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Study of Thermal Aggregation of Globulin from Common Buckwheat (*Fagopyrum esculentum* Moench) by Size-Exclusion Chromatography and Laser Light Scattering

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The heat-induced aggregation of common buckwheat (*Fagopyrum esculetum* Moench) globulin (BWG) was studied using size-exclusion chromatography (SEC) combined with on-line multiangle laser light scattering (MALLS) and quasielastic light scattering (QELS). The unheated BWG was found to exist mainly as a hexamer, with an estimated weight-average molecular weight (M_w) of 342 000, close to that deduced from the genomic cloned data of 13S buckwheat globulin. The QELS data predicted that the hexamer exists as two annular trimeric rings (diameter ~ 10.8 nm) placed on top of each other, forming an oblate cylinder (height ~ 9.1 nm). Upon heating, hexamers and trimers were dissociated and then associated to form extended small aggregates, finally forming compact, large macroaggregates. *N*-Ethylmaleimide would favor macroaggregate formation and increased the molar masses and hydrodynamic radii of the soluble aggregates, suggesting a different aggregation process in the presence of the sulfhydryl-blocking agent. A plot of log hydrodynamic radius versus log molar mass showed changes in the slope during heat treatment, suggesting conformational transformation in the heat-denatured and aggregated BWG molecules.

KEYWORDS: ***Fagopyrum esculentum* Moench; buckwheat globulin; thermal aggregation; laser light scattering; quasielastic light scattering**

INTRODUCTION

Common buckwheat (*Fagopyrum esculentum* Moench) is an underutilized pseudocereal with high nutritional value (*1*, *2*). Buckwheat seeds contain 10−12.5% proteins, 65−75% starch, 2−2.5% fat, and 2−2.5% minerals (*3*). Buckwheat is also rich in polyphenols and can be used as a good source of dietary rutin, which is not found in other pseudocereals or cereals (*4*, *5*). Buckwheat protein has a high biological value due to a well-balanced amino acid composition that is rich in lysine and arginine (*1*). According to net protein utilization (NPU), buckwheat protein is classified close to animal proteins and considered to have excellent supplementary value to cereal grains (*6*). The salt-soluble protein, buckwheat globulin (BWG), represents the major Osborne fraction of buckwheat seed proteins (*7*) and has been classified as a legumin-like storage protein (*8*). It contains six nonidentical monomers, each of which is composed of one large acidic and one small basic polypeptide, linked by disulfide bonds (*2*, *9*). The six monomers are linked by noncovalent forces to form the hexamer, similar to legumins (*8*, *10*, *11*). BWG is of interest as an ingredient for fabricated foods, since it possesses good nutritional value (*1*) and exhibits promising functional properties, such as emulsion-forming and stabilizing capacity (*12*). Thermal aggregation and gelation are important functional properties of food proteins, and a detailed study of these properties is critical in developing protein products to meet the requirements for food applications.

Using differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy, BWG has been shown to form aggregates at temperatures below its denaturation temperature of 100.4 °C (*13*). The physicochemical properties and microstructure of heat-induced BWG aggregates have also been evaluated by techniques such as DSC, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE), sucrose gradient ultracentrifugation and transmission electron microscopy (TEM) (*14*). In this investigation, thermal aggregation of BWG will be studied using size-exclusion chromatography combined with on-line multiangle laser light scattering and dynamic quasielastic light scattering (SEC−MALLS−QELS). The SEC−MALLS system is a useful technique for monitoring changes in proteins during heat treatment and for characterization of protein aggregation behavior (*15*, *16*). MALLS can accurately measure the molecular weight (M_w) and root-mean-square (rms) radii of molecules in solution (including soluble aggregates) without dependence on column calibration or reference standards. An additional benefit of such measurement is that it can reveal whether the protein exists as a monomer, dimer, or a higher aggregate. Moreover, it is possible to follow conformational changes in the protein during heat treatment (*17*). For globular proteins with diameters below 10 nm, dynamic

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light scattering is an appropriate technique, since it can determine the hydrodynamic radii (r_h) of molecules from 1 to 500 nm (*17*). Recently, we used SEC−MALLS−QELS to monitor the aggregation process of oat globulin, the first time that a plant protein with limited solubility was studied by such a technique (*18*). In this study, the results of M_w, r_h, and the conformation of BWG and its aggregates will be compared with the data deduced from the cloned data, gel filtration, and transmission electron microscopy. Moreover, the conformational characteristics of native and heated BWG will be deduced from the slopes of the log−log plots of r_h versus M_w.

MATERIALS AND METHODS

Preparation of Buckwheat Globulin. Buckwheat flour with 84.6% carbohydrate, 10.7% protein, 2.9% lipid, and 1.8% ash on dry weight basis (*14*) was obtained from Nikkoku Flour Milling Co. Ltd. (Japan) and was defatted by Soxhlet extraction with hexane. BWG was extracted from the defatted buckwheat flour using 0.5 M NaCl buffer in a ratio of 1:10 (w/v) according to the Osborne fractionation scheme (*19*). The protein content of BWG was 93.9% (dry basis), as determined by the micro-Kjeldahl method (*20*) using a nitrogen-to-protein conversion factor of 5.53 (*21*).

Preparation of Buffer-Soluble Heat-Induced Aggregates. BWG dispersions (0.5%, w/v) were prepared in 50 mM phosphate buffer at pH 7.4, containing 1.0 M NaCl. Each tube containing an aliquot (1 mL) of the protein solution was heated for different time periods in a thermostatically controlled water bath. Two heating temperatures, 92 and 100 °C, were selected on the basis of our differential scanning calorimetric (DSC) study (*13*, *14*), which showed the denaturation temperatures (T_d) of the native BWG and the major purified polypeptide (23−25 kDa) to be 100 and 92 °C, respectively. Heated samples were cooled in an ice bath for 5 min and then filtered through a 0.22-μm filter (Millipore Corp., Bedford, MA), and 50 μL of the filtrate was injected into the chromatographic system.

High Performance Size-Exclusion Chromatography Combined with Multiangle Laser Light Scattering and Quasielastic Light Scattering. The high-performance size exclusion chromatographic (HPSEC) system consisted of an in-line degasser, a pump, an ultraviolet (UV) detector, and a differential refractive index (RI) detector (Hewlett-Packard, Palo Alto, CA). Two TSK columns (G4000 PW_{XL} + G6000 PW_{XL}) (TOSOH Corp., Montgomeryville, PA) were connected in series with fractionation range of 2−300 and 40−8000 kDa, respectively. The columns were eluted with phosphate buffer (50 mM) containing 1.0 M NaCl (pH 7.4) filtered with a 0.22 μm filter. The flow rate was 0.5 mL/min.

The multiangle laser light scattering detector (DAWN EOS, Wyatt Technology Corp., Santa Barbara, CA) was placed directly before the RI detector and after the HPLC columns and UV detector to avoid backpressure on the RI cell. Chromatographic data were collected and processed by the ASTRA software, version 4.81.07 (Wyatt Technology Corp., Santa Barbara, CA). Bovine serum albumin (BSA) monomer was used for normalizing various detectors' signals relative to the 90° detector's signal. Dynamic light scattering measurement was performed on-line in the K5 flow cell using a quasielastic light scattering (QELS) detector. An optical fiber receiver was mounted in the read head of one of the MALLS detectors (detector 13 in this work), 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. Molecular masses and hydrodynamic radii were determined according to our previous study (*18*). All experiments were performed in triplicates, and reproducible data with standard deviations less than 10% were obtained.

Statistical Analysis. Analysis of variance and Duncan's multiple range tests were performed at the $p \leq 0.05$ level, using a standard statistical software package.

RESULTS AND DISSCUSSION

Chromatographic Profile of Native BWG. The elution profiles of unheated BWG, detected by UV, MALLS, and QELS detectors are shown in **Figure 1**. In the UV profile of the unheated native BWG (0 min), three partially resolved peaks were observed (**Figure 1A**). Peak 3 represents the major fraction of BWG, probably the hexamer. Peak 2 may correspond to the trimer, and peak 1 may represent a mixture of acidic and basic polypeptides. Two peaks at lower elution volume were detected in the MALLS profile (**Figure 1B**). Peak 4, a minor shoulder of peak 3, could be the dimeric form of the BWG hexamer. Very large-sized materials were eluted with the void volume (peak 5), which was invisible to the UV detector. The appearance of this peak could represent protein molecules aggregated during the lyophilization process (*22*). The elution pattern resembles that of oat globulin (*18*), probably attributed to their similarity in molecular structure.

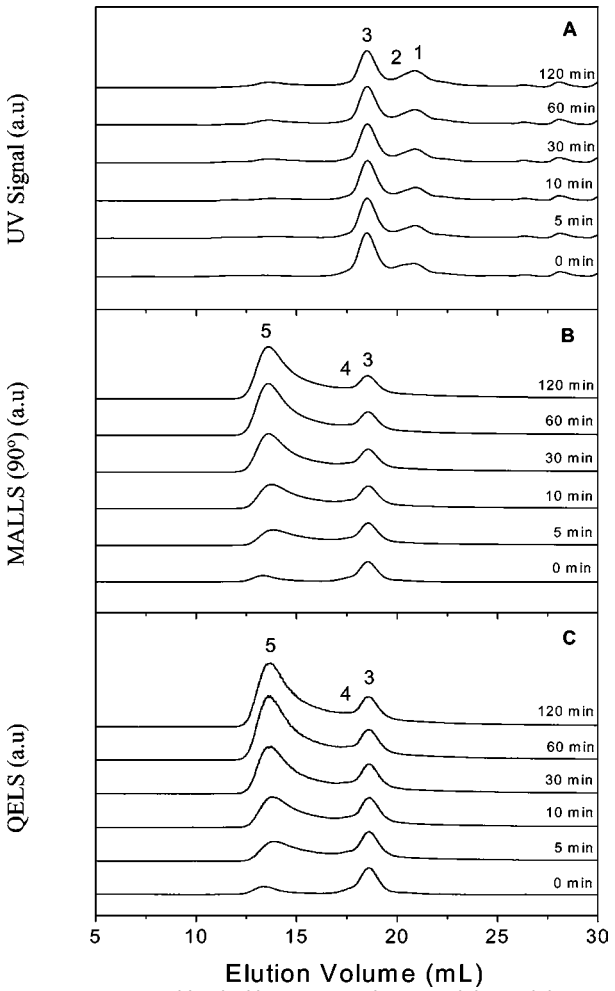


Figure 1. HPLC combined with MALLS and QELS: (**A**) UV, (**B**) MALLS (at 90°), and (**C**) QELS elution profiles of buckwheat globulin (in 50 mM phosphate buffer with 1.0 M NaCl, pH 7.4) heated at 92 °C for various time intervals.

Similar elution patterns were observed in the MALLS and QELS profiles of unheated BWG (**Figure 1C**). Since the light scattering signal is proportional to the concentration of the products and molecular mass and is strongly dependent on particle radius, a small amount of large size materials in the sample would give a large response by the light scattering detector, although their amount, as measured by the UV response, is very small (*17*). The UV detector is much more sensitive to small-sized molecules than the MALLS detector, and therefore, peaks 1 and 2 were detected only in the UV profile.

Chromatographic Profiles of Heated BWG. Upon heating at 92 °C, the size of peak 5 in the MALLS profile (**Figure 1B**),

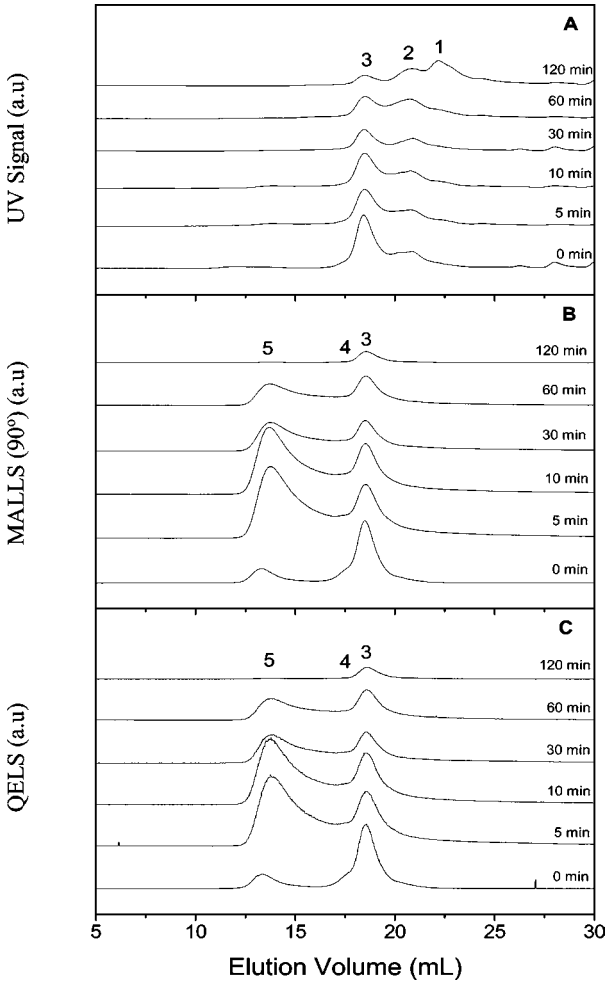


Figure 2. HPLC combined with MALLS and QELS: (**A**) UV, (**B**) MALLS (at 90°), and (**C**) QELS elution profiles of buckwheat globulin (in 50 mM phosphate buffer with 1.0 M NaCl, pH 7.4) heated at 100 °C for various time intervals.

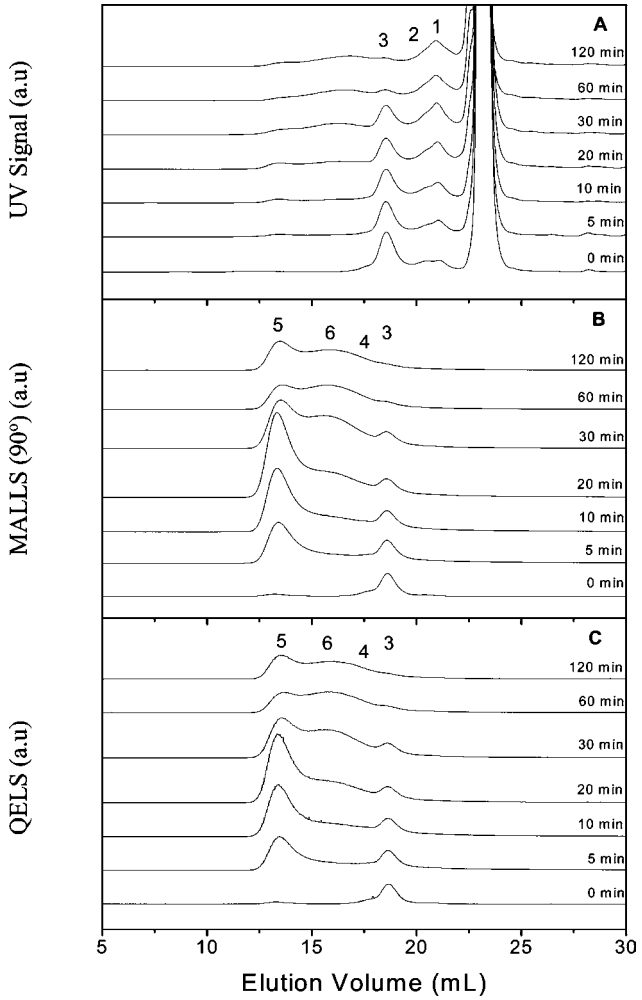


Figure 3. HPLC combined with MALLS and QELS: (**A**) UV, (**B**) MALLS (at 90°), and (**C**) QELS elution profiles of buckwheat globulin (in 50 mM phosphate buffer with 10 mM *N*-ethylmaleimide and 1.0 M NaCl, pH 7.4) heated at 100 °C for various time intervals.

representing the amount of soluble aggregates, increased with increasing heating time. The peak was initially invisible in the UV profile and was then shown as a very small peak after extended heating (60 and 120 min). The growth of aggregates accelerated with increasing heating time as long as native proteins were still present in the solution. With the fractionation range of the SEC columns, large-sized soluble aggregates were eluted in the void volume (13 mL), and peak 5 can therefore be used to show the formation of soluble aggregates. The data suggest that large soluble aggregates were formed from BWG hexamer and its dimeric form during heating. As shown in **Figure 1A**, heating did not cause much change in peaks 1 and 2.

The UV, MALLS, and QELS elution profiles of BWG heated at 100 °C for various time periods are shown in **Figure 2**. From the UV profile, the amount of hexamers (the size of peak 3) was shown to decrease, accompanied by an increase in peak 1 when the heating time was increased (**Figure 2A**), suggesting dissociation of the oligomers into monomers. The MALLS profiles show a marked increase in the size of peak 5 when BWG was heated for 5 and 10 min (**Figure 2B,C**). Upon further heating, peak 5 was progressively decreased, together with further decreases in peak 3 (**Figure 2B**). There was complete disappearance of peak 5 after heating for 120 min. The results suggest that with increasing heating time, more BWG hexamers were aggregated and then associated rapidly to form larger and more stable insoluble aggregates. These insoluble aggregates cannot be analyzed by the SEC system, as they were removed by centrifugation.

Our DSC data show that BWG has exceptionally high thermal stability with a T_d close to 109 °C in 1.0 M NaCl (*13*). The present results indicate that BWG could aggregate well below its T_d, similar to that observed in oat (*18*) and red bean globulin (*23*). The present study also demonstrates the dissociation of BWG hexamers into trimers and monomers, and the formation of insoluble aggregates occurred at a much faster rate at 100 °C than at 92 °C.

Effect of *N*-Ethylmaleimide on Chromatographic Profiles. Since BWG, similar to other legumin-like proteins, contains disulfide-linked polypeptides in each monomer, the importance of SH−SS interchange reactions on thermal aggregation is worth studying. This can be assessed by the use of an SH-blocking agent, NEM, in the buffer. The SEC elution profiles of BWG heated at 100 °C in the presence of 10 mM NEM are shown in **Figure 3**. NEM has high UV absorbance and was shown as a huge peak in the elution volume of the columns. For unheated BWG, an UV profile similar to that without NEM was observed (**Figure 3A**), suggesting that the native structure remained unchanged in the presence of the reagent. With increasing heating time, the size of peak 3 (hexamers) was decreased, with a concomitant progressive increase in peak 1 (dissociated

molecules) (**Figure 3A**). The MALLS profiles (**Figure 3B**) show that the rate of reduction in peak 3 was increased when compared to samples heated in the absence of NEM (**Figure 2B**), and there was almost complete disappearance of the hexamers after 60 min of heating. Peak 5 was markedly increased, reaching a maximum at around 20 min, followed by a progressive decrease with extended heating. When compared to samples heated in the absence of NEM (**Figure 2B**), there was a much more pronounced increase in the size of peak 5. In the presence of the additive, the MALLS profiles also showed the appearance, after 20 min of heating, of a new peak (peak 6) between peak 4 and peak 5. The QELS profiles (**Figure 3C**) are similar to those of the MALLS profiles.

The data demonstrate that when heated in the presence of NEM, BWG hexamers were dissociated into smaller M_w molecules (peak 1), similar to that observed in the absence of the additive. However, NEM seems to promote more dissociation and association of the hexamers, leading to the formation of a larger quantity of macroaggregates (peak 5), including some intermediates with smaller molecular size (peak 6). The results suggest that the blocking of free SH groups in BWG, or the prevention of SH−SS interchange reaction, would favor macroaggregate formation. Hence, SH−SS interactions seem to play only a minor role in thermal aggregation of BWG, since NEM cannot prevent the formation of either soluble or insoluble aggregates. It has been reported that the inability of NEM to inhibit aggregate formation under restricted disulfide exchange may be attributed to the fact that SH and SS residues have propensities to be buried in the hydrophobic regions of the protein molecules (*24*).

NEM has been shown to affect thermal aggregation of β-lactoglobulin, which appeared to aggregate via a different route when the thiol groups were blocked (*25*). The decrease in thiol content and the subsequent decrease in disulfide bond formation in the presence of NEM may increase molecular flexibility and enhance interactions via nonspecific bonding (*25*). In contrast, NEM was found to inhibit the formation of insoluble aggregates in soy glycinin by blocking free SH groups and preventing SH−SS interactions (*26*, *27*). For oat globulin, NEM did not prevent protein precipitation, but the formation of soluble aggregates was hindered (*28*). In studying thermal aggregation of potato patatin, heating in the presence of NEM also did not prevent the formation of dimers, trimers, and subsequent large aggregates (*29*). Compared to previous investigations, the use of SEC−MALLS−QELS system in this study can provide more accurate and detailed information on the influence of NEM on thermal aggregation of proteins.

Determination of Molecular Mass. For the unheated BWG, the M_w were estimated as 104 000, 342 000, 613 000, and 3 567 000 for peaks 2, 3, 4, and 5, respectively. The determined M_w of the native BWG hexamer (342 000) is close to the value (335 000) estimated from gel filtration chromatography calibrated with protein standards (*9*). The value is also within the range of M_w for over 20 11S globulins isolated from dicotyledonous and monocotyledonous plants, typically in a narrow range of 300−370 kDa (*9*). Recently, several genomic clones corresponding to 13S buckwheat globulin have been characterized (*30*−*33*). The acidic and basic polypeptides were found to contain 293 and 191 amino acids, respectively (*30*), and the calculated M_w of 13S buckwheat globulin was 342 258. The present value (342 000) is therefore in very good agreement to that deduced from cloned data.

The MALLS elution profile of BWG heated at 100 °C for 30 min overlaid with the estimated molar mass is shown in

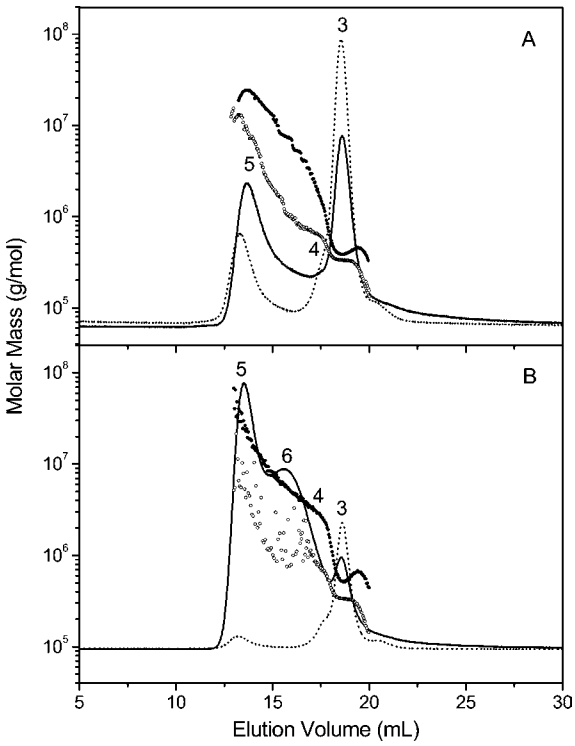


Figure 4. Relationship between molar mass and elution volume of the unheated (○) and heated (at 100 °C for 30 min) (●) buckwheat globulin in 50 mM phosphate buffer with 1.0 M NaCl, pH 7.4. MALLS (at 90°) elution profiles of unheated (dotted line) and heated (solid line) buckwheat globulin are included. (**A**) Without NEM and (**B**) with 10 mM NEM.

Figure 4A. Heating caused a slight increase in M_w and showed a concave slope across peak 3, suggesting less homogeneous populations of BWG molecules due to the formation of small aggregates. The molar mass slope across peaks 4 and 5 declined sharply and were not parallel to the *x*-axis, indicating formation of a heterogeneous population of aggregates with different sizes across the peak. The molar mass in peak 5 increases markedly upon heating, as large soluble aggregates were formed. BWG heated at 92 °C exhibited a similar profile (data not shown). Similar molar mass distribution was observed in the presence of NEM (**Figure 4B**), except that the unheated sample shows a more scattered distribution of M_w across peaks 5 and 6, suggesting a more heterogeneous population of molecules in the presence of NEM. Similar M_w distribution was also observed in β-lactoglobulin (*34*) and oat globulin (*18*), in which the molecular mass of heat-induced aggregates was found to increase with increasing heating time and temperature.

The changes in M_w for BWG heated at 100 °C are shown in **Figure 5**. There was a rapid increase in the estimated M_w for peak 3 and 5, followed by a leveling off after 10 min. Similar changes in M_w were also observed for peak 3 when BWG was heated in the presence of NEM (data not shown). For peak 5, the M_w of the unheated sample was more than twice that in the absence of the reagent, suggesting the promotion of self-association to BWG molecules to form larger macroaggregates. At any time period, the estimated M_w of peak 5 components was much higher than that recorded when BWG was heated in the absence of NEM (data not shown). The new peak (peak 6) between peaks 3 and 5 has an estimated M_w value (5 570 000 Da) intermediate between those of the two peak components, and a progressive but slight decline in M_w was observed during heat treatment (data not shown). The data confirm that NEM has an influence on the aggregation process of BWG, similar

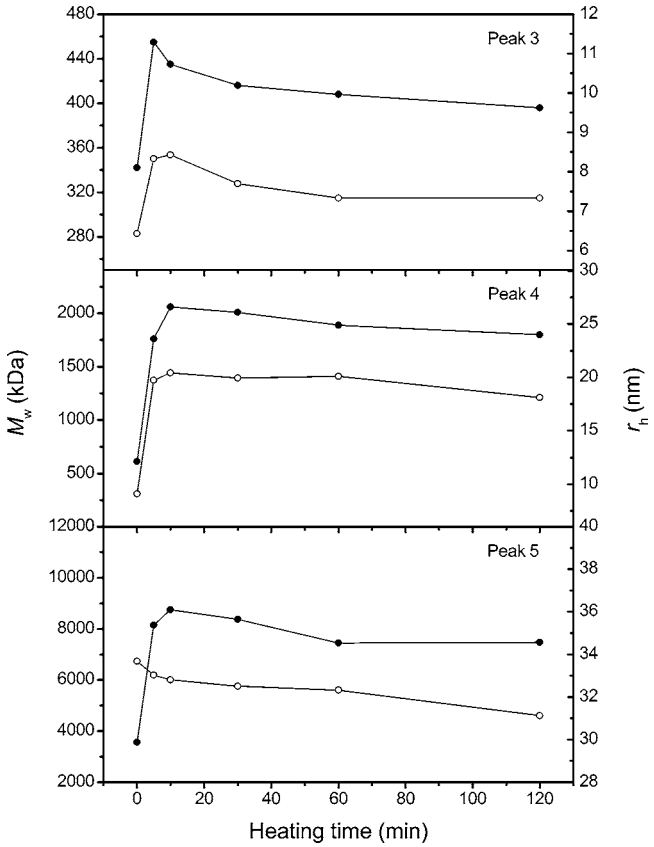
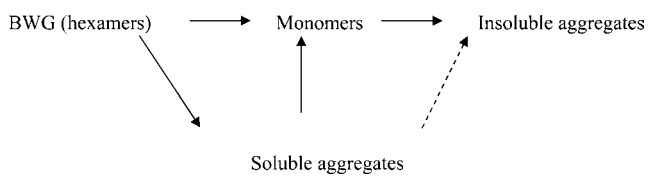


Figure 5. Changes in molar mass (M_w) (●) and hydrodynamic radius (r_h) (○) of BWG (in 50 mM phosphate buffer with 1.0 M NaCl, pH 7.4) heated at 100 °C for various time intervals.

to that observed in β-lactoglobulin, in which the blocking of SH groups led to a change in the route of aggregation (*25*).

In the thermal aggregation of oligomeric proteins such as soy glycinin (*27*), oat globulin (*18*, *28*), and broad bean legumin (*35*), thermal changes may include association and dissociation of the oligomer, formation of soluble aggregates and their dissociation into constituent polypeptides, reassociation of the polypeptides, formation of high molecular weight macroaggregates, and the ultimate formation of insoluble aggregates. For soy glycinin, heat-induced changes were proposed to include the formation of soluble aggregates, dissociation of the soluble aggregates to acidic and basic polypeptides and formation of insoluble aggregates from dissociated basic polypeptides, whereas acidic polypeptides remain soluble (*27*). Similar dissociation of soluble aggregates was also reported in oat globulin (*28*), particularly at higher temperatures, but the predominant product was monomer, with little acidic and basic polypeptides. It was also suggested that SH−SS interchange is limited in oat globulin, and heating only disrupts noncovalent bonds linking the monomers into soluble aggregates (*28*). In the present study, dissociation of BWG and association to high M_w aggregated molecules were also observed.

On the basis of the results, the following scheme for the thermal coagulation of BWG, similar to that of oat globulin (*28*), is proposed:



At the early stage of heating, a small portion of the oligomeric BWG associates to form soluble aggregates that may dissociate into monomers upon further heating. The major proportion of the hexamers could dissociate into monomeric polypeptides upon heating. Insoluble aggregates may be formed from partially denatured monomers through protein−protein interactions or from oligomers via a disruption of the quaternary structure.

Determination of Hydrodynamic Radius. The estimated r_h values for unheated BWG ranged from 5.4 to 33.7 nm, decreasing with increasing elution volume. The nonidentical hexamers of BWG were found to have r_h in the range of 6.3−6.7 nm (data not shown). The average r_h of BWG hexamer and trimer is 6.4 and 5.4 nm, respectively. In the case of 11S globulins, the arrangement of the six monomers can be approximated by a two-layered parallel hexagonal model (*36*, *37*) or a trigonal antiprism model (*38*, *39*). With a two-layered parallel hexagonal model (*36*, *37*), trimer of seed globulins appears as a flat disk about 11.8−12.2 nm in diameter ($2r_h$ = diameter). Electron microscopic and small-angle X-ray scattering studies of soybean glycinin (*36*), sunflower helianthinin (*38*, *39*), rapeseed cruciferin (*39*), and broad bean legumin (*40*) demonstrated that the dimensions of all 11S globulins are in the range of 10.4−12.6 nm in diameter and 7.5−9 nm in height.

From the present data, the diameters of the native BWG trimer and hexamer were determined as 10.8 nm (2 × 5.4 nm) and 12.8 nm (2 × 6.4 nm), respectively. The stacking of two trimeric rings may lead to a slight increase in diameter as the BWG monomer contains nonidentical polypeptides (acidic and basic polypeptides). The height of the hexamer could be deduced from the r_h of the dimeric form of hexamer (9.1 nm), assuming that the two hexamers are stacked on top of each other, forming an oblate cylinder with height (18.2 nm) larger than diameter (12.8 nm). Both the estimated diameter and height of BWG hexamer are within the range of other seed 11S globulins. Similar quaternary configuration has also been demonstrated in oat globulin based on QELS data (*18*), suggesting that some level of structural homology exists between globulins from buckwheat and oats.

The MALLS elution profile of BWG heated at 100 °C for 30 min overlaid with the estimated hydrodynamic radius is shown in **Figure 6A**. The pattern resembles that of changes in molar mass (**Figure 4**). Upon heating, the r_h values of BWG in peaks 3 and 4 were increased, but the r_h of large soluble aggregates (peak 5) remained unchanged when compared to the unheated samples. BWG heated at 92 °C exhibited a similar profile (data not shown). A similar trend was also observed when BWG was heated in the presence of NEM (**Figure 6B**), although a different pattern of increase in r_h (concave vs convex shape) was observed between peaks 3 and 5 (including the new peak, peak 6). More scattering of the r_h values in this region was observed in the unheated sample when compared to the profile ran in the absence of the additive, similar to the molar mass profiles (**Figure 4A,B**). The data again suggest a different route of aggregation for BWG when the SH groups were blocked.

The changes in r_h of BWG heated at 100 °C during heat treatment are shown in **Figure 5**. The r_h for peak 3 was initially increased, which suggest unfolding of the protein molecules to assume an extended structure, followed by a leveling off upon further heating. There was little change in r_h for peak 5, despite marked increases in M_w, suggesting the formation of large aggregates with a compact structure. The high M_w aggregates may be formed from the association of dissociated molecules, which were tightly bound by covalent intermolecular disulfide linkages and noncovalent forces such as hydrophobic interac-

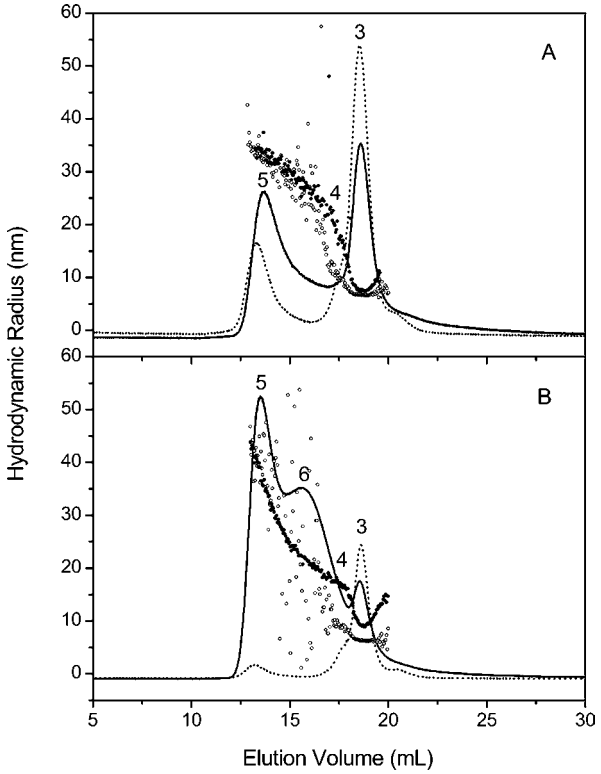


Figure 6. Relationship between hydrodynamic radius and elution volume of the unheated (○) and heated (at 100°C for 30 min) (●) buckwheat globulin in 50 mM phosphate buffer with 1.0 M NaCl, pH 7.4. MALLS (at 90°) elution profiles of unheated (dotted line) and heated (solid line) buckwheat globulin are included. (**A**) Without NEM and (**B**) with 10 mM NEM.

tions and hydrogen bonding, leading to decreases in r_h. Hence, there was no net increase or decrease in r_h, resulting in the formation of large aggregates with compact conformation.

Increases in r_h of peak 3 components were also observed in the presence of NEM (**Figure 6B**). For peak 5, the unheated control exhibited an r_h value (41.1 nm) considerable larger than that (33.7 nm) of the control (no NEM), probably due to partial protein unfolding. Heating did not cause marked changes in peak 5 (data not shown). The component of the new peak (peak 6) has an r_h value (22.8 nm) intermediate between those of components of peaks 3 and 5, and no marked changes in r_h were observed during heating (data not shown).

Our transmission electron microscopic (TEM) study (*14*) revealed that native BWG molecules exist as uniform compact globules with diameter of 11.7−12.5 nm, close to the diameter (12.8 nm) estimated by MALLS. The TEM data showed the formation of strandlike structures in the heat-induced BWG aggregates, consistent with the increases in r_h value of BWG hexamers (peaks 3 and 4) upon heating, suggesting extended conformation. The large soluble macroaggregates that eluted at peak 5 may exhibit a compact conformation as the M_w increased with fairly constant r_h value upon heating (**Figure 5**). These large compact globular macroaggregates were also observed in the TEM micrographs (*14*).

Conformational Characteristics of Native and Heated BWG. Previous studies reported that a log−log plot of r_h versus M_w permits the extraction of information on molecular conformation (*15*). The slope of the line can indicate whether the molecule is random-coiled (slope = 0.5−0.6), rodlike (slope = 1), or spherical (slope = 0.33). The steepness of the slope can also be related to the conformation of the molecules, with

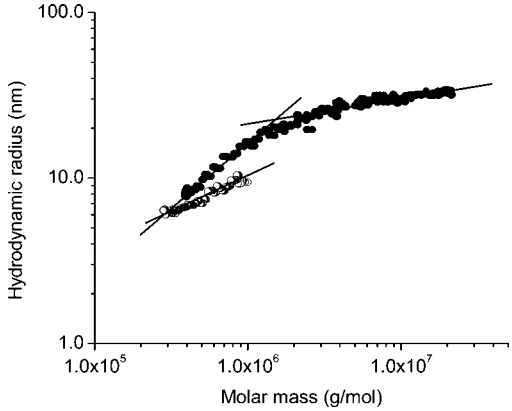


Figure 7. Plot of log hydrodynamic radius (r_h) as a function of log molar mass (M_w) for unheated (○) and heated (at 100 °C for 60 min) (●) buckwheat globulin (in 50 mM phosphate buffer with 1.0 M NaCl, pH 7.4).

Table 1. Conformation Slope of Buckwheat Globulin (in 50 mM phosphate buffer, 1.0 M NaCl at pH 7.4) Heated at 100 °C for Different Times[a]

heating time (min)	peak 3 (BWG hexamers)	peak 5 (BWG aggregates)	heating time (min)	peak 3 (BWG hexamers)	peak 5 (BWG aggregates)
0	0.38 ± 0.03 a	ND[b]	30	0.52 ± 0.04 b	0.29 ± 0.01 b
5	0.49 ± 0.04 b	0.41 ± 0.02 a	60	0.52 ± 0.03 b	ND
10	0.50 ± 0.04 b	0.29 ± 0.01 b			

[a] Mean of triplicate determinations ± SD. Means in a column bearing the same letter are not significantly different ($p > 0.05$). [b] Not determined.

extended structures giving larger slopes than compact structures. **Figure 7** presents a plot of log r_h as a function of log M_w for unheated and heated (100 °C for 60 min) BWG. For unheated native BWG, the plot yielded a straight line with a slope of 0.38 (**Table 1**), which is close to that expected for a spherical molecule. Heating caused an increase in the slope at the same region of the plot, suggesting unfolding of the BWG hexamers and the formation of random-coiled molecules (with larger slope). Estimation of the slope of peak 3 during heat treatment (**Table 1**) shows a significant increase in the slope from 0.38 (spherical) to around 0.50 (random-coiled). At the higher M_w portion of the plot, corresponding to large soluble aggregates (peak 5), a decline in the slope was observed (**Figure 7**). Slope determination of the heated samples shows a rapid decline from 0.41 (5 min of heating) to 0.29 (after 10 and 30 min of heating) (**Table 1**), suggesting the formation of compact spherical structure from an initially more extended conformation. The findings further confirm that the large soluble aggregates assume a more compact conformation than the native BWG. Slopes of small peaks (such as peak 5 at 0 and 60 min and peak 3 at 120 min) were not determined due to inaccuracy in the measurements.

On the basis of the information obtained, it can be summarized that native BWG exists mainly as a hexamer with an estimated molecular weight of 342 kDa, close to that deduced from the genomic cloned data of 13S buckwheat globulin. The hexamer was predicted to exist as two annular trimeric rings, each with a diameter of 10.8 nm placed on top of one another, forming an oblate cylinder with a height of about 9.1 nm. The diameter of the BWG hexamer determined by QELS (12.8 nm) was close that (11.7−12.5 nm) estimated by TEM. Moreover, the compact globular conformation of BWG molecules observed by TEM has been confirmed by the log−log plot of r_h versus

M_w, giving a slope (0.38) close to that expected for a spherical molecule. It is interesting to note that the molecular structure of BWG is very close to that of oat globulin, with comparable dimensions (*18*).

In conclusion, this study showed that thermal aggregation of BWG occurred at a faster rate at 100 °C than at 92 °C. Similar to other oligomeric proteins with complex quaternary structures, heating caused both dissociation and association of BWG, with subsequent aggregate formation. The SEC−MALLS data showed that NEM promoted the formation of macroaggregates with higher M_w and r_h values than those of the control (no NEM), and the route of aggregation seems to be altered, suggesting that the blocking of the SH groups in BWG may favor the involvement of other noncovalent forces in aggregation, leading to the formation of larger aggregates. Similar to our previous study on oat globulin (*18*), the present findings demonstrate that the SEC−MALLS−QELS system is a reliable tool to measure the absolute molar masses and hydrodynamic radii of globular proteins and to monitor thermal aggregation of seed storage proteins with complex quaternary structures. Moreover, conformational changes in protein molecules during thermal aggregation can also be predicted from these measurements. Since heating is a common manufacturing step applied to protein foods, more detailed information on the relationship between heat treatments and the size and shape of heat-induced aggregates is essential for process optimization to produce products with desired organoleptic characteristics.

ABBREVIATIONS USED

BWG, buckwheat globulin; SEC, size-exclusion chromatography; MALLS, multiangle laser light scattering; QELS, quasielastic light scattering; UV, ultraviolet; RI, refractive index; M_w, molecular weight; r_h, hydrodynamic radius; DSC, differential scanning calorimetry; TEM, transmission electron microscopy; NEM, *N*-ethylmaleimide; T_d, denaturation temperature; SH−SS, sulfhydryl−disulfide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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