Effects of Enzymatic Hydrolysis of Fava Bean Protein Isolate by Alcalase on the Physical and Oxidative Stability of Oil-in-Water Emulsions

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ABSTRACT: Fava bean protein isolate (FBPI) was hydrolyzed by Alcalase with different degrees of hydrolysis (DHs), and the role of hydrolysates in oil-in-water (O/W) emulsion stability was investigated. Four emulsions, DH0, DH4, DH9, and DH15, were prepared by 1% (w/v) FBPI hydrolysates with different DHs (0% as the control and 4, 9, and 15%) and 5% (w/v) purified rapeseed oil. The emulsions were monitored for physical and oxidative stability at 37 °C for 7 days. DH4 and DH0 exhibited better physical stability than DH9 and DH15, indicated by droplet size, morphology, and Turbiscan stability index. More importantly, FBPI hydrolysates with DH of 4% most effectively inhibited lipid oxidation (i.e., formation of conjugated dienes and hexanal) while maintaining protein oxidative stability compared to the native and extensively hydrolyzed FBPI. Higher DHs (9 and 15%) induced unduly decreased surface hydrophobicity and increased surface load, which might negatively affect the emulsifying activity. FBPI hydrolysates with DH of 4% had suitable molecular weight for better interfacial layer stability, increased surface net charge for more repulsive electrostatic force, and increased hydrophobicity for better adsorption at the interface and, therefore, may serve as potential natural emulsifiers to maintain both physical and oxidative stability of O/W emulsions.

KEYWORDS: fava bean protein, Alcalase hydrolysis, emulsion, stability

INTRODUCTION

Proteins are widely added to food products that are formulated as oil-in-water (O/W) emulsions as emulsifiers to facilitate droplet breakdown and maintain physical stability.1 As a result of economic cost and sustainability issues, growing research interests focus on plant proteins as substitutes for animal proteins.2 Fava bean (Vicia faba L.) is rich in protein (27−34% of the dry weight) and widely grown as a result of easy cultivation, making it an excellent source of plant protein.3 A few studies reported the use of native plant proteins, including fava bean, lentil, and pea proteins, as emulsifiers.4,5 Fava-bean-protein-stabilized emulsion displayed relatively lower physical stability than lentil-protein-stabilized emulsion, which may be attributed to differences in surface hydrophobicity or steric interactions.6 Various modifications on fava bean protein isolate (FBPI), such as heating,6 transglutaminase treatment,7 acetylation,8 and high-pressure treatment,9 have been reported to improve its emulsifying functionality. However, these reports generally did not take into account the oxidative stability of emulsion.

Lipid oxidation is a common problem in O/W systems and causes a loss of product quality.10 Proteins not only serve as emulsifiers in the emulsion system but also affect lipid oxidation in different ways: unadsorbed proteins can bind metal ions and scaveng free radicals; adsorbed proteins might repel cationic metal ions or bring metal ions to interfacial layers, depending upon the surface charges; adsorbed proteins may also act as a physical barrier and sterically constrict the interaction between metal ions and the lipid droplet.10 Moreover, increasing evidence suggests that food protein oxidation itself is associated with various diseases.11 Therefore, it is important to consider the oxidative stability of both lipid and protein and include oxidative stability as an index for assessing emulsifying functionality.

Enzymatic hydrolysis has been reported as a safe, simple, and economical way to improve emulsifying activities and antioxidant activities of plant proteins.12−14 Hydrolysis could increase the plant protein solubility and surface hydrophobicity and expose more buried hydrophobic groups, thus improving the adsorption at the O/W interface and emulsifying capability.15 In addition, protein hydrolysates show better hypoallergenic and high-tolerance properties than native proteins.16 Alcalase is a commercial enzyme preparation from Bacillus licheniformis, which consists primarily of subtilisin A; subtilisin A is an endopeptidase with broad actions, preferably cleaving terminal hydrophobic amino acids.17 Alcalase-derived hydrolysates not only have higher antioxidant activities than those from other peptidases but also are more resistant to digestive enzymes.18 However, it should be stressed that insufficient or extensive hydrolysis might impair the functionality of proteins.19 Therefore, it is critical to determine the optimum degree of hydrolysis (DH).

To the best of our knowledge, it is not clear how Alcalase hydrolysis affects the emulsifying capability of FBPI and the physical and oxidative stability of FBPI-stabilized emulsions. Therefore, the aims of this study were to (1) investigate how Alcalase treatment affects physiochemical properties and emulsifying activity of FBPI, (2) determine how DH affects the physical and oxidative stability of O/W emulsion, and (3)
explore the relationship among physical stability and lipid and protein oxidation in O/W emulsion. This study provides new knowledge about characteristics of plant protein applicable as natural emulsifiers.

MATERIALS AND METHODS

Materials. Fava beans (cultivar ‘Divine 2012’) were grown at Viikki Experimental Farm of the University of Helsinki in Finland. Alcalase (Alcalase 2.4 L FG) with 2.22 AU/g activity (determined by a Folin phenol method\(^{36}\) with FBPI as the substrate) was provided by UNIVAR (Vantaa, Finland). Rapeseed oil was purchased from a local store. Bovine serum albumin (BSA), guanidine hydrochloride, sodium dodecyl sulfate (SDS), and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich (Steinheim, Germany). Ethanol, ethyl acetate, hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium phosphate buffer (SPB), and sodium azide were purchased from Merck (Darmstadt, Germany). All chemicals employed in this study were of analytical grade.

Extraction of FBPI. FBPI was extracted as previously described.\(^3\) Briefly, fine fava bean flour was obtained by an ultracentrifugal mill (2M 200, Retsch, Germany). Then, FBPI was extracted by three repeated acid precipitations (pH 4.5 with 2 M HCl) and alkaline dissolutions (pH 8.0). Finally, the solution was dialyzed against water, lyophilized, and stored at −20 °C. The yield of FBPI was 89%, determined by the Biuret method.

Protein Hydrolysis. FBPI was hydrolyzed by Alcalase with DHs of 4% (DH4-FBPI), 9% (DH9-FBPI), and 15% (DH15-FBPI) as previously described, with minor modifications.\(^12\) Briefly, Alcalase (0.01 AU/g of protein) was added to 5% (w/v) FBPI dispersions pre-equilibrated at pH 8.0 and 50 °C. Then, the reaction was maintained at 50 °C and pH 8.0 by the continuous addition of 0.2 M NaOH. The total added volume of NaOH to reach each target DH was calculated from the equation DH = (b/h) × 100 = ((B × N)/MP) × (1/α) × (1/hₐ) = 100, where DH is the percent ratio of the number of peptide bonds cleaved (b) to the total number of peptide bonds in the protein substrate (hₐ, assumed to be 7.8 mequiv/g of protein for fava bean protein\(^11\)), B is the base consumption (mL), N is the normality of the base, MP is the mass of the protein (g), and α is the average degree of dissociation of the α-NH₂ amino groups released during the hydrolysis, which is assumed to be 0.885 at pH 8.0 and 50 °C.\(^12\) After target DHs were reached, hydrolysis was stopped by heating immediately at 80 °C for 20 min to inactivate the enzyme.\(^12\)

Finally, the supernatant containing hydrolysates was recovered by centrifugation at 5000g for 20 min at 4 °C. A FBPI dispersion treated with heat-inactivated Alcalase served as a control (DHF-FBPI). A FBPI dispersion without Alcalase served as a native FBPI control.

Physicochemical Properties of FBPI Hydrolysates. Electrophoresis. Sodium dodecyl sulfide polyacrylamide gel electrophoresis (SDS–PAGE, NuPAGE 12% Bis-Tris, Invitrogen) was performed under reducing conditions.\(^7\) Mixtures of 0.2% (w/v) native FBPI or DH0/DH4/DH9/DH15-FBPIs and NuPAGE LDS sample buffer at 1:1 were heated in boiling water for 3 min. Then, 10 μL of treated samples and 4 μL of Novex sharp pre-stained protein standards were loaded into gel lanes. The electrophoresis was carried out at 200 V for 60 min. Protein bands were stained using Coomassie Brilliant Blue. The molecular weights (MWs) of unknown proteins were estimated via regression between the log of standard MWs and the relative mobility of the protein markers.

Surface Charge (ζ Potential). The overall surface charges of DH0/DH4/DH9/DH15-FBPI dispersions (0.2%, w/v) at pH 8.0 were determined by measuring the electrophoretic mobility (Uₑ) on a Zetasizer Nano-ZSP90 instrument (Malvern Instruments, Westborough, MA, U.S.A.) as previously described.\(^18\) Surface charge hydrophobicity of DH0/DH4/ DH9/DH15-FBPIs was measured using the 8-anilino-1-naphthalene-sulfonic acid (ANS) assay, with minor modifications.\(^13\) Briefly, sample dispersions were diluted with 35 mM SPB to give five gradient concentrations ranging from 0.005 to 0.025% (w/v). Then, ANS (8 mM in 35 mM SPB at pH 8.0, Sigma-Aldrich) was added to the dilution at 1:100 (v/v), followed by vortexing for 5 s and incubation for 5 min. Fluorescence intensity (FI) was then measured at Ex/Em = 390/470 nm (slit = 2.5 nm) by a luminescence spectrometer (LS 55, PerkinElmer, Waltham, MA, U.S.A.).

Protein Solubility. DH0/DH4/DH9/DH15-FBPI dispersions (1%, w/v) were stirred at pH of 8.0 for 10 min and then centrifuged at 12000g at 20 °C for 20 min. The protein solubility was expressed as a percentage of supernatant protein (by the Biuret method) over total protein.

Preparation of Emulsions. Four emulsions, DH0, DH4, DH9, and DH15, were prepared using DH0/DH4/DH9/DH15-FBPIs, respectively. The emulsions consisted of 1% (w/v) DH0/DH4/DH9/ DH15-FBPIs and 5% (w/v) purified rapeseed oil by chromatography\(^15\) (no detectable residual tocopherols). Briefly, protein dispersions in deionized water were blended with oil using a homogenizer at 13 500 rpm and then processed using a M-110Y microfluidizer equipped with 75 μm Y-type F207Y and 200 μm Z-type H30Z chambers (Microfluidics, MFIC Corp., Westwood, MA, U.S.A.) at 600 bar for 10 min. Sodium azide (0.02%, w/v) was added to inhibit microbial growth. Finally, 20 mL of emulsion was transferred to vials and analyzed by a Turbiscan (Lab Expert analyzer, Formulation, France) for daily measurements of emulsion stability. The rest of the emulsion was equally divided into three sealed vials and stored at 37 °C in the dark with constant magnetic stirring. Samples stored in the three sealed vials were collected on days 0, 1, 4 and 7 to determine the physical and oxidative stability.

Physical Properties of the Emulsions. Droplet Size. The size distribution of emulsion droplets was determined at room temperature after appropriate dilution on a laser light scattering instrument (Mastersizer 3000, Malvern Instruments, Ltd., Worcestershire, U.K.). The mean droplet diameters were expressed as Sauter diameters ($d₅₂$).

Morphology. A drop of emulsion was placed on a slide glass and observed using an optical microscope equipped with an AxioCam camera under a 100× objective (Axio Scope A1, Carl Zeiss, Oberkochen, Germany).

Turbiscan. The physical stability of emulsion was monitored by a Turbiscan Lab Expert analyzer (Formulation, France) for 7 days at 25 °C. The vials containing 20 mL of emulsion were scanned from the bottom to the top by a light beam emitted in near-infrared light (λ = 880 nm). Detectors that moved synchronously along the same height measured the intensity of transmitted and backscattered light at 180° and 45°, respectively. The analysis of stability was carried out as a variation of the change of backscattering (ΔABS) calculated as the difference between the backscattering intensity at 0 h and a given time. The Turbiscan stability index (TSI) was calculated with Turbiscan software, version 1.2, and an increase in TSI indicated decreased system stability.\(^24\) TSI is the sum of all of the scan differences in the measuring cell calculated on the basis of the changes in backscattering values and sample height.\(^25\)

Protein Adsorption Fraction ($F_{ads}$) and Surface Load ($Γ$). Unadsorbed and adsorbed proteins were recovered as previously described, with minor modifications.\(^25\) Briefly, 5 mL of emulsion was centrifuged at 35000g for 60 min at 4 °C. The top cream phase containing adsorbed proteins was carefully collected and dispersed in 5 mL of 25 mM SPB (pH 8.0). The bottom aqueous phase and precipitates containing unadsorbed proteins were collected for measurement of the protein content by the Biuret method.

Protein adsorption fraction ($F_{ads}$) refers to the fraction of protein adsorbed onto the droplets and was calculated as follows:\(^26\) $F_{ads} = (C_1 - C_{eq})/C_1 × 100$, where $C_1$ is the initial protein concentration per unit volume of emulsion (kg/m³) and $C_{eq}$ is the unadsorbed protein concentration per unit volume of emulsion (kg/m³). The surface load ($Γ$) was calculated as follows: $Γ = (Γ_i - C_{eq}) × d_j/6d_0$, where $Γ_i$ and $C_{eq}$ are the same as those in the equation for $F_{ads}$, $d_i$ is the...
mean droplet diameter determined as described below, and $\Phi$ is the oil volume fraction (0.05).

**Lipid Oxidation.** Lipid oxidation was evaluated by formation of conjugated dienes (CDs) and hexanal as previously described. Briefly, CDs were extracted by isooctane/isopropanol (2:1, v/v) and then centrifuged. Then, the upper organic phase was collected and diluted with isooctane and measured for absorbance at 234 nm. Hexanal was measured by headspace solid-phase microextraction combined with gas chromatography–mass spectrometry (HS-SPME–GC–MS).

**Protein Oxidation.** Carbonyl Content. The carbonyl content was determined by the 2,4-dinitrophenylhydrazine (DNPH) assay, with minor modifications. Briefly, 400 μL of emulsion was treated with 0.8 mL of 0.3% (w/v) DNPH (Sigma-Aldrich) in 3 M HCl or 3 M HCl (as the blank) for 30 min. Then, the mixture was precipitated by 400 μL of 40% TCA. The pellet was collected by centrifugation at 5000 g for 5 min, washed 3 times with ethanol/ethyl acetate (1:1 v/v), dried with nitrogen, and dissolved in 6.0 M guanidine hydrochloride. Then, the absorbance was measured at 280 and 370 nm. The carbonyl content was calculated by the following equation:

$$\text{carbonyl content (nmol/mg of protein)} = \frac{\text{Abs}_{280} - \text{Abs}_{270} (\text{blank})}{22000[\text{Abs}_{340} - (\text{Abs}_{370} - \text{Abs}_{370} (\text{blank})) \times 0.43]} \times 10^6$$

where 22 000 is the molar extinction coefficient and 0.43 is the coefficient for removing potential hydrazine interference at 280 nm.

Free Sulfhydryl Content. Free sulfhydryl was determined using the 5,5′-dithio-2-nitrobenzoate (DTNB) assay, with minor modifications. Briefly, 1 mL of emulsion was mixed with 5 mL of acetone and centrifuged at 3000 g for 15 min. The pellet was dried by nitrogen and then dissolved in 5 mL of 0.1 M Tris–HCl (pH 8.0). After that, 1 mL of the solution was removed for determining the protein content by the Biuret method. Another 1 mL was treated with 250 μL of 10 mM DTNB (Sigma-Aldrich) in 0.1 M Tris–HCl (pH 8.0) for 30 min. Absorbance at 412 nm was read before and after incubation for determining the free sulfhydryl content with a molar extinction coefficient of 14.150 M$^{-1}$ cm$^{-1}$.

Tryptophan Fluorescence. The loss of tryptophan fluorescence was measured as another index for protein oxidation. Briefly, the emulsion or adsorbed or unadsorbed protein was diluted to 20 μg of protein/mL of water. Emission spectra of tryptophan fluorescence were recorded from 310 to 400 nm at Ex of 295 nm with a slit width of 7 nm and speed of 180 nm/min. Tryptophan standards was used as quality control for the fluorescence measurements.

**Statistical Analysis.** Statistical data analysis was conducted on SAS software (version 9.4, SAS Institute, Cary, NC, U.S.A.). To compare treatment effects among groups, one-way analysis of variance (ANOVA) and Tukey’s post hoc testing was used. To compare time-dependent changes in each group, repeated measure (RM) one-way ANOVA with Tukey’s or Dunnett’s test was used. The hydrolysis was performed once for each DH, and the resulting hydrolysates were used to prepare three batches of emulsions at each DH. Data were expressed as the mean ± standard deviation (SD), and the significant level was set at $\alpha = 0.05$.

### RESULTS AND DISCUSSION

**Changes of the FBPI Structure and Physicochemical Properties Induced by Alcalase Hydrolysis.** Alcalase treatment induced significant changes in the FBPI structure indicated by SDS–PAGE. The native FBPI showed three main bands with MWs of ~37, ~21, and ~50 kDa (lane 1 of Figure 1), which corresponded to $\alpha$- and $\beta$-subunits of legume-like 11S globulins and their intermediary subunit, respectively. DH0-FBPI showed a pattern similar to native FBPI, indicating that the inactivation of Alcalase was complete and the presence of Alcalase did not affect the SDS–PAGE pattern (lane 2 of Figure 1). DH of 4% noticeably reduced the three main bands, with concomitantly increasing appearance of protein bands with MW of <18 kDa (lane 3 of Figure 1). Further hydrolysis with DHEs of 9 and 15% produced bands that were all under MW of 15 kDa. This indicated that Alcalase effectively cleaved FBPIs. Our preliminary experiments showed that the physicochemical properties of FBPI hydrolysates with DHEs of 1, 2, and 3% followed a pattern similar to DH of 4%. However, DH of 4% showed higher surface hydrophobicity, which is critical for emulsifying functionality. Thus, 4% was chosen as the lowest degree of hydrolysis.

In accordance with reduced molecular size, Alcalase hydrolysis improved the FBPI solubility by 6–10% at pH 8 (Table 1). The highest solubility was found with DH15-FBPI. This might be because smaller peptides produced by hydrolysis can form stronger hydrogen bonds with water and become more soluble. Alcalase hydrolysis also increased the electronegativity of FBPI at pH 8 (Table 1). This might be attributed to the increased number of peptides and exposure of ionizable amino acids, according to Mahmoud et al. In addition, Paulson and Tung proposed that dissociation of the carboxylic group at pH 8 by enzymatic hydrolysis could produce more carboxylate ions (COO$^-$), thus increasing electronegativity as well. This increase in net charge could, in turn, improve protein solubility as a result of the higher repulsive electrostatic force between the molecules.
DH of 4% led to an 18% rise in hydrophobicity (Table 1). In contrast, DHs of 9 and 15% resulted in markedly decreased hydrophobicity. Limited hydrolysis by Alcalase has a preferred specificity for hydrophobic peptides and increases the hydrophobicity by exposing more of the embedded hydrophobic amino acid residues to the solvent.13,35 On the other hand, extended hydrolysis led to decreased hydrophobicity, which could be attributed to enzymatic breakdown of hydrophobic areas and the reburying of exposed hydrophobic residues via hydrophobic interactions as previously suggested.3,14

**Physical Stability of Emulsions.** The changes in droplet size were shown in Table 2, as an index for physical stability of emulsions. From day 0 to day 7, the droplet sizes ($d_{3,2}$) of DH9 and DH15 emulsions greatly increased by more than 10-fold. In contrast, the levels of $d_{3,2}$ in DH0 and DH4 emulsions were maintained during 7 days of storage. This was in accordance with the microscopy results (Figure 2). DH0 and DH4 emulsions displayed homogeneous distribution on the first day and appeared to remain relatively stable after 7 days of storage. In contrast, visible droplet coalescence was observed in DH9 and DH15 emulsions after 7 days. To better characterize the phenomena of destabilization, Turbiscan backscattering data were plotted against sample height over time. With the DH9 emulsion taken as an example (Figure 3A), the apparent deviations among the scans at the same time point on different days were observed. The level of $\Delta S$ continued to decrease during storage, suggesting flocculation or coalescence. This was in accordance with larger fat globules shown by microscopy observation. Meanwhile, the $\Delta S$ signal increased at the top of the sample vial, suggesting that a concomitant creaming took place. We further determined the changes in the TSI value, which reflects the destabilization of emulsions by summing up variations, including creaming, coalescence, and/or flocculation.36 In comparison to DH9 and DH15, DH0 and DH4 emulsions showed much lower TSI values during 7 days of storage, indicating better physical stability (Figure 3B).

To understand the underlying cause of differences in physical stability as a result of different DHs, the protein adsorption fraction ($F_{ads}$ %) in emulsions was determined (Table 3). On day 0, DH4 emulsion tended to have a 12% higher $F_{ads}$ value than DH0 emulsion ($p = 0.06$), suggesting increased surface coverage at the interfacial layer. DH15 emulsion exhibited the highest $F_{ads}$ value. However, the $F_{ads}$ values in DH9 and DH15 emulsions were decreased by 12 and 17% by day 7, suggesting a dramatic release of protein from the O/W interface. The decreased physical stability in DH9 and DH15 emulsions could be further explained by the surface load ($\Gamma$). The surface load corresponds to the mass of emulsifier required to cover a unit area of droplet surface (usually expressed as mg/m$^2$); smaller values of $\Gamma$ indicate greater effectiveness of an emulsifier because it could cover greater O/W interfacial area at a fixed amount.20 In comparison to FBPI hydrolysates with DHs of 4 and 0%, which had similar $\Gamma_c$ of $\sim$4 mg/m$^2$, hydrolysates with DHs of 9 and 15% had $\Gamma_c$ of $\sim$10 mg/m$^2$, indicating relatively lower emulsifying capacity (Figure 3C). Our results are in agreement with previous findings showing that moderate hydrolysis could produce flexible peptides with increased hydrophobicity, which could facilitate the anchor to the O/W interface and, thereby, improve the emulsifying property.13,37,38 On the other hand, smaller peptides produced by extensive DH can be more readily desorbed than intact proteins as a result of the high net charge and reduced adsorption layer thickness.37,39 Collectively, the changes in droplet size, TSI, and microscopy structure of the emulsions suggested that moderate hydrolysis of proteins can produce polypeptides with a suitable molecular size and hydrophobicity to stabilize the emulsions, while extensive hydrolysis would negatively affect the emulsifying ability.

**Oxidative Stability of Emulsions.** Oxidative stability is critical for the quality of emulsion-based foods, but it is an underestimated index for assessing emulsifying functionalities of plant proteins.10 We monitored lipid and protein oxidation during storage and investigated the association between physical stability and oxidative stability of emulsions stabilized by FBPI hydrolysates. Progression of lipid oxidation took place in all emulsions, as indicated by increased formation of CDs above the baseline after 4 days of storage and reached a maximum at day 7 with increases of 1.9–4.0-fold (Figure 4A). DH4 emulsion displayed significantly lower CDs on days 4 and 7 compared to DH0 emulsion. In contrast, DH9 and DH15 had a similar or greater level of CDs than DH0 and DH4 emulsions. The formation of hexanal showed similar trends as CDs, with DH4 emulsion being the least oxidized (Figure 4B).

### Table 1. Physiochemical Properties of FBPI Hydrolysates (Mean ± SD)$^{14}$

| solubility (%) | $\zeta$ potential (mV) | surface hydrophobicity ($\Gamma_{0-ANS}$) |
|----------------|------------------------|----------------------------------------|
| DH0-FBPI       | 75.0 ± 1.5 a           | −419 ± 0.9 a                           |
| DH4-FBPI       | 79.2 ± 2.3 ab          | −430 ± 1.1 b                           |
| DH9-FBPI       | 81.4 ± 0.1 ab          | −447 ± 3.7 c                           |
| DH15-FBPI      | 82.6 ± 2.0 b           | −514 ± 1.6 d                           |

$^{14}$FBPIs were incubated with Alcalase (0.01 AU/g of protein) at 50 °C and pH of 8.0 with DHs of 4% (DH4-FBPI), 9% (DH9-FBPI), and 15% (DH15-FBPI). DH0-FBPI represented inactivated Alcalase-treated FBPI. Significant differences were denoted by different letters, determined by one-way ANOVA with Tukey’s test ($p < 0.05$).

### Table 2. Mean Droplet Sizes of Emulsions Stabilized by FBPI Hydrolysates (Mean ± SD)$^{14}$

| time (day) | $d_{3,2}$ (nm) |
|------------|----------------|
|            | DH0           | DH4           | DH9           | DH15          |
| 0          | 57 ± 1 a      | 52 ± 1 a      | 155 ± 12 b    | 107 ± 1 b    |
| 1          | 117 ± 1 a     | 71 ± 1 a      | 1240 ± 185 b  | 1363 ± 45 b  |
| 4          | 116 ± 4 a     | 144 ± 27 a    | 1679 ± 12 b   | 1603 ± 52 b  |
| 7          | 234 ± 70 a    | 111 ± 16 a    | 1568 ± 332 b  | 2097 ± 1480 b |

$^{14}$DH0, DH4, DH9, and DH15 emulsions were prepared with 1% (w/v) control FBPI or hydrolyzed FBPIs with DHs of 4, 9, and 15% and stored at 37 °C in the dark for 7 days. Different lowercase letters indicated significant group differences on each day, determined by one-way ANOVA with Tukey’s test ($p < 0.05$). Different capital letters indicated significant differences among different days within each group, determined by RM one-way ANOVA with Tukey’s test ($p < 0.05$).
Figure 2. Microscopic pictures of emulsions at days 0 and 7. DH0, DH4, DH9, and DH15 emulsions were prepared with 1% (w/v) control FBPI or hydrolyzed FBPIs with DHs of 4, 9, and 15% and stored at 37 °C in the dark for 7 days.

Figure 3. (A) ΔBS in DH9 emulsion, (B) TSI, and (C) surface load ($\Gamma_{sat}$) in all emulsions stored at 37 °C in the dark for 7 days. DH0, DH4, DH9, and DH15 emulsions were stabilized by 1% (w/v) control FBPI or hydrolyzed FBPIs with DHs of 4, 9, and 15% and stored at 37 °C in the dark for 7 days. Significant differences ($p < 0.05$) were denoted by different letters.

Table 3. Protein Adsorption Fraction ($F_{ads}$, %) in Emulsions Stabilized by FBPI Hydrolysates (Mean ± SD)*

| time (day) | DH0        | DH4        | DH9        | DH15       |
|------------|------------|------------|------------|------------|
| 0          | 20.0 ± 1.1 aA | 22.4 ± 0.9 abA | 18.7 ± 1.4 aA | 27.0 ± 1.8 bA |
| 1          | 26.9 ± 0.6 abA | 20.2 ± 2.5 cA | 9.5 ± 1.2 dB | 24.8 ± 1.7 abA |
| 4          | 21.7 ± 12.2 aAB | 22.4 ± 10.8 aA | 3.2 ± 0.9 cB | 10.2 ± 1.7 bB |
| 7          | 28.5 ± 10.0 aAB | 21.7 ± 9.0 aA | 6.5 ± 1.9 bB | 9.8 ± 1.7 bB |

*DH0, DH4, DH9, and DH15 emulsions were prepared with 1% (w/v) control FBPI or hydrolyzed FBPIs with DHs of 4, 9, and 15% and stored at 37 °C in the dark for 7 days. Different lowercase letters indicated significant group differences on each day, determined by one-way ANOVA with Tukey’s test ($p < 0.05$). Different capital letters indicated significant differences among different days within each group, determined by RM one-way ANOVA with Tukey’s test ($p < 0.05$).
These results suggested that moderate hydrolysis with Alcalase improved the ability of FBPI to maintain stability toward lipid oxidation in O/W emulsions. It has been proposed that moderate hydrolysis might improve the antioxidant function of native proteins and the hydrolysates could more effectively scavenge free radicals and chelate transition metal ions, therefore slowing lipid oxidation in emulsions.\textsuperscript{18} Similarly, fish protein hydrolyzed by Alcalase to DHs of 3 and 4\% exhibited improved oxidative stability in O/W emulsion.\textsuperscript{40}

Because adsorbed and unadsorbed proteins in emulsions play different roles in inhibiting lipid oxidation at the expense of protein oxidation,\textsuperscript{1,10} we further determined tryptophan FI in adsorbed and unadsorbed protein recovered by ultracentrifugation. Figure 4C. The percentage change of tryptophan FI from day 0 to day 7 in adsorbed and unadsorbed protein recovered by ultracentrifugation, (C) carbonyl content, (E) free sulphydryl content, and (F) tryptophan FI in emulsions. DH0, DH4, DH9, and DH15 emulsions were prepared with 1\% (w/v) control FBPI or hydrolyzed FBPIs with DHs of 4, 9, and 15\% and stored at 37 °C in the dark for 7 days. Different letters indicated significant group differences on each day, determined by one-way ANOVA with Tukey’s test ($p < 0.05$). ($\star$) Significant differences from day 0 within each group, determined by RM one-way ANOVA with Dunnett’s test ($p < 0.05$). ($\#$) Significant difference between adsorbed and unadsorbed protein within each group, determined by the $t$ test ($p < 0.05$).
contribution of unadsorbed protein to oxidative stability seems to be dominant. However, it should be stressed that protein oxidation might occur during the isolation of adsorbed and unadsorbed proteins. Advances in measuring protein oxidation in real-time might provide more insight into the respective roles of adsorbed and unadsorbed protein on lipid oxidation.

Dietary protein oxidation is linked to in vivo protein oxidation and contributes to aging and age-related diseases, such as Alzheimer’s disease, Parkinson’s syndrome, rheumatoid arthritis, muscular dystrophy, cataractogenesis, etc. Extensive hydrolysis induced more protein oxidation as DH9 and DH15 emulsions displayed a higher level of protein carbonyls, less free sulfhydryl groups, and lower tryptophan FI compared to DH0 emulsion (panels D–F of Figure 4). However, protein oxidation in DH9 and DH15 emulsions did not seem to convey protective effects against lipid oxidation because these emulsions also had more lipid oxidation products (panels A and B of Figure 4). This suggested that the inhibition of lipid oxidation by proteins at the expense of protein oxidation may also depend upon the physical stability. Although DH4 emulsion had greater initial protein oxidation than DH0 emulsion, the progression of protein oxidation in DH4 emulsion was the slowest among all emulsions. By day 7, the carbonyl content increased by 56% in DH4 emulsion, whereas there were 237, 132, and 71% increases in DH0, DH9, and DH15 emulsions, respectively. In addition, DH4 emulsion had lower carbonyl but greater tryptophan content than DH9 and DH15 emulsions, indicating better oxidative stability.

The DH seemed to be a critical factor for the emulsifying functionality of FBPI hydrolysates. Hydrolysis with DHs of 9 and 15% induced unduly decreased surface hydrophobicity and higher surface load \( (Γ_0') \), which eventually led to decreased physical stability. In addition, the DH9 and DH15 emulsions had larger droplet sizes than the DH0 and DH4 emulsions. A larger droplet size might promote the emulsions to cream (suggested by the backscattering data, ΔBS), which could expose the oil droplet more directly to oxygen in the headspace. Moreover, extensive hydrolysis produced increased negative surface charges of FBPI. It is possible that an increased negative charge might attract pro-oxidative cationic metal ions. Therefore, extensive hydrolysis not only negatively affected the emulsifying capacity of FBPI but also led to decreased emulsion stability. On the other hand, moderate hydrolysis (DH of 4%) produced a suitable lower molecular mass with a more flexible peptide structure, which allowed for greater mobility at the interface and better penetration into oil, moderately increased surface charges that produced increased repulsive electrostatic force, and increased hydrophobicity for better emulsifying capacity and steric stabilization. These resulted in a more effective and stable interfacial barrier indicated by a decreased surface load \( (Γ_0') \) and a constant protein adsorption fraction \( (F_{ads}) \) in DH4 emulsion during storage. More importantly, FBPI hydrolysates with DH of 4% significantly inhibited the lipid oxidation in emulsions without impairing protein oxidative stability.

In summary, this study demonstrated that moderate Alcalase hydrolysis (DH of 4%) on FBPI improved both physical and oxidative stability of hydrolysate-stabilized O/W emulsions, as evidenced by the homogeneous droplet size, lower TSI, and markedly reduced CDs and hexanal production during storage. Hydrolysis at DH of 4% produced a suitable lower molecular mass that could result in a more flexible peptide structure, increased surface charge, and hydrophobicity that favored emulsifying activity. On the other hand, undue hydrolysis should be avoided because it might negatively affect the emulsifying activity of FBPI and the oxidative stability of the emulsion.

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