Microwave heating enhances antioxidant and emulsifying activities of ovalbumin glycated with glucose in solid-state

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Abstract The aim of this study was to characterize the properties of ovalbumin (OVA) after glycated with glucose under microwave heating. For this purpose, microwave at 480 and 640 W power levels were used for heating the OVA-glucose system in solid-state for 0, 5, 10, 15, 20 and 25 min, respectively. The results indicated that the protein molecular weight was increased after glycated with glucose under microwave treatment, the pH of the system was decreased with the increase of microwave treatment power and time, while the UV absorbance, browning intensity, antioxidant activities as well as the emulsifying activity and emulsion stability of the Maillard reaction products (MRPs) were increased in according with the raise of microwave treatment power and time. The reaction time of microwave treatment is much shorter than those using traditional methods, suggesting that microwave irradiation is a novel and efficient approach to promote Maillard reaction (MR) in dry state and improve protein antioxidant and functional properties.

Keywords Ovalbumin · Maillard reaction · Microwave heating · Antioxidant activities · Emulsifying activities

Introduction

Maillard reaction (MR) is one of the most common and important chemical reactions occurring in food processing, cooking and storage. It provides tastes, smells and colors that are desired to a variety of foods. MR is initiated by the condensation of the

reducing-end carbonyl group of reducing sugars, aldehydes or ketones and free primary amine group of amino acids, peptides, proteins or any nitrogenous substance (Plaza et al. 2010). Glycation by MR has been considered as a safe way of modifying proteins compared to the other methods like chemical and enzymatic modification (Huang et al. 2012). The functional properties as well as the structure of proteins can be changed by conjugation with carbohydrates (Tang et al. 2011). In addition, the antioxidant activities of proteins can be enhanced by MR (Lingnert and Eriksson 1980).

There are two main factors that influence the protein-saccharide MR. One involves the internal factors, such as temperature, pH, amino:carbonyl ratio, relative humidity and the intrinsic properties of the reactants (Oliver et al. 2006). The other one is external processing methods, such as conventional heating methods, ultrasonic (Shi et al. 2010), sub-critical water treatment (Plaza et al. 2010), pulsed electric field (Guan et al. 2010) and microwave irradiation (Guan et al. 2006).

Microwaves are electromagnetic waves with frequencies between 0.3 and 300 GHz. Microwave irradiation has attracted lots of attention due to its convenience and fast speed. During microwave heating, the electromagnetic energy interacts with the materials at the molecular level, where the electromagnetic energy is transferred and converted to heat through the motion of the molecules, resulting in rapid heating of the materials. Compared with the conventional thermal heating of materials proceeds via conduction, convection, or radiation of heat from the surfaces of the material, microwave-based heating often requires much shorter heating time (Aslan and Geddes 2008). The efficiency of microwave in dramatically reducing reaction times (reduced from days and hours to minutes and seconds) has recently been proven in several different fields (Larhed and Hallberg 2001; De la Hoz et al. 2007; Li and Yang 2008; Lidström et al. 2001; Obermayer et al. 2009). Another notable advantage of microwave heating over conventional heating is selective heating of materials

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within a mixture or a composite. Microwaves can selectively couple to the nonmetallic material with a higher dielectric loss factor (Aslan and Geddes 2008). In the past 2 decades, microwave has been diversely applied in a number of fields, such as microwave assisted extraction of secondary metabolites from plants (Zhang et al. 2011; Bayramoglu et al. 2008; Chen et al. 2008; Chen et al. 2007; Cravotto et al. 2008; Mitra et al. 2012; Shao et al. 2012), microwave pretreatment to promote biomass conversion (Saha et al. 2011; Abeywickrama et al. 2013), microwave-assisted digestion of proteins (Hahn et al. 2009; Hauser et al. 2008; Ha et al. 2011), microwave baking (Sumnu et al. 2005; Chavan and Chavan 2010), microwave drying of fruits and vegetables (Zhang et al. 2006; Mitra et al. 2012; Agrahar-Murugkar and Jha 2010; Chandra and Samsher 2006; Kar et al. 2004), etc. Therefore, microwave is a promising approach in food and bioproducts processing.

Guan et al. (2006) described that microwave could significantly accelerate the MR of soya protein isolate with sugars. Under high-power microwave irradiation, non-covalent bonds and the disulfide bonds in soy protein isolate molecules are disrupted to cause subunit disaggregation and unfolded peptide chains, and even protein disaggregation and unfolding, so that the probability of the effective collisions between proteins and saccharide molecules are enhanced. Moreover, the activation energy of graft reaction can be reduced markedly, thus increasing the reaction selectivity, so drastic acceleration of the reaction rates can be achieved (Guan et al. 2011). However, these studies were conducted in a buffer solution. Microwave radiation uses a heating mechanism that rotates and vibrates the electric dipole of target molecules. Therefore, microwave radiation is preferentially absorbed by water molecules (Tsubokura et al. 2009). To decrease the effect of indirect heating by water molecules, we apply the microwave heating energy to the OVA-glucose mixture in solid-state. In this work, the MR of OVA-glucose in solid-state by microwave heating at two normal power levels (480 and 640 W) which are most popular in China was monitored, and the antioxidant activities as well as emulsifying properties of MRPs were also evaluated.

Materials and methods

Materials

OVA, 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), o-phthaldialdehyde (OPA), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS], Trolox and L-lysine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucose was obtained from Tianjin reagent company (Tianjin, China). All other chemicals were of analytical reagent grade.

Maillard reaction

OVA and glucose were fully solubilized in 50 mM phosphate buffer (pH 8.0) at protein/glucose weight ratio of 1:1, and then freeze-dried. The freeze-dried samples were then equilibrated in saturated KBr solution (relative humidity (RH) 79 %) at room temperature for 1 day. The graft reaction was carried out in a microwave oven (Galanz) (G80F20CN2L-B8(RO) Model, 2450 MHz, 800 W, Guangdong galanz co., Ltd., Foshan, China) and power levels used were 480 and 640 W. Two hundred milligrams of samples were incubated in capped-glass tubes for 0, 5, 10, 15, 20 and 25 min, respectively, and a digital thermometer was used to detect the temperature of each sample after MR immediately. Then samples were placed in an ice bath to cool down to 4 °C and stored at −20 °C prior to further analysis.

Electrophoresis

SDS-PAGE was performed using 5 % stacking gel and 12 % running gel with a vertical gel electrophoresis unit. The lyophilized OVA-glucose mixture and glycosylated OVA samples treated with different microwave power and time were dissolved in water at a concentration of 1 mg/mL and boiled for 10 min. Sample solution (15 µL) was loaded into each well. The electrophoresis was carried out at 15 mA. After separation, protein bands were stained using Coomassie Brilliant Blue R-250 (0.2 %) in 25 % methanol and 10 % acetic acid. Destaining was performed using 40 % methanol and 10 % acetic acid.

Measurement of UV absorbance, browning color and pH

The UV absorbance and browning of the OVA-glucose conjugate were measured according to the method of Ajandouz et al. (2006). Samples were dissolved with distilled water to a final concentration of 1.6 mg/mL and the absorbance was measured at 294 and 420 nm using a spectrophotometer (TU-1900 PuXiTongYong, Beijing, China). The pH was measured using a pH meter (Model SP-71, METTLER TOLEDO, Inc., Shanghai, China).

Antioxidant activity assay

Determination of reducing power

The reducing capacity of a compound can be used to evaluate its potential antioxidant activity (Huang et al. 2004). During the reducing power assay, the presence of reductants in the tested samples result in reducing Fe^{3+} /ferricyanide complex to the ferrous form (Fe^{2+}). The Fe^{2+} can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Yoshimura et al. 1997). Increasing absorbance of the reaction mixture at 700 nm indicates an increase in the reducing power.

The reducing power of the glycosylated OVA samples was determined according to the method of Gu et al. (2010) with modifications. One milliliter of samples (1.25 mg/mL) was mixed with 1.0 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 1.0 mL of 1 % potassium ferricyanide ($K_3Fe(CN)_6$). The reaction mixtures were incubated in a water bath at 50 °C for 20 min, followed by the addition of 1.0 mL of 10 % trichloroacetic acid after cooling to room temperature. The mixtures were then centrifuged at 8000 g using a centrifuge (TGL-10C, Anke, Shanghai, China) for 10 min at 25 °C. Two milliliters of the supernatant was mixed with 2.0 mL of distilled water and 400 μ L of 0.1 % $FeCl_3$. The absorbance of the reaction mixture was measured at 700 nm with TU-1900 spectrophotometer. All measurements were conducted in triplicate and the results were the average of three measurements and expressed as absorbance units (AU).

Determination of DPPH radical-scavenging activity

The ability of MRPs to quench reactive species by hydrogen donation was measured through the DPPH radical scavenging activity assay. As a stable free radical, DPPH can accept an electron or hydrogen radical to become a stable diamagnetic molecule, which is widely used to evaluate radical scavenging activity. The antioxidants can react with DPPH, a deep-violet colored stable free radical, converting it into a yellow colored α, α -diphenyl- β -picrylhydrazine. The discoloration of the reaction mixture can be quantified by measuring the absorbance at 517 nm, which indicates the radical-scavenging ability of the antioxidant (Braca et al. 2001).

DPPH radical-scavenging activity was determined according to the method of Lertittikul et al. (2007) with a slight modification. One milliliter of 0.1 mmol/L DPPH in ethanol was added to 3.0 mL of the sample (1 mg/mL). The mixture of OVA and DPPH-ethanol solution was then allowed to stand in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm using a TU-1900 spectrophotometer. The control was prepared in the same manner, except that de-ionized water was used instead of OVA sample. DPPH radical-scavenging activity was calculated by the following formula (Singh and Rajini 2004):

$$\text{Radical scavenging activity (\%)} = \left(1 - \frac{A_s}{A_c}\right) \times 100 \quad (1)$$

where A_s is the absorbance value of sample, A_c is the absorbance value of the control.

Trolox equivalent antioxidant capacity assay

The ABTS radical cation ($ABTS^{\bullet+}$) was produced by the method of Re et al. (1999). To measure radical scavenging

capacity, 80 μ L of the sample (2.5 mg/mL) was mixed with 3920 μ L of the radical solution. Absorbance was monitored at 734 nm after incubating the mixture at 37 °C for 10 min. The ABTS radical scavenging activity was calculated by the following equation:

$$\text{Scavenging activity (\%)} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100 \quad (2)$$

The standard curve was linear between 0 and 25 mM Trolox. Trolox equivalent antioxidant capacity (TEAC) = $\text{slope}_{\text{sample}} / \text{slope}_{\text{control}}$. Results were expressed in μ mol Trolox equivalent (TE)/g freeze-dried sample (Liu and Kitts 2011).

Determination of Emulsifying Activity Index (EAI) and Emulsion Stability Index (ESI)

EAI was determined according to the method of Pearce and Kinsella (1978), as modified by Cameron et al. (1991). For emulsion formation, 15 mL of 0.1 % (w/v) sample solutions and 5 mL of corn oil were homogenized in an Ultra-Turrax T25 digital homogenizer (IKA Co., Germany) at 9500 rpm for 2 min. Fifty microliters of emulsion was taken from the bottom of the homogenized emulsion, immediately after homogenization, and diluted (1:100, v/v) in 0.1 % (w/v) SDS solution. After 5 s of mixing in a vortex, the absorbance of dilute emulsions was read at 500 nm using a TU-1900 spectrophotometer. EAI and ESI values were calculated using the following equations:

$$\text{EAI (m}^2/\text{g)} = \frac{2 \times 2.30 \times A_0 \times DF}{c \times \Phi \times (1 - \theta) \times 10000} \quad (3)$$

$$\text{ESI (min)} = \frac{A_s}{A_0 - A_{10}} \times 10 \quad (4)$$

where DF is the dilution factor (100), c is the initial concentration of protein (g/mL), Φ is the optical path (0.01 m), θ is the fraction of oil used to form the emulsion (0.25), and A_0 and A_{10} are the absorbances of diluted emulsions at 0 and 10 min, respectively. Measurements were performed in triplicate.

Statistical analysis

The results were reported as mean $\pm$ standard deviation (SD). Error bars indicate standard deviation of three measurements. The significance of differences among mean values was determined using one-way analysis of variance (ANOVA), using SPSS version 16.0 (SPSS Institute, Chicago, USA), with a significance level of 0.05.

Fig. 1 SDS-PAGE profile of glycated and unglycated OVA samples. Lanes: M, protein markers; 0, lyophilized OVA-glucose mixture; 1–5, OVA glycated with glucose at 480 W of microwave heating for 5, 10, 15, 20 and 25 min, respectively; 1'–5', OVA glycated with glucose at 640 W of microwave heating for 5, 10, 15, 20 and 25 min, respectively

Results and discussion

SDS-PAGE analysis

The SDS-PAGE pattern of lyophilized OVA-glucose mixture and glycated OVA samples at different microwave power levels and time are shown in Fig. 1. As expected, with the heating time increased, the bands of OVA glycated with glucose were shifted upward which indicated that the protein molecular weight was increased. Increase in the molecular mass by glycation under microwave heating in dry state has already been reported (Tsubokura et al. 2009), and our results corresponded with this report. However, no significant differences between samples heated at 480 W and 640 W were found in the mobilities on the SDS-PAGE. It was also noticed that different amount of high molecular weight aggregates were appeared after 5 min of microwave heating both at 480 and 640 W (over 200 kDa, lanes 2–5 and 2'–5'). High molecular weight aggregates occurred in OVA-saccharide glycation reaction were mainly caused by covalent cross-linking through sugar-lysine amino carbonyl and inter-molecular disulfide bonds (Sun et al. 2004). The appearance of high molecular weight bands in our SDS-PAGE suggested that microwave heating could also induce protein aggregation.

Changes of pH

The changes in the pH of MRPs derived from OVA-glucose system in solid-state as a function of microwave power and heating time were shown in Fig. 2. At both power levels, 480 and 640 W, the pH of OVA-glucose MRPs was decreased as the heating time was extended up to 25 min. Upon microwave heating, glucose can partially degrade to formic and acetic acid, leading to a decreased level of pH (Rufián-Henares et al. 2006). The decrease in pH could also be attributed to the reaction of amines to form compounds of lower basicity (Van Boekel and Brands 1998). In addition, the consumption of the amino group by MR could shift the mixture into more acidic condition. Taken together, the pH decrease was caused by three factors, the consumption of amino group, formation

of acids and conversion of amines to basic compounds (Liu et al. 2008). At higher power, 640 W, the pH exhibited a significant faster decreasing rate than that at lower power of 480 W. The results are consistent with the observation by Guan et al. (2011), who reported that the soy protein isolate-saccharide graft reaction rate was increased with the elevated microwave power.

Changes in UV absorbance and browning intensity

The color of the samples was a direct and easy indication of MR progress. UV-vis absorbance at 294 nm was generally used to monitor the MR rate (Ajandouz et al. 2006). The final stage of the browning reaction was monitored by the increase in absorbance at 420 nm.

The changes of A_{294} and browning intensity of MRPs samples with different microwave power (480 and 640 W) and time (0, 5, 10, 15, 20 and 25 min) were shown in Fig. 3. At either microwave power level (480 or 640 W), both the A_{294} and A_{420} of MRPs was increased significantly in according with the heating time except the first 5 min at 480 W of microwave heating. The values under 640 W of microwave

Fig. 2 Changes in pH of samples treated with different microwave power and time

Fig. 3 UV-vis absorbance of Maillard reaction products. 0, lyophilized OVA-glucose mixture; 1, 2, 3, 4 and 5, OVA-glucose with 480 W of microwave heating for 5, 10, 15, 20 and 25 min, respectively; 1', 2', 3', 4' and 5', OVA-glucose with 640 W of microwave heating for 5, 10, 15, 20 and 25 min, respectively

heating were apparently higher than those under 480 W of microwave heating, indicating a much faster reaction rate stimulated by higher microwave power. The pH value of the reaction system was considered to affect MR significantly (Lertittikul et al. 2007). As shown in Fig. 2, the pH of the solution remained higher than 7.4 despite of decreasing constantly during microwave heating process. The browning intensity of MRPs showed a similar trend with the pH of the system as indicated by the calculated correlation coefficient ($R^2=0.9246$) (Fig. 8a). In alkaline condition, Schiff-base can be formed and promote the MR further. Consequently, the brown components of MR system can be produced quickly (Gu et al. 2009). During the development of brown color caused by the MR, caramelisation can also occur. Caramelisation reactions contribute to overall non-enzymatic browning, especially in the alkaline pH ranges (Ajandouz et al. 2006; Benjakul et al. 2005).

Antioxidant activity assay

Reducing power

As shown in Fig. 5, before microwave treatment, OVA-glucose sample showed negligible reducing power. Under 480 W microwave heating, the reducing power increased slowly for the first 5 min which could be due to the relatively low temperature (Figs. 4 and 5), and then it was increased drastically with the temperature promotion. At 640 W of microwave heating, the reducing power exhibited a much higher increase rate compared to 480 W heating. This might be ascribed to the higher power applied, the faster reaction rate was achieved within the same reaction time (Guan et al. 2011).

Fig. 4 Temperature of samples treated with different microwave power and time

The correlation coefficient with the browning intensity is fairly high ($R^2=0.8232$) (Fig. 8b), suggesting that there is good correlation between the reducing power and browning intensity. The positive correlation between the antioxidant activity and browning has been found in many MR systems where the formation of antioxidants is the prevalent event during processing (Manzocco et al. 2000; Huang et al. 2012). Several compounds with reducing activity can be generated during MR, including thermolysis of amadori in the primary phase of MR (Hwang et al. 2001), heterocyclization and caramelisation of sugars (Charurin et al. 2002). Microwave-irradiation can induce similar reactions in OVA-glucose system in solid state, generating reductive compounds which contribute to the significantly increased reducing power.

Fig. 5 Reducing power of samples treated with different microwave power and time

DPPH radical-scavenging activity

The changes in the DPPH radical scavenging activity of MRPs derived from OVA-glucose system, as a function of microwave power and time are shown in Fig. 6. The DPPH radical scavenging activity of non-treated OVA-glucose sample was as low as 24.7 %. It increased drastically in the first 15 min microwave heating at 480 W, while nearly reached a plateau after that time. The activity of MRPs at 640 W of microwave treatment was much higher than that at 480 W of microwave treatment in the first 15 min. The results indicated that MRPs formed in the OVA-glucose system in solid state using microwave heating were free radical inhibitors. These results are in accordance with that of Huang et al. (2012), who found that the OVA-glucose conjugates had strong DPPH radical-scavenging activity. It is reported that either intermediates or the final brown polymer can function as hydrogen donors. In addition, sugar caramelization can also contribute to the antiradical activity measured by DPPH test (Benjakul et al. 2005). It has been suggested that the browning compounds formed during the MR, which are primarily composed of melanoidins, are major contributor to the radical-scavenging capacity (Wang et al. 2011). In the first 15 min, 640 W of microwave heating formed more melanoidins during the latter stages of the MR than those formed at 480 W, which could be inferred from the browning results from Fig. 3. So the DPPH scavenging activity of MRPs at 640 W was much stronger than that at 480 W.

Trolox equivalent antioxidant capacity

As shown in Fig. 7, the TEAC of the sample increased significantly with extended heating time both at 480 and 640 W of microwave treatment. The values at 640 W were

Fig. 6 DPPH radical-scavenging activity of samples treated with different microwave power and time

Fig. 7 The TEAC of samples treated with different microwave power and time

much higher than those at 480 W, which reached to as high as 412 ± 15 μmol Trolox/g at 25 min. The correlation is again very strong with the browning intensity ($R^2=0.9702$) (Fig. 8c). Similar results were obtained in experiments of Rufián-Henares and Morales (2007), who reported that there existed correlations between melanoidins formed during the latter stages of the MR and results of the ABTS radical cation assay. At 640 W of microwave heating, stronger MR extent could generate larger quantities of melanoidins and heterocycles in the advanced stage of MR, which are the major contribution of the antioxidant activity (Huang et al. 2012). Compared to the untreated sample, the TEAC value enhanced almost 500 % after 640 W of microwave irradiation for 25 min. In addition, the MRPs of this study had much higher TEAC than the amino-glucose systems treated by subcritical water extraction conditions (Plaza et al. 2010) and α -lactalbumin conjugation with a rare sugar (D-allose) and two alimentary sugars (D-fructose; D-glucose) through MR in dry state at 50 °C and 55 % relative humidity for up to 48 h (Sun et al. 2006). This suggests that the glycation reaction of OVA-glucose in solid-state by microwave heating could be a better method for generating antioxidants for food preservation.

Emulsifying properties

Egg albumen powder offered a number of benefits over liquid eggs in terms of convenience, shelf life and microbiological safety. The ability of egg albumen proteins to form heat-induced gels as well as stable foams by air incorporation and emulsifying properties, which makes egg albumen proteins applicable in a large variety of processed foods. Maillard-type reactions have been used to improve egg albumen proteins functional properties, and it is considered to be suitable to food application because of its safety (Xu et al. 2012). The

Fig. 8 Linear correlations between browning intensity and (a) pH (b) reducing power (c) TEAC

emulsifying ability of a protein emulsifier depends on its ability to form adsorption films around the oil globules and to lower the interfacial tension at the oil–water interface.

Emulsion stability is the capacity of emulsion droplets to remain dispersed without separation by creaming, coalescing, and flocculation (Kuan et al. 2011).

As shown in Fig. 9a, at both microwave power levels (480 and 640 W), the EAI of OVA-glucose system was increased in according with microwave heating time. At 480 W of microwave treatment, the EAI increased from 228 to 338 m^2/g . Similarly, at 640 W of microwave treatment, the EAI increased slightly higher than those at 480 W, from 228 to 348 m^2/g . The tendency of emulsion stability (Fig. 9b) was almost the same with the EAI (Fig. 9a), at both microwave power levels (480 and 640 W). The ESI of samples gradually increased with increasing microwave heating period, with a slightly faster rate at 640 W than at 480 W. The results were in accordance with Achouri et al. (2005), who found that glycation of 11S-rich glycinin fraction was effective in enhancing its emulsifying activity, especially at the early and middle stages of the MR. As emulsifying properties of

Fig. 9 Emulsifying activity index (a) and emulsion stability index (b) of samples treated with different microwave power and time

glycated protein were positively related to the glycation extent (Moreno et al. 2002), which could be enhanced by temperature promotion (Oliver et al. 2006), so the EAI and ESI of the samples heated at 640 W were higher than those at 480 W could be due to the higher temperature as presented in Fig. 4. It is noted that microwave heating will cause inhomogeneous distribution of temperature. It is inevitable that the inside temperature will be much higher than surface temperature during microwave heating. To minimize this effect, all the samples treated by microwave were kept under the exact same conditions with the same weight and volume. Figure 4 illustrates the temperature changes under two different powers of microwave heating. The trends of the temperature increase are similar to the changes of antioxidant activities. Therefore, forming of MRPs is most likely the results of increased temperature generated by microwave heating.

Compared to the previous reports, the glycation time of this study is just 25 min, much shorter than the incubation of kidney bean vicilin-glucose system at 60 °C for 10 h (Tang et al. 2011) and soy 11S glycinin-glucose system at 50 °C for 48 h (Achouri et al. 2005). Moreover, the increased percentage of EAI was 52.8 % (from 228 to 348 m²/g) in this study, much higher than the value of incubation of kidney bean vicilin-glucose system (18.4 %, from 190 to 225 m²/g) and soy 11S glycinin-glucose system (22.0 %, from 12.75 to 15.55 m²/g). These results suggest that the glycation reaction of OVA-glucose in solid-state by microwave heating could be an effective method for improving protein functional properties.

Conclusion

In this study, OVA–glucose mixture in solid-state was subjected to microwave heating at 480 and 640 W for 0 to 25 min. The results showed that the protein molecular weight was increased after glycated with glucose under microwave treatment, the pH of the system was decreased with the increase of microwave treatment power and time, while the UV absorbance, browning intensity, antioxidant activities as well as the emulsifying activity and emulsion stability of the MRPs were increased in according with the raise of microwave treatment power and time. The reaction time of microwave treatment is much shorter than those using traditional methods, suggesting that microwave irradiation is a novel and efficient approach to promote MR in solid state and improve protein antioxidant and functional properties. Our future work will be focused on the identification of the structure for the active compounds and the formation mechanisms of OVA–glucose conjugate in microwave field.

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