

Glycation of ovalbumin after high-intensity ultrasound pretreatment: Effects on conformation, IgG/IgE binding ability and antioxidant activity

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Abstract

BACKGROUND: Ovalbumin (OVA), a protein with excellent nutritional and processing properties, is the major allergen of hen egg white. Previous study showed that high-intensity ultrasound treatment increased the IgG and IgE binding abilities by unfolding the conformational structure of OVA. It may allow modifying the IgG and IgE binding of OVA by combining high-intensity ultrasound with other methods, such as glycation, a promising method for the improvement of protein properties.

RESULTS: Glycation with mannose (M) after ultrasound pretreatment at 0-600 W significantly reduced the IgG and IgE binding abilities and dramatically enhanced the antioxidant activity of OVA-M conjugates, with the lowest values of IgG and IgE binding and highest values of antioxidant capacity observed at 600W. The polyacrylamide gel electrophoresis showed that the molecular weight of OVA-M conjugates with ultrasound pretreatment increased more than

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non-pretreatment sample, implying ultrasound pretreatment promoted glycation. The α -helix content and UV absorption of OVA were observably increased while β -sheet content, intrinsic fluorescence and surface hydrophobicity were notably decreased, indicating the tertiary and secondary structure of OVA were markedly changed.

CONCLUSION: High-intensity ultrasound pretreatment can be conducive to reducing the IgG and IgE binding abilities and enhancing the antioxidant activity of OVA-M conjugates. Therefore, glycation combined with high-intensity ultrasound pretreatment might be a promising method for producing hypo-allergenic and high-antioxidant OVA products.

Keywords: ovalbumin; IgG binding; IgE binding; glycation; ultrasound; antioxidant activity

INTRODUCTION

Hen egg, one of essential daily food, is widely used in food industry because of its excellent nutritional and functional properties. However, about 1-3% of children are suffering egg allergy,¹ leading to the limitation of egg application in food industry. For egg white allergic patients in the world, the most useful method is to eliminate the egg allergens completely. Nevertheless, the egg derived allergic compounds widely exist in food products, and it is very hard to avoid them totally.² Therefore, it is necessary to develop a satisfying method for producing egg hypoallergenic compound.

Ovalbumin (OVA), accounts for about 54% of egg white protein, is a glycoprotein with 385 amino acids and has a molecular weight of about 45 kDa.³ Moreover, it becomes an experimental model of allergy because of its ready availability in quantities. Numerous studies have been done to change egg antigenicity and allergenicity by various treatments, including physical treatments, such as heating,⁴ γ -irradiation,⁵ ultrasound⁶ and chemical treatments, such as glycation⁷ and enzymatic hydrolysis.⁸ However, single physical modification could not significantly decrease the allergenicity by modifying both conformational and linear epitopes. Chemical treatments, for instance, enzymatic hydrolysis, has some sensory defects (such as bitterness) and might cause some potential disordered metabolism.⁹ Among them, glycation has been considered as a satisfying way of modifying proteins compared to other methods.¹⁰

Glycation, the first step of the Maillard Reaction (MR), which generally takes place between proteins and reducing sugars, is one of the most common and important chemical reactions occurring in food processing and storage. This non-enzymatic reaction is often used

to modify food proteins for improving the functional properties, such as solubility, gelling capacity, and emulsifying properties.¹¹ Recently, it has been used to modify potential allergenicity of protein by the covalent binding with reducing sugar. Ilchmann *et al.*¹² and Heilmann *et al.*¹³ found that OVA enhanced T-cell immunogenicity after glycation at 50 °C for 6 weeks. Ma *et al.*⁴ reported the IgE binding ability of OVA decreased while IgG binding increased after glycation under wet-thermal condition (at 60 °C for 30 min and 60 min). Also, they depicted that the IgE binding ability of OVA increased after glycation (at 60 °C for 30 min) in different concentrations of sodium carbonate-bicarbonate buffer (pH 9.6).⁴ Jimenez-Saiz *et al.*⁷ showed glycation under dry-thermal condition (at 50 °C for 96 h) enhanced the IgG binding ability while reduced the IgE binding to OVA. However, few studies investigated other functional properties (such as antioxidant activity) under the corresponding conditions, and limited studies applied combined processing methods for OVA desensitization.¹⁴

High intensity ultrasound is an emerging non-thermal technology that uses a collective force of high temperature, high pressure, microjet, shear force, shock wave and turbulence.¹⁵ It was reported that high-intensity ultrasound promoted MR of Glycin/Glucose and glycin/maltose systems.^{16, 17} and efficiently enhanced the wet-heating glycation content and antioxidant activity of β -lactoglobulin.¹⁸ Our previous work found that high intensity ultrasound treatment increased the IgG and IgE binding capacities by unfolding OVA structure.⁶ But the effect of high intensity ultrasound pretreatment combining with glycation on conformational structure, IgG and IgE binding abilities and antioxidant activity of OVA need further study.

In order to assess the influence of glycation combined with ultrasound pretreatment on the structure, IgG binding, IgE binding and antioxidant activity of OVA, as well as to expound upon their relationships, in this work, OVA was pretreated by high intensity ultrasound followed by glycation with mannose (M) under dry-heating condition. The structure changes were characterized through circular dichroism, ultraviolet absorption and fluorescence spectroscopy. The IgG and IgE binding abilities were determined by competitive ELISA and inhibition ELISA. The antioxidant activity was evaluated by reducing power, DPPH scavenging activity and trolox equivalent antioxidant capacity (TEAC).

MATERIALS AND METHODS

Glycation combined with ultrasound pretreatment

The OVA was isolated and purified from fresh hen eggs and the purity was more than 98%.⁶ Then it (10 mg mL⁻¹, dissolved in phosphate buffered saline (PBS), pH 8.0) was placed in a beaker to be treated with ultrasound probe (NingBo Scientz Biotechnology Co. Ltd., Ningbo, China, Model: JY98-□DN, frequency 20 kHz, power 950 W). The solution was irradiated with an ultrasonic wave directly from the probe with a vibrating titanium tip of 15 mm. Samples were treated for 15 min at 0 W, 400 W and 600 W. The pulse duration of on-time and off-time were 3 s and 7 s, respectively. The samples were treated in ice bath with processing temperature below 20 °C. Then the mannose was added at an OVA/mannose mass ratio of 1:2. After mixed evenly, 100 µL of each samples was lyophilized and incubated for 3 h at 65 °C and 0.79 water activity in a desiccator. Then all glycation samples were dialyzed to remove

mannose, adjusted to 1 mg mL^{-1} and stored at 4°C for less than 48 h. Untreated OVA was used as control and named OVA-N. OVA heated at glycation conditions without mannose was used as another control and named OVA-H. The glycated samples pretreated at 0, 400 and 600 W by ultrasound were named OVA-MH, OVA-M-400 and OVA-M-600, respectively.

Gel electrophoresis

SDS-PAGE (with β -mercaptoethanol) was performed to estimate the molecular weight of protein, 5% stacking gel and 10% separating gel were used.¹⁹ The Coomassie Brilliant Blue R-250 and middle molecular weight protein marker (10-75 kDa) were used as dye and standard, respectively.

Far-UV circular dichroism spectroscopy

Far-UV (190-240 nm) circular dichroism (CD) spectroscopy was performed to define the secondary structure of OVA samples. The CD spectra of OVA (0.1 mg mL^{-1} in 0.02 mol L^{-1} sodium phosphate buffer, pH 7.4) were collected by using a MOS-450 spectropolarimeter (Bio-Logic SAS, Claix, French) with path length, bandwidth and step resolution at 1.0 mm. The data were showed as molar residue ellipticity ($[\theta]$, $\text{deg cm}^2 \text{ dmol}^{-1}$), and the contents of different secondary structures were analyzed with DichroWeb.

UV absorption spectroscopy

UV absorption spectrum was performed by Hitachi spectrophotometer (UV-2910, Hitachi, Tokyo, Japan). The OVA solution (0.5 mg mL^{-1}) was scanned from 240 to 320 nm at a scan

speed of 800 nm min⁻¹.

ANS fluorescence and intrinsic fluorescence spectroscopy

ANS fluorescence was used to measure the surface hydrophobicity of OVA according to the method of Matulis and Lovrien²⁰ with modifications. The volume ratio of sample (0.5 mg mL⁻¹) and ANS solution (0.008 mol L⁻¹) was 200:1. The relative fluorescence intensity was determined by Hitachi spectrofluorimeter (F-7000, Hitachi, Tokyo, Japan). The excitation wavelength was 390 nm and the emission spectrum was scanned from 400 to 600 nm at a speed of 1200 nm min⁻¹. The bandwidth, excitation and emission slits were all 5.0 nm. The voltage of photomultiplier was 400 V. For intrinsic fluorescence, the concentration of OVA was 0.2 mg mL⁻¹. The emission spectrum was scanned from 300 to 420 nm at a speed of 1200 nm min⁻¹ with excitation wavelength set at 280 nm. The other parameters were as same as that of ANS fluorescence measurement.

IgG and IgE binding abilities of OVA

Polyclonal antisera against OVA were obtained from young (three months old, about 2.0 kg) Japanese male rabbits (Permission No. SCXK(Gan)-2014-0005).⁶ Human sera were collected from four egg allergy patients (numbered P1-P4), whose specific IgE levels were 15.4, 22.5, 29.8 and 64.6 kU L⁻¹, respectively. The IgG and IgE binding of OVA samples was evaluated by inhibition ELISA.²¹ Firstly, 100 µL of 2 µg mL⁻¹ untreated OVA in 0.05 M PBS (pH 7.4) was coated into each well of a 96-well microplate overnight at 4 °C. After washing with PBST (0.5 mL Tween-20 in 1.0 L of 0.02 mol L⁻¹ PBS, pH 7.4) for 5 five times, it was blocked for 1

h at 37 °C with 50 mg mL⁻¹ skimmed milk in PBS. Then 50 µL antisera (1:12,800 pooled rabbit antisera (diluted to) or pooled human antisera (diluted to 1:8)) and 50 µL OVA samples (inhibitors) were added after washing. It was incubated for 1 h at 37 °C. After washing, 100 µL of goat anti-human IgE-HRP conjugate or goat anti-rabbit IgG-HRP conjugate (diluted to 1:5,000 in PBST) was added and incubated for 30 min at 37 °C. Then 100 µL of TMB solution was added after washing. And it was incubated for 15 min at 37 °C. Finally, 50 µL of 2 M sulfuric acid was added to stop the reaction and the absorbance was measured at 450 nm using a microplate reader (HF2000, Huaan Magnech, Beijing, China). The inhibition rate was calculated using the following equation: Inhibition = $(1 - B / B_0) \times 100\%$, where B and B₀ are the absorbance values of the well with and without the inhibitor, respectively. IC₅₀ is the concentration of inhibitors that causes a 50% inhibition of antibody binding (µg mL⁻¹).

Determination of reducing power

The reducing power of OVA samples was determined according to the method of Medjkouh *et al.*²² with modifications. Phosphate buffer (1.0 mL, 0.2 mol L⁻¹, pH 6.6) and 1% potassium ferricyanide (1.0 mL) were mixed with 1.0 mL of OVA samples. After incubation in a water bath (50 °C, 20 min), 1.0 mL of 10% trichloroacetic acid was added and the mixture was centrifuged at 8000 g for 10 min at 25 °C. Then 2.0 mL of supernatant was mixed with 2.0 mL of distilled water and 0.4 mL of 0.1% FeCl₃. The absorbance of the reaction mixture was measured at 700 nm and expressed as absorbance units.

Determination of DPPH radical-scavenging activity

The DPPH radical-scavenging activity was determined according to the method of Huang *et al.*¹⁰ with slight modifications. Firstly, 4 mL of 0.2 mmol L⁻¹ DPPH in methanol solution was mixed with 1 mL of OVA samples. After incubation at room temperature for 30 min in the dark, the absorbance at 517 nm was measured. The milli-Q water was used as the control at the first step. DPPH radical-scavenging activity was calculated using following formula: Radical scavenging activity (%) = $(1 - A_s / A_c) \times 100$, where A_s is the absorbance value of sample, and A_c is the absorbance value of the control.

Trolox equivalent antioxidant capacity assay

The ABTS radical cation (ABTS^{•+}) was produced by the method of Huang *et al.*²³ To measure radical scavenging capacity, 80 μ L of the sample or trolox was mixed with 3.92 mL of the radical solution. Absorbance was monitored at 734 nm after 10 min of incubation at 37 °C. The ABTS radical scavenging activity was calculated by the following equation: Inhibition (%) = $(1 - A_d / A_b) \times 100$, where A_d is the absorbance value of sample, and A_b is the absorbance value of the control. The standard curve was linear between 0 and 25 mmol L⁻¹ Trolox. TEAC = slope_d / slope_b. Results were expressed in mmol Trolox equivalent (TE) g⁻¹ freeze-dried sample.²⁴

Statistical analysis

All experiments were carried out in triplicate, and all results were presented as mean value $\pm$ standard deviation (SD). The statistical analysis was performed by analysis of variance (ANOVA) with SPSS 19.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Molecular weight analysis

As shown in **Figure 1**, the band of OVA-MH (around 47 kDa) moved further compared with that of OVA-N (about 45 kDa). However, the bands of OVA-M-400 and OVA-M-600 (almost 48 kDa) moved further than that of OVA-MH. It indicated that the molecular weight of OVA increased after glycation, and ultrasound pretreatment might increase the glycation sites and finally promoted the glycation of OVA. It was reported that ultrasound pretreatment improved the incorporation of galactose to BSA.²⁵ In our previous work, we found that ultrasound treatment could make OVA unfolded.⁶ The unfolding of OVA might expose glycation sites, and finally improve the glycation. Therefore, glycation could increase the molecular weight of OVA, and ultrasound promoted glycation.

Secondary structure analysis

The far-UV CD spectra of OVA samples are shown in **Figure 2**. It depicted that the negative extreme around 208 nm and 222 nm decreased and the positive maximum around 193 nm increased, indicating the increase of α -helix after glycation with mannose. It consistent with the secondary structure content shown in **Table 1**. There are no significant changes in secondary structure of OVA after heating without mannose under dry state ($P < 0.05$), indicating heating under dry state had little influence on the secondary structure. Matsudomi *et al.*²⁶ also found that OVA kept the same secondary structure after dry-heating at 80 °C for 7 days. It might be caused by the low water activity, which limited the movement of OVA

molecule. However, when OVA was glycosylated with mannose, the α -helix content of OVA increased from 23.7% (OVA-N) to 28.1% (OVA-MH) while the β -sheet content decreased from 25.7% (OVA-N) to 19.0% (OVA-MH). It indicated that OVA was unfolded after glycation, and the α -helix content might increase mainly at the expense of β -sheet content. This was consistent with the result reported by Liu *et al.*¹¹, who claimed that glycation increased the α -helix content and mainly at the expense of β -sheet or random coil content. In addition, glycation could make OVA conformation to a more stable state, the α -helix is more stable than other second structure,²⁷ which might explain why the α -helix content was increased after glycation. Furthermore, OVA-M-600 had more α -helix content (31.2%) and less β -sheet content (16.7%) than OVA-MH (28.1% for α -helix content, 19.0% for β -sheet content), this could be due to the enhanced glycation caused by ultrasound pretreatment, leading to the formation of more α -helix. However, the result was opposite to reported in a previous study,⁴ wherein they showed the β -sheet content increased after glycation. It might be due to different glycation conditions, including water activity, pH, reaction temperature and time.

UV absorption spectra analysis

The UV absorption spectra of OVA samples are shown in **Figure 3A**. Compared with untreated OVA, OVA heated without mannose showed a slight increase in UV absorbance at 280 nm, and the glycosylated OVA displayed much higher UV absorbance at 280 nm. And glycosylated OVA pretreated by ultrasound had higher value than that without ultrasound pretreatment. It suggested that dry-heat could partially unfold OVA structure and make Trp

and Tyr residues exposed to the surface of OVA molecule. According to the report of Zhang *et al.*,²⁸ glycation could result in an increased exposure of Trp and Tyr residues to the solvent medium. Moreover, ultrasound pretreatment could make OVA unfolded and promote glycation, which made more Trp and Tyr residues exposed. That might be the reason for the higher UV absorbance of OVA-M-400 and OVA-M-600 OVA than OVA-MH.

Intrinsic fluorescence and ANS fluorescence spectra analysis

Figure 3B shows the changes in the intrinsic fluorescence spectra of OVA samples. The maximum fluorescence intensity of OVA increased from 852 (OVA-N) to 911 (OVA-H) after dry-heated without mannose. The result is similar with UV absorption analysis, implying that dry-heating made OVA partially unfolded, leading to the exposure of Trp and Tyr residues. However, when OVA was glycated, the maximum fluorescence intensity of OVA dramatically decreased, and OVA-M-600 had lower maximum fluorescence peak value than OVA-M and OVA-M-400. It might be resulted from the quenching of fluorescence intensity caused by the exposure of Trp residues to solvent.²⁹ In addition, a red shift of the maximum emission wavelength (from 343 nm to 347 nm) was observed, indicating the polarity of OVA was changed. The result was also similar to some reports,^{10, 30, 31} which showed the significant decrease of intrinsic fluorescence intensity and red shift of fluorescence spectra of protein after glycation with reducing sugar.

The changes in the ANS fluorescence spectra of OVA samples are shown in **Figure 3C**. When OVA was dry-heated without mannose, the maximum fluorescence intensity of OVA significantly increased, indicating the increase of surface hydrophobicity of OVA due to

protein partially unfolding. However, the maximum fluorescence intensity of OVA declined from 50.3 (OVA-N) to 30.6 (OVA-MH), 28.7 (OVA-M-400) and 25.6 (OVA-M-600), respectively. The result was in accordance with the report of *Corzo-Martínez et al.*,³² who found that the hydrophobicity of β -LG was decreased after glycation. The decrease of hydrophobicity might be resulted from the blocking of Lys and/or Arg residues by mannose during glycation. Because ANS could also strongly bind cationic groups, such as Lys and Arg residues.³³ Ultrasound pretreatment could promote Lys and/or Arg residues conjugated with mannose, make ANS bind less cationic groups and finally lead to the decrease of surface hydrophobicity. Accordingly, OVA-M-400 and OVA-M-600 had lower surface hydrophobicity than OVA-MH.

Effect of glycation combined with ultrasound pretreatment on the IgG and IgE binding abilities of OVA

Figure 4 shows the changes of IgG and IgE binding of OVA treated under different conditions. As shown in **Figure 4A**, the IC_{50} value of OVA-H ($1.91 \mu\text{g mL}^{-1}$) was lower than that of OVA-N ($3.10 \mu\text{g mL}^{-1}$). However, when OVA was glycated, the IC_{50} value increased to $10.03 \mu\text{g mL}^{-1}$ (OVA-MH), $17.98 \mu\text{g mL}^{-1}$ (OVA-M-400) and $28.02 \mu\text{g mL}^{-1}$ (OVA-M-600), respectively. It indicated that glycation significantly decreased IgG binding capacity of OVA and ultrasound treatment promote the reduction of IgG binding ability. **Figure 4B** depicts the changes of IgE binding to OVA treated under different conditions. The results were similar to that of IgG binding ability. Compared to OVA-N, OVA-H had lower IC_{50} value while glycated OVA had higher IC_{50} value, indicating dry-heating without mannose enhanced the IgE binding

ability and glycation decreased it. Similar result was reported that the IgG binding of α -lactalbumin and β -lactoglobulin was significantly decreased after glycation.³⁴ However, Ma *et al.*⁴ showed that glycation enhanced the IgG binding of OVA. The opposite results might be caused by different glycation conditions, including protein concentration, glycation mode, temperature and time.

Relationship between IgG/IgE binding ability and structure of OVA

OVA has five allergic IgE linear epitopes, including ³⁸Leu-⁴⁹Thr, ⁹⁵Asp-¹⁰²Ala, ¹⁹¹Glu-²⁰⁰Val, ²⁴³Val-²⁴⁸Glu and ²⁵¹Gly-²⁶⁰Asn.³⁵ Some of them contain the residues of Lys or Arg. As we know, the aldehyde group of mannose reacts with ϵ -amino group of Lys or Arg during glycation. Glycation could modify or mask the IgG and IgE epitopes by the covalent attachment of reducing sugar.³⁶ OVA has 20 Lys and 15 Arg residues, which are the main glycation sites. The increase of molecular weight implied that some Lys and/or Arg reacted with mannose. It makes antibody unable to recognize them and finally leads to the decrease of IgG and IgE binding ability. Ultrasound pretreatment could result in the unfolding of OVA, increase of glycation sites and finally promoted the reduction of the IgG and IgE binding. Therefore, glycation combined ultrasound pretreatment dramatically decreased the IgG and IgE binding ability by modifying IgG and IgE epitopes of OVA.

The conformational changes of OVA after glycation combined with ultrasound pretreatment would be another reason should be responsible for the reduction of IgG and IgE binding. The changes in conformational structure of OVA after glycation were reflected by the increase of UV absorbance, reduction of intrinsic fluorescence intensity and surface hydrophobicity.

However, ultrasound pretreatment promoted these changes, which resulted in further increase of UV absorbance and decrease in intrinsic fluorescence intensity and surface hydrophobicity. The glycation influenced both the local region and the distant area.³⁷ These conformational changes might alter the conformation of antigenic and allergenic epitopes, and lead to the reduction of IgG and IgE binding capacities. In addition, hydrophobic forces play a major role in the binding of IgG or IgE with protein. The decrease in surface hydrophobicity of OVA makes it be more difficult for IgG or IgE to bind with OVA. Therefore, the significant reduction on IgG and IgE binding of OVA induced by glycation with ultrasound pretreatment was resulted from the modification of allergenic epitopes and the alteration of OVA conformational structure.

Antioxidant activity analysis

The antioxidant activity was evaluated by reducing power, DPPH radical-scavenging activity and TEAC. As shown in **Figure 5A**, the un-glycated OVA samples had negligible reducing power. After glycated with mannose, the reducing power of OVA-M conjugates dramatically increased. And OVA treated by glycation with ultrasound pretreatment exhibited a much higher reducing power than non-pretreated ones. **Figure 5B** depicted the changes of DPPH radical-scavenging activity of OVA samples, which exhibited a concentration-dependent manner. The activity increased drastically at the concentration (0.1-0.5 mg mL⁻¹), while it nearly reached nearly a plateau after concentration was over 0.5 mg mL⁻¹. There was no significant difference between OVA-N and OVA-H, indicated that dry-heating could hardly change the DPPH radical-scavenging activity of OVA. However, the

scavenging activity of OVA dramatically increased after glycation, and OVA-M-400 and OVA-M-600 had stronger scavenging activity than OVA-MH at low concentrations. Similar results are showed in **Figure 5C**, the TEAC of OVA-N was almost equal to that of OVA-M (0.53 ± 0.02 mmol trolox g^{-1}). However, it was significantly increased after glycation. And the OVA-M-600 had the strongest TEAC (2.73 ± 0.11 mmol trolox g^{-1}), which is over 4-fold higher than that of OVA-N (0.49 ± 0.02 mmol trolox g^{-1}).

The result was in accordance with other reports,^{10, 38} in which the antioxidant activity of OVA was increased after glycation by microwave heating (480 W and 640 W for up to 25 min) and wet-heating (120 °C for 20 min). Some compounds, such as intermediates and sugar caramelization, with antioxidant potential can be generated during MR, including thermolysis of Amadori products in the primary phase of MR.^{16, 39, 40} Moreover, high molecular weight MR products exhibited strong reducing power.⁴¹ Cavitation of ultrasound treatment could produce hydroxyl radical, which promoted the glycation.⁴² The result above indicated that glycation combined with ultrasound pretreatment could dramatically enhance the antioxidant activity of OVA and ultrasound pretreatment promoted the enhancement. Therefore, glycation combined with ultrasound pretreatment could be a promising method for generating antioxidants for food processing.

4. Conclusions

This study revealed that the structural changes closely correlated with the decrease in IgG/IgE-binding ability and increase in antioxidant activity of OVA induced by glycation combined with high-intensity ultrasound pretreatment. Moreover, high-intensity ultrasound

pretreatment promoted the reduction of IgG and IgE binding capacities and the enhancement of antioxidant activity of OVA-M conjugates. Then, it might be concluded that glycation combined with ultrasound pretreatment might be a promising method for the development of OVA-derived hypoallergenic compounds. However, the glycation sites and content under the condition should be explored by mass spectrometry to explore the mechanism of glycation combined with ultrasound pretreatment reduces the IgG and IgE binding of OVA at molecular level.

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Table 1 Effect of glycation combined with ultrasound pretreatment on the content (%) of OVA secondary structures

samples	α -Helix	β -Strand	Turn	Unordered
OVA-N	23.7 ± 0.1^a	25.7 ± 0.2^a	21.8 ± 0.2^a	28.8 ± 0.4^a
OVA-H	24.3 ± 0.3^a	24.6 ± 0.6^a	21.6 ± 0.3^a	29.5 ± 0.8^a
OVA-MH	28.1 ± 0.3^b	19.0 ± 0.7^b	23.1 ± 1.3^a	29.7 ± 0.2^a
OVA-M-400	29.5 ± 0.4^c	18.8 ± 0.7^b	21.5 ± 0.8^a	30.2 ± 0.5^a
OVA-M-600	31.2 ± 0.5^d	16.7 ± 0.3^c	21.8 ± 0.8^a	30.2 ± 1.2^a

Values followed by different letter (a-d) in the same column are significantly different

($P < 0.05$).

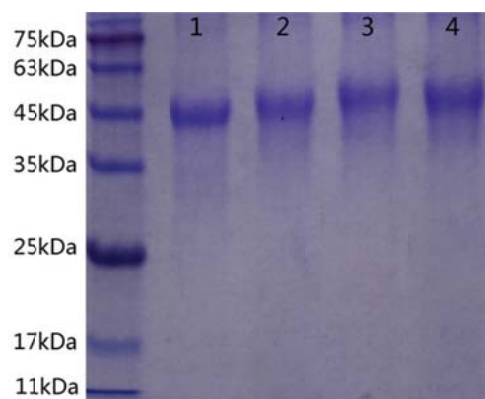


Figure 1. Effect of glycation combined ultrasound pretreatment on the molecular weight of OVA samples. Band 1-4 were represented OVA-N, OVA-MH, OVA-M-400 and OVA-M-600, respectively.

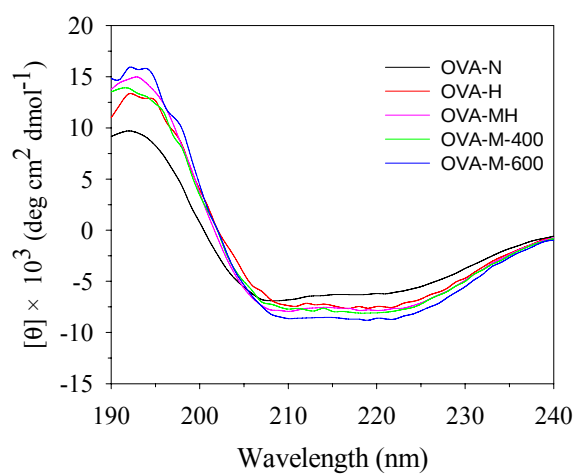


Figure 2. Effect of glycation combined ultrasound pretreatment on the far-UV circular dichroism spectra of OVA samples.

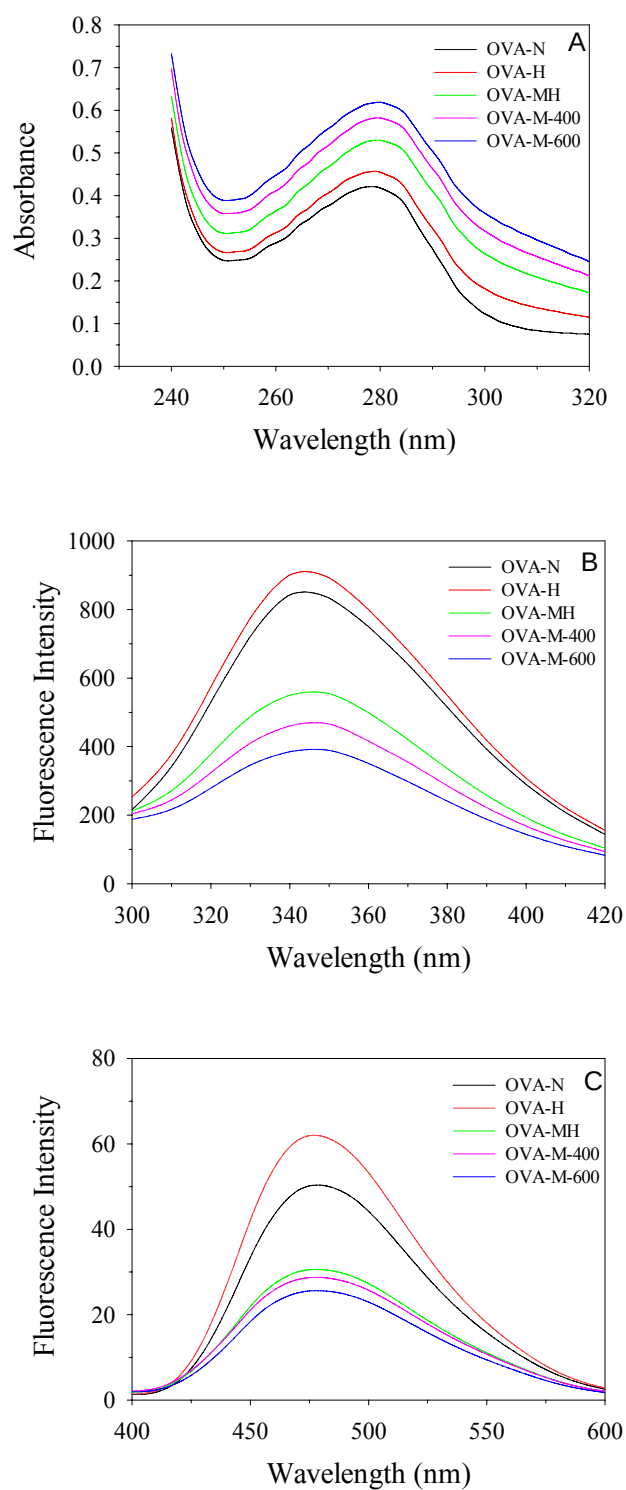


Figure 3. Effect of glycation combined ultrasound pretreatment on UV absorption (A) and intrinsic fluorescence spectra (B) and ANS fluorescence spectra (C) of OVA samples.

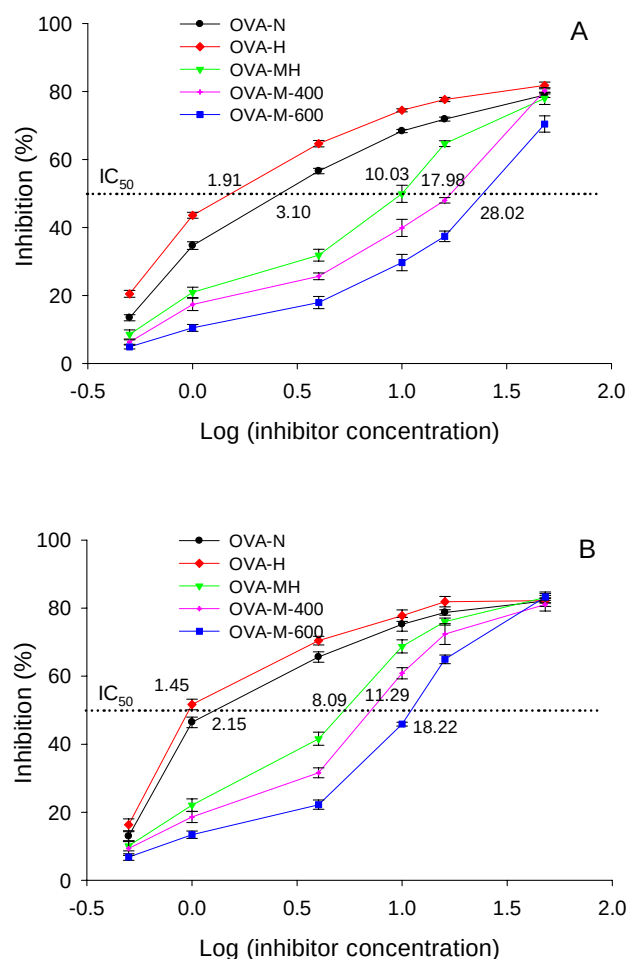


Figure 4. Effect of glycation combined ultrasound pretreatment on the IgG (A) and IgE (B) binding ability of OVA samples. The IgG and IgE binding of OVA samples were determined by inhibition ELISA. IC_{50} is the concentration of inhibitors that causes a 50% inhibition of antibody binding ($\mu\text{g mL}^{-1}$). Pooled rabbit anti-OVA-sera (50 μL per well) were incubated separately with 0.5, 1, 5, 10, 40, 48 $\mu\text{g mL}^{-1}$ of corresponding OVA samples as inhibitors. Pooled human anti-OVA-sera (50 μL per well) were incubated separately with 0.5, 1.5, 5, 15, 30, 60 $\mu\text{g mL}^{-1}$ of corresponding OVA samples as inhibitors.

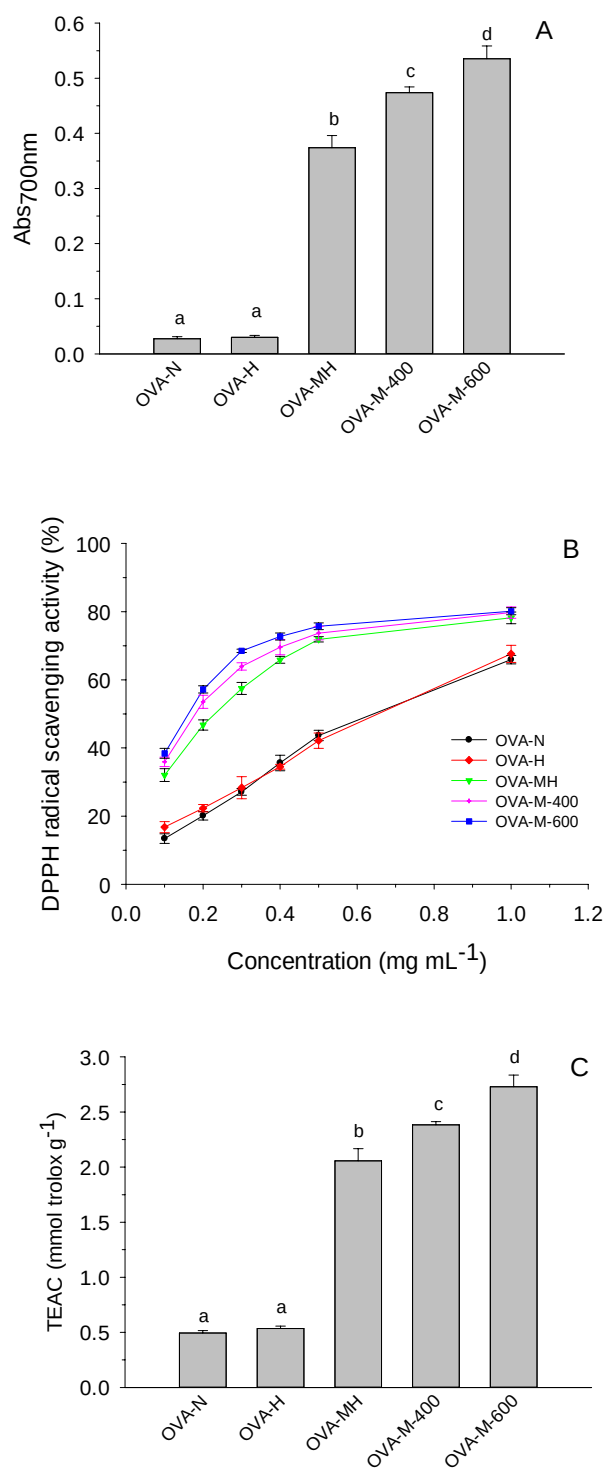


Figure 5. Effect of glycation combined ultrasound pretreatment on the antioxidant activity (reducing power (A), DPPH radical-scavenging activity (B) and TEAC (C)) of OVA samples.

Error bars show mean standard deviation of three determinations. Means with different letters

(a-d) in the bars are significantly different ($P < 0.05$).