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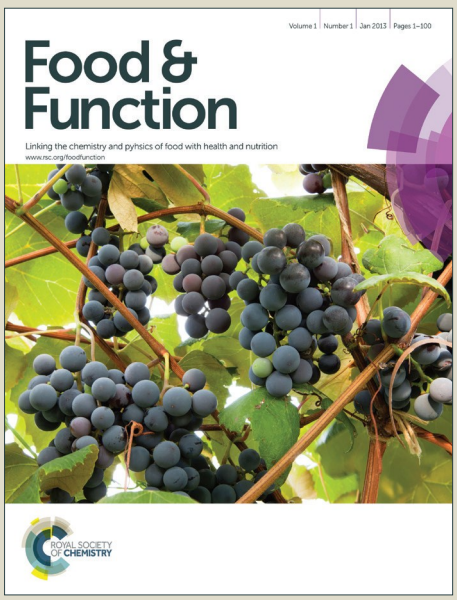

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Protective effects of rice dreg protein hydrolysates against hydrogen peroxide-induced oxidative stress in HepG-2 cells

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Abstract

In this paper, the effects of rice dreg protein hydrolysates (RDPHs) obtained by various proteases on hydrogen peroxide-induced oxidative stress in HepG-2 cells were investigated. Cell cytotoxicity was evaluated through the aspects of cell viability, ROS level, antioxidant enzyme activity, and production of malondialdehyde (MDA). Cell apoptosis was assessed by flow cytometry. Molecular weight distribution was analyzed by gel permeation chromatography, and amino acid composition was measured using an automatic amino acid analyzer. The survival of cells and the activities of superoxide dismutase (SOD) and Glutathione peroxidase (GSH-Px) were significantly increased through the pre-incubation of HepG-2 cells with RDPHs before H_2O_2 exposure. Additionally, these pretreatments also resulted in a reduction in ROS and MDA levels. As a result, apoptosis and loss of mitochondrial membrane potential of the HepG-2 cells were alleviated. Furthermore, the protective effects of protein hydrolysates obtained by various proteases were noticeably distinct, in which RDPHs prepared by alkaline protease showed higher antioxidant activities. The difference in the protective effects might be attributed to the specific peptide or amino acid composition. Therefore, enzymatic hydrolysis with different enzymes studied here could attenuate H_2O_2-induce cell damage, and the type of protease greatly influenced the anti-oxidative activity. Particularly, optimum use of Alcalase could produce peptides with higher antioxidant activity.

Key words: Rice dreg protein; Protease type; Antioxidant activity; Hydrogen peroxide; HepG-2 cells

1. Introduction

Rice dreg protein (RDP), which contains up to 50% of protein in its dry form[1], is a rice by-product produced during the starch extraction process. RDP is gaining a lot of interest in food industry due to its unique nutritional value and nutraceutical properties. It is also gaining interest because it is plentiful and readily available[2, 3]. However, these protein residues are normally used as low-cost animal feed because of their low digestibility and poor solubility at a neutral PH[4]. In order to overcome these defects, protease enzymatic protein modification can be applied. The functional properties of RDP can be improved through the proteases treatment[5]. Several researchers have found that RDP is also a potential protein resource needed to improve the biological activities of RDP, such as its anti-oxidative and ACE-inhibitory properties[6, 7]. These studies indicated that RDPH might serve as an inexpensive and efficient dietary source of protein for human nutrition.

Although the antioxidant activities of rice protein have been documented through chemical tests[8, 9], the particular effects of hydrolyzed rice protein on cells with radical-initiated oxidative damage have not been studied extensively. To the best of our knowledge, influence of enzyme type on the effects of hydrolysates against H_2O_2 induced cell damage has never been reported.

Generally, equilibrium between the generation and elimination of reactive oxygen species (ROS) in normal cell systems is maintained by the antioxidant system. However, when ROS generation grows beyond the capacity of the cellular antioxidant system, or when the normal antioxidant defenses of the cell are inhibited, such

balance is broken, and oxidative stress occurs. Oxidative stress-induced cell damage causes many human chronic diseases such as many cardiovascular diseases, aging, neurodegenerative diseases, diabetes, and cancer[10]. Human HepG-2 cells, which are well-differentiated transformed cell lines from hepatic origins，have been used for the development of cell-based bioassays for food antioxidant activity analysis[11-13]. Hydrogen peroxide (H_2O_2) is thought to be a particularly important contributor to oxidative stress. Therefore, treating HepG-2 with hydrogen peroxide could serve as a model for evaluating the antioxidant activity of food. In addition, studies supported the protective role that natural antioxidants might play in controlling and mitigating oxidative stress-induced diseases[13-15].

In this study, five commercially available and low-cost proteases were selected to hydrolyze the RDP. The RDPHs prepared with various enzymes were evaluated for their protective effects against hydrogen peroxide-induced oxidative stress in HepG-2 cells by considering cytotoxicity and cell viability, intracellular ROS level, activities of antioxidant enzymes, lipid peroxide level, apoptosis, and the related mechanisms.

2. Materials and methods

2.1. Materials

Rice dreg protein (82.9% protein, 7.9% water, 1.35% ash, 6.25% lipid, 1.60% sugar) was provided by Shanyuan Biotechnology Co. LTD (Wuxi, China). Alcalase (2.4 L), Neutrase (0.8 L), Flavourzyme, and Protamex (2.4 AU/g) were purchased from Novozymes (Beijing, China). Trypsin was obtained from Sinopharm Chemical Reagent Co. LTD. HepG-2 cells were purchased from the Institute of Biochemistry

and Cell Biology, SIBS, CAS (Shanghai, China). Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and other cell culture materials were purchased from Gibco BRL, LifeTechnologies (USA). A Cell Counting Kit-8 (CCK-8), a Reactive Oxygen Species Assay Kit, Malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) assay kits were all purchased from Beyotime Biotechnology Co. LTD (Shanghai, China). An Annexin V-FITC Apoptosis Detection Kit, and a Mitochondrial membrane potential assay kit with JC-1 were also obtained from Beyotime Biotechnology Co. LTD (Shanghai, China). A Reactive Oxygen Species Assay Kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). These and all other chemicals and reagents were of analytical grade or higher.

2.2. Preparation of RDP hydrolysate with various enzymes.

Five commercially available proteases were chosen to hydrolyze the RDP. The hydrolysis followed the conditions given in parenthesis: Alcalase 2.4 L (pH 8.5, 55 ℃), Neutrase 0.8 L (pH 7, 45 ℃), Protamex (PH 7, 50 ℃), Flavourzyme (pH 6, 50 ℃), Trypsin (PH 8, 50 ℃).

The RDP was stirred into distilled water (5% [w/v]) for 30 min at the optimum temperature for each enzyme. Reactions were then carried out with proteases for 2 h at each enzyme's respective optimum hydrolysis conditions as described above. The enzyme to substrate (E/S) ratio was 1:100 (w/w) and the pH level of the slurry was kept constant with 1 M NaOH. The resulting hydrolysates were heated in a boiling water bath for 10 min to inactivate the enzyme. After cooling, the hydrolysates were

each adjusted to a pH level of 7.0 and centrifuged at 10000 g for 20 min. The solutions were then freeze-dried and stored at -20 ℃ before use.

2.3. Analysis of cell cytotoxicity and viability

Cell culture: HepG-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), which contains 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100μg/mL streptomycin under conditions of 5% CO_2 and 37 °C in an incubator.

Cells were inoculated into a 96-well plate (4×10^5 cells/mL for cytotoxicity analysis and 1×10^5 cells/mL for viability analysis) and incubated at 37 °C in a CO_2 incubator for a specific amount of time (72 h for cytotoxicity analysis and 24 h or 48 h for viability analysis). After that, 10 μl of the prepared CCK-8 solution was added into each well of the plate and then incubated for another 4 h. Absorbance was measured at 450 nm by a microplate reader (M5, Molecular Devices, USA). The percentage of cell growth inhibition and cell viability was expressed as the following equation:

$$\text{Cell Cytotoxicity} = (A_{control} - A_{treated})/A_{control} * 100\%$$

$$\text{Cell Viability} = A_{treated}/A_{control} * 100\%$$

2.4. Measurement of intracellular reactive oxygen species (ROS)

The level of ROS was determined using the Reactive Oxygen Species Assay Kit. HepG-2 cells were incubated with five RDPHs (1 mg/ml) for 48 h and then 0.4 mM H_2O_2 was added for 4 h. After washing the cells with PBS three times, DCFH-DA (10 mM) was added and the cells were incubated for 30 min at 37 °C in the dark. The DCF fluorescence of the treated cells was measured by a laser scanning confocal

133 microscope (LSM 710, Carl Zeiss AG, German). Relative DCF fluorescence was
134 provided directly by the apparatus.

135 **2.5. Measurements of SOD, MDA and GSH-Px**

136 The assay for superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px),
137 catalase (CAT) and m-alondialdehyde (MDA) was carried out suing commercial
138 assay kits (Beyotime Biotechnology Co. LTD, Shanghai, China). All the steps were
139 taken in strict accordance with the kit-specified method. Briefly, SOD activity was
140 assayed by detecting the concentration of formazan dye (450 nm) formed from WST-8
141 after it reacted with superoxide ions produced from the xanthine–xanthine oxidase
142 system. One unit of SOD activity was defined as the inhibition rate when the above
143 response reached 50%. GSH-Px was detected through the catalytic oxidation of
144 glutathione using t-Bu-OOH as peroxide. Then the presence of glutathione reductase
145 could catalyze the reduction of the GSSH generated above to GSH and the oxidation
146 of the NADPH to NADP+, which could be monitored at 340 nm. One unit of GSH-Px
147 was defined as the 1 μmol NADPH oxidized in 1 min. The assay for CAT was based
148 on its ability to scavenge H_2O_2. The content of MDA was determined by measuring
149 the absorbance of MDA-TBA reacted by MDA and TBA at 450 nm.

150 **2.6. Cell apoptosis and mitochondrial transmembrane potential (ΔΨm) analysis**

151 An Annexin V-FITC apoptosis detection kit was used to evaluate the apoptosis of
152 the cells. Briefly, after the HepG-2 cells were pre-incubated with RDPHs for 48 h, 0.4
153 mM H_2O_2 were added and the cells were incubation for another 4 h. The treated cells
154 were harvested by trypsinization, washed with PBS once, and centrifuged to collect

the cell pellet. Then they were stained with 5 μL Annexin V-FITC, and 10 μL propidium iodide (PI) for 15 min at room temperature in the dark. The cells were analyzed on a flow cytometer (FACSCalibur, BectonDickinson, USA).

The HepG-2 cells were harvested after the above treatment and the mitochondrial transmembrane potential (ΔΨm) was examined using a Mitochondrial Membrane Potential assay kit with JC-1. In brief, the collected cells were incubated with 0.5 mL of a JC-1 working solution for 20 min at 37 °C in the dark, then washed twice with a JC-1 staining buffer, and re-suspended in 0.5 mL of PBS. Flow cytometry (FACSCalibur, BectonDickinson, USA) was used to analyze the cells.

2.7. Determination of molecular weight distribution

The molecular weight distribution of the RDPHs was measured by gel permeation chromatography (1260 Infinity, Agilent Technologies, USA) on a TSKgel2000 SW XL column (7.8 mm i.d. × 300 mm; Tosoh, Tokyo, Japan) with a UV detector at 220 nm. The following describes the mobile phase: acetonitrile/water/trifluoroacetic acid was 45/55/0.1 (V/V), at a flow rate of 0.5 ml/min. The following four protein standards were taken to make a reference curve: cytochrome C (12.5 kDa), bacitracin (1450 Da), tetrapeptide GGYR (451 Da), and tripeptide GGG (189 Da) (Sigma St. Louis, MO, USA).

2.8. Amino acid analysis

200 mg of RDPHs were hydrolyzed in 8 ml of 6 M HCl and heated in a sealed tube for 24 h at 110 °C. After being evaporated under nitrogen at 60 °C, the hydrolysates were diluted with water to 100 mL and then filtered. Amino acid analysis

of the filtrate was measured using an automatic amino acid analyzer (L-8800, Hitachi, Japan).

2.9. Statistical analysis

Data were analyzed using IMB SPSS statistics 2.0 software. The differences between the mean values of samples were determined using the least significant difference (LSD) test at a level of 0.05.

3. Results and discussion

3.1. Cell viability of HepG-2 cells injured with H_2O_2

A reliable in-vitro cellular model was required to represent what occurs in the human body more accurately[16]. In this study, the H_2O_2-induced injury on HepG-2 cells was assessed. As shown in Figure 1A and Figure 1B, time-dependent and dose-dependent decreases in cell viability were indicated by CCK-8 assays in cells exposed to H_2O_2. Furthermore, considering the state of cell growth and sensitivity to the H_2O_2, pre-culture of the cells before H_2O_2 injury for 48 h was better than 24 h. After pre-culture for 48 h, the cell viability decreased to 53.95% ± 2.08 when the HepG-2 cells were then treated for another 4 h with 0.4 mM H_2O_2 (Figure 1A and 1B). When treatment time or H_2O_2 concentration increased, the cell viability decreased slightly. From the previous studies, H_2O_2-injuried cellular models were established as the cell viability reduced by 50%~70%[5, 7]. Therefore, further experiments were carried out with 0.4 mmol/l of H_2O_2 for 4 h.

3.2. Cytotoxic and proliferative effects of RDPHs on HepG-2 cells

Firstly, the CCK-8 assay was used to examine whether or not RDPHs (1 mg/ml)

199 alone would cause cell death. Figure 1C shows that the cytotoxicity levels of RDPHs
200 obtained by five different proteases were all within 10%, suggesting that there was no
201 toxicity to cells at the concentration used in this study[17]. Then, HepG-2 cells were
202 pre-treated with RDPHs over various periods of time (24 h and 48 h) in the
203 concentration of 1 mg/ml before H_2O_2 incubation (0.4 mmol/l for 4 h). As shown in
204 Figure 1D, pretreatment with RDPHs prior to H_2O_2 exposure markedly increased the
205 cell viability of HepG-2 cells when compared to the cells treated with H_2O_2 alone ($P <$
206 0.05). The protective effects were more pronounced when cells were pre-treated with
207 various RDPHs for 48 h. Samples hydrolyzed with Alcalase and Trypsin showed the
208 strongest inhibitory effect against H_2O_2-induced cytotoxicity, while after 48 h of
209 pretreatment, the Flavourzyme hydrolysate was the least efficient, with cell viability
210 values of 86.33% ± 2.78, 85.80% ± 2.19, and 60.13% ± 1.22, respectively. This
211 indicated that the RDPHs had the potential to protect HepG-2 cells against the injury
212 induced by H_2O_2. The differences in the ability to suppress H_2O_2-induced cell death
213 might be attributed to the different proteases employed[18]. During hydrolysis, enzymes
214 with special action sites generated a wide variety of smaller peptides and free amino
215 acids. Moreover, peptide chain length, size, level, and composition of free amino
216 acids greatly influenced the antioxidant activities of the hydrolysates[19, 20].
217 Hydrolysates of Alcalase and Trypsin seemed to contain more anti-oxidative peptides
218 than the other hydrolysates.

219 **3.3. RDPHs inhibited the ROS formation induced by H_2O_2**

220 The intracellular content of ROS provides insights into the anti-oxidative activity

of RDPHs. In order to assess the levels of intracellular ROS in RDPHs-treated HepG-2 cells, a DCFH-DA fluorescent probe was used. The fluorescent signal of RDPHs prepared with various proteases for 2 h are shown in Figure 2A. There were significant differences in the fluorescent signals of RDPHs obtained with various proteases. In decreasing order they were: Alcalase > Trypsin > Neutrase > Protamex > Flavourzye. Furthermore, the signal for each of the hydrolysates-protected groups was considerably lower than that of the H_2O_2-induced group ($p < 0.05$). The results show that RDPHs can efficiently protect the cells from intracellular ROS damage induced by H_2O_2. Additionally, RDP enzymatic hydrolysis by Alcalase showed the highest scavenging activity of intracellular ROS. In contrast, the use of Flavourzye released the least scavenging groups when compared to the other proteases. Images of the HepG-2 cells treated with different proteases demonstrate the same result (Figure 2B). Lower fluorescent signals indicate more scavenging of free radicals. As can be seen, the brightness group was the one treated with H_2O_2 alone. However, the average brightness of the Alcalase-treatment group tended to be more muted than that of the groups pre-treated with the other four kinds of RDPHs. These results provide evidence that rice protein hydrolysates can effectively reduce oxidative damage induced by H_2O_2. Generally, exogenous treatment with H_2O_2 can induce abnormal accumulation of intracellular ROS and damage cellular antioxidant defenses[21]. Furthermore, excess ROS results in protein and lipid oxidation, causes destruction of nuclear DNA and mitochondrial integrity, and ultimately leads to cell death[22, 23]. However, pre-treatment of cells with RDPHs at the concentration of 1 mg/ml

dramatically abrogated these negative impacts by reducing the ROS levels in cells, indicating that the anti-apoptosis properties of the RDPHs was related to ROS scavenging. Moreover, when cells were pre-treated with the same amount of added hydrolysates, the considerable differences among RDPHs might be due to the fact that RDP was more susceptible to the enzymatic attack by Alcalase, resulting in a release of anti-oxidative amino acids and a change in ROS scavenging ability, as previously reported[24]. The peptides released from RDP by various enzymes also reveal distinct peptide chain lengths (Table 1), causing a discrepancy in the ability to enter cells and play a protective role[20].

3.4. Effects of RDPHs on the activities of antioxidant enzymes and lipid peroxide levels in H_2O_2-treated HepG-2 cells

As mentioned above, the levels of intracellular ROS increased sharply after the H_2O_2 damage (Figure 2A). Nevertheless, the redundant ROS could be eliminated by several pivotal antioxidant enzymes, including SOD and GSH-Px[25, 26]. As shown in Figure 3A, the activities of SOD and GSH-Px in HepG-2 cells that were exposed to H_2O_2 at 0.4 mM for 4 h were reduced by 50.59% and 49.07%, respectively, when compared to the control group. However, pretreatment with RDPHs significantly attenuated the loss of enzyme activity in H_2O_2-treated cells. An H_2O_2-induced decrease in SOD activity was restored by 40.93% in HepG-2 cells pretreated with Alcalase hydrolyzed RDP, which was higher than the restoration obtained by other RDPHs. Similar to the results seen for SOD, GSH-Px and CAT activity rose significantly in HepG-2 cells that were pretreated with RDPHs when compared with

the control group, and the highest activity was observed for the Alcalase hydrolysates (Fig.3B and C). On the other hand, Malondialdehyde (MDA), a product of lipid peroxides induced by reactive oxygen species, was also detected. As can be seen in Figure 3D, cells that were subject to H_2O_2 stress had a marked increase in the intracellular MDA (nearly 4-fold vs the control group). However, the overproduction of MDA induced by H_2O_2 was significantly inhibited when the cells were pre-treated with RDPHs. Again, the Alcalase hydrolysates showed the strongest inhibitory effect, with the MDA content decreased by 49.28% compared to the control group. The addition of hydrolysates obtained by the other four kinds of enzymes (Trypsin, Neutrase, Protamex, and Flavourzye) reduced the content of MDA by 46.80%, 30.73%, 22.07%, and 10.94%, respectively, when compared to the control group.

In summary, excessive ROS readily results in damage of the biomolecules within the cell, causes proteins to denature and aggregate along with the collapse of cell membrane, and eventually leads to cell apoptosis[27]. Pre-incubation with RDPHs clearly activates the intracellular antioxidant system, facilitates the expressions of antioxidant enzymes, and thus protects the cells against H_2O_2-induced damage by scavenging intracellular ROS. Additionally, the decrease of MDA content indicates that RDPHs protects the fragile cell membrane from oxidative damage, inhibits the lipid peroxidation and, thus, prevents reactive oxygen from pouring into the cells[28]. One of the probable reasons for the inhibition of the H_2O_2-induced oxidative stress by RDPHs is found in the antioxidant activities of some peptide fractions[29]. During the same hydrolysis time, RDPs were substantially fragmented into peptides by the

287 cleavage reaction of the various proteases[5]. Moreover, there were differences in the
288 exposure of polypeptide chains among the hydrolysates. Stronger inhibitory activity
289 was observed for the hydrolysates prepared by Alcalase and Trypsin, indicating that
290 both contain more essential amino acids and significantly smaller peptides, which
291 greatly enhances the antioxidant properties and thereby effectively inactivates free
292 radicals, than the other hydrolysates.

293 **3.5. Effects of RDPHs on apoptosis of H_2O_2-treated HepG-2 cells**

294 The degree of apoptosis was determined by the Annexin V-FITC/PI assay based
295 on flow cytometry. In Figure 4A, normal cells are seen in the lower left quadrant.
296 Cells in the lower right quadrant are classified as early apoptotic. The cell population
297 in the upper right quadrant has been described as advanced apoptotic or necrotic.
298 When compared to the control group, H_2O_2-injured HepG-2 cells increased the
299 apoptosis rate from 6.64% to 44.98%. However, the ratio of apoptosis was
300 significantly decreased in response to RDPH pretreatment when compared with the
301 control group, indicating clearly that pre-incubation of HepG-2 cells with RDPHs
302 protects the cells against H_2O_2-induced apoptosis. In terms of protective efficiency,
303 Alcalase and Trypsin hydrolysates were found to be the most efficient, while
304 Flavourzyme protease hydrolysates were the least efficient, with apoptosis rates of
305 18.33%, 19.52%, and 38.13%, respectively, after 48 h of incubation. On the other
306 hand, accumulation of intracellular ROS leads to a profound alteration in
307 mitochondrial function. This is closely related to a steep fall in the level of
308 mitochondrial membrane potential[30]. To assess the change of $\Delta\psi m$ during apoptosis

induced by H_2O_2 in HepG-2 cells, flow cytometric analysis was carried out using JC-1. In addition, a decrease of the red/green ratio indicates dissipation of the mitochondrial $\Delta\psi m$. As shown in Figure 4B, a marked drop in the mitochondrial membrane potential was observed when cells were exposed to 0.4 mM H_2O_2 for 4 h (the red/green ratio decreased from 2.62 ± 0.09 to 0.68 ± 0.7). Pre-incubation with various hydrolysates significantly reduced the changes in $\Delta\psi m$ induced by H_2O_2, indicating that H_2O_2-induced mitochondrial membrane depolarization was partly suppressed by pretreatment with RDPHs. The inhibiting effect of RDPHs prepared with Neutrase, Protamex, and Flavourzyme were relatively low, with a red/green ratio of 1.63 ± 0.12, 1.48 ± 0.11, and 1.08 ± 0.07, respectively. The inhibitory effect of RDPHs hydrolysed with Alcalase and Trypsin were much higher than those hydrolysed with other proteases, mainly due to their ability to scavenge high levels of intracellular ROS (Figure 2B). Generally, H_2O_2 can diffuse freely into and out of cells and tissues, destroying the intracellular environment, and ultimately leading to cell death via apoptosis[25, 30]. In fact, cell apoptosis is one of the most easily demonstrable factors for oxidation-induced changes. Furthermore, mitochondrion plays a fundamental role in the apoptotic process, and the level of mitochondrial membrane potential ($\Delta\psi m$) is considered to be an indicator of apoptosis[31, 32]. Our results reveal that RDPHs can efficiently attenuate H_2O_2-induced apoptosis and inhibit the decrease of the mitochondrial membrane potential. These positive effects can be attributed to the hydrolysates' capability to neutralize H_2O_2-induced oxidative stress to some extent (Figure 2) and to ensure the membrane integrity of the mitochondria by avoiding

oxidation of the cell membrane (Figure 3C). Alcalase and Trypsin may be a better choice for the production of peptides with anti-oxidative properties. Apart from high peptide levels resulting from enzymatic hydrolysis, specific amino acid composition may also be a vital factor for this choice, because both of the indicators are closely correlated with oxidation resistance.

3.6. Molecular weight distribution and amino acid composition of RDPHs

RDPHs obtained by various proteases were found to exhibit antioxidant abilities by protecting the cells against H_2O_2-induced damage, and the protective effects were noticeably distinct. This phenomenon may be associated with peptide length, which is closely related to biological activities[33]. Smaller peptides with low molecular weights resulting from enzymatic hydrolysis were more activated. Table 1 shows the molecular weights of peptides hydrolysed by different proteases for 2 h. The peptides released from RDP by various enzymes were mainly composed of low molecular-weight peptides (< 3 kDa). Meanwhile, differences in the distribution of molecular weights were observed among the hydrolysates prepared by various proteases. For Alcalase, the percentages for < 3 and 1-3 kDa fractions were 95.24% and 17.18%, respectively, while only half of the peptides hydrolyzed by Flavourzyme had a molar mass lower than 1kDa. The neutral protease hydrolysates were mainly composed of 1-3 and < 1 kDa fractions (22.17% and 70.39% for Neutrase, 20.19% and 68.9% for Protamex). In addition, the use of Trypsin also produced small peptides, with 93.34% of the peptides falling in the range of 120 to 3,000 Da. This result indicates that Alcalase was much more efficient at producing smaller peptides than

were the other proteases.

On the other hand, amino acid composition is considered to be critical to the antioxidant properties of the hydrolysates. The amino acid composition of RDPHs at 2 h of hydrolysis time is indicated in Table 2. The RDPHs are rich in Asp, Glu, Arg, phe, pro, and Leu, most of which reportedly are related to antioxidant activities either in their free forms or as residues in proteins and peptides[34]. Moreover, after hydrolysis with different enzymes, the amino acid compositions of RDPHs were noticeably distinct, reflecting the differences in exposure of the terminal amino groups. As shown in Table 2, the content of amino acids related to anti-oxidation (take Asp and Glu as representative)[24] was higher for the alkaline protease hydrolysates (Alcalase 22.76 g/100g and Trypsin 22.48 g/100g) when compared with the neutral protease hydrolysates (21.58 g/100g for Neutrase; 20.96 g/100g for Protamex). In addition, the use of Flavourzyme resulted in the lowest amount of Asp and Glu (14.85 g/100g).

These outcomes, in combination with the data for the H_2O_2-induced cell damage model, suggests that peptide chain lengths and amino acids could greatly influence the antioxidant activities of the hydrolysates, and that Alcalase may be the best choice for the production of peptides with anti-oxidative properties.

4. Conclusion

This study demonstrated that the anti-oxidative peptides prepared from five commercially available and low cost proteases were highly capable of inhibiting H_2O_2-induced oxidative damage in human HepG-2 cells. This protection was associated with the ability to neutralize H_2O_2-induced ROS, thereby enhancing certain

antioxidant enzymes, protecting the fragile cell membrane from oxidative damage, and alleviating cell apoptosis. In addition, the protective effects were significantly influenced by the type of enzyme used for hydrolysis. Samples hydrolyzed with Alcalase and Trypsin showed the strongest protective effects against H_2O_2-induced cytotoxicity, while the Flavourzyme hydrolysate was the least efficient, possibly because of the difference in molecular weight distribution and amino acid composition. This work provided the foundation to produce peptides with high antioxidant activity.

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Fig. 1. (A) (B) Cell viability in H_2O_2-injuried HepG-2 cell. Cells were challenged with H_2O_2 in 0.4 mM concentration for 2 h, 4 h and 6 h respectively, or for 4 h in concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 mM. (C) The cytotoxic effects of RDPHs on HepG-2 cells. Cells were co-cultured with RDPHs for 72 h and measured by CCK-8 analysis. (D) Proliferative effects of RDPHs on HepG-2 cells. Cells were pre-incubated with RDPHs (1.00 mg/mL) for 24 h or 48 h prior to treatment with 0.4 mmol/L H_2O_2 for 4 h. After the treatment, cell viability was determined by CCK-8 analysis. Data were shown as means ± S.D.

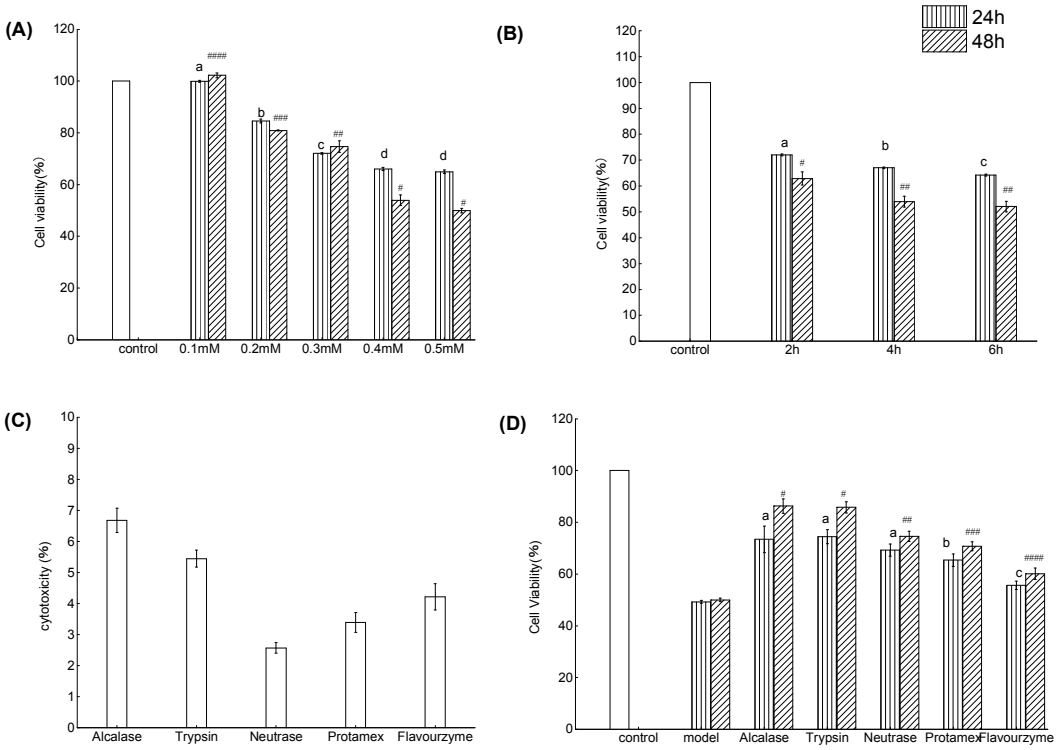


Fig. 2. Effect of RDPHs on intracellular ROS level. HepG-2 cells were pretreated with RDPHs for 48 h before treatment with 0.4mM H_2O_2 for 4 h. Then cells were exposed to DCFH-DA for 30 min. DCF fluorescence of treated cells were measured by laser scanning confocal microscope. Data were shown as means ± S.D.

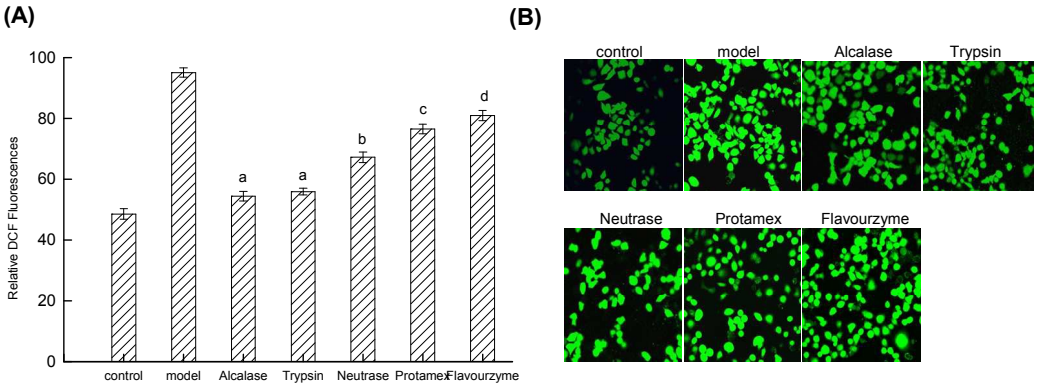


Fig. 3. The effect of RDPHs on SOD, GSH-Px, CAT and MDA activity in H_2O_2-treated HepG-2 cells. RDPHs were added to the culture 48 h prior to H_2O_2 addition, then cells were incubated with 0.4 mM H_2O_2 for 4 h. Data were shown as means ± S.D.

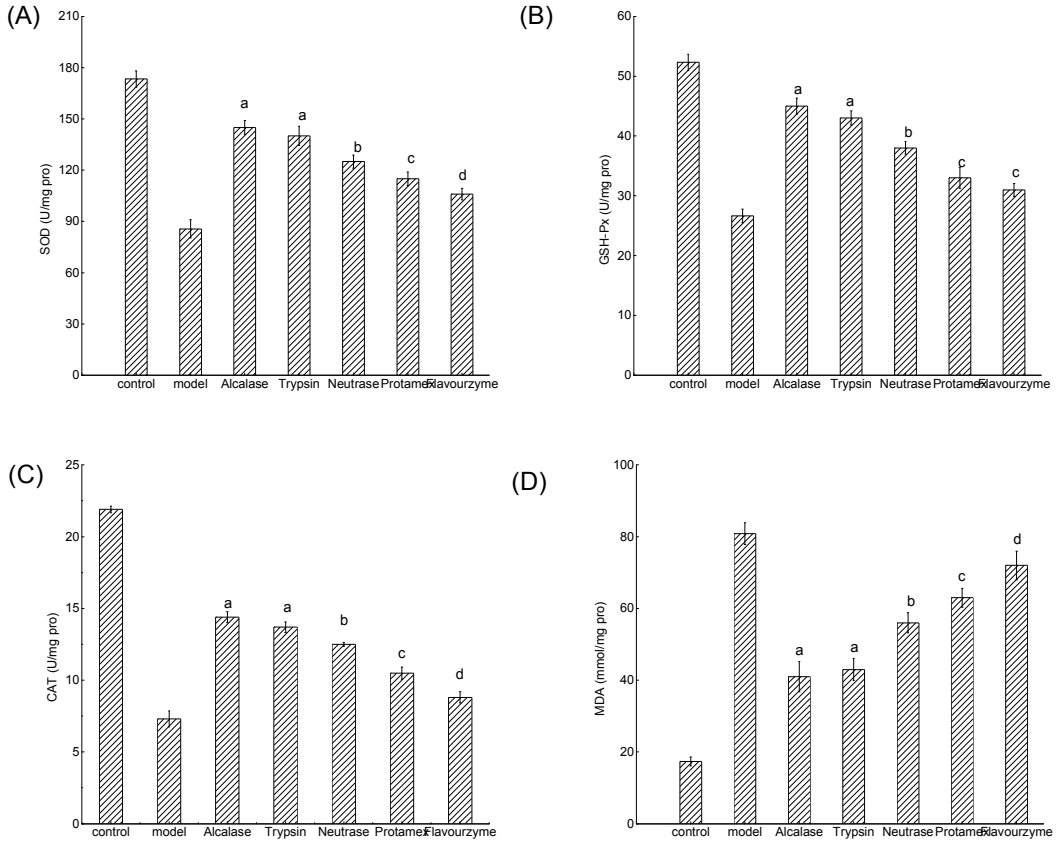
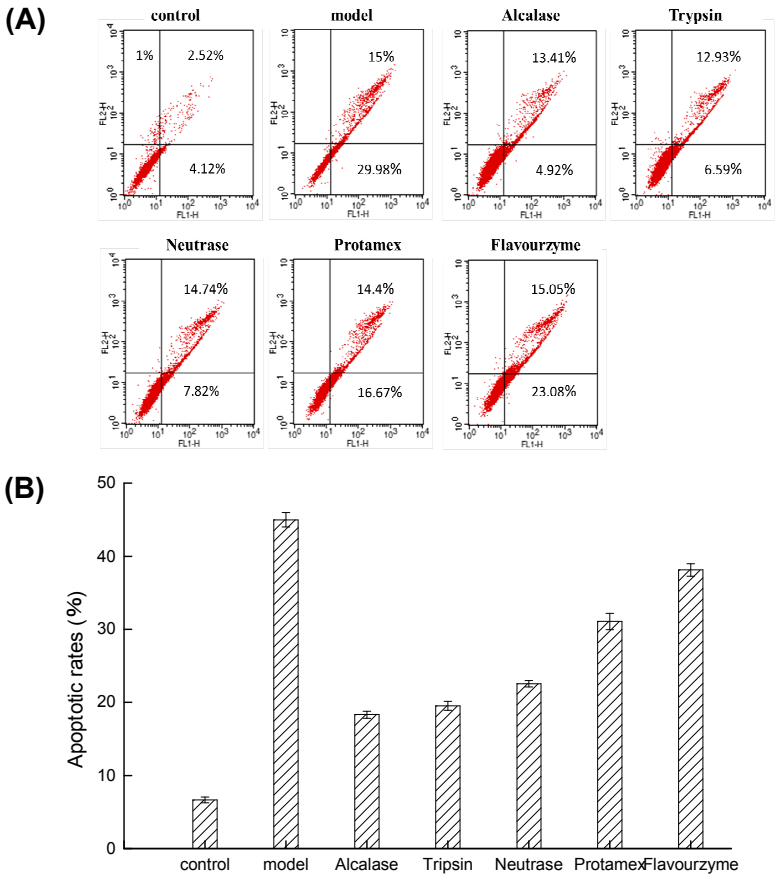


Fig. 4. Protective effects of RDPHs against H_2O_2-induced apoptosis in HepG-2 cells. The cells were pretreated with RDPHs for 48 h before treatment with 0.4 mM H_2O_2 for 4 h. Then, cells were measured by Flow cytometric. (A) Apoptosis detection: Annexin V-FITC assay of HepG-2 cells. (B) Alterations of mitochondrial membrane potential (Δψm) detection : JC-1 assay of HepG-2 cells. Data were shown as means ± S.D.



537 Table 1. The molecular weight distribution profiles of RDPHs

Protease	Percentage of RDPHs fractions (%)			
	>5kD	3-5 kD	1-3 kD	<1 kD
Alcalase 2.4L	1.68±0.03	3.09±0.08	17.18±0.13	78.06±0.15
Trypsin	2.94±0.07	3.72±0.09	16.39±0.15	76.95±0.09
Neutrase 0.8L	2.25±0.10	5.19±0.06	22.17±0.07	70.39±0.21
Protamex	6.17±0.09	4.74±0.12	20.19±0.11	68.9±0.11
Flavourzyme	13.67±0.11	9.64±0.09	27.08±0.09	49.62±0.08

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567 Table 2. Amino acid composition (g/100 g of protein) of RDPHs.

Amino acid	RDPHs prepared by five enzymes				
	Alcalase 2.4L	Trypsin	Neutrase 0.8L	Protamex	Flavourzyme
Asp	8.26±0.17	8.12±0.09	8.05±0.05	7.66±0.04	5.32±0.12
Glu	14.50±0.10	14.36±0.22	13.54±0.15	13.30±0.16	9.53±0.32
Ser	3.03±0.10	3.15±0.16	3.17±0.14	3.02±0.11	2.04±0.15
His	1.95±0.06	2.09±0.06	1.95±0.08	1.90±0.04	1.22±0.09
Gly	3.68±0.05	3.79±0.14	3.83±0.04	3.76±0.06	2.47±0.07
Thr	2.57±0.06	2.65±0.04	2.60±0.04	2.53±0.06	1.69±0.04
Arg	7.12±0.13	7.07±0.19	6.97±0.05	6.76±0.06	4.42±0.22
Ala	4.21±0.13	4.29±0.08	4.20±0.26	4.12±0.19	3.01±0.27
Tyr	2.87±0.17	3.18±0.13	3.02±0.07	3.05±0.06	2.26±0.13
Cys-s	0.49±0.32	0.46±0.12	0.45±0.19	0.43±0.28	0.10±0.33
Val	5.38±0.07	5.44±0.04	5.04±0.07	4.93±0.08	3.20±0.08
met	1.61±0.03	1.53±0.07	1.38±0.07	1.41±0.10	0.50±0.04
Phe	4.28±0.09	4.35±0.05	4.22±0.07	4.16±0.14	2.70±0.19
Ile	3.71±0.10	3.74±0.05	3.52±0.06	3.43±0.08	2.27±0.29
Leu	6.20±0.03	6.27±0.10	5.95±0.11	5.77±0.11	3.90±0.11
Lys	2.88±0.43	3.00±0.35	2.91±0.49	2.87±0.33	2.16±0.75
Pro	3.55±0.07	3.45±0.13	3.34±0.04	3.34±0.06	2.44±0.04

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