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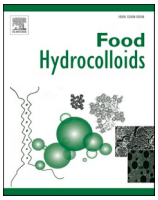

Acylation modification and/or guar gum conjugation enhanced functional properties of pea protein isolate

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ABSTRACT

There has been an increasing demand for diverse and more functional plant-based protein ingredients for food uses. This study aims to improve the functional properties of pea protein isolate through acylation and/or conjugation with guar gum and investigate the physicochemical characteristics of the modified proteins. Acylated pea proteins were prepared by reacting with acetic anhydride (AA) or succinic anhydride (SA) at 0.3 or 0.6 g of AA or SA per g protein, respectively. Guar gum-pea protein conjugates were prepared by incubating the mixture at a mass ratio of 1:20 and 1:40 at 60 °C for 24 h, respectively. Acylated-guar gum-conjugated pea proteins were also prepared to investigate their synergistic effects. Both conjugated and acylated pea proteins showed significantly improved oil holding capacity of up to 2.20 ± 0.05 and 2.09 ± 0.03 g oil/g protein, respectively, compared to the unmodified protein (1.03 ± 0.02 g oil/g). The acylated pea protein also had greater water holding capacity of up to 7.01 ± 0.31 g water/g protein compared to the unmodified protein (3.57 ± 0.05 g water/g). Emulsion capacity and stability were improved up to 96–100% and 95–100%, respectively, for the modified proteins (e.g., 1:20 conj., SA0.3/0.6, AA 0.3/0.6 conj., SA 0.3/0.6 conj.). The suspensions prepared with 9% acetylated pea protein formed firm gels. Sequential acylation and conjugation of pea proteins demonstrated more beneficial and synergistic effects on the water holding capacity and emulsifying properties. However, the *in vitro* gastrointestinal digestibility of the modified pea proteins decreased compared to that of the control pea protein. Overall, the acylated and conjugated pea proteins possessed superior functional properties that could be used as novel food ingredients in meat alternative or beverage applications.

1. Introduction

There has been an increasing demand for plant-based proteins worldwide (Boye, Zare, & Pletch, 2010; Lin et al., 2017). Pea (*Pisum sativum L.*) is one of the most widely cultivated pulse legumes in the world, and it has been utilized in human's diet for thousands of years. Pea protein has significant nutritional advantages such as providing essential amino acids and being associated with health benefits such as reduction of LDL (low density lipoprotein) cholesterol (Rigamonti et al., 2010), anti-inflammatory activity (Ndiaye, Vuong, Duarte, Aluko, & Matar, 2012), modulating intestinal bacterial activities (Świątecka, Świątecki, Kostyra, Marciniak-Darmochwa F, & Kostyra, 2010). Pea protein has been used to produce bioactive peptides with both antioxidant activity and angiotensin I-converting enzyme inhibitor activity (Roy, Boye, & Simpson, 2010). Additionally, pea protein hydrolysates showed beneficial effects on lowering blood pressure (Li et al., 2011). Pea protein has gained great attention in the food and beverages industries as a potentially alterative protein to animal protein for human foods.

Pea contains 20–25% protein, and pea protein contains many essential amino acids, especially that it is rich in lysine, approximately 6.3 g/100 g protein in raw pea (Khattab, Arntfield, & Nyachoti, 2009). Legumin (11S protein) and vicilin (7S protein) are the two major globulin proteins in pea (Burger & Zhang, 2019; Tamnak, Mirhosseini, Tan, Ghazali, & Muhammad, 2016). So far, the utilization of pea protein as a food ingredient is still very limited, partially due to their less-desirable functionalities. For example, pea protein contains high percentage of globulin fraction (49–81%) (salt soluble protein), which showed low solubility in aqueous food system (Tzitzikas, Vincken, De Groot, Gruppen, & Visser, 2006). Commercial pea protein is commonly subjected to harsh processing conditions, which may lead to protein denaturation and further reduce protein solubility (Tamnak et al., 2016). Other functionalities that are associated with solubility may also be impaired, such as water holding capacity, foaming capacity/stability, and

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emulsifying capacity/stability.

To overcome these limitations, previous studies have been conducted to improve pea protein functional properties through chemical modifications. Conjugation between protein and polysaccharide is a popular modification approach, which builds chemical linkages between the protein and polysaccharide via the condensation of carbonyl and ε-amino group at the initial stage of Maillard reaction (Burger & Zhang, 2019). The conjugation reaction enables the protein to be covalently linked with hydrophilic polysaccharide, which enhances protein solubility and emulsifying properties (Guo, Su, Yuan, Mao, & Gao, 2019; Tamnak et al., 2016). Pea protein conjugated with gum Arabic showed improved solubility as well as emulsifying properties (Zha et al., 2019). Additionally, the conjugation reaction mitigated the beany flavor of pea protein. Other studies also showed that pea protein conjugated with propylene glycol alginate (Guo et al., 2019) and pectin (Tamnak et al., 2016) had significantly improved functional properties.

Besides protein-polysaccharide conjugation, acylation is another chemical modification method that has been studied. Succinic anhydride and acetic anhydride are commonly used in the acylation modification of proteins. Acylation is a nucleophilic substitution reaction between acylating agents (e.g., succinic/acetic anhydride) with protein amino acid residues (particularly lysine), resulting in improved functional properties. A previous study demonstrated that acetylation and succinylation of pea protein improved emulsifying properties, foaming, and water holding capacity (Johnson & Brekke, 1983). Acylation modification has also been employed on other proteins, such as faba bean (Jens-Peter Krause, Ralf Mothes, & Schwenke, 1996), chickpea (LIU & HUNG, 2008), and mung bean (El-Adawy, 2000).

Guar gum is derived from endosperm of *Cyamopsis tetragonoloba*, and it is a water soluble polysaccharide (Hamdani, Wani, Bhat, & Siddiqi, 2018). Guar gum is widely used in the food industry due to its excellent water absorption and stabilizing and thickening properties (Karaman, Kesler, Goksel, Dogan, & Kayacier, 2014). This study aims to improve pea protein functional properties in terms of water/oil holding capacity, foaming and emulsion properties, gelation, and solubility through acylation or/and conjugation with guar gum and understand the physicochemical characteristics and *in vitro* gastrointestinal (GI) digestibility of the modified proteins.

2. Materials and methods

2.1. Materials

Pea protein (83% protein content) was supplied by Roquette (Geneva, IL, USA). Guar gum (DeJa' GF Foods, Plain City, OH, USA) and soybean oil were purchased from Amazon. Acetic, succinic anhydrides, 8-anilinonaphthalene-1-sulfonic acid (ANS), β-mercaptoethanol, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of modified pea protein

Acylated pea proteins were prepared by reacting the protein with acetic anhydride (AA) or succinic anhydride (SA) at 0.3 or 0.6 g of AA or SA per g protein in distilled water at 10 wt% protein concentration, respectively. The protein slurry was adjusted to pH 8 using 5 M NaOH and mixed for 1 h at room temperature to allow reaction. After that, the sample was transferred into a dialysis bag (3500 MW cut-off, Thermo Fisher Scientific, Waltham, MA, USA) for dialysis against distilled water at 4 °C for 48 h to remove the residuals of acetic and succinic acids and salts. The distilled water used during the dialysis was changed every 10 h. Then, the modified protein dispersion was lyophilized. All the dried protein powders were kept at 4 °C till further analysis.

The guar gum-pea conjugates were prepared through a wet heating Maillard reaction. Mixture of guar gum and pea protein (1:20 or 1:40 wt ratio) or acylated pea protein (1:20) was dispersed in distilled water at 5 wt% concentration, respectively. The mixture was mixed for 15 min at room temperature and then incubated in a water bath at 60 °C with continuous mixing for 24 h. After that, the sample was lyophilized. All the dried protein powders were kept at 4 °C till further analysis.

2.3. Functional properties

Protein functional properties including solubility, water holding capacity, oil holding capacity, and foaming capacity and stability were measured following our previous methods (Shen, Tang, & Li, 2021) without modification. Emulsion capacity and stability were evaluated similarly to Shen et al. (2021), except that 1.0 g protein was dispersed in 50 mL 50:50 mixture of distilled water and soybean oil, instead of using 1.75 g protein.

The least gelation concentration (LGC) of pea proteins was evaluated following a previous method (Ogunwolu, Henshaw, Mock, Santros, & Awonorin, 2009) with minor modifications. The protein was added into 10 mL distilled water in 15 mL centrifuge tubes and thoroughly mixed to obtain a concentration from 2 to 20% (w/v). The protein suspension was heated at 100 °C for 1 h, cooled under running cold tap water, and refrigerated at 4 °C for 2 h. The LGC was considered as the concentration of protein dispersion that would not fall when the centrifuge tube was inverted.

2.4. Browning reaction during protein conjugation

The measurement of browning reaction was conducted following our previous method (Shen, Chen, & Li, 2018). UV absorbances at 304 and 420 nm are considered as an indicator of the Amadori compound (Wang & Ismail, 2012) and melanoidin (Martinez-Alvarenga et al., 2014) formation in protein-carbohydrate conjugates. The conjugated pea protein (50 mg) was dispersed in 4 mL distilled water in a centrifuge tube, which was vortexed for 10 s and further vigorously mixed for 30 min. After that, the dispersion was centrifuged at 10,000×*g* for 10 min. The supernatant was obtained and analyzed using a double beam spectrophotometer (VWR UV-6300PC, VWR International, Radnor, PA, USA) at 304 and 420 nm.

2.5. Free amino group

Free amino group content of the modified pea proteins was measured following a previous method (Zha, Dong, Rao, & Chen, 2019). One milliliter of protein sample solution (5 mg/mL) was added with 1 mL of 4% $NaHCO_3$ and 1 mL of 0.1% TNBS (2,4,6-trinitrobenzene sulfonic acid) in a centrifuge tube. The mixture was incubated in a water bath at 40 °C for 2 h. After that, 1 mL of 10% (w/v) sodium dodecyl sulphate (SDS) was added to the mixture to solubilize the protein. Finally, the reaction was terminated by adding 0.5 mL 1 N HCl. The protein mixture was cooled at room temperature for 15 min, and absorbance at 340 nm was measured using the double beam spectrophotometer (VWR UV-6300PC). L-leucine was used as a standard to establish the calibration curve.

2.6. Surface hydrophobicity and fourier transform infrared spectroscopy (FTIR)

Surface hydrophobicity information and FTIR spectra of the modified pea proteins were collected according to our previous method without modification (Shen et al., 2021).

2.7. Circular dichroism (CD) spectroscopy

Secondary structures of pea proteins were determined by using a Jasco J-815 circular dichroism spectrophotometer (Jasco Analytical Instruments, Easton, MD). The protein sample was dissolved in distilled water, which was further diluted to a certain concentration that could fit

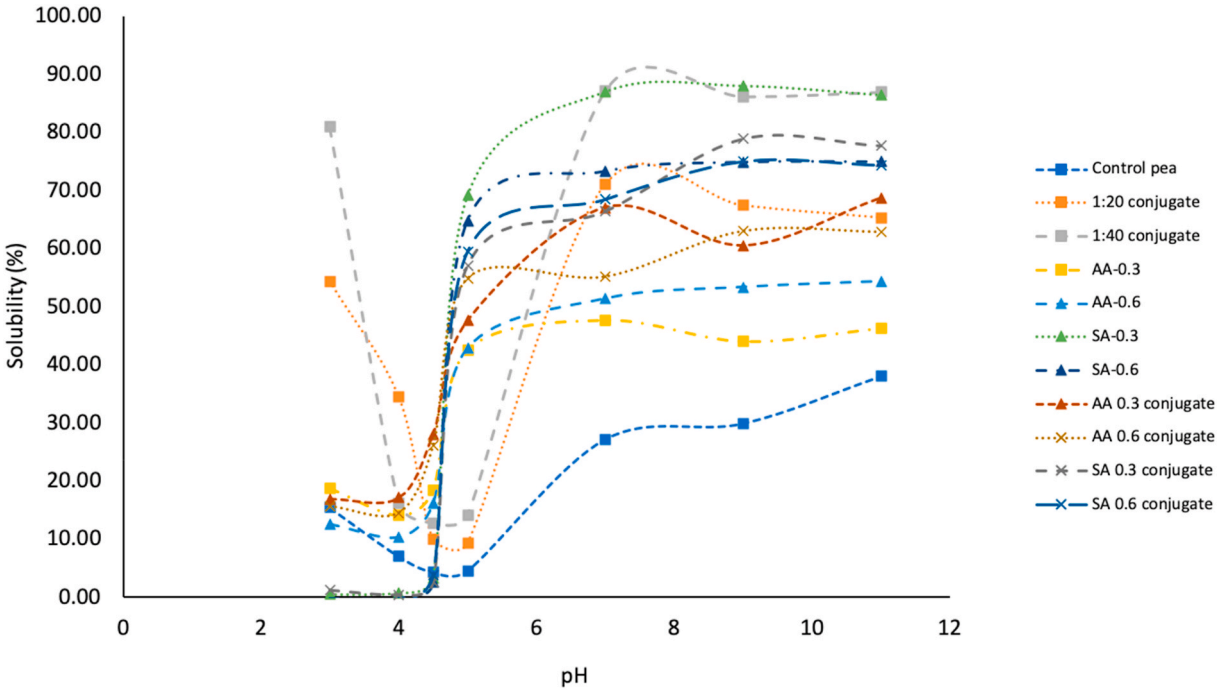

Fig. 1. Solubility of pea and modified pea proteins.

into the scanning regions. The protein solution was scanned from 190 to 250 nm. The following parameters were used: step interval 1 nm, acquisition duration 50 nm/min, and bandwidth 0.5 μm. The data were recorded and corrected by subtracting the water blank. The data of protein secondary structure was estimated using BeStSel (Micsonai et al., 2018).

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

SDS-PAGE of the modified proteins under reducing condition was performed according to our previous method (Chen et al., 2019), except that the protein sample (5 mg/mL) was extracted using 1% SDS/sodium phosphate buffer (pH 7.0) with 2% β-mercaptoethanol, instead of deionized water.

2.9. Free sulfhydryl (SH) content

The measurement of free SH groups was conducted following the method from a literature (Lagrain, Brijs, Veraverbeke, & Delcour, 2005). Protein solution (5 mg/mL) was prepared by dissolving the protein in 0.05 M sodium phosphate sample buffer (pH 6.5), which consisted of 2% SDS (v/v), 3.0 M urea, and 1.0 mM tetrasodium ethylenediamine tetraacetate. Five mL of the prepared solution was added with 500 μL of 0.1% (w/v) DTNB Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid), followed by mixing vigorously for 45 min, and centrifugation at 10,000×*g* for 3 min. The absorbance was measured at 412 nm using the spectrophotometer (VWR UV-6300PC). Glutathione was used as a standard to establish the calibration curve.

2.10. In vitro gastrointestinal digestibility

In vitro digestibility of the proteins were determined following a simulated gastric and intestinal digestion method from literature (Wen, Li, Gu, Wang, & Wang, 2019; Wu, Taylor, Nebl, Ng, & Bennett, 2017) with some modifications. Briefly, 50 mg of protein was first dispersed in 20 mL of simulated gastric fluid solution, which contains 2.5 mM $CaCl_2$, 35 mM NaCl, and pepsin (182 U/mg protein). The protein solution was acidified with HCl to pH 2, and digestion was continued at 37 °C for 1 h in a water bath shaker. *In vitro* intestinal digestion was then carried out by adding 4 mL of simulated intestinal fluid containing 7.6 mM $CaCl_2$, 20.3 mM Tris, 7.4 mM bile salts, trypsin (40 U/mg protein), and chymotrypsin (0.5 U/mg protein) to the protein solution after the 1 h gastric digestion. The pH of the protein solution was adjusted to 7 before incubating the sample in the water bath shaker for 2 h. The digestion was stopped by heating the solution in boiling water for 5 min, cooled down, and centrifugated at 3780×*g* for 5 min. The supernatant was diluted with 100 mM sodium bicarbonate (1: 200, v/v), which was further mixed with OPA reagent (100 mM sodium tetraborate, 0.01% SDS, 0.05 mg/mL OPA, and 0.05 mg/mL DTT) (1:50, v/v). Finally, 200 μL of the solution was added in a 96-well plate, and the fluorescence was determined using a plate reader (excitation at 340 nm, emission at 450 nm) (BioTek, Synergy H1 Hybrid, Highland Park, Winooski, VT, USA). L-Leucine was used to establish a calibration curve. The DH% (degree of hydrolysis) was calculated according to the literature (Wen et al., 2019) with h_{total} factor of 7.8 based on soy (Nelsen et al., 2001).

2.11. Statistical analysis

All the experiments were carried out in at least two replicates. Kruskal-Waillis non-parametric test and Conover-Iman procedure were used to analyze the specific sample pairs for stochastic dominance ($p < 0.05$) among the treatments using Python 3.6 package scipy. stats (Python code and example are available in the Supplementary Document). The results are presented as mean ± standard deviation.

3. Results and discussion

3.1. Protein solubility

Protein solubility is considered as one of the most critical functionalities in food applications, because it is associated with many other functional properties, such as hydration, foaming, and emulsifying properties. Generally, all the modified pea proteins had greatly improved solubility compared with the unmodified pea protein above the isoelectric point (pI, around pH 5) (Fig. 1). Guar gum-pea conjugates

Table 1
Functional properties of pea proteins.

	WHC (g H_20/ g protein)	OHC (g oil/g protein)	EC (%)	ES (%)	LGC (%)
Pea	3.57 ± 0.05^{f}	1.03 ± 0.02^{d}	45.08 ± 1.44^{dc}	39.66 ± 0.76^{d}	18^{a}
1:20 mix	5.20 ± 0.20^{cd}	1.07 ± 0.01^{d}	96.69 ± 0.99^{c}	67.86 ± 5.02^{c}	13^{d}
1:40 mix	4.09 ± 0.07^{de}	1.06 ± 0.01^{d}	67.54 ± 1.95^{c}	54.46 ± 1.02^{cd}	15^{b}
1:20 conj.	3.61 ± 0.11^{ef}	2.02 ± 0.05^{bc}	98.75 ± 0.56^{b}	94.73 ± 0.58^{bc}	11^{e}
1:40 conj.	2.67 ± 0.06^{f}	2.20 ± 0.23^{ab}	95.57 ± 0.56^{c}	60.67 ± 1.73^{c}	15^{b}
AA 0.3	7.01 ± 0.31^{ab}	1.72 ± 0.01^{cd}	41.60 ± 1.06^{d}	34.79 ± 3.58^{e}	9^{g}
AA 0.6	5.03 ± 0.06^{d}	1.63 ± 0.03^{d}	38.48 ± 1.87^{d}	33.72 ± 3.26^{e}	11^{e}
SA 0.3	5.68 ± 0.25^{c}	2.09 ± 0.03^{b}	99.00 ± 0.39^{b}	96.65 ± 0.59^{b}	14^{c}
SA 0.6	6.31 ± 0.65^{bc}	1.88 ± 0.05^{c}	99.14 ± 0.31^{b}	95.63 ± 0.66^{b}	14^{c}
AA 0.3 conj.	5.79 ± 0.21^{b}	1.76 ± 0.02^{cd}	100.00±0^{a}	53.73 ± 1.23^{d}	9^{g}
AA 0.6 conj.	7.78 ± 0.15^{a}	1.85 ± 0.05^{c}	100.00±0^{a}	100±0^{a}	7^{h}
SA 0.3 conj.	3.74 ± 0.24^{ef}	2.18 ± 0.11^{ab}	100.00±0^{a}	99.08 ± 0.34^{a}	10^{f}
SA 0.6 conj.	10.91 ± 0.63^{a}	2.88 ± 0.05^{a}	100.00±0^{a}	98.69 ± 0.55^{a}	10^{f}

Note: WHC: water holding capacity; OHC: oil holding capacity; EC: emulsion capacity; ES: emulsion stability; LGC: least gelation concentration. *Means with different letters for each functional attribute denote significant differences ($p < 0.05$).

(1:20 and 1:40) also showed much higher solubility below the pI, while the solubility of the acylated pea proteins was much lower below pH 5, especially that the succinylated pea proteins were barely soluble. Thus, we can conclude that conjugation modification with polysaccharide is highly effective in improving protein solubility. This is because when protein is conjugated with hydrophilic polysaccharide at the early stage of Maillard reaction, protein hydration properties are improved, therefore, enhancing the solubility (Du et al., 2013).

The succinylated pea protein had relatively higher solubility than the acetylated pea protein when the pH was greater than 5, and it had lower solubility when the pH was less than 5. This could be explained by the fact that the succinylation process replaced the ammonium groups from lysine residues, which resulted in fewer hydrophilic cation groups to counterbalance the protein-protein hydrophobic interactions. Therefore, protein-protein interaction was stronger below the pI, which reduced its solubility. When the pH was above 5, the replacement of ε-amino group of lysine with negatively charged carboxyl groups enhanced the interaction between protein-water, and promoted the intra- and intermolecular charge repulsion, thus, resulting in unfolding and dissociation of the quaternary structures and increased solubility (Anaya Castro et al., 2019; Arogundade et al., 2013; El-Adawy, 2000; Mirmoghtadaie, Kadivar, & Shahedi, 2009). Lower solubility of the acetylated pea proteins than the succinylated pea proteins above the pI was due to stronger aggregation between the unfolded protein via hydrophobic interactions (Yin, Tang, Wen, & Yang, 2009a). Our result was in agreement with other studies on the acylation of African yam bean protein (Arogundade et al., 2013), mung bean protein (El-Adawy, 2000), oat protein (Mirmoghtadaie et al., 2009), and rice protein (Du et al., 2013).

3.2. Water/oil holding capacities

Water and oil holding capacities (WHC, OHC) determine the water/oil retention of the proteins and protein-water/oil interactions and affect texture and quality of food products. The WHC is also associated with other protein functional properties, such as solubility, emulsifying properties, and gelation. The physical mixture of guar gum-pea (1:20) had significantly higher WHC than the conjugated (1:20) and unmodified pea proteins (Table 1). Guar gum is a high molecular weight polysaccharide and strongly interacts with water, acting as a thickening agent (Karaman et al., 2014), and the higher WHC was achieved by its stronger water binding ability. Higher concentration of guar gum (1:20 vs. 1:40) resulted in higher WHC for the simple guar gum/protein mixture and the conjugated proteins, because more hydrophilic polysaccharides enhanced the affinity between protein and water molecules. However, the WHC of the conjugated protein was not obviously improved compared with the unmodified protein, which was probably related to the surface hydrophobicity of the proteins (Table 2). Conjugated proteins with decreased surface hydrophobicity showed stronger WHC (Amid, Mirhosseini, Poorazarang, & Mortazavi, 2013). Arogundade et al. (2013) and Lillard, Clare, and Daubert (2009) also reported that protein-polysaccharide conjugation did not increase the WHC of African yam bean and whey proteins; however, Wang, Zhang, Zhang, Ju, and He (2018) reported that conjugated rapeseed protein had significantly increased WHC. Overall, the WHC of conjugated protein depends on the conjugation conditions, degree of conjugation, types of polysaccharide, and its surface hydrophobicity.

The WHC of acetylated and succinylated pea proteins increased significantly compared with the unmodified and conjugated pea proteins (Table 1). Acylation modification unfolds the protein and alters protein electrical charge distribution, resulting in enhanced hydrophilic binding site of the protein molecules (El-Adawy, 2000). With increased concentration of acylation agents, there was no significant difference for the WHC of the succinylated pea proteins, but WHC of the acetylated protein decreased due to the conversion of protein net positive charge to

Table 2
Surface hydrophobicity, free S–H content, and protein secondary structures.

Protein samples	Surface hydrophobicity	Free S–H (μmol/g protein)	α-helix (%)	β-sheet (%)	β-turn (%)	random coil (%)
Pea	72,543 ± 3,720^{a}	5.42 ± 0.22^{a}	17.17 ± 1.37^{a}	23.97 ± 1.53^{a}	1.17 ± 1.53^{a}	57.67 ± 1.33^{a}
1:20 mix	116,861 ± 2,343^{b}	4.56 ± 0.45^{ab}	33.97 ± 9.85^{b}	32.53 ± 11.04^{ab}	/	33.53 ± 20.75^{ab}
1:40 mix	160,597 ± 5,462^{c}	5.09 ± 0.13^{ab}	21.23 ± 2.22^{ab}	25.60 ± 3.03^{a}	/	53.17 ± 4.81^{a}
1:20 conj.	152,126 ± 7,239^{bc}	5.06 ± 0.17^{ab}	42.87 ± 5.99^{b}	13.97 ± 7.74^{a}	/	43.17 ± 13.58^{b}
1:40 conj.	178,954 ± 6,750^{c}	5.49 ± 0.00^{a}	53.47 ± 9.60^{b}	15.63 ± 4.17^{a}	/	30.90 ± 10.76^{b}
AA 0.3	18,885 ± 2,336^{d}	0.87 ± 0.06^{b}	19.90 ± 0.40^{a}	20.10 ± 3.93^{a}	16.40 ± 0.82^{ab}	43.60 ± 5.02^{ab}
AA 0.6	35,482 ± 2,255^{ae}	0.86 ± 0.02^{b}	10.63 ± 2.49^{a}	34.00 ± 7.53^{b}	15.87 ± 2.57^{ab}	41.10 ± 2.56^{b}
SA 0.3	33,416 ± 3,151^{e}	5.69 ± 0.82^{a}	31.93 ± 9.76^{b}	55.83 ± 8.33^{b}	/	12.30 ± 16.86^{b}
SA 0.6	52,467 ± 3,024^{a}	4.17 ± 0.68^{ab}	18.47 ± 2.59^{ab}	51.97 ± 6.52^{b}	/	29.53 ± 7.91^{b}
AA 0.3 conj.	24,606 ± 1,666^{ed}	0.82 ± 0.00^{b}	18.37 ± 2.77^{a}	26.07 ± 3.52^{b}	15.40 ± 4.25^{ab}	40.20 ± 1.73^{b}
AA 0.6 conj.	21,801 ± 1,685^{d}	0.84 ± 0.08^{b}	10.63 ± 4.54^{a}	31.50 ± 2.10^{ab}	16.87 ± 3.49^{b}	40.73 ± 1.01^{ab}
SA 0.3 conj.	109,611 ± 2,506^{b}	5.28 ± 0.32^{a}	25.90 ± 4.99^{b}	45.40 ± 9.69^{b}	/	28.70 ± 14.56^{b}
SA 0.6 conj.	94,011 ± 3,939^{a}	3.55 ± 0.43^{ab}	15.33 ± 0.85^{a}	34.50 ± 3.69^{ab}	/	50.23 ± 4.46^{ab}

*Means with different letters in each column denote significant differences ($p < 0.05$).

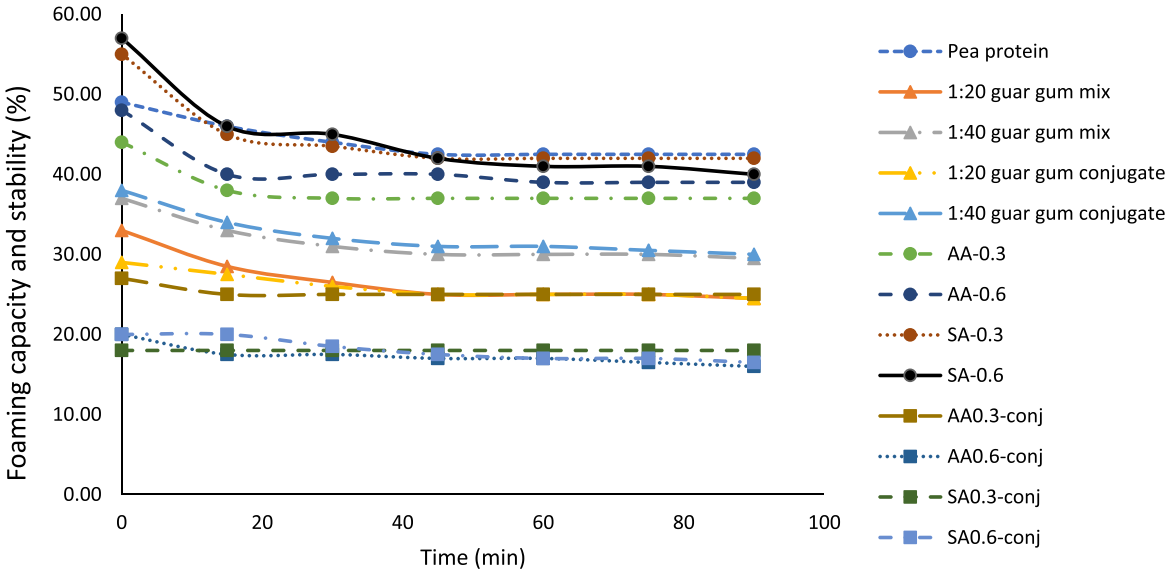

Fig. 2. Foaming capacity and stability of pea and modified pea proteins.

neutral charge. Furthermore, AA-0.3 exhibited higher WHC than SA-0.3. The succinylated protein had higher solubility than the acetylated protein (Fig. 1); therefore, more succinylated proteins were dissolved in water instead of absorbing and holding the water. In addition, sequential acylation and conjugation had synergistic effect on WHC, especially for SA-0.6 conjugate, which exhibited the highest WHC of 10.91 g water/g protein among all the modified proteins.

All the modified proteins (i.e., conjugation, acylation, and sequential modification) had significantly higher OHC compared with the unmodified pea protein (Table 1). The conjugation modification had a greater effect on increasing the OHC, because the heat treatment during the protein-polysaccharide conjugation altered and unfolded the protein structure and exposed more hydrophobic amino acid residues of the protein. Overall, the succinylated pea proteins exhibited higher OHC than the acetylated pea proteins, while there was no significant difference for OHC among the modified proteins with different levels of the same modifier. In addition, the protein from sequential acylation and conjugation (SA 0.6 conj) showed the highest OHC among all the protein samples. Protein OHC could be affected by many factors, such as protein surface area, ratio of hydrophilicity/hydrophobicity, protein net charge, etc.

3.3. Emulsifying properties

Overall, most modified pea proteins exhibited significantly higher emulsion capacity (EC) and emulsion stability (ES) compared with the unmodified pea protein, except for AA 0.3/0.6 (Table 1). Generally, the guar gum-pea protein conjugates had higher EC and ES compared with the simple mixtures at the same gum concentration, indicating that the protein-polysaccharide interactions induced through Maillard reaction are crucial in improving the emulsifying activity of the protein. Higher gum concentration in the modified proteins (1:20 conj vs. 1:40 conj) resulted in greatly enhanced emulsion stability (94.7% vs. 60.7%), which was attributed to the hydrophilicity of the polysaccharide. Conjugation of guar gum and protein caused the formation of strong solvated layer at the oil-water interface, which favored the steric stabilization of the emulsion oil droplet (Keowmaneechai & McClements, 2002). The absorbed layer of conjugated protein has more effective steric stabilization of emulsion droplets than the unmodified protein (Du et al., 2013).

Acetylation and succinylation had distinct effects on the EC and ES of pea protein. The EC and ES of AA 0.3/0.6 were significantly decreased, while the EC and ES of SA 0.3/0.6 were significantly increased compared with the unmodified pea protein (Table 1). The addition of longer aliphatic groups by succinylation increased the protein-water interaction (El-Adawy, 2000; Johnson & Brekke, 1983), and exposed more hydrophobic residues of the protein; therefore, the emulsifying properties were significantly improved. The emulsifying properties were also positively related to protein solubility (Fig. 1). The succinylated protein could form more stable layers around the oil droplets to facilitate their interaction with aqueous phase because of higher solubility, and the emulsifying properties of the acetylated pea proteins were limited due to a lower solubility. Sequential acylation and conjugation modifications had exceptional synergistic effects on the emulsifying properties of the proteins, achieving nearly 100% EC and ES, except for AA 0.3 conjugate. The results showed that modification of protein structures by adding appropriate functional groups is highly effective in enhancing its functional properties (Du et al., 2013).

3.4. Foaming properties

Important characteristics of protein foaming properties include foaming capacity (FC) and foaming stability (FS). Foaming capacity is determined by the amount of interfacial area that can be created by the protein, and it is highly related to protein hydrophobicity, while foaming stability indicates its ability against stress during a certain period of time (Lam, Can Karaca, Tyler, & Nickerson, 2018). Foam formation is dependent on the interfacial film that is formed by the proteins and the ability to maintain the air bubble in the suspension and slow down the coalescence rate (Shen et al., 2021). In this study, most of the modified pea proteins showed decreased FC and FS compared with the unmodified pea protein, except for SA 0.3/0.6 (Fig. 2). The conjugated proteins had much lower FC and FS than the acylated proteins. The higher FC of succinylated pea proteins may be attributed to their smaller molecular size and better solubility, so they could be more rapidly absorbed during the whipping process to generate more foams compared with the conjugated proteins with higher molecular weight and lower solubility (Aluko, McIntosh, & Reaney, 2001; Zhao et al., 2013).

When comparing different guar gum-pea protein conjugates, the 1:40 conjugate exhibited better FC and FS than the 1:20 conjugate; however, the foaming properties of both conjugates were weaker than that of the unmodified protein. The results implied that the addition of high

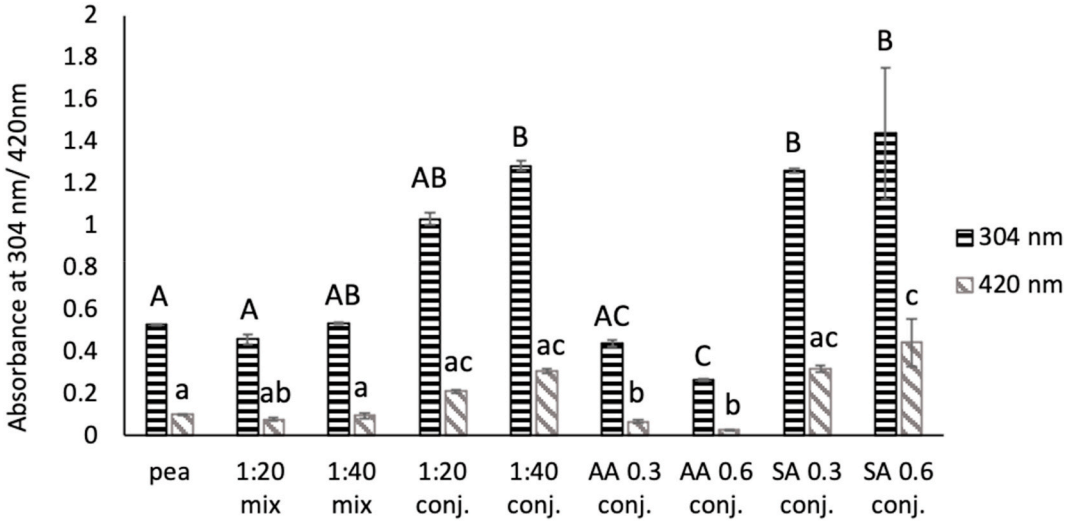

Fig. 3. Browning reaction in modified pea proteins. *Means with different lowercase or capital letters denote significant differences ($p < 0.05$).

molecular weight polysaccharide conjugated with the protein does not help in improving foaming properties. Other studies also found that some excessive modification of proteins could cause foam destabilization and poor stability due to the increase of net charge density, reduce the protein-protein interaction in the foam lamellae, and prevent the formation of elastic film in the air-water interface (Arogundade et al., 2013; Mirmoghtadaie et al., 2009).

3.5. Gelation property

Protein gelation is important in determining the texture, quality and sensory attributes of many foods (Foegeding & Davis, 2011). Overall, gelation properties of all the modified pea proteins were significantly enhanced with lower least gelation concentration (LGC) values compared with the unmodified pea protein (Table 1). The 1:20 protein conjugate had significantly decreased LGC compared with the simple protein-gum mixture (1:20), and both of them had better gelation properties than the 1:40 conjugate and mixture. This is because the addition of higher amount of hydrocolloid improved gel thickening function of the protein (Saha & Bhattacharya, 2010), and unfolding of the protein through conjugation enhanced protein hydrophobic interaction in the formation of more stable gel network, reducing the amount of proteins required for gel formation (O'Kane, Vereijken, Gruppen, & Van Boekel, 2005). Wang et al. (2018) reported that only moderate degree of conjugation of rapeseed protein with dextran could improve the gelation properties, while excessive conjugation decreased gelation properties, because additional static space was created between the conjugated protein molecules with polysaccharide coating, which inhibited protein hydrophobic interaction (Liu, Zhao, Zhao, Ren, & Yang, 2012). The acetylated pea proteins exhibited significantly lower LGC values, and thus better gelation properties, compared with the succinylated proteins. During the acetylation process, the protein was unfolded and disulfide crosslinking was enhanced (Schmandke et al., 1981), improving the gelation properties. Furthermore, sequential acetylation and conjugation dramatically decreased the LGC, especially for the AA 0.6 conjugate, which formed stable gets at only 7% concentration. The result demonstrated that synergistic effect occurred when combining both modifications.

3.6. Browning reaction

The relative amount of browning compounds generated during the conjugation reaction in the modified proteins was measured based on the absorbance at 304 nm (early intermediate Amodari compounds) and

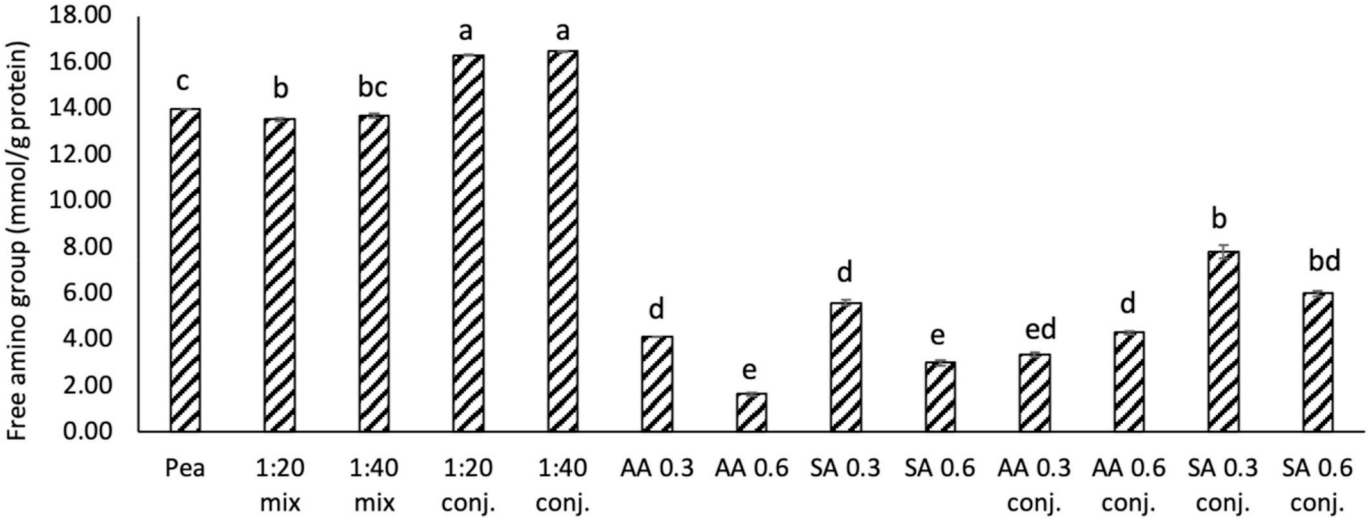

Fig. 4. Free amino group content of pea and modified pea proteins. *Means with different letters denote significant differences ($p < 0.05$).

420 nm (final Maillard reaction products), respectively (Shen et al., 2018b). Generally, the conjugated proteins had significantly higher absorbance at 304 nm compared with the unmodified protein (Fig. 3), but the absorbances at 420 nm were similar, which implied that majority of the protein-polysaccharide conjugates belongs to the early intermediates of Maillard reaction products. The 1:40 conjugate had relatively higher absorbance at 304 nm than the 1: 20 conjugate. This may be caused by the formation of more browning compounds with higher amount of proteins in the 1:40 conjugate during the Maillard reaction. Browning reaction depends on the conjugation conditions, such as reaction temperature, time, and the ratio of protein/polysaccharides (Zha et al., 2019). The simple guar gum-pea protein mixtures and unmodified protein had similar absorbance at 304 and 420 nm, because conjugation reaction was not expected for the mixtures as they were prepared at room conditions by simply mixing (Fig. 3).

3.7. Free amino group content

The amount of available free amino group is another indicator of the degree of protein acylation and guar gum-protein conjugation. The acylated proteins had significantly lower amount of free amino group compared with the unmodified pea proteins (Fig. 4). This is because the acylation reaction mainly occurred between the acylating agent and free amino groups of the proteins, although reactions could also occur with other amino acid residues such as cysteine, tyrosine, serine and/or threonine (Lee, Groninger, & Spinelli, 1981). The succinylated proteins had a significantly higher amount of free amino group than the acetylated proteins with the same amount of acylation agent. When AA and SA were added at the same weight amount, more intensive reactions were expected for AA because of its higher molar ratios to protein and stronger reactivity. Although conjugation reaction occurred between carbonyl groups of polysaccharides and amino groups of protein, the amount of free amino group of the conjugated proteins was not reduced compared with that of the unmodified protein. This was caused by the interfered absorbance of guar gum molecules that was overlapped with the absorbance of the conjugated proteins during free amino measurement. In addition, we used a much lower amount of polysaccharide relative to the protein (1:20 and 1:40); therefore, relatively much less amount of free amino group was consumed during the conjugation modification.

3.8. Surface hydrophobicity

Surface hydrophobicity of protein is dominated by the hydrophobic amino acid group residues available at the surface of protein. The guar gum-pea protein conjugates had greatly larger ($p < 0.05$) surface hydrophobicity compared to the unmodified pea protein (Table 2). This is because the inclusion of polysaccharide to the protein led to protein unfolding and exposure of more hydrophobic residues. However, the surface hydrophobicity of 1:20 conjugate was lower than that of the 1:40 conjugate, which may be attributed to the intrinsic hydrophilicity of the polysaccharide. Both the acetylated and succinylated pea proteins had significantly lower surface hydrophobicity than the unmodified pea protein, although higher level of modifier resulted in slightly higher surface hydrophobicity (Table 2). Acylation modification of the protein introduced succinyl and acetyl groups onto the protein, which increased the electronegativity and enhanced the electronic repulsion, and this prevented ANS probe from binding to the protein hydrophobic area, thus showing decreased surface hydrophobicity. A similar trend was reported for acylated oat proteins (Zhao et al., 2017). Relatively higher surface hydrophobicity was observed for the succinylated protein compared with the acetylated protein with the same amount of modifier (Table 2), which is because of the more hydrophobic nature of the succinic group than the acetic group. Furthermore, the conjugated SA 0.3 and SA 0.6 had significantly higher surface hydrophobicity than the unmodified pea protein and succinylated proteins, which indicated that the conjugation had stronger effect in improving the hydrophobicity.

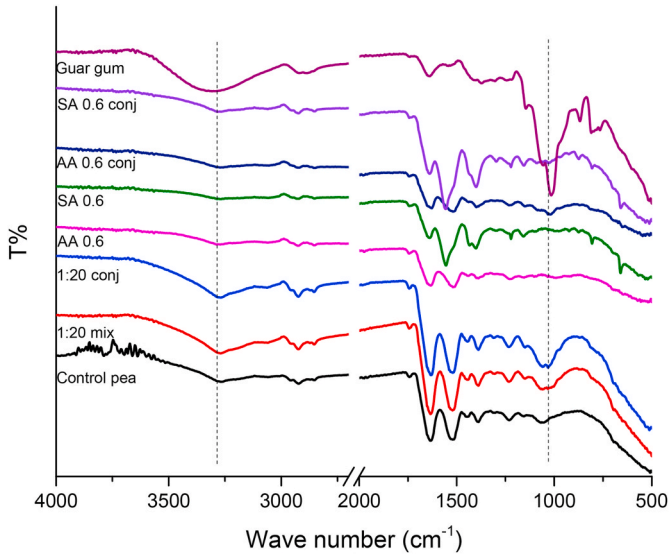

Fig. 5. FTIR spectra of pea and selected modified pea proteins.

3.9. FTIR

Fourier transform infrared spectroscopy is useful in identifying protein functional groups and secondary structures after modification. The bands in the regions of 3700–3200 cm^{-1} and 1100 - 1000 cm^{-1} denote the hydroxyl group and C–O stretching vibration, respectively (Du et al., 2013). There were obvious differences when comparing the conjugated proteins with the unmodified protein (Fig. 5). After protein conjugation with guar gum, it showed more intensive bands at 3700 - 3200 cm^{-1} than the unmodified pea protein and the sequential acylated and conjugated proteins (AA 0.6/SA 0.6 conjugates) (Fig. 5). A strong band at 1100 -1000 cm^{-1} was attributed to –OH bending vibration in the conjugated protein. Acylation modification greatly altered the protein secondary structures, which was related to the bands of amide I, II and III, attributed to 1635 cm^{-1}, 1546 cm^{-1} and 1450-1240 cm^{-1}, which defined the C═O stretching, N–H deformation, C–N stretching and N–H bending vibrations, respectively (Du et al., 2013; Pirestani, Nasirpour, Keramat, Desobry, & Jasniewski, 2018).

3.10. Circular dichroism (CD) spectroscopy

Secondary structures of the modified pea proteins including α-helix, β-sheet, β-turn, and random coil obtained from CD are summarized in Table 2. The unmodified pea protein consisted of 17.17% of α-helix, 23.97% of β-sheet, 1.17% of β-turn, and 57.67% of random coil, and random coil accounted for the majority of the secondary structures. The conjugated proteins (both 1:20 and 1:40 conjugates) had significantly higher amount of α-helix, but lower amounts of β-sheet and random coil compared with the unmodified pea protein. Du et al. (2013) reported a slight decrease in α-helix and β-sheet structures, but an increase in random coil in the rice protein conjugated with *k*-carrageenan. Liu et al. (2012) reported that the amount of both α-helix and random coil of peanut protein-dextran conjugates was decreased, while β-sheet structure was increased. The secondary structural differences could be attributed to the different protein types, reaction conditions, and the ratio of polysaccharide to protein. The acetylated pea protein had relatively lower amount of α-helix but much higher amount of β-turn structure. The succinylated pea protein possessed significantly higher amount of β-sheet structure but lower amount of random coil compared with the unmodified or conjugated pea proteins. Our results confirmed that conjugation and acylation can greatly alter protein secondary

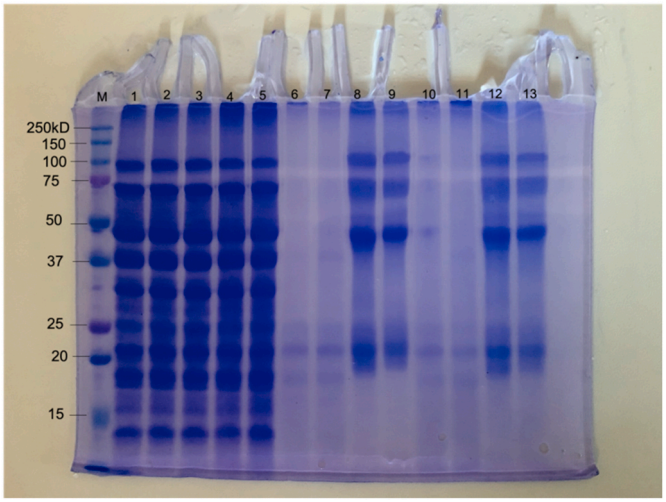

Fig. 6. Electrophoretic patterns of pea and modified pea proteins under reducing condition: Lane M-molecular weight marker; Lane 1: pea; Lane 2: 1:20 guar gum mix; Lane 3: 1:40 guar gum mix; Lane 4: 1:20 guar gum conjugate; Lane 5: 1:40 guar gum conjugate; Lane 6: AA 0.3; Lane 7: AA 0.6; Lane 8: SA 0.3; Lane 9: SA 0.6; Lane 10: AA 0.3 conjugate; Lane 11: AA 0.6 conjugate; Lane 12: SA 0.3 conjugate; Lane 13: SA 0.6 conjugate.

structures and further affect the functional properties.

3.11. SDS-PAGE

Globulins, including both legumin (11S) and vicilin (7S), are the major storage protein in pea. There was no obvious difference when comparing the SDS-PAGE bands of the guar gum-pea conjugates and the unmodified pea protein (Fig. 6). This result was expected, because extremely small amount of polysaccharide relative to the protein was used for the conjugation modification, and changes of protein molecular size could not be observed from the electrophoresis. The succinylated proteins exhibited more intensive bands compared with the acetylated proteins. Although a strong solvent (i.e., SDS/sodium phosphate buffer) was used to dissolve the protein samples prior to the electrophoresis analysis, the acetylated protein still showed very low solubility due to the greatly reduced electronegativity by introducing acetic functionality, which is consistent with the solubility result (Fig. 1). The 11S is a hexameric protein consisting of acidic (40 kDa) and basic (20 kDa) subunits, and the 7S is a glycosylated trimeric cluster consisting of three subunits, with molecular weight of 47.3, 33.3, and 28.7 kDa, respectively (Chéreau et al., 2016; Pirestani, Nasirpour, Keramat, & Desobry, 2017), all of which were observed on the SDS-PAGE under the reducing condition. The band at around 100 kDa was attributed to lipoxygenase (Barać et al., 2011) and may also indicate the formation of newly crosslinked protein structures during processing.

3.12. Free sulfhydryl (SH) group

The content of free sulfhydryl group in pea and modified pea proteins is summarized in Table 2. There was no significant difference for the free SH content between guar gum-pea protein conjugates and the unmodified pea protein, indicating that no or very minimal disulfide crosslinking occurred during the conjugation. Acetylated pea proteins (both AA 0.3/0.6 and AA 0.3/0.6 conjugates) had significantly lower free SH content compared with the unmodified protein, implying intensive disulfide crosslinking during acetylation modification. It was reported that conjugation reaction reduced the free sulfhydryl groups in pea, whey, and rapeseed proteins, respectively (Wang & Arntfield, 2016; Wang & Ismail, 2012; Wang et al., 2018), because heat treatment during the Maillard reaction promoted the formation of disulfide linkages. The different result from our study was attributed to the different conjugation conditions, such as reaction temperature, time, and ratio of polysaccharide to protein.

3.13. In vitro GI digestibility

The *in vitro* GI digestibility of pea and the modified pea proteins was indicated by the degree of hydrolysis, and the results are presented in Fig. 7. Overall, the conjugated (1:20 conj and 1:40 conj) and acylated pea proteins (AA 0.6, SA 0.3, SA 0.6) showed decreased protein digestibility, while the digestibility of AA 0.3 was not significantly different compared with the control pea protein. The digestibility of the conjugated pea proteins was also decreased, because the conjugated protein had higher molecular weight, which became less accessible to

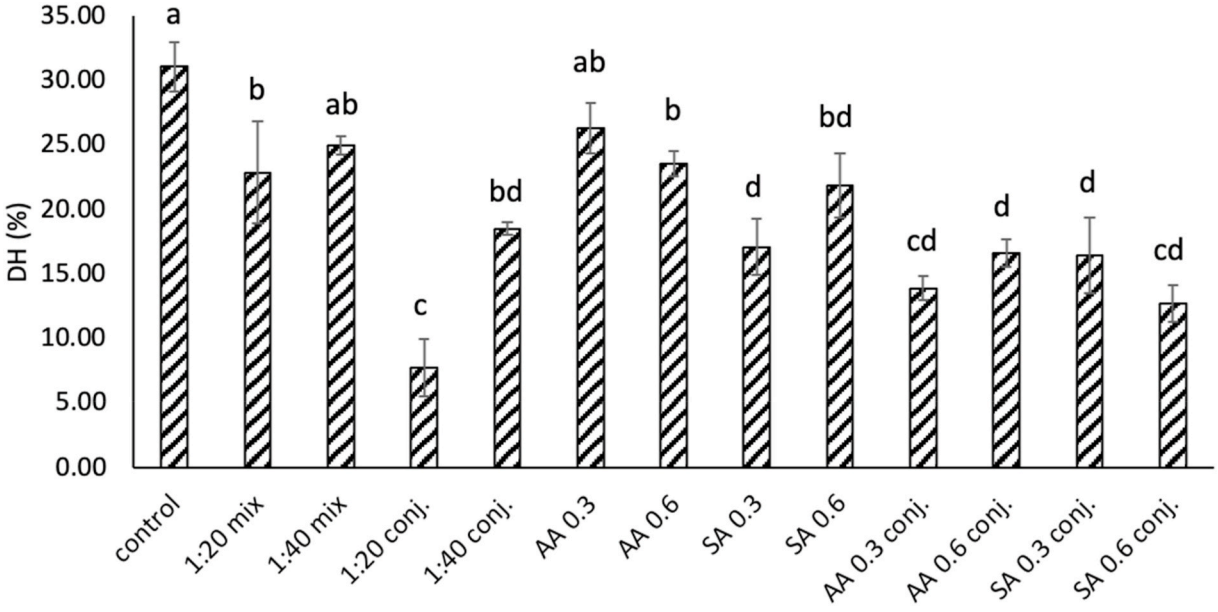

Fig. 7. *In vitro* gastrointestinal digestibility in terms of degree of hydrolysis (DH) of pea and modified pea proteins. *Means with different letters denote significant differences ($p < 0.05$).

the digestive enzymes. However, some literatures (Siu & Thompson, 1982; Yin et al., 2009a; Yin, Tang, Wen, & Yang, 2009b) reported that the acylated proteins had increased digestibility compared with control protein, and this was attributed to their better solubility and unfolded molecular structures during modification.

4. Conclusions

In this study, modified pea proteins were prepared by acylation and/or conjugation through reacting with acetic anhydride (AA) or succinic anhydride (SA) and incubating the guar gum-pea protein mixtures to induce Maillard reaction, respectively. Both conjugated and acylated pea proteins demonstrated significantly improved OHC, and the acylated pea protein also had much greater WHC. The EC and ES of the modified proteins were improved by up to 112% and 140%, respectively, compared to the unmodified protein. Sequential acylation and conjugation of pea proteins demonstrated more beneficial and synergistic effects and further enhanced the WHC, OHC, emulsification and gelation properties, which could be used as novel plant protein ingredients for different applications. However, the *in vitro* GI digestibility of the modified pea protein was decreased compared to the control protein. Future research is necessary to conduct safety evaluation of the chemically modified proteins and further understand protein nutritional changes during the modification.

CRediT author statement

Yanting Shen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Yonghui Li: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that there is no known conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2021.106686.

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