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Conjugation of Sodium Caseinate and Gum Arabic Catalyzed by Transglutaminase

JOHN FLANAGAN† AND HARJINDER SINGH*

Riddet Centre, Massey University, Palmerston North, New Zealand

Conjugation of the milk protein sodium caseinate and a protein-containing polysaccharide, gum arabic, was achieved through the use of the cross-linking enzyme transglutaminase. The extent of conjugation was monitored by size exclusion separation coupled with a multiangle laser light scattering detector. The elution times of gum arabic solutions incubated with transglutaminase were unchanged over time, whereas incubation of sodium caseinate with transglutaminase resulted in shorter elution times as reaction time increased, indicating the formation of cross-linked caseinate polymers. However, when mixtures of caseinate and gum arabic were incubated with transglutaminase, the elution times were decreased markedly, indicating conjugation between the protein and polysaccharide. The molecular masses of the conjugates increased from ∼950 to 1600 kDa. This method of protein−polysaccharide conjugation offers noticeable advantages over previously used methods, and the conjugates produced may exhibit unique functional properties.

KEYWORDS: Conjugates; milk protein; gum arabic; size exclusion; light-scattering

INTRODUCTION

Proteins and polysaccharides perform many noteworthy functions in foods. Both contribute to the structural and textural properties of foods through their aggregation and gelation behavior (*1*). In addition, proteins are known for their surface active properties, that is, their ability to adsorb to an air/water or oil/water interface, and polysaccharides for their water-holding and thickening properties. By covalently linking proteins and polysaccharides it may be possible to combine their individual traits and thus produce food ingredients with a wider range of functional properties.

Two principal methods have previously been used for conjugation of proteins and polysaccharides. Conjugates may by formed enzymatically using the oxidoreductase family of enzymes (EC 1.*X*.*X*.*X*), which catalyze the oxidation of the phenolic group of Tyr residues with carbohydrate groups containing phenolic residues, such as cereal arabinoxylans (*2*). Tyrosine-containing peptides have also been conjugated with ferulic acid (*3*) and with whey proteins through the use of three different oxidoreductases (*4*). However, the industrial application of the oxidoreductase reaction is limited by the cost and availability of the oxidoreductase enzyme.

Alternatively, the Maillard or dry-heating reaction can be used to chemically form protein−polysaccharide conjugates (*5*−*13*). The Maillard-type reaction is restrictive in terms of the times (5−15 days) and temperatures (50−70 °C) required to produce protein−polysaccharide conjugates, in addition to uncertainty about the possible formation of toxic side-products, coupled with possible losses in nutritional value and problems with color and flavor changes. Whereas proteins and polysaccharides may also be conjugated through other chemical means, enzymatic conjugation is preferred due to possible toxic side reactions and adverse public opinion to the use of chemicals in foods and food ingredient production.

Many polysaccharides contain residual protein; for example, gum arabic, guar gum, and locust bean gum all contain low levels of protein. Gum arabic [∼2% protein, depending on source (*14*)] consists of, among other subunits, a glycoprotein and an arabinogalactan protein. As gum arabic is a useful emulsifier (*15*−*17*), it may be elucidated that the glycoprotein exists on the outside of the molecule to assist in the stabilization of oil/water interfaces. Furthermore, the susceptibility of the proteinaceous component of gum arabic to hydrolysis adds further weight to the possibility of the glycoprotein being on the periphery of the gum molecule (*18*).

In this study, we investigate a new method for the conjugation of proteins and polysaccharides (containing residual protein) through the use of a cross-linking enzyme, transglutaminase (EC 2.3.2.13). Transglutaminase, through its ability to covalently cross-link Gln and Lys residues, has been found to improve the functional properties of various proteins, including sodium caseinate (*19*, *20*), whey proteins (*21*), and soy protein isolate (*22*). The amino acid composition of the proteinaceous part of gum arabic reveals Lys residues (*23*), which may participate in the formation of transglutaminase-catalyzed cross-links, depending on the orientation of the glycoprotein within the gum arabic molecule. Therefore, it may be possible to covalently conjugate

* Corresponding author (telephone +64-6-3504401; fax +64-6-3505655; e-mail h.singh@massey.ac.nz).

† Present address: Glanbia Innovation Centre, Carlow Road, Kilkenny, Ireland.

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protein molecules to protein-containing polysaccharides via transglutaminase-catalyzed cross-links.

MATERIALS AND METHODS

Sodium caseinate (ALANATE 180) was supplied by Fonterra Co-operative Group Ltd., Auckland, New Zealand. This product contained ~96% dry matter, of which about 94% was protein, 1.38% was sodium, and 0.06% was calcium. Gum arabic (GA) was obtained from CNI (Rouen cedex, France; product code IRX 40830) or from Bronson and Jacobs Ltd. (Auckland, New Zealand). Both spray-dried gum arabic powders contained a maximum of 15% moisture and 4% ash. Transglutaminase was an Ajinomoto product (Activa MP), with a stated activity of 100 units/g, and was obtained from Kerry Ingredients (Auckland, New Zealand). All of the chemicals used were of analytical grade and were obtained from either BDH Chemicals (BDH Ltd., Poole, U.K.) or Sigma Chemical Co. (St. Louis, MO), unless specified otherwise.

In all experiments, sodium caseinate (5 wt %) and gum arabic (10 wt %) stock solutions were prepared in Milli-Q water, 0.02 wt % sodium azide added, and the solutions were allowed to hydrate overnight at room temperature.

Two different HPLC procedures were used to characterize the formation of conjugates. Initial experiments were conducted using a gel filtration column (dimensions: 96 cm length × 1.4 cm internal diameter) packed with Sephacryl 400 (Amersham Biosciences, Auckland, New Zealand). Analyses of gum arabic (from CNI) control experiments were conducted by adding transglutaminase (1% w/v) to a 10 wt % gum arabic solution and taking 1 mL samples at certain time intervals for loading onto the column. All samples were prefiltered through a 0.45 μm filter. The running buffer used contained 0.5 M NaCl, and the flow rate was 0.5 mL/min. Absorbance at 218 nm was monitored using a UV absorbance detector (GBC Scientific Equipment Ltd., Victoria, Australia). Subsequently, gum arabic (pH 5.2) and sodium caseinate solutions (pH 6.7) were combined to give a final concentration of 5 wt % gum arabic and 0.5 wt % sodium caseinate, and the pH was adjusted to 5.6. Transglutaminase (0.5% w/v, approximately corresponding to an enzyme powder preparation/protein ratio of 1:1, taking into consideration the protein content of gum arabic) was then added, samples were taken at defined time intervals, and UV monitoring was performed as described.

In a second set of experiments, a high-performance size exclusion chromatography (HPSEC) system combined with multiangle laser light scattering (MALLS) was used to measure the molecular weight (M_w) and radius of gyration (R_g) of gum arabic (from Bronson and Jacobs Ltd.) and the conjugated products. Separation was carried out by size exclusion chromatography on a Shodex column (Tokyo, Japan) attached to a GBC HPLC system (GBC Scientific Equipment Ltd.). A solution containing 0.25 M NaCl, prefiltered through a 0.025 μm filter (Millipore Corp.), was used as an eluant buffer. Sample injection volume was 50 μL, and nominal flow rate was 0.5 mL/min. The chromatography system consisted of a Shodex OHpak SB-806 HQ column, a UV absorbance detector (GBC Scientific Equipment Ltd.) operating at 218 nm, a DAWN-DSP MALLS photometer (Wyatt Technology, Santa Barbara, CA) fitted with a helium−neon laser (λ = 632.8 nm) and a K-5 flow cell, and a GBC RI detector (RI 2000 model, Auckland, New Zealand). The light scattering photometer was calibrated with filtered toluene and normalized with BSA (Sigma). The BSA was also used to determine the volume delay between the detectors. The light scattering data were processed using the RI concentration detector, and the M_w and R_g were calculated with a first-order Debye fit, using a specific refractive index increment (dn/dc) value of 0.141 cm^3/g (*24*) and a second virial coefficient (A_2) of 0.

A 5 wt % gum arabic solution was prepared at pH 5.2, and sodium caseinate and transglutaminase were both added to 0.03 wt %. The pH of this mixture was adjusted to 5.6. Every 2 h further additions of sodium caseinate were added to give 0.06, 0.09, 0.15, and 0.25 wt % sodium caseinate in the final mixture, while a 1 mL aliquot was taken, and was filtered through a 0.45 μm filter prior to injection onto the HPSEC−MALLS system. A control experiment was run in tandem, with an aliquot of water added instead of transglutaminase.

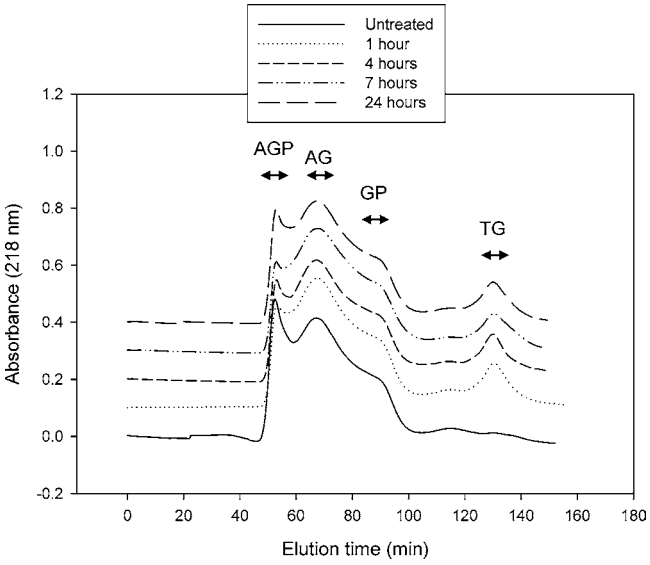

Figure 1. Elution profiles (Sephacryl 400) of 10 wt % gum arabic solutions incubated with transglutaminase (1% w/v) for different time intervals. Peaks are identified as arabinogalactan protein (AGP), arabinogalactan (AG), glycoprotein (GP) and transglutaminase (TG). Profiles have been offset by 0.1 absorbance unit for comparison purposes.

RESULTS AND DISCUSSION

Although transglutaminase has been used to cross-link proteins from various sources, the possibility of employing transglutaminase to cross-link protein-containing polysaccharides does not appear to have been considered previously. As stated earlier, many gums contain residual protein, and following comparison of the protein content of different gums, the amino acid sequences, and the position of the protein moiety in these gums, we decided to focus our attention on gum arabic.

Initial experiments were conducted to determine if transglutaminase could catalyze Gln−Lys cross-links between gum arabic molecules (**Figure 1**). Untreated gum arabic eluted as expected and gave an elution profile similar to that described previously in similar experiments using Sephacryl 400 and Sephacryl 500 as the separating medium (*18*, *25*, *26*). Due to the polydispersity of the gum arabic used, the separation of the peaks was not ideal and there was some overlap of the individual components (**Figure 1**). However, by comparison of the elution profile of the untreated gum arabic to previous work (*14*), the peaks can be attributed to the different components present within gum arabic. The first peak to elute at 50 min is the arabinogalactan protein (AGP) peak, followed closely by the arabinogalactan (AG) peak (66 min), which comprises ~90% of the gum, yet contains very little protein. The glycoprotein (GP) peak component of the gum arabic does not appear to have separated well from the other components and possibly lies at ~90 min. The peak that appears at 130 min is probably the transglutaminase itself, which is reported to have a molecular mass of 37.8 kDa (*27*). After incubation with transglutaminase for 24 h at a relatively high enzyme/substrate ratio (approximately 5:1, considering gum arabic has a protein content of ~2%), little change in the elution profiles was observed, apart from a decrease in the height of the AGP peak. Decreases in the peak height of the AGP fraction of gum arabic as shown using Sephacryl 400 following prolonged heating at 62 °C have been reported previously (*28*), although this effect has never been reported in gum arabic solutions held at room temperature. The absence of changes in the elution times of the major gum

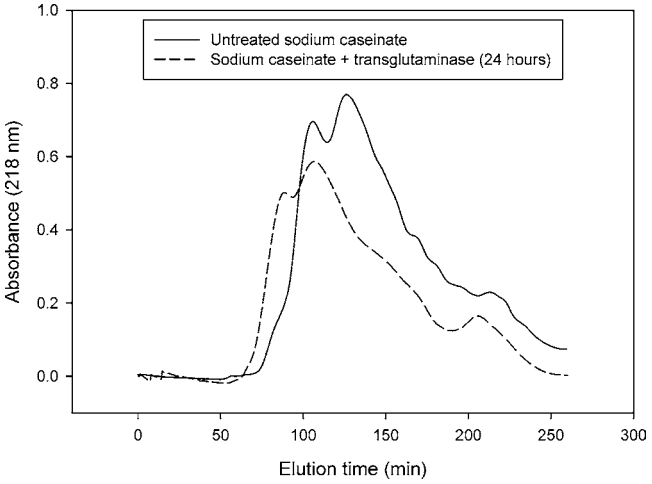

Figure 2. Elution profiles (Sephacryl 400) of 0.5 wt % sodium caseinate solutions incubated with transglutaminase (0.5% w/v) for 0 and 24 h.

arabic components following incubation with transglutaminase indicates that cross-linking most likely did not occur between gum arabic molecules.

A second control experiment comprising a sodium caseinate solution (0.5 wt %) that had been incubated with transglutaminase (0.5% w/v) for 24 h at room temperature, pH 6.8, was also analyzed using the Sephacryl 400 column (**Figure 2**). The increase in molecular mass of casein following incubation with transglutaminase is evident by the shift in the elution profile to shorter elution times. In addition, the peak at 130 min in the untreated sodium caseinate sample was greatly reduced, indicating the loss of caseinate due to the formation of transglutaminase-induced oligomers and polymers. The increase in molecular mass of casein following treatment with transglutaminase has been reported previously using size exclusion chromatography (*29*), and the results are similar to those reported herein. It must be noted that following extensive transglutaminase-induced cross-linking, the cross-linked polymers did not elute prior to 67 min under the current conditions.

Subsequently, sodium caseinate was mixed with gum arabic to give final concentrations of 0.5 and 5% (w/v), respectively, and transglutaminase was added to 0.5% (w/v) enzyme preparation. The resulting elution profiles from the Sephacryl 400 column are presented in **Figure 3**. Prior to mixing, gum arabic was pH adjusted to 5.2; if the pH was left unchanged at the normal reconstitution pH of gum arabic (pH ~4.7) the caseinate aggregated upon mixing, due to proximity to its p*I* (pH 4.6). After the gum arabic and caseinate solutions had been mixed, the pH was adjusted to 5.6. At this pH, there was no evidence of electrostatic interactions, which may occur at slightly lower pH values (pH <5.4) between the negatively charged gum arabic and positive patches on the protein surface (*30*). However, it is likely that interactions between sodium caseinate and gum arabic may be better facilitated where the electrostatic interactions between the two biopolymers are reduced by lowering the pH. In addition, although the optimum pH of the enzyme is pH 7, it retains activity at pH 5.6 (*31*). Transglutaminase concentration was set at 0.5% (w/v) to allow close monitoring of the reaction progression.

The elution profile of the untreated sample is the result of additive absorbances from the protein component of the gum arabic and from the sodium caseinate. A wavelength of 218 nm is required to detect the relatively low protein concentration in gum arabic; unfortunately, at this wavelength, sodium caseinate absorbs strongly, and thus there is some overlap

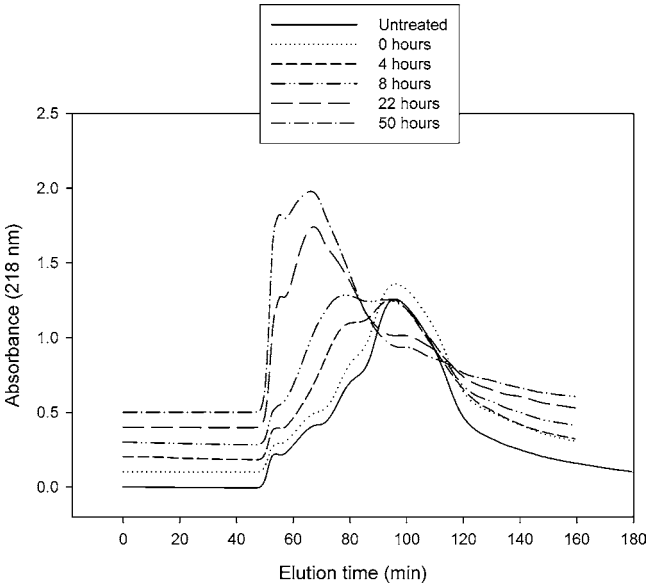

Figure 3. Elution profiles (Sephacryl 400) of mixtures of 5 wt % gum arabic and 0.5 wt % sodium caseinate incubated with transglutaminase (0.5% w/v) for different time intervals. Profiles have been offset by 0.1 absorbance unit for comparison purposes.

between the elution profiles of the gum arabic and sodium caseinate after 66 min of elution time.

As incubation time with transglutaminase increased up to 8 h, a considerable difference in the elution profiles of the gum arabic/sodium caseinate mixtures was observed. The mixtures eluted earlier, indicating an increase in molecular mass. After 22 and 50 h of incubation with transglutaminase, the increase in molecular mass was most evident, with a large portion of the sample eluting prior to 70 min (**Figure 3**). The results indicate that transglutaminase induced the formation of cross-links between sodium caseinate and gum arabic, as when sodium caseinate alone was incubated with transglutaminase, the sample did not elute prior to 66 min (**Figure 2**), and gum arabic was not susceptible to cross-linking by transglutaminase (**Figure 1**). It is likely, given the large shift in the elution profile following incubation of the gum arabic/sodium caseinate mixture with transglutaminase for 50 h, that the cross-linked material formed is composed of large aggregates of sodium caseinate, cross-linked to one, or more, gum arabic molecules; cross-linking of an individual casein molecule to an individual gum molecule is unlikely to induce such large changes in the elution profiles.

To allow for a more accurate characterization of the molecular weight of the conjugates produced following cross-linking of sodium caseinate and gum arabic, a Shodex size exclusion column in conjunction with a MALLS system was employed. Initial experiments found results similar to those obtained with the Sephacryl separaton system: no changes in elution times of the gum arabic components and an elution time of 16.5 min for the cross-linked sodium caseinate (data not shown). A slightly modified experimental procedure was also used to eliminate the possibility that the increase in molecular mass was due to some electrostatic interaction between sodium caseinate and gum arabic. Sodium caseinate was added in increasing concentrations to a solution containing gum arabic in the presence, and absence, of transglutaminase, and the UV and MALLS responses were followed over the elution time (**Figures 4**−**6**).

The separation achieved between gum arabic and sodium caseinate using the Shodex size exclusion column appeared to

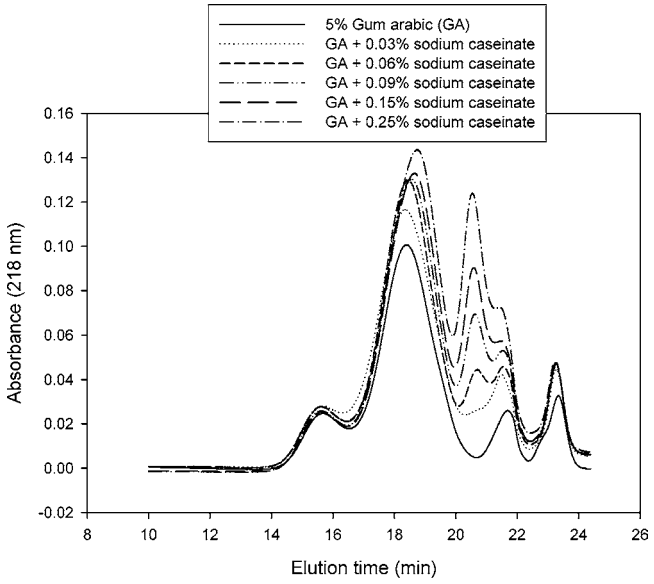

Figure 4. Elution profiles (Shodex SB-806 HQ) monitored by UV of gum arabic mixed with increasing concentrations of sodium caseinate in the absence of transglutaminase.

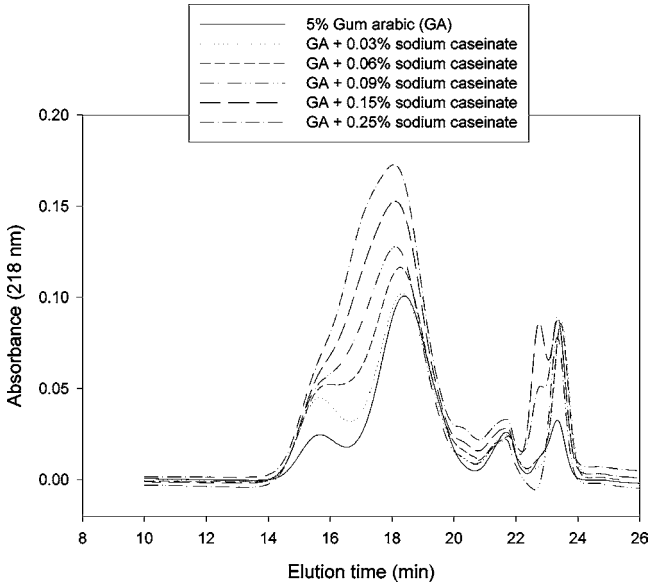

Figure 5. Elution profiles (Shodex SB-806 HQ) monitored by UV of gum arabic mixed with increasing concentrations of sodium caseinate in the presence of transglutaminase.

be superior to the separation achieved using the Sephacryl 400 column as the addition of casein did not overlap with the absorbance of gum arabic between 14 and 18 min of elution time (**Figure 4**). The elution pattern of gum arabic using the Shodex column was also slightly different from that obtained using Sephacryl 400, in that the first peak to elute was of smaller intensity than the second peak. The difference in elution profiles between the two different separation systems could be due to the different separation media used and/or the difference in the properties of the gum arabic samples used. The elution profiles of gum arabic using a Shodex column do not appear to have been reported previously.

In the absence of transglutaminase, an increase in the peak height was observed at 20.5 min, along with an increase in the peak area at 19 min; these relate to the increased concentration of sodium caseinate in the sample. No changes were evident in

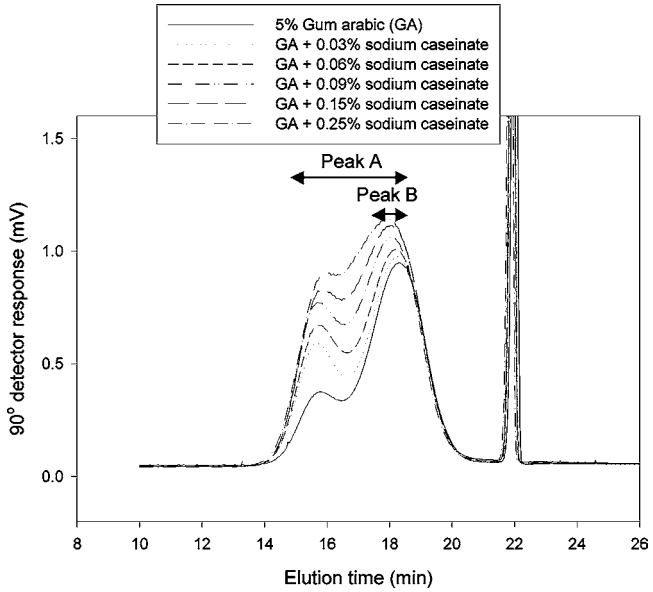

Figure 6. Elution profiles (Shodex SB-806 HQ) monitored by light scattering (90° detector) of gum arabic mixed with increasing concentrations of sodium caseinate in the presence of transglutaminase. For generation of molecular mass values, the data were treated as either one peak (peak A) or by focusing on the 18.5 min peak (peak B).

the elution patterns between 14 and 18 min (**Figure 4**), indicating no interaction between sodium caseinate and gum arabic. In the presence of transglutaminase, however, major shifts in the elution pattern were evident as the concentration of sodium caseinate in the system increased (**Figure 5**). A larger proportion of the material eluted earlier, with an increase in the size of the peak at 15.8 min. At the highest sodium caseinate concentration, the individual peaks had merged. However, the most conclusive evidence for the involvement of the casein molecules in the transglutaminase-catalyzed cross-links is the nonappearance of the casein peak at 19 as well as 20.5 min.

The response from the 90° detector of the MALLS system was recorded over the elution time for the system incubated with transglutaminase, and the results are presented in **Figure 6**. As the concentration of sodium caseinate in the sample increased, the height of the peak at 15.8 min increased and gradually merged with the larger peak at 18.5 min. The elution profiles from the MALLS system of the samples without transglutaminase did not change on addition of different concentrations of sodium caseinate (data not shown). The molecular masses and radii of gyration of the conjugates formed were subsequently calculated from the elution profiles in conjunction with the RI profiles (data not shown) as one peak, either centered on the 18.5 min peak (peak B; from 18.0 to 19.0 min) or treating the entire profile as one peak (peak A; from 15.0 to 19.0 min), and the results were compared to the molecular masses of the mixtures without transglutaminase (**Figure 7**).

Regardless of treating the data as a single peak (peak A) or by focusing on peak B at 18.5 min, the calculated molecular masses of the conjugates in the system increased either from 831 to 1203 kDa (peak A) or from 949 to 1640 kDa (peak B) as the concentration of sodium caseinate increased from 0.03 to 0.15 wt %. The molecular masses of the untreated samples remained relatively constant at different sodium caseinate concentrations, regardless of data treatment. In addition, the calculated R_g values of the non-cross-linked sample remained relatively unchanged at different added sodium

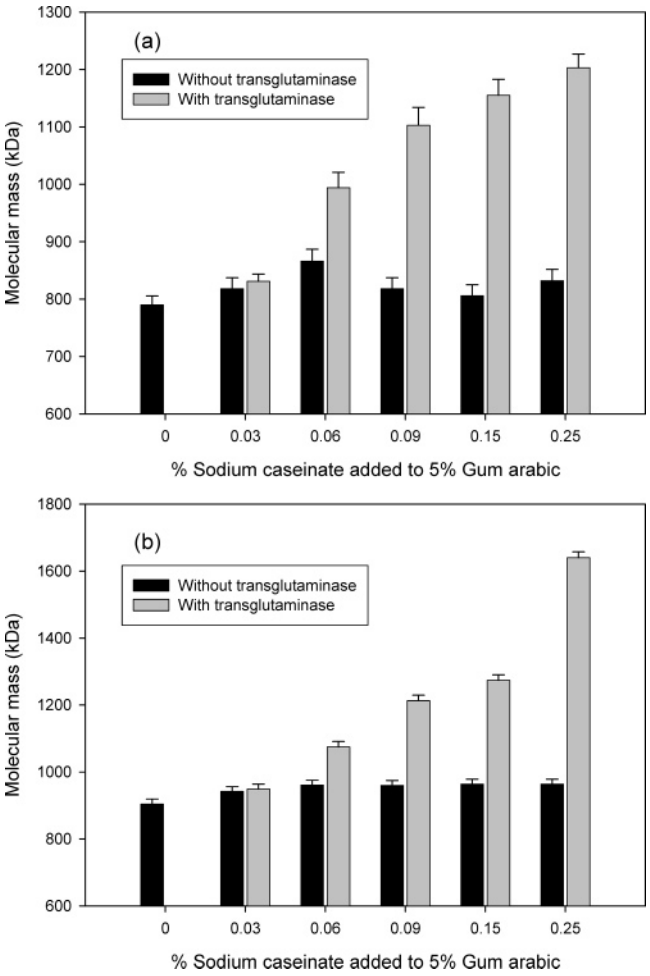

Figure 7. Molecular mass of gum arabic and gum arabic/sodium caseinate conjugates at different additions of sodium caseinate, with and without transglutaminase. Molecular mass was calculated using MALLS and RI data either from peak A (**a**) or from peak B (**b**). See **Figure 6** for details.

caseinate concentrations, whereas in the presence of transglutaminase, an increase in calculated R_g values was observed (data not shown).

Regarding the nature of the cross-linked conjugates, it appears that many different species are being formed, and this is expected in such a heterogeneous system. Given the large increases in molecular mass, even at low sodium caseinate concentrations (0.06% w/v), it is possible that sodium caseinate molecules, either individually or in a cross-linked polymer, are acting as a covalent link between two gum arabic molecules. Conjugation of cross-linked sodium caseinate polymers to a single gum arabic molecule would not cause such substantial increases in molecular mass.

These results show, for the first time, the possibility of using a commercially available enzyme, transglutaminase, to catalyze cross-links between a protein-containing polysaccharide, gum arabic, and a food protein, sodium caseinate. Although other cross-linking methods (chemical, Maillard-induced, oxidative enzymes) have been used previously, they are hindered by the possible formation of toxic side reactions and the availability of oxidative enzymes. The Maillard method of forming conjugates has resulted in protein−polysaccharide conjugates with improved functional properties (*5*, *6*, *11*, *12*). Future work in our laboratory will investigate the functional properties of the novel conjugates described herein.

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