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Effect of ultrasound on the structure and functional properties of transglutaminase-crosslinked whey protein isolate exposed to prior heat treatment

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ABSTRACT

Effects of ultrasound treatment (20 kHz, 41–45 W cm^{-2}, 0, 20, 40 or 60 min) on the physicochemical, functional properties and elements of the secondary structure of transglutaminase (TGase)-crosslinked whey protein isolate (WPI) exposed to prior thermal treatment (75 °C, 15 min) were investigated. The largest molecular size of proteins in the TGase-crosslinked WPI was observed after the ultrasound and thermal pre-treatment (HU-WPI-TGase). HU-WPI-TGase had the maximum intrinsic fluorescence intensity, with highest loss of free amino groups. Ultrasound-treated WPI (U-WPI) showed more (13%) emulsifying activity and more (63%) foaming ability than untreated WPI, but HU-WPI-TGase had higher foam stability and lower emulsifying activity than U-WPI. FTIR analysis indicated that ultrasound, heat treatment and TGase cross-linking had effects on the β-sheet, β-turn and random coil of WPI. The outcomes from this study show a potential application in providing novel functional ingredients for the dairy industry.

1. Introduction

Whey protein isolates (WPIs) are widely used as food ingredients due to their good gelation, emulsifying, foaming and solubility characteristics (Ahmadi, Razavi, & Varidi, 2017). The functional properties of whey protein can be altered by physical modifications that include ultrasound, heat and microwave treatment (Uluko et al., 2015), chemical modifications that include glycosylation (Fu & Zhao, 2016), acylation and phosphorylation (Abaee, Madadlou, & Saboury, 2017; Lawal & Adebowale, 2004) and enzymatic modifications (Agyare, Addo, & Xiong, 2009). Compared with other three modification methods, enzymatic modification typically has high efficiency and specificity and is conducted under mild reaction conditions.

Transglutaminase (TGase, EC 2.3.2.13) catalyses an acyl–transfer reaction between the ε-amino group of peptide-bound lysine residues and γ-carboxyamide group of peptide-bound glutamine residues, resulting in the formation of ε-(γ-glutamyl) lysine bonds (Faergemand, Otte, & Qvist, 1997). Several reports have found that food proteins, including soy protein isolate (SPI), wheat gluten protein and peanut protein, are modified by TGase, which could improve their thermal stability, gel properties and other functional properties (Qin et al., 2016, 2017). However, WPI is not a good substrate for the action of TGase due to the rigid globular structure of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG). Therefore, WPIs need to be denatured to increase the enzymic accessibility (Rodriguez-Turienzo, Cobos, & Diaz, 2013).

In recent years, it is reported that individual ultrasound or heat treatment improves the enzymatic cross-linking degree of native proteins. For instance, it was validated that high intensity ultrasound (20 kHz, 400 W) increased the enzymatic cross-linking degree and 40 min ultrasound treatment of SPI significantly increased the amount of high molecular weight polymers of TGase-crosslinked SPI (Hu et al., 2015). These results were probably due to the cavitation and shear stress of ultrasound treatment made more reaction sites of proteins exposed, leading to an acceleration in the cross-linking reaction (Arzeni, Pérez, & Pilosof, 2012). Furthermore, ultrasound has many advantages such as being safe, non-toxic, and environmentally friendly, and it is already applied in the dairy processing industry.

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There has been a lot of work on the ultrasound treatment of whey proteins. Sonication reduced the denaturation enthalpy of the whey protein concentrate (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011), and the heat-set gel of ultrasound-treated whey protein concentrate (WPC), showed higher gel strengths and water holding capacity than that of non-sonicated WPC (Zisu et al., 2011). Moreover, ultrasound treatment with a 20-kHz horn could reduce the viscosity and increase the heat stability of WPC (Ashokkumar et al., 2009). Ultimately, ultrasound appears as an effective way to enhance the functional properties of whey protein.

In addition to ultrasound, heat treatment contributes to the enzymatic cross-linking, by unfolding the protein molecules and thus disrupts the protein structure and exposes its reaction sites (Al-Saadi, Shaker, & Ustunol, 2014). It is reported that after ewes' milk protein was heated at 85 °C for 15 min, TGase could cross-link ewe protein to form more polymers, leading to an increase of enzymatic cross-linking degree (Rodriguez-Nogales, 2005). The above-mentioned work has proved that ultrasound or heat treatment could lead to an enhancement of the enzymatic cross-linking degree of TGase-crosslinked food proteins.

However, it is not known whether the combination of thermal and ultrasound pre-treatment will better improve the cross-linking degree of TGase-crosslinked WPI and more significantly, enhance the functional properties of cross-linked WPI. In this study, the impact of ultrasound had on properties of thermally pretreated WPI. The properties assessed included cross-linking degree, functionality and changes in the elements of the secondary structure of TGase-crosslinked WPI. This would potentially provide a foundation for development of a novel food ingredient with excellent functional properties for food industry. In addition, it is also expected that an effective practical way of protein modification will be established and applied for industrial production.

2. Materials and methods

2.1. Materials

Whey protein isolate (93.5% protein content) was purchased from Mullins Whey Inc. (Mosinee, WI, USA). TGase (1000 U g^{-1} enzyme activity) was purchased from Yiming Biological Products Co., Ltd (Qinxing, Jiangsu, China). Sodium dodecyl sulphate (SDS), Tris and trifluoroacetic acid (TFA) were purchased from SigmaAldrich (St. Louis, MO, USA). Potassium bromide was purchased from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). *o*-phthalaldehyde (OPA) was purchased from Beijing Biodee Biotechnology Co., Ltd (Beijing, China). Bovine serum albumin (BSA) and β-LG were purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). Commercial α-LA powder (91.6% protein content) was gifted from Davisco Foods International (Eden Prairie, MN, USA). All other chemicals were of analytical grade.

2.2. Preparation of ultrasound-treated TGase-crosslinked WPI exposed to prior thermal treatment

WPI dispersions (50 mg mL^{-1}) were prepared by adding deionised water and then gently stirring for 2 h. WPI solutions were adjusted to pH 7.0 by using 1 mol L^{-1} NaOH. Subsequently, the dispersions were heated at 75 °C for 15 min and then cooled at 25 °C. Afterwards, the heat-treated dispersions were subjected to ultrasound for 0, 20, 40 and 60 min (pulse duration of on-time 1 s and off-time 2 s) using an ultrasound processor model JY92-2D (Ningbo Scientz Biotechnology Co. Ltd, Zhejiang, China) with a net power output of 600 W at a frequency of 20 kHz. The corresponding ultrasound intensity ranged from 41 to 45 W cm^{-2}. The samples were immersed in an ice bath to maintain treatment temperature around 25 °C.

TGase (30 U g^{-1} protein) was added into the WPI solutions, which were previously treated with ultrasound and thermal pre-treatment. Sample mixture was incubated for 4 h at 50 °C and afterwards immediately heated at 75 °C for 15 min to inactivate the enzyme. Then, sample solutions were cooled to room temperature in the ice water and lyophilised as TGase-crosslinked WPI with the ultrasound and thermal pre-treatment (HU-WPI-TGase).

The experimental design included heat treatment (2 levels: none and 75 °C for 15 min), sonication (4 levels: 0, 20, 40 or 60 min) and TGase cross-linking (2 levels: none and 50 °C for 4 h). Three control experiments with ultrasound-treated TGase-crosslinked WPI (U-WPI-TGase), ultrasound-treated WPI (U-WPI) exposed to prior thermal pre-treatment (HU-WPI) or U-WPI, were also carried out.

2.3. Molecular size distribution

Molecular size distribution of samples was estimated by size exclusion chromatography (SEC), using a TSK G 2000 SW (7.5 mm × 60 cm, 1 μm, TosoHaas, Montgomeryville, PA, USA) with a TSK guard column (7.5 mm × 7.5 cm). All sample dispersions were diluted to a final concentration of 5 mg mL^{-1} using distilled water and then filtrated with 0.45 μm membrane and injected to a series of bottles. The columns were equilibrated and eluted with 30% acetonitrile solution containing 0.1% TFA at a flow rate of 0.5 mL min^{-1}. The detection wavelength of UV detector was 280 nm. The method was calibrated using native α-LA, β-LG and BSA as standards.

2.4. Measurement of intrinsic fluorescence spectrum

The intrinsic fluorescence scanning of samples was detected using a Fluorphotometer (F4500, Hitachi, Tokyo, Japan). Samples were diluted with 0.01 mol L^{-1} phosphate buffer (pH 7.0) to a final concentration of 0.1 mg mL^{-1}. The excitation wavelength was set at 280 nm and emission wavelength was scanned from 290 to 420 nm. The slit width of excitation and emission were set at 5 nm and scan rate was 240 nm s^{-1}.

2.5. Determination of free amino groups

The free amino groups of the samples were determined using the OPA method of Church, Swaisgood, Porter, and Catignani (1983). The OPA reagent was freshly prepared before used. Samples were diluted to a final concentration of 5 mg mL^{-1}. Three millilitres of OPA reagent were mixed with 100 μL of each diluted sample and then put in the dark for 5 min at room temperature. Absorbance was recorded at 340 nm. Reagent blank was made using distilled water instead of samples. Free amino groups content of sample was calculated from its absorbance using a calibration curve obtained with L-leucine (0.1–0.5 mg mL^{-1}) as a standard.

2.6. Measurement of emulsifying properties

The emulsifying properties of samples were determined according to the method of Pearce and Kinsella (1978). To prepare emulsions, 1 mL of soybean oil was mixed with 3 mL of diluted sample (with a concentration of 0.5 mg mL^{-1}) and homogenised at 10,000 rpm for 2 min, using a speed homogeniser (IKA, T18 digital Ultra-turrax, Germany). Subsequently, 50 μL of the freshly prepared emulsion was taken out from the bottom of the tube at 0 and 10 min, respectively, and dispersed into 5 mL 0.1% SDS. The absorbance was measured at 500 nm with 0.1% SDS as the blank. Emulsifying activity index (EAI, M^2 g^{-1}) and emulsion stability index (ESI, %) of homogenised emulsions were then calculated.

2.7. Measurement of foaming properties

Foaming ability and foam stability were determined according to the method proposed by Ahmadi et al. (2017) with some modifications. Twenty millilitres of protein suspension (V_c) at a concentration of 50 mg mL^{-1} were pipetted into a 100-mL volumetric cylinder, and then homogenised by using a speed homogeniser (IKA, T18 digital Ultra-turrax, Germany) at the speed of 10,000 rpm for 2 min at 25 °C. After homogenisation, the total volume of protein dispersion and foam was immediately recorded at 0 min (V_{f0}). After the mixture was kept around 25 °C for 30 min, the total volume of the foam and protein dispersion was recorded (V_{f30}). Foaming ability and foam stability were calculated using the following equation.

$$\text{Foaming ability}\ (\%) = \left[\left(V_{f0} - V_c\right)\Big/V_{f0}\right] \times 100 \quad (1)$$

$$\text{Foam stability}\ (\%) = \left[\left(V_{f30} - V_c\right)\Big/\left(V_{f0} - V_c\right)\right] \times 100 \quad (2)$$

2.8. Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectra of samples were measured using a FTIR spectroscopy instrument (Nicolet, Madison, USA) at a full wavelength scan (4000–400 cm^{-1}). Two mg of each sample was mixed with 200 mg potassium bromide (KBr) into uniform powders by an agitate mortar. And the powders were pressed into thin slices under high light. Thirty two scans were made at a selected resolution of 4 cm^{-1}. Quantitative analysis of the changes in secondary structure of samples was performed in amide I band (1700–1600 cm^{-1}) by a peak fitting procedure described by (Jiang et al., 2015). And the baseline correction, deconvolution, second derivative and peak-separation analysis were carried out using an Omnic 8.0 software and Peak Fit 4.12 software. And α-helix (1650–1660 cm^{-1}), β-sheet (1610–1640 and 1670–1690 cm^{-1}), β-turn (1660–1670 and 1690–1700 cm^{-1}) and random coil (1640–1650 cm^{-1}) were assigned to different peaks in the second derivative spectra (Qin et al., 2016). The peaks were identified and fitted with a Gaussian function using the peak fit procedure (Grewal, Huppertz, & Vasiljevic, 2018).

2.9. Statistical analysis

Results were expressed as means with the standard deviation and analysis of variance (ANOVA) was performed by using the SPSS system software 22.0 (SPSS Inc., Chicago, IL, USA). Significant differences ($P < 0.05$) among means from triplicate or more between individual means were identified by Duncan's multiple range test. Analysis including the determination of free amino group, EAI, ESI, foaming capacity, foam stability and FTIR were run in at least triplicate. Fluorescence spectra, molecular size distribution were also carried out in duplicate.

3. Results and discussion

3.1. Analysis of size exclusion chromatography

Chromatogram of untreated WPI showed three major peaks, presented in Fig. 1C and D. And the retention times of the three peaks were 24.63 min, 29.15 min and 31.48 min, corresponding to BSA, β-LG and α-LA, respectively. The molecular size distribution of HU-WPI (Fig. 1C) and U-WPI (Fig. 1D) did not materially change as the ultrasound time was increased. This meant that ultrasound or initial heat and subsequent ultrasound pre-treatment could not change the molecular size of WPI regardless of the ultrasound time. Furthermore, the peak height of formed polymers increased and the peak height of β-LG and α-LA decreased in the U-WPI-TGase with the ultrasound time increased from 0 to 60 min (Fig. 1B). The molecular size of these polymers were close to BSA (an approximate mass of 66 kDa), mainly in the form of trimers and tetramers of β-LG or α-LA. This indicated that ultrasound treatment could enhance TGase cross-linking reaction of WPI. It was also validated that ultrasound treatment of α-LA could induce its production of large molecular conjugates and their amount and size were dependent of ultrasonic time (Yuan et al., 2018).

Molecular size distribution of HU-WPI-TGase is shown in Fig. 1A. During the cross-linking reaction of TGase-crosslinked WPI, initial heat and subsequent ultrasound could remarkably induce the generation of WPI polymers with higher molecular size and lead to a more substantial decrease of β-LG and α-LA than single ultrasound treatment (Fig. 1B). Meanwhile, the formed polymers of HU-WPI-TGase also had a positive correlation with the ultrasound time. Similarly, it was also shown that the 40 min high intensity ultrasound treatment could remarkably enhance the amounts of high molecular polymers of TGase-crosslinked SPI (Hu et al., 2015). This indicated that combination treatment of heat and ultrasound could more effectively enhance the cross-linking degree of TGase-crosslinked WPI.

3.2. Analysis of fluorescence spectra

The fluorescence intensity of HU-WPI-TGase (Fig. 2A), U-WPI-TGase (Fig. 2B), HU-WPI (Fig. 2C) and U-WPI (Fig. 2D) increased with the ultrasound time increased from 0 to 60 min. This might be due to the fact that ultrasound pre-treatment exposed some fluorescent amino acid residues, mainly the tryptophan residue (Jia et al., 2010). Tryptophan residue was more sensitive to solvent polarity than other amino acid residues (tyrosine, phenylalanine), so the fluorescence quantum yield of tryptophan could be used to identify the changes of protein structure (Zhao, Dong, Li, Kong, & Liu, 2015). HU-WPI-TGase and U-WPI-TGase had higher fluorescence intensity than HU-WPI and U-WPI. This showed that the TGase cross-linking reaction might be beneficial in exposing the tryptophan residues of WPI. However, the fluorescence intensity of HU-WPI-TGase was even higher than that of U-WPI-TGase. This phenomenon indicated that heat treatment could further cause the exposure of more tryptophan residues and hydrophobic groups outside the molecules, thus leading to an increase in the fluorescence intensity of proteins (Li et al., 2016).

A red shift in the fluorescence emission wavelength indicates the exposure of the fluorescent amino acid into a hydrophilic environment and led to the changes in protein conformation (Próchniewicz & Strzeleckagolaszewska, 1971). Compared with HU-WPI and U-WPI, the wavelength of fluorescence spectra of the HU-WPI-TGase and U-WPI-TGase moved from 338.6 to 342.6 nm (red shift) after incubated with TGase, which meant the WPI conformation had changed and WPI was denatured by the TGase cross-linking reaction.

3.3. Changes of free amino groups

The TGase reaction leads to a decrease in the number of free lysine ε-amino groups in a protein (Gan, Cheng, & Easa, 2009). Effects of ultrasound treatment (0, 20, 40 or 60 min) on free amino groups of HU-WPI-TGase, U-WPI-TGase, HU-WPI and U-WPI are shown in Fig. 3. Free amino groups of HU-WPI-TGase and U-WPI-TGase significantly ($P < 0.05$) decreased with increased ultrasound time, and the loss of free amino groups was the highest when the ultrasound times were 40 and 60 min. This also indicated that the prolonged ultrasound treatment could significantly enhance the

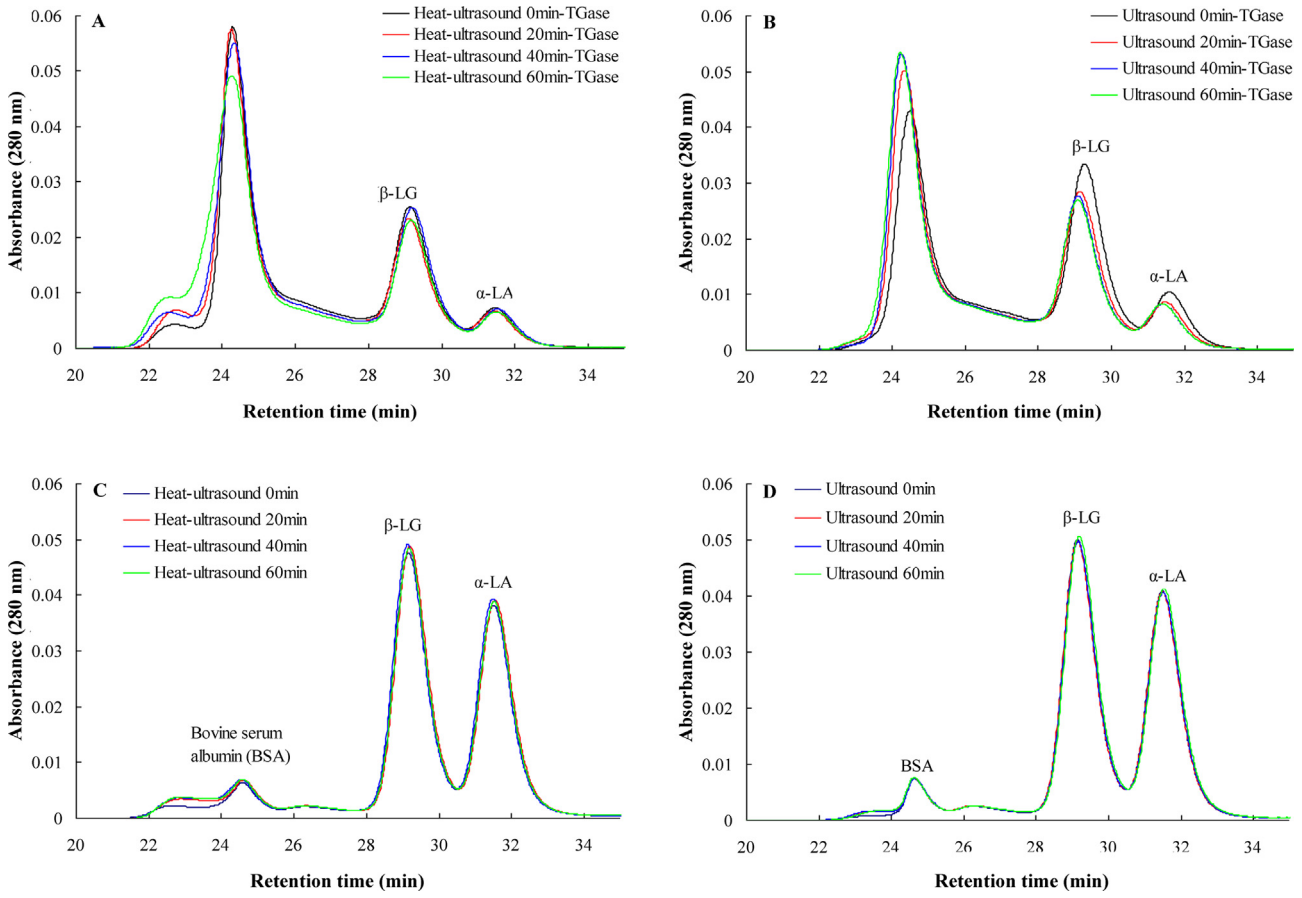

Fig. 1. Effect of ultrasound treatment (0, 20, 40 or 60 min) on size exclusion chromatograms (β-LG, β-lactoglobulin; α-LA, α-lactalbumin) of TGase-crosslinked WPI with: A, ultrasound and thermal pre-treatment (HU-WPI-TGase); B, ultrasound-treated TGase-crosslinked WPI (U-WPI-TGase); C, U-WPI exposed to prior thermal pre-treatment (HU-WPI); D, U-WPI (all at pH 7.0 and 50 °C for 4 h).

degree of TGase-catalysed cross-linking (Fig. 1A and B). However, there was no significant ($P > 0.05$) change in the amount of free amino groups between HU-WPI-TGase and U-WPI-TGase. In addition, the free amino groups of U-WPI and HU-WPI significantly ($P < 0.05$) increased with ultrasound times in the range 20–60 min, and ultrasound treatment was helpful in providing more free amino groups for WPI reaction with TGase. This might be due to the fact that cavitation effects of the ultrasound unfolded the protein molecules, thereby exposing more amino groups (Mu, Zhao, & Bao, 2010).

3.4. Emulsifying ability and emulsion stability

Emulsifying ability and emulsion stability are important functional properties of protein. Emulsifying ability is determined by the maximum oil quantity that can be emulsified by a quantity of protein, while emulsion stability is determined by the phase separation rate of emulsion into water and oil (Pearce & Kinsella, 1978). The emulsifying ability of HU-WPI-TGase, U-WPI-TGase, HU-WPI, U-WPI increased significantly ($P < 0.05$) with the increased ultrasound time from 0 to 60 min (Fig. 4A). Prolonged ultrasound treatment might expose more hydrophobic groups of WPI, leading to greater absorption at the oil/water interfaces (Camino, Pérez, & Pilosof, 2009). Jambrak, Lelas, Mason, Krešić, and Badanjak (2009) also reported that ultrasound treatment could increase the emulsifying ability of soy protein. Furthermore, emulsifying ability declined in the following order of U-WPI, HU-WPI, U-WPI-TGase and HU-WPI-TGase, respectively. High molar mass molecules and the extended protein structures might slow the adsorption of proteins and promote their aggregation on the interface of the emulsion, thus leading to the lower emulsifying properties in cross-linked caseins (Jiang & Zhao, 2012). Compared with the other three groups, HU-WPI-TGase sample had the lowest emulsifying ability. This was due to the fact that HU-WPI-TGase contained the most high molecular size polymers (Fig. 1A), hindering the interfacial absorption of the whey proteins and being easily aggregated in the interfacial areas of the emulsion. This result was consistent with Qiu et al. (2017), who reported that TGase-treated arachin had low emulsifying properties.

Fig. 4B shows the emulsion stability of U-WPI, HU-WPI, U-WPI-TGase and HU-WPI-TGase. Compared with untreated WPI, U-WPI and HU-WPI had the highest emulsion stability with the ultrasound time of 20 and 60 min, respectively. The emulsion stability of U-WPI-TGase also increased ($P < 0.05$) after ultrasound treatment. This indicated that ultrasound treatment contributed to improve emulsion stability of WPI samples. Furthermore, HU-WPI-TGase and U-WPI-TGase had higher emulsion stability than that of HU-WPI and U-WPI. The poor flexibility of protein might lead to lower oil/water interface adsorption capacity and prevent the oil droplets from reaggregating, thus increasing the emulsion stability of protein (Marcoa & Rosell, 2008). In this test, TGase cross-linked WPI had more rigid interface than non-enzymic cross-linked WPI, leading to the increase of its emulsion stability. Agyare et al. (2009) also reported that the emulsion stability of wheat gluten hydrolysate enhanced after a treatment with TGase at 55 °C for 1 h.

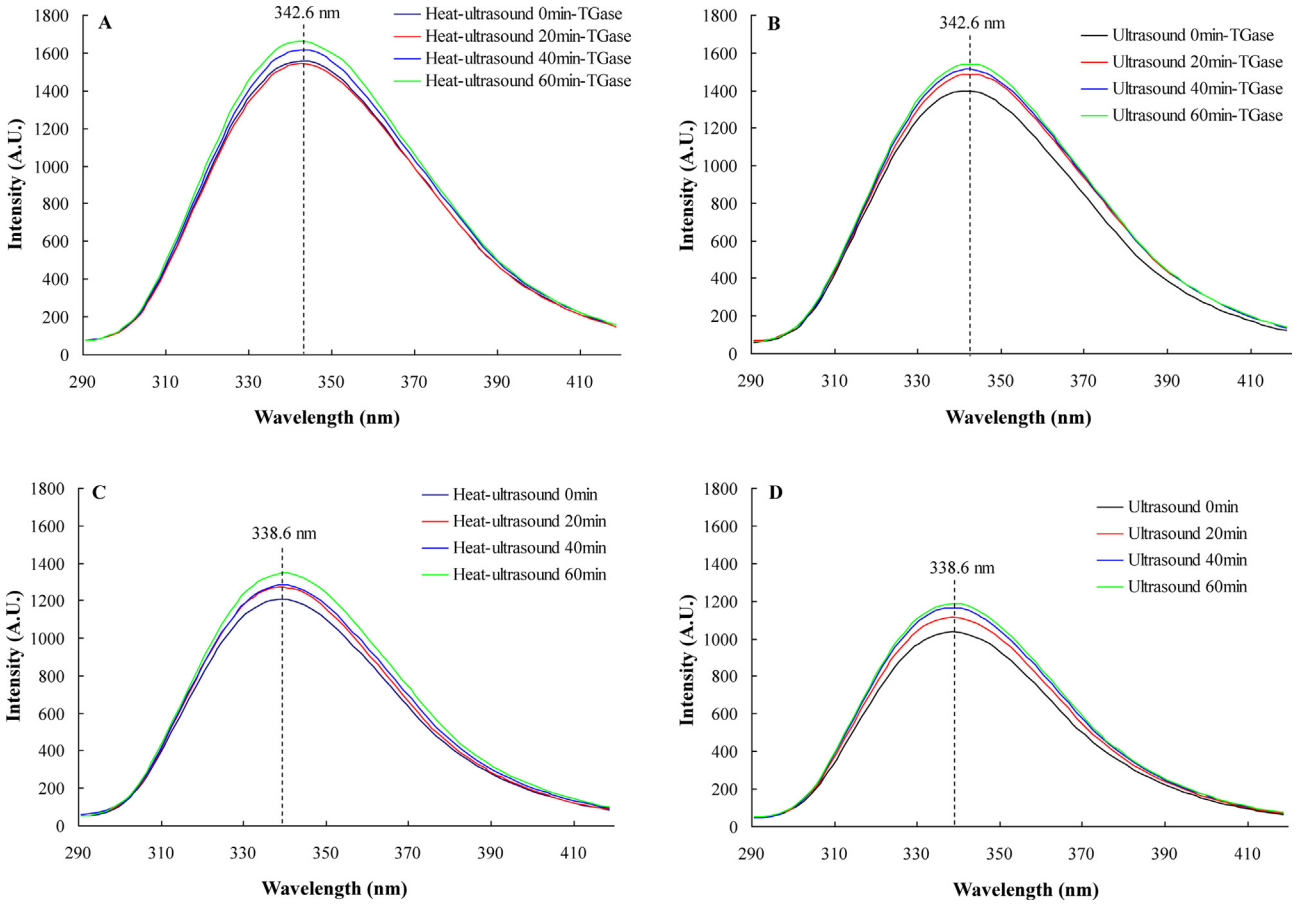

Fig. 2. Effect of ultrasound treatment (0, 20, 40 or 60 min) on intrinsic fluorescence spectrum of: A, HU-WPI-TGase; B, U-WPI-TGase; C, HU-WPI; D, U-WPI (all at pH 7.0 and 50 °C for 4 h). Arrow corresponds to the wavelength of maximum intensity.

3.5. Foaming ability and foam stability

Foaming ability of HU-WPI-TGase, U-WPI-TGase, HU-WPI and U-WPI is shown in Fig. 5A. The foaming ability of the four sample groups significantly ($P < 0.05$) increased as the ultrasonication time was extended from 0 min to 60 min. This result was in accordance with those of Jambrak, Mason, Lelas, Herceg, and Herceg (2008), who found that ultrasound could enhance

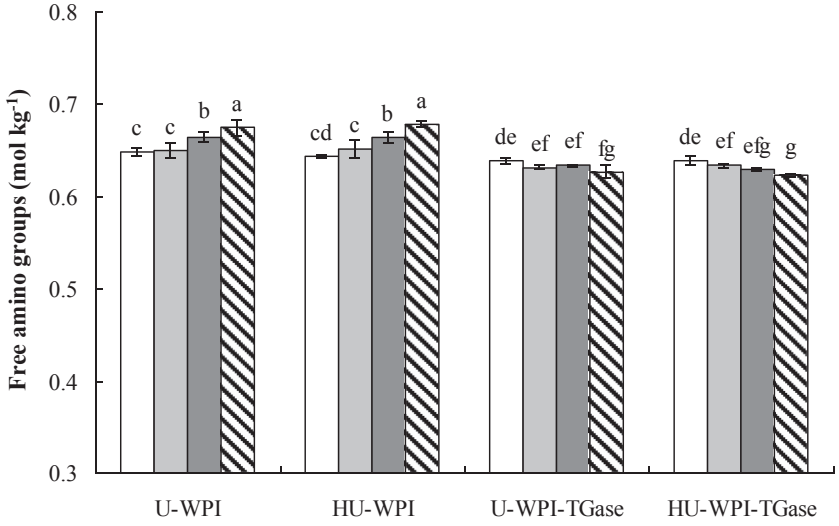

Fig. 3. Effect of ultrasound treatment (□, 0 min; ■, 20 min; ■, 40 min; ▨, 60 min) on free amino groups of HU-WPI-TGase, U-WPI-TGase, HU-WPI and U-WPI at pH 7.0 and 50 °C for 4 h. Error bars represent the standard deviation of the mean of triplicate experiments; different letters indicate significant difference ($P < 0.05$).

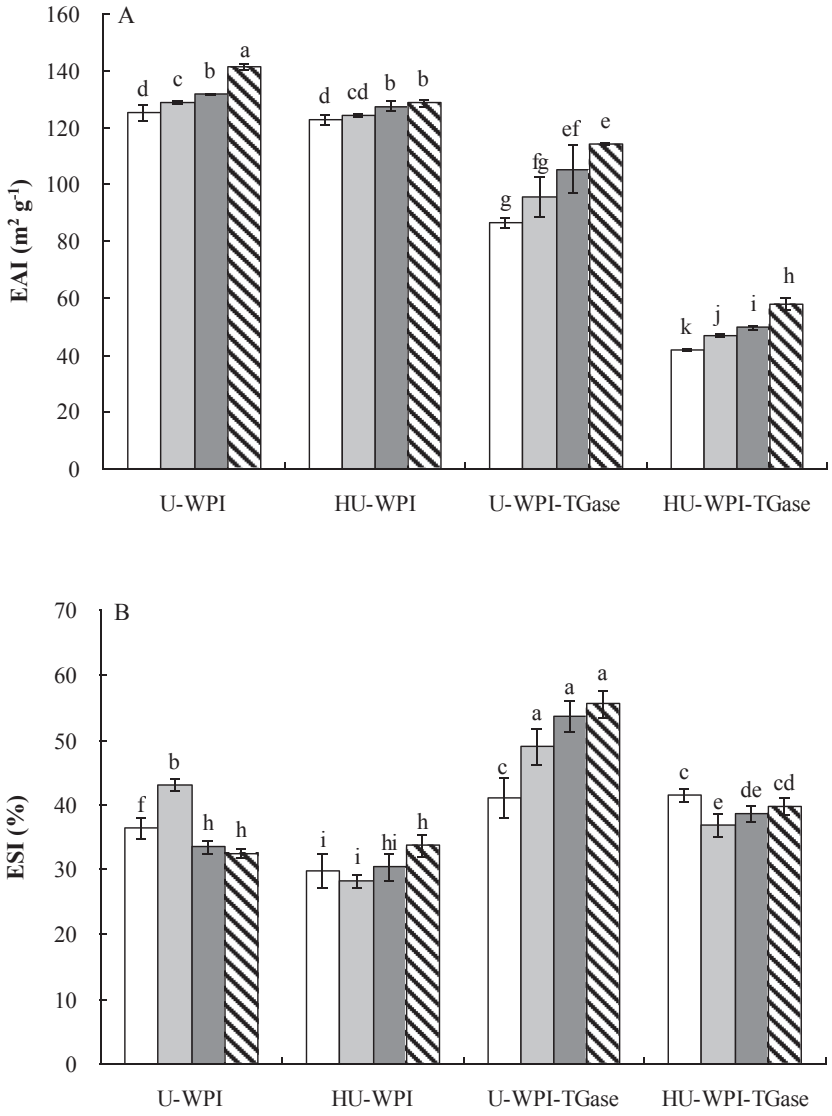

Fig. 4. Effect of ultrasound treatment (□, 0 min; ▩, 20 min; ■, 40 min; ▧, 60 min) on (A) emulsifying ability and (B) emulsion stability of HU-WPI-TGase, U-WPI-TGase, HU-WPI and U-WPI at pH 7.0 and 50 °C for 4 h. Error bars represent the standard deviation of the mean of triplicate experiments; different letters indicate significant difference ($P < 0.05$).

the foaming ability of whey protein suspensions. This increase of foaming ability might be due to the ultrasound homogenisation that probably led to a more uniform distribution of protein and fat particles (Knorr, Zenker, Heinz, & Lee, 2004). Under the same conditions of ultrasound time, HU-WPI had the highest foaming ability compared with other three groups. This meant the combination of heat and ultrasound treatment might improve the foaming ability of protein. Furthermore, the foaming ability of TGase-crosslinked WPI was lower than that of WPI treated without TGase cross-linking. TGase-catalysed cross-linking led to an increase in polymers and viscosity of WPI, which disabled the protein particles to be absorbed on the air–water interface and then affected the foaming ability of proteins (Ahmadi et al., 2017).

Foam stability of HU-WPI-TGase, U-WPI-TGase, HU-WPI and U-WPI is shown in Fig. 5B. The foam stability of four sample groups significantly ($P < 0.05$) increased as the ultrasonication time was extended from 0 min to 60 min. Jambrak et al. (2008) reported that foam stability of WPI and WPC improved after 20 kHz probe treatment. In addition, it was observed that with the ultrasound amplitude and ultrasound time increased, the foam stability of whey protein increased (Tan, Chin, Yusof, Taip, & Abdullah, 2015). This might be due to the ultrasound treatment leading to an increase in the surface activity of proteins at the solution–air interface. Meanwhile, TGase cross-linking could increase the foam stability of WPI. The foam stability in HU-WPI-TGase was also higher than that in U-WPI-TGase. This might be due to the formation of more ε-(γ-glutamyl)lysine bonds and covalent bonds in the HU-WPI-TGase, which appears to be a booster agent in foam stability (Ahmadi et al., 2017).

3.6. Fourier transform infrared spectroscopy

The amide I region (1600–1700 cm^{-1}) of the FTIR spectra of HU-WPI-TGase, U-WPI-TGase, HU-WPI and U-WPI at ultrasound 0 min (Fig. 6A) and ultrasound 60 min (Fig. 6B) were used to illustrate the changes in secondary structure of proteins. Deconvoluted Fourier transform infrared (FTIR) spectra of HU-WPI-

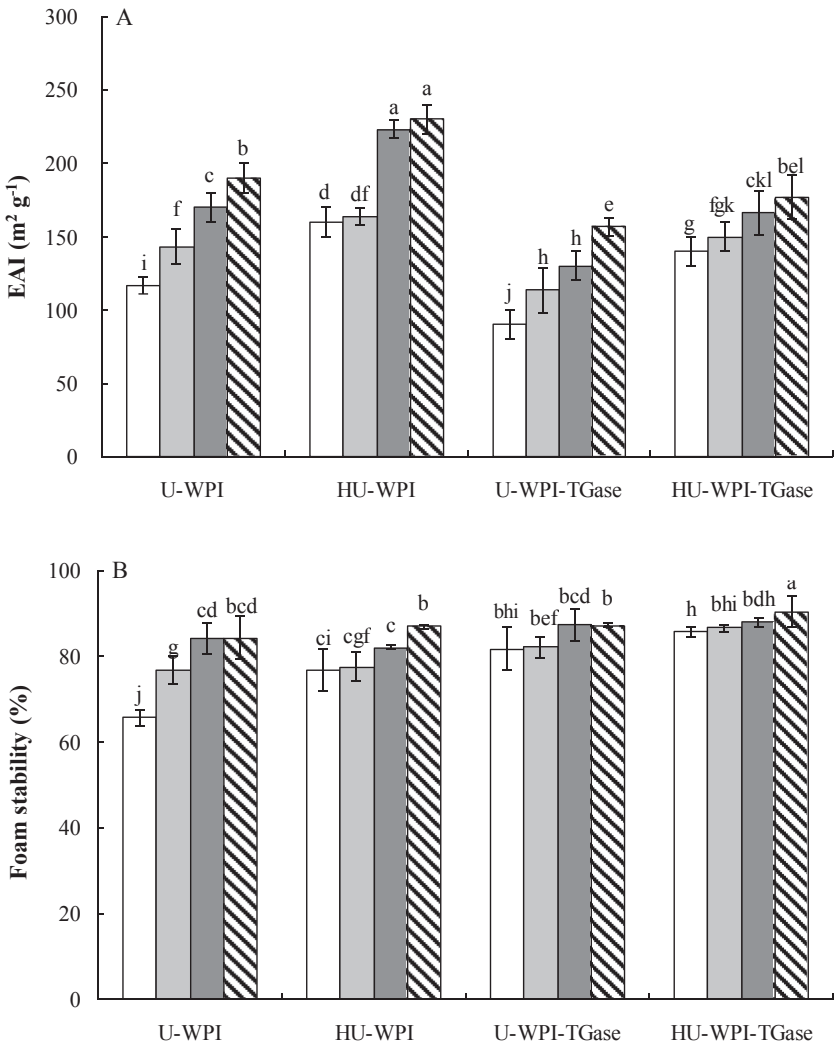

Fig. 5. Effect of ultrasound treatment (□, 0 min; ▩, 20 min; ■, 40 min; ▧, 60 min) on (A) foaming ability and (B) foam stability of HU-WPI-TGase, U-WPI-TGase, HU-WPI and U-WPI at pH 7.0 and 50 °C for 4 h. Error bars represent the standard deviation of the mean of triplicate experiments; different letters indicate significant difference ($P < 0.05$).

TGase (ultrasound 60 min) sample in the amide I region is shown in Fig. 6C, which included eight components: 1616.1, 1626, 1635.8, 1646, 1655.7, 1666.2, 1676.7 and 1687.1 cm^{-1}. Quantitative analysis of the secondary structure changes was achieved by curve fitting of the amide I band, shown in Table 1. There was no difference in the α-helix composition of all samples. This indicated that α-helix (main structure in native α-LA) could not be changed after the treatment of ultrasound, thermal treatment and TGase cross-linking. Generally, α-LA (Ca^{2+} binding protein) containing four intramolecular disulphide bonds, was far more stable than β-LG during the heat treatment (Qi & Onwulata, 2011). Similarly, α-helix structure could also not be changed in thermally denatured β-LG cross-linked with TGase, compared with native β-LG (Eissa, Puhl, Kadla, & Khan, 2006).

However, compared with untreated WPI, there was a significant ($P < 0.05$) increase from 35.80 to 39.18% in the β-sheet of TGase-crosslinked WPI. This showed that the WPI conformation could change after cross-linked by TGase, which was also validated by the red shift of the fluorescence emission wavelength (Fig. 2A and B). Cando, Borderías, and Moreno (2016) also reported that TGase cross-linking resulted in a significant increase in β-sheet composition of surimi proteins that were pretreated by high pressure processing. Furthermore, β-LG was known to be a β-sheet protein (Grewal et al., 2018; Hamada, Segawa, & Goto, 1996). Compared with unheated WPI, the β-sheet composition of heat-treated WPI remarkably decreased from 35.80 to 33.31% ($P < 0.05$), being attributed to the denaturation of β-LG (Grewal et al., 2018). Moreover, after WPI was treated by ultrasound for 60 min, the amount of its β-sheet remarkably increased from 35.80 to 37.93% ($P < 0.05$). Therefore, the β-sheet of WPI could be altered by heat, ultrasound and TGase cross-linking. This meant the conformation of β-LG molecule might be changed and partially unfolded, facilitating the exposure of hydrophobic residue buried in the interface between monomers in natural proteins (Qi & Onwulata, 2011).

Additionally, after ultrasound treatment for 60 min, the amount of random coil in HU-WPI-TGase increased from 20.42 to 25.35% ($P < 0.05$) compared with untreated WPI, meaning that the proteins had become more disordered, probably due to a conversion of β-turns into random coils (Qin et al., 2016). And the interior unfolding of the β-LG molecule, also caused an increase in random coil structures (Boye, Ismail, & Alli, 1996).

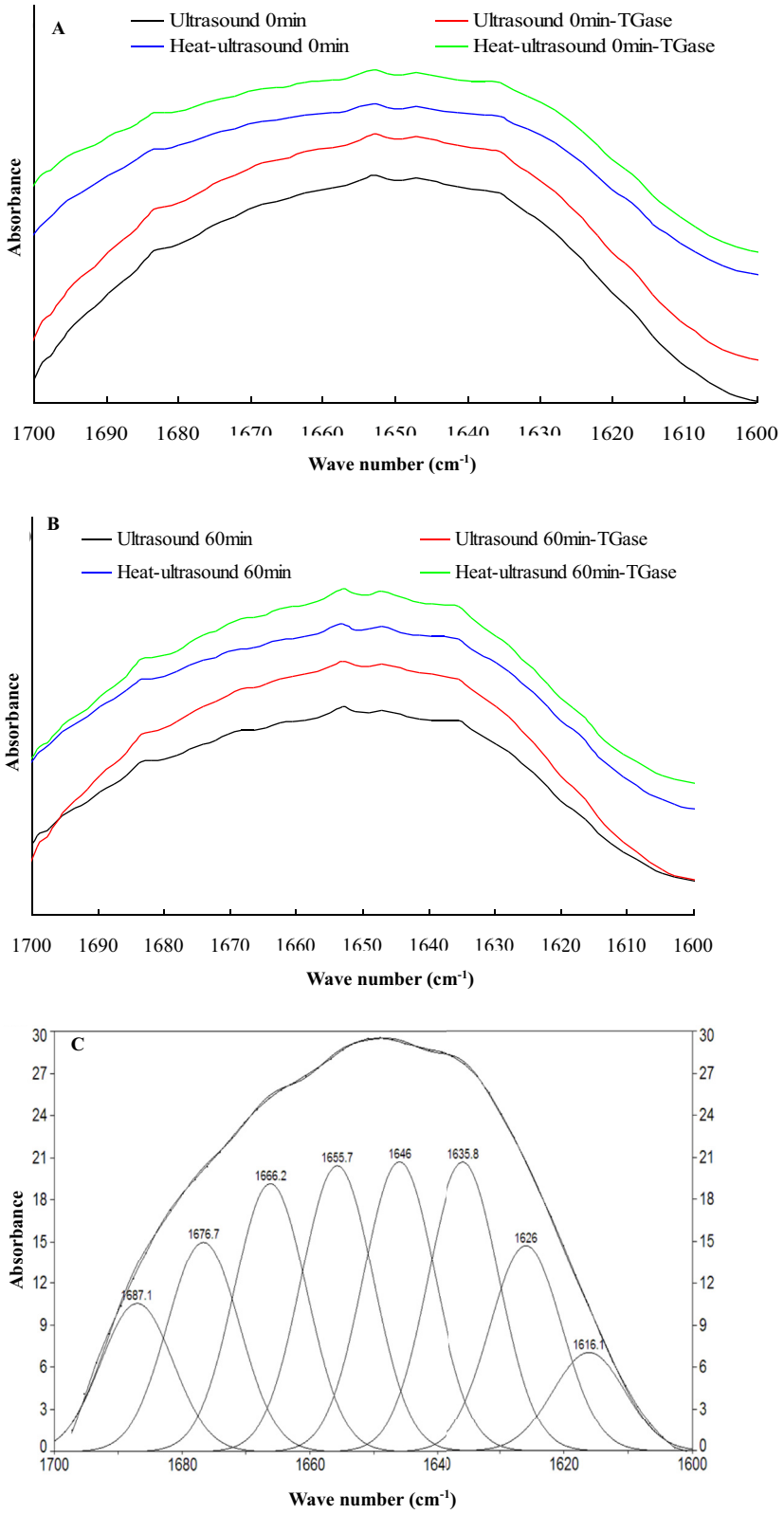

Fig. 6. Effect of non-ultrasound (A; 0 min) or ultrasound treatment (B; 60 min) on Fourier transform infrared (FTIR) spectra of HU-WPI-TGase, U-WPI-TGase, HU-WPI and U-WPI at pH 7.0 and 50 °C for 4 h. Panel C shows deconvoluted FTIR spectra of HU-WPI-TGase (ultrasound 60 min) sample in the amide I region.

Table 1
Secondary structure analysis of all samples in amide I region.[a]

Samples	Secondary structure composition (%)			
	α-helix 1650–1660 cm^{-1}	β-sheet 1610–1640 cm^{-1} 1670–1690 cm^{-1}	β-turn 1660–1670 cm^{-1} 1690–1700 cm^{-1}	Random coil 1640–1650 cm^{-1}
U-WPI-0 min	14.23 ± 0.00^{a}	35.80 ± 0.26^{ef}	29.55 ± 0.03^{b}	20.42 ± 0.28^{g}
U-WPI-60 min	13.40 ± 1.01^{a}	37.93 ± 0.47^{c}	27.34 ± 0.97^{d}	21.33 ± 0.14^{f}
HU-WPI-0 min	13.54 ± 0.47^{a}	33.31 ± 0.33^{g}	31.89 ± 0.14^{a}	21.27 ± 0.00^{e}
HU-WPI-60 min	13.36 ± 0.30^{a}	35.08 ± 0.95^{f}	28.92 ± 0.79^{c}	22.65 ± 0.46^{d}
U-WPI-TGase-0 min	13.33 ± 1.01^{a}	39.18 ± 0.14^{b}	24.22 ± 1.21^{ef}	23.27 ± 0.07^{c}
U-WPI-TGase-60 min	13.49 ± 1.02^{a}	40.36 ± 0.37^{a}	21.83 ± 0.78^{f}	24.32 ± 0.21^{b}
HU-WPI-TGase-0 min	13.85 ± 0.50^{a}	36.35 ± 0.47^{de}	25.54 ± 0.94^{e}	24.25 ± 0.01^{b}
HU-WPI-TGase-60 min	14.93 ± 0.16^{a}	37.09 ± 0.54^{d}	22.65 ± 1.05^{f}	25.35 ± 0.56^{a}

[a] Regions are: α-helix, 1650–1660 cm^{-1}; β-sheet1610–1640 cm^{-1}1670–1690 cm^{-1}; β-turn, 1660–1670 cm^{-1}; 1690–1700 cm^{-1}; random coil, 1640–1650 cm^{-1}. Values represent the means ± standard error (n = 3); different superscript letters in the same column show a significant difference ($P < 0.05$).

4. Conclusions

In this study, the use of the combination of ultrasound and thermal pre-treatment for enhancement in the cross-linking degree of TGase-catalysed WPI and an evaluation in change of its structure and functional properties is presented for the first time. Of four samples tested, the formation of the largest molecular size polymers was found in HU-WPI-TGase. Emulsifying activity, foaming ability and foam stability in HU-WPI-TGase increased as a function of the ultrasonic time. This indicates that HU-WPI-TGase would have a potential application as emulsifier and foam stabiliser in the processing and storage of food products. In future work, it will be further investigated whether or not other physical pre-treatments, including superfine grinding or high-pressure homogenisation, increase the cross-linking degree of TGase-catalysed WPI and improve its functional properties.

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