Effect of ultrasound pretreatment on structural, physicochemical, rheological and gelation properties of transglutaminase cross-linked whey protein soluble aggregates

Tiehua Zhang, Yanli Zhao, Xiner Tian, Jing Liu, Haiqing Ye*, Xue Shen*

Department of Food Science, College of Food Science and Engineering, Jilin University, Changchun 130062, China

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ABSTRACT

A solution (10%, w/v) of whey protein soluble aggregates (WPISA) was pretreated with high-intensity ultrasound (HUS, 20 kHz) for different durations (10–40 min) before incubation with transglutaminase (TGase) to investigate the effect of HUS on the structural, physicochemical, rheological, and gelation properties of TGase cross-linked WPISA. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) results showed that HUS increased the amounts of high-molecular-weight polymers/aggregates in WPISA after incubation with TGase. HUS significantly increased \( P < 0.05 \) the degree of TGase-mediated cross-linking in WPISA, as demonstrated by a reduction in free amino group contents. HUS significantly increased \( P < 0.05 \) the particle size, intrinsic fluorescence intensity, and surface hydrophobicity of TGase cross-linked WPISA, but had no significant impact \( P > 0.05 \) on the zeta-potential or total free sulphydryl group content of TGase cross-linked WPISA. The apparent viscosity and the consistency index of TGase cross-linked WPISA were significantly increased by HUS \( P < 0.05 \), which indicated that HUS facilitated the formation of more high-molecular-weight polymers. HUS significantly increased \( P < 0.05 \) the water holding capacity and gel strength of glucono-δ-lactone (GDL)-induced TGase cross-linked WPISA gels. The results indicated that HUS could be an efficient tool for modifying WPISA to improve its degree of TGase-mediated cross-linking, which would lead to improved rheological and gelation properties.

1. Introduction

Whey protein, which is a mixture of proteins including β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA), lactoferrin (LF), immunoglobulins (Igs), lactoperoxidase (LP), and glycomacropeptide (GMP), is a co-product of cheese making. Whey protein has become a common ingredient in many food formulations due to its high nutritional value and versatile functional properties, such as foaming, emulsification, gelation, and flavour binding properties. These available properties can be tailored or enhanced by modification for a particular purpose. Several strategies have been developed to improve its physicochemical and functional properties, including heat-induced polymerization [1,2], chemical modifications [3], enzymatic treatments [4,5], and new nonthermal processing technologies (e.g., high pressure, pulsed electric field, gamma irradiation, and ultrasound) [6]. In particular, enzymatic modification is an effective and green approach because of its high efficiency, specificity, and mild reaction conditions. The functional performance of whey protein must be continuously explored, improved and designed for specific uses to remain competitive in the food ingredient market [7]. Furthermore, whey protein modifications offer new opportunities to expand the use of these proteins in food and even non-food applications.

Transglutaminase (TGase, EC 2.3.2.13) can catalyse the acyl transfer reaction between the γ-carboxamide group of a peptide or protein-bound glutaminyl residue (acyl donor) and various primary amines (acyl acceptor) to form intra- and/or intermolecular cross-links in protein molecules [8]. When the ε-amino group of protein-bound lysyl bond acts as an acyl acceptor, the covalent cross-links are formed through ε-(γ-glutamyl) lysine isopeptide bonds. TGase has been reported to cross-link a number of food proteins to improve their gel properties and heat stability [8,9], including soy protein, wheat gluten, and whey protein. However, native β-lactoglobulin and α-lactalbumin, the main components of whey proteins, are compact globular proteins [4], and are therefore less susceptible to cross-linking reactions with TGase.

* Corresponding authors at: Jilin University, NO. 5333 Xian Road, Changchun, Jilin province, China.
E-mail addresses: yehq@jlu.edu.cn (H. Ye), shenxue417@163.com (X. Shen).

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Therefore, exposure of the enzyme-targeted sites can facilitate the cross-linking reactions [10]. Some trials have been undertaken in attempts to improve the degree of cross-linking, involving approaches such as heat treatment [4], superfine grinding treatment [11], the addition of a reducing agents [4], and ultrasound treatment [12].

Among these methods, high intensity ultrasound (HUS, 10–1000 W cm$^{-2}$, with a frequency of 20–100 kHz) has been extensively used to modify the food proteins in the food industry [13]. HUS may exert its effects on the food proteins through acoustic cavitation, which refers to the formation, growth, and collapse of cavitation bubbles in liquids, leading to changes in the surrounding pressure and temperature due to the highly localized energy that is released within the bubbles, along with the emission of light (sonoluminescence), shockwaves, the turbulent motion of the liquid and radicals [14,15]. Due to cavitation effects, HUS can disrupt inter- or intramolecular interactions among protein molecules, expose some target regions, and potentially promote enzymatic cross-linking reactions [16]. For example, Hu et al. [16] successfully increased the degree of enzymatic cross-linking TGase cross-linked SPI with high intensity ultrasound (20 kHz, 400 W). Ahmadi et al. [12] found that moderate ultrasound treatments (20 kHz, 500 W, 2.5 min) were favourable for modifying whey protein structures to make them more susceptible to TGase (2 U g$^{-1}$ pr), which resulted in improved functional properties. In addition to HUS treatment, heat treatments are also used to expose whey protein reaction sites to accelerate enzymatic cross-linking. Jiang et al. [17] combined HUS and heat treatments to expose whey protein reaction sites to accelerate enzymatic cross-linking. They found that ultrasound treatments (20 kHz, 41–45 W cm$^{-2}$, for 0, 20, 40, or 60 min) that were performed after thermal treatments increased the degree of TGase-catalysed cross-linking in WP and improved its foaming properties and emulsifying activity as a function of the ultrasonication time. However, its gelation properties are not well understood, and little is known about the effects of HUS on the physicochemical, rheological, and gelation properties of on TGase-crosslinked WPISA. We have found that HUS, when performed after thermal aggregation, can disrupt the associated noncovalent interactions and expose some active groups that were previously located in the interior of the whey protein soluble aggregates (WPISA), which suggested that the possibility of applying HUS to improve the degree of TGase-catalysed cross-linking in WPISA [18]. Moreover, we also showed that HUS of WPISA increased the gelation properties of cold-set gels induced by glucono-δ-lactone (GDL) [19]. The objective of this study was to investigate the effect of HUS (20 kHz, ~69 W cm$^{-2}$, 10–40 min) on the structural, physicochemical, rheological and gelation properties of transglutaminase cross-linked WPISA.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI), containing 93.14% of protein, 0.36% of fat, 4.79% of moisture, 1.6% of ash and 0.7% of lactose, was purchased from Fonterra (Auckland, New Zealand). Transglutaminase (200 U g$^{-1}$ enzyme activity) was purchased from Yuanye Biological Technology Co., LTD (Shanghai, China). Glucono-δ-lactone (GDL) was purchased from Hui Yang Biological Technology Co., (China). All other chemicals used in this study were reagent grade and purchased through Sigma (St. Louis, MO, USA). The water used in this study was filtered using a Millipore Milli-Q$^\text{TM}$ water purification system (Millipore Corp., Milford, MA, USA).

2.2. Preparation of whey protein soluble aggregates

WPI solutions (10%, w/v; protein: 9.31% w/v) were dissolved in deionized Milli-Q water and stirred (1500 rpm) for 2 h at room temperature. Solutions were kept at 4 °C overnight, equilibrated at room temperature for 2 h, preheated at 80 °C for 15 min (pH 7.0) while stirring in a water bath, and cooled to room temperature as described previously [20].

2.3. Ultrasound treatment

Ultrasound treatment was performed using an ultrasonic processor (VCX800, Vibra cell, Sonics, USA) as we previously described [18]. Briefly, WPISA samples were treated with an ultrasound probe (20 kHz) for 10, 20, and 40 min (10 s: 5 s work/rest cycles) at 30% amplitude. The corresponding ultrasound intensity was ~69 W cm$^{-2}$. Samples were immersed in an ice-water bath to remove the heat generated by ultrasound treatment.

2.4. Enzymatic cross-linking of WPISA

The samples, pretreated with HUS, were adjusted to pH 7.5 and then cross-linked by TGase (10 U g$^{-1}$ protein) at 50 °C for 4 h while stirring. TGase was inactivated by heating at 75 °C for 15 min before adjustment to pH 7.0. Then, sample solutions were cooled to room temperature in the ice water.

2.5. SDS-PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions using a Mini Protein Tetra Cell (Bio-Rad Laboratories, Inc., Hercules, CA). The samples were separated using a 12% separating gel at the voltage of 120 V, stained with Coomassie bright blue fast staining solution (Betymeon Biological Technology Co., LTD, China) for 2 h, and destained in deionized Milli-Q water. The prestained protein marker with a 10–180 kDa was applied to estimate MW of tested-protein. The gel was visualized using a Bio-Rad automatic gel imaging system.

2.6. Free amino groups

The free amino group content of samples was determined by the orthophthalaldehyde (OPA) method [21]. The protein samples were diluted to 1 mg/mL with deionized Milli-Q water. Diluted sample solutions (200 µL) were mixed with 4 mL of OPA reagent and incubated in the dark for 5 min at 25 °C. Absorbance was measured using a spectrophotometer (UV-2550, ShimadzuCorp., Tokyo, Japan) at 340 nm. The deionized water instead of the diluted protein sample was used as the reagent blank. The free amino group content of the sample was calculated from the calibration curve, using l-leucine (25–120 µg/mL) as standard.

2.7. Determination of particle size

Particle size was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS 90 (Malvern Instruments, UK). The samples were diluted 100-fold with deionized Milli-Q water. Diluted sample solutions (200 µL) were mixed with 4 mL of OPA reagent and incubated in the dark for 5 min at 25 °C. Absorbance was measured using a spectrophotometer (UV-2550, ShimadzuCorp., Kyoto, Japan) at 340 nm. The deionized water instead of the diluted protein sample was used as the reagent blank. The free amino group content of the sample was calculated from the calibration curve, using l-leucine (25–120 µg/mL) as standard.

2.8. Determination of zeta-potential

Zeta-potential (ζ, mV) of all samples were determined as described previously [18]. The samples were diluted (0.5%, v/v) with deionized Milli-Q water and analyzed at a scattering angle of 173°. ζ was calculated based on the Henry equation [22].

2.9. Determination of intrinsic fluorescence spectrum

The fluorescence intensities of samples were measured using a fluorescence spectrophotometer (RF-5301, Shimadzu Corp., Tokyo, Japan). The protein concentration was 0.2 mg/mL for fluorescence
samples (4 mL) were mixed with 20 μL of fluorescent probe as previously described [18]. The samples were surface hydrophobicity

2.10. Determination of the 1-anilino-8-naphthalenesulfonate (ANS) surface hydrophobicity

The surface hydrophobicity of samples was determined by ANS fluorescent probe as previously described [18]. The samples were diluted with 10 mM phosphate buffer (pH 7.2). The diluted protein samples (4 mL) were mixed with 20 μL of ANS solution (8 mM in 10 mM phosphate buffer, pH 7.2). The fluorescence intensity (excitation wavelength: 365 nm, emission spectrum in the range of 400–650 nm) was measured using an RF-5301 spectrofluorometer (Shimadzu Corp., Tokyo, Japan). The surface hydrophobicity index \(H_0\) of samples was calculated from the initial slope of the fluorescence intensity versus protein concentration plot of serial dilutions.

2.10.1. Determination of free sulfhydryl group (SH)

The free SH measurements were carried out by modifying the method of Segat et al. [23]. Briefly, each sample was diluted to 1% (w/v) with deionized water. The diluted samples (0.5 mL) were mixed with Tris-glycine buffer for measuring surface free SH content and the same buffer containing 8 M urea to determine the total free SH groups. Then, 20 μL of Ellman’s reagent was added to the buffer, incubated in the dark for 15 min at 25 °C, and measured at 412 nm using a UV–Vis spectrophotometer (UV-2550, Shimadzu, Tokyo, Japan). The free sulfhydryl content was calculated by the extinction coefficient of 13600 M\(^{-1}\) cm\(^{-1}\).

2.11. Determination of rheological properties

Rheological property analyses were measured using a rheometer (DHR-1, TA Instrument, USA) equipped with a steel parallel plate geometry (diameter = 40 mm, sample, thickness = 1 mm). Flow ramp analyses were performed at shear rate ranging from 0.1 s\(^{-1}\) to 1000 s\(^{-1}\) at 25 °C. Apparent (shear) viscosity was recorded as a function of shear rate.

2.12. Determination of gelation properties

The samples were mixed with GDL (1.0 g/100 mL) with gentle stirring for 2 min and maintained at 25 °C for 2 h for gel formation. Then, the gels were stored at 4 °C for 12 h until further analyses.

The water holding capacity (WHC) of gel samples were determined according to a method [24] with some modifications. A total of 5 mL protein samples were acidified in a 10 mL centrifuge tube as described above, centrifuged (Avanti J-E, Beckman, USA) at 8000g for 30 min at 4 °C. The water was drained out using a syringe, and the residual water was removed by dry filter paper carefully. WHC was calculated as the ratio of the weight of gel remaining in the centrifuge tube to the initial gel weight.

The gel strength analysis was performed on a Brookfield CT3 texture analyzer (Brookfield, USA), equipped with a TA10 probe (diameter: 12.7 mm). The compression speed was set at 0.5 mm/s with a 0.5 mm test distance and a 4.0 g payload. The gel solubility was measured as we previously described [19]. Briefly, gel samples were dissolved in Tris-glycine buffer (pH 8.0) with (B) or without (A) 8 M urea, incubated at 37 °C in a shaking water bath for 12 h, then subjected to centrifugation (20,000g for 15 min) at 25 °C. The protein concentration in the supernatant was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, China).

2.13. Statistical analyses

All experiments were performed in triplicates. Statistical analyses were carried out using IBM SPSS Statistics 23. A One-way ANOVA and post-hoc analyses were used to compare the data of different groups. Results were expressed as mean ± standard deviation (SD) and considered significantly different when \(P < 0.05\) at 95% level of confidence.

3. Results and discussion

3.1. SDS-PAGE analysis

SDS-PAGE was performed to investigate changes in molecular weight (MW) changes in WPISA after incubation with TGase. Fig. 1 shows the effect of ultrasound pretreatment on the MW changes of TGase cross-linked WPISA. Two main bands that corresponded to α-lactalbumin (α-la) and β-lactoglobulin (β-lg) monomers were observed over the lane of native WPI. There were no significant differences in the electrophoretic bands between WPI (Lane N) and WPISA (Lane C), which may be because SDS-PAGE was performed under reducing conditions, resulting in disruption of most physical and chemical bonds in WPISA to promote lager aggregate dissolution in the presence of 2-mercaptoethanol and/or dithiothreitol [25]. Disulphide exchange and bonding (sulphydryl–disulphide interchange reactions) play an essential role in forming whey protein aggregates upon heating [26]. Compared to WPISA (Lane C), the high-molecular-weight polymers (Lane 0 min) with a MWs ranging from 40 to 170 kDa were observed, but the corresponding β-lg and α-la contents decreased, which indicated that TGase cross-linked WPISA was formed through intramolecular and/or intermolecular cross-linking reactions via isopeptide bond formation. r- (γ-glutamyl) lysine isopeptide bonds have been reported to be insensitive to 2-mercaptoethanol on the basis of SDS-PAGE analysis [27]. The bands obtained under HUS treatment at 10 min (Lane 10 min), 20 min (Lane 20 min), and 40 min (Lane 40 min) were darker than those at 0 min (Lane 0 min) within the 40–170 kDa range but were lighter at the positions corresponding to β-lg and α-la, which indicated that HUS treatment may expose more reaction sites subjected to TGase, resulting in the formation of larger quantities of high-molecular-weight polymers. These results suggested that HUS increased the degree of enzymatic cross-linking. Our previous study [18,19] showed that HUS could disrupt the noncovalent interactions among WPISA molecules and reduce the particle size of WPISA, cause partial unfolding to expose some active/functional groups previously buried within the interior of protein molecules, change protein conformation and increase protein solubility, all of which may contribute to promoting the TGase-mediated cross-linking reaction of WPISA. Ahmadi et al. [12] used HUS as an alternative to heating
denaturation and increased the susceptibility of whey protein concentrates to TGase treatment. Jiang et al. [17] found that the largest molecular size of the proteins in the TGase-crosslinked WPI was observed after the ultrasound (41–45 W cm\(^{-2}\)) and thermal pretreatment (75 °C, 15 min). Qin et al. [28] reported that HUS pretreatment facilitated the TGase enzymatic reaction of wheat gluten, as was demonstrated by the formation of greater molecular weights of the aggregates. There were no significant changes in the electrophoresis bands among different HUS treatment times, which indicated that no additional high-molecular-weight polymers were formed, when HUS times were increased from 10 to 40 min. Furthermore, some high-molecular-weight polymer aggregates (HMWA in Fig. 1) were observed after cross-linking with TGase, and HUS treatment increased HMWA levels. A similar finding was reported by Hu et al. [16], who found that the HUS treatment of soy protein isolates markedly increased the amounts of high-molecular-weight polymer aggregates after incubation with TGase for 40 min.

### 3.2. Analysis of free amino groups analysis

Fig. 2 shows the effects of HUS pretreatment on the free amino groups of TGase cross-linked WPISA. The free amino group contents of WPISA decreased significantly (\(P < 0.05\)) after incubation with TGase for 4 h, which indicated that TGase-crosslinked WPISA formed, as was demonstrated by SDS-PAGE. TGase can catalyse the intra- and/or intermolecular cross-links among protein molecules by forming covalent isopeptide bonds between protein-bound glutaminyl and lysyl residues, and thus decrease the free amino group contents [29]. HUS significantly decreased (\(P < 0.05\)) the free amino group contents of WPISA after cross-linking by TGase. This result indicated that HUS treatment could substantially enhance the degree of TGase-catalysed cross-linking reactions. In a previous study [18], we found that HUS treatment could disrupt the noncovalent interactions between WPISA molecules and expose more active regions to the TGase reaction that were previously buried within the interior of the WPISA, thereby resulting in a reduction of the free amino group content of proteins. However, the contents of free amino groups of TGase-crosslinked WPISA did not change significantly (\(P > 0.05\)) when the HUS time was increased from 10 to 40 min. This may be because HUS treatment for 10 min exposed most TGase reaction sites of WPISA from the interior of the protein to its surface, which resulted in no significant change in free amino groups when the HUS time was increased to 40 min.

### 3.3. Particle size and zeta-potential analysis

The TGase cross-linked WPISA formed when WPISA was incubated with TGase at 50 °C for 4 h. The effects of HUS treatment (e.g., 0, 10, 20 and 40 min) on the particle size distributions of TGase cross-linked WPISA are shown in Fig. 3A. Z-average analysis shows the effects of HUS treatment on the average particle size of WPISA after incubation.
with TGase (Fig. 3B). All the samples exhibited a nearly unimodal size distribution with Z-average sizes between 10 and 100 nm (Fig. 3A). A small peak also appeared near 1000 nm, which was possibly due to the formation of larger protein aggregates after the cross-linking reaction. These results agree with the gel-electrophoresis profiles (some high-molecular-weight polymer aggregates in Fig. 1). Compared to the WPISA, the particle size distribution of TGase cross-linked WPISA became broader, which indicated that the cross-linking reaction occurred between the WPISA and TGase. Some intermolecular covalent TGase cross-linked WPISA was formed, as demonstrated by a significant increase in the D_z of WPISA from 51.3 ± 1.31 nm to 66.2 ± 0.67 nm after incubation with TGase (P < 0.05). The TGase can catalyse cross-linking reactions among protein molecules including intra- and intermolecular cross-links by forming ε-(γ-glutamine) lysine isopeptide bonds [4], thus producing more polymers and aggregates. It has been reported that the TGase-induced covalent cross-linking has no effect on the particle size of caseins when the reaction was mainly in intramicellar (intramolecular) [30,31]. After HUS treatment, the particle size distribution of TGase cross-linked WPISA generally shifted to the right, which demonstrated that the particle size of TGase cross-linked WPISA apparently increased with HUS treatment. There was a significant increase in the D_z of TGase cross-linked WPISA after HUS treatment (P < 0.05). This was likely attributed to ultrasonic cavitations resulting in a high shear force that disrupted the associated noncovalent interactions [18] and contributed to the exposure of some of the reactive lysine and glutamyl residues [10] that were previously buried within the interior of the WPISA. HUS did not significantly impact the D_z of TGase cross-linked WPISA as treatment time increased from 10 to 40 min (P > 0.05), which was in agreement with the SDS-PAGE results.

The effects of HUS on the zeta-potential of TGase cross-linked WPISA are shown in Fig. 3C. The absolute zeta-potential values of all samples were higher than 20 mV, indicating colloidal stability [32,33]. WPISA showed a negative zeta-potential value of approximately −41.9 mV at neutral pH, which was lower than that of WPISA (~27.9 mV) determined in our previous study [18], possibly due to the salt concentrations associated with two pH adjustments and heating protocols. It has been suggested that several factors can affect the zeta-potential of proteins, including salt concentrations, pH, and temperature [34]. The TGase-mediated cross-linking reaction had no significant impact (P > 0.05) on the zeta-potential of WPISA. Furthermore, HUS did not cause significant (P > 0.05) changes in the zeta-potential values of TGase cross-linked WPISA.

### 3.4. Conformational changes analysis

Fluorescence spectroscopy of whey protein samples was performed by selectively exciting the tryptophan residues to evaluate the changes in tertiary structures [35]. TGase-mediated cross-linking increased the fluorescence intensity of WPISA, which indicated that TGase-mediated cross-linking was beneficial for the exposure of some fluorescent amino acid residues, mainly the tryptophan residues [17]. HUS treatment increased the fluorescence intensity of TGase cross-linked WPISA (Fig. 4A). It is likely that HUS exposed more active regions that were sensitive to TGase and increased the degree of cross-linking, which
resulted in the exposure of more tryptophan residues of WPISA. There were no significant increases in fluorescence intensities of TGase-crosslinked WPISA as HUS times increased from 10 to 40 min. However, Jiang et al. [17] reported that the fluorescence intensity of HU-WPI-TGase increased as the ultrasound time increased from 0 to 60 min. These contradictions regarding fluorescence intensity may be due to the HUS intensity and protein pretreatment conditions. A redshift of the emission maximum (from 332 to 334 nm) was observed after TGase-mediated cross-linking (Fig. 4B), which indicated that the fluorescent amino acids (mainly Trp residues) were exposed to the solvent and the conformational and/or dynamic changes in proteins occurred at the tertiary structural level [36].

Fig. 4C shows that the concentrations of the surface free SH in WPISA decreased significantly ($P < 0.05$) from 8.82 to 6.73 μmol SH g$^{-1}$ protein after incubation with TGase. There were no significant changes in the total free SH contents of WPISA after cross-linking with TGase ($P > 0.05$). The TGase-mediated cross-linking reaction may cause conformational changes in WPISA and the formation of some high-molecular-weight polymers (seen from Fig. 1) by WPISA intermolecular ε-(γ-glutamyl) lysine cross-links and thus result in some surface free SH groups being buried into the interior of WPISA molecules that were no longer measurable as surface free SH groups [16]. HUS treatment ($\geq$10 min) significantly decreased the content of the surface free SH ($P < 0.05$) but had no significant effect on the total free SH contents ($P > 0.05$). There were no significant changes ($P > 0.05$) in the surface free SH contents with HUS times increasing from 10 to 40 min. This may be because the increase in TGase-mediated cross-linking degree in WPISA was responsible for the reduction of the surface free SH in WPISA. According to our previous findings [18], HUS could disrupt associated noncovalent interactions and expose buried sulfhydryl groups of WPISA, resulting in increase in surface free SH. In this study, HUS treatment had a significant ($P < 0.05$) impact on the surface free SH content via the cavitation effect on promoting the cross-linking reaction instead of acting directly on cross-linked WPISA. These results indicated that the TGase-mediated cross-linking reaction contributed to burying some surface free SH in the interior of WPISA molecules, while neither HUS treatment nor TGase-mediated cross-linking had a significant impact ($P > 0.05$) on the total free SH content.

Surface hydrophobicity, which is an index of the content of hydrophobic groups on the surface of protein molecules, can reflect the protein conformation changes [37]. The surface hydrophobicity of WPISA increased significantly ($P < 0.05$) after cross-linking with TGase for 4 h (Fig. 4C), which indicated that TGase-mediated cross-linking reactions may be beneficial for exposing the hydrophobic amino acid residues of WPISA. HUS significantly increased ($P < 0.05$) the $H_b$ values of TGase cross-linked WPISA, which may be due to its improvement of the TGase-mediated cross-linking reaction. There was no significant increase ($P > 0.05$) in $H_b$ for TGase treated WPISA with increasing ultrasound times from 10 to 40 min. HUS can produce high-intensity shock waves, shear forces and turbulence, which expose more active sites of WPISA and contribute to the TGase-mediated cross-linking reaction, which would thus increase the degree of cross-linking and expose some hydrophobic amino acid residues of WPISA.

![Fig. 5](image-url) Effects of HUS pretreatment (0, 10, 20 or 40 min) on rheological properties of TGase-treated WPISA. A, apparent viscosity; B, infinite-shear-rate viscosity; C, shear stress; D, consistency index and flow behaviour index. a–c and A–C, means in the graph indicated by different letters are significantly different ($P < 0.05$), according to the least significant difference (LSD) multiple range.
3.5. Analysis of rheological properties

Trios software was used to analyse the flow properties (e.g., viscosity vs. rate and stress vs. rate) of all the samples to determine the best-fitting flow model. Two initial pick points were removed during model fitting to reduce the operational errors in the sample loading and scraping. In viscosity vs. rate mode, the Cross model, Carreau model, and Sisko model exhibited higher fitting abilities than the Carreau-Yasuda and Williamson models, as indicated by their higher $R^2$ values (>0.99) (data not shown). The apparent viscosities of all samples are shown in Fig. 5A. The viscosities of all samples decreased rapidly in the low shear rate region and decreased gradually in the high shear rate region until reaching an infinite-shear-rate viscosity ($\eta_\infty$) (Fig. 5B). $\eta_\infty$ is suitable for indicating the sample characteristics because it is less susceptible to weak interactions during sample measurement and preparation [38]. All samples exhibited typical pseudoplastic and non-Newtonian behaviours with the shear rate ranging from 1 to 1000 s$^{-1}$. It has also been reported that whey protein solutions exhibit a shear-thinning flow behaviour at concentrations ranging from 10 to 24% [39]. TGase cross-linked WPISA showed a higher apparent viscosity than WPISA. HUS treatment (e.g., 10, 20 and 40 min) significantly increased ($P < 0.05$) the apparent viscosity of TGase cross-linked WPISA. This result may be attributed to the fact that the formation of high-molecular-weight polymers after cross-linking causes higher apparent viscosities [11–40,41]. However, there were no significant increases in the apparent viscosity of TGase cross-linked WPISA as the HUS time was increased from 10 to 40 min.

In the stress vs. rate mode, the Herschel-Bulkley model ($R^2 > 0.9999$) and power law model ($R^2 > 0.999$) exhibited higher fitting abilities than the Casson model ($R^2 > 0.99$) and Bingham model ($R^2 > 0.99$), as indicated by their higher $R^2$ values (data not shown). The shear stresses of all samples showed increasing trends as the shear rates increased from 1 to 1000 s$^{-1}$ (Fig. 5C). The consistency index ($K_{HB}$) and rate index ($n$) of the Herschel-Bulkley model that were obtained from fitting the flow (stress vs. rate) curves are shown in Fig. 5D. The $K_{HB}$ value of the WPISA solution increased significantly ($P < 0.05$) after cross-linking with TGase ($P < 0.05$), while the rate index, $n$, of the WPISA solution decreased significantly ($P < 0.05$) after cross-linking with TGase. After incubation with TGase, some TGase cross-linked polymers formed (as seen from Fig. 1), which probably contributed to the $K_{HB}$ and rate index changes [11]. HUS treatment ($\geq 10$ min) significantly increased the $K_{HB}$ values of TGase cross-linked WPISA solutions ($P < 0.05$). However, there were no significant increases in the $K_{HB}$ values of TGase-crosslinked WPISA with increasing HUS times from 10 to 40 min, which was in accordance with...
the degree of TGase-treated WPISA cross-linking. HUS treatment disrupted associated noncovalent interactions among WPISA molecules and exposed a greater number of active regions to the TGase reaction, leading to the formation of greater numbers of high-molecular-weight polymers of TGase cross-linked WPISA (both inter- and intramolecular bonds), favouring changes in the flow behaviour of WPISA solutions.

3.6. Gelation property analysis

WHC and gel strength are two critical parameters of gel properties. WHC can reflect the ability of the food matrix to stabilize water molecules through capillary effects [4]. Gel strength is vital for the mouthfeel of gel food. As shown in Fig. 6, all samples had high WHC values (>82%) and gel strength values (>300 g). The WHC values of the GDL-induced WPISA gels increased significantly from 83% to 86% after incubation with TGase for 4 h (Fig. 6A). Some previous studies have shown that TGase-mediated cross-linking can increase the WHC values of gels produced from different kinds of food proteins, including soy proteins [42], pea proteins [43], wheat gluten [9], and whey protein [41]. TGase-mediated cross-linking had a significant effect on the gel strength of WPISA (Fig. 5B). After incubation with TGase for 4 h, the gel strengths increased significantly (P < 0.05) from 300 to 334 g. It is likely that intermolecular covalent cross-links in the protein molecules contributed to the increased gel strengths [16]. HUS treatment (10–40 min) significantly increased the WHC and gel strength of GDL-induced TGase cross-linked WPISA gel (P < 0.05), indicating that HUS pretreatments had a beneficial effect on the TGase and protein reactions, which facilitated the formation of a dense gel network favoring the binding of more water in the gel. However, there were no significant increases (P > 0.05) in the WHC values and gel strengths of GDL-induced TGase cross-linked WPISA gels when the HUS time increased from 10 to 40 min, which agreed with the degree of cross-linking of TGase-treated WPISA.

As mentioned previously, the formation of disulfide bonds through the oxidation of free SH groups (2SH → S-S reaction) plays an essential role in forming a 3D network structure during gel formation [19]. All the gel samples had low contents of surface free SH groups, and there were small changes among different samples (Fig. 6C). HUS pretreatment (e.g., 10, 20 and 40 min) significantly decreased the surface free SH content of GDL-induced TGase cross-linked WPISA gel (P < 0.05), which remained unchanged after a 10 min HUS treatment, suggesting that the increase in the TGase crosslinking degree induced by HUS may be the main reason for the reduction in surface free SH. After cross-linking with TGase for 4 h, the total free SH content of GDL-induced WPISA gels decreased significantly (P < 0.05), indicating that more disulfide bonds were formed during/after the gelation process resulting from the decrease in total free SH content in the TGase-treated WPISA. However, there were no significant changes in the total free SH content of the gel samples under HUS pretreatment (P > 0.05). It has been reported that disulfide bonds, electrostatic and non-covalent interactions are involved in the three-dimensional networks of gels [44]. The changes in protein interactions in the gels were measured according to gel solubility in different buffers. As shown in Fig. 6D, all the gel samples could dissolve in buffer A and buffer B, which indicated that both electrostatic interactions and noncovalent interactions were important driving forces of the gel network because of the disrupting effects of buffers against their target bonds [45,46]. The solubilities of WPISA gels in buffers A and B decreased significantly (P < 0.05) after incubation with TGase for 4 h. HUS treatment (10–40 min) significantly decreased (P < 0.05) the solubility of GDL-induced TGase cross-linked WPISA gel in buffer A, whereas they had no significant effects on solubility in buffer B (P > 0.05). All the gel samples exhibited low solubility values in buffer B, which indicated that most of the protein molecules in the gel network were linked by covalent bonds.

4. Conclusions

High-intensity ultrasound pretreatment of WPISA markedly improved the TGase-mediated cross-linking degree, as demonstrated by the reduction in free amino group contents. The apparent viscosity and consistency index of TGase cross-linked WPISA were significantly increased by HUS, which indicated that HUS facilitated the formation of greater numbers of high-molecular-weight polymers after the cross-linking reaction. Furthermore, HUS increased the amount of high-molecular-weight polymers of TGase cross-linked WPISA, as demonstrated by SDS-PAGE results. HUS treatment can cause partial unfolding to expose some active/functional groups that were previously buried within the interior of the WPISA to TGase-mediated reactions, thereby facilitating TGase-mediated cross-linking reactions. In addition, HUS increased the gel properties of glucono-δ-lactone (GDL)-induced TGase cross-linked WPISA gels. In conclusion, HUS is a useful technology for modifying WPISA to improve the TGase-mediated cross-linking reaction, thereby expanding the application of whey proteins in the food industry.

CRediT authorship contribution statement

Tiehua Zhang: Writing - original draft. Haiqing Ye: Supervision. Xue Shen: Writing - review & editing, Supervision.

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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