Part I

Angiotensin I-Converting Enzyme Inhibitory Proteins and Peptides from the Rhizomes of Zingiberaceae Plants

Abstract

Ammonium sulphate cut protein extracts, and their pepsin hydrolysates, from the rhizomes of 15 plants in the Zingiberaceae family were screened for their *in vitro* angiotensin I- converting enzyme inhibitory (ACEI) activity. The protein extract from *Zingiber ottensi* had the highest ACEI activity (IC_{50} of 7.30×10^{-7} mg protein/mL), and was enriched for by SP-Sepharose chromatography with five NaCl step gradients (0, 0.25, 0.50, 0.75 and 1 M NaCl collecting the corresponding five fractions. The highest ACEI activity was found in the F75 fraction, which appeared to contain a single 20.7 kDa protein, suggesting enrichment to or near to homogeneity. The ACEI activity of the F75 fraction was moderately thermostable (-20-60 °C), showed >80% activity across a broad pH range of 4-12 (optimal at pH 4-5), and appeared as a competitive inhibitor of ACE (K_i of 9.1×10^{-5} mg protein/mL). For the pepsin hydrolysates, that from *Zingiber cassumunar* revealed the highest ACEI activity (IC_{50} of 0.38 ± 0.012 mg/mL), was enriched to a single active hexapeptide by RP-HPLC with a strong ACEI activity (IC_{50} of 0.011 ± 0.012 mg/mL) and acted as a. competitive inhibitor of ACE (K_i of 1.25×10^{-6} mg protein/mL).

Keywords: angiotensin I-converting enzyme; proteins; peptides; Zingiberaceae plant

Introduction

Hypertension, one of the most common worldwide diseases, is a chronic medical condition in which the resultant elevated blood pressure can damage the health. There are many associated risk factors, such as strokes, heart disease, chronic renal failure or aneurysm disease [1]. There are many predisposition factors, such as a sedentary lifestyle, stress and visceral obesity, of hypertension, which are not restricted to the aged and

elderly [2]. The angiotensin I-converting enzyme (ACE, EC.3.4.15.1) plays a key physiological role in the control of blood pressure, in the Renin-Angiotensin System (RAS) [3], which mediates control of the extracellular volume (i.e. that of the blood plasma, lymph and interstitial fluid) and arterial vasoconstriction. ACE catalyses the conversion of the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II and also degrades bradykinin, leading to the systematic dilation of the arteries and decrease in arterial blood pressure [4]. Some of the ACE inhibitor (ACEI) peptides result in a decreased formation of angiotensin II and decreased blood pressure. For this reason, many studies have been directed towards the attempted synthesis of functional ACEIs without side-effects, such as captoprill or alacepril, which are currently used in the treatment of hypertensive patients [5]. There is a strong trend towards developing natural ACE inhibitors (ACEI) for the treatment of hypertension.

Bioactive proteins and peptides have physiological properties and in recent times several studies have been done on identifying and optimizing the isolation of biopeptides from both plant and animal sources [6-8]. These peptides are generated both *in vivo* and *in vitro* from the proteolytic hydrolysis of food proteins. Peptides with a wide range of regulatory effects have been discovered, including modulation of the immune defence, increased nutrient uptake, neuro-endocrine information transfer, antihypertensive, antithrombotic, antimicrobial, antigastric and opioid activity. These peptides have been discovered in a diverse array of sources, including snake venom, spinach, whey proteins and mushrooms. However, the only legumes that have been investigated for biopeptides to the best of our knowledge are chickpeas, peas, cowpeas and soybeans; although it is assumed that since peanuts have a similar protein profile as these legumes then they will have similar biological activities too.

Zingiberaceae is a family of flowering plants consisting of aromatic perennial herbs with creeping horizontal or tuberous rhizomes [9]. The family is comprised of more than 1300 species, being found in the tropics of Africa, Asia and the Americas, and having its greatest diversity in Southeast Asia. Many species are important medical plants, spices or ornamental plants and include folklore usage for hypertension. Although new bioactive proteins and peptides are discovered and characterized year-by-year, novel bioactive protein peptides (in particular from herbal origins) are still needed because of

their attractive identities, such as in oral administration of "natural" medicines. The objective of this study was to investigate the ACEI activity of proteins and their pepsin-derived peptides from Zingiberaceae rhizomes.

Materials and Methods

Plant materials

The fresh rhizomes of 15 *Zingiberaceae* species were purchased from Chatuchak park market in Bangkok, Thailand. The samples were quickly taken to laboratory and kept in dark 4 °C room until used.

Chemical materials

ACE (E.C. 3.4.15.1) from rabbit lung, bovine serum albumin (BSA), hippuric acid, hippuryl-L-histidyl-L-leucine (HHL), and pepsin (E.C. 3.4.23.1) from porcine gastric mucosa were purchased from Sigma Chemicals Co. (USA). SP-sepharose fast flow was purchased from Amersham Biosciences, (Sweden). 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.

Preparation of the Zingiberaceae rhizomes extract

Rhizomes of Zingiberaceae plants (1.5 kg wet weight) were peeled, cut into small pieces and then homogenized in 5 L of PBS (0.15 M NaCl / 20 mM phosphate buffer, pH 7.2) using a blender (Philips, HR2061, Indonesia) at the highest blending speed (no. 6) until an apparently (visible by eye) homogenous texture was obtained. All contents were stirred using a low speed agitator (IKA Labortechnik, RW 20 DZM, Germany) with a 45° angled, 50 mm × 4 fin propeller at middle speed overnight at 4 °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 was then harvested and ammonium sulphate added, with stirring, to 80% saturation and then left with stirring overnight at 4 °C. The precipitate was collected from the suspension by centrifugation at 15,000 × g for

30 min with discarding of the supernatant. The pelleted material was then dissolved in PBS, dialyzed against three changes of 5 L deionized water and then freeze dried.

ACEI activity assay

ACEI activity was measured according to the method of [10]. The solution (50 μL) of the ammonium sulphate cut fraction of the crude proteins extract of Zingiberaceae rhizomes was mixed with 50 μL of ACE (25 mU/mL) was pre-incubated at 37 °C for 10 min, after which time the mixture was re-incubated with 150 μL of substrate (10 mM HHL in PBS) for 30 min at 37 °C. The reaction was then stopped by adding 250 μL of 1 M HCl. The hippuric acid was extracted with 500 μL of ethylacetate. After centrifugation at 15,000 × g and 4 °C for 15 min, 200 μL of the upper layer was transferred into a test tube, and evaporated in a vacuum at room temperature. The hippuric acid was dissolved in 500 μL of distilled water, and the absorbance was measured at 250 nm using an UV-spectrophotometer. A standard curve was constructed using a series of hippuric acid standards of known concentration to quantify the released hippuric acid in the assay mix. The concentration of Ace inhibitor requied to inhibit 50% of the ACE activity under the above assay conditions was defined IC_{50}.

Protein purification

A 200 mg aliquot of the ammonium sulphate cut protein powder was re-dissolved in 20 mL of deionised water and applied 5 mL at a time into a 5 mL loop of automatic liquid chromatography system (AKTA prime, Amersham Pharmacia Biotech, Sweden) with a 15 cm length glass column filled with swelled SP-Sepharose fast flow gel. The mobile phase used to equilibrate and apply the sample was 20 mM Tris-HCl, pH 7.2, whilst the column was eluted at a flow rate of 2 mL / min with the replacement of this buffer with 1 M NaCl as a stepwise gradient (change every 10 mL elutant) of 25, 50, 75 and 100% (v/v) 1 M NaCl. Ten ml fractions were collected and the protein contents of each fraction were monitored by evaluation of the absorbance at 280 nm and by a Bradford assay (see protein concentration section). 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 dialyzed at 4 °C overnight against three changes of 5 L of deionised water, and the dialysate was freeze-dried and kept at -20 °C until use.

Protein concentration

The protein content was determined by Bradford's procedure [11]. Bovine serum albumin (BSA) was used as the standard with four different concentrations between 5-20 μg/mL to construct the calibration curve. Each sample was serially two-fold diluted with deionised water and then 50 μL aliquots of each dilution were transferred into each well of a microtiter plate and 50 μL of Bradford's reagent added to each well. The plate was shaken for 5 min and then left for 10 min before reading the absorbance at 595 nm using an ELISA plate reader. The obtained OD was calculated for the protein concentration using the linear equation computed from the standard curve. During the column chromatographic separations, the elution peak profiles of proteins were determined by measuring the absorbance at 280 nm.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and size estimation

Discontinuous reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared with 12.5% and 5% (w/v) acrylamide separating and stacking gels, respectively, according to the procedure of Laemmli [12]. 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 (5 mg / lane) 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 staining.

Effect of temperature on the ACEI activity

The effect of temperature on the ACEI activity was determined by incubating the ACEI samples in 20 mM phosphate buffer (pH 7.2) at various temperatures (-20-90 °C at 10 °C intervals) for 30 min, cooling to 4 °C and then assaying the residual ACEI activity (ACEI activity assay section). The activities attained are reported as the relative activities compared to that of the control, and then assaying the residual ACE activity with 100% and 0% activity controls. In all cases, ACE was used and at least three replicates were done for each assay.

The pH-dependence of the ACEI activity

Incubating the ACEI active samples in buffers of broadly similar salinity levels but varying in pH from 2-14 was used to assess the pH stability and the pH optima of the ACEI. 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 ACEI was mixed in each of the different buffer-pH compositions, and then left for 30 min at room temperature prior to assaying for ACEI activity (ACEI activity assay section). The activities attained are reported as the relative activities compared to that of the control, and then assaying the residual ACE activity with 100% and 0% activity controls. In all cases, ACE was used and at least three replicates were done for each assay.

Protein identification

In situ (in gel) trypsinization

The sample preparation process followed the published method of Tiptara et al. [13]. Each band in the electrophoretic gel was excised, cut into small pieces (~1 mm^3), and washed with 100 mL deionized water. The gel pieces were destained by adding 200 mL 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 mL acetonitrile for 15 min prior to drying in a vacuum centrifuge. Then 50 mL of a 10mM DTT solution in 100 mM NH_4HCO_3 was added, and the proteins were reduced for 1 hr at 56 °C. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and gels were incubated for 45 min at room temperature in the dark. The solution was then removed, the gel pieces were dehydrated in acetonitrile, and the solvent was evaporated off before adding 10 mL of a trypsin solution (proteomics grade, Sigma) (10 ng/mL in 50 mM NH_4HCO_3). After allowing the gel plug to swell for 15 min at 4 °C, 30 mL of 50 mM NH_4HCO_3 was added and the digestion allowed to proceed at 37 °C overnight. The supernatant was then harvested following centrifugation at 10,000 ×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.

LC-MS/MS and peptide blasting

The likely amino acid sequence of each internal fragment of the trypsinized material was analyzed by liquid chromatography (LC)/mass spectroscopy (MS)/MS. The extracted tryptic peptides were then subjected to LC-nano electrospray ionization (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 [14].

Production and isolation of ACEI peptides

The 80% ammonium sulphate cut fraction of proteins from Zingiberaceae rhizomes were dissolved in PBS (1 mg/mL) and digested by the addition of 500 µL pepsin (500 U/mL in 0.1 M HCl) for 90 min at 37 °C. The pepsin digestion was then adjusted with KOH to pH 7.2, boiled for 15 min and then centrifuged at 15,000 × g at 4 °C for 30 min [15]. The supernatant was harvested and an aliquot screened for ACEI activity.

For those samples positive for ACEI activity, they were further fractionated by RP-HPLC on a C-18 Shimpak column (250 × 4.6 mm) using (A) 0.1% (w/v) trifluoroacetic acid (TFA) and (B) 70% (v/v) acetonitrile (CH_3CN) in water containing 0.05% (w/v) TFA at a flow rate of 0.7 mL/min and linearly changing from a A:B (v/v) ratio of 100:0 to 14.3:85.7 in 60 min. The peptides were detected at 230 nm. Individual fractions were dried using a speed vacuum, rehydrated in water and then assayed for ACEI activity.

Estimation of kinetic parameters

The rate of hydrolysis of HHL by ACE over an HHL concentration range of 0.05-0.5 mM in the presence or absence of various concentrations of the test compounds (the enriched F75 ACEI positive fraction from *Z. ottensii* rhizomes and the pepsin peptide hydrolysates from *Z. cassumunar* rhizomes) were measured. Then, the data from enzyme assays was subjected to double-reciprocal (Lineweaver-Burk) plot analysis to determine the likely inhibition mode of the enriched F75 ACEI positive fraction and peptide hydrolysates.

Statistical analysis

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

Results and discussion

Screening for ACEI in plant samples

In this study we screened the ammonium sulphate cut fractions from the rhizome extracts of 15 Zingiberaceae plant species for ACEI activity. The IC_{50} values were calculated from the regression equation obtained from evaluation of different concentrations of each test extract (Table 1). Of the 15 species screened, five were found to be positive for ACEI activity with four having a broadly similar level of moderate ACEI activity, as defined by a low IC_{50} value (Table 1). However, the fifth species, the extract from *Z. ottensii*, had a considerably higher ACEI activity with an approximately two-log order lower IC_{50} value ($7.30\times10^{-7} \pm 0.01$ mg/mL) than the other four positive samples. Therefore, although all five positive plant extracts are candidates for future studies, here the rhizome extract of the highest ACEI activity, *Z. ottensii*, was selected for further studies.

Table 1. The *in vitro* ACEI activity in the ammonium sulphate cut fractions of rhizome protein extracts from 15 Thai species from within the Zingiberaceae family[a].

Plant species	ACEI activity as IC_{50} value (μg/mL)	
	Rhizome protein[b]	Pepsin hydrolysate[c]
Alpinia galanga (Linn.) Swartz.	ND	ND
Boesenbergia pandurata Roxb.	$2.43 \times 10^{-5} \pm 0.02$	0.43 ± 0.032
Curcuma aeruginosa Roxb.	ND	ND
Curcuma amarissima Roscoe.	ND	ND
Curcuma aromatica.	$6.97 \times 10^{-5} \pm 0.01$	ND
Curcuma longa Linn.	ND	ND
Curcuma sp. (Kan-ta-ma-la)	ND	ND
Curcuma xanthorrhiza Roxb.	ND	ND
Curcuma zedoaria (Berg) Roscoe.	$7.63 \times 10^{-5} \pm 0.02$	ND
Hedychium coronarium.	ND	ND
Kaempferia galanga Linn.	ND	ND
Zingiber cassumunar	$2.10 \times 10^{-5} \pm 0.01$	0.38 ± 0.012
Zingiber officinale Roscoe.	ND	ND
Zingiber ottensii Valeton.	$7.30 \times 10^{-7} \pm 0.01$	1.01 ± 0.019
Zingiber zerumbet Smith.	ND	ND

[a]Data are shown as the mean ± 1 SEM and are derived from three replicate enrichments. [b]Rhizome protein and [c]Pepsin hydrolysate represents [b]the 80% saturation ammonium sulphate cut fraction of the rhizome protein extract and [c]the pepsin digest of this extract. ND = Not detected

Purification of ACEI with ion exchange chromatography

The ACEI activity from the ammonium sulphate cut fraction of *Z. ottensii* rhizome proteins was enriched to apparent homogeneity using SP-Sepharose column chromatography in 20 mM phosphate buffer (pH 7.2) and eluted in the same buffer with a 0, 0.25, 0.5, 0.75 and 1 M NaCl stepwise gradient. The five fractions, one per step

gradient of NaCl, containing proteins that eluted from the SP-Sepharose column were screened for ACEI activity (Figure 1). Five distinct protein peaks were isolated, one per fraction, being the F0 (unbound) fraction, and the bound proteins that were then eluted at 0.25 (F25), 0.5 (F50), 0.75 (F75) and 1.00 (F100) M NaCl. Most of the protein appeared to be unbound with decreasing amounts being bound with increasing adhesion to the column (increasing NaCl levels to elute it), except for F50 (more protein than expected under the aforementioned trend), such that the F75 and especially F100 protein peaks were very small (Figure 1). However, ACEI activities were not detected in the F50 and F100 fractions with most ACEI activity (just over 90%) being detected in the F75 fraction (Figure 1 and Table 2). Thus, the F75 ACEI fraction was selected for further characterization, but it is noted that the lower ACEI activities observed in the F0 (unbound) and F25 fractions may represent different and interesting ACEI components, albeit at lower levels, and so are for future evaluation.

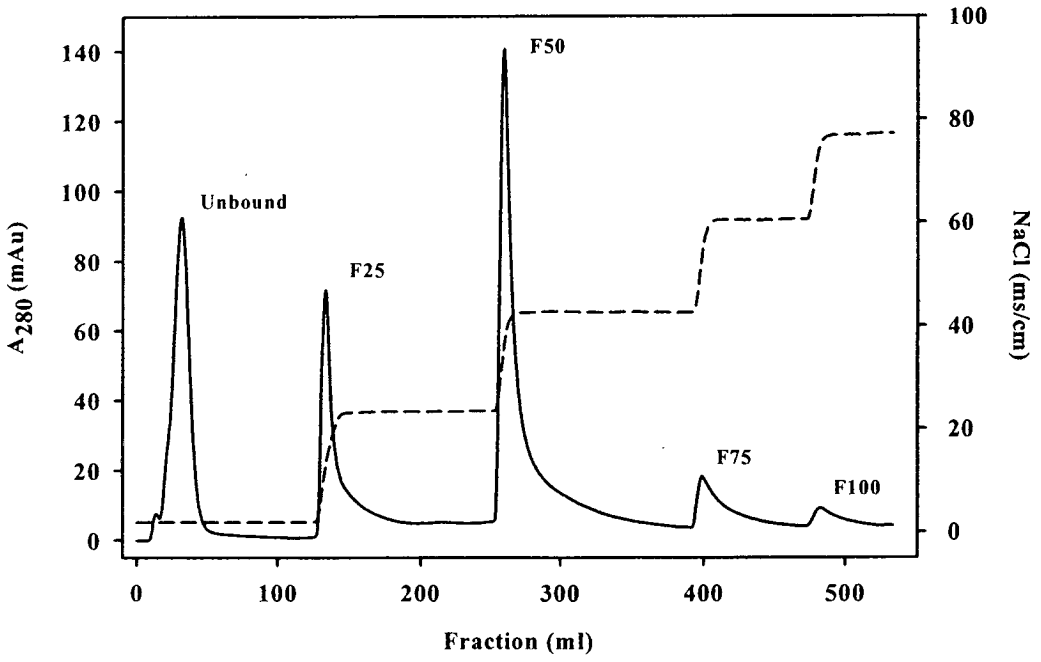


Figure 1. SP-Sepharose chromatogram of the eluted proteins from the loaded ammonium sulphate cut fraction of *Z. ottensii* rhizome proteins (50 mg) with a stepwise NaCl gradient elution (0.00, 0.25, 0.50, 0.75 and 1.00 M) with the protein peaks obtained in each NaCl gradient correspondingly labelled F0 (unbound), F25, F50, F75 and F100. Chromatograph shown is representative of 3 independent enrichments.

Table 2. The protein yield and the ACEI in each enriched fraction[a].

Fraction	ACEI activity	
	IC_{50} (μg/mL)	Maximal inhibition (%)
F0	0.0035 ± 0.02	66.7 ± 0.02
F25	0.0012 ± 0.02	79.9 ± 0.02
F50	ND	ND
F75	0.00063 ± 0.01	85.0 ± 0.03
F100	ND	ND

[a]Data are shown as the mean ± 1 SEM and are derived from three replicate enrichments
ND = Not detected

Molecular weight determination by reducing SDS-PAGE

The protein fractions with ACEI activity from each enrichment stage were analyzed for purity and protein pattern by reducing SDS-PAGE resolution (Figure 2). The implication that the enriched (post-SP-Sepharose) F75 fraction was a relatively homogenous protein preparation was supported by the presence of a single band after reducing SDS-PAGE analysis, and gave an estimated size of about 20.7 kDa (Figure 2). That an apparent high level of purity was attained by just the two steps of ammonium sulphate precipitation and step SP-Sepharose chromatography purification is of relevance since this is easier, has a lower time and purification cost, and should avoid the significant yield losses seen with multiple processing steps [16-19].

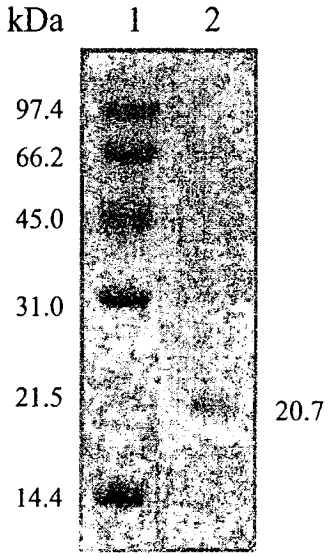


Figure 2. Reducing SDS-PAGE analysis of the enriched (post-SP-Sepharose) F75 fraction. Lane 1, molecular weight standards (10 μg); Lane 2, F75 fraction (5 μg). Gel shown is representative of 3 independent samples.

Characterization of the ACEI activity

Temperature resistance determination

The thermal stability profile of the ACEI active enriched F75 fraction from *Z. ottensii* is shown in Figure 3A. The relative activity of this ACEI was stable over a relatively wide temperature range (>90% ACEI activity at -20-60 °C at a 30 min exposure), with more than 50% and 30% relative activity being retained after 30 minute exposure at 70 and 80-90 °C, respectively. The higher temperature and longer incubation time range may have caused a change in the ACEI protein structure at regions that are involved in binding to ACE. A similar thermal stability has been observed for the proteolytic α-glucosidase inhibitor from the rhizomes of *Z. ottensii* with a high degree of stability over 0-65 °C that then decreased at higher temperatures [20].

pH resistance of the ACEI activity

The residual ACEI activity, as a relative % inhibition, as a function of the pH was largely unaffected, giving a broad pH range of activity from 3-12, with near optimal ACEI activity at pH 4-5 and 8-11. This broad pH range for the ACEI activity makes it a

potentially excellent enzyme for the food and pharmaceutical industry. Although changing the F75 protein preincubation buffer to alter the pH revealed a slight buffer-dependent affect, especially at pH 6, nevertheless the ACEI activity of fraction F75 was still slightly reduced at pH 6-8 and lost at pH 2 independent of the buffer-dependent affects (Figure 3B). That a significantly lower ACEI activity was seen in the sodium acetate than in the potassium phosphate buffer at pH 6 suggests some inhibitor-ion interactions might block or slow down the ACEI activity at such pH values. These are the potential pitfalls in all, including this ACEI activity, enzyme assays and also in potential biotechnological applications where changing buffers is difficult or expensive (except, perhaps, for immobilized enzymes).

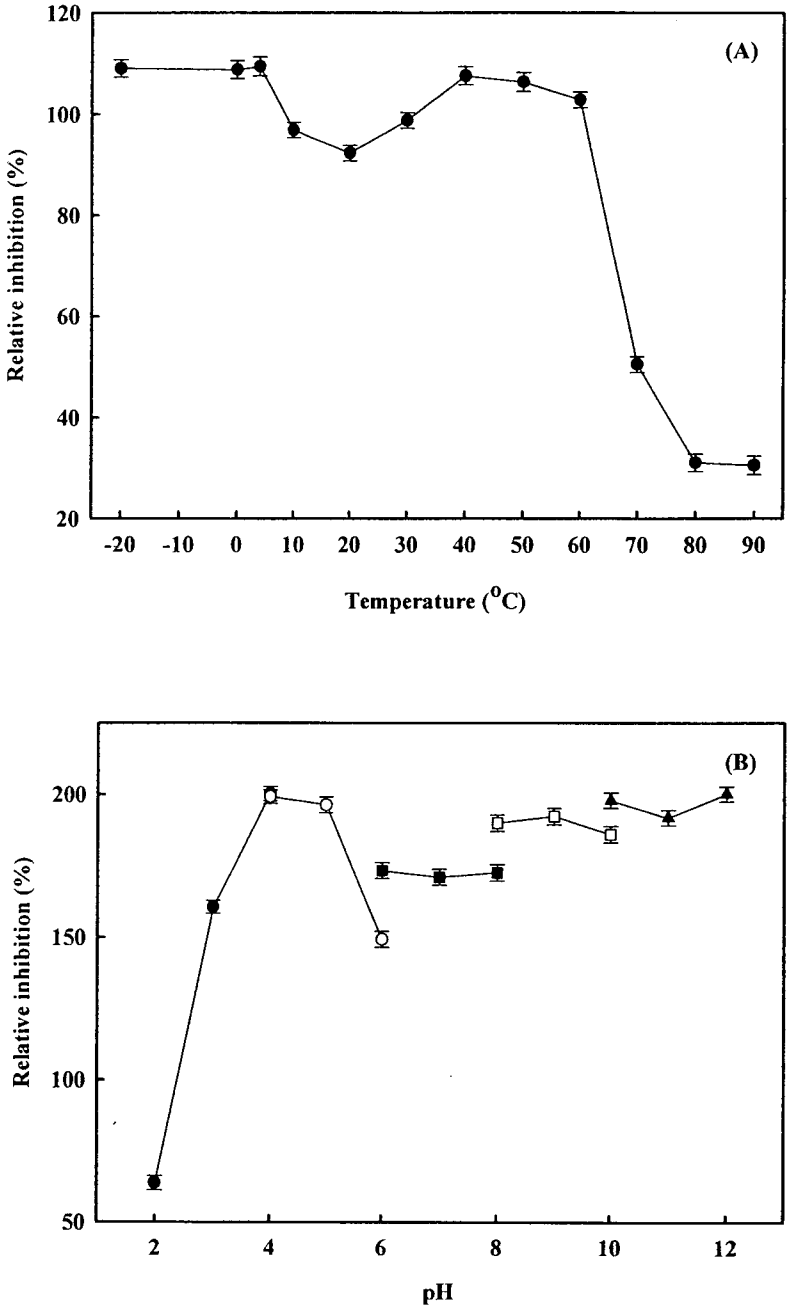


Figure 3. (A) Thermostability of the ACEI activity from the F75 enriched fraction from *Z. ottensii* rhizomes. The assay was performed in 20 mM phosphate buffer pH 7.2 at the indicated temperatures for 30 min. (B) pH stability of the ACEI activity from the F75 enriched fraction from *Z. ottensii* rhizomes. Pretreatment (at a 30 min) was with (closed circle) glycine-HCl (pH 2-4), (open circle) sodium acetate (pH 4-6), (open square) potassium phosphate (pH 6-8), (closed square) Tris-HCl (pH 8-10) and (triangle) glycine-NaOH (pH 10-12) buffers (all 20 mM). Data are shown as the mean $\pm$ 1 SEM and are derived from triplicate experiments. Means with a different lowercase latter are significantly different ($p<0.05$; Duncan's multiple means test).

Mechanism of inhibition

The potential inhibition mode of the ACEI from the *Z. ottensii* enriched F75 fraction was analyzed by double reciprocal (Lineweaver-Burk) plots (Figure 4A). From the double reciprocal (Lineweaver-Burke) plot, with HHL as the substrate, ACE as the active enzyme and F75 added to the enzyme mixture at various concentrations, the kinetics demonstrated a likely competitive inhibition mechanism, with a K_i value of 9.1×10^{-5} mg/mL. Thus, the F75 fraction inhibits the ACE enzyme by competing with the HHL substrate for the active site.

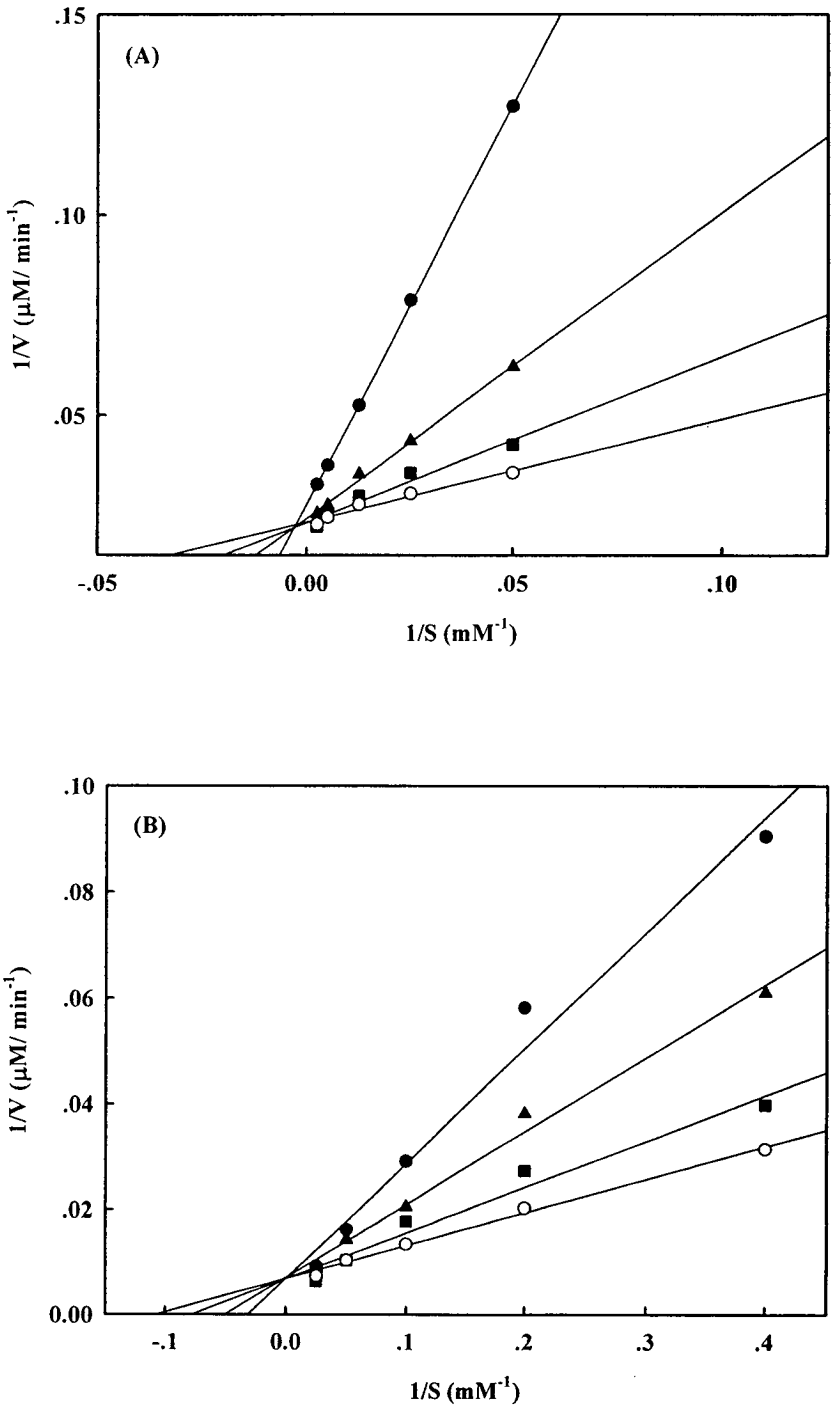
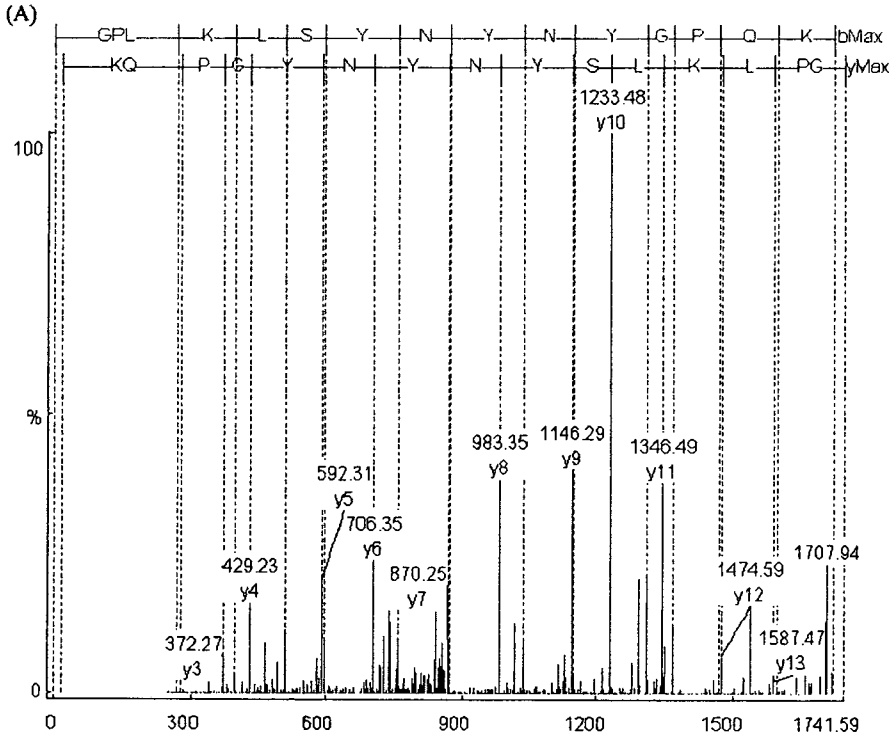


Figure 4. (A) Lineweaver-Burk plots derived from the inhibition of ACE by the ACEI from *Z. ottensii* rhizomes. ACE was treated with each stated concentration of hippuryl-L-histidyl-L-leucine (0.05-0.5 mM). (B) Lineweaver-Burk plots derived from the inhibition of ACE by the ACEI peptide from *Z. ottensii* rhizomes. ACE was treated with each stated concentration of hippuryl-L-histidyl-L-leucine (0.05-0.5 mM).

Potential ACEI protein identification

Amino acid sequences of the tryptic peptide fragments were deduced by LC-MS/MS analysis. Five sequences (GPLKLSYNYNYGPQK, GNQAVFNR, HLFQQDGELVDLNMMR, YGGYNYGAPGK, and TNAENEVTLK) were gained from software analysis (*De novo* deducing). All fragments were aligned to those homologs available in the NCBI GenBank and UniProt databases, but no single protein matched more than one of these five fragments. In BLASTp searches of the GenBank and UniProt nr database only one of the obtained peptide sequences, GPLKLSYNYNYGPQK, revealing any significant amino acid sequence similarity to the available plant sequences, having 12-13 / 15 identical amino acids to a part of a plant chitinase homolog (Figure 5). Whilst the TNAENEVTLK matched to keratin, and so could be a contamination, this still leaves the other three peptides that match to fungal, bacterial and insect proteins. Thus the identity of the ACEI component of F75, and indeed if it truly is enriched to homogeneity is equivocal and awaits further evaluation.



(B)

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

Figure 5. (A) LC/MS/MS spectra of the tryptic digest of the F75 enriched fraction for the sequence GPLKLSYNYNYGPQK. (B) Amino acid sequence alignment of the tryptic fragment of the F75 fraction from *Z. ottensii* rhizomes with some of the other chitinase sequences that showed the highest sequence identity in BLASTp searches of the NCBI and SwissProt databases. Accession codes (UniProt/GenBank) are shown.

Zingiberaceae plants protein hydrolysate containing ACEI peptides

The ammonium sulphate cut protein fraction extracted from Zingiberaceae plant rhizomes were digested with pepsin and then the ACEI activity was determined. ACEI activity was detected in 3 plant species of the pepsin protein hydrolysates from the 15 plant species (Table 1). Of these 3 positive protein hydrolysates, the highest ACEI activity (lowest IC_{50} value) was observed for *Z. cassumunar* (IC_{50} = 0.38 ± 0.012 μg/mL) Therefore, this pepsin hydrolysate was further enriched by RP-HPLC separation of the peptides on a C-18 column using the TFA/CH_3CN solvent system. The chromatographic profile (Figure 6) indicated the presence of peptide peaks in each oaf the three collected fractions (1-3), which were collected every 10 min and then assayed for ACEI activity. Fraction 3 (eluted at 30-40 min), with a single main elution peak, and perhaps three minor trace peaks, exhibited the highest ACEI activity with an IC_{50} value of 0.011 ± 0.012 μg/mL.

The ACEI peptides derived from *Z. cassumuna* were then identified by MALDI-TOF mass spectrometer. The peptide in fraction 3 which appeared as the most potent ACEI fraction, revealed a m/z of 522.23, supporting the presence of a single (dominant) peptide of 521.23 Da. The amino acid sequence of the peptide was determined by Tandem Mass spectrometry and found to be Pro-Ala-Glu-Gly-His-Ser. The amino acid sequence of this peptide supported the importance of Gly-His-Ser at the carboxyl terminal. However it remains to synthetically synthesise this hexapeptide and show it has the ACEI activity, let alone then synthesise any different carboxy terminal sequence versions to test this notion. The amino acid sequence of the peptides derived from the Rieske iron-sulphur protein from potato mitochondria were unique [21], making comparisons between them and this one difficult. Likewise, the ACEI peptide glycinin, the 11S globulin of soybean (*Glycine max*), had a different amino acid sequence of Val-Leu-Ile-Val-Pro with a MW of 577.9 Da [22].

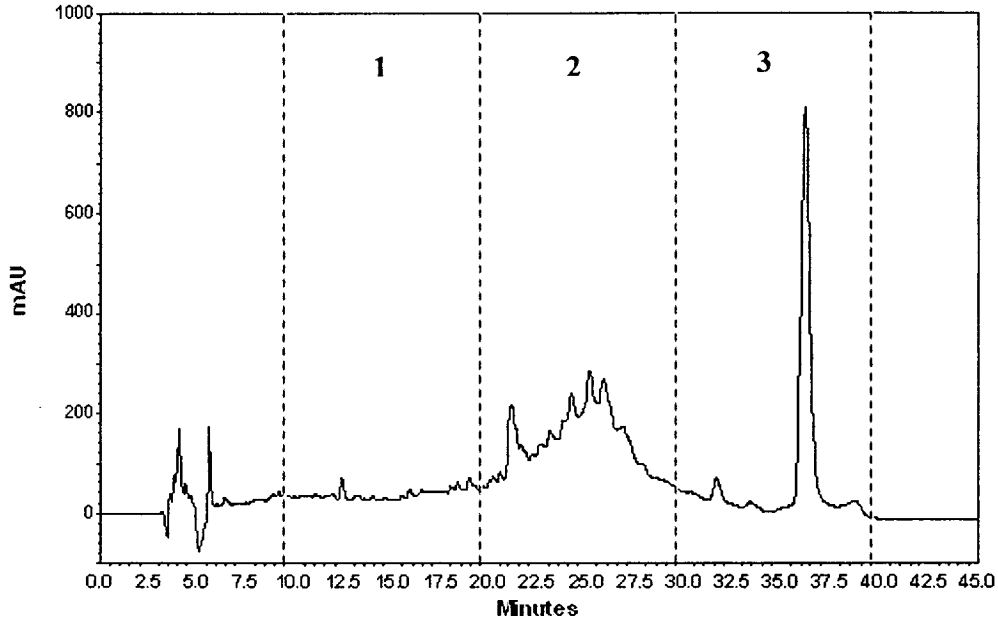


Figure 6. RP-HPLC profile of the protein hydrolysates with pepsin from *Z. cassumunar* rhizomes. Dotted vertical lines show the collected fraction boundaries (every 10 minutes) and within that the fraction number (F1-F3). Profile shown is representative of 3 independent samples.

Kinetic parameters of the ACEI F3 hexapeptide

The ACEI activity of the F3 hexapeptide (Pro-Ala-Glu-Gly-His-Ser) was studied to elucidate the mechanism of action of the peptide using hippuric acid release as a measure of the initial velocity of the ACE. Lineweaver-Burk plots were used to estimate the mode of ACEI. The effect of the fraction F3 hexapeptide inhibitor concentration on the varying substrate concentration indicated that peptide was a competitive inhibitor with a K_i value of 1.25×10^{-6} mg protein (Figure 4B).

Conclusions

In conclusion, screening of the crude ammonium sulphate cut protein extracts, and their pepsin derived peptides, from the rhizomes of plants in the Zingiberaceae family for potential ACEI activity is a potentially promising means for the isolation of

new natural ACEI proteins and peptides. Here, as an example, proteins from *Z. ottensii* showed the highest ACEI activity (IC_{50} of $7.30 \times 10^{-7} \pm 0.01$ mg/mL), whilst the pepsin derived peptides of the same extracts showed a higher ACEI activity the highest ACEI activity (IC_{50} of 0.011 ± 0.012 μg/mL) in *Z. cassumunar*. Moreover, both the protein and peptide fractions were easily enriched to separate fractions by simple procedures. For proteins, the main ACEI activity containing fraction was enriched to apparent homogeneity by just a simple discontinuous moderate cation exchange chromatography leading to three ACEI positive fractions. The main ACEI activity was ascribed to a 20.7 kDa protein, possibly a chitinase homolog, and revealed a strong ACEI activity as a competitive inhibitor of ACE (K_i was 9.1×10^{-5} M), that was active over a broad pH range and moderately thermostable. For the peptides, they were easily resolved to single peptides by RP-HPLC and revealed a strong ACEI active hexapeptide (Pro-Ala-Glu-Gly-His-Ser). This *in vitro* study represents the starting point for the discovery and evaluation of novel natural ACEI proteins or peptides and further research will be performed to evaluate the *in vivo* anti-hypertensive activities.

References

1. Guyton, A.C., and Hall, J.E. (2006) *Textbook of Medical Physiology*. 11th ed. China. Elsevier Saunders, Philadelphia.
2. Egan, B.M., Basile, J.N., and Lackland, D.T. (2004) *Hypertension: Hot Topics*. Hanley & Belfus, Inc., Philadelphia.
3. Ganten, D., Unger, T., and Lang, R.E. (1984) Arzneimittelforschung. 34, 1391-1398.
4. Kostis, J.B., and DeFelice, E.A. (1987) *Angiotensin converting enzyme inhibitors*. Alan R. Liss., Inc., New York.
5. Brown, N.J., and Vaughan, D.E. (1998) Circulation. 97, 1411-1420.
6. Pihlanto, A., and Korhonen, H. (2003) Adv. Food Nutr. Res. 47, 175-276.
7. Kitts, D.D., and Weiler, K. (2003) Curr. Pharm. Des. 9, 1309-1323.
8. Möller, N.P., Scholz-Ahrens, K.E, Roos, N., and Schrezenmeir, J. (2008) Eur. J. Nutr. 47, 171-182.
9. Larsen, K. (1980) Nat. Hist. Bull. Siam. Soc. 151-169.
10. Je, J.Y., Park, P.J., Kim, E.K., and Ahn, C.B. (2009) Food Chem. 113, 932-935.

11. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
12. Laemmli, U.K. (1970) Nature. 227, 680-685.
13. Tiptara, P., Petsom, A., Roengsumran, S., and Sangvanich, P. (2008) J. Sci. Food Agric. 88, 1025-1034.
14. Mortz, E., Vorm, O., Mann, M., and Roepstorff, P. (1994) Biol. Mass Spectrom. 23, 249-261.
15. Arihara, K., Nakashima, T., Mukai, T., Ishikawa, S., and Itoh, M. (2001) Meat Sci. 57, 319-324.
16. Demir, Y., Güngör, A.A., Duran, E.D., and Demir N. (2008) Food Tech. Biotechnol. 46, 286-291.
17. Rameshwaram, N.R., and Nadimpalli, S.K. (2008) J. Chromatogr. B. 861, 209-217.
18. Wang, S., Ng, T., Chen, T., Lin, D., Wu, J., Rao, P., and Ye, X. (2005) Biochem. Biophys. Res. Commun. 327, 820-827.
19. Ye, X.Y., and Ng, T.B. (2001) J. Protein Chem. 20, 353-359.
20. Tiengburanatam, N., Boonmee, A., Sangvanich, P., and Karnchanatat, A. (2010) App. Biochem. Biotechnol. 162, 1938-1951.
21. Emmermann, M., Clericus, M., Braun, H.P., Mozo, T., Heins, L., Kruft, V., and Schmitz, U.K. (1994) Plant Mol. Biol. 25, 271-281.
22. Mallikarjun, G.K.G., Gowda, L.R., Rao, A.G., and Prakash, V. (2006) J. Agric. Food Chem. 54, 4568-4573.

Part II

An alpha-glucosidase inhibitory activity of thermostable lectin protein from *Archidendron jiringa* Nielsen seeds

ABSTRACT

Inhibitors of α-glucosidase from natural resources that inhibit the digestion of carbohydrate polymers into monosaccharides in the gut are used in the treatment of insulin-independent diabetes mellitus type II. *Archidendron jiringa* belongs to pea family of leguminous plants, some of which are a source of interesting bioactivities, including α-glucosidase inhibitory (GI) activity. A novel GI lectin was enriched from the seeds of the Djenkol bean, *A. jiringa*, to apparent homogeneity by 90% saturation ammonium sulfate precipitation and ConA-Sepharose affinity column chromatography. This lectin had an IC_{50} value for GI activity of 0.031 ± 0.02 mg/mL, an estimated molecular mass of 35.7 kDa, of which 15.8% was carbohydrate, was thermostable up to 80 °C for 70 min, showed an optimum activity within the pH range of 8.0 - 10.0, and a high activity with some divalent cations such as Cu^{2+} and high levels (50 - 100 mM) of Zn^{2+} and Fe^{2+}. The sequence of an internal 16 amino acid fragment of the protein showed 100% identity to the mannose-glucose specific lectin precursor of *Dioclea guainensis*. The GI lectin had a high specific interaction with α-glucosidase (affinity constant = 9.3773×10^{-7} s^{-1}, K_s = 0.0241 s^{-1}, $K_a = 2.39 \times 10^3$ $s^{-1}M^{-1}$ and K_d = 0.0117 M).

Keywords: α-Glucosidase inhibitors; Lectin; *Archidendron jiringa*

INTRODUCTION

Diabetes mellitus (types 1 and 2) is recognized as a serious global health problem, often resulting in substantial morbidity and mortality. Type 2 diabetes mellitus is a group of disorders characterized by hyperglycemia and associated with microvascular (retinal, renal and possibly neuropathic), macrovascular (coronary and peripheral vascular) and neuropathic (autonomic and peripheral) complications. Unlike type 1 diabetes mellitus,

the patients are not absolutely dependent upon insulin for life, even though many of these patients ultimately are treated with insulin. The management of type 2 diabetes mellitus often demands combined regimes, including diet and/or medicines. The regulatory drugs administered in such cases include sulfonylurea and biguanide, as well as insulin. Besides the use of multiple approaches, α-glucosidase inhibitors (GIs) are one of the alternative therapeutic approaches. The inhibition of intestinal α-glucosidases delays the digestion and absorption of complex carbohydrates and consequently suppress postprandial hyperglycemia (Puls et al., 1977). Furthermore, other benefits of GIs, such as reducing triglycerides (Lebowitz, 1998) and postprandial insulin (Johnston et al., 1994) levels and anti-HIV activity (Bridges et al., 1994; Fischer et al., 1996a, 1996b), have been reported.

Herbal medicine, also called botanical medicine or phytomedicine, refers to the use of any plant's seeds, berries, roots, leaves, bark or flowers for medicinal purposes and has long been practiced outside of conventional medicine, in folklore (herbal) treatments. Such ancient remedies are becoming of more interest to conventional medicine as up-to-date analysis and research show their value in the treatment and prevention of disease. Indeed, plants have been used for medicinal purposes long before recorded history. Recently, the World Health Organization (WHO) estimated that 80% of people worldwide rely on herbs for the prophylactic or remedial treatment of at least some serious ailments. Increasing public dissatisfaction with the cost of prescription medications, combined with an interest in returning to natural or organic remedies, has led to an increase in the use of herbal medicines (Bruneton, 1995).

α-glucosidase is a very important enzyme responsible for the hydrolysis of dietary disaccharides into absorbable monosaccharide in microbial system and in small intestine of animal digestive system. Glucosidases are not only essential for carbohydrate digestion but it is also very important for processing of glycoproteins and glycolipids and are also involved in a variety of metabolic disorders and other diseases such as diabetes (Jenkins et al., 1981). Inhibition of α-glucosidase activity in animal guts decreases the blood glucose levels via delaying digestion of poly- and oligo-saccharides to the absorbable monosaccharides (McCulloch et al., 1983). Thus, GI testing is useful for screening plants that could be used for blood glucose treatment. Previous studies have revealed that the GI activity of cyanidin-3-galactoside, a natural anthocyanin, can be used in combination

with acarbose for the treatment of diabetes (Adisakwattana et al., 2009). Some GI active substances have been developed from bacterial sources to pharmaceutical applications, such as acarbose (glucobay®) from *Actinoplane* sp. 5 (Shinoda et al., 2006), voglibose (basen®) from *Streptomyces hygroscopicus* var. *limoneus* (Chen et al., 2006) and miglitol (glyset®) from *S. roseochromogenus* (Lee et al., 2002). Thus, considerable effort has been made to search for more effective and safe GIs from natural materials to develop physiologically functional foods to treat diabetes mellitus.

The treatment goal of diabetes patients is to maintain near normal levels of glycemic control, in both the fasting and post-prandial states. Many natural resources have been investigated with respect to the suppression of glucose production from dietary carbohydrates in the gut or glucose absorption from the intestine (Matsui et al., 2007). α-Amylase catalyses the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and various oligosaccharides whilst α-glucosidase further breaks down the disaccharides into simpler monosaccharides that are then readily available for intestinal absorption. The inhibition of the activity of these enzymes in the digestive tract of humans is considered to be an effective means to control this type of diabetes by diminishing the absorption rate of glucose through reducing the conversion rate of complex carbohydrates by these enzymes (Hara and Honda, 1990). Therefore, effective and nontoxic inhibitors of α-amylase and α-glucosidase have long been sought. In addition, GIs have wide applications for the treatment of other carbohydrate mediated diseases in addition to diabetes (Fujisawa et al., 1991), such as cancer (Humphries et al., 1986; Pili et al., 1995), Alzheimer disease (Kivipelto et al., 2005), and certain forms of hyperlipoproteinemia and obesity (Mahley et al., 1999). Since α-glucosidase is required for the breakdown of carbohydrates to absorbable monosaccharides at the intestine, then GIs are usually used to prevent or medically treat type II diabetes (Non-insulin-dependent diabetes mellitus (NIDDM). These inhibitors combine with the intestine alpha-glucosidase and block the uptake of postprandial blood glucose. (Holman, 1998)

Archidendron jiringa Nielsen (Fabaceae: Mimosoideae), known as the Jenkol bean or Luk Nieng tree, is a leguminous tree that is found in Indonesia, Malaysia and Thailand, and is economically important with diverse uses, including as a vegetable (young shoots) and pulse or food flavoring agent (seeds), medicine (leaves), source of

dye for silk (pods) and timber for craft work and firewood (Ong and Norzalina, 1999). Given the abundance of this commercial species, and especially the abundant seed production (1,000-4,000 seeds per tree per year), then the aim of this research was to study the GI activity of *A. jiringa* seeds in relation to their proteinaceous content.

MATERIALS AND METHODS

Biological material

The fresh seeds of *Archidendron jiringa* were purchased from the local market in Bangkok, Thailand. Thus, the exact cultivar, geographical location and season of cultivation are not known and so the effect of such variations within the species in enzyme isoforms or levels is not addressed here. Plants were identified and voucher specimens (BKF130216) deposited at Botany Section Bangkok Herbarium. The human blood was obtained from the blood donation office of The Thai Red Cross Society, Bangkok, Thailand. All other non-human animal blood was supplied from the Division of Production and Supply, National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand.

Chemicals and reagents

Concanavalin A Sepharose (ConA Sepharose) was purchased from Sigma Chemicals Co. (USA). Methyl-α-D-glucopyranoside was purchased from Fluka (Germany). The reagents used in SDS-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 biochemicals and chemicals used in the investigation were of analytical grade.

Extraction of GI from seeds of *A. jiringa*

One kilogram of *A. jiringa* seeds was homogenized in and defatted in acetone at 4 °C (200 mL aqueous acetone per 10 g seed). The insoluble material was then removed by vacuum filtration and extracted overnight at 4 °C with 20 volumes of TBS (20 mM Tris-HCl buffer, pH 7.2, plus 150 mM NaCl). The suspension was then clarified by filtration through double-layered cheesecloth followed by centrifugation at 15,000 × g for 30 min. The clarified supernatant was harvested and ammonium sulfate added, with stirring, to

90% saturation and left with stirring overnight at 4 °C. The precipitate was harvested by centrifugation at 15,000 × g for 30 min, discarding of the supernatant, and dissolved in TBS prior to being dialyzed against 3 changes of 5 L of water and then freeze-dried.

GI activity

The assay method was modified from that reported previously (Boonmee et al., 2007). GI activity was evaluated at every step of the enrichment procedure. Twenty μL of α-glucosidase (1 U/mL) in TB (20 mM Tris-HCl buffer, pH 7.2) was mixed with 10 μL of the test protein sample and 60 μL of TB and then incubated at 37 °C for 10 min before 10 μL of 1 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) in TBS as substrate was added. After incubation at 37 °C for 35 min, the reaction was stopped by the addition of 100 μL of 0.5 M Na_2CO_3. The GI activity was determined by measuring the release of the yellow *p*-nitrophenol at 400 nm, and calculated as follows;

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

Purification of GI from seeds of *A. jiringa*

ConA-Sepharose was pre-equilibrated with TBS and transferred to a 1.6 × 20 cm column. The ammonium sulphate cut fraction, resolvated in TBS, was then applied to the column (10 mL at a total protein concentration of 2 mg/mL) and run in at a flow rate of 1.5 mL/min. The column was then washed with TBS at the same flow rate, collecting 10 mL fractions, until the A_{280} fell to <0.05. The bound proteins, including lectins, were then eluted from the column, using TBS supplemented with 0.2 M methyl-α-D-glucopyranoside as the competitor, at the same flow rate and collecting 10 mL fractions. Fractions were assayed for α-glucosidase inhibitory activity (assay for α-glucosidase inhibition activity section), and those found to contain α-glucosidase inhibitory activity were pooled, dialyzed (3.5 kDa cut-off tubing) against TB and concentrated by freeze dry to 50 mg/mL ready for further analysis.

Protein concentration

The protein content was determined by Bradford's procedure (Bradford, 1976). BSA was used as the standard with four different concentrations between 5 - 20 μg/mL to construct the calibration curve. Each sample was serially two-fold diluted with deionized

water and 50 μL aliquots of each dilution were transferred into each well of a microtiter plate to which 50 μL of Bradford's reagent was added per well. The plate was shaken for 5 min and then left for 10 min before reading the absorbance at 595 nm using an ELISA plate reader. The obtained OD was calculated for the protein concentration using the linear equation computed from the BSA standard curve. During the column chromatographic separations, the elution peak profiles of proteins were determined by measuring the absorbance at 280 nm.

Carbohydrate determination

The phenol-sulfuric acid technique was slightly modified from the reported procedure (Dubois et al., 1956), by scaling up and using glucose as the standard. The enriched GI fraction was serially diluted and 500 μL aliquots of each dilution was transferred into 15 ml glass tubes, to which 500 μL of a 4% (w/v) phenol solution was added, thoroughly mixed and then left at room temperature for 5 min. Next, 4 mL of conc. H_2SO_4 was added into each tube, carefully mixed using a vortex mixer and 100 μL aliquots transferred into the well of a microtitre plate and the absorbance read at 492 nm. The obtained data was used to calculate the sugar content as glucose equivalents using the standard curve developed from five different concentrations of glucose (range 10 - 50 μg/mL) analyzed by the same procedure. Glucose (50 μg/mL) in deionized water and deionized water alone were used as the positive and negative controls, respectively, in the assay.

IC_{50} determination

The half maximal inhibition concentration (IC_{50}), as the concentration of the protein sample that inhibited 50% of the maximal α-glucosidase enzyme activity, was evaluated by using two-fold dilutions of each test protein sample and then proceeding as described in above.

Hemagglutination assays

Serial twofold dilutions of the purified lectin in TBS (50 μL) were incubated with 50 μL of rabbit erythrocyte suspension in 96-well U-shaped microtiter plates and the agglutination was scored after 1 h at room temperature. The hemagglutination unit (HU) was expressed as the reciprocal of the highest lectin dilution showing detectable visible erythrocyte agglutination, and the specific activity was calculated as HU/mg protein. The

hemagglutination activity was assayed separately, in the same manner as above, against erythrocytes from rabbits, rats, mice, guinea pigs, geese, sheep and the four human ABO blood groups.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared with 15% and 5% (w/v) acrylamide separating and stacking gels, respectively. Tris-glycine buffer pH 8.3 containing 0.1% (w/v) SDS was used as the electrode buffer. Discontinuous SDS-PAGE in reducing conditions was performed according to the procedure of Laemmli (Laemmli, 1970). Samples to be analyzed were treated with reducing sample buffer and boiled for five 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 (10 mg/lane) were co-resolved in adjacent lanes and used to determine the subunit molecular weight of the purified protein(s). After electrophoresis, proteins in the gel were visualized by staining with Coomassie Brillion blue R-250.

Effect of temperature on the GI activity and thermostable of the enriched GI fraction

The effect of temperature on the GI activity was determined by incubating the enriched GI protein fraction in TB at various temperatures (-20 - 90 °C at 10 °C intervals) for 30 min. The thermostable of the GI activity was investigated by incubating the enriched GI protein fraction sample at 70, 80 and 90 °C in TB for the indicated fixed time intervals (10-120 min), cooling to 4 °C and then assaying the residual GI activity with 100% and 0% activity controls, as described in above.

The pH-dependence of the GI activity of the enriched GI fraction

Incubating the enriched GI protein fraction samples in buffers of broadly similar salinity levels, but varying in pH from 2-14 was used to assess the pH stability and the pH optima of the GI. The buffers used were (all 20 mM) 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 enriched GI protein fraction was mixed in each of the different buffer-pH compositions, or TB as the control, and then left for 1 hour at room temperature.

Next, the samples were adjusted back to TB and assayed for GI activity. The activities attained were expressed as relative to that of the control, which was set as 100% activity.

Effect of divalent metal ions on the GI activity

The effect of preincubation of the enriched GI protein fraction with six different divalent metal cations on the resultant GI activity was evaluated as follows. The enriched GI protein fraction (1 mg/mL) was incubated for 10 h with one of Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} at one of the five different concentrations (5 - 100 mM), plus the control (0 mM divalent cation) in TB with continuous shaking and was then tested for GI activity as described in above using at least three replicates for each assay. The activities attained were expressed as relative to that of the control, which was set as 100% activity.

Mechanism of the inhibition

To evaluate the inhibition mode of the enriched GI protein against α-glucosidase, the PNPG solution at one of 0.025-0.2 mM, as the substrate, was added to the α-glucosidase (1 U/mL) in TB in the presence of 0, 0.05 and 0.075 mg/mL of the enriched GI protein fraction sample. The remaining α-glucosidase activity, and thus the GI activity, was determined as outlined in above. The inhibition type was determined by Lineweaver-Burk plots, where v is the initial velocity and [S] is the substrate concentration used.

Internal amino acid sequence of GI by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS)

Each band in the electrophoretic gel was excised, cut into small pieces (ca. 1 mm^3) and washed with 100 μL deionized water. The sample preparation process then followed the published method of Mortz (Mortz et al. 1994), with the trypsinization using 100 ng of proteomics grade trypsin (Sigma) in 40 μL of 50 mM NH_4HCO_3 at 37 °C overnight. The supernatant was then harvested following centrifugation at 15,000 × g 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 supernatant and taken to dryness.

The likely amino acid sequence of each internal fragment of the trypsinized peptide was then 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 (http://www.matrixscience.com) search of the NCBI database (http://blast.ncbi.nlm.nih.gov). The following criteria were used in the Mascot search: (i) trypsin cleavage specificity with up to three missed cleavage sites, (ii) cysteine carbamidomethyl fixed modification, (iii) methionine oxidation variable modifications, (iv) ± 0.2 Da peptide tolerance and MS/MS tolerance and (v) ESI-TRAP fragmentation scoring (Mortz et al. 1994).

Biospecific interaction determination by surface plasmon resonance (SPR)

Biospecific interactions, between the enriched GI protein from the seeds of *A. jiringa* and α-glucosidase enzyme, were evaluated on an Autolab ESPRIT system. This is a biosensor system based on the principle of SPR. The various concentrations of protein sample in TB were coupled to a certified grade 11 MUA gold plate. The unreacted groups, on the surface of the gold, were then blocked with ethanolamine. All measurements were performed in the presence of 1 U/mL α-glucosidase enzyme.

RESULTS AND DISCUSSION

Enrichment of the GI activity in the seeds of *A. jiringa*

The present report represents the first investigation of the purification of a GI from *A. jiringa* seeds. *A. jiringa* seeds were homogenated and defated to form a crude soluble extract and, after extraction in TBS followed by 90% saturation ammonium sulphate precipitation and dialysis, the GI was purified in a single step by ConA-Sepharose affinity column chromatography, yielding a single apparent GI at ~3.3% (w/w) of the total starting seed weight. The ConA-Sepharose affinity column chromatography of the ammonium sulfate cut fraction resulted in two factions, an unbound fraction that eluted with the TBS wash through, and did not show any detectable GI activity, and a bound fraction that eluted with the presence of 0.2 M methyl-α-D-glucopyranoside that had a strong GI activity with an IC_{50} of 0.031± 0.02 mg/ ml (Figure 1). Note that although the use of ConA-Sepharose affinity column chromatography assumes the GI component will be a lectin, in this case no non-lectin GI components were excluded since the unbound fraction after affinity chromatography did not show any detectable GI activity. Lectin GIs are known from kidney beans (*Phaseolus vulgaris*), with GI activity levels of 70.6% and 77.1% for the cooked and raw beans, respectively (Shi et al., 2007).

This suggests a degree of thermostable, at least in the environment of the intact bean. Moreover, protein extracts of *Sesbania grandiflora* flowers prepared from 60% and 90% saturation ammonium sulphate precipitation revealed GI activity levels of 49.6% and 82.1%, respectively (Boonmee et al., 2007).

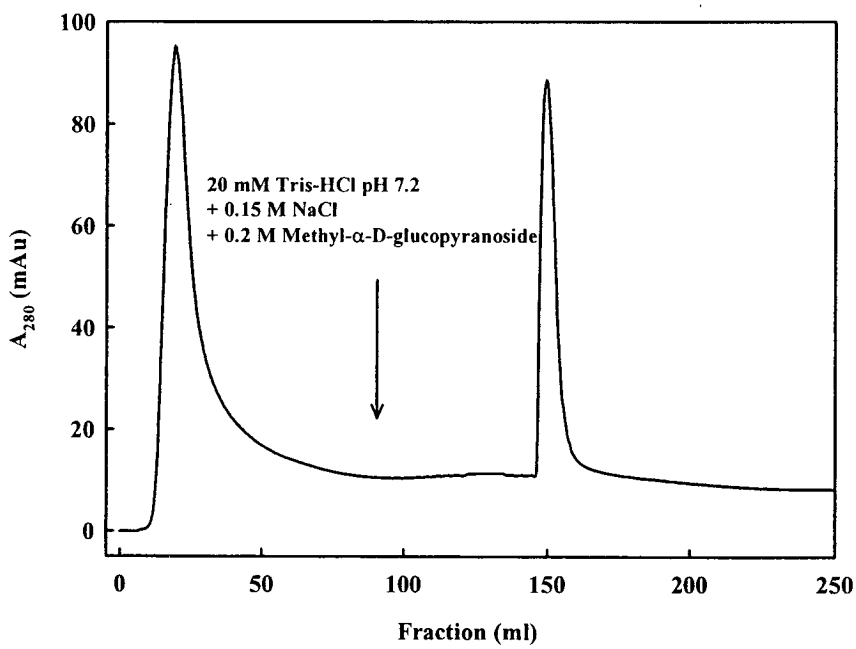


Figure 1. Affinity chromatogram showing the enrichment of GI active lectins from the ammonium sulphate cut fraction of proteins from *A. jiringa* seeds on a ConA-Sepharose column (1.6 × 20 cm) equilibrated and then washed (0-120 mL) with TBS. Lectin was then eluted with TBS containing 0.2 M methyl-α-D-glucopyranoside (120-250 ml) at a flow rate of 1.5 mL/min. The chromatogram shown is representative of three such repeats.

Affinity chromatography presents advantages in relation to other conventional methods due to its specificity and consequentially the reduced number of enrichment steps, typically giving a higher product yield and purity (Goldenberg, 1989). As such it is widely used in the purification of glycoproteins. For example, the mannose-glucose specific lectins from the tepary bean (*Phaseolus acutifolius*) and mulberry, *Morus* sp.

(Rosales: Moraceae) seeds were purified by ConA-Sepharose based affinity column chromatography (Absar et al., 2005). However, in some contrast, there have been reports that affinity chromatography could not be applied successfully for the purification of some lectins. For instance, the isolation of the lectin from *Dolichose biflorus* with *N*-acetyl-galactosamine (NAG) immobilized to Sepharose was not successful, due to the substitution of the binding site at the C-6 hydroxyl group of carbohydrate in the matrix. Rather, these lectins were resolved by affinity electrophoresis, a combination of affinity and conventional chromatography (Borrebaeck & Etzler 1980).

Modern isolation procedures generally employ affinity chromatography on an insoluble carbohydrate derivative (Goldstein & Hayes, 1978). Such insoluble, naturally occurring, or chemically modified substances, such as insoluble hog gestic musin (Etzier & Kabat, 1970), chitin (Shankar et al., 1976), arabinogalactan (Majumdar & Surolia 1978), sephadex (Wang et al., 1974).) and agarose or sepharose, have also been employed as affinity matrics for purification of interesting lectins. For example, the mannose-glucose specific lectins from the seeds of the tepary bean (*Phaseolus acutifolius*) and mulberry, *Morus* sp. (Rosales: Moraceae), seeds were purified by a ConA Sepharose based affinity chromatography column (Richard et al., 1990; Absar et al., 2005). Another example is the lectin from ground elder (*Aegopodium podagraria*) rhizomes which also could not be purified by Gal-NAG-Sepharose, but by an affinity chromatography of erythrocyte membrane protein immobilized on cross-linked agarose (Peumans et al., 1985).

Assay for Hemagglutinating Activity

A. jiringa GI showed no specificity in its ability to hemagglutinate erythrocytes from either different human blood groups (A, B, AB and O) or from rabbits, rats, mice, guinea pigs, geese and sheep. However, hemagglutinating activity against mouse and guinea pig erythrocytes were numerically the lowest, whilst that for human group O and sheep were the highest (Table 1). In this respect, it is similar to previous studies of lectin from Egyptian *Pisum sativum* seeds (Sitohy et al., 2007) and *Bauhinia monandra* (Coelho & Silva, 2000). Several lectins demonstrate a preference in agglutinating one or more types of human, or certain animal erythrocytes, such as *Sphenostyles stenocarpa* lectin that demonstrated a high agglutination of human blood type O (Machuka et al., 1999),

Hevea brasiliensis lectin that preferentially agglutinated rabbit erythrocytes (Wititsuwannakul et al., 1998), and *Talisia esculenta* lectin that demonstrated a preference for human blood type AB (Freire et al., 2002). The classical, and still the simplest way, to detect the presence of a lectin in biological material are to prepare an extract from the desired material and examine its ability to agglutinate erythrocytes. For agglutination to occur, the lectin must bind to the surface of the erythrocytes and form a cross-bridge between them. There is, however, no simple relationship between the amount of lectin bound and agglutination. This is because agglutination is affected by many factors, amongst them being the accessibility of receptor sites, and is also influenced by the external conditions of temperature, cell concentration and mixing. This difference in the agglutination activity may be due to the nature of the glycoproteins protruding on the cell surface, which are weakly or not totally recognized by the lectin.

Table 1. Hemagglutinating activity of *A. jiringa* seed GI against human and animal erythrocytes

Erythrocyte source	Agglutination (titer)[a]
Mouse	2^3
Rat	2^4
Guinea pig	2^3
Goose	2^4
Sheep	2^5
Rabbit	2^4
Human Type A	2^4
Human Type B	2^4
Human Type O	2^5
Human Type AB	2^4

[a]Titer is defined as the reciprocal of the end point dilution causing detectable agglutination of erythrocytes. The initial amount of *A. jiringa* GI used in these assays was 100 μg and was serially diluted 1:1 (v/v) for all subsequent dilutions. Data shown are the mean ± 1 S.D. and are derived from 3 repeats.

Molecular weight determination

The potential purity of the enriched GI lectin *A. jiringa* seeds α-glucosidase inhibitor proteins extract was evaluated at each step of the purification using SDS-PAGE under reducing conditions (Figure 2). The ammonium sulfate cut fraction showed many protein bands of a medium molecular weight from ~14-45 kDa, including a band at about 35.7 kDa. After the ConA-Sepharose affinity chromatography purification step this 35.7 kDa band was absent in the unbound fraction, but in the bound fraction a significant increase in the intensity of the 35.7 kDa band (estimated size) was seen, and only this band, suggesting a high degree of likely purity. The 35.7 kDa band, as in this fraction, was subsequentially found to contain 15.84% sugar by Dubois's method which is high compared to other reported GI lectins, such as that from the Chinese evergreen chinkapin lectin, *Castanopsis chinensis*, at 5.8% (Wong et al., 2008) or *Arundu donex* at 2.1% (Kaur et al., 2005). It remains plausible that during the enrichment procedures prior to and during ConA-Sepharose chromatography, residual endoglycanase activity, in conjunction with the preferential binding of the natural glycoprotein isoforms to the ConA resin, would select for purified glycoprotein of lower carbohydrate content than the real level. Conversely, we may have enriched for high carbohydrate content isoforms by the use of the ConA-Sepharose affinity chromatography.

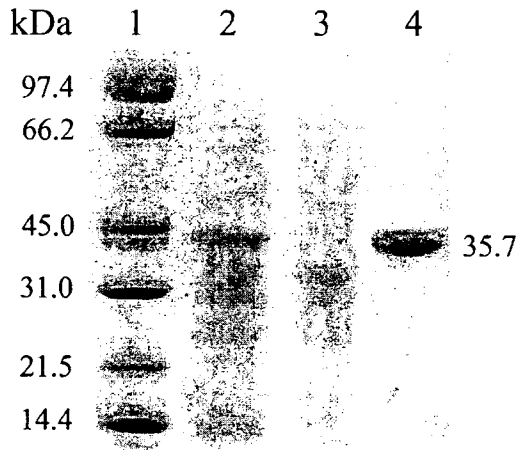


Figure 2. Reducing SDS-PAGE analysis of the enrichment of GI-active lectin from the seeds of *A. jiringa.* Lane 1, molecular weight standards (10 μg); Lane 2, 90% saturation ammonium sulphate cut fraction (10□ μg); Lane 3, the non-bound ConA-Sepharose fraction (10□ μg); Lane 4, the eluted ConA-Sepahrose-bound fraction (10□ μg) as the enriched GI lectin fraction. Gels shown are representative of 3 independent enrichments.

Effect of temperature on the GI activity and thermostable of the enriched GI lectin

No significant changes in the inhibition activity of the enriched GI fraction was seen when pretreated for 30 min within the temperature range of -20 - 80 °C, but at 90 °C the observed GI activity was essentially abrogated (Figure 3A). This is a very broad temperature range for GI activity. Previously, the mannose/glucose-specific lectin from *C. chinensis* (CCL) was reported to be stable up to 60 °C for 30 min, but above this temperature the activity declined (Wong et al., 2008). In accord with the observed high levels of GI activity at up to 80 °C, the thermal stability of this enriched GI lectin at 70 °C, 80 °C and 90 °C for up to 120 min was evaluated (Figure 3B). At 70 °C the GI activity was stable for 20 min and then declined gradually to ~30% after 120 min. At 80 °C, an initial faster rate of loss of GI activity with time was seen in the first 10 min, but then it remained stable at ~50% activity until 70 min before the declining to 30% activity after 120 min, the same level as at 70 °C. However, in some contrast, the level of GI activity at 90 °C declined rapidly to less than 20% at 20 min and was abrogated by

about 100 min (Figure 3B). The thermal stability observed for this *A. jiringa* protein is comparable to that already reported for some other thermostable proteins treated under similar conditions (Konozy et al., 2003; Oliveira et al., 2002), but the only thermophilic lectin previously isolated to date is from *Momordica charantia*, which has a maximal activity at 55 °C (Toyama et al., 2008).

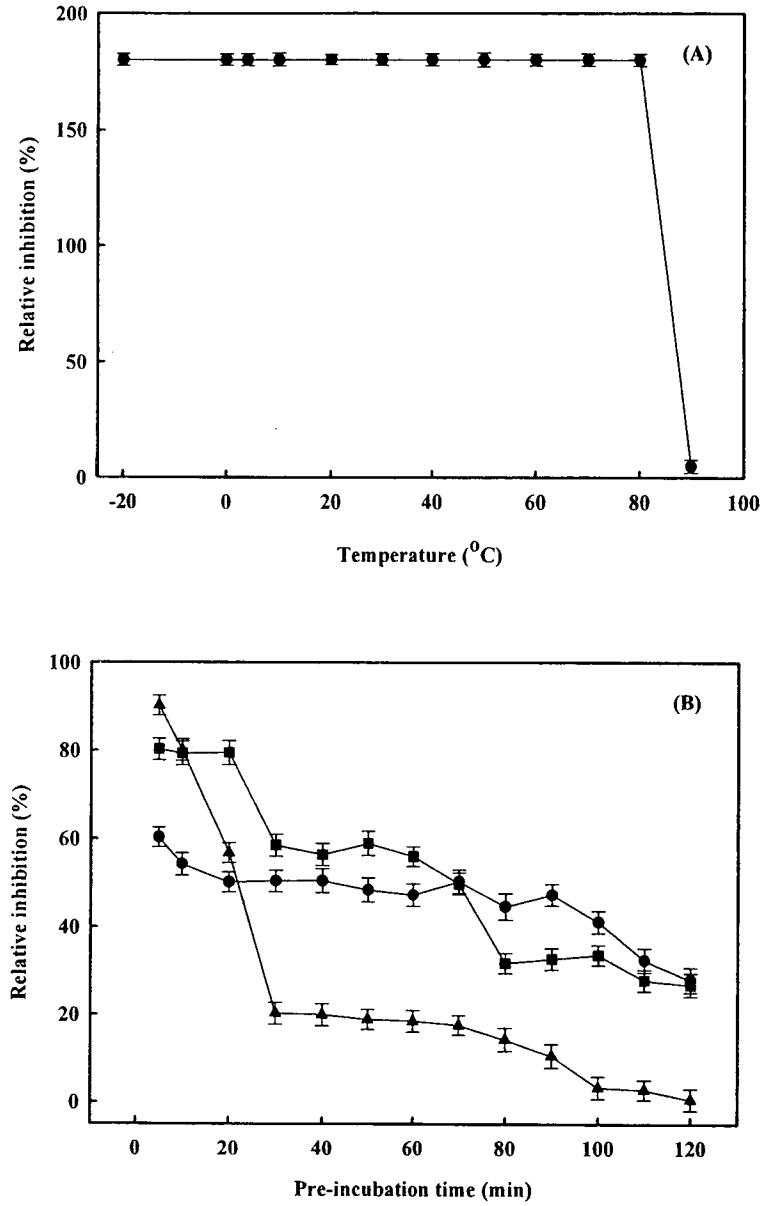


Figure 3. (A) Effect of the pretreatment temperature on the GI activity of the enriched GI lectin fraction towards α-glucosidase. (B) Thermostable with increasing pretreatment time of the enriched GI lectin fraction at (●) 70 °C, (■) 80 °C and (▲) 90 °C on the subsequent GI activity against α-glucosidase. For both panels (A) and (B), the data are shown as the mean ±1 SD and are derived from three repeats. Means with a different lower case letter are significantly different ($p<0.05$; Kruskal Wallis tests).

Effect of pH on the GI activity

The enriched GI lectin fraction displays a high GI activity at the relatively narrow alkaline pH range of 8-10, which would include the environment of the small intestine, with essentially no activity at pH 2-7, which includes include the stomach environment, and pH 11-12 (Figure 4A). The stability of the GI activity of the enriched GI lectin preparation at pH 8-10 was then evaluated over a 120 min preincubation period, where no significant difference in the broad level of GI activity with time was noted over the 120 min at pH 8 and 9, and with only a slight decline at pH 10 after 100 min (Figure 4B). Thus, this lectin GI inhibitor has high stability at pH 8-10. The high thermostable temperature range, up to 80 °C for 70 min, and pH optimum and stability or GI activity within the pH range of 8.0-10.0 (which is compatible with the site of gut alpha-glucosidase) suggests its good potential for therapeutic use in foods as well as a supplement oral pill.

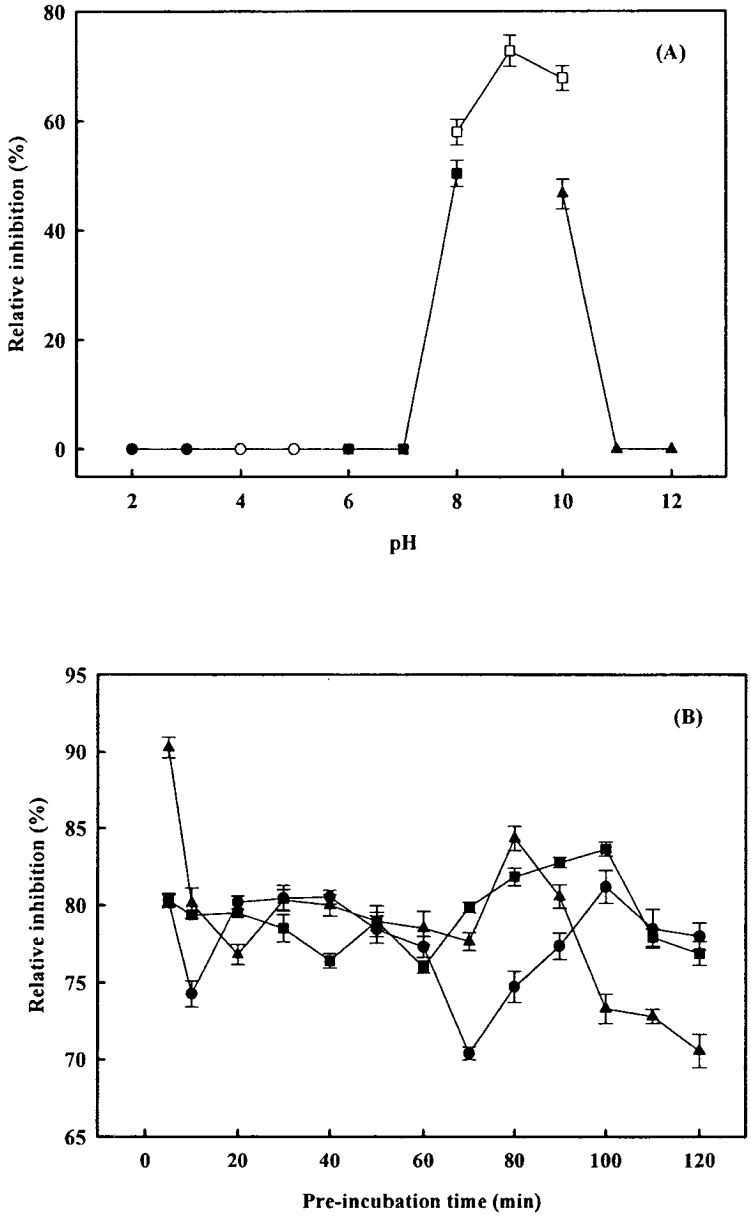


Figure 4. The effect of pH pretreatment on the GI activity of the enriched GI lectin fraction against α-glucosidase. The following buffer systems (all 20 mM) were used: (•) glycine-HCl (pH 2.0-4.0), (○) sodium acetate (pH 4.0-6.0), (■) potassium phosphate (pH 6.0-8.0), (□) Tris-HCl (pH 8.0-1 0.0) and (▲) glycine-NaOH (pH 10.0-12.0). (B) The pH stability with increasing pretreatment time of the enriched GI lectin fraction at pH (•) 8.0, (■) 9.0 and (▲) 10.0 on the subsequent GI activity against α-glucosidase. For both panels (A) and (B) the data are shown as the mean ± 1 SD and are derived from three repeats. Means with a different lower case letter are significantly different ($p<0.05$; Kruskal Wallis tests).

Effect of divalent metal ions on the GI activity of the enriched GI lectin

The effect of divalent cations on the GI activity of the enriched lectin fraction from *A. jiringa* seeds was evaluated with six different divalent metal ions. Cu^{2+} at 5-50 mM, but not at 100 mM, and Fe^{2+} at 50 and 100 mM, but not below this, were found to support and stimulate, respectively, the GI activity. Zn^{2+} offered weak support for the GI activity, increasing slightly as its concentration increased over the evaluated range (5-100 mM), whereas Mn^{2+} and Mg^{2+} offered at best only weak support for the GI activity at all concentrations tested and Ca^{2+} was unable to support any, or was inhibitory to, GI activity at 5-25 mM and weak at 50 and 100 mM (Table 2). That some divalent cations appear to be essential for the GI activity of this protein, with good and weak support from Cu^{2+} and Zn^{2+}, respectively, and Fe^{2+} being stimulatory at higher concentrations (50 and 100 mM), whilst oters maybe inhibitory could be important in terms of potential therapeutic use of this lectin as a food additative, as well as in formulating oral administration pills.

Table 2. The effect of divalent metal cations on the GI activity of the enriched GI lectin from *A. jiringa* seeds[a].

Concentration	Metal salt					
(mM)	Mg^{2+}	Mn^{2+}	Fe^{2+}	Zn^{2+}	Ca^{2+}	Cu^{2+}
5	47.8±0.028	45.0±0.020	6.8±0.013	49.9±0.053	No inhibition	99.0±0.015
10	42.4±0.005	51.4±0.701	21.8±0.002	55.5±0.115	No inhibition	98.8±0.043
25	55.5±0.750	49.1±0.051	48.1±0.007	67.6±0.034	No inhibition	98.4±0.025
50	41.5±0.040	44.6±0.045	116.3±0.146	73.1±0.004	52.0±0.005	94.8±0.003
100	45.1±0.065	56.4±0.042	137.0±0.127	74.4±0.102	55.0±0.044	56.0±0.112

[a]Data are shown as the mean ± 1 SD and are derived from 3 repeats. Means followed by a different superscript lower case letter are significantly different ($p<0.05$; Kruskal Wallis tests)

Mechanism of inhibition

The catalytic kinetic studies for α-glucosidase activity, with different substrate and enriched GI lectin fraction concentrations were initially analyzed using Lineweaver-Burk plots (Figure 5) and then Eaddie-Hoftsee plots (data not shown). Both the maximal velocity (V_{max}, y-intercept) and the Michaelis-Menten constant (K_m, slope of the trend lines) decreased with increasing concentrations of the enriched GI lectin fraction, and so this GI acted as a non-competitive inhibitor of α-glucosidase. Non-competitive inhibitors do not compete with the substrate to bind to the active region of the free enzyme, but bind to enzyme-substrate complex, resulting in an enzyme-substrate inhibitor complex. For this reason, inhibition cannot be overcome by increasing the concentration of substrate. When the concentration of the GI lectin fraction was plotted against $1/V_{max}$ (observed), a K_i value for the GI lectin of 1.887 μg/mL was obtained via non-linear regression using the least squares difference method.

The GI activity of different compounds is described in the literature (Kim et al., 2005; Shim et al., 2003; Tadera et al., 2006). α-Glucosidase was effectively inhibited by naringenin, kaempferol, luteolin, apigenin, (+)-catechin/ (-)-epicatechin, diadzein and epigallocatechin gallate (Tadera et al., 2006). These flavonoids exhibited a mixed and close to non-competitive type of inhibition on the yeast α-glucosidase. However, a combination of non-competitive and uncompetitive inhibition was observed in the study of α-glucosidase inhibition of pine bark extract against yeast *S. cerevisae* α-glucosidase (Kim et al., 2005), whilst non-competitive inhibition of α-glucosidase was reported for the *Rhus chinensis* extract, a Korean herb traditionally used in the treatment of type 2 diabetes in Korea (Shim et al., 2003).

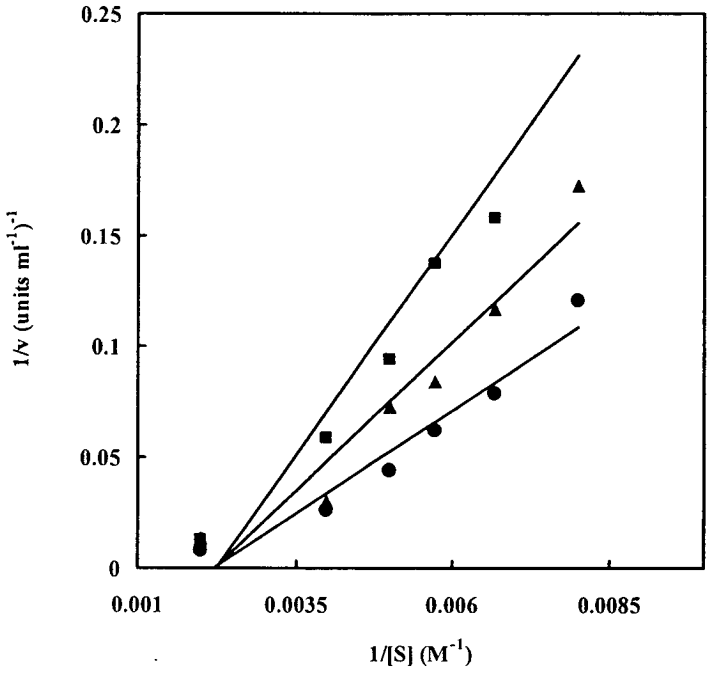


Figure 5. Lineweaver-Burk plots derived from the inhibition of α-glucosidase by the enriched GI lectin fraction from *A. jiringa* seeds. α-Glucosidase was treated with each indicated concentration of PNPG solution (one of 0.025-0.2 mM) in presence of the enriched GI lectin fraction at (●) 0, (■) 0.05 and (▲) 0.075 mg protein/mL. Data are shown as the mean ± 1 SD, derived from three repeats.

Potential identification of the GI lectin

The sequence analysis of a partial internal fragment of the enriched GI lectin from *A. jiringa* seeds, obtained by in gel digestion with trypsin and subsequent sequence analysis with LC-MS/MS, revealed a peptide fragment with the likely sequence **VSSDG SPQGS SVGR** (Figure 6A). Comparisons to all protein sequences in the SwissProt database using BLASTp searching identified this fragment as a likely homolog of the lectin precursor from the mannose-glucose specific lectin family, with 16/16 (100%) identical amino acids to the lectin precursor from the common bean, *Dioclea guianensis*, and 12-15/16 identical residues with other members of this lectin family (Figure 6B).

(A)

		1				5				10				15				Accession Number
Archidendron jiringa lectin		R	V	S	S	D	G	S	P	Q	G	S	S	V	G	R	A	
Lectin precursor (*Dioclea guianensis*)	68	R	V	S	S	D	G	S	P	Q	G	S	S	V	G	R	A	83	A9J248
Concanavalin-A precursor (Con A) (*Canavalia gladiata*, Sword bean)	69	R	V	S	S	N	G	S	P	Q	G	S	S	V	G	R	A	84	P14894
Concanavalin-A precursor (Con A) (*Canavalia ensiformis*, Jack bean)	69	R	V	S	S	N	G	S	P	Q	G	S	S	V	G	R	A	84	P02866
Lectin alpha chain (*Dioclea guianensis*)	159	-	V	S	S	S	G	D	P	Q	G	S	S	V	G	R	A	173	P81637
Lectin alpha chain (*Dioclea rostrata*)	159	-	V	S	S	S	G	D	P	Q	G	N	S	V	G	R	A	173	P58908
Lectin alpha chain (*Cratylia floribunda*)	158	R	V	S	-	N	G	S	P	Q	S	N	S	V	G	R	A	172	P81517
Mannose/glucose-specific lectin (*Cratylia mollis*, Camaratu bean)	156	R	V	S	-	N	G	S	P	Q	S	N	S	V	G	R	A	170	P83721
Mannose-specific lectin (*Chimonanthus praecox*)	101	L	Y	S	S	Q	G	S	A	I	W	S	S	K	T	W	Q	117	A2SVT1

(B)

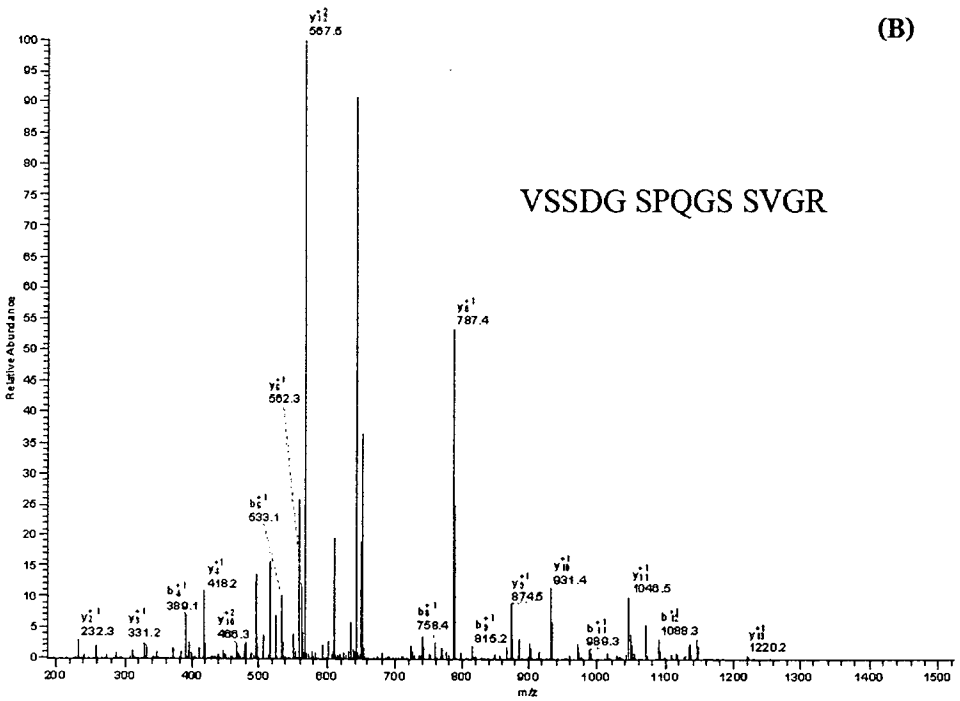


Figure 6. (A) Amino acid sequence of a 16-residue internal tryptic peptide of the enriched GI lectin from *A. jiringa* seeds. Comparisons are made with some of the other lectins from the mannose-glucose specific lectin family that showed the highest sequence homology in BLASTp searches of the NCBI and SwissProt databases. Shaded regions represent regions of identity. (B) LC/MS/MS spectra of the tryptic digest of the enriched GI lectin used to derive the data in (A) above.

Biospecific interaction of the GI lectin with α-glucosidase

Biospecific interactions, between the enriched GI lectin from the seeds of *A. jiringa* and α-glucosidase, were evaluated on an Autolab ESPRIT system. This biosensor system is based on the principle of SPR. The various concentrations of the enriched GI lectin in TB were coupled to a certified grade 11 MUA gold plate and the unreacted groups on the surface of the gold were then blocked with ethanolamine. The blank channel used phosphate buffer as a control and all measurements were analyzed by 1 U/mL α-glucosidase enzyme. The GI lectin was found to have a specific interaction with α-glucosidase with an affinity constant = 9.3773×10^{-7} s^{-1}, $K_s = 0.0241$ s^{-1}, $K_a = 2.39 \times 10^3$ $s^{-1}M^{-1}$ and $K_d = 0.0117$ M (Figure 7). The presented analytical system, based on SPR, is a valuable tool for the characterization of GIs and especially their binding-domains, which can be done by analyzing the initial binding rate and calculating the K_d value.

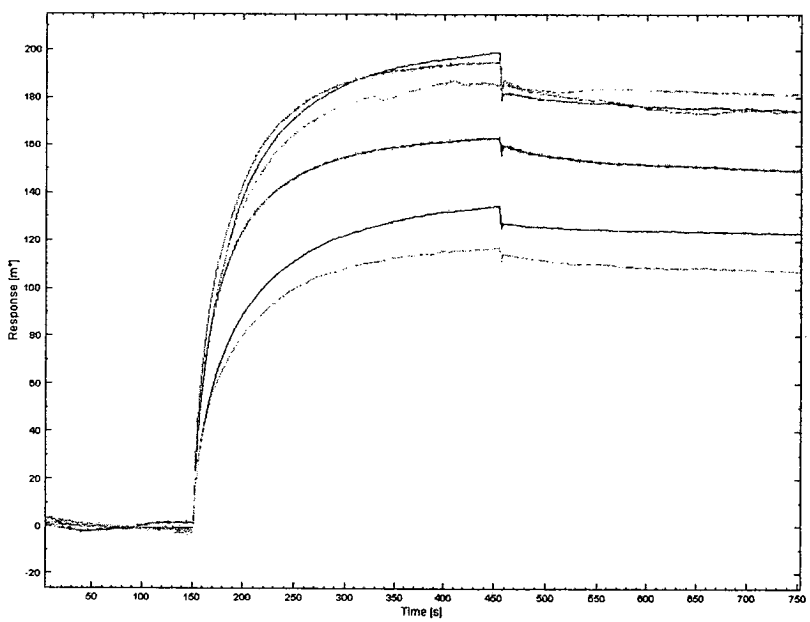


Figure 7. Representative sensorgram interactions of the enriched GI lectin sample at concentrations from 1.4-28 μg, and analyzed with the α-glucosidase enzyme as probe (1 U/mL).

CONCLUSION

The high thermostable temperature range, up to 80 °C for 70 min, and pH optimum and stability or GI activity within the pH range of 8.0-10.0 (which is compatible with the site of gut α-glucosidase) of purified lectin from *A.jiringa* seed suggests its good potential for therapeutic use in foods as well as a supplement oral pill.

REFERENCES

Absar N, Yeasmin T, Raza MS, Sarkar SK and Arisaka F (2005). Single step purification, characterization and N-terminal sequences of a mannose specific lectin from mulberry seeds. Protein J. 24: 369-377.

Adisakwattana S, Charoenlertkul P and Yibchok-Anun S (2009). alpha-Glucosidase inhibitory activity of cyanidin-3-galactoside and synergistic effect with acarbose. J. Enzyme Inhib. Med. Chem. 24: 65-69.

Borrebaeck C and Etzler ME (1980). A study of binding properties of the isolectins from the Dolichos biflorus plant using affinity electrophoresis. FEBS Lett. 117: 237-240.

Boonmee A, Reynolds CD and Sangvanich P (2007). alpha-glucosidase inhibitor proteins from *Sesbania grandiflora* flowers. Planta Med. 73:1197-1201.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Bridges CG, Brennan TM, Taylor DL, McPherson M and Tyms AS (1994). The prevention of cell adhesion and the cell-to-cell spread of HIV-1 in vitro by the a-glucosidase 1 inhibitor, 6-O-butanoyl castanospermine (MDL 28574). Antiviral Res. 25: 169-175.

Bruneton J (1995). Pharmarcognosy, phytochemistry, medicinal plants. Hatton CK, translator, Lavoisier.Publishers, Paris.

Chen X, Zheng Y and Shen Y (2006). Voglibose (Basen, AO-128), one of the most important alpha-glucosidase inhibitors. Curr. Med. Chem. 13: 109-116.

Coelho LCBB and Silva BR (2000). Simple method to purify milligram quantities of the galactose-specific lectin from the leaves of *Bauhinia monandra*. Phytochem. Anal. 11: 295-300.

Dubois M, Giles K, Hamilton JK, Robers PA and Smith F. (1956) Colorimetric method for determination of sugars and related substance. Anal. Chem. 28: 350-356.

Etzier ME and Kabat EA (1970). Purification and characterization of a lectin (plant hemagglutinin) with blood group A specificity from *Dolichos biflorus*. Biochem. 9: 869-877.

Fischer PB, Karlsson GB, Butters TD, Dwek RA and Platt FM (1996). N-butyldeoxynojirimycin-mediated inhibition of human immunodeficiency virus entry correlates with changes in antibody recognition of the V1/V2 region of gp120. J. Virol. 70: 7143-7152.

Freire MGM, Gomes VM, Corsini RE, Machado OLT, De Simone SG, Novello JC, Marangoni S and Macedo MLR. (2002). Isolation and partial characterization of a novel lectin from *Talisia esculenta* seeds that interferes with fungal growth. Plant Physiol. Biochem. 40: 61-68.

Fujisawa Y, Sasaki K and Akiyama K (1991). Increased insulin levels after OGTT load in peripheral blood and cerebrospinal fluid of patients with dementia of Alzheimer type. Biol. Psychiat. 30: 1219-1228.

Goldenberg DP (1989). Circularly permuted proteins. Protein Eng. 2: 493-495.

Goldstein IJ and Hayes CE (1978). The lectins: carbohydrate-binding proteins of plants and animals. Adv. Carbohydr. Chem. Biochem. 35: 127-340.

Hara Y and Honda M (1990). The inhibition of α-amylase by tea polypphenols. Agric. Biol. Chem. 54: 1939-1945.

Holman RR (1998). Assessing the potential for α-glucosidase inhibitors in prediabetic states. Diabetes Res. Clin. Pract. 40: s21-s25.

Humphries MJ, Matsumoto K, White SL and Olden K (1986). Inhibition of experimental metastasis by castanospermine in mice: blockage of two distinct stages of tumor colonization by oligosaccharide processing inhibitors. Cancer Res. 46: 5215-5222.

Jenkins DJ, Taylor RH, Goff DV, Fielden H, Misiewicz JJ, Sarson DL, Bloom SR and Alberti KG (1981). Scope and specificity of acarbose in slowing carbohydrates absorption in Man. Diabetes. 30: 951- 954.

Johnston PS, Coniff RF, Hoogwerf BJ, Santiago JV, Pi-Sunyer FX and Krol A (1994). Effects of the carbohydrase inhibitor miglitol in sulfonylurea-treated NIDDM patients. Diabetes Care. 17: 20-29.

Kivipelto M, Ngandu T, Fratiglioni L, Viitanen M, Kareholt I, Winblad B, Helkala EL, Tuomilehto J, Soininen H and Nissinen A (2005). Obesity and vascular risk factors at midlife and the risk of dementia and Alzheimer disease. Arch. Neurol. 62: 1556-1560.

Kaur A, Singh J, Kamboj SS, Sexana AK, Pandita RM and Shamnugavel M (2005). Isolation of an N-acetyl-D-glucosamine specific lectin from the rhizomes of *Arundo donax* with antiproliferative activity. Phytochem. 66: 1933-1940.

Kim YM, Jeong YK, Wang MH, Lee YH and Rhee HI (2005). Inhibitory effect of pine extract on alpha-glucosidase activity and postprandial hyperglycaemia. Nutrition. 21: 756-761.

Konozy EHE, Bernardes ES, Rosa C, Faca V, Greene LJ and Ward RJ (2003). Isolation, purification and physicochemical characterization of a D-galactose-binding lectin from seeds of *Erythrina speciosa*. Arch. Biochem. Biophys. 410: 222-229.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage, T4. Nature. 227: 680-685.

Lebowitz HE (1998). α-Glucosidase inhibitors as agents in the treatment of diabetes. Diabetes Rev. 6: 132-145.

Lee A, Patrick P, Wishart J, Horowitz M and Morley JE (2002). The effects of miglitol on glucagons-likepeptide-1 secretion and appetite sensatiuons in obese type 2 diabetics. Diabetes Obes. Metab. 4: 329-335.

Machuka JS, Okeola OG, Van Damme EJM, Chrispeels MJ, Van Leuven F and Peumans WJ (1999). Isolation and partial characterisation of galactose-specific lectins from African yam beans, *Sphenostyles stenocarpa* Harms. Phytochemistry 51: 721-728.

Mahley RW, Huang Y and Rall SCJr (1999). Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia): questions, quandaries, and paradoxes. J. lipid Res. 40: 1933-1949

Majumdar T and Surolia A (1978). A large scale preparation of peanut agglutinin on a new affinity matrix. Prep. Biochem. 8: 119-131.

Matsui T, Tanaka T, Tamura S, Toshima A, Tamaya K, Miyata Y, Tanaka K and Matsumoto K (2007). Alpha-glucosidase inhibitory profile of catechins and theaflavins. J. Agric. Food Chem. 55: 99-105.

McCulloch DK, Kurtz AB and Tattersall RB (1983). A new approach to the treatment of nocturnal hypoglycemia using alpha-glucosidase inhibition. Diabetes Care. 6: 483-487.

Mortz E, Vorm O, Mann M and Roepstorff P (1994). Identification of proteins in polyacrylamide gels by mass spectrometric peptide mapping combined with database search. Biol. Mass Spectrom. 23: 249-261.

Oliveira MDL,. Andrade CAS, Santos-Magalhães NS, Coelho LCBB, Teixeira JA, Carneiro-da-Cunha MG and Correia MTS (2008). Purification of a lectin from *Eugenia uniflora* L. seeds and its potential antibacterial activity. Lett. Appl.Microb. 46: 371-376.

Ong HC and Norzalina J (1999). Malay herbal medicine in Gemenceh, Negri Sembilan, Malaysia. Fitoterapia. 70: 10-14.

Peumans WJ, Nsimba-Lubaki M, Peeters B and Broekaert WF (1985). Isolation and partial characterization of a lectin from ground elder (*Aegopodium podagraria*) rhizomes. Planta. 164: 75-82.

Pili R, Chang J, Partis RA, Mueller RA, Chrest, FJ and Passaniti A 1995. The alpha-glucosidase I inhibitor castanospermine alters endothelial cell glycosylation, prevents angiogenesis, and inhibits tumor growth. Cancer Res. 55: 2920-2926.

Puls W, Keup U, Krause HP, Thomas G and Hofmeister F (1977). Glucosidase inhibition: a new approach to the treatment of diabetes, obesity, and hyperlipoproteinaemia. Naturwissenschaften. 64: 536-537.

Richard C, Narendra P, Singh K, Shade RE, Murdock LL and Bressan RA (1990). Isolation and partial characterization of a seed lectin from tepary bean that delays bruchid beetle development. Plant Physiol. 93: 1453-1459.

Shankar IPN, Wilkinson KD and Goldstein IJ (1976). An *N*-acetyl-D-glucosamine binding lectin from *Bandeiraea Simplicifolia* seeds. Arch. Biochem. Biophys. 177: 330-333.

Shi J, Xue SJ, Kakuda Y, Ilic S and Kim D (2007). Isolation and characterization of lectins from kidney beans (*Phaseolus vulgaris*). Proc. Biochem. 42: 1436-1442.

Shim YJ, Doo HK, Ahn SY, Kim YS, Seong JK and Park IS (2003). Inhibitory effect of aqueous extract from the gall of *Rhus chinensis* on alpha-glucosidase activity and postprandial blood glucose. J. Ethanopharmacol. 85: 283-287.

Shinoda Y, Inoue I, Nakado T, Seo M, Sassa M, Goto SI, Awata T, Komoda T and Katayama S (2006). Acarbose improves fibrinolytic activity in patients with impaired glucose tolerance. Metabolism. 55: 935-939.

Sitohy M., Doheim M and Badr H (2007). Isolation and characterization of a lectin with antifungal activity from Egyptian *Pisum sativum* seeds. Food Chem. 104: 971-979.

Tadera K, Minami Y, Takamatsu K and Matsuoka T (2006). Inhibition of alpha-glucosidase and alpha-amylase by flavonoids. J. Nutr. Sci.Vitaminol. 52: 149-153.

Toyama J, Tanaka H, Horie A, Uchiyama T and Akashi R (2008). Purification and characterization of Anti-H lectin from the seed of *Momordica charantia* and the inter-specific differences of hemagglutinating activity in Cucurbitaceae. Asian J. Plant Sci. 7: 647-653.

Wang JL, Becker JW, Reeke GNJ and Edelman GM (1974). A crystalline lectin from *Vicia faba*. J. Mol. Biol. 88: 259-262.

Wititsuwannakul R, Wititsuwannakul D and Sakulborirug C (1998). A lectin from the bark of the rubber tree (*Hevea brasiliensis*). Phytochemistry 47: 183-187.

Wong JH, Chan HYE and Ng TB (2008). A mannose/glucose-specific lectin from Chinese evergreen chinkapin (*Castanopsis chinensis*). Biochim. Biophys. Acta. 1780: 1017-1022.

Part III

INHIBITION OF NITRIC OXIDE PRODUCTION IN THE MACROPHAGE-LIKE RAW 264.7 CELL LINE BY PROTEIN FROM THE RHIZOMES OF ZINGIBERACEAE PLANTS

ABSTRACT

Nitric oxide (NO) plays a key role in the pathogenesis of inflammation and has been implicated in endotoxin-induced tissue injury. *Zingiberaceae* is a family of indigenous plants of tropical regions, many of which have traditionally been used as anti-inflammatory agents. Here, the ability of crude protein extracts from the rhizomes of 15 *Zingiberaceae* species to inhibit NO production in the RAW 264.7 cell line after costimulation with lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) was evaluated. The crude protein extract of *Zingiber ottensii* Valeton exhibited the highest inhibitory activity, with an IC_{50} value of 38.6 ± 0.34 μg protein/mL, and also suppressed the LPS and rmIFN-γ mediated increase in the iNOS, IL-6 and TNF-α mRNA transcript expression levels, suggesting the interference was mediated at the transcriptional level. This strong anti-inflammatory activity may have the potential to be developed as a therapeutic compound. Analytical SDS-PAGE and mass spectrometry revealed four main protein bands, including a likely lectin, superoxide dismutase and cysteine protease in the fractions related to the antioxidant activity.

Key words: Nitric Oxide, Macrophage RAW 264.7, Zingiberaceae Plants

INTRODUCTION

Nitric oxide (NO) is a molecule that plays a key role in the pathogenesis of inflammation and is an effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues.[1] NO is also an important component of the antineoplastic and antimicrobial armament of macrophages.[2] This highly labile and noxious gas is produced in large and sustained quantities by

macrophages following exposure to a variety of immunological and inflammatory mediators.[3] In mammals, NO is synthesized by the oxidation of L-arginine, a reaction that is catalyzed by inducible nitric oxide synthase (iNOS) in phagocytes, such as macrophages. NO, as well as its peroxynitrite product ($ONOO^-$) generated as O_2^- produced by phagocytes reacts with nitric oxide, induces apoptosis in neighboring cells. Macrophages are induced to synthesize and release NO by stimulation with lipopolysaccharide (LPS) and / or interferon-gamma (IFN-γ). LPS is the major component of the cell wall of Gram-negative bacteria that functions in protecting the bacteria from chemical attack. It is a prototypical endotoxin that induces innate immune responses in animals.[4-7] In addition, reactive oxygen species (ROS) propagate inflammation by stimulating release of these mediators, which stimulate recruitment of additional macrophages at the inflammatory site. It is believed that persistent inflammatory cells recruitment, repeated generation of ROS and pro-inflammatory mediators, and continued proliferation of genomically unstable cells contribute to neoplasic transformation and ultimately result in tumor invasion and metastasis.[8]

The family Zingiberaceae is a world-wide distributed family that is comprised of more than 1500 species, distributed throughout tropical Africa, Asia and the Americas. It is now estimated that Thailand has about 25 genera and some 250 species.[9] Zingiberaceae is well known for its medicinal and economic significance, furnishing as it does a number of spices and condiments. The various plant tissues of ginger are rich in volatile aromatic compounds, with the majority of the essential oils being extracted from the leaves, stems and rhizomes. Currently, other species of Zingiberaceae have become the subjects of extensive research into the various antioxidant compounds present in their leaves and rhizomes, based upon their previous ethnomedical use in order to validate and expand upon the native knowledge about folk medicines and to search for new potential candidates for modern drug investigations and development. [10-14]

Proteins play many essential roles in cells but also can act as biological mediators between cells or species. As antimicrobial agents, these natural products may exert their effect on bacterial cell membranes and/or specific protein targets, [15-17] and various proteins have been developed as therapeutic drugs due to their specific targeting, high efficacy and low side effects such as Mannose-binding proteins derived from

Hippeastrum hybrid and *Galanthus nivalis* which can inhibit human immunodeficiency virus (HIV) replication and was selected for drug-resistant viruses. [18] In this study, the anti-inflammatory properties of the crude protein extracts from rhizomes were evaluated from fifteen *Zingiberaceae* plant species in terms of modulation of the response of the macrophage-like RAW 264.7 cell line after costimulation with LPS and recombinant murine (rm)IFN-γ as an *in vitro* model of inhibition of inflammation.

MATERIALS AND METHODS

Materials

The fresh rhizomes from plant species in the Zingiberaceae family *Alpinia galanga* (UBUPH00015), *Boesenbergia pandurata* Roxb. (BKF73995), *Curcuma aeruginosa* Roxb. (BKF301), *Curcuma amarissima* Roscoe , *Curcuma aromatica* (BK60395), *Curcuma longa* L. (BKF90655), Curcuma sp. (kun-ta-ma-la), *Curcuma comosa* (BKF97298), *Curcuma zedoaria* Rosc. (UBUPH486), *Hedychium coronarium* Roem. (BKF2008026), *Kaempferia galanga* L. (BK59948), *Zingiber cassumunar* (BK63895), *Zingiber officinale* Rosc. (BKF 118527), *Zingiber ottensii* Valeton. (BKF60689) and *Zingiber zerumbet* L. (BKF67428) were periodically (April 2010 - May 2010) purchased from Chatuchak park market in Bangkok, Thailand. The RAW 264.7 (ATCC TIB-71) murine macrophage-like cell line was obtained from the American Type Culture Collection (USA). 1,1-diphenyl-2-picrylhydrazyl (DPPH), DMEM medium, fetal bovine serum (FBS), penicillin-G, streptomycin sulphate, HEPES, sodium pyruvate, LPS (from *Escherichia coli*), rmIFN-γ, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), N-(1-Naphthyl)ethylene-diamine dihydrocholide (NED) were purchased from Sigma Chemicals Co. (USA). All other biochemicals and chemicals used in the investigation were of analytical grade.

Preparation of the crude protein from Zingiberaceae rhizomes

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 °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 °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 three changes of 5 L of water at 4 °C and then freeze dried. This is referred to as the "ammonium sulphate cut fraction".

Cell culture

The RAW 264.7 cell line maintained in complete medium (CM) (DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin-G, 0.4 mg/ml streptomycin sulphate, 1% (w/v) sodium pyruvate and 1% (w/v) HEPES) at 37 °C in a humidified atmosphere with 5% (v/v) CO_2. For routine maintenance in culture (passage), cells were seeded in non-tissue culture treated dishes at approximately 10% confluency and grown to approximately 80% confluency, which typically took two days. The old medium was aspirated, the cells were gently rinsed with PBS and then dislodged by gently scraping with a rubber policeman and then harvested by centrifugation at 15,000 × g for 5 min. After that, the PBS was removed, the cell pellet was resuspended in CM and the viable and total cell density evaluated by using Trypan blue dye exclusion on an improved Neubauer hemocytometer. The following equation was then used to calculate the viable cell numbers.

Total cell count (cells/mL) = the number of cell counted in 16-large squares × 2 × 10^4

For storage, cells were harvested and resuspended in cold freezing media (CM plus 10% (v/v) DMSO) at 2 × 10^6 cells/ ml, aliquoted at 1 mL per cryogenic tubes and transferred to -80 °C immediately overnight and then into liquid nitrogen. To initiate cultures from frozen stocks the cells were thawed at 37 °C, transferred into 10 mL of serum free DMEM media and centrifuged at 15,000 × g for 5 min. The cell pellet was resuspended in CM, plated as cultured as above except that after the cells had adhered (approximately 6 h), the old medium was replaced.

Pretreatment of RAW 264.7 cells

RAW 264.7 cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 100 µL CM and were incubated overnight at 37 °C in 5% (v/v) CO_2. After that the medium was replaced with a fresh CM supplemented with solvent alone (negative control), the crude protein extract at the desired concentration or parthenolide at 2.5 µg/mL (positive control) and was incubated for 1 h. Then NO production was stimulated by the addition of 100 ng/ml LPS and 10 ng/mL rmIFN-γ, and incubated for 18 - 24 h.

Determination of nitric oxide (NO) production from RAW 264.7 cells

NO production was determined indirectly by measuring the nitrite concentration in the cell culture supernatants. Sodium nitrite was used as the standard at concentrations ranging from 0 - 100 µM. To 50 µL of the culture supernatant was added 50 µL of sulfanilamide in 96 - well plate and then incubated at room temperature for 10 min in the dark. Then 50 µL of NED solution was added and incubated for a further 10 min at room temperature in the dark (Griess reagent). The absorbance at 540 nm was measured using a microplate reader. To eliminate the interaction between the sample and the Greiss reagent, the NO concentration in the culture medium without the cells was also measured, and this value was subtracted from that obtained with the cells. The concentration that inhibited the LPS-stimulated NO production by 50% (50% inhibitory concentration: IC50) was determined from the dose-response curve. [19-21]

MTT assay for the measuring of cell proliferation

RAW 264.7 cell proliferation was measured using the MTT assay. Cells were plated at density of 1×10^4 / well in each well of a 96 - well plate and then 100 µL of a 5 mg/ml MTT solution (in PBS) was added per well. After incubating at 37 °C in 5% (v/v) CO_2 for 4 h, 100 µL of isopropanol containing 0.04 N HCl was added and mixed thoroughly to lyse all the cells and dissolve the purple formazan crystals. The absorbance at 540 nm was measured using a microplate reader.

$$\% \text{ viability} = \frac{(\text{O.D. test average}) - (\text{O.D. blank average})}{(\text{O.D. control average}) - (\text{O.D. blank average})} \times 100$$

Total RNA isolation from RAW 264.7 cells

RAW 264.7 cells (4×10^5 cell/well) were seeded in a 6-well culture plate and allowed to adhere overnight at 37 °C and 5% (v/v) CO_2. After that, cells were treated with solvent (negative control), crude protein at the desired concentrations or 2.5 μg/mL parthenolide (positive control) for 1 h.[22] Then, cells were stimulated with 100 ng/mL LPS and 10 ng/mL rmIFN-γ. After 6 h incubation, to harvest the total RNA, 1 mL of TRI®zol reagent was added to each 96 - well culture plate and incubated at room temperature for 5 min. This was followed by extraction with 200 μL of chloroform with mixing for 15 sec and incubating at room temperature for 2 - 3 min. Phase separation was completed by centrifugation at 12,000 × g at 2 - 8 °C for 15 min and the upper aqueous layer harvested, from which the total RNA was precipitated by the addition of 500 μL of isopropanol, mixing and leaving at room temperature for 10 min. The total RNA was then harvested by centrifugation as above, washed with 1 mL of 75% (v/v) ethanol, air dried and redissolved in 20 μL of RNase free water at 55 - 60 °C for 10 min before being stored at -80 °C until use. The amount of RNA in the sample was evaluated using the Qubit® fluorometer and Quant-iT™ RNA Assay Kit (Invitrogen), according to the manufacturer's recommendation.

Detection iNOS, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) mRNA by reverse transcription (RT-PCR)

Each 0.2 μg aliquot of the total RNA from each experimental condition was subjected to reverse transcription in a final reaction volume of 20 μL comprised of 0.2 μg total RNA, 10 nM random hexamer primer, 1 × RT buffer, 250 μM of each dNTP, 0.5 U/mL RNase inhibitor and 10 U reverse transcriptase. The reverse transcription was performed at 25 °C for 10 min, 42 °C for 60 min and 70 °C for 10 min. The resulting cDNA was then used as the template for subsequent PCR amplification of iNOS, IL-6, TNF-α and β-actin gene fragments. β-actin, assumed to be constitutively expressed, was used as the internal standard. The oligonucleotide primers used for the amplification of each gene fragment are listed in Table 1. PCR was performed in a total reaction volume of 25 μL and consisted of 2 μL of the cDNA as template, 1 × PCR reagent mix (Takara, Japan) and 200 μM of each primer. Amplification was performed for 30 cycles, except

for β-actin which was amplified for 25 cycles, using a Takara PCR Thermal Cycler Dice TP600 (Takara, Japan) at 98 °C for 30 s, 60 °C for 30 s, and 74 °C for 1 min. The 580 base pairs (bp) of iNOS, 475 bp of IL-6, 239 bp of TNF-α and 514 bp of β-actin DNA fragments obtained were separated through a 1.5% (w/v) agarose - TBE gel electrophoresis. The bands of DNA were detected by ethidium bromide staining for 10 min and were observed under UV transillumination using gel documentation system (Bio-Rad; Hercules, CA).

TABLE 1 Oligonucleotide primers used in experiments

Primer	GenBank Accession Number	Sequence (5' to 3')	Reference
β-actin forward	NM_001101	ACCAACTGGGACGACATGGAGAA	Palaga et al. 2008[50]
β-actin reverse	NM_001101	GTGGTGGTGAAGCTGTAGCC	Palaga et al. 2008[50]
iNOS forward	NM_010927	CCCTTCCGAAGTTTCTGGCAGCAGC	Barnholt et al. 2009[51]
iNOS reverse	NM_010927	GGCTGTCAGAGCCTCGTGGTCTTGG	Barnholt et al. 2009[51]
IL-6 forward	NM_010548	CATGTTCTCTGGGAAATCGTGG	Palaga et al. 2008[50]
IL-6 reverse	NM_010548	AACGCACTAGGTTTGCCGAGTA	Palaga et al. 2008[50]
TNF-α forward	NM_013693	CCTGTAGCCCACGTCGTAGC	Barnholt et al. 2009[51]
TNF-α reverse	NM_013693	TTGACCTCAGCGCTGAGTTG	Barnholt et al. 2009[51]

Measurement of free radical scavenging capacity using DPPH assay

The measurement of the free radical scavenging capacity was carried out using the DDH assay according to the method of Sharma and Bhat (2009)[23] with slight modifications. Briefly, the crude protein extract and vitamin C (positive control) were pipetted into each tube and then covered with aluminum foil. The appropriate concentration of the test sample (200, 100, 50, 25, 20, 10, 5 or 0 μg/mL) was added followed by 800 μL of a 200 μM DPPH solution in absolute ethanol. The mixture was shaken vigorously and incubated at 37 °C for 30 min. Then, 200 μL of the mixture was added per well of a 96 - well plate. The absorbance was measured at 517 nm using a microplate reader. The percentage of DPPH radical scavenging activity was calculated as follows;

$$\% \text{ DPPH radical activity} = [(A_{DPPH}-A_{sample})/A_{DPPH}] \times 100$$

Total amino acid analysis

Acid hydrolysis

Five milliliters of HCl 6N was added (5 mg protein/mL HCl) and mixed. The tube was flushed with nitrogen for 1 min to remove air. Hydrolysis was then carried out at 110 °C for 22 h. The internal standard (10 mL of 2.5 mM L-α-amino-*n*-butyric acid in HCl 0.1 M) was added and diluted with water to 250 ml. The solution was filtered with 0.20 μm filter and was then derivatized with 6 aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Flour reagent). It was then heated in a heating block at 55 °C, for 10 min. Heating converts a minor side product of tyrosine to a major mono-derivatized compound. Total amino acid content was determined by high performance liquid chromatography.

Chromatographic conditions

Chromatographic separation was carried out in a Waters Alliance 2695 with heater amino acid analysis Hypersil Gold column C_{18}. The column was thermostatted at 35 ± 1 °C and the flow rate was 1.0 ml/min. The injection volume was 5 μL. Mobile phase A consisted of sodium acetate buffer pH 4.90 and 60% acetonitrile.

Protein content determination

Protein contents were determined by the Bradford assay,[24] using 5, 10, 15 and 20 μg/mL of bovine serum albumin (BSA) as the standard to construct the calibration curve. For each serial two-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 (100 mL contains: 10 mg Coomassie Brilliant Blue G-250 and 10 mL of 85% (v/v) phosphoric acid, dissolved in 95% (v/v) ethanol) was added to each well. The plate was shaken (Biosan, OS-10, Latvia) for 5 min and then left for 10 min before reading the absorbance at 595 nm using an ELISA plate reader (Biotek Synergy HT, Biotek instrument, USA). The obtained OD was converted to the protein concentration using the linear equation computed from the standard curve.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS - PAGE)

The SDS - PAGE was prepared with 0.1% (w/v) SDS in 15% and 5% (w/v) acrylamide separating and stacking gels, respectively. Tris - glycine buffer pH 8.3 containing 0.1% (w/v) SDS was used as the electrode buffer. Discontinuous SDS -

PAGE in reducing conditions was performed according to the procedure of Laemmli (1970),[25] and 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 adjacent lanes and used to determine the subunit molecular weight of the purified protein(s). After electrophoresis, proteins in the gel were visualized by staining with Coomassie Brilliant Blue R-250 in 20% (v/v) ethanol, 20% (v/v) acetic acid in deionized water overnight, and then destained in several changes of the same solution without the dye until the blue color was absent from the non-lane part of the gel. Relative molecular weights were achieved by comparison of their Rf values with a graph of the Rf values of the standards against their weights (kDa).

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

The sample preparation process followed the method of Mortz et al. (1994).[26] Each single band from the SDS-PAGE resolution of the crude protein 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 °C. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and gels were incubated for 45 min at room temperature in the dark. The solution was then removed, the gel pieces were dehydrated in acetonitrile and the solvent evaporated off before adding 10 µL of a trypsin solution (1:250 U / mg) proteomics grade, Sigma) (10 ng / µL in 50 mM NH_4HCO_3). After allowing the gel plug to swell for 15 min at 4 °C, 30 µl of 50 mM NH_4HCO_3 was added and the digestion allowed to proceed at 37 °C overnight. The supernatant was then harvested following centrifugation at 10,000 × g 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 by Nitrogen gas.

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.[26]

Statistical analysis

All determinations, except for NO activity, were done in triplicate, and the results are reported as the mean + 1 standard error of the mean (SEM). Regression analyses and calculation of IC_{50} values was done using GraphPad Prism Version 4.00 for Windows (GraphPad Software Inc.).

RESULTS AND DISCUSSION

Inhibition of NO production in LPS and rmIFN-γ costimulated RAW264.7 cells

The crude protein extracts of fifteen Zingiberaceae rhizomes were assayed for their inhibitory activity against NO production in the macrophage-like RAW 264.7 murine cell line that was costimulated with LPS and rmIFN-γ. Of the 15 plant species tested (as rhizome extracts), the protein extracts of four were sufficiently active at these concentrations in inhibiting the NO production that an IC_{50} value could be determined (Table 2). Of note is that at these concentrations, including for these four plant extracts, no effect on the viability of RAW 264.7 macrophages was observed, as determined by the MTT assay and visual observations with tryphan blue exclusion, indicating no cellular toxicity (data not shown). The crude protein of *Z. ottensii* exhibited the highest activity against NO production with an IC_{50} value of 38.6 ± 0.34 μg/mL. It has been reported that the ethanol extract of the dried rhizomes of *Z. officinale* have analgesic, anti-inflammatory and hypoglycemic effects upon mice and rats.[27] However, although *C. comosa*, and *C. longa* (along with *C. xanthorrhiza* which was not evaluated here) have long been used for the treatment of anti-inflammatory, anti-cancer, anti-microbial and

anti-oxidation effects,[28-29] no significant inhibition of NO production was detected in this system, perhaps indicating an alternative mechanism of action for these plant extracts.

TABLE 2 Inhibition on NO production in RAW 264.7 cells and DPPH radical scavenging activity of crude protein from fifteen selected plants in the Zingiberaceae family.

Scientific name	IC_{50} value (μg protein/mL)	
	Inhibition on NO production	DPPH radical scavenging activity
Alpinia galanga (Linn.) Swartz.	ND	ND
Boesenbergia pandurata Roxb.	ND	ND
Curcuma aeruginosa Roxb.	54.6 ± 0.31	1.020 ± 0.069
Curcuma amarissima Roscoe.	ND	1.419 ± 0.005
Curcuma aromatica	52.7 ± 0.96	9.849 ± 4.698
Curcuma longa Linn.	ND	2.404 ± 0.021
Curcuma sp. (kan-ta-ma-la)	ND	2.554 ± 0.528
Curcuma xanthorrhiza Roxb.	ND	0.953 ± 1.051
Curcuma zedoaria (Berg) Roscoe.	ND	3.136 ± 0.210
Hedychium coronarium	41.8 ± 0.27	0.254 ± 0.003
Kaempferia galanga Linn.	ND	ND
Zingiber cassumunar	ND	2.040 ± 0.066
Zingiber officinale Roscoe.	ND	1.064 ± 0.020
Zingiber ottensii Valeton.	38.6 ± 0.34	1.101 ± 0.056
Zingiber zerumbet Smith.	ND	2.036 ± 0.328

*ND = not detected

DPPH free radical scavenging (antioxidant activity)

The antioxidant properties of crude protein from Zingiberaceae plants were determined using the DPPH assay. The results, summarized as the IC_{50} values of DPPH radical scavenging activities of the rhizome crude protein extracts for the 15 plant species, are shown in Table 2. All Curcuma and Zingiber species tested (seven and four, respectively) were positive, and indeed of the 15 species, only three showed no significant free radical scavenging ability. With respect to the 12 positive cases, the *H. coronarium* extract exhibited the highest activity at an IC_{50} value of only 1.37-fold higher

than that of the vitamin C positive control, followed by *C. comosa*, *C. aeruginosa*, *Z. officinale* and *Z. ottensii* with IC_{50} values of five- to six-fold higher than that of the vitamin C. The remaining positive plant extracts decreased in activity (as IC_{50} values) in the order *C. amarissima*, *Z. zerumbet*, *Z. cassumunar*, *C. longa*, *Curcuma sp.*, *C. zedoaria*, and *C. aromatic*, respectively, with IC_{50} values from 7.62- to 53-fold higher than vitamin C (0.186 ± 0.009 μg/mL). No significant correlation was found for DPPH at $P < 0.005$ (data not shown), except for *C. zedoaria* and *C. aromatic*, and so the crude protein had DPPH radical scavenging activities that broadly equate with vitamin C. It has previously been reported that the seed coat extract of *T. indica* contains a polyphenolic flavonoid that displays antioxidant properties. An inhibitory effect of the seed coat extract of *T. indica* on NO production *in vivo* and *in vitro* has also been reported.[30] Correlation between the DPPH bleaching activity of herbal extracts and some antioxidant properties of natural products have been observed.[31-32] Taken together, DPPH and oxygen free radicals interact with phospholipids and protein thiol groups. The herbal extract, considered as a mixture of antioxidant components, prevented the oxidative changes induced by oxygen free radicals.

Effect of crude protein of *Z. ottensii* on iNOS, IL-6 and TNF-α mRNA expression levels in LPS and IFN-γ-stimulated RAW 264.7 cells

From the crude protein extracts from the rhizomes of fifteen Zingiberaceae species, the crude protein of *Z. ottensii* exhibited the highest level of free radical scavenging activity and a reasonable (fifth highest) inhibitory activity against NO production (Table 2). In order to determine the mechanism of the inhibition of LPS and IFN-γ co-induced NO synthesis by the crude rhizome protein(s) of *Z. ottensii*, we examined the effect of the crude protein compared to that for parthenolide (positive control) on the expression level of iNOS, IL-6 and TNF-α gene transcripts. LPS is known to stimulate the RAW 264.7 cell line to produce NO and enhance iNOS, IL-6 and TNF-α transcript expression levels.[33] Both the crude rhizome protein and parthenolide strongly decreased the LPS and rm-IFN-γ mediated increase in the cellular iNOS, IL-6 and TNF-α mRNA levels, but had no significant affect alone (Figure 1). However, the concentration of parthenolide (and so likely IC_{50} value) was much lower than the concentration of the crude rhizome protein. Of course the crude rhizome protein is likely diluted out by the

presence of other proteins, and so a direct comparison of effective inhibition and a real IC_{50} value must await purification of the relevant protein(s) from the rhizome extract.

Since iNOS is induced in response to various pro-inflammatory cytokines, including INF-γ, TNF-α and IL-6, and mediates several inflammatory responses,[34] these results then correlate well with the results of NO production, and iNOS IL-6 and TNF-α transcript expression levels. Furthermore, modulation at either the transcriptional and/or post-transcriptional levels could also involve the inhibition of gene expression. Previous reports have identified that the regulation of iNOS activity mainly occurs at the transcriptional level, where IRF-1 is an essential transcription factor that can bind to the iNOS promoter.[35] In the RAW 264.7 cell line, disruption of IRF-1 gene resulted in the loss of NO production and barely detectable iNOS expression levels in response to stimulation.[36] Moreover, it has been reported that the inhibition of iNOS mRNA expression might involve the blockade of NFκB, an essential transcription factor for iNOS gene transcription.[34, 37]

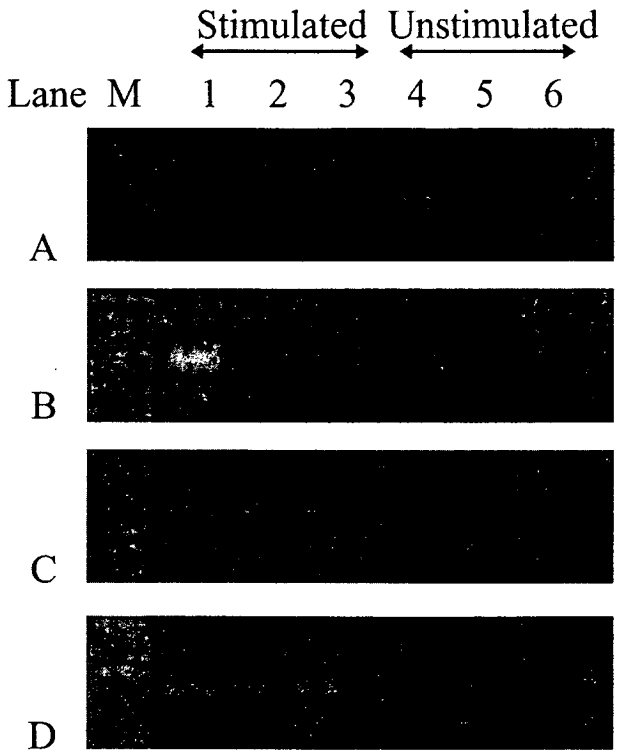


FIGURE 1 Effect of the crude rhizome protein extract of *Z. ottensii* on the iNOS, IL-6 and TNF-α transcript expression levels in LPS (100 ng/mL), and IFN-γ (10 ng/mL) co-stimulated RAW 264.7 cells. (A) β-actin, (B) iNOS, (C) IL-6 and (D) TNF-α. For all panels; Lane 1: LPS + rmIFN-γ, Lane 2: LPS + rmIFN-γ and crude rhizome protein extract (200 ng/mL), Lane 3: LPS + rmIFN-γ and parthenolide (2.5 ng/mL; positive control), Lane 4: no addition, Lane 5: crude rhizome protein extract (100 ng/mL), Lane 6: parthenolide (2.5 ng/mL). Lane M; 100 bp molecular markers. Images shown are representative of that from 3 independent repeats.

Total amino acid contents of crude protein of *Z. ottensii*

The total amino acid contents of crude protein of *Z. ottensii* are shown in Table 3 (on a dry weight basis). The aspartic acid had the highest contents of the total essential amino acid (2.42 mg/100mg), followed by glutamic acid, proline, leucine, arginine, lysine, phenylalanine, valine, threonine, isoleucine, glycine, tyrosine, serine, alanine and histidine, respectively. It has previously been reported that the anti-inflammatory

properties of soy high in this type of protein.[38] These results are similar to those reported by Song et al. (2008).[39] The total amino acid of soy presented a large amount of glutamic acid followed by aspartic acid, arginine, alanine, glycine and serine, and proline. Among the essential amino acids, leucine presented the highest, followed by lysine, isoleucine, valine, threonine, tyrosine, phenylalanine and histidine. In lower amounts was cysteine and methionine but these each amino acid contents slightly lower than those reported.

TABLE 3 Amino acid contents of crude protein of *Z. ottensii* (mg/100 mg dry weight)

Amino acids	Contents (mg/100mg)
Aspartic acid	2.42
Serine	0.65
Glutamic acid	1.82
Glycine	0.72
Histidine	0.31
Arginine	1.25
Threonine	0.77
Alanine	0.42
Proline	1.29
Tyrosine	0.71
Valine	0.79
Lysine	1.17
Isoleucine	0.73
Leucine	1.29
Phenylalanine	0.88

Protein identification

The crude proteins from the *Z. ottensii* rhizome extract were separated by SDS - PAGE (Figure 2), revealing four clear dominant bands. The fourth and largest band was excluded from further analysis because contain enough amount, whilst the other three, marked Z1, Z2 and Z3 in Figure 2, were excised and subjected to in-gel trypsin digestion. The resultant mixture of tryptic peptides was evaluated with tandem mass spectrometry, and the tandem mass spectra so obtained were used to deduce the amino acid sequences of each principal peptide using the Masslyxn software. The sequences of peptides were

compared against existing known proteins using the MS-Blast algorithm to search the NCBI database.

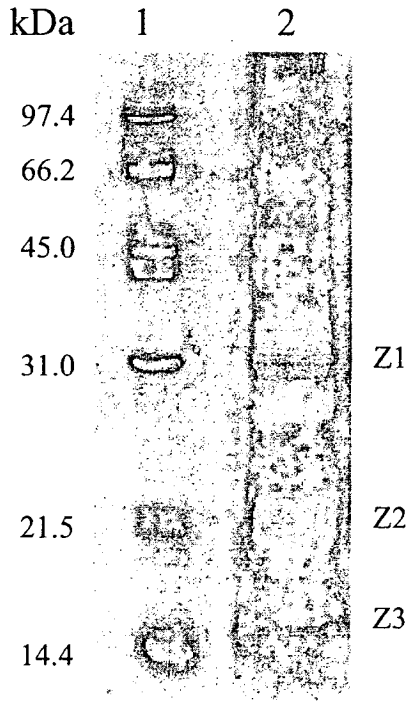


FIGURE 2 Reducing SDS-PAGE resolution of the crude protein extract from the rhizomes of *Z. ottensii*. Lane 1, molecular weight standards; lane 2, 30 μg of the crude protein extract from the rhizomes of *Z. ottensii*. Gel shown is representative of that from 3 independent repeats.

For the Z1 protein, the MS-Blast result revealed five cysteine proteases (Figure 3) from various plants with a total HSP score of more than the threshold score (HSP =170). These homologous cysteine proteases have a MW of 28 - 35 kDa, which compares well with the ~31.0 kDa of band Z1. This enzyme family is known to play a role in plant growth, development and senescence. Most plant cysteine proteases belong to the papain and legumain families. Recently, this enzyme family has been reported from three members of the ginger family, in *C. longa*,[40] *C. aromatica*,[41] and *Z. offinale*,[42] and this ginger protease is used as a food improver and anti-inflammatory agent. That the cysteine protease in the four members of Zingiberaceae, *C. comosa, C. longa, C. aromatica* and *Z. offinale,* show different molecular weights is interesting as, for example, should they

represent the same gene (rather than different isoforms from different loci), then they may be useful as a protein (allozyme) or genetic (PCR or PCR - RFLP amplicon size) marker to classify specific species in this family in the future.

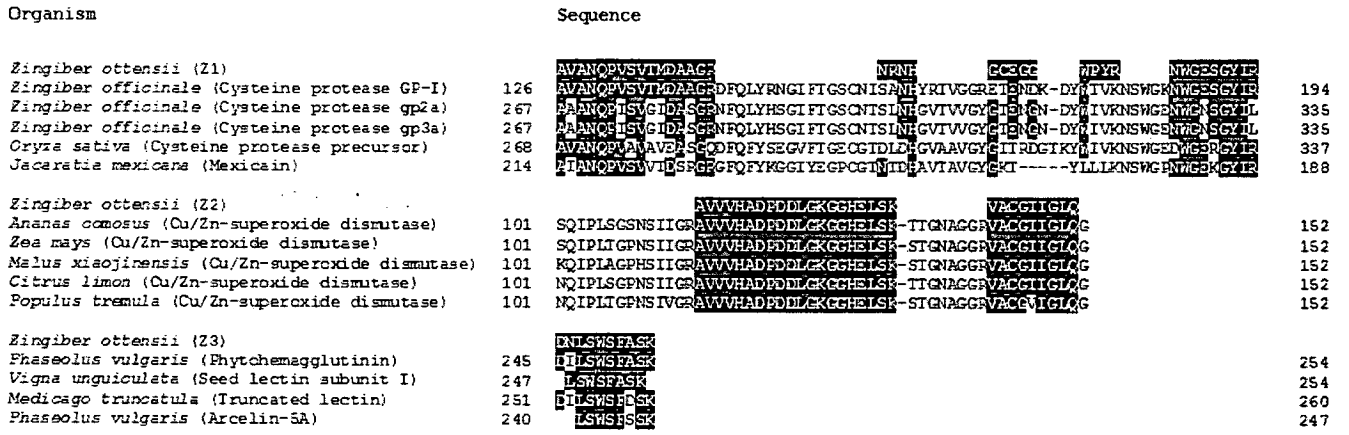


FIGURE 3 Amino acid sequence from the tryptic fragments of the three *Z. ottensii* rhizome crude proteins (Z1, Z2 and Z3 in figure 2). Comparisons are made with other cysteine proteases, Cu/Zn-SOD, and lectin from the others family that showed the highest sequence homology in BLASTP searches of the NCBI and SwissProt databases.

Interestingly, an antioxidant protein was found in the gel. Superoxide dismutase (SOD), a class of enzymes that convert the reactive superoxide radical into oxygen and hydrogen peroxide, was identified as the likely component of band Z2 (Figure 3). This result is in accord with the recent report of an antioxidant activity and isolation of a SOD homologue from *C. comosa*.[43] Indeed, SOD homologues have also been reported in other Zingiberaceae plant species, such as *C. longa*,[44] and *C. zedoaria*.[45] Their current biotechnological applications have mainly been in cosmetic products to reduce free radical levels that otherwise cause skin damage,[46] and so the discovery of an antioxidant enzyme may suggest some benefit for *Z. ottensii* in the natural product based cosmetic industry, but this will depend upon their relative specific activity or ease of enrichment.

Moreover, comparisons of the amino acid sequences of the tryptic peptides obtained from the Z3 protein, at ~15 kDa, using BLASTp and tBLASTx searching identified this fragment as a potential homolog of a lectin precursor from the common

bean, *Phaseolus vulgaris* L. (Fabales: Fabaceae)[47] although only one small peptide region of 10 amino acids was mapped (Figure 3). Thus, the Z3 band, from *Z. ottensii* rhizomes, may be a homolog of other members of the leucoagglutinating phytohemagglutinin precursor family, but this awaits confirmation. A mannose binding lectin with a molecular mass of 13.4 kDa has been also isolated from *C. zedoary*.[48] In addition, six homologous lectin proteins of various molecular masses (8.84 - 32.8 kDa) were found in *C. aromatica*.[41] Most of them are mannose binding lectins. With respect to high throughput protein identification, agglutinin was also found to be present in the *C. longa* 2-D IEF-SDS-PAGE protein profile.[49]

CONCLUSION

In conclusion, the data presented in this study demonstrates a significant inhibitory activity by the crude rhizome protein extract of four out of fifteen Zingiberaceae plants against NO production in the macrophage-like RAW 264.7 cell line that was costimulated by LPS and IFN-γ. The crude protein of *Z. ottensii* exhibited the highest activity against NO production with an IC_{50} value of 38.6 μg protein/mL, and also had a reasonably good free radical scavenging activity with an IC_{50} value of 1.101 μg protein/ml. In addition, the crude rhizome protein extract of *Z. ottensii* almost totally inhibited the ability of LPS and IFN-γ costimulation to increase the cellular iNOS, IL-6 and TNF-α mRNA levels which are potent pro-inflammatory cytokines. The protein patterns showed four main protein bands, including a potential lectin, cysteine protease and superoxide dismutase (SOD), that are related with the antioxidant activity. These results suggest that the crude protein of *Z. ottensii* possesses a strong anti-inflammatory activity and has the potential to be developed as a therapeutic compound.

REFERENCES

1. Ignarro, L.J. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *J. Physiol. Pharmacol.* **2002**, *53*, 503-514.
2. Coleman, J.W. Nitric oxide in immunity and inflammation. *Int. Immunopharm.* **2001**, **1**, 1397-1406.

3. Fang, F.C. Mechanisms of nitric oxide-related antimicrobial activity. *J. Clin. Invest.* **1997**, *99*, 2818-2825.
4. Bjorkbacka, H.; Fitzgerald, K.A.; Huet, F.; Li, X.M.; Gregory, J.A.; Lee, M.A.; et al. The induction of macrophage gene expression by LPS predominantly utilizes Myd88-dindependent signaling cascades. *Physiol. Genomics.* **2004**, *19*, 319-330.
5. Fitzgerald, K.A.; Rowe, D.C. Golenbock, D.T., Endotoxin recognition and signal transduction by the TLR4/MD-2 complex. *Microbes Infect.* **2004**, *6*, 1361-1367.
6. Griffith, O.W.; Stuehr, D.J. Nitric oxide synthases: properties and catalytic mechanism. *Ann. Rev. Physiol.* **1995**, *57*, 707-736.
7. Peck, O.M.; Fan, H.; Tempel, G.E.; Teti, G.; Halushka, P.V.; Cook, J.A. *Staphylococcus aureus* and lipopolysaccharide induce homologous tolerance but heterologous priming: Role of interferon-gamma. *Shock* **2004**, *21*, 254-260.
8. Khan, N.; Afaq, F.; Mukhtar, H. Cancer chemoprevention through dietary antioxidants: progress and promise. Antioxid. Redox. *Signal* **2008**, *10*, 475-502.
9. Larsen, K. A preliminary checklist of Zingiberaceae in Thailand. *Thai Forest Bull. (Botany)* **1996**, *24*, 35-49.
10. Chien, T.Y.; Chen, L.G.; Lee, C.J.; Lee, F.Y.; Wang, C.C. Anti-inflammatory constituents of *Zingiber zerumbet*. *Food Chem.* **2008**, *110*, 584-589.
11. Jatoi, S.A.; Kikuchi, A.; Gilani, S.A.; Watanabe, K.N. Phytochemical, pharmacological and ethnobotanical studies in mango ginger (*Curcuma amada* Roxb.; Zingiberaceae). *Phytother. Res.* **2007**, *21*, 507-516.
12. Lu, Y.; Zhong, C.X.; Wang, L.; Lu, C.; Li, X.L.; Wang, P.J. Anti-inflammation activity and chemical composition of flower essential oil from *Hedychium coronarium*. *Afr. J. Biotechnol.* **2009**, *8*, 5373-5377.
13. Mau, J.L.; Lai, E.Y.C.; Wang, N.P.; Chen, C.C.; Chang, C.H.; Chyau, C.C. Composition and antioxidant activity of the essential oil from *Curcuma zedoaria*. *Food Chem.* **2003**, *82*, 583-591.
14. Victorio, C.P. Therapeutic value of the genus Alpinia, Zingiberaceae. *Rev. Bras. Farmacogn.* **2011**, *21*, 194-201.
15. Butler, M.S. The role of natural product chemistry in drug discovery. *J. Nat. Prod.* **2004**, *67*, 2141-2153.

16. Newman, D.J.; Cragg, G.M.; Snader, K.M. Natural products as a source of new drugs over the period 1981-2002. *J. Nat. Prod.* **2003**, *66*, 1002-1037.
17. Stone, M.J.; Williams, D.H. On the evolution of functional secondary metabolites (natural products). *Mol. Microbiol.* **1992**, *6*, 29-34.
18. Balzarini, J.; Van Laethem, K.; Hatse, S.; Froeyen, M.; Peumans, W.; Van Damme, E.; et al. Carbohydrate-binding agents cause deletions of highly conserved glycosylation sites in HIV GP120 - A new therapeutic concept to hit the achilles heel of HIV. *J. Biol. Chem.* **2005**, *280*, 41005-41014.
19. Hashiba, K.; Yokoyama, K.; Wakabayashi, H.; Hashimoto, K.; Satoh, K.; Kurihara, T.; et al. Inhibition of LPS-stimulated NO production in mouse macrophage-like cells by azulenes. *Anticancer Res.* **2004**, 24, 3939-3944.
20. Yokoyama, K.; Hashiba, K.; Wakabayashi, H.; Hashimoto, K.; Satoh, K.; Kurihara, T.; et al. Inhibition of LPS-stimulated NO production in mouse macrophage-like cells by tropolones. *Anticancer Res.* **2004**, 24, 3917-3922.
21. Nishishiro, M.; Arikawa, S.; Wakabayashi, H.; Hashimoto, K.; Satoh, K.; Yokoyama, K.; et al. Inhibition of LPS-stimulated NO production in mouse macrophage-like cells by azulenequinones. *Anticancer Res.* **2004**, 25, 4157-4164.
22. Wangthong, S.; Palaga, T.; Rengpipat, S.; Wanichwecharungruang, S.P.; Chanchaisak, P.; Heinrich, M. Biological activities and safety of Thanaka (*Hesperethusa crenulata*) stem bark. *J. Ethnopharmacol.* **2010**, *132*, 466-472.
23. Sharma, O.P.; Bhat, T.K. DPPH antioxidant assay revisited. *Food Chem.* **2009**, *113*, 1202-1205.
24. Bradford, M.M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248-257.
25. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, 227, 680-685.
26. Mortz, E.; Vorm, O.; Mann, M.; Roepstorff, P. Identification of proteins in polyacrylamide gels by mass spectrometric peptide mapping combined with database search. *Biol. Mass Spectrom.* **1994**, *23*, 249-261.

27. Ojewole, J.A.O. Analgesic, antiinflammatory and hypoglycaemic effects of ethanol extract of *Zingiber officinale* (Roscoe) rhizomes (Zingiberaceae) in mice and rats. *Phytother. Res.* **2006**, *20*, 764-772.
28. Claeson, P.; Panthong, A.; Tuchinda, P.; Reutrakul, V.; Kanjanapothi, D.; Taylor, W.C.; Santisuk, T. Three non phenolic diarylheptanoids with anti-inflammatory activity from *Curcuma xanthorrhiza*. *Planta Med.* **1993**, *59*, 451-454.
29. Lechtenberg, M.; Quandt, B.; Nahrstedt, A. Quantitative determination of curcuminoids in Curcuma rhizomes and rapid differentiation of *Curcuma domestica* Val. and *Curcuma xanthorrhiza* Roxb. by capillary electrophoresis. *Phytochem. Anal.* **2004**, *15*, 152-158.
30. Komutarin, T.; Azadi, S.; Butterworth, L.; Keil, D.; Chitsomboon, B.; Suttajit, M. Extract of the seed coat of *Tamarindus indica* inhibits nitric oxide production by murine macrophages *in vitro* and *in vivo*. *Food Chem. Toxicol.* **2004**, *42*, 649-658.
31. Feng, L.; Tan, L.N.; Liu, Y.; Jiang, J.; Jiang, W.D.; Hu, K.; et al. Influence of dietary zinc on lipid peroxidation, protein oxidation and antioxidant defence of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquac. Nutr.* **2011**, *17*, E875-E82.
32. Naczk, M.; Towsend, M.; Zadernowski, R.; Shahidi, F. Protein-binding and antioxidant potential of phenolics of mangosteen fruit (*Garcinia mangostana*), *Food Chem.* **2011**, *128*, 292-298.
33. Lee, C.G.L.; Demarquoy, J.; Jackson M.L.; O'Brien, W.E. Molecular cloning and characterization of a murine LPS-inducible cDNA. *J. Immun.* **1994**, *152*, 5758-5767.
34. Jung, K.H.; Ha, E.; Kim, M.J.; Won, H.J.; Zheng, L.T.; Kim, H.K.; et al. Suppressive effects of nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression by *Citrus reticulata* extract in RAW 264.7 macrophage cells. *Food Chem. Toxicol.* **2007**, *45*, 1545-1550.
35. Martin, E.; Nathan, C.; Xie, Q.W. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J. Exp. Med.* **1994**, *180*, 977-984.
36. Kamijo, R.; Harada, H.; Matsuyama, T.; Bosland, M.; Gerecitano, J.; Shapiro, D.; et al. Requirement for transcription factor irf-1 in NO synthase induction in macrophages. *Science* **1994**, *263*, 1612-1615.

37. DiazGuerra, M.J.M.; Velasco, M.; MartinSanz, P.; Bosca, L. Evidence for common mechanisms in the transcriptional control of type II nitric oxide synthase in isolated hepatocytes - Requirement of NF-kappa B activation after stimulation with bacterial cell wall products and phorbol esters. *J. Biol. Chem.* **1996**, *271*, 30114-30120.

38. Hernandez-Ledesma, B.; Davalos, A.; Bartolome, B.; Amigo, L. Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *J. Agric. Food Chem.* **2005**, *53*, 588-593.

39. Song, Y.S.; Frias, J.; Martinez-Villaluenga, C.; Vidal-Valdeverde, C.; Gonzalez de Mejia, E. Immunoreactivity reduction of soybean meal by fermentation, effect on amino acid composition and antigenicity of commercial soy products. *Food Chem.* **2008**, *108*, 571-581.

40. Nagarathnam, R.; Rengasamy, A.; Balasubramanian, R. Purification and properties of cysteine protease from rhizomes of *Curcuma longa* (Linn.). *J. Sci. Food Agric.* **2010**, *90*, 97-105.

41. Tiptara, P.; Petsom, A.; Roengsumran, S.; Sangvanich, P. Hemagglutinating activity and corresponding putative sequence identity from *Curcuma aromatica* rhizome. *J. Sci. Food Agric.* **2008**, *88*, 1025-1034.

42. Choi, K.H.; Laursen, R.A. Amino-acid sequence and glycan structures of cysteine proteases with proline specificity from ginger rhizome *Zingiber officinale*. *Eur. J. Biochem.* **2000**, *267*, 1516-1526.

43. Boonmee, A.; Srisomsap, C.; Karnchanatat, A.; Sangvanich, P. An antioxidant protein in *Curcuma comosa* Roxb. rhizomes. *Food Chem.* **2011**, *124*, 476-480.

44. Kochhar, S.; Kochhar, V.K. Identification and characterization of a super-stable Cu-ZnSOD from leaves of turmeric (*Curcuma longa* L.). *Planta* **2008**, *228*, 307-318.

45. Loc, N.H.; Diem, D.T.H.; Binh, D.H.N.; Huong, D.T.; Kim, T.G.; Yang, M.S. Isolation and characterization of antioxidation enzymes from cells of zedoary (*Curcuma zedoaria* roscoe) cultured in a 5-L bioreactor. *Mol. Biotechnol.* **2008**, *38*, 81-87.

46. Diehl, C.; Lipozencić, J.; Ledić-Drvar, D. The basis of topical superoxide dismutase antipruritic activity. *Acta Dermatovenerol. Croat.* **2009**, *17*, 25-39.

47. Hoffman, L.M.; Donaldson, D.D. Characterization of two *Phaseolus vulgaris* phytohemagglutinin genes closely linked on the chromosome. *EMBO J.* **1985**, *4*, 883-889.

48. Tipthara, P.; Sangvanich, P.; Macth, M.; Petsom, A. Mannose-binding lectin from *Curcuma zedoaria* Rosc. *J. Plant Biol.* **2007**, *50*, 167-173.

49. Chokchaichamnankit, D.; Subhasitanont, P.; Parichartanakul, M.; Sangvanich, P.; Svasti, J.; Srisomsap, C. Proteomic alterations during dormant period of *Curcuma longa* rhizomes. *J. Proteomics Bioinform.* **2009**, *2*, 8.

50. Palaga, T.; Buranaruk, C.; Rengpipat, S.; Fauq, A.H.; Golde, T.E.; Kaufmann, S.H.E.; et al. Notch signaling is activated by TLR stimulation and regulates macrophage functions. *Eur. J. Immunol.* **2008**, *38*, 174-183.

51. Barnholt, K.E.; Kota, R.S.; Aung, H.H.; Rutledge, J.C. Adenosine blocks IFN-gamma-induced phosphorylation of STAT1 on Serine 727 to reduce macrophage activation. *J. Immunol.* **2009**, *183*, 6767-6777.

Part IV

Zingipain, a ginger protease with acetylcholinesterase inhibitory activity

Abstract

In order to search for new Acetylcholinesterase inhibitors (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). Gelatin-degrading zymography showed that the AChEI containing band also contained cysteine 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 mM but inhibited at 10 mM. In contrast, Hg^{2+} and EDTA were very and moderately strongly inhibitory. 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* and a nine amino acid long fragment that was 100% identical to Actinidin Act 2a, suggesting the preparation was heterogeneous. AChEI exhibited non-competitive inhibition of AChE for the hydrolysis of acetylthiocholine iodide with a K_i value of 9.31 mg/mL.

Keyword: acetylcholinesterase inhibitor, ginger protease, Zingipain

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 [1]. 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 [2], short term recall [3, 4], and long term memories as well as the speed of processing [5]. Although aging is always associated with a decline in physiological functions, it is not necessarily associated with diseases [6]. 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 [7]. On the other hand, pathological aging is always associated with non-normal factors, such as diseases and brain trauma [8]. 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 [9]. 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 [10]. 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 [11].

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 [12-15]. Four commercial AChEIs approved by the US Food and Drug Administration (US FDA) and presently used in AD therapy are tacrine [16-17], donepezil, galanthamine [18], and rivastigmine [19]. Although all commercial AChEIs are effective in AD treatment, they have undesirable side effects and are very expensive. Therefore, new alternative AChEIs is always still in demand. Many pharmaceutical industries have invested effort and capital to evaluate natural potential sources of drugs for the prevention and treatment. In traditional folklore medicine, many herbs have been used as a nerve tonic and to improve cognitive function. Therefore, such folklore based medicinal plants reputed as a nerve tonic and used for memory improvement are very attractive candidates as potential sources of new drugs to heal and prevent those neurodegradative diseases.

The Zingiberaceae is a well-known plant family in Southeast Asia and many species were used in traditional folklore medicine for the effective treatment of several diseases. Furthermore, they are perennial herbs widely existed and cultivated in Thailand as well as other tropical regions in Asia. They have been commonly used as medicinal plants and spices in Thailand. The rhizomes of these plants possess diverse biological activities, including anti-microbial [20-21], anti-ulcer [22-23], anti-inflammatory [24], anti-oxidant [25], cytotoxic and anti-tumor [26-29], vasorelaxant [30], anti-spasmodic [31], anti-hepatotoxic [32] and anti-depressant activities [33-34]. Although there have been many reports concerning the active chemical constituents of these plants including the rhizomes, and some biological activities of these species, but almost of them were non-proteinaceous compounds and only a few reports have focused on the bioactive peptides or proteins from these plants.

In deed, 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 [35]. Besides, the 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 biotechnology industries [36]. The objective of this study was to investigate *in vitro* AChEI activities of proteins from some *Zingiberaceae* species. Here, using azocasein digestion as the bioactivity readout for protease activity to guide the purification direction, we report on the enrichment of a protease from some *Zingiberaceae* species, and its likely identity, as deduced by tryptic peptide sequencing coupled with homology searches.

Material and methods

Plant materials

The fresh rhizomes of 15 *Zingiberaceae* species were purchased from Chatuchak park market in Bangkok, Thailand. The samples were quickly taken to laboratory and kept in dark 4 °C room until used.

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.

Preparation of the Zingiberaceae rhizomes extract

The rhizomes (1.5 kg wet weight) of the 15 selected plant species were peeled, minced (~ 10 × 10 × 10 mm), and then homogenized in 5 L PBS (20 mM phosphate

buffer (pH 7.2) containing 0.15 M NaCl) using a blender and then overnight left at 4 °C with continuously stirring. The suspension was then filtered through double-layered cheesecloth followed by centrifugation at 15,000 × g for 30 min. Clear supernatant ("crude homogenate") was harvested and proper amount of ammonium sulfate was added, with stirring, to make 80% saturation and then overnight left at 4 °C with stirring. The precipitate was collected from the suspension by centrifugation at 15,000 × g for 30 min. The pelleted materials were then re-dissolved in PBS, dialyzed (using 3,500 MWCO dialysis tube) against 3 changes of 5 L of water at 4 °C and then freeze dried. This will be referred to as the "ammonium sulphate cut fraction".

Acetylcholinesterase inhibition assay

The AChEI activity was measured *in vitro* by a modified Ellman's method [37]. The assay contained 125 µL of 3 mM DTNB, 50 µL of 50 mM Tris-HCl buffer (pH 8.0) with 0.1% (w/v) BSA, the plant extract at different concentrations were diluted in 50 mM Tris-HCl buffer (pH 8.0), 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 at 37 °C for 1 hr. The enzymatic reaction of AChE which hydrolyses the acetyl group in ATCI to yield thiocholine that can then react with DTNB to form 5-thionitrobenzoate, a colored anion readable at 415 nm. The percent inhibition was calculated using following formula: [(control absorbance – sample absorbance) / control absorbance] × 100, where the control is the reaction mixture without AChE. The concentrations of 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.

Protease activity assay

The method in determination of protease activity in solution was modified from that previously reported [38]. 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 °C for 30 min. Next, 500 µL of a 5% (w/v) trichloroacetic acid solution was added, thoroughly mixed and left at 37 °C for 30 min before 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 was added, the content was thoroughly mixed, and the

absorbance was read at 340 nm with a spectrophotometer. The protease activity of sample was calculated in relation to a trypsin standard curve as protease units. Increasing the absorbance by one unit was considered as one unit of activity [39].

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. × 15 cm length) pre-equilibrated with 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 (protein content determination section) as well as AChEI and protease activities (acetylcholinesterase inhibition assay, and protease activity assay section, respectively). The fractions containing corresponding activity were pooled, dialyzed, and freeze-dried, and then kept at -20 °C until use. Each fraction is known as the "post-DEAE-cellulose AChEI x fraction", where x stands for unbound, F25, F50, F75 and F100 which referred to unbound and bound fractions eluted at 0.25, 0.5, 0.75 and 1.0 M NaCl, respectively.

Protein content determination

For evaluation of protein levels in the DEAE-cellulose column chromatography step, 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 [40], 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, the plate was shaken for 5 min and then left for 10 min before reading at 595 nm using an ELISA plate reader. The obtained OD was converted to protein concentration using the linear equation computed from the standard curve.

Determination of the protein pattern by native-PAGE

The protein from each step of the purification was further analyzed under its native protein pattern according to the method of Bollag [41], 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.

Zymography of gelatin-containing native-PAGE

To test for the presence of protease activity by zymography [42] 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 °C for overnight. The gels were developed using Coomassie blue R-250 solution and the positive protease activity result was appeared as white band against a blue background.

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 [43]. Samples to be analyzed were treated with reducing sample buffer and boiled for 5 min prior to apply into 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 Coomassie blue R-250 staining.

Effect of temperature on the AChEI activity

The effect of temperature on the AChEI activity was determined by incubating the samples in 20 mM phosphate buffer (pH 7.2) at various temperatures (-20-90 °C at 10 °C intervals) for 30, 60, 90 or 120 min. After that, all were cooled to 4 °C and then the residual AChEI activities were assayed as the method described in acetylcholinesterase inhibition assay section. The attained activities were normalized using a complete

uninhibit mixture (set as 100% inhibition) as positive control and maximum inhibit mixture as negative control. The inhibitory activities are reported as relative activities compared to these two experimental sets. The AChE was used in all cases and at least three replicates were done for each assay.

The pH-dependence of the AChEI activity

Incubating the AChEI in buffers of broadly similar salinity levels but varying in pH from 2-14 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 (acetylcholinesterase inhibition assay section). The activities attained were compared relative to that of the positive and negative controls (complete inhibit and complete non-inhbibit sets) and so are reported as the % relative activity. The AChE was used in all cases and at least three replicates were done for each assay.

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 with one of these divalent cation salts; Ca^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} (all as chlorides), Cu^{2+} or Zn^{2+} (as sulphates); or EDTA at 0, 5 or 10 mM final concentration for 30 min with continuously shaking. After that, the AChEI activity was determined as described (acetylcholinesterase inhibition assay section) using at least three replicates for each assay.

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.

Protein identification

In situ (in gel) trypsinization

The sample preparation process followed the published method of Tiptara et al. [44]. Each band in the electrophoretic gel was excised, cut into small pieces (~1mm^3), and washed with 100 mL deionized water. The gel pieces were destained by adding 200 mL 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 mL acetonitrile for 15 min prior to drying in a vacuum centrifuge. Then 50 mL of a 10 mM DTT solution in 100 mM NH_4HCO_3 was added, and the proteins were reduced for 1 hr at 56 °C. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and gels were incubated for 45 min at room temperature in the dark. The solution was then removed, the gel pieces were dehydrated in acetonitrile, and the solvent was evaporated off before adding 10 mL of a trypsin solution (proteomics grade, Sigma) (10 ng/mL in 50 mM NH_4HCO_3). After allowing the gel plug to swell for 15 min at 4 °C, 30 mL of 50 mM NH_4HCO_3 was added and the digestion allowed to proceed at 37 °C overnight. The supernatant was then harvested following centrifugation at 10,000 ×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.

LC-MS/MS and peptide blasting

The likely amino acid sequence of each internal fragment of the trypsinized material was analyzed by liquid chromatography (LC)/mass spectroscopy (MS)/MS. The extracted tryptic peptides were then subjected to LC-nano electrospray ionization (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 [45].

Statistical analysis

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

Results and discussion

Screening for ACEI in plant samples

The IC_{50} calculated by the regression equation obtained from evaluation of each test extract with different concentrations has been presented in Table 1. Of six crude homogenates screened, all gave AChEI activity positive with good inhibitory activity (low IC_{50} values) that were observed for *Boesenbergia pandurata*, *Hedychium coranarium* and *Zingiber officinale* (Table 1). Screening of the ammonium sulphate cut fraction found that five of fifteen species revealed positive AChEI activity, and three of these were different species from those 9 positive species in crude homogenates screening. Of note is that of the screened two species (*Z. officinale* and *Curcuma amarissima*), in both a crude homogenate and an ammonium sulphate cut fraction, 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 it was thus selected for further enrichment of the AChEI activity and evaluation.

Protease activity was found in 14 of 15 tested Zingiberaceae species extracts , as evaluated using the method modified from Iversen & Jorgensen (1995) [38], with the 15th species, *B. pandurata*, gave negative result (Table 1). The level of protease activity varied some 66-fold in the crude homogenate between the 14 positive species, ranging from lowest in *C. zedoaria* to highest in *Alpinia galanga*, with most actitities falling within 20-120 U / mg protein range. However, in all bar two cases (*C. zeodaria* and *K. galanga* which remained the same and 1.91-fold higher, respectively), the protease specific activity was significantly decreased (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, for example a loss of essential cofactors, which is still 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 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 ± 0.39	56.6 ± 0.13
Boesenbergia pandurata Roxb.	24.3 ± 0.37	ND	ND	ND
Curcuma aeruginosa Roxb.	ND	ND	212.8 ± 0.09	120.2 ± 0.04
Curcuma amarissima Roscoe.	348.4 ± 0.10	466.1 ± 0.58	34.5 ± 0.05	10.8 ± 0.09
Curcuma aromatica.	ND	ND	21.6 ± 0.04	9.74 ± 0.25
Curcuma longa Linn.	67.2 ± 0.78	ND	66.8 ± 0.05	9.71 ± 0.02
Curcuma sp. (kan – ta – ma – la)	ND	ND	20.6 ± 0.02	15.6 ± 0.04
Curcuma xanthorrhiza Roxb.	ND	704.3 ± 0.41	33.3 ± 0.12	8.84 ± 0.08
Curcuma zedoaria (Berg) Roscoe.	ND	393.9 ± 0.19	9.69 ± 0.07	9.99 ± 0.02
Hedychium coronarium.	25.7 ± 0.63	ND	59.6 ± 0.06	11.3 ± 0.11
Kaempferia galanga Linn.	112.9 ± 0.75	ND	24.2 ± 0.02	46.3 ± 0.08
Zingiber cassumunar	ND	ND	42.0 ± 0.11	14.4 ± 0.26
Zingiber officinale Roscoe.	22.4 ± 0.52	137.3 ± 0.55	76.9 ± 0.04	21.6 ± 0.11
Zingiber ottensii Valeton.	ND	265.7 ± 0.40	113.4 ± 0.10	33.9 ± 0.24
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

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 4 °C 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 with the same buffer of the 0, 0.25, 0.5, 0.75 and 1 M NaCl stepwise concentration. The protein containing fractions eluted from the DEAE-cellulose column were screened for protease and AChEI activity (Figure 1). Four distinct protein peaks were isolated, the unbound fraction and the bound ones were then eluted at 0.25 (F25), 0.5 (F50) and 0.75 (F75) M NaCl. The protein amounts eluted with higher salt concentration than 0.5 M tend to be decreased (such as smaller peak of F75 and trace amount or absence of F100) (Figure 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 1 and Table 2). Thus, the post-DEAE-cellulose unbound AChEI fraction was selected for further characterization.

Table 2. The protein yield and the AChEI and protease activities in each enriched fraction.[a]

Fraction	AChEI activity		Protease activity (U/mg protein)
	Protein yield (μg)	Maximal inhibition (%)	
Crude extract[b]	151.1 ± 0.05	92.4 ± 0.02	76.9 ± 0.04
Crude protein[c]	798.5 ± 0.12	76.7 ± 0.07	21.6 ± 0.11
Fraction unbound	72.3 ± 0.03	88.4 ± 0.02	786.3 ± 0.45
Fraction F25	30.5 ± 2.30	ND	ND
Fraction F50	24.1 ± 1.90	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

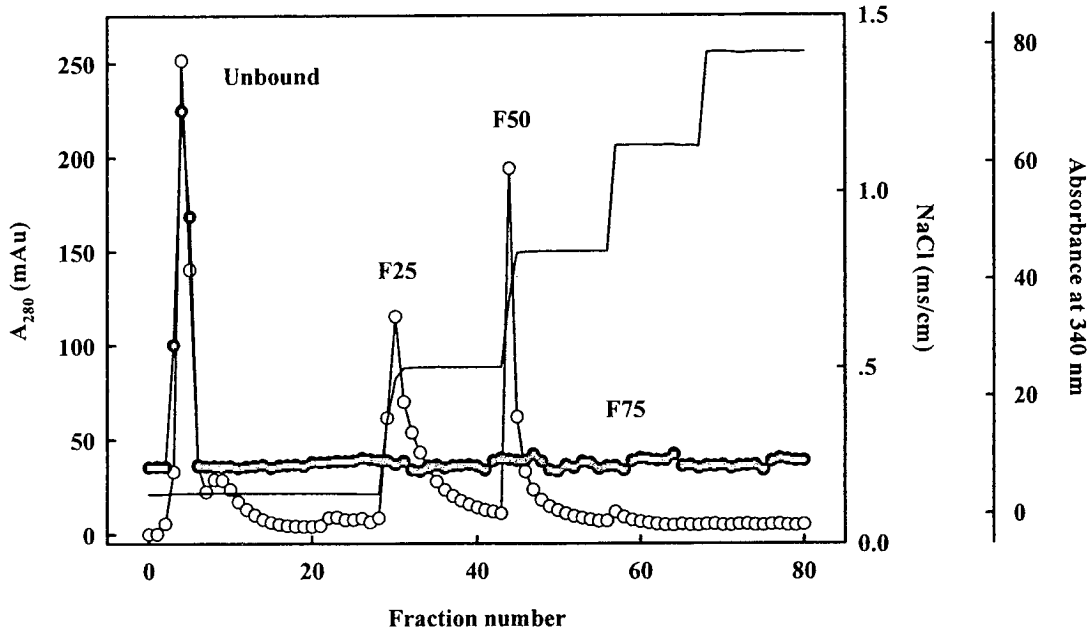


Figure 1. DEAE-cellulose chromatogram of the ammonium sulphate cut fraction of *Z. officinale* rhizome proteins (50 mg) with stepwise NaCl elution (0.00, 0.25, 0.50, 0.75 and 1.00 M). Fractions were assayed for (○) absorbance at 280 nm and (●) protease activity. Profile shown is representative of three independent trials.

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 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 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, giving an estimated molecular weight of about 35.5 kDa (Figure 2B). This molecular size is in good agreement with evident previously published biochemical characteristics for zingipain with a reported the size of about 34.8 kDa [46]. This thus indicated that high purity protein was obtained by just single step chromatography

purification. This procedure seems suitable for zingipain purification from *Zingiberaceae* rhizomes due to its easier, lower time and cost spend, and can avoid the significant yield losses during multiple processing steps [47-50].

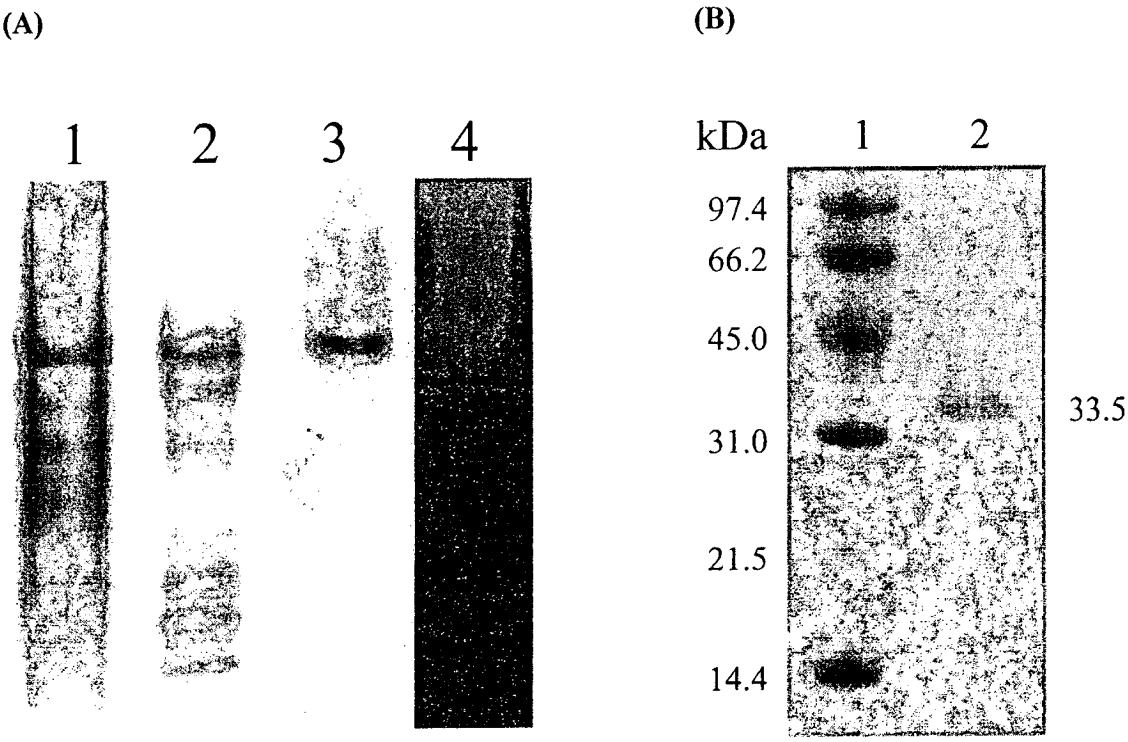


Figure 2. (A) Coomassie blue stained non-denaturing PAGE of the *Z. officinale* rhizome protein from each step of enrichment. Lanes 1-4 show 20 μg of total protein from (1) the crude homogenate, (2) the 80% saturation ammonium sulphate cut fraction and (3) the post-DEAE-cellulose unbound fraction and (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 representativ

Characterization of the AChEI activity

Current research upon 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 since they provide more chances in selecting more effective and stable inhibitors that are usable under a large variety of environmental conditions. Moreover, the knowledge of their structural features is fundamental to understanding the inhibitor-enzyme interactions and allows novel approaches in the use of synthetic or modified inhibitors for drug design. The maximum AChEI activity of purified protein (post-DEAE-cellulose unbound fraction) from *Z. officinale* is 88.4 ± 0.02 % at 72.3 μg/10 mL which can be converted to 0.0072 mg/mL or 7.2 μg/mL. This is extremely stronger than the moderate inhibitory level of small molecule AChE inhibitor reported by Mukherjee et al. [51] in *Fumaria capreolata* (96.89 ± 0.17 % inhibition at 1 mg/mL concentration) and comparable to the strongest one reported by the same authors in *Tabernaemontana divaricata* (93.50 ± 0.37 % at 0.1 μg/mL). The purified protein gave IC_{50} of 137.3 ± 0.55 μg/mL which lower than the activity of quecetin in *Agrimonia pilosa* (estimately equal to) 5.98 μg/mL [52]. From these results, it can be said that among over reported 180 herb species (with almost all active substances are small molecules) our protein locates at relatively high AChEI activity in range and thus has highly feasibility to become one of hopeful drug for neurodetradative disease therapy in future.

Temperature resistance determination

The thermal stability profile of the enriched AChEI (post-DEAE-cellulose unbound fraction) from ginger has shown in Figure 3. The relative activity of this AChEI was stable over a relatively wide temperature range (-20-70 °C at a 30 min exposure) with more than 60% relative activity being retained at 90 °C for 30 min. Except for at 90 °C, increasing the incubation time from 30 to 60 min resulted to only slightly decrease in AChEI activity by temperature, but prolong preincubation (exposure) time to 90 or 120 min resulted to further decrease in AChEI activity. However, these relatively thermostable characters can be durable up to 60 °C. At the temperature range between -20 to 60 °C, the AChEI covers the range from 100 to 40% activities. However, no activity was observed after 120, 90 and 60 mins at 70, 80 and 90 °C incubations, respectively.

One possible reason was the higher temperature and longer time incubation may cause 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 [53].

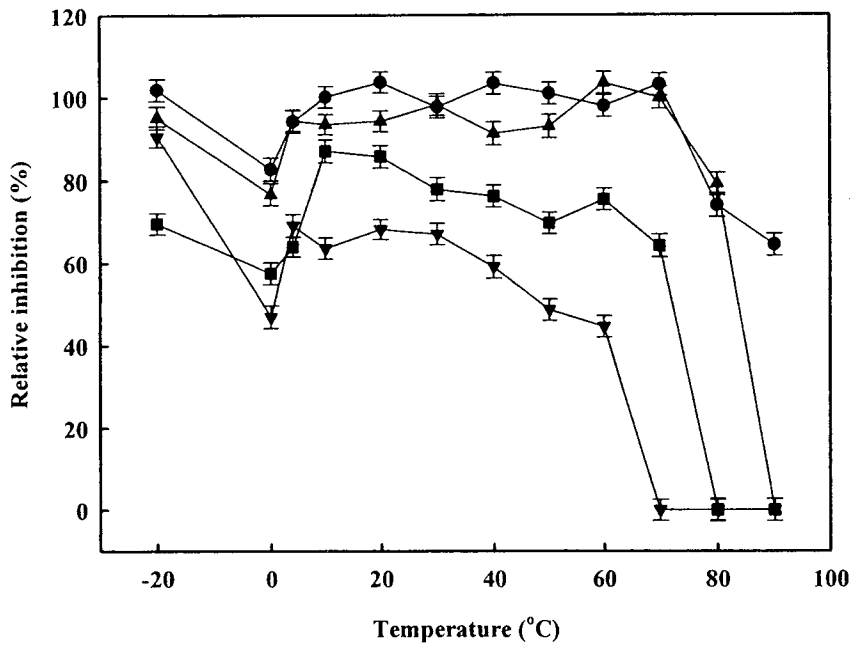


Figure 3. Thermostability of the enriched AChEI from *Z. officinale* rhizomes (post-DEAE-cellulose unbound fraction). The assay was performed in 20 mM phosphate buffer pH 7.2 at various temperatures for (●) 30, (▲) 60, (■) 90 and 120 (▼) min. Data are shown as the mean ± 1 SEM and are derived from triplicate experiments.

pH resistance of the AChEI activity

The residual AChEI activity, as a relative % inhibition, in function of given pH was largely unaffected under a broad pH optimum. This makes the becomes an enzyme excellent for food and pharmaceutical processing. However, some buffer-dependent affects were seen, especially at pH 10.0 (Figure 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).

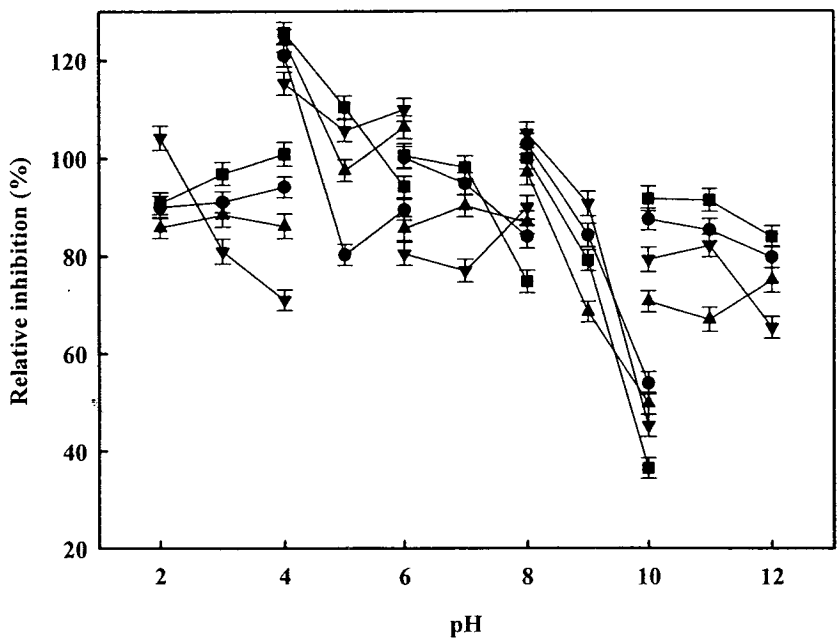


Figure 4. pH stability of enriched AChEI from *Z. officinale* rhizomes (post-DEAE-cellulose unbound fraction). The assay was performed in the following buffer systems (all 20 mM); glycine-HCl (pH 2.0-4.0), sodium acetate (pH 4.0-6.0), potassium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.0-10.0) and glycine-NaOH (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.

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 divalent metal cation salts on the AChEI activity is shown as a relative % in Table 3. The presence of $Mn^{2+}$and Cu^{2+} ions at 1-10 mM clearly and significantly stimulated the AChEI activity which was highest at 5-10 mM. Perhaps

these two metal ions stabilize this AChEI protein providing more suitable conformational structure. Although Zn^{2+} offered weak AChEI activity stimulation 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+} that being 100% loss activity at 5 and 10 mM. AChEI diminishing effect by Hg^{2+} is probably due to 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 deteriorate of the AChEI activity, consistent with the apparent ability of divalent metal ions, such as Mn^{2+}, to stimulate the AChEI activity.

Table 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

Mechanism of inhibition

The inhibition mode of the AChEI from ginger was analyzed by double reciprocal (Lineweaver-Burk) plots (Figure 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 [54]. Tolserin, the novel experimental AD therapeutic agent, inhibits AChE in a non-competitive manner [55]. 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 [56]. Other studies also suggest that the Aβ aggregating property of AChE during the onset of AD can be inhibited by non-competitive inhibitors [57]. 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 wider functional range of medicinal properties that each plant can offer, whereas pharmaceutical drugs are usually designed to attack only a single target [58].

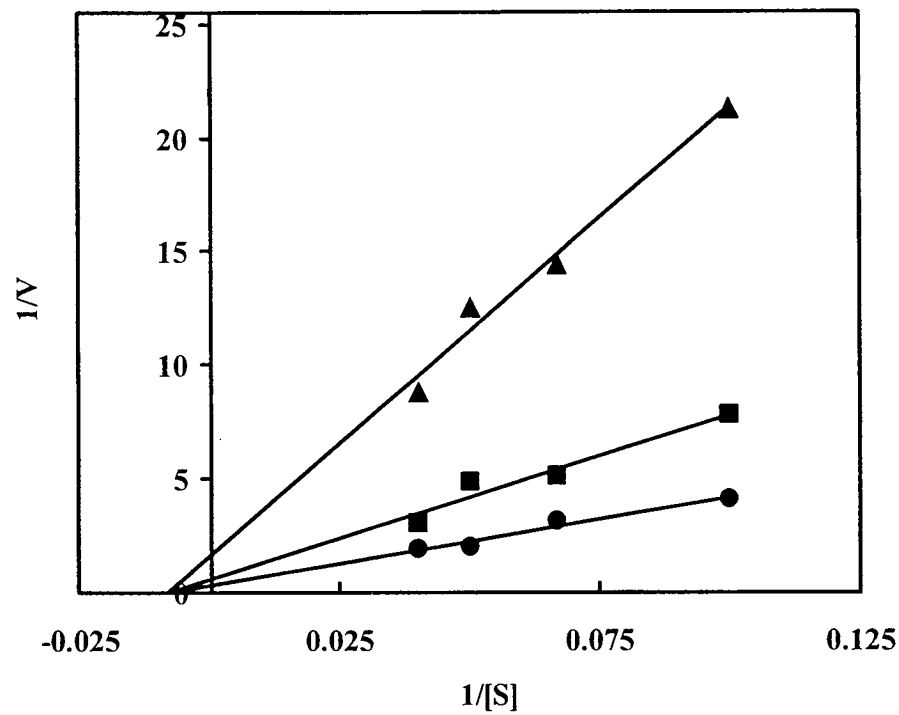


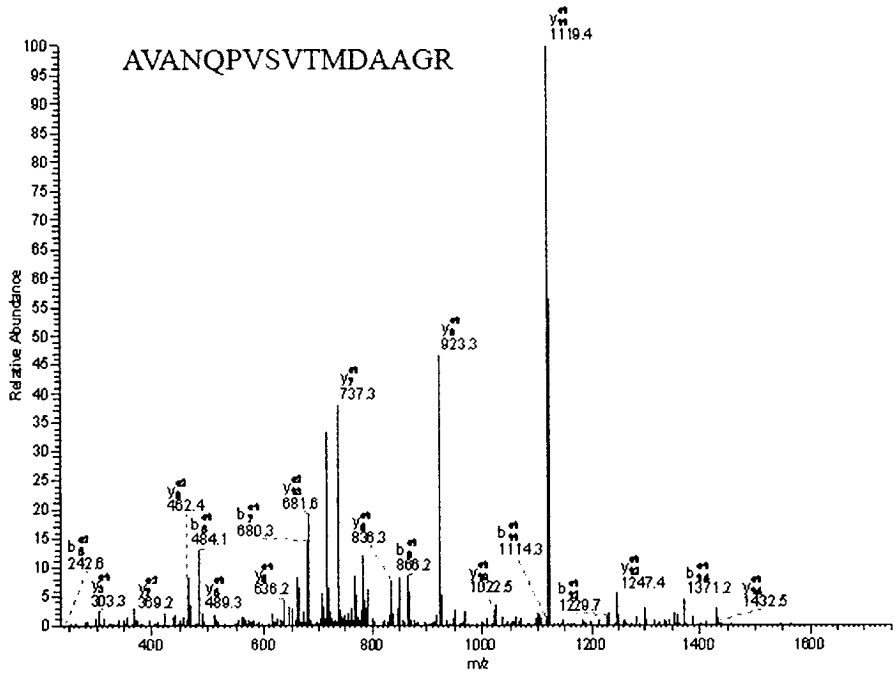
Figure 5. Lineweaver-Burk plots derived from the inhibition of AChE by the AChEI from *Z. officinale* rhizomes. AChE was treated with each stated concentration of ATCI (0.04-0.4 mM) in the presence of the AChEI at (●) 0, (■) 0.2 and (▲) 0.5 mg protein/mL.

Potential AChEI protein identification

Amino acid sequences of the tryptic four fragments were deduced by LC-MS/MS analysis. Five sequences (AVANQPVSVTMDAAGR, GCEGG, WPYR and ENAHVVSIDSYR) were gained from software analysis (*De novo* deducing). All fragments were aligned to those homologs available in the NCBI GenBank and UniProt databases. The longest sequence obtained, AVANQPVSVTMDAAGR (Figure 6 (A)) was also BLASTp searched against the GenBank and UniProt database alone, revealing 100% amino acid sequence similarity to parts of zingipain-2, zingipain-1, cysteine protease GP2a and cysteine protease GP2b (all from *Z. officinale* rhizome) for the first peptide. The remaining three fragments, GCEGG, WPYR and ENAHVVSIDSYR, 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 larger peptide sequence, unbound is likely to be a member of the cysteine protease family (Figure 6B), 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.

(A)



(B)

Accession number	Organism		Sequence	
	Zingiber officinale (unbound)		AVANQPVSVTMDAAGR	
P82473	*Zingiber officinale* (Cysteine proteinase GP-I)	125	KAVANQPVSVTMDAAGRD	142
Q5ILG5	*Zingiber officinale* (Cysteine protease gp3a)	202	KAVANQPVSVTMDAAGRD	212
P82474	*Zingiber officinale* (Cysteine proteinase GP-II)	125	KAVANQPVSVTMDAAGRD	142
Q5ILG7	*Zingiber officinale* Cysteine protease gp2a	266	KAVANQPVSVTMDAAGRD	283
A6N8F8	*Elaeis guineensis*(Cysteine proteinase)	265	KAVANQPVSVAIEAGGRE	282
B9R777	*Ricinus communis* (Cysteine protease)	265	KAVANQPVSVAIEAGGRE	282
P25250	*Hordeum vulgare* (Cysteine proteinase EP-B2)	261	RAVANQPVSVAVEASGKA	278
Q7X750	*Glycine hispida*(Cysteine proteinase)	252	KAVANQPVSVAIDAGGSD	269
Q84M27	*Helianthus annuus*(Cysteine protease-3)	249	RAVANQPVSIAIDAGGLN	266
Q1EPL9	*Triticum aestivum* (Cysteine proteinase)	365	KAVANQPVSVGIDASGKA	282

Figure 6. (A) Amino acid sequence from the tryptic fragments of the enriched AI protein fraction. Comparisons are made with other plant proteins that showed the highest sequence homology in BLASTp and tBLASTn searches of the NCBI and SwissProt databases. Shaded regions represent regions of identity. (B) LC/MS/MS spectra of the tryptic digest of the enriched AI protein used to derive the data in (A) above.

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.

References

1. Harman, D. (1998) Ann. N.Y. Acad. Sci. 854, 1-7.
2. James, L.E., Fogler, K.A. & Tauber, S.K. (2008) Psychol. Aging 23, 657-664.
3. Gilchrist, A.L., Cowan, N. & Naveh-Benjamin, M. (2008) Memory 16, 773-787.
4. Gazzaley, A., Sheridan, M.A., Cooney, J.W. & D'Esposito, M. (2007) Neuropsychology 21, 532-539.
5. Park, D.C., Lautenschlager, G., Hedden, T., Davidson, N.S., Smith, A.D. & Smith, P.K. (2002) Psychol. Aging 17, 299-320.
6. Anton, B., Vitetta, L., Cortizo, F. & Sali, A. (2005) Ann. N.Y. Acad. Sci. 1057, 525-535.
7. Christensen, H.(2001) Aust. New Zeal. J. Psychiatr. 35, 768-775.
8. Reese, C.M., Cherry, K.E. & Copeland, A.L. (2000) Aging Neuropsychol. Cognit. 7, 1-8.
9. Nussbaum, R.L. & Ellis, C.E. (2003). N. Engl. J. Med. 348, 1356-1364.
10. Evans, D.A. (1990) Millbank Quarterly 68, 267-269.
11. Perry, E. K. (1986) Brit. Med. Bull. 42, 63-69.
12. Almkvist, O., Darreh-Shori, T., Stefanova. E., Spiegel, R. & Nordberg, A. (2004) Eur. J. Neurol. 11, 253-261.
13. Persson, C.M., Wallin, A.K., Levander, S. & Minthon, L. (2009) BMC Neurol. 9, 7.

14. Rosler, M., Anand, R., Cicin-Sain, A., Gauthier, S., Agid, Y., Dal-Bianco, P., Stahelin, H.B., Hartman, R. & Gharabawi, M. (1999) *Brit. Med. J.* 318, 633-638.
15. Winblad, B., Engedal, K., Soininen, H., Verhey, F., Waldemar, G., Wimo, A., Wetterholm, A.L., Zhang, R., Haglund, A. & Subbiah, P. (2001) Neurology 57, 489-495.
16. Arrieta, J.L.A.F. (1998) Age and Ageing 27, 161-179.
17. Qizilbash, N., Whitehead, A., Higgins, J., Wilcock, G., Schneider, L. & Farlow, M. (1998) J. Amer. Med. Assoc. 280, 1777-1782.
18. Jones, R.W. (2003) Int. J. Geriatr. Psych. 18, 7-13.
19. Polinsky, R.J. (1998). Clin. Ther. 20, 634-647.
20. Yamada, Y., Kikuzaki, H. & Nakatani, N. (1992) J. Antibac. Antifun. Agents. 20, 309-311.
21. Hiserodt, R.D., Franzblau, S.G. & Rosen, R.T. (1998) J. Agric. Food Chem. 46, 2504-2508.
22. Al-Yahya, M.A., Rafatullah, S., Mossa, J.S., Ageel, A.M., Al-Said, M.S. & Tariq, M. (1990) Phytother. Res. 43, 112-114.
23. Matsuda, H., Pongpiriyadacha, Y., Morikawa, T., Ochi, M. & Yoshikawa, M. (2003) Eur. J. Pharmacol. 471, 59-67.
24. Araujo, C.A.C. & Leon, L.L. (2001) Mem. Inst. Osw. Cruz. 96, 723-728.
25. Selvam, R., Subramanian, L., Gayathri, R. & Angayarkanni, N. (1995) J. Ethnopharmacol. 47, 59-67.
26. Itokawa, H., Morita, H., Sumitomo, T., Totsuka, N. & Takeya, K. (1987) Planta Med. 53, 32-33.
27. Murakami, A., Jiwajiinda, S., Koshimizu, K. & Ohigashi, H. (1995) *Cancer Lett.* 95, 137-146.
28. Murakami, A., Toyota, K., Ohura, S., Koshimizu, K. & Ohigashi, H. (2000) J. Agric. Food Chem. 48, 1518-1523.
29. Pal, S., Choudhuri, T., Chattopadhyay, S., Bhattacharya, A., Datta, G.K., Das, T. & Sa, G. (2001) Biochem. Biophys. Res. Comm. 288, 658-665.
30. Othman, R., Ibrahim, H., Mohd, M.A., Awang, K., Gilani, A.H. & Mustafa, M.R. (2002) Planta Med. 68, 655-657.

31. Ammon, H.P.T. & Wahl, M.A. (1991) Planta Med. 57, 1-7.
32. Hikino, H., Kiso, Y., Kato, N., Hamada, Y., Shioiri, T., Aiyama, R., Itokawa, H., Kiuchi, F. & Sankawa, U. (1985) J. Ethnopharmacol. 14, 31-39.
33. Noro, T., Miyase, T., Kuroyanagi, M., Ueno, A. & Fukushima, S. (1983) Chem. Pharm. Bull. 31, 2708-2711.
34. Yu, Z.F., Kong, L.D. & Chen, Y. (2002) J. Ethnopharmacol. 83, 161-165.
35. Schaller, A. (2004) Planta 220, 183-197.
36. Kaneda, M. & Tominaga, N. (1975) J. Biochem. (Tokyo) 78, 1287-1296.
37. Ellman, G.L., Courtney, D., Andres, V.Jr. & Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88-95.
38. Iversen, S. & Jorgensen, M. (1995) Biotechnol. Tech. 9, 573-576.
39. Chandrashekar, K. & Gujar, G.T. (2003). Indian J. Exp. Biol. 42, 164-173.
40. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
41. Bollag, D.M., Rozycki, M.D. & Edelstein, S.J. (1996) Protein methods (2^{nd} ed.), *Wiley-Liss Inc.*, USA.
42. Ambili, M. & Sudhakaran, P.R. (1998) Indian J. Biochem. Biophys. 35, 317-320.
43. Laemmli, U.K. (1970). Nature 227, 680-685.
44. Tiptara, P., Petsom, A., Roengsumran, S. & Sangvanich, P. (2008) J. Sci. Food Agric. 88, 1025-1034.
45. Mortz, E., Vorm, O., Mann, M. & Roepstorff, P. (1994) Biol. Mass Spectrom. 23, 249-261.
46. Adulyatham, P. & Owusu-Apenten, R. (2005) J. Food Sci. 70, C231-C234.
47. Demir, Y., Gungor, A.A., Duran, E.D. & Demir, N. (2008) Food Tech. Biotechnol. 46, 289-291.
48. Wang, H. & Ng, T. (2002). Peptides 23, 1025-1029.
49. Ye, X., Ng, T., Tsang, P. & Wang, J. (2001). J. Protein Chem. 20, 367-375.
50. Rameshwaram, N. & Nadimpalli, S. (2008). J. Chromatogr. B 861, 209-217.
51. Mukherjee, P., Kumar, V., Mal, M. & Houghton, P. (2007) Phytomedicine 14, 289-300.
52. Mankil, J. & Moonsoo, P. (2007) Molecules 12, 2130-2139.

53. Tiengburanatam, N., Sangvanich, P., Boonmee, A. & Karnchanatat, A. (2010) Appl. Biochem. Biotechnol. 162, 1938-1951.

54. Alhomida, A.S., Al-Rajhi, A.A., Kamal, M.A. & Al-Jafari, A.A. (2000) Toxicology 147, 33-39.

55. Kamal, M.A., Greig, N.H., Alhomida, A.S. & Al-Jafari, A.A. (2000) Biochem. Pharmacol. 60, 561-570.

56. Bartolini, M., Bertucci, C., Cavrini, V. & Andrisano, V. (2003) Biochem. Pharmacol. 65, 407-416.

57. Choudhary, M.I., Nawaz, S.A., ul-Haq, Z., Lodhi, M.A., Ghayur, M.N., Jalil, S., Riaz, N., Yousuf, S., Malik, A., Gilani, A.H. & ur-Rahman, A. (2005). Biochem. Biophys. Res. Commun. 334, 276-287.

58. Mills, S., & Bone, K. (2000). Principles and practice of phytotherapy: Modern herbal medicine, Churchill Livingstone, Edinburgh, UK.

PROJECT OUTPUT

1. Yodjun, M., Karnchanatat, A., and Sangvanich, P.* (2012) Angiotensin I-converting enzyme inhibitory proteins and peptides from the rhizomes of Zingiberaceae plants. *Applied Biochemistry and Biotechnology* 166: 2037-2050.
2. Virounbounyapat, P., Karnchanatat, A. and Sangvanich, P.* (2012) An alpha-glucosidase inhibitory activity of thermostable lectin protein from *Archidendron jiringa* Nielsen seeds. *African Journal of Biotechnology* 11: 10026-10040.
3. Chantaranothai, C., Palaga, T., Karnchanatat, A., and Sangvanich, P.* (2013) Inhibition of nitric oxide production in the Macrophage-like Raw 264.7 cell line by protein from the rhizomes of Zingberaceae plants. *Preparative biochemistry and biotechnology* 43: 60-78.
4. Rungsaeng, P., Sangvanich, P., and Karnchanatat, A.* (2013) Zingipain, a ginger protease with acetylcholinesterase inhibitory activity. *Applied Biochemistry and Biotechnology* 170: 934-950.

*Coressponding author
Tel.:0-2218-7637; Fax: 0-2252-6920
E-mail: spolkit@chula.ac.th, polkit@gmail.com