Effect of limited enzymatic hydrolysis on structure and emulsifying properties of rice glutelin

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Abstract

The influence of the degree of hydrolysis (DH) on the structure, solubility, rheology, and emulsifying properties of rice glutelin was investigated. Structural properties were characterized using analysis of molecular weight distribution, surface hydrophobicity, intrinsic fluorescence, and Circular Dichroism (CD) spectra. Protein hydrolysis changed molecular weight, increased flexibility, altered surface hydrophobicity, and increased solubility. Oil-in-water emulsions were prepared from rice glutelin with different DH (0.5%, 2%, and 6%) and their stability to storage, pH, salt, and thermal processing was assessed. The storage, pH, salt, and temperature stability of the emulsions increased with decreasing hydrolysis. Emulsions prepared with 2% DH rice glutelin were stable over a range of environmental conditions: pH 7-9; NaCl < 100mM (pH 7); temperatures < 90 °C (pH 7, 0 mM NaCl) studied. These results will facilitate the formulation and production of natural emulsion-based products using rice glutelin as an emulsifier.

Keywords: rice glutelin; enzymatic hydrolysis; emulsion; stability;

1. Introduction

The nature of the emulsifier used to formulate oil-in-water emulsions plays an important role in determining their structural, physicochemical and functional properties, such as droplet size, charge, interactions, stability, rheology, appearance, and digestibility (McClements, 2015). A wide variety of emulsifiers is available for utilization within the food industry, including synthetic surfactants, proteins, polysaccharides, and phospholipids (Kralova & Sjoblom, 2009). Nevertheless, there is currently considerable interest in the utilization of natural emulsifiers in foods due to consumer demands for natural products and clean labels (Ozturk & McClements, 2016). Many types of food proteins are amphiphilic molecules that can be used as emulsifiers to stabilize food emulsions (E. Dickinson, 2003; Eric Dickinson, 2011; Qian, Decker, Xiao, & McClements, 2011; Qiu, Sun, Zhao, Cui, & Zhao, 2013). The physicochemical and functional properties of emulsions depend on the interfacial characteristics of the protein molecules at the droplet surfaces, *e.g.*, adsorption kinetics, surface load, packing, thickness, electrical charge, rheology, and hydrophobicity (E. Dickinson, 2003; Murray, 2002). At present, the majority of protein-based emulsifiers used in the food industry are derived from dairy sources *e.g.*, whey proteins and caseins (Adjonu, Doran, Torley, & Agboola, 2014; Ozturk et al., 2016; Wilde, 2009). However, there is interest in developing plant-based alternatives to these proteins due to allergenicity, economic, sustainability and dietary issues (Karaca, Low, & Nickerson, 2015; Lam & Nickerson, 2013; Ozturk et al., 2016).

In this study, we focused on the emulsifying properties of proteins derived from rice, a highly abundant staple food. Rice protein is rich in essential amino acids, has a high nutritive value, lowers cholesterol, and is hypoallergenic (Du et al., 2013; Qiang Zhao, Selomulya, et al., 2012). Previously, rice proteins have been reported to have good emulsifying properties under acidic conditions (Romero et al., 2012), but not under neutral conditions (Liu et al., 2011), which was attributed to differences in the aggregation state of the proteins under different pH conditions. The functional

properties of proteins can often be improved by modification of their molecular structure and conformation using physical treatments (such as heating (Tang & Ma, 2009) or sonication (L. Jiang et al., 2014)) , chemical treatments (such as deamidation (Paraman, Hettiarachchy, & Schaefer, 2007) or glycosylation (Du et al., 2013; Li et al., 2009)), or enzymatic treatments (Liu et al., 2011; Zheng et al., 2015). Moderate heating has been shown to significantly enhance the functional properties of vicilin-rich protein isolates (such as solubility, emulsifying activity, and foaming activity), while excessive heating has been shown to reduce these properties (Tang et al., 2009). Enzymatic treatments are often preferred for this purpose due to the mild reaction conditions required, and the high degree of specificity in altering molecular properties (Guan, Yao, Chen, Shan, & Zhang, 2007). In the current study, we focused on using hydrolytic enzymes to alter the degree of hydrolysis (DH) of rice glutelin so as to improve its solubility and emulsifying properties.

A number of previous studies have examined the factors that influence the emulsifying properties of rice protein hydrolysates. Studies have shown that combining rice protein hydrolysates (DH= 7.8%) with a non-ionic surfactant (Tween 20) can lead to the formation of more stable emulsions than possible with the protein hydrolysates alone (Qiang Zhao, Xiong, et al., 2012). Moreover, these combined rice protein hydrolysates/Tween 20 systems have been shown to have good antioxidant activity in emulsions (Cheetangdee & Benjakul, 2015). However, the utilization of a synthetic surfactant to improve the functional attributes of rice protein hydrolysates is undesirable for products that required an all-natural label. Some studies have shown that rice protein hydrolysates can adsorb to oil droplet surfaces but that they are not very effective in preventing coalescence, which may be because the interfacial layers formed are relatively thin and mobile (Adjonu et al., 2014; Lam et al., 2013). In general, emulsion stability depends on the effectiveness of the emulsifier at producing small droplets during homogenization, and then preventing their aggregation during storage or when environmental conditions are changed (McClements, 2015; Qian et al., 2011).

The ability of rice glutelin to form and stabilize emulsions is likely to depend on the degree of hydrolysis, since this will affect the molecular and functional characteristics of the hydrolyzed proteins. We therefore compared the influence of different degrees of hydrolysis on the molecular weight distribution, conformation, surface hydrophobicity, and functionality (solubility, rheology, and emulsifying properties) of rice glutelin. In addition, the impact of different environmental conditions (pH, ionic strength, and temperature) on the stability of the emulsions formed was examined so as to elucidate conditions where the proteins could be effectively used as ingredients in foods. This study is important for the identification of natural hypoallergenic protein emulsifiers that can be used in the development of label-friendly emulsion systems.

2. Materials and Methods

2.1. Materials

Rice protein (90.3 wt%, dry basis) was kindly provided by Golden Agriculture Biotech Company Limited (Jiangxi, China). Sodium chloride, sodium azide, trypsin, and other chemicals were of analytical grade and were obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO).. Double-distilled water was used throughout to prepare all solutions and emulsions.

2.2. Extraction of rice glutelin

Rice glutelin was extracted from rice protein based on its differential solubility in different solutes using a procedure described previously (Ju, Hettiarachchy, & Rath, 2001; Likitwattanasade & Hongprabhas, 2010). Rice protein was washed with 5% NaCl solution, 70% ethanol and distilled water until no protein was detected in the washing liquor. The final glutelin was recovered by centrifugation at 3000 g for 30 min, freeze-dried, and stored at -20 °C. The protein content of rice glutelin was 93.42% (dry basis) as determined by the Kjeldahl method ($N\% \times 5.95$) (Qiang Zhao, Selomulya, et al., 2012).

2.3. Preparation of rice glutelin hydrolysates

Rice glutelin was hydrolyzed by trypsin at an optimized enzyme/substrate ratio according to preliminary experiments. 2 g of rice glutelin was dispersed in 30mL deionized water and stirred for 1h. After pH and temperature adjustments (pH=8, 50 °C), Trypsin was added. Inactivation of the enzyme was achieved by heating to 95 °C for 10 min followed by immediate cooling in water to room temperature. The mixture was adjusted to pH 7.0, followed by centrifugation (TGL-20B, Anting Scientific Instrument Factory, Shanghai, China) at 4800 g for 10 min and the supernatant was freeze-dried. The hydrolysates formed had 0.5%, 2% and 6% DH, and were therefore referred to as DH0.5RG, DH2RG and DH6RG, respectively.

2.4. Estimation of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the number of hydrolyzed peptide bonds (h) relative to the number of peptide bonds per unit weight (h_{tot}) expressed as a percentage, was determined by using the pH-stat method (Adler-Nissen, 1986) and calculated according to the following equation:

$$DH = \frac{h}{h_{tot}} \times 100\% = \frac{BN_b}{\alpha h_{tot} M_p} \times 100\% \quad (1)$$

Where B is base consumption (mL), N_b is the molarity of the base used, M_p is the mass (g) of the protein, h_{tot} is the theoretical overall number of peptide bonds in the protein substrate (7.40 meq/g rice protein) (Qiang Zhao, Xiong, et al., 2012), and α is the average degree of dissociation of the α -NH₂ groups expressed as:

$$\alpha = \frac{10^{pH-pK_a}}{1 + 10^{pH-pK_a}} \quad (2)$$

Here pH is the value at which the enzyme hydrolysis was conducted, and pK_a is the average pK_a for α -NH₃⁺ groups on the protein. The value of α was 0.885 at pH 8 and 50 °C treatment (Adler-Nissen, 1986; García-Moreno et al., 2014).

2.5. Structural characteristics of hydrolyzed rice glutelin

2.5.1. Molecular weight distribution

A high performance size exclusion chromatography (SEC-HPLC) system (Agilent 1260 series) equipped with an appropriate column (Shodex KW804) was used according to the methods described previously (Liu et al., 2011) with some modification. Protein samples were dissolved in 100 mM phosphate buffer (pH 7.0) containing 20 mg/mL SDS. The supernatants were collected after centrifuged at 4800 g for 10 min. All protein solutions were filtered through 0.45 μ m cellulose acetate membranes (Millipore, Billerica, MA) before use. The eluent used was 200 mM phosphate buffer (20mg/mL SDS, pH 7.0) at a flow rate of 1 mL/min used at room temperature. The UV absorbance at 220 nm was performed to monitor protein elution.

2.5.2 Sodium dodecyl sulfide polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of protein samples was performed according to a method described previously (Laemmli, 1970) by using 12% separating gel and 5% stacking gel. Protein samples were mixed with the sample buffer: 0.5 M Tris-HCl, 10% glycerol, with or without 5% β -mercaptoethanol (β -ME), and 1% (w/v) bromophenol blue (pH 6.8). The mixtures were heated at 100 °C for 10 min, and then centrifuged at 10,000 rpm for 30 s. A sample containing 40 μ g of protein was loaded into each well, and subjected to electrophoresis at a voltage of 60 V in the stacking gel and 80 V in the separating gel until the tracking dye reached the bottom of the gel. Then the gels were stained for 2 h using 0.05% Coomassie Brilliant Blue R-250 in acetic acid/methanol/water solution (46:227:227, v/v/v) and destained for 6 h with methanol/acetic acid/water solution (50:75:875, v/v/v).

2.5.3. Surface hydrophobicity

The surface hydrophobicity (H_0) of the proteins was determined as reported previously (Zheng et al., 2015) using 1-anilino-8-naphthalene sulphonate (ANS) as a hydrophobic fluorescence probe. Protein solutions were diluted with 10 mM sodium phosphate buffer (pH=7.0) to give concentrations ranging from 0.01 mg/mL to 0.1

mg/mL. 20 μ L of ANS solution (8 mM) was added to 2 mL of sample solution and incubated in the dark for 20 min at ambient temperature. An ANS blank was also prepared, which consisted of 2 ml buffer and 20 μ L of the ANS probe. The fluorescence intensity was measured using the excitation and emission wavelengths of 390 and 470 nm, respectively. The excitation and emission slits used were both 2.5 nm. The initial slope of the relative fluorescent intensity vs. sample concentration (mg/mL) plots was evaluated by linear regression analysis and used as an index of H_0 . Each spectrum was corrected with respect to the corresponding blank.

2.5.4. Intrinsic fluorescence

The fluorescence emission spectrum of samples was performed using a fluorescence spectrofluorimeter (F-7000, Hitachi, Tokyo, Japan) according to a method described previously (Avramenko, Low, & Nickerson, 2013) with a slight modification. 0.01mg/mL of protein samples were prepared using 10 mM phosphate buffer (pH 7). The intrinsic fluorescence spectra were then obtained using an excitation wavelength of 280 nm and acquiring emission spectra between 300 and 500 nm. The excitation and emission slit were both adjusted to 2.5 nm during these measurements. The fluorescent intensity (FI) was reported as a function of emission wavelength. All fluorescence experiments were performed in triplicate.

2.5.5. Circular dichroism studies

The far-UV CD spectra were determined on a Bio-Logic MOS-450 CD spectrometer (French Bio-Logic SAS, Claix, French) according to a method described previously (Du et al., 2013) using a 1.0 mm path length quartz cuvette. 0.1 mg/mL of protein solutions were prepared by dissolving samples in 10 mM phosphate buffer (pH 7.0). All the samples were scanned from 190 to 250 nm and the spectrum of the protein-free buffer was extracted from that of the sample. The analysis of the CD spectroscopic data was carried out using the online SELCON3 method in DICHROWEB.

2.6 Protein solubility

Protein solubility was analyzed using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) with bovine serum albumin as standard. An aqueous solution (1%, w/v) of protein sample was stirred magnetically at 25 °C for 30 min, and then the pH was adjusted to 3 to 10 using either 0.5 M HCl or 0.5 M NaOH. After 30 min of stirring, the pH was readjusted if necessary. The dispersions were centrifuged at 4800 g for 15 min, and the protein content of the supernatant was analyzed. Protein solubility was expressed as the amount of protein in the supernatant relative to the total protein content in the dispersion. All measurements were conducted in triplicate.

2.7. Emulsion preparation

An emulsifier solution was prepared according to a method described earlier (Qiu, Zhao, & McClements, 2015) with some slight modifications. The protein samples were dispersed into 10 mM phosphate buffer (pH 7.0) and stirred for 3 h to ensure complete hydration. Then the solution was centrifuged at 4000 g for 15 min and the final supernatant was collected. A coarse emulsion was prepared by pre-homogenizing 10 mL of corn oil with 90 mL of emulsifier solution (5 mg/mL) using a high-shear mixer (M133/1281-0, Biospec Products Inc., ESGC, Switzerland) at 12000 rpm for 2 min, and then emulsified using a Microfluidizer (NCJJ-0.007/200, Langfang Tongyong Machinery Manufacturing Co., Ltd, China) for 3 times at 80 MPa. 0.01% (w/w) of sodium azide was then added to the emulsions to prevent microbial growth.

2.8. Emulsion characterization

2.8.1 Flow behaviour measurements

The rheological properties of the samples were characterized according to the methods described previously (Qiangzhong Zhao et al., 2015) using a dynamic shear rheometer (Paar Physica rheometer, MCR 302, Anton Paar GmbH, Germany) with a parallel-plate geometry (20 mm diameter and 1 mm gap) at 25 °C. The extra sample around the edge of the plates was trimmed off, and a thin layer of mineral oil was applied to prevent moisture loss of the samples during measurements. The shear stress

was then measured as the shear rate was increased from 5 to 100 s⁻¹. The power law model was used to calculate the flow behaviour index and consistency coefficient of the samples:

$$\tau = K\dot{\gamma}^n \quad (3)$$

Here, τ is the shear stress (mPa); K is the consistency coefficient (mPa sⁿ); $\dot{\gamma}$ is the shear rate (s⁻¹); and n is the flow behaviour index.

2.8.2. Particle size and surface charge (ζ -potential) measurement

The particle size and ζ -potential of the emulsions were measured by dynamic light scattering (NicompTM 380 ZLS, PSS Nicomp, Santa Barbara, CA, USA). Prior to measurement, the emulsions were diluted 100-fold with the working buffer to avoid multiple scattering during measurements. Duplicate measurements were performed at 25 °C on each sample.

2.8.3. Influence of environmental stress on emulsion stability

The emulsions were exposed to a number of environmental stresses to simulate the conditions that they may experience during their application in commercial products:

2.8.3.1 Influence of pH

Freshly produced emulsions were placed into 50 mL beakers and each sample was adjusted to different pH values (3-9) by adding either HCl or NaOH solutions. The samples were then transferred to 10 mL test tubes and stored at 25 °C for 24 h prior to particle size and ζ -potential analysis.

2.8.3.2 Influence of NaCl

Freshly prepared emulsions were transferred into 10 mL test tubes, and then varying ratios of 6M NaCl solution and buffer solution were added to obtain samples with various final NaCl concentrations (0-300mM). The samples were vortexed and stored for 24 h at 25 °C prior to particle size and ζ -potential determination.

2.8.3.3 Influence of temperature

Freshly prepared emulsions were transferred into 10 mL test tubes, which were

then were placed into a water bath set at different temperatures (30-90 °C) for 30 min and cooled to ambient temperature immediately. The samples were vortexed and stored for 24 h at 25 °C prior to particle size and ζ -potential determination, and for 15 days prior to visual observation of creaming stability.

2.9 Statistical analyses

All the experiments were conducted in triplicate and the data are expressed as means values $\pm$ standard deviation (SD). Significant differences between means ($p < 0.05$) were studied by one-way ANOVA test using SPASS 19.0 (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1 Molecular weight distribution of glutelin hydrolysates

Size exclusion chromatography was used to determine the molecular weight distribution of the native rice glutelin (RG) and the rice glutelin hydrolysates (RGH) (Fig.1). The RG chromatogram was characterized by two major peaks with a molecular weight > 678 kDa (P1) and 44 kDa (P2). The P1 peak has previously been attributed to large macromolecular complexes polymerized by disulfide bonds (Liu et al., 2011; Utsumi, 1992). The P2 peak was most likely to be the α -subunit and β -subunit of RG. Upon hydrolysis, the area of the P1 peak significantly decreased, suggesting that RG was hydrolyzed by trypsin. The P1 peak was actually larger for the RG with DH 6% than with DH 0.5% and 2%, which suggests the formation of soluble peptide aggregates at high degrees of hydrolysis. Hydrolysis-induced aggregation of glutelin was also reported previously (Barac et al., 2012), indicating that protein aggregates could be produced during hydrolysis or during thermal inactivation of the enzyme. Furthermore, after hydrolysis the P2 peak disappeared, whereas two new peaks (P3 and P4) appeared. This suggested that RG was hydrolyzed into small peptides.

3.2 SDS-PAGE analysis

The polypeptide profiles of protein samples were measured under reducing and

non-reducing conditions to investigate changes of RG composition (Fig. 2). Under non-reducing conditions, the RG sample showed three major subunits (around 30, 20 and 12 kDa) (Fig.2a). The 30 and 20 kDa subunits correspond to the acidic α -subunit and alkaline β -subunit of RG, respectively. The 12 kDa fraction corresponds to prolamin, which is known to be an integral part of RG (Likitwattanasade et al., 2010; Shabbir, Anjum, Zahoor, & Nawaz, 2011; Zarins & Chrastil, 1992). Furthermore, high molecular weight (> 260 kDa) aggregates of RG were also observed at the top of the stacking and separating gels. Upon hydrolysis, the bands at 20 kDa and 30 kDa disappeared accompanied by appearance of new subunits < 14 kDa. These results support the conclusion that the protein structure changed upon treatment with trypsin (Avramenko et al., 2013). An increase of DH induced a shift of the RG band towards lower molecular weight regions. DH0.5 RG had major fractions with molecular weights <15 kDa while DH6 RG had more fractions with molecular weights <12 kDa. Our results match with previous studies (Avramenko et al., 2013), that reported that smaller lentil protein isolate sub-units were produced upon treatment with trypsin under non-reducing conditions, while few differences in SDS-PAGE profiles were observed for hydrolyzed samples.

The SDS-PAGE profiles measured under reducing conditions (Fig. 2b) were fairly similar to those measured under non-reducing conditions (Fig. 2a). However, some of the larger molecular weight fractions were not observed under reducing conditions, which suggested that some of the larger protein aggregates were disrupted in the presence of β -ME. These results suggest that these large aggregates were held together by disulfide bonds in both the native and hydrolyzed rice glutelin.

3.3 Surface hydrophobicity (H_0)

It is widely recognized that the surface hydrophobicity of proteins plays an important role in determining their conformation and functional properties (S. Damodaran, Parkin, & Fennema, 2007). The H_0 values of RG and RGH with different DH were therefore quantified (Fig. 3a). The H_0 value of the hydrolysates initially increased with increasing hydrolysis (0 to 2% DH), but then it decreased at the highest

DH value (6% DH). The increase in H_0 can be attributed to the fact that limited hydrolysis of protein was accompanied by partial unfolding of the protein structure and exposure of hydrophobic sites previously buried within the interior of the protein (Avramenko et al., 2013; Jung, Murphy, & Johnson, 2005). An increase in H_0 of proteins due to partial hydrolysis with trypsin has been reported previously (Guan et al., 2007), as has a decrease in H_0 upon further hydrolysis (Jung et al., 2005). According to previous studies (Avramenko et al., 2013; Jung et al., 2005), the decrease in hydrophobicity could be due to (1) enzymatic hydrolysis breakdowns hydrophobic areas that may contribute to surface hydrophobicity; (2) reducing the surface area of the hydrophobic groups exposed to the surrounding water by protein-protein aggregation. In summary, the RGH with a DH of 2% had the highest H_0 value.

3.4 Intrinsic fluorescence

Fluorescence intensity measurements provide an indication on the extent of exposure of aromatic amino acids to water, which is related to changes of protein tertiary conformation (Cui, Zhao, Yuan, Zhang, & Ren, 2013; Roger, 2004). The emission fluorescence spectroscopic for RG and RGH as a function of DH were therefore measured (Fig. 3b). The fluorescence intensities of RGH were found to be higher than that of RG, which can be attributed to the fact that more of the aromatic groups were exposed to water and more available to emit fluorescence (Roger, 2004; Sponton, Perez, Carrara, & Santiago, 2014). In agreement with the surface hydrophobicity measurements, the fluorescent intensity of the hydrolysates with 6% DH was lower than that with 2% DH, which suggests that less aromatic groups were exposed to water. This effect can again be attributed to hydrophobic attraction of the polypeptides and peptides at high degrees of hydrolysis, since the hydrophobic groups were re-buried within the interior of larger aggregates (Avramenko et al., 2013). A similar result has been reported for peanut protein isolate treated with alcalase (G. Zhao, Liu, Zhao, Ren, & Yang, 2011).

3.5 CD spectra analysis

A deeper understanding of RG and RGH secondary structure was obtained using CD spectra (Table 1). Compared with the native RG, the β -sheet regions in the RGH decreased from 30.9% to 21.1% by hydrolysis with DH of 6%, whereas the contents of random coil and α -helix regions increased from 29.7% to 38.6% and from 17.7% to 22.8%, respectively. It has been reported that the β -sheet structures (“trains”) are relatively stable, whereas the α -helix, β -turn, and random coil structures (“loops”) are relatively flexible and open (Srinivasan Damodaran, 2007; Yong, Yamaguchi, & Matsumura, 2006). This would suggest that enzyme hydrolysis led to a more extended form of the RG secondary structure, which was in agreement with the results of the intrinsic fluorescence measurements. According to previous studies (Yong et al., 2006), the secondary structure in wheat gluten becomes more flexible form after treatment with protein-glutaminase, with decreasing β -sheet regions and increasing α -helix, β -turn and random coil regions. Proteins with more flexible structures adsorb more easily to oil-water interfaces, and may therefore be better emulsifiers (Z. Jiang et al., 2015).

3.6 Protein solubility

Good water-solubility of proteins is often a prerequisite for their proper functioning as emulsifiers, and so we measured the influence of pH on the water solubility of the native protein and hydrolysates (Fig. 3c). The solubility of all samples exhibited U-shape curves, with minimal values around pH 5 for RG (2.2%) and around pH 4 for the hydrolysates (60-71%). Overall, the solubility of the hydrolysates was much higher than the native proteins, and the solubility increasing with increasing DH. The lower solubility at intermediate pH values can be attributed to the reduction in the electrostatic repulsion between the proteins around their isoelectric point (Du et al., 2012). The shift in the isoelectric point after partial hydrolysis has also been reported in earlier studies, and may be attributed to differences in the types and numbers of charged groups on the proteins after hydrolysis (Jamdar et al., 2010; Ochiai, Kamata, & Shibasaki, 1982). The poor solubility of RG may due to

the large rigid macromolecule structure, with subunits linked by multiple intermolecular and intramolecular disulfide bonds and hydrophobic interactions (Paraman et al., 2007). The disulfide bond content of glutelin has been reported to decrease after hydrolysis, which may lead to a decrease in protein aggregation and improvement in protein solubility (Zheng et al., 2015). The solubility of RGH was significantly higher than that of RG at all pH values ($p < 0.05$). Partial hydrolysis of other types of glutelin has also been reported to increase water-solubility, such as corn glutelin (Zheng et al., 2015) and barely glutelin (Xia, Bamdad, Gänzle, & Chen, 2012). Partial hydrolysis of hemp protein isolate has also been reported to improve its water-solubility (Yin et al., 2008). The improved solubility of RGH can be attributed to the reduction of molecular weight, the unfolding of the peptide chains, and the release of soluble aggregates from insoluble aggregates or precipitates (Ghribi et al., 2015; Zheng et al., 2015).

3.7 Emulsion stability

In this section, the influence of various environmental stresses on the physical state of oil-in-water emulsions was studied. Preliminary experiments showed that RG could not form stable emulsions. Instead, these systems rapidly separated into separate oil and aqueous phases, which suggested that the water-insoluble RG was unsuitable as an emulsifier. Consequently, only the three RGH (0.5, 2, and 6% DH) were used for the emulsion stability studies.

3.7.1 Storage stability

Changes in the size of the droplets in the emulsions were measured throughout 7 days of storage at ambient temperature (Fig. 4). The initial mean diameter of the droplets formed after homogenization increased with increasing hydrolysis: 0.5% DH (221 nm) < 2% DH (233 nm) < 6% DH (253 nm). This suggests that the protein hydrolysates with a lower DH were able to adsorb to the droplet surfaces more rapidly during homogenization, or that they were more effective at preventing recoalescence inside the homogenizer (Hakansson, Tragardh, & Bergenstahl, 2009). During storage

there were distinct differences between the ability of the hydrolysates to prevent particle growth. There was a slight increase in mean particle diameter over time for the 0.5% DH samples, little change for the 2% DH samples, and a relatively rapid increase for the 6% DH samples (Fig. 4). This result suggests that there was an optimum degree of hydrolysis required to prevent droplet aggregation during storage. A similar finding was reported for hydrolysates formed from whey proteins (Euston, Finnigan, & Hirst, 2001). It should be noted that the most likely origin for the measured increase in particle size over time was due to flocculation, as there was a corresponding increase in the viscosity of the samples (Section 3.7).

3.7.2 pH

The influence of pH on the mean particle diameter and ζ -potential of the different emulsions was measured (Fig. 5). All the emulsions exhibited fairly similar trends in the particle size versus pH profiles: extensive droplet aggregation occurred from pH 3 to 5, but the emulsions were stable from pH 7 to 9 (Fig. 5a). Visual observation of the samples also showed that the emulsions separated into a transparent serum layer at the top and a white cream layer at the bottom from pH 3 to 5, but were stable to creaming at pH 7 to 9 (data not shown). The creaming instability at lower pH values can be attributed to the fact that the creaming velocity increases with particle size (McClements, 2015). These results are consistent with previous studies that have reported that RG emulsions are highly unstable to aggregation and creaming near the proteins isoelectric point (Du et al., 2013).

To provide some further insights into the mechanism of emulsion instability, the electrical characteristics of the protein-coated droplets were measured at different pH values (Fig. 5b). For all the emulsions, the ζ -potential went from highly positive at low pH to highly negative at high pH, with a zero change at around pH 4.3. This finding can be attributed to the fact that the isoelectric point of the adsorbed layer of RG hydrolysates was around pH 4.3, which was in agreement with the solubility results. At low pH values, the H^+ concentration is relatively high, which causes the carboxyl groups ($-COOH$) and amino groups ($-NH_3^+$) to become protonated leading to a net

positive charge. Conversely, at high pH values, the H^+ concentration is relatively low, which causes the carboxyl groups ($-COO^-$) and amino groups ($-NH_2$) to be non-protonated leading to a net negative charge. At the isoelectric point, the number of positive and negative groups is balanced. The interfacial layers formed by globular proteins are known to be relatively thin, and so the major type of interactions preventing droplet aggregation is electrostatic repulsion (Qian et al., 2011). Thus, the reduction in the net droplet charge at pH values around the isoelectric may have promoted droplet flocculation since then the electrostatic repulsion was not large enough to overcome the van der Waals attraction (Ozturk, Argin, Ozilgen, & McClements, 2015).

3.7.3 Ionic strength

The influence of ionic strength on particle size and ζ -potential of the different emulsions (pH 7, ambient temperature) was measured (Fig. 6). All the emulsions were destabilized by addition of NaCl, as seen by an increase in the mean particle diameter. Droplet aggregation can be attributed to the ability of the counter ions (Na^+) in the salt to screen the electrostatic repulsion between the negatively charged droplets (Salminen & Weiss, 2014). When the NaCl concentration was above a critical level, the electrostatic repulsion could no longer overcome the attractive interactions (van der Waals and hydrophobic) between the droplets, thereby leading to aggregation. The oil droplets coated by the 2% DH rice glutelin showed the least change in particle size with increasing salt content in the range 0 to 100 mM NaCl.

The addition of NaCl led to a slight reduction in the magnitude of the negative charge on the droplets (Fig. 6b), which can again be attributed to electrostatic screening effects (Israelachvili, 2011).

3.7.4 Temperature

The effect of temperature on the mean particle diameter, ζ -potential, and creaming stability of the emulsions (pH 7, 0 mM NaCl) was measured (Fig. 7). The emulsions containing oil droplets coated by rice glutelin with a low level of hydrolysis (0.5 and 2%

DH) were relatively stable to thermal processing, with little change in mean particle diameter from 30 to 90 °C (Fig. 7a). Conversely, there was an appreciable increase in the particle size of the emulsions stabilized by the 6% DH rice glutelin upon heating. The increase in droplet aggregation in this sample may have occurred because of a decrease in the electrostatic repulsion between the droplets (Zhang, Wu, Yang, He, & Wang, 2012), or due to an increase in the exposure of hydrophobic groups during heating leading to an increase in electrostatic attraction and/or formation of disulfide bonds (Charoen et al., 2011). The ζ -potential measurements support the notion that there was an alteration in the interfacial composition or structure at the droplet surface for the 6% RH samples (Fig. 7b). In these emulsions there was an appreciable change in the ζ -potential of protein-coated droplets upon heating, but there was little change in the other samples. The reduction in the magnitude of the electrical charge on the 6% DH samples could have led to a reduction in electrostatic repulsion between the droplets, thereby promoting droplet flocculation. This change in electrical charge may have occurred because of a change in interfacial properties due to rearrangement or desorption of the peptide molecules at the droplet surfaces. These processes could have also led to an increase in surface hydrophobicity, which could also promote flocculation. A higher degree of hydrolysis may have led to smaller peptides that were more easily desorbed from the droplet surfaces. Interestingly, RGH with the lowest degree of hydrolysis (0.5 and 2% DH) did not show an appreciable change in ζ -potential or stability upon heating. Consequently, these hydrolysates may be more suitable for applications where thermal stability is required.

Visual observation of the emulsions supported the particle size measurements. The emulsions containing RGH with low levels of hydrolysis (0.5 and 2% DH) were relatively stable to creaming, whereas those with high levels of hydrolysis (6%) exhibited distinct phase separation (Fig. 7c). Indeed, heating caused these emulsions to form a watery layer at the bottom and a curd-like cream layer at the top.

3.8 Emulsion Rheology

The rheology of food emulsions is important in many practical applications, and

so the viscosity-shear rate profiles of the emulsions were measured using a shear rheometer during storage at ambient temperature for 7 days (pH 7, 0 mM NaCl). The resulting flow curves were then fitted to the power law model, and the consistency coefficient (K) and flow behaviour index (n) were calculated (Table 2). The flow behaviour index (n) is 1 for a Newtonian fluid and less than 1 for a shearing thinning fluid. Initially, all the emulsions had a relatively low consistency coefficient and exhibited weak shearing thinning behaviour. After 7 days of storage, there was little change in the K and n values of the emulsions stabilized by the 2% DH rice glutelin, suggesting that it was stable to droplet aggregation, which is in agreement with the particle size measurements for this sample (Fig. 4). Conversely, there was an appreciable increase in the consistency index of the other two emulsions, and shear-thinning behaviour was observed. The phenomenon is indicative of extensive droplet flocculation in the emulsions (Qiu, Zhao, Decker, & McClements, 2015; Taherian, Britten, Sabik, & Fustier, 2011; Tang & Liu, 2013), and is again consistent with the observed increase in particle size in these emulsions during storage (Fig. 4).

4. Conclusions

This study demonstrates that the stability of emulsions prepared using rice glutelin hydrolysates as emulsifiers depends on the degree of hydrolysis of the protein molecules. Hydrolysis of glutelin made the protein structure more flexible, and transformed the insoluble aggregates present in the native protein to soluble aggregates in the hydrolyzed protein. The surface hydrophobicity and intrinsic fluorescence of the hydrolysates first increased and then decreased as the degree of hydrolysis increased, which was attributed to exposure of buried hydrophobic groups to the outer surface of the molecule followed by protein aggregation through hydrophobic interaction. The rice glutelin hydrolysates were found to have much higher solubility than that of the original glutelin across the entire pH range studied. However, oil droplets stabilized by the rice glutelin hydrolysates were highly unstable to aggregation at pH values around the isoelectric point of the adsorbed proteins, and

upon addition of salt. These results highlighted the importance of electrostatic interactions in stabilizing the protein-coated droplets against aggregation. The rice glutelin with 2% DH gave emulsions that had the best storage stability and that were most resistant to salt addition and thermal processing. Overall, these results suggest that hydrolysis of native rice glutelin can be used to improve its functional properties so that it can be used as an effective emulsifier. However, further research is needed to improve the stability of the emulsions over a wide range of environmental conditions.

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700

Table 1 Secondary structure content of native and hydrolyzed rice glutelin (RG and RGH) determined by circular dichroism.

DH	Secondary structures (%)			
	α -helix	β -sheet	β -turn	Random coils
RG	17.7	30.9	21.7	29.7
0.5	20.6	24.1	19.2	36.1
2	21.6	22.5	19.2	36.7
6	22.8	21.1	17.5	38.6

Table 2 The mean particle diameter, consistency coefficient (K) and flow behavior index (n) of oil-in-water emulsions stabilized by native or hydrolyzed rice glutelin. Different letters in the same row for each parameter are significantly different ($p < 0.05$).

Samples	K(mPa•s)		n	
	0 day	7 days	0 day	7 days
DH0.5	1.65±0.08 ^a	2.39±0.08 ^b	0.95±0.03 ^a	0.93±0.02 ^a
DH2	2.96±0.10 ^a	3.06±0.06 ^a	0.94±0.01 ^a	0.94±0.01 ^a
DH6	3.02±0.12 ^a	19.90±0.83 ^b	0.94±0.01 ^b	0.67±0.01 ^a

Figure Captions:

Fig. 1. Molecular weight distribution profiles of native and hydrolyzed rice glutelin (RG and RGH, respectively).

Fig. 2. SDS-PAGE profiles of RG and RGH under (a) non-reducing and (b) reducing conditions.

Fig. 3. Physicochemical properties of rice glutelin samples with different degrees of hydrolysis: (a) surface hydrophobicity; (b) intrinsic fluorescence; and (c) solubility.

The values reported represent means $\pm$ standard deviations ($n = 3$) and different superscript letters indicate significant differences ($P < 0.05$).

Fig. 4 Influence of storage time on the mean particle diameter of oil-in-water emulsions stabilized by hydrolyzed rice glutelin.

Fig.5 Influence of pH on the characteristics of oil-in-water stabilized by hydrolyzed rice glutelin: (a) mean particle diameter; (b) ζ -potential.

Fig. 6 Influence of NaCl on the characteristics of oil-in-water stabilized by hydrolyzed rice glutelin: (a) mean particle diameter; (b) ζ -potential.

Fig. 7 Influence of temperature on the characteristics of oil-in-water stabilized by hydrolyzed rice glutelin: (a) mean particle diameter; (b) ζ -potential; and (c) photographs of emulsions after 15 days storage.

Fig.1

Fig.1. Molecular weight distribution profiles of native and hydrolyzed rice glutelin (RG and RGH, respectively).

Fig.2a

Fig.2. SDS-PAGE profiles of RG and RGH under (a) non-reducing and (b) reducing conditions.

Fig.2b

Fig.2. SDS-PAGE profiles of RG and RGH under (a) non-reducing and (b) reducing conditions.

Fig.3a.

Fig.3. Surface hydrophobicity (a), intrinsic fluorescence (b), solubility (c) of RG with different DH. The values reported represent means ($n = 3$) $\pm$ SDs and different superscript letters indicate significant difference ($P < 0.05$)

Fig.3b.

(b)

Fig.3. Surface hydrophobicity (a), intrinsic fluorescence (b), solubility (c) of RG with different DH. The values reported represent means ($n = 3$) $\pm$ SDs and different superscript letters indicate significant difference ($P < 0.05$)

Fig.3c.

Fig.3. Surface hydrophobicity (a), intrinsic fluorescence (b), solubility (c) of RG with different DH. The values reported represent means ($n = 3$) $\pm$ SDs and different superscript letters indicate significant difference ($P < 0.05$)

Fig.4

Fig.4 Influence of storage time on the mean particle size of RGH emulsion.

Fig.5a

(a)

Fig.5 Influence of pH on the characteristics of oil-in-water RGH emulsions (a) mean particle size, (b) ζ -potential.

Fig.5b

Fig.5 Influence of pH on the characteristics of oil-in-water RGH emulsions (a) mean particle size, (b) ζ -potential.

Fig.6a

Fig.6 Influence of NaCl on the characteristics of oil-in-water RGH emulsions (a) mean particle size, (b) ζ -potential.

Fig.6b

Fig.6 Influence of NaCl on the characteristics of oil-in-water RGH emulsions (a) mean particle size, (b) ζ -potential.

Fig.7a

Fig.7 Influence of temperature on the characteristics of oil-in-water RGH emulsions

(a) mean particle size, (b) ζ -potential and (c) photographs of emulsions with different temperature after 15 days storage.

Fig.7b

Fig.7 Influence of temperature on the characteristics of oil-in-water RGH emulsions

(a) mean particle size, (b) ζ -potential and (c) photographs of emulsions with different temperature after 15 days storage.

Fig.7c

Fig.7 Influence of temperature on the characteristics of oil-in-water RGH emulsions

(a) mean particle size, (b) ζ -potential and (c) photographs of emulsions with different temperature after 15 days storage.

“Effect of limited enzymatic hydrolysis on structure and emulsifying properties of rice glutelin”

Xu et al

Food Hydrocolloids

Graphical Abstract