Ultrasound-Assisted Transglutaminase Catalysis of the Cross-Linking and Microstructure of αs-Casein, β-Casein and κ-Casein

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Abstract: The effects of ultrasonic treatment (UT)-assisted transglutaminase (TGase) catalysis on the physicochemical properties of individual αs-casein (αs-CN), β-casein (β-CN), and κ-casein (κ-CN) were investigated. After 60 min of incubation at 30 °C, αs-CN, β-CN, and κ-CN were cross-linked with TGase (6.0 units/mL), and high molecular weight polymers (>200 kDa) were formed. The use of TGase in conjunction with UT (20 kHz, power of 400 W, and amplitude 20%) led to an increase in the rate of αs-CN, β-CN, and κ-CN polymerization compared to the individual casein that contained TGase but did not undergo UT. SDS-PAGE scrutiny showed that the intensities of αs-CN, β-CN, and κ-CN incubation with regard to TGase and UT at 30 °C for 60 min noticeably decreased to 5.66 ± 0.39, 3.97 ± 0.43, and 26.07 ± 1.18%, respectively (p < 0.05). Particle size analysis results indicated that the molecule size appropriation for the cross-linking of αs-CN, β-CN, and κ-CN ranged from 6000 to 10,000 nm after 60 min incubation with TGase and UT. Transmission electron microscopy investigation showed network structures of cross-linking αs-CN, β-CN, and κ-CN were formed from αs-CN, β-CN, and κ-CN, respectively. As our results show, the comprehensive utilization of TGase and UT will be a superior method for the polymerization of αs-CN, β-CN, and κ-CN.

Keywords: αs-casein; β-casein; κ-casein; ultrasound; transglutaminase; polymerization

1. Introduction

The crude component of milk contains 3.7% fat, 3.3% protein, and 4.9% carbohydrates. Milk proteins are classified into two major categories: casein and whey proteins. For milk proteins, the casein content is the highest, accounting for 80%, and it shows lipophilicity. Whey proteins constitute 20% of total milk proteins and show amphiphilic features [1]. The casein family is the first and most abundant and consists of several fractions, such as αs-CN, β-CN, and κ-CN, and most of them exist in colloidal particles known as casein micelles [2]. The casein component is a complex mixture of the four common caseins in a proportion of approximately 5:4:1 [3,4]. The second protein group is whey proteins, which comprise...
β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) [5]. Milk is used to make a variety of dairy products, such as yogurt and cheese. These have been made using a traditional method made from milk using an enzyme such as TGase (EC 2.3.2.13), which is generally used to improve the performance of dairy products [6–8].

TGase catalyzes acyl transfer reactions between the ε-amino group of lysine and the γ-carboxamide groups of glutamine. Casein is a fabulous substance for TGase to form covalent inter- or intramolecular cross-links [9]. There is a wealth of glutamine and lysine in milk proteins. In food industry development, TGase is considered to be the most efficient enzyme that enhances the strength of protein gels and the polymerization of milk proteins [10]. Usually, it unfolds the protein sufficiently because of the adsorption activity, and then the enzyme enters to stimulate the polymerization reaction. The heat stability of casein, gelation, rheological, solubility, viscosity, and renneting properties can be changed by TGase [11]. Additionally, TGase can be processed into cheese, which is utilized to improve the characteristics of cheese manufacturing products [12].

Waves that are generated by mechanical waves have a frequency that is higher than the ridgeline of human hearing (14–20 kHz), which is called ultrasound. UT emits high-intensity and high-frequency sound or waves, causing violent collisions between particles in the liquid [13–15]. Recently, ultrasound technology has become popular for enhancing the character and safety of foods around the world [16]. Usually, there is a nonthermal treatment that involves ultrasonic technology for food processing. Studies have demonstrated the benefits of using ultrasound technology in food processing; for example, ultrasound waves can promote structural modifications and faster transfer of protein molecular weight by physical, chemical, enzymatic, or their combination treatments in food proteins [17]. In addition, aggregates with a determined particle size can be obtained, and at the same time, the determined functional properties are changed [18,19]. In the milk industry, the application of ultrasound in the milk industry includes homogenizing, reducing particle size, uniformity, and extending the shelf life of milk [20].

Previous studies have been performed on the polymerization behavior of whey proteins and casein in dairy products. There are multitudinous studies on the effect of TGase on casein [21,22]. Likewise, several studies on the effect of TGase on whey proteins have been reported [23,24]. Additionally, the effect on the coagulation characteristics induced by rennet in goat milk has been investigated. However, information on the relative susceptibility and polymerization of individual αs-CN, β-CN, κ-CN with TGase treatment is insufficient by ultrasound. Therefore, the objective of this study was to investigate the effect of UT on the physicochemical properties of TGase-induced milk proteins.

2. Materials and Methods

2.1. Preparation of Milk Protein Samples

Raw milk was produced from healthy Holstein cows from a local dairy farm in Taipei. For the experiment, skim milk (29.1 mg protein/mL) was prepared by separating the fat from whole milk by centrifugation (5000×g, 20 min). Individual milk proteins, including αs-CN, β-CN, κ-CN, αs-LA, and β-LG, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TGase from Streptoverticillium mobaraense was obtained from Ajinomoto Co. Inc. (Activa TG-B, solution form; Ajinomoto Co. Inc., Tokyo, Japan).

2.2. Preparation of Milk Samples with/without TGase and UT

Investigations have been performed on the effects of TGase and UT on the cross-linking of milk proteins. The UT has an operating frequency of 20 kHz, an amplitude of 20%, and a power of 400 W by a probe-type ultrasonic sensor (Sonifier®S-450D Digital Cell Disruptor, USA). The milk samples with/without TGase (6.0 units/mL) and with/without UT were incubated at 30 °C for 60 min, and then the samples were heated to 80 °C for 5 min to inactivate the TGase. To investigate the effects of UT on the cross-linking of TGase-induced individual αs-CN, β-CN, κ-CN, αs-LA and β-LG, the individual casein samples (2 mg) with TGase (6.0 units/mL) and with/without UT were incubated at 30 °C
for 0, 10, 20, 40, and 60 min, and the samples were heated to 80 °C for 5 min to inactivate the TGase. The experiment was conducted under one of three conditions: (1) skim milk proteins without TGase, (2) skim milk proteins with TGase and with/without UT, and (3) individual milk protein with and with/without UT. The experiments on the samples were performed in triplicate.

2.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

SDS-PAGE was used to analyze milk samples with/without TGase and UT for the study. Milk samples were shown by using stacking gel (5%) and separating gel (12.5%). For each sample, 0.3 mL of sample buffer, which comprises bromophenol blue (0.02%), β-mercaptoethanol (5%), SDS (2%), glycerol (10%), and Tris-HCl (70 mM, pH 6.8), was mixed with the 0.1 mL of a sample. The samples were then heated to 95 °C for 5 min. Five microliters of sample and protein markers (10–170 kDa) were loaded on an SDS-PAGE gel. Separation was performed in the separating gel at 90 V.

2.4. Gel Staining and Image Analysis

After electrophoresis, Coomassie blue dye was used to stain the gels. The stained gels were scanned by an EPSON perfection V39 image scanner and then analyzed by Gel-Pro Analyzer software (version 4.0, Media Cybernetics, Inc., Rockville, MD, USA). SDS-PAGE profiles were used to evaluate milk protein cross-linking induced by ultrasound-assisted cross-linking of milk proteins with TGase.

2.5. Particle Size Analysis of Polymerized Individual Caseins with TGase and UT

To investigate the effects of TGase and UT on the polymerization of individual caseins, αs-CN, β-CN, and κ-CN were investigated. The z-average diameter of the colloidal dispersion was measured with a nanoparticle analyzer (SZ-100Z, HORIBA Instruments, Inc., Kyoto, Japan). Before analyzing and measuring the samples, they were diluted with 0.05 M phosphate buffer solution (pH 6.8) to avoid multiple scattering effects. The experiments on the sample were performed in triplicate.

2.6. Transmission Electron Microscopy (TEM) Analysis

The freshly prepared colloidal dispersion was diluted with pH 6.8 water, dropped onto a carbon film grid, and dried in air. The TEM experiment was conducted on a JEM-1400 microscope (JEOL Ltd., Japan) with an acceleration voltage of 200 kV. Images were taken on film at 20,000–50,000 × magnification.

2.7. Statistical Analysis

The results were performed utilizing SAS® version 9.4 (SAS Institute, Cary, NC, USA), and the data are shown as the mean ± standard deviation. One-way analysis of variance was used to calculate the significant differences between treatments. Each treatment was measured three times, and the statistical significance level was set to p < 0.05.

3. Results and Discussion

3.1. SDS-PAGE Analysis of TGase and UT on the Cross-Linking of Milk Proteins

The impact of TGase (6.0 units/mL) and UT on the cross-linking of milk protein was studied. Skim milk tests were hatched with/without TGase and UT for 60 min at 30 °C before analysis by SDS-PAGE (Figure 1). Compared to the skim milk samples without TGase and UT for 60 min at 30 °C (Figure 1, L1), the polymerization of a portion of αs-CN, β-CN and κ-CN by TGase into higher molecular weight proteins (>200 kDa) was observed (Figure 1, L2). This finding indicates that αs-CN, β-CN, and κ-CN polymerization occurred in the presence of TGase. On the other hand, αs-CN, β-CN, and κ-CN are good substrates for TGase. Pierro et al. (2010) reported that TGase was used to obtain cheese, and the results of SDS-PAGE indicated that mainly β-CN was involved in the TGase-catalyzed cross-links occurring in cheese [25]. Hsieh et al. (2012) reported that TGase forms intermolecular
and intramolecular cross-links by forming isopeptide bonds. Furthermore, no significant changes in α-LA and β-LG were observed in the milk sample with TGase [26]. This result indicates that α-LA and β-LG tend to have low cross-linking efficiency during the reaction with TGase and are poor substrates. Jovanović et al. (2005) reported that whey proteins, for example, α-LA and β-LG, are poor substrates for TGase [27]. It has been proven that α-LA and β-LG tend to have lower cross-linking efficiency and poor substrates during the reaction with TGase due to their compact spherical structure [28]. These findings are related to the results of Paramban et al. (2016), who studied the cross-linking of milk proteins with TGase [29]. Cozzolino et al. (2003) reported that TGase treatment conferred a decreased protein content to derived whey. Moreover, further additions of whey to milk in the presence of TGase during the manufacturing process led to whey protein-enriched cheese curd. This cross-linking reaction was verified by SDS-PAGE, which showed a high molecular weight band accompanied by a decrease in the density of caseins and whey proteins [30]. These also suggested that the polymers (molecular weights >200 kDa) contained caseins and a portion of whey proteins. Pierro et al. (2010) reported that the use of TGase in the dairy industry increased the cheese yield by 4%. In addition, the protein content in the cheese significantly increased from 33.8% to 39.5% [25]. Furthermore, Mahmood (2009) suggested that TGase improved the properties of soft cheese manufactured from milk by enhancing the cross-linking reaction among milk proteins. Sensory evaluation of the cheeses showed that the TGase-treated cheese was superior to the untreated cheese throughout the eight-day storage period [31]. We also noticed that the combined use of TGase and UT for 60 min improved the polymerization of αs-CN, β-CN, and κ-CN, which made the polymerization of milk protein faster than that of the sample containing TGase alone (Figure 1, L3). Therefore, the use of TGase in conjunction with UT increased the rate of αs-CN, β-CN, and κ-CN polymerization compared with the proteins that contain TGase but without UT. Zhao et al. (2014) reported that the application of UT in the milk industry includes extending the shelf life of milk and improving the polymerization performance [20]. Furthermore, Zhang et al. (2016) report that cold set gels from TGase-catalyzed soy protein isolate could form at low temperatures [19]. Qin et al. (2016) also indicated that ultrasound pretreatments significantly increased the water holding capacity and gel strength, resulting in more homogeneous and denser networks of TGase-induced soy protein isolate gels [14]. Therefore, ultrasound is useful in promoting the gelation properties of TGase-induced protein gels, and their applications could be expanded in the food industry.

![Figure 1](image_url). SDS-PAGE image showing skim milk incubated with/without TGase (6.0 units/mL) and with/without UT at 30 °C for 60 min. (L1) Milk, (L2) milk with TG, and (L3) milk with TGase and UT. M = protein marker. The experiments on the sample were performed in triplicate.
3.2. SDS-PAGE Analysis of TGase and UT on the Cross-Linking of Individual Milk Proteins

TGase-containing individual αs-CN, β-CN, κ-CN, α-LA, and β-LG were also incubated with/without UT for 60 min at 30 °C (Figure 2). SDS-PAGE indicated that a portion of the αs-CN, β-CN, and κ-CN bands decreased when 6.0 units/mL TGase was added (Figure 2A). However, αs-CN, β-CN, and a portion of the κ-CN bands almost disappeared after 60 min of incubation with TGase and UT (Figure 2B). The results showed that the total intensities of αs-CN, β-CN, and κ-CN after 60 min of incubation decreased to 6.0%, 3.3%, and 26.2%, respectively. The disappearance of αs-CN, β-CN, and κ-CN confirmed the formation of cross-linked αs-CN, β-CN, and κ-CN, and these cross-linked proteins were observed at the top of the SDS-PAGE gels. Furthermore, no cross-linked α-LA and β-LG were observed on the gels with TGase and UT after 60 min of incubation. These results indicate that after 60 min of reaction with TGase and UT, α-LA and β-LG have little or no cross-linking. The TGase-containing individual αs-CN, β-CN, and κ-CN with/without UT at 30 °C for 0, 10, 20, 40, and 60 min were also investigated (Figure 3). The SDS-PAGE images showed αs-CN (32 kDa), β-CN (26 kDa), and κ-CN (25 kDa) after reaction with TGase (6.0 units/mL) for 60 min. Initially, the casein sample containing TGase did not change significantly during the 0 min incubation period, which indicates that the cross-linking of αs-CN, β-CN, and κ-CN did not occur immediately (Figure 3A,C,E). However, cross-linked αs-CN, β-CN, and κ-CN were observed after 40, 20, and 60 min of treatment with TGase, respectively. Note that we also observed cross-linked αs-CN, β-CN, and κ-CN following treatment with both TGase and UT after 20, 10, and 20 min of incubation (Figure 3B,D,F).

Figure 2. SDS-PAGE image showing individual αs-CN, β-CN, κ-CN, α-LA and β-LG treated with TGase (6.0 units/mL) and UT at 30 °C for 60 min. (A) TGase, (B) with TGase and UT. M = protein marker. The experiments on the sample were performed in triplicate.

Densitograms relating to the SDS-PAGE investigations of TGase-containing individual αs-CN, β-CN, κ-CN, α-LA and β-LG at 30 °C for 10, 20, 40, and 60 min were also analyzed (Figure 4). The intensities of β-CN with TGase alone after 0, 10, 20, 40, and 60 min of incubation decreased to 90.23 ± 1.29, 49.63 ± 2.81, 15.94 ± 1.38, and 4.62 ± 1.54%, respectively. As shown in Figure 4B, the intensities of αs-CN, β-CN, and κ-CN noticeably decreased when both TGase and UT were used in combination compared with those of the samples that contained TGase alone, after 10, 20, 40, and 60 min of incubation, and decreased to 15.47 ± 0.64, 7.28 ± 0.98, 4.96 ± 0.17, and 3.97 ± 0.43%, respectively. Similar results were obtained with TGase-containing individual αs-CN and κ-CN with/without UT at 30 °C for 10, 20, 40, and 60 min (Figure 4A,C). Hsieh et al. (2012) reported that the cross-linking reaction of β-CN was faster than κ-CN and αs-CN in milk by TGase induction [26]. β-CN contains more prolyl residues than other caseins, which gives it a more open structure.
that is flexible and disordered and accelerates enzymatic reactions. Therefore, our results revealed that UT accelerated the TGase-polymerized reactions of $\alpha_s$-CN, $\beta$-CN, and $\kappa$-CN. TGase is an acyltransferase that forms intermolecular and intramolecular cross-links by forming isopeptide bonds between glutamine and lysine residues, thereby causing a cross-linking reaction with milk proteins [9]. Furthermore, UT promotes the formation of protein molecules and aggregates during the enzymatic cross-linking process [19].

Figure 3. SDS-PAGE image showing individual $\alpha_s$-CN, $\beta$-CN and $\kappa$-CN treated with TGase (6.0 units/mL) and UT at 30 °C for 0, 10, 20, 40 and 60 min. (A) $\alpha_s$-CN with TGase, (B) $\alpha_s$-CN with TGase and UT, (C) $\beta$-CN with TGase, (D) $\beta$-CN with TGase and UT, (E) $\kappa$-CN with TGase, and (F) $\kappa$-CN with TGase and UT. M = protein marker. The experiments on the sample were performed in triplicate.
3.3. Particle Size Analysis of Individual Caseins Treated with TGase and UT

We examined the effects of TGase and UT on individual αs-CN, β-CN, and κ-CN polymerization, and individual αs-CN, β-CN, and κ-CN were incubated with TGase (6.0 units/mL) and UT at 30 °C for 60 min (Figure 5). The mean particle size of αs-CN with TGase and UT resulted from a 0–400 nm particle yield of 94% and a 6000–10,000 nm particle yield of 6% for 0 min (Figure 5A). For 60 min, the mean particle size of αs-CN with TGase and UT were decreased to a 69% yield for particles of approximately 0–400 nm and increased to a 31% yield for particles of approximately 6000–10,000 nm (Figure 5B). These results indicated that TGase polymerized the aggregated αs-CN to form high-molecular-
weight polymers after 60 min of incubation. We noticed that the particles of β-CN with TGase and UT decreased from 15% to 0% yield for particles of approximately 400–6000 nm and increased from 0% to 13% for particles of approximately 6000–10,000 nm after 60 min of incubation (Figure 5C,D). Chen et al. (2020) reported that it is the most sensitive to the response induced by TGase [32]. Furthermore, the particle size of κ-CN with TGase and UT decreased from a 4% to 2% yield for particles of approximately 100–6000 nm and increased from a 1% to 2% yield for particles of approximately 6000–10,000 nm, after 60 min of incubation (Figure 5E,F). Hence, the aggregated β-CN and κ-CN were polymerized into cross-linked β-CN and κ-CN after 60 min of incubation. Kieliszek et al. (2014) reported that TGase catalyzes acyl transfer between acyl donors and acyl acceptors, leading to the formation of covalent cross-links of milk proteins [9]. Moreover, a similar result was obtained by Qin et al. (2016) [14]. This finding is attributed to the individual αs-CN, β-CN, and κ-CN particle protein formation of small aggregates during TGase and UT processing. This sonication results from the formation of an ultrasound-induced aggregate, which is a noncovalent bond with hydrogen bonding, and reduces electrostatic and hydrophobic interactions [33].

Figure 5. Particle size distribution of the individual αs-CN, β-CN, and κ-CN treated with TGase (6.0 units/mL) and UT at 30 °C for 0 and 60 min. (A) αs-CN for 0 min, (B) αs-CN for 60 min, (C) β-CN for 0 min, (D) β-CN for 60 min, (E) κ-CN for 0 min, and (F) κ-CN for 60 min. The experiments on the sample were performed in triplicate.

3.4. Microstructures of the Individual Caseins Treated with TGase and UT

According to the above results of the particle size distribution of individual αs-CN, β-CN, and κ-CN, the particle yield of cross-linked αs-CN, β-CN, and κ-CN (6000–10,000 nm) was observed to be in the following order: κ-CN < β-CN < αs-CN. Therefore, the microstructures of individual αs-CN, β-CN, and κ-CN with TGase (6.0 units/mL) and UT at
30 °C for 60 min were investigated (Figure 6). As shown in Figure 6A, aggregated αs-CN was observed in the TEM image (Figure 6A). The cross-linked network structure of αs-CN was formed from the aggregated αs-CN after 60 min of incubation (Figure 6B). The results showed that TGase combined with UT to form αs-CN polymerizations. Qin et al. (2016) reported that UT can accelerate the efficiency and cross-linking of TGase to milk protein, leading to the formation of more aggregated, compact, and homogeneous particles [14]. Nguyen and Anema (2010) reported that the UT mark can cause violent collisions between particles in liquid food [34]. It can also promote the enhanced binding of water in the protein gel network, thereby having a beneficial effect on the water retention capacity [20]. In addition, aggregated β-CN (Figure 6C) and κ-CN (Figure 6E) were also observed in the TEM images. The cross-linked network structures of β-CN (Figure 6D) and κ-CN (Figure 6F) were formed from the aggregated αs-CN after 60 min of incubation.

![Figure 6](image-url)

Figure 6. Transmission electron microscopy of the individual αs-CN, β-CN and κ-CN treated with/without TGase (6.0 units/mL) and UT at 30 °C for 0 and 60 min. (A) αs-CN, (B) αs-CN with TGase for 60 min, (C) αs-CN with TGase and UT for 60 min, (D) β-CN, (E) β-CN with TGase for 60 min, (F) β-CN with TGase and UT for 60 min, (G) κ-CN, (H) κ-CN with TGase for 60 min, and (I) κ-CN with TGase and UT for 60 min. The experiments on the sample were performed in triplicate.

3.5. Reaction Scheme for the TGase and UT on the Polymerization of αs-CN, β-CN and κ-CN

UT-assisted polymerization of individual αs-CN, β-CN, and κ-CN induced by TGase was examined in this study. Based on the SDS-PAGE and particle size data, TGase (6.0 units/mL) and UT-induced individual αs-CN, β-CN, and κ-CN polymerization after 60 min of incubation was observed at the following rate: κ-CN < αs-CN < β-CN. The particle yields of cross-linked αs-CN, β-CN, and κ-CN (6000–10,000 nm) were also observed in the following order: κ-CN < β-CN < αs-CN. Therefore, a reaction scheme for the effect
of polymerization of individual $\alpha_s$-CN, $\beta$-CN, and $\kappa$-CN induced by TGase and UT was proposed, as shown in Figure 7. The particle size distribution of individual $\alpha_s$-CN was incubated with TGase and with UT at 30 °C for 0 and 60 min (Figure 7A). As shown in Figure 7A, the particle size distribution of aggregated $\alpha_s$-CN was a 0–400 nm particle yield of 94% and a 6000–10,000 nm particle yield of 6%. A portion of these aggregated $\alpha_s$-CN was polymerized into cross-linked $\alpha_s$-CN after 60 min of incubation. The particle yield of cross-linked $\alpha_s$-CN (6000–10,000 nm) was increased from 6% to 31%. Moreover, a portion of aggregated $\beta$-CN (0–6000 nm) was polymerized into cross-linked $\beta$-CN (6000–10,000 nm) after 60 min of incubation (Figure 7B). A similar polymerization scheme was also observed in the results of $\kappa$-CN, and a portion of these aggregated $\alpha_s$-CN was polymerized into cross-linked $\alpha_s$-CN after 60 min of incubation. The particle yield of cross-linked $\kappa$-CN (6000–10,000 nm) was increased from 1% to 2% (Figure 7C). Therefore, this scheme shows that the cross-linking of $\beta$-CN catalyzed by TGase and UT is faster than the cross-linking of $\alpha_s$-CN and $\kappa$-CN. However, the particle yield of cross-linked $\alpha_s$-CN (6000–10,000 nm) was higher than that of $\beta$-CN and $\kappa$-CN.

**Figure 7.** Reaction scheme for the TGase and UT on the polymerization of individual $\alpha_s$-CN, $\beta$-CN and $\kappa$-CN induced by TGase and UT. (A) $\alpha_s$-CN, (B) $\beta$-C, and (C) $\kappa$-CN.
4. Conclusions

We verified the effects of TGase and UT on the polymerization of individual αs-CN, β-CN, and κ-CN. Our results revealed that individual αs-CN, β-CN, and κ-CN are excellent substrates for TGase, while individual α-LA and β-LG are poor substrates for TGase. The combination of TGase and UT on the polymerization of individual αs-CN, β-CN, and κ-CN was faster than that of TGase alone. SDS-PAGE analysis shows that under the conditions of TGase and UT, the cross-linking reaction of β-CN is faster than the cross-linking reaction of αs-CN and κ-CN. Furthermore, the particle size distribution results suggested that the particle yield of cross-linked αs-CN was higher than that of β-CN and κ-CN. Our results show that the combination of TGase and UT for 60 min significantly improves the polymerization of αs-CN, β-CN, and κ-CN, which indicates that UT accelerates TGase-polymerized reactions.

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**References**

1. Lopes, A.S.; Garcia, J.S.; Catharino, R.R.; Santos, L.S.; Eberlin, M.N.; Arruda, M.A.Z. Cloud point extraction applied to casein proteins of cow milk and their identification by mass spectrometry. *Anal. Chim. Acta* 2007, 590, 166–172. [CrossRef] [PubMed]
2. Farrell, H.M., Jr.; Jimenez-Flores, R.; Bleck, G.T.; Brown, E.M.; Butler, J.E.; Creamer, L.K.; Hicks, C.L.; Hollar, C.M.; Ng-Kwai-Hang, K.F.; Swaisgood, H.E. Nomenclature of the proteins of cows’ milk—sixth revision. *J. Dairy Sci.* 2004, 87, 1641–1674. [CrossRef]
3. Jensen, H.; Poulsen, N.; Andersen, K.; Hammershøj, M.; Poulsen, H.; Larsen, L.B. Distinct composition of bovine milk from Jersey and Holstein-Friesian cows with good, poor, or noncoagulation properties as reflected in protein genetic variants and isoforms. *J. Dairy Sci.* 2012, 95, 6905–6917. [CrossRef]
4. Wang, C.; Xie, Q.; Wang, Y.; Fu, L. Effect of ultrasound treatment on allergenicity reduction of milk casein via colloid formation. *J. Agric. Food Chem.* 2020, 68, 4678–4686. [CrossRef]
5. McDermott, A.; Visentin, G.; De Marchi, M.; Berry, D.P.; Fenelon, M.A.; O’Connor, P.M.; Kenny, O.A.; McParland, S. Prediction of individual milk proteins including free amino acids in bovine milk using mid-infrared spectroscopy and their cor-relations with milk processing characteristics. *J. Dairy Sci.* 2016, 99, 3171–3182. [CrossRef]
6. Buettner, K.; Hertel, T.C.; Pietzsch, M. Increased thermostability of microbial transglutaminase by combination of several hot spots evolved by random and saturation mutagenesis. *Amino. Acids.* 2011, 42, 987–996. [CrossRef] [PubMed]
7. Giosafatto, C.; Rigby, N.; Wellner, N.; Ridout, M.; Husband, F.; Mackie, A. Microbial transglutaminase-mediated modification of ovalbumin. *Food Hydrocoll.* 2012, 26, 261–267. [CrossRef]
8. Özer, B.; Guyot, C.; Kulozik, U. Simultaneous use of transglutaminase and rennet in milk coagulation: Effect of initial milk pH and renneting temperature. *Int. Dairy J.* 2012, 24, 1–7. [CrossRef]
9. Kielszek, M.; Misiewicz, A. Microbial transglutaminase and its application in the food industry. A review. *Folia Microbiol.* 2014, 59, 241–250. [CrossRef]
10. Ridout, M.J.; Paanen, A.; Mamode, A.; Linder, M.B.; Wilde, P.J. Interaction of transglutaminase with adsorbed and spread films of β-casein and κ-casein. *Colloids. Surf. B Biointerfaces* 2015, 128, 254–260. [CrossRef]
11. Sayadi, A.; Madadlou, A.; Khosrowshahi, A. Enzymatic cross-linking of whey proteins in low fat Iranian white cheese. *Int. Dairy J.* 2013, 29, 88–92. [CrossRef]
12. Moon, J.H.; Hong, Y.H.; Huppertz, T.; Fox, P.F.; Kelly, A.L. Properties of casein micelles cross-linked by transglutami-nase. Int. J. Dairy Technol. 2009, 62, 27–32. [CrossRef]

13. Kwiatkowska, B.; Bennett, J.; Akunna, J.; Walker, G.; Bremner, D.H. Stimulation of bioprocesses by ultrasound. Biotechnol. Adv. 2011, 29, 768–780. [CrossRef]

14. Qin, X.-S.; Luo, S.-Z.; Cai, J.; Zhong, X.-Y.; Jiang, S.-T.; Zhao, Y.-Y.; Zheng, Z. Transglutaminase-induced gelation properties of soy protein isolate and wheat gluten mixtures with high intensity ultrasonic pretreatment. Ultrason. Sonochemistry 2016, 31, 590–597. [CrossRef]

15. Vikhu, K.; Mawson, R.; Simons, L.; Bates, D. Applications and opportunities for ultrasound assisted extraction in the food industry—A review. Innov. Food Sci. Emerg. Technol. 2008, 9, 161–169. [CrossRef]

16. Jiang, Z.; Wang, C.; Li, T.; Sun, D.; Gao, H.; Gao, Z.; Mu, Z. Effect of ultrasound on the structure and functional properties of transglutaminase-crosslinked whey protein isolate exposed to prior heat treatment. Int. Dairy J. 2019, 88, 79–88. [CrossRef]

17. Cui, Q.; Wang, G.; Gao, D.; Wang, L.; Zhang, A.; Wang, X.; Xu, N.; Jiang, L. Improving the gel properties of transgenic microbial transglutaminase cross-linked soybean-whey mixed protein by ultrasonic pretreatment. Process. Biochem. 2020, 91, 104–112. [CrossRef]

18. Gordon, L.; Pilosof, A.M.R. Application of High-Intensity Ultrasounds to Control the Size of Whey Proteins Particles. Food Biophys. 2010, 5, 203–210. [CrossRef]

19. Zhang, P.; Hu, T.; Feng, S.; Xu, Q.; Zheng, T.; Zhou, M.; Chu, X.; Huang, X.; Lu, X.; Pan, S.; et al. Effect of high intensity ultrasound on transglutaminase-catalyzed soy protein isolate cold set gel. Ultrason. Sonochemistry 2016, 29, 380–387. [CrossRef] [PubMed]

20. Zhao, L.; Zhang, S.; Uluko, H.; Liu, L.; Lu, J.; Xue, H.; Kong, F.; Lv, J. Effect of ultrasound pretreatment on rennet-induced coagulation properties of goat’s milk. Food Chem. 2014, 165, 167–174. [CrossRef]

21. Hinz, K.; Huppertz, T.; Kelly, A.L. Susceptibility of the individual caseins in reconstituted skim milk to cross-linking by transglutaminase: Influence of temperature, pH and mineral equilibria. J. Dairy Res. 2012, 79, 414–421. [CrossRef]

22. Smolenski, G.; Haines, S.; Kwan, F.Y.-S.; Bond, J.; Farr, V.; Davis, S.R.; Stelwagen, K.; Wheeler, T.T. Characterisation of Host Defence Proteins in Milk Using a Proteomic Approach. J. Proteome Res. 2007, 6, 207–215. [CrossRef] [PubMed]

23. Moatsou, G.; Hatzinaki, A.; Samolada, M.; Anifantakis, E. Major whey proteins in ovine and caprine acid wheys from indigenous greek breeds. Int. Dairy J. 2005, 15, 123–131. [CrossRef]

24. Zhong, Q.; Wang, W.; Hu, Z.; Ikeda, S. Sequential preheating and transglutaminase pretreatments improve stability of whey protein isolate at pH 7.0 during thermal sterilization. Food Hydrocoll. 2013, 31, 306–316. [CrossRef]

25. Di Pierro, P.; Marinelli, L.; Sorrentino, A.; Giosafatto, C.V.L.; Chianese, L.; Porta, R. Transglutaminase-Induced Chemical and Rheological Properties of Cheese. Food Biotechnol. 2010, 24, 107–120. [CrossRef]

26. Hsieh, J.-F.; Pan, P.-H. Proteomic Profiling of the Coagulation of Milk Proteins Induced by Chymosin. J. Agric. Food Chem. 2012, 60, 2039–2045. [CrossRef] [PubMed]

27. Jovanović, S.; Barać, M.; Maćej, O. Whey proteins-properties and possibility of application. Mljetkarstvo 2005, 55, 215–233.

28. Tang, C.-H.; Ma, C.-Y. Modulation of the thermal stability of β-lactoglobulin by transglutaminase treatment. Eur. Food Res. Technol. 2007, 225, 649–652. [CrossRef]

29. Rahila, M.P.; Kumar, R.; Mann, B.; Koli, P.S. Enzymatic Modification of Milk Proteins for the Preparation of Low Fat Dahi. J. Food Process. Preserv. 2015, 40, 1038–1046. [CrossRef]

30. Cozzolino, A.; Di Pierro, P.; Marinelli, L.; Sorrentino, A.; Masi, P.; Porta, R. Incorporation of whey proteins into cheese curd by using transglutaminase. Biotechnol. Appl. Biochem. 2003, 38, 289–295. [CrossRef] [PubMed]

31. Mahmoud, W.A. Effect of microbial transglutaminase on soft cheese properties. Mesop. J. Agric. 2009, 37, 19–27.

32. Chen, C.C.; Chen, L.Y.; Chan, D.S.; Chen, B.Y.; Tseng, H.W.; Hsieh, J.F. Influence of microbial transglutaminase on physico-chemical and cross-linking characteristics of individual caseins. Molecules 2020, 25, 3992. [CrossRef] [PubMed]

33. Munir, M.; Nadeem, M.; Qureshi, T.M.; Leong, T.S.; Gamlath, C.J.; Martin, G.J.; Ashokkumar, M. Effects of high pressure, microwave and ultrasound processing on proteins and enzyme activity in dairy systems—A review. Innov. Food Sci. Emerg. Technol. 2019, 57, 102192. [CrossRef]

34. Nguyen, N.H.; Anema, S.G. Effect of ultrasonication on the properties of skim milk used in the formation of acid gels. Innov. Food Sci. Emerg. Technol. 2010, 11, 616–622. [CrossRef]