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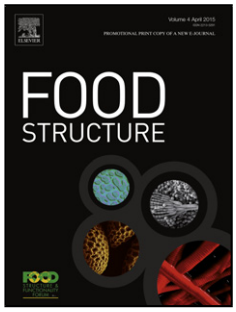

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HEAT TREATMENTS OF DEFATTED SOY FLOUR: IMPACT ON PROTEIN STRUCTURE, AGGREGATION, AND COLD-SET GELATION PROPERTIES

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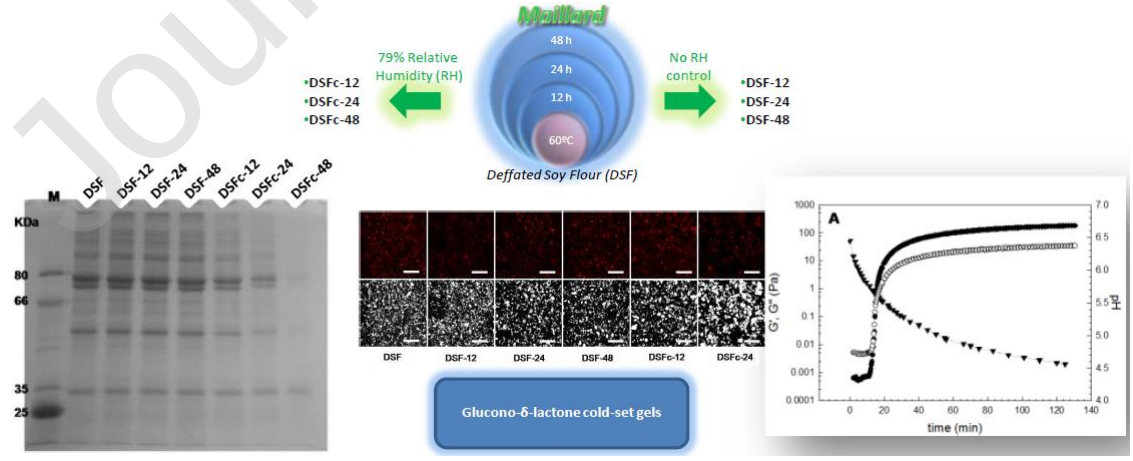
Graphical abstract



Highlights

- Maillard reaction was promoted in defatted soy flour under mild conditions
- Glycation was more evident under relative humidity control and for longer times
- Antitryptic activity of defatted soy flour decreased due to glycation
- Protein glycation was accompanied with protein aggregation and denaturation
- Glycation could not affect acid gelation as long as the protein solubility prevails
- Protein solubility loss led to weaker cold-set gels with bigger pores

Abstract

This study reveals that mild heat treatments of defatted soy flour promote Maillard reaction and modify its protein techno-functional properties such as solubility, aggregation, and cold-set gelation. Glycation was promoted by treatments of defatted soy flour (DSF) at 60ºC for 12, 24 and 48 h with and without relative humidity control (RHC and WRHC, respectively) at 79%. All samples presented a significant increase of glycation extent (GE), reaching the higher value after 48 h at RHC. Despite all samples presented a similar protein denaturation degree, the GE increment was accompanied by a decrease of antitryptic activity. Protein solubility (PS) of DSF remained constant for treated samples WRHC. However, PS decreased progressively with the treatment time at RHC. SDS-PAGE of soluble proteins revealed a positive relation between band intensities and PS. Despite sample dispersions showed a protein particle size increment with treatment time, further aggregation after heat-treatments at 100ºC produced a similar protein size distribution among samples. Rheological and microstructural studies of cold-set gels of samples obtained WRHC revealed no changes in the maximum elastic modulus (G'_{max}) and a slight increase of its pore sizes. However, samples obtained with RHC showed cold-set gels with a progressive G'_{max} decrease with the

treatment time, which could be related to a coarser gel microstructure. In the more extreme condition, the sample obtained after 48 h at RHC showed a total loss of gelation capability. These results can be used to address the development of new tofu-like food products with different rheological properties.

Keywords: glycation; humidity control; protein solubility; cold gelation

1. Introduction

Consumption of soy-based foods is increasing because of reported beneficial effects on nutrition and health. These effects are promoted by its protein and dietary fiber content and include the lowering of plasma cholesterol, prevention of diabetes and obesity, and protection against bowel and kidney disease (Friedman & Brandon, 2001; Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006; Xiao, 2011). In this context, and taking into account its low cost and functionality, defatted soy flour has become an interesting ingredient for the food industry (H.-H. Liu & Kuo, 2016).

Defatted soy flour contains more than 50% w/w of proteins and 35% w/w of carbohydrates in dry basis (Erickson, 2015). Soy proteins include 20% of biologically active proteins, such as α-amylase, lectin, urease, Kunitz and Bowman-Birk trypsin inhibitors (KTI and BBTI respectively), and 80% of storage proteins, which mostly include 7S and 11S globulins (β-conglycinin and glycinin, respectively). These latter are precipitable at pH 4.5 (Cheftel, Cuq, & Lorient, 1989). Furthermore, soy main carbohydrates include sucrose, oligosaccharides (raffinose, stachyose, and verbascose) and structural polysaccharides that are comprised of dietary fiber (cellulose, pectin-like acidic polysaccharides and hemicelluloses) along with mannans, galactans, and xyloglucans (Karr-Lilienthal, Kadzere, Grieshop, & Fahey, 2005).

On the other hand, the Maillard reaction has received much attention in recent years for protein modification in order to endow food proteins with improved functional properties. Besides the natural reactivity of carbohydrates and proteins, this reaction is promoted by the increase of temperature, water activity between 0.5 and 0.8, pH between 6.0 and 9.0 and the presence of salts (J. Liu, Ru, & Ding, 2012). Despite most of non-enzymatic glycation studies were performed with milk proteins and different sugars (Ding, Valicka, Akhtar, & Ettelaie, 2017; Hiller & Lorenzen, 2010; Jiménez-Castaño, López-Fandiño, Olano, & Villamiel, 2005; J. Liu et al., 2012; Q. Liu, Kong, Han, Sun, & Li, 2014; Spotti et al., 2014; Sun et al., 2011; S. Zhang, Gong, Khanal, Lu, & Lucey, 2017), there are numerous studies about soy protein glycation which also show an improvement in their functional properties (J. Liu et al., 2012; Xu et al., 2010; Xue, Li, Zhu, Wang, & Pan, 2013; X. Zhang et al., 2012). However, these latter focuses in soy protein isolate as starting material and carbohydrates which are not naturally present in soy flour.

The aim of the present work was to study the effect of different controlled heat treatments of defatted soy flour, as a strategy for protein modification via the Maillard reaction, on the structural, aggregation and gelling behavior of its constituent proteins. As a new approach, for the glycation reaction, we employed soy own carbohydrates which are naturally present in soy flour. As defatted soy flour contains active trypsin inhibitors, antitryptic activity after heat treatments was also assessed. It is expected that this protein modification will modify their behavior during cold-set gelation because of steric effects and hydrophilicity provided by carbohydrate moieties, which may result in cold-set gels with different rheological properties. These results can be used as the basis for the development of new tofu-like food products.

2. Materials and Methods

2.1. Materials

Glucono-δ-lactone (GDL), *o*-phthalaldehyde (OPA), *β*-mercaptoethanol (β-ME), benzoyl-DL-arginine-*p*-nitroanilide (BAPA), dimethyl sulfoxide (DMSO) and bovine serum albumin (BSA) were purchased from Sigma Co. (MO, USA). Other laboratory grade reactants were purchased by Anedra (Research AG, San Fernando; Argentina). Distilled water was always used in all assays, and all other chemicals were analytical grade reagents.

Defatted soy flour (DSF) without a thermal inactivation process was provided by Bunge Argentina S.A. (Puerto San Martín, Santa Fe, Argentina) and before use it was ground in a high-speed smashing machine (Chincan FW model; China) and sieved (ASTM-E-11-81 Zonytest; Buenos Aires, Argentina) to assure a maximum particle size of 500 μm of diameter. The chemical composition, expressed in dry basis, was: (55.1 ± 0.4) % w/w crude protein (micro Kjeldahl method, N×6.25) (Nkonge & Ballance, 1982), (7.7 ± 0.1) % w/w total ash~~es~~ (dry-ashing at 550 ± 5 °C), (20.6 ± 0.6) % w/w insoluble dietary fiber, (3.3 ± 0.2) % w/w soluble dietary fiber and (10.0 ± 0.2) % w/w oligosaccharides (AOAC 2009.01 method (McCleary et al., 2012)). The moisture content was (8.4 ± 0.8) % w/w. According to the provider, the total oil content was less than 1.0% w/w.

2.2. Heat treatment of DSF

DSF was subjected to heat treatments at (60 ± 2) ºC in a forced draft oven (Memmert; Schwabach, Germany) at controlled relative humidity (RH) at standard atmospheric pressure in a desiccator containing a saturated KBr solution (RH=79%) (Xu et al., 2010), and at standard atmospheric pressure with no RH control, during 12, 24 and 48 h

(samples DSFc-12, DSFc-24, DSFc-48 and DSF-12, DSF-24, DSF-48, respectively). For comparative purposes, DSF with no heat treatment was used as a control sample.

2.3. Sample characterization

2.3.1. Protein solubility (PS)

Aqueous dispersions were prepared by dispersing the samples in distilled water (3.0 % w/w crude protein) and centrifuged at 10,500 × g during 20 min (Beckman Coulter GS-15R centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). Protein content was determined in the supernatants (soluble protein) according to the Micro Kjeldahl method (N×6.25) (Nkonge & Ballance, 1982). PS was expressed as g of soluble protein per 100 g of dry sample.

2.3.2. Glycation extent (GE)

As reported by Liu et al., free amino groups determination based on the *o*-phthaldialdehyde (OPA) assay is an effective way to know the glycation extent by Maillard reaction (J. Liu et al., 2012). Therefore, the glycation extent was determined according to this spectrophotometric method, which is based on the reaction of OPA and β-ME with primary amino groups (Church, Swaisgood, Porter, & Catignani, 1983; Hernandez & Alvarez-Coque, 1992). Each sample was dispersed in 1.0 % w/v SDS, heated at 100ºC for 30 min, cooled in an ice-water bath and subsequently centrifuged at 800×g for 30 min (Beckman Coulter GS-15R centrifuge, Beckman Coulter Inc.; Fullerton, CA, USA). Supernatants were mixed in a volume ratio of 7:60 with the OPA reagent (8.0 mM OPA, 0.2 % v/v β-ME, 50 mM sodium tetraborate, 1.0 % w/v sodium dodecil sulfate, SDS) and the absorbance at 340 nm was read after 2 min incubation at room temperature. 8.0 mM L-lysine hydrochloride in 1.0% w/v SDS was used as the stock solution to perform the calibration procedure (Hernandez & Alvarez-Coque,

1992). Finally, the nitrogen content was determined according to the Micro Kjeldahl method (Nkonge & Ballance, 1982). The GE was then calculated for each sample as a percentage of reactive-Lysine (RL, in g Lys/16 g N) loss respect to the control as follows:

(1) $$GE\,(\%) = 100\% \times (1 - \frac{RL_S}{RL_C})$$

where RL_S and RL_C are RL (g/16 g N) for the sample and the control (DSF without treatment), respectively.

2.3.3. Trypsin inhibitor activity (TIA)

The antitryptic activity was determined according to the method of Liu and Markakis with slight modifications (K. Liu & Markakis, 1989). The sample extraction was performed by magnetic stirring with distilled water (0.1% w/v) for 30 min. The sample dispersion was mixed with an equal volume of 50 mM Tris-HCl buffer (pH 8.2) with 10 mM $CaCl_2$ and then filtered through a Whatman No. 2 paper. The filtrate was diluted with distilled water so that 1 mL corresponded to 30–70% trypsin inhibition. The diluted filtrate (1.0 mL) was then mixed with 2.0 mL of BAPA 0.92 mM (in DMSO) and 0.5 mL of trypsin solution (50 μg/mL in 1.0 mM HCl with 2.5 mM $CaCl_2$). After 20 min of incubation at 37ºC, the reaction was stopped by adding 30% w/w acetic acid and absorbance at 410 nm was read (A_{410}^{S}). In order to measure the trypsin activity in the absence of inhibitors, the diluted filtrate was replaced with 1 mL of distilled water; and the corresponding absorbance was A_{410}^{R}. Defining a trypsin unit as an absorbance increase at 410 nm of 0.01 under the assayed condition, the TIA was expressed in trypsin units inhibited per milligram of dry sample (TUI/mg) and calculated as follows:

(2) $$TIA\,(TUI/mg\,dry\,sample) = \frac{[A_{410}^{R} - A_{410}^{S}]}{V_F \times C_F} \times 100$$

where V_F is the volume of diluted filtrate (mL) and C_F is sample concentration in the dilute filtrate (mg dry sample/mL).

2.3.4. Differential Scanning Calorimetry (DSC)

Sample dispersions (30% w/w in 1.0 M NaCl) were hermetically sealed in standard hermetic pans. Thermograms were obtained at a 5 ºC/min heating rate in the range of 20-120 ºC using a Q200 calorimeter (TA Instruments Waters LLC; New Castle, DE, USA). An empty pan was used as reference. Partial and total denaturation enthalpy (ΔH, J/g protein) were obtained from each thermogram.

2.3.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All samples were evaluated by SDS-PAGE in reducing and non-reducing conditions in 4.0% and 13.0%, stacking and running gel concentration respectively, in a vertical gel system according to Laemmli (Laemmli, 1970). Samples were dispersed in distilled water (0.1% w/w) by magnetic stirring for 30 min. In order to evaluate the soluble fraction, dispersions were centrifuged at 10,000 × g for 20 min at 20ºC. Sample dispersions or their corresponded supernatant were mixed with sample buffer in a 1:1 volume ratio and heated at 95ºC for 20 min. Finally, SDS-PAGE gels obtained were scanned for further analysis. Densitometric analysis of the developed bands was performed using Image J Software (version 1.485) (Abràmoff, Magalhães, & Ram, 2004).

2.4. Confocal laser scanning microscopy (CLSM) of sample dispersions

In order to evaluate whether the protein particle size of sample dispersions could be affected by the different heat treatments of DSF at 60ºC, and also by the subsequent

heat treatment of sample dispersions at 100ºC for 5 min (as the first step for cold-set gelation), several images of each system were obtained by CLSM. Each sample dispersion was prepared in distilled water at 3.0% w/w crude protein and stirred for 30 min at room temperature. When corresponded, sample dispersions were heated at 100ºC for 5 min and immediately cooled in an ice-water bath. A small aliquot of Rhodamine B solution (0.05 mg/mL) was added to the dispersions in order to reach a final concentration of 0.002 mg/mL. 85 μL of Rhodamine B-stained dispersion was placed in compartments of LAB-TEK II cells. Digital images (10 for each system) were taken with a 40.0 × objective in a confocal laser scanning microscope (TE2000E Model, Nikon Instruments Inc., USA). All acquired images (1024×1024 pixel resolution) were storage in tiff format for further analysis. Each image is the mean image of several ones in order to avoid the random noise generated by the electronic components of the equipment. Finally, in order to perform a quantitative analysis of sample dispersions, protein particle size histograms of each system were obtained after thresholding operation with Image J software (Abràmoff et al., 2004) and Bone J plugin (Doube et al., 2010).

2.5. Rheological and microstructural analysis of cold-set gels

As described in the previous section, sample dispersions were prepared in distilled water at 3.0% w/w crude protein and stirred for 30 min at room temperature. As a first step for cold-set gelation, protein denaturation was promoted by heating at 100ºC for 5 min and the dispersion was immediately cooled in an ice-water bath to avoid further aggregation and precipitation. Acid gelation was induced by the addition of solid GDL in the heat-treated sample dispersions in order to achieve a final concentration of 1.5% w/w. Rheological properties were determined in a stress and strain controlled rheometer

(AR-G2 model, TA Instruments Waters LLC; New Castle, DE, USA) using a plate geometry (diameter 40 mm). Temperature was fixed at 25ºC with and controlled with a recirculating bath (Julabo model ACW 100, Germany) associated with the rheometer. Measurements were performed each 20.8 s during 120 min with a constant oscillation stress of 0.1 Pa and a frequency of 0.1 Hz. The Lissajous figures at various times were plotted to ensure that the measurements of storage or elastic modulus (G') and loss or viscous modulus (G'') were always obtained within the linear viscoelastic region. The G'-G'' crossover times (t_{gel}) of acidified systems were considered as the gel times and the pH at t_{gel} was also determined considering the pH value at the G'-G'' crossover (pH_{gel}) (Braga, Menossi, & Cunha, 2006). Also, the maximum storage modulus (G'_{max}) was determined. Once the system reached the equilibrium, a frequency sweep step was performed from 0.1 to 10 Hz.

In order to evaluate the microstructure of cold-set gels, Rhodamine B was added to sample dispersions after the heat-treatment at 100ºC. Then, acid gelation was induced by GDL addition, and 85 µL of each Rhodamine B-stained dispersion was quickly placed into compartments of LAB-TEK II cells. These systems were stored in a temperature-controlled room at 25ºC before image acquisition by CLSM. A pore size distribution of each image was obtained after thresholding operation as previously described in Section 2.4.

2.6. Statistical Analysis

All determinations were performed at least in triplicate and results were expressed as mean ± standard deviation. The statistical analysis was performed by analysis of variance (ANOVA) and Tukey test with Sigma Plot software (11.0 trial version).

3. Results and Discussion

3.1. Sample characterization

3.1.1. Protein solubility and glycation extent

Protein solubility is an important characteristic for a functional application viewpoint such as gelation (Cheftel et al., 1989). Results obtained for PS for all assayed samples are shown in Table 1.

Table 1

DSF presented a PS in distilled water which represented 28% of the total protein content (Section 2.1). This is consistent with the presence of insoluble protein aggregates at isoionic pH (6.94 ± 0.01), which could be in part produced by the oil extraction with *n*-hexane during the soybean industrial processing (Erickson, 2015). On the other hand, all samples obtained after heat treatment of DSF without RH control did not show a significant decrease of PS ($p>0.05$). For a better understanding, the moisture of the starting material was measured in function of time of the heat treatments (data not shown). Results showed that without RH control almost total dehydration was reached before 12 h of heating. Therefore, the moisture loss rate of the starting material might be fast enough to promote molecular movement constraints and to prevent protein-protein interaction that might lead to a decrease of PS. In contrast, when the heat treatment was carried out with RH control (79%) the PS gradually decreased with the increase of heating time. Undoubtedly, the presence of water molecules in this condition allowed protein-protein interactions that promoted the formation of insoluble aggregates. This is in agreement with the moisture measurement as a function of treatment time at RH control: sample moisture increased from 8% w/w (initial DSF moisture content, Section 2.1) to 20% w/w after 12 h and remained constant throughout the rest of the heat treatment.

As described earlier in Section 2.3.2, the OPA assay was performed in order to determine the glycation extent promoted by the Maillard reaction. Table 1 shows the GE (%) parameter obtained for all samples, which is based on the reactive lysine loss of each sample assayed respect to RL of the starting material (DSF). In general, all samples obtained after treatments at (60 ± 2) ºC presented a significant increase of GE by the Maillard reaction. DSF obtained after 12 h of heat treatment without RH control (DSF-12) showed (15.0 ± 0.2)% of glycation. However, 12 h later there was not a significant increase of GE and only a slight increase was observed after 48 h (17.25 ± 3.40 and 17.19 ± 1.27 GE (%) for samples DSF-24 and DSF-48, respectively). This may be explained taking into account the initial moisture content of DSF (8%), which corresponds to a water activity level that might promote the Maillard reaction between soy proteins and reducing carbohydrates commonly present in DSF. As stated before, DSF is almost dehydrated after 12 h of heat treatment, and therefore, water activity is not high enough to promote further glycation.

When heat treatment was performed with RH control, GE gradually increased with treatment time, reaching the higher value at 48 h. What is more, DSFc-48 presented a GE ~40% higher than its counterpart at no RH control (24.04 ± 1.74 against 17.19 ± 1.27 GE (%) for samples DSFc-48 and DSF-48, respectively). In this condition, water activity remains high enough to promote the Maillard reaction throughout the heat treatment. This is consistent with the moisture content of samples during the treatment at RH control (from 8% to 20%), as explained before.

3.1.2. Differential scanning calorimetry (DSC) and trypsin inhibitor activity (TIA)

DSC assays were performed with the aim of monitoring the denaturation degree of proteins in DSF during the heat treatments. All samples showed three endotherms

centered at ~71 °C (peak I), ~92 °C (peak II), and ~109 °C (peak III). According to Sorgentini & Wagner (1999), KTI is the most thermally unstable soy protein, presenting the lowest denaturation temperature. On the other hand, the thermal stability of whey soy proteins (KTI, and lectin) is slightly affected by ionic strength. Instead, when NaCl concentration increase from 0 to 1M, β-conglycinin, and glycinin present a significant higher thermal stability, presenting a temperature transition increment of 17ºC and 20ºC, respectively. In this context, peak I, II and III of our defatted soy flour dispersions in NaCl 1M may correspond to KTI, lectin/β-conglycinin, and glycinin, respectively (Sorgentini & Wagner, 1999).

Figure 1

Figure 1 represents the partial and total enthalpies obtained from DSC thermograms and TIA for DSF and samples obtained after heat treatment without (A) and with (B) RH control. At no RH control, partial enthalpy corresponding to peak I (ΔH_I) and peaks II + III (ΔH_{II+III}) decreased after 12 h without further changes (Figure 1 A), revealing some protein denaturation. This behavior is in good correlation with TIA and with the fact that after 12 h there were no important changes in PS and GE due to moisture loss kinetics (Section 3.1.1). It is interesting to point out that dry heating has no significant reducing effect on TIA, even at more than two times the temperature we used in DSF heat treatments (Barać, Stanojević, Jovanović, & Pešić, 2004). Therefore, the TIA loss after 12 h of mild heating at 60ºC may be attributed to a change in the configuration of a key region of the trypsin inhibitors due to glycation processes rather than a common thermal protein denaturation process.

At RH control (Figure 1B), ΔH_I decreased after 24 h whereas ΔH_{II+III} decreased in a similar extent as no RH control. Taking into account that storage globulins (β-

conglycinin and glycinin) are originally localized in the protein bodies of soybean, it is expected that glycation (and the consequent protein denaturation) involves more accessible proteins such as KTI, BBTI, and lectin among other biologically active proteins (Erickson, 2015). This could explain the significant and progressive decrease of TIA to ~25% its initial value after 48 h, which is correlated with GE (%) values (Section 3.1.1).

3.1.3. SDS-PAGE

Figure 2A shows the SDS-PAGE pattern of soluble proteins present in the supernatants obtained from sample dispersions of DSF and DSF obtained after heat treatments with and without RH control at different times.

Figure 2

In all cases, the load volumes were always the same. Line 1 to 7 show the typical band of AB subunit of glycinin (~56 kDa) and α, α' and β subunits of β-conglycinin (~79 kDa, ~72 kDa and ~47 kDa, respectively) (Arrese, Sorgentini, Wagner, & Anon, 1991; H.-H. Liu & Kuo, 2016). Also, the band at ~33 kDa corresponds to the naturally glycated subunit of lectin (Sorgentini & Wagner, 1999).

In general, band intensities of line 1 (DSF) prevail almost identical from line 2 to 4. This is in agreement with no PS changes among samples obtained after heat treatments without RH control (Table 1). On the other hand, bands from line 5 to 7 (DSFc-12, DSFc-24 and DSFc-48 samples) became less and less perceptible, which is in a good correlation with a PS decrease and a GE increase. This behavior was also reported by other authors for the Maillard reaction between glycinin and β-conglycinin with cross-linking agents (Yasir, Sutton, Newberry, Andrews, & Gerrard, 2007) and between milk proteins and several alimentary sugars after heat treatments (Chevalier, M Chobert,

Dalgalarrondo, Choiset, & Haertlé, 2002; Hiller & Lorenzen, 2010; Q. Liu et al., 2014), suggesting that protein constituents form cross-linked products of larger MW. This large protein aggregates could be noticed in SDS-PAGE of non-centrifuged dispersions as a more intense smearing toward the top of the concentration and stacking gel (data not shown).

It is worth highlighting that the perception loss mentioned above is not equal for all bands. For a detailed analysis, Figure 2B shows the absolute integrated OD of the main noticeable bands obtained by SDS-PAGE (Figure 2A). Clearly, for DSFc-12, DSFc-24 and DSFc-48 samples, the lectin subunit band does not tend to disappear in the same extension than α, α' and AB bands. This may be attributed to the carbohydrate moiety of this naturally glycated protein. Several authors reported that glycation promotes a decrease in the propensity of protein aggregation processes during heating because the presence of hydrophilic saccharides improve the affinity between proteins and water molecules and restrict protein-protein interactions (Xue et al., 2013; X. Zhang et al., 2012). Furthermore, Jiménez-Castaño et al. (2005) reported a protective effect of glycation between β-lactoglobulin and maltodextrin towards a decrease of β-lactoglobulin solubility due to self aggregation processes under heat treatment at 60ºC. As reported before, samples DSFc-12, DSFc-24 and DSFc-48 presented a protein solubility loss in the order DSF< DSFc-12< DSFc-24< DSFc-48. Therefore, this may be related to the formation of insoluble aggregates involving glycinin and β-conglycinin subunits and, in a progressive and lesser extent, lectin ones.

It is important to highlight that the behavior of lectin subunit stated above is not observed for β-conglycinin ones probably because β-conglycinin is assembled in protein bodies along with glycinin (Erickson, 2015). Therefore, β-conglycinin subunits might

be more predisposed to form insoluble protein aggregates during the heat treatment of DSF at 60ºC.

3.2. Protein particle size distribution of sample dispersions

As described in Section 2.4, protein particle size distributions were obtained by quantitative analysis of CLSM images of each sample dispersion (3.0% crude protein) (Figure 3).

Figure 3

All sample dispersions presented visible protein particles from a 1-2 μm to 25 μm (Figure 3). Interestingly, the global protein particle sizes increase when the treatment time of DSF at 60ºC increased, and in a greater extent, upon RH control at 79%. These increments might be related to the GE increase, but correlate better with the PS decrease (Table 1). As discussed above, at RH control, protein interactions promote the formation of insoluble protein aggregates. In contrast, at no RH control, the absence of water molecules limits protein diffusion and molecular movement, which is crucial for protein interaction and aggregation.

Figure 4 shows CLSM images of sample dispersions after heat treatment at 100ºC. All samples showed higher protein particle sizes than their counterpart before heat treatment. Evidently, protein denaturation at 100ºC promoted further protein aggregation. This protein global size increase was by far more evident in samples obtained without RH control at 60ºC and in the control sample DSF. This difference may be explained by the fact that these samples showed a higher content of soluble protein capable of participating in further aggregation processes (Table 1). It is also interesting to notice that this more evident global size increase after the heat treatment at

100ºC conducted to a similar protein particle size distribution among all samples assayed.

Figure 4

3.3. Cold-set gels

All sample dispersions (3.0% w/w crude protein), except for DSFc-48, formed cold-set gels after the heat treatment at 100ºC for 5 min and GDL addition (1.5% w/w). These gelling systems showed a similar G' and G'' modulus evolution upon acidification. At the early stage of acidification (at initial times) G' and G'' remained in small values and the dispersion showed a liquid-like behavior, since G'<G''. After some time, both moduli suddenly increase and reach a plateau value, where G'>G'', indicating a solid-like behavior and thus, a protein network formation. Is at the beginning of this latter stage where G' equals G'', allowing the crossover time obtention (t_{gel}). As an example, Figure 5A shows G', G'', and pH values as a function of time of DSF control sample during cold-set gelation. Also, in order to elucidate the physical structure of the gels obtained, a frequency sweep step was performed when the system reached the equilibrium (Stading & Hermansson, 1990). In this context, Figure 5B shows G' and G'' behavior upon frequency changes (0.1 to 10 Hz) of DSF gelled system.

Figure 5

Figure 5B shows that both moduli increase with frequency. As an attempt to performing a more detailed analysis, the frequency dependence of G' was quantified for each cold-set gel mechanical spectrum by fitting the power law equation, Eq. (1), given below (Bi, Li, Wang, Wang, & Adhikari, 2013).

$$G' = K'\omega^{n'} \quad (1)$$

where ω, K' and n' are the angular frequency, the power law constant and the frequency exponent, respectively. All gelled systems showed n'>0 (data not shown), revealing the physical nature of cold-set gels, which is in contrast to covalent gels (n=0) (Stading & Hermansson, 1990). However, these values showed no significant differences among samples ($p>0.05$) ranging from 0.120 to 0.131, indicating only a same frequency dependence of G'.

As described in Section 2.5, G'-G'' crossover times (t_{gel}), pH at t_{gel} (pH_{gel}) and maximum storage modulus (G'_{max}) parameters were obtained for each gelling system and are shown in Table 2.

Table 2

After glycation, the amount of protein positive-charged lysyl residues is reduced (Liu & Zhong, 2013). Because of the fact that charged amino acid residues are mostly on the protein surface, glycation of ε-amino of lysyl residues by reducing saccharides is expected to reduce the amount of the protein net surface charge at a certain pH over the isoelectric point (pI) and/or the protein pI (Cabodevila, Hill, Armstrong, De Sousa, & Mitchell, 1994; Liu & Zhong, 2012; Liu & Zhong, 2013). Furthermore, the greater net charge on the protein, the greater electrostatic repulsion between molecules, thus preventing the interactions required to form a gel matrix (Totosaus, Montejano, Salazar, & Guerrero, 2002). In this context, all heat-treated DSF samples with GE from 13% to 21% (Table 1), were expected to present a lower pH_{gel} since constituent proteins would present a higher negative net charge and require lower pH values to aggregate and constitute the cold-set gel network. However, all these samples did not show significant differences ($p>0.05$) in pH_{gel} (Table 2), indicating that any changes produced in protein net charge by glycation did not significantly change the global electrostatic stability of soy proteins.

As expected, since cold-set gelation of all samples was set at a constant temperature of 25ºC, at the same crude protein, and GDL concentration, the pH decrease rate was the same for all systems (data not shown). In all cases, the minimum pH value was 4.5 ± 0.2, similar to the isoelectric point of glycinin and β-conglycinin. These latter facts may explain the no significant t_{gel} changes ($p > 0.05$) among samples (Table 2).

It is important to notice that although all samples obtained after DSF treatment at 60ºC showed an increase in protein denaturation (Section 3.1.2), hydrothermal treatments at 100ºC of their aqueous dispersions promoted 100% protein denaturation (data not shown). Therefore, changes in G'_{max} may be affected only by glycation extent and protein solubility.

Samples DSFc-12 and DSFc-24 formed cold-set gels with a lower G'_{max} than DSF in the order DSF > DSFc-12 > DSFc-24 (Table 2). Moreover, sample DSFc-48 showed a total loss of DSF gelation capability (Table 2). In addition, the microstructural analysis showed that this elasticity loss was accompanied by a noticeable increase of the pore sizes in the order DSF < DSFc-12 < DSFc-24 (Figure 6).

Figure 6

It is known that during cold-set gel formation, protein-protein interactions, required for the gel network assembly, involve mainly hydrophobic interactions instead of covalent ones (Campbell, Gu, Dewar, & Euston, 2009). As mentioned above, since lysyl residues are mostly on the protein surface (Liu & Zhong, 2012), a GE increase may promote a decrease of the protein surface hydrophobicity. This behavior was previously reported by Gu et al (2009) and Li et al (2016) for soy proteins glycated with different sugars. These changes in terms of protein surface hydrophobicity could explain the G'_{max} decrease due to a decrease of protein-protein aggregation by hydrophobic interactions. As a result, a coarser and weaker gel structure may be formed (Table 2 and Figure 6).

In addition, Liu & Zhong (2012) reported that protein glycation may provide steric hindrance against protein aggregation. This behavior may also promote the formation of weaker gels. This effect was reported by Gu et al. (2009) for soy protein isolates glycated with glucose and lactose. In a similar way, Spotti et al. (2014) reported that dextran-conjugated whey proteins presented heat-induced gels with lower G' values than mixed dextran/whey protein systems, indicating a difficulty of conjugates to participate in protein-protein association by disulfide bonds and/or hydrophobic interactions during the gel network assembly. Finally, in a more recent study, Zhang et al. (2017) reported that G' values of sodium caseinate GDL-induced gels were significantly lower in conjugated samples with maltodextrin in comparison with mixtures of maltodextrin/sodium caseinate.

Another important factor that can be responsible for the substantially and gradually decrease G'_{max} with the heat treatment time of DSFc samples (Table 2), is the gradually decrease of PS (Table 1). The formation of insoluble protein aggregates may induce a decrease of the local protein concentration, and thus a decrease of interparticle interactions during the gelation process, conducting to weaker gels with bigger pores (Figure 6). Furthermore, these insoluble aggregates may interfere with protein entanglement by disrupting gel network. This disrupting effect was reported by Liu, Chien & Kuo (2013). These authors found that smaller and more uniform fiber particle sizes in aqueous dispersions of soy flour improved the firmness of cold-set gels.

Last but not least, another factor that can promote a weaker cold-set gel formation with a coarser microstructure in DSFc samples can be related to the protein composition of the soluble protein fraction. In a previous study, we reported that a higher relative content of whey soy protein isolate (composed mostly of lectin and KTI) in a mixture with soy protein isolate at a constant protein concentration of 3% promoted the

formation of cold-set gels with bigger pore sizes with lower G'_{max} values (Ingrassia et al., 2013). Therefore, the relative lectin enrichment in soluble fractions with treatment time mentioned above (Section 3.1.3) may also be related to the aforementioned effect. On the other hand, cold-set gels obtained from samples glycated without RH control did not showed significant differences in G'_{max} ($p>0.05$) in comparison with DSF control sample (Table 2) despite their significant GE values (Table 1) and the slight increase in their cold-set gel pore sizes (Figure 6). Therefore, the decrease on protein surface hydrophobicity and/or the steric hindrance effects as a consequence of glycosylation mentioned above could not explain these last results. Thus, the GE of these samples may not be high enough to produce a critical change on the cold-gelling behavior of DSF soy proteins. Evidently, the main factor influencing the cold-set gelation properties of DSF after the heat treatment at 60ºC is the PS change, which in turn could be affected by the RH control.

All these findings are interesting from a technological application point of view and could be used as a starting point for further research.

4. Conclusions

Despite the fact that heat treatments of DSF were performed under mild conditions, the Maillard reaction promoted soy protein glycation with soy own carbohydrates. This was more evident under RH control and for longer treatment times. The GE increment was accompanied by a decrease of antitryptic activity. However, PS and DSC studies revealed that protein aggregation and denaturation processes occur in parallel. Considering our results, protein glycation might not affect the cold-set gelation behavior of DSF as long as PS prevails without significant changes. At RH control, a progressive and significant loss of PS with treatment time is also reflected as a progressive

weakening of cold-set gels along with a coarser microstructure, and, in extreme conditions, as a total loss of the capability of forming gels. Four phenomena may contribute to this behavior. Firstly, the formation of insoluble aggregates would sequestrate soluble protein which otherwise may be involved in network assembly during acidification after GDL addition. Secondly, for soluble glycated proteins, a decrease in surface hydrophobicity and a higher steric hindrance would reduce protein-protein interactions during the network assembly upon acidification. Thirdly, the presence of insoluble protein aggregates could interfere with protein entanglement by disrupting the gel network. Finally, and according to previous studies, a progressive higher relative content of soy lectin with treatment time in the soluble fractions (as shown by SDS-PAGE analysis) may also promote the formation of weaker cold-set gels with a coarser microstructure.

On the other hand, it is important to point out from a nutritional viewpoint that trypsin inhibitor activity decreased significantly mainly due to a glycation effect rather than protein denaturation. In this context, these results show that there is a balance between the inactivation of antitryptic activity and the rheological characteristics of the final cold-set gels obtained from heat-treated defatted soy flour.

These findings may allow the design of new tofu-like gel products with particular rheological characteristics by monitoring the glycation extension through time and RH control of the heat treatment.

Industrial relevance

This study evidence that mild heat treatments of defatted soy flour may promote not only the Maillard reaction but also a significant decrease of its antitryptic activity along with protein denaturation and aggregation processes. In addition, cold-set gels obtained

from these heat-treated defatted soy flours showed different rheological characteristics which underscore the differential contribution of this type of protein modification. Therefore, these findings may be used as the basis for the design of new tofu-like products.

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FIGURES CAPTIONS

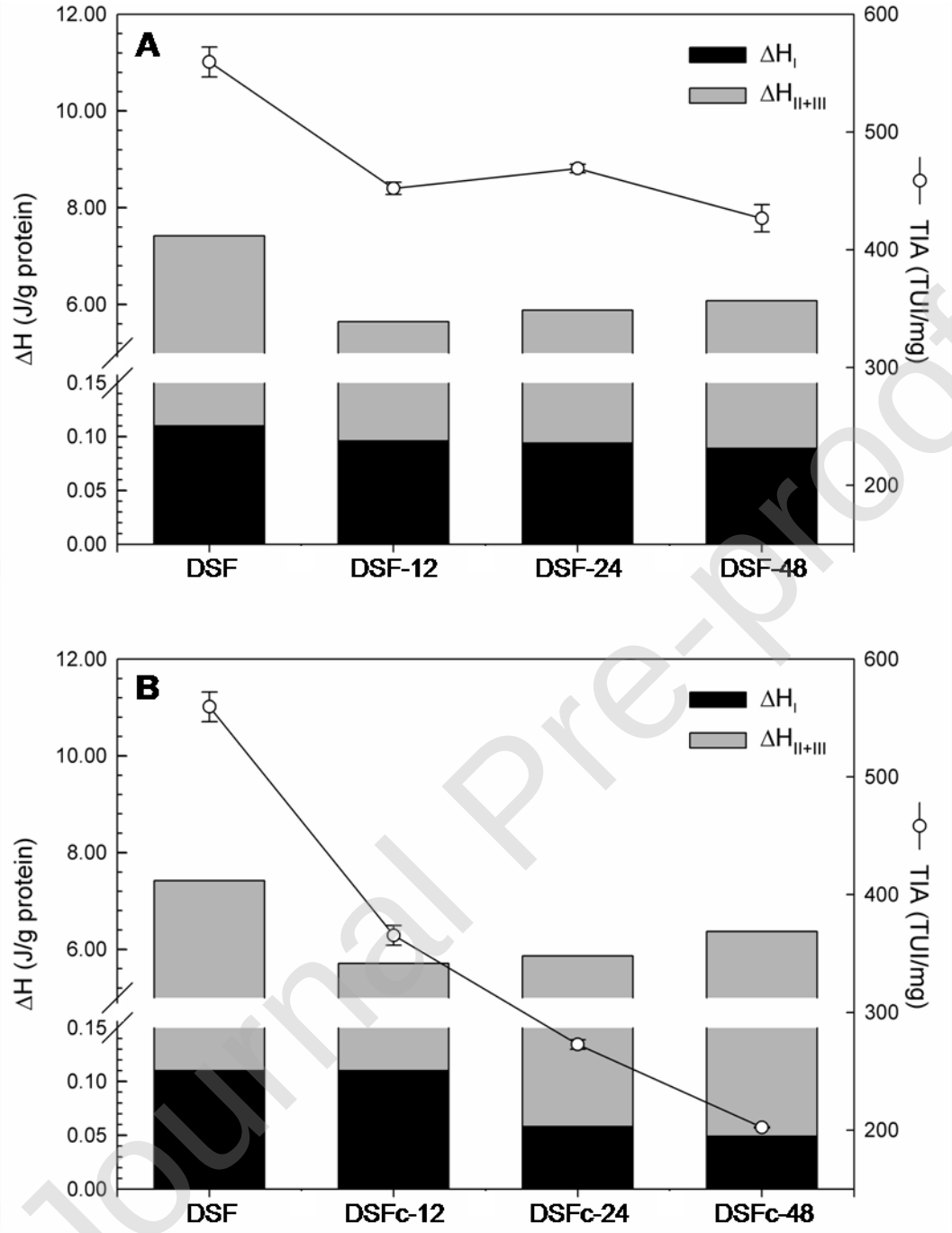

Fig. 1. Partial enthalpy for Peaks I and Peaks II +III (ΔH_I and ΔH_{II+III}, J/g protein) and total enthalpy (ΔH, J/g protein) obtained from DSC thermograms and Trypsin Inhibitor Activity (TIA, TUI/mg) of defatted soy flour (DSF) after heat treatment at (60 ± 2) ºC for 12, 24 or 48 h without (A) and with 79% relative humidity control (B): (samples

DSF-12, DSF-24 and DSF-48, and DSFc-12, DSFc-24, DSFc-48, respectively). DSF is the control sample (without heat treatment). ΔH_I and ΔH_{II+III} values presented a standard deviation < 5%.

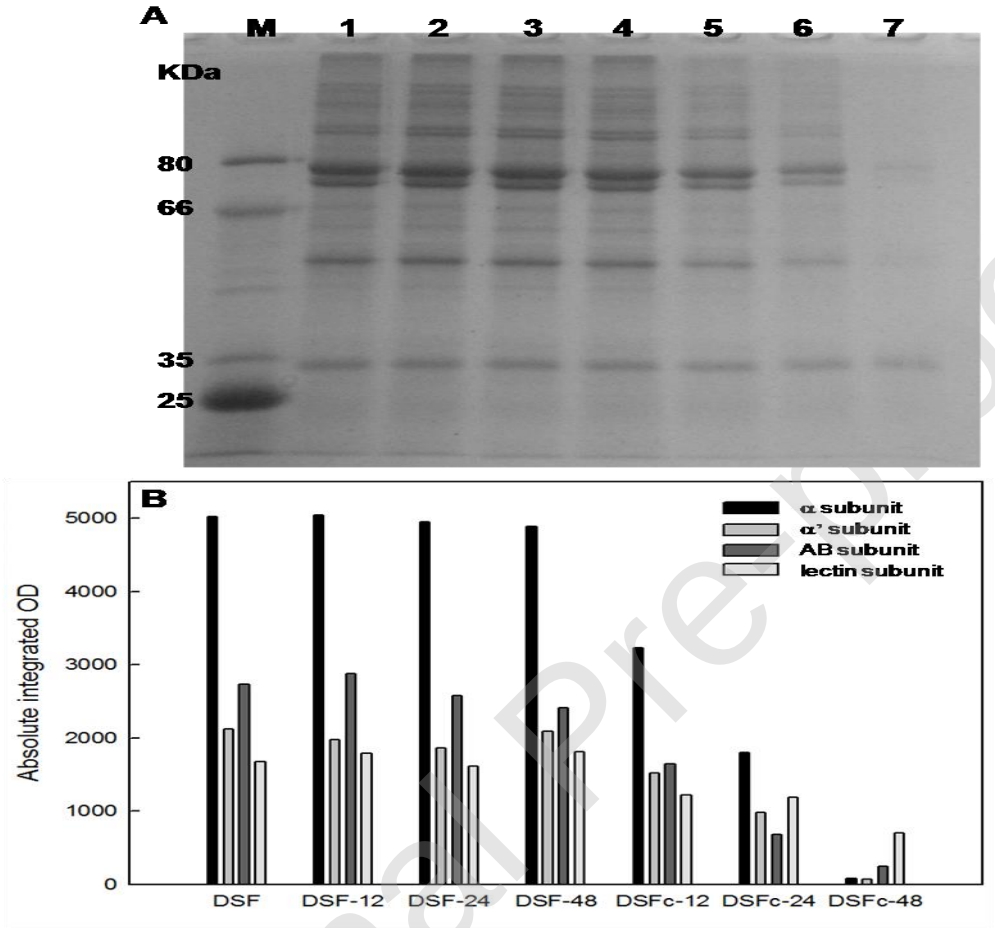

Fig. 2. (A) SDS-PAGE of the supernatants of sample dispersions (0.1% w/w). The labeled lanes are (M) molecular weight markers, (1) DSF, (2) DSF-12, (3) DSF-24, (4) DSF-48, (5) DSFc-12, (6) DSFc-24 and (7) DSFc-48. (B) Absolute integrated optical density (DO) for α and α' subunit of β-conglycinin, AB subunit of glycinin and L subunit of lectin.

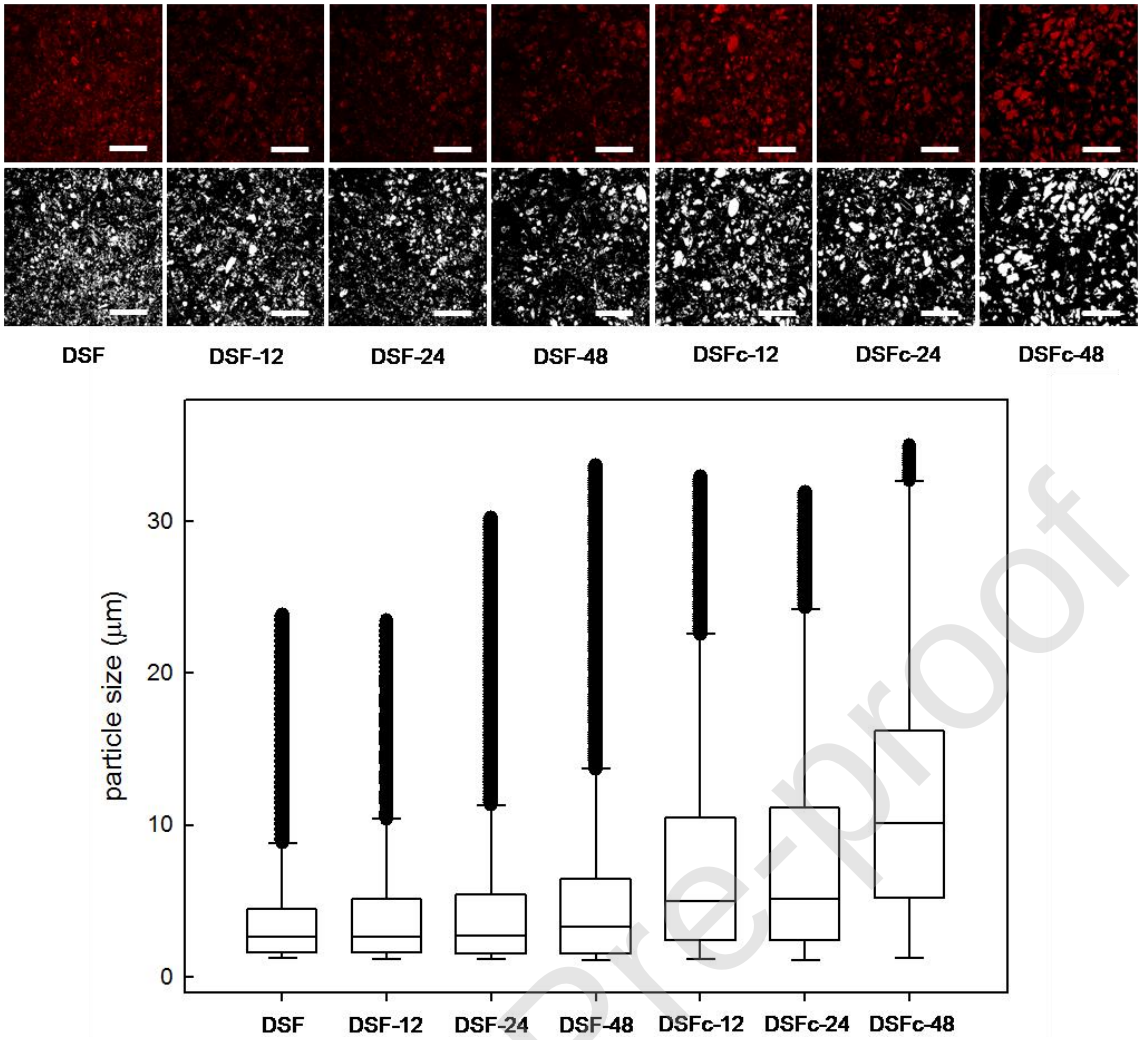

Fig. 3. Protein particle size distribution obtained from CLSM images (on top) of defatted soy flour (DSF) and heat-treated DSF dispersions (3.0% w/w crude protein) after thresholding process. Processed images obtained after threshold are also shown below their corresponded CLSM images. Image scale bars correspond to 150 μm. Heat treatment of DSF was performed at (60 ± 2) ºC for 12, 24 or 48 h without and with relative humidity control (79%): samples DSF-12, DSF-24 and DSF-48, and DSFc-12, DSFc-24, DSFc-48, respectively. DSF is the control sample (without heat-treatment).

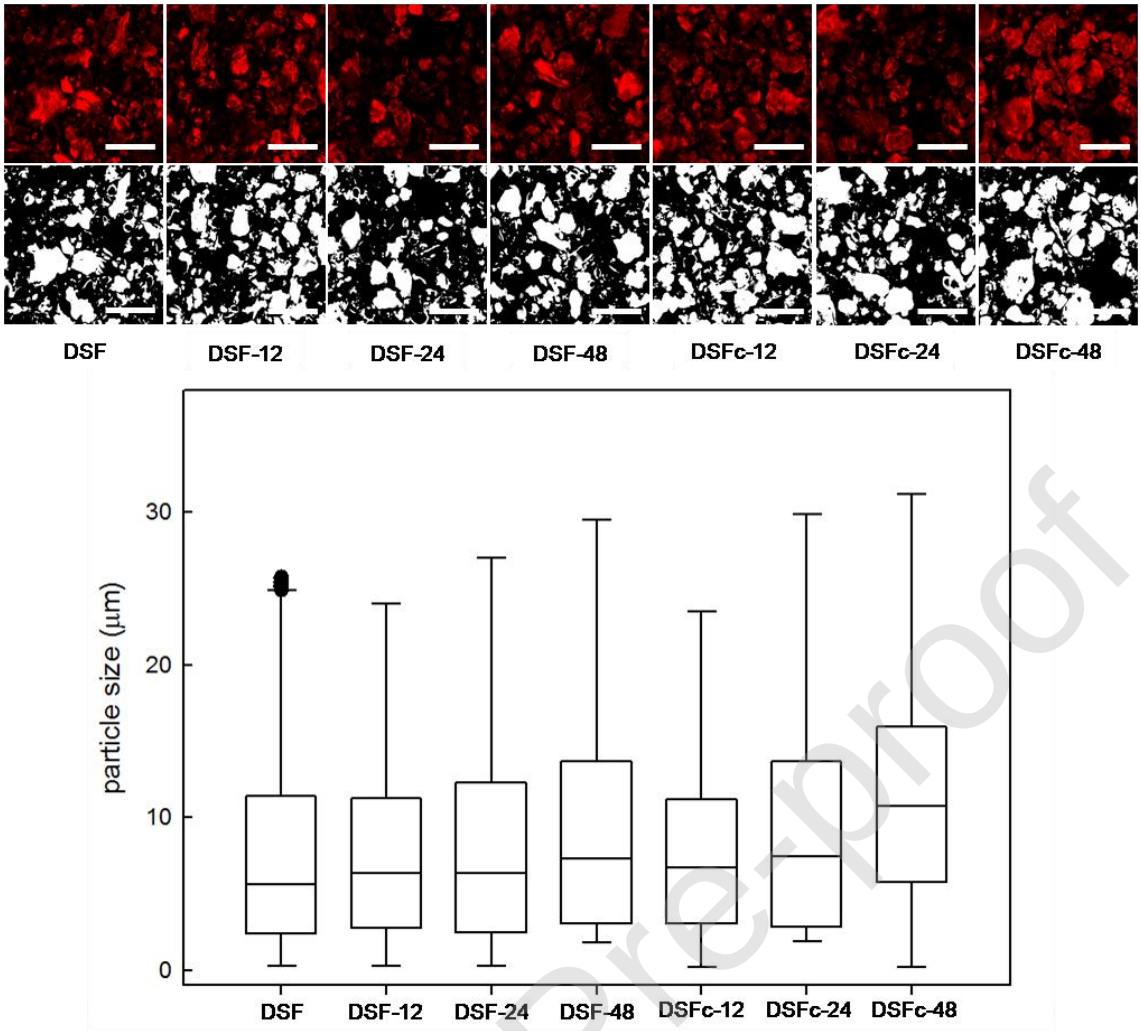

Fig. 4. CLSM images (on top) and their corresponded protein particle size distribution of sample dispersions (3.0% w/w crude protein) after hydrothermal treatment at 100ºC for 5 min. Processed images obtained after threshold are also shown below their corresponded CLSM images. Image scale bars correspond to 50 μm.

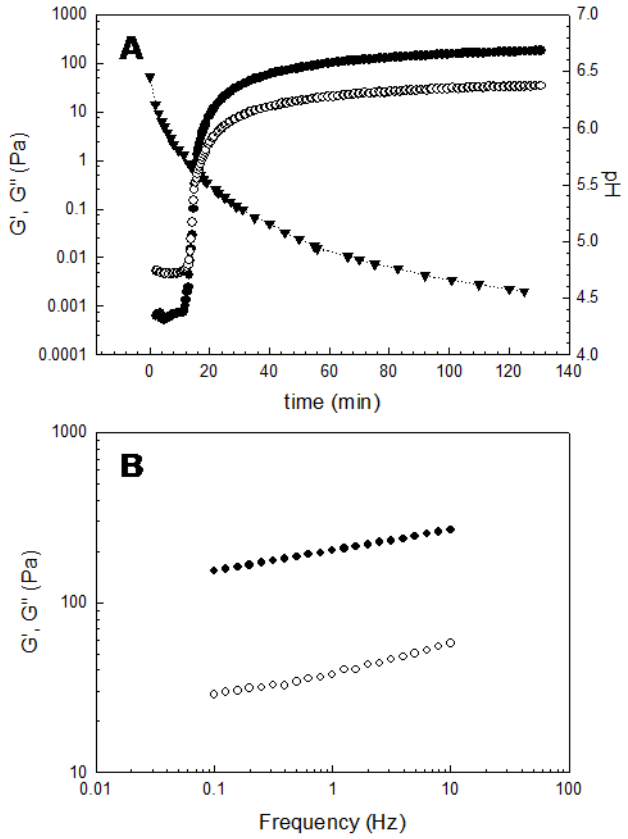

Fig. 5. (A) Rheological parameters G' (●) and G'' (○) modulus vs. time for defatted soy flour (3.0% w/w crude protein) after GDL addition (1.5% w/w) at 25ºC. (B) Angular frequency dependence of G' and G'' once system reached the equilibrium.

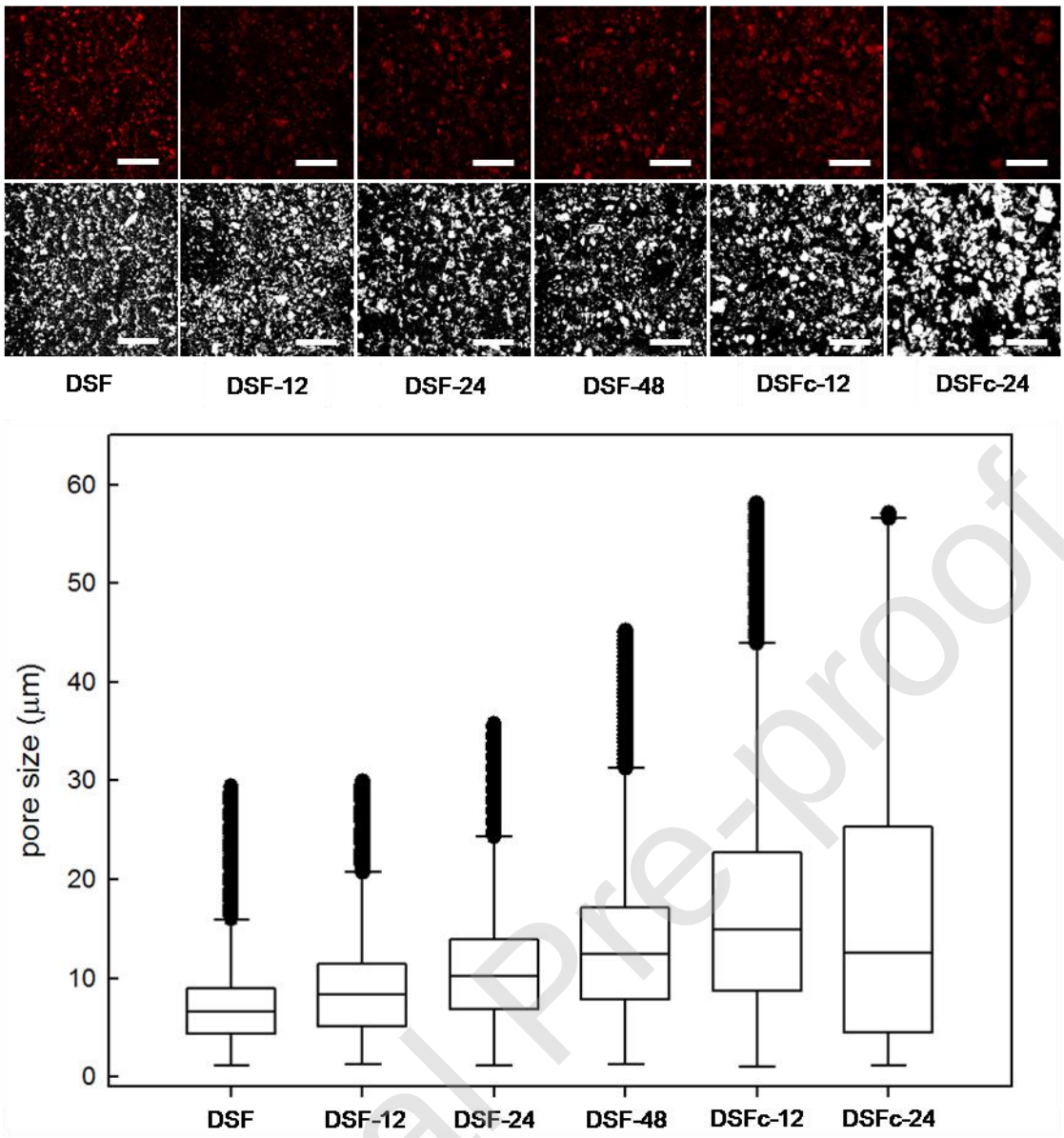

Fig. 6. CLSM images (on top) and pore size distributions of cold-set gels obtained from defatted soy flour (DSF) and heat-treated defatted soy flour dispersions (3.0% w/w crude protein) at 25 ºC after hydrothermal treatment at 100ºC for 5 min and GDL addition (1.5% w/w). Processed images obtained after threshold are also shown below their corresponded CLSM images. Image scale bars correspond to 150 μm.

TABLES

Table 1

Protein solubility in distilled water (PS, g soluble protein/100g sample) and Glycation Extent (GE, %) of samples obtained after thermal treatment of defatted soy flour (DSF) at (60 ± 2) ºC for 12, 24 or 48 h without and with relative humidity control (79%) (samples DSF-12, DSF-24 and DSF-48, and DSFc-12, DSFc-24, DSFc-48, respectively). DSF is the control sample (without heat-treatment).

Sample	PS (g/100g)	GE (%)
DSF	26.15 ± 1.13*,a	0.00
DSF-12	27.47 ± 0.19[a]	15.03 ± 0.20[a]
DSF-24	27.94 ± 0.51[a]	17.25 ± 3.40[a,b]
DSF-48	27.14 ± 0.82[a]	17.19 ± 1.27[b]
DSFc-12	20.10 ± 1.14[b]	13.35 ± 0.20[c]
DSFc-24	15.25 ± 0.25[c]	21.43 ± 5.17[d]
DSFc-48	6.88 ± 0.28[d]	24.04 ± 1.74[d]

*Mean value ± standard deviation (n=3). Means within the same column following by different letters are significantly different ($p<0.05$)

Table 2. Rheological parameters: t_{gel}, pH_{gel} y G'_{max} obtained from aqueous dispersions of heat-treated defatted soy flour (3.0% crude protein w/w) during acid induced gelation at 25ºC by GDL addition (1.5% w/w). DSF is the control sample (without heat-treatment).

Sample	t_{gel} (min)	pH_{gel}	G'_{max} (Pa)
DSF	14.4 ± 0.8*,a	5.66 ± 0.02[a]	159 ± 6[a]
DSF-12	16.9 ± 0.3[a]	5.5 ± 0.2[a]	176 ± 20[a]
DSF-24	16.0 ± 0.03[a]	5.67 ± 0.04[a]	180 ± 10[a]
DSF-48	16.0 ± 0.9[a]	5.56 ± 0.04[a]	200 ± 40[a]
DSFc-12	15.5 ± 0.1[a]	5.7 ± 0.1[a]	125 ± 10[b]
DSFc-24	19 ± 4[a]	5.70 ± 0.05[a]	74 ± 5[c]
DSFc-48	ND†	ND	ND

*Mean value ± standard deviation (n=3). Means within the same column following by different letters are significantly different ($p<0.05$). †ND = no detected.