Changes in physical, chemical and functional properties of whey protein isolate (WPI) and sugar beet pectin (SBP) conjugates formed by controlled dry-heating

Phoebe X. Qi^{a,I,*}, Yingping Xiao^{a,b,I} and Edward D. Wickham^a

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Abstract

A Maillard type reaction in the dry state was utilized to create conjugates between whey protein isolate (WPI) and sugar beet pectin (SBP) to achieve improved functional properties including solubility, colloidal stability and oil-in-water emulsion stability. To optimize the reaction conditions, mixtures of WPI and SBP at varying weight ratios, 3:1, 2:1 and 1:1, were heated at 60 °C, 79% RH for 72 hrs. Changes in the physical properties, *i.e.* solubility, and chemical compositions, *i.e.* free sulfhydryl and amine contents were assessed using chemical assays. Bradford assay results demonstrated that the protein solubility of WPI increased significantly, ~20% by reacting with lower levels of SBP (3:1 and 2:1) whereas the total powder solubility was not affected until an equal ratio of SBP was used, and the reduction was only ~15%. The free sulfhydryl (SH) and primary (NH₂) and secondary amine (NH) contents of WPI were also mitigated by conjugating with SBP. The formation of the covalent conjugates between WPI and SBP was confirmed by SDS-PAGE gel electrophoresis through staining for both proteins and glycoproteins. The UV-VIS spectroscopy showed significant changes in the molecular electronic transition states of both WPI and the feruloyl moieties of SBP upon interaction and conjugation. Measurements of ζ -potential, particles size distribution and average particle size demonstrated that the WPI-SBP conjugates prepared at the weight ratio of 3:1 were most effective at stabilizing oil-in-water emulsions than other ratios used in this work.

Keywords: Maillard reaction; O/W emulsion stability; whey protein isolate; sugar beet pectin; conjugation; UV-Vis spectroscopy

Introduction

Once a waste product from the cheese manufacturing and an environmental burden, whey proteins have increasingly been recognized as an invaluable food and non-food ingredient for their undeniable health benefits and versatile functional properties. Research in the past few decades has provided a large body of evidence on the numerous physical, chemical, nutritional and biological prominences that the whey protein components sustain (de Wit, 1998; Smithers, 2015). Combined with the fast growing processing and manufacturing technologies, whey proteins have found their way in a diverse and expanding range of food products, including sports beverages, nutritional supplements, infant formula, pharmaceutical formulations, and non-food such as personal care products. Despite these developments, whey proteins are still excluded by certain formulation conditions and processing operations which prevent their potentials to be fully realized (Cayot & Lorient, 1997; Foegeding, Davis, Doucet, & McGuffey, 2002; Wijayanti, Bansal, & Deeth, 2014). These impediments include reduced solubility and thermal stability (de Wit & Kessel, 1996; Kazmierski & Corredig, 2003; Pelegriane & Gasparetto, 2005; Ryan & Foegeding, 2015), and diminished emulsion stability (Demetriades, Coupland, & McClements, 1997; Fachin & Viotto, 2005; Hunt & Dalgleish, 1994; McClements, Monahan, & Kinsella, 1993; Moon & Mangino, 2004) caused by protein denaturation, polymerization and aggregation.

These techno-functional issues have led increasing research effort to the Maillard-type reaction to create conjugates between whey proteins and carbohydrates including mono- and polysaccharides (de Oliveira, dos Reis Coimbra, de Oliveira, Zuñiga, & Garcia Rojas, 2016; Dickinson, 2009, 2015; Evans, Ratcliffe, & Williams, 2013). Using this simple modification

process, progress has been made in improving the emulsifying and gelation properties as well as the thermal stability of the end products. These research results demonstrated that when whey proteins were dry-heated at controlled temperature, relative humidity (RH) and reaction time with appropriate weight ratios of polysaccharide, a significant improvement in the functional properties (*e.g.* emulsion and gelation) was observed in the conjugated products. These polysaccharides included dextran (Akhtar & Dickinson, 2003; Jiménez-Castaño, Villamiel, & López-Fandiño, 2007; Shekaripour, Aminlari, Niakosari, & Eskandary, 2014; Spotti, *et al.*, 2014), maltodextrin (Akhtar & Dickinson, 2007; Martinez-Alvarenga, *et al.*, 2014), xanthan gum (Benichou, Aserin, Lutz, & Garti, 2007), carboxymethylcellulose (Kika, Korlos, & Kiosseoglou, 2007; Koupantsis & Kiosseoglou, 2009) and pectin (Einhorn-Stoll, Ulbrich, Sever, & Kunzek, 2005; Mishra, Mann, & Joshi, 2001; Neiryneck, Van der Meeren, Bayarri Gorbe, Dierckx, & Dewettinck, 2004; Schmidt, *et al.*, 2016; Xu, Wang, Jiang, Yuan, & Gao, 2012).

A by-product of the sugar manufacturing, sugar beet pulp is a rich source of carbohydrates and contains 67 - 80% of potentially high valued plant cell wall polysaccharides (Oosterveld, Beldman, Schols, & Voragen, 1996). Nearly 19 - 25% of these polysaccharides are pectin (Sun & Hughes, 1999), which is of particular interest to us because creative utilization of sugar beet pulp will increase its economic value over its current primary use as an animal feed. SBP is commonly used as an emulsifier because of its high content of the proteinaceous materials believed to be bound to the side chains through covalent linkages (Evans, *et al.*, 2013; Fishman, Chau, Qi, Hotchkiss, & Yadav, 2013; Funami, *et al.*, 2007; Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003; Siew & Williams, 2008; Williams, *et al.*, 2005). Furthermore, SBP is also rich in feruloyl groups, both as free ferulic acid (Ferreira, Diez, Faulds,

Soliveri, & Copa-Patino, 2007; Qi, Chau, Fishman, Wickham, & Hotchkiss, 2014; Wicker, *et al.*, 2014) and as ester links covalently attached to the neutral sugar side chains (Guillon & Thibault, 1990; Micard, Renard, & Thibault, 1994; Oosterveld, Beldman, Schols, & Voragen, 2000; Oosterveld, *et al.*, 1996; Rombouts & Thibault, 1986). These unique feruloyl groups make SBP an excellent antioxidant and bioactive agent for food and non-food applications (Graf, 1992; Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002; Maxwell, Belshaw, & Waldron, 2012).

Despite these demonstrated attributes of SBP, modifying whey protein using SBP has received little attention, especially through the Maillard-type reaction. To our best knowledge, the only published report (Xu, *et al.*, 2012) involved preparing the whey protein isolate (WPI)-SBP conjugates by dry-heating (79% RH) at varying weight ratios (20:1, 10:1, 5:1, 2:1, 1:1 and 1:2), temperatures (80, 90 and 100 °C) and times (0 to 9 hr). The resulting products were evaluated in the β -carotene emulsions stabilization experiments and the conjugates formed at 1:2 (80 °C for 5 hr) were found to be the most effective in stabilizing the emulsions against freeze-thaw treatment.

The Maillard reaction involves an initial condensation of a reducing sugar with an amino group of the protein, namely the ϵ -amino group of the lysine, the ζ -amino group of the arginine residues and the N-terminal amino group. A range of reactions takes place subsequently, and leads, via the formation of Schiff base and the Amadori rearrangements, to the formation of advanced Maillard reaction products (AMREPs) depending on the nature of the reactants, pH, reaction temperature and time, and humidity (Ames, 1998). It is reported (Einhorn-Stoll, *et al.*, 2005; Mishra, *et al.*, 2001; Schmidt, *et al.*, 2016; Wang & Ismail, 2012) that the conjugation

between whey proteins and polysaccharides via the Maillard reaction causes changes in the chemical composition and physical properties such as solubility, protein conformation, and thermal stability. A myriad of the functional properties were greatly improved in polymerized whey protein as a result of these conjugation reactions (Klemaszewski & Kinsella, 1991; Monahan, McClements, & Kinsella, 1993). However, a fundamental understanding is still lacking regarding the physical and chemical changes that occurred in the protein as a result of Maillard conjugation with the polysaccharides. Undoubtedly, these changes were responsible for the observed gross improvement in the functional properties.

In addition to conjugating whey proteins and polysaccharides by utilizing the Maillard-type reaction, successful attempts have also been made in forming complexes through non-covalent interactions such as electrostatic, hydrogen binding and hydrophobic interactions under various conditions (Alizadeh-Pasdar, Nakai, & Li-Chan, 2002; Dickinson, 2008; Evans, *et al.*, 2013; Jones, *et al.*, 2011; Mishra, *et al.*, 2001; Qi, Chau, *et al.*, 2014). When combined, proteins and polysaccharides are expected to form either soluble complexes or coacervates depending on the conditions used (de Kruif, Weinbreck, & de Vries, 2004; Schmitt & Turgeon, 2011; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003; Turgeon, Schmitt, & Sanchez, 2007).

In this work, we investigate quantitatively the changes that occurred in the physical properties and chemical compositions of whey protein isolate (WPI) conjugated with varying ratios of sugar beet pectin (SBP) through a dry-state Maillard-type reaction, and to correlate these changes with the improved functional properties as observed in the resulting conjugates. Rather than a typical heating time of several weeks, a shortened reaction time was used to limit the reaction propagation and to reduce excessive browning and the formation of possibly

harmful, toxic and even carcinogenic compounds (de Oliveira, *et al.*, 2016; Ledl & Schleicher, 1990; Stadler, *et al.*, 2002).

2. Materials and Methods

2.1. Materials

All reagents including NaCl, sodium phosphate (monobasic and dibasic), sodium citrate, sodium benzoate, DL-dithiothreitol, 5,5'-dithio-*bis*-(2-nitrobenzoioc acid) (DTNB, Ellman's reagent), β -mercaptoethanol ($\geq 99\%$, BME) and ninhydrin reagent used in these studies, unless otherwise noted, were of the analytical grade or 'ACS certified' from Sigma-Aldrich (St. Louis, MO). Cold-pressed Valencia orange oil was supplied by Citrus and Applied Essences Ltd., Belcamp, MD. The Polytron homogenizer (Model PT 10/35) was purchased from Brinkmann, Westbury, NY and the EmulsiFlex-B3 high-pressure homogenizer was purchased from Avestin Inc., Ottawa, Canada. Tris Ultra Pure was purchased from ICN Biomedicals, Inc. (Cleveland, OH). The electrophoresis reagents including the NuPAGE® Novex® 12% Bis-Tris Gels (NP0341), the NuPAGE® LDS Sample Buffer (4X, NP0007), the SimplyBlue® SafeStain (LC6060) and the Pierce® Glycoprotein Staining Kit (24562) were supplied by Thermo Fisher Scientific (Waltham, MA). The low molecular weight marker (LMWM) was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ).

The whey protein isolate (WPI) powder (Provon 190) used in this work was purchased from Glanbia Nutritionals, Inc. (Twin Falls, ID). The components of the spray-dried WPI, according to the manufacturers' specifications are: protein 89.6%; fat 2.5%; moisture 2.8%, and ash 3.3%.

Sugar beet pectin (SBP, Lot# 30091) was donated by CP Kelco (San Diego, CA). The composition of the pectin was determined using previously published methods (Qi, Wickham, & Garcia, 2014; Yoo, Fishman, Savary, & Hotchkiss, 2003): galacturonic acid (GalA) $58.5 \pm 5.4\%$;

degree of methyl esterification (DME), $57.4 \pm 1.0\%$; degree of acetyl esterification (DAE), $21.7 \pm 0.1\%$; and neutral sugar, $37.3 \pm 1.1\%$. The feruloyl moieties in SBP, bound and free, were estimated to be $0.88 \pm 0.15\%$ based on the calculations obtained from the UV-VIS spectra of SBP and pure ferulic acid (Qi, Chau, *et al.*, 2014).

2.2. Preparation of WPI-SBP conjugates by dry-heating

The WPI-SBP conjugates were prepared by dry-heating at $60\text{ }^{\circ}\text{C}$ in a controlled relative humidity (79%) using saturated KBr solution for 72 hrs, as described separately (Qi, Xiao, & Wickham, 2017).

2.3. Determination of the extinction coefficient of sugar beet pectin (SBP) at 325 nm

The extinction coefficient of SBP in 25 mM sodium phosphate (PBS), pH 6.75 was determined by carefully measuring the weight and volume of completely dissolved SBP solution ($\sim 5.0\text{ mg/mL}$). After an overnight stirring at room temperature ($20\text{ }^{\circ}\text{C}$), the solution was centrifuged at $21,400\times g$ (also at $20\text{ }^{\circ}\text{C}$) in a Sorvall Legend XTR bench top centrifuge (Thermo Scientific, Germany) for 30 min and a small amount of pellet was discarded. The supernatant was filtered through a $0.45\text{-}\mu\text{m}$ syringe-driven filter (Millex HV, PVDF, Millipore Corp., Billerica, MA). The volume (V, mL) of the filtered SBP solution was recorded. The UV-VIS spectrum (450-200 nm) (Cary 100 Bio UV-VIS spectrometer, Agilent Technologies, Böblingen, Germany) of the SBP solution were collected, and an absorbance value at 325 nm ($A_{325\text{ nm}}$) was obtained using a quartz cuvette with 0.5-cm path length (L) and the PBS buffer as a reference. The cuvette was then washed 3x with Milli-Q water. All washes were combined with the SBP

solution and freeze-dried. An equal volume (V) of the PBS buffer was also lyophilized to account for the salt contribution from the buffer. The weights of the lyophilized SBP (W_{SBP} , mg) and the buffer salt (W_{buffer} , mg) were used to calculate the extinction coefficient of SBP at 325 nm ($\epsilon_{325 \text{ nm}}$, mL • mg⁻¹ • cm⁻¹) using the following equation:

$$\epsilon_{325} = \frac{A_{325}}{\frac{(W_{\text{SBP}} - W_{\text{buffer}})}{V} \times L} \quad (1)$$

2.4. Determination of total powder and protein solubility

Total solubility (%) of the dry-heated WPI and SBP powder in water was determined by suspending the sample (~100 mg) of known weight (W_p) in ~10 mL Milli-Q water at room temperature for 4-12 hrs. The total weight of the suspension was recorded to be W_{total} (mg), and centrifuged at 24,000×g (also at 20 °C) in a Sorvall RC-5B centrifuge (DuPont Co., Wilmington, DE) for 30 min. The supernatant was filtered through a 0.45-μm syringe-driven filter (Millex HV, PVDF, Millipore Corp., Billerica, MA). A fraction of the filtered supernatant with known weight (W_{Tsol} , mg) was then lyophilized. The weight of the freeze-dried powder is denoted as W_{sol} , and the total solubility is calculated as the following:

$$\text{total solubility (\%)} = \frac{W_{\text{sol}}/W_{\text{Tsol}}}{W_p/W_{\text{total}}} \times 100 \quad (2)$$

Protein solubility was determined using a modified Bradford method (Basch et al., 1985). β-LG at varying concentrations, from 0.1 to 1.0 mg/mL, was used to build a standard curve, using $\epsilon_{280} = 0.96 \text{ mL} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ (Townend, Winterbottom, & Timasheff, 1960). The assay

solvent contained 0.1 M sodium phosphate, 0.01 M sodium citrate, 50 mM DTT and 4.0 M urea (pH 7.0). The centrifuged and filtered supernatant from the total solubility experiments (described above, ~10 mg/mL) was diluted by 10x using the assay solvent. Fifty microliters (50 μ L) of the diluted sample was added to 2.5 mL prepared dye reagent (according to the directions from the manufacturer). Total protein content in the dry-heated WPI-SBP samples was estimated by taking the sum of total protein in WPI (89.6%) and in SBP, 4.78%.

All solubility (%) experiments were repeated three times, and an average and standard deviations were obtained.

It should be pointed that it was reported previously (Alkorta, Llama, & Serra, 1994) that the presence of pectin (from citrus and apple), at 10 mg/mL, interfered with the determination of protein concentration by Bradford assay. The concentration of SBP present in the samples of this work, even at 1:1 ratio, was well below 0.5 mg/mL. Therefore, the interference introduced by SBP was considered negligible.

2.5. Determination of soluble sulfhydryl and disulfide bond contents

Total free sulfhydryl concentration was determined according to the method by Ellman (Ellman, 1959) in combination with a modified procedure provided by the supplier (Sigma, St. Louis, MO). Twenty milligrams (20 mg) of each dry-heated WPI and SBP sample were dissolved in 1 mL reaction buffer, which contained 0.1 M sodium phosphate, 8.0 M urea and 1.0 mM EDTA (pH 8.0). After centrifugation at 18,500 xg (at room temperature) for 30 min, the supernatant was filtered through a 0.45- μ m syringe-driven filter (Millex HV, PVDF, Millipore Corp., Billerica, MA). Two hundred fifty microliters (250 μ L) of this sample solution (~20 mg/

mL) and 25 μ L of 1.0 mM 5,5'-dithio-*bis*-(2-nitrobenzoioc -) (DTNB, Ellman's reagent) assay solution were pipetted into 1.125 mL of the reaction buffer. The reaction mixture was incubated in the dark for 20 min at room temperature (20 °C), and the absorbance was then recorded at 412 nm against a blank of 1.125 mL reaction buffer containing 50 μ L of 1.0 mM DTNB reagent and 250 μ L of Milli-Q water. A molar extinction coefficient $\epsilon_{412} = 14,290 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for TNB (the colored product from the reaction between -SH and DTNB) (Riddles, Blakeley, & Zerner, 1983) was used. The final free sulfhydryl concentration (μ M) was normalized against the total amount of protein (mg) contained in each sample. All assay experiments were performed in triplicate.

2.6. Determination of soluble primary and secondary amine content

The Sigma Aldrich product N 7285 ninhydrin reagent solution (2%) (Starcher, 2001) was used to determine the concentration of the primary and secondary amines in the soluble fraction of the dry-heated WPI samples in the absence and presence of SBP. A stock solution of leucine at concentration of 50 mM in 0.05% glacial acetic acid was used to prepare the standard curve according to the manufacturer's instructions, and the absorbance was recorded at 570 nm. The concentration of the stock solution for each sample was prepared at $\sim 5.0 \text{ mg/mL}$ in water as described above, in section 2.5. Appropriate dilutions, 10 to 20 times, were made from the stock solution using 0.05% glacial acetic acid to obtain reasonable absorbance reading in the range of 0.5 - 1.0 at 570 nm for each assay. The result from the assay yielded concentration of the sum of NH and NH_2 (mM), and was then normalized against the total amount of protein present in each sample as estimated spectrophotometrically (described in section 2.8).

2.7. Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of all dry-heated WPI-SBP samples was carried out using pre-cast mini gels (10 wells), NuPAGE® Novex® 12% Bis-Tris Gel (NP0341BOX). The electrophoresis was performed under both native and reducing conditions using β -mercaptoethanol (BME, 20% stock solution). All samples were prepared by dissolving the powder at 2.5 mg/mL concentration in the diluted (4x with Milli-Q water) sample buffer NuPAGE® LDS (4X, NP0007). All sample solutions containing 1X sample buffer and BME (50 mg/mL, when used) were first vortexed and then centrifuged at 21,400 xg for 30 min. Ten microliters (~25.0 μ g) of supernatant from each sample solution were loaded into each well of the gel. The running buffer was NuPAGE® Tris-Acetate SDS Running Buffer (20X) (LA0041) and used according to the procedures by the manufacturer. The LMWM was also treated in the absence and presence of BME before use. Gel was stained with SimplyBlue® SafeStain (LC6060) for 3 hrs followed by destaining in a solution containing 30% methanol and 10% acetic acid to the desired color density level. For glycoprotein staining, the procedure as recommended by the manufacturer (Thermo Fisher Scientific, Inc.) of the Pierce® Glycoprotein Staining Kit (#24562) was followed using both the positive (horseradish peroxidase) and negative (soybean trypsin inhibitor) controls provided by the supplier.

2.8. Determination of WPI and SBP concentrations in dry-heated mixtures

Unless otherwise noted, the stock solutions for the spectroscopic studies in this work including WPI, WPI-dh, SBP, SBP-dh, and dry-heated WPI and SBP mixtures were prepared at a concentration of 5.0 mg/mL in 25 mM sodium phosphate buffer, pH 6.75. Each sample was

stirred in the buffer for a few hours at room temperature (20 °C), and then followed by centrifugation and filtration as described in section 2.3. The UV-VIS spectra (450-200 nm) of 5 times diluted solutions (in the same buffer) were recorded using a Cary 100 Bio UV-VIS spectrometer (Agilent Technologies, Böblingen, Germany). The absorbance values at 330, 325 and 278 nm were recorded using a quartz cuvette with 1.0-cm path length (L) and the phosphate buffer as a reference. The concentration of WPI, WPI-dh, SBP and SBP-dh was then calculated based on the Beer-Lambert law: $C=A/\epsilon L$ using the extinction coefficient $\epsilon_{278}=1.046$ mL•mg⁻¹•cm⁻¹ (Mahmoudi, Mehalebi, Nicolai, Durand, & Riaublanc, 2007) and $\epsilon_{325}=0.763$ mL•mg⁻¹•cm⁻¹ (determined in this work, section 2.3.) for WPI and SBP respectively. The absorption at 278 nm for WPI and WPI-dh was corrected for light scattering ($A_{330} \gg 0.0$) using $A_{278}(\text{corrected}) = A_{278} - 1.986 \times A_{330}$ (Grimsley & Pace, 2004).

Because of the considerable absorption contribution at 278 nm by SBP and a much lesser degree at 325 nm by WPI, the contribution of SBP and WPI to the mixture was estimated by taking the ratios of A_{278} and A_{325} for SBP and WPI in their respective absorption spectrum in 25 mM phosphate buffer, pH 6.75, to yield $(A_{278}/A_{325})^{\text{SBP}}$ and $(A_{325}/A_{278})^{\text{WPI}}$, which are 0.56 and 0.015 respectively. The concentrations of WPI and SBP in each mixture were calculated as the following:

$$C_{\text{WPI}} = \frac{A_{278} - A_{325} \times (A_{278}/A_{325})^{\text{SBP}}}{\epsilon_{278}^{\text{WPI}} \times L} \times \text{DF} \quad (3)$$

$$C_{\text{SBP}} = \frac{A_{325} - A_{278} \times (A_{325}/A_{278})^{\text{WPI}}}{\epsilon_{325}^{\text{SBP}} \times L} \times \text{DF} \quad (4)$$

Where DF stands for dilution factor, and L (1.0 cm) is the cell path length of the cuvette used in the UV-VIS experiments.

All concentrations (mg/mL) determined by UV-VIS spectroscopy were repeated three times, and an average and standard deviation were obtained.

2.9. Electrophoretic mobility measurements

All samples studied in this work, including WPI, WPI-dh, SBP and their conjugates (3:1, 2:1 and 1:1) were dissolved at 2.0 mg/ml in 33 mM sodium phosphate buffer (I=50 mM, pH 6.75). Electrophoretic mobility (20 °C) of these solutions was determined by the Zetasizer Nano Z system (Malvern Instruments, Worcestershire, U.K.). Samples were inserted in plastic folded capillary cells (Malvern Instruments) possessing two metal electrodes at the capillary ends. The instrument operates by measuring the velocity of the colloidal particles when a pulsed electric field is applied using the Laser Doppler Velocimetry technique. The electrophoretic mobility was measured with a Zetasizer NanoZS (Malvern Instruments). ζ -Potential was converted into millivolts (mV) by the software (v.7.03), also provided by Malvern Instruments.

2.10. Emulsion preparation

Emulsions were prepared in a buffer system containing 33 mM sodium phosphate, and 0.1% (w/w) sodium benzoate (a preservative), pH 6.75. The stock solution of each emulsion was prepared by slowly adding the calculated amount (11.2 mg/g) of WPI, SBP and the dry-heated mixture a little at a time with vigorous stirring to the buffer at room temperature and then gently

stirring overnight to produce a hydrated, well dissolved and homogeneous solution. The samples for oil-in-water emulsions were prepared in triplicate for each sample at 1:4 for emulsifier to oil weight ratio by taking 9.0 g of above emulsifier stock solution (~100 mg adjusted to WPI only according to calculated concentration in Table 1) and 4% (400 mg) of Valencia orange oil to obtain the final total 10.0 g solution (using the above buffer) in a glass vial. No weighting agent was added during emulsion preparation to avoid the effects of such agent on the emulsification process. The solution was vortexed and then homogenized using a polytron bench top homogenizer equipped with a 12 mm diameter head (Brinkmann, Switzerland, PT 10/35) at 20,000 rpm for 30 sec. The above-homogenized emulsion was then passed through the EmulsiFlex-B3 high-pressure homogenizer (Avestin Inc., Canada) at 20,000 psi homogenization pressure 3 times to yield the final emulsion concentrate. The emulsions were stored at 60 °C for the duration of the experiment.

2.11. Emulsion droplet distribution and size measurements

The oil droplet distribution measurements were made using the same phosphate buffer (pH 6.75) as stated above. The pH value for each emulsion was recorded to be varying from 6.22 (for 1:1) to 6.68 for WPI controls, which were considered relatively close to the buffer pH. At a given time interval, the emulsion was mixed and a 100 µL aliquot was dispersed in Milli-Q water and used in the funnel of a HORIBA Laser Scattering Particle Size Distribution Analyzer LA-950 (Horiba Ltd., Kyoto, Japan). Particle size distribution calculations were made on the volume basis. Refractory index of the oil and water were kept 1.450 and 1.333 respectively. The emulsifying stability was assessed by the particles poly-dispersion and the

value of the volume-weighted average droplet diameters. The average droplet size was measured at time 0 (immediately after preparation) and after storage at 60 °C for 3 and 7 days.

2.12. Statistical Analysis

All results were represented as the means of triple measurements. To evaluate the differences among various samples, one-way analysis of variance (ANOVA) was performed using StatPlus software (AnalystSoft, Walnut, CA). Pairwise comparison between samples was carried out using a two-tailed Student t-test with unequal variance. *P* values of < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Effects of dry-heating and SBP on the solubility of WPI-SBP conjugates

The total powder and protein solubility of the dry-heated WPI in the absence and presence of varying amount of SBP were assessed by weight difference (Eq. 1) and the Bradford assay method respectively. Figure 1A compares the results and shows clearly that mild dry-heating condition (60 °C, 79% RH and 72 hrs) decreased the total solubility of WPI by ~5% compared to the WPI control. SBP was nearly completely soluble under similar experimental conditions (10 mg/mL in water). The pH of the solution, however, was ~3.5, making it **inappropriate to be compared** with all other samples (pH ~6.0 at the same concentration). Therefore, it was excluded from Fig. 1A. The presence of SBP at lower weight ratios, 3:1 and 2:1, appeared to have negligible effect ($P > 0.05$) on the total solubility of the mixture compared to WPI-dh, when SBP was absent. At an equal amount of SBP (WPI:SBP=1:1), the total solubility of the heated mixture dropped considerably, by ~17% and ~12% compared to WPI and

WPI-dh respectively. This could be caused by the overall limited solubility of SBP in water (~2%).

Figure 1B shows the protein solubility of WPI dry-heated at various ratios of SBP. It is clear that the protein solubility was reduced slightly, ~3% ($P < 0.01$), by dry-heating alone (WPI-dh) due to protein denaturation compared to the control, WPI. Interestingly, the presence of SBP, at lower ratios, 3:1 and 2:1, increased the protein solubility dramatically, by 17% and 20% compared to WPI respectively. This can be attributed to the conjugates formed between the protein components of WPI including β -LG, α -LA, IgG and BSA and the limiting amount of SBP were more soluble than unreacted WPI. It should be noted that these interactions are only of chemical conjugation in nature because the assay conditions used in this work would disrupt any forms of physical interactions, *e.g.* electrostatic and hydrophobic interaction. **More importantly, the molecular structure of WPI, both the secondary and tertiary levels, was significantly stabilized by interacting and conjugating with a lower level of SBP (3:1 and 2:1), as demonstrated in our subsequent spectroscopic studies (Qi, *et al.*, 2017). In addition to the highly soluble and sustainable conjugates formed between WPI and SBP, *i.e.* in the form of FRET, the basic residues (Arg, Lys and His) and the aromatic residues (Phe, Tyr and Trp) of the unreacted WPI appeared to have become more susceptible and accessible to the binding of the Coomassie Brilliant Blue G-250 dye (the Bradford assay reagent) than the WPI control. Further research is currently being carried out in our laboratory to provide an in-depth understanding of this remarkable improvement on the solubility of WPI (by SBP).**

Increasing SBP to an equal level as WPI (1:1), however, led the protein solubility to drop to a comparable level to that of the dry-heated WPI (WPI-dh). This is anything but surprising.

First of all, the fraction of soluble protein should be strictly affected by the total powder solubility (Fig. 1A). Secondly, other chemical changes **also** took place, *i.e.* loss of free SH and primary amine levels, as a result of the conjugation reactions (discussed below), which would **impact negatively** on the protein solubility. We proposed two different interaction and reaction schemes (Qi, *et al.*, 2017) for the reactions at higher WPI and SBP ratios (3:1 and 2:1) versus that occurred at an equal level (1:1). Nevertheless, the soluble fraction of WPI was evidently held steady by reacting with SBP despite the heat-induced denaturation effect in this case (1:1), demonstrating the overall positive effect of reacting with SBP on the solubility and stability of **WPI at all levels**.

The concentrations of WPI and SBP (mg/mL) in the stock solutions (total ~5 mg/mL in 25 mM phosphate, pH 6.75) were estimated using Eqs. 3 and 4 as described in section 2.8 and are given in Table 1. It should be acknowledged that the source of error in obtaining the concentrations (listed in Table 1) largely resided in the assumption that the extinction coefficients for WPI and SBP (at 278 nm and 325 nm respectively) remained unchanged in the conjugates from their unmodified states. Nevertheless, it is important to obtain an estimation of the concentration of each component in the mixtures using the approximation procedure outline in this work.

3.2. Effects of dry-heating and SBP on the chemical composition of WPI

The effect of dry-heating in the absence and presence of SBP on the chemical composition of WPI was studied by DTNB and ninhydrin assay methods respectively (Figure 2). Figures 2A and 2B show a considerable loss in both free sulfhydryl (SH) and primary (NH₂) and

secondary (NH) amine contents (normalized by the total amount of protein present) as a result of dry-heating, in WPI-dh. This is apparently caused by protein denaturation and increased level of disulfide bonds (S-S), both intra- and intermolecular among different proteins in whey, especially involving β -LG and α -LA upon heating. The presence of SBP, at relatively low weight ratios, 3:1 and 2:1, evidently mitigated the losses of soluble sulfhydryl (SH) and available primary and secondary amines compared to the dry-heated control ($P < 0.05$), WPI-dh. Higher level of SBP (1:1) caused the SH to decrease to the level below that of WPI-dh, indicative of increased protein denaturation and aggregation. Because the assay was performed using the soluble fraction of the reacted mixtures, it is therefore not surprising that the effect of SBP on the free SH content of WPI and conjugates formed with SBP mirrored the protein solubility results (Fig. 1B). On the other hand, the concentration of primary (NH₂) and secondary (NH) amine of WPI and conjugates didn't suffer much reduction by an equal weight ratio of SBP. This is consistent with the notion that the level of conjugates formed was limited by the available WPI, as noted above.

The concentrations of NH₂ and NH in WPI were apparently preserved by the presence of all levels of SBP. This observation further attests the protective effect of SBP, particularly at lower ratios, on WPI against heat treatment by forming conjugates either covalently through a Maillard type of reaction. It should be pointed out that the ninhydrin assay method used in this work is not sensitive enough to detect exclusively the reduction in the primary amine level (NH₂) of WPI caused by the Maillard reaction with SBP, in which case, any remaining (unreacted) NH₂ and resulting NH (of the products) were also included.

The SDS-PAGE electrophoretic profiles of dry-heated WPI at varying ratios of SBP with and without the use of the reducing agent (β -mercaptoethanol), stained with Coomassie blue (for

proteins) are shown in Figures 3A and 3B respectively. Under non-reducing conditions (Fig. 3A), all samples, both in the absence (Lane 2) and presence of SBP (Lanes 3, 4 and 5) displayed the formation of heat-induced high molecular weight species, *i.e.* aggregates formed among the proteins of WPI and conjugated complexes between WPI and SBP, which were all soluble but too large to migrate into the gel, except for the WPI control (Lane 1). In addition, large molecular weight species within the range of the gel separating capacity were also visible in all heat-treated samples (Lanes 2-5). Reduction of the disulfide bonds (S-S) by BME (Fig. 3B) caused the amount of species with extremely high molecular weight that remained in the stacking gel (Fig. 3A) to decline significantly, especially for WPI-dh (Lane 2) and at low level of SBP (3:1, Lane 5). Soluble complexes and conjugates formed at higher ratios of SBP, 1:1 and 2:1 are clearly visible in lanes 3 and 4, suggesting the formation of covalent compounds (between WPI and SBP) of much higher molecular weight.

The SDS-PAGE electrophoretic profiles of dry-heated WPI at varying ratios of SBP with and without the use of the reducing agent (β -mercaptoethanol, BME), stained for glycoproteins, are shown in Figures 4A and 4B respectively. These gel electrophoretograms clearly showed the formation of high molecular weight species in all samples (lanes 5, 6 and 7) due to conjugation reaction between WPI and varying ratios of SBP. In addition, a low level of glycoproteins (Fig. 4A), the faint band in stacking gel, was also detected for WPI and WPI-dh, lanes 1 and 3 respectively. These glycoproteins were likely formed between whey protein and residual minimal level of lactose during the WPI manufacturing process. Similar to the gel stained with Coomassie blue shown in Fig. 3B, reduction with BME significantly reduced the amount of high molecular weight species (lanes 1 and 3, Fig. 4B) to a nearly undetectable level. The high

molecular weight glycoproteins resulted from conjugation between WPI and SBP through dry-heating still remained distinctively visible in lanes 5, 6, and 7 (Fig. 4B) upon reduction.

3.3. Interactions between WPI and SBP induced by dry-heating probed by UV-VIS

The UV-VIS absorption spectra (450-200 nm) of the dry-heated mixtures of WPI and SBP with varying weight ratios (3:1, 2:1 and 1:1) along with the control samples, WPI, WPI-dh, SBP, and SBP-dh are shown in Figure 5A. At constant protein concentration (0.40 mg/mL, ~16.0 μ M using assumed average molecular weight of 25 kDa for WPI), the intensity of the main WPI absorption band at the region ~280 nm increased upon heating (WPI-dh), demonstrating further solvent exposure of the aromatic residues, namely, Tyr and Trp. In addition, appreciable light scattering in the range of 300-400 nm was also observed compared to the unheated control, WPI, due to heat-induced denaturation, aggregation and low level of glycosylation (discussed below).

To assess the effect and degree of chemical conjugation as well as physical interactions between WPI and SBP, the near UV absorption spectra of WPI and SBP in the dry-heated mixture were obtained by subtracting the appropriate amounts of SBP and WPI from that of the mixtures (Table 1) accordingly. The resulting calculated absorption spectra of WPI and SBP (in the mixtures) are shown in Figures 5B and 5C respectively, and compared with the experimental spectra of the controls including the untreated (WPI and SBP) and dry-heated controls, WPI-dh and SBP-dh, all at an equivalent experimental concentrations, 0.40 and 0.45 mg/mL for WPI and SBP respectively. The presence of SBP (at all weight ratios used) clearly prevented the aromatic residues (Tyr and Trp) of WPI from becoming further solvent exposed, as in the case of dry-

heated WPI (WPI-dh). This is evidenced by the nearly identical absorption band intensity (at 280 nm) of the calculated WPI in the mixture compared to that of the unheated control, WPI (Fig. 5B). A weak but obvious broad absorption band (relative to WPI) centered around 366 nm was revealed in the presence of all levels of SBP (Fig. 5B), likely caused by the advanced Maillard reaction end products (AMREPs) between WPI and SBP (Suarez, Rajaram, Oronsky, & Gawinowicz, 1989).

The interactions between WPI and SBP induced by dry-heating also drastically impacted the near-UV absorption spectrum of SBP as well. Figure 5C illustrates changes in the absorption behavior of SBP, and shows a strong dependence on the amount of WPI used when the concentration of SBP was kept constant at 0.45 mg/mL. At relative higher ratios of WPI to SBP used in this work, 3:1 and 2:1, an absorption band at 303 nm showed prominent intensity, nearly equivalent to that of the main absorption peak of SBP (at 325 nm). Furthermore, both this absorption band (~303 nm) and the shoulder band in the area of 350-400 nm saw reduced intensity as the amount of SBP increased, suggesting decreased level of conjugates at equal weight ratio of WPI:SBP (1:1). Perhaps, the presence of excess level of SBP inhibited the formation of AMREPs. Previous work on the WPI-dextran conjugation (Zhu, Damodaran, & Lucey, 2008) and the sodium caseinate-maltodextrin conjugates (Morris, Sims, Robertson, & Furneaux, 2004) appeared to also attribute the absorption band at 303 nm to the Schiff base, the initial product during the Maillard reaction.

The interactions between WPI and SBP as probed by UV-VIS spectroscopy in this work are expected to be both covalent (*e.g.* Maillard reacted conjugates) as well as non-covalent in nature including hydrophobic, between aromatic side-chains of the WPI proteins and proteinous

moieties of SBP, and local electrostatic interactions (Qi, Chau, *et al.*, 2014). Whereas conditions used in all chemical assays (as above) inevitably disrupt most physical and intermittent interactions, spectroscopic techniques such as UV-Vis are capable of detecting interactions of all types. The effect of physical interaction on the UV-Vis behavior of WPI and SBP can be analyzed by comparing the UV-Vis spectra of simple mixtures (data not shown) at varying weight ratios with that of the controls, WPI and SBP at appropriate concentrations. As demonstrated previously (Qi, Wickham, *et al.*, 2014), the physical interactions between β -LG, a major whey protein, and SBP caused significant changes for each partner on the molecular level. An in-depth analysis (Qi, *et al.*, 2017) of the UV-Vis spectra of their simple mixtures showed the Tyr and Trp residues of WPI as well as the ferulic acid moieties of SBP were deeply involved in the interactions. Our results were in agreement with the work published by Chevalier *et al.* (Chevalier, Chobert, & Dalgalarondo, 2002) on β -LG glycosylated by various simple sugars using near-UV CD spectroscopy, in which they reported disruption in the environment of aromatic side-chains upon glycation.

3.4. Effect of conjugation on the electrophoretic mobility

The ζ -potential (mV) of the samples studied in this work at pH 6.75 is given in Figure 6. Naturally, SBP exhibited higher colloidal stability than the protein (WPI and WPI-dh). Figure 6 also showed clearly that the conjugation between WPI and SBP increased the colloidal stability ($P < 0.05$) than unreacted WPI and SBP. This increase was directly proportional to the level of SBP used, and reached the highest at the weight ratio of 2:1 (25% SBP). Further increase in the level of SBP, to 1:1, failed to result in greater ζ -potential values ($P > 0.05$). Despite these

moderate ζ -potential values (< 30 mV measured using the conditions specified), the colloidal stability was improved significantly by conjugating WPI and SBP compared to that of WPI (unheated and heated). Further research is needed to find the optimal ratios between WPI and SBP that would yield maximum colloidal stability. Among the conditions explored in this work, the ratio of 2:1 between WPI and SBP appeared to give rise to the most stable colloidal system.

3.5. *Oil-in-water (O/W) emulsion stability*

The average droplet particle diameters in the emulsion systems containing WPI, WPI-dh, SBP and their conjugates at varying ratios, 3:1, 2:1 and 1:1, measured immediately after the emulsions (Day 0) and after storage at 60 °C up to a week at ~pH 6.7 is presented in Figure 7. The elevated storage temperature (60 °C) instead of room temperature was chosen in order to complete the experiments in a reasonable amount time. The WPI, WPI-dh and SBP showed poor emulsion stability with increasing droplet size as a function of storage time. The dry-heating reacted conjugates at varying WPI:SBP ratios, produced emulsion droplets of smaller size at Day 0 in comparison to unconjugated WPI and WPI-dh, but bigger relative to SBP. This is presumably because the levels of WPI used in the conjugation reactions were all in excess (including 1:1), and the denatured WPI would behave in a similar fashion as the WPI control in such an emulsion system. As time progressed, it is evident that all conjugates yielded much smaller droplets than the proteins or SBP alone, and the droplet diameter was reduced to ~ 1.0 μm for 3:1, and less than 1.5 μm for 2:1 and 1:1 samples upon extended storage (7 days). The oil-in-water emulsions under the conditions employed were stabilized by the conjugating systems at all reaction ratios. Moreover, the droplet size continued to decline during the storage period, such

as the case in the 3:1 and 1:1 samples. At the storage temperature (60 °C) and time (7 days) used, the unreacted WPI likely experienced an increased level of denaturation, making it more susceptible for further physical interactions with the protein moieties and the feruloyl groups of SBP (Qi, Chau, *et al.*, 2014). In addition, the conjugation reaction between WPI and SBP was also continuing, contributing to the apparently increasing oil droplet emulsion stability. There was no visible creamy layer separation over the storage period of one week in the emulsions prepared with the conjugates, unlike the emulsions of WPI alone which showed a creamy layer separation in a few days (visual observation). As suggested by Dickinson (Dickinson, 2009) and Yadav (Yadav, Parris, Johnston, Onwulata, & Hicks, 2010) for other polysaccharide-protein conjugates, the improved emulsion stabilizing ability of WPI-SBP conjugates might be due to the increased steric and electrostatic repulsion between the bulky hydrophilic polysaccharide moiety around the tiny oil droplets, which protect them against flocculation and coalescence. It is believed that the notably increased protein solubility in 3:1 and 2:1, assessed in this work, played an important role in their increased emulsion stability. To further understand this apparent improved O/W emulsion stability by the WPI-SBP conjugates compared to the unreacted WPI and SBP, we have carried out systematic spectroscopic studies. Results were published in another manuscript (Qi, *et al.*, 2017) in which a mutual stabilization effect was observed for both WPI and SBP in the conjugating and interacting systems formed at ratios of 3:1 and 2:1.

Conclusions

It has been shown in this work that whey protein isolate (WPI) reacts and interacts with varying weight ratios of sugar beet pectin (SBP) through controlled dry-heating (60 °C, 79%

RH). These interactions are expected to be both covalent, through a Maillard-type reaction, and non-covalent, local hydrophobic and electrostatic in nature. Protein solubility was increased by nearly 20% at lower ratios of SBP, 3:1 and 2:1 as a result of the conjugation reaction, whereas the total powder solubility showed little effect by SBP at less than 25% (3:1). Similarly, reacting with SBP also mitigated changes in the chemical composition of WPI, including free sulfhydryl content and available primary and secondary amine level. The UV-VIS spectroscopy revealed the presence of interacting complexes between WPI and SBP involving the aromatic side-chains of WPI and the feruloyl groups of SBP. These findings established a fundamental understanding on changes in the chemical composition and molecular electronic transition state in WPI caused by interacting and reacting with SBP. Significant improvement in colloidal stability and oil-in-water (O/W) emulsions was observed in the conjugates, especially at the 3:1 ratio, compared to the controls as well as other ratios used in this work. Further research using various spectroscopic techniques has been conducted to shed light on the roles of WPI and SBP in these observed improvements in solubility and emulsion stability. Results have been published (Qi, *et al.*, 2017) separately .

Acknowledgements

The authors would like to acknowledge Dr. Madhav P. Yadav and Ms. Stefanie Simon of USDA-ARS-ERRC for their technical assistance in the emulsion preparation and the subsequent particle size measurements.

Figure Captions

Figure 1. A) Total powder solubility (%); and B) protein solubility (%) of dry-heated WPI and SBP mixtures in Milli-Q water and room temperature (20 °C) as determined by weight difference and Bradford assay methods respectively.

Figure 2. A) Free sulfhydryl content (SH) (μM per mg protein); and B) the total concentration of primary and secondary amine (NH and NH_2) (mM per mg protein) of dry-heated WPI in the absence and presence of SBP at varying weight ratios as determined by the DTNB and ninhydrin methods respectively. All data were collected in triplicate with standard deviations plotted.

Figure 3. SDS-PAGE analyses of dry-heated WPI and SBP mixtures. Gels were stained with coomassai blue (SimplyBlue® SafeStain). The gels were run at A) non-reducing, and B) reducing (with 5% BME) conditions respectively. Samples were loaded in the lanes as the following: 1) WPI; 2) WPI-dh; 3) WPI:SBP = 1:1; 4) WPI:SBP = 2:1; and 5) WPI:SBP = 3:1. The total amount of protein loaded in each lane was estimated to be $\sim 25 \mu\text{g}$.

Figure 4. SDS-PAGE analyses of dry-heated WPI and SBP mixtures. Gels were stained for glycoproteins using the Glycoprotein Staining Kit (Thermo Fisher Scientific, Inc.). The gels were run at A) non-reducing, and B) reducing (with 5% BME) conditions respectively. Samples were loaded in the lanes as the following: 1) WPI; 2) SBP; 3) WPI-dh; 4) SBP-dh; 5) WPI:SBP =

3:1; 6) WPI:SBP = 2:1; and 7) WPI:SBP = 1:1. Both “+” (horseradish peroxidase) and “-” (soybean trypsin inhibitor) controls were also included.

Figure 5. A) UV-VIS spectra of dry-heated WPI and SBP mixtures in 25 mM phosphate buffer, pH 6.75. The concentration of WPI was kept at 0.40 mg/mL in all solutions, and the concentrations of SBP were calculated accordingly based on Table 1. The concentration of SBP and SBP-dh was 0.45 mg/mL. B) Calculated UV-VIS spectra of WPI (at 0.40 mg/mL) by subtracting the appropriate level of SBP from each mixture. The experimental spectra of WPI and WPI-dh were also plotted as a comparison. C) Calculated UV-VIS spectra of SBP (fixed at 0.45 mg/mL) by subtracting the varying amount of WPI from each mixture according to Table 1. The experimental spectra of SBP and SBP-dh were compared (in solid and dotted grey lines).

Figure 6. ζ -Potentials (mV) of WPI, SBP and the Maillard reacted products (2.0 mg/mL in 33 mM phosphate buffer) at 20 °C. Error bar represents standard deviation (*SDV*) of a set of four repeated measurements.

Figure 7. Comparison of the volume-weighted average droplet diameter of emulsions composed of 4% orange oil and 1% emulsifier (w/w) in 33 mM sodium phosphate and 0.1% sodium benzoate buffer, ~pH 6.3. The emulsifiers used are the following: WPI, WPI-dh, SBP, and the conjugates at 3:1, 2:1 and 1:1. The measurements were taken immediately after making emulsions (Day 0), after storage at 60 °C for 3 and 7 days. Data are an average of three trials $\pm$ standard deviation.

REFERENCES

- Akhtar, M., & Dickinson, E. (2003). Emulsifying properties of whey protein-dextran conjugates at low pH and different salt concentrations. *Colloids and Surfaces B: Biointerfaces*, 31(1-4), 125-132.
- Akhtar, M., & Dickinson, E. (2007). Whey protein–maltodextrin conjugates as emulsifying agents: An alternative to gum arabic. *Food Hydrocolloids*, 21(4), 607-616.
- Alizadeh-Pasdar, N., Nakai, S., & Li-Chan, E. C. (2002). Principal component similarity analysis of Raman spectra to study the effects of pH, heating, and κ -carrageenan on whey protein structure. *Journal of Agricultural and Food Chemistry*, 50(21), 6042-6052.
- Alkorta, I., Llama, M. J., & Serra, J. L. (1994). Interference by pectin in protein determination. *Food Science and Technology-Lebensmittel-Wissenschaft & Technologie*, 27(1), 39-41.
- Ames, J. M. (1998). Applications of the Maillard reaction in the food industry. *Food Chemistry*, 62(4), 431-439.
- Benichou, A., Aserin, A., Lutz, R., & Garti, N. (2007). Formation and characterization of amphiphilic conjugates of whey protein isolate (WPI)/xanthan to improve surface activity. *Food Hydrocolloids*, 21(3), 379-391.
- Cayot, P., & Lorient, D. (1997). Structure-Function Relationships of Whey Proteins. In: Damodaran, S. & Paraf, A. (Eds.), *Food Proteins and Their Applications, Volume 80 of Food Science and Technology* (Vol. 80, pp. 225-256). New York, NY: Marcel Dekker, Inc.
- Chevalier, F., Chobert, J.-M., & Dalgalarondo, M. (2002). Maillard glycation of β -lactoglobulin induces conformation changes. *Food / Nahrung*, 46(2), 58-63.

- de Kruif, C. G., Weinbreck, F., & de Vries, R. (2004). Complex coacervation of proteins and anionic polysaccharides. *Current Opinion in Colloid & Interface Science*, 9(5), 340-349.
- de Oliveira, F. C., dos Reis Coimbra, J. S., de Oliveira, E. B., Zuñiga, A. D. G., & Garcia Rojas, E. E. (2016). Food protein-polysaccharide conjugates obtained via the Maillard reaction: A review. *Critical Reviews in Food Science and Nutrition*, 56(7), 1108-1125.
- de Wit, J. N. (1998). Marschall Rhone-Poulenc Award Lecture. Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science*, 81(3), 597-608.
- de Wit, J. N., & Kessel, T. (1996). Effects of ionic strength on the solubility of whey protein products. A colloid chemical approach. *Food Hydrocolloids*, 10(2), 143-149.
- Demetriades, K., Coupland, J. N., & McClements, D. J. (1997). Physical properties of whey protein stabilized emulsions as related to pH and NaCl. *Journal of Food Science*, 62(2), 342-347.
- Dickinson, E. (2008). Interfacial structure and stability of food emulsions as affected by protein-polysaccharide interactions. *Soft Matter*, 4(5), 932-942.
- Dickinson, E. (2009). Hydrocolloids as emulsifiers and emulsion stabilizers. *Food Hydrocolloids*, 23(6), 1473-1482.
- Dickinson, E. (2015). Colloids in Food: Ingredients, Structure, and Stability. *Annual Review of Food Science and Technology*, 6(1), 211-233.
- Einhorn-Stoll, U., Ulbrich, M., Sever, S., & Kunzek, H. (2005). Formation of milk protein-pectin conjugates with improved emulsifying properties by controlled dry heating. *Food Hydrocolloids*, 19(2), 329-340.

- Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, 82(1), 70-77.
- Evans, M., Ratcliffe, I., & Williams, P. A. (2013). Emulsion stabilisation using polysaccharide-protein complexes. *Current Opinion in Colloid & Interface Science*, 18, 272-282.
- Fachin, L., & Viotto, W. H. (2005). Effect of pH and heat treatment of cheese whey on solubility and emulsifying properties of whey protein concentrate produced by ultrafiltration. *International Dairy Journal*, 15(4), 325-332.
- Ferreira, P., Diez, N., Faulds, C. B., Soliveri, J., & Copa-Patino, J. L. (2007). Release of ferulic acid and feruloylated oligosaccharides from sugar beet pulp by *Streptomyces tendae*. *Bioresource Technology*, 98(8), 1522-1528.
- Fishman, M. L., Chau, H. K., Qi, P. X., Hotchkiss, A. T., Jr., & Yadav, M. P. (2013). Physico-chemical characterization of protein-associated polysaccharides extracted from sugar beet pulp. *Carbohydrate Polymers*, 92(2), 2257-2266.
- Foegeding, E. A., Davis, J. P., Doucet, D., & McGuffey, M. K. (2002). Advances in modifying and understanding whey protein functionality. *Trends in Food Science & Technology*, 13(5), 151-159.
- Funami, T., Zhang, G., Hiroe, M., Noda, S., Nakauma, M., Asai, I., Cowman, M. K., Al-Assaf, S., & Phillips, G. O. (2007). Effects of the proteinaceous moiety on the emulsifying properties of sugar beet pectin. *Food Hydrocolloids*, 21(8), 1319-1329.
- Graf, E. (1992). Antioxidant potential of ferulic acid. *Free Radical Biology and Medicine*, 13(4), 435-448.

- Grimsley, G. R., & Pace, C. N. (2004). Spectrophotometric determination of protein concentration. *Current Protocols in Protein Science*, 3.1.1-3.1.9.
- Guillon, F., & Thibault, J.-F. (1990). Oxidative cross-linking of chemically and enzymatically modified sugar-beet pectin. *Carbohydrate Polymers*, 12(4), 353-374.
- Hunt, J. A., & Dalgleish, D. G. (1994). Effect of pH on the stability and surface composition of emulsions made with whey protein isolate. *Journal of Agricultural and Food Chemistry*, 42(10), 2131-2135.
- Jiménez-Castaño, L., Villamiel, M., & López-Fandiño, R. (2007). Glycosylation of individual whey proteins by Maillard reaction using dextran of different molecular mass. *Food Hydrocolloids*, 21(3), 433-443.
- Jones, O. G., Handschin, S., Adamcik, J., Harnau, L., Bolisetty, S., & Mezzenga, R. (2011). Complexation of β -lactoglobulin fibrils and sulfated polysaccharides. *Biomacromolecules*, 12(8), 3056-3065.
- Kazmierski, M., & Corredig, M. (2003). Characterization of soluble aggregates from whey protein isolate. *Food Hydrocolloids*, 17(5), 685-692.
- Kika, K., Korlos, F., & Kiosseoglou, V. (2007). Improvement, by dry-heating, of the emulsion-stabilizing properties of a whey protein concentrate obtained through carboxymethylcellulose complexation. *Food Chemistry*, 104(3), 1153-1159.
- Kikuzaki, H., Hisamoto, M., Hirose, K., Akiyama, K., & Taniguchi, H. (2002). Antioxidant properties of ferulic acid and its related compounds. *Journal of Agricultural and Food Chemistry*, 50(7), 2161-2168.

- Klemaszewski, J. L., & Kinsella, J. E. (1991). Sulfitolysis of whey proteins: effects on emulsion properties. *Journal of Agricultural and Food Chemistry*, 39(6), 1033-1036.
- Koupantsis, T., & Kiosseoglou, V. (2009). Whey protein–carboxymethylcellulose interaction in solution and in oil-in-water emulsion systems. Effect on emulsion stability. *Food Hydrocolloids*, 23(4), 1156-1163.
- Ledl, F., & Schleicher, E. (1990). New aspects of the Maillard reaction in foods and in the human body. *Angewandte Chemie International Edition in English*, 29(6), 565-594.
- Leroux, J., Langendorff, V., Schick, G., Vaishnav, V., & Mazoyer, J. (2003). Emulsion stabilizing properties of pectin. *Food Hydrocolloids*, 17(4), 455-462.
- Mahmoudi, N., Mehalebi, S., Nicolai, T., Durand, D., & Riaublanc, A. (2007). Light-scattering study of the structure of aggregates and gels formed by heat-denatured whey protein isolate and β -lactoglobulin at neutral pH. *Journal of Agricultural and Food Chemistry*, 55(8), 3104-3111.
- Martinez-Alvarenga, M. S., Martinez-Rodriguez, E. Y., Garcia-Amezquita, L. E., Olivas, G. I., Zamudio-Flores, P. B., Acosta-Muniz, C. H., & Sepulveda, D. R. (2014). Effect of Maillard reaction conditions on the degree of glycation and functional properties of whey protein isolate–maltodextrin conjugates. *Food Hydrocolloids*, 38, 110-118.
- Maxwell, E. G., Belshaw, N. J., & Waldron, K. W. (2012). Pectin – An emerging new bioactive food polysaccharide. *Trends in Food Science & Technology*, 24, 64-73.
- McClements, D. J., Monahan, F. J., & Kinsella, J. E. (1993). Disulfide bond formation affects stability of whey-protein isolate emulsions. *Journal of Food Science*, 58(5), 1036-1039.

- Micard, V., Renard, C. M. G. C., & Thibault, J. F. (1994). Studies on enzymic release of ferulic acid from sugar-beet pulp. *LWT - Food Science and Technology*, 27(1), 59-66.
- Mishra, S., Mann, B., & Joshi, V. K. (2001). Functional improvement of whey protein concentrate on interaction with pectin. *Food Hydrocolloids*, 15(1), 9-15.
- Monahan, F. J., McClements, D. J., & Kinsella, J. E. (1993). Polymerization of whey proteins in whey protein-stabilized emulsions. *Journal of Agricultural and Food Chemistry*, 41(11), 1826-1829.
- Moon, B. K., & Mangino, M. E. (2004). The effect of preheating on solubility and emulsion stability of whey protein concentrates. *Milchwissenschaft-Milk Science International*, 59(3-4), 165-169.
- Morris, G. A., Sims, I. M., Robertson, A. J., & Furneaux, R. H. (2004). Investigation into the physical and chemical properties of sodium caseinate-maltodextrin glyco-conjugates. *Food Hydrocolloids*, 18(6), 1007-1014.
- Neiryneck, N., Van der Meeren, P., Bayarri Gorbe, S., Dierckx, S., & Dewettinck, K. (2004). Improved emulsion stabilizing properties of whey protein isolate by conjugation with pectins. *Food Hydrocolloids*, 18(6), 949-957.
- Oosterveld, A., Beldman, G., Schols, H. A., & Voragen, A. G. (2000). Characterization of arabinose and ferulic acid rich pectic polysaccharides and hemicelluloses from sugar beet pulp. *Carbohydrate Research*, 328(2), 185-197.
- Oosterveld, A., Beldman, G., Schols, H. A., & Voragen, A. G. J. (1996). Arabinose and ferulic acid rich pectic polysaccharides extracted from sugar beet pulp. *Carbohydrate Research*, 288(0), 143-153.

- Pelegrine, D. H. G., & Gasparetto, C. A. (2005). Whey proteins solubility as function of temperature and pH. *LWT - Food Science and Technology*, 38(1), 77-80.
- Qi, P. X., Chau, H. K., Fishman, M. L., Wickham, E. D., & Hotchkiss, A. T., Jr. (2014). Investigation of molecular interactions between β -lactoglobulin and sugar beet pectin by multi-detection HPSEC. *Carbohydrate Polymers*, 107(17), 198-208.
- Qi, P. X., Wickham, E. D., & Garcia, R. A. (2014). Structural and thermal stability of β -lactoglobulin as a result of interacting with sugar beet pectin. *Journal of Agricultural and Food Chemistry*, 62(30), 7567-7576.
- Qi, P. X., Xiao, Y., & Wickham, E. D. (2017). Stabilization of whey protein isolate (WPI) through interactions with sugar beet pectin (SBP) induced by controlled dry-heating. *Food Hydrocolloids*, 67, 1-13.
- Riddles, P. W., Blakeley, R. L., & Zerner, B. (1983). [8] Reassessment of Ellman's Reagent. *Methods in Enzymology*, 91(Part 1), 49-60.
- Rombouts, F. M., & Thibault, J.-F. (1986). Feruloylated pectic substances from sugar-beet pulp. *Carbohydrate Research*, 154(1), 177-187.
- Ryan, K. N., & Foegeding, E. A. (2015). Formation of soluble whey protein aggregates and their stability in beverages. *Food Hydrocolloids*, 43, 265-274.
- Schmidt, U. S., Pietsch, V. L., Rentschler, C., Kurz, T., Endress, H. U., & Schuchmann, H. P. (2016). Influence of the degree of esterification on the emulsifying performance of conjugates formed between whey protein isolate and citrus pectin. *Food Hydrocolloids*, 56, 1-8.

- Schmitt, C., & Turgeon, S. L. (2011). Protein/polysaccharide complexes and coacervates in food systems. *Advances in Colloid and Interface Science*, 167(1-2), 63-70.
- Shekaripour, F., Aminlari, M., Niakosari, M., & Eskandary, M. H. (2014). Effect of Maillard-based conjugation with dextran on the functional properties of whey protein. *Innovation in Food Science and Technology*, 5(4), 1-8.
- Siew, C. K., & Williams, P. A. (2008). Role of protein and ferulic acid in the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry*, 56(11), 4164-4171.
- Smithers, G. W. (2015). Whey-ing up the options—Yesterday, today and tomorrow. *International Dairy Journal*, 48, 2-14.
- Spotti, M. J., Martinez, M. J., Pilosof, A. M. R., Candioti, M. C., Rubiolo, A. C., & Carrara, C. R. (2014). Influence of Maillard conjugation on structural characteristics and rheological properties of whey protein/dextran systems. *Food Hydrocolloids*, 39, 223-230.
- Stadler, R. H., Blank, I., Varga, N., Robert, F., Hau, J., Guy, P. A., Robert, M.-C., & Riediker, S. (2002). Food chemistry: Acrylamide from Maillard reaction products. *Nature*, 419(6906), 449-450.
- Starcher, B. (2001). A ninhydrin-based assay to quantitate the total protein content of tissue samples. *Analytical Biochemistry*, 292(1), 125-129.
- Suarez, G., Rajaram, R., Oronsky, A. L., & Gawinowicz, M. A. (1989). Nonenzymatic glycation of bovine serum albumin by fructose (fructation). Comparison with the Maillard reaction initiated by glucose. *Journal of Biological Chemistry*, 264(24), 3674-3679.

- Sun, R. C., & Hughes, S. (1999). Fractional isolation and physico-chemical characterization of alkali-soluble polysaccharides from sugar beet pulp. *Carbohydrate Polymers*, 38(3), 273-281.
- Townend, R., Winterbottom, R. J., & Timasheff, S. N. (1960). Molecular interactions in β -lactoglobulin. II. Ultracentrifugal and electrophoretic studies of the association of β -lactoglobulin below its isoelectric point. *Journal of the American Chemical Society*, 82(12), 3161-3168.
- Turgeon, S. L., Beaulieu, M., Schmitt, C., & Sanchez, C. (2003). Protein-polysaccharide interactions: phase-ordering kinetics, thermodynamic and structural aspects. *Current Opinion in Colloid & Interface Science*, 8(4-5), 401-414.
- Turgeon, S. L., Schmitt, C., & Sanchez, C. (2007). Protein-polysaccharide complexes and coacervates. *Current Opinion in Colloid & Interface Science*, 12(4-5), 166-178.
- Wang, Q., & Ismail, B. (2012). Effect of Maillard-induced glycosylation on the nutritional quality, solubility, thermal stability and molecular configuration of whey protein. *International Dairy Journal*, 25(2), 112-122.
- Wicker, L., Kim, Y., Kim, M.-J., Thirkield, B., Lin, Z., & Jung, J. (2014). Pectin as a bioactive polysaccharide – Extracting tailored function from less. *Food Hydrocolloids*, 42(Part 2), 251-259.
- Wijayanti, H. B., Bansal, N., & Deeth, H. C. (2014). Stability of whey proteins during thermal processing: A review. *Comprehensive Reviews in Food Science and Food Safety*, 13(6), 1235-1251.

- Williams, P. A., Sayers, C., Viebke, C., Senan, C., Mazoyer, J., & Boulenguer, P. (2005). Elucidation of the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry*, 53(9), 3592-3597.
- Xu, D., Wang, X., Jiang, J., Yuan, F., & Gao, Y. (2012). Impact of whey protein–beet pectin conjugation on the physicochemical stability of β -carotene emulsions. *Food Hydrocolloids*, 28(2), 258-266.
- Yadav, M. P., Parris, N., Johnston, D. B., Onwulata, C. I., & Hicks, K. B. (2010). Corn fiber gum and milk protein conjugates with improved emulsion stability. *Carbohydrate Polymers*, 81(2), 476-483.
- Yoo, S. H., Fishman, M. L., Savary, B. J., & Hotchkiss, A. T., Jr. (2003). Monovalent salt-induced gelation of enzymatically deesterified pectin. *Journal of Agricultural and Food Chemistry*, 51(25), 7410-7417.
- Zhu, D., Damodaran, S., & Lucey, J. A. (2008). Formation of whey protein isolate (WPI) - Dextran conjugates in aqueous solutions. *Journal of Agricultural and Food Chemistry*, 56(16), 7113-7118.

Table 1. Average concentrations (mg/mL) of WPI and SBP in the stock solutions (at 5.0 mg/mL in 25 mM PBS buffer, pH 6.75) as described in section 2.8 and estimated spectrophotometrically using Eqs. 3 and 4. Results were obtained from triplicate measurements*.

	WPI	SBP
WPI	4.06 ± 0.06^a	-
WPI-dh	3.53 ± 0.08^b	-
SBP	-	4.91 ± 0.09^a
SBP-dh	-	4.68 ± 0.08^b
WPI:SBP=3:1	3.63 ± 0.05^b	1.23 ± 0.03^c
WPI:SBP=2:1	3.24 ± 0.07^c	1.55 ± 0.05^d
WPI:SBP=1:1	2.24 ± 0.10^d	2.63 ± 0.03^e

* Data not sharing the same superscript letter were significantly different ($P < 0.05$) within the same category (WPI and SBP).

Figure 1.

Figure 2.

Figure 3A.

Figure 3B.

Figure 4A.

Figure 4B.

Figure 5.

Figure 6.

Figure 7.