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ORIGINAL RESEARCH

Combined milk gel generated with a novel coagulating enzyme by *Virgibacillus* sp. SK37, a moderately halophilic bacterium

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The hydrolysis of milk proteins by the recombinant AprX-SK37 protease and the changes in the rheological properties of the milk gel generated with AprX-SK37 and glucono-δ-lactone (GDL) were investigated. The AprX-SK37 and rennet selectively hydrolysed κ-casein to yield a 16-kDa band, while subtilisin hydrolysed all of the casein components. Milk treated only with AprX-SK37 formed softer gel. Storage modulus (G′) values of the combined gels increased with GDL concentrations up to 7 g/L. High tan δ was observed in the combined gel at 8.75 g/L GDL alongside syneresis. AprX-SK37 is a promising milk-clotting enzyme when combined with an optimal GDL concentration.

Keywords Casein hydrolysis, Milk-clotting enzyme, Dynamic rheology, Combined milk gel.

INTRODUCTION

Gelation of milk protein is the crucial step during the manufacture of cheese, yoghurt and other dairy products. Milk gels can generally be formed through enzyme-catalysed reactions, acidification, heat treatment or combinations of these methods (Lucey 2001). Calf chymosin (EC 3.4.23.4) modifies the electrostatic and hydrophobic balance to stabilise casein micelles (Horne 1998); it cleaves the peptide bond at Phe_{105}-Met_{106} of κ-casein selectively, which causes a reduction of net negative charge and steric repulsion. Consequently, the aggregation of casein micelles is initiated (Rao *et al.* 1998). Because the production of calf chymosin is limited, recombinant chymosins have been expressed in *Aspergillus niger* var. awamori, *Kluveromyces marxianus* var. lactis, *Pichia pastoris* or *Bacillus subtilis* var. natto and have been widely used for cheese production in many countries (Kappeler *et al.* 2006; Vallejo *et al.* 2008; Chen *et al.* 2010).

Microbial milk-clotting enzymes are an alternative to expensive calf rennet and its recombinant counterparts. Sources of fungal enzymes include *Penicillium citrinum* (Abdel-Fattah *et al.* 1972), *Mucor pusillus* (Nouani *et al.* 2009), *Myxococcus xanthus* 422 (Poza *et al.* 2004) and others. The chestnut blight fungus (*Cryphonectria parasitica*) cleaves the Ser_{104}-Phe_{105} bond (Jacob *et al.* 2011), while the *Rhizomucor* protease hydrolysed mostly the β-casein (Broome *et al.* 2006). These proteases exhibit broad specificity towards κ –casein, which lead to the formation of more bitter peptides (Agboola *et al.* 2004). Reports also indicate that bacterial proteases have milk-clotting abilities. Proteases from various species of *Bacillus* have been reported to have this activity, including *Bacillus licheniformis* 5A1 (Esawy and Combet-Blanc 2006), *Bacillus sphaericus* (El-Bendary *et al.* 2007), *B. licheniformis* USC13 (Ageitos *et al.* 2007) and *B. subtilis*, which is isolated from fermented red bean (natto-red bean) (Chang *et al.* 2012). Several bacilli-derived enzymes exhibit drawbacks, such as high levels of nonspecific thermostable hydrolysis, which might lead to a low yield and a bitter product after storage.

Recently, a serine protease from the subtilase superfamily, AprX-SK37, was isolated from the genomic library of *Virgibacillus* sp. SK37, which is a moderately halophilic bacterium isolated from Thai fish sauce (Phrommao *et al.* 2011). The enzyme has been cloned and successfully overproduced in an *Escherichia coli* system. AprX-

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SK37 demonstrated its potential as a milk-clotting enzyme, especially in conjunction with a small quantity (1.75 g/L) of glucono-δ-lactone (GDL); the amount of added GDL is ~10 times less than the dose used in acid-induced gels. A number of studies have investigated the properties of combined gels induced by the synergistic actions of rennet and GDL (Lucey *et al.* 2000, 2001) or rennet and a starter bacteria (Tranchant *et al.* 2001). Milk clotting enacted under decreased pH conditions was induced by neutralising the negative charges to decrease the electrostatic repulsion and steric stabilisation (Lucey and Singh 1997). A good balance between enzymatic action and the rate of acidification was critical for governing the rheological properties and microstructure of the gel (Veith *et al.* 2004). Because the ability of milk-clotting enzymes to destabilise casein micelles seems source-dependent, it is important to understand the gelation phenomenon induced by a combination of recombinant AprX-SK37 and GDL. The aim of this study was to elucidate the milk-clotting ability and proteolytic activity of recombinant AprX-SK37. In addition, the combined effects of the AprX-SK37 and GDL on the hydrolysis and rheological changes of the milk proteins were systematically investigated.

MATERIALS AND METHODS

Materials

Recombinant AprX-SK37 from *Virgibacillus* sp. SK37 was expressed by *E. coli* BL21, as previously described (Phrommao *et al.* 2011). The enzyme was purified to homogeneity with a Ni-NTA resin (Qiagen, Düsseldorf, Germany). Subtilisin A, chymosin, bovine κ-casein and GDL were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Commercially available low-fat (LF) milk (skim milk powder-free, 2% (w/w) fat) was obtained from the Suranaree University of Technology (SUT) Dairy Farm. The pasteurisation conditions were >75 °C for 16 s and were carried out with a commercial plate heat exchanger (Sondex Inc., Louisville, KY, USA).

Enzyme activity assay

The protein content of LF milk and the three enzymes (AprX-SK37, subtilisin and chymosin) were determined with a Bradford assay (Bradford 1976) that used bovine serum albumin as a standard. The protein content of LF milk from different production lots was routinely measured. The pH of commercial LF milk in all lots was 6.7 ± 0.1. Ten microlitre of enzyme solution was added to a 190 μL reaction mixture that also contained 0.2% (w/v) azocasein, 100 mM Tris-HCl (pH 6.7) and 10 mM $CaCl_2$. The reaction was incubated at 37, 55, or 60 °C for chymosin, AprX-SK37 or subtilisin, respectively. The reaction was halted by adding 60 μL of 20% trichloroacetic acid (TCA). The supernatant was collected via centrifugation at 10 000 *g* and the addition of an equal volume of 2.5 N NaOH. Subsequently, the absorbance of solution was measured at 405 nm. The unit activity was defined as the amount of enzyme necessary to effect an increase 0.01 OD_{405} in 1 min.

Effect of temperature on milk proteolysis by AprX-SK37

To commercially acquired LF milk was added 0.26 U AprX-SK37 and 3.5 g/L GDL. The mixture was incubated at temperatures ranging from 30 to 65 °C for either 30 min or 2 h. Subsequently, the reactions were stopped by heating at 100 °C for 5 min. Small aliquots were taken to investigate the extent of the hydrolysis of the milk proteins with SDS-PAGE.

Hydrolysis patterns generated in milk proteins by various enzymes

The hydrolysis of LF milk proteins (20 ± 3 mg/mL) with chymosin, AprX-SK37 and subtilisin at 0.26 U was carried out under the optimum conditions for each enzyme as follows: 37 °C, pH 6.0; 55 °C, pH 5.8; and 60 °C, pH 8.5, for rennet, AprX-SK37 and subtilisin, respectively. Aliquots were withdrawn every 2 h and were immediately heated to 100 °C for 5 min to inactivate the enzyme. The hydrolytic patterns of the milk proteins were analysed by SDS-PAGE.

Another set of aliquots was also removed to determine the oligopeptide content. The aliquot was combined with cold 20% TCA and incubated on ice for 30 min. Subsequently, the mixture was centrifuged at 12 000 *g* for 15 min. The TCA-soluble oligopeptide content was determined as reported by Lowry *et al*. (1951) with tyrosine as a standard.

Hydrolysis of κ-casein

κ-Casein was dissolved in deionised water to attain a concentration of 2 mg/mL, and the protein content was confirmed with a Bradford assay. The reaction mixture contained 20 μg of κ-casein, 50 mM Tris-HCl (pH 6.7), 10 mM $CaCl_2$ and 0.013 U of either AprX-SK37 or rennet. The reactions were incubated at 37 or 55 °C for chymosin or AprX-SK37, respectively. Aliquots were removed at intervals of 5, 10, 20, 30, 60 and 120 min and were treated at 100 °C for 5 min to stop the hydrolytic reaction. The hydrolysis of κ-casein was monitored with SDS-PAGE.

SDS-PAGE

The samples were mixed with an equal volume of 2× treatment buffer (0.125 M Tris-HCl, 4% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 0.2 M dithiothreitol (DTT) and 0.02% (w/v) bromophenol blue) and boiled at 100 °C for 5 min; subsequently, the samples were subjected to electrophoresis with 15% and 4.5% acrylamide in the separating and stacking gel, respectively, according to procedure detailed by Sambrook and Russel (2001). The electrophoresis was performed under a constant 15 mA current for

45 min, followed by 30 mA for 1.5 h in a tank buffer (25 mM Tris-HCl, 196 mM glycine, pH 8.3 and 0.1% (w/v) SDS). After the electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 and destained in a destaining solution (25% (v/v) methanol and 10% (v/v) acetic acid). Protein molecular weight markers (Fisher Scientific, Pittsburgh, PA, USA) were β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp98I (25.0 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa).

Dynamic rheological measurement

Dynamic oscillatory measurements were performed with an AR-G2 2000-ex (TA Instruments, New castle, DE, USA) rheometer equipped with a parallel plate (60 mm diameter) and a Peltier temperature control system. Changes in the storage (G') and loss (G'') moduli, as well as the loss tangent (tan δ) during gelation, were recorded.

The samples were prepared on ice. GDL was added to the LF milk to attain final concentrations of 0.45, 1.75, 3.5, 4.81, 5.25, 7 and 8.75 g/L, followed by the immediate addition of the recombinant AprX-SK37 at 0.26 U. The mixture was vigorously shaken with a vortex mixer for at least 20 s and transferred to the rheometer. A metallic cover with mineral oil filling its buffer trap was placed on the system to minimise evaporation. The samples were measured at 55 °C, a frequency of 1 Hz and an applied strain of 0.08, which were determined to be within the linear viscoelastic range. The measurements were recorded at every 60 s over 40 min. Subsequently, the samples were cooled to 4 °C, and a frequency sweep at 0.01–5 Hz was carried out at the same applied strain.

Changes in the pH of the milk/GDL mixture during gelation

Changes in the pH during the enzyme/acid-induced gelation were monitored. The LF milk/GDL mixtures, which contained various concentrations of GDL, were incubated in a 55 °C water bath; aliquots were collected at varied time intervals. Each aliquot was immediately immersed in an ice water bath. The pH was measured with a pH meter (UB-10 Denver Instrument Co., Arvada, Colorado, USA) when temperature of the sample reached 26 ± 1 °C.

Statistical analyses

All experiments were carried out independently twice using milk from two different lots. The chemical analyses were conducted in duplicate for each replication. Analysis of the variance (ANOVA) was performed with $P \leq 0.05$ as the significance level. Significant differences between the mean values were measured with Duncan's multiple range test (DMRT). Statistical analysis was performed by using the SPSS package (SPSS 17.0 for windows; SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Hydrolysis of milk proteins

AprX-SK37, which is an intracellular serine protease, was isolated from the genomic library screening of *Virgibacillu*s sp. SK37 and constructed in *E. coli* DH10B (Phrommao *et al.* 2011). This bacterium was originally isolated from fermented Thai fish sauce and was found to produce several extracellular proteases with high activity. Unlike the AprX-SK37, those extracellular proteases did not display any milk-clotting activity. When the LF milk was hydrolysed by the recombinant AprX-SK37, the degradation of protein presumably to be κ-casein (κ-CN) was noticed first; this process provided a protein that was approximately 16 kDa (called 16-kDa CN, Figure 1a). This 16-kDa CN appeared at temperatures greater than 40 °C after a 30 min of hydrolysis reaction. After 2 h of hydrolysis, the 16 kDa CN band had a higher intensity when observed at temperatures from 30 to 55 °C (Figure 1b). In all cases, the most intense 16-kDa CN band was observed at 55 °C, based on visual examination. This temperature was considered optimal for the milk-clotting activity of the AprX-SK37 and was used in later experiments. The enzyme exhibited optimal azocaseinolytic activity at pH 9.5 and 55 °C, indicating an alkaliphilic enzyme (Phrommao *et al.* 2011). The isolation of milk-clotting proteases has not been reported

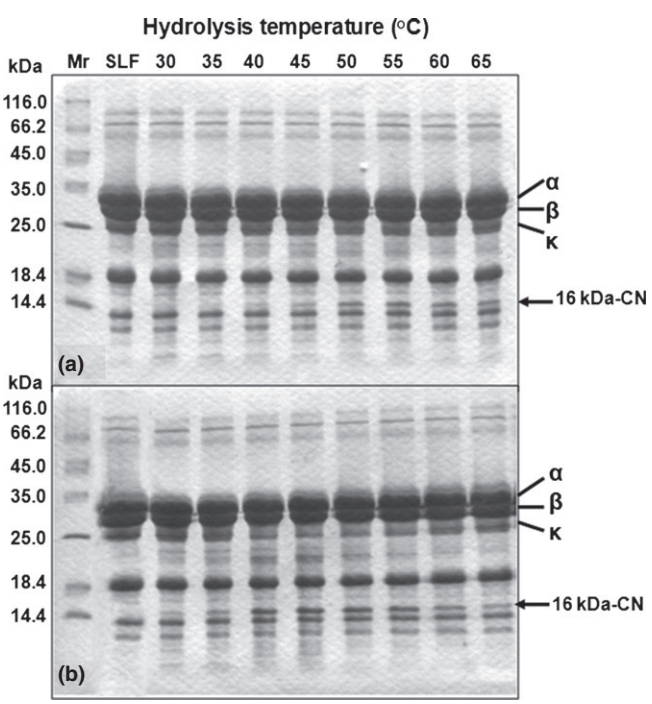

Figure 1 SDS-PAGE analysis of the temperature-dependent hydrolysis of milk proteins by AprX-SK37 (holoenzyme) activity. All reactions contained 3.5 g/L of GDL. Reactions were incubated at the given temperatures for (a) 30 min and (b) 2 h. Mr, molecular weight marker; SLF, SUT low-fat milk. The three major casein (CN) subunits (α-, β- and κ-CN) are marked. The 16 kDa-CN bands generated during digestion are arrowed.

previously with *Virgibacillus*. However, milk-clotting enzymes have been isolated from *Bacillus* sp., which is the genus most similar to *Virgibacillus* sp., and have been classified as either neutral or alkaline serine proteases. The sources of these enzymes include *B. licheniformis* 5A1 (Esawy and Combet-Blanc 2006), *B. sphaericus* (El-Bendary *et al.* 2007) and *B. licheniformis* USC13 (Ageitos *et al.* 2007).

Chymosin is known to selectively hydrolyse κ-CN, yielding para κ-CN with molecular mass of 16 kDa and glycomacropeptide (GMP) (Trujillo *et al.* 1997). Degradation of a protein band with apparent molecular mass of approximately 24 kDa commenced after 1 min of rennet addition and increased with prolonged reaction time in concomitant with an increase in band intensity of a 16-kDa protein (Figure 2a). Thus, a 24-kDa protein was likely to be κ-CN and its proteolysis induced by rennet resulted in formation of para κ-CN with apparent molecular mass of 16 kDa. Although molecular mass of bovine κ-CN is reported to be 19 kDa, its SDS-PAGE pattern typically showed higher molecular mass due to its glycoprotein nature (Farrell *et al.* 2004; Egito *et al.* 2007). Sugar moieties associated with κ-CN reduced the SDS binding, leading to lower electrophoretic mobility.

The recombinant AprX-SK37 also selectively hydrolysed κ-CN to produce a 16-kDa protein after 5 min of reaction, and the intensity of the protein band reached its maximum after 20 min (Figure 2b). Coincidentally, the 16-kDa CN was the same size as the para-κ-CN obtained from the reaction with chymosin (Figures 2a,b). The formation of milk gels mediated by chymosin and AprX-SK37 were also visually observed after 1 and 20 min, respectively, in the presence of 3.5 g/L GDL. Unlike the rennet-catalysed reaction, small fragments with masses less than 14 kDa were detected after 40 min during the AprX-SK37-catalysed reaction (Figure 2b). This result indicates that the nonspecific digestion of CN by the recombinant AprX-SK37 occurs after prolonged reaction times. Interestingly, there was no evidence indicating that the recombinant AprX-SK37 caused the extensive hydrolysis of milk proteins other than κ-CN, even after 120 min. Therefore, the recombinant AprX-SK37 selectively hydrolysed κ-CN in the presence of other milk protein components, and the enzyme seemed to cleave the renneting macropeptide spitting site (Phe_{105}-Met_{106}), in addition to other peptide bonds. In contrast, a nonspecific hydrolytic pattern was detected when using subtilisin (Figure 2c). Subtilisin randomly hydrolysed the milk proteins into small fragments very rapidly; most of CN subunits disappeared within 1 min of digestion.

To confirm the limited activity of AprX-SK37 towards κ-CN, commercially purified κ-CN was used as a substrate for both AprX-SK37 and rennet. The κ-CN was susceptible to hydrolysis by the recombinant AprX-SK37 to yield a para-κ-CN-like protein with a size comparable to that of the renneting-produced para-κ-CN (Figures 3a,b). However, the rate of the κ-CN hydrolysis by AprX-SK37 was slower than with chymosin. Therefore, the 16-kDa CN produced from the AprX-SK37-mediated hydrolysis of κ-CN is called the '16-kDa-para-κ-CN-like protein'. GMP and aglyco-macro-

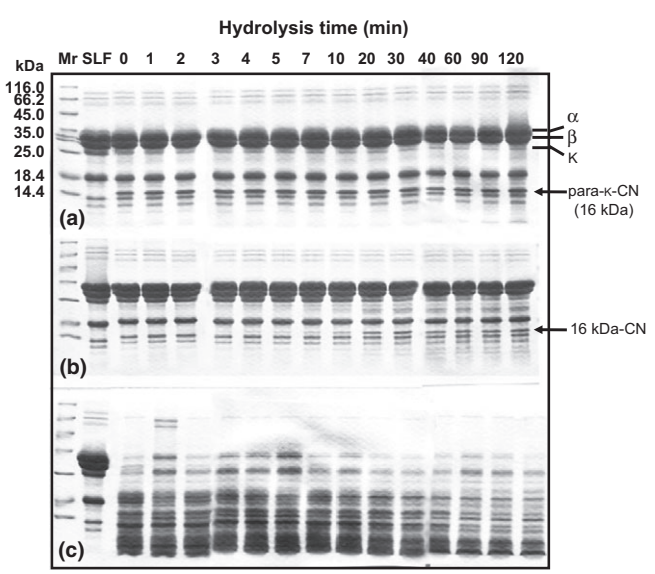

Figure 2 SDS-PAGE analysis of the patterns of milk protein hydrolysis with (a) chymosin at 37 °C, (b) AprX-SK37 (holoenzyme) at 55 °C and (c) subtilisin at 60 °C as a function of time. All enzymes were used with 0.26 U. Acronyms were as indicated in Figure 1a. The presence of rennet-produced para-κ-CN at 16 kDa is marked with an arrow.

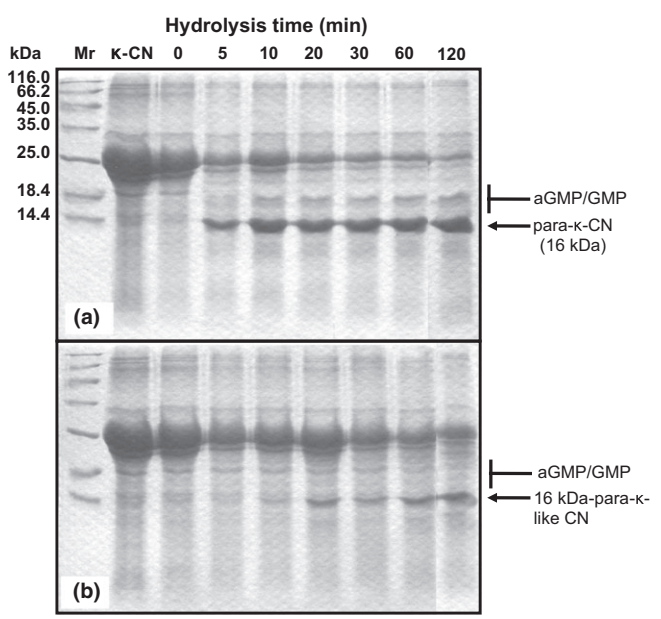

Figure 3 SDS-PAGE hydrolytic patterns of commercial bovine κ-casein promoted by (a) chymosin at 37 °C and (b) AprX-SK37 (holoenzyme) at 55 °C as a function of time. Mr, molecular weight marker; κ-CN, intact κ-CN. The para-κ-CN and para-κ-like CN are marked with an arrow. The presence of glycol-macropeptides (GMP) and aglyco-macropeptides (aGMP) is highlighted.

peptide (aGMP) fragments are also products from a specific cleavage of κ-CN at Phe_{105}-Met_{106}. Theoretical average molecular mass of GMP and aGMP is 7.5 kDa (Mollé and Léonil 2005). But higher molecular masses of 14–28 kDa are typically observed on SDS-PAGE due to self-association of GMP (Farı′as *et al.* 2010). These bands normally appear with high broadness and irregular shape on SDS-PAGE because GMP containing negative-charged sialic acid, a carbohydrate moiety, has low affinity to negatively charged Coomassie Brilliant Blue dye. Therefore, smear bands around 18 kDa observed in both AprX-SK37- and rennet-catalysed reactions are assumed to be GMP and aGMP (Figures 3a,b). Band intensity of GMP/aGMP increased with reaction time in rennet-catalysed sample (Figure 3a), but was not obvious in the reaction catalysed by the AprX-SK37 (Figure 3b). This observation indicates the common cleavage site of κ-CN between these two enzymes, but the extent of κ-CN hydrolysis by rennet appears to be greater. Gallagher *et al.* (1994) reported that most of the *B. subtilis* proteases hydrolysed β-CN and α-CN to yield small peptide fragments with molecular masses under 10 kDa. In addition, *B. subtilis* var (natto) proteases extensively hydrolyse β-CN and α-CN, generating 15 kDa and sub-10 kDa peptides, respectively (Chen *et al.* 2010). The complete digestion of milk proteins by subtilisin was observed in this study. Because AprX-SK37 is a subtilisin-like enzyme in subtilase superfamily, this result may indicate that there is a marked difference between the substrate specificity of AprX-SK37 towards milk proteins and the specificity of the other members in this superfamily.

Peptidase activity

Subtilisin demonstrated the highest peptidase activity towards LF milk proteins ($P \leq 0.05$, Table 1). This result was validated by the severely degraded milk proteins observed during SDS-PAGE (Figure 2c). However, rennet and AprX-SK37 displayed lower peptidase activities ($P \leq 0.05$). These enzymes' peptidase activity towards LF was approximately 2–3 times lower than with subtilisin. The activity of the recombinant AprX-SK37 at pH 6.5–7, which is the physiological pH of cows' milk, was limited; less than 5% of its maximal activity was observed when azocasein was used as the substrate (Phrommao *et al.* 2011). This result agreed with limited amount of milk proteolysis promoted by this enzyme (Table 1). High proteolytic activity in a milk-clotting enzyme is generally not acceptable because it catalyses the cleavage of peptide bonds and produces high content of bitter peptides.

Table 1 Peptidase activity of various proteases towards milk proteins at 0.26 U*

Enzyme	*TCA-soluble oligopeptide (mM)*
AprX-SK37	1.73[b]
Chymosin	2.82[b]
Subtilisin	8.21[a]

Different superscripts indicate the significant difference ($P \leq 0.05$), $n = 4$.

*Milk hydrolysis was carried out at 37, 55 and 60 °C, as well as pH 6.0, 5.8 and 8.5 for rennet, AprX-SK37 and subtilisin, respectively.

Dynamic rheological properties of combined gels

At concentrations of 0 and 0.45 g/L GDL, milk gels were not formed in the presence of the recombinant AprX-SK37, corresponding to the low G′ values or the high tan δ over the course of the incubation (Figures 4a,b). The G′ values increased and tan δ decreased when the concentration of GDL increased (Figures 4a,b). Changes in the G′ values during the 55 °C incubation exhibited three different profiles, depending on the GDL concentration. Profile I, which was at 1.75–4.81 g/L GDL, illustrated an exponential trend with a maximal G′ < 1 Pa; profile II exhibited a sigmoidal curve with a maximal G′ > 1 Pa at 5.25–7.00 g/L GDL;

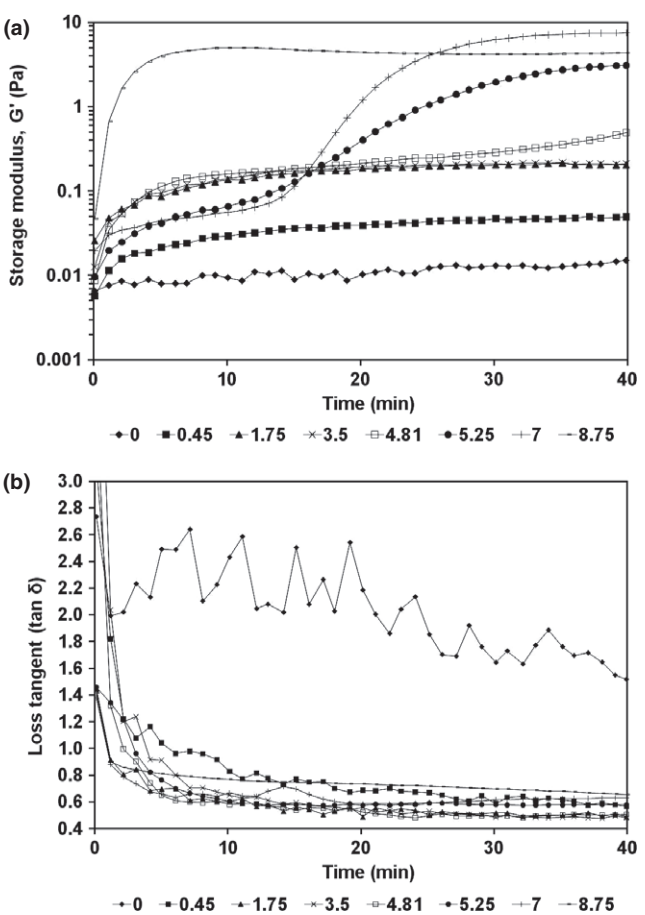

Figure 4 Effect of the recombinant AprX-SK37 and 0–8.75 g/L GDL addition on (a) storage modulus (G′) and (b) the loss tangent (tan δ) of combined gels at 55 °C. Each value represents mean of two independent samples.

and profile III revealed a sharp exponential curve with a maximal G′ > 1 Pa at 8.75 g/L GDL. In profile I and III, the G′ values increased sharply and reached a plateau after approximately 10 min, while in profile II, the G′ values gradually increased during the first 15 min of incubation and abruptly increased thereafter to reach the maximum G′ value after 30 min of incubation. Similar changes in G′ were also reported during the coagulation of acidifying milk, which were reported by Tranchant *et al.* (2001).

The pH of the samples decreased with an increased concentration of GDL. In the absence of and at 0.45 g/L GDL, the pH values of the milk samples were 6.48–6.5 during heating. The aggregation of the casein micelles in these samples was mainly caused by the destabilisation of the κ-CN by the recombinant AprX-SK37 protease, as well as the heat treatment. The cleavage of κ-CN at pH 6.5 occurred to a lesser extent to limit the coagulation of the milk protein. The limited cleavage also corresponded to the predominant viscous component, which had higher tan δ (>1) values (Figure 4b). As the GDL concentration increased to 4.81 g/L, the pH of the milk gel decreased from 6.67 to 5.86 over the first 5 min and gradually decreased to pH 5.54 within 40 min. Horne (1998) reported that the dissolution of the colloidal calcium phosphate at low pHs induced the destabilisation of casein micelles and promoted coagulation. This demineralisation, in conjunction with the dissociation of the casein micelles induced by AprX-SK37, led to a reduction of the electrostatic repulsion and steric stabilisation, which caused coagulation, and the increase in the G′ values observed at 4.81 g/L GDL (Figure 4a,b).

When the GDL content increased to 5.25–7 g/L, a sigmoidal change in G′ was observed. The pHs of these samples decreased to 5.6 within 5 min and reached 5.3 in 40 min. The extent of demineralisation and charge neutralisation of the hydrolysed para-CN was more prominent due to the lower pH. The AprX-SK37-promoted hydrolysis of κ-CN and the low pH caused by the GDL at 5.25–7 g/L synergistically promoted coagulation, resulting in a sharp increase in G'; the highest G' was attained at 40 min for the sample with 7 g/L GDL. At 8.75 g/L GDL, changes in the dynamic rheological parameters of the combined gel resembled those of the sample with only 8.75 g/L GDL (data not shown). This result implied that the rheological changes were mainly induced by GDL with only minimal action by the AprX-SK37. At this concentration of GDL, the pH of the system decreased to 5.4 within 5 min and the final pH after 40 min was 5.0. This low pH range destabilised structure and activity of the AprX-SK37. In addition, this pH approached the isoelectric region for caseins and whey proteins, which is near pH 5.3–4.6 (Tranchant *et al.* 2001). The predominant acidification and the minimal κ-CN hydrolysis generated more protein–protein interactions, which ultimately resulted in syneresis. The highest tan δ values were also observed over the course of the incubation of the gel with 8.75 g/L GDL ($P \leq 0.05$, Figure 4b). The GDL concentration and rate of acidification (pH decrease) played an important role in the rheological changes of the combined gel, as described by Tranchant *et al.* (2001).

The elasticity of the combined gels was measured by using frequency sweep mode. A linear change in the G′ values within a selected frequency range for all of the samples suggested that the samples would display elastic behaviour. The G′ values of both the combined gel and the GDL-induced gels increased with the GDL concentration ($P \leq 0.05$, Figure 5). The G′ values of the combined gels with 1.75–7.00 g/L GDL were higher than the analogous GDL-induced gels ($P \leq 0.05$). The use of AprX-SK37 in combination with GDL-induced better milk coagulation than the application of GDL alone. The combined gels generated from rennet and GDL also demonstrated higher elasticity than those induced by the acids alone (Lucey *et al.* 2000; Li and Dalgleish 2006).

CONCLUSIONS

The recombinant AprX-SK37, which is an intracellular serine protease, was isolated from the genomic library screening of *Virgibacillus* sp. SK37 and constructed in *E. coli* DH10B. This enzyme selectively hydrolysed κ-CN and produced a 16-kDa CN protein that exhibited the same mass as the para-κ-CN obtained from the renneting reaction. The gel made from AprX-SK37 (without acidification by GDL) presented as a liquid-like material with a tan δ greater than 1, while gels containing an additional 1.75–4.81 g/L of GDL generated a solid-like material. Combined gels made with AprX-SK37 and GDL exhibited higher G′ values than the GDL-induced gels. Our results suggested that AprX-SK37 could be a promising milk-clotting agent when combined with GDL.

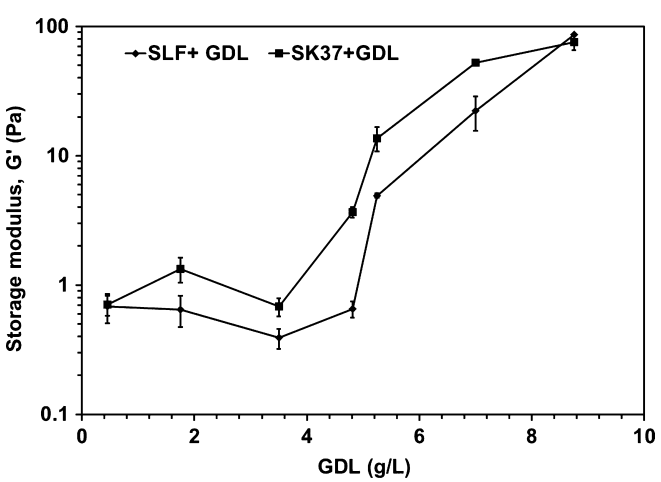

Figure 5 Storage moduli (G′) of the gels at various GDL concentrations measured at 5 Hz, 4 °C. Each value represents mean of two independent samples.

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REFERENCES

Abdel-Fattah A F, Mabrouk S S and ElHawwary N M (1972) Production and some properties of rennin-like milk-clotting enzyme from *Penicillium citrinum*. *Journal of General Microbiology* **70** 151–155.

Agboola S, Chen S and Zhao J (2004) Formation of bitter peptides during ripening of ovine milk cheese made with different coagulants. *Lait* **84** 567–578.

Ageitos J M, Vallejo J A, Sestelo A B F, Poza M and Villa T G (2007) Purification and characterization of a milk-clotting protease from *Bacillus licheniformis* strain USC13. *Journal of Applied Microbiology* **103** 2205–2213.

Bradford M M (1976) A dye binding assay for protein. *Analytical Biochemistry* **72** 248–254.

Broome M C, Xu X and Mayes J J (2006) Proteolysis in cheddar cheese made with alternative coagulatants. *Australian Journal of Dairy Technology* **61** 85–87.

Chang C T, Wang P M, Hung Y F and Chung Y C (2012) Purification and biochemical properties of a fibrinolytic enzyme from *Bacillus subtilis*-fermented red bean. *Food Chemistry* **133** 1611–1617.

Chen M T, Lu Y Y and Weng T M (2010) Comparison of milk-clotting activity of proteinase produced by *Bacillus subtilis var, natto* and *Rhizopus oligosporus* with commercial rennet. *Asian Australasian Journal of Animal Sciences* **23** 1369–1379.

Egito A S, Girardet J M, Laguna L E, Poirson C, Molle D, Miclo L, Humbert G and Gaillard J L (2007) Milk-clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine κ-casein. *International Dairy Journal* **17** 816–825.

El-Bendary M A, Moharam M E and Ali T H (2007) Purification and characterization of milk clotting enzyme produced by *Bacillus sphaericus*. *Journal of Applied Sciences Research* **3** 695–699.

Esawy M A and Combet-Blanc Y (2006) Immobilization of *Bacillus licheniformis* 5A1 milk-clotting enzyme and characterization of its enzyme properties. *World Journal of Microbiology & Biotechnology* **22** 197–200.

Farı′as M E, Martinez M J and Pilosof A M R (2010) Casein glycomacropeptide pH-dependent self-assembly and cold gelation. *International Dairy Journal* **20** 79–88.

Farrell H M Jr, Jimenez-Flores R, Bleck E M, Brown G T, Butler J T, Creamer L K, Hicks C L, Hollar C M, Ng-Kwai-Hang K F and Swaisgood H E (2004) Nomenclature of the proteins of cows' milk —sixth revision. *Journal of Dairy Science* **87** 1641–1674.

Gallagher J, Kanekanian A D and Evans E P (1994) Hydrolysis of casein: a comparative study of two proteases and their peptide maps. *International Journal of Food Science & Technology* **29** 279–285.

Horne D S (1998) Casein interactions: casting light on the black boxes, the structure in dairy products. *International Dairy Journal* **8** 171–177.

Jacob M, Jaros D and Rohm H (2011) Recent advances in milk clotting enzymes. *International Journal of Dairy Technology* **64** 14–33.

Kappeler S R, van den Brink H M, Rahbeck-Nielsen H, Farah Z, Puhan Z, Bech Hansen E and Johansen E (2006) Characterization of recombinant camel cymosin reveals superior properties for the coagulation of bovine and camel milk. *Biochemical and Biophysical Research Communications* **324** 647–654.

Li J and Dalgleish G D (2006) Controlled proteolysis and the properties of milk gels. *Journal of Agricultural and Food Chemistry* **54** 4687–4695.

Lowry O H, Rosenbrough N J, Farr A L and Randall R J (1951) Protein measurement with the Folin Phenol Ragent. *Journal of Biological Chemistry* **193** 265–275.

Lucey J A (2001) The relationship between rheological parameters and whey separation in milk gels. *Food Hydrocolloids* **15** 603–608.

Lucey J A and Singh H (1997) Formation and physical properties of acid milk gels: a review. *Food Research International* **30** 529–542.

Lucey J A, Tamehana M, Singh H and Munro P A (2000) Rheological properties of milk gels formed by a combination of rennet and glucono-d-lactone. *Journal of Dairy Research* **67** 415–427.

Lucey J A, Tamehana M, Singh H and Munro P A (2001) Effect of heat treatment on the physical properties of milk gels made with both rennet and acid. *Journal of Dairy Research* **11** 559–565.

Mollé D and Léonil J (2005) Quantitative determination of bovine k-casein macropeptide in dairy products by liquid chromatography/electrospray coupled to mass spectrometry (LC-ESI/MS) and liquid chromatography/electrospray coupled to tandem mass spectrometry (LC-ESI/MS/MS). *International Dairy Journal* **15** 419–428.

Nouani A, Belhamice N, Slamani R, Belbraouct S, Fazouane F and Bellal M M (2009) Extracellular protease from *Mucor pusillus*: purification and characterization. *International Journal of Dairy Technology* **62** 112–117.

Phrommao E, Yongsawatdigul J, Rodtong S and Yamabhai M (2011) A novel subtilase with NaCl-activated and oxidant-stable activity from *Virgibacillus* sp. SK37. *BMC Biotechnology* **11** 65.

Poza M, Prieto-Alcedo M, Sieiro C and Villa T G (2004) Cloning and expression of *clt* genes encoding milk-clotting proteases from *Myxococcus xanthus* 422. *Applied and Evironmental Microbiology* **70** 6337–6341.

Rao M B, Tanksale A M, Ghatge M S and Deshpande V V (1998) Molecular and Biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews* **62** 597–635.

Sambrook J and Russel D W (2001) *Molecular Cloning: A Laboratory Manual*, pp. A8.40–A8.49. New York: Cold Spring Harbor Laboratory.

Tranchant C C, Dalgleish D G and Hill A R (2001) Different coagulation behaviour of bacterologically acidified and renneted milk: the importance of fine-tuning acid production and rennet action. *International Dairy Journal* **11** 483–494.

Trujillo A J, Guamis B and Carretero C (1997) Proteolysis of goat casein by calf rennet. *International Dairy Journal* **7** 579–588.

Vallejo J A, Ageitos J M, Poza M and Villa T G (2008) Clonning and expression of buffalo active chymosin in *Pichia pastoris*. *Journal of Agricultural and Food Chemistry* **56** 10606–10610.

Veith B, Herzberg C, Steckel S *et al.* (2004) The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. *Journal of Molecular Microbiology and Biotechnology* **7** 204–211.

