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Preparation, Separation and Antioxidant Properties of Hydrolysates Derived from *Grifola frondosa* Protein

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Abstract

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In order to take full advantage of *Grifola frondosa* resources, the protein (GFP) was extracted from *G. frondosa* fruiting body and digested using six different proteases (papain, neutrase, alcalase, trypsin, pepsin, and Protamex) to produce the antioxidative hydrolysates. The trypsin hydrolysate prepared at 1.5 h possessed the strongest antioxidant potential among different hydrolysates, which was separated into four major fractions by ultrafiltration membranes with different molecular weight cut-off (MWCO), that are GFHT-1 (MW > 10 kDa), GFHT-2 (MW = 5–10 kDa), GFHT-3 (MW = 3–5 kDa), and GFHT-4 (MW < 3 kDa). In succession, the *in vitro* antioxidant activities of the four fractions were further evaluated, including the scavenging effect on DPPH, ferrous ion chelating effect, reducing power, and ability to inhibit the autoxidation of linoleic acid. The results demonstrated that all fractions are effective antioxidants and comparably GFHT-4 has the highest antioxidant activity. Then GFHT-4 was separated to two major peptide fractions by gel filtration chromatography, and the two fractions were located at 2385 Da (F-1) and 1138 Da (F-2), respectively.

Keywords: mushroom; trypsin antioxidant activity; peptides

The oxidation of lipids and proteins of food products would reduce consumer acceptability of foods by causing undesirable consequences in appearance, texture, flavour, and nutritional quality, as well as possible production of toxic compounds (Lund *et al.* 2011). Consumption of these potentially toxic products may contribute to a wide range of human diseases, including cancer, arteriosclerosis, and other aging related diseases (Sarmadi & Ismail 2010). To prevent foods from undergoing such deterioration and to provide protection against serious diseases, it is very important to inhibit lipid peroxidation occurring in foodstuffs. Therefore, antioxidants are now increasingly added to foods to reduce oxidation in the food industry (Choe & Min 2009). Many artificial antioxidants exhibit strong antioxidant activity against several oxidation systems, but they are suspected to be carcinogenic at sizeable intake. So it is necessary to search and develop antioxidants from naturally available sources that are more effective and safe.

In the past several years, many hydrolysed proteins from many plant and animal sources have been found to possess antioxidant activity, such as soybean (Gibbs *et al.* 2004), peanut protein (Chen *et al.* 2007), milk casein (Blanca *et al.* 2007), fish protein (Chen *et al.* 2012a), and so on. Therefore, they are widely used in food products as natural antioxidants. Despite these previous investigations, little information is yet available on an antioxidant hydrolysate from mushroom proteins. *Grifola frondosa* is one of the most popular edible mushrooms, which has been under a large-scale artificial cultivation and increasingly consumed in China and other Asian countries. Many researches focused on polysaccharides produced by *G. frondosa* due to their biological activity (Wasser 2011; Chen *et al.* 2012a). However, to the best of our knowledge,

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the research on *G. frondosa* protein has not been reported. In this paper, the antioxidant activity of hydrolysates of *G. frondosa* protein obtained with six different proteases and of fractionated hydrolysates prepared using UF membranes was investigated by various assays, and the molecular weight distribution of the peptide fraction with the highest antioxidant activity was evaluated.

MATERIAL AND METHODS

Material and reagents. Fruiting bodies of *G. frondosa* were provided by Chestnut Research and Development Center of Qianxi County (Qianxi, China). The ultrafiltration (UF) membrane system was from Tianjin Motimo Membrane Technology Co., Ltd. (Tianjin, China). Alcalase and neutrase were purchased from Novozymes Biotechnology Co. Ltd. (Kalundborg, Denmark). Papain, trypsin, Protamex, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethylenediaminetetraacetic acid (EDTA), butylated hydroxyanisole (BHA), thiobarbituric acid (TBA), trichloroacetic acid (TCA), and Sephadex G-15 were purchased from Sigma Chemical Co. (St. Louis, USA). Pepsin was purchased form Junsei (Saitama, Japan). Linoleic acid (~99%) was purchased from Fluka Chemical Co. (St. Gallen, Switzerland).

Preparation of GFP and GFHs. *G. frondosa* protein (GFP) was extracted from *G. frondosa* fruiting body using a previously described method (Chen *et al.* 2014). Then GFP was hydrolysed by six different proteases separately. The optimal reaction conditions of six proteases are taken from the supplier and shown in Table 1.

Dried GFP was suspended in distilled water to obtain a 5% (w/v) protein solution and was preheated at 90°C for 20 min, then the protease was added to the protein solution after the temperature and pH were properly adjusted. During hydrolysis, the pH of the solution was maintained constant by addition of 1.0 M NaOH or 1.0 M HCl. After hydrolysis, the hydrolysate obtained at different times was immediately put in a boiling water bath for 10 min to inactivate protease, then filtered, concentrated, vacuum-dried, and stored at –18°C until the time for use.

Determination of the degree of hydrolysis (DH). DH was defined as the percent ratio of the number of peptide bonds broken to the total number of peptide bonds in the substrate studied, and the calculation formula was expressed as below: DH = α-amino nitrogen content/total nitrogen content. The α-amino nitrogen was determined by the formol titration method (Nilsang *et al.* 2005). The total nitrogen content was determined by Kjeldahl method (the conversion factor is 6.25 for each protein) (Liu *et al.* 2007).

Fractionation of GFHs by ultrafiltration. GFH with the highest antioxidant activity was fractionated according to molecular weights (MW) using three different molecular weight cut-off membranes (10, 5, and 3 kDa). Then four fractionates were separated and designated as GFH-1 (MW >10 kDa), GFH-2 (MW 5–10 kDa), GFH-3 (MW 3–5 kDa) and GFH-4 (MW < 3 kDa). All peptide fractions recovered were lyophilised in a freeze drier and stored at –18°C for use.

Hydroxyl radical (•OH) scavenging activity. The •OH scavenging activity was investigated using the method described by Wang *et al.* (2008) with some minor modifications. The sample was dissolved in 10 ml of distilled water at the concentration of 2 mg/ml. The sample solution (0.25 ml) was mixed with 2.0 ml of 0.2 M phosphate buffer (pH 7.4), containing 0.1 mM EDTA, 0.1 mM $FeSO_4 \cdot 7H_2O$ and 1.75 mM deoxyribose, then 0.25 ml of 1.0 mM ascorbic acid and 0.25 ml of 10 mM H_2O_2 were added into the reaction solution. The reaction was incubated at 37°C for 10 min in a water bath and then 1.0 ml of 1% TBA and 2.0 ml of 2.8% TCA were added to the mixture. The mixture was boiled for 10 min and cooled on

Table 1. Optimal reaction conditions of six proteases

Protease	Activity (U/g)	Optimal conditions		
		pH	temperature (°C)	E/S (% w/w)
Papain	3.5×10^4	6.5	45	0.5
Neutrase	3.2×10^4	7.0	40	0.5
Alcalase	5.9×10^4	8.0	55	0.3
Trypsin	4.5×10^4	7.5	45	0.4
Pepsin	1.8×10^4	2.0	37	1.0
Protamex	2.6×10^4	7.0	50	0.6

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ice. The absorbance of the mixture was measured at 532 nm. Deionised water instead of sample was used as blank. The •OH scavenging activity was calculated according to the following formula:

$$\text{Scavenging (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

where: A_0 – absorbance of the blank; A_1 – absorbance in the presence of the test sample

DPPH radical (DPPH•) scavenging activity. The DPPH• scavenging activity was measured by the previous method with some modifications (Chen *et al.* 2012). Amount of 1 ml of sample (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/ml) was mixed with 1.0 ml of phosphate buffer (0.02 M, pH 6.0) and 1.0 ml of 0.2 mM DPPH-ethanol solution. The mixture was shaken and left for 30 min at room temperature, and the absorbance of the resulting solution was measured at 517 nm. BHA was used as positive control. The scavenging percentage of DPPH• was expressed according Eq. (1), where A_0 – absorbance of 0.2 mM DPPH without sample; A_1 – absorbance of 0.2 mM DPPH with sample.

Ferrous ion chelating effect. The ferrous ion chelating activity was measured as reported (Dinis *et al.* 1994) by measuring the formation of ferrous iron-ferrozine complex. Five millilitres of the sample in different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/ml) were mixed with 0.1 ml of 2.0 mM $FeSO_4$ and 0.2 ml of 5.0 mM ferrozine solution, the mixture was left to stand for 10 min at room temperature, and then the absorbance was determined at 562 nm. EDTA was co-assayed as a positive control. The ferrous ion chelating activity was calculated according to Eq. (2):

$$\text{Chelating ability (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (1)$$

where: A_0 – absorbance of the control (deionised water, instead of sample); A_1 – absorbance of the test sample mixed with reaction solution

Reducing power assay. The reducing power was measured by the procedure described by Oyaizu (1986). Briefly, 2 ml of the sample in different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/ml) were added to 2.0 ml of 0.2 M phosphate buffer (pH 6.6) and 2.0 ml of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50°C in a water bath for 20 minutes. Then 2.0 ml of 10% (w/v) TCA was added to the reaction mixture, which was then centrifuged at 3000 *g* for 10 minutes. The supernatant (2.0 ml) was mixed with 2.0 ml of distilled water and 0.4 ml of 0.1% (w/v) ferric chloride in a test tube. After reaction for 10 min, the absorbance of the resulting solution was measured at 700 nm. For comparison, the corresponding concentration of ascorbic acid was also tested.

Lipid peroxidation inhibition assay. The lipid peroxidation inhibition activity was measured in a linoleic acid model system according to the method of Chen *et al.* (1996) with some modifications. Briefly, 2.0 ml of 0.1 M phosphate buffer (pH 7.0), and 2.0 ml of 50 mM linoleic acid in ethanol (99.5%) were mixed in a test tube. Samples were added the phosphate buffer maintaining the total volume at 4.0 ml. The tubes were sealed tightly with silicone rubber caps and kept in the dark at 60 ± 1°C to accelerate oxidation. The antioxidant activities were determined by the ferric thiocyanate (FTC) method described by Osawa and Namiki (1981) with a slight modification. At 24-h regular intervals, to 0.1 ml of the reaction mixture was added 4.7 ml of 75% (v/v) ethanol, 0.1 ml of 30% (w/v) ammonium thiocyanate, and 0.1 ml of 20 mM ferrous chloride in 3.5% (v/v) HCl. After exactly 3 min, absorbance of the coloured solution was measured at 500 nm. Distilled water and BHA were used as control and standard antioxidant, respectively.

Determination of molecular weight distribution. The GFHT fraction with the highest antioxidant activity was analysed for molecular weight distribution by gel filtration chromatography. The peptides were loaded onto a Sephadex G-15 column (1.6 × 50 cm; Pharmacia, Uppsala, Sweden), eluted with deionised water at a flow rate of 0.5 ml/min, and 3-ml fractions were collected and monitored at 220 nm. A molecular weight calibration curve was obtained from the following standards: insulin (5733 Da), bacitracin (1422 Da), Gly-Gly-Tyr-Arg (451 Da), and L-tyrosine (181 Da).

Statistical analysis. Experimental results were mean ± SD of three measurements. All the data were subjected to analysis of variance and Duncan's multiple range tests, using the SPSS v. 11.0 system (SPSS Inc., Tulsa, USA). Significant levels were defined as probabilities of 0.05 or less.

RESULTS AND DISCUSSION

Preparation of protein hydrolysates and their hydroxyl radical scavenging ability. DH and antioxidant ability of the hydrolysates were monitored for a 5-h period for each protease (Figure 1). It obvi-

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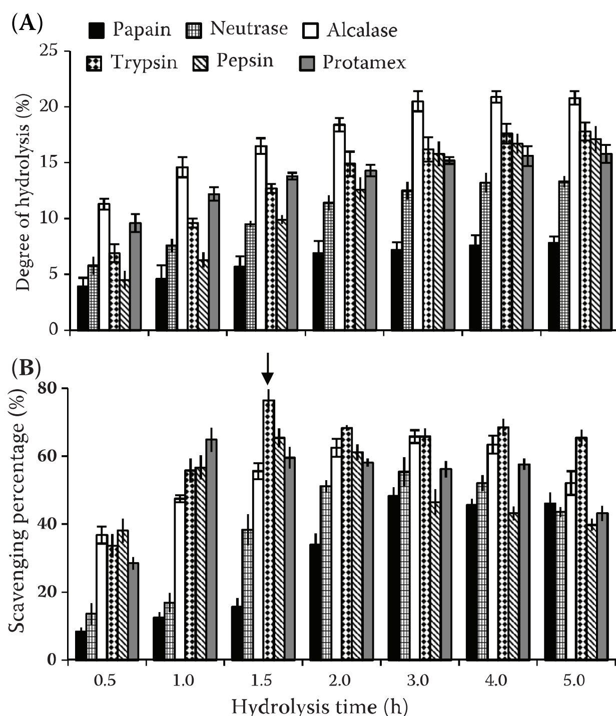

Figure 1. Hydrolysis process of *G. frondosa* protein treated with different proteases (**A**) and hydroxyl radical scavenging ability of *G. frondosa* protein hydrolysates prepared using six proteases (**B**). The values are means ± SD of triplicate measurements

ously showed that there was no correlation ($P > 0.05$) between hydrolysis time and antioxidant activity. The hydrolysis of GFP with proteases proceeded at a high rate during the initial 1 h and thereafter slowed down, and alcalase showed the highest DH values (20.5 ± 0.9% at 3 h), followed by trypsin (17.6 ± 0.8% at 4 h), then pepsin (16.7 ± 0.9% at 4 h), Protamex (15.2 ± 0.3% at 3 h), nutrase (13.2 ± 0.9% at 4 h), and papain (7.2 ± 0.7% at 3 h). However, among the hydrolysates, trypsin derived GFH prepared at 1.5 h showed the strongest antioxidant activity (76.5 ± 3.2%), which was significantly higher than that of the same dose of GFHs derived from the other five proteases ($P < 0.01$). This result indicates that the antioxidant activity of the hydrolysates is inherent to their characteristic amino acid sequences of peptides depending on protease specificities.

In order to obtain a hydrolysate or peptide fraction with both the desired molecular size and antioxidant properties, the trypsin derived GFH (GFHT) prepared at 1.5 h was further separated by using three kinds of UF membranes (10, 5, and 3 kDa MWCO membranes) according to the molecular size and four kinds of permeates (GFHT-1, GFHT-2, GFHT-3, and GFHT-4) were obtained.

DPPH radical scavenging ability of UF fractions. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. When DPPH encounters a hydrogen-donating substance, the radicals would be scavenged and the absorbance is reduced (Shimada *et al.* 1992). Based on this principle, the scavenging effects of GFHTs and BHA on DPPH radicals at various concentrations were measured and the results are shown in Figure 2.

In the test concentration range from 0.5 mg/ml to 3.0 mg/ml, the scavenging activity of GFHTs on DPPH radicals increased with increasing concentrations, and GFHT-4 showed a significantly higher activity than that of the other fractions ($P < 0.05$), which showed about 89.6% scavenging effect on the DPPH radicals at 2.5 mg/ml, close to the effect (88.6%) of 1.5 mg BHA/ml. This revealed that the GFHTs, especially GFHT-4, possibly contained substances that are hydrogen donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

Ferrous ion chelating effect of UF fractions. Figure 3 shows the ferrous ion chelating activity for GFHTs and EDTA. As shown in the figure, all GFHTs exhibited a strong ferrous chelating activity, even though lower than did EDTA. And obviously, the GFHT-3 exhibited the strongest chelating capacity compared to other UF fractions, followed by GFHT-4, GFHT-2, and GFHT-1 successively. But over the concentration of 2.5 mg/ml, there were no statistical differences between GFHT-4 and GFHT-3 in ferrous ion chelating activities ($P < 0.05$). Because the molecular weight of GFHT-4 and GFHT-3 is lower than that of GFHT-1 and GFHT-2, this obtained result suggested that the metal chelating activity of the UF

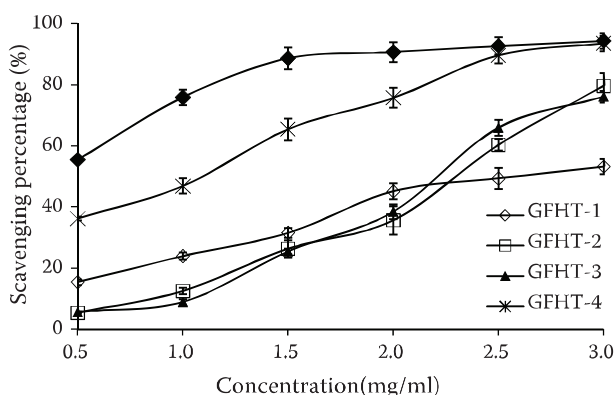

Figure 2. DPPH radical scavenging activity of UF fractions from GFHT with BHA. The values are means ± SD of triplicate measurements

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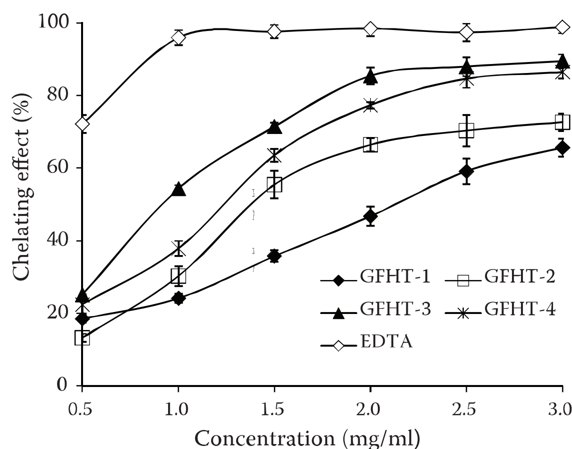

Figure 3. Ferrous ion chelating effect of UF fractions from GFHT. The values are means ± SD of triplicate measurements

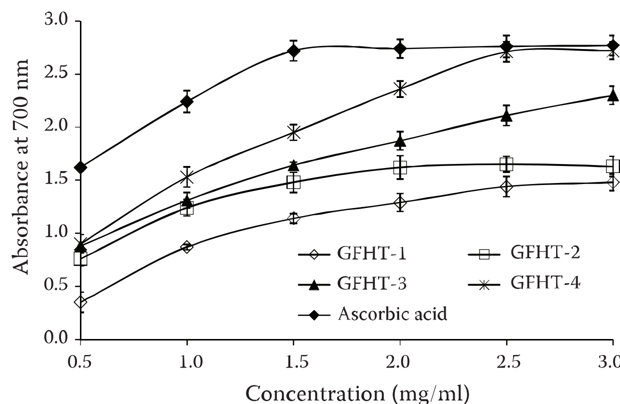

Figure 4. Reducing power of UF fractions from GFHT. The values are means ± SD of triplicate measurements

fraction hydrolysates may be related to their amino acid composition rather than to the peptide chain length (Sami *et al* 2014).

Reducing power of UF fractions. The results of the reducing power of GFHTs and ascorbic acid are shown in Figure 4. In the test concentration range from 0.5 to 3.0 mg/ml, the reducing power of GFHTs increased with increasing concentrations, and significant differences ($P < 0.05$) in reducing power were found between the four fractions in the order of GFHT-4 > GFHT-3 > GFHT-2 > GFHT-1. GFHT-4 displayed significantly higher reducing power than that of the other fractions ($P < 0.05$), which reached a peak value 2.71 at 2.5 mg/ml, it was close to the value (2.72) of 1.5 mg ascorbic acid/ml. These results indicated that GFHTs could act as good electron donors, so they can react with free radicals, thus terminating the free radical chain reaction.

Antioxidant activity in linoleic acid emulsion. The antioxidant activities of GFHTs at 0.5 mg/ml, against the linoleic acid oxidation, were investigated and compared to BHA. As shown in Figure 5, the peroxides of linoleic acid accelerated notably when incubated at 60°C (control), and reached the highest concentration on the fifth day. The peroxides were unstable and they gradually decomposed into secondary metabolites, so their content was decreased on the sixth day. Compared with the control, GFHT fractions showed strong inhibitory effects and significantly prolonged the induction period of autoxidation of linoleic acid. From the FTC results, significant differences ($P < 0.05$) in inhibitory effects were found between the four fractions in the order of GFHT-4 > GFHT-3 > GFHT-2 > GFHT-1. The inhibitory effect of GFHT-4 was found equivalent to the commonly used synthetic antioxidant BHA (0.5 mg/ml) ($P > 0.05$). This result showed that higher antioxidant activities of GFHT-4 are thought to be due to the low molecular weight as it can be easily adsorbed or loosely localised at the oil/water interface where oxidation takes places and thereby reduce radical-mediated LPO (Sun *et al.* 2011).

Molecular weight distribution of GFHT-4. Considering that GFHT-4 was found to possess the highest antioxidant activity, this fraction was analysed for molecular weight distribution (Figure 6). The chromatographic data indicated that GFHT-4 was composed of two major fractions, named F-1 and F-2, respectively. Molecular weight of the two fractions was determined as 2385 Da (F-1) and 1138 Da (F-2).

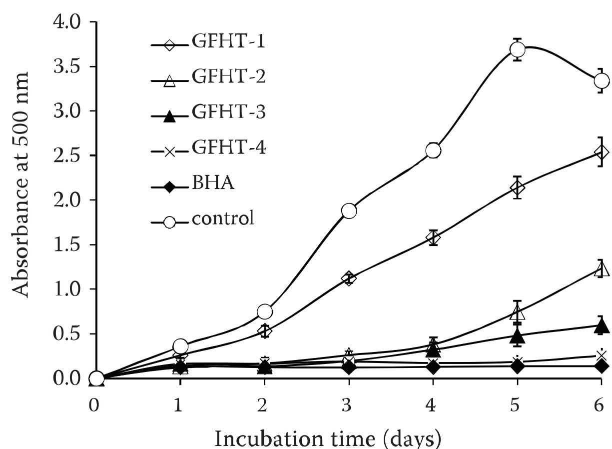

Figure 5 Antioxidant effects of UF fractions from GFHT on linoleic acid emulsion in the FTC method. The values are means ± SD of triplicate measurements.

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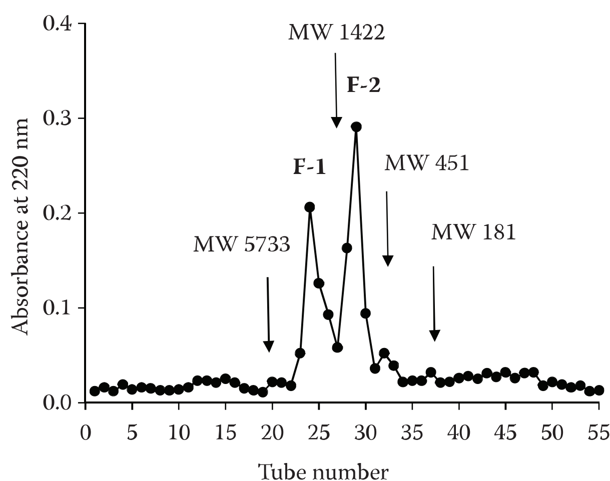

Figure 6. Molecular weight distribution of GFHT-4 with the highest antioxidant activity. Molecular weight for each peak: 2385 Da (F-1) and 1138 Da (F-2)

It was reported that the antioxidant activity of hydrolysates is dependent on their molecular weight distribution (Moure *et al.* 2006). The results of this study showed that the peptide fractions with molecular weight less than 3000 Da were associated with higher antioxidant activity. These findings are consistent with some other studies (Kim *et al.* 2007; Sun *et al.* 2011) and well support the fact that functional properties of antioxidant peptides are highly affected by properties such as molecular mass.

CONCLUSIONS

In the present study, *G. frondosa* protein hydrolysates obtained by six proteases were found to possess antioxidant activity. Among the six proteases, trypsin was more effective than other proteases in preparing GFHs with antioxidant activity. We separated hydrolysed GFHTs with different MW distributions using the UF system and among these GFHT-4 exhibited the highest antioxidant activity, including the scavenging effect on DPPH, ferrous ion chelating effects, reducing power, and ability to inhibit the autoxidation of linoleic acid. Finally, GFHT-4 was separated to two major peptide fractions by gel filtration chromatography, and the two fractions were located at 2385 Da (F-1) and 1138 Da (F-2), respectively. GFHT-4 would be expected to protect against oxidative damage in living systems in relation to aging and carcinogenesis. Therefore, it could be used as a promising natural antioxidant for application in food, cosmetic and medicine industries. But further research should be done in order to purify and identify the antioxidative peptides.

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