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**Original article**

# Use of exogenous streptokinase to accelerate proteolysis in Cheddar cheese during ripening

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**Abstract –** A semi-purified preparation of streptokinase, a plasminogen activator from *Streptococcus uberis,* was added to cheesemilk at levels of 0.1%, 0.25% or 0.50% (v/v) to assess its use for the activation of plasminogen and, if this occurred, to evaluate the effects of the resultant increase in plasmin activity on proteolysis in Cheddar cheese during ripening. Streptokinase successfully activated plasminogen in cheese to plasmin, as indicated by increases in plasmin activity and concomitant decreases in plasminogen levels; plasmin activity increased with increasing addition of streptokinase. Increased plasmin activity accelerated proteolysis in cheese during ripening; increased breakdown of β-casein and concomitant increases in levels of $\gamma_1$-, $\gamma_2$- and $\gamma_3$-caseins were observed, using urea polyacrylamide gel electrophoresis, in experimental cheeses compared to control cheeses. Peptide profiles of 70% ethanol-soluble fractions, analysed by reversed-phase HPLC, showed greater production of hydrophobic peptides eluting in the region of 55–60 min in cheeses made from milk with added streptokinase compared to control cheese, which was attributed to increased plasmin activity. The increased plasmin activity did not affect the levels of total free amino acids. In conclusion, addition of streptokinase to cheesemilk resulted in activation of plasminogen to plasmin, which accelerated proteolysis in Cheddar cheese during ripening.

**Cheddar cheese / streptokinase / plasmin / proteolysis / ripening**

**Résumé – Addition de streptokinase au lait de fabrication pour accélérer la protéolyse du fromage Cheddar durant l'affinage.** Dans le but d'évaluer les effets d'un accroissement de l'activité de la plasmine sur la protéolyse au cours de l'affinage du fromage Cheddar, une préparation partiellement purifiée de streptokinase (activateur du plasminogène extrait de *Streptococcus uberis)* a été ajoutée au lait de fabrication aux doses de 0,1 %, 0,25 % ou 0,50 % (v/v). Les résultats obtenus ont confirmé l'activation du plasminogène en plasmine dans le fromage proportionnellement à la dose ajoutée de streptokinase. L'accroissement de l'activité plasminique ainsi obtenue, déterminée par comparaison des fromages expérimentaux et des fromages témoins par électrophorèse sur gel de polyacrylamide en milieu urée, se traduisait par une hydrolyse accrue de la caséine β et un accroissement concomitant des caséines $\gamma_1$, $\gamma_2$ et $\gamma_3$. L'analyse par HPLC en phase inverse des fractions solubles dans l'éthanol à 70 % a également montré une concentration accrue, dans les fromages essais, de peptides hydrophobes élués dans la zone 55–60 min, résultant vraisemblablement d'une action plasminique. Par contre, aucune différence n'a été observée dans les teneurs en acides aminés

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libres des deux séries de fromages. En conclusion, il est possible d'accélérer la protéolyse du fromage Cheddar au cours de l'affinage par addition au lait de fabrication de streptokinase.

**Plasmine / protéolyse / affinage / Cheddar / streptokinase**

## 1. INTRODUCTION

Plasmin (a serine proteinase; EC 3.4.21.7) is secreted into blood as its inactive form, plasminogen. The principal function of plasmin in blood is to degrade fibrin clots [16]; plasmin gains entry into milk via blood and is the principal indigenous proteinase in milk. A complex plasmin/plasminogen system exists in milk and blood, comprising of plasmin, plasminogen, plasminogen activators (PA), plasmin inhibitors, and inhibitors of PA. Plasmin acts on caseins in the order $\beta = \alpha_{s2} >> \alpha_{s1}$ casein and has a preference for bonds of the type Lys-X and, to a lesser extent, Arg-X [4, 13].

Proteolysis is the most important biochemical change occurring during the ripening of most hard cheese varieties, including Cheddar, and plays a pivotal role in the development of cheese texture and flavour. Plasmin is a significant agent of proteolysis in Cheddar cheese [12]; addition of exogenous plasmin to cheesemilk before manufacture of Cheddar cheese has been demonstrated to accelerate proteolysis during ripening [9]. However, a recent study on addition of plasmin to cheesemilk for the manufacture of Mozzarella-type cheese showed loss of plasmin in cheese whey [26], thus suggesting that use of exogenous plasmin may be an uneconomical way to accelerate proteolysis.

Since plasmin is secreted as its inactive form, plasminogen, which is activated in blood and milk by the action of various PA, there exists the possibility of accelerating proteolysis in cheese by activating plasminogen to plasmin using exogenous PA. In milk, the principal PA are urokinase type (u-PA) and tissue type (t-PA), which are both serine proteinases. Hydrolysis of the $Arg_{557}$-$Ile_{558}$ bond is responsible for activation of bovine plasminogen to plasmin [24]. Successful attempts have been made to accelerate proteolysis in cheese by increasing plasmin activity through addition of exogenous urokinase to the milk used to produce Cheddar cheese [3], Swiss cheese [6], ultrafiltered Havarti and Saint Paulin cheeses [5].

In addition to the above types of PA, several micro-organisms produce PA, the role of which is believed to be to enable these organisms to exploit the plasmin activity of the host to degrade fibrin and other matrix proteins, which facilitates bacterial colonisation of deep tissue sites [14]. *Streptococcus uberis*, a common cause of bovine mastitis, secretes a streptokinase which is able to activate bovine plasminogen but not human or porcine plasminogen [19]. Streptokinase is a dimeric protein with molecular mass of approximately 30 $kg \cdot mol^{-1}$. During mastitis, *S. uberis* increases plasmin activity in milk via streptokinase, resulting in hydrolysis of the caseins, which, in turn, assists the growth of *S. uberis* in milk by liberation of peptides that may contain essential amino acids. Streptokinase forms a 1:1 stoichiometric complex with plasminogen, inducing a conformational change of the serine proteinase domain of plasminogen, which renders the proteinase active without prior proteolytic cleavage. The active streptokinase-plasminogen complex is then able to convert other plasminogen molecules to plasmin by proteolysis [15].

Streptokinase from *S. uberis* has not been used in cheese studies to date as a means of increasing plasmin activity and thereby accelerating proteolysis during ripening. The aim of this study was to investigate the activation of bovine plasminogen to plasmin by adding streptokinase from *S. uberis* to Cheddar cheesemilk and to study the effects of consequent increases in plasmin activity on proteolysis in Cheddar cheese during ripening.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of streptokinase

*S. uberis* streptokinase was produced by the genetically-engineered strain, *Lactococcus lactis* AMJ798, which is *Lc. lactis* MG1363 containing the plasmid pAMJ315. This plasmid includes the gene for streptokinase from *S. uberis* [14] fused to the signal peptide, SP310mut2, which was inserted downstream of the P170 promoter [8]. Strain AMJ798 was grown in a benchtop fermentor (Applikon, Schiedam, The Netherlands) containing 1 L of the defined medium LM5-50 [8], and pH was maintained at 6.5 using KOH. Cells were removed by centrifugation, and the supernatant was sterilized by filtration using Millipak 40 filter with a pore size of 0.22 μm (Millipore, Bedford, USA) and concentrated to 300 mL by ultrafiltration using a Prep/Scale TFF cartridge equipped with a 10 $kg \cdot mol^{-1}$ cut-off membrane (Millipore). The activity of *S. uberis* streptokinase was confirmed by the streptokinase assay of Johnsen et al. [14].

### 2.2. Cheese manufacture

Raw whole milk for cheese manufacture (Pilot plant, University College, Cork) was pasteurised at 72 °C for 15 s. As determined in preliminary trials, streptokinase was added to cheesemilk at levels of 0.1%, 0.25% or 0.5% (v/v); cheese made from milk without addition of streptokinase acted as the control. Four batches of Cheddar cheese (one control and three experimental) were manufactured from 15 L of milk on two occasions (Trials A and B) by a conventional cheesemaking procedure, using *Lc. lactis* ssp. *lactis* 317 (Microbiology Department, University College, Cork) as starter and Maxiren 180 (DSM Food Specialties, Delft, The Netherlands) as coagulant. The cheeses were pressed overnight, vacuum packed and ripened at 9 °C; samples were subsequently taken from the cheeses after 1, 60, 120 and 180 d. The cheeses were grated immediately after sampling and stored at –20 °C until analysis.

### 2.3. Compositional analysis

Moisture and protein contents of cheese were measured according to standard methods [20]; salt content was determined by the potentiometric method of Fox [10]. The pH of cheese:water slurries (1:2) was measured. All compositional analyses were performed in triplicate.

### 2.4. Assessment of proteolysis

pH 4.6-Soluble extracts were prepared from the cheeses [18]; the pH 4.6-insoluble fractions were freeze-dried, while the pH 4.6-soluble fraction was sub-fractionated using 70% (v/v) ethanol, as described by Sousa and McSweeney [27]. Both the ethanol-soluble and -insoluble sub-fractions were freeze-dried.

Soluble nitrogen (SN) content of the pH 4.6-soluble fractions was determined by the macro-Kjeldahl method. Freeze-dried pH 4.6-insoluble fractions were analysed by urea-PAGE (12.5% T, 4% C, pH 8.9) according to the method of Andrews [1], as modified by Shalabi and Fox [25]. Gels were stained as described by Blakesley and Boezi [7], destained in distilled water and scanned on a flatbed scanner (HP Scanjet 6300C, Singapore). Densitometric analysis was performed on the scanned image using gel analysis software (Total Lab 1D, Nonlinear Dynamix, Newcastle-upon-Tyne, UK). Peak volumes of individual bands, obtained by densitometric analysis of gels, were used as variables for principal component analysis (PCA), performed using a covariance matrix with the SPSS statistical package (SPSS for Windows, Version 10.0, SPSS Inc, Chicago, USA). Groupings on the score plots were obtained by performing hierarchical cluster analysis on the data.

Peptide profiles of 70% ethanol-soluble fractions were determined as described by Sousa and McSweeney [27] and data obtained were analysed by multivariate statistical techniques. The data for the factor reduction analysis were obtained by visually recognising the peaks and taking peak heights as variables. Factor reduction analysis was

**Table I.** Compositions* of Cheddar cheeses made from milk with different levels of added streptokinase.

| | Level of streptokinase (%, v/v of cheesemilk) | pH | % Fat | % Moisture | % Protein | % Salt |
|---|---|---|---|---|---|---|
| Trial A | 0.00 | 5.12 ± 0.02 | 33.5 ± 0.3 | 37.69 ± 0.13 | 25.12 ± 0.54 | 1.03 ± 0.00 |
| | 0.10 | 5.09 ± 0.01 | 32.5 ± 0.0 | 38.61 ± 0.09 | 24.92 ± 0.17 | 0.89 ± 0.02 |
| | 0.25 | 5.13 ± 0.02 | 32.5 ± 0.0 | 37.69 ± 0.19 | 25.01 ± 0.53 | 0.92 ± 0.00 |
| | 0.50 | 5.10 ± 0.03 | 32.5 ± 0.3 | 38.04 ± 0.41 | 24.54 ± 0.15 | 0.92 ± 0.02 |
| Trial B | 0.00 | 5.13 ± 0.01 | 33.0 ± 0.0 | 38.17 ± 0.34 | 23.79 ± 0.46 | 0.90 ± 0.00 |
| | 0.10 | 5.12 ± 0.04 | 33.0 ± 0.0 | 37.57 ± 0.32 | 23.13 ± 0.35 | 1.00 ± 0.01 |
| | 0.25 | 5.15 ± 0.00 | 33.0 ± 0.0 | 36.92 ± 0.06 | 23.26 ± 0.16 | 1.00 ± 0.02 |
| | 0.50 | 5.11 ± 0.02 | 33.5 ± 0.3 | 36.74 ± 0.44 | 23.75 ± 0.11 | 1.07 ± 0.00 |

* Means ±standard deviations of triplicate analysis.

performed on the data using the covariance matrix for determination of principal components [22] using the SPSS statistical package.

Total free amino acids in the pH 4.6-soluble fractions were determined by the trinitrobenzenesulphonic acid (TNBS) method, as described by Polychroniadou [21].

### 2.5. Plasmin assay

The cheeses were assayed for plasmin activity and plasminogen levels by a modification of the method of Richardson and Pearce [23]. Grated cheese (3 g) was mixed with 2% (w/v) trisodium citrate (27 mL) and stomached for 5 min in a laboratory stomacher (Colworth Stomacher 400, Seward Laboratory, London, UK). The resultant homogenate was equilibrated at 37 °C for 15 min before centrifuging at 27 000 *g* for 30 min at 4 °C. The supernatant beneath the fat layer was assayed for plasmin activity using *N*-succinyl-alanyl-phenylalanyl-lysyl-7-amido-4-methyl-comarin (Suc-Ala-Phe-Lys-AMC) as substrate. Plasminogen-derived activity in the supernatant was determined after activating plasminogen to plasmin using 0.2 $U \cdot mL^{-1}$ urokinase (Fluka Laboratories, Buchs, Switzerland). Plasmin activity in cheese was expressed as plasmin units $g^{-1}$ cheese (where 1 unit was defined as the activity necessary to release 1 nmole of 7-amido-4-methyl-coumarin From Suc-ala-Phe-Lys-AMC per min at pH 7.5 and 25 °C).

## 3. RESULTS AND DISCUSSION

The compositions of the cheeses (control and experimental) made in Trials A and B are shown in Table I. Within each trial, the composition of control and experimental cheeses was similar, indicating that addition of streptokinase did not influence gross composition. Moreover, addition of streptokinase to cheesemilk did not change the rate of decrease in pH in the vat (not shown). The final pH was similar for all cheeses in both trials, while the moisture content was typical of Cheddar cheese [3]. The salt content was lower than the normal levels in Cheddar cheese (i.e., 1.5%), but values were similar in all cheeses.

Plasmin activity in cheese increased with level of addition of streptokinase (Fig. 1a) with concomitant decreases in the level of plasminogen (Fig. 1b) and plasminogen: plasmin ratio (Fig. 1c). A decrease in plasminogen: plasmin ratio indicates increased conversion of plasminogen to plasmin. Most activation of the plasminogen to plasmin by streptokinase occurred on the day of manufacture, as indicated by results for plasmin activity, plasminogen levels and plasminogen:plasmin

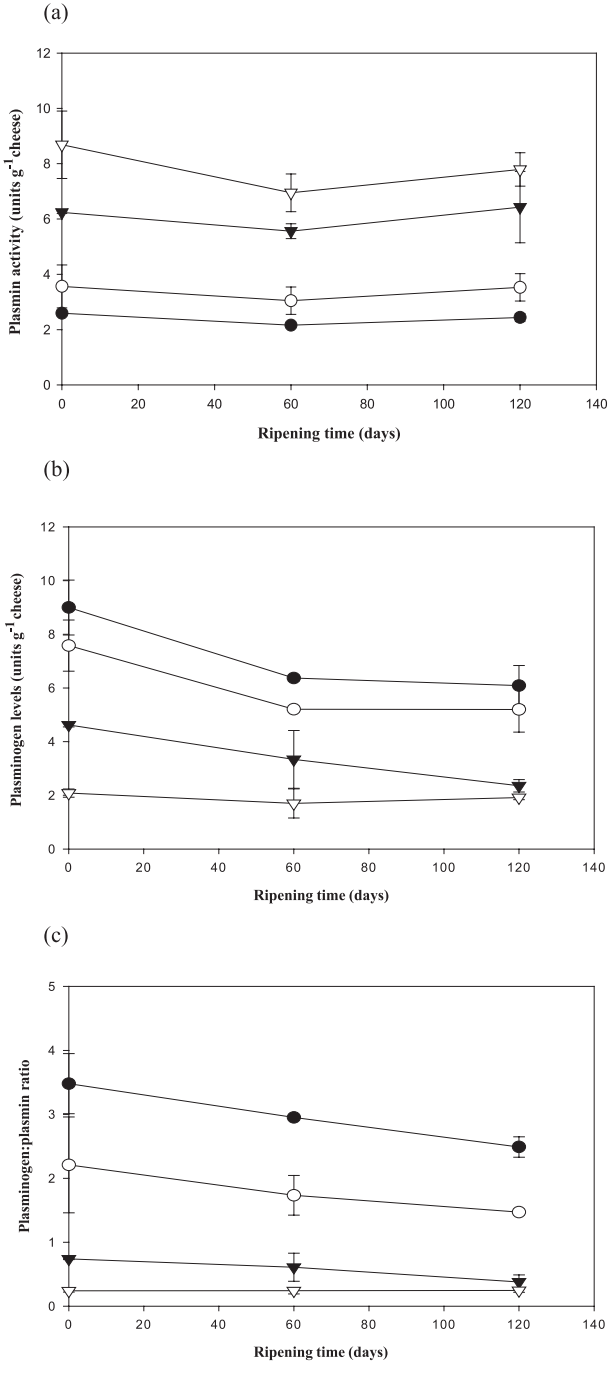


**Figure 1.** Changes in (a) activity of plasmin, (b) plasminogen levels (measured as plasmin activity after activation with 0.2 U·mL$^{-1}$ urokinase) and (c) plasminogen:plasmin ratios during ripening of cheeses made from milk supplemented with 0 (●), 0.10% (○), 0.25% (▼) or 0.50% (▽) streptokinase (v/v). Values are means ± standard deviations of data from triplicate analyses of cheeses from 2 separate trials.

ratio in the cheeses at day 1. After day 1, little further plasminogen activation occurred during ripening. These results were in agreement with the findings of Barrett et al. [3] who found that most of the activation of plasminogen by urokinase occurred on the day of cheese manufacture. The effects of trial and stage of ripening on plasmin activity, plasminogen levels and plasminogen:plasmin ratios were found to be not significant ($P > 0.05$) by ANOVA while treatment had a significant effect ($P < 0.05$) (results not shown). Upadhyay et al. [28] used a streptokinase-producing strain as a starter culture in manufacture of Cheddar cheese and found increased plasmin activity in cheese manufactured using that strain, with most of the activation of plasminogen also occurring on the day of cheese manufacture.

Urea-PAGE electrophoretograms of pH 4.6-insoluble fractions of control and experimental cheeses indicated progressive breakdown of $\alpha_{s1}$- and β-caseins in all cheeses as ripening progressed (not shown). With increased level of addition of streptokinase, increased breakdown of β-casein was observed, with a concomitant increase in the levels of $\gamma_1$-, $\gamma_2$- and $\gamma_3$-caseins, which are breakdown products from β-casein by the action of plasmin [4]. With increasing levels of streptokinase added to cheesemilk, no differences were observed in breakdown of $\alpha_{s1}$-casein, as would be expected, since $\alpha_{s1}$-casein is less susceptible to attack by plasmin than β-casein [2].

Visual observations of breakdown of caseins on urea-PAGE gels were confirmed by densitometric analysis of urea-PAGE electrophoretograms. Pixel intensities (peak volume) of individual bands corresponding $\gamma_1$-, $\gamma_2$-, $\gamma_3$- and β-caseins were plotted against ripening time (Figs. 2a–d). There was a gradual decrease in concentration of β-casein over ripening in all cheeses, including the control; proteolysis of β-casein in the control cheese was due principally to indigenous plasmin activity. With increased levels of streptokinase added to milk, the rate of degradation of β-casein increased proportionately and concentrations of $\gamma_1$-, $\gamma_2$- and $\gamma_3$-caseins also increased. Levels of $\alpha_{s1}$-casein in cheeses did not differ between control and experimental cheese (results not shown).

Data obtained from densitometric analysis were analysed by factor reduction analysis for principal components (PCs), using the peak volume of separated bands as variables; the score plot obtained by PCA is shown in Figure 3. Cheese samples were clearly separated on the basis of age of cheese and experimental treatment; PC 1, which explained 75.18% of total variation, separated the samples based on the ripening time, while PC2 (which explained 19.01% of total variation), separated samples based on treatment. As the level of streptokinase added increased, the samples separated well on PC2 at all stages of ripening; in particular, the control cheese separated well from the cheese made with highest level of streptokinase (0.5%). Based on the loading plot, the probable reason for the separation of samples on PC2 was due to reduction in the band intensity of β-casein and concomitant increases in the intensity of $\gamma_1$-, $\gamma_2$- and $\gamma_3$-caseins.

Levels of pH 4.6-soluble N (SN), expressed as % of total N (TN), after 60 and 120 d of ripening are shown in Table II. With increasing levels of streptokinase, the levels of pH 4.6-SN/TN in cheeses generally increased proportionately. Differences in levels of pH 4.6-SN after 60 and 120 d of ripening (Δ WSN), an index of the rate of proteolysis in the cheese, increased as level of streptokinase increased. Production of pH 4.6-SN in cheese is a result of the activity of chymosin and, to a lesser extent, plasmin [11]. However, urea-PAGE analysis of control and experimental cheeses showed no differences in the levels of $\alpha_{s1}$-casein, which is the substrate most susceptible to chymosin action, or breakdown products from $\alpha_{s1}$-casein. Hence, this increase in the levels of pH 4.6-SN must be attributed to the increased plasmin activity due to activation of plasminogen by streptokinase.

Peptide profiles of lyophilised 70% ethanol-soluble fractions were analysed by

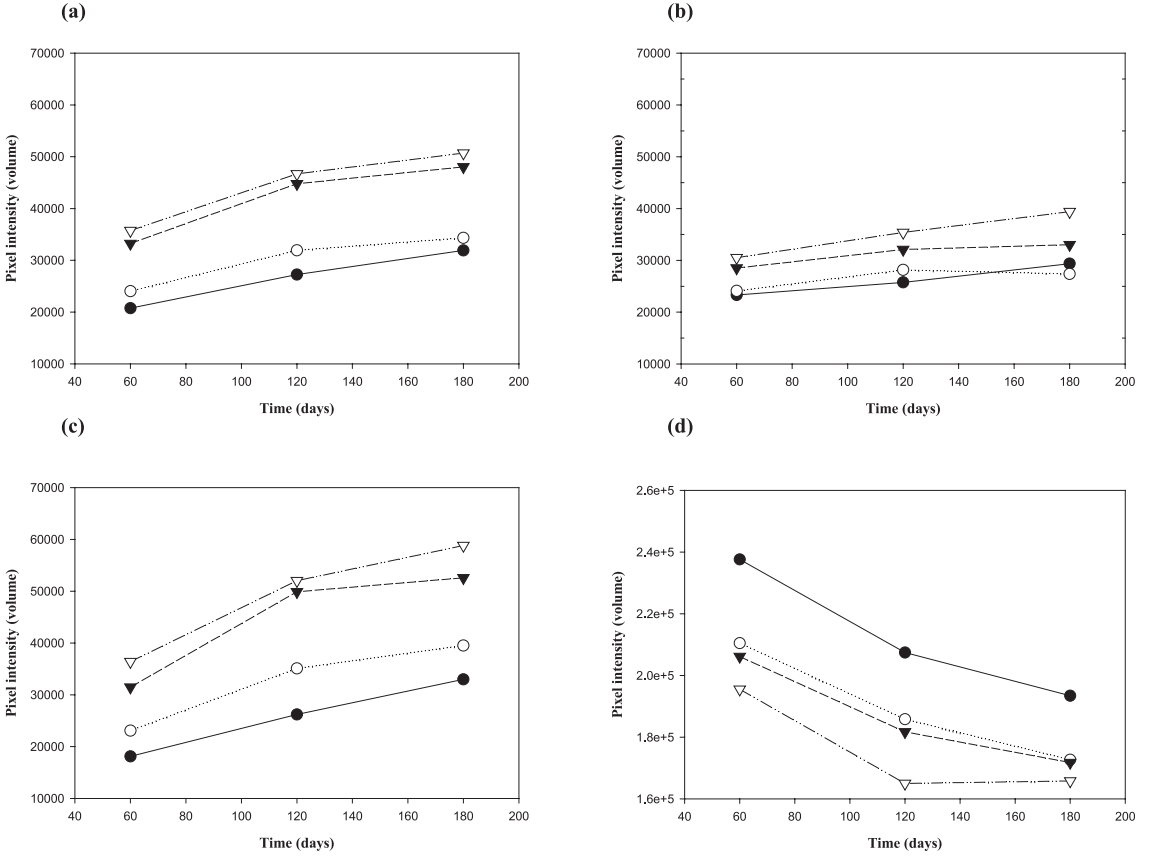


**Figure 2.** Levels of (a) $\gamma_1$-casein, (b) $\gamma_2$-casein, (c) $\gamma_3$-casein and (d) β-casein in Cheddar cheese (Trial A) made from milk supplemented with 0 (●), 0.10% (○), 0.25% (▼) or 0.50% (▽) streptokinase (v/v) at 60, 120 or 180 d, as determined by densitometric analysis of urea-polyacrylamide gel electrophoretograms.

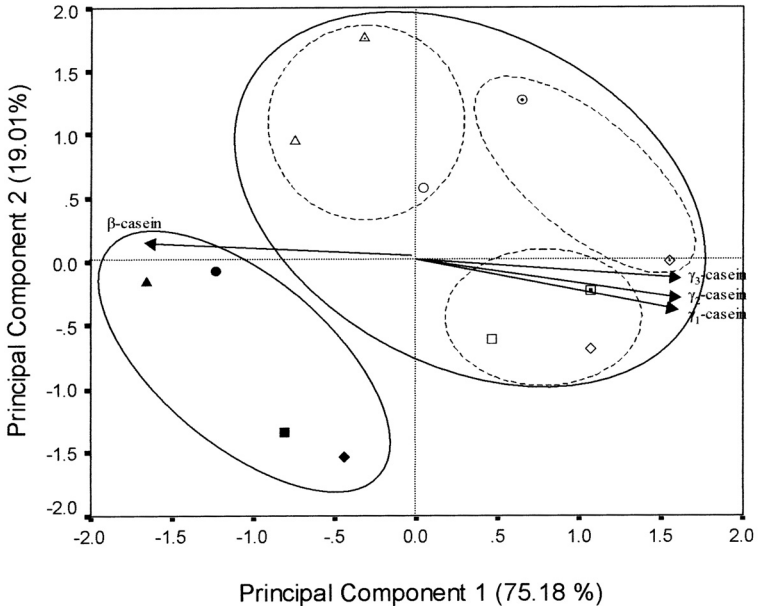


**Figure 3.** Score plot obtained by principal component analysis of densitometric data from urea polyacrylamide gel electrophoresis of Cheddar cheese (Trial A) from milk supplemented with 0 (triangles), 0.10% (circles) 0.25% (squares) or 0.50% (diamonds) streptokinase (v/v) after 60 (closed symbols); 120 (open symbols) or 180 (symbols with dot) d of ripening. Groupings are based on the results of Hierarchical Cluster Analysis (not shown). Arrows indicate loading vectors for β- and γ-caseins.

**Table II.** Levels of pH 4.6-soluble N (expressed as % of total N) in control and experimental cheeses after 60 and 120 d of ripening.

| | Levels of streptokinase (%, v/v of milk) | Time (days) | | |
|---|---|---|---|---|
| | | 60 | 120 | Δ WSN* |
| Trial A | 0.00 | 13.88 | 18.52 | 4.64 |
| | 0.10 | 14.42 | 20.15 | 5.73 |
| | 0.25 | 14.63 | 20.37 | 5.74 |
| | 0.50 | 15.86 | 21.71 | 5.85 |
| Trial B | 0.00 | 14.10 | 19.73 | 5.63 |
| | 0.10 | 15.92 | 21.68 | 5.76 |
| | 0.25 | 15.54 | 21.44 | 5.90 |
| | 0.50 | 16.72 | 23.78 | 7.06 |

* Difference in levels of pH 4.6-soluble nitrogen between 60 and 120 d of ripening.

reversed-phase HPLC; results for Trial A at 180 d are shown in Figure 4. Chromatograms of cheese from other trials and ripening times yielded generally similar trends, and are not shown. In all chromatograms, differences were apparent between levels of peptides

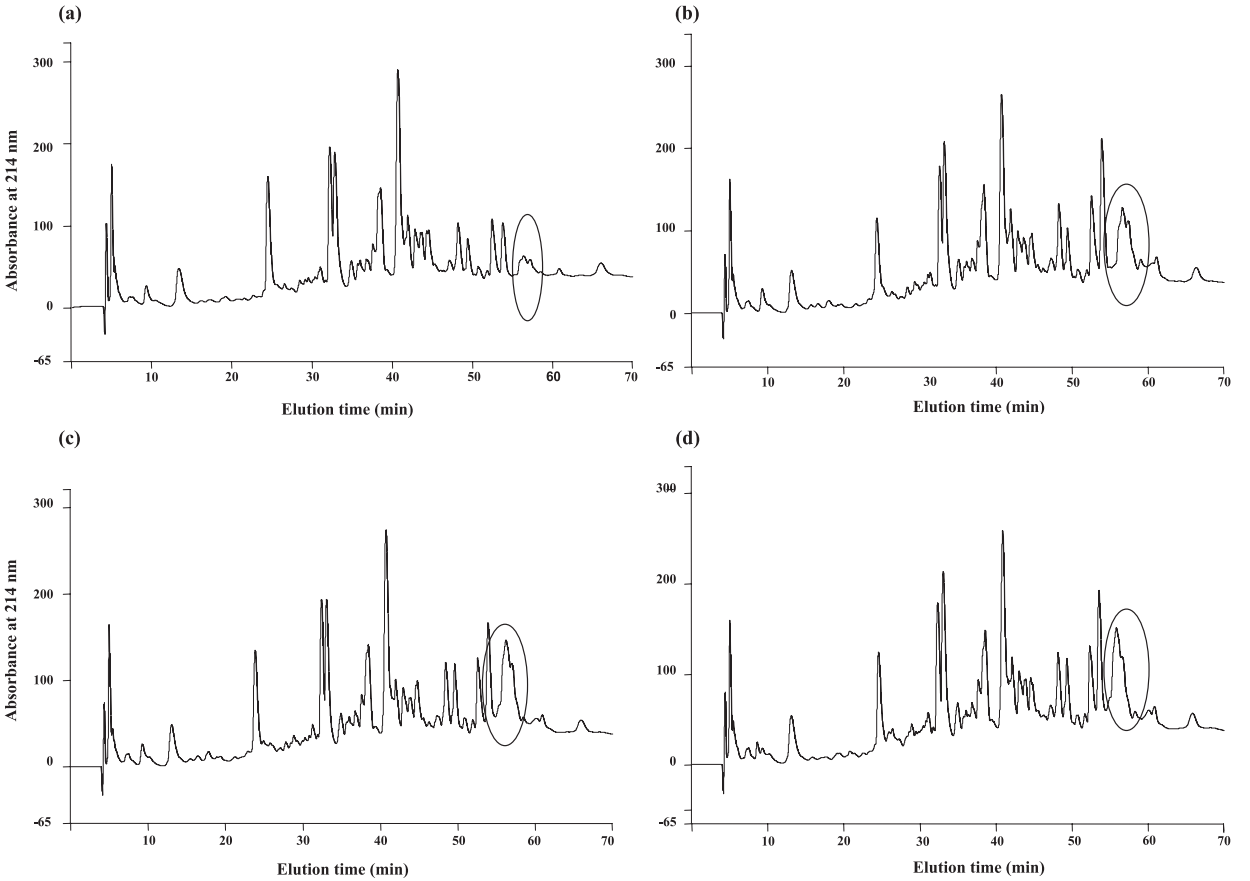


**Figure 4.** Reversed-phase HPLC chromatograms of 70% (v/v) ethanol-soluble sub-fractions of pH 4.6-soluble extracts of Cheddar cheese (Trial A) made from milk supplemented with (a) 0, (b) 0.10%, (c) 0.25% or (d) 0.50% streptokinase (v/v) at 180 d of ripening.

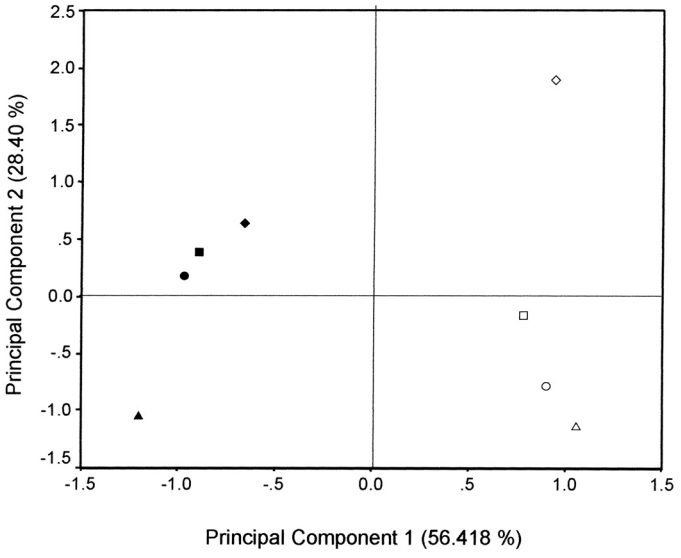


**Figure 5.** Score plot obtained by principal component analysis of reversed-phase HPLC chromatograms of 70% (v/v) ethanol-soluble fractions from Cheddar cheese made from milk supplemented with 0 (triangles), 0.10% (circles), 0.25% (squares) or 0.50% (diamonds) streptokinase (v/v) for Trial A (closed symbols), and B (open symbols) after 180 d of ripening.

eluting between 55–60 min (marked in Figs. 4a–d). Proteose peptones are degradation products produced from β-casein by the action of plasmin [2]; they are soluble at pH 4.6 and are very hydrophobic. Hence when 70% ethanol-soluble fractions are analysed by reversed phase-HPLC, proteose peptones elute at high acetonitrile concentrations. Kelly and O'Donnell [17], when studying proteolysis in Quarg, suggested that the peptides eluting between 55 to 60 min in reversed phase-HPLC chromatograms (under conditions similar to those used in this study) were produced by the action of plasmin. In the present study, the level of peptides eluting in this region increased with level of streptokinase added to the cheesemilk; this further confirmed accelerated breakdown of β-casein as a result of increased plasmin activity due to addition of streptokinase.

Factor reduction analysis was performed on chromatographic data to assess the effect of streptokinase addition; a total of 67 peaks were considered as variables. When the samples were subjected to multivariate statistical analysis (Fig. 5), PC1, which separated the samples based on trial, explained 56.1% of total variation, while PC2, which separated samples based on treatment (level of addition of streptokinase), explained 28.4% of total variation.

Levels of total free amino acids, as determined by the TNBS method, increased over ripening; however, they did not differ between control and experimental cheeses (not shown). Plasmin has little effect on the production of free amino acids but is responsible for production of intermediate-sized peptides [9], which are precursors for formation of free amino acids by action of starter peptidases.

## 4. CONCLUSIONS

Addition of streptokinase from *S. uberis* to cheesemilk effectively increased conversion of plasminogen to plasmin in cheese, hence increasing plasmin activity in Cheddar cheese. Increased plasmin activity accelerated proteolysis of β-casein, with a

concomitant increase in the concentrations of $\gamma_1$-, $\gamma_2$- and $\gamma_3$-caseins, as shown by urea-PAGE analysis and subsequent densitometric analysis of the gels. Increased breakdown of β-casein resulted in differences in the peptide profiles of ethanol-soluble fractions, where more hydrophobic peptides (probably proteose peptones) were produced with increasing level of streptokinase. Increased plasmin activity did not affect total levels of free amino acids. In conclusion, addition of streptokinase to cheesemilk for manufacture of Cheddar cheese accelerated conversion of plasminogen to plasmin and, hence, accelerated proteolysis in Cheddar cheese during ripening.

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