Introduction

Free radicals or reactive oxygen species, ROS, including superoxide radicals, hydroxyl radicals, and hydrogen peroxide, are often generated as byproducts of the body's metabolic process or from exogenous factors such as atmospheric pollutants and from transitional metal catalysts (Sreedhar et al., 2010). Oxygen free radical can initiate peroxidation of lipids which in sequence stimulated glycation of protein, inactivation of enzyme systems, decreased membrane fluidity and DNA mutations(Aqil et al., 2006)The oxidative damage derived from free radicals may be related to aging, degenerative and other diseases, such as atherosclerosis, cancer, inflammation, neurodegeneration and rheumatoid arthritis. Even though human body and other organisms have antioxidant defense and repair systems to protect them against oxidative damage, the systems are lacking to prevent all the damage. So antioxidant compounds in human diets or natural sources are of great notice as likely protective agents to help human body diminish oxidative damage. Many natural antioxidants have already been isolated from different kinds of plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs. (Mau et al., 2002). Many mushrooms are also rich sources of antioxidant compounds. As previous studies, many mushrooms are good sources of antioxidants. This report summarized antioxidant activity of mushrooms briefly

1. Antioxidant activity was determined by the 1,3 diethyl-2-thiobarbituric acid (DETBA) The antioxidant activity was expressed as the percentage of lipid peroxidation with a control containing no mushroom extract being 100%. A higher percentage indicated a lower antioxidant activity

- Huang, 2000. He found that methanolic extracts from the medicinal mushroom *Antrodia camphorate* (Chang-Chih) showed excellent antioxidant activities as evidences by 5.32-5.78% of lipid peroxidation at 1 mg/ml. *Agaricus brazei* (Brazillian mushrooms), showed a high antioxidant activity(26.0% of lipid peroxidation) at 1 mg/ml.
- Lin, 1999. At 1.2 mg/ml, only *Dictyophor indusata*(basketstinkhorn) showed excellent antioxidant activity (2.26% of lipid peroxidation). *Grifola frondosa* (maitake) showed a relatively high antioxidant activity (29.8% of lipid peroxidation), whereas *Hericium erinaceus* (lion'smane) and *Tricholoma giganteum* (white matsutake) showed moderate antioxidant activitys (48.45 and 67.02% of lipid peroxidation, respectively). Commercial mushrooms at 1.2 mg/ml, *Flammulina velutipes* (winter mushrooms), *Lentinula edodes* (shiitale), *Pleurotus cystidiosus* (abalone mushrooms), and *Pleurotus ostreatus* (tree oyster mushrooms) showed moderate to high anioxidan activities (24.71-62.30% of lipid peroxidation).

- Mau et al.,2001 He reported that methanolic extracts from ear mushrooms, including black, red, jin, snow, and silver ears, showed low to moderate antioxidant activity (57.71-71.5% lipid peroxidation) at 1 mg/ml.
- Gezer et al., 2006. Inhibition value of ethanolic *Ramaria flava* extracts, BHA and α-tocopherol standards were found to be 94.7, 98.9 and 99.2%, respectively, at 160 µg/ml).
- Turkoglu et al., 2007.The 50% of inbition value for *Russula delica* ethanol extract seem to be fairly significant when compared to commonly used synthetic antioxidant BHT, BHA and α-tocopherol. (IC 50 = 207.09 µg/ml ethanolic extract was necessary to obtain 50% of DPPH degradation)

2. Scavenging effect on 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH assay) ،

- Mau et al., 2002. Scavenging effects of methanolic extracts from medicinal mushrooms on DPPH radical increased with the increased concentrations. At 0.64 mg/ml, scavenging effects were 67.6-74.4% for *Ganoderma lucidum* (Ling-chih), *Ganoderma tsugae*(Sung-shan-chih) and 24.6% for *Coriolus versicolor*(Yun chih) . It was anticipated that scavenging effects would be excellent for Ganoderma and higher for *C.versicolor* at concentrations >0.64 mg/ml. However the scavenging effects of BHA and α-tocopherol at 20 mM(3.6 and 8.6 mg/ml) were 96 and 95% respectively.
- Huang, L.C. found that excellent scavenging effects(96.3-99.1 and 97.1%) were observed with methanolic extracts from *A.camphorata* and *Brazillian* mushrooms at 2.5 mg/ml, respectively.
- Lin, H.-C. reported that at 0.64 mg/ml, the methanolic extract from stinkhorn scavenged DPPH radical by 92.1% whereas scavenging effects of methanolic extracts from other specialty mushrooms were 63-67.8%. At 0.64 mg/ml, the methanolic extract from three oyster mushrooms scavenged DPPH radical by 81.8%, whereas scavenging effects of extracts from other commercial mushrooms were 42.9-69.9%
- Mau et al., 2002. revealed at 1 mg/ml, methanolic extracts from black and red ear mushrooms scavenged DPPH radical by 94.5% at 0.4 mg/ml and 95.4% at 3 mg/ml, respectively. However, silver ear mushrooms were not effective in scavenging DPPH radical (71.5% at 5 mg/ml).
- Chirinang & Intarapichet 2009. *P. ostreautus* possessed more antioxidant than *P.sajor-caju.* The EC50 of *P.ostreatus* and *P.sajor-caju* water extracts were 11.56 and 13.38 mg/ml, respectively, while those of the ethanol extracts were 31.75 and 58.44 mg/ml, respectively.

Organisms have an inclusive assortment of antioxidant defense mechanisms to diminish free radical formation or reduce their damaging effects. These include proteins such as superoxide dismutase and catalase to decay superoxide and peroxidase respectively and essential radicals scavengers like ascorbic acid and glutathione peroxidase reductase, vitamin E (tocopherols and tocotrienols), vitamin C etc. apart from many dietary components. An enhanced antioxidant status helps to minimize the oxidative damage and thus to delay or prevent pathological changes. Potential antioxidant therapy should be therefore included either as natural free radical scavenging antioxidant enzymes or as agents which are able of augmenting of the antioxidant enzymes.

Selenium is an essential trace element in the human body, an important part of the antioxidant enzymes that protect cells against the effects of free radicals that are produced during the normal oxygen metabolism. Many mushrooms are also good sources of Se .Our bodies have developed defenses, which can damage cells and contribute to the development of some chronic diseases. Se is an essential trace element for both human beings and animals that has received considerable attention(Chen and Berry 2003;Tp 1998; Tapiero and others 2003). The essentiality of this element is due to the requirement for the 21st amino acid, selenocysteine, which is used for the synthesis of about a dozen selenoenzymes, including glutathione peroxidase(reducing peroxides), iodothyronine deiodinases (regulating thyroid hormone acitivity), and thioredoxin reductases(regenerating antioxidant systems)(Arner and Holmgren 2000; Brigelius-Flohe, 1999; Crack and others 2001). Compared with these well characterized enzymes, many functions of growing number of selenoproteins remain unclear (Chen and Berry 2003). Se also is needed for the proper functioning of the immune system, and appears to be a key nutrient in counteracting the development of virulence and inhibiting HIV progression to AIDS. In addition to incorporation as slenocystein, selenium can replace sulfur in methionine forming selenomethionine, which can also be incorporated nonspecifically into proteins in place of methionine(Behne and Kyriakopoulos 2001). Therefore Se functions as a redox centre, for instance when the selenoenzyme, thioredoxin reduxtases, reduces nucleotides in DNA synthesis and helps control the intracellular redox state, or when the Se-dependent iodothyronine deiodianese produce active thyroid hormone from inactive precursor. The best known example of this redox function is the reduction of hydrogen peroxide and damaging lipid and phosphlipid hydroperoxides to harmless products(water and alcohols) by the family of selenium-dependent glutathione peroxidases. This function helps to maintain membrane integrity, protects prostacyclin production, and reduces the likelihood of propagation of further oxidative damage to biomolecules such as lipids, lipoproteins and DNA with the associated increased risk of conditions such as atherosclerosis and cancer. Because Se can only be obtained from food, it seems to be very important for human beings to find dietary sources. The Se content of plants

varies tremendously according to its concentration in soil, which varies regionally. There are regions in which the Se concentration in the soil is very low. These include arid regions in Australia, northeast and south central China, northern North Korea, Napal and Tibet. Among people living in these low-Se region, there is a high incidence of Se-related diseases such as Keshan disease and cancer. Cancer is an uncontrollable growth and spread of cells may agent almost any tissue of the body. Lung, colorectal and stomach cancer are among the five most common cancers in the world for both men and women. The World Health Organization (WHO) has reported that more than 10 million people are diagnosed with cancer every year. With that high prevalence of cancer cases, researching for naturally occurring agents, such as Se, which may inhibit cancer development, is becoming an important objective for scientists. The first demonstration of the functional role of Se came in 1973, when Rotruck and his colleages established the biochemical basis for the role of Se in the glutathione peroxidase enzyme; this enzyme is responsible for preventing damage caused by oxidative stress. To date, the use of Se has been examined worldwide in human clinical trials. The first human intervention trials to prevent cancer with Se were performed in China. Se was used for synthesis selenoproteins.

Selenocysteine(Sec) has a similar structure to cysteine, with an atom of Se taking the place of Sulfure . Proteins that include a selenocyteine are called seleno proteins. The general pathway of selenoprotein synthesis involves four cell genes, selA, selB, selC, and selD. There are four steps in this mechanism. The first step involves tRNA(selC), which gets charged with serine. SelA then converts this serine to selenocysteine in step two. Afterwards, selD provides a Se donor, and the selB translation factor recognizes the selenocystely-tRNA and then delivers it to the UGA on the ribosome.

Table 1: Known selenoproteins that carry out nutritional functions of selenium

Selenoproteins
- Glutathione peroxidases(GPx1, GPx2, GPx3, GPx4)
- (Sperm) mitochondrial capsule selenoprotein
- Iodothyronine deiodinases (three isoforms)
- Thioredoxin reductase (probably three isoforms)
- Selenophosphate synthetase, SPS2
- Selenoprotein P
- Selenoprotein W
- Prostate epithelial selenoprotein (15 kDa)
- DNA-bound spermatid selenoprotein (34 kDa)
- 18 kDa selenoprotein

Mushrooms are defined as macrofungi with a distinctive and visible fruiting body that may be above or below ground. According to this definition, a large percentage of the fungi belonging to class Basidiomycetes and some fungi of class Ascomycetes are classified as mushrooms. The function of fungi in natural is degradation organic matter in animal waste products and plants litter into inorganic matter. Geologically, mushrooms existed on the earth even before man appeared on it, as evidenced from the fossils records of the lower cretaceous period. It is rather clear that human used the mushroom as food for a long time. Mushrooms are also good medicines because they have been found effective against cancer, cholesterol reduction stress, insomnia, asthma, allergies and diabetes(Wani et al.,2010). High Proteins amount were detected in mushrooms, they can be used to help protein malnutrition in vegetarian. They work as functional foods are used as nutrient supplements to enhance immunity in the form of tablets. Mushrooms two proteins, a sialic acid binding lectin and a laccase have also been previously identified from *H.erinaceum*. Other than the polysaccharide β-glucan and 2 proteins identified from these mushrooms, there are only a few report on other expressed protein components. Proteins are key to study as they have miscellaneous functions in the cell and are essential components involved in life activity of an organism from the cradle to the vital. Naturally, proteins are dynamic, some are constitutively expressed, and others are only expressed at specific time or conditions during the life cycle.(Horie et al., 2008). Mushrooms are a customary Asian medicine and also used as food for a long time. Currently, three species of medicinal mushrooms are commercially available in Taiwan, namely, *Ganoderma lucidum* (Curtis: Fr.) Karsten (Ling-chi or Reishi), *Ganoderma tsugae Murrill* (Sung-Shan-ling-chih), and *Coriolus versicolor* (Fr.) Quel.(Yun-chih or turkey tail). However, there are many species of commercial and edible mushrooms in Asia.

Mushrooms are used as food resource, contribute in material cycles, and have possibility of variable use in bioindustry. So far, studies on mushrooms have focused mostly with polysaccharides derived from cell wall and some specific proteins, but a proteomics-scale investigation aimed at creating a mushroom(s) proteome is still deficient. In this study investigates the antioxidant activity as well as proteins of some locally available edible mushroom in Phitsanulok, Loei and Trang Provinces of Thailand to provide a deeper insight in future.

Objective of this study

1. To compare profile of proteins of cultivated and natural mushrooms.
2. To study antioxidant activity of cultivated and natural mushrooms in vitro for application in future.

Materials and methods

Materials

Mushrooms samples

Cultivated and natural Lentinus mushrooms were collected from Phitsanulok, Loei and Trang Province. The cultivated Lentinus mushrooms, Horm (*Lentinula edodes* (Bull.) Singer), Lom (*Lentinus squarrosulus* Mont.) and Kornkaow (*Lentinus squarrosulus* Mont.) were purchased from farm in Phitsanulok and market in Loei Provinces. Natural Lentinus mushrooms, Lom mushrooms were perchased from market in Phitsanulok or Kradang *(Lentinus polychrous lev.)* mushrooms were collected from forest in Trang Province. The mushrooms were dried at 40 °C for 24 hours, grounded to fine powder using domestic electric grinder and kept at -50 °C until used for experiments.

Lentinula edodes (Bull.) Singer

Lentinus polychrous lev.

Lentinus squarrosulus Mont.

Chemicals

- Acrylamide (GE Healthcare,Sweden)
- Agarose (GE Healthcare,Sweden)
- Ammonium persulfate (Plusone, Amersham Bioscience,Sweden)
- Ammonium thiocyanate (BIO BASIC Inc., USA)
- Butylated hydroxyanisol(BHA) (Sigma Chemicals (St. Louis, MO).
- BSA strandard (Amersham Bioscience ,Sweden)
- Bromophenol blue (USB Corporation,USA)
- 3-[(3-chlamido propyl)-dimetylammonio]-1-propano sulfonate (CHAPS)(USB Corporation ,USA)
- Bio-Safe Coomassie (Bio-RAD Laboratories,USA)
- Dimethyl sulfoxide (Sigma- Alidrich,USA)
- 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Chemicals (St. Louis, MO).
- Dithiothreitol (DTT) (USB Corporation,USA)
- Ethanol (Lab scan analytical science,USA)
- Fetal bovine serum (FBS) (Gibco,USA)
- Ferrous chloride (BIO BASIC Inc., USA)
- Glycerol (ACS,USA)
- Hydrogen peroxide (Merck Schuchardt OHG,Germany)
- Iodoacetamide (GE Healthcare,Sweden)
- IPG buffer pH 3-10 (GE Healthcare,Sweden)
- IPG strip pH3-10NL 7 cm. (GE Healthcare,Sweden)
- linoleic acid (Sigma- Alidrich,USA)
- Methanol (Lab scan analytical science,USA)
- 2-mercaptoethanal (GE Healthcare,Sweden)
- Sodium dodecyl sulfate (SDS) (Amersham Bioscience, Sweden)
- Sodium chloride (Carlo ERBA reagenti,France)
- Sodium hydrogen carbonate (Fisher Scientific ,U.S.)
- Sodium hydroxide (Carlo ERBA reagent,France)
- Sodium carbonate (sigma, USA)
- Sodium EDTA (Bio-RAD Laboratories,USA)
- Sodium thiosulfate (Sigma, USA)
- α-tocopherol (Sigma Chemicals (St. Louis, MO).
- Tetramethylethylenediamine (TEMED) (USB Corporation, USA)
- Thiourea (Etton TM sample preparation kit reagent, GE Healthcare,Sweden)
- Tris hydroxymethyl aminomethane (USB,USA)
- Urea (USB Corporation, USA)

Methods

1. Extraction of total protein using Tris buffered phenol

Grind 1g of mushrooms to a powder with liquid nitrogen and transfer the tissue to 15 ml tube. Add 2.4 ml of chilled tris pH 8.8 buffered phenol and 2.4 ml of chilled extraction buffer and vortex immediately until all tissue is wet and place sample on ice. Mix samples and solution well for 30 min at 4° C and then centrifuge 10 min at 5,000 g at 4 °C. Transfer the phenol phase to a new tube and back extract aqueous phase again. Precipitate phenol extracted proteins by adding five volumes of cold 0.1 M ammonium acetate in 100% methanol (chilled at -20° C) to phenol phase. The solution were mixed and incubated at -20° C for at least 1 hour or overnight. The samples were centrifuged and collect the precipitate. Pellet was washed by adding five milliliters of cold 0.1 M ammonium acetate in methanol (-20° C) and resuspend sample. The resuspended sample was placed at -20° C for at least 15 minutes, then centrifuge 20 minutes, 20,000 g, 4° C. The sample was washed with cold 0.1 M ammonium acetate in methanol, 80% acetone, and cold 70% methanol, respectively. The samples were dried and resuspened the dried pellet immediately in homogenization buffer(0.2 M Tris-HCl buffer, pH 7.8, containing 5 mM EDTA·2Na, 14 mM 2-ME, 10%(v/v)glycerol, and 2 EDTA-free proteinase inhibitor tablets(Roche) per 100 ml of buffer solution in MQ H_2O or IEF buffer. The samples were used for protein quantification by Bradford method and stored at -80° C until analyzed by 1-DGE and 2-DGE.

To effectively solubilize the protein pellet, sodium dosesyl sulfate(SDS)-sample buffer[2.5x, 62 mM Tris(pH 6.8) containing 10%(v/v) glycerol, 2.5%(w/v) SDS, and 5%(v/v) 2-ME, pH 6.8] was added to mixture, followed by vortexing and centrifugation of the sample at 15,000 rpm for 15 min(4° C). The supernatant was used for protein determination by a Bradford solution, and stored in aliquots at -80 ° C until analyzed by 2D-PAGE.

2. Two-Dimensional Gel Electrophoresis

2-DGE was carried out using precast IPG strips (7 cm, pH 3-10NL) on an IPGphore unit (GE Healthcare Bio-Science AB, Upsala, Sweden) followed by 12.5% SDS-PAGE on a vertical electrophoresis unit. The volume carrying 99 μg of total protein was mixed. To visualized the protein spots, the 2D gels were stained with colloidal CBB G-250. Protein patterns in the gel were recorded as digitized images using a Image Scan III (GE Healthcare Bio-Science AB, Upsala, Sweden). ImageMaster 2D Platinum software ver.7.0 (GE Healthcare) was used for downstream analysis of detected protein spots on 2D gels.

3. Preparation of mushrooms extract

3.1 Methanolic mushroom extracts

Fine powder of each mushrooms 10 g was extracted by stirring with 100 ml of methanol at 25 °C at 20 g for 24 hours and filtering through Whatman No.4 filter paper. The residue was then extracted with two additional 100 ml portion of methanol. The combined methanolic extracts were then rotary evaporated at 40 ° C to dryness. The dried extract was used directly for analyses of antioxidant components or redissolved in methanol to a concentration of 20 mg/ml and stored at 4° C for further use.

3.2 Hot water extracts

Fine powder of each mushrooms 10 g was extracted by stirring with 100 ml of hot water at 80 °C for 24-48 hours and filtering through Whatman No.1 filter paper. The filtrate was lyophilized and stored at 4° C for further use.

4. Free radical scavenging assay DPPH(1,1-Diphenyl-2-picrylhydrazyl)

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-colored methanol solution of 1,1- diphenyl-2-picrylhydrazyl. Each methanolic extract of mushroom was mixed with 1 ml of methanolic solution containing DPPH (Sigma) radical, resulting in a final concentration of 0.2 mM DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm. BHA and α-tocopherol were used as controls. The percent inhibition was calculated from the following equation:

% inhibition = [(Absorbance of control–Absorbance of test sample) /absorbance of control] x100

5. Ferric thiocyanate(FTC) method

The standard method was followed from Kikuzaki and Nakatani was used. A mixture of 4 mg of plant extract in 4 ml of absolute ethanol, 400 µl of 2.52% linolenic acid in absolute ethanol and 800 µl of 0.05 M phosphate buffer (pH 7.0), was placed in a vial with a screw cap and then placed in a dark oven at 40 °C. The solution in each sample tube was removed 33 µl every 6 hours until 72 hours. Thirty three microlitres of 30% ammonium thiocyanate and one milliliters of 70% ethanol were added in the collected samples in interval 6 hours. Precisely 3 minutes after the addition of 33 µl of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of red color was measured

at 500 nm every 6 hours until one day after the absorbance of control reached its maximum(72hours). Butylated hydroxyanisol(BHA) and α-tocopherol were used as positive control.

6. Total selenium content in mushrooms

For determination of total selenium by ICP-MS, an agilent 7500 C Japan was employed with in house method based on AOAC (2000)Ch.9, 999.10. Approximately mushroom powder was digested by using HNO_3 and H_2O_2. To complete digestion, samples were covered with a watch glass and heat at reflux on a hot plate. Three replicated of each sample were prepared and analyzed. The reagent blank was digested in the same manner. The method of external calibration was applied for the quantitation of the selenium present in the sample.

7. Total phenolic compound in mushrooms

The amount of phenolics in the cultivated and natural muhrooms were determined with Folin-Ciocalteu reagent using the modified method of Spanos and Wrolstad. Briefly, to 25 µl of each sample (3 replicates), 400 µl of Folin-Ciocalteu reagent (1:10) and 800 µl of $NaHCO_3$ (7.5%, w/v) were added and the resulting mixture was incubated at dark for 60 minutes. The absorbance of all samples was measured at 765 nm. Results were expressed as milligrams of gallic acid equivalent/L

8. Determination of protein by Bradford solution

This method was performed by applied Bradford, M.M. (1976). Prepare standard concentrations of BSA of 1, 5, 7.5 and 10 µ g/ml. Add samples protein extracts about 20 µl to separate tubes (use microcentrifuge tubes) and add 1.0 ml of Bradford solution to each tube. Wait 2 minutes and read the absorbance of each standard and sample at 595 nm. Plot the absorbance of the standards vs their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.