

# Physicochemical properties of whey protein isolate and alkaline soluble polysaccharide from sugar beet pulp conjugates formed by Maillard reaction and genipin crosslinking reaction: A comparison study

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## ABSTRACT

This study aim to investigate the effect of alkaline soluble polysaccharide from sugar beet pulp (ASP2) grafted with whey protein isolate (WPI) by two linking models (grafting on amino group or carbonyl group) on its emulsifying properties. Results demonstrated that the  $d_{4,3}$  value of WPI, M-AW, M-AA, G-AW and G-AA stabilized emulsions was 0.18  $\mu\text{m}$ , 0.28  $\mu\text{m}$ , 0.72  $\mu\text{m}$ , 0.56  $\mu\text{m}$  and 0.83  $\mu\text{m}$ , respectively, suggesting the higher emulsifying activity of the products prepared by Maillard reaction compared with the products obtained from genipin crosslinking reaction. After storage, the  $d_{4,3}$  increment was 1.05  $\mu\text{m}$ , 0.21  $\mu\text{m}$ , 0.31  $\mu\text{m}$ , 0.2  $\mu\text{m}$  and 0.15  $\mu\text{m}$  for WPI, M-AW, M-AA, G-AW and G-AA stabilized emulsions, respectively, indicating that the new generated polymers held stronger emulsifying stability compared with WPI. However, the aggregates emerged in high calcium emulsions system indicated that grafting with WPI could not efficiently reduce the sensitivity of ASP2 to calcium.

## 1. Introduction

Emulsion system is widely used for encapsulating and delivering hydrophobic bioactivity substances, such as curcumin, fisetin, and dihydromyricetin (Cao et al., 2020; Zhong, Farag, Chen, He, & Xiao, 2022; Zhang et al., 2021). Polysaccharides with amphiphilic nature, such as gum arabic, sugar beet pectin, soybean soluble polysaccharide and corn fiber gums, are great options for constructing food grade emulsion system (Nakauma et al., 2008). The amphiphilicity of polysaccharides mainly originates from the protein moiety which is covalent-linked with the polysaccharide chain and considered to be the anchor for polysaccharides to adsorb on oil surfaces. In prior study, some researchers suggested that the emulsifying activity of polysaccharide was positively correlated with its protein content (Akhtar et al., 2002). However, with more in-depth studies into the polysaccharide emulsification mechanism, researchers found that the emulsifying activity of polysaccharides was not entirely due to the absolute content of protein in the polysaccharides but depended on the

protein content being effectively absorbed on the oil surface (Williams et al., 2005). Indeed, since the main structural element of amphiphilic polysaccharides is hydrophilic, and the polysaccharide chain is not fully expanded in usual, the adsorption ability of protein moiety in the polysaccharides onto the oil surface is varied. Previous studies showed that more hydrophobic amino acid proteins seem to possess better emulsifying properties (Ai, Meng, Lin, Zhang, & Guo, 2020). Therefore, the contribution of protein to the emulsifying properties should be further studied.

Chemical grafting is a common method for enhancing the activity of natural products (Teng, Mi, Cao, & Chen, 2022). Whether it is to study the contribution of protein to the amphiphilic nature of polysaccharide or enhance the polysaccharide emulsifying properties, it is a direct and common method for the construction of polysaccharide-protein-conjugates (PPC) by linking polysaccharides with exogenous proteins such as bovine serum protein, whey protein isolate (WPI) and sodium caseinate. Due to its low cost and an easily controlled reactive process, the Maillard reaction has become a common method for producing PPC.

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Previous studies showed that the hydrophobic anchors of pectin could be effectively increased by grafting protein onto the C1 position of galacturonic acid (GalA) backbone or neutral side chain through the Maillard reaction (Wefers, Bindereif, Karbstein, & van der Schaaf, 2018). Moreover, due to the increased molecular weight (MW) and decreased zeta potential, the stability of the emulsions stabilized by PPC, faced freeze thawing, heating or ionic strength change, have been significantly improved (Guo, Guo, Yu, & Kong, 2018). Recently, genipin crosslinking as a novel method for constructing PPC through covalent binding has attracted attention. Several published works have demonstrated that the emulsifying properties of polymers were amended after genipin crosslinking (Lin, Guo, Ai, Zhang, & Yu, 2020). Since genipin is a hydrolyzed product of gardenoside, it is regarded as a natural crosslinker. Unlike the Maillard reaction, the crosslinking reaction caused by genipin is spontaneous in an aqueous solution at ambient temperature, and occurs in the C3 and C11 positions of genipin, which is a nucleophilic substitution with primary amine groups. Based on the above crosslinking mechanism, genipin possesses the ability to crosslink two polymer molecules that contain  $-NH_2$ , such as protein and chitosan in the C3 and C11 positions of genipin. In theory, when the genipin content in the crosslinking system is enough, the “-protein-genipin-protein-genipin-protein-” network structure will be shaped. However, which method is preferred — the Maillard reaction or genipin crosslinking reaction — for constructing PPC is still unknown.

ASP2 is an amphiphilic polysaccharide which is extracted from depectinized sugar beet pulp by NaOH- $H_2O_2$  treatment. Previous studies demonstrated ASP2 possessed great emulsifying stability and could be used as natural food emulsifiers for encapsulating and delivering bioactive substances (Ai, Guo, Lin, Zhang, & Meng, 2019; Teng et al., 2021). However, due to the insufficiency of hydrophobic groups of ASP2, the emulsifying activity of ASP2 is unsatisfactory. Therefore, in order to improve the emulsifying properties of ASP2 and enhance the value of sugar beet pulp in food industry, in this study, WPI, as an exogenous protein, was grafted on ASP2 to increase the content of the hydrophobic groups by the Maillard reaction and genipin reaction, respectively. Based on the above, we further studied the effect of exogenous protein linked with polysaccharide through different reactive sites on the physicochemical characteristics of produced polymers, particularly the emulsification performance.

## 2. Materials and methods

### 2.1. Materials and reagents

Sugar beet pulp was provided by Lvxiang beet sugar company (Xinjiang, China), and the ASP2 preparation process was according to the previous report (Ai, Guo, Lin, Zhang, & Meng, 2019). WPI powder was purchased from Davisco Foods International Inc. (Le Sueur, MN, USA) and contained 97.6% protein, 0.4% fat, and 2% ash (dry basis) according to the information supplied by the manufacturer. Medium-chain triglycerides (MCT) were purchased from Britz Networks Sdn. Bhd (Melaka, Malaysia). Reagents used for chemical analysis were analytical grade, while reagents used for chromatographic analysis were of high-performance liquid chromatography (HPLC) grade.

### 2.2. ASP2-WPI conjugates preparation

#### 2.2.1. Maillard reaction

The process of ASP2-WPI conjugate prepared by the Maillard reaction was according to the method described by (Guo, Guo, Yu, & Kong, 2018) with some modifications. Briefly, ASP2 powder and WPI powder were dissolved in distilled water with a concentration of 1% w/w, respectively. After complete dissolution, ASP2 solution was mixed with WPI solution with a ratio of 9:1, and pH value of the mixed solution was adjusted to 7 using 500 mM HCl or 500 mM NaOH and freeze dried. A desiccator which contained saturated KBr solution was placed in an

80 °C oven for 2 h to maintain a constant relative humidity of 79%. Then, the freeze dried mixture was grounded to pass through a 100-mesh screen and incubated in the above desiccator for 9 h. The product was recorded as M-AW.

For investigating the self-Maillard reaction occurred in ASP2, a sample named M-AA which completely consisted of ASP2 was prepared by non-adding WPI in the above process.

#### 2.2.2. Genipin crosslinking reaction

The preparation process of ASP2-WPI conjugate through genipin crosslinking reaction was modified from the method of previous studies (Lin, Yu, Ai, Zhang, & Guo, 2020) as following steps: (a) ASP2 powder and WPI powder were dissolved in distilled water to prepare solutions with a concentration of 2% w/w, respectively; (b) ASP2 solution was mixed with WPI solution with a ratio of 9:1; (c) the pH value of mixture was adjusted to 7; (d) genipin powder was added in the mixture with stirring to reach a concentration of 10 mM; (e) the mixture was put in a 25 °C constant water bath with stirring for 12 h; (f) three times volume of absolute ethanol was added in the mixture for terminating the reaction; (g) the precipitate was collected by centrifugation at  $10,000 \times g$  for 10 min, and washed twice with 95% ethanol and then air-dried at 45 °C for 24 h until samples reached a constant weight. The product was recorded as G-AW.

For investigating the self-genipin-crosslinking reaction of ASP2, a sample named G-AA which completely consisted of ASP2 was prepared by non-adding WPI in the above process.

### 2.3. Degree of glycosylation (DG)

The degree of glycosylation of samples was measured by the 2, 4, 6-trinitrobenzene sulfonic acid method described as Guo et al. (Guo, Guo, Yu, & Kong, 2018) with some modifications. In brief, WPI, ASP2, M-AW and M-AA powder were dissolved in distilled water to prepare solutions with a concentration of  $0.5 \text{ mg}\cdot\text{mL}^{-1}$  (WPI),  $5 \text{ mg}\cdot\text{mL}^{-1}$  (M-AW),  $1 \text{ mg}\cdot\text{mL}^{-1}$  (ASP2) and  $1 \text{ mg}\cdot\text{mL}^{-1}$  (M-AA), respectively. Then, 1 mL  $\text{NaHCO}_3$  solution (4% w/v, pH 8.5) and 1 mL 0.1% 2, 4, 6-trinitrobenzene sulfonic acid solution were added to 1 mL sample solution. After incubation at 40 °C for 2 h, 1 mL SDS solution (10% w/v) and 0.5 mL 1 M HCl were added to terminate the reaction. The absorbance of the solution was measured at 344 nm. The DG of M-AW was calculated as:  $\text{DG} (\%) = (A_{\text{WPI}} - A_{\text{M-AW}}) / A_{\text{WPI}} \times 100$ , and the DG of M-AA was calculated as:  $\text{DG} (\%) = (A_{\text{ASP2}} - A_{\text{M-AA}}) / A_{\text{ASP2}} \times 100$ , where A was the respective absorbance values, and the corresponding subscript represented sample name.

### 2.4. Solubility

The solubility of samples was determined according to a previous method reported by Al-Hakkak and Al-Hakkak (2010) with minor modification. Samples were dissolved in distilled water with the concentration of 1.5% w/w at ambient temperature. The pH value of each sample solutions was adjusted to 7.0 by 500 mM NaOH. Then, the sample solutions were centrifuged at 4 °C and  $10,000 \times g$  for 30 min. The supernatant was collected and filtered through a  $0.45 \mu\text{m}$  PES filter and freeze-dried. The solubility was calculated as  $\text{Solubility} (\%) = (C_1 / C_2) \times 100$ , where  $C_1$  was the weight of freeze-dried supernatant and  $C_2$  was the weight of initial sample.

### 2.5. Amino acid analysis

The amino acid analysis was according to the HPLC method as described in previous study (Liu, Pi, Guo, Guo, & Yu, 2018).

### 2.6. Fourier transform infrared spectroscopy (FTIR)

A vector 33 spectrometer (Bruker, Germany) was used to determine

the Fourier transform infrared spectra of the samples. Prior to analysis, samples were collected using the KBr pellet method as described in the previous study (Yu et al., 2022). Then, the FTIR spectra were measured in transmission mode with a resolution of  $4\text{ cm}^{-1}$  resolution from 400 to  $4000\text{ cm}^{-1}$  (Shi et al., 2021; Liu et al., 2018).

## 2.7. Molecular weight

The molecular weight distribution of samples was determined by high-performance size-exclusion chromatography as described by Ai et al. (Ai, Guo, Lin, Zhang, & Meng, 2019). The Mw value of samples were calculated by constructing a calibration curve using dextran criterion (from 400 kDa to 4.4 kDa) and the Empower 2.0 software (Waters Corp., USA).

## 2.8. Atomic force microscope

Micro shape of polymers was observed by an atomic force microscope (AFM) instrument (Multimode 8, Bruker, USA). The measurement process was according to our previous study (Guo, Wang, Fang, Pan, & Yu, 2018). Briefly, samples were dissolved in ultrapure water and diluted to a final concentration about  $3\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ . Before observation,  $2\text{ }\mu\text{L}$  sample solution was pipetted onto a freshly cleaved mica sheet, and air-dried at ambient temperature for 30 min. Then, the mica sheet was scanned in tapping mode using aP-S10 probe made of silicon nitride. Obtained data were processed into 2D and 3D images by the Nanoscope Analysis software (version 1.70, Bruker).

## 2.9. Zeta potential and interfacial tension

The zeta potential of the polymers was measured using a Nano-Zetasizer (Malvern Instruments Ltd, UK). Polymers were dissolved and diluted with ultrapure water to a final concentration of  $2\text{ mg}\cdot\text{mL}^{-1}$ . The pH value of polymer solutions was adjusted to 3.5 by 0.5 M NaOH or 0.5 M HCl, and then, each solution was filtered through a  $0.45\text{ }\mu\text{m}$  PES filter. Each polymer solution was filling into the folded capillary cell (DTS1070, Malvern) at ambient temperature to obtain the zeta potential data of samples.

Interfacial tension of samples was performed by an optical drop shape tensiometer (OCA-20, DataPhysics, Germany) as described in previous study (Lin, Yu, Ai, Zhang, & Guo, 2020). In brief, 1% w/v sample aqueous solution with the pH value of 3.5 was prepared and filled in a syringe. The syringe was immersed in MCT and pushed to form a pendant drop ( $25\text{ }\mu\text{L}$ ) of sample solution. The shape of pendant drop was imaged by a CCD camera. Then, the interfacial tension of samples was calculated by measuring the shape of pendant drop using SCA 20 software (DataPhysics, Germany) according to the Young-Laplace equation.

## 2.10. Emulsion preparation

The oil-in-water emulsions prepared in this study contained 10% w/w MCT as disperse phase, 1% w/w polymer as emulsifier, 0.1% sodium benzoate as preservative, and 88.9% w/w as continuous phase. The emulsion preparation process was according to our previous report (Ai, Meng, Lin, Tang, & Guo, 2022).

## 2.11. Emulsion characterization

### 2.11.1. Emulsion droplet size distribution

The droplet size distribution and volume-weighted mean diameter ( $d_{4,3}$ ) value of emulsions were determined using a MasterSizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). The relative refractive indices of water and MCT were 1.330 and 1.450, respectively. The fresh emulsions were measured within 10 h after preparation, while the stored emulsions were measured after storage at  $60\text{ }^\circ\text{C}$  for seven days.

### 2.11.2. Rheological property

Flow curves of the emulsions stabilized by polymers were performed at shear rates from 0.01 to  $150\text{ s}^{-1}$  with a gap of 1 mm at  $25\text{ }^\circ\text{C}$  using a strain-controlled ARES rheometer (TA Instruments, New Castle, USA) equipped with parallel plate (40 mm). The steady shear viscosity of emulsions was calculated as the viscosity value at the shear rate of  $10\text{ s}^{-1}$  according to (Nakauma et al., 2008).

### 2.11.3. Morphology of oil droplet

The morphology of oil droplet was observed using an optical microscope (XPH300Z, Changfang Optical instrument Ltd., China). Before observation, the emulsion was diluted 5 times using distilled water with vortex shaking. Then,  $5\text{ }\mu\text{L}$  diluted emulsion was pipetted onto the glass slide and coated with glass coverslips and imaged by a digital camera.

## 2.12. Statistical analysis

The entire experiments were independently replicated three times, unless otherwise stated. The data was evaluated using a one-way ANOVA which was performed using the SPSS 21.0 software (IBM Corp., USA). Significant differences between the means were identified using Duncan's multiple range tests, and the differences were considered to be statistically significant when  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Solubility and degree of glycosylation

The solubility of samples is shown in Fig. 1a. Both the solubility of G-AW (82.3%) and G-AA (77.6%) were significantly lower than ASP2 (96.1%) or WPI (99.8%) ( $p < 0.05$ ). This suggests that some insoluble components were generated after the genipin crosslinking reaction. Generally, due to the effect of thermal denaturation and polymerization, the solubility of the Maillard reaction product would be lower than the raw materials (Tammak, Mirhosseini, Tan, Ghazali, & Muhammad, 2016). However, there was no significant difference in the solubility of M-AW and M-AA compared with ASP2 or WPI. These phenomena could be attributed to the following two aspects: firstly, the dosage of WPI (10%) used in this study was much lower than that of previous studies (from 25% to 50%) (Qi, Xiao, & Wickham, 2017). Secondly, the produced insoluble substances are related to the degree of reaction. Evidence has shown that a short ( $\leq 9\text{ h}$ ) Maillard reaction time does not significantly reduce the solubility of PPC (Guo et al., 2019).

The DG of M-AW and M-AA before the reaction (0 h) was 2.5% and 0.6%, respectively (Fig. 1b). The glycosylation in the sample before reaction may be produced during the procedures of freeze-drying, crushing, and storage. After reaction, the DG of M-AW (24.8%) was significantly higher than that of M-AA (7.6%) ( $p < 0.05$ ). It has been reported that the degree of Maillard reaction was related to many factors, such as polysaccharide types, protein content, and reaction parameters (Abd El-Salam & El-Shibiny, 2018). Thus, the low DG of M-AA may be due to the low protein content in M-AA reaction system.

### 3.2. Amino acid analysis

Both genipin crosslinking and Maillard reaction between protein and polysaccharide were based on the free amino group in the protein. As shown in Table 1, the amino acid composition of proteins in WPI and ASP2 were varied. The main amino acid of WPI measured in this study was leucine, aspartic, and glutamic. The amino acid composition of ASP2 was similar to that of SBP reported in previous work (Liu, Pi, Guo, Guo, & Yu, 2019), and the main amino acids of ASP2 were aspartic, glutamic and glycine.

As shown in Table 1, the proportion of the most amino acids of produced polymers was only changed slightly when compared with ASP2. However, some other amino acids, such as threonine,

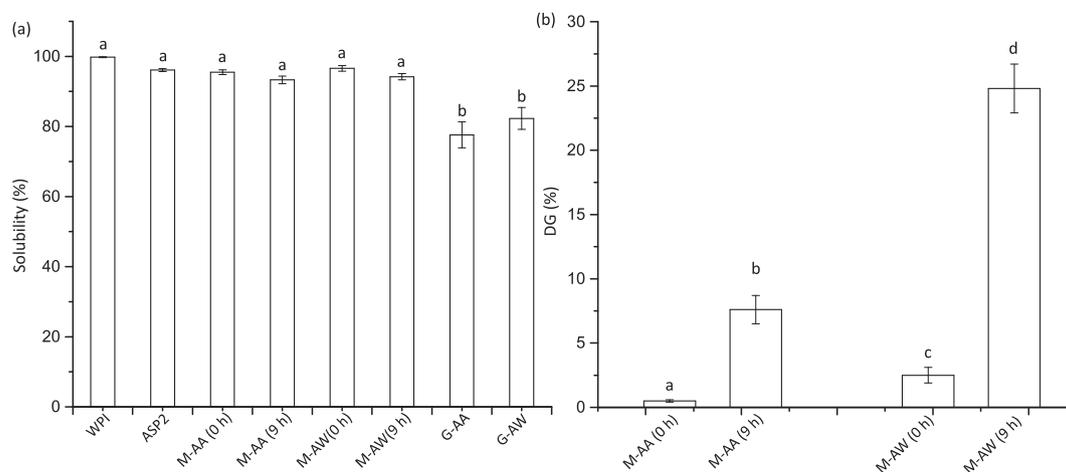


Fig. 1. The solubility of ASP2-WPI conjugates (a) and the degree of glycosylation of M-AA and M-AW (b).

hydroxyproline and glycine, were significantly different from ASP2, of which lied in between WPI and ASP2. It could be attributed to the addition of WPI which possessed much different proportion of these amino acids. Notably, after the Maillard reaction, the lysine ratio in products was significantly lower raw material ( $p < 0.05$ ). According to a previous report, the Maillard reaction tends to occur in the amino of lysine rather than leucine (Wefers, Bindereif, Karbstein, & van der Schaaf, 2018). Therefore, the decrease in lysine could be attributed to the fact that lysine was involved in the Maillard reaction, and changes of other amino acids may be caused by the addition or thermal degradation of WPI. In theory, the addition of WPI would result in the increase of the lysine ratio in M-AW, which is significantly lower than that of M-AA ( $p < 0.05$ ). These phenomena were due to the relatively higher DG of M-AW, which used more lysine to participate in the Maillard reaction.

Interestingly, the changes in the ratio of amino acids after genipin crosslinking reaction were similar to that of the Maillard reaction, indicating that lysine is not only the key binding site for the Maillard reaction, but also the genipin crosslinking reaction. According to Lee et al. (Lee, Lim, Bhoo, Paik, & Hahn, 2003), all the glutamine, arginine, and lysine could be the reactive sites of genipin crosslinking, but the ratio of glutamine and arginine was not significantly decreased after reaction in this study. From the aspect of the molecular structure, the  $\epsilon$ -amino group of lysine binds with a saturated carbon, producing higher chemical activity when compared with glutamine and arginine (Lin, Yu, Ai, Zhang, & Guo, 2020; Silva, Saint-Jalmes, de Carvalho, & Gaucheron, 2014). When the amino acids, which are not free form, existed as structural elements of polymers, different chemical activity would result in the  $\epsilon$ -amino group of lysine as a prior reactive site of polymers.

### 3.3. FTIR

The FTIR of samples is shown in Fig. 2a. It is easy to observe that the wave pattern of M-AW, M-AA, G-AW and G-AA possess the characteristic absorption peaks of polysaccharides with strong absorption bands at  $3471\text{ cm}^{-1}$  ( $-\text{OH}$ ),  $2935\text{ cm}^{-1}$  ( $-\text{C}-\text{H}$ ) and  $1138\text{ cm}^{-1}$  ( $-\text{C}-\text{O}-\text{C}-$ ) (Vieira et al., 2022). The difference between WPI and these PPC is reflected mainly in amide I bands and amide II bands (from  $1500\text{ cm}^{-1}$  to  $1700\text{ cm}^{-1}$ ) (Ouyang et al., 2019). These bands are sensitive to the changes of protein function groups, including the stretching vibration of  $-\text{C}=\text{O}$ ,  $-\text{C}-\text{N}$ , and the bending vibration of  $-\text{N}-\text{H}$ . A new absorption emerged absorption in amide I bands (from  $1500\text{ cm}^{-1}$  to  $1560\text{ cm}^{-1}$ ) of G-AA, G-AW and M-AW. The new absorption was attributed to the addition of WPI by  $-\text{N}-\text{H}$ , and suggested a newly generated  $-\text{C}-\text{N}$  between polysaccharide and protein, which was in agreement with the previous study (Schwaighofer et al., 2018). Notably, there was a redshift in the spectrum of G-AA and G-AW, indicating the protein structure was

changed by breaking the hydrogen bond association structure (Chen, Xie, Li, Xiong, & Li, 2022).

### 3.4. Molecular weight

As shown in Fig. 2b, the molecular weights of M-AA, M-AW, G-AA, G-AW were 277, 323, 491 and 758 kDa, respectively, significantly higher than that of initial ASP2 (238 kDa) as described in (Ai, Meng, Lin, Tang, & Guo, 2022). These changes indicated that the polymerization phenomenon occurred in the modification process. The molecular weight distribution of polymers is shown in Fig. 2b, to explore the molecular weight changes in detail. WPI presented as a narrow unimodal pattern with the elution time between 35 min and 39 min, suggesting WPI possessed a good uniformity of the molecular weight distribution. After calculation, the molecular weight of WPI was 17 kDa, which was similar to a previous study (Lin, Guo, Ai, Zhang, & Yu, 2020). However, the molecular weight distribution of produced polymers displayed as bimodal or multimodal pattern in the range from 21.5 min to 37.5 min, which suggested these polymers were comprised of multi-components with different molecular weights. There was a new generated peak from 22 min to 24 min in G-AA, G-AW, and M-AW, which meant new macromolecular polymers were formed by covalent-bonding after reaction. There was a peak that ranged in the elution time from 35 min to 39 min in M-AW, indicating that a part of WPI existed in M-AW as a free form. However, G-AW, which was also grafted with WPI, did not contain this peak, suggesting the added WPI was participatory in the genipin crosslinking reaction.

### 3.5. Afm

The morphology of PPC was observed by AFM, as shown in Fig. 3. Due to the relatively small molecular structure of WPI, it is shaped like a very small sphere composed of multiple WPI monomers (Fig. 3 a). A small number of linear molecules with branched structures can be observed in M-AA and M-AW (Fig. 3b and c). These molecular configurations may be caused by the following: the polysaccharide chain became longer after glycosylation and thus tended to present as linear conformation, or, the covalent linkage between protein and polysaccharide have changed the inter-/intra- molecular interaction (such as hydrogen bond, Van der Waals' force, and electrostatic force) which had a significant impact on its configuration. Previous study demonstrated that the intermolecular interaction among polysaccharides may cause its configuration tend to shape as aggregated rather than linear in aqueous solution (Zhang et al., 2018). Thus, when the intermolecular interaction in polysaccharides changed, the aggregated polysaccharides will likely tend to unfold and present as linear configuration. Since the protein

**Table 1**  
Ratio of amino acid compositions of WPI, ASP2 and their conjugates.

ID	WPI	ASP2	G-AA	G-AW	M-AA	M-AW
Aspartic	13.99 ± 0.2a	10.24 ± 0.2b	11.21 ± 0.4b	13.06 ± 0.6a	11.05 ± 0.3b	13.12 ± 0.5a
Glutamic	12.25 ± 0.2a	11.53 ± 0.3a	11.71 ± 0.5a	12.04 ± 0.5a	12.01 ± 0.6a	12.54 ± 0.6a
Hydroxyproline	0.10 ± 0.0a	7.75 ± 0.2b	7.55 ± 0.5bd	5.28 ± 0.4c	7.47 ± 0.4bd	6.59 ± 0.4d
Asparaginate	n.d. <sup>b</sup>	n.d.	n.d.	n.d.	n.d.	n.d.
Glutamine	3.20 ± 0.1a	2.66 ± 0.0b	2.93 ± 0.2bc	2.97 ±	3.03 ± 0.2bc	3.33 ± 0.2c
Histidine	1.01 ± 0.1a	n.d.	n.d.	0.13 ± 0.0b	n.d.	0.15 ± 0.0b
Arginine	1.82 ± 0.1a	3.77 ± 0.1b	3.68 ± 0.2b	2.68 ± 0.3c	3.55 ± 0.2b	3.30 ± 0.3bc
Serine	8.62 ± 0.2a	8.73 ± 0.2a	8.64 ± 0.3a	8.70 ± 0.4a	9.32 ± 0.5ab	9.71 ± 0.4b
Threonine	7.83 ± 0.2a	5.43 ± 0.2b	5.88 ± 0.3bc	6.53 ± 0.5c	6.28 ± 0.3c	6.96 ± 0.3c
Proline	2.59 ± 0.1a	4.35 ±	3.94 ± 0.2b	3.11 ± 0.3a	4.95 ± 0.5d	4.34 ± 0.3b
Alanine	0.12 ± 0.0a	0.16 ± 0.1a	0.25 ± 0.1a	0.23 ± 0.1a	0.18 ± 0.1a	0.15 ± 0.1a
Glycine	5.59 ± 0.2a	11.24 ± 0.3b	10.69 ±	9.52 ± 0.3c	10.67 ± 0.4bc	9.73 ± 0.5c
Valine	4.71 ± 0.2a	9.62 ± 0.1b	9.43 ± 0.2b	7.75 ± 0.3c	9.11 ± 0.5b	8.26 ± 0.4bc
Methionine	1.02 ± 0.1a	n.d.	n.d.	0.11 ± 0.0b	n.d.	0.09 ± 0.0b
Tryptophan <sup>c</sup>	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Leucine	16.43 ± 0.0a	7.35 ± 0.1b	8.38 ± 0.2c	11.67 ± 0.4d	7.19 ± 0.3b	8.07 ± 0.3bc
Isoleucine	5.11 ± 0.2a	4.16 ± 0.3b	4.96 ± 0.4ab	4.80 ±	3.86 ± 0.3b	4.28 ± 0.4b
Phenylalanine	3.75 ± 0.0a	4.03 ± 0.0a	3.93 ± 0.1a	4.32 ± 0.3a	3.85 ± 0.2a	3.41 ± 0.3a
Cysteine	0.24 ± 0.0a	n.d.	n.d.	0.11 ± 0.0b	n.d.	0.15 ± 0.0b
Tyrosine	2.25 ± 0.1a	3.58 ±	3.92 ± 0.2b	3.50 ±	3.38 ± 0.2bc	3.09 ± 0.3c
Lysine	9.37 ± 0.3a	5.31 ± 0.2b	2.90 ± 0.3 cd	3.62 ±	4.10 ± 0.4e	2.88 ± 0.3c

<sup>a</sup>Data are expressed as mole percentage of the detected amino acids and presented as mean ± standard deviation. Mean value in the same row with different letters (a–e) indicate significant ( $p < 0.05$ ) differences among samples.

<sup>b</sup> n.d. means not detectable.

<sup>c</sup> Decomposed by acidic hydrolysis during sample preparation.

moiety of ASP2 was connected to the neutral sugar side chain (Funami et al., 2011), WPI which crosslinked with ASP2 molecules by genipin would extend the size of the side chain. As a result, the configuration of G-AA and G-AW tended to take shape as spherical spots on the mica sheet (Fig. 3d and e). This phenomenon has also been found in modified sugar beet pectin (Lin, Yu, Ai, Zhang, & Guo, 2020). In addition, two ASP2 molecules could be combined by using a genipin molecule as a bridge. This effect may induce the crosslinking of multi-molecules, so that more aggregates could be observed on the mica sheet.

### 3.6. Interfacial tension and zeta potential

As shown in Fig. 4, the interfacial tension of the oil–water interface ( $22.7 \text{ mN}\cdot\text{m}^{-1}$ ) was significantly reduced ( $p < 0.05$ ) by the addition of WPI ( $9.3 \text{ mN}\cdot\text{m}^{-1}$ ), M-AA ( $14.6 \text{ mN}\cdot\text{m}^{-1}$ ), M-AW ( $10.4 \text{ mN}\cdot\text{m}^{-1}$ ), G-AA ( $14.2 \text{ mN}\cdot\text{m}^{-1}$ ), and G-AW ( $10.8 \text{ mN}\cdot\text{m}^{-1}$ ), respectively. A previous study has demonstrated that grafting with protein could improve the amphipathy of polysaccharide (Kutzli, Griener, Gibis, Grossmann, &

Weiss, 2020). Notably, in this study, the interfacial tensions of M-AA and G-AA were significantly lower than that of M-AA and G-AA ( $p < 0.05$ ), suggesting the addition of exogenous protein would enhance the interfacial activity of polysaccharides regardless of the linking model.

Zeta potential is a key parameter in forecasting electrostatic repulsion among polymers in an aqueous solution. As shown in Fig. 4, all the polymers carried a negative charge in an aqueous solution with the pH value of 3.5 except WPI, which carried a positive charge, and decreased with the order of: WPI (25 mV), M-AW ( $-11.3 \text{ mV}$ ), M-AA ( $-21.5 \text{ mV}$ ), G-AW ( $-22.4 \text{ mV}$ ) and G-AA ( $-25.1 \text{ mV}$ ). It indicated that both the PPC constructed by the Maillard reaction and genipin crosslinking possessed a lower charge quantity compared with native ASP2 ( $-33.2 \text{ mV}$ ). In the case of M-AW, previous studies have demonstrated that the Maillard reaction could occur in both the neutral sugar side chain and the GalA chain (Guo, Guo, Yu, & Kong, 2018; Wefers, Bindereif, Karbstein, & van der Schaaf, 2018). When GalA was involved in the reaction, the charge quantity of the free carboxyl group would reduce. Additionally, the free form WPI in the M-AW solution would carry a positive charge at the pH of 3.5, and then the positive charge would be neutralized by the negative charge generated from GalA. As a result, the zeta potential of M-AW was relatively lower than others. In particular, the crosslinking between proteins by genipin would reduce the free amino acid group, which had a positive charge in an aqueous solution, thereby the charge neutrality would be relieved. However, the zeta potential of G-AA was significantly lower than that of the initial ASP2. This inconsistency between fact and theory probably originated from the molecular weight and conformation changes which buried the free carboxyl groups from polysaccharide surface and then suppressed the charge of carboxyl groups.

### 3.7. Rheological property

Emulsion viscosity plays a vital role in maintaining emulsion stability. As shown in Fig. 4b, the viscosity of polymer-stabilized emulsion at the shear rate of  $10 \text{ s}^{-1}$  was  $12.08 \text{ mPa}\cdot\text{s}$  (M-AA),  $13.52 \text{ mPa}\cdot\text{s}$  (M-AW),  $12.57 \text{ mPa}\cdot\text{s}$  (G-AA),  $12.31 \text{ mPa}\cdot\text{s}$  (G-AW) and  $10.31 \text{ mPa}\cdot\text{s}$  (WPI), respectively. The relatively higher viscosity of the emulsion stabilized by G-AA or G-AW could be interpreted as the increasing of molecular weight of polymers after genipin crosslinking. Previous works have demonstrated that the higher molecular weight of emulsifier would result in the highly emulsion viscosity (Lin, Yu, Ai, Zhang, & Guo, 2020). Similarly, emulsion stabilized by WPI possessed the lowest viscosity which could be also attributed to its lowest molecular weight among polymers. Besides, the viscosity of emulsions is also affected by inter-/intra- molecular forces, such as hydrogen bond and electrostatic force. This could be interpreted as M-AA possessing a relatively lower molecular weight, but M-AW stabilized emulsion holds relatively higher viscosity, which originated from the electrostatic force among free form WPI and free carboxyl group polysaccharides. Albano et al. (2020) have reported a similar effect in PPC prepared by the Maillard reaction.

### 3.8. Emulsifying activity

The emulsifying activity of polymers was evaluated by the  $d_{4,3}$  value, oil droplet morphology and distribution of the 10% w/w oil-in-water emulsion which was emulsified by using samples as emulsifiers. As shown in Fig. 5a, all the oil droplets of fresh emulsion presented as bimodal distribution and shaped as sphere with different sizes. It could be observed that the bimodal distribution displayed as a minor peak ( $>1 \mu\text{m}$ ) adjacent to a main peak. The  $d_{4,3}$  value of WPI, M-AA, M-AW, G-AA and G-AW stabilized emulsion was  $0.18 \mu\text{m}$ ,  $0.72 \mu\text{m}$ ,  $0.28 \mu\text{m}$ ,  $0.83 \mu\text{m}$  and  $0.56 \mu\text{m}$ , respectively. It indicated that WPI possessed prior emulsifying activity among the above polymers, and G-AA displayed activity that was inferior to the emulsifying activity of initial ASP2 ( $0.61 \mu\text{m}$ ) (Ai, Meng, Lin, Tang, & Guo, 2022). There was no new generated protein anchor after crosslinking by genipin, which might be a possible

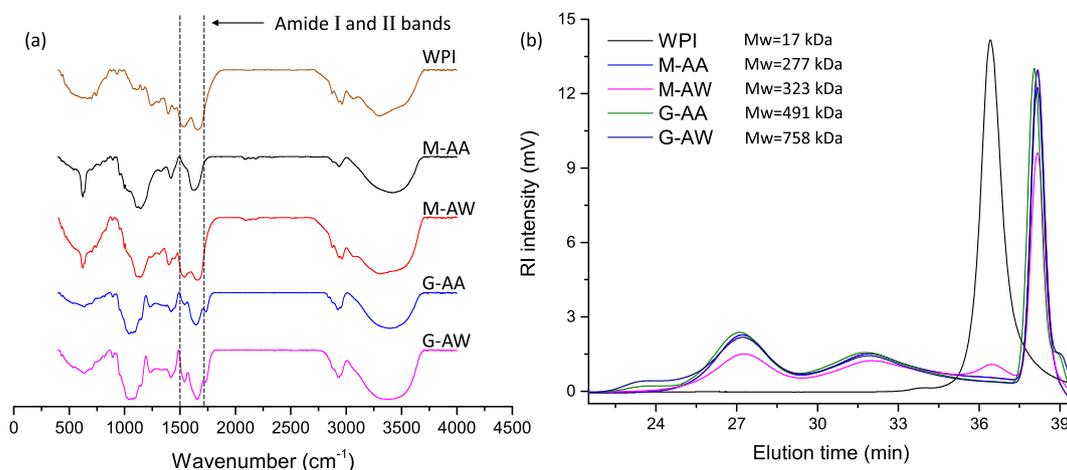


Fig. 2. FTIR spectra of WPI and ASP2-WPI conjugates (a) and Molecular weight distribution profiles of WPI and ASP2-WPI conjugates (b).

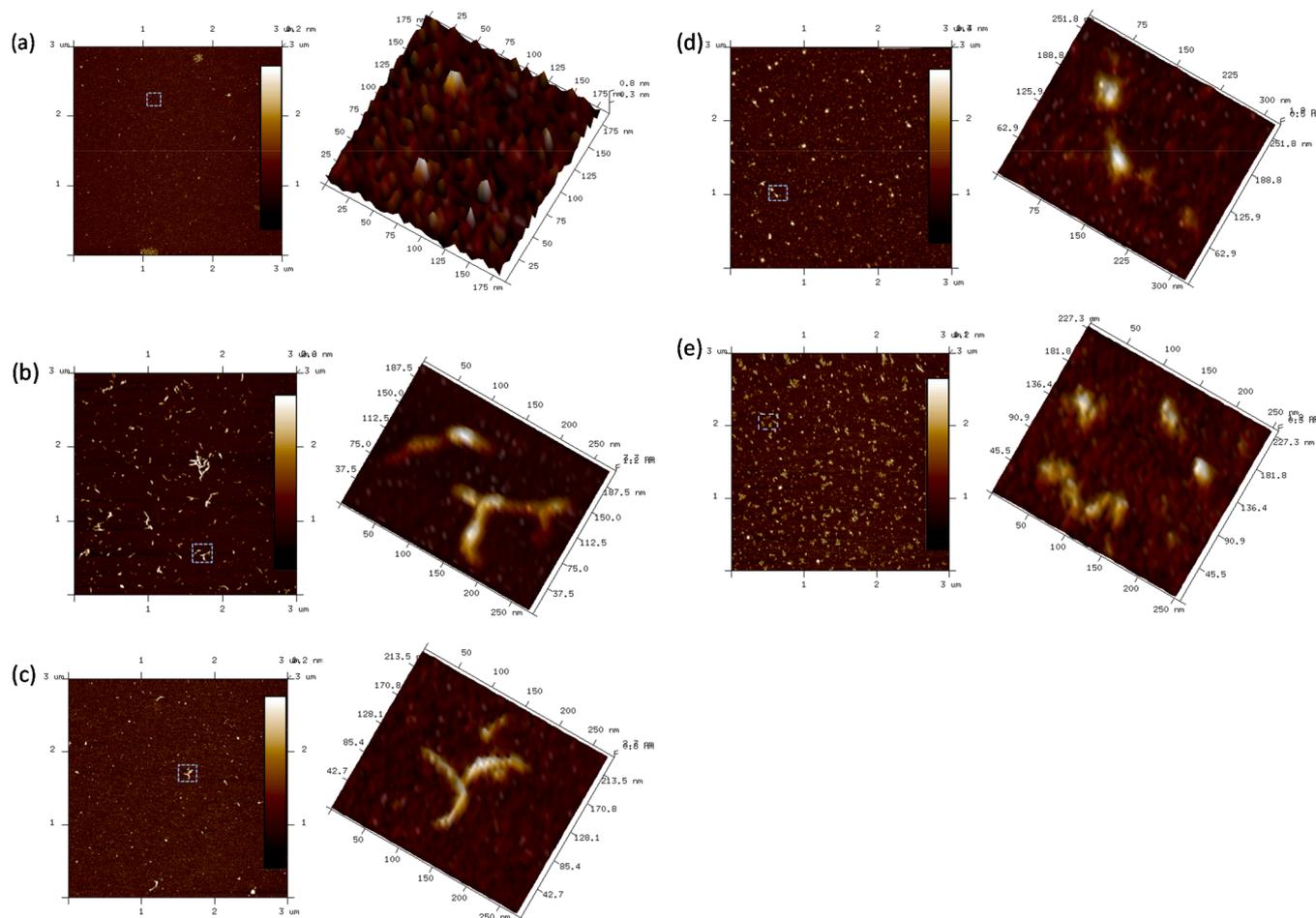


Fig. 3. Atomic force microscopy images of molecular morphology of polymers. (a) WPI; (b) M-AA; (c) M-AW; (d) G-AA; (e) G-AW.

explanation for the inferior emulsifying activity of G-AA, combining two protein anchors in to one. The increased molecular weight of G-AA also retarded its rate of absorbing onto the oil surface. Thus, the quantity of protein anchors would be reduced, resulting in poorer emulsifying activity. Similarly, although the reaction degree in M-AA was low, the protein moiety of M-AA involved in the in Maillard reaction would also reduce the amount of protein moiety from M-AA surface, thereby the emulsifying activity of M-AA was lower than initial ASP2. M-AW stabilized emulsion hold the minimum  $d_{4,3}$  value among all the PPC

stabilized emulsions. This emulsifying activity improvement of M-AA could be due to the following: Firstly, the combination of the amino group from WPI and the carbonyl group from ASP2 would increase the covalent-linked hydrophobic anchor on M-AW, so that the polymers could more easily attach to the oil droplet surface. Secondly, the free form WPI in the aqueous solution could be electrostatically bound with ASP2 at the pH value of 3.5, then, the free form WPI could absorb on the oil surface as an emulsifier. Previous works have demonstrated that PPC prepared by electrostatic bonding possessed better emulsifying activity

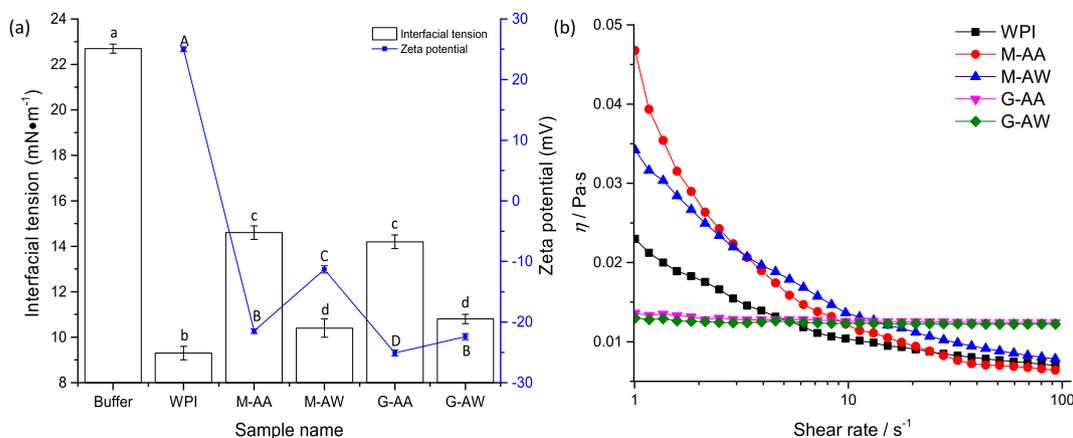


Fig. 4. Zeta potentials and interfacial tensions of WPI and ASP2-WPI conjugates (a) and flow curve of the emulsions stabilized by WPI and ASP2-WPI conjugates (b).

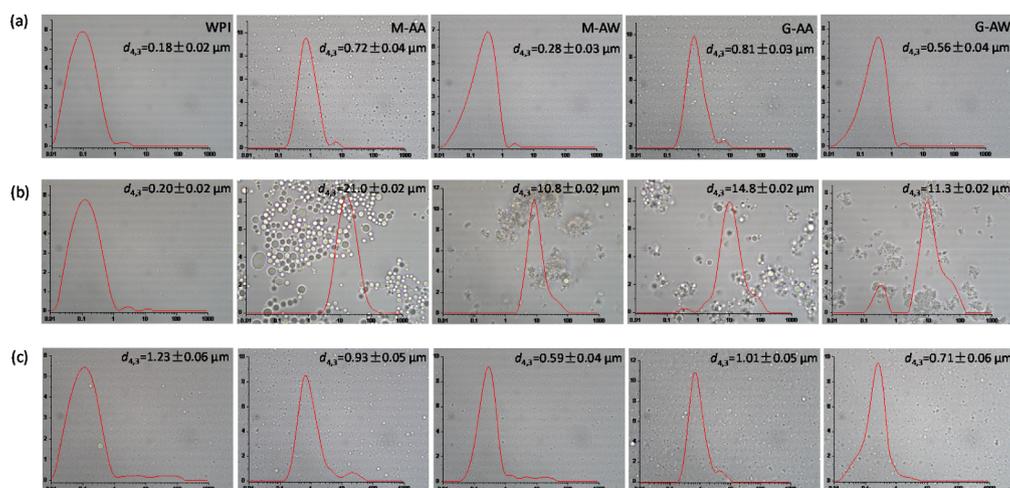


Fig. 5. The droplet size distribution, microscope images and  $d_{4,3}$  value of emulsions stabilized by WPI and ASP2-WPI conjugates. (a) fresh emulsion; (b) fresh emulsion after adding 50 mM CaCl<sub>2</sub>; (c) stored emulsion.

than polysaccharide only (Guzey & McClements, 2007). In the case of G-AW, the addition of WPI did not increase the number of hydrophobic anchors but extended their length or even decreased the anchors quantity the phenomenon occurring in G-AA. However, in this case, the G-AW also presented better emulsifying activity than native ASP2. It suggested that the extended protein anchor may find it easier to approach the oil surface and come into effect by stabilizing the oil–water interface. A previous study has reported a similar result that the emulsifying properties of sugar beet pectin could be amended by genipin crosslinking with bovine serum protein (Lin, Guo, Ai, Zhang, & Yu, 2020).

### 3.9. Calcium sensitivity

The emulsifying property of ASP2 which is negatively charged in an aqueous solution, is unstable in a calcium-containing food system, shaping the calcium bridge between calcium and free carboxyl groups through electrostatic bonding (Ai, Meng, Lin, Tang, & Guo, 2022). This feature limited the application of ASP2 in food systems. On the contrary, when WPI was positively charged in an aqueous solution at a pH of 3.5, its emulsifying activity was barely influenced by calcium, a factor attributed to the electrostatic repulsion between WPI and calcium. As a result, WPI stabilized emulsion prepared in a calcium-containing system possessed a significantly lower  $d_{4,3}$  value of 0.2  $\mu\text{m}$  ( $p < 0.05$ ) (Fig. 5b).

As shown in Fig. 5b, the  $d_{4,3}$  value of each emulsion stabilized by PPC was 0.2  $\mu\text{m}$ , 21.0  $\mu\text{m}$ , 10.8  $\mu\text{m}$ , 14.8  $\mu\text{m}$  and 11.3  $\mu\text{m}$  for WPI, M-AA,

M-AW, G-AA and G-AW, respectively. Theoretically, the free amino group of exogenous protein of PPC will electrostatically bind with the free carboxyl group of polysaccharide resulting in competitive inhibition to calcium. Moreover, the free amino group of protein combined with polysaccharide could occur in the carbonyl of the free carboxyl group when the Maillard reaction was executed, thereby reducing the electrostatic binding site of calcium. However, in practice, it is hard to effectively overcome the calcium bridge effect between polysaccharides and proteins by the preparation of PPC. Otherwise, the  $d_{4,3}$  value of PPC stabilized calcium-containing emulsion would not be dozens of times higher than that of non-calcium emulsion, and obvious aggregates (Fig. 5b) could not be observed in the stabilized emulsions by PPC. Therefore, the data in this study demonstrated that both the presence of exogenous protein combined with polysaccharide using both the Maillard reaction and genipin crosslinking reaction could not contribute enough to maintain good emulsifying activity for the produced PPC to stabilize the oil–water surface in a high calcium system.

### 3.10. Emulsion stability

The stability of emulsions was evaluated by the  $d_{4,3}$  value increment after storage at 60 °C for seven days. As shown in Fig. 5c, the  $d_{4,3}$  value of WPI stabilized emulsion was increased from 0.18  $\mu\text{m}$  to 1.23  $\mu\text{m}$ . The oil droplet distribution of WPI stabilized emulsion showed that the peak area of oil droplet size >1  $\mu\text{m}$  became larger, and more big size oil droplets could be observed under a microscope. However, the  $d_{4,3}$

increment of stored PPC stabilized emulsion was 0.21  $\mu\text{m}$ , 0.31  $\mu\text{m}$ , 0.2  $\mu\text{m}$  and 0.15  $\mu\text{m}$  for M-AA, M-AW, G-AA and G-AW, respectively, which were significantly lower than that of WPI ( $p < 0.05$ ). The relatively inferior emulsifying stability of WPI could be attributed to its relatively thinner hydrated layer compared with polysaccharides, which has been reported in the previous study (Haar, 2011; Setiowati, Wijaya, & Van der Meeren, 2020). Generally, the key parameters for maintaining emulsion stability referred to steric hindrance, electrostatic repulsions, and viscosity (Lin, Wang, Meng, & Guo, 2021). However, in this study, it seems that the emulsion viscosity played a decisive role in maintaining emulsion stability, but the electrostatic repulsion among oil droplets was a secondary consideration. The strong evidence was that M-AW possessed the relatively lower zeta potential but held comparable emulsifying stability with other produced polymers. In theory, the zeta potential of M-AW was significantly lower than other polymers, the relatively lower electrostatic repulsion in M-AW stabilized emulsion could result in oil droplets that more easily tended to close together and aggregate. However, in fact, M-AW stabilized emulsion possessed similar stability to M-AA, G-AW or G-AA stabilized emulsions, since there was no significant difference in  $d_{4,3}$  value increment between M-AW and others ( $p > 0.05$ ). An explanation for the above phenomenon was that the M-AW stabilized emulsion viscosity had to a great extent off-set the performance of negative effects from the lower electrostatic repulsion.

#### 4. Conclusion

ASP2, extracted from sugar beet pulp, was an amphiphilicity polysaccharide with low protein content. This study successfully prepared polymers constructed by WPI-ASP2 crosslinking and ASP2 self-crosslinking through Maillard and genipin crosslinking reactions. According to different grafting models, produced polymers presented varied physicochemical properties. No matter how the WPI was linked with ASP2, the additive WPI would enhance the emulsifying activity of ASP2, while the self-crosslinking products possessed a comparatively reduced emulsifying activity. Notably, the grafting site would significantly affect the interfacial properties of produced PPC; in other words, a protein linked with a polysaccharide at the carbonyl group could endow a better emulsifying activity to PPC compared with being linked with an amino group. However, the calcium-sensitive feature of ASP2 would not be amended after modification. Due to the viscosity effect, the emulsifying stability of PPC was higher than WPI.

Notes.

The authors declare no competing financial interest.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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