PERFORMANCE OF THREE COMMERCIAL LIPASES IN A MODEL ENZYME MODIFIED CHEESE SYSTEM

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ABSTRACT

The effects of Amano 'R' (from *Penicillium roqueforti*), Palatase (from *Mucor miehei*) and kid lipase (from kid goat) activity on hydrolysis of triglycerides in a constant enzyme modified cheese (EMC) base have been investigated. The effects of incubation time, temperature, enzyme concentration, pH, water activity (a_w) and salt-in-moisture content on enzyme activity were studied. Under the same conditions (0.15% enzyme, 30°C, 24 h), Palatase and Amano 'R' showed a greater extent of hydrolysis (total free fatty acids) than Kid lipase. The total free fatty acids (FFAs) released by Palatase, Amano 'R' and Kid lipase were 224, 188 and 20.5 mmol FFA. kg EMC.$^{-1}$, respectively. The optimum temperature for hydrolysis by Amano 'R', Palatase and Kid lipase was around 30°C, 55°C and 45°C respectively. Amano 'R' was very heat sensitive, compared to the other two enzymes. Hydrolysis increased with increasing initial pH. The optimum pH's determined for Amano 'R', Palatase and Kid lipase were 7.5, 8.0 and 5.5 respectively. Enzyme activity decreased slightly as water activity decreased and salt-in-moisture content increased, for all enzymes. The incubation time and enzyme concentration showed the expected trend.

At all process conditions, a high percentage (about 55%) of the fatty acids released by kid lipase was butyric acid. Both Palatase and Amano 'R' were relatively non selective and released large amounts of all fatty acids. Compared to Palatase, Amano 'R' selectively released a higher percentage of butyric acid (about 15% compared to 10%). Generally, the rate of release of butyric acid was greater at lower incubation temperatures for all enzymes. Also, the percentage of butyric acid release decreased with increasing initial pH for Palatase lipase.

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CHAPTER 1

INTRODUCTION

The use of cheese flavour in various food products is commonly accepted. As a consequence, the cheese industry faces an increasing demand for cheese with high flavour intensity from the prepared food industries. Many prepared food products require sources of cheese such as Cheddar, Swiss, Blue, Romano and others which impart typical flavour characteristics. The main alternatives to the use of natural cheese flavour are high-intensity cheese flavour concentrates, such as enzyme modified cheese (EMCs).

A number of compounds have been identified as being characteristic flavour components of certain natural cheese varieties. These flavours can be produced by procedures such as use of specific enzyme systems. To obtain pure compounds a detailed knowledge of the reaction systems is vital (Grueb and Gatfield, 1989) and is probably cost-prohibitive. Therefore, enhancement of the major flavour pathways that occur in natural cheese presently provides the most economic route to the production of intense cheese flavours.

Free fatty acids in cheese can contribute directly to flavour, but threshold values of the individual fatty acids in cheese are not known and are difficult to estimate. Further breakdown of fatty acids and reactions with other components of the maturing cheese are likely to occur, and may contribute to the formation of additional flavour components (Siezen and Van den Berg, 1994). An increase in the amount of free fatty acids in cheese is possible

by inducing lipolysis in the milk or dairy system. This technique is also used during the manufacture of enzyme modified cheese (EMC).

The degree and contribution of lipolysis to cheese flavour varies considerably between cheese varieties (Fox, 1993, Kilcawley, *et al.*, 1998). It is also concluded that for some EMC types, the flavour profile or intensity are proportional to the degree of lipolysis and release of low molecular weight free fatty acids (FFAs), as with Romano or Provolone type EMCs.

The effect of lipase on cheese flavour formation has been evaluated by a number of researchers (Dziezak, 1986). It is reported that various lipases could be selected to give the intensity of flavour required in the final product. A wide range of lipases are commercially available from a number of sources, mainly animal and microbial. The correct choice of lipase is extremely important since the FFA and flavour profiles generated vary significantly with the type of lipase used (Kilara, 1985). The flavour compounds, and their relative concentrations may also vary, depending on the conditions used to manufacture the product (Moskowitz and Noelck, 1987).

The objective of this study is to develop and collect information on the technology of controlled lipolysis in EMC production using commercially available food grade lipases. The plan is to characterise Amano 'R', Palatase and Kid lipase effects on a constant cheese substrate (EMC base) in terms of the amounts of fatty acid (FA) released by hydrolysis and the percentages of FAs released, expressed as a percentage of the total of that particular FA bound initially in the milk triglyceride, and as a percentage of the total free fatty acids (FFAs).

Total free fatty acids, expressed as mmoles per kg of EMC base, indicates the concentration of free fatty acids accumulating in the model EMC system. Total free fatty acid concentration is an indicator of EMC flavour intensity. The percentage of fatty acid released expressed as a percentage of the total amount of that particular fatty acid originally esterified in the milk fat triglyceride, indicates the "extent" of the lipase reaction (degree of hydrolysis). The percentage of fatty acid released, expressed as a percentage of the total free fatty acids released, indicates the specificity of a particular lipase. Lipase specificity impacts on the flavour profile of an EMC.

The enzyme activities will be investigated at different processing variables, namely the incubation time, temperature, enzyme concentration, pH, water activity and salt-in-moisture to find out their impact on hydrolysis of triglycerides in EMC base. Where reaction conditions are described as being optimal, this refers to the conditions required to achieve maximum enzyme activity.

The EMC base to be used is an immature processed cheese, which has undergone proteolysis but not lipolysis. A better understanding of the influence of process variables on the lipase activity and the resultant free fatty acid (FFA) profiles will provide useful information on EMC manufacture and suggest ways to produce new varieties of enzyme modified cheese.

CHAPTER 2

LITERATURE REVIEW

2.1 What is Enzyme-modified cheese

Cheese that has been treated by enzymes to enhance the flavour or a significant portion of the flavour profile can be called enzyme modified cheese (EMC). It provides the food manufacturer with a strong cheese note in a form that is cost effective, nutritious and natural (Moskowitz and Noelck, 1987). EMC may have a flavour profile which is quite different from that of a natural cheese and yet on dilution with a suitable bland base, it will provide the desired cheese note in the product (Sutherland, 1991, Kilcawley, *et al.*, 1988).

Enzyme modification is based on the same chemical changes that occur in traditional processes during cheese ripening. The enzymes used are hydrolytic, not oxidative, and the incubation time is relatively short, requiring days instead of months to complete the process. As a result, the cost of warehousing or ageing are almost eliminated, and the process can be carefully controlled to achieve the specific changes and flavours required.

Cheese flavour development is a subject of intense study. Cheese flavour is derived mainly from proteolysis, glycolysis and lipolysis, the extent of which varies according to the cheese variety (Fox, 1989, 1993; Wilkinson, 1993). Proteolysis is the most complex of the three primary events during cheese ripening. It contributes to cheese ripening by developing a texture change by breakdown of the protein network. The small peptides and free amino acids

which are formed contribute to the flavour of cheese and also serve as substrate for various flavour-generating reactions (McGugan *et al.*, 1979; Aston and Dulley, 1982; Aston and Creamer, 1986; Fox and Wallace, 1997). Lipolysis produces fatty acids from milk fat which is also an important element for the development of cheese flavours. The breakdown products of lactose by glycolysis may also essential for the production of good quality cheese (Fox *et al.*, 1990).

The cheese ripening process is accelerated dramatically in the manufacture of EMC. High flavour intensity EMC can be produced in a matter of days by careful addition of enzymes to the curd or by addition to melted cheese, followed by a controlled incubation process. A wide variety of flavours can be produced using enzyme technology and, a number of different EMC flavours are commercially available including mild, medium, and sharp Cheddar, Colby, Swiss, Blue, Provolone, Romano, Mozzarella and Parmesan.

2.2 Role of lipases

The enzymes used commercially to hydrolyse milk fat are lipases and esterases. Lipases hydrolyse triglycerides, diglycerides, and monoglycerides present at an oil-water interface. Esterases are most active on water soluble substrates. The reaction seldom goes to completion and hydrolysis of a triglycerides by lipase will yield di- and monoglycerides and free fatty acids.

Lipases are used to enhance the flavour in cheese and cheese related products as reviewed in several papers (Stead, 1986; Deeth and Fitz-Gerald, 1987; Arnold *et al.*, 1975; Fox and

Guinee, 1987; Siezen and Van den Berg, 1994). The liberated free fatty acids are not only functioning as flavour components themselves, but are also precursors for the formation of other flavour components (Martinez-Castro *et al.*, 1991; Moskowitz and Noelck, 1987).

2.2.1 Lipolysis and flavour development

Lipolysis refers to the hydrolysis of triglycerides, diglycerides and monoglycerides to yield free fatty acids (FFAs) and flavour precursors by lipolytic enzymes (Fox, 1981; Aston and Creamer, 1986; Birschbach, 1994). The fat in cheese can undergo degradation *via* lipolysis (enzymatic) or oxidation (chemical). The degree of lipid oxidation in cheese is limited, probably due to the low redox potential of cheese and the presence of natural antioxidants (Fox and Wallace, 1997). The enzymatic hydrolysis of lipids is significant in the formation of flavour in cheese. Seitz (1990) outlined the possible routes by which milk lipids could be converted to different flavour compounds (Figure 2.1). They found that hydroperoxides and

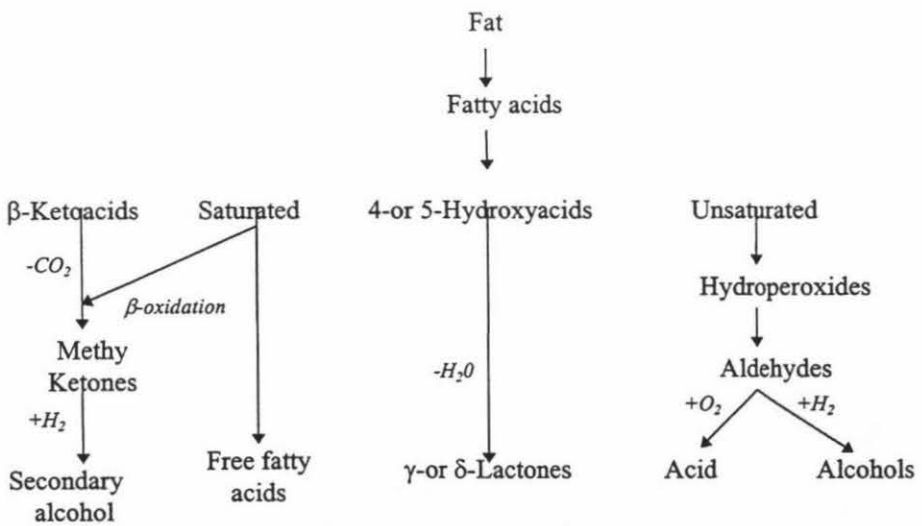

Figure 2.1: Formation of flavour compounds from milk lipids

(Seitz, 1990, p. 3683)

lactones are formed through hydrolysis and subsequent cyclization of hydroxy fatty acids. These compounds are an important flavour components in several cheeses.

The liberated free fatty acids are major contributors to the characteristic flavour of cheese (Arnold *et al.*, 1975). This is confirmed by Fox and Wallace (1997). Furthermore, the relative ratios of fatty acids are related to flavour quality and intensity (Moskowitz and Noelck, 1987; Woo and Lindsay, 1984). There is a clear trend that the C4:0 and possibly C6:0 fatty acids are of major importance for desirable flavours (Harboe, 1994; Deeth and Fitz-gerald, 1987). However, the degree of lipolysis varies among cheese varieties, for instance, 6 meq FFA/100g in Gouda cheese to 45 meq FFA/100g in Danish Blue (Gripon, 1993).

The characteristic Italian cheese flavour is directly related to the amounts of butyric acid released by lipolysis (O'Connor *et al.*,1996; Moskowitz and Noeleck, 1987). The aroma typical for Provolone cheese is related to the content of both butyric acid and glutamic acid, and the flavour characteristics for Romano cheese are related to the butyric acid only (Long and Harper, 1956).

In Blue cheese 20% of the fat may be hydrolysed to medium and long chain fatty acids, between C8:0 and C14:0 which are oxidised to methyl ketones and in turn are reduced to secondary alcohols, the former being important components of blue cheese flavour (King and Clegg, 1979; Moskowitz and Noelck, 1987; Gripon, 1993). It is reported that, octenol is an important flavour compound in Camembert cheese (Moskowitz and Labelle, 1981; Eaton, 1994).

2.2.2 The use of lipases for flavour production of different varieties of cheese

Lipases are used mainly for Italian cheese, Italian cheese types and enzyme modified cheese (Harper and Long, 1956). Further, it is known that lipases are also used for a large range of other cheese varieties. Lipases use is growing in New Zealand as a wide range of varieties of cheese is manufactured.

(a) Italian cheese

Lipase is very often used for the production of Italian cheese varieties. Harper and Long (1956) studied the butyric acid formed by lipase of different origins and found that Kid rennet paste and Kid pregastric lipase gave equally good cheese quality when used for commercial Provolone cheese. This was also confirmed by Huang and Dooley, 1976; Shahani *et al.*, 1976; Woo and Lindsay,1984. Richardson *et al.* (1971) characterised lamb gastric lipase in relation to pregastric lipase for Italian cheese production and found that Provolone made by gastric lipase was of higher organoleptic quality than cheese made with pre-gastric lipase. It is also reported that, lipase secreted by the fungus *Mucor miehei* produces flavour close to Italian type cheese (Huang and Dooley, 1976).

(b) Cheddar cheese

Various workers have studied hydrolysis of milk fat during ripening of Cheddar cheese. Ohren and Tuckey (1964), describe the addition of pregastric esterase to pasteurised milk, to produce Cheddar cheese containing more free fatty acid and with improved flavour.

However, microbial lipases can also used for accelerated ripening of Cheddar cheese. Arbige *et al.* (1986) used a novel lipase from *Aspergillus oryzae* for Cheddar cheese. Good results were obtained and a high retention of the lipase in the curd was demonstrated. Also an accelerated development of proper Cheddar flavour was observed. This lipase is unusual as it preferentially releases C6:0 to C10:0 FFAs from triglycerides. However, Wilkinson *et al.* (1992) showed that this enzyme preparation contained substantial proteolytic activity with adverse effects on flavour and texture when added to accelerate the ripening of natural Cheddar cheese.

Kosikowski (1974) also reviewed the potential of microbial lipases in a continuous cheese process, reporting good flavour enhancement in Cheddar cheese from several fungal lipase preparations. However, Richardson *et al.* (1971) indicated that Cheddar cheese was organoleptically preferred when gastric lipase preparations were included in their manufacture. The addition of lipases to commercial Cheddar is used only to a very limited extent.

(c) Feta cheese

Pregastric lipase or microbial lipase is often used for production of Feta cheese when it is produced from cows milk instead of ewe or goats milk (Arnold *et al.*, 1975; Kilara, 1985). The use of various pregastric lipase products for feta cheese has been reported by Ethymion and Maltick (1964). The results showed that the desired, mild rancid flavour of feta was associated with free fatty acids from C2:0 through C10:0, whereas objectionable rancid flavour was due to higher levels of free fatty acids higher than C10:0.

(d) Blue cheese

Several authors have recently studied the production of blue cheese flavour based on the addition of lipase and protease extracts from *Penicillium roqueforti* (Revah and Lebeault, 1989; King and Clegg, 1979; Dziezak, 1986; Moskowitz and Noelck, 1987) to the curd or to granular curd. In many cases favourable blue cheese was produced.

(e) Enzyme-modified cheese (EMC)

Lipase is a very important enzyme for production of EMC, especially for enhancing and accelerating the Romano, Provolone or blue type cheese flavour. The levels of free fatty acids in EMC are increased by a factor of 5-25 compared to a two month old cheese.

Kosikowski and Iwasaki (1975) found that EMC made by addition of pregastric or microbial lipase to Cheddar resulted in a pronounced cheese flavour, low bitterness but often strong rancidity. Furtado *et al.* (1984) produced a Camembert cheese flavour concentrate by addition of calf pregastric lipase and surface ripening by *Penicillium caseicolum*. Significant acceleration of intense cheese flavour was obtained.

However, the production of Cheddar-flavoured EMC is slightly more difficult than other varieties, because of the absence of identified specific key components (Moskowitz and Labelle, 1981; Eaton, 1994). However, some compounds and process parameters are known to have a role in the generation of good Cheddar flavour and it is useful to be aware of these (Fox, 1993).

2.3 Characteristics of lipases

The fundamental difference between lipases and esterases is in their action at fat-water interface (Villeneuve and Foglia, 1997). Esterases function in a homogenous aqueous environment and hydrolyse water-soluble substrates. Lipases or triacylglycerol lipases, are enzymes that have a strong preference for triacylglycerides, particularly when this substrate is located in a lipid-water interface. In this interface the substrate can be split a thousand times faster than in free solution (Brockman, 1984; Drewenda *et al.*, 1992; Kilara, 1985).

Lipases and esterases also exhibit functional differences. These differences are based on the factors that affect enzyme activity and their substrate specificity. Both aspects are discussed in this subsection.

2.3.1 Factors affecting lipase activity

In general, lipases, like most enzymes, are very active only over a limited range of pH and temperature values. Pre-gastric esterase has been reported to have a temperature optimum of 28°-30°C for calf, 32°-34°C for kid goat and lamb enzymes, while other reports indicate the temperature optimum for all pre-gastric enzymes to be 35°-42°C range (Nelson *et al.*, 1977). On the other hand, Sweet *et al.*(1984) reported that the optimum temperature for calf pre-gastric lipases is approximately 45°C. By contrast, the temperature optima for calf and lamb lingual lipases were 35° to 40°C and 25° to 35°C, respectively (O'Connor and Manuel, 1993). Most microbial lipases exhibit temperature optima at approximately 45°C (Birschbach, 1994). In another study, with respect to temperature optimum, most lipases are optimally active

between 30° and 40°C (Shahani, 1975). On the other hand, lipolytic rancidity in foods held as low as -29°C has also been reported (Kilara, 1985).

The optimal pH of hydrolysis of different enzymes depend upon the substrate used, the presence of salts and the kind of emulsifiers used (Kilara, 1985 and Wills *et al.*, 1965). Milk lipase has been shown to have an optimum pH of 9 (Shahani, 1975), while several microbial lipase exhibit pH optima for activity between 5.6 and 8.5. Arnold *et al.*(1974), reported that the pH optima for *Penicillium roqueforti* lipase (Amano 'R') is about pH 9 while Moskowitz *et al.* (1977) reported a pH optima for *Mucor miehei* lipase (Palatase, Novo Industries) is about 8.5.

The optimal pH of hydrolysis of tributyrin by calf and lamb lingual lipases was 6.9 and 6.6, respectively (O'Connor et al., 1993). However, the pH optima for kid goat and lamb pregastric esterases are 4.5 to 6.0, with complete inhibition of activity below pH 2.4 and above pH 7.8 (Nelson *et al.*, 1977; Kilara, 1985).

Salts affect lipase activity in different ways. Sodium chloride up to 7 mM concentration increased hog pancreatic lipase activity while bovine pancreatic and milk lipases do not show such an effect (Shahani, 1975). Sodium chloride, potassium chloride and calcium chloride appear to have little or no effect on the activity of microbial lipases (Moskowitz *et al.*, 1977). Desnuelle and Nord (1969) reported that commercial preparations of pre-gastric esterases show an apparent increase in activity, possibly due to altering interfacial charge effect by sodium chloride.

2.3.2 Specificity of lipolytic enzymes

Lipolytic enzymes exhibit several types of specificity which affect their role and significance in flavour development. Nelson, (1972) pointed out that the most significant factors in the development of useful lipolytic technology are the discovery and application of a variety of lipase systems with differing specificities for:

- fatty acid chain length
- types of glyceride molecule
- the physical condition of the substrate

The rate of enzyme activity on various triglyceride substrates is commonly used to compare lipolytic enzymes. Khan *et al.* (1967), reported that milk lipase exhibited greater activity on tributyrin substrate than on triolein substrate, while *Achromobacter lipolyticum* lipase exhibited the opposite relative activities. Shipe (1951) observed that *Penicillium roqueforti* lipase hydrolysed tributyrin, tricaproin, tricaprylin, and tripropionin in decreasing order whereas *Aspergillus niger* lipase hydrolysed tricaprylin faster than the other three substrates. This observation is also confirmed by Eitenmiller *et al.* (1970). Morris and Jezeski (1953) have reported that *Penicillium roqueforti* lipase was only about one-third as active on butter fat as on tributyrin, and that its activity decreased as the molecular weight of the esterified fatty acids increased. Siewert and Otterby (1968), Pitas and Jensen (1970) reported that pregastric esterase is specific for glycerides containing short chain fatty acids. With a series of triglycerides containing butyric acid, greatest activity was observed on lower molecular weight triglycerides. Farnham *et al.* (1956) reported differences in the relative activities of pregastric esterases on milk fat, with calf esterase showing greater lipolysis than either lamb

or kid esterases. A comparable study of calf and lamb lingual lipases using triolein as substrate showed that calf lingual lipase is a more effective catalyst than lamb lingual lipase (O'Connor and Manuel, 1993).

Another type of substrate specificity is the relative activity of lipolytic enzymes on triglyceride versus mono- and di-glyceride substrate. Table 2.1 compares the activities of four types of lipolytic enzyme on tri-, di-, and mono-butyrin (Richardson and Nelson, 1967). Pregastric esterase showed no activity on the monobutyrin and very limited activity on dibutyrin whereas pancreatic lipase maintained high activity on the mono- and diglyceride substrates.

Table 2.1: Ratio of enzyme activity on monoglyceride and diglyceride substrates compared to activity on tributyrin

Enzyme	Tributyrin	Dibutyrin	1-Monobutyrin
Pregastric esterases	1.00	0.04	00
Pancreatic lipase	1.00	0.74	0.95
Fungal lipase	1.00	0.26	0.15
Milk lipase	1.00	0.35	0.12

(Richardson and Nelson, 1967, p. 1061)

Most of the lipases studied are capable of hydrolysing a variety of fat and oil and synthetic mono-, di-, and tri-glycerides (Kilara, 1985). Glycerol esters are the preferred substrate.

According to Kilara (1985), unsaturated fatty acids were hydrolysed from triglycerides more rapidly than saturated fatty acids by pancreatic lipase. It was implied therefore that pancreatic lipase exhibited fatty acid specificity. However, Nelson (1972) reported that the pancreatic lipase is non specific in its action. Systematic studies, however, revealed that the unsaturated fatty acids were preferentially hydrolysed because they were located at the *Sn* 1, and 3 or primary positions i.e. the lipase demonstrates positional specificity and not fatty acid specificity (Kilara, 1985).

Different groups of lipases possess a specificity for the type and position (1-, 2- or 3-) of the fatty acids (Kilara, 1985 and Macrae, 1983). Most lipases distinguish between long and short chains and some lipases even specifically recognise one particular unsaturated fatty acid. Concerning the positional specificity, some lipases are able to hydrolyse both the so called ∝-linked (1- and 3- position) and the β-linked (2-position) fatty acids, while other lipases only hydrolyse the ∝-linked fatty acids (Kilara, 1985). No lipases have been found that distinguish between fatty acids at the 1- and 3-positions in the triglycerides (Siezen and Van den Berg, 1994).

Microbial lipases can be divided into two groups based on their positional specificity. One group is non-specific and releases fatty acids from all three positions of the glycerol moiety (Kilara, 1985). Lipases from *Geotrichum candidum*, *Corynebacterium acnes*, *Penicillium cyclopium, Chromobacterium viscosum*, and *Staphylococcus aureus* are non-specific lipases. The non-specific lipases cause complete breakdown of triglycerides to free fatty acids and glycerol.

The second group of lipases based on specificities hydrolyse fatty acids located at the *sn* 1, and *sn* 3 positions preferentially, to give free fatty acids and di- and mono-glycerides as reaction products. The 2-monoglycerides in particular and sometimes the 1,2 or 2,3-diglycerides are unstable and undergo acyl migration to yield 1,3-diglycerides and 1-monoglycerides. Therefore prolonged incubation of substrates with such enzymes can eventually yield a complete breakdown of a triglycerides to free fatty acids and glycerol (Desnuelle and Nord, 1969). Many microbial lipases are 1,3 specific, e.g. lipases derived from *Aspergillus niger*, *Mucor javanicus*, *Rhizopus arrhizus*, *Rhizopus delemar*, and *Pseudomonas fragi* (Kilara, 1985).

Some lipases also show fatty acid specificity in some instances. It is believed that chain length of fatty acids involved an exert effect on the rate of lipolysis. Pancreatic lipase is believed to be more specific towards short-chain than long-chain fatty acids with tripropionic and tributyric being hydrolysed at maximum rates (Kilara, 1985). Similarly, pre-gastric esterases release large amounts of short-chain fatty acids (such as butyric and caproic acids) from milk fat as well as from synthetic glycerides (Kilara, 1985). Nelson (1972) reported that the relative specificity of calf esterase for short-chain fatty acids, particularly butyric acid, from milk fat is apparent. Similar data was presented by Jensen (1971). However, the highest concentration of butyric acid is produced by kid pregastric esterases and kid rennet paste, when compared with other pre-gastric esterases (Huang and Dooley, 1976). Generally, animal pregastric lipases mainly release short-chain fatty acids, especially butyric acid (Kwak *et al.*, 1989; Harper, 1957).

The Table 2.2 compares the free fatty acids released by eight different lipolytic enzyme preparations (Harper, 1957). These data indicate that pancreatic lipase hydrolysed predominantly higher molecular fatty acids whereas the lipase from an *Aspergillus* spp. hydrolysed primarily lower chain length fatty acids. The high specificity of the pregastric esterases (lingual lipases) for the release of butyric acid is also apparent from these data. The impact of each of these enzymes on flavour is the net result of the combined factors of activity and specificity toward the substrate.

Table 2.2: Selective liberation of individual free fatty acids from milk fat by eight different lipase preparations

Lipases	Total free fatty acids -% (micro equivalent basis)				
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0 & higher*
Imported crude Kid rennet paste	32.8	11.3	7.1	11.8	33.6
Domestic purified calf rennet paste	10.7	3.1	trace	trace	86.5
Calf PGE	36.7	8.9	4.8	10.7	39.0
Kid PGE	44.4	15.2	7.6	12.3	21.5
Lamb PGE	48.1	8.6	14.2	9.3	19.8
Aspergillus lipase	43.1	18.9	20.2	17.5	trace
Milk lipase	13.5	8.2	10.2	8.7	60.0
Pancreatic lipase	8.4	2.1	trace	trace	89.1

(Harper, 1957, p. 556)

The microbial lipases also show fatty acid specificity. Huang and Dooley (1976) reported that the lipase from *Aspergillus niger* shows higher specificity towards short-chain fatty acids (C4:0 to C8:0). In contrast, *Mucor miehei* lipase preferentially hydrolysed ester bonds containing fatty acids longer than myristic acid (C14:0) (Lee and Rickansrud, 1979). Some microbial lipases are specific for particular fatty acids. Lipase from *Geotrichum candidum* is reported to be specific for long-chain fatty acids containing a *cis* double bond at the *n*-9 position (De Greyt and Huyghebaert, 1995).

Another characteristic of substrate specificity is related to the physical form of the substrate. Richardson and Nelson (1967) compared the relative activities of four types of lipolytic enzymes on triacetin solution and emulsion substrates as reported in Table 2.3.

Table 2.3: Activities of various enzymes in triacetin solution (5%) and emulsion (15%) at pH 6.2

Enzyme	Activity (units/min)		
	Solution (s)	***Emulsion (e)***	***Ratio e/s***
Pregastric esterase	2.8	2.0	0.71
Pancreatic lipase	2.6	5.8	2.2
Fungal lipase	0.2	4.4	22.0
Milk lipase	4.6	2.2	0.48

(Richardson and Nelson, 1967, p. 1061)

The interdependence between the physical form of the substrate and the specificity of the lipolytic enzyme used is an extremely important consideration in designing systems for the application of lipolytic enzymes for flavour development in dairy manufacturing.

GENERAL DISCUSSION

Overall, a number of key factors are involved in EMC production and these can be summarised as follow; type and specificity of protease enzyme used, type and specificity of lipase enzyme used, and processing parameters (e.g. pH, temperature).

Different EMC flavours can be produced using various lipase enzymes such as microbial and animal lipases. However a detailed knowledge of the enzymatic reactions under various conditions is important to produce consistent EMC product. Comparative data on enzyme specificity and enzyme activity indicate a wide variety of flavour effects are possible from various commercial enzyme preparations.

A number of research groups have published in the application of lipolytic enzymes to flavour development in EMC and different cheese products, but most of the information is patented. There is limited information available on lipase biochemistry including lipase specificity and effect of process condition on lipase activity, such as enzyme concentration, water activity, salt content, moisture content etc.. Therefore, it is highlighted the requirement for further research into cheese flavour development from the same initial substrate. This thesis looks at the impact of lipase activity on the EMC process. The impact of commercially available lipases, namely Amano 'R', Palatase and kid lipase are defined in a model EMC system.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cheddar 'P'

Cheddar 'P' is an immature natural cheese which has young body structure. It was manufactured at the Dairy Research Institute (NZDRI), kept at 2°C for 14 days and then stored below -10°C until required for the production of the enzyme modified cheese (EMC) base.

3.1.2 Emulsifying salts

Trisodium citrate and disodium citrate were used for emulsification of the EMC base.

3.1.3 Proteolytic enzymes

Protease enzyme Promod 215P and peptidase enzyme Flavorpro 192P were used for the proteolysis of the EMC base. Both enzymes were supplied by Biocatalysts, Midglanorgan, UK.

3.1.4 Lipase enzymes

Three different preparations of commercial lipase enzymes were used in this to study.

(a) Palatase 20000 L

Palatase 20000 L is a purified 1,3 specific lipase from M*ucor miehei* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* micro-organism. This enzyme complies with FAO/JECFA and FCC recommended specifications for food grade enzymes, and was supplied by Novo Industries, Dk-2880, Bagsvaerd, Denmark. Palatase 20000 L is standardised to 20,000 Lipase Units/g (LU/g). This commercial liquid was added directly to the EMC base. It was stored in 5°C to maintain its maximum activity.

(b) Amano 'R'

Amano 'R' is a fungal enzyme which derived from *Penicillium roqueforti*. This is a 1-,3-specific lipase. The enzyme was manufactured by Amano Pharmaceutical Company Ltd., Nagoya, Japan. Amano 'R' is standardised to 900-1100 Lipase Units/g (Lu/g). This commercial powder was dissolved in milli-Q water to give a 50% (w/w) solution. This solution was added to the EMC base. It was stored in 5°C to maintain it's maximum activity.

(c) Kid lipase

Kid lipase is derived from the lingual (tongue and salivary) glands of young (kid) goats. The enzyme was manufactured by Rhodia Food Ingredients, Wisconsin, USA. Kid lipase is standardised to 42 Lipase Units/g (Lu/g). This commercial powder was dissolved in Milli-Q water to give a 75% (w/w) solution. This solution was added to the EMC base. It was stored in 5°C to maintain its maximum activity.

3.2 Compositional Analysis

The composition of the EMC base was analysed before proteolysis and after proteolysis. The results are given in Appendix 1, Table 1 and 2.

3.2.1 Moisture content

The moisture content of the EMC base was determined after drying in an oven at 105°C for 16 hours. Weight loss on drying, as a percentage of total initial weight was determined as moisture (NZDRI Method ACCA12, New Zealand Dairy Research Institute (NZDRI), 1991).

3.2.2 Salt content

The salt content was determined by the Volhard method (NZDRI Method ACCA28, NZDRI, 1991). The organic matter in the sample was destroyed by boiling with nitric acid in the presence of a known amount of standard silver nitrate. The silver nitrate reacted with the chloride in the sample to form silver chloride which precipitated. Excess silver nitrate was then back titrated with standard potassium thiocyanate using ammonium ferric sulphate as the indicator.

3.2.3 Fat content

Fat content was measured by the Werner Schmid method (NZDRI Method ACCA03, NZDRI, 1991). Approximately 1g of cheese sample was digested with hydrochloric acid, ethanol was added and the acid/ethanol solution was extracted with diethyl ether and light petroleum. The solvent was removed by distillation, and the substances which were soluble in light petroleum were determined.

3.2.4 Total nitrogen

The total nitrogen content of cheese samples was determined by digestion of the sample in a block digester followed by automatic distillation and titration according to the Kjeldahl method (NZDRI Method ACCA 043, NZDRI, 1991).

3.2.5 Non protein nitrogen

Cheese samples were prepared by the method of Aschaffenberg and Drewry,1959 (NZDRI Method ACCA07l, NZDRI, 1991). The sample was dissolved and the protein precipitated by 12% trichloroacetic acid. The nitrogen remaining in the filtrate was determined by the Kjeldahal method (NZDRI Method ACCA 07, NZDRI, 1991).

3.3 Microbial analysis

The EMC base was analysed for total aerobic plate count, coliforms, sulphite reducing clostridia, staphylococci-coagulase +ve and thermophiles after incubating for 3 to 5 days at 30°C.

3.3.1 Total aerobic plate count

A sample of EMC was mixed with milk plate count agar, and incubated aerobically at 30°C for 72 hours. The number of colonies on selected plates were counted (NZTM 2, Microbiological Methods Manual, Section 43, NZDRI, 1997).

3.3.2 Coliform

This method is based on IDF Standard 73A, 1985. A portion of EMC sample was mixed with violet red bile agar, overlaid with the sample agar, and incubated aerobically at 30°C for 24 h (NZTM 2, Microbiological Methods Manual, Section 48, NZDRI, 1998).

3.3.3 Sulphite reducing clostridia

A sample of EMC was held at 80°C for 10 min to eliminate vegetative cells. The samples was then cooled, mixed with differential reinforced clostridial agar and overlaid with plain 2% agar. After incubation with anaerobic conditions at 37°C for 24 h the number of colonies were counted (NZTM 2, Microbiological Methods Manual, Section 59, NZDRI, 1996).

3.3.4 Coagulase-positive *Staphylococcus aureus*

A sample of EMC was spread onto the surface of Baird Palker agar and, optionally, also on rabbit plasma fibrinogen agar and incubated aerobically at 37°C for 24 hours (NZTM 2, Microbiological Methods Manual, Section 47, NZDRI, 1996).

3.3.5 Thermophilic bacteria

A sample of EMC was mixed with milk plate count agar and incubated aerobically at 55°C for 48 hours in a humid environment. The number of thermophilic bacteria per gram is calculated from the number of colonies counted on selected plates (NZTM 2, Microbiological Methods Manual, Section 60, NZDRI, 1996).

3.4 Determination of proteolysis

The protein in the EMC base was hydrolysed to large peptides, small peptides, amino acids and small organic molecules through the action of proteolytic enzymes added during manufacture (Schormaller, 1968; O,keeffe et al., 1976 and Fox, 1989). The degree of hydrolysis was measured by a alkaline urea polyacrylamide gel electrophoresis (PAGE).

PAGE was carried out using 11% polyacrylamide gel in a vertical Bio-Rad model Mini-PROTEAN II electrophoresis cell by a method described in the Gel Electrophoresis Manual, 1998, Food Science Section, NZDRI.

The sample was prepared by extracting the fat from 0.5 g of EMC base, dissolving in the remaining part of the base in the alkaline urea sample buffer, and centrifuging for 10 min at 4°C at 10000 rpm. Any fat that remains was removed from the top of the solution. Electrophoresis runs were conducted for 1.4 h at a constant voltage of 210 v, 70 ma current and 6.5 w power per gel. The concentration of the samples applied to the gel was such as to allow casein breakdown components to appear in the electrophoretic patterns. A rennet casein standard was also run.

Gel lanes were scanned by densitometer. The relative protein concentration was calculated from integration on gel image by Image Quant software. Relative mobility was assigned to the various protein components as an aid to their identification. The results are given in Appendix 1, Table 3 and Figure 1.

3.5 Determination of fatty acid composition of triglycerides

Methyl esters of fatty acids were prepared by direct trans-esterification of an anhydrous fat sample with sodium methoxide/methanol. Individual fatty acids were determined by gas chromatography of the methyl esters. A Certified Reference Material (AMF) was used as the primary standard for calibration (Method ACGC 1.1, Version 2, NZDRI, 1996). The results are given in Appendix 1, Table 4.

3.6 Determination of free fatty acids

The overall procedure involved the isolation of the fat and the free fatty acids (FFAs) from the EMC base, and then the separation of the FFAs from the fat using aminopropyl solid phase extraction (SPE). This was followed by separation and quantification of individual FFAs by gas liquid chromatography (GLC).

(a) Cheese extraction

Cheese sample was warmed to 40°C and 1g weighed accurately. The sample was mixed thoroughly with 10 ml DEE/heptane 1:1 and the following addition made:- 6g oven dried sodium sulphate, 0.1 ml of C13 internal standard solution (2mg/ml) and 0.3 ml of 5M sulphuric acid . The sample was capped and shaken using a vortex mixer, and then centrifuged at 10000 rpm, 4°C for 5 minutes. The supernatant was removed to 16 ml kimax tubes containing 1 g sodium sulphate. The remaining pellet was washed once with 3 ml DEE/heptane 1:1 and all supernatant were combined.

(b) Amino propyl solid phase extraction (SPE)

500 mg amino propyl columns were conditioned with 10 ml of heptane. The DEE/heptane cheese extract (Total amount for non lipolysed EMC base and 1 ml for lipolysed EMC base) was run slowly through the columns using a vacuum manifold. The column was then washed successively with 3 ml of chloroform/IPA (2:1). Up to this point eluates, including those containing neutral lipids were discarded. Free fatty acids were eluted from the columns with 2 ml of 6% formic acid in DEE. The eluate was collected in a small GC vials and capped.

(c) Gas chromatography analysis

The FFA were analysed by using a gas liquid chromatograph (Shimadzu, model GC-17 A), fitted to a GC 1000 (FFAP) capillary column (30 m long, 0.53 mm internal diameter glass column, 0.25 μm film thickness). The GC was equipped with a flame ionisation detector (FID) which was supplied with flows of 50 ml/min hydrogen, nitrogen and 500 ml/min air. Operating conditions were as follows; Injector temperature 220°C, Detector temperature 275°C, temperature rate 10°C/min, Equilibrium time 0.2 min and total programmed time 40 min. The GC was conditioned for 1 h at 220°C before use, and it was programmed from 100° to 220°C at 10°C/min during each analysis.

One microlitre of sample was injected with an auto sampling syringe. The injection was splitless for 1 min; then the spliter opened with a ratio of 5:1. The entire GLC run required 40 minutes for the analysis of C4:0 to C18:2 FFAs. Chromatographic peaks were identified by retention time, and peak areas were integrated by Shimadzu Class-VP computer software.

3.7 Determination of volatile compounds

The level of volatile compounds in the EMC base was determined before lipolysis. About 5.5 g of a melted cheese sample were placed in a wide-mouth Dionex sample bottle, and tightly closed with a Teflon-faced silicon septum. The bottle was held at 37°C for 10 min in a temperature controlled water bath prior to head space sampling. Samples were stirred using a submersible magnetic stirrer. The volatile compounds in the head space were extracted for 20 min using a solid phase micro extraction (SPME) device (SPME; Supelco, Bellefonte, PA) equipped with a polydimethyl- siloxane/divinylbenzene coated fibre (65μm thickness). The SPME fibre was inserted into the head space through the Teflon septum.

Volatile compounds that were adsorbed onto the SPME fibre were thermally-desorbed at 220°C for 33 s in the injector port of a GC-Mass spectrometer (GC-MS). Identification of volatile compounds was based on GC-MS analysis and matching with standard compounds in the computer data-base. The results are shown in Appendix 1, Table 6

3.8 Preparation of Enzyme Modified Cheese base

The enzyme modified cheese (EMC) base was prepared in a Stephan high shear cooker, which is a 25 L vessel with a variable speed scraper (55 to 1500 rpm). It is jacketed and heated by direct injection of steam. The Stephan vessel was sterilised by direct injection of steam for 15 min. and then the following ingredients were added to the vessel.

Grated cheddar 'P'	- 18kg
tri Sodium Citrate	- 0.36kg
di Sodium Phosphate	- 0.36kg
Deionised water	- 5.28kg

The ingredients were heated to 82°C using direct steam injection and allowed to stand at this temperature for 2 min for pasteurisation. Then the slurry was allowed to cool to 37°C within 2.5 h. Once 37°C was reached, the protease enzyme preparation (Promod 215P -25g and Flavorpro 192P- 15 g) was added and allowed to incubate at 37°C for 4 h. Finally, the enzyme was inactivated by heating to 95°C for 5 minutes using direct steam injection.

The EMC base was analysed for moisture, salt, non protein nitrogen, total nitrogen, fat, degree of proteolysis and FFA content before proteolysis and after proteolysis. All the results are given in Appendix 1.

3.9 Treatment to inhibit microbial growth during enzyme incubation

A preliminary experiment was carried out to determine the most effective method to inhibit microbial growth in the EMC base. Samples were treated with the antibacterial agent Nisin (5% solution of Nisaplin in 0.01M sterilised hydrochloric acid), followed by Tindalisation. Tindalisation was carried out by heating the sample to 30° C for 2 h, then heating to 70°C for 1.5 h and finally cooling to 30°C. This heating and cooling process was done twice. The following experiments were undertaken:

(1) 100g of EMC base was incubated at 30°C for 3 days without any anti microbial treatment. Another 100g sample was neither treated nor incubated.

(2) 0.5 ml of 5% Nisin in HCl was added to 100g of EMC base, to give 250IU Nisin/ml of cheese

(3) 1 ml of 5% Nisin in HCl was added to 100g of EMC base, to give 500IU Nisin/ml of cheese

(4) 1 ml of 5% Nisin in HCl solution was added to 100g of EMC base, followed by Tindalisation.

(5) 100g of EMC base was treated by Tindalisation

The microbial plate count results, after above treatments are given in Table 3.1. The results indicated that the best treatment to inhibit microbial growth during incubation was to add, 1 ml of 5% Nisin in HCl solution to 100g of EMC base, to give 500IU Nisin/ml of cheese, and followed by Tindalisation.

Table 3.1: Microbial plate count results by after various treatments which were carried out to inhibit microbial growth during enzyme incubation

Sample	Microbial plate count (cfu/g)				
	APC	*Coliform*	*SRC*	*SC+ve*	*Thermophiles*
Without Nisin treatment*	$1.2*10^7$	<1	<1	<10	$>10^6$
Without Nisin, no incubation	<1	-	-	-	-
250 IU/ml Nisin*	10^7	-	-	-	-
500 IU/ml Nisin*	$>10^6$	-	-	-	-
500 IU/ml Nisin, Tindalisation*	110	-	-	-	-
Tindalisation only*	10^6	-	-	-	-

APC = *Aerobic plate count*

SRC = *Sulphite reducing clostridia*

SC+ve = *Staphylococci coagulase+ve*

* *Experimental conditions: Three day incubation at 30 ℃*

3.10 Lipolysis of Enzyme Modified Cheese base

The enzyme activity of the three different lipolytic enzymes, Palatase 20000 L, Kid lipase and Amano 'R' were investigated using the EMC base as the substrate.

100g samples of Tindalised EMC base, each containing 500Iu/ml Nisin, were heated at 40°C in a temperature controlled water bath for 1 h. The lipase enzyme was added to each 100g sample in a laminar flow cabinet. Samples were stirred well using sterilised stainless steel forceps. The enzyme treated samples were kept in temperature controlled water baths for incubation. At the end of the incubation period about 10 g of sample was collected and the enzyme was inactivated by heating at 95°C for 5 min in a closed water bath. 1 g of lipolysed sample was weighed into a Nalgane tube which was stored at ˉ20°C until FFA analysis.

The effect of different parameters on the activity of the three enzymes was investigated. The parameters studied were, incubation time, temperature, enzyme concentration, pH, water activity and salt-in-moisture. Each parameter was investigated under conditions where all other variables were held constant.

3.10.1 Incubation time and temperature

The different incubation temperatures and times that were used in the experiments are given in Table 3.2. The enzyme concentration was set constant for each enzyme (Table 3.2). The initial pH, water activity and salt content of the EMC base were held constant (pH 5.7, Aw 0.97 and salt 2.41%).

Table 3.2: The different incubation times, temperatures and enzyme concentrations used for the experiments

Type of Enzyme	Time range (h)	Temperature range (°C)	Enzyme concen. (% w/v)
Palatase	8, 24, 32, 48, 56, 72	30, 35, 40, 45, 50, 55, 60	0.15
Kid lipase	8, 24, 48, 72	30, 35, 40, 45, 50	1.0
Amano 'R'	4, 8, 24, 48, 72	20, 25, 30, 35, 40, 45, 50, 55, 60	1.0

3.10.2 Enzyme concentration

The different enzyme concentrations that were used in the experiments are given in Table 3.3. The incubation time and temperature were set constant for each enzyme (Table 3.3). The water activity (a_w), pH and salt content of the EMC base were also held constant (a_w 0.97, pH 5.7 and salt 2.41%).

Table 3.3: The different enzyme concentrations, incubation times and temperatures used for the experiments

Type of Enzyme	Enzyme concentration (% w/v)	Temperature (°C)	Time (h)
Palatase	0.075, 0.15, 0.3	45	24
Kid lipase	0.5, 1.0, 2.0, 4.0, 8.0	40	45
Amano 'R'	0.075, 0.15, 0.3, 1.0, 2.0, 4.0	30	24

3.10.3 Initial pH

The different initial pH levels of the EMC base that were used in the experiments were 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The pH of the EMC samples was adjusted using solutions of 50% NaOH and 30% HCl. The samples were heated to 45°C and stirred on a magnetic stirrer during pH adjustment. A pH meter with a calomel electrode was used to measure pH.

For the experiments, the incubation time, incubation temperature and enzyme concentration were held constant for each enzyme (Table 3.4). The water activity (a_w) and salt content of the EMC base were held constant (a_w 0.97 and salt 2.41%).

Table 3.4: The different incubation times, temperatures and enzyme concentrations used for the experiments

Enzyme	Temperature (°C)	Time (h)	Enzyme concentration (w/v)
Palatase	45	24	0.15
Kid lipase	40	48	1.0
Amano 'R'	30	24	1.0

3.10.4 Water activity

The different water activities of the EMC base that were used in the experiments were; 0.89, 0.91, 0.93, 0.95, 0.97. Initially the effect of the humectants sucrose and sorbitol on the water activity ($a_{w)}$ of the EMC base was measured using a water activity meter (CX2-Decagen) and a calibration graph was plotted to relate humectant % to water activity($a_{w)}$. The calibration graphs are shown in Figure 3.1 (using sucrose) and Figure 3.2 (using sorbitol). The EMC base was then adjusted to different a_w using sorbitol.

For the experiments, the incubation time, temperature and enzyme concentration as were held constant for each enzyme (Table 3.4). The water activity (a_w), pH and salt content of the EMC base were also held constant (a_w 0.97, pH 6.5 and salt 2.41%).

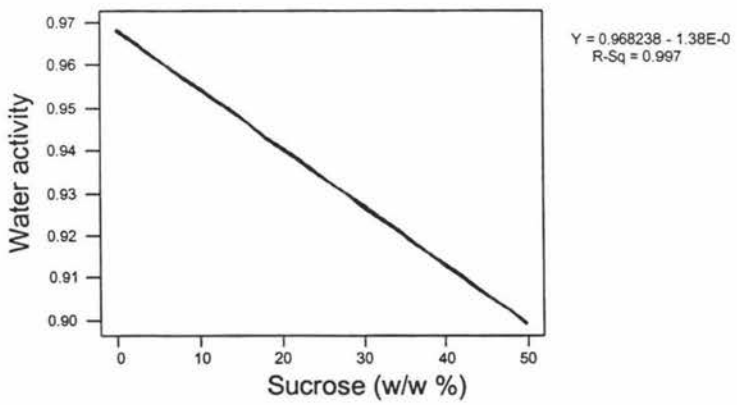

Figure 3.1: Effect of sucrose content on water activity of EMC base at pH 6.5

(Calibration graph)

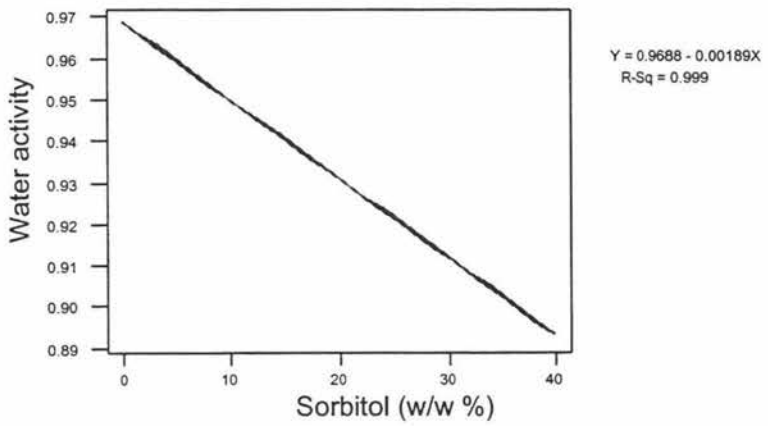

Figure 3.2: Effect of sorbitol content on water activity (a_w) of EMC base at pH 6.5

(Calibration graph)

3.10.5 Salt levels

The different salt in moisture levels of the EMC base that were used in the experiments were; 2.4%, 4.8%, 7.2% and 9.6%. The salt levels of the EMC samples were adjusted using sodium chloride (BDH -Analar grade). The samples were heated to 45°C and salt was added while stirring with a magnetic stirrer.

The incubation time, temperature and enzyme concentration were held constant for each enzyme (Table 3.4). The water activity (a_w) and initial pH of the EMC base were also held constant (a_w 0.97 and pH 6.5).

CHAPTER 4

RESULTS AND DISCUSSION

Amano'R' lipase activity on enzyme modified cheese

This chapter describes the effect of a number of parameters; incubation temperature, time, enzyme concentration, pH, water activity (a_w) and salt-in-moisture on the activity of Amano 'R' lipase on the triglycerides in the enzyme-modified cheese (EMC) base. The effect of these parameters on Amano 'R' activity was defined in terms of the amounts of fatty acid (FA) released by hydrolysis and the percentages of FAs released, expressed as a percentage of the total of that particular FA bound initially in the milk fat triglyceride, and as a percentage of the total free fatty acids (FFAs).

4.1 Incubation time

The effect of incubation time on the amounts of individual FAs released from the triglycerides in the EMC base is shown in Table 4.1. As expected, the extent of hydrolysis increased as the incubation time increased. After 72 h the amounts of individual FAs released were in the order C16:0>C18:1>C:4>C:14>C18:0>C6:0>C10:0>C12:0>C8:0>C18:2. The amounts of these released FAs (or free fatty acids), show a pattern which is similar to the FA composition of the triglycerides in the EMC base, (C16:0>C18:1>C14:0>C18:0>C4:0>C6:0>C10:0> C12:0>C8:0>C18:2, see Appendix 1, Table 4). The exception is butyric acid (C4:0) which tended to be hydrolysed to a greater extent.

Table 4.1: The effect of incubation time on the amounts of individual fatty acids released by Amano 'R' lipase*

Time	Free fatty acid (mmol FFA. kg EMC^{-1})										
(h)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FF*
0	0.13	0.05	0.05	0.10	0.12	0.23	0.63	0.51	0.80	0.03	2.65
4	24.9	10.9	4.2	7.1	5.9	16.5	41.7	15.7	29.9	0.5	157.3
8	31.8	14.0	5.6	9.6	8.1	22.4	56.5	20.3	39.0	0.6	208.0
24	58.3	24.3	9.8	16.2	14.0	40.3	101.5	38.7	69.5	1.8	374.4
48	62.2	26.1	10.9	18.2	16.3	47.3	119.2	43.7	78.2	2.0	424.0
72	74.7	31.7	14.0	23.6	21.5	62.3	151.8	56.7	100.7	2.8	539.8

* ***Experimental conditions: 1% Amano 'R', 30°C***

The rate of release of FAs from the triglycerides in the EMC base was greater during the first four hours of incubation and was approximately 40 mmol FA. kg $EMC^{-1}.h^{-1}$ (Figure 4.1). Thereafter the rate gradually slowed with time. A similar trend was shown for butyric acid, which had a rate of release of 6 mmol FA. kg $EMC^{-1}.h^{-1}$ during the first four hours of hydrolysis.

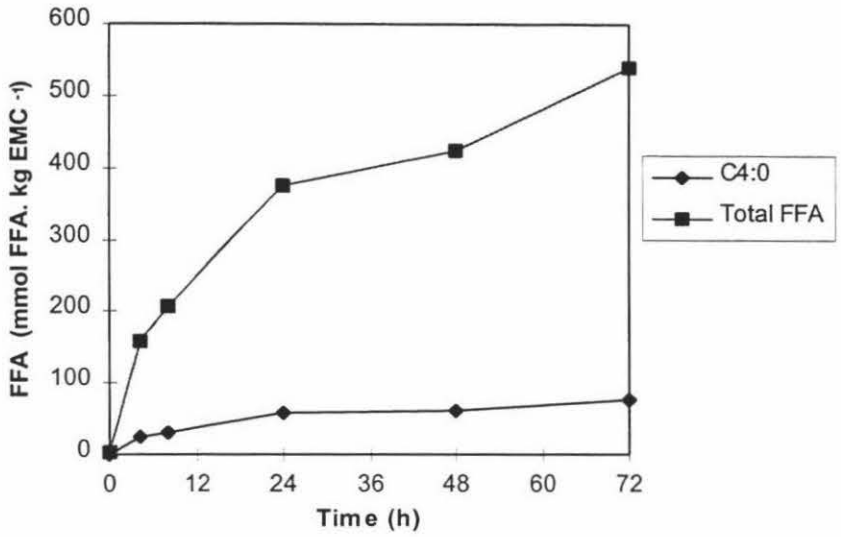

Figure 4.1: The effect of incubation time on the amount of butyric acid and total free fatty acids released by hydrolysis *(Experimental conditions: 1% Amano 'R', 30°C)*

The percentages of each individual FA (expressed as a percentage of the total of that particular FA) that were released by hydrolysis are presented in Table 4.2. The overall percentage of the FAs released was high, 65% after 72 h. Short-chain FAs (C4:0 to C10:0) were released to a greater extent than long-chain FAs (C12:0 to C18:0); the percentages after 72 h for C4:0, C6:0, C16:0 and C18:1 were 86%, 78%, 63% and 65% respectively.

This suggests that the Amano'R' enzyme shows some specificity towards short-chain FAs. This trend is supported by Eitenmiller *et al.*, 1970. who showed that, Amano 'R' lipase (*Penicillium roqueforti* lipase) released FAs from tributyrin, tricaprylin, tricaprin, tripropionin and triolein in decreasing order. Similarly, Huang and Dooley (1979), reported that the lipase from *Aspergillus niger* shows higher specificity towards short chain FAs (C4:0 to C8:0).

Table 4.2: The effect of incubation time on the percentages* of individual fatty acids released by Amano 'R' lipase#

Time	Free fatty acid (%)										
(h)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0	0.14	0.13	0.24	0.29	0.35	0.23	0.26	0.60	0.52	0.29	0.33
4	28.7	26.6	20.9	20.6	17.2	16.4	17.3	18.7	19.4	5.6	18.6
8	36.7	34.3	27.9	27.6	23.9	22.3	23.4	24.1	25.3	6.7	24.6
24	67.3	59.4	49.0	46.6	41.1	40.2	42.0	46.0	45.1	19.0	44.4
48	71.9	63.8	54.6	52.5	48.0	47.1	49.4	51.9	50.7	20.5	50.8
72	86.3	77.6	69.7	68.1	63.3	62.1	62.9	67.4	65.3	29.0	65.1

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 1% Amano 'R', 30°C***

The percentages of each individual FA (expressed as a percentage of the total free fatty acids) that were released by hydrolysis at different times during the incubation period are shown in Figure 4.2 (short-chain FAs) and Figure 4.3 (long-chain FAs). The percentage of butyric acid (C4:0) released by hydrolysis decreased slightly from 16% to 14% as the incubation time increased while C16:0 increased slightly, from 26% to 28%. All other FAs showed even smaller changes.

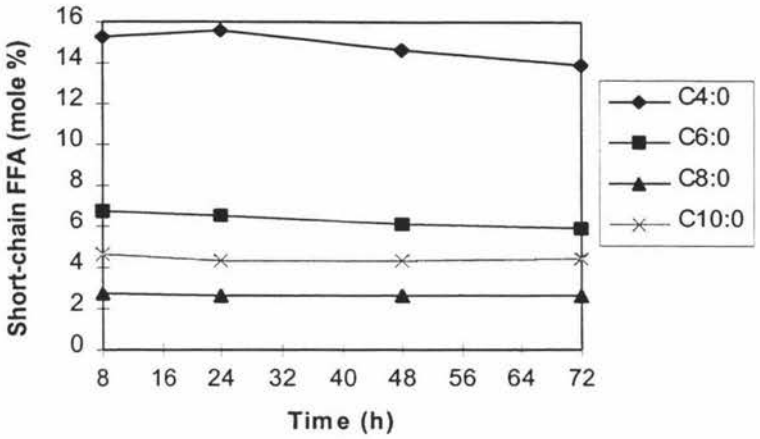

Figure 4.2: The effect of incubation time on the percentages of short-chain fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis

(Experimental conditions: 1% Amano 'R', 30°C)

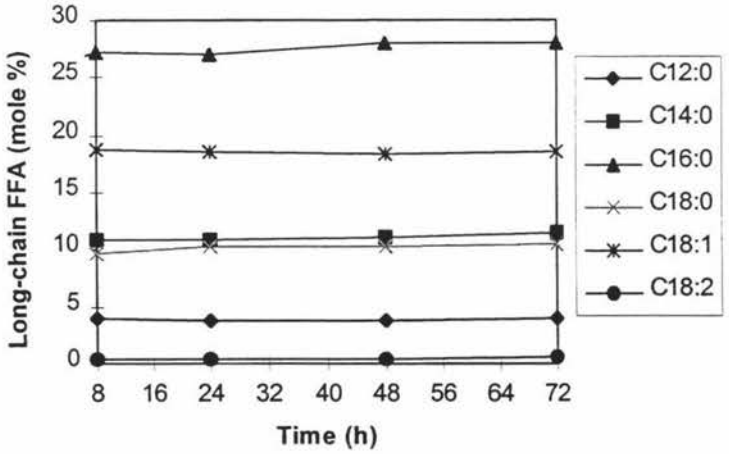

Figure 4.3: The effect of incubation time on the percentages of long-chain fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis

(Experimental conditions: 1% Amano 'R', 30°C)

4.2 Incubation temperature

The effect of incubation temperature on the amounts of individual FAs released from the triglycerides in the EMC base is shown in Table 4.3. The total amount of FAs released over the 24 h incubation period varied markedly with temperature (Figure 4.4). Large amounts of FAs (350 to 370 mmol FA. kg EMC^{-1}) were released over the temperature range 25° to 35°C. However, the amounts decreased sharply as the temperature increased from 40°C to 60°C and only about 30 mmol FA. kg EMC^{-1} were released at 60°C. A similar pattern was observed for individual FAs (Table 4.3).

Table 4.3: The effect of incubation temperature on the amounts of individual fatty acids released by Amano 'R' lipase*

Temperature (°C)	Free fatty acid (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
20	46.8	20.8	8.7	14.6	12.6	35.1	83.8	30.1	60.3	1.6	314.5
25	57.1	23.9	9.9	16.4	14.3	40.9	103.0	38.5	72.4	1.9	378.3
30	58.3	24.3	9.8	16.2	14.0	40.3	101.5	38.7	69.5	1.8	374.4
35	50.7	21.8	9.0	15.5	13.7	40.2	99.0	34.7	65.4	1.9	351.9
40	41.4	17.6	7.3	12.6	12.2	34.4	83.0	29.7	54.7	1.4	294.2
45	20.3	9.7	4.0	7.2	6.5	19.4	49.6	18.4	34.4	0.5	170.2
50	12.1	5.8	2.4	3.9	3.8	11.2	31.1	11.6	21.3	0.5	103.6
55	7.3	3.3	1.4	2.0	2.0	6.0	18.0	6.5	12.2	0	58.7
60	4.4	1.9	0.8	1.1	1.0	3.3	9.6	2.9	4.9	0	29.9

* ***Experimental conditions: 1% Amano'R', 24 h***

The results suggest that at temperatures above 35°C enzyme deactivation occurs. The degree of deactivation increased as the temperature increase from 40° to 60°C. Thus the effective operating temperature for Amano 'R' is in the range of 25° to 35°C. However, this lipase is more unusual compare to other microbial lipases. According to the Birschbach (1994), most microbial lipases exhibit temperature optima at approximately 45°C.

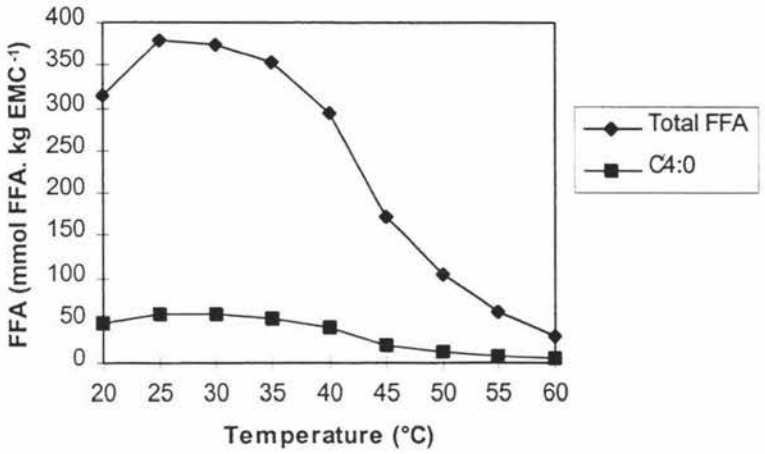

Figure 4.4: The effect of incubation temperature on the amounts of butyric acid and total fatty acids released by hydrolysis *(Experimental conditions: 1% Amano'R', 24 h)*

The percentages of each individual FA (expressed as a percentage of the total of that particular FA), that were released by hydrolysis at different incubation temperatures are shown in Table 4.4. The results show a similar pattern to that described above. Large percentages of individual FAs were released from the triglycerides over the temperature range 25° to 35°C with the percentages decreasing sharply as the temperature was increased from 40° to 60°C. At all incubation temperatures, the short-chain FAs, C4:0 and C6:0, were

released to a greater extent than the long-chain FAs (C12:0 to C18:2). For example, at an incubation temperature of 30°C, the percentages of C4:0 and C16:0 released were 67% and 42% respectively.

Table 4.4: The effect of incubation temperature on the percentages* of individual fatty acids released by Amano 'R' lipase#

Temperature (°C)	Free fatty acid (%)										
	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	Total FFA
20	54.0	51.0	43.6	41.9	37.2	35.0	34.7	35.8	39.1	17.1	37.4
25	65.9	58.6	49.2	47.2	42.1	40.8	42.7	45.7	47.0	20.3	45.1
30	67.3	59.4	49.0	46.6	41.1	40.2	42.0	46.0	45.1	19.0	44.4
35	58.5	53.5	45.1	44.5	40.4	40.1	41.0	41.2	42.4	19.9	42.2
40	47.8	43.1	36.5	36.3	35.8	34.3	34.4	35.3	35.5	14.4	35.4
45	23.5	23.8	20.2	20.8	19.1	19.3	20.6	21.9	22.3	5.3	20.9
50	14.0	14.1	12.0	11.2	11.1	11.1	12.9	13.8	13.8	5.1	12.8
55	8.5	8.0	6.9	5.8	5.8	6.0	7.5	7.7	7.9	0.0	7.2
60	5.0	4.7	3.8	3.2	3.1	3.2	4.0	3.5	3.2	0.0	3.6

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 1% Amano 'R', 24 h***

The percentages of four FAs (each expressed as a percentage of the total FFAs), that were released by hydrolysis at different temperatures, are shown in Figure 4.5. The data for all FAs are given in Appendix 2, Table 1.

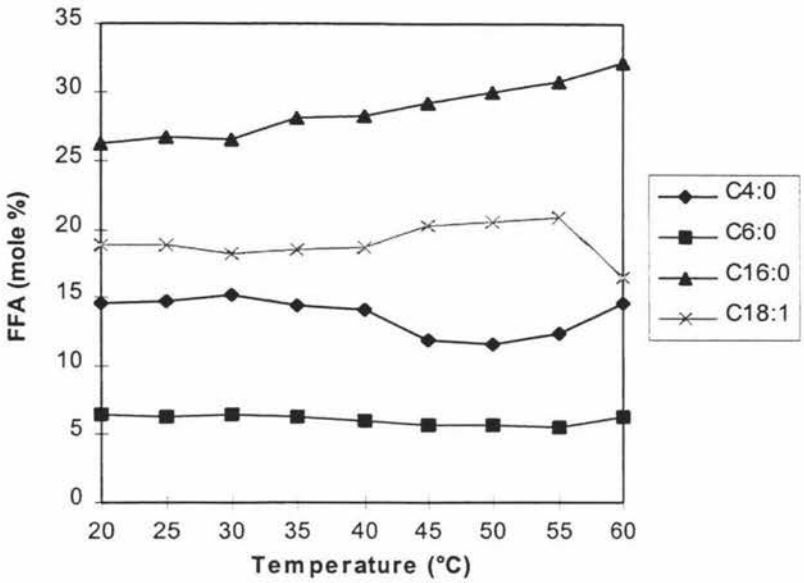

Figure 4.5: The effect of incubation temperature on the percentages of C4:0, C6:0 C16:0 and C18:1 FAs (expressed as percentages of the total FFA) that were released by hydrolysis *(Experimental conditions: 1% Amano 'R', 24 h)*

As the incubation temperature increased from 20° to 40°C there were only slight changes in the percentages of the FAs that were released by hydrolysis (Figure 4.5). In contrast, there were some significant variations in the percentages of these FAs at temperatures greater than 40°C; notably, the percentage of butyric acid dropped. However, these later results should be treated with caution as significant deactivation of the enzyme was taking place in the temperature range 45° to 60°C.

The relationship between the percentage of butyric acid released by hydrolysis (expressed as a percentage of the total FFA) and the total amount of FAs released (i.e. total FFAs) at different incubation times is shown in Figure 4.6. It can be seen that, the rate of release of butyric acid

by hydrolysis is greater at low incubation temperatures (20°C) than at high temperatures (40°C). Furthermore, the percentage of butyric acid reached a maximum and thereafter, decreased slightly.

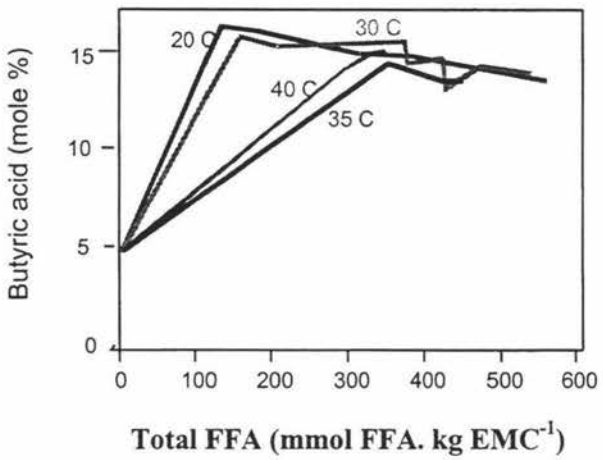

Figure 4.6: The relationship between the percentage of butyric acid that was released by hydrolysis (expressed as a percentage of the total FFA) and the total FFA concentration ***(Experimental conditions: 1% Amano 'R', 0 to 72 h)***

4.3 Enzyme concentration

The effect of Amano 'R' concentration on the amounts of individual FAs released from the triglycerides in the EMC base is shown in Table 4.5. The total amount of FAs released over the 24 h incubation period increased from 130 to 430 mmol FFA. kg EMC^{-1} as the enzyme concentration increased from 0.075% to 2.0% (Figure 4.7). However, the amount increased only slightly as the enzyme concentration increased from 2.0% to 4.0%. A similar pattern was observed for individual FAs with the short-chain FAs (C4:0 to C10:0) showing virtually no increase as the Amano 'R' concentration increased from 2.0% to 4.0%.

Table 4.5: The effect of Amano 'R' concentration on the amounts of individual fatty acids released by hydrolysis*

Enzyme concentration (%)	Free fatty acids (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0.075	23.5	9.6	3.4	5.1	4.2	12.6	35.0	13.9	25.5	0.4	133.3
0.15	29.7	12.1	4.5	7.4	6.2	19.0	51.2	20.8	37.1	0.8	188.7
0.3	39.6	16.4	6.4	10.8	9.4	27.6	71.3	26.3	48.4	1.0	257.1
1.0	53.6	22.7	9.2	15.7	13.9	40.9	100.0	36.6	66.9	1.7	361.2
2.0	60.1	25.5	11.0	18.6	17.0	49.8	121.9	45.9	81.5	2.2	433.5
4.0	59.3	25.6	11.3	19.3	18.1	53.3	130.7	49.4	86.2	2.3	455.3

* *Experimental conditions: 30 °C, 24 h*

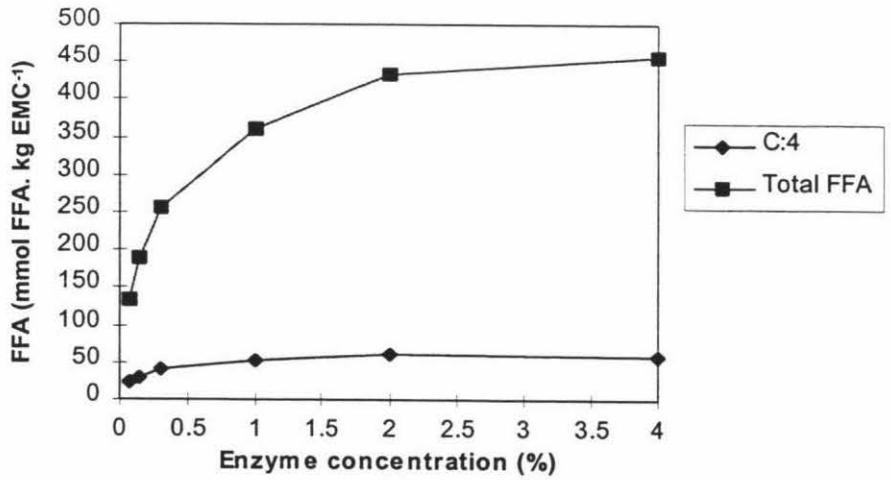

Figure 4.7: The effect of Amano 'R' concentration on the amounts of butyric acid and total fatty acids released by hydrolysis *(Experimental conditions: 30 °C, 24 h)*

The data indicate that an Amano 'R' concentration of about 2.0% is optimal for the hydrolysis of triglycerides in EMC.

The percentages of each individual FA (expressed as a percentage of the total of that particular FA), that were released by hydrolysis at different enzyme concentrations are presented in Table 4.6. The percentages of total FAs released increased from 16% to 52% as the enzyme concentration increased from 0.075% to 2.0%. At all enzyme concentrations, the short-chain FAs, C4:0 and C6:0 were released to a greater extent than the long-chain FAs (C12:0 to C18:0); the percentages at 2% concentration for C4:0, C6:0, C16:0 and C18:1 were 69%, 62%, 50% and 53% respectively.

Table 4.6: The effect of Amano'R' concentration on the percentages* of individual fatty acids released by hydrolysis#

Enzyme Concentration(%)	Free fatty acid (%)										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0.075	27.2	23.5	16.8	14.8	12.4	12.6	14.5	16.6	16.5	4.3	15.6
0.15	34.3	29.7	22.3	21.4	18.1	19.0	21.2	24.7	24.0	8.8	22.5
0.3	45.7	40.1	31.7	31.1	27.5	27.5	29.5	31.2	31.4	10.9	30.6
1.0	61.9	55.5	46.1	45.2	40.9	40.8	41.4	43.5	43.4	17.5	43.1
2.0	69.4	62.4	54.9	53.6	50.0	49.6	50.5	54.6	52.9	22.7	52.3
4.0	68.5	62.7	56.2	55.6	53.3	53.1	54.1	58.7	55.9	24.3	55.4

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 30 °C, 24 h***

The percentages of four FAs (each expressed as a percentage of the total FFAs) that were released by hydrolysis at different enzyme concentrations, are shown in Figure 4.8. The data for all FAs are given in Appendix 2, Table 2. As the enzyme concentration increased from 0.075% to 0.3% the percentages of C4:0 decreased from 17.5% to 15% while C16:0 increased from approximately 26% to 27.5%. Further increases in enzyme concentration produced a slight variations in the percentages of these two FAs. All other FAs were more or less constant. Above the optimal Amano 'R' concentration (about 2.0%) none of the FAs showed any appreciable variation.

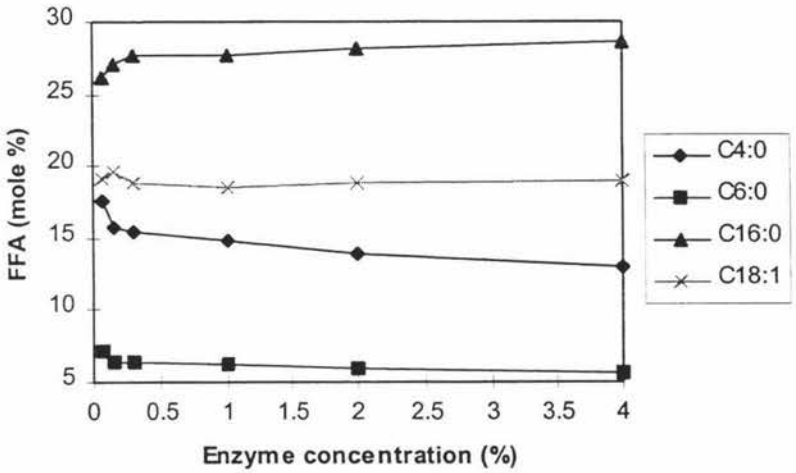

Figure 4.8: The effect of Amano'R' concentrations on the percentages of C4:0, C6:0 C16:0 and C18:1 FAs (expressed as percentages of the total FFA) that were released by hydrolysis *(Experimental conditions: 30°C, 24 h)*

4.4 Initial pH

The effect of the initial pH of the EMC base (i.e. pH prior to incubation) on the amounts of individual FAs released from the triglycerides in the EMC base is shown in Table 4.7. The total amount of FAs released over the 24 h incubation period increased from 290 to 500 mmol FA. kg EMC^{-1} as the initial pH was increased from 5.0 to 7.5 (Figure 4.9). However, the amounts of FAs released decreased markedly below pH 5.0 with only 50 mmol FA. kg EMC^{-1} was released at pH 4.0. A similar pattern was observed for individual FAs (Table 4.7).

Table 4.7: The effect of pH on the amounts of individual fatty acids released by Amano 'R' lipase*

pH	Free fatty acid (mmol FFA. kg EMC^{-1})										
	C4:o	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
4	4.4	2.9	1.5	2.5	2.3	6.6	16.8	6.2	10.9	0.3	54.4
4.5	27.1	11.8	5.1	8.9	7.9	24.3	63.8	24.4	44.1	1.2	218.6
5	41.8	17.8	7.4	12.7	11.0	33.1	81.7	29.5	53.7	1.4	290.2
5.5	47.7	20.7	8.5	14.5	12.7	37.7	92.0	35.3	64.0	1.5	334.6
5.7	53.6	22.7	9.2	15.7	13.9	40.9	100	36.6	66.9	1.7	361.2
6	55.2	23.5	9.8	16.7	15.0	43.6	105.4	40.6	73.1	1.9	384.8
6.5	58.7	26.1	11.1	18.8	17.2	47.6	113.5	42.0	76.5	2.0	413.5
7	63.3	28.1	12.1	20.9	18.7	54.4	131.3	49.8	90.1	2.3	471.0
7.5	71.4	30.9	12.8	21.5	19.5	57.0	138.3	51.9	94.3	2.4	500.0
8	67.7	30.3	12.8	22.0	20.6	59.1	142.4	53.4	97.4	2.5	508.2

* ***Experimental conditions: 1% Amano 'R', 30°C, 24 h***

These results suggest that the optimal pH range for this enzyme is about 7.5. However, Arnold *et al.*(1974), reported that the pH optima for *Penicillium roqueforti* lipase is about 9.0. It should be noted that the EMC base has a pH of 5.7 and this was the pH at which several of the experiments were carried out (e.g. incubation time, temperature).

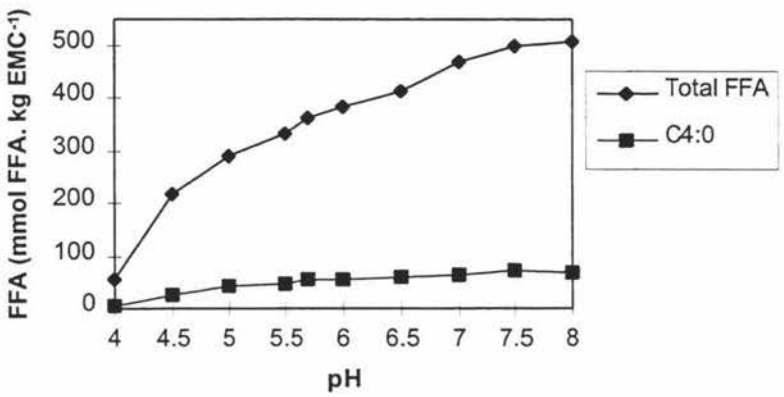

Figure 4.9: The effect of pH on the amounts of butyric acid and total fatty acids released by hydrolysis *(Experimental conditions 1% Amano 'R', 30 °C, 24h)*

A probable reason for the decrease in the rate of hydrolysis as the pH is decreased is the denaturation of the enzyme at low pH. Another reason for the change may be due to a change in the strength of the emulsion. At the iso-electric point of the cheese proteins (pH 4.6), they have no net charge, minimal solubility and minimal interaction with other proteins. This may lead to the weakening of the fat emulsion (Shimp, 1985) which in turn may lead to a slower rate of hydrolysis since it is known that lipases have greater activity on emulsified substrates than in free solution (Kilara, 1985; Brockman, 1984; Drewenda *et al.*, 1992). Conversely, as the pH is increased towards pH 7.0 proteins become better emulsifying agents (Shimp, 1984) and thus, the extent of FAs hydrolysed is increased. This explanation is in good agreement

with those reported by Richardson and Nelson (1967), who observed that fungal lipase activity was significantly higher in emulsified substrate than in free solution.

The percentages of each individual FA (expressed as a percentage of the total of that particular FA) that were released by hydrolysis at different pH values are given in Table 4.8. The results show a similar pattern to that described above. The percentages of individual FAs that were released from the triglycerides increased steadily as the pH values increased from 5

Table 4.8: The effect of pH on the percentages* of individual fatty acids released by Amano 'R' lipase#

pH	Free fatty acid (%)										
	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	Total FFA
4	5.0	7.1	7.6	7.3	6.8	6.6	7.0	7.4	7.0	3.6	6.9
4.5	31.3	28.9	25.4	25.7	23.2	24.2	26.4	28.9	28.6	12.2	26.9
5	48.3	43.6	36.8	36.7	32.5	33.0	33.9	35.0	34.8	14.8	34.8
5.5	55.1	50.6	42.7	41.8	37.4	37.6	38.1	41.9	41.5	16.0	40.2
5.7	61.9	55.5	46.1	45.2	40.9	40.8	41.4	43.5	43.4	17.5	43.1
6	63.7	57.7	49.0	48.2	44.1	43.5	43.7	48.2	47.4	19.4	46.2
6.5	67.8	63.9	55.4	54.1	50.6	47.4	47.0	50.0	49.6	20.5	49.5
7	73.1	68.7	60.3	60.2	55.1	54.3	54.4	59.1	58.5	23.7	57.0
7.5	82.4	75.6	64.2	61.9	57.5	56.8	57.3	61.7	61.2	25.4	60.0
8	78.2	74.1	63.8	63.4	60.6	58.9	59.0	63.5	63.2	25.7	61.6

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 1% Amano 'R', 30°C, 24 h***

to 7.5. At all pH values above 4.5, the short-chain FAs, C4:0 and C6:0 were released to a greater extent than the long-chain FAs (C12:0 to C18:0). For instance, at pH 7.5, the percentages of C4:0 and C16:0 hydrolysed were 82% and 57% respectively. This again suggests that the enzyme shows some specificity towards short-chain FAs.

The percentages of four FAs (each expressed as a percentage of the total FFAs) that were released by hydrolysis at different pH levels, are shown in Figure 4.10. The data for all FAs are given in Appendix 2, Table 3. As the pH increased from 5 to 8 there were only slight changes in the percentages of the FAs that were released by hydrolysis (Figure 4.10). In contrast, there were significant variations in the percentages of C4:0 and C16:0 at pH lower than 5. These changes may be tied up with the deactivation of the enzyme which was taking place in the pH range 4.0 to 4.5.

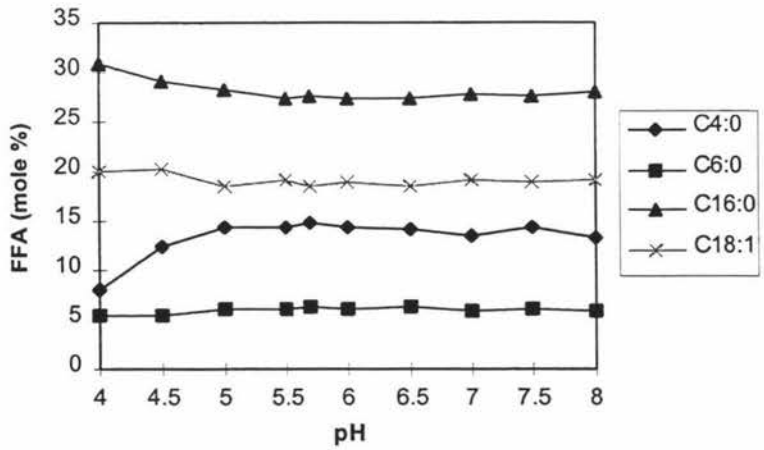

Figure 4.10: The effect of pH on the percentages of C4:0, C6:0 C16:0 and C18:1 fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis *(Experimental conditions: 1% Amano 'R', 30 °C, 24 h)*

4.5 Water activity (a_w)

The effect of water activity (a_w) on the amounts of FAs released is shown in Table 4.9. The total amount of FAs released over the 24 h incubation period increased from 335 to 385 mmol FA. kg EMC^{-1} as the water activity increased from 0.89 to 0.97 (Figure 4.11).

The reduction in the water activity of the EMC base will cause a reduction in unbound water, which could possibly lead to a lowering of the solubility of milk protein. This in turn could cause a weakening of the fat emulsion and a slower rate of hydrolysis as discussed in the previous section. This explanation fits the observed trend although the variation in rate of lipolysis is relatively small.

Table 4.9: The effect of water activity (a_w) on the amounts of individual fatty acids released by Amano 'R' lipase*

a_w	Free fatty acids (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0.89	49.5	21.5	8.8	14.6	12.7	37.2	93.4	33.6	63.2	1.3	336.1
0.91	50.2	21.6	8.9	14.7	13.0	38.1	92.1	32.6	61.7	1.4	334.3
0.93	53.3	22.5	9.2	15.3	13.3	40.0	100.7	37.5	67.2	1.7	360.8
0.95	55.8	24.4	10.1	16.9	14.7	42.9	106.2	39.2	71.3	2.0	383.5
0.97	53.8	23.4	9.8	16.4	14.8	43.1	108.4	39.6	73.5	1.7	384.6

* ***Experimental conditions: 1% Amano 'R', pH 6.5, 30°C, 24 h***

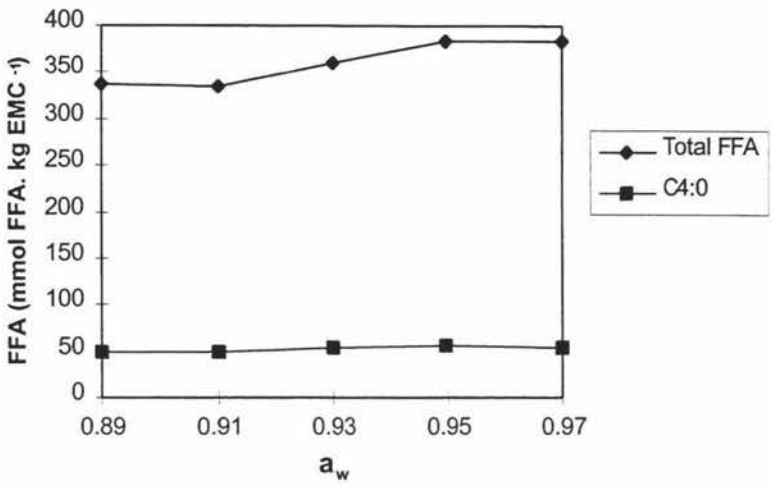

Figure 4.11: The effect of water activity (a_w) on the amounts of butyric acid and total fatty acids released by hydrolysis ***(Experimental conditions 1% Amano'R', pH 6.5, 30°C, 24h)***

The percentages of each individual FA (expressed as a percentage of the total of that particular FA) that were released by hydrolysis at different water activities are presented in Table 4.10. Large percentages (40 to 65%) of individual FAs were released from the triglycerides over the a_w range 0.89 to 0.97. At all water activities, the short-chain FAs, C4:0 and C6:0 were released at a greater extent than the long-chain FAs (C12:0 to C18:2).

Table 4.10: The effect of water activity (a_w) on the percentages* of individual fatty acids released by Amano 'R' lipase#

a_w	Free fatty acid (%)										
	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	Total FFA
0.89	58.0	53.5	44.7	42.7	38.0	37.7	39.2	40.5	41.6	13.8	40.7
0.91	58.9	53.8	45.1	43.0	38.7	38.5	38.8	39.3	40.7	14.4	40.4
0.93	62.6	56.0	46.9	44.7	39.9	40.6	42.4	45.3	44.3	17.8	43.9
0.95	65.6	60.7	51.1	49.5	44.2	43.5	44.8	47.5	47.1	20.8	46.7
0.97	63.4	58.5	49.8	48.3	44.5	43.9	45.8	48.0	48.6	17.9	47.3

* *expressed as a percentage of the total of that particular FA*

\# *Experimental conditions: 1% Amano 'R', pH 6.5, 30°C, 24 h*

The percentages of individual FAs (each expressed as a percentage of the total FFAs) that were released by hydrolysis showed little variation over the range of water activities 0.89 to 0.97 (The data are given in Appendix 2, Table 4).

4.6 Salt-in-moisture content

The effect of salt-in-moisture content on the amounts of individual FAs released from the triglycerides is shown in Table 4.11. The total amounts of FAs released over the 24 h incubation period remained constant as the salt-in-moisture increased from 2.4% to 4.8%. The FAs then decreased from 390 to 340 mmol FA. kg EMC^{-1} as salt-in-moisture increased from 4.8% to 9.6% (Figure 4.12).

Table 4.11: The effect of salt-in-moisture content on the amounts of individual fatty acids released by Amano 'R' lipase*

Salt (% in moisture)	Free fatty acid (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
2.4	54.9	23.9	10.0	16.8	15.1	44.0	110.6	40.4	75.0	1.7	392.3
4.8	56.1	23.8	9.9	16.9	15.0	45.0	110.0	41.0	74.7	1.8	394.3
7.2	51.1	22.4	9.4	16.0	14.4	42.7	104.9	37.0	68.2	1.8	367.9
9.6	46.9	20.8	8.7	14.5	13.3	40.2	97.8	34.8	62.8	2.9	342.9

* *Experimental conditions: 1% Amano 'R', pH 6.5, 30°C, 24 h*

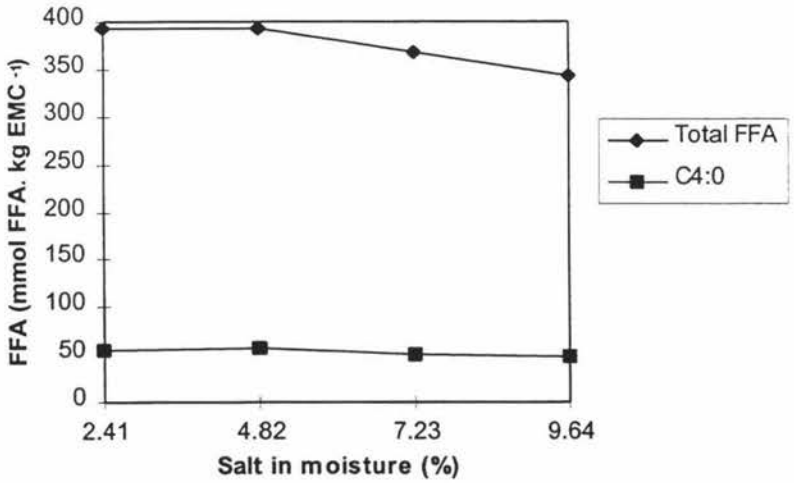

Figure 4.12 :The effect of salt-in-moisture content on the amounts of butyric acid and total fatty acids released by hydrolysis *(Experimental conditions: 1% Amano 'R', pH 6.5, 30°C, 24 h)*

The slight decrease in extent of hydrolysis with increasing salt content could possibly be due to a slight denaturation of the enzyme at high salt concentration or substrate limitation, since water is a substrate in the reaction. Also Moskowitz *et al.* (1977) reported that sodium chloride appears to have little or no effect on the activity of microbial lipases.

The percentages of each individual FA (expressed as a percentage of the total of that particular FA) that were released by hydrolysis at different salt-in-moisture contents are shown in Table 4.12. Reasonably large percentages of individual FAs were released from the triglycerides over the entire salt range 2.4% to 9.6% with the percentages decreasing slightly as the salt-in-moisture content increased. Again the short-chain FAs, C4:0 and C6:0, were released at a greater extent than the long-chain FAs (C12:0 to C18:2).

Table 4.12: The effect of salt-in-moisture content on the percentages* of individual fatty acids released by Amano 'R' lipase#

Salt (% in moisture)	Free fatty acids (%)										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
2.4	63.4	58.5	49.8	48.3	44.5	43.9	45.8	48.0	48.6	17.9	47.3
4.8	64.8	58.3	49.5	48.6	44.3	44.9	45.6	48.7	48.5	18.8	47.4
7.2	59.0	54.7	47.1	46.1	42.5	42.6	43.5	43.9	44.3	19.0	44.3
9.6	54.2	51.0	43.4	41.8	39.1	40.1	40.5	41.4	40.8	30.2	41.4

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 1% Amano 'R', pH 6.5, 30°C, 24 h***

The percentages of individual FAs (each expressed as a percentage of the total FFAs), that were released by hydrolysis at different salt-in-moisture contents, are given in Appendix 2, Table 5. As the salt content increase from 2.4% to 9.6%, neither short-chain FFAs (C4:0 to C10:0) nor long-chain FFAs (C12:0 to C18:2) showed any appreciable variations.

SUMMARY

These Investigations show that incubation temperature and pH of the substrate (EMC base) have a marked effect on the activity of the Amano 'R' lipase on the EMC base. The optimal temperature range was 25° to 35°C and the amount of FAs released by hydrolysis decreased substantially as the incubation temperature increased from 40°C to 60°C. The optimum pH was 7.5 and there was a marked decreased in enzyme activity as the pH of the EMC base dropped below pH 5.0.

The impact of incubation time and enzyme concentration showed the expected trends, with the amounts of FAs that were released by hydrolysis increasing as time increased and as concentration increased. There were relatively small decreases in Amano 'R' activity as water activity decreased and as salt concentration increased.

The variation in Amano 'R' activity can be largely explained in terms of deactivation of the enzyme under certain experimental conditions (e.g. at temperatures above 40°C) and possibly variation in the emulsifying power of the milk protein in the EMC base at different pH values (e.g. at pH 4.6) and water activities.

The results showed that under optimum conditions, Amano 'R' lipase caused considerable lipolysis which released both short-chain and long-chain FAs in the EMC base. However, for all experiments, the Amano 'R' enzyme showed some specificity towards short-chain FAs. The percentages of C4:0 and C6:0 released by hydrolysis were always greater than the corresponding percentages of the long-chain FAs. Incubation temperature also had a slight effect on the rate of release of C4:0, with relatively greater amounts released at lower temperatures.

The amounts of C4:0 and C6:0 that were released, relative to the other FAs will influence the flavour profile and intensity of cheese (Deeth and Fitz-Gerald, 1987 and Harboe, 1994). According to the several studies the lipases secreted by *Penicillium roqueforti* (Amano 'R') are major contributors to the development of Blue cheese flavour (Revah and Lebeault, 1989; King and Clegg, 1979; Dziezak, 1986; Moskowitz and Noelck, 1987).

CHAPTER 5

RESULTS AND DISCUSSIONS

Palatase enzyme activity on Enzyme-Modified Cheese

This chapter describes the effect of a number of parameters; incubation temperature, time, enzyme concentration, pH, water activity (a_w) and salt-in-moisture on the activity of Palatase 20000 L lipase on the triglycerides in the enzyme-modified cheese (EMC) base. The effect of these parameters on Palatase activity was defined in terms of the amounts of fatty acids (FAs) released by hydrolysis and the percentages of FAs released, expressed as a percentage of the total of that particular FA originally bound in the triglyceride, and as a percentage of the total free fatty acids (FFAs).

5.1 Incubation time

The effect of incubation time on the amounts of individual FAs released from the triglycerides in the EMC base is shown in Table 5.1. As expected, the extent of hydrolysis increased as the incubation time increased. After 72 h the amounts of individual FAs released were in the order C16:0>C18:1>C14:0>C18:0>C4:0>C6:0>C10:0>C12:0>C8:0>C18:2. The amounts of these released FAs, which are the same as the FFAs, show a pattern which is similar to the FA composition of the triglycerides in the EMC base.

Table 5.1: The effect of incubation time on the amounts of individual fatty acids released by Palatase lipase*

Time	Free fatty acid (mmol FFA. kg EMC^{-1})										
(h)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0	0.1	0.1	0.0	0.1	0.1	0.2	0.6	0.5	0.8	0.0	2.8
8	15.8	10.5	6.3	9.6	9.2	24.0	69.0	25.2	43.9	1.6	215.1
24	32.9	20.7	10.6	16.6	16.4	42.7	110.6	33.7	58.0	1.7	343.9
32	45.4	27.1	13.1	20.7	19.3	49.9	121.2	35.4	64.3	1.5	397.9
48	48.7	28.3	13.6	22.5	22.4	60.6	148.8	43.6	85.9	3.3	477.6
56	53.3	29.2	13.8	23.3	23.4	61.7	150.2	44.1	84.4	3.4	486.9
72	60.7	31.8	14.3	24.1	24.2	67.6	167.5	51.5	99.8	4.0	545.6

* ***Experimental conditions: 0.15% Palatase, 45 °C***

The rate of release of FAs from the triglycerides in the EMC base was greatest during the first eight hours of incubation and was approximately 27 mmol FFA. kg EMC.$^{-1}$ h^{-1} (Figure 5.1). Thereafter the rate gradually slowed with time. A similar trend was shown by butyric acid, which had a rate of release of 2 mmol FFA. kg EMC.$^{-1}$ h^{-1} during the first eight hours.

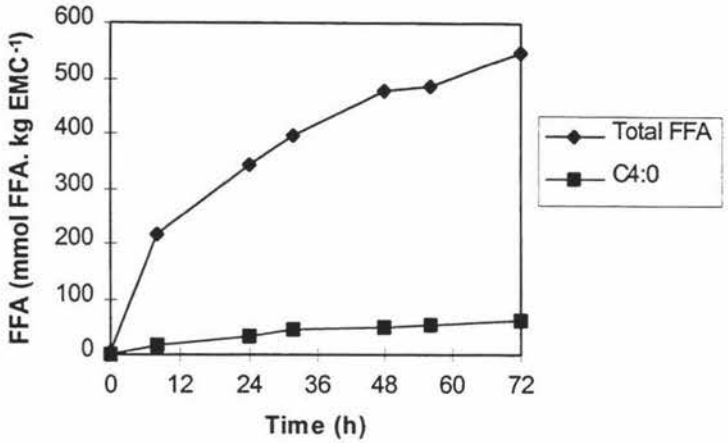

Figure 5.1: The effect of incubation time on the amounts of butyric acid and total fatty acids released by hydrolysis *(Experimental conditions: 0.15% Palatase, 45 °C)*

The percentages of each individual FAs (expressed as a percentage of the total of that particular FA) that were released by hydrolysis are presented in Table 5.2. The overall percentage of the FAs released was high, 85% after 72 h. However, butyric acid (C4:0) was released at a lower extent than other FAs; the percentage after 72 h for C4:0, C6:0, C16:0 and C18:1 were 64%, 80%, 88% and 90% respectively. Moreover, FAs longer than C14:0 (C16:0 to C18:1) were hydrolysed to a greater extent than the rest of the FAs (C4:0 to C14:0).

This suggests that the Palatase enzyme shows some specificity towards long-chain FAs especially C16:0, C18:0 and C18:1. This trend is supported by Lee and Rickansrud, 1979 who showed that, Palatase (*Mucor miehei* lipase) enzyme preferentially hydrolysed ester bonds containing FAs longer than myristic acid (C14:0). Also a long chain FAs specificity was reported for the lipase secreted by *Geotrichum Candidum* (De Greyt and Huyghebaert,1995).

Table 5.2: The effect of incubation time on the percentages* of individual fatty acids released by Palatase lipase#

Time	Free fatty acid (%)										
(h)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
8	13.41	19.7	25.6	21.81	23.73	18.86	26.3	36.1	29.6	53.7	26.87
24	26.73	39.3	44.7	41.41	41.7	36.94	45.2	48.3	45	32	43.14
48	44.74	61.7	64.5	62.81	61.93	60.33	70.2	72.9	69.1	51.2	66.86
72	63.72	80.2	80.9	81.37	80.39	78.47	88.4	86.4	90.2	65.2	85.11

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 0.15% Palatase, 45 °C***

The percentages of each individual FA (expressed as a percentage of the total FFAs) that were released by hydrolysis at different times during the incubation period are shown in Figure 5.2 (short-chain FAs) and Figure 5.3 (long-chain FAs). The percentages of butyric acid (C4:0) and caproic acid (C6:0) released by hydrolysis increased slightly as the incubation time increased, from 8 to 32 h, while long chain FAs (C16:0, C18:0, C18:1) decreased slightly over the 32 h incubation period. However, the percentages of FAs which longer than C14:0, were very high, compared to FAs shorter than C14:0, at any given time. For example, at an incubation time 72 h, the percentages of C4:0, C6:0, C16:0 and C18:1 were 11%, 6%, 31% and 18% respectively.

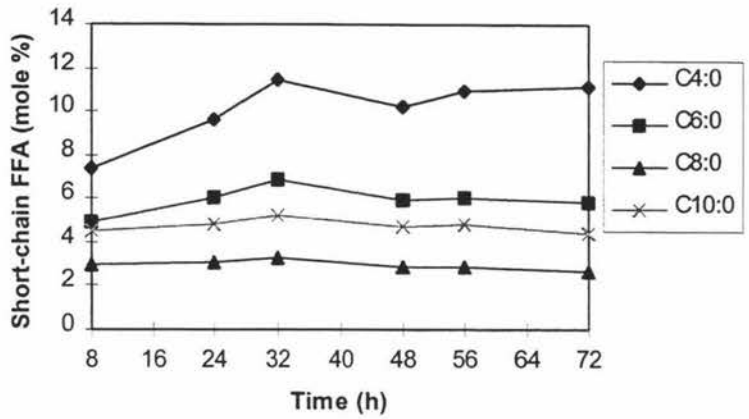

Figure 5.2: The effect of incubation time on the percentages of short-chain fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis ***(Experimental conditions: 0.15% Palatase, 45°C)***

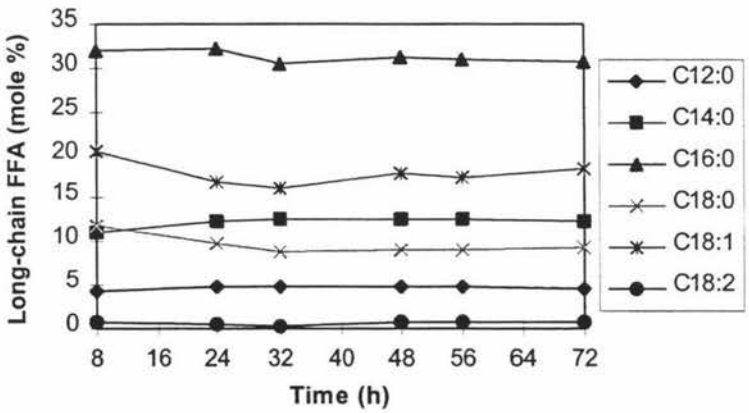

Figure 5.3: The effect of incubation time on the percentages of long-chain fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis ***(Experimental conditions: 0.15% Palatase, 45°C)***

5.2 Incubation temperature

The effect of incubation temperature on the amounts of individual FAs released from the triglycerides in the EMC base is shown in Table 5.3. The total amounts of FAs released over the 24 h incubation period varied markedly with temperature (Figure 5.4). The amount increased gradually as the temperature increased from 30°C to 40°C, and thereafter increased rapidly to 55°C. Large amounts of FAs (509 to 540 mmol FA. kg EMC^{-1}) were released over the temperature range 55° to 60°C. However, the amounts decreased above 55°C. This was obvious at 48 h incubation period (Figure 5.4). A similar pattern was observed for individual FAs.

Table 5.3: The effect of incubation temperature on the amounts of individual fatty acids released by Palatase lipase*

Temperature (°C)	Free fatty acid (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FAA*
30	24.0	13.8	6.7	9.0	7.8	20.9	70.3	28.9	41.5	1.1	224.1
35	30.1	16.7	8.4	11.4	11.6	25.4	71.5	24.7	43.3	0.9	244.0
40	22.3	14.2	7.5	11.6	11.8	30.4	84.9	28.8	49.6	1.6	262.8
45	32.9	20.7	10.6	16.6	16.4	42.7	110.6	33.7	58.0	1.7	343.9
50	29.1	19.8	10.9	18.2	17.8	49.8	141.8	49.8	93.5	3.9	434.5
55	34.1	23.1	12.4	21.2	21.2	59.8	163.5	59.5	107.4	6.6	508.9
60	31.5	24.4	13.7	23.5	23.7	66.4	177.4	60.0	114.2	4.9	539.9

* ***Experimental conditions: 0.15% Palatase, 24 h***

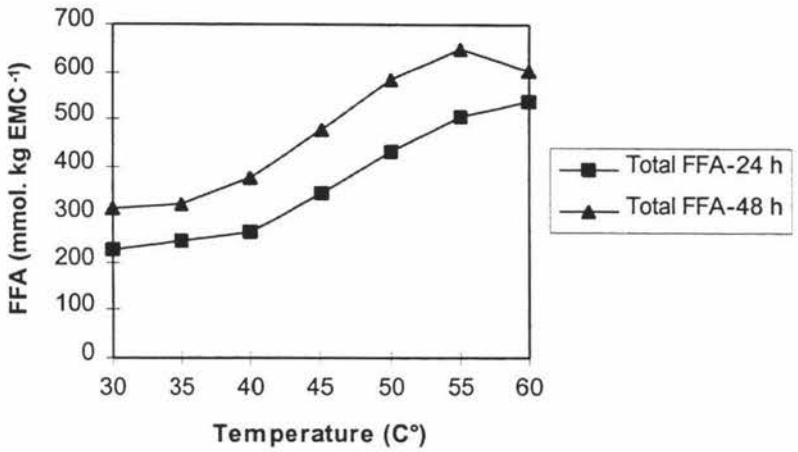

Figure 5.4: The effect of incubation temperature on the amounts of total fatty acids released by hydrolysis at 24 h and 48 h incubation periods *(Experimental conditions: 0.15% Palatase)*

The results suggest that at temperatures above 55°C enzyme deactivation occurs with the degree of deactivation increasing as the incubation time increased from 48 to 72 h and temperature increased from 55°C to 60°C. Also, the Palatase enzyme activity is very high at 40°C to 55°C. Thus the effective operating temperature range for Palatase is 40°C to 55°C. This observed trend is supported by Birschbach (1994), who reported that the most microbial lipases exhibit temperature optimal at approximately 45°C.

The percentages of each individual FA (expressed as a percentage of the total of that particular FA) that were released by hydrolysis at different incubation temperatures are shown in Table 5.4. The results shows a similar pattern to that described above. Large percentages of individual FAs were released from the triglycerides over the temperature range 55° to 60°C. At higher incubation temperatures (40°C to 60°C), the FAs which are lower than C14:0

(C4:0 to C14:0) were released at a lower extent than the FAs which are higher than C14:0 (C16:0 to C18:1). For example, at an incubation temperature of 55°C, the percentages of C4:0, C6:0, C16:0 and C18:1 were 39%, 57%, 68%, 70% respectively. In all cases, the lowest percentage was observed in butyric acid (C4:0). Long chain FA selectivity (C16:0 to C18:0) of Palatase enzyme was more apparent at higher temperatures.

Table 5.4: The effect of incubation temperature on the percentages* of individual fatty acids released by Palatase lipase#

Temperature	Free fatty acid (%)										
(°C)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
30	27.8	33.9	33.4	25.9	23.1	20.8	29.1	34.3	26.9	11.4	27.8
35	34.8	40.8	41.8	32.9	34.2	25.3	29.6	29.4	28.1	9.6	29.4
40	25.7	34.7	37.6	33.5	34.8	30.3	35.2	34.2	32.2	17.0	33.0
45	26.7	39.3	44.7	41.4	41.7	36.9	45.2	48.3	45.0	32.0	43.1
50	33.5	48.4	54.5	52.5	52.5	49.7	58.7	59.1	60.6	41.2	56.0
55	39.3	56.5	62.0	60.9	62.5	59.7	67.7	70.7	69.7	69.2	65.6
60	36.4	59.7	68.5	67.6	69.9	66.2	73.5	71.3	74.1	51.5	69.8

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 0.15% Palatase, 45°C, 24 h***

The percentages of four FAs (each expressed as a percentage of the total FFAs), that were released by hydrolysis at different temperatures, are shown in Figure 5.5. The data for all FAs are given in Appendix 3, Table 1. As the incubation temperature increased from 30° to 50°C

the percentages of C4:0 decreased (from 11% to 7%) while C18:1 increased from approximately 18% to 21%. All other FAs show smaller decreases or increases. Over the optimal temperature range, 50° to 60°C, neither C4:0 nor C16:0 showed any appreciable variation.

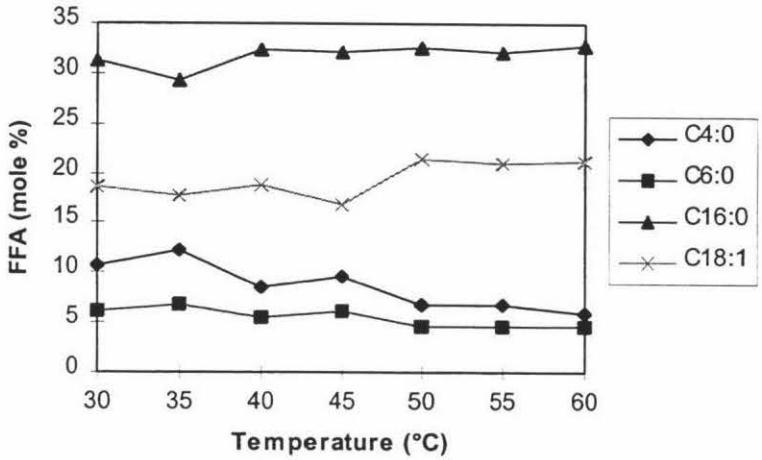

Figure 5.5: The effect of incubation temperature on the percentages of C4:0, C6:0, C16:0 and C18:1 fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis *(Experimental conditions: 0.15% Palatase, 24 h)*

The relationship between the percentage of butyric acid released by hydrolysis (expressed as a percentage of total FFA) and the total amount of FAs released (i.e. Total FFAs) at different incubation times is shown in Figure 5.6. It can be seen that during the initial stages of incubation, when the total FFAs are relatively low, the rate of release of butyric acid by hydrolysis is very low. Furthermore, there is a greater release of C4:0 at low incubation temperatures (30° to 35°C) than at high temperature (50° to 60°C). It can be concluded from

these data that butyric acid formation is selectively increased at lower temperatures. The increasing temperature has a detrimental effect on butyric acid formation in EMC.

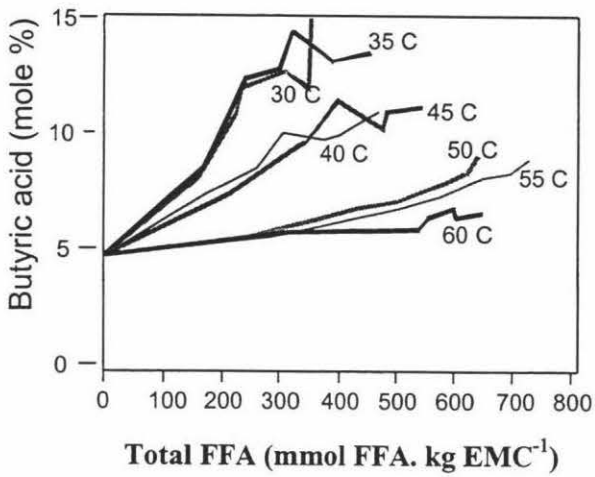

Figure 5.6: The relationship between the percentage of butyric acid that was released by hydrolysis (expressed as a percentage of the total FFA) and the total FFA ***(Experimental conditions: 0.15% Palatase, 0 to 72 h incubation period)***

5.3 Enzyme concentration

The amounts of individual FAs released from the triglycerides in the EMC base at various enzyme concentrations are shown in Table 5.5. The total amount of FAs released over the 24 h incubation period increased from 223 to 344 mmol FFA. kg EMC^{-1} as the enzyme concentration increased from 0.075% to 0.15% (Figure 5.7). The amounts were fairly constant as the enzyme concentration increased from 0.15% to 0.3%. A similar pattern was observed for individual FAs.

Table 5.5: The effect of Palatase concentration on the amounts of individual fatty acids released by hydrolysis*

Enzyme concentration (%)	Free fatty acid concentration (mmol FA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0	0.1	0.1	0.0	0.1	0.1	0.2	0.6	0.5	0.8	0.0	2.7
0.075	16.2	11.6	7.0	11.0	10.8	29.6	69.4	27.2	38.0	2.0	222.7
0.15	32.9	20.7	10.6	16.6	16.4	42.7	110.6	33.7	58.0	1.7	343.9
0.3	27.3	16.9	9.4	15.6	15.0	41.5	102.4	37.0	58.3	2.3	325.7

* *Experimental conditions: 45 °C, 24 h*

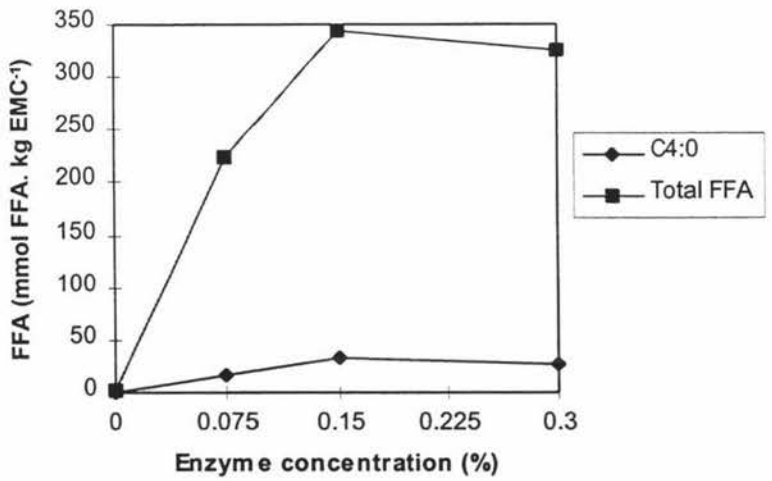

Figure 5.7: The effect of Palatase concentration on the amounts of butyric acid and total fatty acids released by hydrolysis *(Experimental conditions: 45 °C, 24 h)*

The data suggest that a Palatase concentration of 0.15% is optimal for the hydrolysis of triglycerides in EMC.

The percentages of each individual FA (expressed as a percentage of the total of that particular FA) that were released by hydrolysis at various enzyme concentrations are presented in Table 5.6. The overall percentage of the FAs released increased from 28% to 42% as the enzyme concentration increased from 0.075% to 0.15%. At all enzyme concentrations, the butyric acid (C4:0) was released at a lower extent than other FAs (C6:0 to C 18:1). For example, at 0.15%, the percentages of C4:0 and C16:0 were 38% and 46% respectively.

Table 5.6: The effect of Palatase concentration on the percentages* of individual fatty acids released by hydrolysis #

Enzyme Concentration (%)	Free fatty acid (%)										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0.075	18.7	28.3	34.8	31.6	31.8	29.5	28.7	32.4	24.6	20.8	28.1
0.15	38.0	50.7	52.9	47.9	48.3	42.6	45.8	40.0	37.6	17.4	42.4
0.3	31.5	41.4	47.1	44.9	44.1	41.4	42.4	43.9	37.8	23.9	40.9

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 45 °C, 24 h***

The percentages of four FAs (each expressed as a percentage of the total FFAs), that were released by hydrolysis at different enzyme concentrations, are shown in Figure 5.8. The data for all FAs are given in Appendix 3, Table 2. As the enzyme concentration was increased from 0.075% to 0.15% the percentages of C4:0 increased (from about 7% to 10%) while all other FAs showed only small decreases or increases. From 0.15% to 0.3%, none of the FAs showed any appreciable variation (Figure 5.8).

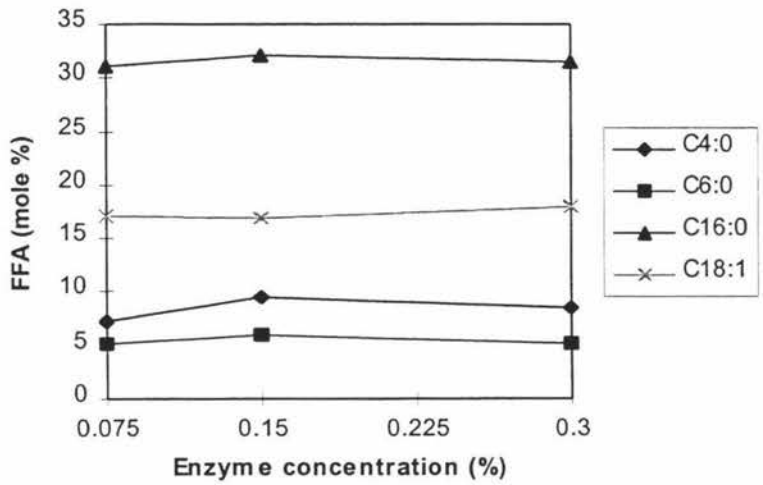

Figure 5.8: The effect of Palatase concentration on the percentages of C4:0, C6:0, C16:0 and C18:1 fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis *(Experimental conditions: 45 ℃ 24 h)*

5.4 Initial pH

The effect of the initial pH of the substrate (i.e. pH prior to incubation) on the amounts of individual FAs released from the triglycerides in the EMC base is given in Table 5.7. The total amount of FAs released over the 24 h incubation period increased from 233 to 663 mmol FA. kg EMC^{-1} as the pH increased from 4.5 to 8 (Figure 5.9). However, a very slight increase

Table 5.7: The effect of pH on the amounts of individual fatty acids released by Palatase lipase*

pH	Free fatty acid (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
4	28.4	15.1	7.1	10.5	9.7	24.6	68.0	24.4	43.9	1.1	232.8
4.5	26.6	14.1	6.8	10.2	9.6	24.4	67.8	26.8	45.8	1.0	233.0
5	29.0	16.5	8.2	13.1	12.3	33.1	88.9	33.9	61.3	1.5	297.9
5.5	29.5	18.7	9.8	15.6	14.9	39.9	100.2	33.8	63.4	0.9	326.9
5.7	27.8	16.8	8.8	14.5	14.5	41.5	104.6	39.8	73.7	2.9	345.1
6	37.1	23.8	12.0	19.1	17.9	48.0	116.2	38.0	71.8	2.0	385.9
6.5	43.1	29.0	14.0	22.4	21.0	55.0	127.1	42.4	80.1	2.3	436.5
7	46.0	32.1	15.6	26.1	24.6	66.1	155.3	49.7	94.3	3.0	512.8
7.5	42.0	30.4	15.0	25.8	25.7	73.1	179.1	64.0	123.4	5.0	583.4
8	50.6	36.6	17.8	31.0	29.5	84.8	200.3	69.8	137.3	5.2	663.0

* ***Experimental conditions: 0.15% Palatase, 45 °C,24 h***

in FAs which are lower than C14:0 (C4:0 to C12:0) were observed from pH 4.5 to 5.7; then there was a rapid increase up to pH 8. Furthermore, FAs which are longer than C14:0 (C14:0 to C18.1) increased rapidly from pH 4.5 to 8. In contrast, the extent of hydrolysis remained almost the same at pH range 4.5 to 4. (Table 5.7).

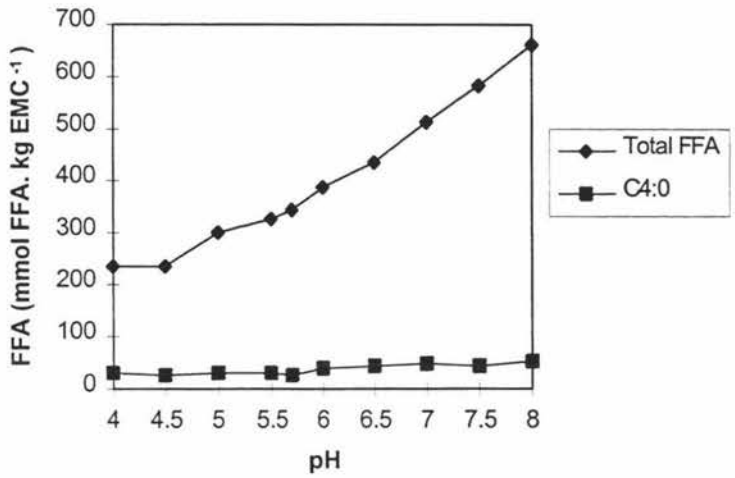

Figure 5.9: The effect of pH on the amounts of butyric acid and total free fatty acids released by hydrolysis *(Experimental conditions 0.15% Palatase, 45 °C, 24 h)*

These results suggest that the optimal pH range for this enzyme may be above pH 8 and that enzyme deactivation may occur to a some degree (partial deactivation) at lower pHs (below 4.0). The EMC base has a pH of 5.7 and this is the pH at which most experiments have been carried out. This appears to be different to the optimal initial pH. This observation is in good agreement with those reported by Moskowitz *et al.* (1977) who found the pH optimum for Palatase (*Mucor miehei* lipase) is pH 8.5.

A possible reasons for the change in the rate of lipolysis with pH may be due a change in the strength of the emulsion as a result of minimal solubility in protein (as noted in Chapter 4, Section 5.4). Furthermore, as the pH is decreased from pH 4.5 to 4 (below the iso-electric point of the casein) protein become also a better emulsifying agents and thus, the extent of hydrolysis is remained almost the same without regards to the decreasing pH.

The percentages of each individual FA (expressed as a percentage of the total of that particular FA), that were released by hydrolysis at different pH values are shown in Table 5.8. The results show a similar pattern to that described above. The percentages of individual FAs that were released from the triglycerides increased as the pH values increased from 4.5 to 8.0. At all pH values above 5.0, the butyric acid (C4:0) was released at a lower extent than the all other FAs (C6:0 to C18:1). However, the percentages of short-chain FAs (C6:0 to C12:0) were increased markedly as the pH values increased from 6 to 8. At optimal pH about 8, FAs (C6:0 to C18:1) were released at a approximately equal amounts. For example, at a pH 8, the percentages of C4, C6, C16 and C18.1 hydrolysed were 58%, 90%, 83% and 89% respectively. Similarly, at lower pH range 4.5 to 4, all FAs were released at a almost equal amounts (Table 5.8). This results suggest the at higher pH (above 6) the Palatase enzyme specificity changed and become more non-specific to FAs.

Table 5.8: The effect of pH on the percentages* of individual fatty acids released by Palatase lipase#

pH	Free fatty acid (%)										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
4	32.8	37.0	35.5	30.3	28.4	24.5	28.2	29.0	28.4	11.9	28.3
4.5	30.7	34.5	34.0	29.2	28.3	24.3	28.1	31.9	29.7	10.9	28.7
5	33.5	40.5	41.1	37.6	36.1	33.0	36.8	40.3	39.7	15.9	37.2
5.5	34.1	45.7	49.1	45.0	43.9	39.8	41.5	40.2	41.2	9.5	40.7
5.7	32.1	41.1	43.8	41.8	42.6	41.4	43.3	47.3	47.8	30.8	43.9
6	42.9	58.2	59.7	55.1	52.6	47.9	48.1	45.2	46.6	20.6	47.6
6.5	49.8	71.1	69.9	64.5	61.7	54.8	52.7	50.4	52.0	24.1	53.4
7	53.1	78.6	77.9	75.2	72.4	65.9	64.3	59.1	61.2	31.2	63.4
7.5	48.5	74.5	74.7	74.2	75.6	72.9	74.2	76.1	80.0	52.1	74.3
8	58.4	89.7	89.1	89.4	86.9	84.5	83.0	83.0	89.1	54.7	83.8

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 0.15% Palatase, 45 °C, 24 h***

The percentages of four FAs (each expressed as a percentage of the total FFAs), that were released by hydrolysis at different pH, are shown in Figure 5.10. The data for all FAs are given in Appendix 3, Table 3. As the pH increased from 4 to 7.5 the percentages of C4 decrease (from about 12% to 7%) while all other FAs (C6 to C18.2) percentages showed small increase or decreases. At optimal pH about 8, non of the FAs showed any appreciable variations.

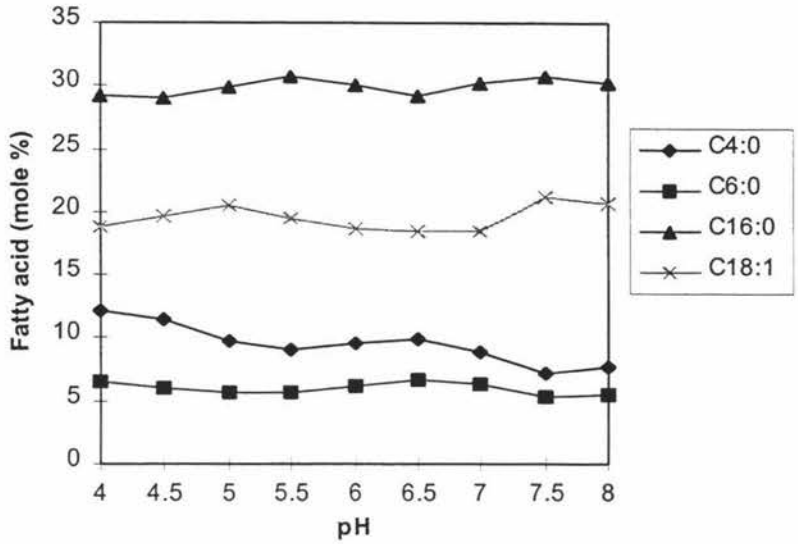

Figure 5.10: The effect of pH on the percentages of C4:0, C6:0, C16:0 and C18:1 fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis ***(Experimental conditions: 0.15% Palatase, 45°C, 24 h)***

5.5 Water activity (a_w)

The effect of water activity (a_w) on the amounts of individual FAs released from the triglycerides in the EMC base is shown in Table 5.9. The total amount of FAs released over the 24 h incubation period increased slightly from 399 to 479 mmol FA. kg EMC^{-1} as the water activity increased from 0.89 to 0.93 (Figure 5.11). However, the amounts decreased slightly as the a_w increased from 0.93 to 0.97 and only about 425 mmol FA. kg EMC^{-1} were released at a_w 0.97. A similar pattern was observed for individual FAs (Table 5.9).

Table 5.9: The effect of water activity (a_w) on the amounts of individual fatty acids released by Palatase lipase*

a_w	Free fatty acid (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0.89	38.7	23.2	11.3	17.8	17.2	48.0	122.1	42.3	76.8	1.6	399.0
0.91	39.8	24.9	12.4	19.9	19.1	53.3	133.9	45.1	83.4	2.3	434.1
0.93	43.1	26.7	13.1	21.8	20.9	59.7	145.9	51.4	94.1	2.5	479.2
0.95	37.5	24.3	12.0	20.0	19.5	56.0	138.0	47.0	88.4	2.9	445.7
0.97	39.4	25.0	11.7	19.2	18.3	52.3	130.5	44.4	81.3	2.3	424.5

* *Experimental conditions: 0.15% Palatase, pH 6.5, 45°C, 24 h*

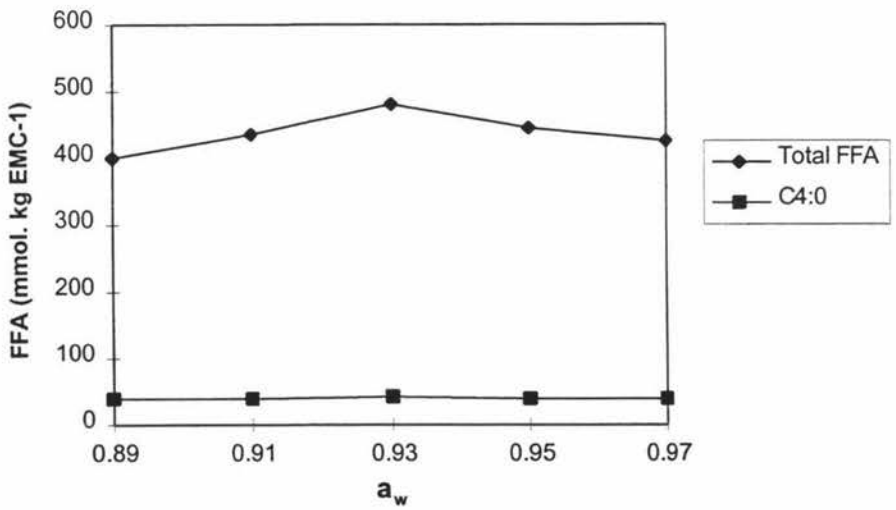

Figure 5.11: The effect of water activity (a_w) on the amounts of butyric acid and total fatty acids released by hydrolysis *(Experimental conditions: 0.15% Palatase, pH 6.5, 45°C, 24 h)*

The results suggest that the water activity has no major effect on hydrolysis of triglycerides. The reducing water activity in EMC may lead to a weak fat emulsion thereby decrease the rate of lipolysis (as noted in Chapter4, Section 4.5). This explanation fits the observed trend although the variation in rate of lipolysis is relatively small.

The percentages of each individual FA (expressed as a percentage of the total of that particular FA), that were released by hydrolysis at different water activities (a_w) are shown in Table 5.10. Large percentages (50 to 60%) of individual FAs were released from the triglycerides over the a_w range 0.89 to 0.93. At all a_w levels, the butyric acid (C4:0), were released at a lower extent than the other FAs (C6:0 to C18:1). For example, at an a_w of 0.93, the percentages of C4:0 and C16:0 released were 50% and 61% respectively.

Table 5.10: The effect of water activity (a_w) on the percentages* of individual fatty acids released by Palatase lipase#

Aw	Free fatty acid (%)										
	C4:0	***C6:0***	***C8:0***	***C10:0***	***C12:0***	***C14:0***	***C16:0***	***C18:0***	***C18:1***	***C18:2***	***Total FFA***
0.89	44.7	56.7	56.3	51.3	50.6	47.9	50.6	50.3	49.8	16.2	49.6
0.91	45.9	60.9	62.1	57.3	56.2	53.2	55.5	53.6	54.1	23.7	54.1
0.93	49.8	65.4	65.2	62.8	61.6	59.5	60.5	61.0	61.0	26.2	59.9
0.95	43.3	59.5	59.9	57.7	57.4	55.9	57.2	55.9	57.4	30.1	56.0
0.97	50.0	67.3	64.2	60.8	59.3	57.4	59.5	58.1	58.0	26.4	58.1

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 0.15% Palatase, pH 6.5, 45 °C, 24 h***

The percentages of individual FAs (each expressed as a percentage of the total FFAs) that were released by hydrolysis showed little variation over the range of water activities 0.89 to 0.97 (the data are given in Appendix 3, Table 4).

5.6 Salt-in-moisture content

The effect of salt-in-moisture content on the amounts of individual FAs released from the triglycerides is shown in Table 5.11. The total amount of FAs released over the 24 h incubation period decreased slightly with salt-in-moisture contents from 457 to 406 mmol FA. kg EMC^{-1} as salt increased from 2.4% to 9.6% (Figure 5.12).

Table 5.11: The effect of salt-in-moisture content on the amounts of individual fatty acids released by Palatase lipase*

Salt (% in moisture)	Free fatty acid (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
2.4	34.0	24.8	12.4	20.8	20.0	57.0	142.3	50.4	92.6	2.8	457.1
4.8	31.5	22.5	11.3	19.1	18.3	53.0	134.2	49.1	92.2	3.4	434.6
7.2	28.6	19.8	10.0	16.9	16.6	49.1	121.1	42.1	76.7	2.1	382.9
9.6	29.8	21.8	11.0	18.3	17.7	51.4	128.3	45.1	81.0	2.1	406.4

* ***Experimental conditions: 0.15% Palatase, pH 6.5, 45°C, 24 h***

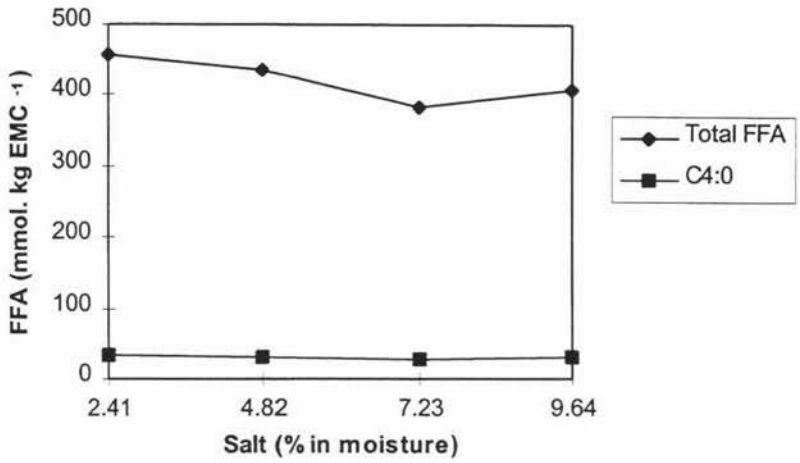

Figure 5.12: The effect of salt-in-moisture content on the amounts of butyric acid and total fatty acids released by hydrolysis ***(Experimental conditions: 0.15% Palatase, pH 6.5, 45 °C, 24 h)***

The slight decrease in extent of hydrolysis with increasing salt content could possibly be due to a slight deactivation of the enzyme at high salt concentrations.

The percentages of each individual FA (expressed as a percentage of the total of that particular FA), that were released by hydrolysis at different salt content are shown in Table 5.12. Reasonably large percentages of individual FAs were released from the triglycerides over the entire salt range (2.4% to 9.6%) with the percentages decreasing slightly as the salt content increased. Again the butyric acid was released at a lower extent than the other FAs (C6:0 to C18.1).

Table 5.12: The effect of salt-in-moisture content on the percentages* of individual fatty acids released by Palatase lipase#

Salt (% in moisture)	Free fatty acid (%)										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
2.41	39.3	60.8	62.0	60.0	58.9	56.9	59.0	60.0	60.2	29.0	58.0
4.82	36.4	55.1	56.3	55.0	54.0	52.9	55.7	58.4	59.9	35.6	55.5
7.23	33.1	48.4	50.0	48.8	48.8	49.0	50.2	50.1	49.8	22.0	48.7
9.64	34.4	53.4	55.2	52.7	52.3	51.3	53.2	53.7	52.6	22.0	51.6

* *expressed as a percentage of the total of that particular FA*

\# *Experimental conditions: 0.15% Palatase, pH 6.5, 45 °C, 24 h*

The percentages of individual FAs (each expressed as a percentage of the total FFAs), that were released by hydrolysis at different salt-in-moisture contents are given in Appendix 3, Table 5. As the salt content increased from 2.41% to 9.64% neither short-chain nor long-chain FAs showed any appreciable variation.

SUMMARY

The results have shown that the incubation temperature and pH of the EMC base have marked effect on the activity of the Palatase enzyme. Generally, the amounts of FAs increased as the pH of the EMC base increased from 4.5 to 8 while optimal pH was above 8. There was a marked increase in enzyme activity as the temperature increased from 40°C to 55°C while the optimal temperature range was 55°C. The incubation temperature, time and enzyme

concentration showed the expected trends with the amounts of FAs that were released by hydrolysis increasing as the time increased and as concentration increase. There was a relatively small decrease in hydrolysis with decreasing water activity and increasing salt-in-moisture content.

Palatase enzyme showed high resistance to certain reaction conditions such as temperature and pH. The variation in activity due to deactivation can be seen at above 55°C and possibly far below pH 4. Also, the emulsifying power of the milk protein in the EMC base at different pH values and water activities could explain the change of enzyme activities.

Generally, data indicate that, under optimum condition (0.15% enzyme concentration), Palatase enzyme caused higher degree of lipolysis, there by released significantly large amounts of both short-chain FAs (C4:0 to C10:0) and long-chain FAs (C12:0 to C18:2) in the EMC base. In some experiments the Palatase enzyme showed some specificity towards FAs, those longer than C14:0. This trend is in good agreement with those reported by Lee and Rickansrud (1979). Furthermore, this specificity was more apparent in different incubation time and temperature trials. However, this trend is not significant in other trials, with incubation at various enzyme concentrations, pHs, a_w and salt content, at constant temperature and incubation time.

For all experiments the Palatase enzyme released lower percentages of butyric acid than corresponding percentages of other FAs (C6:0 to C18:1). It was reported that the flavour profile or intensity is proportional to the degree of lipolysis and the relative ratios of FFA

(Moskowitz and Noelck, 1987). And also, there is a clear trend that the butyric acid and possibly caproic acid are of major importance for desirable flavours (Fitz-Gerald *et al.*, 1987 and Harboe, 1994). Therefore, the favourable flavour of butyric acid may be influenced by higher amounts of other FAs.

The incubation temperature and time appear to affect the rate of release of C4:0 with greater amounts released at lower temperature and longer incubation time. Therefore, lower temperature (30°C to 35°C) and longer incubation time may also have a favourable influence on the flavour profile and intensity of Palatase enzyme. Huang and Dooley (1976) reported that a Palatase (*Mucor miehei* lipase) gave flavour notes closely resembling those of Italian cheese.

CHAPTER 6

RESULTS AND DISCUSSION

Kid lipase activity on enzyme modified cheese

This chapter describes the effect of a number of parameters; incubation temperature, time, enzyme concentration, pH, water activity (a_w) and salt-in-moisture on the activity of Kid lipase on the triglycerides in the enzyme modified cheese (EMC) base. The effect of these parameters on Kid lipase activity was defined in terms of the amounts of fatty acids (FAs) released by hydrolysis and the percentages of FAs released, expressed as a percentage of the total of that particular FA originally bound in the milk triglyceride, and as a percentage of the total free fatty acids (FFAs).

6.1 Incubation time

The effect of incubation time on the amounts of individual FAs released from the triglycerides in the EMC base is shown in Table 6.1. As expected, the extent of hydrolysis increased as the incubation time increased. After 72 h the amounts of individual FAs released were in the order C4:0>C6:0>C10:0>C8:0>C12:0>C14:0>C16:0>C18:1>C18:0>C18:2. The amounts of these released FAs, which are the same as the FFAs, show a pattern which is completely different to the FAs composition of the triglycerides in the EMC base (order C16:0>C18:1>C14:0>C18:0> C4:0>C6:0>C10:0>C12:0>C8:0>C18:2). The short-chain FAs (C4:0 and C6:0) were released at a much higher rate than other FAs (C8:0 to C18:2). However, long-chain FAs (C12:0 to C18:2) were released in trace amounts.

Table 6.1: The effect of incubation time on the amounts of individual fatty acids released by kid lipase*

Time	Free fatty acids (mmol FFA. kg EMC^{-1})										
(h)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0	0.13	0.05	0.04	0.10	0.12	0.23	0.63	0.51	0.80	0.03	2.65
8	12.7	4.7	1.1	1.6	1.2	1.2	1.5	trace	trace	trace	23.9
24	25.2	9.5	2.2	3.3	2.3	2.0	2.4	1.2	1.5	trace	49.6
48	38.8	13.1	2.9	4.5	3.3	2.8	2.8	0.8	1.7	trace	70.7
72	48.7	16.4	4.0	5.9	4.2	3.6	3.4	1.6	2.1	trace	89.9

* ***Experimental conditions: 1% Kid lipase, 40°C***

The rate of release of FAs from the triglycerides in the EMC base was higher during the first eight hours of incubation and was approximately 3 mmol FA. kg $EMC.^{-1}$ h^{-1} (Figure 6.1). Thereafter the rate gradually slowed with time. A similar trend was shown by butyric acid, which had a rate of release of 1.6 mmol FA. kg $EMC.^{-1}$ h^{-1} during the first eight hours. Overall, a very low FA rate was observed with Kid lipase. Also according to Farnham *et al.* (1956), the kid lipase showed comparatively lower rate of lipolysis than calf pregastric esterases.

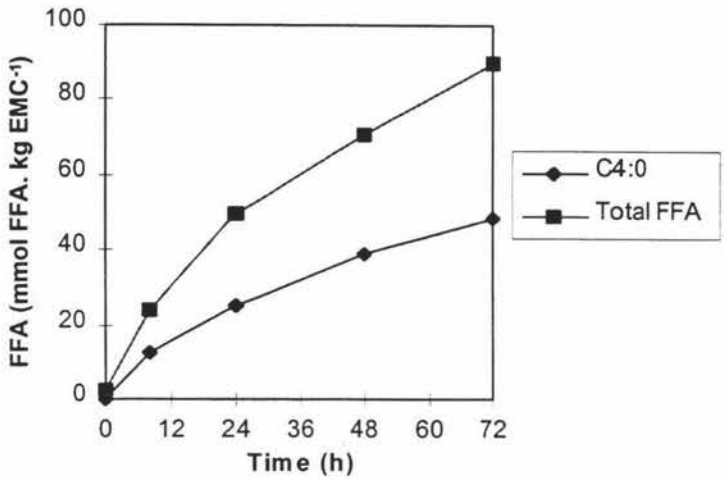

Figure 6.1: The effect of incubation time on the amounts of butyric acid and total fatty acids released by hydrolysis ***(Experimental conditions: 1% Kid lipase, 40 °C)***

The percentages of each individual FA (expressed as a percentage of the total of that particular FA) that were released by hydrolysis are presented in Table 6.2. The overall percentage of the FAs released was very low, 6% after 72 h. Short-chain FAs (C4:0 to C10:0) were released at a greater extent than long-chain FAs (C12:0 to C18:0); the percentages after 72 h for C4:0, C6:0, C16:0 and C18:1 were 56%, 40%, 1% and 1% respectively.

This suggests that the Kid lipase shows very high specificity towards short-chain FAs especially butyric acid. Furthermore it released negligible amounts of long-chain FAs (C14:0 to C18:2). This trend is supported by Kwak *et al.* (1989); Siewert and Otterby (1968); Pitas and Jenson (1970); Kilara (1985), and Harper (1957) who reported that, animal pregastric lipases release mainly short-chain FAs especially butyric acid. And also, Huang and Dooley (1976) confirmed that kid pregastric esterases and kid rennet paste produced the highest

concentration of butyric acid. These results also revealed the low rate of FA release by Kid lipase.

Table 6.2: The effect of incubation time on the percentages* of individual fatty acids released by kid lipase#

Time	Free fatty acid (%)										
(h)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
8	14.6	11.5	5.3	4.6	3.4	1.2	0.6	trace	trace	trace	1.6
24	29.1	23.2	11.1	9.5	6.9	2.0	1.0	1.4	1.0	trace	3.6
48	44.7	32.1	14.7	12.9	9.7	2.8	1.2	0.9	1.1	trace	4.8
72	56.2	40.3	19.8	16.9	12.4	3.6	1.4	1.9	1.4	trace	6.2

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 1% Kid lipase, 40 °C***

The percentages of each individual FA (expressed as a percentage of the total free fatty acids) that were released by hydrolysis at different times during the incubation period are shown in Figure 6.2 (short-chain FAs) and Figure 6.3 (long-chain FAs). The percentage of butyric acid (C4:0) released by hydrolysis increased slightly from 53% to 55% as the incubation time increased to 48 h, while C16:0 decreased slightly from 6% to 4%. All other FAs showed little change.

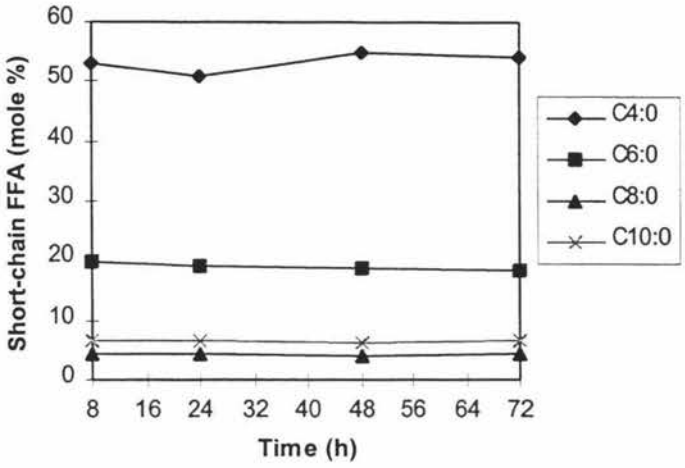

Figure 6.2: The effect of incubation time on the percentages of short-chain fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis

(Experimental conditions: 1% Kid lipase, 45 °C)

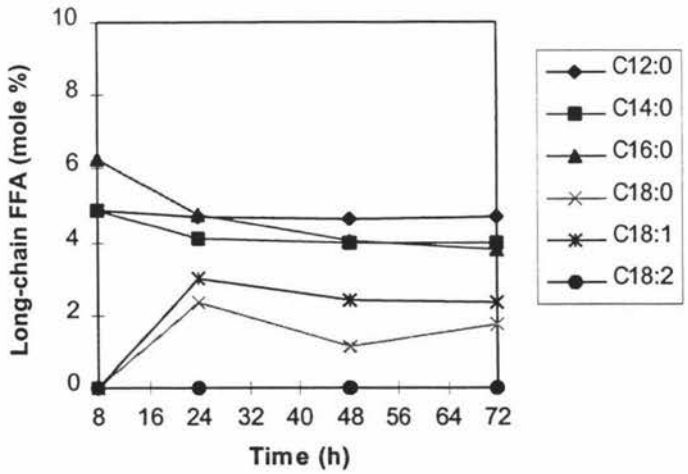

Figure 6.3: The effect of incubation time on the percentages of long-chain fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis

(Experimental conditions: 1% Kid lipase, 45 °C)

6.2 Incubation Temperature

The effect of incubation temperature on the amounts of individual FAs released from the triglycerides in the EMC base is shown in Table 6.3. The total amounts of FAs released over the 48 h incubation period increased gradually with temperature (Figure 6.4). Large amounts of FAs (77 mmol FA. kg EMC^{-1}) were released at the temperature 45°C. However, the amounts tend to decrease as the temperature increased from 45°C to 50°C and only about 71 mmol FA. kg EMC^{-1} were released at 50°C. The C4:0 and C6:0 were the major FAs released over the temperature range (30°C to 50°C) while all other FAs were released in trace amounts (Table 6.3).

Table 6.3: The effect of incubation temperature on the amounts of individual fatty acids released by kid lipase*

Temperature	Free fatty acid (mmol FFA. kg EMC^{-1})										
(°C)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
30	41.5	13.1	2.7	4.2	2.8	2.1	1.9	0.0	0.6	0.0	68.8
35	36.6	12.2	2.8	4.1	2.9	2.4	2.1	0.0	0.2	0.0	63.3
40	38.8	13.1	2.9	4.5	3.3	2.8	2.8	0.8	1.7	0.0	70.7
45	42.5	14.7	3.3	5.0	3.6	3.0	3.0	0.8	1.5	0.0	77.4
50	38.7	13.8	3.0	4.4	3.1	2.6	2.7	0.8	1.7	0.0	70.8

* ***Experimental conditions: 1% Kid lipase, 48 h***

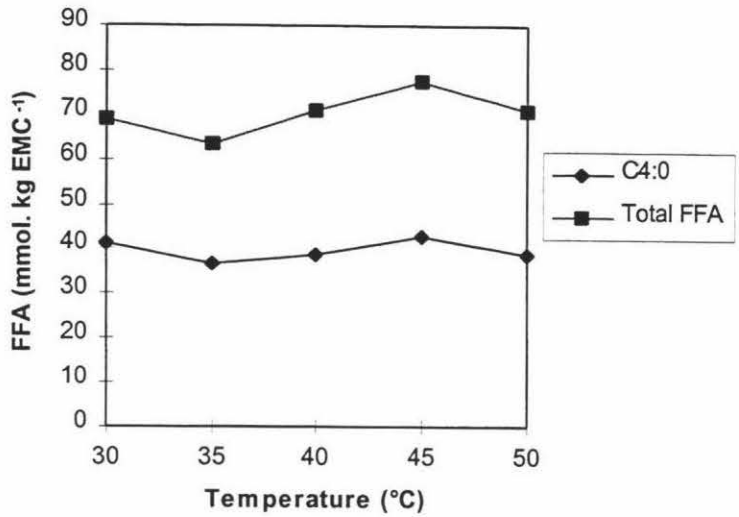

Figure 6.4: The effect of incubation temperature on the amounts of butyric acid and total fatty acids released by hydrolysis *(Experimental conditions: 1% Kid lipase, 48 h)*

The results suggest that at temperatures above 45°C enzyme deactivation occurs with the degree of deactivation increasing as the temperature increases from 45°C to 50°C. Also, the temperature has only a minor impact on extent of hydrolysis over this range. Thus the effective operating temperature range for kid lipase is possibly 30°C to 45°C. This observation is similar to that reported by Nelson *et al.*, (1977) who reported the temperature optima for kid goat and lamb pregastric enzymes to be 35°C to 42°C range. However, Sweet et al. (1984), reported that the optimum temperature for calf pregastric lipases is approximately 45°C.

The percentages each individual FA (expressed as a percentage of the total of that particular FA) that were released by hydrolysis at different incubation temperatures are shown in Table 6.4. The results show a similar pattern to that described above. Large percentages of C4:0 and C6:0 were released from the triglycerides over the temperature range 30°C to 45°C with the percentages decreasing slightly as the temperature was increased from 45°C to 50°C. At all incubation temperatures, the short-chain FAs, C4:0 and C6:0, were released at a greater extent than the long-chain FAs (C12:0 to C18:2). For example, at an incubation temperature of 45°C, the percentages of C4:0 and C16:0 released were 49% and 1% respectively. However, at all temperatures the long-chain FAs (C16:0 to C18:2) were released in trace amounts.

Table 6.4: The effect of incubation temperature on the percentages* of individual fatty acids released by kid lipase#

Temperature	Free fatty acid (%)										
(°C)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
30	47.9	32.2	13.5	12.0	8.2	2.1	0.8	0.0	0.4	0.0	4.3
35	42.3	30.0	13.7	11.8	8.5	2.4	0.9	0.0	0.1	0.0	4.1
40	44.7	32.1	14.7	12.9	9.7	2.8	1.2	0.9	1.1	0.0	4.8
45	49.1	36.0	16.4	14.3	10.7	2.9	1.2	1.0	1.0	0.0	5.3
50	44.7	33.8	15.0	12.6	9.0	2.6	1.1	1.0	1.1	0.0	4.8

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 1% Kid lipase, 48 h***

The percentages of four FAs (each expressed as a percentage of the total FFAs), that were released by hydrolysis at different temperatures, are shown in Figure 6.5. The data for all FAs are given in Appendix 4, Table 1. As the incubation temperatures increased from 30°C to 40°C the percentages of C4:0 decrease (from about 60% to 55%) while all other FAs showed only small decrease or increases. Furthermore, both C4:0 and C6:0 contributed about 80% of the total FFAs. Over the temperature range, 40°C to 50°C, none of the FAs showed any appreciable variation (Figure 6.5).

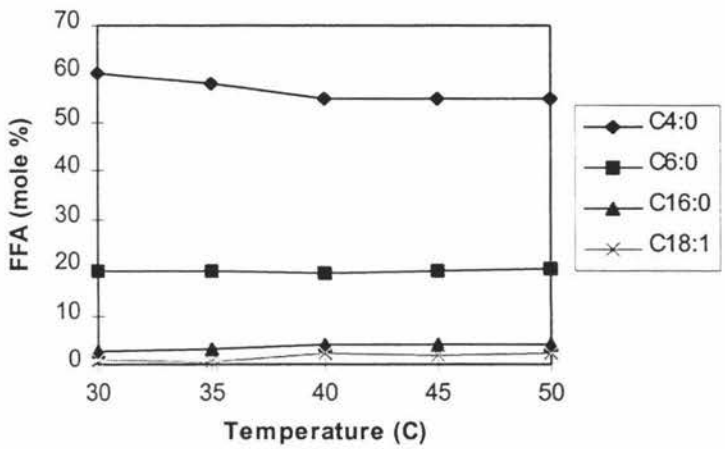

Figure 6.5: The effect of incubation temperature on the percentages of C4:0, C6:0, C16:0 and C18:1 fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis *(Experimental conditions: 1% Kid lipase, 48 h)*

The relationship between the percentage of butyric acid released by hydrolysis (expressed as a percentage of total FFAs released) and the total amount of FAs released (i.e. total FFAs) at different incubation times is shown in Figure 6.6. It can be seen that, the rate of release of butyric acid by hydrolysis is greater at low incubation temperature (30°C to 35°C) than at

high temperatures (40°C to 50°C). Furthermore, the percentage of butyric acid reached a maximum and thereafter remained almost the same at higher temperatures (40°C to 50°C), while it tended to increase at lower temperatures (30°C to 35°C). It can be seen that, butyric acid formation is selectively increased at lower temperatures.

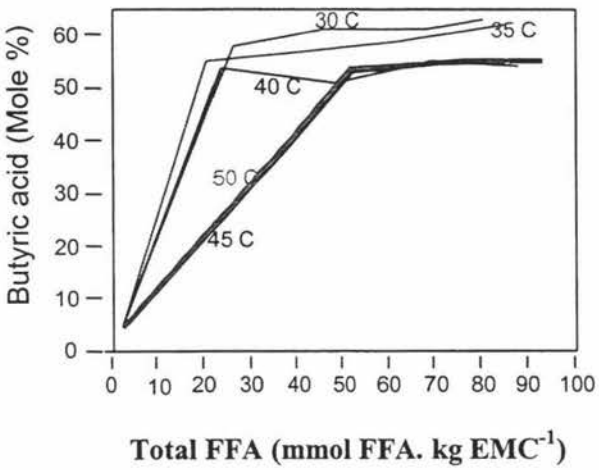

Figure 6.6: The relationship of percentage of butyric acid (expressed as percentages of the total FFA) and total FFA that were released by hydrolysis at various incubation temperatures*(Experimental conditions: 1% Kid lipase, 0 to 72 h)*

6.3 Enzyme concentration

The amounts of individual FAs released from the triglycerides in the EMC base at various enzyme concentrations are shown in Table 6.5. The total amount of FAs released over the 48 h incubation period increased from 57 to 117 mmol FFA. kg EMC^{-1} as the enzyme concentration increased from 0.5% to 4% (Figure 6.7). However, the amount increased only

Table: 6.5: The effect of Kid lipase concentration on the amounts of individual fatty acids released by hydrolysis*

Enzyme concentration (%)	Free fatty acid (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0.5	30.9	10.6	2.4	3.5	2.6	2.3	2.5	0.9	1.3	trace	57.1
1.0	38.8	13.1	2.9	4.5	3.3	2.8	2.8	0.8	1.7	trace	70.7
2.0	53.3	16.9	4.3	6.5	5.0	4.3	4.2	1.0	1.9	trace	97.3
4.0	61.4	19.5	5.4	8.2	6.0	6.3	6.0	1.4	3.1	trace	117.2
8.0	61.7	19.5	5.8	8.6	6.9	7.9	8.1	2.0	3.8	trace	124.4

* *Experimental conditions: 40 °C, 48 h*

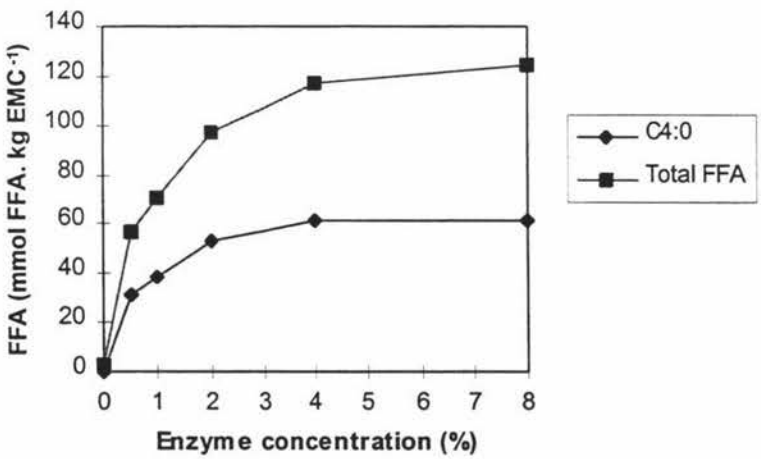

Figure 6.7: The effect of Kid lipase concentration on the amounts of butyric acid and total fatty acids released by hydrolysis *(Experimental conditions: 40 °C, 48 h)*

slightly as the enzyme concentration increased from 4% to 8%. A similar pattern was observed for individual FAs with the short-chain FAs (C4:0 to C10:0) showing virtually no increase as the Kid lipase concentration increased from 4% to 8%. The results suggest that a Kid lipase concentration of 4% is optimal for the hydrolysis of triglycerides in EMC.

The percentages of each individual FAs (expressed as a percentage of the total of that particular FA) that were released by hydrolysis at various enzyme concentrations are presented in Table 6.6. The percentages of total FAs released increased from 4% to 8.5% as the enzyme concentration increased from 0.5% to 4%. At all enzyme concentrations, the short-chain FAs, C4:0 and C6:0, were released at a greater extent than the long-chain FAs

Table 6.6: The effect of Kid lipase concentration on the percentages* of individual fatty acids released by hydrolysis#

Enzyme concentration (%)	Free fatty acid (%)										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0.5	35.7	26.0	11.8	10.2	7.6	2.3	1.0	1.0	0.8	0	3.9
1.0	44.8	32.1	14.7	12.9	9.7	2.8	1.2	0.9	1.1	0	4.8
2.0	61.5	41.5	21.4	18.7	14.6	4.3	1.7	1.2	1.2	0	6.7
4.0	70.9	47.7	26.8	23.5	17.8	6.3	2.5	1.7	2.0	0	8.4
8.0	71.2	47.8	28.9	24.8	20.3	7.9	3.4	2.4	2.4	0	9.3

**** expressed as a percentage of the total of that particular FA***

 # Experimental conditions: 1% Kid lipase, 40 °C, 48 h

(C12:0 to C18:2). For example, at an enzyme concentration of 4%, the percentages of C4:0, C6:0, C16:0 and C18:1 were 71%, 48%, 2.5% and 2.0% respectively. However, at all enzyme concentrations the long-chain FAs (C16:0 to C18:2) were released in trace amounts.

The percentages of four FAs (each expressed as a percentage of the total FFAs), that were released by hydrolysis at different enzyme concentration, are shown in Figure 6.8. The data for all FAs are given in Appendix 4, Table 2. As the enzyme concentration increased from 2% to 4% the percentages of C4:0 decreased from 55% to 52%, while all other FAs showed smaller decrease or increases. Above the optimal enzyme concentration (about 4%) none of the FAs showed any appreciable variation.

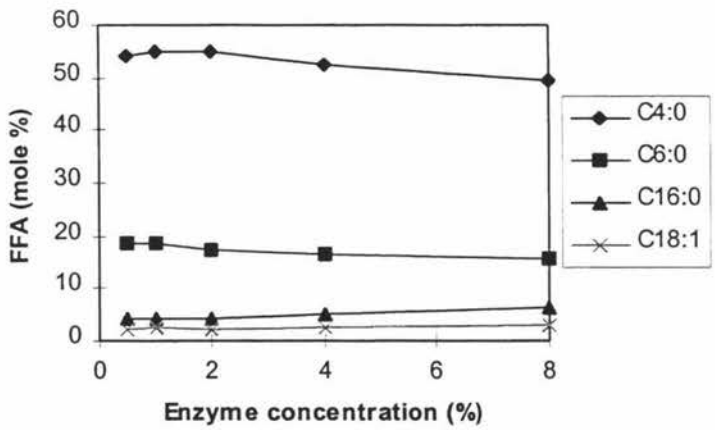

Figure 6.8: The effect of enzyme concentration on the percentages of C4:0, C6:0, C16:0 and C18:1 fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis *(Experimental conditions: 1% Kid lipase, 40 °C, 48 h)*

6.4 Initial pH

The effect of the initial pH of the substrate (i.e. pH prior to incubation) on the amounts of individual FAs released from the triglycerides in the EMC base is shown in Table 6.7. The total amount of FAs released over the 48 h incubation period increased from 40 to 75 mmol FFA. kg EMC^{-1} as the pH increased from 4.5 to 5.5 (Figure 6.9). Similarly, the amounts of FAs released increased markedly from 40 to 60 mmol FA. kg EMC^{-1} at below pH 4.5 to 4.

Table: 6.7: The effect of initial pH on the amounts of individual fatty acids released by kid lipase*

pH	Free fatty acid (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
4	31.3	11.2	2.8	4.2	3.0	2.3	2.6	0.8	1.4	0.0	59.5
4.5	20.4	7.6	1.9	3.0	2.1	1.8	1.9	0.4	0.6	0.0	39.7
5	27.5	9.5	2.3	3.4	2.5	2.2	2.2	0.4	0.8	0.0	51.0
5.5	41.8	14.3	3.4	5.1	3.6	2.9	2.5	0.5	0.8	0.0	74.9
5.7	38.8	13.1	2.9	4.5	3.3	2.8	2.8	0.8	1.7	0.0	70.7
6	40.7	14.2	3.4	5.1	3.7	3.2	3.1	0.6	1.2	0.0	75.1
6.5	38.9	13.9	3.3	4.9	3.5	3.1	3.1	0.7	1.0	0.0	72.3
7	35.7	12.9	3.0	4.4	3.0	2.6	2.8	0.6	1.0	0.0	66.0
7.5	3.6	2.1	0.5	0.7	0.4	0.7	1.0	0.0	0.0	0.0	8.9
8	1.2	0.8	0.3	0.4	0.3	0.4	0.8	0.0	0.0	0.0	4.3

* ***Experimental conditions: 1% Kid lipase, 40°C, 48 h***

Large amounts of FAs (75 to 72 mmol FA. kg EMC^{-1}) were released over the pH range 5.5 to 6.5. However, the amounts decreased sharply as the pH increased from 6.5 to 8 and only about 4.3 mmol FA. kg EMC^{-1} were released at pH 8. A similar pattern was observed for individual FAs (Table 6.7).

These results suggest that the optimal pH range for this enzyme is 5.5 to 6.5 and that enzyme deactivation is marked at higher pH's (greater than pH 6.5). The degree of enzyme deactivation increased markedly as the pH increased from 6.5 to 8 with only about 4.3 mmol FA. kg EMC^{-1} released at pH 8. The observed trend is similar to that reported by Nelson *et al.* (1977) and Kilara (1985) who found that pH optima for kid goat pregastric esterases is 4.5 to 6.0, with complete inhibition of activity below pH 2.4 and above pH 7.8. The EMC base had a pH of 5.7 and this is the pH at which most experiments were carried out. This appears to be the optimal pH.

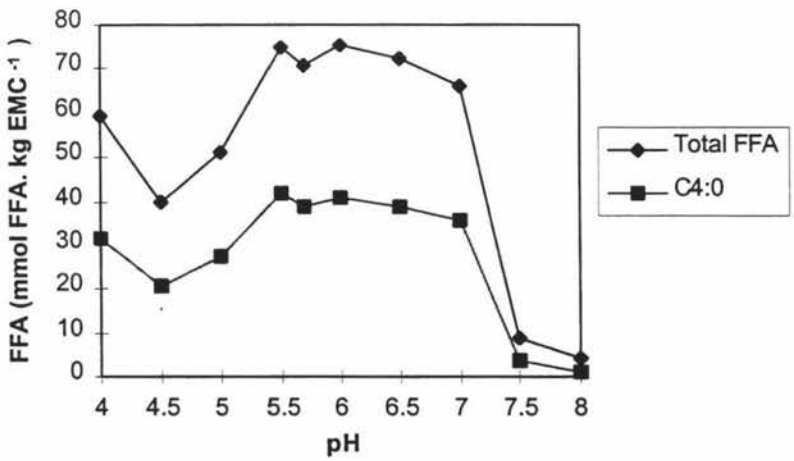

Figure 6.9: The effect of initial pH on the amounts of total fatty acids and butyric acid released by hydrolysis *(Experimental conditions: 1% Kid lipase, 40 °C, 48 h)*

A possible reason for the change in the rate of lipolysis with pH may be due to a change in the strength of the emulsion as discussed in Chapter 4, Section 4.4. According to that explanation, the lowest lipolysis at pH 4.5 was due to the weakening of fat-water emulsion as a results of poor protein solubility. Conversely, as the pH increases or decreases from the iso-electric point of the cheese protein (4.6), the protein become better emulsifying agents and thus, lipolysis increased.

The percentages of each individual FA (expressed as a percentage of the total of that particular FA), that were released by hydrolysis at different pH values is shown in Table 6.8. The results show a similar pattern to that described above. The percentages of individual short-chain FAs (C4:0 to C12:0) that were released from the triglycerides increased markedly as the pH values increased from 4.5 to 5.5 and remained almost the same over the pH range 5.5 to 6.5. At all pH values below pH 7, the short-chain FAs, C4:0 and C6:0 were released at a relatively higher extent than the other FAs (C8:0 to C18:2). For example, at a pH of 6.5, the percentages of C4:0 and C16:0 released were 45% and 1.3% respectively. However, at all pH values, the long-chain FAs (C14:0 to C18:2) were released in trace amounts. This again suggest the enzyme shows very high specificity towards short-chain FAs mainly C4:0 and C6:0.

Table 6.8: The effect of initial pH on the percentages* of individual fatty acids released by kid lipase #

pH	Free fatty acid (%)										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
4.0	36.1	27.3	13.8	12.2	8.8	2.3	1.1	0.9	0.9	0	4.2
4.5	23.6	18.5	9.6	8.5	6.3	1.8	0.8	0.5	0.4	0	2.8
5.0	31.8	23.3	11.3	9.9	7.4	2.2	0.9	0.5	0.5	0	3.5
5.5	48.2	35.0	17.0	14.8	10.7	2.8	1.1	0.6	0.5	0	5.0
6.0	47.0	34.7	17.0	14.7	10.9	3.2	1.3	0.7	0.8	0	5.1
6.5	44.9	34.1	16.4	14.2	10.2	3.1	1.3	0.8	0.7	0	4.9
7.0	41.2	31.7	14.8	12.6	8.9	2.6	1.2	0.7	0.7	0	4.5
7.5	4.1	5.2	2.6	1.9	1.2	0.7	0.4	0	0	0	0.7
8.0	1.4	1.8	1.6	1.2	0.9	0.4	0.3	0	0	0	0.4

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 1% Kid lipase, 48 h***

The percentages of four FAs (each expressed as a percentage of the total FFAs), that were released by hydrolysis at different pHs, are shown in Figure 6.10. The data for all FAs are given in Appendix 4, Table 3. The percentage of C4:0 released by hydrolysis increased from 51% to 56% as the pH was increased from 4.5 to 5.5. Thereafter the percentage decreased slightly (56% to 54%), up to pH 7. All other FAs showed little change over the pH range 4 to 7 (Figure 6.10). In contrast there were some significant variations in the percentages of these

FAs at pHs greater than 7. However, these later results should be treated with caution as extensive deactivation of the enzyme was taking place in the pH range 7 to 8.

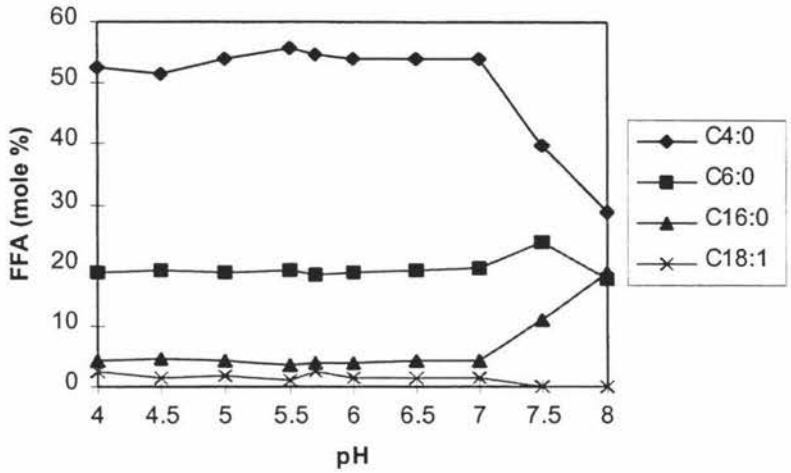

Figure 6.10: The effect of initial pH on the percentages of C4:0, C6:0, C16:0 and C18:1 fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis *(Experimental conditions: 1% Kid lipase, 40 °C, 48 h)*

6.5 Water activity (a_w)

The effect of water activity (a_w) on the amount of individual FAs released from the triglycerides in the EMC base is shown in Table 6.9. The total amounts of FAs released over the 48 h incubation period increased slightly from 73 to 81 mmol FA. kg EMC^{-1} as the water activity increased from 0.89 to 0.95 (Figure 6.11).

Table: 6.9: The effect of water activity (a_w) on the amounts of individual fatty acids released by kid lipase*

a_w	Free fatty acid (mmol FA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0.89	38.6	14.3	3.4	4.7	3.0	2.7	3.7	1.2	1.7	0.0	73.3
0.91	43.5	15.1	3.2	4.4	2.8	2.4	3.1	0.7	1.0	0.0	76.2
0.93	42.7	15.0	3.4	4.8	3.1	2.7	3.1	0.7	0.9	0.0	76.4
0.95	45.0	15.8	3.6	5.1	3.2	2.8	3.3	0.6	1.1	0.0	80.5
0.97	38.9	14.6	3.3	4.5	2.8	2.2	2.4	0.5	0.8	0.0	69.9

* ***Experimental conditions: 1% Kid lipase, pH 6.5, 40C, 48 h***

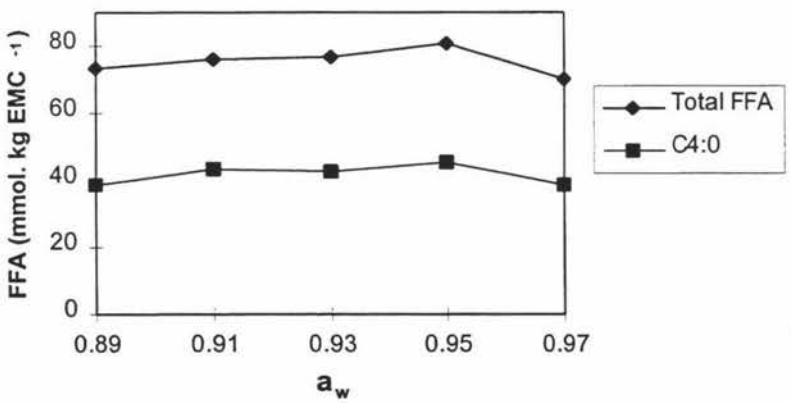

Figure 6.11: The effect of water activity (a_w) on the amounts of total fatty acids and butyric acid released by hydrolysis *(Experimental conditions: 1% Kid lipase, pH 6.5, 40°C, 48 h*

The percentages of each individual FA (expressed as a percentage of the total of that particular FA), that were released by hydrolysis at different water activities are shown in Table 6.10. Large percentages (35% to 50%) of C4:0 and C6:0 were released from the triglycerides over the a_w range 0.89 to 0.97 while all other FAs were released in trace amounts.

Table 6.10: The effect of water activity (a_w) on the percentages* of individual fatty acids released by kid lipase#

a_w	Free fatty acid (%)										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
0.89	45.0	35.3	17.2	13.6	8.9	2.7	1.6	1.4	1.1	0	5.2
0.91	50.7	37.2	16.3	12.7	8.3	2.4	1.3	0.9	0.7	0	5.1
0.93	49.8	37.1	17.0	14.0	9.2	2.8	1.3	0.8	0.6	0	5.1
0.95	52.6	39.1	18.2	14.8	9.6	2.8	1.4	0.8	0.7	0	5.4
0.97	45.5	36.1	16.5	13.2	8.4	2.2	1.0	0.6	0.5	0	4.7

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 1% Kid lipase, pH 6.5, 40 °C, 48 h***

The percentages of individual FAs (each expressed as a percentage of the total FFAs) that were released by hydrolysis showed little variation over the range of water activities 0.89 to 0.97 (the data are given in Appendix 4, Table 4). However, the percentages of C4:0 increased from 53% to 57% over the water activity 0.89 to 0.91 (Figure 6.12).

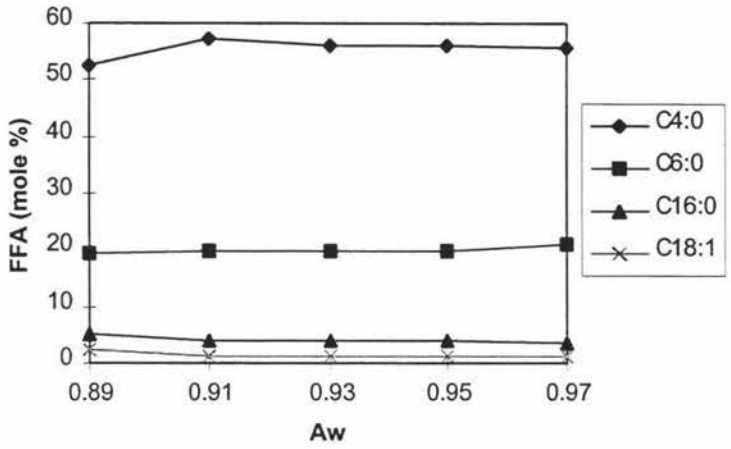

Figure 6.12: The effect of water activity on the percentages of C4:0, C6:0, C16:0 and C18:1 fatty acids (expressed as percentages of the total FFA) that were released by hydrolysis ***(Experimental conditions: 1% Kid lipase, pH 6.5, 40°C, 48 h)***

6.6 Salt-in-moisture content

The effect of salt content on the amounts of individual FAs released from the triglycerides is given in Table 6.11. The total amounts of FAs released over the 48 h incubation period decreased slightly, from 71 to 61 mmol FA. kg EMC^{-1} as salt increased from 2.4% to 7.2% respectively. The amounts were much constant as the salt in moisture content increased from 7.2% to 9.6% (Figure 6.13). A similar pattern was observed for C4:0 and C6:0 while other FAs were released in trace amounts.

The slight decrease in rate of hydrolysis with increasing salt content could possibly be due to a slight denaturation of the enzyme at high salt concentration. However, this observation is some what in disagreement with those reported by Desnuelle and Nord (1969), who reported

Table: 6.11: The effect of salt in moisture content on the amounts of individual fatty acids released by kid lipase*

Salt (% in moisture)	Free fatty acid (mmol FFA. kg EMC^{-1})										
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
2.4	39.6	14.9	3.3	4.6	2.9	2.2	2.5	0.5	0.8	0.0	71.3
4.8	38.6	14.0	3.1	4.4	2.7	2.2	2.3	0.4	0.7	0.0	68.3
7.2	34.3	12.4	2.8	3.9	2.4	2.0	2.1	0.3	0.5	0.0	60.8
9.6	34.7	12.5	2.8	3.9	2.5	2.2	2.3	0.3	0.5	0.0	61.6

* *Experimental conditions: 1% Kid lipase, pH 6.5, 40 °C, 48 h*

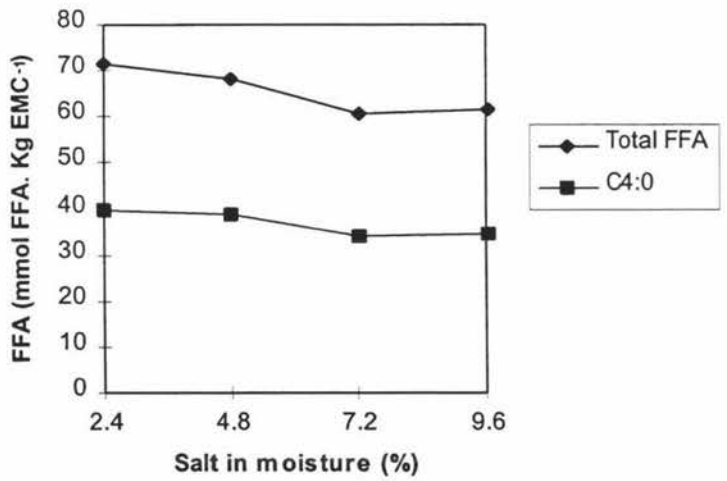

Figure 6.13: The effect of salt-in-moisture content on the amounts of total fatty acids and butyric acid released by hydrolysis *(Experimental conditions: 1% Kid lipase, pH 6.5, 40 °C, 48 h*

that pregastric esterases show an apparent increase in activity, possibly due to altering interfacial charge by sodium chloride.

The percentages of each individual FA (expressed as a percentage of the total of that particular FA) that were released by hydrolysis at different salt levels are shown in Table 6.12. Reasonably large percentages of C4:0 and C6:0 were released over the entire salt range (2.4% to 9.6%). Again the short-chain FAs, C4:0 and C6:0, were released at a greater extent than the long-chain FAs (C12:0 to C18:0). However, the long-chain FAs (C14:0 to C18:2) were released in trace amounts.

Table 6.12: The effect of salt-in-moisture on the percentages* of individual fatty acids released by kid lipase#

salt	Free fatty acid (%)										
(% in moisture)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*	*Total FFA*
2.4	45.5	36.1	16.5	13.2	8.4	2.2	1.0	0.6	0.5	trace	4.7
4.8	44.3	34.0	15.2	12.5	8.0	2.2	1.0	0.4	0.4	trace	4.4
7.2	39.4	30.2	13.7	11.1	7.2	2.0	0.9	0.4	0.3	trace	3.9
9.6	39.8	30.5	13.7	11.0	7.2	2.2	0.9	0.4	0.3	trace	4.0

* ***expressed as a percentage of the total of that particular FA***

\# ***Experimental conditions: 1% Kid lipase, pH 6.5, 40°C, 48 h***

The percentages of individual FAs (each expressed as a percentage of the total FFAs), that were released by hydrolysis at different salt levels are given in Appendix 4, Table 5. As the salt content increase from 2.4% to 9.6%, neither short chain FFAs nor long chain FFAs percentages showed any appreciable variation.

SUMMARY

The results obtained from these experiments demonstrated that pH of the EMC base has a marked effect on lipolysis, whereas temperature has minor impact on the activity of the Kid lipase. The optimum pH range was 5.5 to 6.5. The amount of FAs released by hydrolysis decreased rapidly as the pH increased from 6.5 to 8.0. There was a slight increase in Kid lipase activity as the temperature increase from 30°C to 45°C while it tended to decrease at above 45°C. The incubation time and enzyme concentration showed expected trends with the amounts of FAs released by hydrolysis increasing as time increased and as concentration increased. Water activity and salt content had a very slight impact on Kid lipase activity. The enzyme activity decreased slightly as water activity decreased and as salt-in-moisture increased.

The variation in Kid lipase activity is mainly due to deactivation of the enzyme which is more apparent at higher pH's (above 6.5) and above 45°C. The variation in enzyme activity at lower pH's and lower water activities can be explained due to variation in the emulsifying power of the milk protein in the EMC base.

The results showed that Kid lipase exhibits lower levels of total lipolysis under most experimental conditions (i.e. 1.0% enzyme concentration, 48 h incubation) which have been used for most experiments. However, the activity can be increased largely by using optimum enzyme concentration (4.0%) and also by prolonged incubation period.

The most important property of Kid lipase is its high specificity towards short-chain FAs, mainly on C4:0. In all experiments it released large amounts of C4:0 and considerable amounts of C6:0 and only trace amounts of long-chain FAs (C10:0 to C18.2). This trend is in accordance with Huang and Dooley (1976); Harper (1957) and Kwak *et al.*, 1989. The butyric acid (C4:0) and possibly caproic (C6:0) are of major importance for desirable flavours in EMC (Moskowitz and Noelck, 1987).

As observed in other enzymes (Amano 'R' and Palatase), there is a greater rate of C4:0 released at lower incubation temperatures than higher temperatures. Therefore, these conditions may also enhance the favourable flavour characteristic of Kid lipase. Many researchers have confirmed that kid lipase produced Italian-type-cheese flavours (Huang and Dooley, 1976; O'Connor *et al.*, 1996; Shani *et al.*, 1976; Woo and Lindsay, 1984; Harper and Long, 1956).

CHAPTER 7

PROPERTIES OF AMANO 'R', PALATASE AND KID LIPASES

This chapter provides a comparison of the effect of experimental parameters such as incubation time, temperature, initial pH and enzyme concentration on the activities of the three enzymes, Amano 'R' lipase, Palatase lipase and kid lipase.

7.1 Extent of hydrolysis

For the three enzymes the amounts of individual FAs released from the triglycerides in the EMC base are shown in Table 7.1. For the same experimental conditions (0.15% enzyme, 30°C, 24 h) the total amounts of FAs released were similar for Palatase and Amano 'R' lipases but markedly different for kid lipase. The total amounts of FAs release were 224, 188 and 20 mmol FFA. kg EMC.$^{-1}$ for Palatase, Amano 'R' and kid lipase respectively.

The amounts of individual FAs released by Palatase and Amano 'R' lipase were broadly similar. However for the kid lipase they were much lower, with only very small amounts of long chain FAs released.

Table 7.1: The amounts of individual fatty acids released by Amano 'R'*, Palatase* and Kid lipase*

Fatty acids	Fatty acid composition of triglycerides in EMC (mmol FA. kg EMC^{-1})	Free fatty acid (mmol FFA. Kg EMC^{-1})		
		Palatase	*Amano 'R'*	*Kid lipase*
C4:0	86.6	24.0	31.5	11.6
C6:0	40.8	13.8	13.1	4.0
C8:0	20.0	6.7	4.9	0.8
C10:0	34.7	9.0	8.2	1.2
C12:0	34.0	7.8	6.9	0.9
C14:0	100.3	20.9	19.9	0.8
C16:0	241.4	70.3	50.7	1.0
C18:0	84.1	28.9	19.2	0.0
C18:1	154.1	41.5	32.7	0.0
C18:2	9.6	1.1	0.7	0.0
Total	805.7	224.1	187.7	20.4

* ***Experimental conditions: 0.15% enzyme, 30°C, 24 h***

The percentages of short-chain FAs (expressed as a percentage of the total FFAs), that were released by the three enzymes are shown in Table 7.2. The percentages of butyric and caproic fatty acids released were much higher for Kid lipase than for the other two lipases (Figure 7.1). However, the Amano 'R' releases relatively higher amounts of butyric acid than Palatase. The high specificity for short-chain FAs shown by kid lipase may have important commercial considerations because it is the short-chain FAs have a major influence on the flavour of cheese (Deeth and Fitz-Gerald, 1987 and Harboe, 1994).

Table 7.2: The percentages* of short-chain fatty acids released by Amano 'R'#, Palatase# and Kid lipase#

Lipase	Free fatty acid (mole %)				
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0 to C18:2*
Amano 'R'	16.8	7.0	2.6	4.4	69.3
Palatase	10.7	6.2	3.0	4.0	76.1
Kid	57.0	19.7	4.0	6.0	13.3

* *expressed as a percentage of the total FFAs*

\# *Experimental conditions: 0.15% lipase, 30℃, 24 h*

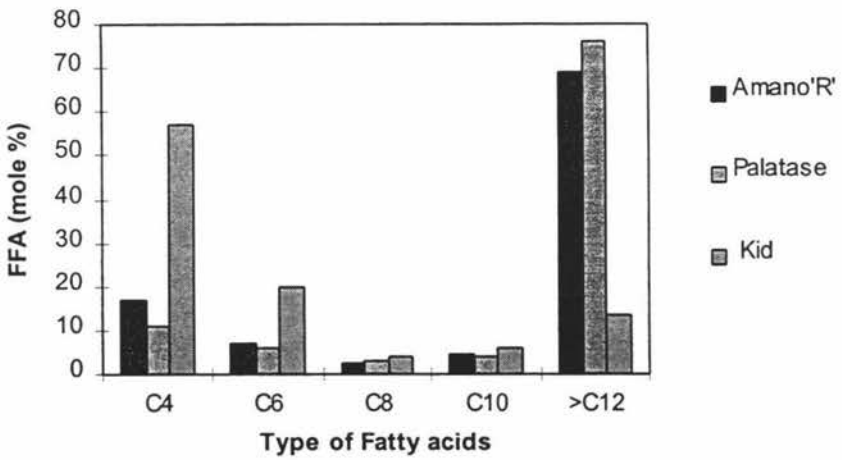

Figure 7.1: FFA percentages (expressed as a percentages of the total FFAs) resulted from Amano 'R', Palatase and Kid lipase *(Experimental conditions: 0.15%-enzyme, 30℃, 24 h)*

7.2 Effect of key processing variables on extent of hydrolysis

7.2.1 Incubation time

For each lipase, the effect of incubation time on the total amount of FAs released by hydrolysis followed the expected pattern (Figure 7.2). The extent of hydrolysis increased as the incubation time increased while the rate of release of FAs was greatest during the early stages of incubation.

The percentages of FAs (expressed as a percentages of the total FFA) were also affected by incubation time. For example, the percentage of butyric acid (C4:0) released by hydrolysis increased slightly as the incubation time increased, for Palatase lipase (Figure 7.3). In contrast, the percentage of C4:0 released by Amano 'R' lipase decreased slightly with time while kid lipase released approximately the same percentage of C4:0 over the 72 h period.

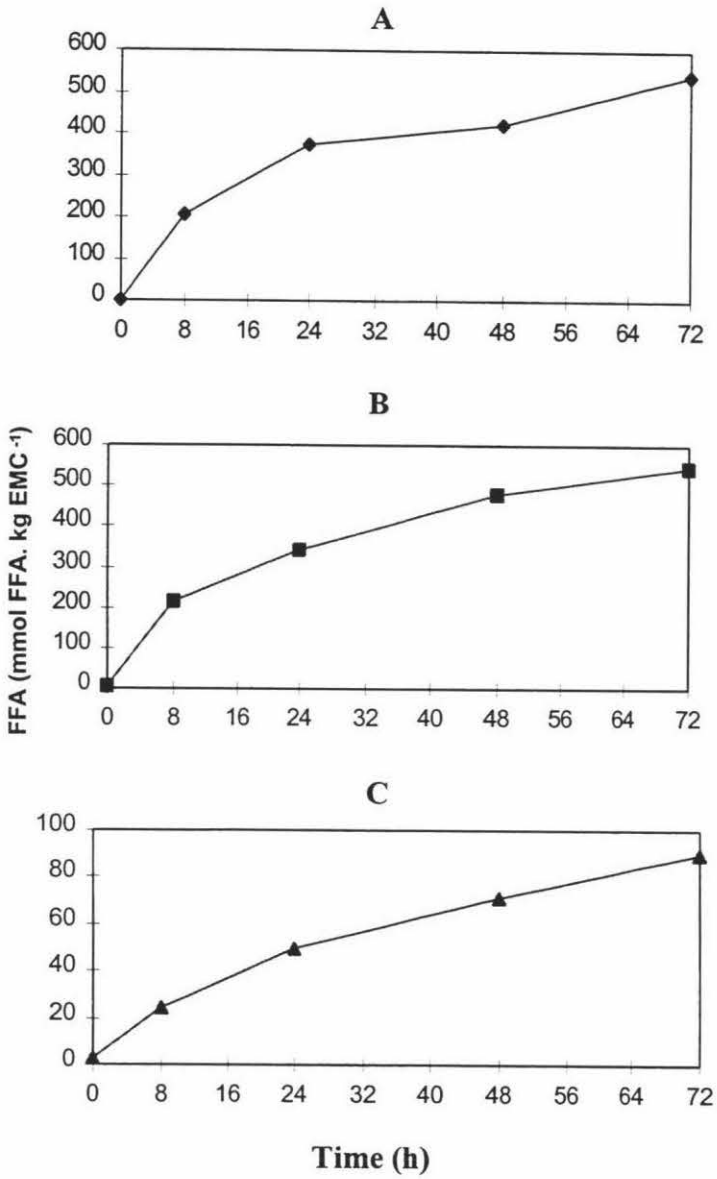

Figure 7.2: The effect of incubation time on the total amount of fatty acids released by hydrolysis: ***(A) Amano 'R'- 1%, 30℃ (B) Palatase - 0.15%, 45℃ (C) Kid lipase - 1%, 40℃***

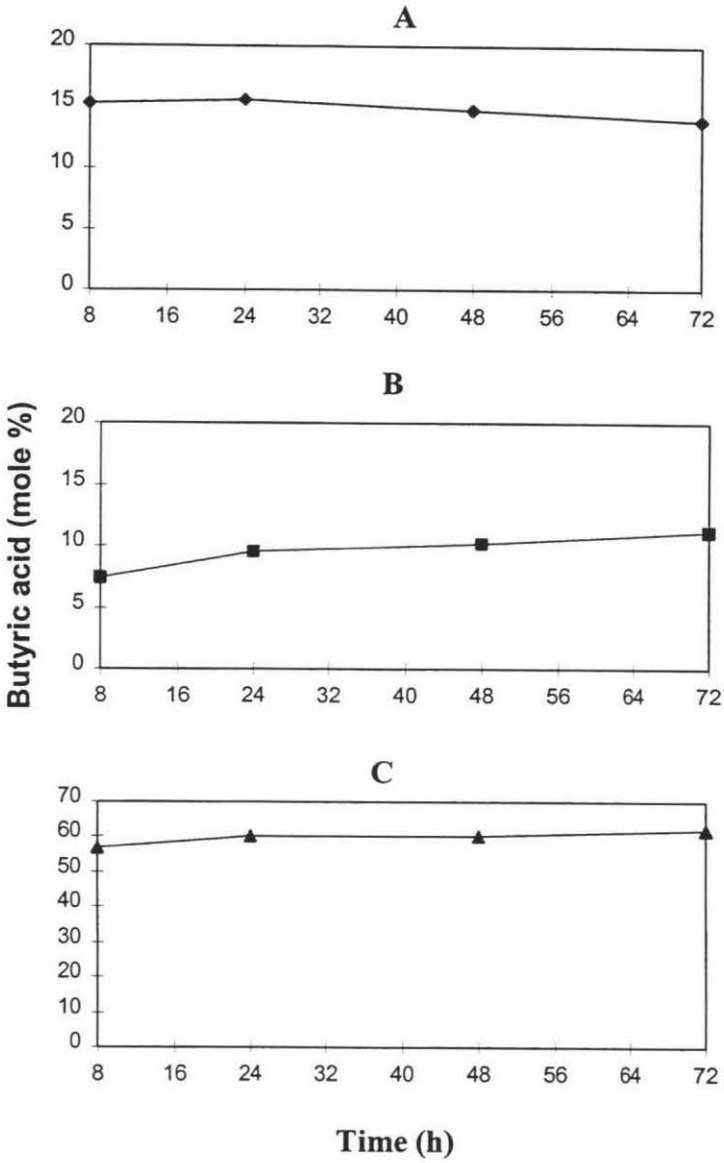

Figure 7.3: Effect of time on the percentage of Butyric acid (expressed as percentages of the total FFA) that were released by hydrolysis *(A) Amano 'R'- 1%, 30°C (B) Palatase - 0.15% , 45°C (C) Kid lipase - 1%, 40°C*

7.2.2 Temperature

The effect of incubation temperature on the total amounts of FAs released by hydrolysis varied depending on the lipase (Figure 7.4). For Palatase lipase the amounts of FFAs increased from about 260 to 500 mmol FFA. kg EMC.$^{-1}$ as the temperature increased from 40°C to 55°C, while for Amano 'R' lipase there was a decrease from about 290 to 50 mmol FFA. kg EMC.$^{-1}$ as the temperature increased from 40° to 55°C. For kid lipase there was a little variation in the extent of hydrolysis over the temperature range 30° to 50°C.

There is an interesting relationship between the percentage of butyric acid released by hydrolysis (expressed as a percentage of the total FFA) and the extent of lipolysis (total FFA). For each enzyme, the rate of release of butyric acid during the initial stages of lipolysis, is greater at lower incubation temperatures (20° to 30°C) than at higher incubation temperatures, with Palatase showing the largest change in rate (Figure 7.5).

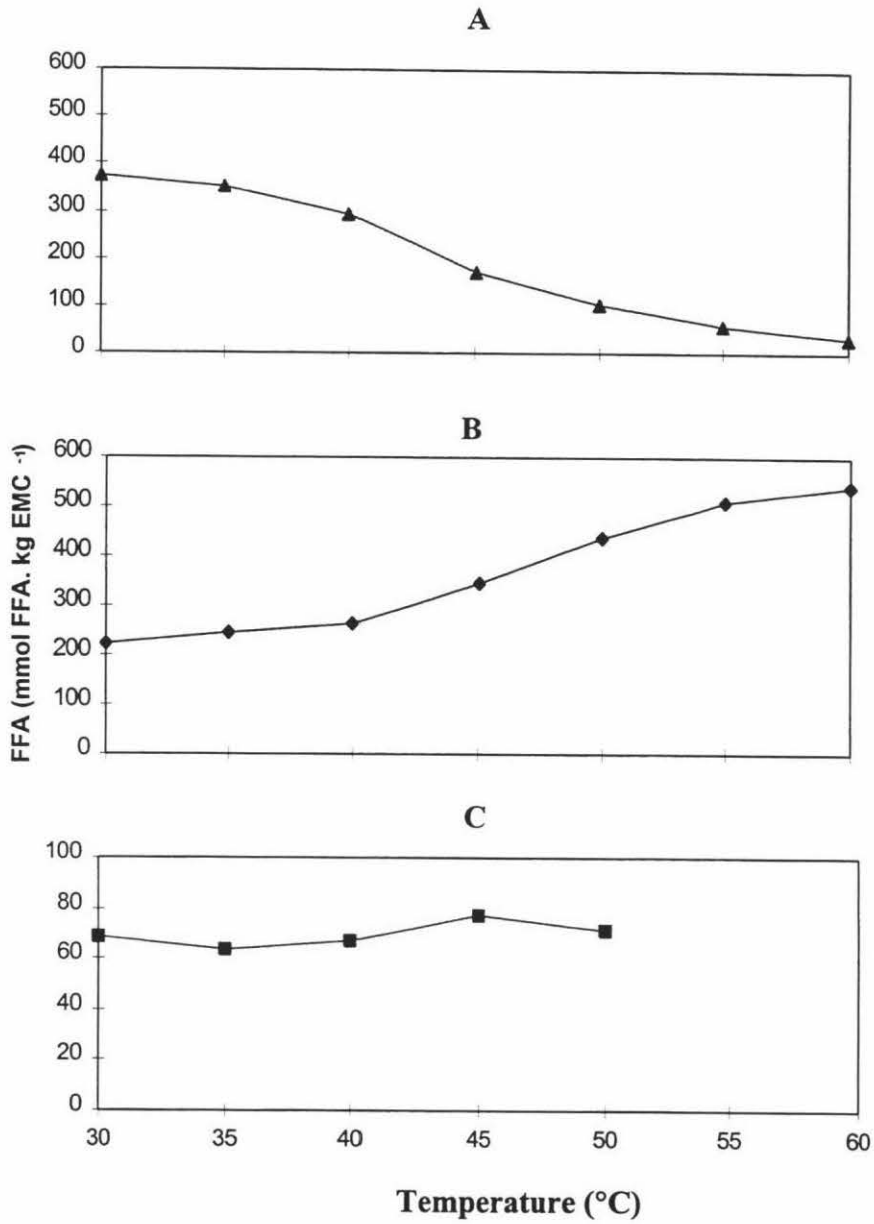

Figure 7.4: The effect of incubation temperature on the total amounts of fatty acids released by hydrolysis *(A) Amano 'R'- 1%, 24 h (B) Palatase - 0.15%, 24 h (C) Kid - 1%, 48 h*

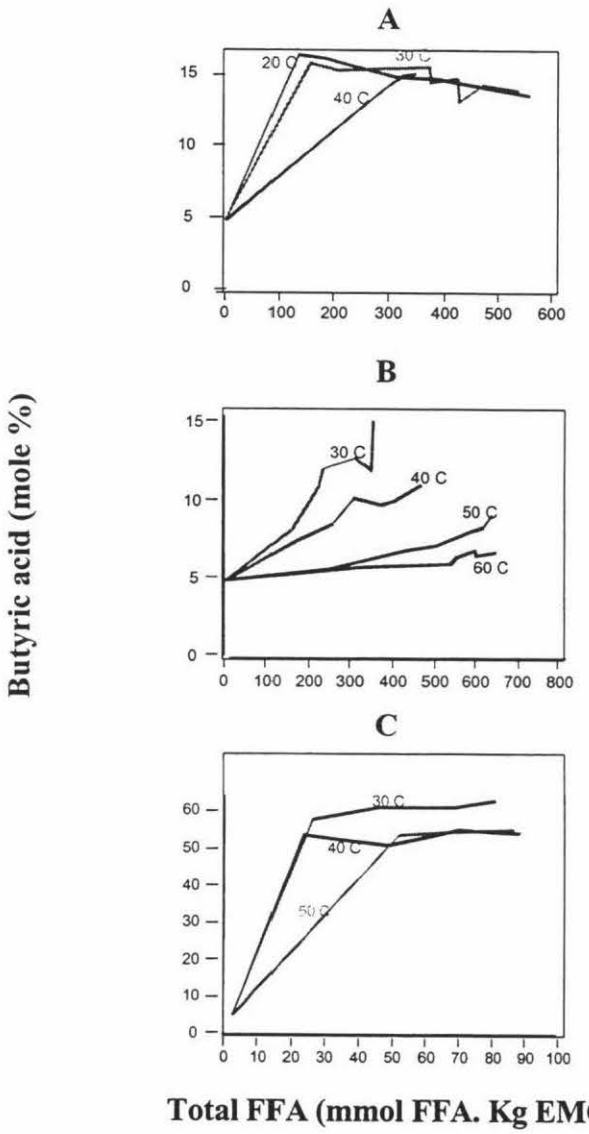

Figure 7.5: **The relationship between the percentage of butyric acid that was released by hydrolysis (expressed as a percentage of the total FFA) and the total free fatty acid (A) 1% Amano 'R', 0- 72 h (B) 0.15% Palatase, 0-72 h (C) 1% kid lipase, 0-72 h**

7.2.3 Initial pH

The initial pH of the substrate influences the extent of hydrolysis of triglyceride in the EMC base and results in a different optimum pH range for each enzyme (Figure 7.6). For both Palatase and Amano 'R' lipases, the total amount of FAs released by hydrolysis increased steadily as the pH increased, from 4.5 to 8.0 and from 4.5 to 7.5 respectively. The kid lipase had an optimum pH range of 5.5 to 6.5. The activity of the Amano 'R' lipase decreased markedly below pH 4.5 while the kid lipase was almost completely inhibited at pH values greater than 7.0.

The percentage of butyric acid (expressed as a percentage of the total FFAs) that was released by Amano 'R', Palatase and Kid lipase was affected by the initial pH of the EMC base (Figure 7.7). For both Amano 'R' and kid lipase, the percentage of C4:0 released decreased significantly at pH values below 5.0 and above 7.0 respectively. In each case these changes may be tied up with the inactivation of the lipase. On the other hand, the percentages of butyric acid released by Palatase decreased from 12% to 8% as the pH of the substrate increased from 4.0 to 8.0, showing that the extent of hydrolysis of C4:0 is pH dependent.

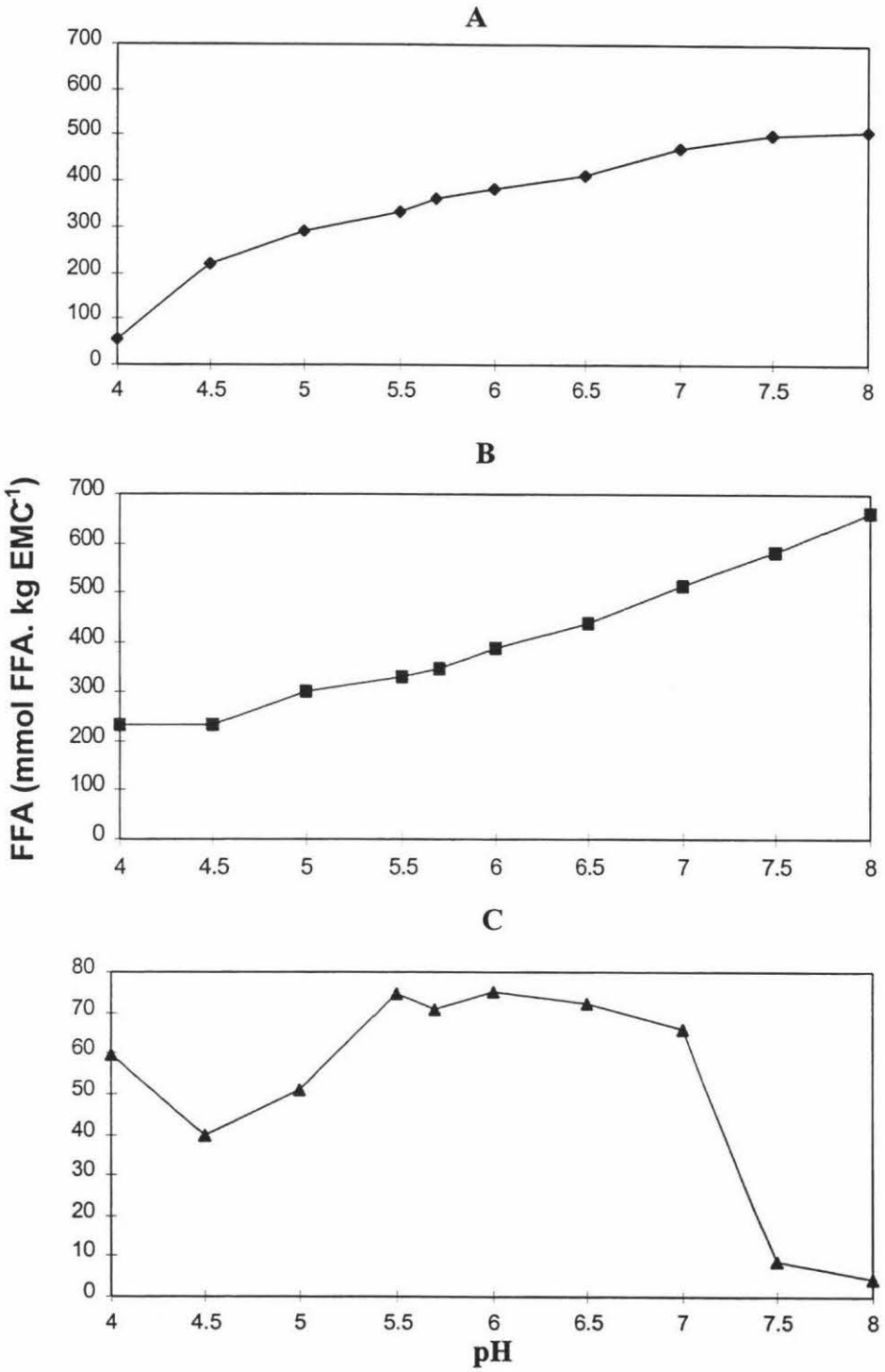

Figure 7.6: The effect of pH on the amounts of total fatty acids released by hydrolysis (A) Amano 'R'- 1%, 30°C, 24 h (B) Palatase - 0.15%, 45°C, 24 h (C) Kid lipase - 1%, 40°C, 48 h

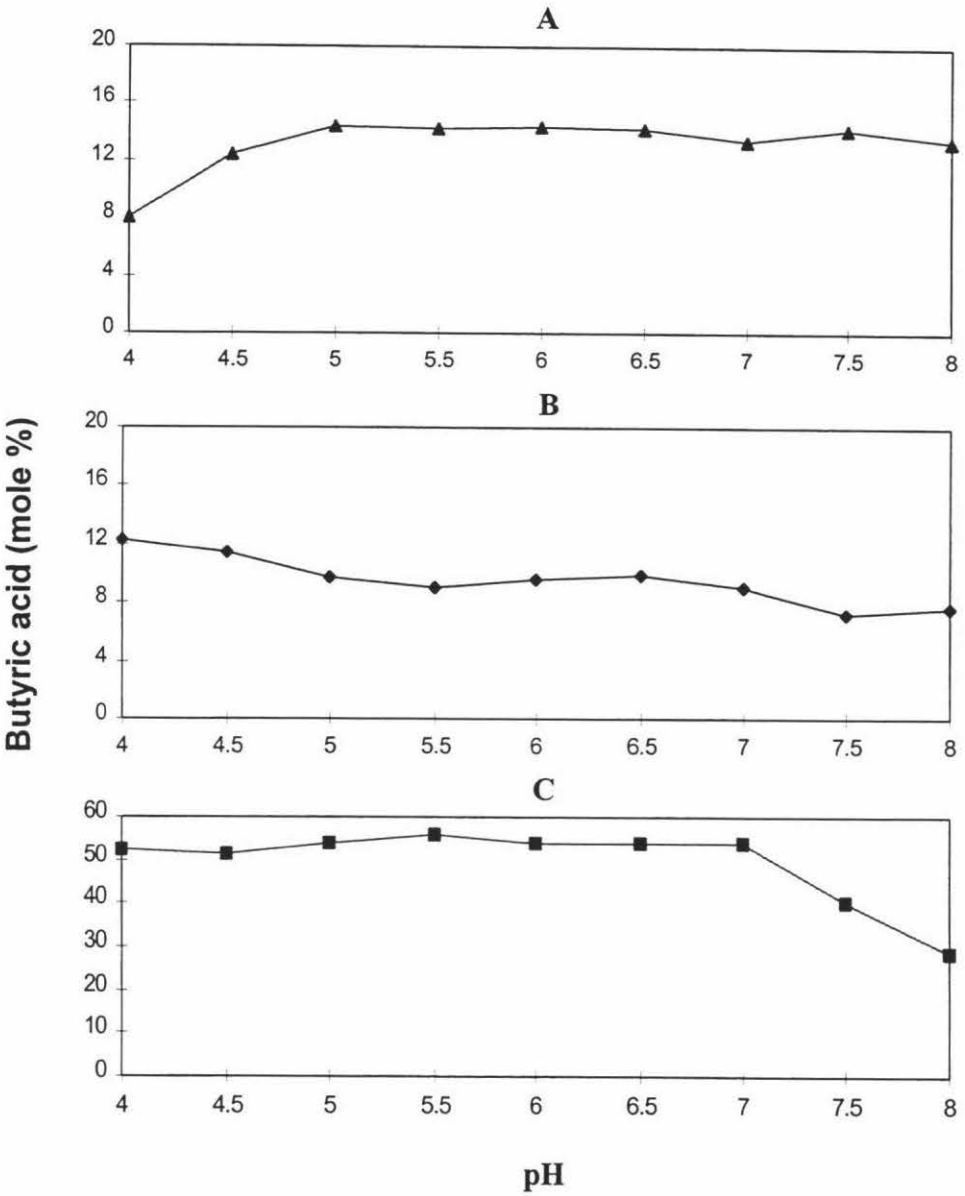

Figure 7.7: The effect of initial pH on the percentage of butyric acid (expressed as a percentage of the total FFA) that was released by hydrolysis (A) Amano 'R'- 1%, 30°C, 24 h (B) Palatase - 0.15% , 45°C, 24 h (C) Kid lipase - 1%, 40°C, 48 h

7.2.4 Enzyme concentration

The effect of enzyme concentration on the total amounts of FAs released by hydrolysis followed the expected pattern (Figure 7.8). The extent of hydrolysis increased in different rate as the enzyme concentration increased for each enzymes. The enzymes shows different optimum concentrations, which is 2.0%, 0.15% and 4.0% for Amano 'R', Palatase and kid lipase respectively. The enzymes released different amounts of total FAs at optimum concentration which are 430, 340 and 120 mmol. FFA. kg EMC^{-1} for Amano 'R', Palatase and kid lipase respectively.

The percentages of FAs (expressed as a percentage of the total FFA) were also affected by enzyme concentration. For example, the percentage of butyric acid (C4:0) released by hydrolysis increased slightly up to optimum concentration, for Palatase lipase (Figure 7.9). In contrast, the percentage of C4:0 released by Amano 'R' lipase decreased slightly up to optimum enzyme concentration while kid lipase released approximately the same percentage of C4:0.

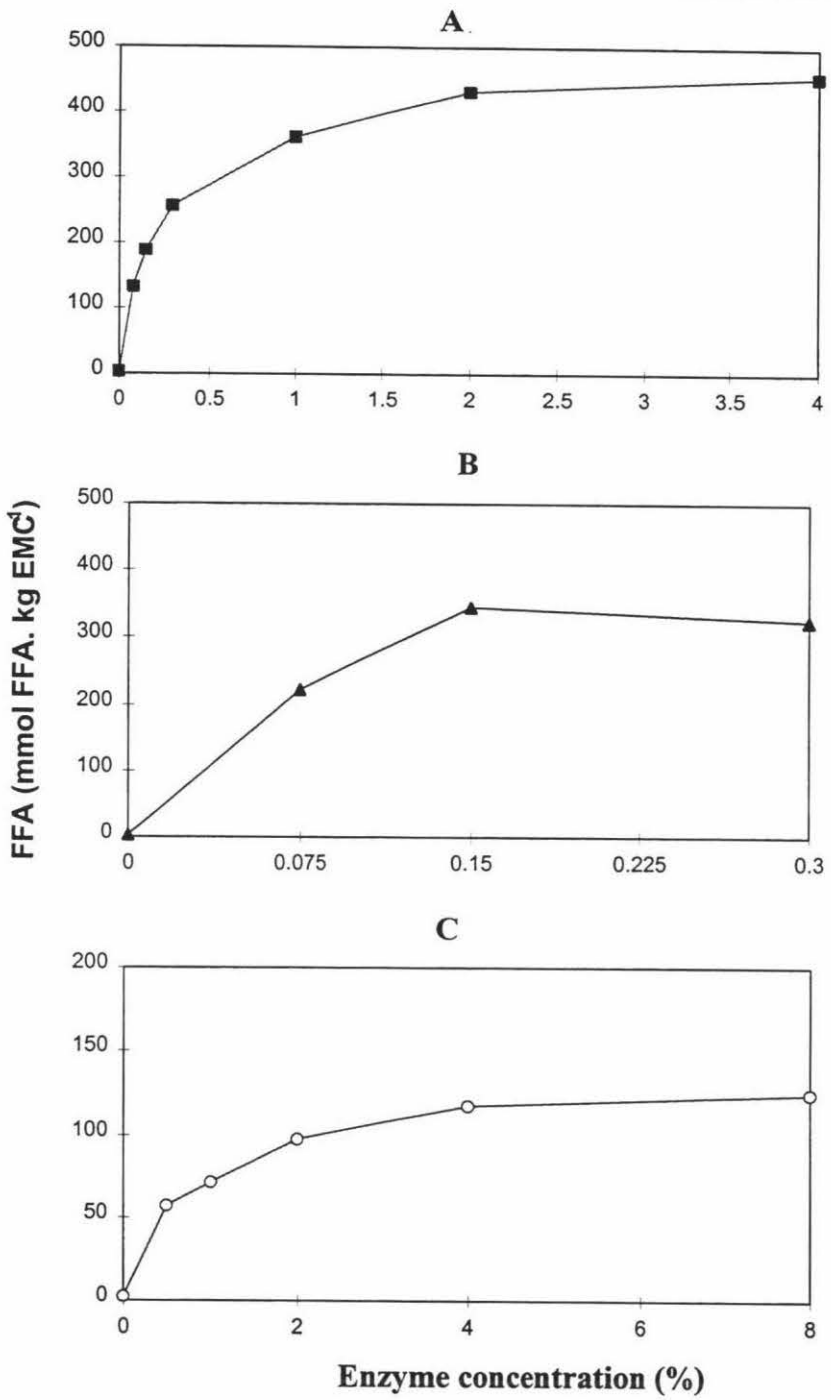

Figure 7.8: The effect of enzyme concentration on the total amount of fatty acids released by hydrolysis (A) Amano'R'-30°C, 24 h (B) Palatase - 45°C, 24 h (C) Kid lipase 40°C, 48 h

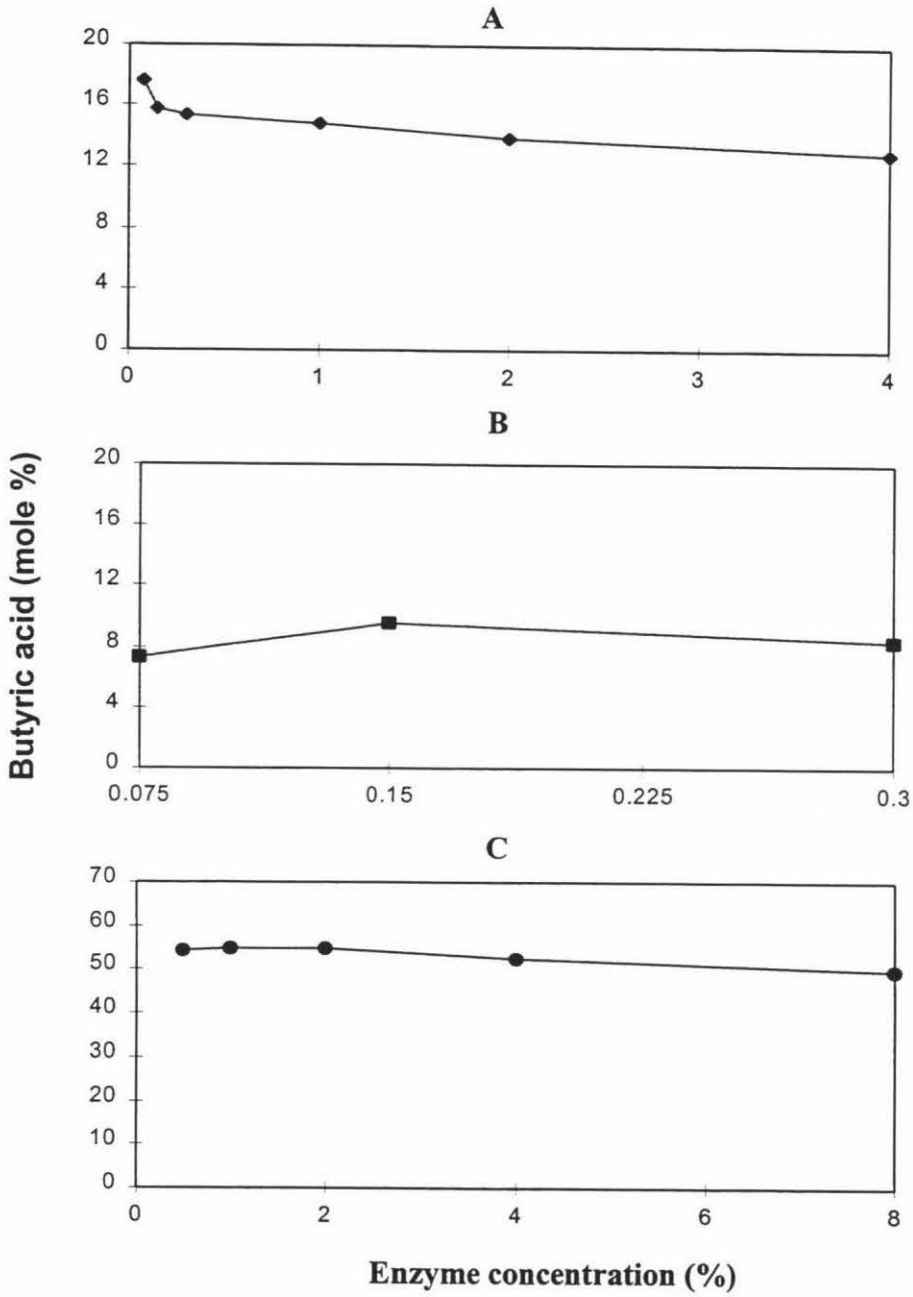

Figure 7.9: The effect of enzyme concentration on the percentage of Butyric acid (expressed as a percentage of the total FFA) that was released by hydrolysis (A) Amano'R'-30°C, 24 h (B) Palatase -45°C, 24 h (C) Kid lipase -40°C, 48 h

CONCLUSIONS

The effects of process parameters on Amano 'R', Palatase and Kid lipase activity were studied for their impact on hydrolysis of triglycerides in EMC base. The initial pH and temperature had the greatest effect on the extent of hydrolysis. An increase in incubation temperature increased hydrolysis, as did a pH increase. Salt-in-moisture content and water activity had a minor impact on hydrolysis.

The amounts and the percentage of individual fatty acids (FAs) released by hydrolysis is dependent on the type of enzyme and the reaction conditions used. Kid lipase shows specificity for short-chain FAs (C4:0 to C10:0), especially butyric acid. Amano 'R' shows some selectivity for short-chain fatty acids but Palatase shows some selectivity for long chain FAs (C16:0 to C18:1). Generally, incubation temperature has an inverse relationship on the rate of release of butyric acid (C4:0). This is true for all three enzymes. The percentage of butyric acid released by Palatase decreased as the pH of the EMC base increased. The amount of these enzymes used for hydrolysis is dependent on the process conditions used, will affect flavour intensity and may also affect flavour profile by changing free fatty acid percentages. Different EMC flavour profiles can be produced by hydrolysis of triglycerides from the same initial substrate using different commercial lipases and process conditions.

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APPENDICES

Appendix 1 : Characteristics of enzyme modified cheese base

Table 1: Gross composition of the EMC base before and after proteolysis

(on a wet weight basis)

Composition (% w/w)	Before proteolysis	After proteolysis
Moisture	50.6	57.3
Dry matter	49.4	42.7
salt in moisture	3.08	2.41
Fat	25.0	21.3
Total Nitrogen	2.86	2.49
Non Protein N	0.33	0.96

Table 2: Gross composition of EMC base before and after proteolysis

(on a dry weight basis)

Composition (% w/w)	Before proteolysis	After proteolysis
salt in moisture	3.16	3.23
Fat	50.61	49.88
Total Nitrogen	5.79	5.83
Non Protein N	0.68	2.25

Table 3: Degree of proteolysis in EMC base after 4 h incubation with protease.

Casein components	% Remaining	Degree of proteolysis (%)
αs_1	trace	100
αs_{1i}	trace	100
αs_2	trace	100
β-casein	3.9	96
γ-casein	70.6	29.4
Total protein	15.1	84.9

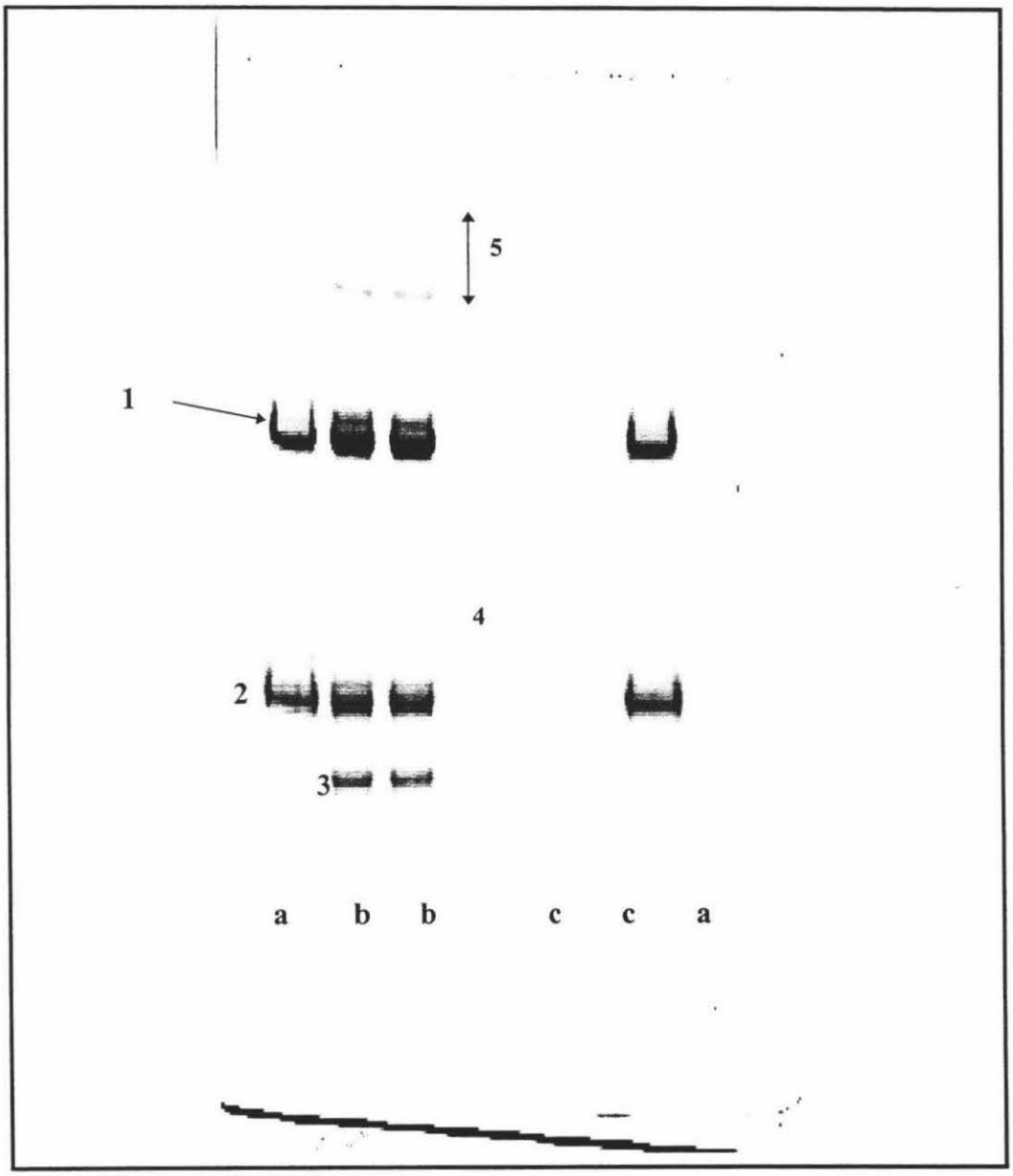

Figure 1: **Gel electrophoretic pattern of 72 hour incubated enzyme modified cheese base with protease enzyme (a) *Rennet casein standard (b) EMC base before proteolysis (c) EMC base after proteolysis***

(1) β-casein, (2) αs_1 (3) αs_{1i} (4) αs_2 (5) γ-casein

Table 4: Fatty acid composition in triglyceride of fat in enzyme modified cheese base

Fatty acid	Fatty acid composition *(mmol FA. kg EMC^{-1})*
C4:0	86.6
C6:0	40.8
C8:0	20.0
C10:0	34.7
C12:0	34.0
C14:0	100.3
C16:0	241.4
C18:0	84.1
C18:1	154.1
C18:2	9.6
Total	805.7

Table 5: Free fatty acid composition of the enzyme modified cheese base before and after proteolysis

Type of FFA	Free fatty acid (mmol FFA. kg EMC^{-1})	
	Before proteolysis	*After proteolysis*
C4:0	0.23	0.16
C6:0	0.05	0.06
C8:0	0.04	0.06
C10:0	0.06	0.11
C12:0	0.05	0.10
C14:0	0.10	0.25
C16:0	0.18	0.63
C18:0	0.10	0.21
C18:1	0.13	0.60
C18:2	0.04	0.01
Total FFA	1.81	2.81

Table 6: Peak area of identified volatile compounds extracted by SPME from head space of EMC base before lipolysis

Volatile compounds	Peak Area
Pentanol	1347829
2-Butanone, 3-hydroxy	3034300
2-Pentanone	3413877
2-Heptanone	13681287
2-Nonanone	5212821
Butyrolactone	884470
Pentanal,2-methyl	1158113
Benzaldehyde	466874
Acetic acid	1891704
Butyric acid	1469134
Hexanoic acid	2570637
Methylacetylene	738434

Appendix 2: Experimental data - lipolysis by Amano 'R' lipase

Table 1: The effect of incubation temperature on the percentages* of individual fatty acids released by hydrolysis#

Temperature (°C)	Free fatty acid (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
20	14.6	6.5	2.7	4.5	3.9	10.9	26.1	9.4	18.8	0.5
25	14.8	6.2	2.6	4.2	3.7	10.6	26.7	10.0	18.8	0.5
30	15.3	6.4	2.6	4.2	3.7	10.6	26.6	10.1	18.2	0.5
35	14.4	6.2	2.6	4.4	3.9	11.4	28.1	9.8	18.6	0.5
40	14.1	6.0	2.5	4.3	4.1	11.7	28.2	10.1	18.6	0.5
45	11.9	5.7	2.4	4.3	3.8	11.4	29.2	10.8	20.2	0.3
50	11.7	5.6	2.3	3.7	3.6	10.8	30.0	11.2	20.6	0.5
55	12.5	5.6	2.3	3.4	3.3	10.3	30.7	11.1	20.8	0.0
60	14.6	6.3	2.6	3.7	3.5	10.9	32.2	9.8	16.5	0.0

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 1% Amano 'R', 24 h*

Table 2: The effect of enzyme concentration on the percentages* of individual fatty acids released by hydrolysis#

Enzyme concentration (%)	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
0.075	17.6	7.2	2.5	3.9	3.2	9.5	26.2	10.4	19.1	0.3
0.15	15.7	6.4	2.4	3.9	3.3	10.1	27.1	11.0	19.6	0.4
0.3	15.4	6.4	2.5	4.2	3.6	10.7	27.7	10.2	18.8	0.4
1.0	14.8	6.3	2.6	4.3	3.8	11.3	27.7	10.1	18.5	0.5
2.0	13.9	5.9	2.5	4.3	3.9	11.5	28.1	10.6	18.8	0.5
4.0	13.0	5.6	2.5	4.2	4.0	11.7	28.7	10.8	18.9	0.5

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: Amano 'R'- 30 °C, 24 h*

Table 3: The effect of pH on the percentages* of individual fatty acids released by hydrolysis#

pH	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
4	8.0	5.3	2.8	4.6	4.2	12.1	30.8	11.4	20.0	0.6
4.5	12.4	5.4	2.3	4.1	3.6	11.1	29.2	11.1	20.2	0.5
5	14.4	6.1	2.5	4.4	3.8	11.4	28.2	10.2	18.5	0.5
5.5	14.3	6.2	2.6	4.3	3.8	11.3	27.5	10.5	19.1	0.5
6	14.3	6.1	2.6	4.4	3.9	11.3	27.4	10.5	19.0	0.5
6.5	14.2	6.3	2.7	4.5	4.2	11.5	27.4	10.2	18.5	0.5
7	13.4	6.0	2.6	4.4	4.0	11.6	27.9	10.6	19.1	0.5
7.5	14.3	6.2	2.6	4.3	3.9	11.4	27.7	10.4	18.9	0.5
8	13.3	6.0	2.5	4.3	4.1	11.6	28.0	10.5	19.2	0.5

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 1% Amano 'R', 30°C, 24 h*

Table 4: The effect of water activity (a_w) on the percentages* of individual fatty acids released by hydrolysis#

a_w	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
0.89	14.7	6.4	2.6	4.4	3.8	11.1	27.8	10.0	18.8	0.4
0.91	15.0	6.5	2.7	4.4	3.9	11.4	27.6	9.7	18.5	0.4
0.93	14.8	6.2	2.6	4.2	3.7	11.1	27.9	10.4	18.6	0.5
0.95	14.5	6.4	2.6	4.4	3.8	11.2	27.7	10.2	18.6	0.5
0.97	14.0	6.1	2.5	4.3	3.9	11.2	28.2	10.3	19.1	0.4

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 1% Amano 'R', pH 6.5, 30°C, 24 h*

Table 5: The effect of salt in moisture content on the percentages* of individual fatty acids released by hydrolysis#

Salt- (% in moisture)	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
2.4	14.0	6.1	2.5	4.3	3.9	11.2	28.2	10.3	19.1	0.4
4.8	14.2	6.0	2.5	4.3	3.8	11.4	27.9	10.4	19.0	0.5
7.2	13.9	6.1	2.6	4.4	3.9	11.6	28.5	10.0	18.5	0.5
9.6	13.7	6.1	2.5	4.2	3.9	11.7	28.5	10.2	18.3	0.8

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 1% Amano 'R', pH 6.5, 30°C, 24 h*

Appendix 3: Experimental data - Lipolysis by Palatase lipase

Table 1: The effect of incubation temperature on the percentages* of individual fatty acids released by hydrolysis#

Temperature (°C)	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
30	10.7	6.2	3.0	4.0	3.5	9.3	31.4	12.9	18.5	0.5
35	12.4	6.8	3.4	4.7	4.8	10.4	29.3	10.1	17.7	0.4
40	8.5	5.4	2.9	4.4	4.5	11.6	32.3	11.0	18.9	0.6
45	9.6	6.0	3.1	4.8	4.8	12.4	32.2	9.8	16.9	0.5
50	6.7	4.5	2.5	4.2	4.1	11.5	32.6	11.5	21.5	0.9
55	6.7	4.5	2.4	4.2	4.2	11.8	32.1	11.7	21.1	1.3
60	5.8	4.5	2.5	4.3	4.4	12.3	32.9	11.1	21.2	0.9

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 0.15% Palatase, 24 h*

Table 2: The effect of enzyme concentration on the percentages* of individual fatty acids released by hydrolysis#

Enzyme concentration (%)	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
0.075	7.3	5.2	3.1	4.9	4.9	13.3	31.1	12.2	17.0	0.9
0.15	9.6	6.0	3.1	4.8	4.8	12.4	32.2	9.8	16.9	0.5
0.3	8.4	5.2	2.9	4.8	4.6	12.8	31.5	11.4	17.9	0.7

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: Palatase- 45 °C, 24 h*

Table 3: The effect of pH on the percentages* of individual fatty acids released by hydrolysis#

pH	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
4	12.2	6.5	3.1	4.5	4.1	10.6	29.2	10.5	18.8	0.5
4.5	11.4	6.0	2.9	4.4	4.1	10.5	29.1	11.5	19.6	0.4
5	9.7	5.6	2.8	4.4	4.1	11.1	29.9	11.4	20.6	0.5
5.5	9.0	5.7	3.0	4.8	4.6	12.2	30.7	10.4	19.4	0.3
5.7	8.1	4.9	2.5	4.2	4.2	12.0	30.3	11.5	21.4	0.9
6	9.6	6.2	3.1	5.0	4.6	12.4	30.1	9.8	18.6	0.5
6.5	9.9	6.7	3.2	5.1	4.8	12.6	29.1	9.7	18.4	0.5
7	9.0	6.3	3.0	5.1	4.8	12.9	30.3	9.7	18.4	0.6
7.5	7.2	5.2	2.6	4.4	4.4	12.5	30.7	11.0	21.1	0.9
8	7.6	5.5	2.7	4.7	4.5	12.8	30.2	10.5	20.7	0.8

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 0.15% Palatase, 45 °C, 24 h*

Table 4: The effect of water activity (a_w) on the percentages* of individual fatty acids released by hydrolysis#

a_w	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
0.89	9.7	5.8	2.8	4.5	4.3	12.0	30.6	10.6	19.2	0.4
0.91	9.2	5.7	2.9	4.6	4.4	12.3	30.8	10.4	19.2	0.5
0.93	9.0	5.6	2.7	4.6	4.4	12.5	30.5	10.7	19.6	0.5
0.95	8.4	5.5	2.7	4.5	4.4	12.6	31.0	10.6	19.8	0.6
0.97	9.3	5.9	2.8	4.5	4.3	12.3	30.7	10.5	19.2	0.5

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 0.15% Palatase, pH 6.5, 45 °C, 24 h*

Table 5: The effect of salt-in-moisture content on the percentages* of individual fatty acids released by hydrolysis#

Salt (% in moisture)	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
2.4	7.4	5.4	2.7	4.6	4.4	12.5	31.1	11.0	20.3	0.6
4.8	7.3	5.2	2.6	4.4	4.2	12.2	30.9	11.3	21.2	0.8
7.2	7.5	5.2	2.6	4.4	4.3	12.8	31.6	11.0	20.0	0.5
9.6	7.3	5.4	2.7	4.5	4.4	12.6	31.6	11.1	19.9	0.5

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 0.15% Palatase, pH 6.5, 45°C, 24 h*

Appendix 4: Experimental data -lipolysis by Kid lipase

Table 1: The effect of temperature on the percentages* of individual fatty acids released by hydrolysis#

Temperature (°C)	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
30	60.3	19.1	3.9	6.1	4.1	3.0	2.7	0.0	0.8	0.0
35	57.8	19.3	4.3	6.5	4.5	3.9	3.3	0.0	0.4	0.0
40	54.8	18.5	4.2	6.3	4.7	4.0	4.0	1.1	2.4	0.0
45	55.0	19.0	4.3	6.4	4.7	3.8	3.8	1.1	1.9	0.0
50	54.7	19.5	4.2	6.2	4.3	3.7	3.9	1.1	2.4	0.0

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 1% Kid lipase, 48 h*

Table 2: The effect of enzyme concentration on the percentages* of individual fatty acids released by hydrolysis#

Enzyme concentration (%)	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
0.5	54.2	18.6	4.2	6.2	4.5	4.1	4.4	1.5	2.3	0
1.0	54.8	18.5	4.2	6.3	4.7	4.0	4.0	1.1	2.4	0
2.0	54.8	17.4	4.4	6.7	5.1	4.4	4.3	1.0	1.9	0
4.0	52.4	16.6	4.6	7.0	5.1	5.4	5.1	1.2	2.6	0
8.0	49.6	15.7	4.7	6.9	5.6	6.4	6.5	1.6	3.0	0

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: Kid lipase- 40°C, 48 h*

Table 3: The effect of pH on the percentages* of individual fatty acids released by hydrolysis#

pH	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
4.0	52.6	18.7	4.6	7.1	5.0	3.8	4.3	1.3	2.4	0.0
4.5	51.4	19.1	4.8	7.5	5.4	4.5	4.8	1.1	1.4	0.0
5.0	54.1	18.7	4.4	6.7	4.9	4.3	4.4	0.8	1.6	0.0
5.5	55.8	19.1	4.5	6.9	4.9	3.8	3.4	0.6	1.0	0.0
5.7	54.8	18.5	4.2	6.3	4.7	4.0	4.0	1.1	2.4	0.0
6.0	54.1	18.8	4.5	6.8	4.9	4.3	4.1	0.8	1.6	0.0
6.5	53.8	19.2	4.5	6.8	4.8	4.2	4.2	0.9	1.4	0.0
7.0	54.0	19.6	4.5	6.6	4.6	4.0	4.2	0.9	1.6	0.0
7.5	39.9	23.7	5.9	7.5	4.5	7.6	10.8	0.0	0.0	0.0
8.0	28.6	17.6	7.4	10.0	7.4	10.3	18.7	0.0	0.0	0.0

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 1% Kid lipase, 40°C, 48 h*

Table 4: The effect of water activity on the percentages* of individual fatty acids released by hydrolysis#

a_w	Free fatty acids (mole %)									
	C4:0	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
0.89	52.7	19.5	4.6	6.4	4.1	3.7	5.1	1.6	2.3	0.0
0.91	57.1	19.8	4.2	5.7	3.7	3.2	4.0	1.0	1.3	0.0
0.93	55.9	19.7	4.4	6.3	4.1	3.6	4.1	0.9	1.2	0.0
0.95	55.9	19.6	4.5	6.3	4.0	3.4	4.1	0.8	1.4	0.0
0.97	55.6	20.8	4.7	6.5	4.0	3.1	3.4	0.7	1.1	0.0

* *expressed as a percentage of the total free fatty acid*

\# *Experimental conditions: 1% Kid lipase, pH 6.5, 40°C, 48 h*

Table 5: The effect of salt in moisture content on the percentages* of individual fatty acids released by hydrolysis#

Salt	Free fatty acids (mole %)									
(% in moisture)	*C4:0*	*C6:0*	*C8:0*	*C10:0*	*C12:0*	*C14:0*	*C16:0*	*C18:0*	*C18:1*	*C18:2*
2.4	55.6	20.8	4.7	6.5	4.0	3.1	3.4	0.7	1.1	0.0
4.8	56.5	20.4	4.5	6.4	4.0	3.2	3.4	0.5	1.0	0.0
7.2	56.4	20.4	4.5	6.4	4.0	3.3	3.5	0.5	0.8	0.0
9.6	56.2	20.3	4.5	6.2	4.0	3.6	3.7	0.5	0.8	0.0

* ***expressed as a percentage of the total free fatty acid***

\# ***Experimental conditions: 1% Kid lipase, pH 6.5, 40 °C, 48 h***