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# Susceptibility of the individual caseins in reconstituted skim milk to cross-linking by transglutaminase: influence of temperature, pH and mineral equilibria

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The susceptibility of total casein and the individual caseins in reconstituted skim milk to transglutaminase (TGase)-induced cross-linking was studied as a function of incubation temperature (5–40 °C), pH (5·0–7·0) and mineral addition. Within the ranges studied, the level of total casein cross-linked increased with increasing temperature, pH and concentration of added trisodium citrate, whereas adding calcium chloride had the opposite effect. These effects can be largely related to the effects of these parameters on TGase activity. In addition, the parameters were also found to influence the susceptibility of κ-casein, and to a lesser extent β-casein, to cross-linking, whereas the susceptibility of $\alpha_{s1}$-casein was not affected. The susceptibility of κ-casein to cross-linking increased with increasing temperature and calcium chloride addition, but decreased with increasing pH and citrate content, whereas the susceptibility of β-casein to TGase-induced cross-linking decreased with increasing temperature, but was not affected by other parameters. These findings highlight the fact that selection of environmental conditions during cross-linking can be applied to tailor the surface, and hence possibly colloidal stability, of casein micelles in TGase-treated milk.

**Keywords:** Casein micelles, transglutaminase, cross-linking, micelle structure.

Many technologies for improving the functionality of milk proteins are currently being investigated, with the aim of facilitating a reduction in ingredient cost, fat content or caloric content, or achieving an improved texture or stability of the final product. One technology which has received considerable attention over the past two decades is the enzymatic cross-linking of milk proteins. The main enzyme used for this purpose is transglutaminase (TGase), which catalyses inter-molecular cross-linking of protein molecules through the formation of isopeptide-bonds between lysine and glutamine residues. Of the milk proteins, the caseins are particularly susceptible to TGase-induced cross-linking due to their natively-unfolded structure; the whey proteins, in contrast, require pre-denaturation to facilitate TGase-induced cross-linking (for reviews see Jaros et al. 2006; Huppertz, 2009).

TGase-induced cross-linking has strong effects on the functionality of caseins. For instance, acid-induced coagulation of milk is enhanced (Anema et al. 2005; Huppertz & De Kruif, 2008), leading to improved textural properties of yoghurts prepared from TGase-treated milk (see Jaros et al. 2006) with the extent of the improvement in textural properties being strongly dependent on the degree of cross-linking (Jaros et al. 2010). In contrast, TGase treatment impairs the rennet-induced coagulation of milk (Lorenzen, 2000a; O'Sullivan et al. 2002a; Huppertz & De Kruif, 2007a; Huppertz, 2011). Furthermore, TGase treatment strongly influences the heat stability of unconcentrated and concentrated milk (O'Sullivan et al. 2001, 2002b; Huppertz & De Kruif, 2008) and improves the ethanol stability of milk (Huppertz & De Kruif, 2007b). Most of these effects can be related to TGase-induced changes in the colloidal stability of the casein micelles, in particular to changes in the properties of the casein 'brush' stabilising the micelles. Furthermore, TGase treatment also improves the stability of casein micelles against dissociation due to chelation of micellar calcium or improved solvent quality (O'Sullivan et al. 2002a; Smiddy et al. 2006; Huppertz et al. 2007; Huppertz & Smiddy, 2008).

In contrast to the abundance of information available on the opportunities for improving the functional properties of milk proteins with TGase, comparatively little is known

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about the factors influencing the degree and particularly the pattern of cross-linking of the proteins in milk by TGase. Several articles, mostly using TGase treatment of milk at 30–40 °C, have reported that κ-casein and β-casein are the most susceptible of the caseins to TGase-induced cross-linking, whereas the $\alpha_s$-caseins are least susceptible to TGase-induced cross-linking (Sharma et al. 2001; Smiddy et al. 2006; Hinz et al. 2007; Huppertz & De Kruif, 2007a, b).

However, when solutions of individual caseins are treated with TGase at 25 °C, the order of susceptibility to cross-linking is $\alpha_{s1}$-casein ≈ β-casein > κ-casein (Ikura et al. 1980) and, in sodium caseinate at 37 °C, the susceptibility of the caseins to cross-linking is κ-casein > $\alpha_s$-casein > β-casein (Tang et al. 2005). The susceptibilities of individual caseins to TGase-induced cross-linking in the casein micelles have been related to the location, and hence accessibility, of the respective caseins in the micelles (Smiddy et al. 2006), with κ-casein and, to a lesser extent, β-casein are predominant at the micellar surface, whereas the $\alpha_s$-caseins are largely found in the micellar core. However, casein micelle structure is extremely dynamic and is influenced by a wide number of readily adjustable parameters, most notably temperature, pH and the mineral balance in milk (Holt & Horne, 1996; De Kruif, 1999, De Kruif & Holt, 2003; Dalgleish, 2011). The changes in micelle structure induced by adjusting such parameters may also influence the accessibility of TGase to its substrate proteins. In this study, the influence of temperature, pH and added calcium or citrate on the degree and pattern of cross-linking of caseins in reconstituted skim milk was studied.

## Materials and Methods

### *Sample preparation*

Low-heat skim milk powder (NILAC, NIZO food research, Ede, The Netherlands) was reconstituted in demineralised water at a level of 10% (w/w), and stirred for 24 h at 5 °C to ensure complete hydration of the casein micelles and equilibration of the mineral balance. Sodium azide (0·5 g/l) was added to prevent microbial growth. Milk pH was adjusted to 5·50, 5·75, 6·00, 6·25 and 6·50 with 2 M HCl or to 6·75, 7·00 and 7·50 with 2 M NaOH. The influence of added salts was investigated by adding either 1 M $CaCl_2$ or solid trisodium citrate to aliquots of reconstituted skim milk at concentrations of 0–10 mmol/l for $CaCl_2$ or 0–25 mmol/l for trisodium citrate. In each case, the pH was readjusted to 6·6 following addition. For each treatment described above, three independent samples were prepared.

### *Incubation with TGase*

Activa WM TGase (declared activity 100 units/ g; Ajinomoto Co., Tokyo, Japan) was added at a level of 0·5 g/l to all milk samples, which were then incubated for 0–24 h at 5, 20, 30 or 40 °C; unless otherwise stated, incubation temperature was 30 °C. TGase-induced cross-linking of caseins was stopped by mixing samples with a reducing buffer containing, among others, urea and dithiothreitol (see below) before reversed-phase high performance liquid chromatography (RP-HPLC) analysis.

### *Reversed-phase high-performance liquid chromatography analysis*

The degree and pattern of TGase-induced cross-linking was determined by RP-HPLC. To stop TGase-induced cross-linking of milk proteins, samples were mixed with 3 volumes of a reducing buffer containing 0·1 mol/l Bis-Tris, 8 mol/l urea, 13 g/l trisodium citrate and 40 g/l dithiothreitol. The mixture was left at room temperature for 60 min, after which it was diluted further by the addition of 4 volumes of a 6 mol/l urea solution adjusted to pH 2·5 with trifluoroacetic acid (TFA). Prior to analysis, samples were filtered through 0·22-μm filters (Millipore, Billerica, MA, USA). Analytical RP-HPLC was carried out at 30 °C using a 250 × 4·6 mm Widepore $C_{18}$ column (Bio-Rad Laboratories, Richmond, CA, USA), with a $C_{18}$ cartridge (Bio-Rad Laboratories) as a guard column. Solvent A was a mixture of acetonitrile–water–TFA (20:980:1, v/v/v) and solvent B contained these components at a ratio 900:100:0.8 (v/v/v). The gradient used was adapted from that of Visser et al. (1991) and the absorbance of the eluent was monitored at 220 nm.

The peaks corresponding to the caseins were identified in the chromatograms according to Visser et al. (1991). A typical example of a RP-HPLC chromatogram of the milk samples before and after incubation with TGase for various times at 40 °C is shown in Fig. 1. Three casein fractions were distinguished: κ-casein, $\alpha_{s1}$-casein and β-casein. Peak areas for each of these three casein fractions were determined by integration. Although $\alpha_{s2}$-casein could be detected in the chromatograms, reliable quantification of the peak areas for this protein was not possible and therefore not included in this study. With increasing incubation time, progressive reductions in the peak areas of all caseins were observed (Fig. 1). Changes in the peak area for any individual casein fraction were taken to be the result of TGase-induced cross-linking, as this would result in the caseins not eluting at their original retention times, or, if the aggregates are sufficiently large, being retained in the filter during sample preparation. The residual peak area of a casein after *x* h incubation was expressed as a percentage of the peak area of that casein at 0 h; i.e., the percentage residual κ-casein refers to is defined as follows:

$$\%\ residual\ \kappa\text{-}casein_{xh} = 100 \times \frac{\text{peak area } \kappa\text{-casein}_{xh}}{\text{peak area } \kappa\text{-casein}_{0h}}$$

Consequently, the percentage of cross-linking κ-casein can be expressed as:

$$\%\ \text{cross-linked } \kappa\text{-casein}_{xh} = 100 - \%\text{residual } \kappa\text{-casein}_{xh}$$

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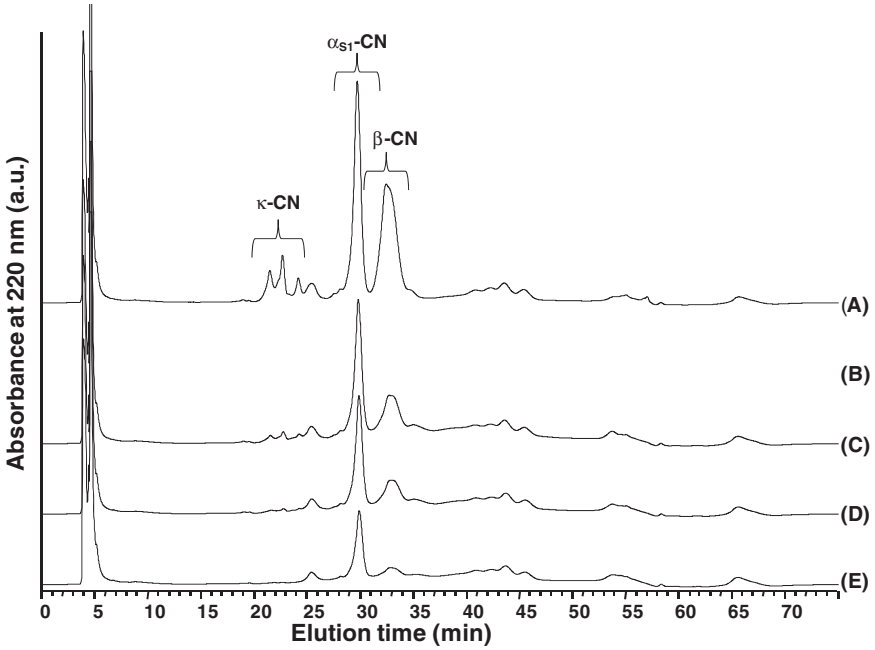


**Fig. 1.** RP-HPLC chromatogram of reconstituted skim milk incubated with TGase at 40 °C for (a) 0, (b) 2, (c) 4, (d) 8 or (e) 24 h. The peaks corresponding to the different casein fractions are identified in chromatogram

Total casein was expressed as the sum of the peak areas of $\alpha_{s1}$-, β- and κ-casein, and calculations of the percentage of residual native and cross-linked total casein were carried out as outlined above. Each RP-HPLC result was generated as an average of the data from three samples and the reliability of the results was confirmed by the fact that for each data point, the coefficient of variation was < 10% of the actual value.

## Results

### *Influence of incubation temperature*

The influence of incubation temperature (5, 20 or 40 °C) on the cross-linking of total casein by TGase is shown in Fig. 2a, while its influence on the susceptibility of $\alpha_{s1}$-, β- and κ-casein to TGase-induced cross-linking in reconstituted skim milk is shown in Fig. 2b–d. As expected, both higher temperatures and longer incubation periods resulted in a higher degree of total cross-linking of casein (Fig. 2a). Incubation temperature also markedly affected the susceptibility, as judged from the slopes of the lines, of the individual caseins to cross-linking. The proportion of cross-linked κ-casein at a given concentration of cross-linked total casein increased with incubation temperature (Fig. 2b); this suggests that the susceptibility of κ-casein in reconstituted skim milk to TGase-induced cross-linking increases with increasing temperature. No influence of temperature on the degree of cross-linking of $\alpha_{s1}$-casein, expressed as a function of the degree of cross-linked total casein, could be observed (Fig. 2c), suggesting that the susceptibility of $\alpha_{s1}$-casein to TGase-induced cross-linking is unaffected by incubation temperature. Finally, the susceptibility of β-casein to TGase-induced cross-linking showed a trend opposite to that observed for κ-casein, i.e., β-casein showed highest susceptibility to TGase at 5 °C and this decreased steadily as a function of increasing incubation temperature (Fig. 2d). The order of susceptibility of the individual caseins to cross-linking was β-casein > $\alpha_{s1}$-casein > κ-casein at 5 °C, whereas it was β-casein > κ-casein ≈ $\alpha_{s1}$-casein at 20 °C and β-casein ≈ κ-casein > $\alpha_{s1}$-casein at 40 °C (Fig. 2).

### *Influence of pH*

The influence of milk pH on the susceptibility of caseins in milk to TGase-induced cross-linking is shown in Fig. 3. The pH range 5·5–7·5 was chosen for studying the influence of pH on TGase-induced cross-linking because it is practically-applicable in dairy processing and does not induce micellar flocculation. Throughout incubation, the level of total cross-linked casein was higher at higher incubation pH (Fig. 3a), which is in line with the optimum pH of TGase being around 7·0–7·5. The susceptibility of κ-casein in milk to TGase-induced cross-linking decreased with increasing pH (Fig. 3b), whereas that of $\alpha_{s1}$-casein (Fig. 3c) or β-casein (Fig. 3d) was not affected by pH. The order of susceptibility of the individual caseins in milk to TGase-induced cross-linked gradually changed from κ-casein > β-casein > $\alpha_{s1}$-casein at pH 5·5 to β-casein > κ-casein > $\alpha_{s1}$-casein at pH 7·5 (Fig. 3b–d).

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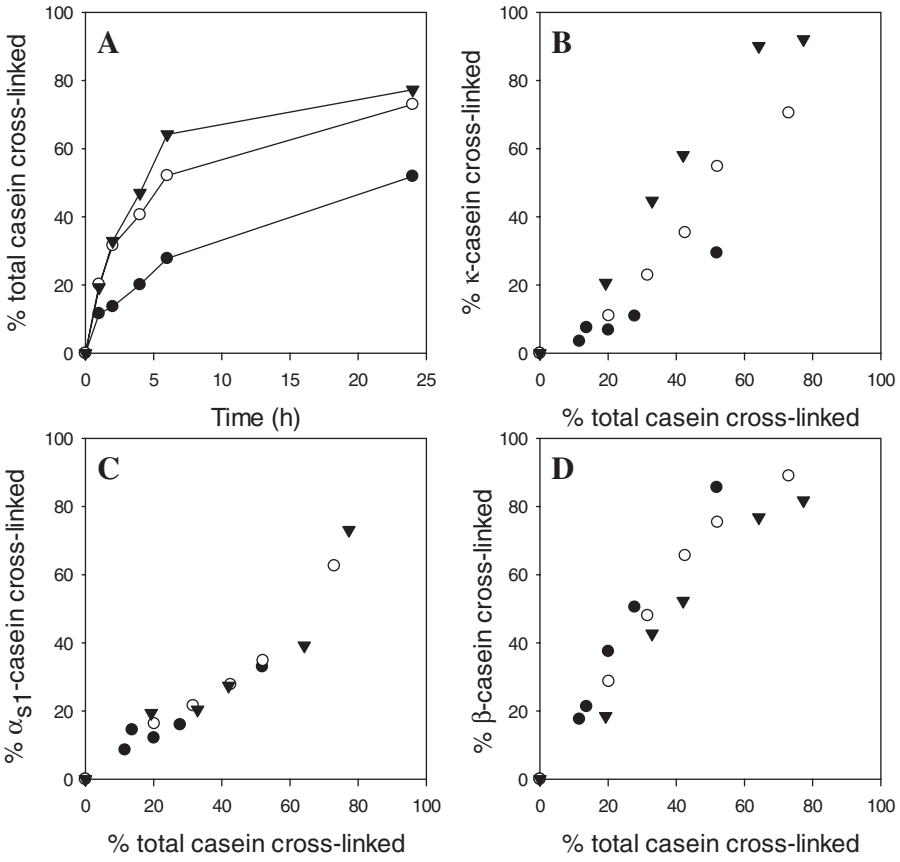


**Fig. 2.** Cross-linking of (a) total casein as a function of incubation time and (b) κ-casein, (c) $\alpha_{s1}$-casein and (d) β-casein, expressed as a percentage of total cross-linked casein, in reconstituted skim milk on incubation with TGase at 5 (●), 20 (○) or 40 (▼) °C. Values are means of data from three individual experiments. The coefficient of variation was <10% of the actual value for each data point

*Influence of adding calcium chloride or trisodium citrate*

Up to 10 mM $CaCl_2$ was added to reconstituted skim milk to evaluate its influence on the cross-linking of caseins by TGase (Fig. 4a–d). To avoid additional effects due to the decrease in pH on addition of $CaCl_2$, the pH of all samples was readjusted to 6·6 after addition of $CaCl_2$. The degree of total cross-linked casein was found to decrease slightly with increasing level of added $CaCl_2$ (Fig. 4a). In contrast, susceptibility of κ-casein to TGase-induced cross-linking increased with increasing concentration of added $CaCl_2$. For $\alpha_{s1}$-casein and β-casein, the susceptibility to TGase-induced cross-linking in milk was not affected by $CaCl_2$ (Fig. 4c, d). The order of susceptibility to TGase induced cross-linking changed from β-casein > κ-casein > $\alpha_{s1}$-casein for milk with no added $CaCl_2$ to κ-casein > β-casein > $\alpha_{s1}$-casein for milk with 10 mM added $CaCl_2$.

The influence of the addition up to 25 mM of the calcium-chelating agent trisodium citrate was also investigated (Fig. 5). As for $CaCl_2$, the pH of samples was readjusted to pH 6·6 following addition of citrate. In general, the addition of citrate showed opposite effects compared with those observed for $CaCl_2$. The degree of cross-linking of total casein increased with increasing level of added citrate (Fig. 5a), whereas for κ-casein, a clear reduction in the susceptibility to TGase-induced cross-linking was observed with increasing concentration of added citrate (Fig. 5b). For $\alpha_{s1}$-casein (Fig. 5c) and β-casein (Fig. 5d), no differences in susceptibility to TGase-induced cross-linking were observed as a result of adding trisodium citrate. The order of susceptibility to TGase-induced cross-linking changed from β-casein > κ-casein > $\alpha_{s1}$-casein in milk without added trisodium citrate to β-casein > $\alpha_{s1}$-casein > κ-casein in milk with 25 mM added citrate (Fig. 5b–d).

## Discussion

The susceptibility of total casein and the individual caseins to TGase-induced cross-linking is influence by a wide variety of physicochemical properties (Figs. 2–5). A summary of these trends is provided in Table 1; the cross-linking of total casein and of κ-casein was strongly affected by all parameters studied. Susceptibility of β-casein was affected to a lesser extent, whereas the susceptibility of $\alpha_{s1}$-casein to TGase-induced cross-linking in milk was unaffected by parameters studied (Table 1). This suggests that control of

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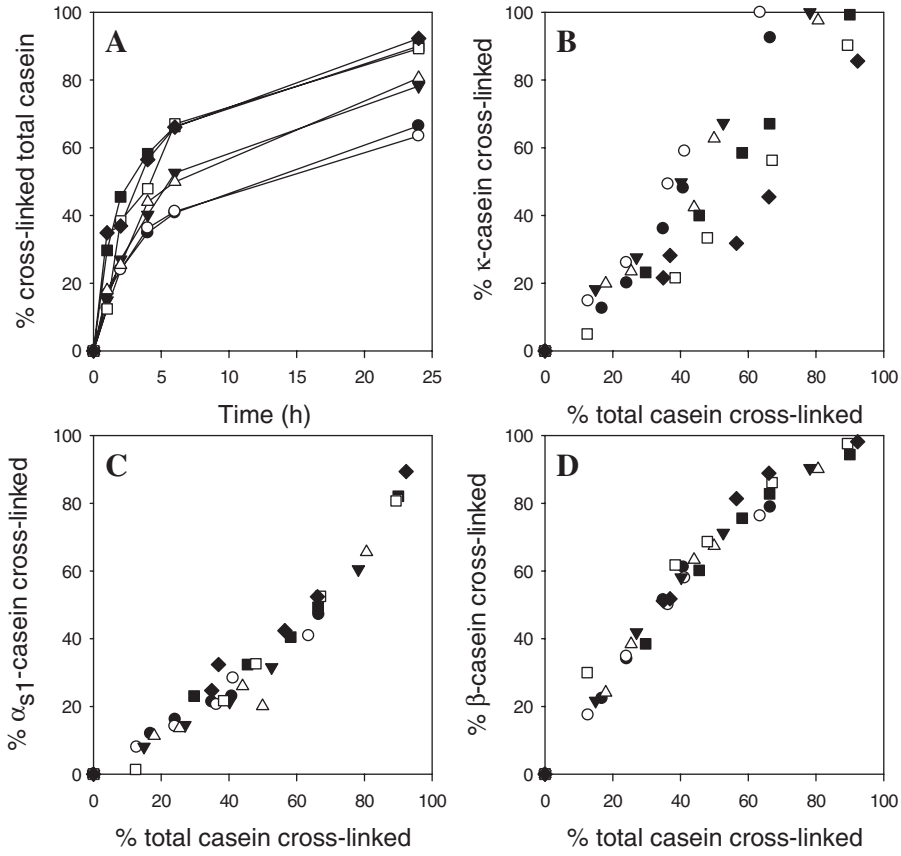


**Fig. 3.** Cross-linking of (a) total casein as a function of incubation time and (b) κ-casein, (c) $\alpha_{s1}$-casein and (d) β-casein as a percentage of total cross-linked casein, in reconstituted skim milk, during incubation at 30 °C following adjustment to pH 5·5 (●), 6·0 (○), 6·25 (▼), 6·5 (△), 6·75 (■), 7·0 (□) or 7·5 (♦). Values are means of data from three individual experiments. The coefficient of variation was < 10% of the actual value for each data point

physiochemical parameters can be used to tailor the degree of cross-linking of caseins and that of the caseins on the surface of the casein micelles. Effects of the variables studied on the cross-linking of total casein can be related to both the influence on TGase activity as well as enhanced or reduced substrate accessibility. In the case of susceptibility of individual caseins to TGase cross-linking, their expression as a function of total casein cross-linked (as shown in Figs. 2–5b–d) omits TGase activity as a parameter of consideration. This thus shows a direct measure of the influence of the studied parameters on the susceptibility of the individual caseins to TGase-induced cross-linking.

The increased cross-linking of total casein with increasing temperature (Fig. 2a) and pH (Fig. 3a) is in line with expected trends for temperature and pH in terms of activity of TGase; TGase activity has been shown to increase with temperature in the range 5–50 °C and be highest around pH 7·0 (Seguro et al. 1996). Likewise, the decrease in cross-linking of total casein with increasing amount of added calcium chloride (Fig. 4a) is in line with the reported decrease in TGase activity with increasing calcium concentration (Kütemeyer et al. 2005). The increased degree of total cross-linking of casein with increasing level of the calcium chelator trisodium citrate added (Fig. 5a) appears to be a logical extension of the results of Kütemeyer et al. (2005). Furthermore, the addition of citrate to milk leads to partial disruption of casein micelles (Udabage et al. 2000; Smiddy et al. 2006) and may also increase susceptibility of caseins to cross-linking, analogous to the higher susceptibility to cross-linking observed for non-micellar casein, i.e., sodium caseinate, compared with micellar casein (Lorenzen, 2000b).

The order of susceptibility of the individual caseins to cross-linking at 30–40 °C, i.e., κ-casein ≈ β-casein > $\alpha_{s1}$-casein (Figs. 2–5) is in line with previous studies (Sharma et al. 2001; Smiddy et al. 2006; Hinz et al. 2007; Huppertz & De Kruif, 2007a, b) despite the fact that, of the individual caseins, κ-casein is the least susceptible to TGase-induced cross-linking (Ikura et al. 1980). This suggests that it is not the actual properties of κ-casein but its ready accessibility on the micellar surface that makes it the most susceptible to cross-linking. However, as shown in Figs. 2–5 and summarized in Table 1, deviations are observed when TGase-induced cross-linking of micellar casein was studied under different conditions. As it is particularly the susceptibility of κ-casein, located on the micelle surface, which is affected by variables

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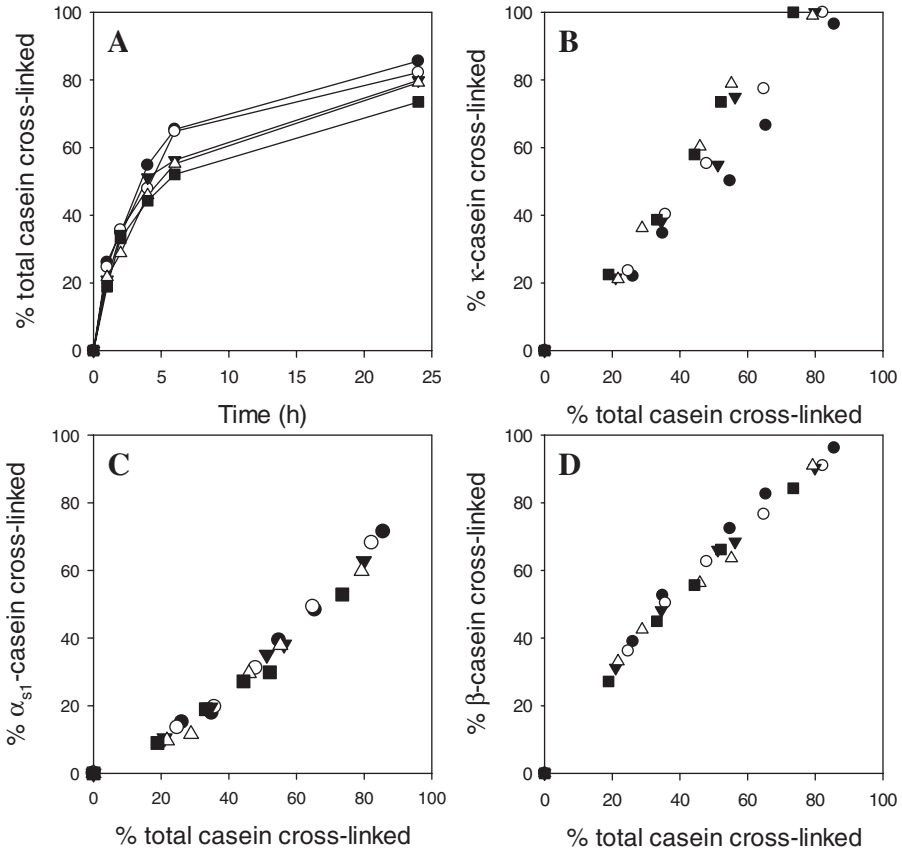


**Fig. 4.** Cross-linking of (a) total casein as a function of incubation time and (b) κ-casein, (c) $\alpha_{s1}$-casein and (d) β-casein as a percentage of total cross-linked casein, in reconstituted skim milk, during incubation at 30 °C in the presence of 0 (●), 2 (○), 5 (▼), 7 (△) or 10 (■) mM $CaCl_2$. Values are means of data from three individual experiments. The coefficient of variation was <10% of the actual value for each data point

studied, whereas $\alpha_{s1}$-casein, located in the core of the micelles, is not affected, it appears that the trends summarized in Table 1 are primarily a result of the dynamic nature of the casein micelle surface.

The increased susceptibility of β-casein and reduced susceptibility of κ-casein to TGase-induced cross-linking at low temperature can be related to effects of temperature on casein micelle structure. NMR spectra of casein micelles show that the mobile part at 20–40 °C consists predominantly of the caseinomacropeptide (CMP) part of κ-casein (Rollema & Brinkhuis, 1989). As a result, κ-casein is readily accessible to TGase for cross-linking. When milk is cooled to 5 °C, considerable amounts of β-casein dissociate onto the micelle surface (De Kruif & Roefs, 1996) and into the milk serum (Davies & Law, 1983; Dalgleish & Law, 1988). This results in an increased accessibility of β-casein to TGase, as a result of which cross-linking is facilitated (Fig. 2). In addition, the presence of β-casein at and around the micellar surface at 5 °C can also hinder cross-linking of κ-casein, either due to the fact that β-casein is more preferentially cross-linked by TGase than κ-casein (Ikura et al. 1980) or due to steric hindrance for TGase with respect to its accessibility to κ-casein caused by the presence of β-casein at the surface. Cold dissociation of $\alpha_s$-casein is limited (Davies & Law, 1983; Dalgleish & Law, 1988) and, as a result, cross-linking of $\alpha_s$-casein by TGase is virtually independent of the temperature at which the cross-linking reaction is carried out (Fig. 2).

The increased susceptibility of κ-casein to TGase-induced cross-linking with decreasing pH (Fig. 3), in agreement with the results of Moon et al. (2009), is probably due to the fact that a decrease in pH results in a reduced solvency of the κ-casein, due to a progressive loss of net-negative charge; this eventually leads to collapse of the κ-casein hairs on the micelle surface but, in the pH range studied here, full collapse does not occur (De Kruif & Zhulina, 1996; De Kruif, 1999). In this state of reduced repulsion, accessibility of κ-casein on the micelle surface is likely to be increased and the susceptibility of κ-casein to TGase-induced cross-linking thus also increased (Fig. 3).

The increased susceptibility of κ-casein and reduced susceptibility of β-casein to TGase-induced cross-linking with increasing level of added calcium can also be related to effects of added calcium chloride on the casein distribution of milk. In milk without additives or pH adjustment, the casein micelle surface consists primarily of κ-casein but also partially of β-casein (Holt & Horne, 1996), whereas some of the β-casein is also non-micellar (Davies & Law, 1983;

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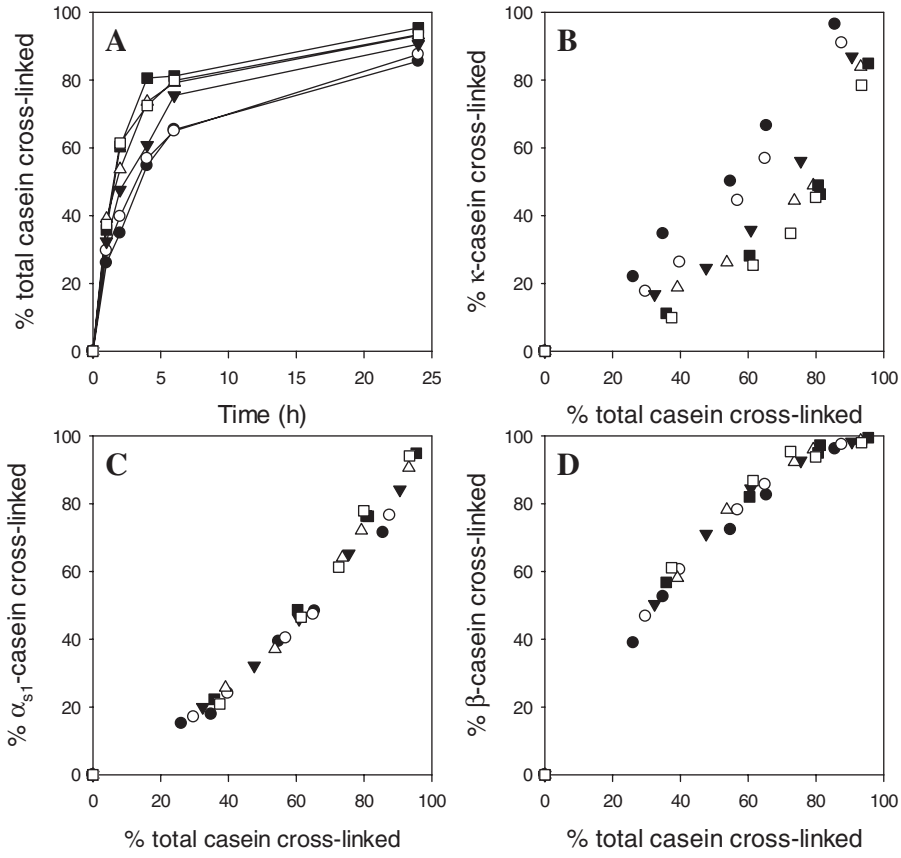


**Fig. 5.** Cross-linked total casein (a) as a function of incubation time and cross-linked κ-casein (b), $\alpha_{s1}$-casein (c) and β-casein (d) as a percentage of total cross-linked casein in reconstituted skim milk, during incubation with TGase at 30 °C in the presence of 0 (●), 5 (○), 10 (▼), 15 (△), 20 (■) or 25 (□) mM trisodium citrate. Values are means of data from three individual experiments. The coefficient of variation was <10% of the actual value for each data point

**Table 1.** Influence of physicochemical parameters studied on the susceptibility of total casein or the individual caseins to TGase-induced cross-linking. Susceptibility for individual casein fractions is expressed relative to the fraction of total casein cross-linked (see text). Trends displayed are as a function of an increase in the respective physicochemical variable, with the boundary conditions studied indicated between brackets. ↑ = increase in susceptibility, – = no change in susceptibility, ↓ = decrease in susceptibility

| | Total casein | $\alpha_{s1}$-casein | β-casein | κ-casein |
|---|---|---|---|---|
| Temperature (5–40 °C) | ↑ | – | ↓ | ↑ |
| pH (5·5–7·5) | ↑ | – | – | ↓ |
| Calcium chloride (0–10 mM) | ↓ | – | – | ↑ |
| Trisodium citrate (0–25 mM) | ↑ | – | – | ↓ |

Dalgleish & Law, 1988). The addition of calcium chloride results in the reassociation of non-micellar casein, particularly β-casein, with the casein micelles (Udabage et al. 2000) and removal of β-casein from the micelle surface (Holt & Horne, 1996), which enhances the susceptibility of κ-casein to cross-linking, analogous to the aforementioned effect of increasing temperature. Likewise, the decreased susceptibility of κ-casein to cross-linking with increasing level of citrate may be attributed to release of caseins from the core of the micelle, and hence reduced accessibility to κ-casein for cross-linking (Fig. 5).

## Conclusion

This systematic study has demonstrated how the degree of TGase-induced cross-linking of the individual caseins in reconstituted skim milk varies with incubation temperature, pH and salt concentrations. In particular, the cross-linking of proteins on the micellar surface was strongly dependent on environmental conditions. These findings offer opportunities to tailor the surface, and hence colloidal properties, of TGase-treated casein micelles by selection of conditions at which incubation is carried out and thus offers a tool for modifying functional properties such as rennet or acid gelation or heat-induced coagulation of milk.

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