Investigation of the molecular interactions between β -lactoglobulin and low
methoxyl pectin by multi-detection High Performance Size Exclusion
Chromatography

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Abstract

To achieve controlled physiochemical, structural, and consequently, functional properties of interacting systems containing β -lactoglobulin (β LG) and commercial standardized low methoxyl pectin (LMP, DE 36.8%), solution conditions were established and studied at the weight ratio of 3:1 (w/w), pH $\sim$ 6.50. Online multi-detection High Performance Size Exclusion Chromatography (HPSEC) was used to characterize the physicochemical properties of the interacting biopolymer fractions in detail. Results showed that upon direct mixing, $\sim$ 6.1% (w/w) of native dimeric β LG molecules formed complexes with LMP molecules of two sizes, namely $[M_w] \sim 250$ and 55 kDa. The interactions produced complexes of altered shapes and hydrodynamic properties compared to those of β LG and LMP controls. Heat-induced (80 °C, 10 min) denaturation of β LG greatly increased its participation in the complexes as aggregates, to $\sim$ 10.4%, which appear to favor the LMP molecules of higher $[M_w]$. Pre-heating LMP, however, impeded its binding to β LG and increased the amount of unbound β LG as a result. The effect of sucrose on the interactions between β LG and LMP was also investigated using a set of similarly treated samples but with sucrose removed from LMP. These studies demonstrated that sucrose greatly facilitated the complex formation, notably between the pre-heated β LG and LMP. The structures of the complexes were found to be less flexible and less open in the presence of sucrose than its absence. It is suggested that sucrose contributed greatly to the stabilization of the heat-induced β LG aggregates as they interact with LMP through a preferential exclusion mechanism.

Highlights

- ~6.1% native dimeric β LG forms complexes with LMP with preference for the smaller $[M_w]$
- ~10.3% heat-treated aggregated β LG forms complexes with LMP of larger $[M_w]$
- Heat treatment of LMP impeded its binding ability to β LG at β LG:LMP=3:1 (w/w)
- Sucrose (~9%) facilitates the interactions between pre-heated β LG and LMP
- Complexes formed between β LG and LMP are more compact in the presence of sucrose

Keywords

β -lactoglobulin; low methoxyl pectin; HPSEC; hydrodynamic properties; molecular interactions;
sucrose

Introduction

Recent years have seen growing interest in the interactions between proteins and polysaccharides when used in food, pharmaceutical and cosmetic products (Dickinson, 1998; Doublier, Garnier, Renard, & Sanchez, 2000; Evans, Ratcliffe, & Williams, 2013). Among the many uses, protein-polysaccharide mixtures are often found in processed foods, such as fat substitutes (Lobato-Calleros, Vernon-Carter, Sanchez-Garcia, & Garcia-Galindo, 1999), meat analogues (Ramirez, Uresti, Velazquez, & Vazquez, 2011), protein recovery (Serov, Antonov Yu, & Tolstoguzov, 1985), edible films and packaging (Porta, Mariniello, Di Pierro, Sorrentino, & Giosafatto, 2011; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Conjugates between proteins and polysaccharides have been created via forming covalent bonds by crosslinking agents (Chen, *et al.*, 2003; Jung & Wicker, 2012; Mariniello, *et al.*, 2014) and by the Maillard type reactions (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016; Evans, *et al.*, 2013; Oliver, Melton, & Stanley, 2006). A large body of literature (Dickinson, 2015; Evans, *et al.*, 2013; Jones & McClements, 2010, 2011; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003) also exists regarding the formation of water-soluble complexes consisting of oppositely charged globular proteins and polysaccharides because of their potential applications in targeted transport of biologically active compounds. However, much less attention (Cooper, Dubin, Kayitmazer, & Turksen, 2005; McClements, 2006; Rodriguez-Patino & Pilosof, 2011) has been paid to the interactions between protein and polysaccharides when under unfavorable conditions, *i.e.* at a pH and ionic strength when both partners carry net negative charges (pH > pI).

Among the numerous protein and polysaccharide complexes investigated to date, the interaction between β -lactoglobulin (β LG), the most abundant whey protein of cow milk, and pectin, a cell-wall anionic polysaccharide, has been regarded 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, well studied structural features, and broad-ranging applications in the food and nutraceutical industries (Cho, Batt, & Sawyer, 1994; Liang & Subirade, 2010; Ron, Zimet, Bargarum, & Livney, 2010; Teng, Li, Luo, Zhang, & Wang, 2013). Recently, it has been demonstrated that nanoparticles formed between β LG and pectin (Izadi, Divsalar, Saboury, & Sawyer, 2016) hold the promise as a delivery vehicle for oral colon cancer drugs.

Pectins are a class of complex polysaccharides found in the cell walls of higher plants. For centuries, they have been widely used as thickeners, emulsifiers, texturizers and stabilizers in jams and jellies, fruit juices, desserts and fermented dairy products (Axelos & Thibault, 1991; Oakenfull, 1991; Tamine, Hassan, Farnworth, & Toba, 2007). In addition to their superior functional properties, pectin and pectin-derived oligosaccharides have been recognized as emerging prebiotics for their ability to modulate microbiota (Blatchford, *et al.*, 2013; Gómez, Gullón, Yáñez, Schols, & Alonso, 2016), lower blood cholesterol levels, especially the low-density lipoprotein cholesterol fractions, and thus reduce the risk for coronary heart diseases (Jenkins, Kendall, Axelsen, Augustin, & Vuksan, 2000).

Commercial pectins are usually extracted from apple pomace or citrus peels (Rolin, 2002). These high methoxyl pectins (HMP) usually have a degree of methyl esterification (DE) of 70-80% (May, 1990; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). Pectins of lower ester content (LMP), $DE \leq 50\%$, can be prepared by controlled deesterification, often with acid or ammonia in an alcoholic media (Axelos, *et al.*, 1991). Both HMP and LMP can undergo gelation by various mechanisms (Fraeye, Duvetter, Dounghla, Van Loey, & Hendrickx, 2010;

Thakur, Singh, Handa, & Rao, 1997). The formation of gels with HMPs requires low pH (~3) and high sugar content (~65%). These are governed by a combination of hydrogen bonding and hydrophobic interactions (Oakenfull, 1991). In contrast, LMPs may form gels in the presence of calcium or other divalent metal ions over a wide range of pH with or without sugar (Grant, Morris, Rees, Smith, & Thom, 1973). Because of their relatively low sugar content, LMP gels are often found in low calorie and dietetic foods (May, 1990).

Several successful attempts have been made in preparing biopolymers through complex formation between proteins and polysaccharides for various applications (Dickinson, 2008; Evans, *et al.*, 2013; Jones & McClements, 2008; Jones, *et al.*, 2011). Most published work so far, however, has largely focused on complexes formed under acidic pH conditions, *i.e.* pH < pI (of the protein) when the protein and polysaccharide were expected to interact through electrostatic forces. The binding stoichiometry at pH 4 where 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 low methoxyl pectin at neutral pH, on the other hand, has not been fully characterized. Previously, we studied the interaction between β LG and sugar beet pectin at near neutral pH (Qi, Chau, Fishman, Wickham, & Hotchkiss, 2014). At the experimental conditions studied, both β LG and LMP carry an overall negative charge (Cooper, *et al.*, 2005). When β LG and LMP are combined, the lowering in binding free energy is largely driven by entropy released upon counter-ion binding (de Kruif, Weinbreck, & de Vries, 2004). Additionally, enthalpy is also expected to contribute significantly as well. This apparent net charge asymmetry in the participating pair (β LG and LMP) 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 charge patches are more effective and stronger than the repulsive charges (Becker, Henzler, Welsch, & Ballauff, 2012; da Silva, Lund, Jonsson, & Akesson, 2006; Park, Muhoberac, Dubin, & Xia, 1992; Yigit, Heyda, Ballauff, & Dzubiella, 2015). Furthermore, other weaker forms of interactions including localized hydrophobic interaction and hydrogen binding may also play a role in stabilizing the interacting complexes.

It has been known (Kim, *et al.*, 2003; Lee & Timasheff, 1981) that a neutral sugar, such as sucrose, can significantly increase the thermal and conformational stability of globular proteins in aqueous solution by preferential exclusion of the sucrose from the protein surface (Arakawa & Timasheff, 1982; Kim, *et al.*, 2003). Recent studies (Barnett, *et al.*, 2016) further showed that these preferential interactions between osmolyte, *e.g.* sucrose and protein might be specific to the different regions of the protein. Neutral sugars may affect the native and denatured states of the protein differently (Barnett, *et al.*, 2016). At sufficient concentrations, they tend to reduce protein unfolding and aggregation by increasing the unfolding free energy *in vitro* (Lee, *et al.*, 1981). The presence of sucrose also was found to strengthen the rheology and structure of the low-methoxyl pectin gels during gelation (Fu & Rao, 2001), and to increase the glass transition temperature of dairy protein gels and emulsions (Rogers, Roos, & Goff, 2006; Schorsch, Jones, & Norton, 2002; Semenova, Antipova, & Belyakova, 2002) as well.

The objectives of this study are to establish experimental conditions for interacting systems containing food-grade β -lactoglobulin (β LG) and commercially standardized low methoxyl pectin (LMP), to achieve controlled physiochemical, structural and consequently functional properties, and to gain an understanding of the role that sucrose plays on these interactions. The molecular and hydrodynamic details involved in various soluble complexes

148 formed between β LG and LMP at pH ~6.50 in the presence and absence of sucrose were
149 characterized. We employ High Performance Size Exclusion Chromatography (HPSEC) in
150 combination with a serial online multi-detection system capable of measuring UV-VIS
151 absorption, Multi-angle Light Scattering (MALLS), refraction index (RI), and intrinsic viscosity
152 (DPV) simultaneously. The knowledge gained in this study should be useful in designing
153 formulations of food products containing dairy proteins and pectin with targeted functional
154 properties.

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 of the powder (expressed as dry weight percent unless otherwise indicated) was: > 95% protein. Ninety percent of it 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 between 51.2% of genetic variants A and 48.8% of B, respectively.

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

Low methoxyl citrus pectin (LMP), labeled DE 36.8%, was a gift from CP Kelco, a Huber company (www.cpkelco.com). The composition of the pectin was determined to contain galacturonic acid (GalA), $56.4 \pm 4.55\%$; degree of esterification (DE), $29.2 \pm 0.90\%$; degree of amidation (DA), $0.1 \pm 0.01\%$; and neutral sugar was $36.6 \pm 1.1\%$ using previously published methods (Yoo, Fishman, Savary, & Hotchkiss, 2003). As reported previously (Fishman, *et al.*,

2015), the total protein content of the LMP was determined to be $1.17 \pm 0.01\%$ by amino acid composition analysis.

Since the LMP sample was standardized with sucrose by the supplier, the LMP sample without sugar, denoted as LMP(NS), was prepared and used as a comparison in this work. The sugar was removed by stirring 5.5 g of LMP in 100 g of 70% isopropyl alcohol (IPA) and Milli-Q water solution overnight. The suspension was passed through a Whatman paper filter (Grade 541, Sigma-Aldrich, St. Louis, MO). The precipitated pectin was removed from the filter and dried overnight at room temperature in a vacuum oven.

2.2. Preparation of Sample Solutions

Dried β -lactoglobulin (β LG) and low methoxyl pectin, LMP or LMP (NS) (DE 36.8%) sample was dissolved at 12.0 mg/ml and 4.0 mg/ml respectively in a solvent system containing 50 mM NaNO_3 and 0.01% NaN_3 (pH 6.50). β LG was easily soluble at the specified concentration, and the pH was about 6.60. Dissolving LMP and LMP(NS), on the other hand, required overnight stirring, followed by centrifugation at $27,000 \times g$ at room temperature (20°C) for 20 min in a Sorval RC-5B centrifuge (DuPont Co., Wilmington, DE). Only the LMP or LMP (NS) supernatant was used in all experiments. The pH was measured to be 3.35, which was then adjusted carefully with 1.0 NaOH to be ~ 5.0 . All β LG and LMP 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 LMP or LMP(NS) or mixtures, β LG+LMP and β LG+LMP(NS) at the concentration of 3.0 mg/mL (β LG) and 1.0 mg/mL (LMP or LMP(NS)) respectively.

Pre-heating treatment involved holding each solution of β LG, LMP and LMP(NS) at the concentration of 12.0 mg/ml (protein) and 4.0 mg/ml (pectin) respectively at 80 °C for 10 min in a water bath to yield β LG-h, LMP-h and LMP(NS)-h, and heated mixtures [β LG+LMP]-h and [β LG+LMP(NS)]-h. Each heated solution was then allowed to cool to room temperature prior to subsequent analysis or further combination. The following mixtures of pre-heated β LG and LMP or LMP(NS) were prepared by mixing the two components of untreated or pre-heated LMP at the final concentration of 3.0 mg/ml (for β LG) and 1.0 mg/ml (for LMP or LMP(NS)): β LG-h+LMP, β LG-h+LMP-h, β LG-h+LMP(NS) and β LG-h+LMP(NS)-h. All solutions were made fresh prior to each use.

2.3. Electrophoretic Mobility Measurements

The ζ -potential of β LG, LMP and various β LG+LMP mixtures was determined (at 20°C) from the electrophoretic mobility measurement using the Zetasizer Nano ZS system (Malvern Instruments Ltd., Worcestershire, UK) and converted by applying the Henry equation. The solvent, same as in the HPSEC experiments, *i.e.* 50 mM NaNO₃ and 0.01% NaN₃, pH 6.50, was used for calibration prior to each measurement. Samples containing 3.0 mg/mL β LG or/and 1.0 mg/mL LMP were inserted in plastic, folded capillary cells (Malvern Instruments) possessing two metal electrodes at each end of the capillary. The instrument measures the velocity of the colloidal particles when a pulsed electric field is applied using the Laser Doppler Velocimetry technique. ζ -potential was converted into millivolts (mV) by the software, also supplied by Malvern Instruments. All electrophoretic mobility values were analyzed with at least three sample replicates and are presented as the mean $\pm$ *s.e.m.* (standard error of the mean).

2.4. High Performance Size Exclusion Chromatography (HPSEC)

The solutions containing 3.0 mg/mL β LG and/or 1.0 mg/mL LMP or LMP(NS), pre-heated (β LG-h, LMP-h, and LMP(NS)-h), various mixtures (β LG+LMP, β LG-h+LMP, β LG-h+LMP-h, β LG+LMP(NS), β LG-h+LMP(NS), and β LG-h+LMP(NS)-h) as well as mixed then heated together ([β LG+LMP]-h and [β LG+LMP(NS)]-h), were characterized by HPSEC (1200 Series, Agilent Technologies, Santa Clara, CA). The solvent delivery system consisted of a vacuum degasser, an auto sampler and a pump. The mobile phase was 0.05 M NaNO_3 and 0.01% NaN_3 (pH $\sim$ 6.50). The injection volume was 200 μL , and the flow rate was held at 0.7 mL/min. All samples were run in triplicate. Two guard columns (TSK-GEL[®] PW_{XL} 6.0 mm ID x 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 x 300 mm, 13 μm particle size, Tosoh Bioscience, Tokyo, Japan), and the other before the detectors. The column set was heated in a water bath at 35 $^{\circ}\text{C}$, connected online and in series to a UV-1260 Infinity spectrophotometer (Agilent Technologies, Santa Clara, CA), HELEOS II multi-angle laser light scattering photometer (MALLS) (Wyatt Technology, Santa Barbara, CA), model 255-V2 differential pressure viscometer (DPV) (Wyatt) and a differential refractive index (RI) detector (Wyatt). The UV/VIS, MALLS, DPV and RI detectors were aligned with bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO) and normalized with narrowly monodispersed pullulan P-50 (Shodex STANDARD P-82, JM Science, Grand Island, NY). The percentage mass of a particular biopolymer in each sample was analyzed by integrating the chromatographs into 3 or 4 individual elution peaks. The volume ranges of elution were set as 18.2-23.5, 23.5-25.1, 25.1-27.6, and 30.8-32.1 mL for peaks 1, 2, 3 and 4 respectively. Peak 4 was attributed to the monosaccharide fragments in pectin plus the added

sugar (sucrose). It was determined to be $8.75 \pm 0.19\%$ and $1.10 \pm 0.05\%$ for LMP and LMP(NS) respectively in the samples used in this work (1.0 mg/mL). The total amount of pectin, excluding sucrose, was thus estimated to be $\sim 16.2\%$ for the samples containing LMP and $\sim 23.8\%$ for those containing LMP(NS). The percentage of mass fraction (%Mass Fraction) for each elution peak was then obtained by taking the ratio of each integrated peak area over the sum of all peak areas including the sugar peak (#4). To compare the level of β LG (%) bound to LMP among various samples, the ratio (w/w) of the total β LG complexed to the total amount of LMP present was calculated as the following:

$$\text{Bound } \beta\text{LG:LMP} = \frac{75\% (\text{total } \beta\text{LG}) - \% \text{Mass Fraction (unbound } \beta\text{LG, peak 3)}}{\text{Total LMP present (\%)}}$$

The ASTRA software (v.6.1.1.17, Wyatt Technology, Santa Barbara, CA) was used in the analysis of the chromatographs. 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 LMP (Fishman, *et al.*, 2015) respectively. The extinction coefficient for each wavelength was determined from the RI concentration of each individual sample.

2.5. Statistical Analysis

All results were represented as the means of triple measurements. To evaluate the difference in ζ -potential 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.

Results and Discussion

3.1. Electrophoretic Mobility Properties of Interacting Systems of β LG and LMP

The ζ -potential of the β LG and LMP interacting system is given in Figure 1. As shown, both LMP and LMP(NS) possess far greater colloidal stability than β LG in the solvent and pH conditions used in this work. The combination of β LG (3.0 mg/mL) and LMP (1.0 mg/mL) clearly produced a biopolymer system, β LG+LMP and β LG+LMP(NS), with significantly increased colloidal stability compared to β LG alone. It should be pointed out that the difference between β LG+LMP and β LG+LMP(NS) is statistically negligible as indicated by the pairwise t-test comparison ($P > 0.05$).

Although pre-heating treatment at 80 °C for 10 min caused little change in the ζ -potential of LMP and LMP(NS), it more than doubled the value for β LG. This suggested significant structural changes in the β LG molecules as a result of thermal denaturation and aggregation (Qi, Chau, *et al.*, 2014). The various combinations of pre-heated β LG, *i.e.* β LG-h, with either untreated (LMP and LMP(NS)) or pre-heated LMP (LMP-h and LMP(NS)-h), yielded colloidal systems with greatly increased stability compared to both the native and the pre-heated β LG (β LG-h) alone ($P \ll 0.001$ by pairwise comparison). However, little difference was observed between β LG-h+LMP and β LG-h+LMP-h, and between β LG-h+LMP(NS) and β LG-h+LMP(NS)-h as well. This indicated that pre-heating pectin only caused negligible effect on the stability of the biopolymer systems formed between β LG and LMP, unlike pre-heated β LG. A similar level of stability improvement was also achieved for [β LG+LMP]-h and [β LG+LMP(NS)]-h in which β LG and LMP or LMP(NS) were first combined, then heated.

The effect of added sugar (sucrose), commonly used to standardize commercial pectin products by the manufacturers (Fishman, *et al.*, 2015), was also assessed. The colloidal stability was compared between mixed systems containing β LG and LMP and mixtures included β LG and LMP(NS). As demonstrated in Fig. 1 and analyzed by ANOVA, the interacting systems formed between β LG and LMP (~8.75% sucrose) appeared to be less sensitive to the heat treatment of β LG and LMP, $P > 0.05$, with both β LG and β LG-h excluded in the analysis. On the contrary, the absence of sucrose caused the colloidal stability to be significantly different among all mixtures ($P \ll 0.01$), namely the series of samples containing β LG and LMP(NS). This held true regardless whether β LG and β LG-h were included in the analysis. Fig. 1 points to a potential stabilization effect of sucrose on the interacting systems between β LG and LMP. An in-depth investigation of this effect was carried out using HPSEC, as discussed below.

3.2. Interaction Between β LG and LMP by HPSEC

To quantitatively characterize the molecular and hydrodynamic properties of the interacting complexes between β LG and pectin, both the original (LMP) and sucrose removed pectin (LMP(NS)), multi-detector HPSEC experiments on the various mixed systems, untreated, pre-heat treated and mixed, then followed by heating were carried out. The results of these experiments were compared with similar experiments using β LG, LMP and LMP(NS) alone, untreated and pre-heated as controls.

Representative chromatograms for samples LMP, LMP(NS), β LG+LMP, β LG+LMP(NS), β LG-h+LMP, β LG-h+LMP(NS), [β LG+LMP]-h and [β LG+LMP(NS)]-h are compared in Figures 2A-D as detected by all four online detectors, UV (at 278 nm), refractive index (dRI), 90° light scattering (LS 90°), and differential viscosity (DP) respectively. For

reasons of brevity, the chromatograms for samples β LG-h+LMP-h and β LG-h+LMP(NS)-h are not included as they do not show appreciable difference than those of β LG-h+LMP and β LG-h+LMP(NS). The HPSEC analysis of β LG agreed with our previously published results under the same experimental conditions (Qi, Chau, *et al.*, 2014) in which only the dimeric form of β LG was observed.

Figs. 2A-D illustrated that high molar mass species elutes between 18 and 24 mL in all samples, shown by the presence of considerable UV absorbance at 278 nm (Fig. 2A), refractive index (Fig. 2B), light scattering (Fig. 2C) and intrinsic viscosity (Fig. 2D). As reported in our previous studies (Fishman, *et al.*, 2015), the LMP used in this work contained 1.17% proteins. The majority of these resided in the high molecular weight fractions. The amino acid composition of these protein moieties indicated significant presence of Tyr and Phe residues in LMP, and possibly Trp as well, which are attributable to the low but significant level of absorption at 278 nm in the UV-Vis spectrum (data not shown). The chromatographs of LMP were analyzed as two peaks, 1 and 2, in which the elution volume ranged from 18.2 to 23.5 and 23.5 to 25.1 mL respectively.

Table 1 summarizes the percentage of mass fraction (%) for each peak recovered experimentally from the various mixtures of biopolymers, weight average molecular weight ($[M_w]$, kg/mol) and weight average intrinsic viscosity ($[\eta_w]$, mL/g). The average polydispersity (M_w/M_n and M_z/M_n), the Mark–Houwink-Sakurada (MHKS) exponents (a), and the average shape of space that each group of species occupies were also obtained simultaneously and are given in Table 2. The standard deviations based on a set of triplicate measurements are given in parentheses. Table 1 showed that peak 1 consists of about 24% of the total biopolymer molecules recovered from the total LMP injected with $[M_w] \sim 250$ kDa and $[\eta_w] \sim 600$ mL/g,

whereas the remaining (peak 2) ~41% are of much lower weight average molecular weight and intrinsic viscosity, $[M_w] \sim 55$ kDa and $[\eta_w] \sim 190$ mL/g. Consistent with our previous HPSEC studies on LMP (Fishman, *et al.*, 2015), the LMP sample (DE 36.8%) used in this work contained about 35% sucrose (in peak 4). Table 2 suggested that the biopolymers in peak 1 are polydispersed compact spheres, and those in peak 2 are monodispersed rigid rods on average based on the analysis of the MHKS exponents (a) (Harding, 1997) (Table 2). It however has been shown previously (Fishman, *et al.*, 2015) that LMP is composed of rigid rods, segmented rods, kinked rods and spheres. It should be noted therefore that the classification of the average biopolymer shapes (Table 2) was only a close approximation because of the complex and diverse nature of the biopolymers studied in this work. The correlation between the MHKS exponent (a) and the shape of polymer molecules was established using monodispersed model synthetic polymers (Harding, 1997). It is only reasonable to assume that natural biopolymers such as pectin and pectin-protein complexes possessed a vastly variety of sizes and shapes. Nevertheless, analysis from the MHKS exponent (a) yielded invaluable information about the average shapes of the global conglomerates (Table 2), and provided a useful way to carry out comparable studies in this work.

Table 1 demonstrated clearly that the simple addition of β LG to LMP significantly reduced the relative fraction of the high molar mass species (in peak 1) by 10%, to ~14%. An increase of more than 70 kDa in $[M_w]$ (to ~320 kDa) and a sharp drop in $[\eta_w]$ to 370 mL/g from $[\eta_w] \sim 595 \pm 10$ mL/g in peak 1 for LMP, suggesting approximately two dimeric β LG molecules were bound per LMP molecule in this group. The biopolymers in peak 2 accounted for ~8.4% of the total species eluted and with markedly elevated $[M_w] \sim 153$ kDa, and reduced $[\eta_w] \sim 30$ mL/g from ~190 mL/g, indicating an increased level of β LG in this group of complexes, an equivalent

of at least additional three dimeric β LG molecules per LMP molecule on average. This redistribution (%Mass Fraction) between peaks 1 and 2 was evidently the result of interacting complexes formed between β LG and LMP, which tend to boost the share of the biopolymers with higher molar mass. Occupying nearly 69% of the total eluted species, the unbound dimeric β LG dominated the β LG+LMP system, and maintained the values of $[M_w]$ and $[\eta_w]$ for peak 3 similar to that of native β LG. The net amount of β LG bound to LMP was therefore estimated to be about 6%, and an equivalent of ratio of bound β LG:LMP=0.37:1 (in peaks 1 and 2 combined).

Furthermore, as shown in Table 2, the interactions between β LG and LMP occurred upon direct combination also significantly altered the hydrodynamic properties of the LMP and β LG from the single component system that each existed. Instead of compact spheres and rigid rods in the LMP only system as discussed above, interacting with β LG caused the complexes in the β LG+LMP system to become largely random coils, polydispersed and monodispersed for peak 1 and 2, respectively. Changes to the unbound dimeric β LG were even more dramatic due to the presence of LMP as it was transformed into a monodispersed shape that resembles more of a random coil on average than a highly compact sphere, as expected for a native form β LG. This was in good agreement with our previously published spectroscopic studies (Qi, Wickham, & Garcia, 2014) on the interactions between β LG and sugar beet pectin, in which β LG molecules underwent denaturation upon binding to pectin. As it was reported previously (Kim, *et al.*, 2003; Lee, *et al.*, 1981), high concentration of sucrose (> 0.5 M) could stabilize globular proteins by preferential exclusion. The presence of sucrose (~8.75%) in the mixture of β LG+LMP may not be sufficient to stabilize the molecular structure of β LG from the denaturing effect of LMP.

Figure 3 contains the molar mass distribution curves obtained by refractive index (dRI) for LMP, LMP(NS), β LG+LMP, β LG+LMP(NS), β LG-h+LMP, β LG-h+LMP(NS),

[β LG+LMP]-h and [β LG+LMP(NS)]-h in which the molar mass ($\log(M_w)$, g/mol) is compared among all species as a function of elution time/volume. Evidently, the interactions of β LG with LMP and LMP(NS), as in samples β LG+LMP and β LG+LMP(NS), produced biopolymers with increasing molar mass than LMP and LMP(NS) alone spanning regions of peaks 1 and 2. In addition, peak 2 area saw the most pronounced surge in both the size of the biopolymers and the noise level of the curves (due to increased light scattering) for the interacting systems, indicating that the binding of β LG prefers the group of LMP with lower $[M_w]$. Figure 3 also demonstrated that the presence of added sucrose in LMP appeared to facilitate the interaction between native β LG and the group of LMP with higher $[M_w]$ (peak 1). It, however, did not show appreciable effect on the binding of β LG to that of LMP with smaller $[M_w]$ (peak 2 region).

3.3. Effect of Pre-heating Treatment of β LG and LMP on the Interacting Complexes

It has been demonstrated previously (Qi, Chau, *et al.*, 2014) that the amount of β LG involved in the interactions with sugar beet pectin was increased by pre-heating β LG at 80 °C for 10 min. In this work, the HPSEC studies revealed that pre-heated β LG, β LG-h, contained 6.3% high $[M_w]$ ($\sim 1,600$ kDa) species (peak 1) due to heat-induced denaturation and aggregation formed by intermolecular S-S bonds. These aggregates were highly polydispersed and behaved more like compact spheres on average (Table 2). The pre-heating treatment of LMP, the LMP-h sample, on the other hand, didn't cause the physiochemical properties of LMP to change noticeably in both peaks 1 and 2, as shown in Figures 3A-C and Table 1. The only significant ($P < 0.01$) difference is that pre-heating increased the $[M_w]$ of those pectin molecules in peak 1 by 22 kDa (Table 1).

The combination of preheated β LG and untreated LMP, namely sample β LG-h+LMP, increased the fractions of interacting complexes, notably in peak 1 to 19% (Table 1) from 14% in the untreated mixture, β LG+LMP. The relative mass fraction of peak 2 remained roughly the same for both mixtures. The amount of unbound dimeric β LG (peak 3) was reduced to 65% from 69% (in β LG+LMP). The extremely high $[M_w]$ ($\sim 1,355 \pm 14$ kDa) for peak 1 was consequently the result of binding interactions between the heat-induced β LG aggregates and LMP of high $[M_w]$. Additionally, Table 1 attested that there was lessened involvement of LMP of high $[M_w]$ in this group of complexes compared to that of β LG+LMP due to the significantly reduced intrinsic viscosity, $[\eta_w] \sim 290$ from 414 mL/g. Similarly, the biopolymers in peak 2 also contained declined level of LMP with $[\eta_w] \sim 20$ as opposed to 30 mL/g for that of β LG+LMP. The weight average molar mass remained roughly the same, $[M_w] \sim 164$ kDa, suggesting that the complexes in peak 2 might consist of heat-treated β LG dimers or oligomers rather than large aggregates. As a result, the total amount of β LG bound to LMP was estimated to be $>10\%$. Thus the level of β LG bound to LMP increased to a ratio of 0.63:1. This ratio was nearly double the amount that in the untreated mixture. Pre-heating β LG also changed the hydrodynamics properties of the interacting complexes with LMP when compared to the untreated mixed pair. This is most notable for the complexes in peak 2, which assumed the average shape of LMP, namely monodispersed rigid rods, as shown in Table 2.

Combining β LG and LMP, both pre-heated, *i.e.* sample β LG-h+LMP-h, led to a reduced $[M_w] \sim 1,193 \pm 13$ kDa for peak 1 compared with $\sim 1,355 \pm 14$ kDa for β LG-h+LMP. The physiochemical properties of the interacting complexes in peak 2 including their distributions (%Mass Fraction), $[M_w]$, $[\eta_w]$, polydispersities and average shapes were roughly

indistinguishable. Despite the nominal loss (~2%) in the unbound β LG (peak 3), the ratio of β LG in the complexes relative to the total LMP, dropped to 0.51 from 0.63 as was the case of β LG-h+LMP sample (Table 1). This loss is indicative that pre-heat treatment of LMP impeded the complex formation between β LG-h and LMP. This is especially true for the complexes containing LMP with the highest $[M_w]$ (*i.e.* peak 1).

Heat treatment of the mixture of β LG and LMP, sample $[\beta$ LG+LMP]-h, on the other hand, significantly increased the $[M_w]$ of the complexes in both peaks 1 and 2, to nearly 2,000 and 1,350 kDa respectively. The relative distributions of these two peaks also shifted to 19% for peak 1, a 5% gain, and a nearly equal loss for peak 2, to ~5% from that of the β LG+LMP sample. The total amount of unbound β LG remained unchanged, at ~68%, suggesting that heat treatment of the mixture didn't increase the total level of complex formed between β LG and LMP. The intrinsic viscosity $[\eta_w]$ for peak 1 underwent a large reduction, to 334 ± 5 from 414 ± 3 mL/g, indicating a greatly enhanced level of β LG (as oligomers or aggregates) in this group of complex. Changes in $[\eta_w] \sim 33 \pm 3$ for peak 2 from 30 ± 0.5 mL/g for β LG+LMP, appeared insignificant ($P > 0.05$). This demonstrated that heating didn't change the involvement of LMP in these complexes in peak 2. The total ratio of β LG in the binding complexes (relative to the total LMP) increased noticeably when heated together to 0.43 from 0.37 for the pair without heat treatment (Table 1), and this gain clearly occurred in peak 1 rather than peak 2.

Interestingly, instead of dimers as in its native state, the unbound β LG apparently was transformed into tetramers (peak 3) with $[M_w] \sim 75.7 \pm 4.0$ kDa when subjected to heating in the mixture with LMP. This is likely due to the denaturation of dimeric β LG in the presence of LMP, as discussed above. Furthermore, these unbound random coil-like shaped tetrameric β LG molecules are highly polydispersed, unlike those samples without heat treatment. The binding

complexes of high molar mass formed in peaks 1 and 2 in [β LG+LMP]-h mostly assumed the shape of polydispersed random coils and monodispersed rigid rods respectively (Table 2).

3.4. The Effect of Sucrose on the Structure of LMP

The effect of added sucrose in the commercial LMP on the interactions between β LG and LMP was examined by comparing systems with LMP and with LMP(NS) (sucrose removed LMP). The chromatographs in Figs. 2A-D were essentially superimposable for the two kinds of pectin. The analysis from the HPSEC studies revealed less than 4% sugar (peak 4) remained in LMP(NS) compared to ~35% in LMP. The relative distributions of peaks 1 and 2 are 37% and 63%, and 34% and 66% for LMP and LMP(NS), respectively. The molar mass distribution curves obtained from the refractive index detector (dRI) for LMP and LMP(NS) in Fig. 3 were nearly identical as a function of elution volume. The molecular characteristics (Table 1) and hydrodynamic properties (Table 2) also demonstrated insignificant difference between LMP and LMP(NS).

Figures 4A-D compare changes that occur in the radius of gyration (R_{g_z}) and hydrodynamics radius ($R_{h_{zv}}$) as a function of elution volume. The sucrose removed LMP (LMP(NS)) seemed to have larger R_{g_z} values overall, especially, for those molecules with extremely large and small $[M_w]$ (Fig. 4A). As shown by Burchard (Burchard, 1996), taking the ratio of R_{g_z} and $R_{h_{zv}}$, $\rho (= R_{g_z}/R_{h_{zv}})$ provides information on the structure of macromolecules which can change with concentration. Figure 5 displays plots of $\rho = R_{g_z}/R_{h_{zv}}$ as a function of elution volume, and shows clearly that pectin molecules became more open and flexible in solution when sucrose was absent (LMP(NS)). This is particularly true for the lower $[M_w]$ fraction (peak 2), and for part of peak 1, to a lesser extent. The resulting weight average ρ value

was thus expected to be slightly higher for the sucrose removed LMP, which was consistent with our previous findings (Fishman, *et al.*, 2015). We propose that preferential exclusion of sucrose from the LMP surface or preferential solvation of sucrose (Fishman, Cooke, Chau, Coffin, & Hotchkiss, 2007) may be attributable to the less open and less flexible structure, in a similar fashion as the effect of sucrose on proteins (Kim, *et al.*, 2003; Lee, *et al.*, 1981).

Similar to LMP with sucrose (~9% as determined for LMP solution), pre-heating LMP in its absence, LMP(NS)-h, did not appear to cause the physiochemical properties of LMP(NS) to change noticeably.

3.5. The Effect of Sucrose on the Interactions Between β LG and LMP

The combination of β LG and LMP(NS) also produced biopolymers with increased $[M_w]$ in both peaks 1 and 2 compared to LMP(NS), as shown in Table 1. When compared with β LG+LMP, however, the $[M_w]$ for peaks 1 and 2, 298 ± 3 and 122 ± 8 kDa, were about 39 and 30 kDa lower respectively. This reduction approximated about one less bound dimeric β LG molecule in the complexes in peaks 1 and 2. It suggested diminished binding capability of LMP(NS) to β LG (see Fig. 3). Consistent with this observation, the ratio of bound β LG to total LMP also saw a drastic reduction, from 0.37 to 0.25 (Table 1) for the sucrose depleted LMP pair. Furthermore, the decrease in $[\eta_w]$ to 361 ± 1 from 414 ± 3 mL/g indicated a declined involvement of LMP when combined with β LG in the absence of sucrose in peak 1. On the other hand, a slight increase in $[\eta_w]$ to 35.2 ± 1.0 from 29.9 ± 0.5 mL/g in peak 2 suggested a small boost in the level of LMP in β LG+LMP(NS) when compared with β LG+LMP. These opposing effects of sucrose on peaks 1 and 2 lead to the overall reduced ratio of bound β LG to LMP for β LG+LMP(NS).

Table 2 illustrated that the hydrodynamic properties of the interacting complexes between β LG and LMP (peaks 1 and 2), as well as the unbound dimeric β LG (peak 3) remained essentially unaffected by sucrose.

Figures 4B and 5 showed greater R_{gz} values for the complexes formed between β LG and LMP in peak 1 in the absence of sucrose than in its presence. The reverse was true, however, for the complexes in peak 2. Because the difference in $R_{h_{zv}}$ was negligible for both peaks, a greater ρ for peak 1 and a lower ρ value for peak 2 were obtained. Such behavior of ρ for peak 1 suggested that the structure of the complexes is more flexible and open when sucrose was depleted, similar to the cases of LMP(NS) and LMP (discussed above). In contrast, the complexes in peak 2 are less flexible and less open for β LG+LMP(NS) than for β LG+LMP. This indicated that the binding interactions between the native dimeric β LG and LMP of smaller $[M_w]$ were rather insensitive to sucrose, as supported by past research (Arakawa, *et al.*, 1982).

The complexes formed from the combination of pre-heated β LG (β LG-h) and LMP(NS) also displayed increased $[M_w]$ for both peaks 1 and 2 compared to the untreated pair, β LG+LMP(NS). They were 900 ± 6 and 159 ± 6 kDa respectively. The intrinsic viscosities $[\eta_w]$ for these biopolymers were 297 ± 3 and 24.8 ± 2.0 mL/g respectively. Both these values were lower than those for β LG+LMP(NS), which revealed increased involvement of β LG in these complexes. The ratio between bound β LG and total LMP (Table 1) was estimated to be 0.36, an increase from 0.25 as found for β LG+LMP(NS).

When β LG-h+LMP(NS) was compared with complexes formed in β LG-h+LMP, it showed that the $[M_w]$ of peak 1 dropped considerably, namely by over 400 kDa. This decrease in $[M_w]$ suggested reduced binding capability of β LG aggregates to the group of LMP molecules with higher $[M_w]$ in the absence of sucrose. Whereas the $[M_w]$ for peak 2 (within the standard

deviations) remained roughly unchanged by sucrose. The $[\eta_w]$ values also increased noticeably for both peaks 1 and 2, indicating expanded levels of LMP in these two groups of complexes. Consequently, the ratio between bound β LG and total LMP (0.36, Table 1) saw a drastic decline from that of β LG-h+LMP (0.63). This apparently attested that sucrose (~8.75%) facilitated the interaction between β LG-h and LMP. Moreover, Table 2 shows that sucrose bears little effect on the hydrodynamic properties of these complexes between β LG-h and LMP.

Figures 4C and 5 illustrated that Rh_{zv} retained much lower values for the complexes formed in β LG-h+LMP(NS) than in β LG-h+LMP, especially in peak 1. Nevertheless, little difference in Rg_z was observed in peak 1 and only a slight increase in peak 2 in the absence of sucrose. As a result, slightly yet clearly elevated ρ values were reached for all complexes formed in β LG-h+LMP(NS), in both peaks 1 and 2. This implies that the structure of these interacting biopolymers, random coil like and rigid rod shapes, are more flexible and open in the absence of sucrose than its presence.

When both pre-heated β LG and LMP(NS) were combined to produce sample β LG-h+LMP(NS)-h, the resulting complexes acquired lowered $[M_w] \sim 761 \pm 7$ and 138 ± 7 kDa for both peaks 1 and 2 than those formed in β LG-h+LMP(NS), $[M_w] \sim 900 \pm 6$ and 159 ± 6 kDa respectively. The intrinsic viscosities were essentially the same for each peak in both samples. This resembled to the cases of the analogous samples in the presence of sucrose, as discussed in Section 3.3. Although the amount of unbound β LG (peak 3) was nearly identical, ~8.0%, the ratio of bound β LG to the total LMP dropped to 0.30 from 0.36 in β LG-h+LMP(NS) (Table 1). This loss demonstrated that pre-heat treatment of LMP(NS) also hindered its ability to interact with β LG-h, in much the same manner as in β LG-h+LMP-h (when sucrose was present). Such impediment is particularly pronounced for the complexes in peak 1.

3.6. The Effect of Sucrose on Heated β LG and LMP Mixtures

Heat treatment of the mixture of β LG and LMP(NS), sample [β LG+LMP(NS)]-h, yielded complexes with significantly increased $[M_w] \sim 4,335 \pm 322$ and $2,197 \pm 69$ kDa in peaks 1 and 2 respectively. Both peaks constituted species with the highest weight average molar mass observed among all samples in this work. The total amount of unbound β LG was determined to be $\sim 67.4\%$ (Table 1), which is a noticeable reduction from 68.9% for the mixture without heating, β LG+LMP(NS). This suggested that heat treatment increased the level of β LG in the complexes slightly. The intrinsic viscosities ($[\eta_w]$) were also decreased to 322 ± 9 and 38.0 ± 0.2 mL/g from 361 ± 1 and 35.2 ± 1.0 mL/g, mL/g for peaks 1 and 2 in the unheated pair respectively. These reductions further indicated an elevated degree of participation by β LG (as oligomers or aggregates) in these complexes. As a result, the ratio of bound β LG to LMP(NS) increased noticeably to 0.32 from 0.25 for the β LG+LMP(NS) (Table 1). Table 2 showed that the binding complexes formed in [β LG+LMP(NS)]-h are random coil-like shapes on average.

Comparison between samples [β LG+LMP(NS)]-h and [β LG+LMP]-h revealed that the complexes possessed greatly increased $[M_w]$ in the absence of sucrose than its presence ($\sim 8.75\%$) (Table 1). The intrinsic viscosities ($[\eta_w]$) remained statistically unchanged ($P > 0.05$) despite the diminished ratio of bound β LG to LMP, which is 0.32 for [β LG+LMP(NS)]-h compared to 0.43 for [β LG+LMP]-h (Table 1). This declined binding of β LG to LMP(NS) suggested that the β LG aggregates (prompted by heating) propagated into species of even higher molecular weight in the absence of sucrose, which may not be conducive for forming complexes. The binding complexes of high molar mass formed in [β LG+LMP(NS)]-h assumed random coil-

like shapes in both peaks 1 and 2 (Table 2). This is in clear contrast to the complexes formed in the presence of sucrose ([β LG+LMP]-h), notably for peak 2, in which case the rigid rod shapes on average were adopted.

Furthermore, the weight average molecular weight [M_w] $\sim 120 \pm 2.0$ kDa for peak 3 of [β LG+LMP(NS)]-h, *i.e.* the unbound β LG corresponded to approximately 3.5 dimeric β LG molecules. Whereas the unbound β LG existed in the form of tetramers with [M_w] $\sim 75.7 \pm 4.0$ kDa in the presence of sucrose, namely sample [β LG+LMP]-h. We hypothesize that this apparent oligomerization of β LG dimers is likely due to the intermolecular crosslinking among the (heat induced) denatured dimeric β LG in the absence of sucrose. Therefore, it is evident that sucrose played an important role in stabilizing β LG against heat treatment in the mixture with LMP. Additionally, these unbound oligomeric β LG molecules are polydispersed and assumed the shapes of random coils on average.

Figure 4D showed nearly an equal amount of increase in both R_{gz} and $R_{h_{zv}}$ for the complexes formed between β LG and LMP(NS) upon heating compared to those between β LG and LMP. The resulting ρ values were essentially identical for both samples (Fig. 5). The effect of sucrose on the structures of the complexes was therefore insignificant. The flexibility and openness of these complexes are inversely proportional to their weight average molar mass ([M_w]).

Conclusions

It has been demonstrated that soluble complexes between β LG and LMP were formed when combined at 3:1 (w/w) at near neutral pH (~ 6.5). The resulting biopolymers possessed increased weight average molecular weights ([M_w]) and altered molecular shapes compared to the

controls, β LG and LMP, in all cases. Heat-induced (80 °C for 10 min) denaturation of β LG greatly enhanced the participation of β LG (as aggregates) in the interacting complexes. Pre-heating treatment of LMP, in contrast, impeded its ability to bind β LG despite negligible effect on the hydrodynamic properties. Furthermore, heat treatment of the mixture of β LG and LMP significantly increased the sizes of the resulting complexes, but failed to boost the level of interactions appreciably. It was found that sucrose, commonly found in commercial LMP, caused the LMP molecule to become less flexible and less open (at ~35%). When present (~8.75%) in the mixtures containing β LG and LMP, sucrose greatly facilitated the interactions between heat-induced β LG aggregates and LMP. Presumably, sucrose contributed to the stabilization of β LG aggregates through preferential exclusion prior to or coincide with their interactions with LMP. It however showed a far less drastic effect on the interactions between native dimeric β LG and LMP. Additionally, results also indicated that sucrose played an important role in maintaining the molecular structures of the complexes formed between β LG aggregates and LMP less flexible and less open.

Figure Legends

Figure 1. ζ -Potentials (mV) of various β LG and LMP solutions (0.05 M NaNO_3 , and 0.01% NaN_3 , pH 6.50) at 20 °C. Error bar represents standard error of the mean (*s.e.m.*).

Figure 2. Superimposed HPSEC chromatograms of various β LG and LMP mixtures as detected by online (A) UV 278 nm; (B) Refractive Index (dRI); (C) 90° Light Scattering (LS); and (D) Intrinsic Viscosity (DP) respectively. The concentration was 3.0 and 1.0 mg/mL for β LG and LMP respectively. The y-axis was normalized to unity in each graph.

Figure 3. Comparison of molar mass distributions of biolymers in β LG and LMP mixtures as analyzed from the refractive index detector (dRI) of the HPSEC system. The concentration of the mixture was 3.0 and 1.0 mg/mL for β LG and LMP respectively.

Figure 4. Changes in radii for the interacting systems containing β LG and LMP as a function of elution volume. The *rms* radius of gyration (R_{gz}) and hydrodynamic radius ($R_{h_{zv}}$) of (A) LMP and LMP(NS), (B) β LG+LMP and β LG+LMP(NS), (C) β LG-h+LMP and β LG-h+LMP(NS), and (D) [β LG+ LMP]-h and [β LG+LMP(NS)]-h.

Figure 5. Comparison of ρ ($R_{gz}/R_{h_{zv}}$) values as a function of elution volume for the interacting systems containing β LG and LMP (as indicated in black lines), and those consisted of β LG and LMP(NS) (in grey lines).

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800 Table 1. Molecular characteristics of β LG (3.0 mg/ml) and LMP (1.0 mg/ml) as studied by
801 HPSEC^a.

Sample	Elution Peak # ^b	%Mass Fraction ^c	Bound		
			β LG:LMP (w/w) ^d	[M _w] (kDa)	[η _w] (mL/g)
β LG	3	93.5(1.0)		34.2(0.6)	4.1(0.3)
β LG-h	1	6.3(0.1)		1,606(39)	10.1(2.0)
	3	84.2(0.1)		36.0(0.7)	4.4(0.1)
LMP	1	24.6(0.4)		246(6)	595(10)
	2	41.2(0.2)		55.5(3.0)	191(4)
LMP-h	1	23.7(0.1)		268(4)	590(7)
	2	40.4(0.5)		52.4(0.2)	191(2)
β LG+LMP	1	14.1(0.1)	0.37	339(10)	414(3)
	2	8.4(0.1)		153(12)	29.9(0.5)
	3	68.9(0.1)		35.6(0.5)	4.3(0.1)
β LG-h+LMP	1	19.4(0.2)	0.63	1,355(14)	289(1)
	2	7.4(0.1)		164(12)	20.2(1.0)
	3	64.7(0.4)		35.4(0.9)	4.4(0.2)
β LG-h+LMP-h	1	17.6(0.2)	0.51	1,193(13)	292(3)
	2	6.7(0.1)		165(1)	22.9(2)
	3	66.7(0.3)		34.9(0.3)	4.5(0.1)
$[\beta$ LG+LMP]-h	1	18.6(0.1)	0.43	1,927(82)	334(5)

	2	4.9(0.1)		1,342(108)	33.3(3)
	3	68.0(0.2)		75.7(4.0)	4.4(0.2)
LMP(NS)	1	32.7(1.0)		257(11)	594(20)
	2	62.9(1.0)		53.1(2.0)	193(7)
LMP(NS)-h	1	35.5(0.7)		263(6)	594(10)
	2	62.0(1.0)		47.2(2)	215(4)
β LG+	1	23.9(0.1)	0.25	298(3)	361(1)
LMP(NS)	2	6.1(0.1)		122(8)	35.2(1)
	3	68.9(0.1)		34.7(0.4)	4.6(0.1)
β LG-h+	1	24.7(0.2)	0.36	900(6)	297(3)
LMP(NS)	2	7.8(0.8)		159(6)	24.8(2)
	3	66.5(0.1)		35.2(0.3)	4.5(0.4)
β LG-h+	1	23.1(0.1)	0.30	761(7)	300(1)
LMP(NS)-h	2	8.0(0.1)		138(7)	25.8(1)
	3	67.9(0.3)		34.6(0.4)	4.5(0.1)
[β LG+	1	26.0(0.1)	0.32	4,335(103)	322(9)
LMP(NS)]-h	2	5.6(0.1)		2,197(69)	38.0(0.2)
	3	67.4(0.1)		120(2)	4.6(0.2)

^aAll values were averaged between a set of triple refractive index (dRI) measurements.

Standard deviations from the triplicate analyses are given in parenthesis.

^bPeak # was assigned as described in the Materials and Methods section.

^cPercentage (%) of an integrated peak area over the total of all eluted peak areas.

^dEstimated as described in the Materials and Methods section.

807 Table 2. Hydrodynamic properties of interacting systems of β LG (3.0 mg/ml) and LMP (1.0
808 mg/ml) as studied by HPSEC^a.

Sample	Elution Peak #	M _w /M _n	M _z /M _n	<i>a</i>	Average shape of space occupied ^b
β LG	3	1.03(0.01)	1.08(0.02)	0.299(0.20)	Compact sphere
β LG-h	1	6.28(0.10)	63.2(6.0)	0.103(0.005)	Compact sphere
	3	1.03(0.01)	1.07(0.02)	0.276(0.20)	Compact sphere
LMP	1	1.41(0.02)	2.29(0.1)	0.484(0.01)	Compact sphere
	2	1.16(0.03)	1.40(0.2)	2.79(0.5)	Rigid rod
LMP-h	1	1.55(0.08)	2.77(0.3)	0.441(0.004)	Compact sphere
	2	1.25(0.01)	1.47(0.02)	2.29(0.06)	Rigid rod
β LG+LMP	1	1.83(0.03)	5.90(0.70)	0.729(0.01)	Random coil ^c
	2	1.06(0.02)	1.12(0.03)	0.828(0.10)	Random coil
	3	1.03(0.01)	1.07(0.01)	0.727(0.20)	Random coil
β LG-h+LMP	1	2.07(0.01)	4.28(0.08)	0.734(0.02)	Random coil
	2	1.09(0.02)	1.18(0.03)	2.39(0.10)	Rigid rod
	3	1.03(0.01)	1.10(0.03)	0.788(0.20)	Random coil
β LG-h+	1	3.05(0.03)	4.21(0.09)	0.770(0.02)	Random coil
LMP-h	2	1.08(0.02)	1.18(0.03)	2.27(0.40)	Rigid rod
	3	1.02(0.01)	1.06(0.02)	1.07(0.20)	Random coil
[β LG+	1	1.47(0.01)	3.41(0.09)	0.796(0.03)	Random coil

LMP]-h	2	1.06(0.02)	1.14(0.01)	1.57(0.20)	Rigid rod
	3	1.25(0.02)	2.11(0.08)	0.548(0.01)	Random coil
LMP(NS)	1	1.45(0.04)	2.48(0.30)	0.459(0.02)	Compact sphere
	2	1.18(0.02)	1.36(0.03)	2.42(0.20)	Rigid rod
LMP(NS)-h	1	1.49(0.02)	2.61(0.10)	0.435(0.01)	Compact sphere
	2	1.17(0.02)	1.36(0.04)	2.15(0.03)	Rigid rod
β LG+	1	1.61(0.01)	4.11(0.10)	0.888(0.06)	Random coil
LMP(NS)	2	1.03(0.00)	1.06(0.01)	1.02(0.40)	Random coil
	3	1.02(0.01)	1.07(0.02)	0.924(0.10)	Random coil
β LG-h+	1	1.78(0.02)	3.00(0.04)	0.879(0.02)	Random coil
LMP(NS)	2	1.05(0.02)	1.10(0.03)	2.73(0.10)	Rigid rod
	3	1.02(0.01)	1.06(0.01)	1.21(0.08)	Random coil
β LG-h+	1	1.75(0.07)	2.93(0.09)	0.888(0.04)	Random coil
LMP(NS)-h	2	1.05(0.01)	1.09(0.01)	3.30(0.10)	Rigid rod
	3	1.02(0.02)	1.04(0.03)	1.21(0.20)	Random coil
[β LG+	1	1.93(0.01)	5.16(0.05)	0.587(0.02)	Random coil
LMP(NS)]-h	2	1.04(0.01)	1.08(0.01)	0.695(0.30)	Random coil
	3	1.39(0.01)	2.82(0.01)	0.541(0.01)	Random coil

^aAll values were averaged between a set of triple refractive index (dRI) measurements.

Standard deviations from the triplicate analyses are given in parenthesis.

^bAssessed based on the Mark-Houwink-Sakurada exponent values (a) (Harding, 1997).

^cThe term “random coil” used in this table represents a close approximation to the synthetic

model polymers from which the correlation with the MHKS exponent (a) was established.

814 Figure 1.

816 Figure 2.

821 Figure 3.

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Weight average molecular characteristics of interacting systems containing β LG (0.3 mg/mL) and LMP (0.1 mg/mL) as studied by HPSEC. Data were analyzed by integrating the chromatographs as peaks 1, 2, 3 and 4. (A) Experimental % Mass Fraction and estimated bound β LG:LMP (w/w); (B) weight average molar mass ($[M_w]$); and (C) weight average intrinsic viscosity ($[\eta_w]$). Error bar represents standard deviation of a set of triple RI measurements.

Figure Legends

Figure 1. ζ -Potentials (mV) of various β LG and LMP solutions (0.05 M NaNO_3 , and 0.01% NaN_3 , pH 6.50) at 20 °C. Error bar represents standard error of the mean (*s.e.m.*).

Figure 2. Superimposed HPSEC chromatograms of various β LG and LMP mixtures as detected by online (A) UV 278 nm; (B) Refractive Index (dRI); (C) 90° Light Scattering (LS); and (D) Intrinsic Viscosity (DP) respectively. The concentration was 3.0 and 1.0 mg/mL for β LG and LMP respectively. The y-axis was normalized to unity in each graph.

Figure 3. Comparison of molar mass distributions of biolymers in β LG and LMP mixtures as analyzed from the refractive index detector (dRI) of the HPSEC system. The concentration of the mixture was 3.0 and 1.0 mg/mL for β LG and LMP respectively.

Figure 4. Changes in radii for the interacting systems containing β LG and LMP as a function of elution volume. The *rms* radius of gyration (R_{gz}) and hydrodynamic radius ($R_{h_{zv}}$) of (A) LMP and LMP(NS), (B) β LG+LMP and β LG+LMP(NS), (C) β LG-h+LMP and β LG-h+LMP(NS), and (D) [β LG+ LMP]-h and [β LG+LMP(NS)]-h.

Figure 5. Comparison of ρ ($R_{gz}/R_{h_{zv}}$) values as a function of elution volume for the interacting systems containing β LG and LMP (as indicated in black lines), and those consisted of β LG and LMP(NS) (in grey lines).