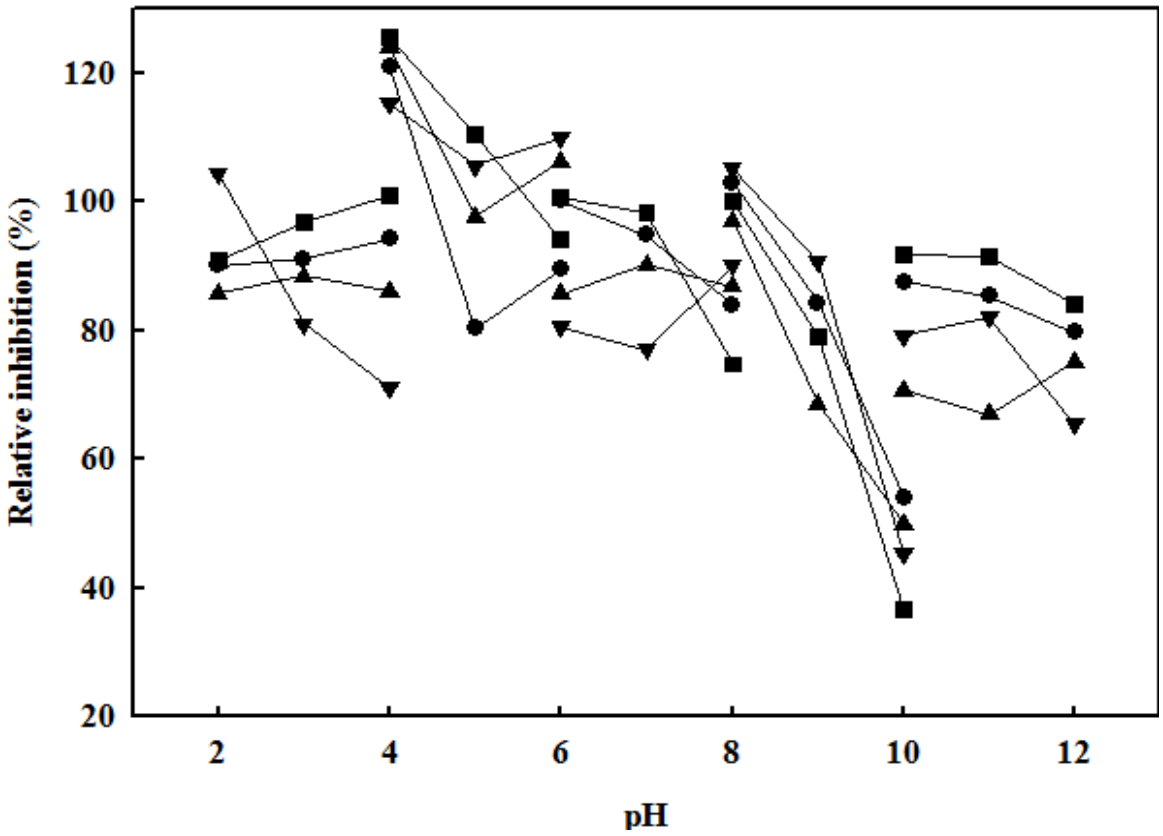

Figure 4.4 pH stability of enriched AChEI from *Z. officinale* rhizomes (post-DEAE-cellulose unbound fraction). The assay was performed in the following buffer systems (all 20 mM); glycine-HCl (pH 2.0 - 4.0), sodium acetate (pH 4.0 - 6.0), potassium phosphate (pH 6.0 - 8.0), Tris-HCl (pH 8.0 - 10.0) and glycine-NaOH (pH 10.0 - 12.0) at various temperatures for (●) 30, (▲) 60, (■) 90 and 120 (▼) min. Data are shown as the mean ± 1 SEM and are derived from triplicate experiments.

4.4.3. Effect of metal ions on AChE inhibitory activity

The activity assayed in the absence of metal ions was recorded as 100% and the effect of the addition of various bivalent metal cation salts on the AChEI activity is shown as a relative % in Table 4.3 The presence of $Mn^{2+}$and Cu^{2+} ions at 1 – 10 mM clearly and significantly stimulated the AChEI activity and was highest at 5 – 10 mM. Perhaps these two metal ions stabilize this AChEI protein in a more suitable conformational structure. Although Zn^{2+} offered weak stimulation of the AChEI activity at 1 mM, this was negated to essentially no effect at 5 and 10 mM, whilst Mg^{2+}, Ca^{2+}, Hg^{2+} and Fe^{2+}, that caused some stimulation at 1 mM, inhibited the AChEI activity at 10 mM, and this was especially marked for Hg^{2+} being 100% loss of activity at 5 and 10 mM. That Hg^{2+} inhibited the AChEI activity is suggestive of

the presence of at least one sulfhydryl group, most likely a cysteine amino acid residue at the active site. The divalent metal ion chelating agent EDTA at 1, 5 and 10 mM showed a marked inhibition of the relative AChEI activity, consistent with the apparent ability of divalent metal ions, such as Mn^{2+}, to stimulate the AChEI activity.

Table 4.3 Effect of bivalent metal cations on the AChEI activity on the enriched post-DEAE-cellulose unbound protein fraction from *Z. officinale* rhizomes[a].

Reagent	Relative inhibition (%)		
	1 mM	5 mM	10 mM
Ca^{2+}	119.5 ± 0.008	129.6 ± 0.083	67.8 ± 0.046
Cu^{2+}	176.4 ± 0.004	200.4 ± 0.011	199.0 ± 0.006
Fe^{2+}	122.2 ± 0.065	103.5 ± 0.107	69.4 ± 0.099
Mg^{2+}	123.9 ± 0.057	100.5 ± 0.071	76.7 ± 0.034
Mn^{2+}	154.0 ± 0.029	163.9 ± 0.072	164.2 ± 0.029
Zn^{2+}	139.7 ± 0.047	123.7 ± 0.090	96.3 ± 0.099
Hg^{2+}	125.2 ± 0.032	0.0 ± 0.0	0.0 ± 0.0
EDTA	40.6 ± 0.051	45.3 ± 0.082	39.9 ± 0.061

[a]Data are shown as the mean ± 1 SEM and are derived from 3 replicate enrichments

4.4.4. Mechanism of inhibition

The inhibition mode of the AChEI from ginger was analyzed by double reciprocal (Lineweaver-Burk) plots (Figure 4.5). The K_m value, with ATCI as the substrate and AChE as the active enzyme, was 130.6 mM with a V_{max} of 3.41 mM / min. When the AChEI was added to the enzyme mixture at various concentrations, the kinetics demonstrated a non-competitive inhibition mechanism with a K_i value of 9.31 mg / ml. Thus, this AChEI might compete with ATCI for binding at substrate binding site of AChE or it might combine with either AChE or with AChE-ATCI. In the case of high concentrations of ATCI, the extract may bind to the secondary binding site of AChE. This notion is supported by the decreasing V_{max} values observed as the concentration of the AChEI was increased. The K_i value suggests that the extract had a low affinity for AChE.

One of the most important anti-ChE drugs, tacrine, has been shown to have both competitive and non-competitive inhibitory activities on AChE (Alhomida *et al*., 2000). Tolserin, the novel experimental AD therapeutic agent, inhibits AChE in a non-competitive manner (Kamal *et al*., 2000). Non-competitive type inhibitors have been put forward as model candidates for inhibiting AChE-induced Aβ aggregation due to their ability to bind to the peripheral anionic site (Bartolini *et al*., 2003). Other studies also suggest that the Aβ aggregating property of AChE during the onset of AD can be inhibited by non-competitive inhibitors (Choudhary *et al*., 2005). The ginger AChEI kinetics found in the present study indicates a putative non-competitive mechanism that may have a novel therapeutic potential for AD. One of the main benefits of phytotherapy is the wide range of medicinal properties that each plant can offer, whereas pharmaceutical drugs are usually designed to attack only a single target (Mills and Bone, 2000).

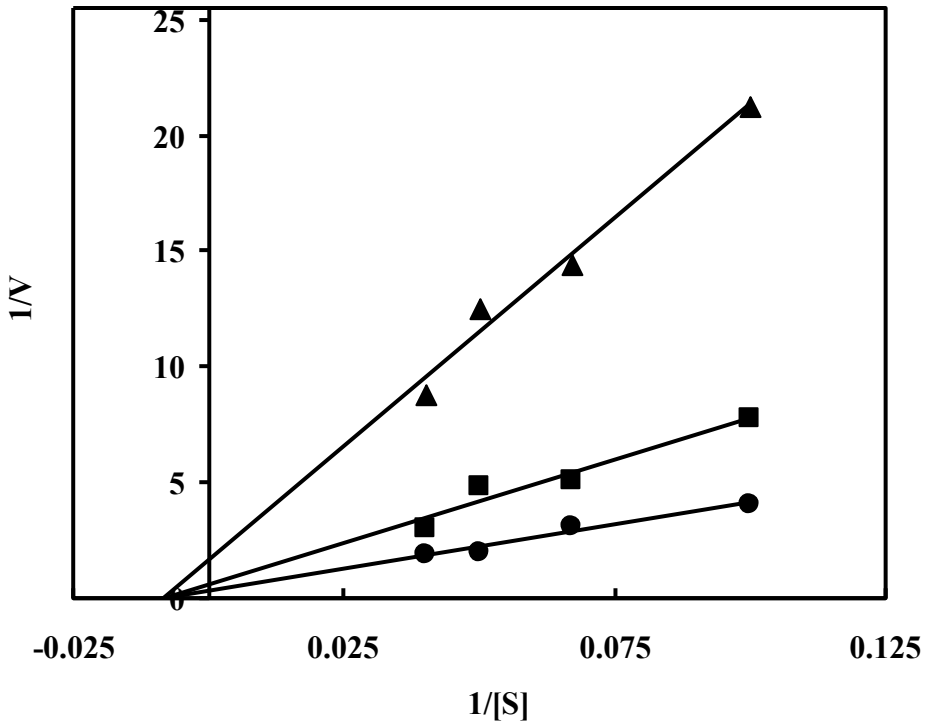

Figure 4.5 Lineweaver-Burk plots derived from the inhibition of AChE by the AChEI from *Z. officinale* rhizomes. AChE was treated with each stated concentration of ATCI (0.04 - 0.4 mM) in the presence of the AChEI at (●) 0, (■) 0.2 and (▲) 0.5 mg protein/ml.

4.5. Potential AChEI protein identification

Amino acid sequences of the tryptic peptide fragments were deduced by LC-MS/MS analysis. Five sequences (AVANQPVSVTMDAAGR, NRNH, GCEGG, WPYR and ENAHVVSIDSYR) were gained from software analysis (*De novo* deducing) (Figure 4.6). All fragments were aligned to those homologs available in the NCBI GenBank and UniProt databases. The longest two sequences obtained, AVANQPVSVTMDAAGR and ENAHVVSIDSYR, were also BLASTp searched against the GenBank and UniProt nr database alone, revealing 100% amino acid sequence similarity to that of part of zingipain-2, zingipain-1, cysteine protease GP2a and cysteine protease GP2b (all from *Z. officinale* rhizome) for the first peptide. 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 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, unbound is likely to be a member of the cysteine protease family (Figure 4.6), 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 the enriched protein being a cysteine glycoprotease opens up the ability to evaluate the reasons for the other observed results

Accession number	Organism		Sequence	
	Zingiber officinale (unbound)		AVANQPVSVTMDAAGR ENAHVVSIDSYR	
P82473	*Zingiber officinale* (Cysteine proteinase GP-I)	125	KAVANQPVSVTMDAAGRDFQLYRNGIFTGSCNISANHYRT	164
Q5ILG5	*Zingiber officinale* (Cysteine protease gp3a)	202	KAVANQPVSVTMDAAGRDFQLYRSGIFTGSCNISANHALT	234
P82474	*Zingiber officinale* (Cysteine proteinase GP-II)	125	KAVANQPVSVTMDAAGRDFQLYRSGIFTGSCNISANHALT	164
Q5ILG7	*Zingiber officinale* Cysteine protease gp2a	266	KAVANQPVSVTMDAAGRDFQLYRSGIFTGSCNISANHALT	305
A6N8F8	*Elaeis guineensis*(Cysteine proteinase)	265	KAVANQPVSVAIEAGGREFQLYHSGIFTGRCGTDLDHGVV	304
B9R777	*Ricinus communis* (Cysteine protease)	265	KAVANQPVSVAIEAGGREFQFYQSGIFTGRCGTALDHGVA	304
P25250	*Hordeum vulgare* (Cysteine proteinase EP-B2)	261	RAVANQPVSVAVEASGKAFMFYSEGVFTGECGTELDHGVA	300
Q7X750	*Glycine hispida*(Cysteine proteinase)	252	KAVANQPVSVAIDAGGSDFQFYSEGVFTGDCSTELNHGVA	291
Q84M27	*Helianthus annuus*(Cysteine protease-3)	249	RAVANQPVSIAIDAGGLNFQFYSQGVFNGACGTELNHGVA	288
Q1EPL9	*Triticum aestivum* (Cysteine proteinase)	365	KAVANQPVSVGIDASGKAFMFYSEGVFTGECGTELDHGVA	304

Figure 4.6 Amino acid sequence from the tryptic fragments of the AChEI from *Z. officinale* rhizomes (post-DEAE-cellulose unbound fraction). Comparisons are made with other cysteine proteases from the cysteine protease family that showed the highest sequence identity in BLASTp searches of the NCBI and SwissProt databases. Accession codes (UniProt/GenBank) are shown.

CHAPTER V

CONCLUSION

ACh is one of the most important neurotransmitters in the central and peripheral nervous systems, and the inhibition of AChE has been proposed as a biomarker for neurotoxicity. According to the data presented here, based upon screening for protease activity in Zingberaceae plants that could also be used as AChEI, a protease from ginger (*Z. officinale*) rhizomes showed the best AChEI activity. Therefore, to achieve an effective therapeutic use, in this work, we identified and partially characterized a this AChEI containing protease factor and determined its effective AChEI activity, and the temperature and pH dependence on its stability. Since only one such AChEI activity was followed in this report from 15 such plants, these plants may offer great potential for the treatment of different diseases, including AD, and their anti-AChE properties introduce them as promising candidates for more detailed *in vitro* and *in vivo* studies.

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APPENDICES

APPENDIX A

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

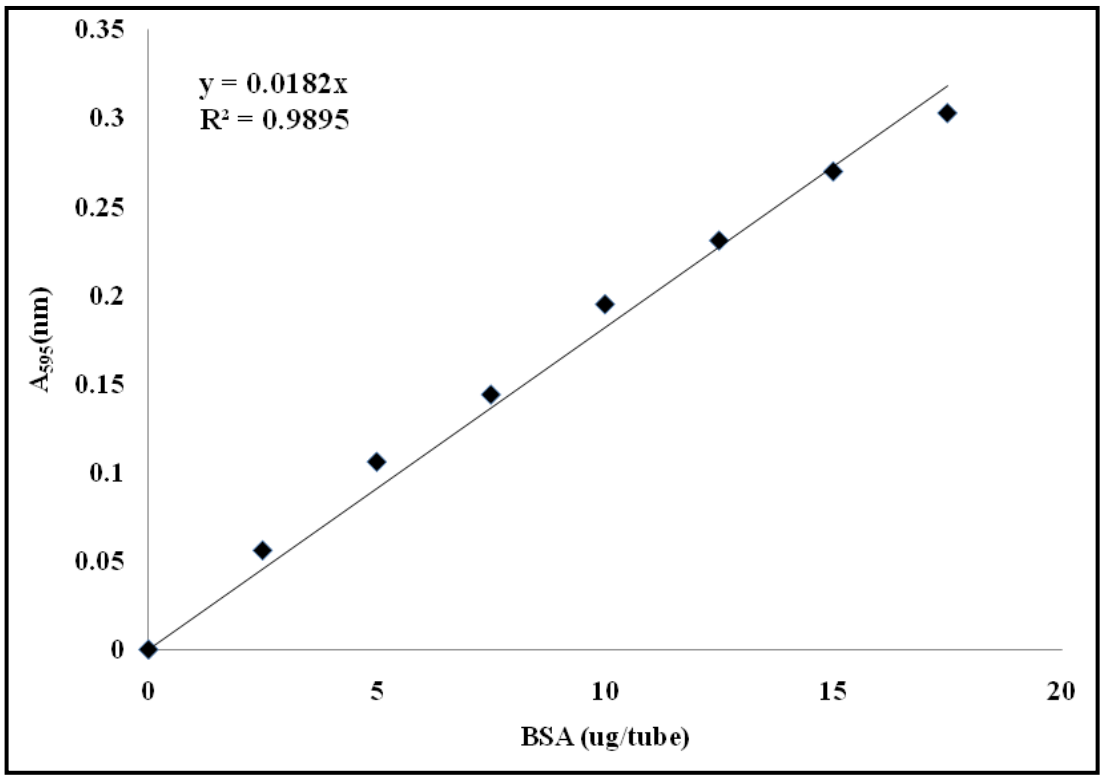
Solution A	10.0	ml
Solution B	5.0	ml
Distilled water	5.0	ml
10% Ammonium persulfate	100	µl
TEMED	10	µl

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

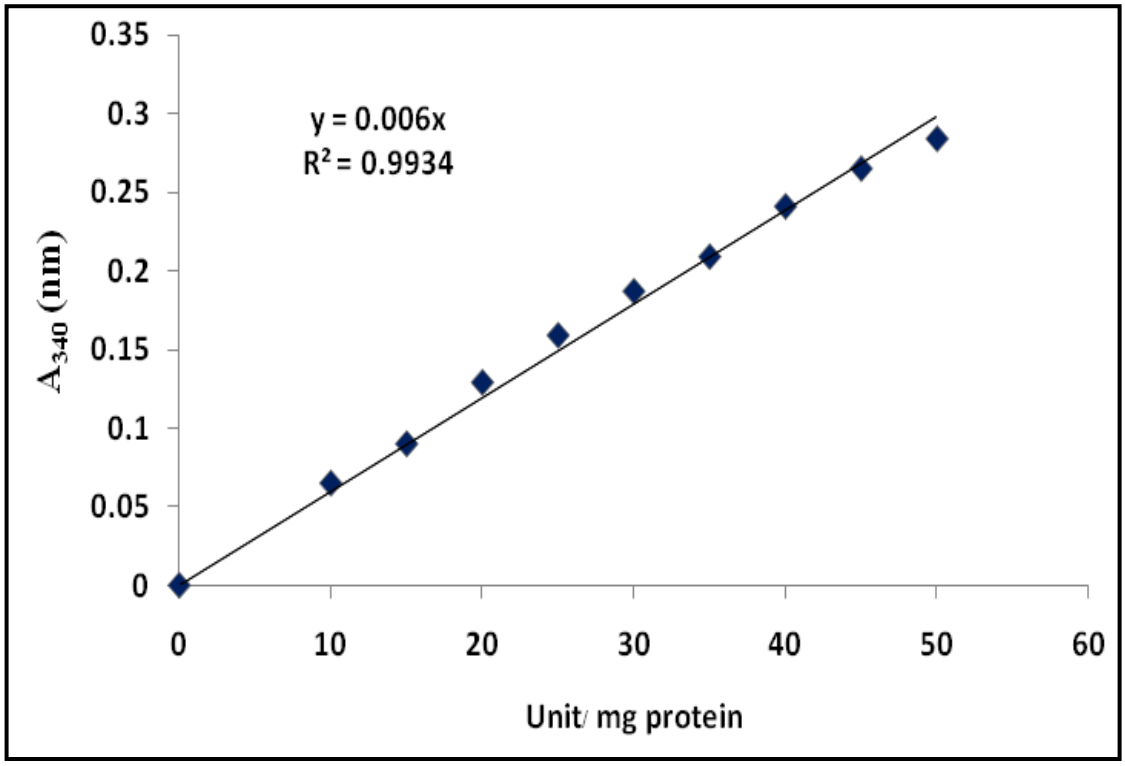
APPENDIX B

Calibration curve for protein determination by Bradford method



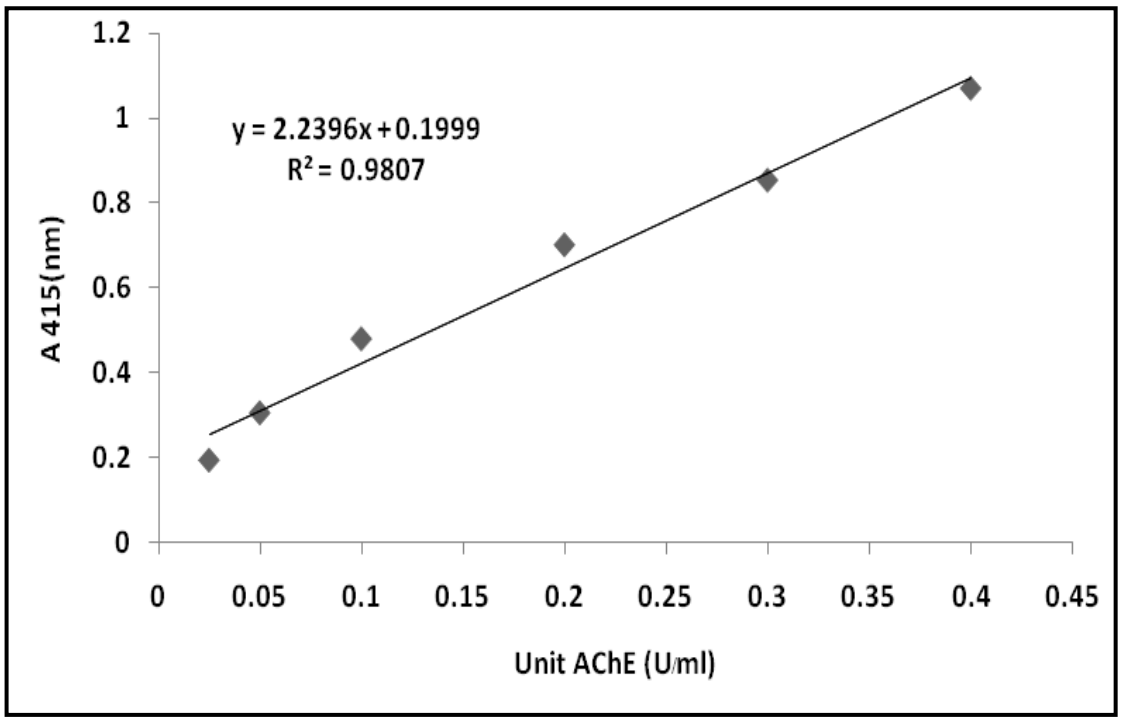
APPENDIX C

Calibration curve for trypsin standard



APPENDIX D

Calibration curve for AChE determination by Ellman's method



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

BIOGRAPHY

Miss Porlin Rungsaeng was born on September 23, 1986 in Bangkok, Thailand. She graduated with a Bachelor Degree of Science from Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2008. She had been studies for a Master Degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2008.

Academic presentation;

1) Rungsaeng, P., Sangvanich, P., and Karnchanatat, A. Screening for Acetylcholinesterase Inhibitory Activity from the Extract of the Rhizomes of Zingiberaceae Plants, Pure and Applied Chemistry International Conference (PACCON2011), 5-7 January 2011, Bangkok, Thailand.
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