Regular Article

Adsorption of rapeseed proteins at oil/water interfaces. Janus-like napins dominate the interface

Eleni Ntone a,b, Tessa van Wesel a, Leonard M.C. Sagis c, Marcel Meinders b,d, Johannes H. Bitter a, Constantinos V. Nikiforidis ⋆ a

a Biobased Chemistry and Technology, Wageningen University and Research, Bornse Weilanden 9, P.O. Box 17, 6708 WG Wageningen, The Netherlands
b TiFN, P.O. Box 557, 6700 AN Wageningen, The Netherlands
c Laboratory of Physics and Physical Chemistry of Foods, Wageningen University, Bornse Weilanden 5, 6708 WG Wageningen, The Netherlands
d Food and Biobased Research, Wageningen University and Research Centre, P.O. Box 17, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands

Graphical abstract

Abstract

Plants offer a vast variety of protein extracts, typically containing multiple species of proteins that can serve as building blocks of soft materials, like emulsions. However, the role of each protein species concerning the formation of emulsions and interfaces with diverse rheological properties is still unknown. Therefore, deciphering the role of the individual proteins in an extract is highly relevant, since it determines the optimal level of purification, and hence the sustainability aspects of the extract. Here, we will show that when oil/water emulsions were prepared with a rapeseed protein extract containing napins and cruciferins (in a mass ratio of 1:1), only napins adsorbed at the interface exhibiting a soft solid-like rheological behavior. The dominance of napins at the interface was ascribed to their small size (radius \( r = 1.7 \text{ nm} \)) and its unique Janus-like structure, as 45% of the amino acids are hydrophobic and primarily located at one side of the protein. Cruciferins with a bigger size (\( r = 4.4 \text{ nm} \)) and a more homogeneous distribution of the hydrophobic domains couldn't reach the interface, but they appear to just weakly interact with the adsorbed layer of napins.

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1. Introduction

Oil-in-water emulsions are soft materials broadly used in a variety of areas, like food, pharmaceutical, cosmetic and paint industry...
To stabilize an interface between the two immiscible liquids, the adsorption of surface-active molecules is needed. These molecules decrease the surface tension and can impart various types of surface rheological behavior (viscous, viscoelastic fluid-like or viscoelastic solid-like behavior) to a surface [2,3]. Some of the most commonly used classes of surface-active species are low molecular weight surfactants, amphiphilic polymers, colloidal particles.

To meet the sustainability demands for a circular economy, the use of biobased surface-active molecules and preferably of plant origin is essential [4,5]. Several plant-based surfactants are currently used as emulsifiers in commercial applications, especially in food, such as polar lipids [4] and proteins, but these are mostly limited to a small number of specific sources, like soybeans and peas [6]. There are many other protein sources available in plants, that are currently being investigated for their potential to be used to design functional interfaces.

One of the reasons that plant proteins are not yet more widely exploited as emulsifiers, emerges from the high molecular complexity of plant matrices [7]. In seed cells, for example, proteins are organized in structures called protein bodies, that coexist with other structures such as oleosomes/lipid droplets, polysaccharides and phenols. The intricacy of plant cells requires intensive treatment to extract the proteins, such as pressing, use of organic solvents, and high-alkaline conditions [7,8,9]. These extraction steps can induce extensive changes in the protein structure, leading to a decrease in the capability of proteins to adsorb at the surface and decrease the surface tension [10]. Another reason that plant proteins are not yet broadly used, is the fact that they are always present in complex mixtures of proteins with different functionalities, which are difficult to purify [6]. These bottlenecks can cast doubt on the environmental benefit of substituting synthetic and animal-based emulsifiers with purified plant proteins [11].

However, very recent studies have shown that extensive purification of plant proteins can be avoided since mildly purified plant protein mixtures are also effective in decreasing the surface tension and behave similarly to purified proteins [8,12,13]. For instance, mildly extracted sunflower protein mixtures with 50% protein content have shown similar interfacial and emulsifying properties to purified sunflower proteins [8]. In a different study, native pea flour containing only 20% protein also showed similar interfacial properties compared to concentrated pea protein systems (55 wt% in protein) [13]. Additionally, even low protein content (6.3 wt%) in a cellulosic material derived in simple steps from leaves, could reduce the surface tension and efficiently stabilize oil-in-water interfaces [14].

Even so, the exact mechanism of interface stabilization is still unknown as the protein mixtures contain different species of proteins. The adsorption at the interface of different types of plant proteins results in the formation of interfaces with diverse rheological properties [2,4] which is very relevant for the targeted applications. Thus, it is essential to decode the role of each protein species present in these extracts on the interface stabilization mechanism. This knowledge would allow us to determine the optimal level of purification based on the desired functionality, and hence the sustainability aspects of the plant extracts.

The diversity in the emulsifying and interfacial properties that plant proteins offer, in combination with the abundance of plant feedstock, makes plant protein mixtures promising emulsifiers with advanced functionalities. Furthermore, the fact that plant proteins are already interfacially active when they are present in a mildly purified mixture, allows us to investigate and exploit the properties of unique protein structures that in other cases would be difficult to purify.

Therefore, in this study we investigated the interfacial and emulsifying properties of a rapeseed protein mixture (40 wt% proteins and 10 wt% oleosomes) which is mainly composed of two different types of proteins; cruciferins and napins in a mass ratio of 1:1. Napins are low molecular weight albumins of around 17 kDa with a rather unique structure, with 45% of its amino acids being hydrophobic and mainly located in one distinct domain [15]. On the other hand, cruciferins are hexamers with a molecular weight of around 300 kDa with the hydrophobic domains widely distributed amongst the protein’s surface [16]. Due to their differences in physicochemical properties, these proteins can differ in their interfacial properties, while when present in mixtures, competitive adsorption can occur. So far, there are only a few studies on the differences on the interfacial properties of pure napins and cruciferins but not when present in native mixtures. Hence, we aimed to understand the mechanism of the oil/water interface stabilization when using the protein mixture, by deciphering the role of each protein on this stabilization mechanism.

2. Materials and methods

2.1. Materials

The rapeseed protein mixture was extracted from untreated Alize rapeseeds stored at −18 °C. All chemicals used were of analytical grade and were purchased from Sigma Aldrich (St Louis, MO, USA).

All experiments and the subsequent analysis were performed at least in duplicates.

2.2. Extraction process

The protein mixture was extracted as described in our previous work [17]. Briefly, dehulled rapeseed particles were dispersed in deionized water at a ratio of 1:8 (w/w) and kept at room temperature (around 20 °C) for 4 h under continuous stirring using a head stirrer (EUROSTAR 60 digital, IKA, Staufen, Germany). The pH of the dispersion was maintained at 9.0 during the soaking time using NaOH (0.5 M). Afterwards, the dispersion was blended for 2 min at maximum speed with a kitchen blender (HR2093, Philips, Netherlands). The slurry was then filtered using a twin-screw press (Angelia 7500, Angel Juicer, Naarden, The Netherlands). The filtrate was collected, the pH was adjusted to pH 9.0, and centrifuged (10,000 g, 30 min, 4 °C) (Sorvall Legend XFR, ThermoFisher Scientific, Waltham, MA, USA). We obtained a cream layer (oleosomes-rich), a serum (protein mixture (RPM)) and a pellet (fiber-rich) which were collected separately. The protein mixture was freeze-dried (Alpha 2–4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and stored at −18 °C until further use.

2.3. Isolation of napins

For the isolation of napins, the extracted protein mixture was first dialyzed with two coupled dialfiltration cassettes of 100 kDa cut-off (Hydrosart, Sartorius, Göttingen, Germany). This step was necessary to remove the high molecular weight non-protein compounds as well as cruciferins which have a molecular weight of 300 kDa. Then, to remove the low molecular weight compounds, the filtrate containing the napins (<100 kDa) was collected and further concentrated by ultrafiltration and then diluted 1:1 with NaCl (0.08 M) to avoid protein precipitation. The mixture was pumped through two coupled dialfiltration cassettes (cut-off 5 kDa; membrane area 0.2 m²) (Hydrosart, Sartorius, Göttingen, Germany) for 6 cycles until a transparent filtrate was obtained. In the last cycle only, deionized water was used to remove any remaining salt. The retentate which consisted of napins was freeze-dried and stored at −18 °C until further use.
2.4. Physicochemical characterization of the protein fractions

2.4.1. Composition analysis

We determined the protein content of the protein mixture and the napin isolate on dry-matter weight basis using the dumas method (FlashEA 1112 Series, Thermo Scientific, Waltham, Massachusetts, US); d-methionine (≥98%, Sigma Aldrich, Darmstadt, Germany) was used as a standard and as a control. Cellulose (Sigma Aldrich, Darmstadt, Germany) served as blank. A nitrogen–protein conversion factor of 5.7 (calculated based on amino acid sequence) was used and the protein content was calculated using:

\[
PC(\text{wt}%) = 100 \times \left( \frac{NC \times 5.7}{M} \right)
\]

(1)

here PC is the protein content, NC is the nitrogen content, and M is the mass of the dry sample.

The oil content (OC) of the mixtures was calculated on a dry-matter weight basis using Soxhlet extraction. The oil was extracted for 7 h with petroleum ether (40–60 °C) as a solvent. The oil content after extraction was calculated using:

\[
OC(\text{wt}%) = 100 \times \left( \frac{M_o}{M} \right)
\]

(2)

here M_o [g] is the mass of the extracted oil.

The concentration of phenolic compounds was determined using the Folin-Ciocalteu reagent. The reduction (at alkaline pH) by the phenolic compounds of phosphomolybdate and phosphotungstate present in the reagent resulted in a color change. A dispersion of the protein extract was prepared at a concentration of 0.5 mg/mL. The concentration of the reaction product was determined based on the absorbance of the solution at 725 nm. We used tannic acid solution (0.0–0.1 mg/mL in water) to obtain a calibration curve.

To determine the ash content 1 g of the protein extract was placed in an air oven at 550 °C for 24 hrs and the weight loss during calcination was measured.

The carbohydrate content was determined by subtraction of the protein, oil, phenolic and ash content from the total dry mass of the extract.

2.4.2. Electrophoresis (SDS-PAGE)

The qualitative and quantitative analysis (protein profile) of the protein fractions and of the emulsion interface and continuous phase was carried out using SDS-PAGE. Each sample was mixed with a sample buffer (NuPAGE™ LDS, Thermo- Fisher, Landsmeer, the Netherlands) to achieve a final protein concentration of 1.0 mg/mL. Then, 10.0 µL of protein marker (PageRuler™ Prestained Protein Ladder, 10–180 kDa, ThermoFisher, Landsmeer, the Netherlands) and 15 µL of the sample were loaded onto the gel (NuPAGE™ Novex® 4–12% Bis-Tris Gel, ThermoFisher, Landsmeer, the Netherlands). MES running buffer (NuPAGE™ MES SDS Running Buffer, ThermoFisher, Landsmeer, the Netherlands) was added to the buffer chamber. The gel was washed with water and stained (Coomassie Brilliant Blue R-250 Staining Solution, Bio-Rad Laboratories B.V., Lunteren, the Netherlands) for 5 h under gentle shaking. Thereafter, the gel was washed with water and destained (destaining solution of 10% ethanol and 7.5% acetic acid in deionized water) overnight under gentle shaking.

The gels were scanned with a GS-900 Calibrated Densitometer (Bio-Rad Laboratories, California, US) and the intensity of the bands was analyzed with Image Lab software.

2.4.3. Particle size and ζ-potential of napin isolate

The number-based mean diameter and the ζ-potential of the napin isolate at pH 7 were determined using a ZS Nanosizer (Malvern Instruments, Ltd, Worcestershire, U.K.). The intensity-based diameter was also determined and turned out to be similar to the number-based. The napin isolate was dispersed in deionized water in a ratio of 1:100 and the pH was adjusted to 7.0 prior to the measurements. The equilibration time was set to 120 s and the temperature to 25 °C.

2.4.4. Emulsion preparation

The protein mixture was used to stabilize 10.0 wt% oil-in-water emulsions. The mixture was dispersed in deionized water, standardized at different protein concentrations (0.2–1.5 wt%). The pH was adjusted to 7.0 and the dispersions were stirred for 3 h at 20°C with a magnetic stirrer at 300 rpm (2mag magnetic e motion, 2mag AG, Munich, Germany) to allow hydration and solubilization of the proteins. Subsequently, the dispersions were sheared using a disperser (Ultra-Turrax, IKA®, Staufen, Germany) at 8000 rpm for 30 s. Next, 10.0 wt% rapeseed oil was slowly added to the dispersion and sheared for 1 min at 10,000 rpm. The formed coarse emulsion was further processed with high-pressure homogenizer (GEA®, Niro Soavi NS 1001 L, Parma, Italy) 5 times at 250 bars.

The emulsions with napin isolate were prepared as described above using 0.35 wt% napin isolate and 10.0 wt% oil.

2.5. Emulsion characterization

2.5.1. Particle size distribution

The particle size distribution of the emulsions was determined by laser diffraction (Malvern Instruments, Ltd, Worcestershire, UK) using a refractive index of 1.47 for rapeseed oil. The emulsions were diluted with deionized water at a ratio of 1:100. To determine the individual droplet size of the emulsions, we added sodium dodecyl sulfate (SDS) (1.0 wt%) to the sample in a ratio of 1:1. The measurements were reported as the surface $d_{3,2} = \Sigma n_i d_i^2/\Sigma n_i$ mean diameter where $n_i$ is the number of droplets with a diameter of $d_i$.

2.5.2. ζ-potential measurements

To measure the charge of the emulsions stabilized by the protein mixture under different pH conditions, we performed titration using a ZS Nanosizer (Malvern Instruments, Ltd, Worcestershire, U.K.). The 10 wt% oil emulsion (at 0.7 wt% protein) were diluted with deionized water in a ratio of 1:100. The equilibration time was set to 120 s and the temperature to 25°C. The ζ-potential of the samples was measured at a pH ranging from 2 to 12.

2.5.3. Confocal laser scanning microscopy

The structure of the protein mixture and of the emulsions stabilized with the protein mixture or with the napin isolate was observed using a Confocal Laser Scanning Microscope with a water immersion objective at 63x magnification (Leica SP8-SMD microscope, Leica Microsystems, Wetzlar, Germany). A 0.1 wt% protein mixture dispersion and 10.0 wt% oil emulsions with 0.7 wt% protein concentration or 0.35 wt% napin isolate were stained with fluorescent dyes in a ratio of 1:200 to visualize the structural components; Nile red was used for staining the oil phase (excitation wavelength $\lambda = 488$ nm) and fast green for the proteins ($\lambda = 633$ nm).

2.5.4. Transmission electron microscopy

The emulsions stabilized with 0.7 wt% protein mixture and 0.35 wt% napin were investigated with transmission electron microscopy. The emulsions stabilized with the protein mixture
were washed three times to remove the unadsorbed and non-interacting proteins by centrifugation at 10,000 g (30 min, 4°C) in 15 mL tubes at pH 9.0. A cream layer (interface) and a serum phase (continuous phase) were obtained. The serum was drained by making holes in the tube. The cream was collected and resuspended (1:10 w/w) in deionized water at pH 9.0. The centrifugation step was repeated for two more times under the same conditions. After the third centrifugation the cream was collected again and analyzed.

For the sample fixation, dehybridization and polymerization: The samples were mixed 1:1 in 3% LMP agarose at 40 °C. Once this hardened in the fridge the sample/LMP gel was cut into approximately 1 mm by 1 mm cubes. These cubes were fixed using 2.5% glutaraldehyde (EMS) for 1 h after which they were washed for 3 times with 0.1 M phosphate/citrate buffer. The cubes were then fixed again, this time with 1% osmium tetroxide (EMS). After this step the samples were washed 3 times with deionized water. Thereafter dehydration with ethanol was applied, substituting the deionized water for 30%, 50%, 70%, 80%, 90% and 100% ethanol (10 min. for each step). Once the samples were in 100% ethanol, this was substituted by Spurr embedding liquid (EMS) in 3 steps: 2:1, 1:1, 1:2 (ethanol:Spurr, 30 min. per step). Then the sample was left in 100% Spurr for 1 h. The Spurr was refreshed once more and the sample was left overnight in Spurr. The next day one more incubation of 1 h in fresh Spurr was done after which the samples were polymerized for 8 h at 70°C to harden the Spurr and the samples in it.

For the sample sectioning and imaging: Once the samples were hardened in the Spurr, they were sectioned by first trimming the samples using a Leica EM Rapid (Leica Microsystems, Vienna, Austria), after which the sample was sectioned using the Leica Ultramicromote UC7 Rapid (Leica Microsystems, Vienna, Austria), sectioning the sample into 70 nm thin coupes. These coupes were collected with formvar film 150 mesh copper TEM grids. The grids containing sample were loaded into the JOEL-JEM 1400 Plus-120 kV TEM (JEOL, Massachusetts, USA) with an EM-11210SQCCH specimen quick change holder. The samples were analyzed at spot size 1, at 120 kV.

2.5.5. Interfacial protein composition

To qualitatively determine the amount of proteins adsorbed at the interface, the prepared emulsions were centrifuged at 10,000 g (30 min, 4°C) in 15 mL tubes at pH 9.0 to remove any unadsorbed proteins. A cream layer (interface) and a serum phase (continuous phase) were obtained. The serum was drained by making holes in the tube. The cream was collected and resuspended (1:10 w/w) in deionized water. The centrifugation step was repeated for two more times under the same conditions. After the third centrifugation the cream was collected again. The protein profile and the intensity of the protein bands were determined with electrophoresis as described in section 2.4.2.

2.5.6. Estimated radius of napins and cruciferins

We also estimated the spherical radius of napins and cruciferins $r_{estim}$ using [18]:

$$r_{estim} = \sqrt{\frac{3V_p}{4\pi}} \frac{m}{M_w} \text{[nm]}$$

(3)

where $V_p$ is the volume of the protein, equal to $V_p = \frac{M_p}{M_w}$ [nm$^3$], and $M_w$ [Da] equals the molecular weight of proteins (17 and 300 kDa for napins and cruciferins, respectively).

2.6. Dynamic interfacial properties

2.6.1. Interfacial tension

The ability of the protein fractions to reduce the interfacial tension of an oil/water interface was measured using an automated drop tensiometer (ADT, Tracker, Teclis-instruments, Tassin, France). An oil droplet with a surface area of 10.0 mm$^2$ was created at the tip of a rising-drop capillary. Stripped rapeseed oil was used. The droplet was immersed in the protein mixture dispersion standardized at 0.01 wt% protein content at pH 7.0. Since napins comprise 50% of the total protein content of the mixture, the dispersion of the napin isolate was standardized at 0.005 wt% proteins. The interfacial tension $\gamma$ was continuously monitored for 3.3 h (12,000 s) at 20°C until it reached equilibrium.

2.6.2. Dilatational interfacial rheology

After the equilibrium state was reached (end of 3.3 h), oscillatory dilatational interfacial rheology was used to characterize the interfacial elastic ($E''_d$) and viscous ($E'_d$) moduli as a function of frequency and deformation amplitude. For the frequency sweeps, the droplet was subjected to a range of oscillation frequencies $\omega$ (0.005–0.1 Hz) at constant deformation (5% amplitude). Regarding the amplitude sweeps, the droplet was subjected to sinusoidal deformations with an amplitude of 5–20% of its original surface area at a constant frequency (0.01 Hz). Each amplitude consisted of a series of 5 cycles followed by a rest period of 5 cycles. The interfacial tension and area changes were recorded during the oscillations, and the dilatational elastic ($E''_d$) and viscous ($E'_d$) moduli were obtained according to:

$$E'_d = \Delta \gamma \frac{g_0}{\Delta A} \cos \delta$$

(4)

$$E''_d = \Delta \gamma \frac{g_0}{\Delta A} \sin \delta$$

(5)

3. Results and discussion

3.1. Composition and physicochemical properties of the coarse protein mixture

Dehulled and not-defatted rapeseeds (18.7 ± 0.6 wt% protein and 41.9 ± 0.9 wt% oil) [17] were used to extract the protein mixture. We extracted the proteins using the method that we have previously developed [17] to avoid the variability of the rapeseed cake’s processing and the possible effect of the non-protein components present in the cake on the functionality of the proteins. The extracted protein mixture was composed of 39.7 ± 3.1 wt% proteins and 11.5 ± 1.3 wt% oil, 6.2 ± 0.1 wt% phenolic compounds and 7.8 ± 0.3 wt% ash content. The carbohydrate content was determined by subtraction of the above components from the total mass (34.8 wt%).

To establish the composition of the protein mixture, electrophoresis (SDS-PAGE) and confocal microscopy analysis (CLSM) were used (Fig. 1A and 1B). The protein electropherogram (Fig. 1A) shows the protein species present in the mixture as separated based on their molecular weight, under reducing and non-reducing conditions. Both rapeseed storage proteins, napins (~17 kDa) and cruciferins (~20–70 kDa) were present in the protein mixture [17]. Based on the intensity of the protein bands the mass ratio of napins to cruciferins was 1:1.

Apart from a high amount of proteins, the protein mixture also contained oil, since no defatting was applied before the extraction. It has been previously reported that in mildly extracted oilseed protein extracts, the oil is present in the form of oleosomes/lipid
droplets [8,17]. To confirm this, confocal microscopy was carried out. The confocal image (Fig. 1B) shows the dispersion of the protein mixture at pH 7.0 stained for the oil (red color) and the proteins (green color). Spherical oil droplets of 0.2–3.0 μm and protein clusters were present. The presence of oil droplets and absence of free oil confirmed our previously reported observation that oleosomes were present in the protein mixture [17]. The size of the oleosomes corresponded to the native individual size of the oleosomes in the rapeseed cell embryos [19], proving that they were not damaged during the extraction. It has been previously reported that when protein mixtures with oleosomes are extracted, storage proteins are forming a film around the oleosomes by interacting with the oleosome membrane proteins through hydrophobic and van der Waals attractive forces [20]. This film provides additional protection to the oleosomes against coalescence [21] which even withstand the drying processing step [8].

3.2. Emulsions with the protein mixture

To evaluate the emulsifying properties of the protein mixture, different protein concentrations were used (standardized on protein content) to stabilize a 10.0 wt% oil-in-water emulsion at pH 7.0. Fig. 2A presents the individual droplet size ($d_{43}$) of the emulsions after the addition of SDS, as a function of protein concentration at time zero and after storage for 7 days. The droplet size decreased with increasing protein concentration and reached a plateau (1.0–1.5 μm) at protein concentrations above 0.7 wt% (protein-rich regime). When the protein concentration was below 0.7 wt%, bigger droplets were formed (2.0–5.0 μm) with higher size variations between samples. In the protein-poor regime (<0.5 wt%) the lower amount of proteins, in combination with the weak electrostatic repulsion at pH 7 (the zeta-potential of the droplets equals ~5 mV) (Fig. 2C), led to more re-coalescence during homogenization, and hence larger droplet sizes [22]. The values for the size of the individual oil droplets reported here at 0.7 wt% proteins, were similar to the ones reported when dairy proteins (i.e. sodium caseinate) were used at similar protein to oil ratio [23].

To further investigate the microscopic picture of the emulsions we used confocal microscopy. Fig. 2B displays the confocal image of the 10 wt% oil emulsion at the protein-rich regime (0.7 wt%) stained for the red oil and the proteins (green color). The image shows clusters of aggregated oil droplets (shown as red) of 20–30 μm size with small protein clusters (shown as green) between the oil droplets. The presence of aggregated oil droplets is due to weak repulsive electrostatic forces present at pH 7 (zeta potential of emulsion equals to ~5 mV) (Fig. 2C) that cannot overcome the attractive Van der Waals interactions. Despite the emulsion droplet aggregation, which induces more droplet–droplet
interactions, no coalescence of the individual oil droplets was observed over time (Fig. 2A).

Insipite of the complexity in composition the “impurities” present in the protein extract, like phenols and oleosomes, did not affect the emulsifying ability of the proteins. In our previous study we showed that the phenolic compounds present do not interact with the proteins and therefore they do not influence their functional properties [17]. Additionally, the oleosomes and their associated membrane components (i.e. proteins and phospholipids) are not expected to have a notable impact on the interfacial [24] and emulsifying properties of proteins [8,25] especially since they are present in very low concentration in the amount of extract used for the emulsions.

As the protein mixture contained initially two types of proteins, cruciferins and napins in a 1:1 mass ratio, we investigated whether both types of proteins were absorbed at the oil/water interface of the emulsions using electrophoresis. For this analysis we chose the emulsion in the protein-rich regime (at 0.7 wt% proteins) where the droplet size is mainly determined by the settings of the homogenizer and not by the amount of proteins present. The electrophoreogram (Fig. 3) shows the protein profile of the interface and the continuous phase of the 10.0 wt% oil emulsion stabilized with 0.7 wt% proteins. The results showed that at the oil/water interface almost exclusively napins were present, while excess of napins and cruciferins were present at the continuous phase. In a different study when separated napins and cruciferins were used to stabilise oil/water emulsions, it was reported that napins were more efficient emulsifiers than cruciferins [26]. However, it is the first time that a mixture of napins and cruciferins is used and solely napins were identified at the interface.

The fact that only one type of protein is adsorbed at the interface when a protein mixture is used is not common. So far studies on mixtures of different types of proteins (i.e. dairy proteins or egg yolk proteins) have mainly reported co-adsorption of the proteins at the interface, although the ratios between proteins at the interface typically differ from their ratio in the bulk [27–29].

To confirm whether napins were efficient emulsifiers in the absence of cruciferins, we isolated the napins (protein content of 84.0 ± 1.0) (protein profile provided in supplementary material) and used them to stabilize a 10.0 wt% oil/water emulsion. For comparison with the protein mixture, the same concentration of napins as present in the protein mixture was used.

Fig. 4A displays the volume-based droplet size distribution of the emulsion (after the addition of sds) stabilized by napins at 0.35 wt% protein concentration. For comparison we also included the individual droplet size distribution of the emulsion stabilized by the protein mixture at 0.7 wt% proteins (of which 0.35 wt% corresponds to napins). The emulsion stabilized with the protein mixture showed a monomodal droplet size distribution with an average droplet size ($d_{43}$) of 1.4 ± 0.1 µm. The droplet size distribution of the emulsions stabilized by napins was also monomodal with an average droplet size ($d_{43}$) of 3.4 ± 0.14 µm. The individual droplet size of the emulsion stabilized by napins did not change upon storage over 7 days (data not shown), showing that the concentration of napins at the interface was sufficient to form a dense layer that ensured stability against coalescence.

Fig. 4B shows the confocal image of the emulsions stabilized by napins stained for the oil (red color) and the proteins (green color). Droplets aggregates were present (aggregate size of around 30–
in the emulsion. The droplet aggregation was assigned to the low charge of the napin molecules adsorbed at the interface (-10.7 ± 1.3 mV), which led to weak electrostatic repulsion between the oil droplets. No protein clusters were present between the oil droplets, in contrast to the emulsions stabilized with the protein mixture (Fig. 2B). Napins were present as single molecules at pH 7.0 (hydrodynamic diameter of 4.7 ± 0.6 nm), thus no napin aggregates were formed.

The individual droplet size as well as the aggregate size of the emulsions stabilized by napins was bigger than in the emulsions stabilized with the protein mixture where cruciferins were also present. This led to the hypothesis that cruciferins present in the protein mixture had an indirect role in the formation of the droplets. Since we observed protein clusters in between the oil droplets in the emulsion stabilized with the protein mixture but not in the emulsion stabilized with napins, we hypothesized that these protein clusters were cruciferins, that form aggregates due to their hydrophobic character. When these cruciferin aggregates are present in the intervening continuous phase between the oil droplets, they might increase the local viscosity. This higher local viscosity could cause higher shear during homogenization or could impede thin film drainage between the colliding droplets [30,31] which both eventually could lead to smaller droplet sizes.

To understand the arrangement of the proteins at the oil/water interface and continuous phase in the emulsions stabilized by the protein mixture or the napin isolate, we used transmission electron microscopy. The micrograph (Fig. 5) shows the emulsion droplets stabilized by the protein mixture (Fig. 5A-C) or by the napin isolate (Figure D-E) at different magnifications. The images showed oil droplets with a homogeneous similar interface for both emulsions (Fig. 5C, F). However, in the emulsions stabilized with the protein mixture, high-density dark areas in between the oil droplets (Fig. 5-A-C) were also present, which probably corresponded to cruciferin aggregates.

3.3. The interfacial behavior of single napin molecules and the rapeseed protein mixture (RPM)

3.3.1. Interfacial tension

The interfacial behavior of the isolated napin molecules and the protein mixture was further investigated using oscillating drop tensiometry (Fig. 6).

Fig. 6 shows the dynamic interfacial tension reduction of the oil/water interface as a function of time for the protein extract (0.01 wt%) or napin isolate dispersions (0.005 wt%). The interfacial tension was reduced from ~25 mN/m to a value of ~9 mN/m in 1.2 10^4 s, for both cases. Both protein systems showed a short lag time of around 1 sec (Fig. 6, inset) before a further reduction of the ten-

![Fig. 5. TEM images of 10.0 wt% O/W emulsions stabilized with the protein mixture (0.7 wt% proteins) after removal of the unadsorbed proteins (A-C) and of emulsions stabilized with napins (0.35 wt%) (D-F). Magnifications: A) x12000, B) x3000, C) x10000, D) x500, E) x2000, F) x12000.](image-url)
sion occurred. The similar behavior of the two protein systems confirmed that cruciferins present in the protein mixture did not co-adsorb at the interface and also, did not displace napins over time. In a previous report, cruciferins were found to have lower surface activity in comparison to napins, leading to a reduction of the interfacial tension only to 15 mN/m [32].

The surface activity of a protein depends on different parameters such as the charge, molecular size and amphiphilicity of a protein, which influence the rate of diffusion towards the interface, and adsorption at the interface [33]. The protein mixture containing both napins and cruciferins, had a low zeta-potential (-15 mV) at the pH value of the emulsions (pH 7.0) [17]. Despite weak electrostatic repulsive forces large insoluble clusters were not formed and both proteins were soluble at this pH (supplementary material), indicating that the attractive forces between the proteins (hydrophobic, dipole) were also weak.

The diffusion of the protein molecules from the bulk to the interface is the first prerequisite for the adsorption of proteins [34]. According to Einstein’s equation (Equation (6)), the diffusion coefficient of the proteins (D) is proportional to Boltzman’s constant (k_B) and temperature (T), while it is inversely proportional to the viscosity (η) and the particle radius. Since T and η are constant, the difference in diffusion rate of napins and cruciferins was determined only by the difference in radii of the molecules [35].

\[
D = \frac{k_B T}{6\pi\eta r}
\]  

(6)

According to Equation (3), napins have an estimated radius \( r_{\text{est}} \) of 1.7 nm while cruciferins in their hexameric form have a radius of \( r_{\text{est}} = 4.4 \) nm. Therefore, the diffusion coefficient of napins is three times larger than that of cruciferins, which allowed them to diffuse faster towards the interface.

After diffusion, the proteins may have to overcome an energy barrier to adsorb [36,37]. The height of the energy barrier and thus the probability to adsorb is related to the structure of the proteins and the arrangement of the apolar domains at the oil/water interface [34,37,38]. By looking at the molecular structure of napins and cruciferins one can observe significant differences in structure and distribution of the hydrophobic domains. Napins are albumsins with a molecular weight of around 15–17 kDa, having a spherical particle shape while cruciferins are hexameric globulins of around 300 kDa [39]. Cruciferins, in their hexameric form, have the hydrophobic amino acids broadly distributed amongst their peptide chains and as is the case with most proteins, are buried inside the core [16]. The intramolecular hydrophobic attractive interactions contribute to a compact protein structure by holding two trimers together to form the hexamer structure [34]. Cruciferins are considered to have a three-fold higher surface-exposed hydrophobicity than napins (347 vs 104 (S_0^2) [39]. However, 45% of the amino acids of the peptide chain of napins are hydrophobic, and more importantly, they are concentrated mostly on one exposed side of the protein [15]. The structures of napins and cruciferins are sketched in Fig. 7.

The fact that there are such distinct domains on the protein, one hydrophobic and one hydrophilic, make napins resemble amphiphilic Janus particles [40]. Janus particles possess two distinct surface regions with each region having a different affinity for the solvents (i.e. water and oil) [41]. These two distinct surface regions have different wettability [40], and as it has been shown previously, lead to a higher surface activity than a homogeneous particle [41,42].

Thus, the higher interfacial activity of napins compared to cruciferins could be attributed to two main reasons; i) their smaller size which allowed faster diffusion towards the interface and ii) their large exposed hydrophobic domain which permitted them to overcome the energy barrier and adsorb at the interface. For the above reasons, cruciferins were prevented from co-adsorbing at the interface and also, could not displace napins over time.

3.3.2. Dilatational interfacial rheology

To gain a further insight into the rheological properties of the oil/water interface after adsorption of napins, and the possible effect of cruciferins on these properties, we employed interfacial dilatational rheology. In Fig. 8A the dilatational moduli (\( E'_d, E''_d \)) of the interface stabilized by either the protein mixture or the napin isolate are plotted as a function of increasing oscillation frequencies (\( \omega \)) on a double logarithmic scale. The results showed a higher elastic (\( E'_d \)) than viscous modulus (\( E''_d \)) in both samples.

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**Fig. 7.** Sketch of napins and cruciferins (trimer and hexamer) structure represented as molecular surface, showing the distribution of hydrophobic (red) and hydrophilic (green) domains. (Images retrieved from the RCSB PDB[15,16]). [For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.]
In both interfaces, the elastic modulus ($E_d'$) showed a minor increase upon increasing frequency while the viscous modulus displayed the contrary behavior (minor decrease). This behavior suggests a very minor dependency of the interparticle interactions at the interface upon oscillatory frequencies. By fitting a power law model through the data of $E_d'$, an exponent of around 0.1 was found. This, together with the low value for the loss tangent ($E_d''/E_d'$) for both interfaces, indicate a soft solid-like behavior.[43]

The moduli were slightly higher for the interface stabilized by the protein mixture compared to the one stabilized by napins. This result points out that the main response at the interface stabilized by the protein extract comes from napins, while there is a minor effect of the cruciferins present in the bulk which appear to interact with the adsorbed layer of napins.

To further investigate if any structure that is present at the interface will be affected when the amplitude of deformation is increased, we studied the dependency of the moduli on deformation amplitude in amplitude sweeps. Fig. 8B shows the dilatational moduli ($E_d'$, $E_d''$) of the interface stabilized by the protein mixture or the napin isolate as a function of amplitude. The elastic modulus ($E_d'$) was higher compared to the viscous ($E_d''$) modulus in both samples. Overall, the elastic moduli were relatively low compared to interfaces stabilized by other commonly used proteins, like whey protein isolate (WPI) (~30 mN/m vs > 40 mN/m) [44,45], which implied that the in-plane interactions between the adsorbed napin molecules were relatively weak. This response indicates that mainly attractive interactions, like van der Waals and hydrogen bonds took place after adsorption. Although sulfur-containing amino acids are involved in the structure of napins, the weak in-plane interactions at the interface show that it is unlikely that a significant amount of covalent sulfur bonds is formed after adsorption. If these bonds were important, a much stiffer response and a stronger dependence on strain amplitude would be observed (i.e. a more profound decrease in $E_d'$ as amplitude increases), a common phenomenon for interfaces stabilized by WPI.

It is important to emphasize that at the interface stabilized by the napin isolate the moduli were independent of the amplitude of deformation. On the other hand, when the interface was stabilized by the protein mixture, the elastic modulus was somewhat higher at low amplitude and showed a minor decrease upon increasing the amplitude of deformation. When the amplitude reached 20%, both systems had the same elastic and viscous moduli. The higher modulus at low amplitude implies that cruciferins are most likely interacting with the primary layer of napins. The decrease of the modulus upon increasing the amplitude shows that this interaction must be weak and is completely overcome at larger deformations (20%). Thus, the interactions at the interface were dominated by napins. This observation, in combination with the fact that we did not see adsorption of cruciferins at the interface, allows us to conclude that cruciferins remain in the bulk and most likely, they just weakly bind to the napins primary layer.

To understand these interactions in more detail, Lissajous plots were used which provide information about the behavior of the interfacial network upon extension and compression. Fig. 8C shows...
the Lissajous plots of the interface stabilized by the protein mixture or napin isolate where the surface pressure is plotted against deformation (Fig. 8C). At low amplitude (10%) the plots for both interfaces had a narrow ellipse shape. This shape indicates an almost linear, predominantly elastic response of the interface, without noticeable asymmetries, pointing out a weak solid structure [46]. The interface stabilized by the protein mixture showed slightly higher surface pressure upon compression (bottom left quadrant of the plot, at ΔA/Δθ = -0.1) compared to the interface stabilized only by napins (3.1 vs 2.4 mN/m). We assign this difference to the additional weak interactions of cruciferins with the adsorbed napins at the interface. At 20% amplitude of deformation the response of both the interfaces was similar and showed that the surface pressure increased from around 3 mN/m at 10% amplitude to 6 mN/m at 20% amplitude upon compression (bottom left quadrant of the plot, at ΔA/Δθ = -0.2), showing a minor strain hardening behavior. The bottom left part of the ellipse had a pointy tip, indicating that upon compression of the proteins the system was jammed. This behavior suggests weak in-plane attractive interactions at the interface [44]. As napins had low charge at pH 7.0 (-10.7 ± 1.3 mV), strong electrostatic repulsion was prevented and thus, weak in-plane attractive forces occurred. Therefore, the main rheological response came from the interactions of napins with the oil phase through its distinct hydrophobic domain [43]. As a result of all the above interactions, a soft-solid like structure of the interface was formed. By comparing our Lissajous plots with the ones recently reported for WPI at similar amplitude (20%) one can clearly observe that WPI-stabilized interfaces give a plot of wider shape at the lower left part [44,45]. This is a result of yielding of the surface microstructure upon compression and the start of the extension, which shows a much stronger structure [44,45] than the one we report here for the napin-stabilized interface.

4. Conclusions

In this research we investigated the stabilization mechanism of oil/water interfaces when using a rapeseed protein mixture, in which napins and cruciferins co-exist in a 1:1 mass ratio. Our findings suggest that when making oil/water emulsions with the protein mixture, competitive adsorption of napins occurred at the interface. The higher interfacial activity of napins was attributed to their small size (radius 1.7 nm) which allowed them to diffuse to influence the work reported in this paper.

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.

Acknowledgments

The project is organized by and executed under the auspices of TiFN, a public-private partnership on pre-competitive research in food and nutrition. The authors have declared that no competing interests exist in the writing of this publication. Funding for this research was obtained from Fromageries Bel S.A., Nutricia Research B.V., PepsiCo Inc., Unilever R&D Vlaardingen B.V., the Netherlands Organisation for Scientific Research (NWO) (ALWTF.2016.024) and the Top-sector Agri&Food. We gratefully acknowledge Helene Mocking-Bode and Irene van den Hoek for the technical assistance.

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