

High intensity ultrasound enhances the IgG and IgE binding of ovalbumin

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Abstract

BACKGROUND: Hen's egg is widely used in food industry due to its excellent nutritional and processing properties, and Ovalbumin (OVA) is the major allergen of hen's egg white. Ultrasound has been widely used in food processing industry, but effect of high intensity ultrasound on the immunoglobulin G (IgG) binding, immunoglobulin E (IgE) binding and conformational structure of OVA remains unclear.

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RESULTS: Ultrasound treatment at 600 - 800 W dramatically increased the IgG and IgE binding of OVA with the highest values observed at 600 W. The free sulphydryl content, surface hydrophobicity and UV absorption were gradually increased when the power was improved from 200 to 600 W, implying the occurrence of molecule unfolding. When the power was 800 W, the binding ability was slightly decreased associated with the aggregation of OVA molecule. Polyacrylamide gel electrophoresis analysis implied that ultrasound treatment at 800 W could induce the aggregation of OVA molecule through disulfide bonds and/or hydrophobic interaction.

CONCLUSION: High intensity ultrasound can increase the potential allergenicity of OVA. Therefore, high intensity ultrasound processing of some egg products alone may improve the risk of allergenic reaction of egg allergy patients to some extent.

Keywords: ovalbumin; egg allergy; IgG binding; IgE binding; ultrasound; structural change

INTRODUCTION

Food allergy is an increasingly recognized problem due to its prevalence in general population.¹ During the past decade, the number of people with food allergy and the severity of these symptoms has continuously increased, this attracts a growing attention on the allergenicity of food allergy.^{2,3}

Hen's egg, one of essential food processing ingredients, is widely used in the food industry due to its excellent nutritional value and processing properties. However, it can cause anaphylaxis by ingestion, inhalation or skin contact, which accounts for 1.6 - 8.0% of food allergy in infants and young children, and ranks among the top allergic foods.⁴ Currently, many processing methods have been applied to investigate the effect of processing on the allergenicity of egg allergens, such as thermal processing, glycation, fermentation, hydrolysis, γ -irradiation and high pressure.^{3,5-7} Lee *et al.*⁵ revealed that the antigenicity and allergenicity of OVA were significantly decreased by γ -irradiation treatment at 3 ~ 10 kGy. Lopez-Exposito *et al.*⁶ described that enzymatic hydrolysis combined with high pressure treatment could significantly reduced the IgG and IgE binding ability of OVA. Ma *et al.*⁷ reported the glycation and heating at 60 °C for 30-120 min decreased the IgE reactivity. However, the great majority of researches focus on exploring methods reducing the allergenicity of food allergies, whether the commonly used processing technology will increase the allergenicity of egg white or not is usually ignored.

High intensity ultrasound, with a frequency of 16 - 100 kHz and intensity of 10 - 1,000 W cm⁻², is a non-thermal technology for food production.⁸ The main effect of ultrasound

is owed to the cavitation phenomenon, which could generate chemical reactions and violent physical forces, including extreme high temperature, high pressure, microjet, shear force, shock wave and turbulence.⁹ Compared with conventional thermal approaches, high intensity ultrasound has been considered as a promising processing in the food industry due to its potential in the development of green, relatively mild, time-saving, energy saving and targeted processes to improve the quality and safety of foods.¹⁰

Recently, high intensity ultrasound has been used in various food processing areas, including freezing, substance extraction, homogenizing, crystallization, bacterial inactivation, etc.¹¹ It is employed to improve food properties, such as emulsibility, gelation, antioxidation, foamability, etc. Most researches about high intensity ultrasound are mainly aimed to inactivate microbe and improve functional properties,¹² literatures about the effect of high intensity ultrasound on the allergenic property of food proteins are limited. *Li et al.*¹³ showed that high intensity ultrasound (20 kHz, 300 W for 15 min) decreased by 18.7% of the allergenicity of soybean protein isolate (SPI). However, *Stanic-Vucinic et al.*¹⁴ discovered that high intensity ultrasound (135 W cm⁻¹ for 5 ~ 60 min) had a minor effect on the IgE-binding properties of β -lactoglobulin. *Tammineedi et al.*¹⁵ found that high intensity ultrasound (20 kHz, 500 W for 5 ~ 15 min) had no noticeable influence on IgE-binding capacity of α -casein, β -lactoglobulin and α -lactalbumin. Therefore, the effect of high intensity ultrasound is varied with different food proteins. it is hereby necessary to explore the immunoactivity alternation of egg proteins induced by high intensity ultrasound.

Egg allergens mainly exist in egg white, including ovomucoid, ovalbumin (OVA), ovotransferrin and lysozyme.¹⁶ OVA, the main allergen (ca. 54% of the egg white protein) in egg, is a glycoprotein with 385 amino acids with molecular weight of about 45 kDa, about half of the amino acids are hydrophobic and nearly one-quarter are charged.¹⁷ It becomes an ideal experimental model of allergy due to its availability. Therefore, in this study, high intensity ultrasound was used to treat OVA to assess the effect of high intensity ultrasound treatment on the IgG and IgE binding ability, along with structural properties of OVA, and to expound their relationship.

MATERIALS AND METHODS

Preparation of OVA

The crude OVA was isolated from fresh chicken egg white by ammonium sulfate precipitation and isoelectric precipitation.¹⁸ Then it was subjected to ion exchange chromatography on a QAE-Sepharose fast flow column (1.6×20 cm) using a protein purifier explorer QT-1D (Qite, Shanghai, China). Phosphate buffer (PB, 50 mM, pH 6.0) was used as the buffer, and PB (50 mM, pH 6.0) containing 0.5 M NaCl was used as eluant. The eluting peaks were collected, and determined by polyacrylamide gel electrophoresis (PAGE). After determination and collection, OVA was dialyzed over water for 24 h, freeze-dried and stored at -20°C until used.

Ultrasound treatment

A Scientz JY98-□DN ultrasound producer (20 kHz, 15 mm diameter titanium probe, NingBo Scientz Biotechnology Co. Ltd., Ningbo, China) was used to process OVA

solution (10 mg mL^{-1}). Samples were treated at different power (0, 200, 400, 600 and 800 W) for 15 min with 3 s of on-time and 7 s of off-time. To avoid the effect of high temperature, the real-time temperature of all samples were measured and kept below 20°C by placing the glass beaker in ice bath. In this experiment, the temperature of all samples after ultrasound were $15\text{--}20^\circ\text{C}$. After ultrasound treatment, all samples were stored at 4°C for less than 24 h or stored at -20°C until analyzed. The ultrasonic intensity at 200 W, 400 W, 600 W and 800W was 37 - 41, 55 - 58, 65 - 70, and 80 - 86 W cm^{-1} , respectively.

Preparation of antisera against OVA

Animal polyclonal antisera against OVA were obtained from young (3 months old, about 2.0 kg) Japanese male rabbits (Permission No. SCXK(Gan)-2014-0005). Rabbits were immunized by ear venous injection with 1 mL purified OVA (1 mg mL^{-1} , pH 7.4), which was emulsified with an equal volume of Freund's complete adjuvant for first injection while Freund's incomplete adjuvant for remaining injections. Antisera titer was monitored by indirect ELISA (enzyme-linked immunosorbent assay). Serum from a non-immunized rabbit was used as negative control. The rabbits were bled when the titer value was over 10,000, the antisera were obtained by centrifugation and stored at -80°C .

Human sera was collected from four patients (numbered P1–P4) with documented clinical history and a positive skin prick test (SPT) to hen's egg. The specific IgE levels was 15.4, 22.5, 29.8 and 64.6 kUA L^{-1} (determined by ImmunoCAP), respectively. According to clinical record, all patients had atopic allergy (such as hives) to hen's egg. Serum from a patient with no allergy to hen's egg was used as negative control. All serum

samples were supplied by PlasmaLab International (Everett, W.A., USA) and stored at -80 °C.

IgG binding ability of OVA

The IgG binding to OVA was measured by the indirect competitive ELISA with rabbit polyclonal antisera.¹⁹ Firstly, 96-well microplate (Corning, NY, USA) was coated with 100 μL of 2 $\mu\text{g mL}^{-1}$ OVA, and incubated overnight at 4 °C. Then it was blocked by 50 mg mL^{-1} skimmed milk in PBS (10 mmol L^{-1} , pH 7.4) at 37 °C for 1 h. After five times of washing with PBST (PBS with 0.5 mL L^{-1} Tween-20), 50 μL of either standard OVA (0.5 - 32 $\mu\text{g mL}^{-1}$) or diluted OVA samples and 50 μL of IgG antisera (diluted to 1:12,800) were added and incubated at 37 °C for 1 h. Then 100 μL of goat anti-rabbit IgG-HRP conjugate (diluted to 1:10,000 in PBST) was added, and incubated at 37 °C for 1 h. After washing, 100 μL of TMB solution was added, and incubated at 37 °C for 15 min. Then 50 μL of 2 mol L^{-1} sulfuric acid was added to stop the reaction. Finally, absorbance at 450 nm was measured using a HF2000 microplate reader (Huaan Magnech, Beijing, China). The IgG binding ability of OVA was calculated from a standard curve of OVA with linear logarithmic correlation in the range of 0.5 - 32 $\mu\text{g mL}^{-1}$. All analyses were carried out in triplicate, the averaged values were finally converted to mg mL^{-1} through multiplying by dilution factor.

IgE binding ability of OVA

The IgE binding to OVA was determined by indirect ELISA with egg allergic patients' sera.²⁰ Firstly, the polystyrene microplate was coated with 100 μL of 2 $\mu\text{g mL}^{-1}$ OVA

samples (including untreated and ultrasound treated OVA) and incubated overnight at 4 °C, followed by blocking with 50 mg mL⁻¹ skimmed milk at 37 °C for 1 h. After five times of washing with PBST, 100 µL of IgE antisera (diluted 1:32) was added and incubated at 37 °C for 30 min. Then 100 µL of goat anti-human IgE-HRP conjugate was added and incubated at 37 °C for 30 min. Then, the color development, reaction termination and absorbance measurement were performed following the steps of indirect competitive ELISA protocol described above. The absorbance at 450 nm reflects the IgE binding ability of OVA.

Determination of free sulphhydryl content

Free sulphhydryl content was determined by the Ellman's method.¹² Ellman's reagent was prepared by dissolving 4 mg of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reagent in 1 mL of Tris-glycine buffer (0.086 mol L⁻¹ Tris, 0.09 mol L⁻¹ glycine, 4 mmol L⁻¹ EDTA, pH 8.0) and centrifuged at 10,000 × *g* for 15 min before used. Then 0.4 mL of OVA samples were added to 0.6 mL of phosphate buffer (0.1 mol L⁻¹, pH 8.0) containing 0.01 mL of Ellman's reagent. After incubation at 25 °C for 20 min, absorbance at 412 nm was measured against a blank. The micromoles of free SH contents per gram soluble protein were calculated using the extinction coefficient of 13,600 M⁻¹ cm⁻¹.

Measurement of surface hydrophobicity

ANS was used as probe to measure the surface hydrophobicity of OVA.²¹ The ratio of sample (1mg mL⁻¹) and ANS solution (8 mmol L⁻¹) was 400:1 (V/V). The relative fluorescence intensity was determined by Hitachi F-7000 spectrofluorimeter (Tokyo,

Japan) using a quartz cuvette with path length of 10 mm. The excitation wavelength was 390 nm and the emission spectrum was scanned from 420 to 600 nm at a scan speed of 240 nm min⁻¹. The bandwidth was 5 nm, and the excitation and emission slits were 2.5 nm.

UV absorption spectrum analysis

UV absorption spectrum analysis was performed by UV-2910 spectrophotometer (Hitachi, Tokyo, Japan). The concentration of OVA was 1 mg mL⁻¹. The UV absorption spectra were scanned from 240 to 400 nm at a scan speed of 800 nm min⁻¹.

Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to estimate the change of the molecular weight of OVA. The concentration of stacking gel and separating gel was 50 g L⁻¹ and 12 g L⁻¹, respectively. Proteins were stained with Coomassie Brilliant Blue R-250, and the molecular weights were determined by using Thermo middle molecular weight protein marker (10 - 180 kDa) as standard. Native PAGE was done without SDS and β -mercaptoethanol in gels and sample buffer.

Secondary structure analysis

Far-UV (190–250 nm) circular dichroism (CD) spectroscopy was performed to define the secondary structure of OVA treated with ultrasound. The CD spectra of OVA (0.1 mg mL⁻¹) were collected using a MOS-450 spectropolarimeter (French Bio-Logic SAS,

Claix, French) with the path length of cylindrical quartz cuvette of 1 mm. The bandwidth and step resolution were set at 1 nm. Every sample was scanned for 3 times at a scan rate of 100 nm min⁻¹. The data was shown in terms of molar residue ellipticity ([θ], deg cm² dmol⁻¹), and the content of different secondary structures were analyzed with DichroWeb.

Statistical analysis

The statistical analysis was measured by Analysis of Variance (ANOVA) with SPSS 19.0 (SPSS Inc., Chicago, USA). A value of $p < 0.05$ was considered significant. All tests were done in triplicate and data were expressed as mean $\pm$ SD (standard deviation).

RESULTS AND DISCUSSION

Effect of ultrasound treatment on the IgG and IgE binding abilities of OVA

The IgG and IgE binding abilities were assessed by ELISA with rabbit polyclonal antibodies and egg allergy patients' sera, respectively. As shown in Fig. 1(a), the IgG binding ability of OVA were gradually enhanced with ultrasound power increasing from 200 W to 600 W. However, it was slightly decreased when **ultrasonicated** at 800 W, but it was still much higher than that of native OVA. **The effect trends of ultrasound on the IgE binding ability was similar to that of IgG, shown in Fig. 1(b). The highest IgE binding ability of OVA was detected at 600 W, whereas it was slightly decreased when the power was increased to 800 W.** Fig. 1(b) showed that the influence of ultrasound treatment on the interaction of OVA and IgE from different patients was similar although the binding ability of IgE from different patients was different. Above results indicated that

ultrasound treatment could enhance the IgG and IgE binding abilities of OVA.

The results were inconsistent with that reported in some previous studies, for example, Li *et al.*^{13, 22} found that high intensity ultrasound at 300 W for 15 min or 800 W for 30 ~ 180 min could significantly decrease the allergenicity of shrimp and SPI. While, Stanic-Vucinic *et al.*¹⁴ indicated that β -lactoglobulin ultrasonicated at 135 W cm⁻¹ for 5-60 min had a minor change on the IgE binding ability because the sequential allergenic epitopes of β -lactoglobulin might be sonication resistant and more important than conformational epitopes. Tammineedi *et al.*¹⁵ found ultrasound treatment (500 W for 5-15 min) with 60 °C preheating had no significant influence in IgE binding ability of α -casein, β -lactoglobulin and α -lactalbumin. The different results might be resulted from the difference in molecular weight, stability of conformational structures, the exposure of IgG and IgE epitopes, as well as the different processing temperature.²³ Ultrasound could produce extreme high pressure and shear force, which might influence the conformational structure and finally result in the alternations of IgG and IgE binding.^{20, 24} Therefore, in order to show the relationship between structure and IgG and IgE binding abilities of OVA, the corresponding conformational alternations of OVA induced by ultrasound treatment were investigated in subsequent experiments.

Free SH content analysis

Sulphydryl groups are considered to be the most reactive functional group in proteins. OVA contains six cysteines with a single disulfide bond between Cys73 and Cys120.²⁵ The free SH contents of OVA induced by different ultrasound treatments are shown in Fig. 2. The free SH content increased with increasing ultrasound power in the range of 0 -

600 W, with the value ranged from $3.3 \mu\text{mol g}^{-1}$ (untreated OVA) to $10.8 \mu\text{mol g}^{-1}$ (600 W, 15 min). The increase of free sulphhydryl content might be attributed to the exposure of sulphhydryl groups to the surface of OVA molecules resulting from the unfolding of molecular structure.²³ It is also possible that ultrasound treatment breaks disulfide bonds, leading to the formation of SH groups and improvement of free sulphhydryl as a consequence of cavitation phenomenon (turbulent flow, high pressure and shear force, et al.).²⁶ In addition, the maximum free SH content in OVA samples was lower than the reported value of $40 \mu\text{mol g}^{-1}$,²⁷ indicating the partial unfolding of OVA molecule induced by ultrasound. Molecular unfolding and increased free SH groups content caused by ultrasound treatment were also detected on SPI.²⁶

However, when treated at 800 W for 15 min, the free SH content of OVA was decreased to $9.6 \mu\text{mol g}^{-1}$, this may be explained by the susceptible oxidization of free SH groups resulted from the cavitation-generated hydrogen peroxide.²⁸ Furthermore, instantaneous high pressure and high temperature produced by ultrasound might induce the aggregation of protein through forming new S-S bonds caused by SH/S-S interchange reactions.²⁴ Therefore, the content of free SH groups of OVA ultrasonicated at 800 W for 15 min was decreased.

Surface hydrophobicity analysis

The surface hydrophobicity is generally used to reflect the number of hydrophobic groups exist on the surface of protein molecule, and to characterize the molten globule state and aggregates of proteins. Effect of ultrasound treatment on the surface hydrophobicity of OVA is shown in Fig. 3(a), which gives the same change trend with

that of free SH content, and the surface hydrophobicity of ultrasonicated samples were largely enhanced compared to the native OVA. The fluorescence intensity of OVA was gradually improved as the ultrasonic power was increased from 200 to 600 W, indicating an increasing surface hydrophobicity. This suggests that ultrasonic treatment could cause the unfolding of OVA molecular, and thereby resulted to the exposure of hydrophobic groups and regions that were originally buried inside the molecules. Higher surface hydrophobicity induced by high intensity ultrasound was also detected on soy protein isolate and BSA.^{26, 28} However, the fluorescence intensity started to decrease when the power was over 800 W. This may be that prolonged ultrasound treatment induces the aggregation of OVA, this aggregation protects the hydrophobic regions of protein molecules and results to the reduced surface hydrophobicity.²⁹

UV absorption spectrum analysis

The UV absorbance of protein in the near-UV region depends on the residues of aromatic amino acid, including tryptophan, tyrosine and phenylalanine, tryptophan is mainly responsible for the near-UV region.³⁰ Effect of ultrasound treatment on the UV absorption spectra of OVA is shown in Fig. 3(b). Compared with untreated OVA, the absorption intensity of OVA treated from 200 W to 600 W was gradually increased with increasing ultrasound power, suggested that tryptophan residues were gradually exposed to the surface of protein molecule.²⁰ This may be explained by the partial unfolding of OVA, it was also revealed by the increased surface hydrophobicity (Fig. 3(a)). However, when OVA was treated at 800 W for 15 min, the absorption intensity of OVA was slightly decreased, implying the followed burying of some tryptophan residues resulted from the

aggregation of protein. Yang M, et al. indicated that the aggregation through hydrophobic and thiol/disulfide interactions could make the tryptophan residues buried between the molecules.³¹ Thus, the decrease of UV absorption intensity of OVA treated at 800 W for 15 min was possibly due to the aggregation of OVA molecule.

Secondary structure analysis

Far-UV CD spectroscopy was used to determine the content of secondary structure of OVA. The far-UV CD spectra of ultrasound treated OVA samples are shown in Fig. 5 and the proportions of secondary structure are shown in Table 3. The CD spectrum of OVA treated at 200 W was almost identical to that of the unheated protein. It indicated that ultrasound treatment at 200 W had insignificant effect on the secondary structure of OVA because of low output intensity, but it could change the tertiary structure of OVA. Similar result was found by Xiong *et al.*³², who found that ultrasound with low intensity (34–48 W cm⁻²) could not alter the secondary structure of OVA but changed the tertiary structure of OVA.

When ultrasound power was increased from 400 W to 600 W, the values of negative peak at 222 nm were gradually decreased, which implied that the α -helix content of OVA was progressively increased. Since the secondary structure of protein not only depends on the local sequence of amino acids but also on the interactions between different parts of molecule, the above results might indicate that ultrasound treatment can disrupt these interactions, leading to the changes of secondary structure.²⁹ Similar phenomenon has also been found in soy protein isolate and whey protein concentrate ultrasonicated at 400 - 600 W.^{26, 33} However, when ultrasound power was increased to 800 W, the α -helix

content was significantly decreased and the β -strand content was increased. It might be owe to the aggregation of OVA treated at 800 W. Sonication-induced aggregates had high β -content, and proteins with more native α -helical structure showed more β -structure with a concomitant decrease in α -helix structure in the aggregates.³⁴ Therefore, the far-CD results indicated that ultrasound treatment at low power (200 W) could not change the secondary structure of OVA, and middle power (400 W and 600 W) would increase the α -helix component, decrease the β -stand and random coil component, however, ultrasound at high power (800 W) resulted to increased β -stand content, but decreased α -helix content.

Molecular weight distribution analysis

The native-PAGE of OVA upon exposure to ultrasound at 0 - 800 W for 15 min is shown in Fig. 4(a). There was no significant difference between untreated OVA (lane 1) and OVA treated at 200 - 600 W (Lane 2 - 4). However, some bands in the stacking gel and the beginning of separating gel in lane 5 (OVA treated at 800 W) were observed. The result implied that OVA was aggregated after sonication at 800 W. The SDS-PAGE was also performed to assess the effect of ultrasound on the molecular weight of OVA. As shown in Fig. 4(b), insignificant difference between the bands of untreated and treated OVA samples was observed, suggesting that ultrasound treatment could not alter the molecular weight. In addition, OVA ultrasonicated at higher intensity might aggregate through hydrophobic interaction and/or covalent bonds that could be disrupted by denaturants, such as SDS and β -mercaptoethanol.³⁵ Jiang *et al.*²⁹ found that higher intensity ultrasound treatment at 150 and 300 W for 12 min could induce the aggregation

of black bean protein isolate.²⁹ The results further explain why free SH groups and surface hydrophobicity of OVA ultrasonicated at 800 W were decreased.

Relationship between IgG binding, IgE binding and structure of OVA

The antigenicity and allergenicity of protein is related to the integrity of antigenic and allergenic determinant, namely, epitopes, which depends on the structure of protein. The epitopes consist of sequential and conformational epitopes. Only epitopes on the protein surface could be recognized by the specific antibody. OVA was the dominant allergen for the IgE antibodies from atopic patients, and IgE antibodies preferred to recognizing sequential epitopes of OVA.³⁶ In this study, the IgE sera were obtained from atopic patients. The results in Fig. 4(b) showed that ultrasound did not change the molecular weight of OVA, indicating the sequential epitopes of OVA were not destroyed. Therefore, the changes of IgE binding to OVA induced by ultrasound treatment were mainly related to the exposure or hide of sequential epitopes.

The IgG and IgE epitopes are widely spread along the whole sequence and conformation of OVA, and were composed primarily of hydrophobic amino acids consisted of β -sheet and β -turn structures containing polar and charged residues. Thus, the allergenicity of OVA was determined by the conformational structure. When OVA ultrasonicated below 600 W, the free SH content, surface hydrophobicity, UV absorbance and α -helix content were **increased** with the increase of ultrasound intensity, indicating the partially unfolding of the conformational structure of OVA. The unfolding of protein could make more epitopes buried inside the native molecule exposed on the surface of the molecule, and finally led to the increase of IgG and IgE binding abilities.^{20, 36}

However, when OVA was treated at 800 W, the decrease of free SH content, surface hydrophobicity, UV absorbance, α -helix content and a concomitant increase of β -strand content suggested that OVA was aggregated. In addition, the results in Fig. 4 proved that OVA were aggregated through hydrophobic interaction and/or thiol/disulfide interaction.²⁹ The aggregation of OVA might make some epitopes reburied inside the inter-molecules and finally resulted in the decrease of allergenicity. Therefore, the alternations of allergenicity were closely related to the molecular unfolding and aggregation, exposure of free sulphhydryl groups and hydrophobic groups, et al..

CONCLUSION

High intensity ultrasound could significantly enhance the IgG and IgE binding of OVA, which had a close relation to the conformational structure of OVA. The IgG and IgE binding ability was enhanced with increasing ultrasound power at the range of 200 ~ 600 W due to the partially unfolding of OVA. While the binding ability was slightly decreased as a consequence of the aggregation of OVA when the power was at 800 W. Therefore, high intensity ultrasound processing of some egg products alone might improve the risk of allergenic reaction of egg allergy patients. However, the unfolding of OVA molecules and exposure of IgG and IgE epitopes, may give us a chance to modify the epitopes and reduce the potential allergenicity of OVA by combining other methods with high intensity ultrasound.

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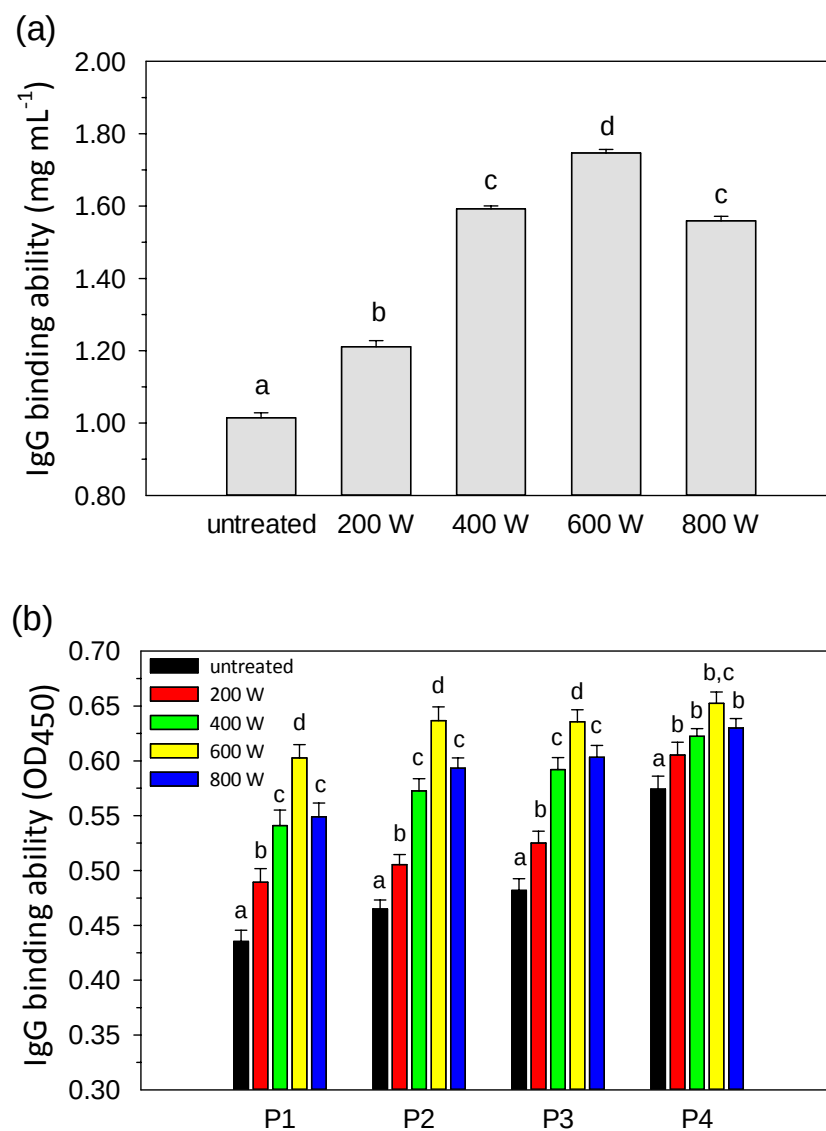


Figure 1. Effect of high intensity ultrasound on IgG binding ability (a) and IgE binding ability (b) of OVA. P1–P4 refers to the egg allergy patients numbered 1, 2, 3 and 4, respectively. Means with different letters (a-d) on the same patients are significantly different ($p < 0.05$).

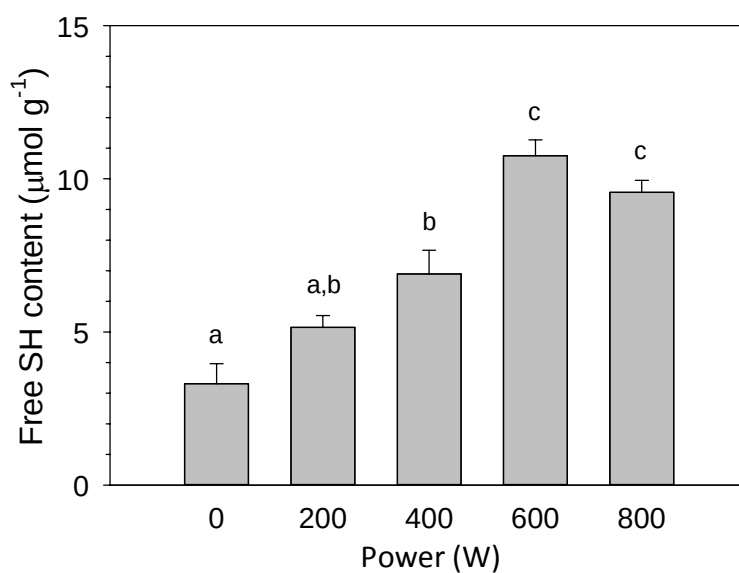


Figure 2. Effect of high intensity ultrasound on the content of free SH groups of OVA. 0 W represents the untreated sample, all other samples were treated for 15 min. Different letters in the bars mean significantly different ($p < 0.05$).

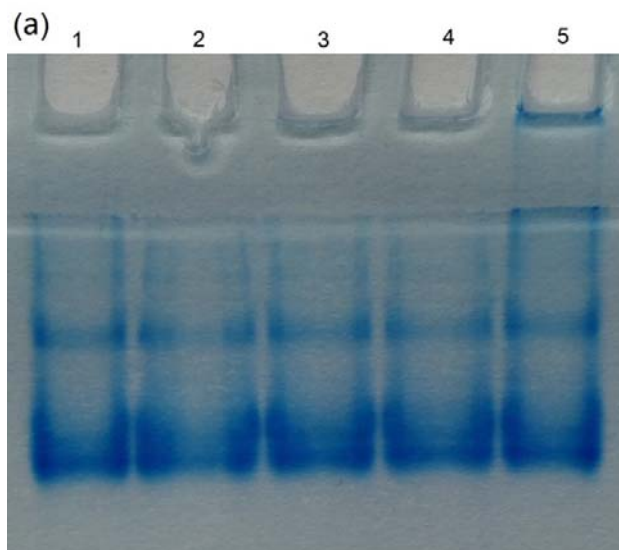


Figure 3. Effect of high intensity ultrasound on surface hydrophobicity (a) and UV absorption (b) of OVA. 1 to 5 represents OVA treated at 0, 200, 400, 600 and 800 W, respectively.

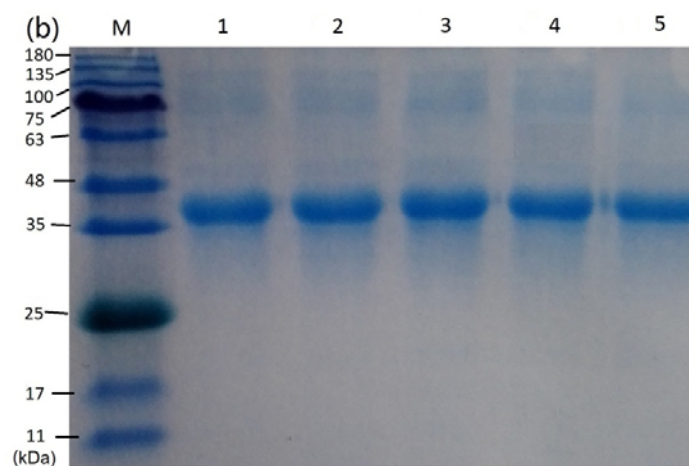


Figure 4. Effect of high intensity ultrasound on the molecular weight of OVA. Native-PAGE (a) and SDS-PAGE (b) of OVA treated at different ultrasound intensity for 15 min. Lane 1-5 represents OVA treated at 0, 200, 400, 600 and 800 W, respectively. The molecule weight of Marker (represented by M) ranges from 11 kDa to 180 kDa.

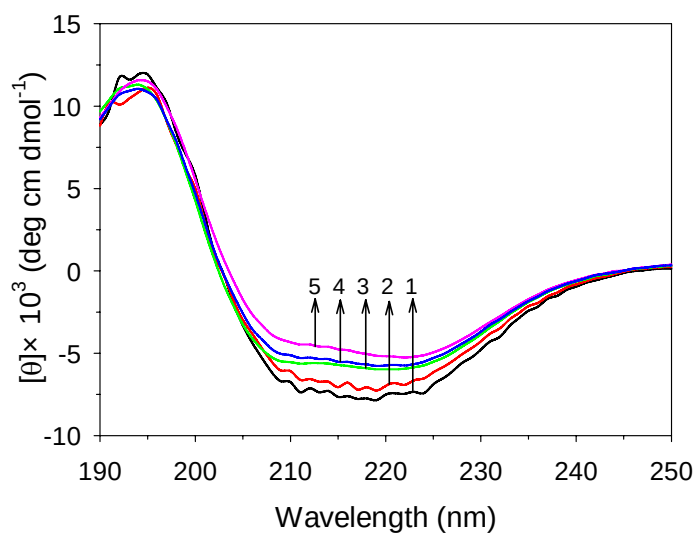


Figure 5. Effect of high intensity ultrasound on far-UV CD spectra of OVA. 1 to 5 represents OVA treated at 0, 200, 400, 600 and 800 W, respectively.

Table 1. Effect of high intensity ultrasound on the content (%) of secondary structures of OVA

Power(W)	α -Helix	β -Strand	β -Turn	Random Coil
0	22.2 $\pm$ 0.5 ^a	26.5 $\pm$ 0.5 ^a	22.6 $\pm$ 0.8 ^a	28.8 $\pm$ 0.1 ^a
200	22.5 $\pm$ 0.9 ^a	25.3 $\pm$ 0.4 ^{a,b}	24.0 $\pm$ 0.5 ^{a,b}	28.0 $\pm$ 0.1 ^a
400	26.1 $\pm$ 0.7 ^b	25.6 $\pm$ 0.2 ^a	24.1 $\pm$ 0.7 ^{a,b}	24.3 $\pm$ 0.5 ^b
600	29.6 $\pm$ 0.4 ^c	24.4 $\pm$ 0.4 ^b	23.0 $\pm$ 0.5 ^a	23.0 $\pm$ 0.6 ^b
800	16.6 $\pm$ 0.4 ^d	35.4 $\pm$ 0.3 ^c	22.1 $\pm$ 0.3 ^a	25.9 $\pm$ 0.5 ^c

Values followed by different letter (a-c) in the same column mean significantly different ($p < 0.05$).