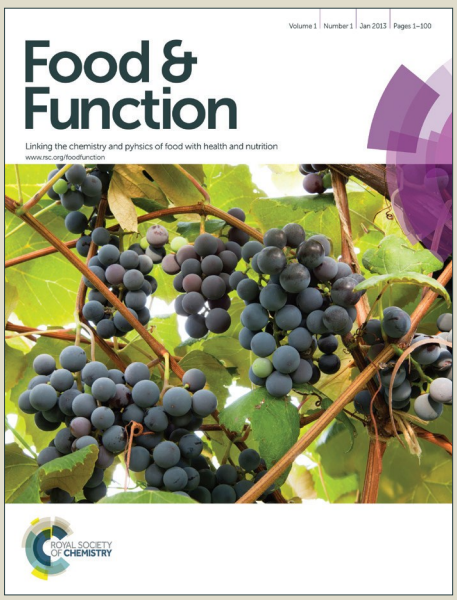
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Maillard Reaction of Shrimp by-products protein hydrolysate: Chemical changes and inhibiting effects of reactive oxygen species in human HepG2 cells

Fengchao Zha, [a,$] Binbin Wei,[a,$] Shengjun Chen,[b] Shiyuan Dong,[a]* Mingyong Zeng[a], Zunying Liu [a]

[a] College of Food Science and engineering, Ocean University of China, Qingdao, Shandong 266003, China

[b] South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, 510300, China

*Corresponding author. Tel: +86 532 8203 2400; fax: +86 532 8203 1908. E-mail: dongshiyuan@ouc.edu.cn; [$]These authors equally contributed to the study.

Abstract

Recently, much attention has been given to improving antioxidant activity of protein hydrolysates via Maillard reaction, but little is known about the cellular antioxidant activity of Maillard reaction products (MRPs) from protein hydrolysate. We firstly investigated chemical characterization and cellular antioxidant activity of MRPs in shrimp (*Litopenaeus vannamei*) by-products protein hydrolysate (SBH)-glucose system at 110 ℃ for up to 10 h of heating. Solutions of SBH and glucose were also heated alone as controls. Maillard reaction greatly resulted in the increase of hydroxymethylfurfural (HMF) and browning intensity, high molecular weight fraction, and reduction of the total amino acid in SBH with the heating time, which correlated well with free radical scavenging activity of MRPs. MRPs had stronger inhibiting effects on oxidative stress of human HepG2 cells than the original SBH, and its cellular antioxidant activity strongly correlated with free radical scavenging activity, but less affected by brown intensity and HMF level. The caramelization of glucose partially affected the HMF level and free radical scavenging activity of MRPs, but it was not relate to the cellular antioxidant activity. The cellular antioxidant activity of MRPs for 5 h of heating time appeared to reach a maximum level, which was mainly due to carbonyl ammonia condensation reaction. In conclusion, Maillard reaction was a potential method to increase the cellular antioxidant activity of shrimp by-products protein hydrolysate, but the higher HMF levels and the lower amino acid in its MRPs should also be considered.

Keywords: Maillard reaction, shrimp, protein hydrolysate, reactive oxygen stress, antioxidant activity

1. Introduction

In China, the output of shrimp from aquatic breeding was up to 2,634,383 tonnes in 2012, 55% of which was Pacific White shrimp (*Litopenaeus vannamei*).[1] Generally, the shrimp by-products from shrimp processing account for approximately 30–40 % of the total shrimp population, and this waste has a real potential for pollution and disposal problems. Nowadays, the potential for producing functional protein hydrolysates from shrimp by-products has also been suggested by recent researchers.[2] The Maillard reaction is involved in the formation of brown pigments via the condensation of a carbonyl group of reducing sugars, aldehydes or ketones with the amine group of amino acids (such as amino acids, peptides and proteins) or any nitrogenous compound in heated foods or model system.[3] Development of the Maillard reaction can be assessed by monitoring the formation of some new compounds, such as the Amadori compounds formed exclusively via MR, hydroxymethylfurfural (HMF) derived from both the MR and caramelization, and browning polymers.[4] Furosine, originated in the early stage of the MR from the interaction of the ε-amino groups of lysine with glucose, lactose and maltose, have been considered a useful indicator of the degree of damage during the initial steps of the MR in cereal products or milks.[5, 6] HMF has been used to evaluate the extent of the MR during processing and storage as it is produced from the middle-stage of MR.[4] However, the formation of Amadori compounds in the MR, such as furosine and N-(2-furoylmethyl) amino acids, resulted in a decrease of protein digestibility and

lysine bioavailability, and HMF is considered as a potentially harmful substance in thermally processed food.[7, 8]

It is well known that Maillard reaction products (MRPs) produced in both heat-treated food systems and in sugar–amino acid model systems have antioxidant activity.[9] In recent years, some research has shown that Maillard reaction could effectively improve the antioxidant activity of proteins hydrolysate.[10] Some MRPs of protein hydrolysates from soybean,[11-12] peanut,[13] mechanically deboned chicken residue[14] and lactoglobulin[15] have stronger antioxidant activity in both chemical model and food systems. Furthermore, the measurement of antioxidative activity using certain reaction conditions may be different from one assay to another, and the antioxidant activity must be determined using various assays. The use of cell model systems is closer to in vivo.[16] Some MRPs from heating rice starch and glycine,[17] biscuit melanoidin[18] and coffee melanoidin,[19] have shown antioxidant effects on some cells induced damage.

Despite these previous investigations, little is known about the formation of HMF and furosine in protein hydrolysate Maillard reaction system and the cellular antioxidant activity of their MRPs. Therefore, the objectives of this research were to investigate the chemical changes including furosine, HMF, the amino acid, browning development and molecular weight distribution of shrimp by-products (*Litopenaeus vannamei*) protein hydrolysate reacting with glucose, and their protective effects against reactive oxygen stress (ROS) in HepG2 cells. As biomarkers for antioxidant capacity, the intracellular ROS level-, and cytotoxicity-induced by

2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) were used. The structural changes of samples after the Maillard reaction were also analyzed through the FI-IR spectrum. In this way, the present findings will help to provide the theoretical basis for improvement of antioxidant activity of functional foods containing protein hydrolysates via Maillard reaction.

2. Materials and Methods

2.1 Materials

The shrimp by-products, containing shrimp head, shell and tail, generated from processing of Pacific White shrimp (*Litopenaeus vannamei*), was donated by Meijia Group, Ltd. (Shangdong, China). The human liver carcinoma cell line HepG2 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Dulbecco modified Eagle's minimal essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, Hank's buffered saline solution (HBSS) and trypsin-EDTA were purchased from Hyclone (Thermo scientific). Alcalase 2.4 L, fluorescein, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2', 7'-dichlorofluorescein diacetate (DCFH-DA), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), AAPH, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade or better.

2.2 Preparation of Maillard reaction products (MRPs)

The shrimp by-products were ground in a blender (Precision DS-1, Shanghai, China),

and then mixed with deionized water at a ratio of 1:3 (w/v). The resultant mixture was subjected to protein hydrolysis (3.6 mL Alcalase / 100 g protein) by Alcalase 2.4 L at pH 8.0 and 60 ℃ in a stirred water bath. The pH of the mixture was maintained a constant pH using 2 M NaOH. After 3 h of hydrolysis, the solution was subsequently heated to 90 ℃ for 5 min to inactivate the enzyme, followed by centrifugation at 10000 g for 10 min. Finally, shrimp protein by-products hydrolysates (SBH) was freeze-dried, and kept at −18 ℃ before further analysis.

MRPs were prepared using the method of Laroque et al.[20] with some modifications. SBH at a protein concentration of 18.43 mg mL^{-1} was dissolved in 50 mM phosphate buffer solution (pH 6.5) and glucose was added at the concentration of 35.3 mg mL^{-1} (equivalent to approximately 1 : 4 mole ratio of free amino group residues to sugar carbonyl groups). The solution (3 mL) was kept in 10 mL screw-cap tubes and heated in an oil bath at 110 ℃ for up to 10 h.

2.3 Measurement of HMF and furosine

HMF was monitored using the method of Zappalà et al.[21] with a slight modification. Samples (5mg mL^{-1}) were filtered on 0.45 μm filter and used for HPLC analysis (Agilent 1100, USA). The column was an Agilent Zorbax SB-C_{18} (250 × 4.6, 5 μm). The elution was done isocratically with a mixture of 90% water containing 1% acetic acid and 10% acetonitrile using a flow rate of 1 mL min^{-1} and an injection volume of 20 μL. The UV detector was set at 282 nm. HMF was quantified using the external standard method within 0.1-50 mg L^{-1}.

Furosine was determined using the methods of Rufián-Henares et al.[22] with some

modifications. One milliliter of the sample was hydrolyzed with 7 mL of 9.08 M HCl at 120 ℃ for 23 h in a Pyrex screw-cap vial with PTFE-faced septa. High-purity N_2 gas was bubbled through the solution for 2 min. The hydrolysates were filtered with a medium-grade paper filter. A 0.5 mL portion of the filtrate was applied to a Sep-pak C_{18} cartridge (Millipore) pre-wetted with 5 mL of methanol and 10 mL of deionized water and was then eluted with 3 mL of 3 M HCl and evaporated with rotary evaporator at 65 ℃. Dried sample was dissolved in 1 mL of a mixture of water, acetonitrile and formic acid (95:5:0.2), and 20 μL of the resulting solution was introduced into a HPLC system (Agilent 1100, USA) equipped with a C8 column (250 × 4.6 mm Alltech furosine dedicated; Alltech, Nicolasville, KY). The mobile phase was 5 mM sodium heptane sulphonate including 20 % of acetonitrile and 0.2 % of formic acid and at a flow rate of 1 mL/min. The UV/VIS detector was set at 280 nm, and furosine was quantified by the external standard method within 0.05-1.5 mg L^{-1}.

2.4 Determination of amino acids

The amino acid composition of MRPs were determined according to the method of Li et al.[23] After hydrolysis of the sample with 6 M HCl at 110℃ for 22 h, the amino acid content was determined by an HPLC system (Agilent 1100, USA) with on-line pre-column derivation by o-phthaldialdehyde and 9-fluorenylmethyl chloroformate (for proline analysis). Analysis was performed on a Hypersil C18 column (4.6 mm × 150 mm, 5 μm). The UV detector was set at a wavelength of 338 nm. The column temperature was 30 ℃. Gradient elution was used. The gradients were formed with

20 mM sodium acetate (A) and 20 mM sodium acetate: methanol: acetonitrile (1:2:2 V/V/V; solvent B). The elution profile was: 0-10 min, 50% B; 10-20 min, 50-100% B; 20-25 min, 100-50% B. The flow rate was 1.0 mL/min.

2.5 Browning intensity of MRPs

The absorbance of samples was measured using a UV-VIS 2550 spectrophotometer (Shimadzu, Kyoto, Japan) at 420 nm.[24] Appropriate dilutions were prepared in order to obtain an absorbance value of less than 1.5.

2.6 Molecular weight distributions

Molecular weight distributions of samples were determined by Gel Permeation Chromatography using a HPLC system (Agilent 1100, USA) according to the method of Dong et al.[25] A TSK gel 2500 SWXL column (300 mm ×7.8 mm, Tosoh Co., Tokyo, Japan) was equilibrated with 50 % acetonitrile (v/v) in the presence of 0.1 % trifluoroacetic acid. The samples were applied to the column and eluted at a flow rate of 0.5 mL/min and monitored at 280 nm at room temperature. A standard calibration curve was obtained from the retention time of the following standards: cytochrome C (12,500 Da), insulin (5734 Da), bacitracin (1450 Da), and glutathione (309.5 Da), hippuric acid (179.2 Da).

2.7 Chemical-based antioxidant activity assay

Chemical-based antioxidant activity was determined by using DPPH scavenging activity and the oxygen radical absorbance capacity (ORAC) method.[26] ORAC assay measures the peroxyl radical reaction to completion by combining both inhibition percentage and inhibition time of the free radical quenching process. ORAC assay

was estimated according to the procedure reported by Dávalos et al.[27] The results were expressed in μmol equivalents of trolox per g dry matter.

2.8 Cellular antioxidant activity

HepG2 cells were cultured in DMEM, containing 10% (v/v) FBS, 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin at 37 ℃ in 5% CO_2. After being seeded in 6-well microplates (**10^4 cells mL^{-1}**) for 12 h, the cells were washed with PBS twice for cell viability and oxidation damage assay.

2.8.1 Cell viability For evaluation the protective effect of samples against oxidative stress, cells were pre-treated with DMEM medium containing 2.8 mM AAPH for 14 h, and then the medium was discarded and fresh medium with the different concentrations of MRPs were added, and followed by incubation for an additional 6 h. Cell viability was estimated using the MTT assay. Briefly, cells were pre-treated with 2.8 mM AAPH for 14 h, and then the culture was incubated with MRPs (1000 μg mL^{-1}) for another 6 h. Furthermore, the cells were treated with MTT (0.5 mg mL^{-1}) for 4 h at 37 ℃. The formazan precipitate was then dissolved in 100 μL DMSO and absorbance at 570 nm was measured using a Benchmark microculture plate reader (Bio-Rad, CA). The cell viability in stressed cells was expressed as percent of nonstressed and unsupplemented (US) HepG2 cells.

2.8.2 Measurement of intracellular ROS concentration Intracellular ROS concentration was monitored quantified by the DCFH assay according to the method of Esmaeili et al.28 with slight modifications. DCFH-DA (25 μM) in absolute ethanol was kept in the dark at −20 °C until used. Cells were pre-treated with 2.8 mM AAPH

for 16 h, and MRPs were added and incubated for another 6 h. Cells were treated with 50 μL 25 μM DCFH-DA for 1 h, then the compound-treated cells were washed twice with HBSS to remove the extracellular compounds, and DCFH-DA fluorescence was detected using a spectrofluorometer with the excitation and emission wavelengths at 485 and 530 nm, respectively. Both excitation and emission slits were set to 1 nm. Cellular ROS concentration was expressed as the fluorescence intensity quantified by the DCFH assay using a microplate reader.

2.9 Fourier transform – infrared (FTIR) measurement

FTIR spectra were obtained from discs containing 1 mg of sample in approximately 100 mg of potassium bromide (KBr). An FTIR spectrophotometer (Bruker Equinox-55, Germany) was used in the experiment. All spectra were recorded within a range from 400 to 4000 cm^{-1} with 4 cm^{-1} resolution and 32 scans.

2.10 Statistical analyses

The data obtained in cell cultures are reported as the mean ± standard deviation (SD) of six replicate determinations, data from other analysis are presented as the mean ± SD of triplicate determinations. The least significant difference (LSD) mean comparison was performed using the SPSS software program (SPSS Inc., Chicago, IL, USA) to compare the mean differences ($p < 0.05$).

3. Results and discussion

3.1 Formation of furosine and HMF, Browning development of MRPs

Fig. 1A shows the furosine levels of MRPs as a function of heating time. The furosine concentration in MRPs showed a rapid increase and reached the maximum levels

within 2 h of heating, and no significant decrease was observed between 2 h to 5 h; subsequently, the furosine levels showed a gradual decrease for up to 10 h of heating. The results suggest that most of the furosine formed in the early stages of MR and then degraded subsequently. Moreover, the contents of furosine in MRPs keep stable for 2-5 h of heating, possibly implying that the synthesis and decomposition of the furosine was close. The results was not consistent with Charissou et al.,[29] who reported that changes of the furosine level in model systems as a heating time followed a bell-shape curve pattern.

. The HMF content in MRP samples and glucose heated alone gradually increased as a function of heating time (**Fig. 1B**). The HMF level for glucose heated alone for 10h of heating accounted for 42 % of the corresponding value of MRP samples. There are a lot of studies about HMF levels in various foods.[30] The maximum levels of HMF (150 mg/kg) in SBH–Glu system for up to10 h of heating are close to that of processed almonds and lower than beverage powder with coffee (286 mg/kg).[31]

The formation of melanoidin, which causes brown coloration via MR, is categorized as the final stage of MR. **Fig. 1C** shows browning development for SBH-Glu as a function of heating time. Browning intensity of SBH-Glu increased slightly within the first 1 h of heating. Subsequently, it increased faster with the further heating. When the glucose was heated alone, browning development due to glucose caramelisation was found to account for less than 10% of that of MRPs.

Generally, the caramelisation mainly occurs at very high temperatures and alkaline pH values.[24] The present results suggest that the caramelisation of glucose partially affect

formation of HMF from SBH–Glu model system, while barely browning development.

3.2 Molecular weight distribution

Molecular weight (MW) distribution analysis was to gain knowledge concerning the mechanism of polymer formation of the MR. **Table 1** shows changes in MW of MRP samples as a function of heating time. The molecular weight of the main peaks of the hydrolysates was lower than 2000 Da, indicating that the relative proportion of <1000 Da fraction was more than 75%. For MRP samples, the amount of MW 1000-2000 Da (medium molecular weight, MHW) and >2000 fractions (high molecular weight, HMW) increased significantly as the heating time prolonged, and the corresponding fractions of MW <1000 Da (low molecular weight, LMW) showed a continuous decrease ($p < 0.05$). After 10 h of heating, the percentage of HMW fraction increased by 30.38%. When SBH was heated alone, the HMW fractions decreased significantly with heating time (data not shown), and the percentage of HMW fractions decreased by 9.2% for 10 h of heating time, suggesting that higher temperature lead to thermal degradation of SBH. Overall, both peptide degradation and crossing-linking process were able to occur in the heating process of SBH-Glu. Some similar results were also reported that peptide degradation and cross-linking of soy protein hydrolysates or peanut hydrolysates occurred simultaneously during MR.[11, 13] Additionally, the thermal degradation of soy protein hydrolysates during MR had a leading role in the decrease of molecular weight.[11] From the present results, the increase of HMW fractions of MRP samples might be mainly based on peptide cross-linking.

3.3 Changes in amino acids composition

The amino acid composition is a very important index used to measure the nutritive quality of food. Amino acid analysis of MRPs (Fig. 2) showed that the total amino acids decreased dramatically after 5h of heating, followed by no significant changes with further heating ($p > 0.05$). After 5 h of heating, the loss of the total amino acid and the essential amino acid in MRPs was 32.37 % and 33.56 %, respectively. The loss of the amino acid might be caused by thermal degradation of amino acid to form volatile compounds in thermal reaction, or the cross-linking between sugars and free amino acids or bound amino acids to form nonvolatile compounds.[11, 32] From the present results, Maillard reaction greatly results in the loss of amino acid of shrimp by-products hydrolysates.

3.4 Free radical scavenging activity

The free radical-scavenging capacity of MRPs were assessed by two methods: the ORAC and DPPH assay (**Fig. 3**). As for the SBH heated alone, the free radical-scavenging capacity by the two antioxidant assays showed no pronounced increase during the heating time (data not shown). A dramatic increase ($p < 0.05$) in the DPPH scavenging activity was detected for SBH-Glu for up to 2 h of heating. Subsequently, DPPH scavenging activity significantly increased for 2-7 h of heating ($p < 0.05$) and reached the maximum levels for 7 h of heating, and no significant difference was observed between 7 h and 10 h of heating ($p > 0.05$). As shown in **Fig. 3B**, the ORAC value for SBH-Glu showed a rapid increase and reached the maximum levels within 5 h of heating, and no significant changes were observed after 5 h of

292 heating ($p > 0.05$). These results were similar to those previously reported, where
293 MRPs from sugar-amino acid, -peptide and -protein model systems showed the
294 capacity of radical scavenging activity,[33] confirming the findings of others that
295 antioxidant capacity of MRPs varies considerably according to the reaction time.[34]
296 Furthermore, DPPH scavenging activity for the glucose heated alone accounted for 49%
297 of that of MRPs for 10 h of heating, but the ORAC value only accounting for less than
298 10% of the ORAC value of MRP. The result suggested that the caramelisation
299 reaction of glucose played more roles in antioxidant activity of MRPs by DPPH assay,
300 compared to that of ORAC assay. Our previous study also found that the heated
301 glucose at 90 ℃ for 18 h had stronger DPPH scavenging activity.[15]

302 **3.5 Cellular antioxidant activity of MRPs**

303 The human hepatoma HepG2 cell line is widely used for biochemical and nutritional
304 studies as a cell culture model of human hepatocytes because these cells retain their
305 morphology and most of their function in culture. In addition, HepG2 is a reliable
306 model through which many dietary antioxidants and conditions can be assayed with
307 minor inter-assay variations.[18] Moreover, DCFH-DA is widely used to measure
308 oxidative stress in cells due to the high sensitivity of this fluorescence-based assay.
309 DCFH-DA penetrates into cells and is hydrolysed to DCFH by intracellular esterases,
310 and the presence of intracellular reactive oxygen species (ROS) can oxidise DCFH to
311 form fluorescent DCF used as an index of the overall oxidative stress within cells.[35]
312 Firstly, HepG2 cells were supplemented with MRPs at 50−1000 μg mL^{-1} medium
313 concentration, and possible cytotoxic effects were assessed by MTT assays. Neither

SBH nor its MRPs for up to the 1000 μg mL^{-1} medium concentration have any cytotoxic effects (data not shown). Furthermore, glucose heated alone did not have the ability to inhibit effects on reactive oxygen species in human HepG2 cells (data not shown).

The cell viability against AAPH-induced damage of HepG2 cells increased with MRPs concentration ranging from 50 to 1000 μg mL^{-1}, and MRPs concentration at 1000 μg/ml for cell viability was the strongest (date not shown). Therefore, MRPs (1000 μg mL^{-1}) protection effects from AAPH-induced damage of HepG2 cells by measuring cell viability and intracellular ROS content in the media was shown in **Fig. 4**. The cell viability (**Fig. 4A**) due to the genotoxicity of AAPH on HepG2 cells showed no significant increase ($p > 0.05$) within 1 h of heating for SBH-Glu compared to non-heated SBH-Glu, and then a significant increase and reached the maximum levels at 5 h of heating. In addition, the cell viability for SBH-Glu at 5 h and 10 h of heating was significantly higher than that of nonstressed and unsupplemented (US) cells. This same results were obtained by Chung et al.,[17] who reported that the cell viability due to the genotoxicity of H_2O_2 on Caco-2 cells for MRPs (1000 μg mL^{-1}) from rice starch with different dextrose and glycine was higher 26% than that of untreated cells. Jiang et al.[33] reported that ribose or lactose-bovine casein peptide MRPs (0.02-0.2 mg mL^{-1}) had no any cytotoxicity on Caco-2 cells, and slightly stimulated the proliferation of Caco-2 cells. As for our present study, the MRPs was a complicated mixture, possibly containing products from different stages of Maillard reaction[34], cross-linking peptide[11], and minor non-reacted glucose and

shrimp by-products hydrolysates. We assumed that some substances in MRPs have protection effect against AAPH-induced damage of HepG2 cells, and others maybe enhance cell growth.

Moreover, the addition of the MRP samples to the cells treated with AAPH significantly suppressed the ROS generation compared to the original SBH (**Fig. 4B**). The ROS generation showed a significant decrease within the 5h of heating, and no significant difference was observed between 5 h to 10 h of heating ($p > 0.05$).

From the present results, the genotoxicity of AAPH on HepG2 cells could be alleviated when HepG2 cells had taken up MRP samples after AAPH exposure, indicating the therapeutic activity of MRPs on HepG2 cell against oxidative stress. Generously, the concentration of MRPs (1000 μg mL^{-1}) with cellular antioxidant activity is a bit high, and its application as a medicine improving the human health was limited. Furthermore, the MRPs maybe provide nutritional, flavor and other biological effect besides its cellular antioxidant activity.[36]

3.6 Changes in FTIR spectra

FTIR spectra of modified protein MRPs are commonly accompanied by secondary structure changes which are expressed in the amide bands of the spectra.[37] For carbohydrates, a series of overlapping peaks located in the region of 1180–953 cm^{-1} results from vibration modes such as the stretching of C–C and C–O and the bending mode of C–H bonds.

Due to the relatively greater cellular antioxidant activity of Maillard reaction products from SBH-glucose at 5 h of heating (SBH-Glu-5), the FTIR of SBH-Glu-5 was

358 further analyzed (**Fig. 5**). The SBH-Glu-5 showed the strongest absorptions of the
359 regions of 1600–1690 cm^{-1} and 1420-1400 cm^{-1}, possibly indicating that the changes
360 of C=O and C–N stretching from amide were related to some structural
361 characterization of SBH-Glu-5. The absorptions of the region of 1180–953 cm^{-1} were
362 stronger in SBH-Glu-5, indicating that there seemed to the saccharide attached to
363 SBH.[38]

364 **3.7 Relationships between antioxidant activity and chemical properties**

365 The correlation coefficients (r) of chemical properties and antioxidant activities of
366 MRPs are shown in **Table 2**. The high positive linear correlations ($r > 0.83$, $p < 0.05$)
367 were observed between HMF formation, the high molecular weight fraction,
368 browning development and free radical scavenging activity, and between the free
369 radical scavenging activity and cellular antioxidant activity. The high molecular
370 weight fraction was positively correlated with the cell ROS concentration, while not
371 with cell viability. In addition, the browning development, HMF formation and
372 furosine level did not significantly correlate with the cellular antioxidant activity ($r <$
373 0.82, $p > 0.05$).

374 Many investigators have correlated the radical-scavenging activity with browning
375 pigments (melanoidins) and high molecular weight fraction, while others have not.
376 The AAPH peroxyl radical scavenging capacity of MRPs from sugar-amino acid
377 model systems at 121 ℃ for 90 min were significantly and positively correlated with
378 browning products,[34] and the radical-scavenging activity from MRPs in a whey
379 protein isolate and sugars system correlated well with browning intensity.[39] The high

molecular weight MRPs separated by ultrafiltration in sugar-amino acid system consistently showed the greatest antioxidant potential in chemical assays.[40] As for the antioxidant activity in cell system, Chung et al.[17] reported that the antioxidant activity of MRPs prepared by heating a mixture of rice starch with different dextrose and glycine in Caco-2 cells were coincidental with an increase in its browning intensity. However, Ruiz-Roca et al.[41] found that the antiradical activity of MRPs generated by heating glucose-lysine mixtures for 15 - 90 min did not correlated with browning development, while increasing in early periods of the reaction (15 and 30 min of heating) and decreased thereafter. Our previous research also showed that MRPs from heated casein peptide and glucose against inhibiting lipid oxidation in fish oil-in-emulsions were not related to the browning intensity[42]. These conflicting data on the antioxidant activity of MRPs may be a result of the complicated components of melanoidin fractions, as well as the assays used to measure these activities.

In the present study, we found that the brown development, HMF level and high molecular weight correlated well with free radical scavenging activity of MRPs, implying that MRPs produced brown pigments and peptide cross-linking in the intermediate-to-late stage of the MR play an essential role in free radical scavenging activity. The browning development and HMF formation did not significantly correlate with the cellular antioxidant activity, suggesting that the protective effects on AAPH-induced oxidative stress of human HepG2 cells in present of MRPs was less affected due to its brown intensity and HMF level. In addition, the free radical scavenging activity correlated well with cellular antioxidant activity, which MRPs are

thought to be important in preventing oxidative damage and diseases related to free radicals.[43]

4. Conclusions

In this study, our results highlighted that protein hydrolysates via Maillard reaction could enhance the cellular antioxidant properties and radical scavenging activity. The free radical scavenging activity determined by DPPH and ORAC assays was mainly related to its brown intensity, HMF level and high molecular weight fraction, while the protective effects on AAPH-induced oxidative stress of human HepG2 cells in present of shrimp by-products Mallard reaction products were less affected due to its brown intensity or HMF level. These Maillard reaction products showed significant potential as antioxidants, especially preserving cellular damage when exposed to ROS. The potential of Maillard reaction products applications in foods is immense and is expanding in the neutraceutical field.

However, Maillard reaction produces contained high HMF levels, which are considered as potentially harmful substances, but also resulted in the loss of nutritional quality. The understanding about benefit and risk of Maillard reaction products with complex composition and chemical structures according to our present study are still limited. Therefore, future research should obtain the main active fraction isolated from Maillard reaction products and further evaluating the risk and benefit of the main active fraction combined with determining antioxidant activity in *vivo* before conclusions can be drawn.

Acknowledgments

This research was funded by Grant 2010-30901121 and 2012-31270038 from the National Natural Science Foundation of China and NYBJG201207 Key Laboratory of Aquatic Product Processing, Ministry of Agriculture, P.R. China.

References

1. Bureau of Fisheries, Ministry of Agriculture of the people's Republic of China, China fishery statistical yearbook, China agriculture press, 2013, pp28-30.
2. I. W. Y. Cheung and E. C. Y. Li-Chan, *Food Chem.*, 2010, **122**, 1003–1012.
3. J. S. Kim and Y. S. Lee. *Food Chem.*, 2009, **116**, 846–853.
4. J. A. Rufián-Henares and C. Delgado-Andrade, *Food Res Inter,* 2009, **42**, 94-400.
5. C. Delgado-Andrade, J. A. Rufián-Henares and F. J. Morales, *Food Chem*, 2007, **100**, 725–731.
6. T. T. Le, B. Bhandari, J. W. Holland and H. C. Deeth, *J Agric Food Chem*, 2011, **59**, 12473–12479.
7. F. J., Morales, S. Martin, Ö. C. Açar, G. Arribas-Lorenzo and V. Gökmen, *Eur Food Res Technol*, 2009, **228**, 345-354.
8. E. Capuano and V. Fogliano, *LWT-Food Sci. Technol.*, 2011, **44**, 793-810.
9. X. M. Chen and D. D.Kitts, Ann. *N.Y. Acad. Sci.*, 2008, **1126**, 220–224
10. X. Hong, J. Meng and R. R. Lu, *J. Sci. Food Agric.*, 2015, **95**, 66–71
11. P. Liu, M. Huang, S. Song, K. Hayat, X. Zhang, S. Xia and C. Jia, *Food Bioprocess Technol.*, 2012, **5**, 1775-1789.
12. M. Huang, P. Liu, S. Song, X. Zhang, K. Hayat, S. Xia, C. Jia and F. Gu, *J. Sci. Food Agric.*, 2011, **91**, 710–720.
13. G. Su, L. Zheng, C. Cui, B. Yang, J. Ren and M. Zhao, *Food Res. Inter.*, 2011, **44**, 3250-3258.

14. W. Sun, M. Zhao, C. Cui, Q. Zhao and B. Yang. *Meat Sci.*, 2010, **86**, 276–282.
15. S. Y. Dong, A. Panya, M. Y. Zeng, B. C. Chen, D. J. McClements and E. A. Decker. *Food Res. Inter.* 2012, **46**, 55-61.
16. R. D. Bernardini, P. Harnedy, D. Bolton, J. Kerry, E. O'Neill, A. M. Mullen and M. Hayes, *Food Chem.*, 2011, **124**, 1296-1307
17. S. Y. Chung, Y. K. Lee, S. H. Han, S. W. Lee and C. Rhee. *Starch/Stärke*, 2012, **64**, 921–928
18. M. A. Martín , S. Ramos, R. Mateos, J. A. Rufián-Henares, F. J. Morales, L. Bravo and L. Goya, *J. Agric. Food Chem.*, 2009, **57**, 7250-7258.
19. L. Goya , C. Delgado-Andrade, J. A. Rufián-Henares, L. Bravo and F. J. Morales. *Mol. Nutr. Food Res.* 2007, **51**, 536-545.
20. D. Laroque, C. Inisan, C. Berger, E. Vouland, L. Dufossé and F. Guérard, *Food Chem*. 2008, **111**,1032-1042.
21. M. Zappalà, B. Fallico, E. Arena and A. Verzera, *Food Control*, 2005, **16**, 273-277.
22. J. A. Rufián-Henares, B. Guerra-Hernández and E. García-Villanova, *Inter. J. Dairy Technol.*, 2002, **55**, 121-126.
23. Y. Li, F. Zhong, W. Ji, W. Yokoyama, C. F. Shoemaker, S. Zhu and W. Xia, *Food Hydrocolloids*, 2013, **30**, 53-60
24. E. H. Ajandouz, L. S. Tchiakpe, O. F. Dalle, A. Benajiba and A. Puigserver. *Food Chem. Toxicol.* 2001, **66**, 926–931.
25. S. Y. Dong, M. Y. Zeng, D. F. Wang, Z. Y., Liu, Y. H. Zhao and H. C. Yang, *Food Chem*, 2008, **107**, 1485-1493.
26. C. Delgado-Andrade, J.A. Rufián-Henares, F. J. Morales. *J. Agric. Food Chem.*, 2005, **53**, 7832–7836.
27. A. Dávalos, C. Gómez-Cordoves and B. Bartolomé, *J. Agric. Food Chem*. 2004, **52**, 48–54.
28. M. A. Esmaeili, A. Sonboli and N. M. Ayyari. *Food Chem.*, 2010, **121**, 148-155.
29. A. Charissou, L. Ait-Ameur and I. Birlouez-Aragon. *J. Agric. Food Chem*. 2007, **55**, 4532-4539.

30. E. Capuano and V. Fogliano, *LWT-Food Sci. Technol.*, 2011, **44**, 793-810.
31. K. Abraham, R. Gürtler, K. Berg, G. Heinemeyer, A. Lampen and K. E. Appel, 2011, Mol. *Nutr. Food Res.*, **55**, 667–678
32. X. Lan, P. Liu, S. Xia, C. Jia, D. Mukunzi, X. Zhang, W. Xia, H. Tian and Z. Xiao, *Food Chem.*, 2010, **120**, 967-972.
33. Z. Jiang, L. Wang, W. Wu and Y. Wang. *Food Chem.*, 2013, **141**, 3837–3845
34. X. Chen and D. D. Kitts. *J. Food Sci.* 2011, **76**, 831-837.
35. H. Wang and J. A. Joseph, *Free Radical Bio. Med.*, 1999, **27**, 612–616
36. H. Y. Wang, H. Qian, and W. R. Yao, *Food Chem.*, 2011, 128, 573–584
37. Y. Joubran, A. Mackie and U. Lesmes, *Food Chem.*, 2013, **141**, 3796–3802
38. F. Gu, J. M. Kim, S. Abbas, X. Zhang, S. Xia and Z. Chen, *Food Chem.*, 2010, **120**, 505–511.
39. W. Wang, Y. Bao and Y. Chen, *Food Chem.*, 2013, **139**, 355–361.
40. D. D. Kitts, X. Chen and H. Jing, *J. Agric. Food Chem.*, 2012, **60**, 6718–6727.
41. B. Ruiz-Roca, M.P. Navarro and I. Seiquer, *J. Agri. Food Chem.*, 2008, **56**, 9056-9063.
42. S. Y. Dong, B. B. Wei, B. C. Chen, D. J. Mcclements and E. A. Decker, *J. Agric. Food Chem.*, 2011, **59**, 13311–13317.
43. H. Y. Wang, H. Qian and W. R. Yao, *Food Chem*, 2011, **128**, 573–584

Tables

Table 1. Changes in molecular weight distribution of shrimp by-product protein hydrolysate-glucose (SBH-Glu) as a function of heating time. Each value is expressed as the mean ±SD (n = 3) [1].

Heating time (h)	Molecular weight (Da)		
	>2000	1000-2000	<1000
0 h	5.30 ±0.29^{d}	19.62 ±0.82^{d}	75.05 ±3.77^{a}
1 h	5.55 ±0.37^{d}	20.76 ±1.06^{c}	74.26 ±4.55^{a}
2 h	6.14 ±0.30^{c}	22.70 ±2.15^{b}	71.17 ±2.73^{ab}
3 h	6.46 ±0.28^{b}	23.89 ±1.89^{ab}	69.65 ±4.47^{bc}
5 h	6.59 ±0.21^{ab}	24.38 ±2.13^{a}	68.04 ±4.23^{c}
7 h	7.06 ±0.76^{a}	24.35 ±2.78^{a}	68.46 ±4.67^{c}
10 h	6.91 ±0.38^{a}	25.58 ±2.11^{a}	66.51 ±3.65^{c}

Means in same column with different letters are significantly different (P < 0.05).

Table 2. Correlation coefficients (r) of chemical properties and antioxidant activities of MRPs from shrimp by-product protein hydrolysate and glucose (SBH-Glu).

Models	[a]A_{420}	Cell viability	Cellular ROS concentration	[c]Furosine	[d]HMF	DPPH	ORAC	HMW
[b]Cell viability	0.75							
[b]Cellular ROS concentration	0.82	-0.89*						
Furosine	0.054	0.26	-0.43					
HMF	0.93*	0.72	-0.80	0.09				
[e]DPPH	0.90*	0.79	-0.95*	0.37	0.92*			
[f]ORAC	0.94*	0.84*	-0.95*	0.21	0.89*	0.96*		
[g]HMW	0.90*	0.73	-0.92*	0.27	0.88*	0.97*	0.97*	
[h]Amino acids	-0.91*	-0.81	0.96*	-0.28	-0.87*	-0.96*	-0.98*	-0.97*

*significant correlation at $P < 0.05$; A_{420}, browning intensity, visible absorbance at 420 nm; [b]cell viability or cellular reactive oxygen species (ROS) concentration by AAPH-induced oxidative stress of human HepG2 cells; [c]furosine contents in MRPs; [d]hydroxymethylfurfural (HMF) level in MRPs; [e]DPPH scavenging activity; [f]Oxygen radical absorbance capacity; [g]high molecular weight; [h]changes of amino acids composition.

Figure Captions

Fig. 1 Effects of heating time on furosine contents (A), hydroxymethylfurfural (HMF) formation (mg/kg sample) (B) and browning development (expressed as absorbance at 420 nm × dilution factor) (C) for shrimp by-products protein hydrolysate and glucose (SBH-Glu) and/or Glu. Bars indicate the standard deviation from triplicate determinations.

Fig. 2 The changes of amino acids composition in Shrimp by-products protein hydrolysate-glucose (SBH-Glu) during Maillard reaction. Bars indicate the standard deviation from triplicate determinations. Bars indicate the standard deviation from triplicate determinations.

Fig. 3 Effects of heating time on the antioxidant activity of shrimp by-product protein hydrolysate and glucose (SBH-Glu), and glucose heated alone as measured by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) assays. The results were expressed in μmol equivalents of trolox per g sample. Bars indicate the standard deviation from triplicate determinations.

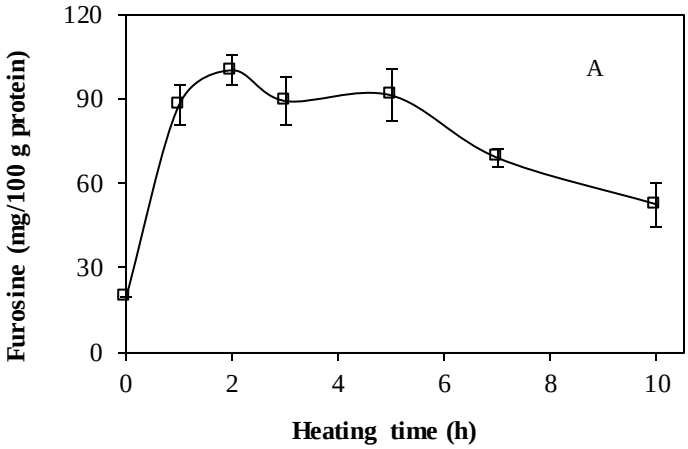
Fig. 4 The cell viability (A) and cellular reactive oxygen species (ROS) concentration (fluorescence intensity) (B) by AAPH-induced oxidative stress of human HepG2 cells in the presence of shrimp by-products protein hydrolysate and glucose (SBH–Glu) as a function of the heating time. Control: HepG2 cells only exposed to 2.8 mM AAPH; US: nonstressed and unsupplemented HepG2 cells. Cell viability is expressed as percent of US cells (assigned as 100%). Bars indicate the standard deviation from six determinations. Different letters in the figure indicate significant differences ($p < 0.05$).

Fig. 5 The Fourier Transform Infrared Spectroscopy (FTIR) of shrimp by-products protein hydrolysate (SBH) and SBH-glucose Maillard reaction products at 5 h of heating (SBH-Glu-5).

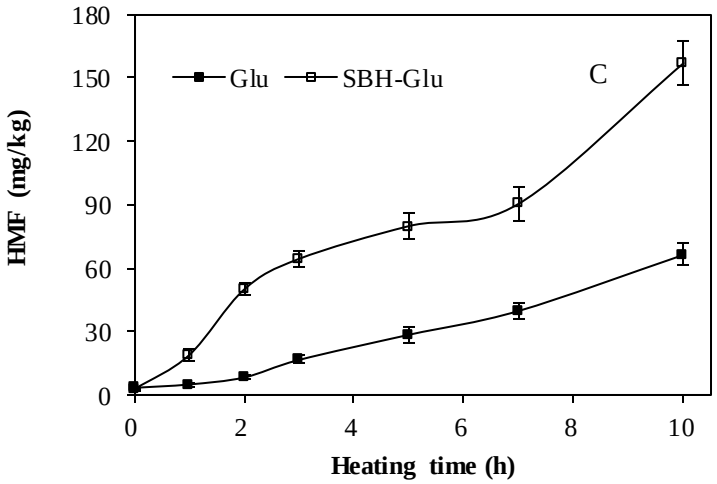
591 **Figures**

592

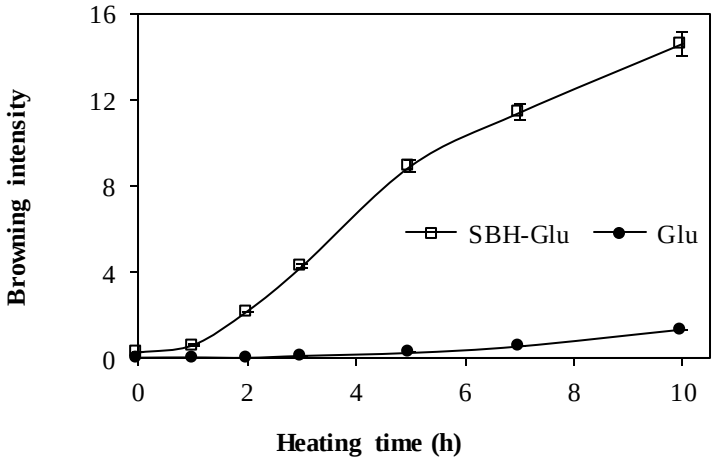
593 **Fig. 1**



594



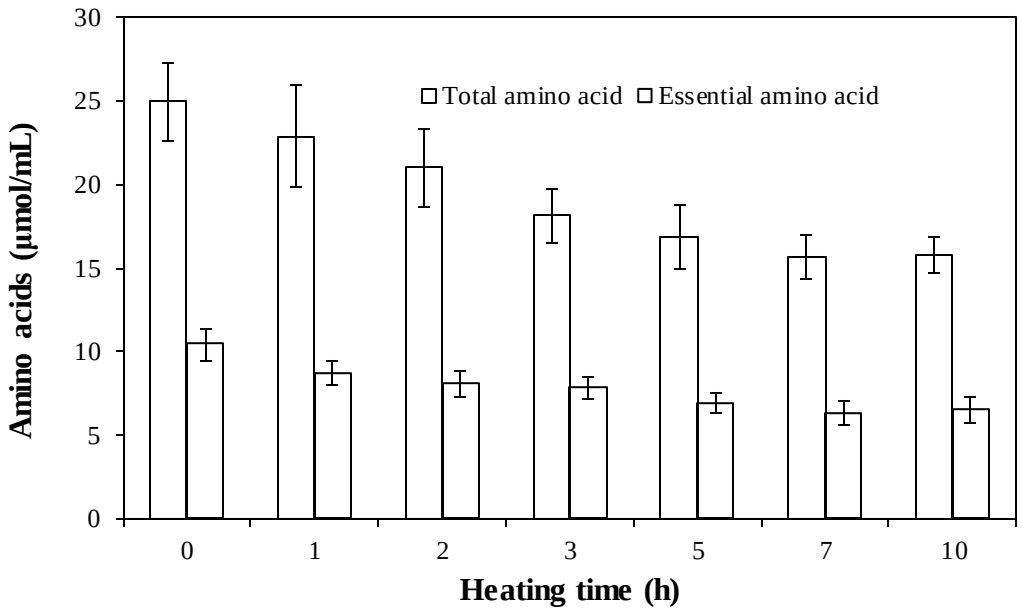
595



596

597

598 Fig. 2



599

600

601

602

603

604

605

606

607

608

609

610

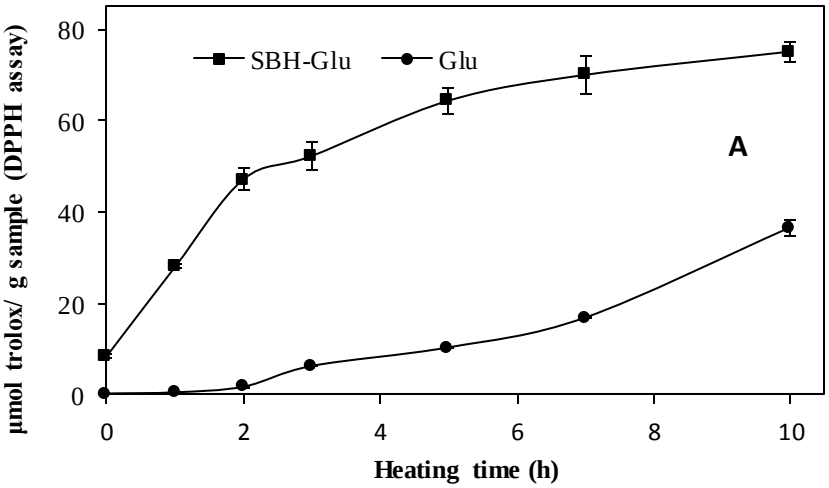
611

612

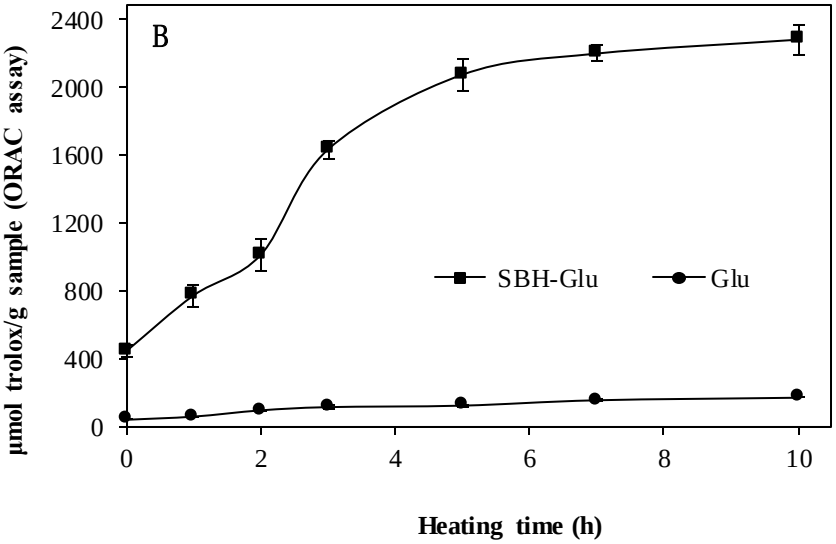
613

614 Fig. 3

615



616



617

618

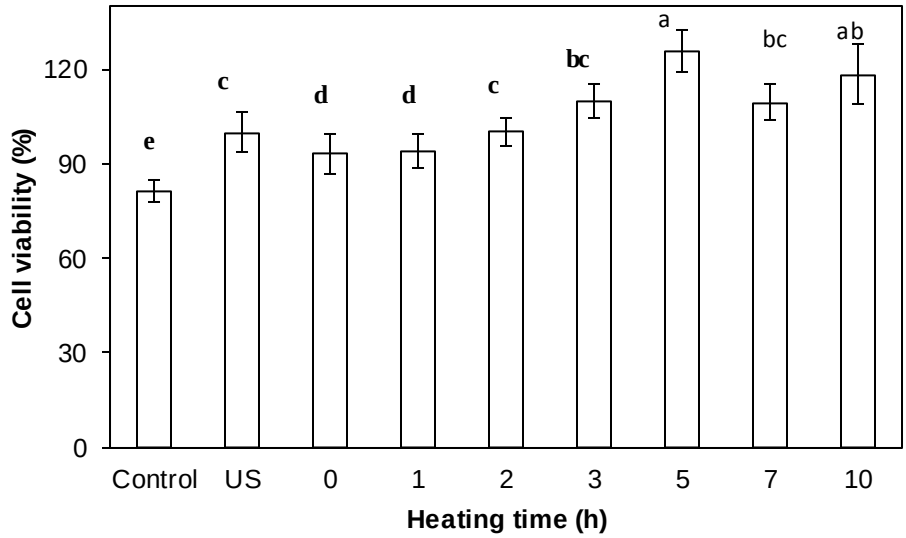
619

620

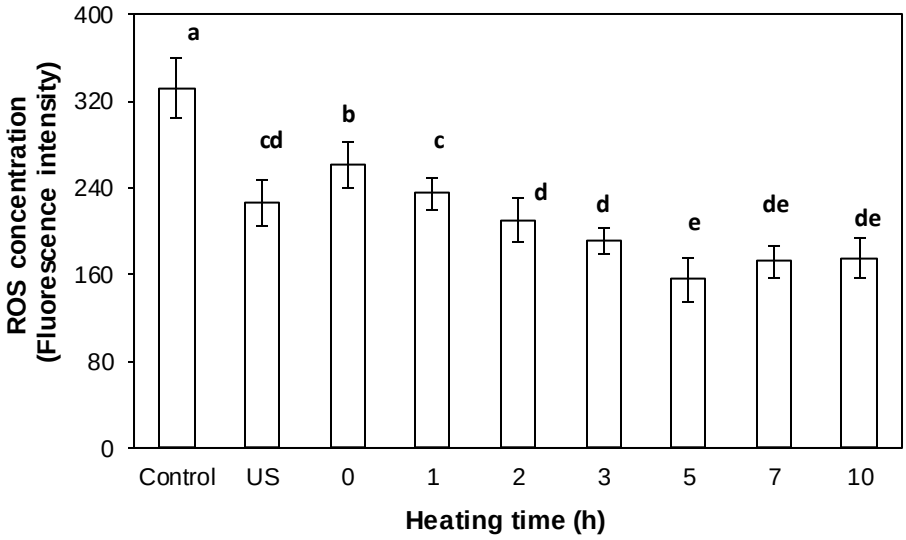
621

622 Fig. 4

623



624



625

626

627

628

629

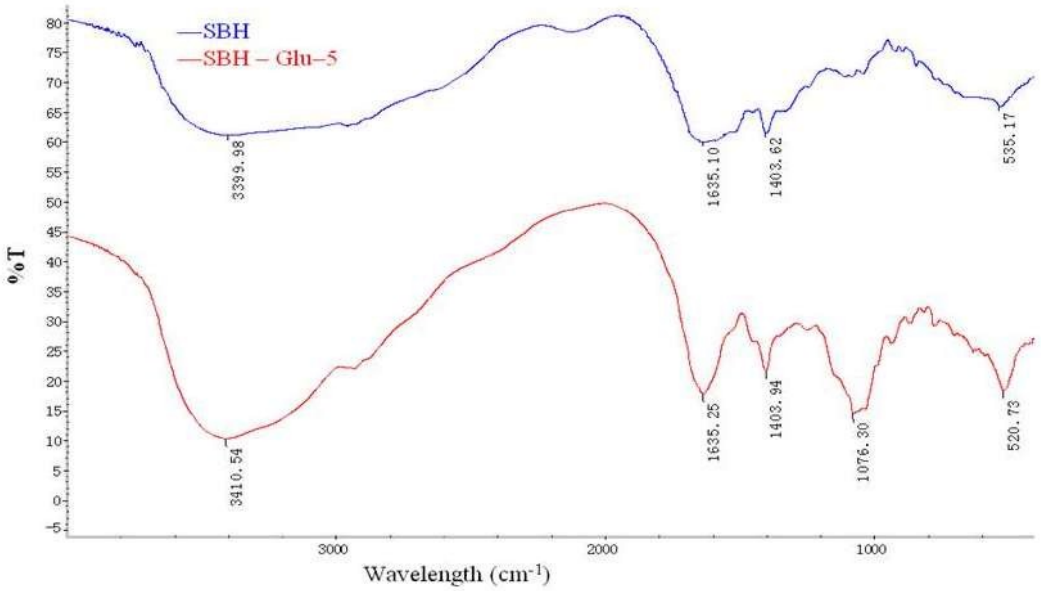
630

631

632

633 Fig. 5

634



635

636

637

638