Thermodynamic Parameters of β-Lactoglobulin–Pectin Complexes Assessed by Isothermal Titration Calorimetry

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Isothermal titration calorimetry (ITC) was used to determine the binding constant, stoichiometry, enthalpy, and entropy of β-lactoglobulin/low- and high-methoxyl pectin (β-lg−LM- and HM-pectin) complexes at 22 °C and at pH 4. The binding isotherms revealed the formation of soluble intrapolymer complexes (C1) further followed by their aggregation in interpolymer complexes (C2). The interaction between β-lg and LM- or HM-pectin in C1 and C2 occurred spontaneously with a Gibbs free energy around −10 kcal/mol. The C1 were enthalpically driven, whereas enthalpic and entropic factors were involved in the C2 formation. Because ITC did not allow the dissociation of different enthalpic contributions, the values measured as pectin and β-lg interacted could partially be attributed to conformational changes. The C1 had a binding stoichiometry of 8.3 and 6.1 β-lg molecules complexed per LM- or HM-pectin molecule, respectively. The C2 had about 16.5 and 15.1 β-lg molecules complexed per LM- and HM-pectin, respectively.

KEYWORDS: β-Lactoglobulin; pectin; interactions; isothermal titration calorimetry

INTRODUCTION

The β-lactoglobulin (β-lg) is a small globular protein of 18 350 Da. The β-lg has a well-known structure containing mainly β-sheets, some β-turns, and one α-helix (*1*). Its quaternary structure is influenced by pH, mineral content, and temperature. This protein is monomeric at pH below 3 or above 8, dimeric at neutral pH, and found in both states between pH 4 and 6 (*2*). The β-lg may form a few octamers around pH 4.5 (*3*), but some authors are doubtful about the existence of such an assembly (*2*, *4*). Pectin is a cell-wall polysaccharide, generally extracted from apple pomace or citrus fruits. This anionic polysaccharide is essentially made up of D-galacturonic residues (D-galA). High-methoxyl (HM) pectin occurs when more than 50% of its carboxylic groups are esterified, whereas low-methoxyl (LM) pectin is found when less than 50% of these groups are esterified (*5*).

The interactions between proteins and polysaccharides can lead to the formation of complexes. These complexes, or coacervates, have many applications, including fat substitution (*6*), protein separation (*7*), microencapsulation (*8*), and enzyme immobilization (*9*). The nature of protein/polysaccharide complexes is influenced by entropic factors, such as the structure, and the molecular weight of biopolymers. The complexes are also influenced by enthalpic forces, which are regulated by the protein/polysaccharide ratio and the nature and density of charges on the biopolymers. Many studies on protein/polysaccharide interactions have been carried out (*10*−*13*). Ultrafiltration used with destabilizing agents showed that interactions between β-lg and pectin occurred through ionic and hydrogen bonds, whereas the hydrophobic interactions were negligible (*14*). The measurement of thermodynamic parameters, such as binding constant, enthalpy, entropy, and binding stoichiometry of interactions, is essential to accurately evaluate binding conditions. Binding parameters from biopolymer systems are sometimes hard to quantify because of the heterogeneity of the natural material. Consequently, few quantitative studies have been carried out on the interactions between β-lactoglobulin (β-lg) and polysaccharides (*15*). Thermodynamic quantities involved in biopolymer interactions are often indirectly determined through noncalorimetric experimental methods (*16*). These methods involve the calculation of thermodynamic parameters from theoretical relationships.

Enthalpy variations (i.e., gain and loss) are the easiest thermodynamic parameters to measure during complexation. Isothermal titration calorimetry (ITC) is currently the only technique used to directly measure the enthalpy for many ternary mixtures (*16*). ITC was used to study the interaction between β-lg and LM- or HM-pectin at pH 4 in 5 mM of sodium phosphate buffer. To our knowledge, this technique has not yet been used to carry out a binding study involving two biopolymers having heterogeneous molecular weights.

MATERIALS AND METHODS

Materials. Bovine β-lg A and B (3X crystallized) was purchased from Sigma-Aldrich Canada Ltd (Oakville, On, Canada) as 95% pure protein. Low-methoxyl pectin (DE 28.3%, apparent molecular weight 94.3 kDa) and high-methoxyl pectin (DE 73.4%, apparent molecular weight 118 kDa) were donated by Hercules Copenhagen A/S (Copenhagen, Denmark). All reagents were of analytical grade and used as received.

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Preparation of Solutions. To eliminate excess salts, β-lg and LM- and HM-pectin were dialyzed against deionized water for 24 h at 4 °C. Afterward, β-lg and LM- or HM-pectin were dialyzed extensively in the same beaker against a 5-mM phosphate buffer (pH 4) to equilibrate the pH and ionic strength of the solutions. The weak concentration of the buffer was used to prevent dissociation of β-lg and pectin complexes, which are very sensitive to ionic strength as demonstrated earlier (*14*). The dialysis buffer was preserved for ITC experiments. The dialysis was critical to avoid inaccuracies inherent to buffer mismatching. Ionic strength or pH differences could have lead to modifications in the dimer/monomer equilibrium of the β-lg (*2*, *17*). The β-lg, LM-, and HM-pectin solutions and buffer were filtrated through a 0.22-μm membrane to eliminate insoluble particles. The concentration of dialyzed β-lg was measured with the bicinchoninic acid protein assay (*18*). The LM- and HM-pectin concentrations were assayed with the phenol-sulfuric acid method (*19*). A dialysis buffer was used as solvent to adjust the LM- or HM-pectin and β-lg solutions to around 10 and 1500 μM (monomeric equivalent), respectively. All solutions were degassed 10 min at 20 °C prior to ITC measurements by means of a device provided with the ITC apparatus.

Isothermal Titration Calorimetry (ITC). An isothermal titration calorimeter (VP-ITC, Microcal Inc., Northampton, MA) was used to measure enthalpy values. After setting the temperature of solutions at 20 °C in the degassing device, the apparatus was equilibrated at 22 °C for at least half an hour before the measurement. The LM- or HM-pectin solution in the 1.445-mL reaction cell was titrated with 50 successive 5-μL injections of β-lg solution. Each addition lasted 12 s with an interval of 800 s between consecutive injections. This long resting time between injections was necessary to reach thermodynamic equilibrium between injections. This equilibrium was attained when no more energy was released or absorbed in the reaction cell. The solution in the reaction cell was stirred at 310 rpm throughout the experiments. The heats of dilution from the blank titration of the β-lg into the buffer solution and the buffer into pectin solution were subtracted from the raw data. Measurements were carried out in duplicates.

Thermodynamic parameters, including binding constant (K), enthalpy (ΔH), entropy (ΔS), and binding stoichiometry (N) (molar ratio monomeric β-lg/pectin), were calculated by iterative curve fitting of the binding isotherms. The "2-binding-site" model from the software Microcal ORIGIN provided with the ITC apparatus was used. The Gibbs free energy was calculated from the equation $\Delta G = \Delta H - T\Delta S$.

Overlapping Binding Site Model. An earlier binding study, involving the β-lg and pectin under similar experimental conditions, was carried out using a capillary electrophoresis (CE) method. The free β-lg was separated from the complexed β-lg by means of an electric field (*15*). Raw data obtained with CE were subsequently treated with the mathematical overlapping-binding-site model (*20*).

The overlapping binding site model is a modified Scatchard plot, which takes into account the large size of the ligand (protein) relative to the macromolecule (pectin) and the nonspecific nature of the complexation. By means of steric hindrance, the complexed proteins could prevent other ligand molecules from linking to potential binding sites. Consequently, the number of carboxylic groups on the pectin is not directly equivalent to the number of potential binding sites. According to the overlapping binding site model, complexation between ligand and macromolecule including interacting ligand molecules can be expressed as follows:

$$\frac{\nu}{L} = K(1 - n\nu) \cdot \left(\frac{(2\omega - 1)(1 - n\nu) + \nu - R}{2(\omega - 1)(1 - n\nu)}\right)^{n-1} \cdot$$
$$\left(\frac{1 - (n+1)\nu + R}{2(1 - n\nu)}\right)^2, R = \sqrt{(1 - (n+1)\nu)^2 + 4\omega\nu(1 - n\nu)}$$

where L is the free ligand concentration, K is the binding constant, n is the average size of the binding sites, ω is the cooperativity parameter (interaction between ligand molecules), and ν is the binding density (number of β-lg complexed per interacting unit). The cooperativity parameter (ω) can be negative as the interactions are repulsive (anticooperative) or positive for attractive interactions (cooperative).

The binding density of the overlapping binding site model has to be lower or equal to 1. Because more than one protein molecule is expected to complex on a pectin molecule, the galacturonic acid residue was used as the interacting unit instead of the whole pectin molecule used with the ORIGIN software.

ITC calorimetric data were transformed to obtain the binding density "ν" and free β-lg concentration "L" values (*21*). For each injection, the volume in the cell "vol" (L), the energy released "Q" (J), the enthalpy ΔH (J/mol), the β-lg amount "Xc" (mol), the β-lg concentration "ΔXt" (M), and the pectin concentration "Mt" (M) were calculated. Consequently, the maximum energy value "$Qmax$" (J), the complexed ligand concentration "Xb" (M), and the complexed β-lg concentration "Xt" (M) were calculated as follows:

$$Q_{max} = \Delta Xt_1 \cdot \Delta H \cdot \text{vol}$$

where ΔXt_1 is the β-lg concentration after the first injection

$$Xb = (\Delta Xt \cdot Q)/Q\text{max}$$

$$Xt = Xc/\text{vol}$$

Given the apparent molecular weights and the fact that pectin contains 80% of D-galA residues, the average content of D-galA residues was estimated at 420 and 507 per LM- and HM-pectin molecule, respectively. The binding parameters L (M) and ν (β-lg molecules complexed / D-galA residue) were calculated as follows:

$$L = Xt - Xb$$

$$\nu = (Xb \cdot N_A \cdot \text{vol})/Mt \cdot \text{vol} \cdot N_A \cdot \text{D-galA}$$

where N_A is the Avogadro number and D-galA is the average content of galacturonic acid residues in a pectin molecule.

The nonlinear curve fitting of ν/L) plotted against ν was used to obtain K, n, and ω values. The JMP IN software was used for the curve fittings and statistics (SAS Institute Inc, USA). The ITC binding data according to the overlapping binding site model were then compared to CE data.

RESULTS

Raw results from ITC measurements of the β-lg−LM-pectin complexes are presented on the thermogram at the top of **Figure 1**. The peak areas correspond to the energy released by the cell containing the pectin at each β-lg injection. Because all the injections throughout the analysis released energy, the complexation between β-lg and LM-pectin is considered as exothermic.

Saturation of the LM-pectin with the β-lg was reached at a β-lg/LM−pectin molar ratio of ∼26:1, as the energy released in the cell became null. The injections liberated about 2−3 μcal/s in the first third of the analysis. The energy released was relatively low, considering that ITC analysis should have had at least 5−10 μcal/s peaks in the first two-thirds of the thermogram (*16*). However, the 0.1-μcal sensitivity of the VP-ITC model used provided accurate results with only 3−5 μcal/s liberated. It was hard to improve signal intensity through higher component concentrations because of the high viscosity brought on by the pectin. Moreover, the β-lg−pectin complexes increased the overall viscosity of a solution, as was demonstrated earlier (*22*). This higher viscosity required a faster stirring, leading to unacceptable baseline distortion. The higher viscosity also reduced the dispersion of β-lg in the cell, which in turn, drastically slowed the thermodynamic equilibrium between injections. Decreasing the number of injections to increase their volume did not improve results, as complex binding isotherms were better described with many small injections rather than a few large ones.
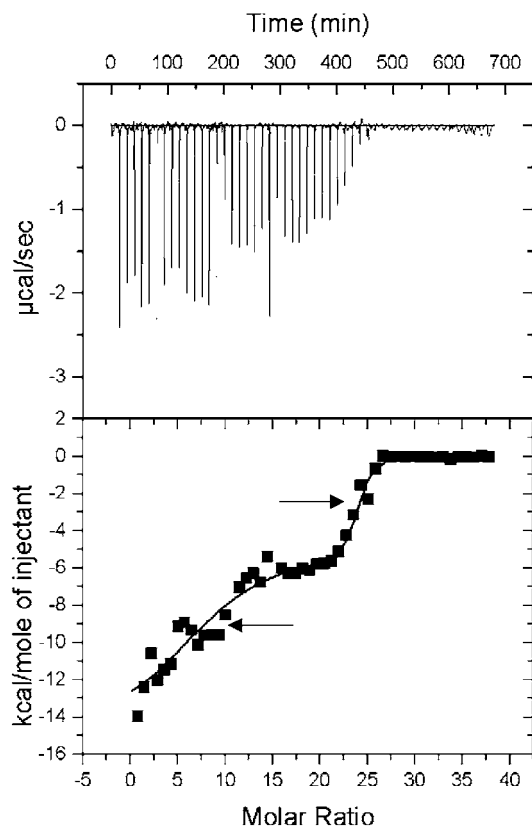
Figure 1. Thermogram (top) and binding isotherm (bottom) of the β-lg complexing with LM-pectin at pH 4 in 5 mM sodium phosphate buffer. Arrows indicate the 2 inflection points.

Figure 2. Thermogram (top) and binding isotherm (bottom) of the β-lg complexing with HM-pectin at pH 4 in 5 mM sodium phosphate buffer.

The binding isotherm of β-lg−LM-pectin complexes is the integrated areas of heat peaks plotted against the molar ratios of components (**Figure 1**; bottom panel). Although the software integrates the peak areas automatically, it was recommended to adjust the baseline and to integrate the peaks manually for more accurate results. This binding isotherm had two inflection points, which are generally indicative of two types of independent binding sites on the macromolecule. Two types of binding sites have been observed in an earlier binding study involving the protein p85, a subunit of phosphoinositide 3-kinase, and the tyrosyl phosphopeptides pY740 and pY751 (*23*).

Because of the nonspecific nature of interactions and because the carboxyl group was the only interacting group on the pectin, the two inflection points are more likely indicative of two different steps involved in the β-lg−pectin complexation rather than two different binding sites. For that reason, the expression "2-site model" was replaced here with the more appropriate "2-step model". In the first step, the amount of β-lg complexed on pectin decreased with each injection until pectin was saturated corresponding to the first plate. Then, the second step began with a plate where the same amount of β-lg molecules interacts with each injection. Then, the pectin gradually saturated with β-lg until no more energy was released. The x coordinates of inflection points correspond to the sum of stoichiometry values of β-lg−LM-pectin complexes at each step. ITC measurements showed that the titration of β-lg with salt solutions was exothermic, due to the shift in dimer/monomer equilibrium toward the dimer state (*17*). Blank titrations carried out were negligible, showing that titration did not modify the equilibrium of dimeric β-lg at pH 4 or the pectin aggregation. Thus, the prior dialysis was effective to equilibrate the ionic strength and pH of phosphate buffer, β-lg, and pectin solutions.
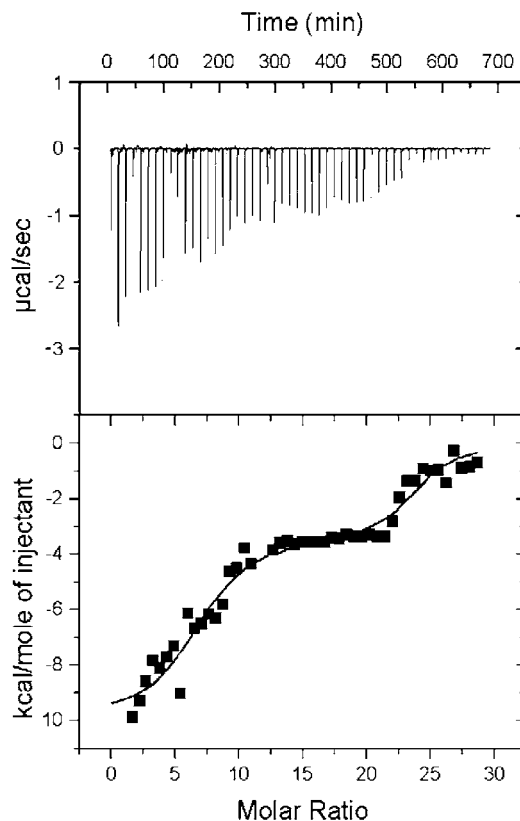
The thermogram at the top of **Figure 2** shows raw data obtained for β-lg−HM-pectin complexes. First injections of β-lg in HM-pectin released between 2 and 3 μcal/s, as with LM-pectin. The complexation between β-lg and HM-pectin was also exothermic. The binding isotherm of β-lg/HM-pectin had two inflection points, which indicates that two steps are also involved in the complexation process, as it was the case for LM-pectin (**Figure 2**). The saturation of the HM-pectin with the β-lg was reached at a molar ratio of about 24:1. There were no significant differences between the binding isotherms of β-lg interacting with LM- or HM-pectin. About 45% of the total interaction time was needed to achieve the first step, while 55% was required for the second step. The difference between the analysis lengths is explained by the initial HM-pectin concentration (9.43 μM), which was higher than that of LM-pectin (7.33 μM). Therefore, more injections were needed for the β-lg/HM-pectin mixture to reach equivalent molar ratios to β-lg−LM-pectin.

The curve fitting of the binding isotherms of β-lg−LM- and HM-pectin with the Microcal ORIGIN software was also used to calculate the binding stoichiometry of complexes, the binding constant, the enthalpy, the entropy, and the Gibbs free energy. The enthalpy is the only parameter that is model independent and corresponds to the energy released per mole of interacting β-lg. At high c value (see calculations below), the enthalpy on the binding isotherm can be approximated by the energy released on the first injections where all the β-lg molecules injected are complexed on the pectin. In the subsequent injections, pectin molecules become increasingly saturated with β-lg, and only a part of the protein molecules injected is complexed, which explains the lowering of the energy released. Enthalpy values correspond roughly to −14 and −10 kcal/mol of β-lg injected for the LM- and HM-pectin, respectively (**Figure 1**).

Because of the two inflection points, both binding isotherms obtained were characteristic of two different events in the

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Table 1. Thermodynamic Parameters of β-lg–LM- or HM-Pectin Complexes at pH 4 in 5 mM Sodium Phosphate Buffer Determined with the Microcal ORIGIN Software[a].

| thermodynamic parameters	β-lg/LM-pectin		β-lg/HM-pectin	
	step 1	step 2	step 1	step 2
N	8.3 ± 0.9	16.5 ± 1.0	6.1 ± 0.5	15.1 ± 0.7
K (M^{-1})	$(4.48 \pm 2.6) \times 10^7$	$(4.5 \pm 3.0) \times 10^6$	$(1.9 \pm 1.0) \times 10^7$	$(6.3 \pm 3.5) \times 10^5$
ΔH (kcal mol^{-1})	-12.6 ± 1.94	-5.5 ± 0.7	-10.0 ± 0.6	-3.6 ± 0.5
$T\Delta S$ (kcal mol^{-1})	-2.5 ± 2.8	3.5 ± 0.5	-0.2 ± 0.04	4.1 ± 0.4
ΔG (kcal mol^{-1})	-10.4 ± 4.8	-9.1 ± 1.2	-9.9 ± 0.6	-7.7 ± 0.9

[a] Results are the mean of duplicates.

reaction cell. Thus, the model of the ORIGIN software appropriate to fit binding isotherms with two inflection points was used to calculate the thermodynamic parameters of the two steps (**Table 1**).

There were about 8 β-lg molecules complexed on the LM-pectin at the first step and 16 at the second step. Six β-lg were complexed on the HM-pectin at the first step, which was significantly lower than what was obtained at the first step on the LM-pectin. The amount of β-lg complexed at the second step was not significantly different from the value obtained for the LM-pectin. The binding constants with LM- and HM-pectin at the first step were not statistically different. However, the second step had lower binding constants, which were about 10 times lower than the ones obtained during the first step. Enthalpy values were negative on the first and second steps for both pectins. The enthalpies, as a result of the interaction between β-lg and LM- and HM-pectin were higher at the first steps (-12.6 ± 1.94 and -10.0 ± 0.6 kcal mol^{-1}, respectively) than at the second steps (-5.5 ± 0.7 and -3.6 ± 0.5, respectively). The entropy values of complexes containing LM- and HM-pectin multiplied by the temperature were slightly negative at the first step (-2.5 ± 2.8 and -0.2 ± 0.04 kcal mol^{-1}, respectively) and positive at the second step (3.5 ± 0.5 and 4.1 ± 0.4 kcal mol^{-1}, respectively). The Gibbs free energy values were all negative, and no significant differences were observed between interactions at the first or at second step or between LM- or HM-pectin. Given its lower molecular weight, the LM-pectin would have reacted more with the β-lg than the HM-pectin did. However, there was no statistical evidence for that tendency.

When the binding constant and stoichiometry are known prior to an ITC experiment, the c value is calculated to determine the initial macromolecular concentration in the cell (*24*). The c value can be calculated using the binding constants of the β-lg–LM- and HM-pectin complexes to verify whether the initial concentrations of pectin in the cell were adequate. The c value is unitless and is calculated as follows:

$$c = K \cdot M_{\text{tot}} \cdot N$$

where K is the binding constant, M_{tot} is the pectin concentration in the analysis cell at the start of the experiment, and N is the binding stoichiometry. The ideal c value should be between 1 and 1000, and preferably between 10 and 100. At high c values, the binding isotherm has a square shape, which limits the use of fitting models. As a result, the slope of the curve can only be used to calculate the enthalpy of the reaction. Results obtained with ITC (K and N from **Table 1**) were used to verify if the pectin concentrations was appropriate. The c value was calculated for each step. Calculated c values for the β-lg–LM-pectin complexes were 1437 and 498 for the first and second steps, respectively, and 905 and 93 for the HM-pectin for the first and second steps, respectively. All the c values were in the
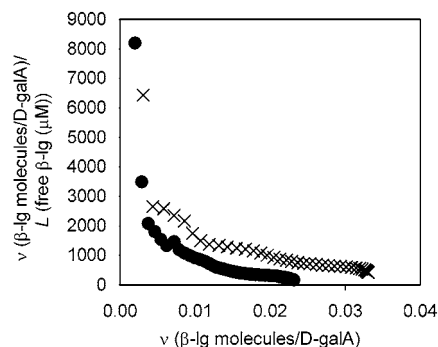
Figure 3. Scatchard plots of the β-lg–LM-pectin ($\times$) and HM-pectin ($\bullet$) mixtures at pH 4 in 5 mM sodium phosphate buffer.

suitable range, except for the first step of the β-lg–LM-pectin, which exceeded 1000. However, the high LM-pectin concentration in the cell was justified by the need to reach an acceptable signal on the ITC apparatus. The c values around 1000 could explain the relatively high error margin observed in the binding constants K calculated with the nonlinear fitting model. Moreover, these results show that higher concentrations used would have increased the error margin on the binding parameters calculated.

The binding isotherms generated by the ORIGIN software were also treated with the overlapping binding site model (*20*). Using this model, the binding stoichiometry, binding site size, binding constant, and cooperativity values obtained from the ITC measurements for the second step of β-lg–LM- and HM-pectin complexation were calculated. Binding parameters for the first step were not calculated, because not enough points were available for the nonlinear fitting (**Figure 3**). Therefore, the non linear least-squares fitting was achieved for values of ν between 0.015 and 0.025 and between 0.010 and 0.030 for complexes with LM- and HM-pectin, respectively

Results obtained with the overlapping binding site model followed the same tendencies observed with the ORIGIN software. The overlapping model made it possible to calculate the binding site size covered by 1 β-lg molecule, which was 14 and 22 D-galA residues for LM- and HM-pectin, respectively. The interpolymer complexes had a binding constant of 2128 M^{-1} with LM-pectin, which was higher than the value 1233 M^{-1} obtained with the HM-pectin. One can see that the binding constants calculated with the overlapping binding model were much lower than those obtained with the ORIGIN software. In fact, the overlapping binding site model uses the main repetitive unit of the pectin (D-galA residues) in the calculation as the interacting unit, whereas the ORIGIN software considers the whole pectin molecule. Consequently, the amount of interacting units is much higher in the overlapping binding model, which leads to lower binding constants. The β-lg complexed with LM- or HM-pectin did not show any significant cooperative behavior.

DISCUSSION

The negative Gibbs free energy values show the spontaneous character of the interaction between β-lg and LM- or HM-pectin. However, the relatively long period needed for the system to reach equilibrium between injections showed that the β-lg–pectin complexes have a slow binding kinetics.

The existence of two steps involved in the β-lg–LM- and HM-pectin complexation process is in agreement with the Tainaka theory (*25–26*), which states that the complexes are formed in two steps passing from the molecular to the aggregated state. The Tainaka theory is adapted from the Veis-Aranyi theory *(27)* and can be applied to systems regardless of their charges density. The first step would correspond to the formation of soluble intrapolymer complexes (C1) between β-lg and pectin molecules. The second step would imply the aggregation of these intrapolymer complexes forming insoluble interpolymer complexes (C2). The isoenthalpic plateau observed in the binding isotherms ends when the β-lg/pectin ratio required to induce intrapolymer complex aggregation is reached. A light scattering study carried out on poly(vinyl alcohol) sulfate/ proteins salt free systems at different ratios revealed that intrapolymer complexes did not aggregate as long as proteins were in excess (*28*).

The lower stoichiometries and higher binding constants of C1 compared to C2 are the result of lower levels β-lg in the cell that can interact with pectin and the higher proportion of complexed β-lg on the pectin, respectively. The first step was obviously enthalpically driven principally because of the direct interaction between the β-lg and pectin ionized carboxylic groups, which were shown to be located mainly in the smooth region of the pectin (*5*). The unfavorable entropy observed at the first complexation step is in agreement with the Tainaka theory, which states that C1 have poor conformational entropy. It was previously shown that the formation of protein–polyelectrolyte complexes was driven by electrostatic interactions that could be partially encountered by a loss in polyelectrolyte conformational freedom (*29*).

According to the Tainaka theory, the process called complexation begins at the second step, where C1 aggregates to form C2. Results show that enthalpic and entropic contributions support this second step. The favorable enthalpic contribution would result from the interaction of β-lg with the residual net negative charge on the C1. The overlapping of intrapolymer complexes leads to an electrostatic energy gain resulting from the increased ion density in the overlapped domain (*25, 26, 30*). The positive entropic contribution at the second step is essentially the product of the rearrangement of C2 in the solution and of the dilution of the concentrated phase containing the C2 (*27, 31, 32*). The presence of C1 and C2 was also observed with the BSA/PDMDAAC (bovine serum albumin/poly(dimethyldiallylammonium chloride) mixture (*33, 34*).

The enthalpy values measured with ITC are the sum of contributions coming from the interaction between β-lg and pectin, conformational changes, and aggregation of molecules (*16*). Hence, the enthalpy value measured could have been either overestimated or underestimated, depending on the possible conformational changes in the molecules.

Monte Carlo simulations showed that at low ionic strengths, a polyelectrolyte chain would wrap around an oppositely charged spherical particle (*35–36*). The entropically favorable release of counterions and water molecules at the time of complexation (*31–32*) may have been compensated by the negative entropy arising from conformational changes in the pectin. The exothermic reaction observed between maltodextrin and SDS can be explained by conformational changes in the maltodextrin (*37*).

Measures with circular dichroism revealed the partial disappearance of the β-lg α-helix as it interacts with the acacia gum at pH 4.2 (*38*). Conformational changes are also suspected on the ribulose diphosphate carboxylase and on the 11s broad bean globulin, once complexed with the LM-pectin (*12*). A contribution to enthalpy could also come from the possible aggregation of proteins around pH 4. To verify the accuracy of the binding parameters obtained with ITC, the calorimetric data treated with the overlapping binding site model were compared to those obtained with CE in a previous study (*15*).

The total stoichiometry values for ITC and CE were 25 and 23 monomers of β-lg complexed per LM-pectin molecule, respectively. However, the stoichiometry values calculated for the β-lg–HM-pectin complexes were higher with ITC (21) than with CE (13). This could be explained by a partial dissociation of β-lg–HM-pectin complexes during the CE analysis due to the electric field. The lower binding constant obtained at the second step is indicative of weaker interactions in β-lg–HM-pectin interpolymer complexes compared to those obtained with LM-pectin. A previous study using ultrafiltration and destabilizing agents demonstrated that β-lg–HM-pectin interpolymer complexes were more likely dissociated with higher pH values or ionic strengths than β-lg–LM-pectin complexes (*14*). The binding constant at the second step of the β-lg–LM- and HM-pectin complexes obtained with ITC were 2128 and 1233 M^{-1}, respectively, compared with 1431 and 857 M^{-1}, respectively, calculated with capillary electrophoresis for the same mixture (*15*). The binding constant values obtained by ITC were higher than those found with capillary electrophoresis. This binding parameter calculated from enthalpy values may have been overestimated because of conformational changes involved in pectin and β-lg molecules as they are complexing. The binding site sizes calculated with ITC (14 and 22 D-galA) were similar to those obtained with capillary electrophoresis (12 and 20 D-galA) for the LM- and HM-pectin, respectively (*15*). Contrary to what was observed with capillary electrophoresis, the β-lg complexed with LM- or HM-pectin did not show any significant cooperative behavior (*15*). The complexes analyzed with capillary electrophoresis were T as the β-lg and pectin solutions were mixed before the acidification. The ITC complexes were M, because β-lg and pectin solutions were at pH 4 before mixing. The T and M complexes were shown to have cooperative and noncooperative behaviors respectively (*39*).

CONCLUSIONS

Isothermal titration calorimetry is a useful tool for carrying out binding studies with biopolymers. The low cost of the food biopolymers makes them ideal molecules for studies in ITC, which requires a substantial amount of sample. ITC was used to characterize the formation of β-lg–LM- and HM-pectin complexes. The complexation is believed to occur in two steps, as predicted by the Tainaka theory. Enthalpic contribution from conformational changes in pectin and β-lg molecules may have lead to an overestimation of binding parameters. Further studies are needed to dissociate enthalpic contributions to from the interactions between β-lg and pectin and from conformational changes. Experiments with laser light scattering are presently carried out to determine the β-lg–LM- and HM-pectin complexes' formation mechanism and structure.

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