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Simultaneous use of transglutaminase and rennet in milk coagulation: Effect of initial milk pH and renneting temperature

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ABSTRACT

The objective of the present study was to assess competitive interactions between transglutaminase (TGase) and rennet during rennet coagulation of skim milk. Rennet coagulation was achieved at two different renneting temperatures (30 °C and 34 °C), three initial milk pH levels (6.5, 6.3 and 6.1) and two TGase concentrations (0.6 U g^{-1} protein and 1.8 U g^{-1} protein). Results of the relative caseinomacropeptide in serum and degree of polymerization revealed that TGase influenced both the primary and secondary phases of rennet coagulation, respectively. Overall, the higher the renneting temperature and the lower the TGase level, the lower were the yields of the rennet gels. The coagulation times (t_c) of the gels decreased with decreased initial milk pH and increased coagulation temperature. The optimum initial milk pH, coagulation temperature and TGase concentration were determined to be 6.3, 30 °C and 1.8 U g^{-1} protein.

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1. Introduction

Transglutaminase (TGase; EC. 2.3.2.13), a transferase, is widely distributed in body secretions and most animal tissues (Sharma, Lorenzen, & Qvist, 2001). The function of this enzyme is to catalyse an acyl transfer reaction between the γ-carboxyamide group of peptide or protein-bound glutamine (an acyl donor) and primary amines (an acyl acceptor), including the ε-amino group of lysine residues (Liu & Damodaran, 1999). As a result of this specific reaction, new intra- and intermolecular crosslinks are formed, leading eventually to the modification of the structure of proteins. Milk proteins can be used as substrates by TGase. Although the open structure of caseins makes them readily available for the TGase action, whey proteins are not good substrates for this enzyme unless they are denatured (Sakamoto, Kumazawa, & Motoki, 1994; Schorsch, Carrie, Clark, & Norton, 2000; Traorè & Meunier, 1992). Faergemand, Otte, and Qvist (1997) showed that the cross-linking of whey proteins was facilitated in the presence of dithiothreitol, a reducing agent, although Nieuwenhuizen, Dekker, Groneveld, Koster, and De Jong (2004) demonstrated that β-lactoglobulin may not need any structural modification for the activity of TGase.

The role of TGase in the improvement of the textural quality of low-fat or non-fat yoghurt has been well demonstrated (Faergemand, Sorensen, Jorgensen, Budolfsen, & Qvist, 1999; Lorenzen, 2002; Özer, Kırmacı, Öztekin, Hayaloğlu, & Atamer, 2007). The application of TGase in cheese technology seems promising, since similar results in terms of increased gel strength and reduced syneresis not unlike those found in applications in yoghurt can be expected.

In the case of cheese manufacture, higher heat treatment than pasteurization affects the renneting properties of milk due to the heat-induced interaction between whey proteins and κ-casein (Bönisch, Heidebach, & Kulozik, 2008). According to Lorenzen (2000), both incorporation of TGase prior to renneting and heat-induced interaction between whey proteins and κ-casein result in a surface sealing effect on casein micelle that sterically slows down the cleavage of caseinomacropeptide (CMP) from the casein micelle, resulting in retardation in the enzymatic phase of renneting. Conversely, O'Sullivan, Kelly, and Fox (2002) observed that the release of CMP was inhibited both in skim milk and whey protein free milk incubated with TGase prior to renneting. This observation contradicts the surface sealing theory proposed by Lorenzen (2000). O'Sullivan et al. (2002) postulated that TGase did not interfere with the secondary phase of renneting, but the primary stage of renneting was influenced by this enzyme. Recently, Bönisch et al. (2008) found that protein cross-linking by a TGase preparation containing glutathione (TGase+GSH) positively affected the rennet coagulation of casein. GSH is a food grade

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reducing agent that stimulates thiol–disulphide interaction reactions. Bönisch, Lauber, and Kulozik (2007) demonstrated that milk proteins could well be cross-linked by TGase in the presence of GSH without the need to pre-heat treatment beyond pasteurization. This observation is critical for TGase application in cheese-making. TGase can be incorporated into cheese-making either prior to or simultaneously with rennet addition. Bönisch et al. (2008) showed that the application of TGase+GSH and rennet simultaneously was more effective in preventing the reduction in rennet coagulation properties of skim milk. Generally, TGase-induced crosslinks can lead to potential positive effects on rennet-gel strength. On the other hand, it can lead to the deterioration of CMP release during the enzymatic phase of renneting (Bönisch et al., 2008; Huppertz & de Kruif, 2007).

While the TGase reaction can be expressed in terms of a degree of polymerization (DP), the rennet reaction can be measured by means of CMP release. In this context, the reaction conditions such as renneting temperature and initial milk pH could directly affect the DP and the level of CMP released due to the combined action of TGase and rennet. Although the temperature optimum of rennet and TGase reaction is in the same region of around 45–50 °C, the temperature-dependent reaction kinetics seems to differ when the temperature is lowered. The release of CMP by means of rennet and the cross-linking of this fraction by TGase correlate with the subsequent gel development and strength. Therefore, it is essential to understand the role of renneting temperature and initial milk pH on the combined action of TGase and rennet on milk proteins based on the physical properties of the resulting gel and yield. The present study aimed to investigate to what extent the reaction conditions in terms of enzymatic reaction products affect the gel strength and yield of rennet gels. This is particularly important for the manufacture of semi-hard brined cheese types (e.g., Feta or Turkish white-brined cheese) with improved yield and physical characteristics.

2. Materials and methods

2.1. Chemicals

Acetonitrile, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate), sodium phosphate and hydrochloric acid (HCl, 37%) were obtained from Sigma–Aldrich (Steinheim, Germany); perchloric acid, urea, 1,4-dithiothreitol (DTT), sodium chloride (NaCl) and sodium hydroxide (NaOH) from Merck (Darmstadt, Germany); citric acid from Chem-Lab NV (Zedelgem, Belgium) and trifluoroacetic acid (TFA) from Thermo Scientific (Rockford, IL, USA). All chemicals used were of analytical grade.

2.2. Skim milk, milk serum and micellar casein suspension

Pasteurized skim milk (with a protein concentration of 3.4% (w/w)) was supplied from a local dairy. The pasteurization of the skim milk was carried out at 74 °C for 30 s. Casein and whey protein free milk serum was obtained by a combination of both microfiltration (nominal pore size 0.1 μm, filtration area 1.68 m^2, model Invensys APV, Unna, Germany) and ultrafiltration (cut off 25 kDa, filtration area 3 m^2, model DSS, Nakskov, Denmark) according to the procedure established by Bönisch, Tolkach, and Kulozik (2006). Skim milk was microfiltered at 55 °C and the microfiltration permeate containing whey proteins was further subjected to ultrafiltration at 55 °C. The major whey proteins were kept in the retentate while the permeate containing lactose, soluble milk salts and low molecular mass milk compounds (the so-called milk serum) was re-circulated to microfiltration unit in diafiltration mode. After six circulation steps, a whey protein-free micellar casein suspension was obtained in the microfiltration retentate.

Micellar casein suspension was prepared in milk serum (protein level of 3%, w/w). The pH of the skim milk and casein suspension were adjusted to 6.5, 6.3 or 6.1 by adding citric acid (9%, w/v).

2.3. Transglutaminase

In the study, the Ca^{+2}-independent microbial transglutaminase (TGase+GSH preparation Activa®YG) was used. The specific activity of the enzyme was 100 U g^{-1} powder and the enzyme preparation contained 8% GSH. The enzyme was kept in a −20 °C chest freezer until used. The enzyme preparation was provided by Ajinomoto Foods Europe (Hamburg, Germany).

2.4. Rennet

The renneting of skim milk and casein suspension was achieved by means of Naturen™ Premium 145 rennet preparation consisting of chymosin (80%) and pepsin (20%) (Chr. Hansen, Nienburg, Germany). The declared activity of the enzyme was 140 IMCU mL^{-1}. The concentration of rennet for the clotting reaction was 0.02% (v/w).

2.5. Experimental design

The rennet and TGase+GSH were used simultaneously. To monitor the role of TGase+GSH on renneting properties of skim milk and casein suspension under different incubation temperatures and initial pH levels, two different TGase+GSH concentrations (0.6 and 1.8 U g^{-1} of protein), two incubation temperatures (30 °C and 34 °C) and three initial pH levels (6.5, 6.3 and 6.1) were examined.

2.6. Rheological experiments

The rheological properties of renneted skim milk were measured using a dynamic oscillatory rheometer (AR 1000N rheometer, TA Instruments, New Castle, Delaware, USA), fitted with a stainless steel cone (diameter 4 cm and 2° angle) and a plate. The temperature of the plate was kept constant at the set temperature with a heating system fitted to the instrument. After the pH adjustment, rennet (0.02%, v/w) and TGase+GSH (0.6 U g^{-1} or 1.8 U g^{-1} protein) were added to the pasteurized skim milk simultaneously, and the milk was stirred for 30 s. Then, the milk was immediately loaded on to the rheometer and the sample was rested for 3 min before commencing the measurement. The storage modulus (G′) was monitored throughout 60 min at the set temperature (30 °C or 34 °C). The frequency and strain applied were 1 Hz and 3%, respectively. The point at which the storage modulus exceeded 1 Pa was considered as coagulation time (t_c). The storage modulus after 60 min reaction time ($G'_{60\ min}$) was accepted as the gel strength (Wang, Bulca, & Kulozik, 2007). Rheological experiments were done in triplicate.

2.7. Determination of yield

The yield of TGase+GSH treated gels was determined using a centrifugation method. Rennet was added to skim milk samples with or without TGase+GSH treatment in 50 mL test tubes. The tubes were incubated in a water bath at 30 °C or 34 °C for 60 min. Then the tubes were centrifuged at 3000 × g at 20 °C for 15 min using a Heraeus Sepatech Biofuge 28 RS (Osterode, Germany). After centrifugation, the supernatant was carefully drained and the yield was calculated according to equation (1):

$$\text{Yield}[\%] = \frac{m_{gel}}{m_{milk}} \times 100\% \quad (1)$$

where; m_{milk} and m_{gel} were the mass of milk before centrifugation and gel after centrifugation, respectively.

2.8. Determination of caseinomacropeptide content

Determination of caseinomacropeptide (CMP) was carried out according to Bönisch et al. (2008). Casein suspensions (100 g) were equilibrated at 30 °C or 34 °C. Rennet (0.02%, v/w) and TGase+GSH (0.6 U g^{-1} or 1.8 U g^{-1} of protein) were added simultaneously to the suspension and thereafter 4 mL of the suspension was transferred to test tubes. The tubes were incubated at 30 °C or 34 °C for different reaction times (0, 0.5, 1, 2, 3, 5, 10, 20, 30, 60, 90 min) and the reaction was stopped by applying perchloric acid (1 mL of 15%, w/w). Next the test tubes were centrifuged at 3000 × *g* at 20 °C for 15 min (Heraeus Sepatech Biofuge 28 RS). The CMP content in the serum was determined using reversed-phase high performance liquid chromatography (RP-HPLC) following the method of Thomä, Krause, and Kulozik (2006). The reference sample for maximal attainable CMP concentration was prepared without TGase+GSH. All measurements were in duplicate and the CMP concentrations were calculated as follows:

$$C_{CMP}[\%] = \frac{C_{CMP,sample}}{C_{CMP,max}} \times 100\% \quad (2)$$

with C_{CMP}: CMP content in serum; $C_{CMP,\ sample}$: CMP concentration measured by RP-HPLC and $C_{CMP,\ max}$: maximal CMP concentration.

2.9. Determination of total protein

Total protein content of skim milk and casein suspension was determined by the Dumas method (Wiles, Gray, & Kissling, 1998) using a nitrogen analyzing system Leco FP 528 (Leco Instrumente GmbH, Moenchengladbach, Germany). Total protein content was calculated by multiplying the total nitrogen values by 6.38.

2.10. Determination of whey protein

To determine the whey proteins, an Agilent 1100 HPLC system and an UV detector, together with a PLRP-S 300 Å 8 μm column (Latek, Eppelheim, Germany) was used. After diluting the samples with distilled water, the pH was adjusted to 4.6 by HCl, and the coagulated aggregates were removed by filtration through folded filters (Dassel, Germany). The samples were further filtered using a membrane filter (0.45 μm; Macherey–Nagel, Düren, Germany) and 20 μL of the sample was injected into the HPLC column. The temperature of analyses and flow rate were 40 °C and 1 mL min^{-1}, respectively. The detection was carried out at 226 nm wavelength (Beyer, 1990).

The concentration of major whey proteins C_{WP} was calculated using the following equation:

$$C_{WP} = C_{\alpha-La} + C_{\beta-LgA} + C_{\beta-LgB} \quad (3)$$

where $C_{\alpha-La}$ is the concentration of α-lactalbumin, $C_{\beta-LgA}$ the concentration of β-lactoglobulin A, and $C_{\beta-LgB}$ the concentration of β-lactoglobulin B.

The degree of whey protein denaturation (DD_{WP}) was calculated using the following equation:

$$DD_{WP}[\%] = \left(1 - \frac{C_{WP,GSH}}{C_{WP,native}}\right) \times 100\% \quad (4)$$

where $C_{WP,\ GSH}$ is the concentration of GSH-treated whey proteins and $C_{WP,\ native}$ the concentration of native whey proteins.

The GSH concentration in skim milk ranged between 0.0 mM and 0.6 mM.

2.11. Gel permeation chromatography

The degree of polymerization (DP) was determined by means of the ÄKTA HPLC system with a variable wavelength P900 UV detector, together with a Superdex™ 200 10/300 GL gel filtration column (Amersham Biosciences, Freiburg, Germany) (Lauber, Henle, & Klostermeyer, 2000; Walter, 1995). The elution of the column was achieved at room temperature at a flow rate of 0.5 mL min^{-1} with the elution buffer (pH 6.8). The elution buffer contained 0.1 M sodium phosphate, 6 M urea, 0.1 M sodium chloride, and 0.1% (w/v) CHAPS. Protein samples were dissolved in an elution buffer plus 1% DTT to a final concentration of 0.3%. The mixture of the sample and the buffer was kept overnight at 4 °C to achieve full reduction of the disulphide bonds. The mixture was then filtered using a 0.45 μm membrane filter (Schleicher & Schuell, Dassel, Germany) and 50 μL samples were loaded into the system. The detection was carried out at 280 nm. Data obtained were processed using the Unicorn 4.11 software from Amersham Biosciences (Freiburg, Germany). The DP was calculated using the following formula:

$$DP[\%] = \frac{\sum A_{dimer,trimer,polymer}}{\sum A_{monomer,dimer,trimer,polymer}} \times 100\% \quad (5)$$

The results were expressed as ΔDP ($DP_{ti}-DP_{t0}$); where DP_{ti} was the degree of polymerization at the time of measurement and DP_{t0} was the degree of polymerization prior to TGase addition.

2.11.1. Statistical analyses

The data were analyzed with one-way analysis of variance. The differences between the groups were determined according to the Least Significance Test (LSD). Statistical analysis was performed using the SPSS version 9.0 (SPSS Inc., Chicago, IL, USA). The experiment was repeated three times ($n = 3$).

3. Results and discussion

3.1. Gelation profiles of cross-linked skim milk proteins

Variations in the gelation profiles of the cross-linked skim milk proteins during a 60 min gelation period at 30 °C as a function of initial milk pH and TGase+GSH concentrations are presented in Fig. 1. Overall, with the decrease in milk pH, the storage modulus (G′) of the gels increased remarkably ($P < 0.05$). Increasing TGase + GSH levels from 0.6 U g^{-1} protein to 1.8 U g^{-1} protein led to a significant reduction in the G′ values of the gels cross-linked at 30 °C. A similar trend was obtained for the gels coagulated at 34 °C, with higher values of G′ than at 30 °C (results not shown).

At both renneting temperatures (30 °C and 34 °C), the coagulation times (t_c) of the samples containing TGase+GSH and the controls decreased significantly with decreasing milk pH from 6.5 to 6.1 (Fig. 2). At a higher concentration of TGase+GSH (1.8 U g^{-1} protein), the coagulation times of the skim milk samples were higher than for the samples supplemented with lower levels of TGase+GSH (0.6 U g^{-1} protein). This indicates an impact of the TGase cross-linking on the hydrolysis of κ-casein as stated by Lorenzen (2000) and O'Sullivan et al. (2002). The decrease in milk pH from 6.6 to 6.0 was reported to affect the primary phase of casein hydrolysis and, therefore, reduce the rennet coagulation time (Fox, Guinee, Cogan, & McSweeney, 2000). The increase in the renneting temperature from 30 °C (Fig. 2A) to 34 °C (Fig. 2B)

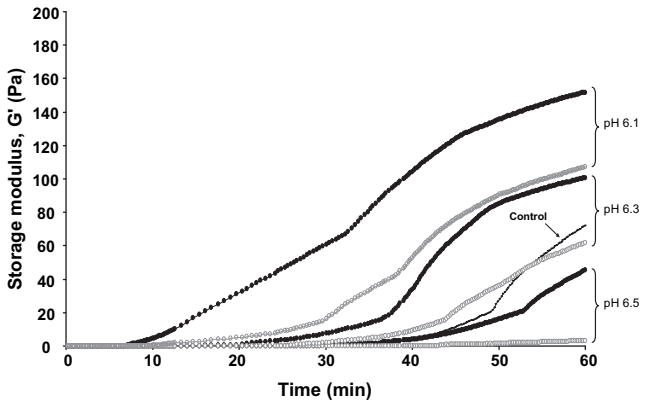

Fig. 1. Gelation profiles of the cross-linked skim milk proteins coagulated at 30 °C throughout 60 min of coagulation. Thin black, thick black and grey lines show control (no TGase+GSH addition at pH 6.5), low (0.6 U g^{-1} protein) and high (1.8 U g^{-1} protein) TGase concentrations, respectively.

resulted in a decrease in the coagulation time of the samples, but the overall profile of coagulation remained similar. The principal effect of the set temperature is on the secondary, non-enzymatic phase of coagulation, which usually occurs at temperatures above 18 °C (Fox et al., 2000). The coagulation time decreases at >18 °C to a minimum at 40–45 °C and then increases again due to the denaturation of rennet. The storage moduli of the skim milk samples after 60 min reaction time ($G'_{60\ min}$) were also affected by the initial milk pH, renneting temperature and TGase+GSH concentration (Fig. 2). At 30 °C (Fig. 2A), the $G'_{60\ min}$ values of the skim milk samples supplemented with TGase+GSH at a level of 0.6 U g^{-1} protein increased with the decrease in initial milk pH, reaching from 45.2 Pa (pH 6.5) to 100.6 Pa (pH 6.3) and 151.7 Pa (pH 6.1). A similar trend with lower figures was noted for the samples supplemented with TGase+GSH at a higher concentration (1.8 U g^{-1} protein). At 34 °C (Fig. 2B), the $G'_{60\ min}$ values of the TGase-treated samples were higher than those treated at 30 °C. The TGase+GSH containing samples had lower $G'_{60\ min}$ values than the control samples at the same initial pH levels. The TGase+GSH concentrations negatively affected the $G'_{60\ min}$ values. Overall, the higher the TGase+GSH concentration, the lower the $G'_{60\ min}$ values. Interestingly, a very weak gel was obtained in the sample containing TGase+GSH at a higher level (1.8 U g^{-1} protein) at pH 6.5 (Fig. 1). The TGase concentration-dependent variation in the $G'_{60\ min}$ values indicates that TGase+GSH interfered with the secondary phase of rennet coagulation. Similar results were reported by Bönisch et al. (2008) who demonstrated that with an increase in the extent of protein cross-linking, the rennet-induced aggregation of para-κ-casein micelles was reduced, resulting in lower $G'_{60\ min}$ values. The same authors also demonstrated that no measurable gel was obtained at ≥5.0 U g^{-1} TGase levels at 38 °C when the enzyme was simultaneously added with rennet. This may be attributed to either inhibition of κ-casein hydrolysis (O'Sullivan et al., 2002) or steric modification of the casein micelle due to extensive cross-linking which was expected to alter the secondary stage of rennet coagulation (Bönisch et al., 2008).

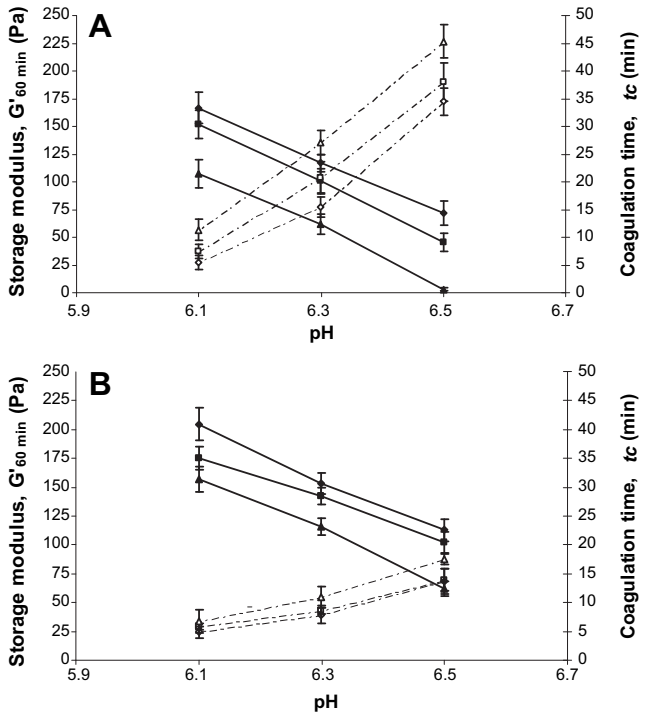

Fig. 2. Variations in the storage modulus after 60 min reaction time ($G'_{60\ min}$) (closed symbols) and rennet coagulation time (t_c) (open symbols) of the gels treated with TGase+GSH ad rennet at temperatures of (A) 30 °C and (B) 34 °C: (◆, ◇) control; (■, □) 0.6 U g^{-1} protein TGase+GSH; (▲, △) 1.8 U g^{-1} protein TGase+GSH. The error bars indicate standard deviation ($n = 3$).

3.2. Effect of cross-linking on the yield of skim milk gels after centrifugation

The yield of the rennet gels after centrifugation was determined to see how TGase-mediated gel properties may have affected their serum binding properties of the gels. The effects of TGase+GSH concentration, initial milk pH and coagulation temperature on the yield of the gels after centrifugation are presented in Fig. 3. The decrease in initial milk pH led to decreases in the yield values of the samples and among the control samples at different pH levels. The highest yield was obtained in the sample renneted at 30 °C and at pH 6.5 (24.4%). Therefore, this sample was considered as a reference for further discussions. At 30 °C, the yield of the gels supplemented with TGase+GSH (0.6 U g^{-1} protein) at pH 6.5, 6.3 and 6.1 were 26.0%, 23.4% and 20.6%, respectively. These figures increased to 31.8%, 29.3% and 26.6%, respectively in the samples with triple the TGase+GSH concentration at 1.8 U g^{-1} protein. The trend of variation in yield values was independent of the renneting temperature, but the yields at 34 °C were found to be significantly lower than that at 30 °C ($P < 0.05$), being slightly more remarkable at lower TGase+GSH levels. Overall, the release of serum from the gel after centrifugation was reduced with TGase+GSH incorporation. The increased yield may well be attributed to the increase in the serum binding capacity of the gels with altered physical properties

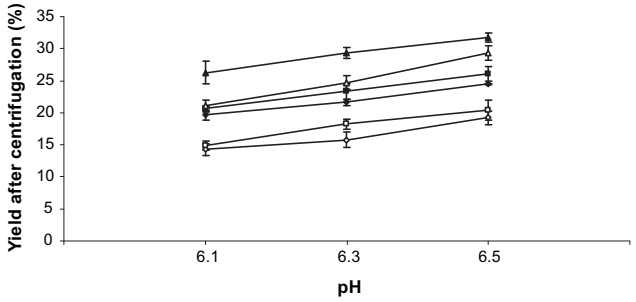

Fig. 3. Variations in the yield of the gels treated with TGase+GSH and rennet at (A) 30 °C (closed symbols) and (B) 34 °C (open symbols): (◆, ◇) control; (■, □) 0.6 U g^{-1} protein TGase+GSH; (▲, △) 1.8 U g^{-1} protein TGase+GSH. The error bars indicate standard deviation ($n = 3$).

by TGase, as stated by Bönisch et al. (2008). In the present case, the milk was heat treated at 74 °C for 30 s and this heating was not expected to cause significant whey protein denaturation. Additionally, the concentration of the reducing agent (GSH) in TGase preparation was too low to lead to denaturation in whey proteins. Therefore, it is fair to assume that the differences between the gel yields were mainly connected with the action of TGase on milk proteins.

The yield may also be affected by levels of whey proteins in the gels, having been increased as a result of heat treatment. In the present case, the heat treatment to milk was low enough (at 74 °C for 30 s) not to cause any denaturation in whey proteins. Additionally, the concentration of the reducing agent (GSH) in TGase preparation was fairly too low to lead to denaturation in whey proteins. Similarly, the TGase+GSH preparation was simultaneously added with rennet and the reaction time between TGase+GSH and whey proteins was too short to stimulate denaturation reactions at a considerable level. Therefore, a negligible level of denaturation in whey proteins triggered by GSH was expected in this case. This was further supported by determining C_{WP} in serum levels as a function of milk pH, renneting temperature and TGase+GSH concentrations. The C_{WP} in serum after centrifugation was between 92.2% and 97.5% (results not shown). Overall, at a higher renneting temperature the C_{WP} in the gels were slightly higher than at a lower temperature. The concentration of TGase+GSH was found to be ineffective on the C_{WP} ($P > 0.05$). The C_{WP} in the serum is affected by the method of TGase incorporation. Cozzolino et al. (2003) and Di Pierro et al. (2010) incorporated TGase into cheese immediately after cutting the coagulum instead of milk, and observed a remarkable increase in the total protein content of the curd. On the contrary, when TGase was used simultaneously with rennet in cheese production, the protein level remained almost unchanged, but the water content of the cheese increased (Di Pierro et al., 2010). Radošević, Tonković, Gregurek, Kos, and Šušković (2007) showed that treatment of TGase prior to rennet coagulation for 8 h at 11 °C resulted in reduced whey syneresis and increased yield in probiotic cheese curd.

3.3. Effect of TGase+GSH on hydrolysis of caseinomacropeptides during rennet coagulation

The relative CMP concentration in the serum phase was determined to monitor the effect of TGase+GSH on the primary phase of rennet coagulation. The determination of relative CMP concentration in the serum phase was done on micellar casein instead of skim milk, so that the initial CMP level was kept constant, as recommended by Bönisch et al. (2008). This also made it possible to investigate the effect of TGase+GSH on casein micelle at a molecular level. The changes in the relative CMP concentrations in the serum phase of casein suspension treated with TGase+GSH at levels of 0.6 U g^{-1} protein or 1.8 U g^{-1} protein during rennet coagulation at 30 °C are shown in Fig. 4A and Fig. 4B, respectively. A marked increase in the CMP concentration in the serum was noted immediately after the addition of rennet, followed by an almost steady state after a 20 min reaction time period. Although the shapes of the curves were similar, the CMP concentrations of casein suspensions were affected by the initial pH, TGase+GSH concentration and rennet coagulation temperature. At 30 °C, in the casein suspension supplemented with TGase+GSH at lower concentration (0.6 U g^{-1} casein) the relative CMP concentrations in serum phase at the end of the coagulation period (90 min) were 80.8%, 84.4% and 89.6% for the initial milk pH values of 6.5, 6.3 and 6.1, respectively (Fig. 4A). These figures increased to 84.0%, 87.3% and 91.2% when the casein suspension

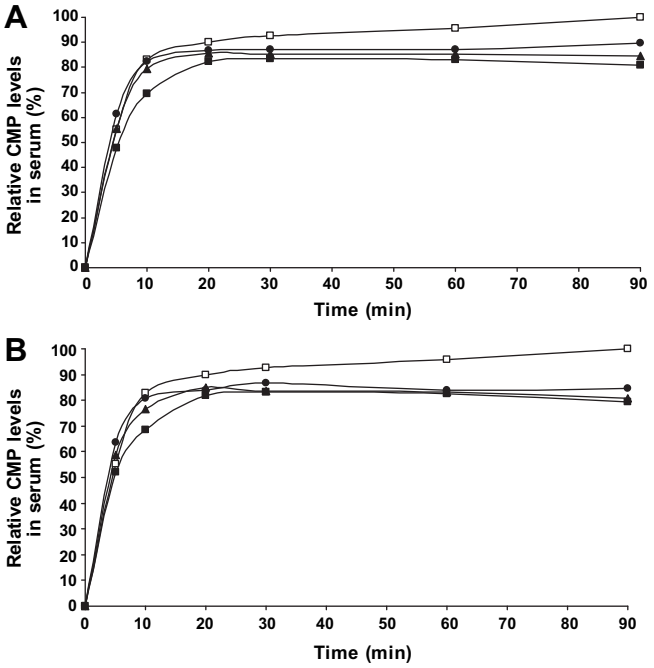

Fig. 4. Variation in relative caseinomacropeptide (CMP) concentrations in serum of the casein gels treated with TGase + GSH at a level of (A) 0.6 U g^{-1} casein and (B) 1.8 U g^{-1} casein as a function of initial pH of casein suspension (3%, w/w). Renneting temperature was 30 °C. Control sample contained no TGase + GSH. Initial pH levels: (□) pH 6.5 (control); (●) pH 6.1; (▲) pH 6.3; (■) pH 6.5. Error bars are less than symbol dimension.

was coagulated at 34 °C (results not shown). Increasing the level of TGase+GSH to 1.8 U g^{-1} casein resulted in decreases in the CMP concentrations in the serum, being more remarkable at lower initial pH values. At 30 °C, the CMP levels in the serum phase were 79.4%, 80.6% and 84.4% for the samples added with rennet and TGase+GSH at pH values of 6.5, 6.3 and 6.1, respectively (Fig. 4B). The sensitivity of CMP towards TGase was reported previously by Tolkach and Kulozik (2005). Therefore, protein cross-linking by TGase+GSH inhibits the separation of CMP from the casein micelle and with the increase in the concentration of TGase+GSH, the CMP level in the serum decreases accordingly during the secondary phase of rennet coagulation (Bönisch et al., 2008; O'Sullivan et al., 2002). In the present case, when the concentration of TGase+GSH was increased from 0.6 U g^{-1} casein to 1.8 U g^{-1} casein, the release of CMP into the serum phase was inhibited by 1.37%–5.24% at 30 °C. A similar trend was noted at a higher rennet coagulation temperature (34 °C), with the exception of the sample coagulated at pH 6.1. A number of hypotheses have been proposed regarding the impact of TGase on the hydrolysis of casein micelle. One possible mechanism is the inhibition of the primary phase of the rennet coagulation by the TGase cross-linking reaction (O'Sullivan et al., 2002). This may be due to more steric hindrance due to the cross-linked κ-casein on the micelle surface for rennet reaction.

In an alternative hypothesis proposed by O'Sullivan et al. (2002), the sterically stabilizing layer of casein micelle is not removed to such a degree allowing for the coagulation of casein micelle, since crosslinks between CMP region of κ-casein and other caseins are still attached to the surface of casein micelle and prevents their release from the micelle (O'Sullivan et al., 2002). A hypothesis of the surface sealing effect of whey proteins on casein micelle proposed by Lorenzen (2002) was excluded since whey protein denaturation was at such a negligible level in the present case.

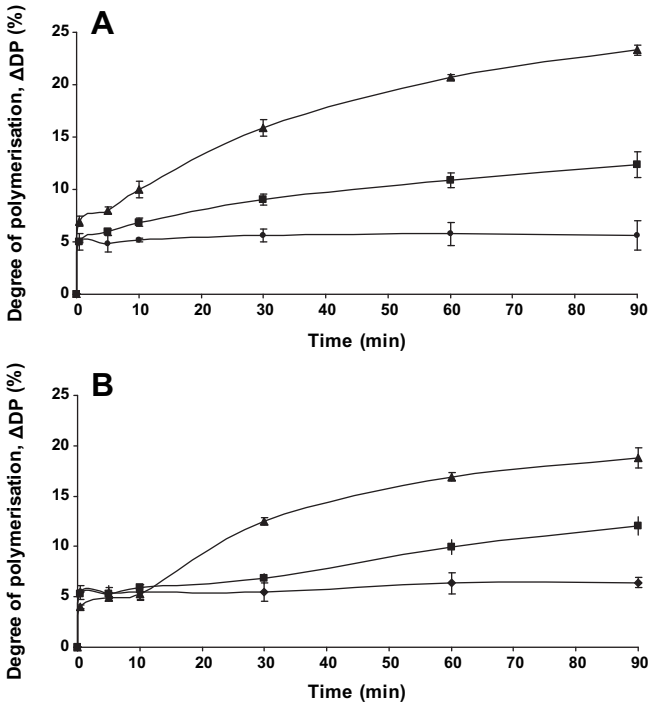

Fig. 5. Variation in degree of polymerization (ΔDP) in casein suspensions treated with TGase + GSH at a level of 0.6 U g^{-1} protein or 1.8 U g^{-1} protein at (A) 30 °C and (B) 34 °C: (▲) pH 6.5, 1.8 U g^{-1} protein; (■) pH 6.5, 0.6 U g^{-1} protein; (●) pH 6.5, control (contained no TGase+GSH). The error bars indicate standard deviation ($n = 3$).

3.4. Impact of TGase-induced cross-linking on degree of protein polymerization of micellar casein suspension

The degree of polymerization of milk proteins in the absence and presence of TGase+GSH was determined by gel permeation chromatography (GPC). The complete dissociation of casein micelle and splitting of disulphide bonds were achieved by adding 6 M urea, CHAPS and DTT prior to analysis. Fig. 5 shows the variation in ΔDP values of the TGase+GSH treated casein suspensions during 90 min incubation at varying incubation conditions. Overall, the ΔDP values were considerably higher at the higher TGase+GSH concentration. Within the first 10 min of coagulation, a low level of polymerization was noted. The polymerization of proteins became more remarkable after this period, coinciding with the completion of the primary phase of rennet coagulation. In Fig. 5, it is clear that the TGase-induced cross-linking of proteins was more effective during the secondary phase of rennet coagulation. This finding contradicts the observation of O'Sullivan et al. (2002) who stated that only the primary phase of rennet action, i.e., cleavage of Phe_{105}-Met_{106} bond of κ-casein, was inhibited by cross-linking of proteins in casein micelles by TGase. Our findings are in agreement with Bönisch et al. (2008) who demonstrated that at TGase levels higher than 0.6 U g^{-1} protein, the relative CMP levels in the serum decreased after 20 min of incubation, proving the greater interference of TGase during the secondary phase of rennet coagulation of casein micelle. The ΔDP values were found to be higher at a higher TGase+GSH concentration. The impact of the incubation temperature on the ΔDP values was limited, with slightly higher values at 30 °C than at 34 °C. Similarly no marginal difference was found between the samples treated with TGase+GSH at different initial pH values, with the exception of enzyme treatment at a higher concentration at pH 6.5 at 30 °C.

4. Conclusions

Transglutaminase (TGase) has been shown to be able to modify caseins through complex pathways. The specific action of TGase on milk proteins has been successfully exploited to improve the texture of low-fat or non-fat yoghurt. Previous studies revealed that the specific action of TGase could be utilized to improve the physico-chemical properties of rennet gels as well as increasing the yield. The present study demonstrated that the specific action of TGase preparation (containing glutathione as a reducing agent, TGase+GSH) in rennet gels was affected by processing parameters including coagulation temperature, TGase+GSH concentration and initial milk pH. Therefore, it is necessary to optimize these parameters before offering incorporation of TGase+GSH into cheese-making (especially for semi-hard brined cheeses). Regarding the physical properties and yield values of the rennet, the optimum renneting temperature, milk pH and TGase+GSH concentration were determined to be 30 °C, pH 6.3 and 1.8 U g^{-1} protein, respectively. Additionally, TGase+GSH was shown to interfere with both primary and, to a greater extent, secondary stages of rennet coagulation, which allows manifold applications in the manufacture of cheese (especially for soft or semi-hard varieties). Further studies should concentrate on the physico-chemical changes (i.e. proteolysis profile, textural changes, etc.) in cheese (especially semi-hard brined cheeses) produced with TGase+GSH during the ripening process.

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