# Influence of Heating on Oil-in-Water Emulsions Prepared with Soybean Soluble Polysaccharide

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The effect of heating on the physicochemical properties of emulsions prepared with soybean soluble polysaccharide (SSPS) was investigated. The emulsions were stable after heating at 90 °C for up to 30 min. Heating at different pH values or in the presence of $CaCl_2$ (<10 mM) did not affect the stability; however, at higher concentrations of calcium ions, the emulsion particle size increased. Two fractions, a high molecular weight (HMF) and a low molecular weight (LMF) fraction, were separated from the crude SSPS preparation by gel fitration. Emulsions prepared with SSPS/HMF (MW = 310−420 kDa) showed little change in size with heating, while the protein impurities of the SSPS/LMF fraction formed aggregates by heating at pH 7. Analysis of the heat-induced aggregation of the two fractions of SSPS suggested that the changes in SSPS functionality with heating can be attributed to the protein impurities (LMF) present in the SSPS.

## INTRODUCTION

A large number of beverage systems are prepared as a concentrated oil-in-water emulsion and then diluted into the final products (*1*). This type of emulsion has to be stable not only under concentrated conditions but also under highly diluted conditions. In addition, as the emulsions are exposed to a variety of temperature conditions during production, storage, transport, or consumption, it is important to understand the changes in the physicochemical properties of the emulsions during heating.

Beverage emulsions are often formulated with surface-active polysaccharides, such as gum arabic or modified starch (*2−6*). Gum arabic from *Acacia senegal* is most commonly used, because of its high dispersability in water and low bulk viscosity and its ability to create a strong protective film around the oil droplet (*6, 7*). Modified starch has also been investigated as an alternative to gum arabic (*3*). These polysaccharides have relatively low surface activity when compared to low molecular weight surfactants or proteins, and they need to be used in a large amount to obtain stable emulsions (*8, 9*). For example, under comparable homogenization conditions, it has been reported that 20% gum arabic or 12% modified starch is required to obtain a stable emulsion containing 12.5% oil (*9*). Furthermore, the excess unabsorbed polysaccharide present in the aqueous phase of the emulsions can promote droplet flocculation, coalescence, or creaming (*10, 11*). The stability of the emulsions prepared with gum arabic or modified starch during

heating in the presence or in the absence of $CaCl_2$ has been investigated (*9*). The diluted emulsions (0.01%) prepared with gum arabic or modified starch were stable to heating up to 90 °C for 20 min, in spite of the presence of 25 mM $CaCl_2$.

Soybean soluble polysaccharide (SSPS) is a polymer extracted from the residual carbohydrate byproduct of making isolated soy protein, okara. SSPS is an acidic polysaccharide containing 18% of galacturonic acid (GalA) (*12*). The main backbone of SSPS consists of homogalacturonan and rhamnogalacturonan, branched by $\beta$-1,4-galactan and α-1,3- or α-1,5-arabinan chains (*12*). SSPS has a pectin-like structure but a more branched conformation. It has been recently shown using static and dynamic light scattering methods that the radius of gyration of the polysaccharide is about 23.5 ± 2.8 nm with a globular shape structure (*13*). It has also been demonstrated that SSPS contains a protein fraction, which plays a major role in its functionality (*14, 15*).

SSPS can be used to stabilize oil-in-water emulsions over a wide pH range (*15*) and at lower concentrations than those reported for other polysaccharides, for example gum arabic or modified starch (*16, 17*). Without heating, the emulsions prepared with SSPS are not influenced by the addition of $CaCl_2$ or NaCl up to 25 mM. The protein fraction associated with SSPS seems to be responsible for anchoring the carbohydrate moieties of the polysaccharide onto the oil/water interface (*14*). SSPS stabilizes the emulsion particles by steric repulsion, as its hydrophilic portion creates a thick, hydrated layer of about 30 nm, which prevents the droplets from coalescing (*15*). The interactions of SSPS at an oil−water interface were also studied in mixed systems containing small molecular weight surfactants (Tween 20 or Tween 80). The thick layer of SSPS was not

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**Table 1.** Analytical Data of Soybean Soluble Polysaccharides

| | | | | sugar composition (mol %)[a] | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Rha | Fuc | Ara | Gal | Xyl | Glc | GalA | sugar[b] (%) | protein[c] (%) |
| SSPS-L | 4.3 | 1.5 | 15.3 | 48.3 | 1.5 | 1.6 | 27.5 | 86.8 | 8.2 |
| HMF | 10.5 | 1.3 | 19.2 | 36.6 | 2.5 | 0.5 | 29.4 | 91.6 | 2.1 |
| LMF | 0.8 | 6.2 | 31.5 | 38.3 | 7.0 | 0.4 | 15.8 | 13.3 | 52.4 |
| SSPS-M | 4.1 | 2.4 | 20.1 | 47.2 | 1.2 | 1.1 | 23.9 | 87.9 | 5.9 |
| HMF | 4.0 | 1.8 | 17.5 | 46.7 | 0.5 | 4.9 | 24.6 | 92.3 | 2.0 |
| b | 1.9 | 2.4 | 40.6 | 40.1 | 0.5 | 2.3 | 12.2 | 15.9 | 53.3 |

[a] Rhamnose (Rha), fucose (Fuc), arabinose (Ara), galactose (Gal), xylose (Xyl), glucose (Glc), galacturonic acid (GalA). [b] The sugar content was measured by phenol–sulfuric acid method. [c] The protein content at 20 °C was measured by Lowry method.

displaced by small molecular weight surfactants (*18*) and was considered to be stable in comparison with that prepared with whey protein or casein. As heating is an important unit operation in the manufacture of food emulsions, the objective of this study was to investigate the effect of heating on the stabilizing behavior of SSPS.

## MATERIALS AND METHODS

Soybean oil was obtained from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO). Hemicellulase (from *Aspergillus niger*) and other analytical grade reagents were also purchased from Sigma Chemical Co. Ltd. (St. Louis, MO). MilliQ water was used in the preparation of all solutions.

Soybean cotyledons, after removal of the hulls and hypocotyls, were powdered and defatted at 40 °C with 5 volumes of *n*-hexane. Proteins and water-soluble substances in the defatted meal were extracted twice at 50 °C at pH 7.0 for 1 h with 11 volumes of water. SSPS (SSPS-L) was extracted from the residue with hot water by heating at 120 °C at pH 3.0 for 2 h. After removal of insoluble materials by centrifugation, the extract was desalted by electrodialysis (Micro acylyzer G1, Asahi Glass Co., Ltd, Tokyo, Japan) and spray-dried (*12*, *15*). Another extraction (SSPS-M) was carried out from another batch of the same residue at 120 °C at pH 4.0−5.0 for 2 h. The differences in sugar and protein composition between the two SSPS types (L and M) are summarized in **Table 1**.

**Fractionation of SSPS.** The high molecular mass fraction (HMF) and low molecular mass fraction (LMF) of SSPS-L and SSPS-M were prepared by preparative gel-filtration chromatography using a Sepharose CL-6B column (2.5 cm × 90 cm; Amersham Biosciences Co., Piscataway, NJ) according to the method described by Nakamura et al. (*14*). Samples were eluted with 50 mM sodium acetate buffer, pH 5.0. Aliquots (15 mL) of 5.0% (w/w) SSPS solutions filtered through a 0.45 μm filter (Millex-HV, Millipore Co. Ltd., Billerica, MA) were injected. Separation was carried out at a flow rate of 0.3 mL/min, and 4 mL of fractions were collected. 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 electrodialysis (Micro acylyzer G1, Asahi Glass Co., Ltd, Tokyo, Japan) and then freeze-dried. The sugar and protein composition of fractionated SSPS are also shown in **Table 1**.

The polysaccharide samples were enzymatically hydrolyzed by Driselase (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) to measure the galacturonate contents. A solution containing 0.1% polysaccharide and 0.1% glycerol (as an internal standard) in a 50 mM sodium acetate buffer at pH 4.0 was hydrolyzed at 35 °C for 48 h with 100 units/mL of Driselase. After passing the reaction solution through a Millipore Molcut II GC filter, the filtrate was directly analyzed for sugar composition by HPLC on a Shodex SUGAR SH-1821 column (*19*) and on a TSKgel SUGAR AXI column (*20*).

The protein concentration was measured with the Lowry method (*21*) with bovine serum albumin as the standard.

**Analysis of Molecular Mass and Amino Acid Sequence of Peptides Comprising of SSPS-M/LMF.** Positive-ion MALDI-TOF/ TOF mass spectrometry was performed to obtain the molecular mass information of proteins or peptides in SSPS-M/LMF using an Autoflex

II TOF/TOF time-of-flight mass spectrometer equipped with LIFT system, and controlled by the Flexcontrol Ver. 2.4 software package (Bruker Daltonics GmbsH, Bremen, Germany). Fifty milligrams of precipitates obtained from SSPS-M/LMF after heating at pH 7 was suspended in 500 μL of Milli Q water containing 1% SDS and dissolved completely with gentle stirring. SDS was removed from the solution by filtering through a ZipTip filter (ZipTip C₁₈; Nippon Millipore Inc., Tokyo, Japan). The protein fraction bound to the filter was then extracted with 100 μL of 90% acetonitrile. One microliter aliquots of the sample solutions were placed on concave flat surfaces of a stainless steel plate, mixed with 1 μL of the matrix solution, the supernatant of a 50% acetonitrile with 0.1% trifluoroacynamic acid (TFA) solution saturated with α-cyano-4-hydroxycynamic acid, and then air-dried. The spectra were obtained using reflector mode, and ions generated by a pulsed laser beam (nitrogen laser, $\lambda = 337$ nm, 5 Hz) were accelerated to 23.5 kV with a delayed extraction (100 ns). To determine the amino acid sequence of SSPS-M/LMF, the precursor ions obtained were accelerated to 8 kV and selected in a time ion gate. The fragments were further accelerated by 19 kV in the LIFT cell (LIFT means "lifting" the potential energy for the second acceleration of ion source), and their masses were analyzed after the ion reflector passage (reflection mode). The masses and amino acid sequences of the peptides were analyzed and assigned by FlexAnalysis, Biotools (Ver. 3.0, Bruker Daltonics GmbH, Leipzig, Germany) and Mascot Database search system (Matrix Science Inc, Tokyo, Japan).

**Effect of Heating on SSPS Solution at Different pH Conditions.** Five grams of whole, HMF, and LMF from SSPS-L or SSPS-M were suspended in 50 g of 20 mM sodium acetate buffer, pH 3−7. The SSPS suspensions were heated at 90 °C for 30 min in a water bath (ThermoNESLAB), cooled immediately to room temperature in iced water, and centrifuged by Optima LE-80K (Beckman Coulter Inc., Fullerton, CA) at 3000*g* for 20 min. Both supernatants and precipitates were separated, collected, and freeze-dried.

**Preparation of Emulsions.** SSPS was slowly added, at room temperature with gentle stirring, to a 20 mM sodium citrate or sodium acetate buffer, containing 0.02% sodium azide as an antimicrobial. The pH of SSPS solutions was adjusted to pH 4 by adding 0.1 M HCl. The pH values indicated in the text refer to the pH of the SSPS solutions before emulsification. SSPS solutions were filtered through a 0.45 μm filter (Millex-HV, Millipore Co. Ltd., Billerica, MA) and mixed with soybean oil, to obtain the final concentration of 20% (w/w) and 4% (w/w) for oil and SSPS, respectively. This concentration of SSPS was chosen because, under the homogenization conditions employed, most SSPS should be adsorbed onto the oil droplets and very little left in the dispersed phase (*15*). All mixes were dispersed using a Power Gen 125 (Fisher Scientific Co. Ltd, Nepean, ON, Canada) for 5 min before homogenization. Emulsions were prepared at room-temperature using a laboratory homogenizer (EmulsiFlex-C5, Avestin Inc., Ottawa, Canada) with two passes at 40 MPa. The results presented are averages of at least two independent experiments.

**Effect of Heating on Emulsion Stability.** Heat treatment was carried out on diluted emulsions. The emulsions prepared were diluted to 0.01% (w/w) in 20 mM sodium acetate buffer containing different concentrations of CaCl₂ (adjust the final concentrations to 0−25 mM), pH 3−7. Five milliliter amounts of the diluted emulsions in sample tubes were heated from 60 to 90 °C for up to 60 min in a temperature-controlled water bath (ThermoNESLAB), cooled immediately to room temperature in iced water and stored at 20 °C for 24 h. The particle size distribution was then measured by the Mastersizer X (Malvern Instruments Ltd., Malvern, England). The presentation code used was 0303, corresponding to a relative refractive index of the particles of 1.06, a sample absorption of 0.001, and a refractive index of the solvent of 1.33. The emulsifying ability was determined by comparing the shape of the distributions and the values of the average particle size (*D*[4, 3]).

The apparent diameter of the emulsion droplets was also measured by dynamic laser light scattering (DLS) using a Malvern 4700 optical system attached to a 7032 correlator at 25 °C. Measurements were all made at a scattering angle of 90°, and all emulsions were diluted at a rate of 0.5 μL emulsion per 4 mL of 20 mM sodium acetate buffer, at pHs ranging from 3 to 7. For each sample, repeated sets of five individual runs were carried out.
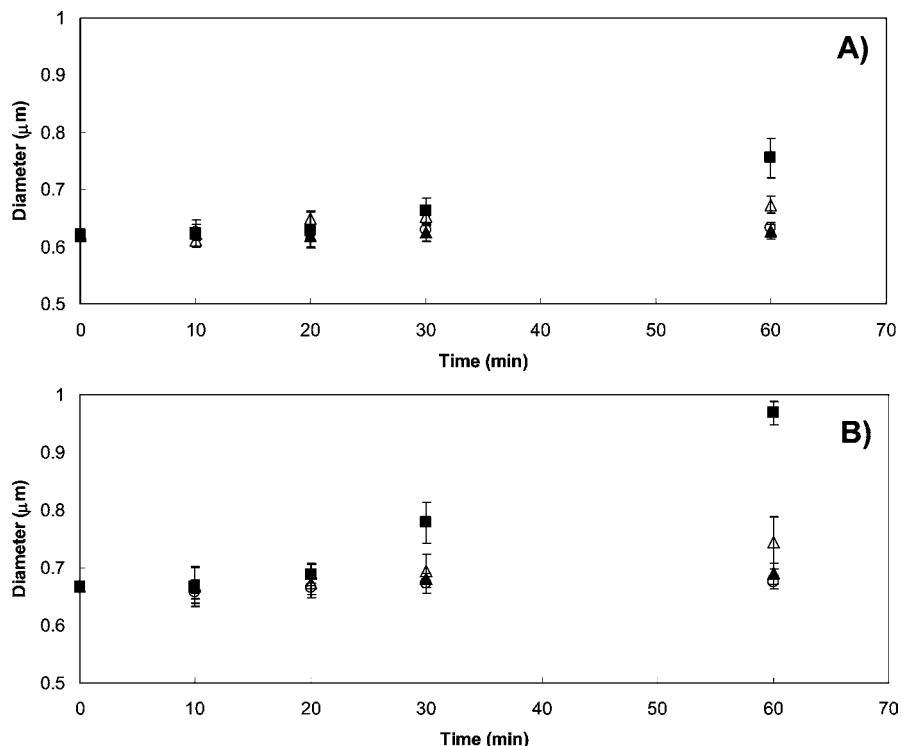
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**Figure 1.** Average particle size of emulsions (*D*[4, 3] measured by Mastersizer X) heated at different temperatures (○: 60 °C, ▲: 70 °C, □: 80 °C, ■: 90 °C) after dilution in water to 0.01% (w/w) at pH 4. The original emulsion contained 20% oil and 4% SSPS-L (A) or SSPS-M (B).

**Table 2.** Mean Droplet Size *D*[4, 3] of Emulsions, Which Were Prepared with SSPS-L or SSPS-M, Measured by Mastersizer after Heated or Nonheated at 90 °C for 30 min at pH 3–7 with or without CaCl$_2$. In Parentheses Are Mean Standard Deviations

| | | nonheated, concn of CaCl$_2$ (mM): | | | | | | heated, concn of CaCl$_2$ (mM): | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 5 | 10 | 15 | 20 | 25 | 0 | 5 | 10 | 15 | 20 | 25 |
| SSPS-L | pH 3 | 0.60 (0.02) | 0.62 (0.03) | 0.66 (0.02) | 0.65 (0.04) | 0.71 (0.02) | 0.76 (0.03) | 0.67 (0.03) | 0.66 (0.03) | 1.57 (0.12) | 1.58 (0.09) | 1.62 (0.13) | 1.70 (0.12) |
| | pH 4 | 0.66 (0.02) | 0.64 (0.04) | 0.68 (0.03) | 0.68 (0.03) | 0.76 (0.02) | 0.78 (0.05) | 0.67 (0.02) | 0.69 (0.04) | 1.24 (0.10) | 1.58 (0.08) | 1.64 (0.14) | 1.71 (0.22) |
| | pH 5 | 0.67 (0.02) | 0.70 (0.03) | 0.75 (0.05) | 0.74 (0.06) | 0.81 (0.04) | 0.86 (0.03) | 0.70 (0.04) | 1.01 (0.09) | 1.88 (0.13) | 1.88 (0.11) | 1.92 (0.24) | 1.98 (0.16) |
| | pH 6 | 0.86 (0.03) | 0.89 (0.06) | 0.92 (0.03) | 0.94 (0.05) | 0.99 (0.04) | 0.98 (0.04) | 1.01 (0.06) | 1.13 (0.09) | 1.89 (0.13) | 1.87 (0.14) | 1.96 (0.17) | 2.09 (0.18) |
| | pH 7 | 0.97 (0.04) | 0.98 (0.05) | 1.00 (0.03) | 1.03 (0.04) | 1.09 (0.09) | 1.06 (0.12) | 1.09 (0.11) | 1.20 (0.09) | 1.53 (0.14) | 1.73 (0.16) | 1.94 (0.20) | 1.97 (0.24) |
| SSPS--M | pH 3 | 0.66 (0.02) | 0.68 (0.02) | 0.71 (0.03) | 0.74 (0.05) | 0.79 (0.03) | 0.81 (0.04) | 0.78 (0.04) | 0.79 (0.03) | 0.83 (0.05) | 0.86 (0.03) | 1.58 (0.11) | 1.66 (0.09) |
| | pH 4 | 0.68 (0.02) | 0.67 (0.04) | 0.72 (0.03) | 0.73 (0.03) | 0.79 (0.05) | 0.82 (0.05) | 0.77 (0.03) | 0.81 (0.04) | 0.86 (0.06) | 0.88 (0.05) | 1.72 (0.18) | 1.81 (0.14) |
| | pH 5 | 0.65 (0.03) | 0.67 (0.04) | 0.74 (0.04) | 0.77 (0.06) | 0.85 (0.02) | 0.91 (0.06) | 0.84 (0.02) | 0.91 (0.02) | 0.96 (0.04) | 0.92 (0.05) | 1.16 (0.09) | 1.80 (0.16) |
| | pH 6 | 0.69 (0.02) | 0.71 (0.02) | 0.84 (0.05) | 0.87 (0.03) | 0.93 (0.08) | 0.95 (0.07) | 0.85 (0.04) | 0.94 (0.06) | 1.03 (0.04) | 1.00 (0.10) | 1.77 (0.11) | 1.89 (0.15) |
| | pH 7 | 0.75 (0.04) | 0.88 (0.05) | 1.07 (0.12) | 1.09 (0.09) | 1.16 (0.16) | 1.24 (0.23) | 1.17 (0.09) | 1.18 (0.07) | 1.66 (0.13) | 1.93 (0.15) | 2.44 (0.17) | 2.88 (0.22) |

**Enzymatic Treatment of SSPS at the Oil/Water Interface.** Aliquots of hemicellulase suspension (400 μL, 60 units as β-galactosidase) were added to a clean cuvette containing the emulsion previously diluted at a rate of 0.5 μL of emulsion per 4.0 mL of 20 mM sodium acetate buffer, pH 7. The changes in droplet size during hydrolysis were measured with the DLS, at 4 min intervals, at 30 °C for 80 min. Emulsions prepared with SSPS-M, SSPS-M/HMF, nonheated, heated at 90 °C for 30 min at pH 7 with or without CaCl$_2$ were tested.

**Microstructural Observations of Emulsions Prepared with SSPS-M.** Phase contrast microscopy observations were carried out after 24 h of storage at refrigeration temperatures. Selected emulsions were analyzed using phase contrast optical microscopy (Olympus BX 60, Markham, Ontario). Emulsions, nonheated, heated, and heated with CaCl$_2$, were gently agitated in their test tube before analysis to ensure homogeneous sampling. Five milliliter amounts of emulsions were diluted with 20 μL of 10 mM imidazole−acetate buffer containing 0.002% sodium azide (to prevent microbial growth) at the same pH of the emulsions. Samples were placed on a microscope slide, covered by a cover slip, and observed using 40× objectives. Images were acquired using a digital camera (Sensys, Carsen Group Inc., Markham,

Ontario) and digital image-processing software (Image-Proplus, Media Cybernetics Inc., MD). All experiments reported were carried out at least in triplicate, and unless otherwise indicated, the average values and standard deviations are reported in Results and Discussion.

## RESULTS AND DISCUSSION

**Effect of Heating on the Emulsions Prepared with SSPS.** The heating stability of emulsions prepared with SSPS-L or SSPS-M was assessed by heating the diluted emulsions (0.01% final oil (w/w) in Milli Q water at pH 4.0. The original emulsions were composed of 20% oil and 4% SSPS (L or M type)) at different temperatures from 60 to 90 °C for up to 60 min. **Figure 1** illustrates the average particle size *D*[4, 3] measured by light scattering (Mastersizer X). Differences in the aggregation behavior were shown between emulsions prepared with SSPS-L and SSPS-M. Heating at 60 or 70 °C for up to 60 min did not affect the average particle size of the emulsions, while aggregation was shown with heating at 80 or 90 °C after 30 min. A higher extent of aggregation seemed to be present in
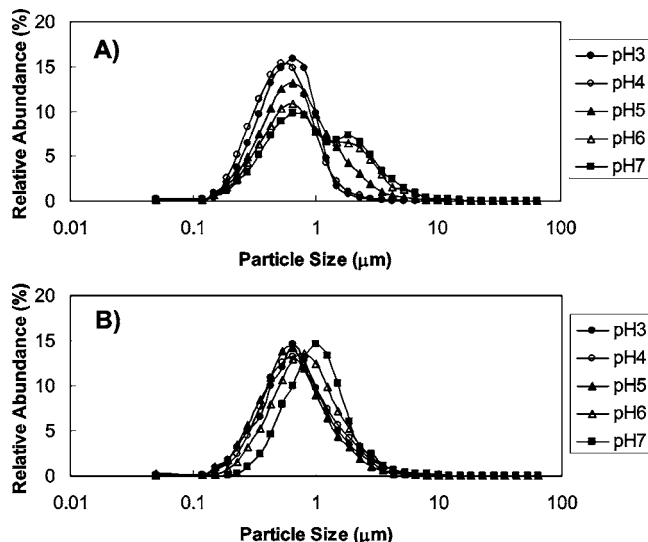
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**Figure 2.** Particle size distribution of diluted emulsions (0.01% oil) heated at 90 °C for 30 min at various pH values. ●: pH 3, ○: pH 4, ▲: pH 5, □: pH 6, ■: pH 7. After heating, the emulsions were immediately cooled to room temperature and stored at 20 °C for 24 h before measurement. (A) Emulsions prepared with SSPS-L. (B) Emulsions prepared with SSPS-M. .



**Figure 3.** Particle size distribution of emulsions prepared with SSPS-L (A) and SSPS-M (B), heated at 90 °C for 30 min after dilution (final concentration 0.01%) in 20 mM sodium acetate buffer containing various concentrations of CaCl$_2$ solution at pH 4. Emulsions were stored at 20 °C for 24 h before measurement. (●) 0 mM CaCl$_2$, (○) 5 mM CaCl$_2$, (▲) 10 mM CaCl$_2$, (□) 15 mM CaCl$_2$, (■) 20 mM CaCl$_2$, (□) 25 mM CaCl$_2$.

the emulsions prepared with SSPS-M. Based on these results, further work was carried out on heating of emulsions at 90 °C for 30 min.

The effect of pH on the heating stability of the diluted emulsions stabilized by SSPS-M and SSPS-L was also assessed by heating at 90 °C for 30 min at different pH values from pH 3 to 7. The particle size distribution of heated emulsions is shown in **Figure 2**. The heating stability of emulsions prepared with SSPS-L was influenced by pH (**Figure 2A**), while less changes were shown for the emulsions prepared with SSPS-M. Emulsions containing SSPS-L showed a monomodal distribution of sizes after heating at pH 3 and 4, while at a higher pH, aggregation occurred. On the other hand, emulsions prepared with SSPS-M showed a monomodal distribution after heating at all pHs, although showing a larger average size at pH 7 (1.22 ± 0.07 μm).

To confirm the results obtained with integrated light scattering (Mastersizer X measurements), the average apparent diameter of the heated emulsions was also measured using DLS at various pH values. The particle diameter of emulsions prepared with SSPS-L increased about 26 nm after heating at 90 °C for 30 min at all pHs. For example, the diameter of the emulsion prepared at pH 3 increased from 416.7 ± 5.3 nm to 442.7 ± 8.6 nm after heating. No significant changes in the average apparent diameter after heating were noted for the emulsions prepared with SSPS-M in the pH range between 3 and 6, with diameter values remaining at about 440 nm. These results confirmed that the heating stability of the emulsions prepared with SSPS varies depending on the type of SSPS, with SSPS-M showing overall a better heating stability than SSPS-L. It has been previously demonstrated (15) that these polysaccharides show a different thickness at the interface (17 versus 35 nm, for SSPS-L and SSPS-M, respectively). The difference in heating stability between the two types of SSPS may be attributed to the differences in the thickness of the adsorbed layer made by SSPS molecules. As SSPS-M, which has higher arabinose and galactose content than SSPS-L (**Table 1**), is considered to have longer neutral chains, the emulsion particle droplets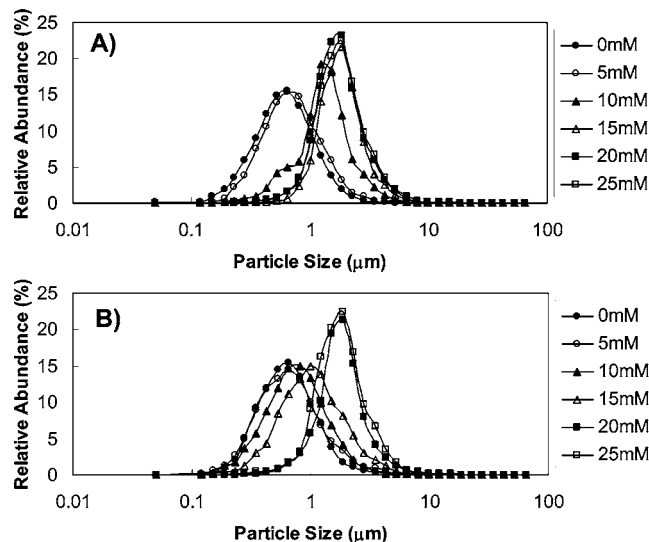 prepared with SSPS-M were suggested to be fully covered with the long galactan and hairy arabinan chains of SSPS and dispersed by stronger steric repulsion than those prepared with SSPS-L even after heating.

**Effect of Calcium on the Heat-Induced Aggregation of Emulsions Prepared with SSPS.** Emulsions were tested for heat stability at various pH values in the presence of varying concentration of CaCl$_2$ (**Table 2**). The 20% oil-in-water emulsions prepared with 4% SSPS-L or SSPS-M were diluted to 0.01% (w/w) in 20 mM sodium acetate containing 5 to 25 mM CaCl$_2$. The diluted emulsions were then heated at 90 °C for 30 min, and the effect of heating and CaCl$_2$ on the particle size distribution at pH 4.0 is summarized in **Figure 3**. The measurements of particle size were carried out 24 h after heating (the emulsions were stored at room temperature). All emulsions after heating showed a monomodal distribution of droplet sizes. It was demonstrated that 10 mM CaCl$_2$ was a critical concentration which affected the average size of the particles.

In the previous report, we assessed the influence of CaCl$_2$ on the particle size of emulsions prepared with SSPS-L or SSPS-M without heating in the concentrated system diluted to 50% (15), and no significant change was observed in mean droplet size of the emulsion for CaCl$_2$ concentrations up to 25 mM. The difference in the critical concentration of CaCl$_2$ may be caused by the difference in dilution factor of the emulsions tested in the two studies. It has been previously suggested (9) for emulsions made with gum arabic or modified starch that changes in the mineral environment affect the changes in the electrostatic interactions between the emulsion droplets. However, no information is available for these emulsions with heating and in the presence of CaCl$_2$. These present results indicate that heating affected the interface of the emulsions, and in the presence of CaCl$_2$ a larger extent of aggregation occurred.

To confirm the measurements by light scattering, phase contrast microscopy was carried out on the emulsions before and after heating. **Figure 4** shows the effect of heating and CaCl$_2$ on diluted emulsions prepared with SSPS-M. Although
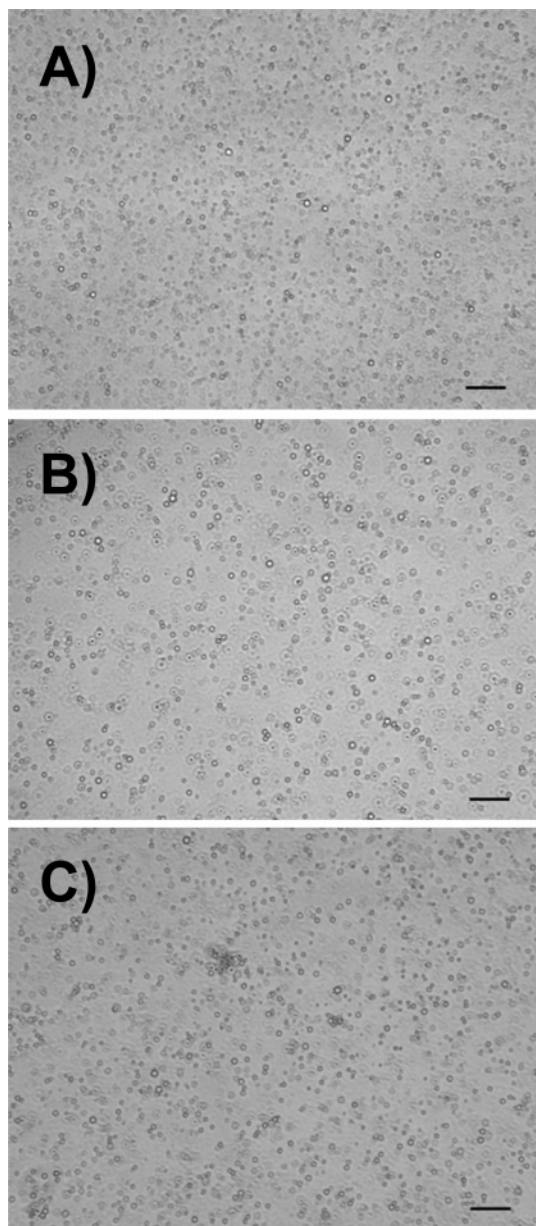
**Figure 4.** Phase contrast microphotographs of 20% oil-in-water emulsions prepared with SSPS-M (pH 7). (A) Nonheated, (B) heated, (C) heated with 20 mM CaCl₂. Scale bar 10 $\mu$m.



**Figure 5.** The amount of insoluble materials resulting from SSPS-L (A) and SSPS-M (B) suspensions (10% (w/w)) after heating at 90 °C for 30 min at various pHs. Values indicated in brackets are protein content (w/ w, as determined by Kjeldhal) for (■) whole SSPS, (□) SSPS/HMF, (■) SSPS/LMF.



**Figure 6.** Particle size distribution of emulsions prepared with SSPS-L/ HMF (A) and SSPS-M/HMF (B), heated at 90 °C for 30 min after dilution (final concentration 0.01%) in 20 mM sodium acetate buffer containing various concentrations of CaCl₂ at pH 7. Emulsions containing (●) 0 mM CaCl₂; (○) 5 mM CaCl₂; (▲) 10 mM CaCl₂; (□) 20 mM CaCl₂.

all emulsion droplets showed similar sizes, a larger extent of flocculation was shown in the heated emulsions in the presence of CaCl₂.

**Effect of Heating on SSPS, SSPS/HMF, and SSPS/LMF Solution.** To better understand the effect of heating on the interfacial changes of emulsions prepared with SSPS, two fractions were prepared from SSPS. It was previously demonstrated that a fraction of low molecular weight can be separated from SSPS, and this fraction negatively affects the emulsifying properties of SSPS (*14*). SSPS/HMF and SSPS/LMF were separated by size exclusion chromatography and tested for stability to heating compared to the whole SSPS fraction. SSPS, SSPS/ HMF, and SSPS/LMF were dissolved in 20 mM sodium acetate buffer, pH 3−7, and the suspensions were heated at 90 °C for 30 min. After being cooled to room temperature, the samples were separated by centrifugation into a soluble and insoluble phase and then freeze-dried. **Figure 5** illustrates the amount of insoluble material obtained after heating at various
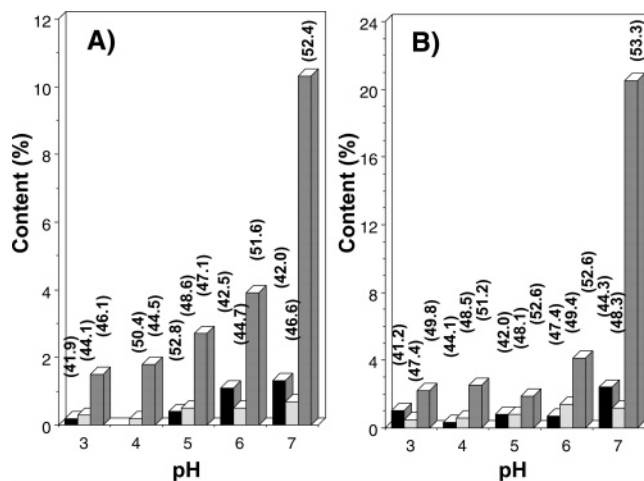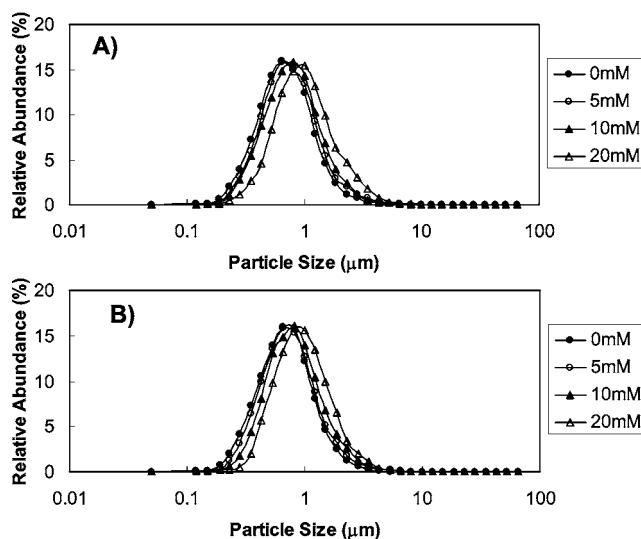
pH values for SSPS-L and SSPS-M and their respective HMF and LMF fraction. In addition, the figure indicates the amount of protein (measured by Kjeldahl, $N \times 6.25$) present in the pellets. It was clearly shown that heating affected the solubility of the protein fraction present in the SSPS, and that heating at pH 7 showed a higher extent of precipitation compared to lower pH values. The amount of insoluble material obtained from the LMF suggested that this fraction does affect the stability of the SSPS-L and SSPS-M. For this reason, the effect of heating on the emulsions prepared with HMF of SSPS-L and SSPS-M was further evaluated.

**Effect of Heating with CaCl₂ on Emulsions Prepared with SSPS/HMF.** Some 20% oil in water emulsions were prepared with SSPS-L/HMF or SSPS-M/HMF and diluted to concentrations of 0.01% (w/w) in 20 mM sodium acetate buffer containing different concentrations of CaCl₂ (0 to 20 mM) at pH 3 to 7 and then heated at 90 °C for 30 min. The average diameter
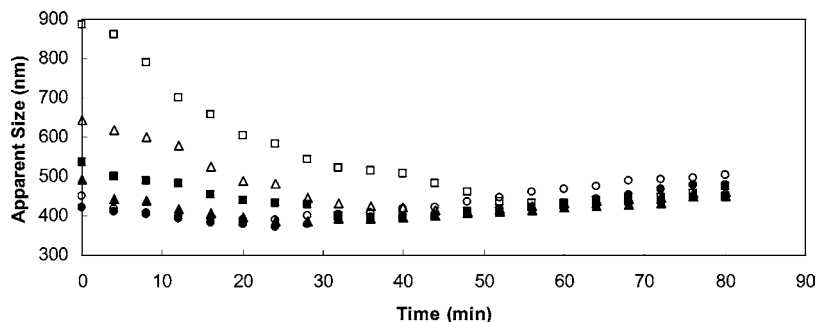
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**Figure 7.** Change in apparent diameter measured by DLS in emulsions treated with hemicellulase. Emulsions were prepared with SSPS-M (empty symbols ○, □, □) or SSPS-M/HMF (solid symbols ●, ▲, ■) and heated at pH 7 at 90 °C for 30 min. Unheated emulsions (○, SSPS-M; ● SSPS-M/HMF); heated emulsions (□, SSPS-M; ▲ SSPS-M/HMF); heated emulsions with 25 mM CaCl₂ (□, SSPS-M; ■, SSPS-M/HMF).
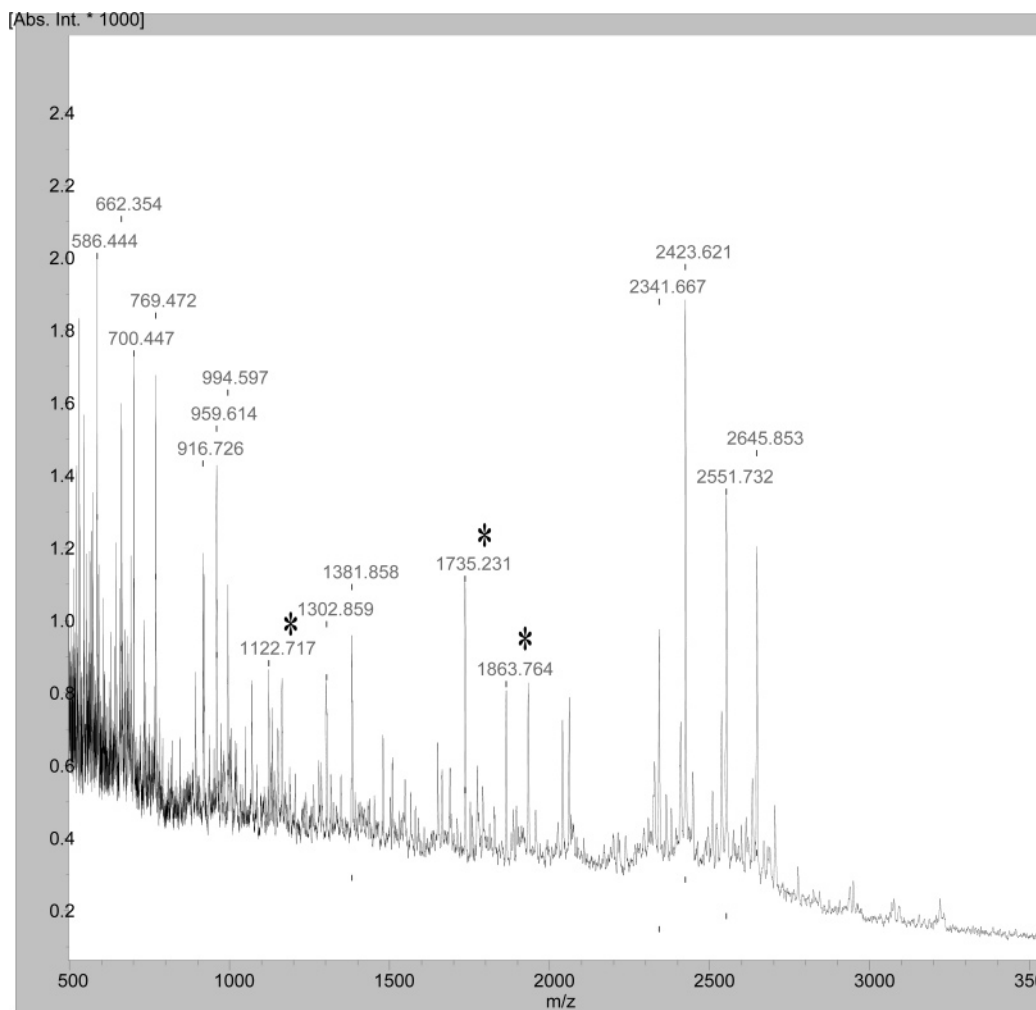


**Figure 8.** Mass spectrum of insoluble materials from SSPS/LMF with heating at 90 °C for 30 min analyzed by MALDI-TOF/TOF mass spectrometer (Autoflex II TOF/TOF) after being dissolved in 1% SDS. *: These fragment ions were analyzed for their amino acid sequences by LIFT-TOF/TOF mode.

**Table 3.** Particle Size $D[4, 3]$ of Emulsions, Prepared with SSPS-L/HMF or SSPS-M/HMF, Measured by Mastersizer after Heated at 90 °C for 30 min at pH 3–7 with CaCl₂. Parentheses Mean Standard Deviations

| | SSPS-L/ HMF, concn of CaCl₂ (mM): | | | | SSPS-M/HMF, concn of CaCl₂ (mM): | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 20 | 0 | 5 | 10 | 20 |
| pH 3 | 0.62 (0.02) | 0.60 (0.03) | 0.64 (0.05) | 0.68 (0.06) | 0.71 (0.04) | 0.69 (0.06) | 0.73 (0.06) | 0.68 (0.08) |
| pH 4 | 0.64 (0.02) | 0.63 (0.04) | 0.68 (0.04) | 0.70 (0.05) | 0.75 (0.08) | 0.80 (0.06) | 0.78 (0.06) | 0.82 (0.10) |
| pH 5 | 0.65 (0.03) | 0.64 (0.03) | 0.69 (0.06) | 0.72 (0.10) | 0.75 (0.06) | 0.77 (0.04) | 0.76 (0.06) | 0.84 (0.09) |
| pH 6 | 0.66 (0.06) | 0.67 (0.05) | 0.69 (0.08) | 0.73 (0.11) | 0.72 (0.02) | 0.74 (0.04) | 0.77 (0.06) | 0.81 (0.10) |
| pH 7 | 0.70 (0.05) | 0.70 (0.04) | 0.79 (0.08) | 0.88 (0.10) | 0.74 (0.06) | 0.75 (0.06) | 0.85 (0.11) | 0.93 (0.18) |

$D[4, 3]$ of the emulsions is summarized in **Table 3**. Emulsions showed a monomodal distribution of particles with a size increase only at pH > 6.0 and in the presence of high concentrations of CaCl₂. As the emulsions showed the highest extent of aggregation at pH 7.0, further work was carried out to characterize the heated particles at this pH. **Figure 6** illustrates
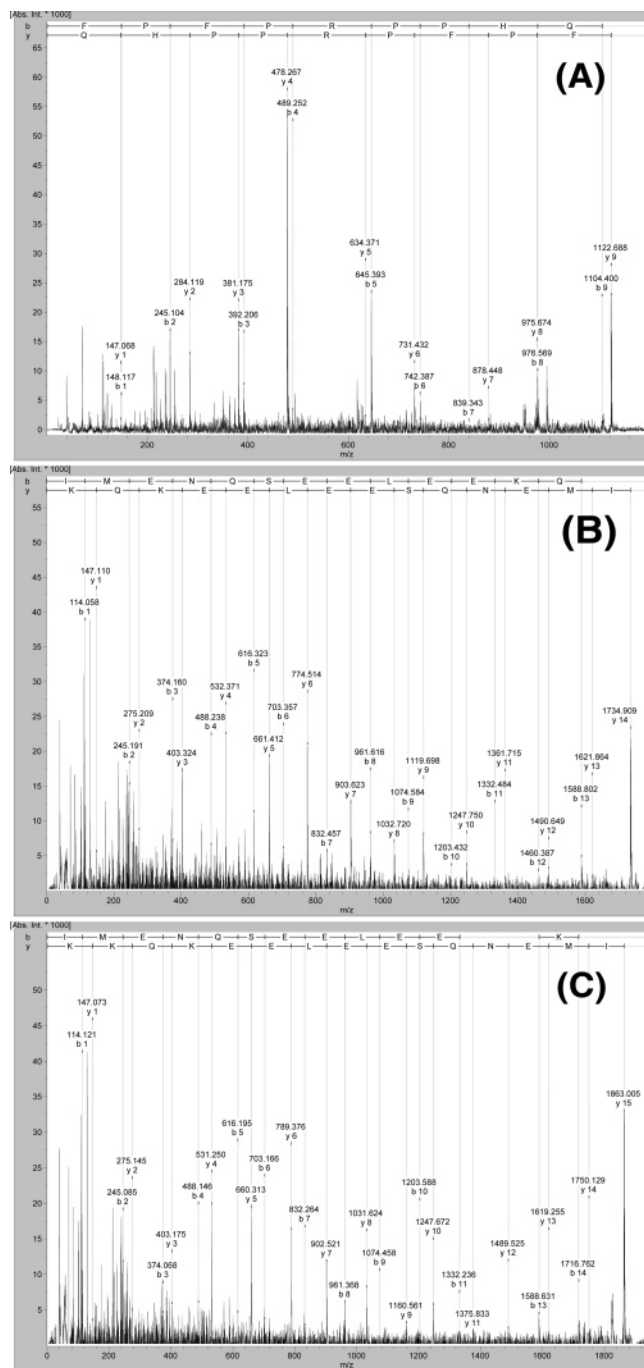
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**Figure 9.** Postsource decay MALDI-TOF/TOF spectra and amino acid sequences assigned to peptide fragments in SSPS-M/LMF. Arrows show the sequences from N and C termini based on $y_m$ and $b_n$ ions, respectively (where *m* and *n* denote positions counted from the C and N termini) that were produced by cleavage of peptide bonds during MS/MS analysis. Amino acids were expressed by a one-letter code.

the particle size distribution of heated emulsions prepared with SSPS-L/HMF (**Figure 6A**) or SSPS-M/HMF (**Figure 6B**). The *D*[4, 3] of heated emulsions prepared with SSPS-L/HMF and SSPS-M/HMF changed from 0.70 μm to 0.88 μm and 0.74 μm to 0.93 μm, in 20 mM CaCl₂.

As indicated in **Table 2**, the average size of the emulsions prepared with the whole SSPS (SSPS-L and SSPS-M) showed an effect of CaCl₂ with heating. To better understand the difference in behavior of whole SSPS and SSPS/HMF, the thickness of the membrane which surrounds the oil droplets was characterized using DLS and treating the oil droplet surfaces

with hemicellulase. The emulsions prepared at pH 7.0 with SSPS-M or SSPS-M/HMF either before or after heating and in the presence of 20 mM CaCl₂ were treated with hemicellulase as previously described (*15*). The enzyme was added to emulsions diluted in buffer at pH 7.0, and the average apparent diameter was measured over time (**Figure 7**). In unheated emulsions (both prepared with SSPS-M or SSPS-M/HMF) the average diameter showed a drop in the average size after about 20 min and then showed a slow increase in size. The change in size before the aggregation has been previously related to the thickness of the adsorbed polymer layer (*14*, *15*). In nonheated emulsions, the size difference between control and hemicellulase-treated emulsion was 59.6 ± 1.8 nm for SSPS-M and 50.4 ± 1.2 nm for SSPS-M/HMF. It could be concluded that the layer thickness between emulsions containing SSPS-M or SSPS-M/HMF were comparable, although the HMF fraction showed a lower average droplet diameter change. After heating, the size difference between control and hemicellulase-treated emulsion was 230.4 ± 12.2 nm for SSPS-M and 104.7 ± 9.4 nm for SSPS-M/HMF. The presence of CaCl₂ affected the average diameter of the emulsions, showing an average at 453.6 ± 13.1 nm and 444.8 ± 8.7 nm, for the unheated emulsions prepared from SSPS-M or SSPS-M/HMF, respectively, while heating showed a significantly higher apparent diameter when the emulsions contained CaCl₂, which confirmed the results described above. The minimum value of droplet size for emulsions prepared with SSPS-M during hemicellulase treatment was 388.8 ± 5.2 nm for nonheated emulsions and 412.4 ± 5.5 or 430 ± 7.8 nm for heated emulsions without or with CaCl₂. Little changes were shown in emulsions prepared with SSPS-M/HMF, where the minimum value reached after hemicellulase treatment was 372.1 ± 3.8 nm, 386.2 ± 4.7 nm, and 391.4 ± 7.6 nm, for unheated, heated, and heated in the presence of calcium, respectively. These results suggested that the LMF fraction of SSPS-M induced aggregation between the emulsion particles or precipitation of SSPS on the surface of emulsions particles via Ca⁺⁺ bridging.

These results indicated that the LMF of SSPS negatively affected the heating stability of emulsions prepared with SSPS. The insoluble material obtained after heating 10% SSPS suspensions contained a high amount of protein (see **Figure 5**), and was dissolved in 1% SDS. These results may suggest that the protein fraction is strongly affected by heat and is sensitive to CaCl₂ and that this fraction caused droplet aggregation on heating of the emulsions at pH 7.0.

**Molecular Masses and Amino Acid Sequences of Peptide Fragments Obtained from SSPS/LMF.** To better understand the composition of the LMF fraction from SSPS, the insoluble material formed after heating of SSPS-M/LMF at 90 °C for 30 min at pH 7 was dissolved in 1% SDS and analyzed by a positive-ion MALDI-TOF/TOF mass spectrometer. The mass spectrum shown in **Figure 8** demonstrated that the LMF fraction separated from SSPS-M is composed of impurities, being composed of less than 3.5 kDa of proteins or polypeptides. The amino acid sequence of some ion signals was also determined by using PSD/ LIFT-MS/MS technique. **Figure 9** shows the three peptide ions in SSPS-M/LMF measured and assigned by Biotools. The amino acid sequences of the ions isolated (with $m/z = 1122.7$, $m/z = 1734.9$, and $m/z = 1863.0$) were as follows: FPFPRPPHQ, IMENQSEELEEKQK, and IMENQ-SEELEEKQKK, respectively. The database search suggested that the first peptide was rich in proline (derived from the α-subunit of β-conglycinin of soybeans, Mascot Score: 56.6). This peptide also contained other hydrophobic and basic amino

acids (phenylalanine and arginine). The other two peptides were rich in glutamic acid (derived from fragments from the 2S albumin subunit of soybeans, Mascot Score: 58.3) and also contained methionine, isoleucine, and leucine (N-terminal) and lysine.

**Conclusions.** The heat-induced aggregation of emulsions prepared with SSPS seems to be mainly related to the presence of low molecular weight impurities, which included proline-rich and glutamic acid-rich peptides. Emulsions prepared with SSPS/HMF (with no LMF impurities) showed much better stability to heating and to $CaCl_2$. These results are the first evidence of the effect of heating on SSPS-stabilized emulsions, and they emphasize the important role played by the protein fractions present in the polymer fraction in affecting the functional properties of the polysaccharide.

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