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Gels and gelled emulsions prepared by acid-induced gelation of mixtures of faba bean (*Vicia faba*) protein concentrate and λ-carrageenan

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ABSTRACT

In this study, gels were successfully prepared at room temperature from mixtures of dry fractionated faba bean protein concentrate (FPC) and λ-carrageenan (λ-CGN), through acidification with glucono-δ-lactone (GDL). At neutral pH, the mixtures were shear thinning liquids, although the shear viscosity increased dramatically with λ-CGN addition. After adding GDL, the gelling kinetics were followed through small amplitude oscillatory rheology for 19 hours, at which point all gels had reached a gel modulus plateau. Elastic moduli for the prepared gels were in the range of 1500 – 4500 Pa, dependent on FPC:λ-CGN ratio and concentrations, and final pH (3.5 – 4). Rheological data further indicated the gels had properties typical of aggregated particle gels, e.g., low yield strains (~1%). All gels showed some syneresis upon centrifugation (2000g), with the least amount of syneresis (15 – 20%) at the highest λ-CGN concentrations (1.5 – 2%). FPC is a good emulsifier, and gelled emulsions were successfully prepared. Inclusion of emulsion droplets had significant impact on the gel network, with ~40% and ~60% increased gel storage modulus at 20% and 30% oil, respectively. Preparing similar formulations using a more extensively processed commercial faba bean protein isolate was also attempted, but this resulted in poor gels with very high syneresis. This indicates that dry fractionation methods may be beneficial to preserve native protein functionality.

1. Introduction

Legumes are staple crops around the world, due to their ease of growth/low cost and good nutritional value. Their ability to fix nitrogen lowers fertilizer demands, and makes them useful in crop rotations to replenish soil nitrogen. The seeds of grain legumes such as various beans, peas and lentils are good sources of protein, but are also rich in fiber, some minerals and vitamins as well as various bioactive phytochemicals that may provide health benefits (Moreno-Valdespino et al., 2020).

In recent years, for both ethical and environmental reasons there has been a growing focus on the replacement of animal derived ingredients with plant based alternatives (Malek et al., 2019). Due to their high protein content and interesting functional properties, flours or protein isolates mechanically prepared from legumes (protein concentrates) or isolated by aqueous extraction and precipitation (protein isolates) may find use as functional food ingredients, partially or fully replacing animal protein in various food products (Singhal et al., 2016).

The main proteins in legumes are water soluble albumins, and salt soluble globulin storage proteins (7S vicilin and/or 11S legumin fractions) (Boye et al., 2010). These globular proteins consist of polymorphic subunits bound together by primarily non-specific hydrophobic interactions; vicilin is a trimer, while legumin is a hexamer (Schwenke, 2001). Legume proteins are relatively high in beta-sheet structures compared to e.g., cereal or animal protein, imparting a high structural flexibility. This aids emulsion stabilization, as the proteins undergo significant conformational changes upon adsorbing to emulsion droplets, exposing hydrophobic residues to the oil phase and forming a highly stable interfacial layer (Sharif et al., 2018; Tang & Sun, 2011). Legume proteins are also commonly used to provide viscosity or texture. Gelation of globular proteins, such as legume protein is usually done through heat denaturation. This exposes hydrophobic residues, which, if the protein concentration, pH and ionic strength are correct, may cause the protein to aggregate into a gel network. Depending on the solution parameters and protein, the resulting gel structure may be more fine-stranded or more particulate (Langton et al., 2020; Urbonaite et al., 2016).

Commercial legume protein isolates usually contain around 90 % protein. However, producing these quite pure protein isolates is a relatively energy intensive process based around aqueous extractions and pH manipulations, that can also lead to protein denaturation or degradation (Singhal et al., 2016; Vogelsang-O'Dwyer et al., 2020). Through dry milling followed by air classification based on particle size/density,

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a protein enriched flour or concentrate can be achieved that retains native protein functionality, while keeping energy usage low (Vogelsang-O'Dwyer et al., 2020). On the negative side, dry fractionation rarely achieves protein concentrations higher than 60–70%. The remaining dry weight is primarily starch granules, oligosaccharides and fiber which can affect the functionality of the protein enriched isolate.

Legume starch granules have gelatinization temperatures around 65°C (Zhang et al., 2019). However, to denature legume proteins, a common step in gelation, boiling is commonly employed. As the protein enriched isolate is dispersed in water and heated above 65°C, starch gelatinization will increase the solution viscosity to a large degree, making processing more difficult (Jiang et al., 2020). If the starch concentration is sufficiently high, starch gelation may also occur. While legume starch gels can have good gel strength and interesting properties (Pelgrom et al., 2015; Vogelsang-O'Dwyer et al., 2020), they may not be desired.

A legume of particular interest is the faba bean (*Vicia faba*, FB). It is a hardy legume that can be grown even in colder climates (Duc, 1997). It has a very high nitrogen fixation capacity, making it an excellent plant for crop rotations (Herridge et al., 2008). It is rich in protein, primarily storage globulins (7S and 11S), with medium/high hydrophobicities when compared with other legume storage proteins making them good emulsifiers (Karaca et al., 2011; Kimura et al., 2008). Traditionally, dry fractionated FB protein concentrate (FPC) has seen limited commercial use, due to the presence of anti-nutrients such as vicine/convicine, that are not removed in dry fractionation (Khazaei et al., 2019). However, new FB cultivars with strongly reduced anti-nutrient levels are being developed, which may open the door for more widespread use of this highly interesting ingredient (Khazaei et al., 2019; Sinha & Kumar, 2018).

Carrageenans (CGNs) are linear sulfated polysaccharides extracted from red seaweeds (Tuvikene, 2021). The three main idealized types are κ-, ι- and λ-CGN, with one, two and three sulfate groups per dimer, respectively. While κ- and ι-CGN can form gels through cross-linking with certain ions, λ-CGN can not. λ-CGN is instead usually used as a thickener, and can be easily dissolved in room temperature solutions, which is why it was chosen for this study. Also, the higher charge density of λ-CGN may lead to stronger electrostatic CGN-protein interactions (Lam & Nickerson, 2014). Many proteins have predominantly positively charged patches even at neutral pH, allowing for attractive interactions with CGN (Gu et al., 2005; Liu et al., 2020a). CGN-protein interactions are employed in e.g., dairy products, where interactions between CGN and casein can improve the product through optimizing texture and binding moisture (Tuvikene, 2021). The acid induced gelation of mixtures of κ-CGN and whey protein aggregates has also been explored (Liu et al., 2020a). As the pH is decreased below a critical point, both protein-protein aggregation and κ-CGN electrostatic interactions contribute to the formation of a particulate gel network of decent mechanical properties (Liu et al., 2020b). Acid-induced gelation of whey protein aggregates with other polysaccharides have also been examined, and the conclusions seem to be that polysaccharides with higher charge densities promote the formation of more homogeneous microstructures upon acidification rather than phase separation (Liu et al., 2020a).

The objective of this study was to examine the functional properties of FPC in regards to gelation. To avoid interference from starch gelatinization, heating was avoided. Instead, the hypothesis was that gels could be formed at room temperature through electrostatic cross-linking with λ-CGN by acidification with glucono-δ-lactone (GDL). GDL provides a slow and gradual drop in pH, allowing for easy mixing and homogeneous acidification throughout the solution/gel. As FB proteins have isoelectric points in the range of 4.5 – 5.5 (Bhatty, 1974; Vogelsang-O'Dwyer et al., 2020), net electrostatic attraction with polyanions are expected at pH values below this. To the authors' knowledge there has not been any reported studies of acid gelation in combined FPC and λ-CGN systems.

Several formulations were prepared, with different concentrations of FPC, λ-CGN and GDL. The amounts of GDL were set to give final pH values in the range of 3.5 – 4, where the FPC and λ-CGN have opposite net charges. The mechanical properties of the systems were examined using rheology, and syneresis was quantified by centrifugation. Formulations with oil were also prepared, to examine if stable gelled emulsions could be formed. Finally, the FPC was compared with a more highly processed FB protein isolate (FPI), to examine if there is any functional benefit to the more gentle dry fractionation protein enrichment over more extensive processing.

2. Materials and methods

2.1. Materials

Two protein concentrates were obtained from faba bean (*Vicia faba* L.) varieties from a screening study organized within the FoodProFuture project (Ertesvåg, 2020). The low tannin cultivar Tiffany was grown in Halden, Norway (2019) and supplied by the Norwegian Institute of Bioeconomy Research (NIBIO), whereas Vertigo, grown at Vollebekk Ås, Norway (2019), was supplied by the Norwegian University of Life Sciences (NMBU).

Dried kernels were cleaned and subjected to an initial milling < 0,5 mm (ZM200, Retsch GmbH, Haan, Germany). For air classification the samples were further disintegrated in a pin mill (100 UPZ, Hosokawa Alpine, Augsburg, Germany) and subjected to separation into a fine and a coarse fraction in an air classification system (Hosokawa Alpine, Augsburg, Germany) operated by moving nitrogen. From a large set of samples one protein enriched fine particle fraction was select from each variety. The Vertigo derived protein concentrate (V-FPC) contained 69 % crude protein (N x 6.25) and 6% starch whereas and the Tiffany protein concentrate (T-FPC) contained 65% protein and 6% starch. V-FPC contained 1.6% sucrose and 6.9% raffinose series oligosaccharides (RSO) whereas T-FPC contained 1.9% sucrose and 6.5% RSO. All percentages on dry weight basis. Both protein concentrates only contained trace amounts of reducing sugars (glucose and fructose).

A third faba bean protein concentrate was a commercial sample produced through a process including a wet extraction, containing 92% protein on dry basis (Hill Pharma, Faba-tein 90%), hereafter denoted as Faba bean Protein Isolate (FPI).

λ-carrageenan (Viscarin® GP 109F) was generously donated by Dupont (Delaware, United States).

Corn oil, glucono-δ-lactone (GDL) and sodium azide (NaN_3) were purchased from Sigma-Aldrich (Germany). All water used was deionized water (18.2 MΩcm, Stakpure OmniaPure, Germany), containing 0.02 wt% NaN_3 as antimicrobial agent.

2.2. Preparation of gels and gelled emulsions

An appropriate amount of water was weighed out in a bottle. Then the protein concentrate or isolate and λ-CGN were added, and the solution was stirred for 1 hour until all solids were properly dissolved or dispersed. After this, the gradual pH mediated gelation was initiated by adding GDL powder and stirring the solution well for 2 minutes.

To prepare gelled emulsions, oil was added to the protein-λ-CGN mixture after the 1 hour of stirring, and the emulsion was formed by using a VDI12 homogenizer (VWR International, Darmstadt, Germany) at 11500 RPM for 1 minute. Then gelation was initiated by GDL addition as described in the previous paragraph.

The formulations that were examined are listed in Table 1. To examine the effect of total amount of protein+λ-CGN, a range of formulations with either 10% or 15% solids were prepared. Above 15% solids it became challenging to properly disperse all the material. To examine the effect of protein:λ-CGN-ratio, the amount of λ-CGN was varied between 0 – 2% for the 15% solids formulations, and 1 – 2% for the 10% solids

Table 1
Formulations used in this study. The number in parenthesis in the first column is the amount of protein in the formulation, calculated by FPC or FPI wt% multiplied by protein % (listed in Section 2.1).

V-FPC wt% (protein wt%)	λ-CGN wt%	Protein/λ-CGN ratio	Water wt%	GDL
13 (8.9)	2	4.5	85	3%, 4% & 5%
13.5 (9.3)	1.5	6.2	85	4% & 5%
14 (9.6)	1	9.6	85	3% & 4%
15 (10.3)	0	-	85	4%
8 (5.5)	2	2.8	90	3% & 4%
9 (6.2)	1	6.2	90	4%
Other FB proteins				
T-FPC: 14.1 (9.2)	1.49	6.2	84.4	4%
FPI: 10.6 (9.6)	1.55	6.2	87.8	3%

formulations. More than 2% λ-CGN was initially tested, but this led to excessive viscosity making mixing difficult.

The formulations with other FB protein sources are formulated to have similar protein:λ-CGN and λ-CGN:water ratios as the 13.5-1.5 V-FPC-λ-CGN formulation. For short, the formulations with V-FPC and λ-CGN are referred to as e.g., 13-2 for the formulation with 13% V-FPC and 2% λ-CGN, and so on. The 13.5-1.5 formulation with 4% GDL was also used to prepare the emulsions, with 20% and 30% corn oil. The GDL amounts listed are added in addition to the other ingredients.

2.3. Rheological measurements

Rheological measurements were performed on a Kinexus Ultra+ rheometer (Malvern Instruments, Worcestershire, UK), using cone (40 mm, 4°, 144 μm truncation gap) plate geometry. Just after addition and mixing of GDL, the solution was added to the rheometer and allowed to equilibrate for 4 minutes before the measurements were started. Total time from addition of GDL to measurement start was 7 minutes for all samples. Then the sample was measured for 19 hours, using small amplitude oscillatory measurements (SAOS) at 1 Hz and 0.1 % strain, with 15 minutes break between each measurement point. After 19 hours, a frequency sweep was run, from 0.1 to 10 Hz at 0.1 % strain, followed by an amplitude sweep at 1 Hz, from 0.1 to 100 % strain. All measurements were performed at a constant 25°C, using a solvent trap system to avoid any evaporation.

Steady shear viscosity measurements were also performed with the same rheometer and geometry. Solutions of protein isolate and λ-CGN were prepared as normal, but before any GDL addition they were added to the rheometer and a shear rate sweep was performed from 0.1 to 10 s^{-1}.

2.4. Syneresis and pH measurements

For syneresis measurement, 1.00 g solution was added to a 2.0 mL Eppendorf tube just after GDL addition. Then the tube was sealed with parafilm and stored at 25°C and 60 RH% for 19 hours. pH in solutions were measured directly. Gels were gelled in 1.5 mL Eppendorf tubes, sealed and stored at 25°C, 60 RH% until the point of pH measurement. All pH measurements were done using a handheld pH meter (pH Spear, Eutech Instruments, Paisley, United Kingdom).

To force syneresis, the tube was centrifuged at 2000g for 20 minutes. The tube with contents was then weighed before and after removal of the supernatant. Percent total syneresis was calculated by taking the change in weight (i.e., weight of supernatant) divided by original sample weight (i.e., 1.00 g).

2.5. Emulsion characterization

Just after emulsion formation, but before addition of GDL, a small amount of the emulsion solution was diluted 1:10 in deionized water.

Table 2
pH of the different V-FPC-λ-CGN formulations before GDL addition. Measured at 25°C. Average of two measurements ± standard deviation. Different superscript letters indicate statistically significant differences in mean pH values ($p < 0.05$).

Formulation	pH
13-2	$6.67 \pm 0.00^{a,b}$
13.5-1.5	$6.63 \pm 0.01^{a,c}$
14-1	$6.63 \pm 0.02^{a,c}$
15-0	6.58 ± 0.01^{c}
8-2	6.73 ± 0.04^{b}
9-1	6.72 ± 0.03^{b}

One drop of this solution was applied to a microscopy slide, and examined with optical microscopy. To measure droplet size, the diluted solution was further diluted to half concentration with deionized water containing 2 wt% sodium dodecyl sulfate (SDS) as deflocculant. Then droplet size measurements were performed using a Mastersizer 3000 (Malvern, UK). Refractive indices were set to 1.330 (water) and 1.470 (dispersed phase), and particle absorption was set to 0.010. The obscuration was between 8 – 12 %.

Droplet size measurements on gelled emulsions were performed similarly, except after dilution 1:10 in deionized water the solution pH was neutralized (with $NaHCO_3$) to dissolve the gel and release the emulsion droplets.

2.6. Statistics

Statistical analyses were performed using IBM SPSS 26 (Armonk, USA). To compare means, one-way analysis of variance (ANOVA) was performed, with Tukey HSD post-hoc test for multiple pairwise comparisons.

3. Results and discussion

Several formulations were prepared with different ratios of V-FPC to λ-CGN, and different amounts of GDL to achieve different final pH values. It was found that going above 2% λ-CGN increased solution viscosity to an impractical point for the currently used preparation procedure, so this was set as a limit.

3.1. FPC-λ-CGN solutions before GDL addition

The pH of all the solutions before GDL addition was measured as described in Section 2.4, and the results are listed in Table 2. It can be seen that the V-FPC had little effect on pH, only causing a slight decrease with increasing amount.

Shear viscosity was measured for the different formulations before addition of GDL, as described in Section 2.3, and the results are shown in

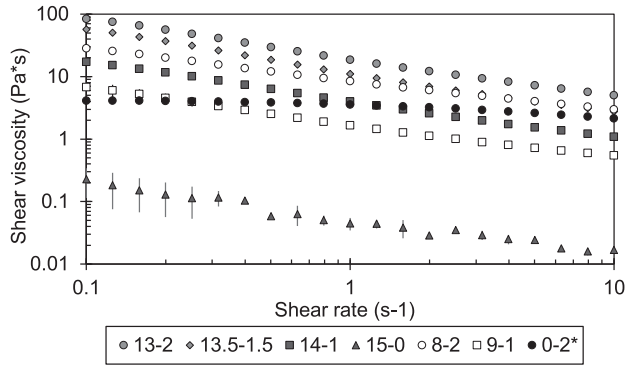

Fig. 1. Shear viscosity measured for different percentages of V-FPC and λ-CGN in water, before acidification with GDL. Each point is an average of 4 – 6 runs, with standard deviation shown as error bars. *0-2 is 2 parts λ-CGN in 85 parts water, i.e., same λ-CGN-water ratio as in 13-2.

Fig. 1. All solutions displayed shear thinning behavior, which is typical for polymer solutions; as shear is increased, the polymer chains untangle and orient themselves along the direction of shear, leading to a decrease in shear viscosity.

The ability of a biopolymer to impart viscosity correlates to some degree with the molecular volume the polymer occupies in the solution (Picout & Ross-Murphy, 2003). This explains why the V-FPC by itself (15-0) imparted very little viscosity, as the majority of the native FB proteins have a compact globular structure (Kimura et al., 2008). In addition, some of the protein remains undissolved at neutral pH (do Carmo et al., 2020; Vogelsang-O'Dwyer et al., 2020). λ-CGN, on the other hand is highly hydrophilic and soluble with an extended conformation in aqueous solution, explaining the large increase in viscosity with its addition. However, the combination of V-FPC and λ-CGN resulted in significantly higher viscosity than λ-CGN by itself, indicating the presence of some V-FPC-λ-CGN interactions even at neutral pH. The isoelectric point of FB protein has been reported to be in the range of pH 4.5 – 5.5 (Bhatty, 1974; Langton et al., 2020; Vogelsang-O'Dwyer et al., 2020). However, despite the net negative charge at pH > pI the proteins may still contain domains of predominantly positive charge that may interact with the sulfate groups of λ-CGN, forming larger structures than can impart higher viscosity. These structures are likely transient and break down as shear rate is increased, explaining the much higher level of shear thinning for the V-FPC+λ-CGN formulations compared to λ-CGN alone. Phase separation of mixtures of globular proteins and CGN at neutral pH (pH > protein pI) due to depletion flocculation has also been reported (Baussay et al., 2006). This could also lead to some aggregation and structure formation. However, no major phase separation or precipitation was observed and all solutions appeared macroscopically homogeneous.

It was also seen that while the solution with only λ-CGN followed the Cox-Merz rule, i.e., $\eta^* = \eta$ at comparable time scales, the solutions containing V-FPC did not, instead showing $\eta^* > \eta$. This further indicates the presence of shear sensitive weak structures at neutral pH (Ikeda & Nishinari, 2001). High levels of shear thinning can make proper homogeneous mixing of a solution more challenging, as the mass away from the impeller at the edges of the mixing tank tend to become more stagnant (Ameur & Bouzit, 2012). However, choosing the proper mixer design will reduce this problem.

3.2. Gelling kinetics and gel properties of FB protein-λ-CGN gels

Gelling kinetics were examined by measuring the development in gel moduli over 19 hours after addition of GDL, using SAOS rheology as described in Section 2.3. The storage modulus (G') and loss modulus (G") over time for all the formulations are shown in Fig. 2 and the final pH values, gel moduli and phase angles are listed in Table 3.

From Fig. 2 it can be seen that for all the systems the storage modulus increased fairly rapidly initially and over time approached a plateau value. The formulation without λ-CGN (15-0) had a similarly shaped curve, but the storage modulus was almost two orders of magnitude below the systems with λ-CGN. As the pH drops towards the protein isoelectric point, the net charge of the protein, and thus also the protein-protein electrostatic repulsion, is reduced. As a result, more short-ranged attractive interactions start dominating, leading to protein aggregation. It has indeed been shown that FB protein solubility is highly pH dependent. While the solubility is fairly good at neutral or slightly alkaline pH values, the solubility quickly drops as the pH is reduced closer to the protein isoelectric point (do Carmo et al., 2020; Vogelsang-O'Dwyer et al., 2020). Precipitation of globular legume proteins through reducing the solution pH to a value close to the isoelectric point (e.g., 4.5 – 5.5) is commonly employed in the production of legume protein isolates, known as isoelectric precipitation (Johnston et al., 2015). The lack of an initial lag phase in the gelling kinetics curves is due to a rapid initial drop in pH from the GDL. The change in pH over time after adding GDL was measured for the 15-0 formulation, and after 7 minutes (i.e., the time between GDL addition and the first SAOS measurement point) was already at ~5.4, within the isoelectric point range for faba bean proteins. After this the pH kept dropping at a gradually decreasing rate, reaching 4.8 after 20 minutes, 4.5 after 1 hour, 4.2 after 2 hours and 4.0 after 5 hours. After ~9 hours, the pH was within ±0.01 of the final pH value.

The low gel moduli values for the 15-0 system show that λ-CGN-interactions were essential to hold the aggregated protein together to form a pronounced gel. The final pH values of the systems were in the range of 3.5–4, as shown in Table 3, all below the isoelectric point of FB protein. At this point, the FB protein is predominantly positively charged, allowing for extensive electrostatic cross-linking between proteins or protein aggregates by the negatively charged λ-CGN.

The formulations with 14% V-FPC and 1% λ-CGN displayed slightly different gelling kinetics curve shapes, with a gradual increase up to a certain point, followed by a small decrease in moduli. This was the formulation with the lowest concentration of λ-CGN relative to V-FPC. As the pH decreased, the net positive charge of the proteins may have become too high relative to the λ-CGN negative charge, facilitating, e.g., formation of protein-λ-CGN complexes instead of interprotein cross-linking. This may also explain why the 14-1 with 4% GDL had lower moduli values than the same formulation with 3% GDL.

For the 13.5-1.5 and 13-2 gels, more GDL, and thus lower final pH seemed to increase the resulting gel modulus, although the effect was only statistically significant for the 13-2 system, between 3% and 4% GDL. The higher amount of λ-CGN in these systems may balance better with the amount of positive charge on the protein in regards to network formation. It seems the ratio between positive protein charges and negative λ-CGN charges is important, and the moduli of the resulting gels can to some degree be tuned by changing the V-FPC:λ-CGN ratio and the final pH. The importance of the ratio between protein and anionic polysaccharide has also been previously described for other similar systems, such as acid-induced whey protein-κ-CGN gels (Liu et al., 2020a).

For each sample, after the 19 hours of gelling kinetics measurements, a frequency sweep was run, as described in Section 2.3. The results are summarized in Fig. 3.

As can be seen from the figure, all systems displayed some frequency dependence. An ideal «true gel» has a relaxation time approaching infinity, which results in constant gel moduli values independent of frequency. The frequency dependence in this case indicates shorter relaxation time, and is typical of gel networks of a more aggregated particle nature rather than homogeneous fine stranded gel networks.

In addition, while G' > G" at all frequencies for all samples, the difference is only approximately one order of magnitude (less for the 15-0 formulation). The 8-2 gels are slightly less frequency dependent than

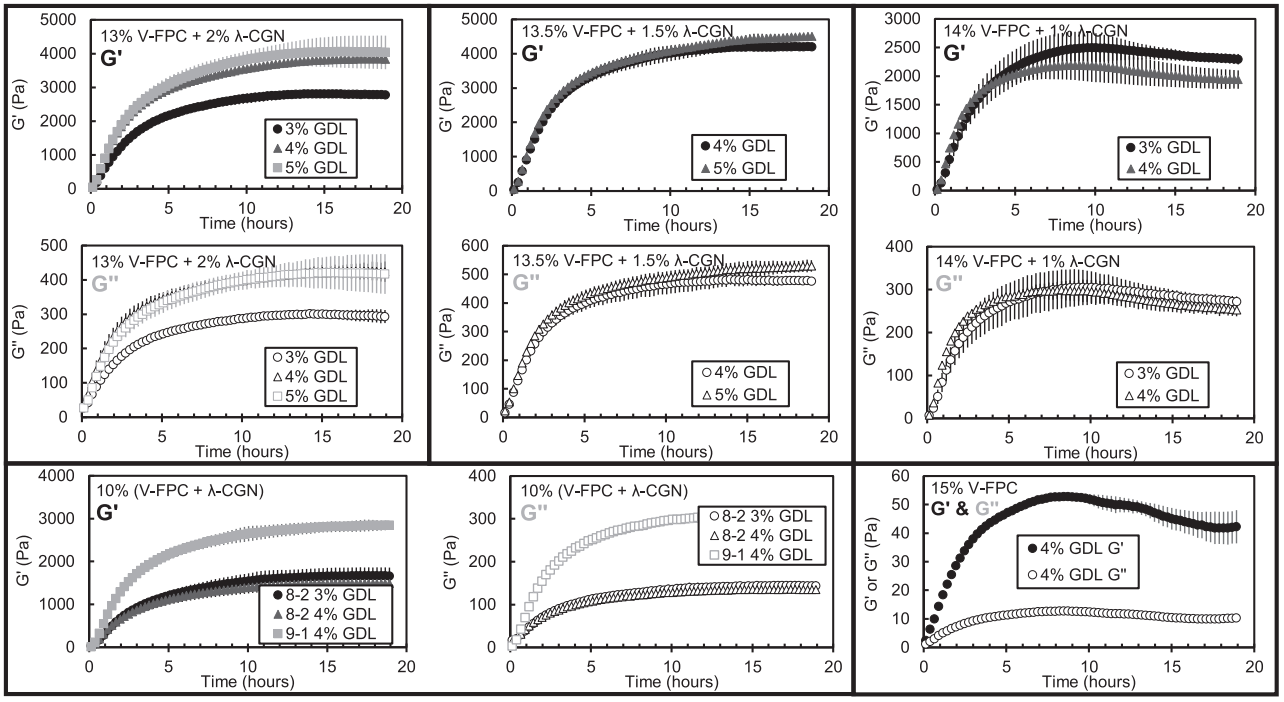

Fig. 2. Development in storage modulus (G') and loss modulus (G") over 19 hours after addition of GDL for the various formulations. Measured at 1 Hz frequency and 0.1% strain. Filled symbols indicate G' values, while empty symbols indicate G" values. Points are average of two runs with standard deviation shown as error bars.

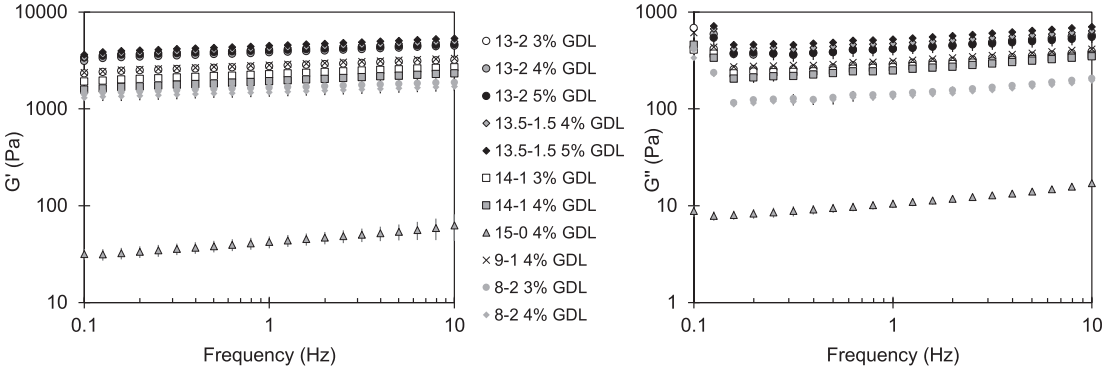

Fig. 3. Frequency sweep results for all formulations after 19 hours of gelling. Performed at 0.1 – 10 Hz, and 0.1% strain. Left: G' values, right: G" values. Each point is the average value of two runs, with standard deviation shown as error bars.

Table 3
Parameters of the different formulations, approximately 19 hours after GDL addition. Average of four values (pH) or two values (rest) ± standard deviation. Different superscript letters in the same column indicate statistically significant values ($p < 0.05$).

Formulation	GDL %	pH	G' (Pa)	G" (Pa)	Phase angle (°)
13-	3	3.97 ± 0.03	2775 ± 4^{a}	293 ± 19^{a,b}	6.03 ± 0.38^{a,b,c,d}
2	4	3.85 ± 0.03	3834 ± 92^{b}	374 ± 41^{b,c}	6.31 ± 0.29^{a,b,c,d}
	5	3.70 ± 0.01	4035 ± 496^{b}	417 ± 58^{c,d}	5.90 ± 0.09^{a,b,c}
13.5-	4	3.87 ± 0.02	4198 ± 6^{b}	476 ± 8^{d,e}	6.47 ± 0.12^{b,c,d}
1.5	5	3.72 ± 0.01	4513 ± 71^{b}	530 ± 20^{e}	6.69 ± 0.14^{c,d}
14-	3	4.05 ± 0.02	2296 ± 92^{a,c}	272 ± 12^{a}	6.75 ± 0.04^{c,d}
1	4	3.85 ± 0.04	1940 ± 161^{c,d}	252 ± 14^{a}	7.41 ± 0.20^{d}
15-0	4	3.88 ± 0.01	60 ± 32^{e}	14 ± 7^{f}	13.6 ± 0.9^{e}
8-	3	3.78 ± 0.02	1660 ± 199^{c,d}	144 ± 11^{g}	4.97 ± 0.22^{a}
2	4	3.55 ± 0.00	1490 ± 158^{d}	135 ± 9^{g}	5.21 ± 0.21^{a,b}
9-1	4	3.64 ± 0.05	2841 ± 105^{a}	312 ± 6^{a,b}	6.27 ± 0.35 ^{a,b,c,d}

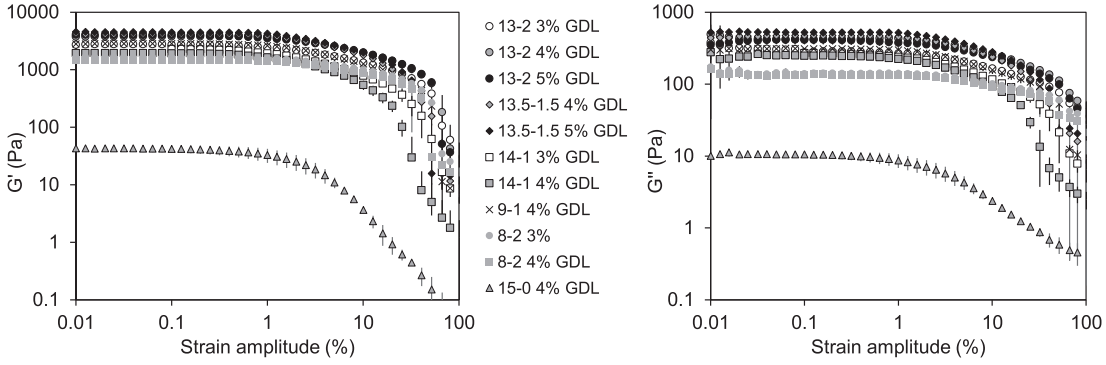

Fig. 4. Amplitude sweeps of the various formulations after 19 hours of gelling. Strain was increased from 0.01 – 100 %, at a constant frequency of 1 Hz. Storage modulus (G') shown to the left, loss modulus (G'') shown to the right. Same legend for both figures. Each point is the average value of two runs, with standard deviation shown as error bars.

the other gels, and also have the largest difference between G' and G'' values. The 8-2 gels have the highest ratio of λ-CGN to V-FPC, which may cause some differences in gel network formation.

In addition to the frequency sweep, an oscillatory amplitude sweep was also run on the samples after 19 hours of gelling, as described in Section 2.3.

From Fig. 4 it can be seen that all formulations started deviating from the initial linear viscoelastic (LVE) region around 1 % strain. This indicates a fairly weak network structure that can only withstand small amounts of shear deformation before starting to fracture. In addition, after the LVE region, the G'' values decreased at a similar gradual rate as the G' values, with G' > G'' up to the highest strains, with no clear brittle failure. Low yield stresses are typical for gel networks consisting of aggregated particles (Chen & Dickinson, 1999; Picout & Ross-Murphy, 2003). Due to the inhomogeneous nature of such gels, deformation stress may be concentrated at certain weak points of the network leading to an early network failure. In addition, the presence of significant amounts of larger solid particles from the V-FPC such as starch granules and insoluble fiber introduces further structural flaws into the network. From Fig. 4 it can be seen that the 8-2 formulations, despite having the lowest initial moduli of all systems (except 15-0), had the longest linear region and slowest moduli reduction afterwards. This system had the highest λ-CGN:V-FPC ratio, and the lowest total amount of V-FPC. This may have led to the formation of a less dense network, explaining the relatively low G', but perhaps also a more homogeneous network due to an excess of λ-CGN to form electrostatic linkages. A more homogeneous network may have less weak links, explaining the slightly increased extent of the LVE region.

3.3. Syneresis

Forced syneresis for the different formulations was measured as described in Section 2.4, and the results are summarized in Fig. 5.

From Fig. 5, it can be seen that all gels here displayed some level of (forced) syneresis. Due to the relatively inhomogeneous nature of particle gels, there will be large pores throughout the gel with poor water holding capacity (Urbonaite et al., 2016). When force is applied to the gel (in this case centrifugation), the water in the large pores will be squeezed out (Mizrahi, 2010). The syneresis liquid for most systems was transparent (not turbid) and had low viscosity (water-like), indicating good network connectivity and little loss of protein and λ-CGN. An exception was the 8-2 formulations, where the syneresis appeared slightly viscous. This system had the highest amount of λ-CGN relative to protein. It may be suggested that the amount of protein may have been insufficient to bind all the λ-CGN to the network, leading to some excess unbound or poorly bound λ-CGN getting lost with the syneresis (Liu et al., 2020b).

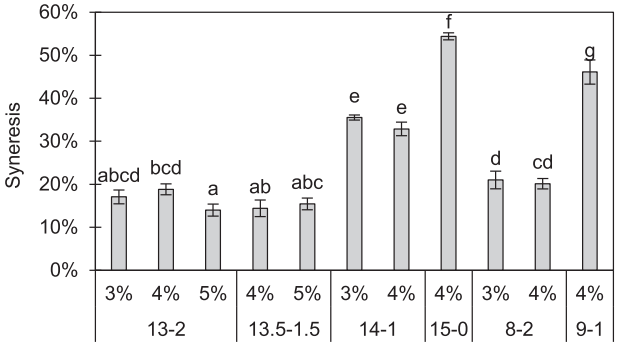

Fig. 5. Syneresis for the different formulations. Measured 20 hours after GDL addition. Each bar is the average of 3 – 4 measurements, with standard deviation shown as error bars. Columns labeled with different lower case letters represent significantly different values ($p < 0.05$).

The parameter with highest effect on the amount of syneresis was the λ-CGN concentration. The highest syneresis was seen for the system without λ-CGN (15-0). The 9-1 system displayed the second highest syneresis, despite relatively high gel moduli values. However, this system had the lowest λ-CGN:water ratio of the λ-CGN-containing systems. The lowest syneresis was seen for the 13-2, 13.5-1.5 and 8-2 systems, which had the highest λ-CGN concentrations. λ-CGN is an extended biopolymer with high charge density and a large molecular volume. This makes it an efficient water binding agent, explaining its positive effect on syneresis (Mizrahi, 2010). Higher levels of λ-CGN may also lead to the formation of a more homogeneous network with smaller pore size, due to the potential for more numerous electrostatic connections between proteins. However, only the 8-2 gels displayed significant differences in rheological frequency and strain response, as shown in the previous section, indicating some changes in network structure. The rheological differences for the 8-2 system may also be due to the excess unbound λ-CGN, indicated by the slightly viscous syneresis, as discussed in the previous paragraph.

For food gels, minimizing syneresis is usually desired, as expulsion of liquid during storage, handling or mastication can have a negative impact on the sensory quality of food products (Mizrahi, 2010). To further reduce syneresis in this system, increasing both V-FPC and λ-CGN concentration might have a positive effect. Increasing only λ-CGN might lead to excess unbound λ-CGN, that would be expulsed along with the water, as seen for the 8-2 system. Increasing both would most likely lead to the formation of a more dense particle network with smaller pores, more resistant to syneresis. However, it could also lead to excessively high viscosities that could cause issues during manufacturing.

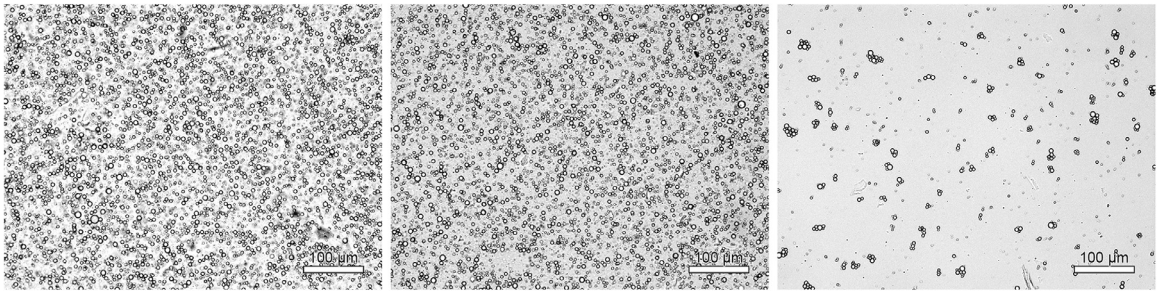

Fig. 6. Photomicrographs of FB protein emulsions. Prepared with 13.5% V-FPC and 1.5% λ-CGN in the water phase, then emulsified with 20% (left) or 30% (middle) corn oil. Diluted 1:10 in deionized water at neutral pH before microscopy. Right is the 30% corn oil emulsion after three weeks of storage as a gelled emulsion, dissolved by neutralization and mixing with deflocculant (SDS).

Table 4
Droplet size characteristics for the V-FPC emulsions with 20 and 30% corn oil. Each value is the average of two replicates with each five measurements ± standard deviation.

Oil	Droplet size initial (µm)		Droplet size after 3 weeks as gelled emulsion (µm)	
wt%	D[4,3]	D[3,2]	D[4,3]	D[3,2]
20	11.6 ± 0.2	4.1 ± 0.0	11.9 ± 0.1	4.5 ± 0.1
30	9.3 ± 0.1	3.5 ± 0.0	10.7 ± 0.1	4.4 ± 0.1

3.4. FPC-λ-CGN gelled emulsion properties

Emulsions were prepared using the system with 13.5 % V-FPC and 1.5 % λ-CGN, gelled with 4 % GDL. This system provided good gel properties and relatively low syneresis, as described in the previous sections. At above 1.5% λ-CGN the solution became too viscous to homogenize properly with the current setup.

To examine whether homogenization itself could affect gel properties, due to e.g., reductions in particle sizes or increased protein solubilization, a sample with the same composition was homogenized without oil present and measured in the same ways.

As shown in Table 4, emulsification with the V-FPC produced droplets with average diameters of a few micrometers. The insoluble particles, such as starch granules, are unlikely to affect the emulsion droplets directly, due to lack of surface activity (Sridharan et al., 2020). However, they will likely scatter some light during the droplet size measurements, meaning the measured emulsion droplet sizes are not completely exact. However, by manual comparison with the photomicrographs shown in Fig. 6(left and middle), the measured values seem reasonable. Fig. 6(left and middle) also shows that there is only sporadic flocculation at neutral pH before GDL addition. The slightly smaller measured droplet sizes for the formulation with 30% oil may in part be due to the dilution of larger particles such as starch granules with addition of more oil. It was also observed that addition of more oil increased the viscosity during homogenization, due to more emulsion droplet interactions. A higher viscosity means more shear forces imparted on the solution at the same homogenization shear rate, which may result in smaller droplets formed.

To remeasure the droplet size after 3 weeks, the gels were dispersed in water, the pH was neutralized and 2% SDS was added as a deflocculant. While this caused the gels to dissolve, significant emulsion flocculation remained, as shown in Fig. 6(right). Increasing the pH further, adding more SDS, or agitating the solution for longer did not disintegrate the flocculates. This indicates that irreversible flocculation between emulsion droplets occur in the gelled emulsions. When globular legume proteins adsorb to emulsion interfaces, they undergo conformational changes eventually forming an extended viscoelastic film at the interface (Tang & Sun, 2011). If the droplets are flocculated as the protein undergoes the conformational changes, the formed viscoelastic film might be able to irreversibly bridge droplets together through, e.g., hydrophobic interactions.

The increase in droplet size as measured after three weeks is likely primarily due to this irreversible flocculation, and the droplets appear stable against coalescence. Strong flocculation may impact the digestion of the emulsions, as it could hinder enzyme access to the interfaces hidden in the flocculates. However, it is possible that gastrointestinal factors, such as proteolytic enzymes, could deflocculate the systems.

Due to the significant amount of insoluble particulate matter in the V-FPC, it was hypothesized that homogenization itself could affect the properties of the system through changing the particle size distribution. However, the homogenized formulation without oil did not show any significant differences from the same formulation without homogenization.

Introducing emulsion droplets into the system led to an increase in gel moduli, as shown in Fig. 7. If there is a direct connection between the dispersed phase and the continuous network, the droplets will contribute their modulus to the composite gel. This is known as the active filler effect, and the result can be either an increase or a decrease in the gelled emulsion modulus with increasing emulsion fraction, depending on whether the droplet modulus is above or below the continuous network modulus, respectively (Dickinson & Chen, 1999). The modulus of emulsion droplets can be approximated with the droplet Laplace pressure, defined as interfacial tension times two divided by droplet radius (van Vliet, 1988). Estimating an interfacial tension of 5 – 10 mN/m based on literature (Félix et al., 2019), and a droplet radius of 2 – 5 µm, the emulsion droplet modulus here would be in the range of 2 – 10 kPa, compared to 4.2 kPa for the gel without oil. This means that while the smaller droplets could contribute positively, the larger droplets might contribute negatively to the composite modulus. However, this theory assumes the emulsion droplets are evenly distributed throughout the system (van der Poel, 1958). If droplets flocculate together, the result can be a significantly higher G' than theory predicts (Chen & Dickinson, 1999; Hattrem et al., 2015).

As previously mentioned, and shown in Fig. 6(right), significant irreversible flocculation remained after dissolving the gelled emulsions after three weeks. This shows that droplet aggregation indeed occurred in the gelled emulsions. It is also possible that the conformational changes in the globular proteins as they adsorb to emulsion droplets could expose more charged residues outwards into the aqueous phase, optimizing λ-CGN interactions and improving the network (Sharif et al., 2018; Tang & Sun, 2011).

As seen in Fig. 7(bottom), the inclusion of oil droplets did not have any large impact on moduli frequency dependence or the extent of the LVE region by strain. For more homogeneous fine-stranded gels, introduction of emulsion droplets may reduce the yield strain, due to introduction of inhomogeneities into the gel (Sala, van Vliet, Stuart, van Aken, & van de Velde, 2009). However, in this case the gel is already a relatively inhomogeneous particulate gel.

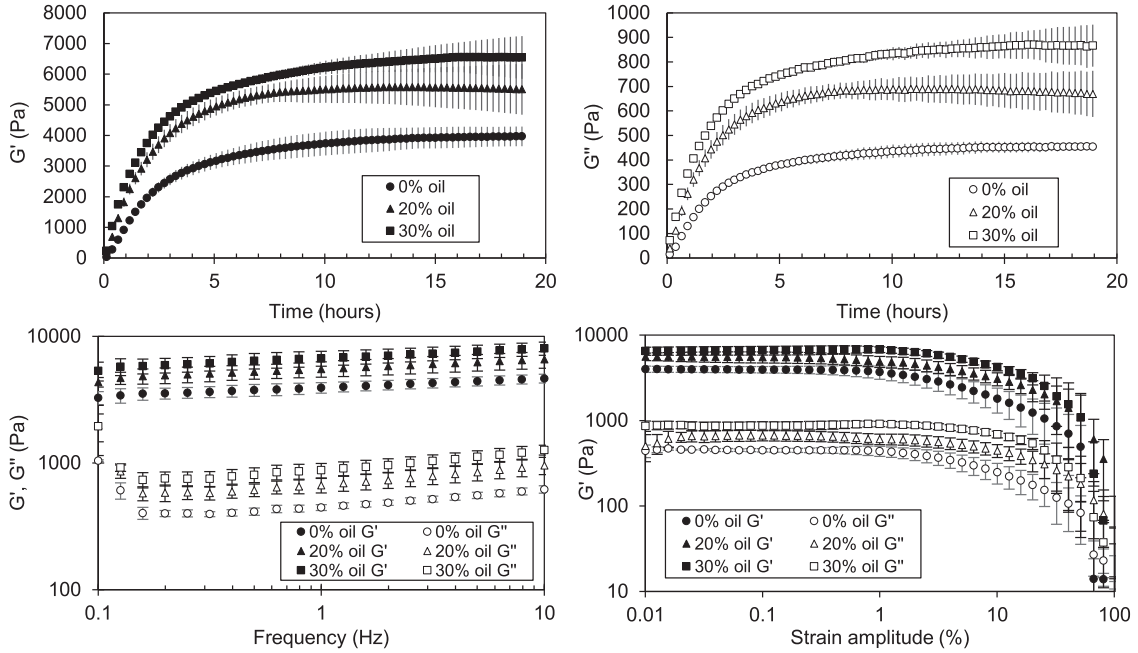

Fig. 7. Rheological data for the homogenized sample without oil, and the two emulsions. Top: Gelling kinetics during 19 hours, G' values (left) and G'' values (right). Bottom left: frequency sweep from 0.1 – 10 Hz at 0.1% strain. Bottom right: amplitude sweep from 0.01 – 100% strain at 1 Hz.

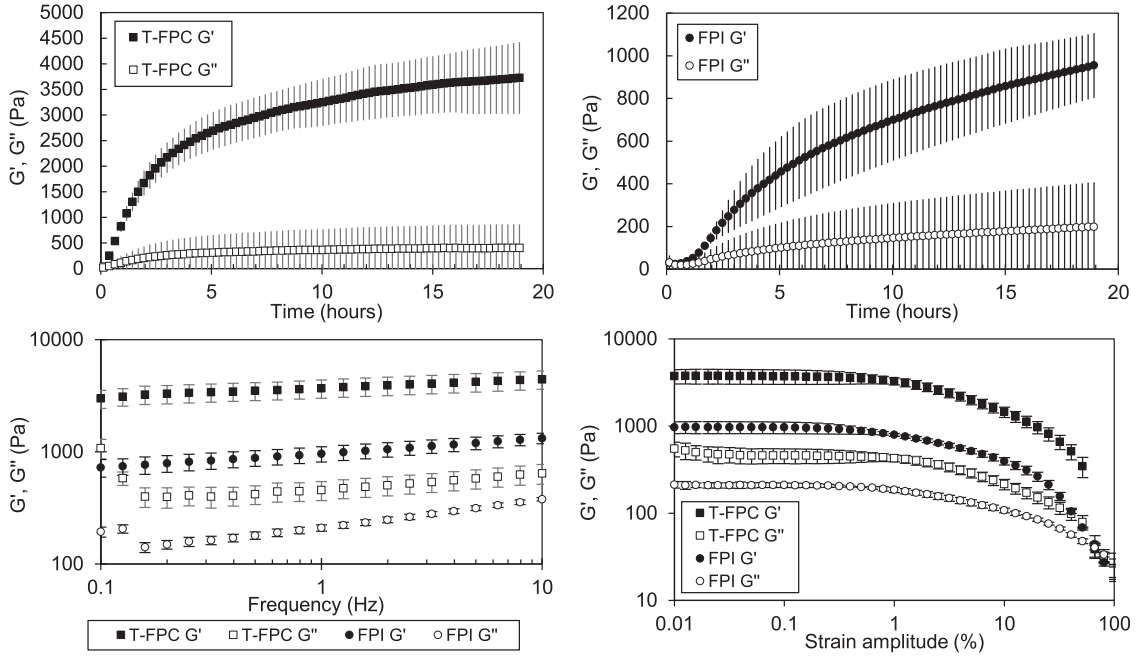

Fig. 8. Rheological data for the formulations with T-FPC and FPI. Top: Gelling kinetics during 19 hours for T-FPC (left) and FPI (right). Bottom left: frequency sweep from 0.1 – 10 Hz at 0.1% strain. Bottom right: amplitude sweep from 0.01 – 100% strain at 1 Hz.

3.5. Similar formulations prepared with other FB protein isolates

As a comparison, formulations were prepared using two other FB protein concentrates/isolates. The formulations were equivalent to the formulation with 13.5% V-FPC and 1.5% λ-CGN, adjusted to have similar protein:λ-CGN ratio as well as similar λ-CGN:water ratio. Both were gelled with 4% GDL. The exact formulations are listed in Table 1.

The FPI formulation resulted in a gel with fairly poor mechanical properties, as shown in Fig. 8 and Table 5. The gelling kinetics curves were somewhat different in shape to the FPC formulations, with a small initial lag period and no clear plateau after 19 hours. Also, the moduli were lower, and G' was only 5 times higher than G''. The formulation made with the FPI also displayed very high levels of syneresis when centrifuged. The expulsed liquid had significant viscosity, indicating significant syneretic loss of λ-CGN (and possibly protein) due to poor network connectivity. To achieve a protein concentration as high as 90%, various wet precipitation methods may be employed. These however commonly lead to some protein denaturation and degradation, with significant changes to native protein functionality (Vogelsang-O'Dwyer et al., 2020). As protein is denatured, hydrophobic residues may be exposed due to disruption of the quaternary and lower structures of the globular proteins, reducing protein solubility and making the protein more

Table 5
Various properties for the gels prepared with FPI and T-FPC instead of V-FPC. Values for V-FPC formulation with 13.5% V-FPC, 1.5% λ-CGN and 4% GDL included for comparison.

	FPI	T-FPC	V-FPC (13.5-1.5)
Before GDL:			
Initial pH	6.93 ± 0.01**	6.64 ± 0.02	6.63 ± 0.01
19 hours after GDL addition:			
pH	3.81 ± 0.01	3.89 ± 0.03	3.87 ± 0.02
G'	955 ± 152**	3723 ± 700	4198 ± 6
G''	209 ± 15**	464 ± 90	476 ± 8
Syneresis	61.8 ± 2.7%**	21.4 ± 1.7%*	14.4 ± 1.9%

*,** = statistically significant difference (* $p < 0.05$, ** $p < 0.01$) from the equivalent formulation with V-FPC (rightmost column).

susceptible to aggregation as interprotein electrostatic repulsion is reduced upon pH reduction. In addition, some highly soluble albumin proteins are typically lost in production of high concentration protein isolates, changing the total protein composition (Vogelsang-O'Dwyer et al., 2020). Overall, these effects may reduce the interaction potential with λ-CGN, explaining the low gel moduli and viscous syneresis for the FPI formulation. However, it is important to note that only one FPI formulation was examined. It is possible that better FPI gels could be achieved through FPI-λ-CGN ratio or pH optimizations.

The T-FPC on the other hand showed very similar properties to the V-FPC in regards to acid induced gelling with λ-CGN. As opposed to the V-FPC, the T-FPC is from a low-tannin FB cultivar. Some tannins can interact with and precipitate proteins, which can reduce protein digestibility and bioavailability. However, as shown here, using dry fractionated FPC from a different strain has little impact on the measured functional properties presented in this study. The only statistically significant difference, a slightly higher syneresis may be due to slight batch differences from the small scale milling and fractionation procedure, such as protein concentration, amount of insoluble particles and particle size.

This again shows that dry fractionation may preserve some interesting native protein functionality, and that this functionality may be lost if other processing methods are employed. It would be of interest to examine if there is a processing method sufficiently gentle to produce a FPI with the functionality to form strong gels with λ-CGN preserved, while also removing the anti-nutrient factors that are present in FB. This may be a requisite for the practical use of this gelling method, until anti-nutrient reduced FB cultivars are more widely available (Khazaei et al., 2019).

4. Conclusions

Dry fractionated FPC in combination with λ-CGN formed gels of decent mechanical properties upon acidification with GDL. The storage modulus values after 19 hours were in the range of 1500 – 4500 Pa, depending on the FPC-λ-CGN ratio and concentration, as well as the final pH (in the range of 3.5 – 4.0). The gels had properties typical of particulate gels, including syneresis and low yield strain. Syneresis was reduced with increasing λ-CGN concentration.

Inclusion of emulsion droplets led to a strengthening of the gel network, likely due to emulsion droplet aggregation. At 30% oil, the gels reached a storage modulus of 6500 Pa, more than a 50% increase from the gel without oil. The gelled emulsions had good emulsion storage stability for at least 3 weeks.

Similar formulations prepared with a processed FPI of higher protein concentration resulted in weak gels with very high syneresis, possibly due to less λ-CGN binding. This shows that some interesting native protein functionality may be lost upon aqueous processing towards protein isolates. Processing based on milling dry fractionation only, which is the method with the smallest environmental footprint, should be the method of choice for gel systems described in this present context.

Declaration of conflicts of interest

The authors declare no conflicts of interest.

Ethics

This work does not include any human or animal experiments

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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