JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Study of Thermal Aggregation of Oat Globulin by Laser Light Scattering

YU ZHAO,† YOSHINORI MINE,‡ AND CHING-YUNG MA*,†

Food Science Laboratory, Department of Botany, The University of Hong Kong, Hong Kong, and Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

The heat-induced aggregation of oat globulin was studied using size-exclusion chromatography (SEC) combined with on-line multiangle laser light scattering (MALLS) and quasi-elastic light scattering (QELS). The unheated oat globulin exists as a mixture of hexamer (>95%), trimer, and dimer forms of hexamer. The molecular weight of the hexamer was estimated by MALLS to be 330 000, close to that deduced from the genomic cloned data of the acidic and basic polypeptides of oat globulin. From QELS measurements, it can be predicted that the hexamer exists as two annular trimeric rings, with a diameter of 11.8 nm, placed on top of each other, forming an oblate cylinder with a height of about 8.5 nm. Upon heating at 100 °C, the oat globulin hexamers and trimers were dissociated into monomers. The heat-denatured monomers, probably assuming an extended structure, were associated to form small aggregates, which were further aggregated to high molecular weight complexes. Upon further heating (60 min), the soluble aggregates were associated to form insoluble aggregates. Aggregation of oat globulin occurred at a much faster rate at 110 °C. The results indicate that the SEC-MALLS-QELS system is suitable for studying thermal aggregation of food proteins.

**KEYWORDS: Oat globulin; thermal aggregation; laser light scattering; quasi-elastic light scattering**

## INTRODUCTION

Oat protein has been shown to possess good nutritional value and functional properties (*1*). Oat globulin, the major storage protein fraction, is an oligomeric protein with a quaternary structure very similar to that of legumin. Oat globulin is made of six monomers or subunits each of which consists of an acidic and a basic polypeptide, with molecular weights of 22 000−24 000 and 32 000−37 000, respectively, linked by disulfide bonds (*2*, *3*). The six subunits are linked by noncovalent forces to form the hexamer, similar to legumins (*4*−*6*).

Thermal aggregation or coagulation is an important property of food proteins influencing their applications in many food systems such as gels and emulsions (*7*, *8*). To fully utilize oat globulin as a functional ingredient in various manufactured foods, its heat-aggregating properties will need to be evaluated. Thermal aggregation of food proteins in aqueous solutions has been widely studied. Most globular proteins have a tendency to aggregate when heated, but the aggregation mechanism varies. For oligomeric proteins with complex quaternary structures, heat may cause association and/or dissociation of the oligomers, and disruption of the quaternary structure itself may result in aggregation (*7*, *8*). We have studied thermal coagulation of oat globulin using size-exclusion chromatography (SEC) and SDS-PAGE (*9*). Oat globulin was found to dissociate into subunits and associate into high molecular weight aggregates.

Further investigation of the aggregation mechanism of oat globulin requires accurate determination of the molecular weight and size distribution of the aggregates. Although molecular weights of proteins can be determined by techniques such as mass spectrometry, SDS-PAGE, and SEC, the use of SEC combined with multi-angle laser light scattering (MALLS) is considered suitable for monitoring the aggregation process (*10*). This system can accurately measure the molecular mass and the root-mean-square radius of macromolecules in solution and provide information about the oligomeric state of the protein directly, without dependence on column calibration or reference standards (*10*). Recently, the SEC-MALLS system has been widely used to study animal proteins such as $\beta$-lactoglobulin (*11*, *12*), sodium caseinate (*13*), bovine serum albumin (*12*), and ovalbumin (*14*). However, only a few plant proteins have been studied with this technique, including wheat protein (*15*, *16*), legumin from *Pisum sativum* (*17*), and soybean glycinin (*18*). Furthermore, there were few reports on using the techniques to study thermal aggregation of proteins, and these were limited to animal proteins (*11*, *14*, *19*, *20*).

In this investigation, in addition to MALLS, an on-line dynamic quasi-elastic light scattering (QELS) system was used, which has the ability to measure the hydrodynamic radius ($r_h$) of molecules with diameter below 10 nm, the lower size limit of MALLS detection (*10*). This should provide a more accurate determination of the oat globulin oligomer and the aggregates

* Corresponding author. Phone: +852-2299-0318. Fax: +852-2858-3477. E-mail: macy@hkucc.hku.hk.
† The University of Hong Kong.
‡ University of Guelph.

10.1021/jf030735y CCC: $27.50 
Published on Web 04/16/2004

since most globular proteins have diameters below 10 nm. The SEC-MALLS-QELS combination allows the simultaneous determination of the molecular weight and the hydrodynamic radius of protein eluting in small and individual slices of the SEC chromatogram.

## MATERIALS AND METHODS

**Oat Globulin.** Oat seeds (variety Hinoat) were grown in the Central Experimental Farm, Ottawa, Agriculture and Agri-Food Canada in 1998, and were dehulled and ground in a pin-mill. The ground groats were defatted by Soxhlet extraction with hexane and stored at −20 °C. Oat globulin was prepared from the defatted ground groats by salt extraction according to a previous study (*21*). Groats were mixed with 1.0 M NaCl at a solvent-to-solid ratio of 10:1 and stirred at room temperature for 30 min. The slurry was centrifuged at 20 000*g* and 4 °C for 30 min, and the residue was extracted twice with 1.0 M NaCl. The combined supernatant was dialyzed exhaustively against distilled water at 4 °C, and the precipitated globulin was separated by centrifugation and freeze-dried.

**Sample Preparation.** Buffer-soluble heat-induced oat globulin aggregates were prepared as follows: 1% (w/v) oat globulin was prepared in 50 mM phosphate buffer, pH 7.4, containing 1.0 M NaCl, and stirred for 60 min at room temperature. Oat globulin was almost completely soluble under such conditions. The solution was centrifuged at 10 000*g* and 4 °C for 10 min, and 1-mL aliquots of the supernatant were then transferred to glass tubes stoppered with marbles. The tubes were heated in a boiling water bath for various time intervals. For experiments conducted at 110 °C, screw cap culture tubes were used and the protein solution was heated in a prevacuum sterilizer (Steris Corp., Mentor, OH). After heating, the tubes were cooled immediately by immersion in an ice bath, and then centrifuged at 10000*g* and 4 °C for 10 min. The pellets containing precipitated protein were discarded and the supernatant, containing the buffer-soluble aggregated protein, was filtered through a 0.22 $\mu$m syringe filter unit (Millipore Corp., Bedford, MA). Aliquots (50 $\mu$L) of the filtrates were injected into the chromatographic system. As shown in the previous study (9), the amount of protein precipitated under these heating conditions ranged from less than 10% at 100 °C to over 70% at 110 °C.

**High-Performance Size-Exclusion Chromatography.** The high-performance size exclusion chromatographic (HPSEC) system consisted of an HP G1379A in-line degasser, a G1312 pump, a G1315 ultraviolet (UV) detector, and a G1362 differential refractive index (RI) detector (Hewlett-Packard, Palo Alto, CA). Two TSK columns (G4000 $PW_{XL}$ + TSK G6000 $PW_{XL}$) were connected in series (TOSOH Corp., Montgomeryville, PA). The fractionation ranges of the two columns were 2 000−300 000 and 40 000−8 000 000 respectively for proteins. The mobile phase (50 mM phosphate buffer, pH 7.4, containing 1.0 M NaCl) was filtered by 0.2 $\mu$m (Whatman International Ltd., Maidstone, England) and then 0.02-$\mu$m filters (Millipore Corp., Bedford, MA). The flow rate was 0.5 mL/min. The RI detector was calibrated over a linear concentration range with sodium chloride standards, operated at room temperature.

**Multi-Angle Laser Light Scattering.** A Dawn EOS photometer (Wyatt Technology Corp., Santa Barbara, CA) was used. The 18 discrete photodetectors were spaced around the flow cell (type K5) and enabled simultaneous measurements over a range from 15° to 160°. Two auxiliary analogue inputs enable interfacing to external detectors such as RI and UV detectors. The instrument was placed directly before the RI detector and after the SEC columns and UV detector to avoid backpressure on the RI cell. Chromatographic data were collected and processed by the ASTRA software (Wyatt Technology Corp., Santa Barbara, CA). Bovine serum albumin monomer (Sigma, St. Louis, MO) was used for normalizing various detectors' signals relative to the 90° detector signal.

**Quasi-Elastic Light Scattering.** Dynamic light scattering measurement was performed on-line in the flow cell using a quasi-elastic light scattering (QELS) meter (Wyatt Technology Corp., Santa Barbara, CA). An optical fiber receiver was mounted in the read head of one of the MALLS detectors (detector 13 in our works), and the fiber was in turn coupled to an avalanche photodiode in an autocorrelator that has been specially modified to accept the signal from the Dawn instrument.

## DATA TREATMENT

**Determination of Molecular Masses.** The relationship between the experimental data and the molecular parameters is described by the following equation (*22*):

$$\frac{R_\theta}{K^*c} = MP(\theta) - 2A_2cM^2P^2(\theta) \quad (1)$$

where here $R_\theta$, the excess Rayleigh ratio, is the excess scattering of the solution compared to that of the solvent alone; $c$ is the mass concentration of the solute molecules in the solvent (g/mL), as measured by the on-line RI detector; $M$ is the weight average molar mass (g/mol); $A_2$ is the second virial coefficient (mol mL/g$^2$); $K^*$ is an optical constant function of the specific refractive index increment d$n$/d$c$; and $P(\theta)$ is the theoretically derived form factor that describes the angular dependence ($P(\theta) \sim \sin^2(\theta/2)$) of the scattered light.

Molecular mass can be determined by solving eq 1. The ASTRA software typically uses the Debye method in the chromatography mode, which gives good results over a wide range of molecular weight. A Debye plot, $R_\theta/K^*c$ vs $\sin^2(\theta/2)$, is first constructed, followed by a polynomial fit of $\sin^2(\theta/2)$ to the data, and molecular mass can be obtained from the intercept at zero angle. As $\theta$ approaches zero, the form factor $P(\theta)$ approaches unity. Therefore, eq 1 becomes

$$\frac{R_{\theta\to 0}}{K^*c} = \frac{R_0}{K^*c} = M - 2A_2cM^2 \quad (2)$$

Sample concentration for each data slice is determined from the RI detector output, assuming a constant d$n$/d$c$ value across the sample peak. For the $i$th slice, the change in concentration of solute, compared to pure solvent, is given by

$$\Delta c_i = \frac{\Delta n_i}{\mathrm{d}n/\mathrm{d}c} = \frac{\alpha_{\mathrm{RI}}(V_i - V_{i,\mathrm{baseline}})}{\mathrm{d}n/\mathrm{d}c} \quad (3)$$

where $\alpha_{RI}$ is the RI detector calibration constant; $\Delta n_i$ is the change in refractive index compare to pure solvent for the $i$th slice; and $V_i$ and $V_{i,\mathrm{baseline}}$ are the RI signal and baseline voltages, respectively. Since the baseline represents pure solvent, $\Delta c_i = c_i$. For a protein that contains no carbohydrate, d$n$/d$c$ is constant (±0.186 mL/g) and nearly independent of its amino acid composition (*23*). During gel permeation chromatography, the protein concentrations are so low that the virial coefficient term ($A_2$) is negligible. Sample concentration ($c$) for each data slice is determined from the RI detector output assuming a constant d$n$/d$c$ value across the sample peak.

**Determination of Hydrodynamic Radius.** The optical fiber of the QELS detector detects wavelets of light that scatter either destructively or constructively, depending on the position of the illuminated molecules (*10*). As the molecules undergo Brownian motion, their relative positions change with time. The autocorrelation function of a monodisperse sample is related to its diffusion constant:

$$G(\tau) = \langle I(t)\rangle^2(1 + \alpha e^{-2D_\tau q^2\tau}) \quad (4)$$

where $\langle I(t)\rangle^2$ is the average intensity squared, $\alpha$ is an instrument constant, $\tau$ is a delay time, $D_\tau$ is the translational diffusion constant, and $q$ is the scattering. By analyzing the correlation function, the Stokes−Einstein relation enables the molecule's hydrodynamic radius ($r_h$) to be determined:

$$r_h = \frac{k_BT}{6\pi\eta D_T} \quad (5)$$

where $k_B$ is Boltzman's constant, $T$ is the absolute temperature, and $\eta$ is the solvent viscosity.

## RESULTS AND DISCUSSION

**Chromatographic Profiles.** The SEC-MALLS-QELS system uses four detectors (LS, QELS, UV, and RI) that are connected

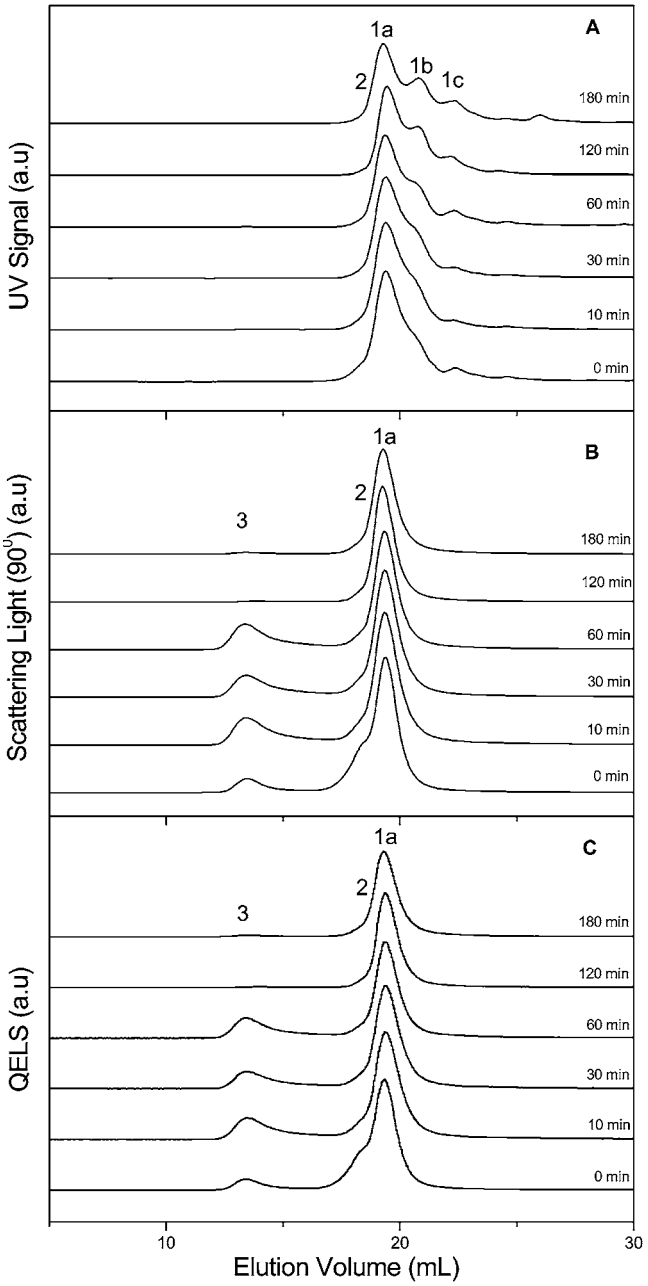


**Figure 1.** HPLC combined with MALLS and QELS: (**A**) UV, (**B**) LS (at 90°), and (**C**) QELS elution profiles of oat globulin (in 50 mM phosphate buffer, pH 7.4, with 1 M NaCl) heated for various time intervals at 100 °C.

in series after the SEC columns. In this study, the RI detector was used as a concentration detector and the UV detector was used as a mass detector.

The elution profiles of oat globulin heated for various time intervals at 100 °C are shown in **Figure 1**. With UV detection, the unheated sample, containing native oat globulin, was partially resolved into four peaks (**Figure 1A**). The pattern resembles that of a previous profile of oat globulin, using a different chromatographic system (*9*). Peak 1a represents the major fraction of oat globulin, probably the hexamer. Peak 1b may correspond to the trimer, and peak 1c may represent a mixture of acidic and basic polypeptides. Peak 2, the shoulder of peak 1a, could be the dimeric form of oat globulin hexamer.

The MALLS profile of native oat globulin shows three peaks (**Figure 1B**). Very large materials were eluted as a small peak at the void volume (peak 3), which was invisible to the UV detector. The appearance of this peak is unlikely due to minute quantities of "impurities" such as dust particles (*11*), but could represent aggregated protein molecules during the lyophilization process (*23*). The light scattering signal is proportional to the products of concentration and molecular mass. Since scattering intensity is strongly dependent on particle radius, a small amount of large materials in the sample would give a large response with the light scattering detector, although their amount, as measured by the UV response, is very small. The MALLS detector is much less sensitive to small molecules, and peaks 1b and 1c were therefore not detected. The QELS elution pattern of unheated oat globulin (**Figure 1C**) is similar to the MALLS profile.

Upon heating at 100 °C, the amount of hexamer, as shown by the size of peak 1a, decreased with a corresponding decrease in peak 2. Peak 1b was present both before and after heating, and after 120 min, peak 1b was separated from peak 1a (**Figure 1A**). With heated for 10, 30, and 60 min, there was a progressive increase in the size of peak 3, detected only by MALLS and QELS (**Figure 1B**,**C**). There was a concomitant progressive decrease in the size of peak 1a and the complete disappearance of peak 2. With the fractionation range of the SEC columns, only very large molecules will be eluted at the void volume, and peak 3 can be used to show the formation of large aggregates. Our data suggest that large aggregates were formed from oat globulin hexamer and its dimeric form during heating at 100 °C. Upon further heating (>60 min), peak 3 disappeared, suggesting the formation of insoluble aggregates (formed from the buffer-soluble aggregates) which cannot be analyzed by the SEC system. Interestingly, no distinct peaks were observed between peaks 2 and 3. The smaller aggregates formed during heating could be highly reactive and associated rapidly to larger and more stable molecules. Similar results were also observed in the aggregation of patatin (*24*).

The elution profiles of oat globulin heated at 110 °C are shown in **Figure 2**. With UV detection, a progressive decrease in peak 1a was observed, and after 30 min of heating, peak 1a was almost completely converted to peak 1b (**Figure 2A**). With MALLS and QELS detection, rapid decreases in peak 1a and disappearance of peak 2 and peak 3 were observed (**Figure 2B**,**C**). The results suggest dissociation of oat globulin hexamers, and the formation of insoluble aggregates was at a much faster rate at 110 °C than at 100 °C.

Studies of thermal aggregation of ovalbumin (*25*), legumin (*26*), and whey proteins (*27*) indicated that a heating temperature above the denaturation temperatures ($T_d$) caused an increase in the degree of polymerization and unfolding in the structure. Oat globulin has exceptionally high heat stability with a $T_d$ at 114 °C in 1.0 M NaCl (*9*, *28*). Our previous differential scanning calorimetric data showed that heating at 100 °C for various time periods caused a progressive decrease in denaturation enthalpy, suggesting protein unfolding, although the extent of changes was much lower than that observed at 110 °C (*29*). Our previous Fourier transform infrared spectroscopic results also showed that aggregation of oat globulin started to occur after heating at 100 °C for 15−30 min and then leveled off (*30*).

**Measurement of Molecular Weight.** The molar mass distribution of unheated oat globulin as a function of elution volume is shown in **Figure 3**. The weight-average molecular mass ($M_w$) was calculated from the combined UV and MALLS signals according to previous workers (*10*, *31*). During the elution of the major component (peak 1a), the molar mass slope was almost parallel to the *x*-axis, suggesting no marked variation

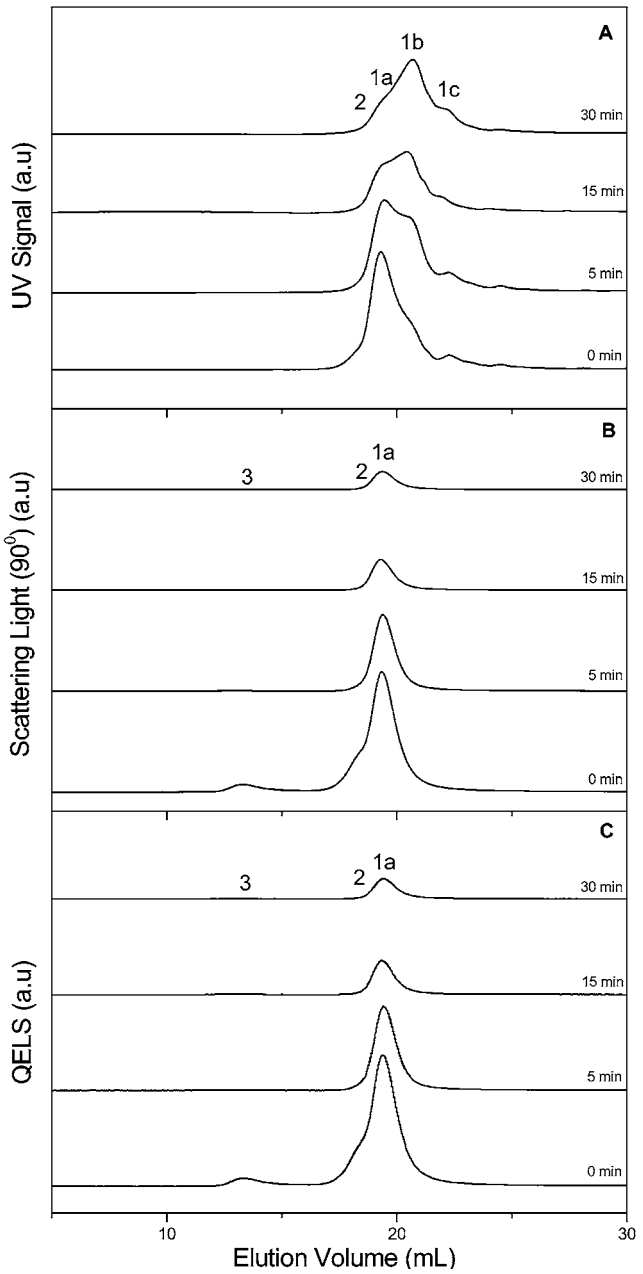


**Figure 2.** HPLC combined with MALLS and QELS: (**A**) UV, (**B**) LS (at 90°), and (**C**) QELS elution profiles of oat globulin (in 50 mM phosphate buffer, pH 7.4 with 1 M NaCl) heated for various time intervals at 110 °C.

in $M_w$ (333 000) across the peak. This indicates that the peak contains a homogeneous population of molecules with respect to $M_w$. At lower elution volumes (peak 2 and peak 3), the molar mass slopes assumed a downward curvature shape, probably due to weak light scattering signals and reduced resolution near the exclusion limit (*32*). The molar mass for slices across peak 1b, peak 2, and peak 3 exhibited broad distributions, with estimated $M_w$ of 100 000−250 000, 480 000−650 000, and 15 000 000−40 000 000, for the three peaks, respectively.

No molar mass data were obtained in the elution volume range between peaks 3 and 2, probably due to the low quantity of material present, making it difficult for the system to be measured accurately (*33*). Since the MALLS signals of peak 1c were also very weak, the ASTRA software could not accurately calculate its molecular mass.

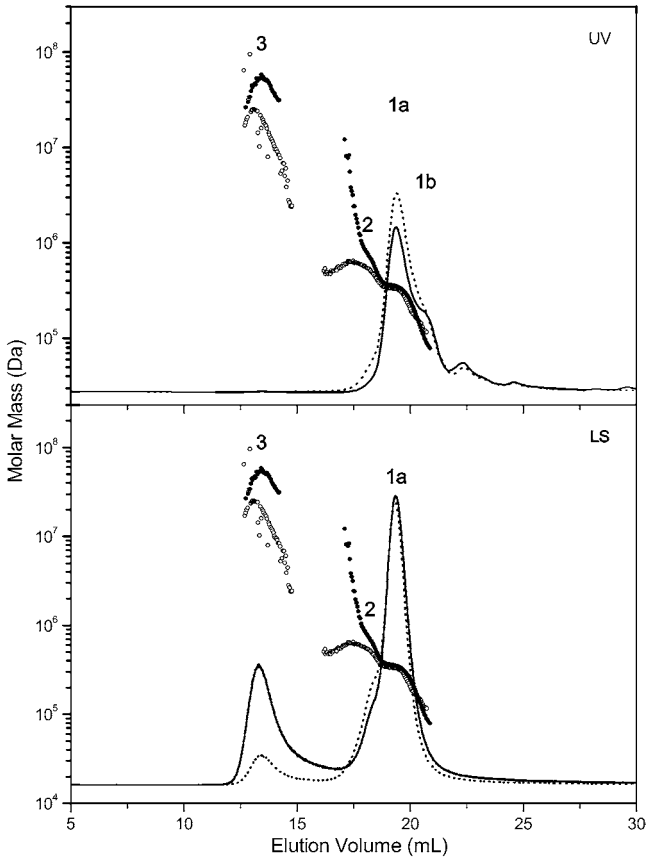


**Figure 3.** Relationship between molar mass and elution volume of the unheated (○) and heated (●) oat globulin (at 100 °C for 60 min). UV and LS (at 90°) elution profiles of the unheated (dotted line) and heated (solid line) oat globulin are included.

Technically, the molecular mass $M_i$ of material eluting in chromatographic slice *i* from light scattering measurement is a weight-averaged molecular mass (*34*). The $M_i$ values can be used together with the concentrations $c_i$ to calculate the molecular mass moments (*35*, *36*). **Table 1** summarizes the weight-average molecular weights ($M_w$), number-average molecular weights ($M_n$), and z-average molecular weights ($M_z$) of oat globulin heated at 100 °C for various time intervals, calculated from the combined RI and MALLS signals. For the unheated oat globulin, $M_w$ was estimated as 188 000, 333 000, 516 000, and 22,200 000 for peaks 1b, 1a, 2, and 3, respectively.

Shotwell et al. (*37*) isolated and characterized several genomic clones corresponding to the 12S globulin of oats. From the published data, the acidic polypeptide was found to contain 293 amino acids ($M_w$ 33 112), and the basic polypeptide has 201 amino acids ($M_w$ 22 758) (*37*). In the present study, $M_w$ of native oat globulin was determined to be 333 000, close to that (335 220) deduced from the cloned data, and slightly lower than the $M_w$ (350 000) determined previously (*9*). The $M_w$ of peak 1b corresponds to the trimer (half of a hexamer). It is known that soybean glycinin hexamers dissociated to trimers under various environmental conditions (*38*, *39*). Some studies demonstrated that the two trimers were held together by hydrophobic interactions to form a hexamer in a two-layered parallel hexagonal model (*40*) or a trigonal antiprism model (*41*, *42*). The estimated $M_w$ of peak 2 was smaller than that of the dimeric form of hexamers. As peak 2 was not fully separated from peak 1a, the estimated $M_w$ could be depressed at the regions where the peaks were overlapping. This is due to the fact that light

**Table 1.** Molar Mass Moments of Oat Globulin Heated for Different Times

| temp, °C | heating time, min | peak 1a | peak 1b | peak 2 | peak 3 |
|---|---|---|---|---|---|
| 100 | 0 | 3.33[a] (3.33,[b] 3.33[c])/ 1.000 ± 0.004[d] | 1.88 (1.87, 1.90)/ 1.010 ± 0.003 | 5.16 (5.14, 5.18)/ 1.003 ± 0.004 | 222 (215, 249)/ 1.116 ± 0.365 |
| | 10 | 3.36 (3.33, 3.38)/ 1.003 ± 0.003 | 1.25 (1.23, 1.26)/ 1.010 ± 0.004 | 6.42 (6.30, 6.55)/ 1.012 ± 0.008 | 229 (231, 268)/ 1.043 ± 0.155 |
| | 30 | 3.42 (3.42, 3.42)/ 1.002 ± 0.002 | 1.08 (1.06, 1.07)/ 1.016 ± 0.004 | 6.44 (6.32, 6.56)/ 1.018 ± 0.009 | 240 (228, 266)/ 1.103 ± 0.299 |
| | 60 | 3.51 (3.49, 3.51)/ 1.002 ± 0.003 | 0.93 (0.92, 1.00)/ 1.007 ± 0.004 | 6.96 (6.80, 7.14)/ 1.024 ± 0.012 | 352 (318, 388)/ 1.076 ± 0.328 |
| | 120 | 3.55 (3.54, 3.54)/ 1.001 ± 0.002 | 0.63 (0.62, 0.65)/ 1.002 ± 0.005 | 5.98 (5.97, 5.99)/ 1.002 ± 0.012 | |
| | 180 | 3.58 (3.56, 3.56)/ 1.000 ± 0.002 | 0.63 (0.63, 0.63)/ 1.001 ± 0.005 | 5.59 (5.61, 5.62)/ 1.002 ± 0.014 | |
| 110 | 5 | 2.50 (2.47, 2.53)/ 1.018 ± 0.013 | | | |
| | 15 | 2.00 (1.95, 2.05)/ 1.016 ± 0.013 | | | |
| | 30 | 1.53 (1.42, 1.63)/ 1.031 ± 0.017 | | | |

[a] Weight-average molecular weight ($10^5$), $M_w$. [b] Number-average molecular weight ($10^5$), $M_n$. [c] z-average molecular weight ($10^5$), $M_z$. [d] Polydispersity ($M_w/M_n$) ± SD.

scattering is measuring the weight-average $M_w$ of all the species present in solution.

The polydispersity ($M_w/M_n$) of separated peaks can also be estimated, which indicates whether the peak is homogeneous with respect to molar mass. The polydispersity of peaks 1a, 1b, 2, and 3 was 1.000 ± 0.004, 1.010 ± 0.003, 1.003 ± 0.004, and 1.116 ± 0.365, respectively (**Table 1**). The data suggest that peaks 1a, 1b, and 2 contained a fairly homogeneous population of protein molecules, whereas the aggregates eluted in peak 3 were relatively polydispersed.

The UV and MALLS elution profiles of heated oat globulin (60 min at 100 °C) overlaid with the estimated molar mass are shown in **Figure 3**. When compared with the unheated control, the slope of molar mass across peak 1a was also parallel to the *x*-axis, indicating a fairly homogeneous population of molecules. In contrast, the molar mass slopes across peak 1b and peak 2 were declining sharply, and assumed a concave shape across peak 3, suggesting less homogeneous populations.

The estimated weight-average $M_w$ of molecules in peaks 1a, 1b, 2, and 3 are summarized in **Table 1**, and the changes in $M_w$ as a function of heating time are also shown in the table. The estimated $M_w$ increased slightly during heating for peak 1a and decreased rapidly for peak 1b. The $M_w$ and polydispersity of oat globulin molecules in peak 2 increased with heating to 60 min, and then declined. There was a rapid increase in $M_w$ for peak 3 upon heating. Upon extended heating (>120 min at 100 °C), the estimated $M_w$ was decreased to 63 000 (**Table 1**), slightly larger than that of the monomers observed by Brinegar and Peterson (*2*) and Matlashewski et al. (*3*). The results suggest that the trimers were dissociated into monomers upon heating. Similar observation was reported by other workers. Chambers et al. (*17*) investigated the dissociation of legumin by salt using laser light scattering, and showed that the hexameric protein was dissociated first to trimers and then to monomers. In our previous study, due to a lack of detailed information on molecular weight distribution, only the dissociation of oat globulin hexamers into monomers was observed during heat treatments (*9*).

The UV and MALLS elution profiles of oat globulin heated at 110 °C for 30 min overlaid with estimated molecular mass are shown in **Figure 4**. From the UV profile, peak 1a was found to decrease rapidly and was changed to a shoulder of peak 1b.

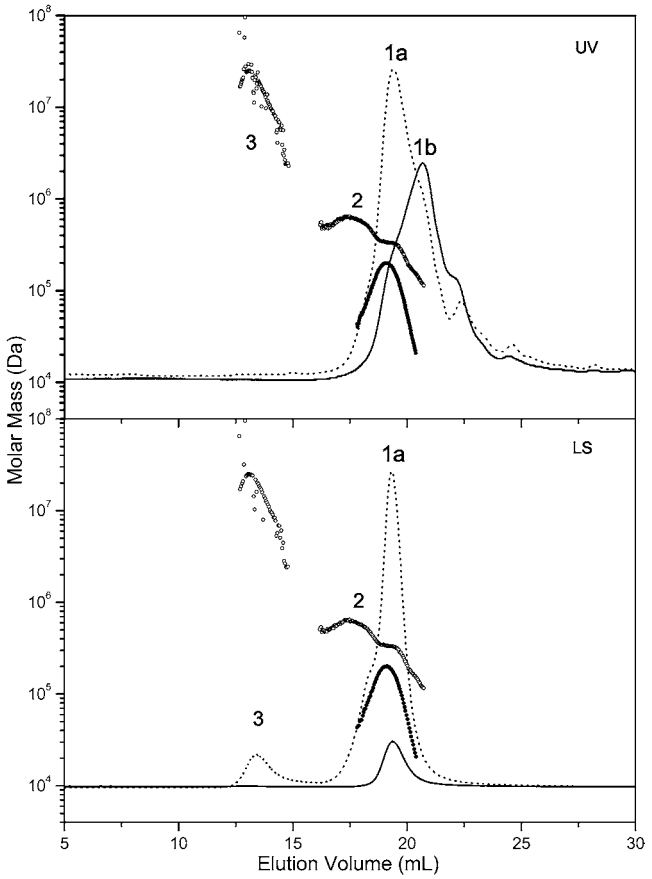


**Figure 4.** Relationship between molar mass and elution volume of the unheated (○) and heated (●) oat globulin (at 110 °C for 30 min). UV and LS (at 90°) elution profiles of the unheated (dotted line) and heated (solid line) oat globulin are included.

From the LS pattern, peak 1a was drastically decreased and peak 2 and peak 3 disappeared. The data suggest that a large quantity of protein was lost due to the formation of insoluble aggregates. The molar mass distribution assumed a concave shape over a much narrower range of elution volume when compared to the pattern at 100 °C (**Figure 3**). The molar mass slope across peak 1a declined sharply and was not parallel to

**Table 2.** Hydrodynamic Radius ($r_h$) of Oat Globulin Heated for Different Times

| temp, °C | heating time, min | peak 1a | peak 1b | peak 2 | peak 3 |
|---|---|---|---|---|---|
| 100 | 0 | 6.2[a] (6.2,[b] 6.2[c]) | 5.9 (5.9, 5.9) | 8.5 (8.5, 8.5) | 41.9 (41.3, 41.8) |
| | 10 | 6.1 (6.1, 6.1) | 6.8 (6.9, 6.8) | 9.6 (9.4, 9.8) | 42.5 (42.2, 42.8) |
| | 30 | 6.1 (6.1, 6.1) | 6.7 (6.8, 6.7) | 9.6 (9.5, 9.7) | 43.3 (43.3, 43.5) |
| | 60 | 6.1 (6.1, 6.1) | 6.7 (6.7, 6.6) | 9.7 (9.5, 9.8) | 44.8 (44.6, 45.0) |
| | 120 | 6.1 (6.1, 6.1) | 5.9 (5.9, 5.9) | 7.1 (8.1, 8.1) | |
| | 180 | 5.9 (5.9, 5.9) | 5.9 (5.9, 5.9) | 7.1 (7.2, 7.0) | |
| 110 | 5 | 5.8 (5.8, 5.8) | | | |
| | 15 | 5.8 (5.8, 5.8) | | | |
| | 30 | 5.8 (5.8, 5.8) | | | |

[a] Radius-average hydrodynamic radius (nm). [b] Number-average hydrodynamic radius (nm). [c] z-average hydrodynamic radius (nm).

the *x*-axis, indicting a mixture of molecules with different sizes in this peak. The estimated $M_w$ of molecules in peak 1a decreased upon heating at 110 °C (**Table 1**), suggesting dissociation of the oat globulin hexamer. The LS signals of peak 1b and peak 2 were so low that their estimated $M_w$ were not accurate. The data again indicate that oat globulin aggregated at a much faster rate at 110 °C than at 100 °C.

Hoffmann et al. (*11*) demonstrated that the molecular mass of heat-induced $\beta$-lactoglobulin aggregates increased with increasing heating time and temperature. For oligomeric proteins with complex quaternary structures, such as soybean 11S glycinin (*39*), and broad bean legumin (*26*), heat-induced changes include association/dissociation of the oligomer, formation of soluble aggregates, dissociation of the soluble aggregates into their constituent subunits/polypeptides, association of the soluble aggregates into insoluble aggregates, reassociation of subunits/polypeptides, and formation insoluble aggregates. Some of these changes were also observed in the present study for oat globulin.

**Determination of Hydrodynamic Radius.** Although the MALLS system can measure the root-mean-square radii ($r_g$) of globular proteins such as oat globulin, the values are generally below the angular variation detection limit of 10 nm, making the determination unreliable. Since dynamic light scattering techniques can accurately measure radii of macromolecules below 10 nm, a quasi-elastic light scattering detector was used to determine the hydrodynamic radii ($r_h$) for native and heat-aggregated oat globulin.

The estimated $r_h$ values of oat globulin in peak 1a, peak 1b, peak 2, and peak 3 are summarized in **Table 2**. The $r_h$ for unheated oat globulin ranged from nearly 6 nm to about 42 nm, decreasing with an increase in elution volume. The $r_h$ of hexamer was 6.2 nm, and that of trimer was 5.9 nm. With a two-layered parallel hexagonal model (*40*, *43*), the trimer of plant seed globulins appears as a flat disk about 11.8 to 12.2 nm in diameter ($2r_h$ = diameter). High-resolution electron microscopic and small-angle X-ray scattering studies of soybean glycinin (*40*), sunflower helianthinin (*41*, *42*), rape seed cruciferin (*42*), and broad bean legumin (*44*) demonstrated that the dimensions of these 11S seed globulins are in the range of 10.4−12.6 nm in diameter and 7.5−9 nm in height. Our data show that the diameter of the native oat globulin trimer is 11.8 nm (2 × 5.9 nm), whereas the diameter of the hexamer is 12.4 nm (2 × 6.2 nm). Since the oat globulin monomer contains nonidentical polypeptides (acidic and basic polypeptides), the stacking of the two trimeric rings may lead to a slight increase in diameter. The height of the hexamer could be deduced from the $r_h$ of the dimeric form of hexamer (8.5 nm), assuming that the two hexamers are stacked on top of each other, with the height (17 nm) larger than the diameter (12.4 nm). Both the estimated diameter and the height of the oat globulin hexamer are within the range of other seed 11S globulins.

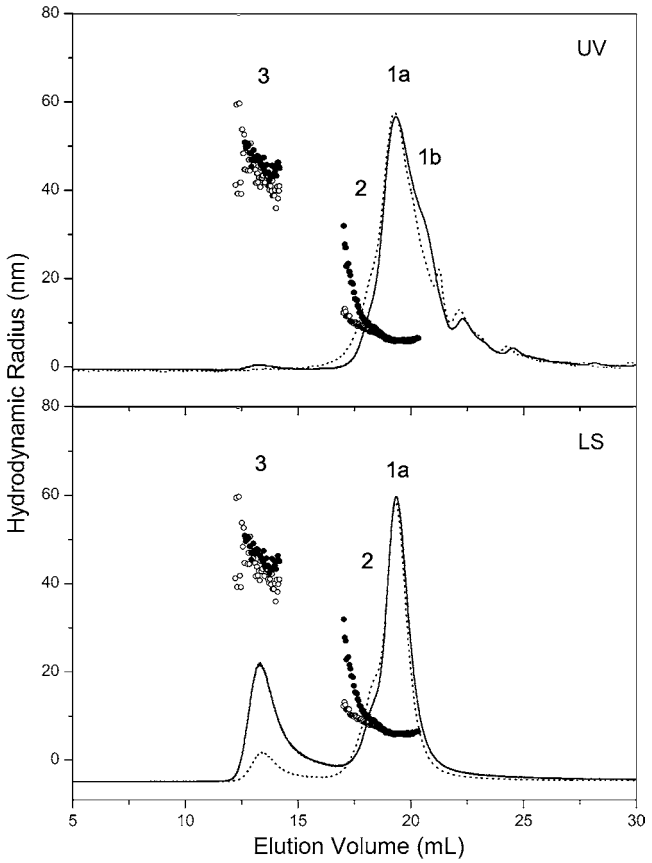


**Figure 5.** Relationship between hydrodynamic radius and elution volume of the unheated (○) and heated (●) oat globulin (at 100 °C for 60 min). UV and LS (at 90°) elution profiles of the unheated (dotted line) and heated (solid line) oat globulin are included.

The changes in $r_h$ of oat globulin during heat treatment at 100 °C are shown in **Table 2**. There was no marked change in the $r_h$ value of peak 1a during heating, suggesting that the oat globulin hexamers were still in globular form during dissociation, similar to that observed in 11S soybean glycinin (*45*). Heating caused an increase in $r_h$ for peak 1b molecules, suggesting marked protein unfolding before the dissociation of trimers into monomers, with a more extended structure. Upon further heating, the $r_h$ of molecules in peak 1b decreased, and after heating for 180 min, the estimated $r_h$ was 5.9 nm. Since the molecular mass measurements show that the trimers were dissociated into monomers, the data suggest that the monomers, with an $r_h$ similar to that of the trimer, should be in an extended conformation.

The UV and MALLS elution profiles of oat globulin heated at 100 °C for 60 min overlaid with estimated hydrodynamic radius are shown in **Figure 5**. The patterns resemble those of changes in molar mass (**Figure 3**). The $r_h$ values of oat globulin in both peak 2 and peak 3 were increased with an increase in heating time up to 60 min, suggesting the formation of aggregates. When heated for 60 min, the $r_h$ of molecules in peak 2 ranged from nearly 6 nm to 24 nm, and decreased upon further heating. The data show that oligomeric oat globulin molecules were associated into large macroaggregates with $r_h$ larger than 24 nm.

## CONCLUSION

The present investigation demonstrates that the SEC-MALLS system can be used to monitor thermal aggregation of plant seed storage proteins with limited solubility, such as oat globulin. When coupled with dynamic light scattering, the dimensions of various oligomeric forms of oat globulin, including the monomer, trimer, and hexamer, as well as the aggregated molecules, can be estimated. Furthermore, conformational changes in the protein molecules during thermal aggregation can be predicted from these measurements. More accurate measurements of the conformation of these molecules would require the determination of parameters such as the ratio of $r_g$ and $r_h$, and are limited by difficulties in accurately measuring $r_g$ of these globular proteins by MALLS.

## ACKNOWLEDGMENT

We thank Dr. V. D. Burrows, Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, for the supply of dehulled oat seeds.

## LITERATURE CITED

(1) Ma, C. Y. Preparation, composition and functional properties of oat protein isolates. *Can. Inst. Food Sci. Technol. J.* **1983**, *16*, 201−205.

(2) Brinegar, A. C.; Peterson, D. M. Separation and characterization of oat globulin polypeptides. *Arch. Biochem. Biophys.* **1982**, *219*, 71−79.

(3) Matlashewski, G. J.; Adeli, K.; Altosaar, I.; Shewry, P. R.; Miflin, B. J. In vitro synthesis of oat globulin. *FEBS Lett.* **1982**, *145*, 208−212.

(4) Millerd, A. Biochemistry of legume seed proteins. *Annu. Rev. Plant Physiol.* **1975**, *26*, 53−72.

(5) Derbyshire, E.; Wright, D. J.; Boulter, D. Legumin and vicilin. Storage proteins of legume seeds. *Phytochemistry* **1976**, *15*, 3−24.

(6) Neilsen, N. C. The structure and complexity of 11S polypeptides in soybeans. *J. Am. Oil Chem. Soc.* **1985**, *62*, 1680−1686.

(7) German, B.; Damodaran, S.; Kinsella, J. E. Thermal dissociation and association behaviour of soy proteins. *J. Agric. Food Chem.* **1982**, *30*, 807−812.

(8) Hermansson, A. M. Aggregation and denaturation involved in gel formation. In *Functionality and Protein Structure*; ACS Symp. Ser. 92; Pour-EI, A., Ed.; American Chemical Society: Washington, DC, 1979; pp 82−165.

(9) Ma, C. Y.; Harwalkar, V. R. Thermal coagulation of oat globulin. *Cereal Chem.* **1987**, *64*, 212−218.

(10) Wyatt, P. J. Combined differential light scattering with various liquid chromatographic separation techniques. In *Laser Light Scattering in Biochemistry*; Harding, S. E., Satelle, D. B., Bloomfield, V. A., Eds.; Royal Society of Chemistry: Cambridge, UK, 1992; pp 35−38.

(11) Hoffman, M. A. M.; Sala, G.; Olieman, C.; de Kruif, C. G. Molecular mass distributions of heat-induced $\beta$-lactoglobulin aggregates. *J. Agric. Food Chem.* **1997**, *45*, 2949−2957.

(12) van Dijk, J. A. P. P.; Smit, J. A. M. Size-exclusion chromatography-multiangle laser light scattering analysis of $\beta$-lactoglobulin and bovine serum albumin in aqueous solution with added salt. *J. Chromatogr. A* **2000**, *867*, 105−112.

(13) Lucey, J. A.; Srinivasan, M.; Singh, H.; Munro, P. A. Characterization of commercial and experimental sodium caseinates by multiangle laser light scattering and size-exclusion chromatography. *J. Agric. Food Chem.* **2000**, *48*, 1610−1616.

(14) Mine, Y. Laser light scattering study on the heat-induced ovalbumin aggregates related to its gelling property. *J. Agric. Food Chem.* **1996**, *44*, 2086−2090.

(15) Bean, S. R.; Lookhart, G. L. Factors influencing the characterization of gluten proteins by size-exclusion chromatography and multiangle laser light scattering (SEC-MALLS). *Cereal Chem.* **2001**, *78*, 608−618.

(16) Rhazi, L.; Cazalis, R.; Aussenac, T. Sulfhydryl-disulfide changes in storage proteins of developong wheat grain: influence on the SDS-unextractable glutenin polymer formation. *J. Cereal Sci.* **2003**, *38*, 3−13.

(17) Chambers, S. J.; Carr, H. J.; Lambert, N. An investigation of the dissociation and denaturation of legumin by salts using laser light scattering and circular dichroism spectroscopy. *Biochim. Biophys. Acta* **1990**, *1037*, 66−72.

(18) Zhang, G. Y.; Hayashi, Y.; Matsumoto, S.; Matsumura, Y.; Mori, T. Molecular species of glycinin in some soybean cultivars. *Phytochemistry* **2002**, *60*, 675−681.

(19) Schokker, E. P.; Singh, H.; Pinder, D. N.; Norris, G. E.; Creamer, L. K. Characterization of intermediates formed during heat-induced aggregation of $\beta$-lactoglobulin AB at neutral pH. *Int. Dairy J.* **1999**, *9*, 791−800.

(20) Tromp, R. H.; Grotenhuis, E. ten; Olieman, C. Self-aggregation of gelatine above the gelling temperature analyzed by SEC-MALLS. *Food Hydrocolloids* **2002**, *16*, 235−239.

(21) Ma, C.-Y.; Harwalkar, V. R. Chemical characterization and functionality assessment of oat protein fractions. *J. Agric. Food Chem.* **1984**, *32*, 144−149.

(22) Zimm, B. H. The dependence of the scattering of light on angle and concentration in linear polymer solutions. *J. Phys. Colloid Chem.* **1948**, *52*, 260−267.

(23) Müller, A.; Pretus, H. A.; McNamee, R. B.; Jones, E. L.; Browder, I. W.; Williams, D. L. Comparison of the carbohydrate biological response modifiers Krestin, schizophyllan and glucan phosphate by aqueous size exclusion chromatography with in-line argon-ion multi-angle laser light scattering photometry and differential viscometry detectors. *J. Chromatogr. B* **1995**, 666, 283−290.

(24) Pots, A. M.; Gruppen, H.; de Jongh, H. H. J.; van Boekel, M. A. J. S.; Walstra, P.; Voragen, G. J. Kinetic modeling of the thermal aggregation of patatin. *J. Agric. Food Chem.* **1999**, *47*, 4593−4599.

(25) Kato, A.; Takagi, T. Estimation of the molecular weight distribution of heat-induced ovalbumin aggregates by the low-angle laser light scattering technique combined with high-performance gel chromatography. *J. Agric. Food Chem.* **1987**, *35*, 633−637.

(26) Zheng, B. A.; Matsumura, Y.; Mori, T. Thermal gelation mechanism of legumin from broad bean. *J. Food Sci.* **1991**, *56*, 722−725

(27) Verheul, M.; Roefs, S. P. F. M. Structure of particulate whey protein gels: effect of NaCl concentration, pH, heating temperature, and protein composition. *J. Agric. Food Chem.* **1998**, *46*, 4909−4916.

(28) Harwalkar, V. R.; Ma, C. Y. Study of thermal properties of oat globulin by differential scanning calorimetry. *J. Food Sci.* **1987**, *52*, 394−398.

(29) Ma, C. Y.; Harwalker, V. R. Studies of thermal denaturation of oat globulin by differential scanning calorimetry. *J. Food Sci.* **1988**, *53*, 531−534.

(30) Ma, C. Y.; Rout, M. K.; Mock, W. Y. Study of oat globulin conformation by fourier transform infrared spectroscopy. *J. Agric. Food Chem.* **2001**, *49*, 3328−3334.

(31) Wen, J.; Arakawa, T.; Philo, J. S. Size-exclusion chromatography with on line light-scattering, absorbance, and refractive index detectors for studying proteins and their interactions. *Anal. Biochem.* **1996**, *240*, 155−166.

(32) Fishman, M. L.; Rodriguez, L.; Chau, H. K. Molar masses and sizes of starches by high performance size-exclusion chromatography with on-line multi-angle laser light scattering detection. *J. Agric. Food Chem.* **1996**, *44*, 3182−3188.

(33) Fredheim, G. E.; Braaten, S. M.; Christensen, B. E. Molecular weight determination of lignosulfonates by size-exclusion chromatography and multi-angle laser light scattering. *J. Chromatogr. A* **2002**, *942*, 191−199.

(34) Tanford, C. Light Scattering. In *Physical Chemistry of Macromolecules*; Wiley: New York, 1961; pp 275−316.

(35) Billingham, N. C. In *Molar Mass Method in Polymer Science*; Wiley: New York, 1977; pp 105−145.

(36) Shortt, D. W. Differential molecular weight distributions in high performance size exclusion chromatography. *J. Liq. Chromatogr.* **1993**, *16*, 3371−3391.

(37) Shotwell, M. A.; Boyer, S. K.; Chesnut, R. S.; Larkins, B. A. Analysis of seed storage protein genes of oats. *J. Biol. Chem.* **1990**, *265*, 9652−9658.

(38) Wolf, W. J.; Briggs, D. R. Studies on the cold-insoluble fraction of the water-extractable soybean proteins. II. Factors influencing conformation changes in the 11S component. *Arch. Biochem. Biophys.* **1958**, *76*, 377−393.

(39) Mori, T.; Nakamura, T.; Utsumi, S. Gelation mechanism of soybean 11S globulin: formation of soluble aggregates as transient intermediates. *J. Food Sci.* **1982**, *47*, 26−30.

(40) Badley, R. A.; Atkinson, D.; Hauser, H.; Oldani, D.; Green, J. P.; Stubbs, J. M. The structure, physical and chemical properties of the soybean protein glycinin. *Biochim. Biophys. Acta* **1975**, *412*, 214−218.

(41) Reichelt, R.; Schwenke, K.-D.; Konig, T.; Pahtz, W.; Wangermann, G. Electron microscopic studies for estimation of the quaternary structure of the 11S globulin (Helianthinin) from sunflower seed (*Helianthus annuus* L.). *Biochem. Physiol. Pflanz.* **1980**, *175*, 653.

(42) Plietz, P.; Damaschun, G.; Muler, J. J.; Schwenke, K. D. The structure of 11-S globulins from sunflower and rape seed. *Eur. J. Biochem.* **1983**, *130*, 315−320.

(43) Marcone, M. F.; Niekamp, F. K.; Maguer, M. L.; Yada, R. Y. Purification and characterization of the physicochemical properties of the albumin fraction from the seeds of *Amaranthus hypochondriacus*. *Food Chem.* **1994**, *51*, 287−294.

(44) Plietz, P.; Zirwer, D.; Schlesier, B.; Gast, K.; Damaschun, G. Shape, symmetry, hydration and secondary structure of the legumin from *Vicia faba* in solution. *Biochim. Biophys. Acta* **1984**, *784*, 140−146.

(45) Nakamura, T.; Utsumi, S.; Mori, T. Network structure formation in thermally induced gelation of glycinin. *J. Agric. Food Chem.* **1984**, *32*, 349−352.

**Received for review October 28, 2003. Revised manuscript received February 17, 2004. Accepted February 26, 2004. The research was supported by a Hong Kong Research Grant Council (RGC) grant (HKU 7223/98M).**

JF030735Y