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Effects of ultrasound synergized with microwave on structure and functional properties of transglutaminase-crosslinked whey protein isolate

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ABSTRACT

In the present study, ultrasound (400 W, U), microwave heating (75 ℃ for 15 min, M) and ultrasound synergized with microwave heating (UM) pretreatments of whey protein isolate (WPI) were applied to investigate and compare their influence on structure, physicochemical and functional characteristic of transglutaminase (TGase)-induced WPI. From the results of size exclusion chromatography, it could be seen that all three physical pretreatments could promote the formation of polymers in TGase cross-linked WPI, whose polymer amounts were increased by the order of U, UM and M pretreatment. Among three physical methods, M pretreatment had the strongest effect on structure and functional characteristics of TGase-induced WPI. Furthermore, compared with TGase-induced WPI, α-helix and β-turn of M−treated TGase-induced WPI (M−WPI−TGase) were reduced by 7.86% and 2.93%, whereas its β-sheet and irregular curl were increased by 15.37% and 7.23%. Zeta potential, emulsion stability and foaming stability of M−WPI−TGase were increased by 7.8%, 59.27% and 28.95%, respectively. This experiment exhibited that M was a more effective pretreatment method than U, UM for WPI, which could promote its reaction with TGase and improve its functional properties.

1. Introduction

As an extracellular catalytic transferase, transglutaminase (TGase, EC 2.3.2.13) can generally be obtained by microbial fermentation, which catalyzes the catalytic acyl transfer reaction, deamidation reaction and cross-linking polymerization between glutamine (as acyl donor) residues and lysine (as acyl receptor) residues in proteins [1]. The structure and functionality of food proteins modified by TGase have been extensively studied, such as soy protein [2], casein [3], whey protein [4,5]. However, native α-lactalbumin and β-lactoglobulin are compact globular proteins [6,7], which prevents their TGase reaction sites from being exposed, resulting in lowering the reaction between TGase and WPI. Therefore, it is necessary to alter the structure of whey protein in order to crosslink it with TGase easily.

Ultrasound (U) has been examined to be a fresh technology that changes the structure and functional characteristic of proteins. In addition, it is an environmentally friendly technology that does not contain exogenous chemical additives [8]. For instance, Cheng et al. [9] found that gel properties of whey protein emulsion gels treated by 20/28 kHz at 10 min were enhanced significantly. Soy protein isolate (SPI) was cross-linked with TGase after being treated by ultrasonic power of 400 W for 40 min, which could enhance the gel characteristic of SPI and increase the embedding rate of riboflavin [10]. It also significantly reduced the release rate of riboflavin in the subsequent digestion process. Nazari et al. [11] proved that solubility, emulsification properties, foaming ability and foaming stability of millet protein concentrate had been significantly enhanced after it was treated by ultrasonic 20 kHz for 5, 12.5 and 20 min. It was reported that ultrasound (40 kHz, 300 W) could be used to promote the gel properties of TGase-induced SPI and wheat gluten mixtures [12]. The effects of ultrasound on liquid systems might be correlated with acoustic cavitation phenomenon, wherein cavitation bubbles were formed rapidly and collapsed violently during sonication [13]. In protein solutions, more reaction positions of proteins were uncovered, which led to an increase in the cross-linking reaction through cavitation effect and shear stress of ultrasound pretreatment [14]. And it is well known that protein could produce massive free radicals after ultrasound pretreatment. Moreover, it was reported that free radicals could cause protein structure modification and oxidative degradation [15].

Microwave (M) is an electromagnetic wave in the frequency range of 0.3–300 GHz. Microwave heating (2450 MHz) is a dielectric treatment method. And it was widely used in the food industry [16]. The potential

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mechanism of M−induced modification is mainly in consideration of the quick development in temperature [17] and the electromagnetic effect [18]. The electromagnetic effect in the microwave field can rearrange the electric field distribution and destroy the electrostatic interaction among the electric residues, thus contributing to the divorce and stability of protein [16]. Besides, it was also reported that the formation of reactive free radicals was also one of the reasons for microwave-induced modification [17]. It has been reported that M could vary the secondary structure of shrimp protein, increase the β-sheet, and lose the β-turn [19]. Polymerization rate of TGase-induced milk protein formed under microwave heating (30 °C) was three times higher than that under the same temperature by conventional heating [20]. Additionally, Qin et al. [21] discovered that M treatment, with the increase of microwave power (0 ~ 700 W), significantly increased the gel strength, water holding capacity and storage modulus of soybean protein and wheat protein induced by TGase. The above-mentioned numerous studies showed that single microwave heating or ultrasound could promote the reaction of TGase and food proteins. Although the mechanisms of ultrasound and microwave are absolutely diverse, scholars have previously demonstrated that synergism between microwave and ultrasound (UM) pretreatments played an essential role in the development of biomaterials [20] and extraction of natural products [22]. Moreover, it was reported that microwave radiation could swiftly interpenetrate into the interior of complicated structures which was unattainable for cavitation, and microwave effects contributed to accelerate structural changes in sonicated proteins. Therefore, UM pretreatment may be a better modification method than individual M and U pretreatments. However, whether synergy treatment of ultrasound and microwave would promote cross-linking reaction of WPI and TGase, or enhance functionality of WPI was still not clarified. Accordingly, the intention of this research was to compare effects of three physical pretreatments (microwave assisted with ultrasound (UM), individual microwave and ultrasound) on structure and functional properties of TGase-induced WPI. The findings will provide technical data supports for the effective protein modification method and preparation of whey proteins with better functional characteristics. Additionally, a novel food constituent which has excellent functional properties was provided for food industry.

2. Materials and methods

2.1. Materials

The WPI (protein content 93.5%) was gained from Mullins Whey Inc. (Mosinee, WI, USA). Transglutaminase (enzyme activity 1000 U/g protein) was obtained from Yiming Biological Products Co., Ltd. D-Lemonene was obtained from MAYA Company. Sodium dodecyl sulfates (SDS), trifluoroacetic acid (TFA), tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). O-Phthalaldehyde was purchased from Biotopped Technology Co., Ltd (Beijing, China). Trichloroacetic acid was obtained from Tianjin Guangfu Fine Chemical Institute. Potassium bromide (KBr) was purchased from Macklin Biochemical Co., Ltd (Shanghai, China). All of the chemicals used in this study were of analytical grade.

2.2. Preparation of WPI obtained from three physical methods

The WPI was dissolved in distilled water and magnetically stirred for 2 h at room temperature to configure a WPI solution at a concentration of 50 mg/mL. Subsequently, 1 mol/L NaOH was used to adjust the pH of WPI suspensions to 7.

2.2.1. Preparation of ultrasound-treated WPI

A XH-300PE ultrasonic microwave combination synthesizer (Beijing Xianghu Science and Technology Development Co., Ltd) was used to sonicate of WPI suspension. Ultrasound treatment (25 kHz) was performed at 400 W for 15 min. In order to retain approximately 22 ± 3 °C during the ultrasonication process, WPI samples were dipped in an ice bath and the program was set to work for 2 min and then to stop for 1 min. The WPI samples after ultrasound treatment were marked as U-WPI.

2.2.2. Preparation of microwave-treated WPI

WPI solutions were heated with individual microwave at 75 °C for 15 min by a XH-300PE ultrasonic microwave combination synthesizer. The microwave-heated samples were subsequently cooled to room temperature and marked as M−WPI.

2.2.3. Preparation of WPI treated with ultrasound and microwave

The power of ultrasound was set to 400 W and its frequency was 25 kHz and microwave heating temperature was set to 75 °C, simultaneously. WPI solutions were loaded into a three-necked flask and heated under ultrasound and microwave worked together at 75 °C for 15 min. During this period, the program of ultrasound synergized with microwave was set to work 2 min-pause 2 min in order to maintain the reaction temperature at 75 °C. The WPI samples were cooled to room temperature after aforementioned treatment subsequently and marked as UM-WPI.

2.2.4. Preparation of TGase-induced WPI exposed prior to physical pretreatments

A 30U/g TGase was added to WPI solutions modified with different physical pretreatments (U-WPI, M−WPI, UM-WPI), and the obtained WPI was placed in a 50 °C water bath for 4 h, to ensure the WPI reacted fully with TGase. After 4 h, They were placed in a 75 °C water bath for 15 min to make TGase inactivity. Afterwards, samples were cooled in an ice water bath to room temperature and freeze-dried. The aforementioned WPI samples were recorded as U-WPI-TGase, M−WPI−TGase and UM-WPI-TGase. Control groups were TGase-induced WPI (WPI-TGase), and WPI that was prepared with three different physical pretreatments.

2.3. Physicochemical properties

2.3.1. Size exclusion chromatography (SEC)

The molecular size distribution of samples was calculated by SEC as described by Hu et al. [23].

2.3.2. Intrinsic fluorescence spectrum analysis

The Intrinsic fluorescence of WPI samples were measured according to the method of Shi et al. [24].

2.3.3. Mensuration of free amino groups

The free amino groups of WPI samples were measured according to the approach of Wang et al. [14].

2.3.4. Zeta-potential analysis

The Zeta-potential of WPI samples was measured according to the approach of Zhou et al. [25]. Zeta-potential of the samples could be determined with a particle size potential analyzer (Zetasizer Nano-ZS90, Malvern Ltd, UK). The sample and control groups were diluted with pH 7.0 PBS buffer (0.01 mol/L) to a concentration of 1 mg/mL.

2.3.5. Fourier transform infrared spectroscopy (FTIR)

The secondary structure changes of WPI samples were mensurated in the light of the method described by Shi et al [26]. The quantitative analysis of the secondary structural changes of protein samples in the amide I band (1700–1600 cm^{-1}) was carried out the peak fitting procedure described by Jiang et al. [27].

2.4. Functional characteristic

2.4.1. Determination of emulsifying properties

The emulsification stability and emulsification activity of the sample

were mensurated according to the method of Jiang et al. [28]. The emulsifying activity (EAI, M^2/g) and emulsifying stability of emulsion were calculated (ESI, %). The formula is as follows:

$$EAI\left(M^2/g\right) = (2 \times 2.303)/[C \times (1-\phi) \times 10] \times A_{500} \times dilution$$

A500 is the absorbance of the samples at 500 nm after 0 min of placement

C is WPI samples concentration before emulsification (mg/mL)

φ is the oil phase fraction of emulsion

$$ESI(\%) = A_{10} \times 100/A_0$$

A_0 and A_{10} are the absorbance measured at 500 min after 0 and 10 min, respectively

2.4.2. Determination of foaming properties

By modifying the method proposed by Wang et al. [14], we determined the foaming characteristics of the samples. The foam property was counted by the following equations. The formulas are as follows:

$$\textbf{Foamingability}(\%) = [(\mathbf{V_{f0}} - \mathbf{V_c})/\mathbf{V_{f0}}] \times 100$$

$$\textbf{Foamingstability}(\%) = [(\mathbf{V_{f30}} - \mathbf{V_c})/(\mathbf{V_{f0}} - \mathbf{V_c})] \times 100$$

Vc is before dispersion, the total volume of WPI samples dispersion and foam.

Vf0 is after dispersion, the entire volume of WPI samples dispersion and foam at 0 min.

Vf30 is after dispersion, the total volume of WPI samples dispersion and foam at 30 min.

3. Results and discussion

3.1. Analysis of size exclusion chromatography

As shown in Fig. 1A, there are three peaks of natural WPI, the retention times of which were 24.32 min, 28.46 min and 30.59 min, corresponding to BSA, β-lactoglobulin and α-lactalbumin, respectively. The WPI pretreated by microwave heating (M−WPI) had a small amount of macromolecular polymers, which was probably due to that M could speed up the rates of protein denaturation by loosening protein structures [18]. The unique heating method of microwave induced the polar molecules to rotate violently, causing them to rub against each other to generate heat and stretch the protein structure. The exposure of the hydrophobic groups changed the molecular structure and promoted cross-linking. Meanwhile, excessive free radicals were generated during microwave heating. Free radicals and rapid elevated temperature accelerated motion of protein molecules, and increased the collision chance between protein molecules, which also probably promoted cross-linking reaction of protein molecules [29]. There was no significant change in the molecular size of the other samples. Potentially, WPI aggregates might be destroyed to form fragments through the ultrasonic field when the ultrasound and microwave synergized.

As shown in Fig. 1B, the three different physical pretreatment methods promoted WPI-TGase crosslinking to form macromolecular aggregates. Among them, there was the most macromolecules and lowest α-lactalbumin and β-lactoglobulin in the group of M−WPI−TGase. This meant that M promoted TGase cross-linking significantly. And ultrasound is mainly through the cavitation effect acting on the WPI [30]. The degree of crosslinking reaction in UM-WPI-TGase was weaker than that in M−WPI−TGase but stronger than that in U-WPI-TGase. It was revealed that microwave pretreatment combined with ultrasound could not have good synergism on the crosslinking reaction of WPI and TGase. Potentially, in the group of UM-WPI-TGase, a steady and uninterrupted cavitation process of ultrasound might somewhat weaken the uniform heating phenomenon that occurred in the course of M treatment. Moreover, U could enhance nonthermal M effects

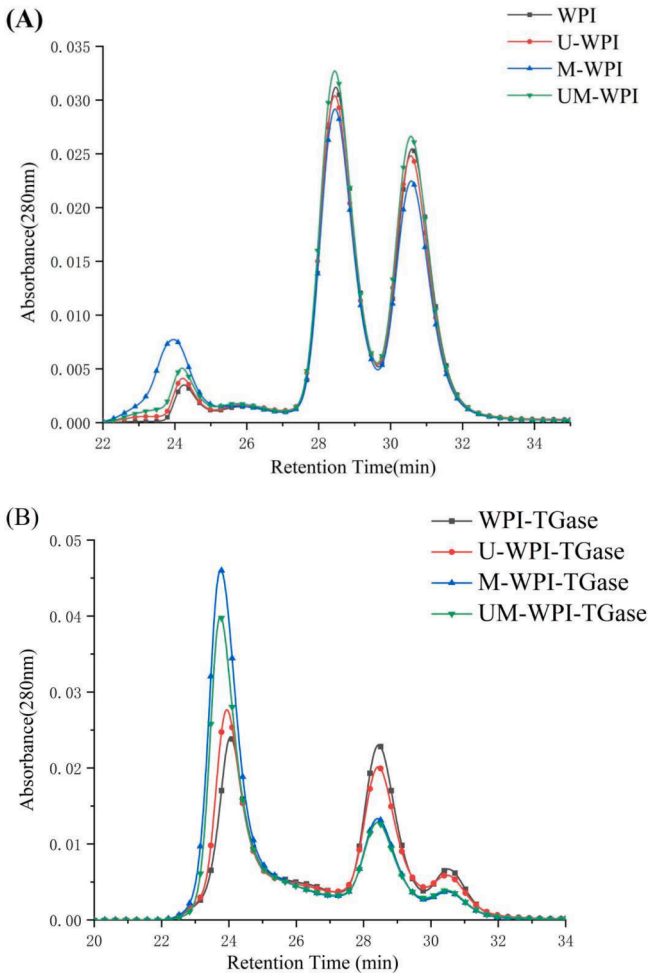

Fig. 1. Effect of physical pre-treatment on molecular weight distribution of TGase cross-linked WPI (A) and WPI (B).

and/or cause other specific effects that stabilized unfolded molecules when the UM were applied to WPI [29]. Therefore, compared with M−WPI, the UM-WPI structure became steady and lowly reactive which made the reaction of UM-WPI and TGase weak. And it was reported that free radicals produced by microwave heating improved irreversible and chemical cross-linking of neighbouring protein aggregates [29]. Additionally, due to the thermal effects of ultrasound, microwave output power in UM-WPI-TGase was prone to be lower than that of M−WPI−TGase in order to keep the same temperature, which further affected the crosslinking reaction of WPI and TGase.

3.2. Intrinsic fluorescence spectrum

The intrinsic fluorescence of protein is mainly produced by tryptophan residues and tyrosine residues. At present, intrinsic fluorescence is widely used in the determination of protein structure and function [31].

As shown in Fig. 2A, the intrinsic fluorescence intensities of three samples were increased at the sequence of M−WPI, UM-WPI and U-WPI. From Fig. 2B, the fluorescence intensity of UM-WPI-TGase was stronger than that of U-WPI-TGase, but weaker than that of M−WPI−TGase. This meant that there were the most tryptophan residues and hydrophobic groups exposed in M−WPI−TGase. It might be explained that microwave heating was transmitted through polar molecular vibration transmission, resulting in an alignment effect of molecular motion, and the effective collision frequency of polar molecules increased [21]. This would potentially unfold the protein structure, exposing more tryptophan residues. Additionally, the acceleration in temperature and the

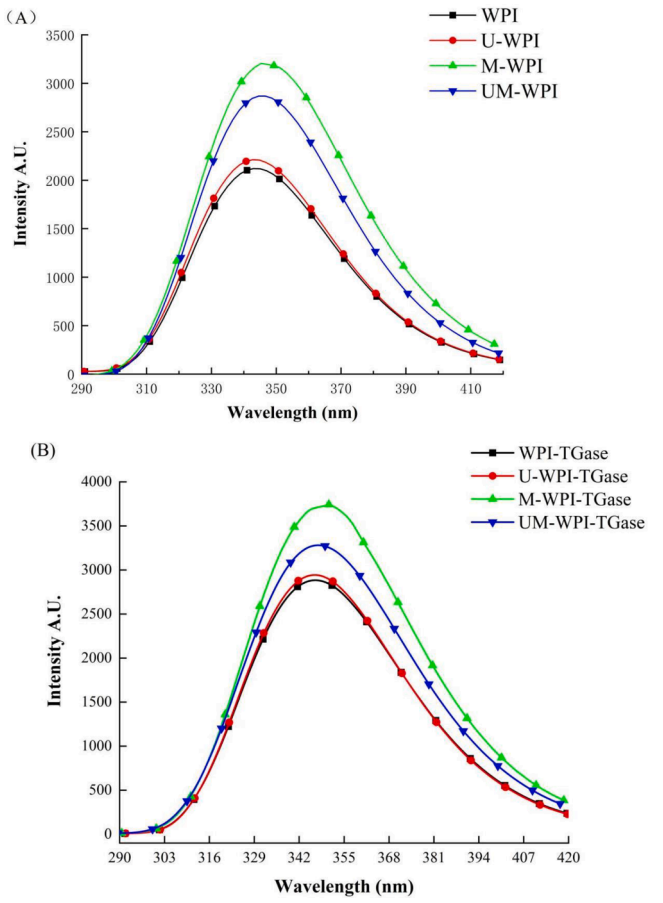

Fig. 2. Effect of physical pre-treatment on intrinsic fluorescence of TGase cross-linked WPI (A) and WPI (B).

slight increase in free radicals caused the partial denaturation of protein, leading to exposure of buried hydrophobic groups and tryptophan residues. However, when ultrasound and microwave were synergized, ultrasound pretreatment could stabilize unfolded protein molecules, which weakened the role of microwave pretreatment.

Furthermore, after the crosslinking of microwave-heated WPI with TGase, its λmax underwent a red shift from 346.6 nm to 350.6 nm, which indicated that WPI structure had been changed after microwave heating and TGase treatment.

3.3. Determination of emulsifying properties

Fig. 3A shows that emulsifying activity (EAI) of M−WPI was significantly increased compared with that of untreated WPI ($P < 0.05$), while EAI of UM-WPI and U-WPI was not changed. This might be that WPI after microwave heating pretreatments exposed a small number of hydrophobic groups, making the WPI easier to adsorb on the surface of oil droplets. Furthermore, EAI of TGase-induced WPI decreased significantly compared with the EAI of WPI ($P < 0.05$) and the order of EAI was U-WPI-TGase > UM-WPI-TGase > M−WPI−TGase. Compared with WPI-TGase, emulsifying activity of U-WPI-TGase, UM-WPI-TGase and M−WPI−TGase was reduced by 14.35%, 29.24%, and by 39.67%, respectively. Potentially, formation of TGase-induced WPI aggregates and its unfolded protein structure, slowed down the adsorption capacity of protein and promoted its aggregation in the interface area [32]. It had also found that large particle protein did not keep flexibility when absorbed on the surface of oil droplets, thus leading to its lower EAI [33].

As it is shown in Fig. 3B, compared with WPI, emulsion stability (ESI) of U-WPI, M−WPI, UM-WPI increased slightly ($P < 0.05$). Moreover,

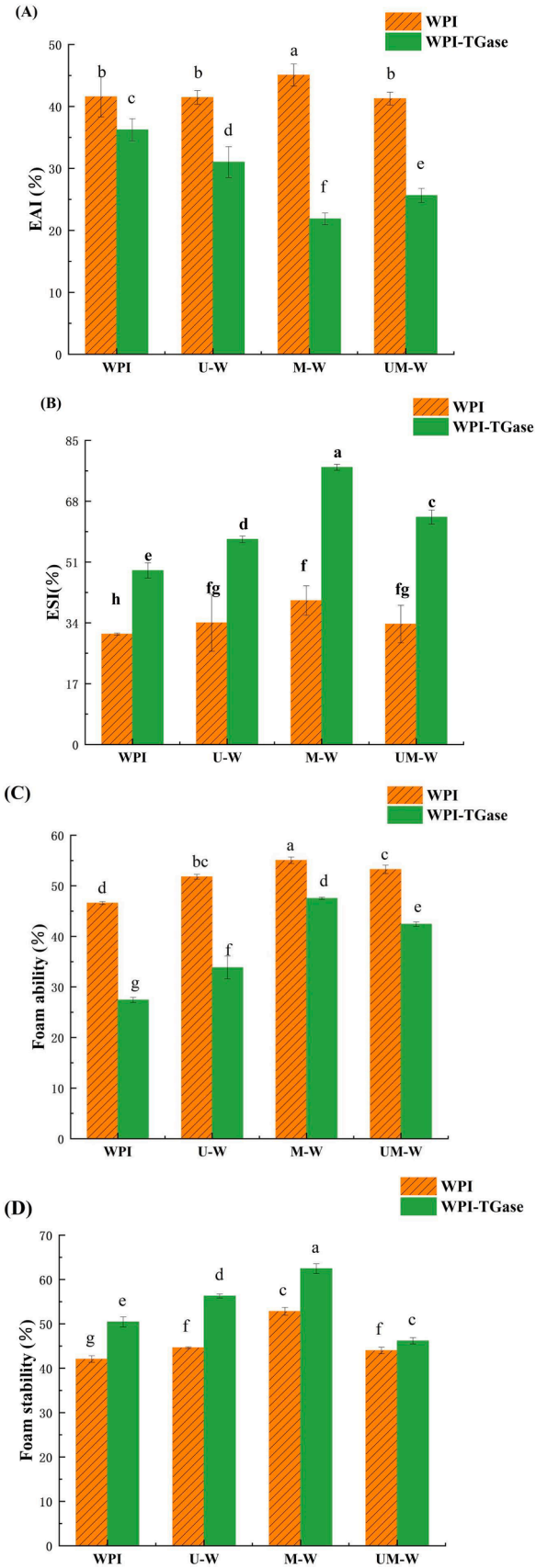

Fig. 3. Effects of physical pre-treatments on EAI (A) and ESI (B) of TGase cross-linked WPI and WPI. And Effects of physical pre-treatments on foaming ability (C) and foam stability (D) of TGase cross-linked WPI and WPI.

TGase-treated WPI exposed to three physical pretreatments, had higher emulsion stability than the corresponding nontreated-TGase WPI ($P < 0.05$). This illustrated that three physical pretreatments combined with TGase could significantly improve the ESI of WPI. After WPI crosslinked with TGase, the produced stable macromolecular polymers, could form a stronger rigid interface film [14] and protein viscosity increased [34], which reduced the oil diffusion of emulsion, leading to its ESI increase. Additionally, the reason was also that the increase in the molecular weight of polypeptide chains might lead to poor flexibility and reduce the protein ability to unfold at the oil–water interface [35], thereby developing the emulsion stability of protein. Meanwhile, compared with U-WPI-TGase and UM-WPI-TGase, M−WPI−TGase had the highest ESI due to its largest aggregates (shown in Fig. 1B).

3.4. Determination of foaming characteristics

Effects of three physical methods on foaming ability (FA) and foaming stability (FS) of TGase-treated WPI and WPI are exhibited in Fig. 3C and 3D, respectively.

As shown in Fig. 3C, compared with WPI, FA of M−WPI, UM-WPI and U-WPI was improved remarkably and M−WPI had the highest FA ($P < 0.05$). Generally, the physical treatment made the protein structure unfold and exposed its hydrophobic group, which induced the protein molecules distributed more evenly on the gas–liquid interface [36].

More importantly, after three physical pretreatment WPI reacted with TGase, its FA decreased ($P < 0.05$). TGase-catalysis brought about expanding polymers and viscosities of WPI, which prevented WPI particles from being absorbed by the air–water interface, thus affecting the FA of proteins [18]. M−WPI−TGase had better FA than UM-WPI-TGase and U-WPI-TGase ($P < 0.05$). Possible explanation was that the thermal effect and field effect of M could promote the deployment and adsorption of protein at gas–water interface readily [37].

It can be seen from Fig. 3D that FS of WPI increased after three physical pretreatments and M−WPI had the highest FS. Potential interpretation was that the maximum electrostatic repulsion of protein after microwave could be conducive to delay foam collapse [36]. Compared with WPI, the FS of WPI-TGase enhanced enormously ($P < 0.05$). Meanwhile, FS was declined according to the order of M−WPI−TGase, UM-WPI-TGase, U-WPI-TGase and WPI-TGase, respectively. It might be due to that during the reaction protein with TGase, lysine and glutamine residues bound covalently to form *iso*-peptide bonds [37], and high molecular weight polymers were generated simultaneously, which stably adsorbed to the gas–water interface. It was reported that molecular weight of the protein was proportional to its FS [38]. Wang et al. [14] also found that foam stability of WPI increased after the macromolecular protein aggregates were produced. Size exclusion chromatography (shown in Fig. 1B) proved M−WPI−TGase had the maximum aggregate among all the TGase-treated samples, thus showing it exhibited stronger FS.

3.5. Determination of free amino groups

Effects of three physical pretreatments on the free amino group of WPI samples are shown in the Fig. 4A. Free amino groups of WPI treated by three physical treatments were increased ($P < 0.05$). This illuminated that all three physical pretreatments could alter the protein structure to expose its free amino groups. The number of free amino groups decreased significantly in WPI-TGase ($P < 0.05$) in Fig. 4A. It indicated that the reaction between TGase and WPI caused free lysine ε-amino groups of proteins to be reduced [39]. It also demonstrated that quantity of free amino groups in rice protein decreased after treatment of different concentrations of TGase [40]. The reaction between an e-amino group on protein bound lysine residues and a γ-carboxyamide group on protein bound glutamine residues led to covalent cross-linking of the proteins by TGase catalyzing [41]. Furthermore, M−WPI−TGase showed the largest consumption of free amino groups among all the

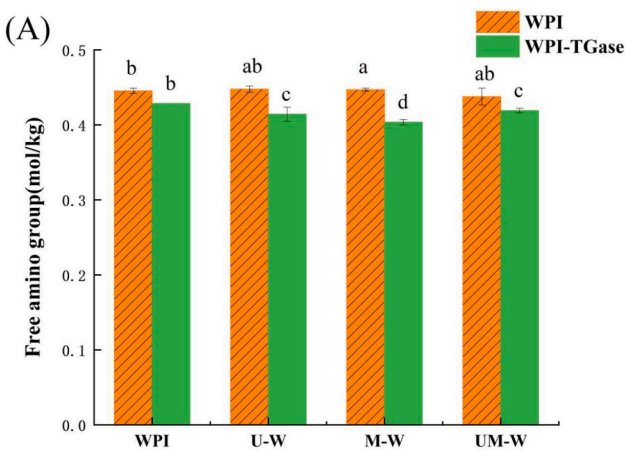

Fig. 4a. Effects of physical pre-treatments on free amino groups of TG cross-linked WPI and WPI.

samples. This indicated that there was the maximum reaction degree of cross-linking in M−WP−TGase. It also illustrated that M−WP−TGase had the maximum larger molecules, which was consistent with the result of SEC as shown in Fig. 1B.

3.6. Zeta potential analysis

Results of effects of three physical pretreatments on Zeta potential of WPI and WPI-TGase are shown in Fig. 4B. All Zeta potential absolute values of WPI pretreated by three physical methods were significantly bigger than those of natural WPI ($P < 0.05$). This illustrated that extension of the protein structure by physical pretreatments, exposed the internal amino acid residues with negative charge, resulting in the increase of its absolute value of Zeta potential. Furthermore, compared with U-WPI and UM-WPI, M−WPI had the highest absolute value of Zeta potential. This phenomenon might associate with the stretching of spherical protein molecules and the exposure of charged groups caused by the thermal effect and field effect of M [42].

Zeta absolute potential values of M−WPI−TGase, UM-WPI-TGase and U-WPI-TGase were all notably upper than those of TGase-untreated WPI ($P < 0.05$). Probably, during crosslinking reaction of WPI and TGase, the acyl donor (glutamine) and the acyl acceptor (lysine) led to the loss of amino groups, which made the numbers of its positive charges reduced [43]. It was discovered that mixed micelles of lactoferrin and casein that reacted with TGase had higher Zeta potential

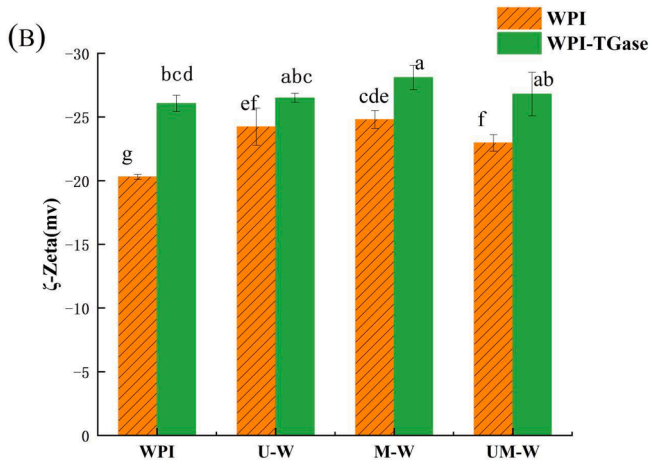

Fig. 4b. Effects of physical pre-treatments on ζ-Zeta of TGase cross-linked WPI and WPI.

absolute value than mixed micelles without TGase [44]. Additionally, the order of Zeta potential absolute value was M−WPI−TGase > UM-WPI-TGase > U-WPI-TGase. Furthermore, it is reported that Zeta potential value of the protein was one of the significant factors affecting protein foaming properties [36]. Generally, protein with the higher absolute Zeta potential value, had better foam performance [36]. This was consistent with the results of the foaming characteristics (shown Fig. 3C and D).

3.7. FTIR spectroscopy

Fig. 6A and B shows that the FTIR spectra of samples the variation in secondary structure in amide I region (1600 cm^{-1}-1700 cm^{-1}). UM-WPI-TGase amide I region is shown in Fig. 6C, which contains seven components: 1686.2, 1674, 1662.2, 1650.8, 1639.8, 1628.7, 1617.3 cm^{-1}. The correspondence between the secondary structure and the absorption peak is shown as follows: a-helix is 1650–1660 cm^{-1}; β-turn is 1660–1700 cm^{-1} 1690–1700 cm^{-1}; β-sheet is 1610–1640 cm^{-1}, 1670–1690 cm^{-1}; Random coil is 1640–1650 cm^{-1}. Table.1 shows blending character of the secondary structure by curvefitting in the amide I region. Usually, α-helix is derived primarily by intramolecular hydrogen bonds between amino hydrogen (NH–) and carbonyl oxygen

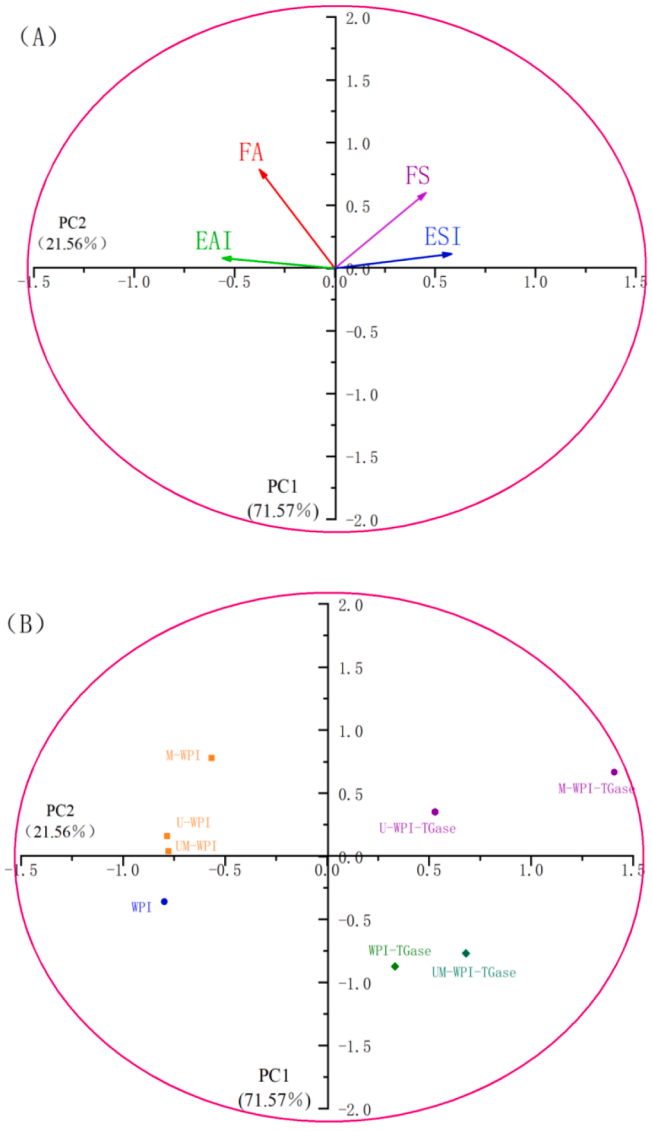

Fig. 5. The load diagram of the functional properties of WPI samples on the principle component (A) and the samples score (B).

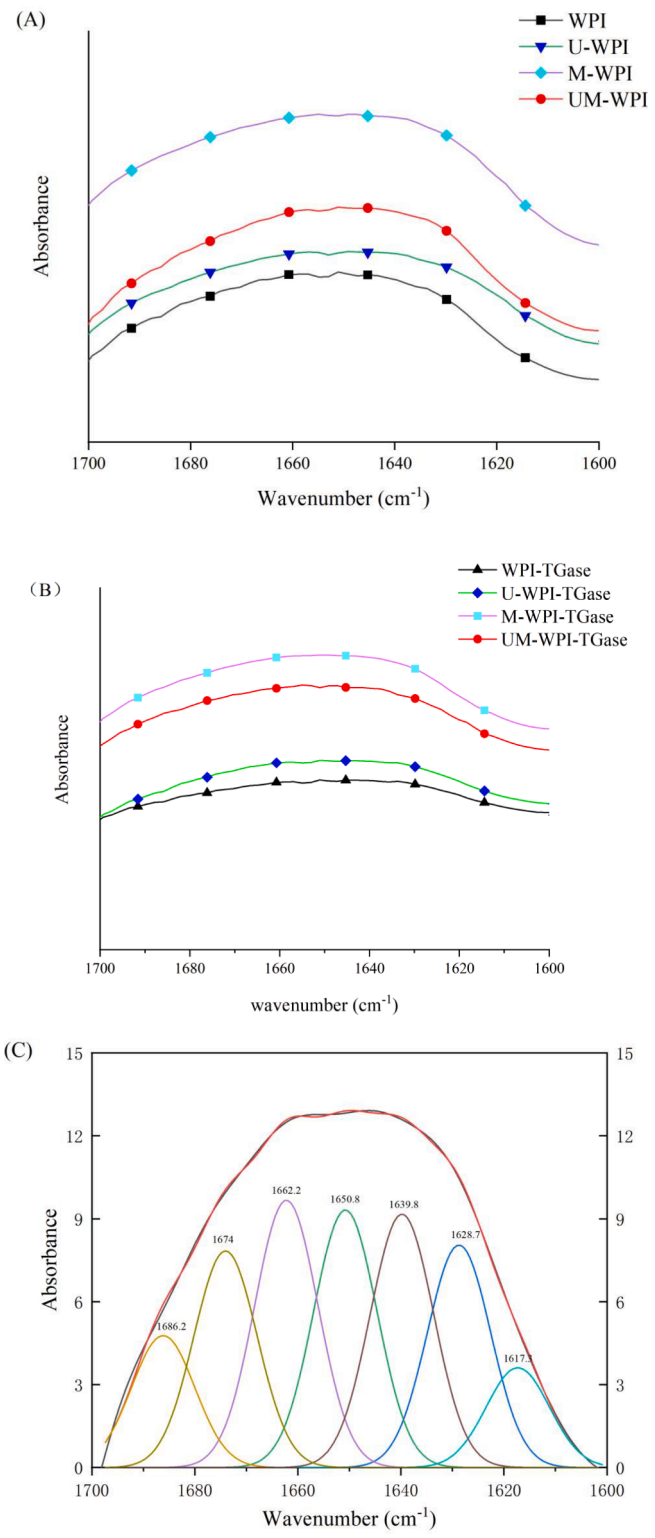

Fig. 6. Effect of three different physical pre-treatments on FTIR of WPI (A) and WPI-TGase (B) in amide I region (1600 cm^{-1}-1700 cm^{-1}). Panel C shows deconvoluted FTIR spectra of UM-WPI-TGase in the amide I region.

(–CO) groups, while β-sheet is steadied through interchain hydrogen bonds between polypeptide chains, β-turn results from faintly hydrogen bonded structures, and random coil is due to unfolded configuration and is connected to protein flexibility [46]. From Table.1, after U, M and UM pretreatment, the α-helix content of WPI decreased by 2.80%, 13.19% and 7.37%, and the β-sheet content increased by 2.78%, 15.37% and

Table 1
Secondary structure analysis of all the samples in amide I region.

Sample	Secondary structure composition (%)			
	α-helix	β-turn	β-sheet	random coil
WPI	18.19 ± 0.05[a]	33.27 ± 0.05[a]	25.64 ± 0.19[e]	22.90 ± 0.03[f]
U-WPI	17.68 ± 0.17[a]	33.09 ± 0.63[a]	25.98 ± 0.17[e]	23.36 ± 0.11[e]
M−WPI	15.79 ± 0.04[c]	32.33 ± 0.03[b]	26.97 ± 0.54[d]	24.85 ± 0.07[d]
UM-WPI	16.85 ± 0.01[b]	32.71 ± 0.14[b]	26.24 ± 0.10[de]	24.40 ± 0.13[d]
WPI-TGase	13.49 ± 1.02[b]	31.09 ± 0.01[b]	26.22 ± 0.20[de]	25.31 ± 0.16[c]
U-WPI-TGase	16.38 ± 0.18[c]	31.87 ± 0.24[c]	26.95 ± 0.2[d]	25.86 ± 0.31[bc]
M−WPI−TGase	12.43 ± 0.27[f]	30.18 ± 0.11[d]	30.25 ± 0.51[a]	27.14 ± 0.20[a]
UM-WPI-TGase	14.18 ± 0.16[d]	31.03 ± 0.09 [cd]	28.62 ± 0.27[c]	26.17 ± 0.44[b]

Values represent the means ± standard error (n = 3); different superscript letters in the same column show a signifificant difference ($P < 0.05$).

9.15%, respectively, implying that originally hydrogen-bonded structures were partly demolished. The augmentation of β-sheet and the decrease of α-helix might be due to the exposure of hydrophobic regions and the destruction of hydrogen bonds [45] during three physical pretreatments. After TGase cross-linking, the α-helix content decreased and the irregular coil content increased for all samples. Furthermore, α-helix content of M−WPI−TGase decreased by 31.67%, and irregular coil content increased by 18.51%. The reaction of TGase catalyzed acyl group destroyed the stability of hydrogen bonds in the arrangement of α-helix structures [14]. The expansion of polypeptide chains exposed hydrogen bonds and hydrophobic groups, and the internal aggregation of molecules formed irregular curl [47], which enhanced the disorder of proteins.

3.8. The principal component analysis (PCA)

The changes in WPI functional characteristics were analyzed and classified according to EAI and ESI, FA, and FS, which were treated by different physical treatments.

PCA results of different samples are displayed in Fig. 5A. The total proportion of principal components 1(PC1) and principal component 2 (PC2) was 93.13%. Amongst them, the variance contribution rate of PC1 was 71.57%, which was positively related to FA, FS, EAI, ESI. PC2 accounted for 21.56%, which was positively related to FS and ESI and negatively related to EAI and FA. And it was also shown that FA and FS occupied a larger percentage in PC1, whereas EAI and ESI occupied a larger percentage in PC2.

As shown in Fig. 5B, three groups of samples (M−WPI, U-WPI, UM-WPI) that had undergone different physical pretreatments were in the second quadrant, and the samples that had not undergone physical pretreatment (WPI) were in the third quadrant. It showed that the three physical treatments had an influence on the functional characteristics of WPI, and M had the most excellent effect on the functional characteristics of WPI. This was in accordance with the scoring results in Table.2. The M−WPI score was the highest among the first four groups (WPI, U-WPI, M−WPI, UM-WPI) scores. Similarly, U-WPI-TGase and M−WPI−TGase were located in the first quadrant, and UM-WPI-TGase and WPI-TGase were located in the fourth quadrant. Among the scores in Table.2, M−WPI−TGase had the highest score, and there was little difference between U-WPI-TGase and UM-WPI-TGase. Comprehensive analysis showed that microwave heating and TGase cross-linking had the greatest impact on functionality of WPI. However, there might be a few possible reasons why individual microwave pretreatment was better than UM. Firstly, ultrasound improved the mass transfer between

Table 2
Scores of WPI after three different processing.

Sample	PC1	PC2	Total Score
WPI	−1.60117	−0.53897	−1.262159301
U-WPI	−1.57496	0.24144	−1.075144408
M−WPI	−1.13757	1.16739	−0.562469565
UM-WPI	−1.56078	0.06226	−1.10362699
WPI-TGase	0.65933	−1.30815	0.189845341
U-WPI-TGase	1.05244	0.52892	0.86726646
M−WPI−TGase	2.80884	1.0002	2.225929908
UM-WPI-TGase	1.35386	−1.1531	0.720349242

phases, mixing and permitting an enhanced homogeneity in the distribution of temperature. And when ultrasound and microwave synergized, the role of the ultrasonic field would stabilize the protein unfolded molecules. Therefore, protein molecules were in the dynamic balance of unfolding and aggregation [30], which caused WPI to crosslink with TGase uncomfortably. In addition, compared with single M pretreatment, the addition of ultrasonic fields attenuated the effects of microwave thermal effects during ultrasound synergized with microwave pretreatment. Besides, it was reported that effectiveness of ultrasound could be lessen at higher than 60 °C [48].

4. Conclusion

Ultrasound synergized with microwave, ultrasonication, and microwave heating pretreatment were compared for the first time to investigate its influence on structure and functional properties of TGase-induced WPI. Three physical pretreatments could remarkably heighten the crosslinking degree of WPI and TGase, and improve WPI functional properties. Moreover, compared to UM-WPI-TGase and U-WPI-TGase, M−WPI−TGase had the maximum molecular size polymers and optimal FS, FA and ESI. This illustrated that M−WPI−TGase had potential to be used in food processing as emulsifiers and foam stabilizers. Combination of microwave heating and TGase was effective to enhance improve functional characteristics of WPI, which will be being potential application in food industry.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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