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Influence of heat treatment on structure, interfacial rheology and emulsifying properties of peanut protein isolate

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Abstract: The influence of heat treatment on the protein size, zeta potential, surface hydrophobicity, secondary structure, interfacial rheology and creaming stability of peanut protein isolate (PPI) was studied. Heat treatment of PPI increased the protein size, surface hydrophobicity and interface diffusion rate, and decreased the protein zeta potential, particularly heat treatment at 80°C for 30 min (PPI-80), which increased the surface hydrophobicity from 117.33 ± 2.77 to 253.24 ± 2.47. Interfacial rheology results demonstrated that the heat treatment promoted the absorption of PPI at the oil-water interface, which might be due to the increase of surface hydrophobicity. In contrast, the heat treatment at 90°C resulted in slightly lower surface hydrophobicity and K_{diff} compared with PPI-80 due to the hydrolysis of partial protein aggregates during high temperature. Moreover, heat-treated PPI showed better emulsifying properties than unheated PPI. These results would be useful to expand the utilization of PPI products in the food processing industry.

Keywords: adsorption; emulsifying property; heat treatment; interfacial rheology; peanut protein isolate

In recent years, due to their properties of oil-water (O/W) interfacial absorption, good emulsification and texture, plant proteins have attracted increasing attention in the fields of food and pharmaceutics (Tergesen 2010; Qin *et al*. 2011; Xiao *et al*. 2014). Compared with animal proteins, plant proteins contain no cholesterol (Stone *et al*. 2001; Qin *et al*. 2011). Peanut protein isolate (PPI) is the most important plant protein second only to soybean protein in nature. PPI is mainly derived from defatted peanut flour (DPF). Compared with soybean protein isolate, PPI has higher nitrogen solubility index, higher soluble protein content and no anti-nutritional factors, and is easy to digest and absorb. Besides, PPI has good emulsifying, foaming, dispersion, gelation and other functional properties. Therefore, the utilization of PPI is highly promising

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in the food industry owing to its higher nutritional value, more desired functional properties and lower cost compared with other proteins (Feng *et al*. 2014).

Heat treatment is widely applied to alter the structure and functional properties of proteins, to enhance their adsorption at the O/W interface, and to promote the formation of a thin and gel-like layer (Nicolai *et al*. 2011). Peng *et al*. (2016) observed that pea protein heated at 95°C for 30 min showed higher surface hydrophobicity and interfacial pressure. Under 120°C heat treatment for 30 min, soybean protein showed enhanced surface activity and rapid intermolecular interactions in the adsorption layer, which is accordance with changes in the dilatational modulus and surface pressure (Wang *et al*. 2012).

Many researchers have studied the effects of isolation, preparation, modification techniques or other treatments on the structure and functional properties of PPI. Wu *et al*. (2009) investigated the effects of different isolation methods from DPF on the functional properties of PPI, including alcohol precipitation and/or isoelectric precipitation, and alkali solution with isoelectric precipitation. Gong *et al*. (2016) also reported the effects of different drying methods on the emulsifying and structure properties of PPI. He *et al*. (2014) and Zhang *et al*. (2014) studied the influence of high pressure treatment on the functional and physicochemical properties of PPI. Li *et al*. (2014) also observed the impacts of ultrasonic treatment and classical heating on the emulsifying properties of the glycation products of PPI and gum arabic or dextran. However, the influence of pre-heating on interfacial properties and structure of PPI has not been reported.

This study mainly investigated the influence of heat treatment on the structure and functional properties of PPI, including size distribution, secondary structure, surface hydrophobicity and interfacial adsorption rheology. Then, PPI-stabilized emulsions were prepared and their performance was evaluated.

MATERIAL AND METHODS

Preparation of PPI dispersions. Peanut protein isolate (PPI) was prepared from defatted peanut flour (DPF) by alkali solution and isoelectric precipitation using a freeze-drying method (Wu *et al*. 2009). The protein content of PPI was 85%. The reagent 1-anilinonaphthalene-8-sulfonate (ANS) was provided by Sigma-Aldrich (St. Louis, USA). Soybean oil was obtained from a supermarket in Wuhan (China). Other chemicals were of analytical grade unless otherwise stated. Milli-Q water was used throughout the experiments.

PPI solutions of 3 wt% were prepared by adding a certain amount of PPI powder into distilled water. The PPI dispersion was then stirred for at least 2 h under gentle agitation (300 rpm). The dispersion was left overnight at 4°C to ensure complete protein hydration and avoid the formation of protein aggregates.

The PPI dispersion was then treated at 70, 80 or 90°C for 30 min, and immediately cooled to ambient temperature. The obtained PPI samples were labelled as PPI-70, PPI-80 and PPI-90, and unheated PPI dispersion was named as PPI.

Size and zeta potential. Because peanut protein isolate has a pH near neutrality, size distribution and zeta potential of pre-heated PPI dispersions were measured by a Zetasizer Nano (Malvern Instruments Ltd., UK) after diluting with Milli-Q water. $D_{4,3}$ isconsidered to be the average particle diameter by volume, which was calculated from the following Equation (1):

$$D_{4,3} = \frac{\Sigma n_i d_i^4}{\Sigma n_i d_i^3} \quad (1)$$

where: n_i – number of droplets of diameter d_i; $n = 3$

Drop size ($D_{4,3}$) and size distribution of the emulsions were evaluated using a laser light diffraction instrument (Malvern 2000; Malvern Instruments, UK). The refractive index of the continuous phase and the dispersed phase was set to 1.333 and 1.45, absorptivity was set to 0.001; pump speed was set to 2000 rpm. Three scans were accumulated and averaged.

Surface hydrophobicity (H_0). Hydrophobicity (H_0) of the PPI was determined by a fluorescence probe using ANS according to the method of Delahaije and Xiong (Delahaije *et al*. 2013; Xiong *et al*. 2016). Three wt% PPI was dissolved in phosphate buffer solutions (PBS) (0.01 M, pH 7.0), then centrifuged at 10 000 rpm for 20 min to remove the insoluble matters. Afterwards, 0.05 wt% PPI was prepared by diluting the bulk PPI dispersion. Fifty microliters of ANS solution (2.4 mM) were added into 5 ml of 0.05 wt% PPI solution. The emission spectrum was tested from 400 to 600 nm by a fluorescence spectrophotometer (Shimadzu RF-5310PC; Shimadzu, Japan). The measurements were performed at 25°C and the excitation wavelength was 385 nm. Emission and excitation slits were set to 5 nm. The area of the fluorescence spectrum was first adjusted by the area

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of the buffer, and the corrected area of the sample represented the hydrophobicity.

Secondary structure. Secondary structure of PPI in solution was analysed using circular dichroism (*CD*) spectroscopy. The concentration of PPI samples was set at 0.05 wt% before analysis. Far-UV spectra of PPI dispersion were measured from 190 to 260 nm using a JASCO J-1500 spectropolarimeter (Jasco analytical instruments, USA) with a 0.1 cm path length quartz cell, a bandwidth of 2 nm and a scan rate of 100 nm/min. The proportion of four secondary structures (α-helix, β-sheet, turn, and unordered coil) was calculated using the CDPro software (Jasco Analytical Instruments, Japan) and Yang equation. Three scans were accumulated and averaged.

Interfacial adsorption rheology. The interfacial rheology of heat-treated PPI was determined according to the previous method (Wang *et al*. 2012). The experiments were performed using the Teclis Tracker (TeclicsScientific, France), and the temperature was controlled at 25°C. MCT（medium-chain fatty acids) were placed in a syringe, and the protein solution (1%) was placed in a cup and was allowed to stand for 3 h to achieve protein adsorption at the oil-water interface, and the surface pressure (π) was calculated by Equation 2:

$$\pi(t) = 2C_0KT\,(D_t/3.14)^{1/2} \quad (2)$$

where: C_0 – concentration in the bulk phase; K – Boltzmann constant; T – absolute temperature; D – diffusion coefficient; t – adsorption time

When the diffusion of proteins at the interface controls the adsorption process, a plot of π against $t^{1/2}$ shows a linear relationship, and the slope of this plot is the diffusion rate (K_{diff}).

Then, measurements of surface dilatational parameters, sinusoidal interfacial compression and expansion were performed by decreasing and increasing the drop volume at 10% of deformation amplitude ($\Delta A/A$) and 0.1 Hz of frequency, and surface dilatational modulus (E) and dilatational elasticity (E_d) were calculated by Equation 3:

$$E = [d\pi/(dA/A)] = -(d\pi/dlnA) = E_d + iE_v \quad (3)$$

where: E – surface dilatational modulus; A – surface area; E_d – dilatational elasticity; E_v – surface dilatational viscosity

Optical microscopy of emulsions. Oil-water emulsions were prepared by mixing 3 wt% PPI dispersion (12 g) and soybean oil (3 g) in a glass bottle, and then homogenized using a high-speed homogenizer operating at 12 000 rpm for 5 minutes.

The microstructures of peanut protein emulsion were visualized using a Nikon Eclipse 80i with a Q-imaging camera with 40× objective. To observe under a fluorescence field, Rhodamine B was used to dye the protein molecules.

Emulsion stability. The emulsion stability was visually evaluated upon quiescent storage for 72 hours. Ten millilitres of each emulsion were stored in a glass tube at ambient temperature. In different storage periods, height of the serum (H_s) and total height of the emulsion (H_t) were recorded. The creaming index (%) was calculated by (H_s/H_t) × 100.

Statistical analysis. All experiments were performed using at least two freshly prepared samples. The results presented are means and standard deviations of these measurements. Statistical differences between samples were calculated using Student's *t*-test for independent samples.

RESULTS AND DISCUSSION

Size distribution and zeta potential of PPI dispersions. In general, the high temperature treatment first causes partial unfolding and aggregation of proteins, and then increases the size of protein particles (Kiokias *et al*. 2007; Wang *et al*. 2012). Figure 1 and 2 show that the particle size of heat-treated PPI dispersion was larger than that of unheated PPI

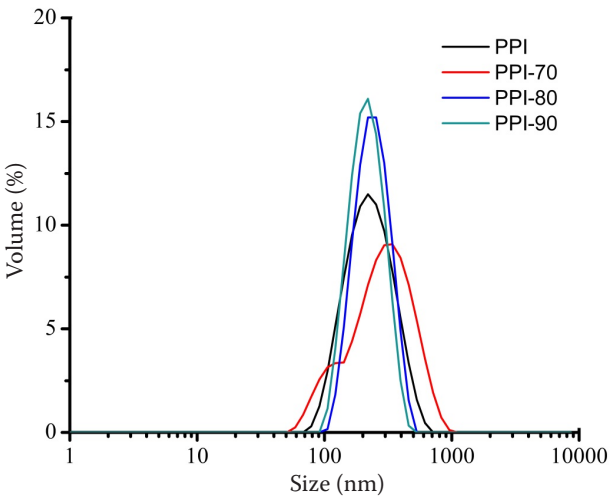

Figure 1. Distributions of protein particle sizes of unheated and heat-treated PPI

PPI – unheated; PPI-70 – heated at 70°C; PPI-80 – heated at 80°C; PPI-90 – heated at 90°C

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dispersion (Figure 1 and 2). The size of unheated PPI was 138 ± 1.32 nm, whereas that of PPI-70, PPI-80, PPI-90 was 176 ± 1.32 nm, whereas that of PPI-70, PPI-80, PPI-90 was 176 ± 4.26 nm, 203 ± 3.15 nm, 176 ± 4.52 nm, respectively. The protein size of PPI-90 was smaller than that of PPI-80, which might be due to the partial hydrolysis of protein aggregates induced by high temperature. These profiles confirmed that the heat treatment led to the aggregation or partial unfolding of proteins. Moro *et al.* (2011), Gonzalo *et al.* (2004) and Petruccelli *et al.* (1995) also confirmed that heat-treated soybean protein could form aggregates and showed a larger droplet size than unheated protein. But when the temperature was increased to 100 or 1200, the droplet size was decreased, which was speculated to be the result of partial hydrolysis of protein aggregates (Wang *et al.* 2012).

The zeta potentials of PPI under different heat treatments are shown in Figure 3. The heat treatment induced a decrease in the zeta potential because the heat treatment led to the aggregation or partial unfolding of proteins and decrease of free SH. However, PPI-90 showed a slightly increased zeta potential compared with PPI-80, which may be associated with the partial hydrolysis of protein aggregates and the conversion of S-S to free SH induced by high temperature.

Surface hydrophobicity. Surface hydrophobicity can be used not only to distinguish the structure changes and predict the surface activity of protein, but also it can influence the interfacial adsorption and emulsifying properties of proteins (Nicolai *et al.* 2011). Figure 4 shows that the H_0 values of heated PPI were higher than those of unheated PPI (117.33 ± 2.77), particularly PPI-80, which had the highest H_0 value (253.24 ± 2.47). Higher surface hydrophobicity of protein after heat treatment can be mainly attributed to the shifting of more hydrophobic groups to the surface, and indicates partial unfolding of protein (Croguennec *et al.* 2007). However, the surface hydrophobicity of PPI-90 was slightly lower than that of PPI-80. This may be attributed to the fact that more hydrophobic groups shifted to the interior of the protein molecules and the increase of free SH groups, which led to an increase in the interaction between ANS (1-anilinonaphthalene-8-sulfonate) and protein and further reduced the hydrophobic interaction between protein molecules. The result is consistent with the zeta potential of protein. Wang *et al.* (2012) and Guo *et al.* (2015) also reported similar results that the

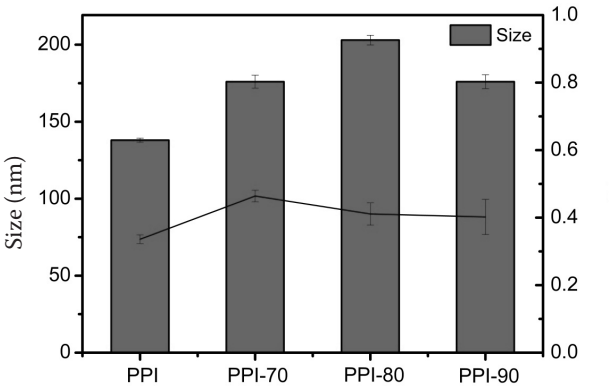

Figure 2. Values of protein particle size and PDI (particle distribution index) of unheated and heat-treated PPI

*For abbreviations see Figure 1

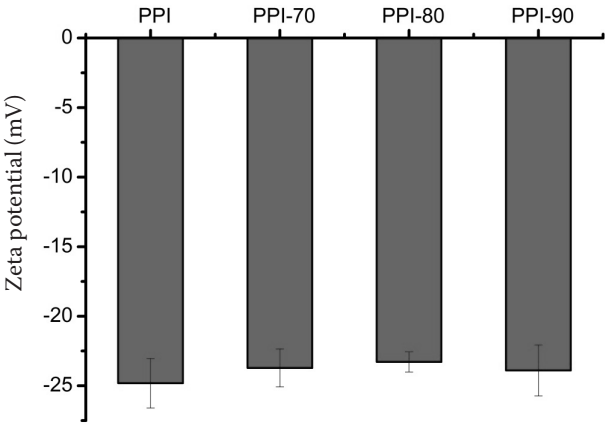

Figure 3. Zeta potential values of unheated and heat-treated PPI

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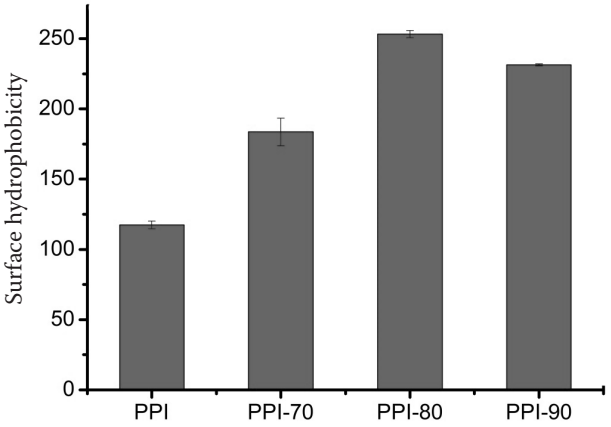

Figure 4. Surface hydrophobicity (H_0) of unheated and heat-treated PPI

*For abbreviations see Figure 1

heat treatment of soybean protein at 90°C resulted in higher H_0, but too high temperature (100 and 120°C) led to a lower H_0 (Wang *et a*l. 2012).

Secondary structure. Figure 5 shows changes in the secondary structure of unheated and heated

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PPI. Table 1 shows the proportions of different secondary structure fractions. All four samples have a peak at approximately 200 nm (unordered coil) and a peak at approximately 222 nm (α-helix). It can be observed that heated PPI had higher proportions of unordered coil structure and α-helix compared with unheated PPI. Moreover, PPI-80 had the smallest proportion of β-sheet structure, confirming that the unfolding degree was the highest. These results are consistent with the changes of particle sizes (Figure 1 and 2) and surface hydrophobicity values (Figure 4) of heated PPI. It has been reported that the changes in secondary structure are highly associated with the emulsifying and foaming properties of protein (Martinez *et al.* 2007). The heat treatment would result in a more flexible conformation because of the partial unfolding of protein, which then leads to the increase of surface hydrophobicity and emulsification. Moreover, previous studies proved that protein aggregation or unfolding contributes to the increased adsorption of protein at the O/W interface (Wang *et al.* 2012).

Interfacial adsorption rheology. Graham *et al.* (1979) proposed that the adsorption kinetics of protein at the *O/W* interface can be illustrated by three steps: first, protein diffusion to the *O/W* interface; second, true adsorption of protein to the interface; and third, reorganization of protein at the interface. Murray *et al.* (2002) reported that the protein adsorption process is often associated with some factors, such as protein hydrophobic structure and protein rearrangement at the *O/W* interface.

Figure 6 shows the change of surface pressure (π) in unheated PPI and heated PPI. The protein adsorption at the interface led to the increase of π-values with adsorption time ($t^{1/2}$). Compared with unheated PPI (K_{diff} = 0.1654), PPI-90 (K_{diff} = 0.1759) displayed similar adsorption kinetics with slightly higher K_{diff}, but PPI-70 showed an obviously lower K_{diff} (K_{diff} = 0.1425) and a slow increase of π value. PPI-80 showed a lower initial π value and a higher K_{diff} (K_{diff} = 0.1933). Compared with PPI-80, PPI-90 exhibited a slightly lower K_{diff}, which is the result of partial hydrolysis of protein aggregates and the increase of zeta potential. These results of K_{diff} may be related to the unfolding and flexible structure formation of heated PPI. It is well known that heat-treated protein is composed of non-aggregated and aggregated protein, and non-aggregated protein is smaller in size than aggregated protein. Thus, the former exhibits higher diffusion coefficients and can rapidly reach

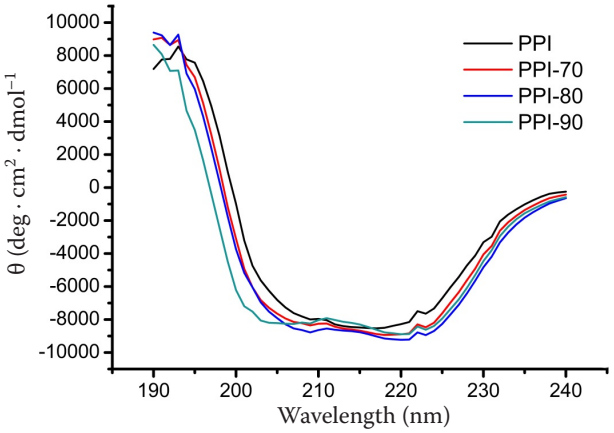

Figure 5. Circular dichroism spectra for dispersions of unheated and heat-treated PPI

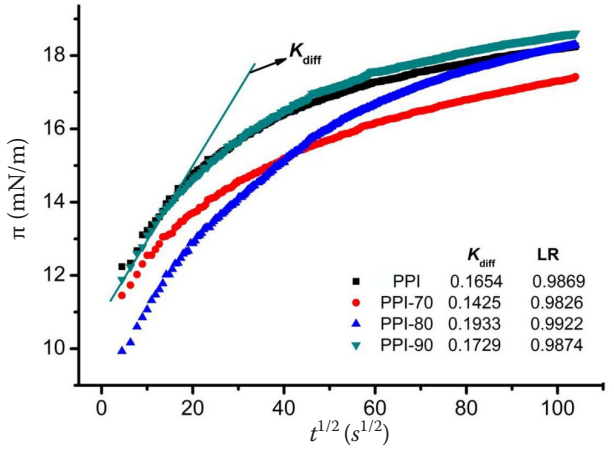

Figure 6. Square root of time ($t^{1/2}$) dependence of the surface pressure (π) of unheated and heat-treated PPI adsorbed layers at the oil-water interface

K_{diff} – diffusion rate; LR – linear correlation coefficient

the *O/W* interface. The first absorbed non-aggregated protein is considered to be responsible for the increase of π value in the first stage of adsorption (Croguennec *et al.* 2007; Mahmoudi *et al.* 2011). In this study, heat-treated protein exhibited a higher surface activity and a more flexible conformation, which then reduced the energy barrier for adsorption and further resulted in higher K_{diff} and π value at long-term adsorption. On the other hand, heat-treated protein can form aggregates and thus cause the increase of droplet size. The increased droplet size leads to the increase of steric hindrance and then inhibits the significant increase of dilatational modulus and interfacial surface pressure.

Figure 7 represents the dynamic elastic modulus of interfacial layers during protein adsorption. The E_d gradually increased with the protein adsorption time, which might be largely related to protein in-

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termolecular contacts and adsorption at the *O/W* interface. PPI-90 and PPI-70 showed slightly lower E_d values compared with unheated PPI; but it was the opposite case for PPI-80. These results explain the fast establishment of intermolecular contacts in the adsorption layer and are consistent with the results of K_{diff} (Figure 7). In fact, the molecular adsorption of protein to the *O/W* interface is associated with protein unfolding, and the consequent formation of intermolecular disulphide crosslinks and a more flexible conformation, which are the reasons for the increase of E_d in PPI-80. Moreover, PPI-90 showed the conversion of S-S to free SH and partial hydrolysis of protein aggregates, which then induced the decrease of dynamic elastic modulus in the interfacial layers during protein adsorption (Kyoko & Tokuji 1975).

The variations of dilatational elasticity (E) with π-value in the surface layer for the adsorption of unheated and heated PPI are displayed in Figure 8. Previous studies suggested that E increased with increasing π, which is related to the interactions among adsorbed protein molecules (Rodriguez Patino *et al.* 1999). It is obvious that heated PPI has a higher E value than unheated PPI at the same π value, especially under heat treatment at 80°C. It might be so because the heat-treated proteins possess higher surface activity. Moreover, this phenomenon suggested that heat-treated protein is absorbed to the *O/W* interface and then forms interfacial films, leading to the increment of E. Similar observations have been reported for other proteins, such as SPI and sodium caseinate (Li *et al.* 2011; Wang *et al.* 2012; Delahaije *et al.* 2014).

Size distribution of emulsions. The size distribution profiles of the emulsions are displayed in Figure 9. The microscopic structure of the emulsion droplets is demonstrated in Figure 10. It can be observed that the emulsion droplet size was significantly lower in the emulsion of heated PPI than in that of unheated PPI (23.48 ± 0.24 μm). Emulsion stabilized by PPI-80 had the smallest particle size of 12.63 μm. Thus, the heat treatment at 80°C for 30 min can be defined as the optimal denaturing conditions to ensure the formation of a more stable final emulsion. Moreover, emulsions of PPI-70, PPI-80 and PPI-90 showed particle aggregation but no flocculation compared with unheated PPI. The particle aggregation of emulsions stabilized by heated PPI may be due to the heat-induced increase of hydrophobic interactions of proteins and adsorption layers at the *O/W* interface. The interfacial layer then acts as an efficient physical barrier to reinforce the stabilization of emulsion. Many previous studies

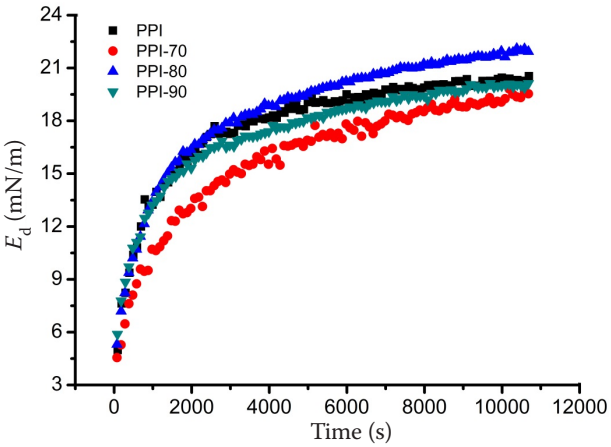

Figure 7. Time (t) dependence of the dilatational elasticity (E_d) of unheated and heat-treated PPI adsorbed layers at the *O/W* interface

*For abbreviations see Figure 1

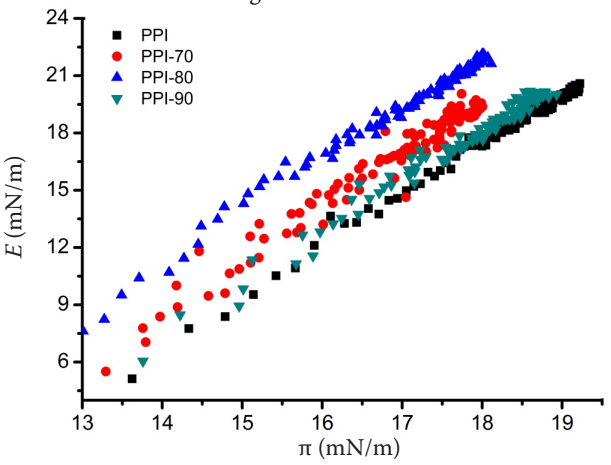

Figure 8. Surface pressure (π) dependence of the surface dilatational modulus (E) of unheated and heat-treated PPI at the *O/W* interface

*For abbreviations see Figure 1

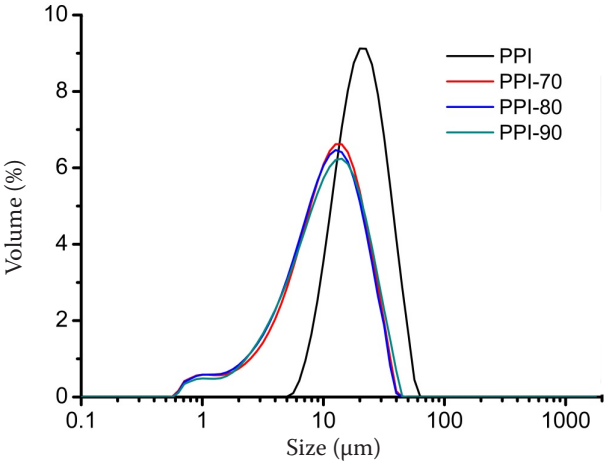

Figure 9. Emulsion particle size distributions for different conditions of unheated and heat-treated PPI

*For abbreviations see Figure 1

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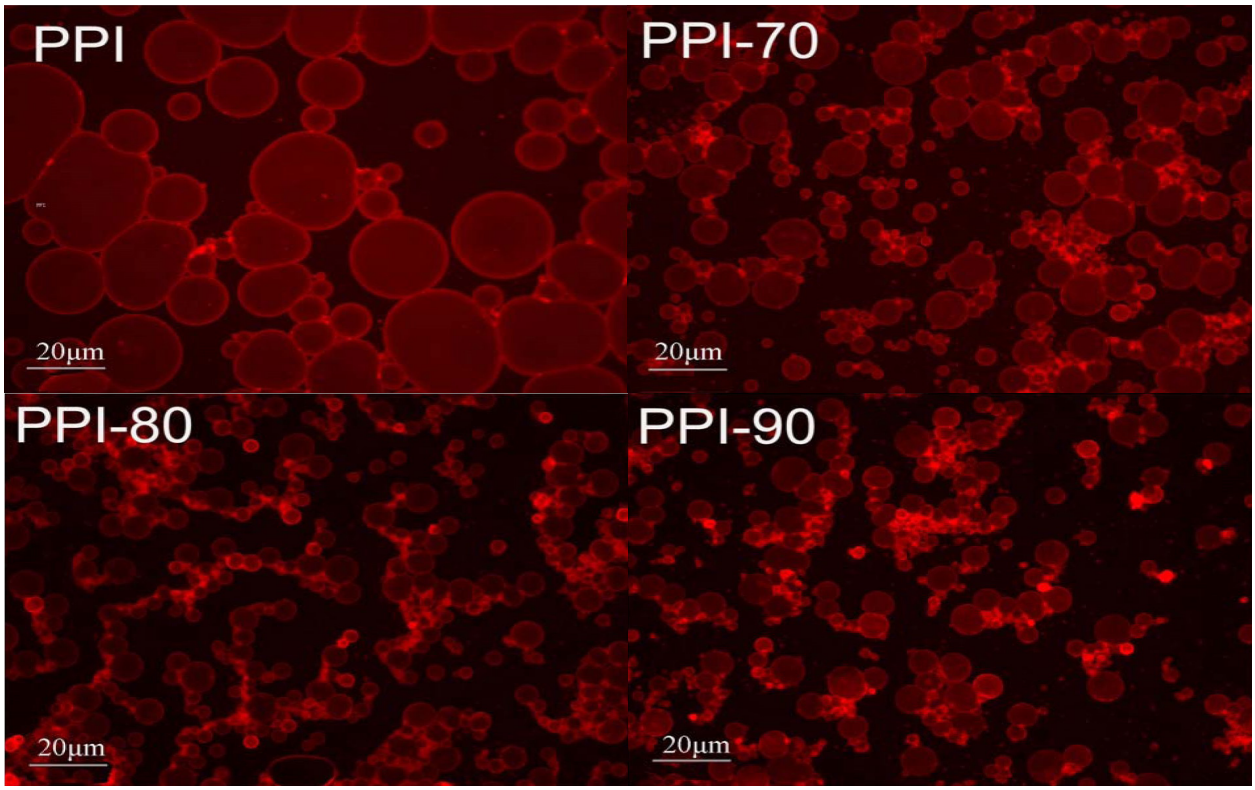

Figure 10. Fluorescence microscopy images with a 40× objective lens of emulsion stabilized by unheated and heat-treated PPI

verified that heat-treated pea protein, soybean protein and WPI showed smaller oil droplet sizes and higher stability of the emulsions than unheated proteins (Li *et al.* 2011; Ruffin *et al.* 2014).

Emulsion stability. The creaming index (CI) indicates the emulsion stability. Figure 11 represents the changes of *CI* for all emulsions within 72-h storage. The emulsions stabilized by heated PPI had higher stability against creaming than those stabilized by unheated PPI. PPI-90 exhibited higher stability against creaming and a larger particle size than PPI-80, which may be so because the higher zeta potential of PPI-90 increased steric hindrance and then improved emulsifying. These results are attributable to the increase of adsorption of particles at the *O/W* interface, which is accompanied by the increase of hydrophobic interactions between protein-coated oil droplets. In addition, the decrease of emulsion droplets can also enhance the emulsion stability (Stokes' law). Similar results were reported for SPI, pea proteins and WPI (Kiokias *et al.* 2007; Wang *et al.* 2012; Peng *et al.* 2016).

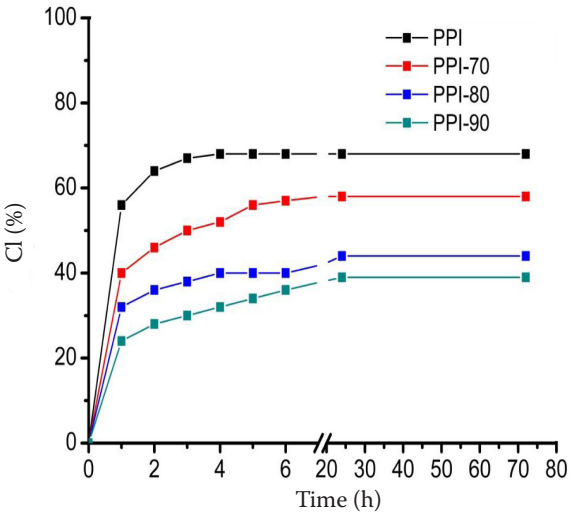

Figure 11. Creaming index (*CI*) for emulsions stabilized by unheated and heat-treated PPI

*For abbreviations see Figure 1

CONCLUSIONS

Heat treatment significantly influences the structure, interfacial adsorption behaviour and emulsifying properties of PPI. Compared with unheated PPI, heated PPI has higher surface hydrophobicity, dilatational modulus and surface pressure. More-

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over, emulsions stabilized by heated PPI exhibit higher creaming stability and smaller particle sizes due to the increase of hydrophobic interactions and adsorption of protein induced by heat treatment, especially heat treatment at 80°C. Important information about the influence of heat treatment on the PPI structure, interfacial behaviour and emulsifying properties is provided.

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