Effect of Transglutaminase Addition on the Physicochemical Properties of Sodium Caseinate and Whey Proteins

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Abstract

In this study, several factors were analyzed in an effort to determine the effects of transglutaminase (TGase) treatment on sodium caseinate (NaCN), α -lactalbumin (α -La), and β -lactoglobulin (β -Lg) polymerization reactions. The results of SDS-PAGE showed that NaCN was slightly hydrolyzed to molecular weights of 50-400 kDa according to activation time. α -La formed high-molecular polymers of 30-300 kDa, whereas β -Lg remained almost completely unhydrolyzed. Melting temperatures of NaCN, α -La with and without TGase were all in the range of $100 \pm 10^\circ\text{C}$ under the endothermic curve, and the melting temperature of β -Lg with TGase was lower than that with TGase. When the proteins were incubated for 3 h with TGase, the micrographic structures showed a small quantity of sediment and broad layers. The final α -La residues remained at a level of 21.38%, and the TGase-treated α -La was confirmed to have undergone a profound loss of mass, to 18.25%. The DPPH-radical scavenging activity of NaCN and β -Lg with TGase treatment was higher than that observed in the untreated sample, while those of α -La increased with concentration.

Key words : milk proteins, transglutaminase, polymerization, field-emission scanning electron microscopy, 1,1-diphenyl-2-picrylhydrazyl-radical scavenging activity

Introduction

Food industries worldwide are currently attempting to develop new ingredients or dairy products with beneficial physical and functional characteristics. Several of the physico-chemical properties of milk proteins are fundamentally modified by the action of TGase, which may have applications in the manufacture of functional proteins for use as novel food ingredients (O'Sullivan *et al.*, 2002). Transglutaminase (TGase, protein-glutamine γ -glutamyl transferase, EC 2.3.2.13) is an enzyme naturally present in most animal tissues and body fluids; it performs a crucial role in blood clot formation. With the recent discovery that this enzyme can be produced from a microbial source (Ando *et al.*, 1989), its applications in the food industry are currently being increasingly investigated (Moon and Hong, 2003; Hwang *et al.*, 2008; Lee and Chin, 2009).

Transglutaminase catalyzes an acyl transfer reaction

between γ -carboxyamide groups of peptide-bond glutamine residues (acyl donor) and the primary amino groups in a variety of amine compounds (acyl acceptor), including peptide-bond ϵ -amino groups of lysine residues. As a consequence of the crosslinking of peptide-bond glutamine and lysine residues, ϵ -(γ -glutamyl)lysine isopeptide bonds and high-molecular weight polymers are formed. In the absence of amine substrates, TGase is capable of catalyzing the deamidation of glutamine residues (Motoki *et al.*, 1986).

Numerous studies have been conducted regarding the effect of TGase on caseins (Ikura *et al.*, 1980; Traore and Meunier, 1991; Sakamoto *et al.*, 1994) and whey proteins (Ikura *et al.*, 1980; Mahmoud and Savello, 1992; Faergemand *et al.*, 1997). Caseins and soya proteins, in which methionine and lysine (respectively) are limiting factors, may be enhanced by such a TGase reaction (Nonaka *et al.*, 1996). TGase treatment applied to sodium caseinate (NaCN) and whey protein isolate (WPI) improves solubility at pH values of 2 and 4; this effect has been attributed to the cross-linking of the individual caseins and whey proteins. The heat stability of NaCN remained nearly unchanged after TGase treatment. After crosslinking by TGase, whey protein evidenced enhanced

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heat stability as compared to the native protein at temperatures in excess of 70°C. Additionally, in the SDS-PAGE results, the main bands of the sample evidenced high mobility and formed bands of molecular weights below 15 kDa after hydrolysis with pepsin followed by TGase-treatment within 10 min of reaction time (Jeong and Hong, 2005; Jeong and Hong, 2006). The cross-linking was conducted using a TGase preparation containing glutathione in order to enhance the reactivity of TGase in unheated or pasteurized milk, allowing for the economic application of the enzyme in the manufacture of cheese (Bonisch *et al.*, 2008). With regard to the range of TGase concentrations used for the assessment of the physical, chemical, and sensory properties of non-fat yogurt, a TGase level of 0.3 gL⁻¹ was considered optimal (Oze *et al.*, 2007). Although the above reports provide us with a good understanding of the technological potential of the TGase reaction in dairy products, information regarding the relative susceptibility of individual milk proteins is currently lacking. Additionally, most of the studies have used indirect means of measuring the crosslinking reactions, and thus no distinction has yet been made between the crosslinking and other TGase-catalyzed reactions.

Understanding the reaction of TGase with caseins and whey proteins is critical to the optimization of the application of TGase in dairy products. In the current study, polymerization reactions, hydrolysis, thermal properties, and FE-SEM microphotographs, as well as [1,1-diphenyl-2-picrylhydrazyl] (DPPH)-radical scavenging activity were employed to characterize the effects of the addition of TGase on the physicochemical properties of NaCN, α -lactalbumin (α -La) and β -lactoglobulin (β -Lg). By focusing the study on the entirety of the functional additives, novel insights can be acquired into the interaction between TGase cross-linking and NaCN, α -La, and β -Lg.

Materials and Methods

Raw materials

NaCN (protein content 94.1%, fat content max. 1.4%) was obtained from Lactoport Deutschland GmbH (Kaltenkirchen, Germany). α -La and β -Lg were acquired from DAVISCO (Minnesota, USA). Samples of NaCN, α -La, and β -Lg were placed in a cold room (-20 ± 1°C).

A Ca²⁺-independent MTGase from *Streptovorticillium mobaraense* was utilized. The enzyme was kindly provided by Ajinomoto Foods (Tokyo, Japan). The microbial TGase preparation Activa®-WM (E.C. 2.3.2.13) has been used previously for the modification of milk proteins.

According to the product specifications, this enzyme preparation is composed of TGase (1%), lactose, and maltodextrin, with a specific enzyme activity of 100 units (U) per g powder. DPPH, dimethyl sulfoxide (DMSO), ethylenediamine-tetraacetic acid (EDTA), and *N*-ethylmaleimide (NEM) were purchased from Sigma Chemicals (St. Louis, MO, USA). Standard molecular markers were obtained from BioRad Laboratories (Richmond, CA, USA). All other reagents were of analytical grade, unless otherwise noted.

Sample preparation

Different milk proteins (5%, w/w) in 0.05 M Tris-HCl buffer (pH 7.0) were carefully added to TGase (1%, v/w) and after 3 h of incubation at 40°C, and the enzyme was inactivated by 5 min of heat treatment at 80°C or NEM. Prior to the addition of the enzyme, the α -La and β -Lg were heated in a temperature range around 37°C for 1 h with 5 mM EDTA, according to the methods developed by Sharma *et al.* (2002). All samples were rapidly cooled to room temperature, lyophilized, and subsequently analyzed.

Enzymatic hydrolysis *in vitro*

The hydrolysis experiments of samples with TGase treatment were adapted from the work of Spellman *et al.* (2003). 5 mL of a 100 mL⁻¹ aqueous solution of proteins was hydrated for 1 h at room temperature with gentle mixing. The protein solution was then pH adjusted to 7.0 with 2.0 N NaOH followed by the addition of trypsin (0.005%), and the mixture was incubated at 40°C for 0, 5, 10, 30, 60, 120, 180, 240 or 300 min. The enzyme was subsequently inactivated by 5 min of heat treatment at 80°C.

To assess their hydrolytic properties, the hydrolysates were analyzed via SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) under the reducing conditions described by Laemmli (1970).

Determination of the degeneration temperature and thermal properities

The thermal denaturation of NaCN, α -La, and β -Lg were monitored using a differential scanning calorimeter (DSC) model 2929 (TA Instrument, USA) with a scan rate of 3°C min⁻¹. DSC was utilized to assess the energy of heat degeneration and kinetics figures at various protein concentrations, pH values, and ion compositions. After the dissolution of each sample into 0.2 M phosphoric acid buffer solution (pH 6.8), each sample was injected into a hermetic pan and sealed with a sample encapsulating press, after which the DSC thermograms

were obtained via heat analysis curves with heating at 3°C/min in a range of 20-180°C. The mass of each sample was evaluated using a thermogravimetric analyzer (TGA-50) system (Shimadzu, Japan). The TGA system was used to measure the mass change of the sample as a function of time or temperature; using the measurement results, the pyrolysis curve of the sample which had already been polymerized could be measured. By measuring the pyrolysis curve, the polymerization and resolution could be compared. When the sample was resolved, the heated velocity was 10°C/min, and the range of temperature was 50-800°C, under a current of nitrogen gas. The control group was used under identical circumstances, measuring only the phosphoric acid buffer solution.

Scanning electron microscopy

Acetone was added to the non-conduction sample to assess the histological polymerization of NaCN, α -La, and β -Lg in TGase-treated samples using field-emission scanning electron microscopy (FE-SEM). A carbon covering was applied three times, and became conductive. The image was then observed using an FE-SEM (S-4700, Hitachi, Japan) in fixed state at a magnification of 600, a phototime of 85 sec, and an acceleration voltage of 15 or 20 kV.

Measurement of antioxidative activity

The antioxidative activity of the hydrolyzed samples was evaluated by the method of Blois (1958). The rate was spectrophotometrically measured (U-2800A, Hitachi, Japan) via the addition of 4×10^{-4} M DPPH into each of the hydrolyzed solutions, all of which were subsequently dissolved into methanol or DMSO at constant concentrations for 30 sec and assessed for extinction rate at 517 nm. The control group was treated with methanol or DMSO instead of samples, and measured in the same fashion. The sample group evidenced reduced electron-donation ability, and the control group was used for data measurement via the addition of each sample. The amount of sample required to reduce the extinction rate by 50% was determined and expressed in terms of EC_{50} (mg/mL), and the measurements were based on the electron donating ability (EDA) of each sample. An experiment using vitamin C as a positive control group was conducted in the same fashion, and the reduction ability of the TG-added and non-TG-added samples was compared. The electron donation ability of the samples were measured by the following equation:

Fig. 1. SDS-PAGE pattern of NaCN, α -La and β -Lg with and without TGase for 3 h of incubation. Lane 1: Molecular weight standard, lane 2: NaCN, lane 3: NaCN with TGase, lane 4: α -La, lane 5: α -La with TGase, lane 6: β -Lg, lane 7: β -Lg with TGase.

$$\text{EDA (\%)} = (1 - A/B) \times 100$$

Where A = Absorption of sample at 525 nm

B = Absorption of control at 525 nm

Results and Discussion

Polymerization of NaCN, α -La and β -Lg

After the reaction with TGase at a rate of 1:100 (Enzyme: Substates), the results from the observation of the polymerization phase and protein fraction changes are shown in Fig. 1.

In the case of NaCN fractions, α_{s1} -, α_{s2} -, β -, and κ -casein levels were all reduced after the polymerization reaction, the range of polymers produced by TGase was between 50-300 kDa, and generated the greatest quantity of milk protein. Sharma *et al.* (2002) previously reported that the α_{s1} - and β -caseins, in particular, appear to be readily cross-linkable, owing to their flexible, random-coil structures and their lack of any disulphide bonds. In SDS-PAGE analyses, more pronounced protein cross-linking was noted under neutral than acidic conditions. It was assumed that, owing to the activity of TGase, electrostatic repulsion was partially negated on the surfaces of the sodium caseinate particles, thereby allowing, for example, hydrophobic interactions between the particles and gel formation (Partanen *et al.*, 2008).

Our analysis with α -La confirmed that the original protein fraction was greatly reduced and resulted in a significant amount of polymer polymerization, and most importantly,

Fig. 2. SDS-PAGE pattern of peptides produced by trypsin hydrolysis of NaCN (A), α -La (B), and β -Lg (C) with TGase. Conditions in terms of reaction time (lane 1: Molecular weight standard, lane 2: NaCN with TGase, lane 3: 5 min, lane 4: 10 min, lane 5: 30 min, lane 6: 60 min, lane 7: 120 min, lane 8: 180 min, lane 9: 240 min, lane 10: 300 min).

confirmed new fractions at approximately 25 kDa and 30 kDa. Transformation by TGase to the apo form of α -La in the molten state was studied under each different circumstance (Gu *et al.*, 2001; Lee *et al.*, 2002; Nieuwenhuisen *et al.*, 2003).

β -Lg yielded a relatively small quantity of polymer as compared to other protein polymers despite three hours' reaction with TGase, in a range between 75 kDa and 250 kDa (Fig. 1). The degree of milk protein crosslinking generated by TGase increased in the following order: NaCN > α -La > β -Lg. β -Lg was unable to cross-link without any reduction in its disulphide bonds (Sharma *et al.*, 2002).

Effect to hydrolysis of cross-linked NaCN, α -La and β -Lg

Fig. 2 shows the hydrolysis behavior of the trypsin treatment applied to the polymerized samples by TGase. As reaction time increased by 5, 10, and 30 min, polymerization in the range of 50-300 kDa was resolved by trypsin, but it remained unclear after 60 min. The low-polymerized fraction increased in the range of 10-15 kDa. Additionally, α s₁-casein was eliminated by 60 min of elapsed time from the beginning of the reaction. After that time, peptides above 15 kDa were resolved and lower-weight molecules were also resolved (Fig. 2A).

α -La was formed as a high-weight molecule by TGase, and appeared on the wells and stacking gel, additionally, a wide variety of polymers in the range of 30-300 kDa were formed. α -La was also hydrolyzed and reduced after two hours. In addition, the new 25 kDa protein band made by TGase was slowly hydrolyzed, and was completely eliminated after one hour, as a single band formed. α -La was hydrolyzed into divided peptides after 30 min, and gradually resolved (Fig. 2B).

Owing to their compact globular structure, whey proteins tend to cross-link less efficiently (Aboumahmoud and Savello, 1990). In a recent study regarding the cross-linking of whey proteins from an industrial whey protein isolate (WPI) and a β -Lg preparation, Faergemand *et al.*, (1997) reported that β -Lg was unable to cross-link without any reduction in its disulphide bonds, whereas α -La could be cross-linked without reduction.

β -Lg does not interact due to its extraordinary protein structure, as has also been reported in the study of Adams *et al.*, (1996); thus, scarcely any effects of TGase were noted in that case. As the hydrolysis reaction was initiated, some of the protein was hydrolyzed to peptides of less than 15 kDa and resolved after 30-60 min. Some quantity of β -Lg re-

Fig. 3. DSC thermograms of NaCN, α -La, and β -Lg with and without TGase heated from 25 to 100°C. Heating rate was 3°C/min under nitrogen gas. Solid lines designate spectra of native and bold lines designate the spectra of TGase treatment groups.

mained after five hours of the final reaction (Fig. 2C).

Degeneration of protein and thermolysis properties

DSC was conducted by heat analysis to evaluate changes in the polymerization states of NaCN, α -La, and β -Lg among the milk proteins. The results are provided in Fig. 3. As the experiment was initiated at 25°C, the curves of all samples evidenced bending. In this region, heating circumstances changed dramatically from a constant temperature state of stand-by to a linear increase mode, and this phenomenon was attributed to an abundance of electronic current. NaCN in an unpolymerized state was degenerated at a temperature near 115°C, and an endothermic peak was detected. In the case of NaCN in polymerization state, a maximum endothermic peak was measured at a temperature of near 90°C, which is lower than that of the former, and the amount was similar. This observed discrepancy in peak temperature may be ascribable to solvent-evaporation, cross-linking, and polymerization. The α -La was degenerated, and evidenced no relation with polymerization at temperatures below 100°C. The degree of denaturation of α -La without TGase was rela-

Fig. 4. TGA thermograms of NaCN, α -La, and β -Lg with and without TGase heated from 50 to 800°C. The heating rate was 10°C/min under nitrogen gas. Solid lines designate the spectra of the native sample, and the Bold lines designate the spectra of TGase treatment. Marking with ♦ indicates a continuous spot on samples' weight loss.

tively higher than in the other samples. The temperature at which β -Lg evidenced maximum heat absorption enthalpy was near 90°C from the original state; it was 120°C in polymerized form, and the degeneration energy absorption degree was lowered substantially after polymerization. Therefore, the degeneration temperature according to the first heat absorption curve (to 200°C) was $100 \pm 10^\circ\text{C}$ at all samples, and the degree of degeneration was similar to or lower than that associated with TGase treatment. In the case of β -Lg, the largest observed difference ($100 \pm 20^\circ\text{C}$) was shown to depend on with or without TGase. According to the report of Boye and Alli (2000), the endothermic reaction of α -La and β -Lg was denatured significantly at ~ 62 and $\sim 75^\circ\text{C}$, respectively. When 100 mM of NaCl was added to 3.0% whey protein isolate, we noted a degeneration of heat absorption at $\sim 87^\circ\text{C}$. The unfolding of proteins affects heat absorption via the disruption of non-covalent or disulfide bonds, while it was degenerated by heat and

absorbed heat (Fitzsimons *et al.*, 2007). As compared with the reports of other researchers, the degeneration temperature of NaCN, α -La, and β -Lg appeared relatively high. This difference was attributed to the particular materials used, including in separation, refining, the whole process, and the production method.

Fig. 4 provides the results of heat stability tests and pyrolysis temperature measurements by TGA in the polymerization state of NaCN, α -La, and β -Lg. On the TGA curve, $\blacklozenge$ refers to the transition point at which the sample's mass loss was constant, and the difference between these two points was expressed in terms of mass loss (step) % and residue material %. In the A section (100-300°C), relatively low temperatures, water or residual solvents, and volatile materials like added oil were initially omitted. At this time, mass loss occurred from original milk protein in the range of 5.88-6.65%, and 93.35-94.12% of it remained. NaCN's polymerized form (bold line) evidenced quite a large loss in mass, but mass loss in the polymerization of α -La and β -Lg was relatively small, at 0.69-0.91%. After this, crystallized water was eliminated from the temperature at near 200°C, the combustion and dissolution reaction was initiated from the B section (300-550°C) to the C section (550-800°C), and these reactions evidenced similar phase without any relation to whether or not NaCN was treated with TGase-nearly 22% of the sample remained. The α -La sample polymerized by TGase at the early phase of division was reduced in a temperature range of 200-300°C, and the mass of the non-TGase-treated group was reduced at temperatures above 300°C. Additionally, the mass loss of 71.35% occurred at 550°C, which is at the end of the B section, and thus the mass loss width of the enzyme-treated group and the non-treated group was 3.06%. 21.38% of the remaining α -La material persisted until the final pyrolysis temperature was reached (800°C). The TGase-treated group evidenced a mass loss of 18.25%, which was a relatively larger amount. β -Lg had a total of 4 pyrolysis transition points, and these occurred in a range between 93.90-94.59%; this was similar to that noted in the TGase-treated samples (50-200°C). In the early A section, the mass loss of the early transition point (5.41-6.1%) was attributed to the evaporation of internal β -Lg molecules. Afterward, mass loss in the TGase-treated group was noted at temperatures up to 300°C, in accordance with the fact that mass loss at the high temperature of 550°C in the final B section was detected at 22.05-22.13%. The loss rate of β -Lg from 50°C to 800°C was 83.11%, whereas the TGase-treated β -Lg evidenced a rate of 16.31%.

Fig. 5. FE-SEM of NaCN, α -La and β -Lg powder without (a, c, e) and with (b, d, f) polymerization by TGase. The FE-SEM structure of freeze-dried NaCN, α -La and β -Lg to which TGase was added and incubated for 3 h at 37°C. The scale bar represents 50 μ m.

Microstructural properties

The SEM images of TGase-added and lyophilized milk proteins are provided in Fig. 5. The NaCN particles featured thick tissues and partial small stoma, and a generally unorganized form with rough surfaces (a). Additionally, TGase-treated NaCN exhibited no small stoma and appeared to have a face organized by enzyme (b). α -La evidenced a circular or cracked particle shape, featuring a soft surface, and some of the particles exhibited small grooves (c). α -La had no stoma, which were observed as fine, wide surfaces after polymerization with TGase. Not only soft-surfaced particles, but also long branch-shaped particles were noted (d). β -Lg featured typical circular and fixed particles and a somewhat rougher surface than was observed for α -La, and was shown to facilitate the development of the fixed form of the particle (e). When the β -Lg is polymerized, it evidenced a form similar to that of α -La, which has a fine surface, but the length was shorter, and one large particle composed of several small particles was detected (f). Similarly, the matrix was readily formed via the formation of a covalent bond between small water-soluble proteins. GL cross-linked polymer material was observed to form a wide surface between proteins.

Fig. 6. Antioxidant activity of NaCN, α -La and β -Lg powder with and without TGase by DPPH radical scavenging method. ¹⁾ NaCN(■), α -La(▲), and β -Lg(●) were treated with T(TGase, □, △, ○), respectively. Mean values and standard deviations(error bars) of three replications are shown.

DPPH free radical elimination ability

The results regarding the polymerization of milk proteins by each concentration are shown in Fig. 6. Antioxidative activity increased with increasing concentrations of milk protein, and the activity was measured in the following order: β -Lg > α -La > NaCN. The sample's EC₅₀ (mg/mL) was > 100 mg/mL, but did not reach a level of 0.011 mg/mL, as compared to the data from the vitamin C positive control group. None of the milk protein groups treated with TGase nor the non-treated group evidenced particularly profound antioxidative activity. However, in the case of NaCN and β -Lg, the TGase-treated group evidenced higher antioxidative activity than the non-treated group. As the concentration of α -La increases, antioxidative activity also increases; however, this increase was not particularly profound, and was similar to that of the TGase-treated group's antioxidative activity, at 10 mg/mL.

Whey protein is rich in cysteine and methionine, both of which are elements of glutathione, and sulfur-containing amino acids result consistently in antioxidative ability and stabilize the DNA when the cell divides (Anderson, 1998).

Conclusion

In this study, we evaluated the physico-chemical properties inherent to the production of TGase-treated dairy ingredients, with added NaCN, α -La, and β -Lg. The results of SDS-PAGE showed that NaCN and α -La induced polymerization at above 37 kDa via cross-linking, but β -Lg was unable to cross-link without reductions in its disulphide bonds. The denaturation temperature of

the samples was quite high, at 90-100°C. After TGase treatment, NaCN and β -Lg were denatured at lower temperatures and absorbed heat, β -Lg evidenced the lowest degree of denaturation, whereas the denaturation temperature of TGase-treated α -La was similar to that of non-heated α -La. The largest reduction of mass was observed in the pyrolysis phase, at approximately 200°C, and 16-17% of NaCN and β -Lg remained after heating to 800°C. In the final reaction section, the results were similar to those observed with the TGase-treated samples. When treated with TGase, α -La lost more mass. Under microscopy, the circular protein structures did not evidence stoma with wide, fine surfaces, after reaction with TGase. The antioxidative activity in the samples was not sufficient to eliminate free radicals, but the TGase-treated β -Lg group evidenced higher antioxidative activity than that of the non-treated group. Moreover, the antioxidative activity of α -La increased in proportion to concentration, and evidenced similar activity to the group treated with 10 mg/mL.

The results of this study demonstrate that NaCN, α -La, and β -Lg polymers treated with TGase are significantly functional materials that exhibit physiological activities. Further studies, including *in vivo* experiments and clinical studies, will be required to evaluate the process by which dairy ingredients are digested and absorbed, as well as their utilization.

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