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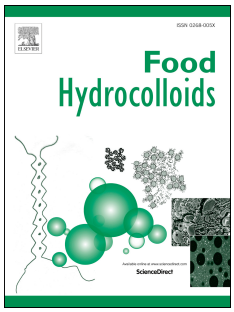

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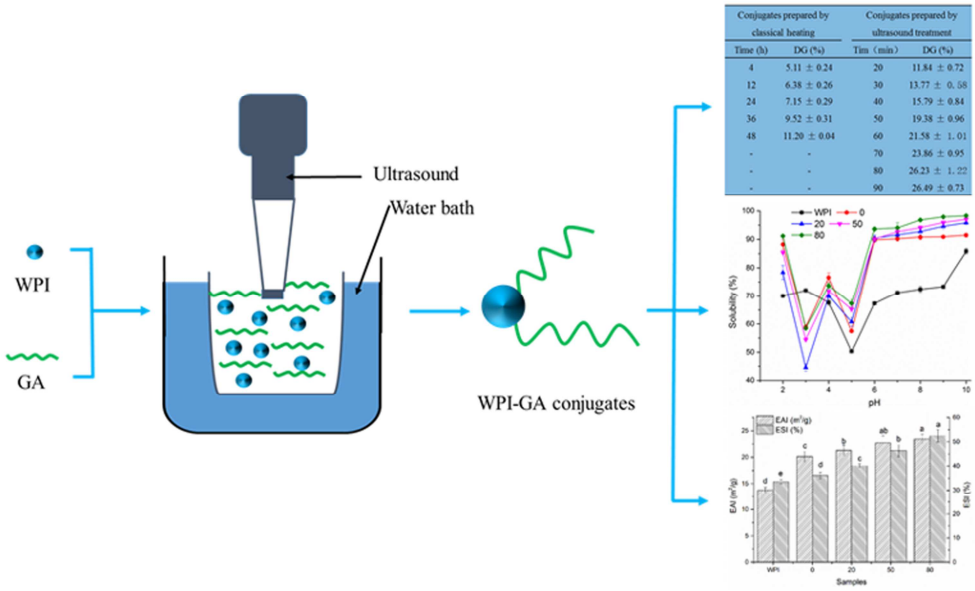

Conjugates prepared by classical heating		Conjugates prepared by ultrasound treatment	
Time (h)	DG (%)	Tim（min）	DG (%)
4	5.11 ± 0.24	20	11.84 ± 0.72
12	6.38 ± 0.26	30	13.77 ± 0. 58
24	7.15 ± 0.29	40	15.79 ± 0.84
36	9.52 ± 0.31	50	19.38 ± 0.96
48	11.20 ± 0.04	60	21.58 ± 1. 01
-	-	70	23.86 ± 0.95
-	-	80	26.23 ± 1. 22
-	-	90	26.49 ± 0.73

Preparation of modified whey protein isolate with gum Acacia by ultrasound Maillard reaction

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Abstract: Effect of ultrasound treatment on whey protein isolate (WPI)-gum Acacia (GA) conjugation via Maillard reaction was investigated. And the physicochemical properties of the conjugates obtained by ultrasound treatment were compared with those obtained by classical heating. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, high-performance size exclusion chromatography and fourier transform infrared spectroscopy provided evidence on the formation of the Maillard type conjugation. Compared with classical heating, ultrasound treatment could accelerate the glycation reaction between WPI and GA. A degree of graft of 11.20 % was reached by classical heating for 48 h, whereas only 20 min was required by ultrasound treatment. Structural analyses suggested that the conjugates obtained by ultrasound treatment had less α-helix content, higher surface hydrophobicity and fluorescence intensity than those obtained by classical heating. Significantly lower level of browning intensity and significantly higher ($p < 0.05$) level of solubility (under alkaline conditions), thermal stability, emulsifying activity and emulsifying stability were observed for the conjugates obtained by ultrasound treatment as compared with those obtained by classical heating.

Keywords: Whey protein isolate; Gum Acacia; Glycation; Ultrasound treatment

1. Introduction

In the last decades, numerous physical, chemical and biochemical methods have been applied to enhance the functional properties of proteins and thus expand their utilization in food industry (Uluko, Liu, Lv, & Zhang, 2016; Zink, Wyrobnik, Prinz, & Schmid, 2016). Among these methods, an increasing number of researchers have begun to conjugate proteins with reducing sugars via Maillard reaction recently. The Maillard reaction involve the covalent attachment of the carbonyl groups of sugars to the free amino acid groups in protein together with the formation of Schiff base and further Amadori compounds (Bi, Yang, Fang, Nishinari, & Phillips, 2017). It occurs naturally and spontaneously under heating without adding extraneous chemicals. Extensive researches have demonstrated that this method of modification was efficient in improving the emulsifying properties, solubility, antibacterial effect and antioxidant effect of proteins (Chevalier,

Chobert, Genot, & Haertle, 2001; Gu, et al., 2010; Karbasi & Madadlou, 2017; Oliver, Melton, & Stanley, 2006). Compared with mono- or disaccharides, conjugation of polysaccharides with proteins leads to a far more significant enhancement in their specific physicochemical and functional properties (Y. Li, et al., 2013; Niu, Jiang, Pan, & Zhai, 2011). In addition, previous reports also indicated that the allergenicity of protein could be effectively masked by glycation with polysaccharides (Corzo-Martinez, Cristina Soria, Belloque, Villamiel, & Javier Moreno, 2010). Therefore, conjugation of protein with polysaccharide has been recognized as one promising chemical approach for protein modification.

Two traditional ways usually used to prepare protein-saccharide conjugates are dry heating and wet heating treatment. The former method involves incubation the protein-saccharide mixture at controlled temperature and relative humidity for a period of tens of hours to several weeks (Guan, Qiu, Liu, Hua, & Ma, 2006). It usually takes a long time which may result a serious degree of browning. Moreover, the reaction extent is uncontrollable and the reaction is limited by the uneven contact between reactants (Zhuo, et al., 2013). Thus this method is not attractive from the industrial viewpoint. The wet heating method involves thermal treatment of a protein-saccharide (mainly monosaccharide and disaccharide) mixture in a buffer solution for tens of minutes to several hours (Guan et al., 2006). Although this method can largely shorten the reaction time, tens of hours is still need for the conjugation of protein with polysaccharide (Zhang, Chi, & Li, 2014). In addition, protein denaturation and aggregation occurs at high temperatures and/or long processing time which may have negative effect on the functionalities of the conjugates (Zhu, Damodaran, & Lucey, 2008). Therefore, new technologies should be applied to improve the efficiency of glycation reaction between protein and polysaccharide.

Some approaches including microwave (Guan, et al., 2006), high pressure (De Vleeschouwer, Van der Plancken, Van Loey, & Hendrickx, 2010), radiation (Muppalla, Sonavale, Chawla, & Sharma, 2012), dynamic high-pressure microfluidization (Huang, et al., 2013) and pulsed electric field (Jian, Wang, Wu, & Sun, 2018) have been applied to speed up the Maillard reaction and minimize the disadvantages of traditional methods. Ultrasound was also used to promote the glycation reaction. However, most experiments were focused on the amino acid-monosaccharide

model system (Corzo-Martinez, et al., 2014; Ong, Seow, Ong, & Zhou, 2015; Yu, Seow, Ong, & Zhou, 2017), few were conducted on the conjugation between protein and polysaccharide. C. Li, Xue, Chen, Ding and Wang (2014) reported that after wet-heating at 80 °C for 24 h, the degree of glycosylation (DG) for peanut protein isolate (PPI)-dextran and PPI-gum Arabic (GA) conjugates were 35.7 % and 31.6 %; however, significantly higher DG values of 45.4 % and 40.6 % were obtained by ultrasound treatment for 40 min. Mu et al. (2010) and C. Li, Huang, Peng, Shan and Xue (2014) not only observed this sonocatalysis effect on the glycation of soy protein isolate (SPI) with GA and PPI with glucomannan, respectively, but also found that the conjugates obtained by ultrasound treatment have better functional properties (e.g., solubility and emulsifying properties) as compared with those obtained by classical heating. It has been suggested that the effective mixing and efficient energy/mass transfer provide by ultrasound was the main reason for shorten the Maillard reaction time (Corzo-Martínez et al., 2014). Besides, ultrasound could change the secondary and tertiary structure of proteins, which not only contribute to decrease the graft reaction time, but also help to improve the functional properties of conjugates (C. Li, Huang, et al., 2014; Mu, et al., 2010).

Whey protein isolate (WPI) is a by-product of cheese whey industry. Due to its high nutritional value and good functional properties, it has great potential in food industry (Khalesi, Emadzadeh, Kadkhodaee, & Fang, 2017). However, its functionalities are sensitive to pH (Teo, et al., 2016), ionic strength (de Wit, 1998) and temperature (LaClair & Etzel, 2010), which prevent the wide application of WPI in diverse food systems and processing technologies. To overcome these techno-functional issues and full realize its potential, many methods were applied to improve its functionality. Glycation base on Maillard reaction was one of the most used methods to modify WPI (Z. Li, Luo, Feng, & Liao, 2013; Qi, Xiao, & Wickham, 2017; Zheng, Luo, Yao, Lu, & Bu, 2014). However, most of those reports were carried out base on the conventional dry heating or wet heating method, reports about the effects of ultrasound treatment on the glycation of WPI were still scarce.

In this work, WPI was grafted with GA with the assistance of ultrasound treatment. The primary structure, secondary structure, intrinsic fluorescence, and surface hydrophobicity of the

glycated WPI were determined and compare with those of classical heating. Furthermore, the solubility, thermal stability as well as emulsifying properties of the conjugates were also evaluated.

2. Materials and methods

2.1. Materials

WPI (according to the manufacturer, the protein content is 93%) was obtained from Hilmar Ingredients (Hilmar, CA, USA). GA was purchased from Aladdin Chemical Reagent Co. (Shanghai, China). 8-Anilino-1-naphthalenesulfonic acid (ANS) was obtained from Sigma–Aldrich, St. Louis, MO, USA. o-Phthaldialdehyde (OPA), sodium tetraborate, sodium dodecyl sulfate, sodium dodecanesulfonate, sodium dihydrogen phosphate, dibasic sodium phosphate, sodium chloride and all other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

2.2 Preparation of the WPI-GA conjugates

Mixture solution of WPI and GA was magnetically stirred at 4 °C overnight. Then 100 mL of the solution was treated by an ultrasound equipment (JY92-IIDN, Ningbo Scientz Biotechnology Co., Ningbo, China) for 40 min with a sequence of 2 s of sonication and 2 s of rest. After that, the solutions were cooled to room temperature and dialyzed at 4 °C for 2 days. Finally, the resulting products were lyophilized and stored at 4 °C for further analysis. Effect of WPI concentration (4 to 12 mg/mL), WPI-GA weight ratio (4:1 to 1:1), initial pH (7 to 12), ultrasound temperature (50 to 90 °C) and ultrasound power (100 to 700 W) on the glycation reaction were investigated. Furthermore, conjugates were also prepared by classical heating.

2.3 Measurement of the degree of glycosylation (DG)

The DG was calculated though determining the decrease of free amino groups in WPI using the OPA method (Xue, Li, Zhu, Wang, & Pan, 2013). The OPA reagent was freshly prepared by mixing 40 mg of OPA (dissolved in 1 mL of ethanol), 25 mL of 0.10 M sodium tetraborate buffer (pH 9.5), 2.5 mL of 20 % (w/w) sodium dodecyl sulfate (SDS) solution, and 0.1 mL of β-mercaptoethanol and finally diluted to 50 mL with distilled water. 0.2 mL of sample solution (2 mg/mL in protein) was mixed with 4 mL of OPA reagent and incubated at 35 °C for 2 min. Then

the mixture was measured at 340 nm by a UV-Vis spectrophotometer (UV-2550, Shimadzu, Tokyo, Japan). The mixture of distilled water and OPA reagent was used as blank. Lysine was used as a standard to calculate the content of free amino groups. DG was defined as follow equation:

$$\mathrm{DG}(\%) = \left(1 - \frac{\text{amine groups after heating (M)}}{\text{amine groups before heating (M)}} \times 100\right) \quad (1)$$

2.4 Measurement of browning intensity

The browning intensity of the conjugate was measured according the method described by Muhoza, et al. (2017). In short, WPI-GA conjugate solutions (2 mg/mL) were diluted 5-fold with 0.1 % (w/v) SDS solution, and then measured at 420 nm by a UV-Vis spectrophotometer (UV-2550, Shimadzu, Tokyo, Japan). The mixture of distilled water and SDS was used as blank.

2.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was carried out on a mini protean® tetra system (Bio-Rad Laboratories, Hercules, California, USA). A 5 % stacking gel and a 12 % separating gel containing 0.1 % SDS were used. Protein solutions (2 mg/mL) were diluted 5-fold with protein loading dye (Sangon Biotech Co., Ltd, Shanghai, China) and then incubated in boil water for 5 min. 8 μL of mixtures were loaded in the gel slots and electrophoresis was performed in Tris-glycine running buffer (pH 8.3). After that, gels were stained with Coomassie Brilliant Blue R250 dye and destained with a solution composed of 40 % methanol and 10 % acetic acid.

2.6 High-performance size exclusion chromatography (HPSEC)

HPSEC was applied to analyze the molecular size distribution of the conjugates by using high-performance liquid chromatography (Waters e2695, US) equipped with a UV/Vis detector (Waters 2489, US) according to the method reported by de Oliveira et al. (2015) with modifications. The mobile phase was 0.05 M PBS (pH 7) containing 0.15 M NaCl. Two columns, TSK Gel 5000 PWXL (7.8 ×300 mm, Tosoh, Tokyo, Japan) and Ultrahydrogel TM 250 column (7.8 ×300 mm, Waters, US) were connected in series and kept at 30 ℃ with a flow rate of 0.5 mL/min. Samples were dissolved in mobile phase solution to obtain a protein concentration of 20 mg/mL and filtered through a 0.45 μm filter before analysis. The injected volume was 20 μL and the absorbance was monitored at 280 nm.

2.7 Fourier transform infrared spectroscopy (FTIR) analysis

Samples (2 mg) were mixed with KBr (0.5 g) and pressed into KBr pellets before measurement. FTIR spectra was collected with an IR spectrometer (Nicolet 5700; Thermo Fisher Scientific, MA, USA), using the absorbance mode in the frequency range of 4000-400 cm^{-1} and a resolution of 4 cm^{-1}.

2.8 Secondary structure analysis

The secondary structures of glycosylated WPI were analyzed using a Circular dichroism (CD) spectroscopy (JASCO J1500, Tokyo, Japan). Briefly, WPI and WPI-GA conjugates solutions were prepared at a protein concentration of 0.25 mg/mL in 0.01 M PBS (pH 7.0). Measurement was carried out by scanning the solutions from 190 to 250 nm at 20 ℃ using a 0.1 cm path length quartz cell. The scan rate was 50 nm/min and the band width was set at 1 nm. The PBS was used as blank and the data were expressed in terms of mean residue ellipticity (θ, degree cm^2 $dmol^{-1}$). The secondary structure were analyzed using DICHROWEB.

2.9 Intrinsic fluorescence analysis

The intrinsic fluorescence spectra of WPI and WPI-GA conjugates were measured by a fluorescence spectrophotometer (Varian Inc., Palo Alto, USA; Model Cary Eclipse). Solutions (0.20 mg/mL in protein, 0.01 M PBS, pH 7.0) were excited at 280 nm (slit =5 nm), and emission spectra were recorded from 300-500 nm (slit = 5 nm) with a scanning speed of 600 nm/min. The PBS was considered as blank.

2.10 Surface hydrophobicity (H_0) measurement

8-Anilino-1-naphthalenesulfonic acid (ANS), as a fluorescence probe, was used to determine the H_0 values of glycated WPI according to the method described by Kato & Nakai (1980). Briefly, samples were dispersed into 0.01 M PBS (pH 7) to obtain a serious of protein concentrations from 0.005 mg/mL to 0.5 mg/mL. Then, 4 mL of each solution was mixed with 50 μL of ANS (0.008 M). After that the mixtures were measured by a fluorescence spectrophotometer (Varian Inc., Palo Alto, USA; Model Cary Eclipse) at 484 nm (emission wavelength) with an excitation wavelength of 365 nm. The index of the H_0 was expressed as the initial slope of FI versus protein concentration.

188 **2.11 Solubility and thermal stability**

189 For protein solubility analysis, the pH of WPI and WPI-GA conjugates solutions (1 mg/mL
190 in protein) were adjusted to 2-10 and stirred at room temperature for 1 h. After readjusted the pH,
191 solutions were centrifuged at 5000 rpm at 20°C for 20 min. Protein concentration of the
192 supernatant was measured using the Bradford method (1976) and calculated by a standard curve of
193 WPI. Solubility of WPI was expressed as a percentage of protein present in supernatant over the
194 total protein content.

195 For thermal stability evaluation, samples were dissolved in 0.01 M PBS (pH 7) to obtain the
196 protein concentration of 1 mg/mL and heated at 50-100 ℃ for 1 h. After quickly cooled to room
197 temperature, the protein solubility was measured as indicated above.

198 **2.12 Emulsifying properties analysis**

199 The emulsifying properties of the conjugates were measured by the method of Pirestani,
200 Nasirpour, Keramat, & Desobry (2017) with modifications. Samples were dispersed in 0.01 M
201 PBS (pH 7) to obtain a protein content of 2 mg/mL. Then, 5 mL of soybean oil was added to 15
202 mL of protein solutions and homogenized for 2 min at 13500 rpm with an Ultra-Turrax
203 homogenizer (Ika, Staufen, Germany). After that, 100 μL of the emulsion was immediately
204 pipetted from the bottom of the container and diluted by 10 mL of 0.1% sodium
205 dodecanesulfonate solution. Another 100 μL of the emulsion was also taken from the bottom of
206 the container after 10 min and diluted by the same volume of sodium dodecanesulfonate solution.
207 The absorbance was recorded at 500 nm with a UV-Vis spectrophotometer (UV-2550, Shimadzu,
208 Tokyo, Japan). Emulsifying activity index (EAI) and emulsion stability index (ESI) were
209 calculated as follows:

210 $$\mathrm{EAI}(m^2/g) = \frac{2\times 2.303\times A_0}{C\times(1-\Phi)\times 10^4} \times \text{dilution factor} \quad (2)$$

211 $$\mathrm{ESI}(\%) = \frac{A_{10}}{A_0} \times 100 \quad (3)$$

212 Where A_0 and A_{10} is the absorbance at 0 min and 10 min, respectively; C is the concentration
213 of protein (g/mL); Φ is the proportion of the oil phase (0.25).

214 **2.13 Statistical analysis**

All experiments were conducted in triplicate and the data were expressed as a mean value with the ± standard deviation (SD). The significant differences ($p < 0.05$) were analyzed by using the SPSS software.

3 Results and discussion

3.1 Effect of process parameters on graft reaction

3.1.1 Effect of ultrasound temperature on graft reaction

As shown in Fig. 1A, the DG value increased when ultrasound at 50-70 °C and then decreased quickly as temperature further increased to > 70 °C. Previous study revealed that the transition temperatures of WPI was about 76 °C (Shen, Fang, Gao, & Guo, 2017). So when ultrasound temperature was lower than 70 °C, increase in temperature may cause more extensive unfolding of protein and therefore improve the effect of ultrasound treatment resulting more reactant groups buried inside of the protein became exposed to the surface. Mu et al. (2010) reported that positive effect on the graft reaction between soy protein isolate (SPI) and gum acacia (GA) was observed for ultrasound temperature at the range of 60 to 90 °C, but high temperature would lead to serious browning of conjugates. C. Li, Huang, et al. (2014) also confirmed that increase in ultrasound temperature (60-80 °C) could give higher DG values for the Maillard-type reaction between peanut protein isolate (PPI) and glucomannan. However, when the temperature was up to 70 °C, greater protein denature and aggregation would occur. In addition, it is also reported that the amount of ultrasound power transferred to a liquid medium decreased drastically at temperatures higher than 70 °C (Raso, Manas, Pagan, & Sala, 1999). Both cause the decrease of the DG value.

3.1.2 Effect of ultrasound power on graft reaction

Fig. 1B showed that with the increase of ultrasound power, the DG value increased first and then decreased sharply. The max DG value was obtained at 500 W. Wang, et al. (2016) reported that the DG of bean protein isolates (BPI) with glucose increased from 150 to 300 W, but decreased at 450 W. Due to its functions of mechanical mass transfer, heating and cavitation, ultrasound was able to support more energy and free amino groups for the process of graft reaction. Meanwhile, local relative translational motions induced by the ultrasound might allow reactive

groups to be brought into closer proximity (Mu et al., 2010). Thus increase the ultrasound power in a proper range was efficient in speeding up the Maillard reaction between protein and saccharide. This positive effect was confirmed by Mu et al. (2010) and C. Li, Huang, et al. (2014) about the glycation of SPI and PPI, respectively. However, too high ultrasound power may result the aggregation of protein (Resendiz-Vazquez, et al., 2017) and thus prevent the graft reaction.

3.1.3 Effect of initial pH on graft reaction

Fig. 1C showed that the DG value increased quickly with the rise of pH from 7 to 11 and then decreased slightly at pH 12. It has demonstrated that initial pH of the system had a marked influence on the cross-linking of proteins via glycation and the reaction took place more effectively at high pH (Lertittikul, Benjakul, & Tanaka, 2007). The reason was that pH strongly influences the proportion of the amino acid in the unprotonated form and thus the initial condensation step of the Maillard reaction is augmented by higher pH. Therefore, many researches about the Maillard reaction between protein/amino acid and saccharide were conducted at strong alkaline condition (Guan, et al., 2010; Sun, Yu, Zeng, Yang, & Jia, 2011; Yu, et al., 2017). In addition, higher pH was farther away from the isoelectric point of the WPI and therefore could cause stronger intramolecular electrostatic repulsions which may resulting more extensive unfolding, more reactant amino groups expose and higher solubility, all these contribute to the improvement of graft reaction. The slight decline of DG value at pH 12 may be because the unfolded proteins at this condition were easy to re-aggregation during ultrasound treatment.

3.1.4 Effect of WPI concentration on graft reaction

Protein concentration is also a main factor influence the Maillard reaction rate. From fig. 1D we can see that the glycation rate increased quickly when the WPI concentration changed from 4 to 10 mg/mL, and then fell sharply. The increase in protein concentration means more reactant groups are available in the system, which may attribute to the increase in DG value. However, too high protein concentration may enhance the stereo-hindrance effect and prevent the molecular movement. In addition, high viscosity of the reaction system caused by the high content of reactant would made the cavitation more difficult to induce and reduce the number of

cavitating bubbles per unit volume, thus weaken the effect of ultrasound (Raso, et al., 1999). Both lead to the decline of the graft reaction speed between WPI and GA.

3.1.5 Effect of WPI-GA ratio on graft reaction

The effect of protein-GA ratio on graft reaction was shown in Fig. 1E. It was obvious that the DG value increased quickly first and then slowly when the ratio of WPI to GA changed from 4:1 to 1:4, followed by declined. The highest DG value appeared at the ratio of 1:4, however, there was no significant difference ($p \geq 0.05$) between it and the values obtained at the ratio of 1:2. It was suggested that more reducing-end carbonyl groups were accessible for interaction with free amino acid groups in protein as the increase in saccharide concentrations, which lead to the improvement of DG value (Zhao, et al., 2016). However, the graft reaction will reaches a saturation point with further increase in saccharide content due to decrease accessibility of the amino groups, leading to slower increase in DG value (Achouri, Boye, Yaylayan, & Yeboah, 2005). Moreover, as a polysaccharide, too high concertation of GA may prevent the reaction glycation by stereo-hindrance effect and weaken the effect of ultrasound due to high viscosity of the reaction system.

3.2 Degree of glycosylation and browning intensity

According to the above results, a temperature of 70 °C, an ultrasound power of 500 W, an initial pH of 11, a WPI concentration of 10 mg/mL, and a WPI-GA ratio of 1:2 were chosen for the following experiments. The DG values of the WPI-GA conjugates prepared by classical heating and ultrasound treatment at different times are shown in Table 1. It was obvious that the degree of glycosylation increased with the reaction time and ultrasound treatment could speed up the graft reaction between WPI and GA. For example, a DG value of 11.20 % was reached by classical heating for 48 h, whereas the same level of DG value would obtained at a much shorter time of 20 min by ultrasound treatment. Due to its effective mixing and efficient energy/mass transfer effect, ultrasound was able to support more energy for the graft reaction and bring the reactive groups into closer proximity, thus accelerate the glycosylation between protein and polysaccharide (Corzo-Martinez, et al., 2014; Mu, et al., 2010). In addition, ultrasound treatment

298 could disrupt the tertiary and quaternary structure of protein, resulting more free amino groups for
299 the reaction (Chen, Chen, Wu, & Yu, 2016).

300 In terms of browning intensity, the grafted WPI have a significantly increase ($p < 0.05$) in
301 browning compared with control WPI (Fig. 2). Besides, the browning intensity of conjugates
302 obtained by classical heating was much higher than that formed by ultrasound treatment with the
303 same level of DG value. Our result was similar to previous reports of Zhang et al. (2014). It may
304 be because ultrasound treatment could reduce the side reactions occurring during the graft reaction.
305 Zhao et al. (2016) also reported that ultrasound pretreatment could decrease the browning intensity
306 of SPI/sugar Maillard reaction products (MRPs) and supposed that ultrasound might interfere with
307 the formation of late MRPs melanoidins through inhibiting polymerization of intermediate
308 products during Maillard reaction. Therefore, ultrasound treatment not only shortens the
309 glycosylation time between WPI and GA, but also effectively suppresses the browning degree of
310 the products. Although Wang et al. (2015) pointed out that the browning of BPI-glucose
311 conjugates prepared by ultrasound treatment is higher than that formed by classical heating, the
312 DG value of the former conjugates were higher.

313 **3.3 Effect of glycosylation on structural properties of WPI**

314 **3.3.1 SDS-PAGE analysis**

315 SDS-PAGE was conducted to confirm the conjugate of WPI with GA. Fig. 3 showed that
316 WPI exhibited three clear bands at about 13.8, 18.0 and 66.0 kDa which represent α-Lactalbumin
317 (α-La), β-lactoglobulin (β-Lg) and bovine serum albumin (BSA), respectively (Liu et al., 2016).
318 For WPI-GA conjugates, the intensity of these bands decreased, and new bands with much higher
319 molecular weight emerged near the top of the gel. With increasing ultrasonic time, the intensity of
320 α-La, β-Lg, and BSA bands decreased gradually, and the bands near the top of the gel increased
321 visibility. In SDS-PAGE analysis, the higher molecular weight of the protein subunits, the lower
322 the mobility of the corresponding bands (Liu et al., 2017). Thus the molar mass distribution of the
323 WPI shifted towards the higher molecular weight and the increased visibility confirmed the
324 formation of WPI-GA conjugates. In addition, the bands of WPI-GA conjugates were highly
325 diffused, which may be as a result of one protein molecule covalently bonded with different
326 numbers of polysaccharides (Alahdad, Ramezani, Aminlari, & Majzoobi, 2009).

3.3.2 HPSEC analysis

To further examine the extent and course the conjugation, HPSEC was also carried out to measure the molecular size changes of glycosylated WPI-GA conjugates. As can been seen from Fig. 4, only one main peak with an elution time of 34.18 min was observed for WPI. But for the WPI-GA conjugates, a new peak was appeared at a shorter elution time. This could be resulted by the formation of covalent conjugates with larger molecular size via Maillard reaction. For ultrasound assisted WPI-GA conjugates, the new peak increased gradually with the increase of ultrasonic time, which indicated that more and more conjugates were obtained. This was consistent with the DG value and SDS-PAGE analysis. The elution time of the new peak for the conjugates obtained by classical heating and ultrasound treatment were about 26.00 min and 27.00 min, respectively. This revealed that the molecular size of the classical heating conjugates was larger than the ultrasound assisted WPI-GA conjugates. It might be because ultrasound treatment could partially degrade WPI, GA, and/or the conjugates. Moreover, the chromatograms of WPI-GA conjugates also indicated the presence of unreacted WPI which correlated well with the SDS-PAGE results as shown in Fig. 3. Similar results were reported by Zhu et al. (2008) and Qi, Xiao, & Wickham (2017) about the glycosylation of WPI.

3.3.3 FTIR analysis

The chemical changes accompanying the MR in WPI-GA conjugate would lead to several changes in the mid-IR spectrum as a result of the consumption of some functional groups and the appearance of others. These functional groups such as NH_2, especially from lysine, may be lost, but the amount of these new groups of MRPs including Amadori compound (C=O), Schiff base (C=N), and pyrazines (C-N) may be increased by the MR (Nooshkam & Madadlou, 2016). As shown in Fig. 5, the peaks around 1651 and 1531 cm^{-1} in the spectrum of WPI, which belong to amide I (80 % C=O stretch) and amide II (60 % N-H bend and 40 % C-N stretch) bands, respectively, are characteristics of proteins (Carbonaro & Nucara, 2010). After glycation with GA, the main absorption bands of WPI at 1651 and 1531 cm^{-1} shifted to 1645 and 1539 cm^{-1}, respectively, accompanied with a decrease of the intensity. This was attributed to the Maillard reaction, which has been proven by previous reports (Abdelhedi et al., 2017; Wang, Bao, & Chen, 2013). For the conjugates obtained by ultrasound treatment, the absorptions at 1645 and 1539 cm^{-1}

were lower than those obtained by classical heating, and decreased with the increase of ultrasonic time. This proved the effect of ultrasound treatment on accelerating the glycation reaction and was consistent with the results of DG, SDS-PAGE and HPSEC analysis. In addition, the absorptions of WPI-GA conjugates in 1050-950 cm^{-1} regions which correspond to side-chain vibrations from protein (Chang & Tanaka, 2002) were stronger than those of WPI, also indicated the alteration of protein structure.

3.3.4 CD analysis

The secondary structure distribution of WPI and WPI-GA conjugates was presented in Table 2. The content of α-helix, β-sheet, β-turn and random coil in WPI secondary structure are 14.2 %, 32.0 %, 22.3 % and 31.5 %, respectively. After glycation, the α-helix and β-sheet contents were decreased while the β-sheet and unordered coil content were increased. This indicated that glycosylation resulted the change in spatial structure and unfolding of WPI molecules. The functional properties of protein were highly correlated with its structure and structural modifications allowing greater conformational flexibility may improve its emulsifying abilities (Martinez, Sanchez, Ruiz-Henestrosa, Patino, & Pilosof, 2007). Thus the increase in unordered structure content may contributes to the improvement of emulsifying properties. For the conjugates prepared by ultrasound treatment, more α-helix content were lost when increase the ultrasound time, which was consisted with the change of DG value as shown in Table 1. Covalent binding of polysaccharide to protein via Maillard reaction involves a condensation between the carbonyl group with a ε-amino group, which is within the α-helix region or its neighbor of proteins (C. Li, Xue, et al., 2014). Thus the decrease in α-helix content suggests that polysaccharides were bound to the protein (Hattori, Nagasawa, Ametani, Kaminogawa, & Takahashi, 1994). With the same level of DG value, the α-helix content of the conjugate formed by ultrasound treatment lost more than those formed by classical heating. However, more evident changes in β-sheet, β-turns and random coil content were observed for the classical heating WPI-GA conjugate. Our results were different with previous reports which indicated that ultrasound assisted protein-polysaccharide conjugates showed more evident change in the distribution of secondary structure (C. Li, Xue, et al., 2014; Mu, et al., 2010; Stanic-Vucinic, Prodic, Apostolovic, Nikolic, & Velickovic, 2013). This may be caused by the difference of the

385 experimental conditions. As ultrasound treatment (S. Li, et al., 2016), heating treatment (Kim,
386 Kim, Yang, & Kwon, 2004) as well as extreme alkaline pH treatment (Chen, et al., 2019) were all
387 effective in changing the secondary structure of protein, the observed secondary structure
388 distribution was the combined effect of these treatment.

389 **3.4.5 Fluorescence analysis**

390 The intrinsic fluorescence spectra of WPI and WPI-GA conjugates was presented in Fig. 6.
391 When excited at 280 nm, the maximum emission intensity (λ_{max}) of WPI was observed at the
392 wavelength of 331 nm, indicating the fluorescent amino acids (tryptophan, phenylalanine and
393 tyrosine) of WPI were present in a "polar" environment (Vivian & Callis, 2001). For the WPI-GA
394 conjugates, the fluorescence intensity increased dramatically and a red-shift of the λ_{max} was
395 observed. This indicates that some of those amino acids groups initially inside the WPI molecule
396 were exposed to the surface (Chen, et al., 2019) and moved towards a more hydrophilic
397 microenvironment (Vivian & Callis, 2001) after glycosylation. C. Li, Huang, et al. (2014), C. Li,
398 Xue, et al. (2014) and Wang et al. (2016) reported that the protein-polysaccharide conjugates
399 obtained by both ultrasonic treatment and classical heating showed lower fluorescence intensity
400 compared with that of native protein. They attributed this phenomenon to the shielding effect of
401 the polysaccharide chain bound to proteins. The pH condition of their reaction system were
402 7.5-8.0 while ours was 11.0, which may be the main reason for the difference between our results
403 with theirs. Because extreme alkaline pH treatment could make the protein unfolding and thus
404 increase its fluorescence intensity (Chen, et al., 2019). With same level of DG value, higher
405 fluorescence intensity was observed for the conjugate prepared by ultrasound treatment, indicating
406 ultrasound treatment could promote the denaturation of protein, resulting further changes of
407 structure (Zhao, et al., 2016). However, the conjugate formed by classical heating showed a
408 stronger red-shift of the λ_{max}. This may be caused by the partially degradation of protein after
409 longer heating treatment at alkaline pH.

410 **3.3.6 Surface hydrophobicity (H_0) analysis**

411 The H_0 values of WPI and WPI-GA conjugates were shown in Fig. 7. It was obvious that H_0
412 values of the conjugates obtained by classical heating were significantly lower than that of the
413 WPI. Similar results was reported by Y. Li, et al. (2013) about the MRPs of rice protein

414 hydrolysates with dextran T20. It may be because that the accessibility of ANS to these groups
415 will be inhibited if polysaccharide was conjugated to protein (Xue, et al., 2013). In addition, the
416 bonding of hydrophilic straight-chain polysaccharide increasing the hydrophilicity on the
417 molecule surface, and thus reducing the exposure of hydrophobic groups buried in
418 intramolecularly (Wang et al., 2015). However, C. Li, Huang, et al. (2014) indicated that the graft
419 reaction between PPI and glucomannan could significantly ($p < 0.05$) increase the H_0 value of PPI.
420 As saccharide modification not only lead to aggregation via cross-linking hydrophobic
421 interactions but also results in the exposure of hydrophobic groups due to protein partial unfolding
422 (Chao, Ma, & Stadtman, 1997). Thus the observed H_0 was caused by the balance of these two
423 effects. The inconsistent phenomena may be caused by experimental conditions, like the different
424 of protein type, reaction temperature, incubation time, pH and so on. Fig. 7 also showed that the
425 H_0 values of conjugates obtained by ultrasound treatment were all significantly ($p < 0.05$) higher
426 than that of WPI and increased with the increase in reaction time. C. Li, Huang, et al. (2014) and
427 Wang et al. (2016) also confirmed that the protein-saccharide conjugates prepared by ultrasound
428 treatment exhibited higher H_0 values than native protein. It may because that the cavitation
429 induced by ultrasound treatment could expose some of the hydrophobic groups initially buried in
430 the interior of protein molecules to the surface and thus increase the H_0 of protein (Zhang et al.,
431 2014). In addition, the pH applied in this work could convert the protein molecules to a state of
432 maximum unfolding and thus enhance the efficient of ultrasound treatment (Lee, et al., 2016).
433 These might explain why protein-saccharide conjugates prepared by ultrasound treatment have
434 higher H_0 value.

435 **3.4 Effect of glycation on the functional properties of WPI**

436 **3.4.1 Solubility analysis**

437 The water solubility of WPI and WPI-GA conjugates at pH ranging from 2 to 10 was shown
438 in Fig. 8. The solubility of WPI was influenced by pH and the lowest solubility was recorded at
439 around pH 5. The solubility of WPI was increased remarkably after glycation with GA for the
440 whole pH ranges except for pH 3. The increase in solubility might be because that the covalent
441 linkage with hydrophilic polysaccharides could enhance the affinity of proteins to a water
442 molecule and hinder their aggregation under unfavorable conditions (Jimenez-Castano,

Lopez-Fandino, Olano, & Villamiel, 2005). The lower solubility of the conjugates at pH 3 may be attributed to interpolymeric complexes formed by strong electrostatic interactions as their zeta potential were lower than that of WPI (data no shown). For the conjugates obtained by ultrasound treatment, the solubility increased with the increase in ultrasound time, which is consistent with the DG value. More and more hydrophilic groups have been bound to the protein may be one main reason for this phenomenon. In addition, ultrasound treatment could unfolding the proteins and break up insoluble protein aggregates into soluble ones, which also contributes to the increase of protein solubility (Mu et al., 2010). With the same level of DG value, the conjugates obtained by ultrasound treatment showed superior solubility at pH range 7-10, but lower solubility at the acidic pH range studied when compared with those obtained by classical heating. Similar results were reported by C. Li, Xue, et al. (2014) and Schmitt, Bovay, & Frossard (2005) about the β-lg-GA conjugates and PPI–GA conjugates, respectively. They supposed that the disappear of NH_2 groups on the protein surface caused by the conjugation with GA is responsible for decrease in solubility at acidic value.

3.4.2 Thermal stability analysis

Fig. 9 showed the solubility changes of the WPI before and after glycated with GA caused by heating. It was obvious that the solubility of WPI increased slightly when heated at 50-70 °C, and then decreased quickly with further increase in heating temperature. The decrease in solubility when the heating temperature above 70 °C was probably caused by greater protein aggregation (Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013). Fig. 9 also revealed that the thermal stability of WPI was improved considerably after conjugation with GA. Similar observations in the improving thermal stability of WPI when conjugated with maltodextrin (Mulcahy, Park, Drake, Mulvihill, & O'Mahony, 2018), α-La when conjugated with GA (de Oliveira, et al., 2015), and β-Lg when conjugated with dextran (Jimenez-Castano, et al., 2005) have been reported. This may attributes to the steric hindrance provided by longer polysaccharide chains to inhibit the aggregation of protein molecules during heating (Enomoto, et al., 2007). For the conjugates obtained by ultrasound treatment, the improvement of thermal stability is directly correlated with the ultrasound time. In other words, higher glycation degree provide better thermal stability, which

implied that glycation played a critical role in protecting the WPI against heat induced denaturation. With the same level of DG value, the conjugates obtained by ultrasound treatment showed better thermal stability than those obtained by classical heating. This may be because the conjugates prepared by ultrasound treatment could glycated with more polysaccharides (C. Li, Xue, et al., 2014) and thus prevent the protein aggregation more efficient than those obtained by classical heating.

3.4.3 Emulsifying properties analysis

One of the most striking characteristics of glycated proteins was their excellent emulsifying properties. Thus the effect of glycation on the emulsifying properties of WPI has been investigated and the results were shown in Fig. 10. As expected, both the EAI and ESI of conjugates were significantly improved as compared with WPI. It was because that covalent bonding of GA to a protein could form a macromolecular stabilizing layer around oil droplets and stabilize them against creaming, flocculation and coalescence by steric repulsion (Pirestani, et al., 2017). This (rather than modification the rheology of continuous phase) was one main reason for the improvement of protein emulsifying properties (Dickinson, 2003). In addition, the improved solubility of the conjugates as presented in Fig. 8 contribute to the enhanced emulsifying properties, too (Pirestani et al., 2017). Moreover, the increase in unordered structure content may be also correlated with the improvement of emulsifying properties (Martínez et al., 2007). For the conjugates obtained by ultrasound treatment, the EAI and ESI values increased with the increase in ultrasound time, which is consistent with the DG value. The reason for this phenomenon might be that the conjugates with high DG value glycated with more GA, had better solubility and higher unordered structure content. With the same level of DG value, the emulsifying properties of conjugates obtained by ultrasound treatment were superior to those obtained by classical heating. This may be because the conjugates prepared by ultrasound treatment could glycated with more polysaccharides (C. Li, Xue, et al., 2014). In addition, ultrasound treatment lead to expose more hydrophobic and thus achieve a new hydrophobic-hydrophilic balance which may in favor of emulsion formation and stabilization (Wang et al., 2015).

4 Conclusion

In conclusion, ultrasound treatment was able to speed up the glycation rate between WPI and GA. The conjugates prepared by ultrasound treatment showed significant lower ($p < 0.05$) level of browning intensity and significant higher ($p < 0.05$) level of solubility (under alkaline conditions), thermal stability, emulsifying activity and emulsifying stability when compared with those prepared by classical heating. This might attributed to the structural modification induced by ultrasound treatment. Therefore, covalent linkage of WPI with GA through ultrasound assisted Maillard reaction might be an ideal method to improve its functional properties and thus expand its utilization in food industry. More investigations on the glycation mechanism between protein and polysaccharides and process optimization under ultrasound treatment need to be carried out in future.

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734 Table 1 DG Values of WPI-GA conjugates prepared by ultrasound treatment and classical heating

Conjugates prepared by classical heating		Conjugates prepared by ultrasound treatment	
Time (h)	DG (%)	Time（min）	DG (%)
4	5.11 ± 0.24	20	11.84 ± 0.72
12	6.38 ± 0.26	30	13.77 ± 0.58
24	7.15 ± 0.29	40	15.79 ± 0.84
36	9.52 ± 0.31	50	19.38 ± 0.96
48	11.20 ± 0.04	60	21.58 ± 1.01
-	-	70	23.86 ± 0.95
-	-	80	26.23 ± 1.22
-	-	90	26.49 ± 0.73

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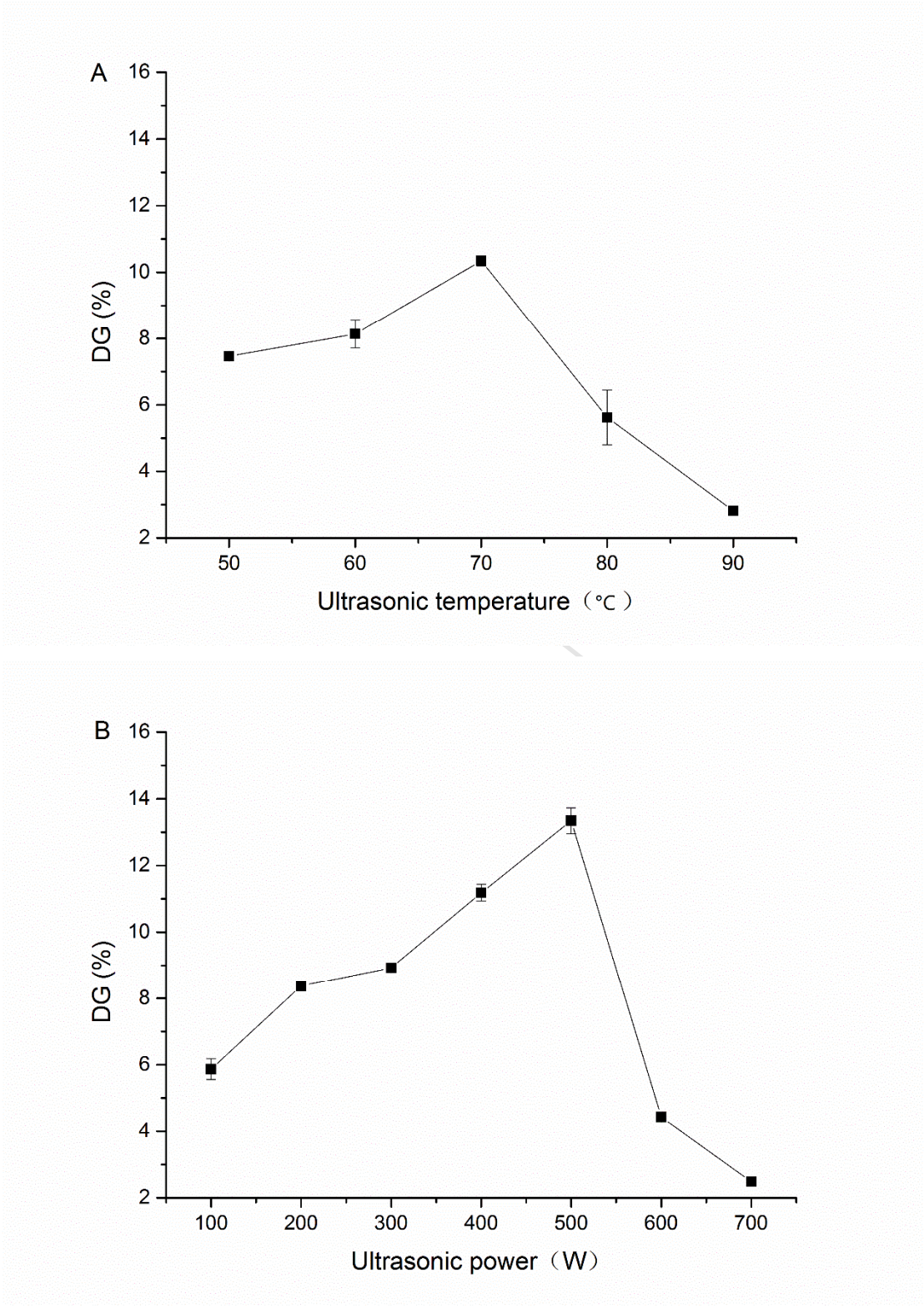
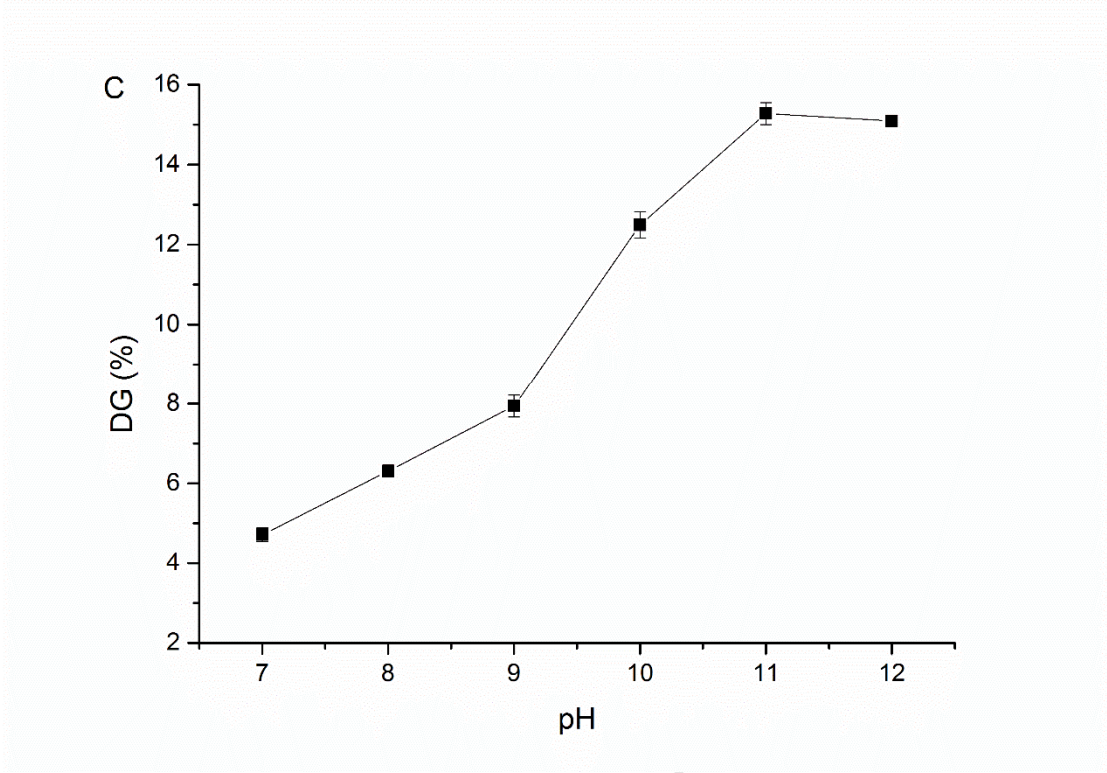
736

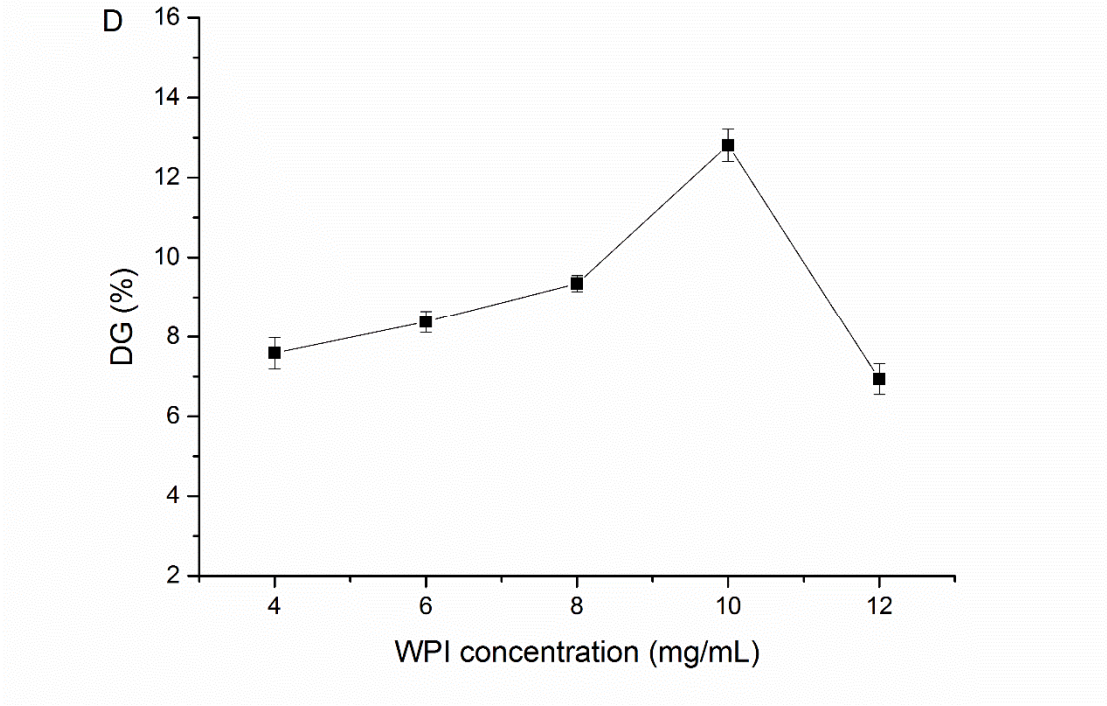
737 Table 2 Secondary structure content (%) of WPI and WPI–GA conjugates

Sample	α- helix	β-sheet	β-turns	Random coil
WPI	14.2	32.0	22.3	31.5
Conjugates obtained by classical heating for 48h	12.5 (-11.97 %)	28.9 (-9.69 %)	24.5 (9.87 %)	34.1 (8.25 %)
Conjugates obtained by ultrasound treatment for 20min	12.2 (-14.08 %)	31.8 (0.63 %)	22.8 (2.24 %)	33.1 (5.08 %)
Conjugates obtained by ultrasound treatment for 50min	11.6 (-18.31 %)	31.7 (-0.94 %)	23.0 (3.14 %)	33.6 (6.67 %)
Conjugates obtained by ultrasound treatment for 80min	10.9 (-23.24 %)	31.2 (-2.50 %)	23.5 (5.38 %)	34.3 (8.89 %)

738 Note: Values in parentheses denote the change in secondary structure of the conjugates compared
739 with WPI.


A
DG (%)
16
14
12
10
8
6
4
2
50
60
70
80
90
Ultrasonic temperature（℃）
B
DG (%)
16
14
12
10
8
6
4
2
100
200
300
400
500
600
700
Ultrasonic power（W）


C
DG (%)
pH
2
4
6
8
10
12
14
16
7
8
9
10
11
12


D
DG (%)
WPI concentration (mg/mL)
2
4
6
8
10
12
14
16
4
6
8
10
12

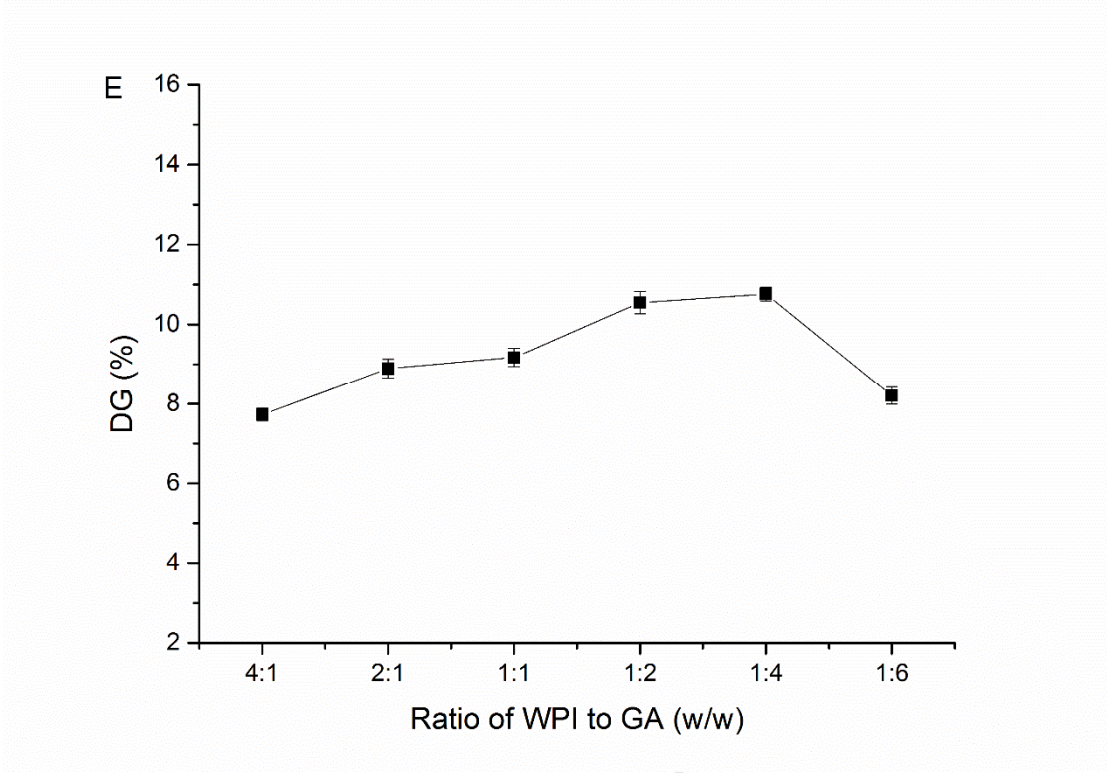


Fig. 1 Effect of ultrasound temperature (A), ultrasound power (B), initial pH (C), WPI concentration (D), and WPI-GA ratio (E) on the graft reaction between WPI and GA.

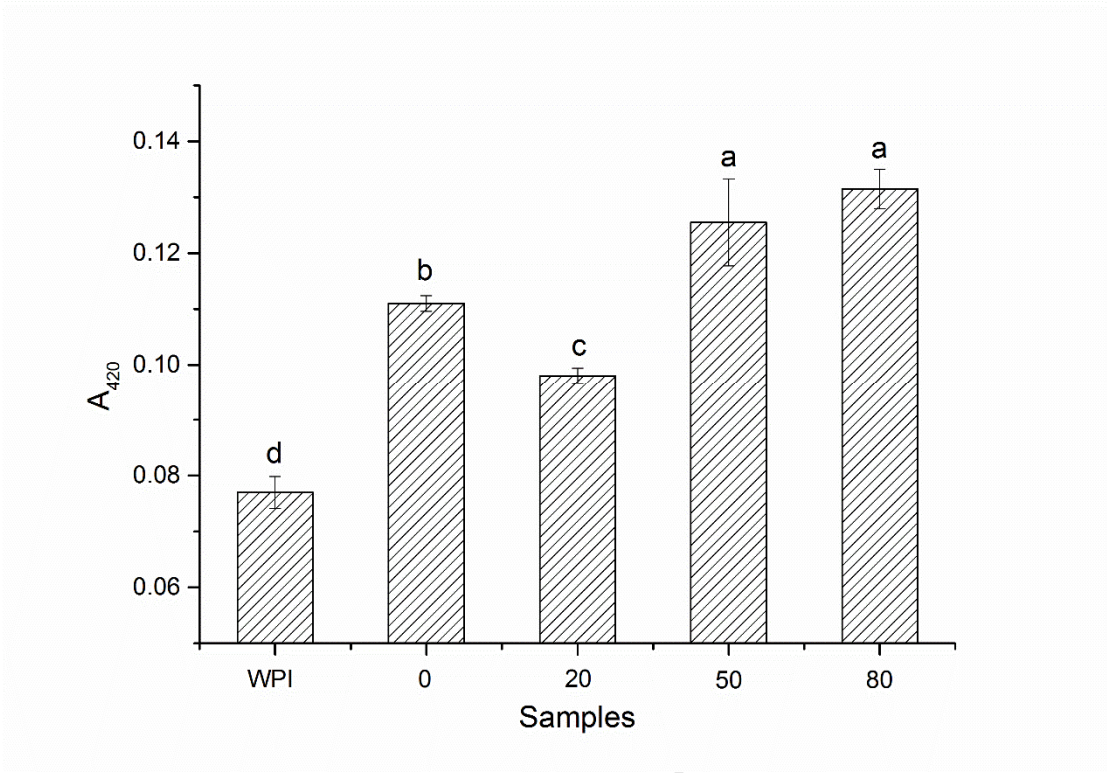


Fig. 2 The browning intensity of WPI and WPI-GA conjugates obtained at different times of ultrasonic treatment. 0 represents WPI-GA conjugates obtained by classical heating for 48 h; 20, 50, and 80 represent the times (min) needed to produce WPI-GA conjugates by ultrasonic treatment.

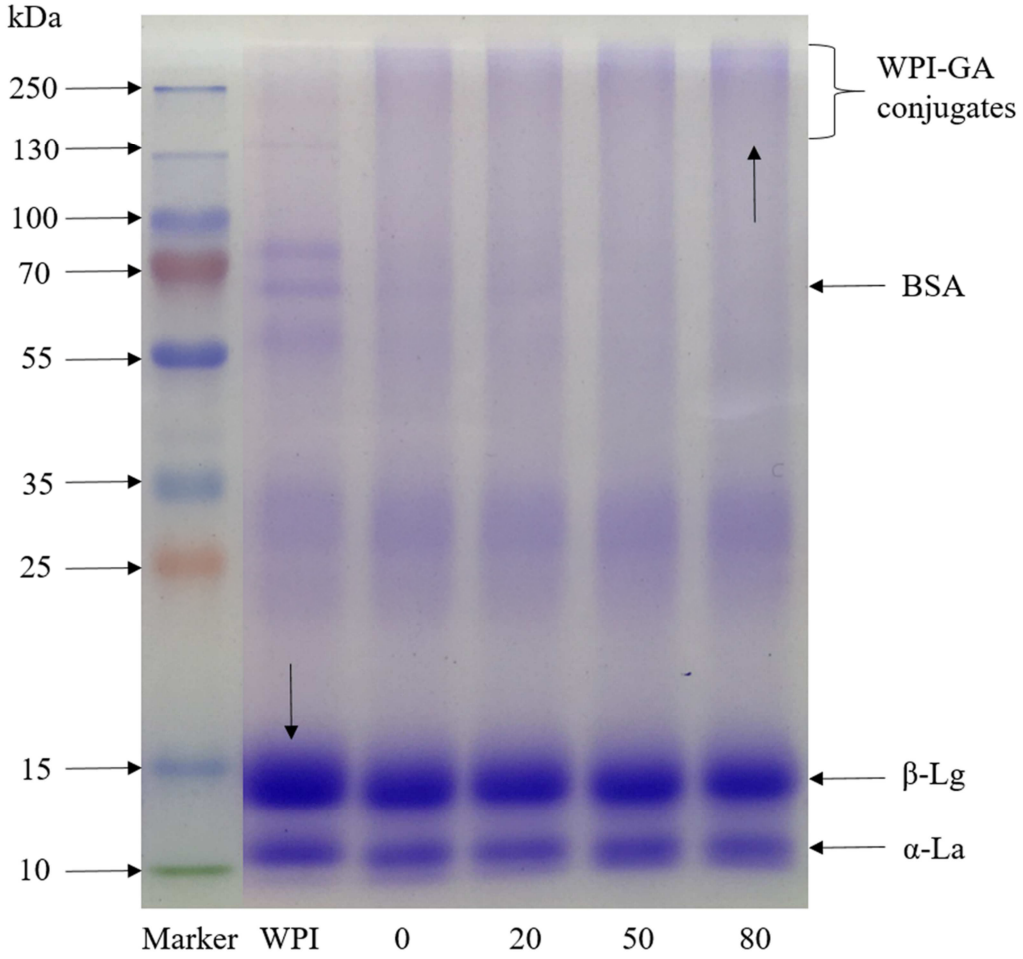


Fig. 3. SDS-PAGE of WPI and WPI-GA conjugates obtained at different times of ultrasonic treatment. 0 represents WPI-GA conjugates obtained by classical heating for 48 h; 20, 50, and 80 represent the times (min) needed to produce WPI-GA conjugates by ultrasonic treatment.

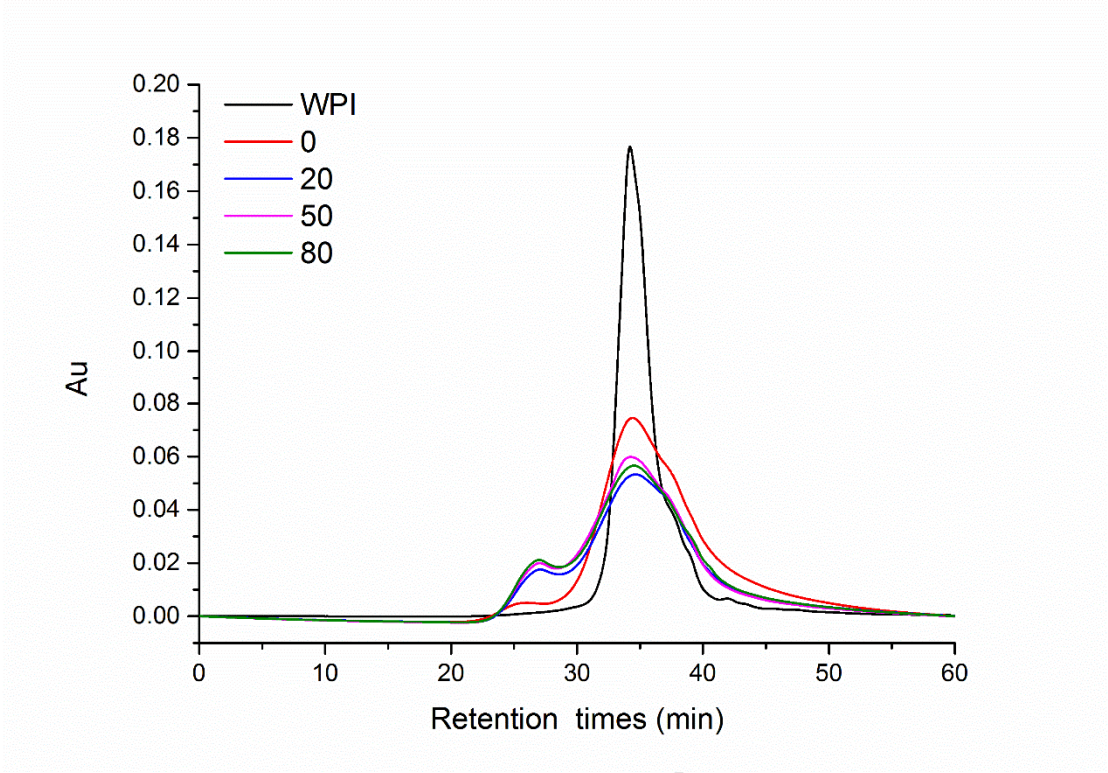


Fig. 4. HPSEC elution profiles of WPI and WPI-GA conjugates obtained at different times of ultrasonic treatment. 0 represents WPI-GA conjugates obtained by classical heating for 48 h; 20, 50, and 80 represent the times (min) needed to produce WPI-GA conjugates by ultrasonic treatment.

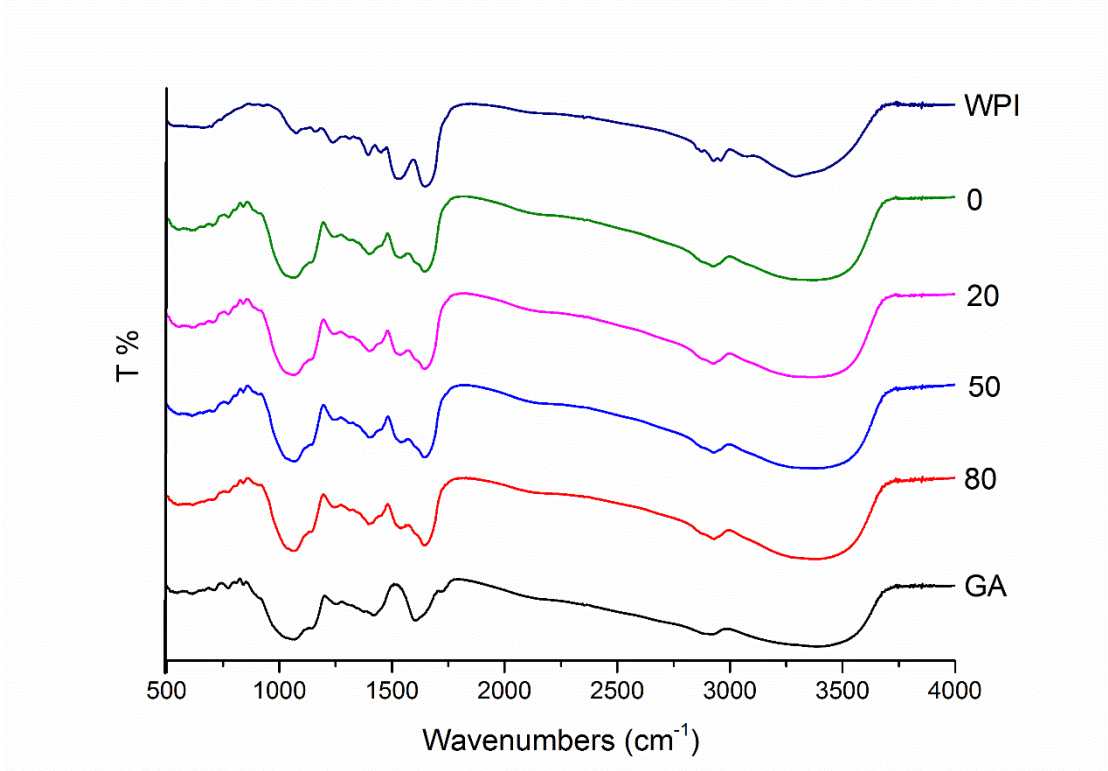


Fig. 5. FTIR spectra of WPI and WPI-GA conjugates obtained at different times of ultrasonic treatment. 0 represents WPI-GA conjugates obtained by classical heating for 48 h; 20, 50, and 80 represent the times (min) needed to produce WPI-GA conjugates by ultrasonic treatment.

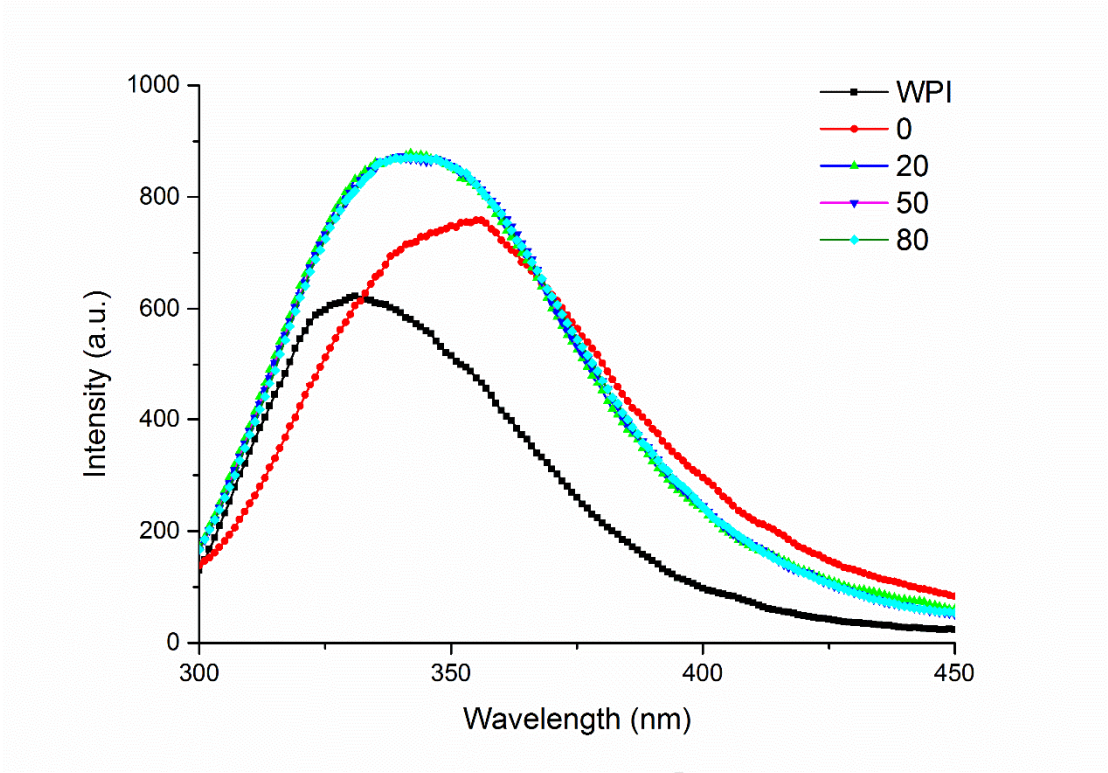


Fig. 6. The intrinsic fluorescence spectra of WPI and WPI-GA conjugates obtained at different times of ultrasonic treatment. 0 represents WPI-GA conjugates obtained by classical heating for 48 h; 20, 50, and 80 represent the times (min) needed to produce WPI-GA conjugates by ultrasonic treatment.

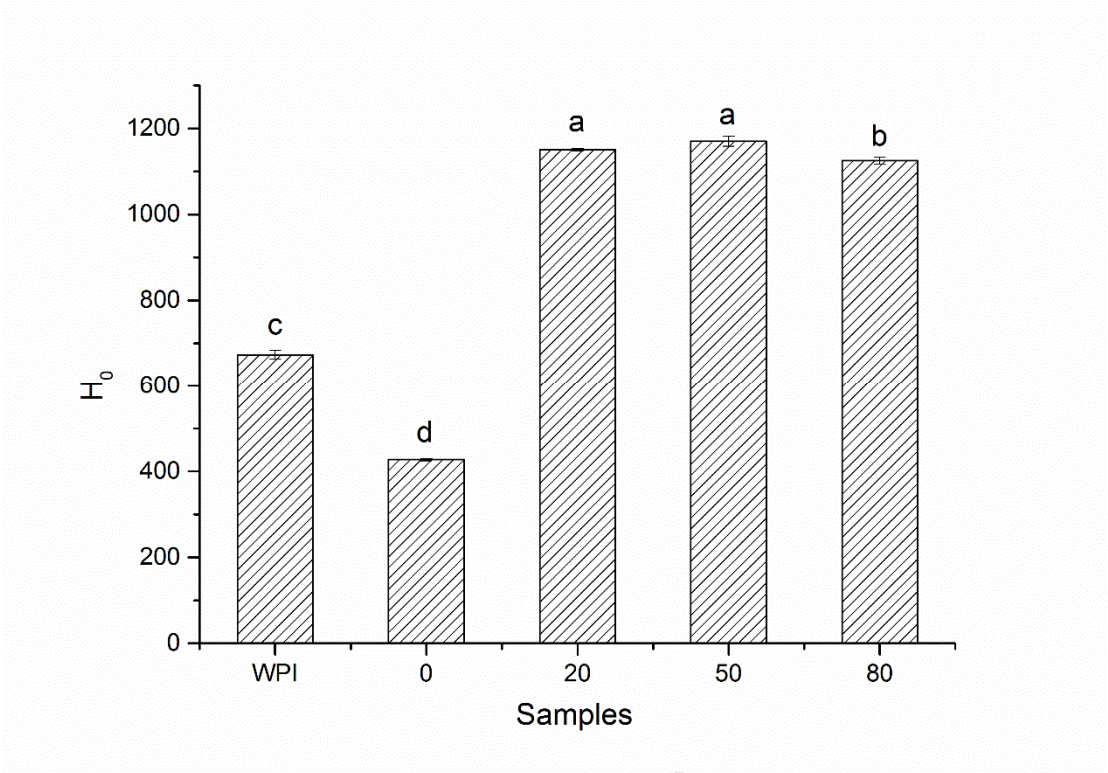


Fig. 7. H_0 values of WPI and WPI-GA conjugates obtained at different times of ultrasonic treatment. 0 represents WPI-GA conjugates obtained by classical heating for 48 h; 20, 50, and 80 represent the times (min) needed to produce WPI-GA conjugates by ultrasonic treatment.

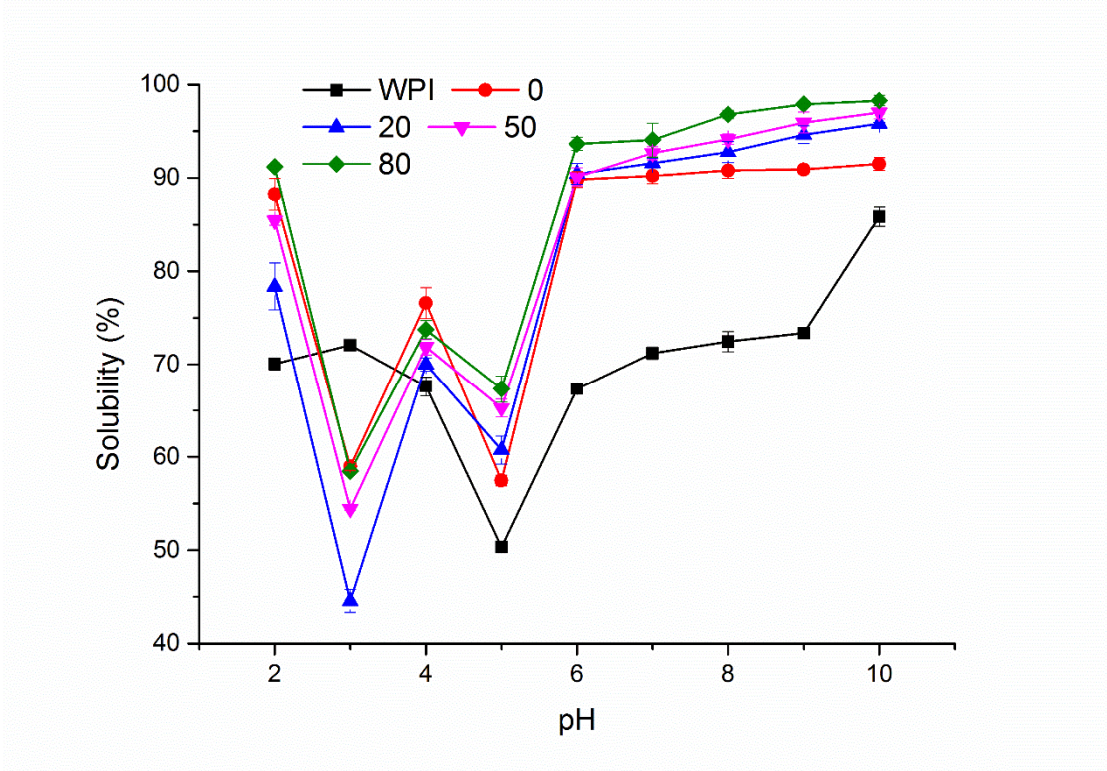


Fig. 8. Solubility of WPI and WPI-GA conjugates in water at different pH values. 0 represents WPI-GA conjugates obtained by classical heating for 48 h; 20, 50, and 80 represent the times (min) needed to produce WPI-GA conjugates by ultrasonic treatment.

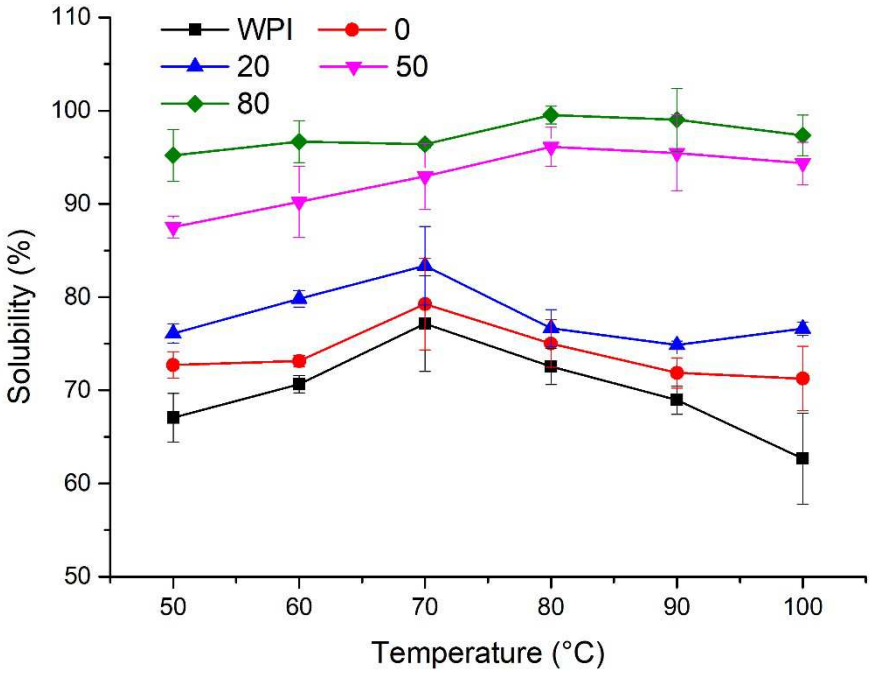


Fig. 9. Effect of temperature on the solubility of WPI and WPI-GA conjugates obtained at different times of ultrasonic treatment. 0 represents the conjugates obtained by classical heating for 48 h; 20, 50, and 80 represent the times (min) needed to produce conjugates by ultrasonic treatment.

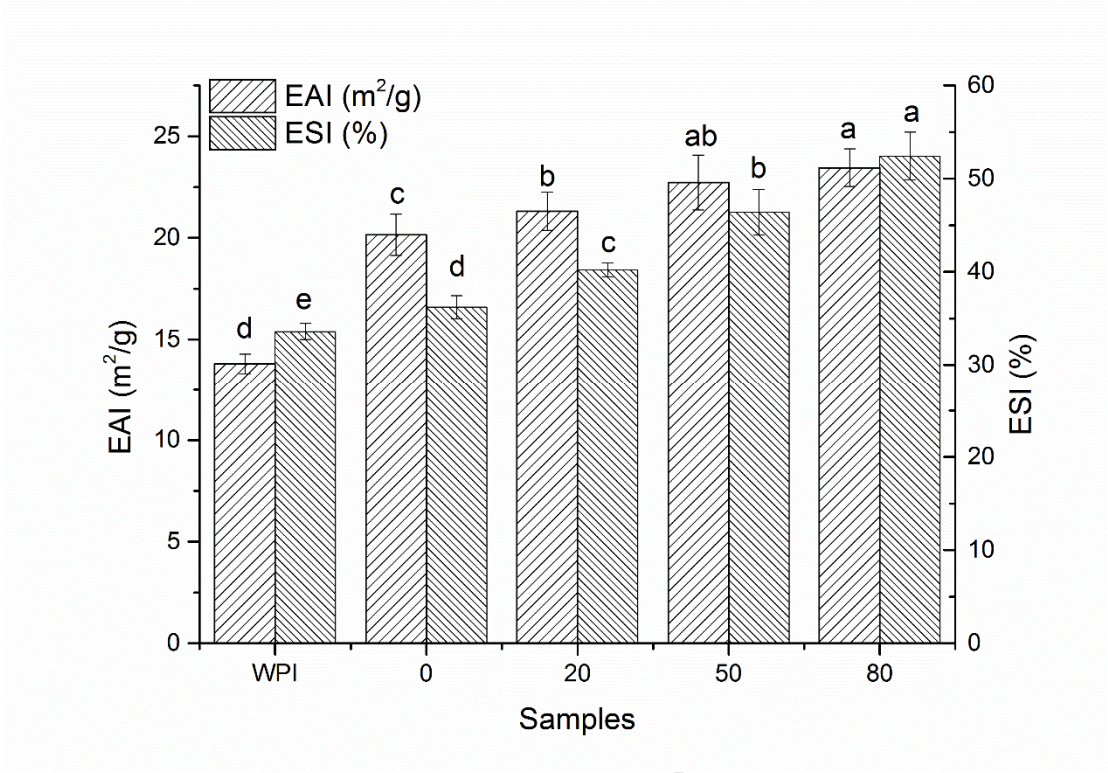


Fig. 10. Emulsifying properties of WPI and WPI-GA conjugates obtained at different times of ultrasonic treatment. 0 represents WPI-GA conjugates obtained by classical heating for 48 h; 20, 50, and 60 represent the times (min) needed to produce WPI-GA conjugates by ultrasonic treatment. Superscripts (a-d) represent significant differences at $p < 0.05$ level.

- Whey protein isolate was glycated with gum Acacia by ultrasound and classical heating.
- Ultrasound could accelerate the glycation reaction.
- Conjugates prepared by ultrasound had lower browning intensity.
- Solubility, heat stability and emulsifying properties of conjugates prepared by ultrasound were improved.