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Conjugation of soy protein isolate (SPI) with pectin: effects of structural modification of the grafting polysaccharide

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ABSTRACT

Recently, there has been a great interest in enhancing the emulsifying properties of soy protein isolate (SPI) by Maillard reaction. As a commonly-used grafting polysaccharide, pectin has proved useful in modifying proteins. However, effects of its structural characteristics on conjugation are still not fully understood. To address this problem, we employed alkaline or/and enzymatic treatments to modify pectin and obtained three modified samples. Structural characteristics of pectin, including the molecular weight, degree of methoxylation and acetylation, and monosaccharide compositions were measured. When conjugated with SPI, pectin with lower molecular weight and less main chains induced higher conjugate yield. Fluorescence intensity and surface hydrophobicity of all conjugates markedly reduced compared to the original SPI, suggesting a more loosened protein structure after Maillard reaction. In this study, the enzymolysis pectin proved an optimum grafting polysaccharide considering the simple preparation procedures and the highest emulsifying properties of its resulting conjugates.

1. Introduction

Soy protein isolate (SPI) is a soy protein product with over 90% protein content and a variety of desirable amino acid components. Aside from its nutritional value, one of the most important applications of SPI in the food industry is to confer emulsifying properties to food matrices. However, SPI is prone to denaturation and aggregation under a range of processing conditions, such as a high temperature, an extreme ironic strength, or a pH near its isoelectric point, which greatly impedes its industrial applications. In this case, many attempts have been made on SPI modification through chemical, physical or enzymatic methods.

As a green chemical modification method, the naturally occurring Maillard reaction can spontaneously take place between protein and carbohydrate at a certain temperature. Since the reactants involved are both from food matrix, the glycosylated protein is a safe additive for the food industry. Furthermore, this simple grafting process has been reported to make progress in enhancing a series of functionalities of protein (Hu, et al., 2020; Kutzli, et al., 2020; Pan, et al., 2020; Zhong, et al., 2019). To construct a properly functionalized Maillard-type product, understandings of the influence of grafting sugar on the properties of the consequent conjugates are necessary. Recent studies into this aspect find that carbohydrates with a larger molecular weight tend to result in conjugates with better emulsifying and stabilizing properties (Klinchongkon, et al., 2019; Pan, et al., 2020; Wang, Gan, Li, Nirasawa, & Cheng, 2019). Generally, a longer grafted sugar chain increases the thickness of the interface film, which could effectively prevent the aggregation of oil droplets. In this case, polysaccharides are increasingly applied in the Maillard reaction.

As a byproduct from juice and essential oil manufacturing, pectin is an acidic polysaccharide with excellent nutritional and functional properties (Chan, Choo, Young, & Loh, 2017). It consists of four main structural domains: homogalacturonan (HG), rhamnogalacturonan I

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(RG-I), rhanmnogalacturonan II (RG-II) and xylogalacturonan (XG) regions. HG is the backbone of pectin and a smooth region comprised of 1, 4-α-D-galacturonic acid (GalA). Carboxyl groups on GalA residues can be partly methyl esterified or acetylated, generating different degrees of methoxylation (DM) and acetylation (DAc) and in turn, leading to diverse functional properties. RG-I as another important structural domain contains a backbone of repeating GalA-rhamnose (Rha) units with a bunch of side chains consisting of galactose (Gal) and arabinose (Ara). RG-II and XG are less common structural regions bound at HG. Recent demand for cost-competitive and biodegradable materials for industrial inputs has made pectin an ideal polysaccharide for protein modification. Better solubility, emulsifying and colloidal properties have been observed for pectin-based Maillard-type products (Klinchongkon, et al., 2019; Murayama, Rankin, & Ikeda, 2020; Qi, Xiao, & Wickham, 2017).

However, from the observed literatures, the source and structure of pectin used for building conjugates vary a lot, and the effects of the structural characteristics of pectin on Maillard reaction and the emulsifying properties of the resulting conjugates are still not fully understood. Schmidt et al. (2016) tried to link the DM of pectin with the emulsifying performance of whey protein isolate-pectin conjugates; results showed that higher conjugate yield was achieved with lower esterified pectin, which enabled the resulting conjugates to stabilize smaller droplets. However, the pectin samples with different DM used in this previous study have different commercial sources, meaning that the differences of other structural characteristics such as molecular weight and monosaccharide compositions might also have played a role in affecting the yield and properties of the resulting conjugates. In view of this, this study selected citrus pectin (CP) as a grafting polysaccharide and employed alkaline or/and enzymatic treatment for modification. By applying controlled modification procedures instead of using different commercial pectin samples, the structural differences brought by different sources could be eliminated, making the results more comparable. β-elimination and saponification are known as two main reactions happening under alkaline conditions, which could effectively decrease the molecular weight, DM and DAc of CP. The pectinase used in this study is an endo-polygalacturonase (endo-PG), which can randomly hydrolyze the α-(1,4)-glycosidic bonds between two de-esterified GalA units. To achieve a higher degradation extent, we also combined the two treatments together. CP was first subjected to alkaline treatment, and then went through enzymatic hydrolysis to produce a significantly lower molecular weight. Structural characteristics of pectin samples, including the molecular weight, DM and DAc, and monosaccharide compositions were measured and compared. CP and three modified pectin (MP) samples were then used for conjugation with SPI under controlled wet-heating conditions. This is the first study, that we know of, to systematically investigate the integrated effects of diverse structural characteristics of pectin on yield, structures and emulsifying properties of its corresponding conjugates, which will further researchers' understanding of the structure–function relationships of pectin-based conjugates and boost the development of research in such areas.

On the other hand, despite the observed better functionalities of the pectin-based conjugates, Maillard reactions involving pectin can be extremely difficult to happen and always end up with low grafting extent. Wang, et al. (2019) compared the conjugation process of deamidated wheat gluten with pectin and maltodextrin; it was revealed that reactions with maltodextrin progressed more rapidly with a higher glycation extent. In our previous study (Ma, Chen et al., 2020), we made attempts to prepare SPI-pectin conjugates under controlled dry-heating conditions; it took up to 5 days to obtain a degree of graft (DG) of over 20%. Thus, another novelty of this study, is to provide a grafting pectin that is capable of not only breaking the structural barriers for Maillard reaction and greatly accelerating conjugation, but also generating conjugates with excellent emulsifying properties. By comparing the structural and emulsifying properties of conjugates prepared with different MPs, the optimum pectin structure will be determined and thus provide reference for material selection in the industry. This will facilitate the industrial use of pectin in Maillard reaction and greatly improve productivity, as well as provide an alternative low-cost additive for the food system.

2. Materials and methods

2.1. Materials

SPI was isolated from defatted soybean meal (Hengrui Food Ltd., Guangzhou, China) based on the method reported in our previous study (Ma, et al., 2019), with a protein content of 96.48 ± 0.36 (%). Citrus pectin (P9135, with a galacturonic acid content of more than 74.0%), standard saccharides and pectinase from *Aspergillus niger* (EC Number 3.2.1.15; optimum temperature: 50 °C, optimum pH: pH 4.0 (according to the supplier)) were obtained from Sigma–Aldrich (Shanghai, China). Sodium-dodecyl-sulfate polyacrylamide gel (SDS-PAGE) gel kit was obtained from Beyotime Biotechnology (Shanghai, China).

2.2. Pectin modification

2.2.1. Alkaline treatment

Alkaline deesterification of pectin was conducted according to Jackson, et al. (2007) with some modifications. CP solution (10 mg mL^{-1}) was placed in an ice water bath. Its pH was adjusted with 1 M NaOH and maintained at pH 12.0 for 4 h. After that, the pH was adjusted to pH 4.0 (the original pH of CP solution) by adding 1 M HCl. The alkali-treated pectin was then dialyzed (MD34; Mw: 3500 Da) in deionized water for 48 h and lyophilized. The resultant MP sample was named as MP1.

2.2.2. Pectinase treatment

Two hundred microliters of pectinase solution (50 mg mL^{-1}) was incubated with 50 mL of CP solution (10 mg mL^{-1}) at pH 4.0 and 50 °C for 10 min. After reaction, the beaker was immediately put in boiling water for 5 min to inactivate the enzyme. The enzymolysis pectin solution was then cooled and dialyzed (MD34; Mw: 3500 Da) in deionized water for 48 h and lyophilized. The resultant MP sample was named as MP2.

2.2.3. Combined alkali-pectinase treatment

First, the alkaline treatment followed the same procedure as described in Section 2.2.1. CP solution (10 mg mL^{-1}) was maintained at pH 12.0 for 4 h in an ice water bath. After that, enzymatic treatment was conducted according to the procedure as described in Section 2.2.2. The pH of the alkaline-treated pectin solution was adjusted to pH 4.0. Then, 50 mL of pectin solution were incubated with 200 μL of 50 mg mL^{-1} pectinase solution at pH 4.0 and 50 °C for 10 min. After reaction, the beaker was immediately put in boiling water for 5 min to inactivate the enzyme. The alkaline-enzymolysis pectin solution was then cooled in an ice water bath, dialyzed (MD34; Mw: 3500 Da) in deionized water for 48 h and lyophilized. The resultant MP sample was named as MP3.

2.3. Structural analysis of pectin samples

2.3.1. Molecular weight and conformational parameters

The weight-average molecular weight (Mw), number-average molecular weight (Mn), polydispersity (Mw/Mn) and root mean square (RMS) radius of gyration of CP, MP1, MP2 and MP3 were determined on a combined *SEC*-MALLS system (Wyatt Technology, Santa Barbara, CA, USA) equipped with two *SEC* columns (Shodex SB-806 HQ and Shodex SB-804 HQ, Showa Denko KK, Tokyo, Japan) following the procedures described in our previous study (Ma, Chen et al., 2020). Briefly, pectin solutions with a concentration of 3.0 mg mL^{-1} were stirred overnight and then filtered through a 0.22 μm membrane prior to injection. NaCl solution (0.2 M, containing 0.02% NaN_3) was used as a mobile phase.

Elution was conducted at a flow rate of 0.75 mL min^{-1} for 90 min.

2.3.2. DM and DAc

The DM and DAc of CP and MPs were determined on the Waters 1525 HPLC system (Waters, Milford, MA, USA) equipped with a C_{18} column (SinoChrom ODS-BP, Elite Corp., Dalian, China) as described in our previous studies (Ma, et al., 2018; Ma, et al., 2016). Five milligrams of pectin samples were added to 1.5 mL of mixture solution consisting of 2 mM $CuSO_4$, 0.3 M NaOH and 4 mM isopropanol (an inner standard). The suspension was kept at 4 °C for 30 min and then centrifuged. The pH of supernatant was adjusted with 0.1 M HCl to pH 2.0. It was then filtered through a 0.45 μm membrane prior to injection. A mixture standard was comprised of methanol (23%, v/v), glacial acetic acid (4%, v/v) and isopropanol (4%, v/v). H_2SO_4 solution (0.4 mM) was prepared as a mobile phase. Elution was conducted at 0.8 mL min^{-1} and 25 °C for 20 min.

2.3.3. Monosaccharide compositions

The monosaccharide compositions of CP and MPs were analyzed on the Waters 2695 HPLC system (Waters, Milford, MA, USA) equipped with a C_{18} column (Zorbax Aclips XDB, Agilent Technologies Inc., CA, USA) using a gradient elution pattern as described in our previous studies (Ma, et al., 2018; Ma, et al., 2016). The mixture standard comprised of manose (Man), rhamnose (Rha), glucuronic acid, GalA, glucose (Glu), galactose (Gal), xylose (Xyl), arabinose (Ara) and fucose (Fuc) was prepared with deionized water; the concentration of each monosaccharide standard was 2.0 mM. Pectin samples were thoroughly hydrolyzed by trifluoroacetic acid at 110 °C for 8 h, and then dried and neutralized. The mixture standard or pectin samples then went through derivatization according to the following procedures: standard/sample was incubated with a mixture solution consisting of 450 μL of 0.3 M NaOH solution and 450 μL of 0.5 M PMP in methanol solution. Fifty microliter of 2.0 mM lactose solution was added to sample solution as an inner standard. Derivatization was conducted at 70 °C for 30 min and then chloroform was added to extract the resulting solution. The final solution was filtered through a 0.22 μm membrane prior to injection.

Two mobile phases were prepared with acetonitrile and 0.05 M KH_2PO_4-NaOH buffer (pH 6.9): phase A, 15% (v/v) acetonitrile; phase B, 40% (v/v) acetonitrile. Analysis of standard and samples was conducted at 25 °C following a gradient pattern as shown below (for phase B): 0 (0 min) → 15% (10 min) → 25% (30 min) → 25% (35 min) → 0 (40 min).

2.3.4. Nuclear magnetic resonance (NMR) spectra

The NMR spectra were measured on a DD2-600 NMR spectrometer (Agilent Technologies Inc., CA, USA) at 25 °C. CP and MPs were measured from deuterium oxide (D_2O) with concentrations from 30 to 50 mg mL^{-1}. Spectra were analyzed by MestReNova (Version 10.0.1, MestreLab Research, Santiago de Compostela, Spain).

2.3.5. Fourier transform infrared spectroscopy (FT-IR) spectra

Pectin samples were mixed with KBr and pressed into pellets. The IR spectra were scanned by the Nicolet 5700 IR spectrometer (Thermo Fisher Scientific, MA, US) from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1}.

2.4. Conjugation of SPI with pectin samples

Conjugation of SPI with CP/MPs was conducted in an aqueous system. SPI and pectin sample at a ratio of 1:1 (w/w) were dissolved in deionized water with a total concentration of 20 mg mL^{-1} at pH 10. The mixtures were reacted at 70 °C for 24 h and then cooled in an ice-water bath to stop the Maillard reaction. DG of the conjugates were measured using the OPA assay as described in our previous studies (Ma, Chen et al., 2020; Ma, Hou et al., 2020). Briefly, OPA reagent was prepared with the following compositions: 1.6 mg mL^{-1} OPA in methanol solution, 50 mM sodium tetraborate, 1% (w/w) SDS solution and 0.4% (v/v) β-mercaptoethanol. Two hundred microliters of sample solution (with a protein concentration of 2 mg/mL) were added to 4 mL of OPA reagent and reacted at 35 °C for 2 min. The absorbance of the mixture was measured at 340 nm. Deionized water was used as the blank. DG was calculated according to Eq. (1):

$$DG = \frac{A_0 - A_t}{A_0} \times 100\% \quad (1)$$

where A_0 and A_t are absorbance of SPI and pectin mixtures and conjugates, respectively.

2.5. Structural and emulsifying properties of the conjugates

2.5.1. SDS-PAGE electrophoresis

Sample solutions were prepared at a constant SPI concentration of 2 mg mL^{-1} and heated with protein loading dye in a boiling water bath for 5 min. The stacking gel (5%) and separating gel (12%) were electrophoresed at 80 V and 120 V, respectively. Gel was stained with Coomassie Brilliant Blue R250 dye and then de-stained with a solution containing 40% methanol and 10% acetic acid.

2.5.2. Intrinsic fluorescence spectra

The freeze-dried samples were dissolved in phosphate buffer at pH 7.0. Intrinsic fluorescence spectra were determined by the Model Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, USA) at a scanning speed of 600 nm min^{-1} with λ_{ex} of 280 nm and λ_{em} of 300–500 nm (slit = 5 nm).

2.5.3. Surface hydrophobicity (H_0)

Sample solutions (4 mL) were prepared by phosphate buffer at pH 7.0 to obtain various SPI concentrations of 0.001–0.400 mg mL^{-1} and mixed with 50 μL of ANS (8 mM). Fluorescence intensity was measured at λ_{ex} of 365 nm and λ_{em} of 484 nm. H_0 was acquired from the slope of the function of fluorescence intensity versus SPI concentration.

2.5.4. Emulsifying activity index (EAI) and emulsifying stability index (ESI)

Measurement of EAI and ESI was based on the method reported by Cameron, Weber, Idziak, Neufeld, and Cooper (1991) and conducted as described in our previous study (Ma, et al., 2019). Samples, including SPI, SPI-CP/MP mixtures and conjugates, were dissolved in deionized water with a protein concentration of 2.5 mg mL^{-1}. The pH of sample solution was adjusted to pH 7.0. Then, 15 mL of sample solution were mixed with 5 mL of blend oil and homogenized at 13500 rpm for 2 min. Immediately after that, 50 μL of the prepared emulsions were mixed with 5 mL of 0.1% (w/v) SDS solution and the absorbance of the mixture was measured at 500 nm. EAI and ESI were calculated based on the following equations:

$$EAI\,(m^2/g) = \frac{2 \times 2.303 \times A_0 \times D}{(1-\varphi) \times c \times 10^4} \quad (2)$$

$$ESI\,(\%) = \frac{A_{10}}{A_0} \times 100\% \quad (3)$$

where A_0 and A_{10} are the absorbance of sample emulsions measured at 0 min and 10 min, respectively; φ is the fraction of the oil phase, c is the initial concentration of protein (g mL^{-1}) and D is the dilution factor.

2.6. Statistical analysis

Data were represented with a mean ± standard deviation of triplicate determinations. Differences between groups were analyzed by one-way ANOVA and Duncan's multiple range tests at a significance level of 95% using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Structural characteristics of pectin samples

3.1.1. Molecular weight, DM and DAc and monosaccharide compositions

The molecular conformational parameters of pectin samples are listed in Table 1. Alkaline treatment can be observed to remarkably decrease the Mw of pectin and its polydispersity by 63.43% and 23.62%, respectively; also, it induced significant reduction in DM and DAc by 85.68% and 34.62%, respectively. As reported by Renard and Thibault (1996), β-elimination and saponification are two main reactions that spontaneously take place in alkaline conditions. The former reaction is known to split the polygalacturonic chain leading Mw to decrease; while the latter reaction can readily cleave the O-acetyl and O-methyl linkages on HG moieties, thus effectively decreasing the DM and DAc of pectin (Bédouet, Courtois, & Courtois, 2003). In this study, these degradation reactions on the HG region were also confirmed by monosaccharide compositions analysis. As shown in Table 1, the alkali-treated pectin showed a decreased content of GalA and an increased content of Rha and Gal; further, compared to the untreated CP, it demonstrated significantly higher ratios of both Rha/GalA and (Gal + Ara)/Rha (which indicated the contents of main chain and side chain of RG-I, respectively). Therefore, results showed an increased proportion of RG-I regions in MP1. However, these variations in the pectin backbone did not induce a smaller molecular size to occur, since there was no significant difference in Rz for CP and MP1. As reported by Bédouet, et al. (2003), β-elimination and saponification are actually two competitive reactions in an alkaline system, which can be controlled by pH and temperature: β-elimination tends to take place at a relatively high temperature and low pH, versus demethylation, which is more enhanced at a low temperature and a high pH. As our alkaline treatment was conducted at pH 12.0 in an ice-water bath, a thorough demethylation reaction readily occurred inducing an extremely low DM of only 7.88%; nevertheless, β-elimination was impeded leading to incomplete degradation of pectin. Therefore, few changes were observed for Rz after alkaline treatment.

The pectinase used in this study is an endo-PG, which can randomly hydrolyze the α-(1,4)-glycosidic bonds between two de-esterified GalA units. The Mw and its polydispersity of MP2 can be seen to significantly reduce from 478.00 kDa to 48.24 kDa, and 2.54 to 1.76, respectively, which was attributed to the efficient enzymatic hydrolysis of pectin HG regions. Compared to β-elimination brought by alkaline treatment, enzymolysis obviously caused much severer degradation in pectin main chains. As shown in Table 1, MP2 showed a 72.40% lower Mw compared to MP1, with a significantly higher ratio of Rha/GalA. Enzymolysis further led MP2 to present a smaller size, causing Rz to significantly decrease from 36.80 nm to 30.15 nm. In fact, as CP used in this study is a high-methoxyl pectin (with a DM > 50%), enzymatic hydrolysis of HG regions can be greatly inhibited by the extensive existence of methoxyl groups; thus, this kind of enzymolysis is still a partial degradation process. On the other hand, as the pectinase did not contain any pectin methyl esterase, the DM and DAc values of MP2 almost remained unchanged compared to the original CP.

In order to obtain an MP with a higher degree of hydrolysis, we subjected CP to alkaline treatment before enzymatic hydrolysis. Theoretically, removal of ester linkages during alkaline treatment can provide a more favorable substrate for the action of endo-PG and in turn, results in a higher degradation extent. This is well confirmed in our study. As expected, the Mw and its polydispersity, and Rz of MP3 were significantly lower than any other pectin samples, with a further decrease to 3.91 kDa, 1.11 and 23.87 nm, respectively, indicating that a more complete enzymolysis reaction happened after alkaline treatment. Analysis of monosaccharide compositions showed that these changes were mainly attributed to hydrolysis on HG regions. As shown in Table 1, the GalA content of MP3 was 50.68% lower than the original CP, whereas the ratios of Rha/GalA and (Gal + Ara)/Rha were significantly increased by 292.31% and 20.22%, respectively.

Table 1
Structural information of pectin samples with different treatments.

Parameters	CP	MP1	MP2	MP3
Molecular weight and conformational parameters				
M_w (kDa)	478.00 ± 0.60^a	174.80 ± 3.40^b	48.24 ± 2.45^c	3.91 ± 0.20^d
M_n (kDa)	188.00 ± 0.10^a	89.85 ± 1.15^b	27.30 ± 0.55^c	3.51 ± 0.16^d
Polydispersity	2.54 ± 0.00^a	1.94 ± 0.01^b	1.76 ± 0.05^c	1.11 ± 0.00^d
R_z (nm)	36.80 ± 0.00^a	34.15 ± 1.25^a	30.15 ± 0.75^b	23.87 ± 1.97^c
DM (%)	54.63 ± 1.01^a	7.88 ± 0.17^b	56.04 ± 1.74^a	7.99 ± 0.25^b
DAc (%)	1.56 ± 0.17^a	1.02 ± 0.05^b	1.58 ± 0.02^a	1.07 ± 0.01^b
Monosaccharide compositions (%)				
Rha	8.85 ± 0.06^d	9.60 ± 0.03^c	10.34 ± 0.08^b	17.16 ± 0.08^a
GalA	68.19 ± 0.31^a	64.92 ± 0.16^b	59.78 ± 0.20^c	33.63 ± 0.08^d
Glu	4.34 ± 0.06^c	4.53 ± 0.12^c	6.66 ± 0.13^b	7.55 ± 0.15^a
Gal	12.44 ± 0.08^d	14.96 ± 0.14^c	16.02 ± 0.08^b	28.72 ± 0.13^a
Xyl	1.38 ± 0.05^c	1.44 ± 0.04^c	1.62 ± 0.01^b	2.56 ± 0.05^a
Ara	3.30 ± 0.04^c	3.00 ± 0.10^d	4.07 ± 0.07^b	7.92 ± 0.06^a
Fuc	1.50 ± 0.09^b	1.55 ± 0.02^b	1.50 ± 0.03^b	2.45 ± 0.08^a
Rha / GalA	0.13 ± 0.00^d	0.15 ± 0.00^c	0.17 ± 0.00^b	0.51 ± 0.00^a
(Gal + Ara) / Rha	1.78 ± 0.02^c	1.87 ± 0.02^b	1.94 ± 0.02^b	2.14 ± 0.02^a

Note: Rha / GalA and (Gal + Ara) / Rha represent molar ratios; values with different italic superscript letters (a–d) in the same row within each parameter indicate significant differences as estimated by Duncan's multiple range test ($P < 0.05$).

3.1.2. ^1H and ^{13}C NMR analysis

Fig. 1A displays the ^1H spectra of CP and 3 MP samples; the corresponding chemical shifts of major signals in GalA are shown in Table A1. In line with the reported ^1H NMR spectra for pectin (Ma & Wang, 2013), featured peaks were distributed in the range of 3.3–5.1 ppm. The spectra of the original CP and MP2 (the enzymolysis pectin) both contain a sharp signal around 3.76 ppm, which is attributed to the protons of $–CH_3$ in the esterified pectin. Winning, Viereck, Nørgaard, Larsen, and Engelsen (2007) reported that the H-2 signal around 3.68 ppm and H-3 signal around 3.96 ppm also yielded a good correlation to esterification, and ascribed this to the overhauser effect between $–CH_3$ and protons positioned in close proximity. For this reason, the proton absorption around these regions in spectra of MP1 and MP3 (*i.e.* pectin samples with alkaline treatment) can be seen significantly lower than those of CP and MP2. Except from the methoxyl group-related absorbs, signals assigned to acetyl groups at 2.12 and 2.01 ppm (Majoros, Keszler, Woehler, Bull, & Baker, 2003; Tamaki, Konishi, Fukuta, & Tako, 2008) also markedly reduced in the spectra for alkaline-treated pectin samples. The α-glycoside linkage of GalA and the pyranose configuration of pectin were not changed with each degradation treatment, as H-1 signals for all the samples were beyond 4.95 ppm and no signals around 5.4 ppm were detected.

The ^{13}C NMR spectra of CP and 3 MPs are displayed in Fig. 1B and the corresponding chemical shifts of predominant signals for galacturonic ring carbons are shown in Table A1. Similar to ^1H spectra, drastic changes in absorptions related to methoxyl group were observed for MP1 and MP3. For these alkaline-treated samples, the signal moved upfield in the region for resonances of esterified and free carboxyl

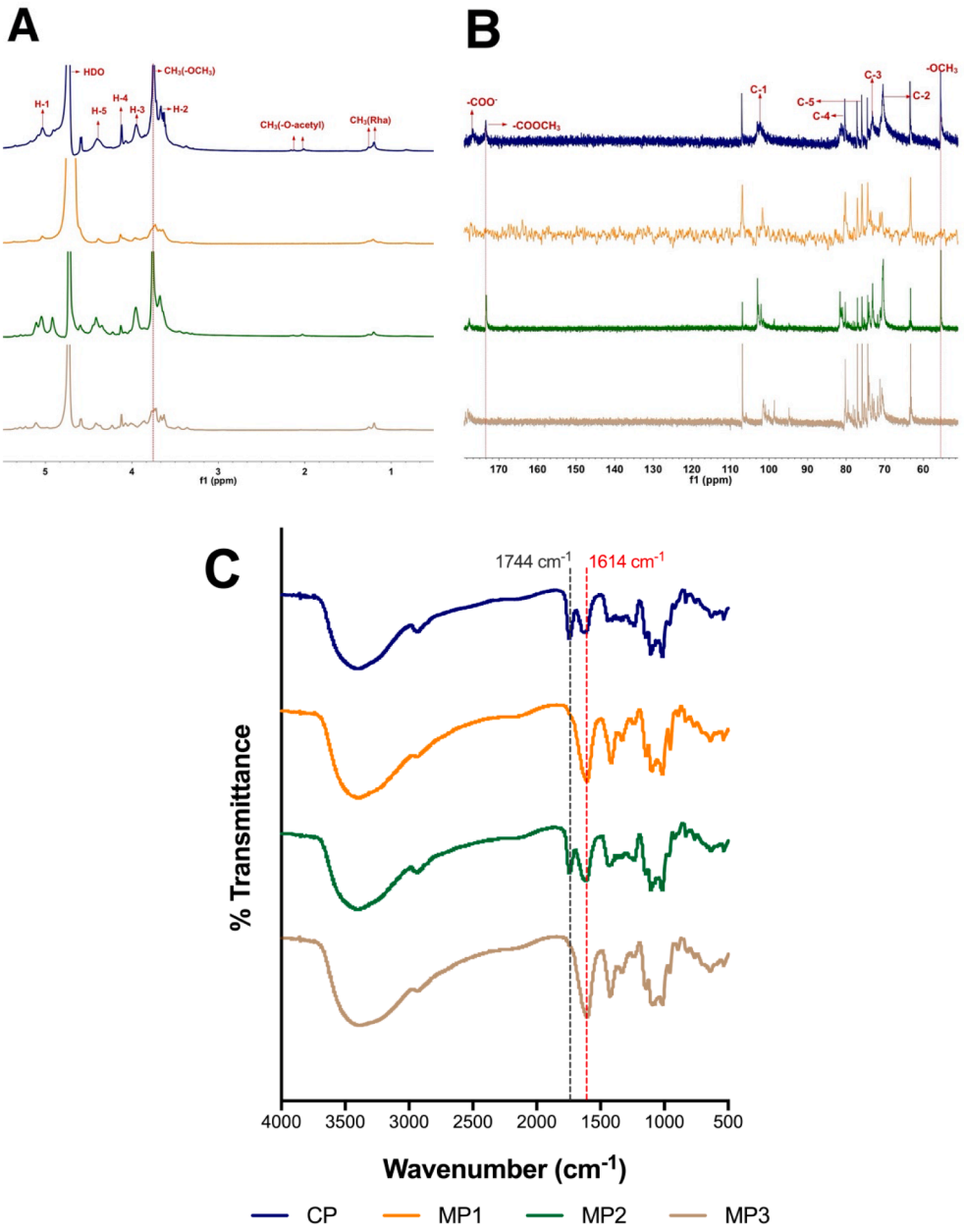

Fig. 1. (A) ^{1}H NMR, (B) ^{13}C NMR and (C) IR spectra of CP, MP1, MP2 and MP3.

groups at 173–177 ppm; furthermore, the resonance of the –CH_3 carbon in esterified GalA around 55.4 was almost undetectable. In conclusion, the NMR spectra for CP and MP2 showed overall similar patterns except for some slight changes in chemical shifts, whereas significant changes in methoxyl group-related signals were observed for MP1 and MP3.

3.1.3. FT-IR analysis

Fig. 1C illustrates the IR spectra of CP and 3 MP samples. The wide band at 3000–3700 cm^{-1} in all samples refers to O—H stretching, attributed to the intra-/intermolecular hydrogen bonds and the absorbed moisture in pectin (Romdhane, et al., 2020). The band around 2932 cm^{-1} is from stretching vibration of C—H, thus, the weakened absorption at this wavelength for MP1 and MP3 was possibly due to the removal of –OCH_3 during alkaline treatment. Saponification of MP1 and MP3 was also evidenced from the almost disappeared absorption at 1744 cm^{-1} and the enhanced absorption at 1614 cm^{-1}, which indicated C═O stretching of methylesterified carbonyl groups and COO– stretching of ionic carboxyl groups, respectively. The carboxylate groups formed in the two alkali-treated MPs also presented a stronger symmetric stretching absorption at 1420 cm^{-1}. Absorption regions oscillating at intervals of 1000–1200 cm^{-1} showed that all pectin samples are rich in uronic acid (Coimbra, Barros, Barros, Rutledge, & Delgadillo, 1998). As can be seen from Fig. 1C, the IR spectra of MP2 was pretty similar to those of the original CP, indicating that enzymatic hydrolysis did not change the primary configuration of pectin, which is in line with our previous results (Ma, et al., 2018; Ma, et al., 2016); while alkaline treatment induced loss of bands belonging to methoxyl groups and enhanced absorptions related to carboxylate groups.

3.2. DG of the conjugates prepared with different pectin samples

Fig. 2 depicts the DG of SPI-pectin conjugates prepared using different pectin samples. The Maillard reactions were carried out under wet heating conditions at pH 10.0 and 70 °C for 24 h, obtaining varying DG of (8.43 ± 0.18)%, (12.29 ± 0.50)%, (44.40 ± 0.31)% and (55.91 ± 1.31)%, for SPI-CP, -MP1, -MP2 and -MP3 conjugates, respectively. This

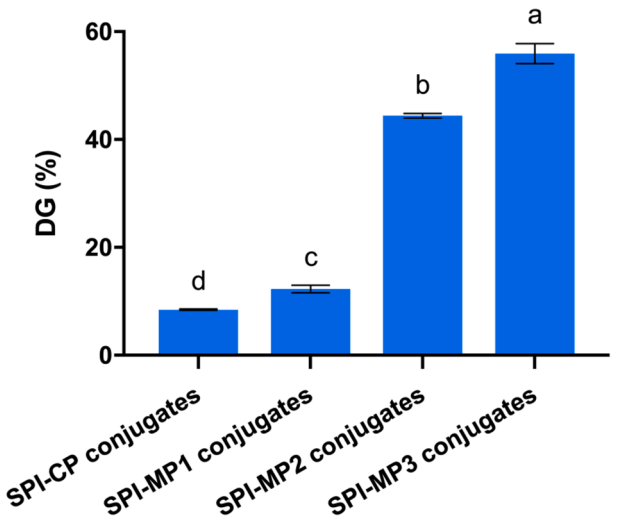

Fig. 2. DG of SPI-CP, -MP1, -MP2 and -MP3 conjugates. Different letters (a–d) above each column indicate significant differences as estimated by Duncan's multiple range test ($P < 0.05$).

significant difference in the grafting extents was indicative of the vital role that structural modification of pectin played in affecting the conjugation behavior. As demonstrated in Table 1, the Mw of CP, MP1, MP2 and MP3 were 478.00, 174.80, 48.24 and 3.91 kDa, respectively, clearly suggesting a negative correlation between the molar mass of pectin and DG of the corresponding conjugates. Li, et al. (2013) reported that the rice peptides tended to show higher degree of substitution in conjugation with glucose and lactose, than maltodextrin and dextran, due to the lower glycation rate in a reaction system containing high-Mw saccharides. A following research (Klinchongkon, et al., 2019) employed color development as an indicator to demonstrate the grafting progress between whey protein isolate and subcritical-water hydrolyzed pectin; it was found that the browning intensity was the highest for the conjugates prepared with the lowest-Mw pectin. These can be seen paralleling to our results. Pectin with a lower Mw has more reducing ends and is less steric, which is more favorable for conjugation. Enzymatic and/or alkaline treatment produced shorter polysaccharide chains and reduced the steric hindrance effect that CP sustained, thus making the reactive groups more accessible for grafting. Likewise, it was shown that DG was also negatively correlated with the content of HG regions (or main chains) in the grafting pectin, indicating that the steric hindrance of pectin was mainly sustained by the cross-linked HG chains instead of the neutral sugar side chains in RG-I region.

On the other hand, some studies have reported that the ester groups in pectin also have an influence on the Maillard reaction (López-Mercado, et al., 2018; Schmidt, et al., 2016). However, the pectin samples with different DM and DAc generally showed very different Mw as well, so currently it is hard to differentiate whether DM and DAc also played a role in affecting the conjugation process. To systematically address this issue, research on controlled degradation methods is still needed in order to obtain more precisely structured fragments.

3.3. Structural and emulsifying properties of the conjugates

3.3.1. SDS-PAGE analysis

The SDS-PAGE profiles for SPI, mixtures of SPI and CP/MPs, and SPI-CP/MP conjugates are shown in Fig. 3. It was demonstrated that all mixture samples (in Lane 2, 4, 6 and 8) showed an almost same SDS-PAGE pattern as the native SPI (in Lane 1). In these samples, the characteristic bands for glycinin and β-conglycinin were clearly shown in the gel, including the acidic polypeptide chains (37–45 kDa) and basic polypeptide chains (18–20 kDa) that compose glycinin, as well as three

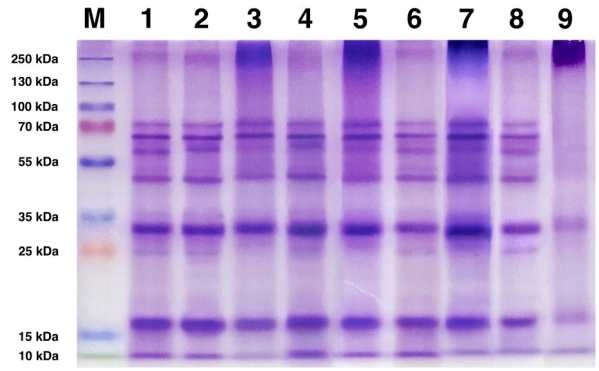

Fig. 3. SDS-PAGE profiles of SPI, mixtures of SPI and different pectin samples, and SPI-pectin conjugates. The amount of protein loaded for each sample is 20 μg. Lane M, molecular weight markers (kDa); Lane 1, SPI; Lane 2, mixtures of SPI and CP; Lane 3, SPI-CP conjugates; Lane 4, mixtures of SPI and MP1; Lane 5, SPI-MP1 conjugates; Lane 6, mixtures of SPI and MP2; Lane 7, SPI-MP2 conjugates; Lane 8, mixtures of SPI and MP3; Lane 9, SPI-MP3 conjugates.

subunits (40–60 kDa) that compose β-conglycinin (Petruccelli & Anon, 1995). As for the conjugate samples, a noticeable new band was formed on the top of Lane 3, 5, 7 and 9, which suggested the formation of SPI-pectin conjugates with too high Mw to migrate into the gel. The top bands of the conjugates formed with MP2 and MP3 displayed an obviously deeper color than those of the SPI-CP/MP1 conjugates, probably due to their higher DG. Furthermore, some bands in the original SPI significantly diminished in Lane 3, 5, 7 and 9, indicating that these peptides had participated in the Maillard reaction. In particular, these featured bands almost disappeared in Lane 9, suggesting that more peptides were grafted in SPI-MP3 conjugates, which was in line with the results of DG measurement. Another thing to note is that although the Mw of MP3 was only 3.91 kDa, there was no clear band in the Mw range of 70–100 kDa for SPI-MP3 conjugates. This phenomenon can be possibly attributed to the reduced steric hindrance and the improved exposure of reactive groups in MP3, which significantly increased the bonding between multiple protein molecules and multiple MP3 molecules. Results can therefore offer credible reasons for the covalent binding between SPI and different pectin samples.

3.3.2. Intrinsic fluorescence and H_0 analysis

As a well-established method for identification of protein tertiary structures, fluorescence spectra of proteins is determined by the amount of fluorophores (the majority of which are reported to be the indole group of tryptophan (Trp)), as well as the microenvironment around them (Miriani, Iametti, Bonomi, & Corredig, 2012). In SPI molecule, Trp is reported to exist in both glycinin and conglycinin (Miriani, et al., 2012); therefore in this study, fluorescence spectra of SPI, mixtures of SPI and CP/MPs, and SPI-CP/MP conjugates were measured to demonstrate changes in SPI conformations during conjugation, which are shown in Fig. 4A. The maximum fluorescence emission (λ_{max}) of the 4 mixture samples kept unchanged at 341 nm. Nevertheless, in four conjugate samples, a modest red shift was shown, with λ_{max} increasing to 343 nm, suggesting that Trp residues were oriented toward a more hydrophilic location after conjugation. Meanwhile, the fluorescence intensity of the conjugates can be seen much lower than that of the original SPI. It has been previously proposed that when proteins are unfolded with a looser structure, more Trp would expose outside to solvent resulting in a more polar microenvironment around chromophores and in turn, an increased λ_{max} (Li, et al., 2019). Furthermore, this unfolded, loosened protein structure would also make chromophores more accessible for fluorescence quenching, and consequently lead to the reduction in the fluorescence intensity (Wang, Zhang, Wang, Xu, & Jiang, 2020).

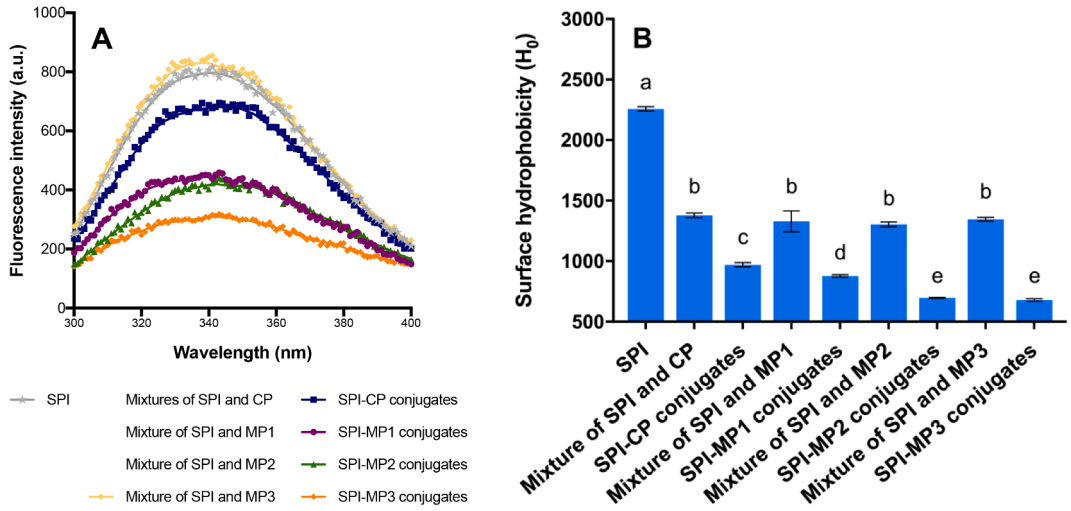

Fig. 4. (A) Intrinsic fluorescence spectra and (B) surface hydrophobicity (H_0) of SPI, mixtures of SPI and different pectin samples, and SPI-pectin conjugates. Different letters (a–e) above each column indicate significant differences as estimated by Duncan's multiple range test ($P < 0.05$).

In addition to the intrinsic fluorescence spectra, H_0 was also evaluated to provide information for SPI structural variations on a basis of the amount of the hydrophobic groups exposed on protein surface, which is shown in Fig. 4B. The simple physical mix with CP or MP significantly decreased the H_0 of the original SPI from 2258 to around 1300, due to the steric-hindrance effect of pectin samples as mentioned before. A further decrease in the protein H_0 was seen for the Maillard-type products. As can be observed from Fig. 4B, the H_0 of the SPI-CP, -MP1, -MP2 and -MP3 conjugates was determined to be 970, 878, 697 and 678, respectively. It has been previously suggested that conjugation with polysaccharides would sterically hinder the way of ANS to the hydrophobic groups inside the conjugates (Zhao, et al., 2016); furthermore, grafting with hydrophilic sugar chains could also enhance the hydrophilicity on SPI surface (Zhou, Wu, Zhang, & Wang, 2017), therefore, the H_0 of protein molecules was markedly decreased after the Maillard reaction. On the other hand, some studies have also reported the similar trend as demonstrated in our results, that the higher the DG, the lower the hydrophobicity (Achouri, Boye, Yaylayan, & Yeboah, 2005; Mu, et al., 2010; Zhao, et al., 2016). However, it is noteworthy that there was no significant difference for the H_0 of SPI-MP2 and SPI-MP3 conjugates, despite the higher DG of the latter conjugates (as shown in Fig. 2). This was speculated to be attributed to the above-mentioned severe degradation on the HG region of MP3 with the combination of alkaline and pectinase; the great loss of long main chains led to the diminished steric hindrance, and in turn, a decreased H_0 of the resultant conjugates.

3.3.3. *Emulsifying properties of the conjugates*

As one of the most important functional properties of protein, emulsifying properties of protein have a direct influence on the textures and sensory quality of food products (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016). In this study, EAI and ESI of SPI, mixtures of SPI and CP/MP, and SPI-CP/MP conjugates were measured and are depicted in Fig. 5. The increased EAI and ESI for SPI and CP mixtures were contributed by the stabilizing properties of CP. However, for the mixtures composed of MP samples, both indices almost kept unchanged compared to those of the original SPI, suggesting that the structural modifications with alkaline or/and enzymatic treatment might have affected the stabilizing properties that pectin sustained. The four conjugates exhibited significantly higher EAI and ESI than the original SPI and four mixtures. It has been well established that when a protein-polysaccharide conjugate is introduced to a water–oil system, protein would absorb at the water–oil interface allowing for a viscoelastic film to occur, while polysaccharide provides steric stabilization for the emulsified droplets via its thickening and gelling properties in the aqueous

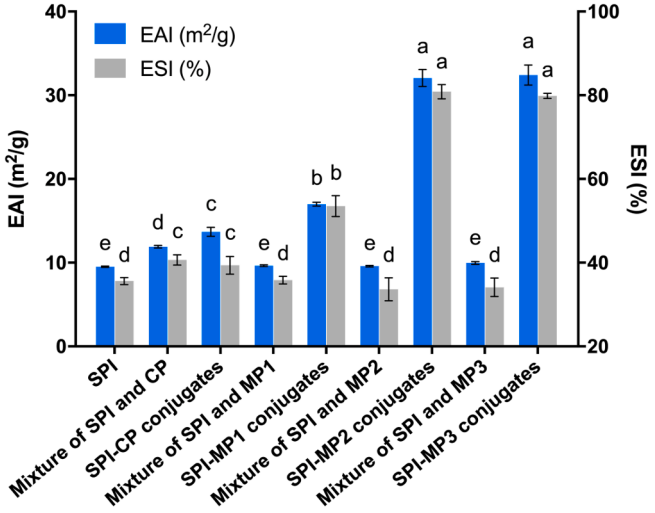

Fig. 5. EAI and ESI of SPI, mixtures of SPI and different pectin samples, and SPI-pectin conjugates. Different letters (a–e) above each column indicate significant differences as estimated by Duncan's multiple range test ($P < 0.05$).

system (Ru, Cho, & Huang, 2009). Thus, the improved EAI and ESI for conjugate samples were attributed to this synergistic behavior.

On the other hand, the emulsifying properties of the conjugates were also found to be greatly influenced by the grafting extent. As can be seen, the EAI and ESI for SPI-CP, -MP1, -MP2 and -MP3 conjugates were 13.68 $m^2\ g^{-1}$ and 39.39%, 16.99 $m^2\ g^{-1}$ and 53.54%, 32.06 $m^2\ g^{-1}$ and 80.84%, 32.41 $m^2\ g^{-1}$ and 79.83%, respectively. For the SPI-CP, -MP1 and -MP2 conjugates, there was a progressive increase in both indices with the increasing DG. As proposed by Li, et al. (2019), as the DG increased, more hydroxyl groups were introduced to the conjugates, leading to the reduced surface tension at water–oil interface. Another reason can be related to SPI structures. As demonstrated in Section 3.3.2, the spatial conformation of SPI was increasingly loosened with the increase in the DG, which could make SPI more readily to spread out at the water–oil interface and thus result in the enhancement of EAI and ESI. However, similar to the results of H_0 analysis, this positive correlation with DG was not applicable to the SPI-MP3 conjugates. Based on these results, we proposed that when proteins are conjugated with pectin, there is an upper threshold for the Mw and HG contents of the grafted pectin, beyond which the steric-hindrance effect would be too strong to

let Maillard reaction occur; but there also is a bottom limit, below which the length of the sugar chains would be too short to sustain a proper steric-hindrance effect. Taking into consideration the productivity and efficacy, in this study, the enzymatic-hydrolyzed pectin (*i.e.* MP2) proved to be an optimum polysaccharide for SPI modification.

4. Conclusions

In this study, we employed alkaline or/and enzymatic treatment to degrade the HG region of CP and obtained three modified pectin samples (*i.e.* MP1, MP2 and MP3) with different structural characteristics. Alkaline treatment efficiently decreased the DM and DAc resulting in MP1 with a Mw of 174.80 kDa; pectinase caused a severe hydrolysis on HG and led to MP2 with a Mw of 48.24 kDa; a combination of alkaline and enzymatic treatment induced a much more complete degradation process resulting in MP3 with a Mw of 3.91 kDa. Structural modification of pectin significantly increased the DG of SPI-pectin conjugates, which was increased with the decrease in pectin Mw and the content of HG regions. SDS-PAGE analysis showed that featured peptides of SPI participated in the Maillard reaction resulting in large-Mw conjugates. Intrinsic fluorescence analysis showed that compared to the original SPI, the fluorescence intensity of all conjugates markedly reduced with a modest red shift, suggesting a more loosened protein structure produced by Maillard reaction. As expected, the conjugation process also caused a lower H_0 and higher EAI and ESI for the conjugates. All these changes were found to be closely related to the Mw and HG content of the grafted pectin samples. Results indicated that an ideal grafting pectin should possess a moderate Mw and a proper HG content, to ensure an efficient Maillard reaction while generating excellent steric-hindrance effects for functional purposes. In this study, the simple enzymatic hydrolysis of pectin proved to be a feasible method for preparation of a desirable grafting polysaccharide.

CRediT authorship contribution statement

Xiaobin Ma: Conceptualization, Funding acquisition, Investigation, Writing – original draft. **Chengdeng Chi:** Software, Formal analysis. **Yunfeng Pu:** Investigation. **Song Miao:** Supervision, Writing – review & editing, Funding acquisition. **Donghong Liu:** Supervision, Funding acquisition.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132876.

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