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Influence of transglutaminase treatment of skim milk on the formation of ε-(γ-glutamyl)lysine and the susceptibility of individual proteins towards crosslinking

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Abstract

Results of transglutaminase reaction on the susceptibility of individual milk proteins in skim milk towards crosslinking and on the distinction between crosslinking and other transglutaminase-catalysed reactions, are presented. In unheated milk, transglutaminase had a small effect on proteins whereas in the preheated milk, considerable crosslinking, deamidation and/or amine incorporation occurred. Direct measurement of the dipeptide ε-(γ-glutamyl)lysine showed that a rapid crosslinking of proteins occurred during the first 30 min of transglutaminase reaction. Results from the SDS and capillary gel electrophoresis showed that although the crosslinking was prevalent during the entire reaction time, most crosslinking occurred during the first 30 min. In both unheated and preheated milk, major reduction in the monomeric forms of κ- and β-caseins occurred due to the reaction with transglutaminase, suggesting that these two proteins were most susceptible to transglutaminase-induced crosslinking. The high susceptibility of κ-casein towards crosslinking is likely to be at least partly due to its peripheral position in the casein micelles, and the high susceptibility of β-caseins is hypothesised to be due to its dynamic nature and thus ease of accessibility in the micelle structure. In addition, it could be shown that only preheated β-lactoglobulin was susceptible to transglutaminase action, while α-lactalbumin was crosslinked with or without preheating to the same extent.

Keywords: Caseins; Whey proteins; Transglutaminase; Crosslinking; Deamidation; Capillary gel electrophoresis; ε-(γ-glutamyl)lysine

1. Introduction

Food industries worldwide, are looking forward to develop new ingredients or dairy products with novel physical and functional characteristics. One of the means of altering the properties of dairy products is via modification of milk proteins using transglutaminase (Motoki & Seguro, 1998). Transglutaminase (protein-glutamine γ-glutamyl transferase, EC 2.3.2.13) is an enzyme naturally present in most animal tissues and body fluids and it plays an important role in the blood-clot formation. With the recent availability of the enzyme from a microbial source (Ando et al., 1989), its applications by food industry are being widely investigated.

Transglutaminase catalyses an acyl transfer reaction between γ-carboxyamide groups of peptide-bound glutamine residues (acyl donor) and the primary amino groups in a variety of amine compounds (acyl acceptor), including peptide-bound ε-amino groups of lysine residues. As a result of crosslinking of peptide-bound glutamine and lysine residues, ε-(γ-glutamyl)lysine isopeptide bonds and high-molecular weight polymers are formed. In the absence of amine substrates, transglutaminase is capable of catalysing the deamidation of glutamine residues (Motoki, Seguro, Nio, & Takinami, 1986).

There are numerous studies on the effect of transglutaminase on caseins (Ikura, Komitani, Yoshikawa, Sasaki, & Chiba, 1980; Traoré & Meunier, 1991; Sakamoto, Kumizawa, & Motoki, 1994) and whey proteins (Ikura et al., 1980; Mahmoud & Savello, 1992;

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Færgemand, Otte, & Qvist, 1997). Due to its high reactivity with casein proteins, its application in dairy products has been widely investigated (Dickinson & Yamamoto, 1996; Færgemand & Qvist, 1997; Lorenzen & Schlimme, 1998; Færgemand, Sørensen, Jørgensen, Budolfsen, & Qvist, 1999; Lorenzen, 2000a, b). Although the above studies give a good understanding of the technological potential of transglutaminase reaction in dairy products, information on the relative susceptibility of individual milk proteins is lacking. Also, most of the studies have used indirect means (e.g. rheological properties) of measuring the crosslinking reactions and therefore no distinction has been made between the crosslinking and other reactions catalysed by transglutaminase.

In order to optimise the performance of transglutaminase in dairy products, it is vital to understand its reaction on the casein micelle and whey proteins in their natural states in milk. Also, there is a need to distinguish between the crosslinking and other reactions catalysed by transglutaminase. The present work is focused on addressing the above two unresolved issues.

2. Materials and methods

2.1. Materials

A Ca^{2+}-independent transglutaminase (Activa MP) was provided by Alsiano Ingredients, Denmark. The transglutaminase was manufactured by Ajinomoto Co., Ltd., Japan using a fermentation process and *Streptoverticillium mobaraense*. The enzyme preparation had an activity of 100 U g^{-1} (measured by hydroxamate method of Folk & Cole, 1966), and contained 1% enzyme. The enzyme has previously been shown to have an optimum activity at 50°C in the pH range 5–8 (Motoki & Seguro, 1998). The enzyme preparation was used in the original form without any further purification.

2.2. Reaction with transglutaminase

Skim milk powder (previously lyophilised from fresh raw skim milk) containing 33.45% protein was reconstituted to 9.7% total solids by dissolving 27.5 g powder in 250 g deionised water. The enzyme preparation was added to 115 mL reconstituted skim milk sample (final enzyme/substrate ratio of 0.1) before or after preheating (85°C, 15 min) and the mixture was incubated at 40°C for 30, 90, 180 or 300 min. The enzymatic reaction was terminated by heating the mixture at 80°C for 2 min. All samples were rapidly cooled to the room temperature, lyophilised and subsequently analysed.

All the chemicals used were of analytical grade unless specified otherwise and were obtained from either Sigma (Sigma Chemicals Co., St. Louis, MO 63178, USA) or Merck (Merck Darmstadt, Germany).

2.3. Estimation of ammonia

The transglutaminase-mediated release of ammonia was monitored using the Boehringer Mannheim ammonia kit (Boehringer Mannheim GmbH, Mannheim, Germany).

One millilitre of sample (with or without transglutaminase) was diluted with 4 mL, 12% trichloroacetic acid and the filtrate from Whatman No. 1 (50 μL) was transferred to a multiwell plate (Elkay, Boston Turnpike Shrewsbury, Massachusetts, USA). To this, 200 μL of a solution containing 2-oxoglutarate, reduced nicotinamide adenine dinucleotide (NADH), glutamate dehydrogenase (GIDH) and water (in proportions as described by the Boehringer Mannheim kit), were added and the mixture held at the room temperature for 20 min. The absorbance of the sample was measured at 340 nm using PowerWave 200 Multiplate Scanning Spectrophotometer (Bio-Tek Instruments, Inc., Highland Park, Winooski, Vermont, USA). A standard curve for NH_4^+ was developed by using the ammonia solution provided with the Boehringer Mannheim kit.

2.4. Determination of ε-(γ-glutamyl)lysine and free amino acids

Determination of ε-(γ-glutamyl)lysine was performed after extensive proteolysis followed by amino acid analysis. As a reference H-γ-Glu-ε-Lys-OH (Bachem Biochemica, Heidelberg, Germany) was used. For proteolysis, the following enzymes were applied: Pronase (Boehringer, Mannheim, Germany, Cat.-No. 165921), Leucinaminopeptidase (Cat.-No. L-1503), Prolidase (Cat.-No. P-6675) and Carboxypeptidase A (Cat-No. C-0261) all obtained from Sigma Chemical Company, Steinheim, Germany.

A sample of transglutaminase-treated or non-treated skim milk containing 50 mg of protein was dissolved in 7.5 mL borat buffer (0.1 mol L^{-1}, pH 8.0) and warmed up to 37°C. The sample was incubated two times for 24 h at 37°C with Pronase (0.4 Um g^{-1} protein). The Pronase action was stopped by heating the sample for 10 min at 100°C. After cooling, proteolysis was continued with Leucinaminopeptidase (0.4 U mg^{-1} protein) and Prolidase (0.45 U mg^{-1} protein) for 24 h at 37°C. After this the sample was incubated with Leucinaminopeptidase (0.4 U mg^{-1} protein) for 24 h and for another 24 h with Carboxypeptidase A (0.2 U mg^{-1} protein) at 37°C. After heating (10 min, 100°C) the hydrolised sample was lyophilised.

Eight milligrams of the lyophilised sample was dissolved in 1 mL of sodium citrate buffer (0.2 mol L^{-1}; pH 2.2) and aliquots of 180 μL were analysed by an ion-exchange technique using an automated analyser (Alpha Plus, Pharmacia, Sweden). Calibration was achieved by running the reference material (ε-(γ-glutamyl)lysine) or a mixture of standard amino acids (AA-S-18, Sigma, Steinheim, Germany). The contents of (ε-(γ-glutamyl)-lysine were calculated as μM g^{-1} protein (Mautner, Meisel, Lorenzen, & Schlimme, 1999).

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Unheated and preheated milk samples, before and after transglutaminase reaction were analysed by SDS-PAGE under reducing conditions using the method described by Færgemand et al. (1997). The separation of protein bands was carried out on a Phastgel homogeneous 12.5% gel using Phastsystem™ (Pharmacia, Allerød, Denmark). The bands were quantified by analysing scanned images of the gels using Scion Image software, version 4.0.1 (Scion Corporation, Maryland, USA).

2.6. Capillary gel electrophoresis

Capillary gel electrophoresis was performed on Waters Quanta 4000 Capillary Electrophoresis System (Waters, Division of Millipore, Milford, MA, 01757, USA) using an untreated silica capillary 50 μm inner diameter. The total length of the capillary was 64.5 cm while the effective length was 56 cm.

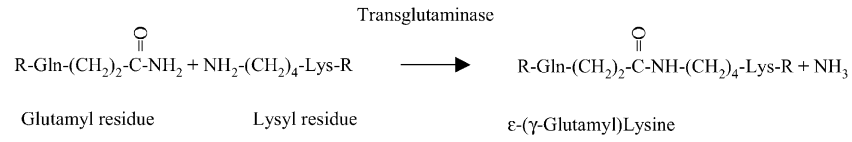
The lyophilised sample (150 mg) was first reconstituted in 1350 μL deionised water and 100 μL of reconstituted sample was mixed with 200 μL of sample buffer (10 M urea and 0.25% dithiothreotol in deionised water). The sample was injected hydrodynamically at the cathodic end at 50 mbar for 5 s. The run buffer consisted of 20 mM sodium citrate buffer, pH 3.0 ± 1 (adjusted with citric acid), containing 6 M urea and 0.02% hydroxypropylmethylcellulose. The separation was carried out at a constant voltage of 25 mV at 45°C for 50 min. The eluted components were detected at 214 nm. Data collection and processing was carried out using Millennium 2010 Chromatography Manager version 2.1 (Waters Chromatography Division, Milford, MA, USA). Between each injection, the capillary was reconditioned by either flushing with the run buffer for 5 min (for preheated milk samples) or with a sequence of 0.1 M NaOH for 2 min, deionised water for 2 min and run buffer for 3 min (for unheated milk samples).

3. Results and discussion

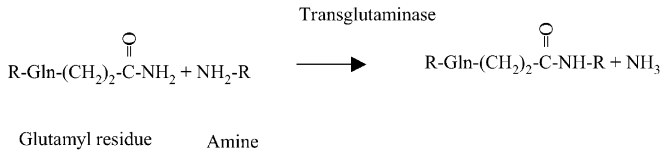
3.1. Ammonia and ε-(γ-glutamyl)lysine

Through an acyl transfer reaction involving protein-bound glutamyl and lysyl side chains, transglutaminase catalyses a protein crosslinking reaction (Motoki & Seguro, 1998). Apart from the crosslinking reaction, the other two reactions that are catalysed by transglutaminase are deamidation and amine incorporation. The reactions catalysed by transglutaminase are shown below.

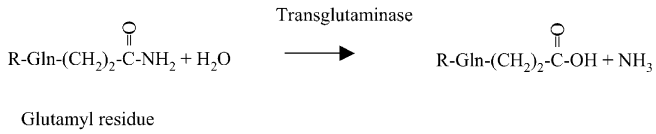
Crosslinking



Amine incorporation



Deamidation



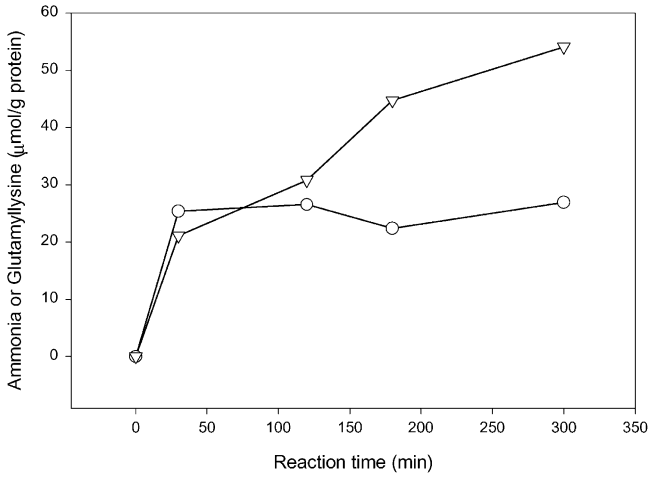


Fig. 1. Effect of transglutaminase reaction time on the amount of ammonia and ε-(γ-glutamyl)lysine contents of preheated (85°C for 15 min) skim milk. The enzyme preparation was added to the skim milk (enzyme/substrate ratio 0.1) and the mixture was incubated at 40°C for 30, 90, 180 or 300 min. The enzymatic reaction was terminated by heating the mixture at 80°C for 2 min. The ε-(γ-glutamyl)lysine content was measured by an ion-exchange chromatography after extensive hydrolyses of peptide bonds with selective peptidases. Triangle symbols represent ammonia while the circle symbols represent ε-(γ-glutamyl)lysine contents.

As all the above reactions result in an increase in free ammonia, measurement of ammonia can be used as a tool to monitor the overall transglutaminase reaction. Skim milk without reaction with the transglutaminase contained approximately 0.4 mM ammonia, which showed no noticeable change with time up to 300 min. The net amount of ammonia generated from the transglutaminase reaction with milk proteins in preheated milk is shown in Fig. 1. From the figure, it appears that the release of ammonia was more rapid during the first 30 min than later in the reaction, suggesting that the available reactive sites were rapidly modified.

Although the measurement of ammonia provides information on the overall transglutaminase reaction, it does not directly measure the crosslinking effect which is of interest when it comes to modifying the functional properties of milk proteins. One of the methods of direct measurement of the crosslinking reaction is the estimation of ε-(γ-glutamyl)lysine, the isopeptide formed during the transglutaminase reaction (Kurth & Rogers, 1984). As this isopeptide is not susceptible to cleavage by proteases that hydrolyse ordinary peptide bonds (Miller & Johnson, 1999), its estimation is possible once other peptides have been broken down. The efficiency of breakdown of other peptides can be judged by the estimation of the free amino acids. Table 1 shows the results for free amino acids obtained from preheated skim milk that was either untreated (0 min) or treated with transglutaminase for varying time intervals. The results from the table suggest that, the transglutaminase-treated samples had reduced amounts of the free amino acids compared with the untreated sample. The biggest reduction in the free amino acids was obtained during the first 30 min of transglutaminase reaction. After 30 min, the further increase in the reaction time caused a little or no change in the free amino acid contents. Overall, there were 20–30% less free amino acids in the transglutaminase-treated skim milk as compared with the untreated milk (Table 1). This result supports the

Table 1
Effect of transglutaminase reaction time on the amount of free amino acids (FAA) in preheated (85°C for 15 min) skim milk. The enzyme preparation was added to the skim milk (enzyme substrate ratio 0.1) and the reaction was carried out at 40°C

Amino acid	FAA (μmol g^{-1} protein) in relation to reaction time				
	0 min	30 min	120 min	180 min	300 min
Asp	156	108	123	127	114
Thr	305	221	243	233	222
Ser	598	439	461	471	420
Glu	356	260	260	294	242
Gly	183	118	98	142	91
Ala	351	241	243	266	227
Cys	16	13	—	12	—
Val	488	378	364	361	400
Met	167	91	100	97	159
Ile	337	235	249	276	229
Leu	657	513	526	527	519
Tyr	331	236	247	263	236
Phe	303	219	216	221	231
His	167	129	133	129	124
Lys	466	335	341	339	331
Arg	174	121	124	136	139
Pro	253	198	180	164	161
Σ (FAA)	5308	3855	3908	4058	3845

finding of Mautner et al. (1999) who found that 15–20% of free amino acids were undetected when protein was hydrolysed with proteases. These undetected amino acids, however, were detectable when hydrolysis was carried out with hydrochloric acid. In the present study, the hydrolysis was confined to proteases only so as to keep the dipeptide ε-(γ-glutamyl)lysine intact.

The amount of ε-(γ-glutamyl)lysine (μM g^{-1} protein) as plotted in Fig. 1, showed that there was a rapid increase in the ε-(γ-glutamyl)lysine content during the first 30 min of the enzyme reaction followed by little or no change after 30 min. Based on this observation, one would be tempted to conclude that almost all the crosslinking occurred during the first 30 min of transglutaminase reaction. However, it appears that due the formation of inter- and intra-molecular ε-(γ-glutamyl)-lysine bonds during the first 30 min, some ε-(γ-glutamyl)lysine bonds may be trapped and thus not accessible for estimation by the method used here. The evidence for such observation is drawn from the fact that 20–30% of amino acids could not be released after reaction with transglutaminase for 30 min. It is likely that 20–30% ε-(γ-glutamyl)lysine dipeptides were underestimated (an amount equivalent to the amount of total underestimated amino acids). Therefore, it appears that although most of the crosslinking occurred during the first 30 min, an accurate estimation of the ε-(γ-glutamyl)lysine content after 30 min may not have been possible.

As suggested previously, transglutaminase catalyses two other reactions (i.e. amine incorporation and deamidation) and all the three reactions generate ammonia (Motoki et al., 1986). As the amounts of ammonia produced during the first 30 min of the transglutaminase reaction is similar to the amount of ε-(γ-glutamyl)lysine (approximately 20 μmol g^{-1} protein), it is likely that the ammonia produced in the early reaction (30 min) is solely due to the crosslinking reaction while in the late reaction, deamidation and/or amine incorporation must occur.

From the above results, it is also obvious that both the methods (i.e. ammonia and ε-(γ-glutamyl)lysine) have their limitations, and thus may still not differentiate among the different reactions after 30 min. Therefore, there is a need to follow other techniques, such as chromatography and electrophoresis to compliment the above methods.

3.2. SDS-PAGE

Reduced SDS-PAGE profiles showed that the thickness of major protein bands (especially α_s- and β-caseins) in unheated milk decreased after 30 min incubation with transglutaminase followed by little or no change with the further increase in the reaction time. In contrast, the thickness of protein bands in the preheated milk although decreased considerably during the first 30 min of reaction, decrease was also noted after 30 min of the reaction time. The reaction of transglutaminase in the preheated milk also resulted in the formation of a new high-molecular weight protein band that resolved in the stacking gel, the thickness of which increased with the reaction time (Fig. 2).

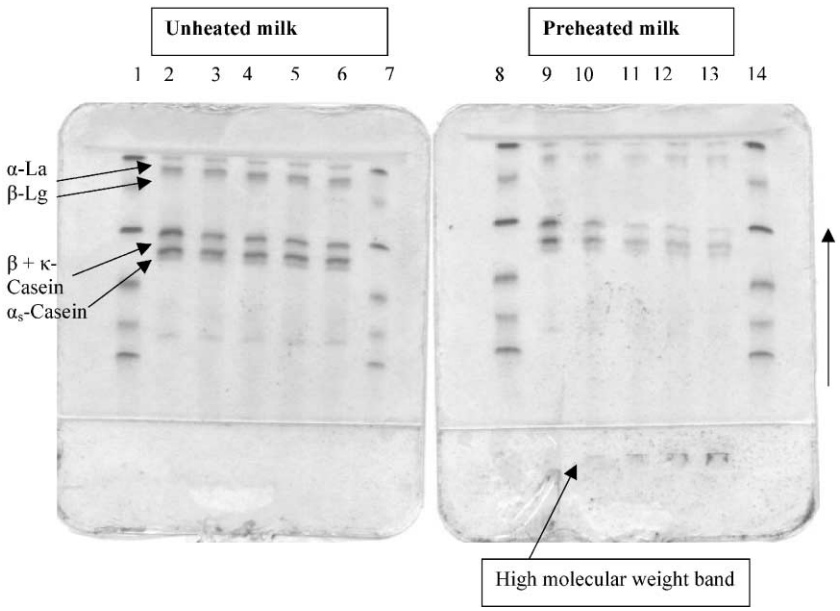


Fig. 2. SDS-PAGE (reduced) patterns of skim milk samples treated with transglutaminase for 0 min (control, lanes 2 and 9), 30 min (lanes 3 and 10), 90 min (lanes 4 and 11), 180 min (lanes 5 and 12) and 300 min (lanes 6 and 13). Lanes 1, 7, 8, and 14 had molecular weight standards (from top to bottom: 14000, 20100, 30000, 43000, 67000 and 94000 Da, respectively). Preheating and incubation conditions are given in Fig. 1.

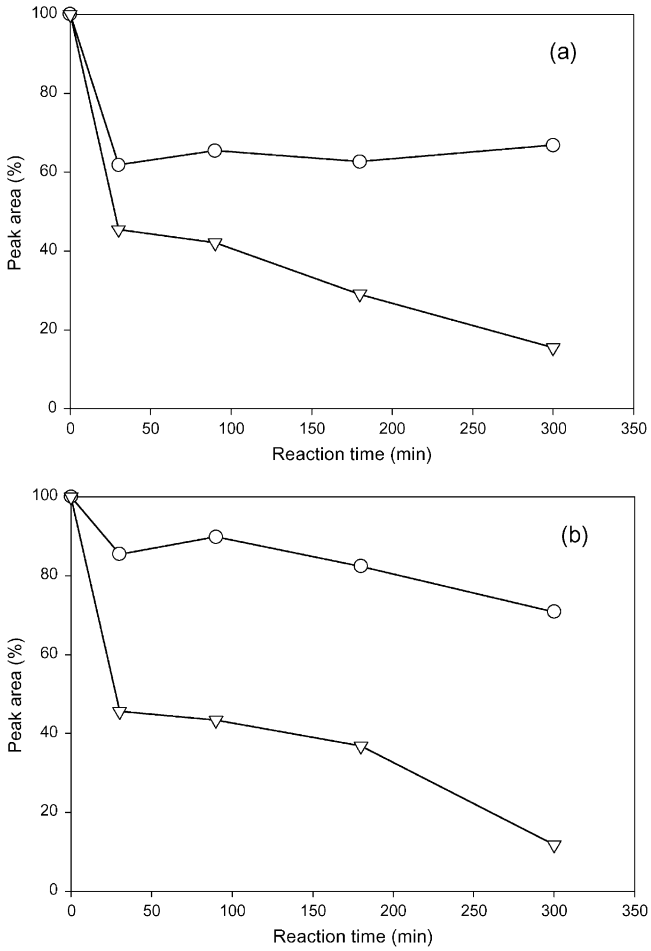


Fig. 3. Effect of transglutaminase reaction time on total casein (sum of α_s-, β- and κ-casein): (a) and total whey proteins (sum of β-lactoglobulin and α-lactalbumin); (b) as resolved in the reduced SDS-PAGE. Circle symbols represent unheated skim milk while the triangle symbols represent preheated skim milk. Preheating and incubation conditions are given in Fig. 1.

Quantitative changes in summed casein and whey proteins are shown in Figs. 3a and 3b. In the unheated milk, the reaction time of 30 min caused a decrease in the intensity of casein bands but further increase in the reaction time caused no further change (Fig. 3a). The preheating of skim milk resulted in a considerable decrease in the intensity of casein (more than 50%) during the first 30 min of transglutaminase reaction. Further increase in the reaction time caused a further decrease in the intensity of casein. During the reaction with transglutaminase, the whey proteins in unheated milk showed only a small decrease with the increase in the reaction time (Fig. 3b). The decrease in the whey protein in preheated milk was more prominent during the first 30 min of reaction followed by a gradual decrease with the increase in the reaction time.

These results suggest that some crosslinking of proteins does occur in the unheated milk (especially during the first 30 min) but a heating step can cause a noticeable increase in the crosslinking of proteins. As the thickness of the new, high-molecular weight protein band increased with the reaction time, it can be suggested that the increased level of crosslinking occurred with the increase in the reaction time.

3.3. Capillary gel electrophoresis (CGE)

The CGE profiles for unheated milks treated with transglutaminase showed only small changes with κ-casein peak showing the most change with the increase in the reaction time (profiles not shown). In the preheated milk, the casein peaks showed a sharp decrease after the first 30 min of transglutaminase reaction followed by a gradual decrease in their peak areas with the increase in the reaction time (Fig. 4). After 30 min of reaction, α_{s1}- and κ-casein peaks (peaks 4–6) could not be resolved well as these peaks merged to form a combined peak. The whey proteins (peaks 1and 2) showed considerable reduction during the first 30 min while afterwards, the changes in the whey protein peaks were small (Fig. 4).

The quantitative analyses of peaks in CGE profiles of unheated and heated milks are presented as graphs in Fig. 5. In unheated milk, the peak area of α_{s1}-casein 8 phosphate (8P) decreased during the first 30 min of reaction with transglutaminase (Fig. 5a). Further increase in the reaction time did not cause any further reduction in the peak area of α_{s1}-casein 8P. In the preheated milk, the incubation time up to 90 min caused a decrease in the peak area of α_{s1}-casein 8P, followed by a slight increase in peak area with time. The slight increase in the peak area after 90 min could be due to an error in the peak baseline detection as the emergence of the very broad peak next to α_{s1}-casein 8P might have interfered and contributed to α_{s1}-casein peak area determination (see Fig. 4).

Peak area of α_{s1}-casein 9P casein in unheated milk showed a trend similar to that obtained for α_{s1}-casein 8P (Fig. 5b). In the preheated milk, the peak area decreased during the first 30 min of reaction. After 30 min, the change in the peak area could not be measured as the peak merged into a new, broad peak that appeared at about 28 min in the elution profile as shown in Fig. 4.

In unheated milk, the reaction with transglutaminase reduced the peak area of α_{s2}-casein during the first 30 min of reaction (Fig. 5c). Further reaction time did not show any change in the peak area of α_{s2}-casein. In the preheated milk, the sharp decrease during the first 30 min was followed by a gradual decrease in the peak area of α_{s2}-casein.

For β-casein A^1 and A^2, reaction with transglutaminase in the heated milk decreased the peak area, with the largest decrease occurring during the first 30 min of reaction (Figs. 5d and e). In the heated milk, the decrease in the peak area of β-caseins was more severe

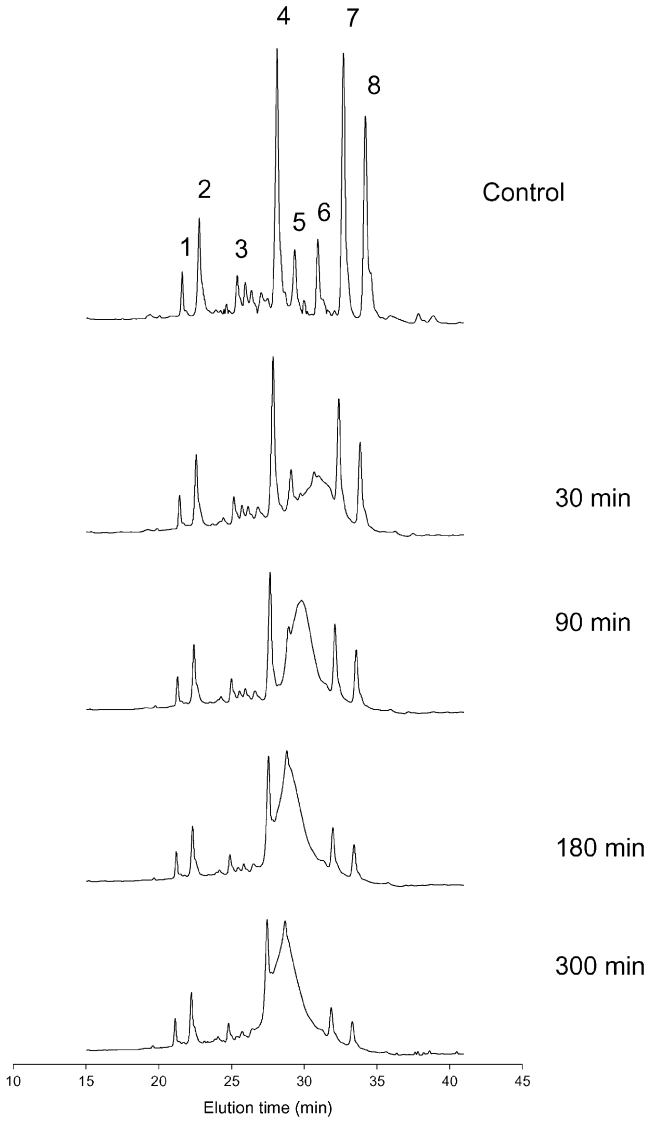


Fig. 4. Capillary gel electrophoresis profiles of preheated skim milk treated with transglutaminase for varying time intervals. The peaks, as identified using pure proteins are α-lactalbumin (peak 1), β-lactoglobulin (peak 2), α_{s2}-casein (peak 3), α_{s1}-casein, 8P (peak 4), α_{s1}-casein, 9P (peak 5), κ-casein (peak 6), β-casein A^1 (peak 7) and β-casein A^2 (peak 8). Preheating and incubation conditions are given in Fig. 1.

than that noticed in the unheated milk. Again, the largest decrease was noticed during the first 30 min of reaction.

Reaction with transglutaminase in unheated milk caused a considerable decrease in the peak area of κ-casein (Fig. 5f). After 300 min of reaction with the transglutaminase, almost no κ-casein was left as a monomer. In the preheated milk, the changes in the peak areas for κ-casein could not be monitored accurately as the new, broad peak formed by the transglutaminase reaction eluted at the same place as the κ-casein.

Without heat treatment, the peak area of β-lactoglobulin was unaltered by the transglutaminase reaction (Fig. 5g). However, when the milk was preheated, β-lactoglobulin showed a gradual decrease with the increase in the reaction time. The peak area of α-lactalbumin decreased during the first 30 min of transglutaminase reaction in both the unheated and preheated milks (Fig. 5h). Further increase in the reaction time had no effect on the peak area of α-lactalbumin.

Thus, the results suggest that in the unheated milk, most reduction in proteins occurred during the first 30 min of the transglutaminase reaction followed by a little or no change, with κ-casein showing the largest reduction followed by β-caseins. As evident from the Fig. 5, the largest reduction in the peak area for all proteins in preheated milk also occurred during the first 30 min of reaction. This was followed by a gradual reduction in the peak areas of all proteins. In preheated milk, the largest reduction in the peak area due to the transglutaminase reaction was observed for β-casein. In the first 30 min, nearly 65% of β-casein was involved in the crosslinking reaction. Further increase in the reaction time caused a further reduction in the peak area of β-casein. After 300 min of reaction, nearly 90% of β-casein was converted from the monomeric form and presumably eluted into the new peak observed at the elution time of 28 min (cf. Fig. 4).

There are no previous studies comparing the relative susceptibility of caseins in the natural casein micelle; however, Traoré and Meunier (1991) while studying the crosslinking behaviour of pure α_s-, β- and κ-caseins, reported that although all the three proteins were susceptible to crosslinking by human placental factor $XIII_a$, κ- and β-caseins were more susceptible than α_s-casein. The low reactivity of α_s-casein towards the human placental factor $XIII_a$ was postulated to be due to the self polymerised structure of α_s-casein that buried the active glutamyl sites. In another study where a guinea pig liver transglutaminase was used, κ-casein was found to be the least reactive (Ikura et al., 1980). Traoré and Meunier (1991) attributed the poor susceptibility of κ-casein towards crosslinking to the poor specificity of the guinea pig liver transglutaminase towards glutamyl residue. It is also likely that κ-casein may have formed polymers in the study by Ikura et al. (1980).

Our results on skim milk would suggest that both κ- and β-caseins in the natural casein micelle are more susceptible to crosslinking than α_s-casein. Even without heating, κ-casein was readily accessible to the enzyme. Preheating of skim milk considerably enhanced the crosslinking effect of transglutaminase on κ- and β-caseins. In milk, preheating causes denaturation of whey proteins and their interactions with casein micelle. Both these heat-induced reactions appear to enhance the susceptibility of proteins to transglutaminase reactions.

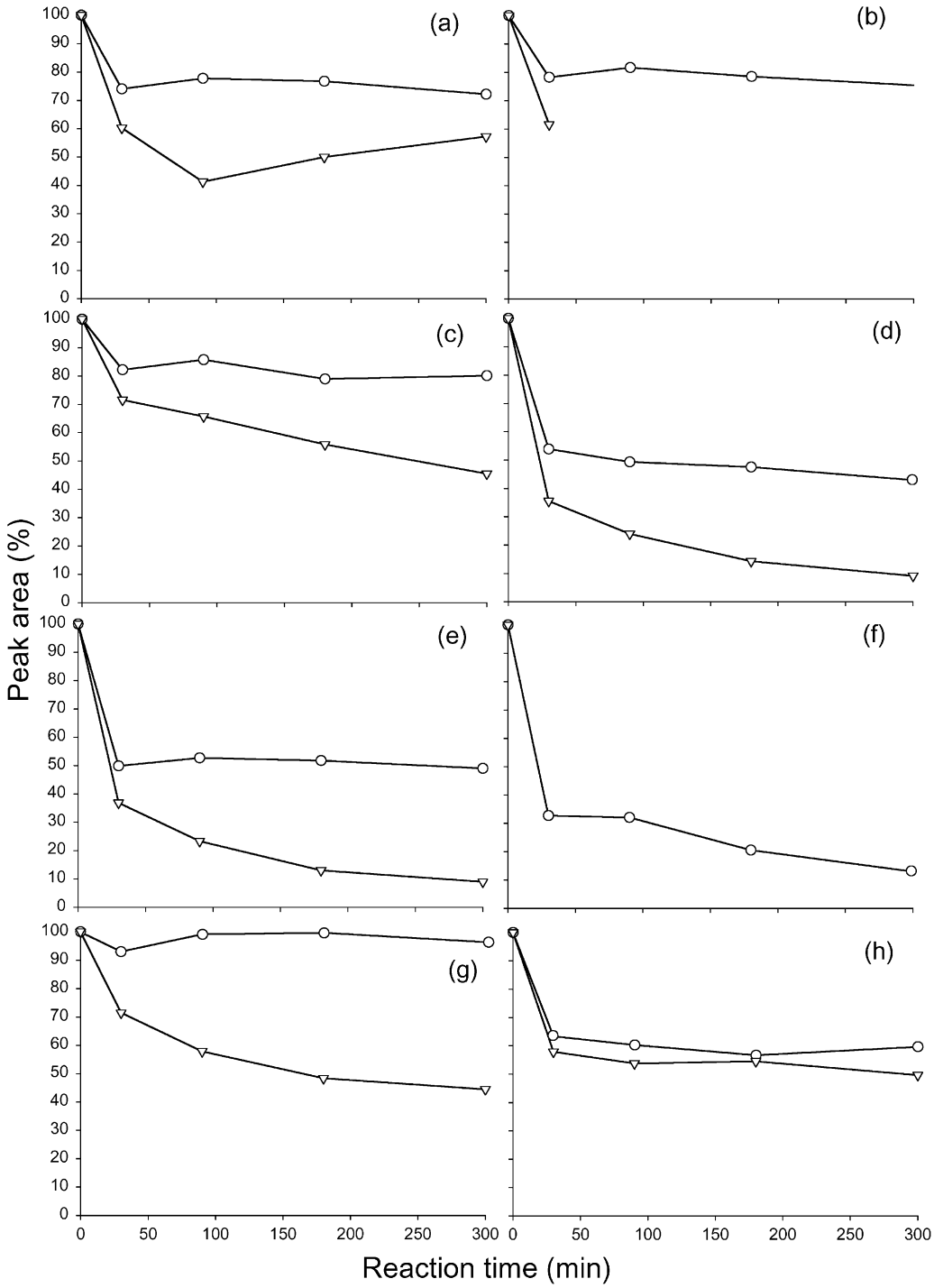


Fig. 5. Peak areas of capillary gel electrophoresis profiles of individual proteins obtained from skim milk treated with transglutaminase. The proteins are α_{s1}-casein, 8P (a), α_{s1}-casein, 9P (b), α_{s2}-casein (c), β-casein A^1 (d), β-casein A^2 (e), κ-casein (f), β-lactoglobulin (g) and α-lactalbumin (h). Circle symbols represent unheated skim milk while the triangle symbols represent preheated skim milk. Preheating and incubation conditions are given in Fig. 1.

The strong reactivity of κ-casein in milk towards the transglutaminase reactions is hardly surprising, as many researchers have presented evidences for the peripheral location of κ-casein in casein micelles. The porous nature of casein micelles probably allows the transglutaminase to infuse into the interior of the micelle. In the interior, it appears that β-casein is more easily accessible than α_s-casein. In the casein micelle structure, it is likely that α_s-casein forms the backbone structure whereas β-casein may be more in a dynamic state. β-casein

contains more prolyl residues than other caseins, which gives it a more disordered, flexible and open structure that could facilitate the enzymatic reaction. Its ability to move in and out of the micelle room temperature ($\sim 20^{\circ}C$), as shown in the studies involving dissociation of the casein micelle (Snoeren, Klok, van Hooydonk, & Damman, 1984; Holt, Davies, & Law, 1986; van Hooydonk, Hagedoorn, & Boerrigter, 1986), may also prove to be a reason for the relative ease of the availability of β-casein for the enzymatic reaction.

4. Conclusions

Crosslinking, deamidation and/or amine incorporation occurred in skim milk when it was treated with transglutaminase. The initial transglutaminase reaction (first 30 min) was dominated by the crosslinking while deamidation and/or amine incorporation were more dominant after 30 min. Milk proteins in unheated skim milk were susceptible to crosslinking by transglutaminase; however, preheating of milk at 85°C for 15 min enhanced the susceptibility of milk proteins towards crosslinking. Crosslinking led to a decrease in the monomeric forms of all proteins and an increase in large molecular weight polymers. In unheated milk, of all proteins, κ-casein was the most susceptible to crosslinking. In preheated milk, of all proteins, β- and κ-caseins were most susceptible to crosslinking.

Concerning whey proteins, it could be shown that β-lactoglobulin was susceptible to transglutaminase action only after heat treatment, while α-lactalbumin was crosslinkable with or without preheating. Detection of ammonia can be used as a tool to monitor the overall transglutaminase reaction; however, the evidence of crosslinking can be obtained by the detection and quantification of the isopeptide ε-(γ-glutamyl)lysine.

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