**Research Paper**

# Impact of alginate-chitosan encapsulated Flavourzyme on peptide and amino acid profiles in Cheddar cheese

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**ABSTRACT**

The effect of alginate-chitosan encapsulated Flavourzyme on the water-soluble and water-insoluble peptides and amino acids released during Cheddar cheese ripening was investigated. Increased and prolonged proteolysis was observed in cheeses incorporated with encapsulated Flavourzyme. Rapid proteolysis and increased accumulation of hydrophobic or high molecular weight peptides was however observed in cheeses with free Flavourzyme compared to control (without Flavourzyme) or cheeses with encapsulated Flavourzyme. Concentration of water-soluble peptides increased with the concentration of encapsulated Flavourzyme in the cheese. Most free amino acids were about 3 times greater in cheese with encapsulated Flavourzyme compared to control cheese after 30 days ripening and about 7 times greater after 90 days ripening. Total amino acid content was highest in cheese with encapsulated Flavourzyme followed by free Flavourzyme treated cheese and lowest in control cheese. Flavourzyme encapsulated in slow release alginate-chitosan matrix can be a potential delivery system for flavour enhancement during cheese ripening.

**Keywords:** *Cheese ripening, Encapsulation, Proteolysis, Peptides*

In long ripening cheese varieties such as Cheddar a range of biochemical processes lead to development of characteristic flavour, aroma and texture over time. Proteolysis, lipolysis and glycolysis are the main biochemical processes occurring in cheese during ripening (McSweeney, 2004). It is possible to achieve accelerated cheese ripening with an understanding of the biochemical processes occurring in cheese during ripening and this subject has been extensively reviewed (Wilkinson, 1993; Law, 2001; Azarnia, Robert and Lee, 2006). Exogenous addition of enzymes is a simple and specific approach for accelerating the rate of biochemical reactions occurring during ripening thus accelerating the formation of flavour compounds and reducing ripening duration. Proteolysis is the main biochemical event occurring

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in Cheddar cheese (McSweeney, 2004) and can be accelerated by addition of proteolytic enzymes. Exopeptidases are known to reduce bitterness, a major flavour defect in Cheddar cheese. Though lipolytic flavours are not dominant in Cheddar cheese, lipolysis of milk fat contributes to the overall flavour in mature Cheddar (Fox and Stepaniak, 1993). Direct addition of enzymes to cheese milk has been reported (Kailasapathy and Lam, 2005) to be disadvantageous due to loss of enzymes in whey, poor enzyme distribution, reduced yield and immediate proteolysis. Encapsulated enzymes can minimise the problems associated with the addition of free enzymes (Wilkinson and Kilcawley, 2005).

The procedure for Flavourzyme microencapsulation in alginate-chitosan for application in cheese ripening; developed and optimized by Anjani, Kailasapathy and Phillips, (2007) has been adopted in this study. Although several techniques have been developed for the assessment and characterization of cheese ripening by qualitative and quantitative determination of chemical compounds formed during cheese ripening, chromatography is one of the most widely used methods. RP-HPLC is commonly used for analysis of peptides (Aguilar, 2004; Seneweera and Kailasapathy, 2011) and amino acids (Cohen, 2001).

In the current study, Flavourzyme a flavour enhancing enzyme with exopeptidase and endoprotease activity was efficiently encapsulated in an alginate-chitosan matrix and incorporated into Cheddar cheese for the evaluation of acceleration of ripening. Proteolysis during ripening was monitored by RP-HPLC analysis of water-soluble and water-insoluble peptides and amino acids.

## Materials and methods

Milli Q (Millipore, Massachusetts, USA) water was used for all water-based preparations in this study unless otherwise mentioned.

### *Cheese preparation incorporating alginate-chitosan encapsulated Flavourzyme*

Alginate-chitosan encapsulated Flavourzyme was made by extrusion using an encapsulator as reported by Anjani *et al*. (2007). Flavourzyme microcapsules with an average encapsulation efficiency of 70% were used in this study. Cheddar cheese preparation was adapted from Madziva, Kailasapathy and Phillips, (2006). Cheese was prepared using Armfield cheese vat (Ringhood, England) from 10L milk; 9L pasteurised skim milk combined with 1L fresh cream (35% milk fat, unhomogenised). Milk was heated to 31°C with constant stirring in the cheese vat followed by addition of $CaCl_2$ (50%; Cheeselinks, Victoria, Australia), 2mL diluted to 20mL with sterile Milli Q water. Starter culture Delvo-Tec LL 50C direct set, deep frozen cultures of *Lactococcus lactis* ssp *cremoris* and/or *Lc. lactis* ssp *lactis* (DSM Food specialities, Sydney, Australia) was added at the rate 0.0185 units per 10L without prior activation. Free or microencapsulated was added to the milk before rennetting. Calf rennet 290 IU/mL (Cheeselinks, Victoria, Australia), 2.5mL diluted to 25mL in sterile Milli Q water was added 10 min after starter addition and the stirring was

stopped after a min and the vat was left undisturbed for 40 min. Curd was cut and left undisturbed for 10 min followed by heating to 38°C over 55 min with gentle stirring until the pH reached 6.40 to 6.45, followed by drainage of whey. The whey was filtered using a cheese cloth to collect cheese curd plus any microcapsules lost in the whey and added back to the vat. The curd was banked up against the walls of the vat for 20 min, allowed to settle and cut into large blocks and turned every 20 min while maintaining the temperature of the curd. Once the pH dropped to 5.40 to 5.45 the curd was cut into 1cm cubes and mixed well with 25g salt and allowed to stand for 10min followed by hooping and pressing overnight under 8kg weights. After about 16h of pressing the curd was sliced and vacuum packed and allowed to ripen at 9°C. Control cheese C with no enzyme, cheese D with free Flavourzyme at the level 0.75LAPU/g milk protein and cheese F75 with microencapsulated Flavourzyme at the level 0.75LAPU/g milk protein were made.

*Monitoring of proteolysis in cheese*

Proteolytic pattern in experimental and control cheeses was established by analysing the water-soluble and water-insoluble peptides and free amino acids by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) (Shimadzu Corporation, Kyoto, Japan) and total amino acids by spectrophotometer.

*Extraction of water-soluble peptides*

All cheeses control (with no added enzymes) and experimental (with Flavourzyme) were grated and 10g was homogenised with 30g water followed by pH adjustment to 4.4.-4.6. This homogenate was held at 40°C for 1h before centrifuging for 30 min at 5°C and 4800rpm. After centrifugation, fat layer was removed and the supernatant was diluted to 100ml with water and filtered through a No. 42 Whatman filter paper. This filtrate was stored at -20°C until RP-HPLC analysis (Verdini, Zorrilla and Rubiolo, 2004).

*Analysis of water-soluble peptides*

Water-soluble extract was filtered through a 0.2µm filter (Sartorius, Melbourne, Australia) and 100µL was injected into Shimadzu LA20 Prominence HPLC system with CBM 20A system controller (Shimadzu Corporation, Kyoto, Japan) fitted with an Alltech Altima $C_{18}$ reverse phase column of length 250mm and 4.6mm internal diameter (Deerfield, USA). Detection was at 220nm using the SPD M20A Photo Diode Array detector and the gradient elution was with solvent A (0.1% v/v trifluoro acetic acid (TFA) in water) and solvent B (0.1% v/v TFA in acetonitrile) with a flow rate of 1.0mL$min^{-1}$.

Gradient: Initially 0% B, isocratic step at 0% B for 5 min then linear step to 50% B in 30 min, isocratic step at 50% B for 5 min (Verdini *et al*., 2004).

*Extraction of water-insoluble peptides*

The precipitate obtained after centrifugation of grated cheese (section 2.2.1) was dissolved in 7M urea (Sigma, Sydney, Australia) and dialysed against distilled water for 48h using a MWCO 6000-8000 membrane then freeze-dried in an α-1-4 freeze drier with controller LDC-1M (CHRIST® Gefriertrocknungsanlagen, Osterode am Harz, Germany) and stored at -20°C until RP-HPLC analysis (Verdini *et al*., 2004).

*Analysis of water-insoluble peptides*

Freeze-dried water-insoluble extract (20mg) (obtained in section 2.2.3) was dissolved in 0.01M imidazole (Fluka, Sydney, Australia) pH 7.0, 0.01M dithioerytritol (Sigma, Sydney, Australia) and 6.6M urea (Sigma, Sydney, Australia), total volume 1.5mL and filtered through a 200µm filter (Sartorius, Melbourne, Australia) and 100µL of this solution was injected into Shimadzu LA20 Prominence HPLC system with CBM 20A system controller fitted with an Alltech Altima $C_{18}$ reverse phase column of length 250mm and 4.6mm internal diameter. Detection was at 220nm using the SPD M20A Photo Diode Array detector and the gradient elution was with solvent A (0.1% v/v TFA in water) and solvent B (0.1% v/v TFA in acetonitrile) with a flow rate of 1.0mL$min^{-1}$.

Gradient: Initial composition 0% B, isocratic step at 0%B for 5 min, linear step to 25% B in 5 min, linear step to 35% B in 30 min, linear step to 50% B in 10 min, isocratic step at 50% B for 10 min (Verdini *et al*., 2004).

*Extraction of amino acids*

To 10mL of the water-soluble extract of cheese (obtained in section 2.2.1), 2mL 15% v/v sulphosalicylic acid (SSA) (Sigma, Sydney, Australia) was added and centrifuged for 30 min at 20°C and 4800rpm. Supernatant was collected and the pH was adjusted to 4.0. This SSA-soluble extract was stored at -20°C until reverse phase ultra performance liquid chromatography (RP-UPLC) analysis (Verdini, Zorrilla and Rubiolo, 2002).

*Derivatization of free amino acids and analysis by RP-UPLC*

Concentration of individual amino acids was measured using the sulphosalicylic acid-soluble extract (obtained in section 2.2.5) at the Australian Proteomic Analysis Facility (APAF, Macquarie University, Sydney, Australia). Free amino acids were analysed using ACQUITY Ultra Performance LC system (Waters Corporation, Milford, MA, USA) with Waters Empower Pro control and analysis software. Waters AccQTag Ultra column (BEH $C_{18}$, 2.1 X 100mm, 1.7µm) was used for all separations. Free amino acid concentrations were determined using pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) followed by separation of the derivatives and quantification by RP-UPLC (Cohen and Michaud, 1993; Cohen, 2001). 5µL of the sample was dried in a Savant Speed Vac centrifugal concentrator, model SC210A (Thermo Savant, Holbrook, NY, USA)

with 10µL of internal standard (α amino butyric acid – AABA). The dried sample was redissolved in 80µL of borate buffer followed by 20µL derivatizing reagent (Waters AccQTag Ultra reagent). The reaction mixture was incubated at 50°C for 10 min in a heating block.

Derivatised amino acid samples were transferred to the auto-sampler. The column and auto-sampler temperatures were maintained at 60°C and 20°C respectively. The sample injection volume and flow rate were 1.0µL and 0.7mL$min^{-1}$ respectively. The mobile phase A consisted of AccQTag Ultra eluent A concentrate (Waters) diluted 1 in 20 with milli Q water. The mobile phase B consisted of AccQTag eluent B (Waters). The wavelength of the UV detection was set at 260nm. Analysis time was 10.2 min per sample. Each sample was analysed in duplicate and the results averaged.

*Determination of total amino acids by spectrophotometer*

Total amino acid concentration of the cheese sample was measured as follows. Water-soluble extract of cheese (100µL) was diluted to 1mL, to this 2mL of Cadmium-ninhydrin reagent was added and this mixture was incubated at 84°C for 5 min, cooled and the absorbance was read at 507nm in a Helios Gamma spectrophotometer (Thermo electron corporation, London, UK) (Folkertsma and Fox, 1992).

*Preparation of cadmium-ninhydrin reagent for the determination of total amino acid concentration by spectrophotometer*

Ninhydrin (0.8g, Aldrich, Sigma, Sydney, Australia) was dissolved in 80mL absolute ethanol (Aldrich, Sigma, Sydney, Australia) and to this 10mL glacial acetic acid (Sigma-Aldrich, Sydney, Australia) was added followed by 1g cadmium chloride (Aldrich, Sigma, Sydney, Australia) dissolved in 1mL water (Folkertsma and Fox, 1992).

## Results

*Assessment of proteolysis*

In Cheddar and related cheese varieties proteolysis is an important factor responsible for development of characteristic flavour and texture (Forde and Fitzgerald, 2000; Upadhyay, McSweeney, Magboul and Fox, 2004). In the current study Flavourzyme, a protease peptidase complex was microencapsulated and added during cheddar cheese preparation and its effect on proteolysis was assessed by monitoring water-soluble and water-insoluble peptides, free amino acids and total amino acids formed during ripening by HPLC. All cheeses were vacuum packed and allowed to ripen at 9°C for 270 days and sampled at intervals of 30 days.

*Peptide profile: water-soluble and water-insoluble*

Comparison of HPLC chromatograms of water-soluble peptides of three cheeses;

control C with no added enzyme, cheese D with free Flavourzyme at a concentration of 0.75 LAPU/g milk protein and cheese F75 with encapsulated Flavourzyme at a concentration of 0.75 LAPU/g milk protein showed higher area for peptides represented by peaks 1, 4, 10, 11, 13, 14, 18, 19, 21 and 22 in cheeses D and F75 compared to C in addition to formation of new peptides represented by peaks 3, 5, 6, 8, 9, 12, 16, 17, 20 and 23 in cheeses D and F75 which were absent in cheese C. At 30 days ripening, peptides represented by peaks 2, 7 and 15 were of similar concentration in all cheeses; C, D and F75 (Figure 1). At this stage of ripening, peptides 1, 2, 5, 8, 10, 11, 18 and 19 are found in relatively higher concentrations in cheese D compared to cheese F75 (Figure 1).

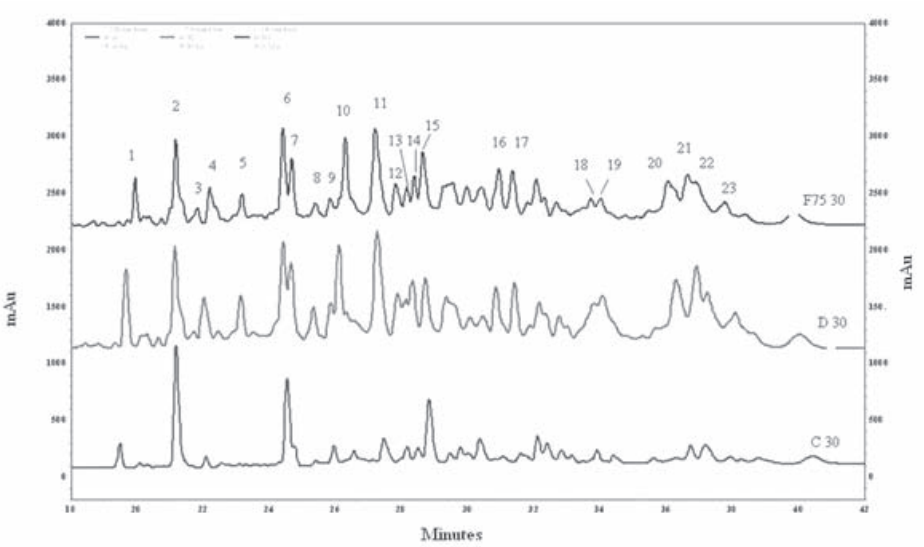


**Figure 1:** Chromatograms of the water-soluble peptides from control cheese (C), cheese with free Flavourzyme (D) and cheese with encapsulated Flavourzyme (F75) extracted after 30 days of ripening

As the ripening progressed to 90 days, the concentration of peptides represented by peaks 1, 2, 6, 11, 12, 14, 19 and 20 was higher in cheeses D and F75 compared to C, while peptides marked by peaks 4, 10 and 15 were at higher concentration in cheese C compared to D and F75. Peptides marked by peaks 3 5, 7, 8, 9, 16, 17, 18, and 21 were found only in cheeses D and F75 but absent in C while peptide 13 was found only in cheese C and absent in experimental cheeses D and F75 (Figure 2). Comparison of water-soluble peptide profile of cheeses D and F75 shows that the peptides represented by peaks 1, 5 and 12 are at higher concentration in cheese F75 compared to D while peptides 4, 10, 14, 16, 18, 19, 20 and 21 are at higher concentration in cheese D compared to F75 while all other peptides appear to be of relatively similar concentration in cheese D and F75 after 90 days ripening (Figure 2).

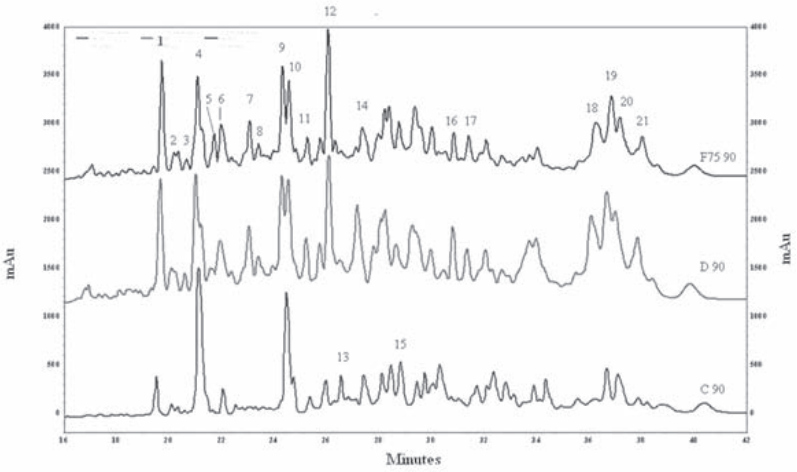


**Figure 2:** Chromatograms of the water-soluble peptides from control cheese (C), cheese with free Flavourzyme (D) and cheese with encapsulated Flavourzyme (F75) extracted after 90 days of ripening

After 180 days ripening, peptides represented by peaks 1, 2, 3, 4, 10, 11, 13, 14, 18, 19 and 20 are in higher concentration in cheeses D and F75 compared to C while peptides represented by peaks 6, 9 and 12 are in higher concentration in cheese C compared to D and F75. Peptides represented by peaks 5, 8, 15, 16 and 17 are found only in cheeses with free and encapsulated Flavourzyme (D and F75) and absent in control cheese while peptide 7 is found only in control cheese C while absent in cheeses D and F75. Comparison of water-soluble peptides of cheeses D and F75 show that the peptides 1 and 14 are at higher concentration in cheese F75 compared to D while peptides 17, 18, 19 and 20 are at higher concentration in

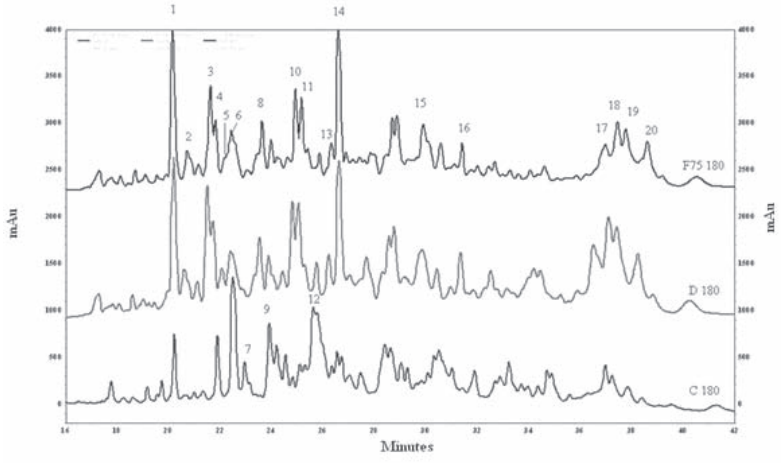


**Figure 3:** Chromatograms of the water-soluble peptides from control cheese (C), cheese with free Flavourzyme (D) and cheese with encapsulated Flavourzyme (F75) extracted after 180 days of ripening

cheese D compared to F75 while all other peptides are at relatively similar concentrations after 180 days ripening (Figure 3).

*Free and total amino acid composition*

Amino acids are largely responsible for the characteristic flavour of cheese (McSweeney and Sousa, 2000; Yvon and Rijnen, 2001). An imbalance in amino acid composition may lead to off-flavours (Habibi-Najafi and Lee, 1996). Comparison of 20 standard amino acid composition of control cheese C and cheese F75 with microencapsulated Flavourzyme at a concentration of 0.75LAPU/g milk protein at 30 days ripening showed that the concentration of all amino acids in F75 was higher than in cheese C; concentrations of serine (Ser), glutamine (Gln), aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), methionine (Met), valine (Val), isoleucine (Ile) and leucine (Leu) in cheese F75 were about 3 times that in cheese C while arginine (Arg) was detected only in cheese F75 (Figure 4). After 90 days ripening, concentration of all amino acids from cheese F75 was ~7-8 times greater than that found in cheese C; asparagine (Asn), serine (Ser), glutamine (Gln), glycine (Gly), glutamic acid (Glu), threonine (Thr), methionine (Met), valine (Val), isoleucine (Ile) and leucine (Leu) from cheese F75 were over 10 times that in cheese

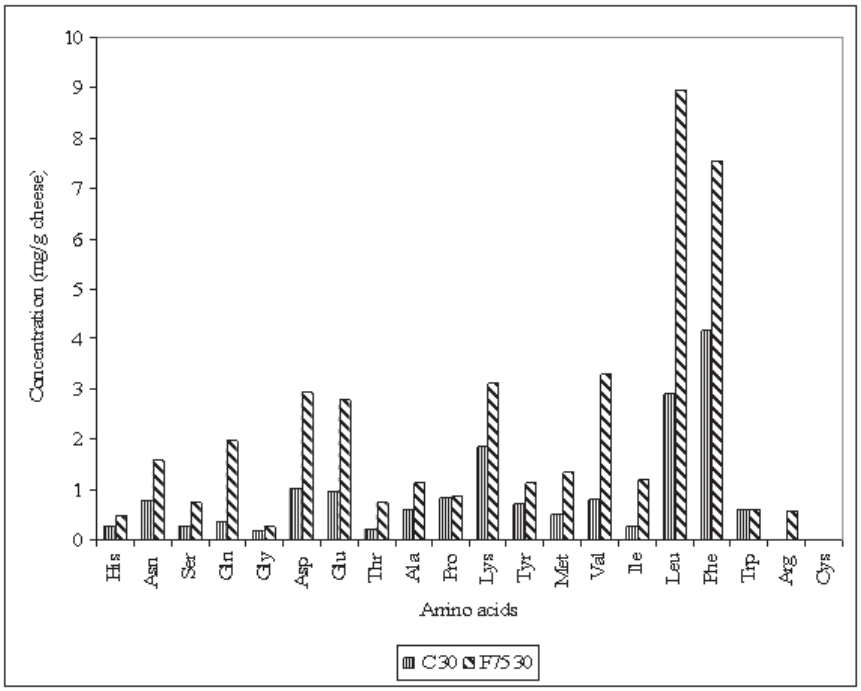


**Figure 4:** Composition of free amino acids from control cheese C with no added enzymes and cheese F75 with microencapsulated Flavourzyme after 30 days ripening

C while arginine (Arg) and cystine (Cys) were detected only in cheese F75 (Figure 5).

Total amino acid concentration in all cheeses increased with the ripening duration and was highest in cheese F75 with 0.75 LAPU Flavourzyme per gram milk protein. Rapid increase in total amino acid concentration was noted after 90 days of ripening (Figure 6).

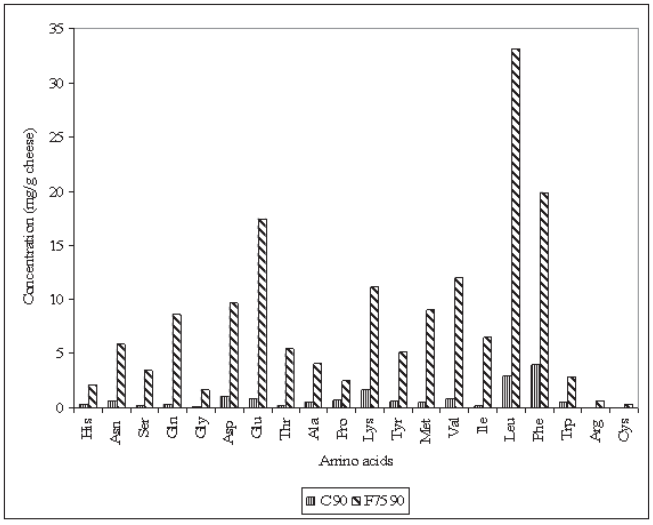


**Figure 5:** Composition of free amino acids from control cheese C with no added enzymes and cheese F75 with microencapsulated Flavourzyme after 90 days ripening

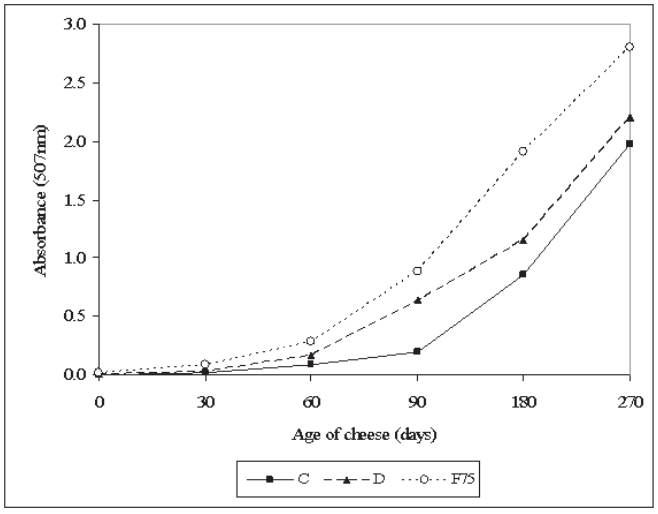


**Figure 6:** Total amino acid concentration monitored in the control cheese C with no added enzymes, cheese D with free Flavourzyme at 0.75LAPU/g milk protein and cheese F75 with microencapsulated Flavourzyme at 0.75LAPU/g milk protein for 270 days at the interval of 30 days

**Discussion**

The comparison of ripening shows clear differences between cheeses with and without added enzymes and also between free and microencapsulated enzymes. Comparison of water-soluble peptide profile of cheeses C, D and F75 after 30 days ripening show that the concentration of peptides in cheeses D and F75 are higher compared to cheese C. There is also release of a larger number of peptides in cheeses D and F75 but absent in C (Figure 1), this can be attributed to the action of Flavourzyme on caseins leading to rapid formation of peptides, which would take longer when cheese ripening occurring naturally (by bacterial enzymes) as seen in cheese C. These results are similar to the results reported by Bergamini, Hynes and Zalazar, (2006) who found increased concentration of several peptides by visual matching of chromatograms of water-soluble extract of a semi-hard cheese with probiotic lyophilized culture compared to control cheese.

Higher concentration of peptides represented by peaks 1, 4, 10, 11, 13, 14, 18, 19, 21 and 22 in cheeses D and F75 compared to C (Figure 1) may possibly be due to faster proteolysis in cheeses D and F75 with free and microencapsulated Flavourzyme respectively. These results are in agreement with reports of increased size-exclusion peak areas of astringent fractions of cheddar cheeses with liposome-encapsulated Flavourzyme added at concentrations of 0.5, 0.75 and 1.0 LAPU/g milk protein at 30 days ripening (Kheadr, Vuillemard and El deeb, 2003). In the current study, presence of peptides 3, 5, 6, 8, 9, 12, 16, 17, 20 and 23 in cheeses D and F75 but absence in C (Figure 1) suggests that these peptides may be the products of hydrolysis of caseins by Flavourzyme in these cheeses. Higher concentration of the peptides 1, 2, 5, 8, 10, 11, 18 and 19 in cheese D compared to F75 (Figure 1) suggests that at 30 days ripening, these peptides may be the products of rapid hydrolysis of caseins by free Flavourzyme in cheese D but due to delayed release of encapsulated Flavourzyme in cheese F75, these peptides appear to be at a lower concentration.

After 90 days ripening, most of the free Flavourzyme added to milk during preparation of cheese D is likely to be lost during whey drainage, but the presence of peptides 1, 2, 6, 11, 12, 14, 19 and 20 at higher concentrations in D and F75 compared to C (Figure 2) suggests that these peptides are possibly the result of immediate hydrolysis of cheese proteins and resistance to further hydrolysis by Flavourzyme and hence continue to be present in higher concentrations even after 90 days ripening in cheese D with free Flavourzyme. These results are in agreement with the reports of higher concentration of size-exclusion peak areas of astringent fractions of Cheddar cheeses with liposome-encapsulated Flavourzyme added at concentrations of 0.5, 0.75 and 1.0 LAPU/g milk protein after 90 days ripening (Kheadr et al., 2003). In the current study, presence of peptides marked by peaks 4, 10 and 15 at higher concentrations in cheese C compared to D and F75 (Figure 2) suggests that these peptides are sensitive to the action of Flavourzyme and hydrolyzed by free and microencapsulated Flavourzyme in cheese D and F75 and thus found in

relatively lower concentrations in these cheeses. Presence of peptides marked by peaks 3 5, 7, 8, 9, 16, 17, 18, and 21 only in cheeses D and F75 but absence in C (Figure 2) indicates that these peptides may be the products of hydrolysis of caseins by Flavourzyme, which is absent in C. Presence of the peptide represented by peak 13 only in cheese C and absence in cheeses D and F75 (Figure 2) suggests that this peptide may be highly susceptible to the action of Flavourzyme and may be hydrolyzed in cheeses D and F75 hence found only in C. Concentration of peptides represented by peaks 1, 5 and 12 at higher concentration in cheese F75 compared to D (Figure 2) suggests that these peptides are products of sustained action of Flavourzyme released from the capsules in cheese F75 whereas in cheese D, most of the free Flavourzyme would be lost in whey as it is water soluble. Higher concentration of peptides 4, 10, 14, 16, 18, 19, 20 and 21 in cheese D compared to F75 (Figure 2) suggests that these peptides may be the products of early hydrolysis by Flavourzyme and may possess the site $Phe_{105}$-$Met_{106}$ or other cleavage sites for Flavourzyme hence appear to be present in lower concentration in cheese F75.

Presence of peptides 1, 2, 3, 4, 10, 11, 13, 14, 18, 19 and 20 at higher concentration in cheeses D and F75 compared to C (Figure 3) is indicative of the prolonged effect of Flavourzyme on cheese proteolysis. Higher concentration of peptides 6, 9 and 12 in cheese C compared to D and F75 and presence of peptide 7 in C but absent in D and F75 suggests that these peptides are sensitive to the action of Flavourzyme and may possess the Flavourzyme cleavage site $Phe_{105}$-$Met_{106}$. Presence of peptides 5, 8, 15, 16 and 17 only in cheese D and F75 but absence in C (Figure 3) indicates that these peptides are products of the action of Flavourzyme on cheese proteins. Highest concentration of peptides 1 and 14 in cheese F75 after 180 days ripening (Figure 3) suggests that these peptides are constantly released due to the prolonged action of encapsulated Flavourzyme on cheese proteins and tend to accumulate during ripening. Presence of peptides 17, 18, 19 and 20 in cheese D at higher concentration compared to F75 suggests that these peptides are produced as early as the day of manufacture and accumulated due to the diminishing impact of free Flavourzyme, (expected to be lost during whey drainage) in cheese D and hence detected even after 180 days of ripening (Figure 3).

Reverse-phase high-performance liquid chromatography (RP-HPLC) involves separation of molecules based on hydrophobicity; solutes are eluted in the order of increasing molecular hydrophobicity (Cliffe and Law, 1990; Aguilar, 2004). Peptides 20, 21, 22 and 23 (Figure 1) appear to be relatively hydrophobic or high molecular weight compared to other peptides as they were eluted after other peptides. These peptides start to accumulate early during ripening and are at high concentration in cheese with free and encapsulated Flavourzyme compared to control C with no added enzyme and these peptides continue to accumulate during ripening and do not appear to be sensitive to the action of Flavourzyme even after 180 days ripening (peaks 17, 18, 19 and 20, Figure 3). In a similar study, Ardo, Lilbaek, Kristiansen, Zakora and Otte, (2007) reported large hydrophobic peptides that elute late in RP-

HPLC in a narrow group (83-89 min) and accumulate in the semi-hard cheese Herrgard during ripening and suggested that these peptides are products of early hydrolysis of â-casein by plasmin. In the current study a similar trend in accumulation of hydrophobic or high molecular weight peptides was observed. These may be the products of â-casein hydrolysis and accumulate during ripening. However in the current study, the retention time of these peaks was found to be 37-40 min. The variation in the retention time of the peaks reported in the current study and that reported by Ardo *et al*. (2007) may be due to variation in the type of cheese, extraction, purification and RP-HPLC analysis. The concentration of water-insoluble peptides appear to be lower in cheese with microencapsulated Flavourzyme compared to control cheese with no enzyme (data not shown) indicating efficient release of the microencapsulated enzyme and its proteolytic activity on cheese proteins.

Amino acids are produced as a result of peptide hydrolysis and are known to contribute to the flavour of cheddar cheese directly and also indirectly by acting as precursors for the production of flavour compounds (Yvon and Rijnen, 2001). From the water-soluble and water-insoluble peptide profiles it was found that the cheeses with free and microencapsulated Flavourzyme showed increased proteolysis compared to control cheese; with no added enzymes. Free amino acid composition of control cheese C and cheese F75; with encapsulated Flavourzyme was compared at 30 and 90 days ripening to assess the exopeptidase activity of Flavourzyme. At both ripening times, levels of all amino acids were at elevated levels in cheese F75 compared to cheese C. The concentration of most free amino acids from cheese F75 was found to be ~1-6 times greater compared to cheese C after 30 days ripening (Figure 4) and ~7-30 times greater after 90 days ripening (Figure 5).

Higher concentration of amino acids in cheese F75 suggests extensive proteolysis due to the action of Flavourzyme. Presence of most amino acids at very high concentration after 90 days of ripening suggests that a large number of amino acids are released around that time, as the amino acids released earlier would be catabolised to smaller compounds by the cheese microflora, but the presence of most amino acids at very high concentration after around 90 days ripening also suggests that these amino acids may be released in high concentrations at this stage of ripening possibly as a result of reduced number of SLAB and low numbers of NSLAB (Fox and McSweeney, 2004) thus with minimal catabolism of amino acids. Release of large number of amino acids especially after 90 days ripening may also be due to the time involved in break down of peptides and subsequent release of amino acids. This observation was also supported by the measurement of total amino acids; the concentration of which increased rapidly after 90 days ripening (Figure 6) and was highest in cheese F75 with microencapsulated Flavourzyme at a level of 0.75 LAPU/g milk protein.

After 30 days ripening, serine (Ser), glutamine (Gln), aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), methionine (Met), valine (Val), isoleucine (Ile) and leucine (Leu) were about 3 times greater in cheese F75 than in cheese C, while

arginine (Arg) was detected only in cheese F75 (Figure 4). Most of these amino acids are hydrophilic and the release of these hydrophilic amino acids indicates the tendency of hydrophilic cleavage of Flavourzyme. Similarly after 90 days ripening, asparagine (Asn), serine (Ser), glutamine (Gln), glycine (Gly), glutamic acid (Glu), threonine (Thr), methionine (Met), valine (Val), isoleucine (Ile) and leucine (Leu) were over 10 times that in cheese C, while arginine (Arg) and cystine (Cys) were detected only in cheese F75 (Figure 5). Most amino acids in C were 10-30% lower compared to F75; these results indicate strong exopeptidase activity of Flavourzyme. At 90 days ripening, Leu, Phe and Lys are the most abundant amino acids in cheese C, where as in cheese F75, Leu, Phe and Glu are the most abundant amino acids. These results agree with Aston and Creamer (1986), McSweeney and Fox (1993) and De Wit, Osthoff, Viljoen and Hugo, (2005) who reported that Leu, Phe and Glu are the most abundant free amino acids in mature Cheddar cheese. Hence release of free amino acids in cheese F75 appears to be similar to standard Cheddar cheese.

While some amino acids in cheese C decreased marginally after 90 days ripening, most were found at similar or higher concentrations. In contrast most amino acids in F75 increased 3-6 folds after 90 days ripening suggesting sustained release of microencapsulated Flavourzyme and its effect on peptide hydrolysis. De Wit et al. (2005) also reported a significant increase in free amino acid content of yeast-inoculated Cheddar cheese from 60-120 days.

The optimum temperature range of Flavourzyme is 30-50°C which is well above the ripening conditions of cheddar cheese. It was assumed that the activity of encapsulated Flavourzyme in the cheese would be reduced during ripening due to low temperature, however extensive proteolysis in all the cheeses with free and encapsulated Flavourzyme suggest that the activity of Flavourzyme was not severely affected even at cheddar cheese ripening conditions and the encapsulated enzymes were efficiently released.

## Conclusion

Increased proteolysis was observed in cheeses with Flavourzyme. Incorporation of free Flavourzyme into Cheddar cheese resulted in immediate proteolysis with accumulation of hydrophobic or large peptides as early as 30 days ripening while cheeses with encapsulated Flavourzyme showed delayed proteolysis with gradual increase in peptide concentration. As the ripening progressed for about 90 days, the concentration of hydrophobic peptides in free Flavourzyme treated cheese was higher compared to cheese with microencapsulated Flavourzyme suggesting added bitterness in the former. Desired rate of release of the encapsulated enzyme can be achieved by careful designing of the alginate-chitosan matrix. Hence can be used for delayed release of the flavour-enhancing enzymes and controlled flavour development during cheese ripening. Extensive proteolysis observed in all the enzyme treated cheeses suggest that the level of enzyme used was higher. Addition of microencapsulated Flavourzyme lead to increased proteolysis even at low

temperature observed during cheese ripening. Incorporation of Flavourzyme in similar alginate-chitosan matrix can be useful for delayed yet sustained release of encapsulated enzymes and hence potentially lead to accelerated cheese ripening. Alginate-chitosan microencapsulated Flavourzyme may also be applied for development of new flavours.

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