Effectiveness of partially hydrolyzed rice glutelin as a food emulsifier: comparison to whey protein

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Abstract

The emulsifying properties of partially hydrolyzed rice glutelin (H-RG, 2% degree of hydrolysis) were compared to those of whey isolate protein (WPI), a commonly used protein-based emulsifier. The surface load of WPI (1% emulsifier, $d_{32} = 167.5$ nm) was 2.8 times lower than that of H-RG (3% emulsifier, $d_{32} = 159.0$ nm). Emulsions containing WPI-coated lipid droplets had better stability to pH changes (2-8), NaCl addition (0-500 mM) and thermal processing (30-90 °C, 0 or 200 mM NaCl). Nevertheless, H-RG emulsions were stable over a range of conditions: pH 6-8; $\text{NaCl} \leq 200$ (pH 7); temperatures ≤ 90 °C in the absence of salt (pH 7); and temperatures ≤ 50 °C in the presence of 200 mM NaCl (pH 7). This study indicates that H-RG may be utilized as a natural emulsifier in the development of label-friendly emulsion-based food products, but that further work is needed to increase the range of applications.

Keywords: rice glutelin; whey protein isolate; emulsion; environmental stress; stability.

1. Introduction

Many kinds of commonly consumed food products are emulsions, such as dairy creamers, dressings, dips, sauces and soft drinks (Dickinson, 2003). Emulsifiers are one of the most important additives for creating stable food emulsions. There are two major categories of emulsifiers used in the food industry: small-molecule surfactants (*e.g.*, Tween-80, monoglycerides, and lecithin) and large-molecule surfactants (*e.g.*, protein and polysaccharides) (Charoen, Jangchud, Jangchud, Harnsilawat, Naivikul, & McClements, 2011; Ozturk, Argin, Ozilgen, & McClements, 2015). Nowadays, consumers are becoming more interested in having food products with “clean” labels, which can be achieved by replacing synthetic or animal-based emulsifiers with plant-based ones (Ozturk, Argin, Ozilgen, & McClements, 2015).

Food proteins are effective emulsifiers, because of their amphiphilic nature, relatively large molecular dimensions, and relatively high electrical charge (Dickinson, 2003; McClements, 2004). Consequently, they can adsorb to oil droplet surfaces during homogenization, and form coatings that protect droplets against aggregation by generating strong repulsive interactions (*e.g.*, steric and electrostatic repulsion). Whey proteins and caseins are the most commonly used protein-based emulsifiers in foods (Khouryieh, Puli, Williams, & Aramouni, 2015). However, because of their **potential allergenicity** (Do, Williams, & Toomer, 2016) and their unsuitability for utilization in vegan products, there is a demand for more label friendly plant-based alternatives. Rice glutelin (RG) is the major storage protein in rice, which is an attractive candidate for a plant-based emulsifier, because of its low allergenicity, ability to reduce cholesterol

levels, and relatively low price (Agboola, Ng, & Mills, 2005; Chrastil, 1992; Fiocchi et al., 2003). Nevertheless, the application of native RG, as an emulsifier, is limited because of its low water-solubility and poor surface activity. Therefore, the native protein must be modified to improve its solubility and emulsifying properties. Enzyme modification has proved to be an effective means of altering the molecular, physicochemical and functional properties of proteins (Avramenko, Low, & Nickerson, 2013; Chen, Chen, Ren, & Zhao, 2011). Limited enzyme hydrolysis of rice protein has been shown to improve its emulsifying properties by increasing its surface hydrophobicity and molecular flexibility (Paraman, Hettiarachchy, Schaefer, & Beck, 2007). Recently, we examined the influence of the degree of enzyme hydrolysis on the ability of rice glutelin to form and stabilize emulsions. We found that the optimum emulsifying properties were obtained for the degree of hydrolysis as 2%, and so a hydrolyzed rice glutelin (H-RG) ingredient with this level of hydrolysis was used in the current study.

In the current study, we compared the ability of H-RG to form and stabilize oil-in-water emulsions, and compared its performance to whey protein isolate (WPI), which is already a commonly used emulsifier in the food industry. In particular, we focused on the influence of environmental stresses that food products may experience during their manufacture, storage and utilization, such as pH, ionic strength and temperature. This study therefore provides valuable information about the potential utilization of natural emulsifiers in the development of label-friendly emulsion-based food and beverage products.

2. Materials and methods

2.1. Materials

Rice glutelin (93.42 wt%, dry basis) was kindly provided by Golden Agriculture Biotech Company Limited (Jiangxi, China). Whey protein isolate (WPI) was purchased from Davisco Foods International Inc. (Le Sueur, MN). As stated by the manufacturer, the protein content was 97.6% (dry basis). Corn oil was obtained from a commercial food supplier (Mazola, ACH Food Companies, Memphis, TN). Double-distilled water was used throughout to prepare all solutions and emulsions.

2.2. Preparation of rice glutelin hydrolysates

Hydrolyzed protein was prepared using trypsin at an optimized enzyme/substrate ratio according to Zhao et al. (2012). 2 g of RG was added to double-distilled water (30 ml) with constant stirring for 1 h. After pH and temperature adjustments (pH=8, 50 °C), trypsin was added. The pH of the suspension was kept constant by the addition of 2.5 M NaOH during the entire period of hydrolysis. After 4.2 min of hydrolysis, the dispersion was heated to 95 °C for 10 min to inactivate the enzyme, followed by adjustment of the pH to 7.0 using HCl or NaOH, and immediate cooling in water to room temperature. The mixture was centrifuged (TGL-20B, Anting Scientific Instrument Factory, Shanghai, China) at 4800 g for 10 min, and the supernatant was freeze-dried. The degree of hydrolysis (DH) was calculated from the amount of base required to maintain a constant pH using the pH-stat method described previously (Adler-Nissen, 1986):

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

where B is base consumption (ml); N_b is the molarity of the base; M_p is the mass (g) of the protein; the value of α was 0.885 at pH 8 and 50 °C treatment (Adler-Nissen, 1986; García-Moreno et al., 2014), and h_{tot} is the theoretical overall number of peptide bonds in the protein substrate (7.40 meq/g rice protein) (Zhao et al., 2012). Rice glutelin with degree of hydrolysis of 2% was finally obtained.

2.3. Characterization of rice protein and its hydrolysates

2.3.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The polypeptide profiles of samples were determined by SDS-PAGE, as described previously (Laemmli, 1970). Protein samples were dissolved in loading buffer that contained β -mercaptoethanol (Solarbio, China) and were heated in boiling water for 5 min, then cooled to room temperature. 20 μ l of sample (1 mg/ml) was loaded on 5% stacking gel and 12.5% separating gel, and subjected to electrophoresis at a constant voltage of 80 V. Then the gels were stained for 2h 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).

2.3.2. Water-solubility

The water-solubility of proteins was analyzed by measuring the protein content using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) with bovine serum albumin as a standard. 1 wt% of protein aqueous solution was stirred magnetically at 25 °C for 30 min, and the pH was then adjusted to the desired value

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 in the supernatant was analyzed. After appropriate dilution, the protein solubility was calculated as the percentage of protein in the supernatant relative to the total protein content. All measurements were conducted in triplicate.

2.4. Emulsion preparation

Protein solutions were prepared by dispersing 0.01 to 8 wt% WPI or H-RG into phosphate buffer (5 mM pH 7.0), and were stirred for at least 3 h. After overnight storage at 4 °C, protein solutions were filtered with a qualitative filter (Fisher Scientific, PA) to remove any insoluble components. The absorbance at 280 nm of the protein solutions was measured before and after filtration, which indicated that a small amount of protein (<2.5% of the total) was lost. 10 wt% oil phase (corn oil) and 90 wt% aqueous phase (protein in buffer) were homogenized using a high-shear mixer at 10,000 rpm for 2 min (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland). The coarse emulsions were passed through a high pressure homogenizer (M110Y, Microfluidics, Newton, MA), with a 75 µm interaction chamber (F20Y) and an operating pressure of 12,000 psi, for 3 times.

2.5. Emulsion stability

The stability of the emulsions was measured after they were subjected to environmental stresses that foods may experience during manufacture, storage and utilization.

2.5.1. pH

Freshly prepared emulsions (pH 7) were distributed into different beakers, and then each sample was adjusted to a different pH value, ranging from 2 to 8 using HCl or NaOH solutions, and then placed in test tubes.

2.5.2. Ionic strength

Freshly prepared emulsions (pH 7) were distributed into different beakers, and then each sample was adjusted to a different ionic strength by adding different amounts of NaCl solution, and then placed in test tubes.

2.5.3. Temperature

The temperature stability of freshly prepared emulsions (pH 7) was measured. Emulsions containing either 0 or 200 mM NaCl were transferred into test tubes, which were then heated in water baths set at different temperatures (30-90 °C) for 30 min. The test tubes were then cooled to room temperature by placing them in ice water.

After exposure to these environmental stresses, samples were stored at room temperature for 24 h prior to determination of their particle size and ζ -potential, and for 7 days prior to visual observation.

2.6. Droplet size and particle charge (ζ -potential) measurement

The particle size distribution was determined using a laser diffraction instrument (Mastersizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Emulsions were diluted with the appropriate buffer solutions (same pH as sample being analyzed) to avoid multiple scattering effects, and stirred at 1200 rpm to ensure complete dispersion. The mean particle diameter is reported as the surface-weighted

mean diameter (d_{32}), since this is inversely related to the specific surface area of the droplets.

The ζ -potential of the emulsions was measured using a particle electrophoresis instrument at 25 °C (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, UK). For the emulsions, the samples were diluted 100-fold with the appropriate buffer (same pH as sample) to avoid multiple scattering during measurements. The refractive indices used were 1.33 for buffer and 1.47 for corn oil.

2.7. Statistical analyses

Each experiment was performed in triplicate, and the results are reported as means values $\pm$ standard deviation (SD). Significant differences between means ($p < 0.05$) were calculated by one-way ANOVA test using statistical analysis software (SPASS 19.0, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Characterization of rice protein and its hydrolysates

3.1.1. SDS-PAGE analysis

SDS-PAGE was performed to investigate differences in the composition of rice glutelin before and after hydrolysis. Under reducing conditions, RG mainly exhibited two clear bands, one that was about 20 kDa, namely alkaline β -subunit, and another one around 33 kDa, namely acidic α -subunit (Figure 1). Another faint band was observed around 53 kDa, which can be attributed to the presence of covalently linked α - and β -polypeptides. This result is in accordance with that reported previously on this protein (Likitwattanasade & Hongsprabhas, 2010), where it was reported that the major

protein subunits of RG were around 20, 30 and 50 kDa.

H-RG exhibited multiple major bands around 23.7, 17.3, and in the range ≤ 16.5 kDa, demonstrating that trypsin hydrolysis fragmented the polypeptide chain into lower molecular weight peptides. These results are in accordance with those reported previously (Avramenko, Low, & Nickerson, 2013; Ghribi, Gafsi, Sila, Blecker, Danthine, Attia, et al., 2015), where it was reported that enzyme hydrolysis cleaved peptide bonds within individual or aggregated proteins to produce smaller peptides or protein subunits. A change in protein structure would be expected to have a significant influence on protein functional properties, such as water-solubility and emulsifying properties.

3.1.2 Water-solubility

The water-solubility of proteins is an important prerequisite for many of their functional attributes. As shown in Figure 2, the solubility-pH profiles of both native and hydrolyzed glutelin were U-shaped, with minimum solubility values around pH 5 and pH 4 for RG (2.2%) and H-RG (66.7%), respectively. This reduction in protein solubility at intermediate pH values can be attributed to a decrease in the magnitude of the electrostatic repulsion between the protein molecules around their isoelectric points (pI) (Du, Jiang, Zhu, Xiong, Shi, Hu, et al., 2012; Sarkar, Kamaruddin, Bentley, & Wang, 2016). Previous studies have also reported a shift in the pI of proteins after partial hydrolysis, which can be attributed to a change in the nature of anionic and cationic groups exposed at the polypeptide surfaces (Jamdar, Rajalakshmi, Pednekar, Juan, Yardi, & Sharma, 2010; Ochiai, Kamata, & Shibasaki, 1982). In general, the

solubility of the hydrolyzed protein was much higher than that of the native protein. The poor water-solubility of the native protein can be attributed to the existence of a rigid polypeptide structure, with subunits linked by high amounts of intermolecular and intramolecular disulfide bonds and hydrophobic interactions (Paraman, Hettiarachchy, & Schaefer, 2007). In our study, the solubility of H-RG was significantly higher than that of RG at all pH values ($p < 0.05$). Similar results have been reported after partial hydrolysis of other types of glutelin, such as corn glutelin (Zheng, Wang, Liu, Sun, Zheng, Wang, et al., 2015) and barley glutelin (Xia, Bamdad, Gänzle, & Chen, 2012). The improved solubility of H-RG was attributed to the reduction in molecular weight, the unfolding of the polypeptide chains, and the release of soluble aggregates from insoluble aggregates or precipitates (Ghribi, et al., 2015). A reduction in the molecular dimensions of the proteins could also lead to a decrease in the van der Waals attraction, as well as an increase in entropy of mixing effects, both of which would favour an increase in solubility.

3.2. Influence of emulsifier type and concentration on emulsion formation

The ability of the hydrolyzed rice glutelin to form stable oil-in-water emulsions was evaluated by comparing its performance to a well-established protein-based emulsifier: whey protein isolate (WPI). Emulsions were formed by passing oil (10 wt%) and aqueous phase (90 wt%) through a microfluidizer at high pressure. The influence of initial emulsifier concentration on the mean particle diameter of the emulsions produced was determined.

For both emulsifiers, the mean particle diameter (d_{32}) decreased as the emulsifier

concentration increased (Figure 3a). For example, as the emulsifier concentration increased from 0.1 to 8%, d_{32} decreased from 2.2 μm to 150 nm for WPI, and from 17.4 μm to 156 nm for H-RG. The droplet diameter *versus* emulsifier concentration profiles could be divided into two regions (Charoen, Jangchud, Jangchud, Harnsilawat, Naivikul, & McClements, 2011; Jafari, Assadpoor, He, & Bhandari, 2008). In region I, d_{32} progressively decreased with increasing emulsion concentration. In this region, the homogenizer is capable of generating smaller droplets, but the minimum droplet size achievable is limited by the total amount of emulsifier present to cover the surfaces of the droplets. In region II, the droplet size remains relatively constant, as there is enough emulsifier present to cover all the droplet surfaces but the homogenizer is not capable of producing any smaller droplets under the operating conditions used (Qian, Decker, Xiao, & McClements, 2011). The mean particle diameters achieved were fairly similar for both emulsifiers at the higher emulsion concentrations used, which is likely because the droplet size was limited by the homogenizer disruptive energy rather than by the emulsifier type (Ozturk, Argin, Ozilgen, & McClements, 2014). Nevertheless, the droplet diameter *versus* the emulsifier concentration profiles were different, which was probably due to a combination of factors related to emulsifier type: the rate of emulsifier adsorption; the reduction in interfacial tension; the modification of interfacial rheology; and the strength of the repulsive interactions between droplets (Jafari, Assadpoor, He, & Bhandari, 2008; Schubert & Engel, 2004).

For many applications of food emulsions, the shape of the particle size distribution is important. Multimodal distributions were observed below 0.5 wt% WPI

and 2% H-RG, whereas monomodal distributions were observed at higher emulsifier levels (Figure 3b-c). Visual observation of emulsions containing relatively low levels of emulsifier indicated that they were highly susceptible to rapid creaming (data not shown), which can be attributed to the relatively large size of the droplets they contained. From a practical and economic standpoint, it is important to use sufficient emulsifier to form an emulsion containing small droplets, without having too much excess emulsifier in the aqueous phase (Ozturk, Argin, Ozilgen, & McClements, 2015). The droplet diameter *versus* the emulsifier concentration profiles indicated that this level was around 1 wt% for WPI ($d_{32} = 167.5$ nm) and around 3 wt% for H-RG ($d_{32} = 150.0$ nm). For the remainder of the studies we therefore utilized these levels to form the emulsions.

The surface load (Γ) of an emulsifier is an important indicator of its effectiveness at forming emulsions. The surface load is the mass of emulsifier required to cover a unit area of droplet surface (mg/m^2), and is given by the following equation (Dickinson, 1992; Mcclements, 2007; Ozturk, Argin, Ozilgen, & McClements, 2015):

$$\Gamma = \frac{C_s \cdot d_{32}}{6\phi} \quad (1)$$

where C_s is the concentration of adsorbed emulsifier present (kg/m^3), d_{32} is the mean droplet diameter produced (m), and ϕ is the disperse phase volume fraction. In our experiments, ϕ was about 0.1 (10% oil), d_{32} was 167.5 nm (WPI) or 159.0 nm (H-RG), and C_s was $10 \text{ kg}/\text{m}^3$ (1 wt% WPI) or $30 \text{ kg}/\text{m}^3$ (3 wt% H-RG). Therefore, the surface load of WPI ($\Gamma \approx 2.79 \text{ mg}/\text{m}^2$) was about 2.8 times smaller than that of H-RG ($\Gamma \approx 7.95 \text{ mg}/\text{m}^2$). This difference can be attributed to the different molecular weights

and packing of the proteins adsorbed to the droplet surfaces. From a practical viewpoint, this means that more H-RG is required to produce an emulsion with a given fat content and droplet size than WPI.

3.3. Influence of environmental conditions on emulsion stability

Practically, emulsion-based foods should remain stable during processing, storage, transportation, and utilization. Food emulsifiers must therefore be able to stabilize emulsions when they are exposed to the environmental conditions typical of a particular product type, such as pH, ionic strength, or temperature changes. We therefore investigated the influence of pH, ionic strength, and thermal processing on the physical stabilities of emulsions stabilized by 1% WPI or 3% H-RG.

3.3.1. Influence of pH

The influence of pH on the ζ -potential, particle size, and creaming stability of emulsions stabilized by the two different proteins was measured (Figure 4). The ζ -potential of both emulsions went from highly positive at pH 2 (+26.9 and +37.1 mV for H-RG and WPI, respectively) to highly negative at pH 8 (-40.7 and -48.8 mV for H-RG and WPI, respectively), with a point of zero charge around pH 4.2 and 4.8 for H-RG and WPI, respectively (Figure 4a). The magnitude of the ζ -potential on H-RG was slightly lower than that of WPI at both high and low pH values, which might be ascribed to differences in the number of anionic and cationic groups they contain. When the pH value was higher than the pI of protein, the carboxyl groups ($-\text{COO}^-$) were negative and the amino groups ($-\text{NH}_2$) were neutral. Conversely, when the pH was appreciably lower than the pI, the carboxyl groups were neutral ($-\text{COOH}$) and the

amino groups were positive (NH_3^+).

The stability of the emulsions to droplet aggregation and creaming was highly pH-dependent (Figure 4b). For WPI, the droplets were stable to aggregation and creaming at relatively low (pH 2 to 3) and high (pH 6 to 8) pH values, but highly unstable at intermediate pH values. This effect can be attributed to the reduction in the magnitude of the ζ -potential on the droplets at pH values around the isoelectric point of the droplets (Figure 4a). As a result, the electrostatic repulsion between the droplets is no longer sufficient to overcome the attractive van der Waals and hydrophobic interactions (McClements, 2004). For H-RG, the emulsions were only stable to aggregation and creaming at relatively high (pH 7 and 8) pH values, and were highly unstable at lower pH values. The extensive amount of droplet aggregation observed in these emulsions at low pH values was surprising, since the relatively large ζ -potential (+27 mV) on the droplets would be expected to generate a strong electrostatic repulsion. This effect may have been due to the fact that the emulsions were initially prepared at pH 7, and then adjusted to other pH values. Consequently, some irreversible droplet aggregation may have occurred when the H-RG-coated droplets passed through the isoelectric point, whereas only reversible aggregation occurred for the WPI-coated droplets. Irreversible aggregation may have occurred due to the formation of strong hydrophobic or disulfide bonds when the droplets came into close proximity. It should also be noted that there might be differences in the isoelectric point of adsorbed and non-adsorbed proteins in emulsions, due to differences in the number and type of ionized groups exposed to the surrounding aqueous phase.

3.3.2. Influence of ionic strength

The magnitude of the ζ -potential of both emulsions decreased with increasing salt concentration (Figure 5a). This effect can be attributed to the accumulation of counter-ions (Na^+) around anionic groups ($-\text{CO}_2^-$) on the droplet surfaces, thereby reducing their net charge, due to electrostatic screening effects. Furthermore, the ionic strength had an appreciable effect on droplet aggregation (Figure 5b). For WPI, there was a slight increase in the mean particle diameter at the highest salt levels used (300 and 500 mM), suggesting that a limited amount of droplet aggregation had occurred. For example, d_{32} increased from about 0.163 μm at 0 mM NaCl to 0.292 μm at 500 mM NaCl. For H-RG, the increase in mean particle diameter was much greater at higher salt levels. For example, in this case, d_{32} increased from around 0.159 μm at 0 mM NaCl to 3.53 μm at 500 mM NaCl. The destabilization of protein-coated oil droplets at high salt levels can mainly be attributed to screening of the electrostatic interactions between them (McClements, 2004), as suggested by the reduction in ζ -potential (Figure 5a). At low NaCl concentrations the electrostatic repulsive forces acting between the droplets are strong enough to overcome any attractive forces (*e.g.*, van der Waals and hydrophobic), thereby inhibiting droplet aggregation (Yang, Leser, Sher, & McClements, 2013). On the contrary, at sufficiently high NaCl levels the attractive forces are larger than the electrostatic repulsive forces, thereby leading to droplet aggregation (Qian, Decker, Xiao, & McClements, 2011). In addition, high levels of salt may alter the solubility of the proteins and peptides in the H-RG (Xu, Liu, & Zhang, 2015), which may cause them to aggregate and alter their ability to form and

306 **stabilize emulsions.** Overall, our results show that WPI-coated oil droplets are more
307 stable at high ionic strength conditions than H-RG-coated ones. This may have
308 occurred for a number of reasons: WPI-coated droplets had higher ζ -potential leading
309 to stronger electrostatic repulsion; WPI formed thicker interfacial layers than
310 hydrolyzed rice glutelin leading to stronger steric repulsion; H-RG-coated droplets had
311 higher surface hydrophobicity leading to a stronger hydrophobic attraction.

312 *3.3.3. Influence of temperature*

313 The influence of heat treatment (30-90 °C, 30 min) on the mean particle diameter
314 and ζ -potential of the emulsions was also examined in the absence or presence of salt (0
315 or 200 mM NaCl). The upper level of NaCl was selected because both the WPI and
316 H-RG emulsions were relatively stable to droplet aggregation at this salt content at
317 room temperature (Figure 5b). The salt was added before the emulsions were thermally
318 processed, because this reduces the electrostatic repulsion between the droplets during
319 heating, which is known to potentially reduce their stability to aggregation (Qian,
320 Decker, Xiao, & McClements, 2011).

321 In the absence of salt, the emulsions prepared using either WPI or H-RG were both
322 stable to heat treatment across the entire temperature range investigated, with no
323 appreciable change in the mean particle diameter or visible evidence of phase
324 separation (data not shown). For example, the mean particle diameters were only about
325 0.163 and 0.167 μm for WPI and H-RG emulsions, respectively, after heating to 90 °C.
326 This suggests that both types of emulsifiers had good heat stability when the emulsions
327 were thermally processed in the absence of added salt. The origin of this stability can

be attributed to the high electrostatic repulsion between the oil droplets at low salt levels. Thus, the repulsive interactions (electrostatic and steric) are strong enough to overcome any attractive interactions (van der Waals and hydrophobic).

In the presence of salt (200 mM), the surface charge for both emulsions showed no significant change after heat treatment over the temperature range studied (Figure 6a). This suggests that there was little alteration in the number or location of the charged groups at the droplet surfaces induced by heating. The WPI-stabilized emulsions remained stable to aggregation when they were held at temperatures of 70 °C or less, but they became highly unstable when they were heated to higher temperatures (Figure 6b). For example, the mean particle diameter increased from around 0.22 µm at 70 °C to around 33 µm at 90 °C. The H-RG-stabilized emulsions only remained stable to aggregation when they were held at temperatures of 50 °C or less, but underwent extensive droplet aggregation when heated to higher temperatures. For example, the mean particle diameter increased from around 0.34 µm at 50 °C to around 10.4 µm at 90 °C. In addition, there was clear evidence of phase separation in the samples, in the test tubes, held at higher temperatures (>50 °C), with a droplet-rich cream layer at the top and a droplet-depleted serum layer at the bottom.

The decrease in the aggregation stability of the emulsions when they were heated above a certain temperature in the presence of salt can be attributed to a change in the nature of the colloidal interactions in the system. Upon heating, the adsorbed globular proteins undergo a conformational change, which leads to exposure of non-polar and sulfhydryl groups, that are normally imbedded in the hydrophobic interior of the

protein (Day, Zhai, Xu, Jones, Hoffmann, & Wooster, 2014; Kim, Decker, & McClements, 2002). Consequently, the surface hydrophobicity of the oil droplets increased, which generate a strong hydrophobic attraction between the droplets. In the presence of salt, the attractive interactions (van der Waals and hydrophobic) are then strong enough to overcome the repulsive interactions (steric and electrostatic), which led to droplet aggregation. The fact that the WPI-coated droplets could be heated to a higher temperature than the H-RG-coated ones before aggregation was observed, suggests that there were differences in their thermal denaturation (T_m) temperatures (Sarkar, Kamaruddin, Bentley, & Wang, 2016). Previously it has been reported that the thermal denaturation temperature of native rice glutelin is around 82.2 °C (Ju, Hettiarachchy, & Rath, 2001) and that of WPI was around 75 °C (Fitzsimons, Mulvihill, & Morris, 2007). The transition temperatures and enthalpy of denaturation of hydrolyzed proteins have also been reported to be lower than that of the native form (Wani, Sogi, Shivhare, & Gill, 2015). Consequently, the thermal denaturation temperature of hydrolyzed rice glutelin may have been lower than that reported for the native form. This phenomenon would then account for the fact that the WPI-coated droplets were stable over a wider temperature range than the H-RG-coated ones.

In summary, the above experiments indicate that H-RG emulsions were stable over a range of environmental conditions: pH 6-8 (0 mM NaCl, 25 °C); $\text{NaCl} \leq 200 \text{ mM}$ (pH 7, 25 °C); temperatures $\leq 90 \text{ °C}$ in the absence of salt (pH 7, 0 mM NaCl); temperatures $\leq 50 \text{ °C}$ in the presence of salt (pH 7, 200 mM NaCl) studied. However,

the emulsions were highly unstable to aggregation at lower pH values, higher ionic strengths, and elevated temperatures. Consequently, there is a need to develop effective strategies to improve the stability of emulsions containing H-RG-coated droplets.

4. Conclusions

In this study, we compared the functional performance of hydrolyzed rice glutelin (H-RG) to that of whey protein isolate (WPI), which is a commonly used dairy protein. The main factors influencing the formation and stability of emulsions produced using either H-RG or WPI as protein-based emulsifiers were studied. The emulsifier content significantly influenced the stability of emulsions showing decrease of mean particle diameter with increasing emulsifier concentration. Both emulsifiers could be used to form oil-in-water emulsions containing relatively small droplets ($d < 200$ nm), but a smaller amount of WPI was required than H-RG. Indeed, the surface load of H-RG ($\Gamma \approx 7.95$ mg/m²) was almost three times higher than that of WPI ($\Gamma \approx 2.79$ mg/m²). In addition, WPI provided better stability to environmental stresses, such as pH changes, high ionic strengths, and heating, than H-RG. Further research is therefore needed to improve the stability of emulsions formed by H-RG so that it can be used in a wide range of food products.

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Figure Captions:

Figure 1. SDS-PAGE of rice glutelin and its hydrolysates in the presence of β -mercaptoethanol.

Figure 2. Water-solubility of RG and H-RG at different pH.

Figure 3a. Influence of protein type and concentration on mean droplet diameter ($d_{3,2}$) of emulsions stabilized by WPI and H-RG.

Figure 3b. Influence of protein type and concentration on the particle size distribution of WPI emulsions.

Figure 3c. Influence of protein type and concentration on the particle size distribution of H-RG emulsions.

Figure 4. Influence of pH (2-7) on ζ -potential (a) and the mean particle diameter ($d_{3,2}$) (b) of corn oil-in water emulsions stabilized by WPI and H-RG.

Figure 5. Influence of NaCl (0-500 mM) on ζ -potential (a) and the mean particle diameter (b) of corn oil-in-water emulsions stabilized by WPI and H-RG.

Figure 6. Influence of temperature (30-90°C) on ζ -potential (a) and the mean particle diameter (b) of corn oil-in-water emulsions stabilized by WPI and H-RG in the presence of 200 mM NaCl.

Figure 1

Figure 2

Figure 3a

(b)

Figure 3c

(c)

Figure 4a

Figure 4b

Figure 5a

Figure 5b

Figure 6a

Figure 6b