Food Hydrocolloids 91 (2019) 174–181

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd

Emulsifying properties of conjugates formed between whey protein isolate and subcritical-water hydrolyzed pectin

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ARTICLE INFO

Keywords:
Hydrolyzed pectin
Superheated water
Oil-in-water (O/W) emulsion
Whey protein isolate
Maillard reaction
Glycosylation

ABSTRACT

This work aimed to evaluate the feasibility of subcritical water-hydrolyzed pectin from passion fruit (*Passiflora edulis*) peel for improving emulsifying property of whey protein at pH near its isoelectric point. The hydrolyzed pectin with different molecular weights (20, 77, and 146 kDa) were conjugated with whey protein isolate by a dry-heating method at 80 °C, 79% RH, for 6, 24, and 48 h. Soluble fraction after the conjugation was analyzed for conjugated molecules by SDS-PAGE and fluorescence spectroscopy. SDS-PAGE showed polydisperse bands from 15 to 250 kDa, which indicated that whey protein isolate-pectin conjugates were formed. Fluorescence spectroscopy also revealed that the highest conjugates yield was obtained from the conjugation with 146 kDa pectin at 6 h. These conjugates were used as emulsifiers in oil-in-water emulsions containing 5% w/w of medium-chain triacylglycerol in an aqueous phase at pH of 5. The smallest oil-droplet size and most stable emulsion was also obtained using the conjugates at 6 h of the dry-heating. Moreover, the conjugates from larger pectins showed significantly higher emulsion stability.

1. Introduction

Pectin is a natural polymer comprises at least 65% galacturonic acid units with partial methyl esterification and various neutral sugars such as arabinose, galactose, rhamnose, and xylose (Rolin, 2002). Pectin is usually extracted from citrus peel, apple pomace, and sugar beet residue by hot diluted acid solution for commercial use (Brejnholt, 2010; Rolin, 2002). However, novel extraction methods to obtain pectin, e.g. enzyme-, microwave-, and ultrasound-assisted extraction as well as subcritical water extraction were also reported (Adetunji, Adekunle, Orsat, & Raghavan, 2017).

Subcritical water, liquid water at temperatures in the range of 100–374 °C under high pressure conditions, has been investigated for valorizing by-products from various plant sources (Khuwijitjaru, 2016). Recently, we reported the extraction of pectin from passion fruit peel using subcritical water which required shorter time for extraction than conventional method, but the obtained pectins were low in molecular weight of 16–37 kDa (Klinchongkon, Chanthong, Ruchain, Khuwijitjaru, & Adachi, 2017). Subcritical water could also effectively hydrolyze pectin into smaller molecules resulting in the increase of reducing ends of the pectin chain (Klinchongkon, Khuwijitjaru, & Adachi, 2017), and decrease of viscosity (Klinchongkon, Khuwijitjaru, & Adachi, 2018).

Whey protein isolate (WPI) has been used as emulsifier in many oil-in-water emulsions products such as whipping cream and ice cream. However, WPI-based emulsions are very sensitive to destabilization especially when the protein is heated or the pH is changed to isoelectric point (pI) (Euston, 2008). Normally, protein precipitates at pI resulting in poor emulsifying property. However, conjugation with pectin could improve the emulsifying properties of WPI at the pH closed to its pI (Jiménez-Castaño, Villamiel, & López-Fandiño, 2007; Schmidt et al., 2016). The reducing end of pectin can react with the ε-lysine amino groups of protein in a Maillard reaction to form hybrid molecules (Wefers, Bindereif, Karbstein, & van der Schaaf, 2018). This conjugation can be achieved either in wet (Diftis, Pirzas, & Kiosseoglou, 2005) or dry states (Einhorn-Stoll, Ulbrich, Sever, & Kunzek, 2005; Neirynck, Van der Meeren, Gorbe, Dierckx, & Dewettinck, 2004; P. X. Qi, Y. P. Xiao, & E. D. Wickham, 2017; Schmidt et al., 2016; Setiowati, Vermeir,

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https://doi.org/10.1016/j.foodhyd.2019.01.005
Received 16 September 2018; Received in revised form 7 December 2018; Accepted 2 January 2019
Available online 04 January 2019
0268-005X/

Table 1
Hydrolysis conditions and properties of subcritical water-hydrolyzed pectins used for preparation of conjugates.

Hydrolysis temperature (°C)	Time [a] (min)	Severity factor	Degree of esterification (%)	Molecular weight (kDa)[b]	Protein (%)[c]
130	14	2.9	57	146	
150	15	4.2	57	77	0.66
170	17	5.6	58	20	

[a] Time to reach the desired temperature.
[b] By size exclusion chromatography method, n = 3.
[c] By Kjeldahl method (N × 6.25), n = 2.

Martins, De Meulenaer, & Van der Meeren, 2016). WPI-pectin conjugate was reported to produce smaller emulsion oil droplets compared to the original WPI at pH of 5.5 (Schmidt et al., 2016) and also showed higher oil-in-water emulsion heat stability (Setiowati et al., 2016). Since the obtained pectins from subcritical water treatment in our previous study (Klinchongkon, Chanthong, et al., 2017; Klinchongkon, Khuwijitjaru, et al., 2017) were of low molecular weight (16–37 kDa), they are expected to be rather ineffective as emulsion-stabilizing agents. However, polysaccharides of low or intermediate molecular weight have been described as being particularly effective in enhancing the emulsifying and emulsion stabilizing properties of protein-polysaccharide conjugates (Akhtar & Ding, 2017). It has been described that there should be a molecular weight optimum for each polysaccharide at which the improvement of the emulsifying properties is largest. If the molecular weight of the polysaccharide is too low, it cannot confer sufficient steric stabilizing properties to the resulting conjugates. If the molecular weight is too high, adsorption of the resulting conjugates to the oil-water interface will be hindered due to a decrease in the diffusion rate (Akhtar & Dickinson, 2007). The optimal molecular weight of the polysaccharide fraction was reported to be 9 kDa for WPI-maltodextrin conjugates (Akhtar & Dickinson, 2007), whereas maltodextrin of 2 and 280 kDa resulted in conjugates with reduced emulsifying properties. For lysozyme-galactomannan conjugates, a molecular weight of the polysaccharide fraction of at least 6 kDa was reported necessary. Galactomannans up to a molecular weight of 24 kDa were investigated (Shu, Sahara, Nakamura, & Kato, 1996). For conjugates formed between β-lactoglobulin and dextran, improving emulsifying properties with increasing molecular weight of dextran between 19 and 150 kDa were reported. Above 150 kDa and up to 2,000 kDa no further improvement could be detected (Dunlap & Côté, 2005).

In addition to the emulsifying properties, the reaction rate of conjugate formation as well as the extent of glycation of the protein are influenced by the molecular weight of the saccharide fraction. For various mono-, oligo- and polysaccharides, studies report that both features increase with decreasing molecular weight of the saccharide (Chen, Liu, Labuza, & Zhou, 2013; Jiménez-Castaño et al., 2007; Ter Haar, Schols, & Gruppen, 2011). Furthermore, it was shown that a higher yield of conjugates could be achieved when WPI reacted with low methyl esterified pectin compared to high methyl esterified pectin. This was due to the fact that the low methyl esterified pectin used in that study also featured a lower molecular weight caused by the industrial preparation process (Schmidt et al., 2016). Emulsions prepared from the resulting conjugates also displayed smaller oil-droplet sizes than emulsions prepared from conjugates formed between WPI and high methyl esterified pectin which was attributed to the mentioned higher conjugate yield.

In this study, the emulsifying performance of subcritical water-hydrolyzed pectin conjugated with WPI was investigated in an oil-in-water (O/W) emulsion system near the pI value of WPI. An impact of the molecular weight of pectin on the emulsifying and emulsion stabilizing properties was expected. For conjugates formed between WPI and low molecular weight pectin (20 kDa) smaller oil-droplet sizes were anticipated whereas conjugates prepared with high molecular weight pectin (146 kDa) should show better emulsion stabilizing properties. Furthermore, higher yields were expected for conjugates prepared from WPI and low molecular weight pectin.

2. Materials and methods

2.1. Materials

Ripe passion fruit was purchased from a local market in Ratchaburi province, Thailand. WPI, which contains 94% protein, 5% moisture, 0.4% lactose, and 0.3% fat contents, was obtained from Fonterra Cooperative Group (Auckland, New Zealand). Medium-chain triacylglycerol (MCT) was kindly provided by Symrise AG (Holzminden, Germany).

2.2. Subcritical water-hydrolyzed passion fruit pectin preparation

Passion fruit pectin was extracted using hot dilute nitric acid and then hydrolyzed in subcritical water using a batch-type reactor as described in Klinchongkon et al. (2018). The subcritical water hydrolysis was operated non-isothermally and the hydrolysis times depended on the desired maximum temperatures (Table 1). To facilitate result comparison, the effects of hydrolyzed time and temperature were expressed as a single factor called severity factor (ln R_o) originally proposed by Overend, Chornet, & Gascoigne (1987) and widely used in subcritical water hydrolysis studies (Khuwijitjaru, Pokpong, Klinchongkon, & Adachi, 2014; Koomyart et al., 2017). Three hydrolysis conditions were performed by controlling the severity factor to obtain the hydrolyzed pectin with different molecular weights. Hydrolysis condition with higher ln R_o resulted in lower molecular weight pectin as shown in Table 1. Molecular weight of the hydrolyzed pectins was determined by size exclusion chromatography on Ultrahydrogel Linear column (7.8 × 300 mm, Waters, Milford, MA, USA) as described by Klinchongkon, Khuwijitjaru, and Adachi (2017). A total of 5 batches for each condition was prepared to obtain approximately 12 g of hydrolyzed pectin sample.

2.3. Preparation of WPI-pectin conjugates

The preparation of WPI-pectin conjugates was carried out by the method described by Schmidt et al. (2016) with some modifications. Subcritical water-hydrolyzed passion fruit pectin (10 g) was dissolved in demineralized water (585 g) using a high-speed rotor-stator-homogenizer (T-25 Ultra-Turrax, IKA, Staufen, Germany) at 5,000 rpm (equals a tip speed of 3.3 m/s) for 2 min. Then, WPI (5 g) was added into the pectin solution and mixed well at 10,000 rpm (equals a tip speed of 6.6 m/s) until the protein was completely dissolved. The mixture was allowed to stand for 3 h at room temperature to allow for hydration, and then adjusted to pH of 7.0 with 10% w/w NaOH solution. The protein-pectin mixture was then frozen at −18 °C overnight before freeze-drying at −80 °C, 20 Pa (Alpha 2–4 LD plus, Christ, Osterode, Germany). The freeze-dried sample was milled into powder that could pass through a sieve with 0.5 mm-holes using a laboratory grinding mill at 6,000 rpm (POLYMIX® PX-MFC 90D, Kinematica, Luzern, Switzerland). Each fine-powder sample (2.5 g) was spread out in a

10 cm-diameter Petri dish which gave the sample thickness around 5–7 mm. Then, the conjugation reaction was induced using a dry-heating method by storing the sample without a cover in a temperature- and humidity-controlled chamber (PR-15, Tabai Espec, Osaka, Japan) at 80 °C under a relative humidity of 79% for 6, 24, and 48 h. Conjugations of WPI with pectin were done in duplicate.

2.4. Determination of solubility of WPI-pectin mixture

Solubility of the WPI-pectin mixture was analyzed by dispersing 2 g of the heated powder (from 2.3) in 100 g of demineralized water using a homogenizer at 5,000 rpm for 2 min. The pH of the dispersion was measured using a pH meter (edge® Multiparameter pH Meter, Hanna Instruments, Woonsocket, RI, USA), and then adjusted to pH of 5.0 with 10% w/w NaOH or HCl solution. The mixture was centrifuged at 4600 rpm for 30 min at 20 °C (Rotanta 460 R, Hettich, Kirchlengern, Germany). The supernatant was carefully separated from the insoluble solid, and both fractions were dried using a freeze dryer. Solubility was calculated by Eq. (1):

$$\text{Solubility}(\%) = \frac{w_t - w_i}{w_t} \times 100 \quad (1)$$

where w_t is weight of the mixture (g), and w_i is weight of the dried insoluble solid (g). The freeze-dried soluble fractions from two replications were pooled because of the limitation of sample amount and used as WPI-pectin conjugates in the next experiments.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was performed according to the method described by Schmidt et al. (2016) with some modifications. WPI-pectin conjugate samples (after centrifugation and freeze-drying, see 2.4) (4 mg/mL) or WPI (2 mg/mL) were dissolved in demineralized water. Each solution (60 μL) was mixed with 20 μL of reducing buffer (Roti®-Load 1, Carl Roth, Karlsruhe, Germany), and then heated at 99 °C for 5 min to denature protein complex. After that, 20 μL of the solution as well as the protein standard solution with molecular sizes in range of 10–250 kDa (Precision Plus Protein™ Dual Color Standards, Bio-Rad Laboratories, Hercules, CA, USA) were loaded onto a precast 4–20% gradient polyacrylamide gel (Mini-PROTEAN® TGX™, Bio-Rad Laboratories). The electrophoresis was conducted at 90 V for 90 min. A Tris-glycine solution (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) was used as running buffer. After electrophoresis separation, the gel was placed in glacial acetic acid for 5 min for fixing proteins on the gel. Then, the proteins were stained using Coomassie brilliant blue solution containing 10% (v/v) acetic acid for 20 min, and destained using 0.5% (w/v) $CuSO_4 \cdot 5H_2O$ in 10% (v/v) acetic acid and 25% (v/v) ethanol solution until the protein bands were visible.

2.6. Fluorescence spectroscopy

Fluorescence spectra were determined using an Infinite 200 PRO microplate reader (Tecan, Crailsheim, Germany) according to the method described in Schmidt et al. (2016). Conjugate samples (50 mg) were dissolved in 20 mL demineralized water. For the emission spectra, the excitation wavelength was set to 368 nm and the emission was recorded from 400 to 580 nm. The emission slits were set to 2 nm and the average of 10 flashes was recorded.

Data are reported as relative fluorescence intensities at 492 nm. For this, the fluorescence intensity at 492 nm of each heat-treated WPI-pectin mixture was divided by that of the untreated WPI-pectin mixture (0 h of dry-heating time). By this, differences caused by the fluorescence of the raw materials were removed. Accordingly, the relative fluorescence intensity of the untreated WPI-pectin mixtures was equal to 1.

2.7. Emulsion preparation

The freeze-dried WPI-pectin conjugate from 2.4 was used as an emulsifier for emulsion preparation. Emulsions composed of 5% w/w of MCT and 0.1% w/w of emulsifier were prepared in duplicate. Low emulsifier and disperse phase concentrations were used due to the limited sample amount available. A continuous phase was prepared by dissolving 0.3 g of the emulsifier in 284.7 g of demineralized water at 30 °C, and adjusted to pH of 5.0 with 10% w/w NaOH or HCl solution. While the continuous phase was homogenized at 5,000 rpm (equals a tip speed of 3.3 m/s), 15 g of MCT was slowly poured into the continuous phase. After adding all MCT, the emulsion was further homogenized at 5,000 rpm for 2 min. Then, the emulsion was transferred to a high pressure homogenizer (Microfluidizer® MF 110 EH, Microfluidics Corporation, Newton, MA, USA) for preparing the fine emulsion. Each emulsion was homogenized at 40, 60, 80, and 100 MPa.

2.8. Oil-droplet size measurement

Oil-droplet size distributions of the emulsions were measured by a laser diffraction particle size analyzer (LA-950, Horiba, Kyoto, Japan). The refractive indexes of 1.333 and 1.473 were used for the continuous and oil phases, respectively. The oil-droplet size was expressed in terms of the Sauter mean diameter, $d_{3,2}$ (μm).

3. Results and discussion

3.1. Confirmation of conjugate formation

3.1.1. Color development, pH change, and solubility of the conjugates

The progress of the Maillard reaction can be monitored by several parameters, such as color development, pH and formation of insoluble reaction products (Belitz, Grosch, & Schieberle, 2009). Upon prolonged combined heating of proteins and polysaccharides, so-called "advanced Maillard reaction products" are formed, that are characterized by their typical brown color (Hiller & Lorenzen, 2010; Neirynck et al., 2004). The intensity of color development can therefore be taken as a measure of the Maillard reaction progress (Einhorn-Stoll et al., 2005). In this work, WPI was heated together with subcritical water-hydrolyzed passion fruit pectins with different molecular sizes for 6–48 h. The color of the resulting powders changed from light brown to dark brown indicating that Maillard reaction proceeded at higher extents at longer heating time (Fig. 1A). (Spotti et al., 2013; Wang & Zhong, 2014; Wang, Bao, & Chen, 2013). As can be seen from Fig. 1A, the increase in browning intensity was largest for the sample of WPI mixed with 20 kDa pectin which was the lowest molecular weight pectin used in this study. In our previous work, we reported that the lower the molecular weight of pectin, the higher the number of reducing end groups (Klinchongkon, Khuwijitjaru, et al., 2017). A larger amount of this reactive carbonyl group can accelerate the rate of the Maillard reaction (Spotti et al., 2013), possibly resulting in a more intensive brown color. The appearances of the soluble fractions and of the powders obtained after freeze-drying of the soluble fractions are shown in Fig. 1B and C,

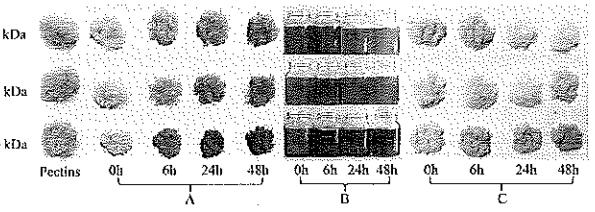

Fig. 1. Color changing of samples: A) samples with conjugation under dry-heating condition at 0, 6, 24, and 48 h, B) soluble fractions at pH 5, and C) freeze-dried soluble fractions at pH 5.

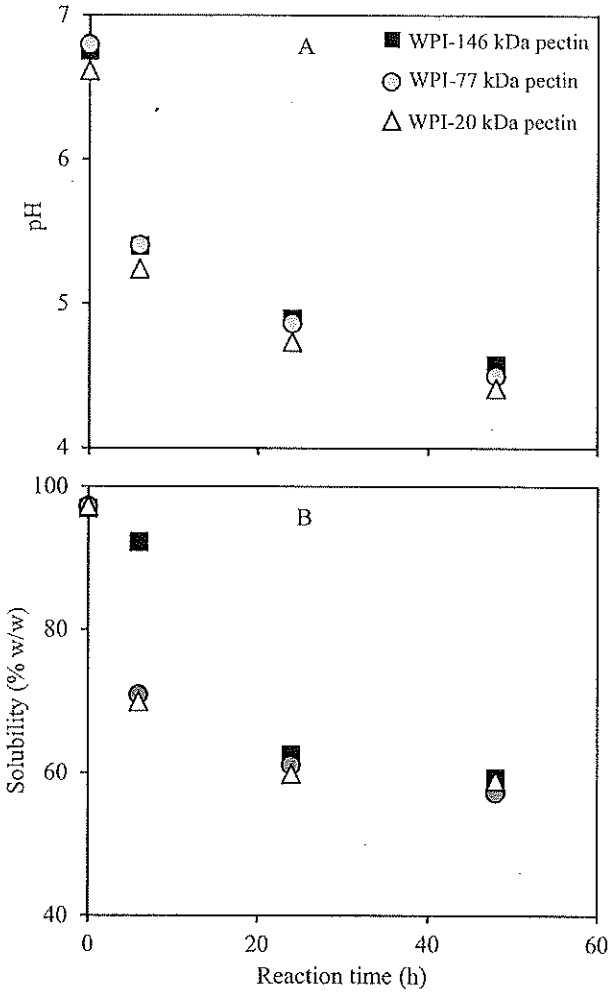

Fig. 2. pH (A) and solubility (B) of WPI-pectin mixtures treated under dry-heating condition at 0, 6, 24, and 48 h.

respectively. It can be seen that the brown color intensities of freeze-dried soluble fractions obviously decreased. This indicated that insoluble brown pigments were partly removed after centrifugation. At the final stage of the Maillard reaction, melanoidins which are high molecular weight brown pigments are produced via polymerization and some of these pigments are completely insoluble in water (Cömert & Gökmen, 2017; Nunes & Coimbra, 2007).

After the dry-heating treatment, the samples were dissolved in demineralized water and the pH of the dispersion was measured. During the Maillard reaction, acid intermediate products, e.g. formic acid, acetic acid, methylglyoxal, glyoxal, and furfural, are formed that can lower the pH of the incubated sample (Brands & van Boekel, 2002, 2003). Fig. 2A shows that the pH value of all WPI-pectin mixtures decreased with heating time from 6.8 to 4.5 after 48 h of heat treatment. The decrease of pH during glycation of WPI and low methoxyl pectin by dry-heating at 60 °C and 74% RH was also reported by Setiowati et al. (2016). However, the measured pH values in that study were much higher (pH 5.6 to 6.2) which was probably due to the less severe reaction conditions.

It is well-known that the solubility of pure WPI after thermal treatment decreases which is caused by the heat-induced formation of insoluble protein aggregates (Einhorn-Stoll et al., 2005). In contrast, the interaction of WPI with pectin and the glycation of whey proteins is hypothesized to change the secondary protein structure which prevents the heat-denaturation and consequently the aggregation of proteins (Einhorn-Stoll et al., 2005; Mishra, Mann, & Joshi, 2001; P. X. Qi, Y. Xiao, & E. D. Wickham, 2017; Xia et al., 2015). Previously, the WPI used in this study showed a decrease in solubility from 97% without heat treatment to 10% after 72 h of dry-heating treatment (Wefers et al., 2018). Fig. 2B shows that all unheated WPI-pectin mixtures (0 h of heating) had a solubility of about 97% independent of the molecular weight of the pectin. This suggests that the Maillard reaction between WPI and pectins did not occur at this stage. Heat treatment for up to 48 h reduced the solubility to around 60% for all investigated pectin types. This value was still markedly higher than the solubility of pure WPI after heat treatment and shows that conjugation of WPI with subcritical water-hydrolyzed pectins gave compounds with higher water solubility. After a heat treatment time of 24 h or more, no difference in solubility between the individual samples could be observed. This indicates that the molecular weight of the pectin does not influence the thermal protective effect provided to WPI by conjugation which is in agreement with previously reported results for WPI-dextran conjugation (Jiménez-Castaño et al., 2007).

3.1.2. SDS-PAGE

The soluble fractions of the heat treated WPI-passion fruit pectin mixtures were characterized by SDS-PAGE analysis under reducing conditions and compared with native WPI and protein standards as shown in Fig. 3. The SDS-PAGE gel indicated that all unheated samples (0 h) showed molecular size distribution patterns similar to native WPI with 4 noticeable bands, corresponding to α-lactalbumin (14 kDa), β-lactoglobulin (18 kDa), β-lactoglobulin dimer (37 kDa), and bovine serum albumin (67 kDa) (Schmidt et al., 2016; Setiowati et al., 2016). For the samples after 6 h of heating, the gel showed polydisperse bands of molecular size distribution from 14 kDa to higher than 250 kDa for all pectin sizes. Polymerization of WPI was shown to not occur under the applied reaction conditions and pectin was documented to not show up in SDS-PAGE analysis with protein staining (Neirynck et al., 2004; Schmidt et al., 2016). Therefore, the observed bands indicate the formation of high molecular weight WPI-pectin conjugates as previously reported by several authors (Einhorn-Stoll et al., 2005; Schmidt et al., 2016; Spotti et al., 2013). Wefers et al. (2018) showed that the result from SDS-PAGE which usually performed as a qualitative analysis was in good agreement with larger molecular weight peaks found by a size-exclusion chromatography with UV detector at 280 nm. Furthermore, the lower the molecular weight of the pectin used for conjugation, the less intense the polydisperse bands seemed. Apparently, less conjugates were present in samples produced from low molecular weight pectin. After longer heat treatment (24 and 48 h) the observed bands were much less intense for all pectin types which resulted from the higher protein precipitation after longer reaction times as mentioned above.

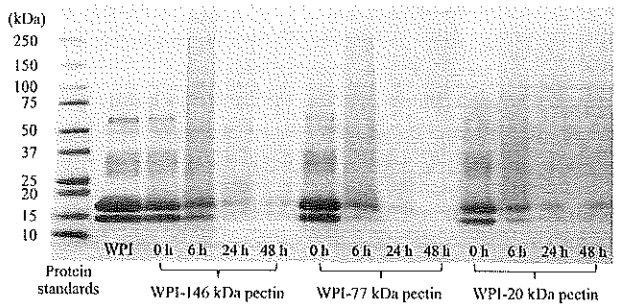

Fig. 3. SDS-PAGE gel of the native WPI, WPI-pectin mixtures (0 h), and WPI-pectin conjugates treated at 80 °C, 79% RH for 6, 24, and 48 h.

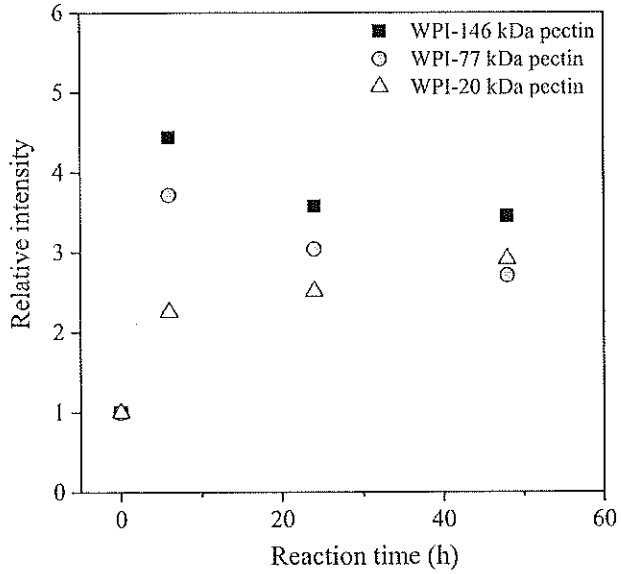

Fig. 4. Relative fluorescence intensity of 0.25% w/v WPI-pectin conjugate solutions as a function of heating time. Intensities were recorded at an excitation wavelength of 368 nm and an emission wavelength of 492 nm.

3.2. Conjugate yield

Conjugate yield was determined by fluorescence spectroscopy as previously described (Schmidt et al., 2016). Here, an increase in relative fluorescence intensity compared to the untreated WPI-pectin mixtures corresponds to the formation of conjugates. Fig. 4 shows that the relative fluorescence intensity increased for all molecular weights of pectin after 6 h of heat treatment, which agrees with the observed high molecular weight bands of the SDS-PAGE analysis. Furthermore, differences can be made out depending on the molecular weight of pectin. After 6 h of heat treatment, the relative fluorescence intensity was highest for high molecular weight pectin, suggesting that there were more conjugates present in the soluble fraction. After 24 h or more, the relative fluorescence intensity of WPI-146 kDa and WPI-77 kDa pectin samples decreased indicating that the conjugate concentration in these samples was lower. This might have been due to degradation of conjugates and an increased formation of insoluble material. For the sample WPI-20 kDa, the relative fluorescence intensity continued to increase with heat treatment time. No maximum intensity was observed within 48 h of reaction time. This is in line with previous results that showed a monotonous increase in the fluorescence maximum for a WPI-52 kDa citrus pectin mixture over a heat treatment time of up to 72 h (Schmidt et al., 2016).

In general, it seems that for WPI-passion fruit pectin, the glycation reaction and the onset of conjugate degradation are faster for high molecular weight pectin (77 and 146 kDa) than for low molecular weight pectin (20 kDa). Literature reports faster reaction rates for lower molecular weight saccharides (Chen et al., 2013; Jiménez-Castaño et al., 2007; Ter Haar et al., 2011). However, these studies mainly investigated oligosaccharides. The polysaccharide dextran with a maximum molecular weight of 20 kDa was the largest investigated molecule (Jiménez-Castaño et al., 2007). Differences to the here reported behavior might derive from the more complex molecular structure of pectin, but more detailed analyses would be necessary to explain the observed reaction behavior.

3.3. Emulsifying performance of whey protein isolate-pectin conjugate

Relatively high negative zeta potentials of WPI-pectin conjugate solutions at pH 5.5 (Schmidt et al., 2016) or emulsions stabilized with pea protein-pectin conjugates were observed (Tamnak, Mirhosseini, Tan, Ghazali, & Muhammad, 2016). The negative zeta-potential might be contributed by carboxyl groups of pectin which give an electrostatic repulsion between oil droplets and good stability of emulsion (Dickinson, 2009). To evaluate the emulsifying performance of conjugates formed between WPI and passion fruit pectin hydrolyzed by subcritical water treatment, a high-energy emulsification method, microfluidization was applied. In order to avoid effects caused by a different conjugate yield in the various samples (see chapter 3.2), emulsions were prepared by using equal amounts of the lyophilized soluble conjugate fraction as emulsifier. By this, the emulsifier concentration was comparable in each prepared emulsion (Wefers et al., 2018).

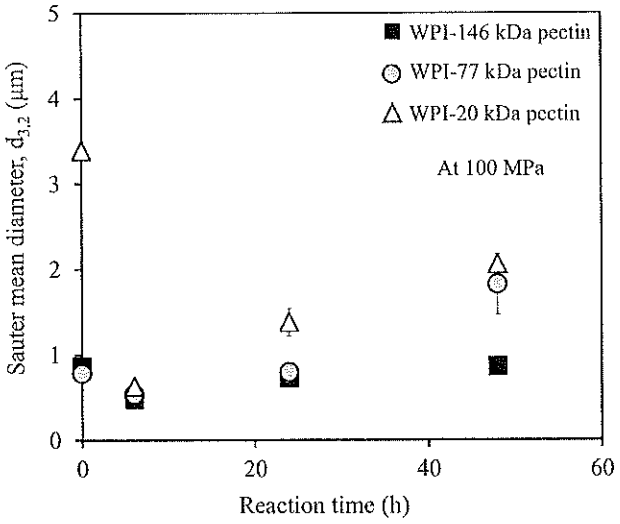

Fig. 5. Sauter mean diameter ($d_{3,2}$) of oil droplet produced from WPI-pectin conjugates obtained from dry-heating of WPI with 20, 77, and 146 kDa subcritical water hydrolyzed pectin at a homogenization pressure of 100 MPa and different heat treatment times.

Fig. 5 shows that all conjugates obtained from 6 h of heating produced emulsions with oil-droplet sizes much smaller than 1.0 μm which is in each case smaller than the untreated WPI-pectin mixture. In addition, all types of conjugates gave oil-droplet sizes smaller than using only hydrolyzed pectin ($d_{3,2}$ = 3.7 − 6.2 μm) or WPI ($d_{3,2}$ > 10 μm) as emulsifiers (data not shown). Nevertheless, mixtures of WPI and higher molecular weight pectin (WPI-77 kDa and WPI-146 kDa) were also very effective in producing submicron droplets which probably resulted from electrostatic complex formation between WPI and pectin (Neirynck et al., 2007).

After longer reaction times, the Sauter mean diameter of oil-droplets in the emulsions prepared from any of the WPI-pectin samples increased.

The observed increase in oil-droplet size was larger for conjugates prepared from low molecular weight pectin (WPI-20 kDa) than for conjugates from high molecular weight pectin (WPI-146 kDa). At any reaction time, conjugates from high molecular weight pectin were able to produce smaller oil droplets than conjugates from low molecular weight pectin. Since these differences were not caused by variations of the conjugate yield, they must originate from the specific properties of the individual conjugate types. According to literature, conjugates formed between protein and low molecular weight polysaccharides should display a faster adsorption to the oil-droplet surface whereas conjugates from high molecular weight polysaccharides should provide better steric stabilization (Chen et al., 2013). In order to investigate both properties for WPI-passion fruit pectin conjugates, emulsification experiments at different homogenization pressures were performed at

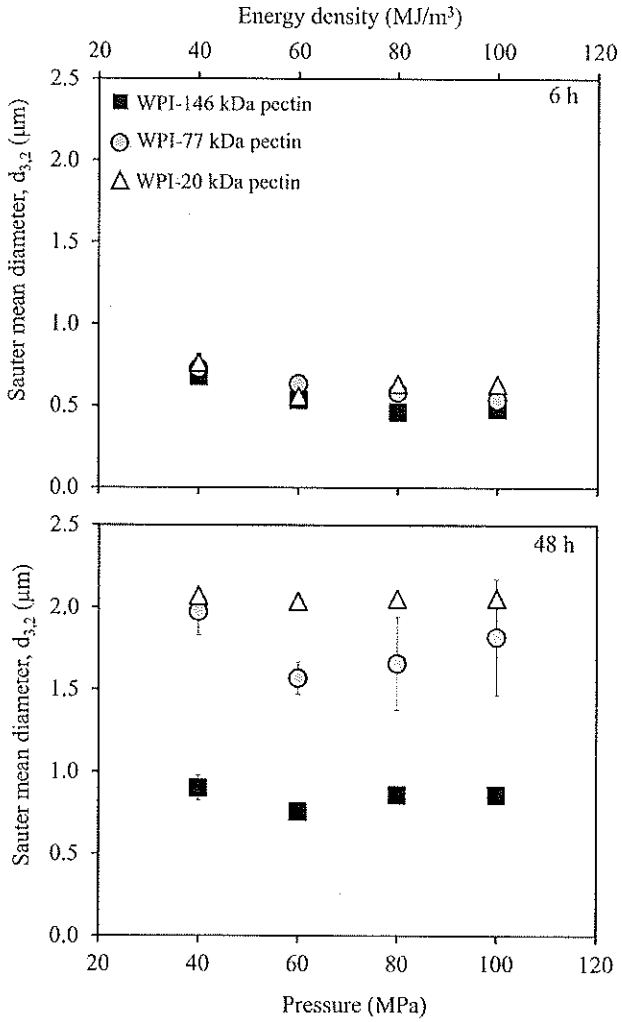

Fig. 6. Sauter mean diameter ($d_{3,2}$) of oil droplet produced from WPI-pectin conjugates obtained from dry-heating of WPI with 20, 77, and 146 kDa subcritical water hydrolyzed pectin at 6 and 48 h and different emulsification pressures.

first.

The homogenization pressure directly correlates with the energy density (E_v) introduced into the system. Furthermore, the dependency of the Sauter mean diameter on the energy density indicates whether an emulsification process is dominated by droplet breakup or coalescence. Negative slopes correspond to a breakup dominated process, whereas positive slopes indicate a strong impact of coalescence on the Sauter mean diameter. The latter is caused by adsorption rates of emulsifiers onto newly created interface that are slower than the collision rates of droplets (Karbstein, 1994; Karbstein & Schubert, 1995; Michael; Stang, Karbstein, & Schubert, 1994; M.; Stang, Schuchmann, & Schubert, 2001). Emulsification were performed at 40, 60, 80, and 100 MPa which correspond to the energy density of 40, 60, 80, and 100 MJ m^{-3}, respectively (Karbstein, 1994; Karbstein & Schubert, 1995). The results in Fig. 6 shows that for all conjugates obtained from 6 h of heating the oil-droplet sizes tended to decrease when the homogenization pressure was increased pointing towards a droplet breakup dominated process. In contrast, emulsions stabilized by conjugates obtained from 48 h of heating did not show a clear positive or negative slope when the homogenization pressure was increased. Droplet breakup and coalescence were at balance showing that adsorption to the interface did not

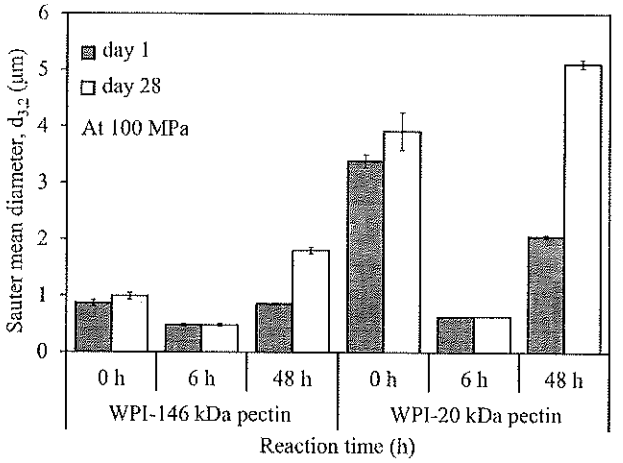

Fig. 7. Sauter mean diameter ($d_{3,2}$) of oil droplet produced from WPI-pectin conjugates obtained from dry-heating of WPI with 20 and 146 kDa subcritical water hydrolyzed pectin on the first day (day 1) and 28 days (day 28) after emulsification.

take place fast enough to stabilize all newly created interface. Furthermore, it can be seen that for all applied homogenization pressures, the Sauter mean diameters stabilized by WPI-20 kDa were larger than for WPI-77 kDa or WPI-146 kDa. This suggests a less efficient droplet stabilization by WPI-20 kDa conjugates which was further investigated by evaluating the long-term stability of the emulsions.

Fig. 7 compares the oil-droplet sizes on the first day (day 1) to the Sauter mean diameter after 28 days of storage (day 28). The droplet in emulsions stabilized by unheated WPI-pectin mixtures only showed a minor increase at day 28. This may be because the pectin portion helped stabilizing the emulsions by steric stabilization (Ngouémazong, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015). Furthermore, stabilizing effects of protein-polysaccharide electrostatic complexes have also been described (Guzey & McClements, 2007). Emulsions stabilized by WPI-146 kDa pectin and WPI-20 kDa pectin conjugates obtained from 6 h of heating showed a high stability as the droplet size did not change over 28 days. In contrast, emulsions prepared from conjugates at 48 h heating time showed a significant increase in droplet size, especially the emulsion produced from WPI-20 kDa pectin conjugate. Einhorn-Stoll, Guyot, and Rittweger (2010) also reported that smaller molecular size pectin showed lower emulsion stabilizing performance. Excellent stability of emulsions prepared from WPI-pectin conjugates was described (Koch, Hummel, Schuchmann, & Emin, 2018; Schmidt et al., 2016), however, the storage period was only 15 days, whereas the one in the present study was over 28 days. Generally, the reduced stabilizing effect of WPI-pectin conjugates after longer heat-treatment time might be caused by degradation reactions affecting both protein and pectin moiety resulting in either less effective interfacial coverage or reduced steric stabilization of droplets (Einhorn-Stoll et al., 2010; Ngouémazong et al., 2015). Therefore, it can be concluded that WPI-pectin conjugates obtained from too small size pectin as well as from too long dry-heat treatment durations possess less efficient emulsifying and stabilizing performance.

4. Conclusions

The present study investigated, for the first time, the emulsifying performance of subcritical water-hydrolyzed pectins conjugated with whey protein isolate via Maillard reaction. The results demonstrated that the conjugates obtained by the dry-heating treatment at 80 °C, 79% RH, 6 h gave small oil-droplet sizes lower than 1.0 μm with excellent stability during 28 days of storage even though the emulsions were prepared at the unfavorable pH of 5.0. In terms of molecular weight

effects, high molecular weight pectin (146 kDa) led to higher conjugate yields than low molecular weight pectin (20 kDa) within the investigated heat-treatment time range of up to 48 h. Regarding the emulsifying and emulsion stabilizing performance, WPI-146 kDa pectin conjugates were able to produce smaller oil-droplets with better long-term stability than WPI-20 kDa pectin conjugates. This effect was more pronounced after longer heat-treatment times. It is suggested that insufficient oil droplet stabilizing properties of the low molecular weight conjugate are responsible for this observation. On the whole, it was demonstrated that subcritical water-hydrolyzed pectin can be used for improving the emulsifying property of whey protein isolate in emulsion with pH close to its isoelectric point.

Acknowledgements

This work was supported by the Royal Golden Jubilee Ph.D. program of the Thailand Research Fund, Thailand (PHD/0055/2557) and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HERP 58/2). P.K. also acknowledges the 2015 Science and Technology Research Grant from Thailand Toray Science Foundation.

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