Regular Article

Sequential adsorption and interfacial displacement in emulsions stabilized with plant-dairy protein blends

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GRAPhICAL ABSTRACT

Hypothesis: Many traditional or emergent emulsion products contain mixtures of proteins, resulting in complex, non-equilibrated interfacial structures. It is expected that protein displacement at oil-water interfaces depends on the sequence in which proteins are introduced during emulsion preparation, and on its initial interfacial composition.

Experiments: We produced emulsions with whey, pea or a whey-pea protein blend and added extra protein post-emulsification. The surface load was measured indirectly via the continuous phase, or directly via the creamed phase. The interfacial composition was monitored over a three-day period using SDS-PAGE densitometry. We compared these findings with results obtained using an automated drop tensiometer with bulk-phase exchange to highlight the effect of sequential protein adsorption on interfacial tension and dilatational rheology.

Findings: Addition of a second protein increased the surface load; especially pea proteins adsorbed to pre-adsorbed whey proteins, leading to thick interfacial layers. The addition of whey proteins to a pea
1. Introduction

Proteins are commonly used to stabilize food emulsions. After adsorption at the oil-water interface, proteins provide steric and electrostatic repulsion that prevents droplets from approaching each other too closely [1]. Protein ingredients used for food applications are never pure from a molecular composition perspective, e.g., whey protein isolate contains mostly bovine serum albumin (BSA), α-lactalbumin (α-lac) and β-lactoglobulin (β-lg); or caseinates contain mostly α-, β- and κ-caseins. As a consequence, when applied in emulsions, mixtures of proteins adsorb at the oil-water interface and contribute to emulsion stability [2]. Different proteins may compete for adsorption during emulsification [3]; [4,5,6], and possibly displace each other too closely [1]. Protein ingredients used for food applications are never pure from a molecular composition perspective, e.g., whey protein isolate contains mostly bovine serum albumin (BSA), α-lactalbumin (α-lac) and β-lactoglobulin (β-lg); or caseinates contain mostly α-, β- and κ-caseins. As a consequence, when applied in emulsions, mixtures of proteins adsorb at the oil-water interface and contribute to emulsion stability [2]. Different proteins may compete for adsorption during emulsification [3]; [4,5,6], and possibly displace each other over time. Displacement of proteins adsorbed at an oil-water interface by surfactants has been studied extensively, for which the orogenic displacement theory has been suggested. According to this theory, displacement of a pre-adsorbed protein film starts at a so-called nucleation site where surfactants can adsorb, and displacement then proceeds from this site [7]. Whether this mechanism also applies to protein displacement by another protein is not known, yet it is highly relevant to understand the mechanisms underlying protein interfacial rearrangements. Several studies were conducted using model interfaces and/or emulsions to characterize protein partitioning between the interface and the aqueous phase, and also to investigate protein-protein interactions. For example, at model interfaces, in a system consisting of a mixture of α-casein and β-casein, the latter predominated at the air-water interface after spontaneous adsorption, but over time the proteins were able to displace each other [8]. When using the egg white proteins ovalbumin and lysozyme, ovalbumin adsorbed at the air-water interface with lysozyme being present as a second layer, independently of the order of addition (e.g., sequentially or as mixture) [9]. The predominant adsorption of ovalbumin was attributed to its higher surface activity, and the interaction of ovalbumin and lysozyme in the interfacial region led to the formation of additional layers. This interaction is fascinating, since the proteins did not interact in the bulk: it therefore implies that the protein conformational changes induced by interfacial adsorption are a pre-requisite for the interaction.

Exchange experiments in emulsions where one protein was adsorbed first, and the second protein added post-emulsification, indicated that α-casein and β-casein were able to displace each other from the interface [10], which is in line with the findings at model interfaces [8]. When using a 1:1 mixture of α-lac and β-lg for emulsification, no preferential adsorption was reported [11,12,13], which would be expected for proteins with similar propensity to adsorb on oil droplets under convective flow. However, in emulsion exchange experiments, pre-adsorbed α-lac was readily displaced by added β-lg, but pre-adsorbed β-lg was displaced only when ten times as much α-lac was added to the continuous phase [14].

It was suggested that the resistance to displacement may be linked to the mechanical properties of the protein layer [4], which can be quantified through parameters such as the interfacial shear viscosity. For instance, interfacial films made of globular whey proteins have a surface shear viscosity that is about $10^3$–$10^4$ times higher than that of flexible casein films [15,16]. Moreover, the relative importance of the elastic and viscous contributions is probably also instrumental: intermolecular attraction between adsorbed proteins, leading to an interconnected solid-like elastic film at the interface [17,18], could be particularly effective at preventing protein displacement, whereas a predominantly viscous behaviour would not be able to do so. Therefore, it is important to characterize in depth the rheological properties of protein-based interfacial layers, which can be performed by oscillatory dilatational deformation experiments. Whey protein stabilized-interfaces have an elastic dilatational modulus (Ed’) that is around 10-fold higher than for caseins [19], which could explain the resistance of adsorbed whey proteins to displacement. Yet, displacement of such globular proteins can be facilitated by increasing the interfacial mobility, or the flexibility of the displacing protein [20].

To address this gap, the present study aims at understanding protein displacement at plant-dairy protein stabilized oil-water interfaces, by varying the order of addition of different proteins in emulsion systems. We investigated protein interfacial displacement in 10 wt% oil-in-water (O/W) emulsions with different interfacial composition and continuous phase protein concentrations. Displacement, as measured in the emulsions systems, is linked to the interface rheological properties before and after displacement which enables us to determine the driving forces for the displacement. As dairy protein source we used whey protein isolate (WPI), and as plant protein source, pea protein isolate (PPI),
2. Materials and methods

2.1. Materials

WPI, purity 97.0–98.4% (BiPro®, Davisco, Switzerland) and PPI, purity 80–90% (NUTRALYS s85F, Roquette, France) were used. Determination of amino acid composition and content, and of protein content are reported in the supplementary information (Table S1). The compositional analysis of the non-protein material present in the commercial PPI is reported in [26]. The soluble protein content was determined using a bicinchoninic acid (BCA) kit with a standard bovine serum albumin (BSA) solution. (Thermo Fisher Scientific, Massachusetts, US). Sunflower oil was purchased from a local supermarket and stripped with Florisil (Sigma-Aldrich, Saint Louis, MO, USA, 20281, Supelco, 100–200 mesh) to remove surface-active impurities, as described previously [27]. MiniPROTEAN gels (12% Mini-PROTEAN™ TGX™ Precast Protein Gels, 10-well comb, 30 µL/well), Bio-safe Coomassie G-250 stain, Laemmli sample buffer, Tris/Glycine/SDS-buffer (running buffer) and precision plus protein standard (Bio-Rad, Richmond, CA, US), Laemmli sample buffer, Tris/Glycine/SDS-buffer (running buffer) and precision plus protein standard (Bio-Rad, Richmond, CA, US), were used for SDS-PAGE analysis. Sodium phosphate dibasic, sodium phosphate monobasic, sodium dodecyl sulfate (SDS) and 2-mercaptoethanol were purchased from Sigma Aldrich and were used for SDS-PAGE analysis. Sodium phosphate dibasic, sodium dodecyl sulfate (SDS) and 2-mercaptoethanol were purchased from Sigma Aldrich and were used for SDS-PAGE analysis. Sodium phosphate dibasic, sodium dodecyl sulfate (SDS) and 2-mercaptoethanol were purchased from Sigma Aldrich and were used for SDS-PAGE analysis.

2.2. Preparation of aqueous phases

WPI (1 or 10 wt%) was dissolved in 10 mM phosphate buffer (pH 7.0) and stirred overnight at 4 °C. PPI was dispersed in the same buffer (6 wt%) and stirred for at least 48 h at 4 °C; the insoluble part was removed by centrifugation (16,000g, 30 min) and the supernatant was collected and centrifuged again under the same conditions to ensure complete removal of the insoluble fraction. The second supernatant was collected, and its protein content was determined with the BCA-assay [28] at 562 nm using a DU 720 UV–vis spectrophotometer (Beckman Coulter, Woerden, the Netherlands), which was about 25% of the total proteins present in the starting suspension. At 10 g/L soluble pea proteins, 0.06 wt % residual fat was present [22]. This supernatant was used for all pea protein-based experiments, and for simplicity is referred to as ‘pea protein solution’ from now on.

2.3. Preparation of emulsions

A coarse emulsion was prepared by mixing 10 wt% stripped sunflower oil with the protein solutions (1 wt%) using a high-speed blender (518N-19C, Ultraturrax R, IKA-Werke GmbH & Co., Staufen, Germany) at 11,000 rpm for 1 min. When protein blends were used, both solutions were stirred by hand before adding the oil. The coarse emulsion was then passed five times through a high-pressure M-110Y Microfluidizer (Microfluidics, Massachusetts, USA) at 400 bars to obtain the final emulsion. The emulsions were diluted with a second protein solution (1 or 10 wt% protein) to obtain a 5 wt% O/W emulsion (Table 1). The emulsions were stored in glass bottles at 4 °C.

2.4. Droplet size distribution

The droplet size distribution of the initial emulsions was measured by static light scattering using a Mastersizer 3000 (Malvern Instruments Ltd.; Worcestershire, UK). The refractive index was 1.465 for the dispersed phase (stripped sunflower oil) and 1.330 for the dispersant (water). An absorption index of 0.01 was applied.

All emulsions were diluted (1:1, v/v) in a 1 wt% SDS solution prior to the measurement, to de-flocculate any flocs present and thereby measure the individual droplet size.

2.5. Determination of surface load and interfacial composition

The surface load in emulsions, i.e., the mass of adsorbed proteins per unit of interfacial area, was determined via three different methods (Fig. 1): via determination of the non-adsorbed protein amount in the continuous phase (method 1), of or the adsorbed protein amount in the creamed phase (method 2) and in the washed creamed phase (method 3).

The continuous phase of the emulsions was separated from the creamed phase by centrifugation at 15,000g for 1 h. The serum phase was collected by making a hole at the bottom of the tube, and its soluble protein content was determined with the BCA-assay. The surface load \( \Gamma \) (ng/m²) was calculated with equation (1).

\[
\Gamma = \frac{C_i d_{3,2}}{6\varphi}
\]  

where \( C_i \) (mg/L) is the adsorbed protein concentration calculated by subtracting the protein concentration in the serum phase from the initial protein concentration of the solution used for emulsion preparation, \( d_{3,2} \) the surface-weighed mean droplet diameter after dilution of the emulsion in 1 wt% SDS, and \( \varphi \) the dispersed phase volume fraction. The calculated surface load corresponds to method 1.

For the surface load determination via the creamed phase (method 2), the amount of adsorbed proteins and the oil content in the cream were determined. The creamed phase obtained after centrifugation was re-dispersed in 1 wt% SDS solution (mass ratio 0.06:1). The resulting mixture was agitated under slow rotation for at least 1 h and then re-centrifuged at 15,000g for 1 h. The aqueous subnatant, containing the proteins that were initially adsorbed, was collected, and protein content determined by the BCA assay. The oil content in the creams was determined by mixing an aliquot of the cream with hexane:isopropanol (3/2 v/v) and water in a mass ratio of 0.02 (sample):1 (organic phase):0.2 (water). The obtained tubes were vortexed three times for 1 min, then agitated under slow rotation for at least one hour before centrifugation (3000g, 5 min). The upper phase was carefully taken out and collected in Eppendorf tubes. Tubes were placed in the fume hood overnight at 40 °C for the hexane to evaporate and weighed subsequently. Eppendorf tubes had preliminary been weighed to determine the amount of extracted oil. The total surface area of the emulsion was calculated based on the oil content and \( d_{3,2} \), and surface load (ng/m²) was calculated from the amount of adsorbed protein and total surface area. For the washed surface load (method 3) the cream was first dispersed in buffer (0.05:1 mass ratio) and agitated under slow rotation for 1 h before the surface load was determined as described before.

### Table 1
Overview of the protein solutions used for emulsification and for post-emulsification addition.

<table>
<thead>
<tr>
<th>Initial protein solution (emulsification)</th>
<th>Second protein solution (post-emulsification)</th>
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<tbody>
<tr>
<td>1 wt% PPI</td>
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Overview of the protein solutions used for emulsification and for post-emulsification addition.
The protein interfacial composition in the washed cream was determined by SDS-PAGE under reducing conditions. The final supernatant obtained via method 3 (Fig. 1) was mixed (0.75:1 v/v) with a pH 6.8 buffer containing Tris-HCl 0.5 M, glycerol 30% w/v, SDS 10% w/v, bromophenol blue 0.5% w/v and 2-mercaptoethanol, vortexed and heated at 95 °C for 5 min in a heating block. Ten microliters protein standard (Biorad, Precision Plus protein™ Standards, Mw 10–250) and 20 µL of the diluted samples in sample buffer were loaded on the gel as dependent duplicates. A running buffer of pH 8.3 consisting of Tris-HCl 25 mM, glycerol 192 mM and SDS 0.1 wt% was used. Electrophoresis was performed in the MiniPROTEAN Tetra Cell (Bio-rad laboratories, USA) at 200 V. After electrophoresis the gels were extensively washed with ultrapure water before staining with Coomassie G-250 for 1 h. Subsequently, the gels were washed with ultrapure water for 12 h before analysis. Gels were scanned and analyzed using a calibrated densitometer (GS-900™, Bio-rad laboratories, USA) and Image Lab software (Bio-Rad laboratories, USA). The molecular weights were determined by point to point regression. For WPI solutions, mainly bovine serum albumin, β-lactoglobulin, α-lactalbumin, and traces of immunoglobulins were found. PPI mainly consisted of convicilin (38–40 kDa), vicilin subunits (~30, ~34, ~47 and ~50 kDa), α-legumin (19–22 kDa) [29]. The pea proteins dissociated in the buffer containing SDS solution and 2-mercaptoethanol, leading to multiple bands in SDS-PAGE. The sum of the subunits is reported for the different pea proteins.

2.6. Automated drop tensiometer measurements

The interfacial tension between stripped sunflower oil and the protein solutions in 10 mM phosphate buffer (pH 7.0) was measured with an automated drop tensiometer (ADT, Tracker, Teclis, Longessaigne, France) at 20 °C. The ADT was equipped with a single-phase exchange device for the continuous phase. We used a rising drop with an area of 30 mm² (i.e., a drop of oil was immersed in a 25-mL glass cuvette filled with the protein solution) using 20-gauge needles. We started with a 0.01 wt% WPI or PPI solution as the continuous phase, and after a first adsorption phase, the second protein solution was rinsed through the cuvette (25 mL) with a flow rate of 10 mL/min, to ensure complete refreshment of the continuous phase. The second protein was allowed to adsorb for one hour before amplitude sweeps with ΔA/A₀ in the range of 0.05–0.3 and a frequency 0.1 Hz, started. Five deformation cycles were applied, after which five rest cycles were applied before the next deformation started. The oscillating surface tension signal was analyzed with a Fast Fourier transform, and the intensity and phase of the first harmonic was used to calculate the dilatational elastic modulus (E_d) and the dilatational viscous modulus (E'_d) according to equations (2) and (3):

\[
E_d = \Delta\gamma \left(\frac{A_0}{A_0}ight) \cos\delta
\]  
\[
E'_d = \Delta\gamma \left(\frac{A_0}{A_0}ight) \sin\delta
\]

Here \( \Delta\gamma \) is the amplitude of the change in interfacial tension, \( A_0 \) the initial droplet area, \( A_A \) the amplitude of change in droplet area, and \( \delta \) the phase shift of the oscillating interfacial tension signal, compared to the induced area change. This first harmonic-based analysis is accurate in the linear response regime. For the higher deformation amplitudes, the response enters the nonlinear viscoelastic regime, in which higher harmonics are present in the surface tension signal. As an alternative, measurements in the nonlinear regime were analyzed by Lissajous plots in which the change in surface pressure (\( \pi = \gamma - \gamma_0 \)) is plotted against the oscillating deformation signal [31] to compare the behavior of the various interfaces.

2.7. Experimental design and data treatment

Each emulsion was characterized for particle size distribution, surface load and interfacial composition at day 0, 1, and 3. This was done for at least two independently prepared emulsions, and means, and standard deviations were calculated from these replicates. Independent t-tests (SPSS Statistics 20, IBM) were performed, using all experimental values, to determine if differences in surface load and interfacial composition occurring in time were significant (\( p < 0.05 \)).
3. Results and discussion

3.1. Surface load

We determined the surface load of the initial emulsions via the continuous phase, the cream and the washed cream (Fig. 2). For all tested proteins, the three methods gave the same trends, with the continuous phase method leading to higher surface loads than those determined from the cream, and from the washed cream. The latter method gave the lowest surface loads: 2.5, 1.4 and 1.5 mg/m² for PPI-, WPI- and WPI-PPI-stabilized emulsions, respectively. These values are in good agreement with values reported for washed creams of WPI-stabilized emulsions, around 1.5–3.2 mg/m² [5]. Although widely used, the surface load determined via the continuous phase is an indirect method [32], and may be overestimated due to the presence of small oil droplets in the continuous phase or to protein precipitation upon centrifugation [5]. Regarding method 2 (analysis of the creamed phase as such), proteins that may be loosely attached to the interface or captured between the oil droplets in the creamed phase would erroneously be considered as adsorbed. Washing of the cream is expected to remove loosely bound proteins, and possibly even those that are present as a secondary layer at the interface. Also, proteins captured in the void fraction of the cream would be removed and thus the lowest values were expected for this technique.

When adding the second protein to the initial emulsions, an increase in surface load was observed for all systems tested (Fig. 3A) which suggests additional association of proteins to the pre-formed interfacial layer; more details on the composition of the interfaces will be given in the next section. As a control experiment, we also measured the surface load of WPI, PPI and WPI-PPI stabilized-emulsions after addition of more of the same protein post-emulsification (Supplementary information, Fig. S1) and also found an increase in the surface load compared to the initial emulsions, both via the cream and washed cream methods. An exception to this trend is the surface load in the WPI-stabilized emulsion with added WPI post-emulsification, determined via the washed cream method (method 3), which perfectly matched the surface load determined in the starting WPI-based emulsion (Supplementary information, Fig. S1). In exchange experiments carried out by others, the surface load of a β-casein-stabilized emulsion increased by about one third after addition of 1 wt% β-lg, as determined via the continuous phase analysis after centrifugation. This increase was explained by multilayer formation and/or by adsorption of the second protein in gaps present in the original interfacial layer [11]. The presence of non-adsorbed proteins in the void fraction of the cream could lead to an overestimation of the surface load. This effect probably explains the lower surface loads determined via the washed cream method, compared to the cream method.

Only a few methods are available to determine the surface load in protein-stabilized emulsions without physical separation of the serum and creamed phases. For example, Granger and co-workers used front-surface fluorescence to determine protein partitioning between the interface and the continuous phase [33]. The location of the proteins could be determined due to the sensitivity of the intrinsic fluorescence of the tryptophanyl residues to aqueous or hydrophobic environments. However, the change in fluorescence emission spectra between adsorbed and non-adsorbed proteins is small for proteins such as β-lg and casein, and this method is therefore not applicable for all proteins [34].

Based on these initial results, we concluded that the surface load determined via the washed cream is the most accurate to quantify the adsorbed protein amount at the oil-water interface (e.g., not present as loosely bound or interstitial proteins), and will therefore be used for the displacement study. It is good to note that over the three-day period, the surface load as determined from the washed cream did not significantly change (Fig. 3B, p < 0.05); more details on the implications in terms of interface composition follow in the next section.

3.2. Interfacial composition

For the PPI-stabilized emulsions, all protein species initially present in the solution were found at the interface (Fig. 4A). The addition of 1 wt% PPI-solution to the PPI-stabilized emulsion led to an increase of all proteins at the interface. Extra adsorption was also found for the other tested systems, e.g., WPI- or WPI-PPI-stabilized emulsion after addition of 1 wt% WPI or 1 wt% WPI-PPI, respectively (Fig. 4B and C). The pea protein-stabilized interface is dominated by the vicilin, as described in detail earlier [22]. Directly after the addition (day 0) of 1 or 10 wt% WPI solution, whey proteins adsorbed at the PPI-stabilized interface (Fig. 4A). Over the three-day period, the adsorbed amount of β-lg significantly increased at the expense of the pea proteins, and at the expense of α-lac that was yet added simultaneously.

In the WPI-stabilized emulsion (Fig. 4B), β-lg was the major protein dominating the interface with 0.99 mg/m². After addition of PPI, almost half of the surface load consisted out of pea proteins, which shows that pea proteins partly displaced the pre-adsorbed whey proteins and adhered to the interface. This is remarkable since proteins did not show specific interactions in bulk, as measured by asymmetric flow field flow fractionation (AF4, supplementary information, Fig. S2). In a previous study on ovalbumin and lysozyme, lysozyme was found to adsorb at an ovalbumin-based interfacial layer as a consequence of interfacial electrostatic complexation, yet without interfacial displacement [9]. However, in contrast to ovalbumin and lysozyme, whey and pea proteins have similar overall charges and electrostatic complexion is thus probably not the reason for the interfacial accumulation of pea proteins. Over time, the concentration of adsorbed β-lg significantly increased from 0.74 to 1.10 mg/m², as well as that of adsorbed legumin (0.24 to 0.31 mg/m²).

In the WPI-PPI blend-stabilized emulsions, proteins from both sources initially co-located at the interface (Fig. 4C). Upon addition of a 1 wt% WPI-PPI solution, the surface load increased mainly due to extra adsorption of pea proteins. This confirms their high affinity for the pre-adsorbed protein layer, as was also found for the addition of pea proteins to a WPI-stabilized emulsion (Fig. 4B) or to a PPI-stabilized emulsion (see also Supplementary information, Fig. S1). When adding pea proteins to the WPI-PPI blend-stabilized emulsion, the interface composition was clearly dominated by pea proteins and no significant changes in interface composition occurred over the three-day period (Fig. 4C). The addition of 1 or 10 wt% WPI solutions also increased the surface load of the WPI-PPI blend-stabilized emulsions however, to a lower extent.
compared to the system where PPI was added. Remarkably, this higher surface load was a result of extra adsorption of pea proteins. This shows the lower affinity of whey proteins for the pre-adsorbed WPI-PPI-layer, compared to pea proteins. After addition of whey proteins (1 or 10 wt%) to the WPI-PPI blend-stabilized emulsions, the β-lg concentration significantly increased at the expense of all other adsorbed proteins (pea proteins and α-lac).

To summarize, we determined that β-lg is able to induce interfacial displacement for the following systems: PPI-stabilized emulsions after addition of 1 and 10 wt% WPI; WPI-stabilized emulsion after addition of 1 wt% PPI; and WPI-PPI-stabilized emulsions after addition of 1 and 10 wt% WPI. We estimated the amount of whey proteins present in the continuous phase of our emulsions immediately after addition of the second protein using the protein concentration as measured in the continuous phase (method 1). A continuous phase whey protein concentration of ~1 g/L was determined for the WPI-PPI-stabilized emulsion after addition of 1 wt% PPI; whereas the WPI-stabilized emulsion after addition of 1 wt% PPI, had ~2.2 g/L whey proteins in the continuous phase. In the former case, β-lg did not displace pea proteins over the three-day period, whereas in the latter case, it did. When comparing with previous work (1:3 w/w WPI-PPI blend-stabilized emulsion) in which no displacement was measured in similar storage conditions, ~0.9 g/L whey proteins was present in the continuous phase [22].

When adsorbed at the oil-water interface, whey proteins are known to form a viscoelastic network linked by intermolecular disulfide bridges involving the free thiol groups of β-lg [35]. Because of this, displacement studies on model interfaces using surfactants showed that β-lg resisted interfacial displacement by Tween 20 better than β-casein, which does not establish such covalent protein-protein interactions [36]. In line with this, it was found that when β-casein was added to a freshly prepared β-lg-stabilized emulsion, it was able to displace 0.5 mg/m² β-lg within 1 h [11], whereas β-casein added to a β-lg-stabilized emulsion preliminarily aged for 3 days could not displace the adsorbed β-lg anymore [4]. When adding β-lg to a β-casein-stabilized emulsion, extra adsorption of the β-lg took place, but no casein displacement was measured [11]. For the two major whey proteins it was found that α-lac was only able to displace ~15% of pre-adsorbed β-lg, when added at a concentration of 10 wt% whereas 1 wt% β-lg could displace ~30% α-lac of a pre-adsorbed film [14]. Due to the limited solubility of the commercial pea proteins, the addition of 10 wt% PPI to the WPI-stabilized emulsion could not be tested and we can only speculate about the outcome. It is expected that at higher pea protein concentrations, higher amounts will adsorb at the WPI-stabilized interface. However, it is unlikely that pea proteins would be able to displace the whey proteins. In general, whey proteins have lower interfacial mobility and form layers with high viscoelastic moduli [4,14], making the protein layer more resistant to displacement compared to other proteins (e.g., pea proteins). This aspect was next further investigated by performing interfacial rheology measurements.

3.3. Interface rheological measurements

It is known that whey protein films have a higher resistance against dilatational deformation compared to pea protein films [19,23]. Since interfacial dilatational rheology (i) is a direct consequence of the interface composition and structure and (ii) is related to emulsion stability [17], it is interesting to probe possible changes in interface rheological properties after addition of the second protein. For this, we used an automated drop tensiometer with external phase exchange. The interfacial tension was recorded over the entire timescale of the experiment (Fig. 5).

For the interfacial layer initially formed with WPI the interfacial tension was 15.9 ± 0.1 mN/m and decreased to 14.1 ± 0.1 mN/m after the phase exchange with the PPI solution (Fig. 5A, the interfacial tension during exchange is reported in the supplementary information, Fig. S3). The opposite effect was found for the interface initially covered with PPI with an interfacial tension of 13.5 ± 0.1 mN/m, where the interfacial tension increased after the phase exchange with the WPI solution to 14.1 ± 0.1 mN/m. The fact that the same interfacial tension was obtained is certainly worth noticing, but does not necessarily mean that the same interfacial structures were formed. In order to investigate the structural
Fig. 4. Interfacial composition measured in the washed cream (method 3) of (A) PPI-stabilized emulsion with no addition, addition of PPI at day 0 and with addition of 1 wt% WPI or 10 wt% WPI at day 0, 1, and 3. (B) WPI-stabilized emulsion without addition, with addition of 1 wt% WPI at day 0 and 1 wt% PPI at day 0, 1 and 3. (C) WPI-PPI-stabilized emulsion with no addition, with addition of 1 wt% WPI-PPI at day 0 and with addition of 1 wt% PPI, 1 wt% WPI or 10 wt% WPI at day 0, 1 and 3. Significance in surface composition changes over time within the sample is indicated by different letters.

Fig. 5. (A) Interfacial tension (γ) during equilibration and amplitude sweeps (ΔA/A0 = 0.05–0.30) for a β-lactoglobulin and a WPI-based interface followed by exchange with PPI (black). B) the dilatational elastic (E′) closed symbols) and viscous (E″ open symbols) moduli as a function of the applied deformation before and after the phase exchange. Adsorbed PPI (0.01 wt%, green diamond) followed by exchange with WPI (0.01 wt%, grey circle); and adsorbed WPI (0.01 wt%, orange square) followed by exchange with PPI (0.01 wt%, black triangle). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
properties of the involved interfacial films further, oscillatory dilatational deformation was applied. The resistance to dilatational deformation is quantified through the elastic ($E_d$) and viscous moduli ($E'_d$), calculated from the dynamic interfacial tension response.

Before phase exchange, the elastic modulus of the WPI-stabilized interface was the highest (30 mN/m at $\Delta A/A_0 = 0.05$, Fig. 5B), in accordance with previous results [19]. Interfaces stabilized by WPI tend to have high moduli, an indicator of relatively strong in-plane interactions between the adsorbed protein molecules. After addition of PPI to the pre-adsorbed WPI, the elastic modulus decreased (16 mN/m at $\Delta A/A_0 = 0.05$), indicating that the film became less stiff. Upon increased deformation amplitude, the elastic moduli decreased to 13 mN/m ($\Delta A/A_0 = 0.3$), showing a low strain dependency. The PPI-stabilized interface had an elastic modulus of 20 mN/m at $\Delta A/A_0 = 0.05$ before phase exchange. So, the layer formed by adding PPI to a pre-adsorbed WPI-stabilized interface had a lower elastic modulus compared to both layers made of the individual proteins. A lower value for $E_d$ and a weak strain dependency of that modulus show that the connectivity typically found for whey protein-stabilized interfaces was decreased by the adsorption of the pea proteins. Addition of WPI to the pre-adsorbed PPI-stabilized interface increased the interfacial elastic modulus. Upon increasing the deformation amplitude, the elastic modulus decreased from 24 mN/m at $\Delta A/A_0 = 0.05$ to 18 mN/m at $\Delta A/A_0 = 0.3$, respectively. Such a strain dependency and increase in elastic modulus indicate that whey proteins contributed to the in-plane-protein interactions, leading to a stiffer interface. The same was reported for mixtures of $\beta$-lg and $\beta$-cas in the air-water interface, that are able to displace each other: when adding a low concentration of $\beta$-lg to a $\beta$-cas solution, the viscoelastic modulus was ten times higher compared to that measured for the $\beta$-cas-stabilized interface alone [37].

To understand the interfacial structure in more detail, the surface pressure as a function of the deformation ($\Delta A/A_0 = 0.3$) was plotted for both phase exchange systems and for the individual proteins (Fig. 6). The so-called Lissajous plots obtained provide information about the interfacial network behavior in dilatational expansion and compression. Furthermore, the plots include possible nonlinear effects, which are otherwise neglected when only calculating the dilatational moduli ($E_d'$ and $E'_d$). A linear shape of the plot indicates an elastic behavior of the interfacial layer, whereas a spherical shape indicates a viscous behavior. A linear viscoelastic response results in an ellipse-shaped plot, whereas non-linear responses result in asymmetric shapes that may give information regarding jamming, buckling, yielding, etc. of the interfacial film [31].

All systems tested gave a predominantly elastic and non-linear response upon 30% deformation (Fig. 6). At the start of expansion ($\Delta A/A_0 = −0.3$) the surface pressure first increased, after which it levelled off towards maximum expansion ($\Delta A/A_0 = 0.3$), upon compression the reverse phenomenon happened, i.e., the surface pressure decreased strongly. This is indicative of interface strain softening in expansion and strain hardening in compression, which is typical for globular protein-stabilized interfaces [38]. The Lissajous plots of individual whey and pea protein-stabilized interfaces have been extensively described elsewhere [19]. In brief, the pointy shape of the plot obtained for the pea protein-based interface when approaching maximum compression (lower left part of the plot) shows that the surface pressure change is the same in compression and subsequent expansion, meaning that only weak in-plane protein interactions are present. Upon expansion, strain softening occurs meaning that the interfacial structure is disrupted. In contrast, the plot obtained for the whey protein-stabilized interface showed a steeper slope upon expansion, which is a result of protein interactions leading to a stiff structure which is gradually, and to a lesser extend compared to the PPI-stabilized interface, disrupted upon expansion.

The interface formed with pre-adsorbed whey proteins, followed by introduction of pea proteins, showed the most viscous response compared to the other interfaces, as concluded from the open shape of the Lissajous plot (Fig. 6), and confirmed by the higher loss moduli $E'_d$ (Fig. 5B). Upon compression, the density of adsorbed proteins increased, which led to an increase in the interactions between the proteins. At the start of the subsequent expansion (lower left corner of the plot), we observe that the slope of the tangent to the plot is significantly lower compared to the interface stabilized by WPI. This indicates that pea proteins adsorbed to the pre-adsorbed whey protein interface, as was also found in the emulsion systems (Fig. 4) and disrupted the adsorbed whey protein network. Although having an increased surface load, the resulting interfacial film is less stiff, and has relatively more viscous response compared to the individual protein-stabilized interfaces. The strain softening in expansion can be explained by the disruption of the interfacial microstructure. Such a strain softening is also found for the control pea protein-stabilized interface (Fig. 6A), but to a much lesser extent than for the whey protein-stabilized interfaces (Fig. 6C). Taking into account (i) the adsorption of pea proteins to a pre-adsorbed whey protein-stabilized droplet (Fig. 4), (ii) the fact that the interfacial tension decreased upon introducing the pea protein solution in the cuvette and (iii) the changes in the surface rheological behavior, it is presumable that pea proteins adsorb in the interfacial layer, thereby decreasing the connectivity and hence the stiffness of the interfacial network.

The interface formed with pre-adsorbed pea proteins, followed by introduction of whey proteins, gave a predominantly elastic response, as seen by the narrow shape of the Lissajous plot.
(Fig. 6B). The slope of the plot is steeper compared to the control pea protein-stabilized interface (Fig. 6A) and it reaches higher surface pressures in expansion. This leads to a higher elastic modulus $E'$ in the first harmonic analysis (Fig. 5B) and points to a somewhat more cohesive structure which shows less softening in expansion. This shows that whey proteins were located at the interface and reinforced the interfacial elasticity. Because pea proteins alone did not form a strong interconnected network, it may be possible for whey proteins to squeeze into holes/defects in the interfacial layer. However, compared to the whey protein-stabilized interface, lower surface pressures were reached and relatively low interactions between the proteins occurred as seen by the pointy shape of the Lissajous-plot upon compression and subsequent expansion (lower left part of the plot). Furthermore, the ‘equilibrium’ interfacial tension increases, but did not reach the same interfacial tension as the whey protein-stabilized interface (Fig. 5A). This suggests that pea proteins still largely contributed to the interfacial structure and, when viewed from a different angle, did not allow whey proteins to form a cohesive film. Whey proteins adsorbed slightly after addition to the pea protein-stabilized emulsion droplets (Fig. 4, day 0) and displaced the adsorbed pea proteins over time. Whey proteins are probably not present at high enough concentrations to allow the formation of an interconnected stiff whey protein network (i.e., lower elastic moduli compared to whey protein alone). The rate of displacement will be enhanced at higher concentrations of added whey proteins (addition of 10 wt% WPI) as this will enhance protein adsorption [39].

Surfactants are known to displace adsorbed proteins since they are more effective in interfacial tension reduction [40]. However, the exact same mechanism cannot be considered to explain the current data, since added whey proteins displace pea proteins (Fig. 4) while leading to an increase in interfacial tension (Fig. 5A, supplementary information Fig. S3). Other things being equal, an increase in interfacial tension is not thermodynamically favorable, so this effect is counterintuitive at first sight. Yet, in view of all thermodynamic driving forces in the system, an increase in interfacial tension may be compensated by thermodynamically favorable conformational protein rearrangements including interactions with the interface and in-plane protein interactions. The energy barrier for adsorption is related to the interactions of the proteins with the interface molecules rather than surface pressure [41]. The interactions may be more favorable for the whey proteins compared to the pea proteins. Furthermore, after adsorbing at the interface, the surface pressure increases. From a critical adsorbed protein concentration on, the surface pressure only increases slightly although the adsorbed amount still increases [37]. At the interface, a concentration dependent two-dimensional aggregation of the protein starts. Interfacial protein aggregation has been described to reduce the interfacial tension due to displacement of water molecules at the interface and/or inclusion of the water molecules in the protein aggregates [42]. Due to protein aggregation, the interfacial region behaves as a disordered viscoelastic solid [43]. One could hypothesize that in case of $\beta$-lg and therefore also, whey proteins, the formation of the highly elastic interfacial layer may not only remove water from the interface but also from the hydrophobic parts of the adsorbed proteins, which would be thermodynamically favorable.

The present results show that oil-water interfaces stabilized by protein blends can be structured and tuned based on the order of addition of the proteins. Interfacial proteins can interact with bulk proteins, leading to thick, viscous interfaces. If the initial layer is stiff enough, no protein displacement is expected and only add-on layers with minimal in-depth insertion are formed. In the case of an initially weak interfacial film, protein displacement may occur, which eventually results in a mixed interface with non-additive rheological characteristics.

4. Conclusion

Plant-dairy protein blend-stabilized emulsions have been gaining interest as both sustainable and functional alternatives to emulsions stabilized by their individual counterparts [22,44,24,25]. However, in protein blend-stabilized emulsions, complex and non-equilibrated interfacial structures are formed and interfacial displacement occurs [22]. The current study focused on whey-pea protein blend-stabilized emulsions. Pea proteins were able to adsorb to the oil-water interface after introduction to a pre-adsorbed whey protein interface, but did not displace the pre-adsorbed proteins over time. We found that the whey proteins, and more specifically $\beta$-lactoglobulin, were able to displace pre-adsorbed pea proteins from the oil-water interface. Displacement seemed to be driven by an increased interfacial elasticity rather than a decrease in the interfacial tension. This may be explained by the removal of water from the adsorbed proteins and interfacial protein-protein interactions that would be thermodynamically more favorable compared to interfacial tension changes. Our results match previous results showing that $\beta$-lactoglobulin is able to displace pre-adsorbed proteins but is not itself displaced easily after adsorption at the oil-water interface [11,14]. A highlight of the current work is that we were able to understand displacement in mixed protein-based systems by combining displacement studies in emulsions and interfacial rheology. Interfacial displacement was measured at relatively low continuous phase whey protein concentrations (>1 g/L), which is much lower than the protein concentrations typically used to stabilize emulsions. As such rearrangements are even more likely to occur with higher protein concentrations in the continuous phase, this implies that the present outcomes are highly relevant when formulating protein blend-stabilized emulsions. Our results therefore highlight the importance of protein dynamics in complex emulsion systems, and thereby open perspectives for the rational structuring of plant-dairy protein blend-stabilized emulsions.

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Emma B.A. Hinderink: Conceptualization, Investigation, Formal analysis, Methodology, Visualization, Writing - original draft. Leonard Sagis: Formal analysis, Supervision, Writing - review & editing. Karin Schroën: Formal analysis, Supervision, Writing - review & editing. Claire C. Berton-Carabin: Methodology, Formal analysis, Supervision, Writing - review & editing.

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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Appendix A. Supplementary data

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