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Maillard Reaction Induced Lactose Attachment to Bovine β-Lactoglobulin: Electrospray Ionization and Matrix-Assisted Laser Desorption/Ionization Examination

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Nonenzymatic attachment of lactose to β-lactoglobulin (β-Lg) was investigated under different conditions. Solubilized conditions, dry environment, and a combination of dry and solubilized environments, were examined for their effects on lactosylation. Temperatures ranging from 50 to 65 °C and time intervals between 1 h and 4 days were used. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry were implemented to examine the reaction products. Maximum attachment efficiency occurred at 65 °C held for 3 h in dry-way conditions. Incubations held for long periods of time under dry-way conditions suggest possible denaturation. Both ESI and MALDI data suggest β-Lg removal in the solubilized samples held for long periods of time. A combination of solubilized and dry environments led to very similar mass spectrogram results over time.

KEYWORDS: β-Lactoglobulin; nonenzymatic; solubilized; dry-way; combination

INTRODUCTION

Covalent attachment of reducing sugars to proteins can be accomplished through early Maillard-type reactions. Resultant products of this reaction are influenced by the conditions in which it is performed. Recent investigations have been performed to attempt to identify the sites of lactose attachment (*1*−*4*), as well as the effects of this modification on the β-Lg molecule (*5*−*7*). One of the effects postulated was the formation of a homodimer. This was thought to be due to interaction of free thiol groups in swollen monomers brought on by the solubilized glycation conditions.

The effects, both functional and biological, of glycation on the β-Lg molecule have also been investigated. Chemical glycosylation has been used to observe effects on surface-active properties (*8*). The effects of carboxymethyl dextran attachment on emulsification have been observed (*9*). An in depth investigation on functional property changes directly related to Maillard-induced glycosylation on both of the major globular proteins of whey has been performed (*10*). Also, Shida et al. (*11*) saw an *E.coli* enterotoxin binding glycoprotein within a proteose peptone fraction corresponding to the molecular weight of β-Lg.

The work described in this paper was intended to show an overall investigation into the many different conditions possibly influencing lactosylation of β-Lg. Although past investigations have examined the same ranges in temperature under solubilized conditions, there has not been examination of the same type of range under dry conditions. This may be due to the inability of current commercial incubators to extend relative humidity control past 55 °C. There has also not been investigation into the possibility of a combinatorial approach to these two environments. Electrospray ionization/mass spectrometry was used to give insight into the number of lactose attachments to each of the β-Lg variants, whereas MALDI/TOF mass spectrometry was used to give an overall examination of sample makeup after treatment.

MATERIALS AND METHODS

Materials. To obtain relatively large amounts of purified β-Lg, a modification to the method of Maté and Krochta (*12*), which was founded on the methodology of Mailliart and Ribadeau-Dumas (*13*), was used. A commercial whey protein isolate (WPI), Alacen 895 (New Zealand Milk Products, Inc.) consisting of 97.4% protein on a dry basis was used as the starting material for β-Lg purification. At room temperature, WPI was dissolved at 15% (w:w) in deionized water, and adjusted to pH 2.0 by adding 2 N HCl. Sodium chloride was added until a concentration of 7% (w:v) was reached. This solution was held for 20 min, after which it was centrifuged at 10000*g* for 20 min. The supernatant was filtered through cheesecloth into Spectra Por dialysis tubing with a cutoff of 6−8 kDa. After extensive dialysis in refrigeration, the supernatant was freeze-dried, vacuum packed, and stored at −20 °C until used.

Glycation Experiments. Glycation to β-Lg was carried out in dry, solubilized, and a combination of dry and solubilized systems.

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For the solubilized glycation experimentation, solutions containing β-Lg (0.15 mM) and α-lactose (15 mM) were made and adjusted to pH 7.2 with 4 M and 1 M NaOH. These solutions were covered and placed in a water bath set to the desired experimental temperature (50, 60, or 65 °C). Solutions were removed at time intervals of 1, 3, 24, 48, and 96 h and dialyzed to remove excess lactose. After dialysis, the samples were freeze-dried. Portions of the freeze-dried sample powder were placed in a Biorad 600-μL thin-wall tube and stored in a freezer until examined by ESI/MS or MALDI/MS.

For dry-way glycation experimentation, the same solutions made for solubilized glycation experimentation were made and then freeze-dried. The resultant powders were then held in a relative humidity (RH) controlled environment equilibrated to 65%. For the 50 °C incubations, a NuAire CO_2 water-jacketed incubator model NU4500 with RH control was used. For the 60 °C and 65 °C incubations, sample powders were placed into a desiccator containing a saturated solution of KI and a temperature/RH indicator preequilibrated to the desired temperature and RH. The desiccator was then placed into a Fisher Scientific Isotemp oven for the predetermined time intervals. After incubation, the samples were then resolubilized, dialyzed, and freeze-dried. Portions of these sample powders were held in the same conditions as the solubilized powders until examined by ESI/MS or MALDI/MS.

For the combination approach experimentation, the conditions of sample preparation used were the same as those used in the solubilized and the dry-way experimentation. Dry-way conditions were performed first at 50 °C for 96 h, after which solubilized conditions were performed at 50 °C for 1, 3, 24, 48, and 96 h.

ESI/MS. Electrospray ionization (ESI) experiments were performed on a Micromass Q-Tof II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal electrospray source (Z-spray) operated in the positive ion mode. Polyalanine and alanine were used for mass calibration for a calibration range of *m*/*z* 100−2000. Salt buffers from the protein samples were cleaned using manual syringe protein traps from Michrom BioResources (Auburn, CA). Proteins were prepared in a solution containing 50% acetonitrile (ACN)/50% water and 0.1% formic acid at a concentration of 50 pmol/μL, and infused into the electrospray source at a rate of 5−10 μL min^{-1}. Optimal ESI conditions were as follows: capillary voltage, 3000 V; source temperature, 110 °C; and cone voltage, 60 V. The ESI gas was nitrogen. Q1 was set to optimally pass ions from *m*/*z* 100−2000, and all ions transmitted into the pusher region of the TOF analyzer were scanned over *m*/*z* 100−3000 with a 1 s integration time. Data were acquired in continuum mode until acceptable averaged data was obtained (10−15 min). ESI data was deconvoluted using MaxEnt I provided by Micromass.

MALDI−TOF/MS. Matrix-assisted laser desorption/ionization−time-of-flight (MALDI−TOF) analysis was performed on a Bruker Reflex III (Bruker, Breman, Germany) mass spectrometer operated in a linear, positive ion mode with a N_2 laser. Laser power was used at the threshold level required to generate signal. Accelerating voltage was set to 28 kV. The instrument was calibrated with protein standards bracketing the molecular weights of the protein samples (typically mixtures of apo myoglobin and bovine serum albumin using doubly charged, singly charged, and dimmer peaks, as appropriate). Salt buffers from the protein samples were cleaned using manual syringe protein traps from Michrom BioResources (Auburn, CA). Samples from the trap were lyophilized and prepared in 0.1% trifluoroacetic acid (TFA) at an approximate concentration of 50 pmol/μL. Sinapinic acid was used as the matrix and prepared as a saturated solution in 50% ACN/0.1% TFA (in water). Allotments of 1 μL of matrix and 1 μL of sample were thoroughly mixed together; 0.5 μL of this was spotted on the target plate and allowed to dry.

RESULTS

Comparisons were made against the initial starting material, WPI, and the β-Lg purified from the WPI. ESI examination of the WPI (**Figure 1**) shows peaks representative of glycated forms of β-Lg present as well as a singly glycated form of α-Lactalbumin. The glycated forms of β-Lg persisted through the purification process. The presence of these glycated species in the control may be due to this WPI's production method or storage environment, or to preexisting glycoforms in the whey before manufacture.

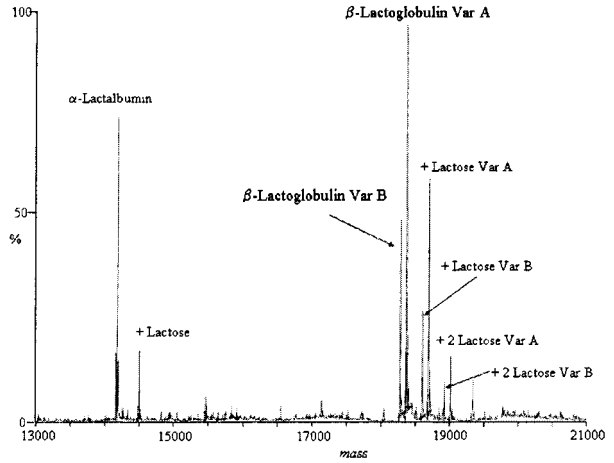

Figure 1. ESI mass spectrogram of commercial WPI.

Table 1. ESI Results for Solubilized Treatments of β-Lg and Lactose: Solubilized (S) Temperatures in °C (50, 60, or 65) and Time Treatments (1–96 h) are Followed by Percentage of Signal for the Number of Glycations in the ESI Mass Spectrogram

	% of total signal for following number of glycations										
treatment	0	1	2	3	4	5	6	7	8	9	10	11
WPI–native	53	31.5	10.5	5								
purified β-Lg	65	35										
S-50–1 hr	51.5	38	10									
S-50–3 hr	52.5	37	10.5									
S-50–24 hr	53.7	35.5	10.8									
S-50–48 hr	57	31.5	11.5									
S-50–96 hr	62	32	6									
S-60–1 hr	51	34	15									
S-60–3 hr	52.1	32.3	15.6									
S-60–24 hr	58.1	30.3	11.6									
S-60–48 hr	70	30										
S-60–96 hr	77	23										
S-65–1 hr	52	33	15									
S-65–3 hr	56	33	11									
S-65–24 hr	75	25										
S-65–48 hr	84	16										
S-65–96 hr	84	16										

Solubilized conditions were the first to be compared; 0.15 mM β-Lg and 15 mM lactose heated at 50, 60, and 65 °C for time intervals of 1, 3, 24, 48, and 96 h. From the data in **Table 1** it can be seen that there was loss of glycoforms. The apparent loss was linear with time, occurring at a faster rate as the temperature increased from 50 to 65 °C.

Because it is unlikely that there was cleavage of the covalent bonds between lactose and amino groups in these samples, it may be inferred that there was further activity within these samples. Peak trends between major species (native, +1, +2...) did not fluctuate, nor were intensities very high to suggest intramolecular modification. This indicated that further activity might be due to cross-linking reactions such as homodimer formation (previously mentioned) or advanced glycation end product (AGE) formation resulting in lysine to arginine bridging. To investigate this, one sample was chosen for comparison with the control (**Figure 2**) to be deconvoluted up to the 40 kDa range using ESI. Results, seen in **Figure 3** , show an increase in dimer content. The exact nature of the dimer is unknown at this time.

The second conditions to be compared were termed dry-way as samples were prepared as powders and relative humidity was controlled at 65%. The same time and temperature treatments

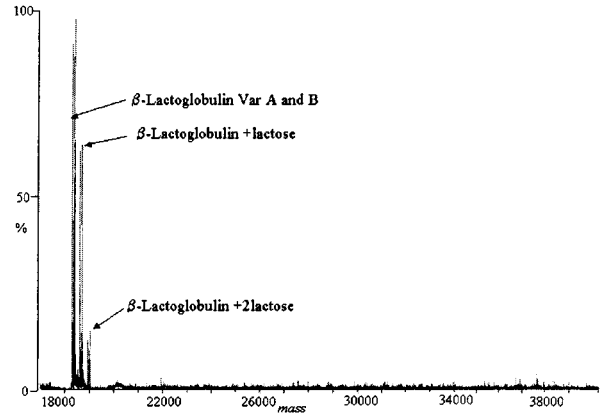

Figure 2. ESI mass spectrogram of starting material scanned from 18 kDa to 40 kDa.

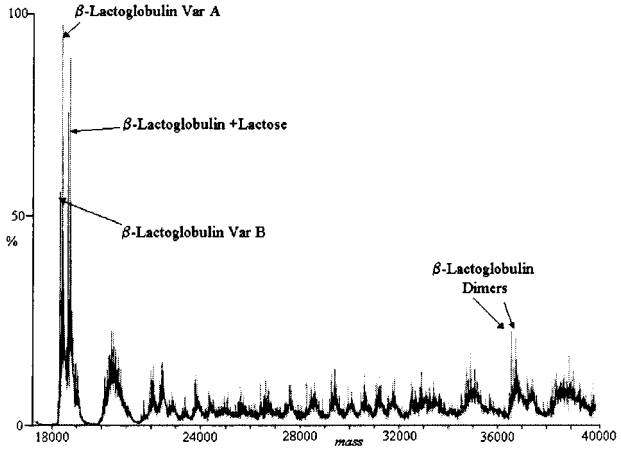

Figure 3. ESI mass spectrogram of solubilized, 60 °C sample held for 96 h scanned from 18 kDa to 40 kDa indicating further interaction between β-Lg monomers.

Table 2. Results for Dry Treatments of β-Lg and Lactose: Dry-Way (D) Temperatures in °C (50, 60, or 65) and Time Treatments (1–96 h) are Followed by Percentage of Signal for the Number of Glycations in the ESI Mass Spectrogram

	% of total signal for following number of glycations											
treatment	0[a]	1	2	3	4	5	6	7	8	9	10	11
WPI-native	53	31.5	10.5	5								
D-50–1 hr	43.9	38.6	17.5									
purified β-Lg	65	35										
D-50–3 hr	40.7	37.3	16.7	5.3								
D-50–24 hr	15.7	31.4	25.1	17.9	10							
D-50–48 hr	8.5	24.2	24.2	21.8	15.3	6						
D-50–96 hr	4.4	16.3	19.5	24.4	19.8	10.7	4.9					
D-60–1 hr	7.7	20.6	30.8	22.5	12.9	5.5						
D-60–3 hr				7.8	16	20.7	23.5	17.8	10.8	3.5		
D-60–24 hr	XX											
D-60–48 hr	XX											
D-60–96 hr	XX											
D-65–1 hr		7.3	17.7	25.3	20.8	17	8.9	3				
D-65–3 hr					5.9	9.7	16.9	22.6	19.9	14.2	7.7	3.2
D-65–24 hr	XX											
D-65–48 hr	XX											
D-65–96 hr	XX											

[a]A mark of XX indicates samples which did not have resolution under ESI conditions used.

that were applied to the solubilized samples were also applied to the dry-way samples. From the results seen in **Table 2** it can be seen that dry-way treatments resulted in numerous lactose attachments increasing with both time and temperature.

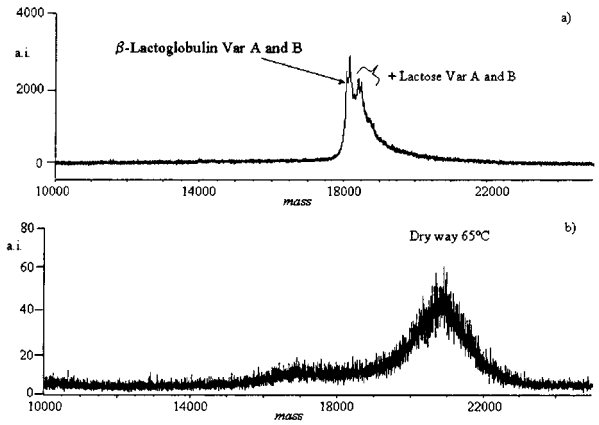

Figure 4. MALDI/MS mass spectrogram of dry-way 65 °C, 48-hour sample (bottom) compared to that of the starting material (top).

Table 3. Results for Combination Dry and Solubilized Treatments of β-Lg and Lactose: Dry-Way Heating Conditions of 50 °C for 96 Hours Were Followed by Solubilized Conditions of 50 °C for 1, 3, 24, 48, and 96 Hours

	% of total signal for following number of glycations											
treatment	0	1	2	3	4	5	6	7	8	9	10	11
WPI-native	53	31.5	10.5	5								
purified β-Lg	65	35										
combined 1 hr	17.4	33.4	30.1	19.1								
combined 3 hr	21.8	30.7	27.6	19.9								
combined 24 hr	21	31.8	28.7	18.5								
combined 48 hr	24.3	32.9	26.3	16.5								
combined 96 hr	22.4	33.9	27.1	16.6								

Dry-way experimentation results did not exactly mimic that of Morgan et al (*4*). The maximum number of glycations seen in the 50 °C, 48-hour sample was 5 compared to a reported average of 7.7. It was not until the 65 °C condition that averages around 7 to 8 glycations could be seen. The species containing the maximum number of glycations observed was at the 65 °C, 3-hour treatment showing 11 lactose attachments. It is unknown at this time why there was a difference in experimental findings. Mass spectrograms of dry-way samples also indicated further intramolecular modification occurring more in the higher temperature incubations.

After 3 h in the 60 °C and the 65 °C conditions, ESI data indicated a loss of protein signal, leading to speculation that denaturation of the protein had occurred. However, as MALDI is more sensitive that ESI, MALDI/MS readings of these same samples showed that there were still fractions of modified β-Lg. MALDI/MS readings similar to those of **Figure 4** were seen for 60 °C and 65 °C samples held for 24 and 48 h. These readings would indicate the presence of species again containing an average of 7 to 8 glycations with maximums around 11 to 12 glycations.

Combination treatment samples yielded interesting results (**Table 3**). Although they did not obtain a higher degree of glycation than most of the dry-way glycations, there was not a decrease in peak intensity seen in the 2, 3, and 4 addition glycoforms over time (**Figure 5**). In comparison to those samples heated in solution this would indicate a stabilization of the glycoprotein to further reactions leading to loss of signal. However, a noticeable difference can be seen in the intensity of peaks occurring between variants and species of β-Lg. This would indicate further intramolecular modification. This further reactivity might be attributed to the dry-way incubation step.

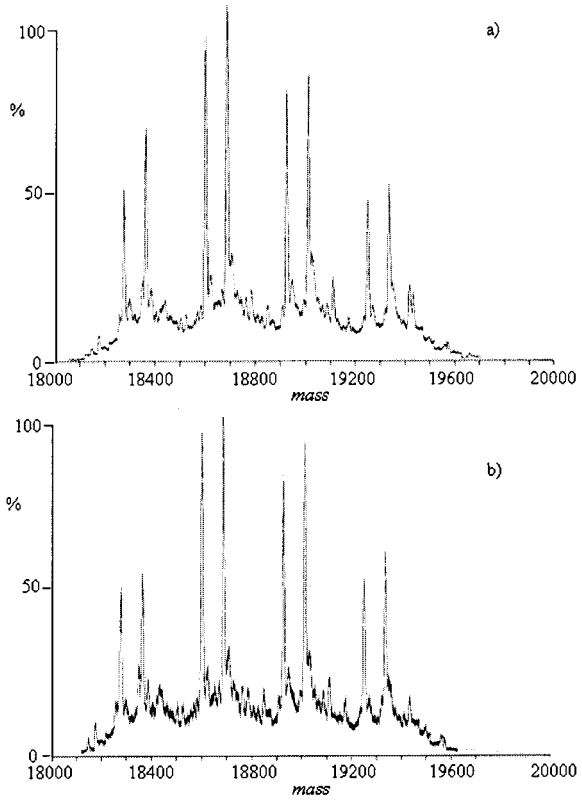

Figure 5. ESI/MS mass spectrogram of combination approach 1-hour (top) and 96-hour (bottom) samples.

DISCUSSION

The results shown from these experiments show a greater propensity for Maillard-induced attachment of lactose molecules under dry conditions. This has implications related to shipping and storage conditions of whey protein concentrates and isolates. Exposure of these products to moderately high temperatures over time might increase their heterogeneous nature, and possibly result in modification of their functionality. Further investigation of the impact of glycation on functionality should be performed.

Maillard-type glycation of proteins might be desired, as resultant products may carry benefits (biological as well as functional) that offset the detrimental loss of lysine availability or any negative impacts on functionality. In this case, optimization of conditions leading to the desired degree of carbohydrate attachment is needed. This work showed optimum attachment under dry-way conditions at 65 °C held for 3 h. Though MALDI/MS results showed the possibility of greater degrees of glycation at longer incubation times at this temperature, the specific types and ratios were unable to be detected using ESI/MS. Also, the presence of further-reacted products must be taken into account as dry-way conditions showed greater propensity for intramolecular modification, and solubilized conditions indicated some form of further reactivity yet to be defined.

The combinatorial approach to glycation leads to further questions regarding sites of lactose attachment and effects on intra/intermolecular modification. Understanding these factors would help in controlling the glycation process and thus controlling the end product.

ABBREVIATIONS USED

AGE, advanced glycation endproduct; B-Lg, β-Lactoglobulin; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; TFA, trifluoroacetic acid; TOF, time-of-flight; WPI, whey protein isolate.

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