INFLUENCE OF COMMERCIAL PROTEASES ON THE PROTEOLYSIS OF ENZYME MODIFIED CHEESE

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Abstract

The influence of four commercial proteases, Protease A, Protease B, Protease C and a two enzyme blend Protease DE, on proteolysis in an enzyme modified cheese (EMC) base has been investigated. Also, a series of preliminary experiments to determine the basic characteristics of the four enzyme preparations in buffer systems has been undertaken.

Generally, the exopeptidase activity of the four enzyme preparations was more stable than the endopeptidase activity of the preparations. The highest enzyme activity for all preparations was given at pH 6.5 and Protease B was found to be sensitive to chelating agents. In addition, Protease B was found to contain at least two exopeptidases.

Residual protease activities in EMC using a 55% moisture cheese base were found to be 0.005%, 0.009%, 0.007% and 0.004% (w/v) for Protease A, Protease B, Protease C and Protease DE, respectively, following inactivation by heating at 95°C heating for 30 minutes.

Under the same incubation conditions (0.15% enzyme at 40°C for 24 h), Protease DE gave greater proteolysis than the three other enzymes and Protease B was the weakest protease. EMC digestion with a combination of proteases was different from that obtained with individual proteases. The combinations of Protease A/Protease C, Protease DE/Protease C, Protease B/Protease C and Protease DE/Protease A showed that the higher the proportion of the former protease in the combinations, the higher the amounts of total amino acids produced in the EMC. The combinations of Protease A/Protease B and Protease B/Protease DE gave greater amounts of total amino acids with the ratio of each enzyme close to 50:50 than with the individual enzymes. With respect to the molecular mass distribution of peptides in the various EMC digestions, Protease DE produced the greatest amount of peptides of 3 or fewer residues and Protease C gave the greatest amount of more medium sized peptides with 11-20 residues. Compared with Protease C, Protease A was more efficient in giving small peptides, while Protease B gave the lowest levels of medium and small peptides, but a high level of free amino acids.

In sensory testing, Protease DE produced EMC with a strong pungent and astringent flavour, Protease C gave bitterness, Protease A gave a sweet flavour at a low concentration but bitter flavours with a high concentration and Protease B produced more savoury flavour without bitterness.

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Chapter 1. Introduction

Today's consumers prefer food full of flavour, especially in low fat food products. Flavour is one of the critical determinants of cheese quality. Cheese flavour is popular and used widely in the food industry. In Cheddar cheese, flavour development usually requires storage for at least 12 months to reach a characteristic full flavour. The cost of storage space and time is a major factor in the overall production cost.

Some investigators indicate that Cheddar cheese has a complicated flavour development process that results from enzymatic reactions (Moskowitz and Noelck, 1987; Fox, 1989a). Nowadays, a number of cheesemakers are using supplementary enzymes to manage Cheddar flavour development to improve targeting of cheese flavour. There are various methods for achieving the extra flavour boost during manufacturing and accelerated ripening, such as adding enzyme to the starter milk or to the curds as whey is drained. However, cheese-flavoured ingredients can be obtained by making enzyme modified cheese (EMC).

Cheese that has been treated enzymatically to improve the flavour or provide a significant portion of the flavour profile is called enzyme modified cheese (Moskowitz and Noelck, 1987). Specific enzymes are added and, as near as possible, conditions are adjusted to optimal for the production of typical cheese flavours. EMC flavour development can take only a few hours or a few days, compared with traditional aging that may require several months or more (Vafiadis, 1996). In addition, the flavour intensity of EMC is approximately 15-30 times that of natural cheese, and it is able to replace up to 75% of the naturally aged cheese used in foods (Anon, 1989). The advantages of enzyme modified cheese therefore include production of a targeted flavour ingredient, avoiding the use of naturally aged cheese with all its inherent costs and production disadvantages, and a reduction of the overall cheese solid content in food products such as low fat, health-orientated products.

Cheese flavour is derived from proteolysis, lipolysis and lactose breakdown. Proteolysis is the most complex and perhaps the most important biochemical event during natural cheese ripening (Fox, 1989a, 1993; Wilkinson, 1993; Kilcawley *et al.*,

1998). Both lipolysis and proteolysis are important in EMC manufacture. The impact of lipases in EMC has been studied (Gunaratne, 1999) and the current project focuses on the influence of proteases on EMC digestion. Proteolysis is the factor that causes softening of the texture of natural cheese during the early stages of ripening and influences cheese flavour development by the formation of amino acids and peptides. The water soluble fraction of cheese contains considerable concentrations of free amino acids that probably contribute to the background flavour of cheese and serve as substrates for various flavour generating reactions. On the other hand, an imbalance of high molecular weight peptide formation may cause bitter flavours. The production of bitterness is the main problem encountered when most commercial proteinases are used to accelerate cheese ripening. Another critical aspect of EMC technology is controlling enzyme activities. Unless they can be inactivated, enzymes may continue to break down the cheese after the desired flavour is developed and thereby produce undesirable flavour. Residual enzyme activity in EMC can also hydrolyse components of the final product in which the EMC is an ingredient.

Proteolytic enzyme activities are influenced by parameters such as incubation time, temperature and enzyme concentration. The purpose of this project is to better understand how these parameters impact on the action of four different proteolytic enzymes, namely Protease A, Protease B, Protease C and the two enzyme blend Protease DE (individually or in combination) in terms of the extent of proteolysis and flavour development of EMC cheese base. High performance liquid chromatography, especially gel filtration chromatography and reversed phase chromatography, were used to profile the molecular mass distribution of peptides and the quantities of amino acids in EMC. These profiles will serve as preliminary maps to aid ongoing efforts to achieve the targeted manipulation of EMC flavour.

Chapter 2. Literature Review

2.1. Cheese flavour development

2.1.1. The compounds of cheese flavour development

The chemistry of cheese flavour is very complex. For example, there are more than 180 compounds that have been identified as contributing to Cheddar cheese flavour, and more than 125 compounds as components of Swiss cheese flavour. Although it is difficult to characterize cheese flavour, any particular cheese-type flavour contains certain significant compounds (Moskowitz and Noelck, 1987).

According to a review by Fox and Wallace, (1998), compounds produced during ripening of natural cheese are at concentrations and relative proportions typical of that variety of cheese. Cheese flavour components can be grouped into three simple types; 1) fat-derived - mainly fatty acids, 2) protein-derived - mainly peptides, 3) methyl ketones (Godfrey, 1996; Fox, 1993; Kosikowski and Iwasaki, 1975). Most of these compounds can be generated in EMC, and their relative concentrations are dependent on the conditions used during manufacture. Therefore, it is very important to understand the basis of cheese flavour development to better understand the chemistry of flavour and flavour development in making enzyme modified cheese. For example, proline and propionic acid are important components in Swiss cheese flavour. In Blue cheese the important components are acetic acid, butanoic acid, acetone, methyl ketones, 2-pentanol, methyl hexanoate, ethyl butanate, 2-nonanol, and free fatty acids. In Camembert, octenol is a significant component (Moskowitz and Noelck, 1987; Kilcawley *et al.*, 1998). Cheddar flavour is complex, comprising a high number of flavour compounds including acetic acid, diacetyl, fatty acids, sulphur compounds, amino acids, and small peptides (West, 1996). Table 2.1 shows the volatile flavour compounds that have been identified in Cheddar cheese.

Table 2.1. Volatile flavour compounds identified in Cheddar cheese

Acetaldehyde	Dimethyl sulfide	3-Methyl-2-butanone
Acetoin	Dimethyl disulfide	3-Methylbutyric acid
Acetone	Dimethyl trisulfiide	2-Nonanone
Acetophenone	δ-Dodecalatone	δ-Octalactone
β-Angelicalatone	Ethanol	n-Octanoic acid
1,2-Butanediol	Ethyl acetate	2-Octanol
2-Butanol	2-Heptanone	2,4-Pentanediol
Butanone	n-Hexanoic acid	2-Pentanol
n-Butyl acetate	n-Hexanol	Pentan-2-one
n-Butyric acid	2-Hexenal	n-Propanol
Carbon dioxide	Isobutanol	Propenal
ρ-Cresol	Isohexanal	n-Propyl butyrate
γ-Decalactone	Methanethiol	Tetrahydrofuran
δ-Decalactone	Methional	Thiophene-2-aldehyde
n-Decanoic acid	Methyl acetate	Thiophene-2-aldehyde
Diacetyl	2-Methylbutanol	2-Tridecanone
Diethyl ether	3-Methylbutanol	2-Undecanone

Seitz (1990) reported that methanethiol was a significant contributor to Cheddar cheese flavour and that H_2S was formed during cheese ripening from cysteine and methionine. Butanone and 2-butanol were found in high concentration in Cheddar cheese; they were produced by *Lactobacillus plantarum* and *L. brevis* (Dumont and Adda, 1979). West (1996) suggested that it is desirable to use salted Cheddar curd to produce Cheddar EMCs to generate the most authentic flavour. This is because the activity of chymosin and the enzymes of starter cultures have already begun breaking down casein to products that contribute to the final flavour (West, 1996; Kilcawley *et al.*, 1998). In addition, the starter produces lactic acid that both contributes to flavour and provides the correct acidity for curd formation; the increase in acidity also helps to reduce the risk of contaminating microorganisms (West, 1996). Microbial spoilage may also be more of a problem in EMC systems in which a lipase is not added because short chain free fatty acids can have anti-microbial activity. According to Parker and

Pawlett (1986), the protein-derived flavour notes of Cheddar are best developed at pH 5-5.5.

Tables 2.2 and 2.3 show volatile and non-volatile compounds, respectively, involved in the flavour of cheese and the microorganisms that produce them (Fox and Wallace, 1998; Dziezak, 1986)

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Table 2.2. Volatile compounds involved in flavour of various cheeses

Compounds	Cheeses	Producing microorganisms
Propionic acid	Swiss	*Propionibacterium shermanii*
Dimethyl sulfide	Swiss	*Propionibacterium shermanii*
Volatile fatty acids (butyric, valeric, *etc.*)	Swiss	*Clostridium butyricum, Lactic (starter) lactococci*
Amines	Cheddar-Limburger	*Lactococcus fecalis, Lactococcus lactis*
Methyl ketones	Blue-veined	*Penicillium roqueforti*
2-pentanol, 1-octen-3-o1	Blue-veined	*Penicillium roqueforti*
Volatile fatty acids	Limburger	*Candida mycoderma*
Carbonyl compounds	Limburger	*Candida mycoderma*
Acetone-acetic acid	Limburger	*Debaryomyces kloeckeri*
Volatile sulfur compounds (H_2S, dimethyl disulfide, methyl mercaptan)	Camembert	*Aspergillus oryzae,* *Lactococcus* and *Lactobacillus,* *Propionibacterium shermanii*
Alcohols Ethanol, 1-propanol, 1-butanol		

Table 2.3. Non-volatile compounds involved in flavour of cheeses

Compounds	Cheeses	Producing microorganisms
Lactic acid	All cheese	*Lactococcus* and *Lactobacillus*
Acid-soluble nucleotides	White mould cheese	*Penicillium caseicolium*
Citric acid	Limburger cheese	*Brevibacterium linens*
Peptides	All cheese	*Lactococcus cremoris, Lactobacillus bulgaricus, L. helveticus, Penicillium roqueforti and P. camemberti*
Amino acids	All cheese	*Lactococcus, Lactobacillus, P. roqueforti and P. camemberti*
Proline, hydroxyproline	Swiss cheese	*Propionibacterium shermanii*

2.1.2. The role of proteolysis in cheese flavour development

Proteolysis, lipolysis and lactose breakdown are the three major processes responsible for cheese flavour development. Of these, proteolysis is perhaps the most complex event during cheese ripening. In fact, proteolysis has been identified as a critical process to determine the rate of flavour and texture development in most cheese varieties. The role of proteolysis in cheese flavour development is considered to be the production of peptides and free amino acids from protein which are not only flavour compounds themselves, but can also give rise to other flavour compounds by further metabolic processes. Consequently, protein breakdown has been used as a crude indication of cheese ripening (Samples *et al.*, 1984). Proteolysis can impact on cheese ripening in four distinct ways: (1) by production of amino acids and peptides, (2) by catabolism of the amino acids produced, (3) by pH change through the formation of NH_3, (4) by texture changes due to breakdown of the protein network (Fox, 1993; Gripon *et al.*, 1991; Kieran and Cogan, 1988). Figure 2.1 shows the formation of flavour

components that can be traced back to protein breakdown (Anon, 1989).

Proteolysis mainly contributes to the softening of the texture of cheese during the early stages of ripening and influences the development of cheese flavour through the formation of amino acids and peptides. Amino acids are substrates for the formation of numerous flavour compounds. Table 2.4 shows the flavour characteristics and flavour threshold of free amino acids themselves (Seitz, 1990; Vafiadis, 1996).

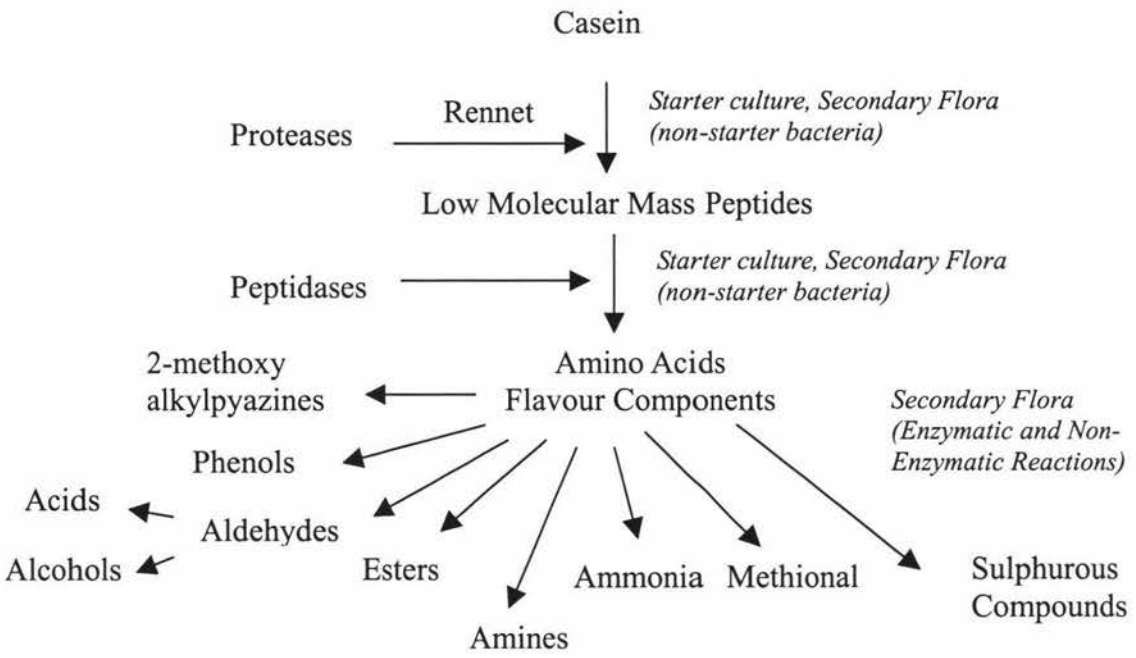


Figure 2.1. Formation of flavour components from protein breakdown

Table 2.4. Taste descriptors and threshold values for taste perception of amino acids

Amino acid	Taste threshold $\mu g\ mL^{-1}$	Conc [a] in Cheddar ($\mu g\ g^{-1}$)	Perceived in Cheddar [b]	Taste			
				Sweet	Sour	Bitter	Umami
Gly	1300	371	–[c]	***[d]			
Ser	1500	1210	–	***			*
Thr	2600	649	–	***		*	
His	200	436	±		***		
Asp	30	606	+		**		*
Glu	50	5075	+		***		**
Arg	500	1737	+			***	
Ala	600	337	–	***			
Met	300	869	+			***	
Lys	500	2330	+	**		**	
Val	400	2022	+			***	
Leu	1900	4610	±			***	
Pro	3000	389	–	***		***	
Phe	900	2400	+			***	
Tyr	-[e]	607	-			***	
Ile	900	466	–			***	
Trp	900	-	-			***	

[a] : Wilkinson *et al.*(1992); 6 month old Cheddar, total concentration = 24.1 mg g^{-1} cheese

[b] : Amino acids in Cheddar are deemed to be perceived if their concentration in a water extract (50 g cheese containing 37% moisture to 100 mL water) is greater than their threshold concentration.

[c] : –, not perceived; ±, possibly perceived; +, definitely perceived.

[d] : *, mild; **, moderate; ***, strong.

[e] : -, not known

Proteolytic agents in cheese originate from five sources: the coagulant, the milk itself (indigenous proteinases) and from the starter, adjunct cultures and adventitious non-starter lactic acid bacteria (Fox, 1993).

In mould-ripened cheeses, further proteolysis results from the proteinases and peptidases of, *e.g.*, *Penicillium roqueforti* and *P. camemberti*.

In fact, the flavours of most of the foods we enjoy arise as a result of enzymes. Recently the main commercial flavour compounds have been chemically synthesized and are identical or similar to the flavour compounds produced by nature. However, consumer demand for healthier and more natural products has created a demand for enzyme-produced flavours, that is flavours that are made by man but using natural means rather than chemical synthesis.

2.2. Technology of enzyme modified cheese

2.2.1. The production of enzyme modified cheese

Enzyme modified cheeses (EMCs) are made by combining cheese curd with selected enzymes and then incubating at a suitable temperature for a defined time to develop an intense cheese flavour or a significant portion of the cheese flavour profile. Emulsifying salts such as sodium phosphate are added prior to enzyme addition, to ensure that the fat and protein form a homogeneous phase.

In general, the various types of EMCs are made using the corresponding immature cheeses (*e.g.*, Mozzarella EMC is made using Mozzarella cheese base) to generate the authentic flavour cheese pastes. In the production of EMCs, the choice of immature cheese and incubation with the proper type of enzyme are critical. During production of EMC the conditions are favourable for the growth of bacteria, so bacterial spoilage can potentially be a serious problem. Therefore, equipment must be sterile and all obvious measures must be considered to avoid contamination (Kilcawley *et al.*, 1998). Figure 2.2 shows the outline of a scheme for EMC production (West, 1996).

Vafiadis (1996) showed that control of time and temperature was very important in order to obtain a consistent EMC product. Unless they are inactivated, enzymes will continue to break down the cheese after the desired flavour is developed, and so generate undesirable flavour and texture before the end of the product's shelf life. Heat treatment is therefore used to inactivate the enzymes. The heating temperature is critical: it is necessary to avoid destroying the desirable flavour by over-heating, but not enough heating will mean that enzymes are not completely inactivated.

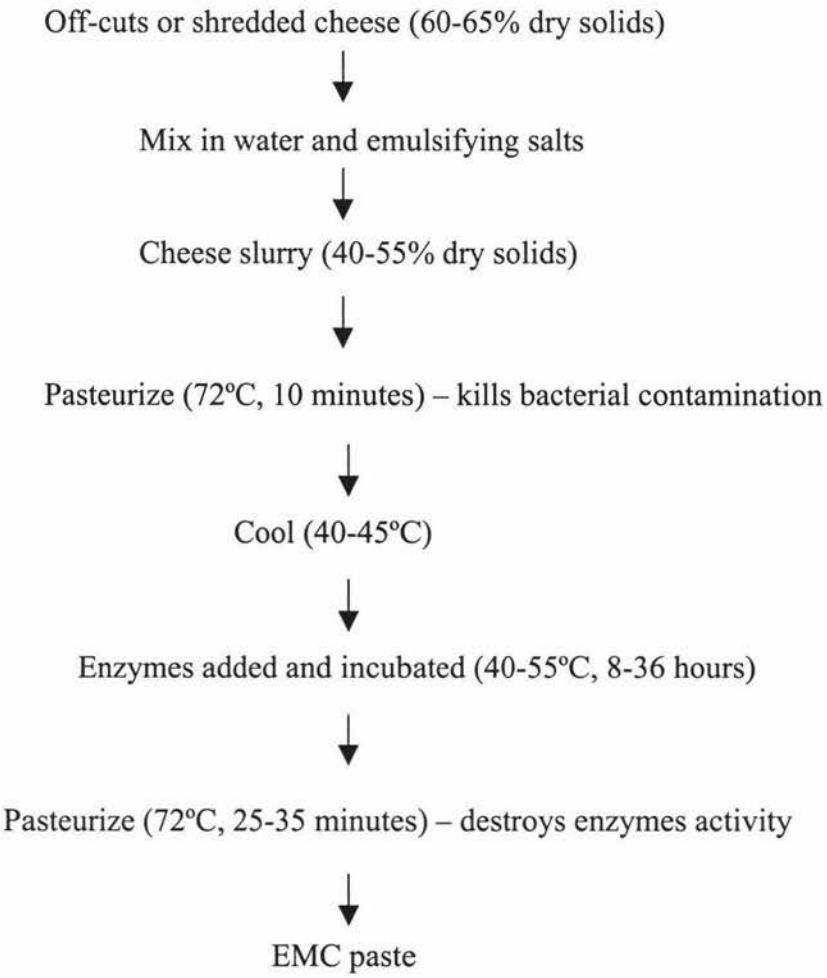


Figure 2.2. Outline of scheme for enzyme modified cheese production

2.2.2. Uses of enzyme modified cheese

The flavour intensity of EMC is approximately 15-30 times that of natural cheese (Anon, 1986; Kilcawley *et al.*, 1998). As EMCs provide a strong cheese note, food technologists only need to use a small amount of EMC to provide the same flavour intensity as larger amounts of regular cheese. They can therefore either increase the cheese flavour of a product without increasing the cheese solid content, or reduce the cheese content to produce "healthier products", while still maintaining the original cheese flavour (Vafiadis, 1996). Therefore, EMCs are considered to be economical, nutritional and natural. EMC flavour is available in

Cheddar, Mozzarella, Romano, Provolone, Feta, Blue, Gouda, Swiss, Emmental, Gruyere, Colby and Brick types (Kilcawley *et al.*, 1998). These cheese flavours have a wide range of applications in salad dressings, dips, soups, sauces, snacks, pasta products, cheese analogues, frozen foods, crackers, cake mixes, biscuits, quiche fillings, cheese spreads, low-fat and no-fat cheese products and cheese substitutes or imitations (Anon, 1996; Kilcawley *et al.*, 1998).

Enzyme modification for flavour development of EMC is based on the same biochemical changes that happen in traditional cheese ripening processes, but using exogenous hydrolytic enzymes to shorten the incubation time.

2.2.3. The proteases involved in EMC production

Although EMC preparations are normally associated with fat-derived flavours, proteolysis is particularly important for certain types of cheese, such as Cheddar flavour. There are two approaches to achieve production of cheese flavours derived from proteolysis. The traditional approach is a one-step process, *i.e.* mixing the substrate and all of the required enzymes together. The other approach is to generate the flavour components separately, then blend these together in the desired ratio.

Fungal proteases are very widely used in EMC manufacture. The reasons for the widespread use of fungal proteases are that not only do they not produce high levels of bitter peptides, but also some of them contain very high levels of both carboxy- and amino- peptidases and will debitter hydrolysates (Hanson, 1990). *Bacillus subtilis* and *Aspergillus* sp. are apparently the source for most of the commercial proteinases (El Soda, 1993). Several researchers have, however, extracted enzymes for EMC manufacture from the following cheese-related microorganisms; *Lactococcus, Lactobacillus, Pediococcus, Leuconostoc, Propionibacterium, Brevibacterium, Micrococcus and Pseudomonas* (Law, 1984; Fox, 1989b; El Soda and Pandian, 1991; El Soda, 1993).

The enzymes extracted from various microorganisms provide for the development of a variety of cheese flavours.

Swiss cheese is made through the use of the culture *Propionibacterium freudenreichii* subsp. *shermanii* to produce propionic acid and free proline

together with acetic acid (Steffen *et al.*, 1993; Eaton, 1994; Kilcawley *et al.*, 1998). *P. shermanii* has weak proteinase activities, but has high peptidase activities, especially proline specific peptidases, and gives significant proteolysis in Swiss cheese. Although proline is an important component of Swiss cheese flavour, it is also a common bitter peptide component. Therefore, it is critical to avoid excess proline-rich peptides during the production of EMC (Moskowitz and Noelck, 1987; Kilcawley *et al.*, 1998).

Most of the flavour and aroma in Camembert cheese is produced by lactic acid bacteria and the secondary mould culture, *Penicillium camemberti*. The extent of proteolysis in Camembert cheese is relatively high: up to 35% total nitrogen in the cheese surface is soluble at pH 4.6 and is therefore made up of small peptides (Gripon *et al.*, 1991; Kilcawley *et al.*, 1998).

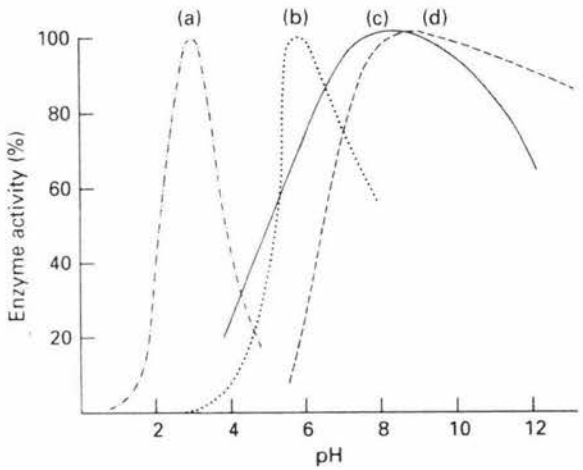
2.2.4. Physical factors affecting enzyme activities

The efficiency of any enzyme added to the curd to accelerate cheese ripening will depend on the following factors: enzyme dosage, ratio of peptidase to proteinase activity, uniform distribution of the enzyme in the curd, and stability of the enzyme in the curd (Wilkinson *et al.*, 1992).

The main physical factors affecting enzyme activity and stability are pH, temperature and salt concentration.

1) Effect of pH

The pH value will affect the ionization state of the amino acids which control the primary and secondary structure of enzymes. Consequently, pH controls the overall activity and stability of enzymes. Figure 2.3 shows pH activity curves of different enzymes (Godfrey, 1996), while Table 2.5 shows pH optima ranges for both these and other important enzymes (Godfrey, 1996).



(a) Acid protease, *Aspergillus niger*.
(b) Neutral protease, *Bacillus subtilis*.
(c) Alkaline protease, *B. licheniformis*.
(d) Superalkaline protease, *B. licheniformis*.

Figure 2.3. The pH activity curves of different microbial proteases (from Godfrey, 1996)

Table 2.5. pH optima ranges for several important enzymes

Enzyme	E.C. classification number	Producing organism	Stability/activity under 'normal' conditions (pH 2 4 6 8 10 12 14)
Pepsin	3.4.23.1	Mammalian	
Acid proteinase	3.4.23.6	*Aspergillus niger*	
Microbial rennet	3.4.23.4	*Mucor miehei*	
Neutral proteinase	3.4.24.4	*Bacillus subtilis*	
Alkaline proteinase I	3.4.21.14	*B. licheniformis*	
Alkaline proteinase II	3.4.21.14	*B. licheniformis*	

....., stability optimum range; _____, activity optimum range (from Godfrey, 1996)

The stability of the enzyme may be affected by a change of pH, because it may cause the irreversible denaturation of the enzyme structure and loss of enzyme activity. It is important to note that many industrial enzyme reactions are not run at a fixed pH value, and slowly drift from a start pH to a terminal pH. The buffering capacity of substrate or the products of reaction is usually involved in controlling the pH value of the system (Godfrey, 1996).

2) Effect of temperature

Temperature is a critical parameter for enzyme activity. The amount of activity given by an enzyme is related to both heat activation and heat inactivation of the enzyme. As the temperature rises, the rate of enzyme activity increases, but so does the rate of inactivation. Table 2.6 shows practical temperature activity ranges of some important enzymes (Godfrey, 1996).

Table 2.6. Temperature optima ranges of some important enzymes

Enzyme	E.C. classification number	Producing organism	Stability/activity under 'normal' conditions Temp.°C 20 30 40 50 60 70 80 90 100 110
Acid proteinase	3.4.23.1	*Aspergillus niger*	
Microbial rennet	3.4.23.6	*Mucor miehei*	
Neutral proteinase	3.4.23.4	*Bacilis subtilis*	
Alkaline proteinase I	3.4.24.14	*B. licheniformis*	
Alkaline proteinase II	3.4.24.14	*B. licheniformis*	

....., stability optimum range; _____, activity optimum range (from Godfrey, 1996)

3) Effect of ionic strength

The influence of ionic strength on the primary phase of rennet coagulation is critical (Alais and Lagrange, 1972; Grufferty and Fox, 1985; Payens and Visser, 1981; Banks and Muir, 1985; Barry and Donnelly, 1980; Aston and Creamer, 1986). For instance, increasing the ionic strength reduces the rate of hydrolysis of the sequence His_{98}-Lys_{112} which contains the necessary determinants for rapid cleavage of the Phe-Met bond in κ-casein. The effect becomes more significant while the reaction pH is increased but this effect is independent of ion type (Fox, 1989b; Visser *et al.*, 1980). Jenkins and Emmons (1983) also reported that NaCl, $CaCl_2$ and $MgCl_2$ (1 mM), stimulated the hydrolysis of κ-casein in the isolated form and in sodium caseinate. Ionic strength can be expected to have a significant effect on the activity of other proteolytic enzymes.

2.2.5. The defects of enzyme modified cheese

Kilcawley *et al.* (1998) reported that proteinases are important in producing Cheddar cheese slurries and EMCs due to their ability to accelerate proteolysis; however, the production of bitterness is a major problem associated with most

commercial proteinases (Kilcawley *et al.*, 1998; Anon, 1989). Most bitter peptides normally contain 3-15 amino acids and are characterized by the presence of a high level of hydrophobic amino acids such as leucine, isoleucine, proline, valine, phenylalanine, tyrosine, and tryptophan.

Adding an enzyme such as a purified protease to an EMC base can therefore result in bitterness due to an imbalance in the formation of high molecular weight peptides. The production of hydrophobic peptides by the action of endoproteases on casein then leads to the bitter off-flavours of EMC which is a common defect. Balancing flavour development is therefore important. Some reports showed that the addition of peptidases can be used to reduce bitterness (Cliffe and Law, 1990; Kristoffersen *et al.*, 1967).

Ney (1979) reported that bitterness in dairy products is related to the average hydrophobicity of peptides or amino acids rather than specific amino acid sequences or peptide chain length. He also described the relationship between Q value (average hydrophobicity) and bitterness of peptides as Q value = $\Sigma\Delta f/ n$ (Δf = hydrophobicity of an individual amino acid; n = number of amino acid residues). Ney (1979) also found that bitterness only occurred when the Q value of a peptide (molecular mass < 6,000 Da) exceeded 1,400.

The water-soluble fraction (WSF) consists of peptides and amino acids, which are responsible for flavour as well as off-flavour intensity in cheese. Reverse-phase HPLC and Liquid Chromatography/Mass Spectrometry are generally used to analyze water soluble peptides of cheese (Park *et al.*, 1993). According to Habibi-Najafi and Lee (1996), addition of *Lactococci* or *Lactobacilli* or their crude extracts helped significantly in reducing bitterness. Also, Law and Wigmore (1982) combined neutrase (sourced from *Bacillus subtilis*) with *Lactococcus* peptidases and this mixture resulted in an acceptable cheese flavour (El Soda, 1993).

According to Godfrey and West (1996), bitterness in EMC can be reduced by controlling protein hydrolysis through the correct balance of endopeptidase and exopeptidase enzymes. Exopeptidases breakdown bitter peptides by cleaving single amino acids or dipeptides from oligopeptides (Pawlett and Bruce, 1996).

Habibi-Najafi and Lee (1996) also reported several attempts have been made to

control the bitterness problem by addition of masking agents to hydrolyzed protein, application of hydrophobic chromatography to absorb bitter peptides, and employment of the transpeptidation reaction.

2.3. Assessment of cheese proteolysis

The measurement of proteolysis during cheese ripening is usually done by determining the quantity of peptides and amino acids extracted from cheese. McSweeney and Fox (1997) broadly classified the methods for cheese proteolysis assessment under two categories: non-specific methods and specific methods. Non-specific methods include the quantitation of nitrogen soluble in various extractants or precipitants and the liberation of reactive functional groups, while specific methods include those which resolve individual peptides or free amino acids, such as electrophoretic and chromatographic methods (McSweeney and Fox, 1997).

Non-specific techniques provide information about the extent of proteolysis and the activity of enzyme, but they provide little information about increases or decreases in peptide levels during ripening. Thus, more attention has been paid to electrophoresis and chromatography in recent years (McSweeney and Fox, 1997).

Fox (1989b) summarized several approaches adopted to monitor proteolysis quantitatively in cheese during ripening: solubility of peptides, measurement of reactive functional groups, enzyme assays, chromatographic techniques and electrophoresis.

2.3.1. Solubility of peptides in various solvents or precipitants

Caseins and proteins in general are insoluble in many solvents, while peptides released from them may be soluble. Therefore, the ratio of soluble to insoluble nitrogen will increase with proteolysis, which is the principle behind many of the techniques used for assessment of proteolysis. A number of solvents and precipitants have been used to extract or fractionate cheese nitrogen, including water, buffers near pH 4.5, NaCl (5%), trichloroacetic acid (TCA) (2%, 5%, 10% or 12%), phosphotungstic acid (5%), sulphosalicilic acid (2.5%), picric acid, ethanol, ethanol-acetone, chloroform-methanol and $CaCl_2$ solution (Fox, 1989b).

2.3.2. Measurement of the liberation of reactive functional groups

Estimation of cheese proteolysis can be done by monitoring the liberation of specific compounds or reactive groups such as amino- or carboxyl- groups. Many functional groups can be used to measure cheese ripening:

1). The formation of ammonia

Ammonia is formed from amino acids. In Camembert or smear-ripened varieties, ammonia is an important product of proteolysis as it is partly responsible for the flavour and texture of these cheese varieties. Many workers (Furtado and Chandan, 1985; Alonso *et al.*, 1987; Zarmpoutis *et al.*, 1996) have indirectly estimated ammonia formation by measuring the increase in the pH of cheeses. However, it has not been used widely as an index of proteolysis (McSweeney and Fox, 1997).

2). The formation of soluble tyrosine and tryptophan

Measurement of the tyrosine / tryptophan content of alcohol-, TCA- or water-extracts of cheese is a well established method for assessing proteolysis. Folin-Ciocalteau reagent and absorbance of UV light (280 nm) are both used to quantify tyrosine and tryptophan. UV light absorbance has been widely used to monitor proteolysis in cheese (McSweeney and Fox, 1997; Fox, 1989b).

3). Dye-binding

Dye binding techniques exploit the isoionic point of proteins reacting with anionic dyes such as amido-black, acid orange 12 and orange G to form an insoluble protein-dye complex. Centrifugation or filtration is used to remove the protein-dye complex and the remaining dye in the supernatant or filtrate is measured spectrophotometrically. Because the amount of dye bound by the protein is proportional to the concentration of protein, the remaining dye is inversely proportional to the protein concentration. However, low molecular weight proteins and peptides react slowly, resulting in poor separation and turbid filtrates or supernatants. Coomassie blue G250 has also been used in protein determination, but Wallace and Fox (1994) found that this technique was not suitable for quantifying water-soluble peptides (Fox, 1989b; McSweeney and Fox, 1997).

4). Free amino groups - colorimetric and fluorimetric methods

Colorimetric and fluorimetric techniques are based on the principle that the amino group that results from the cleavage of a peptide bond can react with several chromogenic or fluorogenic reagents, such as 2, 4, 6-trinitrobenzenesulphonic acid (TNBS), ninhydrin, fluorescamine and *o*-phthaldialdehyde (OPA). The reactions of these reagents with the α-amino group of an amino acid are shown in Figure 2.4 (McSweeney and Fox, 1997; Lemieux, 1990).

a). TNBS

TNBS gives a stoichiometric reaction with primary amines to form a chromophore, which remains attached to the amino acid, peptide or protein and absorbs maximally at 420 nm. In the method, an alkaline pH is optimum for the reaction while a lower pH can stop the reaction. The advantage of the technique is that it is a simple procedure. However, the disadvantage is that the dry TNBS powder is explosive and long storage can result in high blank values (McSweeney and Fox, 1997; Clegg *et al.*, 1982).

b). Ninhydrin

Ninhydrin is widely used to estimate cheese ripening by monitoring the liberation of free amino groups. The technique is based on the spectrophotometric measurement of a chromophore produced from the reaction of ninhydrin with free amino groups. The maximum absorbance is about 507 nm for this method. The major advantage of this technique is that the purple chromophore, Ruhemann's purple, does not remain attached to the protein or peptide, thus there is no precipitate to be clarified before spectrophotometric analysis (McSweeney and Fox, 1997; Pearce *et al.*, 1988). In addition, the technique is more sensitive than TBNS methods.

Many researchers have adopted this technique to assay proteolysis in cheese ripening. Ninhydrin has been widely used to quantify amino groups in chromatographic eluates, particularly in ion-exchange chromatography followed by post-column derivatisation. Cliffe *et al.* (1989) used ninhydrin to measure fractions obtained by reverse-phase chromatography. Folkertsma and Fox (1992) found that Cd-ninhydrin reagent was about five times as sensitive as TNBS for measuring amino acid nitrogen and could be used on citrate-soluble and water-

soluble fractions, but not TCA-soluble fractions.

c). Fluorescamine

The principle of the fluorescamine method is the assay, at 390 nm excitation and 475 nm emission, of the fluorophore produced from the reaction of fluorescamine and primary amino groups. Fluorimetric methods are generally more sensitive than the colorimetric methods for quantification of free amino groups, but have not been used widely. This method has, however, been used to quantify acid-soluble proteins, peptides and amino acids in cheese extracts. The results obtained from this technique have been found to be more consistent than TNBS (Creamer *et al.*, 1985; McSweeney and Fox, 1997).

d). *o*-phthaldialdehyde

The principle of this method is the measurement of a fluorescent complex (1-alkylthio-2-alkylisoindole) formed by the reaction of *o*-phthaldialdehyde, 2-mercaptoethanol and primary amines at 340 nm. The advantage of the method is that it is more convenient than the ninhydrin, TNBS or fluorescamine procedures (Church *et al.*, 1983; McSweeney and Fox, 1997).

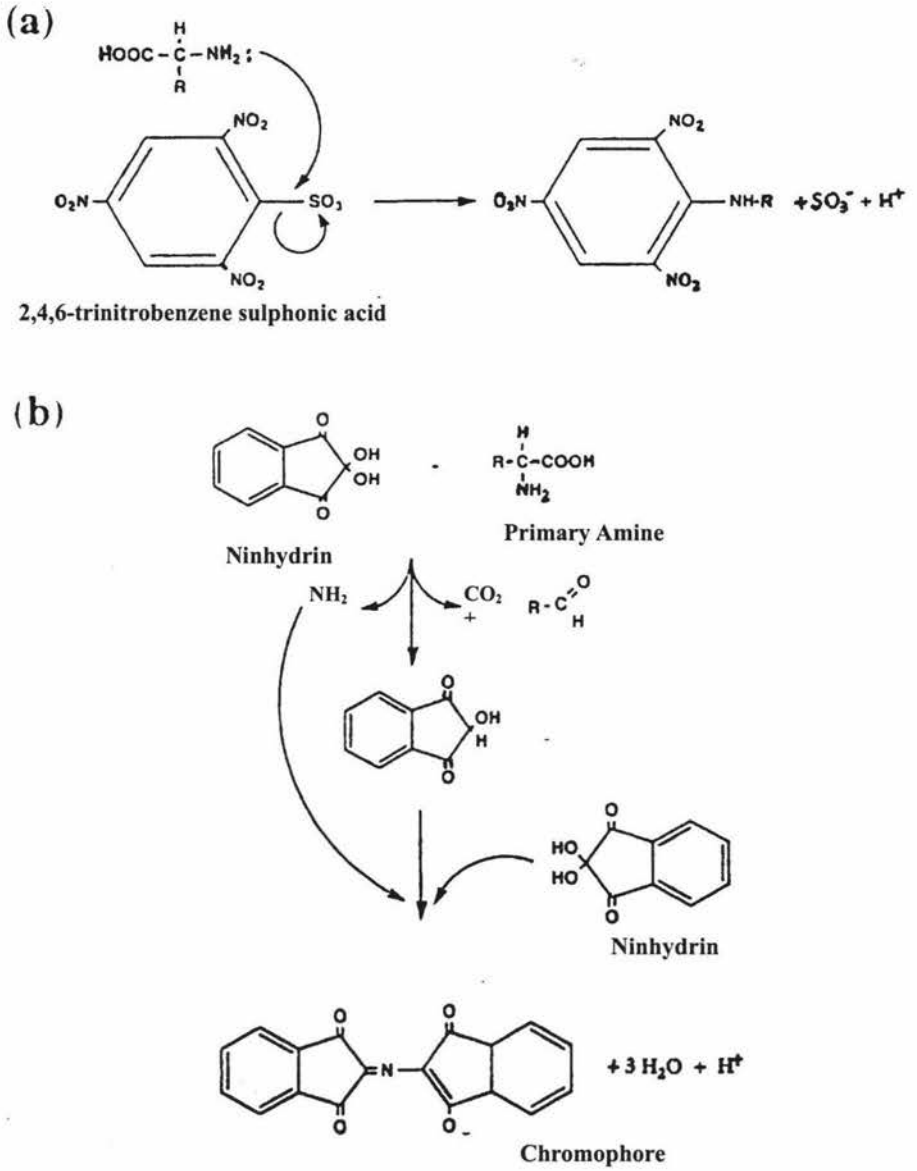


Figure 2.4. Reaction of (a) 2,4,6-trinitrobenzenesulphonic acid, (b) ninhydrin, (c) fluorescamine and (d) *o*-phthaldialdehyde with the α-amino group of an amino acid. (Part 1)

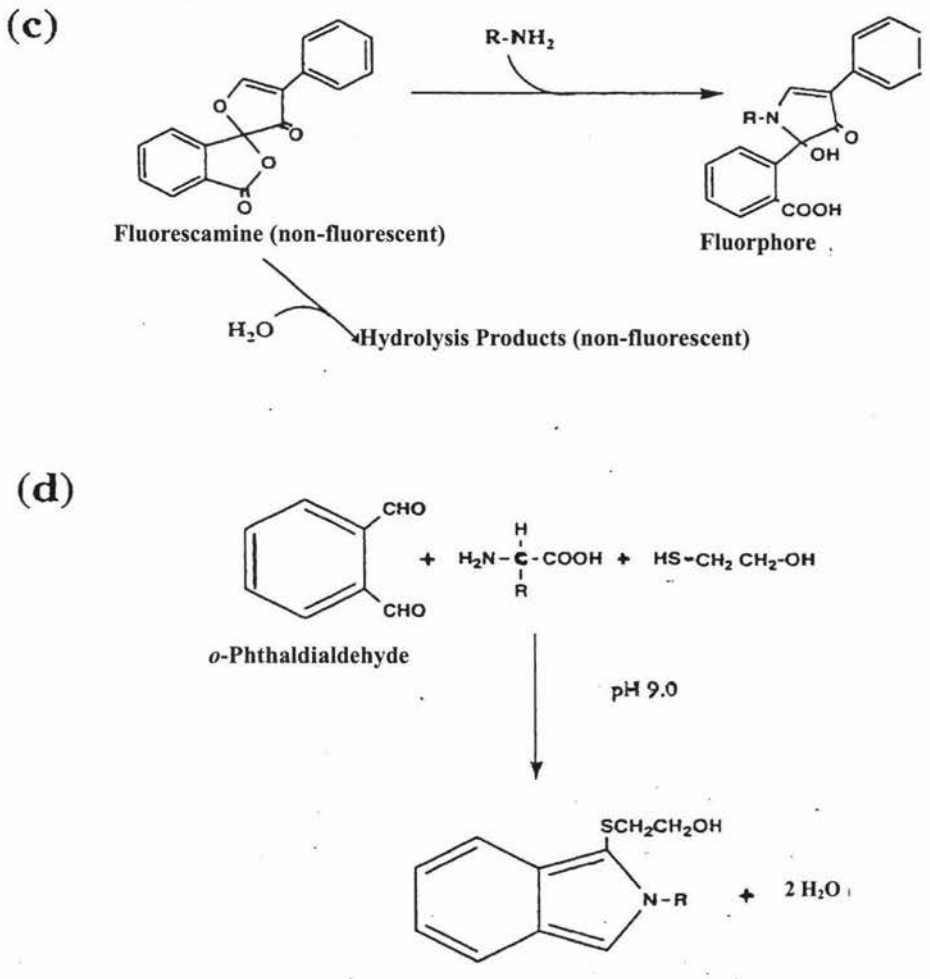


Figure 2.4. **Reaction of (a) 2,4,6-trinitrobenzenesulphonic acid, (b) ninhydrin, (c) fluorescamine and (d) *o*-phthaldialdehyde with the α-amino group of an amino acid. (Part 2)**

2.3.3. Enzyme techniques

L-Glutamic acid (Glu) is an important free amino acid in many cheese varieties as it contributes an "umami" flavour. Many enzyme assays for Glu have therefore been adopted for measuring proteolysis of cheese during ripening (Puchades *et al.*, 1989; McSweeney and Fox, 1997). Puchades *et al.* (1989) used flow injection analysis and glutamate dehydrogenase immobilized on activated glass for the

enzymic assay of Glu. Enzyme activity was estimated from the decrease in NAD^+. McSweeney *et al.* (1993) used a commercial assay kit containing glutamate dehydrogenase to measure free Glu in water extracts of Cheddar. The procedure used pig-heart diaphorase to catalyze the reaction of iodonitrate-triazolium chloride with NADH and formazan, followed by spectrophotometric determination at 492 nm. Using the method, the free Glu content of the cheese was shown to increase with ripening and the proteolysis of cheese could be measured according to the extent of Glu content (McSweeney *et al.*, 1993). This procedure was different from that of Puchades *et al.* (1989), in which NADH was measured directly.

2.3.4. Various forms of chromatography

Molecules can be separated by chromatography according to their physical properties (size, shape and charge), hydrophobic interactions, chemical properties, covalent binding, or biological properties such as biospecific affinity. Table 2.7 shows a number of chromatography techniques that allow the separation of molecules based on these properties (Gölker, 1990).

Table 2.7. Chromatographic methods

Type of chromatography	Principle	Separation mode
Adsorption	Surface binding	Surface affinity
Distribution	Distribution equilibrium	Polarity
Ion exchange	Ion binding	Charge
Gel filtration	Pore diffusion	Molecular size, molecular shape
Affinity	Specific adsorption	Molecular structure
Hydrophobic	Hydrophobic chelation	Molecular structure
Covalent	Covalent binding	Polarity
Metal chelate	Complex formation	Molecular structure

High performance liquid chromatography is widely used to separate or purify peptides and proteins. Size exclusion high performance liquid chromatography (SE-HPLC) and reverse phase high performance liquid chromatography (RP-HPLC) are two techniques that are widely used for the analysis of peptides and proteins.

Size exclusion chromatography, also referred to as gel filtration or gel permeation chromatography, separate molecules by exploiting differences in their apparent molecular sizes. SE-HPLC does not inactivate enzymes and thus is routinely used as an important step in their purification. The widespread used of SE-HPLC can also be attributed to the speed, simplicity and versatility of the method. The column packing materials of SE-HPLC contain particles with well-defined pore sizes. The molecules may flow into and out of the pores as they are carried through the column by the mobile phase. These molecules will be separated by differences in their sizes, or more precisely, hydrodynamic volumes (Phenomenex, 1998). The schematic representation of SE-HPLC is shown in Figure 2.5.

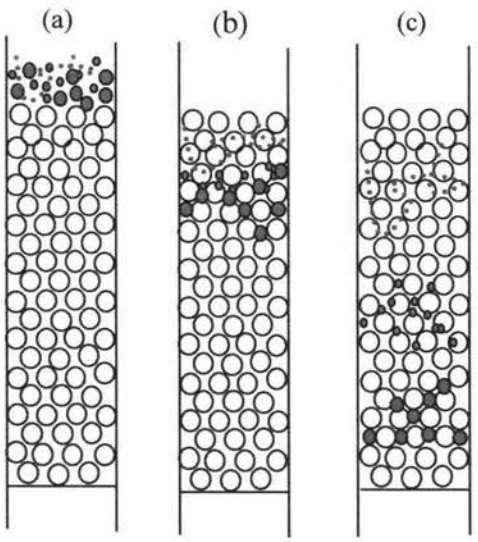


Figure 2.5. Schematic representation of SE-HPLC (Phenomenex, 1998)
Molecules are separated according to size during migration through the gel-filtration matrix as shown in (b) and (c), green colour represents large molecules, red colour medium molecules and blue colour small molecules; large molecules elute from the column first, while small molecules elute from the column last.

In ideal SE-HPLC, there is a linear relationship between the elution volume of a protein and the logarithm of its molecular size. Thus from calibration curves, the molecular weight of an individual protein can be estimated.

RP-HPLC is the most widely used HPLC technique in the separation, purification and study of peptides. The majority of packings in RP-HPLC are silica-based supports containing covalently bonded alkyl chains of different lengths. C18 (n-octadecyl) and C4 (n-butyl) are the most common supports, especially C18 columns, which are often chosen for the analysis of small peptides. Polymer-based reverse phase resins are also used as they have the advantage of increased stability over a wide range of pH values (pH 2 to pH 12). Organic solvents such as acetonitrile are typically used as the mobile phases. Peptides and proteins are separated according to their relative hydrophobicities. It is thought that the polypeptide attaches to the hydrophobic surface and remains bound until a sufficiently high concentration of organic solvent is passed through the column (i.e. organic solvent gradient) and displaces it from the solid support. The elution of solutes from the column is extremely sensitive to minute changes in organic solvent strengths, therefore, shallow organic solvent gradients are powerful resolving tools in polypeptide separations (Phenomenex, 1998).

2.3.5. Various forms of electrophoresis.

Electrophoresis has been widely used to assess the primary proteolysis of casein in cheese. The assessment is based on the fact that proteins and large peptides can be visualized after staining. There are several basic electrophoretic techniques to be found in the literature: isoelectric focusing (IEF), electrophoresis in starch gels, electrophoresis in polyacrylamide gels (PAGE) and two-dimensional (2-D) electrophoresis. PAGE has become the standard technique among these methods. Moreover, electrophoresis in SDS-containing buffers is a standard technique for protein analysis in general biochemistry. Electrophoresis in alkaline urea-containing gels with direct staining by Coomassie blue G250 is widely used in monitoring proteolysis in various cheese varieties. Creamer (1991) reported that using 20% acrylamide gels results in poor resolution of caseins, but it is very suitable for peptides with molecular weights in the range of 1,000~10,000. IEF is a superior electrophoretic technique for resolving proteins

and peptides based on their different isoelectric points and has been particularly useful in studies on genetic polymorphism in milk proteins (McSweeney and Fox, 1997). Creamer (1991) also reported that 2-D-electrophoresis (which combines IEF in one dimension and SDS PAGE in the second) may give good resolution, but there were some problems with reproducibility and obtaining quantitative data, and the method is very time consuming (Creamer 1991). However, recent advances in 2-D techniques may overcome these problems.

A somewhat different electrophoretic technique is capillary electrophoresis (CE). CE separations are based on the net charge on the peptides, and it is also reported as the technique with great potential for the resolution of complex mixtures of peptides. Unlike traditional electrophoretic techniques, CE uses UV absorbance, not staining, to detect peptides. Some advantages of CE over traditional electrophoretic techniques have been reported: CE can monitor small peptides which cannot be visualized by staining and also CE can be quantitative (Lindeberg, 1996; McSweeney and Fox, 1997).

2.4. Overall discussion

While proteolysis is the most complex process in cheese ripening, there is very little literature to be found on the study of EMC proteolysis. However, proteolysis can be assumed to be critical in EMC flavour development. The use of commercial enzymes to produce the proper ratio of amino acids and peptides to achieve desirable cheese flavour is an important issue for the EMC industry.

The objective of this project is to look at protein hydrolysis in EMC digested with the commercial proteases, Protease A, Protease B, Protease C and Protease DE, either individually or in combination. Various methods were used to profile both the extent and the patterns of proteolysis in these EMCs.

Chapter 3. Materials and Methods

This chapter describes various enzyme assay methods, high performance liquid chromatography (size exclusion chromatography and reverse phase chromatography) methods, polyacrylamide gel electrophoresis (alkaline urea PAGE, sodium dodecyl sulphate PAGE and low molecular mass SDS PAGE) methods, enzyme modified cheese base preparation methods and enzyme modified cheese processing. The specific methods for each individual chapter are described in each chapter.

3.1. Enzyme assay

Various enzyme assay methods such as an exopeptidase assay using *p*-nitroanilide or Lysyl aminomethyl coumarin, an endopeptidase assay using FITC-β-casein, and the BODIPY and Bioquant kit protease assays were compared in this study.

3.1.1. Materials

3.1.1.1. Enzymes

Protease A and Protease C are commercial proteases of fungal origin.

Protease B is a commercial blended protease of fungal and bacterial origin.

Protease DE is an in-house blend protease of fungal (D) and bacterial (E) origin.

For commercial reasons the identities of these enzymes can not be disclosed.

3.1.1.2. Substrates

p-Nitroanilide (*p*NA) derivatives of amino acids, Lys-*p*NA (Sigma L-7002), Leu-*p*NA (Sigma L-2504), Gly-*p*NA (Sigma G-4254), Glu-*p*NA (Sigma G-6133) or Pro-*p*NA (Sigma P-5267) used as substrates for exopeptidase assay were from Sigma Chemical Company, St. Louis, USA.

Fluorescein isothiocyanate (FITC) and β-casein were from Sigma Chemical

Company.

Lysyl aminomethyl coumarin (Lys-AMC) from Sigma Chemical Company was used for exopeptidase assay in enzyme modified cheese.

3.1.1.3. Buffer solutions

Bis-tris propane (BTP) and albumin were from Sigma Chemical Company.

Tris base (2-Amino-2- (hydroxymethyl)-1, 3-propanediol) was from Boehringer Mannheim, GmbH, Germany.

MES (2-[N-Morpholino] ethane-sulfonic acid), sodium phosphate (Na_2HPO_4), citric acid and phthalate were from BDH Chemical Company, Poole, England.

3.1.1.4. Enzyme assay stop solutions

Acetic acid glacial (100% w/v) and trichloroacetic acid (TCA) were from BDH Chemical Company, Poole, England.

3.1.1.5. Proteinase assay kits

The BODIPY protease assay kits obtained from Molecular Probes company (Eugene, OR, USA) contain casein derivatives that are labelled with the pH-insensitive green fluorescent BODIPY FL (E-6638) dye.

The Bioquant protease assay kits obtained from MERCK Company (Darmstadt, Germany) contain incubation buffer, enzyme solution, stop solution, substrate solution, coenzyme solution, standard solution.

3.1.2. Assay Methods

3.1.2.1. Exopeptidase assays

For exopeptidase assay using *p*-nitroanilide (*p*NA) derivatives of amino acids, 30 µL enzyme solution were incubated at 37°C for 20 minutes with 200 µL 5 mM substrate (one of Lys-*p*NA, Leu-*p*NA, Gly-*p*NA, Glu-*p*NA or Pro-*p*NA) in 50 mM bis-tris propane (BTP), pH 6.5. Enzyme solutions were 1 g/100 mL Protease A, 0.5 g/ 100 mL Protease B, 2.5 g/ 100 mL Protease C or 0.5 g/ 100

mL Protease DE, all made up in Milli-Q water. At the end of incubation, 1 mL 10% (v/v) acetic acid was added to stop the reaction. After mixing well, the absorbance of the solution was determined at 410 nm (Hitachi U-2000 spectrophotometer, Tokyo, Japan).

For assay of exopeptidase activity with lysyl aminomethyl coumarin (Lys-AMC), 950 μL 0.1 M MES, pH 6.8 (MES buffer) and 200 μL 5 mM Lys-AMC in ethanol/MES buffer (1:1, v/v) were incubated at room temperature with 200 μL enzyme solution (at the same concentrations as above). The fluorescence of the incubation mixture was measured every 15 seconds for 2 minutes (excitation 385 nm, emission 456 nm, slit width 5 nm) in a Perkin Elmer LS50 spectrofluorimeter (Perkin Elmer Limited, Beaconsfield, Buckinghamshire, England). Note that the inclusion of ethanol avoided re-crystallization of Lys-AMC from MES buffer.

3.1.2.2. FITC-β-casein endopeptidase assay

For assay of the various enzymes in buffered solutions, 20 μL enzyme solution was incubated with 20 μL FITC-β-casein (prepared as detailed in Appendix 3.1) and 20 μL 50 mM BTP, pH 6.5, at 37°C for 1 hour. The enzyme solutions were 0.005 g/ 100 mL Protease A, 0.05 g/ 100 mL Protease B, 0.005 g/ 100 mL Protease C and 0.001 g/ 100 mL Protease DE. The reaction was stopped and unhydrolysed substrate precipitated with the addition of 150 μL ice cold 15% (w/v) trichloroacetic acid. The stopped reactions were held on ice for 5 min, then centrifuged at 11,000 *g* for 3 minutes. Using a fresh pipette tip each time, 150 μL was transferred into 3 mL 0.5 M Tris-HCl, pH 8.5. This Tris-HCl buffer was prepared by dissolving Tris base in Milli-Q water and adjusting the pH with concentrated hydrochloride. The fluorescence was then determined at 490 nm excitation, 525 nm emission, 5 nm slit width in a LS50 spectrophotometer.

3.1.2.3. Buffers for the determination of the optimum pH for enzyme activity

The eight buffers used to determine enzyme activity pH profiles were: Na_2HPO_4 (200 mM) and Citric Acid (100 mM) mixed to give buffers at pH 4.5,

pH 5.5 and pH 6.5; Bis-tris propane (50 mM), pH 6.5; phthalate (50 mM), pH 4.5 and pH 5.5; MES (50 mM), pH 5.5 and pH 6.5. The pH values of BTP, phthalate and MES were adjusted with 0.1 M HCl or NaOH solution as required.

3.1.2.4. BODIPY assay

To determine a suitable enzyme concentration for the BODIPY assay, 1 mL from one of four enzyme dilutions (0.001, 0.01, 0,1 and 1 g/ 100 mL, made with Milli-Q water) was diluted to 1 mL with digestion buffer (contained in kit) and incubated with 1 mL BODIPY casein working solution for one hour. As a control, 1 mL of buffer-only (Tris-HCl, pH 7.8 or BTP, pH 6.5) solution (no enzyme) was incubated with 1 mL BODIPY casein working solution. Aluminium foil was used to exclude light from the assay mixture during incubation. The fluorescence was then determined periodically at 590 nm excitation, 645 nm emission, 5 nm slit width in a LS50 spectrophotometer. The data obtained were plotted to show the relationship between fluorescence and protease concentration.

In subsequent studies of enzyme activity using the BODIPY assay, the enzyme concentration from the above method was used.

3.1.2.5. Bioquant assay

To determine a suitable enzyme concentration for the Bioquant assay, each enzyme was made up at five dilutions as follows: 0, 0.0001, 0.0005, 0.001 and 0.005 g/ 100 mL for Protease A; 0, 0.005, 0.01, 0.02, and 0.05 g/ 100 mL for Protease B; 0, 0.0001, 0.0005, 0.01 and 0.02 g/ 100 mL for Protease C and 0, 0.00005, 0.0001, 0.0002 and 0.0005 g/ 100 mL for Protease DE. A 0.1 mL sample of the four enzymes at each of the five dilutions was incubated with 1 mL incubation buffer (supplied in kit) and 0.05 mL dehydrogenase enzyme solution (supplied in kit) at 37°C for 2 hours. 1 mL stop solution (supplied in kit) was added to stop the reaction and the incubation was continued for a further 30 minutes according to the instruction (reason not disclosed by manufacturer). 0.05 mL of the stopped incubation mixture was added to 1 mL substrate solution (supplied in kit) and 0.1 mL coenzyme solution (supplied in

kit) and the absorbance of the solution was determined at 340 nm after exactly 1 minute (A_1) and 3 minutes (A_2). These two readings were completed within 5 minutes of mixing the stopped incubation solution with the substrate and coenzyme solution at 25°C. The change in absorbance per minute at 340 nm $[(A_2 - A_1)/2]$ was calculated for each sample and plotted against enzyme concentration.

In subsequent studies of enzyme activity in EMC using the Bioquant assay, samples were diluted using Tris-HCl/albumin buffer (0.02% (w/v) albumin in 0.42 M Tris-HCl, pH 8.5) to give the enzyme concentration selected from the above study.

3.2. High performance liquid chromatography (HPLC)

Size-exclusion high performance liquid chromatography (SE-HPLC) and reverse phase high performance liquid chromatography (RP-HPLC) were used in this study. HPLC equipment was a Hewlett Packard 1050 modular fitted with an auto-injector. The HPLC system was equipped with a diode array detector and operated by HP Chemstation software (Hewlett-Packard Co., Germany).

3.2.1. Size-exclusion HPLC

3.2.1.1. Materials

Tris buffer, urea, boric acid, ethylene diaminetetra-acetic acid (EDTA), acetonitrile (CH_3CN) and trifluoroacetic acid (TFA) were obtained from BDH Chemicals New Zealand Ltd., Palmerston North, New Zealand.

3.2.1.2. Methods

Proteins and peptides were extracted from EMC samples using an alkaline urea extraction buffer (75 mM Tris-HCl, pH 8.4, containing 6 M urea, 0.09 M boric acid and 2.8 mM EDTA). 0.5 g (±0.0005g) EMC sample was added to 25 mL of extraction buffer and incubated (40°C) for 1 hour. The mixture was then homogenized with an ultraturrex (Janke & Kunkel Co., GmbH, Germany) for 20 seconds at high speed and centrifuged at 9,000 *g* for 10 minutes at 4°C. The supernatant was decanted and stored at -20°C until required for HPLC analysis.

Note that care is required to decant the supernatant because a solid surface layer of fat forms after centrifugation.

SE-HPLC was carried out by injecting 50 μL of extracted sample to a TSKgel G2000 SW_{XL} size exclusion column (Tosoh Corporation, Tokyo, Japan) protected with a TSKgel SW_{XL} guard column (Tosoh Corporation, Tokyo, Japan), both of which were held at a constant 25°C. The sample was eluted from the column with 36% (v/v) CH_3CN / 0.1% (v/v) TFA at a flow rate of 0.5 mL/ minute for 35 minutes. The absorbance of the eluate at 214 nm and 280 nm was monitored continuously. The following series of standard proteins of known molecular mass were analysed using the SE-HPLC column: carbonic anhydrase (29,000 Da), bovine serum albumin (66,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), trypsin inhibitor (20,100 Da), egg white lysozyme (14,300 Da), aprotinin (6,500 Da), insulin (5,730 Da), insulin B-chain (3,400 Da), bacitracin (1,450 Da) and phenylanine (an amino acid; 165 Da). A calibration curve was then constructed relating elution volume with molecular mass.

3.2.2. Reverse phase HPLC

3.2.2.1. Materials

As for Section 3.2.1.1.

3.2.2.2. Methods

EMC samples were extracted for analysis by RP-HPLC using the procedure described in Section 3.2.1.2.

Reverse-phase HPLC was carried out by applying 100 μL of extracted sample to a Vydac C18 column (5 μm, 300 Å pore size; Phenomenex, Torrance, CA, USA). The sample was applied to the column in 0.1 % (v/v) TFA, 0.08 % TFA (v/v) in water and eluted by using an increasing concentration of CH_3CN. The composition of the two solvents and the gradient used are described in Table 3.1. The absorbance of the eluate was continuously monitored at 214 nm and 280 nm.

Table 3.1. The two-solvent gradient used to elute compounds from the RP-HPLC column

Time (minute)	A (0.1% TFA in H_2O)	B (0.08% TFA in CH_3CN)
0	100%	0%
50	50%	50%
52	25%	75%
57	25%	75%
62	100%	0%
67	100%	0%

3.3. Polyacrylamide gel Electrophoresis (PAGE)

Alkaline urea PAGE, high molecular mass SDS PAGE and low molecular mass SDS PAGE were compared in this study work.

3.3.1. Materials

Tris buffer, concentrated HCl, urea, boric acid, ethylene diaminetetra-acetic acid (EDTA), sodium dodecyl sulphate (SDS), glycerol, dithiothreitol, glycine, bromophenol blue, Coomassie brilliant blue R-250, iso-propanol, acetic acid and 2-mercaptoethanol were obtained from BDH chemical company.

Acrylamide, Bis-acrylamide, ammonium persulphate (APS) and TEMED (electrophoresis grade reagents) were from Bio-Rad Co., Hercules, USA.

3.3.2. Methods

3.3.2.1. Alkaline urea PAGE

EMC samples to be analysed by alkaline urea PAGE were extracted using the method given in Section 3.2.1.2. 20 μL of extracted sample was added to 20 μL bromophenol blue (0.4 % w/v) and 20 μL 2-mercaptoethanol. A sample (20

μL) of this mixture was applied to a 12% (w/v) polyacrylamide gel in a Bio-Rad model Mini-Protean II vertical electrophoresis cell. Sample components were separated by applying a constant voltage of 210 v, a current of 70 mA and 6.5 w power per gel for 1.4 hours, after which time the gels were stained by immersion in Coomassie blue solution (0.05% Coomassie brilliant blue R-250, 25% iso-propanol, 75% acetic acid) for 1 hour with gentle agitation. Gels were destained over approximately 20 hours using several changes of destaining solution (iso-propanol, acetic acid, water, 10:10:80). Destained gels were scanned using a densitometer (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

3.3.2.2. Sodium dodecyl sulphate (SDS) PAGE

EMC samples to be analysed by SDS-PAGE were extracted using the method given in Section 3.2.1.2. The extract was diluted 1:1 with SDS sample buffer (Milli-Q water, 0.5 M Tris-HCl buffer pH 6.8, conc. glycerol, 10% (w/v) SDS, 0.4% (w/v) bromophenol blue solution, 20: 5: 4: 8: 1 by vol) and 2-mercaptethanol was then added (20 μL mercaptoethanol per mL). The mixture was heated at 100°C for 4 minutes, 20 μL of the heat-treated sample was applied to a 16% polyacrylamide gel (apparatus described in Section 3.3.2.1.) and a constant voltage of 210 v with a current of 70 mA and 6.5 w power per gel was applied for 0.9 hour. The gels were stained, destained and scanned as described in Section 3.3.2.1.

3.3.2.3. Low molecular mass (LMM) SDS PAGE

EMC samples to be analysed by LMM SDS-PAGE were extracted using the method given in Section 3.2.1.2. The extract was diluted 1:1 with LMM SDS sample buffer (0.5 M Tris-HCl buffer, 10% SDS solution, glycerol, dithiothreitol, bromophenol blue solution (0.4 %), Milli-Q water, 12.5: 10: 15: 0.23: 2: 2.63 by vol) and heated at 100°C for 1.5 minutes. 20 μL of the heat-treated sample was applied to a 20% polyacrylamide gel (resolving and stacking gels were 20% (w/v) and 12% (w/v) acrylamide monomer, respectively), and a constant voltage of 210 v with a current of 70 mA and 6.5 w power per gel was applied for 1.4 hour (apparatus as described in Section

3.3.2.1. 12 mg rennet casein was dissolved in 6 mL LMM SDS PAGE sample buffer for use as a standard.

The gels were stained, destained and scanned as described in Section 3.3.2.1.

3.4. Preparation of Enzyme Modified Cheese (EMC)

3.4.1. Manufacture of EMC base

3.4.1.1. Materials

Young (1 month) grannular Cheddar cheese was obtained from Kiwi Dairy Cooperative Company, Hawera, New Zealand. The cheese was stored at 2°C from 15th October (cheese age was 1 month), 1999 to 15th December, 1999 and then stored at -2°C from 15th December, 1999 to February, 2000.

Disodium phosphate, used as emulsifying salt, was from BDH.

3.4.1.2. Methods

EMC base (55% moisture) was prepared by heating a mixture of 10 kg young grannular cheese, 5 kg water and 0.225 kg disodium phosphate at 95°C in a Stephan high shear cooker and holding at this temperature for 15 minutes. At this point, a sample was taken for a sulphite reducing clostridia test (the results are given in Appendix 3.2).

The slurry was then cooled to 35°C using a water bath and held at this temperature for at least 2 hours. To kill any spores that may have germinated as a result of this treatment, the slurry was then heated from 35°C to 86.5°C within 1.75 hours (the ideal temperature for this purpose is 95°C, but the steamer that was capable of reaching this temperature was unavailable and so a water bath with a maximum temperature of 86.5°C was used). The slurry was finally packed into 500 g pry-off containers. A sample was taken for chemical composition and microbiological tests (the results are given in Appendix 3.4 and 3.2 for chemical composition and micro results, respectively). The EMC base was stored at -2°C until required.

An EMC base with 65% moisture was also made by using 9.4 kg water, 0.3 kg disodium phosphate and 10 kg grannular cheese. The method used was as described above. The results for chemical and micro tests of the 65% moisture EMC base are given in Appendix 3.2 and 3.4.

3.4.2. Determination of microbial contamination of EMC base

Both the EMC bases (55% and 65% moisture) were tested for microbial contamination after incubation at 40°C for 48 hours; with and without the addition of the four commercial proteases (at 0.15% w/v). The results which are given in Appendix 3.2 and 3.3, showed that the EMC with moisture of 65% was more susceptible to contamination by microorganism.

As the microbial contamination was highest in the 65% moisture EMC base after 48 hour incubation and also because the 55% moisture base was closer to the moisture content of the commercial EMC, the 55% moisture base was used for all work in this study.

3.4.3. Manufacture of Enzyme Modified Cheese (EMC)

Commercial enzyme solutions were made up using deionised water at a concentration of 10% (0.1 g/ mL). The enzyme solution was added to 15-20 g EMC base (in a sterile universal bottle) to give a final enzyme concentration of 0.15% (w/v). Immediately following enzyme addition, the EMC base was thoroughly stirred and then incubated at 40°C for 24 h (commercial conditions). After incubation, the enzyme was inactivated by heating the EMC at 95°C for 30 minutes (preliminary inactivation experiment described in Chapter 6). As far as possible, aseptic technique was used throughout the EMC manufacturing process to reduce the risk of microbial contamination.

3.4.4. Chemical composition analyses

3.4.4.1. Moisture content

The moisture content of the EMC base was determined by measuring the weight loss after drying samples in an oven at 105°C for 16 hours (NZDRI Method ACCA12, New Zealand Dairy Research Institute, 1977).

3.4.4.2. Salt content

The salt content of the EMC base was determined according to the Volhard method. Silver nitrate was used to react with the chloride in the sample and form silver chloride which is insoluble. Residual silver nitrate was then titrated with standard potassium thiocyanate using ammonium ferric sulphate as the indicator (NZDRI Method ACCA 28, NZDRI, 1996).

3.4.4.3. Fat content

Fat content was determined by the Werner Schmit method. Hydrochloric acid was used to digest 1 g of sample. Ethanol was then added, and the acid/ethanol solution was extracted with diethyl ether and light petroleum. The mixture was distilled to eliminate solvent and the fatty substances which were soluble in the non-volatile light petroleum were measured (NZDRI Method ACCA 03, NZDRI, 1992).

3.4.5. Microbial analyses

The EMC base was determined for sulphite reducing clostridia, total aerobic plate count, yeast and moulds and coliforms.

3.4.5.1. Sulphite reducing clostridia

EMC samples were held at 80°C for 10 minutes to remove vegetative cells. The spores were then grown using reinforced clostridial agar with anaerobic incubation at 37°C for 24 hours (NZTM 2, Microbiological Methods Manual, Section 59, NZDB, 1996).

3.4.5.2. Anaerobic Plate Count

The Anaerobic Plate Count was carried out using Differential Reinforced Clostridial Agar (DRCA) with incubation at 30°C for 72 hours.

3.4.5.3. Aerobic plate count

The Aerobic plate count was done using Milk Plate Count Agar (MPCA) with a 72 hour incubation at 30°C (NZTM 2, Microbiological Methods Manual,

Section 43, NZDB, 1996).

3.4.5.4. Yeasts and Moulds

Yeasts and moulds were determined using Yeast Extract Glucose Chlor-Amphenicol Agar (DIFCO) with a five day incubation at 25°C (NZTM 2, Microbiological Methods Manual, Section 61, NZDB, 1996).

3.4.5.5. Coliforms

Coliforms were determined using Violet Red Bile Agar (VRBA) with a 24 hour incubation at 30°C (NZTM 2, Microbiological Methods Manual, Section 48, NZDB, 1996).

Chapter 4. Comparison of *in vitro* methods for assay of commercial proteases

4.1. Introduction

Protease is the generic term for two types of enzymes, endopeptidases (*e.g.*, proteinases) and exopeptidases (*e.g.*, aminopeptidases). Endopeptidases can cleave internal peptide bonds to hydrolyse proteins into polypeptides, examples are pepsin, trypsin and chymotrypsin. Exopeptidases such as aminopeptidases, carboxypeptidases and dipeptidases are essential to extensively hydrolyse the protein to small peptides and free amino acids. Extensive enzymic hydrolysis of cheese curd or casein using a combination of endopeptidases and exopeptidases produces a high level of small peptides and free amino acids that impart a number of flavours.

Commercial proteases contain different mixes of proteolytic enzymes. Protease A, Protease B, Protease C and Protease DE are four commercial enzyme preparations of interest in EMC manufacture. The endopeptidase and exopeptidase activities were assayed using various methods in this study. The objective was to better understand the activities of the four commercial enzyme preparations and to compare the advantages and disadvantages of various assay methods for this purpose and for subsequent studies.

The exopeptidase assay was based on the cleavage of *p*NA derived amino acids, using amino acid-derived *p*NA substrates. The proteinase assay was done using various methods, including FITC-β-casein and the BODIPY and Bioquant assay kits.

4.2. Exopeptidase assay using amino acid-derived *p*NA substrate

Exopeptidases cleave N-terminal amino acids from polypeptides. Exopeptidases hydrolyse amino acid-*p*NA substrates, such as Lys-*p*NA, Leu-*p*NA, Gly-*p*NA, Glu-*p*NA and Pro-*p*NA, to give a free amino acid and yellow *p*-nitroaniline.

Therefore, exopeptidase activity can be detected by measuring the *p*-nitroaniline using a spectrophotometer.

4.2.1. Methods

The preliminary experiment to determine the suitable enzyme concentration for exopeptidase assay was carried out. Lysine-*p*NA was used as substrate and various concentrations (1 g/ 100 mL, 0.1 g/ 100 mL, 0.01 g/ 100 mL 0.001 g/ 100 mL and 0.0001 g/ 100 mL) of each commercial enzyme (Protease A, Protease B, Protease C and Protease DE) were used. The methods are described in Section 3.1.2.1. The objective was to determine the enzyme concentration that produced an absorbance (410 nm) of about 1.0 after 20 minutes of incubation in the assay mixture described in Section 3.1.2.1.

An end-point assay and a continuous assay were carried out using Protease A in a trial to ensure all materials worked properly and to determine the correlation between exopeptidase activity and incubation time and enzyme concentration. The end-point assay was done by incubating Protease A exopeptidase for 2, 4, 6, 8, 10 and 15 minutes according to the method in Section 3.1.2.1. The continuous assay was done using 25 µL and 50 µL of Protease A at 1 g/ 100 mL for 5 minutes. 200 µL Lysine-*p*NA was used as substrate for each incubation in this study.

4.2.2. Results and discussion

The optimal concentrations found for each commercial enzyme to produce an absorbance at 410 nm of approximately 1.0 after 20 minutes of incubation using Lys-*p*NA as substrate and determining the absorbance at 410 nm were: Protease A, 1 g/ 100 mL; Protease B, 0.5 g/ 100 mL; Protease C, 2.5 g/ 100 mL; Protease DE, 0.5 g/ 100 mL. Figure 4.1 shows the results of the end-point assay for Protease A exopeptidase activity. A linear relationship between incubation time and exopeptidase activity was obtained.

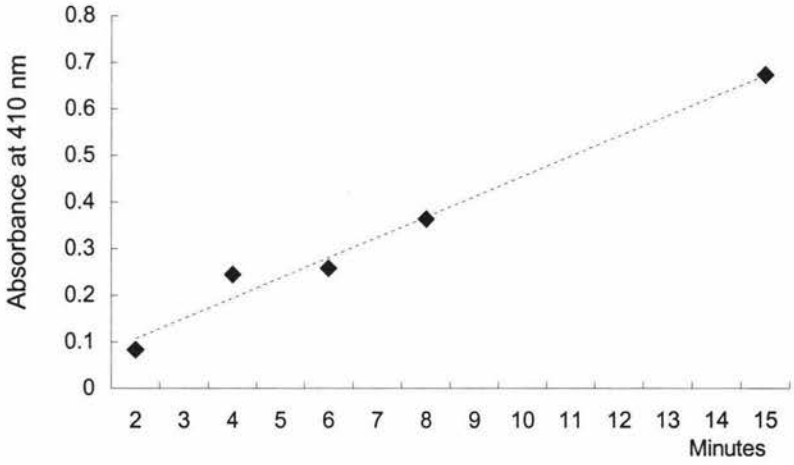


Figure 4.1. End-point assay for Protease A exopeptidase activity

A continuous assay of Protease A revealed that 25 μL and 50 μL of enzyme (1 g/ 100 mL) gave average values for Δ A410 nm of 1.44 x 10^{-2} absorbance (ABS) units/ min and 2.09 x 10^{-2} ABS units/ min. In theory, the reaction rate from 50 μL Protease A should be double that of 25 μL Protease A. A deviation might occur because of the loss of activity resulting from proteolysis of the enzymes themselves.

4.3. Endopeptidase assay using FITC-β-casein

4.3.1. Methods

Fluorescein isothiocyanate (FITC) is a fluorescent compound, which can be linked to lysine residues of proteins such as β-casein and peptides. Since FITC-β-casein is insoluble in TCA (trichloroacetic acid) while small and medium sized FITC-peptides are TCA-soluble, any intact substrate (FITC-β-casein) that remains following hydrolysis can be removed by precipitation with TCA. Enzyme activity can therefore be measured by determining the levels of fluorescent FITC-peptides. This method cannot detect peptides which do not

contain labelled lysine groups. If the substrate used does not contain enough labelled lysine residues, then the enzyme activity does not cause the release of sufficient quantity of FITC-labelled peptides to enable activity to be accurately observed. Therefore, this method is only suitable for assay of peptides which contain labelled lysine groups.

A preliminary experiment to determine a suitable enzyme concentration for an endopeptidase assay using FITC-β-casein was carried out using various concentrations (1 g/100 mL, 0.1 g/100 mL, 0.01 g/100 mL, 0.001 g/100 mL and 0.0001 g/100 mL) of each enzyme as detailed in Section 3.1.2.2. The aim of the preliminary experiment was to determine the enzyme concentration giving a change of fluorescence of about 600 relative fluorescence units (RFU) in 60 minutes at 37°C incubation.

After the optimal enzyme concentration for endopeptidase assay using FITC-β-casein was determined, the endopeptidase activity of Protease A was determined by measuring the fluorescence of assay samples taken at 10, 20, 30, 45 minutes and 1 hour. In this study, Protease A was used as an example to test the linearity of this assay under the conditions used.

4.3.2. Results and discussion

The optimal concentrations found for each commercial enzyme using FITC-β-casein as substrate were: Protease A, 0.005 g/ 100 mL; Protease B, 0.05 g/ 100 mL; Protease C, 0.005 g/ 100 mL; Protease DE, 0.001 g/ 100 mL.

Figure 4.2 shows that when using FITC-β-casein as the substrate for Protease A (0.005g/ 100 mL), the increase in fluorescence was not linear. This might result from few FITC-peptides being produced at the beginning of the incubation with increasing amounts of labelled peptides being produced at later stages. This would result in a non-linear curve as in Figure 4.2.

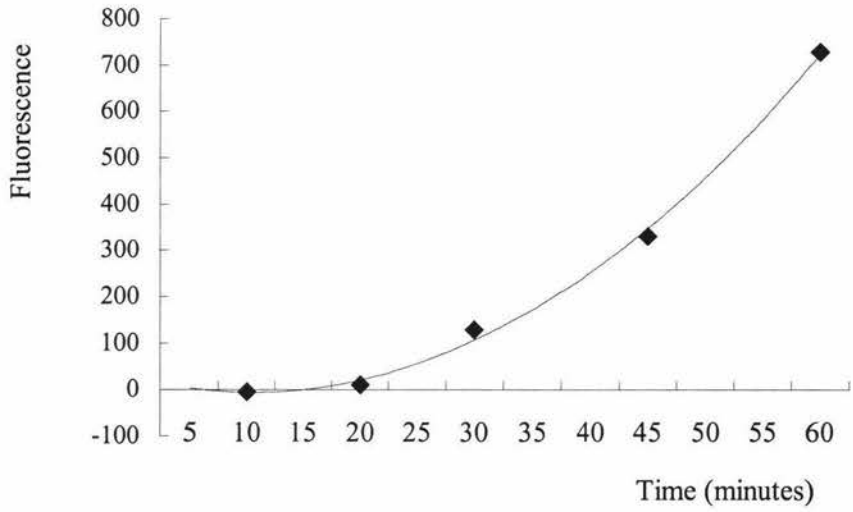


Figure 4.2. Relative fluorescence vs incubation time for the assay of Protease A (0.005 g/ 100 mL) using FITC-β-casein as substrate

4.4. Proteinase kit assays

4.4.1. BODIPY protease assay

4.4.1.1. Methods

The BODIPY assay is based on the release of fluorescent cleavage products from casein derivatives labelled with fluorescent dyes as a result of protease-catalyzed hydrolysis. The BODIPY protease assay was performed as detailed in Section 3.1.2.4 with Protease DE and Protease A being used as the enzymes to evaluate the method at the concentrations of 1 g/100 mL, 0.1 g/100 mL, 0.01 g/100 mL and 0.001 g/100 mL.

4.4.1.2. Results and discussion

The BODIPY assay was tried with Protease DE and Protease A. The results obtained were non-linear. The enzyme solutions were a ten-time dilution series and each dilution was assayed separately. However, the assay results did not show a 10-time relationship between the activity of each dilution. After

incubation for 2 hours, the results obtained from assay of Protease DE showed about a 2-time relationship between the activity of each dilution; while the results of Protease A showed no obvious relationship between the activity of each dilution. In addition, the overall sensitivity of the assay was not as good as that given by the FITC-β-casein assay method. Thus, this assay method was not used for further work. The data obtained from the assay method are shown in Appendix 4.1.

4.4.2. Bioquant protease assay

4.4.2.1. Methods

The Bioquant protease assay is an indirect method for determining protease levels in a sample. It relies on measuring the decrease in the activity of a readily assayed enzyme that is subject to inactivation by the protease in the sample. The amount of protease in the sample is inversely proportional to the residual activity of the second enzyme, which in the Bioquant method is a dehydrogenase. The Bioquant protease assay was performed as detailed in Section 3.1.2.5.

4.4.2.2. Results and discussion

Figures 4.3 - 4.6 show the results of the standard curves obtained using the Bioquant assay for Protease A, Protease B, Protease C and Protease DE, respectively, in a buffer system. The useful ranges of enzyme concentration were 0.0001-0.0005, 0.005-0.02, 0.0001-0.0002 and 0.00005-0.0001 g/ 100 mL for Protease A, Protease B, Protease C and Protease DE, respectively.

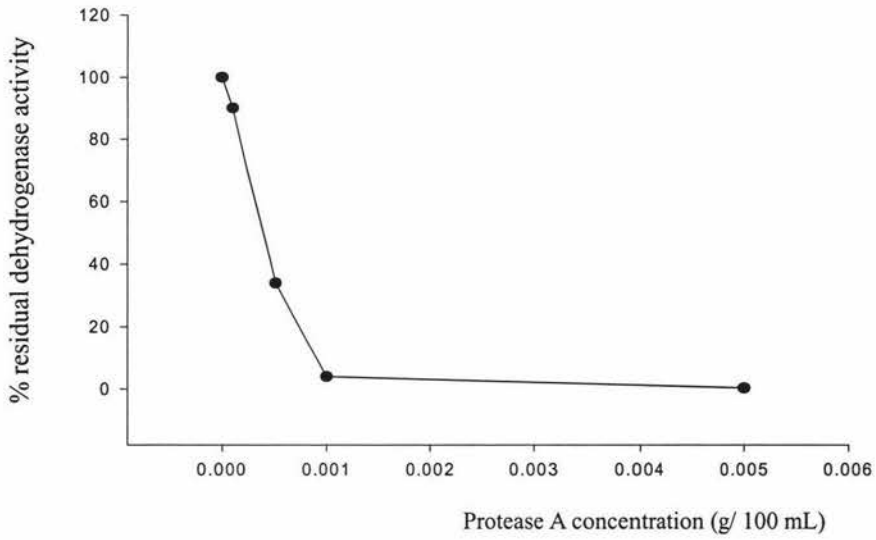


Figure 4.3. Standard curve of Protease A activity against dehydrogenase residual activity using the Bioquant assay

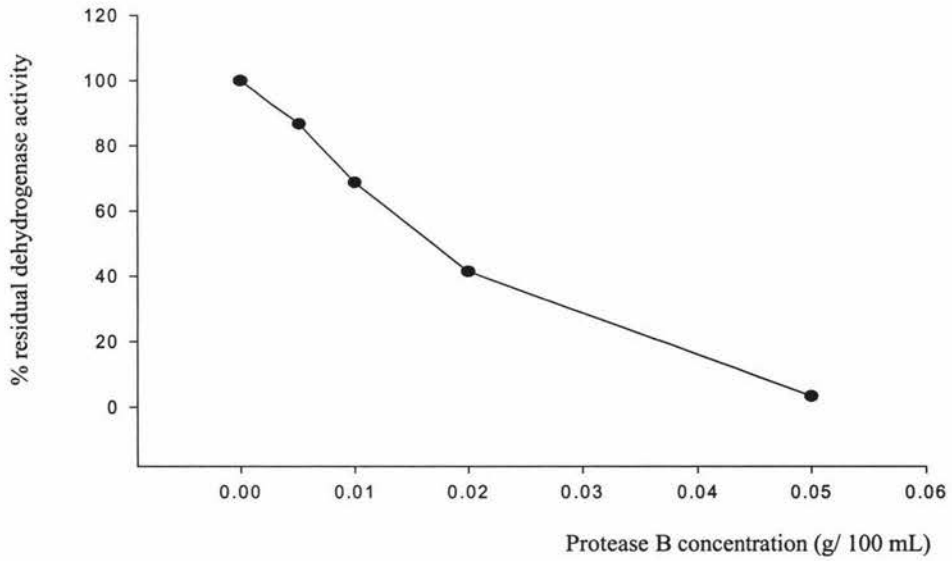


Figure 4.4. Standard curve of Protease B activity against dehydrogenase residual activity using the Bioquant assay

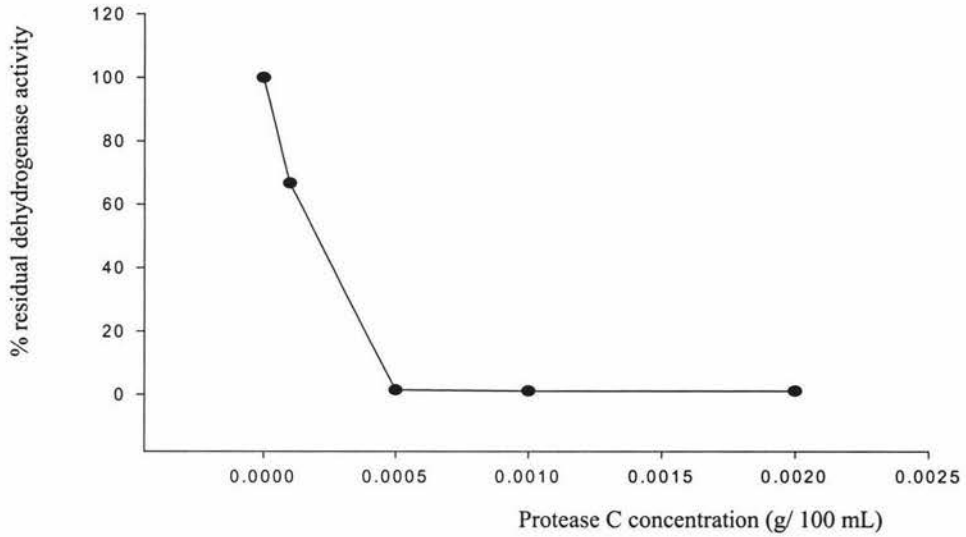


Figure 4.5. Standard curve of Protease C activity against dehydrogenase residual activity using the Bioquant assay

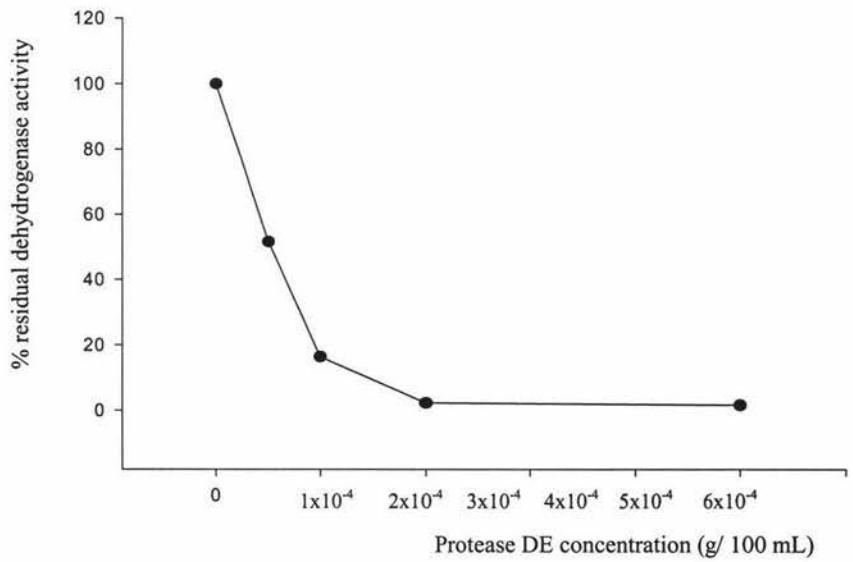


Figure 4.6. Standard curve of Protease DE activity against dehydrogenase residual activity using the Bioquant assay

4.5. Conclusion

The lowest detectable concentrations of each enzyme for the exopeptidase assay in buffer using Lys-*p*NA were determined to be: Protease A, 1 g / 100 mL; Protease B, 0.5 g / 100 mL; Protease C, 2.5 g / 100 mL; Protease DE, 0.5 g / 100 mL.

The lowest detectable concentrations of each enzyme for the endopeptidase assay in buffer using the Bioquant assay method were: Protease A, 0.1~0.5 mg /100 mL; Protease B, 1.0 ~ 5.0 mg / 100 mL; Protease C, 0.1 ~ 0.5 mg / 100 mL; Protease DE, 0.05 ~ 0.1 mg / 100 mL.

These results indicated that for the endopeptidase assay about 10 to 100-time less enzyme was required than for the exopeptidase assay. The assays also gave a comparison of the relative levels of endopeptidase and exopeptidase activity in the enzyme preparations. Protease C and Protease DE had almost the same relative levels of endopeptidase and exopeptidase, while Protease A had relatively less proteinase and the relative level of exopeptidase in Protease B (compared with proteinase) was very high. Table 4.1 shows the ratio of endopeptidase to exopeptidase for each of the four commercial enzymes.

Table 4.1. Assay sensitivity and activity ratios for endopeptidase and exopeptidase of four commercial proteases

Enzyme	Lowest concentration required for endopeptidase assay	Lowest concentration required for exopeptidase assay	Endo- to Exo- ratio
Protease A	0.1 – 0.5 mg / 100 mL	1 g / 100 mL	2000:1
Protease B	1 – 5 mg / 100 mL	0.5 g / 100 mL	100:1
Protease C	0.1 – 0.5 mg / 100 mL	2.5 g / 100 mL	5000:1
Protease DE	0.05 – 0.1 mg / 100 mL	0.5 g / 100 mL	5000:1

Two protease assay kits, the BODIPY and Bioquant kits, were compared with the FITC-β-casein assay. The results from the BODIPY kit assay were extremely unreliable and so the BODIPY method was not used further. The results indicated that the Bioquant kit assay was ten-time more sensitive than the FITC-β-casein assay. However, the method was technically difficult compared with the FITC-β-casein method and very expensive, therefore, the FITC-β-casein assay was used for the remainder of this study.

It should be noted that the enzyme assays in this chapter were conducted in a buffer system and such low levels of enzyme would not be expected to be detectable in EMC with the same assay methods because of the masking effect of the relatively large amount of protein in the cheese base compared with the amount of substrate for the assay.

Chapter 5. Basic characteristics of commercial proteases in buffer systems

5.1. Introduction

Enzyme activity is influenced by factors such as pH, substrate type and the thermostability of the enzyme itself. It is more difficult to measure enzyme activity in a complex environment such as EMC where many additional parameters need to be considered. Hence, a series of preliminary experiments were carried out to better understand the characteristics of Protease A, Protease B, Protease C and Protease DE in a buffer system and to profile their characteristics for further study. These preliminary experiments included: the measurement of thermostability (expressed as half life, *i.e.* the time required to lose half of the initial activity at a given temperature [$T_{1/2}$]), the determination of the practical temperature and pH optima for activity, a comparison of exopeptidase activity toward different amino acid-*p*NA substrates and the storage stability of enzyme solutions. For these experiments, enzyme activity was assayed in buffers instead of in EMCs.

5.2. Temperature optimum for activity of four commercial proteases

Temperature influences the reaction rate and the stability of proteases. The optimal practical temperature was obtained for the exopeptidase and endopeptidase activities of the four commercial proteases. In theory an "optimum" temperature for enzyme activity does not exist, since the optimal temperature will vary with environmental conditions. In practice, however, optimal temperature can be determined for particular environmental conditions, for example, in this case the optimal temperature was determined for a given pH and substrate type.

5.2.1. Methods

For the exopeptidase assay, activity was determined over the temperature range

of 28 to 70°C using Lys-*p*NA as substrate as detailed in Section 3.1.2.1. Endopeptidase was assayed over the temperature range of 28 to 50°C using FITC-β-casein as detailed in Section 3.1.2.2.

5.2.2. Results and discussion

(a) Exopeptidase assay

The relationship between incubation temperature and exopeptidase activity of the four proteases is shown in Figure 5.1. Protease A, Protease C and Protease DE all showed a similar optimal temperature of approximate 60°C under the assay conditions. Protease B appeared to have two optimum temperatures for exopeptidase activity, *i.e.* 33°C and 60°C, which again suggests the presence of at least two exopeptidases in the preparation. The low and high optima correlate with an unstable and a stable exopeptidase, respectively.

(b) Endopeptidase assay

The relationship between incubation temperature and endopeptidase activity of the four proteases is shown in Figure 5.1. The optimal temperatures for endopeptidase activities are lower than those for exopeptidase activities of the four commercial proteases.

The optimal temperatures for the endopeptidase activities of Protease B and Protease DE are the same *i.e.* 37°C. The optimal temperatures for Protease A and Protease C are higher *i.e.* 45°C. At temperatures above the optimum, the activity of Protease A and Protease B decrease more rapidly than do the activities of Protease C and Protease DE. Thus although the optimum temperature for Protease DE was lower than that for Protease A, at 50°C the former enzyme retained more activity than the latter.

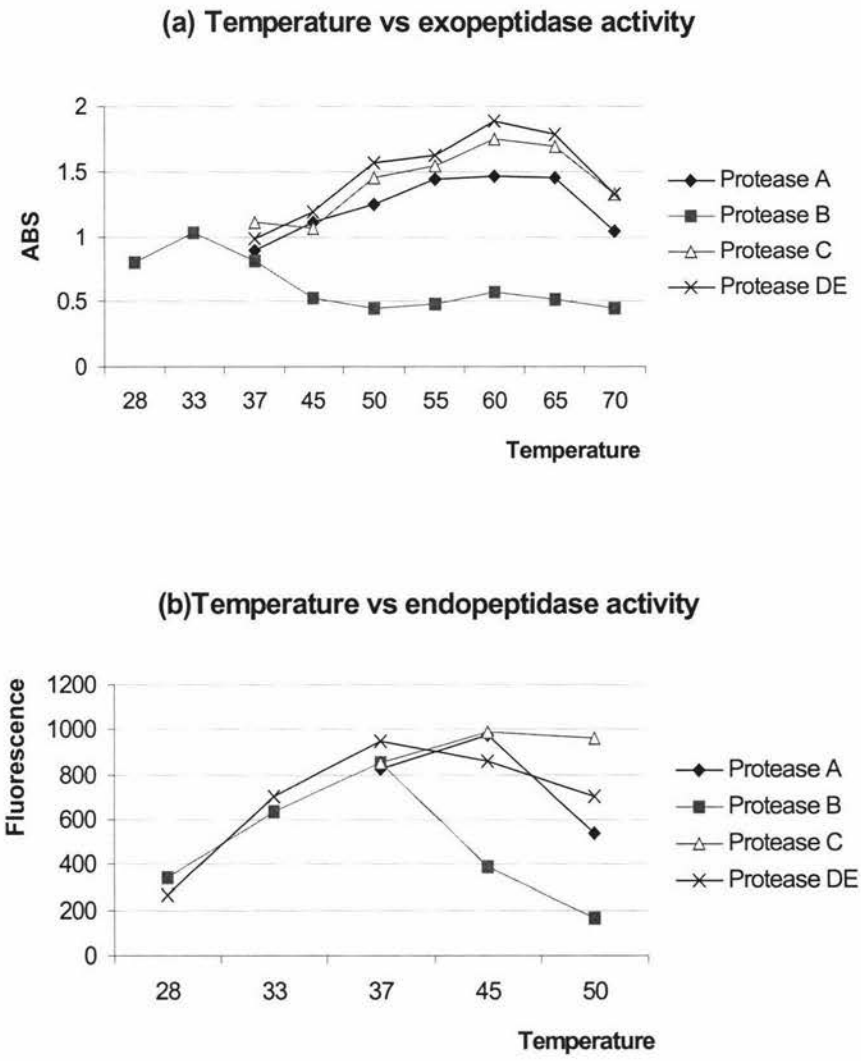


Figure 5.1. The exopeptidase and endopeptidase activities vs temperature for the four commercial proteases

5.2.3. Conclusion

The optimal temperatures for the exopeptidase activities of Protease A, Protease C and Protease DE is 60°C. In contrast Protease B has two optimal temperatures (33°C and 60°C) which indicates that Protease B contained at least two exopeptidases. The optimal temperatures for the endopeptidase activities of Protease A and Protease C (45°C) are different from those of Protease B and Protease DE (37°C).

5.3. The thermostability of commercial proteases

The purpose of this experiment was to better understand the thermostability of Protease A, Protease B, Protease C and Protease DE in buffer systems and to determine the half-life ($T_{1/2}$) of each protease at certain temperatures. The data from this study were used to select suitable temperatures to trial in enzyme inactivation experiments in EMC.

5.3.1. Methods

(a) Half life of exopeptidase activity at different temperatures:

Enzyme concentrations of 1 g/100 mL, 0.5 g/100 mL, 2.5 g/100 mL and 0.5 g/100 mL for Protease A, Protease B, Protease C and Protease DE, respectively, were used in thermostability studies (enzyme concentration was selected on the basis of results in Section 4.2.2). Each enzyme (30 μL) was heated at 40 - 80°C in 5°C increments for 0, 10, 20, 30, 40, 50 and 60 minutes. All incubations were done in triplicate and samples were stored on ice prior to assay. Lys-*p*NA was used as substrate for the exopeptidase assay as detailed in section 3.1.2.1. The results were expressed by plotting the % activity remaining against incubation time. The half-life of the enzyme was determined from a plot of Log_{10} (% activity remaining) vs incubation time (*i.e.* $T_{1/2}$ equals the time at which 50% of the initial enzyme activity was lost). The correlation between Log_{10} % activity remaining and incubation time is linear when the loss of enzyme activity is due to thermal denaturation alone (*i.e.* a first order plot). Where other factors, such as autolysis, are also involved in the loss of activity, the plots are non linear.

(b) Half-life of endopeptidase activity at different temperatures:

Enzyme concentrations of 0.005 g/100 mL, 0.05 g/100 mL, 0.005 g/100 mL and 0.001 g/100 mL for Protease A, Protease B, Protease C and Protease DE, respectively, were used in thermostability studies (enzyme concentration were selected on the basis of results from Section 4.3.2.). Each enzyme (20 μL) was heated at 55 and 60°C for 0, 10, 20, 30, 40, 50 and 60 minutes. All incubations were done in triplicate and samples were stored on ice prior to assay. FITC-β-casein was used as substrate for the endopeptidase assay as detailed in section

3.1.2.2. The results were expressed as a plot of % activity remaining against incubation time. The half-life ($T_{1/2}$) values were calculated as described above (5.3.1 (a)).

5.3.2. Results and discussion

(a) Thermostability of exopeptidase activity

The loss of exopeptidase activity in each of the proteases was determined. As an example the data obtained for Protease A at 80°C are shown in Figure 5.2. These data were replotted in Figure 5.3 as $\log_{10}$ % activity remaining vs incubation time. The initial portion of this plot was linear and therefore the initial half-life could be readily calculated.

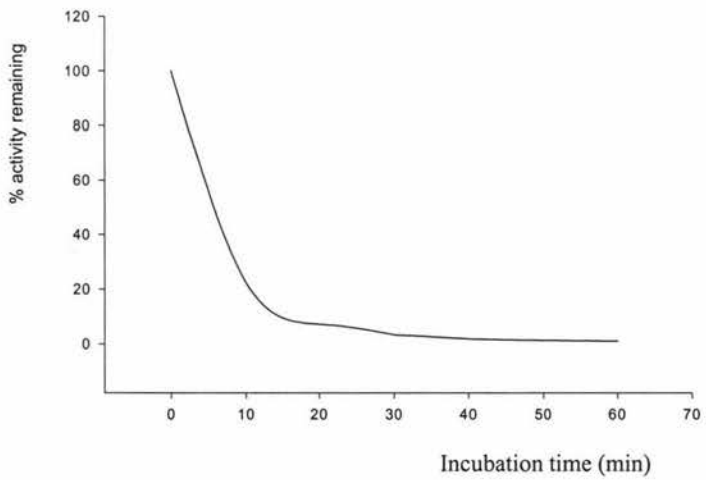


Figure 5.2. % activity vs incubation time at 80°C for exopeptidase activity in Protease A

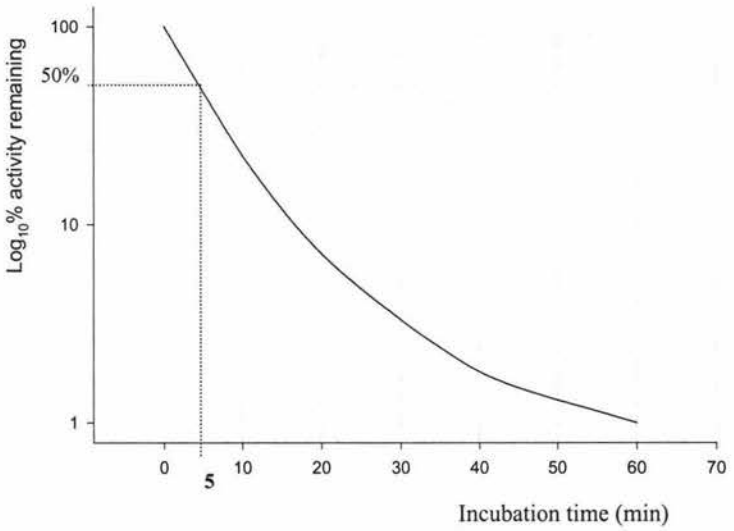


Figure 5.3. Log_{10} % activity vs incubation time at 80°C for exopeptidase activity in Protease A

From Figure 5.3, the initial half life of exopeptidase activity in Protease A at 80°C was calculated to be 5 minutes, *i.e.* 50% of the exopeptidase activity initially present in Protease A was lost in 5 minutes at 80°C.

However, the plot was definitely non-linear at later times which meant that the actual enzyme activity remaining after approximately 20 minutes was greater than the calculated activity based on a half life of 5 minutes. This could reflect the presence of more than one enzyme of different stability, or a more stable form of the enzyme generated on heating.

The results of the half-life studies of exopeptidase activity in Protease A, Protease B, Protease C and Protease DE at all temperatures tested are shown in Table 5.1.

Table 5.1. Thermostability (half-life) of exopeptidase activities of four commercial proteases

Protease	Half life of enzymes at different temperatures						
	40°C	50°C	55°C	60°C	65°C	75°C	80°C
Protease A	[1] ND	ND	ND	[2] No change	ND	ND	5 min
Protease B	23 min	[3]	[3]	[3]	[3]	[4] 20 min	[4] 1.5 min
Protease C	ND	ND	ND	No change	ND	16 min	3 min
Protease DE	ND	ND	ND	No change	ND	11 min	2.5 min

[1] ND, Not determined.

[2] No change: No observable decrease in activity after one hour.

[3] Approximately 75% of the activity was immediately lost when the incubation temperature was reached. The remaining activity (25%) was stable for up to 60 minutes incubation time.

[4] Preheated 60°C for 10 minutes

For the Protease C and Protease DE, the $T_{1/2}$ obtained at 80°C were 3.0 and 2.5 minutes, respectively. The plots of $\log_{10}$ % residual activity vs incubation time were similar to that obtained for Protease A (see Figure 5.3).

However, the results for Protease B showed that it contains at least two different exopeptidases. One exopeptidase was very unstable at 40°C, while the other was completely stable at 65°C for up to 1 hour. To determine the $T_{1/2}$ of the most stable exopeptidase, Protease B was pre-heated at 60°C to inactivate the less stable enzyme. The theromostability of the most stable exopeptidase was then measured at 75 and 80°C. As shown in Table 5.1, the most stable exopeptidase of Protease B is stable for 1 h at 60°C while the $T_{1/2}$ is 20 minutes at 75°C and 1.5 minutes at 80°C.

(b) Thermostability of endopeptidase activity

Figure 5.4 shows the loss of endopeptidase activity in Protease A at 55°C. These data were replotted as $\log_{10}$ of % activity remaining against time in Figure 5.5.

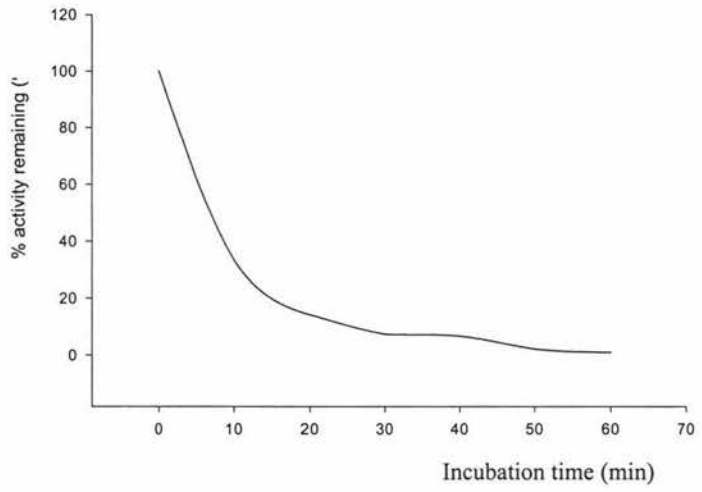


Figure 5.4. % activity remaining vs incubation time at 55°C for endopepdiase activity in Protease A

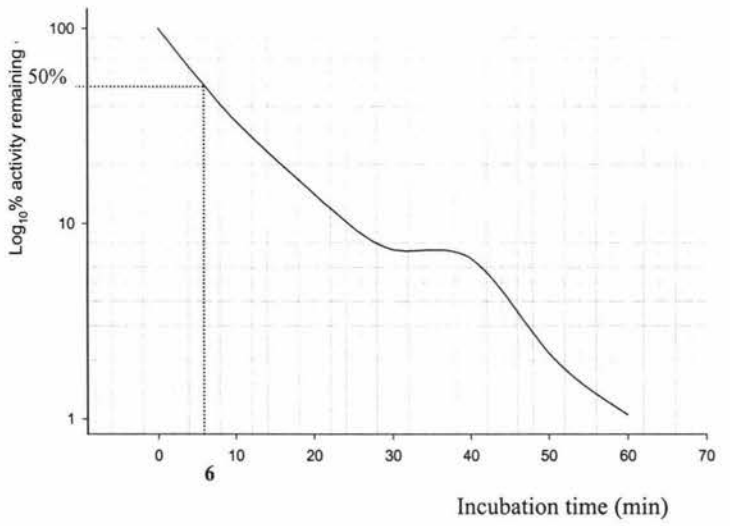


Figure 5.5. Log_{10} % activity vs incubation time at 55°C for endopepdiase activity in Protease A

Figure 5.5 shows that the initial half-life ($T_{1/2}$) of endopeptidase activity in Protease A at 55°C is 6 minutes, *i.e.* 50% of the initial endopeptidase activity was lost in 6 minutes at 55°C.

The $T_{1/2}$ for endopeptidase activity in each of Protease A, Protease B, Protease C and Protease DE at 55°C and 60°C are shown in Table 5.2. The endopeptidase activity of Protease B was more stable than that of the three other proteases at 55°C, but at 60°C all of the enzymes tested had similar stabilities. Protease A was not tested at 60°C because it was so unstable at 55°C.

Table 5.2. Thermostability (half- life) of endopeptidase activities of four commercial proteases

Protease	Half-life of enzymes at different temperatures	
	55°C	60°C
Protease A	6 minutes	ND [1]
Protease B	30 minutes	2.5 minutes
Protease C	8 minutes	2 minutes
Protease DE	12 minutes	3 minutes

[1] ND, not determined.

5.3.3. Conclusion

The thermostability of the exopeptidase activities in Protease A, Protease C and Protease DE was considerately greater than that of the endopeptidase activities. Protease B contained two exopeptidases, the major one of which could be destroyed by heat treatment of 60°C for 10 minutes, while the other was as stable as the exopeptidases in the other commercial preparations.

5.4. The pH optimum activity of commercial proteases

The pH of the environment in which an enzyme is active will influence activity. The relationship between pH and protease activity was therefore determined and the optimum pH for the exopeptidase and endopeptidase activities of the four commercial proteases established.

5.4.1. Methods

To determine the optimum pH of exopeptidase activity, Protease A, Protease C and Protease DE were incubated at 60°C (optimal temperature) and Protease B

at 33°C (optimal temperature of major exopeptidase) in the various buffers detailed in section 3.1.2.3. The method of assay was as detailed in Section 3.1.2.1 using Lys-*p*NA as substrate.

For endopeptidase assay, Protease A and Protease C were incubated at 45°C and Protease B and Protease DE were incubated at 37°C in the various buffers (see Section 3.1.2.3). FITC-β-casein was used as substrate (see Section 3.1.2.2).

5.4.2. Results and discussion

(a) Exopeptidase activity

The exopeptidase activities of the commercial proteases in different pH buffers are shown in Table 5.3.

Table 5.3. The exopeptidase activities of commercial proteases at various pH values

Protease	Exopeptidase activity in different pH buffers			
	Na_2HPO_4 + Citric Acid, pH 4.5	Na_2HPO_4 + Citric Acid, pH 5.5	Na_2HPO_4 + Citric Acid, pH 6.5	BTP pH 6.5
Protease A	0.012 *	0.019	1.118	1.286
Protease B	0	0	0.085	0.279
Protease C	0.007	0.029	1.004	1.438
Protease DE	0.012	0.026	1.015	1.552

* The results are expressed as Δ Absorbance at 410 nm/min.

Thus, within the pH range 4.5 to 6.5 the optimal pH for exopeptidase activities of Protease A, Protease B, Protease C and Protease DE is pH 6.5. It is possible that a higher activity may have been measured if higher pH values had been tested but since pH 6.5 is close to the practical limit for an EMC system, pH values above this were not investigated.

The exopeptidase activity of Protease B in Na_2HPO_4/Citric Acid buffer (Table 5.3) was extremely low. It was possible that Protease B contained a metallo enzyme which requires a metal ion for activity. The Na_2HPO_4/Citric Acid buffer would act as a chelating agent by removing the metal ion which would severely inhibit the activity of the metallo enzyme. In view of this possibility, the pH

optimum of Protease B was investigated further using the non-chelating buffers, phthalate, MES and BTP the assay results are shown in Table 5.4.

Table 5.4. The exopeptidase activity of Protease B in various non-chelating buffers

Activity of Protease B				
Phthalate pH 4.5	Phthalate pH 5.5	MES pH 5.5	MES pH 6.5	BTP pH 6.5
0.006	0.124	0.331	1.156	0.507

The results shown in Table 5.4 support the conclusion that Protease B contained a metallo enzyme. Exopeptidase activity of Protease B in MES pH 6.5 buffer is higher than in the chelating buffers used. Thus a non-chelating buffer should be used when Protease B is assayed in a buffer system.

It should be noted that the exopeptidase activity of Protease B in BTP buffer shown in Table 5.3 is lower than that shown in Table 5.4. This is probably due to the fact that the Protease B preparation used to obtain the earlier solutions had been stored in the liquid state, while that used to obtain the data shown in Table 5.4 was freshly made.

(b) Endopeptidase activity

Table 5.5 shows the results obtained from the endopeptidase assay with FITC-β-casein at different pH values.

Table 5.5. The endopeptidase activities of commercial proteases at various pH values

Protease	Endopeptidase activity in different pH buffers			
	Na_2HPO_4 + Citric Acid pH 4.5	Na_2HPO_4 + Citric Acid pH 5.5	Na_2HPO_4 + Citric Acid pH 6.5	BTP pH 6.5
Protease A	238 [1]	561	784	983
Protease B	0	2.8	23	**377**
Protease C	120	309	897	872
Protease DE	80	135	194	296

[1] results given in RFU/min

The highest endopeptidase activity of the four commercial proteases was given at pH 6.5. At lower pH values lower activity was obtained, with at least 4-fold less activity at pH 4.5 than at 6.5 for Protease A, Protease C and Protease DE.

The endopeptidase activity of Protease B, as with its exopeptidase activity, was far lower in Na_2HPO_4 + Citric Acid buffer than in BTP buffer. This again indicates a chelating agent effect.

To avoid this chelating effect, non-chelating buffers were used in further experiments to determine the effect of pH on Protease B endopeptidase activity. Table 5.6 shows the results.

Table 5.6. The endopeptidase activity of Protease B in various non-chelating buffers

Activity of Protease B				
Phthalate pH 4.5	Phthalate pH 5.5	MES pH 5.5	MES pH 6.5	BTP pH 6.5
100 [1]	710	547	809	798

[1] results given in RFU/min.

Using these buffers it was clear that the highest activity was given at pH 6.5, and that the chelating buffer did indeed have a severe inhibitory effect on the enzyme. The results for the endopeptidase activity of Protease B in BTP buffer reported in Table 5.5 is lower than that reported in Table 5.6. Again this is because the Protease B solution used for the experiments reported in Table 5.5 had been stored as a liquid and that used in the experiments reported in Table 5.6 was freshly made.

5.4.3. Conclusion

The results on the pH effect show that at pH 6.5 the activities of both exopeptidase and endopeptidase of the four commercial proteases is greater than at more acidic pHs. It is clear that for the same amount of activity to be obtained at lower pH values in a given time, up to 5 times more enzyme would be required, or if the same amount of enzyme were used at pH 4.5, the reaction would have to proceed for up to 5 times longer than at pH 6.5. Further, Protease

B is sensitive to chelating agents, and therefore may be partially inhibited in EMC by the emulsifying salts used in EMC manufacture.

5.5. Relative exopeptidase activities of commercial proteases

The levels of exopeptidase activities of the four commercial proteases toward the substrates Lys-*p*NA, Leu-*p*NA, Gly-*p*NA, Glu-*p*NA and Pro-*p*NA were compared in order to determine whether the specificities of the exopeptidases were similar.

5.5.1. Methods

Each of the substrates was used individually at a concentration of 5 mM and incubated at 60°C with Protease A, Protease C and Protease DE and at 33°C with Protease B. The system was buffered at pH 6.5. The assay methods were as detailed in Section 3.1.2.1.

5.5.2. Results and discussion

To readily compare the exopeptidase specificities of the commercial proteases, the activities given by each enzyme against the different amino acid *p*NA substrates were calculated and then expressed relative to the activity given against Lys-*p*NA. The results are presented in Table 5.7.

Table 5.7. The relative exopeptidase activities of the commercial proteases toward different substrates

Protease	Leu-*p*NA	Gly-*p*NA	Glu-*p*NA	Pro-*p*NA
Protease A	11.7 [1]	0.001	0.003	0
Protease B	7.6	8.06	0.02	0.03
Protease C	12.1	0.01	0.002	0.0007
Protease DE	10.9	0.004	0.003	0.0007

[1] For each enzyme the activity is expressed relative to that given against Lys-*p*NA (*i.e.* Lys-*p*NA=1).

The results shown in Table 5.7 indicate that Protease A, Protease C and Protease DE have similar exopeptidase activities towards each of the five substrates used. The activity against Leu-*p*NA was approximately 10 times greater than Lys-*p*NA

for each of these commercial proteases. Activity against the other substrates, particularly Pro-*p*NA, was very low. Protease B was significantly different from the other enzymes; its activity against Gly-*p*NA was as high as that against Leu-*p*NA and nearly 1000 times higher then any of the other enzymes. Protease B activity against Glu-*p*NA and Pro-pNA was at least 10 and 50 times higher, respectively, than that of the other enzymes.

5.5.3. Conclusion

There are two main findings from this experiment. Firstly, Leu-*p*NA is preferred over Lys-*p*NA, Glu-*p*NA, Gly-*p*NA and Pro-*p*NA as substrate by all four commercial proteases. Secondly, it is clear that Protease B contains a different type of exopeptidase from the three other commercial proteases. It should be noted that the incubations with Protease B were done at 33°C, the optimum temperature for the less stable of the two exopeptidases that Protease B appeared to contain. What the specificity profile of Protease B would have been had the incubation been done at 60°C (*i.e.* a temperature at which only the more stable exopeptidase is active) is unknown.

5.6. Influence of storage time on protease activity

Normally the protease activities of the commercial preparation would be expected to decline over prolonged storage. The influence of storage time on exopeptidase and endopeptidase activities of protease solutions at 4°C was determined over one month to clarify how long the protease solutions could be kept.

5.6.1. Methods

Solutions of Protease A, Protease B, Protease C and Protease DE were made up at 1 g/100 mL, 0.5 g/ 100 mL, 2.5 g/100 mL and 0.5 g/ 100 mL, respectively, in Milli-Q water for exopeptidase assay; 0.005 g/100 mL, 0.05 g/ 100 mL, 0.005 g/100 mL and 0.001 g/ 100 mL, respectively, in Milli-Q water for endopeptidase assay. The solutions were stored at 4°C for 1, 2, 3, 4, 7, 8 and 9 days, and exopeptidase and endopeptidase activities determined at each time point using the method detailed in Section 3.1.2.1 and 3.1.2.2, respectively.

5.6.2. Results and discussion

(a) Exopeptidase activity

Table 5.8 shows the changes in levels of exopeptidase activity (Lys-*p*NA as substrate) in the four commercial proteases preparations during storage.

Table 5.8. Influence of storage at 4°C on exopeptidase stability

day \ protease	Protease A	Protease B	Protease C	Protease DE
1	0.9 *	1.2	1.0	1.2
2	0.9	1.0	1.0	1.2
3	0.9	0.9	1.0	1.2
4	0.9	0.7	1.0	1.2
7	0.8	0.3	1.0	1.2
8	0.8	0.3	0.9	1.2
9	0.8	0.3	0.9	1.1

* The results are expressed as Δ Absorbance at 410 nm/ min

As shown in Table 5.8, the exopeptidase activity of Protease A, Protease C and Protease DE remained relatively unchanged after storage at 4°C for 9 days, while that of Protease B decreased dramatically over 7 days. Compared with the three other commercial proteases, Protease B exopeptidase activity is therefore much less stable. It was intended that the exopeptidase activities would be determined after a further 21-day storage (*i.e.* after 30 days), but severe microbial contamination of the storage samples meant that any such assay would be invalid.

(b) Endopeptidase assay

Table 5.9 shows the changes in the levels of endopeptidase activity (FITC-β-casein as substrate) during storage.

Table 5.9. Influence of storage at 4°C on endopeptidase stability

protease / day	Protease A	Protease B	Protease C	Protease DE
1	906 [1]	689	950	1176.3
2	936	735	924	ND [2]
3	829	623	918	1178.9
4	862	576	842	1200.0
7	875	275	867	736
8	811	240	ND	676
9	729	109	330	517

[1] The results are given as RFU/ min.
[2] ND: not determined.

As shown in Table 5.9, the endopeptidase activity of Protease B decreased dramatically after storage for four days and was less stable than the endopeptidase activities of the three other commercial proteases. The endopeptidase activity of Protease A was the most stable of the preparations, although all four preparations were relatively stable for at least four days. Again, the assay after 30 days was prevented because of microbial growth in the storage samples.

5.6.3. Conclusion

For the commercial proteases A, C and DE, the exopeptidase activity was more stable than the endopeptidase during storage of enzyme solutions at 4°C. For Protease B, both the exopeptidase and endopeptidase activities were particularly unstable. It would therefore seem important that solutions of all the commercial proteases, particularly that of Protease B, should be prepared fresh for addition to the EMC base to ensure consistent digestion results.

Clearly, solutions of the four commercial enzyme solutions can not be stored for long periods without antibacterial agents added.

5.7. Overall discussion

The thermostability of the exopeptidases and endopeptidases in the four commercial proteases was shown to be different by comparing their half lives ($T_{1/2}$). The study also showed that Protease B contained two exopeptidases. The exopeptidase activity of Protease B was less stable than that of the three other proteases at 80°C, but the endopeptidase activity of Protease B was more stable than that of three other proteases at 55°C. It should be noted that the stability of the proteases in EMC would be expected to be greater than in buffers due to the protective effect of the EMC base.

Over the pH range studied (pH4.5 to 6.5), the highest exopeptidase and endopeptidase activities for all preparations was obtained at pH 6.5. Further study of the effect of pH was not done, since the parameters used needed to be consistent with real EMC work. For this study, pH values above 6.5 are irrelevant, even though the enzymes assayed would probably be active and present pH optima in the alkaline region. The study also found that Protease B was sensitive to buffer containing chelating agents.

It is recommended that freshly made enzyme solutions are used instead of aged ones due to the decline in enzyme activity during storage. Protease B activity dramatically decreased after four days storage at 4°C. The endopeptidase activities of Protease A, C and DE decreased significantly over a nine-day period, while the exopeptidase activities of these proteases declined to a lesser degree. This effect may in fact be a tool to modify the exopeptidase to endopeptidase activities of the preparation (*i.e.* raise the relative exopeptidase activity by storing the preparation for a few days). However, the preparations were clearly prone to microbial contamination and so antibacterial agents would be needed (or filter sterilization into sterile containers) for such storage.

Chapter 6. Time / temperature parameters for inactivation of commercial proteases in EMC

6.1. Introduction

This chapter describes the determination of the level of inactivation of the four commercial proteases in EMC after heat treatment and the quantification of the percentage of residual enzyme activity. The inactivation protocol was based on the enzyme stability results described in the previous chapter and focused on two critical factors of enzyme inactivation: time and temperature. According to the results obtained in buffer systems, the heating temperature needed to be higher than 80°C. To quantify residual enzyme activity, different assay methods were applied and compared, including the Bioquant assay and size exclusion chromatography.

6.2. Methods

6.2.1. Test tube assay for residual enzyme activity

An enzyme concentration of 0.15% (w/v) for the four commercial proteases was used in EMC digestion at 40°C for 20 hours. The method is detailed in Section 3.4.3. At the end of digestion, 1 mL of each EMC sample was heated according to the following time / temperature conditions: (1) heating at 90°C for 0, 5 and 10 minutes; (2) heating at 95°C for 0, 15 and 30 minutes.

After the heat treatments, residual enzyme activity was determined using Leu-*p*NA or Lys-AMC as substrate for the exopeptidase assay. Because the enzyme concentration in EMC was lower than the detectable enzyme concentration using Lys-*p*NA as substrate, Leu-*p*NA which was the most sensitive substrate shown in Chapter 5 was used as substrate to determine exopeptidase activity. Before measuring exopeptidase activity by determination of absorbance at 410 nm, EMC samples were treated by centrifuging at 4°C for 10 minutes at max speed (10,000 x *g*) and then filtering the supernatant to remove fat and other solids in order to provide a clear solution for measuring absorbance. The method is

detailed as Section 3.1.2.1 for the exopeptidase assay. For the exopeptidase assay using Leu-*p*NA as substrate, the optimum temperatures for exopeptidase activity described in chapter 5, *i.e.* 60°C for Protease A, Protease C and Protease DE assays and both 33°C and 60° for Protease B were used.

The Bioquant assay was also used to measure the residual endopeptidase activity for the heat treated samples. For this assay, initial concentrations were diluted to 0.00025%, 0.01%, 0.0001% and 0.00005% for Protease A, Protease B, Protease C and Protease DE, respectively, with $CaCl_2$/ Tris buffer, pH 8.5. The method is detailed as Section 3.1.2.5.

6.2.2. Assay for residual enzyme activity using size exclusion HPLC (SE-HPLC)

SE-HPLC was also used to quantify the levels of residual protease in EMC. Freshly-prepared enzyme/ EMC slurries were immediately "inactivated" leaving high levels of intact protein that could be easily quantified by SE-HPLC. Subsequent incubation of the "inactivated" EMC sample at the normal EMC manufacturing temperature would lead to small changes in the level of intact protein if some enzyme remained active. These changes would be quantified by SE-HPLC and would be directly proportional to the amount of active enzyme remaining in the "inactivated" sample. By comparing the observed change with the amount of change obtained in a similar EMC sample that had not been "inactivated" and that contained a known amount of enzyme, it was possible to calculate the % residual activity.

An enzyme concentration of 0.15% (w/v) for the four commercial proteases was used to prepare the EMC slurry. The method is detailed in Section 3.4.3. The preparation time required for each enzyme addition to the EMC base was approximately 12 minutes. After addition of enzyme to the EMC base, 3 x 1 mL of "zero time" samples of each EMC were taken and heated, respectively, at 84°C, 90°C and 95° for 30 minutes. The remainder of the EMC samples were incubated at 40°C for 20, 40, 60 and 180 minutes, at which time the samples were inactivated at 95°C for 30 min and then immediately stored at 4°C. One half of the 95°C-heated sample was immediately stored at 4°C (as control sample), while the other half, along with the 84°C- and 90°C-heated samples, were digested at

40°C for a further 24 hours. All of the EMC samples were treated at 95°C for 30 minutes at the end of digestion.

Soluble proteins, peptides and amino acids were extracted from each sample by using alkaline urea buffer. The extracts were used to determined proteolysis by means of size exclusion chromatography (Section 3.2.1).

6.3. Results and discussion

6.3.1. Residual exopeptidase activity

The results obtained for exopeptidase assays using either Leu-*p*NA or Lys-AMC as substrate showed that the methods were not sufficiently sensitive to detect enzyme activity in the samples. The assay methods were not therefore applied further in the study.

6.3.2. Residual endopeptidase activity (using Bioquant assay kit)

Table 6.1 shows the results for the residual enzyme activity obtained using the Bioquant assay.

Table 6.1. Residual endopeptidase activity in heat-treated EMC samples *

Heat treatment of EMC samples	Levels of enzyme activity remaining in EMC after heat treatment			
	Protease A	Protease B	Protease C	Protease DE
90°C for 10 min	3.6%	Not determined	~0%	~0%
95°C for 15 min	~0%	26%	~0%	~0%
95°C for 30 min	~0%	1.6%	~0%	~0%

* Endopeptidase activity measured using the Bioquant assay kit

The results obtained from the Bioquant assay (Table 6.1) show that no activity was left in EMC made with Protease A, Protease C and Protease DE after inactivation at 95°C, but some activity appeared to remain in EMC made with Protease B.

The results presented in Table 6.1 showed that 26% and 1.6% of initial Protease B activity remained in EMC after heating at 95°C for 15 and 30 minutes, respectively. These levels seemed surprisingly high for Protease B residual

activity. Thus, the residual activity assay was repeated on EMC made with Protease B and it was clear from the results that reproducibility was a major problem with the assay. The assay results were therefore considered unreliable and the Bioquant method was shown to be inapplicable for residual enzyme assay.

6.3.3. Residual enzyme activity measured by size exclusion chromatography (SE-HPLC)

All samples described in Section 6.2.2 were extracted using alkaline urea extraction buffer and the extracts were analysed by SE-HPLC as detailed in Section 3.2.1.2. To determine the percentage of residual activity by size exclusion chromatography, the sample that was immediately heat-treated at 95°C for 30 minutes and then kept at 4°C was considered as the zero-time sample for subsequent measurements of residual enzyme activities. Figure 6.1 gives the results for EMC made with Protease B and shows the chromatogram given by the zero-time sample and those obtained from the samples that were inactivated at 84°C, 90°C and 95°C for 30 minutes followed by incubation at 40°C for 24 hours. It is clear from the chromatograms that there was some enzyme activity remaining, even after heating at 95°C for 30 minutes. The chromatograms for EMCs made with Protease A, Protease C and Protease DE are shown in Appendix 6.1.

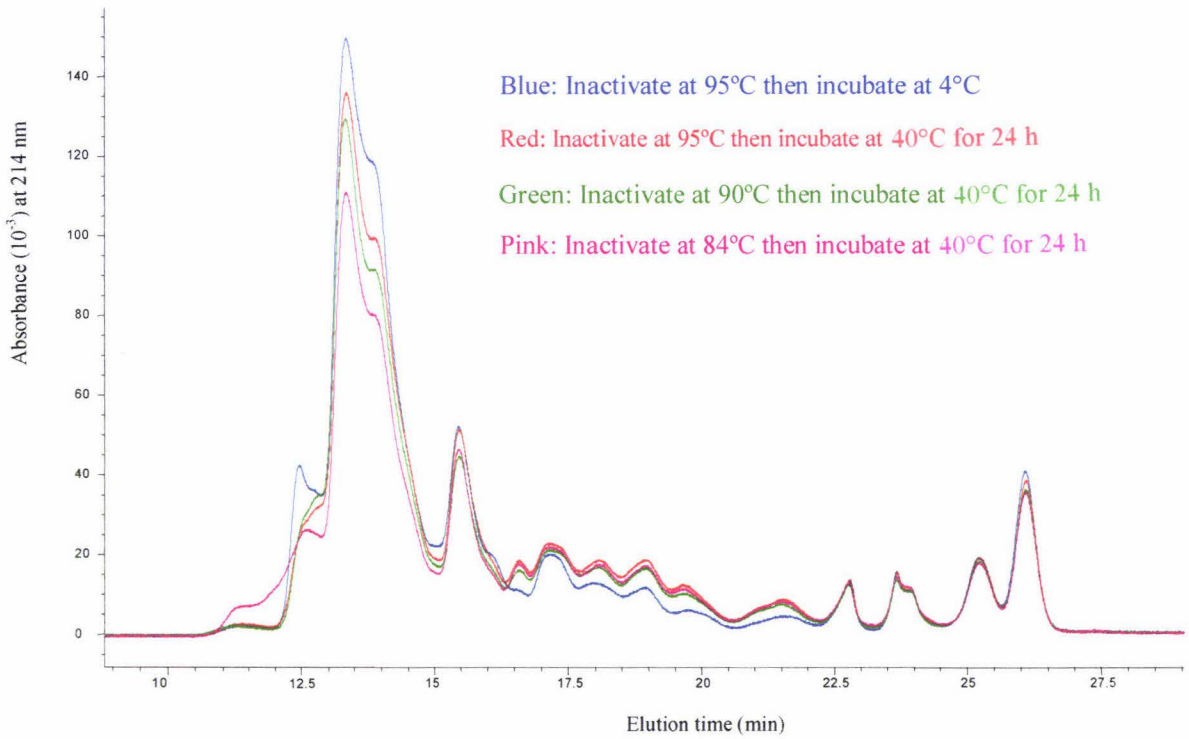


Figure 6.1. SE-HPLC chromatograms obtained for EMC made with Protease B, inactivated at various temperatures and then incubated at 4°C or 40°C for 24 h

The enzyme activity remaining in EMC after heat treatment can be quantified by integrating areas under the early eluting large peaks (12.5-15 min) of the size exclusion chromatograms (contains intact protein and large protein fragments). The peak areas from the "zero-time" sample were compared with the areas of the equivalent peaks given by samples that had been heat treated (84°C, 90°C and 95°C) and further incubated at 40°C for 24 h. A decrease in the peak areas following incubation at 40°C was considered to be the result of residual active enzyme. The integrated peak areas obtained from the EMC samples made with Protease B are shown in Table 6.2.

Table 6.2. Areas of the early eluting peaks of SE-HPLC for EMC samples made with Protease B that have been heat treated

Sample	Integration area of early-eluting peaks	Difference from "zero-time" sample
"zero-time" sample [1]	12,591 [2]	-
95°C heat treatment [3]	11,148	1,443
90°C heat treatment [3]	10,624	1,967
84°C heat treatment [3]	9,557	3,034
EMC, incubated 3 h [4]	9,518	3,073

[1] Freshly-made EMC, heat treated at 95°C and stored at 4°C,

[2] Area given in arbitrary units; peaks eluting from 12.5-15 min,

[3] Freshly-made EMC, heat treated at given temperature and incubated at 40°C for 24 h,

[4] EMC sample, incubated at 40°C for 3 h, then at 95°C for 30 min.

The % residual activity of the enzyme was calculated by comparing the change in peaks areas of heat-treated samples with the change in peak area of a non heat-treated EMC sample incubated at 40°C for 3 h. For this calculation, the peak area change in the non-heat-treated sample was first converted to the value expected from a 24 h incubation at 40°C (*i.e.* by multiplying by a factor of eight). This method was used because at an initial enzyme dose of 0.15% (w/v) the change in peak area (early-eluting peaks) was nearly linear for digestion times of 3 h or less.

$$\% \text{ Residual active enzyme} = \frac{\text{Peak area change for heat-treated sample}}{\text{Peak area change for non-heat-treated sample} \times 8} \times 100\%$$

An example calculation is shown below for the EMC sample that was heat-treated at 90°C for 30 min;

% Residual active enzyme = (1967)/ (3073 x 8) x 100% = 8.0 %

Table 6.3 shows the percent activity remaining for the four commercial proteases in EMC after the different heat treatments.

Table 6.3. Percentage of initial enzyme activity remaining in EMCs after inactivation treatments

	Percentage activity remaining		
	95°C	90°C	84°C
Protease A	3.5	5.4	7.3
Protease B	5.9	8.0	12
Protease C	4.9	6.4	6.4
Protease DE	2.6	2.7	2.75

The data shown in Table 6.3 indicate that there is some activity remaining in all heat-treated samples, even after heating at 95°C for 30 minutes. Protease B retained the highest level of activity while Protease DE retained the lowest level of activity after all three heat treatments. The actual amounts of each enzyme remaining after heating at 95°C for 30 minutes can be calculated from the initial dose of each protease (0.15% w/v). The levels remaining are equivalent to an initial enzyme dose of 0.005%, 0.009%, 0.007% and 0.004% (w/v) for Protease A, Protease B, Protease C and Protease DE, respectively.

6.4. Overall discussion and conclusion

Of the three methods used to estimate the residual activity of the enzymes, SE-HPLC proved to be the most sensitive. This method was not only sufficiently sensitive for detection of the residual enzyme levels, but it was also a very reproducible method. However, a disadvantage of SE-HPLC is that a lot of time is necessary in sample preparation and analysis. As described in chapter 4, the Bioquant assay kit was estimated to be about ten times more sensitive than the FITC-β-casein assay for measuring endopeptidase activity in buffer-based system. However, in EMC systems problems with this method emerged.

The EMC base used in this study had a higher moisture content (55%) than that of a commercial EMC base. Thus, it is likely that the enzymes would be less active if used in a commercial EMC base because the lower water content reduces the rate at which hydrolytic enzymes are able to act. Furthermore, the reduced proteolytic activity would likely increase the stability of the proteases as hydrolysis of the enzymes themselves would be reduced. However, following EMC introduction into a higher water activity product, such as a spread, the amount of enzyme activity obtained could be higher. The maximum heating temperature used in the study was 95°C since higher temperature would likely have a negative impact on EMC products.

This part of the study showed that the commercial enzymes are not inactivated completely even after heating for 30 minutes at 95°C. However, using this heat treatment the remaining activities were relatively minor, equivalent to an enzyme dose lower than 0.009%, but the influence of these levels of enzyme on EMC quality is unknown. Furthermore, the impact of these levels of remaining activity in EMCs on the products with which they are incorporated is also unknown. Further work needs to be done on both of these areas.

Chapter 7. Comparison of PAGE, SE-HPLC and RP-HPLC methods for the determination of proteolysis in EMC

7.1. Introduction

The experiments described in this chapter were undertaken to compare different methods to analyse proteolysis in EMC. The methods included polyacrylamide gel electrophoresis (PAGE), size exclusion HPLC (SE-HPLC) and reverse phase HPLC (RP-HPLC).

In PAGE, molecules are separated on a gel matrix according to charge and molecular size. Alkaline urea PAGE, used to analyse casein-containing materials, separates proteins on the basis of both their size and charge. In sodium dodecyl sulphate (SDS) PAGE, the presence of SDS confers the same charge on all molecules and they are separated on the basis of size alone. Smaller compounds migrate faster than larger compounds. Low molecular mass (LMM) SDS gels contain 10% glycerol to slow diffusion and smaller pore sizes to retard migration, and can therefore be used to separate peptides of low molecular mass.

In SE-HPLC, molecules are generally separated on the basis of size alone. Large molecules cannot penetrate the pores of the packing material and elute first from the column, while small molecules fully penetrate all the pores and are eluted last. With a protein hydrolysate, the first peaks eluted are protein peaks, followed by peptides of decreasing size and finally amino acids. Unlike size exclusion, separation by RP-HPLC is based on the hydrophobic interactions between the sample molecules and the ligands on the chromatographic support. In general, the peaks eluted first are small hydrophilic molecules and those that are eluted last are large, hydrophobic molecules.

7.2. Methods

EMC samples were made using an enzyme concentration of 0.15% (w/v) of the individual commercial proteases at an incubation temperature of 40°C (see section

3.4.3). Samples were taken at 0, 0.5, 4 and 24 h and immediately heat-treated at 95°C for 30 min. the samples were analysed by alkaline urea PAGE, SDS PAGE, LMM SDS PAGE, SE-HPLC and RP-HPLC to determine the extent of proteolysis. The methods are detailed in Sections 3.3 and 3.2 for PAGE and HPLC, respectively.

7.3. Results and discussion

7.3.1 PAGE

Figures 7.1, 7.2 and 7.3 show the separation of proteinaceous material from EMC made with Protease B by alkaline urea PAGE, SDS-PAGE and LMM SDS PAGE, respectively. The corresponding gels obtained for EMCs made with Protease A, Protease C and Protease DE are shown in Appendix 7.1-7.3.

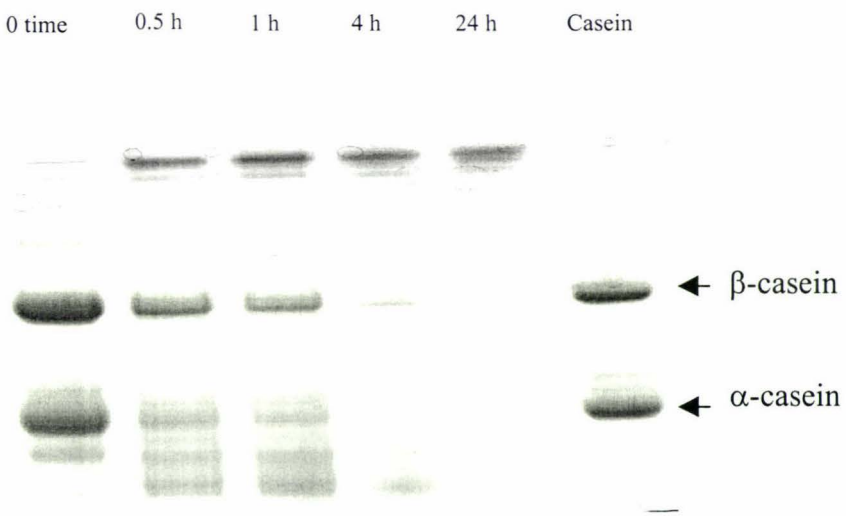


Figure 7.1. Alkaline urea PAGE separation of EMC made with Protease B. EMC digestion times are as indicated. The lane marked "casein" was a sample of a mix of α- and β-casein standards. Bands corresponding to β-casein and α-casein are indicated.

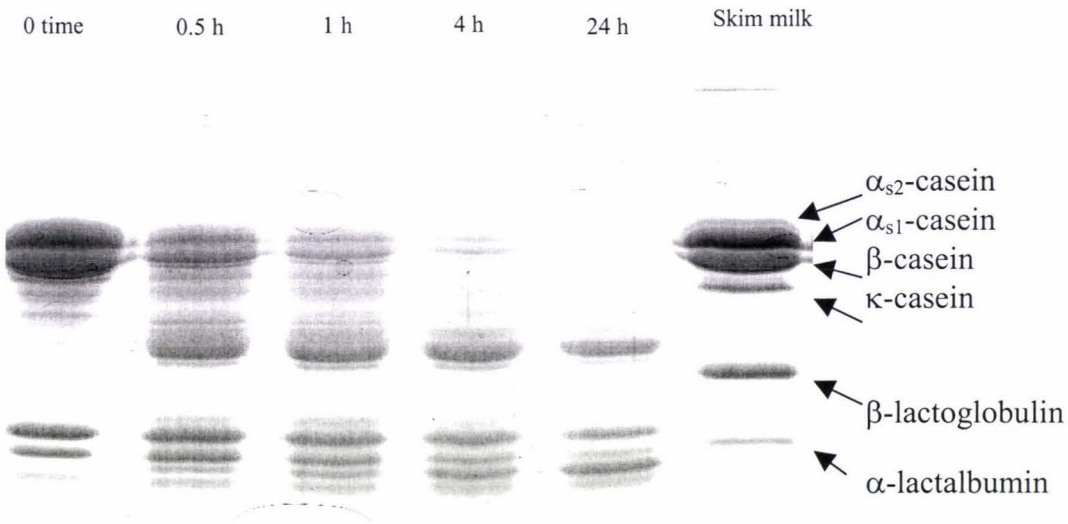


Figure 7.2. SDS PAGE separation of EMC made with Protease B. EMC digestion times are indicated for each lane. Skim milk was used as a source of caseins and whey proteins. Bands corresponding to four casein types, β-lactoglobulin and α-lactalbumin are indicated.

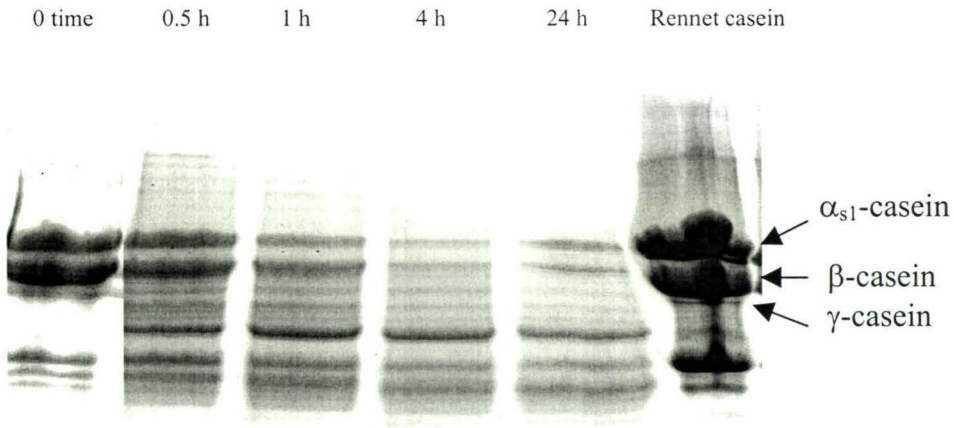


Figure 7.3. LMM SDS PAGE separation of EMC made with Protease B. EMC digestion times are indicated. Rennet casein was used as a source of whole casein and casein fragments. Bands corresponding to α_{s1}-casein, β-casein and γ-casein are indicated.

All the gels show, to a certain extent, the conversion of casein protein to peptides through digestion by Protease B. Figure 7.1 shows that with alkaline urea gels almost all protein bands decrease in intensity after 24-hour digestion, but little definition of products is possible. Figures 7.2 and 7.3 give more detail with respect to the products of digestion. Unlike alkaline urea PAGE, some bands can be seen to increase in intensity through the 24-hour digestion. However, the ability to analyse the products relevant to flavour formation (*i.e.* small peptides) is very limited. One benefit of PAGE analysis is that a quantitative comparison of casein (α–, β–, γ– and κ–) digestion can be made by gel scanning and integration. Table 7.1 shows a comparison of β–casein digestion in EMCs made with Protease A, Protease B, Protease C and Protease DE using the integrated data from scanned alkaline urea gels.

Table 7.1. The percentage of intact β–casein in four EMC digestion samples using alkaline urea gel electrophoresis

Digestion time (hour)	β-casein remaining in EMCs digested with commercial proteases (%)			
	Protease A	Protease B	Protease C	Protease DE
0	100 [1]	100	100	100
0.5	10.7	49.4	9.6	25.5
1	6.1	29.6	7.3	0.4
4	1.7	4.7	2.6	0
24	0	2.4	0.6	0

[1] The integrated data from scanned gels was used to quantitate β-casein levels.

7.3.2 HPLC

Figures 7.4 and 7.5 show SE-HPLC and RP-HPLC results, respectively, of EMC base digested with the four commercial proteases for 4 h.

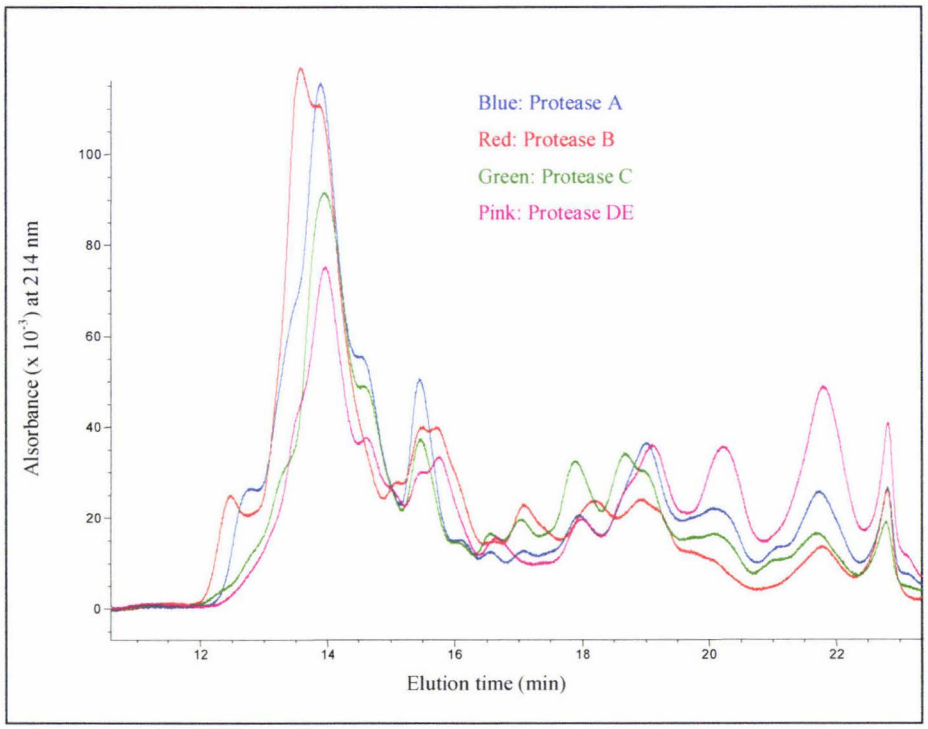


Figure 7.4. SE-HPLC chromatograms of EMC base digested with four commercial proteases for 4 h

The chromatograms shown in Figure 7.4 demonstrate that a clear comparison of proteolysis given by the four commercial proteases can be made with SE-HPLC. Differences in the extent and nature of proteolysis catalysed by the various proteases were clearly evident from differences in some of the peaks. For example, proteolysis by Protease DE generated larger quantities of small peptides (later-eluting peaks) and removed more whole protein (early-eluting peaks) than did proteolysis by the other three proteases. Digestion by Protease B led to the highest level of undigested protein, and the lowest level of small peptides.

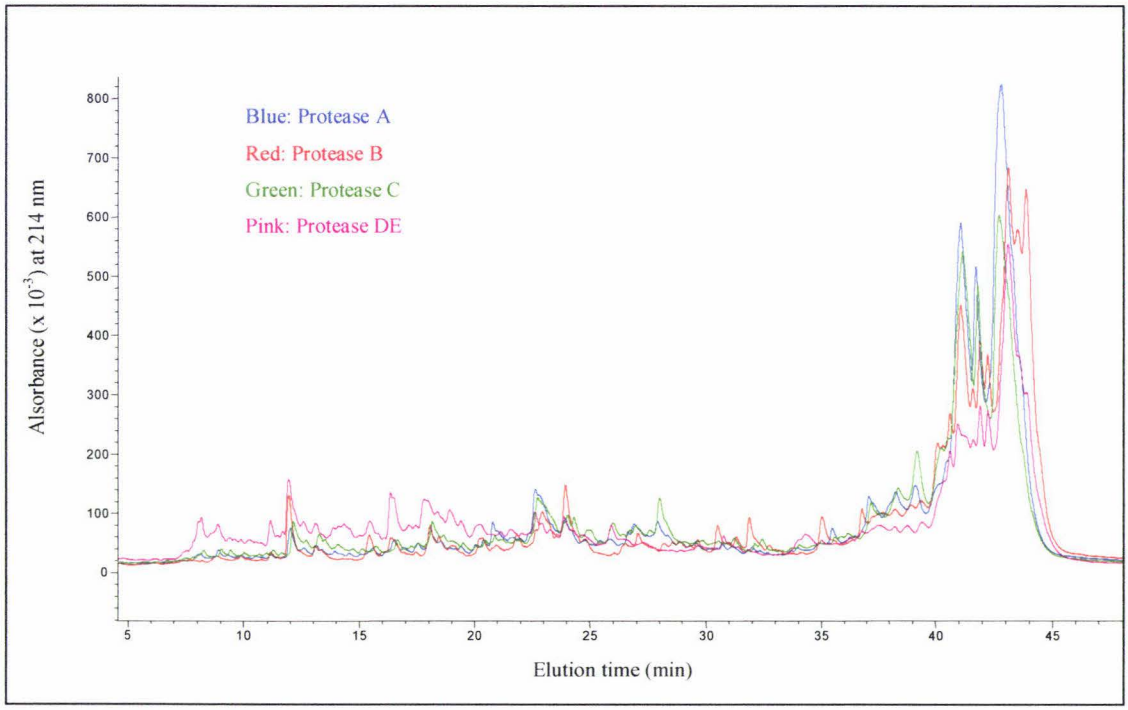


Figure 7.5. RP-HPLC chromatograms of EMC base digested with four commercial proteases for 4 h

It is clear from Figure 7.5 that RP-HPLC gives chromatograms that contain more individual peaks and are therefore more complex than those chromatograms given by SE-HPLC (Figure 7.4). Because of this complexity, quantitation of proteolysis is difficult from the chromatograms given by RP-HPLC.

SE-HPLC and RP-HPLC both have advantages and disadvantages for the study of the digestion of EMC bases. SE-HPLC can be used to give information on the distribution of compounds according to molecular size; RP-HPLC can be used for specific compound identification.

The aim of this study was to profile the molecular size distribution of proteolytic products in EMC made with the four commercial proteases rather than to identify individual compounds produced from EMC proteolysis and therefore SE-HPLC was by far the more useful technique. Further studies on the taste of individual peptides generated through proteolysis in EMC will need to use RP-HPLC techniques.

Appendix 7.4 which was used to determine the approximate retention times for peptides with relative molecular masses of <400 Da (≤ 3 residues), 400-900 Da (4~6 residues), 900-1250 Da (7~11 residues), 1250-2500 Da (11~20 residues), 2500-3500 Da (20~30 residues) and >3500 Da (>30 residues). The amount of material within each molecular mass range was quantified by integrating the chromatogram within the appropriate retention times. The average molecular masses of peptides were statistically calculated using the possible masses of peptides from α_{s1}-, α_{s2}-, β- and *para*-κ-casein taking into account the proportions of these proteins in cheese (about 39% of α_{s1}-casein, 36% β-casein, 10% α_{s2}-casein and 13% *para*-κ-casein). The chromatogram data obtained from SE-HPLC were transformed in this assay to give histogram profiles of molecular mass distribution. Histograms were constructed using the data from 5 EMC digestion times and the 6 molecular mass ranges. The conversions of the chromatograms given by EMC made using Protease B into histogram form are shown in Figure 7.6. The corresponding data for Protease A, Protease C and Protease DE are shown in Figures 7.7, 7.8 and 7.9, respectively.

The data show that Protease DE releases a far greater proportion of tripeptides, and dipeptides than do the other proteases. Over 24 h of digestion, Protease C tends to produce more middle range peptides of 1250~2500 Da than the other proteases , whereas Protease B produces more high mass peptides of molecular size over 3500.

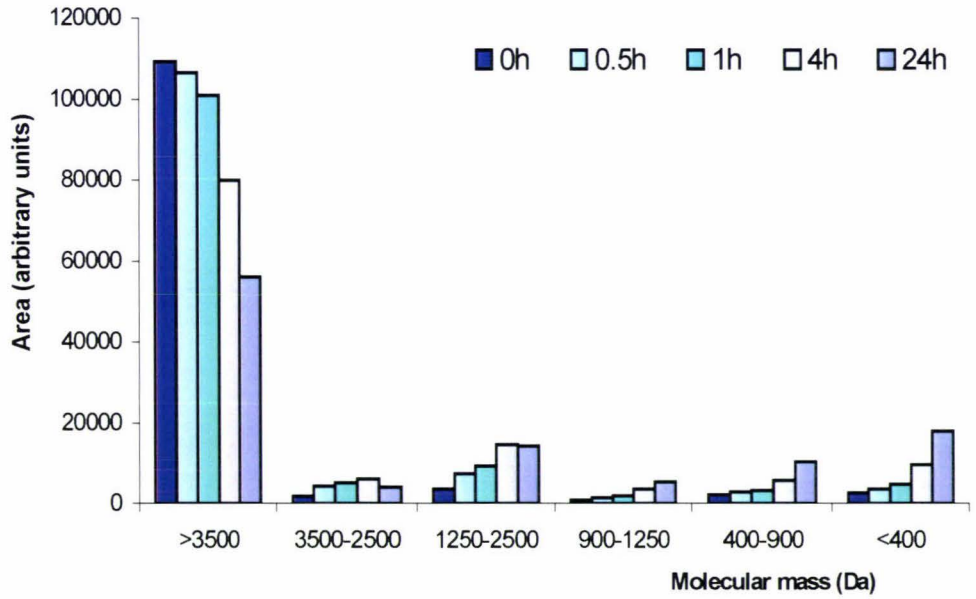


Figure 7.6. The molecular mass profile of compounds in EMC bases digested with Protease B for up to 24 h

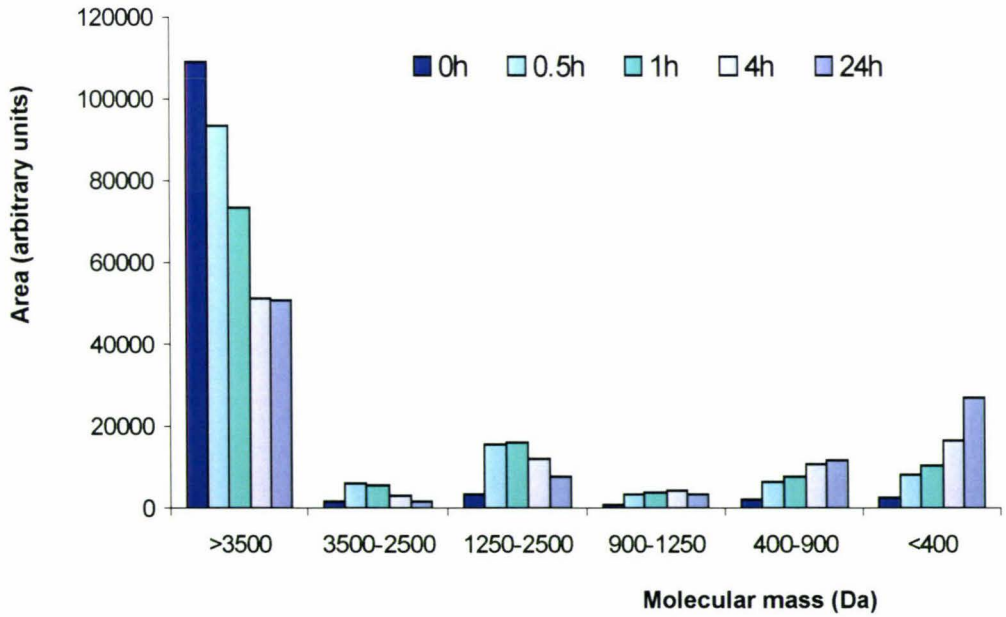


Figure 7.7. The molecular mass profile of compounds in EMC bases digested with Protease A for up to 24 h

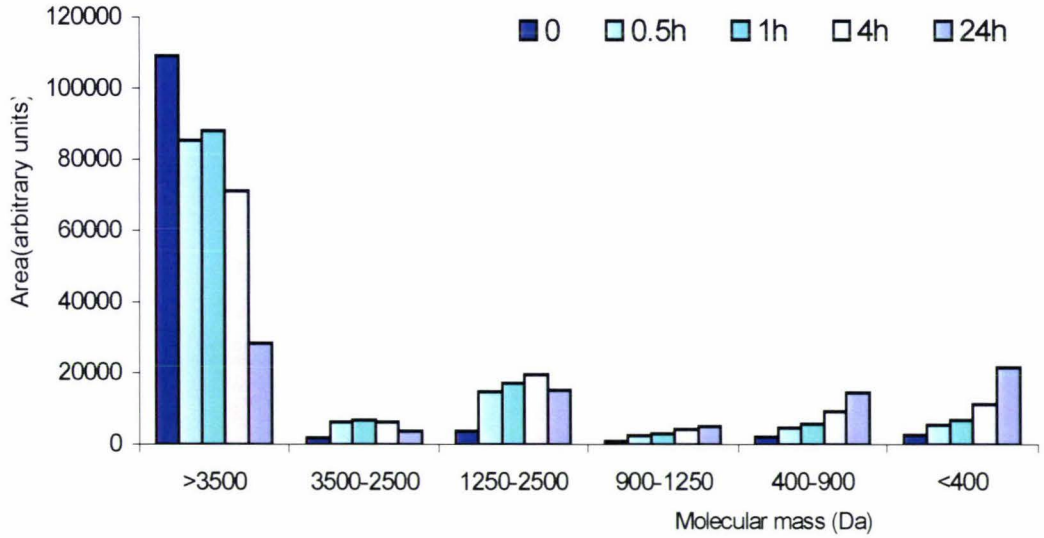


Figure 7.8. The molecular mass profile of compounds in EMC bases digested with Protease C for up to 24 h

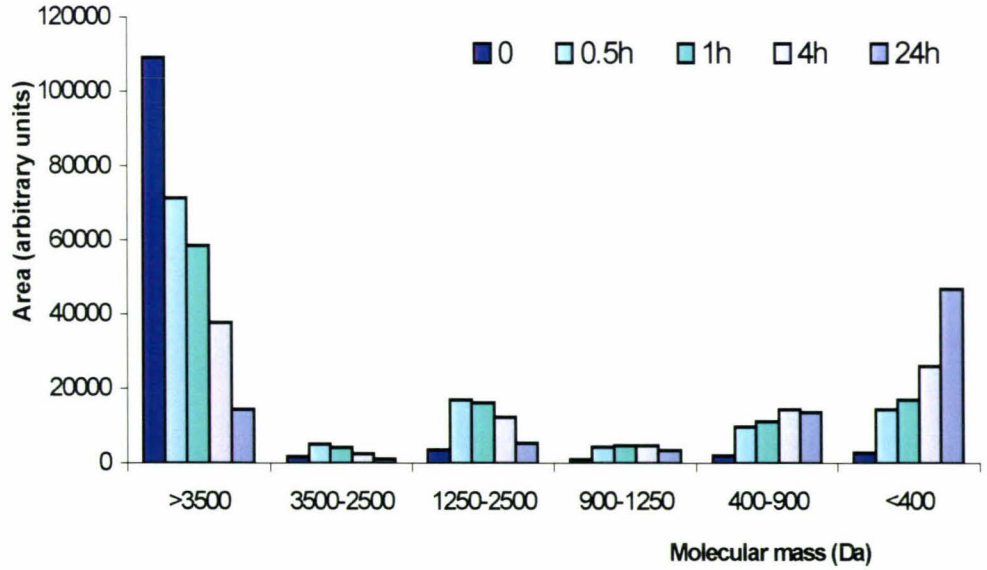


Figure 7.9. The molecular mass profile of compounds in EMC bases digested with Protease DE for up to 24 h

7.4. Overall discussion and conclusion

Both PAGE and HPLC consistently showed that Protease DE was the strongest hydrolysing protease of the four commercial proteases, and that Protease B was the weakest. The histogram derived from the SE-HPLC chromatogram gives clear quantitative data for the relative molecular mass (M_r) profiles of digested products. SE-HPLC allowed a more straight-forward quantification than PAGE, and also allowed analysis across the entire M_r range from largest components to amino acids. One disadvantage of the HPLC methods is that the chromatograms given are the result of detection by absorbance at 214 nm. This wavelength is used mainly to detect peptide bonds. As the number of peptide bonds decreases through hydrolysis, so the level of absorbance decreases, with the result that peaks for small peptides are not as large as peaks for large peptides (at the same molar concentration). Further, amino acids do not contain peptide bonds and therefore, with the exception of aromatic amino acids such as phenylalanine, tyrosine and tryptophan, are not detected.

The different methods for analysing proteolysis give different features. Of the individual components produced by EMC digestion, only caseins and large peptides could be observed by PAGE. Small peptides could not be detected. It is possible that different staining methods could have visualized some of the smaller peptides, but this was not attempted since the resolution would still have been poor. On the other hand, small and large peptides could be quantified using SE-HPLC, whereas individual components could not be detected. RP-HPLC did identify individual components of proteolysis, however the data were too complex for the purposes of this study. This project had the objective of looking at the conversion of caseins in EMC to small compounds that are important to cheese flavour. SE-HPLC allowed ready quantification of this and was therefore used as the preferred method of analysis for further work.

Chapter 8. HPLC analysis of proteolysis in EMC by different combinations of commercial proteases

8.1. Introduction

From the SE-HPLC results presented in Chapter 7, Protease DE tends to release a higher proportion of low mass peptides in EMC, more large peptides remained in EMCs made with Protease B, Protease C produced more medium size peptides and Protease A gave a more general profile. It was necessary to study these trends more fully and also to look at EMC digestion with combinations of proteases. A combination of commercial proteases may give different results from EMC digestion with individual proteases since one protease may provide substrates for the other, and the overall degree and nature of proteolysis would be different due to this synergistic effect. This chapter describes EMC proteolysis by various two-protease combinations with Protease A, Protease B, Protease C and Protease DE. SE-HPLC was used to analyse the products of proteolysis and the data are presented as histograms giving molecular mass distribution of the peptides. Amino acid analysis of the EMC, digested with proteases for 24 h, was also done using pre-column derivatisation of the amino acids and analysis of the products HPLC..

Furthermore, Protease DE comprises two proteases Protease D and Protease E, respectively. The digestion of EMCs with different ratios of Protease E and Protease D was carried out to study the effect of different levels of the two proteases.

Finally, a new batch of Protease B was used in a Protease B /Protease C combination to compare the reproducibility of Protease B batches. This was because of the presence of two exopeptidases in Protease B, one of which was relatively unstable and therefore possibly vulnerable to batch-to-batch variation.

8.2. Protease combinations

The following paired combinations of the commercial proteases were prepared;

Protease A/Protease B, Protease A/Protease C, Protease A/Protease DE, Protease B/Protease C, Protease B/Protease DE and Protease C/Protease DE. The different ratios of the two proteases in each combination are given in Table 8.1. The further combination with different ratios of Protease E and Protease D were prepared with the same ratios.

Table 8.1. The ratios of paired protease combinations used for EMC digestion

Protease	Ratios					
	Blend 1	Blend 2	Blend 3	Blend 4	Blend 5	Blend 6
X	100%	80%	60%	40%	20%	0%
Y	0%	20%	40%	60%	80%	100%

EMC was made with each combination of commercial proteases using 0.15% initial enzyme concentration (*i.e.* the sum of both proteases in each digestion was 0.15%).

To make the combination of two proteases, 0.3 g of each protease were individually dissolved in 3 mL Milli-Q water, then combined together to produce two-protease solutions. Since proteases are proteins that can be degraded by each other, the two protease solutions were mixed together just prior to addition to EMC base. Note that even individual commercial protease solutions can be degraded by its own components. The EMC was incubated for 0.5, 1, 4 and 24 h. The methods of EMC incubation and inactivation are detailed as in Section 3.4.5.

8.3. Peptide analysis by SE-HPLC

8.3.1. Method

SE-HPLC was performed according to the method detailed in Section 3.2.1. The chromatograms were divided into molecular mass (M_r) ranges of < 400 Da (≤ 3 residues), 400-900 Da (4~6 residues), 900-1250 Da (7~11 residues), 1250-2500 Da (11~20 residues), 2500-3500 Da (20~30 residues) and > 3500 Da (>30 residues). The method for determining molecular mass ranges was detailed in Section 7.3.2. The chromatogram data obtained from SE-HPLC were

transformed to give histogram profiles of molecular mass distribution.

It should be noted that strict comparison between different combinations of proteases using these histograms is difficult as the detection system of SE-HPLC measures peptide bonds. Thus peaks containing a given molar concentration of small peptides are smaller than peaks containing the same molar concentration of large peptides.

8.3.2. Results and discussion

Figures 8.1 and 8.2 show as histograms the molecular mass profile of peptides from EMCs digested for 4 h and 24 h with different combinations of proteases. For both Figures 8.1 and 8.2, panels a-c show Protease A in combination with Protease B, Protease C and Protease DE, respectively, panels d-f show Protease B in combinations with Protease A, Protease C and Protease DE, respectively, panels g-i show Protease C in combination with Protease A, Protease B and Protease DE, respectively, and panels j-l show Protease DE in combination with Protease A, Protease B and Protease C, respectively. Note that a quantitative comparison of the different panels within a figure cannot be made because the amount of EMC base used for each enzyme combination was different.

The results of the EMC digestions for individual commercial proteases showed that Protease DE produced more peptides of 3 residues or less (< 400 Da) than did the other proteases, Protease B gave more large peptides of over 30 residues but fewer small peptides, while Protease C gave more medium-sized peptides of 11-20 residues (1250~2500 Da). Protease A was more efficient with respect to producing small peptides and reducing the levels of large peptides than Protease C.

The use of Protease A in combination with other commercial proteases shows quite distinct effects. From Figure 8.1a it can be seen that as the proportion of Protease B increased, the overall levels of digestion decreased. Figure 8.1b shows that as the ratio of Protease C increased, the level of medium-sized peptides with molecular mass 1250~2500 Da (11-20 residues) increased slightly and the quantity of peptides with molecular mass less than 400Da (≤3 residues) and 400-900 Da (4-6 residues) decreased slightly. Figure 8.1c shows that as the

ratio of Protease DE increased the amount of the lower molecular mass peptides of less than 400 Da increased and the level of the large peptides decreased.

Figure 8.1e shows that as the ratio of Protease C is increased in EMC digestions in combination with Protease B, the amount of overall hydrolysis increases and the relative level of medium and short length peptides increases. Figure 8.1f shows that with Protease DE in combination with Protease B, an increase in the ratio of Protease DE leads to an increase in the level of short peptides and a decrease in the level of long peptides while the level of medium length peptides tend to remain the same. Figure 8.1i shows that when in combination with Protease C, an increase in the ratio of Protease DE leads to an increase in overall hydrolysis, a decrease in the levels of mid and large peptides and an increase in the level of small peptides.

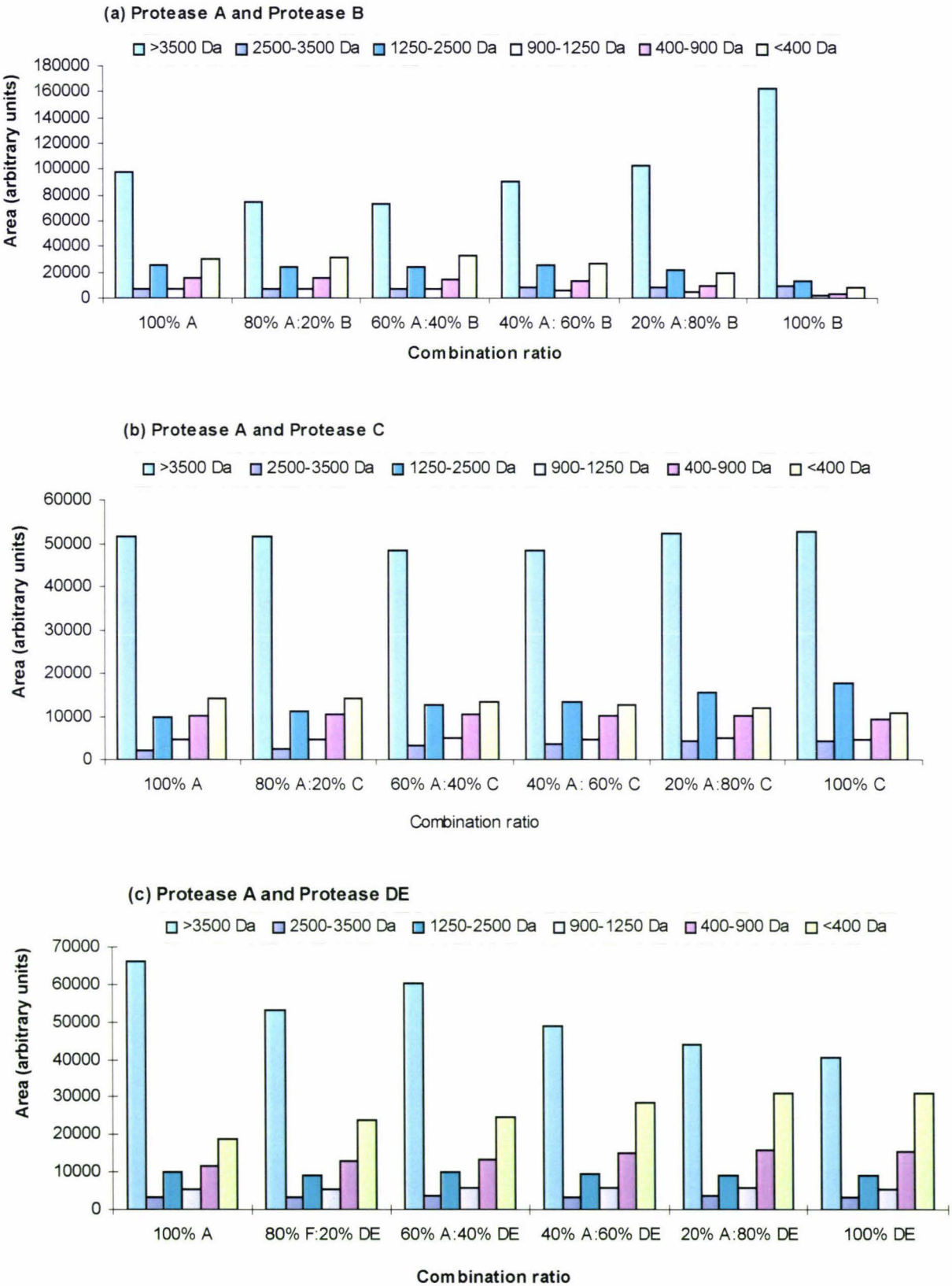
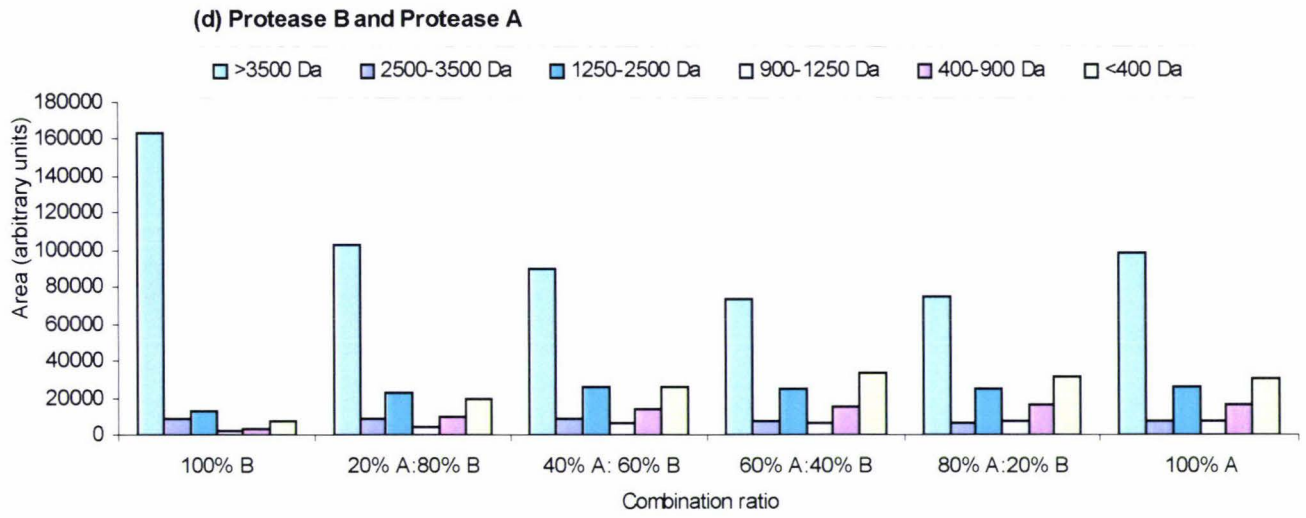
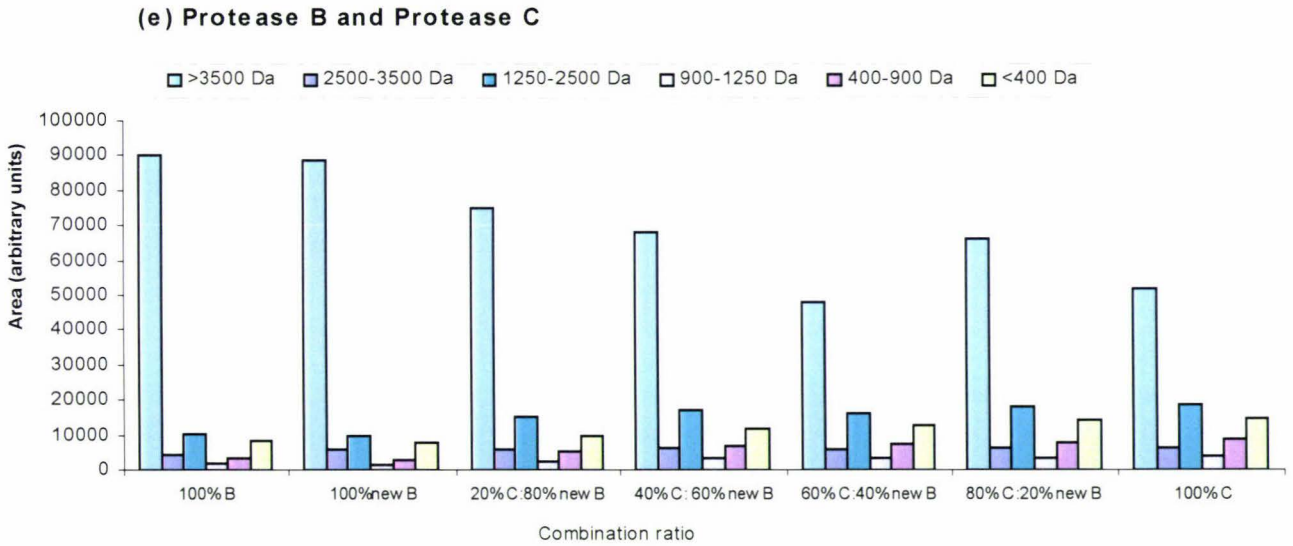


Figure 8.1.I. Molecular mass profiles of peptides from EMC bases digested with Protease A + Protease X for 4 h





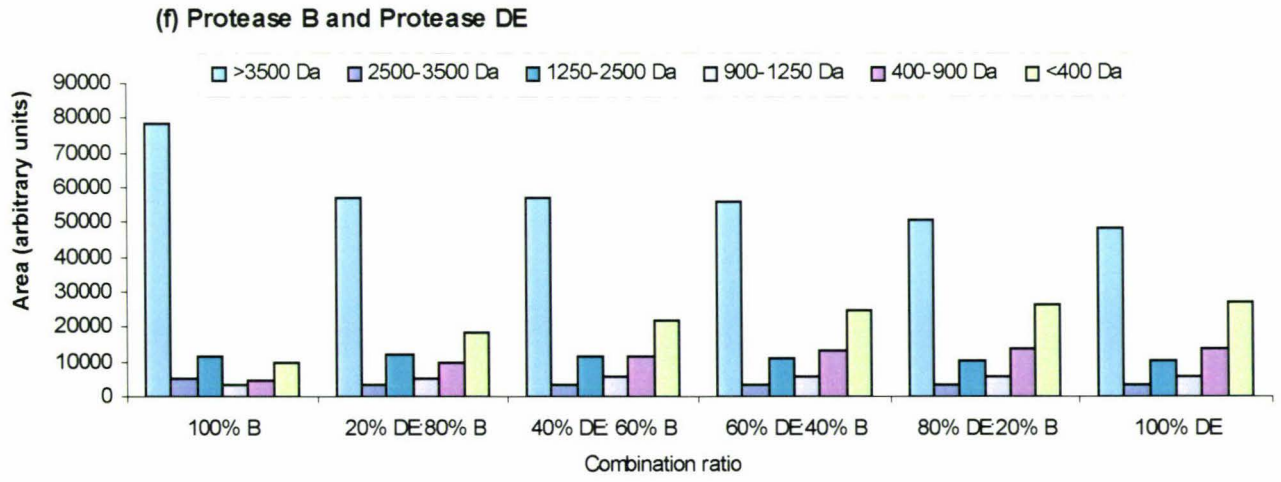
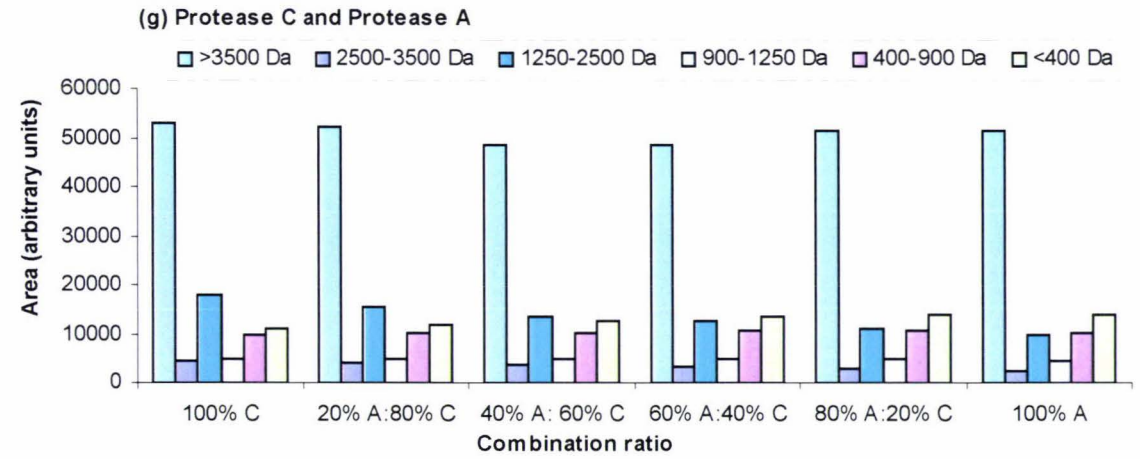
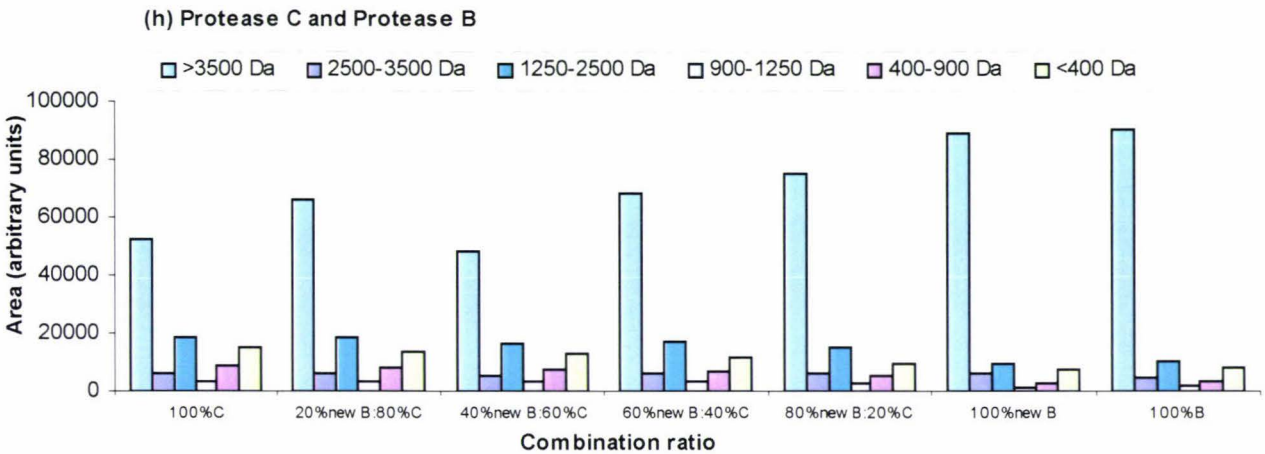


Figure 8.1.II. Molecular mass profiles of peptides from EMC bases digested with Protease B + Protease X for 4 h. Note that the original and new batches of Protease B (B and new B, respectively) are compared in (e)





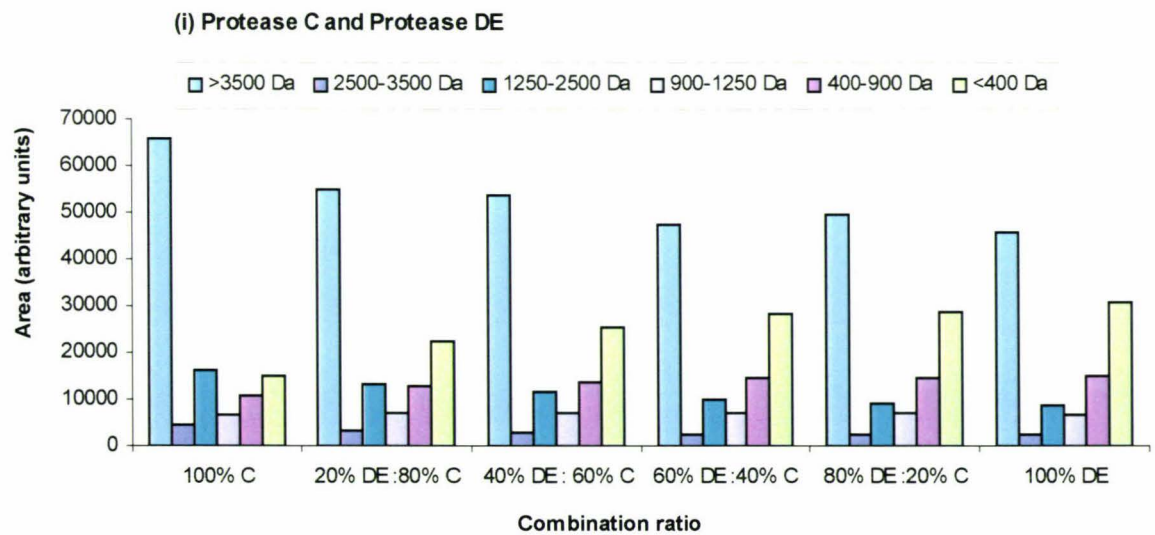
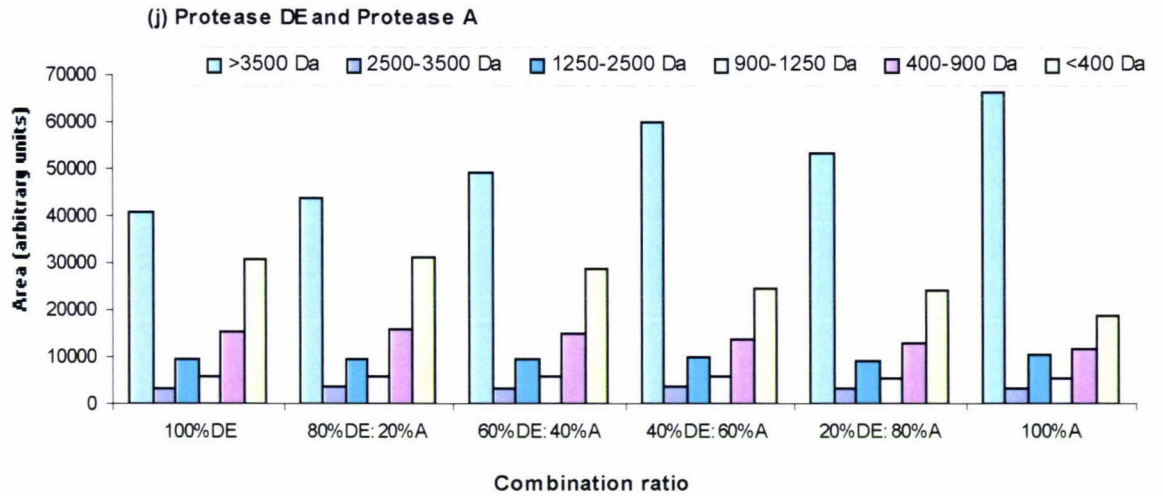
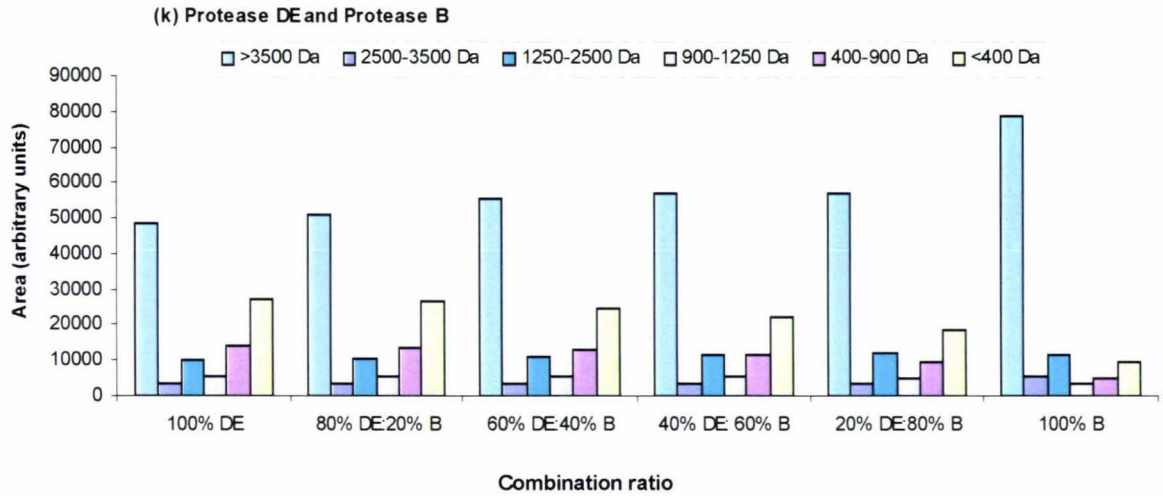


Figure 8.1.III. Molecular mass profiles of peptides from EMC bases digested with Protease C + Protease X. Note that the original and new batches of Protease B (B and new B, respectively) are compared in (h)





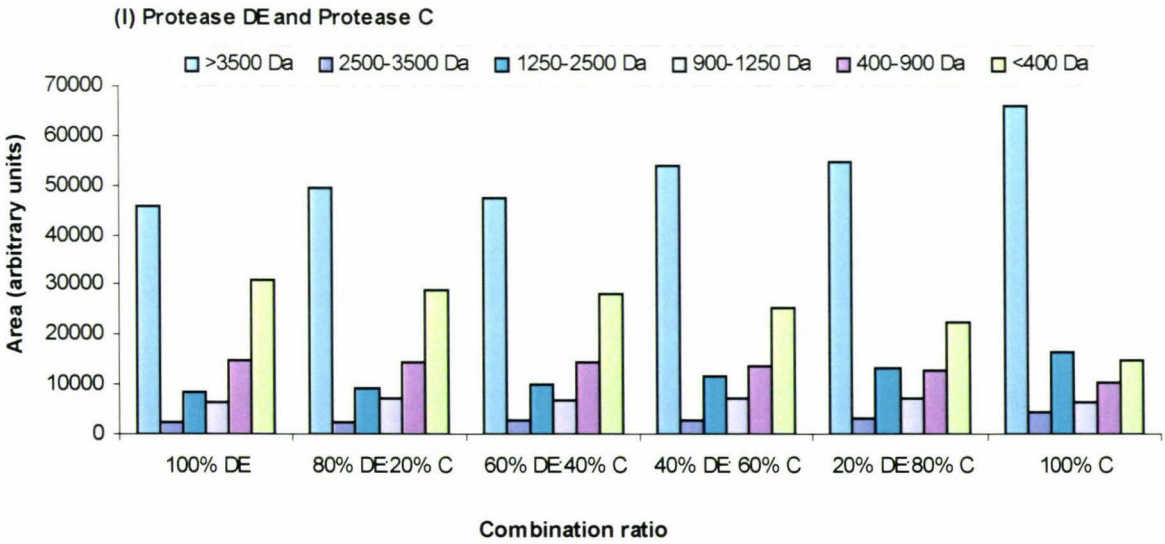
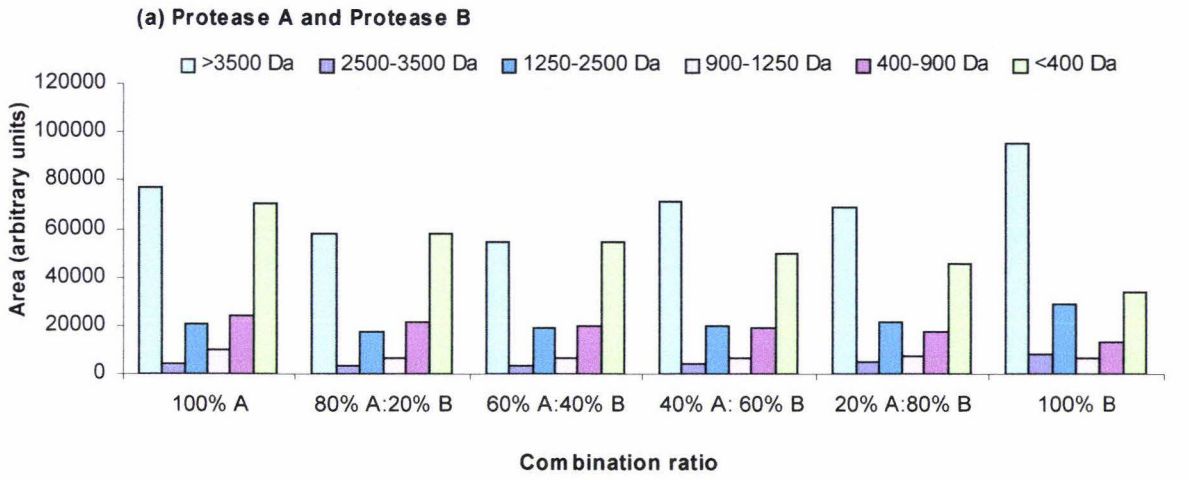


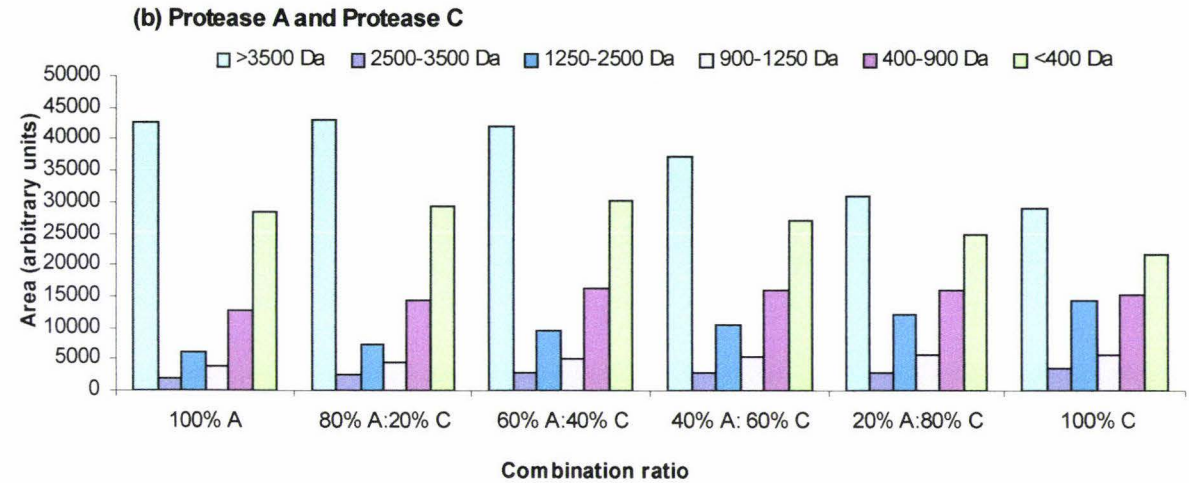
Figure 8.1. IV. Molecular mass profiles of peptides from EMC bases digested with Protease DE + Protease X for 4 h.

For the 24h EMC digestions, the trends shown by the four individual commercial proteases are similar to those shown for the 4 h digestion: Protease A gives relatively high levels of small peptides but significant levels of large peptides remain, with Protease B the levels of mid and small peptides are similar, Protease C gives a relatively high level of mid to small peptides and Protease DE gives extensive hydrolysis of large peptides with low levels of medium sized peptides and high level of small peptides.

For the combinations of commercial proteases, the trends of hydrolysis at 24 h are again similar to those seen at 4 h. The main difference is in the extent of hydrolysis. The level of large peptides decreased whereas the medium and small peptides increased.

With respect to the different batches of Protease B, very little difference in the peptide profiles can be seen after 4 h digestion (the first two histogram sets of Figure 8.1e and the last two histogram sets of Figure 8.1h). After 24 h digestion, however, the tendency for the older batch to give higher level of small peptides is clear (the first two histogram sets of Figure 8.2e and the last two histogram sets of Figure 8.1h).





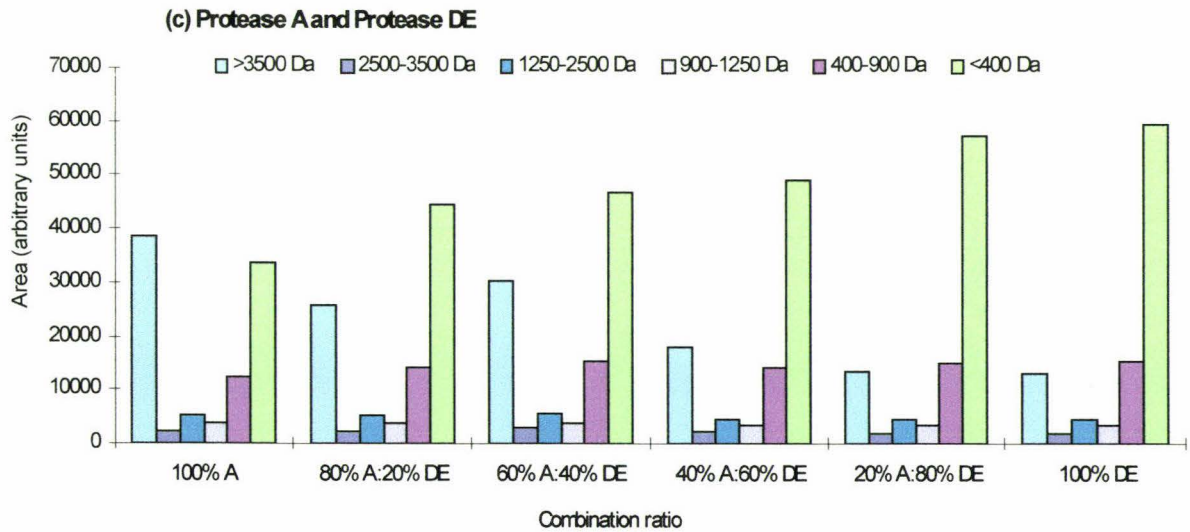
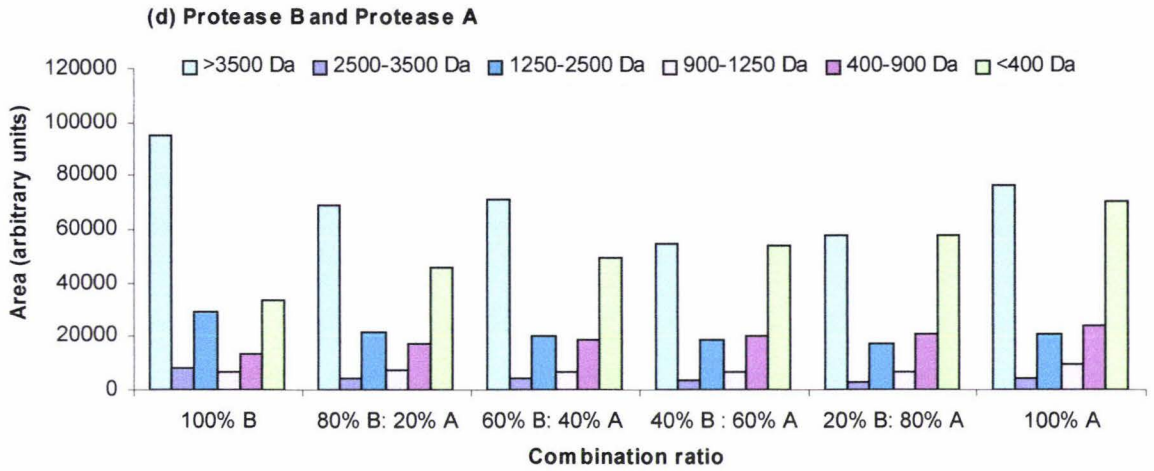
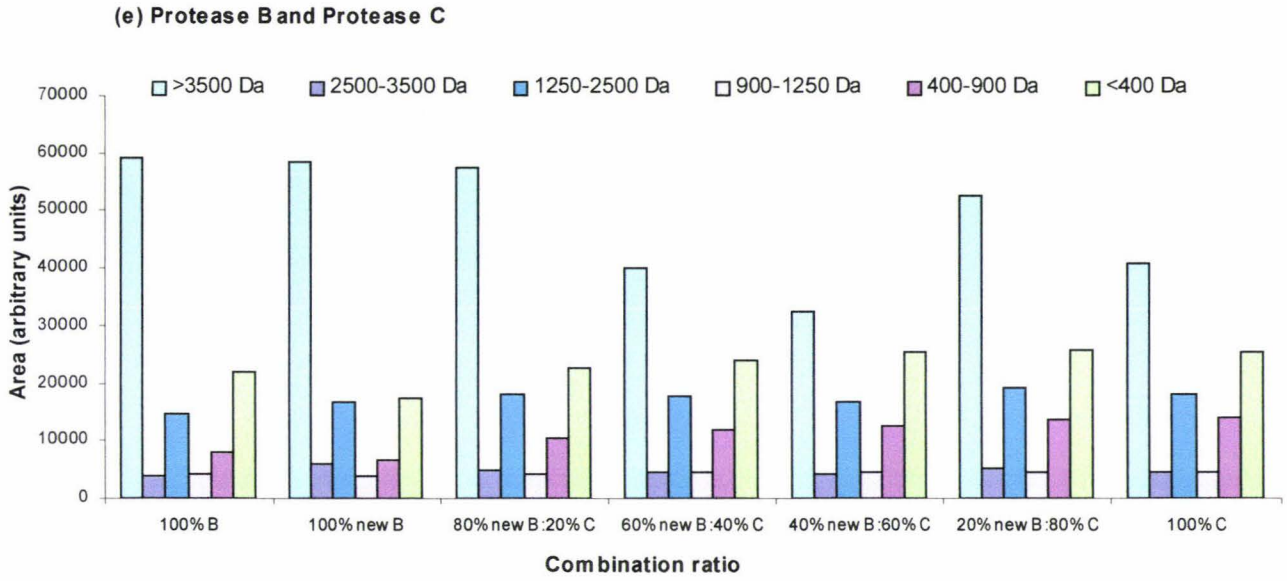


Figure 8.2.1. Molecular mass profiles of peptides from EMC bases digested with Protease A + Protease X for 24 h.





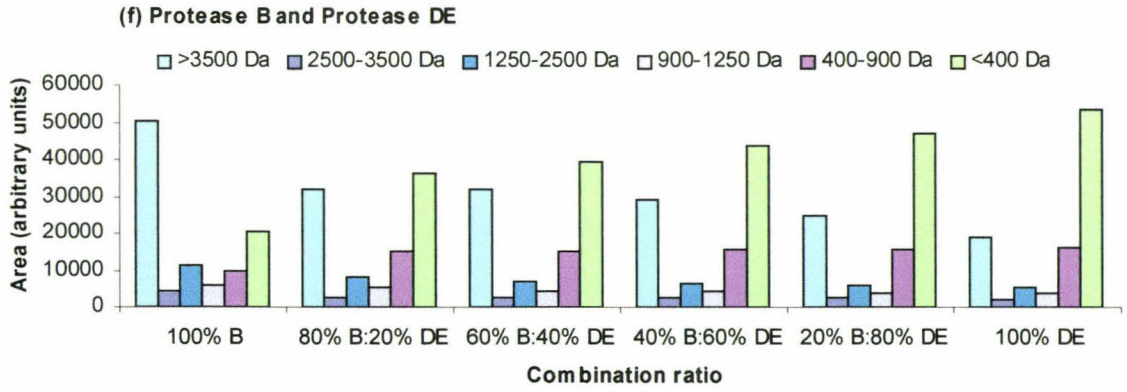


Figure 8.2.II. Molecular mass profiles of peptides from EMC bases digested with Protease B + Protease X for 24 h. Note that the original and new batches of Protease B (B and new B, respectively) are compared in (e)

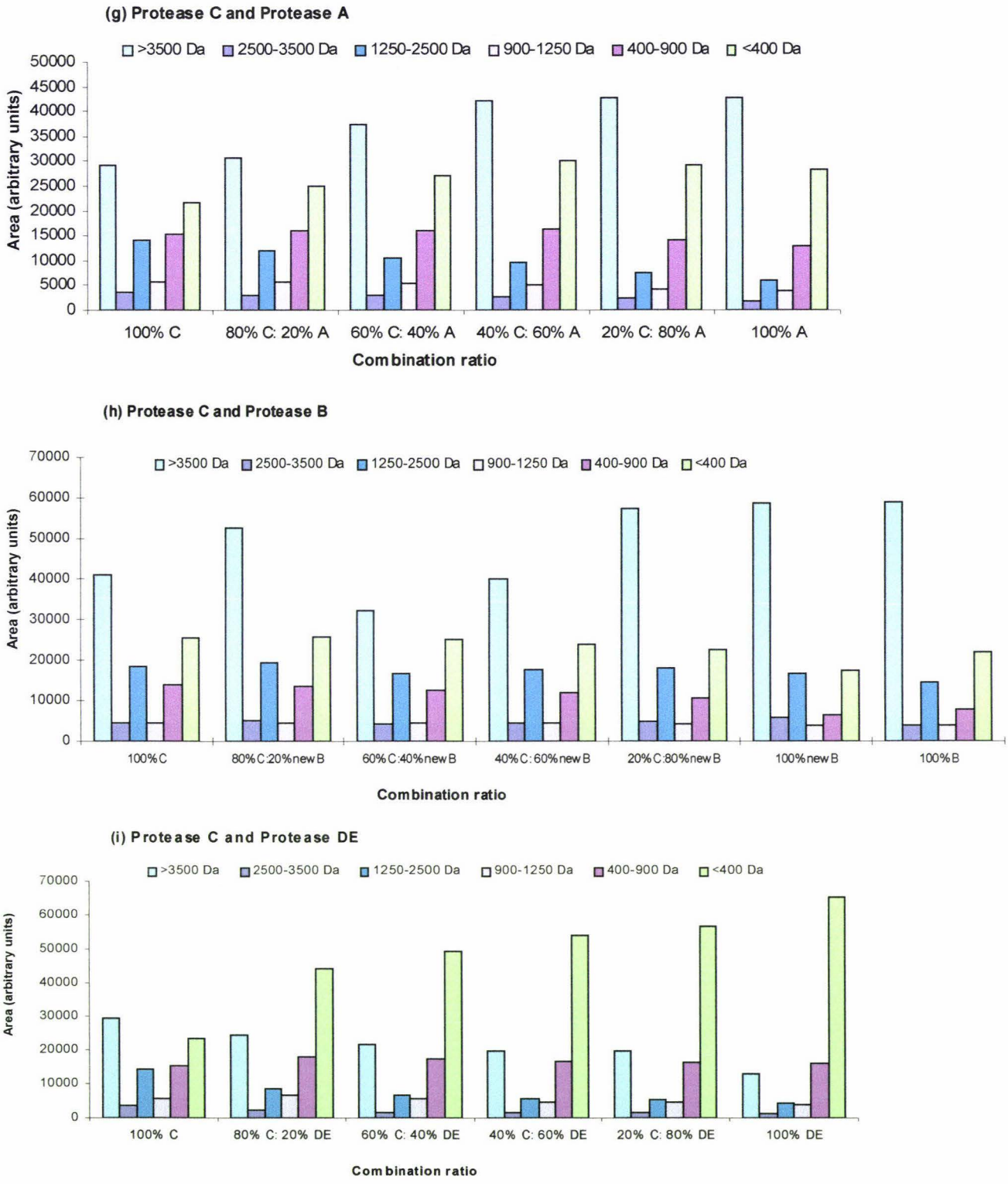


Figure 8.2.III. Molecular mass profiles of peptides from EMC bases digested with Protease C + Protease X for 24 h. Note that the original and new batches of Protease B (B and new B, respectively) are compared in (h)

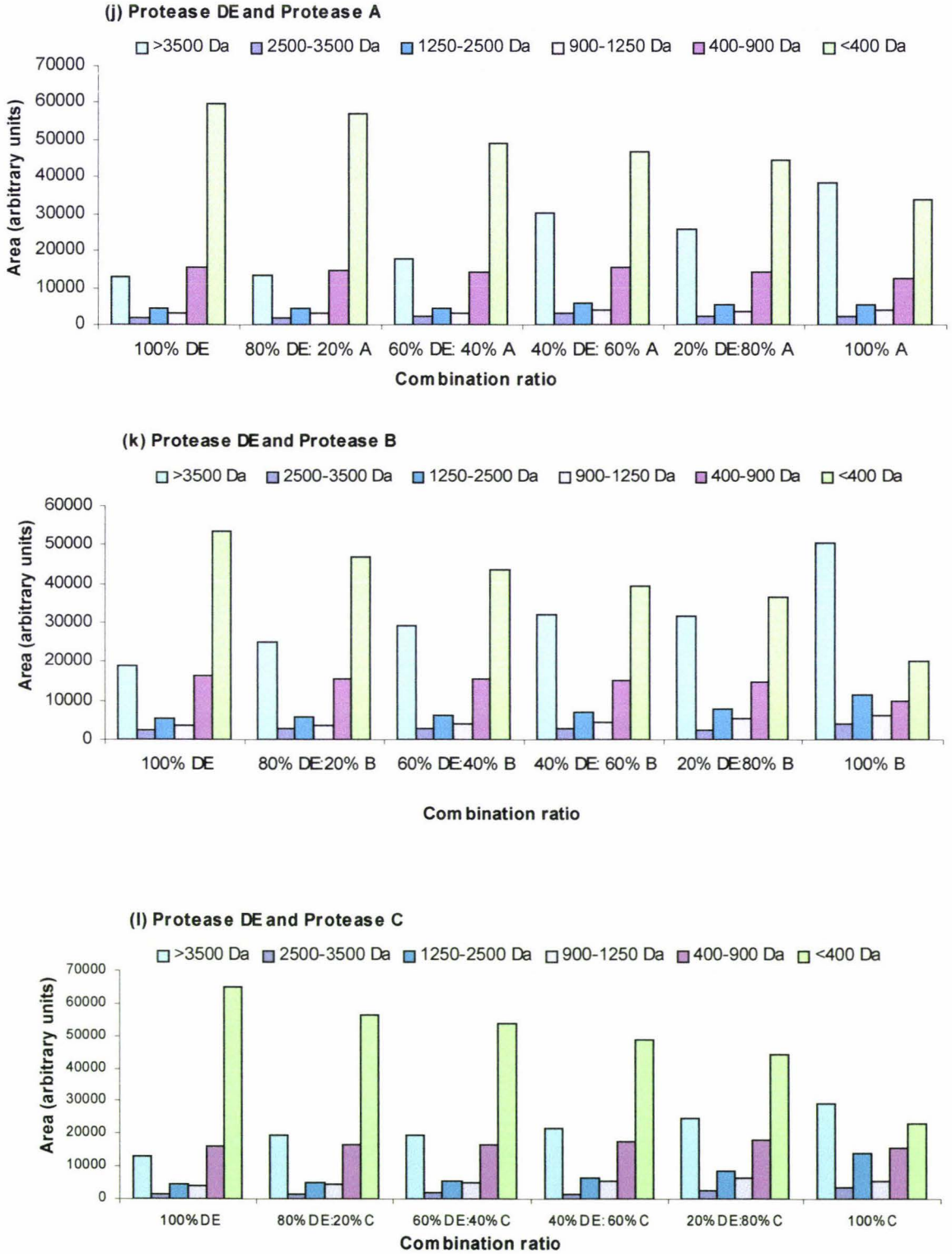


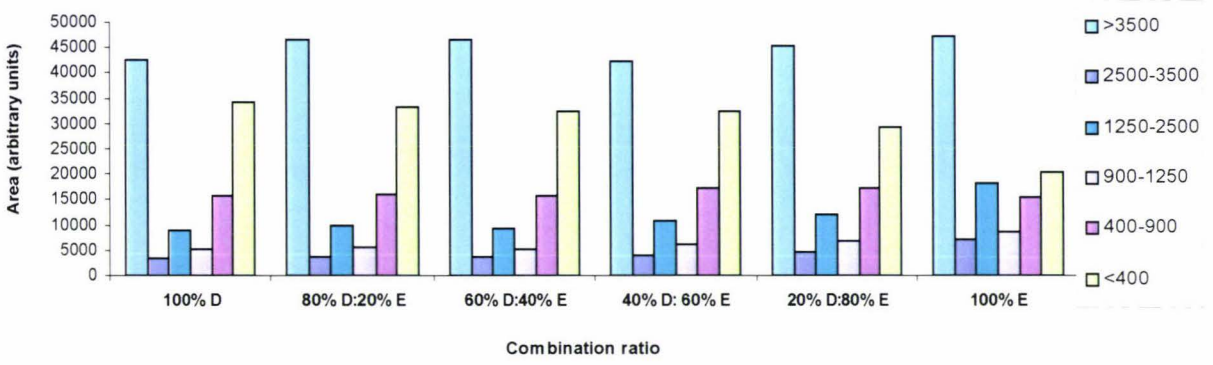
Figure 8.2. IV. Molecular mass profiles of peptides from EMC bases digested with Protease DE + Protease X for 24 h.

A comparison of Figures 8.1b and 8.2b, which show the profiles given by the Protease A / Protease C combination, reveals that after digestion for 4 h the profiles given by the different combination ratios were quite similar, but after 24 h digestion Protease C produced more medium-sized peptides while Protease A produced more smaller peptides. This might result in Protease C producing more bitterness than Protease A. This result indicates that, in some cases, the molecular mass distribution of peptides can change significantly between 4 h and 24 h.

Figure 8.2c shows that Protease DE contains stronger exopeptidase and endopeptidase activities than Protease A since most large compounds were converted to smaller peptides by 24 h. Figure 8.2b reveals that Protease C contains stronger endopeptidase activity since it reduces the level of intact protein more than does protease A. However, protease A contain stronger exopeptidase activity, since it produces larger quantities of small peptides (M_r < 400 Da). Figure 8.2 (a) shows that Protease B contains a significantly weaker endopeptidase activity compared with Protease A. This resulted in higher quantities of larger compounds remaining after 24 h.

Figure 8.3 shows the molecular mass distribution of peptides in EMC digested for 4 h and 24 h with different combinations of the Protease D and Protease E, the proteases that constitute Protease DE. From Figure 8.3 it can be seen that Protease D produces significantly more small peptides than Protease E, while Protease E produces more medium-sized peptides. Therefore Protease D contains strong endopeptidase and exopeptidase activity while Protease E contains less exopeptidase activity. The SE-HPLC chromatograms (Figure 8.4) show that Protease E contains some differences from the other proteases shown in Figure 8.4. This possibly suggests that some of the compounds produced by Protease E are unique.

(a) Protease D and Protease E – 4 h digestion



(b) Protease D and Protease E – 24 h digestion

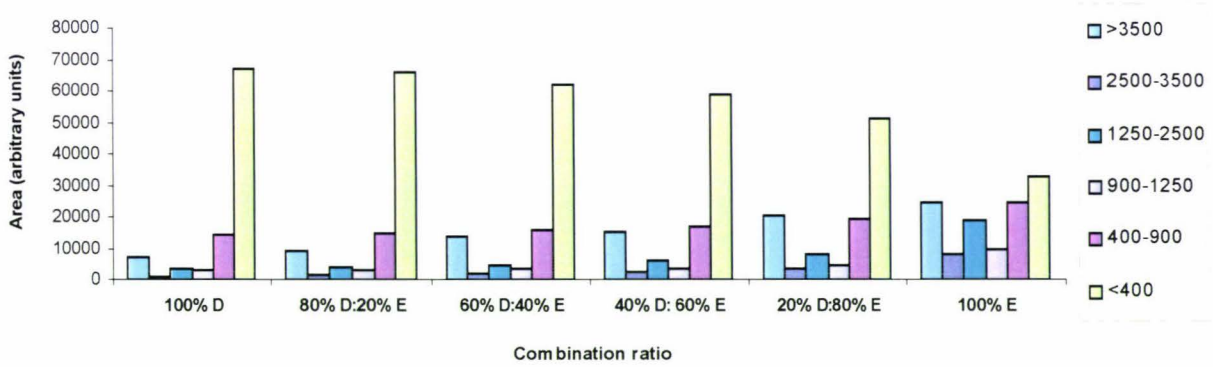


Figure 8.3. Molecular mass distribution of EMC bases digested with Protease D and Protease E

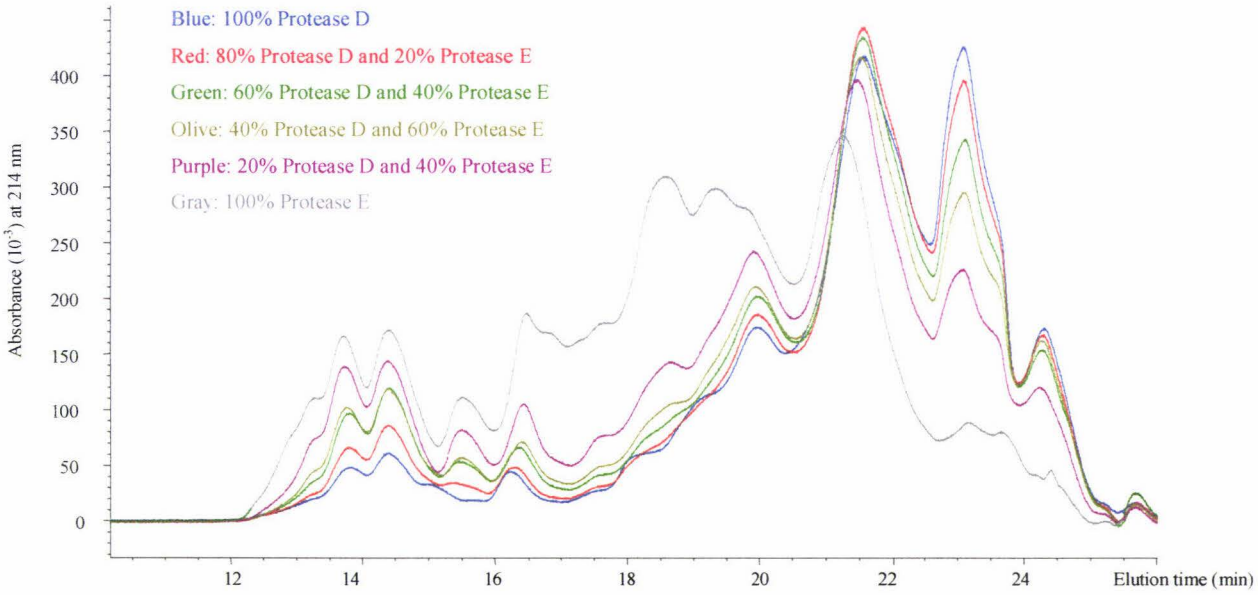


Figure 8.4. Size exclusion chromatograms of EMC bases digested for 24 h with Protease D and Protease E combination

8.1. Amino acid analysis by RP-HPLC

Amino acids also contribute to the flavour of dairy products. Therefore the amino acid composition of EMCs, produced by the various paired-combinations of commercial proteases after 24 h digestion, was determined.

8.1.1. Methods

8.1.1.1. Extraction of amino acids from EMC

Amino acid analysis cannot be done directly on EMC samples as the complex nature of the EMC matrix interferes with the analysis. Thus, the amino acids must first be extracted from the bulk of the EMC matrix. Since urea has a similar structure to arginine and would therefore interfere with amino acid detection, the alkaline urea buffer extraction method was not suitable for these experiments. A water extraction method was therefore applied. 5 mL 0.1% TFA in Milli Q water was added to 0.5 g EMC sample and the mixture was held at 40°C for 30 minutes.

The mixture was then homogenised using an Ultra Turrax (T25) homogeniser with 10G shaft (speed setting high; 24000 rpm) for 1 minute and then centrifuged at 5000 rpm (2000 x *g*) for 10 minutes at 4°C. The supernatant was filtered using a syringe filter (Acrodisc PF, 0.8 micron /0.2 micron) and stored frozen (-20°C).

8.4.1.2. Separation of amino acids from peptides and protein in the water extract

As the method used for amino acid analysis is also sensitive to the presence of peptides, it was first necessary to separate the amino acids from the peptides and proteins in the water extract. This was done using RP-HPLC. In this method the amino acids elute from a RP-HPLC column earlier than the majority of peptides. Samples of each water extract were applied to the RP-HPLC column and an acetonitrile gradient (described below) was used to elute firstly the amino acids, which were collected as one fraction, then the peptides/proteins which were discarded. Since phenylalanine is the last-eluting amino acid (apart from tryptophan) its elution time was used to determine when the amino acids had fully eluted. Because phenylalanine is an aromatic compound, its elution from the column at about 10 min could be detected by absorbance at 214 nm. As there were no significant peptide peaks eluting prior to 17 min, the column eluate was collected for the first 17 min of each run to ensure that all amino acids were recovered. An aliquot (1 mL) of each amino acid sample was freeze dried, dissolved in 150 μL of 0.1M HCl and stored at -20°C until required for amino acid analysis.

The mobile phase gradient used to elute amino acids and peptides from the RP-HPLC column was as follows; 2% B to 15% B in 15 min; 15% B to 75% B in 5 min. Solvent A was 0.1% TFA in water; solvent B was 0.08% TFA in acetonitrile. The column equipment used was as described in Section 3.2.2.

8.4.1.3. Amino acid analysis

Amino acid analysis was done using pre-column, on-line derivatisation with an HP 1100 series HPLC, using an HP AminoQuant column at 40°C. The on-line derivatisation was performed using *ortho*-phthaldialdehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for the secondary

amino acids. The amino acid derivatives were detected by UV absorbance at 338 nm (10 nm slit width). UV detection had to be used rather than fluorescence detection in this study because fluorescence detection equipment was not available. Sixteen of the amino acids were detected in this way: aspartic acid, glutamic acid, serine, histidine, glycine, threonine, alanine, arginine, tyrosine, cystine, valine, methionine, phenylalanine, isoleucine, leucine and lysine. Proline was not determined since it could only be analysed using fluorescence detection.

8.4.2. Results and discussion

Figure 8.5 shows the total amino acid levels in EMCs digested for 24 h with the individual proteases and combinations of proteases. The combinations of Protease A/ Protease C, Protease A/ Protease DE, Protease B/ Protease C and Protease C/ Protease DE gave similar trends in that the total amino acids declined as the level of the second protease was increased. On the other hand, the combinations of Protease A/ Protease B and Protease B/ Protease DE gave a different trend in which the highest total amino acid level was given by a combination of proteases rather than an individual protease. This indicates a synergistic effect between the different enzyme preparations *i.e.*, the peptide products of one enzyme preparation were substrates for the exopeptidases present in the other enzyme preparation.

Figures 8.6 and 8.7 show the amino acid analysis for EMC digested for 24 h with combinations of Protease A/ Protease C and Protease B/ Protease DE, respectively. The data for the paired-combinations of the other commercial proteases are shown in Appendix 8.1. From Figure 8.6 it can be seen that the levels of almost all of the amino acids decreased as the level of Protease C was increased. Leucine and Lysine were the dominant amino acids produced from Protease A/Protease C combinations. Interestingly, the EMC base appeared to contain relatively high levels of glutamate. Figure 8.7 shows a different trend from that of Figure 8.6., *i.e.* in Figure 8.7, the level of each amino acid increase as the combination ratio approaches 40 % Protease DE/60% Protease B.

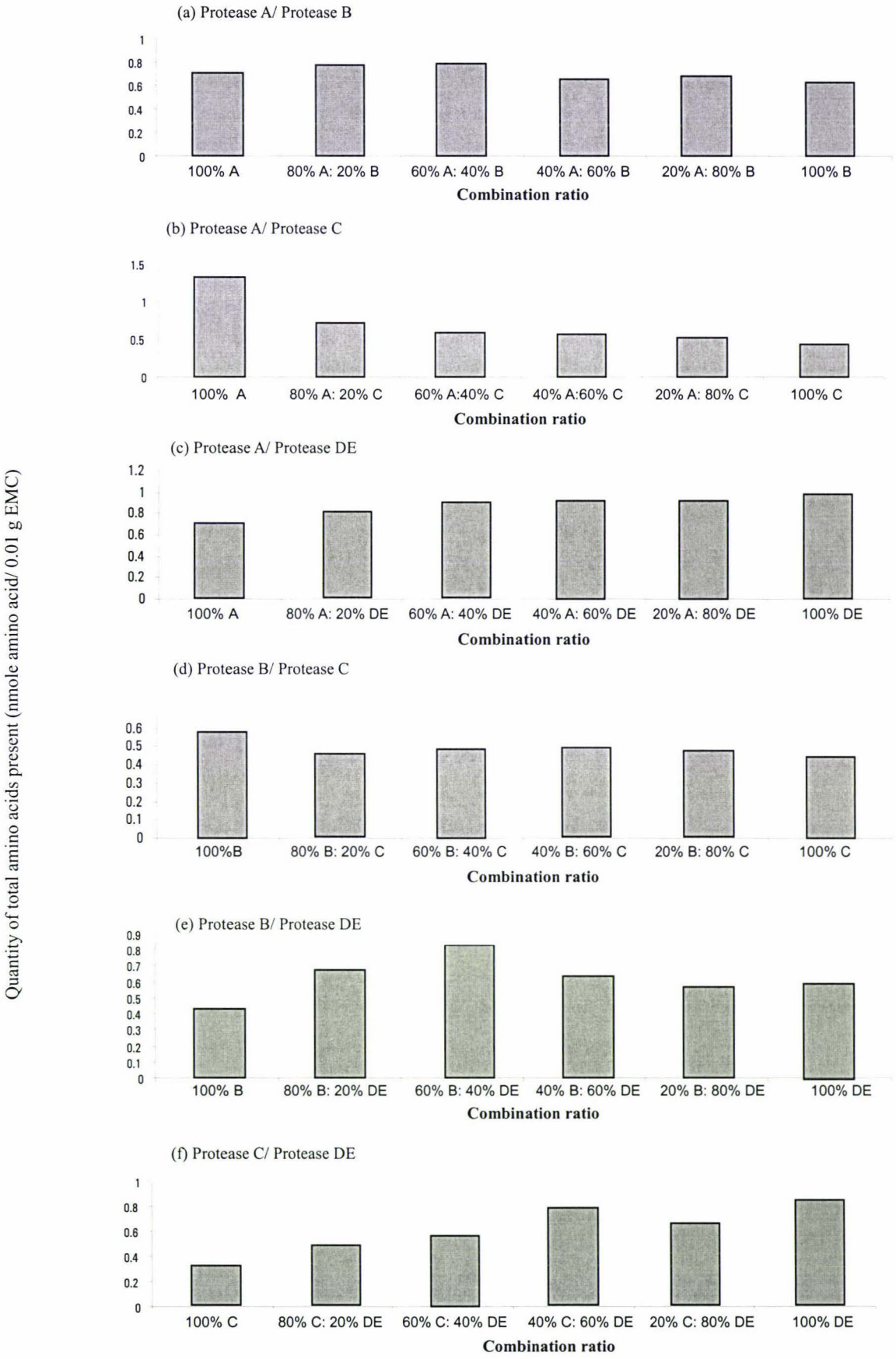


Figure 8.5. Total amino acids in 0.01 g of EMC bases digested for 24 h with different paired-combinations of commercial proteases

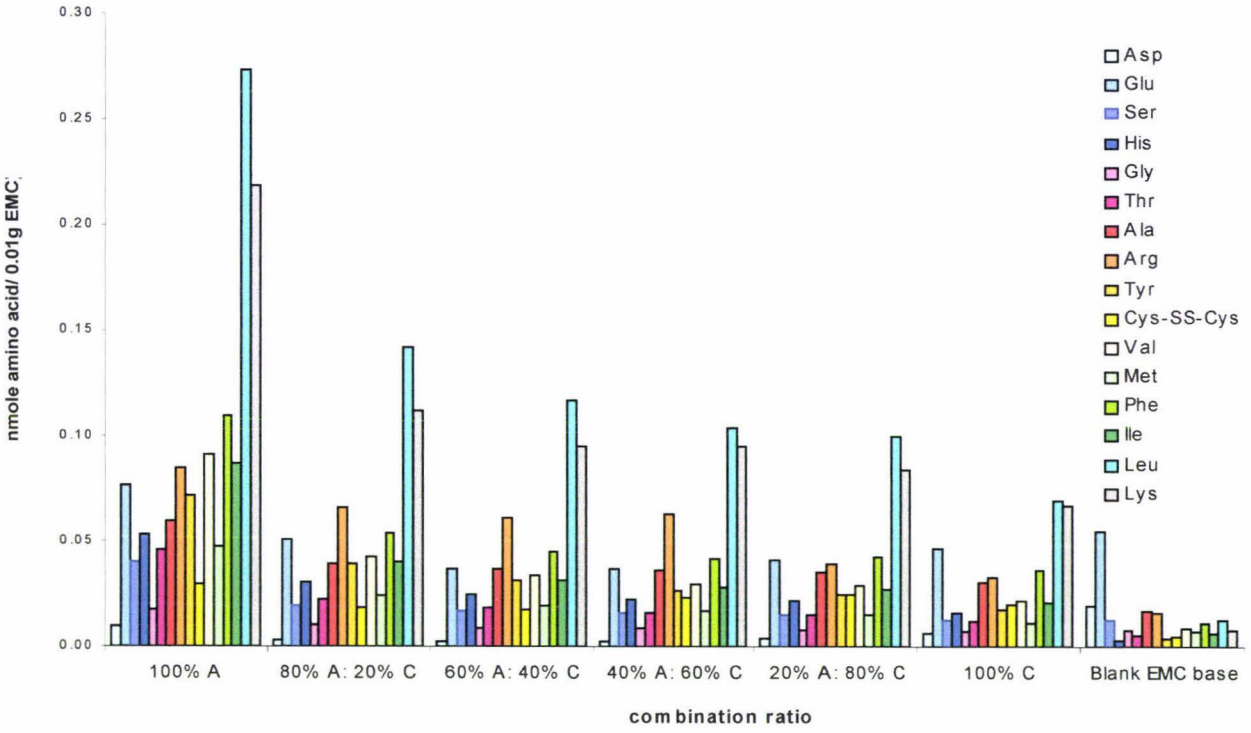


Figure 8.6. Amino acid analysis of EMC bases digested for 24 h with Protease A and Protease C. Note that the data for total levels are given in Figure 8.5b

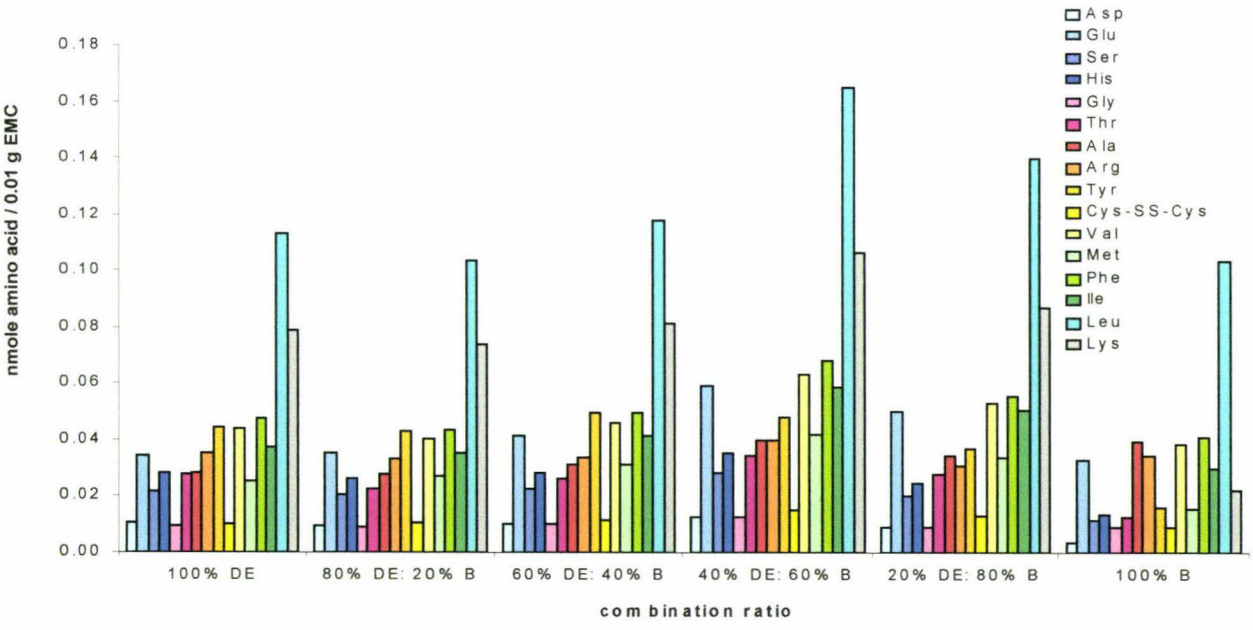
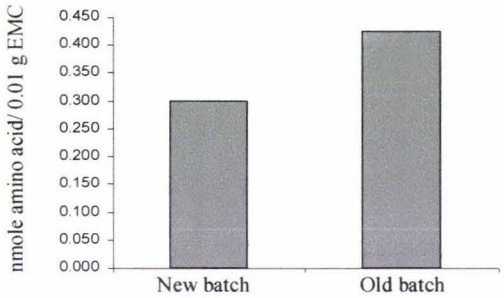


Figure 8.7. Amino acid analysis of EMC bases digested for 24 h with Protease B and Protease DE. Note that the data for total levels are given in Figure 8.5e

Figure 8.8a shows the comparison of the levels of amino acids given by the new and old batches of Protease B. The results indicated that the old batch of Protease B contained more exopeptidase activity than the new batch. Figure 8.8b shows that the new Protease B batch produced more arginine than the old batch, but overall the old batch of Protease B released more amino acids than new one, particularly glutamate and leucine.

(a)



(b)

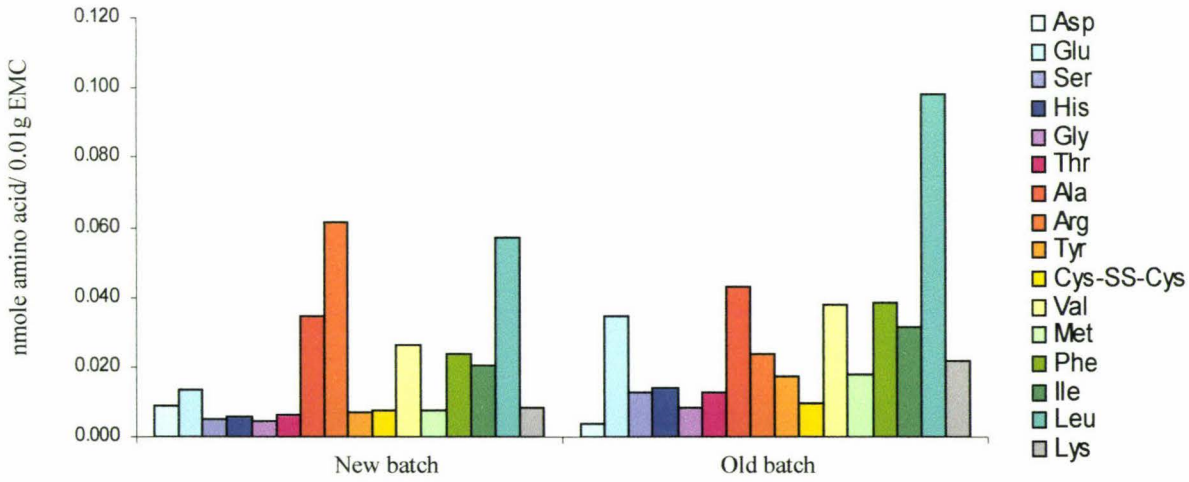


Figure 8.8. Comparison of amino acid levels given by different batches of Protease B. The total amino acid levels are given in (a), levels of individual amino acids in (b).

Figure 8.9 shows the amino acid analysis results for combinations of Protease D and Protease E. The results were consistent with the results obtained from SE-HPLC in that Protease D clearly contains much more exopeptidase activity than Protease E.

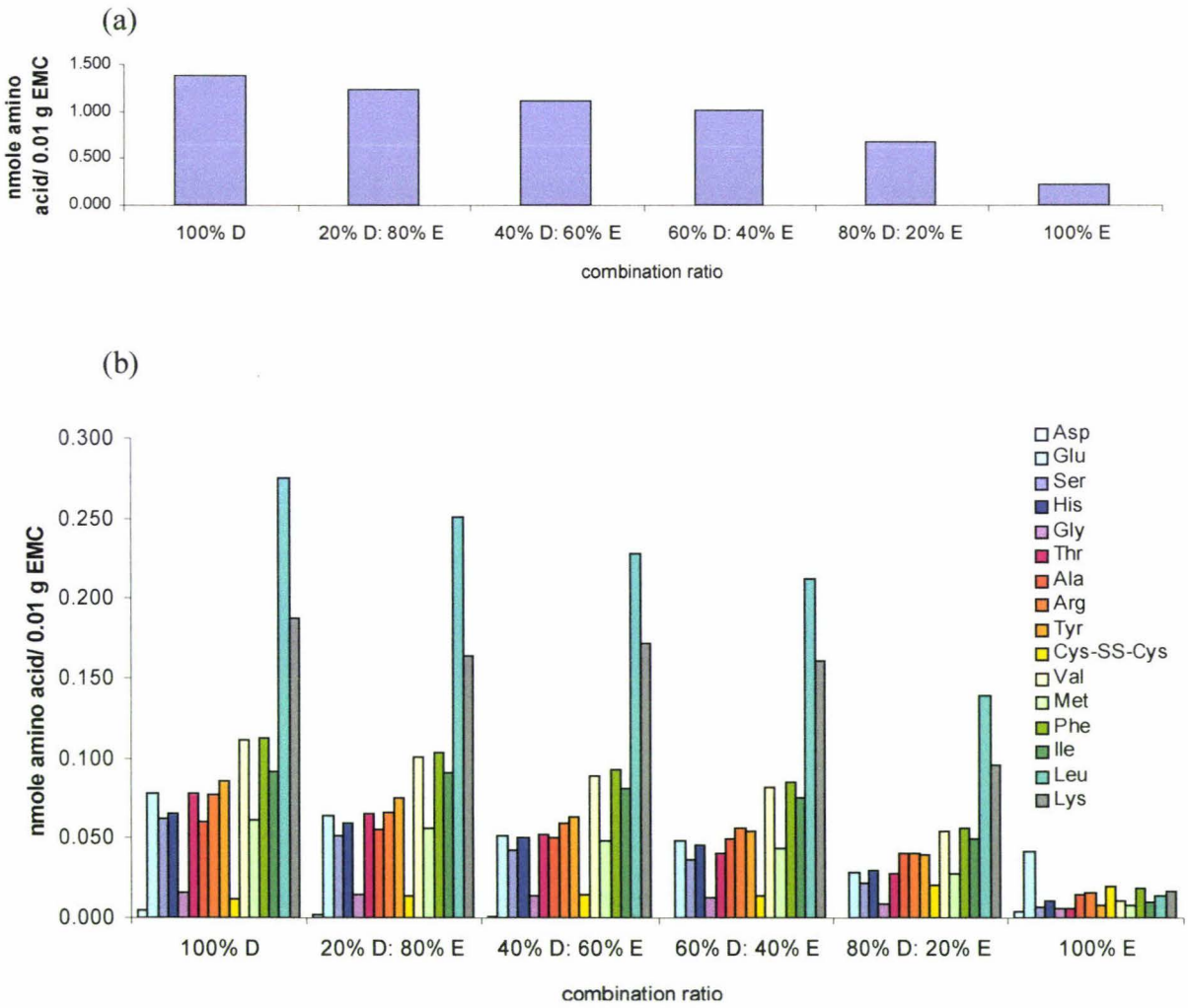


Figure 8.9. Amino acid analysis of EMC bases digested for 24 h with Protease D and Protease E. Total levels of amino acids are given in (a), the levels of individual amino acids in (b).

Table 8.2 shows the predicted flavour characteristics of EMC digested with combinations of Protease B and Protease DE. The figures for sweet, sour and bitter are calculated from the data obtained from amino acid analysis in the present study and the interpretations of amino acid flavour from Seitz (1990) and Vafiadis (1996). These authors suggested that the flavour of Ser, Thr, Gly, Ala, Lys and Pro can be associated with sweetness, His, Asp and Glu give sour flavour, while Arg, Met, Lys, Val, Leu, Pro, Phe, Tyr, Ile, Trp and Thr are bitter. The fact that some amino acids appear in both lists (*e.g.* Pro, Lys, Thr) is related to the perception of the flavour of the amino acids changing with concentration. The percentages in each column of Table 8.2 add up to > 100%, since different flavours can be associated with the same amino acid, *e.g.* Lys is both bitter and sweet (see Table 2.4) and therefore can contribute to both flavour classes.

Table 8.2. Predicted profile of amino acid flavours of EMC digested with Protease B and Protease DE combinations

Amino acid flavour \ *Combination ratio*	100%B[1]	80%B[1]:20%DE	60%B[1]:40%DE	40%B[1]:60%DE	20%B[1]:80%DE	100%DE[1]
Sweet (%)	21.98%	26.43%	26.73%	27.12%	27.78%	27.43%
(nmole/μL)	0.10	0.18	0.22	0.17	0.17	0.15
Sour(%)	11.58%	12.43%	12.95%	12.58%	12.32%	12.66%
(nmole/μL)	0.05	0.08	0.11	0.08	0.07	0.07
Bitter(%)	72.38%	76.21%	75.52%	75.46%	76.08%	75.21%
(nmole/μL)	0.31	0.52	0.63	0.48	0.45	0.42

[1] B: Protease B; DE: Protease DE

It should be noted that an issue about amino acid detection emerged in this study: the results of amino acid analyses in this chapter were not entirely reproducible. The results could be interpreted with only limited confidence, especially between analyses done on different days. It will be necessary to confirm the results using other methods at a later stage, *e.g.* amino acid analysis using ion-exchange chromatography with ninhydrin detection could be used. An advantage of this method is that all 20 of the amino acids can be detected.

The results from Table 8.2 suggest that Protease DE might be expected to produce more sweet and possibly more bitter flavour than Protease B. Following the same procedure, predicted flavour profiles of EMC digested with different combinations of commercial proteases can be calculated (see Appendix 8.2).

These results suggest that Protease DE might produce the most sweetness, followed by Protease A, then Protease C and Protease B the least. Protease B may give more sour flavour than the others. Protease A and DE may result in more bitterness than Protease B and C.

However, the protein-derived flavour of EMC depends not only on free amino acids but also peptides, particularly medium-sized peptides. The peptides might build flavour and also mask the flavour of free amino acids. In addition, the taste threshold concentrations for each component need to be considered in any flavour study. It is recommended that a further study should look at the influence of these factors on EMC flavour.

8.5. Conclusion

The proteolysis in EMC obtained by combining two proteases could be different from that obtained using the individual proteases. Thus adjusting the combination ratio of two (or more) proteases to produce different desirable flavours in EMC becomes an interesting possibility. For example, using Protease B in combination with Protease C to produce more small peptides might reduce the bitterness resulting from the use of Protease C alone. For further work, identification of peptides related to cheese flavour is required to facilitate prediction of which proteases or protease combinations produce desirable flavours in EMC.

Protease DE was the most active protease of the four commercial proteases with almost all the large components being digested after 24 h incubation. Protease DE is composed of Protease D and Protease E. The results indicated there were different exopeptidase and endopeptidase activities in Protease D and Protease E, also Protease D contained much more exopeptidase activity than Protease E.

The results also revealed differences between two batches of Protease B. Firstly, the proportions of individual amino acids released from the different batches were dissimilar, and secondly the older batch of Protease B seemed to produce more total amino acids than the other.

Chapter 9. Sensory impact of proteolysis in EMC produced with different combinations of commercial proteases

9.1. Introduction

The work in this chapter presents an initial study of the effect on flavour of using either individual or combinations of commercial proteases to make EMC. The sensory tests conducted in the study are preliminary work, only crudely examining cheese flavour and tentatively linking the results to the data described in the previous chapter. Thirteen ratios of the combinations described in the previous chapter were selected to carry out the digestion for sensory tests. To taste the EMCs an informal sensory panel of experienced dairy researchers was used. These researchers were experienced in cheese tasting using attribute analysis.

9.2. Methods

Thirteen combinations of proteases were selected as shown in Table 9.1.

Table 9.1. Combinations of proteases selected for generation of sensory samples

Protease A 100%	Protease B[1] 60%: Protease DE 40%
Protease A 60%: Protease B 40%	Protease B[2] 40%: Protease C 60%
Protease A 60%: Protease C 40%	Protease C 100%
Protease A 60%: Protease DE 40%	Protease C 60%: Protease DE 40%
Protease A 40%: Protease C 60%	Protease C 40%: Protease DE 60%
Protease B[1] 100%	Protease DE 100%
Protease B[2] 100%	

[1] Old Protease B batch.

[2] New Protease B batch.

9.2.1. Preparation of EMC

A total enzyme concentration of 0.15% (w/v) for individual enzymes and two-enzyme combinations shown in Table 9.1 was used in EMC digestions at 40°C

for 24 h. Enzymes were inactivated by heating the EMCs at 95°C for 30 minutes. Each EMC was diluted to 5%, 10% and 25% with a bland-tasting white sauce for sensory tests. Undiluted EMC was also available for the tests.

9.2.2. Preparation of white sauce

The white sauce base was previously developed to evaluate cheese products with minimal flavour contribution by the base itself (Blair, 1997). The dry white sauce base was prepared by combining 500 g of cream powder 55 (CP55), 120 g of Elfin high-grade flour and 5 g of salt. For the liquid preparation of white sauce, 125 g of well-mixed dry white sauce base was blended with 450 g of warm tap water with a Bamix blender. The mixture was heated in a microwave on high power for 1 min and then stirred with a Bamix blender. The heating and stirring steps were repeated a further 5 times. Care was required when the mixture was heated to avoid an overflow of sauce from the container.

9.2.3. Preparation of EMC flavour samples

EMC samples were diluted to 5%, 10% and 25% (w/v) by adding 4, 8 and 20 g of inactivated EMC into three labelled bottles and making up to 80 g with white sauce. Each diluted sample was stirred thoroughly with a disposable plastic teaspoon. Note that the samples were prepared and stored at 4°C one day before tasting to allow equilibration between EMC flavours and white sauce base. The samples were reheated in the microwave before tasting by heating gently on 100% power in increments of 30 seconds. Typically 2 minutes was required. This was to ensure that the samples were at ambient temperature before tasting.

The samples from each of the 13 enzyme combinations were labelled from "1" to "13". The diluted EMC samples comprising 5%, 10%, 25% and 100% EMC (undiluted) (w/w) were labelled W, X, Y and Z, respectively. For example, the samples labelled 1W, 1X, 1Y and 1Z corresponded to the 5%, 10%, 25% (w/w) dilution and undiluted EMC sample 1, respectively.

9.3. Results and discussion

Table 9.2 shows the results of the sensory testing, the figures of 5%, 10% and 25% referring to the dilution of EMC in the white sauce.

Table 9.2. Sensory results for EMCs made with different commercial proteases or combinations of proteases.

EMC samples	Flavour descriptions
A[1] 100%	5%[2]: sweet; 25%: bitter
A 60%: B 40%	10%: sweet; 25%: bitter
A 60%: C 40%	10%: savoury; 25%: bitter and pungent
A 60%: DE 40%	5%: sweet; 25%: bitter
A 40%: C 60%	10%: bitter but cheesy
B 100%	10%: savoury, not bitter
B* 100%	10%: sweet and cheesy
B 60%: DE 40%	10%: bitter with mushroom flavour, but cheesy
B* 40%: C 60%	25%: bitter
C 100%	bitter and unpleasant
C 60%: DE 40%	25%: bitter, chemical and astringent flavour
C 40%: DE 60%	25%: bitter but cheesy
DE 100%	25%: pungent, astringent and bitter

[1] A: Protease A, B: Protease B, C: Protease C, DE: Protease DE, B*: new batch of Protease B
[2] 5%, 10%, 25% concentration of EMC in the white sauce.

Individually the enzymes gave different flavours in EMC, and the perception of taste partially depended on the concentration of EMC in the white sauce. EMC made with Protease A and incorporated with the white sauce at 5% gave a sweet flavour, but at higher levels the flavour became bitter. EMC made with Protease C alone seemed to have an unpleasant flavour and this might result from the greater proportion of medium sized peptides. EMC made with Protease B alone (either the new batch or the old batch) gave a savoury cheesy perception and no bitterness. This correlated with the results of HPLC and amino acid analysis where Protease B was shown to give least mid-sized peptides but high levels of amino acids. On the other hand, EMC made with Protease DE had been shown to have undergone the

most proteolysis, but its taste was pungent, astringent and bitter.

Protease A and Protease B in combination gave a sweet flavour when the blend concentration was low (10%), but the bitter flavours dominated when the concentration of EMC in the white sauce was increased to 25%. Similarly, EMC made with a combination of 40% Protease C / 60% Protease A tasted savoury at a concentration of 10% in the white sauce, but at a concentration of 25% the taste was pungent, whereas EMC made with a combination of 60% Protease C / 40% Protease A gave bitter flavour at a concentration of 10% in the white sauce. This implies that a low Protease C / high Protease A combination would produce EMC that gives a savoury flavour when only used in small amounts. Protease B / Protease C and Protease B / Protease DE combinations all gave bitter flavoured EMCs, but some cheesy flavours were found. Both Protease C and Protease DE produced bitter flavours when used individually, but a combination of 40% Protease C / 60% Protease DE gave a more cheesy flavour (with bitterness) and it tasted better than the combination of 60% Protease C / 40% Protease DE. The results showed that EMC flavour could be manipulated by changing both enzyme type and enzyme ratios in the protease combinations.

The sensory results were consistent with the results obtained from amino acid analysis of flavour characteristic profiles. Both results show Protease A producing more sweetness than the other enzymes, Protease DE and Protease C producing bitter, pungent and astringent flavours and Protease B giving the least bitterness.

9.4. Conclusion

Different levels and combinations of the different enzymes resulted in various flavours and the results imply that it may be possible to manipulate desirable cheese flavour for the food industry. In sensory tests, Protease B seemed to give more savoury flavour than the other enzymes and in combination with one of the three other enzymes tended to give more cheesy flavours than the enzymes individually. The combination of Protease C with three other commercial enzymes tended to give more bitterness than other combinations, while Protease DE tended to give a strong pungent flavour.

Chapter 10. Conclusions and Recommendations

Different assay methods and assay kits were evaluated for detecting protease activities in four commercial enzyme preparations. The Bioquant kit was estimated to be 10 times more sensitive than FITC-β-casein fluorescence measurement in detecting residual protease activity in buffer systems, but lack of reproducibility in the EMC systems was a major disadvantage. Ultimately SE-HPLC was evaluated as the most useful and sensitive method to assay residual protease activity in EMC. It was also found that the chromatograms obtained from SE-HPLC were suitable for quantifying the molecular mass distribution of peptides in EMCs digested with the different proteases. Reviewing the literature, PAGE, especially SDS-PAGE, was viewed as the standard technique for proteolysis analysis in general biochemistry (McSweeney, 1996). However, PAGE was found to be unsuitable for this study. Gölker (1990), described gel filtration (SE-HPLC) allowing the separation of molecules based on their molecular size and shape. In this study, SE-HPLC was employed as the method to determine proteolysis of EMC digested with different commercial proteases. RP-HPLC would be more suitable for study of flavours of individual peptides and was useful for obtaining amino acid fractions from the EMCs for amino acid analysis.

Enzyme inactivation is a critical feature in EMC manufacture; incorrect conditions will fail to inactivate the enzymes fully and will make it difficult to reproducibly obtain and maintain the desirable flavours in EMC. However, there are no investigations of residual enzyme activity in EMC to be found in the literature. In this study, after estimating the residual enzyme activity under different inactivation conditions and considering the maximum temperature allowable in the EMC process without destroying cheese flavour, it was decided to use inactivation conditions of 95°C heating for 30 minutes. Residual protease activities in EMC using a 55% moisture cheese base were detected as 3.5% for Protease A, 5.9% for Protease B, 4.9% for Protease C and 2.6% for Protease DE after this treatment. Using an initial enzyme concentration of 0.15% (w/v), this implied that the actual levels of four commercial proteases remaining after inactivation were 0.005%, 0.009%, 0.007%

and 0.004% (w/v) for Protease A, Protease B, Protease C and Protease DE, respectively. Although these levels of enzyme activities remaining in EMC after inactivation at 95°C for 30 minutes are very low, it is not known yet what affect the levels have in flavour (or functionality) of the final products. The moisture level used for commercial EMC is lower than 55% (which was used in this project for practical reasons), therefore, the protease in a commercial preparation of EMC would be more stable but less active. On reconstitution of the EMC with a more liquid product, however, this would mean that a higher level of enzyme activity would be carried over. It is recommended that further study should be conducted to analyze the influence of such low residual enzyme levels on EMC products.

EMC digestion with a combination of proteases is different from that obtained with individual proteases. In some cases, a synergistic effect resulted in more products produced by proteases in combination than when used individually. The results of this study implied that with the combinations of Protease A/Protease C, Protease DE/Protease C, Protease B/Protease C and Protease DE/Protease A, the higher the proportions of the former protease in the combinations, the higher the levels of total amino acids produced in the EMC. In addition, the combinations of Protease A/Protease B and Protease B/Protease DE showed greater levels of total amino acids when the ratio of each enzyme was approximately 50:50 than when the enzymes were used individually. The method for amino acid analysis was using OPA and FMOC which had been employed successfully by Lemieux (1990) and Church (1983) to estimate proteolysis and amino acids in dairy products.

Molecular mass profiles appeared to be a very useful way to present proteolysis of EMC digestion. The profiles are readily obtained by using standard protein and peptide solutions to obtain a molecular mass calibration curve for the size exclusion HPLC column and then integration of the chromatograms within appropriate windows of molecular mass distribution. SE-HPLC chromatograms showed that Protease DE was a stronger protease than the other enzymes and Protease B was the weakest protease. With respect to the molecular mass distribution of peptides in the various EMC digestions, Protease DE produced the highest quantities of peptides of three or less amino acid residues, Protease C gave the highest quantities of more medium sized peptides with 11-20 residues, Protease B gave the lowest levels of

medium and small peptides, yet gave just as high a level of free amino acids and Protease A was more efficient in giving small peptides than Protease C.

The proteolysis profiles may offer the dairy industry a rough map to produce desirable flavours if a correlation between the peptide profiles and amino acid profiles can be obtained. Some initial data on the correlation between the action of the four commercial proteases and cheese flavour development were obtained in this study. From the results of sensory tasting, Protease DE gave a strong pungent and astringent flavour, Protease C tended to give bitterness, Protease A produces EMC that gives a sweet flavour at a low concentration but bitter flavours with a high concentration. Protease B produced more savoury flavour without bitterness. The results of sensory tests have similarities to flavour characteristic results obtained using amino acid analysis that suggested that Protease DE and Protease C released more sour and bitter amino acids, Protease A produced more sweet amino acids and Protease B gave less bitter amino acids. However, the reproducibility of the results from amino acid analysis was an issue. It is recommended that a method that provides more confidence in amino acid detection is required for further study. The overall protein-derived flavour is not only due to free amino acids but also to di- and tripeptides and medium sized peptides. The literature describes the production of bitterness as a major problem associated with most commercial proteases (Kilcawley *et al.*, 1998; Anon, 1989) and how to reduce bitterness by adding peptidase (Cliffe and Law, 1990; Kristoffersen *et al.* 1967) is an important issue. Critical future work would be required to identify individual flavour peptides in EMC products (by RP-HPLC and mass spectrometry), and to identify the protease combinations giving these cheese flavours during EMC production in order to manipulate cheese flavour effectively and predictably.

During this study, it was revealed serendipitously that two batches of Protease B gave different results. One batch of Protease B seemed to produce more total amino acids than the other, but both batches of Protease B gave EMCs that tasted pleasant. This finding indicates that, as EMC production gets more sophisticated and targeted towards predicted flavours, so batch-to-batch variations between commercial protease preparations will become more important. This means that good comparative analysis of the protease batches and an understanding of the impact of

such variation on EMC production will be required.

Proteolysis is considered the most complicated and perhaps the most important biochemical issue during cheese ripening (Fox, 1989a, 1993; Wilkinson, 1993; Kilcawley *et al.,* 1998). From this study, basic proteolysis and amino acid analysis profiles of EMCs made with the four commercial proteases individually and in combination were achieved. This could provide EMC producers a background profile for flavour manipulation with different ratios of protease combinations. In further work, three or four combinations of proteases with different ratios may be required to give appropriate EMC flavour production. Also, the influence of enzyme concentration addition and environmental pH value on the proteolysis and flavour development of EMC are interesting issues and should be conducted in further studies.

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Appendix 3.1 Method for FITC-β-casein preparation

1. Materials:

β-casein (C-6905) and Fluorescein isothiocyanate (FITC) (F-7250) were from Sigma Chemical Company, St. Louis, USA.

Sodium Carbonate, $NaCl{\cdot}2H_2O$ and Tris base (T-1503) were from Boehringer Mannheim, GmbH, Germany.

2. Methods:

0.2 g of β-casein and 0.005 g of FITC were dissolved in 20 mL and 0.5 mL of sodium carbonate NaCl buffer, respectively, and then these two solutions were mixed together and stirred at room temperature in the dark for 2 h. Sodium carbonate-NaCl buffer was made by dissolving 5.3 g of Na_2CO_3 and 22.05 g of $NaCl \cdot 2H_2O$ in Milli-Q water to final volume of 50 mL and using either NaOH or HCl to adjust pH to 9.5. The FTIC-β-casein was first dialysed at 4°C against Milli-Q water containing 1 g decolorising charcoal per litre, then against two 2 L changes of 50 mM Tris-HCl, pH 8.5 and finally against two 2 L changes of 50 mM Tris-HCl, pH 7.2 (12 h per each 2 L volume). The solution was stored at -20°C, protected from light (aluminium foil).

Appendix 3.2 Microbial test results of EMC base prior to incubation

Sample	[c] Anaerobic cfu/g	[d] APC cfu/g	[e] Coliform cfu/g	[f] SRC cfu/g	[g] Y & M cfu/g
[a] 55% EMC base	[h] ND	ND	ND	<1	--
[a] 65% EMC base	ND	ND	ND	27	--
[b] 55% EMC base	<10	<10	<1	<1	<1
[b] 65% EMC base	16	6	<1	4	<1

[a] Samples taken immediately following the first heat step (95°C) of EMC base manufacturing procedure (Section 3.4.1.2.)
[b] Samples taken immediately following the second heat step (86.5°C) of EMC base manufacturing procedure (Section 3.4.1.2.)
[c] Anaerobic count DRCA (Section 3.4.5.2.)
[d] Aerobic plate count (Section 3.4.5.3.)
[e] Coliform VRBA powder (Section 3.4.5.5.)
[f] Sulphite reducing clostridia (Section 3.4.5.1.)
[g] Yeast and moulds (Section 3.4.5.4)
[h] not determined

Appendix 3.3 Microbial test results after EMC incubation for 48 h at 40°C

Sample	moisture	[c] Anaero cfu/g	[d] APC cfu/g	[e] Coliform cfu/g	[f] SRC cfu/g	[g] Y & M cfu/g
[a] Blank EMC	55%	<10	<10	<1	<10	<1
[a] Blank EMC	65%	$>10^5$	$>10^6$	<1	<10	<1
[b] Protease B EMC	55%	$>10^6$	$> 3.3 \times 10^4$	<1	<1	<1
[b] Protease B EMC	65%	$>10^5$	$>10^6$	<1	<1	<1
[b] Protease A EMC	55%	30	20	<1	<1	<1
[b] Protease A EMC	65%	$> 3.1 \times 10^3$	$>10^5$	<1	<10	<1
[b] Protease C EMC	55%	10	20	<1	<1	1
[b] Protease C EMC	65%	$> 3.1 \times 10^3$	$>3.0 \times 10^4$	<1	<10	<1
[b] Protease DE EMC	55%	30	10	<1	<1	<1
[b] Protease DE EMC	65%	$>10^5$	$>10^5$	<1	<10	<1

[a] No enzyme addition to EMC base.
[b] Enzyme addition rate was 0.15% (w/v).
[c] Anaerobic count DRCA (Section 3.4.5.2.)
[d] Aerobic plate count (Section 3.4.5.3.)
[e] Coliform VRBA powder (Section 3.4.5.5.)
[f] Sulphite reducing clostridia (Section 3.4.5.1.)
[g] Yeast and moulds (Section 3.4.5.4)

Appendix 3.4 Chemical composition results of EMC base

Sample	[a] Moisture (% w/w)	pH	[b] Salt (% wt/wt)	[c] Fat (% wt/wt)
EMC base (55% moisture)	57.3	5.81	0.93	23.0
EMC base (65% moisture)	67.1	6.00	0.73	17.8

[a] Moisture in cheese, 16 hours at 105°C.
[b] Salt using autotitrator
[c] Fat by Werner Schmit

Appendix 4.1 The results of commercial protease assays using the BODIPY kits

1. Protease DE protease assay with BODIPY kit buffer (Tris-HCl, pH 7.8), excitation: 590 nm, emission: 645 nm, slit width 5 nm.

concentration / incubation time	Control	0.001 g/100 mL	0.01 g/100 mL	0.1 g/100 mL	1 g/100 mL
0 minute	0.6 [1]	0.6	1.2	2.5	7.2
30 minutes	1.3	1.8	6.1	21.0	40.2
1 hour	1.8	3.9	10.2	27.1	50.2
1.5 hours	1.6	6.7	15.0	33.0	60.0
2 hours	3.3	10.2	20.3	36.2	68.0
Overnight	48.5	70.8	87.0	97.6	99.3

[1] Results given in RFU/min.

2. Protease DE protease assay with BTP buffer pH 6.5, excitation: 580 nm, emission: 625 nm, slit width 5 nm.

concentration / incubation time	control	0.001 g/100 mL	0.01 g/100 mL	0.1 g/100 mL[3]	0.1 g/100 mL[4]	1 g/100 mL
0 minute	2.1 [1]	ND [2]	ND	ND	ND	ND
30 mins	4.1	5.5	8.9	28.7	141.7	109.9
60 mins	5.1	8.2	15.5	45.5	211.9	177.1
105 mins	6.9	12.2	20.3	64.3	268.8	235.3
115 mins	7.7	15.3	25.1	77.1	293.2	280.2
Overnight	10.8	47.3	32.2	179.1	527.7	437.9

[1] Results given in RFU/min.
[2] ND, No determining.
[3] add 1 mL 0.1 g/100 mL concentration enzyme.
[4] dilute from 1 g/100 mL concentration enzyme

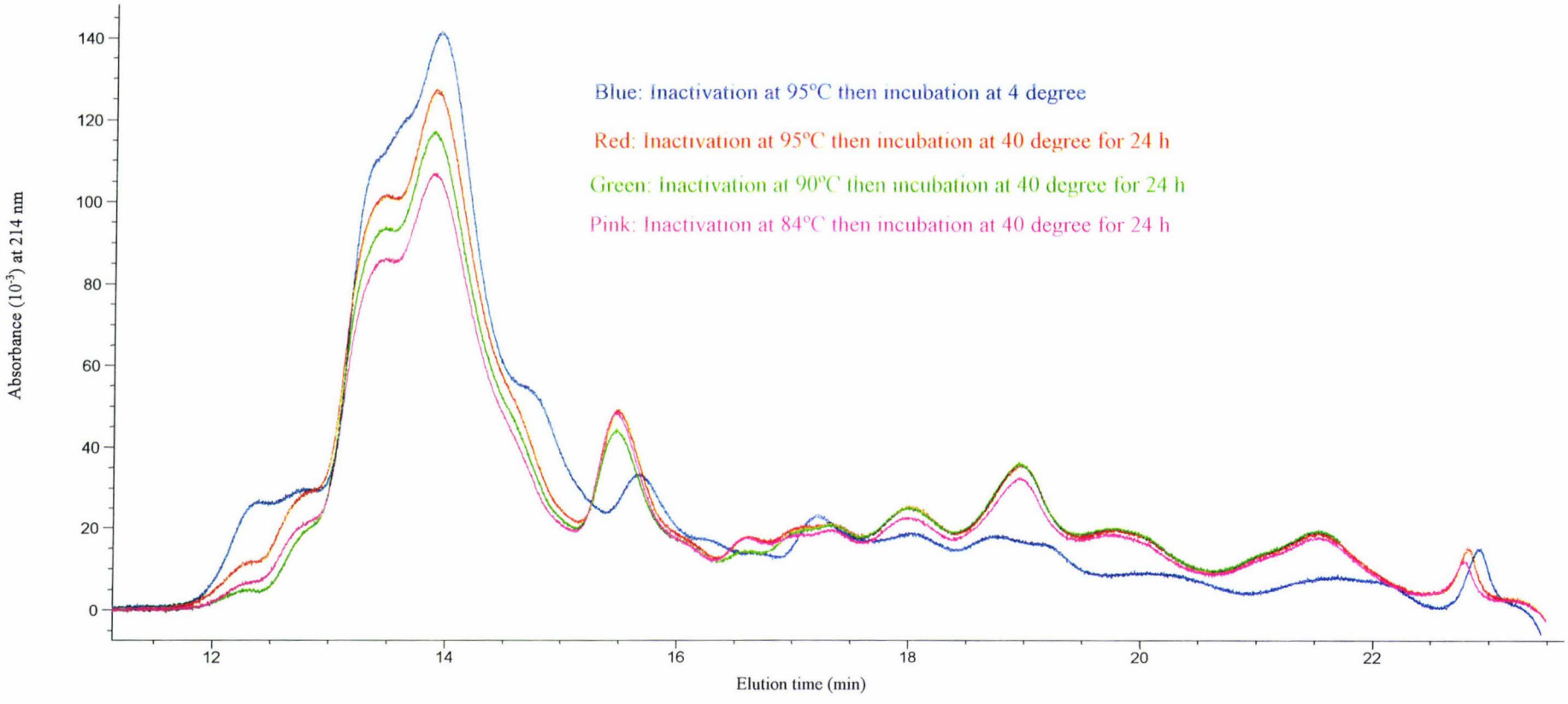
3. Protease A protease assay with BTP buffer pH 6.5.

concentration / incubation time	control	0.001 g/100 mL	0.01 g/100 mL	0.1 g/100 mL[3]	0.1 g/100 mL[4]	1 g/100 mL
0 min	0.12[1]	ND [2]	ND	ND	ND	ND
30 mins	0.93	4.0	5.4	13.4	59.2	50.0
75 mins	1.18	4.3	6.3	11.0	82.2	62.5
90 mins	2.1	4.3	5.9	5.6	60.3	53.2
120 mins	1.3	3.0	3.3	3.0	38.6	39.5
Overnight	1.2	8.5	8.2	6.3	24.7	27.5

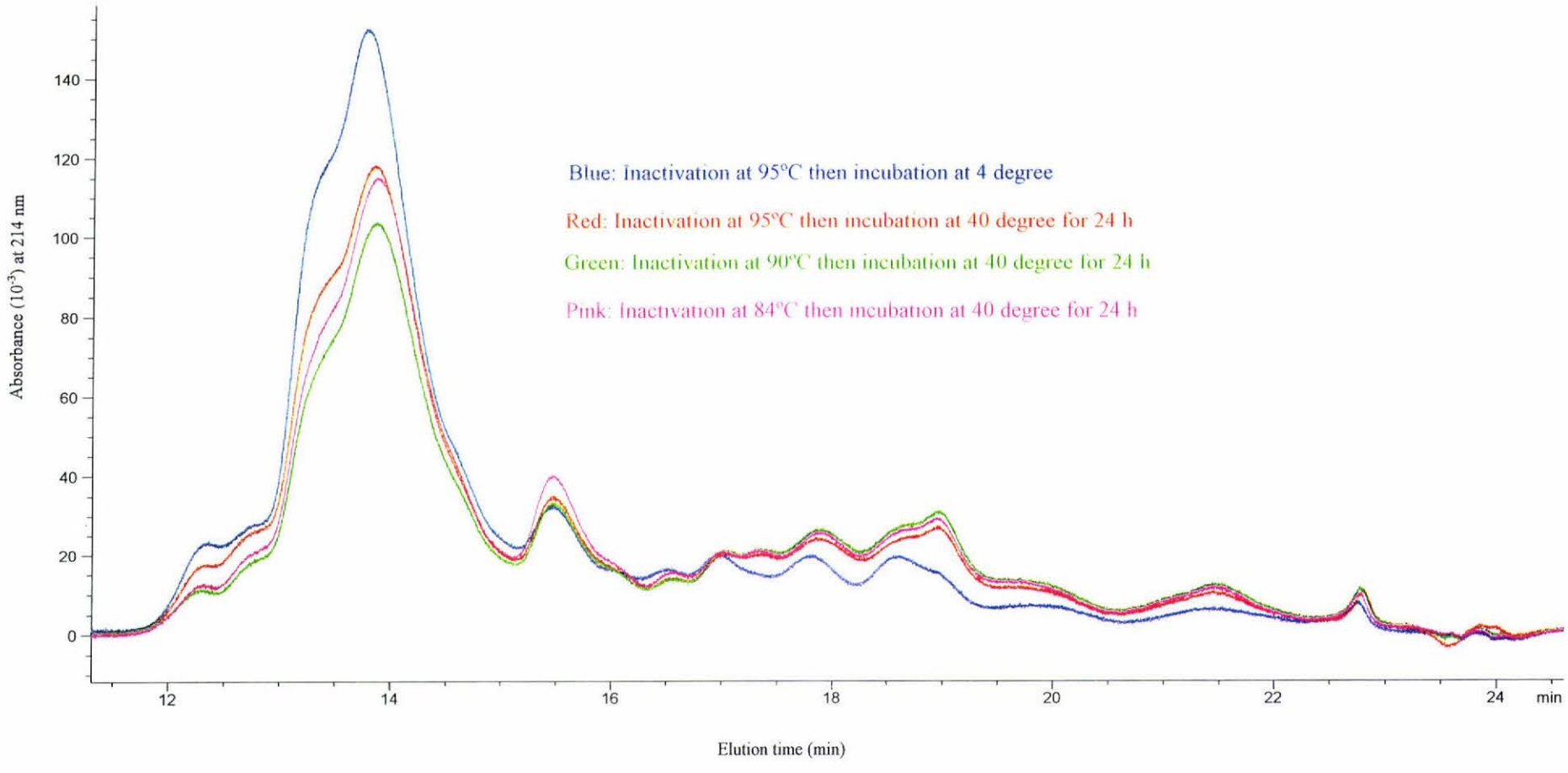
[1] Results given in RFU/min.
[2] ND, No determining.
[3] add 1 mL 0.1 g/100 mL concentration enzyme.
[4] dilute from 1 g/100 mL concentration enzyme

Appendix 6.1. Comparison of Protease A, Protease C and Protease DE inactivation in EMC at various temperatures

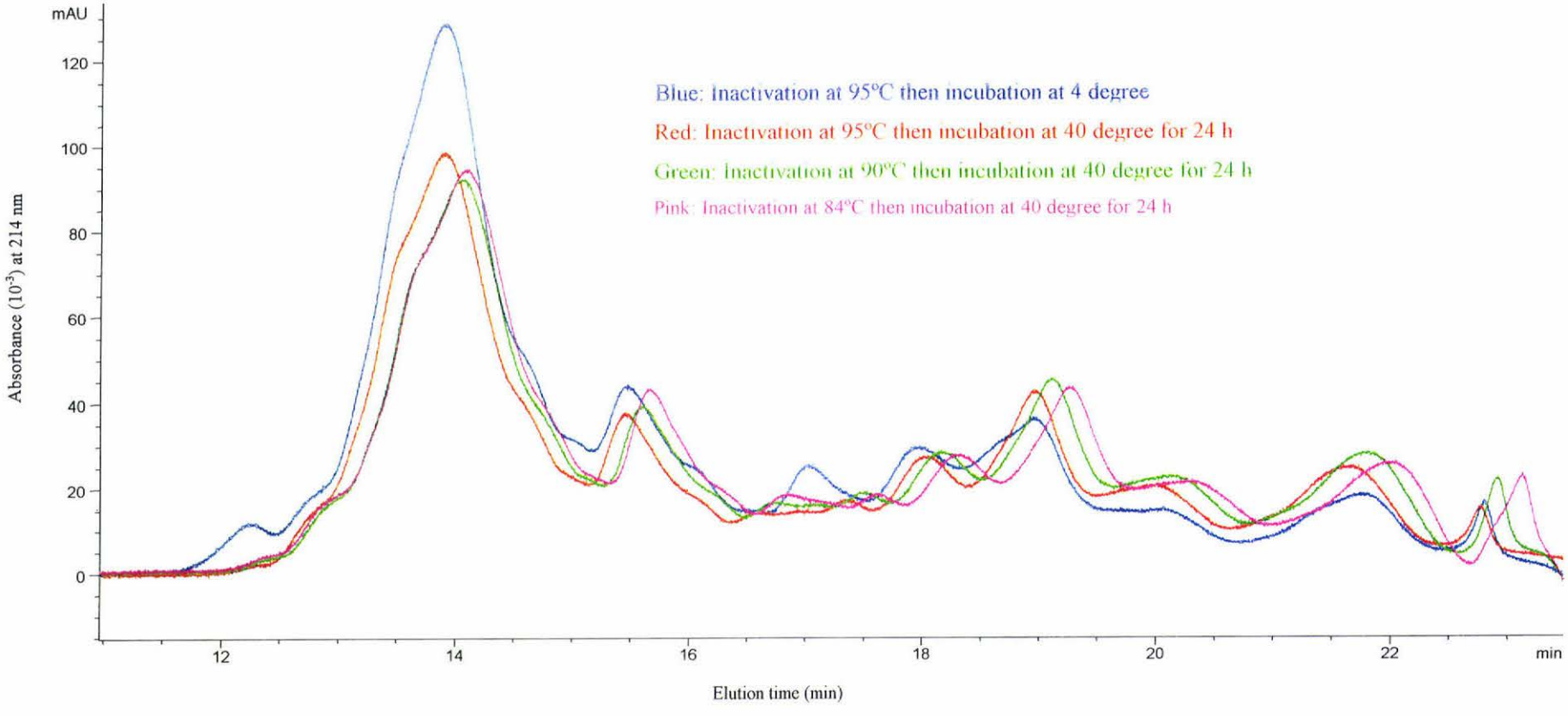
(a) Protease A



(b) Protease C

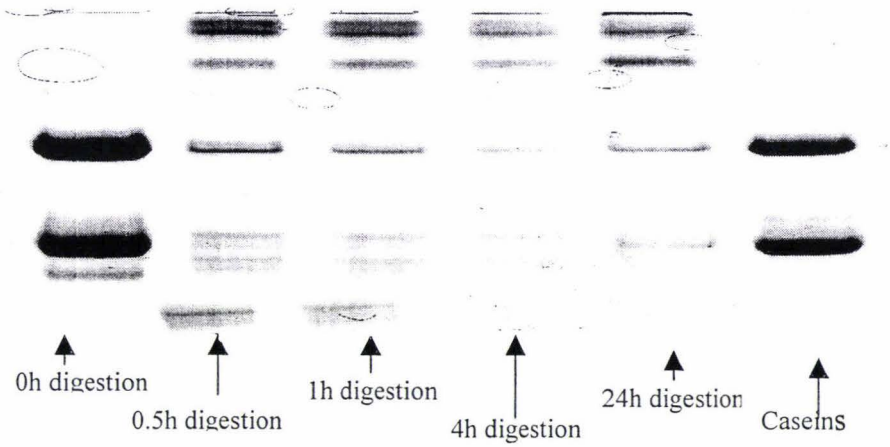

Blue: Inactivation at 95°C then incubation at 4 degree
Red: Inactivation at 95°C then incubation at 40 degree for 24 h
Green: Inactivation at 90°C then incubation at 40 degree for 24 h
Pink: Inactivation at 84°C then incubation at 40 degree for 24 h
Absorbance (10^{-3}) at 214 nm
0
20
40
60
80
100
120
140
12
14
16
18
20
22
24
min
Elution time (min)

(c) Protease DE

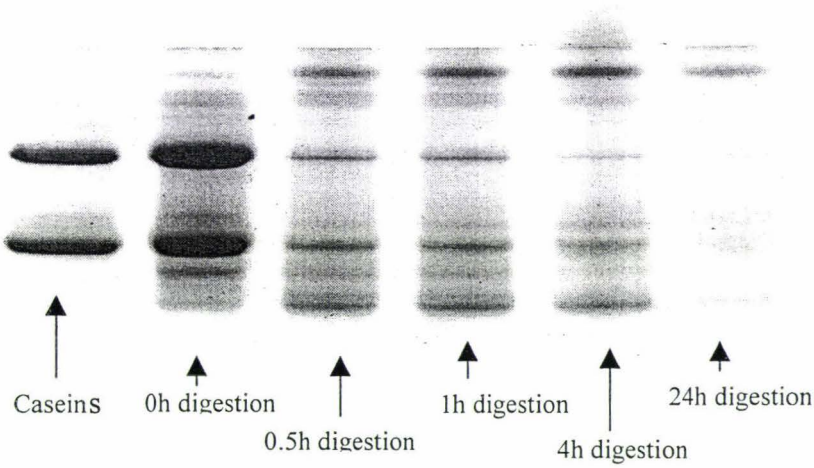


Appendix 7.1 Alkaline urea PAGE results of EMC samples

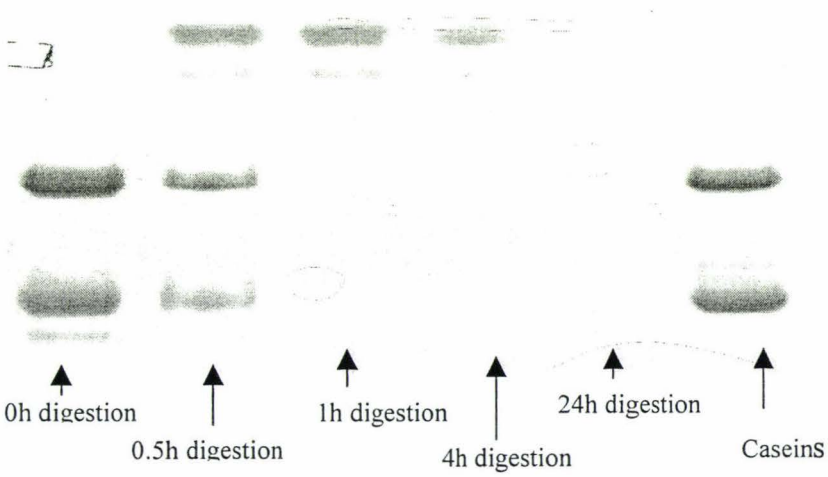
(a) EMC digested with Protease A



(b) EMC digested with Protease C

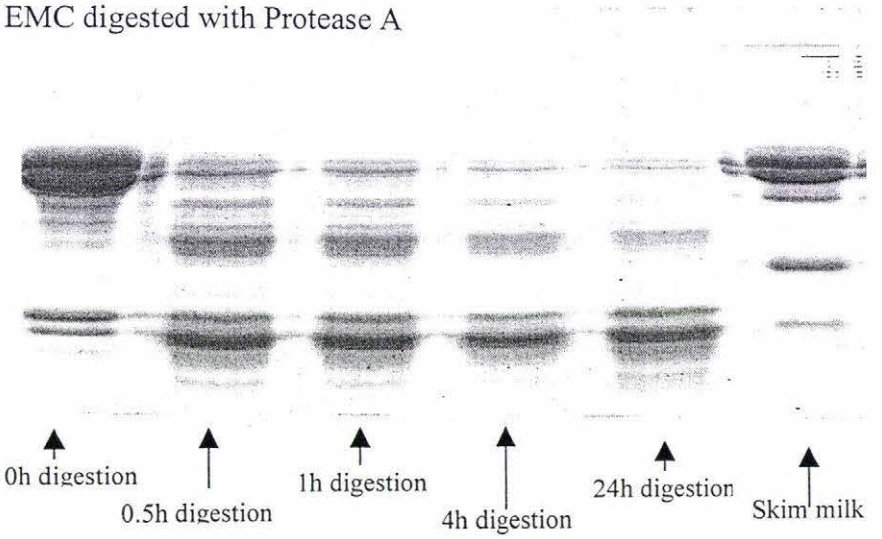


(c) EMC digested with Protease DE

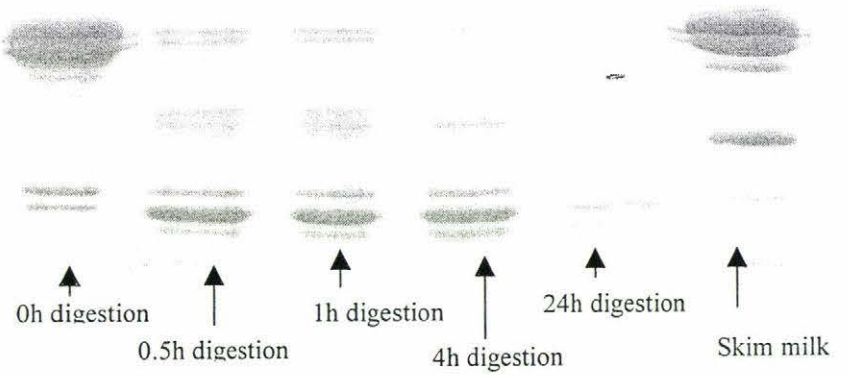


Appendix 7.2 SDS PAGE results of EMC samples

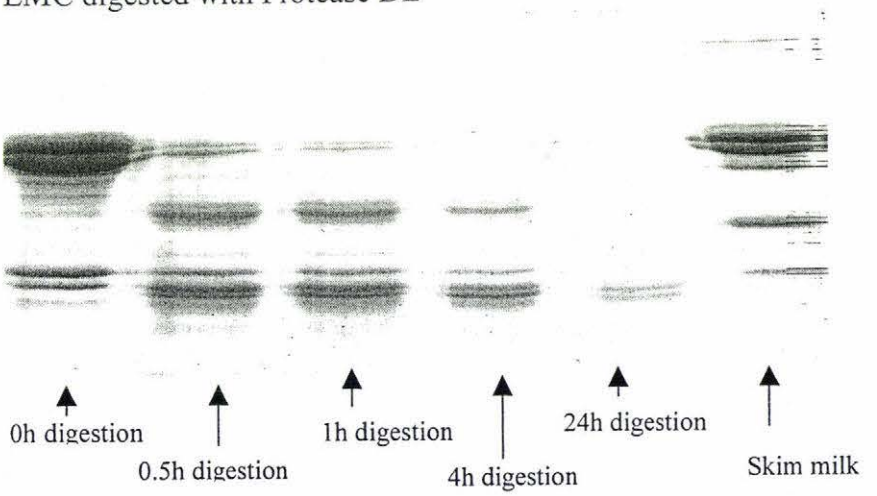
(a) EMC digested with Protease A



(b) EMC digested with Protease C

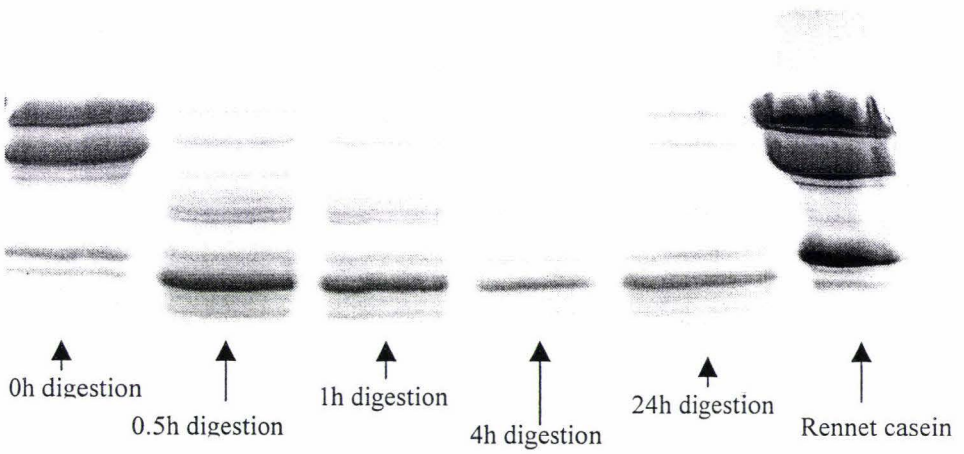


(c) EMC digested with Protease DE

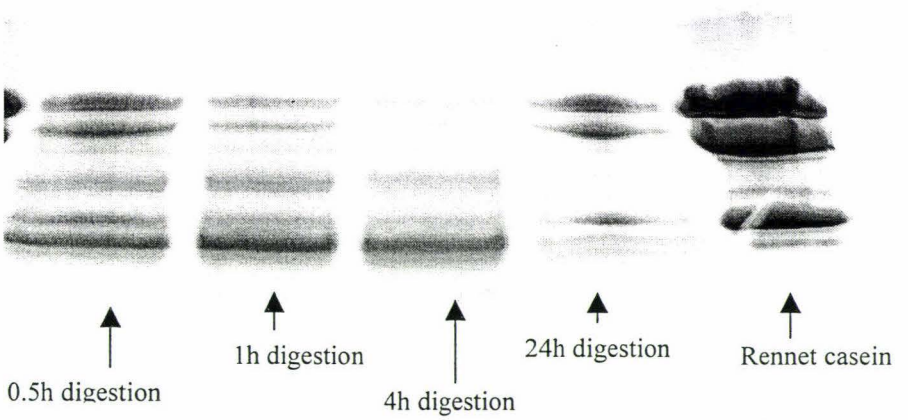


Appendix 7.3 Low molecular mass SDS PAGE results of EMC samples

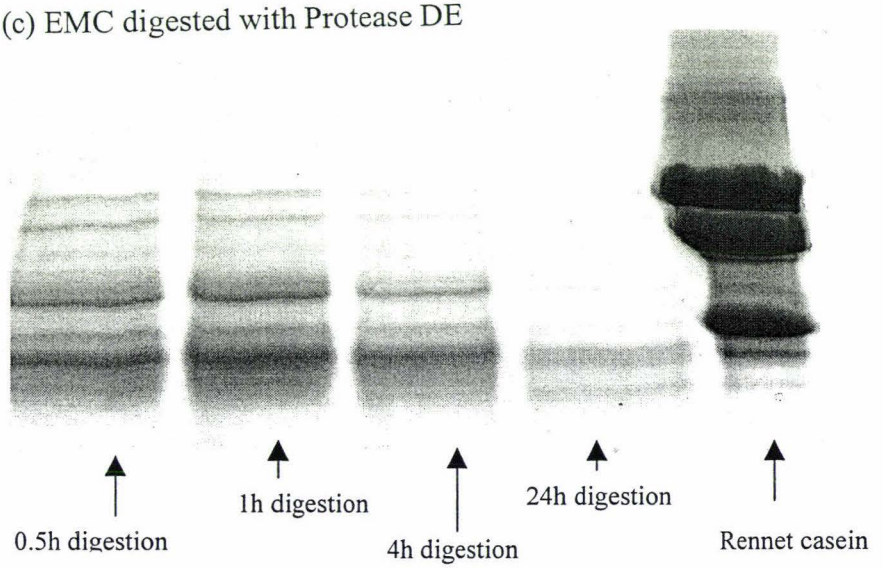
(a) EMC digested with Protease A



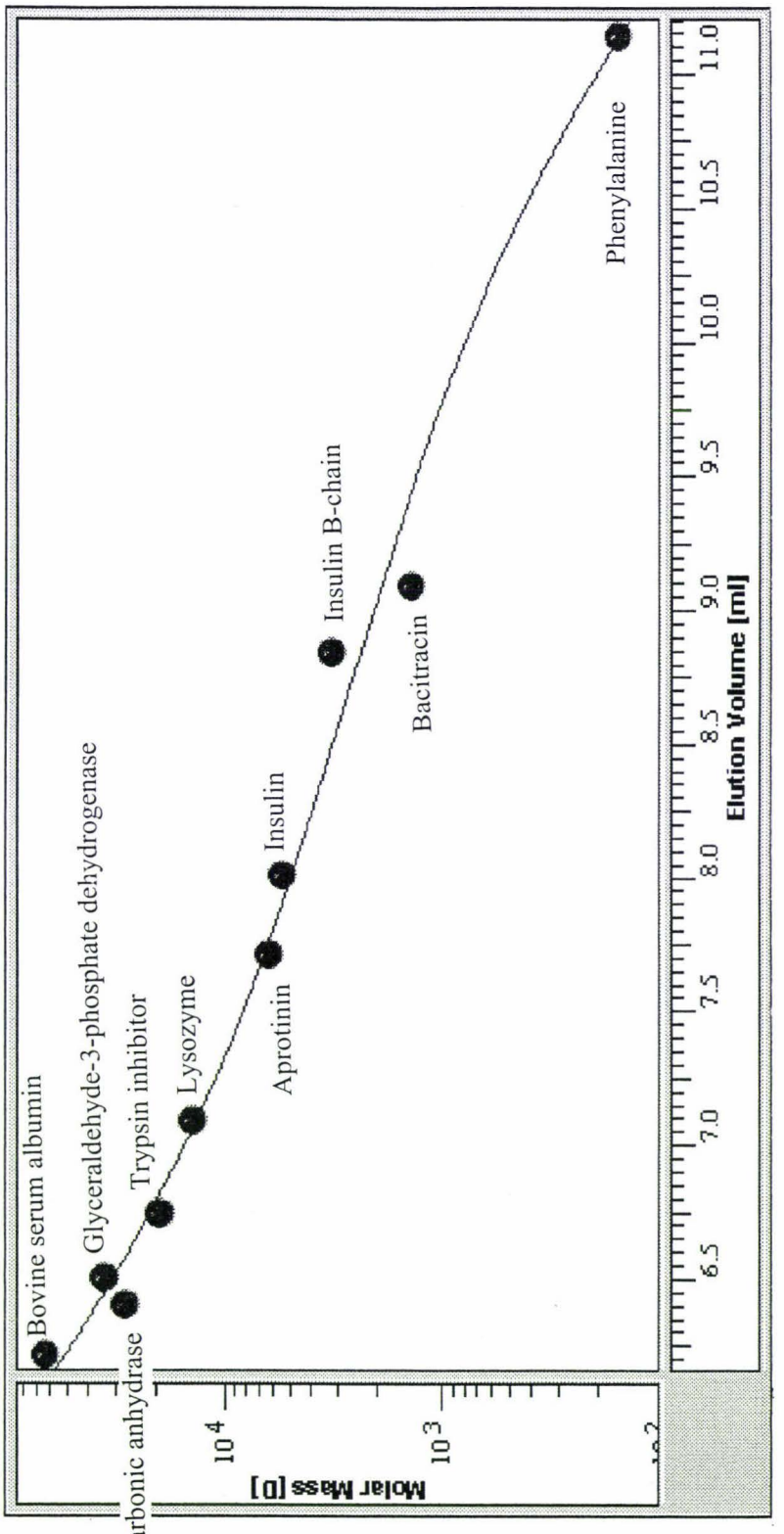
(b) EMC digested with Protease C



(c) EMC digested with Protease DE

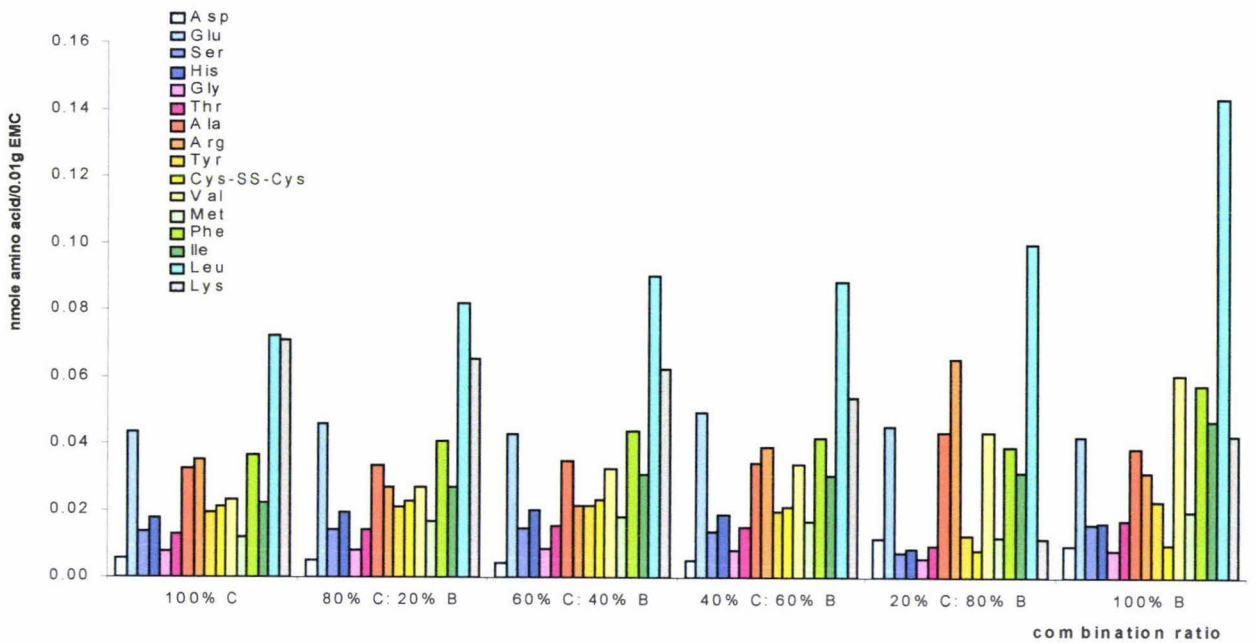


Appendix 7.4 Calibration curve from standard protein solution



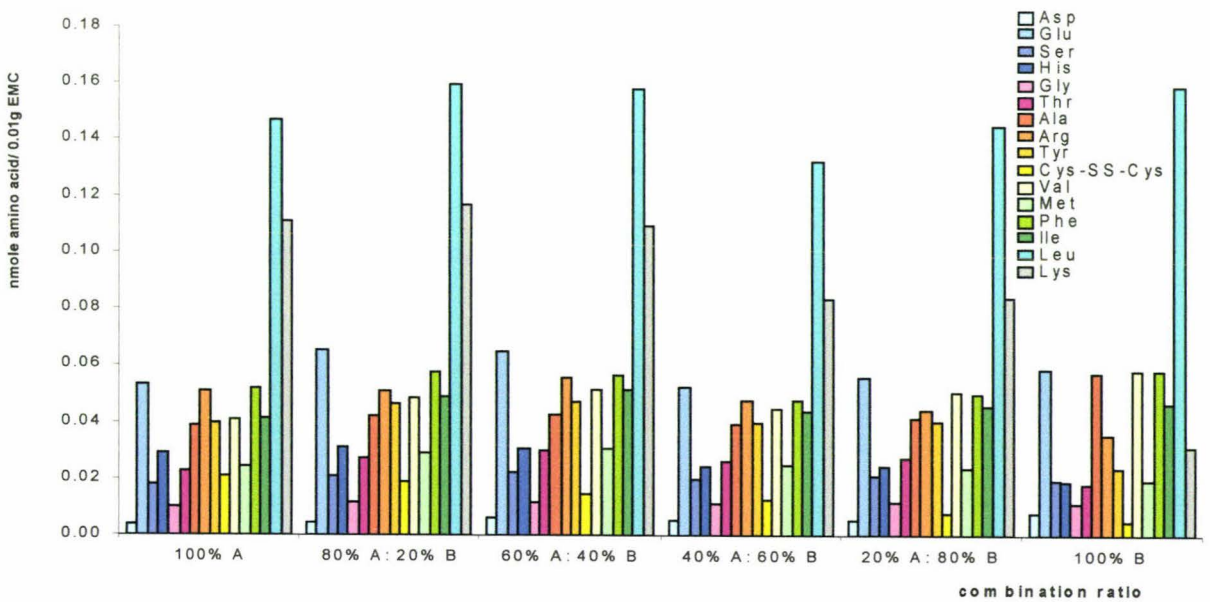
Appendix 8.1. The profiles of amino acid for EMC bases digested with combination proteases

(a) Amino acid analysis for 24 h EMC bases digested with Protease C and Protease B combination



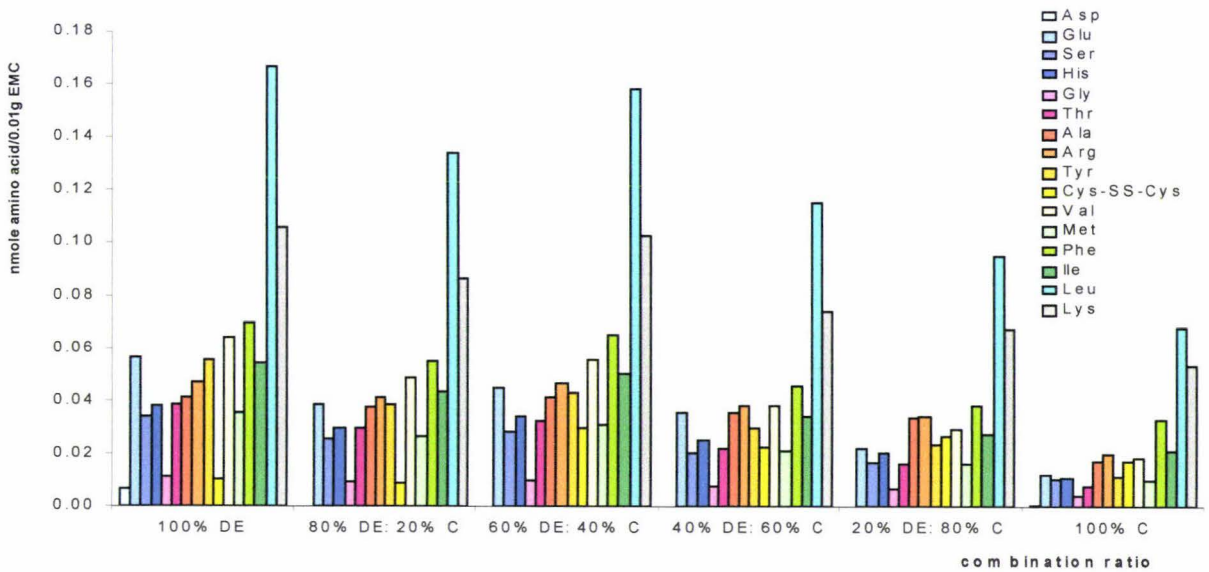
C: Protease C; B: Protease B

(b) Amino acid analysis for 24 h EMC bases digested with Protease A and Protease B combination



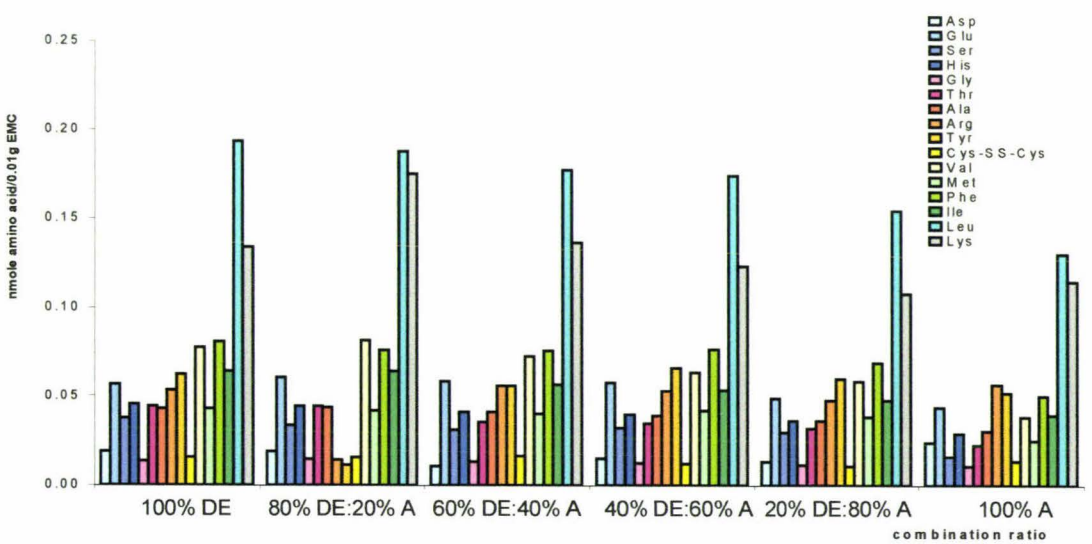
A: Protease A; B: Protease B

(c) Amino acid analysis for 24 h EMC bases digested with Protease DE and Protease C combination



DE: Protease DE; C: Protease C

(d) Amino acid analysis for EMC bases digested with Protease DE and Protease A combination



DE: Protease DE; A: Protease A

Appendix 8.2. Amino acid flavour profiles of commercial protease combinations

(a) Protease A and Protease C combination

Flavour amino acid \ Combination ratio	100%A[1]	80%A:20%C	60%A:40%C	40%A:60%C	20%A:80%C	100%C
Sweet (%)	29.03%	28.45%	29.60%	30.34%	29.79%	30.07%
(nmole/μL)	0.38	0.20	0.18	0.17	0.16	0.13
Sour(%)	10.62%	11.87%	10.79%	10.98%	12.70%	16.19%
(nmole/μL)	0.14	0.08	0.06	0.06	0.07	0.07
bitter(%)	78.15%	75.86%	75.71%	74.19%	71.48%	67.37%
(nmole/μL)	1.03	0.54	0.45	0.42	0.38	0.29

(b) Protease C and Protease B combination

Flavour amino acid \ Combination ratio	100%C	80%C:20%B	60%C:40%B	40%C:60%B	20%C:80%B	100%B
Sweet (%)	30.82%	28.72%	28.06%	25.53%	17.04%	21.04%
(nmole/μL)	0.14	0.14	0.14	0.13	0.08	0.12
Sour(%)	14.94%	14.99%	13.96%	14.93%	14.39%	11.61%
(nmole/μL)	0.07	0.07	0.07	0.07	0.07	0.07
bitter(%)	68.21%	68.40%	69.27%	69.21%	71.42%	75.92%
(nmole/μL)	0.31	0.32	0.34	0.34	0.32	0.44

(c) Protease A and Protease B combination

Flavour amino acid \ Combination ratio	100%A	80%A:20%B	60%A:40%B	40%A:60%B	20%A:80%B	100%B
Sweet (%)	28.55%	28.11%	27.55%	27.43%	27.23%	21.78%
(nmole/μL)	0.20	0.22	0.22	0.18	0.18	0.14
Sour(%)	12.29%	12.95%	13.00%	12.52%	12.61%	13.57%
(nmole/μL)	0.09	0.10	0.10	0.08	0.09	0.09
bitter(%)	75.25%	75.06%	75.41%	74.82%	75.42%	71.70%
(nmole/μL)	0.53	0.59	0.59	0.49	0.51	0.45

(d) Protease DE and Protease C combination

Combination / Flavour amino acid ratio	100%DE	80%DE:20%C	60%DE:40%C	40%DE:60%C	20%DE:80%C	100%C
Sweet (%)	27.66%	28.87%	27.85%	28.20%	29.62%	29.73%
(nmole/μL)	0.23	0.19	0.22	0.16	0.14	0.09
Sour(%)	12.16%	10.48%	10.24%	10.79%	8.94%	7.33%
(nmole/μL)	0.10	0.07	0.08	0.06	0.04	0.02
bitter(%)	76.16%	77.10%	75.59%	73.96%	73.42%	77.09%
(nmole/μL)	0.64	0.50	0.59	0.42	0.35	0.24

(e) Protease DE and Protease A combination

Combination / Flavour amino acid ratio	100%DE	80%DE:20%A	60%DE:40%A	40%DE:60%A	20%DE:80%A	100%A
Sweet (%)	27.73%	33.58%	27.66%	27.00%	27.09%	27.93%
(nmole/μL)	0.27	0.31	0.26	0.24	0.22	0.19
Sour(%)	12.32%	13.34%	11.70%	12.54%	12.25%	13.94%
(nmole/μL)	0.12	0.12	0.11	0.11	0.10	0.10
bitter(%)	76.47%	75.02%	77.66%	76.75%	76.68%	75.87%
(nmole/μL)	0.75	0.70	0.73	0.69	0.61	0.53

A: Protease A, B: Protease B, C: Protease C, DE: Protease DE