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AUTHOR CONTRIBUTIONS

Pedro J. García-Moreno: Conceptualization, Methodology, Formal analysis, Writing - original draft. Jack Yang: Methodology, Formal analysis, Writing - review and editing. Simon Gregersen: Methodology, Writing - review and editing. Nykola C. Jones: Methodology, Formal analysis, Writing - review and editing. Claire C. Berton-Carabin: Methodology, Writing - review and editing. Leonard M.C. Sagis: Methodology, Writing - review and editing. Søren V. Hoffmann: Methodology, Writing - review and editing. Paolo Marcatili: Writing - review and editing. Egon B. Hansen: Writing - review and editing, Funding acquisition, Project administration. Charlotte Jacobsen: Methodology, Writing - review and editing, Funding acquisition, Project administration.

Fish oil-in-water emulsions stabilized with α -helix peptides (weakly interconnected) or β -strand peptides (strongly interconnected) with (top) negative surface charge that attract ferrous ions to the interface and promote lipid oxidation, or (bottom) positive surface charge that repel ferrous ions from the interface and enhance oxidative stability of the emulsions.

The structure, viscoelasticity and charge of potato peptides adsorbed at the oil-water interface determine the physicochemical stability of fish oil-in-water emulsions

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ABSTRACT

We investigated the role of the interfacial properties of ten synthetic potato peptides (previously identified by bioinformatics) to physically and oxidatively stabilize 5 wt.% fish oil-in-water emulsions (pH 7). Peptides $\alpha 10$, $\alpha 12$, $\gamma 1$, $\gamma 75$ and $\gamma 76$ adopted a predominantly α -helical conformation (48-57%) at the interface leading to poor inter-peptides interactions as well as weak and stretchable interfaces ($E_d^* < 12$ mN/m). Peptides $\beta 22$, $\beta 27$, $\gamma 36$ and $\gamma 38$ displayed a significantly higher degree of interfacial inter-peptide interaction, resulting in stiff and solid-like interfaces ($E_d^* = 35-45$ mN/m). $\beta 22$ and $\gamma 36$ peptides re-arranged at the interface adopting a highly β -strand structure (63-65%). Emulsions stabilized with all peptides showed high physical stability during one week ($D_{3,2}$ at day 1 = 0.134-0.175 μ m), except from the ones

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stabilized with β 27 that had creaming after day 1, or β 22 that destabilized during storage. Emulsions stabilized with peptides exhibiting negative surface charge at pH 7 (α 12, β 22, γ 1, γ 75, γ 76, γ 36 and γ 40) (zeta potential: -50.9 to -69.5 mV) showed the lowest oxidative stability due the attraction of cationic metal ions that catalyzed lipid oxidation. In contrast, emulsions stabilized with peptides having positive surface charge at pH 7 (α 10, β 27 and γ 38) (zeta potential: 11.6 to 42.8 mV) showed high oxidative stability (i.e., by repulsion of cationic metal ions), independently of the peptide length, secondary structure at the interface, or viscoelasticity of the interfacial layer. Hence, this work advances our understanding of the relation between interfacial properties of peptide layers and the physicochemical stability of emulsions.

Keywords: peptide emulsifiers, synchrotron radiation circular dichroism, dilatational surface rheology, physical stability, lipid oxidation, omega-3

1. INTRODUCTION

The use of fish oil-in-water emulsions is a common approach to fortify food with omega-3 polyunsaturated fatty acids, yet it requires controlling lipid oxidation during processing and storage (Jacobsen, 2015). The physical stabilization of oil-in-water (O/W) emulsions requires the use of emulsifiers that favor oil droplet disruption and avoid coalescence of the newly formed droplets (McClements & Jafari, 2018). The recent clean label trend in the food industry aims to replace synthetic emulsifiers/surfactants by natural ones. Proteins are natural compounds exhibiting emulsifying activity, which stabilize O/W emulsions by providing steric and/or electrostatic repulsions between oil droplets (McClements & Gumus, 2016).

The use of sustainable plant-based emulsifiers is gaining particular attention (Sharif et al., 2018; Tang, 2017). Therefore, current research efforts focus on enhancing the emulsifying properties of plant-based proteins, with the purpose of progressively employing them to

replace synthetic surfactants and animal-based proteins (Burger & Zhang, 2019; Dai, Bergfreund, Reichert, Fischer, & Weiss, 2019; Felix, Cermeño, & FitzGerald, 2019). Enzymatic hydrolysis is one of the most efficient alternatives to enhance the emulsifying activity of proteins, when: i) the enzyme with the adequate specificity is used, and ii) the required degree of hydrolysis (percentage of peptide bonds cleaved) is achieved (García-Moreno, Guadix, Guadix, & Jacobsen, 2016; Tamm, Herbst, Brodkorb, & Drusch, 2016). Thus, the traditional approach to obtain emulsifier hydrolysates/peptides by enzymatic hydrolysis consists in optimizing these processing variables together with other processing conditions in the production of the emulsions (e.g. pH) (Padial-Domínguez, Espejo-Carpio, Pérez-Gálvez, Guadix, & Guadix, E. M. 2020). On the other hand, the advances in bioinformatics, comprising prediction of functionality which could be combined with *in silico* digestion, allow the development of a highly targeted approach to obtain emulsifying peptides by enzymatic hydrolysis (García-Moreno, Gregersen, et al., 2020; García-Moreno, Jacobsen, Marcatili, Gregersen, Overgaard, Andersen, Sørensen, Hansen, 2020; Hajfathalian, Ghelichi, García-Moreno, Moltke Sørensen, & Jacobsen, 2018).

The peptides released in the enzymatic hydrolysis have smaller size as well as more exposed hydrophobic and ionic groups than the original proteins, and this change in surface hydrophobicity and charge could significantly influence the adsorption and interactions at the interface (Hajfathalian, et al., 2018; Schröder, Berton-Carabin, Venema, & Cornacchia, 2017). Thus, peptides have higher solubility in the aqueous phase, diffuse faster and if they undergo the proper folding can stabilize a larger interfacial area when compared to the parent protein (O'Regan & Mulvihill, 2010; Schröder et al., 2017). Indeed, it is generally reported that the structural organization and intermolecular interaction of peptides at the oil-water interface affect the mechanical properties of the interfacial peptide film (e.g. viscoelasticity), playing a critical role on the physicochemical stability of O/W emulsions (Schröder et al.,

2017; Tamm & Drusch, 2017). It should be also noted that the use of emulsifier peptides exhibiting antioxidant activity is highly desired since they efficiently scavenge free radicals at the O/W interface (i.e., where lipid oxidation is initiated in emulsions), and thus prevent rancidity (García-Moreno et al., 2016; Tamm et al., 2016). Moreover, charge of the interfacial layer is also considered as a main parameter influencing lipid oxidation in emulsions (Berton-Carabin, Ropers, & Genot, 2014). Nevertheless, contradictory results are found in the literature, where some studies suggest a decrease in lipid oxidation at $\text{pH} < \text{pI}$ (i.e., compared to $\text{pH} > \text{pI}$) where the emulsifying protein is positively charged and thus repels metal ions from the interface (Kellerby, McClements, & Decker, 2006), whereas others studies observed an increased in lipid oxidation at $\text{pH} < \text{pI}$ (i.e., compared to $\text{pH} > \text{pI}$) as a consequence of an increase of iron solubility (i.e., despite of the positive charge of the protein interfacial layer) (Villiere, Viau, Bronnec, I., Moreau, & Genot, 2005).

Despite its low protein content (~1-2 wt.%), potato, the world's fourth most important food crop, is an important source for obtaining plant-based protein/peptides with the desired functionality (Schmidt, Damgaard, Greve-Poulsen, Larsen, & Hammershøj, 2018; Waglay, Achouri, Karboune, Zareifard, & L'Hocine, 2019). Indeed, potato starch industry produces upwards of ~240,000 tons/year of potato protein (Alting, Pouvreau, Giuseppin, & van Nieuwenhuijzen, 2011). In our previous studies (García-Moreno, Gregersen, et al., 2020; García-Moreno, Jacobsen, et al., 2020), with the aid of bioinformatics (EmulsiPred: <https://github.com/MarcatiliLab/EmulsiPred>), we identified and then validated, using small-scale assays, the emulsifying and antioxidant activity of peptides embedded in potato proteins present in different side-streams from the potato starch industry. Interestingly, out of the 38 peptides that we tested, we found that eight peptides ($\alpha 10$, $\alpha 12$, $\beta 22$, $\beta 27$, $\gamma 1$, $\gamma 36$, $\gamma 38$, and $\gamma 40$, see Table 1), with different predicted conformation at the oil-water interface (e.g., α -helix, β -strand), provided a decent or better physicochemical stability to fish O/W emulsions

when compared to a control emulsion stabilized with sodium caseinate. However, the *in situ* secondary structure that these peptides adopt when adsorbed at the oil-water interface is still unknown. Moreover, the potential intermolecular peptide interactions at the interface and thus the viscoelasticity of the interfacial peptide layer remain to be characterized.

Hence, this work aimed to investigate the effect of: i) the structure of the aforementioned potato peptides (α 10, α 12, β 22, β 27, γ 1, γ 36, γ 38, and γ 40) at the oil-water interface, and ii) the properties of the peptide interfacial layer, e.g., viscoelasticity and charge, on the physical and oxidative stability of fish oil-in-water emulsions. In addition, two variants of peptide γ 1, namely peptides γ 75 and γ 76 (Table 1), which are favorably released and highly abundant in tryptic hydrolysates from potato industry side-streams (García-Moreno, Gregersen, et al., 2020), were also included in this study. First, synchrotron radiation circular dichroism (SRCD) was used to quantitatively evaluate the changes in the structure of peptides from when they are (a) solubilized in buffer solution, to (b) being adsorbed at the oil-water interface in O/W emulsions. Secondly, we evaluated the mechanical properties of the peptide interfacial film (namely, viscoelasticity) by using dilatational rheology. Finally, we studied the physical and oxidative stability of fish O/W emulsions stabilized with the peptides. Contrary to our previous studies, we produced larger volumes of emulsions by using standard methods employed in industry (high-pressure homogenization), and we evaluated their oxidative stability by specific methods such as determination of lipid peroxides and secondary volatile oxidation products.

2. MATERIALS AND METHODS

2.1 Materials

Synthetic peptides with a purity > 70% were purchased from Pepmic Co., Ltd (Jiangsu, China). Table 1 shows the sequences and properties of the synthetic peptides used as

emulsifiers. Commercial cod liver oil was kindly provided by Vesteraalens A/S (Sortland, Norway) and stored at -40 °C until use. The fatty acid (% , w/w) content of the fish oil was as follows: C14:0 (0.2%), C16:0 (9.4%), C16:1 n-7 (8.6%), C18:0 (2.0%), C18:1 n-9 (16.2%), C18:1 n-7 (4.6%), C18:2 n-6 (1.8%), C18:3 n-3 (0.1%), C20:1 n-9 (12.6%), C20:5 n-3 (9.1%), C22:1 n-11 (5.9%), and C22:6 n-3 (11.1%). α -, β -, γ -, and δ -tocopherol contents were 250 $\pm$ 2, 0 $\pm$ 0, 118 $\pm$ 1, 48 $\pm$ 1 μ g toc/g oil, respectively. The peroxide value (PV) of the fish oil used was 0.12 $\pm$ 0.08 meq/kg oil. The characterization of the fish oil used was determined as described elsewhere (García-Moreno, Guadix, Guadix, & Jacobsen, 2016). Sodium caseinate (Miprodan 30) was provided by Arla Foods Ingredients amba (Viby J, Denmark). Protein content of sodium caseinate was reported by Arla Foods as 92% (Kjeldal-N $\times$ 6.38). Tricaprylin and Brij 97 were purchased from Sigma-Aldrich (Brøndby, Denmark). All other chemicals and solvents used were of analytical grade.

2.2 Interfacial properties of peptides

2.2.1 Determination of secondary structure of peptides by synchrotron radiation circular dichroism (SRCD)

To determine the conformational changes of peptides when adsorbed at the oil-water interface, their secondary structure was determined both in solution and in 5 wt.% O/W emulsions.

2.2.1.1 Preparation of solutions and emulsions for SRCD experiments

For preparing peptide solution and aqueous phases for emulsions, peptides (0.2 wt.%) were dissolved in 10 mM phosphate buffer (pH 7). The peptide-buffer solutions were shaken (100 rpm) for 2 h in a water bath at 50 °C and overnight at room temperature to allow complete solubilization and rehydration of the peptides (when possible). For producing the 5 wt.% O/W emulsions, primary homogenization was done by adding the tricaprylin to the peptide-buffer solution and mixing at 18,000 rpm for 30 s by using a POLYTRON® PT1200E (Kinematic

Inc., New York, USA). Secondary homogenization was done using a sonicator equipped with a P1 probe (Microson XL2000, Misonix, Inc., New York, USA). Emulsions were homogenized at an amplitude of 75% (maximum amplitude of 180 μm), running 2 passes of 30 s with a break of 1 min between passes. During sonication the emulsions were surrounded by iced water to minimize the increase in temperature. An emulsion stabilized with 0.2 wt.% sodium caseinate (Miprodan 30), a milk protein exhibiting excellent emulsifying activity, was also produced as positive emulsifier control. A Brij 97 (0.2 wt.%) stabilized O/W emulsion was also prepared by homogenizing 5 wt.% tricaprylin with 95% aqueous solution (Brij 97 in 10 mM phosphate buffer, pH 7) under the same conditions as described above. This emulsion and dilutions from it were used for SRCD baseline correction.

2.2.1.2 SRCD measurements

SRCD measurements were carried out on the AU-CD beamline at the ASTRID2 synchrotron radiation source, (ISA, Department of Physics & Astronomy, Aarhus University in Denmark). The operation of the SRCD spectrometer was confirmed daily using camphorsulfonic acid for optical rotation magnitude and wavelength (Andrew J. Miles, Wien, & Wallace, 2004). A 0.01 cm path length quartz Suprasil cell (Hellma GmbH & Co., Germany) was used for far-UV SRCD measurements at 25 °C. The baseline was corrected using 10 mM phosphate buffer (pH 7) for peptide solutions and Brij 97 stabilized tricaprylin-in-water emulsion for peptides-stabilized emulsions. The far-UV SRCD spectra were recorded in triplicate from 270 to 170 nm in 1 nm steps, with a dwell time of 2.1 s per point.

For SRCD measurements, caseinate and β 22 solutions were diluted in buffer (2:1, v/v) and (1:1, v/v) respectively to reduce absorbance. All emulsion samples were diluted in buffer (1:1, v/v), except β 22-stabilized emulsion which was further diluted in buffer (1:3, v/v) in order to reduce absorbance and scattering.

2.2.1.3 SRCD data analysis and calculation of peptides secondary structure

The spectra were converted to delta epsilon units using the peptide concentration obtained from absorbance at 205 nm and corrected for scattering where necessary (Nordén B., Rodger, A., & Dafforn, 2010). When light is elastically scattered from particles with a size similar to or smaller than the wavelength, it may not be detected by the detector. Where scattering occurs, it is evident in the absorbance spectrum which is measured simultaneously with the CD spectrum. A long, non-zero sloping tail of the absorbance curve can be fitted using an equation based on Rayleigh scattering, to simulate the effect of scattering over the full spectrum (Nordén B., Rodger, A., & Dafforn, 2010), allowing for a corrected absorbance at 205 nm to be obtained. The sequence of peptides was used to determine the molar extinction coefficient and mean residual weight (Anthis & Clore, 2013). For casein, the sequence of β -casein from bovine was used, which is slightly imprecise since Miprodan 30 also contains α - and κ -caseins. Another effect of light scattering from the emulsions is a change in the underlying CD baseline, due to the very different paths of light through the system compared to normal solution measurements. The Brij 97 stabilized emulsion produced in the same way as for the peptides, enables the light scattering effects to be reproduced and therefore allowing a baseline correction of the peptide stabilized emulsions.

The proportions of each secondary structure components were determined using a web-based calculation server DICHROWEB that incorporates various methods and a wide range of protein spectral databases (Whitmore & Wallace, 2007). The calculation method used in this study was CDSSTR with the reference set SMP180 (Abdul-Gader, Miles, & Wallace, 2011; Sreerama & Woody, 2000). Due to the nature of the emulsions, in particular due the scattering which occurs from the droplets, the accuracy of peptide concentration determination is lower than for those in solution. For emulsions which destabilized before measuring, those stabilized with peptides β -27, γ 38 and γ 40, the error in the concentration determined may be

as high as 25%. However, despite these higher uncertainties for the emulsions, which may affect the absolute values obtained through the secondary structure fitting, relative changes in the structure determination are still credible and the changes in CD spectral shapes from solution to emulsion samples reflecting significant folding changes are clearly observed.

2.2.2 Interfacial adsorption and dilatational rheology

The dilatational surface properties were analyzed using a drop tensiometer (ADT, Teclis IT Concept, Longessaigne, France) at 20 °C. A rising droplet of tricaprylin (used as received) with a surface area of 30 mm² was formed at the tip of the needle (curved G18) in the peptide solution. The peptide solutions were prepared at 0.1 g/L in phosphate buffer (10 mM, pH 7). The samples were stirred for 30 min at 50 °C, and followed by overnight stirring at room temperature (100 rpm), except for β 27 which was stirred at 50 °C for 3 h, and sodium caseinate which was not stirred at 50 °C. The drop contour was monitored and fitted with the Young-Laplace-Gauss equation to obtain the interfacial tension. The interfacial tension of the pure water-tricaprylin interface was found to be 24.0±0.8 mN/m. The change in interfacial tension was monitored for 10,800 s, followed by a frequency and amplitude sweep. The frequency sweep was performed with frequencies between 0.005 to 0.1 Hz at an amplitude of 5%. The amplitude sweep was performed with amplitudes between 5 to 30% at a frequency of 0.02 Hz. The droplet was subjected to five oscillatory cycles at every amplitude or frequency. Via oscillatory dilatational deformation experiments (compression and expansion), the complex surface dilatational modulus was calculated from the first harmonic of the Fourier spectrum of the oscillatory interfacial tension signal. The complex surface dilatational modulus is equal to $E_d^* = E_d' + iE_d''$, where E_d' is the dilatational storage modulus (elastic modulus), and E_d'' is the dilatational loss modulus (viscous modulus). The loss tangent is defined as $\tan\Phi = (E_d''/E_d')$ (Sagis, Humblet-Hua, & Van Kempen, 2014). The measurements were done in triplicate.

2.2.2.1 Rheology data analysis

The results of the amplitude sweeps were analyzed using Lissajous plots of the surface pressure ($\Pi = \gamma - \gamma_0$) versus the deformation $(A - A_0)/A_0$. Here, γ and A are the surface tension and area of the deformed interface, whereas γ_0 and A_0 are the surface tension and area of the non-deformed interface. Plots were drawn for each deformation from the middle three oscillations. For the quantitative analysis of the curves, four moduli were determined according to Van Kempen, Schols, Van Der Linden, & Sagis (2013): $E_{L,E}$, the large strain modulus in extension; $E_{M,E}$, the minimum strain modulus in extension; $E_{L,C}$, the large strain modulus in compression; and $E_{M,C}$, the minimum strain modulus in compression. Using these moduli, the stiffening- or S-factors were calculated: $S_{\text{exp}} = (E_{L,E} - E_{M,E})/E_{L,E}$ for the expansion part of the Lissajous plot, and $S_{\text{com}} = (E_{L,C} - E_{M,C})/E_{L,C}$ for the compression part.

2.3 Production of emulsions and sampling for physicochemical characterization

Batches (200 g) of 5 wt.% fish O/W emulsions (pH 7) stabilized with 0.2 wt.% potato peptides were produced. An emulsion stabilized with sodium caseinate was also produced as control. Synthetic peptides or sodium caseinate (0.2 wt.%) were dissolved in 10 mM sodium acetate – 10 mM imidazole buffer (pH 7). For that, the peptide solutions were shaken (100 rpm) for 2 h in a water bath at 50 °C and overnight at room temperature to allow complete solubilization and rehydration of the sodium caseinate and peptides (when possible). Primary homogenization was done by adding the fish oil slowly to the aqueous phase during mixing at 16,000 rpm (Ystral mixer, Ballrechten-Dottingen, Germany). The fish oil was added during the first minute of mixing, and the total mixing time was 3 min. Secondary homogenization was done on a Microfluidizer (M110L Microfluidics, Newton, MA, USA) equipped with a ceramic interaction chamber (CIXC, F20Y, internal dimension 75 μm). Emulsions were homogenized at a pressure of 9,000 psi, running 3 passes. To accelerate lipid oxidation FeSO_4

was added to the emulsions after homogenization to have 100 μM of FeSO_4 in the emulsion. Sodium azide (0.05 wt.% in the emulsion) was added to prevent microbial growth. Emulsions were stored in 50 mL brown bottles at room temperature (19-20 $^{\circ}\text{C}$) in the dark for 7 days. We prepared a bottle containing 35 g of sample for each type of emulsion and each sampling point for lipid oxidation analyses. Samples were taken at days 0, 1, 3, 5 and 7 for lipid oxidation measurements. Measurements of droplet size distributions were carried out at day 1 and 7, and zeta potential was determined at day 1. Four grams of each emulsion were placed in graduated cylindrical tubes for creaming index (CI) determination.

2.4 Physical characterization of emulsions

2.4.1 Zeta potential

The zeta potential was measured in a Zetasizer Nano ZS (Malvern instruments Ltd., Worcestershire, UK) with a DTS1070 cell at 20 $^{\circ}\text{C}$. Before analysis the emulsions were diluted 2:1000 (v/v) in buffer. The zeta potential range was set to -100 to +50 mV and the samples were analysed with 100 runs. Measurements were done in triplicate.

2.4.2 Droplet size and creaming index (CI)

Droplet sizes were measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). Emulsions were vortexed and diluted in recirculating water (3000 rpm), until reaching an obscuration of 12–15%. The refractive indices of sunflower oil (1.469) and water (1.330) were used as particle and dispersant, respectively. Results are given as the Sauter mean diameter ($D_{3,2}$). Measurements were made in triplicate.

CI was determined on each day during storage without replicates. CI was evaluated as $\text{CI}(\%) = (H/H_o) \times 100$, where H is the height of aqueous phase which is separated at the bottom of the graduated cylindrical tube, and H_o is the height of total emulsion.

2.4.3 Yellowness index (YI)

The YI of the emulsions at day 7 was determined by measuring the color of the emulsions using a spectrophotometer (X-Rite, Inc. Grandville, MI, USA). The instrument was calibrated before each measurement and the results were recorded using the CIE color system profile of L^* (Lightness), a^* (redness/greenness), b^* (yellowness/blueness). YI was calculated according to Francis & Clydesdale (1975): $YI = 142.86 \frac{b^*}{L^*}$. The measurement was performed in triplicate for each sample.

2.5 Oxidative characterization of emulsions

2.5.1 Peroxide value (PV)

Fish oil was extracted from the emulsions according to the Bligh and Dyer method using a reduced amount of the chloroform/methanol (1:1 w/w) solvent (Bligh & Dyer, 1959). Two extractions were made from each sample. PV was determined on lipid extracts using the colorimetric ferric-thiocyanate method at 500 nm as described by Shantha & Decker (1994). Measurements were performed in duplicate.

2.5.2 Tocopherol content

For the determination of the tocopherol content, about 2 g of the chloroform extract from the Bligh and Dyer extraction were evaporated under nitrogen and dissolved in 1 mL n-heptane before being analyzed as described elsewhere (García-Moreno, Guadix, Guadix, & Jacobsen, 2016). Measurements were performed in duplicate.

2.5.3 Secondary volatile oxidation products - Dynamic headspace GC-MS

Approximately 4 g of emulsion and 30 mg internal standard (4-methyl-1-pentanol, 30 µg/g water) were weighed out in a 100 mL purge bottle. To it, 5 mL of distilled water and 1 mL antifoam (Synperonic 800 µL/L water) were added. The bottle was heated in a water bath at 45 °C while purging with nitrogen (flow 150 mL/min, 30 min). Volatile secondary oxidation products were trapped on Tenax GR tubes. The volatiles were desorbed again by heat (200

°C) in an Automatic Thermal Desorber (ATD-400, Perkin Elmer, Norwalk, CN), cryofocused on a cold trap (-30 °C), released again (220 °C), and led to a gas chromatograph (HP 5890IIA, Hewlett Packard, Palo Alto, CA, USA; Column: DB-1701, 30 m x 0.25 mm x 1.0 µm; J&W Scientific, CA, USA). The oven program had an initial temperature of 45 °C for 5 min, increasing with 1.5 °C/min until 55 °C, with 2.5 °C/min until 90 °C, and with 12.0 °C/min until 220°C, where the temperature was kept for 4 min. The individual compounds were analysed by mass-spectrometry (HP 5972 mass-selective detector, Agilent Technologies, USA; electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250). The individual compounds were identified by both MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard) and quantified through calibration curves. The external standards employed were 2-ethylfuran, 1-penten-3-ol, pentanal, (*E*)-2-heptenal, hexanal, (*E*)-2-hexenal, heptanal, (*E,E*)-2,4-heptadienal, nonanal, (*E,E*)-2,6-nonadienal. The standard solutions were directly injected to the Tenax GR tube and dried for 5 min under N₂ (50 mL/min). Measurements were made in triplicate for each sample.

2.6 Statistical analysis

Statgraphics 18 software (Statistical Graphics Corp., Rockville, MD, USA) was used for data analysis. Data were expressed as mean ± standard deviation. For principle component analysis (PCA), peptide codes were selected as objects. Length, amphiphilic score, isoelectric point (pI), interfacial tension, complex surface dilatational modulus (0.02 Hz, 5% amplitude), Sauter diameter ($D[3,2]$) at days 1 and 7, zeta potential, and concentration of volatiles (1-penten-3-ol, 2,4-heptadienal, 2-pentenal and hexanal) at day 7 were the selected variables. Only measured variables that resulted in experimental values for all peptide samples were selected for PCA. The PCA was carried out on the correlation matrix, standardizing each variable by subtracting the mean value and dividing by the standard deviation.

3. RESULTS AND DISCUSSION

3.1 Interfacial properties of peptides

3.1.1 Secondary structure of peptides

Synchrotron radiation circular dichroism (SRCD) is a well-established technique for the determination of protein structure (Wallace, 2009). Particularly, the higher flux of light from a synchrotron (compared to bench-top CD) results in higher penetration and leads to a better signal-to-noise ratio in the far ultraviolet wavelength. This enables, for example, a more accurate determination of the structure of membrane proteins by collecting data to lower wavelengths, also allowing the measurement in the presence of highly absorbing non-chiral components (e.g., lipids) (Miles & Wallace, 2016).

Fig. 1 and 2 show the far-UV SRCD spectra and the secondary structure composition (% α -helix, β -strand, turns and unordered) of potato peptides in solution (pH ~7) and adsorbed at the tricaprylin oil-water interface, respectively. The majority of the peptides in solution had low ellipticity above 210 nm and a negative band at around 195-200 nm, which accounted for a high percentage of disordered structures (~60% of turns and unordered regions) (Fig. 1 and Fig. 2a). However, spectra for α 10, α 12 and γ 1 peptides in solution exhibit a positive peak at 192 nm and the minima at 208 and 222 nm, indicative of α -helical structure (Hoffmann, Fano, & van de Weert, 2016), which was not observed for the other peptides (Fig. 1). In fact, α 10, α 12 and γ 1 had 35, 21 and 18% of α -helical structure in solution, respectively, with approximately half of it regular and the other half distorted (Fig. 2a). The other peptides and caseinate had very low α -helical structure percentage in solution and were richer in β -strand structure compared to α 10, α 12 and γ 1 (Fig. 1 and Fig. 2a). These findings correlated well with our previous results (implied by homology modelling) indicating that: i) α 10, α 12 (fully embedded in α 10) and γ 1 were localized in regions within native patatin with predominantly

α -helical structure, and ii) β 22, β 27, γ 36, γ 38 and γ 40 were localized in regions within native patatin (γ 36), or Kunitz-Type Inhibitor-A (β 27), or Kunitz-Type Inhibitor B (β 22, γ 38 and γ 40) that comprise β -strand structures (García-Moreno, Gregersen, et al., 2020).

Interestingly, the conformation that potato peptides adopted upon adsorption at the tricaprylin oil-water interface was significantly different when compared to their secondary structure in solution (Fig. 1 and 2). The lack of tertiary structure for the short potato peptides investigated explained their considerable conformation change at the oil-water interface allowing the hydrophobic patches of the peptides to interact with the oil (Enser, Bloomberg, Brock, & Clark, 1990). Peptides γ 75 and γ 76 varied their main structural motif from unordered in solution to α -helical at the oil-water interface, while α 12 and γ 1 showed an increase in α -helical content at the interface (Fig. 1 and 2). This increase in α -helicity was accompanied by a reduction of β -strand structures for these peptides when located at the oil-water interface (Fig. 2). The high α -helicity at the oil-water interface was expected for α 12 peptide, which was identified by bioinformatics as an emulsifier peptide adopting amphiphilic α -helix structure (García-Moreno, Gregersen, et al., 2020). Likewise, γ 1 as well as γ 75 and γ 76 (which are truncated versions of γ 1), have hydrophobic and hydrophilic residues distributed in a surface exposed α -helix in the native patatin protein (García-Moreno, Jacobsen, et al., 2020), explaining why they re-arranged in an amphiphilic α -helix conformation at the oil-water interface. Similarly, but to a lower extent, an increase in α -helicity of ~7 to 16% when adsorbed at the tricaprylin oil-water interface was observed for caseinate (Fig. 1 and 2). This is in line with the results reported for β -casein, present in caseinate, where an increase from 5 to 20% was observed (Wong et al., 2012).

On the contrary, β -strand was the major structural motif exhibited by the β 22 and γ 36 peptides when located at the oil/water interface (Fig. 1 and 2). Indeed, β 22 was identified by bioinformatics as an emulsifier potato peptide exhibiting amphiphilic β -strand structure and it

was located in a region of the native protein containing two anti-parallel β -strands (García-Moreno, Gregersen, et al., 2020), which correlated well with the SRCD results. Curiously, peptide $\gamma 36$ re-arranged at the oil-water interface to adopt a β -strand structure to a much higher extent (Fig. 2) than when buried in the native protein (García-Moreno, Gregersen, et al., 2020). The latter indicates the influence of the hydrophobic interactions between the oil and the hydrophobic amino acids, as well as the electrostatic and Van der Waals forces between peptide molecules, on the structure adopted by the peptide in the reduced space available at the interface (Zhai, Day, Aguilar, & Wooster, 2013). Likewise, an increase in β -strand structure when adsorbed at the tricaprylin-water interface (when compared to solution) was reported for globular proteins such as bovine serum albumin (from 2 to 7%), lysozyme (from 15 to 18%) and myoglobin (from 2 to 10 %) (Day et al., 2014).

Secondary structure determination for $\alpha 10$, $\beta 27$, $\gamma 38$ and $\gamma 40$ at the oil-water interface have a higher degree of uncertainty, since emulsions stabilized with these peptides creamed before measuring. Thus, protein concentration in these emulsions could not be as accurately determined. Nonetheless, secondary structure analysis for these peptides at the oil/water interface have been carried out and can also be qualitatively described from their SRCD spectra and by comparison with the SRCD spectra of the peptides in solution. For instance, when comparing $\alpha 10$, $\gamma 38$ and $\gamma 40$ SRCD spectra in solution and at the interface, they are not significantly different (Fig. 1b,j,k). The $\alpha 10$ (Fig. 1b) remains structured with ~50% alpha and beta-type folding, while $\gamma 38$ (Fig. 1j) and $\gamma 40$ (Fig. 1k) largely maintain their unordered and some β -strand structure at the interface. For $\beta 27$, the SRCD spectra at the interface suggest an increase in α -helicity, with small reductions in the amount of unordered and β -strand structure (Fig. 1e).

3.1.2 Interfacial tension, adsorption kinetics and interfacial dilatational rheology

3.1.2.1 Interfacial tension and adsorption kinetics

Fig. 3 shows the interfacial tension at the tricaprylin oil-water interface for potato peptides and caseinate. A rapid decrease in interfacial tension was observed in the first 300 s for all samples, followed by a slower decrease (Fig. 3a). Peptides $\alpha 12$, $\gamma 1$, $\gamma 75$, $\gamma 76$ and caseinate led to a fast initial decrease in interfacial tension, leveling off afterwards (Fig. 3a). Hence, these peptides with mainly α -helical structure at the interface speedily adsorb at the interface, shielding the oil molecules from the water molecules, while they do not considerably further re-arrange at the interface after adsorption. This was confirmed by the semi-logarithmic plots of adsorption kinetics, where in the second and third interfacial tension regimes, after 100 s (Beverung, Radke, & Blanch, 1999), these samples (except for caseinate) did not show a pronounced decrease in interfacial tension (Fig. 3b,d). Among samples with this behavior, caseinate resulted in the most rapid decrease in interfacial tension, leading to the lowest interfacial tension value after 3 h ageing, followed by $\gamma 1$ (Fig. 3a).

On the other hand, $\alpha 10$, $\beta 22$, $\beta 27$, $\gamma 36$, $\gamma 38$, and $\gamma 40$ showed a more gradual decrease in interfacial tension during 3 h of aging, with extensive reduction in interfacial tension after the first interfacial tension regime (>100 s) (Fig. 3). The latter is explained by structural re-arrangement of these peptides at the interface, which increased interfacial coverage by projecting the hydrophobic amino acids in the oil phase and allowed the interfacial tension to be considerably reduced (Beverung et al., 1999; Schröder, Berton-Carabin, Venema, & Cornacchia, 2017). Interestingly, these peptides (except $\alpha 10$) had a considerable percentage of β -strand in their secondary structure at the interface (Fig. 1 and 2), implying that this type of secondary structure, instead of peptide length, might play a significant role in the slower re-arrangement and interaction of the peptides at the interface upon adsorption. It should be also noted that $\beta 27$ drastically reduced interfacial tension in the second and third interfacial

tension regimes, reaching a final value lower than for caseinate (Fig. 3a). Likewise, γ_{40} notably decreased interfacial tension in the third regime, which is associated with further molecular re-arrangement and build-up of multilayers (Beverung et al., 1999), leading to a final value similar to γ_1 . Nevertheless, care should be taken with data obtained for γ_{40} , since the peptide solution tested presented some insoluble particles. The differences observed between these results and those reported in our previous work (García-Moreno, Gregersen, et al., 2020) are due to the different method (e.g., protein solution droplet in oil vs. oil droplet in protein solution), different type of oil (MCT vs. tricaprylin) and different time scale (30 min vs. 3 h) used.

3.1.2.2 Interfacial dilatational rheology

Dilatational rheology enables the characterization of the peptide-based interfacial layers in terms of re-arrangements at the interface as well as exchange processes between the bulk and the interface (Berton-Carabin, Sagis, & Schroën, 2018).

3.1.2.2.1 Frequency sweeps

Frequency sweeps describe the time-dependent behavior of the interfacial layer, allowing the study of short and long time scales by changing the frequency of deformations. We observed for all samples that the storage modulus (E'_d) is larger than the loss modulus (E''_d) (data not shown), which is indicative of predominantly elastic interfacial layers. In fact, all samples had a phase angle $\Phi \leq 16^\circ$, except for γ_{75} ($20 < \Phi < 35^\circ$), which indicate highly elastic interfacial films. Fig. 4a shows that interfacial layers formed by peptides γ_{36} , γ_{38} , β_{22} and β_{27} exhibited the highest complex surface dilatational moduli ($E^*_d = 35\text{--}45$ mN/m), which implies a higher resistance against deformation when compared to γ_{40} ($E^*_d = 16\text{--}17$ mN/m), and then caseinate, α_{10} , γ_{76} , α_{12} , γ_1 and γ_{75} ($E^*_d < 12$ mN/m). The frequency dependency was quantified by a power law function ($E'_d \sim \omega^n$) (Fig. 1S). All the slope (n) values were lower than 0.5, indicating that the exchange of peptides between the bulk water phase and the

interface was not the main contribution to the observed response of the interface, and that other processes had a significant contribution, e.g., in-plane interactions at the interface (Sagis et al., 2019). The latter seemed to be particularly important for interfacial layers formed by $\alpha 10$, $\gamma 36$, $\gamma 38$ and $\gamma 40$ as a result of their much lower slope value when compared with caseinate, $\alpha 12$, $\beta 22$, $\gamma 1$, $\gamma 75$ and $\gamma 76$ peptides (Fig. 1S).

3.1.2.2.2 Amplitude sweeps

Amplitude sweeps allow the study of the strength and degree of linearity of the response of the interfacial layer versus the amplitude of deformation. Fig. 4b shows that in the range of applied amplitudes (5-30%), E_d^* was independent of the amplitude for caseinate, $\alpha 10$, $\alpha 12$, $\gamma 1$, $\gamma 75$, $\gamma 76$ and $\gamma 40$, denoting that these peptides form weak (low E_d^*) and easily stretchable interfaces. This indicated that, independent of peptide length, no or only weak inter-peptide interactions occurred at the interface (Sagis, 2013; Schröder et al., 2017). Conversely, E_d^* decreased with increasing amplitude for interfacial layers formed by $\beta 22$, $\beta 27$, $\gamma 36$ and $\gamma 38$ (Fig. 4b). This indicates that these deformations are in the nonlinear viscoelastic (NLVE) regime and affected the interfacial microstructure (Yang, Thielen, Berton-Carabin, van der Linden, & Sagis, 2020). These results for $\beta 22$, $\beta 27$, $\gamma 36$ and $\gamma 38$ confirmed the formation of solid-like viscoelastic interfacial layers formed by inter-peptide interactions at the interface, which deformed or yielded during elongation or compression of the droplet (Van Kempen et al., 2013). Similar findings were reported for whey proteins, which led to a dense viscoelastic interfacial layer (e.g., by the formation of disulphide bridges between neighboring protein molecules) that also deformed/yielded at increased amplitudes (Schröder et al., 2017). It is worth noting that $\beta 22$, $\beta 27$ and $\gamma 38$ contain one Cys residue in their sequences (Table 1). Free Cys could potentially enable covalent inter-peptide interactions through disulphide bonds, which may form spontaneously in solution following Cys oxidation by e.g. molecular oxygen or other appropriate electron acceptors (Creighton, 1984; Anfinsen, 1973). In addition, these

peptides have low net charge (<1.1 , Table 1), except for $\gamma 38$ (net charge of 4.0, Table 1), which reduces repulsion among adsorbed peptides and those in the bulk. The low net charge favors inter-peptide interactions and the formation of dense interfacial layers (Tamm & Drusch, 2017).

3.1.2.2.3 Lissajous plots

Lissajous plots, which consider higher harmonics of the Fourier spectrum, were used to further characterize the interfacial layers (Van Kempen et al., 2013). Emulsifiers forming stretchable interfaces with low values of E_d^* (<12 mN/m) and linear responses ($\alpha 10$, $\alpha 12$, $\gamma 1$, $\gamma 75$ and $\gamma 76$), resulted in Lissajous plots with a lot of scattering in the data at all amplitudes (Fig. 2S in Supplementary Material). In contrast, Lissajous plots for peptide $\gamma 40$ (E_d^* of 16-17 mN/m) showed an almost straight line (closed plot) at all amplitudes (Fig. 5), suggesting a dominating elastic component of the response at all amplitudes. Similar behavior was observed for caseinate, with slightly more opened plots at higher amplitudes (Fig. 5). Peptides $\beta 22$, $\beta 27$, $\gamma 36$ and $\gamma 38$, forming stronger interfacial layers ($E_d^* = 35-45$ mN/m), showed a response where the elastic contribution dominated at 5% amplitude (straight line-closed plot). However, the Lissajous plots for these peptides started opening up asymmetrically to a larger extent (i.e., when compared to caseinate) at higher amplitudes (Fig. 5). These findings indicated that after adsorption $\beta 22$, $\beta 27$, $\gamma 36$ and $\gamma 38$ started to interact at the interface to form solid-like viscoelastic layers (with a relatively high value for elasticity), and showed significant nonlinear behavior during extension and compression (Sagis et al., 2014). For $\gamma 38$, we observed a slower decrease of E_d^* with amplitude (Fig. 4b), which led to a less wide Lissajous plot at amplitude 30% and indicated a lower increase in the viscous component of the response when compared to $\beta 22$, $\beta 27$, and $\gamma 36$ (Fig. 5). For $\gamma 36$, at 30% deformation, when the interface started expanding from its maximum compressed state (lower left corner of the plot), we first observed a relatively fast increase in the surface pressure, in which the

slope of the curve (the tangential modulus) increased. This indicated intra-cycle strain hardening (e.g., strong inter-peptide interactions at the interface) until a deformation of -0.10 (Fig. 5). At deformations between -0.10 and 0.30, the increase in surface pressure became more gradual, suggesting intra-cycle strain softening ($S < 0$, see Fig. 3Sa). The latter indicated a gradual disruption of the interfacial network and concomitant increase in viscous response of the interface (Schröder et al., 2017). In compression, the plot showed a steeper surface pressure increase when compared to extension, which indicated intra-cycle strain hardening ($S > 0$, see Fig. 3Sb), and could be the result of jamming of the interfacial structure. The significant strain hardening upon compression suggests strong anchoring of the $\gamma 36$ peptide and confirms our earlier assertion that exchange with the bulk phase plays only a minor role in the response for this peptide.

Interfacial layers formed by $\beta 22$ and $\beta 27$ peptides exhibited significantly wider Lissajous plots at 30% deformation than $\gamma 36$, which indicated a more significant distortion of the interfacial structure and resulted in a relatively more viscous behavior (Fig. 5). Starting again from maximum compression (-0.30), $\beta 22$ presented a rather sudden change in slope upon extension at deformation -0.20, which could be an indication of yielding of the structure. A further increase in deformation due to extension (from -0.20 to 0.30) led to a more gradual increase in surface pressure revealing strain softening, again associated with the disruption of the interfacial network and flow of the system (Fig. 5). Upon compression, strain hardening was observed, while there seems to be a slight strain softening in the last part of the compression (-0.20 to -0.30) (Fig. 5). The strain softening in compression could be due to: i) buckling of the interface, ii) the peptides at the interface being pushed into the bulk, or iii) fracturing of the interface followed by layers starting to slide over each other (Sagis et al., 2014). Lissajous plots for $\beta 27$ showed a dominant elastic response at 5%, whereas the plots opened-up at larger deformations indicating an increase of the viscous components with

increasing strain (Fig. 5). We observed strain softening during expansion ($S < 0$, see Fig. 3Sa), while we did not observe any significant strain hardening upon compression, but instead a slight strain softening at the very end of the compression part of the cycle (as observed for $\beta 22$) (Fig. 5).

Altogether, and although our large amplitude results suggest that $\beta 22$, $\beta 27$, $\gamma 36$ and $\gamma 38$, peptides had significant in-plane attractive interactions at the interface with neighboring peptide molecules, resulting in relatively more stiff, solid-like viscoelastic interfacial layers, which showed considerable softening upon extension. On the other hand, and although helical structures are in general more physically rigid than strands, caseinate, $\alpha 10$, $\alpha 12$, $\gamma 1$, $\gamma 75$, $\gamma 76$ and $\gamma 40$ weakly interact at the interface, leading to significantly less stiff and more stretchable layers.

3.2. Physicochemical stability of emulsions

3.2.1 Physical stability

The emulsification performance of the peptides was further investigated by studying the physical stability of fish O/W emulsions stabilized with the peptides (Table 2). As expected, peptides $\alpha 10$, $\beta 27$ and $\gamma 38$ with $pI > 7$ led to emulsions (at $pH \sim 7$) with positive zeta potential values, whereas the rest of peptides with $pI < 7$ resulted in emulsions (at $pH \sim 7$) with negative zeta potential values (Table 2). High net zeta potential values (> 30 mV) were obtained, apart from the emulsion stabilized with $\beta 27$ (11.6 ± 0.9 mV) (Table 2). The latter indicates that $\beta 27$ may not provide sufficient electrostatic repulsion forces to maintain the emulsion physically stable. Indeed, emulsions stabilized with $\beta 27$ or $\beta 22$ (despite the large net zeta potential for the latter emulsion) had trimodal droplet size distributions with peaks centered in 0.15, 0.7 and 1.25 μm , whereas the rest of emulsions had bimodal droplet size distributions with a peak centered in 0.15 μm and a shoulder at 0.5 μm (see Fig. 4S). These results reveal that the

emulsion stabilized with $\beta 27$ had the largest droplet size at day 1 (followed by the emulsion stabilized with $\beta 22$) (Table 2), and creamed after one day of storage (CI= 28%); whereas no creaming was observed for the rest of the emulsions. In addition, peptides $\beta 22$ and $\beta 27$ have a Cys residue in their N- and C-terminus, respectively, that may have led to inter-droplet disulphide bridging negatively affecting physical stability of the emulsions.

Interestingly, emulsions stabilized with $\alpha 12$, $\gamma 75$, $\gamma 76$, $\gamma 38$ and $\gamma 40$ had markedly smaller droplet size at day one when compared to the emulsion stabilized with caseinate (Table 2) (Fig. 3a). It is noteworthy that, in despite of the low peptide concentration used (0.2 wt.%), these emulsions had $D_{3,2}$ values in the range of those reported for 5 wt.% fish O/W emulsions stabilized with 2 wt.% whey, soy or blue-whiting protein hydrolysates (Padial-Domínguez, Espejo-Carpio, García-Moreno, Jacobsen, & Guadix, 2020). This reveals the high emulsifying performance of the assayed peptides. It should be also noted that emulsions stabilized with $\alpha 10$, $\gamma 1$ and $\gamma 36$ did not have markedly different droplet size values when compared to caseinate emulsion at day one (Table 2). In addition, emulsions stabilized with $\gamma 1$, $\gamma 75$ and $\gamma 76$, with small droplet size after production, did not markedly increase their droplet size after one week of storage (Table 2).

Hence, the physical stability of the emulsions is influenced by other factors than only reduction in interfacial tension by the peptides. Nonetheless, it is important that peptides rapidly diffuse to the interface and reduce interfacial tension in the first seconds to allow droplets to break up during homogenization (Fig. 3a). On the other hand, these findings revealed that potato peptides could physically stabilize O/W emulsions by creating i) for $\alpha 12$, $\gamma 1$, $\gamma 75$, $\gamma 76$ and $\gamma 40$: highly stretchable interfaces (with weak inter-peptide interactions) by anchoring at the interface through hydrophobic interactions and adopting a highly amphiphilic α -helical structure (except $\gamma 40$, which is largely unordered), while having an unordered part of the peptide highly charged and fully solvent exposed (i.e., providing steric hindrance and

electrostatic repulsion) (García-Moreno, Gregersen, et al., 2020; García-Moreno, Jacobsen, et al., 2020), or ii) for $\gamma 36$ and $\gamma 38$: stiffer and more solid-like interfaces by adopting amphiphilic β -strand structures that anchor and interact at the interface while also presenting an unordered end of the peptide that provides additional steric hindrance and/or electrostatic repulsive forces. Particularly, our results suggested that the first type of potato emulsifier peptides ($\alpha 12$, $\gamma 1$, $\gamma 75$ and $\gamma 76$), with interfacial protein films with similar properties to caseinate, i.e., elastic and low E^*_d , (Fig. 4 and 5), exhibited superior emulsification performance (e.g., markedly higher physical stability, Table 2).

3.2.2 Oxidative stability

Fig. 6a shows the evolution of peroxide value (PV) of the emulsions during storage. In general, the emulsions had high PV after production, denoting that lipid oxidation occurred during homogenization. Three groups could be differentiated, given in decreasing order for PV at day 0: i) emulsions stabilized with $\gamma 76$, $\gamma 75$, $\alpha 12$ and $\gamma 1$ (17-33 meq O_2 /kg), ii) emulsions stabilized with $\beta 22$ and $\gamma 40$ (~13 meq O_2 /kg), and iii) emulsions stabilized with caseinate, $\alpha 10$, $\beta 27$, $\gamma 36$ and $\gamma 38$ (~10 meq O_2 /kg) (Fig. 6a). Indeed, the emulsions stabilized by the last group of peptides ($\alpha 10$, $\beta 27$ and $\gamma 38$), with a low PV at day 0, increased their PV during storage to a markedly lower extent (PV at day 7: ~20 meq O_2 /kg) when compared to caseinate (PV at day 7: ~32 meq O_2 /kg) and to the rest of peptides (Fig. 6a.). In contrast, emulsions stabilized with $\gamma 76$, $\gamma 75$, $\alpha 12$, $\gamma 1$ and $\beta 22$ peptides showed a sharp increase in PV in the first three days of storage (44-57 meq O_2 /kg), exhibiting a higher rate of decomposition than formation of hydroperoxides from day 1 for $\gamma 76$, $\gamma 75$ and from day 3 for $\alpha 12$, $\gamma 1$ and $\beta 22$ (Fig. 6a.). On the other hand, emulsions stabilized with $\gamma 36$ and $\gamma 40$ reached their maximum PV at day 5 (42-46 meq O_2 /kg) (Fig. 6a). In the same line, the α -tocopherol content of emulsions stabilized with $\alpha 10$, $\beta 27$ and $\gamma 38$ and also caseinate was considerably higher during storage when compared to the rest of the peptides (Fig. 5S). This correlated well with PV

results, suggesting that emulsions stabilized with caseinate, α 10, β 27 and γ 38 were less susceptible to oxidation during storage and thus less α -tocopherol was consumed to protect the oil.

Likewise, emulsions stabilized with caseinate, α 10, γ 38 and β 27 presented the lowest concentration of secondary volatile oxidation products such as 1-penten-3-ol (Fig. 6b) and (*E,E*)-2,4-heptadienal (Fig. 6c). These volatiles were the most abundantly formed in the emulsions (up to 800 ng/g emulsion) and are derived from the oxidation of omega-3 polyunsaturated fatty acids (EPA and DHA). These emulsions also had the lowest content of other secondary volatile oxidation products formed to a lower extent such as 2-ethylfuran, (*E*)-2-pentenal, (*E*)-2-hexenal and (*E,E*)-2,6-nonadienal (all derived from the oxidation of omega-3 fatty acids); hexanal and heptanal (derived from oxidation of omega-6 fatty acids) and nonanal (derived from oxidation of omega-9 fatty acids) (Fig. 6S). Nonetheless, it is worth noting that the emulsion stabilized with β 27 showed a higher content of some volatiles at day 7 (i.e., 1-penten-3-ol (Fig. 6b) as well as (*E*)-2-hexenal and hexanal (Fig. 6S)) when compared to emulsions stabilized with α 10, γ 38 and caseinate. It should be also mentioned that the emulsion stabilized with γ 40 had a lower content of most of volatiles derived from oxidation of omega-3 fatty acids such as (*E,E*)-2,4-heptadienal (Fig. 6c) as well as 2-ethylfuran, (*E*)-2-pentenal and (*E*)-2-hexenal (Fig. 6S) when compared to emulsions stabilized with α 12, β 22, γ 1, γ 75, γ 76 and γ 36.

Taken altogether, the results indicated that the emulsions stabilized with α 12, β 22, γ 1, γ 75, γ 76 and γ 36 had the lowest oxidative stability during storage, followed by the emulsion stabilized with γ 40. On the other hand, emulsions stabilized with α 10, γ 38 and β 27 presented high oxidative stability which was comparable or enhanced (i.e. based on (*E,E*)-2,4-heptadienal) when compared to the oxidative stability provided by caseinate, which exhibits excellent radical scavenging and metal chelating properties (Elias, Kellerby, & Decker, 2008).

It is remarkable that emulsions stabilized with $\alpha 12$, $\beta 22$, $\gamma 75$ and $\gamma 76$ (but not $\gamma 1$ and $\gamma 36$) presented significantly higher values of YI when compared to emulsions stabilized with caseinate, $\alpha 10$ and $\gamma 38$ (Table 2). Increased brown color of the emulsions (high YI) might be related to advanced stages of oxidation where peptide co-oxidation could occur by non-enzymatic browning reactions (Lu, Nielsen, Baron, Diehl, & Jacobsen, 2013). Nonetheless, further research is required to investigate peptide co-oxidation and if they may play a chemical intermediate role in the lipid oxidation radical chain reaction.

In general, these findings are aligned with our previous results suggesting that emulsions stabilized with $\alpha 12$, $\beta 22$ and $\gamma 1$ had low oxidative stability when oxidation was accelerated with temperature and lipid oxidation was measured by electron spin resonance (ESR) (García-Moreno, Gregersen, et al., 2020). However, in our previous study we also found low oxidative stability for emulsions stabilized with $\alpha 10$ and $\gamma 38$ that was not observed in the present work where oxidation was accelerated by adding metal ions. Thus, the interfacial properties, specifically the positive surface charge of $\alpha 10$ and $\gamma 38$ peptides at the interface, played a major role for the enhanced oxidative stability of the emulsions studied in the present work as discussed in the section below.

3.2.2.1 Influence of physicochemical and interfacial properties of peptides on the oxidative stability of emulsions

PCA was performed to gain more insight into the effect of interfacial properties of peptides on the oxidative stability of the emulsions. The first principal component (PC1) explained 55.7% of the variance, while the second principal component (PC2) explained 18.8% (Fig. 7). PC1 was largely described by the concentration of volatiles and amphiphilic score (with positive weights and placed in the right side of the biplot) as well as pI and zeta potential (with negative weights and placed in the left side of the biplot). PC2 was mainly described by peptide length (positive weight and located in the upper part of the biplot) as well as droplet

size and complex surface dilatational modulus (negative weights and placed in the lower part of the biplot) (Fig 7).

PCA clearly indicated that emulsions stabilized with $\alpha 12$, $\gamma 1$, $\gamma 75$ and $\gamma 76$ (placed in the right part of the biplot very close to volatiles) were the most oxidized. These peptides, having α -helicity as the major structural motif when adsorbed at the interface (Fig. 2b), correlated positively with amphiphilic score (Fig. 7). They were also located close to the zero with respect to PC2, which denotes high physical stability (i.e., low droplet size at day 1 and 7) for these emulsions in spite of their poor inter-peptide interactions at the interface leading to weak and highly stretchable (low E^*_d) interfaces (Fig. 4). Moreover, these peptides negatively correlated with zeta potential and pI (Fig. 7), indicating very negative zeta potential values for the corresponding emulsions (Table 2). Thus, the emulsifying behavior showed by these peptides was similar to that exhibited by caseinate leading to a weak and non-dense but elastic and thick interface, which provides high steric hindrance and electrostatic repulsions to physically stabilize the emulsion (Dickinson, 2001). On the other hand, the oxidative stability of the emulsion stabilized with caseinate was significantly higher when compared to the emulsions stabilized with $\alpha 12$, $\gamma 1$, $\gamma 75$ and $\gamma 76$, despite all emulsions having negative zeta potential values (Table 2). A negative surface charge of peptides adsorbed at the interface favors the attraction of metal ions, which catalyze lipid oxidation by decomposing hydroperoxides sitting at the interface (i.e., due to their increased polarity) (McClements & Decker, 2018). Thus, the higher oxidative stability showed by the emulsion stabilized with caseinate can only be explained by its superior radical scavenging and, more importantly, its metal chelating activity that minimized the prooxidant effect of metal ions.

Fig. 7 shows that emulsions stabilized with $\beta 22$, $\gamma 36$ and $\gamma 40$ were centered in the bi-plot with respect to PC1, which implies that they were less oxidized than emulsions stabilized with $\alpha 12$, $\gamma 1$, $\gamma 75$ and $\gamma 76$. It is worth mentioning that although $\beta 22$ and $\gamma 36$ resulted in stiffer and more

solid-like interfaces (Fig. 4), they could not prevent diffusion of metal ions through the interface (i.e., physical barrier effect) due to the considerably lower size of metal ions than the pores present in protein/peptide interfacial layers (Berton-Carabin, et al., 2014). Thus, this finding could be only explained by the superior radical scavenging activity of β 22 and γ 40 (García-Moreno, Gregersen, et al., 2020).

On the other hand, PCA clearly shows that emulsions stabilized with α 10, β 27 and γ 38 (placed in the very left part of the biplot) were the least oxidized during storage and the ones exhibiting the highest zeta potential (i.e., positive zeta potential) (Fig. 7). Although peptides β 27 and γ 38 did not show metal chelating *in vitro*, they exhibit high radical scavenging (García-Moreno, Gregersen, et al., 2020), which indicates that these peptides could retard lipid oxidation by trapping peroxy radicals at the interface where oxidation is initiated in emulsions (McClements & Decker, 2018). Nevertheless, α 10 gives a weak and stretchable interface with poor inter-peptide interactions, that did not exhibit antioxidant activity *in vitro* (García-Moreno, Gregersen, et al., 2020) and also provided high oxidative stability to the emulsion (Fig. 6). Therefore, these findings clearly support that although strength and packing of the interface as well as antioxidant activity of the peptides might be important factors, charge of the interfacial film is the major factor affecting lipid oxidation in physically stable fish oil-in-water emulsions stabilized with potato peptides where oxidation is promoted by metal ions. Positively charged peptides adsorbed at the interface repelled metal ions, minimizing the decomposition of hydroperoxides and enhancing the oxidative stability of the system (Hu, McClements, & Decker, 2003). Thus, the lower positive charge exhibited by β 27 when compared to α 10 and γ 38 (11.6 vs. 30.1 and 42.8 mV) also explained the higher content of 1-penten-3-ol (Fig. 6b) and (*E*)-2-hexenal and hexanal (Fig. 6S) for the emulsion stabilized with β 27 when compared to the emulsions stabilized with α 10 and γ 38. Finally, it is worth mentioning that emulsions with positive zeta potential were obtained at pH ~7, implying that

in all emulsions the solubility of metal ions was the same. Additionally, the potato peptides assayed did not show any in vitro chelating activity (García-Moreno, Gregersen, et al., 2020), meaning that negatively charged potato peptides in the aqueous phase could not potentially chelate metal ions. These circumstances allow the clear elucidation of the effect of peptide charge on oxidative stability of fish oil-in-water emulsions. .

4. CONCLUSIONS

The potato protein-derived peptides assayed provided different physicochemical stabilities to fish O/W emulsions when used as emulsifiers. The differences observed were related to the secondary structure as well as interfacial properties (i.e., viscoelasticity and charge) of the peptides. Emulsions with high physical stability can be produced when using amphiphilic peptides adopting either α -helix or β -strand structures at the interface. Peptides with a predominant α -helix conformation at the interface ($\gamma 1$, $\gamma 75$ and $\gamma 76$) resulted in less stiff and more stretchable interfaces, although they present regions offering steric hindrance and electrostatic repulsions. On the other hand, the physical stability provided by peptides ($\gamma 36$ and $\gamma 38$) re-arranging predominantly as β -strand structures at the interface is explained by stronger inter-peptide interactions that lead to the formation of stiffer interfaces (with a significant degree of softening upon deformation). Interestingly, our results clearly showed that, independently of the interfacial structure and viscoelasticity of the interface, the oxidative stability of fish O/W emulsions, where oxidation is promoted by cationic metal ions, was mainly determined by the surface charge of the peptides. Potato peptides exhibiting high emulsifying activity with $pI > 7$ ($\alpha 10$, $\beta 27$ and $\gamma 38$), but preferentially with $pI > 10$, are desired to confer high oxidative stability to emulsions produced at pH 7 (i.e., positive net charge). Hence, these findings allow the design of O/W emulsions stabilized with peptides with enhanced physicochemical stability and show the potential of potato peptides as

alternative plant-based emulsifiers. Consequently, these results may attract the interest of potato starch industry for the development and optimization of industrial enzymatic and purification processes leading to the production of the desired emulsifier potato peptides.

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AUTHOR CONTRIBUTIONS

Pedro J. García-Moreno: Conceptualization, Methodology, Formal analysis, Writing - original draft. Jack Yang: Methodology, Formal analysis, Writing - review and editing. Simon Gregersen: Methodology, Writing - review and editing. Nykola C. Jones: Methodology, Formal analysis, Writing - review and editing. Claire C. Berton-Carabin: Methodology, Writing - review and editing. Leonard M.C. Sagis: Methodology, Writing - review and editing. Søren V. Hoffmann: Methodology, Writing - review and editing. Paolo Marcatili: Writing - review and editing. Egon B. Hansen: Writing - review and editing, Funding acquisition, Project administration. Charlotte Jacobsen: Methodology, Writing - review and editing, Funding acquisition, Project administration.

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Peptide	Sequence	Protein source	Score*	Number of residues	Mw, g/mol	Purity, %	Net charge (pH 7)**	pI**
α10	KKPVSKDSPETYEEALKRFAKLLSDRKKL	Patatin	2.176	29	3405.9	75.0	4.0	10.4
α12	DSPETYEEALKRFAKLLSD	Patatin	3.037	19	2212.4	80.3	-2.0	4.1
β22	CPFSSDDQFCLKVGV	Kunitz	2.501	15	1644.8	71.3	-1.1	3.7
β27	LNIQFNIPTPKLC	Kunitz	2.213	13	1500.8	77.9	0.9	8.8
γ1	GIKGIIPAIILEFLEGQLQEVDNNKDAR	Patatin	4.146	28	3094.6	83.3	-2.0	4.2
γ75	GIIPAIILEFLEGQLQEVDNNKDAR	Patatin	4.254	25	2796.7	77.1	-3.0	3.8
γ76	GIIPAIILEFLEGQLQEVDNNK	Patatin	3.842	22	2453.8	78.1	-3.0	3.5
γ36	FDVIGGTSTGGLLTAMITTPNENNR	Patatin	3.190	26	2676.9	72.5	-1.0	3.9
γ38	FCLKVGVVHQNGKRRRLALVKDNP	Kunitz	3.168	23	2592.1	78.4	4.0	10.9
γ40	SSDDQFCLKVGVV	Kunitz	3.137	13	1396.6	78.0	-1.1	3.7

* Amphiphilic score obtained by the bioinformatics tool EmulsiPred (<https://github.com/MarcatiliLab/EmulsiPred>) as described in our previous study (García-Moreno, Gregersen, et al., 2020).

** pI and net charge were calculated by using peptide property calculator from INNOVAGEN (Innovagen AB, Lund, Sweden).

Emulsion	Zeta potential (mV)	Droplet size $D_{3,2}$ (μm)		YI (day 7)
		Day 1	Day 7	
Cas	-41.2 $\pm$ 1.7	0.177 $\pm$ 0.008	0.233 $\pm$ 0.006	8.2 $\pm$ 0.8
α 10	30.1 $\pm$ 4.1	0.160 $\pm$ 0.005	0.225 $\pm$ 0.011	13.0 $\pm$ 1.0
α 12	-51.4 $\pm$ 1.8	0.143 $\pm$ 0.001	0.150 $\pm$ 0.001	19.1 $\pm$ 2.6
β 22	-50.9 $\pm$ 1.4	0.190 $\pm$ 0.012	0.457 $\pm$ 0.173	20.0 $\pm$ 0.5
β 27 [†]	11.6 $\pm$ 0.9	0.228 $\pm$ 0.011	0.208 $\pm$ 0.010	-
γ 1	-58.5 $\pm$ 2.1	0.156 $\pm$ 0.016	0.171 $\pm$ 0.005	14.0 $\pm$ 1.3
γ 75	-60.1 $\pm$ 1.5	0.148 $\pm$ 0.001	0.151 $\pm$ 0.003	20.1 $\pm$ 1.7
γ 76	-69.5 $\pm$ 0.3	0.146 $\pm$ 0.001	0.147 $\pm$ 0.003	19.2 $\pm$ 2.7
γ 36	-52.5 $\pm$ 2.0	0.175 $\pm$ 0.001	0.235 $\pm$ 0.019	14.4 $\pm$ 1.3
γ 38	42.8 $\pm$ 1.7	0.147 $\pm$ 0.001	0.192 $\pm$ 0.005	13.8 $\pm$ 0.8
γ 40	-52.3 $\pm$ 1.9	0.134 $\pm$ 0.002	0.138 $\pm$ 0.001	15.4 $\pm$ 0.7

[†] For this emulsion, droplet size was not measured at day 7 but at day 3.

Fig. 1. Far-UV SRCD spectra of potato peptides in solution at pH ~7 (blue line) and adsorbed at the tricaprilyn oil-water interface (red line): a) caseinate, b) α 10, c) α 12, d) β 22, e) β 27, f) γ 1, g) γ 75, h) γ 76, i) γ 36, j) γ 38, k) γ 40. Light scattering of oil droplets in the caseinate- or peptides-stabilised emulsions was corrected using Brij 97-stabilised emulsions.

Fig. 2. The secondary structure composition (% α -helix, β -strand, turns and unordered) of peptides in a) solution, and b) adsorbed at the tricaprylin oil-water interface. The results derived from deconvolution of the respective SRCD spectra. The numbers 1 and 2 in α -helix and β -strand denote regular and distorted structures, respectively.

Fig. 3. Interfacial tension as a function of time for potato peptide solutions of 0.1 g/L (pH 7) at the tricaprylin oil-water interface: a) all peptides in a plot with normal scale in the X-axis, b) α -peptides represented as semilogarithmic plot, b) β -peptides represented as semilogarithmic plot, and c) γ -peptides represented as semilogarithmic plot. Results for caseinate (cas) solution (0.1 g/L, pH 7) were also plotted for comparison. The experiments were performed at 20 °C. The bare tricaprylin oil-water interfacial tension was 24.0 ± 0.8 mN/m.

Fig. 4. a) Complex surface dilatational modulus for tricapyrylin oil-water interfaces stabilized by potato peptides (0.1 g/L, pH 7, 20°C) as a function of: a) frequency (0.005 to 0.1 Hz, amplitude of deformation: 5%), and b) amplitude (5-30%, frequency 0.02 Hz)

*Frequency sweeps were performed from 0.005 to 0.1 Hz, but the software could not fit the data below 0.02 Hz. Thus, only data from 0.02 to 0.1 Hz are shown.

Fig. 5. Lissajous plots obtained during amplitude sweeps (5-30%) of tricaprylin oil-water interfaces stabilized by caseinate, $\beta 22$, $\beta 27$, $\gamma 36$, $\gamma 38$ and $\gamma 40$ potato peptides (0.1 g/L, pH 7, 20 °C).

a)

b)

c)

Fig. 6. Lipid oxidation of 5% fish O/W emulsions (pH 7) stabilized with potato peptides (0.02 wt%) during storage at 20 °C: a) Peroxide value (PV), b) content of 1-penten-3-ol, and c) content of (E,E)-2,4-heptadienal.

Fig. 7. Principle component analysis (PCA) biplot including data on peptide properties, interfacial properties, physical stability and oxidative stability. Letters a, b and g in the peptide names in the plot stand for α , β and γ , respectively. The words “score”, “Zeta”, “IntTens” in the plot refer to amphiphilic score, zeta potential and interfacial tension, respectively.

Highlights

- Interfacial properties of potato peptides determine the stability of emulsions
- Potato peptides with α -helix structure at the interface led to elastic interfaces
- Peptides with β -strand structure at the interface led to more viscous interfaces
- Peptides with negative surface charge led to emulsions having high oxidation
- Peptides with positive surface charge led to emulsions having low oxidation

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: