



Investigation of molecular interactions between β -lactoglobulin and sugar beet pectin by multi-detection HPSEC[☆]



Phoebe X. Qi^{*}, Hoa K. Chau, Marshall L. Fishman,
Edward D. Wickham, Arland T. Hotchkiss

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 E. Mermaid Lane, Wyndmoor, PA 19038, USA

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ABSTRACT

Molecular interactions between β -lactoglobulin (β -LG) and sugar beet pectin (SBP) were studied using online multi-detection high performance size exclusion chromatography (HPSEC) at neutral pH and 50 mM ionic strength. The hydrodynamic properties of various interacting polymer fractions were characterized in detail and compared with those of β -LG and SBP. Results showed that $\sim 6.5\%$ (w/w) of native dimeric β -LG molecules formed complexes with over 35% SBP molecules of varying sizes, 800, 110 and 75 kDa. Although the β -LG molecules bind to SBP molecules of all sizes and shapes, they tend to favor the intermediate (110 kDa) and small sized (75 kDa) SBP molecules. All resulting complexes possess altered shapes and hydrodynamic properties when compared to unbound SBP and β -LG. About half of the interacting β -LG ($\sim 3.5\%$) molecules were thought to bind to a small amount of non-covalently bound feruloyl groups, possibly present in SBP. When pre-heat treated β -LG and SBP were combined, more than 16% of β -LG formed complexes with at least 45% of SBP molecules of varying sizes, $M_w \sim 750$ –800, 110, and 55–80 kDa. The complexes formed between β -LG aggregates and/or oligomers and the large SBP molecules (750–800 kDa) adopt the shape of β -LG aggregates, random coil. Both groups of complexes formed between β -LG intermediate (110 kDa) and small sized (55–80 kDa) SBP take on the shape of rigid rod. It was speculated that half of the interacting heat-treated β -LG molecules ($\sim 8\%$) are complexed with non-covalently bound feruloyl groups in SBP.

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1. Introduction

There has been growing interest in complexes formed between proteins and polysaccharides in recent years, particularly by the food, cosmetics and pharmaceutical industries as these complexes found many uses in stabilizing dairy beverages (Ron, Zimet, Bargarum, & Livney, 2010), in the formulation of emulsion systems (Dickinson, 2008; Evans, Ratcliffe, & Williams, 2013), in the encapsulation of ingredients (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998; Turgeon & Laneuville, 2009), and in delivering protein drugs (George & Abraham, 2006). In addition to numerous studies using crosslinking agents (Chen et al., 2003; Jung & Wicker, 2012a, 2012b; Mariniello et al., 2014) and through Maillard reaction (Evans

et al., 2013; Oliver, Melton, & Stanley, 2006) to create conjugates between proteins and polysaccharides via covalent bonding formation, a great deal of attention (de Kruif & Tuinier, 2001; Dickinson, 2008; Jones & McClements, 2011) also has been paid to complexes formed through various non-covalent interactions including electrostatic, hydrogen bonding, hydrophobic, and steric interactions. These interactions depend greatly on the type, molecular weight, charge density and concentration of the protein and polysaccharide used, and are particularly sensitive to the ionic strength, pH, and temperature of the solution conditions (Cooper, Dubin, Kayitmazer, & Turksen, 2005; de Kruif & Tuinier, 2001).

Among many protein and polysaccharide complexes investigated to date, the interaction between β -lactoglobulin (β -LG), a major whey protein in cow's milk, and pectin, a cell-wall anionic polysaccharide, has been found particularly interesting as a model system (Gancz, Alexander, & Corredig, 2006; Girard, Sanchez, Laneuville, Turgeon, & Gauthier, 2004; Jung & Wicker, 2014; Sperber, Cohen Stuart, Schols, Voragen, & Norde, 2010) to study the formation of complexes between oppositely charged biopolymers. β -LG was chosen because of its easy accessibility, intrinsically interesting structural features, and broad-ranging applications in

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^{*} Corresponding author. Tel.: +1 215 233 6438; fax: +1 215 233 6597.

E-mail address: Phoebe.Qi@ars.usda.gov (P.X. Qi).

the food and nutraceutical industries (Cho, Batt, & Sawyer, 1994; Liang & Subirade, 2010; Ron et al., 2010; Teng, Li, Luo, Zhang, & Wang, 2013).

At room temperature, β -LG is monomeric at acidic (below 3) or alkaline pH (above 8), predominately dimeric at physiological pH and in equilibrium between both states at pH 4–8 (Taulier & Chalikian, 2001). After nearly 80 years of intense research (Sawyer, 2003), the details of the biological function of β -LG remain poorly defined. It is known to bind to a host of hydrophobic ligands, including palmitate (Wu, Perez, Puyol, & Sawyer, 1999), fatty acids and retinol (Cho et al., 1994), which suggests its possible role as a lipid transport protein. Most of these ligands are believed to bind deeply into the central calyx of the protein, and lead to the rearrangement of the “gate area” formed by the two lysyl residues, Lys60 and Lys69. Thus they provide access to ligand binding and release (Qin, Creamer, Baker, & Jameson, 1998). The interaction of phenolic compounds including ferulic acid with β -LG, however, is less well known (Riihimäki et al., 2008), and warrants further investigation.

In addition to the predominately “smooth” homogalacturonan (HG) regions in pectin, α -(1 $\rightarrow$ 4)-linked D-galacturonate and their methyl esters (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995), also common are the “hairy” regions, which are composed of rhamnogalacturonan (RG) units. Pectin from sugar beets often differs from other sources of pectin in that it tends to have a higher degree of acetylation and a higher amount of neutral sugar side chains (rich in hairy regions), and also contains feruloyl groups in these regions (Rombouts & Thibault, 1986). The functional properties of these pectins are determined by the degree of methyl esterification (DM) of the carboxyl group on C-6 of galacturonates and the distribution of these methyl esters, i.e. degree of blockness (DB). Moreover, unlike pectin from citrus peels or apple pomace, sugar beet pectin is less favored as a thickener because of its poor gelling properties, which has been attributed to the presence of acetyl groups and relatively low molar mass (Pippen, McCready, & Owens, 1950). Instead, sugar beet pectin often is used as an emulsifier because of its higher content of the proteinaceous materials believed to be bound to the side chains through covalent linkages (Funami et al., 2007; Williams et al., 2005).

Although successful attempts have been made in studying and preparing biopolymers through complex formation between proteins and polysaccharides for various applications (Jones & McClements, 2008, 2011), most published work so far has largely focused on complexes formed at acidic pH conditions. The binding stoichiometry at pH 4 when coacervates were formed between β -LG and high methoxyl pectin was studied by Girard et al. using isothermal titration calorimetry (ITC) (Girard, Turgeon, & Gauthier, 2003) and small angle static light scattering (Girard et al., 2004). The interaction between β -LG and pectin at neutral pH, on the other hand, is less well known. In this work, molecular interaction between β -lactoglobulin (β -LG) and sugar beet pectin (SBP), commercially available high methoxyl pectin, is studied at near neutral pH and medium ionic strength (50 mM). At the experimental conditions studied, both β -LG and SBP carry an overall negative charge (de Kruijff, Weinbreck, & de Vries, 2004). When combined, the lowering in binding free energy is largely driven more by entropy released upon counter-ion binding (de Kruijff et al., 2004) although enthalpy is expected to contribute significantly as well. This apparent net charge asymmetry in the participating pair would preclude strong interaction between them. However, as demonstrated in this work, the patches of positive charges localized on the surface of the proteins are attracted to the negative charges on pectin. Apparently these localized attractive forces are more effective and stronger than the incompatible ones (Becker, Henzler, Welsch, & Ballauff, 2012). In addition, other weaker forms of interactions including localized hydrophobic interaction and hydrogen binding may also play a role in stabilizing the interacting complexes.

Importantly, there is still a lack of fundamental understanding of the factors that control the size and physicochemical properties of biopolymer particles formed by protein–polysaccharide complexes. Therefore, the purpose of this study was to investigate the molecular and hydrodynamic details involved in the interacting system between β -lactoglobulin (β -LG) and sugar beet pectin (SBP) at physiological relevant conditions, i.e. neutral pH and 50 mM ionic strength, to obtain an accurate and quantitative characterization of the complexes formed. High performance size exclusion chromatography (HPSEC) was used in combination with a serial online multi-detection system capable of measuring UV-absorption, multi-angle light scattering (MALLS), refraction index, and intrinsic viscosity in one run.

2. Materials and methods

2.1. Materials

Purified β -lactoglobulin (β -LG) powder (Lot# JE001-0-415) was kindly donated by Davisco Foods International (Le Sueur, MN). The reported composition (expressed as dry weight percent unless otherwise indicated) of the powder was: >95% protein (of which 90% was β -LG); <3.5% ash; <1.0% fat; <1.0% lactose; <6.5% moisture (wet weight). HPLC analysis using previously published methods developed by the authors' laboratory revealed that the sample contains a total of 93.1% β -LG, distributed evenly between genetic variants A and B, 51.2% and 48.8% respectively.

Sugar beet pectin (SBP, Lot# 30091) was donated by CP Kelco (San Diego, CA). The composition of the pectin was 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 was $37.3 \pm 1.1\%$ using previously published methods (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956; Yoo, Fishman, Savary, & Hotchkiss, 2003). Ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid) content in the pectin was estimated to be 0.0043% (4.3 ppm) by measuring the UV absorbance (Varian Spectrophotometer Bio100 UV-visible, Agilent, Santa Clara, CA). A molar extinction coefficient of $18.6 \text{ mM}^{-1} \text{ cm}^{-1}$ at 322 nm (in ethanol) (Sigma–Aldrich, St. Louis, MO) for ferulic acid was used to obtain the estimation.

Sodium azide (NaN_3) was purchased from Sigma Chemical Co. (St. Louis, MO) and used as a preservative (at 0.01%). Hydrochloric acid solutions were prepared from a 12.1 N hydrochloric acid solution (Fisher Scientific, Fairlawn, NJ). All other chemicals including ferulic acid (trans isomer), sodium nitrate (NaNO_3), and sodium hydroxide (NaOH) (Sigma–Aldrich, St. Louis, MO) were reagent grade and used directly. All solutions were made with Millipore water (double-distilled/de-ionized) and filtered with a 0.45- μm syringe filter (Millex-HV, PVDF, Millipore Corp., Billerica, MA).

2.2. Amino acid and protein composition analysis of SBP

Sugar beet pectin (SBP) used in this work was subjected to amino acid composition analysis. Powdered sample ($\sim 100 \text{ mg}$) was hydrolyzed in 6 N HCl containing a small amount phenol. The hydrolysis flasks were extensively purged of oxygen using a PicoTag workstation (Waters Corp., Milford, MA), and then incubated at 110°C for 20 h. Hydrolyzed samples were filtered, dried under vacuum, and derivatized with AccQFluor reagent (Waters) following the manufacturer's directions. Chromatography was performed using procedures described as ‘system 1’ in Cohen and De Antonis (1994), with α -aminobutyric acid as an internal standard. Separation was achieved using an AccQTag C18 reverse phase column (Waters), and a fluorescence detector was used with 250 nm as the excitation and 395 nm as the emission wavelength.

Hydrolysis, derivitization and analysis of each sample were performed in triplicate. The total protein content of the SBP was determined to be $4.78 \pm 0.08\%$.

2.3. Preparation of stock solutions

Dried protein (β -LG) or pectin (SBP) sample was dissolved at 10.0 mg/mL or 4.0 mg/mL respectively in a solvent system (pH 6.50) containing 50 mM NaNO_3 and 0.01% NaN_3 . β -LG was easily soluble at the specified concentration, and the pH was 6.60. Dissolving SBP, on the other hand, required an overnight stirring and followed by centrifugation at $27,000 \times g$ and 20°C in a Sorval RC-5B centrifuge (DuPont Co., Wilmington, DE) for 20 min. Only the SBP supernatant was removed and the pH was measured to be 5.60. Careful adjustment of this SBP solution was made using 1.0 N NaOH ($\sim 10 \mu\text{L}$ was used) to obtain pH ~ 6.50 . All β -LG and SBP solutions were filtered through a $0.45\text{-}\mu\text{m}$ syringe-driven filter (Millex HV, PVDF, Millipore Corp., Billerica, MA). Appropriate dilution using the same solvent (50 mM NaNO_3 and 0.01% NaN_3) was made to obtain either individual β -LG or SBP or the mixture at the concentrations of 3.0 mg/mL (β -LG) and 1.0 mg/mL (SBP) respectively, either untreated or pre-heated as described below.

Pre-heating treatment involved holding each solution of β -LG and SBP at concentration 10.0 mg/mL and 4.0 mg/mL respectively at 80°C for 10 min in a water bath. Heated solution was then allowed to cool to room temperature prior to subsequent analysis or mixing. The mixture of β -LG and SBP was prepared by either mixing the two untreated solutions to obtain the final solution containing either untreated and/or heat treated individual solutions at 3.0 mg/mL β -LG and 1.0 mg/mL SBP for most experiments unless otherwise noted. All solutions were made fresh prior to each use.

2.4. Electrophoretic mobility measurements

Electrophoretic mobility (20°C) of β -LG (3.0 mg/mL) or/and SBP (1.0 mg/mL) as an individual solution or mixture was determined by the Zetasizer Nano Z system (Malvern Instruments, Worcestershire, U.K.) using the same solvent as used in the HPSEC experiments, i.e. 50 mM NaNO_3 and 0.01% NaN_3 , pH 6.50. 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 by the software which was provided by Malvern Instruments.

2.5. High performance size exclusion chromatography (HPSEC)

The biopolymer solutions containing 3.0 mg/mL β -LG and/or 1.0 mg/mL SBP, individually or mixed and/or pre-heated were characterized by HPSEC (1200 Series, Agilent Technologies, Santa Clara, CA). The solvent delivery system consisted of a vacuum degasser, auto sampler and a pump. The mobile phase was 0.05 M NaNO_3 and 0.01% NaN_3 (pH 6.65). The injection volume was 200 μL , and flow rate was held at 0.7 mL/min. Samples were run in triplicate. Two guard columns (TSK-GEL[®] PW_{XL} 6.0 mm ID $\times$ 4.0 cm L, 12 μm , Tosoh Bioscience, Tokyo, Japan) were used. One was placed before the separation columns, which consisted of a set of three model TSKgel GMPW_{XL} size exclusion columns (7.8 mm $\times$ 300 mm, particle size 13 μm , Tosoh Bioscience, Tokyo, Japan), and the other before the detectors. The column set was heated in a water bath at 35°C , and connected in series to a UV-1260 Infinity spectrophotometer (Agilent Technologies, Santa Clara, CA), HELEOS II multi-angle laser light scattering photometer (MALLS) (Wyatt

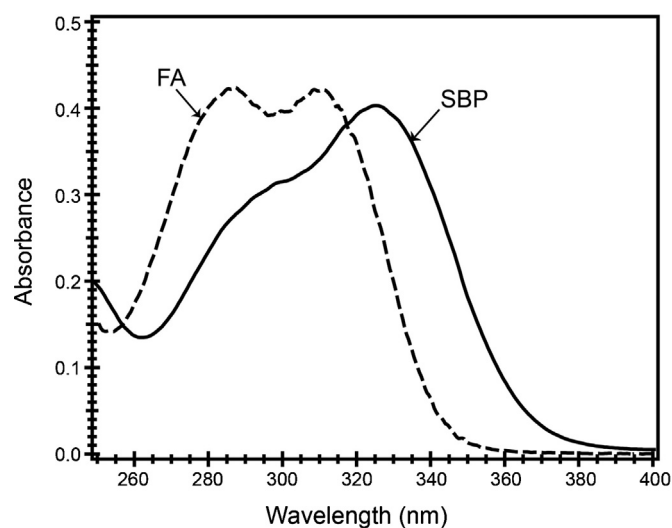


Fig. 1. UV–vis absorption spectra for ferulic acid (FA) and SBP in a solvent containing 0.05 M NaNO_3 and 0.01% NaN_3 at 20°C . The concentration was 0.005 mg/mL and 0.5 mg/mL for FA and SBP respectively.

Technology, Santa Barbara, CA), Model 255-V2 differential pressure viscometer (DPV) (Wyatt) and an RI detector (Wyatt). Narrowly monodispersed pullulan P-50 (Shodex STANDARD P-82, JM Science, Grand Island, NY) was used to calibrate the scattering intensity at the 90° angle. Bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, MO) was used to align all detectors, UV/vis, MALLS, DPV and RI. The percentage of recovery was obtained from the ratio of the mass eluted as determined by integration of the refractometer signal to the mass injected. All signals from the four detectors were analyzed by the ASTRA software (V.6.1.1.17, Wyatt Technology).

All samples were analyzed at UV278 nm and UV325 nm. The refractive index increment (dn/dc) values of 0.180 and 0.132 (mL/g) were used for β -LG (van Dijk & Smit, 2000) and SBP (Fishman, Chau, Qi, Hotchkiss, & Yadav, 2013) respectively. The extinction coefficient for each wavelength was determined from the RI concentration of each individual sample.

3. Results and discussion

3.1. Composition of the sugar beet pectin

The UV–vis absorption spectrum of the SBP (see Fig. 1) in a solvent containing 0.05 M NaNO_3 and 0.01% NaN_3 (pH ~ 6.5) showed a broad absorption band with a maximum at ~ 325 nm and a shoulder of less intensity at ~ 290 nm, and is consistent with the spectrum previously reported for other beet pectin (Williams et al., 2005). Pure ferulic acid in the same solvent exhibited two distinctive sharp absorption peaks at 312 and 287 nm with nearly equal intensity (Fig. 1). This change in the overall absorption behavior of ferulic acid when present in SBP along with the obvious red shift (from 312 to 325 nm) of the main peak provided further evidence for its covalent bonding nature with the pectin, as demonstrated previously by other methods such as mass spectrometry (Levine et al., 2004; Oosterveld, Beldman, Schols, & Voragen, 2000; Ralet, Andre-Leroux, Quemener, & Thibault, 2005). In addition, the presence of ferulic acid of various amounts, from 0.5% to 2.5%, was also reported by Morris, Ralet, Bonnin, Thibault, and Harding (2010) for acid extracted sugar beet pectin fractions and for commercial SBP by Williams et al. (2005).

Similar to previously published results on other sugar beet pectin studies (Williams et al., 2005), the amino acid analysis revealed a considerable amount of Phe and Tyr residues but no

Table 1

ζ -Potential (mV) of β -LG (3.0 mg/mL) and SBP (1.0 mg/mL) solutions (0.05 M NaNO₃, and 0.01% NaN₃, pH 6.65) and 20 °C.

Sample	ζ (mV) ^a	SD ^b
β -LG	−7.33	1.21
SBP	−18.6	0.5
β -LG + SBP	−17.8	0.9
β -LG (heated)	−15.1	1.1
SBP (heated)	−17.5	1.1
β -LG (heated) + SBP (heated)	−18.1	0.4

^a Average values were obtained from a set of five different measurements.

^b Standard deviations (SD) were calculated from a set of five measurements.

detectable level of Trp in the SBP used in this work. However, it is possible that any trace amount of Trp was destroyed during the acid hydrolysis of the sample in the amino acid analysis procedure. These aromatic residues contributed significantly to the broadening of the absorption peak of the SBP, in the area of ~280 nm.

3.2. Electrophoretic mobility as an indicator for possible interactions between β -LG and SBP

The ζ -potential for each solution studied in this work was measured in the same solvent system (0.05 M NaNO₃ and 0.01% NaN₃, pH 6.65 and 20 °C) as was used in the HPSEC studies, and the results are shown in Table 1. As expected and indicated by its highest value that SBP possesses far greater colloidal stability than untreated β -LG at the pH used in this work. The addition of SBP (1.0 mg/mL) to β -LG (3.0 mg/mL) increased the colloidal stability of β -LG, from -7.33 ± 1.21 mV to -17.8 ± 0.9 mV, which is indicative of possible interaction between β -LG and SBP, as further discussed below. On the other hand, one could argue that the addition of β -LG had negligible effect on the colloidal stability of SBP (-18.6 ± 0.5 mV). This is likely due to the insignificant size of β -LG relative to SBP.

Although thermal treatment at 80 °C for 10 min caused little change in the ζ -potential of SBP, it more than doubled the value for β -LG. This increased ζ -potential suggests structural changes in β -LG molecules induced by thermal denaturation and aggregation. The mixture of pre-heated β -LG and pre-heated SBP yielded a colloidal system with nearly equal stability (-18.1 ± 0.4 mV) when compared with the untreated mixture (-17.8 ± 0.9 mV).

3.3. Interaction between β -LG and SBP

To characterize the hydrodynamic properties of the molecular interaction between β -LG and SBP, we carried out multi-detector HPSEC experiments on their mixtures, both untreated and heat-treated. The results of these experiments were compared with similar experiments on β -LG and SBP alone. Table 2 summarizes concentrations determined by UV278 nm, RI and UV325 nm. Also in the table are percentages (%) of mass recovered values (%Rec.). The standard deviations of triplicate measurements are given in parentheses. Weight average molar mass (M_w), polydispersity indexes (M_w/M_n and M_z/M_n), intrinsic viscosity (η_w), the Mark–Houwink–Sakurada (MHKS) exponent (a), z-average radius of gyration (R_{gz}), z-average hydrodynamic radius (R_{hz}) and the calculated molecular shape factor ρ (Burchard, 1996) are included as well. It should be noted that for the mixtures, both β -LG and SBP contribute to 278 nm because of the protein moieties present in SBP, while at 325 nm only the feruloyl groups in SBP are detectable.

Fig. 2 contains HPSEC chromatograms obtained by UV278 nm (A), UV325 nm (B), refractive index (RI) (C), 90° angle light scattering (LS) (D), and intrinsic viscosity (DP) (E) detector. A single component (with 96% recovery) was observed (at elution volume 26.0 mL) for the native β -LG with weight average molecular weight (M_w) ~ 32 kDa, indicating a monodispersed ($M_w/M_n = 1.0$)

and $M_z/M_n \approx 1.0$) dimer of spherical shape. The hydrodynamic radius was 2.8 nm and the radius of gyration was too small to be determined. It should be noted that the experimentally determined M_w was about 3–4 kDa lower than the molecular weight of 36.8 kDa of the “theoretical” dimer, the molar mass of β -LG commonly accepted at neutral pH. This difference in molar mass is probably due to the specific experimental conditions including buffer, pH, and the size exclusion columns used in this work.

For SBP, the chromatograms at UV278 and UV325 nm were virtually superimposable. Although there is certain degree of overlap, a simple deconvolution routine easily identified three main elution peaks in the chromatogram, at volumes of 19.3, 21.4 and 24.0 mL, respectively. These multiple elution peaks are indicative of the heterogeneous nature of the SBP molecules and the prevalence of feruloyl groups present in all three fractions of SBP. This is perhaps not surprising due to the prevalence of ferulic acid in the plant cell walls (Ralet et al., 2005).

As shown in Table 2, a major fraction of SBP, more than 40% was eluted in the first peak (19.3 mL). The M_w was determined to be ~770 kDa for UV278 nm, and ~800 kDa for UV325 nm. This small difference in the measured M_w is likely caused by the presence of protein moieties (with relatively smaller M_w) in beet pectin, and is consistent with previous findings (Fishman et al., 2013). For polymers eluted at 19.3 mL peak, both UV wavelengths gave values of ~40 nm for R_{gz} , ~36 nm for R_{hz} , and M_w/M_n and $M_z/M_n > 1.0$, suggesting these polymers are polydispersed. The MHKS exponent value $a < 0.5$, and $\rho \approx 1.0$ demonstrated that the polymers which eluted at 19.3 mL occupied the space equivalent to that of a compact sphere. These results are in close agreement with previously published work by Fishman, Chau, Cooke, and Hotchkiss (2008).

The weight average molecular weight ($M_w \approx 110$ kDa) and intrinsic viscosity ($\eta_w \approx 236$ mL/g) of the molecules that eluted at 21.4 mL were recovered at about 30%, and also in a close agreement at both detection wavelengths. This group of polymers is monodispersed based on the values of M_w/M_n and $M_z/M_n \approx 1.0$. The MHKS exponent (a) of these polymers is about 0.7, which represents a random coiled shape according to Harding (1997). The ratio between R_{gz} and R_{hz} yielded a relatively high ρ value (~1.7), which also corresponds to a random coil chain based on Burchard's descriptions (Burchard, 1996).

The third elution peak at 24.0 mL (recovered at >12%) included molecules that are of much smaller molecular mass, $M_w \sim 75$ kDa with lowered intrinsic viscosity, $\eta_w \approx 80$ mL/g compared to most other sugar beet pectins characterized previously (Fishman et al., 2013). It is also monodispersed with M_w/M_n and $M_z/M_n \approx 1.0$. The low MHKS exponent a value (≈ 0) suggested this group of polymers occupying the space equivalent to a compact sphere.

It should be pointed out that the total amount of SBP molecules recovered from the three elution peaks is ~87% at UV278 nm wavelength compared to 83% recovered at UV325 nm. The difference of ~4% is consistent with the total protein content of 4.78% in SBP as analyzed by the amino acid analysis. This close agreement provided confidence in the accuracy of the recovery rate determined in this work.

When the untreated β -LG and SBP were combined, three elution peaks at 19.3, 21.4 and 26.0 mL, and four at 19.3, 21.4, 24.0 and 26.0 mL were visible using UV278 and UV325 nm detection wavelengths respectively. Each group of polymers eluted at each peak possessed distinctive weight average molecular weight and hydrodynamic properties, see Fig. 2 and Table 2.

The first elution peak (19.3 mL) dominated the entire distribution with over 40%. Detection at both wavelengths gave extremely large M_w values, in excess of 940,000 kDa, when compared to the SBP molecules eluted in the same volume ($M_w \sim 800$ kDa). The intrinsic viscosity, $\eta_w \approx 350$ mL/g, was approximately the same as that of SBP alone. The complexes formed between SBP and β -LG

Table 2Molecular characteristics of β -LG (3.0 mg/mL) and SBP (1.0 mg/mL) as studied by HPSEC^a.

Sample	UV (nm)	Elution vol. (mL)	%Rec	M_w (kDa)	η_w (mL/g)	M_w/M_n	M_z/M_n	α	R_{gz} (nm)	R_{hz} (nm)	ρ
β -LG	278	26.0	96(1)	32(1)	3.9(0.1)	1.0(0.0)	1.1(0.0)	N/D ^b	N/D	2.8(0.1)	N/D
SBP	278	19.3	44(1)	769(10)	334(4)	1.4(0.0)	1.9(0.0)	0.31(0.02)	42(3)	36(0)	1.2
		21.4	29(1)	108(2)	236(4)	1.1(0.0)	1.1(0.0)	0.72(0.06)	28(5)	16(0)	1.8
		24.0	14(1)	78(10)	80(3)	1.2(0.1)	1.4(0.2)	0.18(0.08)	N/D	9.9(0)	N/D
		19.3	41(0)	812(26)	342(4)	1.4(0.0)	1.9(0.0)	0.27(0.00)	41(2)	37(0)	1.1
β -LG + SBP	325	21.4	30(0)	114(3)	238(4)	1.0(0.0)	1.1(0.0)	0.94(0.15)	29(4)	17(0)	1.7
		24.0	12(0)	75(12)	73(6)	1.0(0.0)	1.0(0.0)	4.3(4.0)	N/D	9.7(0.0)	N/D
		19.2	42(1)	944(28)	347(4)	1.5(0.1)	2.5(0.2)	0.47(0.09)	45(2)	41(1)	1.1
		21.3	44(1)	158(11)	177(2)	1.0(0.0)	1.2(0.2)	1.1(0.1)	44(0)	17(1)	2.6
		26.0	93(0.1)	34(1)	4.4(0.1)	1.0(0.0)	1.1(0.0)	N/D	N/D	2.9(0.0)	N/D
		19.2	40(0)	938(20)	348(2)	1.5(0.0)	2.1(0.1)	0.38(0.04)	42(1)	40(1)	1.0
	278	21.3	29(0)	161(16)	246(6)	1.0(0.0)	1.0(0.0)	1.5(0.4)	36(0)	18(0)	2.0
		24.1	36(1)	91(5)	27(1)	1.8(0.1)	1.2(0.1)	0.45(0.02)	N/D	7.7(0.1)	N/D
		26.0	3.5(1.0) ^c	36(2)	4.1(0.1)	1.0(0.0)	1.0(0.0)	0.14(0.20)	N/D	2.7(0.0)	N/D
		18.7	0.5(0.0)	66,130(7320)	28(4)	1.0(0.0)	1.1(0.0)	0.50(0.20)	44(0)	66(6)	0.67
		22.4	3.7(0.0)	2610(243)	11(1)	3.2(0.3)	7.5(1.0)	0.17(0.02)	48(0)	24(4)	2.0
		26.0	87(1)	51(1)	3.9(0.1)	1.7(0.1)	3.1(0.3)	0.27(0.01)	N/D	3.5(0.1)	N/D
SBP (heated)	278	19.3	41(0)	804(13)	323(2)	1.4(0.0)	1.9(0.0)	0.32(0.00)	42(1)	36(0)	1.2
		21.4	29(0)	114(6)	238(3)	1.0(0.0)	1.1(0.0)	0.65(0.04)	30(3)	17(0)	1.8
		24.0	14(0)	82(14)	83(0)	1.2(0.0)	1.9(0.5)	0.42(0.20)	N/D	11(1)	N/D
		19.3	40(0)	748(6)	330(2)	1.4(0.0)	1.9(0.0)	0.27(0.00)	38(0)	35(0)	1.1
	325	21.4	31(0)	105(5)	234(1)	1.1(0.0)	1.1(0.0)	0.72(0.01)	27(2)	16(0)	1.7
		24.0	13(0)	55(3)	74(1)	1.1(0.0)	1.1(0.0)	0.94(0.10)	N/D	8.9(0.1)	N/D
β -LG (heated) + SBP (heated)	278	19.3	41(1)	2857(158)	326(6)	1.8(0.1)	3.1(0.1)	0.36(0.02)	40(0)	59(4)	0.68
		21.8	56(1)	648(19)	142(2)	1.1(0.0)	1.2(0.0)	2.4(0.1)	35(1)	26(1)	1.3
		26.2	83(0)	42(3)	4.5(0.1)	1.3(0.0)	1.5(0.1)	0.05(0.01)	N/D	3.2(0.1)	N/D
		19.3	41(2)	4276(27)	334(7)	1.4(0.0)	2.1(0.3)	0.40(0.01)	40(0)	62(3)	0.64
	325	21.4	37(1)	850(22)	200(6)	1.0(0.0)	1.0(0.0)	1.2(0.0)	35(1)	30(0)	1.2
		24.0	37(1)	376(4)	29(1)	1.1(0.0)	1.2(0.2)	2.1(0.1)	N/D	13(2)	N/D
		26.0	8.0(0.1) ^c	44(1)	4.4(0.1)	1.0(0.0)	1.0(0.0)	0.084(0.08)	N/D	3.0(0.1)	N/D

^a All values were averaged between triplicate sets of UV and RI measurements. Standard deviations from triplicate analyses are given in parenthesis.^b N/D: not determined.^c Value was taken by the difference between output and input mass measured at UV278 and confirmed by the deconvolution and integration of the peak area.

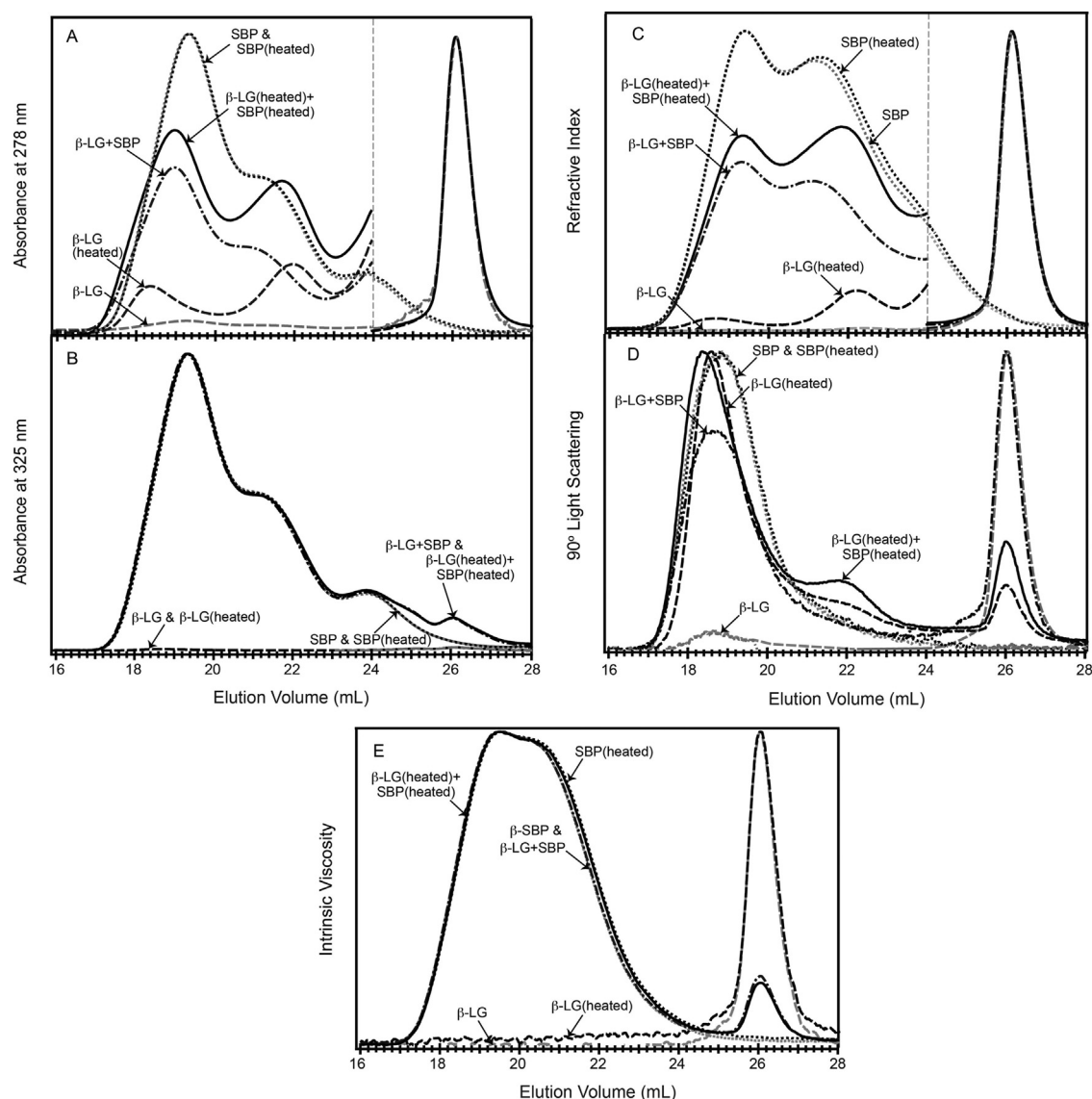


Fig. 2. Superimposed HPSEC chromatograms of β -LG, SBP, β -LG (heated), and SBP (heated) as detected by UV278 nm (A), UV325 nm (B), RI (C), MALLS (D) and DP (E). The concentration was 0.3 and 0.1 mg/mL for β -LG and SBP respectively. The y-axis was normalized to unity in each graph.

are highly polydispersed, $M_w/M_n = 1.5$ and $M_z/M_n > 2.0$. The low MHKS exponent value, $a < 0.5$, indicated a molecular volume equivalent to that of a compact spherical shape (Harding, 1997). The radius of gyration (R_g) and hydrodynamic radius (R_h) yielded a ρ value ~ 1.0 , which also corresponds to homogenous spherical shape according to Burchard (1996). Although further in-depth studies are needed to obtain the binding stoichiometry for the β -LG/SBP complexes, it is evident that interacting with β -LG appeared to cause negligible changes in the shape (the a and ρ values) of the SBP molecules. The resulting complex remained compact and spherical, $a < 0.5$ and $\rho \approx 1.0$. These parameters did not change due, in all likelihood, to the insignificant size of the β -LG dimer compared to that of SBP.

The elution peak at 21.3 mL obtained at both detection wavelengths (in Fig. 2 and Table 2) represented a group of molecular species with $M_w \approx 1,60,000$ kDa. It is 40 kDa larger than SBP alone that is eluted at the same volume. This increase is likely caused by the interaction with β -LG. The measured intrinsic viscosity (η_w) of the complex, however, was 250 mL/g at UV325 nm compared to 180 mL/g at UV278 nm. This may be related to differences in concentrations measured at the two wavelengths. At UV278 nm,

both β -LG and SBP were detectable; resulting in an increase in concentration compared to SBP alone. At UV325 nm, on the other hand, only the SBP molecules containing feruloyl groups were measurable, resulting in a higher intrinsic viscosity value. More importantly, it is apparent that this elution peak obtained at both detection wavelengths represented the same group of interacting complexes between β -LG and SBP. These complexes are monodispersed, $M_w/M_n = 1.0$, $M_z/M_n \approx 1.0$. The MHKS exponent value $a > 1.0$ indicates a shape approximating rigid rod. In addition, their relatively high radius of gyration (R_g), and low hydrodynamic radius (R_h) produced a high ρ value, greater than 2.0, also indicative of a rigid rod shape (Burchard, 1996). The measured M_w and η_w values for this group of interacting complex between β -LG and SBP suggested they might contain one β -LG dimer per SBP molecule of intermediate size (110 kDa).

In addition to the obvious changes observed in the concentration and η_w for the SBP/ β -LG complex involving peak eluted at 21.3 mL, it is interesting to note the overall drastic change in the chromatogram of the mixture (Table 2 and Fig. 2A). Namely, the elution peak at 24.0 mL representing the smallest sized SBP molecules ($M_w \sim 75$ kDa) completely disappeared in the presence of β -LG at

UV278 nm wavelength, or at least it could not be easily resolved. This disappearance demonstrated that the group of SBP molecules with $M_w \sim 75$ kDa was heavily involved in interacting with β -LG and thus became so much bigger that the majority of the polymers in this group merged into peak 21.3 mL. This complexation further contributed to the increased concentration and M_w of the 21.3 mL peak. Any residual unbound SBP molecules ($M_w \sim 75$ kDa), which eluted at the 24.0 mL became relatively insignificant and could not be independently observed, possibly due to the presence of unbound β -LG (93%) in neighboring elution peak at 26.0 mL.

Furthermore, the elution peak at 24.0 mL was not only well resolved in the chromatogram of the mixture measured at UV325 wavelength, but it also acquired a greatly increased presence (36%) when compared to the unbound SBP (12%). The η_w value meanwhile dropped considerably, from 73 to 27 mL/g. This drop indicated that this peak was largely composed of β -LG molecules. Therefore, we concluded that the molecules in the elution peak at 24.1 mL was most likely caused by complexes formed between β -LG and the SBP molecules containing feruloyl groups. This group of SBP molecules, which are relatively small in size, $M_w \sim 75$ kDa, bound to β -LG, and resulted in an additional 20 kDa on average, the equivalent of one β -LG monomer. Our separate spectroscopic analysis further supported the hypothesis that β -LG dimer was disrupted by interacting with SBP (results published elsewhere). Unlike the uncomplexed SBP, this group of complexes is polydispersed, M_w/M_n and $M_z/M_n > 1.0$. The MHKS exponent value ($a \sim 0.5$) suggested this group of polymers is random coil shaped.

It should be noted that the total amount recovered polymers associated with the first three elution peaks at UV325 nm, 105%, exceeded apparently the amount recovered at UV278 nm (86%). In addition to the appreciable changes in the compositions and distributions of various polymers caused by complexation between β -LG and SBP, a small part of the discrepancy may also be due to the inaccuracy in the dn/dc value, particularly for UV325 detection. Because it was not possible to obtain absolutely accurate dn/dc value for each polymer in this work, a constant value for SBP was used for all the elution peaks containing SBP. This approximation in dn/dc may have introduced some small error (Striegel, 2005). Despite of this possible source of error, we believe that the analyses carried out in this work provided a reasonable and valid means for making sound comparisons.

The last elution peak (26.0 mL) in the chromatograms obtained at UV278 nm had a recovery rate of $\sim 93\%$. The molecular weight M_w of ~ 34 kDa and the intrinsic viscosity approximated that of native β -LG, ~ 4.0 mL/g. This group of polymer is highly monodispersed with M_w/M_n and $M_z/M_n = 1.0$ and adopts a compact spherical shape. Clearly, they originate primarily from unbound dimeric β -LG molecules.

Interestingly, a small peak eluted at 26.0 mL in the UV325 chromatogram (Fig. 2B), and occupied $\sim 3.5\%$ of the total β -LG input mass. All hydrodynamic properties were nearly identical to those obtained in the UV278 nm chromatogram except for a slightly increased M_w of 36 kDa when compared to unbound β -LG dimer. This led us to the postulate that this complex was the result of β -LG dimer interacting with non-covalently bound ferulic acid functional group(s), likely present in SBP. This finding has not been reported previously. The total recovery rate determined at UV278 (93%) should therefore include the contribution from this complex. The exact binding stoichiometry and molecular details of the non-covalently bound ferulic acid groups to β -LG remain to be further investigated.

Based on these analyses, the total amount of β -LG participating in forming complexes with various sized SBP molecules was estimated to be $\sim 6.5\%$ (w/w). Furthermore, $\sim 3.5\%$ of these were involved in binding to a small fraction of non-covalently bound ferulic acids possibly present in SBP. By way of

comparison, more than 35% SBP of various sizes formed complexes with β -LG, and the majority, $\sim 20\%$ of these SBP molecules are small sized ($M_w \sim 75$ kDa). At the experimental conditions used (near neutral pH and intermediate ionic strength), both β -LG and SBP are largely negatively charged. Therefore, these complexes are primarily formed through the local positive charges on the protein surface and the negative charges of the pectin. In addition, hydrophobic interaction between the side-chains of β -LG and the neutral sugar moieties of SBP may also play a role.

3.4. Effect of thermal treatment on the hydrodynamic properties of β -LG and SBP

When β -LG was subjected to heat treatment at 80°C , pH 6.50 for 10 min, it undergoes irreversible thermal induced denaturation, intermolecular S–S bond shuffling, aggregation and unfolding, which may be partially reverted (de la Fuente, Singh, & Hemar, 2002). In the HPSEC work reported here, the peak eluted at 18.7 mL (Fig. 2A) contained a small amount (0.5%) of aggregate with extremely high molecular weight. This aggregate apparently is highly monodispersed, M_w/M_n and $M_z/M_n \approx 1.0$, and a random coil in shape ($a \approx 0.5$). Its ρ value of 0.67 met the description of a microgel state due to its large Rh_z value rather than a linear chain (where $\rho \approx 1.5$) according to Burchard (1996). In addition, the total amount recovered ($\sim 91\%$) of the three elution peaks was lower than that of the native β -LG (96%), suggesting $\sim 5\%$ loss in total mass, clearly caused by heat-induced irreversible aggregation, which was insoluble.

The second peak of much lowered weight average molecular weight, $M_w \sim 2610$ kDa, was eluted at volume 22.4 mL with slightly higher recovery rate, 3.7%. This group of polymers was thought to come from multiple β -LG molecules in aggregated form, approximately 140 monomers on average. They are highly polydispersed (M_w/M_n and $M_z/M_n \gg 1.0$) with elevated intrinsic viscosity ($\eta_w = 11$ mL/g) compared to native β -LG dimer ($\eta_w \approx 4.0$ mL/g). The low MHKS exponent value $a = 0.17$ assumes their shape being compact sphere. However, the high Rg_z/Rh_z ratio yielded a ρ value close to 2.0, and pointed it to a rigid rod shape, which clearly is in conflict with that estimated from the MHKS exponent. Heat induced unfolding followed by the aggregation of β -LG could be accounted for the discrepancy and difficulty in using the parameters (a and ρ) to describe the conformational shape of these polymers.

The third elution peak (at 26.0 mL), the main contributor to the HPSEC chromatogram of the heat-treated β -LG, corresponds to a group of polymers with the same intrinsic viscosity (η_w) as that of native β -LG. But its weight average molecular weight was ~ 20 kDa higher than the dimeric β -LG molecule, and constituted to an equivalent of a trimeric β -LG molecule. This polymer may be resulted from intermolecular S–S bond formation between the free SH group in Cys121 of β -LG caused by heating. This trimeric form of β -LG is highly polydispersed, M_w/M_n and $M_z/M_n \gg 1.0$, and remained mainly as a compact spherical shape ($a = 0.27$) with an increased hydrodynamic radius, 3.5 nm compared to 2.8 nm for the native β -LG. Furthermore, if any still remains, likely small amount, the dimeric form of β -LG, the main component of the native protein, was not independently resolved in the heat-treated β -LG.

The chromatograms of heat-treated SBP at both UV278 nm and UV325 nm were completely superimposable in much the same fashion as was the untreated SBP. Three elution peaks at 19.3, 21.4 and 24.0 mL with a total recovery rate at 84% at both wavelengths, $\sim 3\%$ less than the untreated SBP, was likely due to heat-induced degradation (Fishman et al., 2008). Furthermore, this degradation probably occurred in the SBP fraction ($M_w \sim 800$ kDa) that contains protein moieties. The M_w and polydispersed hydrodynamic shapes remained virtually unaffected by the heat treatment, particularly for the first two elution peaks, 19.3 mL and 21.4 mL. Differences

caused by heat treatment were observed for the smallest sized SBP molecules that eluted at 24.0 mL. Namely, there was a significant drop in M_w from ~ 75 to 55 kDa, and a lesser reduction in η_w , by ~ 10 mL/g, as detected at UV325 nm. Such decreases in both M_w and η_w may be due to the heat sensitivity of feruloyl groups present in SBP. In addition, the hydrodynamic shape of these SBP molecules also changed (by heat treatment) from monodispersed compact sphere to polydispersed random coil as indicated by increased MHKS exponent a value (Table 2).

3.5. Interaction between heat-treated β -LG and SBP

Similar to the untreated β -LG and SBP mixture, the chromatogram of the heat-treated mixture included three elution peaks at 19.3, 21.8 and 26.2 mL, and four at 19.3, 21.4, 24.0 and 26.0 mL for detections at UV278 nm and UV325 nm respectively. Each elution peak possessed distinctive weight average molecular weight and hydrodynamic properties, as shown in Fig. 2 and Table 2.

The first elution peak (19.3 mL) detected by both wavelengths represented a group of extremely large molecular weight species, $M_w > 3000$ kDa but with a slightly lower intrinsic viscosity of $\eta_w \sim 330$ mL/g than the equivalent untreated interacting pair with a viscosity of ~ 350 mL/g, suggesting an increased level of involvement of the polymeric form of β -LG in the complex. The M_w measured at UV325 was nearly double of that measured at UV278. This increase may have to do, in part, with a heightened deviation in the dn/dc value from unbound SBP. This deviation may be especially true for UV325 (Striegel, 2005). The MHKS exponent value for these polymers, $a \sim 0.40$, is indicative of a random coil shape. Also these polymers are highly polydispersed with $M_w/M_n > 1.0$ and $M_z/M_n > 2.0$. Although the radius of gyration ($R_{gz} \sim 40$ nm) of this interacting polymer remained roughly the same as that determined for its unheated counterpart, its hydrodynamic radius (R_{hz}) increased by ~ 20 nm, i.e. 1.5 times. As a result, the ρ value was lowered to ~ 0.68 , which is indicative of random coiled polymer chains existing in a microgel state (Burchard, 1996), similar as discussed above on heat-treated β -LG eluted at the same volume. Possibly the elution peak at 19.3 mL with recovery rate of 41% represented a group of interacting polymers between multiple polymeric β -LG molecules and a class of SBP molecules of large M_w (~ 800 kDa). The resulting complex adopted the shape of large heat-induced β -LG aggregates rather than that of SBP, which was the case for its counterpart in the untreated mixture.

Similar to the untreated mixture of β -LG and SBP, the peak which eluted at 21.4 mL gave a different recovery rate (56% vs. 37%), M_w (650 vs. 850 kDa) and η_w (140 vs. 200 mL/g) at the two detection wavelengths (Table 2). This was likely caused by the heavy involvement of the oligomeric form of β -LG molecules that can interact with SBP more effectively than native β -LG. Importantly, this elution peak appears to correspond to an ensemble of complexes between polymeric β -LG and SBP with similar hydrodynamic properties as its counterpart (21.3 mL) in the untreated mixture. These complexes are also monodispersed with M_w/M_n and $M_z/M_n \approx 1.0$, and maintain a rigid rod shape (with $a > 1.0$). An increase, ~ 10 nm, in hydrodynamic radius (R_{hz}) yielded a ρ value > 1.0 , as the radius of gyration (R_{gz}) remained nearly the same, at 35 nm. This increase in ρ value is also indicative of a shape resembling a rigid rod.

Much like the case of the untreated mixture, in addition to the changes involving elution peak at 21.4 mL, the most drastic change had to do with the apparent unresolved elution peak at 24.0 mL when pre-heated SBP and pre-heated β -LG were combined and detected at UV278 nm. The recovery rate for the polymers from the mixture which eluted at 21.3 mL was 56%. This value was also much higher than the 29% of the pre-heated SBP alone. Similar to the discussion above, the polymers eluted at 24.0 mL (as in unbound SBP) likely shifted, if not completely, to 21.3 mL upon

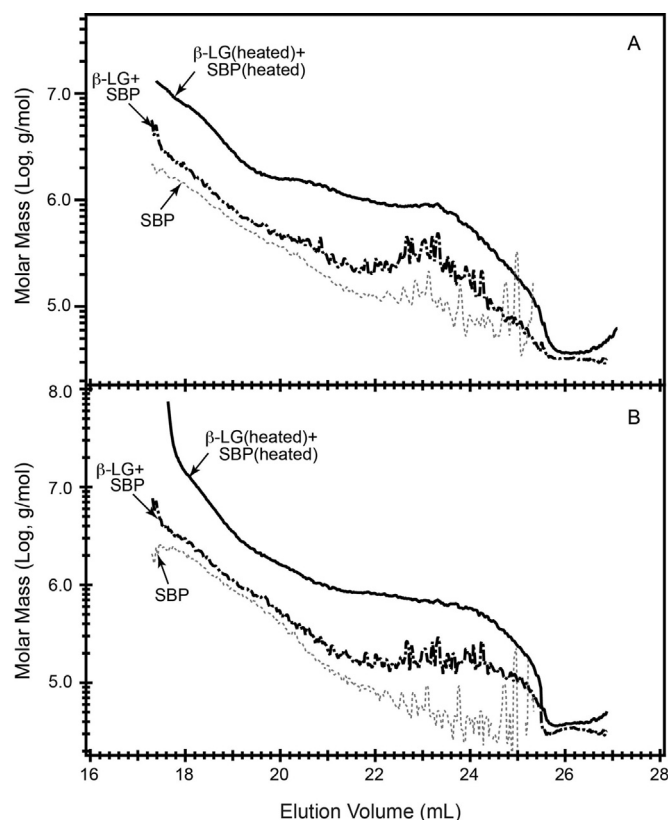


Fig. 3. Comparison of molar mass distributions of SBP, β -LG, SBP and β -LG (heated), and SBP (heated), as analyzed by HPSEC and detected at UV278 nm (A), and (B) RI. The concentration of the mixture was 0.3 and 0.1 mg/mL for β -LG and SBP respectively.

binding to heat denatured β -LG. The peak at 24.0 mL, however, was still measurable at UV325 with a greater presence (37%) than in the unbound SBP (13%). This increase indicated strong involvement of feruloyl groups in SBP in the interaction with β -LG. Even though its M_w (~ 380 kDa) is more than four times greater than its untreated equivalent (~ 90 kDa), its η_w (~ 29 mL/g) is about the same, approximating that of large unbound β -LG aggregates. This elution peak was largely composed of oligomeric β -LG molecules but with direct involvement of the feruloyl groups in SBP molecules. This class of complexes is monodispersed, M_w/M_n and $M_z/M_n \approx 1.0$, and assumes a rigid rod conformation with an increased MHKS exponent a value > 1.0 compared to the unheated pair that eluted at the same volume. The R_{hz} value (~ 13 nm) also nearly doubled than that of the unheated complex (~ 7.7 nm).

At the 278 nm wavelength, the polymers eluted at 26.2 mL from the pre-heat-treated mixture was recovered at $\sim 83\%$. Their molecular weight is small, ~ 42 kDa, and comparable to a mixture of both β -LG dimeric and trimeric forms. This size reduction of the intermolecular aggregates increased their polydispersity, $M_w/M_n = 1.3$ and $M_z/M_n = 1.5$. The restoration of the dimeric form of β -LG, at least partially, from the HPSEC analysis provided further evidence to our other findings by spectroscopic studies. This group of polymers adopts nearly compact spherical shape, $a \approx 0$, but has slightly increased hydrodynamic radius, 3.2 nm, than the native β -LG (2.8 nm). This elution peak also appears to contain a trivial amount of unbound small sized SBP molecules, as well as complexes formed between dimeric and trimeric β -LG molecules with possible free ferulic acid, a similar phenomenon was discussed above in the case of the untreated mixture.

Similar to the untreated mixture, a small peak at 26.0 mL was eluted in the UV325 nm chromatogram (Fig. 2B), and occupied $\sim 8.0\%$ of the total heat-treated β -LG input mass. All hydrodynamic

Table 3

Summary of the hydrodynamic properties of the molecular fractions analyzed by multi-detection HPSEC.

Sample	Elution vol. (mL)	Average %rec.	Approximate M_w (kDa)	Dispersity	Shape of space occupied
SBP	19.3	44	770–800	Polydispersed	Compact sphere
	21.4	29	110	Monodispersed	Random coil
	24.0	14	75	Monodispersed	Compact sphere
β -LG + SBP	19.2	42	940	Polydispersed	Compact sphere
	21.3	44	160	Monodispersed	Rigid rod
	24.1	36	90	Polydispersed	Random coil
	26.0	3.5	34	Monodispersed	Compact sphere
	18.7	0.5	66,000	Monodispersed	Random coil (microgel)
β -LG (heated)	22.4	3.5	2600	Polydispersed	N/D
	26.0	87	50	Polydispersed	Compact sphere
	19.3	41	750–800	Polydispersed	Compact sphere
SBP (heated)	21.4	29	110	Monodispersed	Random coil
	24.0	14	55–80	Polydispersed	Random coil
β -LG (heated) + SBP (heated)	19.3	41	2800–4300	Polydispersed	Random coil (microgel)
	21.8	56	650–850	Monodispersed	Rigid rod
	24.0	37	370	Monodispersed	Rigid rod
	26.0	8.0	44	Polydispersed	Compact sphere

properties including M_w were nearly identical to that obtained at UV278 but are monodispersed M_w/M_n and $M_z/M_n = 1.0$. Once again, this elution peak was expected to be the result of dimeric and trimeric β -LG molecules interacting with non-covalently bound ferulic acid functional group(s) likely present in SBP. The exact amount and the binding stoichiometry remain to be determined. The total recovery rate (83%) determined at UV278 nm should include the contribution from this particular complex. In a study by Jung and Wicker (2012a) using laccase to mediate conjugation between β -LG and SBP, interacting complexes between pre-heat treated β -LG and SBP (in the absence of laccase) were also reported. The results from this work provided further evidence that denatured (not unfolded) protein tends to promote stronger and more effective binding interaction with pectin even though both pectin and protein are largely positively charged in the experimental conditions used.

3.6. Molecular mass distribution of β -LG and SBP complexes

The molecular mass distribution as a function of elution volume is shown in Fig. 3 using UV278 (A) and RI (B), and Fig. 4 using UV325 (A) and RI (B) at UV278 and RI. Clearly, interacting complexes with various M_w were formed between untreated β -LG and SBP as well as individually pre-heated β -LG and SBP as evidenced by increased molecular mass compared to SBP throughout the entire elution profile. Thermal treatment (at 80°C for 10 min) of β -LG and SBP greatly facilitated the interaction and produced complexes with apparently greater molecular mass. As a result, heat-induced denaturation and aggregation of β -LG was partially alleviated, and some of its dimeric form was recovered.

The fraction of SBP molecules with larger average molecular weight (~ 800 kDa) seems to bind β -LG more effectively. This observation is in close agreement with the work of Sperber et al. (2010), and is consistent with the notion that high content of methyl ester in SBP favors the binding with β -LG (at neutral pH) through mostly hydrophobic interaction. In addition, electrostatic interaction between pocketed positive charges in β -LG and negative charges of SBP also is important in the interaction. Furthermore, the protein moieties present in SBP that are mainly associated with the SBP molecules of large M_w (800 kDa) also play an important role in interacting with β -LG. These interactions are likely hydrophobic in nature as well.

Nevertheless, the most significant finding in this work was the constant involvement of SBP with small M_w , ~ 75 kDa, where the majority of feruloyl groups also resides, in binding to both native and denatured, polymeric and oligomeric forms of β -LG. It was speculated that non-covalent bound ferulic acid (FA) moieties in

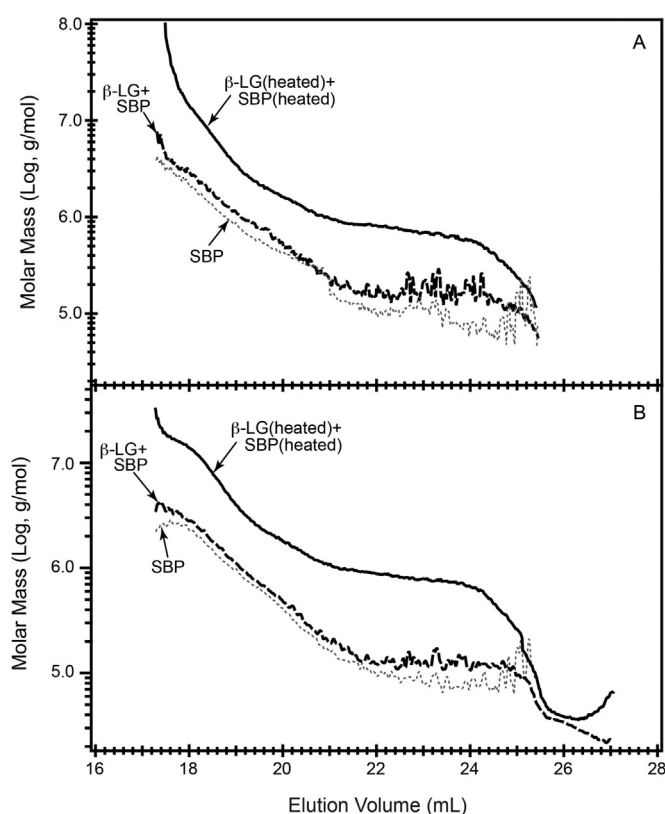


Fig. 4. Comparison of molar mass distributions of SBP, β -LG, SBP and β -LG (heated), and SBP (heated), as analyzed by HPSEC and detected at UV325 nm (A), and (B) RI. The concentration of the mixture was 0.3 and 0.1 mg/mL for β -LG and SBP respectively.

SBP were heightened and released only upon their interaction with both native and heat-denatured β -LG.

4. Conclusions

The hydrodynamic properties of various biopolymer fractions formed through molecular interaction between β -lactoglobulin (β -LG) and sugar beet pectin (SBP) were summarized in Table 3. It has been demonstrated that soluble complexes between β -LG and SBP were formed when combined at physiological relevant conditions (neutral pH and medium ionic strength), resulting in increased molecular weights and altered shapes compared to unbound β -LG and SBP. Moderate heat treatment of the protein and pectin

prior to mixing facilitates the interaction and produced complexes with even greater molecular weights. In addition to the methyl ester groups of SBP, protein moieties, mainly located in the large molecular weight fraction (800 kDa) of SBP, play an important role in interacting with β -LG. Feruloyl groups that exist in all three fractions of SBP molecules are deeply involved in interacting with β -LG. Furthermore, β -LG molecules tend to favor the intermediate (110 kDa) and small sized (75 kDa) SBP.

The most significant finding of this work is the presence of non-covalently bound feruloyl moieties in SBP, which were only released upon their strong interaction with β -LG, in both native dimeric and heat-induced oligomeric/polymeric states.

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