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Molecular characteristics of ovalbumin–dextran conjugates formed through the Maillard reaction

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Abstract

High-performance size exclusion chromatography with multi-angle laser light scattering detection was used to determine the molar mass, molar size, and size distribution of ovalbumin–dextran conjugates formed through the Maillard reaction. SDS–PAGE patterns revealed that dextran and ovalbumin were covalently bonded to each other. Up to 2.5 mol of dextran appeared to bind with 1 mol of ovalbumin, resulting in a conjugate with a maximum molar mass of around 70 kDa. The conjugates had weight-average molar mass values ranging from 84.4 to 105.1 kDa, and root mean square radius values ranging from 19.6 to 26.4 nm. The conjugates prepared by the Maillard reaction were polydisperse and were made more compact by increasing the ratio of dextran bound to ovalbumin.

Keywords: Ovalbumin–dextran conjugates; Multiangle laser light scattering; Size-exclusion chromatography; Molar mass; Molar size

1. Introduction

Important functional properties of proteins relevant to food systems include solubility, viscosity, foaming, gelling, and emulsification (Phillips, Whitehead, & Kinsella, 1994). These functionalities are fundamentally related to intrinsic physicochemical and structural properties of proteins, such as size, net charge, amphipathicity, solubility, and molecular flexibility, and to extrinsic conditions including heat, pH, and charge (Damodaran, 1994). Polysaccharides are utilized as thickeners, binders, and gelling and stabilizing agents in foods. They can also stabilize emulsions due to their viscosity (BeMiller & Whistler, 1996). However, low surface activity of polysaccharides, originating from their high hydrophilicity, limits their capacity to serve as foaming or emulsifying agents themselves.

Chemical and enzymatic modifications of protein have been studied in an effort to enhance protein functionalities. Also, many researchers have investigated the improvement of functionalities using coexisting protein and polysaccharide (Babiker & Kato, 1998; Dickinson & Euston, 1991; Kim, Choi, Shin, & Moon, 2003). In general, the functionalities of protein are increased not by mere coexistence with polysaccharides but by conjugation with a polysaccharide. To make a conjugate, catalyst-mediated methods, transglutaminase, and Maillard-type reactions have been used. Among these methods, the Maillard-type reaction is considered to be most suitable to food application because of its safety.

Egg white proteins are extensively utilized in processed foods. Ovalbumin is the major protein component of egg white. It is an important food ingredient with

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structural functionality including emulsifying properties and foam stability (Li-Chan, Powrie, & Nakai, 1995).

Dextrans are a family of microbial 1 → 6-α-D-glucans derived from *Leuconostoc mesenteroides* with varying proportions of other linkage types (1 → 2-α-, 1 → 3-α-, or 1 → 4-α-branch linkage). Dextrans facilitate the preparation of oil-in-water emulsions and increase their stability, and they have high water-holding capacity. Thus, they can prevent formation of sucrose crystals in syrup and ice crystallization in ice cream as well (Fox, 1992; Robyt, 1998).

Several studies on the conjugates of ovalbumin have been carried out, focusing on the improvement of solubility, heat stability, foaming properties and emulsifying properties (Aoki et al., 1999; Kato, Minaki, & Kobayashi, 1993; Nakamura, Kato, & Kobayashi, 1992).

Size exclusion chromatography (SEC) is generally used to characterize the molecular weight of food materials such as proteins, polysaccharides, or their aggregates. Light scattering (LS) has been used as an absolute method to determine the molar mass (M_w), shape, and conformation of polymers (Billingham, 1977; Wyatt, 1993). However, LS techniques are very sensitive to large-sized materials, which scatter large amounts of light. Also, to obtain information about the conformation of the polymer, a number of angles must be used. The combination of a multi-angle laser light scattering (MALLS) detector with a SEC system and concentration detectors such as UV and differential refractive indexes constitutes a powerful new means for obtaining information about the M_w, *z*-average root mean square radius (R_g), and concentration of material.

Kato, Sasaki, Furuta, and Kobayashi (1990) reported the improved emulsifying properties of an ovalbumin–dextran conjugate prepared with a reaction ratio of 1:5 and the M_w of ovalbumin–dextran conjugate, using a low-angle laser light scattering detector. However, the molecular properties of the conjugates were not characterized in detail. Therefore, the aim of this study was to further characterize the ovalbumin–dextran conjugates by determining the M_w, R_g, and size distribution of the conjugates with different binding ratios using SEC and MALLS.

2. Materials and methods

2.1. Materials

Ovalbumin (Grade V, A-5503) and dextran produced by *L. mesenteroides* with average molar mass of 10 kDa were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical reagent grade.

2.2. Sample preparation

Maillard-type ovalbumin–dextran conjugate was prepared, basically by the method of Kato et al. (1990). Ovalbumin–dextran mixtures in molar ratios of 1:1, 1:2 and 1:3 were dissolved in water and freeze dried. Freeze-dried ovalbumin–dextran mixtures were stored at 60 °C and 79% relative humidity in a desiccator with saturated KBr solution for 10 days. To purify the conjugate, stored samples were dissolved in distilled water to a concentration of 0.3% (w/v) and prefiltered through a 0.45-μm pore membrane filter (Millipore Corp., Bedford, MA, USA). Ultrafiltration was then conducted with a XM-50 membrane (Amicon Inc., Beverly, MA, USA) until half of the initial volume remained, followed by the addition of deionized-distilled water to the original volume. This procedure was repeated three times, and then the retentates were freeze dried and stored at 4 °C.

For the measurement of a specific refraction index increment (d*n*/d*c*), a series of concentrations of the ovalbumin–dextran conjugate was prepared by dissolving in 67 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl and 3 mM sodium azide, and prefiltered with a 0.1-μm pore membrane filter (Millipore Corp., Bedford, MA, USA).

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was carried out by the method set out by Laemmli (Laemmli, 1970) using a 9% acrylamide separating gel and a 5% stacking gel containing 0.1% SDS. Crude ovalbumin–dextran conjugates were dissolved in 10 mM phosphate buffer (pH 7.4) and heated at 100 °C for 3 min in 60 mM Tris–glycine buffer (pH 6.8) with 2% SDS and 14.4 mM 2-mercaptoethanol. Electrophoresis was carried out at a constant amperage of 10 mA for 3 h using an electrophoretic buffer (pH 8.3) of Tris–glycine containing 0.1% SDS. The gels were stained for protein with coomassie brilliant blue R solution (45% methanol, 10% acetic acid) (Bollag, Rozycki, & Edelstein, 1996), and for carbohydrates with periodic acid-Fuchsin solution (Merril & Washart, 1998; Zacharius, Zell, Morrison, & Woodlock, 1969).

2.4. Determination of binding ratio of dextran to ovalbumin

The binding ratio of dextran to ovalbumin was determined by colorimetric methods. The content of ovalbumin was calculated from the absorbance at 595 nm with the Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, Hercules, CA, USA), based on the method of Bradford (Bradford, 1976). The content of dextran was

calculated from the absorbance at 470 nm by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.5. Size exclusion chromatography

The high-performance SEC system consisted of a model PU-2086 Plus (Jasco, Tokyo, Japan), with a 20-μl injector loop (Model 7725, Rheodyne LLC, Rohnert Park, CA, USA), a model degasser (NO-OX Vacuum Station, Alltech Corporate, Deerfield, IL, USA), a differential refractive index detector (Opti-Lab, Wyatt Technology, Santa Barbara, CA, USA), a Shodex OH-Pak guard column, and OHPak 804 and OHPak 802 columns (Showa Denko, Tokyo, Japan). The mobile phase was 67 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl and 3 mM sodium azide (prefiltered with a 0.1-μm filter). The flow rate was 0.3 ml/min.

2.6. Multi-angle laser light scattering measurement

Ovalbumin–dextran conjugate was dissolved in 67 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl and filtered with a 0.1-μm filter. It was injected through a 0.1-μm pore in-line filter to the high-performance SEC system. Light scattering was measured on a DAWN DSP (Wyatt Technology) using a 632-nm laser and a refractive index (RI) detector (Opti-Lab, Wyatt Technology). Photodiodes placed at 18 positions from 26.6° to 144.5° Collected scattered light.

Prior to the measurements, the differential refractive index response factor was measured by injecting a series of known concentrations of NaCl into the detector with a syringe pump. This response factor was obtained from the slope of the linear plot between NaCl concentration and differential refractive index response. The factor to correct the Rayleigh ratio to 90° for instrument geometry was obtained by measuring at 90° the LS intensity of HPLC-grade toluene filtered with a 0.02-μm filter. The responses to LS intensity of the photodiodes arrayed around the scattering cell were normalized to the diode at 90° with monomeric bovine serum albumin, the nominal molar mass of which is 66 kDa.

2.7. Data treatment

The experimental data collected from the DAWN DSP/OptiLab system were processed with ASTRA software (Version 4.90.07, Wyatt Technology). The collected data and the molecular parameters are described by the following equation (Debye, 1944):

$$\frac{K^* c}{R_\theta} = \frac{1}{M_W P(\theta)} + 2A_2 c,$$

where R_θ, the excess Rayleigh ratio that describes the scattering after the concentration of the pure solvent is subtracted, is the light scattered by the pure solution at an angle θ in excess of that scattered by the pure solvent, divided by the incident light intensity. C is the concentration of the solute. M_w is the weight-average molar mass, and A_2 is the second virial coefficient. K^* is an optical constant equal to $4\pi^2 n_0^2 (dn/dc)^2 \lambda_0^{-4} N_A^{-1}$, where n_0 is the refractive index of the solvent at the incident radiation wavelength; λ_0 is the incident radiation wavelength, expressed in nanometers; N_A is Avogadro's number; and dn/dc is the differential refractive index increment of the solvent–solute solution with respect to a change in solute concentration. $P(\theta)$ is the theoretically derived form factor. It can be related to the distance between scattering centers in the molecule by

$$\frac{1}{P(\theta)} = 1 + \frac{16\pi^2}{3\lambda^2} \cdot R_g^2 \cdot \sin^2\left(\frac{\theta}{2}\right) + \cdots,$$

where R_g^2 is the root mean square radius (rms).

At an angle of $\theta = 0$, the molar mass (M_w) can be determined directly from the y-axis of the plot. It depends on the rms radius (R_g^2), independent from molecular conformation. Plotting $K^* c/R_\theta$ against $\sin^2(\theta/2)$ yields values of M_w and R_g^2 from the intercept and the slope.

3. Results and discussion

3.1. Binding of ovalbumin and dextran

SDS–PAGE patterns confirmed that ovalbumin was covalently linked with dextran after 10 days of incubation at 60 °C and 79% relative humidity (Fig. 1). Broad and high molecular-sized bands were also observed in

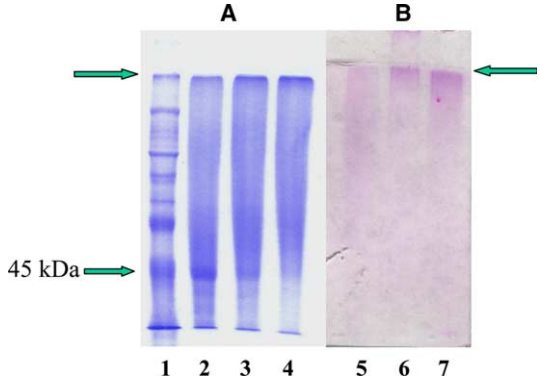

Fig. 1. SDS–PAGE pattern of ovalbumin–dextran mixture after 10 days of Maillard reaction. Panel A, protein stain; panel B, carbohydrate stain: Lane 1, molecular weight standard markers (myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97 kDa; fructose-6-phosphate kinase, 84 kDa; bovine serum albumin, 66 kDa; glutamic dehydrogenase, 55 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa); Lanes 2 and 5, molar ratio 1:1; Lanes 3 and 6, molar ratio 1:2; Lanes 4 and 7, molar ratio 1:3. Arrows indicate the boundary between separating and stacking gels.

both protein and carbohydrate stains at the boundary between stacking and separating gels. This indicates a polydispersed distribution of high-molecular-weight components. In the eletropherogram for all conjugates, a band of remaining ovalbumin that had not taken part in the reaction was observed in samples with reactant ratios (ovalbumin:dextran) of 1:1 and 1:2, but this band was not observed in the 1:3 sample. This result suggests that the amount of ovalbumin was insufficient to react with all the dextran in the 1:3 sample and that all the molecules of ovalbumin participated in the reaction. It implies that at least 2 mol or more of dextran appear to link to 1 mol of ovalbumin. Thus, the molar mass of the conjugate was estimated to be greater than 55 kDa (45 kDa for ovalbumin and 10 kDa for dextran). These conjugates were recovered by ultrafiltration with a membrane with a molecular weight cutoff of 50,000.

The ovalbumin–dextran mixture with a weight ratio of 1:1 and the 1:1 conjugate isolate were applied onto a column of Sephadex G-75, and each fraction was analyzed for protein by measuring the absorbance at 289 nm, and for polysaccharide by measuring the absorbance at 470 nm after color development with the phenol–sulfuric acid method (data not shown). The changes in elution profiles were observed on a Sephadex G-75 column. As ovalbumin was incubated with dextran, significant shifts in the profile peak from a smaller to a higher molecular fraction were observed, suggesting that ovalbumin was covalently attached to dextran to form the ovalbumin–dextran conjugate.

In the yield test, the 1:3 reactant showed the lowest yield (Table 1). This was thought to be due to the discard of unreacted, excess dextran and the increment of insoluble fraction formed in the conjugate.

Maillard-type protein–polysaccharide conjugates are formed between the ε-lysyl amino groups of a protein and the reducing end carbonyl group of a polysaccharide through an Amadori-type linkage (Kato et al., 1993). In the 1:1 reaction system, about 1.5 mol of dextran seemed to bind with 1 mol of ovalbumin. In the 1:2 and 1:3 reaction systems, the ratio of dextran bound with 1 mol of ovalbumin increased to about 2.5 mol (Table 2). These results were presumably due to the steric hindrance occurring around the ε-lysyl amino groups of ovalbumin in the formation of the conjugate. Kim et al. (2003) reported that up to 6.5 mol of galactomannan appeared to bind with 1 mol of BSA. The ratio of galactomannan bound with 1 mol BSA was higher than the ratio of dextran bound with ovalbumin, which could be due to the structural characteristics of galactomannan and dextran. Galactomannan has D-galactopyranose units, linked α-1 → 6 to the β-D-mannan chain (Dey, 1978), whereas dextran has a branched structure. Therefore, galactomannan reacted more readily with BSA than dextran did with ovalbumin.

Table 1
The yield of soluble ovalbumin–dextran conjugates

Molar ratio of ovalbumin to dextran reacted	Yield (%)[a]
1:1	85.0 ± 1.0
1:2	84.1 ± 7.4
1:3	73.9 ± 5.2

[a] Values are means ± SD of three independent experiments. Triplicate measurements were performed for each experiment.

Table 2
Molar binding ratio of ovalbumin and dextran at various reaction ratios

Molar ratio of ovalbumin to dextran reacted	Product molar ratio[a]
1:1	1:1.46 ± 0.23
1:2	1:2.52 ± 0.22
1:3	1:2.56 ± 0.15

[a] Values are means ± SD of three independent experiments. Triplicate measurements were performed for each experiment.

3.2. Weight-average molar mass and root mean square radius of ovalbumin–dextran conjugates

The M_w and R_g of the ovalbumin–dextran conjugates were determined using dn/dc values with a MALLS system. The dn/dc values measured for ovalbumin and the 1:1, 1:2 and 1:3 conjugates were 0.183, 0.162, 0.174 and 0.158, respectively. Fig. 2 shows the chromatograms from the DAWN DSP 90° detector output and the RI signal for the 1:1, 1:2 and 1:3 conjugates. The main peaks of the conjugates appeared earlier than that of native ovalbumin. The chromatogram of the 1:2 conjugate was similar to that of the 1:3 conjugate. This result is consistent with the binding ratios of the conjugates determined by the colorimetric method. The MALLS chromatograms of the 1:2 and 1:3 conjugates showed two partially resolved peaks, but the RI signal showed single peaks. This observation was probably due to a very strong intensity of scattering laser light for the conjugates with very high M_w, even for an extremely small amount. Thus, it could be suggested that conjugates of various molar masses were formed during the Maillard reaction, but that the quantity of conjugates having very high molar masses was very low. A plot of log molar mass versus volume for the conjugates is shown in Fig. 3. The molar masses of the conjugates decreased over the elution volume from 10 to 13 ml, suggesting that the ovalbumin–dextran conjugates prepared by the Maillard reaction were polydisperse. The molar masses of 1:1, 1:2 and 1:3 conjugates eluted from 10 to 13 ml were similar. Kato et al. (1990) reported that the average molar mass of the ovalbumin–dextran conjugate was 200,000. They estimated that 1.6–2.2 mol of dextran (molecular weight, 60,000–90,000) bound with 1 mol of ovalbumin, based on calculations using the phenol–

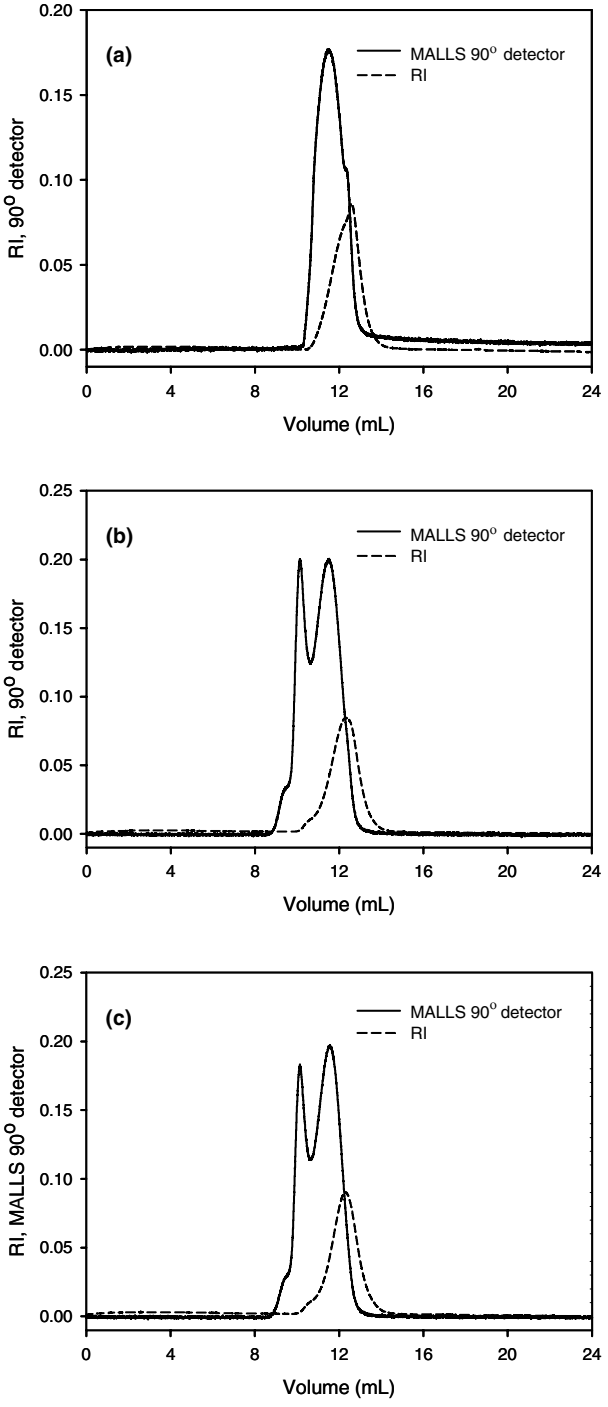

Fig. 2. Chromatograms of the conjugates detected by refractive index detector (RI) and MALLS at the 90° angle: (a) 1:1 conjugate; (b) 1:2 conjugate; (c) 1:3 conjugate.

sulfuric acid method for carbohydrate and UV absorption for protein. Also, the molar mass obtained by the LS method was the same as that obtained by the colorimetric method. Because they used the ovalbumin–dextran mixture in a weight ratio of 1:5, and the molar mass of the dextran was larger than that of the dextran used in this experiment, ovalbumin presumably was

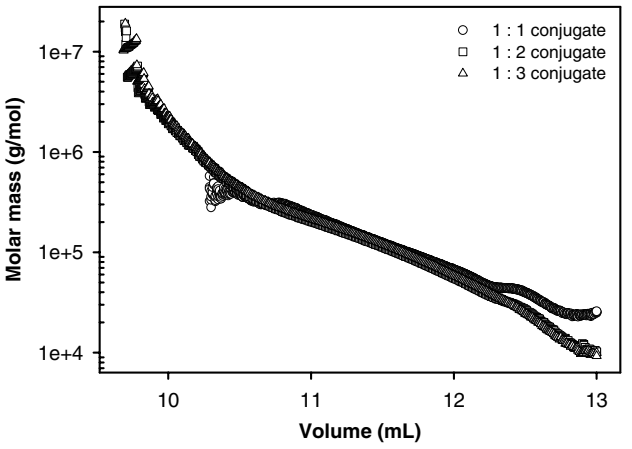

Fig. 3. Plot of log molar mass versus elution volume for the conjugates.

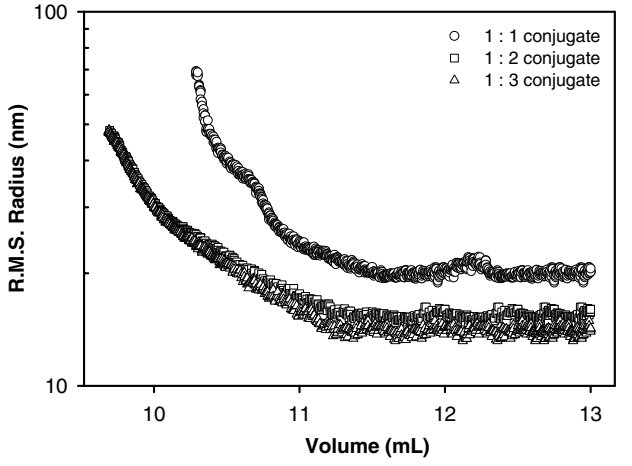

Fig. 4. Plot of log root mean square radius versus elution volume for the conjugates.

hard to aggregate under their experimental conditions. On the other hand, our conditions appeared to favor the formation of ovalbumin aggregates having high molar mass. Therefore, the M_w values of the conjugates were determined to be larger than the values obtained by the colorimetric method. Fig. 4 shows a plot of log root mean square radius versus volume for the conjugates. The radius of the conjugates decreased over the elution volume from 10 to 11.5 ml, but the signals collected from 11.5 to 13 ml were flat. Samples eluted from 11.5 to 13 ml had similar radii but different molar masses, suggesting that they had various shapes. The root mean square radius of the 1:1 conjugate was larger than those of the 1:2 and 1:3 conjugates. Fig. 5 displays the cumulative molar mass and root mean square radius distributions. For 1:1 conjugates, the low 10% weight fraction was below 30,000 g/mol, while the high 10% weight fraction was above 200,000 g/mol. For 1:2 and 1:3 conjugates, the low 10% weight fractions were

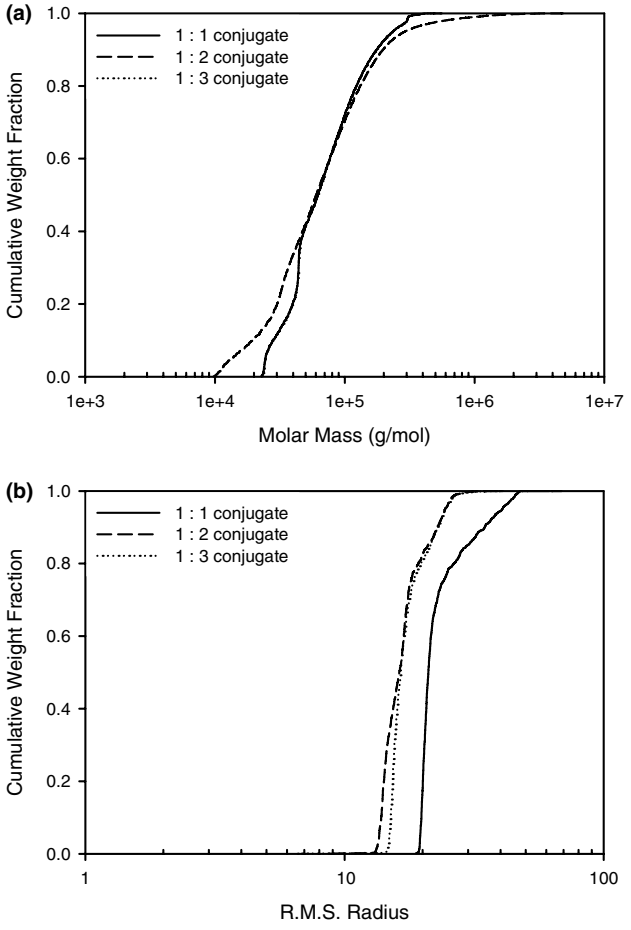

Fig. 5. Cumulative molar mass and root mean square radius distributions for the conjugates: (a) cumulative molar mass distributions; (b) cumulative root mean square distributions.

Table 3
Weight-average (M_w) and number-average (M_n) molar mass and root mean square radius (R_g) of ovalbumin–dextran conjugates[a]

Sample	M_n (g/mol) $\times 10^4$	M_w (g/mol) $\times 10^5$	M_w/M_n	R_g (nm)
1:1	5.423 (0.271)	0.844 (0.025)	1.557 (0.096)	26.4 (4.224)
1:2	3.739 (0.224)	1.028 (0.024)	2.751 (0.195)	20.7 (3.519)
1:3	3.495 (0.280)	1.051 (0.025)	3.007 (0.266)	19.6 (3.724)

[a] Each value is the average of three independent experiments with standard deviation.

10,000 g/mol, but the high 10% weight fractions were 100,000 g/mol. For root mean square radius, all conjugates of about 75% weight fractions had similar radii. A summary of the M_w and R_g values for the conjugates is shown in Table 3. Numerical values of M_w/M_n suggest that the 1:2 and 1:3 conjugates had a wider variety of shapes than the 1:1 conjugate. The results in Table 3 show a similar tendency to the binding molar ratios of all conjugates calculated by the colorimetric method. The dextran that took part in the Maillard reaction had an average molar mass of 10 kDa and was polydisperse; ovalbumin also aggregated during the Maillard reaction. Therefore, larger M_w values were obtained in this instance, as compared to the values determined by the colorimetric method. The R_g of the 1:1 conjugate was larger than those of the 1:2 and 1:3 conjugates. These results suggest that the conjugate became more compact rather than more extended as the ratio of dextran bound to ovalbumin increased.

In conclusion, SEC, combined with MALLS of the ovalbumin–dextran conjugates formed by the Maillard reaction, show that the conjugates had various molar masses and radii. This approach could be used to estimate the conformation of protein–polysaccharide conjugates formed by various methods, such as chemical and enzymatic modifications and the Maillard reaction.

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