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Emulsifying peptides from potato protein predicted by bioinformatics: stabilization of fish oil-in-water emulsions

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ABSTRACT

This work investigated the use of bioinformatics to predict emulsifying peptides embedded in patatin proteins from potato (*Solanum tuberosum*). Six peptides (23-29 amino acids) with potentially different predominant structure at the oil/water interface (e.g. α -helix, β -strand or unordered) were identified within patatin sequences. The interfacial tension between peptides solutions and fish oil as well as the physical and oxidative stability of 5 wt% fish oil-in-water emulsions (pH 7) stabilized with synthetic predicted peptides were evaluated. The peptides predicted to have lower amphiphilic score ($\alpha 1$ and $\alpha 2$) led to emulsions with creaming after production and with low oxidative stability. On the other hand, a half hydrophobic and half hydrophilic peptide ($\gamma 1$), which was predicted to have the highest amphiphilic score, showed a superior ability to reduce interfacial tension (even when compared to casein). $\gamma 1$ -stabilized emulsion was physically stable during storage (48 h at 50 °C) and presented the lowest droplet size ($D_{4,3}=0.518\pm 0.011$ μm). Electron spin resonance (ESR) and Oxygraph results indicated that the type of synthetic peptide used also affected the oxidative stability of fish oil-in-water emulsions differently. Therefore, this study shows the potential of using

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bioinformatics to predict emulsifying peptides, reducing time and cost of extensive screening hydrolysis processes.

Keywords: omega-3, physical stability, oxidative stability, interfacial tension, electron spin resonance, Oxygraph

1. INTRODUCTION

Potato (*Solanum tuberosum*) is the world's fourth most important food crop, after maize, wheat and rice (FAO, 2008). Potatoes, apart from being used directly as a primary food source, are widely employed in Europe for extraction of starch since 10-18% of the total weight is starch (Grommers & van der Krogt, 2009). In the last decade, extraction of protein from side-streams in the potato starch industry has gained increasing interest due to their nutritional and functional properties (e.g. emulsifying, foaming and antioxidant) (Alting, Pouvreau, Giuseppin, & van Nieuwenhuijzen, 2011).

Potato protein (which represent 1-2% of the total potato weight depending on cultivar) is isolated from raw potato fruit juice, which is obtained after rasping and extraction of starch and fiber from the potatoes (Alting, Pouvreau, Giuseppin, & van Nieuwenhuijzen, 2011). Proteins contained in the potato juice are typically divided into three groups: i) patatins (up to 40%) with a molecular weight of 39-43 kDa, ii) protease inhibitors (up to 50%) with a molecular weight of 4.3-20.6 kDa, and iii) other proteins such as enzymes involved in starch synthesis, lipoxygenase and polyphenol oxidase (Schmidt et al., 2018; Alting et al., 2011; Pouvreau et al., 2001). Many of the highly abundant potato proteins have been shown to be located in vacuoles serving as storage proteins (Jørgensen, Stensballe, Welinder, 2011), but the specific protein composition and their abundance in potato tubers depend greatly on the cultivar (Jørgensen, Welinder, Bauw, 2006). Previous studies have reported emulsifying, foaming and antioxidant properties of both crude potato protein isolates as well as patatin and

protease inhibitors fractions (Schmidt et al., 2018; Van Koningsveld et al., 2006; Romero, et al., 2011; Sun, Jiang, & Wei, 2013; Liu, Han, Lee, Hsu, & Hou, 2003).

Gentle methods, such as ethanol precipitation (Van Koningsveld et al., 2006) or initial anion exchange (IEX) followed by hydrophobic interaction chromatography (HIC) (Schmidt et al., 2018), have been developed to isolate proteins from potato juice with reduced protein denaturation compared the classical heat-acid precipitation method. Nevertheless, considerably lower yields are obtained when compared to the traditional method, which yields a 99% recovery of (denatured) protein (Schmidt, 2016). In order to increase its solubility as well as emulsifying and antioxidant properties, enzymatic hydrolysis of denatured potato protein, which increases the exposure of reactive amino acid side chains and/or hydrophobic patches, has been applied (Cheng, Xiong, & Chen, 2010; Wang & Xiong, 2005). The latter is required to shift the application of potato protein from feed to food, leading to high-added value ingredients (e.g. emulsifier/antioxidants).

Previous studies have followed the classical approach of digesting potato protein with different proteases (e.g. subtilisin, pepsin, pancreatin) to various degrees of hydrolysis, testing the emulsifying/antioxidant properties of the hydrolysates, and lastly attempting to identify the bioactive peptides (the latter only for antioxidant peptides) (Udenigwe, Udechukwu, Yiridoe, Gibson & Gong, 2016; Cheng, Xiong, & Chen, 2010; Cheng, Chen, & Xiong, 2010). Alternatively, bioinformatics tools allow the prediction and identification of embedded functional/bioactive peptides, which can be released at specific hydrolysis conditions, reducing time and costs for extensive screening processes (Agyei, Tsopmo, & Udenigwe, 2018). Nevertheless, bioinformatic prediction of especially active food peptides remains largely unexplored until now.

In the light of the above, this work aimed at, for the first time ever, investigating the emulsifying activity of potato peptides predicted by bioinformatics. We limited the initial

search to patatin sequences, as this group of highly homologous proteins is the most abundant in potato juice (Bauw et al., 2006). Six peptide sequences, with potentially different conformation at the oil/water interface (i.e. α -helix, β -strand or unordered), were identified by predicting their emulsifying activity (i.e. hydrophobic vector). Then, the emulsifying properties of synthetic peptides with the predicted sequences were determined in small scale assays. First, the interfacial properties of the peptides was assayed by drop tensiometry. Second, the physical and oxidative stabilities of fish oil-in-water emulsions were evaluated during storage. Last, the *in vitro* antioxidant activity of the peptides was tested.

2. MATERIALS AND METHODS

2.1 Materials

Synthetic peptides (purity > 70% by HPLC) were purchased from Chinapeptide Co., Ltd. (Shanghai, China). Commercial cod liver oil was kindly provided by Maritex A/S (Sortland, Norway), a subsidiary of TINE SA (Oslo, Norway) and stored at -40 °C until use. The fatty acid composition (major fatty acids only) of the fish oil used was C16:0, 9.5%; C16:1, 8.7%; C18:1, 16.3%; C20:1, 12.6%; C20:5, 9.2% and C22:6, 11.4%. The tocopherol content of the fish oil was: α -tocopherol, 200 ± 3 $\mu\text{g/g}$ oil; β -tocopherol, 5 ± 1 $\mu\text{g/g}$ oil; γ -tocopherol, 96 ± 3 $\mu\text{g/g}$ oil and δ -tocopherol, 47 ± 1 $\mu\text{g/g}$ oil. The peroxide value (PV) of the fish oil used was 0.38 ± 0.04 meq/kg oil. The properties of the fish oil used were determined as described elsewhere (García-Moreno, Guadix, Guadix, Jacobsen, 2016). Sodium caseinate (Miprodan 30), which was used as positive control exhibiting excellent emulsifying activity, was provided by Arla Foods Ingredients amba (Viby J, Denmark). Protein content of sodium caseinate was reported as 92% ($N \times 6.38$). Native (non-denatured) potato protein (85% protein, $N \times 6.25$), isolated by a cold-extraction method from the potato juice was provided by KMC (Brande, Denmark). The native potato protein contained 21% patatin (all isoforms) and

67% protease inhibitors (relative molar abundance) determined by LC-MS/MS (unpublished data). By colorimetric SDS-PAGE analysis (relative weight%), the distribution was determined as 35% patatin (approximately 40 kDa) and 58% protease inhibitors (10-25 kDa) (unpublished data). The discrepancy between relative molar abundance and relative weight% can be ascribed to differences in molecular weight and the non-specific nature of SDS-PAGE analysis. Potato protein was used as control to compare emulsifying and antioxidant activity of peptides and raw protein. All other chemicals and solvents used were of analytical grade.

2.2 Bioinformatics

Although protease inhibitors constitute a larger relative proportion of proteins in potato tubers, they are comprised of several sub-classes with high sequence variety. Patatins, however, are highly conserved on the sequence level with 84-96% sequence identity within the Kuras cultivar (Bauw et al., 2006). Consequently, we decided to limit our initial investigation to patatin sequences. From the UniProt Knowledgebase (The UniProt Consortium, 2017), we selected the first nine reviewed isoforms of patatin (out of 34) for analysis. The selected patatin isoforms are identified by the following accession numbers: P15478, P15477, Q3YJT5, P11768, Q2MY60, Q2MY43, Q42502, Q3YJS9, and Q3YJT3. We predicted the emulsifying potential of patatin peptides by calculating the hydrophobic vector of peptides up to a length of 30 amino acids, as function of its secondary structure (i.e. α -helix, β -strand or unordered). The Kyte-Doolittle hydropathic scale was used as a common scale for the hydrophobicity of each amino acid (Kyte & Doolittle, 1982).

2.2.1 α -helix peptides

Alpha-helices have a periodicity of 3.6 amino-acid residues per turn, meaning that each residue corresponds to a 100° or $5/9 \pi$ radians turn. Thus, hydrophobic residues placed alternately three or four residues apart will form a hydrophobic face in a helical peptide.

Hydrophilic residues on the opposite face of the helix would result in the helix having a hydrophilic face as well. A helical hydrophobic vector (A) can be calculated as follows (Eq. 1) (Eisenberg, Weiss, & Terwilliger, 1982):

$$A = \left| \sum_{n=1}^w K(aa_n) \cdot \begin{bmatrix} \cos\left(n \cdot \frac{5}{9}\pi\right) \\ \sin\left(n \cdot \frac{5}{9}\pi\right) \end{bmatrix} \right| \quad (\text{Eq. 1})$$

where $K(aa_n)$ represents the Kyte-Doolittle hydrophobic value, w is the peptide (sequence) length, and n represents the number of a given amino acid in the peptide sequence.

A longer hydrophobic vector, perpendicular to the helical axis, directly correlates with higher amphiphilicity of a peptide in helical conformation (Eisenberg et al., 1982). As increased amphiphilicity in α -helical peptides have been shown to increase emulsifying activity (Saito et al., 1995 and Poon et al., 2001), the α -score from our predictive model (i.e. vector length) directly implies a potentially higher emulsifying activity of the peptide in a helical conformation. According to (Eisenberg et al., 1982), this principle also applies to non-helical peptide structures and can be generalized to amphiphilic peptides in any conformation.

2.2.2 β -strand peptides

Peptides with a β -strand secondary structure have side chains of the amino acids pointing alternatively above and below the plane of the β -strand (e.g. every 180° or π radians). This means that for a β -strand peptide to exhibit amphiphilicity, every second amino acid should be hydrophobic and every other should be hydrophilic (Dexter & Middelberg, 2008). Thus, the hydrophobic vector for β -strand peptides (B) can be calculated as follows (Eq. 2):

$$B = \left| \sum_{n=1}^w K(aa_n) \cdot \begin{bmatrix} \cos(n \cdot \pi) \\ \sin(n \cdot \pi) \end{bmatrix} \right| \quad (\text{Eq. 2})$$

where $K(aa_n)$, w and n are defined as for Eq. 1.

As described for the helical prediction model, a longer hydrophobic vector perpendicular to the β -strand plane (i.e. higher β -score), is directly correlated with higher amphiphilicity and thus a potentially higher emulsifying activity of the peptide in a β -strand conformation.

2.2.3 Half hydrophobic-half hydrophilic peptides (γ -peptides)

A third possibility for a peptide to display amphiphilic properties with emulsifying potential is to have a hydrophobic and a hydrophilic half, allowing the peptide to orient perpendicularly to the interface (Dexter & Middelberg, 2008). These type of peptides could adopt α -helix and β -strand conformations or be unordered. The hydrophobic vector for γ -peptides (G) was calculated as follows (Eq. 3):

$$G = \left| \sum_{n=1}^{\frac{1}{2}w} K(aa_n) - \sum_{n=\frac{1}{2}w+1}^w K(aa_n) \right| \quad (3)$$

where $K(aa_n)$, w and n are defined as for Eq. 1.

As for both α -helical and β -strand peptides, the longer the hydrophobic vector, the higher the amphiphilicity of the peptide implying potentially higher emulsifying activity. However, in contrast to both α -helical and β -strand peptides, the hydrophobic vector for each amino acid is projected in the axial direction of the peptide, thereby making the hydrophobic vector axial (head-to-tail) and not perpendicular to the peptide backbone.

2.3 Interfacial tension – pendant drop method

The dynamic interfacial tension of the peptides at the oil-water interface was determined using an automated drop tensiometer OCA20 (DataPhysics Instruments GmbH, Filderstadt, Germany) at 25°C. Peptides solutions (0.1 wt.%) in 10 mM sodium acetate - 10 mM imidazole buffer (pH 7) were prepared. The peptide solution (water phase) was filled into a syringe with a screwed needle. For each measurement, a small drop of the peptide solution was generated using the automated syringe into a quartz glass cuvette filled with fish oil (oil phase). The image of the drop was recorded with a camera every 10 s for 30 min. The images

were transferred to the drop shape analysis software. Interfacial tension was calculated based on the shape analysis of a pendant drop according to the Young-Laplace equation (Eq. 4):

$$\Delta P = \gamma \cdot \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \quad (4)$$

where ΔP (mN/m²) is the pressure difference across the interface, γ (mN/m) is the interfacial tension and R_1 and R_2 (m) are the principal radii of curvature of the pendant drop. Measurements were carried out in duplicate.

2.4 Production of emulsions

Peptides (0.2 wt.%) were dissolved in 10 mM sodium acetate – 10 mM imidazole buffer (pH 7). The peptide solutions were shaken (100 rpm) overnight at room temperature to allow complete solubilization and rehydration of the peptides (when possible). Primary homogenization was done by adding the fish oil 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 Microson XL2000 sonicator equipped with a P1 probe (Misonix, Inc., New York, USA). Emulsions were homogenized at an amplitude of 75% (maximum amplitude of 180 μ m), 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. A total amount of 2 g of 5 wt.% fish oil-in-water emulsion stabilized with 0.2 wt.% potato peptides was produced.

2.5 Physical stability of the emulsions

2.5.1 Droplet size and creaming

The droplet size distribution of the emulsions was measured immediately after production and after two days of storage at 50 °C by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK). Each emulsion was diluted in recirculating water (3000 rpm), until it reached an obscuration of 12%. The refractive indices of sunflower oil

(1.469) and water (1.330) were used as particle and dispersant, respectively. Results are given in volume mean diameter ($D_{4,3}$). Measurements were made in triplicate. Due to the low volume of emulsions produced, creaming was determined qualitatively by observing the appearance of the emulsions.

2.5.2 Zeta potential

The zeta potential of the emulsions was measured after one day of storage at room temperature in a Zetasizer Nano ZS (Malvern instruments Ltd., Worcestershire, UK) with a DTS1070 cell at 25 °C. Before analysis, the emulsions were diluted (10 μ L emulsion in 5 mL buffer). The zeta potential range was set to -100 to +50 mV and the samples were analyzed with 100 runs. Measurements were done in triplicate.

2.6 Antioxidant activity of peptides and oxidative stability of the emulsions

2.6.1 *In vitro* antioxidant activity of peptides

Peptides were dissolved in 10 mM sodium acetate - 10 mM imidazole buffer (pH 7) at 0.2 wt.%. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, iron (Fe^{2+}) chelating activity and Fe^{3+} reducing power were measured as described elsewhere (García-Moreno et al., 2016). The methods for antioxidant activity assays were modified slightly from the original to adapt for microplate readings using Eon™ microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

2.6.2 Electron spin resonance (ESR)

The oxidative stability of the emulsions was first evaluated by ESR. ESR analysis was carried out using a benchtop MiniScope MS 5000 ESR spectrometer (MAGNETTECH GMBH, Germany). The instrument settings were as follows: magnetic field 320-360 mT, sweep time 60 s, modulation 0.2 mT and frequency 100 kHz. Emulsions were produced as described in section 2.4 and N-tert-Butyl- α -phenylnitron (PBN) (Sigma Aldrich, Søborg, Denmark) was

added to the emulsions as an ethanol solution (concentration of 50 mg/mL) to have 30 mM of PBN in the oil phase of the emulsion. The oxidative stability of the emulsions was determined during 48 h storage at 50 °C and samples were measured at 0, 6, 12, 24, 36 and 48 h. For each sampling point, emulsion samples (50 µL) were transferred to borosilicate capillary tubes before they were introduced to the cavity of the ESR. Analyses were performed in triplicate at room temperature.

2.6.3 Oxygraph system

An Oxygraph system (Hansatech Instrument Ltd., Norfolk, UK) was used to evaluate the oxidative stability of the emulsions by continuously measuring the concentration of dissolved oxygen in the emulsions. The Clark type polarographic oxygen sensor was calibrated daily with air saturated and air depleted water at 30°C and adjusted for atmospheric air pressure. Emulsions (1 mL) were placed in a stirred, water-jacketed cell at 30 °C and a background oxygen uptake rate (R_B) was recorded for 5–10 min. Then, Fe^{2+} was added as a prooxidant through a capillary opening in the cell. 5 µL of a daily prepared 2.5 mg/mL Fe^{2+} solution in distilled water was injected to a final Fe^{2+} concentration of 45 µM. After the injection of Fe^{2+} , a drop in oxygen concentration (ΔO_2) was recorded followed by a constant oxygen uptake rate (R_T). A net oxygen uptake rate (R_N) was calculated as R_T minus R_B . Both R_N and ΔO_2 were employed to evaluate the oxidative stability of the emulsions. Measurements were carried out in duplicate.

2.7 Statistical analysis

Statgraphics 18 (Statistical Graphics Corp., Rockville, MD, USA) was used for data analysis. Data were expressed as mean $\pm$ standard deviation. Firstly, multiple sample comparison analysis was performed to identify significant differences between samples. Secondly, mean

values were compared by using the Tukey's test. Differences between means were considered significant at $p < 0.05$.

2.8 Protein modelling and peptide visualization

In order to determine localization of the predicted peptides within their mature form protein of origin, we modelled the protein structure of Patatin-B2 (UniProt AC# P15477) for $\alpha 1$, $\alpha 2$, and $\alpha 3$ (QMEAN -0,75); Patatin-05 (UniProt AC# Q3YJT5) for $\beta 1$ (QMEAN -0,95); Patatin Group M-1 (UniProt AC# P11768) for $\gamma 1$ (QMEAN -0,73); and Probable inactive patatin-3-Kuras 1 (UniProt AC# Q3YJS9) for $\gamma 2$ (QMEAN -1,34). All models were built with The SwissModel Workspace (Waterhouse et al. 2018) using the X-ray crystal structure of monomeric Patatin-17 (Wijeyesakere, Richardson, & Stuckey, 2016) as structural template (SMTL ID 4pka.1.A). Subsequently, models were visualized in The PyMOL Molecular Graphics System, Version 1.5.0 (Schrödinger, LLC.). For visualization of individual peptides with visible side chains, residues were colored according to the SwissModel hydrophobicity scale (Very hydrophilic residues in blue (R, K, D, E, N, Q, H), partially hydrophilic in purple (P, Y, W, S, T, G), partially hydrophobic in pink (A, M, C, F), and very hydrophobic in red (L, V, I)).

Helical wheel projections were modelled using the HELIQUEST web server (Gautier, Douguet, Antonny, & Drin, 2008). For each model, the mean hydrophobicity $\langle H \rangle$ is calculated using residue-specific hydrophobicity scores (Fauchere, & Pliska, 1983). Furthermore, the mean amphipathic moment $\langle \mu H \rangle$ perpendicular to the backbone axis is calculated (Eisenberg et al., 1982). Both calculated physico-chemical properties are normalized to the length of the peptide.

3. RESULTS AND DISCUSSION

3.1 Prediction of emulsifying potato peptides by bioinformatics

Patatins have been reported to be the most abundant of highly conserved protein families in potatoes and to exhibit superior emulsifying activity when compared to protease inhibitors. These findings were mainly attributed to differences in hydrophobicity between these protein fractions (Schmidt et al., 2018; Bauw et al. 2006). Therefore, nine patatin sequences from *Solanum tuberosum* were extracted from the UniProt knowledgebase (Bateman et al., 2017) and used for prediction of embedded peptides with emulsifying activity.

Emulsifying peptides, which could adopt different secondary structure, present hydrophobic and hydrophilic parts in their molecule. Peptides with α -helix and β -strand secondary structures exhibit facial amphiphilicity (e.g. having hydrophilic and hydrophobic faces in their molecule), which allows them to orientate parallel to the interface. On the other hand, γ -peptides with hydrophobic and hydrophilic halves could orientate perpendicularly to the interface (Dexter & Middelberg, 2008). Interestingly, patatins are highly structured proteins at room temperature with 33% of the residues adopting α -helical and 46% β -stranded structures (Pots, De Jongh, Gruppen, Hamer, & Voragen, 1998). Thus, emulsifying peptides with different secondary structures, and thus potentially different conformation at the oil/water interface, may be embedded within patatin sequences.

Three regions in the patatin sequences showed predicted emulsifying potential for embedded peptides in α -helical conformation, one region showed predicted emulsifying potential for an embedded peptide in β -strand conformation, and two regions had the potential to give half hydrophobic and hydrophilic emulsifying peptides. From each region, the peptide with the highest predicted amphiphilicity was selected for synthesis (Table 1). Three peptides with

potential α -helix ($\alpha 1$, $\alpha 2$ and $\alpha 3$) conformation, one with potential β -strand ($\beta 1$) structure, and two potentially half hydrophobic and half hydrophilic peptides ($\gamma 1$ and $\gamma 2$) were investigated. To investigate the native conformation of the predicted emulsifier peptides, we modelled the structure of the patatin isoform from which the peptides originate (Fig. 1, top). Using a hydrophobic coloring scheme, we also visualized the distribution of amino acids in the peptides (Fig. 1, bottom). This facilitated identification of potential inherent amphiphilicity of the peptides in their native conformation as well as the potential of adopting the predicted emulsifying conformation. Interestingly, all peptides (with the exception of $\alpha 2$, which appear to have a quite amphiphilic antiparallel β -strand conformation), are located in regions of the proteins with predominant α -helical and/or unordered conformation. Especially $\gamma 1$ has a high degree of helical content, as it, in the native conformation, forms a partially buried α -helix, where the N-terminal end (predominantly hydrophobic) is located near the core of the protein, while the C-terminal end (predominantly hydrophilic) is located in a solvent exposed loop region. Furthermore, the central part of $\gamma 1$ appears to be quite amphiphilic by presenting a hydrophobic (red) phase. Although the helical segment of $\alpha 3$ and $\gamma 2$ are significantly shorter, they also appear to be amphiphilic with a well-defined hydrophobic phase. The content of each peptide located in either α -helical or β -strand regions in the native conformation, are summarized in Table 1.

As most of the predicted peptides are found in (partially) helical regions within the native proteins, we decided to visualize the amphiphilic potential using helical wheel projections. *In silico*, we forced the full-length peptide into a helical conformation (Fig. 1S, top). This merely depicts the peptides in an α -helix by projecting helical constraints onto the peptide sequence and does not necessarily correlate with the *in vitro* conformation at the interface. Based on the full-length projections, we selected the segment of each peptide that produced the largest uninterrupted hydrophobic phase and/or largest mean hydrophobic moment (Fig. 1S, bottom).

The calculated mean hydrophobicity $\langle H \rangle$ and mean hydrophobic moment $\langle \mu_H \rangle$ of both full-length and segmented projections are summarized in Table S1. For the full length peptides, $\langle \mu_H \rangle$ is higher for the α -peptides, which is expected based on the parameters used in the prediction model, where similar assumptions are made. Alpha-3 produces the, by far, most amphiphilic helix (highest $\langle \mu_H \rangle$ of all full-length peptides). When considering only the selected segments of each peptide, $\langle \mu_H \rangle$ of $\alpha 3$ increased even further (38% increase relative to full-length $\alpha 3$). Interestingly, $\langle \mu_H \rangle$ for the selected segments of both γ -peptides significantly increased (199% for $\gamma 1$ and 326% for $\gamma 2$) to a level comparable to $\langle \mu_H \rangle$ of the $\alpha 3$ segment.

It is noteworthy that, since the algorithm employed did not normalize the hydrophobic vector according to the peptide length, the predicted emulsifying peptides were between 27 and 29 amino acids long (apart from $\alpha 3$). This was close to the maximum length allowed by the algorithm. The maximum length of 30 amino acid residues was selected because peptides shorter than this diffuse faster and thereby facilitate initial adsorption at the oil/water interface. Moreover, these peptides have increased rate of conformation change at the oil/water interface since they are too small to possess tertiary structure (Enser, Bloomberg, Brock, & Clark, 1990). In addition, our prediction is aligned with the study of Enser et al. (1990) indicating that the emulsifying activity increased considerably for α -peptides between 22 and 29 residues when compared to peptides having 8-18 residues. The authors attributed this fact to an increase in α -helicity of the peptides in aqueous solution when going from 11 to 22 amino acid residues, which correlated well with the higher potential of larger peptides to adopt a larger proportion of well-defined secondary structure due to the flexible nature of peptide termini.

The highest score (e.g. highest predicted amphiphilicity) was found for γ -peptides ($\gamma 1$ and $\gamma 2$) followed by $\beta 1$, $\alpha 2$, $\alpha 1$, and $\alpha 3$ (Table 1). These results indicated that the γ -peptides selected

had more pure hydrophobic and hydrophilic regions when compared to the hydrophilic/hydrophobic faces of α - and β -peptides. The lowest score of $\alpha 3$ obtained by the algorithm, which did not correlate with its mean hydrophobic moment (Table S1), is explained by the lack of normalization for peptide length in the algorithm, as $\alpha 3$ is the shortest of the predicted peptides. Alpha-3 was, however, also selected based on its high content of lysine, which resulted in high pI (e.g. positive net charge at pH 7). On the contrary, the rest of the peptides had a pI lower than 7, which resulted in a negative net charge at pH 7 (Table 1). In emulsions, the charge of the o/w interface may greatly affect oxidative stability, especially when prooxidants such as metal ions are present (Berton-Carabin, Ropers, & Genot, 2014). It is also worth mentioning that the peptides tested do not have any free cysteine, which could be a source of inter-peptide covalent bond formation. That means that any potential self-assembly of peptides in the aqueous phase would be driven by weaker forces (i.e. ionic interactions between charges and hydrophobic interactions) and consequently easily disrupted during homogenization.

3.2 Interfacial tension

Emulsifying peptides have the ability to rapidly adsorb and re-orientate at the interface in order to reduce interfacial tension (García-Moreno et al., 2016). Thus, the evolution of the interfacial tension between fish oil and peptide solutions with time was evaluated (Fig. 2). It was observed that the interfacial tension between fish oil and distilled water was practically constant over time, indicating a minimum influence of the naturally present surface active impurities in the fish oil (e.g. mono- and di-glycerides). However, interfacial tension between fish oil and all peptide solutions showed an initial decrease when compared to oil-water; leveling off at short time (< 5 min) for $\alpha 3$, casein and $\gamma 1$ and after 15 min for $\alpha 1$, $\alpha 2$, $\beta 1$ and potato protein (Fig. 2).

Higher initial and equilibrium values were found for $\alpha 1$, $\alpha 2$, $\beta 1$ and $\gamma 2$ when compared to $\alpha 3$, casein and $\gamma 1$ (Fig. 2). Alpha-1 and $\alpha 2$ peptide solutions were cloudy, indicating that the peptides were not totally soluble in the buffer used and this may explain their higher interfacial tension results (See Fig. 2S in the Supplementary Material). Moreover, $\alpha 3$, that was totally soluble, presented a short length resulting in faster diffusion to the interface, which could explain its lower initial interfacial tension when compared to $\alpha 1$ and $\alpha 2$. Previous studies also pointed out an inverse relation between protein size and adsorption rate, especially in drop tensiometry where adsorption is mainly controlled by diffusion (Jung, Gunes and Mezzenga, 2010; Schröder, Berton-Carabin, Venema, & Cornacchia, 2017).

On the other hand, $\beta 1$ and $\gamma 2$, having similar solubility and size as $\gamma 1$, presented higher initial and equilibrium interfacial tension values when compared to $\gamma 1$ (Fig 1). This may be attributed to the better defined hydrophobic and hydrophilic regions in $\gamma 1$, as indicated by its highest score (Table 1). The effect of peptide amphiphilicity (e.g. the existence of hydrophobic patches that allow the peptides to anchor at the interface), rather than their size, on decreasing interfacial tension, has also been highlighted in literature (Schröder et al., 2017). This also correlates well with the observations for $\alpha 3$ (in comparison to $\alpha 1$ and $\alpha 2$), as the mean helical hydrophobic moment is significantly larger (Table S1), indicating an effect of both size and amphiphilicity. It is also worth noting that $\gamma 1$ resulted in both lowest initial interfacial tension and the lowest equilibrium value of interfacial tension (even when compared to casein, which is an excellent protein emulsifier for oil-in-water emulsions). These results indicate that $\gamma 1$ could be capable of adsorbing faster to the oil–water interface and thereby decrease interfacial tension more rapidly, accounting for an increased rate of droplet breakup, in comparison to the rest of peptides and proteins assayed.

In the case of potato protein, although it presented high initial interfacial tension, it decreased over time resulting in an equilibrium value similar to casein (Fig. 2). This indicated that

potato protein requires longer time to adsorb and rearrange at the interface in order to decrease interfacial tension when compared to casein and $\gamma 1$. As the potato protein is a complex mixture of native proteins spanning a wide range of molecular weights, sizes, and structures, other factors may contribute to the observed result. Smaller proteins and peptides that adsorb poorly but diffuse faster to the interface may be responsible for the high initial surface tension. Over time, larger proteins diffuse to and rearrange at the interface thereby producing strong adsorption that outcompete the smaller proteins and peptides ultimately resulting in a low equilibrium surface tension.

3.3 Physical stability of emulsions

Emulsifying peptides adsorb at the interface and stabilize oil-in-water emulsions by providing steric hindrances and electrostatic repulsions (Cheng, Chen, & Xiong, 2014). Table 2 shows the pH of the emulsions, which ranged from 5.7 to 6.9. At this pH, which was above the pI of the peptides and proteins assayed (apart from $\alpha 3$), the peptides as well as casein and potato protein were negatively charged and led to negative zeta potentials in the emulsions (Table 2). Most of the emulsions, except from $\alpha 3$ and potato protein-stabilized emulsions, showed highly negative surface charges (< -30 mV), which are expected to enhance physical stability by providing strong electrostatic repulsions (e.g. overcoming van der Waals and hydrophobic interactions) (Ghelichi, Sørensen, García-Moreno, & Jacobsen, 2017). Differences in zeta potential are mainly attributed to net charge of peptides (e.g. presence of R, K, D and E residues), which explained the significantly higher negative zeta potential values of $\gamma 1$, $\gamma 2$ and $\beta 1$ when compared to $\alpha 1$ and $\alpha 3$ (Tables 1, 2). Furthermore, surface charge is also affected by other factors like amount of peptides/protein adsorbed at the interface (García-Moreno et al., 2016). For instance, although $\alpha 1$ and $\alpha 2$ showed similar net charge, a significantly higher negative zeta potential was found for emulsions stabilized with $\alpha 2$ when compared to $\alpha 1$. This

might be explained by a lower amount of $\alpha 1$ adsorbed at the interface, since $\alpha 1$ emulsion had larger droplets when compared to $\alpha 2$ emulsion (Table 2). It indicated, for the same total amount of emulsifier and oil volume fraction, less droplets and smaller interfacial area in $\alpha 1$ -stabilized emulsion.

As expected from the interfacial tension results (Fig. 2), significant differences were observed in droplet size (Table 2) and droplet size distributions (see Fig. 3S in Supplementary Material) between the different emulsions. Gamma-1, casein and $\alpha 3$ presented higher emulsifying activity which led to emulsions with significantly smaller droplet size after production when compared to the other (Table 2). Nevertheless, it was observed that emulsion stabilized with $\alpha 3$ totally separated after storage, which could be attributed to its low net zeta potential. This could also be ascribed to the structure of $\alpha 3$ at the interface. The large mean hydrophobic moment may be responsible for a strong initial interaction, which is in agreement with the low interfacial tension. However, a quite small hydrophobic phase and the overall low content of very hydrophobic amino acids (21%) could be responsible for the helix not docking strongly at the interface and being more susceptible to external forces over time. In contrast, emulsions stabilized with casein significantly increased its droplet size due to flocculation and/or coalescence resulting in middle-high creaming. Previous studies highlighted that coalescence in emulsions prepared using ultrasonic equipment was mainly due to the low adsorption rate of emulsifier (e.g. due to the low concentration employed) and the increased likelihood of droplets collision (e.g. due to the high energy density in ultrasonic homogenization) (O'Sullivan, Murray, Flynn, & Norton, 2015). On the contrary, emulsion stabilized with $\gamma 1$ neither increased its droplet size during storage nor had visible creaming (Table 2). This denoted that sufficient peptide was adsorbed at the interface to avoid coalescence and that the initial droplet size was low enough to prevent emulsion physical destabilization (e.g. creaming) (McClements, 2005).

Despite the similar interfacial tension results found for $\alpha 1$, $\alpha 2$, $\beta 1$ and $\gamma 2$ (Fig. 2), the droplet size of these emulsions was significantly different (Table 2). Emulsion stabilized with $\alpha 2$ had the smallest droplet size after production followed by $\beta 1$, and then $\alpha 1$ and $\gamma 2$. The enhanced emulsifying activity of $\alpha 2$ when compared to $\alpha 1$ could be attributed, apart from the better solubility of $\alpha 2$ (e.g. less cloudy solution, see Fig. 2S in Supplementary Material), to the fact that three P residues were present in the sequence of $\alpha 1$. P residues (as well as G residues) are known to have a very low propensity for α -helicity and are usually found in either disordered or turn regions in native proteins (Costantini, Colonna, & Facchiano, 2006). Consequently, high content of either P or G reduces the probability of α -helicity in a peptide, while an increase in α -helicity has been reported to increase emulsifying activity of peptides (Enser et al., 1990). As both $\alpha 1$ and $\alpha 2$ contain several G residues distributed quite evenly throughout the peptide, this may decrease the stability of potential helices formed. If located in a helix, P will, due to the backbone constraints originating from the pyrrolidine side chain, produce a kink in the helix of approximately 26° (Barlow, & Thornton, 1987). If the direction of the kink is unfavorable relative to the curvature of the oil droplet, inclusion of one or more P will weaken the interaction of the helix with the oil thereby decreasing emulsifying potential and/or stability. On the contrary, if the kink is favorable to the curvature of the oil droplet, the inclusion of P will favor the adsorption of the helix at the interface. This may be the case for $\gamma 1$, although further research is needed.

It is worth noting that the emulsions stabilized with $\alpha 1$, $\alpha 2$, $\beta 1$ and $\gamma 2$ had droplet sizes higher than $4.5\ \mu\text{m}$ after storage, resulting in creaming for all these emulsions. It should be also noted that the emulsion stabilized with raw potato protein showed considerably larger droplet size when compared to emulsions stabilized with peptides and casein, leading to phase separation during storage (Table 2). This is in agreement with previous findings indicating

rapid creaming of oil-in-water emulsions stabilized with only potato protein (Cheng, Xiong, & Chen, 2010). Nevertheless, and as indicated by the interfacial tension measurements (Fig. 2), the performance of the potato protein used may improve when using homogenization methods that slowly disrupt oil droplets (e.g. mixing devices such as Stephan mixer). It will provide longer time for potato protein to diffuse and adsorb at the interface in order to stabilize the newly created droplets. This remains to be investigated.

Altogether, the results revealed superior emulsifying activity of the $\gamma 1$ peptide. This correlated well with the highest score predicted for this peptide (Table 1) as well as the lowest initial and equilibrium values of interfacial tension obtained for $\gamma 1$, when compared to the rest of peptides and proteins tested (Fig. 2). γ -peptides with the appropriate axial amphiphilicity have the potential to possibly orientate perpendicularly at the oil/water interface and stabilize oil-in-water emulsions. This may lead to higher packing density at the interface when compared to highly facial amphiphilic peptides (α -helix and β -strand peptides) (Dexter & Middelberg, 2008), which could result in enhanced interfacial structure, although likely demanding a higher amount of peptide to cover the interface due to the direction. However, the localization of $\gamma 1$ in Patatin Group M-1 (Fig. 1) and the potential for producing a (partial) amphiphilic α -helix at the interface, indicates that perpendicular packing at the interface may not be the mechanism of emulsifying action for $\gamma 1$. In fact, the high emulsifying activity may be a sum of several factors. Besides amphiphilic interaction with the oil droplet, the P residue may be placed in a position, where it induces a kink in the helix that makes it bend in accordance with the droplet curvature, thereby increasing the strength of the interaction. The relatively large hydrophobic phase of the amphiphilic helix (assuming only partial helical conformation as outlined for the $\gamma 1_{6-15}$ segment seen in Fig. S1) may be responsible for a strong and durable interaction with the oil droplet. Furthermore, the free and predominantly hydrophilic and highly charged C-terminal of the peptide could be fully solvent exposed thereby producing

steric hindrance and electrostatic repulsion forces, which in turn increases the stability of the emulsion over time. In any case, this needs to be confirmed by studying the *in situ* structure of the peptides at the oil/water interface, which is currently under investigation in our lab.

3.4 Antioxidant activity of peptides and oxidative stability of emulsions

Emulsifying potato peptides, which could also have antioxidant activity, are of special interest. They may retard lipid oxidation in oil-in-water emulsions by exhibiting their antioxidant properties at the oil/water interface (i.e. place where autoxidation is initiated due to the contact between prooxidants and lipids) (García-Moreno et al., 2016). Thus, *in vitro* antioxidant activity of the predicted peptides, as well as oxidative stability of the emulsions stabilized with these peptides, has been investigated in this study.

3.4.1 *In vitro* antioxidant activity of peptides

Patatin, as well as potato peptides, have been reported to exhibit antioxidant activity due to their ability to scavenge free radicals, donate electrons and/or chelate metal ions (Kudo, Onodera, Takeda, Benkeblia, & Shiomi, 2009; Wang & Xiong, 2005; Liu et al., 2003). Fig. 3a shows the ability of the predicted emulsifier peptides, casein and non-hydrolyzed potato protein to scavenge DPPH radical. It was observed that β 1, followed by α 1, showed significantly higher radical scavenging activity when compared to the other peptides. Beta-1 and α 1 present one M residue in their sequence, which is a highly oxidizable amino acid due to its nucleophilic sulfur-containing side chain (Elias, Kellerby, & Decker, 2008). Moreover, β 1 contains up to 4 T residues, three of them in consecutive order, which could enhance radical inhibition by donation of hydrogen from its hydroxyl group. On the other hand, α 1 is the richest peptide in aromatic residues such as F and Y, which have strong radical scavenging ability through direct transfer of electrons (Nwachukwu & Aluko 2019). These findings are in agreement with previous studies reporting short potato peptides (<1 kDa) with

radical scavenging activity, possibly due to the presence of M, T, Y and F residues (Cheng, Chen, & Xiong, 2010; Kudo et al., 2009). It is noteworthy that the potato protein studied showed significantly higher DPPH scavenging activity ($79.6 \pm 3.6\%$ at 2 mg/mL) compared to the predicted peptides and casein. This may be attributed to a higher content of accessible C and W residues in unprocessed potato protein (especially in proteases inhibitors), which exhibit strong antioxidant activity (Cheng, Xiong & Chen, 2010). Higher DPPH scavenging activity for patatin was reported by Liu et. (2003) ($IC_{50}=0.582$ mg/mL) when compared to the potato protein studied in this work, whereas lower radical inhibition was found for patatin containing different isoforms ($IC_{50}=25.63$ mg/mL) (Sun et al., 2013). This may be ascribed to the complexity of the crude potato protein compared to purified patatin.

A considerably low metal (Fe^{2+}) chelating activity was observed for all the peptides, especially when compared to casein, which is known as a good metal chelator (Fig. 3b). Indeed, this correlated well with previous results showing low or no metal chelating activity for potato peptides between 2-4 kDa at concentrations lower than 5 mg/mL (Cheng, Chen, & Xiong, 2010). Nevertheless, short potato peptides (<1 kDa), which have higher negative charge (carboxyl groups)-to mass ratios than large peptides, have been reported to bind metal ions more efficiently (Cheng, Chen, & Xiong, 2010). This is a general trend also found for peptides from other protein sources (e.g. fish or milk), where low molecular weight peptides (<1 kDa) exhibit significantly higher metal chelating activity than large peptides (García-Moreno et al., 2016; O'Loughlin et al., 2014). In any case, it should be highlighted that, besides the lower content of H residues (e.g. which exhibit chelating activity through its imidazole ring) for $\gamma 2$ when compared to $\alpha 1$ and $\alpha 2$ peptides, $\gamma 2$ exhibited significantly higher metal chelating activity when compared to the rest of peptides studied (~3 kDa) (Fig. 3b). This could be attributed to $\gamma 2$ having a higher content of acidic residues with carboxyl groups in the side chain (Nwachukwu & Aluko 2019). Finally, it should be mentioned that the

peptides showed a significantly lower (Fe^{3+}) reducing power at the concentration assayed (2 mg/mL), when compared to casein and potato protein (Fig. 3c). Although there are not relevant data for potato protein in the literature, these low values are not surprising, since, for example, up to 15-30 mg of fish protein hydrolysate per milliliter were required to reach absorbance values above 0.5 at 700 nm (García-Moreno et al., 2016; García-Moreno et al., 2014).

3.4.2 Oxidative stability of emulsions

First, oxidative stability of emulsions was investigated by using ESR spin trapping, where oxidation was accelerated by heating. PBN, which reacts with lipid radicals (e.g. peroxy and alkoxy radicals) yielding more stable radicals (spin adducts), was used as spin trap (Zhou & Elias, 2012). The ESR spectra of PBN-adducts formed in fish oil-in-water emulsions consisted of three broad lines (Fig. 4a), showing the common coupling for nitroxyl radicals. This correlated well with the ESR spectra of PBN-adducts formed in bulk fish oil (Velasco, Andersen, Skibsted, 2005), and indicates that the radicals trapped by PBN were produced in the oil phase of the emulsions. Hence, higher formation of PBN-spin adducts (e.g. measured as the peak-to-peak amplitude of the middle-field line of the ESR spectra), denoted higher lipid oxidation (Andersen & Skibsted, 2008).

Fig. 4b shows the generation of PBN-lipid adducts during storage of fish oil-in-water emulsions at 50 °C. It was found that the emulsion stabilized with $\alpha 1$ was significantly more oxidized after 48 h storage when compared to emulsions stabilized with $\alpha 2$ and casein (which did not show significant differences between them at 48 h, $p > 0.05$). Casein exhibited significantly higher chelating activity and reducing power when compared to $\alpha 1$, which could explain the higher oxidative stability of the emulsion stabilized with casein. Nonetheless, this is not the case for $\alpha 2$, which showed significantly lower radical scavenging and reducing power than $\alpha 1$ (Fig. 3). Therefore, the higher oxidative stability of $\alpha 2$ -stabilized emulsion,

when compared to $\alpha 1$, should be attributed to other properties such as higher adsorption at the interface (as indicated by its higher negative zeta potential but a similar net charge, Table 2). Higher peptide adsorption may lead to more densely packed interfaces, resulting in enhanced interfacial layer by decreasing the accessibility of prooxidants to lipids (García-Moreno et al., 2016). Surprisingly, emulsions stabilized with $\beta 1$, $\gamma 1$, $\gamma 2$ and potato protein showed a decrease in the peak-to-peak amplitude after 6-24 h (see Fig. 4S in Supplementary Material for specific details for $\gamma 1$ -stabilized emulsion). This led to significantly lower intensity after 48h storage at 50 °C for these emulsions when compared to emulsions stabilized with $\alpha 1$, $\alpha 2$ and casein (Fig. 4a). Previous ESR studies on fish oils also reported very low steady state concentrations of PBN spin adducts due to a fast decay of PBN spin adducts as a consequence of the reaction with new radicals to form diamagnetic species (Falch, Velasco, Aursand, & Andersen, 2005; Velasco et al., 2005). Interestingly, emulsions stabilized with $\beta 1$, $\gamma 1$, $\gamma 2$ and potato protein showed brown colour during storage at 50 °C, which was not observed for emulsions stabilized with $\alpha 1$, $\alpha 2$ and casein (see Fig. 5S in Supplementary Material). This may indicate peptide/protein co-oxidation including non-enzymatic browning reactions (Lu, Nielsen, Baron, Diehl, & Jacobsen, 2013), which could be related to advanced stages of oxidation.

Secondly, oxidative stability of emulsions was determined by using the Oxygraph method, where oxidation is accelerated by the addition of Fe^{2+} . Fig. 5a shows the drop in oxygen concentration (ΔO_2) in the emulsions after adding Fe^{2+} . Mozuraityte, Rustad, and Storror (2008) attributed this initial drop in dissolved oxygen to the oxidation of Fe^{2+} to Fe^{3+} by pre-existing lipid hydroperoxides. This results in alkoxyl radicals, which further react with fatty acids leading to lipid radicals and formation of lipid peroxides by oxygen consumption. After equilibrium between Fe^{2+} and Fe^{3+} is reached, a linear oxygen uptake takes place due to the

lower pro-oxidant effect caused by Fe^{3+} (Mozuraityte, Kristinova, Rustad, & Storrø, 2016). Hence, higher ΔO_2 values as well as more negative net oxygen uptake rate (R_N) (Fig. 5b) indicate lower oxidative stability of emulsions.

It was observed that R_N was not significantly different for the emulsion stabilized with casein when compared to the values obtained for emulsions stabilized with either $\alpha 1$, $\beta 1$, $\gamma 1$ or $\gamma 2$. However, significantly more negative R_N values were found for $\alpha 3$, when compared to the rest of the emulsions (Fig. 5b). Hence, the lower oxidative stability of $\alpha 3$ could be attributed to the low physical stability of this emulsion, even soon after preparation, which indicates more accessibility of prooxidants to the oil. Similarly, no significant differences were observed in the ΔO_2 value for emulsion stabilized with casein ($\sim 40 \mu\text{M}$) when compared to emulsions stabilized with $\beta 1$, $\gamma 1$ and $\gamma 2$ (Fig. 5a). This indicates that these emulsions were significantly more oxidatively stable when compared to emulsions stabilized with $\alpha 1$ and $\alpha 2$ ($\sim 50 \mu\text{M}$) (Fig. 5). It can be attributed to: i) higher chelating activity of casein and $\gamma 2$, ii) higher radical scavenging activity showed by $\beta 1$, and iii) higher physical stability of $\gamma 1$. It is noteworthy that Oxygraph results (Fig. 5), where oxidation was measured right after production of the emulsions (still physically stable emulsions), correlated reasonably well with ESR results for $\alpha 1$ and $\alpha 2$, but not for the rest of the emulsions. This may be explained by the different conditions to accelerate oxidation employed (temperature vs. iron addition) as well as the different time scale of the analyses. For instance, no change in color was observed for the emulsions stabilized with $\beta 1$, $\gamma 1$ and $\gamma 2$ during the short time scale of the Oxygraph analysis. This indicates the absence of advanced lipid oxidation products formed through non-enzymatic browning (e.g. carbonyl-amine adducts).

4. CONCLUSIONS

Bionformatics allowed the prediction of peptides with emulsifying activity derived from patatin. The predicted peptides have a potentially different conformation at the interface (α -helix, β -strand or unordered). Lower physical and oxidative stabilities were observed for emulsions stabilized with $\alpha 1$, $\alpha 2$ and $\alpha 3$ peptides, which were predicted to have a low amphiphilic score. Nevertheless, the peptide predicted to have the highest amphiphilic score (a peptide with hydrophobic and hydrophilic halves, $\gamma 1$) showed the highest emulsifying activity. Structural modelling indicated that the mechanism of emulsifying action may, however, be different than expected and also include formation of an amphiphilic helix. Gamma-1 led to the lowest initial and equilibrium values of interfacial tension. This correlated well with the lowest droplet size after production and during storage, and the no or little creaming observed for $\gamma 1$ -stabilized 5wt.% fish oil-in-water emulsion (also when compared to casein-stabilized emulsions). Moreover, the oxidative stability of the emulsions was affected by the type of emulsifier peptide used, which could not be only explained by their different *in vitro* antioxidant properties. For instance, 5wt.% fish oil-in-water emulsions stabilized either with $\gamma 1$ or casein showed similar oxidative stability according to Oxygraph results. On the other hand, ESR results suggested a more advanced oxidation in emulsions stabilized with $\beta 1$, $\gamma 1$ and $\gamma 2$ peptides, which could explain the degradation of PBN spin adducts and the appearance of non-enzymatic browning.

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REFERENCES

- Agyei, D., Tsopmo, A., & Udenigwe, C. C. (2018). Bioinformatics and peptidomics approaches to the discovery and analysis of food-derived bioactive peptides. *Analytical and Bioanalytical Chemistry*, 410(15), 3463-3472.
- Alting, A. C., Pouvreau, L., Giuseppin, M. L. F., & van Nieuwenhuijzen, N. H. (2011). Potato proteins. *Handbook of Food Proteins*, In G. O. Phillips, P. A. Williams (Eds.) Woodhead publishing (pp. 316-334). Cambridge: Woodhead Publishing Limited.
- Andersen, M. L., & Skibsted, L. H. (2008). ESR Spectroscopy for the Study of Oxidative Processes in Food and Beverages. In *Modern Magnetic Resonance* (pp. 1861–1866). Dordrecht: Springer Netherlands.
- Barlow, D. J., & Thornton, J. M. (1987). Helix geometry in proteins. *Journal of Molecular Biology*, 201(201), 601–619.
- Bateman, A., Martin, M. J., O'Donovan, C., Magrane, M., Alpi, E., Antunes, R., ... Zhang, J. (2017). UniProt: The universal protein knowledgebase. *Nucleic Acids Research*, 45(1), D158–D169.
- Bauw, G., Nielsen, H. V., Emmersen, J., Nielsen, K. L., Jørgensen, M., & Welinder, K. G. (2006). Patatins, Kunitz protease inhibitors and other major proteins in tuber of potato cv. Kuras. *Febs Journal*, 273(15), 3569–3584.
- Berton-Carabin, C. C., Ropers, M. H., & Genot, C. (2014). Lipid Oxidation in Oil-in-Water Emulsions: Involvement of the Interfacial Layer. *Comprehensive Reviews in Food Science and Food Safety*, 13(5), 945–977.
- Cheng, Y., Chen, J., & Xiong, Y. L. (2014). Interfacial adsorption of peptides in oil-in-water emulsions costabilized by tween 20 and antioxidative potato peptides. *Journal of Agricultural and Food Chemistry*, 62(47), 11575-11581.

Cheng, Y., Chen, J., & Xiong, Y. L. (2010). Chromatographic separation and tandem MS identification of active peptides in potato protein hydrolysate that inhibit autoxidation of soybean oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 58(15), 8825-8832.

Cheng, Y., Xiong, Y. L., & Chen, J. (2010). Antioxidant and emulsifying properties of potato protein hydrolysate in soybean oil-in-water emulsions. *Food Chemistry*, 120(1), 101-108.

Costantini, S., Colonna, G., & Facchiano, A. M. (2006). Amino acid propensities for secondary structures are influenced by the protein structural class. *Biochemical and Biophysical Research Communications*, 342(2), 441–451.

Dexter, A. F., & Middelberg, A. P. J. (2008). Peptides as functional surfactants. *Industrial and Engineering Chemistry Research*, 47(17), 6391-6398.

Eisenberg, D., Weiss, R. M., & Terwilliger, T. C. (1982). The helical hydrophobic moment: A measure of the amphiphilicity of a helix. *Nature*, 299(5881), 371-374.

Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition*, 48(5), 430–441.

Enser, M., Bloomberg, G. G., Brock, C., & Clark, D. D. (1990). De novo design and structure-activity relationships of peptide emulsifiers and foaming agents. *International Journal of Biological Macromolecules*, 12: 118–124.

Falch, E., Velasco, J., Aursand, M., & Andersen, M. L. (2005). Detection of radical development by ESR spectroscopy techniques for assessment of oxidative susceptibility of fish oils. *European Food Research and Technology*, 221(5), 667–674.

FAO (2008) Statistical crop data.

Fauchere, J. L., & Pliska, V. (1983). Hydrophobic parameters- π of amino-acid side-chains from the partitioning of n-acetyl-amino-acid amides. *European Journal of Medicinal Chemistry*, 18(4), 369–375.

García-Moreno, P. J., Batista, I., Pires, C., Bandarra, N. M., Espejo-Carpio, F. J., Guadix, A., & Guadix, E. M. (2014). Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species. *Food Research International*, 65, 469–476.

García-Moreno, P. J., Guadix, A., Guadix, E. M., & Jacobsen, C. (2016). Physical and oxidative stability of fish oil-in-water emulsions stabilized with fish protein hydrolysates. *Food Chemistry*, 203, 124-135.

Gautier, R., Douguet, D., Antonny, B., & Drin, G. (2008). HELIQUEST: a web server to screen sequences with specific α -helical properties. *Bioinformatics*, 24(18), 2101–2102.

Ghelichi, S., Sørensen, A. -. M., García-Moreno, P. J., Hajfathalian, M., & Jacobsen, C. (2017). Physical and oxidative stability of fish oil-in-water emulsions fortified with enzymatic hydrolysates from common carp (*cyprinus carpio*) roe. *Food Chemistry*, 237, 1048-1057.

Grommers, H. E., & van der Krogt, D. A. (2009). Potato Starch: Production, Modifications and Uses. In G. O. Phillips, P. A. Williams (Eds.) Woodhead publishing (pp. 511–539). Cambridge: Woodhead Publishing Limited.

Jørgensen, M., Stensballe, A., & Welinder, K. G. (2011). Extensive post-translational processing of potato tuber storage proteins and vacuolar targeting. *Febs Journal*, 278(21), 4070–4087.

Jørgensen, M., Welinder, K. G., & Bauw, G. (2006). Molecular Properties and Activities of Tuber Proteins from Starch Potato Cv. Kuras. *Journal of Agricultural and Food Chemistry*, 54(54), 9389–9397.

Jung, J.-M., Gunes, D. Z., & Mezzenga, R. (2010). Interfacial activity and interfacial shear rheology of native b-lactoglobulin monomers and their heat-induced fibers. *Langmuir: The ACS Journal of Surfaces and Colloids*, 26(18), 15366-15375.

Kudo, K., Onodera, S., Takeda, Y., Benkeblia, N., & Shiomi, N. (2009). Antioxidative activities of some peptides isolated from hydrolyzed potato protein extract. *Journal of Functional Foods*, 1(2), 170–176.

Kyte, J., & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology*, 157(1), 105-132.

Liu, Y. W., Han, C. H., Lee, M. H., Hsu, F. L., & Hou, W. C. (2003). Patatin, the tuber storage protein of potato (*Solanum tuberosum* L.), exhibits antioxidant activity in vitro. *Journal of Agricultural and Food Chemistry*, 51, 4389–4393.

Lu, F. S. H., Nielsen, N. S., Baron, C. P., Diehl, B. W. K., & Jacobsen, C. (2013). Impact of primary amine group from aminophospholipids and amino acids on marine phospholipids stability: Non-enzymatic browning and lipid oxidation. *Food Chemistry*, 141(2), 879-888.

McClements, D. J. (2005). *Food emulsions – Principles, practices, and techniques*. Boca Raton: CRC Press.

Mozuraityte, R., Rustad, T., & Storrø, I. (2008). The role of iron in peroxidation of polyunsaturated fatty acids in liposomes. *Journal of Agricultural and Food Chemistry*, 56(2), 537-543.

Mozuraityte, R., Kristinova, V., Rustad, T., & Storrø, I. (2016). The role of iron in peroxidation of PUFA: Effect of pH and chelators. *European Journal of Lipid Science and Technology*, 118(4), 658-668.

Nwachukwu, I. D., & Aluko, R. E. (2019). Structural and functional properties of food protein-derived antioxidant peptides. *Journal of Food Biochemistry*, e12761.

O'Loughlin, I. B., Murray, B. A., FitzGerald, R. J., Brodkorb, A., & Kelly, P. M. (2014). Pilot-scale production of hydrolysates with altered bio-functionalities based on thermally-denatured whey protein isolate. *International Dairy Journal*, 34(1), 146–152.

O'Sullivan, J., Murray, B., Flynn, C., & Norton, I. (2015). Comparison of batch and continuous ultrasonic emulsification processes. *Journal of Food Engineering*, 167, 114-121.

Pots, A. M., De Jongh, H. H. J., Gruppen, H., Hamer, R. J., & Voragen, A. G. J. (1998). Heat-induced conformational changes of patatin, the major potato tuber protein. *European Journal of Biochemistry*, 252(1), 66-72.

Pouvreau L, Gruppen H, Piersma SR, van den Broek LAM, van Koningsveld GA & Voragen AGJ (2001) Relative abundance and inhibitory distribution of protease inhibitors in potato juice from cv. Elkana. *J Agric Food Chem*. 49(6), 2864-2874.

Romero, A., Beaumal, V., David-Briand, E., Cordobés, F., Guerrero, A., & Anton, M. (2011). Interfacial and oil/water emulsions characterization of potato protein isolates. *Journal of Agricultural and Food Chemistry*, 59(17), 9466–9474.

Schmidt, J. M., Damgaard, H., Greve-Poulsen, M., Larsen, L. B., & Hammershøj, M. (2018). Foam and emulsion properties of potato protein isolate and purified fractions. *Food Hydrocolloids*, 74, 367-378.

Schmidt, J. M. (2016). PhD Thesis: Purification and functional properties of potato protein fractions.

Schröder, A., Berton-Carabin, C., Venema, P., & Cornacchia, L. (2017). Interfacial properties of whey protein and whey protein hydrolysates and their influence on O/W emulsion stability. *Food Hydrocolloids*, 73, 129-140.

Sun, Y., Jiang, L., & Wei, D. (2013). Partial characterization, in vitro antioxidant and antiproliferative activities of patatin purified from potato fruit juice. *Food and Function*, 4(10), 1502–1511.

- Udenigwe, C. C., Udechukwu, M. C., Yiridoe, C., Gibson, A., & Gong, M. (2016). Antioxidant mechanism of potato protein hydrolysates against in vitro oxidation of reduced glutathione. *Journal of Functional Foods*, 20, 195-203.
- Van Koningsveld, G. A., Walstra, P., Voragen, A. G. J., Kuijpers, I. J., Van Boekel, M. A. J. S., & Gruppen, H. (2006). Effects of protein composition and enzymatic activity on formation and properties of potato protein stabilized emulsions. *Journal of Agricultural and Food Chemistry*, 54(17), 6419-6427.
- Velasco, J., Andersen, M. L., & Skibsted, L. H. (2005). Electron spin resonance spin trapping for analysis of lipid oxidation in oils: Inhibiting effect of the spin trap α -phenyl-N-tert-butyl-nitron on lipid oxidation. *Journal of Agricultural and Food Chemistry*, 53(5), 1328–1336.
- Wang, L. L., & Xiong, Y. L. (2005). Inhibition of lipid oxidation in cooked beef patties by hydrolyzed potato protein is related to its reducing and radical scavenging ability. *Journal of Agricultural and Food Chemistry*, 53(23), 9186-9192.
- Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., ... Schwede, T. (2018). SWISS-MODEL: Homology modelling of protein structures and complexes. *Nucleic Acids Research*, 46(1), W296–W303.
- Wijeyesakere, Sanjeeva J., Rudy J. Richardson, and Jeanne A. Stuckey (2014). Crystal structure of patatin-17 in complex with aged and non-aged organophosphorus compounds. *PloS one* 9.9: e108245.
- Zhou, L., & Elias, R. J. (2012). Factors Influencing the Antioxidant and Pro-Oxidant Activity of Polyphenols in Oil-in-Water Emulsions. *Journal of Agricultural and Food Chemistry*, 60(11), 2906–2915.

Table 2. Values of pH, droplet size, zeta potential and observations on creaming of the emulsions

Emulsion	Solubility*	pH	Zeta potential (mV)	D _{4,3} (μm)		Observations after production	Observations after 2 days at 50 °C
				Day 0	Day 2 at 50 °C		
α1	Cloudy	6.5	-37.1±2.6 ^d	7.455±1.056 ^d	7.922±0.170 ^{c, ns}	Little creaming	Middle-high creaming
α2	Cloudy	6.3	-51.0±4.2 ^{b, c}	2.606±0.033 ^b	4.532±0.269 ^{b, *}	Little creaming	Middle-high creaming
α3	Soluble	5.7	-13.9±7.0 ^e	0.842±0.128 ^a	n.m.	No creaming	Totally separated
β1	Soluble	6.3	-55.1±2.7 ^{a, b}	4.545±0.163 ^c	15.893±2.813 ^{d, *}	No creaming	Severe creaming with brown aqueous phase
γ1	Soluble	6.3	-62.4±3.5 ^a	0.516±0.010 ^a	0.518±0.011 ^{a, ns}	No creaming	No or very little creaming with brown aqueous phase
γ2	Soluble	6.4	-61.6±2.4 ^{a, b}	8.267±0.079 ^d	8.092±0.789 ^{c, ns}	No creaming	Severe creaming with brown aqueous phase
Casein	Soluble	6.6	-41.8±1.9 ^{c, d}	1.337±0.101 ^a	17.462±0.201 ^{d, *}	No creaming	Middle-high creaming
Potato	Soluble	6.9	-27.3±0.9	57.076±28.245	n.m.	Severe creaming	Totally separated and slight brown aqueous phase

*Solubility of peptides in 10 mM sodium acetate - 10 mM imidazole buffer (pH 7). Soluble: totally soluble; cloudy: not totally soluble. Concentration tested: 0.2 wt.%.
n.m.: emulsions were totally separated and droplet size could not be measured at day 2.

For each column, different letters indicate significant differences between peptide samples ($p < 0.05$). Significant differences between day 1 and 2 are indicated by either *: significantly different ($p < 0.05$), or ns: not significantly different.

TABLES

Table 1. Properties of potato peptides predicted by bioinformatics to have emulsifying activity

Peptide	Sequence	Score*	Accession number	Position	Number of residues	α -helical content**	β -strand content**	Purity***, %	Mw, g/mol	pI****	Net charge**** (pH 7)
$\alpha 1$	AKDIVPFYFEHGPHIFNYSGSIIGPMYDG	35.9	P15477	99	29	52%	0%	98.7	3272.6	5.1	-1.8
$\alpha 2$	HHFVTHTSNGARYEFNLVDGAVATVGDPA	36.4	P15477	197	29	3%	45%	93.7	3083.3	5.8	-1.7
$\alpha 3$	KPVSKDSPETYEEALKRFAKLLS	33.3	P15477 P11768 Q42502 Q3YJT3	352 352 352 340	23	57%	9%	72.4	2636.9	9.4	1
$\beta 1$	LRVQENALTGTTTKADDASEANMELLVQV	42.2	Q3YJT5	318	29	34%	10%	70.3	3118.4	3.8	-3
$\gamma 1$	GIKGIIPAIILEFLEGQLQEVDNNKDAR	51.9	P11768	38	28	72%	0%	99.4	3094.5	4.2	-2
$\gamma 2$	ANMILLVQVGENLLKKSSEDNHETYE	45.3	Q3YJS9	325	27	63%	7%	90.5	3074.4	4.2	-2.9

*Score of mean hydrophobic vector.

**Fraction of residues located in a given secondary structure conformation (within the native protein) as implied by homology modelling using Patatin-17 as structural template.

*** Purity of the synthetic peptides used as received from Chinapeptides.

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

Fig. 1. Localization of predicted emulsifier peptides based on template homology modelling using SwissProt and visualized in PyMOL. Models are made using the identified patatin isoform for the predicted peptides (Table 1) using the X-ray structure of Patatin-17 (SMTL ID 4pka.1.A) as template. Localization of $\alpha 1$ (red), $\alpha 2$ (blue), and $\alpha 3$ (green) in Patatin-B2 (UniProt AC# P15477), $\beta 1$ (red) in Patatin-05 (UniProt AC# Q3YJT5), $\gamma 1$ (red) in Patatin Group M-1 (UniProt AC# P11768), and $\gamma 2$ (red) in Probable inactive patatin-3-Kuras 1 (UniProt AC# Q3YJS9). Beneath, secondary structure of predicted peptides (within the parent proteins) according to the models. Backbone and side chain and coloring according to the SwissModel hydrophobicity color scale (most hydrophobic residues in red and most hydrophilic residues in blue).

Fig. 2. Interfacial tension at the fish oil-aqueous phase interface, with the aqueous phase containing 0.1 wt.% peptides in 10 mM sodium acetate - 10 mM imidazole buffer (pH 7).

Fig. 3. *In vitro* antioxidant capacity of synthetic potato peptides (0.2 wt.% at pH 7): a) DPPH radical scavenging activity, b) Fe²⁺ chelating activity, and c) Fe³⁺ reducing power.

Different letters indicate significant differences between samples ($p < 0.05$)

Peptides names with a, b and g denotes α , β and γ , respectively.

Fig. 4. a) Evolution of peak-to-peak amplitude of PBN-ESR spectra with storage time at 50 °C for the emulsion stabilized with synthetic potato peptide $\alpha 1$. b) PBN-lipid derived spin adducts generation resulting from the oxidation of 5 wt.% fish oil-in-water emulsions stabilized with synthetic potato peptides during storage at 50 °C in the dark.

ESR results for emulsion stabilized with $\alpha 3$ were not shown in Fig. 4b since it totally separated after 6 h at 50 °C giving no intensity when measured at time zero.

Fig. 5. a) Drop in oxygen concentration, and b) net oxygen uptake rate in emulsion stabilized with synthetic potato peptides during iron-mediated oxidation ($45 \mu\text{M}$ of Fe^{2+})

*The determination of oxygen drop in this emulsion was not possible due to the shape of the curve when compared to the rest of the curves obtained (see Fig. 6S in the Supplementary Material).

Different letters indicate significant differences between samples ($p < 0.05$)

Peptides names with a, b and g denotes α , β and γ , respectively.

1 **Highlights**

- 2 • Bioinformatics to identify emulsifying peptides embedded in potato protein
- 3 • Synthetic peptides with different conformation at the o/w interface were tested
- 4 • $\gamma 1$ had superior emulsifying activity than the rest of the peptides and casein
- 5 • The type of peptide used affected the chemical stability of fish o/w emulsions

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: