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Charge State Distribution and Hydrogen/Deuterium Exchange of α-Lactalbumin and β-Lactoglobulin Preparations by Electrospray Ionization Mass Spectrometry

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Charge state distribution (CSD) and hydrogen/deuterium (H/D) exchange of preparations of α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) were investigated using electrospray ionization mass spectrometry (ESI-MS). Storage of α-Lac at pH 3 resulted in substantial changes in its CSD, with the emergence of new ion species and shifts toward higher charge state, indicating less stable conformation. ESI spectra of α-Lac kept at pH 5.5 for 4 days showed stable conformation; however, extending the storage period resulted in substantial changes in CSD and a decrease in the stability of holo-α-Lac (Ca^{2+}-bound form). In comparison to apo-α-Lac, the relative intensity of holo-α-Lac was higher at pH 6.8 but lower at pH 8 during the storage period. β-Lg showed stable CSD at pH 3, substantial changes at pH 5.5, and minor changes at pH 6.8 and 8 during storage. The H/D exchange results demonstrate that the conformation of holo-α-Lac was more stable than that of apo-α-Lac and that the conformation of β-Lg variant B was more stable than that of the β-Lg variant A. Kinetics of H/D exchange indicated that α-Lac and β-Lg fractions obtained from whey protein preparations have the same or improved conformational stabilities compared to those of α-Lac and β-Lg standards. The presence of four or more hexose residues in α-Lac enhanced its conformational stability; the presence of two hexose residues in β-Lg resulted in a less stable conformation.

KEYWORDS: β-Lactoglobulin; α-lactalbumin; charge state distribution; H/D exchange

INTRODUCTION

During the past two decades, considerable effort has been devoted to the structural characterization of food proteins and peptides. As part of this effort, mass spectrometry (MS) is now used to investigate the structure of relatively large protein molecules. The application of MS to large biomolecules has been revolutionized in the past decade with the development of electrospray ionization (ESI) (*1*, *2*). The utility of ESI-MS lies in its ability to generate multiply charged gas phase ions from protein molecules in solution. ESI-MS now plays an important role in studies of the primary (covalent and noncovalent) structure of proteins.

The role of ESI-MS is more than determination of MW of proteins, because a relationship between the structure of proteins in solution under different environmental conditions and their charge state distributions (CSD) in the ESI spectrum has been established (*3*−*5*). Thus, changes in the CSD of a protein can be interpreted in terms of changes in its conformational stability. CSD is sensitive to tuning conditions in the mass spectrometer, to slight variation in pH, and to counterion effect (*6*). Protein conformational changes can also be studied by ESI-MS using amide hydrogen exchange technique. This technique is based on the mass spectrometric measurement of the extent of hydrogen/deuterium (H/D) exchange that occurs in different protein conformers over defined periods of time (*7*, *8*). Higher order structural (secondary and tertiary) features of proteins can affect amide hydrogen exchange rates drastically, thereby forming the basis for estimating, detecting, and locating conformational changes.

Amide hydrogen exchange rates of proteins and peptides are influenced by both acid and base. Therefore, the isotopic exchange rates are highly pH sensitive, and this requires careful control of pH in all H/D experiments (*9*). Exchange rates of amide hydrogen are also affected by inductive and steric effect of the adjacent amino acid side chains but are relatively insensitive to more distant side chains. The effect of neighboring side chains can decrease the amide hydrogen exchange rates by as much as 10-fold, whereas secondary and tertiary structural features of folded proteins may decrease amide hydrogen exchange rates by as much as 10^8 (*9*). In this context, amide hydrogen exchange is considered to be a sensitive probe for

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detecting and locating conformational changes in proteins. The application of H/D exchange to the study of protein conformational stability in solution was first demonstrated by Katta and Chait (*7*) and has since been applied to study protein conformational stability under different environmental conditions. It has been used to study the native state of a protein (*8*), the exchange rates of α-helices and β-sheets (*10*), and the folding pathway of native (*11*) and reduced (*12*) lysozyme and to monitor protein/protein (*13*), ligand/protein (*14*, *15*), and metal/protein interactions (*16*, *17*) and thermal (*18*, *19*) and organic solvent (*20*) unfolding of proteins.

The influence of environmental conditions, such as pH, ionic strength, temperature, processing, and storage time, on the denaturation and functionality of whey proteins is well established. These environmental conditions will alter the molecular structure of whey proteins and thus their functional properties; pH conditions in particular can be expected to affect the CSD of the proteins. Although there is a relative abundance of information in the literature on factors affecting the structural stability of whey proteins, there is relatively limited information on the structural stability of individual β-lactoglobulin (β-Lg) and α-lactalbumin (α-Lac) isolated from different whey protein preparations. In this study, we have used CSD determinations by ESI-MS to monitor changes in the conformational stability of α-Lac and β-Lg as affected by pH and storage time. In addition, the conformational stability of α-Lac and β-Lg fractions from liquid whey (LW), whey protein concentrates (WPC), and whey protein isolates (WPI) was investigated by H/D exchange ESI-MS.

MATERIALS AND METHODS

Materials. Commercial standards of β-Lg (containing variants A and B; L-0130) and α-Lac [containing apo-form (Ca^{2+}-free) and holo-form (Ca^{2+}-bound) of α-Lac; L-6010] were obtained from Sigma-Aldrich Co. (St. Louis, MO). β-Lg and α-Lac fractions were prepared in our laboratory from commercial samples of WPI (>90% protein, René Rivet Inc. Ingredients, Laval, PQ, Canada), WPC (>75% protein, Amcan Ingredients, Lachine, PQ, Canada), and LW (obtained from mozzarella cheese preparation), using the combinational effects of high salt concentration and chelating agents at low pH (*21*). The β-Lg and α-Lac isolated fractions were recovered with purities ranging from 84 to 95% and from 83 to 90%, respectively, depending on the source of whey protein preparations (*21*). Deuterium oxide (D_2O) (>99%) was from Sigma-Aldrich Co. All chemicals were of analytical grade.

CSD-ESI-MS. Solutions (0.5 mg/mL) of α-Lac and β-Lg standards were prepared at pH 3 (1% acetic acid), pH 5.5 (water), pH 6.8 (10 mM ammonium acetate), and pH 8 (10 mM ammonium bicarbonate). The solutions were stored for 0, 2, 4, 6, 8, and 10 days at 4 °C; under these conditions there was no visible deterioration of the samples. A SCIEX API III triple-quadrupole mass spectrometer equipped with a standard atmospheric pressure ionization (API) source and a SCIEX ionspray interface (PE-Sciex, Concord, ON, Canada) was used to generate multiply charged protein ions by spraying the sample solution through a stainless steel capillary. The voltage on the sprayer was set at 4500 V for positive ion production (*22*). The mass scale of the spectrometer was first calibrated with polypropylene glycol and then tuned and recalibrated using lysozyme. All CSD experiments were done in duplicate.

H/D Exchange ESI-MS. α-Lac and β-Lg fractions isolated from WPI, WPC, and LW were dialyzed against distilled water and then further desalted using Centricon-10 filters (Amicon Inc., Beverly, MA); the desalting step was repeated four times to obtain mass spectra with good sensitivity. The desalted samples were then lyophilized and dissolved (1 mg/100 μL) in 10 mM ammonium acetate aqueous solution (pH 6.8). After 1 h, the H/D exchange was initiated by diluting 25 μL of the protein solution in 225 μL of 10 mM deuterated ammonium acetate (1 mg/mL). The amount of labile deuterium atoms in this mixture is 90%. The use of a concentrated solution of protonated proteins in deuterated solution, instead of lyophilized protein in deuterated solution, was based on previous reports that consistent results and sharp peaks were achieved with the former technique (*16*, *22*). The deuterated protein solution was infused into the ionization chamber of the MS from a syringe pump (model 22, Harvard Apparatus, South Natick, MA) at a rate of 2 μL/min. The enclosed ionization chamber of the MS was kept at room temperature (21 ± 2 °C) and atmospheric pressure and was constantly flushed with ultrahigh-purity nitrogen to prevent the back exchange of protein-deuterated atoms by hydrogen in moisture in the laboratory air. Ultrahigh-purity air was used as the nebulizing gas. Under these conditions, exchange of hydrogen by deuterium can occur only in the solution phase, and back exchange of deuterium by hydrogen is negligible in the gas phase (*10*). Collection of the initial MS data was started immediately, usually within 10−20 s of dilution. A small *m*/*z* (mass-to-charge ratio) range (70 units) containing a selected charge state of the molecular ions (ion with maximum intensity) was scanned repeatedly using a step size of 0.2 Da and a dwell time of 10 ms; data were collected and plotted at 30 s intervals during the first 45 min and at much longer intervals for the 60, 2880, 5760, 8640, 11520, and 14400 min periods (*6*). The molecular mass at each time point was calculated from the measured *m*/*z* ratios and the predetermined charge state. **Figure 3** shows representative mass spectra at selected times during the H/D exchange experiment. At each time point (*t*), the average number of unexchangeable hydrogens was determined by subtracting the molecular mass at time *t* from the 90% deuterated molecular mass (*22*). The average number of unexchanged hydrogens was plotted against time to give the H/D exchange curves. All H/D exchange experiments were done in duplicate.

Data Analysis. The apo-form (Ca^{2+}-free) of α-Lac contains 123 amino acid residues and a total of 225 hydrogens (**Table 1**), of which 120 are on the amide backbone, 102 are on the side chains, and 3 are on the two termini; the total numbers of hydrogens in the holo-form (Ca^{2+}-bound) of α-Lac is 223, due to Ca^{2+} binding to the protein molecule. In our experiment, H/D exchange was performed in a solution of 90% D_2O; therefore, total labile exchangeable hydrogens for 90% deuterated apo- and holo-α-Lac are 203 and 201, respectively (**Table 1**). β-Lg (variants A and B) contains 162 amino acid residues and a total of 279 (278 for variant B) hydrogens (**Table 2**), of which 153 are on the amide backbone, 123 (122 for variant B) are on the side chains, and 3 are on the two termini. The total numbers of labile exchangeable hydrogens for 90% deuterated β-Lg (variant A) and β-Lg (variant B) are 251 and 250, respectively (**Table 2**). A single hexose sugar bound to a protein molecule has four exchangeable hydrogens; for a di- or oligosaccharide bound to a protein, the first hexose sugar has three exchangeable hydrogens, and the remaining hexose sugars have four exchangeable hydrogens. Attachment of a sugar residue to a protein molecule results in replacement of one hydrogen atom from the protein molecule. The number of unexchanged hydrogens versus the time in minutes after the addition of D_2O is fitted to the sum of two exponentials as in the equation (*9*, *22*)

$$Y = A \exp^{(-k_1 t)} + B \exp^{(-k_2 t)}$$

where *Y* is the number of unexchanged hydrogens remaining, *t* is the time in minutes after addition of D_2O, and *A* and *B* are the numbers of intermediate and slowly exchanged hydrogens, with exchange rate constants of k_1 and k_2, respectively. The number of hydrogens undergoing exchange at very fast rate (C) was calculated by subtracting total exchangeable hydrogens (90%) from $A + B$. Total amide percent is the sum of (A) and (B) amides, which are the percent of intermediate and slowly exchanged hydrogens from the total number of amide backbone hydrogens in α-Lac (120) and β-Lg (153), respectively.

RESULTS AND DISCUSSION

CSD of α-Lac. Parts A−D of **Figure 1** show the mass spectra of α-Lac standard solutions at pH 3, 5.5, 6.8 and 8, respectively, and stored for 0, 2, 4, 8, and 10 days; the mass spectra for day 6 are not shown to avoid repetition.

Table 1. Hydrogen/Deuterium Exchange Characteristics of α-Lac Standard and α-Lac from Whey Preparations at pH 6.8

origin of α-Lac[a]	total H (100%)	exchangeable H (90%)	unexchanged H after 5 min	unexchanged H after 20 min	unexchanged H after 45 min	k_1[b] (min^{-1})	k_2[b] (min^{-1})	intermediate exchanged H (A)	(A) (%)	slowly exchanged H (B)	(B) (%)	very rapidly exchanged H (C)[c]	(C) (%)	(A) amide[d] (%)	(B) amide[d] (%)	total amide (%)
apo-STD	225	203	38	18	13	0.113	0.001	45	22	14	7	144	71	38	12	50
holo-STD	223	201	58	36	25	0.122	0.012	33	17	42	21	125	62	28	35	63
holo-WPI	223	201	77	38	23	0.095	0.017	65	32	40	20	96	48	54	33	87
holo-LW	223	201	63	45	33	0.072	0.008	28	14	45	23	128	64	23	38	61
holo-WPC + 4Hex	235	212	74	44	24	0.054	0.014	55	26	35	16	122	58	46	29	75
holo-WPC + 5Hex	238	215	82	55	33	0.042	0.012	53	25	41	19	120	56	44	35	79
holo-WPC + 6Hex	242	217	94	68	45	0.038	0.005	65	30	42	19	110	51	54	35	89

[a] apo-form, Ca^{2+}-free; holo-form, Ca^{2+}-bound; STD, standard; WPI, whey protein isolate; WPC, whey protein concentrate; LW, liquid whey; Hex, hexose sugar units. [b] k_1 and k_2 are exchange rate constants of intermediate (A) and slowly (B) exchanged hydrogens, respectively. [c] (C) are the number of hydrogens undergoing exchange at very fast rate. [d] (A) and (B) amides are the percent of intermediate and slowly exchanged hydrogens from the total number of amide backbone hydrogens (120) in α-Lac, respectively.

Table 2. Hydrogen/Deuterium Exchange Characteristics of β-Lg Standard and β-Lg from Whey Preparations at pH 6.8

origin of β-Lg[a]	total H (100%)	exchangeable H (90%)	unexchanged H after 5 min	unexchanged H after 20 min	unexchanged H after 45 min	k_1[b] (min^{-1})	k_2[b] (min^{-1})	intermediate exchanged H (A)	(A) (%)	slowly exchanged H (B)	(B) (%)	very rapidly exchanged H (C)[c]	(C) (%)	(A) amide[d] (%)	(B) amide[d] (%)	total amide (%)
B-STD	278	250	77	46	38	0.244	0.007	98	39	51	21	101	40	64	34	97
A-STD	279	251	74	43	37	0.264	0.005	108	43	47	19	95	38	71	31	102
B-WPI	278	250	79	46	40	0.238	0.005	105	42	50	20	95	38	68	33	101
B-WPI + 2Hex	284	255	75	42	35	0.211	0.006	91	36	46	18	118	46	59	30	90
A-WPI	279	251	74	43	36	0.277	0.007	113	45	49	20	89	36	74	32	106
A-WPI + 2Hex	285	256	72	39	32	0.218	0.006	93	36	43	17	119	47	61	28	89
B-LW	278	250	80	46	40	0.243	0.005	110	44	50	20	90	36	72	33	104
A-LW	279	251	74	43	36	0.271	0.006	114	45	48	19	89	35	74	31	106

[a] B and A are β-Lg variants B and A, respectively. STD, standard; WPI, whey protein isolate; LW, liquid whey; Hex, hexose sugar units. [b] k_1 and k_2 are exchange rate constants of intermediate (A) and slowly (B) exchanged hydrogens, respectively. [c] (C) are the number of hydrogens undergoing exchange at very fast rate. [d] (A) and (B) amides are the percent of intermediate and slowly exchanged hydrogens from the total number of amide backbone hydrogens (153) in β-Lg, respectively.

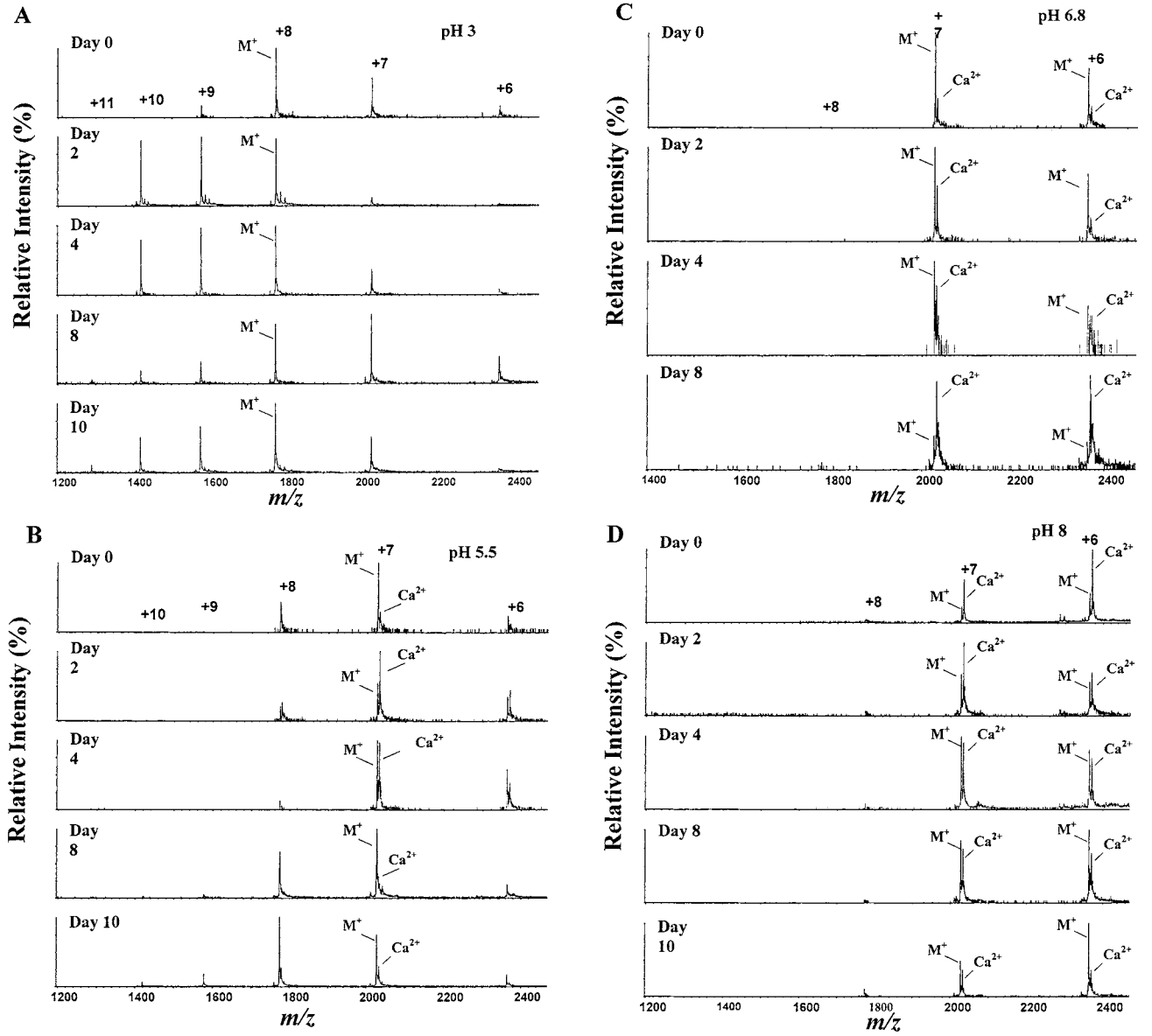

Figure 1. ESI mass spectra of α-lactalbumin at pH 3 (A), 5.5 (B), 6.8 (C), and 8 (D) and stored for 0, 2, 4, 8, and 10 days.

pH 3. Initially, α-Lac at pH 3 exhibited four peaks (+9, +8, +7, and +6), with a maximum charge state of +9 (**Figure 1A**), 8 charges less than the total number of positive charges based on the number of basic residues (arginine, lysine, histidine, and N-terminal). The most intense peak was observed for species +8. Storage for 2 days resulted in the emergence of +10 species with relatively high intensity and a substantial increase in the intensity of the +9 species. This indicates increased solvent accessibility to buried basic amino acid residues due to the less stable conformation of α-Lac. At day 8, a new species (+11) was observed, and its relative intensity increased at day 10, resulting in decreased relative intensities of +10 and +9 peaks (relative intensity is presented as a normalized value of total observed peaks). These results indicate decrease in α-Lac conformational stability during storage.

pH 5.5. At pH 5.5, α-Lac initially exhibited three peaks, with a maximum charge state of +8 (**Figure 1B**), 9 charges less than the total number of basic residues; the most intense peak was observed for species +7. The molecular mass (M_r) obtained for the molecular ion (M^+) was 14178 Da and that for the metal adduct was 14216 Da, representing the apo-form (Ca^{2+}-free) and holo-form (Ca^{2+}-bound) of α-Lac, respectively. It is known that α-Lac is a Ca^{2+} binding protein and the binding of Ca^{2+} stabilizes the structure of native α-Lac (*23*). Storage for 2 days resulted in an increase in the relative intensity of metal adduct (holo-form) (**Figure 1B**). The predominance of the relative M_r of 14216 Da and the narrow CSD suggest that α-Lac retained its native structure under these conditions, for the first 4 days of storage (*24*). Storage for 8 and 10 days resulted in the emergence of species carrying +9 and +10 charges, shifting charge states toward higher values, and a decrease in the relative intensity of metal adduct (holo-form).

pH 6.8. During 8 days, α-Lac at pH 6.8 exhibited only two peaks with charge states + 6 and +7 (**Figure 1C**). The relative intensity of the Ca^{2+} adduct (holo-form) increased, with a decrease in the relative intensity of M^+ (apo-form), indicating stable conformation of α-Lac during storage at pH 6.8. The quality of ESI mass spectra obtained at this pH is not optimal, because basic groups are not fully protonated in solution and the use of ammonium acetate causes a shift in the charge state of a protein toward lower values (i.e., higher *m*/*z* values) (*25*).

pH 8. Initially, α-Lac at pH 8 shows three peaks, with a maximum charge state of +8 (**Figure 1D**); this CSD was essentially unchanged during storage. Unlike the results obtained

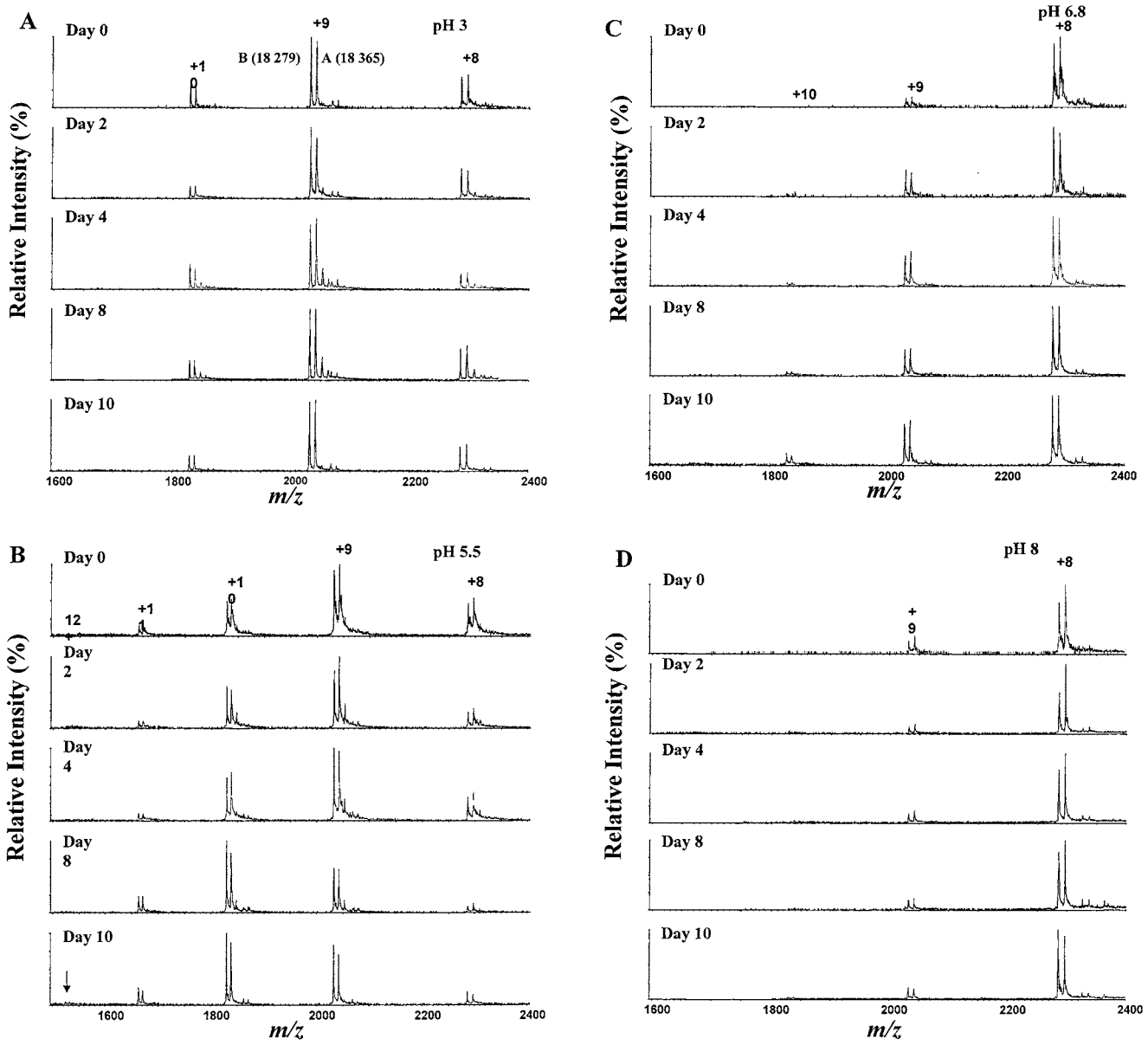

Figure 2. ESI mass spectra of β-lactoglobulin (variants B and A) at pH 3 (A), 5.5 (B), 6.8 (C), and 8 (D) and stored for 0, 2, 4, 8, and 10 days.

at pH 6.8, the relative intensity of the Ca^{2+} adduct (holo-form) decreased with an increase in the relative intensity of M^+ (apo-form), indicating unstable conformation of α-Lac during storage at pH 8.

CSD of β-Lg. Parts A−D of **Figure 2** show the mass spectra of β-Lg standard solutions at pH 3, 5.5, 6.8, and 8 respectively, and stored for 0, 2, 4, 8, and 10 days; the mass spectra for day 6 are not shown to avoid repetition.

pH 3. The spectrum of β-Lg (variants B and A) at pH 3 stored for 10 days exhibited three peaks, with a maximum charge state of +10 (**Figure 2A**), 11 charges less than the total number of basic amino acid residues; the most intense peak was observed for species +9. This CSD was unchanged during storage for both variants, indicating stable conformation of β-Lg at pH 3; this is consistent with results from differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy, which showed that β-Lg has maximum thermal stability at pH 3 (*26*).

pH 5.5. At pH 5.5, β-Lg exhibited four peaks, with a maximum charge state of +11 (**Figure 2B**), 10 charges less than the total number of basic residues; the most intense peak was observed for species +9. Storage for 2, 4, and 6 days resulted in a gradual decrease in the relative intensity of peaks with charges +9 and +8 and a gradual increase in the relative intensity of peaks with higher charge states (i.e., +10 and +11). At day 10, the spectrum of β-Lg shows the emergence of the +12 species, indicating that basic amino acid residues become more accessible to solvent during extended storage.

pH 6.8. At pH 6.8, β-Lg shows only two peaks, with charge states of +8 and +9 at day 0 (**Figure 2C**). At day 2, a new species carrying +10 was observed. After day 4, the relative intensity of charges +9 and +10 increased progressively.

pH 8. At pH 8, β-Lg shows only two peaks, with charge states of +8 and +9 (**Figure 2D**); this CSD was unchanged during storage. The relatively minor changes observed in the mass spectra of β-Lg at pH 6.8 and 8 can be attributed to the inability of ESI to produce multiply charged protonated molecules of native β-Lg in basic solutions, resulting in a shift of protein CSD toward lower values in the higher *m*/*z* range.

H/D Exchange of α-Lac. Figure 3 shows a representative spectrum at charge state +7, of apo- and holo-α-Lac standard incubated in deuterated solution (pH 6.8) at different time

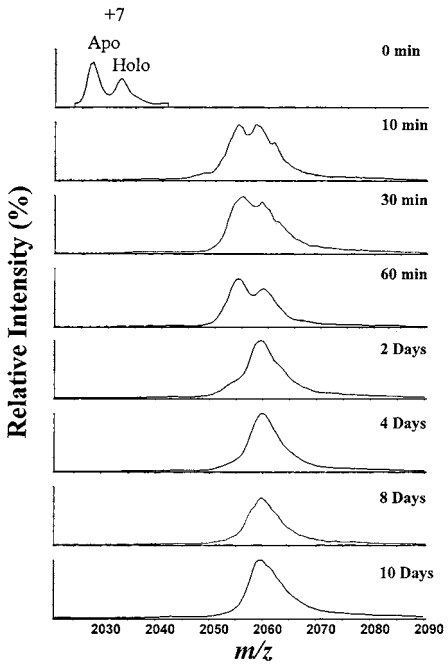

Figure 3. ESI mass spectrum of apo- and holo-α-lactalbumin standard incubated in deuterated solution (pH 6.8) at different time periods.

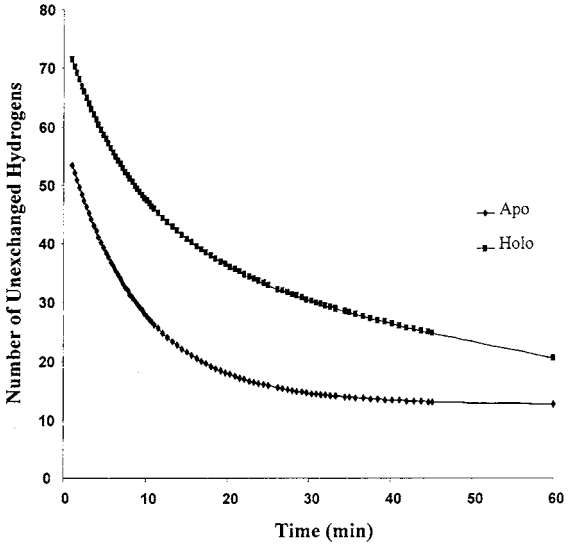

Figure 4. H/D exchange curves of apo- and holo-α- lactalbumin standard at pH 6.8.

periods. **Figure 4** shows the H/D exchange curves for apo- and holo-α-Lac standards at pH 6.8. After 60 s, the total number of unexchanged hydrogens in apo-α-Lac standard was 54 (26.6% unexchanged hydrogens) compared to 72 (35.8% unexchanged hydrogens) in holo-α-Lac standard. The very rapidly exchanged hydrogens are assumed to be on the exposed side chains and on unstructured backbone amides, whereas the remaining exchangeable hydrogens are assumed to be either buried or involved in intramolecular hydrogen bonding (*9*). The difference in extents of unexchanged hydrogens between apo- and holo-α-Lac standard remained constant for the first 20 min (difference of 9.7% unexchanged hydrogens at 5 min and 9% at 20 min, **Figure 4**). At 45 and 60 min, the percentages of unexchanged hydrogens were 5.8 and 4.0, respectively, after which the difference between the two curves remained somewhat constant during the period of 10 days (data not shown).

Table 1 shows the number of intermediate (A) and slowly (B) exchanged hydrogens, with rate constants of k_1 and k_2,

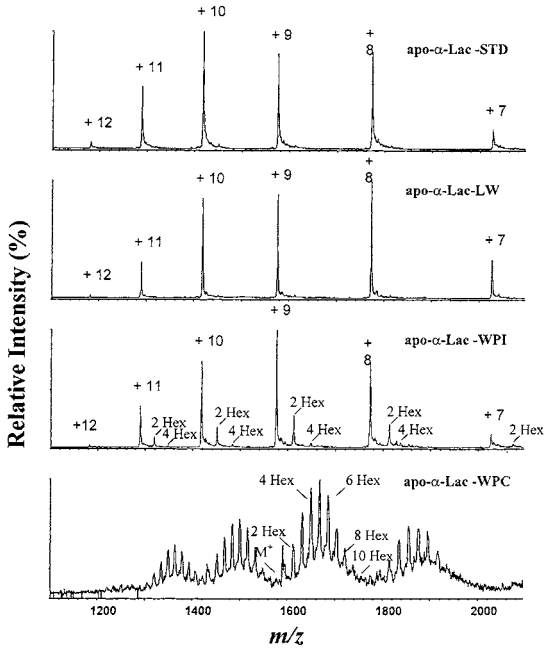

Figure 5. ESI mass spectrum of apo-α-lactalbumin fractions from whey protein isolate (WPI), whey protein concentrate (WPC), and liquid whey (LW).

respectively (Data Analysis section). The number of very rapidly (C) exchanged hydrogens (exchanged during 10−20 s before MS measurements started) was calculated as the total number of exchangeable hydrogens (90%) minus (A) + (B). It has been shown that exchange of hydrogen at exposed side chains and unstructured backbone amides can be essentially complete within minutes in D_2O (pH >5) at room temperature (*27*); therefore, the unexchanged hydrogens in α-Lac remaining after 5 min are expected to be those involved in hydrogen bonding in secondary structure or those on side chains buried inside the hydrophobic core. The results in **Table 1** suggest that (i) 22% of unexchanged hydrogens of apo-α-Lac standard belong to the intermediate (A) exchanging category, (ii) 7% belong to the slowly (B) exchanging category, and (iii) 71% belongs to the very rapidly (C) exchanging category. The results for holo-α-Lac, which contains Ca^{2+}, show 62% of the very rapidly (C), 17% of the intermediate (A), and 21% of the slowly (B) exchangeable hydrogens. These results demonstrate the effect of Ca^{2+} on the stability of the structure of holo-α-Lac, which are consistent with the H/D-MS data of Chung et al. (*28*), who reported that in the absence of Ca^{2+}, protection against hydrogen exchange is decreased. The lower H/D exchange rates in folded proteins can be due to reduced accessibility of solvent to amide hydrogens (*29*) and/or increase in amide hydrogen bond stability (*30*). Therefore, accelerating H/D exchange rates in apo-α-Lac compared to holo-α-Lac suggest that the absence of Ca^{2+} can cause exposure of many amide groups to the solvent or possibly the disruption of many hydrogen bonds. Recently, X-ray (*31*) and NMR (*32*) studies have shown that removal of Ca^{2+} from α-Lac affects mainly the Ca^{2+}-binding region of the protein.

Figure 5 shows spectra of apo-α-Lac fractions isolated from LW, WPI, and WPC. The spectrum of the apo-α-Lac standard was similar to that of apo-α-Lac isolated from LW. The apo-α-Lac from WPI contained two glycated species with two and four hexose sugar units, whereas apo-α-Lac from WPC contained glycated species with 2−11 hexose sugar units. **Table 1** shows the H/D exchange for only the holo-α-Lac, because the relative intensity of its ion signal was higher compared to those of the apo-form. The holo-α-Lac fraction from WPI (holo-α-

Lac-WPI) showed 77 (38%) unexchanged hydrogens after 5 min, which is higher than that of the holo-α-Lac standard (58 or 29%). After 20 min, unexchanged hydrogens in holo-α-Lac-WPI and holo-α-Lac standard were similar. For holo-α-Lac-WPI, the percentage of very rapidly (C) exchanging hydrogens (48%) was less than that for holo-α-Lac standard (62%), and the percentage of intermediate (A) exchanging hydrogens (32%) was greater than that of holo-α-Lac standard (17%). These results, along with increased involvement of the total amide percentage in holo-α-Lac-WPI sample (87%) compared to those of holo-α-Lac standard (63%), suggest that holo-α-Lac-WPI is more protected against hydrogen exchange than holo-α-Lac standard.

Table 1 shows that 14% of unexchanged hydrogens in holo-α-Lac-LW belong to the intermediate (A) exchanging category, 22.5% to the slowly (B) exchanging class, and 64% to the very rapidly (C) exchanging category, with involvement of 61% of the total number of amide backbone hydrogens. These values are comparable to those obtained for holo-α-Lac standard; however, the rates of exchange (k_1 and k_2) of holo-α-Lac LW (0.072 and 0.008 min^{-1}) were much slower than that of holo-α-Lac standard (0.122 and 0.012 min^{-1}). Similarly, the rates of exchange in holo-α-Lac-LW were slower than those of holo-α-Lac-WPI (0.095 and 0.017 min^{-1}). This explains the higher number of unexchanged hydrogens after 20 and 45 min in holo-α-Lac-LW (45 and 33) compared to those of holo-α-Lac standard (36 and 25) and holo-α-Lac-WPI (38 and 23). The increased protection against H/D exchange in holo-α-Lac-LW, compared to holo-α-Lac standard or holo-α-Lac-WPI, can be attributed to fewer structural modifications induced by preparation conditions of α-Lac from LW when compared to preparation conditions of α-Lac from the other sources.

Table 1 shows the number of unexchanged hydrogens after 5, 20, and 45 min and the rates of H/D exchange in glycated holo-α-Lac from WPC (four to six hexose residues). It should be noted that on the basis of the present molecular mass measurements, discrimination between the addition of lactose unit and two hexose residues cannot be achieved. The results show that the numbers of unexchanged hydrogens in holo-α-Lac-WPC increased as the number of hexose residues attached to the protein increased. Also, the exchange rates (k_1 and k_2) of holo-α-Lac-WPC were generally slower, as the numbers of attached sugar moieties increased. Two possible explanations for the observed decrease in the H/D exchange rate in glycated holo-α-Lac-WPC are (i) the hexose residues protect part of the holo-α-Lac protein surface from solvent, thereby delaying exchange [this is evident from the decrease in percentage of very rapidly (C) exchange hydrogens with the increase in number of sugar moieties attached to the protein] and (ii) attachment of four or more hexose residues to the holo-α-Lac protein could result in more stable conformational structure, making local unfolding, and therefore H/D exchange, slower (this is evident from the increase in the percentage of total number of amide backbone hydrogens involved in holo-α-Lac).

H/D Exchange of *β*-Lg. Figure 6 shows the spectra of β-Lg (variants B and A) standard and β-Lg (variants B and A) fractions from LW, WPI, and WPC. The spectrum of β-Lg standard was similar to that of the β-Lg fraction from LW. The β-Lg fraction from WPI was glycated with 2 and 4 hexose sugar units, whereas the β-Lg fraction from WPC was highly glycated with more than 12 hexose units. The high level of glycation in the β-Lg fraction from WPC resulted in complex mass spectra; therefore, H/D exchange experiments were carried out only with β-Lg standard and β-Lg fractions from LW and WPI.

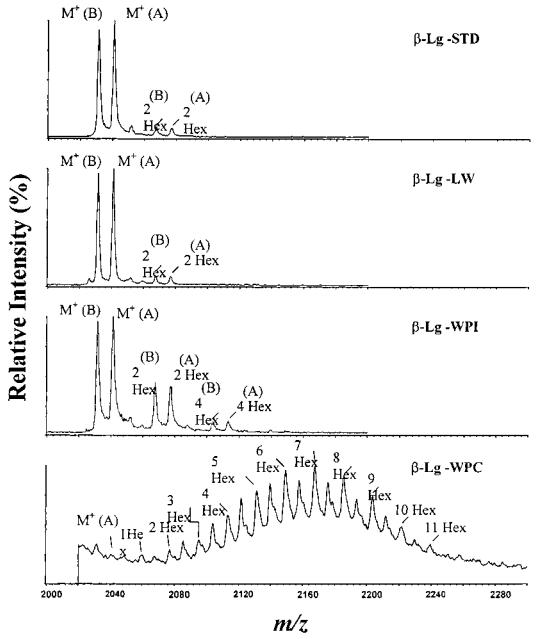

Figure 6. ESI mass spectra of β-lactoglobulin (variants B and A) fractions from whey protein isolate (WPI), whey protein concentrate (WPC), and liquid whey (LW).

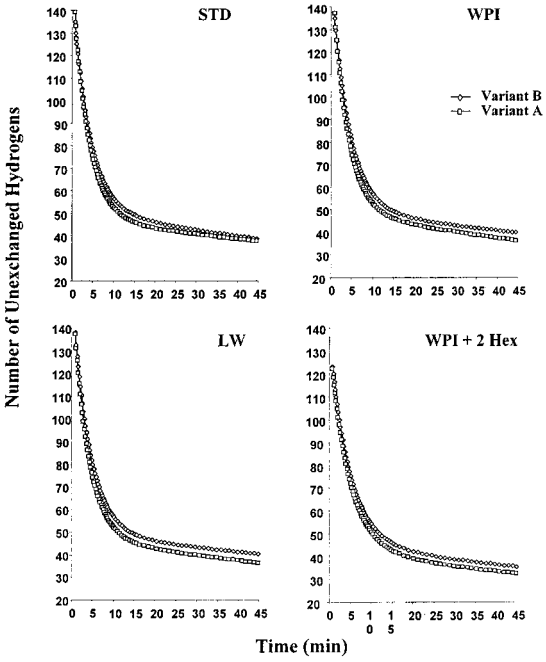

Figure 7. H/D exchange curves of nonglycated β-lactoglobulin (β-Lg) (variants B and A) standard and nonglycated β-Lg (variants B and A) fractions from whey protein isolate (WPI) and liquid whey (LW) and glycated WPI (2 hexose sugar units) at pH 6.8.

Figure 7 shows the H/D exchange curves of nonglycated β-Lg (variants B and A) standard and nonglycated β-Lg (variants B and A) fractions from WPI and LW and glycated (two hexose sugar residues) β-Lg (variants B and A) fraction from WPI at pH 6.8. After 60 s, the total number of unexchanged hydrogens in nonglycated β-Lg variants B and A standard was 140 (56% unexchanged hydrogens). The results in **Table 2** suggest that for variant B (i) 39% of unexchanged hydrogens belong to the intermediate (A) exchanging category, (ii) 21% belong to the slowly (B) exchanging category, and (iii) 40% belong to the very rapidly (C) exchanging category, whereas for variant A (i) 43% unexchanged hydrogens belongs to the intermediate (A)

exchanging category, (ii) 19% belong to the slowly (B) exchanging category, and (iii) 38% belong to the very rapidly (C) exchanging category. The numbers of unexchanged hydrogens in variants B and A after 5 min were 77 (31% unexchanged hydrogens) and 74 (29% unexchanged hydrogens), respectively. After 20 min, the extents of unexchanged hydrogens between variants B and A of β-Lg standard were similar. The rate of exchange in the intermediate category (k_1) of β-Lg (variant A) standard (0.264 min^{-1}) was faster than that of β-Lg (variant B) standard (0.244 min^{-1}). These results indicate that β-Lg variant B standard is more protected against hydrogen exchange than β-Lg variant A standard. These results are consistent with results reported by other workers who showed that β-Lg variant B was more thermally stable than β-Lg variant A (*33*−*35*), and the proteolytic susceptibility of β-Lg variant A was greater than that of β-Lg variant B (*34*).

Figure 7 indicates that β-Lg variant A from WPI (glycated and nonglycated) and LW has fewer unexchanged hydrogens after 5, 20, and 45 min and a faster rate of exchange in the intermediate category (k_1) than variant B, suggesting a more flexible structure. Binding of two hexose sugar residues to β-Lg (variants B and A) from WPI decreased the protection against hydrogen exchange. The number of unexchanged hydrogens after 5, 20, and 45 min in glycated (two hexose sugar residues) β-Lg variant B from WPI was less than that for nonglycated β-Lg (variant B) from WPI (**Table 2**). Glycation of β-Lg variant B from WPI resulted in a decrease in the percentage of intermediate (A) and slowly (B) (36 and 18%) exchanging hydrogens compared to nonglycated β-Lg variant B from WPI (42 and 20%), respectively, and an increase in the percentage of very rapidly exchanging hydrogens (C) in glycated β-Lg variant B (46%) compared to nonglycated β-Lg variant B (38%). Similarly, the binding of two hexose sugar residues to β-Lg variant A from WPI resulted in less protection against hydrogen exchange. One possible explanation for the observed change in exchange behavior is that large conformational change in β-Lg occurred as a result of attachment of two hexose residues, and this could result in accelerated H/D exchange rate. This is evident from the increase in the percentage of very rapidly exchanging hydrogens (C) in glycated β-Lg (variants B and A) from WPI compared to nonglycated β-Lg (variants B and A) from WPI; this increase was more pronounced in glycated β-Lg variant A (**Table 2**). FTIR has revealed that glycation promoted the unfolding of β-Lg via multiple transition pathways leading to a transition state resisting aggregation (*26*). Moreover, Morgan et al. (*36*) showed that glycation led to conformational changes of β-Lg based on fluorescence, susceptibility to pepsin, and immunochemical characterization.

The results demonstrate the capability of CSD of ESI-MS to investigate the conformational stability of α-Lac and β-Lg at neutral pH or lower; however, the information obtained was only qualitative and was of relatively low resolution at basic pH. H/D exchange rates from ESI-MS provided a sensitive measure of conformational stability of the whey proteins. The H/D exchange results demonstrate that the conformation of holo-α-Lac was more stable than that of apo-α-Lac and that the conformation of β-Lg variant B was more stable than that of β-Lg variant A. Kinetics of H/D exchange indicated that α-Lac and β-Lg fractions from whey protein preparations have the same or improved conformational stabilities compared to those of α-Lac and β-Lg standard. The presence of four or more hexose residues in α-Lac enhanced its conformational stability, but the presence of two hexose residues in β-Lg resulted in less stable conformation. These different conformational stabilities of glycated α-Lac and β-Lg could be possibly due to differences in the degree of glycation or different structural effects of glycation on different proteins.

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