



# Analysis of protein-network formation of different vegetable proteins during emulsification to produce solid fat substitutes

Marie-Christin Baune<sup>1</sup> · Sarah Schroeder<sup>1</sup> · Franziska Witte<sup>1</sup> · Volker Heinz<sup>1</sup> · Ute Bindrich<sup>1</sup> · Jochen Weiss<sup>2</sup> · Nino Terjung<sup>1</sup>

Received: 21 August 2020 / Accepted: 29 November 2020 / Published online: 4 February 2021  
© The Author(s) 2021

## Abstract

Plant-based emulsion gels can be used as solid animal fat substitutes for vegan sausages. For this reason, commercially available protein isolates with different amino acid profiles from pea, soy and potato (Pea-1, Pea-2, Soy, Potato) have been tested for their ability to form shape stable emulsions gels at neutral pH and upon heating to 72 °C. In order to obtain emulsion gels that are as solid as possible, the protein concentrations in the continuous phase ( $C_pC$ , 8.0–11.5% (w/w)) and the oil mass fractions (65–80%) were varied. For leguminous proteins, a positive correlation of both parameters on emulsion rigidity was shown, indicating that both, interfacial and protein–protein interactions, are involved in structure reinforcement. Firmness increased with increasing content in cysteine (Pea-1 < Pea-2 < Soy) and the interactions were of electrostatic, hydrophobic and hydrophilic nature. Potato emulsion rigidity was independent of  $C_pC$  and oil content. The emulsions showed a much higher degree in crosslinking, and very low charge density. Temperature-sweep analysis and CLSM revealed that Potato protein gelled as consequence to low temperature stability. Hence, the structure reinforcement in Potato emulsions mainly contributed to the protein network, with 70% oil and  $C_pC$  11.5% forming a hybrid gel with highest firmness. However, gelling of Potato protein also resulted in interfacial adsorption of protein aggregates and reduced interfacial stability with increasing  $C_pC$ . This was demonstrated in the amount of extractable fat which was 2.0 and 0.6% for Pea-1 and 2 emulsions, 6.4% for Soy and 34.4% of total fat for Potato emulsions.

**Keywords** Vegetable protein isolate · Protein network · Emulsion gel · Emulsion stability · Animal fat substitute

## Introduction

In contrast to liquid vegetable oils, animal fats have a solid and elastic structure at room temperature. This difference is due to a high proportion of saturated fatty acids and their incorporation in a network of connective tissue (collagen) [1, 2]. Collagen has an important function during meat processing. It shrinks when heated and it releases soluble protein, gelatin, which forms a structured network during the cooling process [2]. With the development of vegetable-based sausages, the correct fat choice is therefore crucial for texture.

However, the most well-known solid vegetable fat, palm fat, got into criticism since deforestation in order to make way for oil palm plantations destroys the habitat of many species [3]. Taking into account that animal fat substitutes ideally also have structure-giving and elastic properties, plant-based emulsion gels are considered a promising, vegan and environment-friendly option [4, 5].

Emulsion gels are structured emulsions with gel-like, viscoelastic behavior [6]. In emulsion systems, two immiscible or slightly miscible liquids, e.g. vegetable oil and water, are dispersed with the application of mechanical energy [7, 8]. Since emulsions are thermodynamically unstable at the interfacial area, the free energy at the interface increases with time. As a consequence, emulsions are susceptible to creaming, flocculation and coalescence, leading to phase separation over time [9, 10]. Stabilizers, e.g. proteins, can be added to the system to slow down or stop these processes [11]. Proteins can reduce the interfacial tension of oil in water (o/w) emulsions through adsorption at the interface.

✉ Nino Terjung  
n.terjung@dil-ev.de

<sup>1</sup> DIL German Institute of Food Technologies e. V.,  
Prof.-von-Klitzing-Str. 7, 49610 Quakenbrück, Germany

<sup>2</sup> Department of Food Material Science, Institute of Food  
Science and Biotechnology, University of Hohenheim,  
Garbenstraße 21/25, 70599 Stuttgart, Germany

Partial protein denaturation and unfolding cause exposure of internal hydrophobic amino acids that interact with the oil phase and thus increase the interfacial protein concentration [9, 12]. The effect is enhanced the higher the number in hydrophobic amino acids in a protein [13]. Moreover, proteins can extend over the droplet surface because of their large size. Thereby, the protein substructures can interact with different molecular groups of the oil droplets. This allows the formation of strong surface films that are resistant to mechanical stress and, depending on the protein type, can also be resistant to temperature and pH changes [12, 14]. O/W emulsions, based on protein stabilizers, can form two types of emulsion gels: (I) emulsion-filled protein gels, where proteins act as emulsifiers and thickening or gelling agents, and (II) protein-stabilized emulsion gels, where proteins are used as emulsifiers and high oil concentrations cause formation of a particulate gel by dense packing of the protein-covered oil droplets [6, 15]. Rheological properties of emulsion-filled protein gels are determined by the continuous matrix (protein gel) while for protein-stabilized emulsion gels, they are determined by the properties of the network of aggregated emulsion droplets (filler particles) [15]. The aggregation state of the droplets in turn depends on the volume fraction of the filler droplets (> 50%), the size distribution and the form of the droplets [16–18]. Often, a mixture of both extremes is present where textural properties result from the combined structural properties of the protein gel matrix and the filler particles [15, 19].

In the present study, commercially available protein products from potato tubers, soybean and pea seeds were used as emulsifiers. Soy and pea seeds mainly contain salt-soluble 7S and 11S globulins (65–80%) [20, 21]. Globulins are high in lysine but deficient in sulfuric amino acids (SAA), where 11S globulins generally contain slightly higher amounts of SAA than 7S globulins [22]. The ratio 7S/11S is variety dependent [21, 23]. Pea seeds additionally contain a remarkable amount of water-soluble cysteine-rich 2S albumins (20–35%) [20]. Potato tuber proteins are completely different, consisting mainly of patatin (40%) and protease inhibitors (20–50%) [24, 25]. Further distinctions of the proteins are listed in Supplementary Table S1. The techno-functional properties of these proteins have been extensively studied in the past and depend on the protein type, protein concentration, the degree of denaturation, pH, temperature and ionic strength [10, 21, 26–35]. Emulsification properties of proteins additionally depend on the viscosity of the system and protein solubility, as both affect interfacial protein diffusion, on the emulsification technique, surface hydrophobicity of the proteins and their molecular flexibility [13, 36].

Nevertheless, for commercially available proteins, the emulsifying properties are not that well understood as their production process strongly affects their physicochemical status [37] and composition (presence of specific protein types

in a specific ratio). No information on these points is given in the manufacturers' product specifications. This in turn makes it almost impossible to predict whether they are suitable for specific applications. The aim of this study was to identify a commercially available protein isolate from soy, pea or potato that suits best for preparation of pH neutral, heat-resistant (72 °C) and plant-based emulsion gels of type II to be used as solid animal fat substitutes. Neutral pH (6.5) and resistance up to a temperature of 72 °C were chosen as processing parameters, as they correspond to the processing parameters of a meat-based emulsion-type sausage [38]. The working hypothesis was that the use of high amounts of emulsifiers in the continuous phases and oil fractions above 50% cause the formation of o/w emulsion gels with desired properties. To test the hypothesis, emulsions with different types of proteins (Pea-1, Pea-2, Soy, Potato), protein concentrations in the continuous phase ( $C_pC = 8.0$ – $11.5\%$  (w/w)) and oil (canola oil) mass fractions (65–80% (w/w)) were prepared at pH 6.5 and 65 °C. Once the emulsions had reached ambient temperature, they were reheated to 72 °C and then cooled to 4 °C to simulate the scalding process. Based on firmness determined (penetration test), an oil content of 70% (w/w) and  $C_pC$  of 8.0, 10.0 and 11.5% were chosen to compare the rheological properties, particle size and thermodynamic stability (microscopic structure, extractable fat amount) of the emulsion gels. For better understanding of the achieved differences in emulsification and gelling behavior, the surface charge of the emulsion droplets and the interfacial tension and gelling point of the protein suspensions were determined.

## Material and methods

### Materials and compositional analysis

Canola oil (Jeden Tag, ZHG-mbH, Offenburg, Germany), soy protein isolate WILPRO® D150 (LOT: 20160301, Yihai Kerry Protein Industries Co. Ltd., Qinhuangdao, China) (Soy), pea protein isolates EMPRO® E86 (LOT: L000303647, Emsland Stärke GmbH, Emmlichheim, Germany) (Pea-1) plus PISANE® M9 (LOT: N17146O06, Cosucra Group, Warcoing, Belgium) (Pea-2), and potato protein isolate SOLANIC® 300 (LOT: 30TPG530, Avebe, Veendam, Netherlands) (Potato) were used for all analyses. The amino acid profiles of the protein isolates were provided by the manufacturers (Supplementary Table S2). The crude composition was determined following the ASU methods for water (L 06.00-1), ash (F 0014 (EG)), protein (L 17.00-18) and fiber content (L 00.00-18) according to § 64 LFGB [39]. Fat content was determined following the DGF method C-III 19 (00) [40]. Carbohydrate content was calculated in g per 100 g material from mass balance (Eq. 1):

$$\text{Carbohydrates} = 100 - \text{water} - \text{ash}$$

$$- \text{crude protein} - \text{fat} - \text{fibre} \left( \frac{\text{g}}{100 \text{ g}} \right) \quad (1)$$

The protein content was needed to calculate the weight required to achieve the desired protein concentration in solution. It was 80.00, 77.10, 85.40 and 87.40 g/100 g material for Pea-1, Pea-2, Soy and Potato, respectively (Table 1). Protein solubility was analysed using 5% (w/w) protein in aqueous solution at pH 6.5. After 2 h stirring at 20 °C, 200 rpm, and centrifugation, 10 min, 15,000 rcf, 20 °C, protein content of stock solution ( $C_{\text{STOCK}}$ ) and supernatant ( $C_{\text{SN}}$ ) was determined as described above. The proportion of soluble protein was calculated as g per 100 g protein using Eq. 2:

$$\text{Soluble Protein} = \frac{C_{\text{SN}} \times 100}{C_{\text{STOCK}}} \left( \frac{\text{g}}{100 \text{ g}} \right) \quad (2)$$

### Preparation of protein solutions

Aqueous protein suspensions with a protein concentration of 8.0 to 11.5 g protein/100 g suspension (hereinafter referred to as % (w/w)) were prepared with deionized water and stirred at 750 rpm for 5 min on a magnetic stirrer (RCT Basic, IKA®-Werke GmbH & CO. KG, Staufen, Germany). The pH was measured using pH meter FiveGo™ F2 with pH electrode LE 438-IP 67 (Mettler Toledo, Columbus, Ohio, USA). It was adjusted to 6.5 using single drops of 12.5 mol/L NaOH or 8.9 mol/L lactic acid while stirring. The natural pH of the protein solutions at room temperature was 6.3–6.4 for Soy, 6.9 for Pea-1, 7.4–7.5 for Pea-2 and 3.0–3.1 for Potato.

### Protein analysis

#### Temperature-sweep analysis of continuous phase (protein solution)

Protein solutions containing 10% (w/w) protein were prepared as described above. After stirring, the sample was transferred to the oscillatory rheometer AR 2000 (TA Instruments, New Castle, USA). Applied measuring system was plate/plate geometry (upper plate: 50 mm Ø; gap width: 500 µm). Dynamic-mechanical thermal analyses were carried out with controlled deformation rate (125%) and constant frequency (1.0 Hz) in a temperature range from 20 to 98 °C with  $\Delta T = 2.5$  K/min. The storage and loss moduli ( $G'$ ,  $G''$ ), the torque and the dynamic viscosity ( $\eta$ ) were recorded as function of time respectively temperature. The torque-dependent shear stress was automatically calculated by the program. The test was carried out in duplicates.

#### Interfacial tension

Protein solutions containing 10% (w/w) protein were prepared as described above and heated to 65 °C (standard emulsification temperature) or 72 °C (final heating temperature of the emulsion) respectively on a magnetic stirrer while stirring (750 rpm). After cooling, the static interfacial tension was measured in 40 mL crystallizing dishes (Ø 50 mm, VWR International GmbH, Darmstadt, Germany) versus canola oil using the static force tensiometer K100C with ADVANCE software (Krüss GmbH, Hamburg, Germany). Samples were pretempered to 20 °C in a water bath before measurement at 20 °C. As measuring probe a platinum plate (Wilhelmy plate) was used. Measurement took place with a penetration depth of 2 mm, surface detector velocity of 6 mm/min and sensitivity of 0.005 for 60 s. Interfacial tension was determined in triplicates.

**Table 1** Chemical composition of the protein powders

Content (g/100 g)	Pea-1	Pea-2	Soy	Potato
Water	5.59	5.69	4.36	7.44
Ash	3.79	5.88	4.06	< 0.20
Total Fat	8.75	8.01	5.38	3.15
Total Fiber	1.40	2.40	2.80	6.30
Carbohydrates	< 1.00	< 1.00	< 1.00	< 1.00
Crude Protein	80.00	77.10	85.40	87.40
Thereof soluble protein at pH 6.5 (%)	17.10 ± 1.30	25.10 ± 2.31	10.90 ± 0.43	68.40 ± 1.02

Means were determined by duplicate analysis and means ± standard deviation by threefold analysis

## Emulsion analysis

### Preparation of emulsion gels

In order to determine the oil mass fraction and  $C_pC$  at which a maximum emulsion strength can be produced, the effect of protein and oil concentration on the emulsion strength was analyzed first. Therefore, suspensions with a protein concentration of 8.0–11.5% (w/w) were prepared as described above. The suspensions and the canola oil were heated to 65 °C on a magnetic stirrer while stirring at 750 rpm to enhance the emulsifying capacity of the proteins. Once the temperature was reached, emulsification took place. Emulsions with oil mass fraction of 65, 70, 75 and 80% (w/w) were prepared using rotor–stator disperser Ultra Turrax® T25 with dispersion tool S25N 25G (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at 16,000 rpm by slowly adding the oil until a homogenous emulsion with unchanging consistency was obtained. The emulsions were cooled on ice. After room temperature was reached, the emulsions were filled to the top edge of closed polypropylene beakers with a volume of 70 ml, 44 mm diameter and 55 mm height (Sarstedt AG & Co. KG, Nümbrecht, Germany) without large air inclusions. The closed beakers were placed at 90 °C in a convection oven (Joker B 6-23, Eloma GmbH, Maisbach, Germany) for approx. 20 min, until a core temperature of 72 °C was reached. The temperature was monitored in a reference sample using thermometer testo 926 with needle probe TC type T (Testo SE & Co. KGaA, Titisee-Neustadt, Germany). The samples were always stored at 4 °C overnight (approx. 14 h) before analyses were carried out. After the results of texture analysis were available, only emulsions with an oil mass fraction of 70% and comparatively low (8.0%), medium (10.0%) and high (11.5%)  $C_pC$  were produced and used for the subsequent analysis.

All measurements were performed according to the same scheme. The emulsions were stored in the fridge until shortly before the measurement, removed from the fridge and then measured at room temperature without acclimation time if not stated differently.

### Texture Analysis of the Emulsion Gels

Emulsion firmness was measured instrumentally using Texture Analyzer TA XT2 with 25 kg loadcell (Stable Micro Systems Ltd., Godalming, UK) based on ISO 9665. The samples were measured directly in the polypropylene beakers of 70 ml volume, 44 mm diameter and 55 mm height (Sarstedt AG & Co. KG, Nümbrecht, Germany) at room temperature. A cylinder probe with 20 mm diameter was moved into the sample with  $1.0 \text{ mm s}^{-1}$ . The force development until a penetration depth of 10 mm was recorded and pressure strength ( $\sigma$ ) in Pa was calculated using Eq. 3 where  $F$  is the maximum force (in N)

and  $A$  the frontal area of the pistil (in  $\text{m}^2$ ). The texture was measured by five beakers each in three biological replicates ( $n=15$ ).

$$\sigma = \frac{F}{A} (\text{Pa}) \quad (3)$$

### Frequency-sweep analysis of emulsion gels

Rheological properties of Emulsion Gels were analyzed using AR 2000 rheometer (TA Instruments, New Castle, USA). The applied measuring system was a plate/plate geometry (upper plate: 40 mm Ø; gap width: 2,500 µm). The upper sample part was transferred from the beaker to the rheometer plate using a teaspoon. The gap was adjusted without crushing the sample. Frequency sweep tests were carried out at 20 °C, 5 min using a frequency range of 0.1 to 10 Hz at constant shear stress (resulting from constant torque of 100 µN m). The storage and loss moduli ( $G'$ ,  $G''$ ), the shear deformation ( $\delta$ ) and the dynamic viscosity ( $\eta'$ ) were recorded as function of time respectively frequency. The test was carried out with at least two biological replicates each measured twice ( $n=4$ ).

### Extractable fat amount (EFA)

The determination of the EFA was based on the method of Kiemeyer & Schuster [41]. Prior to the analysis the samples were acclimated to room temperature. 20 g of sample (initial weight  $I$ ) was filled in three 250 ml Erlenmeyer flasks, each. 100 mL petroleum ether (Panreac Química SLU, Barcelona, Spain) was added and the flasks were tightly closed. After shaking, 5 min, 180 rpm, the extracts were filtered through a hydrophobic and hydrophilic filter into new flasks to remove water. Again, 100 mL petroleum ether were added and the whole process was repeated. The extracts were filtered in 250 mL round-bottomed flasks with known weight ( $W_0$ ) and the Erlenmeyer flasks were rinsed with additional 25 mL petroleum ether. After complete removal of petroleum ether by rotary evaporation at 40 °C and 700 mbar, the flasks were heated at 104 °C for 3 h to completely remove the solvent, cooled to room temperature and weighed ( $W_m$ ). The EFA was calculated considering the total fat content  $C_F$  (in g/100 g) by Eq. 4 whereby  $m$  is  $W_m - W_0$ . EFA was determined from three biological replicates each measured three times ( $n=9$ ).

$$EFA = \frac{m \times 100 \times 100}{C_F \times I} \left( \frac{g}{100 \text{ g fat}} \right) \quad (4)$$

### Confocal laser-scanning microscopy (CLSM)

Protein in the emulsion was stained by fluorescein isothiocyanate isomer I (FITC; AppliChem GmbH, Darmstadt,

Germany) and fat by Nile red (Fluka® Analytical, St. Gallen, Swiss). CLSM Nikon ECLIPSE E 600 (Nikon Corporation, Tokyo, Japan) with an oil-corrected 60× objective was used for imaging. Fluorescence was excited with Ar laser at 488 nm and He–Ne laser at 543 nm.

### Droplet size and surface charge density of emulsion droplets

To reduce the impact of protein aggregates on the measurement, emulsion samples were creamed prior to charge density analysis. The emulsions were diluted 1:10 in deionized water and stirred for 1 h. After centrifugation, 5 min, 5000 rpm, the cream phase was used to analyze the droplet size distribution and electric potential.

**Droplet size distribution** Oil droplet size of the cream phase was determined using the laser diffraction spectrometer Mastersizer 2000 (Malvern Instruments, Worcester-shire, UK). The cream was dispersed in a wet dispersion unit (Hydro 2000s, Malvern Instruments) using 10 mmol/L  $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10 \text{ H}_2\text{O}$  (VWR, Darmstadt, Germany) until dimming was reached (density 0.92). The volume mean diameter  $D$  [3, 4] ( $\mu\text{m}$ ), surface mean diameter  $D$  [2, 3] ( $\mu\text{m}$ ) and specific surface area (SSA) in  $\text{m}^2/\text{g}$  was automatically calculated by the program. Samples were measured in triplicates.

**Electric potential** The electric potential  $\phi$  of the cream droplets was analyzed by conductometric titration using the CAS charge analyzing system from AFG (AFG Analytic GmbH, Leipzig, Germany). The cream was dispersed in deionized water (1 g/100 g) and 10 mL of the suspension were injected in the fully automatized titration system. Titration was carried out up to the inflection point ( $\phi=0$  mV) with a volume increment of 50  $\mu\text{L}$  and a dose interval of 4 s. As cationic solution 0.001 N poly(diallyldimethylammonium chloride) (polyDADMAC) and as anionic solution 0.001 N poly(vinylsulfonic acid, sodium salt) (PVSA-Na) were used (both from AFG Analytic GmbH, Leipzig, Germany). Samples were measured fourfold.

**Surface charge density** The charge density of the droplet surface  $\sigma$  was calculated as  $\sigma \pm \Delta\sigma$  using Eq. 5 and Gaussian propagation of uncertainty (6).

$$\sigma = \frac{Q}{SSA} = \frac{V(0 \text{ mV}) \times C_{eq} \times F}{SSA} (\text{C}/\text{m}^2) \quad (5)$$

$$\Delta\sigma = \frac{\Delta Q}{SSA} + \frac{Q}{SSA^2} \times \Delta SSA (\text{C}/\text{m}^2) \quad (6)$$

where  $Q$  is the electric charge of the cream in C/g,  $\Delta Q$  is the uncertainty of  $Q$  in C/g,  $V(0 \text{ mV})$  is the volume of the

electrolyte up to the inflection point in L/g cream,  $C_{eq}$  is the normality  $N$  of the electrolyte in mol/L,  $F$  is the Faraday constant, 96485.33212331 C/mol,  $SSA$  is the specific surface area of the cream droplets in  $\text{m}^2/\text{g}$  and  $\Delta SSA$  is the uncertainty of  $SSA$  in  $\text{m}^2/\text{g}$ .

### Statistical analysis

Except for temperature sweep analyses, which were done in duplicate, all results are expressed as means  $\pm$  standard deviation of at least triplicates. Data analysis was performed in Sigma Plot 13 (Systat Software GmbH, Erkrath, Germany). For statistical analysis Two Way Analysis of Variance (ANOVA) with Tukey test ( $P$ -value = 0.05) was used. Detection of significant differences of means of firmness was performed with two independent analyses, one with protein type and protein concentration of the continuous phase ( $C_pC$ ) as variable, and the other with protein type and oil-suspension (o/s) ratio as variable. Statistical analysis of means of oil droplet size and EFA was performed with protein type and  $C_pC$  as variables respectively.

## Results and discussion

### Protein analysis

#### Compositional differences of the proteins

For all three protein types (soy, pea, potato) it was previously shown that the emulsification and gelling behavior strongly depends on the ratio of the individual protein fractions, i.e. 7S and 11S globulins in soy and pea [27, 36, 42, 43] as well as patatin and protease inhibitors in potato protein [33, 35]. However, manufacturers do not specify which protein fractions are present in the purchased products and what isolation process was used. From the amino acid profiles only small differences between Pea-1 and Pea-2 protein become apparent (Supplementary Table S2). Pea-1 showed slightly higher amounts in asparagine/aspartate, glutamine/glutamate, isoleucine, leucine, phenylalanine and lysine, whereas Pea-2 showed higher amounts in sulfuric amino acids (SAA). The amount of hydrophobic amino acids (HAA) was the same. The results of Rubio et al. [44], who analyzed the amino acid profiles of the different pea protein fractions, lead to the suggestion that due to the lower SAA and higher leucine, isoleucine, lysine and acidic amino acid content of Pea-1 a higher content of 7S globulins is present, while Pea-2 has a higher content of 11S globulins and due to the high SAA content this might also be the case for albumins. The amino acid profile of Soy was similar to that of the pea proteins. Noticeable is a slightly higher glutamine/



glutamate, proline and SAA content and a slightly lower arginine, leucine, lysine and valine content. Whereas high SAA content contributes to soy 11S globulins, high amounts in glutamate and proline and low amounts of leucine and valine indicate the presence of 7S globulins [45]. Again, the amount of HAA was the same. Potato showed a completely different amino acid profile with a much higher proportion in SAA and HAA. During the experiments a published patent-application revealed that the potato protein is a protease-inhibitor fraction [46].

For the correct initial weight of the proteins in suspension, the proximate composition and protein solubility of the commercially available products were determined (Table 1). Potato showed the highest protein and fiber content whereas the pea proteins showed the highest oil content. Carbohydrates can also act as stabilizers for emulsions but the final concentration in the emulsions is very low compared to the protein content (Supplementary Table S3) and varies between 0.12 and 0.28 g/100 g emulsion. For Pea-1, Pea-2 and Soy protein the solubility was low at pH 6.5. This is in accordance with literature as the isoelectric point of both proteins is reported to be 4.5 resulting in lowest solubility in the pH range 4–6 [10, 47]. A neutral pH was preferred as the pH of scalded sausages is approx. 6.5 [38]. In addition, the aim was to produce an edible animal fat substitute. Hence, neither an acidic pH (<3.5) nor a basic pH (>8.5) was an option to increase protein solubility as reported in literature [10, 36]. The natural pH of the protein solutions at room temperature was 6.3–6.4 for Soy, 6.9 for Pea-1, 7.4–7.5 for Pea-2 and 3.0–3.1 for Potato. High protein solubility on the one hand is important to achieve higher diffusion rates of proteins to the o/w interface [12, 48]. Though, molecular flexibility and surface hydrophobicity of globular proteins also play an important role during emulsification, while the hydrophobicity can be more directly correlated with the emulsion capacity of the proteins [13]. An increase in surface hydrophobicity generally enhances inter-droplet interaction and prevents the droplet from aggregation [49]. In case of emulsion gels however, the formation of a network structure depends on aggregation of protein subunits (emulsion-filled protein gels) or aggregation of protein-covered oil droplets (protein-stabilized emulsion gels) or a mixture of both (hybrid gels). For the formation of protein aggregates, on the contrary, low protein solubility and charge is advantageous, so that subunits come close to each other for the formation of bonds. The formation of stable emulsion gels, hence, depends on the ability of the protein to aggregate itself, to allow droplet aggregation and to stabilize the interfaces between aggregated emulsion droplets by formation of thin but strong continuous protein films. Therefore, it was first analyzed whether the heating or reheating temperatures of 65 °C and 72 °C cause unfolding of the proteins which

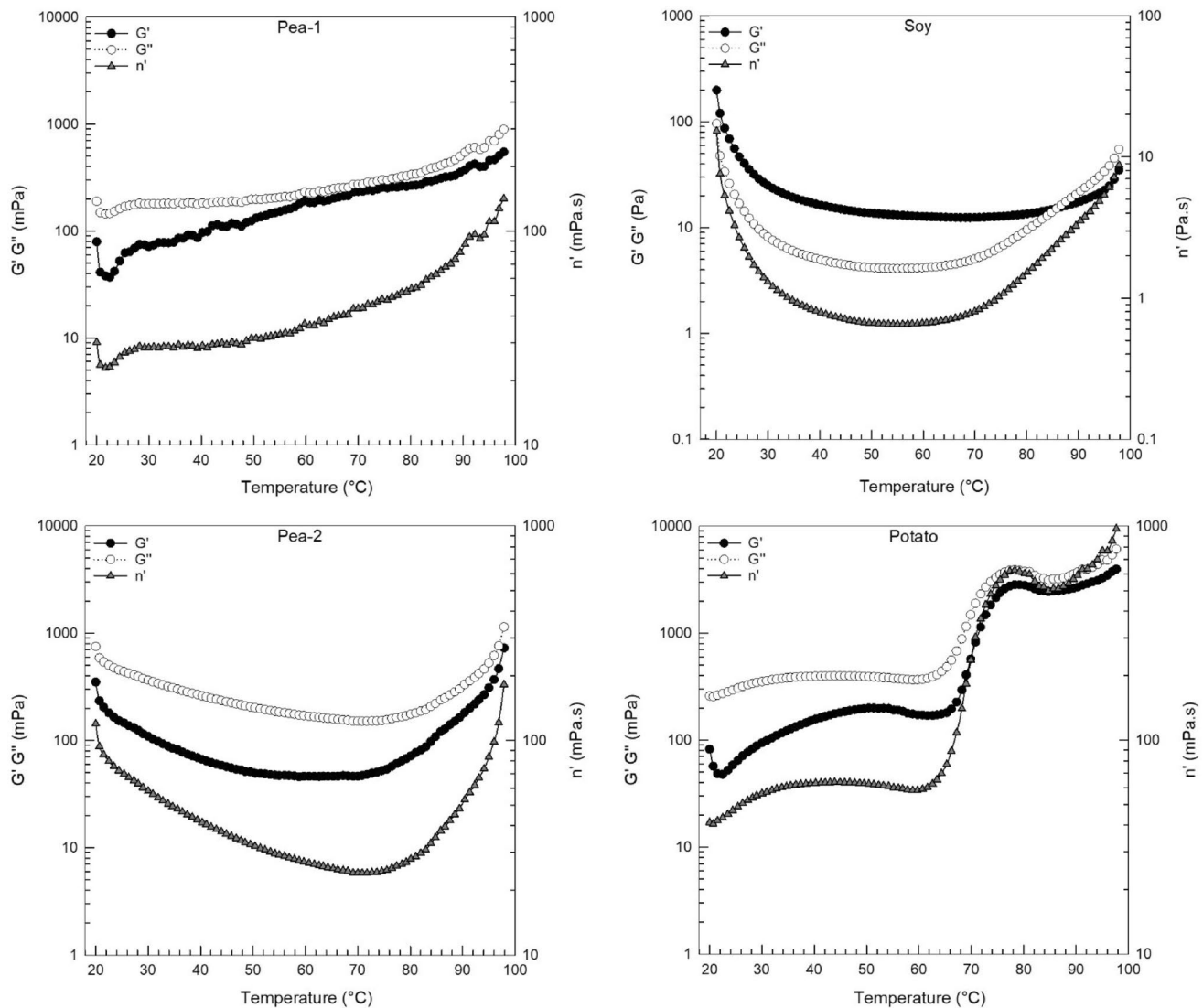
could promote aggregation and protein-gel formation and increase hydrophobicity to lower the interfacial tension.

### Effect of heating on protein denaturation and gelling

To test whether the proteins unfold and may aggregate upon heating to 72 °C, suspensions with 10 g protein per 100 g solution were prepared at room temperature and a dynamic-mechanical thermal analysis was carried out. This protein concentration was selected because it represented approximately the mean of the concentration range studied. The results in Fig. 1 revealed that in Pea-1 protein molecules already unfold above 40 °C. An exponential increase in the dynamic viscosity  $\eta'$  at temperatures above 80 °C and after reaching a maximum at about 90 °C also above 92 °C, indicated that protein denaturation enhanced. As the denaturation temperatures correspond to 7S and 11S globulins as well as 2S albumins (Supplementary Table S1, [10, 26, 36, 47]) it is assumed that all major storage proteins are present, especially 7S globulins. For Pea-2 the curves of the storage modulus  $G'$ , the loss modulus  $G''$  and  $\eta'$  decreased until a minimum was reached (at 71.75 °C) followed by an exponential increase at temperatures above 72 °C. The higher denaturation temperature supports the assumption that 7S globulin content in Pea-2 is lower than in Pea-1. As viscosity further increases exponentially at temperatures above 90 °C 2S albumins may be also present as previously assumed. The results clearly point out that none of the Pea proteins could form a protein gel when heated to 65 or 72 °C. However, the results show that the proteins are more likely to unfold in Pea-1 than in Pea-2.

Soy protein suspensions turned out to show unexpected behavior as they were generally flowable but during the oscillation test  $G'$  appeared to be higher than  $G''$ . In addition, the measured values for Soy suspensions were much higher in comparison to the other proteins. The results can be explained by the phenomenon that, due to the low protein solubility ( $10.9 \pm 0.43\%$ ) and high solid content, particle–particle interactions appear at low temperatures since particle movement at low temperatures is slow. As the temperature increases, particle movement also increases, resulting in a decrease in interactions and measured viscosity. At approx. 70 °C viscosity increases again, most likely as a result of 7S globulin denaturation and at higher temperatures 11S globulin denaturation. At temperatures above 90 °C the slope becomes exponential. Again, gel-formation of the protein cannot be assumed as  $G'$  and  $G''$  curve fitting appears at 98 °C (Fig. 1).

For Potato protein suspensions, the exponential rise of all measured variables above 58.8 °C indicated excessive denaturation of the protein. The denaturation temperature is in line with the denaturation temperature of potato



**Fig. 1** Dynamic-mechanical thermal behavior of the different plant protein suspensions. Plotted are storage ( $G'$ ) and loss moduli ( $G''$ ) in mPa and the dynamic viscosity ( $\eta'$ ) in mPa s as function of tempera-

ture ( $\Delta T = 2.5$  K/min) at constant deformation rate (125%) and frequency (1 Hz). Note that for Soy protein suspension the unit for  $G'$  and  $G''$  is Pa and for  $\eta'$  Pa s

protease inhibitors stated in literature (60–65 °C) [33, 50, 51]. Though, sol–gel transition of the protein suspension is not completely reached, the formation of a protein gel upon cooling and in the presence of oil can be assumed. This assumption is supported as the least gelation concentration of proteins decreases with increasing oil amounts [17]. Therefore, Potato protein could be a candidate to form type I emulsion gels (emulsion-filled protein gels) or hybrid gels, while the leguminous proteins are good candidates to form type II (protein stabilized) emulsion gels at chosen conditions.

### Protein-dependent interfacial tension

Since temperature changes have an influence on the degree of denaturation and hydrophobicity of proteins, the protein surface excess and the interfacial tension are also dependent on temperature [52]. For this reason, the suspensions that contained 10.0% (w/w) protein were heated to 65 °C as normally done prior to emulsification or to 72 °C, which was the reheating temperature of the final emulsions. As shown in Table 2, Potato protein revealed significantly lower interfacial tension values after heating to 72 °C ( $P$  value = 0.017). The interfacial tension of Potato protein was also significantly lower in comparison to Pea-1 ( $P$  value = 0.038),

**Table 2** Interfacial tension of the plant proteins

Heating Temperature (°C)	Interfacial Tension (mN/m)			
	Pea-1	Pea-2	Soy	Potato
65	28.8 ± 0.9	36.3 ± 1.1	33.1 ± 5.3	29.1 ± 1.9 <sup>1</sup>
72	30.3 ± 1.1 <sup>w</sup>	36.9 ± 2.8 <sup>x,y</sup>	29.8 ± 3.2 <sup>x,z</sup>	24.1 ± 1.7 <sup>1,w,y,z</sup>

For measurement protein suspensions with 10 g protein/100 g were prepared, heated (65 or 72 °C), cooled (20 °C) and coated with canola oil

Means ± standard deviation were determined by triplicate analysis. Different superscripts indicate significant differences ( $P < 0.05$ ): <sup>1</sup> within the same sample at 65 °C and 72 °C, and <sup>w–z</sup> between the different proteins at the same temperature

Pea-2 ( $P$  value  $< 0.001$ ) and Soy ( $P$  value = 0.028). Similar observations on the interfacial tension were made for potato protease inhibitors by van Koningsveld et al. [35] upon heating to 80 °C. This indicates that Potato protein was faster adsorbed at the interface and that it increased its hydrophobicity at 72 °C and consequently unfolded to a higher degree, confirming the results in Fig. 1. Additionally, Potato naturally contains more hydrophobic amino acids (Supplementary Table S2). The faster adsorption and the lower temperature stability can be explained by the comparatively small molecule size of potato protease inhibitors (4.3–40 kDa) [25, 53]. Moreover, the Potato protein shows higher solubility (Table 1) which would allow faster adsorption to the interface. However, the solubility of proteins is temperature-dependent and may be reduced after Potato protein aggregate formation, so that no statement can be made in this respect. By contrast, the leguminous proteins showed no significant changes in interfacial tension upon heating to 72 °C, although for Pea-1 protein a slightly rising and for Soy protein a reduction tendency can be seen. The results match with the denaturation behavior (Fig. 1).

To evince protein-dependent differences in emulsion gel formation and to find an oil concentration at which the most solid emulsion gels are formed, the effect of varying oil mass fraction (65–80%) and protein concentration in the continuous phase ( $C_pC$ , 8.0–11.5%) on emulsion rigidity was analyzed first.

## Emulsion analysis

### Effect of protein and oil concentration on emulsion-gel firmness and structure

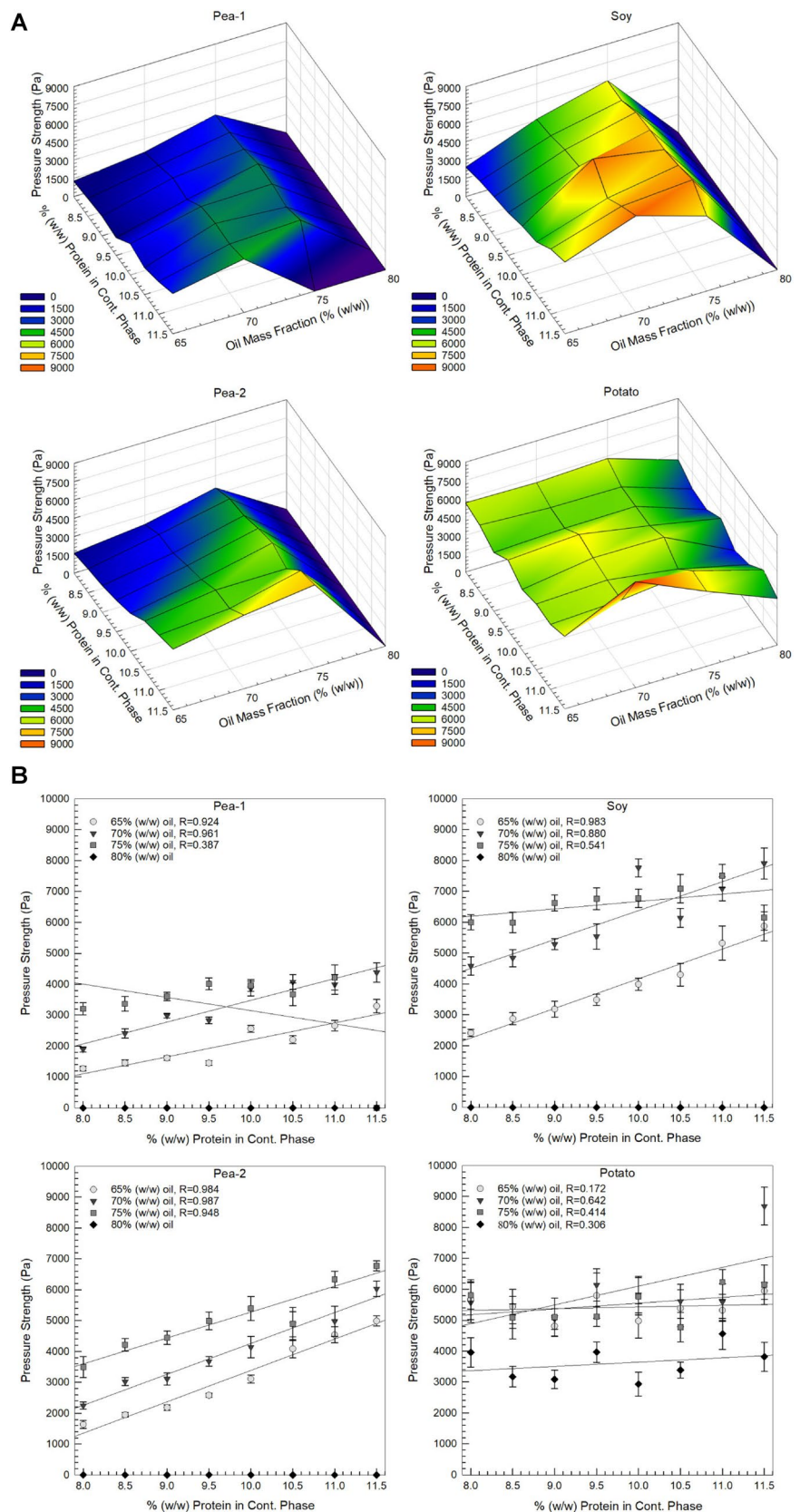
The observed, structural changes of the emulsions are reflected in the texture analysis results (Fig. 2). The oil and protein concentration had a considerable effect on the macroscopic structure (change from a creamy to a stiff texture) of the emulsions prepared with leguminous proteins. An

increase of both resulted in an increase of emulsion firmness which can be explained by the literature findings (I) that with increasing oil content the interfacial area and thus interface-interface interactions increase, and (II) that with increasing protein content the interactions between protein and interface (electrostatic, hydrophobic) and between protein subunits (electrostatic, hydrophilic) increase. This finally results in the formation of protein aggregates that additionally contribute to structure reinforcement [15, 18, 54]. However, significant differences appeared between the three leguminous proteins. Pea-1 emulsions generally had the lowest firmness and the increase stagnated at an oil content of 75% and  $C_pC$  of 9.5% (w/w) which indicates weakening of the structure, e.g. due to coalescence of oil droplets. Most likely the amount of adsorbed protein at the interface is reduced, which is caused by both, reduction of the protein content through increasing oil amounts (see Supplementary Table S3) and either enhanced protein-aggregate formation or lowered protein diffusion in consequence of increasing viscosity which accompanies higher protein concentrations [12, 55]. Pea-2 emulsions showed a higher increase in strength than Pea-1 at all examined oil and protein concentrations. The strength increases strongly with increasing protein concentration. Thus, the ability of Pea-2 to form non-covalent bonds between protein subunits is enhanced in comparison to Pea-1. Soy emulsions had an even higher initial and final firmness than Pea-2 emulsions. In addition, the effect of oil on firmness was even more pronounced in soy. Hence, the ability to form hydrophobic protein-interface bonds and hydrophobic as well as hydrophilic protein-protein interactions that cause protein aggregate formation is even more enhanced if Soy protein is used. Assuming that all proteins are capable of entering into spatial proximity and forming covalent bonds, the difference in the number of covalent bonds is exclusively due to differences in the amino acid profile (Table S2). The amount of HAA is the same in all three proteins. However, no data about availability (surface hydrophobicity) are present. It is also known that uncharged, hydrophilic amino acid residues are involved in hydrogen bonding and hydrophilic adsorption [56, 57]. Since no distinction can be made between glutamate and glutamine, aspartate and asparagine, and other uncharged polar amino acids do not vary in the three legume proteins, cysteine, which has ascending concentration in Pea-1, Pea-2 and Soy, could have a major impact.

At an oil content of 75% and  $C_pC \geq 8.5\%$  structural weakening was observed for Soy emulsions. At this point it is most likely, that coalescence appeared as the formation of protein-aggregates exceeded the adsorption of protein to the interface. The findings were also reflected by the linear regressions ( $R$ ) for pressure plotted against  $C_pC$  (Fig. 2b). By contrast, for emulsion gels prepared with Potato protein, firmness was much higher than of Soy emulsions at low oil



**Fig. 2** Pressure strength of the emulsion gels. Plotted is **a** the maximum pressure  $\sigma$  (Pa) and **b** the linear regression of  $\sigma$  (Pa) as function of protein type, oil mass fraction (65–80% (w/w)) and  $C_pC$  (8.0–11.5% (w/w)). **a** Violet, 0 Pa/instable (coalesced) emulsion; dark blue, 1–1500 Pa; medium blue, 1501–3000 Pa; medium green, 3001–4500 Pa; light green, 4501–6000 Pa; yellow, 6001–7500 Pa; orange, 7501–9000 Pa. **b** oil mass fraction: 65%,  $\circ$ ; 70%,  $\blacktriangledown$ ; 75%,  $\blacksquare$ ; 80%,  $\blacklozenge$  (Color figure online)



mass fraction (65%). In addition, no dependency between firmness and  $C_pC$  was found independent from the oil mass fractions (Fig. 2b). The difference can be explained in the low temperature stability of Potato (Fig. 1) which results in denaturation of the protein upon heating to 65 °C during the emulsification process and finally the formation of a protein-gel network upon cooling. This becomes obvious as no visible structural differences appeared between emulsions that have been reheated to 72 °C or not (Supplemental Figure S1). Hence, the Potato protein forms emulsion-filled protein gels (type I) at the chosen conditions and no protein-stabilized emulsion gels (type II). At an oil content of 65% (w/w) the firmness of Potato emulsions is higher than of leguminous emulsions. It has been previously shown for soy protein that heat-denatured, aggregated proteins result in much higher rigidity of emulsions than non-aggregated proteins [18, 54]. Although different types of proteins are compared, at this oil content the formation of an aggregated Potato-protein networks results in higher firmness than the formation of a Soy-protein stabilized emulsion gel. The increase of  $C_pC$ , which leads to a final protein increase of 0.8–1.2% protein in the emulsion system (Supplemental Table S3), had no further impact on the protein-gel rigidity at any oil content. Hence, the comparatively small amount cannot cause further network reinforcement of the Potato protein. This may be also explained by the findings of Schmidt et al. [33] who could show that potato protease inhibitors as opposed to patatin and protein mixtures, are poor gel formers. Even with increasing oil content, no increase in emulsion strength due to increasing interfacial interactions can be observed. Only at an oil mass fraction of 70% and  $C_pC$  of 11.5% a positive effect on emulsion strength can be monitored. Thus, if enough interfaces connect with increasing oil content, an additional increase in emulsion firmness can be achieved and a hybrid emulsion gel is formed. At very high oil content (80%), the structure of the emulsions is again weakened, most likely due to coalescence.

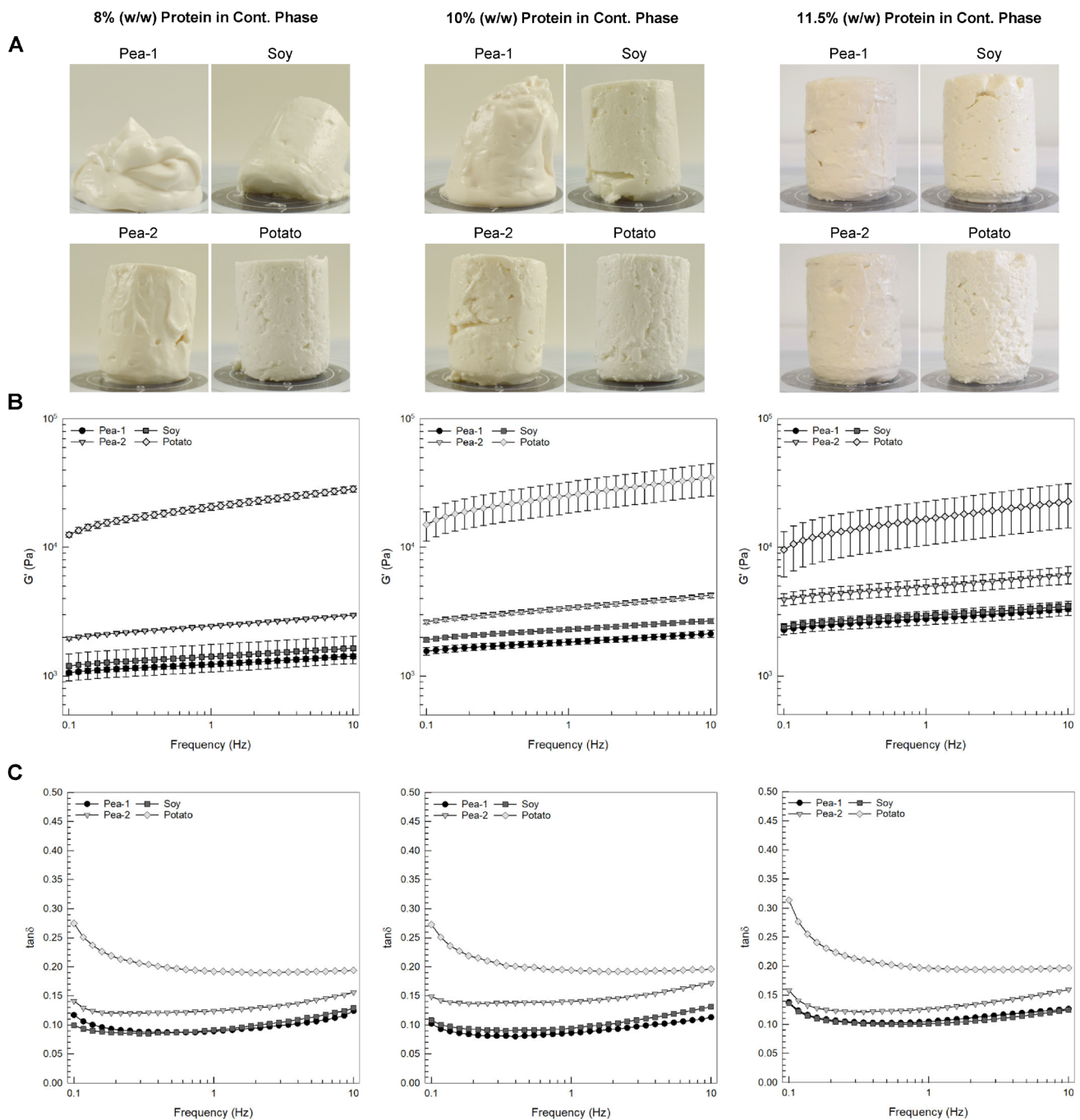
As for Pea-1, Soy and Potato emulsions the highest rigidity was obtained when emulsifying 70% (w/w) oil, this oil mass fraction and comparatively low (8.0%), medium (10.0%) and high (11.5%)  $C_pC$  were chosen to further analyze the effect of the different proteins on the emulsions' rheological behavior and stability.

The effect of reheating to 72 °C on structure is exemplarily shown for emulsions with 70% (w/w) oil in the Supplementary (Figure S1). It can be seen that the reheating step increases the strength of all emulsions with increasing protein content. However, the effect is much more pronounced for Pea-1 and Soy protein than for Pea-2 and Potato. Pea-2 and Potato emulsions possessed a firm structure under all displayed conditions. Potato emulsions only showed a structural difference before and after reheating, independent of the  $C_pC$ , which corresponds to the texture measurement.

The results clearly indicate that with increasing protein content and upon heating additional bonds between all proteins occur but for Potato and Pea-2 most bonds already form during the emulsification process.

### Effect of protein type on viscoelastic behavior of emulsion gels

The emulsions that have been prepared with a  $C_pC$  of 8.0, 10.0 or 11.5% and 70% (w/w) oil are illustrated in Fig. 3a. All emulsions appeared as white and shape stable solids except the emulsions prepared with 8.0% Pea-1 protein (creamy, mayonnaise-like structure) and 10.0% Pea-1 protein (stiffer, creamy mayonnaise-like structure), 8.0% Pea-2 protein (very stiff but creamy mayonnaise) and 8% Soy protein (creamy, mayonnaise-like structure) in the continuous phase. The white appearance is typical for emulsions with droplet in the  $\mu\text{m}$  range, namely macroemulsions [58]. The presence of macroemulsions was confirmed in the CLSM images (see Fig. 4) and droplet size analysis of the cream phase (Fig. 5 and Supplemental Table S4). The volume mean diameter ( $D_{[3,4]}$ ) of Pea-1 and 2 emulsions was approx. 18  $\mu\text{m}$ . That of Soy emulsions varied between 70 and 75  $\mu\text{m}$ . A particle-size in the  $\mu\text{m}$ -range is typical if bridging-flocculation of oil droplets appears and flocculated networks have formed [15, 59]. Solid state properties were proven by oscillatory rheology in the linear-viscoelastic region with a frequency sweep from 0.1 to 10 Hz (Fig. 3b). All emulsions showed a typical curve with low slope.  $G'$  was much higher than  $G''$  (shown by the loss factor  $\tan\delta$  in Fig. 3c) which confirmed that all emulsions had formed crosslinked, viscoelastic gel-like networks. None of the emulsions showed phase crossover ( $G''$  becoming higher than  $G'$ ) which would indicate shear thinning or brittle fracture. In addition, higher  $G'$  values at low frequency indicate a higher degree of crosslinking [60]. For this reason, all Potato protein emulsion gels showed a much higher degree in crosslinking ( $G'$  values at 0.1 Hz) than Pea-2 protein emulsion gels whereas the lowest crosslinking degree was shown for Soy and Pea-1 protein-based emulsion gels. For all leguminous proteins a slight increase in  $G'$  with increasing  $C_pC$  can be observed. This finding confirms the assumption, that with increasing  $C_pC$  more protein-interface and protein-protein interactions appear (non-covalent bonds are formed). Addition of SDS to the emulsions caused the emulsions to become less viscous. Although the effect was less pronounced for Pea-2 protein, this shows that for all emulsion gels the interactions between proteins are based on hydrogen bonds rather than covalent disulfide bridges. Contrary to the texture analysis, Pea-2 emulsions appeared to be firmer than Soy emulsions. This can be explained as the texture analyzer measures macroscopic deformation, while the rheometer measures non-perceptible deformation which provides information on molecular interactions. Other



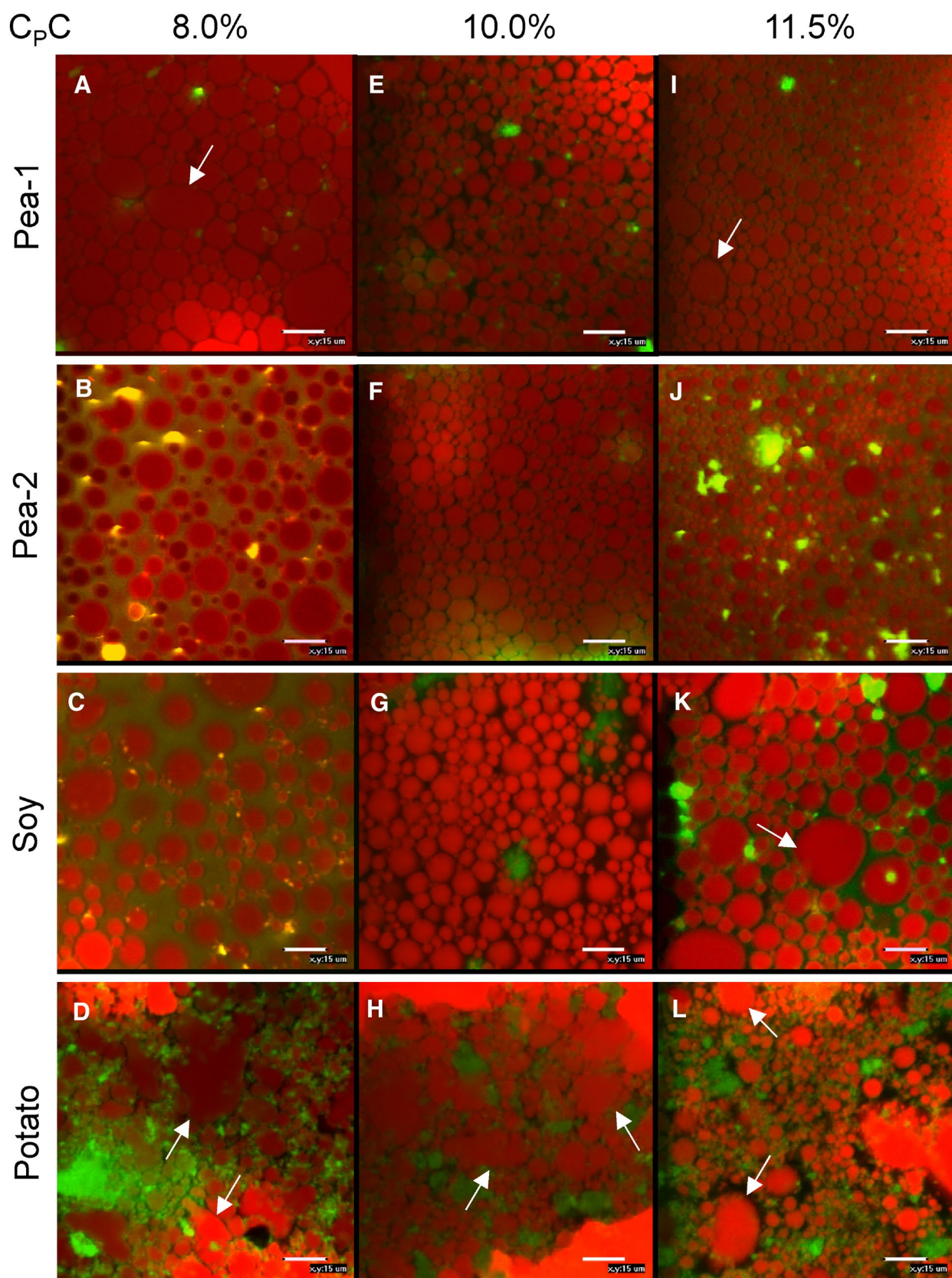
**Fig. 3** Appearance, storage modulus and loss factor of the emulsion gels. **a** Appearance of the emulsion gels in dependence of  $C_pC$  and protein type. Plotted is **b** the storage modulus  $G'$  (Pa) and **c** the loss

factor  $\tan \delta$  of emulsion gels as function of protein type (Pea-1, ●; Pea-2, ▼; Soy, ■; Potato, ◆),  $C_pC$  and frequency (Hz). Emulsion gels were prepared with oil mass fraction of 70%

authors have also reported different properties when applying and comparing the two methods [33]. Firmness of Potato emulsions with  $C_pC$  11.5% decreased which contradicted the result of texture analysis (Fig. 2) and can be explained by the change from an emulsion filled protein gel (type I) to a hybrid gel.

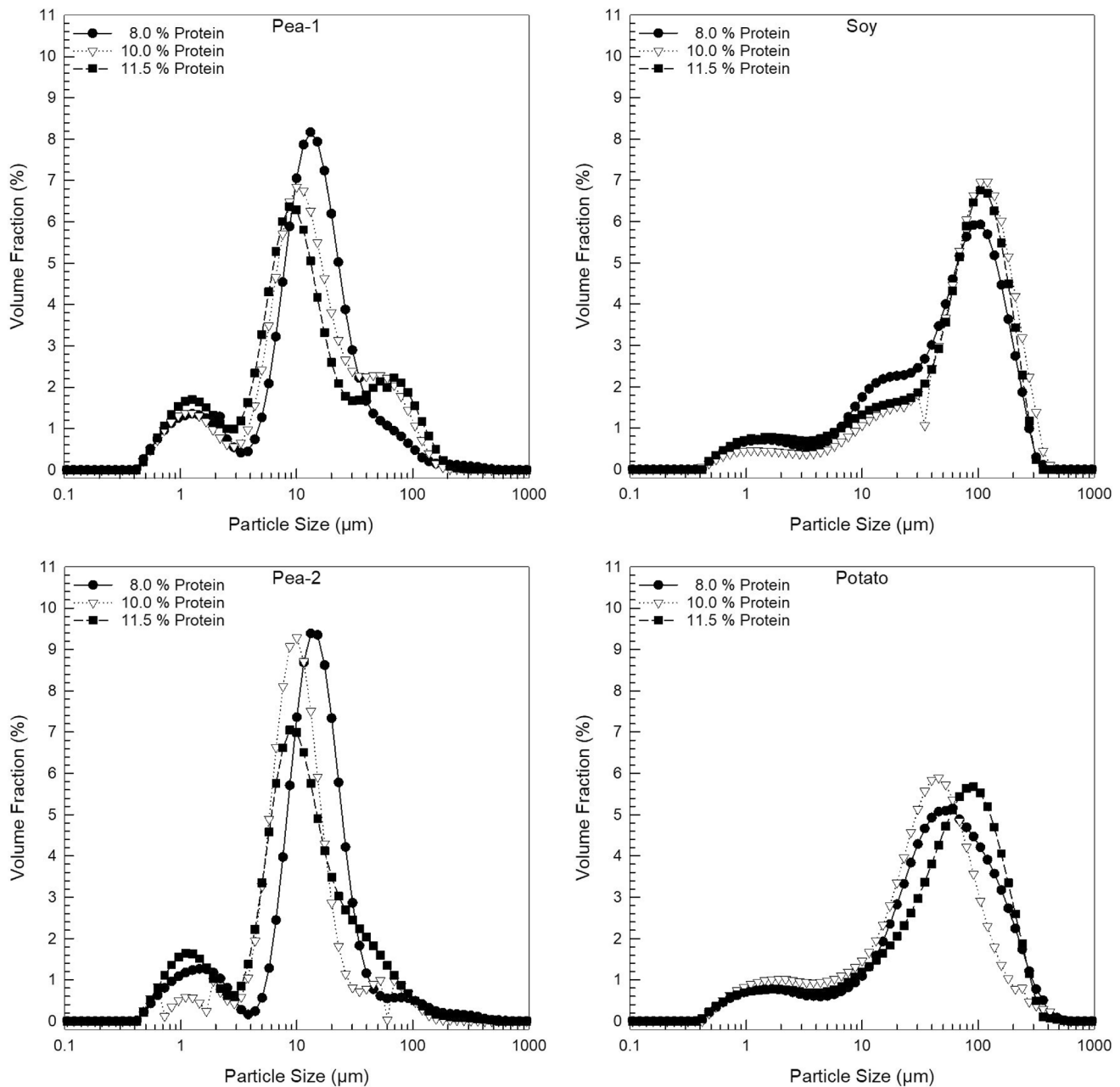
The protein–protein interactions of the Potato protein network mediate enhanced viscoelastic properties in comparison to the networks that rely on aggregated oil droplets. Similar findings have been made for Soy protein by Tang and Liu [18] who could show, that a pre-heated (95 °C) soy protein isolate forms much stronger emulsion gels based on bridging flocculation (in this case a hybrid gels) than





**Fig. 4** CLSM pictures of the emulsion gels. Emulsion gel microstructure in dependence of protein type and  $C_pC$  (8.0%, panel **a–d**; 10.0%, panel **e–h**; 11.5%, panel **i–l**). Emulsion gels were prepared with oil

mass fraction of 70%. Canola oil was stained with Nile Red (red signals) and proteins with FITC (green signals). White arrows indicate coalesced oil droplets. Bars, 15  $\mu m$  (Color figure online)



**Fig. 5** Particle-size distribution of the creamed emulsions. Plotted is the volume fraction (%) of creamed emulsion particles as function of particle size ( $\mu\text{m}$ ) dependent on the protein type (Pea-1, Pea-2, Soy,

Potato) and concentration in the continuous phase (●, 8.0% (w/w); ▼, 10.0% (w/w); ■, 11.5% (w/w))

unheated soy protein isolate that formed gels that depended on bridging-flocculation of oil droplets only (type II emulsion gels). Despite the good viscoelastic properties of Potato emulsions, at low frequency (0.1 Hz)  $\tan\delta$  of Potato protein emulsion gels was 0.27–0.31 (Fig. 3c) with a strong tendency to increase against 1 with lower frequencies. This indicates susceptibility to syneresis [60, 61]. This can be explained by the emulsion structure as in flocculated protein

networks, the stability of the emulsion relies on the stability of the gel-like structure. Therefore, phase separation effects are rather related to syneresis than creaming events [62, 63]. Syneresis can appear as consequence of gel shrinkage. This leads to an increase in capillary diameter (interspace), which reduces the capillary force resulting in serum release. However, to determine this, the emulsions must be stored for a longer period of time.



### Charge distribution of the emulsion droplets

Protein emulsifiers differ in the rate of interface adsorption, in the minimum amount that is required to saturate the droplet surface, and in their ability to form stable films under different environmental conditions [64, 65]. A high charge density as well as well-distributed charge on the film favors electrostatic repulsion that counteracts attractive van der Waals forces, both should stay in balance [66, 67]. While the isoelectric point (pI) of globulins is 4.5 [68, 69] causing a negative charge at pH 6.5, the pI of potato protease inhibitors is between 5.6 and 7.8, depending on the protease inhibitor type [25]. Consequently, the used Potato protein could have both low positive or low negative charge at pH 6.5. To answer this question, the specific surface area (SSA) and charge potential  $\phi$  of the emulsion cream was analyzed and the charge density  $\sigma$  per droplet surface area was calculated (Eqs. 5 and 6). The results in Table 3 show that the Potato protein-covered oil droplets were weakly positive charged, and the charge density was half of that of the pea protein-covered droplets. This indicates that the pI of this specific Potato protein isolate is greater but close to pH 6.5. All leguminous proteins were negatively charged and by far the highest charge density was shown for Soy protein-covered droplets. These findings lead to the assumption that

in Potato protein emulsions gelling of proteins is promoted due to the lower charge density as well as the low heat stability ( $< 65^\circ\text{C}$ ). This causes low electrostatic repulsion of the unfolded proteins at pH 6.5, due to the close location to the pI [67] as well as of the small molecular size (according to literature 4.3–25 kDa) [25, 53, 59, 70].

### Effect of protein type and concentration on emulsion stability

To state about stability of oil droplet interfaces and the emulsions in general, the extractable fat amount (EFA) was determined in dependence of  $C_pC$ . In a stable emulsion with stable interfaces, the oil droplets are strongly encapsulated within the protein layer. Consequently, emulsions with poor or unfavorable protein-interface coating have a high EFA value and less stable interfaces. As shown in Table 4 lowest EFA and therefore highest interface stability was shown for Pea-2 and Pea-1 protein emulsion gels. The EFA of Soy protein emulsion gels was higher but the emulsion interfaces appeared much more stable than those of Potato which can be considered unstable. Compared to the leguminous proteins, the interfacial tension was shown to be significantly reduced by the potato protein (Table 2). However, not quantity but quality of emulsifiers is important to form stable

**Table 3** Droplet surface area and calculated surface charge density

	SSA (m <sup>2</sup> /g)	$\phi(0 \text{ mL})$ (mV)	V(0 mV) (mL/g)	$\sigma$ (mC/m <sup>2</sup> )
Pea-1	1.337 $\pm$ 0.058 <sup>1,2</sup>	– 1314.74 $\pm$ 87.42 <sup>1,2</sup>	2.74 $\pm$ 0.17 <sup>1</sup>	197.73 $\pm$ 20.67 <sup>1,2</sup>
Pea-2	1.547 $\pm$ 0.127 <sup>3,4</sup>	– 1634.25 $\pm$ 56.76 <sup>1,3</sup>	2.78 $\pm$ 0.09 <sup>2,3</sup>	173.39 $\pm$ 20.02 <sup>3,4</sup>
Soy	0.501 $\pm$ 0.103 <sup>1,3,5</sup>	– 1655.50 $\pm$ 467.37 <sup>4</sup>	2.36 $\pm$ 0.32 <sup>2,4</sup>	453.54 $\pm$ 154.50 <sup>1,3,5</sup>
Potato	0.906 $\pm$ 0.114 <sup>2,4,5</sup>	560.50 $\pm$ 132.32 <sup>2,3,4</sup>	0.93 $\pm$ 0.04 <sup>1,3,4</sup>	98.51 $\pm$ 17.12 <sup>2,4,5</sup>

Listed are the specific surface area (SSA) of the creamed emulsion droplets in m<sup>2</sup>/g, the initial charge  $\phi(0 \text{ mL})$  in deionized water (0.1 g/10 g) in mV and respective electrolyte volumes added up to the inflection point V(0 mV) in mL/g cream, as well as the calculated surface charge density  $\sigma$  in mC/m<sup>2</sup>. Emulsions were prepared with  $C_pC$  of 10.0% (w/w) and oil mass fraction of 70%

For SSA means  $\pm$  standard deviation were determined by triplicate analysis. For  $\phi(0 \text{ mL})$  and V(0 mV) means  $\pm$  standard deviation were determined by quadruplicate analysis.  $\sigma \pm \Delta\sigma$  was calculated using Eqs. 5 and 6. Different superscripts within one parameter indicate significant differences between proteins ( $P < 0.05$ )

**Table 4** Extractable fat content of emulsions

$C_pC$ (%)	EFA (g/100 g fat)			
	Pea-1	Pea-2	Soy	Potato
8.0	1.37 $\pm$ 0.33 <sup>v,w</sup>	0.47 $\pm$ 0.15 <sup>x,y</sup>	4.64 $\pm$ 1.65 <sup>v,x,z</sup>	19.44 $\pm$ 8.00 <sup>1,2,w,y,z</sup>
10.0	2.01 $\pm$ 0.40 <sup>v,w</sup>	0.29 $\pm$ 0.06 <sup>x,y</sup>	6.35 $\pm$ 0.80 <sup>v,x,z</sup>	24.69 $\pm$ 2.35 <sup>1,3,w,y,z</sup>
11.5	1.87 $\pm$ 0.46 <sup>v,w</sup>	0.56 $\pm$ 0.16 <sup>x,y</sup>	5.62 $\pm$ 2.79 <sup>v,x,z</sup>	34.36 $\pm$ 2.53 <sup>2,3,w,y,z</sup>

Listed is the extractable fat amount (EFA) of the emulsions in g/100 g fat in dependence of the protein type, and  $C_pC$  (8.0, 10.0, 11.5% (w/w)). Emulsions were prepared with oil mass fraction of 70%

Means  $\pm$  standard deviation were determined by nine single values. Different superscripts indicate significant differences ( $P < 0.05$ ): <sup>1,2,3</sup> within the same protein at different concentrations, and <sup>v–z</sup> between different proteins at the same concentration

interfacial films. Due to the small size and the random coil structure of potato protease inhibitors, as well as the shown aggregate formation by heating (Fig. 1), it can be assumed that the interface is incompletely occupied by the Potato protein, e.g. due to adsorption of protein aggregates. A similar assumption has been made before by van Koningsveld et al. [35] who could show that the interfacial adsorption of potato protease inhibitors increases upon heating to 80 °C while interfacial stability decreased. The result of aggregate adsorption in the formation of a patchy film with pores through which the oil can be extracted. By contrast, globular proteins are known to form more condensed and viscous films with good mechanical properties due to retention of a major portion of their tertiary structure [67, 71]. The  $C_pC$  had no significant influence on EFA of the leguminous proteins ( $P > 0.35$ ) while for Potato protein emulsions the EFA was significantly reduced ( $P$  value  $< 0.001$ ) with decreasing  $C_pC$ . It was described by Bos & van Vliet [72] that the equilibrium protein adsorption at the interface is only achieved if there is no exchange of protein between the interface and bulk solution and if surface tension adjusts instantaneously to the equilibrium for the absorption per  $m^2$ . This is only the case if relaxation processes in or near the interface do not affect surface excess or interfacial tension. It was also shown that at surface coverage between, 0.5 and 1.5 mg protein per  $m^2$  interactions increase while above 1.5 mg  $m^{-2}$  visco-elastic interfaces appear. At 70% (w/w) oil content, the total protein amount varies between 2.4 and 3.4 mg per g emulsion. Taking into account the specific surface area of the emulsions (Table 3) it becomes obvious that for Pea-1 and 2 the amount of protein which is available per surface area is 1.8 to 2.6 respective 1.6 to 2.3  $mg/m^2$ . In case of Soy and Potato the amount of available protein is much higher (4.8–6.8 respective 2.7–2.9  $mg/m^2$ ). Although the amount of available protein does not tell us how much protein is actually adsorbed at the interface, the probability that a high excess of unabsorbed protein will lead to aggregation is much higher in Soy and Potato emulsions. However, in case of Potato it is known that due to gel formation of the protein, aggregates are formed. This reduces the amount of available protein that can be adsorbed at the interface. In addition, the less protein available, the weaker the gel formation. Consequently, the interfaces are more stable at lower protein concentrations as aggregate formation is reduced.

To visualize structural differences between the four emulsion gels, CLSM pictures were taken. The results in Fig. 4 depict similar emulsification results for the leguminous proteins at the chosen conditions. Pea-1 showed larger and coalesced oil droplets at  $C_pC$  8.0% (panel a, white arrow) while the droplets became smaller with increasing  $C_pC$ , indicating

that surface activity of the proteins increased. Comparing the results with the droplet-size distribution (Fig. 5) no change in  $D$  [3, 4] values (Supplementary Table S4) was observed but with increasing  $C_pC$ , the volume of medium-sized droplets shifted towards smaller and larger droplets. This distributional change is typical if oil-droplets start to flocculate and form a network-like structure and has been previously described by Tang & Liu [18]. Similar observations were made for Pea-2 protein. In addition, it can be seen in the CLSM images of Pea-2, that with increasing oil content, the oil-droplet packing became denser and the amount in accumulated protein increased (comparison of Fig. 4, panel b, f, j). This results again confirms that flocculated networks are formed and that with increasing  $C_pC$  both, more interface-protein and more protein–protein interactions, occur which reinforce the structure and confirm the results of texture and oscillation analysis (Figs. 2 and 3). The findings also explain why no concentration dependency was found for EFA as pea proteins formed emulsion gels with stable interfaces independent of the tested protein quantity. In case of Soy protein more densely packed and smaller droplets were observed when increasing  $C_pC$  from 8.0 to 10.0% from CLSM while the particle size increase and coalescence became apparent at  $C_pC$  11.5% (Fig. 4, panel c, g, k). This structural weakening can also be seen in the compression firmness (Fig. 2). From the particle size distribution, at  $C_pC$  10.0% more huge and less smaller droplets were present (Fig. 5) and EFA (Table 4) was slightly but not significantly higher, too. As there were problems with creaming Soy emulsions, it is assumed that the particle-size distribution of Soy may not be representative. We assume that, due to the high excess in protein at  $C_pC$  11.5% protein-aggregation is enhanced resulting in a reduction of Soy protein at the interface which increases interfacial tension and the susceptibility to coalescence which in turn causes the structural weakening as can be seen in CLSM (Fig. 4, panel k).

By contrast to the leguminous protein emulsions, Potato protein emulsions showed many small oil droplets with less dense packing in the CLSM but also a high degree in coalescence (Fig. 4, panel d, h, l, red signals). In addition, the protein appeared as high-density green signals confirming protein aggregation and network formation. The findings confirm that protein-filled emulsion gels with instable interfaces are formed but at  $C_pC$  11.5% no clear structural change to a hybrid gel can be seen (comparison panel h and l). The CLSM results also support that more instable interfaces are formed by Potato in comparison to the leguminous proteins, as coalescence is present to a much higher degree. Coalescence, in addition, is enhanced by the low charge-distribution of the protein (Table 3) as the already irregularly occupied interfaces of the droplets cannot repel each other.

## Conclusion

This work demonstrates that it is possible to form pH neutral, stable and heat resistant (72 °C) emulsion gels without additives from commercial plant protein isolates if the protein is carefully selected. As hypothesized higher rigidity and viscoelastic behavior was achieved by higher internal phases amounts as well as higher protein concentrations but the optimum concentration was strongly protein dependent. Potato formed a class I emulsion gel or at higher oil content and C<sub>p</sub>C 11.5% a hybrid gel with much higher rigidity and G' values than the class II emulsion gels formed by Pea-1, Pea-2 and Soy. Based on the aim of this study, Pea-2 and Soy protein isolate were identified to suit best for preparation of additive-free, solid animal fat substitutes while the Potato protein formed the least stable emulsions although stable gels were formed. Leguminous proteins in general seem to be more promising to prepare shape and long-time-storage stable solid animal fat-substitutes with neutral pH and temperature stability up to 72 °C. However, additional analyses are necessary to provide more precise information on long-term stability. Also the effect of salt, that may diffuse into the emulsion if it is incorporated in food, needs to be carefully studies.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11694-020-00767-9>.

**Acknowledgements** We would like to thank the BMWi for funding and the DIL departments of food physics and analytical chemistry for technical and analytical support.

**Author contributions** All authors contributed to the study conception and design. SS performed the analyses. The first draft of the manuscript was written by M-CB and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Funding** Open Access funding enabled and organized by Projekt DEAL. This IGF Project of the FEI is/was supported via AiF within the programme for promoting the Industrial Collective Research (IGF) of the German Ministry of Economic Affairs and Energy (BMWi), based on a resolution of the German Parliament. Project AiF 18622N.

**Data availability** All raw data are available for reviewers on request.

**Code availability** Not applicable.

## Compliance with ethical standards

**Conflict of interest** Not applicable.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes

were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

## References

1. J.D. Wood, R.I. Richardson, G.R. Nute, A.V. Fisher, M.M. Campo, E. Kasapidou, P.R. Sheard, M. Enser, Effects of fatty acids on meat quality: a review. *Meat Sci.* **66**, 21 (2004)
2. H.-D. Belitz, W. Grosch, P. Schieberle, *Lehrbuch Der Lebensmittelchemie*, 6 Auflage. (Springer-Verlag, Berlin, 2008).
3. E.B. Fitzherbert, M.J. Struebig, A. Morel, F. Danielsen, C.A. Brühl, P.F. Donald, B. Phalan, How will oil palm expansion affect biodiversity? *Trends Ecol. Evol.* **23**, 10 (2008)
4. J. Dreher, C. Blach, N. Terjung, M. Gibis, J. Weiss, Formation and characterization of plant-based emulsified and crosslinked fat crystal networks to mimic animal fat tissue. *J. Food Sci.* **85**, 421 (2020)
5. J. Dreher, C. Blach, N. Terjung, M. Gibis, J. Weiss, Influence of protein content on plant-based emulsified and crosslinked fat crystal networks to mimic animal fat tissue. *Food Hydrocoll.* **106**, 105864 (2020)
6. A.R. Patel, Y. Rodriguez, A. Lesaffer, K. Dewettinck, High internal phase emulsion gels (HIPE-gels) prepared using food-grade components. *RSC Adv.* **4**, 18136 (2014)
7. W. Ternes, *Naturwissenschaftliche Grundlagen Der Lebensmittelzubereitung* (Behr's Verlag, Hamburg, 1990).
8. M.C. Sánchez, M. Berjano, A. Guerrero, E. Brito, C. Gallegos, Evolution of the microstructure and rheology of O/W emulsions during the emulsification process. *Can. J. Chem. Eng.* **76**, 3 (1998)
9. P. Walstra, *Physical Chemistry of Foods* (Marcel Dekker Inc., New York, 2003).
10. A.C.Y. Lam, A. Can Karaca, R.T. Tyler, M.T. Nickerson, Pea protein isolates: structure, extraction, and functionality. *Food Rev. Int.* **34**, 2 (2018)
11. K. Köhler, H.P. Schuchmann, *Emulgiertechnik: Grundlagen, Verfahren Und Anwendungen*, 3rd edn. (Behr's verlag, Hamburg, 2012).
12. R.S.H. Lam, M.T. Nickerson, Food proteins: a review on their emulsifying properties using a structure-function approach. *Food Chem.* **141**, 2 (2013)
13. S. Nakai, L. Ho, N. Helbig, A. Kato, M.A. Tung, Relationship between hydrophobicity and emulsifying properties of some plant proteins. *Can. Inst. Food Sci. Technol. J.* **13**, 1 (1980)
14. S. Tcholakova, N.D. Denkov, I.B. Ivanov, B. Campbell, Coalescence stability of emulsions containing globular milk proteins. *Adv. Colloid Interface Sci.* **123–126**, 259 (2006)
15. E. Dickinson, Emulsion gels: the structuring of soft solids with protein-stabilized oil droplets. *Food Hydrocoll.* **28**, 224 (2012)
16. T. van Vliet, Rheological properties of filled gels. Influence of filler matrix interaction. *Colloid Polym. Sci.* **266**, 6 (1988)
17. J. Chen, E. Dickinson, Viscoelastic properties of heat-set whey protein emulsion gels. *J. Texture Stud.* **29**, 3 (1998)
18. C.-H. Tang, F. Liu, Cold, gel-like soy protein emulsions by microfluidization: emulsion characteristics, rheological and microstructural properties, and gelling mechanism. *Food Hydrocoll.* **30**, 1 (2013)
19. C.K. Reiffers-Magnani, J.L. Cuq, H.J. Watzke, Composite structure formation in whey protein stabilized O/W emulsions. I.

- Influence of the dispersed phase on viscoelastic properties. *Food Hydrocoll.* **13**, 4 (1999)
20. H.E. Schroeder, Quantitative studies on the cotyledonary proteins in the genus *Pisum*. *J. Sci. Food Agric.* **33**, 7 (1982)
  21. K. Nishinari, Y. Fang, S. Guo, G.O. Phillips, Soy proteins: a review on composition, aggregation and emulsification. *Food Hydrocoll.* **39**, 301 (2014)
  22. P.R. Shewry, J.A. Napier, A.S. Tatham, Seed storage proteins: structures and biosynthesis. *Plant Cell* **7**, 7 (1995)
  23. K. Saio, M. Kamiya, T. Watanabe, Food processing characteristics of soybean 11S and 7S proteins. *Agric. Biol. Chem.* **33**, 9 (1969)
  24. A.M. Pots, H.H.J. de Jongh, H. Gruppen, M. Hessing, A.G.J. Voragen, The pH dependence of the structural stability of patatin. *J. Agric. Food Chem.* **46**, 7 (1998)
  25. L. Pouvreau, H. Gruppen, S.R. Piersma, L.A.M. van den Broek, G.A. Van Koningsveld, A.G.J. Voragen, Relative abundance and inhibitory distribution of protease inhibitors in potato juice from cv. Elkana. *J. Agric. Food Chem.* **49**, 2864 (2001)
  26. M.B. Barac, M.B. Pešić, S.P. Stanojević, A.Z. Kostić, S.B. Čabrilo, Techno-functional properties of pea (*Pisum sativum*) protein isolates: a review. *Acta Period. Technol.* **46**, 1–18 (2015)
  27. M.B. Barac, M.B. Pesic, S.P. Stanojevic, A.Z. Kostic, V. Bivolarevic, Comparative study of the functional properties of three legume seed isolates: adzuki, pea and soy bean. *J. Food Sci. Technol.* **52**, 5 (2015)
  28. M. Klost, S. Drusch, Structure formation and rheological properties of pea protein-based gels. *Food Hydrocoll.* **94**, 622 (2019)
  29. C.M.M. Lakemond, H.H.J. de Jongh, M. Paques, T. van Vliet, H. Gruppen, A.G.J. Voragen, Gelation of soy glycinin; influence of pH and ionic strength on network structure in relation to protein conformation. *Food Hydrocoll.* **17**, 3 (2003)
  30. A.H. Martin, M.A. Bos, T. van Vliet, Interfacial rheological properties and conformational aspects of soy glycinin at the air/water interface. *Food Hydrocoll.* **16**, 1 (2002)
  31. J.M.S. Renkema, J.H.M. Knabben, T. Van Vliet, Gel formation by  $\beta$ -conglycinin and glycinin and their mixtures. *Food Hydrocoll.* **15**, 4–6 (2001)
  32. J.M. Schmidt, H. Damgaard, M. Greve-Poulsen, L.B. Larsen, M. Hammershøj, Foam and emulsion properties of potato protein isolate and purified fractions. *Food Hydrocoll.* **74**, 367 (2018)
  33. J.M. Schmidt, H. Damgaard, M. Greve-Poulsen, A.V. Sunds, L.B. Larsen, M. Hammershøj, Gel properties of potato protein and the isolated fractions of patatins and protease inhibitors – Impact of drying method, protein concentration, pH and ionic strength. *Food Hydrocoll.* **96**, 246 (2019)
  34. G.A. van Koningsveld, P. Walstra, H. Gruppen, G. Wijngaards, M.A.J.S. van Boekel, A.G.J. Voragen, Formation and stability of foam made with various potato protein preparations. *J. Agric. Food Chem.* **50**, 26 (2002)
  35. G.A. van Koningsveld, P. Walstra, A.G.J. Voragen, I.J. Kuijpers, M.A.J.S. van Boekel, H. Gruppen, Effects of protein composition and enzymatic activity on formation and properties of potato protein stabilized emulsions. *J. Agric. Food Chem.* **54**, 17 (2006)
  36. H.-N. Liang, C.-H. Tang, pH-dependent emulsifying properties of pea [*Pisum sativum* (L.)] proteins. *Food Hydrocoll.* **33**, 2 (2013)
  37. E.B.A. Hinderink, W. Kaade, L. Sagis, K. Schroën, C.C. Berton-Carabin, Microfluidic investigation of the coalescence susceptibility of pea protein-stabilised emulsions: effect of protein oxidation level. *Food Hydrocoll.* **102**, 105610 (2020)
  38. A. Stiebing, J. Barciaga, U.M. Krell, *Handbuch Fleisch Und Fleischwaren—Technologie, Marketing Und Betriebswirtschaft, Recht*, 18. Aktualisierungslieferung. (B Behr's Verlag GmbH & Co KG, Hamburg, 2007).
  39. Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), *Amliche Sammlung von Untersuchungsverfahren Nach § 64 LFGB, Aktualisierung Februar 2014* (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, Braunschweig, 2014).
  40. Deutsche Gesellschaft für Fettwissenschaft e.V. (DGF), *Deutsche Einheitsmethoden Zur Untersuchung von Fetten, Fettprodukten, Tensiden Und Verwandten Stoffen* (Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 2019).
  41. F. Kielmeyer, G. Schuster, Der Einfluß von emulgatoren auf das Verhalten von Fett im Eismix während des Reifens. *Fette Seifen, Anstrichmittel* **88**, 10 (1986)
  42. S. Utsumi, J.E. Kinsella, Structure-function relationships in food proteins: subunit interactions in heat-induced gelation of 7S, 11S, and soy isolate proteins. *J. Agric. Food Chem.* **33**, 2 (1985)
  43. M. Keerati-u-rai, M. Corredig, Heat-induced changes in oil-in-water emulsions stabilized with soy protein isolate. *Food Hydrocoll.* **23**, 8 (2009)
  44. L.A. Rubio, A. Pérez, R. Ruiz, M.Á. Guzmán, I. Aranda-Olmedo, A. Clemente, Characterization of pea (*Pisum sativum*) seed protein fractions. *J. Sci. Food Agric.* **94**, 2 (2014)
  45. A.L. Riblett, T.J. Herald, K.A. Schmidt, K.A. Tilley, Characterization of  $\beta$ -conglycinin and glycinin soy protein fractions from four selected soybean genotypes. *J. Agric. Food Chem.* **49**, 10 (2001)
  46. J. Bergsma, *Vegan Cheese Analogue* (Coöperatie AVEBE U.A., Brunswick, 2019).
  47. A.M. Hermansson, Physico-chemical aspects of soy proteins structure formation. *J. Texture Stud.* **9**, 33 (1978)
  48. A.C. Karaca, N. Low, M. Nickerson, Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. *Food Res. Int.* **44**, 9 (2011)
  49. N.A. Mustapha, K. Ruttarattanamongkol, S.S.H. Rizvi, The effects of supercritical fluid extrusion process on surface hydrophobicity of whey protein concentrate and its relation to storage and heat stability of concentrated emulsions. *Food Res. Int.* **48**, 2 (2012)
  50. L. Pouvreau, H. Gruppen, G. van Koningsveld, L.A.M. van den Broek, A.G.J. Voragen, Conformational stability of the potato serine protease inhibitor group. *J. Agric. Food Chem.* **53**, 8 (2005)
  51. L. Pouvreau, T. Kroef, H. Gruppen, G. van Koningsveld, L.A.M. van den Broek, A.G.J. Voragen, Structure and stability of the potato cysteine protease inhibitor group (Cv. Elkana). *J. Agric. Food Chem.* **53**, 14 (2005)
  52. E. Ruckenstein, Thermodynamic insights on macroemulsion stability. *Adv. Colloid Interface Sci.* **79**, 59 (1999)
  53. A.C. Altting, L. Pouvreau, M.L.F. Giuseppin, N.H. van Nieuwenhuijzen, *Handbook of Food Proteins* (Woodhead Publishing Limited, Sawston, 2011), pp. 316–334
  54. H. Zhao, B. Yu, Y. Hemar, J. Chen, B. Cui, Improvement of calcium sulfate-induced gelation of soy protein via incorporation of soy oil before and after thermal denaturation. *LWT* **117**, 108690 (2020)
  55. M.A. Tung, Rheology of protein dispersions. *J. Texture Stud.* **9**, 1–2 (1978)
  56. S.A. Lone, K.K. Sadhu, Time-dependent growth of gold nanoparticles: experimental correlation of van der Waals contact between DNA and amino acids with polar uncharged side chains. *J. Phys. Chem. C* **123**, 33 (2019)
  57. S.P. Schwaminger, P.F. García, G.K. Merck, F.A. Bodensteiner, S. Heissler, S. Günther, S. Berensmeier, Nature of interactions of amino acids with bare magnetite nanoparticles. *J. Phys. Chem. C* **119**, 40 (2015)
  58. D.I. Horsup, *Microemulsions and Macroemulsions Behaviour of Systems Containing Oil, Water and Nonionic Surfactants* (University of Hull, Hull, 1991).
  59. J. Glusac, S. Isaschar-Ovdat, B. Kukavica, A. Fishman, Oil-in-water emulsions stabilized by tyrosinase-crosslinked potato protein. *Food Res Int* **100**, 407 (2017)
  60. T.G. Mezger, *Angewandte Rheologie—Mit Joe Flow Auf Der Rheologie-Straße* (Anton Paar GmbH, Graz, 2014).

61. T. Van Vliet, H.J.M. Van Dijk, P. Zoon, P. Walstra, Relation between syneresis and rheological properties of particle gels. *Colloid Polym. Sci.* **269**, 620 (1991)
62. E. Dickinson, Flocculation of protein-stabilized oil-in-water emulsions. *Colloids Surf. B Biointerfaces* **81**, 130 (2010)
63. T. Blijdenstein, A. Van Winden, T. Van Vliet, E. Van der Linden, G. Van Aken, Serum separation and structure of depletion-and bridging-flocculated emulsions: a comparison. *Colloids Surf. A* **245**, 1–3 (2004)
64. D.J. McClements, *Food Emulsions: Principles, Practices, and Techniques* (CRC Press, Boca Raton, 1999).
65. D.J. McClements, Protein-stabilized emulsions. *Curr. Opin. Colloid Interface Sci.* **9**, 5 (2004)
66. J.F. Zayas, *Functionality of Proteins in Food* (Springer, Berlin, 1997), pp. 134–227
67. J. Leman, J.E. Kinsella, A. Kilara, Surface activity, film formation, and emulsifying properties of milk proteins. *Crit. Rev. Food Sci. Nutr.* **28**, 2 (1989)
68. M.C. Puppo, C.E. Lupano, M.C. Añón, Gelation of soybean protein isolates in acidic conditions - effect of pH and protein-concentration. *J. Agric. Food Chem.* **43**, 9 (1995)
69. H. Koyoro, J.R. Powers, Functional properties of pea globulin fractions. *Cereal Chem.* **64**, 2 (1987)
70. A. Romero, V. Beaumal, E. David-Briand, F. Cordobés, A. Guerrero, M. Anton, Interfacial and oil/water emulsions characterization of potato protein isolates. *J. Agric. Food Chem.* **59**, 17 (2011)
71. E.M. Meulenbroek, E.A. Thomassen, L. Pouvreau, J.P. Abrahams, H. Gruppen, N.S. Pannu, Structure of a post-translationally processed heterodimeric double-headed Kunitz-type serine protease inhibitor from potato. *Acta Crystallogr. D Biol. Crystallogr.* **68**(Pt 7), 794 (2012)
72. M.A. Bos, T. van Vliet, Interfacial rheological properties of adsorbed protein layers and surfactants: a review. *Adv. Coll. Interface. Sci.* **91**, 3 (2001)

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.