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Carbohydrate Research 340 (2005) 2637–2644
Carbohydrate RESEARCH

# Molecular characterisation of soybean polysaccharides: an approach by size exclusion chromatography, dynamic and static light scattering methods

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Received 3 June 2005; received in revised form 26 August 2005; accepted 27 August 2005
Available online 29 September 2005

**Abstract**—Water soluble polysaccharides from soybean (SSPS) have a pectin-like structure and are used as stabilisers in acidified beverages. Physicochemical properties such as structure, molecular weight and shape or conformation are primary factors controlling their functional properties. Two soybean polysaccharides, a native SSPS and a modified SSPS treated with β-(1→4)-D-galactosidase (GPase/SSPS) were studied by dynamic and static light scattering (DLS, SLS) and size exclusion chromatography (SEC). Consecutive filtrations using a range of membrane pore size removed a small fraction of macromolecular aggregates from dilute polysaccharide solutions with relatively little effect on the major component molecules as monitored by DLS and SEC measurements. Access to aggregate-free dilute solutions of SSPS and GPase/SSPS allowed the direct measurement of molecular characteristics. SLS results showed that SSPS had a weight average molecular weight of $(645 \pm 11) \times 10^3$ g/mol and a radius of gyration, $R_g$, of $(23.5 \pm 2.8)$ nm. By comparing $R_g$ with the hydrodynamic radius, $R_h$ $(21.1 \pm 0.5$ nm) obtained from DLS, the structural parameter $\rho$ ($R_g/R_h$) was found to be 1.1, suggesting that SSPS has an overall globular shape due to a highly branched structure. The modified SSPS had a significantly lower molecular weight $(287 \pm 18) \times 10^3$ g/mol but a similar radius of gyration $(23.2 \pm 1.7$ nm). The structure parameter $\rho$ of GPase/SSPS was higher ($\rho = 1.3$) because of a smaller hydrodynamic radius $(17.7 \pm 1.8$ nm). This suggests that GPase/SSPS has a much less branched structure yet still differs significantly from a linear random coil conformation ($\rho = 1.7$–$2.0$). The results derived from SLS and DLS are in agreement with the conclusions obtained from a chemical analysis where the reduction of molecular weight of GPase/SSPS was caused by the cleavage of galactan side chains.


*Keywords:* Soybean soluble polysaccharides; Static and dynamic light scattering; Aggregation; Conformation; β-(1→4)-D-Galactosidase

## 1. Introduction

Water soluble soybean polysaccharide (SSPS) is a commercially available food ingredient extracted from soybean cotyledons using high temperatures under weakly acidic conditions.[1] It is soluble in both cold and hot water giving solutions with a relatively low viscosity. The SSPS extracted from soybean cotyledons is composed of D-galactose, L-arabinose, D-galacturonic acid (GalA) and L-rhamnose (Rha). Like pectin, it is an acidic polysaccharide, which is abundant in the peels of citrus fruits. The main backbone of SSPS consists of galacturonan (α-(1→4)-galacturonan), and rhamnogalacturonan, which is composed of a diglycosyl repeating unit, →4)-α-D-GalA-(1→2)-α-L-Rha-(1→. However, the contents of neutral monosaccharides in SSPS are

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doi:10.1016/j.carres.2005.08.012

much higher than those of citrus pectin. SSPS is considered to have a globular structure with long neutral side chains of α-(1→3) and α-(1→5)-arabinan and β-(1→4)-galactan, which are, respectively, composed of 20.7% and 49.8% of total sugars.[2] Recent studies showed soybean polysaccharide had an excellent ability to stabilise acidic milk beverages by preventing milk protein aggregation.[2] The mechanism by which SSPS stabilises milk proteins appears to be different from that of high methoxyl pectin. In the case of high methoxyl pectin, protein particles (mainly casein micelles) are believed to be fully covered by negatively charged pectin molecules, preventing proteins from aggregation due to electrostatic repulsion. In contrast, the existence of high proportion of long side chains in SSPS seems to play a major role in stabilising milk proteins, possibly via a steric effect. In addition, the good emulsifying property of SSPS has also been demonstrated in oil-in-water emulsions.[3] The emulsifying properties of SSPS were found to be dependent on the fine structure and molecular weight of SSPS. The enzymatic degradation of SSPS to remove side chains resulted in oil droplet aggregation, indicating that oil droplet stabilisation occurred mainly via steric repulsion.

Light scattering technique is one of the few absolute methods available for measuring the size and shape of high molecular weight polymers. There are two types of light scattering measurements, static and dynamic light scattering. Static light scattering measures the average total scattering intensity over a selected time period. It provides a convenient method for deriving several molecular parameters simultaneously, including weight average molecular weight ($M_w$), second virial coefficient ($A_2$) and radius of gyration ($R_g$). Dynamic light scattering measures the fluctuation of the intensity over time through the determination of a time-dependent autocorrelation function. An appropriate mathematical approach has to be used to extract meaningful physical parameters, that is, the translational diffusion coefficient, $D$. This translational diffusion coefficient is related to hydrodynamic radius $R_h$ through the Stokes–Einstein relationship for small spheres.

$$D = kT/6\pi\eta_s R_h, \quad (1)$$

where $T$ is absolute temperature, $k$ is the Boltzmann constant, and $\eta_s$ is the solvent viscosity. Cumulant analysis is the most often used method for analysing an autocorrelation function;[4] however, it yields erroneous results when the solution contains particles of multiple size distributions. An example is given by a polysaccharide solution containing both individual molecules and large molecular aggregates.[5] In this case, an alternative approach has to be employed. Several such methods of various complexities have been developed including constrained regularisation (CONTIN),[6] non-negative least squares (NNLS)[7] and double exponential (DE) methods.[7] The objectives of the present study were to determine the molecular characteristics of SSPS compared to a structurally modified SSPS that was obtained by treatment of the polysaccharide with an *exo*-β-(1→4)-D-galactosidase (GPase) to remove galactan side chains. This enzyme specifically hydrolyses β-(1→4)-galactan to give galactose monomers.

## 2. Results and discussion

### 2.1. Sugar composition of SSPS and GPase/SSPS

Table 1 summarises the monosaccharide compositions of the purified SSPS and the β-(1→4)-D-galactosidase treated SSPS (GPase/SSPS) samples. The possible degradation of arabinose and rhamnose from the acid treatment was checked by comparing the results obtained from acid hydrolysis to those from enzymatic hydrolysis. Only a minimal difference was seen as reported in a previous paper.[8] The sum of arabinose and galactose in SSPS accounted for 69.0% of the total sugars compared to 25.4% as the sum of galacturonic acid and rhamnose. In contrast, citrus pectin usually contains ~80% of galacturonic acid. This result is consistent with the previous finding that SSPS are heavily branched by neutral sugars in the forms of arabinan and galactan.[2] Further confirmation of this finding was obtained from the β-(1→4)-D-galactosidase treated sample. The relative galactose content in GPase/SSPS decreased drastically, from 48.5% in the original SSPS to 9.7% in GPase/SSPS. Consequently, the relative content of arabinose increased from 20.5% to 38.2%, indicating that GPase/SSPS still contains a large amount of arabinan side chains.

### 2.2. Optical clarification of sample solutions

**2.2.1. Pre-measurements by dynamic light scattering.** Typical autocorrelation functions obtained from dynamic light scattering measurements on dilute solutions of native and enzymatically treated soybean polysaccha-

**Table 1.** Monosaccharide compositions of soybean polysaccharides (SSPS) and β-(1→4)-D-galactosidase (GPase)-treated SSPS

| Type of SSPS | Sugar composition (mol %) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Rha | Fuc | Ara | Gal | Xyl | Glc | GalA |
| SSPS | 5.5 | 1.1 | 20.5 | 48.5 | 3.5 | 1.0 | 19.9 |
| Gpase/SSPS | 11.3 | 2.0 | 38.2 | 9.7 | 5.9 | 2.7 | 30.2 |

Rha: rhamnose, Fuc: fucose, Ara: arabinose, Gal: galactose, Xyl: xylose, Glc: glucose, GalA: galacturonic acid.

rides are shown in Figure 1. The relaxation processes for both samples took place over a wide range of time indicating a wide distribution of decay rate ($\Gamma$). The autocorrelation functions of SSPS and GPase/SSPS were analysed by several approaches including cumulant analysis, NNLS, CONTIN and double exponential (DE) methods and the results are summarised in Table 2. All these methods fitted the autocorrelation functions reasonably well, yielding small root-mean-square errors (rms). The mean decay rates obtained by NNLS, CONTIN and DE methods were in the same range, with the trend of $\Gamma_{\mathrm{NNLS}} > \Gamma_{\mathrm{CONTIN}} > \Gamma_{\mathrm{DE}}$. However, these results were significantly different from those obtained from cumulant analysis. As shown in Figure 2, except for cumulant analysis, all the other methods revealed a bi-modal distribution of decay rate ($\Gamma 1$, $\Gamma 2$). The NNLS method seemed to give a better resolution of the distribution of the decay rate compared to the CONTIN method. In cumulant analysis, the ratio of the second cumulant to the square of the first cumulant ($u_2/\overline{\Gamma}^2$) provides a measure of the width of the decay rate distribution. This ratio corresponds to the polydispersity index for rigid globular particles. However, for macromolecules, it is also related to the internal segment flexibility. As listed in Table 2, the ratios were high for both SSPS (0.565) and GPase/SSPS (0.475). This explained why cumulant analysis gave a significantly different mean decay rate compared to other methods: cumulant analysis is not applicable to a bi-modal distribution or highly polydisperse samples. As will be seen in the following section, excellent agreement was reached between the results obtained from cumulant analysis and NNLS method on aggregate-free solutions. It was observed that the two peak positions were relatively consistent within experimental error as measured at different angles. The decay rate scaled roughly with $q^2$, where $q$ is the scattering vector. This observation suggests that both decays of autocorrelation function were due to the centre-of-mass diffusion of particles of different mass. The decay rate for the faster motion group ($\Gamma 2$) was 6–8 times higher than that of the slow motion group ($\Gamma 1$). It is not clear at this stage if the group with large particle size is merely molecular aggregates formed from the small molecules, or a species with different chemical structures. Because it is well-known that polysaccharides tend to form aggregates in aqueous systems,[5] it was assumed in this case that these large particles were aggregates although further study is needed to clarify this. The existence of these large particles causes problems for the determination of molecular weight and size of the polysaccharide by static light scattering.

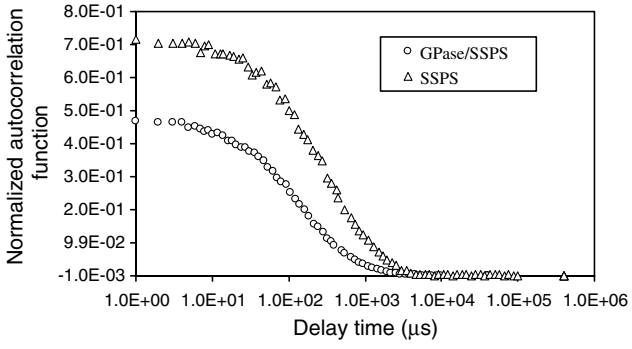


**Figure 1.** Typical autocorrelation functions obtained from dynamic light scattering measurements for SSPS and GPase/SSPS in 50 mM $NaNO_3$ solutions (1 mg/mL). Scattering angle $\theta = 90°$.

In the current paper, using consecutive filtration described below, we successfully removed the large particles from the dilute solutions of SSPS and GPase/SSPS. As examples, Figure 3 and Table 3 illustrate the DLS results measured at 90° from solutions before and after consecutive filtration. Only the major component with the fast decay rate (i.e., small particles) was detected after filtration. The ratio $u_2/\overline{\Gamma}^2$ from cumulant analysis decreased to 0.176 and 0.305 for SSPS and GPase/SSPS, respectively. Consequently, the mean decay rate obtained from cumulant analysis agreed well with values calculated by NNLS and CONTIN methods. Comparing Tables 2 and 3, we can see the mean decay rates for this group are in good agreement before and after filtration. This indicated that the filtration treatment did not cause fractionation of the major component of the samples other than removing the aggregates. The loss of sample during filtration was measured by a differential refractometer. The sample recoveries after filtration were 80% ± 5% and 82% ± 4% for SSPS and GPase/SSPS, respectively.

**Table 2.** Summaries of dynamic light scattering data (90°) for dilute solutions of SSPS and GPase/SSPS analysed by different methods

| Sample | Parameter | Cumulants | NNLS | CONTIN | DE |
|---|---|---|---|---|---|
| SSPS | Mean decay rate $\Gamma$ ($s^{-1}$) | 1502 | 1999 | 1962 | 1727 |
| | $\Gamma 1$; $\Gamma 2$ ($s^{-1}$) | | 487; 3718 | N.S.[a] | 435; 3809 |
| | Rms | $1.401e^{-2}$ | $1.77e^{-2}$ | $8.85e^{-3}$ | $8.95e^{-3}$ |
| | $u_2/\overline{\Gamma}^2$ | 0.565 | | | |
| GPase/SSPS | Mean decay rate $\Gamma$ ($s^{-1}$) | 2841 | 4062 | 3401 | 3168 |
| | $\Gamma 1$; $\Gamma 2$ ($s^{-1}$) | | 931; 5725 | 991; 5254 | 642; 5804 |
| | Rms | $9.45e^{-3}$ | $4.13e^{-2}$ | $3.30e^{-3}$ | $4.26e^{-3}$ |
| | $u_2/\overline{\Gamma}^2$ | 0.475 | | | |

[a] Peaks were not separated.

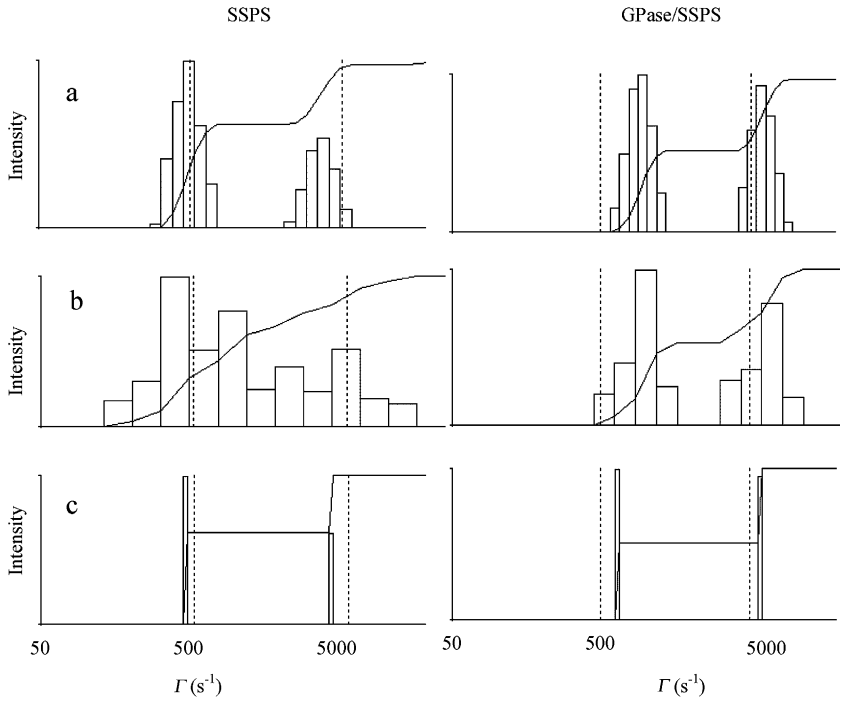


**Figure 2.** Decay rate ($\Gamma$) distributions of 1 mg/mL SSPS and GPase/SSPS in 50 mM $NaNO_3$ solutions obtained from dynamic light scattering data analysed by three models: (a) NNLS; (b) CONTIN and (c) double exponential (DE).

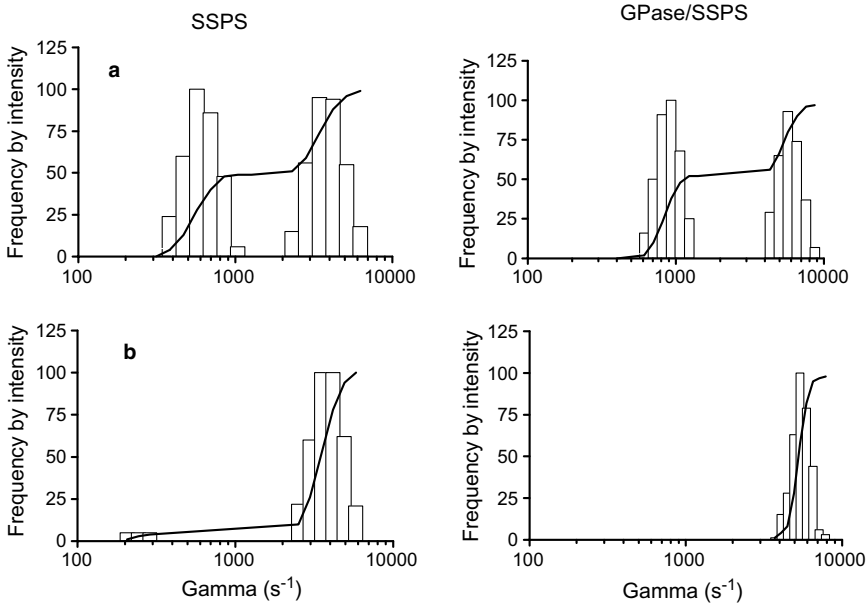


**Figure 3.** Decay rate ($\Gamma$) distributions of SSPS (1 mg/mL) and GPase/SSPS (0.4 mg/mL) in 50 mM $NaNO_3$ solutions before (a) and after (b) consecutive filtration.

**2.2.2. Size exclusion chromatography.** To further clarify the effects of filtration on the sample solutions, the same solutions were tested using size exclusion chromatography (SEC) before and after consecutive filtration. As shown in Figure 4, for both SSPS and GPase/SSPS, the SEC profiles after consecutive filtration were almost coincident with those before filtration. GPase/SSPS showed a slightly wider distribution compared to SSPS. Indeed, the results calculated from HPSEC analysis showed GPase/SSPS had a slightly larger polydispersity index (defined as $P_d = M_w/M_n$) compared to SSPS (Table 4), which is consistent with the dynamic light scattering results (Table 3). The consecutive filtration caused a slight reduction in measured molecular weight

**Table 3.** Summaries of dynamic light scattering data (90°) for dilute solutions of SSPS and GPase/SSPS after consecutive filtration analysed by different methods

| Sample | Parameter | Cumulants | NNLS | CONTIN |
|---|---|---|---|---|
| SSPS | Mean decay rate $\Gamma$ ($s^{-1}$) | 3684 | 3768 | 3865 |
| | Rms | $5.98\,e^{-3}$ | $2.67\,e^{-2}$ | $2.22\,e^{-3}$ |
| | $u_2/\overline{\Gamma}^2$ | 0.176 | | |
| GPase/SSPS | Mean decay rate $\Gamma$ ($s^{-1}$) | 5439 | 6706 | 7019 |
| | Rms | $2.28\,e^{-3}$ | $2.96\,e^{-2}$ | $2.27\,e^{-3}$ |
| | $u_2/\overline{\Gamma}^2$ | 0.305 | | |

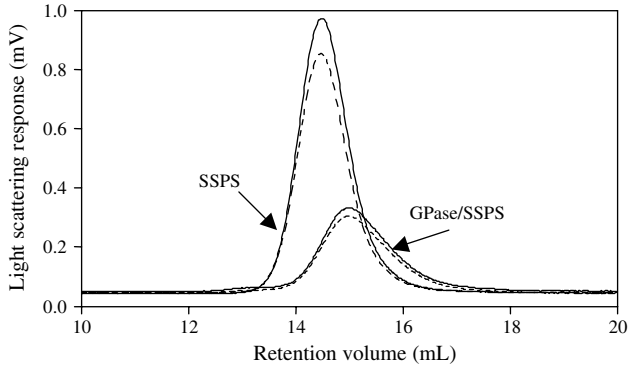


**Figure 4.** HPSEC chromatography of 1 mg/mL SSPS and GPase/SSPS. Solid lines are samples before consecutive filtration and dashed lines are samples after filtration.

and intrinsic viscosity. The reduction was estimated to be 4.8%, 8.5% in molecular weight and 4.4%, 5.5% in intrinsic viscosity for SSPS and GPase/SSPS, respectively. It is reasonable to attribute these reductions to the effect of removing the large aggregates. However, the quality of molecular weight data was significantly improved after filtration as reflected by smaller standard deviations compared to those of un-filtered samples. In combination with DLS data, we are confident in concluding that the current consecutive filtration process did not cause significant fractionation of the polysaccharide sample, aside from removing the small amount of large particles.

## 2.3. Characterisation of aggregate-free samples

**2.3.1. Static light scattering.** Static light scattering measurements were carried out on the aggregate-free solutions after consecutive filtration. Figure 5 illustrates an example of the Zimm plot obtained from SSPS. The weight average molecular weight, radius of gyration and second virial coefficient extracted from the Zimm plots from both samples are summarised in Table 5. Due to the polyelectrolyte character of these samples, it is possible that dilution could lead to expansion or contraction of the molecule. However, it can be seen from the Zimm plots that the angular dependence of the scattered light did not change systematically with decreasing concentration of the polymer, indicating no detectable conformation change of the molecules upon dilution. The second virial coefficients are positive and small for both samples, similar to that for a normal un-charged polysaccharides in a good solvent. The molecular weight values obtained from static light scattering measurement are in fair agreement with those obtained from HPSEC (Table 4). The enzyme treated sample GPase/SSPS had a much lower molecular weight (287 ± 18 kg/mol) compared to the original SSPS (645 ± 11 kg/mol). However,

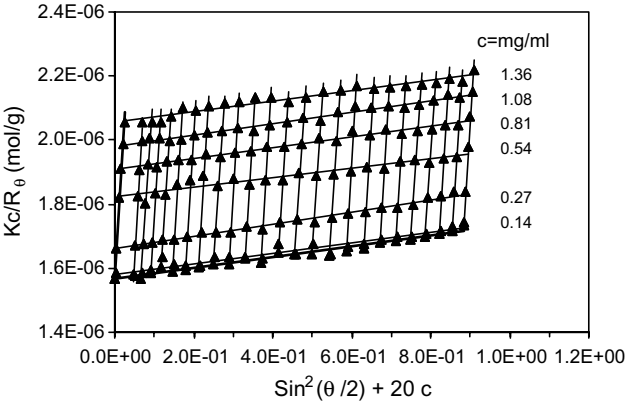


**Figure 5.** An example of static light scattering data presented in a Zimm plot obtained from soybean polysaccharides SSPS in 50 mM $NaNO_3$ solutions.

**Table 4.** Molecular weight ($M_w$) and intrinsic viscosity [$\eta$] of SSPS and GPase/SSPS measured by HPSEC before and after consecutive filtration

| Sample | SSPS | | | GPase/SSPS | | |
|---|---|---|---|---|---|---|
| | Before filtration | After filtration | Reduction[a] (%) | Before filtration | After filtration | Reduction[a] (%) |
| $M_w \times 10^{-3}$ (g/mol) ± SD | 738 ± 27 | 721 ± 17 | 4.8 | 235 ± 14 | 225 ± 10 | 8.5 |
| [$\eta$] (dL/g) ± SD | 0.68 ± 0.02 | 0.65 ± 0.02 | 4.4 | 0.55 ± 0.04 | 0.52 ± 0.02 | 5.5 |
| $P_d$ | 2.0 ± 0.3 | 2.1 ± 0.3 | — | 2.3 ± 0.2 | 2.3 ± 0.1 | — |

[a] Calculated as: $2 \times (P_{before} - P_{after})/(P_{before} + P_{after})$, where $P$ is $M_w$ or [$\eta$], respectively.

**Table 5.** Summaries of static light scattering data on SSPS and GPase/SSPS analysed by Zimm plot method

| Samples | $M_w \times 10^{-3}$ (g/mol) | $R_g$ (nm) | $A_2 \times 10^4$ (cm$^3$ mol/g$^2$) |
|---|---|---|---|
| SSPS | $645 \pm 11$ | $23.5 \pm 2.8$ | $6.5 \pm 1.2$ |
| GPase/SSPS | $287 \pm 18$ | $23.2 \pm 1.7$ | $2.2 \pm 0.4$ |

the radii of gyration for both samples were essentially unchanged ($23.5 \pm 2.8$ nm for SSPS and $23.2 \pm 1.7$ nm for GPase/SSPS, respectively). This resulted in a decrease in apparent segment density ($= M_w/1.33 \times N \times R_g^3$, where $N$ is Avogadro's number) by a factor of 2.2. The current result confirms that the β-(1→4)-D-galactosidase used in this study was specific to galactan. The same conclusion can be drawn from the static light scattering measurements as from sugar analysis, which showed that the reduction in molecular weight was mainly attributed to the reduction of galactan side chains.

**2.3.2. Dynamic light scattering (DLS).** The aggregate-free solutions were further characterised by dynamic light scattering. DLS measurements were carried out on a series of concentrations (typically 0.1–1.5 mg/mL) and in the angular range of 30–130° to consider possible inter-particle interference on scattered light. A minimal angular dependence was observed for solutions in the concentration range studied. As an example, a set of DLS data for 0.8 mg/mL SSPS is shown in Figure 6. The autocorrelation function appeared to decay slightly faster as the detecting angle increased. Similarly, the effects of concentration on DLS data were also small, yet noticeable; the autocorrelation function decayed faster as the concentration decreased. To eliminate the interference of concentration and detecting angle, the DLS data were presented in a format of apparent diffusion coefficient D ($= \Gamma/q^2$) versus scattering vector $q^2$, that is, the so called dynamic Zimm plot, as illustrated in Figure 7. In such a plot, each concentration line was fitted by a linear function and extrapolated to zero degree angle;

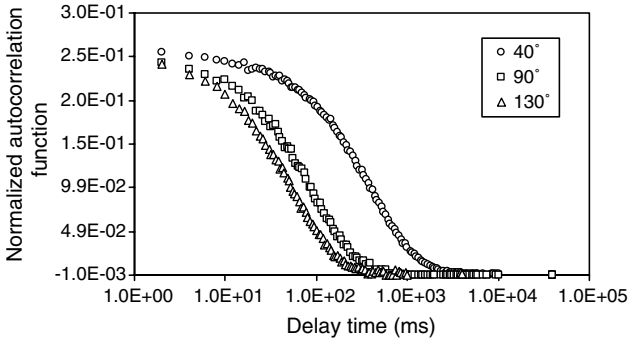


**Figure 6.** Autocorrelation functions for a 0.8 mg/mL solution of β-(1→4)-D-galactosidase treated soybean polysaccharides measured at three detecting angles.

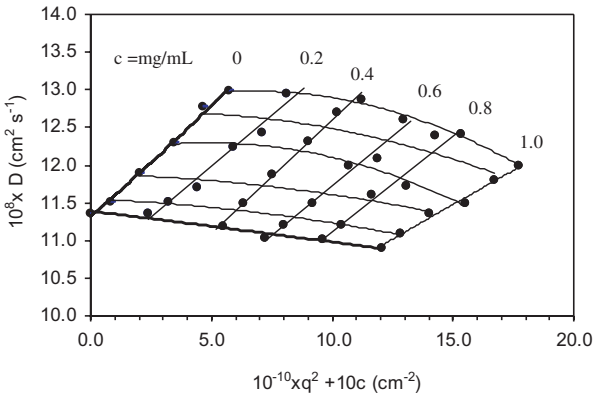


**Figure 7.** An example of dynamic light scattering data presented in a dynamic Zimm plot obtained from soybean polysaccharides in 50 mM $NaNO_3$ solutions.

whereas polynomial regression was used to fit each angle line and extrapolated to zero concentration. From the intercept of the two extrapolation lines, the true translational diffusion coefficient was obtained. They were found to be $11.4 \times 10^{-8}$ and $13.9 \times 10^{-8}$ cm$^2$ s$^{-1}$ for SSPS and GPase/SSPS, respectively. By applying the Stokes–Einstein equation, the hydrodynamic radii $R_h$ were calculated to be $21.7 \pm 1.8$ and $17.7 \pm 1.8$ nm for SSPS and GPase/SSPS, respectively.

**2.3.3. Discussion on molecular shape.** Because static and hydrodynamic dimensions vary characteristically with the structure of the macromolecules, a combination of the two may provide qualitative information on the architecture of the macromolecules.[9] The structure-sensitive parameter $\rho$ is defined as the ratio of radius of gyration $R_g$ obtained from static light scattering to the hydrodynamic radius $R_h$ from dynamic light scattering. The $\rho$ values for some molecular architectures can be found in the literature.[9] Generally, the value of $\rho$ decreases with increasing branching density, but an increase in polydispersity counteracts the effect of branching. The $\rho$ values were found to be 1.1 for SSPS, which is slightly higher than the predicted value for a homogeneous sphere (0.788), but in good agreement with the predicted value for a highly branched globular shape molecule. A significantly higher value of $\rho = 1.3$ was obtained for GPase/SSPS. Because the polydispersity of GPase/SSPS was only slightly higher than that of SSPS (Tables 3 and 4), the increase in $\rho$ value was most likely due to the effect of less branching, although increased polydispersity may also contribute to the difference. However, this $\rho$ value is incompatible with a polydisperse random coil of linear chains (1.7 at $\theta$ condition and 2.1 in good solvent); it is even lower than the predicted values for a monodisperse random coil, which are 1.5 at $\theta$ condition and 1.8 in a good solvent, respectively. This suggests that the enzyme treated polysaccha-

rides still contain a significant amount of branching, although to a less extent. In combination with chemical analysis (Table 1), the remaining branching chains should mainly consist of arabinans.

The intrinsic viscosity is a measure of the hydrodynamic volume occupied by macromolecules in a dilute solution. The intrinsic viscosity of both SSPS and GPase/SSPS were found to be extremely low (Table 4) compared to citrus pectin of similar molecular weight.[13] The degradation of neutral sugar side chains did not appear to reduce the intrinsic viscosity substantially. These observations also support a highly branched spherical structure of soybean polysaccharides.

### 2.4. Conclusion

Consecutive filtrations with a range of pore sizes removed a small fraction of aggregates or super molecules from soybean polysaccharide solutions with relatively little effect on the major population of the molecules. This allowed a direct measurement of the molecular features of the major component. It was found that SSPS exhibited a highly branched globular conformation with a molecular weight of $(645 \pm 11) \times 10^3$ g/mol. In contrast, SSPS treated with β-(1→4)-D-galactosidase had a much lower molecular weight $(287 \pm 18) \times 10^3$ g/mol although it exhibited a similar radius of gyration to the original sample. The reduction of molecular weight was attributed to the removal of galactan side chains rather than the break up of polysaccharide backbone. This leads to a less compact structure of the polysaccharide. The difference in conformation between SSPS and GPase/SSPS may account for, at least partially, their different ability to stabilise proteins in acidified food systems described in a previous paper.[2]

## 3. Experimental

### 3.1. Preparation of soybean soluble polysaccharides (SSPS)

The soybeans used in this study were Glycine max L. crops harvested in 2002 in fields in Indiana, Ohio, and Michigan, USA. Soybean cotyledons, after removal of the hulls and hypocotyls, were powdered and defatted at 40 °C with 5 vol of *n*-hexane. Proteins and water-soluble substances in the defatted meal were extracted twice at 50 °C and pH 7.0 for 1 h with 11 vol of water. SSPS were extracted from the residue (okara) with water by heating at 120 °C and pH 4.0 for 1.5 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.

### 3.2. Preparation of enzyme treated SSPS

An *exo*-glycosidase, β-(1→4)-D-galactosidase (GPase), extracted from the pulp of persimmon fruit (Japanese persimmon cv. Saijyo) was purified according to the method described previously.[10] SSPS was digested by the purified GPase as follows. A 50 g sample of SSPS was dissolved in 5 L of 10 mM sodium acetate buffer, pH 4.5. To this solution, 100 munits/mL of the GPase was added and incubated at 40 °C for 24 h. The polysaccharide solution digested by the enzyme was heated at 90 °C for 20 min to inactivate the enzyme, concentrated to 1/5 vol on a rotary evaporator, dialysed against MilliQ water using an Amicon YM-5 membrane filter (Millipore Co. Ltd, Billerica, MA, USA), and finally freeze dried.

### 3.3. Analysis of sugar composition

To measure galacturonate content, polysaccharide samples were hydrolysed by driselase (Kyowa Hahho Kogyo Inc., Tokyo, Japan), which is a commercial enzyme from *Irpex lacteus*. This enzyme preparation contains a large group of enzyme activities including those of cellulase, pectinase, xylanase, dextranase, laminarinase, amylase and protease, etc. A solution containing 0.1% polysaccharide and 0.1% glycerol (internal standard) in 50 mM sodium acetate buffer, pH 4.0, was hydrolysed 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 analysed by HPLC on a Shodex SUGAR SH-1821 column (7.6 mm × 300 mm; Showa-denko Co. Ltd, Tokyo, Japan) by the method of Matsuhashi et al.[11] To determine the neutral sugar composition, the polysaccharide sample (0.1 wt %) was hydrolysed 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, USA) and analysed as 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.[12]

### 3.4. Light scattering measurements

**3.4.1. Static light scattering.** A multi-angle laser light scattering apparatus from Brookhaven Instruments Co. (New York, USA) was used with a He–Ne laser (632.8 nm) as the light source. Instrument alignment was checked to be satisfactory using well filtered (0.1 μm filter) toluene. Toluene was also used for the calibration of the apparatus and a value of $1.40 \times 10^{-5}$ cm$^{-1}$ was used as the Rayleigh ratio. The sample solution was filtered directly into a cylindrical quartz cell (25 mm in diameter), which was immersed

in a decalin bath at room temperature (21 °C). The measurements were carried out over an angular range of 30–130° and the data were analysed by the Zimm plot method. A specific refractive index increment of 0.15 mL/g was used for soybean polysaccharide in 50 mM $NaNO_3$.

**3.4.2. Dynamic light scattering.** The dynamic light scattering study was performed on the same apparatus as the static light scattering measurements. The measurement was made in self-beat mode detection, at 21 °C over an angular range of 30–130°. Because of the polydisperse nature of polysaccharides, the autocorrelation functions were analysed by a number of approaches using software provided by Brookhaven Instruments (New York, USA). These include the commonly used cumulants and CONTIN methods, as well as the non-negatively constrained least squares (NNLS) and the double exponential (DE) methods. Among those, the NNLS and CONTIN methods provide information on the statistical parameters, that is, the moments that characterise the particle size distribution. However, no information on the size distribution itself could be obtained from the cumulants and double exponential methods.

### 3.5. Size exclusion chromatography

The high performance size exclusion chromatography (HPSEC) system used for this study includes a Shimadzu SCL-10Avp pump and an automatic injector (Shimadzu Scientific Instruments, Inc., Columbia, Maryland 21046, USA), two columns connected in series: a Shodex OH-pak KB-806M (Showa Denko K.K., Tokyo, Japan), and an Ultrahydrogel linear (Waters, Milford, CT, USA). The Viscotec Triple Detectors was the detection system, which consisted of a right angle laser light scattering detector (RALLS), a differential viscometer (DP) and a refractive index detector (RI) (Viscotek, TX, USA). TriSEC software of Viscotec was used for data analysis. The detectors were calibrated by pullulan standards (P-82, JM Science, Inc., NY, USA). A value of 0.15 mL/g was used as the refractive index increment ($dn/dc$) for molecular weight calculation. The columns, DP and RI detectors were maintained at 40 °C. The eluent was 0.1 M $NaNO_3$ containing 0.03% (w/w) $NaN_3$ at a flow rate of 0.6 mL/min.

### 3.6. Estimation of sample loss from filtration

The loss of polysaccharide sample caused by filtration was estimated using a differential refractometer BI-DNDC (Brookhaven Instruments Co., New York, USA), which measures the concentration of polysaccharide. The polysaccharide sample recovery was calculated as the ratio of polysaccharide concentration after filtration to that before filtration.

## Acknowledgements

The authors would like to thank Dr. R. C. McKellar of Agriculture and Agri-Food, Canada, for expert assistance on model fitting of dynamic light scattering data.

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