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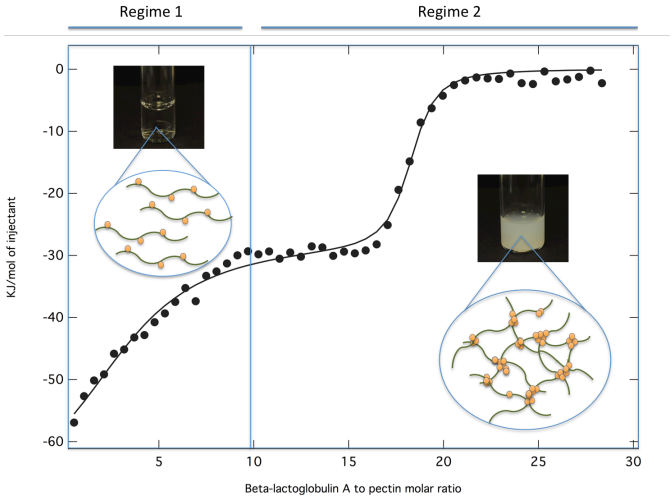
This manuscript reports on the characterisation of assemblies of two model biomacromolecules: a model carbohydrate, pectin, with a model protein, beta-lactoglobulin, in conditions controlled by electrostatic interactions. The pectins used have been modified to control the degree and distribution of charge along the chain. We show in this manuscript that the two biomacromolecules form a hierarchical structure, which depends intimately on the tailored charge distribution on the pectin – and we also notably show that the beta-lactoglobulin significantly rearranges on interaction with the pectin. This provides handles for manipulating these structures, which will prove important for biotechnological applications of these biomacromolecules.



Journal Name

RSCPublishing

ARTICLE

Structural mechanism of complex assemblies: characterisation of beta-lactoglobulin and pectin interactions

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

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Knowledge of how proteins and polysaccharides interact is the key to understanding encapsulation and emulsification in these composite systems and ultimately to understanding the structures of many biological network systems. As a model system we have studied β-lactoglobulin A (βLgA) interacting with pectins of various amounts and distribution patterns of charge. The studies were conducted at pH 4 at minimal ionic strength, where the βLgA and the pectins are oppositely charged, resulting in an electrostatic attraction to each other. Isothermal titration calorimetry (ITC) experiments were performed to determine the thermodynamics associated with βLgA - pectin titration. It was found that βLgA only interacted with pectins with an adequate amount of charge, and that the complexation between βLgA and pectin was a two-step process initially involving binding of the protein to available sites on the pectin, and subsequently binding of the protein onto the bound protein that has previously adsorbed. Circular dichroism (CD) and intrinsic tryptophan fluorescence were also measured of βLgA during its interaction with the pectin samples, and show that the binding leads to significant conformational changes in βLgA. An increase in the turbidity of the solution of the resultant complexes indicates the formation of large-scale interpolymer associations of the primary complexes mediated by protein-rich domains.

Introduction

Complexes formed by proteins and polysaccharides are attractive to both the food industry and material scientists as natural products that can be obtained from commonly available materials and may have improved functional properties compared to the individual polymers[1-4]. For these reasons understanding the interactions between proteins and polysaccharides has become a major focus of research over the last decade[5-8]. Protein-polysaccharide complexes commonly result from electrostatic interactions between oppositely charged macromolecules[9]. Other interactions could include hydrophobic, hydrogen-bonding and steric interactions[10]. Interactions between proteins and polysaccharides depend on the type of polymer and the molar ratio of protein to polysaccharide as well as the state of the solution such as its pH, ionic strength and temperature[5, 11-13].

β-Lactoglobulin (βLg) has been used extensively as a model protein to study the interaction with a range of polysaccharides[14-17]. βLg is the major whey protein in the milk of ruminants and other mammals[18]. It is a small, globular protein that belongs to the lipocalin family and consists of 162 amino acid residues[19]. It has recently been shown to be a dimer over pH range 2.5 to 7.5 when the solution ionic strength is relatively high[20], e.g., 100mM, as would be found in food. The protein features a hydrophobic core made up with eight antiparallel β-sheets, known as the calyx[19]. The major genetic variants of βLg are β-lactoglobulin A and β-lactoglobulin B that differ by two amino acid substitutions, Asp_{64}-Gly_{64} and Val_{118}-Ala_{118}. Although the two variants differ by only two amino acids, this results in differences in multiple relevant physical properties, such as protein charge anisotropy and extent of self-association[19, 21-24]. Therefore, in our study we focus on just one genetic variant, β-lactoglobulin variant A (βLgA).

Pectins, which originate in plant cell walls, are a family of hydrocolloids frequently used in the food industry as natural ingredients for their gelling, thickening and stabilizing properties. The major structure of pectin is a backbone chain composed of 1,4-linked α-D-galacturonic acid (GalA) units. The GalA residues can be methyl-esterified at the C-6 position[25], with un-esterified residues carrying a negative charge on the free carboxylic acid groups when the solution pH is sufficiently high. The physical properties of industrially extracted pectins are largely determined by the un-esterified segments within the pectin molecule[25], and it has been shown that not only the degree of methyl-esterification (DM) but also the degree of blockiness (DB) are important parameters which determine the physical behaviour of individual pectins[25]. Moreover the amount and distribution of methyl-ester groups determine the

charge distributions on the pectin chains: DM determines the overall charge density, while the DB determines the local charge density of the pectin[26]. In our work, a range of pectins with different overall and local charge densities is used to study the effect of charge distributions on pectin's interaction with βLgA.

Thermodynamic studies on βLg and pectin systems have been previously reported[12, 15, 26], however, due to the complexity of the protein-polysaccharide interactions, it is a challenge to elucidate the binding process solely from these data. Our study builds on earlier work by considering a much broader range of pectin charge distributions. The solution conditions used in the study are fixed to pH 4, below the pI of beta-lactoglobulin (when the protein will carry a net positive charge), with low ionic strength, where the electrostatic interactions between the protein and polysaccharide are maximized. Isothermal Titration Calorimetry (ITC) was used to study the thermodynamics associated with binding between βLgA and pectins, and in parallel with ITC experiments, turbidity measurements, circular dichroism (CD) and tryptophan fluorescence measurements were carried out to better understand the thermodynamic results. Based on the experimental results, a βLgA to pectin binding mechanism is proposed.

Experimental

Bovine β-lactoglobulinA (βLgA) with a minimum purity of 90% was purchased from Sigma Aldrich (Missouri, US). Pectins with 75% degree of methyl-esterification and 86% degree of methyl-esterification were purchased from Sigma Aldrich (Missouri, US). Pectins with lower degrees of methyl-esterification (58% and 37%) were a gift from CP Kelco (Georgia, US) and were used as delivered. Each of these pectins was enzymatically de-esterified from highly methyl-esterified pectins using fungal and plant pectin methyl-esterases to create random and block-wise distributed uncharged methyl-ester groups respectively.[27, 28] These pectins were named as: 37R, 37B, 58R, 58B, 75R and 86R, where the numbers indicate the degree of methyl-esterification, and the letters 'R' and 'B' indicate random and block distribution pattern of the uncharged methyl-ester groups. All of the pectins were in the range of molecular weights from 90 kDa – 120 kDa, with the exception of the 86R, which had a molecular weight of 50 kDa. Citric acid, sodium chloride and hydrochloric acid were of analytical grade and used without further purification.

Preparation of β-LactoglobulinA and Pectin Stock Solutions

A stock solution of βLgA was prepared by dissolving the protein in 20 mM citrate buffer with 20 mM NaCl. Pectin stock solutions were prepared by dissolving the polysaccharides in 0.1 M sodium phosphate buffer at pH 4. Both protein and pectin solutions were stirred at room temperature until fully dissolved. The solutions were then stored at 4°C for 24 hrs to ensure complete hydration and equilibration of the biopolymers. In order to eliminate excess salts and avoid buffer mismatch, βLgA and pectin solutions were dialyzed against 5 mM citrate buffer, pH 4 using a 12-14 kDa cutoff dialysis tube (#132-700, Spectra/Por®, California, US) for 18 hrs. The dialysis buffer was replaced every 6 hrs. The stock solutions were further filtered through polyethersulfone membrane filters (Membrane Solutions®, Ohio, US) with 0.22 □m pore size. The concentration of βLgA solution was measured by Nanodrop® spectrophotometer (ND-1000, Thermal Scientific, Wilmington, US) at 278 nm and using protein extinction coefficient[29] of 17,600 cm^{-1} M^{-1}.

Isothermal Titration Calorimetry (ITC)

ITC experiments were performed using a VP-ITC microcalorimeter (MicroCal Inc., Northampton, MA) operated at 25.0 °C. βLgA and pectins in 5 mM citrate buffer, pH 4 were degassed under vacuum for 7 min prior to titration. A βLgA solution was loaded into the injection syringe to titrate with a pectin solution that had been loaded into the calorimeter cell (1.4531 mL). Each titration comprised 50 injections of 5 μL each. In order to achieve equilibrium after each injection, the spacing between injections was 800 s and the solution was stirred with a magnetic flea at 307 rpm. Buffer to buffer, protein to buffer and buffer to pectin controls were also performed, and the heats of dilution for both the protein and pectin solutions were measured and subtracted from the raw ITC data appropriately.

Turbidity Measurements

In order to better understand the kinetics of complex formation between βLgA and various pectins, changes in turbidity during βLgA to pectin titrations were measured under the same experimental conditions as in ITC experiments at pH 4 and 25 °C. A series of βLgA-pectin mixtures were prepared using the stock solutions,with the molar ratio between βLgA and pectin varied from 1 to 21. The optical density of the biopolymer mixtures was measured with an UV/Vis spectrophotometer (U-1100, HITACHI, Japan) at 633 nm. Quartz cuvettes (Hellma, Müllheim, Germany) with 1 cm path length were used for these measurements. 5 mM citrate buffer at pH 4 was used as the blank reference. Turbidity was calculated as: $\tau = (-1/b) \ln (I_0/I_t)$, where b was the optical path length in cm, and I_0 and I_t were the incident and transmitted light intensity respectively. Measurements were carried out in triplicate. Error bars indicate the estimated standard deviation of three different measurements for each sample.

Circular Dichroism

CD spectra of βLgA and its complexes with pectins with different overall and local charge densities were recorded using PiStar-180 spectropolarimeter fitted with a peltier temperature control unit (Applied Photophysics Ltd., Leatherhead, Surrey, UK). The instrument was calibrated with (+) 10-camphorsulphonic acid. βLgA samples were prepared by diluting the stock solution to a final concentration of 5 μM using 5 mM citrate buffer. βLgA and pectin complexes were prepared by mixing 5 μM βLgA and 5 μM pectin solution at certain ratios so that the molar ratios between βLgA and pectin were 1, 5, 10 and 20 respectively. The same set of samples was also prepared using buffer (excluding βLgA) and pectin, so that the CD signal arising from pectin could be subtracted from that obtained from βLgA-pectin complexes. Changes in the secondary structure of βLgA were monitored in the far-UV region from 178 nm to 260 nm with a bandwidth of 1.5 nm and data spacing of 0.5 nm.

For each sample, an accumulation of 7 scans with a scan speed of 10 nm/min was performed. All CD spectra were recorded at 25 °C in nitrogen atmosphere using a quartz cuvette with 1 mm path length (Hellma, Müllheim, Germany). All spectra were corrected by subtracting the baseline and converting to mean residue ellipticity (deg cm^2 $dmol^{-1}$) based on a mean amino acid residual weight of 114 (MRW)[30]. Spectral deconvolution was performed using the CDSSTR[31], CONTILL[32]and K2D[33] algorithms. Reference sets SP175[34] and SMP180[35] were used from the suite of programs available at the online server DICHROWEB[36-38] (http://dichroweb.cryst.bbk.ac.uk).

Intrinsic Fluorescence Measurements

βLgA-pectin mixtures were made by mixing protein: polysaccharide at molar ratios ranging from 1:1 to 20:1. All samples were prepared in 5 mM citrate buffer at pH 4 and 25 °C. Fluorescence measurements were made using a Perkin Elmer LS 55 luminescence spectrophotometer (Buckinghamshire, England, UK). A quartz cell with 1 cm path length was used for fluorescence measurements. Samples were excited at 295 nm in order to minimize the fluorescence contribution from the tyrosine residues. Emission was measured across the range of 310 to 390 nm, with both excitation and emission slit widths set at 2.5 nm.

Results

Isothermal titration calorimetry

ITC at 25.0 ˚C was used to compare the binding energy between βLgA and a wide range of pectins: 37R, 37B, 58R, 58B, 75R and 86R. ITC revealed that the binding between βLgA and pectin with DM less than the 86R was an exothermic process which was consistent with previously published results[12, 15]. Figure 1 shows a typical thermogram obtained from the titration between βLgA and 37B pectin. The titration features a sequence of strong exothermic peaks that gradually decrease in intensity until the exothermic peaks have become endothermic peaks of constant intensity. As reported by others the endothermic process at the end stage of the titration is due to dilution[14, 16]. No exothermic interaction was observed in the βLgA to 86R pectin titration, only endothermic dilution, indicating no binding between these compounds (result not shown).

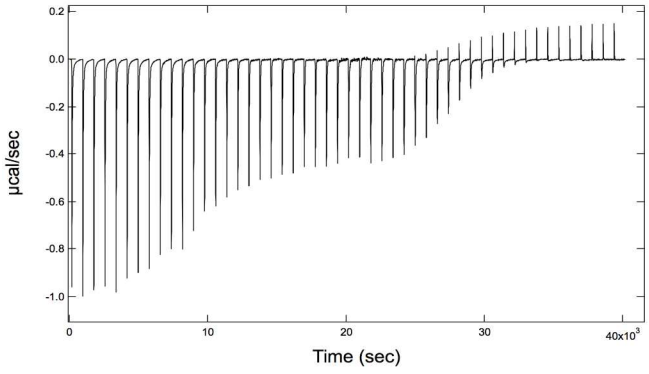

Figure 1 A representative thermogram obtained from βLgA to pectin 37B titration.

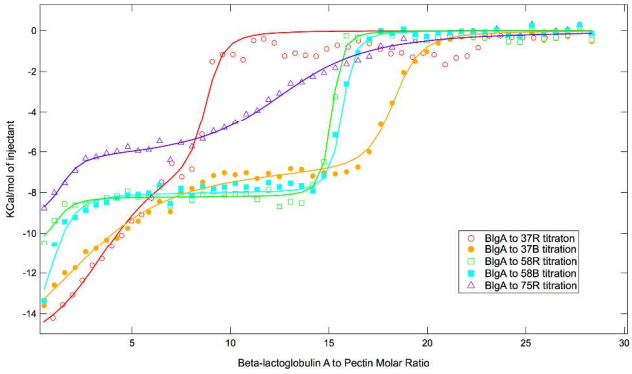

Figure 2 Calculated best-fit (solid lines)for βLgA to pectin titrations using the 'two-types of site' model. Curves are presented as: βLgA to 37R titration (red, open circle), 37B (orange, closed circle), 58R (green, open square), 58B (blue, closed square) and 75R (purple, open triangle).

The binding isotherms obtained from titrations between βLgA and pectins with different degrees of methyl-esterification are shown in Figure 2. The binding between βLgA and pectin is a complicated process which not only includes the protein-polysaccharide binding, but also deformations of the macromolecules such as the bending and folding of the polysaccharide chains, and these all contribute to the energy change in the system. Nevertheless, the shape of the ITC traces obtained from each of the βLgA-pectin titration systems are generally similar, suggesting a common mechanism of interaction between βLgA and all the pectins tested in the study, except 86R which shows no binding at all. We can see that the initial enthalpy change (ΔH_{ini}) measured for titrations between βLgA and 37R, 37B and 58B were similar, around -13 to -14 kCal/mol of injectant. This suggests the presence of high affinity sites on those pectins, and as reported by Bram *et al.*[26] such pectins have similar local charge density at the start of the ITC titrations. The amount of energy released from the first injection during βLgA to 58R titration was around 10 kCal/mol of injectant, less than for the 37R, 37B and 58B, and was the smallest for the βLgA to 75R titration which was around 9 kCal/mol. Therefore, the initial enthalpy change decreases as the degree of methyl-esterification of pectin increases.

A clear plateau can be observed in the ITC curves obtained from the titrations between βLgA and 37B, 58R and 58B pectins, while there was an inflection point in the βLgA to 37R titration curve when the protein/polysaccharide ratio was around 6. For 75R pectin, a steep increase in enthalpy change was observed until the βLgA/pectin molar ratio increased to 3; this was then followed by a plateau until, when the molar ratio reached 8, a steady increase of enthalpy was then observed till the end of the titration. Figure 2 shows that all of the titrations were characterized by two stages, either with or without a plateau region. In order to better understand the thermodynamic changes during different titrations, the raw ITC data were best fitted using a model based on two different types of binding site, provided

by the Microcal Origin Software (Table 1). Thermodynamic parameters, that is the binding constant (K), and enthalpy (ΔH) were calculated by interactive curve fitting of the binding isotherms assuming equilibrium binding. Changes of Gibbs energy (ΔG) were derived from the binding constant, and entropy (ΔS) changes calculated using the equation: $\Delta G = \Delta H - T\Delta S$, where T was the temperature in Kelvin (298 K).

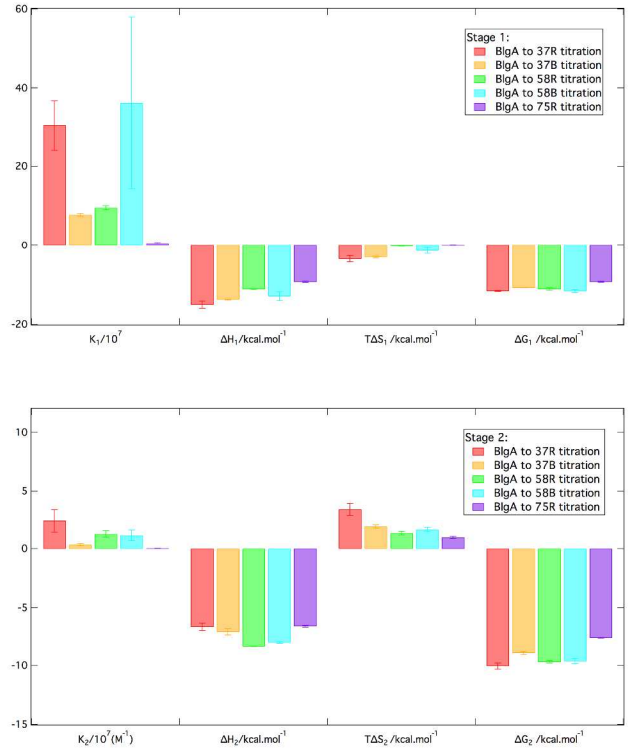

Figure 3 Calculated thermodynamic parameters of two different binding stages. In this graph, different titration systems are represented as: βLgA to 37R titration (red); βLgA to 37B pectin titration (orange); βLgA to 58R pectin titration (green); βLgA to 58B pectin titration (blue); βLgA to 75R pectin titration (purple). Results from the βLgA to 86R titration are not shown since no binding was detected from ITC.

For each titration shown (Figure 3), the calculated ΔG was negative, indicating that the binding between βLgA and pectin is a spontaneous process. Significant higher affinity between the pectin and the protein (larger binding constant K) is observed in the first stage than in the second stage. This is consistent with a model that pictures the first stage, when the pectin molecules carry the highest net charge, to be driven primarily by electrostatic interactions. During the first stage of all five titrations the binding was predominately enthalpically driven, with ΔH values between -9 kCal/mol and -15 kCal/mol, while the binding entropies were slightly unfavourable with TΔS values ranging from 0 to -3.5 kCal/mol. When the titration reached the second stage, the overall free binding energy ΔG decreased only slightly. However, a significant decrease in the enthalpic contribution was observed, offset by a favourable change in entropy. We hypothesise that an unfavourable entropic contribution in the first stage is an indicator of the loss of mobility of the macromolecules upon binding, while a favourable entropic change in the second stage is due to the conformational changes of the biopolymers and the release of counter ions into the bulk solution, as has been previously reported[5, 9, 39].

Turbidity Measurements

During the pectin/protein titration the turbidity of the solution increased, indicating the formation of large complexes in the solution. Results from the turbidity tests are presented in Figure 4 and show that a slight increase in turbidity is observed when initially introducing βLgA into the pectin system indicating the formation of complexes that were large enough to scatter light[40]. For pectins such as 37R, 37B, 58R and 58B, a steady state appeared after the initial rise in turbidity. A significant increase in the turbidity did not occur until the molar ratio increased to greater than 5 for both 37R and 58R, and 15 for 37B and 58B. In contrast, for the βLgA and 75R titration, the turbidity of the solution did not increase as much as for other samples, but remained relatively constant at a low level, while for the 86R pectin system no significant increases in turbidity were observed at any ratio (result not shown).

Comparing the values acquired from different βLgA to pectin mixtures, it can be seen that solutions were generally more turbid in the presence of pectins with randomly distributed methyl-esterified groups than with the block-wise distributed ones. The turbidity measurement results show that more and/or larger complexes were formed with pectins carrying greater charge than pectins with larger extent of methyl-esterification such as 75R. The observed decrease in turbidity at very high protein:pectin ratios (>20:1) suggests the complexes have passed an optimal binding ratio, and is attributed to a decrease in the size or volume fraction of particles caused by the increase in electrostatic repulsion[14] – no detectable precipitation occurred in the system, even on long standing times.

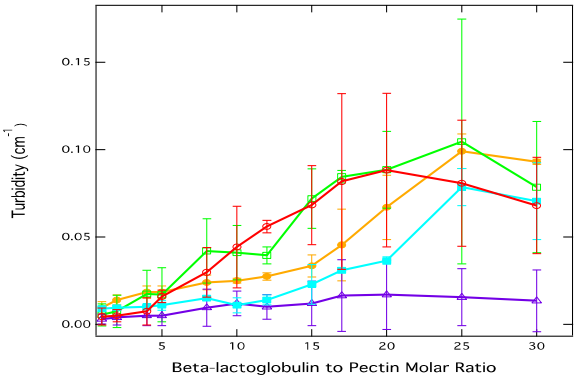

Figure 4 Measured turbidity for βLgA and pectin mixed solutions. Datasets were labelled as: βLgA to 37R titration (red, open circle), 37B (orange, filled circle), 58R (green, open square), 58B (blue, closed square) and 75R (purple, open triangle). Each sample is measured in triplicate, error bars indicate the standard deviation of three different measurements for each sample.

Circular Dichroism

Circular Dichroism (CD) has been widely used as a reliable tool for rapid determination of the secondary structure, folding and binding properties of proteins[41]. In our study, CD was used to measure the effect of pectins on the secondary structure parameters of βLgA. The CD spectra of pectins have a maximum at around 210 nm due to the π to □* transition of the carboxyl group[42]. Therefore, in order to acquire accurate information on the secondary structure change of βLgA in the presence of pectin, the CD spectra of βLgA and pectin mixtures were corrected using the spectra obtained from the corresponding pectin. Figure 5 shows the typical far-UV spectra of βLgA in the absence and presence of 58R pectin obtained from the titration. □-Helical structures typically exhibit negative bands at 222 nm and 208 nm[43], and therefore intensities of the CD signals at these wavelengths can be used to obtain estimates of the □-helical content of peptides or proteins[44]. Figure 5 shows that the CD spectrum of βLgA alone exhibits a typical minimum at 217 nm, due to the presence of the anti-parallel-β-strand structure of the protein, consistent with CD spectrum reported by other groups[30, 45, 46]. However, as the βLgA to pectin titration proceeded, with the βLgA-pectin molar ratio increasing from 1 to 20, significant changes were observed in the absolute values of mean residue ellipticities at both 208 and 222 nm suggesting a decrease in the amount of □-helical structure during the titration. In order to obtain an estimate of the secondary structure content of βLgA in the presence as well as the absence of pectins, all far-UV CD spectra were deconvoluted using the CDSSTR algorithm in combination with reference set SMP180. As a comparison, CONTINLL (with reference set SP175) and K2D methods were also used to deconvolute the CD spectra. Results returned from all three methods were relatively consistent. Percentages of the different secondary structure elements of βLgA found in solution and in complexes with pectins of different overall and local charge densities are listed in Table 1 below.

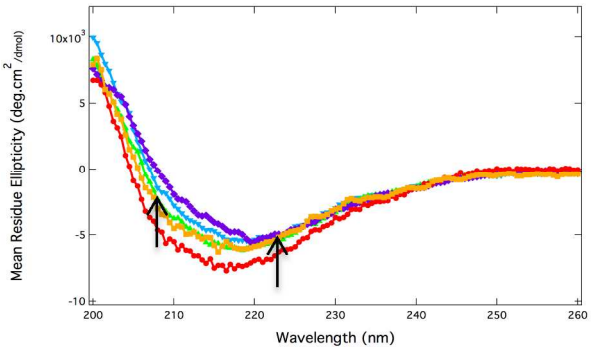

Figure 5 Far-UV CD spectra of βLgA solution (red, circle), βLgA/58R pectin complexes prepared at different βLgA/pectin ratios: 1 to 1 (orange, square), 5 to 1 (green, triangle), 10 to 1 (blue, inverted triangle) and 20 to 1 (purple, diamond). Black arrows indicate the approximate positions of 208 and 222 nm respectively.

For βLgA, the calculated percentage of secondary structure elements was consistent with published literature[45, 47]. CD experiments were conducted under the same conditions as the ITC experiments, namely in 5 mM citrate buffer at pH 4. βLgA /pectin complexes were prepared to mimic different points in the ITC titration experiment by mixing protein:polysaccharide at molar ratios: 1:1, 5:1, 10:1 and 20:1. From Table 1 it can be seen that for the 37R, 37B, 58R, 58B and 75R pectins the percentage of protein □-helical structure reduced significantly as soon as βLgA came into contact with the pectin. This decrease in □ -helical structure was consequently accompanied with a significant percentage increase in the amount of beta-strands. The changes in the proportion of turns and disordered structures were much smaller than the changes in the □ and β secondary structure elements. In contrast to all other mixtures, βLgA exhibited negligible structural changes upon exposure to 86R pectin, which was highly methyl-esterified with a low overall charge density. The total proportion of disordered structure remained approximately constant at each βLgA to pectin molar ratio for all of the pectins tested.

Intrinsic Fluorescence Spectroscopy

β-Lactoglobulin A is a globular protein made up from 162 amino acid residues. There are five cysteine residues with two disulfide bonds between Cys_{66} and Cys_{160}, Cys_{106} and Cys_{119}[18], which stabilize the protein structure. The protein has two tryptophan residues Trp_{19} and Trp_{61}. Trp_{19} is located within the hydrophobic calyx comprised by the anti-parallel beta-strands, while Trp_{61} is in close proximity to the disulfide bond formed between Cys_{66} and Cys_{160} lying close to the protein surface[48]. Conformational changes of βLgA can be monitored by observing the fluorescence of tryptophan residues, as changes in the local environment of the tryptophan residue will lead to changes in the intensity of fluorescence emission as well as a shift in the emission maximum (λ_{max})[47].

Tryptophan fluorescence spectra were collected from the βLgA and pectin system at different protein-polysaccharide molar ratios matching points in the ITC titration experiment. In order to exclude the concentration effect of βLgA, the measured maximum intensity of each sample was corrected by the total βLgA concentration. Changes in tryptophan fluorescence intensity as well as the wavelength of emission maximum from each βLgA-pectin titration were recorded (Figure 6 and Figure 7). For titrations between βLgA and pectins 37R, 37B, 58R and 58B, the fluorescence intensity of tryptophan residues was increased concomitant with a blue-shift in the λ_{max}. For the βLgA-75R pectin system similar changes in fluorescence intensity and λ_{max} were observed during the first half of the titration; however, as the titration proceeded, the fluorescence intensity started to decrease with a red-shift in λ_{max}. This was probably due to the presence of a large amount of native βLgA which had not interacted with 75R pectin. No significant changes in either fluorescence intensity or λ_{max} were observed from the βLgA-86R pectin titration.

Sample	% of α helix	% of β strands	% of turn	% of unordered structure
βLgA (alone)	21±3	39±2	11±1	30±3
βLgA to 37R pectin titration				
βLgA complex (r=1)	5±2	54±6	7±3	32±3
βLgA complex (r=5)	3±1	57±3	8±1	31±2
βLgA complex (r=10)	2±1	57±4	8±1	32±2
βLgA complex (r=20)	2±1	47±4	12±1	37±2
βLgA to 37B pectin titration				
βLgA complex (r=1)	5±1	54±4	8±3	32±2
βLgA complex (r=5)	5±2	53±6	9±2	33±3
βLgA complex (r=10)	4±1	57±5	7±1	32±3
βLgA complex (r=20)	1±1	57±6	9±1	31±4
βLgA to 58R pectin titration				
βLgA complex (r=1)	5±1	52±3	9±2	33±2
βLgA complex (r=5)	5±1	51±5	9±1	34±3
βLgA complex (r=10)	4±1	54±6	8±2	33±3
βLgA complex (r=20)	3±2	52±7	10±2	35±3
βLgA to 58B pectin titration				
βLgA complex (r=1)	5±2	56±5	8±2	32±3
βLgA complex (r=5)	5±2	51±6	9±1	35±4
βLgA complex (r=10)	4±2	54±8	8±2	33±5
βLgA complex (r=20)	3±1	54±3	8±1	34±2
βLgA to 75R pectin titration				
βLgA complex (r=1)	4±2	59±3	7±1	29±2
βLgA complex (r=5)	9±4	44±8	9±2	35±1
βLgA complex (r=10)	4±1	54±5	8±2	33±3
βLgA complex (r=20)	3±2	51±5	10±1	35±3
βLgA to 86R pectin titration				
βLgA complex (r=1)	16±1	38±5	11±3	34±3
βLgA complex (r=5)	18±1	32±6	11±4	37±3
βLgA complex (r=10)	18±2	36±8	11±3	35±6
βLgA complex (r=20)	21±2	29±4	10±5	38±4

Table 1 Percentage of the different secondary structure contents of βLgA free in solution and complexes with pectins with different overall /local charge densities. In the annotation, 'r' represents the βLgA/pectin molar ratio.

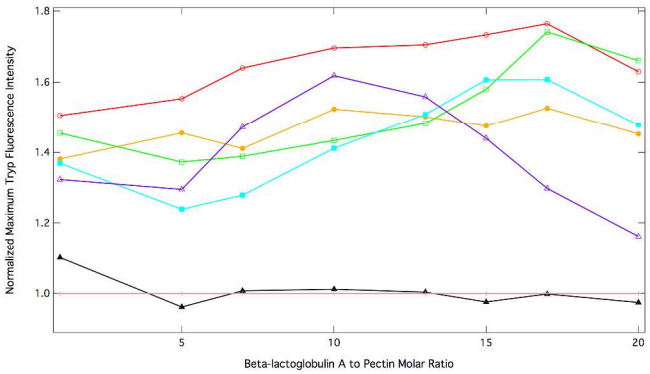

Figure 6 Maximum tryptophan fluorescence intensity observed during βLgA to various pectin titrations. In this graph samples are represented by different coloured lines: βLgA control (pink, cross), βLgA to 37R pectin titration (red, open circle), βLgA to 37B pectin titration (orange, closed circle), βLgA to 58R pectin titration (green, open square), βLgA to 58B pectin titration (blue, closed square), βLgA to 75R pectin titration (purple, open triangle), βLgA to 86R pectin titration (black, closed triangle).

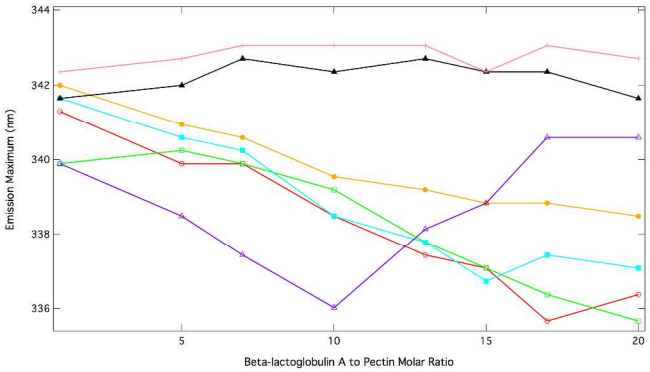

Figure 7 Changes in λ_{max} observed during βLgA titrations into various pectin.systems. In this graph samples were represented by different coloured lines: βLgA control (pink, cross), βLgA to 37R pectin titration (red, open circle), βLgA to 37B pectin titration (orange, closed circle), βLgA to 58R pectin titration (green, open square), βLgA to 58B pectin titration (blue, closed square), βLgA to 75R pectin titration (purple, open triangle), βLgA to 86R pectin titration (black, closed triangle).

Discussion

The degree of methyl-esterification and the distribution of the methyl-esterification (DM and DB) of pectin determines the overall and local charge densities of pectin respectively. In our study, the interactions between βLgA and pectins with different degrees and distributions of methyl-esterification under acidic condition and at low ionic strength were studied. At pH 4, the protein and polysaccharide carry opposite charges – the protein bears a net positive charge and the pectin a negative charge, and hence electrostatic interactions are a major driving force for the protein and polysaccharide complex formation. Consequently, the strength of the interactions between βLgA and pectins are expected to be different depending on the pectin charge densities.

The ITC results reveal that βLgA interacted with pectins with different DM and DB: 37R, 37B, 58R, 58B and 75R, with the negative values of the Gibbs free energy (ΔG) and enthalpy (ΔH) indicating that the binding between βLgA and those pectins was spontaneous and exothermic. Binding isotherms were best fitted using the 'two-types of sites' model. While it has been suggested that such a model is not suitable for describing specific binding interactions between two macromolecules[14], in our case, the interactions between βLgA and pectin are not specific,and crosslinks might be formed between regions that have initially bound a specific motif on the polysaccharide chain. Therefore the '2-types of sites' model might be more appropriately described as a '2-stage binding model[14].

No interaction of βLgA and 86R pectin was detected by ITC, CD spectroscopy or Trp fluorescence. This may be due to several reasons: 1) the overall or local charge density of such pectin was not strong enough to initiate the electrostatic interaction with βLgA, since it is known that the interaction between βLgA and polysaccharides is mostly electrostatically driven during the early stage of the titration[14, 15] and is entropically disfavoured by analogy with the other pectin complexes formed. 2) the non-methyl-esterified sites were significantly separated by the methyl-ester groups, hence the potential binding motifs were too small to accommodate the protein molecule; 3) highly methyl-esterified pectin molecules may have a tendency to self-aggregate prior to the titration[26, 49], so that there would be less sites available for protein interactions.

Since ITC measures only the total energy change of the system and many different physicochemical phenomena could contribute to the overall measured ITC signal, it is difficult to predict the precise molecular events based on ITC results alone. Therefore, circular dichroism and intrinsic tryptophan fluorescence were used to detect the conformational changes associated with βLgA during its interaction with the pectins. Changes in protein secondary structure upon interaction with polyelectrolytes are a common phenomenon[50-52]. The CD results revealed that the secondary structure of βLgA changed when it interacted with pectins from the beginning of the first stage of interaction. During the association the percentage of β-strands increased at the expense of a decrease in □-helix content, while the turns and disordered structures did not change significantly. Borges *et al.*[53] studied the conformational transitions of βLg during its interaction with chitosan. It was demonstrated that βLg underwent conformational relaxation when adsorbed onto chitosan surface, presumably due to the hydrophilic nature of chitosan structure. Therefore, it is reasonable to consider that the observed secondary structure change of βLgA was due to the conformational relaxation of βLgA when interacting with pectin. It is also noteworthy that the change in secondary structure of βLgA is apparently the same in the protein-protein interaction as the protein-pectin interaction.

Due to their aromatic character, tryptophan residues are often found to be fully or partially buried inside the hydrophobic core of proteins[54]. Knowing that the emission maximum of tryptophan is dependent on solvent polarity, and that exposure of the tryptophan residues to a polar environment will lead to a red-shift in the emission maximum[55], we suggest that the observed blue-shift resulted from the exposure of βLgA molecules to a less polar environment as titration proceeded. Recently, Monte Carlo studies

have been applied to a βLgA and pectin system in order to study the complex coacervation between the two molecules[24]. It was found that βLgA had a strong tendency to self-association mainly due to the large negatively charged patches exposed on the protein surface[24]. Further, it is generally accepted that protein aggregation can be a surface induced process[56]. The mechanism of protein aggregation is likely to involve a nucleation step [56, 57] with a seed of one or a few adjacent monomers adsorbing on the surface triggering the growth of protein clusters into three dimensions[56, 58]. Therefore, the observed blue-shift of λ_{max} is explained by the formation of local βLgA domains where the βLgA molecules are buried inside a less polar environment. While each βLgA molecule possesses two Trp residues: Trp_{19} and Trp_{61}, the measured tryptophan fluorescence intensity predominantly arises from Trp_{19} since the fluorescence of Trp_{61} is considerably quenched by the neighboring disulfide bond formed between Cys_{66} and Cys_{160}[59, 60]. The increase in tryptophan fluorescence intensities may imply then that the tryptophan residues move away from particular quenching groups.

Carlsson *et al.* [61] reported Monte Carlo studies on the complexation of an oppositely charged protein and polyelectrolyte. It was pointed out that the formation of protein oligomers could be enhanced with the presence of polyelectrolyte, and such enhancement could be further accelerated at low ionic strength where the electrostatic interaction between the oppositely charged protein and polyelectrolyte were maximized. Considering these simulations, in combination with our ITC, CD and intrinsic fluorescence results, we propose a possible binding mechanism between oppositely charged βLgA and pectin at low ionic strength (Figure 8). From the ITC results, two regimes could be distinguished. The first regime covers from the beginning of the titration to the starting point of the plateau region. In this range, small amounts of βLgA firstly interact with pectins through long-range electrostatic interactions, and at this point, small, soluble 'primary' complexes are formed. The second regime refers to the entire plateau region till the end of the titration where no more heat change is measured. Considering the fact that during the plateau region, each injection releases a similar amount of heat, therefore, we anticipate that the major binding event in this region is a protein-to-protein interaction that leads to the formation of local βLgA domains. In this range, adjacent βLgA molecules start acting as the 'seeding proteins' that then lead to the formation of small protein aggregates.

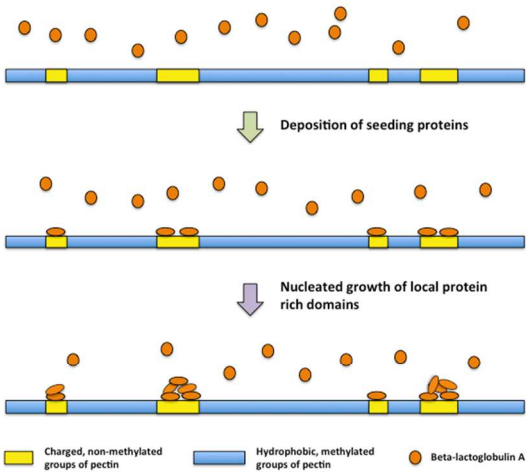

Figure 8 Proposed two-step binding mechanism between βLgA and pectin. The first step involves the deposition of 'seeding proteins' to the charged regions of pectin chain; the second step involves the nucleated growth of β LgA aggregates on top of adjacent seeding proteins, leading to the formation of local β LgA rich domains. Native β LgA in solution is represented by orange circles, the conformational relaxation of β LgA upon interacting with pectin is illustrated by the change in shape from circle to ellipse.

Such protein-protein interactions could produce bridging between two spatially close βLgA molecules that are binding to two separate pectin chains. In this instance, βLgA aggregates act as the junctions between individual 'primary' complexes, and this further lead to the formation of much bigger interpolymer complexes, as observed in turbidity measurements, and eventually a larger complex coacervate. Based on the proposed binding mechanism, the final microstructure of βLgA-pectin complex coacervate should contain several protein-rich domains at the junctions between individual 'primary' complexes (Figure 9), this is consistent with previously published results[62, 63]. For pectins with high overall/local charge densities, more seeding proteins could be deposited initially due to more possible electrostatically favored binding sites, leading to the formation of protein rich domains to a much greater extent. It should be noted that in the ITC profile acquired from βLgA to 75R titration, rather than a fairly immediate stop at the end of titration, a prolonged decrease in the released energy is observed following the plateau region. This might arise because βLgA molecules have difficulties bridging to form bigger aggregates with the decreased number of protein binding sites and increased steric hindrance from hydrophobic regions of pectin.

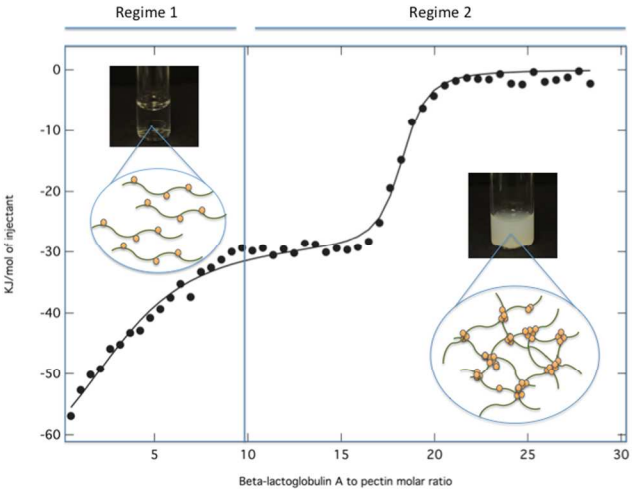

Figure 9 Illustration of the postulated microstructure of βLgA-pectin complex formed during a typical ITC titration. Regime 1 is characterized by the binding of discrete individual βLgA proteins along pectin chains, forming primary complexes. In regime 2, βLgA proteins aggregate on individual pectin chains. These protein aggregates are large enough to enable inter-pectin binding, leading to the formation of much bigger interpolymer complexes and eventually the complex coacervates.

In agreement with ITC results, two regimes could be clearly identified from the measured turbidity results when the protein and the pectin interact: a less turbid region which corresponds to the formation of small, soluble 'primary' complexes and a more turbid region where large bridged complexes are formed. Results from the turbidity measurements support the hypotheses made based on the ITC, CD and Trp fluorescence measurement results. For pectins with high overall charge densities there are more possible protein binding sites on the pectin to start with, the formation of protein aggregates could be promoted, and more 'primary' complexes join together through more protein-rich domains to form large particles that scatter more light. In the case of pectins with low overall charge densities such as 75R pectin, the growth of protein aggregates is hindered due to the limited amount of 'protein seeds' that interact with pectin initially. The turbidity results also showed that for pectins with same DM, the complex formed between βLgA and pectins with randomly distributed methyl-ester groups scatter more light compared to those formed between βLgA and blockwise methyl-esterified pectins. For block methyl-esterified pectins, the charged groups are distributed in blocks, which will lead to the formation of few large protein domains, promoting the formation of tightly bound dense complexes. For randomly methyl-esterified pectins however, the charged groups are randomly distributed, and as a result, the formation of several smaller less compact βLgA aggregates formed discretely along pectin molecules is favoured.

Conclusions

The binding between βLgA and pectins is affected by the overall and local charge densities of the pectin chains. Binding between the macromolecules at pH 4 and at low ionic strengths could only be observed when pectins possessed a sufficient number of non-methyl-esterified groups in a local region; otherwise the binding could not be initiated due to weak electrostatic interactions as well as increased steric hindrance. We propose a binding mechanism to explain the complexation interactions occuring between βLgA and pectin. During the course of titrating βLgA into pectin solutions, small amounts of βLgA molecules are deposited onto pectin chains initially as seeding proteins through electrostatic interactions. This then promotes the large-scale nucleated growth of βLgA aggregates on top of the seeding proteins, ultimately leading to the formation of local protein rich domains that bridge the primary complexes. Based on the proposed binding mechanism, it is anticipated that two different microstructures of βLgA-pectin complexes should be formed throughout the entire ITC titration, as observed. The difference in overall and local charge densities of pectin not only determine the binding affinity and enthalpic and entropic contributions observed from the protein-polysaccharide interaction, but also determine the size and the microstructure of the final assemblies of protein-polysaccharide complexes. Such molecular control of hierachical assembly is of fundamental interest.

Acknowledgements

We wish to thank the Riddet Institute for CoRE funding for this project including a PhD scholarship for AYX and the University of Auckland for a contribution to financial support. We thank Dr Richard Kingston from the University of Auckland for valuable discussions on ITC.

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