Peptide-polysaccharide conjugates with adjustable hydrophilicity/hydrophobicity as green and pH sensitive emulsifiers

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In this study, we used deamidated zein peptide-polysaccharide conjugates as emulsifiers to produce oil in water emulsions. Zein is insoluble in water. Its asparagine and glutamine residues changed to aspartic acid and glutamic acid residues and also zein was degraded during deamidation reaction in alkaline condition. Longer deamidation reaction produced more carboxyl groups and smaller peptides. The peptides with average molecular weights of 7.3, 5.5 and 4.0 kDa were obtained after 36, 96 and 144 h of the deamidation reaction, respectively. Dextran (Mw 16 kDa) and maltodextrin (Mw 3.0 kDa) were separately conjugated to the N-terminals of the peptides via Maillard reaction. The hydrophilicity/hydrophobicity of the peptide-polysaccharide conjugates can be adjusted by deamidation reaction time, number of conjugated polysaccharide molecules, polysaccharide molecular weight, and medium pH. The conjugates, in which about 65.4 e 90.6% of the peptides were conjugated with the polysaccharide, had prominent emulsification ability at acidic condition. The emulsions produced and stored at pH 4.0 and 4.5 were long-term stable. When the emulsions were stored at pH 7.4, at which the carboxyl groups were significantly deprotonated, demulsification happened. The oil layer appearance time changed from 2 days to more than 70 days; the time decreased with the increase of the conjugate hydrophilicity. This study demonstrates that the emulsions produced from the conjugates have pH sensitivity and adjustable stability. The deamidated zein peptide-polysaccharide conjugates, which synchronously possess natural moieties, adjustable hydrophilicity/hydrophobicity, smaller molecular weights and simpler structures compared with protein-polysaccharide conjugates, are excellent and green emulsifiers.© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Emulsifiers are widely used in pharmaceutics and food industries (Bouyer, Mekhloufi, Rosilio, Grossiord, & Agnely, 2012; McClements, 2012; McClements, Decker, & Weiss, 2007; Xu, Yin, Li, & Yao, 2015). Synthetic surfactants, composed of a hydrophilic moiety and a hydrophobic moiety, diffuse rapidly to oil-water interface to reduce the surface tension and stabilize the emulsion droplets (Bouyer et al., 2012; Garti, 1999; Yang, Leser, Sher, & McClements, 2013). Various synthetic surfactants with different hydrophilic-lipophilic balance (HLB) values, thus with different emulsifying abilities and emulsion stabilities, can be obtained by changing the hydrophilic and hydrophobic structures (McClements & Xiao, 2014; Piorkowski & McClements, 2014). The disadvantage of synthetic emulsifiers is their potential toxicity (Kralova & Sjöblom, 2009). Very recently, Chassaing et al. reported that relatively low concentrations of two commonly used dietary emulsifiers, polysorbate-80, derived from polyethoxylated sorbitan and oleic acid, and carbomethyl cellulose, a cellulose derivative, impact the mouse gut microbiota promoting colitis and metabolic syndrome (Chassaing et al., 2015). Proteins can also be used as emulsifiers. Many proteins, such as milk protein and soy protein, are nourishing, nontoxic and low cost, and also possess excellent emulsifying capacity (Day, 2013; McClements, 2004; Singh & Sarkar, 2011). On the other side, the emulsions stabilized by proteins are sensitive to environmental conditions, such as pH, ionic strength, and thermal processing (Delahaije, Gruppen, Giuseppin, & Wierenga, 2015; Dickinson, 2010). To solve this problem, protein-polysaccharide covalent conjugates and protein/polysaccharide electrostatic complexes have been exploited as emulsifiers to produce long-term stable emulsions (Bouyer et al., 2012). Another problem for protein emulsifiers is that proteins diffuse slowly to oil-
water interfaces, and their emulsifying ability and emulsion stability are difficult to control compared with synthetic surfactants, because proteins are natural macromolecules with complex structures (Lam & Nickerson, 2013; Qian & McClements, 2011). Therefore, it is of great interest to develop novel emulsifiers, which combine the advantages of proteins and synthetic surfactants together, that is, synchronously possess natural moieties, adjustable hydrophilicity/hydrophobicity, relatively smaller molecular weights and simpler structures. As we know, peptide generally possesses simpler structure and smaller molecular weight compared with protein, and the peptide with desired hydrophilicity/hydrophobicity, component, and structure can be synthesized chemically and biologically. However, the synthesis and purification of peptide are costly and time-consuming. By now, no investigation on the peptide with adjustable emulsifying ability and emulsion stability was reported.

Zein, a major byproduct of corn starch production, composed of α-zein (19 and 22 kDa), β-zein (14 kDa), γ-zein (16 and 27 kDa) and δ-zein (10 kDa) (Thompson & Larkins, 1989; Zhang et al., 2015). α-Zein accounts for 70–85% of the total zein fraction, and γ-zein accounts for 10–20% as the second most abundant fraction. Zein possesses superior properties of film forming, antioxidation, biodegradability and biocompatibility (Dong, Sun, & Wang, 2004; Fernandez, Torres-Giner, & Lagaron, 2009; Zhang et al., 2015). Zein is insoluble in water and is soluble in 60–85% ethanol solution (Tang et al., 2010; Wu, Luo, & Wang, 2012) because zein is rich in hydrophobic amino acid residues as well as is deficient in basic and acidic amino acid residues (Cabra, Arreguin, Vazquez-Duhalt, & Farres, 2007; Flores, Cabra, Quirasco, Farres, & Galvez, 2010; Shukla & Cheryan, 2001). Zein is absence of tryptophan and lysine (Shukla & Cheryan, 2001). More than 20% of the amino acid residues of zein are asparagine and glutamine (Hu, Peifer, Heidecker, Messing, & Rubenstein, 1982), which can change into aspartic acid and glutamic acid residues via deamidation reaction. The carboxyl groups of zein produced by deamidation reaction increase the solubility of zein in water (Cabra et al., 2007; Flores et al., 2010). During the deamidation reaction, degradation reaction also happens which produces zein peptides with smaller molecular weights. Tang et al. reported that the low molecular weight peptides from Alcalase-treated zein hydrolysate had strong free radical scavenging activity (Tang et al., 2010). Cabra et al. compared the enzymatic, acidic and alkaline deamidation conditions of Z19 α-zein, and they found that the alkaline deamidation condition was better for improving the emulsifying property (Cabra et al., 2007). For deamidated zein, improvement of the emulsification ability is mainly based on the electrostatic repulsive force of the deprotonated carboxyl groups (Flores et al., 2010). At acidic condition, deamidated zein becomes hydrophobic one due to protonation of the carboxyl groups and therefore cannot be an emulsifier. Because of the acidic environment in most food and beverage (Piorkowsk & McClements, 2014), the applications of deamidated zein are limited.

Conjugation of polysaccharide to deamidated zein peptide can increase the hydrophilicity of the peptide. Herein, we produced deamidated zein peptide-polysaccharide conjugates and adopted following ways to adjust the hydrophilicity/hydrophobicity of the conjugates: (1) deamidation degree and degradation degree of the peptide, (2) number of conjugated polysaccharide, (3) polysaccharide molecular weight, and (4) protonation degree of the carboxyl groups. We used the conjugates with adjustable hydrophilicity/hydrophobicity as emulsifiers to produce oil in water emulsions with pH sensitivity and adjustable stability. As illustrated in Scheme 1, after deamidation reaction, the produced peptide is soluble in neutral and alkaline solutions due to the deprotonated carboxyl groups; polysaccharide is conjugated to the primary amine group in N-terminal of the peptide via Maillard reaction; at acidic condition, the conjugate possesses good emulsifying ability; at neutral condition, demulsification happens when the electrostatic repulsion and hydrophilicity of the conjugate are strong enough. This study proves that the deamidated zein peptide-polysaccharide conjugates are green and pH sensitive emulsifiers with adjustable hydrophilicity/hydrophobicity.

2. Experimental

2.1. Materials

Zein from maize was purchased from Sigma (Shanghai, China). Maltodextrin (MAL, DE 20–23) was supplied by DSM Nutritional Products AG (Kaiseraugst, Switzerland). Dextran (DEX, dextran-10) was purchased from Sangon Biotech Shanghai Co., Ltd. (Shanghai, China). Medium chain triglyceride (MCT) for injection was from Avic (Tieling) Pharmaceutical Co., Ltd. (Tieling, Liaoning, China). All other chemicals were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of deamidated zein peptides

Zein was dissolved in a solution containing 0.5 M NaOH and 70% (v/v) ethanol with zein concentration of 10 mg/mL. The solution was incubated at 37 °C under stirring for 6, 36, 72, 96 or 144 h. After the incubation, the ethanol in the solution was immediately removed by rotary evaporation under vacuum. The remaining solution was changed to pH 3.1 by addition of 5 M HCl and then the solution was kept at room temperature overnight to precipitate

Scheme 1. Illustration of the preparation processes of pH sensitive deamidated zein peptide-polysaccharide conjugate and emulsion.
deamidated zein peptide. The precipitate was isolated and then was dissolved in an aqueous solution after addition of 2 M NaOH to reach the final pH of 9.0 at which the peptide was fully deproto-
nated. The resultant peptide solution was freeze-dried to obtain deamidated zein peptide powder.

2.3. Preparation of deamidated zein peptide-dextran conjugate (ZD) and deamidated zein peptide-maltodextrin conjugate (ZM)

Deamidated zein peptide was dissolved in water together with dextran or maltodextrin at the peptide concentration of 10 mg/mL and the polysaccharide concentration of 60 mg/mL. The mixture was adjusted to pH 8.8 and then lyophilized. The lyophilized powder was reacted at 60 °C for 3 h to produce ZD or ZM. The produced conjugates were used directly as emulsifiers without purification. In the following study, for simplifying the description, we used the peptide concentration to indicate the corresponding conjugate concentration.

2.4. Preparation of the conjugate emulsions

ZD or ZM was dissolved in water with 5 mg/mL peptide concentration. The conjugate solution was adjusted to desired pH and then MCT with 10% volume fraction was added. The mixture was pre-emulsified using a homogenizer (FJ200-S, Shanghai Specimen Model Co.) at 10,000 rpm for 1 min, and then was immediately emulsified using a high pressure homogenizer (AH100D, ATS Engineering Inc.) at 800 bar for 4 min. After emulsification, each produced emulsion was divided into 2 parts. One was directly stored at 4 °C and the other was adjusted to pH 7.4 with 1 M NaOH solution then stored at 4 °C to investigate the stability.

For comparison, the individual peptide, individual dextran or the physical mixture of peptide and dextran was used as emulsifier. The peptide concentration was 5 mg/mL and dextran concentration was 30 mg/mL in aqueous solutions; the emulsification condition was the same as the conjugate emulsions described above.

2.5. Molecular weight characterization of dextran, maltodextrin, zein, the peptides and conjugates

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out in the presence of reducing agent on a gel electrophoresis apparatus (JM250, JM-X Scientific Co.) to examine molecular weight change of the zein after deami-
dation reaction. Zein was dissolved in 70% ethanol solution, and the peptide was dissolved in water. Zein or peptide solution was mixed with electrophoretic sample buffer (Cabra et al., 2007), and approximate 0.02 mg zein or peptide was loaded in each lane. The gel was stained with silver (Cabra et al., 2007). BSA (66 kDa), lysozyme (14.4 kDa), insulin (2.35 and 3.4 kDa), and protein marker were used as molecular weight standard.

The molecular weight distributions of the polysaccharides, peptides and conjugates were characterized on a gel permeation chromatography (GPC, Model 1525, Waters) equipped with TSK G4000 and G3000 PWXL columns and a refractive index detector (Optilab T-rEX, WYATT). The mobile phase was 0.05 M pH 7.4 Tris buffer containing 0.15 M NaCl at a flow rate of 1.0 mL/min. Poly-
ethyleneglycol was used as calibration standard.

2.6. Amino acid composition analysis of zein and the peptides

The amino acid compositions of zein and the peptides were analyzed on a HPLC instrument (LC-20A, Shimadzu) after complete degradation of zein and the peptides in 6 M HCl at 110 °C overnight as described in the literature (Fountoulakis & Lahm, 1998).

2.7. Carboxyl content characterization of the peptides

The carboxyl contents of the peptides were analyzed by conductometric titration according to the literature (He, Man, & Shi, 2003). The weighted peptide was dissolved in water. The peptide solution was adjusted to pH 3.0 with HCl solution to pro-
tonate the carboxyl groups completely. The electroconductivity value was recorded after every 20 mL of 0.0500 M NaOH solution was added into the peptide solution.

Table 1
Yield and mole number of the carboxyl groups in per gram of the deamidated zein peptide after the deamidation reaction.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Deamidation reaction time (h)</th>
<th>Yield (%)</th>
<th>Molcarboxyl/Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>6h-zein</td>
<td>6</td>
<td>67.5 ± 6.0</td>
<td>1.16 × 10⁻³ ± 7 × 10⁻⁵</td>
</tr>
<tr>
<td>36h-zein</td>
<td>36</td>
<td>64.5 ± 4.6</td>
<td>1.93 × 10⁻³ ± 8 × 10⁻⁵</td>
</tr>
<tr>
<td>72h-zein</td>
<td>72</td>
<td>62.0 ± 11</td>
<td>2.49 × 10⁻³ ± 1.0 × 10⁻⁴</td>
</tr>
<tr>
<td>96h-zein</td>
<td>96</td>
<td>52.5 ± 2.4</td>
<td>2.69 × 10⁻⁴ ± 8 × 10⁻⁵</td>
</tr>
<tr>
<td>144h-zein</td>
<td>144</td>
<td>56.6 ± 2.1</td>
<td>2.87 × 10⁻⁴ ± 1.3 × 10⁻⁴</td>
</tr>
</tbody>
</table>

Fig. 1. SDS-PAGE analysis of protein marker (lane 1), zein (lane 2), protein marker (lane 3), 6h-zein (lane 4), 36h-zein (lane 5) and 72h-zein (lane 6).
Table 2
Amino acid compositions of zein and the peptides produced by 6, 36, and 72 h of the deamidation reactions.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Molar percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zein</td>
</tr>
<tr>
<td>Asp</td>
<td>4.70</td>
</tr>
<tr>
<td>Glu</td>
<td>21.15</td>
</tr>
<tr>
<td>Ser</td>
<td>6.43</td>
</tr>
<tr>
<td>Gly</td>
<td>2.59</td>
</tr>
<tr>
<td>His</td>
<td>1.15</td>
</tr>
<tr>
<td>Arg</td>
<td>1.05</td>
</tr>
<tr>
<td>Thr</td>
<td>2.76</td>
</tr>
<tr>
<td>Ala</td>
<td>13.32</td>
</tr>
<tr>
<td>Pro</td>
<td>11.38</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.61</td>
</tr>
<tr>
<td>Val</td>
<td>3.73</td>
</tr>
<tr>
<td>Met</td>
<td>1.77</td>
</tr>
<tr>
<td>Cys-Cys</td>
<td>–</td>
</tr>
<tr>
<td>Ile</td>
<td>3.74</td>
</tr>
<tr>
<td>Leu</td>
<td>17.23</td>
</tr>
<tr>
<td>Phe</td>
<td>5.25</td>
</tr>
<tr>
<td>Trp</td>
<td>–</td>
</tr>
<tr>
<td>Lys</td>
<td>0.13</td>
</tr>
</tbody>
</table>

2.8. Amino content characterization of the peptides and conjugates

o-Phthalaldehyde (OPA) assay was used to analyze the primary amine groups of the peptides and conjugates to estimate the degradation of the zein after deamidation reaction and polysaccharide conjugation percent of the peptides after Maillard reaction. OPA reagent was freshly prepared before use as reported previously (Pan, Mu, Hu, Yao, & Jiang, 2006). Zein was dissolved in 70% ethanol solution containing 0.5 M NaOH with zein concentration of 10 mg/mL, and the peptide or conjugate was dissolved in water with the peptide concentration of 10 mg/mL. As described previously (Pan et al., 2006), after mixing 0.1 mL of the zein or peptide or conjugate solution with 2.7 mL OPA reagent and incubating the mixed solution at room temperature for 140 s, the absorbance at 336 nm was measured immediately on an UV–vis spectrophotometer (UV-2550, Shimadzu). The working curve was obtained by analysis of i-leucine standard solutions at the same condition.

2.9. ζ-Potential measurements

ζ-Potentials were measured on a laser light scattering instrument (Zetasizer Nano ZS90, Malvern Instruments) at 25 °C. The samples were prepared as follows: peptide or conjugate was dissolved in aqueous solution with 1 mg/mL peptide concentration, 5 mM NaCl and desired pH value; emulsion was diluted to peptide concentration of 7.5 × 10⁻³ mg/mL, the aqueous solution containing 5 mM NaCl and desired pH value. The ζ-potentials were calculated by the Dispersion Technology Software provided by Malvern according to the Henry equation and Smoluchowski approximation (Pan, Yao, & Jiang, 2007).

2.10. Dynamic light scattering (DLS) measurements

DLS measurements were performed on a laser light scattering instrument (Autosizer 4700, Malvern Instruments). The emulsion samples with the peptide concentration of 7.5 × 10⁻³ mg/mL were prepared freshly before DLS measurement by diluting 5 μL of the emulsion with 3 mL aqueous solution containing the same pH. DLS measurements were performed at the condition of refractive index 1.333 for water and 1.472 for oil, viscosity 0.89, equilibrium time 2 min, during time 4 min, and slit 35 nm. The measurements were carried out at 25 °C and 90° scattering angle. The apparent z-average hydrodynamic diameter (Dh) and polydispersity index (PDI) were obtained by Automatic analysis mode. At least 2 batches of the emulsions were produced at the same condition and were measured to assess reproducibility.

2.11. FTIR measurements

The mixture of peptide and polysaccharide with pH 8.8, peptide concentration of 5 mg/mL and polysaccharide concentration of 30 mg/mL was lyophilized as the mixture sample for FTIR measurement. The FTIR absorption spectra of individual peptide, individual polysaccharide, the mixture as well as the conjugate powder samples were recorded on a FTIR spectrometer (Nicolet 6700, Thermo Fisher) using ATR accessory, resolution of 4 cm⁻¹ and accumulation of 128.

2.12. Transmission electron microscopy (TEM) observations

TEM observations of the emulsions were conducted on an electron microscope (FEI Tecnai G2 TWIN, FEI Company). TEM sample was prepared by depositing diluted emulsion onto a carbon-coated copper grid and drying at room temperature.

3. Results and discussion

3.1. Yields of deamidated zein peptides

More than 20% of the amino acid residues of zein are asparagine and glutamine residues (Hu et al., 1982), which can be hydrolyzed generating carboxyl groups and releasing ammonia (Cabra, Arreguin, Vazquez-Duhalt, & Farres, 2006, 2007). During hydrolysis, the peptide bonds, which are also amido bonds, can be cleaved. In this study, deamidated zein peptides with different deamidation degrees and different molecular weights were obtained by alkaline hydrolysis of zein for desired time. After the deamidation reaction, the peptide was separated by precipitation at pH 3.1 at which the carboxyl groups were fully protonated and the peptide was insoluble in aqueous solution. Table 1 shows that the yield of the peptide after the deamidation reaction decreases with the reaction time. The yield after 6 h of the reaction is 67.5%, and the yield decreases to 50.6% after 144 h of the reaction. This result indicates that after longer deamidation reaction there are more soluble components in pH 3.1 aqueous solution. The soluble components at pH 3.1 would not be used in this study.

3.2. Molecular weights of the peptides

Fig. 1 shows that the zein has a main band of about 20 kDa before deamidation reaction. After 6 and 72 h of the deamidation reaction, the molecular weights of the deamidated zein peptides, 6h-zein and 72h-zein, are smaller than 14 and 4 kDa, respectively. It is notable that the molecular weights measured by SDS-PAGE can only be a reference because the electrophoretic mobility may depend on the protein/peptide structure and component (Cabra et al., 2006). The SDS-PAGE results confirm that the degradation degree of the zein increases with the deamidation reaction time.

3.3. Amino acid compositions of zein and the peptides

As mentioned above, more than 20% of the amino acid residues of zein are asparagine (Asn) and glutamine (Gln) (Hu et al., 1982). During the amino acid assay, zein and the peptides were completely hydrolyzed in 6 M HCl at 110 °C overnight, and all the Asn and Gln residues were converted to aspartic acid (Asp) and...
glutamic acid (Glu) (Fountoulakis & Lahm, 1998). Therefore, the contents of Asn and Gln were measured as the contents of Asp and Glu, respectively. Table 2 shows that after the deamidation reactions, the peptides contain more Glu and Leu, less Ser and Thr compared with zein. There are about 1% (mol/mol) Arg and 0.13—0.38% Lys in the peptides, indicating that the peptides carry very few positive charges. On the contrary, there are 4.10—4.47% Asp and 22.21—24.68% Glu in the peptides, confirming that the peptides have many carboxyl groups after the deamidation reactions.

### 3.4. Carboxyl contents of the peptides

Fig. 2 shows a typical electroconductivity titration curve of the peptide solution as a function of NaOH titrant. The curve can be divided into three parts (He et al., 2003). At pH 3, the solution contained protonated peptide and excess HCl. When NaOH was added, reaction $\text{H}^+ + \text{OH}^- = \text{H}_2\text{O}$ happened. The free $\text{H}^+$ ions were replaced by free Na$^+$ ions. Because the mobility of Na$^+$ was slower than that of $\text{H}^+$, the electroconductivity decreased during the titration (the first part). When the excess HCl was reacted completely, the added NaOH reacted with the carboxyl groups, which produced free Na$^+$ ions and deprotonated peptide molecules, therefore, the electroconductivity increased (the second part). After the carboxyl groups were reacted completely, the added Na$^+$ ions and OH$^-$ ions caused the electroconductivity to increase more rapidly (the third part). The volume of the NaOH solution consumed in the second part, which was obtained by the two intersection points of the three tangents, termed as $V_2 - V_1$, was used to calculate the mole number of the carboxyl groups in per gram of the peptide using the following equation:

$$\text{MolCOOH/\text{gpeptide}} = \frac{C_{\text{NaOH}} \times (V_2 - V_1)}{W_{\text{peptide}}(\text{g})}$$

where $C_{\text{NaOH}}$ was the concentration of the NaOH solution and $W_{\text{peptide}}$ was the weight of the peptide measured. The carboxyl contents of the peptides produced after 6, 36, 72, 96 and 144 h of the deamidation reactions are presented in Table 1. Increasing the reaction time, especially from 6 to 72 h, the carboxyl content increases rapidly, confirming that longer deamidation reaction produces more carboxyl groups, that is, the peptide is more hydrophilic. The result in Table 1 demonstrates that the hydrophilicity of the peptide can be tuned by the deamidation reaction time.

### 3.5. Amine contents and average molecular weights of the peptides

Table 2 shows that the Lys contents are 0.13—0.38% in zein and the peptides, which is consistent with the conclusion reported in the literature that zein is absence of lysine residue (Shukla & Cheryan, 2001). This conclusion indicates that almost all the primary amine groups in zein and the peptides are the N-terminal ones, which means that the mole numbers of the primary amine groups in zein and the peptides are approximately equal to the mole numbers of the zein and corresponding peptides. Table 3 shows that the primary amine content in zein is 7.04 $\times$ 10$^{-2}$ mol/g, measured by OPA assay, from which the average molecular weight of 14.2 kDa was calculated for the zein. As we know, each cleavage of the peptide bond would produce one primary amine group at N-terminal of the peptide. Compared with zein, the primary amine content is about twice after 36 h of the deamidation reaction and further increases with the deamidation time (Table 3). It is notable that the primary amine contents analyzed by OPA assay are for reference only because OPA assay may have deviation for different proteins/peptides (Pan et al., 2006). The average molecular weight calculated from the amine content is 7.3 kDa for 36h-zein, 5.5 kDa for 96h-zein, and 4.0 kDa for 144h-zein as shown in Table 3.

### 3.6. Conjugation of the peptides with polysaccharides

Maillard reaction can link the reducing-end carbonyl group of polysaccharide to the primary amine group in N-terminal or Lys residue of protein/peptide (Hao, Ma, Huang, He, & Yao, 2013; Helou, Jacolot, Niquet-Leridon, Gadonna-Widehem, & Tessier, 2016; Nooshkam & Madadlou, 2016). In this study, dextran and maltodextrin were separately conjugated to the peptides to increase the hydrophilicity of the peptides. The dextran used has $M_w$ (weight-average molecular weight) of 16 kDa and $M_n$ (number-average
molecular weight) of 5.7 kDa, and the maltodextrin used has $M_w$ of 3.0 kDa and $M_n$ of 0.36 kDa, measured by GPC as shown in Fig. S1 of Supplementary data. Each polysaccharide molecule has only one reducing-end carbonyl group that can react with the primary amine group of the peptide (Ho, Ishizaki, & Tanaka, 2000). As discussed above, zein and the peptides are absence of lysine residue therefore the polysaccharides were conjugated to the N-terminals of the peptides.

In order to control the Maillard reaction at the early stage (Fayle & Gerrard, 2002), the reaction was performed at 60 °C and 79% relative humidity for 48 h, and the polysaccharide was excess during the reaction. After the reaction, the color of the product was the same as the color of the mixture before the reaction. The FTIR absorption spectra in Fig. S2 of Supplementary data show that the changes before and after the Maillard reaction are not much, especially for the reaction of the peptide with maltodextrin. Fig. 3 shows the enlarged FTIR absorption spectra of 144h-ZD and the physical mixture of 144h-zein and dextran. The peak at 845 cm$^{-1}$ (N–H bending of primary amine) weakens which indicates the decrease of the primary amine groups after the reaction. The peak at 1110 cm$^{-1}$ increases after the Maillard reaction, which is ascribed to C–N stretching from secondary amine in the Amadori product formed at the early stage of the Maillard reaction (Fayle & Gerrard, 2002; Yang et al., 2015). The intensity around the band at 1650 cm$^{-1}$ increases, due to typical Maillard reaction products formed at the early stage of the reaction, i.e. Schiff’s base imine group (stretching) and enaminol group (stretching) (Fayle & Gerrard, 2002; Wnorowski & Yaylayan, 2003; Yang et al., 2015).

The FTIR result confirms that the reaction between the carbonyl and amine groups was mainly at the early stage. Fig. S1 in Supplementary data and Fig. 4 show the GPC elution curves. The 36h-zein presents two peaks, one is at 17.7 min, and the other is at 20.3 min (Fig. S1). At the same weight ratio of the peptide to the polysaccharide 1:6, the fraction of 36h-ZD at 17 min possesses higher molecular weights compared with the physical mixture of 36h-zein and dextran, and 36h-ZM presents a shoulder peak at 16–18 min compared with the mixture of 36h-zein and maltodextrin (Fig. 4). The polysaccharide is much excess during the reaction, and thus the polysaccharide fraction is much more than the conjugate fraction after the reaction. Although the conjugate signals are overlapped due to the broad molecular weight distributions of 36h-zein and the polysaccharides and also the conjugates, the fractions with higher molecular weights shown in the 36h-ZD and 36h-ZM curves confirm the formation of the conjugates.

Because producing one conjugate molecule would consume one primary amine group and one reducing-end carbonyl group at the early stage of the Maillard reaction (Fayle & Gerrard, 2002), from the decrease of the primary amine groups we obtained the molecular weight contributions of 36h-zein and the polysaccharides and also the conjugates.

Table 4: ζ-Potentials of the peptides and conjugates at different pH values.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ζ-Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 3.5</td>
</tr>
<tr>
<td>36h-zein</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>96h-zein</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>144h-zein</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>36h-ZM</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>96h-ZM</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>144h-ZM</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>36h-ZD</td>
<td>0.43 ± 0.3</td>
</tr>
<tr>
<td>96h-ZD</td>
<td>0.24 ± 0.2</td>
</tr>
<tr>
<td>144h-ZD</td>
<td>1.3 ± 0.3</td>
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</tbody>
</table>
numbers of the carboxyl groups (Table 1) and conjugated dextran molecules (Table 3), the hydrophilicity sequence of ZD is 144h-ZD > 96h-ZD > 36h-ZD. Similarly, the hydrophilicity sequence of ZM is 144h-ZM > 96h-ZM > 36h-ZM. For the peptide with the same deamidation degree, the conjugated maltodextrin molecules in ZM are less than the dextran molecules in ZD as shown in Table 3. Both dextran and maltodextrin are neutral polysaccharides composed of glucose only. It was reported that the 1,6-linked dextran chain is more extended than the 1,4-linked amyllose chain in aqueous solutions (Ruddick & Goodall, 1998). Possibly, the reducing-end carbonyl in the dextran is more accessible than the carbonyl in the maltodextrin and therefore the reaction activity of dextran is higher than the activity of maltodextrin. Besides, the molecular weight of the dextran is much larger than that of maltodextrin, indicating that ZD is more hydrophilic than ZM.

3.7. ζ-Potentials of the peptides and conjugates

Table 4 shows that the peptides and conjugates have pH sensitive ζ-potentials. At pH 3.5, the ζ-potentials are positive ones. The ζ-potentials are negative ones after changing the solution to pH 4.0. At pH 7.4, all the peptides and conjugates carry many negative charges. The data in Table 4 confirm protonation of the carboxyl groups at acidic condition and deprotonation at neutral condition. The charges of the peptides and conjugates carried are strongly dependent on the medium pH, indicating that the hydrophilicity/hydrophobicity of the peptides and conjugates can be adjusted by the medium pH. It is notable that the ζ-potential values shown in Table 4 are for reference only. The reason is that the instrument for ζ-potential measurements is suitable for 5 nm–10 μm particles, and some of the peptides and conjugates in this study may be too small to be measured accurately.

3.8. Preparation and characterization of conjugate emulsions

The conjugates with the peptide concentration of 5 mg/mL in aqueous solutions were used as emulsifiers and MCT with 10% volume fraction was used as oil phase. Firstly, the emulsions were produced at pH 4.0. The reasons are as follows. At pH 4.0, the conjugates are soluble in aqueous solutions, but most carboxyl groups in the conjugates are protonated as shown in Table 4, thus the conjugates are amphiphilic and possess emulsifying ability. The hydrophobic peptide chains enter into oil phase and the conjugated polysaccharide chains make oil droplets dispersible in aqueous solutions. For freshly prepared emulsions, the data in Table 5 show that the droplet size decreases with the increase of the deamidation degree of the conjugate. For the conjugates with the same deamidation degree, ZD emulsion has smaller droplet size than ZM emulsion. These results indicate that more hydrophilic conjugate can produce emulsion with smaller droplets, which is consistent with the conclusion reported in the literature that for amphiphilic macromolecules, more hydrophilic one can stabilize a larger surface area thus can fabricate smaller particles (Li, Jiang, & Wu, 1997; Zhang, Li, Jiang, & Wu, 2000).

As mentioned above, deamidated zein can be an emulsifier at neutral and alkaline conditions (Cabra et al., 2007; Flores et al., 2010), but cannot be an emulsifier at acidic condition. For comparison, we used 36h-zein with the concentration of 5 mg/mL in aqueous solution to produce emulsion at the same emulsification condition as the conjugates. The peptide precipitated after the solution was adjusted to pH 4.0. Flocculation layer and whey layer appeared shortly after emulsification. We also used dextran with 30 mg/mL concentration in aqueous solution as the emulsifier, and oil layer appeared shortly after emulsification. For the physical mixture of 36h-zein and dextran with the same peptide concentration of 5 mg/mL and dextran concentration of 30 mg/mL as the 36h-ZD, precipitation appeared when the mixture was adjusted to pH 4.0; oil, flocculation and whey layers appeared shortly after emulsification. These phenomena clearly indicate that the individual peptide, dextran, and their physical mixture appeared shortly after emulsification. This conclusion is similar to those reported in the literature that conjugation of polysaccharide to protein can increase the solubility of the protein at its isoelectric point (pI), at which protein carries zero net charge, and the conjugate can improve emulsification ability and emulsion stability at pH around the pI of the protein. For example, deamidated wheat protein-dextran conjugates enhanced stabilization of the emulsions at pH 4, which is close to the pI (4.2) of the deamidated wheat protein.

Table 5

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dₜ (nm)</th>
<th>pH 4.0</th>
<th>pH 7.4</th>
<th>pH 4.0</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Freshly prepared</td>
<td></td>
<td>After 70 days at 4 °C</td>
<td></td>
</tr>
<tr>
<td>Dextran</td>
<td>244 ± 1</td>
<td>197 ± 1</td>
<td>Not determined</td>
<td>192 ± 1</td>
<td>Not determined</td>
</tr>
<tr>
<td>36h-zein</td>
<td>207 ± 1</td>
<td>222 ± 3</td>
<td>Not determined</td>
<td>211 ± 1</td>
<td>Not determined</td>
</tr>
<tr>
<td>36h-zein/dextran mixture</td>
<td>182 ± 1</td>
<td>195 ± 2</td>
<td>Not determined</td>
<td>207 ± 1</td>
<td>Not determined</td>
</tr>
<tr>
<td>36h-ZM</td>
<td>211 ± 1</td>
<td>220 ± 1</td>
<td>Not determined</td>
<td>165 ± 4</td>
<td>Not determined</td>
</tr>
<tr>
<td>36h-ZD</td>
<td>203 ± 1</td>
<td>222 ± 3</td>
<td>Not determined</td>
<td>165 ± 4</td>
<td>Not determined</td>
</tr>
<tr>
<td>96h-ZM</td>
<td>191 ± 1</td>
<td>207 ± 1</td>
<td>Not determined</td>
<td>165 ± 4</td>
<td>Not determined</td>
</tr>
<tr>
<td>96h-ZD</td>
<td>183 ± 1</td>
<td>192 ± 3</td>
<td>Not determined</td>
<td>165 ± 4</td>
<td>Not determined</td>
</tr>
<tr>
<td>144h-ZM</td>
<td>214 ± 1</td>
<td>220 ± 1</td>
<td>Not determined</td>
<td>165 ± 4</td>
<td>Not determined</td>
</tr>
<tr>
<td>144h-ZD</td>
<td>167 ± 5</td>
<td>177 ± 6</td>
<td>Not determined</td>
<td>165 ± 4</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

a Oil layer appeared shortly after emulsification.
b Flocculation and whey layers appeared shortly after emulsification.
c Oil, flocculation and whey layers appeared shortly after emulsification.
d The emulsion presented oil layer after 70 days of storage.
e The emulsion presented oil layer after 4 days of storage.

Table 6

<table>
<thead>
<tr>
<th>Sample</th>
<th>ζ-Potential (mV)</th>
<th>Dₜ (nm)</th>
<th>pH 4.5</th>
<th>pH 7.4</th>
<th>pH 4.5</th>
<th>pH 7.4</th>
<th>pH 4.5</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Freshly prepared</td>
<td></td>
<td></td>
<td>After 40 days at 4 °C</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>36h-ZM</td>
<td>–16.0 ± 0.5</td>
<td>–52.7 ± 1.7</td>
<td>202 ± 3</td>
<td>205 ± 1</td>
<td>219 ± 13</td>
<td>Not determined</td>
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<td>36h-ZD</td>
<td>–8.1 ± 0.7</td>
<td>–39.8 ± 2.2</td>
<td>194 ± 2</td>
<td>193 ± 2</td>
<td>220 ± 6</td>
<td>Not determined</td>
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<tr>
<td>96h-ZM</td>
<td>–15.7 ± 1.1</td>
<td>–571 ± 1.3</td>
<td>204 ± 4</td>
<td>194 ± 1</td>
<td>228 ± 9</td>
<td>Not determined</td>
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<tr>
<td>96h-ZD</td>
<td>–7.8 ± 1.3</td>
<td>–38.8 ± 0.5</td>
<td>193 ± 4</td>
<td>196 ± 6</td>
<td>221 ± 8</td>
<td>Not determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>144h-ZM</td>
<td>–20.7 ± 1.4</td>
<td>–60.1 ± 1.8</td>
<td>190 ± 4</td>
<td>Not determined</td>
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<tr>
<td>144h-ZD</td>
<td>–12.4 ± 0.5</td>
<td>–47.0 ± 1.0</td>
<td>192 ± 4</td>
<td>Not determined</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The emulsion presented oil layer after 40 days of storage.
b The emulsion presented oil layer after 3 days of storage.
c The emulsion presented oil layer after 2 days of storage.

As shown in Table 4, the conjugates carry much more negative charges at pH 7.4 than at pH 4.0. After emulsification, each freshly produced conjugate emulsion was divided into 2 parts. One was directly stored at 4°C, and the other was adjusted to pH 7.4 and then stored at 4°C. After 70 days of storage, the emulsions stored at pH 4.0 were still homogeneous, and their droplet sizes were almost the same as those of freshly prepared emulsions as shown in Table 5, suggesting that the conjugates have excellent emulsifying and emulsion stabilizing abilities at pH 4.0. The droplet sizes did not change significantly and the emulsions were homogeneous when the freshly prepared emulsions were adjusted to pH 7.4. After 4 days of storage at pH 7.4, 36h-ZD and 36h-ZM emulsions were homogeneous, whereas the other emulsions presented a transparent oil layer. After 70 days of storage at pH 7.4, 36h-ZD emulsion also presented oil layer, only 36h-ZM emulsion was homogeneous and its size did not change significantly. These results demonstrate that the emulsions have different stabilities at pH 4.0. As discussed above, 144h-ZD, 96h-ZD, 144h-ZM, and 96h-ZM are more hydrophilic than 36h-ZD and 36h-ZM, as well as 36h-ZD is more hydrophilic than 36h-ZM. The emulsion stability of 36h-ZM > 36h-ZD > 96h-ZM, 96h-ZD, 144h-ZM, and 144h-ZD at pH 7.4 indicates that the oil-water interfacial film fabricated at pH 4.0 by more hydrophilic conjugate dissociates faster at pH 7.4.

It was reported that for hydrophobically associating polymeric acids containing carboxyl groups, the hydrophobic effect restricts non-aqueous components into the smallest possible area to minimize disorder within the aqueous phase; the change from a collapsed uncharged coil structure to a charged extended chain depends on the changes in hydrogen ion equilibria (Tonge & Tighe, 2001). Katchalsky and Eisenberg reported that at low pH the molecules of polymethacrylic acid (PMA) were hypercoiled, possessing a compact impermeable form, with increasing pH the molecules uncoiled due to the electrostatic repulsion of the ionized groups (Katchalsky & Eisenberg, 1951). The peptides in this study are the hydrophobically associating polymers containing carboxyl groups. Similarly, the less hydrophilic conjugate forms more compact interfacial film via stronger hydrophobic interaction, whereas, the more hydrophilic conjugate forms less compacted interfacial film at pH 4.0 that facilitates the deprotonation of the film at pH 7.4; subsequently, the film with more charges dissociates faster due to stronger electrostatic repulsion.

The emulsions were also produced at pH 4.5. For freshly prepared emulsions, their \( \zeta \)-potentials are pH sensitive and the droplets carry more negative charges at pH 7.4 than at pH 4.5 (Table 6) due to the pH sensitivity of the conjugates (Table 4). The emulsions were stable after 40 days of storage at pH 4.5 as shown in Table 6 and Fig. 5. When the emulsions were adjusted to pH 7.4 and then stored, oil layer appeared in 144h-ZD and 144h-ZM emulsions after 2 days, in 96h-ZD and 96h-ZM emulsions after 3 days, and in 36h-ZD and 36h-ZM emulsions after 40 days. The photo of the emulsions after 40 days of storage at pH 7.4 shows that the oil volume increases with the increase of the conjugate hydrophilicity (Fig. 5). Besides, whey layer was observable in 144h-ZD, 144h-ZM, 96h-ZD, and 96h-ZM emulsions after 40 days of storage at pH 7.4. Compared with the emulsions produced at pH 4.0, the emulsions produced at...
pH 4.5 are less stable after storage at pH 7.4, especially for 36h-ZM emulsion. These results can be explained by the fact that the conjugates carry more charges at pH 4.5 than at pH 4.0 (Table 4). Therefore, the conjugates fabricate less compact interfacial films at pH 4.5, which facilitate the deprotonation of the films at pH 7.4 resulting in the emulsions less stable.

Fig. 6 shows the TEM images of 144h-2D emulsion produced at pH 4.5 after 147 days of storage at pH 4.5 and pH 7.4. The emulsion stored at pH 4.5 was homogeneous and presented a droplet structure with integrated interfacial film (Fig. 6A). The emulsion stored at pH 7.4 presented a fused droplet structure (Fig. 6B). The TEM images are consistent with the results above that 144h-2D emulsion was stable at pH 4.5, but demulsification happened when the emulsion was adjusted to pH 7.4 and stored.

The study above demonstrates that the conjugates with low concentration can produce stable emulsions at acidic condition and the emulsions have adjustable stability at neutral condition. As we know, oil in water emulsions are a universal system for encapsulation and delivery of hydrophobic drugs and nourishments (McClements & Li, 2010), and most food and beverage are acidic (Piorkowski & McClements, 2014). The deamidated zein peptide-polysaccharide conjugates, which synchronously possess natural moieties, adjustable hydrophilicity/hydrophobicity, smaller molecular weights and simpler structures compared with protein-polysaccharide conjugates, may have potential applications for delivery of hydrophobic drugs and nourishments.

4. Conclusions

Aso and Gln residues of zein changed into Asp and Glu residues and also zein degraded during the deamidation reaction at alkaline condition. Longer deamidation reaction time produced more carbonyl groups and smaller peptide. Dextran and maltodextrin were separately conjugated to the N-terminals of the peptides via Maillard reaction to increase the hydrophilicity of the peptides. The hydrophilicity/hydrophobicity of the conjugates can be adjusted by deamidation reaction time, number of conjugated polysaccharide, polysaccharide molecular weight, and medium pH. The conjugates have excellent emulsification ability at acidic condition. The emulsion stability at neutral condition can be adjusted by the hydrophilicity/hydrophobicity of the conjugates. This study demonstrates that the deamidated zein peptide-polysaccharide conjugates, which combine the advantages of proteins and synthetic surfactants together, are excellent and green emulsifiers.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodhyd.2016.08.028.

References


