JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Study of the Role of the Carbohydrate and Protein Moieties of Soy Soluble Polysaccharides in Their Emulsifying Properties

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The objective of this work was to investigate the role played by the protein fraction of soy soluble polysaccharide (SSPS) during its adsorption at oil/water interfaces. SSPS was separated in a high (HMF; 310 kDa) and low (LMF; 20 kDa) molecular weight fraction by gel filtration. SSPS/HMF consisted of 91.6% carbohydrate and 2.2% protein and showed better emulsifying properties than those of the whole SSPS, whereas SSPS/LMF seemed to affect negatively the adsorption behavior of SSPS. SDS-PAGE of the protein fraction obtained from SSPS/HMF showed a molecular mass of 50 kDa, was composed predominantly of proline (23.1%) and glutamic acid (15.2%), and still contained 8.8% of neutral sugar and 5.3% of uronic acid. Results indicated that not all of the protein material present in SSPS contributes to SSPS functionality but that only the material associated with HMF aids in the adsorption of SSPS onto oil/water interfaces.

KEYWORDS: Soybean; water-soluble polysaccharide; emulsion; protein

INTRODUCTION

Polysaccharides play important roles as thickening, stabilizing, and gelling agents in many foods. In emulsion systems polysaccharides are often used to improve the texture and stability of emulsions. In beverage emulsions, gum arabic from *Acacia senegal* is the most commonly used polysaccharide because of its high water solubility, low bulk viscosity, and ability to create a strong protective film around the oil droplet (*1*−*5*). Gum arabic is described as a highly branched arabinogalactan-protein (*6*−*8*). The structure of gum arabic consists of a core of β-1,3-linked galactose with extensive branching through 3- and 6-linked galactose and 3-linked arabinose. Rhamnose and glucuronic acid are positioned at the periphery of the molecules. Gum arabic contains a small protein fraction, which is thought to play a fundamental role in the adsorption of the polysaccharide at the oil/water interface. This protein is composed predominantly of hydroxyproline and serine and is covalently bound to the reducing end of the polysaccharide chains (*9*, *10*). Both protein and polysaccharide are fundamental to the functional properties of gum arabic: the hydrophobic polypeptide chains anchor the polysaccharide onto the surface of the oil droplet, whereas the hydrophilic polysaccharide portions prevent aggregation by forming a thick charged layer (*11*−*13*).

Most polysaccharides adsorbing at interfaces seem to owe this function to a proteinaceous fraction present (*13*). This was confirmed by recent investigations on pectins from sugar beet (*14*, *15*) or depolymerized citrus pectins (*16*) as emulsifiers in flavor emulsions; their ability to adsorb at the oil/water interface was attributed to the polypeptide residues present, together with the distribution of acetyl groups on the polygalacturonic acid backbone. Although the emulsifying properties of these pectic emulsifiers have been investigated (*16*, *17*), the details of the mechanism of adsorption have yet to be determined.

Soybean water-soluble polysaccharide (SSPS) is an ingredient extracted from the byproduct of the isolation of soybean protein and has been shown to be effective in stabilizing beverage emulsions (*18*) as well as acidic milk beverages (*19*). SSPS is an acidic polysaccharide containing galacturonic acid, and its chemical structure has been characterized (*20*−*22*). SSPS is composed of a main rhamnogalacturonan backbone branched by β-1,4-galactan and α-1,3- or α-1,5-arabinan chains. SSPS may be used in flavor emulsions because of its high water solubility, pH stability, low bulk viscosity, emulsifying properties, and ability to form strong interfacial films (*23*).

Recently we investigated the emulsifying properties of SSPS with particular focus on how different extraction conditions would affect the behavior of the SSPS when adsorbed at oil/water interfaces (*24*). Emulsions prepared with SSPS were stable in the pH range from 3 to 7, and the stability was not affected by pH or ionic strength up to 25 mM NaCl or $CaCl_2$. Although extraction conditions did not seem to affect the stability of the

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10.1021/jf049728f CCC: $27.50
Published on Web 07/24/2004

emulsions, they seemed to dictate the thickness of the layer of SSPS adsorbed onto the oil surface. The hydrophilic portions of SSPS play an important role in the stabilization of the emulsions, by preventing aggregation by steric repulsion. As for the other polysaccharides with interfacial activity (*13*), the protein material present in the SSPS may play a fundamental role in the adsorption of SSPS on oil/water interfaces; however, the details of the anchoring mechanism have not yet been described. The aim of this research was to clarify the function of the protein fraction present in SSPS in the stabilization of oil-in-water emulsions.

MATERIALS AND METHODS

Materials. Soybean oil was obtained from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO). Trypsin (from bovine pancreas, 13600 units/mg), pepsin (from porcine gastric mucosa, 2500−3500 units/mg), pectinase (from *Aspergillus aculeatus*, 2500 units/mL), and other analytical grade reagents were also purchased from Sigma Chemical Co. Ltd. Milli-Q water was used to prepare all solutions.

Preparation of SSPS. Soybean cotyledons after removal of the hulls and hypocotyls were powdered and defatted twice at 40 °C with 5 volumes of *n*-hexane. Proteins and water-soluble substances in the defatted meal were extracted twice at 50 °C and pH 7 for 1 h with 11 volumes of water (*20*). SSPS (extraction type L) was extracted from the residue (okara) with hot water by heating at 120 °C at pH 3 for 2 h. After removal of insoluble materials by centrifugation, the extract was desalted by electric dialysis (Micro acilyzer G1, Asahi Glass Co., Ltd., Tokyo, Japan), and then spray-dried.

Preparation of Emulsions. SSPS was slowly added at room temperature with gentle stirring to 50 mM sodium citrate buffer, pH 4, containing 0.02% sodium azide as antimicrobial. The values of pH indicated in the text refer to the pH of the SSPS solutions before emulsification.

Oil-in-water emulsions (20%) were prepared at room temperature using a laboratory scale homogenizer (EmulsiFlex-C5, Avestin Inc., Ottawa, Canada) with two passes at 40 MPa. Soybean oil was added to the SSPS solutions and then prehomogenized using a Power Gen 125 for 5 min. Results presented are averages of at least two independent experiments.

Emulsion Characterization. The droplet-size distribution of the emulsions was measured using a Mastersizer X (Malvern Instruments Ltd., Malvern, U.K.) with a presentation factor of 0303, corresponding to a relative refractive index of the particles of 1.06, a particle absorption of 0.001, and a refractive index of the solvent of 1.33. The emulsifying ability was assessed by checking the shape of the distribution and the value of the average droplet size ($D[3,2]$ and $D[4,3]$). The droplet size distribution was always measured after 24 h of storage at 20 °C.

Changes in the diameter of the emulsion droplets after the addition of protease or pectinase treatment were determined by dynamic laser light scattering (DLS) using a Malvern 4700 optical system attached to a 7032 correlator according to the method described previously (*24*). Aliquots of protease solution (20 μL, 300 units as pepsin or trypsin) or pectinase solution (400 μL, 60 units as α-polygalacturonase) were added in a clean cuvette containing the emulsion, previously diluted at a rate of 0.5 μL emulsion per 4 mL of filtered buffer (0.22 μm filters, Millex-GV, Millipore Co. Ltd., Billerica, MA). The buffer contained 50 mM sodium citrate adjusted to pH 4 or 7. The changes in droplet size during hydrolysis were measured with the DLS, at 4 min intervals, at 30 °C for 60 min. Pepsin and pectinase treatments were performed at pH 4 according to the same method, whereas trypsin treatments were carried out at pH 7.

Fractionation of SSPS by Gel Filtration Chromatography (GFC). Preparative GFC of SSPS/HMF and SSPS/LMF was performed with a Sepharose CL-6B column (2.5 cm × 90 cm; Amersham Biosciences Co., Piscataway, NJ). Samples were eluted with 50 mM sodium acetate buffer, pH 5. Aliquots (15 mL) of 5% (w/w) SSPS solutions were injected, and separation was carried out at a flow rate of 0.3 mL/min. Four milliliter fractions were collected, and total sugar concentration was measured according to the phenol−sulfuric acid method (*25*). The injection was performed 20 times, and the eluted peaks were collected together to obtain sufficient material for further studies. Fractions of SSPS/HMF and SSPS/LMF were desalted by electric dialysis (Micro acilyzer G1, Asahi Glass Co., Ltd) and then freeze-dried.

Molecular Weight Determination of SSPS Fractions. Analytical GFC of SSPS was performed using HPLC (Waters 600E System, Waters 410 RI detector, and Waters 486 UV detector; Waters Ltd., Toronto, ON, Canada) with a PolySep-GFC-P linear column (7.8 mm × 300 mm; exclusion limit = 20000 kDa as pullulans; Phenomenex, Torrance, CA). Aliquots (20 μL) of 1% (w/w) SSPS were injected, and separation was carried out at a flow rate of 1.0 mL/min in 50 mM sodium acetate buffer, pH 5. The SSPS elution was determined by using both refractive index (Waters 410 RI detector) and UV (Waters 486 UV detector; A_{280nm}) detectors. The molecular weight was determined against standard pullulans (Showa Denko Kogyo Co. Ltd., Tokyo, Japan).

SSPS/HMF was treated with pectinase to partially digest the polysaccharide chains. A 0.2 mL pectinase solution (60 units as a α-polygalacturonase) was added to 20 g of 1% (w/w) SSPS/HMF solution in 20 mM sodium acetate buffer, pH 5. The reaction mixture was incubated at 40 °C for 20 h. The reaction mixture (10 g) was heated at 90 °C for 20 min to inactivate the enzyme and then cooled to room temperature, and aliquots (20 μL) were injected in the HPLC.

Pectinase-treated SSPS/HMF (10 g), not subjected to heat inactivation, was adjusted to pH 7 and treated with trypsin, to determine changes in the molecular weight. Trypsin solution (0.1 mL, corresponding to 2.8 kunits as trypsin activity) was added, and the reaction mixture was incubated at 30 °C for 24 h. The mixture was then heated at 90 °C for 20 min to inactivate the enzyme, and after the mixture had cooled to room temperature, aliquots (20 μL) were injected in the HPLC.

Trypsin Treatment of Whole SSPS. SSPS was treated with trypsin, and the emulsifying property of the hydrolyzed sample was investigated. Trypsin solution (0.5 mL, corresponding to 13.6 kunits as trypsin activity) was added to 80 g of 5% (w/w) SSPS solution in 20 mM sodium acetate buffer, pH 7, and incubated at 30 °C for 1, 3, 6, or 24 h. The reaction mixture was then heated at 90 °C for 20 min to inactivate the enzyme. The hydrolyzed samples were then used to prepare emulsions, by adding 20 g of soybean oil and then following the method described previously. To determine if heating affected the emulsifying properties of the trypsin-treated SSPS, control emulsions were also prepared without the heat-inactivation step.

Further Purification of SSPS/HMF. Pectinase treatment was performed in the SSPS/HMF to digest the polysaccharide chains of SSPS. A 500 μL pectinase solution (300 units as a α-polygalacturonase) was added to 50 mL of 1% (w/w) SSPS/HMF solution in 50 mM sodium acetate buffer, pH 5. The reaction mixture was incubated at 40 °C for 20 h and then heated at 90 °C for 20 min to inactivate the enzyme. Samples were centrifuged at 5000*g* for 20 min at 20 °C (Beckman model J2-21, rotor JA-20, Beckman Coulter Inc., Fullerton, CA), deionized with Bio-Rad AG-1X2 ion exchanger (Nippon Bio-Rad Laboratories, Tokyo, Japan), and then freeze-dried.

Five milliliters of 2% (w/w) pectinase-digested SSPS/HMF dissolved in 20 mM sodium acetate buffer, pH 6, was loaded on a DEAE-Sephacel column (1.0 cm × 5.0 cm; Amersham Biosciences Co.) equilibrated with the same buffer. The column was washed with 20 mM sodium acetate buffer, pH 6, and the bound fractions were eluted with a linear gradient of NaCl from 0 to 0.5 M (in 180 mL) and 60 mL of 1.0 M NaCl at a flow rate of 0.2 mL/min. Fractions (3 mL) were collected, and the peaks were identified as D-1 to D-7. The protein concentration of each fraction was measured by UV absorbance at 280 nm (Beckman model DU640 spectrophotometer, Beckman Coulter Inc.). The sugar concentration was measured according to the phenol−sulfuric acid method (*25*). After 200 μL of sample had been mixed with 200 μL of 5% (w/w) phenol, 1 mL of concentrated sulfuric acid was added quickly and vigorously mixed. After incubation of the mixtures at room temperature for 15 min, the absorbance at 490 nm was measured by the spectrophotometer.

Fraction D-4 obtained by ion exchange chromatography was loaded on a YMC-Pack Protein RP column (4.6 mm × 250 mm; Waters Ltd., Toronto, ON, Canada) equilibrated with 5% CH_3CN solution containing 0.1% TFA. The column was washed with the same solution for 5 min,

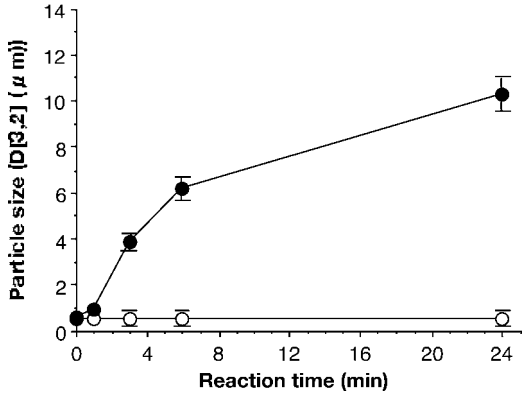

Figure 1. Particle size of the emulsions prepared with SSPS and trypsin-treated SSPS, measured by Mastersizer X: (○) emulsion made with SSPS; (●) emulsion made with trypsin-treated SSPS. Bars indicate values of standard deviation.

and the bound proteins were eluted at a flow rate of 1.0 mL/min with the following CH_3CN gradient: 5−30% of CH_3CN (5−15 min), 30−60% of CH_3CN (15−50 min), and 60−90% of CH_3CN (50−60 min). The protein elution was measured using absorbance at 280 nm. The eluted peaks were separated in five different fractions from RP-1 to RP-5, dried by evaporation, and stored at −20 °C until further analysis.

Analysis of Sugar Composition. Galacturonic acid contents were measured according to the colorimetric method of Blumenkrantz and Asboe-Hansen (*26*). To determine the neutral sugar composition, SSPS (0.1%) was hydrolyzed at 121 °C for 2 h in 2 N trifluoroacetic acid. After removal of the acid by evaporation, the hydrolysates were filtered through a Millipore Molcut II GC filter (Millipore Co. Ltd., Billerica, MA) and analyzed as the borate complexes by HPLC on a TSK-gel SUGAR AXI column (4.6 mm × 150 mm; Tosoh Co. Ltd., Tokyo, Japan) in the presence of ethanolamine (*27*).

Protein and Amino Acid Analysis. Protein content of the sample was measured according to the Lowry method (*28*). Fractions collected by reverse-phase HPLC (RP-2) and SSPS were analyzed the relative molecular weight by SDS-PAGE using 15% gel. The RP-2 fraction (0.5 μg as a protein) and SSPS (10 μg as a protein) dissolved in 50 μL of 20 mM Tris-HCl buffer, pH 6.8, were pipetted into small vials and combined with 30 μL of SDS, 10 μL of 2-mercaptoethanol, and 10 μL of 0.05% bromophenol blue. The vials containing the mixture were placed in a boiling water bath and were stirred vigorously during heating for 5 min. Electrophoresis was performed according to the method of Laemmli (*29*), and proteins were stained with a Coomassie blue staining solution composed of 0.1% phast-gel blue-R, 30% methanol, and 10% acetic acid. The relative molecular weight of the proteins was calculated from the molecular weight standards of phosphorylase B, bovine serum albumin, ovalbumin, bovine carbonic anhydrase, trypsin inhibitor, and lysozyme.

For amino acid analysis (except for cysteine) the powdered samples were hydrolyzed with 6 N HCl containing 0.1% phenol at 110 °C for 24 h. For cysteine analysis, the samples were treated with a performic acid solution at 0 °C for 16 h prior to the hydrolysis by 6 N HCl. The hydrolysates were analyzed using an amino acid analyzer (L-8500A, Hitachi Co. Ltd., Tokyo, Japan). The wavelength for the detection was 570 and 440 nm for proline and hydroxyproline.

RESULTS AND DISCUSSION

Role of Protein in the Emulsifying Property of SSPS. Five different samples were prepared after digestion of SSPS with trypsin for 0, 1, 3, 6, and 24 h. To determine the role of protein in the stabilization of oil-in-water emulsions by SSPS, emulsions were prepared with the partially hydrolyzed SSPS. **Figure 1** illustrates the effect of trypsin treatment on the average droplet size (D[3,2]) of the emulsions made with 4% SSPS and 20% soybean oil at pH 4. The emulsion diameter increased with increasing hydrolysis of the protein fraction in SSPS, indicating

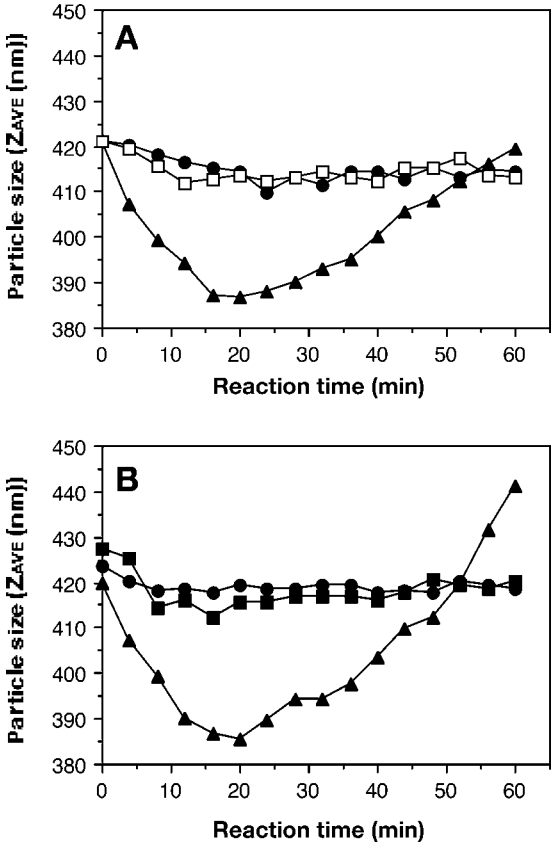

Figure 2. Changes in average particle size of the emulsion droplets treated at pH 4 (**A**) and pH 7 (**B**), measured by dynamic light scattering: (●) no enzyme control; (▲) pectinase-treated emulsion; (□) pepsin-treated emulsion; (■) trypsin-treated emulsion.

a decrease in the emulsifying property of the SSPS fractions. To determine if the heat treatment used to inactivate the trypsin was the cause of the change in functionality, unheated trypsin-treated SSPS fractions were also employed to prepare emulsions, and no differences were shown in the resulting average droplet size (data not shown). These results indicated that the protein plays an important role in the adsorption of SSPS and that partial hydrolysis by trypsin strongly affected the emulsifying behavior. Although peptides were still present in the solution, they were not as efficient in stabilizing the emulsion as the intact SSPS fractions.

The effect of proteases on the adsorbed SSPS were also studied by adding enzymes in diluted emulsions and determining changes in the average droplet diameter using DLS. **Figure 2** shows the effect of pepsin treatment at pH 4 and trypsin treatment at pH 7 on the droplet diameter, compared to the changes caused by the addition of pectinase. There was no size difference between control and protease-treated emulsions, whereas pectinase decreased the droplet diameters of 33.6 ± 2.8 nm at pH 4 and 34.3 ± 3.1 nm at pH 7, as previously reported (*24*). The polysaccharide chains of SSPS cover the surface of the oil droplet with a thick layer, and the addition of pectinase caused a decrease in droplet size up to a minimum value before leading to droplet aggregation. The hydrolysis by pepsin (at pH 4) and trypsin (at pH 7) may be inhibited by the thick interface and by steric interactions caused by SSPS adsorption. This would confirm the hypothesis that the protein adsorbs onto the surface of the oil droplet and acts as an anchor for the SSPS chains. To confirm these results, trypsin treatment (at 30 °C) was also carried out in concentrated systems at pH 7 for 24 h, and no change in particle size distribution was

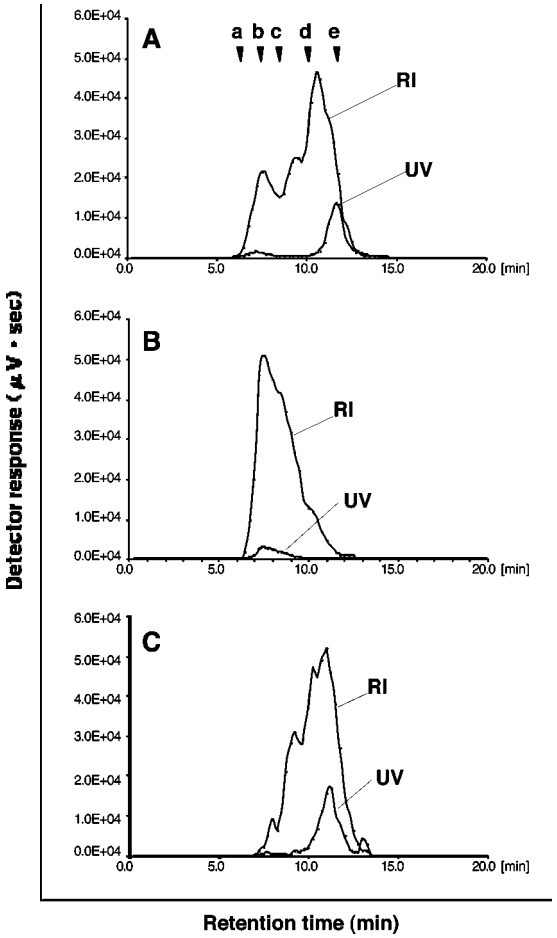

Figure 3. Gel filtration chromatography of (**A**) SSPS, (**B**) SSPS/HMF, and (**C**) SSPS/LMF on a PolySep-GFC-P linear column. Elution was monitored by refractive index and UV detector at 280 nm. Molecular masses (Da) were calculated using standard pullulans (a, 1.60×10^6; b, 3.80×10^5; c, 1.86×10^5; d, 4.80×10^4; e, 1.22×10^4).

observed. Results indicated that the protein fraction of SSPS is not hydrolyzed by trypsin or pepsin after homogenization treatment, once SSPS is adsorbed at the interface.

The present results confirm previous reported models for other polysaccharides (*9*, *10*, *13*). Gum arabic, for example, contains a small amount of protein, and the hydrophobic polypeptide chains are thought to adsorb and anchor the polysaccharide moieties to the surface. The carbohydrate chains of gum arabic inhibit emulsion droplet flocculation and creaming by steric repulsion.

Separation of HMF and LMF from SSPS. Figure 3 shows the elution profile of SSPS and the separated HMF and LMF fractions using GFC on a PolySep-GFC-P linear column. Polysaccharide and protein were measured by refractive index and UV (280 nm) detection, respectively. SSPS eluted in three major fractions (**Figure 3A**) corresponding to 310, 54, and 20 kDa molecular masses (as calculated using pullulan standards). Measurements carried out in parallel using a UV-280 detector indicated that protein was present in the high molecular mass peak (310 kDa) as well as in the low molecular mass fraction (20 kDa).

SSPS could be separated in the two fractions containing protein using GFC using the Sepharose CL-6B column. The elution patterns of the two fractions obtained, one high molecular weight (SSPS/HMF) and one low molecular weight (SSPS/LMF), are depicted in **Figure 3B**,C. The recoveries from SSPS

Table 1. Sugar Composition[a] and Protein Content of SSPS, SSPS/HMF, and SSPS/LMF

	sugar composition (mol %)							sugar (wt %)	protein (wt %)	yield (wt %)
	Rha	Fuc	Ara	Gal	Xyl	Glc	GalA			
SSPS	4.3	1.5	15.3	48.3	1.5	1.6	27.5	86.8	8.2	100.0
SSPS/HMF	10.5	1.3	19.2	36.6	2.5	0.5	29.4	91.6	2.2	20.3
SSPS/LMF	0.8	6.2	31.5	38.3	7.0	0.4	15.8	78.9	13.3	45.8

[a] Rha, rhamnose; Fuc, fucose; Ara, arabinose; Gal, galactose; Xyl, xylose; Glc, glucose; GalA, galacturonic acid.

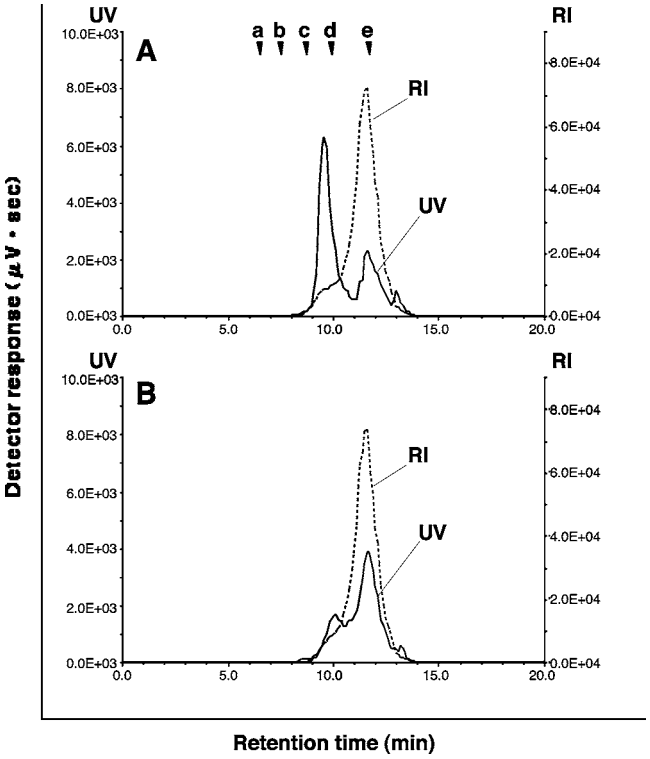

Figure 4. Gel filtration chromatography of SSPS/HMF on a PolySep-GFC-P linear column after treatment with pectinase (**A**) or pectinase followed by trypsin (**B**). Elution was monitored by refractive index and UV detector at 280 nm. Molecular masses (Da) were calculated using standard pullulans (a, 1.60×10^6; b, 3.80×10^5; c, 1.86×10^5; d, 4.80×10^4; e, 1.22×10^4).

of SSPS/HMF and SSPS/LMF were 20.3% (w/w) and 45.8% (w/w), respectively.

The sugar composition, sugar content, and protein content of SSPS, SSPS/HMF, and SSPS/LMF are summarized in **Table 1**. The protein content of SSPS/HMF (2.2%) was lower than that of the original SSPS (8.2%), whereas SSPS/LMF (13.3%) contained a high amount of protein. The sugar composition of SSPS/HMF was similar to that of the whole SSPS, whereas SSPS/LMF showed lower contents of rhamnose (0.8%) and galacturonic acid (15.8%) and higher arabinose (31.5%) than present in whole SSPS.

Molecular Weight Changes of SSPS/HMF with Pectinase and Trypsin. To better understand the effect occurring on the SSPS/HMF with the addition of enzymes, SSPS/HMF fractions treated with pectinase and with trypsin were analyzed by GFC. The elution chromatograms are summarized in **Figure 4**. The molecular weight of the SSPS/HMF decreased after the addition of pectinase, and although RI detection showed only a peak at low molecular weight (compare to **Figure 3B**), the UV elution chromatogram showed two fractions eluting at about 50 and

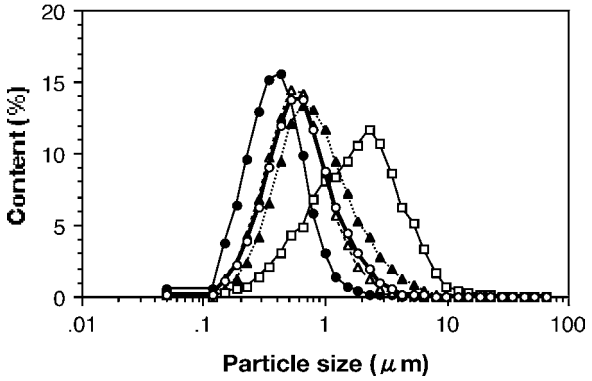

Figure 5. Particle size distribution measured by Mastersizer X of 20% oil-in-water emulsion prepared with 4% SSPS or of a mixture of SSPS/HMF and SSPS/LMF at pH 4: (●) HMF/LMF = 1:0; (△) HMF/LMF = 1:2; (▲) HMF/LMF = 1:3; (□) HMF/LMF = 0:1; (○) intact SSPS.

10 kDa. The decrease in size of SSPS/HMF with pectinase explains the changes in droplet size and destabilization shown in **Figure 2**.

The SSPS/HMF was then further treated with trypsin to determine if the protein fraction present in this purified SSPS was affected by this enzyme. In fact, although **Figure 1** demonstrates that a pretreatment of SSPS with the trypsin strongly affected the emulsifying properties of SSPS, the addition of trypsin to emulsion droplets did not show destabilization (**Figure 2**). The elution chromatogram of SSPS/HMF after trypsin treatment showed a substantial breakdown of the high molecular weight fraction of the proteinaceous material present in the SSPS. These results confirmed the mechanism of stabilization previously hypothesized that the protein portion of SSPS reacts as an anchor to adsorb SSPS molecule onto oil/water interfaces and explained the changes in emulsifying properties after hydrolysis shown in **Figure 1**.

Emulsifying Properties of SSPS/HMF and SSPS/LMF Fractions. It has been previously demonstrated that molecular differences of SSPS lead to differences in the emulsification behavior (*24*). This has also been shown for the emulsifying properties of pectins. For example, Akhtar et al. (*16*) indicated that pectin with a molecular mass of 70 kDa gave the best results in terms of particle size diameter and stability to creaming. However, the emulsifying properties of pectin are affected not only by the molecular weight but also by the amount of protein present (*17*). For this reason, the two fractions obtained by preparative gel permeation chromatography were used to prepare emulsions, to determine which fraction plays a major role during adsorption of SSPS onto the surface of the oil droplets.

The emulsifying properties of SSPS/HMF and SSPS/LMF were evaluated by preparing 20% soybean oil-in-water emulsions at pH 4. The emulsions were prepared with 4% (w/w) of SSPS fractions and compared to emulsions containing whole SSPS. The emulsifying properties of mixtures of SSPS/HMF and SSPS/LMF at different ratios (1:0, 1:1, 1:2, 1:3, 0:1) were also assessed, keeping the final concentration of SSPS to 4%. The droplet size distribution of the various emulsions measured by integrated light scattering is shown in **Figure 5**, and the average particle sizes (D[3,2] and D[4,3]) are summarized in **Table 2**.

The emulsion made with SSPS/HMF showed a monomodal distribution of droplets with an average size (D[3,2]) of 0.38 μm. These emulsions showed a smaller size distribution than did emulsions prepared with SSPS. On the other hand, the emulsion made with SSPS/LMF showed a wide distribution of sizes, with larger average diameters than those reported for 4% SSPS emulsions at pH 4. The average size of the droplets in the emulsions prepared with different ratios of high molecular weight and low molecular weight fractions increased gradually according to the increase of SSPS/LMF content in the mixtures. This result may indicate that the SSPS/LMF, which contains the highest amount of proteinaceus material, has a negative impact on the emulsifying properties of SSPS.

Table 2. Particle Size of the Emulsion Made with Fractionated SSPS Measured by Mastersizer X

	particle size (μm)	
SSPS HMF:LMF	D [3,2]	D [4,3]
1.0:0.0	0.38 (±0.03)	0.52 (±0.04)
1.0:0.5	0.40 (±0.03)	0.54 (±0.04)
1.0:1.0	0.40 (±0.0.5)	0.55 (±0.07)
1.0:1.5	0.49 (±0.06)	0.68 (±0.08)
1.0:2.0	0.54 (±0.05)	0.78 (±0.08)
1.0:2.0	0.68 (±0.08)	0.89 (±0.09)
1.0:3.0	0.67 (±0.09)	1.10 (±0.14)
0.0:1.0	1.12 (±0.18)	2.41 (±0.29)
whole	0.57 (±0.06)	0.86 (±0.08)

The distribution of droplet size and D[3,2] value of emulsions made with the mixed SSPS in the ratio of HMF/LMF = 1:2 were comparable to those of emulsions prepared with the original SSPS. The actual content of HMF and LMF in the SSPS was estimated at approximately this ratio (1:2.3), and it was in agreement with the yield obtained after separation of the two fractions by gel permeation chromatography (**Table 1**).

These results indicated that the emulsifying property of SSPS was carried by the high molecular weight fraction, whereas the low molecular weight fraction seemed to affect negatively the emulsifying properties of SSPS. The small amount of protein present in the SSPS/HMF [2.2% (w/w)] may play an important role, adsorbing at the interface and acting as an anchor for the carbohydrate regions.

This behavior would be similar to what is reported for gum arabic, as its protein fraction anchored to the high molecular weight component adsorbs onto the surface of oil droplet. The protein content and average molecular weight were estimated for gum arabic as <10% and ~250 kDa, respectively (*30*). On the other hand, when compared with gum arabic, the surface-active fraction of SSPS, SSPS/HMF, has a lower protein content and higher molecular weight.

Purification and Isolation of Protein in SSPS/HMF. The carbohydrate chains of SSPS/HMF were treated with commercial pectinase to further characterize the proteinaceus fraction present. The pectinase-digested SSPS/HMF was further purified by ion exchange chromatography on a DEAE-Sephacel column, as shown in **Figure 6**. Seven individual fractions were separated according to the 280 nm absorbance readings (D-1 to D-7). The analytical results of sugar, uronic acid, protein, and the ratio of protein to sugar content were summarized in **Table 3**. Fraction D-1 was rich in sugar content, perhaps because of the monosaccharides and oligosaccharides released during pectinase treatment. On the other hand, the last fraction collected (D-7), which adsorbed strongly to the column, was high in uronic acid (69.6%) and had a low ratio of protein to sugar content (<0.1). D-4 contained the highest ratio of protein to sugar content (8.3) and was further purified by reverse-phase HPLC.

Figure 7 shows the chromatogram for D-4 analyzed by reverse-phase HPLC on a YMC-Pack Protein RP column. This fraction was separated in six major peaks, and five peaks were collected (RP-1 to RP-5). The two major peaks eluting at 2.5 and 53.5 min (RP-1 and RP-5) were not proteinaceous material,

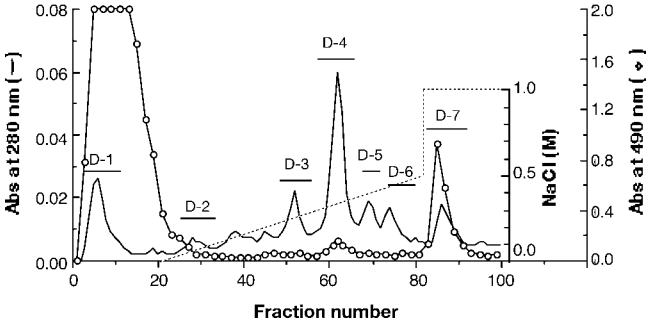

Figure 6. Ion exchange chromatography of pectinase-digested SSPS/HMF on a DEAE-Sephacel column. Protein and carbohydrate were measured by UV absorption (abs at 280 nm) and colorimetric measurement according to the phenol–sulfuric acid method (abs at 490 nm), respectively. Fractions 3–12 (D-1), 24–33 (D-2), 48–55 (D-3), 57–63 (D-4), 67–72 (D-5), 73–79 (D-6), and 83–92 (D-7) were pooled.

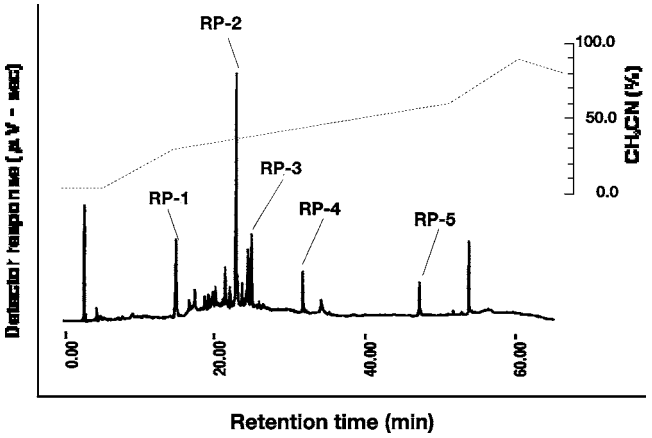

Figure 7. Reverse-phase HPLC of the D-4 fraction on a YMC-Pack Protein RP column. Elution was monitored by UV detector at the absorbance of 280 nm.

Table 3. Analytical Data of the Fractions of Enzyme-Digested SSPS/HMF Separated on a DEAE-Sephacel Column

	total sugar[a] (wt %)	uronic acid (wt %)	protein (wt %)	protein/total sugar
D-1	97.2	6.2	1.9	0.02
D-2	43.1	7.3	48.3	1.12
D-3	15.2	5.8	81.2	5.34
D-4	10.4	8.7	86.3	8.30
D-5	9.9	7.4	81.3	8.21
D-6	15.6	2.5	81.6	5.23
D-7	88.3	69.6	6.2	0.07

[a] Total sugar (wt %) = neutral sugar (wt %) + uronic acid (wt %).

whereas the major protein peak, RP-2, eluted at 22.0 min (concentrated of CH_3CN = 38%). RP-2 was collected, and the molecular mass was analyzed by SDS−polyacrylamide gel (15%) electrophoresis (**Figure 8**) and contrasted with whole SSPS.

RP-2 showed a single band by SDS-PAGE with a molecular mass of 50 kDa, whereas SSPS showed no distinct bands in the electrophoresis. RP-2 was also analyzed for amino acid composition, and **Table 4** shows the amino acid composition, sugar, and galacturonic acid contents of RP-2, compared with those in whole SSPS, SSPS/HMF, and SSPS/LMF. Proline and glutamic acid were predominantly present in RP-2 and SSPS/HMF, whereas the predominant amino acids for SSPS and SSPS/LMF were glutamic acid and aspartic acid. The RP-2 fraction contains the main constituents of SSPS/HMF and could

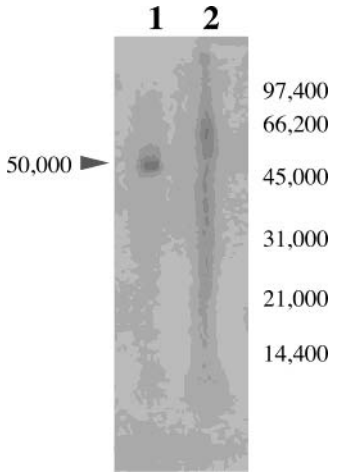

Figure 8. SDS–polyacrylamide gel (15%) electrophoresis of the RP-2 fraction: (lane 1) RP-2 fraction (0.5 μg as a protein); (lane 2) SSPS (10 μg as a protein). Molecular mass standards were phosphorylase B (97400), bovine serum albumin (66200), ovalbumin (45000), bovine carbonic anhydrase (31000), trypsin inhibitor (21500), and lysozyme (14400).

Table 4. Amino Acid Composition and Sugar Content of SSPS and Separate Fractions of SSPS

	SSPS			
amino acid (mol %)	whole	HMF	LMF	RP-2
Asp	10.1	8.1	11.2	6.0
Thr	5.5	5.0	4.8	3.5
Ser	6.0	5.4	6.0	3.2
Glu	18.4	17.0	18.7	15.2
Gly	7.9	5.2	7.6	3.5
Ala	9.2	4.8	8.5	3.0
Val	5.3	4.0	5.2	2.8
Cys	0.5	1.0	0.6	1.2
Met	1.0	2.1	0.9	1.5
Ile	3.2	3.5	3.0	3.4
Leu	5.1	5.2	5.1	4.8
Tyr	1.8	2.9	2.2	2.5
Phe	2.6	3.3	2.6	3.6
Lys	7.0	7.4	7.1	8.2
His	3.2	3.9	3.0	4.6
Arg	4.8	8.0	4.9	8.2
Pro	7.0	12.1	7.2	23.1
Hyp	0.8	1.1	0.6	1.7
total sugar (wt %)	86.8	91.6	78.9	8.8
uronic acid (wt %)	23.9	26.9	12.5	5.3

be the anchor attached to the carbohydrate moieties in SSPS. RP-2 contained 8.8% (w/w) of sugar and 5.3% (w/w) of uronic acid, which constitutes the SSPS main backbone.

Randall et al. (*10*) reported that the predominant amino acids of the protein in gum arabic are hydroxyproline and serine. It was also reported that the polypeptide, mainly composed of hydroxyproline and serine, binds to the polysaccharide chains of gum arabic via *o*-galactosylhydroxyproline (*31*). The low hydroxyproline content of RP-2 (1.7%) or SSPS/HMF (1.1%) and the fact that the reducing end of the polysaccharide chains is galacturonic acid (*22*) may indicate that the linkage between protein and sugar in SSPS is different from that reported for gum arabic.

Conclusions. The role of the protein fraction present in SSPS during adsorption at oil/water interfaces was studied by partial hydrolysis of the protein portion with proteases. In particular, the emulsifying properties of SSPS were affected by a pretreatment of SSPS with trypsin. On the other hand, the diameter of the oil droplets in emulsions stabilized by SSPS did not change after the addition of pepsin (pH 4.0) or trypsin (pH 7.0). These

results indicated that the protein portion of SSPS has an important role during SSPS adsorption. SSPS covers the surface of the oil droplets with a thick layer (*24*), inhibiting the access of the proteases.

The results presented suggest that the hydrophobic protein present in the high molecular weight fraction of SSPS adsorbs at the interface and acts as an anchor for the SSPS chains. The carbohydrate moieties stabilize the droplets against aggregation by steric repulsions.

SSPS contains two different fractions of different molecular masses: 310 kDa (SSPS/HMF; 20% of SSPS) and 20 kDa (SSPS/LMF; 45% of SSPS). These two fractions contain proteinaceous material and a similar carbohydrate composition.

The low molecular weight fraction (SSPS/LMF), although containing the highest amount of protein, did not show good emulsifying properties if used in isolation. On the other hand, the emulsions prepared with SSPS/HMF showed a monomodal distribution with an average particle size smaller than that of emulsions prepared with whole SSPS. Emulsions prepared with increasing amounts of SSPS/LMF in the mixture showed a decrease in emulsifying properties, as the average particle size and the droplet distribution increase with the increase of SSPS/LMF content in the mixtures. The protein fraction in SSPS/HMF was separated by ion exchange chromatography and reverse-phase HPLC. The highest protein fraction, RP-2, showed the relative molecular mass of 50 kDa by SDS-PAGE and a high ratio of proline and glutamic acid. The presence of galacturonic acid in RP-2 indicates that the protein was still covalently bound to a portion of the polysaccharide chains of SSPS.

LITERATURE CITED

(1) Glicksman, M. Gum arabic, In *Food Hydrocolloids*; Glicksman, M., Ed.; CRC Press: Boca Raton, FL, 1982; pp 7−30.

(2) Ray, A. K.; Bird, P. B.; Iacobucci, G. A.; Clark, B. C. Functionality of gum arabic: Fractionation, characterization and evaluation of gum fractions in citrus oil emulsion and model beverages. *Food Hydrocolloids* **1995**, *9*, 123−131.

(3) Kim, Y. D.; Morr, C. V.; Schenz, T. W. Microencapsulation properties of gum arabic and several food proteins: Liquid oil emulsion particles. *J. Agric. Food Chem*. **1996**, *44*, 1308−1313.

(4) McNamee, B. F.; O'Riordan, E. D.; O'Sullivan, M. Emulsification and microencapsulation properties of gum arabic. *J. Agric. Food Chem.* **1998**, *46*, 4551−4555.

(5) Buffo, R. A.; Reineccius, G. A.; Oehlert, G. W. Factors affecting the emulsifying and rheological properties of gum acacia in beverage emulsions. *Food Hydrocolloids* **2001**, *15*, 53−66.

(6) Akiyama, Y.; Eda, S.; Kato, K. Gum arabic is a kind of arabinogalactan-protein. *Agric. Biol. Chem*. **1984**, *48*, 235−237.

(7) Anderson, D. M. W.; Howlett, J. E.; McNab, C. G. A. The amino acid composition of the proteinaceous of *Acacia senagal* gum. *Carbohydr. Res.* **1985**, *2*, 104−114.

(8) Phillips, G. O.; Williams, P. A. Interaction of hydrocolloids in food systems. In *Ingredient Interactions—Effect on Food Quality*; Gaonkar, A. G., Ed.; Dekker: New York, 1995; pp 131−170.

(9) Connolly, S.; Fenyo, J. C.; Vandewelde, M. C. Heterogeneity and homogeneity of an arabinogalactan-protein: Acacia senegal gum. *Food Hydrocolloids* **1987**, *1*, 477−480.

(10) Randall, R. C.; Phillips, G. O.; Williams, P. A. The role of proteinaceous component on the emulsifying properties of gum Arabic. *Food Hydrocolloids* **1988**, *2*, 131−140.

(11) Islam, A. M.; Phillips, G. O.; Sljivo, A.; Snowden, M. J.; Williams, P. A. A review of recent developments on the regulatory, structural and functional aspects of gum arabic. *Food Hydrocolloids* **1997**, *11*, 493−505.

(12) Jayme, M. L.; Dunstan, D. E.; Gee, M. L. Zeta potential of gum arabic stabilized oil-in-water emulsions. *Food Hydrocolloids* **1999**, *13*, 459−465.

(13) Dickinson, E. Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids* **2003**, *17*, 25−39.

(14) Dea, I. C. M.; Madden, J. K. Acetylated pectic polysaccharides of sugar beet. *Food Hydrocolloids* **1986**, *1*, 71−88.

(15) Endress, H. U.; Rentschler, C. Chances and limit for the use of pectin as emulsifier. Part 1. *Eur. Food Drink Rev.* **1999**, Summer, 49−53.

(16) Akhtar, M.; Dickinson, E.; Mazoyer, J.; Langendorff, V. Emulsion stabilisation of depolymerized pectin. *Food Hydrocolloids* **2002**, *16*, 249−256.

(17) Leroux, J.; Langendorff, V.; Schick, G.; Vaishnav, V.; Mazoyer, J. Emulsion stabilizing properties of pectin. *Food Hydrocolloids* **2003**, *17*, 455−462.

(18) Maeda, H. Soluble soybean polysaccharide. In *Handbook of Hydrocolloids*; Phillips, G. O., Williams, P. A., Eds.; CRC Press: Cambridge, U.K., 2000; pp 309−319.

(19) Nakamura, A.; Furuta, H.; Kato, M.; Maeda, H.; Nagamatsu, Y. Effect of soluble soybean polysaccharides on the stability of milk protein under acidic conditions. *Food Hydrocolloids* **2003**, *17*, 333−343.

(20) Nakamura, A.; Furuta, H.; Maeda, H.; Nagamatsu, Y.; Yoshimoto, A. Analysis of structural components and molecular construction of soybean soluble polysaccharides by stepwise enzymatic degradation. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 2249−2258.

(21) Nakamura, A.; Furuta, H.; Maeda, H.; Takao, T.; Nagamatsu, Y. Analysis of the molecular construction of xylogalacturonan isolated from soluble soybean polysaccharides. *Biosci., Biotechnol., Biochem*. **2002**, *66*, 1155−1158.

(22) Nakamura, A.; Furuta, H.; Maeda, H.; Takao, T.; Nagamatsu, N. Structural studies by stepwise enzymatic degradation of the main backbone of soybean soluble polysaccharides consisting of galacturonan and rhamnogalacturonan. *Biosci., Biotechnol., Biochem*. **2002**, *66*, 1301−1313.

(23) Furuta, H.; Maeda, H. Rheological properties of water-soluble soybean polysaccharides extracted under weak acidic condition. *Food Hydrocolloids* **1999**, *13*, 267−274.

(24) Nakamura, A.; Takahashi, T.; Yoshida, R.; Maeda, H.; Corredig, M. Emulsifying properties of soybean soluble polysaccharide. *Food Hydrocolloids* **2004**, in press.

(25) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Robers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *25*, 350−356.

(26) Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem*. **1973**, *54*, 484−489.

(27) Nakamura, A.; Hatanaka, C.; Nagamatsu, Y. Ultraviolet spectrometric determination of neutral monosaccharides by HPLC with ethanolamine. *Biosci., Biotechnol., Biochem*. **2000**, *64*, 178−180.

(28) Lowry, O. H.; Rosebrough, J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(29) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(30) Williams, P. A.; Phillips, G. O. Gum arabic. In *Handbook of Hydrocolloids*; Phillips, G. O., Williams, P. A., Eds.; CRC Press: Cambridge, U.K., 2000; pp 155−168.

(31) Fong, C. W. Q.; Lamport, D. T. A. Gum Arabic glycoprotein is a twisted hairy rope. A new model based on *o*-galactosylhydroxyproline as the polysaccharide attachment site. *Plant Physiol*. **1991**, *96*, 848−855.

Received for review February 18, 2004. Revised manuscript received June 1, 2004. Accepted June 10, 2004.

JF049728F