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Combined effects of ionic strength and enzymatic pre-treatment in thermal gelation of peanut proteins extracts.

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Abstract:

Peanut proteins are mostly composed of arachins and conarachins, globular proteins that can form gels under thermal denaturation or enzymatic treatment. We explore here how ionic strength (0.5 M or 0.8 M) and gelation process (a thermal treatment preceded or not by an enzymatic pre-treatment) affect peanut protein gel properties. Gel formation and final properties are characterized by rheology, and gel structure by confocal microscopy. We show that the ionic strength imposed during protein extraction determines the arachins/conarachins ratio, and that conarachins-rich samples give stronger gels, which is attributed to their higher content in free thiol groups and lysine residues. The gel storage modulus exhibited a power-law dependence with the protein concentration, which exponent depends on the gelation process. Rheological results, together with confocal microscopy imaging, showed that an enzymatic pre-treatment results in denser structures.

Keywords:

Plant proteins, hydrogels, heat-set, transglutaminase, fractal dimension, structure, rheology, elastic modulus, confocal microscopy

1. Introduction

Peanut is a worldwide grown legume of high nutritional interest, characterized by a high energy density, about 550 kcal/100 g (Arya, Salve, & Chauhan, 2016). The major components of peanut are lipids (~50wt%), mostly composed of mono-unsaturated fatty acids (Brown, Cater, Mattil, & Darroch, 1975; Ciquel, 2017) associated with high HDL cholesterol level and improved heart condition (Feldman, 1999). Peanut is also rich in proteins (~25wt%), characterized by an elevated protein amino acid score (PDCAAS), between 0.5 and 0.7 (Suárez López, Kizlansky, & López, 2006). This value is nutritionally equivalent to the one of animal proteins for human health and proper growth (Arya et al., 2016; Toomer, 2017). Peanut proteins also present functional properties for emulsification, foam formation or gelation (Monteiro and Prakash 1994; Yu et al. 2007).

About 90wt% of peanut proteins are globular proteins, classified into three main families: the arachins, the conarachins I, and the conarachins II (Breese, 1917; Monteiro & Prakash, 1994). Arachins make up for about 66wt% of the peanut proteins. SDS-PAGE analysis shows the presence of 4 arachins subunits: 3 acidic subunits of 35 kDa, 37 kDa and 42 kDa and 1 basic subunit of 22 kDa (Hu, Zhao, Sun, Zhao, & Ren, 2011). Conarachins altogether make up for about 30wt% of the peanut proteins. Conarachins I (13wt% of the peanut proteins) are contained in the cytosol while conarachins II (16wt% of the peanut proteins) are contained in protein bodies (the aleurones, that also contain arachins). Their composition differ: conarachin I is a trimer composed of small molecular weight polypeptides (18 kDa, 17 kDa, 15,5 kDa), while conarachin II is a trimer of three 64 kDa units (Toomer, 2017).

In solution, globular proteins behave in their native configuration as colloidal objects stabilized by repulsive interactions (Boire et al., 2019). During food processing, thermal treatment enzymatic action or acidification, may result in protein gelation, giving rise to solid-like behaviors associated with appreciated textural properties (Totosaus, Montejano, Salazar, & Guerrero, 2002).

Thermal treatment requires a temperature higher than the protein denaturation temperature, so that proteins unfold and expose their buried hydrophobic amino acid residues. They subsequently aggregate into a percolating network through covalent and non-covalent interactions. For peanut flour suspensions, a thermal treatment above 90 °C results in gelation, the gels being harder as the flour (and protein) content increases (Davis, Gharst, & Sanders, 2007;

Kain & Chen, 2010). Increasing the ionic strength from 0 M to 0.5 M results in “weaker” gels (Schmidt, Illingworth, & Ahmed, 1978). A pre-treatment of the suspension with transglutaminase delays the gelation during thermal treatment, and results in softer gels (Gharst, Clare, Davis, & Sanders, 2007). A similar behavior is also observed in peanut protein isolate suspensions (Chen, Huang, Wang, Li, & Adhikari, 2016; Jiao, Shi, Liu, et al., 2018).

During enzymatic gelation, protein-protein covalent bonds are formed. For example, the microbial transglutaminase (mTG) forms ϵ -(γ - glutamyl) lysine intra- and inter-molecular bonds (Heck, Faccio, Richter, & Thöny-Meyer, 2013). This procedure was successfully applied on peanut protein isolates (Jiao, Shi, Wang, & Binks, 2018). Besides both arachins and conarachins are involved in the gel network, conarachins are more effectively cross-linked by mTG (Feng et al., 2014; Sun, Xiong, Bu, & Liu, 2012).

Acidification also induces gelation through the reduction of repulsive interactions between proteins as the protein net charge decreases. The storage modulus of peanut protein isolate gels formed by acidification follows a power-law behavior as a function of the concentration, that is reminiscent of fractal structures observed on colloidal gels (Bi et al., 2019).

The relatively low cost of peanuts, their interesting nutritional properties and the gelling capacity of their proteins paves the way for the production of new food products. Recent works reported on the formation of solid-like structures by processing grinded peanuts suspensions with transglutaminase (Basse, D’Huart, Lorand, Dupas, & Saiter, 2018; Patent No. WO 2019/073174 Al, 2018), or by combining a transglutaminase treatment with an heat/high pressure treatment (115 °C, 0.17 MPa) to form tofu-like gels (Guo, Hu, Wang, & Liu, 2018). Textural properties can be tuned by the nature and amount of proteins solubilized in the aqueous phase, that is ionic-strength dependent, and by the gelation process parameters, but this requires a better understanding of processing-structure relationship.

We investigate here the gelation of protein extracts representative of the protein fraction solubilized when a peanut suspension is grinded in salted (NaCl) water. These extracts are obtained at two ionic strengths, 0.5 M and 0.8 M, to produce suspensions with different conarachins/arachins ratio. We first investigate by rheology the gelation of such suspensions through a thermal treatment at 95 °C, preceded or not by an enzymatic pre-treatment, at the two ionic strengths. Then, gel structure is characterized by confocal microscopy.

2. Materials & Methods

2.1 Materials

Dehulled peanuts from a blanched Runner High Oleic variety are bought by bags of 25 kg (Bredabest BV, Netherlands), and stored in plastic containers of 25 kg under vacuum, at 4 °C.

Microbial transglutaminase (EC 2.3.2.13, Activa® EB, activity = 34 U/g) from *Streptoverticillium mobaraense* is supplied by Ajinomoto (Japan).

2.2 Methods

2.2.1 Sample preparation

2.2.1.1 Protein extraction and freeze-drying

Blanched white peanuts are first coarsely grinded for 2-3 min (MC15 grinder from Stephan, Belgium) by batches of about 5 kg. The resulting paste is stored at 4 °C in hermetically sealed containers and used within 4 months. For protein extraction, 295 g of this paste are mixed with 685 g of tap water and 20 or 32 g of sodium chloride (La Baleine Fine Sea Salt, France) to reach an ionic strength (IS) of 0.5 M or 0.8 M, respectively. This suspension is then mixed and finely grinded at room temperature for 15 min at 1100 rpm in a Thermomix TM5 (Vorwerk, Germany), to reach an average particle size of about 25µm.

The suspension is then dispatched in 50 mL centrifuge tubes, and centrifuged at 10 000 g for 2 h (Sigma 3-18K centrifuge from Fisher Scientific, USA). Three fractions were obtained: the supernatant, which contains most of the lipids, the pellet, which contains the insoluble particles, and the intermediate aqueous phase, which contains in particular the solubilized proteins. This intermediate fraction is turbid, suggesting the presence of proteins aggregates or impurities that are not eliminated by centrifugation, however, we choose not to purify further these extracts in order to keep all the solubilized/suspended proteins in the aqueous intermediate phase. The corresponding protein extraction yields, determined with the Kjeldhal method (see below) ranged from 41%±7% w/w (for IS = 0.5 M) to 54%±13% w/w (for IS = 0.8 M) of the total amount of proteins present in the mix. This fraction is collected, frozen at -80 °C, and freeze-dried until complete extraction of the water (Alpha 2-4 LD_{plus} freeze-dryer from Martin Christ, Germany). Freeze-dried protein powders are stored at 4 °C in sealed containers and used within 2 weeks.

2.2.1.2 Protein suspensions preparation

Protein suspensions from 4wt% to 16wt% are prepared by batches of 60 mL from the freeze-dried powder dispersed in distilled water under magnetic stirring for 15 min. To ensure constant ionic strength, suspensions are dialyzed in dialysis bags (Spectra/Por, molecular weight cut-off 3 kDa from Spectrum, USA) against a 0.5 M or 0.8 M NaCl solution (renewed three times) for 48 h. Protein suspensions (pH=6.2±0.2) are then collected and weighed to determine the dilution factor and assess their final protein concentration.

2.2.2 Protein characterization

2.2.2.1 Protein content

Nitrogen contents in the freeze-dried powders are determined in triplicate on 0.1g of sample, with the Kjeldhal method (Speed Digester K-439 from BUCHI, France), using a protein-nitrogen coefficient of 5.46 (Misra, 2001). The mineralization gradient is 300 °C for 20 min, 400 °C for 20 min, and 520 °C for 90min.

2.2.2.2 Total free sulphydryl content

Total free sulphydryl content (TFS) is determined in triplicate using Ellman's reagent. Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid, DTBN) at a concentration of 4 mg/mL is prepared in an urea Tris-glycine EDTA buffer (8 M urea, 0.086 M Tris, 0.09 M glycine, 4 mM EDTA; pH 8.0). 500 µL of the protein suspension with a protein concentration $C_p \approx 5\text{-}10$ mg/mL are mixed with 2.5 mL of buffer and 20 µL of Ellman's reagent. Samples are gently stirred for 15 min, before absorbance measurement at 412 nm (spectrophotometer Carry 100 UV-VIS, Agilent Technologies, USA). The TFS content is calculated using the following equation (Shen, Fang, Gao, & Guo, 2017), with D the dilution factor:

$$[\text{SH}]_{(\mu\text{mol SH / g})} = \frac{73,53 D A_{412}}{C_p}$$

2.2.2.3 Surface hydrophobicity

Surface hydrophobicity is determined in triplicate from fluorescence measurements (Shen et al., 2017), with the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS). Five protein solutions (of 4 mL) were prepared from 0.0025 % to 0.02% w/v in a phosphate buffer (8 mM, pH 7.0). The fluorescence intensity is measured after ANS addition (20 µL), for excitation and emission wavelengths of 390 nm and 470 nm, respectively (spectrophotometer Carry 100 UV-VIS, Agilent

Technologies USA). The relative fluorescent intensity (RFI) is determined as $RFI = (F - F_0)/F_0$, with F the fluorescence of the protein-ANS conjugate, and F_0 the fluorescence of the ANS probe alone. The surface hydrophobicity is calculated from the initial slope of RFI versus protein concentration.

2.2.2.4 Protein profile

SDS-Page gel electrophoresis is performed with a Mini-PROTEAN Tetra System, with Mini-PROTEAN TGX gels and Precision Plus Protein Unstained Standards from Bio-Rad (USA). Protein suspensions ($C_p=5 \mu\text{g}/\mu\text{L}$) are mixed with an equal volume of 2X Laemmli buffer (5% v/v β -mercaptoethanol, 40% v/v glycerol, 12.5% v/v Tris-HCl at 0.5 mol/L (pH=6.8), 20% v/v SDS 10% w/v, 1% v/v of bromophenol blue, and 21.5% v/v distilled water). A volume of 10 μL of sample is then loaded on each lane of the gel. Migration is performed at 200 V in a running buffer composed of 1% w/v SDS, Tris buffer (250 mM) and glycine solution (1.92 M, pH=8.3). After staining with Coomassie brilliant blue R-250 for one hour, gels are thoroughly washed in distilled water and scanned in a GS-800 Calibrated densitometer (Bio-Rad, USA), before image analysis with PDQuest 2-D Gel Analysis Software (v 6-2-1).

2.2.3 Gelation and gel characterization

Protein suspensions are all submitted to a thermal treatment (95 °C for 40 min), preceded or not by an enzymatic pre-treatment with transglutaminase. Whenever used, transglutaminase is added to the suspension under magnetic stirring for 5 min. Its concentration is always 0.3wt%, as this concentration induces the gelation of grinded peanuts suspensions (Basse et al., 2018). The sample naming is specified in Table 1. It distinguishes samples based on (i) the applied process (Thermal Treatment, TT or enzymatic + Thermal Treatment, eTT) and (ii) the Ionic Strength during protein Extraction (E) and (after dialysis) during Gelation (G). Due to the different dilution factors observed during dialysis, the lowest and highest protein concentrations (C_p) slightly differ from one sample to another, as indicate in the table.

2.2.3.1 Rheological characterization

Rheological tests are performed with a stress-controlled rheometer Physica MCR 301 (Anton Paar, Austria) in a Couette cell (sample volume = 25 mL, gap = 1 mm) equipped with a Peltier temperature controller. Gelation is monitored *in situ* under a constant oscillatory strain $\gamma = 1\%$

and at a constant frequency $f = 1$ Hz. Prior tests ensured that such low oscillatory strain did not affect gelation and remain in the linear regime for the final gel characterization. Whatever the gelation process, the suspension is first maintained for 5 min at 25 °C. For samples submitted only to a thermal treatment (TT), the temperature was then increased to 95 °C at a rate of 3.5 °C/min, then hold at 95 °C for 40 min, before being decreased to 25 °C at a rate of 3.5 °C/min. For samples submitted to an enzymatic pre-treatment (eTT), the temperature was increased to 40 °C at 3.5 °C/min, and held for 3 h at 40 °C: at this temperature, the enzymatic activity is about 60% and is stable over time (supplier's data). Then, the temperature evolution follows the TT procedure. Once gelation is achieved, a frequency sweep is performed at a strain $\gamma=1\%$, from $f=0.01$ Hz to $f=100$ Hz, followed by a strain sweep from $\gamma=0.01\%$ to 1000% ($f=1$ Hz).

2.2.3.2 Confocal Laser Scanning Microscopy (CSLM)

Confocal images are obtained with a TCS SP8 AOBS inversed confocal microscope (Leica, Solms, Germany) equipped with a helium-neon laser (458 nm excitation wavelength) and an argon laser (633 nm excitation wavelength). Protein labelling is performed with the commercially available chromophore DyLight 488 nm prepared at 0.5 mg/mL (Thermo Fisher Scientific, Waltham, MA, USA). Fragments of the gels are sampled with a spatula and laid on a 2 μ L drop of chromophore before observation.

3. Results and discussion

3.1 Protein extracts characterization

The protein concentration in the freeze-dried protein powders varied from batch to batch, from 35wt% to 49wt% and from 43wt% to 50wt% when extraction was conducted at 0.5 M and 0.8 M, respectively. In each batch, local heterogeneities were observed, therefore, for each suspension, C_p is determined based on the protein content of the single batch used for its preparation.

The protein composition of the two extracts at 0.5 M and 0.8 M determined by electrophoresis showed 25 bands, from 8 kDa to 250 kDa, assigned to the different subunits of conarachins and arachins as depicted in Figure 1. The band at 64 kDa, attributed to conarachins subunits (Toomer, 2017) is 1.6 times more intense in the proteins extracted at 0.5 M (lanes 2-4) than in the ones extracted at 0.8 M (lanes 5-7). In the opposite, the bands at 22 kDa, 37 kDa and 39 kDa, attributed to arachins subunits, are 3 to 5 times more intense in the samples extracted at 0.8 M

(lanes 5-7) than in the ones extracted at 0.5 M (lanes 2-4). The conarachins/arachins ratio is therefore ~5-8 times larger in the 0.5M extract than in the 0.8 M extract, which is consistent with previous results (Monteiro & Prakash, 1994). Considering this large difference, in the following, the 0.5 M extract will be named the conarachins-rich extract, and the 0.8 M extract the arachins-rich extract.

Thiol group content and surface hydrophobicity are relevant characteristics to assess proteins ability to form disulfide bond and interact through hydrophobic interaction, respectively. As conarachins have 2-3 times more sulfur-containing amino acid than arachins (W. L. Brown, 1942; Wang, 2017), it is expected that the conarachins-rich sample (0.5M extract) contains a higher amount of thiol groups. Indeed, the thiol content of the 0.5 M extract is $17.6 \pm 2.3 \mu\text{mol/g}$, against $11.5 \pm 2.0 \mu\text{mol/g}$ for the 0.8 M extract, these values being close to the one recently determined on Peanut Protein Isolate (X. Sun, Jin, Li, Feng, & Liu, 2018). By contrast, surface hydrophobicity is not significantly different between the two extracts: 248 ± 24 (AU) and 206 ± 27 (AU) for the 0.5 M and 0.8 M extracts, respectively.

3.2 Gelation and rheological properties

3.2.1 Evolution of the rheological properties during gelation

In this section, we discuss the rheological evolution of the sample during gelation, focusing only on the case in which the ionic strength (0.5 M or 0.8 M) imposed for protein extraction is also the one imposed during the gelation process. The latter consists in either (i) a thermal treatment at 95 °C for 40 min (TT process), or (ii) the same thermal treatment preceded by an enzymatic pretreatment with Transglutaminase at 40 °C for 3 h (eTT process).

All samples submitted to the TT process behave as usually observed for globular proteins (Spotti, Tarhan, Schaffter, Corvalan, & Campanella, 2017): in the initial suspension, the loss modulus G'' is higher than the storage modulus G' , then both moduli rapidly increase and reach a plateau with $G' \approx 10G''$ (Figure 2). When the temperature is decreased back to 25 °C, a further increase of the moduli occurs, with $G'_{25^\circ\text{C}} / G'_{95^\circ\text{C}} \approx 2-10$, whatever the gelation process or the ionic strength. It is attributed to an entropic effect (Eleya & Turgeon, 2000) and a reinforcement of hydrogen bonding and intermolecular forces while decreasing the temperature (Chronakis & Kasapis, 1993).

251 If the thermal treatment is preceded by an enzymatic pre-treatment (eTT process), the behavior
252 is profoundly different and depends on both the ionic strength and the protein concentration.

253 At the lowest protein content ($C_p \approx 4$ g/100 g), enzymatic pre-treatment itself is not associated
254 with any rheological signature: both G' and G'' remain constant, with the storage modulus that
255 is almost negligible owing to the liquid-like behavior of the suspension. However, when
256 submitted to the thermal treatment, the protein ability to form a gel is now limited: for the 0.8 M
257 extract, gel formation is inhibited, while for the 0.5 M extract, gelation still occurred but gel
258 strength ($G' \approx 3$ Pa) is lower than without pre-treatment ($G' \approx 6$ Pa).

259 At the highest protein content ($C_p \approx 11-13$ g/100 g), both extracts form a gel. For the 0.5 M
260 extract, gelation occurred during the enzymatic pre-treatment, giving rise to very weak gels
261 ($G' \approx 0.1$ Pa). The following thermal treatment significantly strengthen the gel, and the final
262 modulus is similar to the one obtained with thermal treatment only ($G' \approx 300-450$ Pa). By contrast,
263 for the 0.8 M extract, gelation is not observed during enzymatic pre-treatment (but a slight
264 increase in G' possibly suggests the formation of aggregates). Gelation occurred later on during
265 the thermal treatment, but the final modulus remains below the one of samples only submitted
266 to thermal treatment.

267
268 All gels showed finally a similar rheological signature, with $G'/G'' \approx 10$ and a slight moduli
269 dependence towards angular frequency ω : $G' \sim \omega^a$, with $0.08 < a < 0.2$ for $0.06 \text{ rad.s}^{-1} < \omega < 10 \text{ rad.s}^{-1}$
270 ¹ (results not shown). Strain sweep confirmed that measurements conducted at $\gamma=1\%$ were well
271 in the linear regime, however, we did not observe any evolution of the limit of linearity with the
272 protein concentration, gelation process or ionic strength (results not shown).

273 These first results show that both ionic strength and gelation process play a key role. The 0.5 M
274 extracts (conarachins-rich) exhibit a higher propensity to gel and result in firmer gels than the
275 0.8 M extracts (arachins-rich). The pre-treatment with transglutaminase results in gels with a
276 lower strength, and inhibit gelation at low proteins concentration. In the next sections, we will
277 investigate in details these effects.

3.2.2 Sol-gel transition

The sample gelation or sol-gel transition, determined as the cross-over between G' and G'' , is now investigated.

For suspensions only submitted to thermal treatment, gelation occurred during the temperature ramp, 20 to 30 min after launching the experiments for temperatures ranging from 60 °C to 95 °C. For both ionic strengths, the gel point decreases with the protein concentration C_p (Figure 3, top panels). The higher the C_p , the shorter the time required for the growing protein aggregates to span the entire volume, as observed for other globular proteins (Tobitani & Ross-Murphy, 1997).

For suspensions submitted to an enzymatic pre-treatment, the behavior depends on the ionic strength (Figure 3, lower panels).

For the $E_{0.5}G_{0.5}eTT$ samples (conarachins-rich), the gelation time depends on the protein concentration. At high concentrations ($C_p > 6\text{wt}\%$), most of the samples gelled in the first three hours, during the enzymatic treatment. At low concentrations, ($C_p < 6\text{wt}\%$), gelation only occurred later on, during the thermal treatment.

For the $E_{0.8}G_{0.8}eTT$ samples (arachins-rich), the scenario is completely different. Although it is known that the transglutaminase activity increases with ionic strength (Kütemeyer, Froeck, Werlein, & Watkinson, 2005), gelation always occurred during the thermal treatment (except for one single case). As arachins contain about 15 times less lysine (transglutaminase substrate) than conarachins (Brown, 1942; Wang, 2017), their reactivity under enzymatic treatment is much lower. During the thermal treatment, the gelation time decreases when the protein concentration increases, and the gelation temperature of the 0.8 M extract is higher, for a given concentration, than the one of the 0.5 M.

We also observe that the gelation time does not differ significantly whether gelation occurred at the ionic strength imposed for proteins extraction or at a different one under the experimental conditions investigated.

3.2.3 Fractal-like scaling of the storage modulus

One common pathway for proteins and colloids gelation consists in the fractal aggregation of individual elementary objects eventually forming a three-dimensional network (Ikeda, Foegeding, & Hagiwara, 1999; Wu & Morbidelli, 2001). A rheological signature is the power-law scaling of the plateau storage modulus of the gel with the protein concentration or volume

fraction. This has been observed for peanut-protein gels formed by acidification (Bi et al., 2019), but, as far as we know, never evidenced on heat-set gels of these proteins. Fractal dimensions can be estimated from the power-law exponent, with the following equation (Shih et al., 1990):

$$G' \propto \varphi^{(3+d)/(3-D)}$$

With G' the storage modulus of the gel (in Pa), φ the volume fraction of the proteins, d the fractal dimension of the backbone (between 1.1 and 1.3), and D_f the fractal dimension of the gel.

For samples submitted to a thermal treatment only, the storage modulus indeed follows $G' \sim C_p^x$, with $x=3.4$ (Figure 4). Applying the equation given above, we obtain a fractal dimension $1.74 < D_f < 1.82$, suggesting a diffusion-limited cluster aggregation regime (Lin et al., 1989). The fractal-like scaling is similar for both extracts, however, for a given protein concentration, conarachins-rich samples (0.5 M extracts) exhibit a higher storage modulus than arachins-rich samples (0.8 M extracts). This is attributed to the higher sulfur content of conarachins, resulting in a higher propensity to form disulfide bonds during thermal treatment.

For samples submitted to an enzymatic pre-treatment before the thermal treatment, the power-law scaling of the storage modulus is also found.

For the $E_{0.5}G_{0.5}eTT$ samples, the exponent is $x = 4.6$, which corresponds to a fractal dimension $2.07 < D_f < 2.13$, characteristic of a reaction limited cluster aggregation regime, during which dense clusters are formed (Lin et al., 1989). Beside low concentration samples gelled during the thermal treatment, and high concentration samples gelled during the enzymatic pre-treatment, this does not apparently affect the final modulus, suggesting that the characteristic scale probed during oscillatory shear has the same fractal dimension, resulting from the initial enzymatic cross-linking.

For the $E_{0.8}G_{0.8}eTT$ samples, the range of concentration for which samples gelled is much limited: all samples with $C_p > 7\text{wt}\%$ gelled, but none of the samples for $C_p < 6.1\text{wt}\%$. For $6.1\text{wt}\% < C_p < 7\text{wt}\%$, half of the 6 samples investigated gelled, and the other half did not, suggesting a minimum concentration for gelation $C_g \approx 6.5\text{wt}\%$. As the concentration range in which gel are obtained is reduced, the determination of the fractal exponent is difficult. Still, the data are compatible with a value of 4.6, as for the $E_{0.5}G_{0.5}eTT$ samples. The absence of gelation below $C_g \approx 6.5\text{wt}\%$, contrarily to the $E_{0.5}G_{0.5}eTT$ samples, could result from (i) their lower content in conarachins (rich in sulfur and lysine, both involved in enzymatic and thermal gelation), and (ii) the higher density of the

aggregates formed during enzymatic pretreatment (higher fractal dimension), which occupy less volume but rapidly include most, if not all, of the little amount of conarachins solubilized in these extracts.

It is worth noticing the different behaviors of proteins that were extracted at a certain ionic strength and then gelled at a different ionic strength. $E_{0.5}G_{0.8}TT$ and $E_{0.8}G_{0.5}TT$ samples exhibit a modulus which value lays between the one of their counterpart extracted and gelled at the same ionic strength, e.i. samples $E_{0.5}G_{0.5}TT$ and $E_{0.8}G_{0.8}TT$, respectively. However, if submitted to an enzymatic pre-treatment, the sample gelation is strongly limited. For $E_{0.5}G_{0.8}eTT$ samples, out of 5 samples for which $3.1\text{wt}\% \leq C_p \leq 7.7\text{wt}\%$, only one gelled (for $C_p \approx 5\text{wt}\%$), but exhibited a very weak modulus ($G' \approx 1 \text{ Pa}$). The change in ionic strength, from 0.5 M during extraction to 0.8 M for gelation, may have resulted in the partial insolubilization of the conarachins extracted at 0.5 M, thus reducing the suspension propensity to gel. For $E_{0.8}G_{0.5}eTT$ samples, three samples were prepared with $3.1\text{wt}\% \leq C_p \leq 7.7\text{wt}\%$, and none of them gelled. For $C_p > 7.7\text{wt}\%$, gel moduli are slightly higher to the ones observed for the $E_{0.5}G_{0.5}eTT$ samples. This might result from the partial solubilization of a small fraction of conarachins, initially present in the suspension as insoluble aggregates, thereby reinforcing the gel.

3.3 Gels microstructure

Confocal images of peanut protein gels structure acquired on the samples produced at high protein concentration are presented in Figure 5 (samples prepared at low protein concentration are so weak that they broke in pieces during manipulation). While it is not possible to distinguish between the two extracts at 0.5 M and 0.8 M, it can be observed that applying an enzymatic pretreatment gives a much thinner and denser structure, which is coherent with the fractal dimensions previously determined.

4. Conclusion

Peanut proteins extracted at different ionic strength, 0.5 M or 0.8 M, strongly differ in their conarachins/arachins relative contents. We showed that conarachins, extracted in larger quantities at 0.5 M, strongly contribute to gel formation and gel elasticity. When submitted to a thermal treatment, conarachins-rich samples gelled at a lower temperature and if the thermal treatment is preceded by an enzymatic pre-treatment, their gelation is observed already during the pretreatment (for high enough protein concentration), which is not the case of the arachin-rich samples. For a given gelation process, conarachins-rich samples also give stronger gels. This is attributed to their higher cysteine and lysine contents, two amino-acids involved in protein cross-linking during thermal or enzymatic treatment. Observations in confocal microscopy showed that the enzymatic pre-treatment gives much denser and thinner structures. Indeed, while the fractal dimension of thermally treated samples, estimated from rheological measurement, is about 1.7, it increases to about 2.1 for enzymatically pre-treated samples. These values are characteristic of gels formed in the diffusion-limited and reaction-limited cluster aggregation regimes, respectively. The ionic strength imposed during gelation (in the investigated range) does not strongly modify the gels properties, except if an enzymatic-pre-treatment is applied. In that case, gelation is inhibited if an ionic strength different from the extraction one is imposed, suggesting solubility loss. Ionic strength and transglutaminase pre-treatment therefore constitute two parameters that can be tuned during food processing to generate protein gels with different properties.

CRedit authorship contribution statement

Benoît Basse: Conceptualization, Investigation, Formal analysis, Validation, Visualization, Writing - Original Draft. **Véronique Bosc:** Conceptualization, Methodology, Visualization, Project administration, Supervision, Writing - Review & Editing. **Jean-Marc Saiter:** Conceptualization, Methodology, Visualization. **Monique Chan-Huot:** Conceptualization, Project administration, Funding acquisition, Writing - Review & Editing. **Jean-Pierre Dupas:** Resources. **Marie-Noëlle Maillard:** Conceptualization, Supervision, Writing - Review & Editing. **Paul Menut:** Conceptualization, Methodology, Visualization, Project administration, Supervision, Writing - Original Draft, Writing - Review & Editing.

1 399 **Declaration of Competing Interest**

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4 400 The authors declare that they have no known competing financial interests or personal
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6 401 relationships that could have appeared to influence the work reported in this paper.
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522 **List of Tables**

1 523 Table 1: Classification and protein concentration range (C_p , in g/100g) of the different
2 524 samples.
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Table 1: Classification and protein concentration range (C_p , in g/100g) of the different samples.

Ionic Strength	Gelation process	
	Thermal Treatment only	Enzymatic pre-treatment followed by Thermal Treatment
Extraction and gelation at 0.5 M	$E_{0.5}G_{0.5}$ TT $3.0 \leq C_p \leq 13.5$	$E_{0.5}G_{0.5}$ eTT $3.0 \leq C_p \leq 13.5$
Extraction and gelation at 0.8 M	$E_{0.8}G_{0.8}$ TT $3.4 \leq C_p \leq 12.1$	$E_{0.8}G_{0.8}$ eTT $3.4 \leq C_p \leq 12.7$
Extraction at 0.5 M and gelation at 0.8 M	$E_{0.5}G_{0.8}$ TT $3.0 \leq C_p \leq 7.8$	$E_{0.5}G_{0.8}$ eTT $3.1 \leq C_p \leq 7.8$
Extraction at 0.8 M and gelation at 0.5 M	$E_{0.8}G_{0.5}$ TT $3.2 \leq C_p \leq 10.3$	$E_{0.8}G_{0.5}$ eTT $3.2 \leq C_p \leq 9.3$

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List of Figures

Figure 1: SDS-PAGE patterns (in triplicate) of the proteins extracted at 0.5M (lanes 2-4) and 0.8M (lanes 5-7). Standard protein markers are in lane 1 (molecular weights are indicated on the left). Conarachins II subunits (~64 kDa) and arachins subunits (22-42 kDa) are surrounded.

Figure 2: Evolution of the storage (G' , full symbols) and loss (G'' , empty symbols) moduli during gelation, for proteins extracted (and gelled) at ionic strength 0.5 M (blue) and 0.8 M (red). Top panels: thermal treatment alone (TT, circles). Low panels: enzymatic pre-treatment followed by the thermal treatment (eTT, triangles). Low concentration samples ($C_p = 4$ g/100 g) are on the left and high concentration samples ($C_p = 11$ or 13 g/100 g) are on the right. Temperature profiles (continuous black lines) are plotted with an axis on the right hand side of the graph.

Figure 3: Impact of protein concentration on the gelation time (cross-over between the storage G' and loss G'' moduli), for suspensions submitted to a thermal treatment only (top panel, circles) or to the same thermal treatment preceded by an enzymatic pretreatment (lower panels, triangles). Right panels zoom portions of the left panels during the temperature ramp. Color codes are: $E_{0.5}G_{0.5}$ (blue), $E_{0.8}G_{0.8}$ (red), $E_{0.5}G_{0.8}$ (blue circled with red), $E_{0.8}G_{0.5}$ (red circled with blue). Color lines are guides to the eye.

Figure 4: Storage modulus G' (Pa) as a function of the protein concentration C_p (g/100g) for gels obtained by thermal treatment (top panels, circles) and by the same thermal-treatment preceded by an enzymatic pretreatment (lower panels). Color code are the following: blue: $E_{0.5}G_{0.5}$, red: $E_{0.8}G_{0.8}$, blue circled with red: $E_{0.5}G_{0.8}$, red circled with blue: $E_{0.8}G_{0.5}$. Continuous lines (left panels) represent power-law fits of the data for samples extracted and gel at the same ionic strength, they are reproduced on the right panels to help the reader (the power-law fit obtained on $E_{0.5}G_{0.5}TT$ is reproduced in grey in the lower panel). Gelation does not occur at protein concentrations below the vertical dotted lines (in red and blue).

Figure 5: Images of peanut protein gels ($C_p = 12$ g/100g) obtained by CSLM (magnification x40). Proteins are stained in green. Gelation was obtained by Thermal Treatment (TT, top), or by an enzymatic pre-treatment before the thermal treatment (eTT, bottom). Proteins were extracted and gelled at 0.5M (left) or 0.8M (right). Scale bar are 50 μ m.

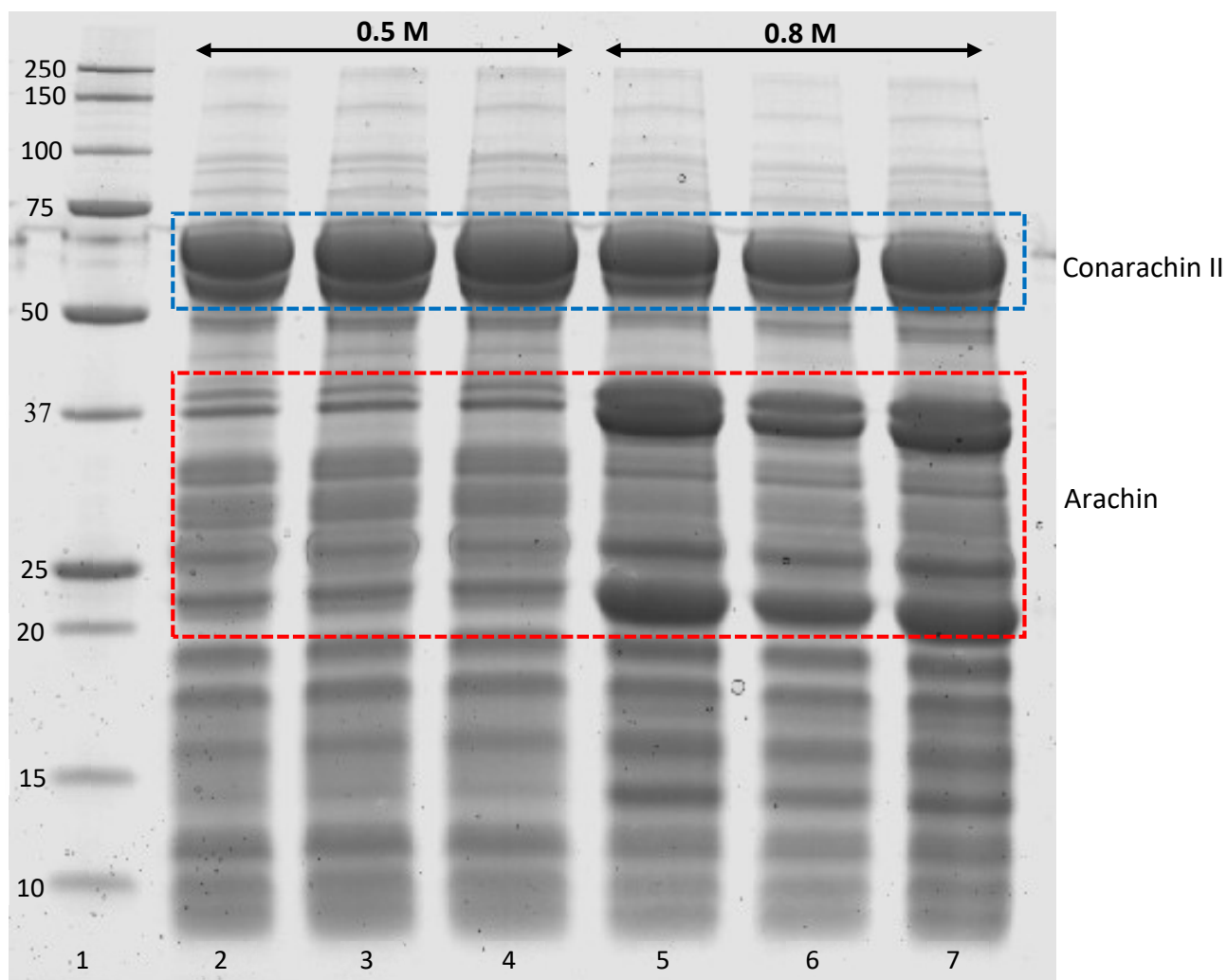


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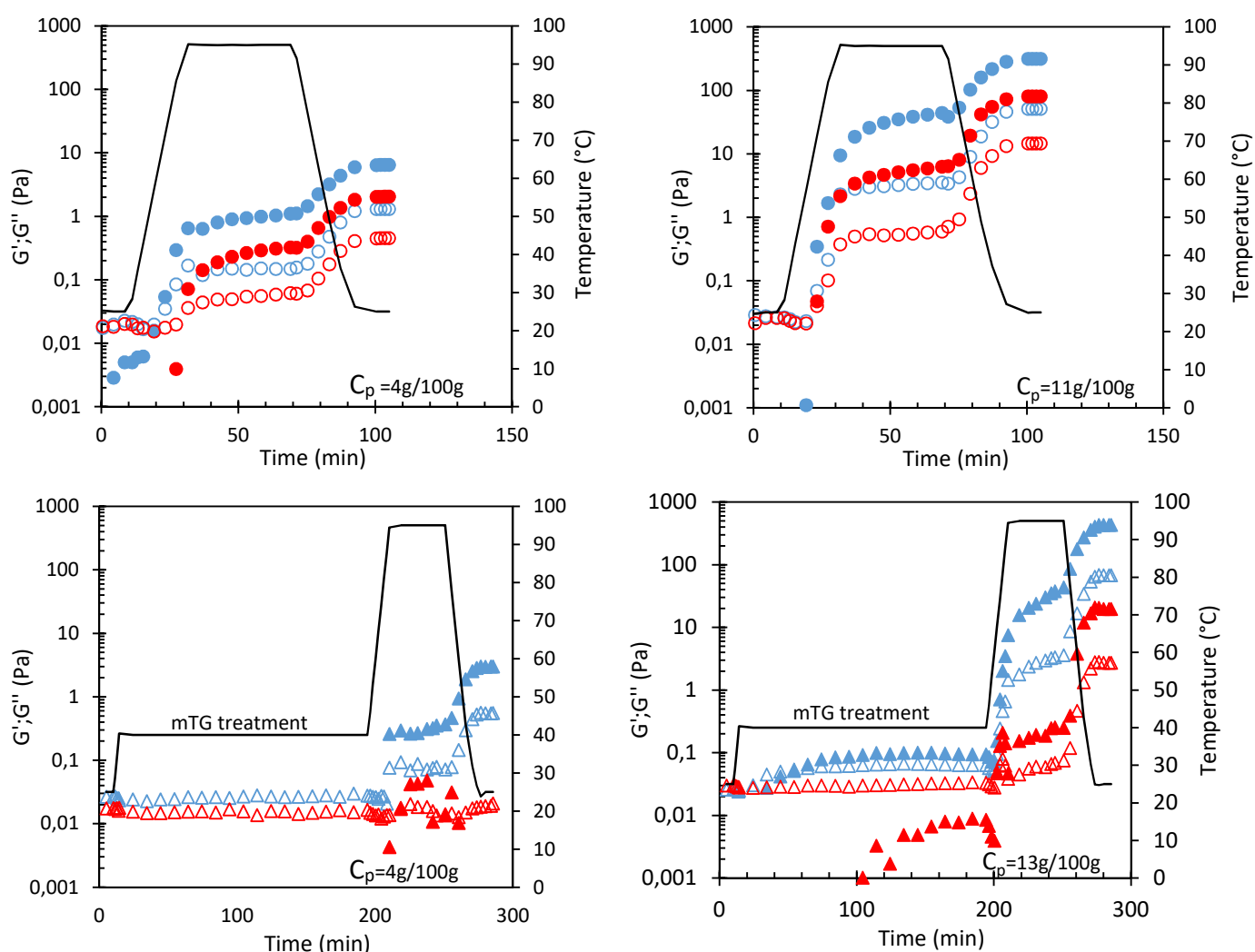


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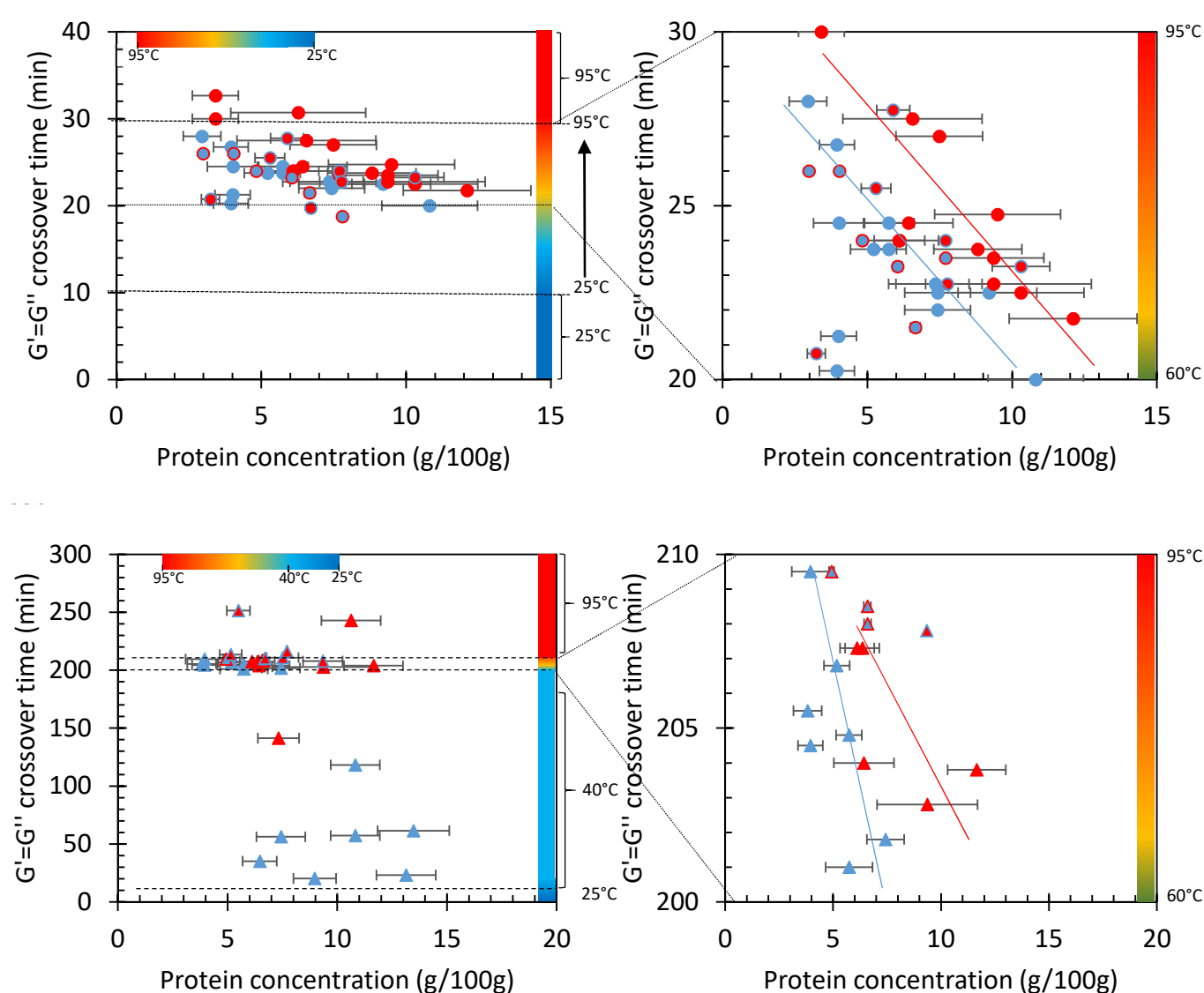


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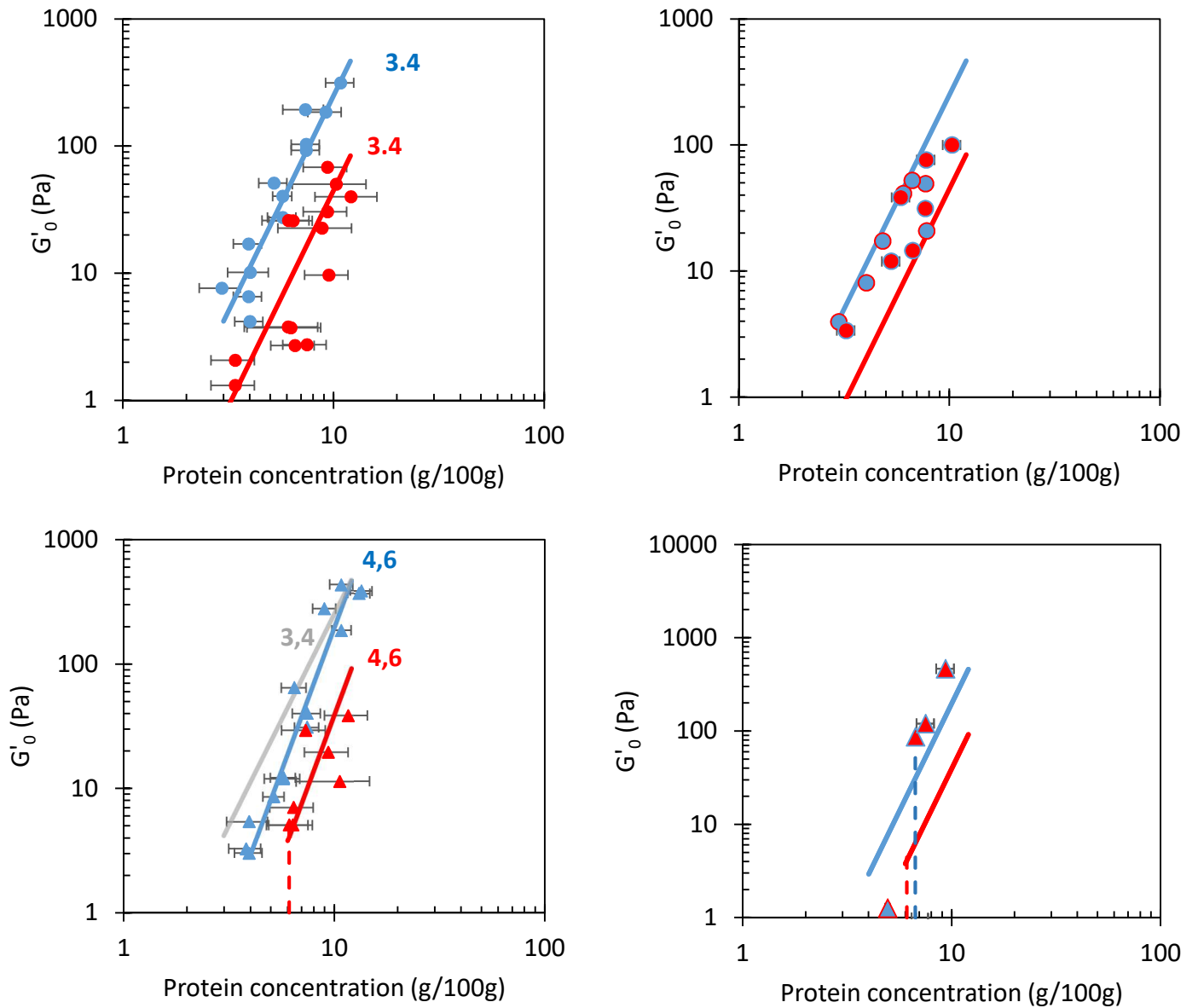


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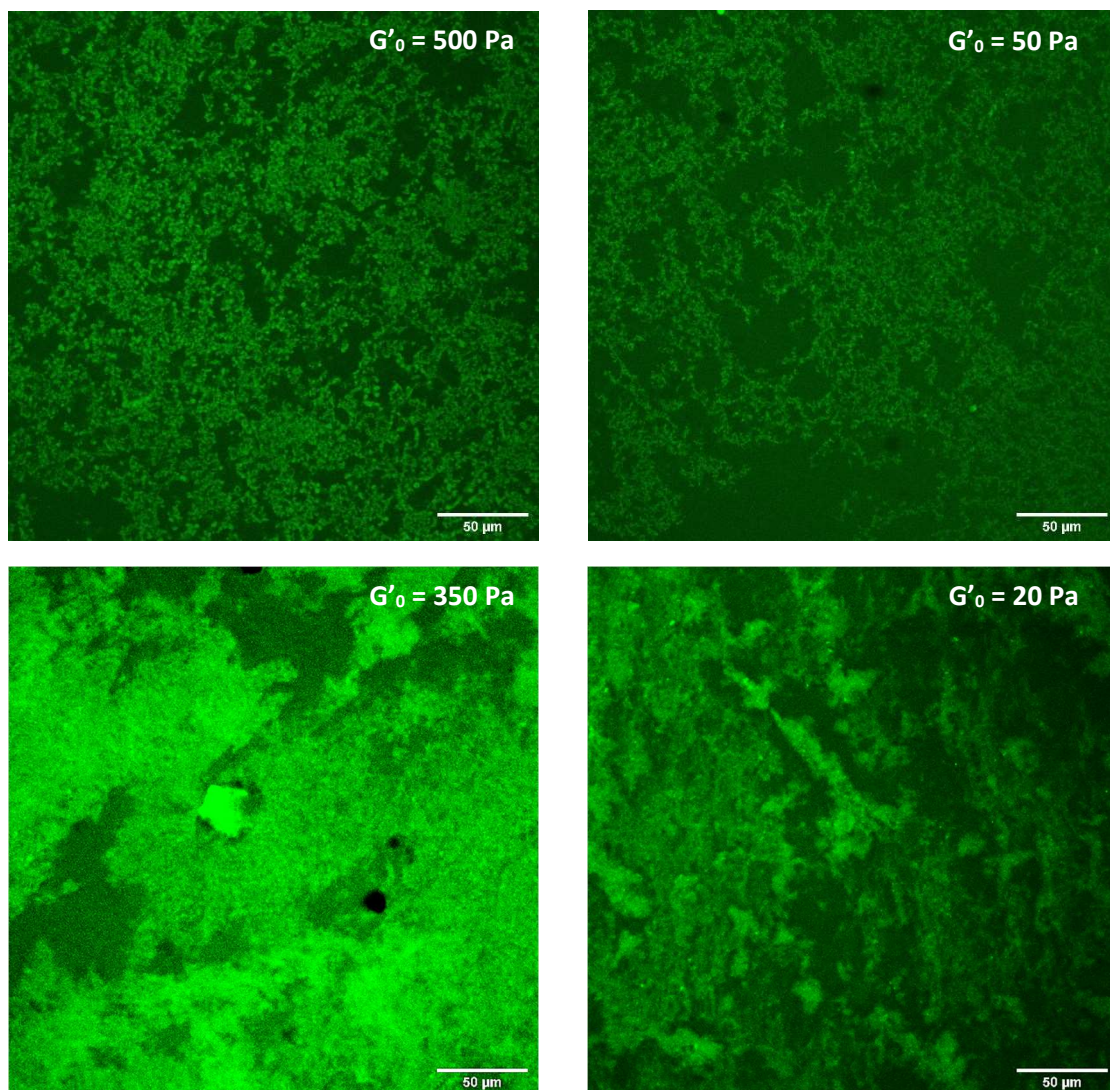
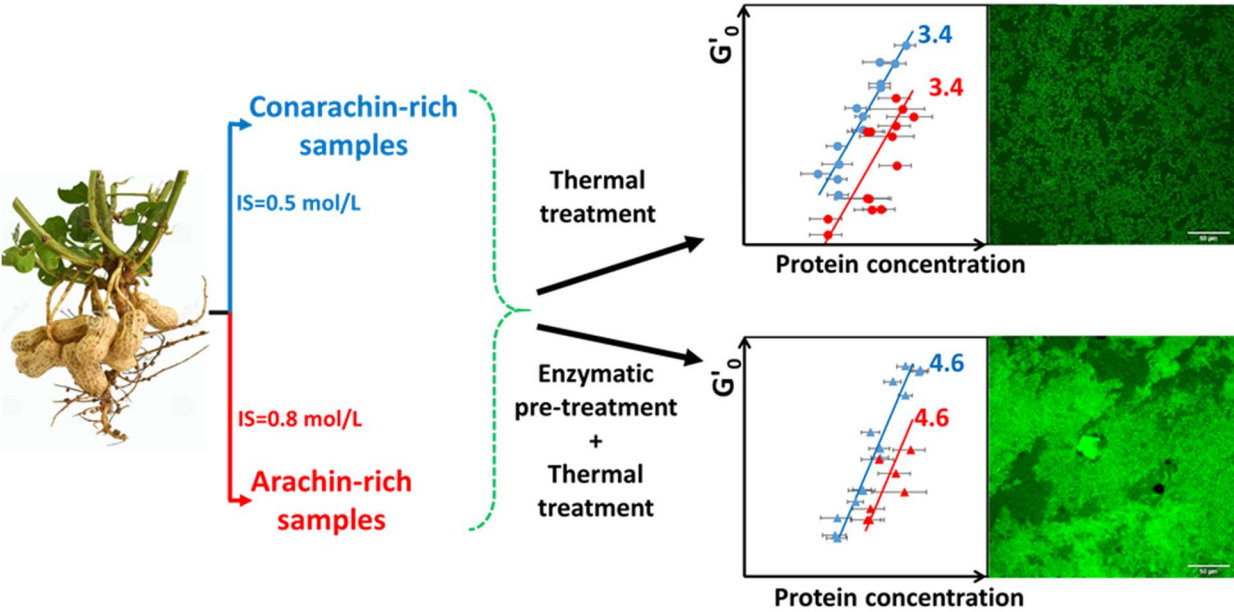


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Declarations of interest: none