

Influence of pectin characteristics on complexation with β -lactoglobulin

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Abstract

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Key words Pectin, β -lactoglobulin, soluble complexes, charge density, charge distribution, complex formation.

Pectin and proteins are both common food constituents. The type of pectin that forms complexes with protein is known to be of great influence on the structure and stability of liquid foods. Therefore, the aim of this thesis is to investigate the influence of the overall charge and local charge density of pectin on the formation of soluble complexes with β -lactoglobulin (β -lg).

Combination of state diagrams and binding isotherms shows that a high local charge density of pectin is a prerequisite to form soluble complexes with β -lg at higher ionic strength. A high overall charge of pectin results in the close proximity of the GalA blocks. Therefore, β -Lg neighbours bind close together on pectin with a high overall charge, which leads to lateral repulsion and hence, maxima in the binding constant and the pH where insoluble complexes form with increasing ionic strength.

The formation of soluble complexes has an enthalpic driving force from electrostatic attraction and an entropic driving force from the release of small counterions from the electric double layer and water molecules from hydrophobic surroundings. A high local charge density, at low ionic strength results in complex formation dominated by an enthalpic driving force. A low local charge density gives a more even distribution between enthalpic and entropic contributions. An increase in ionic strength decreases the enthalpic contribution, with a relative increase in the entropic contribution, supporting the idea of water release from hydrophobic surroundings.

Adsorption from β -lg-pectin mixtures to a hydrophobic surface leads to low adsorption rates due to a low concentration of free protein. Sequential adsorption of β -lg and pectin shows that low overall charge pectin protrudes more into the solution than high overall charge pectin, resulting in a more negative ζ -potential for low overall charge pectin. After sequential adsorption, β -lg is most stable against wash-out with a terminal pectin layer.

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Chapter 1

General introduction

1.1 Introduction

Proteins and polysaccharides are key ingredients in many foods. Proteins perform several critical functions in food systems that contribute to the sensorial properties by thickening, gelation, emulsification, foaming, texturizing, water binding, adhesion and cohesion, and lipid and flavour binding (Damodaran & Paraf, 1997). In addition, their nutritional value is of great importance. Polysaccharides in foods are used for their action as a stabilizer, for water retention, thickener, gelation, emulsifier, but also as dietary fibre (Stephen & Churms, 1995). In food products, proteins and polysaccharides are often both present. When a solution of protein and polysaccharide are mixed, there are three options (see figure 1.1): the protein and polysaccharide are I) co-soluble, giving a stable solution in which both protein and polysaccharide are present individually; II) incompatible, which will give a phase separation into a protein rich and polysaccharide rich phase; III) form complexes, where protein and polysaccharide associate due to electrostatic forces. The formation of complexes between proteins and polysaccharides can be further divided into soluble complexes and insoluble complexes. The phase of aggregated insoluble complexes is also called a coacervate phase (Tolstoguzov, 1991). The complex formation between proteins and polysaccharides can be of great influence on the functional properties the protein and polysaccharide may have in a food. The formation of complexes between protein and polysaccharides will be discussed further in the following paragraphs.

1.2 Complex coacervation

The formation of complexes between proteins and anionic polysaccharides was already studied in the early 20th century by Tiebackx, Bungeberg de Jong and Kruyt (Tiebackx, 1911; Bungenberg de Jong & Kruyt, 1929). They studied the phase separation of gelatin and gum arabic into a concentrated gelatin–gum arabic phase and a phase that contains mainly solvent. This concentrated phase was called the coacervate phase, and the phenomenon of phase separation was called complex coacervation. The complexes are the result of electrostatic attraction between the two

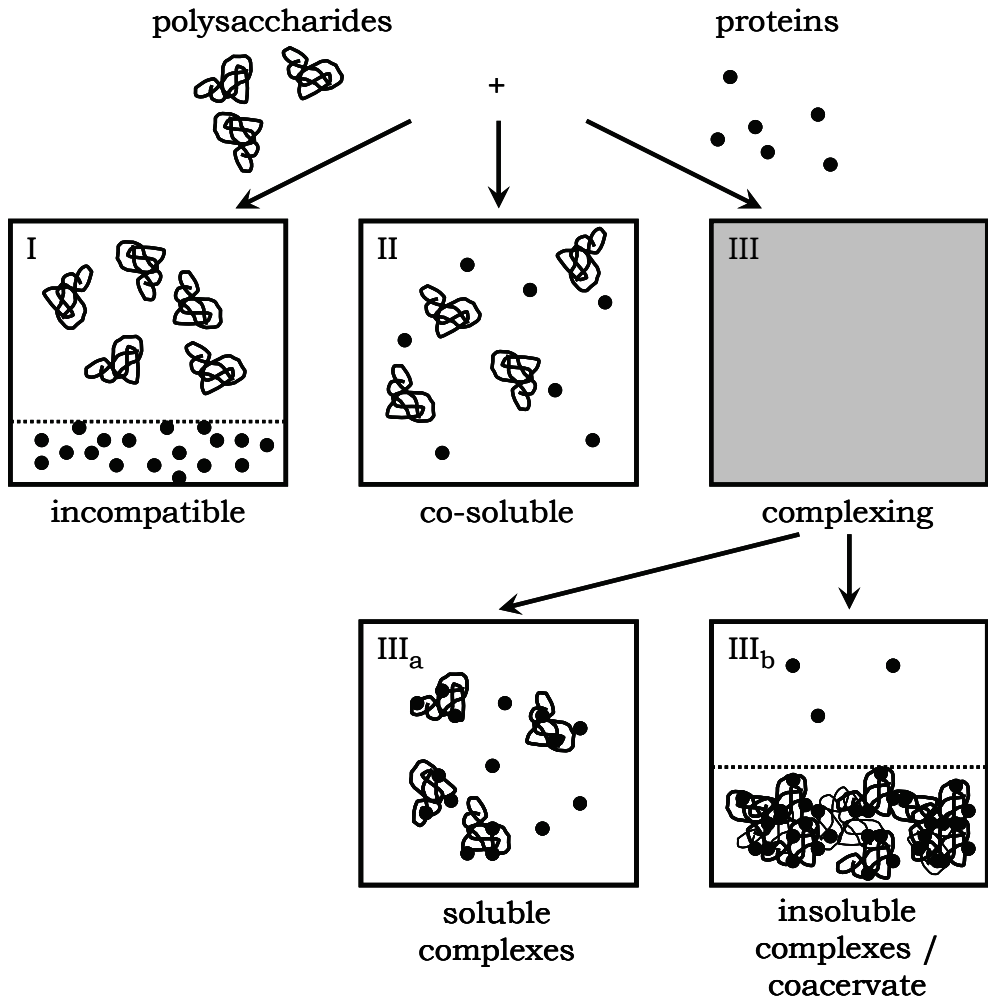


Figure 1.1: Mixing of proteins and polysaccharides can lead to incompatibility, co-soluble polymers or complex formation. Complex formation can be separated in soluble complexes and insoluble complexes, or coacervate (Tolstoguzov, 1991).

biopolymers. As complexes are formed, the opposite charges of the two biopolymers compensate each other. When sufficient protein has bound to the polysaccharide, there is a lack of electrostatic repulsion between the biopolymer complexes. Without the electrostatic repulsion, the complexes aggregate and subsequently phase separate (Mattison, Brittain et al., 1995).

1.3 Complex formation between proteins and polysaccharides

To discuss the complex formation between a protein and an anionic polysaccharide, the formation of complexes between proteins and polyelectrolytes, synthetic or natural, anionic or cationic, may be considered first, as the theory behind the complex formation is universal.

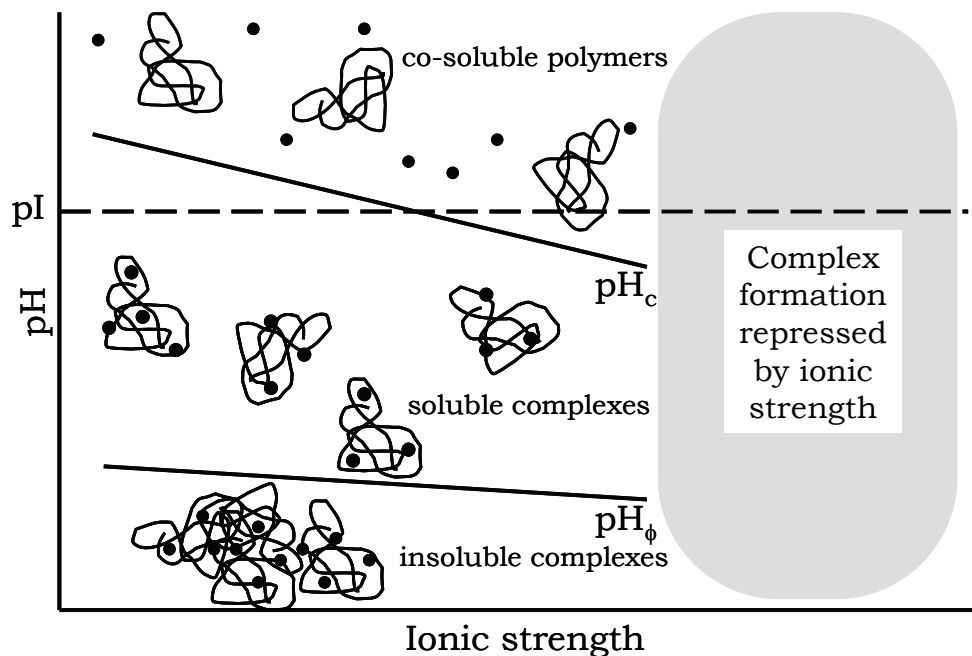


Figure 1.2: Generalized state diagram of protein-anionic polyelectrolyte complex formation. The mentioned pI is that of the protein (partly based on (De Kruif, Weinbreck et al., 2004))

The formation of complexes between proteins and polyelectrolytes is usually driven by electrostatic attraction (Schmitt, Sanchez et al., 1998), but under special conditions, also the release of small counterions from the electric double layer of both the protein and polyelectrolyte can be the driving force for complex formation (Henzler, Haupt et al., 2010). There are three states that can be identified, based on the type of complexes that are present in solution: Co-soluble polymers, soluble complexes, and insoluble complexes (coacervate phase). Figure 1.2 gives a schematic drawing of these three states. The states are separated by two state boundaries: pH_c

in between co-soluble polymers and soluble complexes and pH_{ϕ} for the association of soluble complexes to insoluble complexes (Dubin, Gao et al., 1994). If the pH is changed further into the insoluble complex region, it is possible to resolubilize the coacervate phase into co-soluble polymers. If the state diagram is determined to include the resolubilization of the coacervate phase, pH_{ϕ} is denoted as $pH_{\phi,1}$ and the pH at which the coacervate phase resolubilizes to soluble polymers is denoted as $pH_{\phi,2}$ and would be located below the insoluble complex region in figure 1.2 (Weinbreck, De Vries et al., 2003).

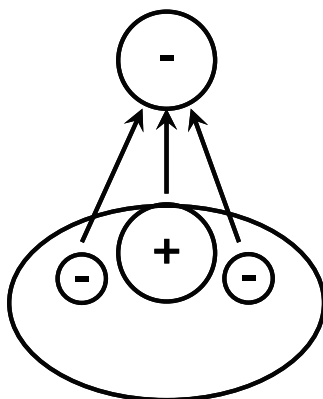


Figure 1.3: Electrostatic attraction and repulsion between a polyelectrolyte and a protein. Due to charge anisotropy on the protein surface there is a short range electrostatic attraction and a long range electrostatic repulsion (Seyrek, Dubin et al., 2003).

pH_c is located close to the iso-electric point (pI) of the protein, usually on the side where protein and polyelectrolyte are still of the same charge (the so called "wrong" side of pI (De Vries, Weinbreck et al., 2003)). This is attributed to charge anisotropy on the protein surface, so that local favourable electrostatic interactions with the polyelectrolyte are possible (Park, Muhoberac et al., 1992). The formation of soluble complexes is driven by electrostatic attraction, so the ionic strength influences the balance between short range electrostatic attraction and long range electrostatic repulsion by reducing the size of the electrical double layer (figure 1.3, (Seyrek, Dubin et al., 2003)). Raising the ionic strength will move pH_c from the wrong side of pI to the correct side (Mattison, Brittain et al., 1995; Weinbreck, De Vries et al., 2003). A theoretical model (De Vries,

Weinbreck et al., 2003; Weinbreck, De Vries et al., 2003) has been developed to predict pH_c as a function of the polyelectrolyte charge density and protein charge anisotropy.

pH_ϕ is always located on the correct side of pI and is generally associated with the overall charge neutralization between protein and polyelectrolyte. The result is a lack of electrostatic repulsion between the individual protein–polyelectrolyte complexes and the individual complexes aggregate to give a macroscopic phase separation (Mattison, Brittain et al., 1995).

1.4 Functionality of protein–polysaccharide complexes

For the functionality of protein–polysaccharide complexes we will focus on food applications. Protein–polysaccharide complexes are used to stabilize acidified milk drinks (AMD), enhance emulsion and foam stability, micro-encapsulation of ingredients, fat replacers and meat analogues (Tolstoguzov, 1991; Schmitt, Sanchez et al., 1998; Vargas, Pastor et al., 2008; Dickinson, 2009; Given Jr, 2009).

1.4.1 Acidified milk drinks

The casein micelles in an AMD are not stable at the pH of the drink, resulting in their flocculation. To prevent the flocculation of the casein micelles, high methyl esterified pectin is added to the drink (Glahn, 1982; Parker, Boulenguer et al., 1994; Kravtchenko, Parker et al., 1995; Boulenguer & Laurent, 2003). The pectin adsorbs onto the surface of the casein micelles by means of attractive electrostatic interactions (Pereyra, Schmidt et al., 1997; Tuinier, Rolin et al., 2002). Pectin stabilizes the casein micelles against flocculation through a combination of steric hindrance and electrostatic repulsion (Glahn, 1982; Kravtchenko, Parker et al., 1995; Tholstrup Sejersen, Salomonson et al., 2007), but also the formation of a weak gel network by the pectin has been suggested (Laurent & Boulenguer, 2003). High methyl esterified pectin is preferred over low methyl esterified pectin as it protrudes more into the solution and hence provides a better steric stabilization (Pereyra, Schmidt et al., 1997). Of the high methyl esterified pectins only the calcium sensitive pectins are

capable of stabilizing the AMD (Glahn & Rolin, 1996; Laurent & Boulenguer, 2003).

1.4.2 Emulsions

An efficient emulsifier rapidly reduces the interfacial tension at the oil-water interface, binds strongly to the interface and protects the oil droplets from coalescence (Dickinson, 2009). Proteins are capable of quickly adsorbing to the oil-water interface, but provide a poor stability towards environmental stresses like pH, ionic strength, heating and freezing (Güzey, Kim et al., 2004). The use of polysaccharides as emulsifiers leads to an emulsion with a good stability to environmental stresses, but polysaccharides are poor at producing small emulsion droplets and often require high concentrations (McClements, 2003). The combination of the two biopolymers seems ideal to obtain an emulsion consisting of small droplets and with good stability towards environmental stresses.

Two different approaches to combine proteins and polysaccharides have been attempted: the formation of covalent complexes between protein and polysaccharides, mainly through a Maillard reaction, and the use of complexes based on the electrostatic attraction between protein and polysaccharide. Covalent complexes of protein and polysaccharide can be considered as a gum arabic analogue, where the protein adsorbs to the interface and covalently bound polysaccharide provides stabilization against coalescence and flocculation by electrostatic repulsion and steric hindrance (Neiryneck, Van der Meeren et al., 2004; Dickinson, 2009). Stable emulsions using electrostatic complexes of proteins and polysaccharides can be separated in bilayer emulsions and mixed emulsions (Jourdain, Leser et al., 2008). Bilayer emulsions are prepared by creating a primary emulsion with a protein as emulsifier. To this primary emulsion, a solution of polysaccharide is added and the emulsion is allowed to equilibrate. After some time the emulsion pH is adjusted to the desired pH (Güzey, Kim et al., 2004; Güzey & McClements, 2007; Jourdain, Leser et al., 2008). Mixed emulsions are prepared using solutions containing both protein and polysaccharide. The protein and polysaccharide co-adsorb to provide an emulsion with improved stability. The presence of polysaccharide on the surface of the emulsion droplets

provides stabilization through steric repulsion between emulsion droplets by and increases the rigidity of the oil–water interfacial membrane (Einhorn-Stoll, Glasenapp et al., 1996; Jourdain, Leser et al., 2008; Gharsallaoui, Yamauchi et al., 2010).

1.4.3 Foam

Foams need stabilization of the air–water interface to protect them from draining. Foams are usually stabilized by proteins (Dickinson, 1998), but stability can be improved by the addition of a polysaccharide that increases viscosity or forms a gel in the stationary phase and thus reduces the drainage of water from the foam (Carp, Baeza et al., 2004; Baeza, Carrera Sanchez et al., 2005). The formation of electrostatic complexes between the protein and polysaccharide can enhance the foam stability, but may also limit the transport of the protein to the surface as the concentration of the fast adsorbing free protein is lowered (Ganzevles, Cohen Stuart et al., 2006). Increased foam stability of protein–polysaccharide complexes, as compared to foam stabilized with only protein, has been reported by several authors (Schmitt, Palma da Silva et al., 2005; Miquelim, Lannes et al., 2010; Schmidt, Novales et al., 2010). The enhanced stabilization is thought to arise from the slowing of the drainage by the presence of the polysaccharide in the Plateau borders and increased viscosity in the continuous phase (Mann & Malik, 1996; Schmidt, Novales et al., 2010), and electrostatic repulsion between the surfaces of the air bubbles (Mann & Malik, 1996).

1.4.4 Micro-encapsulation

Micro-encapsulation, for food ingredients, is performed because a specific ingredient needs to be shielded from its environment. Shielding the ingredient can be necessary to prevent it from degradation, during the shelf life of the product or inside the body, to mask an off flavour, but also when the ingredient is not soluble in water (Schmitt, Sanchez et al., 1998; Guzey & McClements, 2006a; De Vos, Faas et al., 2010; Sagalowicz & Leser, 2010). Micro-encapsulation can be achieved by creating a very rigid, impermeable layer as the stabilizing layer of an emulsion. The ingredient is often in the oil phase of the emulsion, but when it is water soluble, a

W/O/W double emulsion needs to be prepared. The approach to micro-encapsulation is therefore similar to creating emulsions with enhanced stability using proteins and polysaccharides outlined above. Of special interest have been the emulsions using the layer-by-layer approach, where alternating layers of protein and polysaccharide or anionic and cationic polysaccharide are deposited on the oil-water interface. These emulsions have been shown to be very stable under different environmental conditions like pH, salt and temperature (Guzey & McClements, 2006a; Güzey & McClements, 2006).

1.4.5 Fat replacers and meat analogues

Fat replacers and meat analogues are based mainly on the formation of insoluble complexes (coacervate), due to electrostatic attraction between the protein and polysaccharide. Common combinations for fat replacers or meat analogues are whey protein isolate, casein, egg white proteins, gelatin, or soy protein isolate, combined with xanthan gum, alginate, guar, or carrageenan (Schmitt, Sanchez et al., 1998; Laneuville, Paquin et al., 2005). The coacervate phase needs to be broken into small particles in order to mimic the creaminess and melting sensation of fat (Laneuville, Paquin et al., 2000). Meat analogues are based on the fibrous particles that are obtained when protein and polysaccharides are allowed to form complexes under a constant shear force (Soucie & Chen, 1986). Alternatively, the protein can be trapped in a polysaccharide gel matrix by using a polysaccharide that forms a gel under the influence of calcium (Tolstoguzov, Izjumov et al., 1974; Kweldam, 2003)

1.5 Techniques for studying protein-polysaccharide complex formation and characteristics

An excellent and detailed review on techniques that are used to study protein-polyelectrolyte complexes was published by Cooper et al (Cooper, Dubin et al., 2005). The scope of the research described in this thesis limits itself to the formation of soluble complexes between proteins and polysaccharides in solution. Some useful techniques to study these soluble complexes will be outlined in this section. An overview of the techniques is presented in table 1.1.

1.5.1 Turbidimetric titration

Turbidimetric titration is a useful technique to determine the pH where the state boundaries of protein–polyelectrolyte complex formation takes place and requires only a simple spectrophotometer to measure (Park, Muhoberac et al., 1992; Mattison, Brittain et al., 1995; Tsuboi, Izumi et al., 1996; Kaibara, Okazaki et al., 2000; Seyrek, Dubin et al., 2003; Weinbreck, De Vries et al., 2003; Liu, Elmer et al., 2010). Protein and polyelectrolyte are mixed at a pH where they are known to be present as co-soluble polymers. Next, the pH is altered in the direction of the protein pI, to start forming the complexes. This can be done by the addition of acid or base or, to avoid a dilution effect, glucono- δ -lactone (Weinbreck, De Vries et al., 2003). Co-soluble polymers have a very low turbidity, but when the pH comes below pH_c , and soluble complexes are formed, a steady increase in turbidity is observed. When the soluble complexes start to aggregate into insoluble complexes, the increase in turbidity is much more pronounced as the phase separation results in a turbid solution. pH_ϕ can thus easily be identified by the change in the slope of the turbidity curve.

1.5.2 Dynamic light scattering

Dynamic light scattering (DLS) is another technique that can be used for identifying the state boundaries of a protein–polyelectrolyte system and has been used with great success (Seyrek, Dubin et al., 2003; Weinbreck, De Vries et al., 2003; Mekhloufi, Sanchez et al., 2005; Schmitt, Palma da Silva et al., 2005; Tan, Koopal et al., 2009). As the result of particle diffusion, the intensity of scattered light (I_s) fluctuates around a constant, average intensity. DLS uses these fluctuations to determine the hydrodynamic radius (R_H) of the scattering particle. At the same time, the average intensity of scattered light is measured, which can be used in a similar fashion to turbidity measurements to identify pH_c and pH_ϕ . Seyrek et al (Seyrek, Dubin et al., 2003) found, for a system of bovine serum albumin or heparin with hydrophobically modified poly(acrylic acid), that both turbidimetric titration and dynamic light scattering (DLS) gave comparable results for both pH_c and pH_ϕ . Weinbreck et al (Weinbreck, De Vries et al., 2003) found DLS to be more sensitive for a system of whey protein isolate and gum arabic. This difference in sensitivity of the two

Table 1.1: Overview of techniques to characterize soluble complexes of proteins and polysaccharides.

techniques	Information	remarks	reference
Turbidimetric titration	pH _c and pH ₀	Easy technique, also provides information on the insoluble complexes region. Less sensitive than DLS for identification of pH _c	(Park, Muhoberac et al., 1992; Mattison, Brittain et al., 1995)
DLS ¹	pH _c and pH ₀ R _H , I _s	High sensitivity makes it ideal for identifying state boundaries. Additional information by measurement of R _H .	(Seyrek, Dubin et al., 2003; Weinbreck, De Vries et al., 2003)
SLS ¹	R _s , M _w , n	M _w of complexes allows calculation of n. The ratio R _H /R _g provides information about the shape of the complex	(Tsuboi, Izumi et al., 1996; Bowman, Rubinstein et al., 1997)
Proton titration	pH _c , charge regulation	Identification of pH _c (not as sensitive as DLS). Information about shift pK _a values in complex.	(Mattison, Brittain et al., 1995; Wen & Dubin, 1997)
Electrophoretic mobility	Charge of complexes	Charge of the complexes gives information about magnitude of electrostatic repulsion between complexes.	(Kravtchenko, Parker et al., 1995; Mekhloufi, Sanchez et al., 2005)
FACCE ¹	K _a , n	Low sample volume, high sample throughput. Only applicable at pH > pI + 1 to avoid binding of sample to capillary wall	(Gao, Dubin et al., 1997; Le Saux, Varrenne et al., 2006)
Ultra centrifugation	K _a , n	Low sample volume. Binding equilibrium may be disturbed, possibility of protein binding to membrane	(Girard, Turgeon et al., 2002)
ITC ¹	ΔH, K _a , ΔG, ΔS, n	Information about the driving forces of complex formation.	(Girard, Turgeon et al., 2003a; Henzler, Haupt et al., 2010)
DSC ¹	T _m	Shows if complex formation stabilizes or destabilizes the protein tertiary structure	(Zhang, Foegeding et al., 2004; Ibanoglu, 2005)
CD ¹	Protein secondary structure	Complex formation with polyelectrolyte can disturb protein secondary structural elements	(Schmitt, Sanchez et al., 2001; Mekhloufi, Sanchez et al., 2005)
FTIR ¹	Protein secondary structure	Complex formation with polyelectrolyte can disturb protein secondary structural elements	(Schmitt, Sanchez et al., 2001)

¹ DLS dynamic light scattering; SLS static light scattering; FACCE frontal analysis continuous capillary electrophoresis; ITC isothermal titration calorimetry; DSC differential scanning calorimetry; CD circular dichroism; FTIR Fourier transform infrared spectroscopy

techniques could be related to the different charge density of the used polyelectrolyte. The higher sensitivity of DLS compared to turbidimetric titrations makes it the method of choice to determine pH_c and pH_ϕ for protein-polysaccharide systems, as polysaccharides generally have a lower charge density than synthetic polyelectrolytes.

The R_H obtained from DLS experiments can be used to follow the formation of the soluble complexes. The measured R_H is that of the polysaccharide, as small globular proteins do not scatter sufficient light to be measured in a solution with polysaccharides. For a whey protein-gum arabic system, it was found that binding of whey protein to gum arabic reduced the R_H of gum arabic by reducing the internal repulsion. When insoluble complexes are formed, R_H , like the intensity of scattered light, increases dramatically (Weinbreck, De Vries et al., 2003).

1.5.3 Static light scattering

Static light scattering (SLS) allows for the measurement of the radius of gyration (R_g), molecular weight (M_w) and the second virial coefficient (A_2). For this, the intensity of light scattered by the protein-polysaccharide complexes is measured at different angles and at different concentrations to construct a Zimm-plot. From the Zimm-plot R_g , M_w and A_2 can be calculated (Tsuboi, Izumi et al., 1996; Bowman, Rubinstein et al., 1997). The molecular weight of the complex allows for the calculation of the binding stoichiometry (n). When combining the R_H measured using DLS and the R_g from an SLS experiment, the ratio R_H/R_g gives information on the shape of the complexes, where a random coil would give a ratio between 1.3-1.5 (Tsuboi, Izumi et al., 1996). From A_2 it can be determined if the complexes attract ($A_2 < 0$) or repel each other ($A_2 > 0$) (Bowman, Rubinstein et al., 1997).

1.5.4 Proton titration

Proton titration, or potentiometric titration, measures the amount of acid or base that is required to titrate a sample from pH A to pH B, corrected for the background solution. It can be used to measure pH_c (Mattison, Brittain et al., 1995; Girard, Turgeon et al., 2002), but is a considerably more elaborate method than either turbidimetric titrations or

DLS, with a lower sensitivity. Proton titrations are useful to determine a shift in the pK_a of the protein ionic amino acids under the influence of the binding to a polyelectrolyte (Mattison, Brittain et al., 1995; Wen & Dubin, 1997; Kayitmazer, Shaw et al., 2005). Interpretation is straight forward when a polyelectrolyte is used that contains a strong acidic or strong alkaline group as ionic groups, but care should be taken when (bio)polymers are used that consist of weak acidic or basic groups (Fan, Wang et al., 2009).

1.5.5 Electrophoretic mobility

The electrophoretic mobility of a particle may be measured when it is subjected to an external electrical field. From the electrophoretic mobility, the ζ -potential of the particle can be determined. The binding of an oppositely charged protein to a polyelectrolyte compensates the charge of the polyelectrolyte. As the amount of bound protein increases, the ζ -potential approaches zero (Ganzevles, Zinoviadou et al., 2006). From the ζ -potential, it can be estimated whether the particles are sufficiently charged to protect the particles from aggregation by means of electrostatic repulsion (Parker, Boulenguer et al., 1994; Kravtchenko, Parker et al., 1995).

1.5.6 Frontal analysis continuous capillary electrophoresis

Frontal analysis continuous capillary electrophoresis (FACCE) is a method based on capillary electrophoresis (CE). In a CE experiment, a sample is separated according to its charge/mass ratio by injecting a small sample into a capillary, over which an electrical field is applied. For studying the formation of soluble complexes between proteins and polyelectrolytes, the injection of only a small volume of sample will result in the disturbance of the binding equilibrium of protein to the polyelectrolyte, as free and bound protein will move at a different speed as a function of their charge/mass ratio. With FACCE, the sample is injected continuously, so that the concentration of free protein remains constant. The result is an electropherogram with two plateaus: the first plateau is the result of the free protein, the second plateau of both free and bound protein (Gao, Dubin et al., 1997). FACCE is often used for determining the

binding constant and binding stoichiometry, as both the amount of free and bound protein can be determined (Gao, Dubin et al., 1997; Hallberg & Dubin, 1998; Porcar, Cottet et al., 1999; Girard, Turgeon et al., 2003b; Hattori, Bat-Aldar et al., 2005; Le Saux, Varrenne et al., 2006; Seyrek, Dubin et al., 2007).

FACCE is usually performed in a capillary of bare fused silica, which is negatively charged. Therefore, care should be taken to prevent adsorption of positively charged biopolymers (like a protein) to the capillary wall. A good rule of thumb to prevent the adsorption of protein to the capillary wall is: $\text{pH} \geq \text{pI} + 1$ (Gao, Dubin et al., 1997; Porcar, Cottet et al., 1999; Seyrek, Hattori et al., 2004).

1.5.7 Ultracentrifugation

Ultracentrifugation has been employed to measure the amount of free protein in a solution with soluble complexes of protein and polyelectrolyte (Girard, Turgeon et al., 2002, 2003b). A small volume of the solution is centrifuged through a membrane with a molecular weight cut-off that allows the protein, but not the polyelectrolyte to which it binds, to pass the membrane. Care has to be taken not to centrifuge too much of the sample volume over the membrane to not disturb the adsorption equilibrium (Girard, Turgeon et al., 2002). From the measured concentration of free protein and calculated amount of bound protein, a binding isotherm can be constructed. When the free protein concentration is low, this technique can be treacherous, as even low protein binding membranes are known to bind some protein.

1.5.8 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) measures the heat released from the binding of protein to polyelectrolyte. At constant pressure, the released heat equals the enthalpy of binding. In addition, ITC may allow for the determination of a binding isotherm, a binding constant, binding stoichiometry, the Gibbs energy and the entropy change, by means of curve fitting (Kozlov & Lohman, 1998; Girard, Turgeon et al., 2003a; Guzey & McClements, 2006b; Romanini, Braia et al., 2007). This works well for a polyelectrolyte with a constant repeating unit, resulting in uniform binding

sites. For a system with a polyelectrolyte that has a variable repeating unit and hence non-uniform binding sites (like pectin), great care has to be taken into the selection of the model to fit the ITC data, as will be discussed in chapter 4 of this thesis. From the thermodynamic parameters obtained by ITC, the driving force behind the complex formation can be identified. Enthalpic contributions mainly result from electrostatic interaction between oppositely charged ionic groups, hydrogen bridge formation (Girard, Turgeon et al., 2003a; Schmitt, Palma da Silva et al., 2005; Hofs, Voets et al., 2006; Romanini, Braia et al., 2007; Tan, Koopal et al., 2009). Entropic contributions may result from the release of small counterions due to the overlap of electrical double layers, the release of immobilized water, in particular dehydration of hydrophobic moieties, and changes in the conformational entropy of the biopolymers (Tan, Koopal et al., 2009; Henzler, Haupt et al., 2010).

1.5.9 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is used to determine the thermal denaturation temperature (T_m) of a protein. When the protein is in a complex with a polyelectrolyte, the conformational stability may be affected (Ibanoglu, 2005). Structure stabilizing ionic pairs or hydrogen bonds of the protein may be broken by the complex formation or the exposure of buried hydrophobic groups is stabilized by hydrophobic groups on the polyelectrolyte (for instance methyl groups on pectin) (Vardhanabhuti, Yucel et al., 2009; Jones, Decker et al., 2010). A number of studies have been performed on different systems, reporting the stabilization and de-stabilization of the protein, but also no effect on the T_m of a protein by complex formation with a polyelectrolyte (Zhang, Foegeding et al., 2004; Romanini, Braia et al., 2007; Vardhanabhuti, Yucel et al., 2009).

1.5.10 Circular dichroism

Circular dichroism (CD) is a technique that measures the differential adsorption of left and right circularly polarized light. CD can be used to determine the secondary structural elements (α -helix, β -sheet and random coil) of a protein. Upon complex formation of protein and polyelectrolyte,

there may be a change in the secondary structure of the protein (Schmitt, Sanchez et al., 2001; Antonov & Wolf, 2005; Mekhloufi, Sanchez et al., 2005).

1.5.11 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) measures the vibrational frequencies of specific bonds in a molecule. From the FTIR spectrum the type and amount of secondary structural elements can be determined. When the protein binds to a polyelectrolyte, the secondary structure of the protein may be altered, giving rise to a change in the FTIR spectrum of the protein. Identifying which type of secondary structure is altered by the binding to the polyelectrolyte can give information about the location on the protein where binding takes place (Schmitt, Sanchez et al., 2001).

1.6 Biopolymers used in the current study

The research described in thesis deals with the complex formation between β -lactoglobulin (β -lg) and pectin. β -lg is the major whey protein, and often used a model protein for the preparation of emulsions, foams and gels (Dickinson, 1999; Van Vliet, Lakemond et al., 2004; Tcholakova, Denkov et al., 2006). Pectin is an anionic polysaccharide that is best known for its gelling capabilities in jams and jellies, but also finds application as a stabilizing or thickening agent (Voragen, Pilnik et al., 1995). One application of pectin with special interest for this thesis is its capability to stabilize acidified milk drinks against sedimentation, as outlined in paragraph 1.3.1. In the two subsequent sections some structural characteristics of the two biopolymers are discussed in detail.

1.7 Pectin

Pectin is a complex polysaccharide that is found in the primary cell wall and in the middle lamella of plant cells (O'Neill & York, 2003). Pectin plays an important role in the formation of higher plant cell walls, where it influences properties as porosity, surface charge, pH and ion balance (McNeil, Darvill et al., 1984; Fry, 1988). Pectin consists of a so called

"smooth" region of homogalacturonan, and a "hairy" region that consists of a rhamnogalacturonan backbone that is branched with mainly neutral sugars side chains (figure 1.4, (Schols & Voragen, 1996). The side chains of the rhamnogalacturonan in the hairy regions consist mainly of neutral sugars, such as arabinose, galactose and xylose (Voragen, Pilnik et al., 1995).

1.7.1 *Extraction of industrial pectin*

Pectin is isolated, most commonly from citrus or apple peel, by means of an extraction at elevated temperature with a dilute mineral acid, with a pH of approximately 2. Depending on the extraction conditions, the galacturonic acid (GalA) content and the methyl ester content can be varied. The pectin extract is separated from the remaining solid material, and concentrated by vacuum evaporation to prevent undesired pectin degradation due to alleviated temperatures under acidic conditions. Pectin is precipitated from the concentrated liquid by adding an alcohol (mostly iso-propanol). The pectin separates as a gelatinous mass which is recovered, washed, dried and ground (May, 1990; Rolin, 2002).

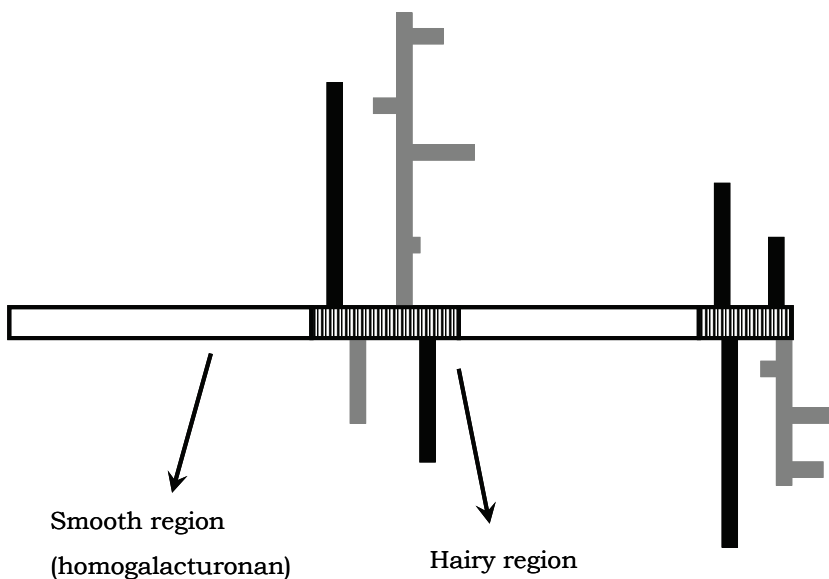


Figure 1.4: Model representation of pectin structure. The smooth region consists of homogalacturonan, the hairy region has a rhamnogalacturonan backbone with different types of branched structures consisting of neutral sugars (Guillotin, 2005)

1.7.2 Composition of industrial pectin

The acidic extraction of pectin removes most of the neutral side chains of the pectin, so that it mainly consists of homogalacturonan. We will therefore only discuss the structural features of the homogalacturonan. The pectin samples have a GalA content of between 70 and 85% (Daas, Boxma et al., 2001), the remainder consists of neutral sugars, methyl esters, acetyl esters, protein, phenols and ash (Kravtchenko, Voragen et al., 1992). Phenols are only a minor fraction (Kravtchenko, Voragen et al., 1992), acetyl esters are usually only present in appreciable amounts in pectins isolated from sugar beets, olives or potato (May, 1990; Vierhuis, Korver et al., 2003). Some protein remains after the extraction, but usually not more than a few percent (Kravtchenko, Voragen et al., 1992; Dickinson, 2003). The methyl esters are present on the carboxylic group at C-6 of GalA (see figure 1.5). Although by weight the methyl esters only represent a few percent of the pectin mass, they are of great influence on the physico-chemical properties of the pectin as discussed below.

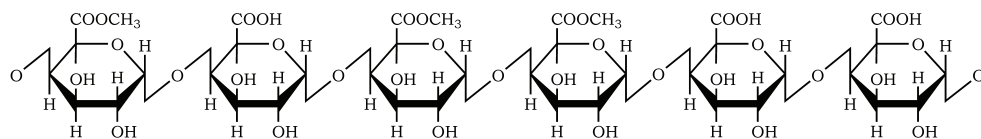


Figure 1.5: Homogalacturonan

1.7.3 Physico-chemical characteristics of pectin

The degree of methyl esterification (DM) of pectin is defined as the percentage of GalA monomers esterified with methanol (Voragen, Pilnik et al., 1995). Pectins are classified into high methyl esterified pectin (HM-pectin) and low methyl esterified pectin (LM-pectin) based on their DM. HM-pectin has a DM of 50% or more, LM-pectin a DM of less than 50% (May, 1990).

The methyl esters of pectin can be distributed in different manners, which greatly affects the physico-chemical behaviour of the pectin. Pectin may form a gel in the presence of calcium. To do so, it requires a block of at least eight to twelve consecutive non-methyl esterified GalA (n-Me-GalA) monomers (Voragen, Pilnik et al., 1995). Pectins that form gels in the presence of calcium are called calcium sensitive pectins, pectins that don't

form gels are called non-calcium sensitive pectins. The distribution of methyl esters over the GalA backbone is characterized by its degree of blockiness (DB). The DB of a pectin is measured by digestion of the pectin with an endo-polygalacturonase from *Kluyveromyces fragiles*. This enzyme can split the homogalacturonan, but requires 4 adjacent n-Me-GalA units to do so (Pasculli, Geraeds et al., 1991; Daas, Meyer-Hansen et al., 1999). The DB of the pectin is defined as the percentage of GalA that is released as mono-, di- or trimer of n-Me-GalA of the total content of n-Me-GalA of the pectin (Daas, Meyer-Hansen et al., 1999). A calcium sensitive pectin will have a high DB, a non calcium sensitive pectin a low DB. From the digests obtained for DB determination, additional information can be obtained about the methyl ester distribution. By looking more closely to the distribution of the mono-, di- and tri GalA released in the digest, an indication is obtained about the length of the GalA blocks. The ratio of GalA oligomers with and without methyl esterified residues, gives information about how close the GalA blocks are together (Daas, Voragen et al., 2000; Daas, Boxma et al., 2001). The DM determines the overall charge of pectin and the DB the local charge density.

The DM and DB of a commercial pectin is the result of the plant source, the extraction process and, possibly, modification by endogenous pectin methyl esterases (PME) (Voragen, Pilnik et al., 1995; Rolin, 2002). The modification by endogenous PME can be of great influence on the functionality of the pectin as it removes methyl esters in a progressive or blockwise manner, thus creating calcium sensitive parts on the pectin. Pectins can be tailored to specific characteristics by treatment with alkali or fungal PME, which removes methyl esters in a random fashion. To remove methyl esters in a blockwise manner plant PME can be used (Limberg, Körner et al., 2000). Smart combination of the two allows for the production of pectins with the desired characteristics for a specific application of the pectin.

1.8 β -Lactoglobulin

β -Lg is the most abundant whey protein from bovine milk, making up more than 50% of a whey protein isolate (Cayot & Lorient, 1997). It is a

small globular protein with a molecular weight of 18.3 kDa (Verheul, Pedersen et al., 1999). The iso-electric point (pI) is 5.1 (Cayot & Lorient, 1997). β -Lg consists of 162 amino acids and has two genetic variants A and B, that differ at positions 64 (Asp/Gly) and 118 (Val/Ala) (Brownlow, Cabral et al., 1997b). β -Lg is folded in a structure with 9 anti-parallel β -strands and a short α -helix (figure 1.6) (Monaco, Zanotti et al., 1987; Sawyer, Kontopidis et al., 1999). β -Lg has an exposed hydrophobic surface pocket in a groove between the β -strand and α -helix (Tavel, Andriot et al., 2008). In solution it is present predominately as a dimer between pH 3.5 and 7.5. Higher order aggregates (mainly octamers) are reported just below the iso-electric point (Kumosinski & Timasheff, 1966; Verheul, Pedersen et al., 1999). The formation of these higher order aggregates is promoted by temperatures below 20°C (Kumosinski & Timasheff, 1966). β -Lg is often used in model studies on the functionality and application of whey protein.

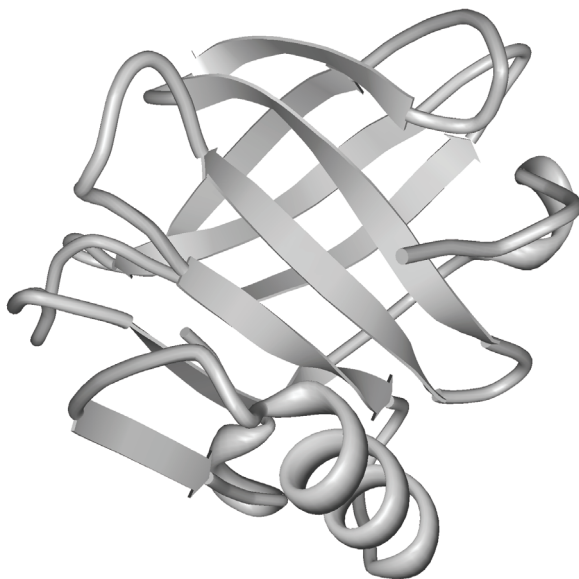


Figure 1.6: Model of the secondary structure of β -lg (Brownlow, Cabral et al., 1997a; Brownlow, Cabral et al., 1997b))

1.9 Aim of the current research

The aim of this thesis is to study the formation of non-covalent complexes of β -lg and pectin in solution, with special emphasis on the

influence of the pectin overall charge and local charge density. Complex formation between proteins and polyions has received considerable attention, including complex formation between the two biopolymers used in this study. The use of charged synthetic polymers allows for variation in the overall charge, the charge density, and the hydrophobic exposure. The downside is that the polymerization reaction doesn't allow variation of the local charge density of the molecule, while keeping the overall charge constant. By selecting the right pectins, the local charge density can be varied, while the overall charge is kept constant, without having to change the polymer backbone. This allows to control the local charge density in a manner that is not available to a synthetic polymer.

1.10 Outline of this thesis

Chapter 1 gives a general introduction on the formation and functionality of complexes between proteins and polysaccharides; an overview of techniques to determine the characteristics of soluble complexes and the two biopolymers chosen for the work in this thesis are reviewed.

In chapter 2 the complex formation between β -lg and pectin in solution is studied as a function of pH, ionic strength and mixing ratio of the two biopolymers. The construction of state diagrams based on dynamic light scattering data identifies the regions where either co-soluble polymers, soluble complexes or insoluble complexes are formed. From the ionic strength dependence of pH_c a local charge density of the pectins is determined. Additionally, proton titrations are performed to obtain information about the interactions between β -lg and pectin inside the formed complexes. From the state diagrams, the region of soluble complexes is chosen for further investigation.

Binding isotherms are described in chapter 3 and fitted with a theoretical model to obtain binding constants and cooperativity parameters. Also, the choice of the theoretical model is discussed, because choosing the correct model and fulfilling the basic assumptions associated with the model are essential for a correct interpretation of the binding

isotherms. The physico-chemical characteristics of the pectins used in this study are discussed in great detail.

Isothermal titration calorimetric experiments of β -lg-pectin complex formation form the basis of chapter 4. Combined with the binding isotherms from chapter 3, molar binding enthalpies are obtained. From the binding constants obtained in chapter 3 Gibbs free energy of binding can be calculated. Combined with the molar binding enthalpies obtained from isothermal titration calorimetry, the driving force behind the complex formation (enthalpic or entropic) is determined.

Chapter 5 investigates the adsorption of β -lg and pectin to a hydrophobic surface as a model system for the adsorption to oil-water or air-water interfaces. From this model system a prediction can be made which combination of β -lg and pectin may be successful in stabilizing emulsions and foams.

Finally, in the general discussion in chapter 6, the combined results are discussed to give a detailed overview of the influence of pectin local charge and overall charge density on the complex formation with β -lg. In chapter 6 also an outlook will be given on the relevance of this information for industrial applications.

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Chapter 2

Influence of the overall charge and local charge density of pectin on the complex formation with β -lactoglobulin

Abstract

The complex formation between β -lactoglobulin (β -lg) and pectin is studied using pectins with different physicochemical characteristics. Pectin allows for the control of both the overall charge by degree of methyl esterification as well as local charge density by the degree of blockiness. Varying local charge density, at equal overall charge is a parameter that is not available for synthetic polymers and is of key importance in the complex formation between oppositely charged (bio)polymers. LMP is a pectin with a high overall charge and high local charge density; HMP_B and HMP_R are pectins with a low overall charge, but a high and low local charge density, respectively. Dynamic light scattering (DLS) titrations identified pH_c , the pH where soluble complexes of β -lg and pectin are formed and pH_ϕ , the pH of phase separation, both as a function of ionic strength. pH_c decreased with increasing ionic strength for all pectins and was used in a theoretical model that showed local charge density of the pectin to control the onset of complex formation. pH_ϕ passed through a maximum with increasing ionic strength for LMP because of shielding of repulsive interactions between β -lg molecules bound to LMP, while attractive interactions were repressed at higher ionic strength. Potentiometric titrations of homo-molecular solutions and mixtures of β -lg and pectin showed charge regulation in β -lg-pectin complexes. Around pH 5.5 to 5.0 the pK_a 's of β -lg ionic groups are increased to induce positive charge on the β -lg molecule; around pH 4.5 to 3.5 the pK_a values of the pectin ionic groups are lowered to retain negative charge on the pectin. Since pectins with high local charge density form complexes with β -lg at higher ionic strength than pectins with low local charge density, pectin with a high local charge density is preferred in food systems where complex formation between protein and pectin is desired.

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2.1 Introduction

The interaction between polysaccharides and proteins influences the stability and structure of foods (De Kruif & Tuinier, 2001). For instance, acidified milk drinks are stabilized against protein sedimentation by the addition of pectins (Glahn, 1982; Parker, Boulenguer et al., 1994; Laurent & Boulenguer, 2003), emulsions can be stabilized (Güzey, Kim et al., 2004; Güzey & McClements, 2007) or destabilized (Dickinson & Pawlowsky, 1998) by the addition of polysaccharides, and foam stability can be influenced by polysaccharide-protein interactions (Nunes & Coimbra, 1998; Schmitt, Palma da Silva et al., 2005; Ganzevles, Zinoviadou et al., 2006).

The attractive and repulsive forces between polysaccharides and proteins may lead to biopolymer incompatibility, (Tolstoguzov, 1991) or complex formation (Tolstoguzov, 2003). Complex formation between (oppositely) charged macromolecules in solution has been widely investigated (De Kruif, Weinbreck et al., 2004; Cooper, Dubin et al., 2005). In a protein-polysaccharide system, generally, the polysaccharide carries negative charge, while the sign of the protein's charge varies with the pH of the solution. The macromolecules are co-soluble when they are both negatively charged (Park, Muhoberac et al., 1992; Mattison, Brittain et al., 1995; Girard, Turgeon et al., 2002; Weinbreck, De Vries et al., 2003). Soluble complexes are formed when the protein binds to the polysaccharide, forming a complex of which the charge has the same sign as the polysaccharide. The soluble complex is stabilized by electrostatic repulsion (Kaibara, Okazaki et al., 2000; Ganzevles, Zinoviadou et al., 2006). This may already occur at the alkaline side of the pI of the protein, due to positively charged patches (Park, Muhoberac et al., 1992) or by charge regulation of the protein-polysaccharide system (Da Silva, Lund et al., 2006). Phase separation occurs when the charge of the protein-polysaccharide complexes approaches neutrality. The lack of electrostatic repulsion between the complexes allows them to aggregate resulting in a macroscopic phase separation (Park, Muhoberac et al., 1992; Xia, Dubin et al., 1993; Mattison, Brittain et al., 1995; Weinbreck, De Vries et al., 2003).

pH_c is the state boundary between soluble polymers and soluble complexes. The onset of complex formation is controlled by the interaction between a single protein molecule and a single sequence of polymer segments and therefore independent of the mixing ratio of protein and polysaccharide (Mattison, Brittain et al., 1995; Weinbreck, Nieuwenhuijse et al., 2004). pH_c shifts to lower pH with an increase of ionic strength, due to shielding of the attractive interactions (Mattison, Brittain et al., 1995; Schmitt, Sanchez et al., 1998; Seyrek, Dubin et al., 2003; Weinbreck, De Vries et al., 2003). For certain combinations of protein and polyelectrolyte pH_c can also pass through a maximum for increasing ionic strength, depending on the balance between attractive and repulsive forces (Seyrek, Dubin et al., 2003). pH_ϕ is the state boundary between soluble complexes and separated phases and depends on the mixing ratio of protein and polysaccharide, as well as on ionic strength (Mattison, Brittain et al., 1995; Wen & Dubin, 1997; Cooper, Dubin et al., 2005). The phase separation is related to charge neutralization in the protein–polysaccharide complex, it implies that varying the ratio between protein and polysaccharide affects pH_ϕ . When ionic strength is increased, pH_ϕ decreases (Mattison, Brittain et al., 1995; Wen & Dubin, 1997; Weinbreck, De Vries et al., 2003).

Pectin is isolated from plant materials by means of an acid extraction. After acid extraction the predominant structure of pectin is α -(1→4) linked D-galacturonic acid (Voragen, Pilnik et al., 1995). These pectins are identified by two parameters: the degree of methyl esterification (DM) of the carboxyl group and the distribution of these methyl esters along the pectin backbone. The distribution of the methyl esters is represented as the degree of blockiness (DB), that can be investigated by digestion of pectin with endopolygalacturonase from *Kluyveromyces fragiles* (Daas, Meyer-Hansen et al., 1999). A high DB value means that the methyl esters are distributed in a blockwise manner, a low value means a random distribution. Glahn and Rolin (Glahn & Rolin, 1996) found that a calcium sensitive high methyl esterified pectin (pectin that forms gel particles when calcium is added, which implies a high DB) is capable of stabilizing acid milk drinks while a non-calcium sensitive high methyl esterified pectin with similar DM showed no stabilization of the acid milk drink.

The work reported in this paper is focused on complex formation between β -lactoglobulin (β -lg) and pectin. β -lg is the major constituent of whey protein and has been shown to dominate the interaction between polysaccharide and whey protein isolate (Weinbreck, De Vries et al., 2003). Pectin is chosen as it provides the possibility of varying the charge density on the polymer: the DM controls the total amount of charge on the polymer, while the DB controls the distribution of the charge. In this study well defined pectins with known DM and DB are used. Studying the interaction of these pectins with β -lg gives insight in the mechanism underlying the stabilizing action that pectin has in many food systems, that are made up of emulsions and foams.

2.2 Experimental

2.2.1 Materials

β -Lactoglobulin (pI 5.2) was purified from bovine milk using a non-denaturing technique described previously by (De Jongh, Gröneveld et al., 2001), with exception that the β -lg was dialyzed extensively against milli-Q water and lyophilized. β -Lg had a purity of over 98% and a ratio of the A:B- β -lg types of approximately 60:40. β -Lg solutions were free of aggregated material as was verified by dynamic light scattering (DLS). Three pectins, low methyl esterified pectin (LMP), high methyl esterified calcium sensitive pectin (HMP_B), and high methyl esterified non-calcium sensitive pectin (HMP_R), were kindly provided by CPKelco (Lille Skensved, Denmark). The pectins originate from lemon and were characterized on degree of methyl esterification and degree of blockiness (DB) (Daas, Boxma et al., 2001). LMP (C30 in (Daas, Boxma et al., 2001)) has a DM of 30 and a DB of 16.5. HMP_B and HM-NCSP were isolated from the same mother pectin on basis of calcium sensitivity. HMP_B (C70 (Daas, Boxma et al., 2001)) has a DM of 70 and a DB of 10.9, HMP_R (C74 (Daas, Boxma et al., 2001)) a DM of 74 and a DB of 1.7. This makes LMP and HMP_B pectins with a blockwise distribution of the methyl esters and HMP_R has a random distribution of methyl esters.

The pectins contained 0.02-0.05 mole of calcium per mole of galacturonic acid. The calcium was removed by washing the pectins five

times with 60% ethanol, containing 5% (v/v) hydrochloric acid. Next, the pectins were washed with 60% ethanol until they were free of chloride (tested with 0.2 % (w/w) silvernitrate). Pectins were subsequently washed with acetone and air dried. This was sufficient for LMP and HMP_B, but HMP_R required further treatment. HMP_R was dissolved to 10g/L and mixed with AG 50W-X4 200-400 mesh ion exchange resin from Bio-Rad Laboratories (Hercules, CA). HMP_R and ion exchange resin were separated by centrifugation and HMP_R was lyophilized. This resulted in pectins that contained less than 0.001 mole of calcium per mole of galacturonic acid.

All chemicals were of analytical grade.

2.2.2 Dynamic light scattering

Dynamic light scattering was performed on an ALV light scattering instrument equipped with a 200mW argon ion laser, tuned at a wavelength of 514 nm. Temperature was controlled by a Haake C3 thermostat and maintained at $25 \pm 0.1^\circ\text{C}$. Measurements were performed at a detection angle of 90° . pH titrations are performed directly in the measurement cell, using a Schott-Geräte computer-controlled titration set-up to control acid addition (0.04M HCl) and cell stirring. Samples were stirred for 30 seconds after acid addition, followed by a rest period of 15 seconds, after which the DLS-measurement was started. pH was measured with a Ag/AgCl glass electrode. The β -lg-pectin mixtures were titrated from their starting pH of 6.8-7.5 to pH 3.

The measured autocorrelation functions were analyzed with the method of cumulants (Stock & Ray, 1985). This method assumes that the scattering particles are spherical and that only the translational diffusion coefficient contributes to the decay of the autocorrelation function. The hydrodynamic radius (R_H) was calculated from the average diffusion coefficient using the Stokes-Einstein relation for spherical particles.

The pectin concentration was kept constant at 0.2 g/L, the β -lg concentration was varied between 0.2 g/L and 1.6 g/L giving weight ratios of 1, 2, 4 and 8 (β -lg/pectin). Ionic strength was set with NaCl to 4, 10, 30, 75 and 300mM. The pH where the intensity of the scattered light starts increasing is taken as pH_c ; pH_ϕ is identified as the pH where R_H shows a sudden strong increase (see figure 2.1), indicating the formation of large

insoluble complexes (Park, Muhoberac et al., 1992; Mattison, Dubin et al., 1998; Weinbreck, De Vries et al., 2003). All samples were measured in duplicate and pH_c and pH_ϕ were reproducible within 0.1 pH-unit. For sake of comparison the intensity of the scattered light is normalized (I_{norm}) for the intensity at pH well above pH_c , where pectin is the sole scattering component. This was done to correct for differences in the intensity in the laser light between different samples.

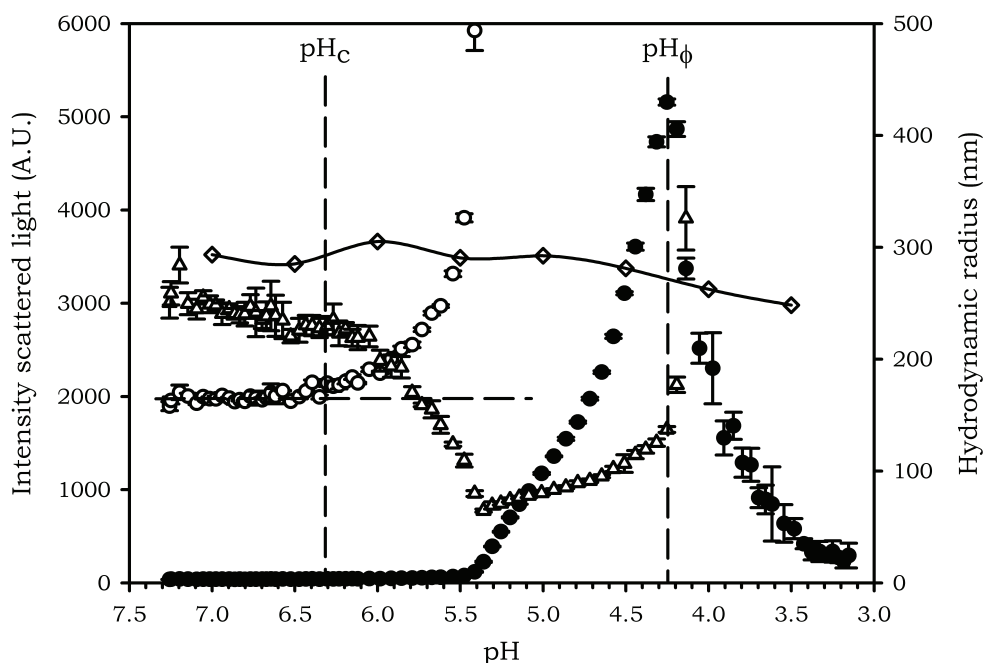


Figure 2.1: pH titration of a mixture of HMP_B (0.2 g/L) and β -lg (0.8 g/L), monitored by DLS. Ionic strength is 4mM. Intensity of scattered light (●), intensity of scattered light $\times$ 50 (○), hydrodynamic radius of the HMP_B- β -lg mixture (△), and hydrodynamic radius of HMP_B (0.2g/L) (◇).

2.2.3 Potentiometric titrations

Potentiometric titration curves were recorded on a Schott-Geräte computer-controlled titration set-up. pH was measured with a Ag/AgCl glass electrode. Titrations were performed under argon atmosphere. Prior to measurement the solutions were flushed with argon for 1 hour at pH 3 and continuous stirring to efficiently remove CO₂. Flushing occurred above the liquid to prevent foam formation. Next, solutions were brought to pH 8

and titrated with 0.1M HCl to pH 3.5, under a continuously refreshed argon atmosphere. Potentiometric titration curves were acquired for β -lg, the three pectins and mixtures of β -lg and pectin. Stock solutions were prepared at 4 g/L for β -lg and 1 g/L for pectin, and diluted to 1 g/L for β -lg and 0.5 g/L for pectin solutions, mixtures of β -lg and pectin were made with a constant pectin concentration of 0.5 g/L at weight ratios of 1, 2 and 4. Ionic strength of the solutions was set with NaCl to 4, 10, 30 and 75mM. Blank solutions were measured for all ionic strengths and subtracted from the samples. To evaluate the influence of β -lg-pectin interaction on potentiometric titration behaviour, ΔH^+ is plotted against pH. ΔH^+ is defined as follows: H^+ consumption of the mixture of β -lg and pectin, minus the H^+ consumption of β -lg, minus the H^+ consumption of pectin, all at the appropriate ionic strength and concentration of biopolymer.

2.3 Results and discussion

2.3.1 State diagrams of β -lg-pectin solutions

Figure 2.1 shows a typical DLS-titration curve, showing the intensity of scattered light (I) and the hydrodynamic radius (R_H) of the scattering particles. The titration starts around pH 7 where both the pectin and β -lg are negatively charged. I and R_H are those of the pectin, as β -lg scattered too little light to be measured. The beginning of the titration curve shows a horizontal line for I , as well as for R_H . The value for R_H is high for a polysaccharide with an approximate molecular weight of 150kDa and caused by a small fraction of the pectin that is in an aggregated form (Jordan & Brant, 1978). When pH_c at a pH of 6.3 is reached, I increases, while R_H decreases. Clearly, this is the result of complex formation as R_H remains constant for the HMP_B blank. The formation of complexes between pectin and β -lg increases the amount of mass in the scattering particle, therewith causing I to rise. This rise is only a fraction of the total increase in I , but can be easily identified by zooming in on the I -axis. The decrease of R_H is caused by the screening of charges by β -lg, which reduces internal repulsion in the pectin molecule. In addition, break up of pectin aggregates under the influence of β -lg molecules can not be excluded. pH_c is above the

iso-electric point of β -lg (5.2), which means that complexes between pectin and β -lg are already formed when both individual polymers carry a net negative charge. This is found for many protein–polyelectrolyte systems (Park, Muhoberac et al., 1992; Weinbreck, De Vries et al., 2003) and also for β -lg–pectin complexes by Girard et al (Girard, Turgeon et al., 2002).

At pH 5.4, when the iso-electric point of β -lg is approached, there is a steep increase in I , while R_H starts sloping up slowly. This indicates that the pectin is being loaded with more β -lg molecules, while still forming soluble complexes. Around pH 4.5 the increase in I becomes even larger. At the same time the increase in R_H becomes a bit steeper, causing the additional increase in I , as I is related to both the mass in the scattering particle as well as R_H . As the pH is decreased further, pH_ϕ is reached at pH 4.2. As this is the pH where insoluble complexes are formed R_H shows a dramatic increase. At pH_ϕ the clear solution becomes turbid and sediments if not stirred. Under these conditions the intensity of the scattered light can not be reliably interpreted due to multiple scattering events.

State diagrams (figure 2.2) displaying the values of pH_c and pH_ϕ for the three pectins and β -lg at different ionic strengths and weight ratios are constructed from the DLS titration curves. The three states are soluble polymers, soluble complexes and insoluble complexes or macroscopic phase separation (I, II and III respectively in figure 2.2). pH_c decreases with increasing ionic strength for all three pectins and shows no correlation with WR, which is in accordance with (Mattison, Brittain et al., 1995). The higher ionic strength screens the charges on β -lg and pectin, which leads to a reduction in the attractive interactions, shifting pH_c to lower pH where the protein carries more positive charge (Mattison, Brittain et al., 1995; Seyrek, Dubin et al., 2003; Weinbreck, De Vries et al., 2003). pH_c is absent for HMP_R at 300mM in the measured pH range.

pH_ϕ increases with increasing WR for all 3 pectins. When there is more β -lg in solution, more β -lg binds to the pectin, resulting in charge neutralization at a higher pH. For LMP pH_ϕ passes through a maximum as a function of ionic strength, whereas for HMP_B and HMP_R pH_ϕ decreases with increasing ionic strength. The overall charge on LMP is much higher than on HMP_B and HMP_R , and therefore LMP needs to associate with more β -lg molecules that have to be packed closer together to reach the point of

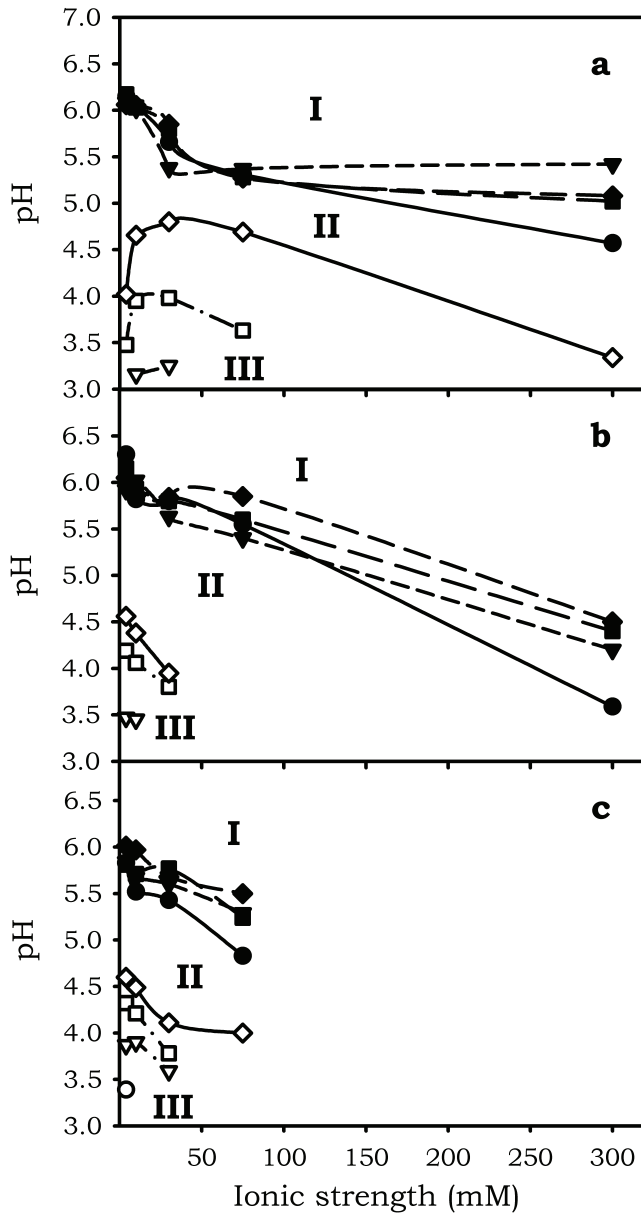


Figure 2.2: State diagrams of β -lg-pectin mixtures. pH_c (solid symbols) and pH_o (open symbols) for LMP (a), HMP_B (b) and HMP_R (c). Weight ratio = 1 ($\bullet, \circ$); 2 ($\blacktriangledown, \triangledown$); 4 ($\blacksquare, \square$) and 8 ($\blacklozenge, \lozenge$). Soluble polymers (I), soluble complexes (II) and insoluble complexes (III). Lines are drawn to guide the eye.

charge neutrality. By increasing the ionic strength, the repulsive forces between the β -lg molecules are reduced, allowing them to be packed closer together, leading to a higher pH_ϕ (Moss, Van Damme et al., 1997; Seyrek, Dubin et al., 2003).

For all three pectins pH_ϕ is absent, within the measured pH range, above a certain ionic strength for the different WR's. Increasing the ionic strength reduces the repulsive forces between the individual β -lg molecules as well as between pectin and β -lg. At the same time it also reduces attractive electrostatic interactions between pectin and β -lg (Seyrek, Dubin et al., 2003). To achieve complete charge neutralization, the pectin needs to bind a certain amount of β -lg molecules. For the combinations of higher ionic strength and lower WR, insufficient amounts of β -lg binds to neutralise the pectin charge within the measured pH range.

Figure 2.3 shows the normalized intensity of scattered light (I_{norm}) for the three pectins at a WR of 8. I_{norm} can be considered as a measure for the amount of mass in the scattering particle (Weinbreck, De Vries et al., 2003). An increase in I_{norm} is thus correlated to β -lg binding to pectin. For LMP (figure 2.3a), I_{norm} increases at the highest pH for an ionic strength of 30mM, followed by 10mM, 4mM, 75mM and 300mM. This agrees with the profile of pH_ϕ in figure 2.2a, where an ionic strength of 30mM results in the highest pH_ϕ . At an ionic strength of 30mM the balance between repulsive and attractive charge interactions between LMP and β -lg is at an optimum for association. Electrostatic repulsion between β -lg molecules, due to an inhomogeneous charge distribution over the β -lg surface, may add to this (Seyrek, Dubin et al., 2003). For an ionic strength of 4mM I_{norm} reaches a lower value than for 10, 30 and 75mM. Although the attractive forces should be at a maximum at 4mM, the repulsive forces between the β -lg and pectin lowers the pH where β -lg and LMP associate. Repulsion between β -lg molecules at pH below pI, prevents the pectin from being fully loaded with β -lg molecules. pH_ϕ is reached at lower pH, where pectin carries less negative charge and β -lg more positive charge to balance the lower number of β -lg molecules associated with the pectin molecules.

For HMP_B and HMP_R the variation in I_{norm} follows the ionic strength: a higher ionic strength implies a lower pH where I_{norm} starts increasing. This is in accordance with figure 2.2 where pH_ϕ decreases with increasing ionic

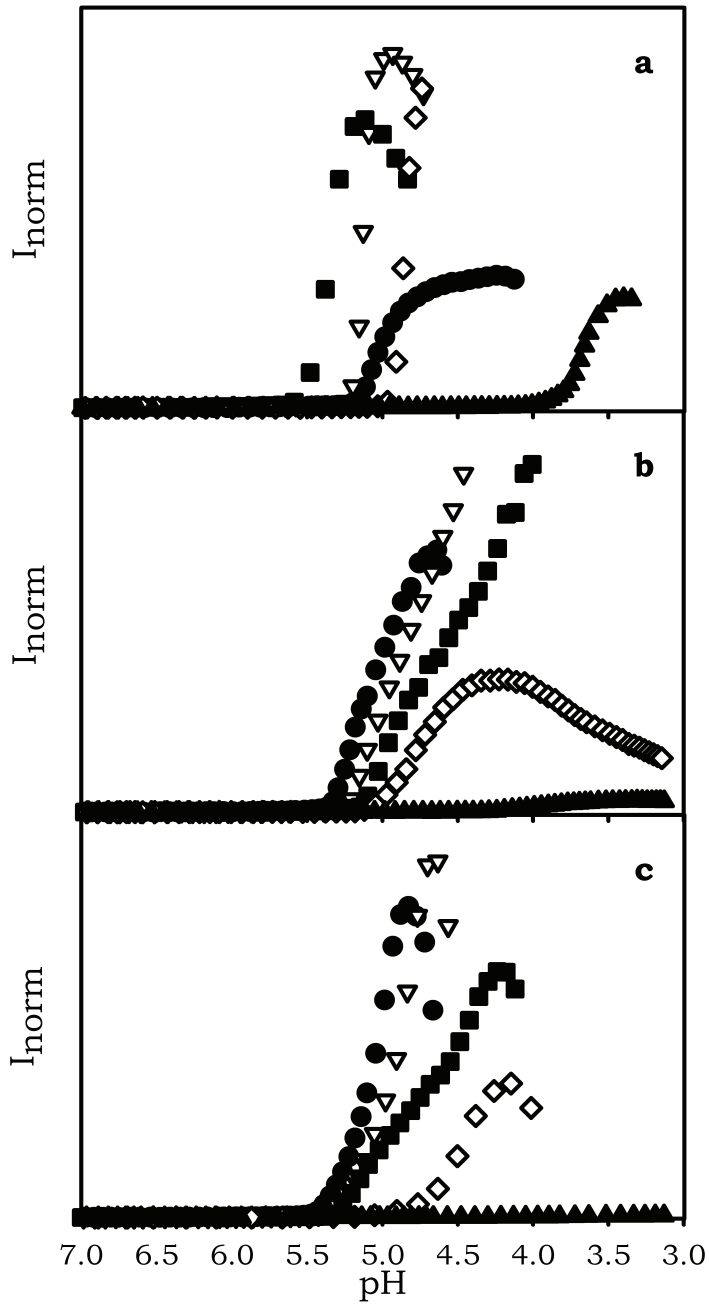


Figure 2.3: Intensity of scattered light, normalized to intensity of scattered light of pectin, for β -lg-pectin mixtures, pectin concentration is 0.2 g/L, weight ratio = 8. a) LMP, b) HMP_B and c) HMP_R. Ionic strength: 4mM (●), 10mM (▽), 30mM (■), 75mM (◇) and 300mM (▲)

strength. At low ionic strength (up to 30mM) there is hardly any difference in the profile of I_{norm} against pH for the two HM-pectins. However, at 75 and 300mM there is a clear difference: at 75mM HMP_B associates with β -lg at a higher pH. At 300mM there is no increase in I_{norm} anymore for HMP_R , where HMP_B still shows a small increase. HMP_B has a blockwise distribution of methyl esters, giving HMP_B regions of high local charge density, like LMP, that show association with β -lg at higher ionic strength than the lower local charge density regions on HMP_R . LMP contains more of these high local charge density regions than HMP_B , resulting in a much larger increase of I_{norm} at an ionic strength of 300mM. It is inferred that the attractive forces between pectin and β -lg are largest when the pectin has parts that carry a high local charge density.

De Vries, Weinbreck et al (2003) developed an analytical estimate for pH_c that includes the effects of protein charge heterogeneity. The theory gives expressions for the dependence of pH_c on ionic strength and the linear charge density of the flexible polyelectrolyte. It neglects many protein and polyelectrolyte structural details, but yields correct order-of-magnitude estimates. The strength of complexation may be summarized by a single parameter: the critical salt concentration, $n_{\text{s,c}}$, above which soluble complexes will only form at pH values below the protein isoelectric point (De Vries, Weinbreck et al., 2003; De Vries, 2004). The critical salt concentration can be estimated. In these estimates, the protein surface is viewed as a randomly charged surface with an average surface charge density σ , as a number density of elementary charges e per unit area, and strong local variations $\Delta\sigma$ around this average value. The polymer is assumed to be an ideal chain consisting of segments with length l_K , carrying v elementary charges per segment. This gives:

$$n_{\text{s,c}} \approx 0.4 \frac{\Delta\sigma v}{l_\text{K}} \quad (2.1)$$

The linear dependence on the polyelectrolyte charge density has also been confirmed in computer simulations (De Vries, 2004). An estimate for the charge density fluctuations on the protein surface at length scales in the order of the polyelectrolyte segment length is:

$$\Delta\sigma^2 \approx -\frac{\mu}{\pi l_K^2} \frac{\partial\sigma}{\partial\text{pH}} \Big|_{\text{pH}=\text{pI}} \quad (2.2)$$

With μ a numerical prefactor, depending on the type of protein.

In figure 2.4 the values for pH_c are averaged for the four weight ratio's and plotted against the ionic strength. At low ionic strength β -lg binds to the pectin above pI . Factors contributing to "complexation at the wrong side of the isoelectric point" may include the heterogeneous protein charge distribution (De Vries, Weinbreck et al., 2003; De Vries, 2004; Da Silva, Lund et al., 2006) and shifts in dissociation equilibria induced by complexation (Biesheuvel & Wittemann, 2005; Da Silva, Lund et al., 2006). From figure 2.4 $n_{s,c}$ can be estimated: HMP_B has a $n_{s,c}$ of 98mM, versus 56mM for HMP_R . LMP is estimated to have a $n_{s,c}$ of 95mM, comparable to HMP_B .

With $n_{s,c}$ estimated from figure 2.4, equation 2.1 and 2.2 can be used to determine v , the amount of elementary charges per pectin segment. For this we estimate l_K to be 2nm (Axelos, Lefebvre et al., 1987); μ was estimated to be 0.35 for β -lg and $\partial\sigma/\partial\text{pH} = -0.25 \text{ nm}^{-2}$ at the isoelectric point (De Vries, Weinbreck et al., 2003). This leads to a local charge density for HMP_R of 1.1 e/nm and 1.9 e/nm for both HMP_B and LMP. These numbers are rough estimates, but can be verified to be in the right range. The length of a galacturonic acid residue is 0.43nm (Rees & Wight, 1971). This gives about 2.5 monomers per nm. With a DM of 30 for LMP, it can carry about 1.8 e/nm . For HMP_B and HMP_R this is about 0.75 e/nm . This clearly shows that the difference between the $n_{s,c}$ of HMP_R and HMP_B , respectively, can be interpreted as being due to a difference in the local charge density of a factor of almost 2, with HMP_B resembling LMP in its local charge density.

2.3.2 Potentiometric titrations

The complexation of a protein with a charged polysaccharide or charged synthetic polymer is of electrostatic nature (Xia, Dubin et al., 1993; Wen & Dubin, 1997; Girard, Turgeon et al., 2002). It has been shown for bovine serum albumin and poly(dimethyldiallylammonium chloride) (PDMDAAC) that complexation leads to a shift in pK_a 's of various

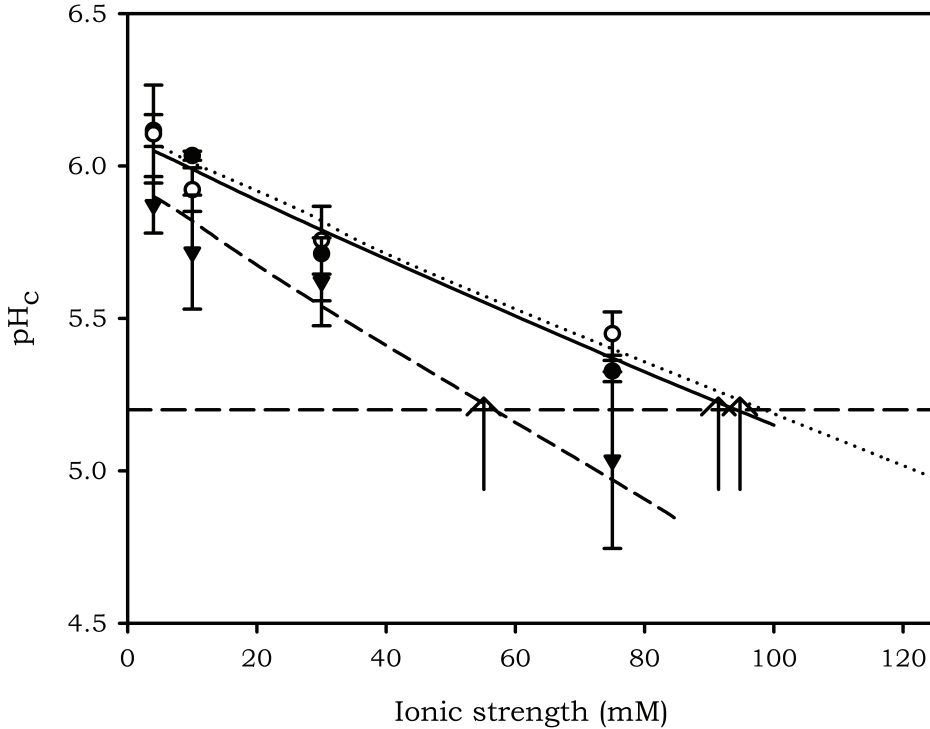


Figure 2.4: Critical pH values (pH_c) versus ionic strength for β -lg-pectin mixtures, for LMP (●), HMP_B (○), and HMP_R (▼). Data are averages for different β -lg/pectin weight ratios (1,2,4 and 8). The dashed horizontal line indicates the iso-electric point of β -lg (5.2). The arrows indicate estimates for the critical salt concentrations at which the pH_c values cross the iso-electric point: about 56mM for HMP_R, about 98mM for LMP and about 95mM for HMP_B

amino acid residues of the protein (Wen & Dubin, 1997). Since the polymer used in these experiments carries its charge on a quaternary ammonium, only the protein is responsible for changes in the potentiometric titration curve. (Wen & Dubin, 1997) and (Mattison, Dubin et al., 1998) showed that the pK_a 's of the protein only start shifting when complexes between protein and polymer are formed, enabling the determination of pH_c from potentiometric titrations. The potentiometric titration of β -lg and pectin is more complex than the titration outlined above as both protein and polysaccharide contain titratable groups in the measured pH range.

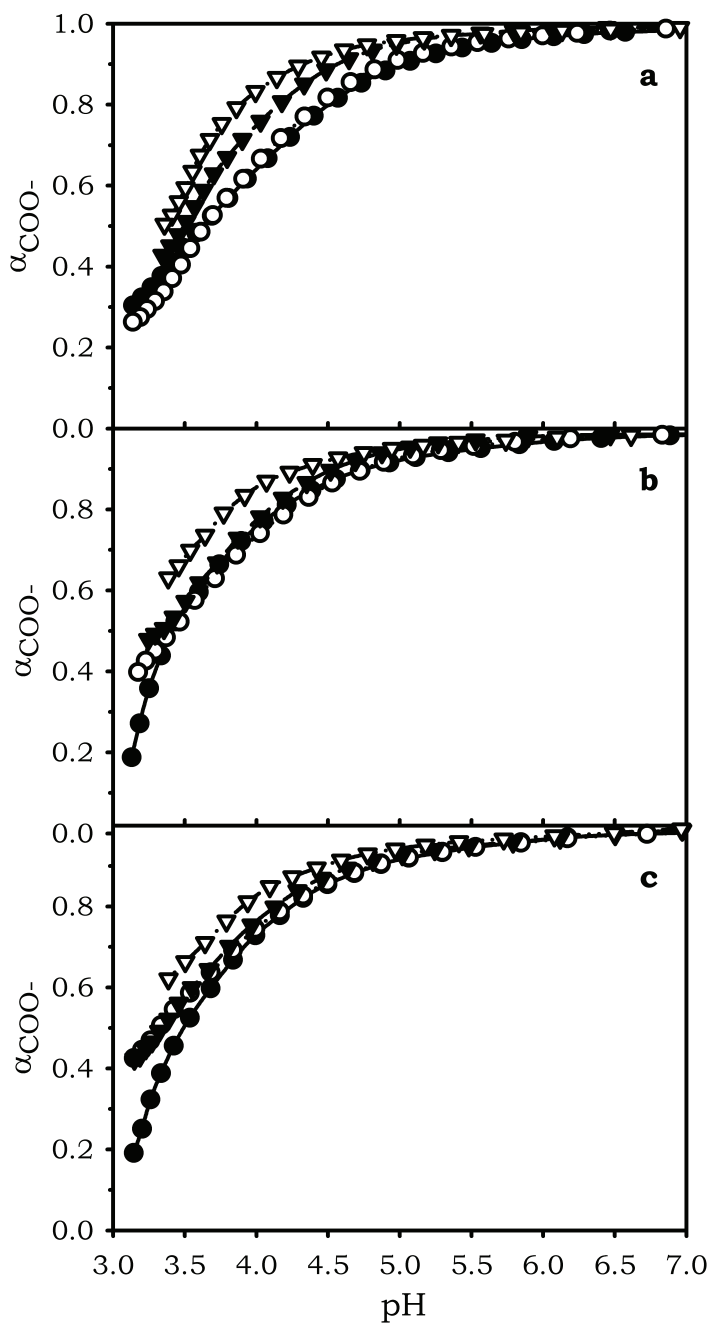


Figure 2.5: Dissociation curves of a) LMP, b) HMP_B and c) HMP_R. Ionic strength: 4mM (●), 10mM (○), 30mM (▼), 75mM (▽)

2.3.2.1 Potentiometric titrations of homomolecular solutions

Potentiometric titration curves of the three pectins are shown in figure 2.5. At ionic strength 4 mM there is a clear difference in the fraction of GalA deprotonated carboxyl groups (α_{COO^-}) between the three pectins. LMP has a higher charge density than HMP_B and HMP_R, giving the GalA residues in LMP a higher pK_a than the GalA residues in HMP_B and HMP_R. This causes α_{COO^-} to decrease at higher pH for LMP than for HMP_B and HMP_R. The profile for α_{COO^-} against pH is similar for HMP_B and HMP_R. Similar observations have been published by (Ralet, Dronnet et al., 2001) who performed potentiometric titrations on pectins de-esterified with fungal and plant pectin methyl esterase to obtain blockwise and random distributions of methyl esters on the pectin. When the ionic strength is increased to 75mM the curves are identical for all three pectins, because of the shielding of the charges on the pectin backbone, lowering the pK_a of the carboxyl group.

Figure 2.6 shows the potentiometric titration of β -lg at various ionic strengths. The curves correlate well with curves published by (Cannan, Palmer et al., 1942). It is assumed that the point of zero charge coincides with the iso-electric point (5.2) of β -lg. Below pI the charge on β -lg increases slightly with increasing ionic strength. This is caused by the shielding of charges on the β -lg molecule, resulting in a minor shift in the pK_a of the ionic groups.

2.3.2.2 Potentiometric titration of β -lg and pectin mixtures

Figure 2.7 shows a titration curve for LMP, β -lg and a β -lg-pectin mixture at a WR of 4 and an ionic strength of 10mM. All the curves have been corrected for H^+ consumption of the background, allowing the summation of the β -lg and LMP curves. When the charged groups of β -lg and pectin do not influence each other, the titration curve of the β -lg-LMP mixture should be identical to the sum of the individual β -lg and LMP curves. This is clearly not the case. This means there is charge regulation of β -lg and/or pectin ionic groups during complexation.

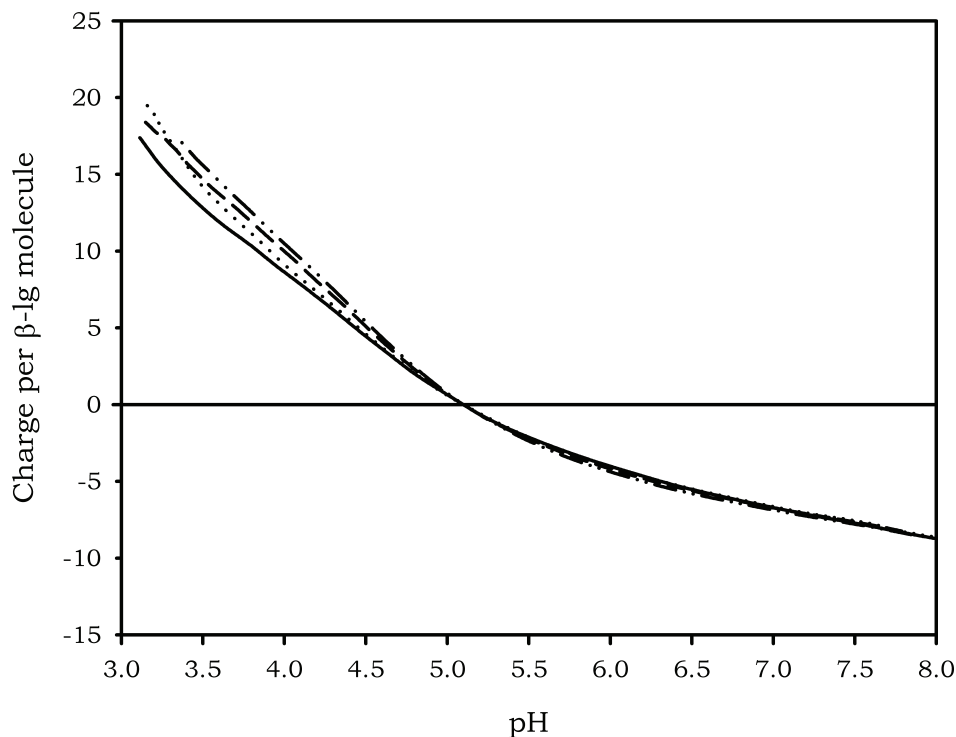


Figure 2.6: Potentiometric titration curve of β -lg. Ionic strength: 4mM (—), 10mM (···), 30mM (---) and 75mM (-·-·-).

From pH 8 to 5.8 no complexes of β -lg and pectin are formed and the H^+ consumption of the mixture is virtually equal to H^+ consumption of the sum of the individual β -lg and pectin (ΔH^+ equals 0, see figure 2.8). This agrees with the findings of (Mattison, Brittain et al., 1995) and (Wen & Dubin, 1997) who report that the titration curve of bovine serum albumin and PDMDAAC only changes beyond pH_c . (Girard, Turgeon et al., 2002) report that β -lg carries more negative charge at pH 7.5 in a solution containing pectin than in the absence of pectin, albeit this being well above pH_c . The reason for this is: The system used by (Girard, Turgeon et al., 2002), as well as in this study, uses a negatively charged polymer (pectin), where (Mattison, Brittain et al., 1995) and (Wen & Dubin, 1997) use a positively charged polymer. Since the sign of the charge of the ionic groups on the polymer is reverse, so is the complexing region.

At $\text{pH} \leq 5.5$ ΔH^+ is positive. The negative field of the LMP negative ionic groups induce a shift in the pK_a values of β -lg ionic groups (aspartic acid and glutamic acid) to a higher pH. This allows complex formation between β -lg and pectin above the pI of β -lg and results in $\Delta\text{H}^+ > 0$. Around pH 5

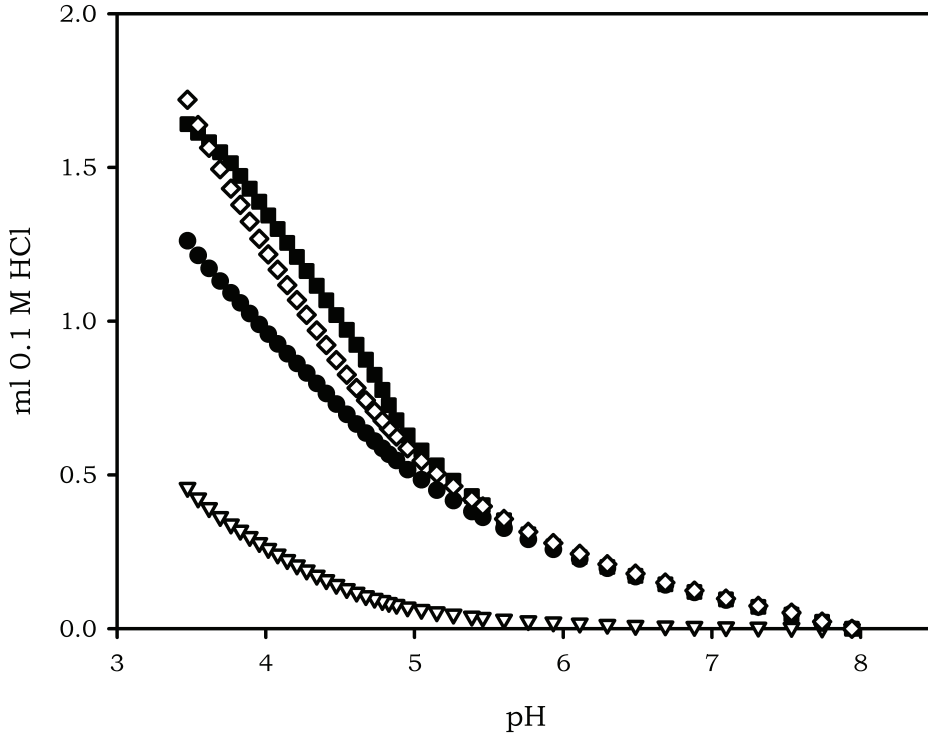


Figure 2.7: Potentiometric titration of β -lg ($\bullet$), LMP (∇), β -lg-LMP weight ratio = 4 ($\blacksquare$) and the summation of the β -lg and LMP titration for a weight ratio of 4 ($\diamond$). Ionic strength is 10 mM, the H^+ consumption of the background has been subtracted from all samples

there is an increase in ΔH^+ . As was already seen in the DLS curves this is the point where the majority of the β -lg binds to the pectin. Below pH 4.5 ΔH^+ reduces, α_{COO^-} for pectin decreases rapidly here (see figure 2.5a). To maintain a sufficient amount of negative charge on the pectin the positive β -lg ionic groups induce the lowering of the pK_a of the pectin ionic groups, resulting in a decrease in ΔH^+ . This decrease could not be observed by (Mattison, Brittain et al., 1995) or (Wen & Dubin, 1997) as they used a cationic polymer containing strong quaternary ammonium groups. (Girard, Turgeon et al., 2002) performed their potentiometric titrations only from

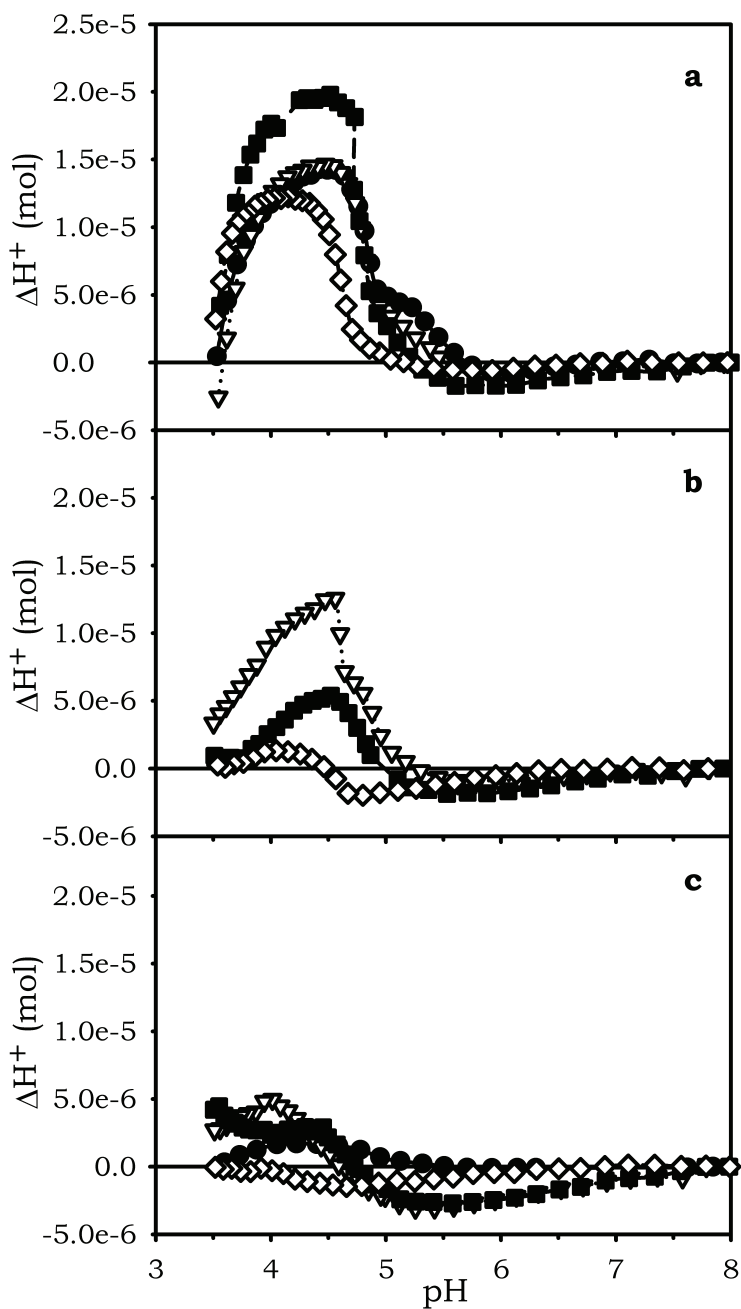


Figure 2.8: Difference in H^+ -consumption (ΔH^+) of β -lg-pectin mixtures. a) LMP, b) HMP_B and c) HMP_R . WR is 4 for all titrations, ionic strength 4mM (●), 10mM (▽), 30mM (■) and 75mM (◇).

pH 7.5 to 4.5, just outside the range to observe this charge regulation phenomenon.

In figure 2.8 ΔH^+ is shown for all three pectins at a WR of 4. The largest change in ΔH^+ for LMP is at an ionic strength of 30mM. The ionic groups of pectin and β -lg need to be close together to influence each other, so ΔH^+ is only influenced by β -lg that is bound to pectin. The DLS measurements also showed that the highest amount of β -lg binds to LMP at this ionic strength. The increase in ΔH^+ for a β -lg–LMP mixture takes place at the lowest pH at an ionic strength of 75 mM. This agrees with the DLS titrations where I_{norm} for 75mM also starts rising at the lowest pH, when excluding 300mM.

The potentiometric titration of HMP_B follows the trend shown in the DLS titrations. At an ionic strength of 10mM the shift in ΔH^+ is largest, followed by 30mM and 75mM. ΔH^+ indicates that the highest amount of β -lg binds at the lowest ionic strength. For an ionic strength of 75mM there is only a small change in ΔH^+ of the HMP_B – β -lg sample. It indicates that at this ionic strength considerably less β -lg binds to the pectin than at lower ionic strengths. The maximum value for ΔH^+ is noticeably lower for HMP_B than for LMP, again showing that LMP has more binding sites than HMP_B . The influence of complex formation between β -lg and pectin on the titration behaviour is smaller for HMP_R as compared to the other two pectins. The profile of the ΔH^+ -curve (figure 2.8c) in case of HMP_R is similar to that of HMP_B and LMP, but the value for ΔH^+ is much lower. The lower local charge density of HMP_R may be the cause that there are less ionic groups involved in the complexation between β -lg and HMP_R compared LMP or HMP_B . At an ionic strength of 75 mM there is hardly any effect on the pK_a of the ionic groups of HMP_R and β -lg. DLS titrations show that complexes are formed under these conditions, so they must either contain very little β -lg or the pK_a 's are hardly influenced.

Table 2.1 compares pH_c values obtained by DLS and potentiometric titration. In general the values for pH_c obtained by DLS are somewhat higher than those derived from the potentiometric titrations. The change in H^+ consumption in the potentiometric titrations is less sensitive to the onset of complex formation as only very few β -lg molecules bind, giving a

very small change in ΔH^+ . This makes DLS titration the preferred technique for measuring pH_c values.

Table 2.1 Comparison of pH_c measured by DLS and potentiometric titration (PT) for β -lg and LMP, HMP_B and HM-NCSP, weight ratio is 4

I (mM)	LMP		HMP _B		HMP _R	
	DLS	PT	DLS	PT	DLS	PT
4	6.2	5.9	6.3	N/A	5.8	5.7
10	6.0	5.8	5.9	5.6	5.7	5.1
30	5.8	5.5	5.8	5.4	5.8	5.4
75	5.3	5.4	5.6	4.6	5.2	4.5

2.4 Conclusions

Pectins with different physicochemical characteristics show different behaviour in their complexation with β -lg. HMP_B and HMP_R have the same DM, but different DB or, for that matter, local charge density. This difference in local charge density has an effect on the sensitivity of complex formation between β -lg and pectin for ionic strength. The blockwise distributed pectin (HMP_B) with high local charge density allows to form complexes at higher ionic strength than a random distributed pectin (HMP_R) having a low local charge density. Low methyl esterified pectin (LMP) has, like HMP_B, a high local charge density and forms complexes with β -lg even at high ionic strength. An analytical estimate for $n_{s,c}$, the salt concentration above which pectin and β -lg only form complexes below the pI of β -lg, indeed revealed comparable local charge densities for LMP and HMP_B, while HMP_R had a local charge density of about half that of LMP or HMP_B. The amount of β -lg that can bind to LMP passes through a maximum for increasing ionic strength. The increase in ionic strength reduces electrostatic repulsion between the β -lg molecules, allowing them to be packed closer together. When ionic strength is increased further the reduced attractive electrostatic interactions between β -lg and LMP lead to a decrease in the amount of β -lg that binds to LMP.

Complex formation between β -lg and pectin is subject to charge regulation in the complexes. At the onset of complex formation (pH_c), around pH 5.0 to 5.5, the pK_a values of the β -lg ionic groups are increased

to yield extra positive charge on the protein which favours complex formation. This leads to an increased in ΔH^+ . At lower pH, 3.5-4.5, the pK_a values of the pectin ionic groups are lowered to retain the negative charge on the pectin. This causes a decrease in ΔH^+ . LMP displays a much larger change in ΔH^+ compared to HMP_B and HMP_R , indicating that more ionic groups are involved in the complex formation. Since LMP has a lower DM than HMP_B and HMP_R there are more binding sites on LMP, resulting in the involvement of more ionic groups as more β -lg binds to the pectin. HMP_B shows more charge regulation than HMP_R . From this it can be concluded that a blockwise distribution of methyl esters leading to regions of high local charge density favours binding.

From the experiments described in this paper it is concluded that for the binding of β -lg to pectin regions of high local charge density are favoured. In food systems where ionic strength is often relatively high and complex formation between protein and pectin is desired, pectin with a high local charge density is therefore preferred. From the physicochemical characteristics of pectin, in particular its degree of methyl esterification and degree of blockiness, it is possible to predict the affinity for complex formation between the pectin and a specific protein.

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Renko de Vries of the laboratory of physical chemistry and colloid science is kindly acknowledged for his help on the calculations to determine the local charge density of pectin through complexation with β -lg.

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Chapter 3

Binding of β -lactoglobulin to pectins varying in their overall and local charge density

Abstract

The formation of complexes between proteins and polysaccharides is of great importance for many food systems like foams, emulsions, acidified milk drinks, and so on. The complex formation between β -lactoglobulin (β -lg) and pectins with a well-defined physicochemical fine structure has been studied to elucidate the influence of overall charge and local charge density of pectin on the complex formation. Binding isotherms of β -lg to pectin are constructed using fluorescence anisotropy, which is shown to be an excellent technique for this purpose, as it is fast and requires low sample volumes. From the binding isotherms the maximal adsorbed amount, binding constant (k_{obs}) and the cooperativity of binding are obtained at different ionic strengths. The Hill model is used to fit the binding isotherms and is shown to be preferable over a Langmuir fit. At pH 4.25, k_{obs} shows a maximum at an ionic strength of 10 mM when using a low methyl esterified pectin (LMP) due to the balance of attractive and repulsive electrostatic forces between β -lg and pectin and β -lg neighbours. For two high methyl esterified pectins, one with a blockwise distribution of methyl esters (HMP_B) and one with a random distribution (HMP_R), this ionic strength maximum is absent and k_{obs} decreases with increasing ionic strength. k_{obs} is found to be largest for LMP and HMP_B and considerably lower for HMP_R. A positive cooperativity is observed for both LMP (above an ionic strength of 45 mM) and HMP_R (above an ionic strength of 15 mM), but not for HMP_B. Positive cooperativity is thought to be caused by a rearrangement of the pectin helix structure caused by binding of β -lg, thus creating new or binding sites with a higher affinity. To attain strong binding of β -lg to pectin it is preferable to use a pectin with a blockwise distribution of methyl esters. When complex formation takes place in high ionic strength media an LMP gives the best results, while at low ionic strength a high methyl esterified pectin with blockwise distribution may give better results, due to reduced electrostatic repulsion between both pectin and β -lg and β -lg neighbours.

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3.1 Introduction

Complex formation between anionic polysaccharides and proteins find their application in a diversity of fields like food industry, biotechnology, medicine, pharmacy, and cosmetics (Schmitt, Sanchez et al., 1998). In the food industry complexes of protein and polysaccharides are used for the stabilization of acid dairy drinks, foams and emulsions, as fat substitutes, meat analogues, purification of macromolecules, or microencapsulation of ingredients (Vanderveen & Glinsmann, 1992; Wang, Gao et al., 1996; Sandrou & Arvanitoyannis, 2000; Laurent & Boulenguer, 2003; Schmitt, Palma da Silva et al., 2005; Gharsallaoui, Roudaut et al., 2007; Güzey & McClements, 2007).

Complexes of proteins and anionic polysaccharides are formed because of attractive interactions between the two biopolymers and can be of an electrostatic or hydrophobic nature (Tribet, 2001). Hydrophobic interactions are limited in systems consisting of proteins and anionic polysaccharides as the alkyl side chain needs to be at least 3–4 carbon atoms long before significant hydrophobic interactions take place (Gao & Dubin, 1999). Electrostatic interactions occur because of opposite charges on both biopolymers. As in a food system, the charge of the polysaccharide is negative, and complex formation takes place below and around the isoelectric point of the protein. If complex formation occurs above the isoelectric point, it is usually attributed to locally positively charged patches on the protein (Park, Muhoberac et al., 1992). Depending on solution parameters, like pH, ionic strength, and concentration of the two biopolymers, either soluble or insoluble complexes are formed. Soluble complexes exist as long as the complexes are sufficiently stabilized against aggregation by electrostatic repulsion. Generally speaking, the phase boundary between soluble and insoluble complexes is found at the pH, ionic strength, and mix ratio of biopolymers that cause the complex of protein and polysaccharide to carry no net charge anymore (Park, Muhoberac et al., 1992; Weinbreck, De Vries et al., 2003; Sperber, Schols et al., 2009).

To compare the formation of complexes between anionic polysaccharides and proteins parameters like composition of the complex, association constant, and the cooperativity of the association are commonly considered. To obtain these parameters isotherms for the binding of the protein to the polysaccharide need to be constructed without disturbing the delicate equilibrium between bound and free protein (Porcar, Cottet et al., 1999). For this, Gao, Dubin et al. (1997, 1998) developed frontal analysis continuous capillary electrophoresis (FACCE). This technique allows the determination of free ligand concentration without disturbing the equilibrium conditions due to continuous injection of fresh sample into the separation capillary. FACCE has been used successfully to construct binding isotherms for different combinations of protein and polyelectrolyte (Porcar, Gareil et al., 1998; Hattori, Kimura et al., 2001; Girard, Turgeon et al., 2003; Seyrek, Dubin et al., 2003; Østergaard, Khanbolouki et al., 2004; Le Saux, Varrenne et al., 2006). The big advantage of this technique is that it uses only small amounts of sample ($< 1 \mu\text{L}$). There is one major drawback to the FACCE technique: To avoid binding of the protein to the negatively charged silica capillary, FACCE can only be used at a solution pH well above the pI of the protein (Gao, Dubin et al., 1997; Porcar, Cottet et al., 1999).

Fluorescence anisotropy (FA) is a technique that measures the polarization of the emitted light after excitation of the sample with polarized light. The fluorescence anisotropy of a fluorophore is, among others, dependent on the diffusion rate and therefore the size of the fluorophore (Lakowicz, 2006). It implies that the fluorescence anisotropy of a fluorophore-containing protein, bound to a polysaccharide, deviates from the anisotropy when it is free in solution. FA has been used successfully in the construction of binding isotherms for protein–DNA complexes (Maleki, Royer et al., 2002; Datta & LiCata, 2003; Arosio, Costantini et al., 2004; Dragan, Frank et al., 2004). There are two big advantages of using fluorescence anisotropy over FACCE: because the measurement is performed directly in the solution, there is no need of separating free and bound protein and the measurement can be performed at any pH, provided that an optically clear sample is measured.

In this work, complexes between pectin and β -lactoglobulin (β -lg) are studied. Pectin is commonly used to stabilize acid dairy drinks and to form gels. Commercially available pectins are isolated from plant materials by means of an acid extraction after which their predominant structure is α -(1 $\rightarrow$ 4)-linked D-galacturonic acid (Voragen, Pilnik et al., 1995). The functionality of these pectins is mainly determined by two parameters: The degree of methyl esterification (DM) of the carboxyl group on C-6 and the distribution of these methyl esters (DB). A pectin with a random distribution pattern of the methyl esters has a low value for DB, a pectin with a blockwise distribution has a high DB (Daas, Meyer-Hansen et al., 1999). The DM determines the overall charge of the pectin, while the DB determines the local charge density.

β -Lg is the predominant protein in whey and used in foods for its emulsifying, foaming and gelation properties (Dickinson, 1998, 1999; Van Vliet, Lakemond et al., 2004) and dominates the complex formation between whey protein isolate and polysaccharides (Weinbreck, De Vries et al., 2003). A better understanding of the binding of β -lg to pectin will help to interpret the influence β -lg-pectin complex formation has on the stability of food structures like foams and emulsions (Ganzevles, Zinoviadou et al., 2006; Güzey & McClements, 2007).

3.2 Materials and Methods

3.2.1 Pectin

Three pectins, a low methyl esterified pectin (LMP), a high methyl esterified calcium sensitive pectin (HMP_B) with a blockwise distribution of methyl esters, and a high methyl esterified non-calcium sensitive pectin (HMP_R) with a random methyl ester distribution, were kindly provided by CPKelco (Lille Skensved, Denmark). The pectins originate from lemon and were characterized by Daas, Boxma et al (2001) on DM and DB. Detailed information for the different pectins can be found in table 1. The pectins had a typical molecular weight of 150 kDa. (Daas, Voragen et al., 2001)

The pectins contained 0.02–0.05 mol of calcium per mole of galacturonic acid (GalA), as determined by flame photometry. The calcium was removed by washing the pectins five times with 60% ethanol,

Table 3.1: Uronic acid content, DM, DB, distribution of mono-, di-, tri-GalA after endo-PG hydrolysis, methyl- to non-methyl esterified peak ratio (Me/n-Me) for LMP, HMP_B, HMP_R and poly-(galacturonic acid) (PGA) (Daas, Boxma et al., 2001)

Pectin	Uronic acid content (%)	DM (%)	DB (%)	Mono-GalA (%)	Di-GalA (%)	Tri-GalA	Me/n-Me
LMP	78.5	30.4	16.5	21	44	35	0.77
HMP _B	84.5	69.8	10.9	35	37	28	0.09
HMP _R	85.3	73.5	1.7	23	60	17	0
PGA	86.0	0	88.2	21	42	37	0

containing 5% (v/v) hydrochloric acid. Next, the pectins were washed with 60% ethanol until they were free of chlorine (tested by addition of 0.2% (w/w) silvernitrate, until no white precipitate is formed). Pectins were subsequently washed with acetone and air-dried. This treatment for removing calcium was sufficient for LMP and HMP_B, but HMP_R required further treatment. HMP_R was dissolved to 10 g/L and mixed with AG 50W-X4 200–400 mesh ion-exchange resin from Bio-Rad Laboratories (Hercules, CA). HMP_R and ion-exchange resin were separated by centrifugation and HMP_R was lyophilized. This resulted in pectins that contained less than 0.001 mol of calcium per mole of GalA.

3.2.2 Preparation of β -lactoglobulin and Alexa Fluor 430 labelled β -lactoglobulin

β -Lg was purified from bovine milk using a non-denaturing technique as described by De Jongh, Gröneveld et al (2001) and was over 99% pure. β -Lg was fluorescently labelled with Alexa Fluor 430 carboxylic acid, succinimidyl ester (A10169, Invitrogen, Breda, The Netherlands). An amine reactive probe, chosen as the only available free thiol group (C121), induces a structural change of β -lg when modified (Jayat, Gaudin et al., 2004). The labelling reaction was carried out according to the "Molecular Probes" protocol (<http://www.probes.com>). Typically, 10 mg of β -lg was dissolved in 1 mL of 0.1 M sodium bicarbonate buffer (pH 8.3). A stock solution of Alexa Fluor 430 was prepared by dissolving 5 mg in 0.5 mL DMSO. The labelling reaction was performed at room temperature by adding 10 μ L of the Alexa Fluor 430 stock solution to the stirred β -lg

solution in 2 μ L portions with a 10 minutes interval. The reaction was performed in the dark. A Total of 20 minutes after the last addition of the Alexa Fluor 430 stock solution, the reaction was stopped by adding 0.1 mL of a 1.5 M sodium hydroxylamine solution (pH 8.5). The conjugate was separated from not reacted reagent using a Sephadex G-25 gel filtration column (17-0851-01, GE Healthcare, Uppsala, Sweden) equilibrated with a 0.1 M sodium acetate buffer (pH 4.25). The degree of labelling was approximately 0.2 mol dye per mol β -lg.

3.2.3 Weak anion-exchange high performance liquid chromatography

Weak anion-exchange high performance liquid chromatography (WAX-HPLC) was performed as described in detail by Guillotin, Van Loey et al (2007). In short, a Dionex Propac WAX-10 column (WAX; 4 $\times$ 250 mm) was attached to an Akta purifier system. A total of 200 μ L of a 5 g/L pectin solution was injected and eluted (1 mL/min) with a linear gradient from 0 to 0.6 M of sodium phosphate (pH 6) for 25 minutes. UV detection is at 215 nm. All elution profiles were baseline corrected for an injection of 200 μ L of water.

3.2.4 Fluorescence anisotropy

Fluorescence anisotropy measurements were carried out on a TECAN Infinite F500 micro plate reader (TECAN, Austria) equipped with an excitation filter of 420 nm (10 nm bandwidth) and an emission filter of 540 nm (10 nm bandwidth) and a Xenon flash lamp with a frequency of 40 Hz. The measurement time after the flash is set at 20 μ s, and per measurement 10 flashes are recorded. Samples were measured in black flat bottom 96-well Fluotrac 200 plates (655076, Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Fluorescence anisotropy is calculated as follows:

$$A = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \quad (3.1)$$

with A the fluorescence anisotropy and $I_{\parallel}$ and $I_{\perp}$ the intensity of the fluorescent light in the parallel and perpendicular direction, respectively (Lakowicz, 2006).

3.2.5 Construction of binding isotherms

Pectin is dissolved in a 20 mM sodium acetate buffer of pH 4.25 to a concentration of 0.5 mg/mL. The ionic strength of the pectin solution is set with sodium chloride to obtain the correct ionic strength (4–300 mM) after mixing with β -lg and buffer. β -Lg (nonlabelled) is dissolved in 20 mM acetic acid. Pectin and β -lg are kept overnight in the fridge to allow for full hydration of the molecules. Prior to experiments the pH is carefully adjusted with 1 M sodium hydroxide to 4.25. Samples of pectin and β -lg (both labelled and nonlabelled) are mixed to obtain weight ratios (mg β -lg/mg pectin) of 0.25 up to 16, all with a pectin concentration of 0.1 mg/mL and an Alexa Fluor 430 labelled β -lg concentration of approximately 1 μ g/mL. Labelled and nonlabelled β -lg are assumed to bind identically to pectin. All points of the binding isotherm are averages of four samples.

Binding isotherms are constructed from the fluorescence anisotropy measurements. The observed fluorescence anisotropy (A_{obs}) is the average of the fluorescence anisotropy of the free (A_f) and bound (A_b) β -lg (Lakowicz, 2006). ΔA is the difference between A_{obs} and A_f :

$$\Delta A = A_{\text{obs}} - A_f = (f_b A_b + f_f A_f) - A_f \quad (3.2)$$

with f_f and f_b the fraction of free and bound β -lg, respectively. As the fluorescence anisotropy is influenced by the viscosity of the solution A_f is determined in a sample that contains both pectin and β -lg and has an ionic strength of 300 mM. At these conditions there is no complex formation between pectin and β -lg (Sperber, Schols et al., 2009) and the measured fluorescence anisotropy is that of free β -lg. The fluorescence anisotropy of bound β -lg, A_b , is determined by taking the maximum value for the fluorescence anisotropy throughout all the experiments. The value for bound β -lg is comparable to the fluorescence anisotropy value for labelled β -lg in the high viscosity medium glycerol. To obtain a molar concentration of β -lg, it is assumed that β -lg adsorbs as a dimer to pectin,

as there are no indications that adsorption to pectin disturbs the monomer–dimer equilibrium of β -lg.

3.2.6 Analysis of binding isotherms

The binding isotherms are fitted with a Langmuir and a Hill adsorption isotherm to obtain a binding constant and a measure for the cooperativity of the system (for the Hill model). Equation 3.3 gives the Hill model used to fit the binding isotherms:

$$\theta = \frac{k_{\text{obs}}^n X^n}{1 + k_{\text{obs}}^n X^n} \quad (3.3)$$

with θ the fractional occupation of binding sites (Γ/Γ_m with Γ_m for the appropriate ionic strength), k_{obs} the observed molar binding constant, n the Hill coefficient, and X the molar fraction of free β -lg (Norde, 2003). For $n < 1$ the binding is anticooperative, for $n > 1$ cooperative and for $n = 1$ the binding is noncooperative and equation 3.3 reduces to the Langmuir model. The Langmuir model assumes reversibility of binding, identical and independent binding sites, and no interaction between the adsorbed molecules. The Hill adsorption allows for interaction between the adsorbed molecules by means of the Hill coefficient.

The fitting procedure is performed using a nonlinear least-squares fit based on the Levenbert-Marquadt algorithm in Origin 5.0 software (Microcal Software, Northampton, MA).

3.3 Results and discussion

3.3.1 Size and distribution of non-methyl esterified galacturonic acid blocks along the pectin backbone.

To determine the block size and block distribution of methyl esters on the pectin backbone several parameters have been determined by Daas, Boxma et al (2001) for the pectins used. These parameters are uronic acid content, DM, DB, distribution of mono-, di-, and tri-GalA after endo-PG digestion, and the ratio between the peak area of methyl ester (Me) containing oligosaccharides and the peak area of the non-methyl esterified (n-Me) oligosaccharides after endo-PG digestion (Me/n-Me ratio). The

details of these parameters can be found in table 3.1. Figure 3.1 shows the elution profile of the pectins on a WAX column to reveal the presence of populations of pectins with different characteristics in the same sample. The combination of all the parameters mentioned above provides a detailed picture of the fine structure of the three pectin samples.

DM determination shows LMP to be a low methyl esterified pectin and HMP_B and HMP_R to be high methyl esterified pectin with virtually identical DM. The DB of HMP_R is very low, indicating that only a very small fraction of HMP_R is *Kluyveromyces fragilis* endo-PG digestible, while for both LMP and HMP_B a considerable amount of n-Me-GalA is present in blocks.

The digestion of pectin with endo-PG gives a distribution between mono-, di-, and tri-GalA oligomers released from the pectin as well as (partially) Me-GalA oligomers. If the distribution of mono-, di-, and tri-GalA resembles that of the distribution of a polygalacturonic acid digest, the blocks can be considered of "infinite length", as is the case for LMP. HMP_B has a reduced amount of di-, and tri-GalA, with an increased amount of mono-GalA. This is indicative for a reduced block length of n-Me-GalA residues. HMP_R has an even bigger reduction of tri-GalA oligomers, mainly in favour of di-GalA. This large decrease in tri-GalA shows that the n-Me-GalA blocks present on HMP_R are small, which is also indicated by HMP_R being a noncalcium sensitive pectin.

The Me/n-Me ratio shows how close endo-PG degradable blocks are together. Endo-PG degradable blocks more than six GalA residues apart will not be detected by this method (Daas, Voragen et al., 2000). HMP_R has a Me/n-Me ratio of 0, implying that the blocks of GalA on HMP_R are more than six GalA residues apart and can therefore be considered isolated blocks. Also HMP_B has a very low Me/n-Me ratio. Although the blocks are much larger than for HMP_R, they are still virtually isolated because of the high DM of HMP_B. LMP has a high Me/n-Me ratio revealing the close proximity of the blocks. This is mainly caused by LMP being a low methyl esterified pectin, causing the blocks to be close together.

The elution on the WAX-column (figure 3.1) reveals the presence of different populations within the pectin sample, as well as information about the size of the blocks in the pectin (Guillotin, Van Loey et al., 2007). Peaks in region I contain the material that does not bind to the column.

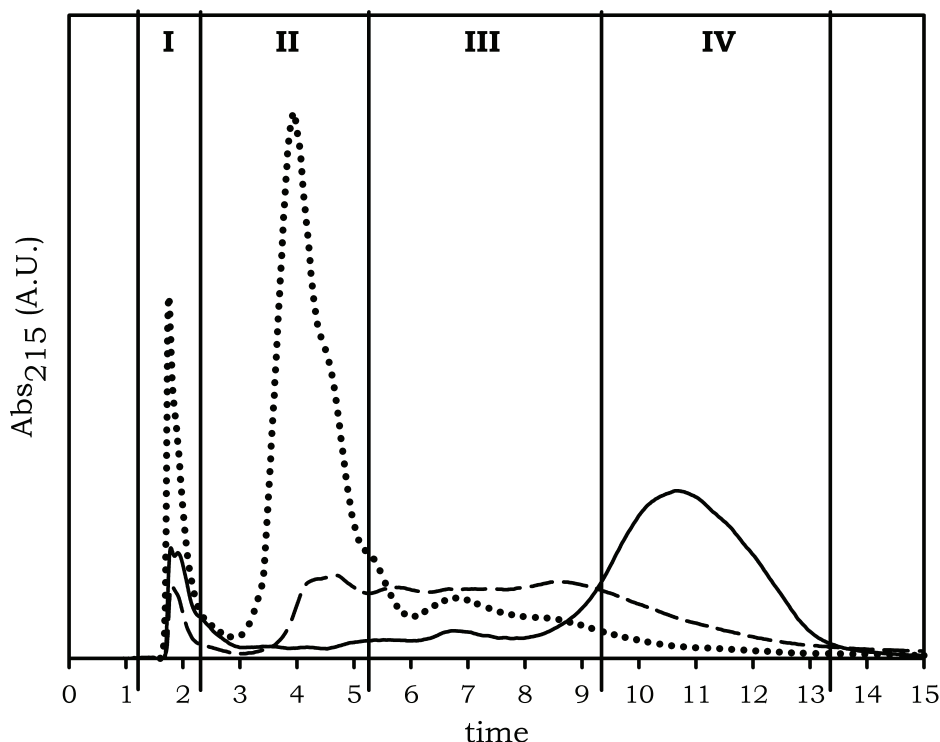


Figure 3.1: WAX elution profile after background correction of LMP (—), HMP_B (---), and HMP_R (···)

For commercial pectin samples, it was found that this peak contains no GalA (Guillotin, Van Loey et al., 2007). Region II contains high methyl esterified, random pectin. HMP_R has a large peak in region II, HMP_B a much smaller one, and for LMP no peak is observed. In region III, high to intermediate methyl esterified pectin with a blockwise distribution is found. A small peak is found for HMP_R, but HMP_B has a continuous peak throughout this region, indicating a distribution in the block length for HMP_B. LMP only starts showing a peak at the end of region III. Region IV is the "PGA"-region, where pectins with large ("infinite") blocks of n-Me-GalA elute. This is the region where virtually all LMP elutes, as well as some HMP_B, while HMP_R has no peak in this area.

The three pectins can thus be characterized as follows: LMP has the lowest DM and, therefore, the most n-Me-GalA groups. The blocksize of n-Me-GalA residues on LMP is large and they are close together. LMP has a

homogeneous blocksize, mainly because of its low DM, as shown by elution on the WAX column. HMP_B and HMP_R have a comparable DM, but differ in the distribution of the methyl esters. The blocks of n-Me-GalA on HMP_B are large, albeit being somewhat smaller than those on LMP as shown by the Me/n-Me ratio. The blocks of n-Me-GalA on HMP_B are considered to be isolated. The distribution on the WAX column shows that HMP_B is a heterogeneous sample, consisting of many different pectins, ranging from HM-random pectin to PGA characteristics. HMP_R is a pectin that contains no large blocks of n-Me-GalA and only very few isolated intermediate blocks of n-Me-GalA.

3.3.2 Binding isotherms

Binding isotherms are measured to investigate the binding of β -lg to pectins having different physicochemical characteristics. The influence of the local charge density and overall charge of the pectin on the complex formation with β -lg is expected to be reflected in the binding isotherms, showing the mass of β -lg bound per unit mass of pectin as a function of β -lg concentration in solution.

Figure 3.2 shows ΔA for β -lg and LMP at pH 4.25 and an ionic strength of 4 mM. At low weight ratio (WR), ΔA is constant at the maximum value, indicating that all β -lg is bound to LMP. When WR increases, ΔA decreases as LMP reaches saturation and not all of the β -lg is bound to LMP. The data for ΔA is used to calculate the fractions of free (f_f) and bound (f_b) β -lg, using equation 3.2. In figure 3.3 binding isotherms for β -lg to LMP, HMP_B, and HMP_R are shown. FA shows to be a reliable and fast technique for measuring binding isotherms between protein and polysaccharide, with the advantage over FACCE that it can also successfully measure binding isotherms below and around the pI of the protein, providing that the sample is optically clear. As the measurements are performed in microtiter plates, sample volumes can be kept at 1 mL or less and the measurement of an entire binding isotherm takes only several minutes.

β -Lg shows a very high affinity for LMP and HMP_B at low ionic strength, as virtually all added β -lg binds to pectin. Only when the pectin binding sites are close to saturation, the concentration of free β -lg increases. For

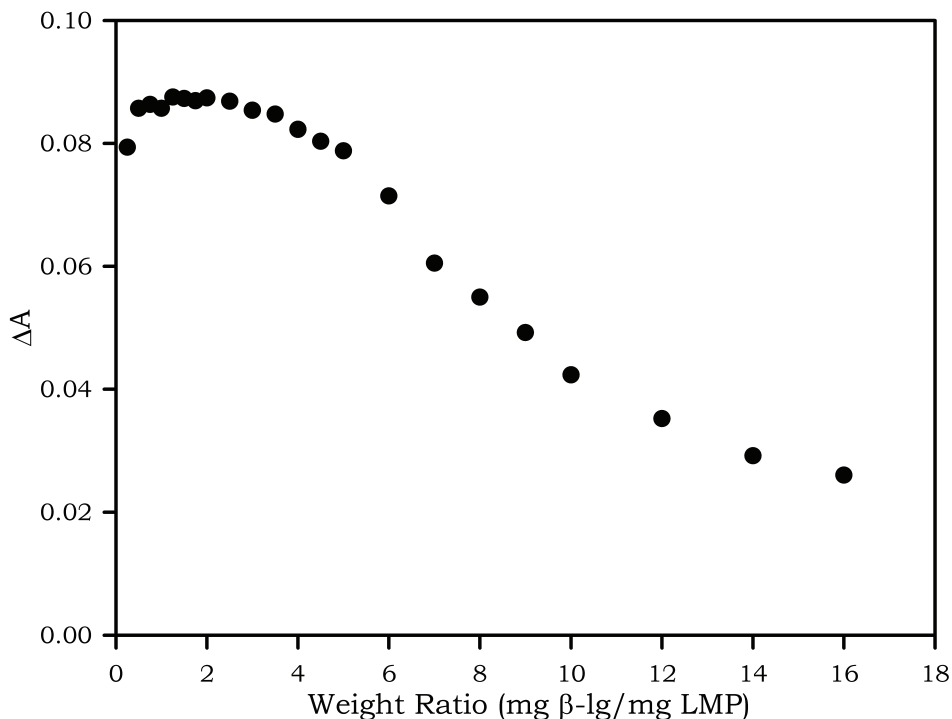


Figure 3.2: Fluorescence anisotropy of β -lg-LMP mixtures at pH 4.25, ionic strength is 4mM.

HMP_R the initial high affinity is lower, most likely by the virtual absence of large blocks of n-Me-GalA. When the ionic strength of the solution is increased, the binding of β -lg to pectin decreases. HMP_R shows the highest sensitivity to ionic strength: at an ionic strength of 60 mM no binding of β -lg to HMP_R takes place anymore. At an ionic strength of 75 mM β -lg no longer binds to HMP_B and for LMP binding of β -lg is suppressed at an ionic strength of 200 mM. This difference in sensitivity for ionic strength was found before by means of dynamic light scattering (DLS) (Sperber, Schols et al., 2009), only at slightly higher ionic strength. The measurements with DLS are extremely sensitive to complex formation: at pH 4.25, the increase in the intensity of scattered light (I_s) at high ionic strength is below 1% of the increase in I_s at low ionic strength. This high sensitivity of the DLS technique cannot be matched by FA, leading to a slightly lowered maximum ionic strength where complex formation is observed.

The maximum amount of β -lg adsorbed per gram of pectin (Γ_m) is the highest for LMP with an Γ_m of 138 $\mu\text{mol/g}$, HMP_B and HMP_R bind similar quantities of β -lg with 84 $\mu\text{mol/g}$ and 82 $\mu\text{mol/g}$, respectively (see table 3.2). Clearly, Γ_m increases when the DM becomes smaller. These Γ_m values are comparable to the Γ_m found by Girard, Turgeon et al (2003), who found, after recalculation to the same units, 122 $\mu\text{mol/g}$ for a LMP (DM 28.3) and 62 $\mu\text{mol/g}$ for a HMP (DM 73.4), even though Girard, Turgeon et al (2003) measured at pH 4.

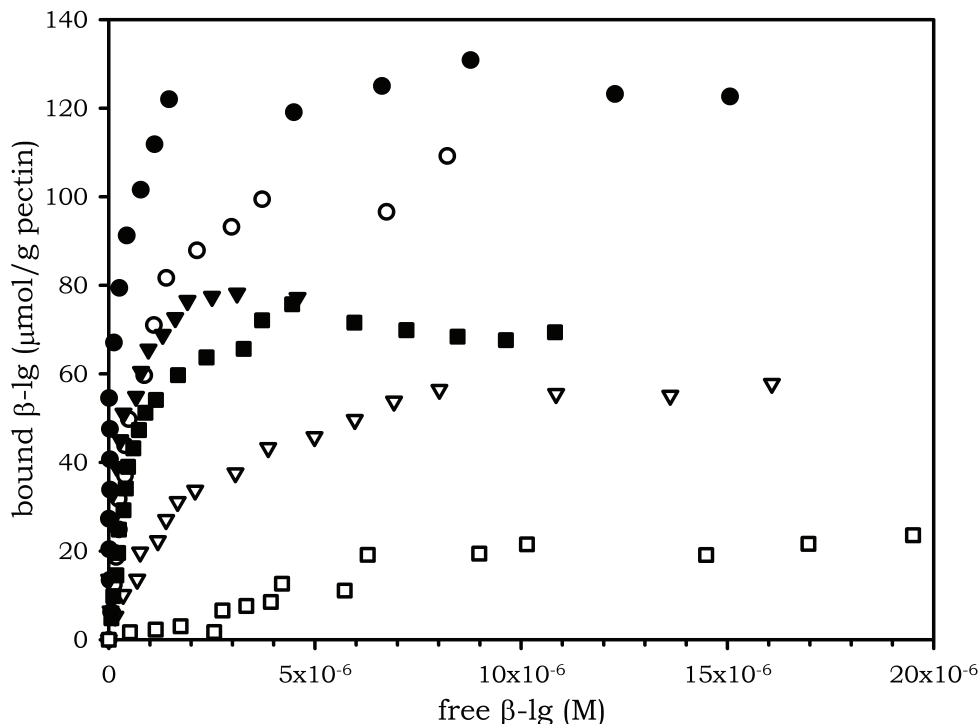


Figure 3.3: Binding isotherm of β -lg to LMP ($\bullet, \circ$), HMP_B ($\blacktriangledown, \triangledown$) and HMP_R ($\blacksquare, \square$) at pH 4.25. Ionic strength is 10mM for filled symbols, and 45mM for open symbols. β -Lg is assumed to bind as a dimer.

When Γ_m is calculated as mol β -lg per mol n-Me-GalA of the pectin, Γ_m is 44 mmol/mol for LMP, 58 mmol/mol for HMP_B, and 64 mmol/mol for HMP_R. The two HM-pectins bind considerably more β -lg than LMP. Gao and Dubin (Gao & Dubin, 1999) found that the minimum length of the alkyl side chain is 3–4 carbon atoms, before hydrophobic interactions start to influence binding of protein to a oppositely charged polymer. This

Table 3.2: Γ_m of β -lg to pectin for all three pectins at pH 4.25, ionic strength as indicated.

I (mM)	LMP		HMP _B		HMP _R	
	Γ_m (μ mol/g pectin)	Γ_m (mol/mol GalA)	Γ_m (μ mol/g pectin)	Γ_m (mol/mol GalA)	Γ_m (μ mol/g pectin)	Γ_m (mol/mol GalA)
4	138.1	0.044	83.1	0.057	82.4	0.064
10	130.8	0.042	78.2	0.054	75.7	0.059
15	127.7	0.041	84.4	0.058	67.8	0.053
20	124.9	0.040	79.1	0.055	57.7	0.045
25	119.3	0.038	74.9	0.052	58.1	0.045
30	119.4	0.038	66.9	0.046	36.4	0.028
45	109.2	0.035	57.8	0.040	23.6	0.018
60	97.6	0.031	50.4	0.035		
75	95.5	0.031				
100	74.3	0.024				
150	40.5	0.013				

observation is substantiated by Girard, Turgeon et al (2002) who found hydrophobic interactions not to be important for pectin- β -lg systems. The difference in adsorption of β -lg per unit of n-Me-GalA for the different types of pectin is therefore not caused by different extents of hydrophobic interactions between β -lg and pectin. The maximum amount of β -lg that binds to pectin is found at or below an ionic strength of 10 mM, where both attractive and repulsive electrostatic forces are largest. LMP has the largest blocks of n-Me-GalA. This prevents all n-Me-GalA residues to participate in binding of β -lg due to electrostatic repulsion between β -lg neighbours, as β -lg molecules cannot be packed "shoulder to shoulder". The blocks of n-Me-GalA on HMP_B are somewhat smaller, but also more isolated than on LMP. This may reduce repulsion between pectin and local negatively charged patches on β -lg, as the presence of Me-GalA residues at the edges of the n-Me-GalA blocks have no repulsive interactions with β -lg. HMP_R binds slightly more β -lg than HMP_B. Both have a similar DM, but the difference in DB allows HMP_R the higher Γ_m : The more even spread of n-Me-GalA on HMP_R leads to less lateral repulsion between β -lg molecules as they are not packed as closely as on LMP or HMP_B.

Γ_m may be estimated based on the dimensions of β -lg and the GalA unit. The β -lg dimer is an ellipsoid with a length of 6.9 nm and width of 3.6 nm (Verheul, Pedersen et al., 1999), the GalA monomer has a length of 0.435 nm (Rees & Wight, 1971). Considering the GalA content and the DM of the pectins, and depending on which diameter of the β -lg dimer is used, Γ_m is estimated to be between 196 and 375 $\mu\text{mol } \beta\text{-lg/g LMP}$, Γ_m between 91 and 175 $\mu\text{mol } \beta\text{-lg/g HMP}_B$, and Γ_m between 81 and 155 $\mu\text{mol } \beta\text{-lg/g HMP}_R$. Only HMP_R reaches the lower boundary of the estimation, LMP and HMP_B bind less β -lg. Because Γ_m is measured at low ionic strength electrostatic repulsion between β -lg molecules will be high, thus lowering the packing density of β -lg on the pectin. Also, the estimation does not take the presence of Me-GalA into account. It is possible that not all n-Me-GalA is participating in the binding of β -lg as their neighbours are of the Me-GalA kind, causing insufficient attraction between binding site on the pectin and β -lg. Because HMP_R comes closest to the estimate, the presence of n-Me-GalA residues in the binding site seems to have less influence than the electrostatic repulsion caused by crowding of β -lg molecules along the pectin chain.

3.3.3 Analysis of binding isotherms

To obtain binding characteristics from the binding isotherms, the reversibility of binding of β -lg to pectin has to be established, as this is a key assumption of most binding models. The reversibility of the binding of β -lg to pectin with respect to dilution was tested by mixing samples of different WR and the same ionic strength. Samples of the same WR, but of different ionic strength were mixed to establish the reversibility with respect to ionic strength. The mixed samples gave the correct values for free and bound β -lg according to their new WR or ionic strength (data not shown) from which it was concluded that the binding of β -lg to pectin is reversible.

The binding isotherms may be fitted with theoretical models to obtain information about the binding constant, k_{obs} , and the cooperativity of binding. Previously, different models were used to describe the binding of a protein to either charged synthetic polymers or polysaccharides: a two class binding site Langmuir fit (Gao, Dubin et al., 1997; Porcar, Cottet et

al., 1999), the Hill model (Porcar, Cottet et al., 1999) and the McGhee–Von Hippel model (McGhee & Von Hippel, 1974, 1976) for overlapping binding sites (Gao, Dubin et al., 1998; Hallberg & Dubin, 1998; Girard, Turgeon et al., 2003; Seyrek, Dubin et al., 2003). The two class binding site Langmuir model, is used when two types of binding sites are present. The analysis of the pectin samples shows that especially HMP_B, but also HMP_R and LMP are not homogeneous in their distribution of methyl esters and hence the size of n-Me-GalA blocks. Additionally, it is difficult to define what is a binding site and how the presence of methyl esters influences the binding of β -lg to pectin. Because of this, it is more appropriate to use an average binding site model, instead of assuming two binding sites.

The McGhee–Von Hippel model was originally developed for protein–DNA binding, but has also been applied to protein–synthetic polyelectrolyte binding by Dubin et al (Gao, Dubin et al., 1998; Hallberg & Dubin, 1998; Seyrek, Dubin et al., 2003) and for β -lg–pectin by Girard, Turgeon et al (2003). This model takes into account that a binding site consists of multiple polymer repetitive units. When the binding sites are being filled, the space in between two bound ligand molecules may be smaller than the binding site of the ligand. The polymer units in between these two ligands are thus inaccessible for a ligand molecule to bind. This inaccessibility of polymer units results in an apparent negative cooperativity (McGhee & Von Hippel, 1974). The McGhee–Von Hippel model assumes a lattice as a linear array of N identical units. This assumption will work well for a polyelectrolyte built of continuously repeating units. Pectin's repeating unit is GalA, which would suit this assumption as well, if it were not for the methyl esters on the carboxyl group that make pectin consisting of two types of units. This makes the McGhee–Von Hippel model poorly applicable to β -lg–pectin systems, as one of the parameters in the model is the number of lattice units that are occupied by one ligand.

The Hill equation is derived from the Langmuir model to allow for cooperative binding and has been used to describe the binding of BSA to synthetic polyelectrolytes with varying length in alkyl side chain (Porcar, Cottet et al., 1999). As no homogeneous binding site can be identified on the pectin, only an average binding constant can be obtained. The cooperativity parameter in the Hill model has an empirical character, only

showing whether or not the binding is cooperative or not. The empirical character of the cooperativity parameter makes it attractive for the β -lg-pectin system where it is difficult to define what the binding site looks like. In view of these considerations, we decided to fit the binding isotherms with the Hill model and, for comparison, with the one site Langmuir model.

Table 3.3: Hill coefficient of the binding of β -lg to pectin, obtained from the Langmuir and Hill model.

I (mM)	n		
	LMP	HMP _B	HMP _R
4	0.8	0.8	0.9
10	0.7	0.9	1.2
15	1.0	1.5	1.6
20	1.1	1.3	1.8
25	1.2	1.3	1.6
30	1.1	1.0	2.3
45	1.2	1.3	2.3
60	1.5	1.2	
75	1.5	0.9	
100	1.9		
150	3.2		

Figure 3.4 shows the Hill fit, but also the fit of the Langmuir model ($n = 1$) to the binding isotherms. As the Hill model is an extension of the Langmuir model, it is more flexible to fit the experimental data and, therefore, may fit the data better than the Langmuir model. Figure 3.5 shows the observed binding constants from the Langmuir and Hill model, the Hill coefficient for the Hill model can be found in table 3.3. The values found for k_{obs} by fitting with either the Langmuir or the Hill model are comparable. The additional information obtained from the Hill model concerning the cooperativity of binding, gives it preference over the Langmuir model.

For LMP, k_{obs} shows a maximum with increasing ionic strength. Seyrek, Dubin et al (2003) also found a maximum in k_{obs} with increasing ionic strength for other protein-synthetic polyelectrolyte combinations. The

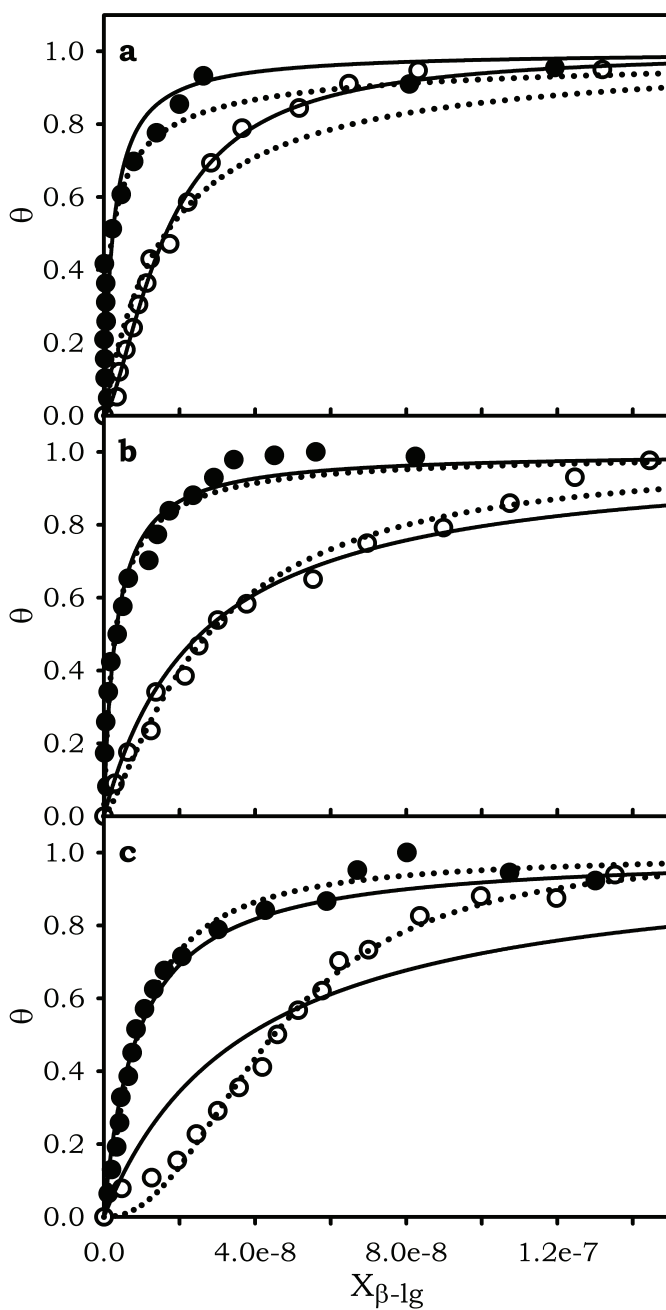


Figure 3.4: Fit of Langmuir (solid lines) and Hill (dotted lines) binding models to a) LMP, b) HMP_B and c) HMP_K at pH 4.25. Ionic strength for LMP: 10mM (●) and 75mM (○), HMP_B: 10mM (●) and 45mM (○) and HMP_K: 10mM (●) and 30mM (○)

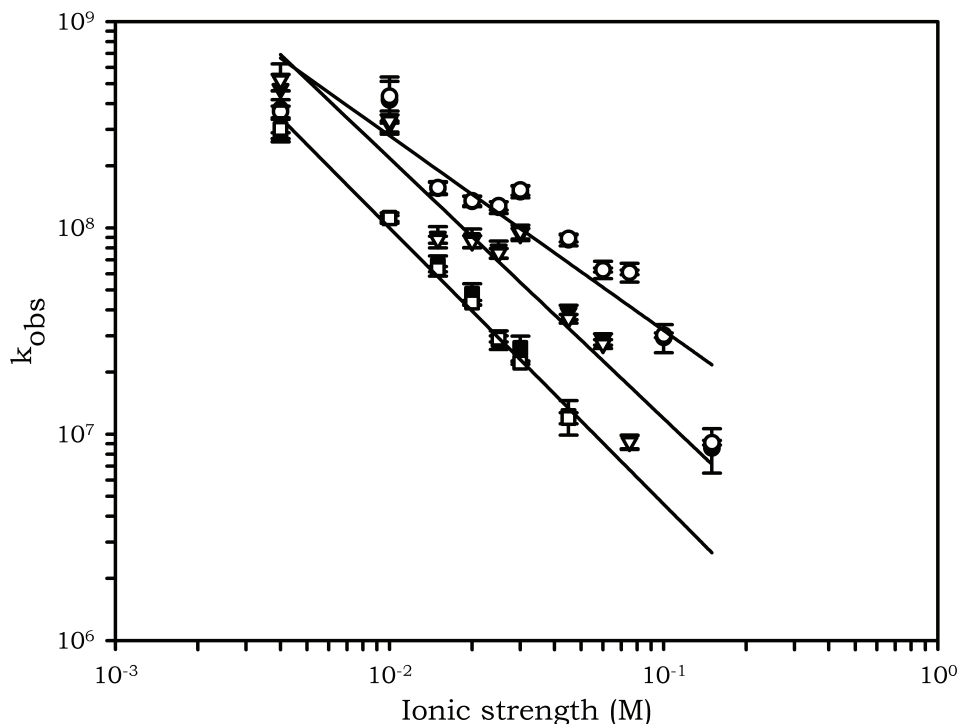


Figure 3.5: Dependency of k_{obs} on ionic strength of β -lg to pectin at pH 4.25 as obtained by the Langmuir (filled symbols) and Hill binding models (open symbols). LMP ($\bullet, \circ$), HMP_B ($\blacktriangledown, \triangledown$) and HMP_R ($\blacksquare, \square$), lines are merely to guide the eye.

maximum of k_{obs} is related to the distribution of positively and negatively charged groups on the protein molecule. The attractive and repulsive electrostatic forces between protein and polyelectrolyte associated with these charges lead to an optimum for binding at a specific ionic strength. For LMP this optimum is situated at 10 mM. The cooperativity parameter, n , is just below unity for the ionic strengths of 4 and 10 mM. When β -lg binds to LMP it will occupy more than one GalA. McGhee and Von Hippel (1974, 1976) showed that this can lead to apparent negative cooperativity, because the amount of n -Me-GalA residues between two bound β -lg molecules is lower than the amount required for binding of a β -lg molecule, making it harder to bind at higher θ . At low ionic strength, in this study 4 and 10 mM, the Debye length is longer, therewith increasing the repulsion between β -lg molecules and, hence, making it more difficult for the next β -lg molecule to adsorb in between two others. Binding isotherms of β -lg and

a LM-pectin were obtained at pH 4 by Girard, Turgeon et al (2003). Girard et al. reported a positive cooperativity at an ionic strength of 5 mM by fitting with the McGhee and Von Hippel model. Apart from the slight difference in pH, this is most likely caused by the use of frontal analysis continuous capillary electrophoresis (FACCE) and not by the choice of a different fitting model. When performing FACCE below the pI of the protein in a bare fused silica capillary, binding of the protein to the negatively charged capillary wall is inevitable (Seyrek, Hattori et al., 2004). In the initial region of the binding isotherm the concentration of free protein is very low, because the majority of β -lg is bound to pectin. The little amount of β -lg that is not bound to pectin binds to the capillary wall, leading to an underestimation of free β -lg at low concentrations of free β -lg. This was observed in our laboratory when trying to employ the FACCE technique to the β -lg-pectin system. Replacing the bare fused silica capillary by a poly(vinyl alcohol) coated capillary did not improve results. An underestimation of the free β -lg concentration at low concentrations of free β -lg leads to an apparent positive cooperativity, as β -lg seems to bind more profoundly after some β -lg is already bound to the pectin. The fluorescence anisotropy measurements, of this study, are not sensitive to this problem.

The binding of β -lg to LMP shows n increasing slowly just above unity for an ionic strength of 15 to 45 mM. At this higher ionic strength the repulsive electrostatic interactions between the β -lg molecules are reduced, allowing a β -lg molecule to adsorb unhindered by its neighbours. Above 45 mM n becomes larger than 1.5. Positive cooperativity for the adsorption of β -lg to pectin can be the result of two effects: attraction between β -lg molecules or a change in the binding sites on pectin creating new binding sites or binding sites of a higher affinity (Norde, 2003). Pectin folds into a helix structure in solution (P  rez, Mazeau et al., 2000). It is possible that binding β -lg to pectin disrupts this helical structure, thus changing the binding sites on pectin and allowing subsequent β -lg molecules to bind more easily. β -Lg molecules are known to form higher oligomers between pH 4.0 and 5.2 (Verheul, Pedersen et al., 1999), these aggregates are favoured at low temperature (<10  C) and low ionic strength, both conditions that are not applicable to the current experimental conditions.

Attraction between β -lg molecules is therefore unlikely to be the cause of the positive cooperativity.

For HMP_B , there is no maximum observed in k_{obs} . With the exception of 4 mM, k_{obs} is lower for HMP_B than for LMP at all ionic strengths. As revealed by analysis of the size and distribution of n-Me-GalA residues, blocks of n-Me-GalA along the HMP_B backbone are relatively isolated, and they are smaller in size than the blocks on LMP. This may be the reason that, at 4 mM, k_{obs} is higher for HMP_B than for LMP. When the size of the n-Me-GalA blocks on HMP_B are of the optimal size for β -lg binding, the repulsion of unfavourable negative charges on β -lg and n-Me-GalA residues outside the binding site may be reduced as on HMP_B these places are occupied by Me-GalA residues. Also, as the binding site is isolated from others, unfavourable electrostatic repulsion between β -lg molecules will be lower for binding to HMP_B than to LMP. This is also a likely reason for the absence of a maximum in k_{obs} with increasing ionic strength: there is not sufficient repulsion between HMP_B and β -lg and between β -lg neighbours to reach an optimum in attractive and repulsive forces with increasing ionic strength. When the ionic strength is subsequently increased these repulsive interactions diminish, making the larger n-Me-GalA blocks on LMP favourable over the smaller blocks on HMP_B . Increasing the ionic strength results in a decrease in k_{obs} . There is some slight negative cooperativity at an ionic strength of 4 and 10 mM, but at higher ionic strength the cooperativity parameter remains close to unity.

HMP_R , like HMP_B , shows no maximum in k_{obs} probably because of similar reasons as outlined above for HMP_B . k_{obs} is also considerably lower at the various ionic strengths. As only a very few intermediate blocks of n-Me-GalA are present on HMP_R , the binding sites on HMP_R do not contain sufficient n-Me-GalA to tightly bind β -lg. A positive cooperativity is observed above an ionic strength of 15 mM, most likely again due to the disruption of the pectin helix by adsorption of β -lg.

3.4 Conclusions

Fluorescence anisotropy is an excellent technique to measure immobilization of proteins and, hence, to determine binding isotherms of

protein to anionic polysaccharides. It has the advantage over FACCE that it can be used at any pH, providing the solution is optically clear. Because the measurements can be performed in microtiter plates, the sample volumes can be kept low and an entire binding isotherm can be measured within a few minutes.

The physicochemical fine structure of the used pectins has been elucidated in detail, revealing that LMP has large blocks of n-Me-GalA of a homogeneous block size that are close together. HMP_B has large blocks of n-Me-GalA that are isolated on the pectin backbone, with a heterogeneous distribution. HMP_R has only very few isolated, relatively small blocks of n-Me-GalA, that are of a homogeneous size.

The Hill equation is preferred over the Langmuir model because of the additional information about the cooperativity of binding. The binding constant, k_{obs} , obtained from the Hill equation and the Langmuir model are comparable. For LMP k_{obs} shows a maximum at 10 mM, due to the balance between attractive and repulsive forces. Cooperativity is shown to increase for binding of β -lg to LMP with increasing ionic strength. At low ionic strength there is a slight negative cooperativity, caused by electrostatic repulsion between neighbouring β -lg molecules. Above 45 mM a positive cooperativity is observed, most likely caused by disrupting the helix fold of pectin, which makes more binding places accessible to β -lg. For HMP_B, there is no maximum in k_{obs} , but at 4 mM k_{obs} reaches a higher value than for LMP. This is caused by the nature of the blocks of n-Me-GalA on HMP_B. The blocks are smaller and of an isolated nature, compared to those on LMP. This leads to less electrostatic repulsion between β -lg and n-Me-GalA residues on pectin, but also between β -lg neighbours and, hence, a higher k_{obs} . The cooperativity coefficient for binding of β -lg to HMP_B is close to unity, indicating noncooperative binding over the entire range of ionic strengths. Binding of β -lg to HMP_R gives the lowest k_{obs} . The random distribution of n-Me-GalA leads to binding sites that have a reduced affinity compared to the binding sites on the other two pectins, probably because of a smaller number of n-Me-GalA residues per site. HMP_R shows positive cooperativity at an ionic strength above 15 mM. The most likely explanation for this is again changes in the helix conformation of pectin, induced by the binding of β -lg.

To strongly bind β -lg to a pectin, it is best to choose a pectin with a blockwise distribution of methyl esters. This leads to binding sites with a higher affinity. When binding in media of higher ionic strengths is required, a low methyl esterified pectin is suited best. At low ionic strength it may be beneficial to use a high methyl esterified pectin with a large blocksize, because the isolated nature of the n-Me-GalA residues reduces the electrostatic repulsion between pectin and β -lg as well as between β -lg neighbours.

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Chapter 4

Influence of overall charge and local charge density of pectin on the complex formation with β -lactoglobulin as assessed by isothermal titration calorimetry

Abstract

The complex formation between β -lactoglobulin and pectins of varying overall charge and local charge density were investigated. Isothermal titration calorimetry experiments were carried out to determine the enthalpic contribution to the complex formation at pH 4.25 and various ionic strengths. Complex formation was found to be an exothermic process for all conditions. Combination with previously published binding constants (Sperber, Cohen Stuart et al., 2009), allows for the determination of the changes in the Gibbs energy and the change in entropy of the system upon complex formation between β -lactoglobulin and pectin. The local charge density of pectin is found to determine the balance between enthalpic and entropic contributions. For a high local charge density pectin, the main contribution to the Gibbs energy is of an enthalpic nature, supported by a favourable entropy effect due to the release of small counterions. A pectin with a low local charge density has a more even distribution of the enthalpic and entropic part to the change of the Gibbs energy. The enthalpic part is reduced due to the lower charge density, while the relative increase of the entropic contribution is thought to be caused by a change in the location of the binding place for pectin on the β -lactoglobulin molecule. The association of the hydrophobic methyl esters on pectin with an exposed hydrophobic region on β -lg results in the release of water molecules from the hydrophobic region and surrounding the methyl esters of the pectin molecule. An increase of the ionic strength decreases the enthalpic contribution, due to the shielding of electrostatic attraction in favour of the entropic contribution, supporting the idea that the release of water molecules from hydrophobic areas plays a part in the complex formation.

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4.1 Introduction

β -Lactoglobulin – pectin complex formation in solution has been used as a model system to study the formation of complexes between oppositely charged biopolymers (Wang & Qvist, 2000; Girard, Turgeon et al., 2002; Kazmierski, Wicker et al., 2003; Ganzevles, Zinoviadou et al., 2006; Sperber, Cohen Stuart et al., 2009). β -Lactoglobulin (β -lg) is chosen as it is the major constituent of whey protein (Verheul, Pedersen et al., 1999) and is used in the food industry for its capability to form gels and to stabilize emulsions and foams (Kinsella, 1984). It is a small globular protein with a well known structure (Sawyer, Kontopidis et al., 1999). It has an iso-electric point of 5.1, a molecular weight of 18.3 kDa and is present predominately as a dimer between pH 3.5 and 7.5, with some higher order aggregates reported around pH 4.7 (Verheul, Pedersen et al., 1999). β -Lg is reported to have a hydrophobic surface pocket in a groove between the β -strands and α -helix (Tavel, Andriot et al., 2008).

Pectin is used by the food industry to stabilize acid milk drinks and in various applications as a gelling and thickening agent (Voragen, Pilnik et al., 1995). An industrially extracted pectin contains mainly galacturonic acid, that can be methyl esterified at the carboxylic acid on C6. Based on their degree of methyl esterification (DM), pectins are divided in low methyl esterified pectins, DM < 50%, (LMP) and high methyl esterified pectins, DM > 50%, (HMP). Further characterization of pectins can be based on their degree of blockiness (DB), which is a measure for the size of the non-methyl esterified GalA (n-Me-GalA) blocks along the pectin backbone (Daas, Arisz et al., 1998). The DB is associated with the calcium sensitivity of pectin, as pectin with larger blocks of consecutive n-Me-GalA residues form gels under lower concentrations of calcium (Thibault & Rinaudo, 1986).

Complexes formed between proteins and polysaccharides are of special interest for the food industry as they are used to stabilize acid dairy drinks (Laurent & Boulenguer, 2003), foams (Schmitt, Palma da Silva et al., 2005; Miquelim, Lannes et al., 2010) and emulsions (Güzey, Kim et al., 2004; Dickinson, 2009), fat replacement (Laneuville, Paquin et al., 2005; Le

Révérènd, Norton et al., 2010), encapsulation of ingredients (Gharsallaoui, Roudaut et al., 2007), and the purification of food or non-food proteins (Ye, 2008). The use of the complexes is not limited to the food industry, they also find application as an antithrombic agent (Seyrek, Dubin et al., 2007), a drug delivery system (George & Abraham, 2006) and packaging material (Shih, 1994).

The thermodynamics of complex formation between proteins and polysaccharides, or in a broader sense polyelectrolytes, may be studied by isothermal titration calorimetry (ITC). ITC measures the enthalpy of binding of the protein to the polyelectrolyte. A distinction can be made between two different systems: Complex formation between oppositely charged and between likewise charged protein and polyelectrolyte. Complex formation between likewise charged protein and polyelectrolyte is found to be an endothermic process due to unfavourable electrostatic interactions, and the complexes are formed by an entropic driving force (Henzler, Haupt et al., 2010). The entropic driving force is assigned to the release of small counter ions from the electrical double layer, due to local oppositely charged patches on the protein (Henzler, Haupt et al., 2010) and hydrophobic interaction, implying the release of water molecules from contact with hydrophobic moieties (Jelesarov & Bosshard, 1999; Lin, Chen et al., 2001). For oppositely charged systems an exothermic process is found, due to favourable electrostatic interactions (Lin, Chen et al., 2001; Girard, Turgeon et al., 2003; Hofs, Voets et al., 2006; De Souza, Bai et al., 2009; Tan, Koopal et al., 2009). In addition to electrostatic interactions, entropic contributions due to the release of small counter ions and the release of water molecules are also reported (Hofs, Voets et al., 2006; Tan, Koopal et al., 2009).

In this study complex formation between β -lg and pectin is studied by means of isothermal titration calorimetry. The combination of the ITC data with binding constants reported in a previous publication (Sperber, Cohen Stuart et al., 2009), allows for the calculation of both the enthalpic and entropic contributions to the Gibbs energy of the complex formation. This study is focused on the influence of the overall charge and local charge density of pectin on the complex formation. The use of various pectins that have been characterized in detail with respect to their physico-chemical

characteristics, provides insight into the mechanism of complex formation between β -lg and pectin.

4.2 Materials and Methods

4.2.1 Materials

Three types of pectin, low methyl esterified pectin (LMP), high methyl esterified calcium sensitive pectin (HMP_B), and high methyl esterified non-calcium sensitive pectin (HMP_R), were kindly provided by CPKelco (Lille Skensved, Danmark). The pectins are isolated from lemon and were characterized in detail by Daas et al.(2001) on degree of methyl esterification and degree of blockiness (DB), see table 4.1. The pectins had a typical molecular weight of 150 kDa (Daas, Voragen et al., 2001).

Table 4.1: Uronic acid content, DM and DB for LMP, HMP_B, HMP_R (Daas, Boxma et al., 2001)

Pectin	Uronic acid content (%)	DM (%)	DB (%)
LMP	78.5	30.4	16.5
HMP _B	84.5	69.8	10.9
HMP _R	85.3	73.5	1.7

The pectins contained 0.02-0.05 mole of calcium per mole of galacturonic acid (GalA) as a result of their isolation process. To remove the calcium, the pectins were washed five times with 60% ethanol, containing 5% (v/v) hydrochloric acid. Next, the pectins were washed with 60% ethanol until they were free of chlorine (tested with 0.2 % (w/w) silvernitrate) and subsequently with acetone and air dried. This treatment was sufficient for LMP and HMP_B, but for HMP_R only 50% of calcium was removed. Therefore, HMP_R was further treated by dissolving it at 10g/L and adding AG 50W-X4 200-400 mesh ion exchange resin from Bio-Rad Laboratories (Hercules, CA). HMP_R and ion exchange resin were separated by centrifugation and HMP_R was lyophilized. This resulted in pectins that contained less than 0.001 mole of calcium per mole of GalA.

β -Lg was purified from bovine milk using a non-denaturing technique as described by De Jongh et al (2001). β -Lg was over 99% pure.

4.2.2 Isothermal titration calorimetry

ITC experiments were performed using a MicroCal MCS-ITC instrument (MicroCal, Northampton, MA). Pectins and β -lg were dissolved to 0.7 g/L and 50 g/L, respectively, in a 20mM sodiumacetate buffer, pH 4.25. The ionic strength of the buffer was set with NaCl. Solution pH was adjusted and the samples were stored at 4°C overnight to ensure full hydration of the polymer chain. The pH was adjusted again if needed and both pectin and β -lg were dialyzed using a 5kDa cutoff dialysis tube for 24 hours at 4°C in the same vessel against the experimental buffer. This dialysis step is necessary to ensure pre-equilibration (Norde & Lyklema, 1978). Solutions were stored at 4°C until they were used for measurement, but never longer than 2 days.

Pectin and β -lg solution were degassed under vacuum just before the measurement. The ITC measurement cell (1.353 mL) was filled with the pectin solution, and titrated with the β -lg solution. ITC measurements were performed at 25°C. The injection volume varied between 2.5 and 6.5 μ L, depending on the amount of heat released by the complex formation between pectin and β -lg. The time between subsequent injections was 500 s, which was found to be sufficient to achieve equilibrium. The solution was stirred at 300 rpm throughout the experiment. The heats of dilution for the pectin solution and the β -lg solution were found to be minimal due to pre-equilibration in the dialysis tubes. Nevertheless, the small heats of dilution were subtracted from the raw experimental data. At constant pressure the heat exchange between the system and the surroundings equals the enthalpy change of the system.

Heat peaks were integrated using Origin software supplied with the MicroCal MCS-ITC instrument. The calculated enthalpy is corrected for the amount of β -lg that actually binds to the pectin using the binding constant, k_{obs} , and Hill coefficient published by Sperber et al (Sperber, Cohen Stuart et al., 2009) to obtain the enthalpy change per mole of bound β -lg. Binding of β -lg to pectin is expressed as a function of the molar ratio r , i.e., the ratio of the molar concentration of the β -lg dimer and the molar concentration of n-Me-GalA residues.

4.3 Results and Discussion

Performing the ITC experiments at pH 4.25 is based on the state diagrams for β -lg and pectin complex formation, published by Sperber, Schols et al (2009). Depending on the type of pectin and the solution ionic strength, at pH 4.25, a large part of the ITC curve is in the region where soluble complexes are formed. It is assumed that in this region the detected heat changes are only due to the binding of β -lg to pectin and not to phase separation of β -lg – pectin complexes. Figure 1a shows a typical thermogram for the β -lg – LMP system. The large repetitive peaks occurring upon each injection of β -lg to LMP reflect an exothermic binding process, i.e., the binding is energetically favourable. The small, positive peaks at the end of the titration are the result of dilution heats when β -lg is injected in a solution of LMP that is already saturated with β -lg. This was confirmed

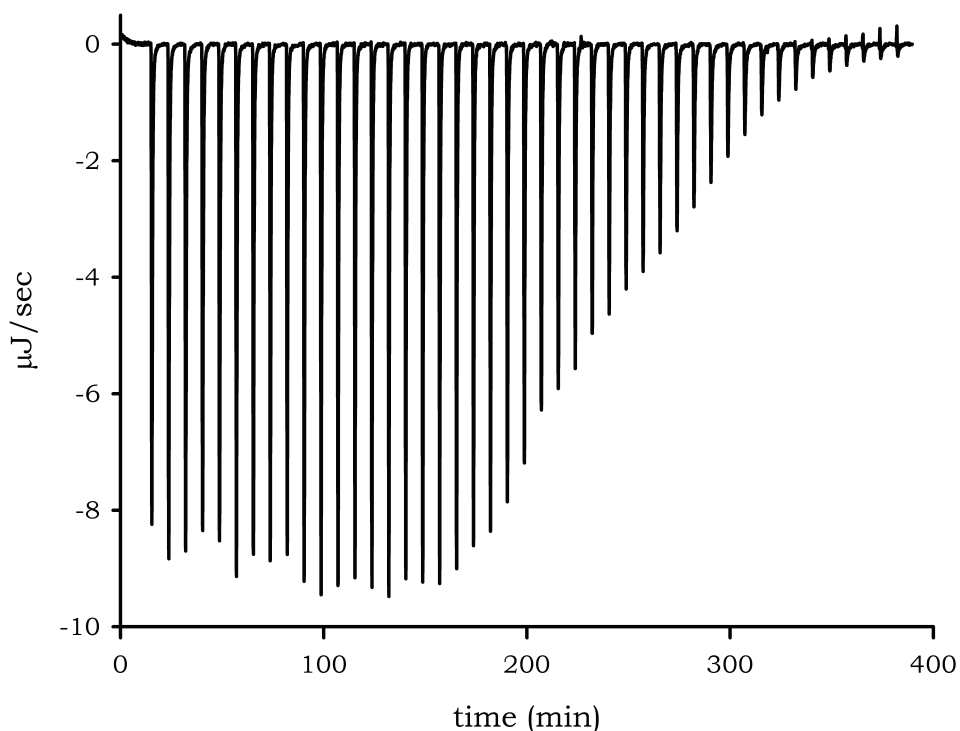


Figure 4.1: Thermogram of the binding of β -lactoglobulin to LMP at pH 4.25 in a 20mM Na-acetate buffer. The ionic strength is set to a total of 10mM using NaCl

by blank titrations. The use of a buffer with a low heat of ionization is important, as the complex formation between β -lg and pectin leads to charge regulation of ionisable groups of both β -lg and pectin, causing expulsion of protons from the complexes (Sperber, Schols et al., 2009).

Figure 4.2 shows an example of the calculated heat release as the result of β -lg binding to pectin, in this case LMP. When the heat release is plotted against r , i.e., when not corrected for the amount of added β -lg that actually binds, a plateau is observed from $r = 0$ to $r = 0.031$. In this initial plateau 65% of the total β -lg binding takes place and virtually all of the added β -lg (>98%) binds due to the high affinity of the binding sites. At $r = 0.031$ a gradual decrease of the heat effect is observed until r reaches

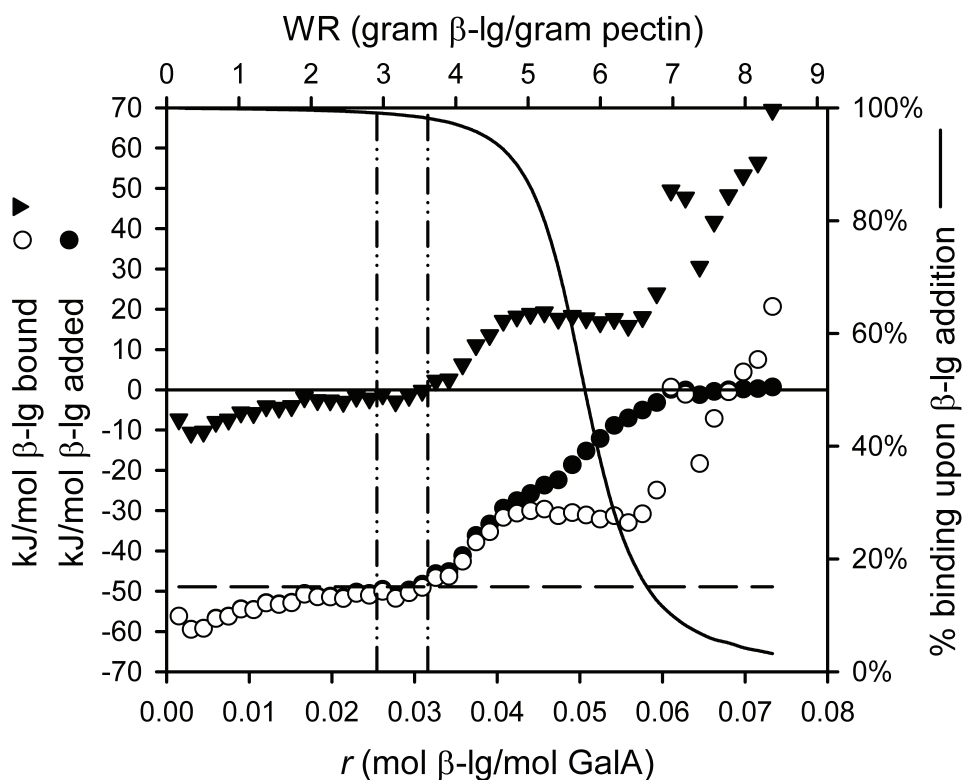


Figure 4.2: Changes in enthalpy (● for raw ΔH , ○ for ΔH corrected for the fraction of β -lg actually binding) entropy (▼, as $T\Delta S$) and Gibbs energy (dashed line) for the binding of β -lactoglobulin to LMP at pH 4.25 in a 20mM Na-acetate buffer. The ionic strength of the buffer is 4mM. The binding isotherm (solid line) is displayed as the percentage of added β -lg that binds. The r where lateral repulsion is calculated to start influencing binding is displayed by the two vertical lines.

0.061, whereafter no more heat is released. Inspection of the binding isotherm learns that this is close to the r -value where saturation of LMP binding sites with β -lg occurs. When these results are adapted for the amount of β -lg that actually binds to LMP, a secondary plateau appears at $0.041 < r < 0.058$. At the start of this secondary plateau, 82% of the β -lg binding sites on LMP are occupied, and at the end this is 98%. At the end of this second plateau there is hardly any heat release anymore. Because in this trajectory of the binding isotherm only a very small fraction of the supplied β -lg binds, the result per mol bound β -lg molecule becomes erroneous and is not reliable for $r > 0.061$.

Commercial pectins were shown to be heterogeneous materials with respect to their inter- and intramolecular distribution of methyl esters (Guillotin, Bakx et al., 2005; Sperber, Cohen Stuart et al., 2009). As β -lg binds to pectin via the negatively charged GalA residues, there will be an optimum to the GalA block length for the binding site. This makes the interpretation of ITC experiments for the complexation of β -lg with pectin treacherous. A change in the amount of released heat is not necessarily caused by saturation of the pectin molecule, but can also be caused by the binding of β -lg to binding sites of a lower affinity. The construction of a binding isotherm from the ITC data, as is often done with the software supplied with the ITC apparatus, should therefore be avoided for this specific type of polysaccharide.

As judged from figure 4.2, up to $r = 0.031$, the binding of β -lg to LMP occurs on binding sites of essentially equal affinity. It is reasonable to assume that these sites have an "infinite" length of GalA residues. After r reaches 0.031, when the heat effect starts to decrease, different scenarios are possible: the binding sites are of a suboptimal length, leading to smaller favourable electrostatic attraction between β -lg by LMP and hence a reduction in the heat release, or the β -lg molecules need to bind closer together, leading to unfavourable lateral repulsion between β -lg neighbours, or a combination of the two. A rough calculation may be performed to verify whether lateral repulsion between β -lg neighbours plays a role. The GalA monomer is reported to have a length of 0.43nm (Rees & Wight, 1971); the β -lg dimer is a prolate ellipsoid with a length of 6.9 nm and width of 3.6 nm (Verheul, Pedersen et al., 1999). Based on the

GalA content of the pectins and DM, the total length of available GalA can be calculated. Electrostatic repulsion takes place when the electrical double layers surrounding the β -lg molecules overlap, that is at a separation of $\leq 2\kappa^{-1}$, where κ^{-1} is the thickness of the electrical double layer. For symmetrical electrolytes κ^{-1} can be estimated by:

$$\kappa = \sqrt{10cz^2} \text{ (nm}^{-1}\text{)} \quad (4.1)$$

with c the salt concentration in M and z the valence of the ions. A minimum and maximum effective size of β -lg is calculated by using either the length or width of the β -lg molecule plus 2 times κ^{-1} . Using these assumptions electrical double layer overlap is calculated to start at r between 0.025 and 0.032, depending on the orientation of the β -lg molecule. As can be seen in figure 4.2, this is roughly at the r -value where the heat release starts to decrease, indicating the onset of lateral repulsion between β -lg neighbours.

From the binding constant (k_{obs}) determined by Sperber, Cohen Stuart et al (2009) the Gibbs energy of binding, excluding the contribution from the configuration entropy associated with the distribution of β -lg along the pectin chain, can be calculated using the following relation:

$$\Delta G = -RT \ln k_{\text{obs}} \quad (4.2)$$

with R is the gas constant and T the temperature (K). The change in Gibbs energy (ΔG), comprises changes in enthalpy (ΔH) and entropy (ΔS) as follows:

$$\Delta G = \Delta H - T\Delta S \quad (4.3)$$

where ΔG is negative for a spontaneous process. The enthalpy change, ΔH , can directly be obtained from the ITC data because, at constant pressure (p), the heat effect $(\Delta q)_p = \Delta H$. The calculations from ΔH and $T\Delta S$ to ΔG are shown in figure 4.2. For the first stage of binding, up to r is 0.031, $\Delta H < \Delta G$, implying, according to equation 3, an unfavourable entropy decrease, as $\Delta S < 0$. This is unexpected as the entropy contribution associated with the binding of oppositely charged polymers is reported to be favourable, due to the release of counterions from the electrical double layer (Ball, Winterhalter et al., 2002; Bharadwaj, Montazeri et al., 2006; Hofs, Voets et

al., 2006; Tan, Koopal et al., 2009). However, as stipulated by Sperber, Cohen Stuart et al (2009), the distribution of methyl esters on the GalA backbone will lead to inhomogeneous binding sites. As k_{obs} has been calculated by fitting the entire binding isotherm, it has an averaged character. Hence, as the most favourable binding sites will be occupied first, for low r , the negative value of ΔG may be underestimated and therefore ΔG consists of an enthalpic component and, most likely, also a favourable entropic term. As r increases, and the enthalpy release decreases, the negative value of ΔG is overestimated and the entropic contribution is probably smaller than shown in figure 4.2. At high r no heat release is measured, although, according to the binding isotherm, binding still takes place. As the binding isotherm was determined under conditions deviating from those of the ITC experiments, i.e., a lower total biopolymer concentration and not using a titration format, significant inaccuracy can be expected in the last part of the curve. Yet, also Tan et al (Tan, Koopal et al., 2009) reported that binding between lysozyme and humic acid at the end of the titration curve, where phase separation of the complexes takes place, is driven by an entropy increase. The main contribution to the entropy gain is attributed to the release of bound water from the surface of the complexes when they phase separate.

Figure 4.3 shows the enthalpy changes for all three pectins at various ionic strengths. For LMP the profile of the enthalpy effect is similar at all ionic strengths, but the magnitude decreases with increasing ionic strength, and, furthermore, binding saturation is reached at lower r -value. The reduced enthalpy change is caused by screening of the electrostatic attraction between the oppositely charged groups on β -lg and GalA, as was also reported for complex formation between oppositely charged polymers by several authors (Hofs, Voets et al., 2006; De Souza, Bai et al., 2009; Tan, Koopal et al., 2009). For higher ionic strength LMP is saturated with β -lg at lower r , which is also related to the decrease in electrostatic attraction due to more efficient screening of electrostatic forces. This causes not all binding sites to be able to bind β -lg sufficiently strong, as shown before by Sperber, Cohen Stuart et al. (2009), who observed a decrease in the adsorption maximum of β -lg to pectin at higher ionic strength.

The titration of HMP_B with β -lg shows a profile different from that of β -lg to LMP. For the first 2 injections the heat release is as large as for LMP. It indicates that in HMP_B less high affinity sites are present. The presence of a small number of high affinity sites on HMP_B is in accordance with the conclusion that at the onset of complex formation the binding sites on LMP

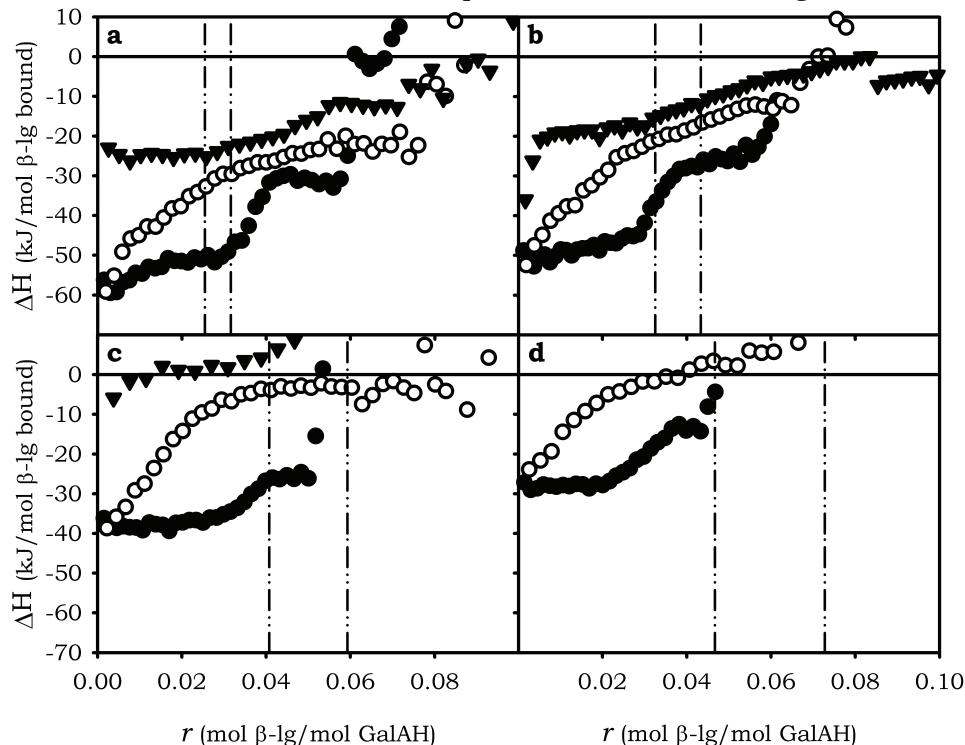


Figure 4.3: Corrected enthalpy change for the binding of β -lactoglobulin to LMP (●), HMP_B (○) and HMP_R (▼) at pH 4.25 in a 20mM Na-acetate buffer. The ionic strength is set to a total of 4 (a), 10 (b), 30 (c) and 75 mM (d) using NaCl. The r where lateral repulsion is calculated to start influencing binding is displayed by the two vertical lines.

and HMP_B have a similar local charge density (Sperber, Schols et al., 2009). There is a gradual decrease of the heat release throughout the titration experiment. This indicates a large variety of binding sites, all with their individual binding constant. At low ionic strength (up to 10mM) binding of β -lg to HMP_B takes place up to a higher r than for LMP. Since the reported binding constants for HMP_B and LMP are comparable (Sperber, Cohen Stuart et al., 2009), this is believed to be caused by the fact that HMP_B contains less binding sites than LMP, so that they have a

larger separation distance along the polymer chain. This allows binding of β -lg at higher r as there is less electrostatic repulsion between β -lg neighbors, leading to a more efficient use of the available binding places.

The enthalpy of β -lg binding with HMP_R is considerably lower than for the binding of β -lg to HMP_B or LMP. The random nature of HMP_R leads to the absence of high affinity sites as are present on HMP_B and LMP. For low ionic strength there is an initial plateau containing binding sites of similar affinity. As for HMP_B , binding of β -lg to HMP_R takes place up to a higher r than for LMP, for the same reason as outlined for HMP_B . The β -lg – HMP_R system is more sensitive to ionic strength compared to HMP_B or LMP. Since the size of GalA blocks on HMP_R are smaller, the reduction of attractive electrostatic forces by increased screening at higher ionic strength leads to a suppression of binding at lower ionic strength. At an ionic strength of 75mM no heat release was detected, and at 30mM only for the first injection, despite the fact that β -lg and HMP_R are reported to form complexes under these conditions (Sperber, Cohen Stuart et al., 2009; Sperber, Schols et al., 2009).

Table 4.2 shows values for the changes in Gibbs energy and enthalpy resulting from the binding of β -lg to pectin. For LMP, ΔG does not differ much from ΔH in the first plateau for ionic strengths of 4 and 10 mM, although also an entropic contribution to the binding of β -lg to LMP is expected in this region due to the release of small counterions. At the final part of the titration, ΔH (after correction for the amount of β -lg that actually binds to LMP) is smaller than ΔG . As discussed above, this may well be due to overestimation of k_{obs} , and, hence, of ΔG in this titration region. At ionic strengths of 30 and 75 mM, ΔG is considerably larger than ΔH . This suggests that part of the interaction between β -lg and LMP at this ionic strength is driven by an entropy gain. It is unlikely that this entropy gain originates only from the release of small counterions, as entropy change due to the release of small counterions should reduce with increasing ionic strength (Henzler, Haupt et al., 2010). Possibly, the reduced electrostatic forces allow β -lg to bind at different places on LMP, causing the release of water molecules that are associated with the hydrophobic methyl groups of pectin. It is also possible that a different part of the β -lg molecule is available for binding to LMP. As β -lg has an

exposed hydrophobic cavity (Tavel, Andriot et al., 2008), binding of LMP in this cavity could result in the release of immobilized water molecules from both β -lg and LMP. Schmitt et al (Schmitt, Sanchez et al., 2001) found a change in the tertiary structure of β -lg upon binding to acacia gum. They reported a decrease in the α -helix content of β -lg when it was in a complex with acacia gum. β -Lg only has one α -helix that is located next to the exposed hydrophobic cavity (Tavel, Andriot et al., 2008).

Table 4.2: Parameters obtained from the ITC data. k_{obs} from Sperber, Cohen Stuart et al (2009). The plateaus are identified in figure 4.3

	I (mM)	$10^{-6} \times k_{\text{obs}}$	ΔG (kJ/mol)	ΔH_{ini} (kJ/mol)	$\Delta H_{\text{Plat},1}$ (kJ/mol)	$\Delta H_{\text{Plat},2}$ (kJ/mol)
LMP	4	366	-48.9	-59.4	-51.4	-30.4
	10	434	-49.3	-52.3	-48.1	-26.0
	30	152	-46.7	-38.1	-37.7	-26.0
	75	60.9	-44.4	-28.8	-27.9	-13.5
HMP _B	4	520	-49.8	-57.2		
	10	330	-48.6	-50.0		
	30	95.7	-45.5	-37.3	-	-
	75	9.2	-39.7	-22.8	-	-
HMP _R	4	302	-48.4	-25.5	-24.6	-12.2
	10	111	-45.9	-36.7	-19.5	-7.0
	30	22.2	-41.9	-5.9	-	-

Finally, it should be mentioned that the ITC experiments are performed at much higher biopolymer concentrations than the binding isotherms experiments, to obtain a good signal to noise ratio. This may introduce uncertainties in the comparison of thermodynamic parameters obtained from the different types of experiments.

For HMP_B, ΔG is larger i.e., more negative, than ΔH for all ionic strengths, with the exception of the first few injections at an ionic strength of 4 and 10mM. These first few injections involve binding sites comparable to LMP. After those first few injections and at higher ionic strength the complex formation between β -lg and HMP_B is therefore driven both by enthalpic and entropic contributions, with the relative contribution of the entropic component increasing with increasing ionic strength. β -Lg binds

on HMP_B in blocks of GalA, like on LMP, but as discussed before, the majority of GalA blocks on HMP_B are not as large as on LMP, resulting in many different types of binding sites having their own individual binding affinities and, hence, leading to a gradual reduction in the release of heat from the onset of the titration curve. β -Lg therefore binds on binding sites that are not isolated from interaction with the methyl esterified groups of HMP_B . This results in the release of hydration water surrounding the hydrophobic methyl group and possibly the hydrophobic cavity of β -lg, which, in turn, raises the entropy of the system.

Complex formation between β -lg and HMP_R comprises, like for HMP_B , an enthalpic and an entropic component. Where for HMP_B initially $\Delta H \approx \Delta G$, for HMP_R the binding of β -lg upon the first addition already comprises an enthalpic component (as the result of electrostatic interactions between the oppositely charged groups) and an entropic contribution (from the release of small counter ions and hydration water surrounding the methyl groups of pectin and, possibly, the exposed hydrophobic region of β -lg). As the ionic strength increases, the enthalpic contribution is fully suppressed and only the entropic component remains.

4.4 Conclusions

The complex formation between β -lg and pectin is driven by both enthalpic and entropic contributions. The enthalpic contribution consists of the electrostatic attraction between oppositely charged groups on β -lg and pectin. The entropic contribution is the result of the release of small counter ions from the electrical double layer surrounding the β -lg and pectin ionic groups, as well as the release of water restricted in its movement surrounding the pectin methyl esters and an exposed hydrophobic region on β -lg.

The balance between the enthalpic and entropic contribution to complex formation depends strongly on the pectin physico-chemical characteristics. The local charge density and overall charge of a pectin determines the ratio between the enthalpic and entropic contribution of complex formation. The complex formation between β -lg and LMP at pH 4.25 is mainly enthalpy driven for ionic strengths up to 10 mM, although

entropic contributions are suspected to be underestimated, due to the averaged character of k_{obs} . At 30 mM and above there is also a considerable entropic contribution to the complex formation. For HMP_B the onset of complex formation is mainly enthalpically driven at ionic strength up to 10mM, but when the high affinity, LMP resembling, binding sites are filled, a considerable entropical contribution is part of the driving force for complex formation as well. $\beta\text{-Lg} - \text{HMP}_\text{R}$ complex formation is driven by an enthalpic and entropic component at ionic strengths up to 10mM. At 30mM the enthalpic contribution is found to be absent and the complex formation is fully entropically driven.

It can be concluded that when $\beta\text{-lg}$ binds to a pectin, the local charge density of the pectin determines the composition of the driving force for complex formation. For a pectin with a high local charge density, such as LMP, the main driving force is of an enthalpic nature, originating from the favourable electrostatic interactions. The complex formation is supported by a favourable entropic contribution, resulting from the release of small counterions. When the local charge density of pectin is lower, like for HMP_R , but also for HMP_B , the entropic contribution to the driving force of complex formation becomes more pronounced. It is postulated that this may be the result of lower electrostatic repulsion between $\beta\text{-lg}$ and pectin, allowing $\beta\text{-lg}$ to bind in such a way that the entropic contribution comprises of both the release of small counterions, as well as immobilized water molecules from the pectins methyl groups and, possibly, from an exposed hydrophobic cavity on $\beta\text{-lg}$. An increase in ionic strength results in an increase of the relative contribution of the entropic component, supporting the idea that the release of water molecules from hydrophobic regions on both pectin and $\beta\text{-lg}$ plays a role in the driving force of complex formation.

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Chapter 5

Adsorption of β -lactoglobulin and pectin to a solid hydrophobic surface

Abstract

Hydrophobized silica is used as a model system to study the adsorption of β -lactoglobulin (β -lg) and pectin to an oil-water interface. Adsorption takes place both from mixtures as sequentially. The adsorption of β -lg and pectin to a hydrophobic surface is investigated at pH 4.25, where soluble complexes are formed over a large range of mixing ratio's. The adsorption to a hydrophobic surface from a mixed β -lg-pectin solution leads to a reduction in the rate of adsorption as compared to a solution of β -lg alone. This can be related to a lower concentration of free β -lg. When low weight ratio's of β -lg / pectin are used, the ζ -potential is lower than for higher weight ratio's, due to charge compensation of β -lg that is bound to pectin. The low rate of adsorption of mixtures of β -lg and pectin hampers emulsion stabilization, as the newly created surface cannot be covered before coalescence of the droplets would occur. Sequential adsorption of β -lg and pectin results in the build up of multi layers of β -lg and pectin. When the multilayer is completed with a layer of pectin with a high local charge density, it is most stable against the wash out of β -lg from the multilayer. When the last layer is of a pectin with a low overall charge, the ζ -potential is lowest. This indicates that high methyl esterified pectin protrudes more into the solution than low methyl esterified pectin. The above suggests that to enhance the stability of an emulsion a primary emulsion of β -lg needs to be prepared to which high methyl esterified, blockwise pectin is added to create a secondary emulsion that is stabilized by a layer of pectin adsorbed on top of the β -lg layer.

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5.1 Introduction

Pectin is widely used in the food industry for its gelling properties in the production of jams and jellies, fruit juice, confectionary products and bakery fillings (Voragen, Pilnik et al., 1995; May, 1997), but pectin is also added to acidified dairy drinks to stabilize the casein micelles from precipitation (Tholstrup Sejersen, Salomonson et al., 2007). Pectin stabilizes the casein micelles by electrosorption to the micelle surface (Pereyra, Schmidt et al., 1997; Marozienne & De Kruif, 2000; Tuinier, Rolin et al., 2002). It was first postulated that the negative charge of the pectin stabilised the casein micelle by electrostatic repulsion (Glahn, 1982), but ζ -potential measurements showed that the charge of the casein micelle-pectin complexes was not sufficient for stabilization and the stabilization occurs by a combination of electrostatic repulsion and steric hindrance (Kravtchenko, Parker et al., 1995). For the stabilisation of acidified milk drinks, the choice of pectin is very important, as different pectin types have different stabilizing properties. The difference in stabilising properties of the pectins is related to the degree of methyl esterification (DM) and distribution of the methyl esters (DB) of galacturonic acid within the pectin backbone. High methyl esterified (HM) pectin is found to be a better stabiliser of acidified milk drinks than low methyl esterified (LM) pectin (Pereyra, Schmidt et al., 1997; Liu, Nakamura et al., 2006). This better stabilization by HM-pectin is explained by the proportion of the pectin molecule that is bound to the casein micelle. A LM-pectin has mainly non-methyl esterified GalA (n-Me-GalA) residues, resulting in the binding of the majority of the pectin molecule to the casein micelle. HM-pectin has much less binding places, resulting in a pectin molecule that protrudes more into the solution and provides a better stabilization of the casein micelle by steric repulsion (Pereyra, Schmidt et al., 1997; Rolin, 2002; Boulenguer & Laurent, 2003). For a long term stability of the acidified milk drink it is important that a weak gel network is formed (Boulenguer & Laurent, 2003). Of the HM-pectins, calcium sensitive pectin is preferred over a non-calcium sensitive pectin as the blocks of n-Me-GalA bind strongly to the casein micelle, while the randomly distributed non-calcium sensitive

pectin hardly binds to the casein micelle (Glahn & Rolin, 1996; Laurent & Boulenguer, 2003).

Emulsions are often stabilised by proteins. Proteins adsorb on the oil-water interface, thereby protecting the emulsion against coalescence (Dickinson, 1999). Protein-stabilised emulsions are often not stable to environmental stresses like pH, salt, heating, freezing or shear forces (McClements, 2003). One way of improving the stability of the emulsion is the addition of a charged polysaccharide to the system (Dickinson, 1994; Güzey, Kim et al., 2004). There are two ways to stabilize the emulsion: a sequential adsorption of protein and polysaccharide, or the preparation of an emulsion from a mixed solution of protein and polysaccharide. Sequential adsorption, or layer-by-layer deposition, is achieved by making a primary emulsion with protein, to which a solution of polysaccharide is added. This gives stable emulsions, although an ultrasound treatment seems to be a prerequisite to prevent flocculated droplets for a system consisting of β -lg and pectin (Güzey, Kim et al., 2004). Emulsions prepared from a mixture of sodium caseinate and dextran sulphate are also stable. No bridging flocculation was observed in these emulsions (Jourdain, Leser et al., 2008). The characteristics of the adsorbed complexes and the structures of the adsorbed layers from sequential adsorption on the interface between oil and water are however still poorly understood (Dickinson, 2008).

To investigate the influence of pectin physicochemical characteristics (overall charge and local charge density) on the adsorption to hydrophobic surfaces, like oil-water or air-water surfaces, of β -lg and β -lg-pectin complexes a model system of hydrophobized silica was used. Sperber et al. (Sperber, Schols et al., 2009) studied the formation of complexes of β -lg and pectin as a function of pH and ionic strength by means of state diagrams. The region where soluble complexes of β -lg and pectin are formed was chosen to study the functionality of β -lg-pectin complexes on hydrophobic surfaces. Furthermore, the possibility of a layer-by-layer deposition of β -lg and pectin from their individual solutions by alternating exposure to solutions of β -lg and pectin was investigated.

5.2 Materials and Methods

5.2.1 Materials

Three pectins, low methyl esterified pectin (LMP), high methyl esterified calcium sensitive pectin (HMP_B), and high methyl esterified non-calcium sensitive pectin (HMP_R), were kindly provided by CPKelco (Lille Skensved, Denmark). The pectins originate from lemon and were characterized by Daas et al.(Daas, Boxma et al., 2001) on degree of methyl esterification and degree of blockiness (DB), see table 1. The pectins had a typical molecular weight of 150 kDa.(Daas, Voragen et al., 2001).

Table 5.1: Uronic acid content, DM and DB for LMP, HMP_B, HMP_R (Daas, Boxma et al., 2001)

Pectin	Uronic acid content (%)	DM (%)	DB (%)
LMP	78.5	30.4	16.5
HMP _B	84.5	69.8	10.9
HMP _R	85.3	73.5	1.7

The pectins contained 0.02-0.05 mole of calcium per mole of galacturonic acid (GalA). The calcium was removed by washing the pectins five times with 60% ethanol, containing 5% (v/v) hydrochloric acid. Next, the pectins were washed with 60% ethanol until they were free of chlorine (tested with 0.2 % (w/w) silvernitrate). Pectins were subsequently washed with acetone and air dried. This was sufficient for LMP and HMP_B, but HMP_R required further treatment. HMP_R was dissolved to 10g/L and mixed with AG 50W-X4 200-400 mesh ion exchange resin from Bio-Rad Laboratories (Hercules, CA). HMP_R and ion exchange resin were separated by centrifugation and HMP_R was lyophilized. This resulted in pectins that contained less than 0.001 mole of calcium per mole of GalA.

β -Lactoglobulin (β -lg) was purified from bovine milk using a non-denaturing technique as described by De Jongh et al.(De Jongh, Gröneveld et al., 2001) β -Lg was over 99% pure.

5.2.2 Hydrophobization of solid surfaces

Surface modification of silicium wafers was performed as previously described by Van der Wielen et al (Van der Wielen, Baars et al., 2000), with

some minor modifications. For the reflectometry measurements, silicium wafers are oxidized by placing them in an oven at 1000°C for approximately 90 minutes. The wafers were cut to size and sonicated for 15 minutes in ethanol. Wafers were subsequently rinsed with ethanol and milli-Q water and dried in a stream of nitrogen. Next, the wafers were cleaned by immersion in a solution of concentrated sulphuric acid and 20% hydrogen peroxide (3:1). Wafers were washed with Milli-Q water and dried in a stream of air. The cleaned, dry wafers were hydrophobized by immersion in a 1% dichlorodimethyl-silane (DCDM-silane) solution in toluene. The strips were removed from the DCDM-silane solution and immersed in toluene. Next, the wafers were washed, first with acetone and second with Milli-Q water. The wafers were stored in Milli-Q water until further use (always within 24 hours).

For the ζ -potential measurements, microscope glass slides were hydrophobized using the same protocol outlined above, except for heating at 1000°C and sonication.

5.2.3 Reflectometry

The adsorbed amount of material on the hydrophobized silica wafers is measured by reflectometry. Reflectometry is a simplified form of ellipsometry, where the changes in intensity of a reflected polarized laser beam are measured. For a detailed description of this technique, we refer to Dijt et al (Dijt, Cohen Stuart et al., 1990). Briefly, the technique can be described as follows: The output signal (S) is defined as the ratio of the parallel ($I_{||}$) and perpendicular intensities ($I_{\perp}$) of the reflected light:

$$S = \frac{I_{||}}{I_{\perp}} \quad (5.1)$$

Due to the adsorption of material, S changes from its original value S_0 to a value $S_0 + \Delta S$. As S is set to zero for S_0 only ΔS is reported. ΔS can be converted to an amount of mass per unit area. For this, the composition of the adsorbed layer needs to be known, as well as the refractive index of the different layers: 3.85 for Si, 1.46 for SiO₂, 1.44 for protein and 1.33 for water. The refractive index increment (dn/dc) for β -lg is 0.181 ml/g (De Feiter, Benjamins et al., 1978). For the pectins the following dn/dc 's were

measured using a Shodex RI-72 instrument: 0.123 ml/g for LMP, 0.121 ml/g for HMP_B and 0.124 ml/g for HMP_R.

The experiments are started by a buffer rinse of the surface. When a stable baseline is obtained, the buffer flow is replaced by a sample flow.

5.2.4 Streaming potential

The streaming potential is measured to calculate ζ -potential of the surface. Two hydrophobized glass slides are mounted in a cell separated by 0.1mm. The sample solution is forced to flow through the cell by a mechanical pressure difference, Δp . The streaming potential (V_s) is measured, from which the ζ -potential can be calculated as follows:

$$\frac{V_s}{\Delta p} = \frac{\epsilon_0 \epsilon \zeta}{\eta K} \quad (5.2)$$

where ϵ_0 is the dielectric permittivity of vacuum, ϵ the dielectric constant of the solvent, η the dynamic viscosity of the solvent and K the conductivity of the cell filled with solution (Elgersma, Zsom et al., 1992). The ζ -potential of the surfaces is determined when the adsorption of material from the solution is complete.

5.3 Results and Discussion

The adsorption of β -lg and pectin, both from mixtures and sequentially, to a hydrophobized silica surface is a model for adsorption phenomena at hydrophobic surfaces in foods like oil-water or air-water interfaces. Fast adsorption is required to be able to stabilize new surface area that is being formed when emulsions are produced (Dickinson, 2003). With reflectometry, information about the rate of adsorption and the adsorbed amount can be determined. To prevent coalescence of emulsions the charge density of the stabilized interface is important and can be determined by streaming potential measurements.

5.3.1 Adsorption of β -lg and pectin from homo-molecular solutions

Figure 5.1 shows the adsorption of β -lg and LMP to hydrophobized silica at pH 4.25. β -lg adsorbs very fast, reaching a plateau value that

corresponds to approximately 1.3 mg/m^2 . β -Lg is known to adsorb quickly to hydrophobic interfaces (Paulsson & Dejmek, 1992). The ζ -potential of the hydrophobized silica surface was determined to be -10.0 mV . Not all of the silanol groups have reacted with DCDM-silane, leaving a negative charge on the hydrophobic surface. As the adsorption experiment takes place below the pI of β -lg, this will increase the adsorption rate due to attractive electrostatic forces between β -lg and the surface. After adsorption of β -lg the ζ -potential of the surface is found to be $+11.7 \text{ mV}$. The adsorbed amount of β -lg can be increased by increasing the ionic strength, which reduces electrostatic repulsion in the adsorbed layer, allowing the β -lg molecules to be packed closer together. Increasing the concentration of the β -lg solution from which the β -lg adsorbs will also increase the adsorbed amount, as the β -lg has less time to adapt its structure, i.e., to "spread" over the surface (Elofsson, Paulsson et al., 1997).

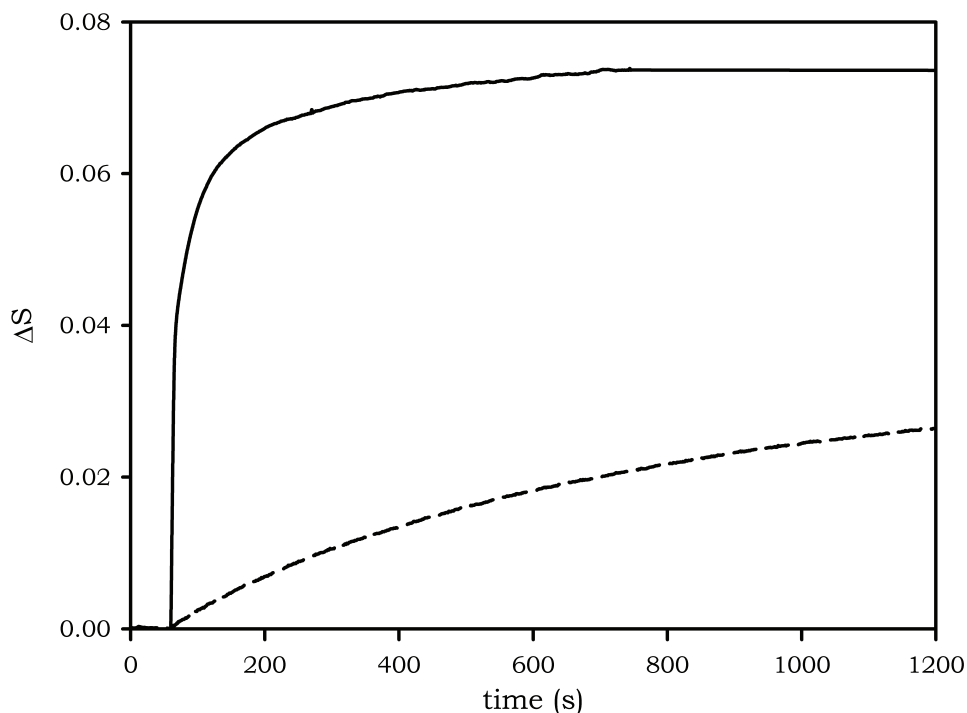


Figure 5.1: Adsorption of β -lg (—) and LMP (---) to hydrophobic surface, pH 4.25, 20 mM NaAcetate, I is set with NaCl to 10 mM. $[\beta\text{-lg}] = 10 \text{ mg/L}$ $[\text{LMP}] = 100 \text{ mg/L}$.

From a solution of LMP only very slow adsorption takes place. The negative charge of both the hydrophobic surface and the pectin slows the adsorption kinetics. Pectin is known to adsorb very poorly to hydrophobic surfaces and to have no emulsifying properties. It is therefore not unlikely that the actual slow adsorption observed is the result of contaminations in the pectin sample, as they are known to contain, for instance, a small amount of protein (Dickinson, 2003). Furthermore, the very slow adsorption kinetics for LMP, and the absence of an adsorption plateau, indicate that the adsorbing component is present at a low concentration. This indicates that the observed adsorption is the result of a contaminant. Since the adsorption is considered to be the result of a contaminant in the pectin sample and has not reached an adsorption plateau after 20 minutes, the surface charge after adsorption was not determined.

5.3.2 Adsorption of β -lg-pectin from mixed solutions

The adsorption on hydrophobic surface from a mixed solution of β -lg and pectin is shown in figure 5.2. For comparison, also the curve of β -lg from figure 5.1 is shown. It is difficult to obtain an adsorbed amount from these graphs, as this depends on the refractive index increment (dn/dc) of both β -lg and pectin. For adsorption from homo-solutions this is easy to do, since the composition of the adsorbed layer is known. With the adsorption from mixed solution both β -lg and pectin may adsorb, but it is unknown in what ratio. In literature, values for the dn/dc for pectin can be found ranging from 0.092 - 0.183 ml/g (Corredig, Kerr et al., 2000; Hunter & Wicker, 2005; Gulfi, Arrigoni et al., 2007). In this study, LMP, HMP_B and HMP_R were found to have nearly identical dn/dc of 0.123, 0.121 and 0.124 ml/g respectively. Possibly this is the result of the extensive washing of the pectins to remove salt ions complexed with the carboxyl groups of the galacturonic acid. Since the pectins all have the same dn/dc , it is possible to qualitatively compare the results for the different samples.

The adsorption from mixed solutions of β -lg and LMP shows a considerable reduction of the adsorption speed, compared to adsorption from a β -lg homo-solution, for both a WR of 2 and 6. Sperber et al (Sperber, Cohen Stuart et al., 2009) published binding isotherms of β -lg and pectin. The same pectins and conditions were used in this study. The

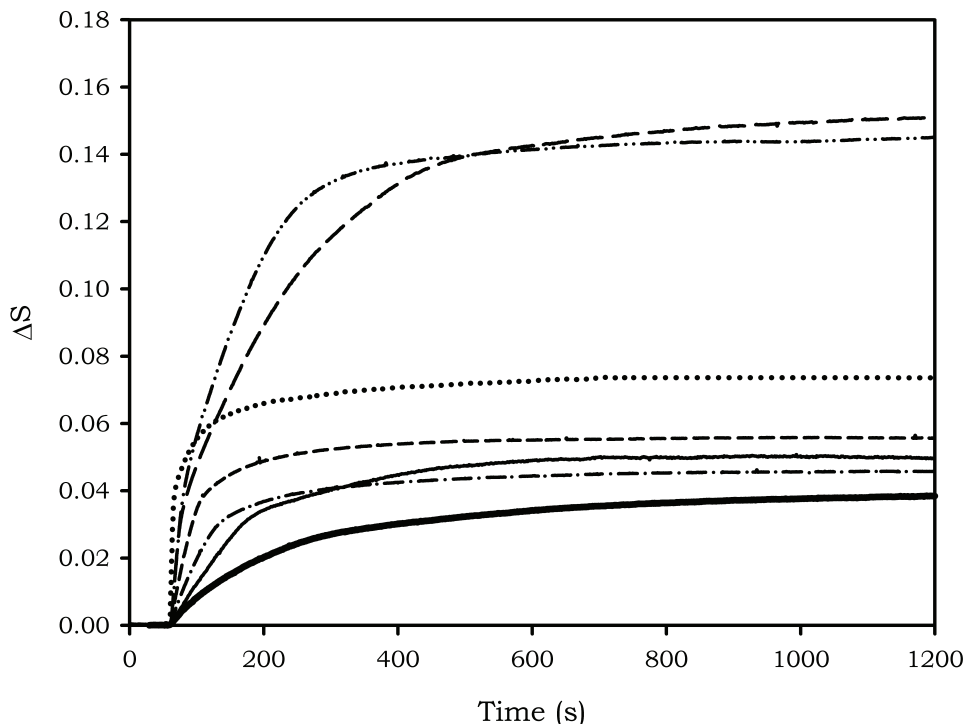


Figure 5.2: Adsorption of β -lg-pectin mixtures. β -lg-LMP, WR 2 (—), WR 6 (—); β -lg-HMP_B, WR 2 (— • —), WR 6 (— • • —); β -lg-HMP_R, WR 2 (— —), WR 6 (— —) and β -lg (• • •) to hydrophobic surface. pH is set to 4.25, using a 20 mM NaAcetate, the ionic strength is set with NaCl to 10 mM. $[\beta\text{-lg}] = 10 \text{ mg/L}$.

binding isotherm shows that for the WR 2 sample the pectin is far from saturation, and the affinity of β -lg for LMP is very high, causing virtually all β -lg to be bound to LMP. Only 0.15% of β -lg was found to be free in solution. For WR 6 saturation of LMP is reached and 27% of the β -lg is present as free β -lg. The result of this considerable lowering of $[\beta\text{-lg}]_{\text{free}}$ is a reduced rate of adsorption, as the adsorption rate is controlled by $[\beta\text{-lg}]_{\text{free}}$. This was also found for the adsorption to air-water interfaces from β -lg-LMP mixtures by Ganzevles et al (Ganzevles, Cohen Stuart et al., 2006), where the change in surface pressure due to the adsorption of β -lg from a β -lg-LMP mixture could be approximated by using a 10 times diluted β -lg homo-solution. The adsorbed amount from the mixture is lower than for adsorption from a β -lg solution alone. Since there is only very slow adsorption of β -lg on the surface, there is a lot of time for the adsorbed β -lg

molecules to rearrange on the surface, leading to spreading of the β -lg molecules on the surface.

The ζ -potential of the surface is -19.9mV for WR 2 and -9.7mV for WR 6. For WR 2, the binding sites on LMP are far from saturated, leaving negative charge on LMP uncompensated by positive charge of the β -lg, thus giving a negative ζ -potential. At WR 6 LMP is close to saturation with β -lg, but the overall charge of the complex is still negative (see also Ganzevles et al(Ganzevles, Zinoviadou et al., 2006)).

For HMP_B the WR 2 sample also shows a considerable reduction in the adsorption rate. From the binding isotherm it follows that 6.5% is present as β -lg_{free}, for WR 6 this is 52%. Although the WR 6 sample shows some reduction in adsorption rate, it is a relatively small reduction, as can be expected from a reduction of $[\beta\text{-lg}]_{\text{free}}$ by only a factor 2. More pronounced is the difference in the amount of adsorbed mass in the WR 6 sample. The signal is approximately two times as high as for adsorption of β -lg alone. This implies that besides a surface coverage of β -lg that is comparable to a surface coverage of β -lg alone, a considerable amount of complexes of β -lg and HMP_B adsorbs on top of the β -lg surface layer. A buffer rinse showed that only approximately 10% of the adsorbed material could be desorbed (results not shown), indicating that the complexes are tightly bound to the β -lg surface layer. The ζ -potential of the surface for WR 2 and 6 are -21.6mV and -13.9, respectively, which may be explained following the same reasoning as for LMP.

The adsorption from mixtures of β -lg-HMP_R is similar to that of mixtures of β -lg-HMP_B. For WR 2 13% of β -lg is not bound to HMP_R, 55% for WR 6. The higher $[\beta\text{-lg}]_{\text{free}}$ compared to HMP_B for WR 2 leads to an increased adsorption rate, compared to the adsorption from a solution of β -lg-HMP_B. The ζ -potential for WR 2 is -22.4mV, for WR 6 it is -10.8mV.

Figure 5.3 gives a model representation of the adsorption from a β -lg solution and from β -lg-pectin mixtures. The adsorption from a solution of β -lg is represented in figure 5.3a. The β -lg adsorbs quickly leaving little time for rearrangement of the protein on the surface to reach the optimal conformation on the surface. Figure 5.3b shows the adsorption from mixtures of β -lg and pectin at a WR of 2. Since the $[\beta\text{-lg}]_{\text{free}}$ is low, adsorption of β -lg is slow allowing the protein to spread on the surface.

The pectin adsorbing onto this β -lg layer is far from being saturated with β -lg, allowing the pectin to cover a larger surface area. Figure 5.3b also represent the adsorption from a solution of β -lg-LMP at WR 6, since $[\beta\text{-lg}]_{\text{free}}$ is also low under those conditions. The resulting pectin layer for LMP at WR 6 will contain more β -lg. Finally, figure 5.3c shows the adsorption from mixtures of β -lg and HMP_B or HMP_R at WR of 6. As the concentration of $[\beta\text{-lg}]_{\text{free}}$ is considerably higher, the adsorption of β -lg is much faster, allowing for limited relaxation on the surface. The pectin is fully saturated with β -lg and the majority of the pectin protrudes into the solution, causing a considerable increase in the adsorbed mass compared to adsorption from a solution of β -lg.

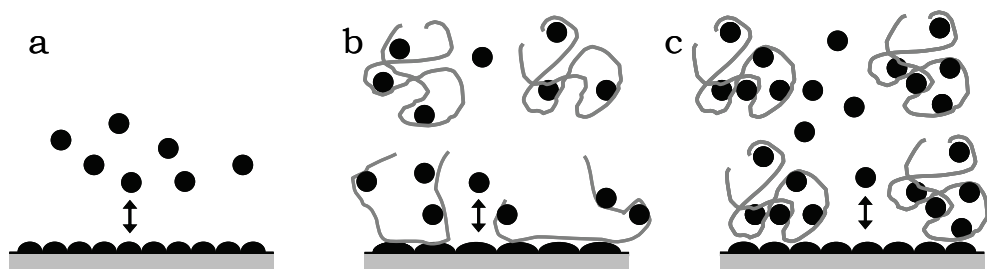


Figure 5.3: Model representation of adsorption on a hydrophobic surface from a solution of a) β -lg b) β -lg-pectin mixture at WR 2 for all pectin and WR 6 for LMP c) β -lg-pectin mixture at WR 6 for HMP_B and HMP_R.

5.3.3 Sequential adsorption of β -lg and pectin

Sequential adsorption of β -lg and pectin onto a hydrophobic surface can be used in the formation of secondary emulsions, which have an improved emulsion stability (Güzey & McClements, 2006), or for encapsulation purposes (De Kruif, Weinbreck et al., 2004). As pectin does not adsorb to a hydrophobic surface the first layer is made of β -lg. In total 4 layers of both β -lg and pectin are deposited, but build-up of more layers was found to be possible.

Figure 5.4 shows the sequential adsorption of β -lg and pectin to a hydrophobic surface. As expected, the first layer of β -lg is identical for all three pectins, as no pectin has been in contact with the surface. A short buffer rinse removes the β -lg solution before pectin is offered to the surface. The ζ -potential of the β -lg layer is +12mV, as found before. After

adsorption of pectin the ζ -potential of the adsorbed layer has become negative: -28mV for HMP_B and HMP_R, but only -17mV for LMP. The difference in ζ -potential of the LMP layer compared to the HMP_B and HMP_R layer is attributed to the orientation of the pectin layer. As LMP contains many more carboxyl groups, its orientation is much "flatter" on the surface than for the two HMP's that protrude farther into the solution. As the loops of two HMP's are not in contact with the β -lg covered surface, their negative charge is not compensated by the positive charge of the protein. As the ζ -potential is measured at the shear plane, more charge compensation by small counterions takes place in a thicker layer. The actual difference in ζ -potential between a terminal layer of one of the two HMP's and LMP may therefore actually be larger than was measured. The

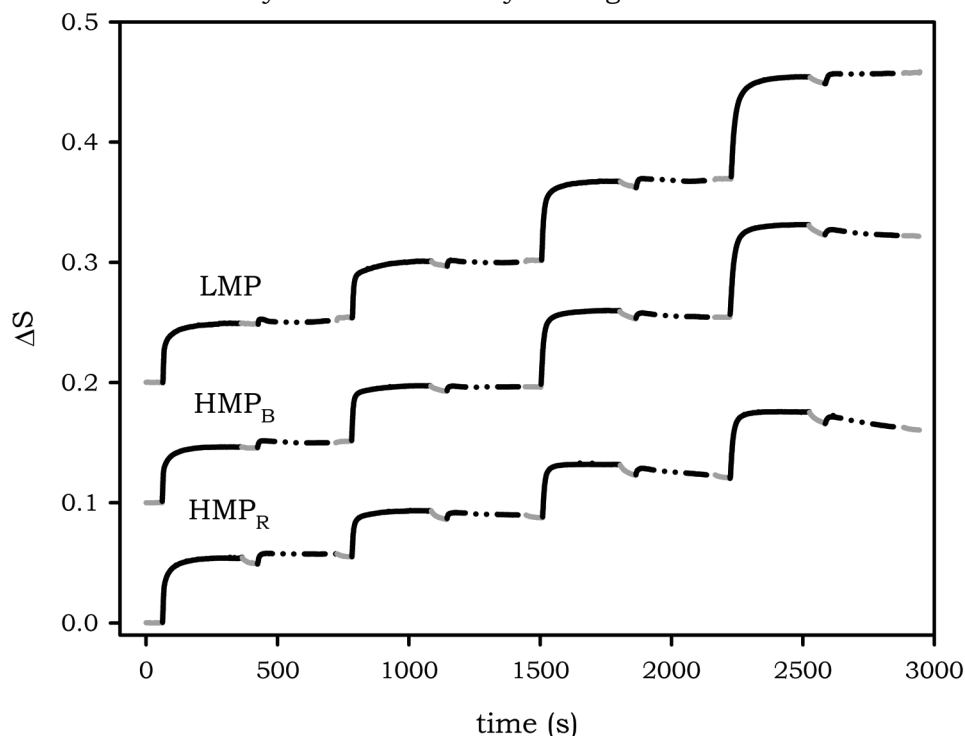


Figure 5.4 Sequential deposition on a hydrophobic surface of β -lg and LMP, HMP_B and HMP_R. [β -lg] = 10 mg/L, [pectin] = 100 mg/L, 20mM NaAcetate buffer pH 4.25, I is set with NaCl to 10 mM. Buffer (—), β -lg (—), pectin (— · · —)

difference in orientation between LMP and HMP was also suggested by Pereya et al for casein-pectin complex formation (Pereya, Schmidt et al.,

1997). The adsorption of pectin on the β -lg layer has only a small effect on ΔS , mainly because of the low dn/dc compared to β -lg, but pectin is adsorbing on the β -lg surface, as indicated by the change of the ζ -potential.

The following layers for LMP all show the same trend, β -lg adsorbs on the LMP layer, LMP on the β -lg layer. The ζ -potential of terminal β -lg layers was determined to be -2mV. This shows that no overcompensation of LMP charge takes place. The terminal LMP layers have a ζ -potential of about -21mV, somewhat lower than the first layer of LMP. As adsorbing LMP needs to share β -lg molecules in order to attach to the existing layers, the charge compensation of β -lg is probably a bit more efficient for the subsequent layers of LMP. From the increase in ΔS , it seems that more β -lg adsorbs in subsequent layers. At the same time, the buffer rinse and LMP adsorption, after the initial plateau, seem to lower ΔS a bit. Although the binding isotherm shows a high affinity of β -lg for pectin at an ionic strength of 10mM, there will still be some desorption when the surface is in a solution that is void of either β -lg, LMP or both. Therefore, when β -lg is subjected for adsorption, it may also replenish some of the β -lg that was washed out during the buffer rinse and LMP adsorption. When comparing the buffer rinse after β -lg and LMP adsorption, the loss of mass is much greater after adsorption of β -lg than after adsorption of LMP, which implies that desorption of β -lg from a saturated LMP layer is much easier than from an unsaturated one.

Sequential adsorption of β -lg and HMP_B shows a pattern similar to that of LMP. The increase in ΔS is comparable to LMP, although the desorption of β -lg seems to be somewhat higher. Since HMP_B has a higher DM than LMP, it should contain less binding sites for β -lg. Since approximately equal amounts of β -lg adsorb to a HMP_B layer as to a LMP layer this hints again to a different orientation of the pectin on the surface, where HMP_B protrudes more into the solution than LMP and thus more HMP_B can adsorb onto the β -lg layer. The desorption of mass after the initial adsorption of HMP_B is caused by the β -lg not being bound as tightly to HMP_B as to LMP (Sperber, Cohen Stuart et al., 2009), allowing it to move through the HMP_B layer easier than for LMP. The ζ -potential of the layers is -28mV for a HMP_B -terminal layer and -3mV for a β -lg terminal layer. So,

also for the β -lg-HMP_B system adsorption of β -lg does not lead to overcompensation of the pectin charge.

The sequential adsorption of β -lg and HMP_R shows a similar pattern to both LMP and HMP_B, although the desorption of β -lg, especially after the adsorption of a β -lg layer, is more pronounced than for the two other pectins. As HMP_R contains much less high affinity binding sites than LMP and HMP_B (Sperber, Cohen Stuart et al., 2009), the desorption of β -lg in a buffer solution or a solution of HMP_R is much easier as compared to LMP and HMP_B. For the ζ -potential a similar trend is observed: the HMP_R layers have a ζ -potential of -28mV, like HMP_B, but the ζ -potential after β -lg adsorption is -8mV. Where the charge of pectin was almost fully compensated for LMP and HMP_B, HMP_R cannot bind sufficient β -lg to compensate all the negative charge on the pectin.

5.4 Conclusions

The stabilization of oil-water or air-water interfaces requires a high rate of adsorption of the surface-active component. In β -lg-pectin systems the surface-active component is β -lg, as pectin has no surface-active characteristics. Pectin and β -lg are shown to form soluble complexes in the pH region roughly between pH 5.5 and 3.5, depending on pectin type and ionic strength. Within this pH range, it is possible to use the formation of complexes between β -lg and pectin to enhance the surface stabilizing capacity of β -lg. To this end, it is important to choose the right type of pectin. LMP, a pectin with a high local charge density and high overall charge is shown to form complexes at the highest ionic strength of the tested pectins. HMP_B, a pectin with a low overall charge, but high local charge density due to a block wise distribution of the methyl esters, also has a good tolerance to ionic strength. HMP_R, with a low overall charge and a low local charge density, has the poorest tolerance to ionic strength. This shows that the ability to form complexes between β -lg and pectin is mainly determined by the local charge density.

Adsorption of β -lg to a hydrophobic surface is quick, but when pectin is added and adsorption has to take place from mixtures of β -lg and pectin, the adsorption is slower. This is caused by a lowering of the

concentration of unbound β -lg, which is confirmed by binding isotherms (Sperber, Cohen Stuart et al., 2009). A reduced adsorption rate was also found by (Ganzevles, Zinoviadou et al., 2006) for adsorption to the air-water interface from mixed β -lg-pectin solutions. At WR of 6 (β -lg to pectin), $[\beta\text{-lg}]_{\text{free}}$ is relatively high and the adsorption is reasonably fast. The ζ -potential of these surfaces is only half compared to when a WR of 2 is used (-11mV vs. -20mV).

Sequential adsorption of β -lg and pectin leads to thick films in which adsorbed layers of β -lg and pectin are stacked in alternate fashion. When a pectin with a high local charge density is used (LMP or HMP_B), the adsorbed layer is much more stable against desorption of β -lg and pectin, than when a pectin with a low local charge density (HMP_R) is used. LMP and the two HM-pectins have a different conformation on the surface: The HM-pectin molecules are thought to protrude more into the solution, due to less binding sites on the pectin, where LMP is more "flat" on the surface.

The speed of adsorption and the surface characteristics make sequential adsorption the preferred technique over the adsorption from mixtures of β -lg and pectin to stabilize interfaces. Pectins with a high local charge density, or blockwise distribution of the methyl ester, have the best tolerance for ionic strength, while pectins with a low overall charge, or high DM protrude most into solution, giving the largest ζ -potential. This shows that to enhance emulsion stability, it would be best to prepare a primary emulsion with β -lg as emulsifier to which a HMP_B solution is added to create a secondary emulsion where the surface is stabilized with a layer of HMP_B on top of the β -lg surface.

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Chapter 6

General discussion and outlook

6.1 Aim

The aim of the research described in this thesis is to investigate the effect of the overall charge and local charge density of pectin on the formation of soluble complexes with proteins. The overall charge and local charge density of pectin have been found to be a determining factor in the ability of pectin to stabilize casein micelles in acid dairy drinks (Glahn & Rolin, 1996; Pereyra, Schmidt et al., 1997; Laurent & Boulenguer, 2003). In many food systems, small globular proteins are used to stabilize interfaces (oil–water, air–water). The functionality of these proteins is influenced by the presence of pectin by the formation of complexes. In the current study a small globular protein, β -lactoglobulin (β -lg), is used, to study the formation of soluble complexes between β -lg and pectin and the influence of these complexes on the functional properties of β -lg. β -Lg is chosen as it is the main constituent of whey protein and as such, it is often used as a model protein for the functionality of whey protein. The main parameters of interest for this thesis are the influence of the overall charge and local charge density of pectin on the formation of soluble complexes and the stability of those complexes at different pH and ionic strength.

First, the influence of pectin physicochemical characteristics on the soluble complex formation with proteins is discussed. Next, the possibilities to tailor pectin samples to give them the desired physicochemical characteristics are presented. Finally, applications of soluble complexes consisting of protein and pectin are discussed, followed by an outlook to possible new applications based on soluble complexes of protein and pectin.

6.2 Influence of pectin overall charge and local charge density on the complex formation with proteins

The work described in this thesis has been performed on a system consisting of β -lg and pectins with varying, but well defined, physicochemical characteristics. A generalized model for the influence of overall charge and local charge density of pectin on the formation of soluble complexes with β -lg is presented. This model is used to predict the

formation of soluble complexes between other food proteins and pectin based on the influence of the overall charge and local charge density of pectin on the formation of complexes with these proteins.

6.2.1 *Pectin physicochemical characteristics*

Three different pectins are used in this thesis: one low methyl esterified pectin (LMP) and two high methyl esterified pectins (HMP). These pectins are discussed in detail in chapter 3 (Sperber, Cohen Stuart et al., 2009b) of this thesis. The two HM-pectins were isolated from the same mother pectin based on their ability to form gels with calcium ions. The fraction that formed a gel was called HMP_B (B for blockwise distribution of methyl esters), the non-calcium sensitive fraction was called HMP_R (R for random distribution of methyl esters). The characteristics of the pectins are summarized in table 6.1.

Table 6.1 Characteristics of pectins used in this study

pectin	overall charge	local charge density	DM ¹	DB ¹
LMP	high	high	30	16.5
HMP _B	low	high	70	10.9
HMP _R	low	low	74	1.7

¹ DM = degree of methyl esterification, DB = degree of blockiness (from Daas, Boxma et al (2001))

6.2.2 *State boundaries for complexes between pectin and β -lactoglobulin*

The formation of soluble complexes of β -lg and pectin is studied with a variety of techniques. Figure 6.1 shows a generalized state diagram for complex formation between β -lg and pectin. pH_c, the pH where soluble complexes are formed, is dependent on the local charge density of pectin: a higher local charge density moves pH_c to higher pH. An increase in ionic strength results in a decrease of pH_c. pH_c is independent of the mixing ratio of β -lg and pectin. pH₀, the pH where soluble complexes are no longer stable and aggregate to form a separate phase, is found to be dependent on the overall charge and to some extent on the local charge density. For a high overall charge pectin the binding sites for β -lg on pectin are so close together that at low ionic strength lateral repulsion between β -lg

neighbours takes place. When the ionic strength is increased the lateral repulsion decreases and pH_ϕ shifts to higher pH. Further increase of the ionic strength leads to a decrease in pH_ϕ as the attractive electrostatic forces diminish as well. For the low overall charge pectins the binding sites for β -lg are sufficiently spaced to be considered isolated from each other and lateral repulsion between β -lg neighbours has no influence on the complex formation. Also, for both low overall charge pectins, an increase in ionic strength leads to a decrease in pH_ϕ .

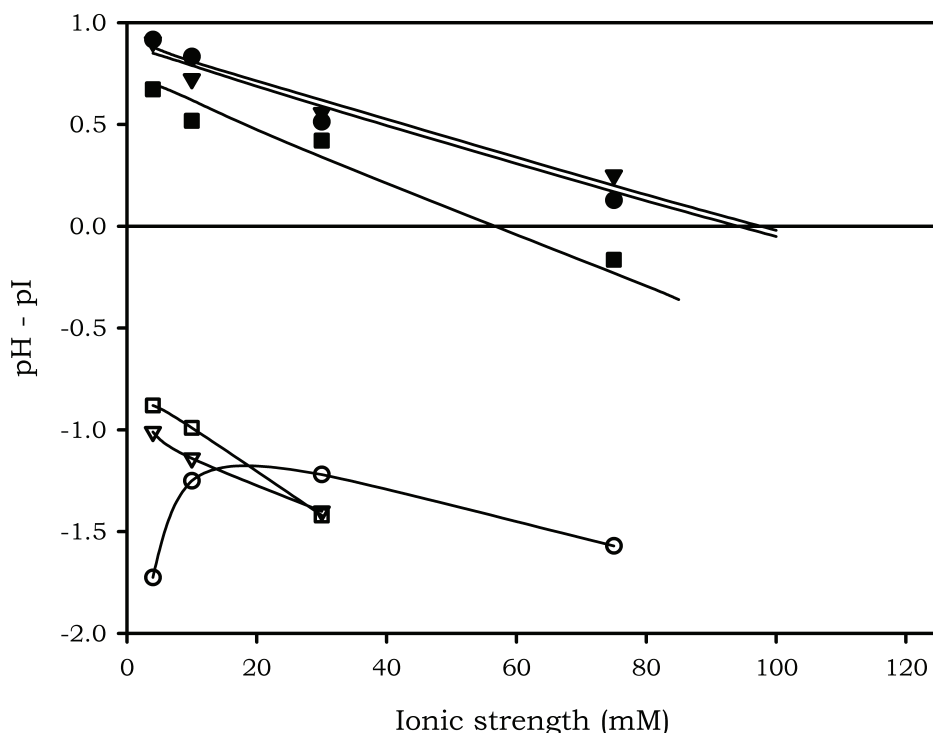


Figure 6.1 Generalized state diagram of complex formation between β -lg pectins with different overall charge and local charge density; LMP ($\bullet, \circ$), HMP_B ($\blacktriangledown, \triangledown$) and HMP_R ($\blacksquare, \square$). Closed symbols are for pH_c and open symbols are used for pH_ϕ .

6.2.3 Mechanism of β -lactoglobulin binding to pectin

In chapter 2 (Sperber, Schols et al., 2009) the local charge density of the binding sites on the three pectins is calculated using a theoretical model developed by De Vries, Weinbreck et al (2003). This model showed

the binding sites at the onset of complex formation to have a local charge density of about 1.9 e/nm for the two pectins with a high local charge density and 1.1 e/nm for the pectin with a low local charge density. The charge density of a block of GalA residues, with a GalA residue length of 0.43 nm (Rees & Wight, 1971), is estimated to be 1.9 e/nm , taking the fraction of deprotonated carboxylic groups (0.8) into account. This means that the optimum binding site for β -lg on pectin consists of a block of only non-methyl esterified GalA residues.

A more detailed study of the soluble complexes at pH 4.25, by means of binding isotherms (chapter 3, (Sperber, Cohen Stuart et al., 2009b)) and isothermal titration calorimetry (chapter 4), reveals that β -lg binds strongest to pectin with a high local charge density. For the high overall charge pectin, LMP, it is found that the binding constant passes through a maximum with increasing ionic strength, due to lateral repulsion between β -lg neighbours. It also showed that a high local charge density is required for binding β -lg at higher ionic strength ($>45\text{mM}$). In figure 6.2 the observations from chapter 2 and 3 (Sperber, Cohen Stuart et al., 2009b; Sperber, Schols et al., 2009) are combined to form a visualisation of influence of the pectin overall charge on the lateral repulsion between β -lg neighbours.

The binding of β -lg to pectin has a main driving force of enthalpic origin from electrostatic attraction between the β -lg and pectin ionic groups. When binding takes place on binding sites with a low local charge density, or at higher ionic strength for pectins with a high local charge density, there is an additional entropic driving force. The entropy gain is the result of the release of small counterions from the electrical double layer and the release of water molecules from hydrophobic moieties on both pectin and β -lg. The entropy gain due to the release of small counterions is also expected to be present for high local charge density pectins at low ionic strength, but was not experimentally proven.

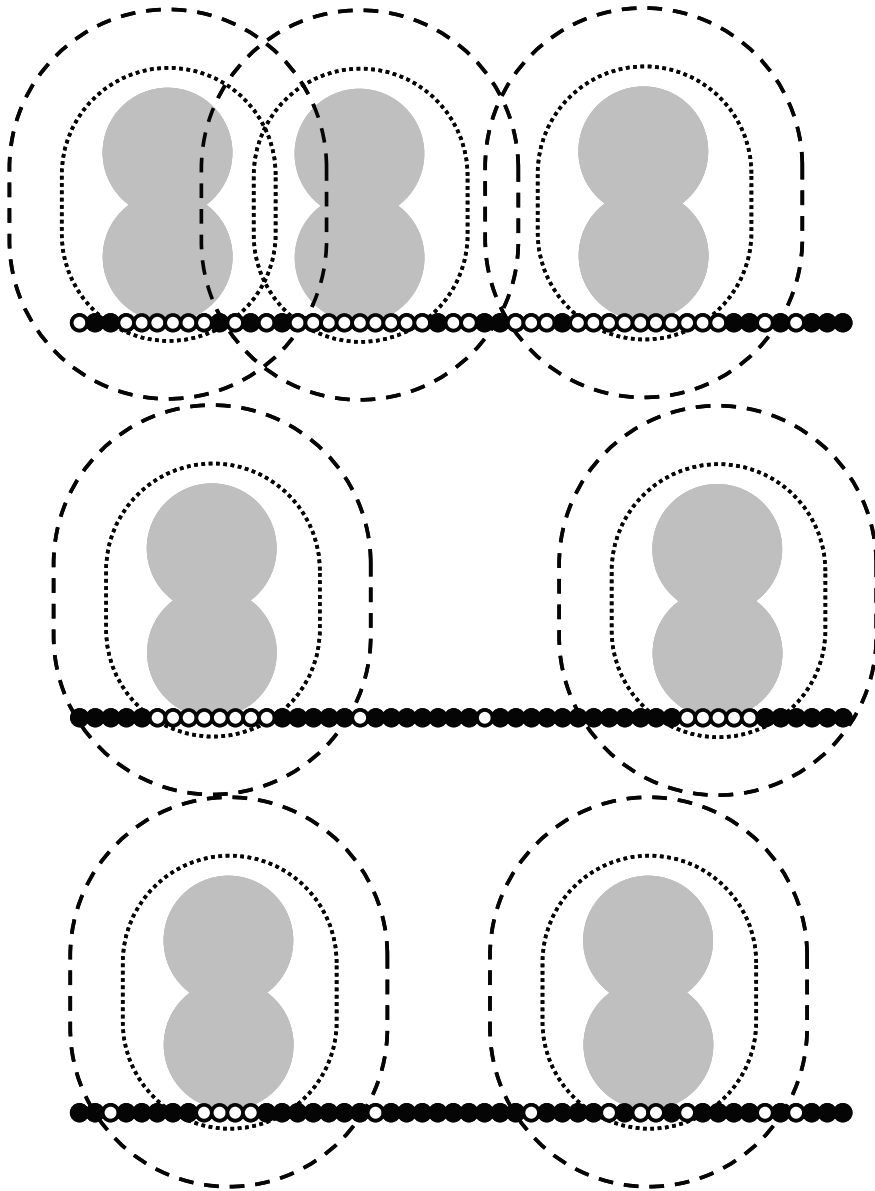


Figure 6.2 Model representation of the binding of β -lg to pectin. In gray the β -lg dimer. The dashed line around the β -lg dimer shows the double layer thickness for low ionic strength (10mM), the dotted line for a medium of high ionic strength (75mM). Pectin is represented as circles; open for non methyl esterified GalA and closed for methyl esterified GalA. On the top a LM-pectin is shown, in the middle a HM-pectin with a blockwise distribution of methyl esters and below a HM-pectin with a random distribution of methyl esters. The drawing is approximately to scale, but due to the charge anisotropy on the β -lg surface, the actual potential fields around the ionic groups will show a different profile.

6.2.4 Complexes between pectin and other small globular proteins

The main prerequisite for the formation of soluble complexes between small globular proteins and pectin is the existence of favourable electrostatic interactions between the two components. Electrostatic attraction between pectin and protein exists when the solution pH is below the pI of the protein. Table 6.2 holds data on food proteins, commonly used on food systems. These proteins could be combined with pectin to form soluble complexes, with the goal to enhance the functional properties, like emulsion or foam stabilization, of the protein by complexation with pectin. The proteins have, with the exception of lysozyme, pI's comparable to that of β -lg. This means that the state diagram presented in figure 6.1 remains relatively unchanged. There will however be variations in the charge anisotropy on the protein surface. This may result in pH_c to shift up for higher charge anisotropy and down for a more even distribution of charge on the protein surface, compared to β -lg. The position of pH_ϕ is defined by the neutralization of the pectin negative charge by the positive charge on the protein. It will most likely shift in the same direction as the pI of the protein compared to the pI of β -lg. The range of $\text{pH}_\phi - \text{pI}$ can change with the pI of the protein. As the pI of the protein becomes lower, so does the negative charge of pectin that needs to be compensated by the protein, shifting the value of $\text{pH}_\phi - \text{pI}$ closer to zero. For proteins with a higher pI than β -lg the non-monotonic behaviour of pH_ϕ for LMP may be increased as the amount of charge on the protein may be higher around pH_ϕ and lateral repulsion between proteins plays a bigger role.

Especially the plant derived proteins may be present in higher order aggregates. Often, they consist of different subunits with an acidic and basic polypeptide. If such a protein is used to form soluble complexes with pectin, it is expected that pectin will bind preferentially to the basic peptide.

There is a considerable variation in the molecular weight of the proteins, especially taking into account that several proteins combine to form higher order aggregates. The persistence length of the pectin chain is in the order of a β -lg monomer, one of the smallest proteins in table 6.2.

This implies that, combined with the fact that charge regulation takes place in the complexes (see chapter 2, (Sperber, Schols et al., 2009)), pectin can adjust its conformation to move away from unfavourable area's. The effect of the size of the protein on the formation of complexes with pectin can be considerable, depending on the type of pectin used. LMP is known to be a poor stabilizer of casein micelles as it covers the surface of the micelle and doesn't protrude into the solution. For HM-pectins tails and loops are created that have a stabilizing effect.

Table 6.2 Overview of physical properties for proteins used in food applications

protein	origin	pI	M _w (kDa)	reference
β-lactoglobulin	bovine milk	5.1	18	(Cayot & Lorient, 1997)
BSA	bovine milk	4.7-4.9	66	(Cayot & Lorient, 1997)
α-lactalbumin	bovine milk	4.5-4.8	14	(Cayot & Lorient, 1997)
caseinate ¹	bovine milk	4.9-5.6	19-25	(Modler, 2000)
ovalbumin	hen egg white	4.5	45	(Mine, 1995)
lysozyme	hen egg white	10.8	14	(Doi & Kitabatake, 1997)
vicilin ²	pea	~5.5	30-70	(Gatehouse, Croy et al., 1981; Sanchez-Monge, Lopez-Torrejón et al., 2004)
legumin ²	pea	4.7	60	(Gatehouse, Croy et al., 1980)
glycinin ²	soy	5.0-6.0	55-65	(Rickert, Johnson et al., 2004; Kuipers, 2007)
β-conglycinin ²	soy	5.0-6.0	50-70	(Sykes & Gayler, 1981; Kuipers, 2007)

¹ Caseinate is present as a higher order aggregation protein

² Plant storage proteins are usually present as higher order aggregates of several subunits

The tolerance of the soluble complexes against ionic strength could be influenced by the protein pI. When the protein pI is higher than the pI of β-lg, the soluble complex region will be at higher pH than for β-lg. At higher pH, the pectic GalA residues have a higher fraction of deprotonation. This leads to a higher overall charge, and subsequently a higher local charge density on the pectin. To compensate this negative charge of the pectin, more positive charge of the protein is needed and although charge regulation in the complexes will take place, the higher charge of the

protein and higher local charge density of the pectin are expected to improve the tolerance towards ionic strength of the system.

6.2.5 Complexes between pectin and casein micelles

For the complex formation of pectin with casein micelles, it is known that the type of pectin is of great influence on the stabilization of the casein micelles in an acidified milk drink. HM-pectins are preferred over LM-pectins. Among the HM-pectins, a pectin with a blockwise distribution of methyl esters provides the best stabilization (Glahn & Rolin, 1996; Pereyra, Schmidt et al., 1997; Laurent & Boulenguer, 2003).

In chapter 5 (Sperber, Cohen Stuart et al., 2009a), the adsorption of β -lg to a hydrophobic solid surface is described. Subsequently, pectin is adsorbed on top of the β -lg layer. This can be seen as a model system for the adsorption of pectin on the surface of the positively charged casein micelles under the conditions of an acidified milk drink (around pH 4.0-4.5). It is shown that both HMP_B and HMP_R pectins adsorb in a different manner than LMP. The HM-pectins protrude more into the solution, resulting in a more negative ζ -potential than for LMP. The ionic strength of milk is considerably higher than the 10mM used in these experiments. Consequently, HMP_R will not be able to adsorb onto the casein micelle at these higher ionic strengths and, therefore, stabilization on the basis of combined electrostatic-steric hindrance is unlikely for a HMP_R.

It was shown, that HMP_B is a heterogeneous sample (chapter 3, (Sperber, Cohen Stuart et al., 2009b)), with parts having a similar local charge density as LMP, while other parts have a lower charge density. This explains the observation of Boulenguer and Laurent (2003) that not all of the added pectin adsorbs on the surface of the casein micelle.

6.3 Influence of pectin bound calcium on the soluble complex formation with β -lactoglobulin

The pectins used in this study were treated to remove residual calcium. In this section the motivation to remove this residual calcium from the pectins will be outlined.

Calcium is used for the isolation of pectin, but it is also present in the plant material pectins are isolated from. The pectins used in this thesis

were tested on their calcium content by flame photometry. The pectins were found to have a calcium content of up to 0.05 mol/mol GalA. As discussed above, and in more detail in chapter 4 of this thesis, the release of small counterions from the GalA electrical double layer is one of the driving forces for the formation of complexes between protein and pectin. In addition, calcium can also bind very specifically on the pectin GalA units to form junction zones, involved in the formation of pectin gels (Voragen, Pilnik et al., 1995). To investigate if pectin bound calcium has an influence on the formation of complexes between β -lg and pectin, calcium free pectins were prepared, to which known amounts of calcium were added.

For LMP and HMP_B washing the pectin powder with an acidified ethanol solution was found to be sufficient, while, surprisingly, for HMP_R the pectin needed to be dissolved in water and mixed with an ion exchange resin (Sperber, Schols et al., 2009). It is not expected that HMP_R binds the calcium more strongly than LMP or HMP_B. When acidified ethanol is used to remove calcium, the pectins are still in a solid form. For reasons not fully understood, the structure of the HMP_R powder doesn't allow for the exchange of calcium in the acidified ethanol slurry.

Figure 6.3 shows the binding isotherm of calcium to the three calcium free pectins. The maximum amount of bound calcium per mole of GalA units is highest for LMP, followed by HMP_B and HMP_R binds the least calcium. The affinity is highest for LMP, again followed by HMP_B and lastly HMP_R. This is, as expected, according to the degree of blockiness of the pectins.

To study the influence of calcium on the formation of complexes between pectin and β -lg, pectins with a calcium content of 0.3 mol/mol GalA were prepared. Next, β -lg was added to a weight ratio of β -lg/pectin of 10 and the free calcium concentration was measured again (β -lg was verified to be calcium free). Table 6.3 shows the results for LMP and HMP_B, as HMP_R did not bind sufficient calcium for an accurate measurement of the change in free calcium concentration before and after addition of β -lg. LMP binds a higher fraction of calcium than HMP_B at pH 4.25, showing that there are more calcium sensitive regions on LMP than on HMP_B. The addition of β -lg releases most of the calcium bound to LMP, but only a fraction of the calcium bound to HMP_B. This directly shows that small

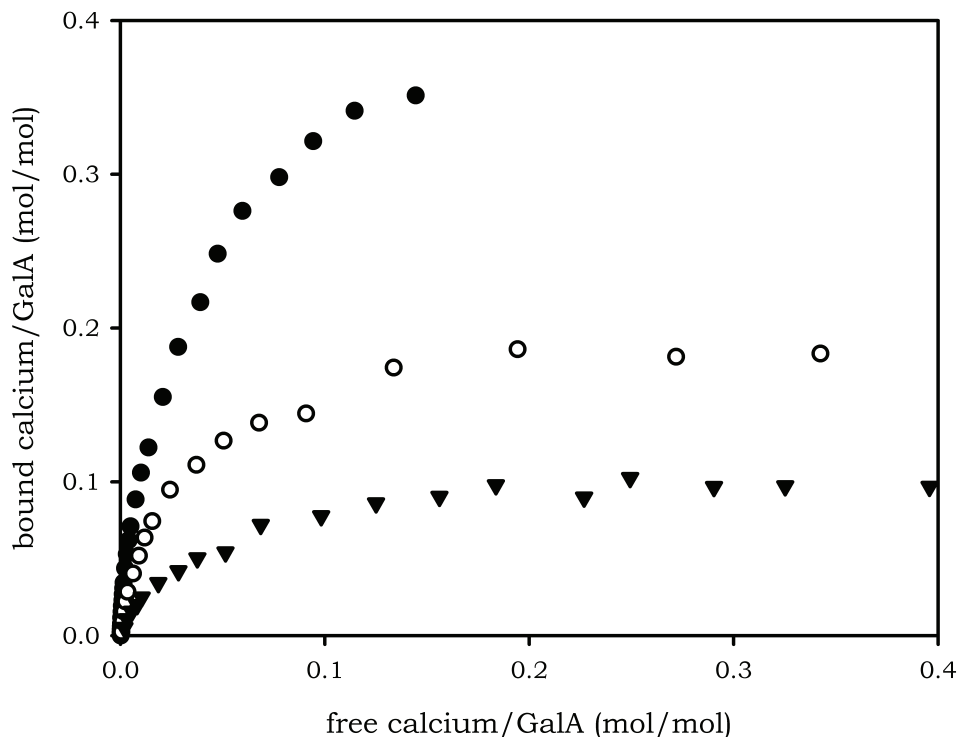


Figure 6.3: Binding isotherm of calcium to calcium free LMP (●), HMP_B (○) and HMP_R (▼) at pH 5.7

counterions are released from the electrical double layer by the formation of complexes between pectin and β -lg, also when they are more specifically bound than monovalent ions like sodium. It also shows that the majority of the calcium on LMP is bound in regions of large blocks of GaA where β -lg preferably binds as well, while HMP_B binds most of the calcium in regions that are not favoured for the binding of β -lg. This confirms that β -lg prefers to bind in regions of high local charge density.

Table 6.3 Release of calcium from pectin by the binding of β -lactoglobulin at pH 4.25

Pectin	Ca ²⁺ added to solution (mol Ca ²⁺ / mol GaA)	% of added Ca ²⁺ bound to pectin	% of bound Ca ²⁺ released by addition of β -lg
LMP	0.30	61	70
HMP _B	0.30	20	10

As mentioned before, the release of small counterions from the electrical double layer of pectin and protein ionic groups is one of the driving forces behind the complex formation. To be in full control of the solution conditions and to be able to compare results between pectins, it was decided to use calcium free pectins in this study.

6.4 Possibilities of tailoring pectins to desired characteristics

The characteristics of pectin being beneficial to form soluble complexes with proteins have been described in section 6.2. To obtain pectins with the desired characteristics for a specific application, several strategies to modify the overall charge (DM) and local charge density (DB) can be followed. Pectins can be chemically or enzymatically de-esterified. A pectin preparation can also be fractionated as a function of the local charge density. Fractionation with calcium can separate a pectin sample in a calcium sensitive and non-calcium sensitive fraction. Anion exchange chromatography can provide a more gradual fractionation.

6.4.1 Chemical de-esterification

Chemical modification of pectin can be performed during the extraction of pectin from the plant material by controlling the extraction conditions (pH, temperature and time). By choosing the proper conditions a DM between 55 and 80 can be obtained (Rolin, 2002). If a DM below 55 is desired, an additional treatment is needed to remove more methyl groups. The possibilities for this are acid hydrolysis or saponification by a base. For saponification, a strong alkali can be used (e.g. NaOH), but temperature should be kept low to avoid β -elimination, especially with high DM-pectins (Rolin, 2002). Chemical removal of methyl esters occurs in a random fashion (De Vries, Hansen et al., 1986). Thus the increase of the degree of blockiness is marginal until a low DM is reached (see figure 6.4). The advantage of a chemical modification is that it acts on all the different populations in the pectin sample. The chemical modification therefore does not create additional populations with a much lower DM than the sample average, which can be beneficial if the pectin is to be used in a calcium rich food, where a calcium induced gel is not desired. Chemical de-

esterification can also be used to increase the overall charge of a high DM pectin with a high DB.

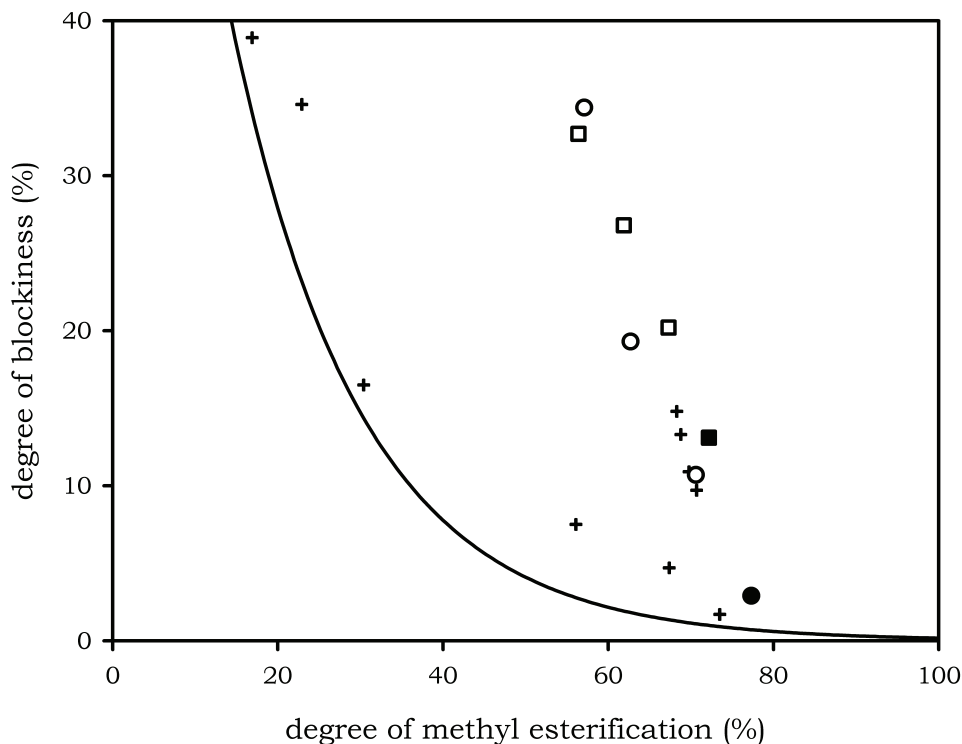


Figure 6.4: Dependency of degree of blockiness on the degree of methyl esterification for several commercial pectins (+) (Daas, Boxma et al., 2001). A commercial DM 77 pectin (●) and a DM 72 pectin (■) were de-esterified with a pPME (○, □). The solid black line represents the relation between DM and DB for randomly de-esterified pectins by means of chemical saponification.

6.4.2 Enzymatic de-esterification

The degree of methyl esterification of pectin can be lowered enzymatically using a pectin methyl esterase (PME). These enzymes hydrolyse the methyl ester bond to release methanol and a GalA residue with a free carboxylic group. There are two types of PME's available: PME's that hydrolyze methyl esters in a processive manner and PME's that hydrolyze methyl esters in a random manner. PME's with a processive mode of action are found in plants (pPME) and PME's with a random mode of action are found in fungi (fPME) (Willats, Orfila et al., 2001; Benen, Van

Alebeek et al., 2003). As a pectin with a blockwise distribution of methyl esters has been shown to form soluble complexes with protein at higher ionic strength than compared to a random distribution, the use of pPME will be discussed.

Two approaches of pectin modification with pPME may be chosen: using the native PME's (mainly for citrus, apple is low in native PME) present in the plant material from which the pectin is isolated (Owens & MacLay, 1948; May, 1990), or adding a pPME once the pectin is isolated (Luzio, Forman et al., 2001). Figure 6.4 shows the DM and DB of commercially available lemon pectins. There is already considerable difference between the samples with respect to the DB at comparable DM. Since no pPME's are added, this is the result of the native pPME's of the citrus fruits.

Adding pPME to an isolated pectin will lower the DM and increase the DB (Daas, Boxma et al., 2001). As can be seen in figure 6.4, the addition of pPME to a HM-pectin increases DB considerably with only a small decrease in DM. This is caused by the mode of action of the pPME, hydrolyzing a series of sequential methyl esterified GalA units on a single pectin molecule, before dissociating and acting again on a new pectin molecule. This results in the lowering of the DM of some pectin molecules, while some other pectin molecules in the preparation are unaltered (Tucker & Seymour, 2002). There is some control that can be exerted on the size of the blocks that are produced by a pPME. The easiest way to achieve this is by increasing the dosage of pPME, which will cause more pectin chains to be altered. When the reaction is stopped at the same DM as for a lower dosage of pPME, there should be more blocks of a shorter average size. It has been shown by Denès, Baron et al (2000) that changing the solution pH affects the mode of action of the pPME. At pH 4.5 short blocks were formed on all pectin molecules, while at pH 7.0 large blocks on only a fraction of the pectin molecules were created.

The use of pPME can be tricky. When using the endogenous pPME of the plant material, it is difficult to control the extent of their action as the starting DM and DB are not known and the reaction can not be monitored. If the pPME is added after down stream processing of isolated pectin, the control of the de-esterification process is much easier as it can be

monitored by the amount of base that is needed to keep the solution pH constant. The use of pPME can create populations differing in DM and DB in the pectin sample, but depending on the application this is not necessarily a disadvantage.

6.4.3 Fractionation of pectin on local charge density

Pectin preparations consist of complex, heterogeneous molecules and many different molecules with different DM and DB may be present (Guillotin, Bakx et al., 2005). Pectins with more homogenous characteristics can be obtained by fractionation. The easiest method for fractionation is based on the calcium sensitivity of pectin. By adding calcium to a solution of pectin, the calcium sensitive pectin, with a more blockwise distribution, precipitates, while the non calcium sensitive pectin stays in solution. The calcium can be subsequently washed out (Glahn, 2000). It has to be taken into account that especially the calcium sensitive fraction is still very heterogeneous in the distribution of methyl esters (Daas, Boxma et al., 2001; Williams, Foster et al., 2003), as was also shown for the two HM-pectins used in this study by weak anion exchange chromatography, where HMP_B eluted as a broad band, indicating the presence of many different local charge densities (chapter 3, (Sperber, Cohen Stuart et al., 2009b)). Fractionation of pectins by calcium sensitivity could be used in combination with pPME de-esterification, to separate the modified pectin from the original, unmodified pectin, as the modified pectin will have blocks of GalA residues that can form junction zones under the influence of calcium ions.

Ion exchange chromatography can also be used for the fractionation of pectins. This technique has been used successfully on analytical and laboratory preparative scale to separate fractions of different DM and DB (Ralet, Bonnin et al., 2001; Guillotin, Bakx et al., 2005). However, ion exchange chromatography is an expensive technique to perform on industrial scale. To use it for fractionation of pectins requires a higher value application than the stabilization of foods.

6.4.4 Amidation of pectin

When pectins are chemically de-esterified, it is also possible to use ammonia as base. Part of the methyl esters are replaced by amide groups in this process (Rolin, 2002). The use of amidated pectins would be interesting in the formation of soluble complexes with proteins as the polar amide group interacts differently than the hydrophobic methyl groups. The amide groups are able to interact with the protein by forming hydrogen bridges, where the methyl esters have a hydrophobic interaction.

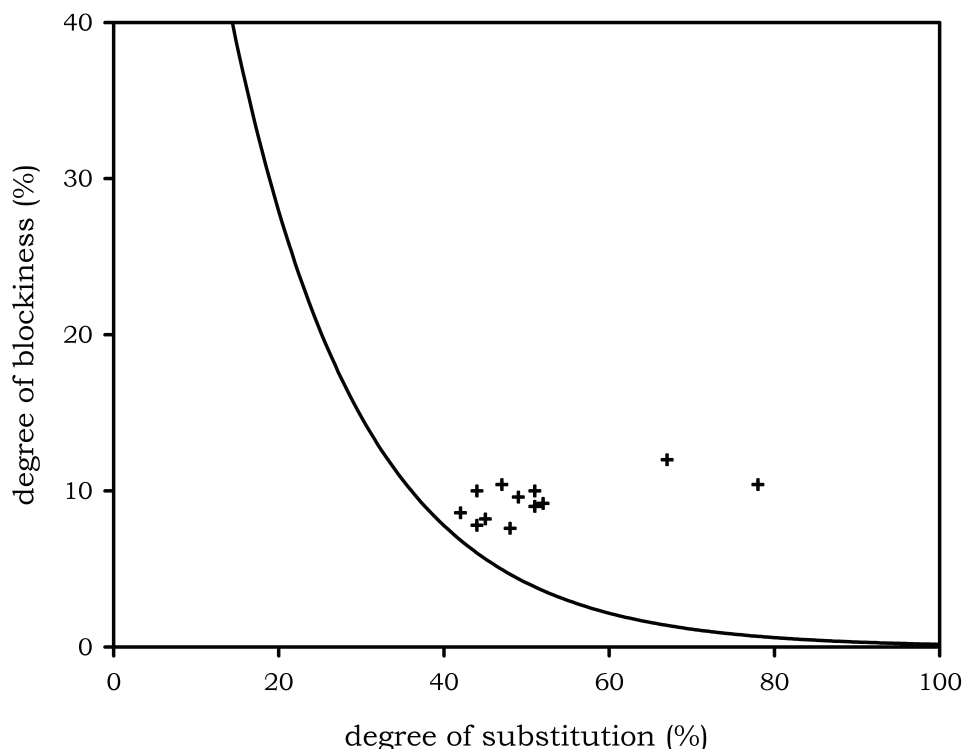


Figure 6.5 Dependency of degree of blockiness on degree of methyl esterification for several commercial, amidated pectins (+) (Guillotin, Van Kampen et al., 2006). The solid line represents the relation between DS and DB for a random distribution of substituents.

There are however limitations to the use of amidated pectins: the fraction of GalA that can be amidated for food use is legally restricted to 25% (Rolin & De Vries, 1990); higher degree of amidation is possible, but results in poor solubility (Reitsma, Thibault et al., 1986). Amidation of

pectin leads to demethylation of pectin and the degree of substitution (both amide groups and methyl esters) of the amidated pectin is usually 50 or lower (Guillotin, Van Loey et al., 2007). The blockiness of these pectins is not much higher than that of random distributed pectins (figure 6.5), as can be expected for a chemical, non-selective process (Guillotin, Van Kampen et al., 2006). Due to the low degree of substitution and the legal restriction on the degree of amidation of amidated pectins, the behaviour is expected to be dominated by the electrostatic interaction between the non-substituted GalA residues and the protein ionic groups. Also, amidated pectins are reported to lower the stability of caseinate by forming inter-pectin hydrogen bridges (Einhorn-Stoll, Salazar et al., 2001), which makes amidation an undesired modification to enhance the functionality of proteins in foods.

6.5 Implications for protein-pectin soluble complex applications

Protein-pectin soluble complexes find their application in the stabilization of acidified milk drinks, foams, emulsions and the encapsulation of ingredients. The choice of the right pectin for an application will be of great influence for the success of the application. To make the correct choice of pectin the results of this thesis can be very helpful.

6.5.1 Acidified milk drinks

The stabilization of acidified milk drinks has already been mentioned shortly in section 6.2.5. Stabilization of AMDs is needed to prevent the casein micelles to aggregate and subsequent whey separation (Tromp, De Kruif et al., 2004). To stabilize AMD's it is important that pectin adsorbs onto the casein micelle. To do so, a high local charge density of the pectin is needed. Several authors have shown that not all of the pectin that is added to a AMD adsorbs onto the casein micelle, and that the free pectin contributes to the stabilization by forming a weak network (Boulenguer & Laurent, 2003; Tromp, De Kruif et al., 2004; Jensen, Rolin et al., 2010). The stabilization of AMD also depends on the ionic strength of the solution as dilution with water destabilizes the AMD (Jensen, Rolin et al., 2010).

This can be directly related to the work in this thesis, where pectins are shown to be heterogeneous (chapter 3, (Sperber, Cohen Stuart et al., 2009b)) and binding of pectin to protein is highly depending on the block size of GalA units and the ionic strength (chapter 2, 3 and 4, (Sperber, Cohen Stuart et al., 2009b; Sperber, Schols et al., 2009)). A too low ionic strength will allow the non-adsorbing pectin to adsorb on the casein micelle as well.

Modification of pectin with a pPME, to create larger blocks of GalA, may provide an improved stabilization of AMDs. The modified pectin molecules with a high local charge density will bind strongly to the casein micelle, the unmodified pectins remain in the serum phase, where it contributes to the stabilization of the AMD through its viscosifying properties.

6.5.2 *Foam stability*

The effect of protein-polysaccharide complexes on foam formation has been studied in detail in the thesis of Ganzevles (2007), where also a table with strategies on how to use protein-polysaccharide complex formation to enhance their functionality is presented. To stabilize foam, there needs to be a free fraction of protein that can adsorb to the air water interface when it is formed. When the surface is stabilized, protein-pectin complexes aid in the stabilization by electrostatic repulsion between the surfaces of the air bubbles. Unbound pectin in the Plateau borders reduces drainage of the foam by an increase in viscosity (Mann & Malik, 1996; Schmidt, Novales et al., 2010). To enhance foam stability it is therefore important to choose a pectin that consists of a mixed population: one fraction that can form complexes with the protein, and another fraction that remains in solution to increase the viscosity in the Plateau borders. Modification of random high methyl esterified pectin with a pPME could yield such a pectin.

6.5.3 *Emulsion stability and encapsulation*

Complex formation of protein with anionic polysaccharides have been shown to enhance emulsion stability (Güzey, Kim et al., 2004; Güzey & McClements, 2007; Jourdain, Leser et al., 2008; Jourdain, Schmitt et al.,

2009). Emulsions can be created by means of a bilayer emulsion or a mixed emulsion. For a bilayer emulsion, first an emulsion is prepared using protein as emulsifier, subsequently, the anionic polysaccharide is added to create the bilayer of protein and polysaccharide. A mixed emulsion is prepared by using a mixture of protein and polysaccharide as emulsifier (Jourdain, Leser et al., 2008).

Since the enhancement of emulsion stability depends on the formation of a complex between protein and pectin at the interface, the use of pectin with a high local charge density would be preferred. As stabilization, like for AMD, also depends on the pectin protruding into the solution, the use of a HM-pectin is required. For the quick stabilization of the oil–water interface, it is important to have free protein in solution for using the mixed emulsion protocol. If the bilayer protocol is used it is also an option to create the emulsion with the solution pH above pH_c and subsequently lower the pH, to adsorb the pectin on the protein layer.

Güzey and McClements (2007) made emulsions using β -lg and pectin (citrus, DM 60). The emulsion was created by making an emulsion at pH 7, that is above pH_c . After the emulsion was created, the pH was lowered to 4, leading to the adsorption of pectin on the β -lg covered emulsion droplets. At pH 4, the emulsion with β -lg and pectin showed a higher resistance against salt induced flocculation than an emulsion with only β -lg. Possibly, the stabilisation of the emulsion can be further enhanced by using pectin with a higher local charge density, to improve the stabilizing capabilities of the β -lg–pectin complexes at higher ionic strengths (>50 mM). Pectin with a lower overall charge, but similar local charge density as currently used, could enhance the stabilization, as a larger proportion of the pectin molecule will not bind to the β -lg surface. This results in an increase in the thickness of the adsorbed pectin layer, as well as the ζ -potential of the layer due to less charge compensation by β -lg. Such pectins could be prepared by using a pPME, followed by fractionation on the charge density by means of calcium sensitivity or anion exchange chromatography to isolate the pectins with a high local charge density.

For the encapsulation of ingredients, which often occurs in an oil phase, similar strategies can be followed as outlined here for emulsions.

6.6 Outlook for future research on protein-pectin complex formation

The results presented in this thesis increase our understanding of the formation of complexes between pectin and proteins, with special emphasis on the pectin overall charge and local charge density. The local charge density of the pectin was shown to be a key parameter in the formation of soluble complexes. To get a more detailed view on the influence of the local charge density, but also of the overall charge of pectin, the work presented in this thesis should be expanded using more pectins, characterized to the same detail. Combination with fractionation of pectins by means of preparative anion exchange chromatography, would allow the use of pectins with a much more narrow distribution of local charge density, providing more detailed information on the influence of the local charge density.

Adsorbing protein to a solid matrix, like small polystyrene beads with a high specific surface area, and subsequently adsorbing pectin on top of the protein layer, would form a great basis to investigate the dependency of the formation of soluble complexes on ionic strength. The non-adsorbing fraction of pectin can easily be separated from the adsorbed fraction by centrifugation and as the ionic strength is stepwise increased, a fractionation of pectin based on the affinity for the charged protein surface can be obtained. The desorbed fractions of pectin can be analyzed on DM, DB, and weak anion exchange chromatography to give an insight in the requirements for pectin to form complexes with protein at specific ionic strengths.

Another important step would be the use of these well characterized pectins in actual applications like emulsions and foams. Currently, research published on the use of pectin in relation to its complex formation with proteins usually does not characterize the pectins beyond their DM, where also the DB of the pectins is shown to be a very important parameter for its functionality. Ganzevles (2007) used well defined pectins in her studies on protein-polysaccharide complexes at the air-water interface, but no actual foams to test foam stability were produced, based on her findings using model systems.

Fluorescence anisotropy (FA) was shown to be an excellent technique to measure binding constants for soluble complexes below the pI of the protein. The advantages of FA over frontal analysis continuous capillary electrophoresis, which is currently used most frequently, are the ability to also measure around and below the protein pI and its high sample throughput. FA is therefore the method of choice to determine binding isotherms, not only for protein–pectin soluble complexes, but for protein–polyelectrolyte soluble complexes in general.

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Summary

Summary

The aim of this thesis is to investigate the influence of the overall charge and local charge density of pectin on the formation of soluble complexes with β -lactoglobulin (β -lg). Pectin is a naturally occurring polysaccharide, used in many food applications, among which the stabilization of acidified milk drinks. After an industrial acid extraction, pectin mainly consists of galacturonic acid (GalA) residues. These GalA residues can be methyl esterified at the carboxylic group on C-6. The amount of methyl esters determines the degree of methyl esterification (DM) and, hence, the overall charge of the pectin. The distribution of the methyl esters defines the degree of blockiness (DB), related to the local charge density of the pectin.

To investigate the influence of overall charge and local charge density of pectin on the soluble complex formation, the three pectins used in this study were characterized in the greatest detail. Chapter 3 combines results from other studies on the same pectins with new analysis to give a detailed structural analysis of the pectins. In short, LMP has a high overall charge and high local charge density with the blocks of non-methyl esterified GalA close together. HMP_B (B for blockwise distribution) has a low overall charge and high local charge density and isolated blocks of non-methyl esterified GalA. Finally, HMP_R (R for random distribution) has a low overall charge and a low local charge density. The few blocks of non-methyl esterified GalA units on HMP_R are also of an isolated nature.

The complexing regions of β -lg and pectin, as function of pH, ionic strength and weight ratio (WR, $[\beta\text{-lg}]/[\text{pectin}]$), are identified in chapter 2 by means of dynamic light scattering (DLS) titrations. From the DLS data, pH_c (state boundary between co-soluble polymers and soluble complexes) and pH_ϕ (state boundary between soluble complexes and insoluble complexes) are identified and state diagrams are constructed. pH_c is found to decrease with increasing ionic strength and to be independent of WR, which is consistent with literature. pH_ϕ increases for increasing WR. For HMP_B and HMP_R, pH_ϕ is found to decrease with increasing ionic strength. For LMP pH_ϕ passes a maximum with increasing ionic strength, due to lateral repulsion between β -lg neighbours as the result of the closeness of

the binding sites. From the state diagrams, pH 4.25 is chosen to be used in further studies of soluble complex formation as throughout a large range of ionic strengths and WR only soluble complexes are formed. The pH_c values are used to estimate the local charge density for the binding sites on the pectins at the onset of complex formation. LMP and HMP_B are found to have similar local charge densities, while HMP_R is found to have a lower local charge density. Proton titrations of β -lg-pectin mixtures show that charge regulation takes place to allow the β -lg and pectin ionic groups to better match each other.

In chapter 3 fluorescence anisotropy is introduced as a technique to measure binding isotherms of proteins to charged polysaccharides. The binding isotherms are fitted with the Hill model to obtain a binding constant and cooperativity parameter. The complex formation shows a strong dependency on local charge density. A high local charge density allows complex formation between β -lg and pectin up to higher ionic strength. LMP shows a maximum in the binding constant at an ionic strength of 10 mM, due to lateral repulsion of β -lg neighbours. The binding of β -lg to pectin is found to be cooperative, which is thought to originate from a rearrangement of the pectin helix structure upon binding of β -lg.

The mechanism of binding is investigated by means of isothermal titration calorimetry (ITC) in chapter 4. Using the binding constants obtained in chapter 3, a Gibbs free energy is calculated. Combination with the enthalpy data obtained by ITC gives values for the enthalpic and entropic contributions to the complex formation. Enthalpic contributions originate from electrostatic interactions between β -lg and pectin, entropic contributions by the release of small counter ions from the electrical double layers and the release of water molecules from hydrophobic regions. The local charge density of pectin is found to determine the balance between enthalpic and entropic contributions. For a high local charge density, the complex formation is dominated by an enthalpic component, supported by an entropic contribution. The enthalpic contribution is smaller for pectin with a lower local charge density. The relative increase in the entropic contribution is thought to arise from the association of the pectin methyl esters to an exposed hydrophobic moiety on the β -lg surface. An increase in the ionic strength lowers the enthalpic

contribution to the driving force, in favour of the entropic contribution, supporting the idea that release of water molecules from hydrophobic areas participates in the complex formation.

The functionality of β -lg-pectin soluble complexes is studied in chapter 5. Hydrophobized silica is used as a model surface for oil-water and air-water surfaces. Pectin is not capable of adsorbing to the hydrophobic surface, while β -lg readily adsorbs. Adsorption of β -lg-pectin soluble complexes results in a reduction of the adsorption rate of β -lg, as most of the β -lg is bound to pectin and not available for adsorption to the hydrophobic surface. This reduced adsorption rate hampers the emulsion stabilizing properties as the newly formed oil-water interface is not covered before coalescence of the emulsion droplets takes place. Sequential adsorption (layer-by-layer) of first β -lg, followed by pectin, and so on, results in the build up of a multilayer system of alternating β -lg and pectin layers. A terminal pectin layer is efficient against the wash out of adsorbed material. Systems consisting of a low overall charge pectin show the most negative ζ -potential, due to tailing and looping of the pectin chains. This suggests that for the formation of a double layer emulsion pectin with a low overall charge would be most suited.

In chapter 6 the results from the experimental chapters are combined to give the following general view. The local charge density of pectin determines the tolerance towards ionic strength: a higher local charge density allows complex formation up to a higher ionic strength. The overall charge influences how close the binding sites on pectin are together. A high overall charge puts binding sites in close proximity, causing pH_0 and the binding constant to pass through a maximum with increasing ionic strength. The knowledge obtained for the β -lg-pectin system is extrapolated to other small globular proteins, as well as larger aggregation proteins, like casein micelles. Strategies to tailor pectins to the desired characteristics of overall charge and local charge density are outlined. Finally, an outlook is given on areas of interest for future research on protein-pectin soluble complexes.

Samenvatting

Samenvatting

Het doel van dit proefschrift is het onderzoeken van de invloed van de totale elektrische lading en de lokale ladingsdichtheid van pectine op de vorming van oplosbare complexen met β -lactoglobuline (β -lg). Pectine is een natuurlijk voorkomend polysacharide, met vele toepassingen in levensmiddelen, zoals de stabilisatie van zure melkdranken. Na een industriële extractie bij lage pH, bestaat pectine voornamelijk uit galacturonzuurresiduen (GalA). De carboxylgroep van GalA kan op C-6 methylesterd zijn. De hoeveelheid methylesters bepaalt de methyleringsgraad (DM) en de totale lading van pectine. De verdeling van de methylesters (DB) bepaalt de lokale ladingsdichtheid van pectine.

Om de invloed van de totale lading en de lokale ladingsdichtheid van pectine op de vorming van oplosbare complexen te bestuderen, worden drie in deze studie gebruikte pectines in detail gekarakteriseerd. Hoofdstuk 3 combineert resultaten uit andere studies op dezelfde pectines met nieuwe analyses om een gedetailleerde structurele karakterisering van de pectines te geven. Samengevat, LMP heeft een hoge totale lading en hoge lokale ladingsdichtheid, met de niet methylesterde GalA blokken dicht bij elkaar. HMP_B (B voor blok verdeling) heeft een lage totale lading en hoge lokale ladingsdichtheid, met geïsoleerde niet methylesterde GalA blokken. HMP_R (R voor willekeurige verdeling) heeft een lage totale lading en lage lokale ladingsdichtheid. De weinige blokken die zich op HMP_R bevinden kunnen als geïsoleerd voorkomend worden beschouwd.

Het gebied waar β -lg en pectine complexen vormen als functie van pH, ionsterkte en gewichtsratio (WR, $[\beta\text{-lg}]/[\text{pectine}]$), wordt in hoofdstuk 2 bepaald door middel van dynamische lichtverstrooiing (DLS) titraties. Met de DLS data worden toestandsgrenzen pH_c (co-oplosbare polymeren en oplosbare complexen) en pH_φ (oplosbare complexen en onoplosbare complexen) geïdentificeerd en toestandsdiagrammen samengesteld. pH_c neemt af met toenemende ionsterkte en is, consistent met de literatuur, onafhankelijk van de WR. pH_φ neemt toe met toenemende WR. Voor HMP_B en HMP_R neemt pH_φ af met toenemende ionsterkte. Voor LMP passeert pH_φ een maximum met toenemende ionsterkte vanwege laterale repulsie tussen β -lg burens door de nabijheid van de bindingsplaatsen. pH 4.25 is gekozen

voor verdere bestudering van oplosbare complexen, omdat over een groot gebied van ionsterkte en WR oplosbare complexen worden gevormd. De pH_c waarden worden gebruikt om de lokale ladingsdichtheid van de bindingsplaatsen bij het begin van complexvorming te bepalen. LMP en HMP_B hebben een vergelijkbare lokale ladingsdichtheid, terwijl voor HMP_R een lagere lokale ladingsdichtheid wordt gevonden. Proton titraties van β -lg-pectine mengsels tonen aan dat er ladingsregulatie optreedt tijdens de complexvorming, zodat de geladen groepen van β -lg en pectine beter bij elkaar passen.

In hoofdstuk 3 wordt fluorescentieanisotropie geïntroduceerd om bindingsisothermen tussen eiwitten en geladen polysachariden te meten. De bindingsconstante en coöperativiteitsparameter wordt uit de bindingsisothermen verkregen door middel van een fit met het Hill-model. De complexvorming vertoont een sterke afhankelijkheid van de lokale ladingsdichtheid. Een hoge lokale ladingsdichtheid faciliteert complexvorming tussen β -lg en pectine bij een hogere ionsterkte. LMP vertoont een maximum in de bindingsconstante bij een ionsterkte van 10 mM, ten gevolge van laterale repulsie tussen β -lg burens. De binding van β -lg aan pectine is coöperatief. Een herschikking van de helixstructuur van pectine wanneer β -lg bindt, wordt gedacht hiervan de oorzaak te zijn.

Het bindingsmechanisme is onderzocht door middel van isothermische titratie-calorimetrie (ITC), zoals beschreven in hoofdstuk 4. De Gibbs vrije energie wordt berekend met de bindingsconstanten uit hoofdstuk 3. Door deze te combineren met de enthalpie data van de ITC, wordt de enthalpische en entropische bijdrage aan de complexvorming verkregen. Enthalpische bijdragen komen voort uit elektrostatistische interacties tussen β -lg en pectine, entropische bijdragen door het vrijkomen van kleine tegenionen uit de elektrische dubbellaag en watermoleculen uit hydrofobe regio's. De lokale ladingsdichtheid van pectine bepaalt de balans tussen de enthalpische en entropische bijdragen. Voor een hoge lokale ladingsdichtheid wordt de complexvorming gedomineerd door een enthalpische bijdrage, ondersteund door een entropische bijdrage. De enthalpische bijdrage wordt lager in geval van een lagere lokale ladingsdichtheid. De relatieve toename van de entropische bijdrage wordt toegeschreven aan de associatie van pectine methylesters met een

blootliggend hydrofoob gebied op het β -lg oppervlak. Een toename in de ionsterkte verlaagt de enthalpische bijdrage aan de drijvende kracht ten faveure van de entropische bijdrage, hetgeen de hypothese van de deelname van het vrijkomen van watermoleculen uit hydrofobe delen bijdraagt aan de complexvorming wordt hierdoor ondersteunt.

De functionaliteit van oplosbare β -lg-pectine complexen wordt beschreven in hoofdstuk 5. Gehydrofobiseerd silica wordt gebruikt als model oppervlak voor olie-water en lucht-water grensvlakken. Pectine adsorbeert niet op het hydrofobe oppervlak, terwijl β -lg snel adsorbeert. Adsorptie van oplosbare β -lg-pectine complexen resulteert in een afname van de adsorptie snelheid van β -lg, omdat het merendeel β -lg gebonden is aan pectine en dus niet beschikbaar voor adsorptie op het hydrofobe oppervlak. Deze gereduceerde adsorptiesnelheid hindert de emulsie-stabiliserende eigenschappen, omdat het nieuw gevormde olie-water oppervlak niet volledig wordt bedekt voordat coalescentie van de emulsiedruppels plaatsvindt. Sequentiële adsorptie (laag-voor-laag) van β -lg, gevolgd door pectine, en zo verder, resulteert in de opbouw van een multilaag van afwisselend β -lg en pectine. Een eindstandige pectinelaag is het efficiëntst tegen het uitwassen van geadsorbeerd materiaal. Het gebruik van een pectine met een lage totale lading leidt tot de meest negatieve ζ -potentiaal, door een lus en staart oriëntatie van pectine ketens. Dit insinueert dat voor de vorming van een dubbellaag emulsie pectine met een lage totale lading het meest geschikt zou zijn.

In hoofdstuk 6 worden de resultaten uit de experimentele hoofdstukken gecombineerd tot een meer algemeen beeld. De lokale ladingsdichtheid van pectine bepaalt de tolerantie voor ionsterkte: een hogere lokale ladingsdichtheid faciliteert complexvorming bij een hogere ionsterkte. Een hoge totale lading heeft tot gevolg dat de bindingsplaatsen dicht bij elkaar zitten, met als gevolg dat pH_0 en de bindingsconstante een maximum passeren voor toenemende ionsterkte. De kennis opgedaan voor het β -lg-pectine systeem wordt geëxtrapoleerd naar andere kleine globulaire eiwitten en grotere geaggregeerde eiwitten zoals caseïne micellen. Strategieën om pectine te modifieren tot de gewenste eigenschappen met betrekking tot totale lading en lokale ladingsdichtheid

worden besproken. Tot slot wordt een blik geworpen op mogelijkheden voor vervolg onderzoek aan oplosbare eiwit-pectine complexen.

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Curriculum Vitae

Bram Laurens Hendricus Maria Sperber was born on September 20th, 1977 in Tilburg, the Netherlands. He attended secondary school at the "Onze Lieve Vrouwe Lyceum" in Breda, the Netherlands and graduated in 1995. In the same year he started his study of Bioprocess technology at the former Agricultural University Wageningen, the Netherlands. During his study he performed a research thesis at the Laboratory of Physical Chemistry and Colloid Science on micelles with complex coacervate core and at the Food Physics group on the rheology and structure of ovalbumin gels. An internship was conducted at the Ian Wark Research Institute, Adelaide, Australia on the kinetics of adsorption of alkanethiols on gold. He graduated in 2001 with a M.Sc. degree. In 2002 he started his Ph.D. thesis combined at the Laboratory of Food Chemistry and the Laboratory of Physical Chemistry and Colloid Science, of which the work is presented in this thesis. From 2007 to 2010 he worked as a scientist on biomass, bioenergy and biofuels, at Food and Biobased Research, Wageningen University and Research Centre, Wageningen, the Netherlands.

List of publications

- Sperber, B. L. H. M., Cohen Stuart, M. A., Schols, H. A., Voragen, A. G. J. & Norde, W. **2009**. Binding of β -lactoglobulin to pectins varying in their overall and local charge density. *Biomacromolecules*, 10(12), 3246-3252.
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Overview of completed training activities

Discipline specific activities

Summer School Glycosciences (VLAG), Wageningen, The Netherlands, 2002

Advanced Food Analysis (VLAG), Wageningen, The Netherlands, 2002

Industrial Proteins (VLAG), Wageningen, The Netherlands, 2003

Bio-Nano Technology (VLAG), Wageningen, The Netherlands, 2005

Food Enzymology (VLAG), Wageningen, The Netherlands, 2005

Industrial Proteins (SenterNovem, IOP-IE), Ede, The Netherlands, 2003

Gums and Stabilisers for the Food Industry, Wrexham, Wales, 2003

Food Chemistry Symposium, Osaka, Japan, 2004

"Macroion complexation: fundamentals and applications", Wageningen, The Netherlands, 2005

Pectin & Pectinases, Wageningen, The Netherlands, 2008

General courses

VLAG introduction week, Bilthoven, The Netherlands, 2002

Scientific writing, Wageningen, The Netherlands, 2003

Optional courses and activities

Preparation PhD research proposal

PhD Study trip, Food Chemistry, USA, 2002

PhD Study trip, Food Chemistry, Japan, 2004

Food Chemistry Seminars, 2002-2006

Food Chemistry Colloquia, 2002-2006

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