



# Modification of grass pea protein isolate (*Lathyrus sativus* L.) using high intensity ultrasound treatment: Structure and functional properties

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# **Effect of ultrasonic pretreatment on the rheology and structure of grass pea protein emulsion gels induced by transglutaminase**

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## **Abstract**

Aqueous solutions of grass pea protein isolate (GPPI) were sonicated at different amplitudes (25, 50 and 75%) and times (5, 10 and 20 min). Stable oil in water emulsions were prepared with corn oil and the sonicated GPPI that were subsequently jellified by enzymatic crosslinking of the proteins with transglutaminase (TGase). The kinetics of gelation was followed by measuring oscillatory shear moduli as a function time and the dynamic mechanical properties were studied using oscillatory shear and penetration measurements. A two-step gelation process was observed for the emulsion gels with a fast stage leading to weak gels followed by a slow stage that strongly reinforced the gels. The first stage was not strongly dependent on the sonication treatment, but was not observed without oil. The gel stiffness and maximum penetration force was systematically larger in the presence of oil and increased generally with increasing sonication time and amplitude, but an optimum time of 10 min was found at 75% amplitude. The water holding capacity increased after sonication in line with the mechanical properties. The thermal stability of sonicated emulsion gels was promoted due to higher intermolecular cross-linking within the gel network. The interactions between the oil and protein in the emulsion gel network were validated by the absorption peaks obtained from FTIR spectra. Although non-sonicated samples clearly displayed large protein aggregates and phase separation, CLSM revealed that the microstructures of sonicated GPPI emulsion gels were almost the same at all sonication amplitudes and times.

**Keywords:** Emulsion; gel; protein; grass pea; sonication; enzyme; gelation kinetics.

## **1. Introduction**

Emulsion gels or emulgels, are gels embedding oil droplets (Li et al., 2021). Emulsion gels are generally prepared by crosslinking the proteins in a protein-stabilized oil-in-water emulsion. They are stable against creaming and may have useful mechanical properties (Lu et al., 2020). Emulsion gels have a wide range of applications in the food industry such as in meat-analogues, reduced-fat food products, and carriers for bioactive ingredients (Li et al., 2021; Mao et al., 2020).

Gel forming ability is an important functional property of legume proteins (Gharibzahedi & Smith, 2020) and the application of legume proteins in the food formulation has attracted considerable attention due to their digestibility and balanced composition of polar, nonpolar and charged hydrophobic/hydrophilic amino acids (Mahdavian Mehr & Koocheki, 2020). However, the compact structure and poor functionality of legume proteins in their native state limit their application in the food industry. Therefore, plant proteins can profit from structural modification to improve their emulsifying and gelation properties (Mozafarpour et al., 2022).

The application of high intensity ultrasound (HIU) to modify the conformation and functional properties of proteins has attracted considerable attention (Kahraman et al., 2022; Y. Li et al., 2020; Ma et al., 2019; Mozafarpour et al., 2022; Xue et al., 2022). High intensity ultrasound (frequency 20-100 kHz) is an efficient, non-thermal and physical technique to cause modification of the secondary and tertiary structure of proteins. Ultrasound waves exert their effects on protein molecules through the collapse of cavitation bubbles in water (Shen et al., 2017) possibly due to high local pressure and temperature, water jets, shock waves and shear forces, which can cause protein molecules to undergo chemical and physical changes (Martínez-Velasco et al., 2018).

The structural unfolding of globular proteins exposes hydrophobic and sulfhydryl groups which reinforce the gel forming ability of protein through intermolecular hydrophobic interactions and disulphide bonds (Liang et al., 2020). It has been reported that electrostatic, hydrophobic and disulphide interactions play an important role in the initial development of pea protein gel network (Sun & Arntfield, 2012). Hu et al. (2015) and Zhang et al. (2016) have shown that ultrasonication facilitates the enzymatic gelation of soy protein isolate with transglutaminase (TGase). TGase is an enzyme that catalyzes acyl-transfer between  $\gamma$ -hydroxylamine groups of glutamine (Gln) and  $\epsilon$ -amino groups of lysine (Lys) leading to the formation of covalent  $\epsilon$ -( $\gamma$ -glutamyl) lysine isopeptide bonds (Qin et al., 2016). HIU treatment can partially unfold the compact structure of protein and expose embedded Gln and Lys groups to the surface of the proteins, promoting subsequent enzymatic reactions. In addition, hydrophobic groups and free SH-groups may become exposed that strengthen the emulsion gel network (Geng et al., 2022; Kumar et al., 2022). Moreover, HIU can improve the emulsifying properties of protein through particle size reduction, which accelerates the protein adsorption at the oil/water interface (Geng et al., 2022).

When soy protein-containing emulsions were sonicated prior to enzymatic gelation, Geng et al. (2022) observed that the shear rate dependent viscosity increased compared to when the emulsion was made by mechanical homogenization. However, as far as we are aware, the effect of different HIU pretreatments of proteins on enzymatic gelation of protein stabilized emulsions has not yet been reported. The objective of this study was to investigate enzymatic gelation of emulsions stabilized by grass pea protein isolate (GPPI) that were sonicated at different amplitudes and for different durations. Recently, we reported on the effect of HIU on the properties of GPPI in aqueous solution (Mozafarpour et al., 2022). It was found that the

amount of protein that sedimented at  $10^4$  g during 15 min and the average particle size decreased after HIU, suggesting that larger protein aggregates were broken up by HIU. The surface hydrophobicity and the amount of free SH groups also increased after HIU. Changes in the secondary structure induced by HIU were observed with circular dichroism. Furthermore, the interfacial tension between water and oil was reduced more by the addition of GPPI when the proteins were more severely treated and the sonicated protein were better emulsifiers. Finally, incubation with TGase led to the formation of gels at lower GPPI concentrations after HIU treatment and the gels were stronger for a given protein concentration. Here, we focus on the effect of HIU on the kinetics of gelation and the dynamic mechanical properties of the emulsion gels in comparison with gels formed at the same protein concentration without oil. The structure and thermal properties of emulsion gels were also investigated.

## **2. Materials and methods**

### **2.1. Materials**

Grass pea seeds and corn oil were obtained from a local market in Mashhad, Iran. Protein extraction and isolation was performed according to the method described elsewhere (Mozafarpour et al., 2022). The protein content of the final grass pea protein isolate was approximately 92%, evaluated by the micro Kjeldahl method ( $N \times 5.8$ ). Commercial TGase was purchased from TAIXIN YIMIN Fine Chemical Industry Co. Ltd. (Jiangsu province, China). Fluorescein and Nile red were purchased from sigma Aldrich Co. All other chemicals and materials were of analytical grade.

### **2.2. Emulsion preparation and characterization**

GPPI was dispersed in distilled water (8% w/v) with 0.02% (w/v) sodium azide and shaken using a roller shaker at room temperature for 2 h. After preparation, corn oil was added to the dispersions, mixed and homogenized using a laboratory homogenizer (Digital Ultra Turrux T-25, IKA instruments, Germany) at 20000 rpm for 3 min to obtain a uniform emulsion ( $\Phi=0.2$ ). The average droplet size and polydispersity index (PDI) of emulsions were measured using a dynamic light scattering particle size analyzer (Cordouan, VASCO 3, France). Samples were diluted with deionized water at a ratio of (1:200 v/v) to prevent multiple scattering particle. Three measurements were done per sample. Zeta potential was measured by a Zeta potential Analyzer (Cordouan Technol, Zetasizer, France) through determining the electrophoretic mobility of emulsion droplets. Emulsions were diluted 200-fold using deionized water. All measurements were done in triplicate.

### **2.3. GPPI emulsion gel preparation**

The emulsion gel was fabricated using the same procedure used to create the emulsion. Corn oil ( $\Phi=0.2$ ) and TGase (50 U/g) were added to the GPPI dispersion (8% w/v), which was then mixed under a vortex and homogenized using a laboratory homogenizer (Digital Ultra Turrux T-25, IKA instruments, Germany).

### **2.4. Rheology**

The gelation kinetics of sonicated and non-sonicated GPPI stabilized emulsion gels were evaluated using a rheometer (AR2000, TA instruments) with plate-plate geometry (diameter = 40.0 mm; gap = 700  $\mu\text{m}$ ). 1 ml of the emulsion containing enzyme was loaded immediately after preparation between the parallel plates. Excess sample around the plates was trimmed off

and the geometry was covered by a thin layer of paraffin oil to prevent water evaporation during the measurement. The temperature was controlled with a Peltier system and set to 37°C. The measurements were performed in the linear viscoelastic region at a constant strain of 3%. Dynamic oscillations with a frequency of 1Hz were applied and elastic modulus ( $G'$ ) and loss ( $G''$ ) were recorded every 30s as a function of incubation time during 20 h. The frequency dependence of the shear moduli was measured between 0.1 to 10 Hz in the linear response regime after the time sweep. Measurements were done on two independently prepared samples for each system, which were found to be the same within  $\pm 10\%$ .

The resistance to penetration of the emulsion gels was determined using a texture analyzer (AMETEK Lloyd, TA Plus instruments Ltd, USA) with a cylindrical probe (diameter: 4 mm). Emulsion gels formed in tubes (40mm×10mm, 5ml) by incubation at 37°C for 20h were penetrated at a constant rate of 1 mm/s and trigger force of 5g to 50% of their original height. The gel strength was taken as the maximum penetration force (Hu, Fan, et al., 2013). Measurements were done on two independently prepared samples for each system, which were found to be the same within  $\pm 5\%$ .

## **2.5. Confocal laser scanning microscopy (CLSM)**

The microstructure of the emulsion gels was analyzed using CLSM instrument Zeiss LSM800 equipped with three Ar-He-Ne lasers with excitation wavelengths of 488nm, 543 nm and 633 nm, respectively (Carl Zeiss Microscopy GmbH, Germany). To find the relative location of the oil phase and protein phase in the emulsion gel structure, Nile red (1mg/ml oil or methanol) and Fluorescein (1mg/ml distilled water) were used before emulsion gel formation to stain the oil and protein phase, respectively. The fluorescence of Fluorescein was excited at 480 nm and detected between 480 and 540 nm. The fluorescence of Nile red was excited at 550 nm and



detected between 550 and 630 nm. An aliquot of fresh emulsion gels was immediately transferred to a single concave microscope glass slides and covered with glass slips and then kept 20 h at 37°C for emulsion gel formation. The CLSM images of emulsion gels were obtained by a 63× magnification water immersed lens (HC ×PL APO 63 × (NA = 1.2). The oil phases dyed with the Nile red are normally red, whereas the proteins phase dyed with the fluorescence are green. In fact, most of the protein coated oil droplets appeared predominantly yellow in the images (green+ red→yellow).

## **2.6. Water holding capacity (WHC)**

The water holding capacity of emulsion gels was evaluated using a method previously described by Liang et al. (2019) with slight changes. 3g of emulsion gel samples were transferred into a weighted centrifuged tube and centrifuged (Sigma, 3-30 k refrigerated centrifuge, Germany) at 10000g for 10 min. Expulsed water was carefully drained off and the residual liquid dried using a paper filter. The centrifuge tubes containing emulsion gel samples were accurately weighed before and after centrifugation. The WHC was calculated as follows:

$$WHC = \frac{W_t - W_r}{W_t} \times 100 \quad (1)$$

where,  $W_t$  is the total weight of water in the gel and  $W_r$  represents the mass of water released after centrifugation. Measurements were done on two independently prepared samples for each system, which were found to be the same within  $\pm 0.5\%$ .

## **2.7. Differential scanning calorimetry (DSC)**

The thermal properties of emulsion gels were evaluated using DSC instrument (DSC 214 Polyma, NETZSCH, Germany). 15 mg of freeze-dried emulsion gel transferred into an

aluminum pan and sealed with an aluminum cover using a tablet press machine. Samples were heated at the rate of 10°C/min from 25 to 150°C. The denaturation temperature peak (Td) and denaturation enthalpy ( $\Delta H$ ) were taken from DSC thermograms. A hermetic aluminum pan was used as a reference.

## **2.8. FT-IR spectroscopy**

Freeze dried emulsion gels were mixed with KBr at a ratio of 1:100 to make a tablet. Infra-red spectra of samples were analyzed using IR spectrometer (Thermo Nicolet Avatar 370 FT- IR, USA) in the region of 4000–400  $\text{cm}^{-1}$  using 64 readings per second at a resolution of 4  $\text{cm}^{-1}$ .

## **2.9. Statistical analysis**

Statistical analysis was performed employing SPSS 26.0 statistical software (2020). The measurements were repeated at least three times. The samples were analyzed by a completely randomized factorial design. Results were expressed as means  $\pm$  standard deviation. The significant differences were analyzed at the P-level of 0.05 using Duncan's test.

# **3. Results and discussion**

## **3.1. Emulsion droplet size and zeta potential**

The average droplet size of emulsion stabilized by GPPI before gel formation are shown in Table 1. The oil droplet size decreased with increasing the ultrasound amplitude and time. The smaller size and uniform distribution of emulsion droplets could enhance the gel properties when the emulsion is converted to the emulsion gel (Yuntao Wang et al., 2020). The decrease in droplet size could be due to the increase in the surface hydrophobicity of ultrasonically

modified GPPI (Mozafarpour et al., 2022), which improves the emulsifying activity of sonicated protein particles and also increases the adsorption of proteins at the O/W interface (Cheng et al., 2021). Qin et al. (2018) also reported that the number of sonicated quinoa protein particles at the interface increased and the droplet size significantly decreased as the sonication time increased.

The PDI of sonicated GPPIs-stabilized emulsions were lower than that of non-sonicated GPPI-fabricated emulsion. Lower PDI values were also observed for emulsions prepared with protein sonicated at higher ultrasound amplitudes i.e., 50 and 75%. The enhanced interfacial activity and intermolecular interactions between protein particles and oil droplets could form more uniform emulsion gels with smaller PDI value (Zhang et al., 2021).

The  $\zeta$ -potential values of emulsions fabricated with native and sonicated GPPIs are shown in Table 1. Both ultrasonic amplitude and time increased the negative surface charge values on the surface of oil droplets. The denaturation and unfolding of protein during ultrasonication exposed more negatively charged amino acids on the particle's surfaces (Jiang et al., 2014). Sha et al. (2021) also observed a slight increase in the negative surface charge for emulsion fabricated by sonicated pea protein isolate.

**Table 1.** Droplet size, PDI and  $\zeta$ -potential values of emulsion stabilized with 1% w/v native and sonicated GPPIs at different sonication amplitudes and times.

Amplitude (%)	Duration (min)	Droplet size (nm)	Zeta potential (mV)	PDI
<b>Control</b>	-	734±50.5 <sup>a</sup>	-2.98±0.73 <sup>i</sup>	0.53±0.06 <sup>a</sup>
<b>25</b>	<b>5</b>	645±48.0 <sup>b</sup>	-3.98±0.51 <sup>h</sup>	0.33±0.07 <sup>b</sup>
	<b>10</b>	642±4.00 <sup>bc</sup>	-6.07±0.57 <sup>g</sup>	0.35±0.06 <sup>b</sup>
	<b>20</b>	569±23.0 <sup>de</sup>	-9.36±1.21 <sup>d</sup>	0.28±0.02 <sup>c</sup>
<b>50</b>	<b>5</b>	572±24.0 <sup>cde</sup>	-6.43±0.34 <sup>f</sup>	0.35±0.06 <sup>b</sup>
	<b>10</b>	515±6.50 <sup>e</sup>	-8.50±0.30 <sup>e</sup>	0.26±0.08 <sup>d</sup>
	<b>20</b>	522±5.00 <sup>e</sup>	-11.03±0.48 <sup>b</sup>	0.16±0.08 <sup>g</sup>
<b>75</b>	<b>5</b>	602±17.0 <sup>bcd</sup>	-9.22±0.07 <sup>d</sup>	0.23±0.08 <sup>e</sup>

<b>10</b>	444±45.0 <sup>f</sup>	-9.63±0.05 <sup>c</sup>	0.19±0.09 <sup>f</sup>
<b>20</b>	423±21.0 <sup>f</sup>	-15.85±1.53 <sup>a</sup>	0.19±0.04 <sup>f</sup>

### 3.2. Gelation kinetics

The enzymatic gelation rate of native and sonicated GPPI emulsions at 37°C was evaluated by measuring the oscillation moduli as a function of time. Fig. 1 shows  $G'$  as a function of time after addition of TGase for the emulsion with proteins treated by ultrasound at 25%, 50% and 75% amplitudes as well as with untreated proteins. Gelation appears to occur in two stages. The first stage started immediately after addition of enzyme, whereas the second stage, which caused the upward inflection in the dependency of  $G'$  on time, was clearly visible only after about 8 h. At 75%, the increase in  $G'$  during the first stage was faster when the proteins were subjected to an ultrasonic treatment for up to 10 min, but it was slower when the treatment lasted for 20 min. This might be due to the oxidation of susceptible SH groups at longer sonication treatment (Mozafarpour et al., 2022).  $G'$  increased faster with increasing the sonication time to up to 20 min at 25% and 50% amplitudes. For a given duration, the first stage of gelation was slower when the sonication amplitude was lower. The subsequent increase of  $G'$  during the second stage occurred at approximately the same rate independent of the ultrasound treatment. The gradual increase in the  $G'$  values of sonicated GPPI emulsion gels at the second stage, reflects the development of exposed  $\epsilon$ -(glutamyl) and lysine covalent cross-linking through the TGase reaction, which reinforces the gel networks (Zhang et al., 2016).

In all cases, the increase of  $G'$  stagnated after about 16 h. We note that in the absence of enzyme,  $G'$  increased very weakly under these conditions, reaching 2 Pa after 20 h for an emulsion fabricated with proteins treated at 75% amplitude for 10 min. This means that if any

crosslinks other than those created by enzymatic crosslinking contributed to  $G'$  value, they were formed only as a result of enzymatic crosslinking. Qin et al. (2016) detected less free SH groups after enzymatic crosslinking of soy and gluten proteins, suggesting that disulfide bridges had been formed during gelation in addition to enzymatic crosslinks.

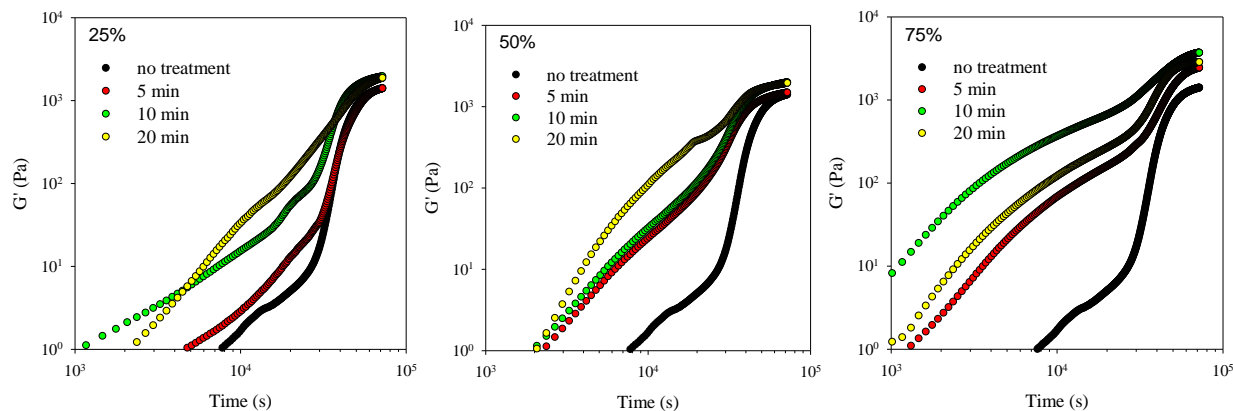


Fig. 1 Evolution of  $G'$  with time during incubation with TGase for emulsions containing GPPI that were sonicated at different amplitudes and times.

In order to understand the contribution of the dispersed oil droplets to the gelation kinetics, we repeated the measurements at 25% and 75% amplitude without oil. In all cases, only the second stage of the gelation was observed (Fig. 2). The onset of gelation was slightly earlier after ultrasonic treatment, but depended little on the duration or amplitude. Most likely, the first stage of the gelation was caused by crosslinking of proteins that were adsorbed at the oil/water interface leading to the formation of a relatively weak network of crosslinked droplets. This network is subsequently reinforced by crosslinking of free proteins in the bulk.

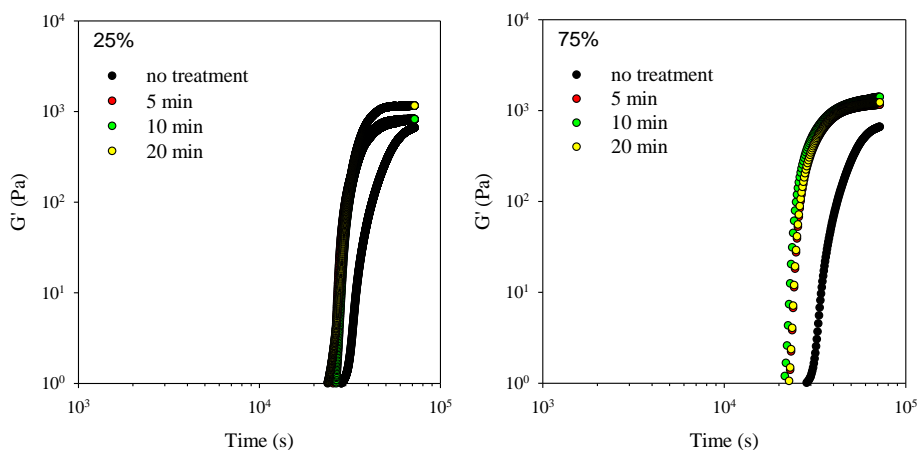


Fig. 2 Evolution of  $G'$  with time during incubation with TGase for oil-free solutions of GPPI that were sonicated at different amplitudes and times.

### 3.3. Shear moduli

The values of  $G'$  close to the inflection point ( $t = 2 \times 10^4$  s) and at the end of the time sweep ( $t = 7.5 \times 10^5$  s) are shown in Fig. 3. It is clear that the contribution of the second gelation stage to  $G'$  is much more important than that of the first stage, leading in most cases to a reinforcement by more than an order of magnitude. As a general trend,  $G'$  was larger for gels formed with more severely treated protein both after the first stage and after the second stage. This implies that the gel network structure strengthened as a result of partial unfolding of interior functional groups and contribution of more covalent and hydrophobic bonds in the gel network (Ben-Harb et al., 2018). The proteins treated at 75% amplitude were the exception, as treatment for 10 min resulted in a stiffer gel than treatment for 20 min. The reduction of  $G'$  value for emulsion gel fabricated from sonicated protein at 75% amplitude for 20 min is due to the oxidation of some free SH groups at stronger HIU treatment (Mozafarpour et al., 2022).

It's interesting to note that compared to the second stage, the relative values of  $G'$  after the first stage depended much more on the ultrasonic amplitude and time. It appears that ultrasound treatment has a greater impact on the rate and degree of enzymatic crosslinking of the adsorbed

proteins. After the first stage, the  $G'$  appears to be strengthened since TGase becomes more accessible to glutamyl and lysyl residues (Wen et al., 2019). In the first stage of gelation, after enzymatic cross-linking, bulk proteins come into close contact and gradually stiffen the gel through intermolecular hydrophobic interactions and disulfide bonds (Djoullah et al., 2018; Shen, Zhao, et al., 2017).

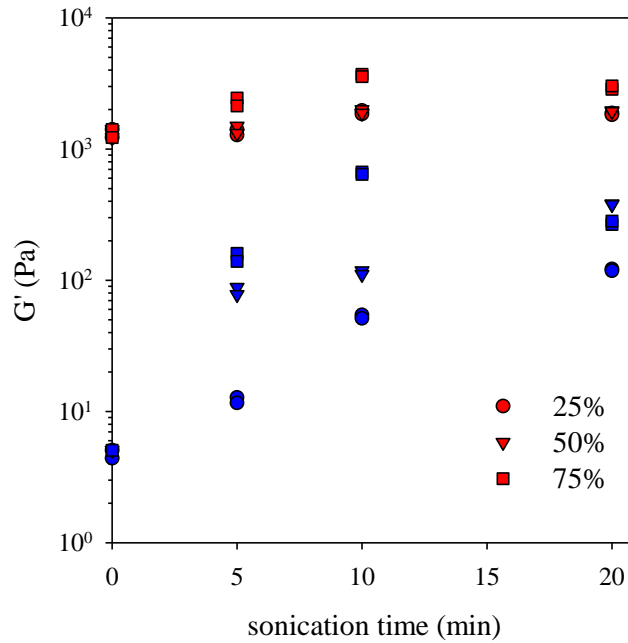


Fig. 3  $G'$  of emulsion gels formed after 8 h (blue symbols) and after 20 h (red symbols) incubation with TGase as a function of the sonication time of GPPI at different amplitudes. For each system, results obtained from two independently prepared samples are shown.

The values of  $G'$  reached after 20 h, for gels formed without oil are compared to those of the emulsion gels in Fig. 4. The  $G'$  values for emulsion gels were systematically twice as high as those for the oil-free gels. A maximum  $G'$  was once more discovered with proteins treated at 75% for 10 min. The observation that the sample treated at 75% amplitude for 10 min had a stronger gel than the protein treated for 20 min at this amplitude, indicates that there is an optimum ultrasonic treatment for obtaining stiff gels when using a crosslinking enzyme such as

TGase. A similar observation was reported by Qin et al. (2016) for gels formed enzymatically with soy and gluten proteins that were treated with ultrasound at different amplitudes and durations.

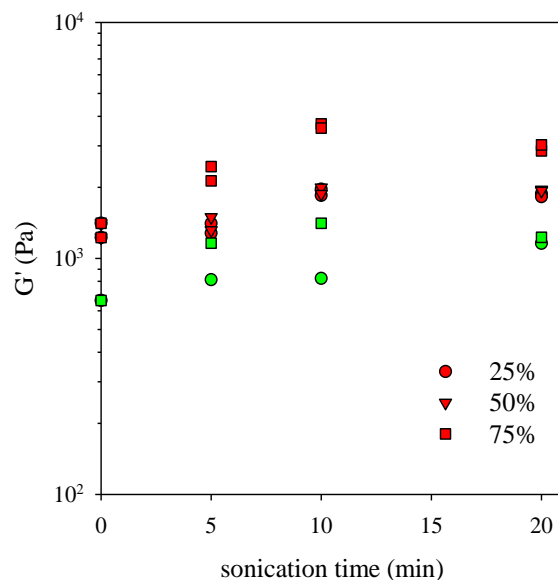


Fig. 4 G' of emulsion gels (red symbols) and oil-free gels (green symbols) formed after 20 h incubation with TGase as a function of the sonication time of GPPI at different.

Frequency sweeps between 0.1 and 10 Hz were done after the time sweep and showed that G' and G'' were almost independent of the frequency in this range, and that G' was more than an order of magnitude larger than G'' (Fig 5), indicating that strongly elastic gels had been formed (Malik & Saini, 2018).



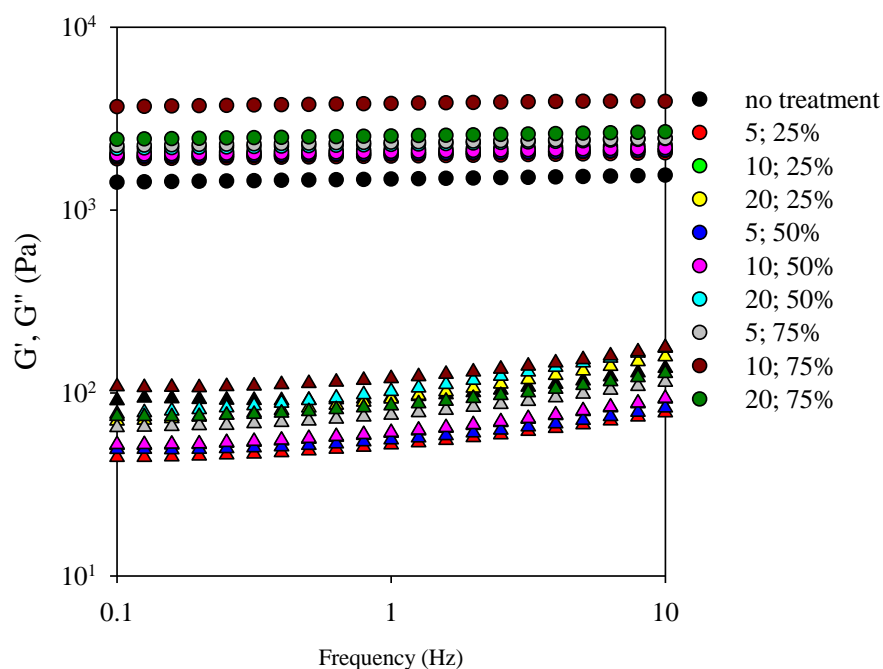


Fig. 5 Frequency dependence of  $G'$  and  $G''$  of emulsion gels formed after 20 h incubation with TGase containing GPPI that was sonicated at different times and amplitudes.

### 3.4. Penetration force

The gel strength of the emulsion gels was characterized by measuring the maximum force during a penetration test as explained in the methods section. The effect of sonication on the force was similar to that on  $G'$  (Fig.6) showing a systematic increase with increasing amplitude and duration except at 75% for which a maximum was found at 10-min. Higher gel strength for the sonicated GPPI emulsion gel is related to the larger number of exposed hydrophobic groups which favors the intermolecular interaction between protein particles and oil droplets in the emulsion gels. Besides, when glutamyl and lysyl residues are exposed through the ultrasonic cavitation effect, the accessibility of TGase to the glutamyl and lysyl residues increases, and consequently, the strength of the gel structure is improved (Wen et al., 2019). The decrease in the gel strength when GPPI was subjected to ultrasonication amplitude of 75% for 20 min

might be due to the oxidation of reactive SH groups at longer sonication time (Mozafarpour et al., 2022). In this case, the emulsion gel breaks more easily when exposed to the external forces.

The similar effect of sonication on  $G'$  and on the maximum penetration force is not surprising if we consider that both are a consequence of the effect of sonication on the crosslink density. We reported a similar effect of the sonication amplitude and duration for these gels without oil elsewhere (Mozafarpour et al., 2022). The absolute values were found to be smaller without oil, but they cannot be compared directly with the results obtained here since in the previous study gels were incubated for only 2 h.

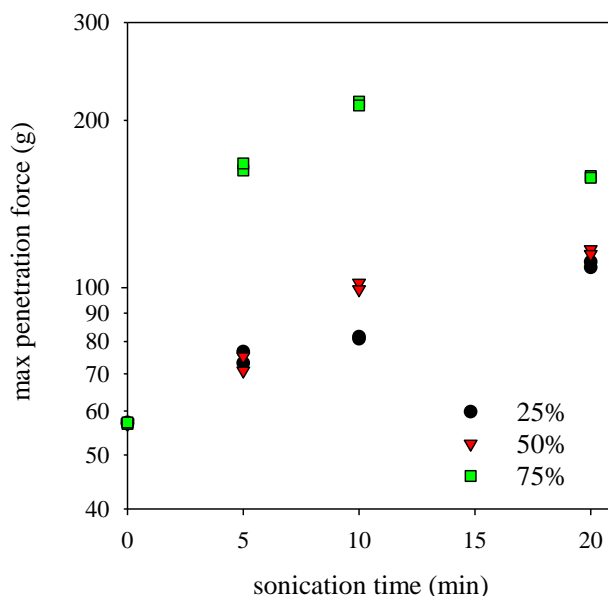
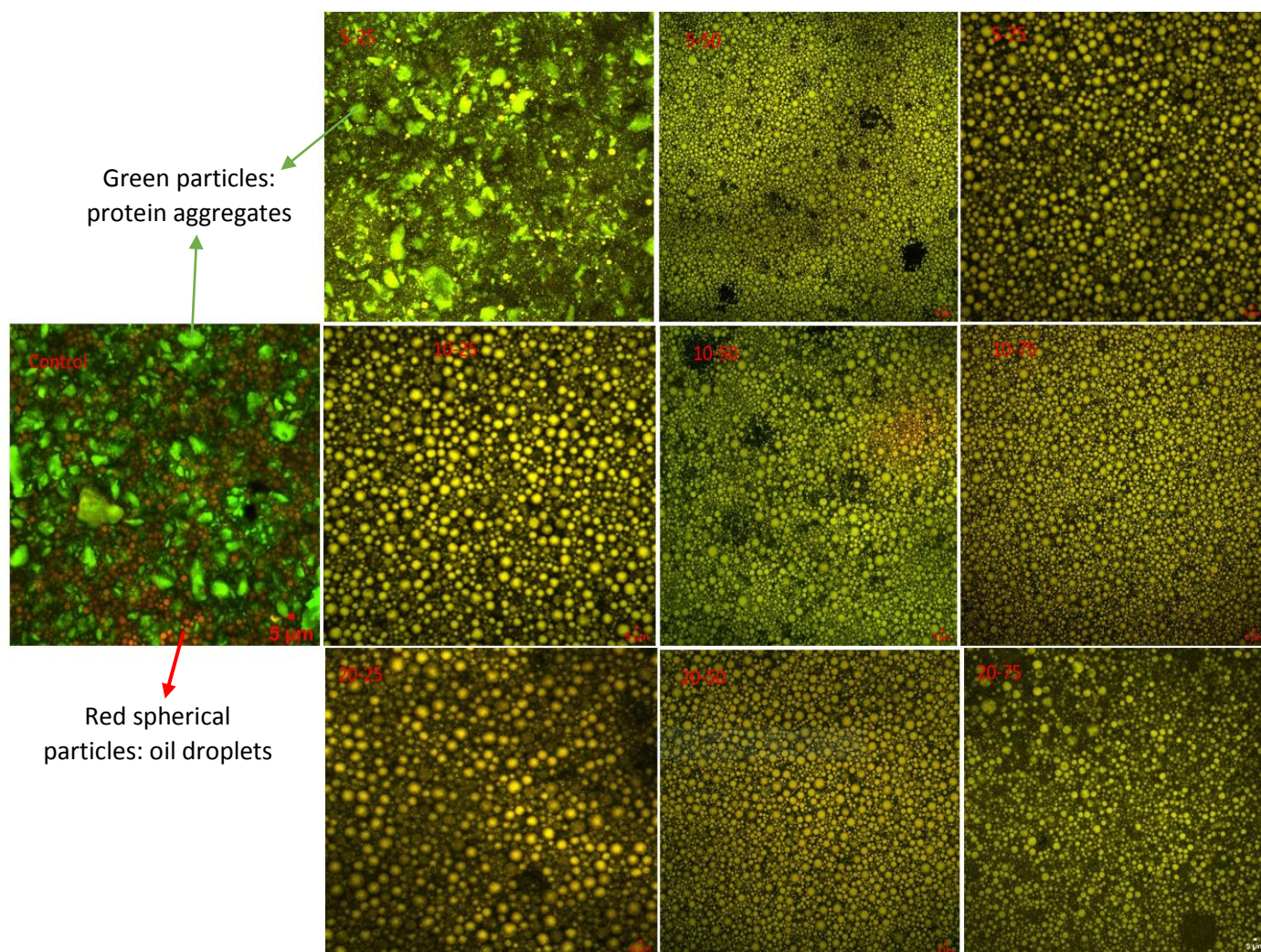


Fig. 6 Maximum penetration force for emulsion gels formed after 20 h incubation with TGase as a function of the sonication time of GPPI at different amplitudes. For each system results obtained from two independently prepared samples are shown.

### 3.5. Microstructure analysis by CLSM

The microstructure of GPPI emulsion gels were analyzed using CLSM technique. Emulsion gel prepared with non-sonicated GPPI showed obvious large protein aggregates and phase separation while those prepared with sonicated GPPIs had homogeneous microstructures with uniform droplet distributions (Fig. 7). Therefore, sonication of GPPI increased the adsorption efficiency of protein to stabilize the oil/water interface due to their higher surface hydrophobicity (Mozafarpour et al., 2022). Moreover, HIU produced smaller oil droplets (Table 1) and formed uniform emulsion gel networks. Geng et al. (2022) also reported that emulsion gels stabilized with sonicated soy protein had uniform gel network. No obvious difference was observed among the microstructure of sonicated GPPI emulsion gels at different amplitudes and times. However, similar to the control sample, sonicated sample at 25% amplitude for 5min, showed larger protein particles and flocculated oil droplets. It seems that the shear energy caused by ultrasonication was insufficient to disrupt large aggregates during the sonication at this low amplitude and sonication time. As a results, sonicated protein particles at higher amplitudes and times could effectively form a dense gel network with embedded oil droplets within its network structure (Mao et al., 2014).

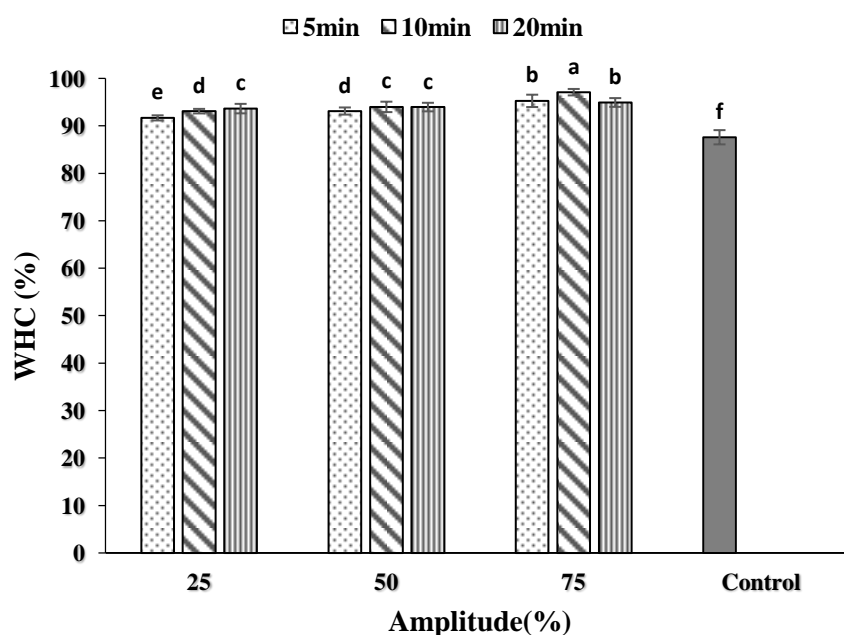


**Figure 7.** Microstructure of emulsion gels fabricated from non-sonicated and sonicated GPPIs at different ultrasonic amplitudes and times.

### 3.6. Water holding capacity

The WHC was 87% for emulsion gels formed with non-sonicated proteins and was improved by sonication (Fig. 8). The effect of varying the amplitude and duration on the WHC was small but followed the same trends as the effect on  $G'$ , with a maximum of 97% with proteins that were sonicated at 75% amplitude during 10 min. The smaller particle size and higher solubility of sonicated GPPIs (Mozafarpour et al., 2022) could enhance the formation of a dense and

uniform gel network which could bind higher amount of water (Hu, Li-Chan, et al., 2013; Zhang et al., 2016). Moreover, the formation of intermolecular covalent bonds between glutamine and lysine residues through the action of TGase enzyme could enhance the formation of dense networks with higher WHC (Liang et al., 2020). As a result, the denser the gel structure, the more difficult it is for the water molecules to be squeezed out by the external forces. Liang et al. (2020) also inferred that TGase can form intermolecular covalent cross-links between glutamyl and lysyl residues, thereby promoting the hydration capacity of whey protein emulsion gel.



**Fig. 8** Water holding capacity of emulsion gels formed after 24 h incubation with TGase as a function of the sonication time of GPPI at different amplitudes. For each system results obtained from two independently prepared samples are shown.

### 3.7. Differential scanning calorimetry (DSC)

All samples exhibited characteristic endothermic peaks (denaturation temperature) centered around 60°C (Table 2). The untreated sample exhibited two thermal transition temperatures and  $\Delta H$  values. The second transition temperature is likely related to the denaturation temperature of the heat resistant small aggregates within the native GPPI which are thermo-resistant during exposure to heat treatment (Khatkar et al., 2018). However, this second transition temperature was not detected in sonicated samples, demonstrating that ultrasonic treatment disrupted the aggregates and hence removed the second transition temperature. The difference in the denaturation temperature ( $T_d$ ) was not considerable among emulsion gels. The highest denaturation temperature ( $T_d$ ) belonged to the sonicated GPPI at 75% for 10 min due to the higher intermolecular disulphide bonds and  $\epsilon$ -( $\gamma$ -glutamyl) lysine residues within this emulsion gel which increases the thermal resistant of protein gel network (Chandrapala et al., 2011; Mozafarpour et al., 2022).

The development of intermolecular interactions during emulsion gel formation was confirmed by increase in the  $\Delta H$  of sonicated GPPI emulsion gels. The higher  $\Delta H$  value highlights the certain degree of intermolecular interactions (hydrogen bonds, hydrophobic interactions and  $\epsilon$ -( $\gamma$ -glutamyl) lysine isopeptide bonds) during enzymatic gelation. Hence, more energy is required to disrupt the structure and denature these highly cross-linked molecules in protein (Lo et al., 2022; Shahbazi et al., 2021). On the other hand, sonicated proteins reinforced the interfacial layer of oil-water; thus, a more stable emulsion gel networks with uniform droplets distributions were formed (Fig. 1). However, the enthalpy slightly reduced for sonicated protein at 75% amplitude for 20 min. This decrease could be due to the oxidation of susceptible functional groups during excessive sonication treatment which can deteriorate the structure and stability of the emulsion gel. As a results, the denaturation of sonicated emulsion gels would

require more heat absorption due to the higher intermolecular cross-linking within the gel network (Zhang et al., 2021).

**Table 2.** denaturation temperature (Td) and enthalpy ( $\Delta H$ ) values of emulsion gels fabricated by sonicated GPPI at different ultrasonic amplitudes and times.

Ultrasound variables			
Amplitude (%)	Time(min)	Td (°C)	$\Delta H(J/g)$
control	-	61.4±0.1 <sup>g</sup>	4.3±0.2 <sup>f</sup>
	-	100.6±0.5 <sup>a</sup>	0.5±0.1 <sup>h</sup>
25	5	63.0±0.2 <sup>de</sup>	3.6±0.2 <sup>g</sup>
	10	63.2±0.2 <sup>d</sup>	7.3±0.2 <sup>d</sup>
	20	63.1±0.4 <sup>d</sup>	7.4±0.7 <sup>d</sup>
50	5	61.9±0.3 <sup>f</sup>	6.3±0.2 <sup>e</sup>
	10	62.8±0.3 <sup>de</sup>	6.1±0.1 <sup>e</sup>
	20	63.7±0.3 <sup>c</sup>	8.7±0.6 <sup>c</sup>
75	5	61.5±0.2 <sup>fg</sup>	9.6±0.2 <sup>b</sup>
	10	65.2±0.3 <sup>b</sup>	10.7±0.1 <sup>a</sup>
	20	62.8±0.3 <sup>de</sup>	9.6±0.1 <sup>b</sup>

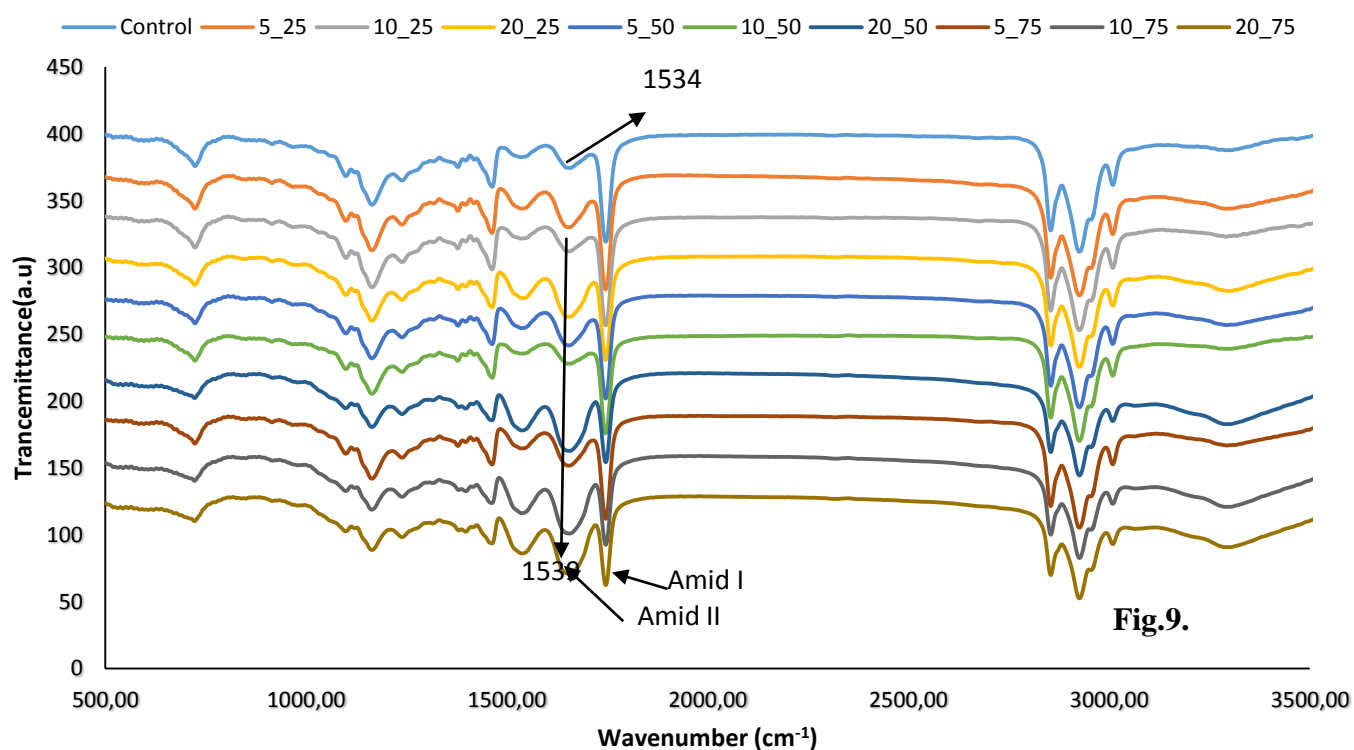
### 3.8. FT-IR spectroscopy

The FT-IR spectra of emulsion gels are shown in Fig. 9. The peak's locations did not change with sonication amplitude or time, indicating that no functional groups had been completely destroyed or created during the ultrasonic treatment (Xue et al., 2022). The main spectral bands

consisted of three intense bands including amid I ( $1700\text{-}1575\text{ cm}^{-1}$ ), amid II ( $1550\text{-}1510\text{ cm}^{-1}$ ) and amid III ( $1238\text{ cm}^{-1}$ ). Amid I region ( $1600\text{-}1700\text{ cm}^{-1}$ ) is mainly associated with the stretching vibration of carbonyl group (C=O), C-N, and N-H bending contributions. Amid I region directly represents the backbone conformation and hydrogen bonding of protein secondary structure (Martínez-Velasco et al., 2018). Ultrasonication of GPPI increased the intensity of amid I region. This increase could be associated to the changes occurred in the secondary structure of protein under sonication. Ultrasonication stretches the molecular structure of the protein and exposes more functional groups to the surface which results in the change in the intensity of amid I region (Yang Wang et al., 2020). Similarly, the changes in transmittance intensity and peak positions for amide I region were previously reported by Malik and Sani (2018) for sonicated sunflower meal.

Compared to the spectrum of non-sonicated emulsion gel, the peak position of amid II bands for sonicated samples at amplitudes of 50 and 75%, shifted from  $1534\text{ cm}^{-1}$  to  $1539\text{ cm}^{-1}$  and their intensities increased. These changes in the amid II bands, indicate the alteration in the secondary structure of GPPI due to the breakup of internal interactions thorough the ultrasonic cavitation effect (Malik & Saini, 2018). The absorption peak at  $1745\text{ cm}^{-1}$  corresponding to the C=O stretching vibration, was also exhibited in the FT-IR spectra of emulsion gels. This peak shows the interactions between the oil and protein in the emulsion gel network (Hu et al., 2021). Sonication had no considerable effect on the peak intensity at  $1745\text{ cm}^{-1}$  and amid III region.





FTIR spectroscopy of emulsion gels fabricated from sonicated GPPIs at different ultrasonic amplitudes and times.

from sonicated GPPIs at

**Fig.9.**

#### 4- Conclusion

Sonicated GPPI emulsion gels exhibited a homogeneous and continuous gel network. Enzymatic gelation of emulsions stabilized by GPPI occurs in two steps. A relatively fast stage leading to relatively weak gels is followed by a much slower stage during which the gels are reinforced by more than an order of magnitude. The first gelation step was not observed in the absence of oil

from which we deduce that it was caused by crosslinking of proteins adsorbed at the oil/water interface. The second step occurred at the same rate with and without oil and was caused by crosslinking of the proteins in the water phase. After the second step, the emulsion gels were systematically stiffer about a factor of two than gels without oil. The gel stiffness, maximum penetration force and water holding capacity increased with increasing sonication amplitude and duration indicating that HIU facilitated enzymatic crosslinking. However, at the higher ultrasound amplitude (75%) the values after 20 min were lower than that sonicated for 10 min, suggesting that there is an optimum HIU treatment to obtain maximum mechanical gel properties, which needs to be taken into account for potential application of these proteins in food products. Higher intermolecular cross-links promoted the thermal stability of emulsion gels. Under ultrasonic treatment, no new protein functional groups had been created or destroyed.

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