HIGH-ENERGY ELECTRON BEAM TREATMENT: A NOVEL APPROACH TO ENHANCE ANTIOXIDANT ACTIVITY IN RICE PROTEINS

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ABSTRACT

In this paper, intracellular antioxidant mechanism of rice protein hydrolysates (RPHs) treated with high energy electron beam irradiation (EBI) was discussed using CAA assay and H_2O_2-induced oxidative damage modelin HepG-2 cell. Changes of RPHs structures were measured by FTIR and CD spectra. Molecular weight distribution was detected by gel permeation chromatography. After EBI treatment, CAA value of RPHs increased from 7.58±0.19 μM QE/g to 11.46±0.16 μM QE/g, indicating that the cellular antioxidant activity of RPHswas improved by EBI. Furthermore, the EB Itreated RPHs has better protective effect on oxidative stressed cells, with cell viability increased to 88.02± 1.18%, while the level of ROS, MDA and LDH decreased by2.05-fold, 49.03% and 2.16-fold, respectively, compared with H_2O_2 damaged group. RPHs pretreated with EBI exerted its antioxidant effect by activating intracellular antioxidant enzymes: the SOD, CAT and GSH-Px activity of cells incubated with EBI treated RPHs were significantly higher than that of the damaged group, which was increased by 100.57 U/mg pro, 16.78 U/mg pro and 21.28 U/mg pro, respectively.The basic structure of RPHs was not changed after EBI treatment, while the secondary structure change and molecular weight reduction induced by EBI result in the antioxidant activity improvement in HepG-2cells.

Keywords: *Rice protein hydrolysates; High energy electron beam; cellular antioxidant activity; HepG-2 cells; structural changes.*

I. INTRODUCTION

Electron beam irradiation (EBI) is a process where food is exposed to high energy electron beam produced by machine sources[1]. The EBI technology can be used continuously in line operation with simple operation and high efficiency. What's more, EBI does not need a radiation source and is more economical and safer than other irradiation methods. Because it is a cold process, EBI could eliminate microbial and fungal contaminants from food with little loss of flavor and nutrients[5]. As an emerging technology, EBI has been successfully applied to decontamination, assisted extraction, and disinfestation for improvement of food quality and safety[6-8]. EBI has also received increasing attention for its ability to improve food proteins, including the following four aspects: improvement on protein functional properties[9]; promotion of crude protein digestibility[10-12]; enhancement of the proteolytic effect via the modification of the protein structure[13]; facilitating the biological activities of proteins[14-15]. It is worth noting that some scholars have recently studied the effect of EBI treatment on the bioactivity of cereal protein hydrolysates, which has opened up a new field for the application of EBI technology. Wang et al. reported that the EBI treatment of pea protein and wheat germ protein results in an increase in the DPPH and $ABTS^+$ radical scavenging activity of their hydrolysates[15-16]. The improved antioxidant activity was related to exposure of hydrophobic groups and production of smaller peptides. Lin et al. demonstrated that the DPPH radical scavenging activity of a corn peptide was effectively improved after irradiation with 3.24 kGy of electron beam. They believed that the enhancement of antioxidant activity was attributed to the improvement of enzymatic hydrolysis effect of high energy electron beam. These studies indicated that EBI technology may be potentially used to prepare peptides with higher antioxidant activity. However, the study on EBI treatment to improve the antioxidant activity of polypeptides remains on the chemical evaluation, and its mechanism in the physiological environment of the body still needs to be studied. Cell model, which takes place within the context of the cellular environment, is a more biologically relevant method for evaluating the antioxidant activity of peptides. To the best of our knowledge, the particular effects of EBI treated cereal protein hydrolysates on cells with radicalinitiated oxidative damage have not been studied extensively.

In recent years, biological materials with antioxidant activity, especially antioxidant peptides, have attracted great attention of researchers because of their low molecular weight, easy absorption and high activity biomaterials[17]. Rice proteins (RPs), as natural cereal proteins, are an excellent source of antioxidative peptides due to their unique nutritional value and hypoallergenic properties. However, as a byproduct produced during the starch extraction process, RPs often undergo denaturation because of high-temperature and highpressure treatments, leading to low hydrolysis degree and protein-peptide yield that limits their applications in food formulations[19-20]. In our previous work, it is found that EBI pretreatment improved significantly ($P < 0.05$) the degree of hydrolysis, increasing the hydrolysis degree value by more than 15.09% at a dose of 30 kGy. In addition, radical scavenging results showed that EBI treatment had effects on antioxidant activity and could increase the DPPH and $ABTS^+$ radical scavenging activity of rice protein hydrolysates (RPHs) by 32.06% and 79.11%, respectively (30 kGy), when compared with nonirradiated RPHs (NRPHs). However, there is no any report on the cellular antioxidant activity of RPHs after EBI treatment. Moreover, no systematic research has been conducted on the protective effect of EBI treated RPHs (ERPHs) against oxidative damage in cells.

Therefore, the objectives of this research were to (1) investigate the effect of ERPHs on HepG-2 cells, which can be reflected by cell morphology and CCK-8 assay; (2) study the impacts of ERPHs on intracellular antioxidant activity, which can be measured by CAA assay; (3) further explore the protective mechanism of ERPHs against oxidative stress in cells, including CCK-8 assay, malondialdehyde (MDA), lactate dehydrogenase (LDH) and antioxidant enzymes activities assay; (4) study the reason why the EBI-treated RPHs can increase the antioxidant activity in HepG2 cells through structural analysis.

II. METHODS AND MATERIALS

2.1. Materials

Rice proteinwas provided by Shanyuan Biotechnology Co. Ltd. (Wuxi, China). Alcalase (2.4 L) is an endopeptidase from Novozymes (Beijing, China). HepG-2 cells were purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other cell culture materials were purchased from Gibco BRL, Life Technologies (USA). Cell Counting Kit-8 (CCK-8), a reactive oxygen species (ROS) assay kit, malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) assay kits were all obtained from Beyotime Biotechnology Co. Ltd. (Shanghai, China).These and all other chemicals and reagents were analytical grade purity.

2.2 Electron beam treatment

Sealed 100g of rice protein (in powder form) in a sterile food-grade polyethylene bag, about 1.0cm thick.The sample was then placed on a conveyor belt and irradiated with electron beam produced by using a high-energy linear acceleratorat 5.0 MeV(AIBANG EB-Tech Co., Ltd., Wuxi, China). The irradiation does dependon the exposure time and was conducted at 30 kGy. The irradiation was performed at room temperature and the sample was stored at -20 °C after irradiation for further use.

2.3 Enzymatic hydrolysis of rice protein

5 g of rice protein was stirred in 100 mL distilled water for 30 min at 55 °C, pH 8.5. The Alcalase 2.4 L was then added and the enzyme to substrate (E/S) ratio was 1:100 (w/w). 1 M NaOH was applied to maintain the pH level during the 2-hour reaction. Two hours later, a water bath for 10 min was used to inactivate the enzyme. The hydrolysates were adjusted to a pH level of 7.0 after cooling and centrifuged at 10 000g for 20 min. The supernatant was then freeze-dried and stored at -20 °C for further use.

2.4 Cell culture and treatment

The HepG-2 cells were propagated in a DMEM nutrient mixture containing FBS (10%), penicillin (100 units/mL), and streptomycin (100 μg/mL)in an incubator (5% CO_2 and 95% air)at 37 °C.

2.5 Determination of cell cytotoxicity and viability

HepG-2 cells were seeded onto 96-well plates (4×10^5 cells/mL) and incubated at 37 °C in a CO_2 incubator overnight. The culture medium was removed and 100 μL of fresh medium containing 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL of RPHs was added to incubate the cells. For cytotoxicity assay, the cells were incubated for 72 h before

CCK-8 assay. For proliferation assay,the cells were incubated for 48 h before exposure to 0.4 mM H_2O_2 for 4 h and then CCK-8 assay was applied.

CCK-8 assay: 10 μL CCK-8 was added to each well and incubated for 4 h. The medium was removed before adding150 μL DMSO to each well. A microplate reader (M5, Molecular Devices, USA) was used to determine the absorbance. The absorption wavelength is 450nm.The cell viability was expressed as the following equation:
Cell viability = $A_{treated}/A_{control}\times 100\%$

2.6 Cell morphology
HepG-2 cells were seeded onto 96-well plates (4×10^5 cells/mL) and incubated at 37 °C in a CO_2 incubator overnight. The culture medium was removed and 100 μL of fresh medium containing 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL of RPHs was added to incubate the cells and cultured for 48 h for morphology analysis.

2.7 CAA assay
HepG-2 cells were seeded onto 96-well plates (4×10^5 cells/mL) and incubated at 37 °C in a CO_2 incubator overnight. The culture medium was removed and 100 μL of fresh medium containing 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL of RPHs and 25 μ M DCFH-DA was added to incubate the cells for 1 h. When the step of PBS wash was applied, the wells were washed with 100 μL of PBS and then 100 μL of HBSS (containing 600 μM ABAP)was applied to the cells. When no PBS wash was done, 100 μL of HBSS (containing 600 μM ABAP) was directly applied to the cells. A microplate reader (M5, Molecular Devices, USA) was used to measure the absorbance at 450 nm (at 37°C). The emission and excitation wavelength were 538 and 485 nm, respectively. The measurement was conducted every 5 min for 1 h. The control group was treated with DCFH-DA and an oxidant while the blank group treated with dye and HBSS without the oxidant. The CAA value at each concentration of RPHs was calculated as follows:
CAA unit = 100 -(∫ SA／∫ CA)× 100
where ∫SA is the integrated area under the sample fluorescence versus time curve. ∫CA is the integrated area under the control fluorescence versus time curve.

The median effective dose (EC_{50}) was calculated from the dose response curve from the ratio of the area under the curve of the sample to that of the control where the CAA unit = 50. In this experiment, quercetin was used as a standard, and cellular antioxidant activitiy (CAA) for RPHs were expressed as micromoles of quercetin equivalents (QE) per gram of protein。

2.8 Intracellular reactive oxygen species (ROS)
To determine the level of ROS,a reactive oxygen species assay kit was used. Briefly, the cells were incubated with culture medium containing 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL of RPHs for 48 h prior to exposing to 0.4 mM H_2O_2 for 4 h. After PBS wash, the cells were further incubated with 10 μM DCFH-DA at 37 °C in the dark for 30 min. Subsequently, a laser scanning confocal microscope (LSM 710, Carl Zeiss AG, Germany) was used to analyze the intracellular DCF fluorescence. The relative DCF fluorescence was obtained directly by the apparatus.

2.9 Layered double hydroxide (LDH)
The LDH leakage was detected with an assay kit. Briefly,the cells were incubated with culture medium containing 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL of RPHs for 48 h prior to exposing to 0.4 mM H_2O_2 for 4 h. After the treatment, the culture medium was gently collected for LDH determination. The adherent cells were washed with PBS twice, lysed with cell lysis buffer, and live intracellular LDH was released into the new supernatant. After there action, each sample was measured at wavelength of 450 nmto monitor the reduction of pyruvic acid which represented the LDH activity. LDHleakage was expressed as the percentage of LDH in the culture medium versus total LDH in the cells.

2.10 SOD, GSH-Px, CAT and MDA
The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and the content of malondialdehyde (MDA) were measured by assay kits. All experiments were carried out in strict accordance with the instructions of the kit. The inhibition rate when the reaction reaches 50% is set as one unit of SOD activity.CAT was determined based on its ability to remove H2O2.1 μmol NADPH oxidized in 1 minis set as one unit of GSH-Px activity. The content of MDA was calculated by the absorbance of the reaction of MDA-TBA with TBA at 450 nm.

2.11Fourier transform infrared spectroscopy (FTIR) analysis
2 mg of protein powder was mixed with KBr and grinded into a pellet. The spectra were recorded using a Nicolet iS10 FTIR spectrometer (ThermoFisher Scientific, Marietta, OH, USA). The test was conducted at a wavenumber range of 4000-400 cm^{-1} at a 2 cm^{-1} resolution.

2.12 Circular dichroism (CD) spectroscopy
RPHs were prepared with PBS (0.01 M,pH 8.0) at a concentration of 0.5 mg/mL.A 0.1 cm path length quartz cell and a 0.2 nm band were used for the measurement. CD spectra of PRHs were recorded using a MOS-450 spectrometer (BioLogic Science Instruments, Ltd., Claix, France).The content of the protein secondary structure was calculated by CDSSTR.

2.13 Molecular weight distribution
The molecular weight distribution of the RPHs was measured usinggel permeation chromatography (1260 Infinity, Agilent Technologies, USA). A TSkgel2000 SW XL column (7.8 mm i.d. × 300 mm; Tosoh, Tokyo, Japan) was used. The flow rate was 0.5 mL/min. The mobile phase proportion was as follows: aceto-nitrile/water/trifluoroacetic acid was 45/55/0.1 (v/v). A molecular weight calibration curve was prepared using the following protein standards: cytochrome C(12.5 kDa), bacitracin (1450 Da), tetrapeptide GGYR (451 Da), and tripeptide GGG (189 Da) (Sigma St. Louis, MO, USA).

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

III. RESULTS AND DISCUSSION

3.1 Cytotoxicity of ERPHs on HepG-2 cells
To ensure the accuracy of the experimental results, the CCK-8 assay was used to evaluate whether samples in the concentration range of 0.20-1.20 mg/mL would cause cell death. Fig. 1 shows that the cell viability of all groups co-cultured with NRPHs and ERPHs was higher than 90%, suggesting that there was no toxic effect on cells in this concentration range[21]. It was also proved that the protective effect of the sample on oxidative damage cells was not achieved by stimulating cell proliferation.

As can be seen in Fig. 1, the HepG-2 cells in control group grew well and kept normal cell morphology. No significant difference was observed in cell morphology between the control group and the experimental group pretreated with NRPHs and ERPHs (1.2mg/mL). The cells kept a good state without number reduction and congregating. The results showed that RPHs before and after EBI treatment could not affect the morphology of HepG-2 cells. Similar images were reported by Zhu et al. [22]

3.2 Cellular antioxidant activity assay
Chemical test is often used to evaluate the in vitro activity of antioxidant substances, such as $ABTS^{+}$ free radical and DPPH free radical scavenging experiments. However, the antioxidant mechanisms of bioactive substances in and out of cells are quite different, thus cell model seems more accurate and effective to evaluate the activity of antioxidant peptides[23]. The cellular antioxidant activity (CAA) assay established by Wolfe and Liu in HepG-2 cells[24], a biologically relevant model, is choose in present work to evaluate the antioxidant activity of rice protein hydrolysates pretreated with EBI. The kinetics of DCFH oxidation in HepG-2 cells by peroxyl radicals generated from ABAP is shown in Fig. 2. The increase in fluorescence from DCF formation was inhibited by RPHs in a dose-dependent manner, as demonstrated by the curves generated from cells treated with NRPHs (Fig. 2C, D) and ERPHs (Fig. 2E, F). In addition, the fluorescence value in cells co-cultured with ERPHs increased more slowly than that cultured with NRPHs, indicating that ERPHs could more effectively remove free radicals generated by ABAP and showed stronger antioxidant activity. On the other hand, stronger oxidation inhibition effect for the samples was seen when no PBS wash was done between antioxidant and ABAP treatments. This indicated that there maybe two ways for the samples to exert their antioxidant effects: partial of the samples act at the cell membrane and cut off the peroxyl radical chain reactions outside the cell; partial of them could be absorbed into the cells and continue to exert antioxidant activity.

To further evaluate the influence of EBI pretreatment on the antioxidant capacity of RPHs in HepG-2 cells, the mean effective concentration (EC_{50}) and CAA value are calculated according to Wolfe's method[24] and the results are presented in Fig.3. Pretreatment of rice proteins with EBI before hydrolysis could effectively reduce the EC_{50}value of the hydrolysates, with the EC_{50} value of ERPHs decreased by 31.37% (no PBS wash) and 33.90% (PBS wash), respectively, when compared with that of NRPHs. The CAA value of irradiated RPHs presented marked increases when compared with the nonirradiated sample, with the value increased from 7.57±0.13 to 11.03±0.12 μM QE/g when no PBS wash was done between antioxidant and ABAP treatments and from 7.58±0.11 to 11.46±0.16 μM QE/g when a PBS wash is employed. The results indicated that EBI pretreatment could be an effective way to improve the cellular antioxidant activity of RPHs. This is mainly due to the fact that EBI treatment enhances the proteolytic effect of rice proteins and produces much smaller peptides that are more likely to enter the cell and clear the peroxyl radical generated by ABAP, thus reduce the EC_{50} value and enhanced the cellular antioxidant activity.
Although difference in antioxidant efficacy of the same samples using the protocols with and without a PBS was existed, the differences was not dramatic, as measured by EC_{50} values for CAA.The comparisons in antioxidant activities may provide information on the uptake degree of the samples by cells. When a PBS wash is employed, compounds must be taken up by the cells to have antioxidant effects, as the PBS will remove compounds that are loosely associated with the membrane. The results imply that RPHs exert antioxidant activity mainly by entering the cell and scavenging the peroxyl radical generated by ABAP intracellularly. Therefore, we explore the antioxidative mechanism of ERPHs in cells in the following work.

3.3 The protection of ERPHs on H_2O_2-induced oxidative damage in HepG-2 cells

H_2O_2 is thought to be a particularly important contributor to oxidative stress[25].HepG-2 cells are well-differentiated transformed cell lines from hepatic origins, have been used for developing cell-based antioxidant activity analysis[26-27]. Therefore, oxidative injury model established by exposing HepG-2 cell to H_2O_2 may serve as a reliable model for exploring the antioxidant activity of food and the related antioxidative mechanism. In this study, HepG-2 cells with a H_2O_2-induced oxidative stress model, constructed in our previous work[28], was used to evaluate RPHs oxidative stress activity. Fig. 3A shows the effects of RPHs on H_2O_2-induced oxidative damage in HepG-2 cells. The viability plunged to50.66 ± 1.72% when the HepG-2 cells were exposed to 0.4 mM H_2O_2 only. All RPHs samples exhibited concentration-dependent alleviation of H_2O_2-induced cell damage. It indicates that the EBI-treated and untreated RPHs have certain protective effects against the oxidative damage induced by H_2O_2 and show the activity of oxidative stress inhibitory. Although the untreated RPHs has significantly increased the cell viability to 81.75 ±1.17% at 1.0 mg/mL, it should be noted that the EBI-treated RPHs, the focus of our current work, was proved to show significant improvement on cell viability of 88.02± 1.18%. The results suggested that the EBI-treated RPHs exhibited extremely stronger cytoprotective effect on HepG-2 cells induced damage by H_2O_2 comparing with untreated RPHs. Pretreatment of rice proteins before enzyme hydrolysis can effectively facilitate the antioxidant activity of RPHs.

To investigate how ERPHs protect HepG-2 cells from H_2O_2-induced oxidative damage, intracellular ROS level was tested using DCFH-DA fluorescent probe and the result is shown in Fig. 3B. The fluorescent signal of DCF liberated in the cells was enhanced by 0.4 mM H_2O_2 treatment, but was significantly inhibited in the cells pretreated with RPHs. All samples exhibited concentration-dependent inhabitation of H_2O_2-induced ROS accumulation. Especially, treatment with RPHs resulted in 1.45-fold (NRPHs) and 2.05-fold (ERPHs) decrease in DCF fluorescence intensity compared with that of the H_2O_2-induced group, suggesting that the EBI treated RPHs had a better reducing effect on ROS generation. Exogenous H_2O_2 causes ROS accumulation in cells, and excessive ROS will attack biological molecules, leading to cell damage[22]. The result reveals that RPHs treated with EBI can remove free radicals in cells more effectively, reduce ROS level and inhibit cell damage caused by ROS, thus achieving better protection effect compared with untreated RPHs.

On the other hand, the MDA content of H_2O_2-induced group was significantly ($P < 0.05$) increased compared with control group (Fig. 3C). This is because excess ROS will attack the biomembrane and cause lipid peroxidation in the cell, producing a large amount of MDA[29]. However, pre-incubation of HepG-2 cells with EBI-treated and untreated RPHs significantly inhibited the overproduction of MDA induced by H_2O_2. When the EBI-treated RPHs concentration was 1.0 mg/mL, the MDA content was significantly ($P < 0.05$) decreased to 32.03 ± 2.77 mmol/mg pro, while the untreated RPHs pretreated group was decreased to 46.71 ± 3.71 mmol/mg pro at concentration of 1 mg/mL compared with the damaged group. Moreover, there was significant ($P < 0.05$) difference between the EBI treated and untreated RPHs in MDA content of RPHs pretreated groups with the concentration of 0.8 mg/mL and 1.0 mg/mL. The results showed that RPHs could reduce cellular lipid peroxidation and thereby protect the fragile

cell membrane from H_2O_2-induced oxidative stress injury. Moreover, EBI treatment could enhance the effect of RPHs on inhibiting cellular lipid peroxidation.

The LDH level, another indicator of cell membrane integrity, was performed to investigate the protective effect of RPHs (Fig. 3D). LDH is a stable enzyme present in cell cytoplasm and it will be released into the cell culture supernatant once the cell membrane is damaged[30]. In cultures exposed to H_2O_2 alone, LDH leakage was increased from 0.36± 0.05 % to 1.32± 0.04 %. The LDH leakage of all protection groups was inhibited by EBI-treated and untreated RPHs pretreatment. The EBI treated hydrolysates showed the stronger inhibitory effect than untreated groups, with the LDH content decreased to 0.61 ± 0.02% at the concentration of 1.0 mg/ml (the LDH content for NRPHs was 0.70± 0.05 %). These data indicated that pretreatment of HepG-2 cells with RPHs could protect against H_2O_2-induced cell membrane damage, which is consistent with the results of MDA determination. Furthermore, the protective effect of RPHs was enhanced by pretreating rice proteins with EBI.

The data suggests that RPHs, with or without EBI treatment, protected HepG-2 cells against H_2O_2-induced oxidative damage probably via eliminating intracellular ROS. The reduction of ROS inhibits lipid peroxidation, ensures the integrity of cell membrane and prevents reactive oxygen from pouring into the cells, thus, improving the cell viability.

3.4 Effects of ERPHs on oxidative damage HepG-2 cells of the related antioxidant enzymes

Cellular enzyme system, as a cellular antioxidant defense system, can eliminate excess ROS and ensure the physiological balance of redox. It is known that SOD can catalyzes the dismutation of superoxide into H_2O_2 and molecular oxygen, and the formed H_2O_2 is converted into O_2 and H_2O by the CAT[31-32]. In addition, GSH-Px is responsible for promoting GSH to eliminate intracellular ROS[33]. To investigate whether the protective effect of ERPHs on H_2O_2-induced oxidative damage of hepg-2 cells is related to the regulation of endogenous antioxidant defense system, the influence of RPHs, with or without EBI treatment, on the intracellular antioxidant enzyme activities of SOD, CAT, GSH-Px in HepG2 cells were determined in present work.

As shown in Fig. 4, a significant ($P < 0.05$) decrease in the levels of SOD, CAT and GSH-Px was observed in the HepG-2 cells exposed to H_2O_2 as compared with the control. However, RPHs treatment encouraged the protein expression of these antioxidative enzymes in comparison with H_2O_2-induced group. In addition, when the sample concentration was 0.2 mg/mL, the NRPHs pretreated group could not increase the levels of SOD and CAT comparing with H_2O_2-induced group, but the ERPHs pretreated group could significantly ($P < 0.05$) increase the levels. The SOD level of ERPHs and NRPHs pretreated groups showed significant ($P < 0.05$) difference at concentration of 0.8 mg/mL and 1.0 mg/mL, while the CAT level showed significance at 0.6, 0.8, 1.0 mg/mL between the EBI treated and untreated RPHs pretreated groups. When the sample concentration was 0.8 mg/mL, there was significant ($P < 0.05$) difference between the EBI treated and untreated RPHs incubated groups in the GSH-Px level. Apparently, the SOD, CAT and GSH-Px activities of cells pretreated by NRPHs and ERPHs were significantly higher than that of the damaged group, especially the HepG-2 cells pretreated by ERPHs had better antioxidant enzyme activity which was increased by 100.57 U/mg pro, 16.78 U/mg pro and 21.28 U/mg pro, respectively.

In addition to scavenging intracellular free radicals, another pathway by which RPHs protects cells from oxidative damage is to stimulate the expression of antioxidant enzymes in cells, as depicted in Fig. 4D. A series of experiments in this work have clearly shown that EBI treatment has a significant effect on the antioxidant activity of RPHs and can enhance its protection against oxidative damage to cells. However, the reason for EBI treatment to enhance antioxidant activity of RPHs need to be further studied.

3.5 Effects of EBI treatment on structure of ERPHs

The structure of EBI-treated RPHs was measured to reveal the reason of improved cellular antioxidant activity. Fig. 5A shows the FTIR spectroscopy of EBI-treated and untreated RPHs. Though the EBI treated RPHs sample had stronger absorbance than untreated samples, it was clear that all the samples had similar FTIR absorption pattern at 4000 to 400 cm^{-1}. The results indicated that EBI treatment would not damage the functional groups, which is consistent with Jin's findings[13]. Moreover, similar absorption curve of NRPHs and ERPHs from 1480 to1200 cm^{-1} (fingerprint area of proteins)[34] and 1300 to 1200 cm^{-1} (C-N single bond vibration)[35] in the FTIR spectra revealed that the primary structures of RPHs was not destroyed by EBI. However, these two samples demonstrated different absorption strength, indicating conformational disparities among the samples. Additionally, the amide I band (1700-

1600 cm-1), amide II band (1560-1535 cm-1) and amide III band (approximately 1240 cm-1) mainly comprises the stretching vibration structure which represents the secondary structure of the protein. Changes in the absorption peak strength of the amide band indicated that the secondary structure of RPHs was affected by EBI.

CD spectra is sensitive to the conformational changes in the secondary structure of proteins. In particular, when the ordered structure of a polypeptide chain changes in the local environment, it is easy to distinguish it from the native state. The CD spectra of EBI treated and untreated RPHs was shown in Fig. 5B. The characteristic peaks of the α-helix appeared at 192 nm (positive peak) and 208 and 222 nm (negative band). A positive peak at 195 nm and a negative band at 215 nm reflect traits of β-helix conformation, otherwise a negative band at 200 nm represents the random coil. Compared to NRPHs, the peaks of ERPHs at 192 nm and 195 nm increased in intensity, while the amplitude at 192 nm had no significant change, indicating that the content of β-sheet increased. EBI treatment shifted the negative band into lower wavelength and increased the absorption strength. These changes suggested the decrease of α-helix and an increase in random coil content[37]. Moreover, the secondary structure content of different samples is shown in Fig.5C.After EBI treatment the content of β-sheet was increased from 18.6% to 19.9%, random coil was increased from 55.6% to 69.3%, but the α-helix was decreased from 10.80% to 5.10%. Thus, results indicate that the EBI processing could promote the α-helix transforming to β-sheet and random coil.A study by Lin et. al pointed that the improvement on antioxidant activity of peptides were resulted from secondary structure changes induced by pulsed electric field[38]. Opening of ordered α-helix exposed more free radicals scavenging sites and lead to improvement of antioxidant activity[39]. Based on the present researches, it could be speculated that EBI induced conformational changes in secondary structure of RPHs resulted in exposure of active site, which can capture free radicals in HepG-2 cells, thus, showing stronger antioxidant activity.

Molecular weight (MW) distribution is measured to further explore the reason of cellular antioxidant activity improvement caused by EBI and the results is shown in Fig. 5D. The MW distributions of RPHs treated with or without EBI treatment were noticeably distinct, reflecting the differences in peptide chain length and terminal amino exposure, which had a great impact on the antioxidant activity of the hydrolysates[40]. The proportion of >1000 Da and 1000-500 Da fractions in ERPHs decreased from 22.92±0.14 % and 29.01±0.42% to 12.13±0.76% and 16.88±0.51%, respectively, when compared with that of NRPHs. In addition, the fraction of <500 Da peptides in ERPHs, which consisted mainly of dipeptide and tripeptide, became larger because of EBI treatment, with the fraction increased from 48.07±0.45% to 70.99 ±0.76%. The results showed that rice protein treated with EBI could produce smaller peptides in the process of enzymatic hydrolysis. Many studies have shown that small peptides have higher antioxidant activity[17, 41-42]. Our previous study found that EBI treatment of rice protein can improve its enzymatic hydrolysis efficiency (data not shown). Based on the above findings, it could be speculated that the increase in the content of small molecular weight peptide in RPHs treated by EBI would increase the active sites which can capture free radical, thus improving the antioxidant capacity of RPHs. In addition, small peptides are more easily taken up by cells, then the ERPHs exerted the antioxidant activity fully[17].

IV. CONCLUSION

As mentioned above, the protective mechanism of EBI treated RPHs on cells with radical initiated oxidative damage was studied. Major conclusions can be drawn from the information presented in this study. (1) EBI technology has been proven to assist enzymatic treatment as a powerful tool for production of antioxidative peptide from rice protein. (2) EBI treated RPHs has no cytotoxic effect on HepG-2 cells. Moreover, the protection of ERPHs on oxidative damage cells was not achieved bystimulatory effect on HepG-2 cell growth. (3)CAA assay showed that EBI pretreatment could enhance the antioxidant activity of RPHs cells in two ways: the first is through acting at the cell membrane and cutting off the peroxyl radical chain reactions outside the cell, the second is by entering into the cells and exerting the antioxidant activity. The second way plays leading role in the cellular antioxidative activity. (4) the intracellular antioxidant mechanism of ERPHs includes eliminating intracellular ROS and enhancing endogenous antioxidant enzyme defense system. (5) the structural analysis revealed that the improvement on antioxidant activity of RPHs is closely related to the secondary structure change and molecular weight reduction induced by EBI. Our results provide a fresh way to produce peptides with better antioxidant activity.

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