Antioxidant and antiproliferative activities of protein hydrolysate from the rhizomes of Zingiberaceae plants

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Abstract: Plant proteins have been investigated for their antioxidant activities, but there are still no reports detailing the antioxidant activity levels of plants in the Zingiberaceae family, which are popular food agents and used in folklore medicine. In this study, the crude rhizome protein extract and associated pepsin/pancreatin protein hydrolysate of 15 plants in the Zingiberaceae family were screened using the DPPH method for antioxidant activity. The protein hydrolysate of *C. zedoaria* possessed the highest antioxidant activity (IC_{50} of 25.7±6.3µg/mL), which was close to that of the reference ascorbic acid (IC_{50} of 22.3±1.8µg/mL). After enrichment by Q Sepharose ion exchange chromatography using a five step elution gradient of increasing NaCl concentration (0, 0.25, 0.5, 0.75 and 1M), the fraction eluting in the 0.5M NaCl (F50) showed the highest antioxidant activity (IC_{50} of 41.78±2.9µg/mL), and was found to have weak *in vitro* cytotoxicity against the HEP-G2 and SW620 cell lines (IC_{50} of 200.8±11.8 and 241.0±9.3µg/mL, respectively), but not the BT474, CHAGO and KATO-3 cell lines. F50 had an estimated molecular weight by MALDI-TOF mass spectrometry of 12,400-12,800 Da.

Keywords: Antioxidant, antiproliferative, protein hydrolysate, Zingiberaceae plants.

INTRODUCTION

Free radicals and reactive oxygen species (ROS) are chemically reactive species because of their unpaired valence shell electrons (Roberts *et al*., 2010). They are normally created in living systems via the electron transport systems of mitochondria, chloroplasts and plasma membranes in the respiration of aerobic organisms (Foyer *et al*., 1997). Although essential in various functions, such as apoptosis and defense against pathogens, or as a signal for certain stress responses, the excessive or inappropriate production and accumulation of ROS and free radicals is damaging and the cause of diseases or reduced fitness in many plants and animals. Within humans, excessive levels of free radicals have been implicated in the cause of many chronic diseases that affect the daily life of people, including arthritis, cancer, atherosclerosis, diabetes, Alzheimer's disease, Parkinson disease and aging (Ames *et al*., 1993). For instance, hydrogen peroxide (H_2O_2) and the super oxide radical ($O_2^{\bullet-}$) are created in the nervous and immune systems, whilst there is an abundant supply of iron in many parts of the human body that can be simply activated and eventually encourage free radical reactions ROS production can be increased by different external stresses and some biotic factors like pathogenic intrusion into the body (Roberts *et al*., 2010; Foyer *et al*., 1997; Ames *et al*., 1993; Halliwell and Gutteridge 1989; Boo Jung 1999). Free radicals can be found in every part of the body, but within acceptable limits. It is the excess levels of free radicals and ROS above these limits that can start chain reactions, which damage cells and finally cause several ailments. The chain reaction of free radicals can easily induce per oxidation of membrane lipids, which is involved in initiation of many diseases (Halliwell 1999; Halliwell and Whiteman 2004). Therefore, although their occurrence within limits is required for correct bodily functions, too many free radicals in the organism are harmful. Oxidative stress, when the production rate of these highly reactive species exceeds the level of their removal, leading to an increase in free radicals concentrations above acceptable limits for that issue, gives rise to many problems in both the short term and chronically. Likewise, the input of excess amounts of free radicals, such as from saturated fat, air pollution and some kinds of drugs, is undesirable for the same reasons.

Antioxidants are compounds that help prevent oxidation from oxidants and are mediated by several mechanisms, including electron donation to the oxidant that changes to a more stable compound that will not react further. Super oxide dismutases (SOD) remove O_2^- by catalyzing its conversion to H_2O_2, while catalases (CAT) in the per oxisomes convert the H_2O_2 into the non toxic H_2O and (at these concentrations) O_2 and so help to eliminate H_2O_2 which is created by SOD. Glutathione per oxidase (GP_x) is also involved in the removal of H_2O_2. Although there are various defense mechanisms in the human body to protect the build up of local or systemic levels of free radicals, which consist of the antioxidant enzymes and

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non-enzymatic compounds, oxidative stress can still happen because living systems are incapable of resisting and scavenging all ROS (Halliwell *et al*., 1995; Sies 1993; Miyake *et al*., 2000; Tseng 1997). Hence, due to excessive amount of oxidants, it can be beneficial or required to ingest additional antioxidants into the body.

Synthetic antioxidants are potentially harmful since they can be adsorbed into and remain in the body for a long period and interfere with the normal non-pathogenic ROS functions, and also damage the liver and can eventually cause cancer (Ito *et al*., 1986). These disadvantages of synthetic antioxidants are not found in natural antioxidants and so these are considered to be interesting alternatives to the commonly used artificial antioxidants, such as butylated hydroxyl toluene, butylated hydroxyl anisole, propyl gallate and tert-butyl-hydroquinone (Grice 1988; Becker 1993). Natural antioxidants, like ascorbic acid (vitamin C), tocopherol (vitamin E), and more recently some diverse phenolic extracts from plants, have been demonstrated to have suitable antioxidant activities (Krings and Berger 2001; Brown and Rice-Evans; 1998). Antioxidative proteins, and their derived hydrolysates and peptides, have attracted a lot of interest in recent years due to their high specific activity and safety. In addition, hydrolyzed proteins are considered as better options than antioxidative enzymes because of their endurance under various conditions, simpler structure, and, especially, are typically without danger in food as following oral administration they are degraded into the harmless natural components of amino acids (Mitsuta *et al*., 1996; Hatate *et al*., 1990; Maw *et al*., 2011).

Zingiberaceae is a well-known family of flowering plants of more than 1300 species that are found in the tropical climates of Africa, Asia and South America. Southeast Asia has the greatest diversity of this family of plants and includes some 200 species (from 20 Genera) within Thailand (Larsen 1980). Within Thailand, some species of these perennial plants have been used as spices, preservatives, and herbs in Thai cooking and traditional folk medicine. Therefore, it is of interest to evaluate the antioxidant activity of the protein hydrolysates extracted from the rhizomes (the most abundant part of the plants and the part most used in folklore medicine and cooking) of these species of Zingiberaceae. Although there has been a fair amount of research into antioxidant compounds from members of the Zingiberaceae, most of them are small molecules, with very few studies about the antioxidative activities of protein hydrolysates. The objective of this study was to screen, purify, determine, and characterize the antioxidative activity of the protein hydrolysates obtained from the rhizomes of different species of Zingiberaceae from Thailand (Jatoi *et al*., 2007; Lu *et al*., 2009; Mau *et al*., 2003; Victorio 2011).

MATERIALS AND METHODS

Chemical and biological materials

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^{o}C room until used.

Chemical materials

Ammonium sulphate, ascorbic acid, bovine serum albumin (BSA), 2,2-Diphenyl-1-picryhydrazyl (DPPH), pancreatin from porcine pancreas, and pepsin from porcine gastric mucosa were purchased from Sigma Chemicals Co. (USA). Q Sepharose fast flow was purchased from Amersham Biosciences, (Sweden). All other chemicals used in the investigation were of analytical grade. The five human tumor derived cell lines, BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon), were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand and were used at a passage of 3-5 in this study.

Preparation of the rhizome extract from each species of Zingiberaceae

For each of the 15 selected species, 1.5kg of rhizome were pared, sliced into small parts and then homogenized using a blender (Philips, HR 2061, Indonesia) in 5 L of phosphate buffered saline (PBS; 0.15M NaCl with 20 mM phosphate buffer pH 7.2) using a mixer and subsequently stirred overnight at 4^{o}C with a magnetic stirrer. Filtration through double-layered cheesecloth was used to separate the suspension from the liquid and then the filtrate was clarified by centrifugation (15,000×g, 30 min, 4^{o}C) and the supernatant harvested. Ammonium sulfate was added to 80% saturation and subsequently stirred overnight at 4^{o}C prior to centrifugation (15,000× g, 30 min, 4^{o}C) with harvesting of the insoluble material (pellet). The pellet was then dissolved in 20mL PBS, dialyzed against excessive amounts of water using dialysis tubing with 3500 Dalton cut-off (SnakeSkin, Thermo scientific Co., Ltd., USA), and lastly freeze dried to yield the crude protein preparation. Prior to use the crude protein preparations were dissolved in 10 to 25mg/mL.

Hydrolysis of crude protein by pepsin and pancreatin

The crude proteins produced from the rhizome of each species were used as a substrate for production of the protein hydrolysate following the method of Torres-Fuentes *et al*., (2011). In brief, each crude protein preparation at 10mg/mL was mixed with pepsin until the final substrate/enzyme (g/g) concentration ratio was 20:1 and adjusted to pH 2.5 by 1 M HCl. The hydrolysis was carried out for 180 min at 37^{o}C with shaking (180 rpm), was and then inactivated by adding 1 M NaOH to pH 7.5. Next, pancreatin was added to a 20:1 (g/g) substrate:

enzyme ratio and shaken (180 rpm) for 180 min at 37°C. The hydrolysis (enzyme reaction) was stopped by heating at 80°C for 20 min. Hydrolysates were clarified by centrifugation (15,000 × g, 30min, 4°C) and kept at -20°C until use. The choice of these two proteases was to crudely mimic that in the human gastro-intestinal tract.

Measurement of free radical scavenging capacity by the DPPH assay

The antioxidant activity of each of the fractions was determined using the DPPH assay by combining 100µL of 200µM DPPH (4mg of DPPH in 100mL of methanol) with 100µL of sample at various concentrations (0-200mg/mL) into 96-well plates and then incubating under dark conditions for 30 minutes at room temperature prior to reading the absorbance at 517nm in a microplate reader (Deng *et al*., 2011). The negative control (blank) used water instead of the protein / protein hydrolysate sample while the positive control was ascorbic acid (0-50mg/mL). The activity is reported as the IC_{50} value, the concentration which inhibits DPPH oxidation by 50% under these conditions (Jatoi *et al*., 2007; Lu *et al*., 2009). The percentage of radical scavenging was determined as in Eq. (1):

$$\% \text{ radical scavenging} = \frac{(A\text{-}Ab)\text{-}(As\text{-}Asb)}{(A\text{-}Ab)} \times 100 \quad (1)$$

Where A is the absorbance of water with DPPH (in methanol), Ab is the absorbance of the blank (water with methanol without DPPH), As is the absorbance of the sample with DPPH (in methanol) and Asb is the absorbance of the sample with water without DPPH.

Enrichment of the protein hydrolysate

A sample (6mL) of the protein hydrolysate (50mg/mL) from *Curcuma zedoaria* (Berg) Roscoe. was injected into a 5mL loop of an automatic liquid chromatography system (AKTA prime, Amersham Pharmacia Biotech, Sweden) with a 15cm length glass column filled with swelled Q Sepharose fast flow gel. The column was equilibrated and the sample loaded in 20mM Tris-HCl, pH 7.2. This buffer was also used as the mobile phase for elution, at a flow rate of 1.5mL/min and collecting 10mL fractions, except that it was replaced by 1M NaCl as a stepwise gradient of 0, 25, 50, 75 and 100% (v/v) 1 M NaCl, each increment step being changed every 100mL of elution. The protein content of each fraction was crudely estimated by determination of the absorbance at 280 nm, and subsequently by the method of Bradford (1976). Data was analyzed and demonstrated by Prime view version 1.00 (Amersham Biosciences). Finally, all fractions in the same peak were pooled together and dialyzed at 4°C overnight with 5L of deionized water for three times in order to remove salts from the substance, freeze-dried, and kept at -20°C until further use.

Protein concentration

During the ion exchange chromatography, the amount of protein in each fraction was crudely estimated by monitoring the absorbance at 280 nm. For other steps in this investigation, the Bradford assay was used for measuring the protein concentration using BSA (0-200µg/mL in 25µg/mL increments) as the reference protein for calibration. Samples were diluted so as to lie within this concentration range (25-200µg/mL). Then, 50µL of the sample was mixed with 50µL of Bradford reagent, shaken for 5 min the absorbance was measured at 595nm. The absorbance was then used in calibration curve to determine the concentration of the sample (Bradford 1976).

In vitro cytotoxicity assay for human malignant cell lines

The *in vitro* antiproliferative / cytotoxic activity of the crude protein or enriched protein hydrolysate was performed on five different human malignant cell lines in tissue culture, comprised of the BT474 (breast), HEP-G2 (hepatoma), CHAGO (lung), SW620 (colon), and KATO-3 (gastric) cell lines. Cells were kept in complete media (CM), composed of RPMI-1640 supplemented with 2.0 mM L-glutamine and 10% (v/v) FCS, at 37°C under 5% (v/v) CO_2. Cells were aspirated, trypsinized, and finally washed prior to being suspended at 5×10^3 cells/µL in CM and seeded at 200µL per well of a 96-well plate and cultured for 1 day. Later, serial dilutions of the *C. zedoaria* crude protein or enriched protein hydrolysate preparation was added into each well at various concentrations (0-25µg/mL in 200µL of CM), combined together and incubated for 3 days. Then, 10 µL of 3-[5,5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium]bromide solution (5mg/mL) (MTT) was added into each well and incubated for 4 h. The media was then gently aspirated off, the cells washed with RPMI-1640 to remove residual media and aspirated off. Subsequently, 150µL of dimethylsulphoxide (DMSO) was added into each well, left for 30 minutes and then aspirated to fully lyse all the cells and dissolve the formazan crystals, and then the absorbance at 540nm was determined by a microtiter reader. Each cytotoxicity assay was done in triplicate with the CH-Liver cell line as the negative control and 10µg/L doxorubicin without PPC as the positive control.

Identification of the molecular weight of the antioxidative protein hydrolysates by MALDI-TOF MS

Matrix-assisted laser desorption/ionization mass spectrometer (MALDI-TOF MS) was used to identify the molecular weight of the active protein hydrolysate. The pooled fraction that possessed the highest antioxidant properties was analyzed by MALDI-TOF MS with a delayed extraction source and a 355 nm pulse nitrogen laser. The MALDI MS was performed in the linear mode with a 20 kV acceleration. The sample was combined with a matrix solution (20mg/mL of sinapinic acid in a 1:1 (v/v) ratio of water: Acetonitrile. For each sample loading, 0.5-1µL was placed on the MALDI target plate. All results are derived from 200 shots. Moreover,

the MALDI-TOF MS was performed in the positive mode.

STATISTICAL ANALYSIS

All data were determined from triplicate repeats and are presented as the mean ± one standard error of mean (SEM) while regression analysis was accomplished by using of Graphpad Prism Version 4.00 for Windows (Graphpad Software Inc.). Statistical tests of significant difference were performed by ANOVA and Duncan's MMT for parametric data, and by Kruskal-Wallis and Mann-Whitney U tests for non-parametric data, with $p < 0.05$ being accepted as significant.

RESULTS

Screening for antioxidative protein hydrolysate in plants samples

The crude protein preparation from the rhizome of each of the 15 selected plant species, and their respective pepsin/pancreatin protein hydrolysates were screened for *in vitro* antioxidant activity using the DPPH assay. These species were selected due to their abundance and their importance in Thai culture as spices, food, and also as medicinal herbs. The results, reported as IC_{50} values (table 1), were calculated from the regression equation derived from the % inhibition versus the concentration of sample. Note that the IC_{50} values reported here can not be directly compared with those reported in other investigations because this value depends on the experimental variables, such as the incubation time, type of solvent, and concentration of DPPH. Thus, we used the positive standard of ascorbic acid (vitamin C), a natural antioxidant, as a reference for comparing the IC_{50} values of samples because ascorbic acid is used in many studies (Heravi *et al*., 2012). The IC_{50} of ascorbic acid in this assay (22.3±1.8µg/mL) was similar to those values reported in other studies, and so the assay and its inherent variables as performed in this investigation is acceptable.

Among the 15 species of plants, which were screened, no significant antioxidant activity was detected in *Alpinia galangal* or *Kaempferia pafiflora*. Of the remaining 13 plants with at least some detectable antioxidant activity in the crude rhizome protein preparation the greatest antioxidant activity was found in *Boesenbergia pandurata* (IC_{50}=27.2±4.2µg/mL), which was close to that for ascorbic acid (22.3 ±1.8µg/mL), followed by *Curcuma sp*. and *Zingiber cassumunar*, respectively, while the lowest antioxidant activity was seen for *C. aeruginosa* (IC_{50} =114.5±8.8µg/mL). However, after enzymatic hydrolysis the *in vitro* antioxidant activity of the protein hydrolysate from *B. pandurata* was dramatically reduced (4.8-fold lower IC_{50} value), making it then in fact the second lowest antioxidant activity of the 13 protein hydrolysates (the lowest was that for *Z. officinale* at no detectable activity).

The formation of the protein hydrolysates generally increased the antioxidant activity (in terms of the IC_{50} value) compared to that observed in the respective parental protein preparation, the two further exceptions in addition to the already noted *B. pandurata* and *Z. officinale*, were *C. aromatic* and, perhaps, *C. amarissima*. That aside, with respect to the protein hydrolysates, the highest *in vitro* antioxidant activity was found for *C. zedoaria* (IC_{50} =25.7±6.3µg/mL), followed by *Curcuma sp.* (IC_{50}=36.3±1.9µg/mL) and *Z. cassumunar* (IC_{50}=38.6±4.9µg/mL), respectively.

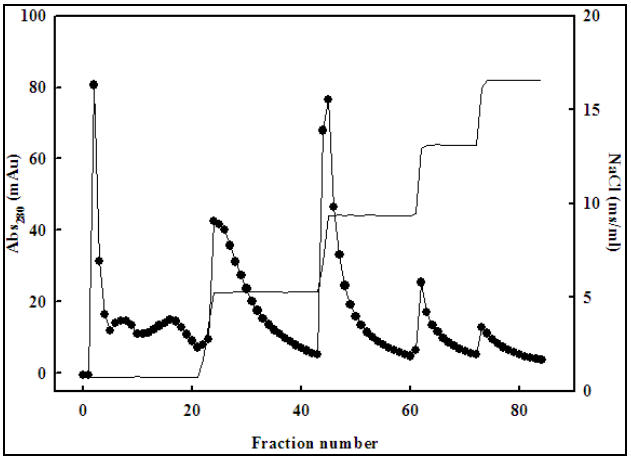

Fig. 1: (**A**) Q Sepharose chromatogram of the protein hydrolysate fraction of *C. zedoaria* rhizome with stepwise NaCl elution (0.00, 0.25, 0.50, 0.75 and 1.00 M). Fractions were assayed for absorbance at 280 nm. Profile shown is representative of three independent trials.

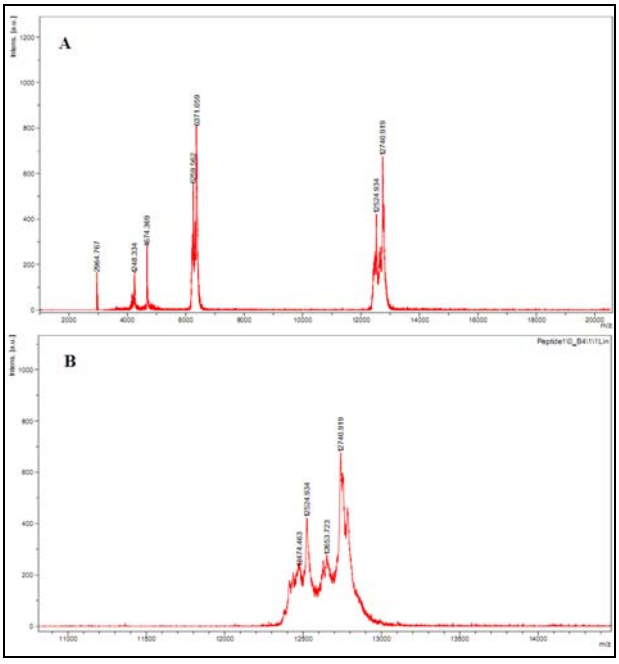

Fig. 2: MALDI-TOF mass spectrum of the protein hydrolysate of *Curcuma zedoaria* showing the (A) whole spectrum and the (B) portion at a m/z ~12,000 Da.

Table 1: The antioxidant activity of crude proteins and protein hydrolysates of 15 species within the Zingiberaceae family[a] and the ascorbic acid reference control.

Plant species	IC_{50} of crude protein (µg/mL)	IC_{50} of protein hydrolysate (µg/mL)
Alpinia galangal (Linn.) Swartz.	ND	ND
Boesenbergia pandurata Roxb.	27.2±4.2	131.8±6.4
Curcuma aeruginosa Roxb.	114.5±8.8	84.5±3.7
Curcuma amarissima Roscoe.	108.7±9.8	127.0±8.2
Curcuma aromatic Salisb.	56.4±2.4	118.2±2.4
Curcuma longa Linn.	109.6±8.6	ND
Curcuma sp.	37.2±0.3	36.3±1.9
Curcuma xanthorrhiza Roxb.	72.7±1.8	39.4±3.2
Curcuma zedoaria (Berg) Roscoe.	57.8±1.6	25.7±6.3
Hedychium coronarium	91.5±4.6	56.1±1.8
Kaempferia pafiflora Linn.	ND	ND
Zingiber cassumunar	50.6±5.9	38.6±4.9
Zingiber officinale Roscoe.	108.8±2.3	ND
Zingiber ottensii Valeton.	66.7±8.0	44.9±4.7
Zingiber zerumbet Smith.	92.0±2.8	54.6±3.6
Ascorbic acid (Vitamin C)	22.3±1.8	

[a]All data are shown as the mean ± 1 SEM and are obtained from three replicated determinations. Means within a column or row with a different lowercase superscript letter are significantly different ($p<0.05$). ND = Not detected

Table 2: DPPH radical scavenging activities (% inhibition and IC_{50} values) of the five separated fractions from the Q Sepharose ion exchange chromatography, as measured at 10µg/mL[a]

Fraction	% inhibition	IC_{50} (µg/mL)
F0	11.0±3.3	ND
F25	2.2±3.1	ND
F50	74.2±0.7	41.8±2.9
F75	5.4±3.3	ND
F100	1.8±1.7	ND

[a]All data are shown as the mean ± 1 SEM and are obtained from three replicated determinations. ND = Not determined as 50% inhibition was not reached at the concentrations tested. (i.e. IC_{50}>100µg/mL)

Therefore, the protein hydrolysate from *C. zedoaria* was chosen for further purification and characterization due to its high antioxidant activity. However, two other species of plants that remain of interest for further study, because of their fairly high *in vitro* antioxidant activity in both the crude protein preparation and the protein hydrolysate, were *Z. cassumunar* and *Curcuma sp.*

Partial purification of protein hydrolysate by ion exchange chromatography

The protein hydrolysate obtained from the pepsin/pancreatic hydrolysis of the *C. zedoaria* rhizome protein preparation was enriched by Q Sepharose ion exchange chromatography. Increasing the ion concentration (Cl^-) in the mobile phase was then used to elute the bound anions by competitive displacement. In this study a five step increasing concentration of NaCl was used and resulted in the five different major peaks, F0, F25, F50, F75 and F100, eluting in the 0, 0.25, 0.5, 0.75 and 1.0M NaCl containing mobile phase, respectively (fig. 1). All fractions in the same eluted protein peak were pooled, concentrated and their antioxidant activity measured by the DPPH assay. Due to the extremely small amount of protein in some peaks, their IC_{50} values could not be calculated directly. All five peaks were adjusted to the same concentration (10 µg/mL) before the *in vitro* antioxidant activity was measured as the % inhibition of the DPPH assay. The F50 fraction (eluted in 0.5M NaCl) showed the best antioxidant activity, with an IC_{50} of 41.8±2.9µg/mL (table 2).

Molecular weight determination of purified protein hydrolysate (fraction F50)

The protein hydrolysate fraction with the highest antioxidant activity (fraction F50 from *C. zedoaria*) was determined for its molecular weight by MALDI TOF mass spectrometry (fig. 2). There were several small MW peaks in the spectrum, such as at a m/z ratio of 2964, 4248 and 4674, but there were only two major regions, which had m/z values of approximately 6,200-6,400 Da

and 12,400-12,800 Da, respectively. Interestingly, the m/z ratio of high peak at 6,259 is about half of the m/z of peak at 12,524 Da and the m/z ratio of peak at 6,371 Da is exactly half of the m/z of the high peak at 12,741 Da. Therefore, the peaks located around 6,200-6,400 Da were considered as simply double charged entities of the same molecule. For this reason, peaks at a m/z =12,400-12,800 Da were considered as the actual molecular weight of compound. In conclusion, the molecular weight of purified protein hydrolysate (fraction F50) was around 12,400 -12,800 Da.

Cytotoxicity assay for human malignant cell lines

The active protein hydrolysate (fraction F50) was evaluated for potential *in vitro* antiproliferative and/or cytotoxic activity (without distinguishing between the two) along with that for the crude protein extract and associated protein hydrolysate and the different Q Sepharose eluted fractions, against five human cancer derived cell lines in tissue culture. All three fractions which had revealed an *in vitro* antioxidant activity in the DPPH assay revealed an insufficient antiproliferative/ cytotoxic activity to be able to determine the IC_{50} value, except for the enriched F50 protein hydrolysate on two cell lines (HEP-G2 and SW620), but with a relatively high IC_{50} values (low activity) of 200.8$\pm$ 11.8 and 241.0$\pm$9.3µg/mL, respectively.

DISCUSSION

The crude protein and protein hydrolysate (hydrolysed by pepsin and pancreatin under virtual condition as in human body of each kind of plants were chosen to be measured the antioxidant activity by DPPH. Fifteen Thai species from the Zingiberaceae family were chosen to be observed in this experiment due to their abundance and their importance in Thai culture as spice, ornament, food, and also as medicinal herb. The results were reported as IC_{50} values which are calculated from the regression equation derived from the % inhibition versus concentration of sample. Actually, the IC_{50} value could not be compared with the IC_{50} value from other investigation because this value depends on a lot of variables such as incubation time, type of solvent, and concentration of DPPH. Thus, the positive standard is necessary and ascorbic acid, natural antioxidant, was used as a reference for comparing the IC_{50} values of samples because ascorbic acid has been one of well known antioxidant and has been used widespread in many studies. The IC_{50} of ascorbic acid obtained was similar to the value that was reported in other studies which revealed that variables chosen in this investigation is quite acceptable.

It has been shown from all of the results that *C. zedoaria* was the plant which occupied largest improved antioxidant activity when it was hydrolysed. This result showed that the consumption of *C. zedoaria* was most beneficial although its crude protein was not that much effective because virtual enzymes were used in the hydrolysis process. The obtained IC_{50} value of crude protein from *Curcuma zedoaria* was only just 57.85±1.61 µg/mL which was not considerably high when comparing with other plants, but then changed to 25.70±6.33µg/mL after it was hydrolysed. Thus, *C. zedoaria* was chosen for further purification and characterization due to its greatly high antioxidant activity.

The IC_{50} value of fraction F50 was increased comparing to the IC_{50} of protein hydrolysate before purifying which might be caused from many reasons such as the loss of active site or active bond due to bond cleavage (Chen *et al.,* 1998; Nagasawa *et al.,* 2001), the loss of crucial amino acid, for instance, Tyr, Trp, Met, Lys, His, and Cys (Rajapakse *et al*., 2005; Wang and Gonzalez de Mejia, 2005; Qian *et al*., 2008), the change in sequence of amino acid (Chen *et al*., 1995; Chen *et al.*, 1996; Rajapakse *et al*., 2005), and the change in the number of amino acid (Li *et al*., 2008). Futhermore, there were some reports showing that protein hydrolysate exhibited better antioxidant activity than purified peptide (Chen *et al*., 1995; Pappenheimer and Volpp, 1992).

5-Fluorouracil (5-FU) was a commercial drug using for treatment of cancer and was reported that it had IC_{50} against HEP-G2 as 82.35±36.97µg/ mL (Mahavorasirikul *et al*., 2010) and had IC_{50} against SW620 as 12.94±0.30µg/mL (Tong *et al*., 2011). Therefore, the IC_{50} value of *C. zedoaria* was comparable to the positive standard against HEP-G2 which showed that the protein hydrolysate from *C. zedoaria* was more specifically active with hepatic cancer than with colorectal cancer while there was no reactivity with other types of cancer such as stomach cancer, breast cancer, and lung cancer.

CONCLUSION

Inappropriate levels of free radicals have been considered as one of major causes for several chronic diseases in humans. Excessive free radicals in living systems that give rise to oxidative stress can be increased by oral consumption of free radical or free radical forming compounds and so additional antioxidants are necessary for consumption into the body or to reduce free radicals in food prior ton their adsorption. Of the 15 species of representative plants from the Zingiberaceae family that were screened by the DPPH assay, *C. zedoaria* showed greatest antioxidant activity with an IC_{50} value of 57.8±1.6 and 25.7±6.3µg/mL for the crude protein and associated protein hydrolysate, respectively. Enrichment of the *C. zedoaria* protein hydrolysate by Q Sepharose ion exchange chromatography yielded lower antioxidant activities in all fractions, the highest being found in fraction F50 (IC_{50} of 41.8±2.9µg/mL). Determination of

the MW of F50 by MALDI-TOF MS analysis gave an approximate size of 12.4-12.8 kDa and it was found to have weak cytotoxic/antiproliferative activity against only two out of five tested human cancer derived cell lines (IC_{50} of 241.0±9.3 and 200.8±11.8µg/mL for SW60 and Hep-G2 cell lines, respectively).

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