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ARTICLE

pH-Stability and Thermal Properties of Microbial Transglutaminase-Treated Whey Protein Isolate

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Whey protein isolate (WPI) was treated to various extents using microbial transglutaminase (MTGase) and changes in pH-stability and thermal stability of its protein components were investigated. The MTGase treatment significantly increased the denaturation temperature (T_d) of β-lactoglobulin in WPI, from 71.84 °C in the untreated sample to 78.50 °C after 30 h of incubation with MTGase. The enthalpy change of denaturation of WPI did not change upon cross-linking, indicating that the increase in T_d was primarily due to covalent cross-linking and not due to an increase in nonpolar interactions within the protein. The surface hydrophobicity (S_o) of the protein decreased upon cross-linking; however, this decrease was not due to burial of the surface hydrophobic cavities in the protein interior, but due to occlusion of the hydrophobic cavities to the fluorescent probes. Fluorescence emission and circular dichroism spectroscopic analyses revealed no major changes in the secondary and tertiary conformations as a result of cross-linking. However, unlike native WPI, the cross-linked protein exhibited a U-shaped pH-stability profile with maximum turbidity at pH 4.0−4.5. The study revealed that even though enzymatic cross-linking significantly improved the T_d of β-lactoglobulin in WPI without causing major structural changes, a reduction in the hydrophilic−hydrophobic balance of the protein surface as a result of elimination of the positive charge on lysyl residues caused precipitation at pH 4.0−4.5.

KEYWORDS: Microbial transglutaminase; whey protein; thermal stability; solubility; cross-linking; hydrolysis

INTRODUCTION

Whey protein is a major coproduct of the cheese industry, and its production from 1999 to 2003 has exceeded 1000 million pounds per year in the United States (*1*). Whey protein is an important food ingredient of high nutritional value and unique functional properties, with applications in food products such as processed meats, bakery products, pasta, ice cream, confectionery, infant foods, spreads, dips, beverages, and dessert items (*2*, *3*). The growing demand for functionally superior and nutritionally excellent novel proteins by the food industry provides potential opportunity for increased utilization of whey proteins in meal replacement products and protein-based beverage applications (*4*, *5*).

However, the functional properties of whey proteins are often impaired by inevitable heat treatments during processing and preservation of whey protein-based food products. Thus, heat sensitivity of whey protein is an important factor that affects the solubility and other functional properties (*2*, *6*). Whey protein thermal denaturation can produce some undesired effects such as deposit formation, which is undesirable in protein beverage-type products but might be desirable in products such as yogurt (*7*). Chemical and physical methods and enzymatic hydrolysis are commonly used to modify whey protein functionality (*8*). More recently, transglutaminase has been used to modify the hydration, gelation, emulsifying, rennetability and heat stability of milk proteins through the cross-linking and deamidation reactions (*9*−*14*).

Transglutaminase (glutaminyl-peptide:amine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme that catalyzes an acyl transfer reaction between γ-carboxyamide of peptide or protein-bound glutamine residue (acyl donor) and a primary amine (acyl acceptor). When an amine group (acyl acceptor) is not present, the enzyme catalyzes hydrolysis of the γ-carboxyamide group of glutaminyl residues, resulting in deamidation (*15*−*17*). The intermolecular cross-links introduced by transglutaminase may improve the functional properties of proteins without compromising their nutritional quality.

However, there is limited published information on the effect of transglutaminase cross-linking on whey protein thermal stability. A few investigations have examined the effect of transglutaminase cross-linking on the heat stability of β-lactoglobulin (*12*, *18*) and other milk proteins (*11*, *13*, *14*). The present study investigated the efficacy of microbial transglutaminase (MTGase) in modifying the thermal and pH-stability properties of whey proteins at neutral and acidic pH commonly employed in beverages applications. The conformational and physicochemical changes of the MTGase-treated WPI were evaluated.

MATERIALS AND METHODS

Materials. Commercial whey protein isolate (WPI) prepared by anion exchange process was obtained from Davisco Foods International, Inc. (Le Sueur, MN). According to the manufacturer, the WPI contained

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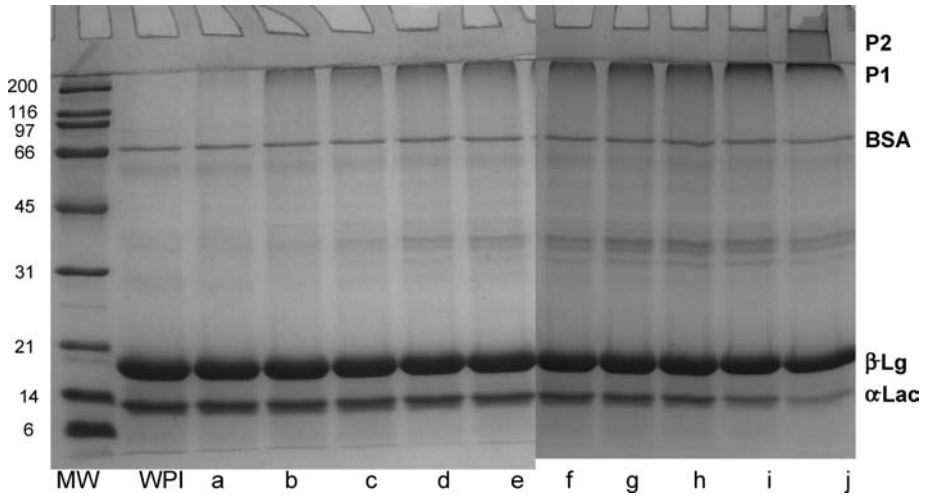

Figure 1. SDS−PAGE patterns of native and MTGase-treated WPI. Lanes: MW = molecular weight standards (in kDa); WPI = native WPI (control); a−j = WPI + MTGase, incubated at 37 °C for 0, 1, 2, 4, 6, 10, 20, 30, 33, and 48 h respectively. α-Lac = α-lactalbumin; β-Lg = β-lactoglobulin; BSA = bovine serum albumin; P1 = polymer 1; and P2 = polymer 2.

>95% protein, 3% moisture, and <1% ash and lactose. Microbial transglutaminase (MTGase) used in this study (Activa-TI, 99% maltodextrin and 1% MTGase) (100 units/g of solid) was donated by Ajinomoto Food Ingredients (Eddyville, IA). The crude enzyme was used without further purification. All chemicals used were at least reagent grade.

WPI Sample Preparation and Transglutaminase Treatment. All reagent solutions were prepared with ultrapure water from the Milli-Q Plus purification system (resistivity = 18.2 MΩ-cm). Protein solutions were prepared by dispersing WPI powder in 10 mM phosphate buffer (pH 7.0), containing 0.002% sodium azide (designated as untreated WPI), and stirring for 2 h at 23 °C. Cross-linking of proteins was performed using MTGase. The MTGase stock solution (50% w/w) contained 5 mM β-mercpatoethanol in order to maintain the active site SH group of the enzyme in the reduced state (*16*). The WPI solution (4.0% protein) was incubated with MTGase (50 units/g of protein substrate) at 37 °C for various times (designated as MTGase-treated WPI). The final concentration of maltodextrin and β-mercpatoethanol in the reaction mixture was 2% and 0.2 mM, respectively. At these low levels, these additives did not affect the thermal properties of WPI. When a specific incubation (reaction) time was reached, the MTGase activity was inhibited by mixing 10 mM NH_4Cl into the reaction solution (*19*).

Differential Scanning Calorimetry (DSC). Changes in thermal stability of untreated and MTGase-treated WPI were measured using a Micro DSC VII (Setaram, Caluire, France). The untreated WPI (4.0% protein) and MTGase-treated WPI (37 °C, 0−48 h) sample solutions were accurately weighed (ca. 620 mg) into preweighed DSC (Hastelloy C276) vessels and securely closed with a stopper. For each run, a DSC vessel containing the buffer for dissolving the protein was used as reference. Samples were heated from 20 to 120 °C at a constant heating rate of 1 °C/min. The denaturation temperature (T_d) and the enthalpy change (ΔH) were determined from the thermogram using the Setsoft software (version 1.40) supplied by the DSC manufacturer. The DSC was calibrated using cyclohexane, phenyl ether, and *o*-terphenyl standards recommended by the DSC manufacturer. Analysis was carried out at least in duplicate for all samples.

Electrophoresis. Changes in the molecular weight of proteins in the untreated and MTGase-treated WPI samples were examined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) according to Laemmli (*20*) with some modifications (*21*). A 12% acrylamide separating gel and a 4% acrylamide stacking gel were used. Protein samples (0.2% protein) were dissolved in an equal-volume sample buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.125 M Tris, pH 6.8) and then heated in boiling water for 3 min. Aliquots of 20 μg of protein per lane were loaded onto the gel. Electrophoresis was run with a Mini-PROTEAN 3 Cell (Bio-Rad Laboratories, Hercules, CA). A cocktail protein standard consisting of proteins of 6.5−200 kDa was also run. Molecular weights (MW) of unknown proteins were estimated from the regression line obtained by plotting the log (MW) versus relative mobility (R_f) of the protein bands.

pH-Stability and Surface Hydrophobicity Measurements. The pH-stability profile of untreated and MTGase-treated WPI were determined according to the procedure of Zhu and Damodaran (*22*) by measuring the turbidity of a 0.1% protein solution at 500 nm at various pHs, namely, pH 2.0−3.5 (with 10 mM phosphate buffer), pH 4.0−5.5 (with 10 mM acetate buffer), and pH 6.0−7.0 (with 10 mM phosphate buffer). The surface hydrophobicity of untreated and MTGase-treated WPI samples was determined by the method of Hayakawa and Nakai (*23*) using ANS (1-anilino-8-naphthalenesulfonate magnesium salt) as the fluorescent probe. The relative fluorescent intensity (RFI) of each sample solution was measured using model LS-5 fluorescence spectrophotometer (Perkin-Elmer Corporation, Norwalk, CT) with excitation (390 nm) and emission (470 nm) slits set at 5 nm. Triplicate samples were measured.

Fluorescence Measurement. The intrinsic fluorescence emission spectra of untreated and MTGase-treated WPI samples were determined according to Tang et al. (*14*) using a model LS-5 fluorescence spectrophotometer (Perkin-Elmer Corporation, Norwalk, CT) with 5 nm emission and excitation slit. The test samples (0.05% protein in 10 mM phosphate buffer, pH 7.0) were excited at 280 nm, and the emission intensities were measured from 300 to 450 nm. Triplicate samples were measured.

Circular Dichroism Measurement. Far and near-ultraviolet (UV) circular dichroism (CD) of native (in 10 mM phosphate buffer, pH 7.0) and MTGase-treated WPI were measured at 25 °C using a Stopped Flow Circular Dichroism spectrometer (model 202SF, Aviv Instruments Inc., Lakewood, NJ). A 0.1 cm path length quartz cell and 0.015% protein solution was used for far-UV CD measurement, and a 1.0 cm path length cell and 0.1% protein solution were used for near-UV CD measurement. Three scans of each sample were averaged, and mean residue ellipticity [θ] values, expressed as deg cm^2 $dmol^{-1}$, were calculated using a value of 115 for the mean residue molecular weight. All the CD spectra were corrected by subtracting the spectrum of a blank containing phosphate buffer (baseline). The secondary structure contents were estimated from the far-UV CD spectra using the CDESTIMA program based on the method of Chang et al. (*24*), and smoothing was done using SM spline.

Statistical Analysis. Experimental design was completely randomized with repeated measures. Data were analyzed using the general linear model procedures of SAS (Statistical Analysis System version 9.1) software (SAS, Cary, NC). Analysis of variance (ANOVA) was performed to determine treatment effect. When significant treatment effects ($P < 0.05$) were found, their means were separated by the Tukey test.

RESULTS AND DISCUSSION

Figure 1 shows the time course of MTGase reaction on the extent of cross-linking of WPI. The SDS−PAGE of untreated WPI under reducing conditions (sample a) showed three main bands corresponding to molecular weights (MW) ~13.8 kDa,

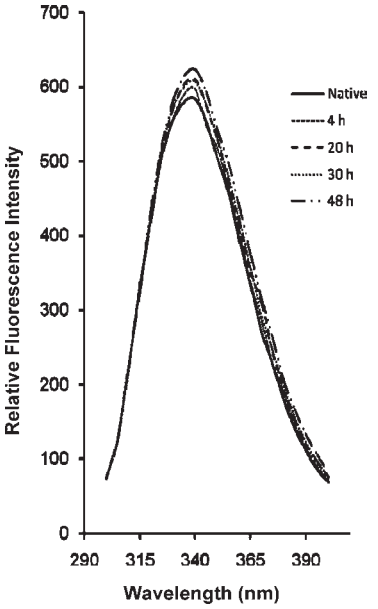

Figure 2. Fluorescence spectra of native and MTGase-treated WPI.

~18.3 kDa, and ~66.6 kDa, attributed to α-la, β-lg, and bovine serum albumin (BSA) monomers, respectively. Following MTGase treatment new protein bands corresponding to MW ~31.6, ~36−40, and > 200 kDa appeared (**Figure 1**, lanes b−j). These new protein species appear to be homo- and/or hetero-dimers and polymers of whey proteins, except for the ~40 kDa band that was assigned to MTGase enzyme (*16*). The two faint bands (~31.6 and 36 kDa) were assigned to α-la/β-lg and β-lg dimers, respectively, because 31.6 kDa is the approximate sum of the MW of α-la monomer (13.8 kDa) plus β-lg monomer (18.3 kDa). The new higher MW band at the top of the resolving gel (> 200 kDa, P1) was whey protein polymers generated by MTGase-catalyzed cross-linking, which is in agreement with previous reports (*19*). The intensity of this high-MW band increased with MTGase reaction time, and after 48 h a part of the polymer (P2) grew in size too large to migrate into the stacking gel. It should be noted that during the time course of the cross-linking reaction, changes in the intensity of the β-lg monomer band was nominal whereas that of α-la decreased rapidly and almost disappeared completely after 48 h. This suggested that α-la was the main substrate in WPI for MTGase, and the high MW polymers were predominantly made up of α-la polymers. However, although polymerization of β-lg appeared to be insignificant, it is quite possible that β-lg might still be involved in intramolecular cross-linking as well as in dimerization reactions.

Conformational Changes. *Fluorescence Emission Spectra.* **Figure 2** shows the intrinsic emission fluorescence spectra of native and MTGase-treated WPI samples. The λ_{max} of fluorescence emission of native WPI was at 340 nm with excitation at 280 nm, which is typical for Trp residues in proteins (*25*). The λ_{max} of fluorescence did not shift significantly after treatment with MTGase, indicating that the dielectric environment of Trp residues in WPI was not altered in any significant way. However, there was a slight increase in fluorescence emission intensity in the case of 30 and 48 h MTGase-treated samples as compared to untreated WPI. This indicated that at longer reaction time some of the Trp chromophores were relocated into a more nonpolar environment. This might be due to polymerization-induced changes in the microenvironment and/or occlusion of Trp residues from the aqueous environment (*14*, *26*).

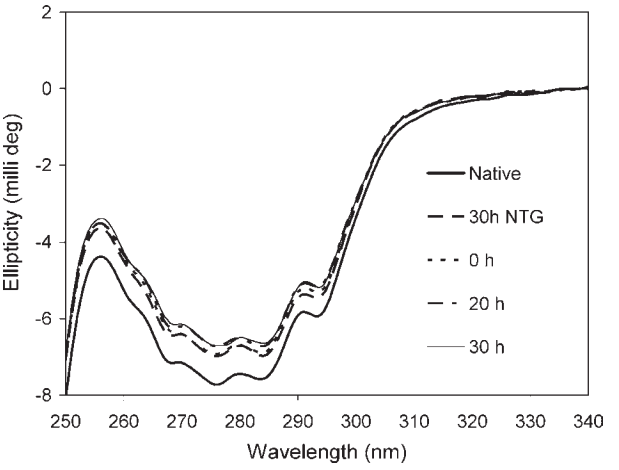

Figure 3. Near-UV circular dichroism spectra of native and MTGase-treated WPI.

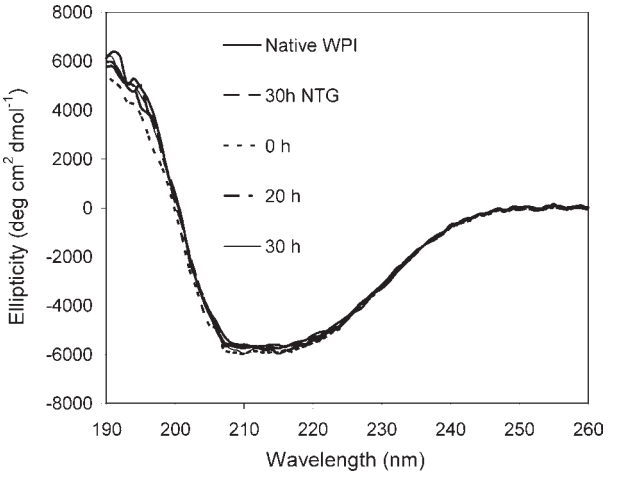

Figure 4. Far-UV circular dichroism spectra of native and MTGase-treated WPI.

CD Spectra. **Figure 3** shows the near-UV CD spectra of untreated and MTGase-treated WPI at 25 °C in 10 mM phosphate buffer (pH 7.0). In proteins, the chromophores of interest in the near-UV absorption range (250−340 nm) include the aromatic amino acid side chains and disulfide bonds (weak broad absorption bands centered around 260 nm). The main feature of the near-UV CD spectrum of the untreated WPI was the strong negative peak at around 276 nm attributed to Tyr residues. In addition, the peaks and shoulders between 284−305 and 255−270 nm suggested fine structure around Trp and Phe residues, respectively (*27*, *28*). The diminution in the peak intensity following MTGase treatment reflected subtle changes in the tertiary structure, which affected the environment of aromatic amino acid (Phe, Tyr, and Trp) side chains (*29*). A decrease in near-UV CD signals in the 276−305 nm range was indicative of burial of Trp residues more into a nonpolar environment. This agreed with changes in the fluorescence emission data (**Figure 2**).

The far-UV CD spectra (190−240 nm) of untreated and MTGase-treated WPI at 25 °C in 10 mM phosphate buffer (pH 7.0) are presented in **Figure 4**. The far-UV CD spectrum of native WPI exhibited characteristic features of β-sheet proteins, displaying a strong positive band in the 190−200 nm region and a negative band in the 207−215 nm region. Analysis of the CD spectrum of untreated WPI using the CDESTIMA program (*24*) gave secondary structure estimates of 12% α-helix, 60% β-sheets,

Table 1. Secondary Structure Estimates (%) of Native and MTGase-Treated Whey Protein Isolate Calculated from Far-UV Circular Dichroism Spectra

sample[a]	α-helix	β-sheet	β-turns	random coil
native WPI (control)	12.0	60.0	3.5	24.5
30 h NTG	11.5	60.5	3.5	24.5
MTGase-treated WPI				
+ MTGase, 0 h	14.0	50.0	8.0	28.0
+ MTGase, 20 h	11.5	60.0	3.5	25.0
+ MTGase, 30 h	11.5	60.5	3.0	25.0

[a] The MTGase enzyme treatment (50 units/g of protein) in 10 mM phosphate buffer (pH 7.0) was performed at 37 °C. Values were means of three replicate measurements at 25 °C. 30 h NTG = native WPI incubated at 37 °C for 30 h (no MTGase).

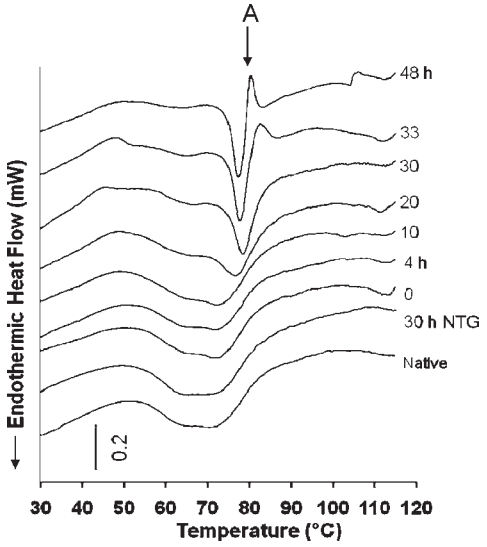

Figure 5. DSC thermograms of native and MTGase-treated WPI. Native WPI = WPI alone (control), 30 h NTG = native WPI incubated at 37 °C for 30 h (no MTGase), 0 h = WPI + MTGase (0 h), 4 h = WPI + MTGase (4 h), 10 h = WPI + MTGase (10 h), 20 h = WPI + MTGase (20 h), 30 h = WPI + MTGase (30 h), 33 h = WPI + MTGase (33 h).

3.5% β-turns, and 24.5% random coil (**Table 1**). This is in agreement with previous reports of bovine β-lg (the major whey protein) being predominantly an antiparallel β-sheet protein (*28*, *30*, *31*). The far-UV-CD spectrum of WPI did not change significantly after the MTGase treatment. The secondary structure estimates did not change significantly even in the 30 h MTGase-treated sample (**Table 1**). Coussons et al. (*29*) also reported insignificant changes in the far-UV CD spectrum of β-lg following transglutaminase incubation for 24 h at 25 °C (pH 5.5 and 7.6).

The results of fluorescence emission and far- and near-UV CD spectroscopic studies indicated that MTGase-catalyzed cross-linking caused only subtle changes in the tertiary structure of the proteins in WPI without major alterations at the secondary structure level.

Differential Scanning Calorimetry. The thermal properties of untreated and MTGase-treated WPI (37 °C, 0−48 h) are shown in **Figure 5**. The DSC thermogram of untreated WPI exhibited a broad endothermic transition with a maximum at 71.8 ± 0.3 °C and a shoulder at ∼64 °C, corresponding to denaturation temperature (T_d) of β-lg and α-la, respectively. These denaturation temperatures are in good agreement with 71.9 and 64.3 °C, respectively, previously reported by Boye and Alli (*32*). It should be noted that the T_d of β-lg and α-la in WPI that had been incubated at 37 °C for 30 h with no MTGase (labeled as 30 h NTG) was essentially the same as that of untreated WPI (**Table 2**).

Table 2. Enthalpy of Denaturation and Thermal Denaturation Temperature of Untreated and MTGase-treated Whey Protein Isolate[a]

sample[a]	T_d (°C)	ΔH (J/g)
native WPI (control)	71.84 ± 0.31^{g}	9.61 ± 0.28^{b}
30 h NTG	$71.76 + 0.16^{g}$	$11.06 + 0.08^{a}$
MTGase-treated WPI		
+ MTGase, 0 h	72.12 ± 0.14^{fg}	9.54 ± 1.08^{b}
+ MTGase, 4 h	72.43 ± 0.16^{ef}	9.44 ± 0.04^{b}
+ MTGase, 6 h	72.55 ± 0.36^{ef}	9.32 ± 0.34^{b}
+ MTGase, 10 h	72.78 ± 0.11^{e}	9.43 ± 0.02^{b}
+ MTGase, 20 h	75.95 ± 0.11^{d}	9.43 ± 0.25^{b}
+ MTGase, 24 h	76.89 ± 0.36^{c}	9.63 ± 0.29^{b}
+ MTGase, 30 h	78.50 ± 0.21^{a}	8.77 ± 0.41^{b}
+ MTGase, 33 h	77.90 ± 0.32^{b}	7.55 ± 0.43^{c}
+ MTGase, 48 h	77.32 ± 0.03^{c}	7.36 ± 0.17^{c}

[a] The MTGase enzyme treatment (50 units/g of protein) in 10 mM phosphate buffer (pH 7.0) was performed at 37 °C for 0−48 h. Values were expressed as mean ± standard error of three replicate measurements (For details, see Materials and Methods). 30 h NTG = native WPI incubated at 37 °C for 30 h (no MTGase). T_d = peak maximum temperature (°C); ΔH = enthalpy (J/g); pH = sample pH before DSC analysis. [a−g]Means in the same column without a common letter differ significantly ($P < 0.05$).

Following MTGase treatment, the T_d of β-lg increased moderately during 0−10 h incubation with MTGase. Thereafter, the T_d increased greatly and reached 78.5 ± 0.2 °C after 30 h incubation with MTGase (**Figure 5**, **Table 2**). This represented a significant ($P < 0.05$) net increase in T_d of 6.7 °C. In contrast, there was no significant change ($P < 0.05$) in the enthalpy of denaturation (ΔH) up to 30 h incubation with MTGase. However, incubation with MTGase for more than 30 h caused an appreciable decrease in ΔH and a marginal decrease in T_d (**Table 2**). It should be noted that although there was no significant difference between the T_d of untreated WPI and WPI incubated for 30 h with no MTGase (labeled "30 h NTG"), the ΔH of the latter was significantly higher than the former as well as those of the MTGase-treated samples (**Table 2**).

The enthalpy change associated with protein denaturation primarily reflects the energy needed to disrupt noncovalent interactions to cause the transformation of the protein from the native state to a denatured state. Previously, Privalov and Khechinashvili (*33*) reported that for several globular proteins solution conditions (such as pH) that caused an increase in T_d also caused a linear increase in ΔH up to ∼100 °C, and they showed that this linear temperature-dependence of enthalpy change was not related to a net change in the number of hydrogen bonds, but mainly related to the number of contacts between nonpolar groups within a protein: the higher the number of nonpolar contacts, the greater was $d\Delta H/dT_d$.

The above dictum, that is, the ΔH is linearly and positively dependent on T_d, did not seem to be applicable to untreated WPI and MTGase-treated WPI. For instance, comparison of the thermal properties of untreated WPI and the "30 h NTG" WPI indicated that, even though the T_d of these samples were almost the same, the ΔH of the former (9.61 J/g) was significantly ($P < 0.05$) lower than that of the latter (11.06 J/g). This suggested that whey proteins did undergo subtle conformational changes during 30 h incubation at 37 °C and those conformational changes apparently increased only the number of hydrogen bonding interactions but not the number of contacts between intramolecular nonpolar groups. Since breakdown of hydrogen bonds is a cooperative process, which occurs within a narrow range of temperature, the T_d remained mostly unaffected by the increased number of hydrogen bonds; however, the ΔH, which is

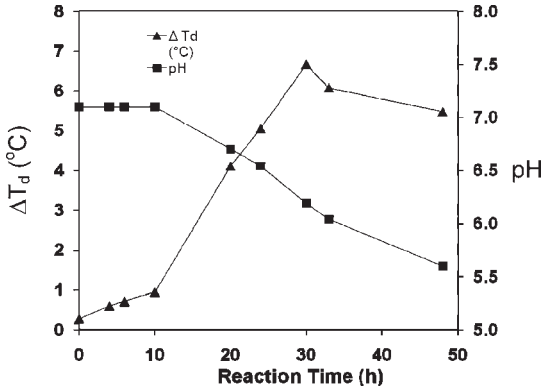

Figure 6. Changes in denaturation temperature (ΔT_d) and pH (before DSC heating) of MTGase-treated WPI, as a function of MTGase reaction time.

related to the heat energy needed to break bonds, increased (**Table 2**).

On the other hand, in the case of MTGase-treated samples, the T_d increased from 71.8 to 78.5 °C as the cross-linking reaction time was increased from 0 to 30 h; however, the ΔH did not change significantly ($P < 0.05$) during that period. In this case, the rise in T_d without a rise in ΔH cannot be attributed to an increase in intramolecular nonpolar interactions because that should cause an increase in ΔH as well. Therefore, it is reasonable to suppose that the increase in T_d might be mainly due to cross-linking.

It was noted during the course of incubation with MTGase that the pH of the samples decreased from the initial value of 7 to a final value of 6.2 after 30 h incubation, while the T_d increased to a maximum value during that period (**Figure 6**). The decrease in pH with reaction time was essentially due to elimination of the positive charge on the ε-amino group of lysyl residues as a result of cross-linking with glutamine residues. This caused release of protons from $-NH_3^+$ groups, resulting in the decrease of pH. It is probable that the increase in T_d might be due partly to the drop in pH of the solution. Previously, Park and Lund (*34*) had reported that the T_d of β-lg increased by 1.5 °C when the pH was changed from 7.0 to 6.0. More recently, Baeza and Pilosaf (*35*) reported that the ΔT_d of β-lg at pH 6 versus pH 7 was about 3 °C. We found that the T_d of β-lg in our untreated WPI sample at pH 6.2, the pH at which the MTGase-treated sample had the highest T_d (**Figure 6**), was 74.5 °C. This value was about 2.5 °C higher than the T_d of β-lg in the untreated WPI at pH 7.0, but was about 4.2 °C lower than the T_d of β-lg in the 30 h MTGase-treated sample at pH 6.2. Thus, of the total ΔT_d of 6.7 °C of β-lg in the 30 h MTGase-treated sample at pH 6.2, about 4.2 °C ought to be attributed to MTGase-catalyzed covalent cross-linking and the remaining 2.5 °C was due to the pH effect.

It should be pointed out that the endothermic peak that shifts from 71.8 to 78.5 °C (**Figure 5**, 30 h sample) belongs to β-lg. Yet, the SDS−PAGE profile (**Figure 1**) showed that the majority of β-lg in the 30 h MTGase-treated WPI was not polymerized but remained as monomers. Therefore, the shift in the T_d of β-lg monomer must arise from intramolecular cross-linking in β-lg.

At longer reaction times, that is, 33 and 48 h, both T_d and ΔH of β-lg decreased (**Table 2**). The decrease in ΔH might be attributed to protein aggregation reactions that gave rise to the exothermic peak (marked as "A" at 83 °C) in the DSC thermogram. It should be noted that even though all the other samples were also fully denatured above their respective T_d, the exothermic peak occurred only in the MTGase-treated samples

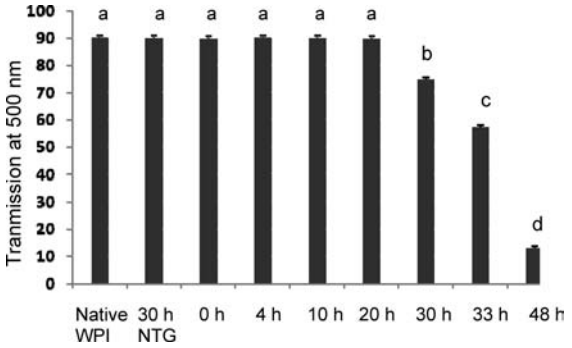

Figure 7. Transmittance at 500 nm of native and MTGase-treated WPI solutions measured after heating to 120 °C in the DSC vessel. Values are means of three replicates. [a−c]Means of verticals bars without a common letter differ significantly ($P < 0.05$).

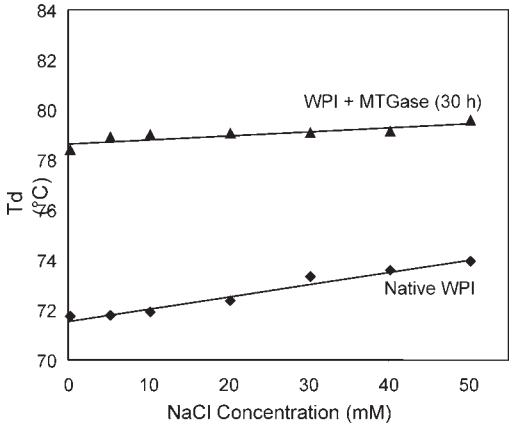

Figure 8. Effect of added NaCl on the denaturation temperature of native and MTGase-treated (30 h incubation) WPI.

incubated for ≥33 h (**Figure 5**). Incidentally, the pH of the MTGase-treated samples incubated for ≥33 h was below 6 and reached a value of 5.6 after 48 h (**Figure 6**). Taken together, the data in **Figures 5** and **6** suggested that heat-induced aggregation of whey proteins, which caused exothermic heat flow at 83 °C (**Figure 5**), occurred only when the pH of the MTGase-treated sample was ≤6.0. To confirm if aggregation did take place in the 33 and 48 h MTGase-treated samples during the DSC run, protein solution was removed from the DSC vessel after each run and the turbidity at 500 nm was measured. The untreated and 0−20 h MTGase-treated WPI samples exhibited 90% transmittance even after the DSC run up to 120 °C, but the transmittance of the 33 and 48 h samples was only about 57% and 13%, respectively (**Figure 7**), confirming exothermic aggregation of denatured WPI.

Figure 8 shows the effect of NaCl concentration (5−50 mM) on thermal properties of untreated and MTGase-treated (30 h) WPI samples. The NaCl concentration range represents the typical range used in most protein beverage-type products. The ΔT_d increased linearly with NaCl concentration for both untreated and MTGase-treated (30 h) WPI. At 50 mM NaCl, the ΔT_d for the untreated WPI was about 2.2 °C, whereas it was about 1.2 °C for the MTGase-treated (30 h) WPI (**Figure 8**). These results indicated that a combination of 30 h incubation with MTGase and addition of 50 mM NaCl would increase the T_d of WPI from 71.9 to 79.7 °C, which was about 9 °C above the typical pasteurization temperature, but about 3 °C below the hot filling/packing temperature used in the manufacture of protein beverages. The onset temperature of denaturation for the 30 h

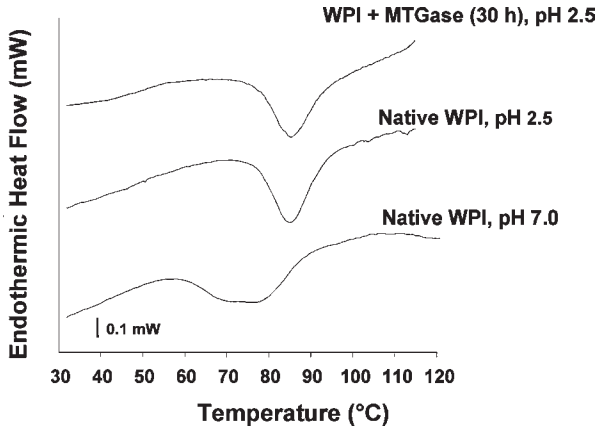

Figure 9. Influence of acidic pH (pH 2.5) on the denaturation temperature of native and MTGase-treated (30 h incubation) WPI.

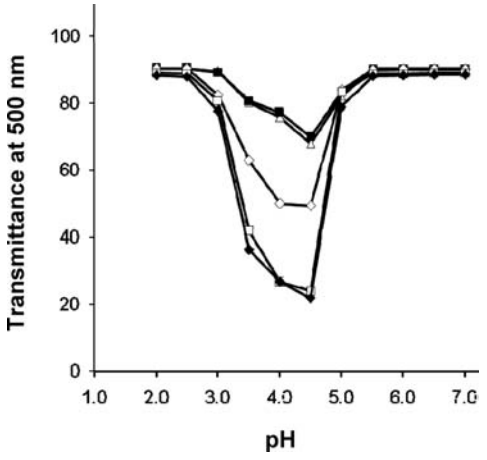

Figure 10. pH-Stability profile of untreated and MTGase-treated WPI. Δ, Native WPI; ■, 30 h NTG; ◇, 0 h sample; □, 20 h sample; ♦, 30 h sample (NTG = sample incubated at 37 °C for 30 h without transglutaminase).

MTGase-treated sample in the presence of 50 mM NaCl was about 72 °C. Since this onset temperature is about 1 °C higher than the pasteurization temperature, it should also minimize the extent of denaturation of β-lg.

Whey proteins, especially β-lg, are known to be highly heat stable in acidic pH (*36*). To determine if treatment of WPI with MTGase further enhanced its heat stability at acid pH, WPI was first incubated with MTGase for 30 h at pH 7.0, and then its thermal denaturation temperature was determined at pH 2.5 (**Figure 9**). The T_d and ΔH of untreated WPI at pH 2.5 was found to be 85.29 °C and 9.78 J/g, respectively, whereas that of the 30 h MTGase-treated sample was 85.77 °C and 7.2 J/g, respectively. It should be noted that although the T_d of untreated WPI increased dramatically at pH 2.5 compared to that at pH 7.0, the ΔH at both pH 7.0 and 2.5 was almost the same (**Table 2**). In the case of the 30 h MTGase-treated WPI, the ΔH decreased from 8.77 J/g at pH 6.2 (**Table 2**) to 7.2 J/g at pH 2.5. These differences in the enthalpy of denaturation suggest that although both the untreated and the MTGase-treated samples undergo thermal transition at about the same temperature, their initial and final thermodynamic states at pH 2.5 were not exactly the same, probably as a consequence of cross-linking.

pH-Stability. pH-stability is an important parameter for applications in protein beverages. Therefore, the effect of MTGase treatment on the pH-stability of WPI was evaluated

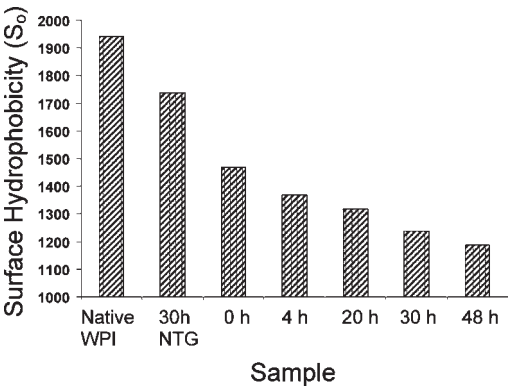

Figure 11. Surface hydrophobicity (S_o) of native and MTGase-treated WPI samples. Error bars represent standard error (n = 6). $^{a-c}$Means of vertical bars without a common letter differ significantly ($P < 0.05$).

in the pH range 2.0−7.0 (**Figure 10**). The untreated WPI exhibited very good stability in the pH range 2.0−7.0. This is attributable to the hydrophilic nature of water-accessible surfaces of native α-la and β-lg (*22*). However, after MTGase treatment (20 and 30 h) the pH-stability profile of WPI decreased, showing a pronounced minimum at about pH 4.5. The pH-stability experiment was not conducted on the 48 h MTGase-treated sample because it was visibly turbid.

The change in the pH-stability profile of MTGase-treated WPI compared to the untreated WPI ought to be related to a change in the hydrophilicity−hydrophobicity balance of the protein surface that came into contact with the surrounding solvent. In light of the fact that the fluorescence and CD spectroscopic data (**Figures 2**−**4**) did not suggest major structural changes in WPI, partial elimination of positively charged lysine residues as a result of cross-linking could be the reason for a decrease in the hydrophilicity−hydrophobicity balance of the protein surface. This might de facto promote hydrophobic interaction between protein molecules as the pH was lowered toward the pK (4.6) of the carboxyl groups.

Surface Hydrophobicity. **Figure 11** shows changes in surface hydrophobicity (S_o) of WPI as a function of MTGase reaction time. The relative S_o decreased with increase of MTGase reaction time. For instance, the S_o of native WPI was 1652 ± 11.9 but decreased significantly ($P < 0.05$) to 1278.6 ± 23.1 after 30 h incubation with MTGase. The decrease in S_o suggested partial burial or occlusion of hydrophobic cavities or clefts on the protein surface. However, in spite of the decrease in S_o with MTGase treatment, there was an increase in hydrophobic character of the protein surface following MTGase cross-linking as indicated by the pH-stability profile (**Figure 10**). The apparent contradiction may be reconciled by recognizing that these two techniques probe different aspects of the protein surface (*22*). It has been shown that binding of fluorescent probes, such as ANS, to proteins occurs only at well-defined hydrophobic cavities on the protein surface (*37*). These cavities are not accessible to water and do not contribute to the solubility characteristics of the protein, but are accessible to nonpolar ligands. The existence of a hydrophobic cavity (calyx) in β-lg and a hydrophobic box structure in bovine α-la are well-known. These may also act as the binding sites for fluorescent probes. Thus, the data in **Figures 10** and **11** indicate that while surface hydrophobic cavities of whey proteins were partially occluded by MTGase cross-linking, causing an apparent decrease in S_o, the hydrophobic character of the protein was nevertheless increased as a result of the elimination of positively charged lysyl residues.

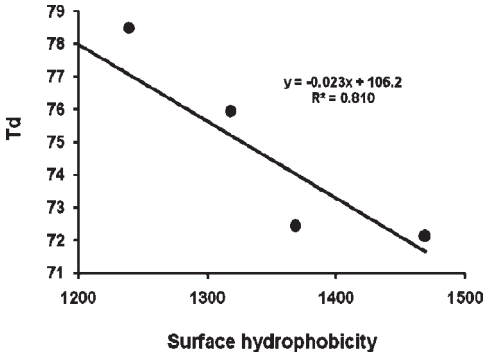

Figure 12. Relationship between thermal denaturation temperature (T_d) and surface hydrophobicity of MTGase-treated samples.

Previously, Privalov and Khechinashvili (*33*) had shown that structural changes that cause increased exposure of hydrophobic surfaces to the aqueous environment decreased thermal stability; conversely, those changes that buried hydrophobic surfaces in the interior promoted thermal stability. If this is true, then one would expect a negative correlation between T_d and S_o of MTGase-treated WPI. This is shown in **Figure 12**. In accordance with the theory, the increase in T_d was accompanied by a decrease in S_o.

The results of this study revealed that although MTGase-catalyzed cross-linking significantly increased the thermal stability of the protein without involving major structural changes in whey proteins, it altered the pH-stability profile of whey proteins with minimum solubility in the pH range 4.0−4.5. This was mainly due to elimination of the positive charge on lysyl residues, which altered the hydrophilic−hydrophobic balance of the protein surface, resulting in precipitation at pH 4.0−4.5. This study revealed that MTGase-catalyzed cross-linking cannot be used to improve the stability of WPI in acidic protein beverage applications.

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