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Characterization of the Surface-Active Components of Sugar Beet Pectin and the Hydrodynamic Thickness of the Adsorbed Pectin Layer

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The fraction of sugar beet pectin (SBP) adsorbed onto limonene oil droplets during emulsification has been isolated, and its chemical and physicochemical characteristics have been determined. While the SBP sample itself was found to contain 2.67 and 1.06% protein and ferulic acid, respectively, the adsorbed fraction contained 11.10% protein and 2.16% ferulic acid. The adsorbed fraction was also found to have a higher degree of acetylation, notably at the C2 position on the galacturonic acid residues, and was also found to contain a higher proportion of neutral sugars, which are present in the ramified side chains of the pectin molecules. The thickness of the layer of SBP adsorbed onto polystyrene latex particles was studied by dynamic light scattering and was found to increase with increasing surface coverage. It was found to have a value of ~140 nm at plateau coverage, which closely corresponded to the hydrodynamic diameter of the pectin chains. The adsorbed layer thickness was found to be sensitive to pH and the presence of electrolyte. The thickness at a surface coverage of ~20 mg/m^2 in the absence of electrolyte at pH ~4 was 107 nm and at pH 8.8 was 70 nm, while at pH ~4 in the presence of 10 mM NaCl the thickness was found to be 70 nm. It was concluded that the SBP molecules form multilayers at the surface due to electrostatic interaction between the positively charged protein moieties and the galacturonic acid residues. The removal of calcium from the SBP had no effect on the adsorbed layer thickness; hence, multilayer formation due to calcium ion cross-linking was considered unlikely.

KEYWORDS: Sugar beet pectin; surface active components; adsorbed layer; hydrodynamic thickness

INTRODUCTION

There has been considerable interest in recent years in the potential use of sugar beet pectin (SBP) as a replacement for gum Arabic in the stabilization of oil-in-water emulsions for the beverage industry. Pectins consist of linear chains of (1→4)-linked α-D-galacturonic acid residues, which may be esterified, with a small percentage of rhamnose units, which give rise to branches consisting of neutral sugars, particularly galactose and arabinose (*1*−*3*). SBP differs from other pectins in that it has a higher proportion of branched regions, a higher acetyl content on the C2 and/or C3 positions on the galacturonic acid residues, and also has phenolic esters (notably ferulic acid) attached to galactose and arabinose units in side chains. These differences may be responsible for the fact that unlike pectins from citrus peel and apple pomace, SBP does not form gels. Pectins also contain a small percentage of protein (typically ~2%), which has been linked to their ability to stabilize emulsions. It has been shown that the pectin fractions that adsorb onto the oil droplets are significantly enriched in protein (~8−21%) (*4*), and it is envisaged that the proteinaceous components anchor the molecules to the oil−water interface, while the carbohydrate components protrude out into the aqueous phase and provide an electrosteric barrier that prevents droplet aggregation. It is interesting to note that Leroux et al. (*4*) have also shown that the acetyl content of the adsorbed fraction is higher than in the bulk material, and we have previously reported that for SBP, the adsorbed fraction was also enriched in ferulic acid. This paper sets out to further establish the chemical and physicochemical nature of the SBP fraction responsible for its emulsification properties and also to determine the interfacial characteristics of the adsorbed SBP layer.

EXPERIMENTAL PROCEDURES

Materials. The SBP sample was provided by Degussa Texturant (France), and details of its source and extraction have been reported

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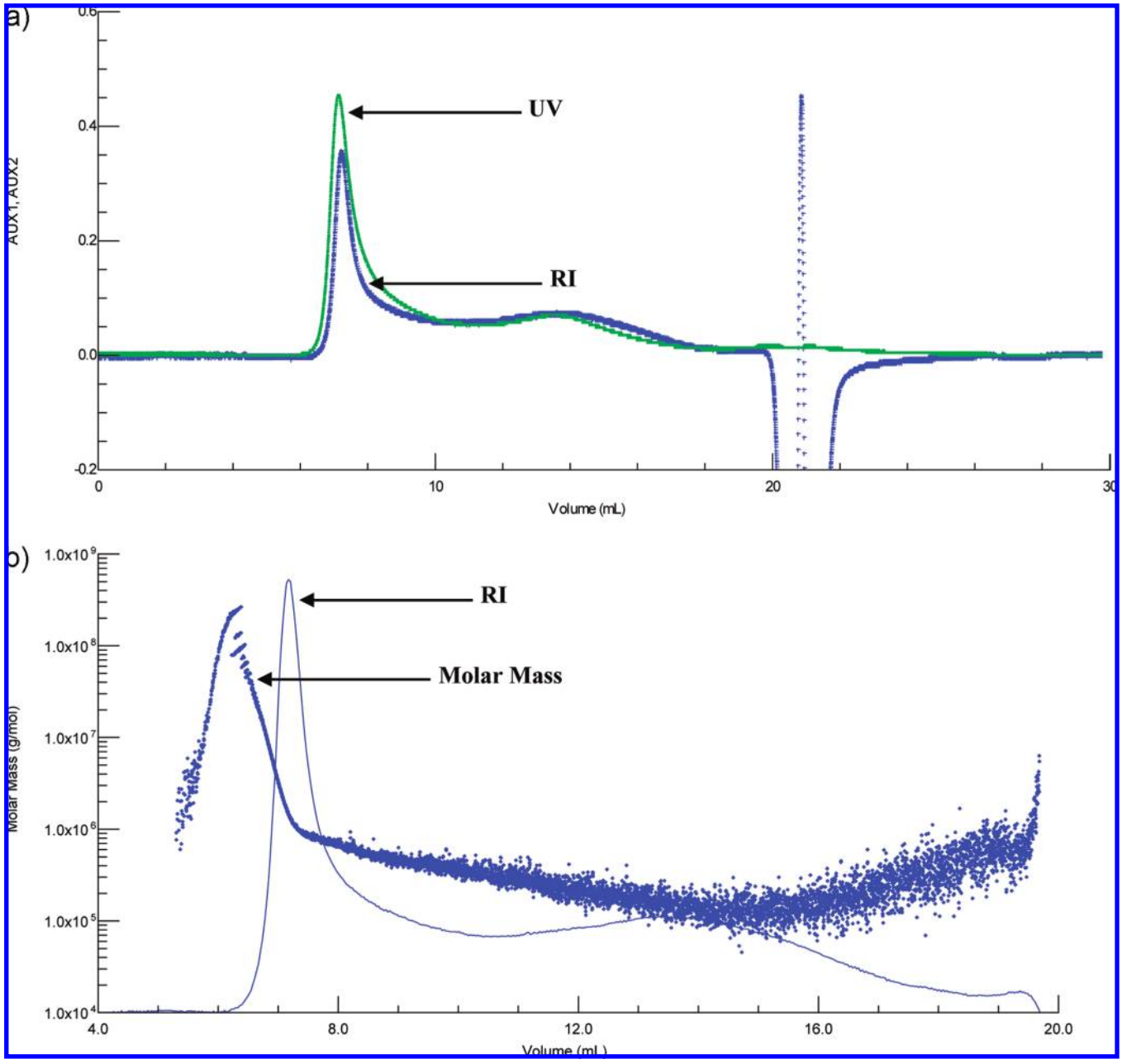

Figure 1. (**a**) GPC elution profile of the SBP showing RI and UV at 310 nm signals. (**b**) GPC elution profile of the SBP showing RI and molecular mass as a function of elution volume.

previously (*5*). A proportion of the sample was treated to remove calcium by dissolving (1% w/w) in an aqueous solution containing 11.69 g of sodium chloride (2 M). One gram of 0.1 M ethylenediaminetetraacetic acid was added to complex any calcium ions present, and the solution was left overnight on a roller-mixer at room temperature. The pectin was precipitated with 60% w/w propan-2-ol (IPA) and then redissolved into 40 g of distilled water. The insoluble material was removed by centrifugation at 2500 rpm at 20 °C for 10 min. Sodium chloride (2 M) was added to the solution, which was then left overnight on a roller-mixer at room temperature. The sodium chloride was removed by precipitating the pectin with IPA, centrifuging, and redissolving. This was repeated three times. Finally, the precipitated pectin was redissolved into 40 g of distilled water and freeze-dried. This procedure was found to remove >85% of the calcium ions present.

Protease enzyme, type XIV (4.7 units/mg solid), which is described as an "unusually nonspecific protease", and polystyrene latex (10% dispersion of particles of 0.3 μm diameter) were obtained from Sigma-Aldrich. Sodium dodecyl sulfate (SDS) and IPA were obtained from Fisons Laboratory and Fischer Scientific, respectively.

Methods. *Isolation of the Adsorbed Pectin Fraction.* An emulsion containing 320 g of 2% (w/w) SBP solution and 80 g of limonene oil was prepared by mixing in a 500 mL bottle and subjecting the bottle to shaking for 30 s immediately prior to homogenization in a 600 mL beaker using a Silverson L4R homogenizer with a medium-perforation emulsor screen at maximum speed for 5 min. The emulsion was left at room temperature for 24 h before being fully separated into aqueous and oil phases by centrifugation at 2500 rpm at 25 °C for 10 h or longer. The aqueous phase containing the nonadsorbed fraction of SBP at the bottom of the centrifuge tube was carefully separated with a needle connected to a syringe. This nonadsorbed fraction was freeze-dried and analyzed. The oil phase containing the adsorbed fraction of the SBP was collected into a 500 mL bottle, and SDS solution (~2.8 g of SDS) was added to desorb the pectin from the surface of the oil droplets. The amount of SDS solution added was such that the amount of the oil phase was 20% of the total weight. This mixture was homogenized using a Silverson L4R homogenizer for 1 min and left at room temperature for 24−48 h before it was centrifuged at 2500 rpm at 25 °C until complete separation of the oil and the aqueous phases. The

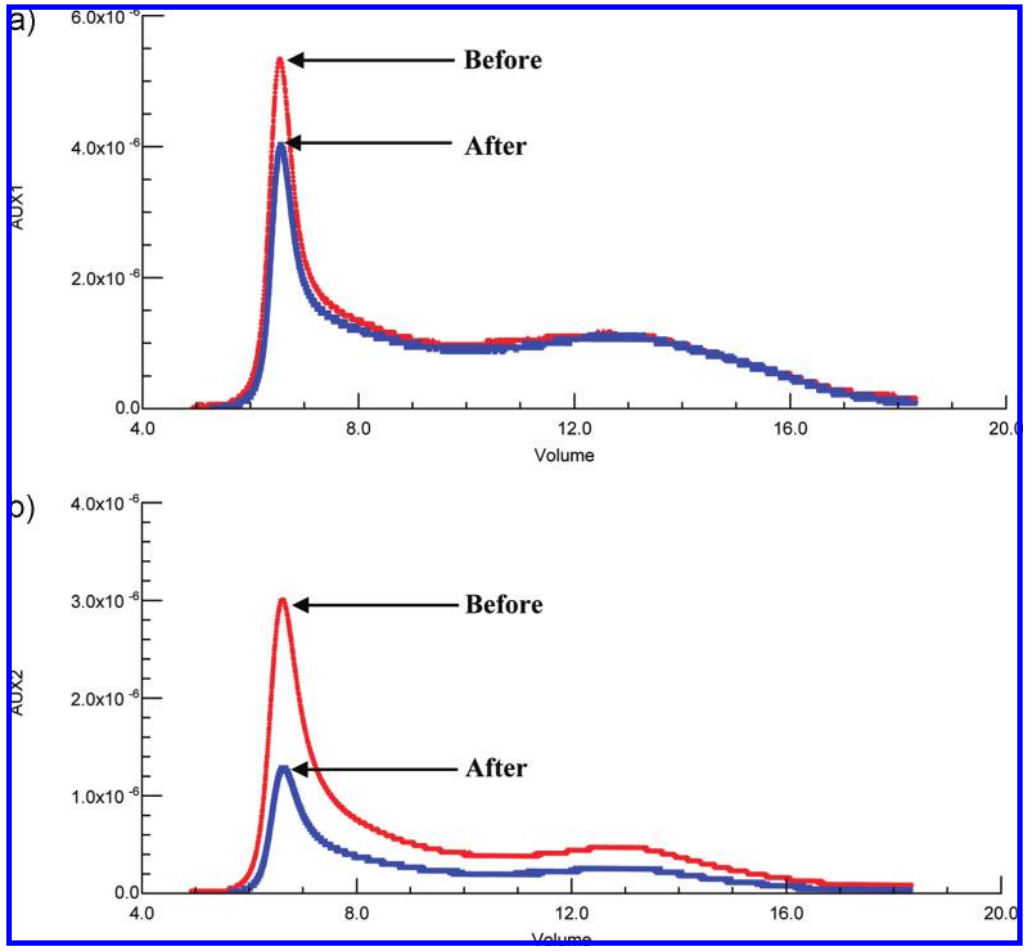

Figure 2. (**a**) RI elution profiles of the SBP sample before and after emulsification with 0.10% w/w initial (before emulsification) polymer concentration. (**b**) UV elution profiles of the SBP sample at 310 nm before and after emulsification with 0.10% w/w initial (before emulsification) polymer concentration.

Table 1. Characterization of the Whole SBP Sample and the Adsorbed and the Nonadsorbed Fractions[a]

SBP characteristics	whole SBP	adsorbed SBP	nonadsorbed SBP
calcium (%)	0.42	ND	ND
molecular mass (M_w) ($\times 10^5$ g/mol)	7.53	ND	4.86
protein by amino acid analysis (%)	2.67	11.10	ND
protein by Kjeldahl method (%)	4.01	13.12	2.24
ferulic acid (%)	1.06	2.16	ND
DE (%)	82.06	53.10	83.70
relative acetyl content	1.00	1.08	0.64
acetylation ratio of C3 to C2	1:2.3	1:4.9	1:1.2
uronic acid (%)	76.81	46.95	64.34
rhamnose (%)	4.15	3.08	2.44
arabinose (%)	5.42	4.02	5.74
galactose (%)	8.88	8.48	8.70
glucose (%)	1.08	0.47	0.87
uronic acid to neutral sugar ratio	3.93: 1	2.92: 1	3.62: 1
uronic acid to rhamnose ratio	18.5: 1	15.2: 1	26.4: 1

[a] ND, not determined.

Table 2. Amino Acid Profiles of the Whole SBP Sample and the Adsorbed SBP Fraction[a]

	SBP	
amino acids (%)	whole sample	adsorbed fraction
hydroxyproline	0.47	2.15
aspartic acid	0.15	0.51
threonine	0.17	0.72
serine	0.17	0.74
glutamic acid	0.25	0.84
proline	0.16	0.65
glycine	0.10	0.34
alanine	0.12	0.37
cystine	0.01	0.30
valine	0.19	0.73
methionine	0.00	0.13
isoleucine	0.06	0.20
leucine	0.10	0.33
tyrosine	0.16	1.05
phenylalanine	0.07	0.23
histidine	0.15	0.58
tryptophan	ND	ND
lysine	0.21	0.77
arginine	0.11	0.46
total protein (%)	2.67	11.10

[a] ND, not determined.

adsorbed SBP fraction in the aqueous phase at the bottom of the centrifuge tube was carefully separated from the oil phase using a needle connected to a syringe and precipitated with IPA (~80% w/w) and collected after brief centrifugation. The recovered pectin was then repeatedly subjected to dissolution (~3−4% w/w) followed by precipitation with IPA (~80% w/w) to remove SDS and was dried at room temperature.

Characterization of SBP and the Adsorbed and Nonadsorbed Fractions. Sugar Composition. The monosaccharide sugar composition was determined by hydrolyzing samples in 1 M H_2SO_4 at 100 °C for 2 h followed by high-performance anion-exchange chromatography (HPAEC) analysis as described by Wood et al. (*6*). The degree of esterification (DE) was determined by Fourier transform infrared (FTIR) spectroscopy. SBP FTIR spectra were recorded using a Golden-gate, Diamond single reflectance ATR in a FTS 700 FT-IR spectrometer

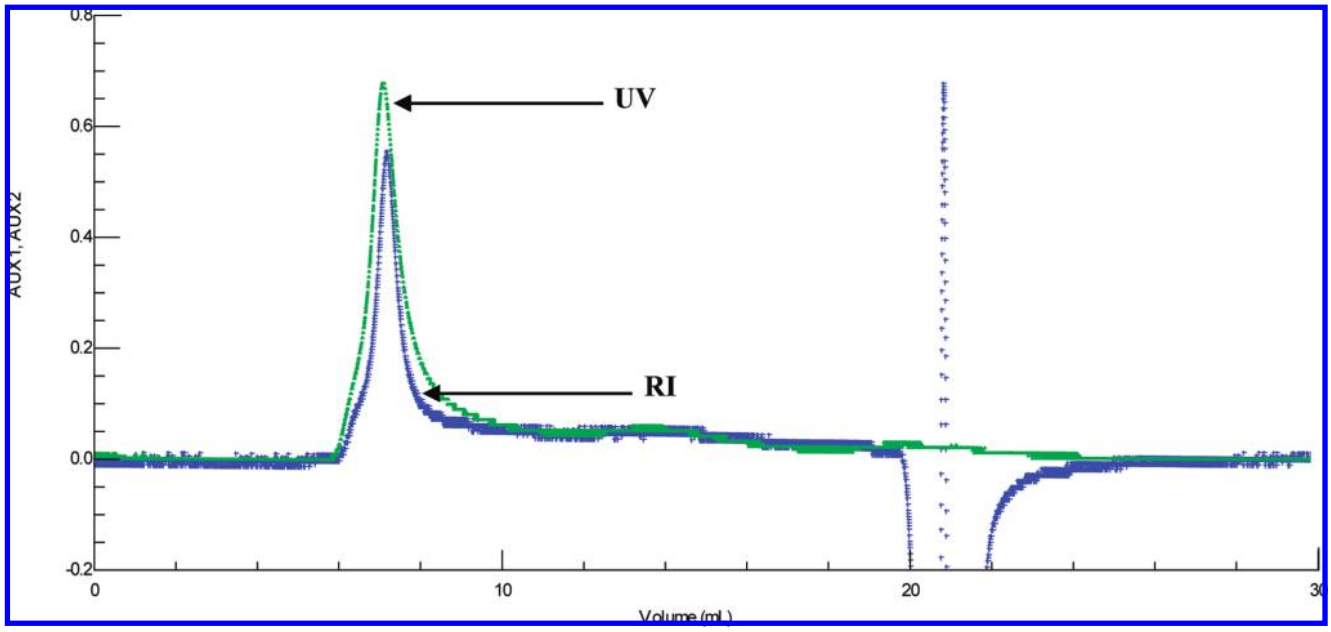

Figure 3. RI and UV (310 nm) GPC elution profiles of the adsorbed SBP fraction.

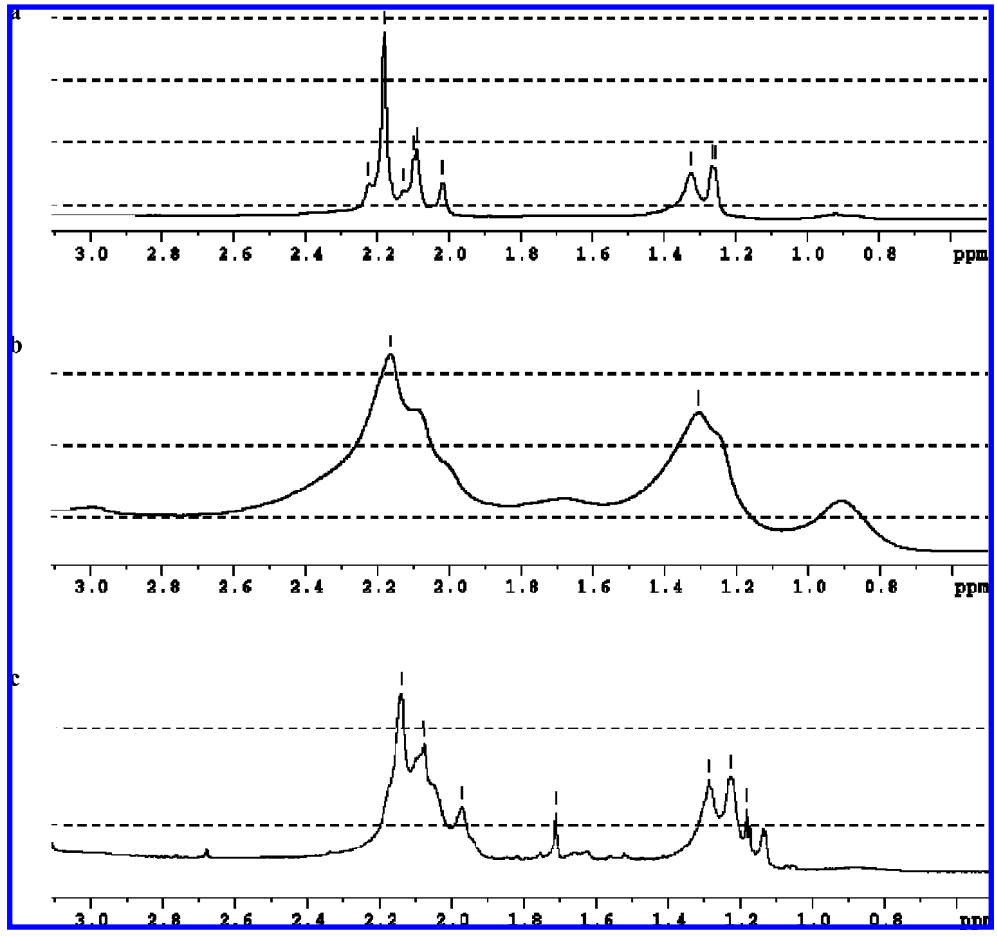

Figure 4. Proton NMR spectra of the whole SBP sample (**a**), the adsorbed SBP (**b**), and the nonadsorbed SBP (**c**) fractions.

equipped with a DTGS detector (DIGILAB, Randolph, MA) at the absorbance mode from 4000 to 400 cmK1 (midinfrared region) at a resolution of 4 cmK1 with 128 coadds. The DE was obtained from the ratio of the area under the band at 1730 cmK1 corresponding to the number of esterified carboxylic acid groups to the total area of the bands at 1730 and 1600 cmK1 corresponding to the number of total carboxylic groups (*7, 8*). The uronic acid contents of the samples were colorimetrically determined by the *m*-hydroxydiphenyl assay (*9*) using galacturonic acid as the standard.

Protein Content. The protein contents of the samples were calculated based on two different techniques, namely, Kjeldahl analysis and amino acid analysis. Kjeldahl analysis was carried out using the NA2100 Nitrogen and Protein Analyzer using the factor of 6.24 for conversion of nitrogen to protein. The amino analysis was carried out by Alto Biosystems Ltd. (Birmingham, United Kingdom).

Ferulic Acid Content. The ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid) content in the samples was determined by measuring the UV absorbance at a wavelength of 310 nm (Shidmazu UV−visible

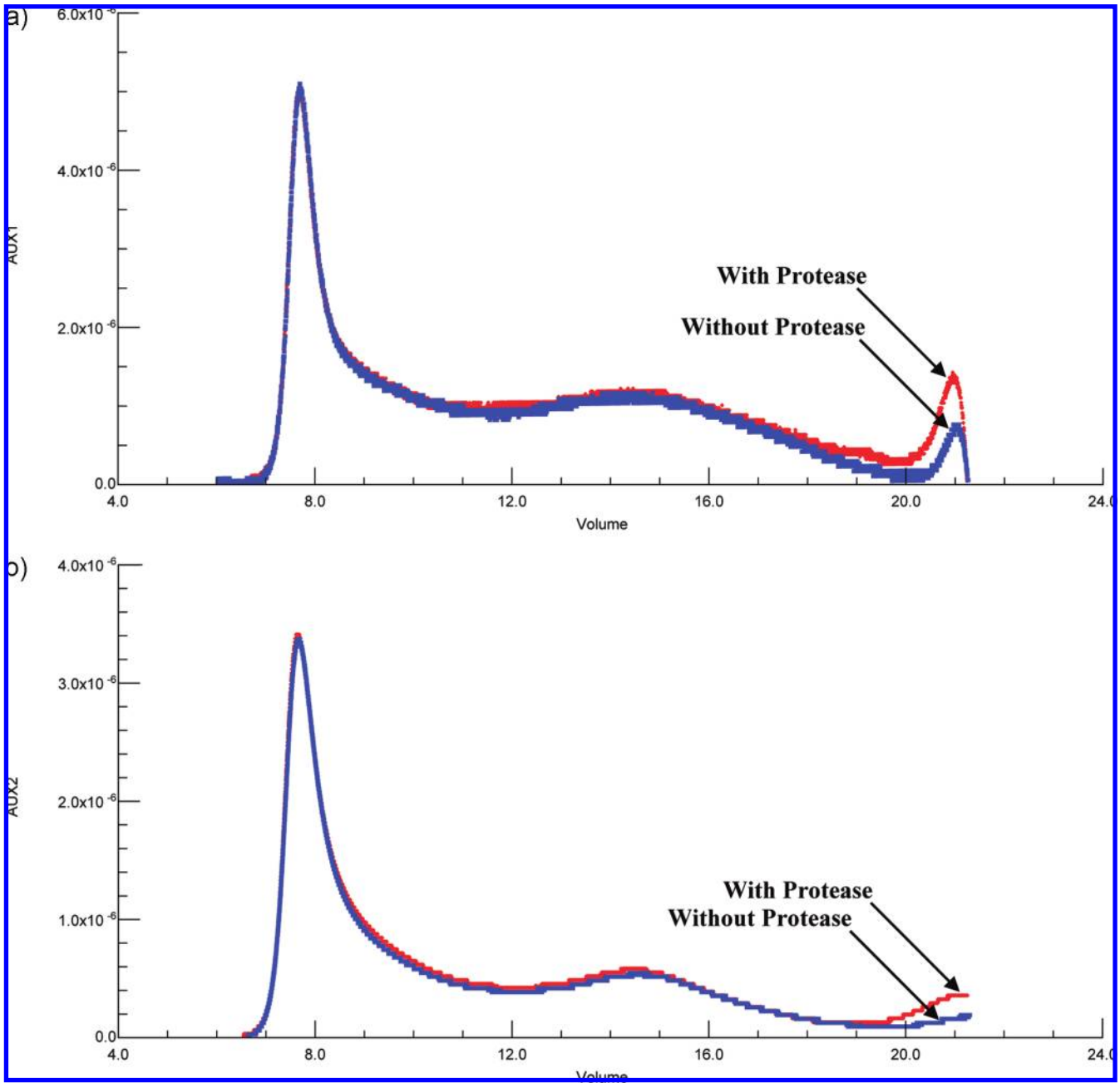

Figure 5. (**a**) GPC RI elution profiles of the SBP with and without protease treatment (46 h). (**b**) GPC UV at 310 nm elution profiles of the SBP with and without protease treatment (46 h).

Recording Spectrophotometer UV240, Graphicord). A calibration curve was constructed using ferulic acid obtained from Aldrich.

NMR Analysis. Proton NMR spectra were recorded on a Bruker AMX 500 FT spectrometer. All samples were dissolved in deuterium oxide (D_2O) at 90 °C for 3 h before NMR analysis.

Molecular Mass Distribution. The molecular mass distribution of the pectin samples was determined using gel permeation chromatography (GPC), coupled to multiangle laser light scattering (MALLS; DAWN DSP Laser Photometer, Wyatt Technology Corp.), refractive index (RI; Optilab DSP Interferometric Refractometer, Wyatt Technology Corp.), and ultraviolet (UV; Agilent 1100 series) detectors. A 100 μL amount of 0.1% polymer solution was passed through a Superose 6 column at a flow rate of 0.5 mL/min with 0.1 M NaCl as the eluent. The eluent was passed through a degasser (Gastorr 153) before being pumped using a Knauer HPLC Pump K-501 into the GPC system. All samples were filtered through a 0.45 μm nylon filter prior to injection to the GPC system. A dn/dc value of 0.131 mL/g for pectin was used, and the data were analyzed using the Debye fitting method.

Treatment with Protease and the Influence on Emulsification Properties. A buffer solution containing 61% volume 0.1 M disodium hydrogen phosphate and 39% volume sodium dihydrogen phosphate was prepared, and the pH was adjusted to 7.5 using 1 M NaOH. This was used to prepare a 2% w/w SBP solution and 3.2 mM enzyme solution. The two solutions were mixed in a 90 and 10% volume ratio, respectively, and kept under constant shaking of 50 rpm/min at 37 °C in a water bath. A control sample was prepared with the same SBP sample and buffer solution but without the protease enzyme. The water bath shaker was covered with aluminum foil to maintain the temperature and avoid evaporation. Sampling was carried out after 24, 48, 96, and 196 h, and the molecular mass distribution was determined using GPC coupled with multiangle light scattering and RI and UV detectors.

Adsorbed Layer Thickness. A 1% w/w stock solution of SBP was prepared and left overnight on a roller-mixer at room temperature. A series of solutions of different concentrations ranging from 0.01 to 0.8% in water and in 0.2 M NaCl were prepared from the stock solution by dilution with water and NaCl, respectively. A 0.1% w/w amount of

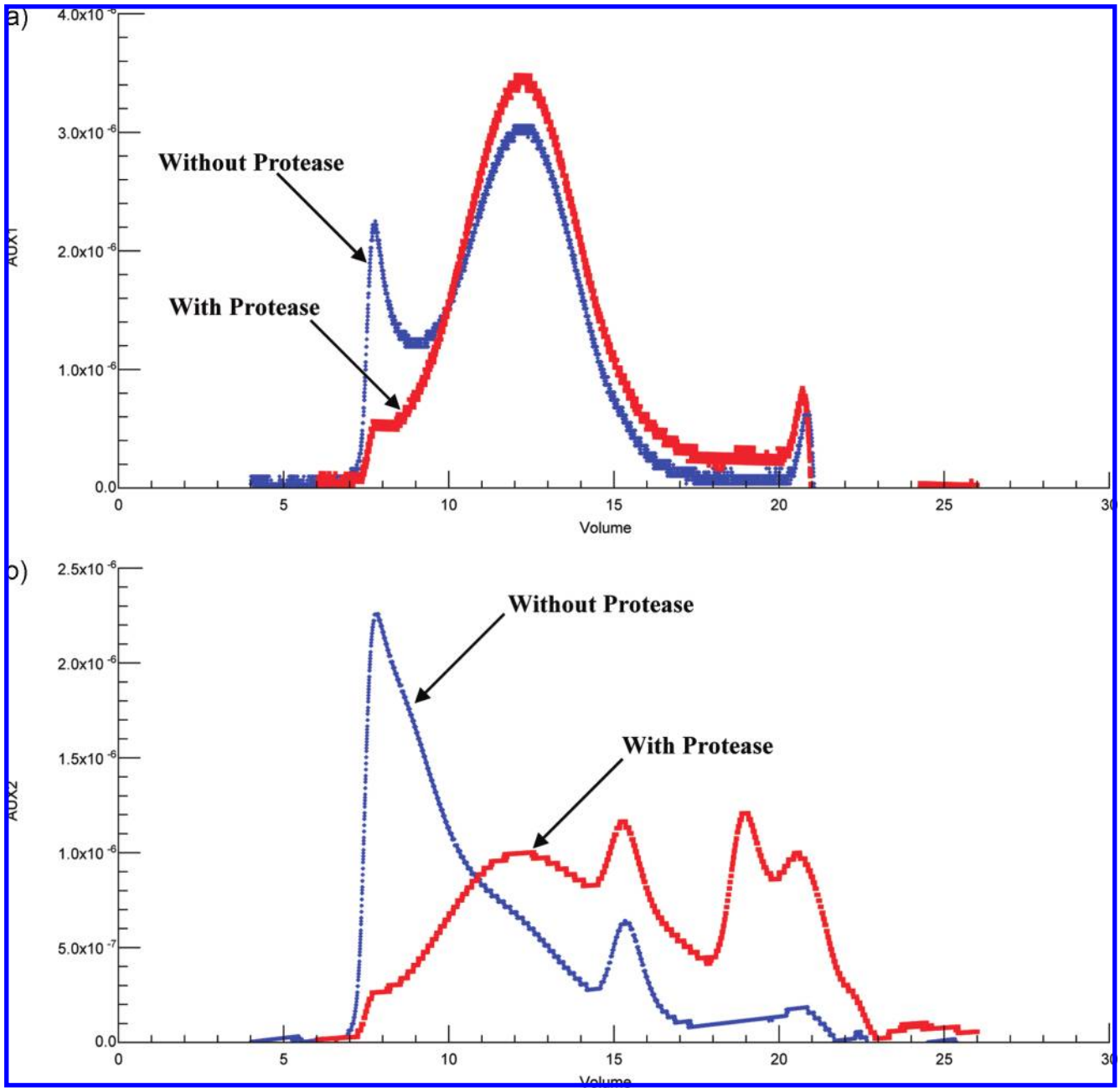

Figure 6. (**a**) GPC RI elution profiles of the GA with and without protease treatment (52 h). (**b**) GPC UV at 214 nm elution profiles of the GA with and without protease treatment (52 h).

polystyrene latex dispersion was prepared by diluting the 10% dispersion with distilled water.

The sizes of the polystyrene latex particles and SBP molecules at different concentrations with and without NaCl were measured by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments). The particle sizes were also measured for the polystyrene latices with SBP adsorbed at varying surface coverages, and the thickness of the adsorbed pectin layer was calculated. All solutions/dispersions were at pH ~4.0, and all values reported are the average of at least 10 subruns.

RESULTS AND DISCUSSION

Characterization of Whole SBP and the Adsorbed and the Nonadsorbed Fractions. *Molecular Mass Distribution.* The RI and UV (310 nm) GPC elution profiles of the SBP sample are presented in **Figure 1a**, and the M_w distribution is presented in **Figure 1b**. The intensity of the RI signal is proportional to the concentration of the eluting species, while the UV absorbance at 310 nm is proportional to the concentration of ferulic acid eluting. The first point to note is that the RI and UV profiles indicate that the material is polydisperse. The weight average molecular mass was found to be 7.53×10^5 g/mol. The fact that the RI and UV profiles are very similar indicates that the ferulic acid is distributed fairly evenly throughout the molecular mass species present.

The RI and UV (310 nm) GPC elution profiles of the SBP sample before and after adsorption are shown in **Figure 2a**,**b**, respectively. The difference in the intensity of the profiles before and after adsorption is due to a proportion of the SBP adsorbing onto the oil droplets. Over the full range of molecular mass species adsorbed, there appears to be a slight preference for adsorption of higher molecular mass molecules. This is demonstrated by the decrease in the weight average molecular mass, from 7.53×10^5 to 4.86×10^5 g/mol (**Table 1**).

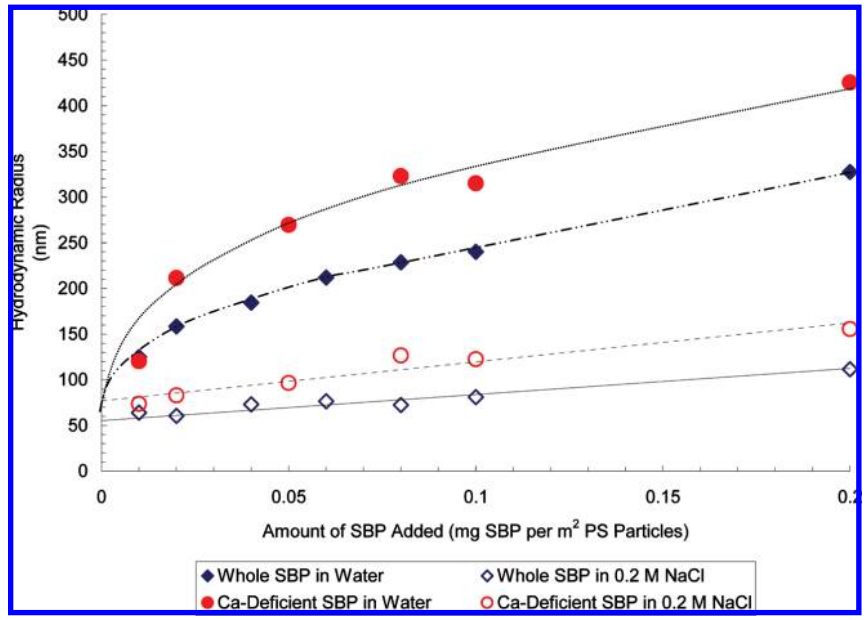

Figure 7. Hydrodynamic radii of the whole SBP and the Ca-deficient SBP in water and in 0.2 M NaCl.

The RI and UV (310 nm) elution profiles of the adsorbed SBP are presented in **Figure 3** and confirm the polydisperse nature of the material. Comparison of **Figure 3** to **Figure 1a** reveals that the adsorbed SBP fraction had similar molecular mass distribution to that of the whole SBP sample. We were unable to determine a definitive value for the molecular mass of the adsorbed SBP fraction as the recovered material was particularly difficult to redissolve completely after IPA precipitation and drying at room temperature. Furthermore, sample recovery following GPC analysis was only ~50%.

Chemical Analysis. The molecular masses, the protein, ferulic acid, acetyl, and calcium contents, the DE, and the sugar composition of the original, nonadsorbed, and adsorbed SBP materials are presented in **Table 1**. The amount of protein in the adsorbed fraction is seen to be ~11%, which is significantly higher than in the original starting material (~2.7%). The results confirm earlier findings (*4, 5, 10*). The amino acid compositions of the starting material and the adsorbed SBP are presented in **Table 2**. The main amino acids are hydroxyproline, glutamic acid, tyrosine, and lysine, and their relative proportions remain fairly constant in the two samples. It is also noted that the adsorbed fraction contained approximately twice as much ferulic acid as compared to the whole sample (**Table 1**). The ratio of galacturonic acid residues (which make up the pectin backbone) to neutral sugars (which are present in the side chains) is seen to be significantly lower for the adsorbed SBP sample as compared to the original (whole) SBP sample, indicating that a greater proportion of the neutral sugar side chains are adsorbed. This is also supported by the fact that the adsorbed fraction has a lower galacturonic acid to rhamnose ratio. Interestingly, the DE of the galacturonic acid residues is significantly lower in the adsorbed fraction.

The proton NMR spectra of the whole SBP sample and the adsorbed and the nonadsorbed fractions are shown in **Figure 4**. In general, all three samples exhibited similar spectra with peaks at ~2.0−2.2 and ~1.1−1.4 ppm. These regions correspond to signals from the acetyl CH_3 (*11*) and the methyl groups of L-rhamnose (*12*), respectively. While there were about four or five reasonably sharp acetyl peaks obtained for the whole SBP sample and the nonadsorbed fraction, the signal peaks for the adsorbed fraction were broad. Renard and Jarvis (*11*) reported four distinct peaks in the acetyl CH_3 region and assigned one each to the C2 and the C3 for single acetylation and the other two for C2 and C3 double acetylation. It was concluded that in the acetylation region the peak for single acetylation at higher chemical shift was likely to be associated with the C2 position. From our spectra, it is noted that the acetyl signal peak intensity for the adsorbed SBP fraction was similar to that of the whole SBP sample but was ~40% higher than in the nonadsorbed fraction (**Table 1**). Integration of the acetyl peaks in **Figure 4** also shows different C2 and C3 acetylation patterns for the three samples. The signal intensity ratio for C3 to C2 acetylation was 1:2.3 for the whole SBP sample, 1:4.9 for the adsorbed fraction, and 1:1.2 for the nonadsorbed fraction. In summary, therefore, it is noted that the adsorbed fraction has a higher acetyl content than the nonadsorbed SBP fraction and also a significantly greater proportion of acetyl groups at C2. Leroux et al. (*4*) also found the adsorbed component to have a higher acetyl content and reported that the emulsification efficiency was greater for pectin of high acetyl contents as compared to those with low acetyl contents.

Influence of Enzyme on Pectin Structure. It has been previously shown that the molecular mass of gum Arabic is significantly reduced on treatment with proteolytic enzyme. This has led to the conclusion that a certain fraction within the gum [the high molecular mass arabinogalactan−protein complex (AGP)] has a "wattle blossom type" structure in which carbohydrate blocks are attached to a common polypeptide chain (*13*). Removal of the protein has been shown to reduce the emulsification efficiency; hence, it has been postulated that the protein is the key component in the emulsification process. Recently, Kirby et al. (*14*), using atomic force microscopy (AFM), reported the presence of linear pectin chains, branched pectin chains, and pectin−protein complexes in SBP extracts and speculated that the protein component was attached at one end of the pectin molecules. To gain further insight into the nature of the protein−carbohydrate linkage, we have carried out proteolytic enzyme hydrolysis experiments. The RI and UV elution profiles of SBP before and after enzyme treatment are presented in **Figure 5a**,**b**. It is clear that there is no change in the molecular mass profile after enzyme treatment, which is contrary to the results reported by Funami et al. (*15*). They found that the molecular mass of SBP decreased from 517000 to 254000 g/mol, and the protein content decreased from 1.56 to

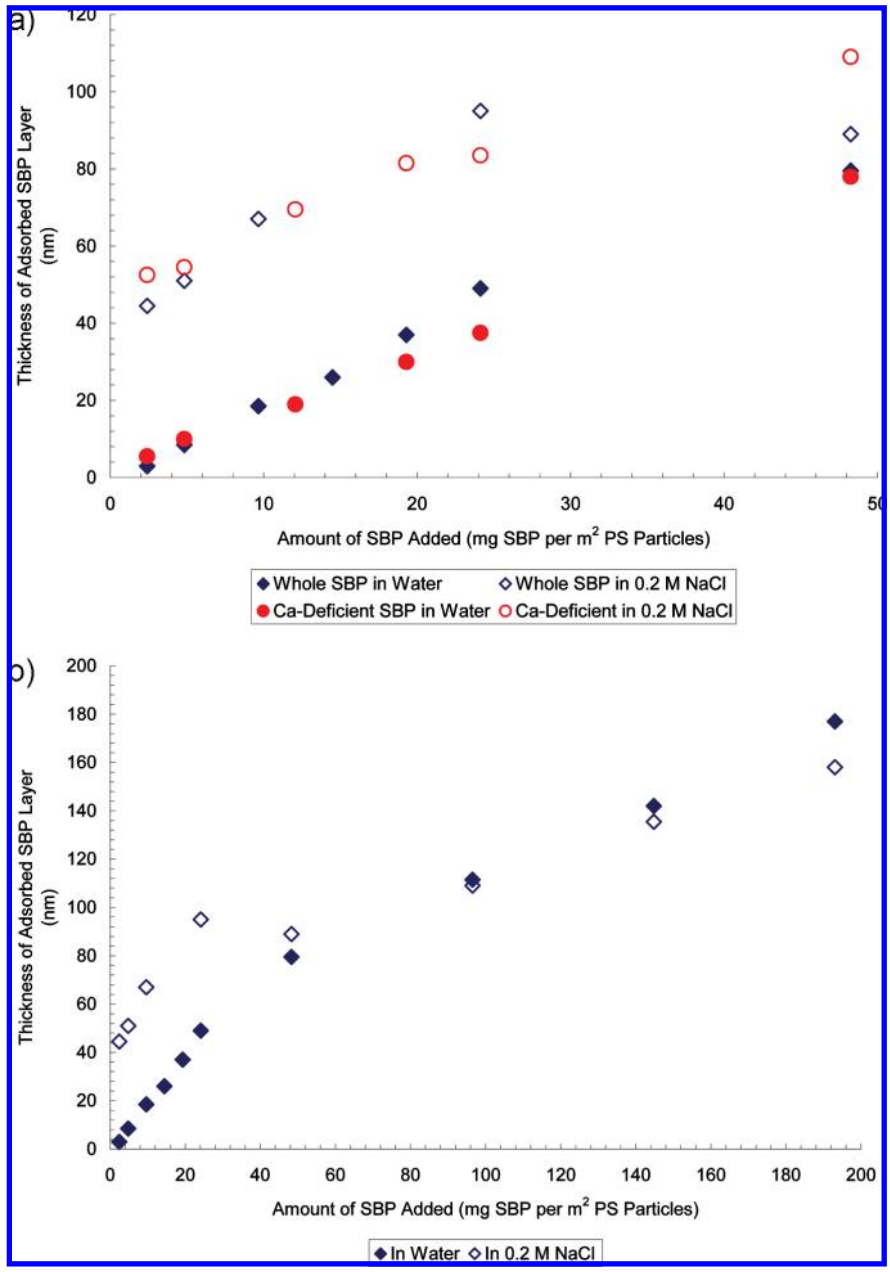

Figure 8. (**a**) Hydrodynamic thicknesses of the adsorbed SBP and the Ca-deficient SBP layers in water and in 0.2 M NaCl as a function of the amount of SBP added. (**b**) Hydrodynamic thicknesses of the adsorbed SBP layer in water and in 0.2 M NaCl as a function of the amount of SBP added.

0.13% after enzyme treatment. Most importantly, protease-treated SBP was found to have lost its emulsification properties. It was concluded that the protein moieties in SBP are bound to the high molecular mass carbohydrate residues based on the findings that the UV peak at 214 nm shifted to a lower molecular mass regime after the enzymatic treatment. It should be pointed out that both galacturonic acid and ferulic acid also absorb at 214 nm and will contribute to the elution peak. It should also be noted that Funami et al. (*15*) heated their sample at pH 3 at 100 °C for 10 min to stop the enzyme reaction prior to freeze-drying. We analyzed the solution immediately after incubation without any further treatment. The fact that the SBP was not degraded by the enzyme is not clear but may be due to the fact that the protein components are sterically inaccessible [this would mean it is unlikely that they are attached to the ends of the chains as suggested by Kirby et al. (*14*)] or it may be due to the nature of the amino acid sequence. It has been found that many AGPs are not degraded by enzyme. To be confident that the enzyme was functioning under the conditions adopted for the experiment, we performed a similar investigation on a gum Arabic sample. The RI and UV (214 nm) elution profiles before and after enzyme treatment are presented in **Figure 6a**,**b**. Clearly significant degradation occurred with the loss of the high molecular mass AGP peak at an elution volume of ~8 mL.

We have carried out some experiments investigating the emulsification properties of both SBP and gum Arabic before and after enzyme treatment and will be reporting the results in a separate paper. The key finding was that whereas enzyme treatment significantly affected the emulsification properties of gum Arabic, this was not the case for SBP, confirming the fact that the proteinaceous component was still attached.

Adsorbed Layer Thickness. It has been previously shown that the amount of pectin adsorbed at plateau coverage at the limonene−oil−water interface is of the order of ~10 mg/m^2 (*10, 16*). If we assume that the molecules adsorb maintaining their solution conformation, we calculate that the amount adsorbed at monolayer coverage would be of the order of 1

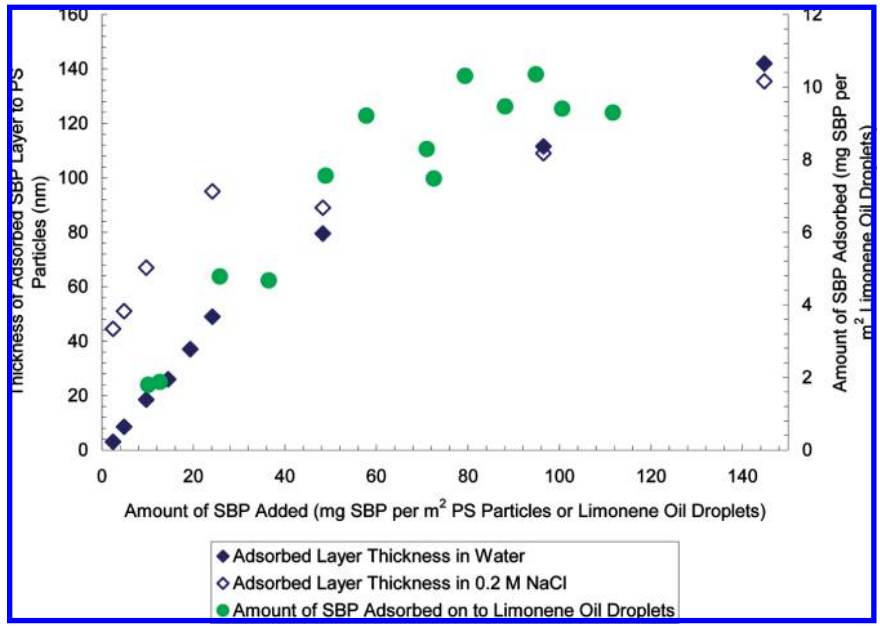

Figure 9. Thicknesses of adsorbed SBP layer on polystyrene latex (in water and in 0.2 M NaCl) and the amount of SBP adsorbed onto limonene oil droplets.

mg/m^2. This indicates, therefore, that either the molecules adsorb with long dangling tails protruding out into solution and/or that multilayer adsorption occurs. Kirby et al. (*14*) proposed that the protein moieties, which they suggested were at one end of the pectin molecules, adsorbed onto the surface of the oil droplets and that the polysaccharide chains extended out as long tails into solution. As a consequence, it was decided to measure the thickness of the adsorbed polymer layer. It was not possible to determine the adsorbed layer thickness of the pectin onto limonene oil droplets because of the relatively large droplet size as compared to the adsorbed polymer layer thickness and also because of the relatively broad distribution in droplet size. It was, therefore, decided to undertake measurements using monodisperse polystyrene latex (PS) particles as a model system.

The hydrodynamic radii, Rh, of the molecules of the original SBP sample and the calcium-deficient sample were initially determined alone in water and 0.2 M NaCl by dynamic light scattering as a function of SBP concentration. The results are presented in **Figure 7**. The values are significantly higher in the absence of 0.2 M NaCl as expected due to intramolecular electrostatic repulsions, which cause the molecular chains to expand. In the presence of 0.2 M NaCl, these repulsions are screened and the chains become more compact. The values were also higher for the calcium-deficient sample than for the original material. This is presumably a consequence of the fact that the divalent calcium counterions are more effective at screening than sodium counterions. The Rh value obtained by extrapolation to infinite dilution was found to be ~65 nm. The thickness of the adsorbed polymer layer was determined for the original and calcium-deficient SBP samples by measuring the PS particle hydrodynamic radius with and without SBP adsorbed. The results are plotted in **Figure 8a** (at low surface coverages) and **b** (at high surface coverages) as a function of pectin addition. The adsorbed layer thickness at low pectin additions (**Figure 8a**) is seen to be greater in the presence of electrolyte than in water as is commonly found for polyelectrolyte adsorption (*17*). It is attributed to the screening of interactions between adsorbed polymer segments enabling the molecules to adopt a more extended conformation with loops and tails protruding into solution and also by screening interactions between molecules in solution and on the surface thus enabling a greater number of molecules to adsorb. At higher pectin additions (**Figure 8b**), the pectin itself contributes to the overall ionic strength and results in an increase in the amount adsorbed.

The adsorbed layer thickness was found to increase from ~4 nm at 2 mg SBP added/m^2, reaching a value of ~140 nm at 145 mg SBP added/m^2, which corresponds to the plateau value obtained for the adsorption onto the limonene oil droplets (**Figure 9**). At low surface coverages, the lower values obtained are due to the fact that there is less pectin adsorbed and/or more probably that the molecules tend to lie relatively flat on the surface as is commonly observed for polymers generally (*18*). It is noted that the adsorbed layer thickness at plateau coverage is similar to the hydrodynamic diameter of the pectin molecules (~130 nm); hence, it is evident that a significant proportion of the polymer segments protrude out into solution. To account for the high adsorption capacities reported, it is likely that multilayer adsorption occurs. This could arise from cross-linking of pectin chains at the interface through calcium ions. However, removal of the calcium from the pectin had no effect on the adsorbed layer thickness (**Figure 8a**); hence, calcium cross-linking of pectin chains is unlikely. An alternative explanation is that electrostatic complexes are formed at the interface through interaction of positively charged protein moieties within the pectin chains and negatively charged galacturonic acid residues. We have determined the adsorbed layer thickness for the calcium-deficient pectin at pH 3.7 where any proteinaceous components are likely to be positively charged and able to form electrostatic complexes with the pectin galacturonic acid residues and at pH 8.8 where they are likely to be negatively charged and unable to interact. It was found that the adsorbed layer thickness (at 20 mg added SBP/m^2) was 107 and 70 nm for pH 3.7 and pH 8.8, respectively, which tends to support the hypothesis of electrostatic complex formation at the interface at the lower pH. We have also measured the adsorbed layer thickness at pH 4.0, 7.4, and 9.4 (at 20 mg added SBP/m^2) in the presence of 10 mM NaCl, which would tend to reduce electrostatic complex formation. The adsorbed layer thicknesses were found to be 70, 70, and 60 nm for pH 4.0, pH 7.4, and pH 9.4, respectively. The very much lower value for the adsorbed

layer thickness at pH 4.0 in the presence of electrolyte as compared to pH 3.7 in the absence of electrolyte also supports our hypothesis.

Conclusions. It has been shown that the SBP fraction that adsorbs at the surface of limonene oil droplets is rich in protein and ferulic acid and also contains a higher proportion of neutral sugars, which are assumed to be present in the ramified side chains of the pectin molecules. It is proposed that the high levels of protein present in the molecules (~11%) give rise to multilayer adsorption at the interface due to electrostatic complexation between the positively charged protein moieties within the SBP chains and the negatively charged galacturonic acid residues.

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