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Thermophilic Enzymes and Their Impact on Milk Powder during Storage

A thesis presented in partial fulfilment of the requirement for the degree of Doctor of Philosophy at the University of Waikato

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2000

Abstract

Milk powder quality and storage life can be compromised by functionality and flavour defects. These defects can be the result of chemical or biochemical reactions, such as the Maillard (browning) reaction, proteolysis and lipolysis. The New Zealand dairy industry has set specifications for thermophile numbers in milk powders that have been used for many years as an indicator of microbial quality. However, any correlation between the numbers of these bacteria and product quality is anecdotal, and there is little supportive scientific evidence.

This research has set out to: (1) survey the cause of the problem for off-flavours in milk powder storage, mainly whole milk powders (WMPs) because they are the largest powder-product, and most likely to have problems associated with flavour; (2) develop assay methods for detecting possibly very low levels of protease and lipase in the WMPs; and finally (3) determine enzymatic effects in the WMPs during storage.

The study has focused on the detection of protease and lipase activities. Several sensitive and commonly used assay methods were applied in reconstituted milk made from different batches of commercial WMPs. Both protease and lipase activities were detected, and the lipase activity was quantified at 0.7-1.1 U/g powder (*p*NP caproate unit), but the level of protease activity is yet to be accurately determined.

A lipase was semi-purified from a WMP using Phenyl Sepharose hydrophobic chromatography. The enzyme is most active at 60°C under the conditions used, and has similar substrate specificity to the lipases produced by seven *Bacilli* (isolated from a

milk powder production stream. Although the source of the powder lipase is yet to be identified, data suggest that it is most likely from a thermophilic bacterium.

Preliminary studies on the characteristics of the proteases and the lipases produced by the seven *Bacilli* showed that the proteases differ between isolates in term of pH optima and heat-stabilities, so do the lipases. The lipases are more heat-stable than the proteases in a buffer system. Under the protection of milk proteins, these enzymes can survive the heat-treatments applied in the milk powder manufacture process, and retain activities in the powder.

Further studies on WMPs spiked with thermophilic proteases and lipases, or taken directly from the commercial process have confirmed that the proteases and the lipases are active in the powder (at moisture < 5%) during storage. Proteolysis was observed as the increase of 1% trifluoroacetic acid (TFA) soluble protein fragments and the decrease of solubility, and lipolysis was shown as the release of free fatty acids (FFA). The lipases showed the same specificities in a buffer system and in the powder. The levels of enzymatic products increase with increasing storage temperatures and time. Proteolysis resulted in poor solubility of WMP within one week of storage at 37°C. Lipolysis resulted in the levels of FFA, especially short chain butyric and caproic acids, exceeding the organoleptic threshold values under the same storage conditions.

Nevertheless, none of the defects observed showed any correlation with thermophile numbers – one of the current specifications for quality control of WMP. Therefore, it is not thermophiles *per se* that affect milk powder quality during storage, it is the heat-stable enzymes, from whatever source.

Acknowledgments

First, I would like to thank my three supervisors Professor Roy Daniel, Dr. Tim Coolbear and Mr. George Miller who have given me the opportunity, the invaluable support and guidance throughout the project.

Thanks also go to many people who provided their friendly support to me throughout my time at the Thermophile Research Unit at the University of Waikato and the Microbiology, Nutrition and Enzyme Science Section at the New Zealand Dairy Research Institute (NZDRI). I would like to thank Dr. J. Bragger and Ms. H. S. Toogood (University of Waikato) and Dr. C. Schmidt-Dannert (University of Stuttgart, Germany) for providing the thermophilic enzymes for the powder-spiking work; Dr. J. R. Reid and Mrs. S. Harvey (NZDRI) for running the mass spectra; Mr. A. Dodge (NZDRI) for water activity test; Mrs. G. N. T. Truong (NZDRI) for carrying out milk powder functionality tests; Mrs. R. L. Hirst (NZDRI) for carrying out transmission electron microscopy; Microbiology Service Group (NZDRI) for testing thermophiles; Mr. T. Loo (Institute of Molecular BioScience, Massey University) for protein sequencing. I also like to thank all the dairy factories that provided milk powder samples for the study.

I would like to express my thanks for financial support in the form of a Graduate Researcher in Industry Fellowship between the Foundation for Research, Science and Technology, the University of Waikato and Anchor Products.

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Abbreviations

BSA	bovine serum albumin
BTP	bis-Tris propane
Caps	3-[Cyclohexylamino]-1-propanesulfonic acid
DEE	diethyl ether
EDTA	ethylenediaminetetra acetic acid
FFA	free fatty acids
FITC	fluorescein isothiocyanate
FTC-casein	fluorescein thiocarbamyl-casein
GLC	gas liquid chromatography
GC/MS	gas chromatography mass spectrometry
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
IAA	iodoacetic acid
IPA	*iso*-propyl achohol
IEF	isoelectric focusing
Mes	2-(N-morpholino) ethanesulfonic acid
Mops	3-[N-morpholino] propanesulfonic acid
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulphonyl fluoride
*p*NA	*p*-nitroaniline
*p*NP	*p*-nitrophenyl
PVDF	polyvinylidene difluoride
RAPD-PCR	random amplified polymorphic DNA primer chain reaction
RP-HPLC	reverse phase high performance liquid chromatography
Suc-AAPF-*p*NA	N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide

SPME	solid phase microextraction
SDS	sodium dodecyl sulphate
SPE	solid phase extraction
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
Tris	Tris (hydroxymethyl) methylamine
TSB	tryptic soy broth
WMP	whole milk powder

Fatty Acids General Information

Nomenclature (IUPAC name)	Trival name	Chain length	M_r	Composition in NZ milkfat (w/w %)
n-Butanoic	Butyric	$C_{4:0}$	88	3.9
n-Hexanoic	Caproic	$C_{6:0}$	116	2.5
n-Octanoic	Caprylic	$C_{8:0}$	144	1.5
n-Decanoic	Capric	$C_{10:0}$	172	3.2
n-Dodecanoic	Lauric	$C_{12:0}$	200	3.6
n-Tetradecanoic	Myristic	$C_{14:0}$	228	11.1
n-Hexadecanoic	Palmitic	$C_{16:0}$	256	27.9
n-Octadecanoic	Stearic	$C_{18:0}$	284	12.2
Octadec-9-enoic	Oleic	$C_{18:1}$	282	21.1
Octadeca-9:12-dienoic	Linolenic	$C_{18:2}$	280	1.4

Amino Acids Letter Code

Amino acid	Single letter code	Amino acid	Single letter code
Alanine (Ala)	A	Leucine (Leu)	L
Arginine (Arg)	R	Lysine (Lys)	K
Asparagine (Asp)	N	Methionine (Met)	M
Aspartic acid (Asn)	D	Phenylalanine (Phe)	F
Cysteine (Cys)	C	Proline (Pro)	P
Glutamine (Glm)	Q	Serine (Ser)	S
Glutamic acid (Glu)	E	Threonine (Thr)	W
Glycine (Gly)	G	Tyrosine (Tyr)	Y
Histidine (His)	H	Tryptophan (Trp)	W
Isoleucine	I	Valine (Val)	V

Terminology

"Esterase, lipase, lipase activity or lipolytic activity" has been used for the activity found in various esterase/lipase in whole milk powders. But esterases/lipases are a group of enzymes that are not as clearly defined as other groups of enzymes on their classification or biochemical characteristics. Generally speaking, esterases hydrolyse carboxyl ester bonds of water-soluble substrates (*e.g.* short chain triacylglcyerols, $C_{4:0}$-$C_{8:0}$), while lipases act on the same bonds of water-insoluble emulsified substrates with a chain length $>C_{8:0}$ (Tsujita *et al.*, 1990). However, some enzymes from *P. aeruginosa* and *B. subtilis* have activities on triacylglcyerols in both solution and emulsion (Jaeger *et al.*, 1994). In this thesis, "lipase" has been used for both esterase and lipase activity because of industry familiarity with the term.

Assays of lipase activity have been carried out using both water-soluble and insoluble *p*-nitrophenyl (*p*NP) esters and triacyglycerols in the thesis. But the reader needs to bear in mind that many 'pure' proteins display esterase activity on short chain *p*NP esters (see results in Table 3.3). Such activity could not be reliably measured since the negative control proteins all originated from bovine milk and therefore may contain contaminating lipase. Even if this 'non-specific' activity is very low in assays of milk powders, the quantity of protein exhibiting it is likely to be very large compared to the enzyme quantity, therefore any such activity present could compromise the assay results. The lipase activity results obtained in milk powders may be due to a combination of the lipase activity and any non-specific activity of the milk proteins. The reason for persisting with this short chain *p*NP ester assay in the face of this difficulty is that this assay is more sensitive than any other and because this "short chain" activity is likely to lead to the production of "off" odours and flavours during milk powder storage.

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Chapter 1 Protease and Lipase in Milk and Dairy Products: A Review

1.1 Introduction

Bovine milk is a biologically active product. Around 50 to 60 different enzymatic activities have been reported in clean, freshly drawn milk (Farkye, 1991; Muir, 1996a). Neither the enzyme types nor their distributions are constant in milk during the season. Influencing factors include the breeds and the age of the cows, the stage of lactation, the diet and the health status of the cows (Farkye, 1991; Deeth and Fitz-Gerald, 1995). However, only a few of the milk endogenous enzymes (*i.e.* enzymes in unprocessed milk) have a substantial impact on the quality and shelf-life of milk and dairy products (Muir, 1996a).

Apart from endogenous enzymes, milk (raw or processed) will also contain enzymes arising from contaminating bacteria. These bacteria can be classified according to their optimal growth temperatures (Singleton and Sainsbury, 1987): thermophiles (> 45°C), mesophiles (20-45°C) and psychrophiles (≤ 15°C). Many spoilage microorganisms found in milk and dairy products are mesophiles and thermophiles. If a bacterium can grow at 7°C or less irrespective of its optimum growth temperature, it is often called 'psychrotroph' (Suhren, 1989).

Adams *et al.* (1975) reported that 70 to 90% of raw milk samples contained psychrotrophs capable of producing heat resistant proteases. Craven and Macauley (1992) reported that 86.8% of psychrotrophic bacteria isolated from pasteurised milk after storage at 4°C were *Pseudomonas*. On the other hand, many mesophiles and

thermophiles commonly found in milk and dairy products are *Bacilli* including *B. lichenifomis*, *B. coagulans*, *B. subtilis*, *B. circulans* and *B. stearothermophilus* (Chopra and Mathur, 1984; Morgan *et al.*, 1997). In fact many *Pseudomonas* and *Bacillus* are mesophiles, because they can grow at 7°C or below, so they are often called psychrotrophs. These two species of bacteria have been identified as the major proteolytic and lipolytic flora in milk and dairy products (Law, 1979; Cousin, 1982; Law and Mabbitt, 1983; Ewings *et al.*, 1984; Sharma *et al.*, 1984; Walker, 1988; Champagne *et al.*, 1994; Matta and Punj 1996). These organisms release heat-stable enzymes that can cause spoilage at very low levels and abroad range of temperatures in the final products during storage (Adams *et al.*, 1975; Cogan, 1977; Griffiths *et al.*, 1981; Chopra and Mathur, 1984; Fairbairn and Law, 1986; Craven and Macauley, 1992; Choi and Jeon, 1993; Deeth and Fitz-Gerald, 1995).

Chopra and Mathur (1984) found that thermophilic *Bacilli* isolated from milk and dairy products can produce high levels of extracellular proteases in a tryptone and yeast-extract medium. In a review, Priest (1977) noted that *Bacilli* synthesise a large variety of extracellular enzymes, such as proteases and lipases, the maximal synthesis of which normally occurred in the late exponential and early stationary phases of growth before sporulation. There is a strong evidence that the synthesis of extracellular serine protease is associated with sporulation (Priest, 1977). Siezen and Leunissen (1997) reported that *Bacilli* commonly produce subtilases, members of the superfamily of subtilisin-like serine proteases.

Garcia-Armesto and Sutherland (1997) reported the temperature characteristics of psychrotrophic and mesophilic *Bacilli* isolated from milk. Majority of the psychrotrophic species (*Pseudomonas*) will probably not survive pasteurisation of

62.8°C for 30 min (low temperature long time: LTLT) or 71.5°C for 15 s (high temperature-short time: HTST; Varnam and Sutherland, 1994) and ultra-heat treatment (UHT, 135°C to 150°C for 1-4 s; Griffiths *et al.*, 1981; Suhren, 1989). In contrast, mesophiles and thermophiles (*Bacilli*) can withstand these processes. Therefore, heat-resistant mesophile and thermophile numbers have been used for many years as criteria for product quality in UHT-milk and milk powders all over the world (Hammer *et al.*, 1995). However, little information suggests any direct correlation between the heat-resistant bacterial numbers and the keeping quality of long-term storage products, such as milk powders.

One distinguishing character of milk powder compared with any other dairy product is its low water content, which is often expressed as moisture content (percentage of water by weight) or water activity (a_w). In pure water, $a_w = 1$ (Walstra and Jenness, 1984). Milk (at an a_w of 0.99, equivalent to a moisture content of 87%) is made to powder (at an a_w of 0.1-0.2, equivalent to a moisture content of 1.5-4.5%) by pasteurisation, evaporation and spray-drying (Pisecky, 1997; Walstra *et al.*, 1999). It is generally accepted that microbial growth can no longer take place at the levels of water content in milk powders, while chemical reactions, such as Maillard reaction (non-enzymatic browning) and lipid autoxidation may still occur (Walstra and Jenness, 1984). However, there is no quantitative information on biochemical reactions, such as enzymatic degradation, in the milk powder (Muir, 1996c).

This review focuses on the possible presence of proteases and lipases in milk and milk powders, detection methods for the enzymes, the characteristics of the enzymes and their effects during storage.

1.2 Proteases in Milk and Dairy Products

1.2.1 Protease Classification

Proteases are generally classified into four groups on the basis of their mechanisms of action: (1) serine proteases, such as plasmin [EC 3.4.21.7] from bovine plasma and subtilisin [EC 3.4.21.14] from *B. licheniformis* or *B. subtilis*; (2) cysteine proteases, such as Cathepsin B [EC 3.4.22.1] from bovine spleen; (3) aspartic proteases, such as Cathepsin D [EC 3.4.23.5] from bovine spleen; and (4) metallo-proteases, such as thermolysin [EC 3.4.24.4] from *B. thermoproteolyticus* (Neurath, 1989; Bond, 1989). Selected inhibitors can differentiate these proteases. Diisopropyl phosphofluoridate (DFP) or phenylmethy-sulphonyl fluoride (PMSF) inhibit serine proteases, E-64[1] inhibits cysteine proteases, pepstatin inhibits aspartic proteases, and 1,10-phenanthroline or ethylenediaminetetra-acetic acid (EDTA) inhibit metallo-proteases (Neurath, 1989; Salvesen and Nagase, 1989).

1.2.2 Endogenous Proteases in Milk

In bovine milk, proteases are either endogenous or arise from contaminating microorganisms. Native proteolytic enzyme in milk was first reported in 1897 (Babcock and Russell, 1897). Following further studies, the milk native proteases have been identified into two groups – milk alkaline proteinase (MAP, a serine protease; Reimerdes, 1981) and milk acidic proteinase (Cathepsin D, an aspartic protease; Kaminogawa and Yamauchi, 1972; Larsen *et al.*, 1996). These milk native proteases arise from mammary tissue cells, blood plasma or leukocytes (Farkye, 1991).

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[1]E-64: L-trans-epoxysuccinyl-leucylamido-(4-guanido)-butane

MAP is the most thoroughly studied all milk endogenous proteases. MAP (found in milk) and plasmin (isolated from bovine blood) have been shown to be the same enzyme on the basis of many characteristics, such as optima and stabilities of pH, heat stabilities, sensitivities to various inhibitors, molecular masses, specificities on caseins and identical sequences (Grufferty and Fox, 1988; Benfeldt *et al.*, 1995).

The plasmin system has five elements: plasmin, plasmin inhibitors, plasminogen, plasminogen activators and inhibitors of plasminogen activators (Grufferty and Fox, 1988). Plasmin occurs mainly as plasminogen – an inactive zymogen form (Rollema *et al.*, 1981). It is derived from blood serum, but is associated with casein micelles rather than being found in the soluble whey fraction of the milk (Reimerdes, 1983; Farkye, 1991). Plasmin activity has been observed in milk fat globule membranes (MFGM) only because of contamination of casein in the fat globules (Politis *et al.*, 1992; Bendelft *et al.*, 1995). Since plasmin is mainly present in its inactive form plasminogen, so plasminogen is also associated with casein micelles (Grufferty and Fox, 1988; Politis *et al.*, 1992; Benfeldt *et al.* 1995). The rest of elements in the plasmin system, such as plasminogen activators are found predominantly in casein micelles and somatic cells (Grufferty and Fox, 1988; Verdi and Barbano, 1991; White *et al.*, 1995). Plasmin inhibitors and plasminogen activator inhibitors only occur in the serum phase of milk (Grufferty and Fox, 1988; Bastian and Brown, 1996).

Mastitis can increase proteolytic activity in milk, particularly after initial infection corresponding to increases of somatic cell counts (Saeman *et al.*, 1988). Auldist *et al.* (1995) reported that milk from mastitic cows has a significantly greater plasmin activity, and a lower level of plasminogen than milk from healthy cows. This phenomenon has been explained as the plasmin system depends on the activator-

inhibitor balance, and the balance in mastitic milk is in favour of activation (Fang and Sandholm, 1995).

Plasmin is a serine protease with trypsin-like activity. It has a molecular mass of ~ 100 k Dalton (kDa) by gel filtration (Dulley, 1972), and 81 to 83.2 kDa by calculating amino acid sequence of blood plasmin (Grufferty and Fox, 1988). Plasminogen was shown to have a molecular mass of 85 kDa on SDS-PAGE (Benfeldt *et al.*, 1995).

Plasmin has maximal activity at temperature of 37°C and pH of 7.5-8.0 (Fox, 1981; Reimerdes, 1983). It hydrolyses all milk caseins with a preference for α_{s1}- and β-caseins (Andrews and Alichanidis, 1983), and has preferential specificity for bonds on the C-terminal side of Lys and Arg residues, cleaving Lys-X bonds much faster than Arg-X bonds (Visser, 1981). Preferential cleavage of β-casein by plasmin is at positions Lys^{28}-Lys^{29}, Lys^{105}-His^{106} and Lys^{107}-Glu^{108} to give 3 β-casein fragments called γ_1-, γ_2- and γ_3-caseins (Visser, 1981). However, the enzyme has no effect on α-lactalbumin and β-lactoglobulin (Visser, 1981). Plasmin is inhibited by DFP, Hg^{2+}, Zn^{2+}, Cu^{2+} and soybean trypsin inhibitors (Fox, 1981).

Half-life ($t_{1/2}$; time required for a 50% reduction of the initial activity) and D-value (time required for a 90% reduction of the initial activity) both have been used for comparison of the enzyme heat-stability. Three elements of the plasmin system have been reported to be heat-stable. Plasmin retained 90% of activity after heating at 60°C for 30 min at pH 6.8 (Dulley, 1972), and has a calculated $t_{1/2}$ of 35.3 ± 3.6 min at 70°C in milk (Richardson, 1983), while plasminogen has a calculated $t_{1/2}$ of 33.3 min (D value of 111 min) at 70°C in milk (Richardson, 1983). Plasminogen activator has a calculated

$t_{1/2}$ of 32.8 min (D value of 109 min) at 70°C in milk (Lu and Nielsen, 1993), and a $t_{1/2}$ of 10 min at 80°C (D value of 33.2 min) in casein solution (Richardson, 1983).

The high heat-stabilities of the plasmin elements in milk are because of the protection of milk proteins, mainly caseins (Humbert and Alais, 1979; Grufferty and Fox, 1988). The caseins can protect plasmin from irreversible inactivation – the enzyme unfolds during heat; then refolds back to an active form after cooling below the denaturation temperature (Metwalli *et al.*, 1998).

Severe heat-treatment of 115°C for 20 min or 120°C for 15 min is required for milk products to be completely protected from proteolysis by plasmin (Driessen and van der Waals, 1978; Humbert and Alais, 1979). It should be noted that some researchers observed that plasmin activity in milk increased by 30-40% after heating at 72°C for 15 s (Humbert and Alais, 1979; Fox, 1981). This was explained as the result of destruction of the inhibitors of plasminogen activators (Fox, 1981; Grufferty and Fox, 1988).

Other milk endogenous proteases, such as cysteine and aspartic proteases originate from polymorphonuclear (PMN) leukocytes in milk (Christensen *et al.*, 1995; McSweeney and Fox, 1995). Cathepsin D has been found mainly in whey fraction (Larsen *et al.*, 1996).

Cathepsin D is an aspartic protease. It has a pH optimum at ~ 4.0 and a molecular mass of 36 kDa (Kaminogawa and Yamauchi 1972). It can degrade all milk proteins except β-lactoglobulin (Larsen *et al.*, 1996). Inhibitor *p*-chloromercuribenzoate (PCMB) can partly inhibit Cathepsin D activity. Heating at 70°C for 10 min (in a buffer) or pasteurisation at 65°C for 30 min (in skim milk) can completely inactivate Cathepsin D

(Kaminogawa and Yamauchi, 1972; Grieve and Kitchen, 1985). Because of its low heat-stability, Cathepsin D has not been regarded as an important enzyme in milk and dairy products (Grieve and Kitchen, 1985).

1.2.3 Bacterial Proteases in Milk

Three main sources of bacterial contamination have been found in raw milk: cow's teats, milking and storage equipment, and the interior of the udder (Law and Mabbitt, 1983). For example, Prabha and Shankar (1994) found that teat swabs and utensil rinsings contained the highest percentage of proteolytic and lipolytic psychrotrophs when compared with the microflora in soil, water and feed. Protease production by psychrotrophs is normally at late exponential or stationary phase of growth (Islam and Blanshard, 1973; Griffiths, 1989). Majority of *Pseudomonas* species found in milk produce only one type of proteases: neutral metallo-proteases that require divalent cations for activity (Zn^{2+}) and stability (Ca^{2+}) (Fairbairn and Law, 1986). These proteases generally have temperature optima of 30-45°C, and pH optima of 6.5-8.0 (Fairbairn and Law, 1986). Triantafyllidou and Roussis (1999) reported that a proteinase from *Pseudomonas* appeared to prefer β-casein over α_s-casein.

On the other hand, *Bacilli* are widely distributed in the environment and can be introduced into milk and dairy products during production, handling and processing (te Giffel *et al.*, 1996). An addition problem with *Bacilli* is that they can easily sporulate during milk powder process and establish persistently in factory equipment, which may lead to post-contamination to the milk powders (Stadhouders *et al.*, 1982).

Many researchers have carried out studies on the *Pseudomonas* and *Bacillus* species isolated from milk and milk products under laboratory conditions. A typical

thermostable *Pseudomonas* metallo-protease MC60 (isolated from raw milk), has a temperature optimum of 35°C, a pH optimum of 7.5, and a molecular mass of ~ 48.5 kDa on SDS-PAGE. This protease has many similar properties to a classical heat resistant metallo-protease thermolysin (from *B. thermoproteolyticus rokko*), such as the content of divalent ions (Zn^{2+} for activity and Ca^{2+} for stability), lack of sulphydryl groups, molecular mass and a high content of hydrophobic amino acid residues (Fairbairn and Law, 1986). Chopra and Mathur (1985) reported that two proteases RM-67 I and II from *B. stearothermophilus* (isolated from raw milk) have the same pH of 8.0 and temperature optima of 70°C. However, RM-67 I is a metallo-protease with a molecular mass of ~ 67.6 kDa, and RM-67 II is a serine protease with a molecular mass of ~ 20 kDa.

The thermostability of microbial proteins (enzymes) generally shows a positive correlation with the optimum growth temperature of the source microorganism (Owusu *et al.*, 1991). However, proteases from *Pseudomonas* showed an ability to retain 55 to 65% activity after heat-treatment of 77°C for 17 s, and 20 to 40% activity after 140°C for 5 s in buffers (Griffiths *et al.*, 1981). Adams *et al.* (1975) reported that proteases produced by 10 different psychrotrophic strains all survived heat-treatment of 149°C for 10 s in buffers. The protease MC60 was reported to have a calculated D-value of 304 min ($t_{1/2}$ of 91.5 min) at 74°C in a buffer (Cogan, 1977). The *P. fluorescens* protease P26 had a D-value of 950 min ($t_{1/2}$ of 286 min) at 71.4°C, while another *Pseudomonas* protease 21B appeared to be less stable with a D-value of 160 min ($t_{1/2}$ of 48.2 min) at 74°C (Cogan, 1977). Triantafyllidou and Roussis (1999) reported an extracellular *P. fluorescens* TR2 neutral metallo-proteinase (from raw milk) retained 75% activity in a buffer after heat-treatment for 60 s at 100°C. A *B. cereus* protease retained 9.5% activity in a buffer after 15 min at 60°C (Islam and Blanshard, 1973). Crude protease

solutions (from cultures of *B. stearothermophilus* and *B. licheniformis*) showed no loss of activity after heat-treatment of 70°C for 10 min (Chapra and Mathur, 1984). A protease produced by *B. stearothermophilus* (found in skim milk powder) retained 100% activity in a buffer after heat-treatment for 20 min at 70°C (Chapra and Mathur, 1984). Purified RM-67 I metallo-protease remained 100% activity in a buffer with the presence of Mn^{2+} after heated for 30 min at 65°C, but the purified RM-67 II serine protease only remained 20% activity after the same heat-treatment (Chapra and Mathur 1985).

Heat-stability data obtained with purified proteases in buffer solutions suggest that they could survive milk processing. Barach *et al.* (1976) reported that inactivation of crude protease was slower in milk than in buffer. This inactivation was independent of initial enzyme concentration. Higher stability of protease in milk and milk salts-buffer was confirmed by Fox (1981). However, the heat-stability of protease is complicated in milk, and some anomalous behaviour has been noted. The rate of inactivation for some psychrotroph proteinases in milk was greater at 55-60°C than expected (Barach *et al.*, 1976). The protease molecule undergoes a reversible loss of catalytic activity and greater susceptibility to autolysis at 55°C. This apparently results in an aggregation of the altered protease with casein micelles to form stabilised enzyme-casein complex (Barach *et al.*, 1978). Therfore, heating at lower teperatures is more effective in inactiviating protease in milk than higher temperatures.

Calcium ions can also stabilise proteases – protecting the enzymes against both thermal denaturation and autolysis (Daniel *et al.*, 1995). The calcium ions can give the protein more flexibility during denaturation. The enzymes can recover the active conformation

through Ca^{2+} salt bridges after the temperature returns below the denaturation range (Fairbairn and Law, 1986).

In summary, proteases present in milk are unlikely to be destroyed by any heat-treatments applied during the process, and will remain in the final product (Muir 1996c).

1.3 Lipases in Milk and Dairy Products

1.3.1 Lipase Classification*

Lipases (triacylglycerol acylhydrolase [EC 3.1.1.3]) are a group of enzymes that act at lipid-water interfaces and hydrolyse triacylglycerols (TG) to diacylglycerols (DG), monoacylglycerols (MG), free fatty acids (FFA) and glycerol (Thomson *et al.*, 1999). With excess water, hydrolysis is favoured; in the absence of water or in trace amount, condensation (esterification and transesterification) is dominant (Kotting and Eibl, 1994).

The lipases are classified based on their specificities towards TG as presented in Fig. 1.1 (Macrae, 1983). The first group is non-specific, and releases FFA and glycerols from all three positions of the glycerol moiety. This group completely hydrolyses TG to FFA and glycerols. The second group only releases FFA from the outer sn-1 and sn-3 positions of glycerol moiety to give 1,2-DG, 2,3-DG, 2-MG and FFA. Because 1,2-DG, 2,3-DG, and especially 2-MG are chemically unstable and undergo acyl migration to 1,3-DG, 1-MG and FFA (Macrae, 1983; Jaeger *et al.*, 1994). Prolonged incubation of the TG with 1,3-specific lipases can result in complete hydrolysis of TG to FFA and glycerols. The third group only act on special structure of acylglycerols (*e.g.* containing a particular double bond), and has not been found in any bacterial lipases (Jaeger *et al.*,

* See Terminology Section xiv

1994). Nevertheless, the classification of the lipases has to be treated with caution since the stereo-specificity of lipases can change as results of changing substrate compositions during lipolysis (Jaeger *et al.*, 1994).

1.3.2 Endogenous Lipases in Milk

Milk and dairy products can contain milk endogenous liproprotein lipase (LPL [EC 3.1.1.34]) and carboxylester hydrolases (esterases), and microbial lipases and esterases derived from contaminating microorganisms. As little detailed information is available on milk native esterases (Deeth and Fitz-Gerald, 1995), this section focuses only on LPL.

LPL is synthesised in the mammary gland secretory cells, but it is not apparently associated with the milkfat globule membrane (MFGM) upon secretion, but with casein (Brockerhoff and Jensen, 1974). Addition of a chelator or removal of Ca^{2+} by dialysis can release the LPL from the casein micelles into the solution (Olivecrona and Bengtsson-Olivecrona, 1991). Casein micelles are large aggregates of phosphoproteins held together by Ca^{2+} bridges and hydrophobic interactions (Walstra *et al.*, 1999). The lipase-casein association is a hydrophobic interaction, which is confirmed by addition of dimethylformamide (DMF) for dissociation of the complex (Deeth and Fitz-Gerald, 1995). Increasing salt concentration, or adding heparin can also release the enzyme from casein micelles. This indicated that the association is also via electrostatic interaction between the positive charges on the LPL and the negative charges on the caseins (Olivecrona and Bengtsson-Olivecrona, 1991; Deeth and Fitz-Gerald, 1995).

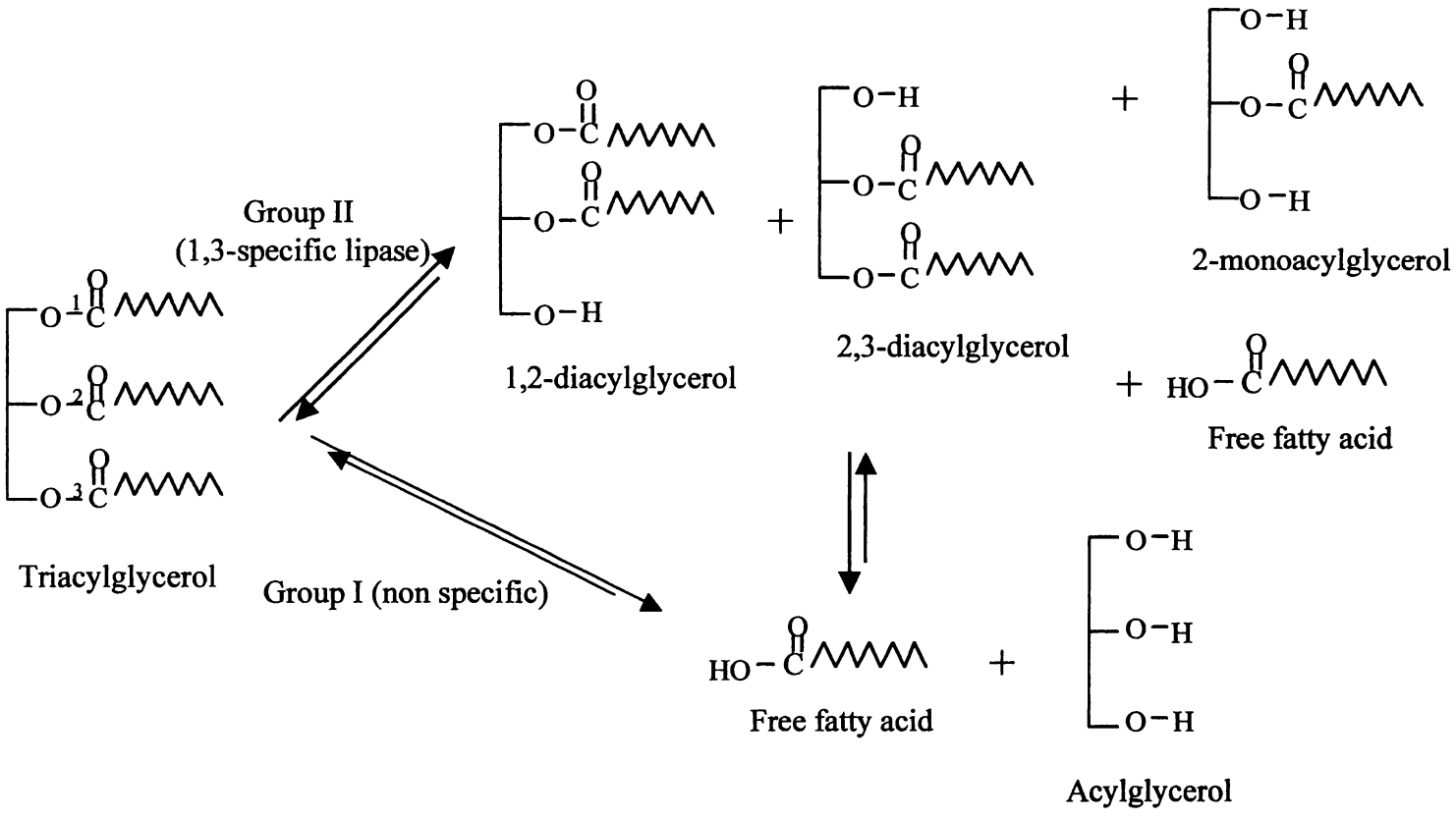

Figure 1.1 Classification of lipases based on position specificity (Macrae, 1983).

Levels of LPL in milk is affected by the breed, the stage of lactation, feed and nutrition of the cows, season and milk production, and even related to milking machines (Deeth and Fitz-Gerald, 1976). LPL is not normally active against milk lipids because they are efficiently packaged in and protected by milkfat globules (Deeth and Fitz-Gerald, 1995). However, the activity of LPL can be induced by disrupting the milkfat globules with sonication, agitation, homogenisation, temperature changes and addition of blood serum or heparin (Deeth and Fitz-Gerald, 1976; Cartier and Chilliard, 1989; Kon and Saito, 1997).

LPL is a dimer of two glycoprotein chains of ~ 41.7 kDa, and contains 8.3% carbohydrate. It has a pI of ~ 9.0 (Olivecrona and Bengtsson, 1984), a pH optimum of 8-9, and a temperature optimum of 35-40°C (Brockerhoff and Jessen, 1974).

LPL can form large aggregates regardless of the ionic strength and retain an active conformation (Olivecrona and Bengtsson, 1984). It has no specific structural requirements on milk lipids, but prefer the sn-1 over the sn-3 position (Olivecrona and Bengtsson, 1984). A typical LPL activity is 1 μmole FFA/min/mL in bovine milk, at 37°C and pH 7.0 with addition of activator (apoliproprotein CII from blood serum) (Olivecrona and Bengtsson, 1984). Under the conditions of 25°C and pH 8.5, 1 mg LPL can release 700 μmole/min FFA (Olivecrona and Bengtsson, 1984). Humbert *et al.* (1997) reported that LPL has a K_m of 0.51 mM for *p*-nitrophenyl butyrate at 37°C and pH 7.6. However, LPL activity depends on a balance between the activator (apoliproprotein CII) and the inhibitor (proteose-peptone fraction 3) in milk (Deeth and Fitz-Gerald, 1995).

LPL has a lipid-binding region as shown by the binding to deoxycholate – a specific anionic detergent that only binds to proteins with lipid-binding regions (Olivecrona and Bengtsson, 1984). This has three effects on LPL: increased solubility; desorption from lipid-droplets; and enhancement of stability (Olivecrona and Bengtsson, 1984). Monoacylglycerols and long chain FFA can have the same effect as the detergent to stabilise LPL, but they also compete for the enzyme binding sites from milk lipids which result in product inhibition (Olivecrona and Bengtsson, 1984). Albumin can destabilise LPL by absorbing FFA from the LPL. So, with an excessive amount of albumin (*e.g.* a ratio of albumin:FFA of 6-7:1), hydrolysis is inhibited (Olivecrona and Bengtsson, 1984).

LPL is a relatively unstable enzyme, and can be inactivated by inhibitors, heat, light, acids and oxidising reagents (Brockerhoff and Jensen, 1974; Deeth and Fitz-Gerald, 1995). Inhibitors such as 1 mM DFP and 0.5 mM diethyl *p*-nitrophenyl phosphate (DNP) can destroy LPL activity completely (Brockerhoff and Jensen, 1974). Heating skim milk for 20 min at 65°C, or exposure to light at 254 nm or 366 nm for 30 min can destroy LPL (Sundheim and Bengtsson-Olivecrona, 1987). It is generally accepted that HTST pasteurisation (72°C for 15 s) almost completely inactivates LPL. However, Deeth and Fitz-Gerald (1995) reported that a more severe heat-treatment of 85°C for 10 s is needed to destroy the LPL activity completely in milk.

1.3.3 Bacterial Lipases

The mechanisms of lipolysis, assay systems, genetics, structures and applications of bacterial lipases have been reviewed extensively by Jaeger *et al.* (1994). Microbial lipases are very diverse in their enzymatic properties and substrate specificities, which make them very attractive for industrial applications. Most bacterial lipases are

extracellular and produced during the late log and early stationary phases of growth (Fox and Stepaniak, 1983; Stuer *et al.*, 1986; Sidhu *et al.*, 1998).

Culture conditions, such as temperature, pH, nitrogen and lipid sources, concentration of inorganic salts and availability of oxygen, influence not only the properties of the lipase produced but also the ratio of extracellular and intracellular lipase (Sugiura, 1984; Aires-Barros *et al.*, 1994). Lipase production can be stimulated by lipids, such as milkfat and olive oil (Aires-Barros *et al.*, 1994). Polysaccharides, such as glycogen, hyaluronate, laminarin, pectin B and gum arabic can enhance the production of extracellular lipase (Sugiura, 1984). Milk is a good medium both for psychrotrophic bacteria growth and their lipases production (Stead, 1986).

Pseudomonas and *Bacillus* species commonly found in milk and milk products introduced in Section 1.2.3 also produce lipases, and hence have attracted most attention. Matta and Punj (1999) reported 48 out of 100 raw milk samples contained lipolytic psychrotrophic spore forming bacteria. These bacteria are strains of *B. cereus, B. polymyxa, B. licheniformis, B. circulans, B. subtilis, B. laterosporus* and *B. coagulans*. However, *B. cereus* is the predominant species in this case. Some characteristics of lipases from *Pseudomonas* and *Bacillus* are listed in Table 1.1.

In general, *Pseudomonas* and *Bacillus* lipases have molecular masses ranging from 12 to 135 kDa, and pH optima between 7.0 and 9.0. Most of them have specificity to the sn-1 and sn-3 specificity on TG. It is also possible that many microorganisms produce two or more extracellular lipases which hydrolyse different chain length fatty acids, especially short chain fatty acids (Macrae, 1983). Erdmann *et al.* (1991) listed substrate specificities for 68 lipases from several sources.

Table 1.1: Properties of some bacterial lipases

Lipase	Mr (kDa)	PH optimum	Temp. optimum	Stability	Specificity	Sensitivity to inhibitors/agents	Reference
P. fluorescens AFT36 (refrigerated bulk milk)	-	8.0	35°C	$T_{1/2}$ = 6.8 min in synthetic milk salts, 65°C; 0.2 min in phosphate buffer, 65°C	K_m = 3.65 mM tributyrin	~ 50% left, 4 M urea, 2 d, 21°C; ~ 25% left, 6 M urea, 33 d, 4°C	Fox and Stepaniak, 1983
P. fluorescens 2D (raw milk)	135	8.5	40°C	D = 14 min, 120°C in buffer ($t_{1/2}$ = 4.2 min)	sn-1 and sn-3 specific	11% left, 10 mM EDTA[1] & 9% left 10 mM Hg^{2+}, 1 h, room temperature	Makhzoum *et al.*, 1996
P. fluorescens 33 (pasteurized milk)	52	7.5-8.5	45°C	> 40% left, 10 min, 50-60°C ($t_{1/2}$ > 4.2 min)		9% left, 1 mM Hg^{2+} & 13% left 1 mM Zn^{2+}, overnight, 0°C	Kumura *et al.*, 1993
Pseudomonas Pf-lip1 (raw milk)	50	9.0	45°C	No data available	trimyristate; milkfat sn-1 and sn-3 specific		Koka and Weimer, 1999
Pseudomonas Pf-lip2 (raw milk)	12	9.0	35°C	53% left, 30 min, 62.5°C ($t_{1/2}$ = 32.8 min)	*p*NP propionate		Koka and Weimer, 1999
B. stearothermophilus recombinant *B. brevis*	29	7.5	37°C	< 10% left, 45 min, 65°C ($t_{1/2}$ < 13.6 min)		4% left, 1 mM IAA[2], 8% left, 1 mM PCMB[3] & 0% left, 1 mM PMSF[4], 5 min, 37°C	Amaki *et al.*, 1992
Bacillus sp. Wai28A5 (thermal field)	-	8.7	70°C	$T_{1/2}$ = 60 min, 70°C $T_{1/2}$ = 12 min, 85°C	maximum activity with 0.4 mM *p*NP palmitate		Janssen *et al.*, 1994

Table 1.1 continued

B. stearothermophilus Tok19A1 (thermal field)	~ 50	7.0	60°C	$t_{1/2}$ = 170 h, 60°C	*p*NP caprylate	stong inhibition by PMSF, $HgCl_2$, eserine, diethylprycarbonate, PCMB	Wood *et al.*, 1995
B. thermocatenulatus BTL-1 (DSM)	16	7.0-8.0	60-70°C	$T_{1/2}$ = 30 min, 60°C	*p*NP caprate and laurate; sn-1 and sn-3 specific	Moderate detergent stability	Schmidt-Dannert *et al.*, 1997
B. thermocatenulatus BTL-2 (cloned)	43	8.0-9.0	60-70°C	48.5% left, 30 min, 60°C ($t_{1/2}$ = 28.7 min)	tributyrin; sn-1 and sn-3-specific	80% left, 30% methanol, propanol and acetone, 1h, 30°C; deactivated immediately with 1% SDS[5], Tween 20, or Tween 80	Schmidt-Dannert *et al.*, 1996b; Rua *et al.*, 1997
B. stearothermophilus L1 (cloned)	43	9.0-10.0	60-65°C	stable, 30 min, 50°C	*p*NP caprylate; tripropionin	13% left, 1 mM Cu^{2+}; 2% left, 1% SDS, 30 min, 30°C	Kim *et al.*, 1998
Bacillus sp. THL027 (seaside restaurant)	69	7.0	70°C	> 80% left, 1 h, 60-75°C ($t_{1/2}$ > 3.1 h)	triacylglycerols ($C_{4:0}$ to $C_{12:0}$); sn-1 and sn-3 specific	Sensitive to 1 mM EDTA	Dharmsthiti and Luchai, 1999
B. themoleovorans ID-1 (hot spring)	34	7.5	70-75°C	$T_{1/2}$ = 1 h, 60°C; $T_{1/2}$ = 30 min, 70°C	tricaprylate	30% left 1 mM SDS, 10 min, 65°C	Lee *et al.*, 1999

[1]EDTA: ethylenediaminetetra acetic acid.

[2]IAA: iodoacetamide.

[3]PCMB: *p*-chloromercuribenzoic acid.

[4]PMSF: phenylmethylsulphonyl fluoride.

[5]SDS: sodium dodecyl sulphate.

Unlike LPL, crude bacterial lipases are capable of hydrolysing milk lipids (Deeth and Fitz-Gerald, 1995). Some microbial lipases hydrolyse DG and MG faster than TG (Macrae, 1983).

Two characteristics of lipases have been attracting most research attention: temperature optimium and heat-stability. Most of *Bacillus* lipases have higher temperature optimia and more heat-stable than *Pseudomonas* lipases (Table 1.1). The lipases produced by *Thermus* and *Bacillus* are active at 80°C, and even at 100°C in a cell-bound form (Jaeger *et al.*, 1994). However, if any of the lipases listed in Table 1.1 were present in milk, all would survive pasteurisation process of 72°C for 15 s.

Little information is published about the *Bacillus* lipases found in milk and dairy products, but the *Pseudomonas* lipases found in milk have been under extensive study. Crude psychrotrophic *Pseudomonas* lipases (produced in skim milk) retained 55.6-100% and 75-100% activity after heat-treatment of 63°C for 30 min (Law *et al.*, 1976) and 100°C for 30 s (Fitz-Gerald *et al.*, 1982), respectively. Andersson *et al.* (1979) also reported that a *P. fluorescens* SIK W1 lipase had a D-value of 23.5 min ($t_{1/2}$ of 7.1 min) at 100°C in skim milk. Fox and Stepaniak (1983) reported that the extracellular lipase from *P. fluorescens* AFT36 was more heat-stable in a synthetic milk salts solution than in phosphate buffer. The *Pseudomonas* lipase 33 showed the highest thermostability in a casein micelle suspension except in the temperature range of 60-80°C, and was completely inactivated in a synthetic milk salts solution containing whey proteins at the same temperature range (Kumura *et al.*, 1993). The thermostability of lipase in skim milk consisted of a balance of thermal stabilisation of the casein micelle suspension and thermal destabilisation of whey proteins (Kumura *et al.*, 1993).

Lipases and proteases are often produced concomitantly by the bacteria. The minimum inactivation for proteases was at 55°C, but was at 60-80°C for lipases (Fox and Stepaniak, 1983), so the lipases are more heat-stable than the proteases (Griffiths *et al.*, 1981). Prolonged heat treatment to milk at relatively low temperatures (~ 55°C) may be more effective in inactivating bacterial protease than a short period at high temperatures (Barach *et al.*, 1976). But this treatment has no effects in inactivating the bacterial lipases in milk, and lipolysis will occur in the final products during storage (Fitz-Gerald *et al.* 1982).

Lipases are not only extremely heat-resistant, but also stable against chemical denaturation. This is because they have a high content of hydrophobic amino acids and disulfide bridges that provide a compact structure in the molecules, which are not easily denatured by changes in the external environment (Andersson *et al.*, 1979). Polysaccharides with divalent cations (*e.g.* Ca^{2+}) also stabilise the enzyme molecule (Andersson *et al.*, 1979). With the addition of 10 mM $CaCl_2$, the lipase was shown to have comparable heat-stability in buffer and milk synthetic salts solution (Fox and Stepaniak, 1983). The importance of calcium binding sites for maintaining bacterial lipase structure was reported by Shibata *et al.* (1998).

Shamsuzzaman *et al.* (1986) reported that freeze-drying or spray-drying without heating had little effect on either LPL or *P. fluorescens* B521 lipase. However, heating at 70°C for 2 min inactivated the LPL, but did not affect the *Pseudomonas* lipase. They concluded that spray-drying after heating had no significant effect on the bacterial enzyme in making non-fat milk powder.

It is well known that most enzyme preparations are more stable at low water content than in aqueous media because the enzymes become more rigid at low water content and therefore more heat-stable. A heat-stable *Pseudomonas* B52 lipase showed no loss of activity after high-heat treatment, and the activity remained unchanged in the freeze-dried powder even after storage for 2 months at 20°C (Shamsuzzaman *et al.*, 1986). The *Bacillus* lipases are more heat-stable than the *Pseudomonas* lipases, therefore the *Bacillus* lipases will be more likely to retain activity in milk powders even after prolonged storage.

1.3.4 Some Lipase Sequence Data

A variety of lipases have been purified and biochemically characterised, their genes cloned and sequenced. Some partial amino sequences are listed in Table 1.2.

Data from partial N-terminal sequence showed differences amongst the milk LPL, the *Pseudomonas* lipases and the *Bacillus* lipases. The lipases from *Pseudomonas* species showed the biggest diversity. On the contrary, the lipases from *Bacillus* showed significant homologies between species. However, Dartois *et al.* (1992) reported that the alignment of *B. subtilis* 168 lipase showed significant homologies with several lipases from *Pseudomonas* species in the N-terminal region.

A pentapeptide Gly-X-Ser-X-Gly (X – an amino acid) is conserved as the "substrate binding site" for most of bacterial and mammalian lipases. An Ala replaces the first Gly in the pentapeptide from the *Bacillus* lipase. This replacement could be a common feature for *Bacillus* lipases (Schmidt-Dannert *et al.*, 1997). The serine residue is also shown to be involved in triacylglycerol hydrolysis (Kordel *et al.*, 1991) as lipases act as

serine hydrolases with a Ser-His-Asp triad in the active site similar to serine protease (Kordel *et al.*, 1991).

Table 1.2: Partial amino acid sequences of some lipases

Lipase	Partial N-terminal amino acid sequence (⇒)	Putative active-site	Reference
Bovine liproprotein lipase (LPL)	GSKRN LSKDH CKVFI VPSKG KQLYS MKERS	GYSLG (132-136)	Olivecrona and Bengtsson-Olivecrona, 1991
Pseudomonas sp.	LSANK LRNAH QRYVS VPSTE FLSTL GFVQN	GHSHG (79-84)	Nishioka *et al.*, 1990
Pseudomonas fragi	RTSAG ASGRA MHNIT DLHDL PYDSR IVQGL	GHSQG (81-85)	Nishioka *et al.*, 1990
Pseudomonas LS107d2	SIVIG EGSLG VVGVL TVSDG GFSIV TDGGV	GHSLG (207-211)	Johnson *et al.*, 1992
P. aeruginosa	LSANK LRNAH QRYVS VPSTE FLSTL GFVQN	GHSHG (78-82)	Jaeger *et al.*, 1994
P. fluorescens B52	GIVVG ETWLG GHGVG VLTVS DAGFT LVTDA	GHSLG (205-209)	Tan and Miller, 1992
P. fluorescens	CRIPG WPRRT TACTP AATPA RSCWG TPQYG	GHSLG (87-91)	Jaeger *et al.*, 1994
B. subtilis 168	NTNQG GGNLG EKILS NVQSS YLLGI HGVGH	AHSMG (106-110)	Dartois *et al.*, 1992
B. pumilus	NTNQG GGNLG EKIYG KVQSS TLLGI HGVGH	AHSMG (75-79)	Jaeger *et al.*, 1994
B. thermocatenulatus BTL-2	PRLSA LQEAL RLYFA RIDFS PNPDV GIVEL	AHSQG (111-115)	Schmidt-Dannert *et al.*, 1997
B. stearothermophilus L1	PRLSA LQEAL RLYFA RINFS PNPDV GIVEL	AHSQG (111-115)	Kim *et al.*, 1998

Sequence homologies between enzymes do not necessarily mean similarities in biological characteristics, *e.g.* the *B. stearothermophilus* L1 lipase and the *B. thermocatenulatus* BTL-2 lipase have 94% similarity in sequence (365 amino acids out

of 388 residues), but the L1 lipase differs from BTL-2 lipase in respect to PMSF inhibition, substrate specificities on *p*-nitrophenyl esters of fatty acids and triacylglycerols (Kim *et al.*, 1998).

Hydrophobic amino acids Ile, Val, Leu, Phe, Met and Ala comprised 39.0% and 38.7% of the BLT-1 and BTL-2 lipases sequences, respectively (both from *B. thermocatenulatus*) (Schmidt-Dannert *et al.*, 1997). The high content of hydrophobic amino acids lead the lipases to have a strong tendency to form aggregates, and result in a strong adsorption to hydrophobic surfaces (Macrae, 1983; Schmidt-Dannert *et al.*, 1997). This character is clearly shown during purification of the lipases (Jaeger *et al.*, 1994; Schmidt-Dannert *et al.*, 1996).

1.4 Protease and Lipase Detection in Milk and Dairy Products

Heat-stability data indicates that milk and dairy products will contain proteases and lipases even after pasteurisation, UHT-treatments and spray-drying. The key issue is to have assay methods that can detect the levels of the enzymes in milk and dairy products, then define product quality. Enzyme activity can be detected in two ways: one is to measure the break down of substrates, such as milk proteins and lipids; and the another is to measure the accumulation of products, such as protein fragments, peptides and FFA. Synthetic chromogenic or fluorescent labelled substrates, such azocasein, *p*-nitroanilide derivatives and FTC-caseins are often used for the detection of the protease activity, while *p*-nitrophenyl esters of fatty acids and 4-methylumbelliferyl triacylglycerols are often used for the detection of the lipase activity. During hydrolysis, release of coloured or fluorescent products can be measured conveniently

using a spectrophotometer or a fluorimeter. Common protease and lipase assay methods are discussed in the next two sections.

1.4.1 Protease Detection

Protease assay methods initially relied on the detection of changes in proteins and peptides content in the sample, such as Kunitz (Kunitz, 1947) and Lowry (Lowry *et al.*, 1951) assays. Later, reagents, such as fluoresecamine, trinitribenzene sulfonic acid (TNBS) and o-phthaldialdehyde (with β-mercaptoethanol), were developed to detect the levels of α-amino groups in the sample (Chism *et al.*, 1979; McKellar, 1981; Church *et al.*, 1983; Humbert *et al.*, 1990). However, the problem of low sensitivity still remains. In the last ten years, more sensitive assays such as enzyme-linked bioluminescent, fluorescent, immunological and radiometric assays have been developed. Assay methods for plasmin and some bacterial proteases are summarised in Table 1.3.

For assaying plasmin, three different assay methods gave similar minimum detectable level of pure plasmin in a buffer system. When used to assay skim milk samples, all methods face the problem of milk protein interference (Rollema *et al.*, 1983; Dupont *et al.*, 1997). Overall, the spectrophotometric assay is convenient and can be easily performed in a factory. However, authors did not rule out proteases other than plasmin may also be present in milk and act on the same substrate Val-Leu-Lys *p*NA (Rollema *et al.*, 1983).

A reverse phase high performance liquid chromatography (RP-HPLC) method was reported to detect milk native aspartic protease – Cathepsin D, using a synthetic

heptapeptide at 37°C and pH 3.2, but no quantitative results were obtained (O'Driscoll *et al.*, 1999).

Table 1.3: *Protease assay methods*

Methods	Assay substrates and conditions	Minimum detection level	Reference
Plasmin			
Spectrophotometric assay	VLK-*p*NA; 37°C; 7 min; pH 7.4	3 ng/mL plasmin	Rollema *et al.*, 1983
Fluorimetric assay	Suc-APL-amido methyl coumarin; 25°C; 2-5 min; pH 7.5	1.6 ng/mL plasmin	Richardson and Pearce, 1981
ELISA	Antibody; room temperature; 1.5 h; pH 9.0	4 ng/mL plasminogen	Haissat *et al.*, 1994
	Monoclonal antibodies; room temperature; 5 h; pH 7.2	5 ng/mL plasmin	Dupont *et al.*, 1997
Bacterial protease			
Spectrophotometric assay	Folin-Ciocalteau (Phenol) reagent; 35°C; 10 min	2 μmole/mL/min tyrosine	Hull, 1947
	o-phthaldialdehyde (OPA); 37°C; 10 min; pH 7.8	7 nmole/mL/min Leu-Gly	Church *et al.*, 1983
Fluorimetric assay	Fluorescamine; 37°C; 30 min; pH 6.7	80 nmole/mL/min Leu-Leu	Chism *et al.*, 1979
	FTC-casein; 37°C; 60 min; pH 8.0	1 nmole/mL/min tyrosine	Sutherland, 1993
Bioluminescence assay	Luciferin, adenosine triphosphate; 25°C; 5 min	30 nmole/mL/min tyrosine	Sutherland, 1993
ELISA	IgG anti-*Pseudomonas* AFT 36 protease; room temperature; 2.5 h	1 nmole/mL/min tyrosine	Matta and Kanwar, 1997
Radiometric assay	[^{14}C]casein; 30°C; 30 min; pH 7.5	0.5 nmole/mL/min tyrosine	Christen, 1987

For bacterial protease assays, the oldest spectrophotometric assay used Folin-Ciocalteau Phenol reagent to detect tyrosine, but has the lowest sensitivity (Table 1.3). Comparing the two methods used for determination of α-amino groups, the spectophotometric assay using OPA was 3.8 times more sensitive than the fluorimetric assay. Other researchers also reported using TNBS to quantify α-amino groups for protease activity in milk (McKellar, 1981; Humbert *et al.*, 1990), but the assay is subjected to interference, *e.g.* Maillard reaction products (Chism *et al.*, 1978).

Chromogenic substrates, such as azocasein (Mitchell and Ewings, 1985) and Hide Powder Azure (HPA; Cliffe and Law, 1982) were also used for protease assay in milk. The azocasein method only detected protease activity when 0.03 U/mL *P. fluorescens* (1 unit was defined as the increase of 0.01 absorbance at 366 nm per h) was added into the milk (Mitchell and Ewings, 1985). The sensitivity of HPA assay was calculated to be 165 nmole/mL/min tyrosine at pH 7.0 and 37°C according to Matta and Kanwar (1997), which is the same low sensitivity as Hull's method (Hull, 1947). McKellar (1984) reported that although the HPA method was 6-7 times more sensitive than the TNBS method in skim milk, it was more expensive.

Protease assay methods have become far more sensitive since the late 1980's. The radiometric method was slightly sensitive than the fluorescent assay using FTC-casein, but is 1,000 times more sensitive than Hull's method (Hull, 1947). However, it is the most expensive method, and involves radioisotope material that requires special care. The immunoassay method gave similar sensitivity to the fluorescent assay, but the process to raise antibody is quite complicated. An advantage of the method is that it

might be applied to milk samples under field conditions (Matta and Kanwar, 1997). The bioluminescent assay was 360 times less sensitive that the fluorescent assay.

RP-HPLC method has also been applied for detection of proteolysis in UHT-milk. The method was said to distinguish between the protein breakdown products of plasmin and bacterial proteases, but no quantitative results were given (Lopez-Fandino *et al.*, 1993).

A new protease assay kit – Bioquant (Merck 1.05852.001, Merck KGaA., Frankfurter, Darmstadt, Germany) was released in 1999. It claimed a minimum detectable level of 2 μg/mL Alcalase® (Novo Industrial A/S, Bagsvaerd, Denmark) in milk at 37°C and pH 7.0, equivalent to 0.05 μmole/min/mL FITC at 40°C and pH 7.2 using FTC-β-casein (our unpublished method). Other fluorescent methods were reported to detect nanogram levels of protease in a broad pH range from 2 to 11 using biotinylated gelatin (Koritsas and Atkinson, 1995) or BODIPY[2] dye labelled casein (Schade *et al.*, 1996; Jones *et al.*, 1997). However, these methods not only require complex processes for labelling the substrates, but also require a special fluorescent polarisation instrument (Schade *et al.*, 1996), and these methods have not yet been applied to assay milk samples. The fluorescent assay using FTC-casein remains the most promising assay method for the detection of protease activity in milk.

1.4.2 Levels of Proteases in Milk and Dairy Products

Pasteurised skim milk contains 0.14-0.73 μg/mL plasmin and 0.55-2.75 μg/mL plasminogen, respectively (Richardson and Pearce, 1981). Rollema *et al.* (1983)

[2]BODIPY: 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid

reported a similar level of plasminogen at 0.7-2.4 μg/mL in raw milk. Bovine skim milk contains 0.4 μg/mL Cathepsin D (Larsen *et al.*, 1996).

Celestino *et al.* (1997a and b) reported that whole milk powder and UHT milk contained 65.5-84.1 U/g and 8.7 U/mL protease activity, respectively (1 unit was defined as the release of 1 nmole/min/mL Leu-Leu 37°C and pH 6.7) using the method of Chism *et al.* (1979; see Table 1.3 for details).

1.4.3 Lipase Detection

Common methods for the detection of microbial lipases have been reviewed extensively by Thomson *et al.* (1999), but the focus here is on the methods being applied to milk and dairy products. Details of some lipase assay conditions and sensitivities are listed in Table 1.4.

The methods for assaying lipase in milk samples are very diverse which makes the comparison between assays very difficult. One semi-quantitative method was developed using tributyrin-agar plates (Fryer *et al.*, 1967). Later methods began to quantify FFA using solvent-extraction and followed by titration with an alkaline solution (Castberg *et al.*, 1975; Deeth *et al.*, 1975). Assays have been developed further using chromogenic (Versaw *et al.*, 1989; Humbert *et al.*, 1997) and fluorescent-labelled substrates (Stead, 1983). Blake *et al.* (1996) developed a reflectance colorimetric assay using *p*-nitrophenyl caprylate for lipase activity in turbid samples, such as milk.

Table 1.4: Lipase assays in milk

Methods	Assay conditions	Sensitivity	Reference
Titration	Tributyrin; 30 min, 37°C, pH 8.5	est. 23 μmole/min/mL FFA	Castberg *et al.*, 1975; Humbert *et al.*, 1997
Spectrophotometric assay	β-naphthyl caprylate; 40°C; 30 min; pH 7.2	est. 0.1 μmole/h/mL β-naphthol (= 0.002 μmole/min/mL)	Versaw *et al.*, 1989
	*p*NP butyrate; 37°C; 30 min; pH 7.6	est. 0.025 μmole/min/mL *p*-nitrophenol	Humbert *et al.*, 1997
Reflectance colorimetric assay	*p*NP caprylate; 37°C; 10 h; pH 7.2	0.005 μmole/mL/min oleic acid	Blake *et al.*, 1996
Fluorimetric assay	4-methylumbelliferyl oleate; 25 or 37°C; 30 min; pH 6.5	est. 30 pmole/min/mL 4-methylumbelliferone	Stead, 1983

However, the main problem for all lipase assays is the interference of milk lipids (Stead, 1983; McKellar and Cholette, 1986; Blake *et al.*, 1996). Addition of 2% milkfat reduced the measurable level of lipase activity by 92%, and with 18% milkfat, no lipase activity could be detected (McKellar and Cholette, 1986). Therefore centrifugation is an essential step for removal of the milk lipids before assay, but it could also result in 20% loss of lipase activity (Stead, 1983). Bile salts (Stead, 1983) and clarifying reagent (Humbert *et al.*, 1997) have been applied to counteract these effects. On the other hand, FFA are also seen to cause product inhibition by competing for the binding sites from the substrates (Blake *et al.*, 1996).

Methods for FFA determination can be titration (Castberg *et al.*, 1975; Deeth *et al.*, 1975) or gas liquid chromatography (GLC; Deeth *et al.*, 1983; de Jong and Badings, 1990). The titration assay is regarded as straightforward, and requires little or no sophisticated equipment (Thomson *et al.*, 1999). While the GLC method is very

sensitive (*e.g.* 5-10 ppm depending on the chain length of the FFA), but requires special equipment and time consuming. In comparison, the spectrophotometric assay is simple, rapid and gives quantitative results, but it is not suitable for turbid samples, such as milk. Improvements have been made using a reflectance colorimeter (Blake *et al.*, 1996) or introducing clarifying reagents (Humbert *et al.*, 1997). Although the reflectance colorimetric assay is simple, rapid and can be used directly in milk, it requires special instrument. The advantage of the fluorimetric assay is having the highest sensitivity (Stead, 1983; Thomson *et al.*, 1999), but the disadvantage is the substrate instability at high assay temperatures. The assay also faces reduction of the fluorescent intensity by milk components through quenching (Stead, 1983). So there is no preferred method for the detection of lipase activity in milk samples.

1.4.4 Levels of Lipase in Milk and Dairy Products

Olivecrona *et al.* (1975) reported that bovine milk contains 1.5-2.5 U/mL LPL (1 unit was defined as the release of 1 μmole/min FFA at 25°C). A later study reported a lower level of LPL at 0.8 U/mL in milk (at 37°C; Hohe *et al.*, 1984). Casein contains 73.4% LPL activity, followed by serum at 24.1%, while sloughed membrane material (fluff) and MFGM contained less than 2.5% (Hohe *et al.*, 1984). Reddy *et al.* (1986) reported that 0.5-2.0 mg LPL activity was obtained from 1 L fresh bovine milk. This is a LPL to protein ratio between 1:17,000 and 1:68,000.

Raw milk was reported to have 0.08-0.09 U/mL lipase activity (1 unit was defined as the release of 1 μmole/min pyrenebutyric acid from pyrenyl triacylglcyerols at 25°C and pH 7.7; Liodakis *et al.*, 1991; Celestino *et al.*, 1996). Jandal (1996) reported a similar level of lipase activity in raw milk at 3.78 μmole FFA/h/mL (equivalent to 0.06

μmole/min/mL FFA at 37°C). Humbert *et al.* (1997) reported that raw milk contains 0.06-0.12 U/mL milk (1 unit was defined as the release of 1 μmole/min *p*-nitrophenyl from *p*NP-butyrate at 37°C and pH 7.6).

Whole milk powder and reconstituted UHT milk contain 0.65-0.81 U/g and 0.10-0.13 U/mL lipase activity, respectively (Celestino *et al.*, 1997a and b).

1.5 Changes in Milk and Milk Powder during Storage

Severe heat treatment (*e.g.* UHT) and subsequent packaging (under vacuum or nitrogen) are used to extend the shelf-lives of dairy products (Renner, 1988). WMPs generally have 6-month shelf-life at room temperature. To have a 12-month shelf-life, they should be packed in cans under vacuum or with nitrogen (Celestino *et al.*, 1997a). The shelf-lives of intermediate and long-life dairy products are largely determined by chemical deterioration or enzymatic degradation (Muir, 1996b). Muir (1996c) believed that even if extracellular enzyme had been very active during processing, degradation would be slow in powders because of low water content. However, no quantitative information is available on this aspect of powder quality.

Functionality and flavour changes in milk powders normally are the result of multiple effects – combinations of physico-chemical and enzymatic reactions (Prisecky, 1997). Langeveld *et al.* (1995) suggested that enzyme-producing bacteria might have been killed during the process, but their enzymes would still be present and active in the final product. Milk endogenous LPL is killed by pasteurisation, but the endogenous protease (plasmin) and contaminating bacterial enzymes (proteases and lipases) will still remain active in the final products. Celestino *et al.* (1997a and b) proved the prediction of

Stadhouders *et al.* (1982) that proteases and lipases survived the milk powder process first, then the UHT treatment afterward, and remained active in the reconstituted UHT milk. The enzymes remained active even after further 6 months storage at 25°C. Changes caused by the heat-stable proteases and lipases in milk and milk powders during storage will be discussed next.

1.5.1 Physico-chemical Changes

Kieseker and Clarke (1984) reported decreases in pH and heat-stability, and an increase in solubility index (SI) for non-fat milk powder (moisture content < 4%) after storage for 12 months at temperatures between 30°C and 40°C. For instance, the SI of the high-heat powder[3] increased from 0.2 mL to 1.0 mL after storage for 6-8 months at 40°C. Use of these powders may cause sedimentation in recombined products (Kieseker and Clarke, 1984). The pH of reconstituted milk from high-heat powders declined from 6.78 to 6.66 after they were stored for 18 months at 40°C (Kieseker and Clarke, 1984). Celestino *et al.* (1997a and b) reported the similar results in WMPs and reconstituted UHT milks (made from stored WMPs) after storage for 6 months at 25°C.

1.5.2 Proteolysis during Storage

Changes in casein-nitrogen and non-protein-nitrogen (NPN) are typical indicators of proteolysis in milk (Renner, 1988). These can lead to microstructural changes, and increases in sediment and viscosity in UHT milk. The changes become more severe with increasing storage temperature (Renner, 1988).

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[3]Definition of heat-treatments: 82°C for 30 min for high-heat powder; 79°C for 3 min for medium-heat powder; and 74°C for 15 s for low-heat powder.

The increase of NPN and the formation of para-κ-casein are generally caused by bacterial protease (Renner, 1988), while the decrease of casein-nitrogen and the formation of γ-casein are induced by plasmin (Visser, 1981; Renner, 1988). They can be distinguished by changes occurring in milk: plasmin causes a serum layer at the bottom of milk (Visser, 1981); the bacterial proteases first lead to a sediment at the bottom of milk, and later completely clear the milk (Kohlmann *et al.*, 1991). Tyrosine levels have been used as an indicator for defects, such as 'unclean', 'sour', 'bitter' and 'putrid' taste in milk and dairy products since an increase of tyrosine level is associated with an increase in proteolysis (Juffs, 1975).

The effects of plasmin in UHT milk are not fully understood because the mechanisms of age thinning and gelation remain unresolved (Bastian and Brown, 1996). No gelation was observed with the addition of protease inhibitors DFP and aprotinin in unconcentrated UHT skim milk after storage for 9 months at 20°C (de Koning *et al.*, 1985). Enright *et al.* (1999) reported that formation of sediments in UHT skim milk during storage appeared closely linked to plasmin activity. Proteolysis was reported to cause decrease in heat-stability of proteins and increase in fouling layer in the heat exchanger (Champagne *et al.*, 1994).

A level of *Pseudomonas* protease MC60 at 0.89 U/mL (1 unit was defined as the production of 1 μg/mL tyrosine per 24 h at 45°C and pH 7.5) can cause bitterness in UHT skim milk after storage for 14 to 32 days at 40°C. When it reaches 18 U/mL, the milk can only be kept for less than 3 days (Adams *et al.*, 1975). Gebre-Egziabher *et al.* (1980) reported that UHT milk developed flavour defects within 7 days at room temperature with 2 U/mL psychrotrophic protease (1 unit was defined as the production

of 1 μg/mL tyrosine per 24 h at 40°C and pH 6.7). If the level reached 9.8 U/mL, it resulted in unclean flavour and bitterness, and subsequently coagulated the milk in less than 3 days. To ensure 1-year shelf-life of UHT milk, Adams *et al.* (1975) recommended that milk should contain less than 0.1 U/mL protease. Similarly, to have a 5-month shelf-life at 23°C, studies have shown that UHT milk should not exceed the upper limit of 0.03 U/mL protease (1 unit was defined as increase of 0.01 absorbance at 366 nm per h at 37°C and pH 7.5), while to have a 3-month shelf-life at 30°C, the UHT milk should contain less than 0.06 U/mL protease (Ewings *et al.*, 1985).

1.5.3 Lipolysis during Storage

Lipolysis can impact on both desirable and undesirable flavours in dairy products. Short-chain fatty acids, such as butyric acid ($C_{4:0}$), caproic acid ($C_{6:0}$) and caprylic acid ($C_{8:0}$) lead to the development of sharp and tangy flavours, while medium-chain fatty acids, such as capric ($C_{10:0}$) and lauric acid ($C_{12:0}$) tend to have an unclean soapy taste. Long-chain fatty acids, such as myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) have little contribution to flavours (Al-Shabibi *et al.*, 1964; Vulfson, 1994).

FFA are not only the major flavour compounds in dairy products, but also the precursor for other flavour ingredients, such as acetoacetate, β-keto acids, methyl ketones, flavour esters and lactones (Vulfson, 1994). Unsaturated fatty acids released during lipolysis are susceptible to oxidation to aldehydes and ketones which give rise to off-flavours as 'oxidized card-boardy (tallowy), or metallic' (Shipe, 1980). Other unpleasant flavours, such as 'rancid, butyric, bitter, unclean, soapy and astringent' have been described in milk and milk products as consequence of lipolysis (Deeth and Fitz-Gerald, 1995).

Freshly secreted milk from a healthy udder contains total FFA of 0.49 μmole/mL (de Jong and Badings,1990; Deeth and Fitz-Gerald, 1995). Pasteurised milk has slightly lower value of FFA at 0.37 μmole/mL (personal communication, Brown, J. C. S.), while UHT milk has a slightly higher value of FFA at 0.64 μmole/mL (Choi and Jeon, 1993). However, the difference could be due to the differences between methods, or different sources of milk. Each individual level of fatty acid is listed in Table 1.5.

Table 1.5: *Level of individual free fatty acids in various milk samples*

Free fatty acid	Milk[1] (μmole/mL)	Pasteurized milk[2] (μmole/mL)	UHT milk[3] (μmole/mL)
Butyric $C_{4:0}$	0.04	0.08	0.15
Caproic $C_{6:0}$	0.03	0.03	0.05
Caprylic $C_{8:0}$	0.02	0.04	0.03
Capric $C_{10:0}$	0.02	0.02	0.04
Lauric $C_{12:0}$	0.03	0.03	0.03
Myristic $C_{14:0}$	0.04	0.03	0.05
Palmitic $C_{16:0}$	0.12	0.06	0.11
Stearic $C_{18:0}$	0.05	0.04	0.05
Oleic $C_{18:1}$	0.14	0.04	0.12
Linolenic $C_{18:2}$	-	0.00	0.01
Total FFA	0.49	0.37	0.64

[1]de Jong and Badings, 1990.
[2]Brown, J. C. S. and Holland, R. unpublished results.
[3]Choi and Jeon, 1993.

'Acid degree value' (ADV) is commonly used to express the levels of FFA as milli-equivalent (meq) per L milk or per 100 g fat. It is one of the standard terminologies of International Dairy Federation (IDF). However, caution should be taken when using ADV value to express the level of FFA because the method has low recoveries for short chain FFA, so the results underestimate the levels of FFA causing off-flavours (Duncan

and Christen, 1991). The ADV can be converted to 'μmole/mL' using the formula: FFA (μmole/mL) = 0.62 ADV (meq/L or meq/100 g-fat) + 0.07 (Deeth *et al.*, 1975). This allows comparison to be made with the given FFA threshold value. A typical FFA flavour threshold in milk is 1.2 to 1.5 meq/100 g-fat (0.8-1.0 μmole/g) for trained experts, and 2.0 to 2.2 meq/100 g-fat (1.3-1.4 μmole/g) for average consumers (Stead, 1986). Levels of individual FFA as the threshold value in milk and rancid milk are listed in Table 1.6.

Table 1.6: *Threshold levels of FFA in milk and rancid milk*

FFA ID.	Threshold value[1] in skim milk (μmole/mL)	Found[2] in rancid milk (μmole/mL)
Butyric $C_{4:0}$	028	0.70
Caproic $C_{6:0}$	0.12	0.30
Caprylic $C_{8:0}$	0.05	0.15
Capric $C_{10:0}$	0.04	0.24
Lauric $C_{12:0}$	0.09	0.20
Total FFA	0.53	1.59

[1]Kinsella (1969).

[2]Based on a total FFA of 2 meq/L n milk (Deeth and Fitz-Gerald, 1995).

Lipases have been used extensively in the dairy industry for hydrolysis of milkfat to produce 'desirable' flavours, such as flavour enhanced cheeses, enzyme modified cheese (EMC), acceleration of cheese ripening and lipolysed butterfat and cream (Vulfson, 1994). However, hydrolysis (lipolysis) as little as 1-2% milk lipids will give a rancid or "lipolyzed" flavour to milk (Olivecrona and Bengtsson-Oliverona, 1991). Rancidity caused by lipolysis is more pronounced than bitterness caused by proteolysis (Celestino *et al.*, 1997a and b). FFA increased from 2.63 to 3.95 meq/100 g-fat (1.8-2.5 μmole/g) in WMP and from 1.19 to 1.50 meq/L (0.8-1.0 μmole/mL) in reconstituted

UHT milk, respectively, after storage for 6 months at 25°C (Celestino *et al.*, 1997a and b).

Raw milk quality affects WMP quality, which consequently affects reconstituted UHT milks quality. The UHT milk made from the WMP made of old raw milk showed lower flavour scores than the ones made from the WMP made of fresh raw milk (Celestino *et al.*, 1997a). Further, the longer the storage of WMP, the lower the flavour score of the reconstituted UHT milk (Celestino *et al.*, 1997b). Skim milk powder was significantly affected with respect to sensory properties during long term storage at 32°C – it began to develop off-flavours after storage for 6 months, and became unacceptable by 24 months (Renner, 1988). Kieseker and Clarke (1984) also reported that the flavour of non-fat milk powder become unacceptable after storage for only 6 months at 40°C. However, the authers did not explain the causes for off-flavour.

1.6 The Aim of This Study

Milk powder quality and storage life can be compromised by functionality and flavour defects arising from the activities of proteases and lipases. There are several potential sources for these enzymes in the milk powder production process. The enzymes can be milk native enzymes, or bacterial enzymes secreted or released by psychrotrophic, mesophilic and thermophilic bacteria during the process.

As psychrotrophs and mesophiles are likely to be killed by pasteurisation, so thermophiles has become the commercial focus. Specification of thermophile[4] numbers

[4]Thermophiles are defined by the industry as a group of bacteria that grow aerobically at elevated temperatures of 55°C or above, and they are largely comprised of *Bacillus* species (NZTM Manual Section 60.0).

as an indicator of the microbial quality of milk powder process has been applied for years. However, a correlation between the numbers of heat-resistant bacteria and the product quality is anecdotal, and there is little supportive scientific evidence.

This research focuses on thermophilic enzymes – proteases and lipases in particular, rather than thermophilic organisms. Thermophilic enzymes do not exclusively originate from thermophiles – even psychrotrophs and mesophiles can yield thermophilic enzymes.

The study began with the development of assay methods for the detection of heat-stable proteases and lipases in WMPs. This was necessary as the literature review showed limited information on the levels of protease and lipase in WMPs.

Second, the study attempted to identify the heat-stable enzymes in the WMP. It was assumed that the most likely origin for a thermophilic enzyme would be a thermophilic bacterium. The reason for this was that most of enzymes from psychrotrophic bacterial have maximum activity at a temperature range from 30 to 45°C, and lower heat-stabilities than the enzymes from thermophiles. Further, the growth and secretion of enzymes in elevated temperatures in milk process streams were more likely to be from thermophiles than psychrotrophs, *e.g.* in biofilms and fouling layers. Therefore studies were also carried out on proteases and lipases produced by seven *Bacilli* (found in milk powder production streams) under laboratory conditions.

Finally, storage trials were carried out on various WMPs including some deliberately spiked with thermophilic *Bacillus* enzymes. These trials were undertaken to determine

the likely or possible effects of enzymes in WMP during storage at < 4% moisture content (equivalent to a_w of ~ 0.23).

Chapter 2 General Methods

All general chemicals and reagents were from BDH (Palmerston North, New Zealand). Commonly used assay buffers were from Sigma-Aldrich Pty Ltd. (Sydney, Australia). All enzyme assays were carried out either in duplicate or triplicate. If the error between each reading point and the average is above 10%, the assay was repeated.

2.1 Enzyme Assays

2.1.1 Protease Assays

2.1.1.1 Casein Plate Assay

The casein-plate method is based on measuring the diameter of a clearing zone or precipitation in a casein-agar plate, which is generated as a result of proteolysis. The clearing zone results from soluble products of casein breakdown, while precipitation results from formation of para-κ-casein. By comparing the size of the zone with that produced by a known amount of protease, the assay can be semi-quantitative. This method is adapted from Cowan and Daniel (1982a).

Casein (10 g, Hammarsten, BDH) and 12 g agar (DIFCO) were dissolved in 1 L deionised water and autoclaved at 121°C for 15 min (15 psi). Following autoclaving, the medium was cooled to 45°C. To the cooled medium, 200 mg Penicillin-G was added. The medium was kept under constant stirring, ~ 20 mL medium were poured into a sterile petri-dish using the PourMatic MP-1000 (New Brunswick Scientific, Edison, NJ, USA) to achieve even thickness. When the medium was set, up to nine 6-mm internal diameter wells were made in the plate with a cork-borer.

A 50 μL sample (enzyme solution or milk) was added to each well in duplicate and incubated at 40°C or 55°C for 24-48 h. The inter-diameter of the ring was measured. Protease activity was determined by comparing the inter-diameter of the zones formed to a known amount of Alcalase® (a serine protease, subtilisin A from *Bacillus licheniformis*, NOVO Industrial A/S, Bagsvaerd, Denmark), and an average of duplicate value was used in the standard plot.

2.1.1.2 Kunitz Assay

The Kunitz assay is based on measuring the phenolic group of tyrosine and phenylalanine, and the indolic group of tryptophan which have maximum absorbance at 280 nm (Kunitz, 1947). The proteolysis products of peptides and amino acids were separated from intact protein using 15% TCA and the absorbance of soluble material measured at 280 nm.

WMP was reconstituted to 10% (w/v) and incubated in 0.1 M Hepes buffer, pH 7.0, containing 0.2 mg/mL Penicillin-G at 40°C and 60°C for up to 100 h. The reaction was stopped by adding 1.4 mL 15% TCA to 0.1 mL sample in triplicate. The samples were kept on ice for 10 min, centrifuged at 10,000 x *g* for 10 min, and the absorbance of middle layer measured at 280 nm. The unit of protease was calculated equivalent to the amount Alcalase® present in the sample under the same assay conditions. The standard plot was an average of triplicate.

2.1.1.3 Azocasein Assay

The azocasein assay method is based on the Kunitz assay principle, which uses chromogenic derivatives of proteins, such as azocasein or azoalbumin instead of casein,

and measures TCA-soluble product at 420 nm. The method is adapted from Cowan and Daniel (1982b).

For the assay, 200 µL 0.1% azocasein (Sigma A2765, Sigma Chemical Co., St. Louis, MO, USA), in 0.1 M Hepes buffer, pH 7.0, and 200 µL sample were mixed and incubated at 55°C in triplicate. Samples were taken at each sampling point by adding 1 mL 15% TCA to each tube, then kept on ice for 10 min, centrifuged at 10,000 x *g* for 10 min, and the absorbance of supernatants read at 420 nm. A thermophilic bacterial metallo-protease, thermolysin (Sigma P1512), was used as the positive control. Protease activity was calculated equivalent to thermolysin using an average of the standard curve obtained in triplicate.

2.1.1.4 p-*Nitroanilide Assay*

The *p*-nitroanilide assay is a spectrophotometric assay in which proteolysis is determined by the breakdown of colourless *p*-nitroanilide-linked peptides into the yellow compound *p*-nitroaniline (*p*NA) which absorbs at 400 nm. The method is adapted from Sarath (1996) and Toogood (1998).

N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Suc-AAPF-*p*NA, Sigma S7388) was prepared at 1 mM by dissolving 1.87 mg of Suc-AAPF-*p*NA in 0.3 mL ethanol (10% of total volume), then adding 0.1 M Hepes buffer, pH 7.0, to give a final volume of 3 mL. A 50 µL Suc-AAPF-*p*NA was dispensed in a 100 µL cuvette and warmed to 40°C for 2 min, then 50 µL sample was added, mixed well and the absorbance at 400 nm was measured continuously in a Perkin-Elmer Lamba #B Spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) fitted with a thermoelectric cell holder.

One unit of protease was defined as the release of 1 μmole/min *p*-nitroaniline (*p*NA) under the assay conditions.

To determine the assay extinction coefficient, *p*NA was dissolved at concentrations of 0-0.20 μmole/mL in 0.1 M Hepes buffer in duplicate, pH 7.0, and the absorbance measured at 400 nm at 40°C. The measured absorbance was plotted against *p*NA concentrations. The extinction coefficient of *p*NA, obtained from the slope of the plot, was 11.2 $mM^{-1}cm^{-1}$.

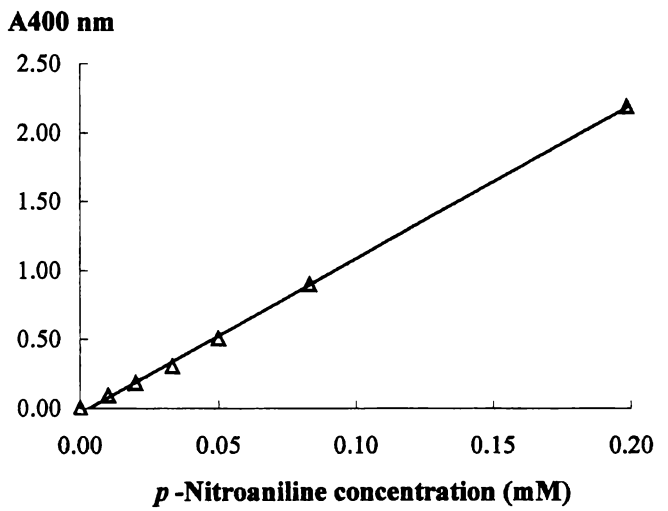


Figure 2.1: Absorbance of different concentrations of *p*-nitroaniline at 400 nm at 40°C. Each data point represents an average of duplicate. The slope of the line gives an extinction coefficient of 11.2 $mM^{-1}cm^{-1}$.

2.1.1.5 Fluorescent Assay

The fluorimetric assay measures the release of fluorescent peptides from fluorescein isothiocyanate-labelled casein [fluorescein thiocarbamoyl casein (FTC-casein)]. The fluorescent-peptides releases due to proteolysis are separated from the intact substrate using 15% trichloroacetic acid (TCA). This method is adapted from Twining (1984).

FTC-β-casein was prepared as follows: 0.2 g β-casein (Sigma C6905) was dissolved with stirring in 20 mL 50 mM Na_2CO_3 buffer, pH 9.5, containing 150 mM NaCl. Fluorescein isothiocyanate (0.005 g; FITC Isomer I, Sigma F7250) was dissolved in 0.5 mL of the same buffer and slowly added to the casein solution. The mixture was stirred in the dark at room temperature for 2 h, and carefully transferred to a dialysis bag with a 3,500 Da molecular mass cut-off (The Spectrum Companies, Laguna Hills, CA, US). The FTC-β-casein solution was dialysed in 2 L deionised water containing 2 g active charcoal, in the dark for 12 h, with one change of water, followed by two changes of 2 L 50 mM Tris-HCl buffer, pH 8.5, and finally with two changes of 2 L 50 mM Tris-HCl buffer, pH 7.5. The dialysed solution was carefully removed from the dialysis bag, put through a 0.22 μm filter in 2 mL lots into glass vials and stored in the dark at -20°C.

For assay, 20 μL FTC-β-casein was dispensed into a 1.5 mL Eppendorf tube, and 20 μL 0.1 M Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) buffer, pH 7.0, 20 μL sample and 12 μg penicillin-G (0.2 mg/mL, Sigma PEN-NA) were added. After mixing thoroughly, the mixture was incubated at 40°C for 0.5 to 72 h, then 150 μL 15% TCA was added. The stopped reaction was chilled on ice for 10 min and then centrifuged at 10,000 x *g* for 5 min. To 150 μL supernatant, 3 mL 0.5 M Tris-HCl buffer (pH 8.5) was added, mixed well, and the fluorescence read at 490 nm excitation/525 nm emission (slit width 5 nm) on a Luminescence Spectrometer LS50B (Perkin-Elmer Ltd.). One unit protease was defined as the release of 1 μmole/min FITC under the described assay conditions. The assay was done in triplicate.

The extinction coefficient of FITC was determined by dissolving different amounts of FITC (0 to 0.16 μmole/mL) in 50 mM Tris-HCl buffer in triplicate, pH 7.5. To a tube

containing 40 µl 0.1 M Hepes buffer, pH 7.0, 150 µL chilled 15% TCA, 20 µL FITC solution was added. The reaction was kept on ice for 10 min and then centrifuged at 10,000 x *g* for 5 min. A 150 µL sample of the supernatant was added to 3 mL 0.5 M Tris-HCl buffer, pH 8.5, mixed well and the fluorescence read at 490 nm excitation/525 nm emission. A plot of fluorescence against FITC concentration was constructed. The extinction coefficient (17089 $mM^{-1}cm^{-1}$) for the assay was determined from the slope of this plot.

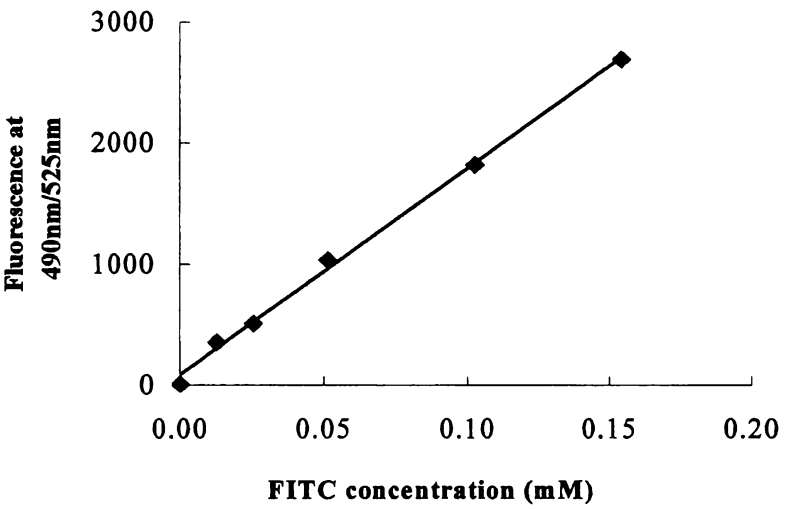


Figure 2.2: Fluorescence of different concentrations of fluorescein isothiocyanate in 0.5 M Tris-HCl, pH 8.5, at excitation 490 nm/emission 525 nm. Each data point represents an average of triplicate. The slope of the line gives the extinction coefficient of 17089 $mM^{-1}cm^{-1}$.

2.1.1.6 Protease Separation and Detection using PAGE

Protease samples were separated on a 10-15% gradient Native PhastGel or PhastGel IEF (Amershan Phamacia Biotech, Uppsala, Sweden) (see later Sections 2.3.2 for details), and the gels were stained for activity using an agarose overlay. This method is adapted from Sarath *et al.* (1996).

To produce the overlay, 0.6 g agarose (SeaPlaque, FMC BioProducts, ME, USA) was dissolved in 40 mL of 0.1 M Hepes buffer, pH 7.0, by heating in a 700 watts National microwave oven (Matsuchita Electric Industrial Co. Ltd., Japan) at medium power for 2 min. The agarose solution was cooled to 45°C, and 8 mg Penicillin-G was added together with 5 mL of 1 mM Suc-AAPF-*p*NA in Hepes buffer. It was mixed by sonicating at 50% duty cycle for 2 min using a VibraCell (Sonic & Material Inc., Danbury, CT, USA). The medium was poured on top of the gel to form an even layer. The overlaid gel was then incubated in a humidity chamber at 40°C until the appearance of a yellow band indicating the release of *p*NA by proteolysis.

2.1.2 Lipase Assays

Lipase hydrolyses milk lipids of mono-, di- and triacylglycerols to FFA and glycerols. As the literature review indicated that the milk lipids will interfere with the assay, so pretreatment-steps, such as centrifugation, are necessary to remove the milk lipids before assay.

2.1.2.1 Removing Lipids from Whole Milk Powders

WMP was reconstituted to 100 mL at 30% (w/v) in deionised water at 50°C and mixted for 5 min using a household hand blender. The milk was then centrifuged at 6,500 x *g* for 20 min at 15°C. The middle-layer was collected by filtering through a cheese-cloth; and the top fat-layer was washed twice by sonicating continuously for 2 min at 50% duty cycle in 30 mL 50°C deionised water using a VibraCell, followed by centrifugation and filtration. All middle layers were combined to give a 10% (w/v) skim milk by adding deionised water to 300 mL.

2.1.2.2 Tricaproin-plate Assay

The tricaproin-plate assay measures the release of caproic acid ($C_{6:0}$) by lipolysis from tricaproin as a result of lipolysis. The caproic acid forms clearing zone around wells in an opaque tricaproin-agar plate. By comparing the size of the clearing zone with that given by a known amount of lipase, the assay can be semi-quantitative. This is a method adapted from Fryer *et al.* (1967).

Tricaproin-plates were prepared as follows: 0.3865 g tricaproin (Sigma T-0888) was dissolved in 5 mL ethanol, then disbursed into 1 L 0.1 M Mops buffer (pH 7.6) to form a stable emulsion using an Ultra-turrax T25 (Janke & Kunkel Co. GmbH, IKA Labortechnick, Staufen, Germany) at 8,000 rev/min for 3 min, and then 12 g agar added. The emulsion was heated in a microwave at a medium heat until the agar completely melted, then cooled to 45°C, and 200 mg Penicillin G added and mixed. Medium of ~ 20 mL was poured into each sterile petri-dish. After the agar set, up to nine 6 mm inter-diameter wells were made using a cork-borer.

For assay, 50 µl sample in duplicate was added to each well in the plate, and incubated at 55°C for 24 to 48 h. The inter-diameter of clearing zone was recorded. Lipase activity was quantified using the standard curve obtained from A^m lipase in duplicate (from *B. stearothermophilus* A^m; see later Section 5.3.3).

2.1.2.3 Titration Assay

The titration assay method measures the release of FFA due to lipolysis. The FFA was first extracted using a solvent mixture, then quantified using NaOH (Castberg *et al.* 1975).

Milk samples of 2 mL each were added to tubes containing 2 mL 0.1 M H_2SO_4 with 1 M NaCl, then mixed with 5 mL of diethyl ether/heptane (1:1, v/v). The solvent/sample mixture was vortexed thoroughly, then centrifuged at 4,000 x *g* for 5 min, and the top layer retained. To 3 mL top layer, 1 mL phenolphthalein indicator (0.2 g/L in isopropyl alcohol) was added, and titrated using 10 mM NaOH in methanol in an Autotitrator system (TTT80 Titrator, TTA80 Titration assembly and ABU 80 Autoburette; Radiometer A/S, Copenhagen, Denmark) until the solution turned bright pink. The amount of NaOH used was recorded.

To determine the extinction coefficient of the assay, caproic acid ($C_{6:0}$) was dissolved in the same solvent mixture at concentrations of 0 to 20 μmole/mL. A 3 mL lot of each concentration of caproic acid-solvent mixture was titrated using 10 mM NaOH. The concentration of caproic acid was plotted against the volume of NaOH. The slope of the line gave the extinction coefficient of 0.36 $mM^{-1}mL$ for the assay. One unit of lipase was defined as FFA released equivalent to 1 μmole/h caproic acid under the assay conditions.

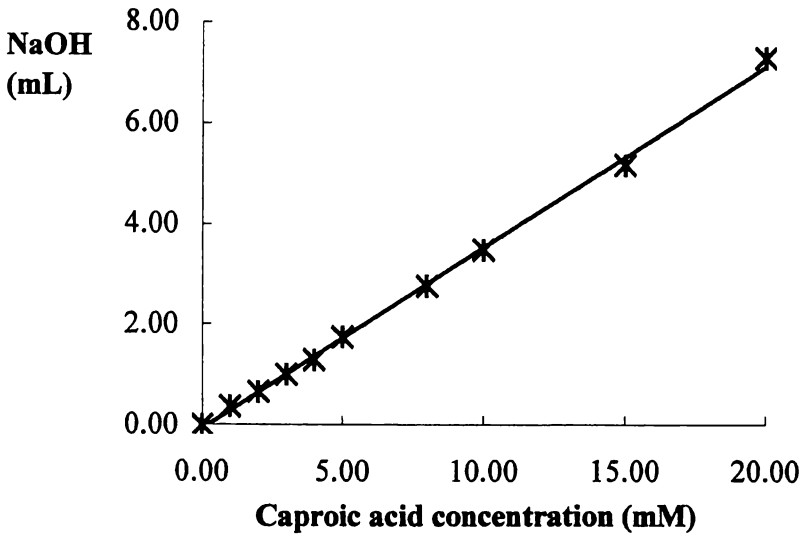


Figure 2.3: The amount of NaOH (10 mM in methanol) required to neutralise different concentrations of caproic acid in the titration assay. Each data point represents an average of triplicate. The slope of the line gives an extinction coefficient of 0.36 $mM^{-1}mL$.

2.1.2.4 Assay with p-*Nitrophenyl Esters of Fatty Acid*

The *p*-nitrophenyl assay is based on the spectrophotometric measurement of the release of the yellow compound *p*-nitrophenol (*p*NP) from *p*-nitrophenyl esters of fatty acids as the result of lipolysis (Winkler and Stuckmann, 1979). The chain length of the esters of fatty acids used depends on the lipase specificity. The assay is a discontinuous assay, the reaction was stopped by adding a stop-reagent, and the absorbance measured at 400 nm (Janssen *et al.*, 1994).

The assay was carried out in triplicate. *p*-Nitrophenyl esters of fatty acids from $C_{4:0}$ to $C_{16:0}$ were used as substrate. First, the *p*NP substrate was dissolved in 1 mL ethanol at 1 mg/mL, then disbursed into 8 mL 0.1 M Mops (3-[N-Morpholino] propanesulfonic acid) buffer, containing 5 mM $CaCl_2$ and 0.1% Triton X-100, by sonicating continuously for 1 min using the VibraCell. The pH of buffer was adjusted to 7.2 in each assay temperature. To 0.9 mL substrate emulsion, 0.1 mL sample was added, then

mixed thoroughly and incubated at 40-70°C for 0.5 to 20 h. When a definite yellow colour had developed, the reaction was stopped by adding 0.5 mL stop-reagent. The tubes were chilled on ice for 10 min, centrifuged at 10,000 x *g* for 5 min and the absorbance measured at 400 nm. One unit of lipase was defined as the release of 1 μmole/min *p*NP under the assay conditions.

To prepare stop reagent, 20 mL 2.8% $CuCl_2 \cdot 2H_2O$ was slowly added to 40 mL 6.85% $Na_3PO_4 \cdot 12H_2O$ with continuous stirring. The mixture was centrifuged at 3,000 x *g* for 5 min at room temperature. The supernatant was discarded; the pellet was washed twice with 100 mL 1.91% $Na_2B_4O_7 \cdot 10H_2O$ by vortexing and centrifugation. Finally the pellet was suspended in 100 mL washing buffer containing 6 g NaCl. Triton X-100 (10 % v/v) was included in the final stop-reagent if it is used for assay samples containing fat. The stop-reagent was stored for at least 2 days and mixed well before use.

To determine the extinction coefficient of *p*-nitrophenol (*p*NP) under the assay conditions, different concentrations of *p*NP (0 to 0.06 μmole/mL) were dissolved in ethanol. To 100 μL *p*NP, 0.9 mL 0.1 M Mops buffer, pH 7.0, containing 5 mM $CaCl_2$ and 0.1% Triton X-100 and 0.5 mL stop-reagent were added. The mixtures were centrifuged at 10,000 x g for 5 min, and the absorbance read at 400 nm. The absorbance was plotted against *p*NP concentration. The slope of this plot gave an extinction coefficient of 12.6 $mM^{-1}cm^{-1}$.

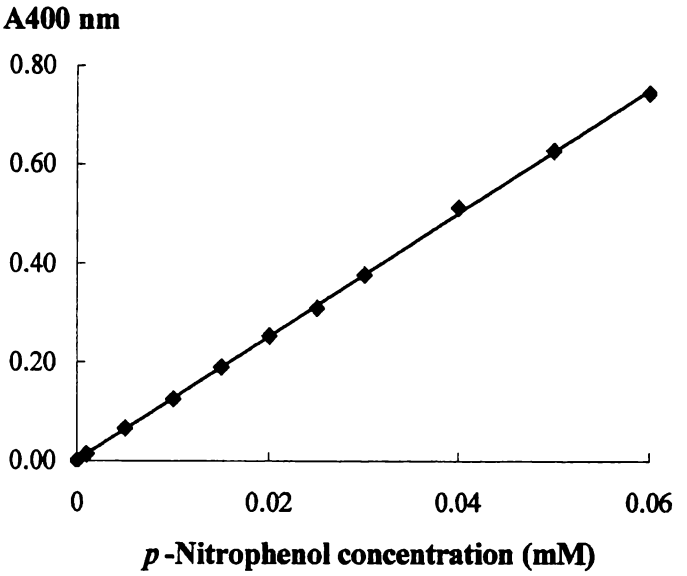


Figure 2.4: Absorbance of different concentrations of *p*-nitrophenol at 400 nm. Each data point represents an average of triplicate. The slope of line gives the extinction coefficient of 12.6 $mM^{-1}cm^{-1}$.

2.1.2.5 Separation and Detection of Lipase Activity using PAGE

This is a method adapted from Amaki *et al.* (1992). Samples containing lipase activity were separated by polyacrylamide gel electrophoresis (PAGE) using a 12.5% gel in a Mini-PROTEAN II system (Bio-Rad Laboratories, Hercules, CA, USA) following the method detailed in later Section 2.5.3. Lipase activity was identified in one of two ways:

1. Natrive PAGE: a final concentration of 0.01% *p*NP ester of fatty acid was incorporated into the gel before polymerisation. After electrophoresis, the gel was rinsed with 20 mL deionised water for 10 min, then incubated at 40°C for 1 to 10 h. Development of yellow bands indicate *p*NP release due to lipolysis.

2. SDS PAGE: after electrophoresis the gels were soaked in 20 mL 20 mM bis-Tris propane (BTP) buffer, pH 7.2, for 2 h with continuous shaking and three changes of

fresh buffer every 30 min. An activity overlay was made by dissolving 0.6 g agarose in 40 mL BTP buffer, heating slowly in the microwave until the agarose was melted. The agarose was cooled to 45°C, 8.0 mg Penicillin-G and 5 mL 1 mg/mL *p*NP ester in ethanol were added. The mixture was sonicated at 50% duty cycle for 2 min, and the resulting emulsion was poured over the gel to form an even layer. The overlaid gel was incubated in a humidity chamber at 40°C until the appearance of yellow bands indicates *p*NP release due to lipolysis.

2.2 Protein Assays

2.2.1 Bicinchoninic Acid Assay

Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent kit (PIERCE, Rockford, IL, USA). BCA reacts with the cuprous cation in the protein and forms a purple product, which absorbs optimally at 562 nm. The macromolecular structure of the protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) are the major factors affecting the colour formation. The assay was carried out at 37°C for 30 min. A standard curve was established in triplicate in the range of 0-1.0 mg/mL protein using bovine serum albumin (BSA). The detail of the method is described in PIERCE file 23225.

2.2.2 BioRad Assay

The BioRad assay method is based on the Bradford assay (Bradford, 1976). The acidic and basic groups of proteins form complexes with Coomassie Brilliant Blue G-250 dye which result in a change of dye colour from red to blue. The absorbance is read at 595 nm.

To 0.1 mL sample, 1 mL of 5-fold diluted BioRad protein assay dye reagent concentrate was added and mixed. The absorbance of the resulting solution was measured at 595 nm after standing for 2 min. BSA (0 to 1.0 mg/mL) was used in triplicate as the standard.

2.2.3 Lowry Assay

The Lowry assay method is based on Lowry *et al.* (1951). Under alkaline conditions, copper oxidises aromatic amino acids of proteins, and turns the Folin-Ciocalteau reagent (phosphomolybdicphosphotungstic acid) to heteropolymolybdenum blue. The absorbance is then measured at 750 nm.

Reagent A was prepared by slowly adding 10% (w/v) Na_2CO_3 to an equal volume of 0.1% (w/v) $CuSO_4 \cdot 5H_2O$ containing 0.2% (w/v) potassium tartrate, with continuous mixing. The reagent was stored below 10°C (stable for 6 months).

Reagent B was prepared when required by adding one part of Reagent A to 2 parts 5% (w/v) SDS and one part 0.8 M NaOH.

For assay of protein concentration, 1 mL Reagent B was added to 1 mL sample in triplicate, mixed, and left to stand for 10 min at room temperature. After standing, 0.5 mL 5-fold diluted Folin-Ciocalteau phenol reagent (BDH 19058 2P, Palmerston North, New Zeland) was added, mixed well and incubated at room temperature for 30 min. The absorbance of the resulting solution was measured at 750 nm. A 1:1 mixture of β-casein (Sigma C6905) and α-casein (Sigma C6780) at concentration range from 0-0.16 mg/mL was used as the standard.

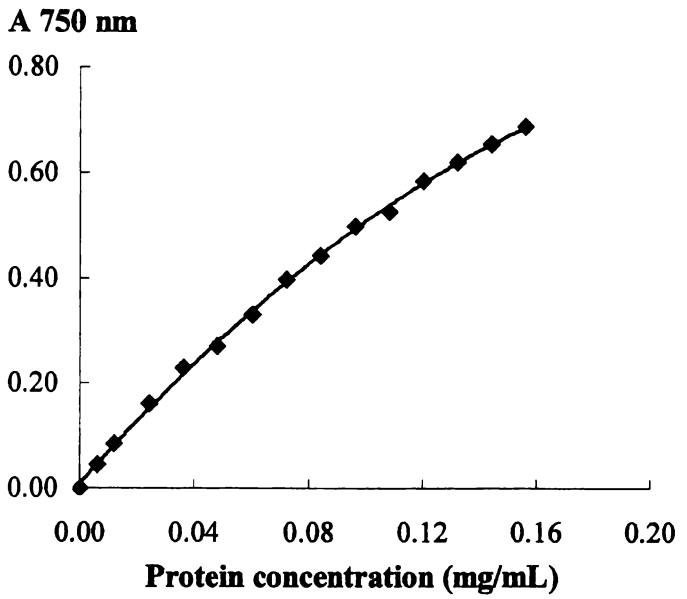


Figure 2.5: The absorbance given in the Lowry assay at different protein concentrations using a 1:1 mixture of β-casein (Sigma C6905) and α-casein (Sigma C6780). Each data point represenst an average of triplicate.

2.3 Peptide and Protein Separation

2.3.1 Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Peptides differ in size, charge and hydrophobicity, therefore can be separated using reverse phase high performance chromatography (RP-HPLC). After separation, each peptide can be identified using a 476A protein sequencer (Appiled Biosystem, Foster City, CA, USA) for N-terminal amino acid analysis and a triple quadrupole API 300 mass spectrometry (Perkin-Elmer-Sciex, Thornhill, Ontario, Canada) for mass analysis. This is a method adapted from Reid (1994).

For separation, WMP was reconstituted to give 150 mL 10% (w/v) skim milk (method details see Section 2.1.2.1), then divided into three portions of 50 mL. To the first 50 mL, 10 mg Penicillin-G was added; to the second 50 mL, the same amount of Penicillin

G as well as one Complete™ protease inhibitor cocktail tablet (Boehringer Mannheim 1697498, Boehringer Mannheim GmbH, Germany) was added, then treated with 1 mM pepstatin.. The third 50 mL was autoclaved at 121°C for 30 min. All milk samples were incubated at 40°C and 60°C for up to 100 h. The reaction was stopped by adding 0.5 mL 3% trifluoroacetic acid (TFA) to 1 mL milk. The samples were chilled on ice for 10 min, centrifuged at 10,000 x *g* for 10 min and filtered through a 0.45 μm filter.

RP-HPLC uses a Vydac C_{18} column (25 cm x 10 μm, AllTech 218TP, AllTech Associates Inc., Deerfield, IL, USA) connected to a Hewlett-Packard Series 1050 High Performance Liquid Chromatograph system (Hewlett-Packard GmbH, Waldbronn, Germany). The column was first equilibrated with a gradient of 95% buffer A (0.1% TFA in Milli-Q water) and 5% buffer B (0.08% TFA in 100% acetonitrile) at a flow rate of 1 mL/min. Samples of 100 μL each were injected onto the column at a flow rate of 1 mL/min, then eluted using buffer B in a three-step-gradient: (1) from 5% to 45% buffer B over 40 min; (2) to 75% B over 5 min and (3) maintained at 75% B for further 5 min. Peptides and proteins were monitored at 214 nm and 280 nm.

2.3.2 Polyacrylamide Electrophoresis (PAGE)

Electrophoresis is a method that separates proteins and large peptides based on their charge density (charge to mass ratio) in an electric field using polyacrylamide gel. With the addition of a cationic detergent, such as sodium dodecyl sulphate (SDS), all proteins become identically negative charges, and are separated according to size (Hames, 1990).

2.3.2.1 PhastGel

For native PhastGel electrophoresis, 0.02% phenyl bromide blue tracking dye was added to 10 μL sample. Samples of 1 or 6 μL (depending on the protein concentration) were applied to a 10-15% gradient PhastGel (Amersham Pharmacia Biotech 17-0540-01) on a PhastSystem (Amersham Pharmacia Biotech, Uppsala, Sweden). The gel was electrophoresed using native buffer strips according to PhastSystem Separation File No.120 (Amersham Pharmacia Biotech).

For SDS PhastGel, 8 μL sample was mixed with 2 μL 5 x sample buffer (see later Section 2.3.2.3 for details) and boiled for 5 min, then electrophoresed on a 10-15% gradient PhastGel using SDS buffer strips according to the PhastSystem separation file No.110 (Amersham Pharmacia Biotech).

2.3.2.2 Isoelectric Focusing (IEF)

For isoelectric focusing, 6 μL sample containing 0.02% phenyl bromide tracking dye was applied to PhastGel IEF 3-9 (Amersham Pharmacia Biotech 17-0543-01), and 6 μL isoelectric focusing calibration standards (broad pI kit, Amersham Pharmacia Biotech 17-0471-01) were used as markers. The gel was electrophoresed according to the manufacturer's instructions described in PhastSystem Separation file No. 100 (Amersham Pharmacia Biotech) until the standard reached the cationic zone of the gel and turned red.

2.3.2.3 BioRad Mini PROTEAN II Cell System

SDS gels are prepared according to the recipe shown in Table 2.1 (Hames, 1990) using BioRad Mini-PROTEAN II Cell system. Ammonium persulphate and TEMED were

added to the mixture after the mixture was degassed for at least 30 min. The stacking gel mixture was prepared the same way, and poured on top of the resolving gel.

For preparation of native gels, SDS was omitted, and the volume difference was made up with deionised water.

Table 2.1: *Recipe for two 12.5% SDS gels using Bio-Rad Mini-PROTEAN II Cell*

Reagents	12.5% Resolving Gel (mL)	4% Stacking Gel (mL)
Acrylamide/Bis (30% T, 2.67% C)	8.30	1.30
Milli-Q Water	6.40	6.10
1.5 M Tris-HCl, pH 8.3	5.00	-
0.5 M Tris-HCl, pH 6.8	-	2.50
20% SDS	0.10	0.05
10% Ammonium persulphate (freshly made)	0.10	0.05
TEMED	0.01	0.01

Note: all reagents are BioRad electrophoresis-grade. To make Native-PAGE, Milli-Q water is substituted for SDS. To make a different percentage resolving gel, adjust the volume between acrylamide and Milli-Q water.

The running buffer was prepared as a 5 x concentrate comprising 125 mM Tris, 1 M glycine and 0.5% (w/v) SDS, pH 8.3. For running native gels, SDS was omitted.

The sample buffer was prepared as a 5 x concentrate containing 0.3 M Tris-HCl, pH 6.8, with 0.01% bromophenol blue, 10% SDS, 50% glycerol and 25% β-mercaptoethanol. For native gels, SDS and mercaptoethanol were omitted.

For electrophoresis, 20 μL sample was mixed with 5 μL 5 x sample buffer, boiled for 5 min (for SDS-PAGE only, not for native gels or activity overlay) and electrophoresed

using a BioRad Mini-PROTEAN II system. Broad molecular range rainbow markers (10 μL; RPN 756, Amersham Pharmacia Biotech) were used as markers. The gel was electrophoresed at a constant voltage of 140 V until the tracking dye reached the bottom of the gel.

2.3.2.4 Gel Staining

After electrophoresis, gels were stained with either Coomassie blue or silver stain (Hames, 1990).

For Coomassie blue stain, the gel was first placed in 0.1% (w/v) Coomassie blue R250 in 40% (v/v) methanol for 30 min, destained with 40% (v/v) methanol containing 10% (v/v) acetic acid for 15 min, and finally transferred to 10% (v/v) ethanol containing 5% (v/v) acetic acid until blue protein bands were visible in the clear background of the gel. The gel was equilibrated in 3% (w/v) glycerol for 24 h and dried using a BioRad gel-drier.

For silver stain, the method described by the manufacturer (Bio-Rad 161-0443) was followed. The gel was first fixed with 40% (v/v) methanol for 40 min, then washed twice in 10% (v/v) ethanol containing 5% (v/v) acetic acid for 20 min.. The gel was then soaked in 10 x diluted oxidizer for 1 min, rinsed with Milli-Q water until the yellow colour disappeared, then soaked in 10 x diluted Silver Stain Reagent for further 30 min, and rinsed with Milli-Q water for 2 min. The gel was finally developed in developer until brown protein bands appeared clearly. At this point, developing was stopped by the addition of 5% (v/v) acetic acid. The gel was preserved in 3% (w/v) glycerol.

2.3.3 Western Blotting-Protein Blotting

2.3.3.1 Protein Blotting

The Western blotting method was adapted from Hayes (1998). Proteins were transferred from SDS gels onto polyvinylidene difluoride (PVDP) membrane (Bio-Rad 162-0180) using a BRL Mini-V 8•10 vertical gel electrophoresis apparatus system (Life Technologies Inc., Gaithersburg, MD, USA).

After electrophoresis, the gel was soaked in 10 mM Caps (3-[Cyclohexylamino]-1-propanesulfonic acid) buffer, pH 11, containing 10% (v/v) methanol (transfer buffer) for at least 30 min. The PVDP membrane was cut to the gel size, soaked in methanol first for 3 min, then in the transfer buffer for 30 min. Two pieces of filter paper (cut to the gel size) with two transfer pressure pads were soaked in the transfer buffer for 30 min.

The protein transfer sandwich was formed using a piece of filter paper, the gel, the PVDP membrane, and another piece of filter paper in respective order. Two transfer pressure pads were carefully placed onto the outer sides of the filter papers. The sandwich was carefully rolled with a glass rod several times to remove any air bubbles between the layers. The sandwich was electrophoresed at constant current of 30 mA overnight at 4°C to achieve good protein transfer.

2.3.3.2 Membrane and Gel Staining

The PVDP membrane was stained with 0.1% (w/v) Coomassie blue in 40% (v/v) methanol for 2-3 min, and destained in 40% methanol until blue protein bands clearly appeared against the white background of the membrane. The membrane was air-dried. The transferred gel was stained with Coomassie blue to check the efficiency of transfer.

2.4 Analysis of Lipids, Free Fatty Acids and Volatile Compounds

2.4.1 Lipid Identification using Thin Layer Chromatography

Different groups of lipids, such as mono-, di- and triacylglycerols can be separated based on differences in their physical properties using thin layer chromatography (TLC). The method uses an aluminium-backed 0.2 mm silica gel plate (Merck Silica Gel 60 F_{254}, Merck kGaA, Darmstadt, Germany) with a mixture of solvent system. The method was obtained from Dr. N. Robinson (personal communication).

WMP (1 g) was dissolved in 10 mL of hexane/*tert*-butyl methyl ether (TBME) (1:1, v/v) containing 0.3 mL sulphuric acid (5 M) and 1 g Na_2SO_4. The extract was centrifuged at 10,000 x *g* for 5 min to remove the protein components, and the top layer was obtained for TLC analysis.

TLC plate was first cut to the required size according to the numbers of samples. The loading spots were marked on the plate at 1.0 cm above the bottom of gel and 0.7 cm apart from the side and each other. The plate was pre-run in a chloroform/*iso*-propyl achohol (IPA) (2:1, v/v) solvent mixture until the solvent front reached 2 cm below the top of the plate, then air-dried.

Samples of 5 µl each was loaded on each marked spot on the TLC plate. A TLC-standard-mixture containing mono-olein, 1,3-diolein, triolein and methyl oleate (18-1-A TLC reference standard, Nu-Chek-Prep Inc. MN, USA) was dissolved in 10 mL hexane/TMBE with addition of 10 mg 1,2-dipalmitin (Sigma D2135) and 10 mg linoleic acid (Sigma L1376), and 5 µL was used on TLC plate.

The loaded TLC-plate was run in a toluene/acetone (9:1,v/v) solvent mixture of until the solvent front reached 3 cm below the top of the plate. After being air-dried, the plate was stained with 10% Cu_2SO_4 (w/v) in 8% H_3PO_4 (v/v) for 2 s, air-dried again for 20 min, then heated on a hot plate, until yellow spots of acylglcyerols and free fatty acdis (FFA) appeared.

2.4.2 Analysis of Free Fatty Acids using GLC

FFA are the products of lipolysis, which can be separated from milk lipids using solid phase extraction (SPE), then quantified by gas liquid chromatography (GLC) against knowing standards. The method is adapted from de Jong and Badings (1990) and Brown and Holland (1999).

2.4.2.1 Sample Preparation

Lipids and FFA were first extracted from the samples by a solvent mixture before SPE. During the extraction, internal standard of tridecylic acid ($C_{13:0}$, Sigma T0502) was used to determine the recovery of the SPE process. The amount of $C_{13:0}$ added is depended on the amount of FFA in the sample (see Table 1.5 for reference).

For milk powders, 2 g sample was added with 2 g oven-dried Na_2SO_4, 50 µl 2 mg/mL $C_{13:0}$, 10 mL diethyl ether (DEE)/heptane (1:1, v/v) mixture and 0.3 mL 5 M H_2SO_4. The mixture was vortexed for 1 min, then centrifuged at 10,000 x *g* for 5 min at 4°C. The upper layer containing lipids and FFA was carefully transferred into a 16 mL Kimax tube containing 1 g oven-dried Na_2SO_4. The lower layer was extracted two more times with 3 mL DEE/heptane. All upper layers were combined for SPE. If less

than 2 g of sample was used, solvents and other chemicals were reduced following the same proportion.

For milk samples, 10 mL sample was added with 0.3 mL 5 M H_2SO_4, 10 mL ethanol, 25 µl 2 mg/mL $C_{13:0}$, and 10 mL DEE/heptane (1:1, v/v). For an enzymatic reaction, the samples was reduced to 2 mLwith 0.3 mL 5 M H_2SO_4, 2 mL ethanol, 25 µl 2 mg/mL $C_{13:0}$ and 5 mL DEE/heptane (1:1, v/v). The extract was vortexed for 1 min, then centrifuged at 10,000 x *g* for 5 min at 4°C. The upper layer was transferred to a Kimax tube containing 2 g oven-dried Na_2SO_4.

2.4.2.2 Solid Phase Extraction (SPE) using Aminopropyl (NH_2) Column

A solid phase column contains Aminopropyl (a weak ion-exchanger) solid phase matrix was used for separating FFA from the mixture of the lipids and the FFA . The size of columns and the amount of solvent depend on the total amount of FFA in the sample. In general, the maximal loading capacity was 15 mg total FFA for a 200-mg column, and 30 mg for a 500-mg column. The 200-mg Isolute NH_2 columns (International Sorbent Technology, Mid Glamorgan, UK) were used for most of milk powder and milk samples.

The SPE columns were connected to a 16-port-Vacuum Manifold (AllTech) and pre-conditioned with 3.0 mL heptane. After loaded all the extract, the columns were washed with 1.5 mL chloroform/IPA (2:1, v/v) to remove all the lipids, then washed with 0.5 mL 6% HCOOH in DEE/heptane (1:2, v/v). The FFA were finally eluted from the N_2H column using 1 mL 6% HCOOH and kept directly into 2 mL glass vials, and

vacuum was applied to collect the residue FFA in the coulmn-void volume, and the samples were stored at -20°C prior for GLC analysis.

2.4.2.3 Gas Liquid Chromatograph (GLC)

FFA were separated using a 30 m x 0.53 mm i.d., 1.2 μm film thickness ET-1000 Econo-Cap polyethylene glycol ester capillary column (AllTech 19688) connected to a Shimadzu gas liquid chromatography GC-17A equipped with a AOC-20i auto-injector/auto-sampler fitted with a GC 15A liner (Shimadzu Corporation, Kyoto, Japan). Samples of 1 μL were injection onto the column at a flow of 6.3 mL/min helium under splitless mode for 1 min, then transferred to a split mode at 5:1 ratio for 39 min. The column was temperature-programmed from 100°C to 245°C at 10°C/min and held at 245°C for 24 min. The injector port temperature was 220°C; and the flame ionization detector temperature was 250°C with flows of 50 mL/min air and 500 mL/min hydrogen as detector gases.

To quantify the FFA in the sample, the column was calibrated with a standard mixture of eleven even-carbon-number fatty acids (see abbrevation for details) plus the internal standard $C_{13:0}$ prepared at 0.100 mg/mL each in 6% HCOOH in DEE/heptane (1:2, v/v). The standards are $C_{2:0}$, (ethanoic or acetic acid, Sigma 15177-7), $C_{4:0}$, (Sigma B10350-0), $C_{6:0}$, (Sigma C2250), $C_{8:0}$ (Sigma C2875), $C_{10:0}$ (Sigma C1875), $C_{12:0}$ (Sigma L4250), $C_{14:0}$ (Sigma M3253), $C_{16:0}$ (Sigma P5585), $C_{18:0}$ (Sigma S4751), $C_{18:1}$ (Sigma O1008) and $C_{18:2}$ (Sigma L1376).

2.4.3 Identification of Volatile Compounds

Volatile compounds derived from WMPs during storage were identified using GC/MS. The volatile compounds were first absorbed onto a poly-dimethylsiloxane divinylbenzene (PDMS/DVB) fibre (Supelco 5-7310, Supelco Inc., Bellefonte, PA, USA), then separated using gas chromatography and identified by mass spectrometry (GC/MS). This method was obtained from Ms. M. M. Hayes (personal communication).

2.4.3.1 Sample Preparation

Samples of 6 g each were sealed in 20-mL glass vials with parafilm, incubated at 37°C for 10 min, shaken for 30 s, then a 0.65 μm thickness partially crosslinked PDMS/DVB fibre was exposed into the headspace of sample for 20 min using a SPME holder (Supelco 57330-U).

2.4.3.2 GC/MS Analysis of Volatile Compounds

The fibre containing the volatile compounds was exposed at injection port for 30 s in a Shimadzu Gas Chromatograph GC-17A system using a 30 m x 0.32 mm id. x 0.25 μm film thickness EC-WAX (Carbowax AT-WAX) Econo-Cap polyethylene glycol capillary column (AllTech 19654). The volatile compounds were separated under a pressure of 54 kPa with a flow of 2.4 mL/min helium as the carrier gas in connected to a Shimadzu Mass Spectrometer QP-5000 system. The temperature gradient was from 50°C to 80°C at 3°C/min, then increased to 120°C at 5°C/min, finally to 220°C at 20°C/min and held at 220°C for 6 min. The injection port temperature was 220°C, and the interface temperature was 230°C.

Mass Spectrometry was carried out using electron impact ionization (EI) scan mode at 70 eV. Scanning rate was at 2 scans/s in a range from 40 to 350 M/Z (threshold 1000), while the detector gain was set at 1.25 kV. Volatile compounds were identified by matching mass spectra in the Japanese National Institute of Standard and Technology (NIST) Library Databases NIST62 and NIST12.

2.5 *Bacilli* Identification and Growth Media for Enzymes Production

2.5.1 Random Amplified Polymorphic DNA-PCR

Seven *Bacilli* were identified from milk powder production streams using the Random Amplified Polymorphic DNA-PCR (RAPD-PCR) technique (Morgan *et al.*, 1997; Ronimus *et al.*, 1997). These isolates were used for proteases and lipases production in order to obtain some characteristic information. The initial cultures were obtained from the Thermophile Research Unit Culture Collection (University of Waikato, New Zealand). After subculturing many times, the RAPD-PCR was used to confirm the cultures remained the same to the initial ones.

For extracting the DNA, a loopful of each culture was streaked onto a tryptic soy broth agar (TSB) plate and incubated for 24 h at 55°C. The cells were harvested from the TSB plate using ~ 5 mL 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl and 20 mM EDTA, and then centrifuged at 4,000 x *g* for 10 min. The cell pellet was resuspended in 1-4 mL sterile Milli-Q water (depended on the size of the cell pellet) and boiled for 10 min. The DNA was recovered by centrifugation at 4,000 x *g* for 10 min, and supernatant obtained. The DNA was quantified at 260 nm (1 absorbance at A260 nm = 50 μg/mL DNA).

RAPD-PCR was performed in a 25 μL-reaction mixture containing 37.5 ng DNA, 50 pmole primer OPR-13 (**GGACGACAAG**, Operon Technologies), 1.25 U *Ampli*Taq (Perkin Elmer), and 5 nmole dNTP (Boehinger Mannheim) in 10 mM Tris buffer containing 4 mM $MgCl_2$, 50 mM KCl and 0.001% gelatin (Ronimus *et al.*, 1997). The amplifying process was started at 94°C for 225 s, followed by 35 cycles of 94°C for 15 s, 36°C for 15 s and 72°C for 2 min sequentially, and completed with a step of 72°C for 4 min.

A 20 μL RAPD-PCR reactant was mixed with 5 μL gel loading buffer[#] and separated on 1.5% agarose gel in TBE buffer (Tris 54.0 g, boric acid 27.5 g, 20 mL 0.5 M EDTA to 1 L of deionised water, pH 8.0) using BRL Horizon^{TM} 20•25 system (Life Technologies Inc.) at a constant voltage of 120 V until the tracking dye reached the cationic side of the gel. The gel was stained with 0.5 μg/mL ethidium bromide for 45 min and destained in water for 45 min. DNA bands were detected under ultraviolet light.

2.5.2 Media for Enzyme Production from the Seven *Bacilli*

Five different media were used to check the production of proteases and lipases from the seven *Bacilli*. All media were autoclaved at 15 psi (121°C) for 15 min unless specified.

[#]Gel loading buffer contains 0.25% bromophenol blue and 40% (w/v) sucrose (Molecular Cloning – A Laboratory manual, 2nd edition, Sambrook, Fritschi and Maniatis).

2.5.2.1 Tryptic Soy Broth (TSB) and Agar media

Tryptic soy broth (30 g, DIFCO 0370-17-3) was dissolved in 1 L deionised water, then autoclaved. For plates, 12 g agar (DIFCO 0140-01) was added. The pH of the media was adjusted to 7.3.

2.5.2.2 C-medium

This is a medium adapted from Chopra and Mathur (1985). To make 1 L C-medium, 5 g tryptone (DIFCO 0123-17-3), 1.5 g yeast extract (BBL 4311929), 5 g NaCl, 1 g soluble starch and 2.5 g K_2HPO_4 were dissolved in 1 L deionised water, and pH was adjusted to 7.2, and autoclaved.

2.5.2.3 Reconstituted Skim Milk

Skim milk powder (NZDRI, Palmerston North, New Zealand) of 100 g was disolved in 900 mL of deionised water, and autoclaved at 10 psi (116°C) for 15 min.

2.5.2.4 B. brevis *medium*

This is a medium adapted from Amaki *et al.* (1992). To make 1 L *B. brevis* medium, 30 g polypeptone and 5 g yeast extract were dissolved in 970 mL deionised water. The pH of the medium was adjusted to 7.3 and autoclaved. Glucose of 10 g was in 30 mL deionised water, then filtered through a 0.22 μm sterile filter to the sterile medium.

2.5.2.5 Claudia medium

This a medium adapted from Schmidt-Dannert *et al.* (1997). To make 1 L Claudia medium, 3.25 g nutrient broth (DIFCO 0003-17-8) and 1 g $CaCl_2$ were dissolved in 980 mL deionised water, and pH was adjusted to 7.1. Olive oil (15 g) was mixed with 20

mL deionised water containing 10 g gum arabic, then added to the medium in droplets to form an emulsion by homogenising using the Ultra-Turrax, and autoclaved.

2.6 Whole Milk Powder Storage Trials

2.6.1 WMP Spiked with Enzymes from the Seven *Bacilli*

WMP batch number IH30 (Anchor Products, Morrinsville, New Zealand) was reconstituted to 40% (w/v) in deionised water. The reconstituted milk was mixed with a cocktail of the proteases and lipases produced by seven *Bacilli* (isolated from the milk powder production streams) under laboratory conditions. A Waring commercial blender (Watson Victor Ltd., Wellington, New Zealand) was used to blend the milk and enzyme mix at high speed continuously for 2 min, and then freeze-dried.

After freeze-drying, the paste was crushed to < 500 μm using a mortar and pestle, and a 500 μm sieve. The sample was tested for total moisture content using a Karl-Fisher titrator at Anchor Products Morrisville. The powder contained final concentrations of 0.45 FITC U/g protease and 0.01 *p*NP palmitate U/g lipase with a moisture of 1.73% (est. $a_w < 0.10$; Presicky, 1997).

The powder was packed in a foil bag with minimum headspace, along with the same batch of commercial WMP without any treatment at a moisture content of 2.75 % (est. a_w of 0.12), and stored at 37°C for 60 days. Samples were taken at 2-week intervals and stored at -20°C prior for further analysis.

2.6.2 WMP Spiked with Pure Thermophilic Enzymes

The same batch of WMP used in Section 2.6.1 was reconstituted to 1125 mL deionised water at 40% (w/v). To 375 mL, 21 U (*p*NP palmitate units) thermophilic lipase BTL-2 was added; to another 375 mL milk, 2800 U (FITC units) thermophilic AK1 protease was added. The third 375 mL milk without anything added was served as the control. All three milk samples were freeze-dried. After freeze-drying, the pastes were ground and sieved to give particles < 500 μm as described in Section 2.6.1.

Water activities (a_w; tested by Mr. A. Dodge, NZDRI) of samples were 0.08, 0.11 and 0.08, respectively, for the control, protease spiked and lipase spiked samples. In order to match the a_w levels in the freeze-dried samples with the level of normal commercial WMP (a_w ~ 0.22), the powders were stored in three separate desiccators over a saturated potassium acetate solution (a_w of 0.23 at 22°C) at room temperature in the dark for 4 days. The final a_w were 0.31, 0.34 and 0.24 for the control, protease spiked and lipase spiked samples, respectively. The samples were then stored in air-tight foil bags at 37°C for 60 days.

2.6.3 WMP spiked with A^m Lipase

The WMP used in Section 2.6.1 was reconstituted to 250 mL at 40% (w/v) in deionied water using a hand-blender by mixing continuously for 2 min, then divided into four portions of 62.5 mL. To three portions, crude A^m esterase was added at 0.5 U (#1), 1.5 U (#2) and 3.5 U (#3) (*p*NP palmitate units). The fourth portion (#4) was without any enzyme added. All four milk samples were then freeze-dried and ground to particles < 500 μm. A_w of samples were 0.36 (#1), 0.15 (#2), 0.11 (#3) and 0.28 (#4).

Samples of #1 and #4 were put to storage. Samples #2 and #3 were rehydrated in a desiccator containing saturated $MgCl_2$ at 37°C, for 2 days to give a_w of 0.30. All powders were stored in air-tight foil bags at 37°C for 60 days.

2.6.4 Commercial WMP Storage and Analysis

Five batches of commercial WMPs containing different levels of thermophiles were packed in air-tight foil bags with minimum headspace and stored at three different temperatures of room temperature (22°C), 37°C and 55°C. Approximately 100 g of each sample was taken at different time points over the storage period, and stored at -20°C prior to analysis. Analysis were: peptide release by RP-HPLC (Section 2.3.1), FFA release by GLC (Section 2.4.2), volatile compound release by GC/MS (Section 2.4.3), and lipase activity by a combination of Phenyl Sepharose extraction (see later section Section 4.3.5) and *p*NP assay (Section 2.1.2.4).

To determine changes of pHs in the powder, 1 g powder was dissolved in 8 mL 50°C Milli-Q water and cooled to room temperature for measurement.

Chapter 3 Assay Method Development: Detection of Protease and Lipase in Milk Powder

3.1 Introduction

One of the goals for this project was to develop simple and quick assay methods that can detect protease and lipase activities in WMP. Ultimately, these assays could be used in routine factory analysis to track enzyme levels during milk powder (MP) manufacture, allowing better assessment of product quality.

Historically, thermophiles numbers have been and still are used to monitor the microbial integrity in the milk powder processing. It has been set as one of the specifications for defining MP quality. Although thermophiles have been readily blamed for defects in MP during production and storage, there is little scientific evidence linking thermophile numbers with MP quality.

However, thermophiles as such do not affect MP quality, it is the enzymes produced by the thermophiles. MP production involves several heat steps of pasteurisation (72°C for 15 s), evaporation and spray drying, so only heat-stable enzymes will retain activity through out the process. Although many enzymes from mesophiles and psychrotrophs are heat-stable, enzymes from thermophiles are more robust and therefore are more likely to be a potential problem.

The most commonly found thermophiles in the MP process, such as *B. subtilis*, *B. licheniformis* and *B. stearothermophilus* not only are well-known spore formers (Harwood and Archibald, 1990), but also are good producers of extracellular proteases

and lipases (Chapra and Mathur, 1984 and 1985; Wood *et al.*, 1995). While thermophilic vegetative cells may die or sporulate during MP storage, it is unlikely that the enzymes will lose their activity – in fact the enzymes are usually more stable in low water content because they have restricted flexibility, therefore become more rigid and have a compact conformation that resist the denaturation process (Sumner and Myrback, 1950; Cowan and Daniel, 1982b; Bell *et al.*, 1995; Broos *et al.*, 1995; Cowan, 1997).

In order to explore the role of thermophile-derived proteases and lipases in MP quality, assays are needed to detect the enzyme levels in the MP. In this study, several thermophilic proteases and lipases were used to develop assays in a system manipulated under conditions similar to milk or in milk. They are (1) thermolysin (a typical metallo-protease, from *B. thermoprotelyticus rokko*, Sigma P1512); (2) Alcalase® (subtilisin A, from *B. licheniformis*); (3) AK1 (a serine protease, from a *Bacillus sp.*, a gift from Ms. H. S. Toogood, Thermophile Research Unit, University of Waikato, New Zealand); and (4) A^m lipase (an lipase, from *B. stearothermophilus* A^m, a gift from Dr. J. Bragger, Thermophile Research Unit, University of Waikato, New Zealand). The most sensitive assay was then applied to measure protease and lipase activities in reconstituted whole milk made from WMP.

3.2 Detection of Protease in Whole Milk Powders

3.2.1 Comparative Sensitivity of Common Protease Assay Methods

3.2.1.1 Casein-plate Assay

The casein-plate assay has been used for the detection of proteolytic bacteria for many years. It is simple assay and can be easily performed in a factory, therefore was selected. Alcalase® was used to determine the sensitivity of the assay under the

conditions modified from Cowan and Daniel (1982a). Each diluted enzyme solution of 50 μL (0-33.0 FITC U/mL) was applied in duplicate to wells in the casein-plate, and incubated at 40°C for 24 h (detailed in Section 2.1.1.1). The same set of enzyme solutions was also assayed for protease activity using the FTC-β-casein assay (detailed in Section 2.1.1.5). The assay results are presented in Figs. 3.1, 3.2 and 3.3.

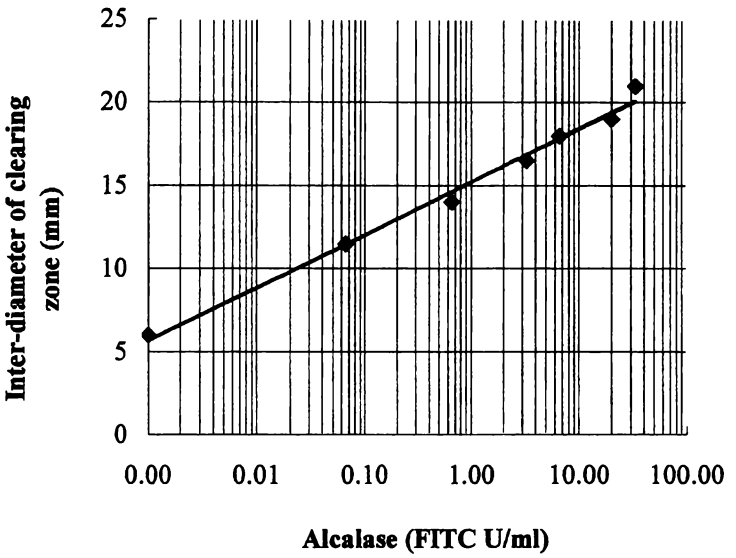


Figure 3.1: The relationship between the concentration of Alcalase® and the inter-diameter of clearing zone in a casein-plate. The assay was at 40°C for 24 h. Each data point represents an average of duplicate. The formula obtained is $y = 1.4\text{Ln}(x) + 15.2$ (R^2=0.99).

The increase in inter-diameter of the clearing zones in the casein-plate assay gave a linear logarithmic relationship to the amount of the Alcalase® in the sample (Fig. 3.1). Each sample required 24 h to obtain results with the casein-plate assay, but only 1 h with the fluorescent assay (Fig. 3.2).

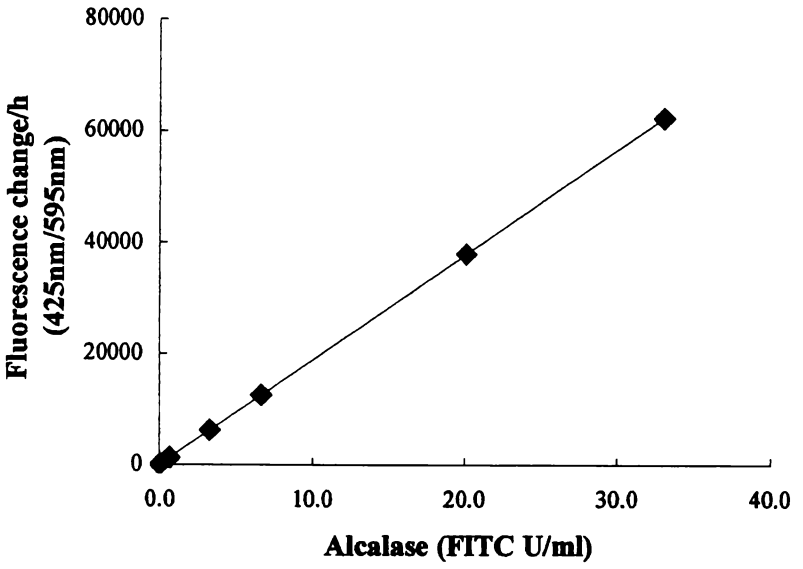


Figure 3.2: Rate of fluorescence release from FTC-β-casein by different concentrations of Alcalase®. The assay was in 0.1 M Hepes buffer, pH 7.0, at 40°C. Each data point represents an average of triplicate. The formula obtained is $y = 1893.3x - 0.2$ ($R^2 = 1.00$).

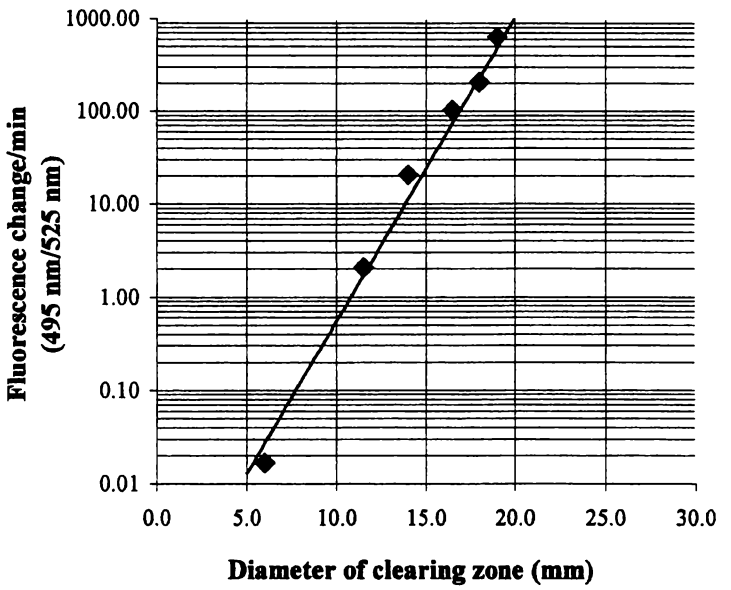


Figure 3.3: Correlation between the diameter of clearing zone in casein-plate assay and the fluorescence release in FTC-β-casein assay. Data points were obtained from Figs. 3.1 and 3.2. The formula obtained is $y = 0.0003e^{0.75x}$ ($R^2 = 0.98$).

Correlations between the casein-plate assay and the fluorescent assay were showed in Fig. 3.3. If a sample contains 0.7 U/mL protease, it will release 1325 arbitrary unit of FITC in 1 hour using the fluorescent assay, but only gives 0.6 mm clearing zone on the casein-plate in the same time, which is very difficult to measure. The minimum amount of protease required to detect appearance of para-κ-casein in the casein-plate is 100 FITC mU/mL using pure protease. Although it should be possible to raise the minimum detectable level of protease to 70 mU/mL using the method of Cowan and Daniel (1982a), it did not appear sensitive enough to warrant further study. On the other hand, if used to measure protease activity in a sample containing caseins, the sensitivity of the casein-plate assay will expect to be even lower.

3.2.1.2 Kunitz Assay

This is a method based on the phenolic groups of tyrosine, tryptophan and phenylalanine give maximum absorbance at 280 nm. The assay desinged to measure 15% TCA soluble protein fragments. It is a rapid assay and can be readily performed in a factory situation, so was selected for assessment.

WMPs were processed to give 10 mL 10% (w/v) skim milk following the method detailed in Section 2.1.2.1. To 5 mL each skim milk, 0.5 to 50.0 FITC mU/mL Alcalase® were added and incubated at 40°C. At the time ranging between 10 min to 3 h, 100 μL samples were removed and added to 1.4 mL chilled 15% TCA to stop the reaction. The samples were kept on ice for 10 min, then centrifuged at 10,000 x *g* for 10 min, and the absorbance of the supernatant read at 280 nm (modified method from Section 2.1.1.2). The results are presented in Fig. 3.4.

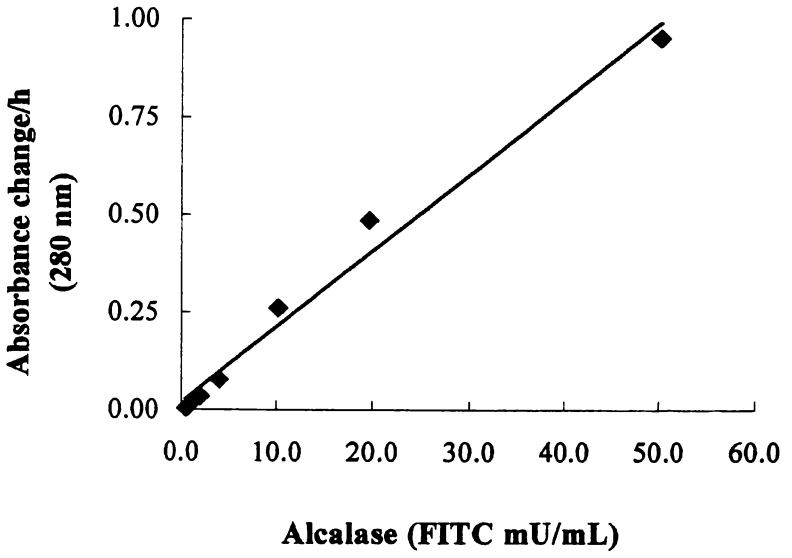


Figure 3.4: Rate of hydrolysis of 10% reconstituted skimmed milk by different concentrations of Alcalase® shown as Δ280 nm/h. Each data point represents an average of triplicate. The formula obtained is y = 0.02x + 0.02 (R^2 = 0.98).

The absorbance increase at 280 nm gave a linear relationship with the amount of Alcalase® present in the assay (Fig. 3.4). The assay detects the least level of protease equivalent to 1.0 FITC mU/mL in 3 h. The assay is 800 times more sensitive than the casein-plate assay. The advantage of the assay is that milk proteins do not interfere with the assay, but it will not detect any proteolysis break down products that do not contain 15% TCA soluble tyrosine, tryptophan and phenylalanine fragments.

3.2.1.3 Azocasein Assay

The azocasein assay is based on the release of coloured peptide from an azo-dye labelled proteins – in this case azocasein. It is a simple method and can be conveniently measured using a spectrophotometer in a dairy factory. Thermolysin of 0-3.5 FITC mU/mLwas used to determine the sensitivity of the assay. The assay was carried out

using azocasein as the substrate in triplicate in 0.1 M Hepes buffer, pH 7.2, 40°C for 6.5 h (detailed in Section 2.1.1.3). The results are presented in Fig. 3.5.

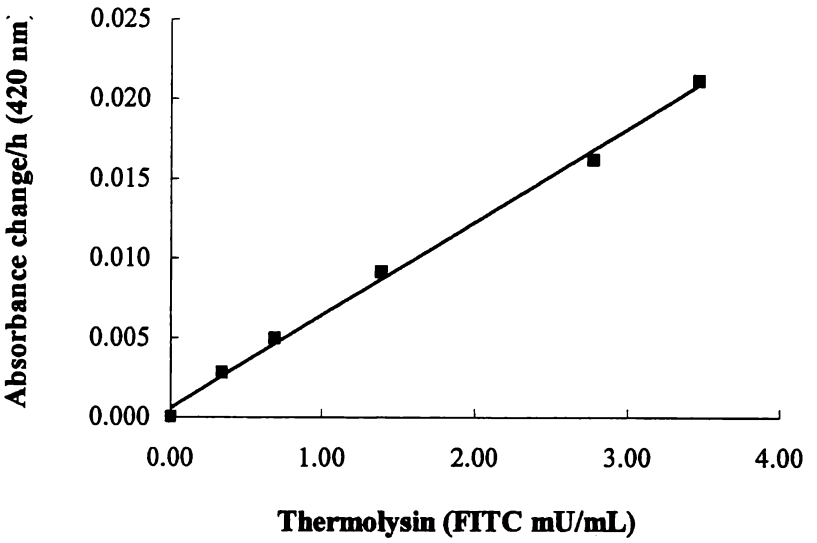


Figure 3.5: Rate of azocasein hydrolysis by different concentrations of thermolysin shown as Δ420 nm/h. Each data point represents an average of triplicate. The formula obtained is $y = 0.006x + 0.001$ ($R^2 = 1.00$).

The increase in absorbance at 420 nm gave a linear relationship to the concentration of thermolysin (Fig. 3.5). It can detect a minimum level of 0.35 FITC mU/mL protease in 6.5 h using a pure protease. The assay is 77 times more sensitive than the casein-plate assay, and nearly the same sensitivity as the Kunitz assay. On the other hand, the sample was pure protease solution and did not contain any milk proteins, so when assay milk samples, the actual sensitivity will expect to decrease.

3.2.1.4 Assay using p-*Nitroanilide Derivatives*

Peptide substrate specificities of proteases were reviewed by Daniel *et al.* (1995). N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Suc-AAPF-*p*NA) was reported to be readily cleaved by *Bacillus* proteases (Toogood, 1998). Alcalase® is a typical serine protease

from *B. licheniformis* and commercially available, therefore was selected for the assay. The Alcalase® was diluted to 0.01 to 0.1 FITC mU/mL according to the fluorescent assay result, then assayed in duplicate for activity in 0.1 M Hepes buffer, pH 7.0, at 40°C, for 10 min to 9 h using Suc-AAPF-*p*NA as the substrate, with and without addition of 0.78% β-casein (method detaled in Section 2.1.1.4). The results are presented in Fig. 3.6.

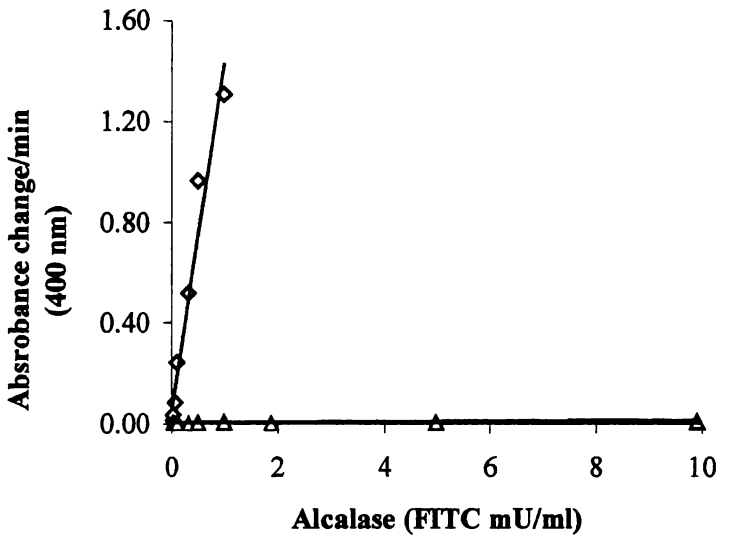


Figure 3.6: Rate of *p*-nitroaniline release from Suc-AAPF-*p*NA by different concentrations of Alcalase® shown as Δ400 nm/min. Assay was in 0.1 M Hepes buffer, pH 7.0, at 40°C. Each data point represents an average of duplicate. (◊) 1 mM Suc-AAPF-*p*NA only; (Δ) 1 mM Suc-AAPF-*p*NA with 0.78% β–casein (Sigma C6905). The formulae obtained are for (◊) $y = 1.38x + 0.07$ ($R^2 = 0.95$); and (Δ) $y = 0.001x + 0.001$ ($R^2 = 0.82$).

Data in Fig. 3.6 showed a linear relationship between the rate of absorbance increase and the concentration of Alcalase®. The assay can detect a minimum level of protease equivalent to 0.1 FITC mU/mL in 9 h in a system contained 0.78% β-casein. It is 2,660

times sensitive than the casein-plate, and 3 times sensitive than the Kunits and the azocasein assays.

However, the assay substrate is very specific which is cleaved only by certain types of proteases, so it does not detect all protease activity in the sample. This is later highlighted by the results given in Section 5.4.3 that the F/G protease, produced by F/G *B. licheniformis* (isolated from a milk powder production stream) did not cleave Suc-AAPF-*p*NA.

3.2.1.5 Fluorescent Assay

The fluorescent assay is a method using fluorescein isothiocyanate (FITC)-labelled casein to assay protease activity. It is a very sensitive assay and can detect nanogram and subnanogram amounts of proteases, such as subtilisin and thermolysin (Twining, 1984). It was therefore chosen to define the sensitivity in an assay containing milk caseins. Alcalase®, protease AK1 and thermolysin were assayed in triplicate in two assay systems (method detailed in Section 2.1.1.5): one system contained only FTC-β-casein; the other comprised a mixture of FTC-β-casein with either 1.67% milk powder (estimated protein content of 0.5%) or 0.5% α-casein and 0.5% β-casein. The milk powder used was free of protease, as determined using the same method (personal communication, Dr. T. Coolbear). The results are presented in Figures 3.7, 3.8 and 3.9.

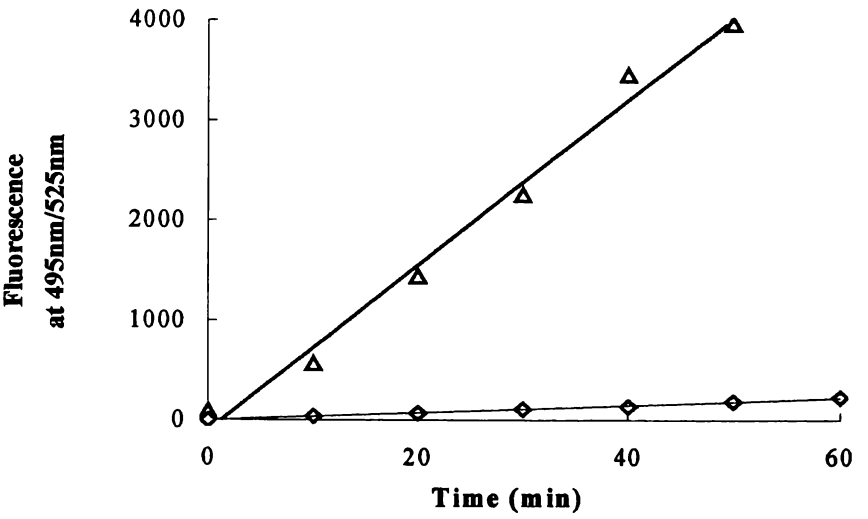


Figure 3.7: Release of fluorescence from FTC-β-casein over time by Alcalase® (46.2 FITC mU/mL). Assay was in 0.1 M Hepes buffer, pH 7.0 at 40°C: (Δ) using 0.33% FTC-β-casein only; (◊) using 0.33% FTC-β-casein with 1.67% milk powder (estimated protein content of 0.5%) with no detectable protease activity. Each data point represents an average of triplicate.

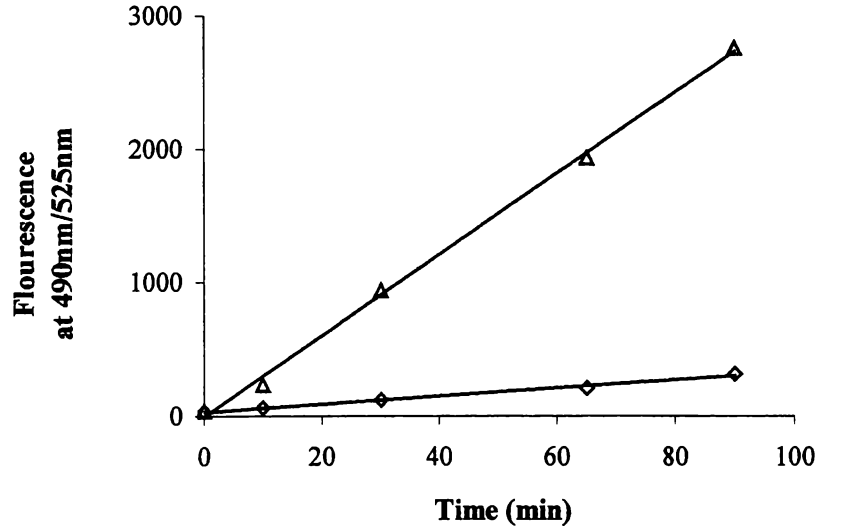


Figure 3.8: Release of fluorescence from FTC-β-casein over time by protease AK1 (18.1 FITC mU/mL). Assay was in 0.1 M Hepes buffer, pH 7.0 at 40°C: (Δ) using 0.33% FTC-β-casein; (◊) using 0.33% FTC-β-casein with 0.5% α-casein (Sigma C6780) and 0.5% β-casein (Sigma C6905). Each data point represents an average of triplicate.

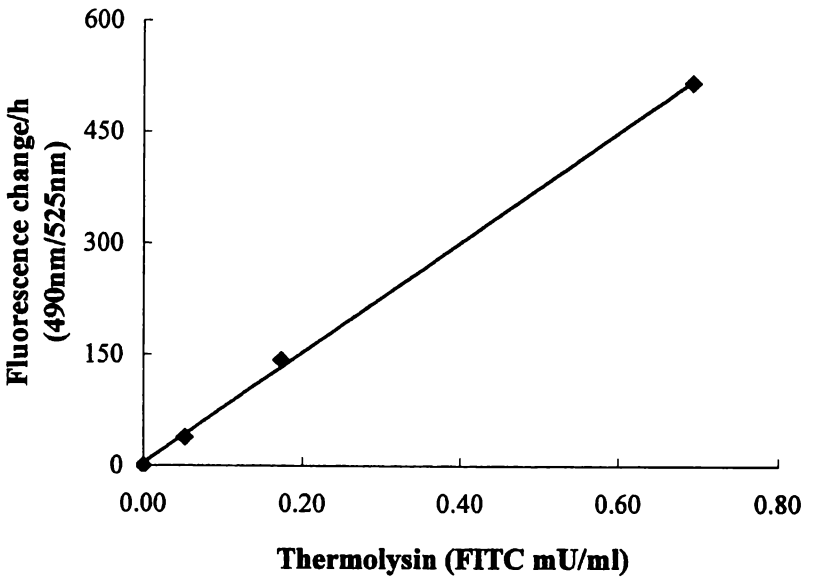


Figure 3.9: Rate of hydrolysis of a mixture of 0.33% FTC-β-casein and 0.45% β-casein (Sigma C6905) by different concentrations of thermolysin (Sigma P 1512). Assay was in 0.1 M Hepes buffer, pH 7.0 at 40°C. The β-casein gives an estimated casein content equivalent to whole milk powder reconstituted to 2% (w/v). The formula obtained is y = 747.4x + 3.8 (R^2 = 1.00). Each data point represents an average of triplicate.

FITC release was linear in two assay systems: FTC-β-casein alone and FTC-β-casein plus 1.67% milk powder or 0.5-1.0% casein (Fig. 3.7 to 3.9). The rate of FITC release was 10 to 20 times less in the assay contained both the FTC-β-casein and the milk proteins than the one contained only FTC-β-casein, but the total protein concentration was only increased 2-3 fold (comparing the slopes of the lines in Fig. 3.7 and in Fig. 3.8). This indicated that the protease acted preferentially on the milk proteins rather than the FTC-β-casein. Therefore, when assaying protease activity in milk powder samples, the presence of milk proteins will markedly reduce the sensitivity of the assay. Despite this, the assay can still detect protease activity at < 0.1 mU/mL (one unit is defined as the release of 1 μmole/min FITC under the assay conditions) in a system

containing FTC-β-casein and the caseins (Fig. 3.9). At this level, results can be obtained after 23 h.

The assay is 1,000 times more sensitive than the casein-plate assay. Furthermore, the assay can be quantitative relative to pure proteinase, as the reduction in apparent activity due to the caseins' interference from the milk powder can be calculated.

3.2.1.6 SDS-PAGE

SDS-PAGE is a method that separates proteins based on their charge density in an electric field. Some of samples taken in Section 3.2.1.2 were analysed on 16.5% SDS gel. The samples were diluted 20 times with deionised water. To 10 μL each diluted sample, 2.5 μL 5 x sample buffer was added, then boiled for 5 min and electrophoresed. The gels were silver stained afterwards (methods detailed in Section 2.3.2). The results are presented in Fig. 3.10 and Table 3.1.

Table 3.1: *Protein density on SDS-PAGE (data from Fig. 3.10)*

Protein ID.	R_f[1]	Relative density[2](%) with Alcalase® at			
		0.5 mU/mL	1.0 mU/mL	2.0 mU/mL	4.0 mU/mL
Band 1	0.1	73	110	35	4
Band 2	0.4	271	207	181	0
Band 3	0.5	63	85	83	11
Band 4	0.7	144	122	211	85
Band 5	0.8	163	135	149	52
Band 6	0.9	273	206	344	131

[1]Distance of migration relative to the dye front.

[2]Density of each band was measured using BioRad Fluor-S Multilmager system. The density at zero time was taken as 100%. Sample incubated at 40°C for 3 h with 0.5-2.0 mU/mL Alcalase®; and 1.5 h with 4.0 mU/mL Alcalase®.

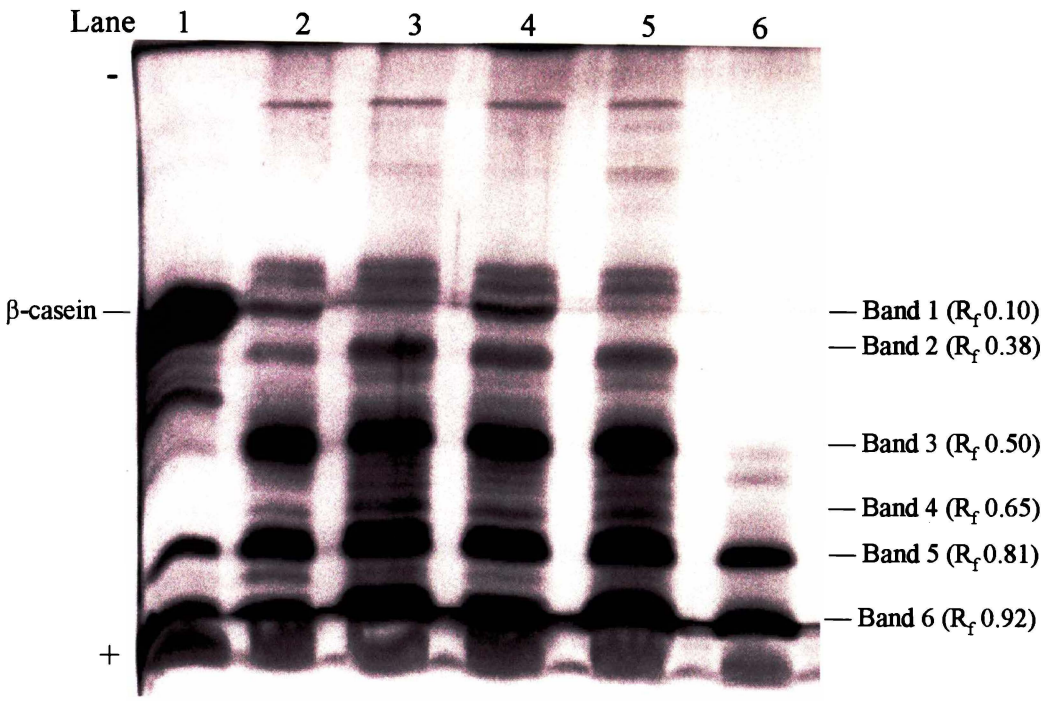


Figure 3.10: Protein fragments on 16.5% SDS-PAGE after silver staining. Samples were 10% skim milk incubated with various concentrations of Alcalase® at 40°C. Lane 1: β-casein (Sigma C6905); lane 2: 10% skim milk at time zero; lanes 3, 4 and 5: incubated for 3 h with 0.5, 1.0 and 2.0 FITC mU/ml Alcalase®, respectively; lane 6: incubated for 1.5 h with 4.0 FITC mU/ml Alcalase®.

Large protein bands decreased in intensity, and ultimately disappeared over time, while some small protein bands became denser and some began to appear (Fig. 3.10), especially the protein fragments at R_f between 0.50-0.65. Quantitative values were given in Table 3.1 expressed as the percentage of the protein density relative to zero time. For example, protein band 1 (at R_f 0.10) migrated the same distance as the β-casein became denser first (lane 4 in Fig. 3.10), and then completely disappeared only after incubation of 1.5 h at 40°C with 4.0 FITC mU/mL Alcalase® (lane 6 in Fig. 3.10). The least estimated level of protease detected on SDS-PAGE is equivalent to 0.5 FITC

mU/mL. The method is very difficult to obtain exact quantitative value in relationship to the protease concentration, but does not face the problem of milk protein interference.

3.2.1.7 RP-HPLC

Reverse phase high performance liquid chromatography (RP-HPLC) separates polypeptides based on their hydrophobicity using a C_{18} reverse phase column. Samples obtained in Section 3.2.1.2 were analysed for peptide production using this method. To 0.5 mL each sample, 0.25 mL 3% TFA was added, then centrifuged at 10,000 x *g* for 10 min at 4°C. Supernatant obtained by filtering through a 0.45 μm filter, and then subjected to peptide analysis using RP-HPLC (method detailed in Section 2.3.1). The results are presented in Fig. 3.11.

The RP-HPLC chromatograms showed that peak areas increased with increasing Alcalase® concentration and with less incubation time as proteolysis proceeded. With the highest protease concentration of 4 mU/ml, proteolysis resulted in further hydrolysis of large 1% TFA soluble fragment at retention time between 25-40 min (Fig. 3.11). The changes were in proportion to the Alcalase® concentration. The assay does not face the problem of milk protein interference, but it is difficult to quantify because of the difficulties of finding the standards.

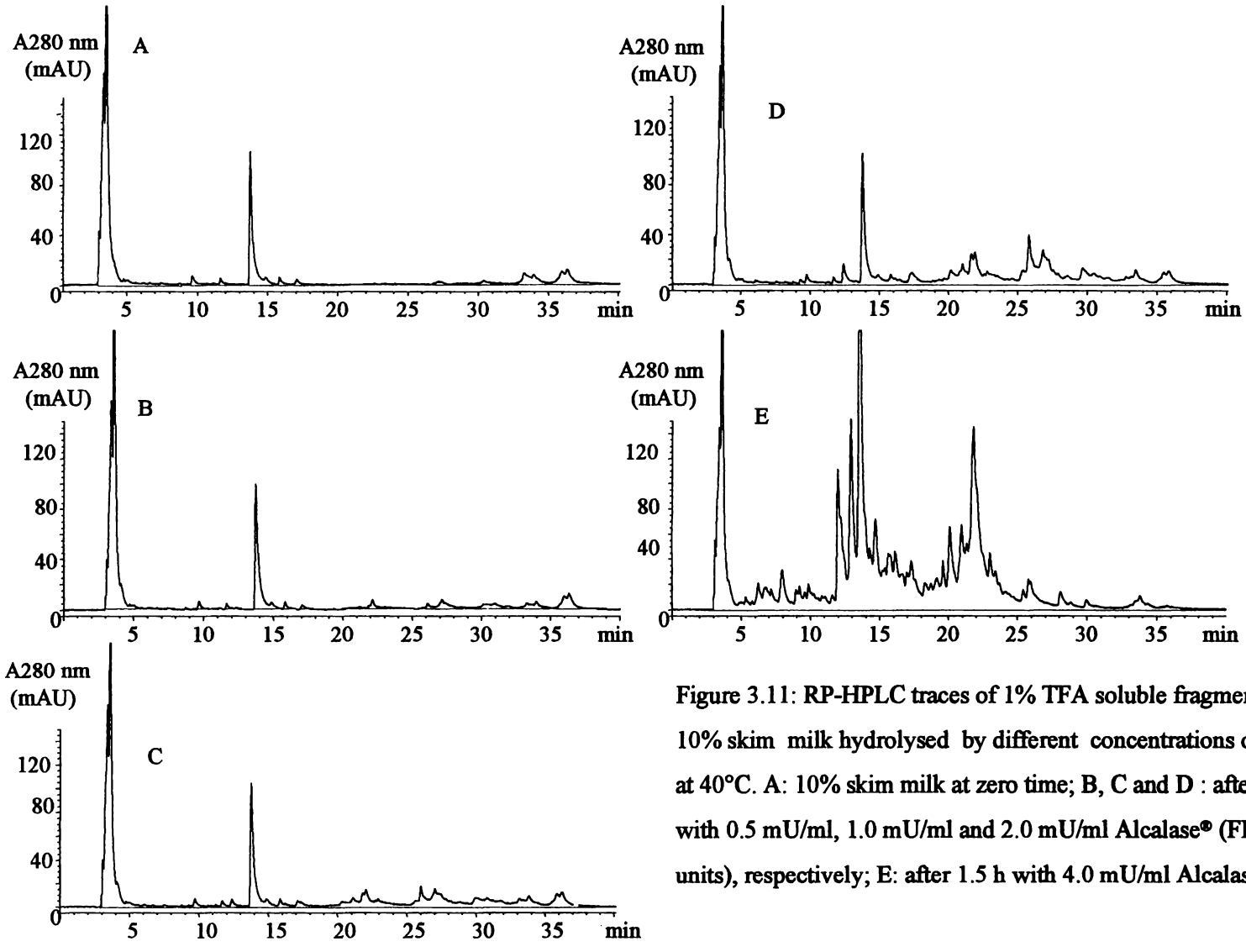


Figure 3.11: RP-HPLC traces of 1% TFA soluble fragments from 10% skim milk hydrolysed by different concentrations of Alcalase® at 40°C. A: 10% skim milk at zero time; B, C and D : after 3 h with 0.5 mU/ml, 1.0 mU/ml and 2.0 mU/ml Alcalase® (FITC units), respectively; E: after 1.5 h with 4.0 mU/ml Alcalase®.

3.2.2 Summary of Protease Assay Methods

The protease assay methods performed in Section 3.2.1 are summarised in Table 3.2.

Table 3.2: Comparison of protease assay methods

Assay Method	Substrate concentration	Assay time and temp.	Minimum detectable protease[1](mU/mL)	Changes (arbitrary unit/h)
Casein-plate	1%	24 h, 40°C	100	0.5
Kunitz	2.7%	3 h, 40°C	1.0	0.04
Azocasein	0.05%	6.5 h, 55°C	0.35	0.003
***p*NA derivative**	1.1%	9 h, 40°C	0.5	0.09
Fluorescent	0.83%	23 h, 40°C	0.1	78.5
SDS-PAGE	2.7%	3 h, 40°C	0.5	Not quantified
RP-HPLC	2.7%	3 h, 40°C	0.5	Not quantified

[1]One unit of protease was defined as the release of 1 μmole/min FITC under the assay conditions (method detailed in Section 2.1.1.5). Other assay results were calculated to FITC units using the data obtained in Section 3.2.1.

The fluorescent assay seems the most sensitive quantitative assay for detection of commercial proteases in a milk system, followed by the *p*NA derivative-assay, and the Kunitz assay or the azocasein assay. This is also proved by the data obtained on A^m protease in later section 5.4.4 that FTC-β-casein gave the highest ratio between V_{max} and K_m. The casein-plate was 1,000 times less sensitive than the fluorescent assay. The SDS-PAGE and RP-HPLC methods are very sensitive, but was not quantified in this occasion. Azocasein method seemed to have a potential to increase substrate concentration in order to improve the sensitivity since it gave the lowest K_m value (see later Section 5.4.4). However, if the substrate concentration is 1000 x K_m, the velocity can only increase about 2-fold.

All the assay methods have advantages and disadvantages. Although the fluorescent assay was the most sensitive assay, it requires a fluorimeter that is not normally possessed by a factory. The *p*NP assay has limitations with respect to assay temperatures and substrate selectivity. If a protease does not have this specificity, it will not be detected.

The azocasein and Kunitz assays can be performed readily using a spectrophotometer which is commonly found in dairy factories. However, these assays are less sensitive in comparison with the fluorescent and *p*NA assays, and may not detect the low levels of protease activity in WMPs. The SDS-PAGE and RP-HPLC assays are moderately sensitive, but cannot easily give quantitative results, and require special equipment. The advantage of these two assay methods is that they show patterns of the milk proteins breakdown, which could be correlated with functionality changes in milk powders.

3.2.3 Application of Protease Assays to Whole Milk Powders

3.2.3.1 Casein-plate Assay

Five batches of WMPs were processed to 20 mL 10% (w/v) skim milk (Section 2.1.2.1). Samples of 50 μL were assayed for protease activity using the casein-plate assay (details in Section 2.1.1.1).

No clearing zone was formed in any of the samples tested, even after incubation for 72 h at 55°C (data not shown). The method is too insensitive to detect any protease activity in the WMPs.

3.2.3.2 Kunitz Assay

Five batches of WMPs were made to 10 mL skim milk each following the method described in Section 2.1.2.1, and each sample was divided into three portions of 5 mL. The first 5 mL was treated with 1 mg Penicillin G; the second 5 mL was treated with the same amount of Penicillin G and 8 mg of the Complete™ protease inhibitor cocktail tablet at room temperature for at least 30 min. The third 5 mL was autoclaved at 121°C for 30 min. All samples were then assayed for protease activity using the Kunitz assay at 40°C and 60°C for up to 100 h (Section 2.1.1.2). A loopful of sample at every sampling point was streaked on TSB plate and incubated at 55°C for 24 h to check for any bacterial growth.

Three out of five samples treated with Penicillin G showed 0.003-0.010 increase in absorbance unit at 280 nm per 24 h at 40°C. No changes were detected in the sample treated with both Penicillin G and the protease inhibitor cocktail. No changes were detected at 60°C. No bacterial growth was detected in any of samples on TSB plates. However these minute changed detected here were probably within experimental error and in practice, the Kunitz assay can not detect any protease activity in the WMPs.

3.2.3.3 Assay using Suc-AAPF-pNA

WMP batch GD03 was processed to 200 mL 2.5% (w/v) skim milk following the method described in Section 2.1.2.1. One lot of 100 mL was ultrafiltered through a 100 kDa cut-off membrane (YM100, Amicon Inc., Berverly, MA, US). The other 100 mL was first passed through a 0.1 μm hollow-fibre filter (Amicon), then ultrafiltered with a 3 kDa cut-off membrane (Amicon YM3). Protease activity was assayed using Suc-AAPF-*p*NA in 0.1 M Hepes buffer, pH 7.0, containing 1% azide, at 40°C for 48 h

(method detailed in Section 2.1.1.4). Samples of 1 mL each were also plated on TSB plates and incubated at 40°C for 24 h to check for any bacterial growth.

Release of *p*NA was detected in all three fractions: YM100 permeate, YM100 retentate and Hollow fibre plus YM3 retentate. However, the absorbance changes were extremely low: 0.0004-0.0016 increase absorbance per h at 400 nm . The calculated level of protease were 0.6 FITC mU/mL and less using the formula obtained in Fig. 3.6 ($y = 0.001x + 0.001$). No bacterial growth was detected in all the samples. The release of *p*NA was likely to be the result of proteolysis.

One point to note is that the two different filtration steps (using a 100 kDa membrane or a 0.1 μm hollow fibre followed by a 3 kDa membrane) did not make any difference in terms of detecting protease activity, and more than 85% of the absorbance increase was detected in the YM 100 retentate. This suggested that the majority of protease activity was associated with a high molecular mass component. This could possibly be due to an unusually large proteinase, a multimeric enzyme or an association of the enzyme with substrate – *i.e.* with casein aggregates. The last explanation is highly likely because milk caseins are very hydrophobic and often form aggregates amongst themselves and with other milk components (Walstra *et al.*, 1999).

The assay has detected very low level of protease activity in WMPs. But the practical application of the assay method is limited in a factory situation because of the cost and limitation of the substrate.

3.2.3.4 Fluorescent Assay

Several batches of WMPs were processed to 10 mL of 2% skim milks first (method detailed in Section 2.1.2.1). One set of 5 mL each was treated with 1 mg Penicillin G only; the other set was treated with the same amount of Penicillin G and 8 mg Complete™ protease inhibitor cocktail tablet. All milk samples were then assayed for protease activity in 0.1 M Hepes buffer, pH 7.0, at 40°C for up to 72 h using the fluorescent assay (Section 2.1.1.5). Both FTC-α-casein and FTC-β-casein were used as substrates. The milk powder with no detectable protease activity (Section 3.2.1.5) was used as the control. Samples were also streaked on TSB plates and incubated at 40°C for 24 h to check for bacterial growth.

There were 0.03-0.12 nmole/h/mL FITC produced in the samples treated with Penicillin only, but none in the samples treated with both the Penicillin G and the protease inhibitors cocktail. The minute changes were possibly a result of proteolysis by the proteases because no bacterial growth was detected at any sampling points on TSB plates. However, the actual level of protease activity is < 0.1 mU/mL thermolysin equivalent using the formula obtained in Fig. 3.9.

One of the samples, batch GD03, was also assayed at 25°C, 40°C, 55°C and 70°C for 48 h in the same assay system using either FTC-β-casein or FTC-α-casein as the substrate. No release of fluorescence was detected. Therefore, in practical terms although the assay is the most sensitive one, it still could not detect the level of protease activity in the WMPs due to milk native substrate (caseins) competition (see Section 3.2.1.5).

The question still remains as to whether a more sensitive assay is actually required –this is dependent on whether such low levels of protease in the powders have any

detrimental effect on MP quality, *i.e.* on functionality or flavour. Therefore, other methods for assaying protease activity in the WMP are investigated further in the following sections.

3.2.3.5 SDS-PAGE

The skim milk samples obtained in Section 3.2.3.2 were also analysed for protease activity using SDS-PAGE. Samples of 20 μL each were diluted 20 fold with deionised water. To each 10 μL diluted sample, 2.5 μL 5 x sample buffer was added, boiled for 5 min, then separated on 16.5% SDS-PAGE (Section 2.3.2). The gels were silver-stained to identify any milk protein breakdown fragments due to proteolysis. The results are presented in Fig. 3.12.

Changes in densities of protein bands (R_f of 0.50 and 0.79) were detected in three reconstituted skim milks (made from batches IH30, II12 and DI05) treated only with Penicillin G only after incubation of 100 h at 40°C. The changes were obvious in sample DI05: disappearance of protein band at R_f 0.50 and appearance of protein bands at R_f between 0.64 and 0.90. No changes were detected in the samples treated with Penicillin G as well as the Complete™ protease inhibitor cocktail at either 40°C (Fig. 3.12) or 60°C (data not shown).

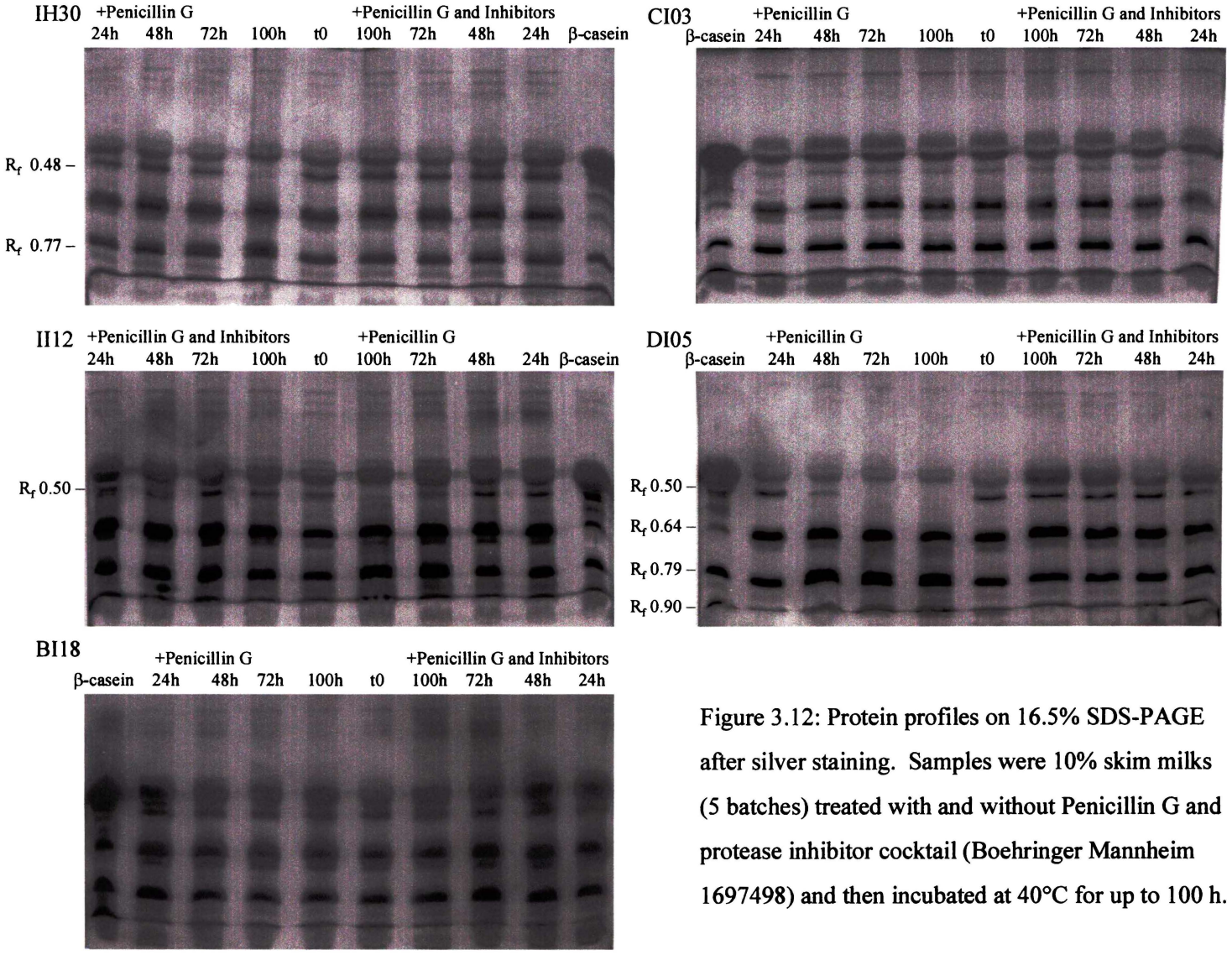


Figure 3.12: Protein profiles on 16.5% SDS-PAGE after silver staining. Samples were 10% skim milks (5 batches) treated with and without Penicillin G and protease inhibitor cocktail (Boehringer Mannheim 1697498) and then incubated at 40°C for up to 100 h.

The results confirmed the findings in Sections 3.2.3.3 and 3.2.3.4 that protease activity was present in the WMPs, which was successfully prevented by the protease inhibitor cocktail. No change was detected in the autoclaved samples (data not shown). This indicated that perhaps sterilisation of 121°C for 30 min was effective to prevent further proteolysis in the WMPs. If there is any remaining protease activity, it may not be detected using the method. Although the SDS-PAGE method was difficult to give quantitative results because of finding the right standards, it confirmed the protease activity presence in WMPs. The assay also has advantage of being able to use higher assay temperatures and does not have the problem of interference from milk proteins since they are the actual substrates in the assay. However, if assay at temperature above 60°C, it could face problem of antibiotic instability.

3.2.2.6 RP-HPLC

Samples obtained in Section 3.2.3.2 were also analysed for peptide production by RP-HPLC. Samples of 0.5 mL each were treated with 0.25 mL 3% TFA, centrifuged at 10,000 x *g* for 10 min at 4°C, and supernatants were obtained. The supernatants of 100 µL each were analysed using the RP-HPLC method (Section 2.3.1). The results from the samples made of WMP II12 and DI05 are presented in Figs. 3.13 and 3.14, and the rest were not shown because they were identical to Figs. 3.13 and 3.14.

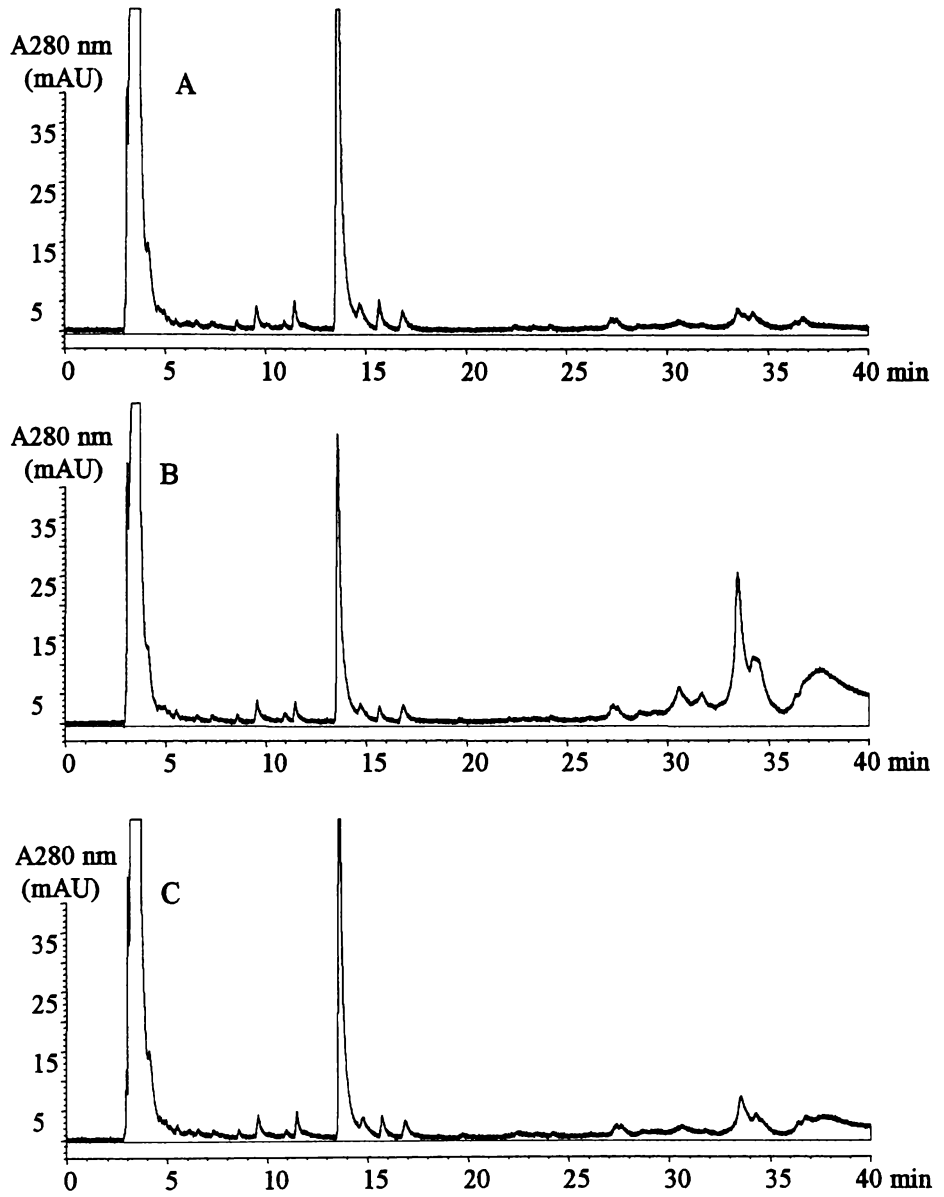


Figure 3.13: RP-HPLC traces of 1% TFA soluble fragments from 10% skim milk made from WMP batch II12. A: sample t0; B: sample treated with Penicillin G and incubated at 40°C for 97.5 h; C: sample treated with Penicillin G and Complete™ protease inhibitor cocktail, then incubated at 40°C for 97.5 h.

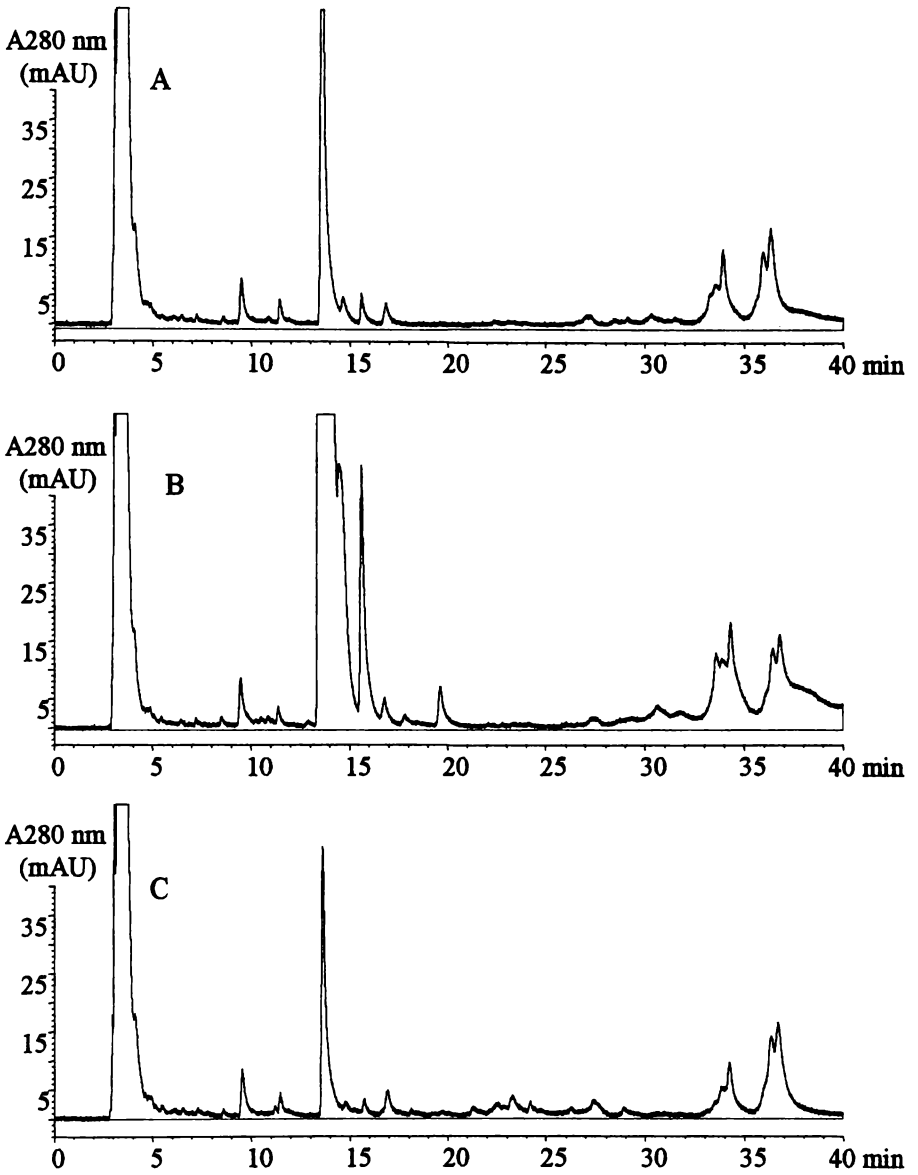


Figure 3.14: RP-HPLC traces of 1% TFA soluble fragments from 10% skim milk made from WMP batch DI05. A: sample t0; B: sample treated with Penicillin G and incubated at 40°C for 72 h; C: sample treated with Penicillin G and Complete™ protease inhibitor cocktail, then incubated at 40°C for 72 h.

Increases in peak areas of 1% TFA soluble fragments (at retention times between 27 and 40 min) were detected in all samples treated only with Penicillin G (shown as examples in Figs. 3.13 and 3.14). No change was detected in the samples treated with both Penicillin G and the protease inhibitor cocktail except sample DI05 after 100 h. It is possible that the antibiotic was no long effective to prevent the bacterial growth.

The results confirmed the finding in Sections 3.2.3.3, 3.2.3.4 and 3.2.3.5 that protease activity was present in the WMPs. For example, the reconstituted milk DI05 showed a huge increase in peptide peak areas at retention time between 13 and 16 min on RP-HPLC after 72 h (Fig. 3.14b), and in parallel a completely disappearances of protein bands at R_f of ~ 0.50 and appearances of smaller protein bands at R_f between 0.60 and 0.90 on SDS-PAGE (Fig. 3.12). The peptide peaks could be identified using automated N-terminal sequence analysis and mass spectrometry, but they were not identified in this occasion, so could not be correlated to protein bands shown on SDS-PAGE.

3.2.4 Summary of Protease Assay in Whole Milk Powders

Six assay methods were applied for the determination of protease activity in the commercial WMPs. Protease activity was shown by the SDS-PAGE and RP-HPLC methods. However, the most sensitive fluorescent assay could not give a quantitative value. The inconsistence in assay results and lack of sensitivity are caused by milk proteins (mainly caseins) interference, as has been reported in several protease assays in milk (Rollema *et al.*, 1983; Dupont *et al.*, 1997).

The advantages and disadvantages of each assay were discussed in details in Section 3.2.2. However, there is not an assay that could determine the actual level of protease activity in whole milk powders. Question of whether the present level of protease

activity will affect milk powder storage properties will be investigated further in later Chapters 6 and 7.

3.3 Detection of Lipase Activity in Whole Milk Powders

3.3.1 Comparative Sensitivity of Common Lipase Assay Methods

3.3.1.1 Tricaproin-plate Assay

The tricapoin-plates was described in Section 2.1.2.2. Based on the *p*NP caproate results in later Section 3.3.1.2, the A^m lipase (from *B. stearothermophilus* A^m isolated from a milk powder process stream) was diluted to 0.01-7 U/mL in 0.1 M Mops buffer, pH 7.6, containing 5 mM $CaCl_2$ and 0.1% Triton X-100. Each sample (50 μL) was applied in duplicate to wells in the tricaproin-plate. The plates were incubated at 55°C for 24 h. Formation of the clearing zones were measured. The results are presented in Fig. 3.15.

The tricaproin-plate assay gave a linear logarithmic relationship between the amount of the A^m lipase and the inter-diameter of the clearing zone (Fig. 3.15). It can detect a minimum level of 433 *p*NP mU/mL lipase. Part of the problem is that the plates cannot be incubated at above 55°C because of the agar melting temperature. This could possibly be overcome using Gelrite with which can be incubated at 70°C. However, it only increases sensitivity by 3-fold (personal communication, Dr. T. Coolbear).

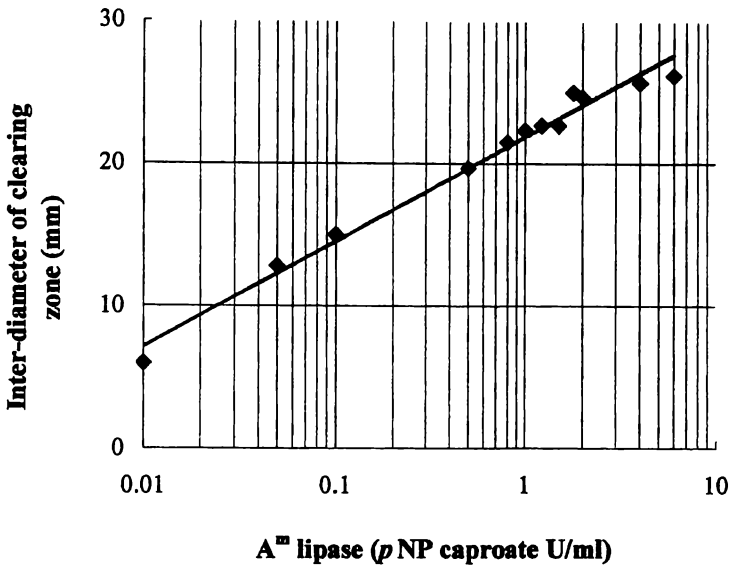


Figure 3.15: The relationship between the concentration of A^m lipase and the diameter of the clearing zone in a tricaproin-plate. The assay was at 55°C for 24 h. Each data point represents an average of duplicate. The formula obtained is $y = 3.2Ln(x) + 21.9$ ($R^2 = 0.98$).

3.3.1.2 Assay using p-*Nitrophenyl Esters of Fatty Acids*

The method was detailed in Section 2.1.2.4. Information obtained in later chapters indicated that both the lipase activity (subsequently found in the WMP) and the lipases produced by the seven *Bacilli* (isolated in milk powder production streams) have specificities on shorter chain *p*NP esters of fatty acids over longer chain ones (see later Sections 4.5.4 and 5.5.4). Therefore *p*NP caproate ($C_{6:0}$) was selected as the substrate, and A^m lipase was used to as the control.

Proteases such as Alcalase® (from *B. licheniformis*) and plasmin, are commonly known to have esterase activities, therefore were checked for activity against *p*NP $C_{6:0}$. As the results described in the later Sections 4.2.1 and 4.5.1 suggested that the lipase activity in WMPs is association with milk caseins, so several commercial preparation of caseins were also checked for esterase activity. This was to find a negative control.

Different caseins were dissolved at 30 mg/mL in 0.1 M Mops buffer, pH 7.6, containing 0.1% Triton X-100. All samples were assayed for lipase activity using the method detailed in Section 2.1.2.4. Commercial protease were also assayed using FTC-β-casein as the substrate at 40°C for 4 h (method detailed in Section 2.1.1.5). No protease activity was detected in any of the casein samples. The lipase assay results are presented in Fig. 3.16 and Table 3.3.

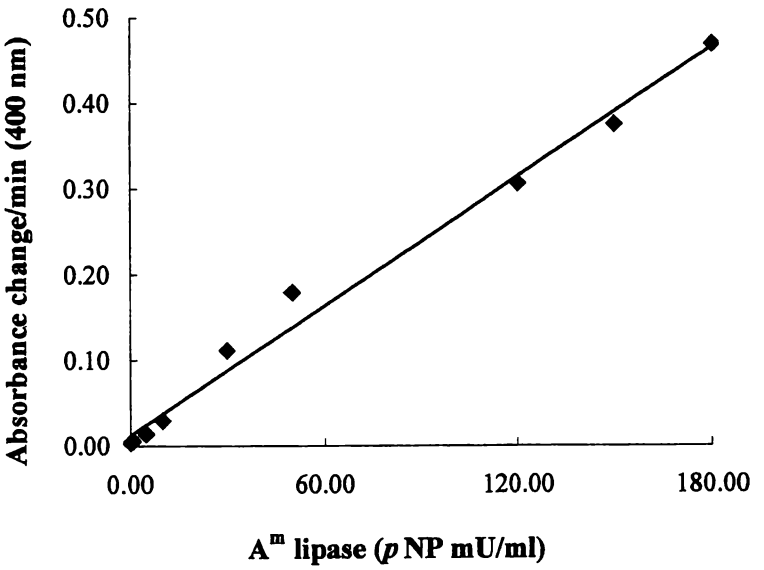


Figure 3.16: Rate of *p*-nitrophenol release from *p*-nitrophenyl caproate by different concentrations of A^m lipase shown as Δ400 nm/min. Assay was in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ and 0.1% Triton X-100 at 60°C for 30 min. Each data point represents an average of triplicate. The formula obtained is $y = 0.0025x + 0.012$ ($R^2 = 0.99$).

Table 3.3: Lipase activity for A^m preparation, commercial proteases and caseins

ID.	Source	ΔA400nm/min	Protein[1] (mg/mL)	Specific activity[2] (mU/mg-protein)
A^m lipase	University of Waikato	1.56 (10^{-2})	1.72	108,300
Plasmin	Boehringer Manheim 602370	0.51	0.77	53
Alcalase®	Novo Industrial A/S	0.33 (10^{-2})	4.3	87,500
α-Casein	Sigma C6780	0.11	22.9	6
β-casein	Sigma C6905	0.11	27.9	5
κ-casein	Sigma C0406	0.11	20.6	7
β-casein	NZDRI	0.11	23.4	6
Casein[3]	NZDRI	0.11	10.3	13

[1]Protein was assayed using the BioRad method (Section 2.2.2).

[2]Lipase was assayed using *p*NP caproate in 0.1 M Mops buffer, pH 7.2, containing 5 mM CaCl2 and 0.1% Triton X-100, at 60°C for 10 to 30 min (Section 2.1.2.4); one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions.

[3]Obtained from raw milk using 6 M HCl.

The increase in absorbance at 400 nm gave a linear relationship with the concentration of A^m lipase in a buffer system (Fig. 3.16). It can detect minimum lipase activity at 0.3 *p*NP mU/mL, and showed the highest specific activity of 108 U/mg, which is 1.2 and 2000 times of Alcalase® and plasmin, respectively, and between 8,000-20,000 times higher than the milk caseins.

However, two proteases tested also contained high levels of lipase activity, which was as expected hence *Bacilli* are known to produce protease and lipase concomitantly. All casein preparations also contained low level of activity at between 6 and 13 mU/mg (Table 3.3). The activity could be non-specific (*i.e.* due to the esterase activity of the casein itself), or "true" arsing from the presence of traces of lipase activity because all casein preparations originated from bovine milk. But the quantity of protein exhibiting

this activity is very large compared to the enzyme quantity, so even if the 'non-specific' activity is very low, it could compromise the assay results in milk powders. However, further work carried out in later Chapter 4 has shown that the activity is true enzyme activity because the specific activity from a milk powder preparation is 12-33 times higher than the caseins preparation, and the activity was inhibited by PMSF. Lipolytic enzymes have a catalytic triad of Ser-His-Asp and act as serine proteases. Nevertheless, the possibility of non-specific activity in milk proteins was not investigated further on this occasion, therefore the lipase activity results obtained are a combination of true lipase activity and perhaps any non-specific activity present in milk proteins. The assay is simple and quick which gives results within 30 min. However, it is clearly not suitable for determination of samples containing protease activity.

Caution must also be taken when interpreting the results in terms of lipase activity. As the assay uses a short chain ester of $C_{6:0}$, it does not measure true lipase activity. Lipolytic enzymes have been defined as 'long chain fatty acid ester hydrolases" or "any esterase capable of hydrolysing esters of oleic acid" (Brockman, 1984). The long chain fatty acids are water insoluble, therefore form an interface between the aqueous phase and the lipid phase. The fundamental difference between a lipase and an esterase is based on the ability to be activated by interfaces. When the monomeric concentration of lipid in aqueous solution reaches maximum saturation (an emulsion), a lipase will act. The esterase, on the other hand, acts on monomeric substrates following Michaelis-Menten kinetics with the maximum reaction rate being reached long before the solution becomes an emulsion (Jaeger *et al.*, 1994). However, lipases from bacteria of *P. aeroginosa* and *B. subtilis* can degrade both emulsion and monomeric substrates. So the definition for the bacterial lipases should not be solely based on the interfacial activation behaviour, but also on the ability to hydrolyse emulsions of long-chain

acylglycerols (lipids; Jaeger *et al.*, 1994). For the detection of lipase activity, the assay should be performed not only on *p*NP esters of fatty acids, but also on lipids.

The reason for persisting with the short chain *p*NP esters of fatty acid assay in the face of all the difficulties is that the assay is more sensitive than any other methods. The results are likely to indicate the specific activity of lipases that lead to the production of "off" odours and flavours during milk powder storage (See later Section 6.2.1).

3.3.2 Summary of Lipase Assay Methods

Both the tricaproin-plate and *p*NP esters of fatty acids assays are common methods for the detection of lipolytic activities. The triacaproin-plate assay has a minimum detection level of 433 *p*NP U/mL in 24 h, while the *p*NP ester assay can detect as low as 0.3 mU/mL in 30 min. So the tricaproin-plate assay is 1,400 times less sensitive than the *p*NP esters of fatty acids assay, but it is simple and can be readily performed for determination of lipolytic bacteria in a factory. Although the *p*NP esters of fatty acids assay is quick and more sensitive, it can obtain false positive results due to contaminating protease activity. Because of the difficulty of obtaining a negative control, the results of this assay should interpreted with caution.

3.3.3 Application of Lipase Assays to Whole Milk Powders

As the literature indicated the methods for the detection of lipolytic activities in milk samples are very diverse, plus the possible non-specific activity arise from milk proteins, therefore not only the methods in Section 3.3.2 were applied for the determination of lipolytic activity in WMPs, but also other methods such as the titration (Casterberg *et al.*, 1979) and the GLC methods (de Jong and Baldings, 1990) were also used to determine lipase activity in milk powders.

3.3.3.1 Tricaproin-plate assay

Five batches of WMPs were made to 50 mL 10% (w/v) whole milk by dissolving 5 g powder to 50 mL deionised water at 50°C. Because milk proteins and fat have been reported to interfere with the assay (see Section 1.4.3 for reference), so the same amount of skim milk samples were obtained following the method detailed in Section 2.1.2.1. Each sample was divided into two lots of 25 mL: one was treated with 5 mg Penicillin G; and the other was autoclaved at 121°C for 30 min. Samples (50 μL) were analysed for lipase activity in duplicate using the tricapoin-plate assay at 55°C for 48 h (Section 2.1.2.2). The results are presented in Table 3.4.

Table 3.4: *Detection of lipolytic activity in reconstituted milk samples on tricaproin-plates*

Sample ID.	Diameter of clearing zone (mm) using			
	Whole milk		Skim milk	
	+ Penicillin G	Autoclaved	+ Penicillin G	Autoclaved
IH30	9.0	9.0	8.4	8.3
II12	9.5	8.4	8.0	8.1
BI18	9.5	8.3	8.1	7.9
CI03	9.0	8.0	8.1	8.0
DI05	9.3	8.3	8.6	7.9

Every sample showed a clearing zone in the tricaproin-plate, and the sizes of the zones formed using the skim milk samples are generally smaller than those formed using the whole milk (Table 3.4). The calculated values for lipase activity is 12.6-19.5 mU/mL using the formula obtained [$y = 3.2\text{Ln}(x) + 21.9$] in Fig. 3.15. They are well below the minimum detection level of 433 mU/mL using a pure lipase in the same assay (Section 3.3.1.1). One reason for this is that milk lipids are known to cause interference. Secondly, it can have artefact in the tricaproin-plate by self-forming clearing zone

which was reported by Fryer (1991). The later is more likely because the level of lipases measured here was nearly 22-34 times less than the detection limit of the assay.

It should be noted that some of the lipases produced by the seven *Bacilli* (found in the milk powder process streams) showed no activity on the tricaproin-plates (see later Table 5.14). If these enzymes are present in the WMPs, they will not be detected using the assay.

3.3.3.2 Titration Assay

All samples obtained in Section 3.3.3.1 were also analysed for FFA production using the titration method. Monopalmitin was used as substrate in skim milk sample. It was first dissolved in ethanol, then disbursed into the skim milks at 1 mM using the Ultra-turrax T-25 1 at 8,000 rev/min for 1 min, and treated with 0.2 mg/mL Penicillin G. All milk samples were then incubated at 60°C for up to 72 h. The release of FFA was extracted by a solvent mixture, and then titrated in triplicate using the titration method (Section 2.1.2.3). Samples were streaked on TSB plates and incubated at 55°C for 24 h to check for bacterial growth.

The total FFA released was calculated to be 0.14-0.26 μmole/mL per 72 h using the formula $y = 0.36x$ obtained from Figure 2.3. These values are half or less than half of the minimum reliable detection limit of 0.50 μmole/mL acid for the assay, so the assay could not be used to detect the level of lipase activity in WMPs. No bacterial growth was detected at any sampling points.

3.3.3.3 p*NP Assay*

Milk samples obtained in Section 3.3.3.1 were also assayed for lipase activity following the method detailed in Section 2.1.2.4. The same assay was repeated 4 times. Later data suggested that the lipase activity was associated with milk caseins in WMPs (Sections 4.2.1 and 4.5.1). Reagent such as N,N-dimethylformamide (DMF) was reported to dissociate the hydrophobic interactions between the milk caseins and lipase (Deeth and Fitz-Gerald, 1995), therefore was used for the assay to try to find the 'true' lipase activity. To 1 mL each sample, 0.1 mL DMF was added, mixed well and then set at room temperature for 1 h. The samples were then centrifuged at 10,000 x *g* for 10 min at 4°C, and the middle layer retained for assay. The assay was carried out 4 times, and average values of 4 assay results ars presented in Table 3.5. All samples were also assayed for lipase activity in triplicate using the method of Humbert *et al.* (1997).

Table 3.5*: Assay lipase activity in reconstituted milk samples using* p*NP caproate*

Sample ID.	Lipase activity[1](mU/mg-protein)					
	Whole milk			Skim milk		
	Penicillin G	DMF	Autoclaved	Penicillin G	DMF	Autoclaved
IH30	1.6	1.9	1.6	3.6	2.8	3.8
II12	3.1	1.8	1.7	3.0	2.0	2.7
BI18	2.0	1.0	0.8	3.8	2.1	3.3
CI03	1.9	1.9	1.7	3.6	2.3	3.0
DI05	1.4	0.7	0.7	2.8	2.3	2.8

[1]Lipase activity was assayed using *p*NP caproate in 0.1 M Mops buffer, pH 7.2, containing 0.1% Triton X-100, at 60°C for 30 min; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4). The protein content was assayed using BioRad protein assay method (Section 2.2.2).

The results showed that all samples had low levels of lipase activity (Table 3.5), and they are similar values to the casein preparations (Table 3.3). However, the skim milk

samples gave almost twice the value than the whole milk samples. This indicated milk lipids' competition and interference. As autoclaved and DMF-treated showed no effect on the lipase activity in milk samples, the question of a negative control remains unanswered (Section 3.3.1.2). Therefore the lipase activity results are a combination of the lipase activity and any non-specific activity of the milk proteins. The milk caseins also cause other interference in the assay, seen as cloudiness in the samples even after centrifugation with the stop-reagent, but it can be overcome by diluting the samples 10 fold in assay buffer.

The method of Humbert *et al.* (1997) did not detect any lipase activity. The clarifying reagent resulted in the pH of samples exceeding 13, which resulted in the release of *p*NP within 5 min, gave absorbance > 2.0 in both blanks and samples, and masked any changes that might be due to the enzyme activity (data not shown).

3.3.3.4 Analysis of Lipase activity using the GLC Method

The milk samples obtained in Section 3.3.3.1 were analysed for FFA production using the GLC method (detailed in Section 2.4.2). The results from one sample BI18 are presented in Fig. 3.17.

No change in the total level of FFA was detected in any samples either treated with Penicillin G or autoclaved, except the one made of powder batch BI18 (Fig 3.17). The sample showed release of FFA that corresponded to that addition of lipid, *e.g.* palmitic acid was predominantly released (4.1 μmole/mL/h) in the sample with addition of the monoplamitin (Fig.3.17d), in comparison, caproic acid ($C_{6:0}$) was predominantly released (0.4 μmole/mL/h) from the sample containing milk nature lipids (Fig. 3.17b).

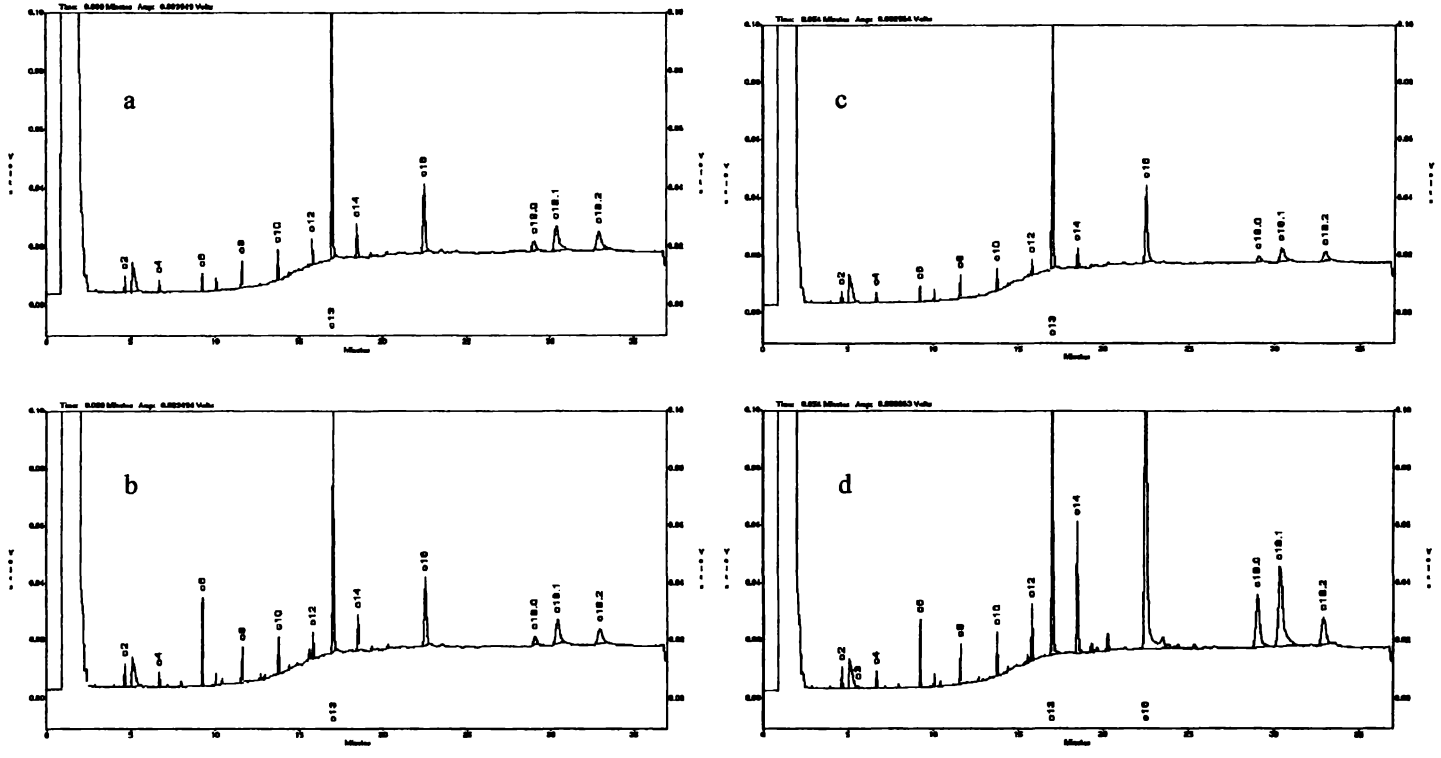


Figure 3.17: GLC traces of 10% reconstituted milk made of WMP batch BI18. (a) whole milk at zero time; (b) as (a) after 72 h incubation at 60°C; (c) skim milk with addition of 1 mM monopalmitin at zero time; (d) as (c) after 72 h incubation at 60°C. $C_{13:0}$ was added as the internal standard.

Even though the assay conditions are the same, the rate of hydrolysis is 10 times faster for monoacylglcerols than for tricaproin. The results correlate well with the characteristics of semi-pure A^m lipase (produced by one of the seven *Bacilli* found in the milk powder processing streams) on lipids, it prefers mono- and 1,2-diacylglycerols instead of triacylglcyerols (see later Section 5.5.6.2). Microbial lipases are reported to have these characteristics (Macrae, 1983).

However, the levels of lipase cannot be determined directly using the GLC method, and it is not a practical assay in a factory situation because of special equipment.

3.3.4 Summary of Lipase Assay in Whole Milk Powders

Four methods have been applied to determine lipolytic activity in the reconstituted milk samples made from WMPs. The advantage and disadvantages of the tricaproin-plate and the *p*NP ester assay were discussed in Section 3.3.2. The titration assay can not detect any lipase activity in WMPs. Although the GLC method is very sensitive (5-10 ppm) and can distinguish between short and long chain FFA since the short chain FFA have more effects on odour and flavour. However, it will not detect the lipase that has little activity on milk triacyglycerols. So there is not a method that could detect the level of lipase activity in WMPs.

3.4 Summary

Several sensitive assays described in this chapter confirmed that there were protease and lipase (esterase) activities in the WMPs. However, the levels of these enzymes are very low: lipase activity is between 0.7-1.1 *p*NP caproate U/g powder (calculated from data obtained in Table 3.5); and the actual level of protease is yet to be determined. The results are not surprising, as the literature indicated that the most sensitive protease assay is subject to the milk protein interference. Further, there is no suitable assay recorded in the literature for assaying lipolytic activity in milk samples where milkfat interference is a problem.

Although the levels of the proteases and lipases activities are very low, the enzymatic effects in reconstituted milk samples are obvious. Protease hydrolysed milk proteins to peptides as shown by SDS-PAGE and RP-HPLC. Lipase hydrolysed milk lipids to FFA as shown on GLC. The production of the peptides and short chain FFA will alter functionality and flavour, respectively, of reconstituted products made from WMPs. The details of enzyme effects on the WMP during storage will be discussed later in Chapter 7. Overall, determination of enzyme levels in WMPs is clearly important, and would give direct indication of WMP quality.

Chapter 4 Separation and Preliminary Characteristics of Proteases and Lipases from Milk Powder

4.1 Introduction

The assay development work detailed in Chapter 3 indicated that the assay limitations and the low level of enzymes did not provide precise results as anticipated. Even the most sensitive protease assay could not determine the level of protease activity in WMPs because of interference from milk proteins. SDS-PAGE and RP-HPLC methods can visually detect proteolysis, but are not readily quantitative. With the lipase assay, low levels of lipase activity were detectable. However, problems remain, such as interferences from milk lipids and milk proteins. These difficulties led to the investigation of enzyme extraction and concentration steps in search of more confident assay steps to determine the levels of protease and the lipolytic activities in WMPs.

The levels of the protease and lipase activities in WMPs are extremely low, about 10,000 times less than an enzyme from a bacterial source. However, these enzymes can cause changes in reconstituted products made from the WMPs. As the literature indicated that same type of the enzymes could show the same substrate specificities, so common assay methods could not differentiate them. Therefore, further work on the separation of the enzymes is important, this could give the opportunity to identify the source of the enzymes. The results from Chapter 3 indicated that most of sensitive assay methods could not define the initial level of the protease or lipase. Therefore, the separation work described in this chapter was mainly focused on obtaining pure enzyme for sequencing, rather than optimising yield of any particular separation step.

Several commonly used protein separation methods, such as acid precipitation, hydrophobic interaction (including different hydrophobicity matrixes), ion-exchange chromatography and electrophoresis were explored. Since Phenyl Sepharose has been used frequently for separating protease and lipase from bacterial cultures (Schmidt-Dannert *et al.*, 1996; Kim *et al.*, 1998; Toogood, 1998) and because of its good recovery compared with other matrixes, so it was selected for separation of the enzymes from WMPs. The conditions affecting the Phenyl Sepharose hydrophobic interaction, such as pH, temperature, contact time and the types of salts were investigated.

Finally, preliminary studies on the characteristics of semi-purified lipase from a WMP were undertaken. For this work a buffer system was used, so extrapolation of the results obtained to milk systems or to a homogeneous enzyme preparation should to be with caution.

4.2 Preliminary Steps for Separating Enzymes from WMPs

4.2.1 Acid-Precipitation

WMP batch GD03 was dissolved in 100 mL deionised water at 10% (w/v) at 50°C, and then adjusted pH to 4.6 using 10% (v/v) acetic acid. The acidified milk was centrifuged at 4,500 x *g* for 20 min at 4°C, and the supernatant obtained. The supernatant was further diafiltered (3 kDa cut-off) against 400 mL 0.1 M Mops buffer, pH 7.0, to a 20-fold concentration, then freeze-dried, giving 2.1 g material. The pellet was also freeze-dried, giving 4.1 g material. Samples of 0.15 g each of the freeze-dried materials were dissolved in 1.5 mL deionised water, and assayed for protease and lipase activities using azocasein and *p*NP palmitate assays, respectively (Sections 2.1.1.3 and 2.1.2.4). The results are presented in Table 4.1.

Table 4.1: *Acid-precipitation of protease and lipase activities from WMP*

Sample ID.	Protein[1] (mg)	Protease[2] (mU/mg-protein)	Lipase[3] (mU/mg-protein)
Pellet	192.5 (88%)[4]	0.12 (93%)	2.56 (96%)
Supernatant	27.5 (12%)	0.06 (7%)	0.73 (4%)

[1]Protein was assayed using Lowry method (Section 2.2.3).

[2]Protease activity was assayed using azocasein in 0.1 M Hepes buffer, pH 7.0, at 40°C for 9 h; the units were calculated using the formula obtained in Fig. 3.5 (Section 3.2.1.3).

[3]Lipase activity was assayed using *p*NP palmitate in 0.1 M Mops buffer, pH 7.0, at 40°C for 40 min; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4).

[4]Figures in parentheses indicate the percentage of protein or enzyme activity in the pellet and supernatant (100% is taken as pellet and supernatant combined).

The results indicated that while acid-precipitation removed most of the milk proteins from the sample, it also removed 93.2% and 96.1% of the protease activity and the lipase activity, respectively (Table 4.1). This possibly indicated that the enzymes in the milk powder are insoluble at pH 4.6, or they are associated with the milk caseins since whey proteins are soluble at pH 4.6. Association between milk lipase and milk caseins was previously reported by Olivecrona and Bengtsson (1984). This interaction is possibly hydrophobic since milk caseins are very hydrophobic and often form aggregates (Walstra *et al.*, 1999).

4.2.2 Other Methods for Removal Caseins

The acid-precipitation treatment resulted in removing not only the milk proteins, but also most of the protease and the lipase activities because little protease or lipase activity could be detected in the soluble whey fraction. Since the starting material contains extremely large quantity of milk caseins, it is useful to find any separation step that can remove most of milk proteins and retain enzyme activity. Ultra-centrifugation and calcium coagulation steps have been commonly used to separate caseins from whey

proteins (Walstra *et al.*, 1999). These two methods were also explored briefly to see if they had the potential to remove milk proteins, and yet retain the enzymes in solution. Milkfat is known to interfere with ultra-centrifugation, so the WMP was first defatted before further process.

WMP of GD03 was processed to 10% (w/v) 60 mL skim milk (method detailed in Section 2.1.2.1), then divided into 3 lots of 20 mL each. The first 20 mL was ultracentrifuged at 100,000 x *g* for 60 min at 4°C, and the supernatant retained. The second 20 mL was adjusted to pH 5.9 using acetic acid, 0.6 mL 2 M $CaCl_2$ was added, mixed, then centrifuged at 6,500 x *g* for 20 min at 4°C to obtain the supernatant. The third 20 mL was adjusted pH to 4.6 using 10% (v/v) acetic acid, and the supernatant obtained the same way as the second sample. All supernatant were then assayed for protease and esterase activities using FTC-β-casein and *p*NP caprylate, respectively (Sections 2.1.1.5 and 2.1.2.4).

No protease activity was detected in any of samples. The results of lipase activity are present in Table 4.2.

The ultra-centrifugation and calcium-coagulation methods improved the assay, and both methods gave 5-fold purification, and they are better than acid-precipitation in terms of purification factors and recovery rates (Table 4.2). The ultra-centrifugation method seemed to be a good first step for separating lipase activity from the WMP given the highest recovery, but was limited by the capacity at each spin. Since the level of lipase activity was extremely low and a large amount of milk would have to be processed at one time, so the method was not used further. Although the calcium-precipitation step

was much more convenient than ultra-centrifugation, it did not recover any protease activity, so it was abandoned.

Table 4.2: Methods for removing caseins

Sample treatment	Protein[1] (mg/mL)	Lipase activity[2]		
		Activity (mU/mg-protein)	Purification factor	Recovery (%)
➢ 10% skim milk	15.8	0.9	-	100
➢ 100,000 x *g* supernatant	1.5	4.6	5.1	52
➢ 0.12 M $CaCl_2$, pH 5.9 & 6,500 x *g* supernatant	1.2	4.9	5.4	36
➢ pH 4.6 & 6,500 x *g* supernatant	0.4	0.5	0.6	2

[1]Protein was assayed using the BCA method (Section 2.2.1).

[2]Lipase activity was assayed using *p*NP caprylate ($C_{8:0}$) in 0.1 M Mops buffer, pH 7.0, containing 5 mM $CaCl_2$ and 0.01% Triton X-100, at 40°C for 40 min; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4).

The results showed once again that any separation steps that can remove the caseins also removed a considerable amount of lipase activity from the sample. This confirmed the hypothesis given in Section 4.2.1 that lipase activity is associated with the caseins in WMPs.

4.3 Phenyl Sepharose Hydrophobic Interaction

The simple physical and chemical methods, such as acid-precipitation, ultra-centrifugation or calcium-coagulation steps described in Section 4.2, did not give a quick solution for separation of the protease and lipase activities from the WMP. The next logical step was to determine if any chromatographic steps could be applied to remove the milk proteins and recover the enzymes more efficiently.

Preliminary experiments with ion-exchange, gel filtration and hydrophobic interaction matrixes showed that only hydrophobic interaction had potential, therefore Phenyl Sepharose was used as the hydrophobic matrix in the screening, and compared with other hydrophobic interaction matrixes before a final decision was made on its further use.

4.3.1 Selection of Hydrophobic Matrix for Separating Lipase

Phenyl Sepharose hydrophobic chromatography was compared with other hydrophobic matrixes available to look for the most efficient separation matrix. Five 1 mL columns of Butyl-, Phenyl- and Octyl- Sepharose, and Phenyl- and Alkyl-Superose (Amersham Phamacia Biotech) were first equilibrated in 10 mL 20 mM BTP buffer, pH 7.2, containing 2 M NaCl at a flow rate of 0.5 mL/min. Samples were made to 2 M NaCl, and 2 mL of each sample was applied to each column. The columns were washed with 6 mL equilibrating buffer, then eluted with a 15 mL linear gradient from 2 to 0 M NaCl. Fractions of 1 mL were collected and assayed for protease and lipase activities using the FTC-β-casein and *p*NP caproate (method detailed in Sections 2.1.1.5 and 2.1.2.4). No protease activity was detected. The lipase assay results are presented in Table 4.3.

The results in Table 4.3 showed that Phenyl Superose was the best matrix for purifying lipase activity from the WMP, but gave unacceptably low recovery. Phenyl Sepharose gave the second best purification factor and 90% recovery. On this basis, Phenyl Sepharose was selected for further purification work.

Table 4.3: Comparison of hydrophobic matrixes for separating lipase from WMP

Matrix	Lipase[1] (mU/mg-protein)	Purification factor	Recovery (%)
Starting material	5	-	100
Phenyl Superose	15	3.0	16
Alkyl Superose	4	0.8	98
Butyl Sepharose	5	1.0	75
Phenyl Sepharose	8	1.6	90
Octyl Sepharose	5	1.0	51

[1]Lipase activity was assayed using 0.01% *p*-nitrophenyl caproate in 0.1 M Mops, pH 7.2, containing 5 mM $CaCl_2$ and 0.1% Triton X-100, at 60°C for 2 h; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4). Protein was assayed using BioRad protein assay (Section 2.2.2).

4.3.2 Effect of pH, Temperature and Contact Time on Phenyl Sepharose

WMP batch IH30 was made to 30% (w/v) skim milk in 1500 mL deionised water following the method detailed in Section 2.1.2.1, then divided into ten lots of 150 mL each. Six samples were adjusted to pH 7.0, two were adjusted to pH 5.5, and the rest to pH 8.5 using either acetic acid or NaOH (detailed in Table 4.4). All ten samples were mixed with 10 mL Phenyl Sepharose in glass beakers with constant stirring using magnetic stirrers, for either 30 min or 120 min. Samples of pH 5.5 or pH 8.5 were treated at 22°C only, and samples of pH 7.0 were treated at 4°C, 22°C and 50°C.

The Phenyl Sepharose matrixes were first washed with deionised water until the wash solution became clear. The enzymes were recovered by washing the Phenyl Sepharose matrix with 30 mL of 60% ethanediol for 30 min at room temperature with constant stirring, and then filtered through No. 4 filter paper. The enzyme solutions were further dialysed (Spectrum 3.5 kDa cut-off) against 10 L deionised water overnight, and ultrafiltered (YM3) to give a 15-fold concentration. The concentrated enzyme solutions

were assayed for protease and lipase activities using Suc-AAPF-*p*NA and *p*NP palmitate, respectively (Sections 2.1.1.4 and 2.1.2.4). The results are presented in Table 4.4.

Table 4.4: *Conditions for separation of protease and lipase activities using Phenyl Sepharose*

pH	Temp. (°C)	Contact time (min)	Protease activity[1]		Lipase activity[2]	
			Total activity (mU)	Recovery (%)	Total activity (mU)	Recovery (%)
7.0	4	30	0.30	85.7	0.69	75.8
7.0	4	120	Not detected	-	Not detected	-
7.0	22	30	0.35	100.0	0.17	18.7
7.0	22	120	0.12	34.3	0.91	100
7.0	55	30	Not detected	-	Not detected	-
7.0	55	120	0.10	28.6	0.47	51.6
8.5	22	30	0.04	11.4	0.04	4.4
8.5	22	120	Not detected	-	Not detected	-
5.5	22	30	Not detected	-	0.80	87.9
5.5	22	120	Not detected	-	0.77	84.6

[1]Protease activity was assayed using Suc-AAPF-*p*NA in 0.1 M Hepes buffer, pH 7.0 at 40°C for 18 h; one unit was defined as the release of 1 μmole/min *p*NA under the assay conditions (Section 2.1.1.4). Percent recovery was calculated relative to that obtained at pH 7.0, 22°C and 30 min.

[2]Lipase activity was assayed using *p*NP palmitate in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ at 40°C for 18 h; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4). Percent recovery was calculated relative to that obtained at pH 7.0, 22°C and 120 min.

The results showed that Phenyl Sepharose gave the highest recovery (0.35 mU) of protease activity at pH 7.0, 22°C (room temperature) with a contact time of 30 min. In comparison, the total recovery of the lipase activity was the highest (0.91 mU) at pH 7.0, 22°C and a contact time of 120 min (Table 4.4). The conditions obtained on the

separation of the protease and lipase activities from the WMP were similar to those reported by Toogood (1998) and Kim *et al.* (1998).

4.3.3 Effect of Salt Type and Concentration on Phenyl Sepharose

The results obtained in Section 4.3.2 defined the conditions of pH, temperature and contact time for separation of both protease and lipase activities from WMP using Phenyl Sepharose. The hydrophobic interaction generally requires the presence of salting-out ion, such as NaCl and $(NH_4)_2SO_4$, which can decrease the availability of water molecules in solution and increase the surface tension to enhance the hydrophobic interaction (Roe, 1996), so they were selected for further studies.

WMP batch II12 was made to 2.5% (w/v) skim milk in 80 mL deionised water following the method described in Section 2.1.2.1. Four samples of 10 mL each were made to NaCl concentration of 1 M, 1.5 M, 2.0 M and 2.5 M, and the other four were made to $(NH_4)_2SO_4$ concentration of 0.3 M, 0.5 M, 1 M and 1.3 M. All samples were mixed at room temperature for 30 min, then centrifuged at 6,500 x *g* for 20 min at 4°C, and the supernatant retained. All supernatants were treated 0.2 mg/mL Penicillin G, then assayed for protease and lipase activities using FTC-β-casein and *p*NP palmitate, respectively (Sections 2.1.1.5 and 2.1.2.4). No protease activity was detected in any of the samples. The lipase assay results are presented in Table 4.5.

The results in Table 4.5 showed that NaCl at 2.0 and 2.5 M, and $(NH_4)_2SO_4$ at 1.3 M gave similar purification factor. The higher concentration the salt, the better the purification of lipase using Phenyl Sepharose. However, higher salt concentrations give rise to problems such as inactivation of the enzymes and interference with assays. Using the data obtained large-scale purification was explored.

Table 4.5: *Effect of salts on separation of lipase activity using Phenyl Sepharose*

Supernatants from salt precipitation	Lipase activity[1] (mU/mg-protein)	Purification factor
10% skim milk	0.013	-
+ 1 M NaCl	0.024	1.8
+ 1.5 M NaCl	0.032	2.5
+ 2.0 M NaCl	0.035	2.7
+ 2.5 M NaCl	0.035	2.7
+ 0.3 M $(NH_4)_2SO_4$	0.018	1.4
+ 0.5 M $(NH_4)_2SO_4$	0.026	2.0
+ 1.0 M $(NH_4)_2SO_4$	0.026	2.0
+ 1.3 M $(NH_4)_2SO_4$	0.036	2.8

[1]Lipase activity was assayed using *p*NP palmitate in 0.1 M Mops buffer, pH 7.0, containing 5 mM $CaCl_2$ and 0.2 mg/mL Penicillin G, at 40°C for 15 h; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4). Protein was assayed using the BioRad method (Section 2.2.2).

4.3.4 Large-scale Separation using Phenyl Sepharose

WMP batch II12 was made to 10% (w/v) skim milk in 8 L deionised water following the method detailed in Section 2.1.2.1, and divided into two lots of 4 L. One lot was made to 2.5 M NaCl, the other was made to 0.5 M $(NH_4)_2SO_4$ (because of slightly higher recovery). Both samples were mixed with the different salts at 4°C for 30 min, then centrifuged at 10,000 x *g* for 20 min at 4°C, and the supernatants retained by filtering through a cheese-cloth.

Two 1 L Phenyl Sepharose columns (A and B) were equilibrated at flow rate of 100 mL/min with 4 L 20 mM BTP buffer, pH 7.2, containing 2.5 M NaCl in the case of column A or 0.5 M $(NH_4)_2SO_4$ in the case of column B.

After loading the supernatant from skim milk treated with 2.5 M NaCl, column A was washed with 4 L 20 mM BTP, pH 7.2, containing 2.5 M NaCl, then eluted in 3 steps: (1) 2.5 L 0.5 M NaCl; (2) 2.5 L deionised water; and (3) 2.5 L 30% ethanediol.

After loading the supernatant from skim milk treated with 0.5 M $(NH_4)_2SO_4$, column B was washed with 4 L 20 mM BTP, pH 7.2, containing 0.5 M $(NH_4)_2SO_4$, then eluted in 3 steps: (1) 2.5 L 0.25 M $(NH_4)_2SO_4$; (2) 2.5 L deionised water; and (3) 2.5 L 30% ethanediol.

Fractions of 60 mL each were dialysed (Spectrum 3.5 kDa cut-off) against 5 L deionised water overnight, then concentrated to a final volume of 3 mL using a UF stirred cell (Amicon) fitted with a YM3 membrane. The concentrated fractions were assayed for protease and lipase activities using FTC-β-casein and *p*NP palmitate, respectively (details see Sections 2.1.1.5 and 2.1.2.4). The results are presented in Table 4.6.

The results showed that protease activity could be detected after large-scale purification through Phenyl Sepharose chromatography, and both 2.5 M NaCl and 0.5 M $(NH_4)_2SO_4$ treatment gave similar purification (Table 4.6). The recovery of the protease can not be calculated because the initial level is not known. For lipase separation, the ethanediol fraction from column B using 0.5 M $(NH_4)_2SO_4$ gave a marginally higher recovery of lipase activity than that from column A using 2.5 M NaCl, so it was selected as one of the methods for determination of the lipase activity in WMPs.

Table 4.6: *Separation of protease and lipase using 2.5 M NaCl or 0.5 M $(NH_4)_2SO_4$ on with Phenyl Sepharose*

Assay	Fractions	Phenyl Sepharose	
		+ 2.5 M NaCl	+ 0.5 M $(NH_4)_2SO_4$
Protease activity[1]	Starting material	Not detected	Not detected
(mU/mg-protein)	Salt fraction	Not detected	Not detected
	Water Fraction	0.002	Not detected
	Ethanediol fraction	0.050	0.048
Lipase activity[2]	Starting material	0.01	0.01
(mU/mg-protein)	Salt fraction	0.01	0.02
	Water Fraction	0.04	0.05
	Ethanediol fraction	0.30	0.35
	Total recovery (%)	90	95

[1]Protease activity was assayed using FTC-β-casein in 0.1 M Hepes, pH 7.0, containing 0.2 mg/mL Penicillin G at 40°C for 15 h; one unit was defined as the release of 1 μmole/min FITC under the assay conditions (Section 2.1.1.5). Protein was assayed using the BioRad method (Section 2.2.2). Since the initial level of protease activity was not detected, so the total recovery could not be calculated and not listed in the table.

[2]Lipase activity was assayed using *p*NP palmitate in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ and 0.2 mg/mL Penicillin G, at 40°C for 15 h; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4). Total recovery was calculated relative to the total activity detected in the starting material.

4.3.5 Phenyl Sepharose Method for Determination of Lipase Activity in WMP

The data obtained in Sections 4.3.2 to 4.3.4 indicated that Phenyl-Sepharose with 0.5 M $(NH_4)_2SO_4$ gave a high purification factor and good recovery for separating lipase activity from WMPs. Although $(NH_4)_2SO_4$ interfered with the assay when using short chain *p*NP esters of fatty acids (data not shown), it was still preferred because of its high recovery, and a dialysis step was added to eliminate the interference.

Other experiments showed that 25% acetonitrile gave the same recovery and purification as that gained using 30% ethanediol, and was easier to handle. Therefore, acetonitrile was used instead of ethanediol in further work.

The usefulness of Phenyl Sepharose preparative extraction step for assay lipase activity in milk powders was tested on five batches of WMPs that had been under storage trial at room temperature (22°C), 37°C and 55°C for different times. These powders were made to 8% (w/v) skim milks (method detailed in Section 2.1.2.1) in 25 mL 20 mM BTP buffer, pH 7.2, and 1.65 g $(NH_4)_2SO_4$ (0.5 M) was added and mixed at room temperature for 30 min. The milk samples were then centrifuged at 10,000 x *g* for 20 min at 4°C, and supernatant of ~25 mL each retained.

A series of 10 mL Phenyl-Sepharose columns were connected to a 16 port manifold and equilibrated with 20 mL 20 mM BTP, pH 7.2, containing 0.5 M $(NH_4)_2SO_4$. The supernatant were loaded onto the columns at a flow rate of ~ 1.5 mL/min. The columns were washed with 30 mL of the equilibration buffer, then eluted with 20 mL Milli-Q water followed by 30 mL 25% acetonitrile.

The two eluents (Milli-Q water and acetonitrile) were pooled and dialysed (Spectrum 3,500 Da cut-off) against 4 L deionised water for 8 h with one change of fresh deionised water after 4 h. Samples were retrieved from the dialysis bags, and assayed for protease and lipase activities using FTC-β-casein and *p*NP caproate, respectively (details see Sections 2.1.1.5 and 2.1.2.4). No protease activity could be detected in any of the samples. The results of an average of duplicate extraction for lipase activity are presented in Table 4.7.

Table 4.7: *Assay lipase activity in WMP after Phenyl Sepharose*

Lipase activity[1] (mU/mg-protein)	IH30	II12	BI18	CI03	DI05
Initial	0.9	0.9	1.3	1.0	1.0
After storage at 22°C[2]	0.9	1.0	1.2	1.0	1.0
37°C x 8 month	1.0	0.9	1.2	1.0	1.1
55°C x 2 month	0.9	0.8	1.2	1.0	0.9

[1]Lipase activity was separated in duplicate from WMP samples using Phenyl-Sepharose, then assayed using *p*NP caproate in 0.1 M Mops, pH 7.2, containing 5 mM $CaCl_2$, at 60°C for 30 min; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4). Protein was assayed using the BioRad method (Section 2.2.2).

[2]WMPs had been stored at 22°C for: 28 months for IH30, 16 months for II12, 11 months for BI18, 10 months for CI03; and 9 months for DI05.

The results showed that low levels of lipase activities were present in all the WMPs, and remained the same levels even after 2-month storage at 55°C (Table 4.7). Enzymes are well known for high stability at low water content because the enzymes become more rigid and have compact conformations which is more resist to heat-denaturation process (Bell *et al.*, 1995; Broos *et al.*, 1995; Cowan, 1997).

There were differences between the results given in Table 4.7 and Table 3.6. Although preliminary results showed good recovery using the Phenyl Sepharose, it was obvious that loss had occurred during extraction and diafiltration. Ont other possibility is that acetonitrile might have a negative effect (inactivation) on the lipase activity which needs to be checked out. The extraction procedure is technically complex and subject to variability, so further work is required to develop a simple, reproducible assay that can be used in a factory.

4.4 Ion-exchange and Gel filtration for Further Purification of Lipase

As mentioned earlier, different bacteria can produce enzymes that show similar similar specificity with general assay methods. Thus it is not possible to determine the source of an enzymes in WMP simply by looking at the enzyme characteristics. The approach required is to obtain a sufficient amount of pure protein for N-terminal sequencing, then to match it with a known N-terminal sequence of corresponding enzyme, and therefore identify the source of the enzyme. So further purification of the powder lipase was carried out.

To a Mono Q HR10/10 column (Amersham Pharmacia Biotech), 10 mL active fraction (15 mg/mL protein) obtained in Section 4.3.4 was applied at a flow rate of 2 mL/min in 20 mM BTP buffer, pH 7.2. The column was washed with 40 mL loading buffer, then eluted with a 200 mL linear gradient from 0 to 1 M NaCl. Fractions of 4 mL were collected and assayed for lipase activity using *p*NP palmitate.

Active fractions of 50 mL pooled from several Mono Q runs were diafiltered against 200 mL deionised water using a 400 mL stirred cell fitted with a YM10 membrane to a final 15 fold concentration. The concentrated fraction of 0.5 mL was applied onto a HR 10/30 Superose 12 column (Amersham Pharmacia Biotech) equilibrated with 20 mM BTP, pH 7.2, at a flow of 0.5 mL/min. Fractions of 1 mL were collected and assayed for lipase activity using *p*NP palmitate (method detailed in Section 2.1.1.4). The results are presented in Table 4.8.

Table 4.8: Purification of lipase using ion-exchange and gel-filtration

Separation steps	Lipase[1] (mU/mg-protein)	Purification	Recovery (%)
Starting material vs Phenyl Sepharose	0.30	-	-
Mono Q	1.20	4	95
Gel filtration	2.50	8	70

[1]Lipase was assayed using *p*NP palmitate in 0.1 M Mops buffer, pH 7.0, containing 5 mM $CaCl_2$, and 0.2 mg/mL Penicillin G, at 40°C for 15 h; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4). Protein was assayed using the BCA method (detailed in Section 2.2.1).

The ion-exchange step using Mono Q gave a 4-fold purification and excellent recovery for further purification of lipase after Phenyl Sepharose hydrophobic chromatography. The gel filtration step gave further 2-fold purification. The total purification factor was 250 fold from a specificity of 0.01 mU/mg in 10% (w/v) skim milk to a 2.50 mU/mg semi-purified lipase preparation. Several preparations of lipase obtained by a combination of Mono Q and gel filtration were pooled and ultra-filtrated using a YM3 membrane to a 5-fold concentration. This semi-purified lipase concentrate is termed 'powder lipase' in subsequent work.

4.5 Preliminary Characteristics of the Powder Lipase

4.5.1 Molecular Mass

Samples of 20 μL each of powder lipase preparation were mixed with 5 μL 5 x sample buffer and separated on 12.5% gels using SDS-PAGE. After electrophoresis, the gels were activity overlayed at room temperature overnight using *p*NP palmitate (Section 2.1.2), and subsequently stained with Coomassie blue. Samples showing activity on the overlays were electrophoresed the second time, then blotted onto PVDF membrane for N-terminal sequencing (Sections 2.3.3). The results are presented in Fig. 4.1.

The yellow spots on SDS-PAGE gels indicated the presence of activity at 30 kDa, 45 kDa, 50 kDa, 60 kDa, 65 kDa and 85 kDa (calculated according to the rainbow markers). The protein of 30 kDa gave the same sequence as β-casein, while 60 kDa and 65 kDa proteins gave the same sequence as α_{s2}-casein (Fig. 4.1b). The normal molecular mass for α_{s2}-casein and β-casein are ~ 25 kDa and ~ 24 kDa, respectively (Walstra *et al.*, 1999). The 85.0 kDa protein was later confirmed to contain β-casein mainly by boiling the sample for 5 min with the sample buffer containing 5% β-mercaptoethanol before electrophoresis (data not shown). Unfortunately, the amount of and 45 kDa (Fig. 4.1d) and 50 kDa (Fig. 4.1b) proteins were insufficient to give any sequence.

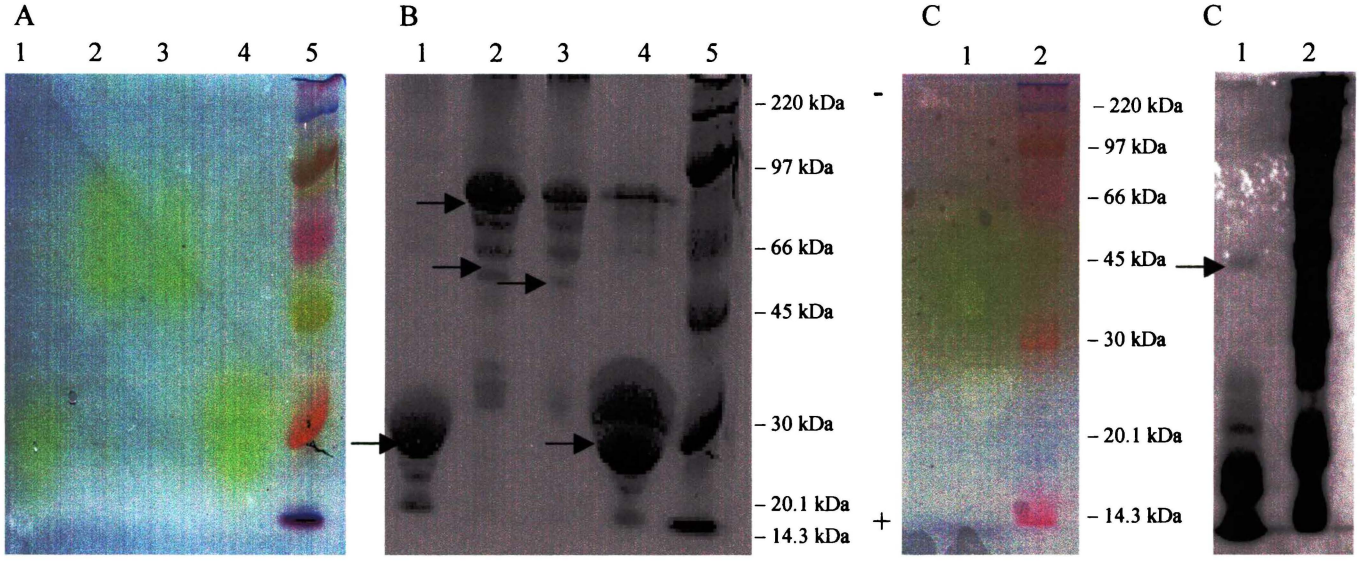


Figure 4.1: Powder lipase activity overlay on 12.5% SDS-PAGE using *p*-nitrophenyl palmitate (room temperature overnight) and Coomassie stained. (A): activity overlay-lane 1 and 4-Mono Q active fraction 1; lane 2 and 3 -Mono Q active fraction 2; lane 5- rainbow markers (Amersham Pharmacia Biotech. RPN 756). (B) as (A) but Coomassie blue stained. (C): activity overlay-lane 1-gel filtration active fraction; lane 2- rainbow markers; (D) as (C) but Coommassie stained. Yellow spots indicated lipase activity , arrows indicate molecular mass.

The results can be explained in a number of ways. It was possible that the lipase molecule had a similar size to the milk caseins, and therefore migrated to the same position as the milk caseins. The result of the N-terminal sequencing would be explained by the amount of enzyme protein being far less than the amount of casein. Secondly, the powder lipase could be a small molecule. A 1570 Da thermostable esterase from *B. stearothermophilus* has been reported by Simoes *et al.* (1997). Lipolytic enzymes are known to have the characteristic of forming aggregates (Rua *et al.*, 1997). All milk caseins are hydrophobic molecules (Walstra *et al.*, 1999), therefore formation of self aggregates or associated with the lipase are highly possible. Since the samples were treated with SDS, but not with reducing agent or boiling, so the interactions between the powder lipase and the milk caseins would have remained, and resulted in no separation (from the milk caseins) on SDS-PAGE. If reducing agent and boiling were used, no activity was recovered after electrophoresis, so it was not possible to investigate the problem further in the time available.

A fraction from a separate preparation process showed *p*NP caproate activity in the test tube, but the 46 kDa protein band failed to show activity on SDS-PAGE using an activity overlay. The protein gave a N-terminal sequence of **EETQA GCYCL XFGPC DLR**, but did not show any homology with any known lipase or esterase sequence reported in the Blast database.

4.5.2 Temperature Profile for Powder Lipase

The semi-purified powder lipase obtained in Section 4.4 was assayed over a temperature range from 37°C to 90°C to determine the temperature for maximum activity under the assay conditions. The assay was using *p*NP palmitate in 0.1 M Mops buffer, containing

5 mM $CaCl_2$ for 30 min (details in Section 2.1.2.4). The pH of buffer was adjusted to 7.0 at each assay temperature. The results are presented in Fig. 4.2.

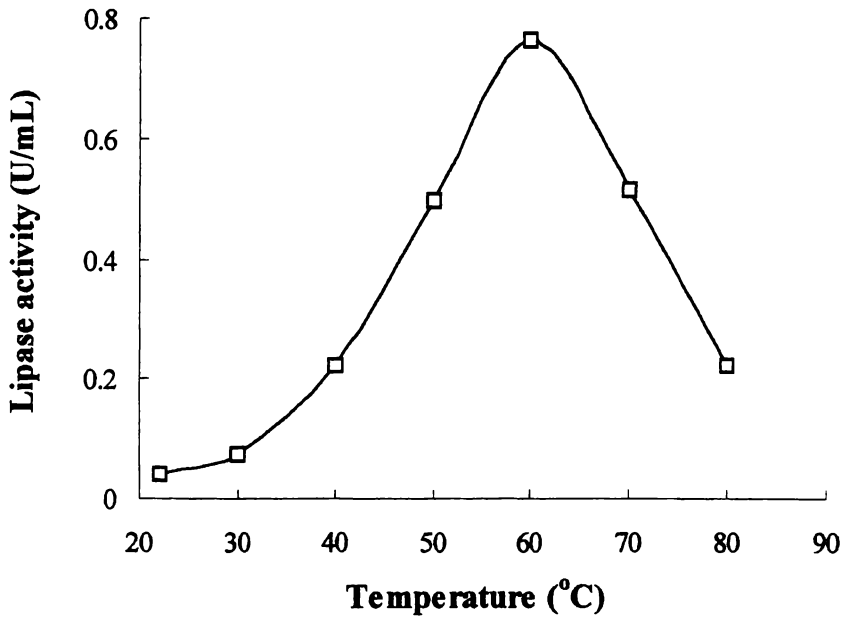


Figure 4.2: Temperature effect on the powder lipase activity. Assay was with *p*NP plamitate in 0.1 M Mops, pH 7.2, containing 5 mM $CaCl_2$, for 30 min at temperature range from 22°C to 80°C. Each data point represents an average of triplicate.

Under the assay conditions used, the powder lipase gave maximally active at 60°C (Fig. 4.2). Literature indicated that *Pseudomonas* lipases (mesophilic) are mostly active at 30°C to 45°C, and the *Bacillus* lipases (thermophilic) are at 60 to 75°C (see Table 1.1 for details). So it is likely that the powder lipase is from a thermophilic bacterium because stabilities and activities of bacterial enzymes generally correlate with the optimum growth temperature of the source microorganism (Owusu *et al.*, 1991).

4.5.3 pH Optimum of Powder Lipase

The optimum pH for activity of the semi-purified powder lipase obtained in Section 4.4 was determined. The assay was with *p*NP palmitate at 60°C for 30 min in 0.1 M buffers: tri-sodium citrate (pH 3.2 to 5.2), Mes buffer (pH 5.9), Mops buffer (pH 6.8), Tris-HCl buffer (pH 7.2) and Caps buffer (pH 9.2 to 10.1). The results are presented in Table 4.9.

Table 4.9: *Powder lipase activity at different pH*

Assay buffers	Assay pH at 60°C	Lipase activity[1](%)
trisodium citrate	3.2	12
	4.2	68
Mes	5.2	77
	5.9	75
Mops	6.8	100
Tris	7.2	90
Caps	9.2	68
	10.1	42

[1]Lipase activity was assayed using *p*NP palmitate in buffers of 0.1 M tri-sodium citrate (pH 3.2 to 5.2), 0.1 M Mes (pH 5.9), 0.1 M Mops (pH 7.2), 0.1 M Tris-HCl (pH 7.2) and 0.1 M Caps (pH 9.2 to 10.2), at 60°C for 30 min (Section 2.1.2.4). Activity of 0.53 U/mL was taken as 100%.

The powder lipase showed maximum activity at pH 6.8 (Table 4.9). Literature review showed that both *Pseudomonas* lipases and *Bacillus* lipases commonly have pH optima between 6.8 and 9.0 (see Table 1.1 for details).

4.5.4 Specificity of Powder Lipase on *p*NP Esters of Fatty Acids

The substrate specificity of the semi-purified powder lipase obtained in Section 4.4 was determined using *p*NP butyrate ($C_{4:0}$), *p*NP caproate ($C_{6:0}$), *p*NP caprylate ($C_{8:0}$), *p*NP caprate ($C_{10:0}$), *p*NP laurate ($C_{12:0}$), *p*NP myristate ($C_{14:0}$), *p*NP palmitate ($C_{16:0}$) and *p*NP

stearate ($C_{18:0}$) as substrates. All assays were carried out in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$, at 60°C for 30 min (Section 2.1.2.4). The results are presented in Fig. 4.3.

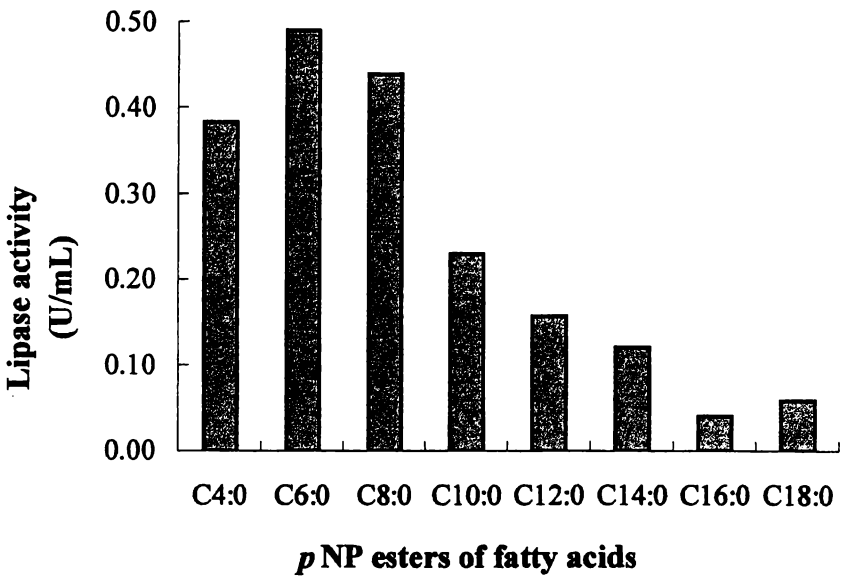


Figure 4.3: The powder lipase substrate specificity on *p*-nitrophenyl esters of fatty acids. Assay was in triplicate in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ at 60°C for 30 min using *p*NP esters of butyrate ($C_{4:0}$), caproate ($C_{6:0}$), caprylate ($C_{8:0}$), caprate ($C_{10:0}$), laurate ($C_{12:0}$), myristate ($C_{14:0}$), palmitate ($C_{16:0}$) and stearate ($C_{18:0}$).

The powder lipase had specificity for short chain *p*-nitrophenyl esters of butyrate, caproate and caprylate, with the highest activity towards *p*NP caproate (Fig. 4.4). This indicated that the powder lipase seems to be an esterase. However, lipases from *P. aeruginosa* and *B. subtilis* have shown activities in both emulsions and monomeric forms of substrates (Jaeger *et al.*, 1994), so further studies of this powder lipase on triacylglcyerols are required to define its character.

4.5.5 Inactivation of Powder Lipase Activity

A separated powder lipase preparation from WMP II12 was checked for its heat-stability. The sample (containing ~ 1.5 mg/mL protein) was packed in 9 lots of 10 mL. One lot was autoclaved at 121°C for 30 min. The second lot was treated with 50 mM PMSF at room temperature overnight. The third lots of samples were treated with 1 FITC U/mL Alcalase® at 37°C overnight, then heated in an oil bath at 140°C for 2 h. The remaining samples were either heated in the sealed glass tubes or open universal bottles in the oil bath or in an drying-oven set at 90-120°C for 18 to 30 h (details see Table 4.9). All samples were then assayed for lipase activity using *p*NP caproate in 0.1 M Mops, pH 7.2, containing 5 mM $CaCl_2$, at 70°C for 15 min (details in Section 2.1.2.4). The results are presented in Table 4.10.

Table 4.10: *Inactivation of powder lipase activity*

Treatments		Remaining activity[1](%)
Untreated	control	100
Inhibitor	50 mM PMSF, overnight	56
Autoclaved	121°C, 30 min	87
Heated in an oil bath	90°, 24 h	73
	120°C, 30 h	27
	120°C, 30 h (5-fold diluted)	6
Heated in an oven	110°C, 18 h	25
Protease digestion/heated	Alcalase® + 140°C, 2 h	52
	Alcalase® + 140°C, 2 h + 1 mM PMSF	35
	Alcalase® + 140°C, 2 h + 50 mM PMSF	0

[1]Activity was assayed using *p*NP caproate in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$, at 70°C for 15 min; one unit was defined as the release of 1 μmole *p*NP per min under the assay conditions (Section 2.1.2.4). Activity value of 170 mU/mg was taken as 100%. Protein was assayed using BCA method (Section 2.2.1). The activity of unheated sample at 170 mU/mg-protein was taken as 100%. Data in the table represent an average of 3 determination in triplicate assays.

First, the lipase activity results obtained in Table 4.9 could be a combination of the lipase activity and any non-specific activity of the milk proteins as indicated in Section 3.3.1.2). However, the sample used here (1) had a specific activity of 170 mU/mg-protein (protein concentration of 1.5 mg/mL), which is 12-33 times higher than the 'pure' caseins of 5-13 mU/mg-protein (protein concentration of 20-30 mg/mL); (2) the activity in the presence and absence of PMSF has brought more confidence to the results because lipolytic enzymes are known to have a catalytic triad of Ser-His-Asp that acts as serine proteases.

Although the possibility of non-specific activity in milk proteins could not be eliminated from the assay system, the majority of activity is true lipase activity. However, assay values of less than 10% should be treated with caution.

The results showed that heat-treatment alone could not completely inactivate the lipase activity in the crude preparation. It retained 27% of initial activity after heat-treatment 120°C for 30 h in a buffer system. When the sample was diluted 5 times, the same heat-treatment of 120°C for 30 h had reduced the activity to 6%. This indicated that the stability of enzyme was related to the protein concentration in the sample. It has been reported by Kumura *et al.* (1991) that milk casein will protect enzymes from heat denaturation. The data obtained in Section 4.5.1 indicated that the lipase activity is strongly associated with caseins by hydrophobic interaction. The hydrophobic interactions will become stronger with increasing temperatures, so the lipase conformation could be held more tightly inside the casein micelles, and remain intact. As the caseins molecules are known to be very heat-stable, and conformational changes are very slow even at temperature above 100°C (Walstra *et al.*, 1999). As the protection from casein slowly disappeared, the enzyme was slowly denatured. The sample

✱ See Terminology Section xiv

contained 1.5 mg/mL protein, which is 18 times less than the protein concentration in milk, undoubtedly the lipase activity will be more heat-stable in milk. This has been reported by Fox (1989) that lipase from psychrotrophs are more stable in a synthetic milk serum or a milk system than in a buffer system. If the heat stable enzymes are in milk, it is very difficult to destroy them completely (Stead, 1986). The data in Table 4.10 indicate that any heat treatments in the milk powder process probably have little impact on the powder lipase activity. To destroy the lipase activity completely in a buffer, a combination of heat with a high concentration of PMSF is required.

Other treatments, such as protease (Alcalase®) digestion plus heating at 140°C for 2 h reduced the lipase activity only by 48%. The activity was destroyed only when treated further with high concentration of 50 mM PMSF. Although assays have shown that most of milk proteins were hydrolysed by Alcalase® (data not shown), it seems to have little effect on the lipase activity in the sample. This is possibly due to the contaminating esterase from Alcalase® (as shown in Table 3.5). It had been hoped that protease digestion would have released the lipase for the casein-lipase complex. However, the contamination of esterase in Alcalase® (and other caseinolytic proteases) showed that it is not a suitable approach for separating lipase activity from WMP.

4.5 Summary

The data obtained in this chapter showed that there is probably only one type of lipases (esterases) present in WMP. This was indicated mainly by the substrate specificity, where a clear preference of short-chain *p*-nitrophenyl esters of fatty acids was found. However, this does not necessary mean that all the activity was from one bacterium, because the lipase (esterase) from different species of bacteria can have similar substrate specificity. The powder lipase showed the highest activity at 60°C and pH 6.8.

Several semi-purified fractions from WMP (through 3-step chromatography) showed lipase activity at 30 kDa, 45 kDa, 50 kDa, 60 kDa, 65 kDa and 85 kDa on SDS-PAGE using *p*NP palmitate as the substrate. Most of these protein bands gave the N-terminal sequences of β-casein or α_{s1}-casein. It is probable that no N-terminal sequence of the enzyme protein band could be obtained because of the extremely low amounts in comparison with milk caseins. There is at least 10,000 times more non-enzyme protein to be removed from the starting material than from a bacterial culture. One unique N-terminal protein sequence obtained from an apparently homogenous protein preparation with lipase (esterase) activity, but this sequence showed no homology with any known proteins in the database, and no match with a bacterial lipase/esterase could be found.

The information obtained in the literature review (Section 1.3.3) indicated that lipases from *Pseudomonas* species commonly have a temperature optimum of 30-45°C, while those from *Bacillus* species are mostly active at 60-75°C. The temperature and heat-stability data obtained in the powder lipase indicated that it is likely to come from a thermophilic bacterium. However, it is debatable whether the enzyme is an esterase or a lipase, because the definition here is not clear-cut. Further studies in the specificities of this enzyme on triacylglcyerols will help to clarify the issue.

The most important points arising are that protease and lipase are present in WMP, and active. The lipase activity seems extremely heat-stable in a buffer system. It will not be destroyed by any of the heat treatments applied in the milk powder processes, such as pasteurisation or UHT, and consequently has the potential to affect WMP quality during storage.

Chapter 5 Production and Characterisation of *Bacillus* Protease and Lipase

5.1 Introduction

Seven *Bacilli* (in order of predominance) A^m and C^m (*B. stearothermophilus*), D^m and F/G (*B. licheniformis*), B. sub. (*B. subtilis*), B^m (*B. stearothermophilus*) and F^{ta} (*B. licheniformis*) were identified from milk powder production streams using the RAPD-PCR technique (Morgan *et al.*, 1997). Protease and lipase activities found in milk powders are likely to be derived from these organisms, or at least have similar characteristics to the enzymes from them. In order to enable a comparison to be made between the characteristics of the enzymes from powders and those from the *Bacillus* contaminants, the enzymes from the *Bacilli* have to be characterised.

As discussed earlier, the difference between an esterase and a lipase is not clearly defined. Generally speaking, the esterases hydrolyse carboxyl ester bonds of water-soluble substrates, while the lipases act on the same bonds of water-insoluble emulsified substrates (Tsujita *et al.*, 1990). As short chain triacylglcyerols are slightly soluble in aqueous solution, it can be said that the esterases hydrolyse acyl esters of short chain $C_{4:0}$ to $C_{8:0}$, and the lipases hydrolyse longer chain esters. However, some lipolytic enzymes from *P. aeruginosa* and *B. subtilis* have activities on triacylglcyerols in both solution and emulsion (Jaeger *et al.*, 1994). This is also the case for the enzymes produced by the seven *Bacilli* found in the milk powder production stream. However, these enzymes are called "lipase" here.

In the first stage of experimentation, proteases and lipases were produced from the seven *Bacilli* in several media selected to enhance enzyme production under the laboratory conditions. The enzymes were harvested by centrifugation and semi-purified using the chromatography methods detailed in Chapter 4. The semi-purified proteases and lipases from the seven *Bacilli* were characterised in buffer systems. While the data obtained are indicative, extrapolating results to a milk system, or to a homogeneous enzyme preparation, needs to be with caution.

5.2 Protease and Lipase Production from the Seven *Bacilli*

To determine protease and lipase production in a given medium, the seven *Bacilli* were inoculated in five different media described in Section 2.5.2. *B. licheniformis* and *B. subtilis* were incubated at 37°C, and *B. stearothermophilus* was incubated at 55°C. The proteases and the lipases produced were named after the isolates (*e.g.* A^m lipase from strain A^m). Some of enzymes were partially purified and characterised.

5.2.1 Production of Extra- and Intracellular Enzymes

Seven *Bacilli* were grown in 100 mL C-medium (Section 2.5.2) with shaking (110 rpm) for 24 h. Extracellular enzymes were harvested from the cultures by centrifugation at 4,500 x *g* for 20 min at 4°C. The supernatants obtained were diafiltered using a YM3 membrane to a final concentration of 12-fold. The cell pellets were washed twice with 10 mL saline (0.8% NaCl) by vortexing and centrifugation. Washed cells were resuspended in 5 mL saline, and the intracellular enzymes were released into solution by disbursing the cells on ice using a Vibra cell (Sonics & Materials Inc., Danbury, Connecticut, US) at 50% duty cycle for 2 min. The suspension was then centrifuged at 4,500 x *g* for 20 min at 4°C, and the supernatant retained. The suspensions were

assayed for protease and lipase activities using FTC-β-casein and *p*NP palmitate, respectively (Sections 2.1.1.5 and 2.1.2.4). The results are presented in Table 5.1.

Table 5.1: *Extra- and intracellular enzyme produced by the seven* Bacilli *in C-medium*

Enzyme ID.	Protease activity[1] (%)			Lipase activity[2] (%)		
	total (mU)	Extra-cellular	Intra-cellular	total (mU)	Extra-cellular	Intra-cellular
A^m	4.7	Not detected	100	18.5	68	32
B^m	12.0	32	68	240.4	48	52
C^m	44.9	84	16	53.3	86	14
D^m	5.0	67	33	10.0	92	8
F/G	1718.0	91	9	3.5	71	29
F^{ta}	5028.0	96	4	13.9	29	71
B. sub.	8033.0	98	2	55.0	95	5

[1]Protease activity was assayed using FTC-β-casein in 0.1 M Hepes buffer, pH 7.0, at 40°C for 3 h; one unit was defined as the release of 1 μmole/min FITC under the assay conditions (Section 2.1.1.5). Total (extra- and intra-cellular) activity was counted as 100%.

[2]Lipase was assayed using *p*NP palmitate in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ at 40°C for 3 h; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4). Total (extra- and intracellular) activity was counted as 100%.

The results showed that the seven *Bacilli* produced both extra- and intracellular proteases and lipase in C-medium. C^m, D^m, F/G, F^{ta} and *B. subtilis* isolates produced more extracellular protease than intracellular protease, while A^m and B^m isolates produced more intracellular protease than extracellular protease. In the case of lipase production, A^m, C^m, D^m, F/G and *B. sub*tilis isolates tended to produce more extracellular lipase than the intracellular lipase, while B^m and F^{ta} isolates tended to produce more intracellular lipase (Table 5.1). Differences in the levels of enzymes produced could be due to different growth rates in the C-medium, or different characteristics of the isolates.

5.2.2 Protease and Lipase Production in Five Media

The seven *Bacilli* were inoculated in five media described in Section 2.5.2, and incubated at 37°C and 55°C for up to 24 h with shaking (110 rpm). The cultures were harvested by centrifugation at 4,500 x *g* for 20 min at 4°C, supernatants obtained and assayed for protease and lipase activity using FTC-β-casein and *p*NP palmitate, respectively (Sections 2.1.15 and 2.1.2.4). The results are presented in Table 5.2.

The seven *Bacilli* showed different characteristics in terms of protease and lipase production in the same medium (Table 5.2). In general, two *B. licheniformis* (F/G and F^{ta}) and *B. subtilis* (B. sub.) isolates produced more proteases than the three *B. stearothermophilus* (A^m, B^m and C^m) isolates and one *B. licheniformis* (D^m) isolate. In the case of lipase production, A^m, B^m, C^m and D^m isolates produced slightly more lipase than F/G, F^{ta} and *B. subtilis* isolates. All isolates preferred skim milk as the growth medium and for enzymes production. However, the behaviour of these organisms in terms of enzyme production may be different in a milk powder process than in a medium under laboratory conditions.

5.2.3 Enzyme Production for Powder Spiking Experiment

TSB medium was selected instead of skim milk medium to eliminate any milk caseins interference in the assay. Each of seven *Bacilli* was innoculated in four 500 mL TSB medium (air/medium ratio of 3) with shaking (110 rpm) for 24 h. The extra- and intracellular enzymes were harvested by the method described Section 5.2.1, then pooled together and diafiltered with 2 L deionised water to a 14-fold concentrate using a YM3 membrane. The preparations were then freeze-dried for future experiments in which powders would be spiked with the enzymes. The results are presented in Table 5.3.

Table 5.2: *Growth and production of protease and lipase by the seven* Bacilli

Isolates	Medium	A650 nm	Final pH	Protease[1](mU/mL)	Lipase[2](mU/mL)
A^m	TSB+0.1% starch	0.47	5.22	0.01	0.20
	C-medium	0.75	5.02	ND[3]	0.05
	10% skim milk	8.30	5.30	1.03	0.18
	Claudia medium	1.72	6.40	ND	0.13
	B. brevis medium	0.86	5.12	ND	0.08
B^m	TSB+0.1% starch	0.47	5.15	0.02	0.11
	C-medium	0.49	5.88	0.06	0.003
	10% skim milk	2.00	5.45	ND	0.74
	Claudia medium	1.63	8.75	ND	0.05
	B. brevis medium	0.68	5.37	ND	0.01
C^m	TSB+0.1% starch	1.66	5.69	0.07	0.07
	C-medium	0.78	5.29	0.01	0.10
	10% skim milk	2.80	5.35	ND	0.16
	Claudia medium	0.93	8.97	ND	0.01
	B. brevis medium	0.95	6.11	ND	0.03
D^m	TSB+0.1% starch	0.26	6.90	ND	0.07
	C-medium	0.67	5.71	0.001	0.25
	10% skim milk	0.90	5.77	0.33	2.62
	Claudia medium	0.54	6.40	ND	0.37
	B. brevis medium	0.43	5.39	ND	0.11
F/G	TSB+0.1% starch	1.13	6.45	0.08	0.05
	C-medium	1.76	7.46	0.24	0.01
	10% skim milk	5.04	7.63	1.69	1.11
	Claudia medium	2.06	7.48	53.1	0.12
	B. brevis medium	1.91	6.89	ND	0.001
F^{ta}	TSB+0.1% starch	1.19	7.01	0.48	0.08
	C-medium	1.59	7.64	0.68	0.03
	10% skim milk	4.92	7.66	3.83	0.38
	Claudia medium	2.30	7.73	53.1	0.14
	B. brevis medium	1.91	7.98	1.69	0.10
B. sub.	TSB+ 0.1% starch	2.09	7.26	0.19	0.15
	C-medium	2.03	7.77	1.06	0.24
	10% skim milk	4.78	7.02	$1.3x10^7$	22.50
	Claudia medium	2.00	8.51	$6.9x10^3$	6.68
	B. brevis medium	2.08	7.42	$3.1x10^4$	6.56

[1]Protease activity was assayed using FTC-β-casein in 0.1 M Hepes buffer, pH 7.0, at 40°C for 0.5-3 h; one unit was defined as the release of 1 μmole/min FITC under the assay conditions (Section 2.1.1.5).

[2]Lipase activity was assayed using *p*NP palmitate in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ at 40°C for 40 min; one unit was defined as the release of 1 μmole/min *p*-nitrophenol under the assay conditions (Section 2.1.2.4).

[3]ND: not detected

Table 5.3:Production of protease and lipase for powder spiking experiment

Enzymes	A[m]	B[m]	C[m]	D[m]	F/G	F[m]	B. sub.	Total
Protease[1](U)	2.01	0.43	11.47	0.85	6.02	3.92	198.29	222.97
Lipase[2](U)	0.52	0.81	0.49	0.28	0.39	0.37	3.23	6.09

[1]Protease was assayed using FTC-β-casein in 0.1 M Hepes buffer, pH 7.0, at 40°C for 0.5-3 h; one unit was defined as the release of 1 μmole/min FITC under the assay conditions (Section 2.1.1.5). Total activity was listed.

[2]Lipase was assayed using 0.01% *p*NP palmitate in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ at 40°C for 1 h; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4). Total activity was listed.

The results in Table 5.3 were consistent with the results obtained in Table 5.2 that different *Bacillus* produced different levels of protease and lipase in the same medium. The TSB medium is rich in tryptone, this may have enhanced protease production. The enzymes were also used for spiking trial in later Section 6.2.

5.2.4 Free Fatty Acids Release in Whole Milk by the Seven *Bacilli*

To determine the production of lipase during growth, the seven *Bacilli* were grown in 10% (w/v) sterile whole milk made from WMP batch IH30. A loopful of each isolate grown 20 h on a TSB plate was inoculated into 10 mL sterile whole milk. After 20 h incubation, the culture was transferred into 150 mL whole milk in a 600 mL flask and incubated with a rotation at 110 rpm for up to 40 h. Growth was monitored by pH changes. Lipase production was quantified by FFA analysis using the GLC method (Section 2.4.2). The results are presented in Figs. 5.1a and b.

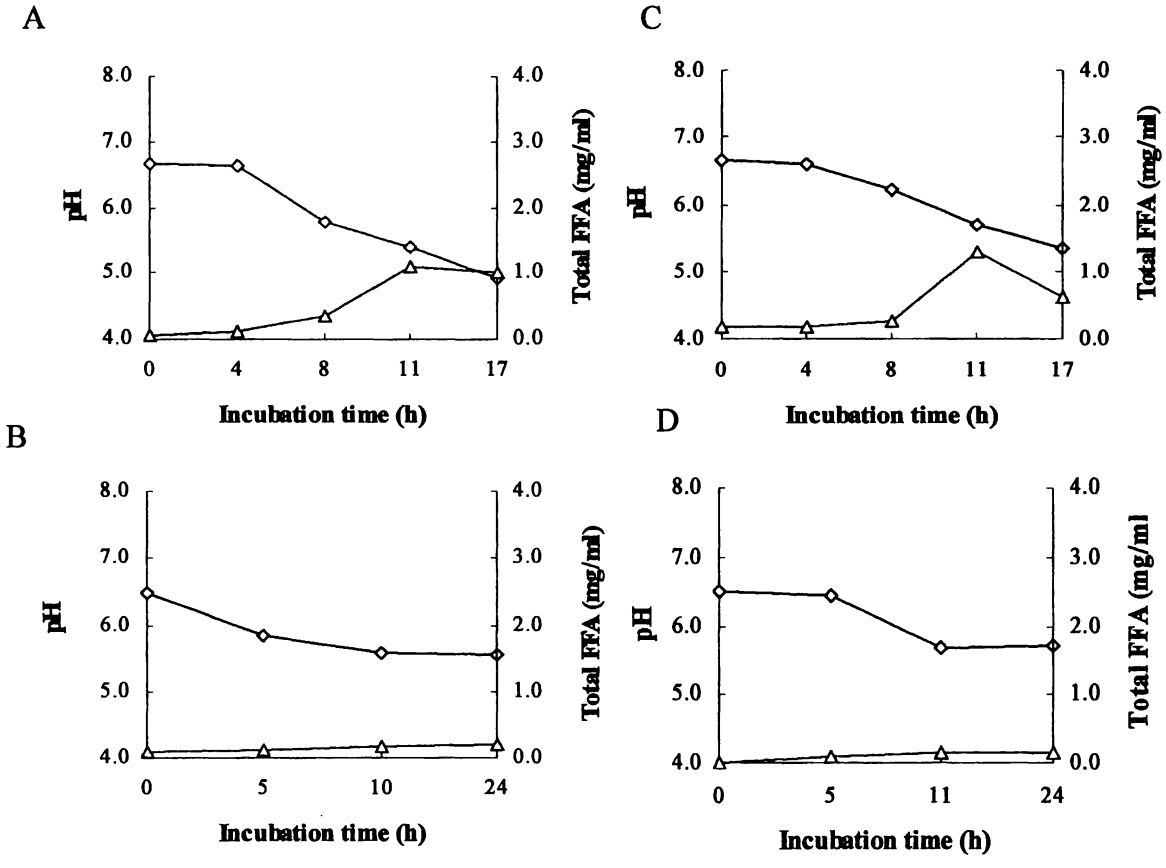
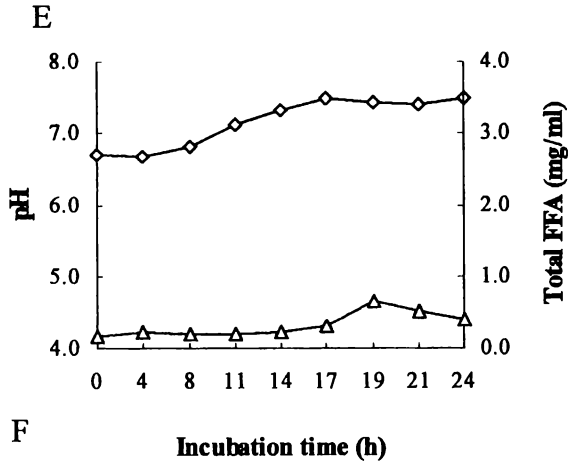
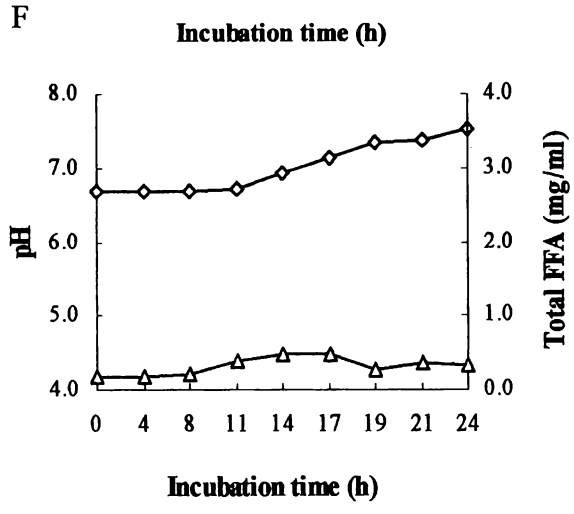


Figure 5.1: Changes in (◊) pH and (Δ) total FFA in 10% reconstituted whole milk inoculated at 55°C with (A) *B. stearothermophilus* A^m; (B) *B. stearothermophilus* B^m; (C) *B. stearothermophilus* C^m; (D) *B. licheniformis* D^m.





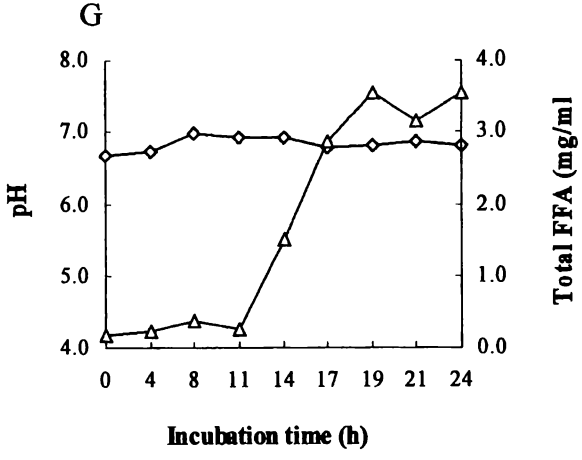


Figure 5.2: Changes in (◊) pH and (Δ) total FFA in 10% reconstituted whole milk at 55°C with (E) *B. licheniformis* F/G; (F) *B. licheniformis* F^{ta}; (H) *B. subtilis*.

Two trends in pH changes were seen: one was a decrease in the reconstituted whole milk samples inoculated with A^m, B^m, C^m and D^m isolates; and the other was an increase in milk samples inoculated with F/G, F^{ta} and *B. sub.* isolates (Figs. 5.1a and b). Levels of FFA released were different amongst the isolates, *B. subtilis* released 2 to 6 times more total FFA than any other isolates, but still kept a constant pH of milk at 6.7 and the milk appeared normal. Decreases in pH were very fast in the milk samples inoculated with A^m, B^m C^m and D^m isolates. The milk reached pH < 5.0 within 10 h inoculation, and showed curdling at the same time. This might be caused by the release of acetic acid (data not shown). Although proteolysis can also cause milk curdling, it will not result in significant drop of pH in milk.

The results showed that the different *Bacilli* behaved differently in the same environment, in this case whole milk. This implies that simply monitoring changes in bacterial numbers, such as thermophile counting, will not give any indication of product quality. This suggests that methods not only can measure the enzyme levels, but also detect the actual enzyme effects, such as FFA release, will be a better indicator for milk powder quality during processing and for storage.

5.2.5 Cultivation of Lipase from the Seven *Bacilli*

The seven *Bacilli* were grown modified C-medium. To 1 L C-medium, 1.0 g of partially hydrolysed milkfat (Industrial Research, 1994) with 10 g gum arabic were homogenised into the medium using the Ultra-turrax. at 8,000 rev/min for 3 min, then autoclaved. Each of 10 mL skim milk culture (20 h at 55°C) was inoculated into 200 mL above medium (air/medium ratio of 9:1) with shaking (110 rpm) at 55°C for 20 h. Lipases were recovered from the cultures by homogenising for 1 min using the Ultra-turrax, then centrifuging at 4,500 x *g* for 20 min at 4°C, and supernatant obtained. Each

obtained. Each supernatant was diafiltered to 20-fold concentrate using a YM10 membrane, and retained for further enzyme characteristics studies.

5.2.6 RAPD PCR-Confirmation of *Bacillus* Isolates

The seven *Bacilli* detailed in Section 5.1 were subcultured numerous times during the production of enzymes. Repeated subculturing can lead to a change in the characteristics of the strains due to either mutation or selection. The PCR method was used to check if the working cultures in the experiments all remained identical to the original cultures, so that legitimate comparisons could be made.

In order to maintain culture identity, the RAPD-PCR technique (detailed in Section 2.5.1) was applied to compare the sets of DNA from the working cultures with the ones from the initial isolates every six months. One set of results is presented in Fig. 5.3.

The working cultures proved to be identical to the original cultures throughout the study (Fig. 5.3). The RAPD-PCR technique was therefore useful not only to identify the isolates in milk powder, but also to ensure consistency between working cultures and original cultures.

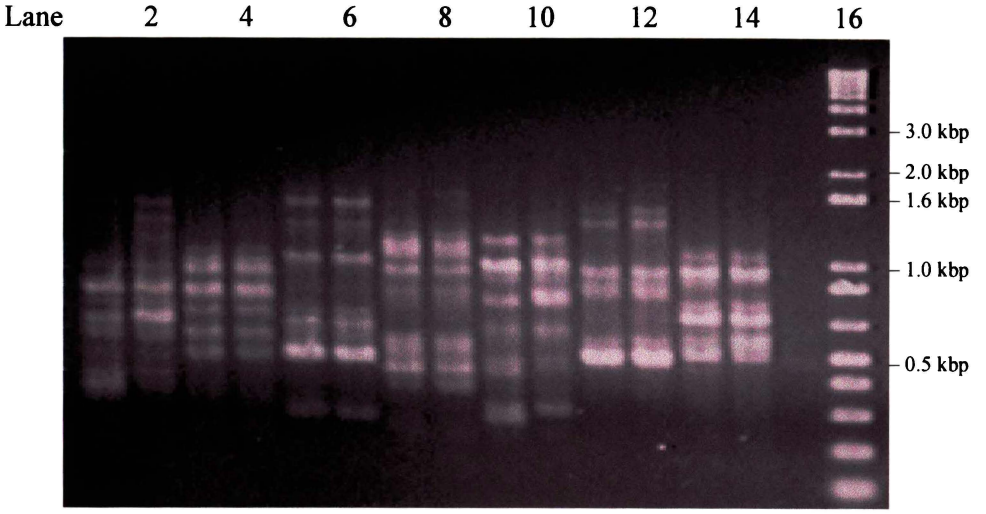


Figure 5.3: The DNA of seven *Bacilli* isolated on 1.5% agarose gel: Lane 1 and 2 – subculture and original A^m, respectively; lane 3 and 4 – subculture and original B^m, respectively; lane 5 and 6 – subculture and original C^m, respectively; lane 7 and 8 – original and subculture F/G, respectively; lane 9 and 10 – original and subculture D^m, respectively; lane 11 and 12 – original and subculture F^{ta}, respectively; lane 13 and 14 – original and subculture *B. sub.*, respectively; lane 15 – negative control; and lane 16 DNA markers.

5.3 Purification of *Bacillus* Protease and Lipase

5.3.1 Preliminary Purification of *Bacillus* Enzymes from Skim Milk Medium

The seven *Bacilli* were inoculated in 150 mL skim milk, and grown with shaking (110 rpm) for 24 h. Enzymes were harvested from the cultures by centrifugation at 4,500 x *g* for 20 min at 4°C, and supernatants obtained. To 20 mL each of the supernatants, 0.8 mL of 0.5 M Tris-HCl buffer was added to give a pH 7.2. The samples then were loaded onto multiple 5 mL Q Fast Flow (Amersham Pharmacia Biotech 17-0510-01) ion-exchange columns at a flow rate of 1.5 mL/min using a 16-port manifold (AllTech). The columns were washed with 20 mL 20 mM Tris buffer, pH 7.2, then eluted in 3 steps: (1) 20 mL 0.5 M NaCl; (2) 20 mL 1 M NaCl; and (3) 20 mL 2.5 M NaCl. Fractions of 5 mL were collected and assayed for protease and lipase activity using

FTC-β-casein and *p*NP palmitate, respectively (methods detailed in Sections 2.1.1.5 and 2.1.2.4). The results are presented in Table 5.4.

Table 5.4: *Separation of Bacillus protease and lipase from skim milk medium*

Protease	Initial activity[1] (mU/mg-protein)	After QFF[2] (mU/mg-protein)	Purification factor	Recovery (%)
A^m	0.8	1.9	2.4	71
F/G	0.5	1.0	2.0	77
F^{ta}	1.5	1.5	1.0	50
B. sub.	42.0	35.8	< 1.0	25
Lipase	**Initial activity[3] (mU/mg-protein)**	**After QFF (mU/mg-protein)**	**Purification factor**	**Recovery (%)**
A^m	0.4	1.1	2.8	89
B^m	0.5	0.7	1.4	79
C^m	0.3	0.5	1.7	110
D^m	0.4	0.4	1.0	40
F/G	0.6	0.4	< 1.0	28
F^{ta}	0.2	0.2	1.0	41
B. sub.	2.0	0.2	< 1.0	2

[1]Protease was assayed using FTC-β-casein in 0.1 M Hepes buffer, pH 7.0, at 40°C for 3 h; one unit was defined as the release of 1 μmole/min FITC under the assay conditions (Section 2.1.1.5). B^m, C^m and D^m isolates had no detectable protease activity and were not listed in the table. Protein was assayed using the BioRad method (Section 2.2.2).

[2]QFF: Q Sepharose fast flow anion-exchange.

[3]Lipase was assayed using *p*NP palmitate in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ at 40°C for 11 h; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4).

The results showed that Q Fast Flow ion-exchange was not a good step for separating certain types of *Bacillus* protease and lipase produced in skim milk medium (Table 5.4). Purification was achieved on the lipases produced by A^m, B^m and C^m at 1.4 to 2.8 fold. This suggested that they might have different isoelectric points from the lipases produced by other isolates. However, the differences amongst the proteases were unclear at this stage, because three out of the seven isolates had no detectable protease

activity using the most sensitive fluorescent assay. This might be growth related (see Table 5.2 for details).

5.3.2 Partial Purification of Protease from C-medium

From the seven *Bacilli*, *B. stearothermophilus* (A^m), *B. licheniformis* (F^{ta}) and *B. subtilis* (B. sub.) were selected for protease production and characteristics comparison. *B. stearothermophilus* A^m was grown in 8 L C-medium (Section 2.5.2) using a 10 L Fermenter (Thermophile Research Unit, University of Waikato) connected to a LH Series 210 control unit. The fermentation was carried out at an agitation of 200-300 rpm and O_2 flow of 4-10 cm^3/min at 55°C. *B. licheniformis* F^{ta} and *B. subtilis* were grown in the same manner but at 37°C. Growth was monitored by measuring the absorbance at A650 nm. Protease production was monitored at the same time. Cultures of 1 mL each were centrifuged at 10,000 x *g* for 5 min at 4°C, and supernatant obtained for protease assay using Suc-AAPF-*p*NA (Section 2.1.1.4). When the protease activity reached a maximum, the cultures supernatant were obtained by passing through a 0.1 μm Hollow-fibre. To 8 L supernatant, 1.2 kg NaCl was added, mixed for 30 min at room temperature and then loaded onto a 1 L Phenyl Sepharose column at a flow rate of 100 mL/min. The column was washed with 5 L 2.5 M NaCl and protease was eluted in 3 steps: (1) 3 L 0.5 M NaCl; (2) 4 L deionised water; and (3) 2 L 50% ethanediol.

Fractions of 1 L were collected and assayed for protease activity. The active fractions were pooled and ultrafiltered using a PLBC Prep/Scale-TFF cartridge with 3 kDa cut-off cellulose membrane (Millipore CDUF-001LB, Millipore Corporation, Bedford, MA, US) to a 15-fold concentration.

The active fraction of 40 mL (~ 0.3 mg/mL protein) each from F^{ta} and B. sub. proteases, respectively, was further purified using a Mono S HR10/10 column (Amersham Pharmacia Biotech 17-0557-01). For F^{ta} protease, 50 mM Mes buffer, pH 6.5, was used, whereas for B. sub. protease, 50 mM Mes buffer, pH 5.5, was used. In both cases, the column was run at a flow rate of 1 mL/min. Each column was washed with 20 mL of the appropriate buffer, then eluted with a 50 mL linear gradient from 0 to 1 M NaCl in the buffer. Fractions of 2 mL were collected and assayed for protease activity.

The most active fractions were pooled and ultrafiltered 20 fold using a YM1 membrane. Samples of 4 μL each were mixed with 1 μL 5 x sample buffer, boiled for 5 min and electrophoresed on 12% SDS-PhastGels. The gels were silver-stained. The same amount of each sample was further separated using isoelectric focusing (IEF) on PhastGel IEF 3-9. Proteins can be separated by charge in a continuous pH gradient until it reaches a pH that the total charge of the protein is zero and the pH is that of isoelctric point (pI) of the protein (Dunn, 1989). The IEF gels were then activity overlay to identify protease (Sections 2.1.1.6 and 2.3.2). The results are presented in Table 5.5 and Fig. 5.4.

The results showed that the proteases produced by the *Bacilli* were purified 9-46 fold after one single Phenyl Sepharose hydrophobic interaction step, which also gave good recovery of more than 80% (Table 5.5). Therefore, Phenyl Sepharose was a good matrix for purification of the proteases. With a further ion-exchange step resulted in the homogeneous preparations of the F^{ta} and B. sub proteases on SDS-PAGE.

Table 5.5: Proteases purification from C-medium

Fractions	Protease[1]		
	A[m] (*B. steraothermophilus*)	**F[ta]** (*B. licheniformis*)	**B. sub.** (*B. subtilis*)
Culture supernatant (U/mg)	0.2	1.5	0.1
Phenyl Sepharose (U/mg)	1.9	14.2	4.0
Purification Factor	9	9	40
Recovery (%)	93	81	105
Mono S (U/mg)	Not tested	26.7 (pH 6.5)	8.1 (pH 5.5)
Purification Factor	-	2	2
Recovery (%)	-	35	114
est. pI[2]	6.0	8.0	9.0
est. molecular mass[3]	Not tested	35 kDa	29 kDa

[1]Protease was assayed using Suc-AAPF-*p*NA in 0.1 M Hepes buffer, pH 7.0, at 40°C for 5-10 min; one unit was defined as the release of 1 μmole/min *p*NA under the assay conditions (Section 2.1.1.4). Protein was assayed using the Lowry method (Section 2.2.3).

[2]pI of the protein was calculated according to the position of the pI standards (Sections 2.1.16 and 2.3.2).

[3]Molecular mass of the protein was calculated according to the migration distance of the markers on SDS-PAGE (Section 2.3.2).

However, proteases produced by the three different species of *Bacilli* (found in the milk powder production streams) were different in both pI and molecular mass (Fig. 5.4). This is also indicated by the results obtained in later Section 5.4. This shows that simply testing thermophile numbers will not give any indication of enzyme effects on product quality.

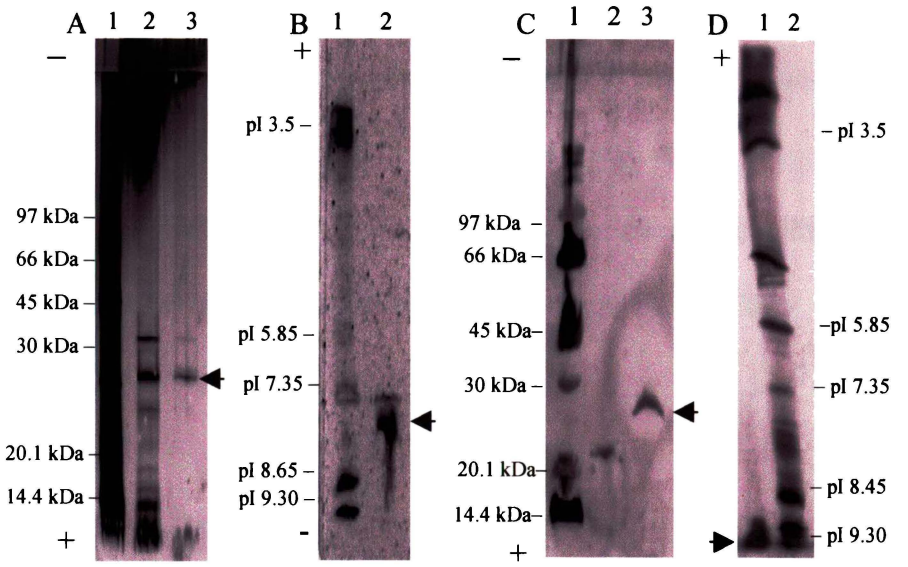


Figure 5.4: Electrophoresis of F^{ta} and B. sub. proteases (Mono S fractions) on 10-15% SDS PhastGel and PhastGel IEF, and silver-stained. (A) SDS PhastGel – lane 1: LMW 14 -97 kDa markers (Amersham Pharmacie Biotech); lane 2 and 3 F^{ta} protease Mono S fraction 1 and 2, respectively. (B) PhastGel IEF – lane 1: broad pI (3.5-9.3) kit (Amersham Pharmacia Biotech); lane 2: protease F^{ta} Mono S fraction 2. (C) SDS PhastGel – lane 1: LMW markers; lane 2 and 3 B. sub. protease Mono S fraction 1 and 2, respectively. (D) PhastGel IEF – lane 1: B. sub protease Mono S fraction 2; lane 2: pI standards.

5.3.3 Partial Purification of A^m Lipase

A crude A^m lipase preparation (36 U in 6 mL; a gift from Dr. J. Bragger, The University of Waikato) was applied onto a Mono Q HR 10/10 column (equilibrated with 20 mM BTP buffer, pH 7.2) at a flow rate of 1 mL/min. The column was washed with the equilibration buffer, then eluted with a 100 mL linear gradient of 0 to 1 M NaCl at a flow rate of 1 mL/min. Fractions of 1 mL were collected.

The active fractions were pooled, 2 M NaCl was added and applied onto a Phenyl-Superose HR 5/5 column (Amersham Pharmacia Biotech) at a flow rate of 0.5 mL/min.

The Phenyl Superose column was washed with the equilibration buffer containing 2 M NaCl, then eluted with a 35 mL linear gradient of 2 to 0 M NaCl, followed by a one-step elution of 10 mL 30% acetonitrile. The active fractions were pooled, dialysed (Spectrum 3.5 kDa cut-off) against 5 L deionised water overnight. The semi-purified A^m lipase was retrieved and assayed using *p*NP caproate (Section 2.1.2.4). The results are presented in Table 5.6.

Table 5.6: *Purification of A^m lipase*

Fractions	Protein[1] (mg/mL)	Lipase activity[2] (U/mg)	Purification factor
Starting material	0.300	20	-
Mono Q	0.030	80	4
Phenyl Superose	0.001	240	12

[1]Protein was assayed using the BCA method (detailed in Section 2.2.1).

[2]Lipase was assayed using *p*NP caproate in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ and 0.1% Triton X-100, at 60°C for up to 10 min; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4).

The results showed that the A^m lipase was purified 12 fold using two chromatography steps of ion-exchange and hydrophobic interaction (Table 5.6). Ion-exchange chromatography gave similar results here and in Table 5.4, although the enzyme was prepared from different media. The active fractions were pooled and used for further characteristics studies.

5.4 Preliminary Characterisation of Protease from the Seven *Bacilli*

5.4.1 Classification of Protease from the Seven *Bacillus*

Proteases are commonly classified into four groups of serine, cysteine, aspartic and metallo-proteases distinguished by the nature of their active sites. Inhibitors such as PMFS and 1,10-phenanthroline are commonly used to identify serine and metallo-

proteases, respectively (Neurath, 1989). The literature review indicated that *Bacilli* commonly produce both serine and metallo-proteases (Priest, 1977; Daniel *et al.*, 1995).

The types of proteases were determined from each of the seven *Bacilli* using crude preparation obtained in Section 5.2.3. The assays were performed in the presence and absence of 12.5 mM PMSF or 4 mM 1,10-phenanthroline, and in a mixture of these two inhibitors. The enzymes were first treated with the inhibitors at room temperature for 30 min, then assayed for protease activity using FTC-β-casein (Section 2.1.1.5). Thermolysin was used as a positive control. The results are presented in Table 5.7.

Table 5.7: *Classification of protease from the seven Bacilli*

	Activity[2] (%) with			
Protease ID.	control (mU/mL)	+ 12.5 mM PMSF[2]	+ 4 mM 1,10-phenanthroline	12.5 mM PMSF and 4 mM 1,10-phenanthroline
Thermolysin	3.6	22	0	0
A^m	1.0	26	21	0
B^m	0.2	42	21	0
C^m	0.8	43	6	0
D^m	0.4	33	77	8
F/G	0.3	0	66	2
F^{ts}	0.3	2	88	0
B. sub	1.1	40	25	0

[1]Proteases were first treated with the inhibitors at room temperature for 30 min, then assayed assayed using FTC-β-casein in 0.1 M Hepes buffer, pH 7.0, at 40°C for 30 min. One unit was defined as the release of 1 μmole/min FITC under the assay conditions (Section 2.1.15). Activity without any treatment were taken as 100%.

[2]PMSF: phenylmethylsulphonyl fluoride.

The data shown in Table 5.7 indicated that all of the *Bacillus* proteases were susceptible to PMSF. However, they can be divided into four groups: (1) A^m, B^m, and *B. sub*; (2)

C^m; (3) F/G and F^{ta}; and (4) D^m. Groups (1) and (2) were sensitive to the zinc chelator, while groups (3) and (4) were more resistant. C^m was very sensitive to 1,10-phenanthroline, and in this respect was very similar to thermolysin. There was only one type of protease produced by F/G and F^{ta} isolates – a serine protease sensitive to PMSF. The 1st and 4th groups produced two types of proteases – a serine and a metallo-protease, because only a combination of inhibitors stopped the activity completely. The results support the finding from Priest (1977) that *Bacilli* generally produce extracellular serine or metallo proteases with very few exceptions. The results also support the finding in Table 5.5 that the F^{ta} protease is different from the A^m and B. sub. proteases, not only on its pI and molecular mass, but also on its mechanism for activity.

5.4.2 pH Optima of the *Bacillus* Proteases

The effect of pH on the activities of the seven *Bacillus* proteases obtained in Section 5.2.3 was determined in 0.1 M potassium phosphate buffers (KH_2PO_4 and K_2HPO_4) over the pH range 4.5 to 11.5 using FTC-β-casein at 40°C for 3 h (Section 2.1.1.5). The results are presented in Table 5.8.

Most of the proteases had an alkaline pH optimum, except B. sub. which had a pH optimum at 7 or just below (Table 5.8). In general, bacterial proteases have a broad pH range for activity, and commonly have an alkaline pH optimum (Daniel *et al.*, 1995). Generally speaking milk has a pH of 6.7, so the proteases will be quite active in milk.

Table 5.8: *pH Optima for the activities of the seven Bacillus proteases*

Assay pH	Protease activity[1](%) for						
	A^m	B^m	C^m	D^m	F/G	F^{ta}	B. sub.
Activity[2] (mU/mL)	1.1	0.2	1.1	0.7	0.5	0.5	1.1
4.5	61	69	36	49	24	51	43
5.0	88	71	38	49	26	55	93
6.0	71	71	49	59	29	64	97
7.0	89	86	75	57	57	60	100
8.0	100	99	96	100	83	85	87
9.0	80	98	100	51	87	89	83
10.0	79	98	99	47	88	95	78
11.0	73	95	96	37	90	95	74
11.5	68	100	91	41	100	100	60

[1]Protease activity was assayed using FTC-β-casein in 0.1 M phosphate buffer, pH 4.5 to 11.5, at 40°C for 3 h (detailed in Section 2.1.1.5).

[2]Activity values were taken as 100%.

5.4.3 Heat-stability of the Seven *Bacillus* Proteases

The heat-stabilities of the seven *Bacillus* proteases (obtained in Section 5.2.3) were determined using enzyme solutions containing ~ 3 mg/mL protein. The enzyme solutions were first heated at 50°C to 90°C for 10 min, quickly chilled on ice, and then assayed for protease activity. Assays were carried out using Suc-AAPF-*p*NA (Section 2.1.1.4). Because the F/G protease did not cleave Suc-AAPF-*p*NA, it was assayed using FTC-β-casein instead (Section 2.1.1.5). The results are presented in Table 5.9.

The seven *Bacillus* proteases showed different heat-stabilities. The enzymes from A^m, B^m, C^m and D^m isolates withstood heating at 80°C for 10 min, and retained 19 to 58% of initial activities, therefore were considered more heat-stable than the proteases from F/G, F^{ta} and *B. sub.* isolates (Table 5.9). The proteases from B^m, C^m and D^m isolates

appeared to lose about 40%, 70% and 50% of their initial activity, respectively, at a relatively low temperature, but they were not further affected at higher temperatures.

Table 5.9: *Heat-stabilities of seven* Bacillus *proteases*

Heat-treatments	Protease activity[2](%) for						
	A^m	B^m	C^m	D^m	F/G	F^m	B. sub.
Activity[1] (mU/mL)	1.0	0.3	0.5	0.2	0.3	3.0	2.7
Untreated	100	100	100	100	100	100	100
50°C	114	99	85	57	98	110	170
60°C	119	84	29	70	98	63	53
70°C	75	59	35	64	70	6	47
80°C	19	24	24	58	4	6	4
90°C	3	24	22	64	3	6	5

[1]Activity values were taken as 100%.

[2]Proteases were first heated at 50°C to 90°C for 10 min, chilled on ice, and then assayed using Suc-AAPF-*p*NA in 0.1 M Hepes buffer, pH 7.0, at 40°C for 1-3 h, except for F/G protease which was assayed using FTC-β-casein in 0.1 M Hepes buffer, pH 7.0, at 40°C for 5 h min (Sections 2.1.1.4 and 2.1.1.5).

The low-heat inactivation phenomenon has been reported in the inactivation of psychrotrophic proteases in milk (Barach *et al.*, 1976 and 1978). This could suggest the presence of more than one enzyme, one of these being the most stable of all the enzymes tested. The inhibitor results also indicated that B^m and D^m had two proteases present. This is shown in the heat-stability data by one protease losing its activity, and the other remaining active. For C^m protease, it is possible that a conformational change in the enzyme resulted in higher stability, as there was no other evidence for more than one enzyme.

5.4.4 Kinetics of A^m Protease

The V_{max} and K_m of semi-purified A^m protease obtained in Section 5.3.2 was determined using azocasein and FTC-β-casein at 40°C, and Suc-AAPF-*p*NA at 40°C and 20°C (Sections 2.1.1.3, 2.1.1.4 and 2.1.1.5). The results are presented in Table 5.10 and Figs. 5.5 to 5.8.

Table 5.10: *V_{max} and K_m of A^m protease on FTC-β-casein and Suc-AAPF-*pNA

Substrate	Assay Temperature	V_{max}(x 10^{-4})	K_m(mM)	V_{max}/K_m
Azocasein	40°C	0.83 (Δ420 nm/min)	0.11[1]	7.5
FTC-β-casein	40°C	7.00 (mM/min)	0.27[2]	25.9
Suc-AAPF-*p*NA	40°C	3.45 (mM/min)	0.23	15.0
Suc-AAPF-*p*NA	20°C	2.30 (mM/min)	1.22	1.9

[1]The K_m for azocasein was 0.26% and calculated to be 0.11 mM with respect to casein (Mr of 23,000 Da).

[2]The K_m for FTC-β-casein was 0.62% and calculated to be 0.27 mM with respect to casein (Mr of 23,000 Da).

The A^m protease gave similar K_m using all three substrates at 40°C (Table 5.10). The results showed that the protease recognised all the substrates equally regardless of the substituted peptide groups. The FTC-β-casein assay gives the highest ratio between V_{max} and K_m, therefore is the "best" assay substrate, followed by Suc-AAPF-*p*NA at 40°C. The results are consistent with the finding from Section 3.2.1 that the fluorescent assay was the most sensitive method for detecting protease activity in milk powders.

However, the azocasein gave the lowest K_m value, indicated that azocasein has the highest affinity for the protease. Even if it is made to 1000 K_m, the velocity can only increase 2-fold, which will probably not increase the assay sensitivity by much.

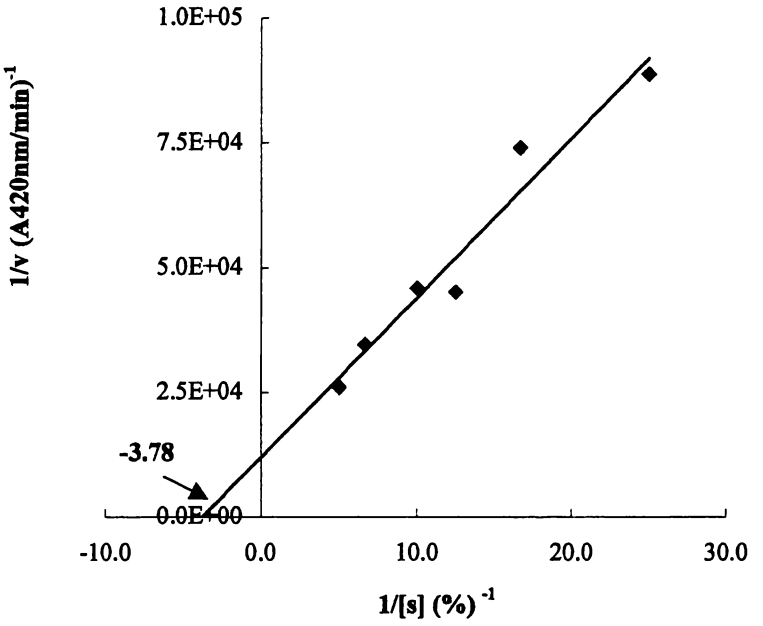


Fig. 5.5: Double reciprocal (1/*v* versus 1/[s]) Lineweaver-Burk plot of the A^m protease using azocasein as substrate at 40°C. Each data point represents an average of triplicate.

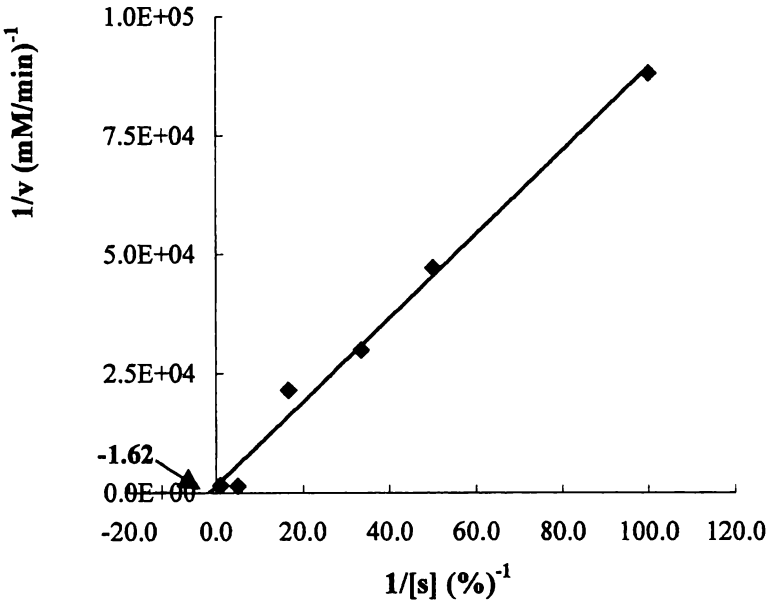


Fig. 5.6: Double reciprocal (1/*v* versus 1/[s]) Lineweaver-Burk plot of the A^m protease using FTC-β-casein as substrate at 40°C. Each data point represents an average of triplicate.

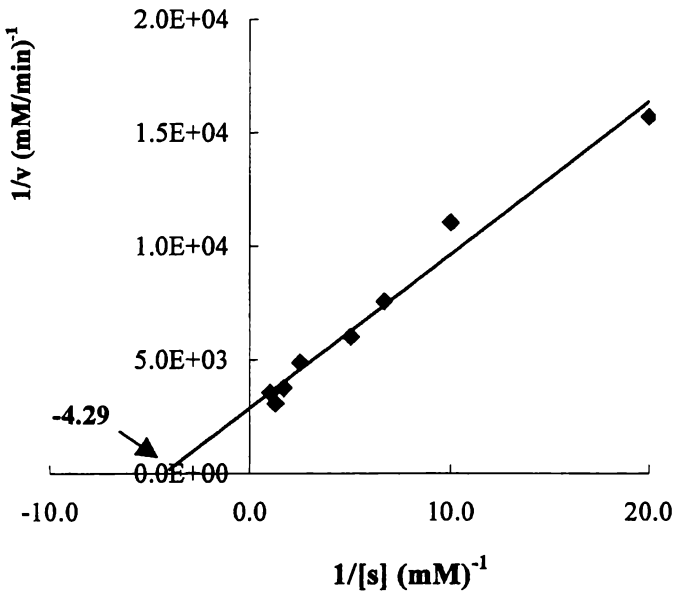


Fig. 5.7: Double reciprocal (1/*v* versus 1/[s]) Lineweaver-Burk plot of the A^m protease using Suc-AAPF-*p*NA as substrate at 40°C. Each data point represents an average of duplicate.

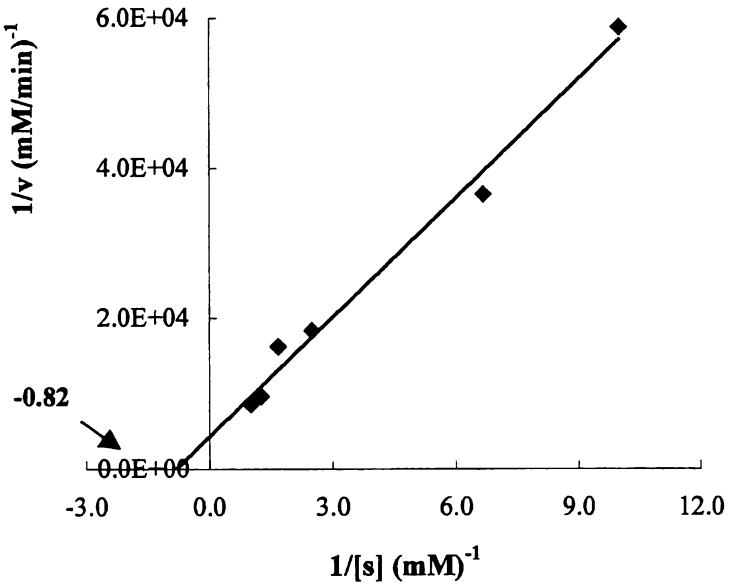


Fig. 5.8: Double reciprocal (1/*v* versus 1/[s]) Lineweaver-Burk plot of the A^m protease using Suc-AAPF *p*NA as substrate at 20°C. Each data point represents an average of duplicate.

5.5.1 *Bacillus* Lipase Sensitivity to Inhibitors

Bacterial lipases have been reported to be sensitive to various inhibitors, such as PMSF, PCMB (Stead, 1986) and heavy metal ion Hg^{2+} (Handelsman and Shoham, 1994). The lipases obtained from the seven *Bacilli* in Section 5.2.5, were first treated with 1 mM of each the inhibitors at room temperature for 60 min, then assayed for lipase activity using *p*NP caproate in 0.1 M Mops buffer, pH 7.2, containing 0.1% Triton X-100, at 60°C for up to 15 min (Section 2.1.2.4). The results are presented in Table 5.11.

Table 5.11: *Effects of inhibitors on lipase activity from the seven* Bacilli

Inhibitors	Lipase activity (%)						
	A^m	B^m	C^m	D^m	F/G	F^{ta}	B. sub.
Activity[1] (U/mL)	0.19	0.11	0.08	0.11	0.09	0.10	0.10
Control	100	100	100	100	100	100	100
1 mM PMSF[2]	58	88	82	78	85	37	38
1 mM $HgCl_2$	93	105	97	91	106	73	95
1 mM PCMB[3]	100	106	102	90	90	72	87

[1]Lipase activity was assayed using *p*NP caproate in 0.1 M Mops buffer, pH 7.2, containing 0.1% Triton X-100 at 60°C for 10 to 15 min. One unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4). Activity values were taken as 100%.

[2]PMSF: Phenylmethylsulphonyl fluoride.

[3]PCMB: *p*-Chloromercuribenzoic acid.

The results showed that all lipases were sensitive to PMSF, and relatively less sensitive to PCMB and the heavy metal Hg^{2+} (Table 5.11). The lipases from A^m, F^{ta} and B. sub. isolates were more susceptible to PMSF (42-63% loss of activity) than those from B^m, C^m, D^m and F/G isolates (12-23% loss of activity). The data indicated that there might be at least two types of lipases produced by the seven *Bacilli*. The first group may contain a Ser in the active sites which was partially blocked by the serine inhibitor PMSF. However, these were crude enzymes preparations, therefore it is possible that

proteases were produced concomitantly that gave esterases activity as indicated in Section 5.2.2.

5.5.2 pH Optima of Lipases from the Seven *Bacilli*

The lipases obtained in Section 5.2.5 were also assayed at between pH 4.5 and 9.0 in 0.1 M Na-citric acid, bis-Tris and bis-Tris-propane (BTP) buffers at 60°C for 10-30 min using *p*NP caproate as the substrate (Section 2.1.2.4).. The results are presented in Table 5.12.

Table 5.12: *Lipase activity in various pH buffers*

Assay	Lipase activity[1](%) for						
pH	A^m	B^m	C^m	D^m	F/G	F^{ta}	B. sub.
Activity[2] (U/mL)	0.27	0.11	0.08	0.11	0.09	0.19	0.10
4.5	2	0	0	0	0	0	0
5.0	52	0.7	0	0.5	0	3	0
6.0	100	30	29	21	22	100	85
7.0	69	100	100	100	100	53	100

[1]Lipase activity was assayed using *p*NP caproate in 0.1 M Na-citric acid, bis-Tris and bis-Tris propane buffer at pH between 4.5 and 9.0, at 60°C for 10-30 min (Section 2.1.2.4). Because of substrate autolysis at 60°C and pH ≥ 8.0, so no activity could not be determined at pH ≥ 8.0, so the results were not listed in the table.

[2]Activity values were taken as 100%.

Because *p*NP caproate is unstable at high pH and temperatures, results at pH ≥ 8 and 60°C could not be obtained. Therefore, the pH optima of *Bacillus* lipases other than A^m and F^{ta} are not clear.

5.5.3 Heat-stability of Lipases from the Seven *Bacilli*

The seven *Bacilli* lipases obtained in Section 5.2.5 were assessed for heat-stability at ~ 3 mg/mL protein concentration. The enzyme solutions were first heated at 50°C to 90°C for 10 min, then chilled on ice immediately and assayed for activity. The assay was carried out using *p*NP caproate in 0.1 M Mops buffer, pH 7.0, containing Triton X-100 at 60°C for 10 to 15 min (Section 2.1.2.4). The results are presented in Table 5.13.

Table 5.13: *Thermostability of the seven* Bacillus *lipases*

Treatment	Lipase activity[1](%) for						
	A^m	B^m	C^m	D^m	F/G	F^{ta}	B. sub.
Activity[2] (U/mL)	0.19	0.11	0.08	0.11	0.09	0.10	0.10
Unheated	100	100	100	100	100	100	100
50°C	99	103	99	96	94	98	83
60°C	100	104	100	95	95	75	58
70°C	99	97	94	97	91	64	42
80°C	18	98	90	97	82	64	40
90°C	15	97	89	97	87	62	34
cal. $t_{1/2}$ (min) at 90°C	3.7	227.6	59.5	227.6	49.8	14.5	6.4

[1]Lipase was heated at various temperature for 10 min, then chilled on ice and assayed using *p*NP caproate in 0.1 M Mops buffer, pH 7.2, containing 0.1% Triton X-100 at 60°C for 10 to 15 min; one unit was defined as the release of 1 μmole/min *p*NP under the assay conditions (Section 2.1.2.4).

[2]Activity values were taken as 100%.

All *Bacillus* lipases retained more than 90% of initial activity after heat-treatment of 70°C for 10 min in a buffer system, except F^{ta} and B. sub. Four of them were even more heat stable – B^m, C^m, D^m and F/G retained more than 85% of the initial activity even after heat-treatment of 90°C for 10 min, and also see calculated $t_{1/2}$ values (Table 5.13).

It is known that protease and lipase are often produced concomitantly by the bacterium. The proteases produced by F^{ta} and *B. sub.* isolates retained 6% and 5% of activity, respectively, after heating at 90°C for 10 min (Table 5.8), while the lipases from these two isolates retained 62% and 34% of activities, respectively, after the same heat-treatment, so the lipases from the seven *Bacilli* were more heat-stable than their proteases. Although the A^m lipase was the least stable, it retained 15% of initial activity after 90°C for 10 min, which is more stable than its protease. All the lipases were therefore more heat-stable than the proteases from the same organism, which is in agreement with the findings of Andersson *et al.* (1979).

The heat-stability data were obtained in buffer systems, and gave comparable high $t_{1/2}$ even at 90°C. A *Pseudomonas* lipase was reported to have a $t_{1/2}$ of 4 min at 100°C in skim milk (Andersson *et al.*, 1979), probably be due to protection by milk caseins (Kumara *et al.*, 1993). Stead (1986) also reported that once lipase from the growth of psychrotrophic bacteria is present in milk, it is not possible to remove it completely. If the heat-stable *Bacillus* lipases are present in milk, they will clearly survive pasteurization (72°C for 15 s), evaporation and spray-drying processes and remain in WMPs. These enzymes will consequently have the potential to generate flavour defects in WMPs (see later Chapters 6 and 7).

5.5.4 Specificity on *p*-Nitrophenyl Esters of Fatty Acids

The seven *Bacillus* enzymes obtained in Section 5.2.5 were assayed using *p*-nitrophenyl esters of butyrate, caproate, caprylate, caprate, laurate, myristate, palmitate and stearate. Assays were carried out in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ and 0.1% Triton X-100 at 60°C for 10 to 150 min (Section 2.1.2.4). They were also assayed

using tricaproin-plates (method detailed in Section 2.1.2.2). The results are presented in Table 5.14.

Table 5.14: *Hydrolysis of p-nitrophenyl esters of fatty acids and tricaproin by the Bacillus lipases and powder lipase*

*p*NP esters of fatty acids	Lipase activity[1] (%) for							
	A^m	B^m	C^m	D^m	F/G	F^{ta}	B. sub	Powder lipase[2]
Activity[3] (U/mL)	1.74	0.36	0.22	0.26	1.52	0.81	0.24	3.20
butyrate ($C_{4:0}$)	100	100	100	100	57	100	100	78
caproate ($C_{6:0}$)	68	61	54	75	36	50	21	100
caprylate ($C_{8:0}$)	89	18	15	20	76	1	1	90
caprate ($C_{10:0}$)	76	6	5	7	100	ND[4]	ND	47
laurate ($C_{12:0}$)	15	8	8	8	74	3	ND	32
myristate ($C_{14:0}$)	3	3	4	3	37	3	ND	32
palmitate ($C_{16:0}$)	1	2	3	2	23	1	ND	25
stearate ($C_{18:0}$)	ND	1	2	ND	9	1	ND	8
Tricaproin-plate[5] (U/mL)	9.2	est. 0.02	ND	ND	est. 0.10	ND	ND	ND

[1]Assay was carried out in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ and 0.1% Triton X-100, at 60°C for 10 to 150 min using pNP esters of butyrate, caproate, caprylate, caprate, laurate, myristate, palmitate and stearate (Section 2.1.2.4).

[2]Data were from Fig. 4.3.

[3]Activity values were taken as 100%

[4]ND: Not detected.

[5]Assay used tricaproin-plate at 55°C for 24 h, and calculated using the formula y = 3.2Ln (x) +21.9 obtained in Fig. 3.15.

The seven *Bacillus* lipases had different specificities towards *p*-nitrophenyl esters of fatty acids (Table 5.14). The A^m lipase had good activity against *p*NP esters up to $C_{10:0}$. The B^m, C^m and D^m enzymes showed a typical esterase activity in preference for short chain esters up to $C_{6:0}$, and less than 20% of activities up to $C_{12:0}$. The F/G lipase was non-selective and capable of hydrolysing all esters regardless the chain length. This

was confirmed later using partially hydrolysed milkfat (see later Section 5.5.5). Both F^{ta} and B. sub. enzymes were different from the rest that only hydrolysed esters up to $C_{6:0}$. They are probably due to contamination of protease activity since these two isolates produced high levels of proteases in the same medium (Section 5.2.2).

Results obtained on the tricaproin-plate indicated that the lipases from the seven *Bacilli* were different: A^m, B^m and F/G produced lipases which hydrolysed tricaproin; but the ones from C^m, D^m, F^{ta} and *B. sub.* did not hydrolyse the tricaproin. However, some of the isolates had only low levels of activity and it was possible that the sensitivity of assay was too low to detect the amount of lipase activity in the sample. This was, in fact, proved in the next section, where all lipases were produced and able to hydrolyse milk lipids after prolonged incubation.

5.5.5 Specificity of *Bacillus* Lipase on Milk Lipids

Natural milkfat contains 98.3% triacylglycerols (TG), with minor contributions from diacylglycerols (DG: 0.3%) and monoacylglycerols (MG: 0.03%) (Walstra *et al.*, 1999). Some microbial lipolytic enzymes are known to have higher activity on partially hydrolysed milk TG (Macrae, 1980). The *Bacillus* lipases obtained in Section 5.2.5 were therefore checked for activity on a partially hydrolysed milkfat (Industrial Research Ltd., 1994) containing 16.2% MG, 27.6% DG (a mix of 1,2- and 1,3-DG) and 54.5% TG.

For assay, 1.6 g partially hydrolysed milkfat was dissolved in 4 mL ethanol, then disbursed in 25 mL 0.1 M Mops buffer, pH 7.2 (containing 5% gum arabic, 0.1% Triton X-100 and 0.2 mg/mL Penicillin G) by sonicating for 2 min continuously using the Vibra cell. To 3 mL of each lipase sample, 1 mL of milkfat emulsion was added, and

then incubated at 60°C for up to 43 h. Samples of 1 mL were withdrawn at different time points and stopped by addition of 0.1 mL 5 M H_2SO_4. The milkfat was then extracted by 1 mL hexene/*tert*-methyl butyl ether (1:1, v/v) by centrifugation at 10,000 x *g* for 5 min, and the top layer was collected. The top layer was then separated on the TLC plate following the method detailed in Section 2.4.1. The results are presented in Fig. 5.9.

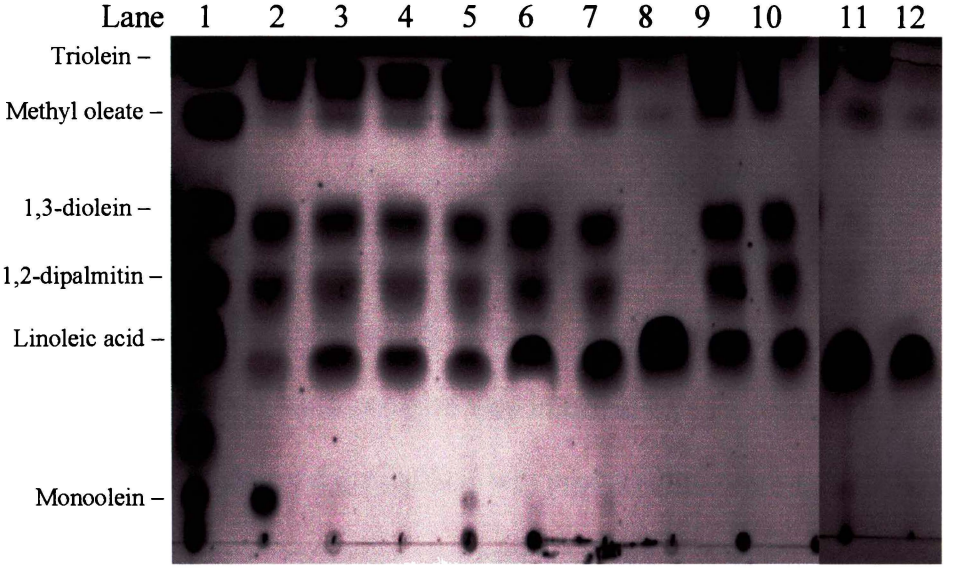


Figure 5.9: Changes in milk lipids composition on Thin Layer Chromatography (TLC). Samples were milk lipids incubated at 60°C with approximately 0.07-0.10 U/mL (*p*NP caproate) of seven *Bacillus* lipases. Lane 1: TLC standard (Nu Chek 18-1A); lane 2: milk lipids (no enzyme added) at zero time. After 45 h, lane 3: milk lipids; lane 4: + A^m lipase; lane 5: + B^m lipase; lane 6: + C^m lipase; lane 7: + D^m lipase; lane 8: + F/G lipase; lane 9: + F^{ta} lipase; lane 10: + B. sub. lipase; lane 11 and 12: milk lipids incubated for 10 and 20 min, respectively, with F/G lipase.

Lipolysis was detected shown as decreases in intensity of milk lipids' spots and increases in intensity of total FFA spots after prolonged incubation (Fig. 5.9). These indicated that all seven *Bacilli* had produced lipases. There were two trends: the A^m, B^m, F^{ta} and B. sub. lipases preferred 1,2-DG over other types of milk lipids; and the C^m,

D^m, and F/G lipases were rather non-specific. The F/G lipase showed much higher activity, shown as complete hydrolysis of MG, 1,2-DG and 1,3-DG after only 10 min incubation at 60°C, and complete hydrolysis of TG after further 10 min incubation. This indicated that the F/G enzyme is actually a true lipase.

Although the majority of milk lipids are TG, it is possible that a true lipase like F/G, can hydrolyses the milkfat rapidly to DG and MG which become substrates for the esterases. Since F/G has been found the predominant contaminate in raw and pasteurised milk. It is possible that the F/G lipase can generate by-products in milk, which will lead to continuation of lipolysis during the milk powder process, and result in "off" odour and flavour during storage. The data again proved that simply testing thermophile numbers does not give any information that correlates to product quality, since different strains of thermophiles will produce different types of enzymes. A single lipase may not have particular consequences, but a combination of lipases could be problematical.

It is fair to predict that the initial quality of milk is also important in terms of its lipid composition and integrity since this will influence susceptibility to lipolysis to great extent. Prior damage (*e.g.* production of MG and DG) to milkfat will lead to high level of lipolysis.

5.5.6 Preliminary Characterisation of the A^m Lipase

5.5.6.1 Molecular Mass and N-terminal Sequence

The semi-purified A^m lipase obtained in Section 5.3.3 was concentrated 10-fold using a Microcon 10 (Amicon, 10 kDa cut-off). To 20 μL sample, 5 μL 5 x sample buffer was

added, and then separated on 12.5% SDS-PAGE. After electrophoresis, activity overlay was applied on the gel (Section 2.1.2.5).

After confirmation of the active protein band by the activity overlay, the sample was re-electrophoresed and transferred onto a PVDF membrane for N-terminal sequencing (Section 2.3.2). The results are presented in Fig. 5.10.

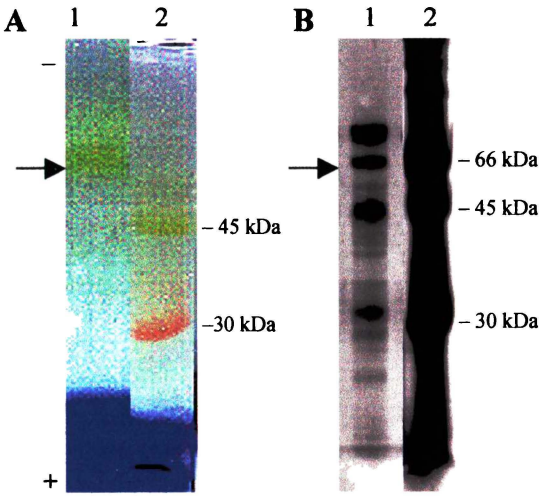


Figure 5.10: Electrophoresis of the A^m lipase (vs Phenyl Superose fraction) on 12.5% SDS-PAGE. (A) An activity overlay using *p*NP caproate at room temperature for 30 min: lane 1 – A^m lipase; and lane 2 – rainbow markers (Amersham Phamacie Biotech RPN756); arrow indicated the yellow band of the release of *p*NP due to lipolysis. (B) Coomassie stain of Western Blotting gel (A) onto PVDF membrane; the lipase band was indicated by the arrow.

The yellow band indicated by the arrow showed the release of *p*NP due to lipolysis. It is a single band has an estimated molecular mass of 67 kDa on SDS-PAG (Fig. 5.10). It gave partial N-terminal sequence as **MERTV VETRY GSLRG**. It has homology with a mesophilic esterase produced by a *B. stearothermophilus* Tok19A isolated from a New Zealand therm field (Wood *et al.*, 1995).

5.5.6.2 A^m Lipase Specificity on Synthetic Triacylgycerols

Specificity of the A^m lipase on synthetic triacylglycerols was determined using different concentrations of synthetic tributyrin (Sigma T8626), tricaproate (Sigma T0888) and tripalmitate (Sigma T5888). The TG was emulsified by sonicating at 50% duty cycle continuously for 2 min in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ and 0.5% gum arabic. The assay was carried out at 65°C for 10 to 60 min. The fatty acids were analysed using the GLC method (Section 2.4.2). The results are presented in Fig. 5.11.

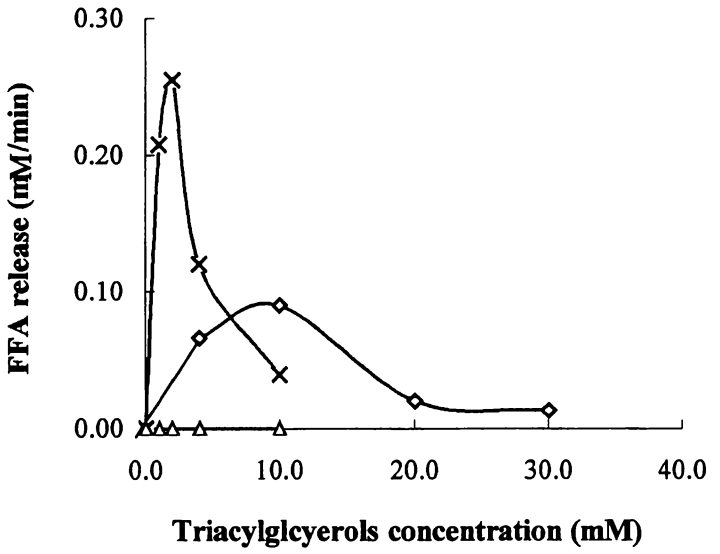


Figure 5.11: The rate of fatty acid release from different concentrations of synthetic triacylglcyerols by the A^m lipase. Substrates were (◊) tributyrin; (×) tricaproin; and (Δ) tripalmitin. The assay was in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ and 0.5% gum arabic, at 65°C for 10 to 60 min. Each data point represents an average of duplicate.

The A^m lipase was active on the short chain synthetic substrates tributyrin and tricaproin, but not on tripalmitin (Fig. 5.11). Increasing concentrations of short chain triacylglcyerols resulted in a decrease in fatty acid release. These results indicate that the A^m enzyme is in fact an esterase, as increasing substrate concentration resulted in

formation of emulsions, and no activity was shown on the longer chain triacylglcyerol. The melting point of tripalmitin is 66.4°C (Creamer and MacGibbon, 1996), so it would have remained solid therefore as an emulsion in the buffer at 65°C, therefore was not cleaved by the A^m esterase. The characteristic of A^m esterase is very similar to the lactic starter lipases reported by Deeth and Fitz-Gerald (1995).

5.5.6.3 A^m Lipase Specificity on Milk Lipids

The A^m enzyme preparation was assayed using natural milk lipids of MG, 1,2-DG, 1,3-DG and TG (separated from the partial hydrolized milkfat; gifts from J. C. S Brown, NZDRI). Milk lipids were dissolved in 0.1 mL ethanol using 0.084 g MG, 0.020 g 1,2-DG, 0.084 g 1,3-DG and 0.084 g TG. Each substrate was disbursed into 2.5 mL 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ and 0.5% gum arabic using the Vibra cell. After addition of 2 *p*NP U/mL A^m lipase each, all samples were incubated at 65°C for up to 3 h. FFA were extracted from 0.5 mL samples and analysed using the GLC method (Section 2.4.2). The results are presented in Fig. 5.12.

The A^m lipase showed the highest specificity on 1,2-DG, followed by MG, with little activity against 1,3-DG and TG (Fig. 5.12). The results correlated well with the TLC results obtained in Section 5.5.5. Therefore, if 1,2-DG are present, the A^m lipase can hydrolyse them rapidly. This emphasises the point of lipid quality in the milk being processed probably being the major determining factor in powder quality with respect to lipolysis.

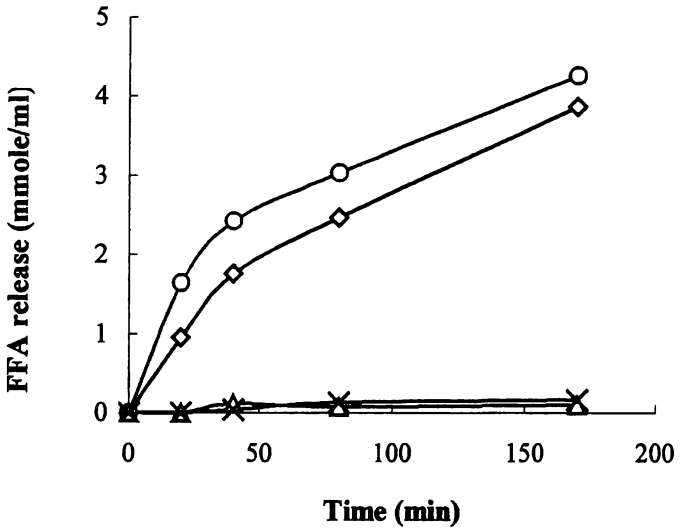


Figure 5.12: Release of total fatty acids from natural milk lipids with time by the A^m lipase. Substrates were (◊) monoacylglycerols; (o) 1,2-diacylglycerols; (Δ) 1,3-diacylglycerols; and (×) triacylgycerols. Assay was carried out in 0.1 M Mops buffer, pH 7.2, containing 5 mM $CaCl_2$ and 0.5% gum arabic, at 65°C for up to 3 h. Each data point represents an average of duplicate.

5.6 Summary

The seven *Bacillus* strains, isolated from milk powder production streams, all produced extracellular and intracellular proteases and lipases (esterases) under the laboratory conditions. These strains preferred milk as the medium for growth and enzyme production. Data from the preliminary characteristics studies showed that different *bacillus* species produce different types of proteases and lipases. These enzymes are heat-stable, as expected, and the lipases are more heat stable than the proteases. For example, A^m enzyme preparation retained 15% initial lipase activity, while only 2.8% protease activity after heat-treatment of 90°C for 10 min in a buffer system; the enzymes from B^m, C^m and D^m were even more stable as shown more than 85% lipase activity and 64% of protease activity retained after the same heat-treatment. The heat stability of these enzymes will be higher in milk because milk proteins will protect them

from heat denaturation. Therefore, if these enzymes are present in the raw milk or produced during the process they will not be destroyed by the typical heat-treatments applied in the milk powder process.

The characteristics studies showed that there were at least two types of proteases being produced – serine- and metallo-proteases. This is quite common for *Bacillus* species. All the proteases had neutral to alkaline pH optima. The kinetic data indicated that FTC-β-casein was the 'best' substrate for assaying proteases, giving the highest V_{max} to K_m ratio.

The lipases (esterases) of the *Bacillus* strains can also be divided into two groups according to their specificities on *p*-nitrophenyl esters: A^m, B^m, C^m, D^m, F^{ta} and B. sub. have specificities on the short chain esters; and F/G was equally effective against longer chain *p*NP esters. The specificity of the first group was identical to the lipase activity found in the whole milk powder (the 'powder lipase', Section 4.5.4), and therefore the 'powder lipase' could originate from a *Bacillus*, except strain F/G. This hypothesis could be tested by obtaining N-terminal sequence of the powder lipase, then comparing the N-terminal sequences of the *Bacillus* lipases (esterases) obtained (*e.g.* A^m lipase sequence, Section 5.5.6.1). This would determine whether they are the same proteins or closely related, but the attempts to obtain the N-terminal sequence of the powder lipase were unsuccessful, so the definitive source of the 'powder lipase' is therefore still unknown.

Further characterisation work on the A^m enzyme confirmed that it is a lipase, which prefer mono- and 1,2-di- and short chain tri-acylglcyerols. The F/G enzyme was

enzyme was apparently a true lipase, and had activity against all milk lipids regardless of their structures.

All data obtained in this chapter suggested that the seven *Bacilli* showed different characters even in the same medium, so the test of thermophile numbers will not give any direct correlation to product quality nor to the spoilage potential. There is a strong probability that establishing tests for different *Bacillus* strains will be an important improvement in specifications, but the essential factor is still to find ways of determining actual enzyme levels in the process and in powders.

Chapter 6 Changes in Enzyme-Spiked Whole Milk Powder during Storage

6.1 Introduction

Many research groups have reported flavour and functionality changes in pasteurized or UHT milk caused by proteolysis or lipolysis during storage (Kishonti, 1975; Chapra and Mathur 1984; Renner, 1988; Choi and Jeon, 1993; Celestino *et al.*, 1997b). However, in contrast, evidence of proteolysis and lipolysis occurring in storage of milk powder is limited and mainly anecdotal.

The nature of milk powder is very different from other dairy products because of the low water content. Water content in milk powders is often expressed as moisture content, which is the total amount of water present relative to dry matter (grams of water per gram of dry matter). However, various types of food may have the same moisture content, but they could differ significantly in perishability. Another way of expressing water content is water activity (a_w) which relates to the amount of free water. Although a_w is not a totally reliable predictor, it is better than moisture content (Fennema, 1996).

Following Lewis's thermodynamic law, a_w is f/f_o, the fugacity of the escape tendency of a solvent from solution divided by the fugacity of the pure solvent (Fennema, 1996). Because there is less than 1% difference between f/f_o and p/p_o or RVP (*i.e.* relative vapour pressure = the water vapour pressure divided by the air pressure saturated with water), so $a_w = p/p_o$ (Fennema, 1996). The true a_w is often lower than the predicted value because of dissociation of compounds from water, presence of larger molecules

and water becoming non-solvent (Walstra *et al.*, 1999), therefore a_w only approximates to RVP (Walstra and Jessen, 1984). The RVP is related to the equilibrium relative humidity (ERH). ERH is measured at 30°C and used as a_w in this thesis.

Moisture content and storage temperature are the main factors for determination of a_w in a milk powder. Milk powders are generally stored at ambient temperature which can range from 20°C to 50°C depending on where the products are marketed (Pisecky, 1997). The composition and the state of the individual components in milk powder also play an important role to a_w. At $a_w < 0.20$, casein is the main water absorber. When a_w is within the intermediate range of 0.20-0.60, the physical state of lactose becomes the dominant factor. When a_w is > 0.60, salts have a marked influence. Milkfat, however, has no effect on a_w. Overall, differences in a_w in milk powders are often due to the state of proteins and the physical state of lactose (Pisecky, 1997).

It is recognised that most bacterial growth is inhibited at $a_w \sim 0.9$, and yeast and mould are inhibited at a lower a_w of 0.88-0.80. Good quality WMP has a maximum moisture of 3.0% (est. $a_w = 0.20 \pm 0.03$; Pisecky, 1997; Walstra *et al.*, 1999). Under normal storage conditions, therefore, little or no microbial growth is expected in milk powders. However, physico-chemical changes may still take place, *e.g.* Maillard reactions, lipid oxidation and crystallization of carbohydrates (Renner, 1988; Pisechy, 1997; Celestino *et al.*, 1997).

To study the impact of thermophilic enzymes on WMPs during storage, known amounts of lipases and proteases were added to 40% (w/v) reconstituted whole milk made from

WMPs, and then freeze-dried to remake to a powder form. Heat-stable *bacillus* lipases and proteases were used.

The enzyme-spiked WMPs were stored at 37°C (the medium temperature range) which allowed experimental results to be obtained within a reasonable time frame. FFA and peptides released as a result of lipolysis and proteolysis, respectively, were analysed after 2 months of storage.

6.2 WMP Spiked with the Enzymes Produced by the Seven *Bacilli*

The experiment was set to determine whether the lipases and the proteases produced by the seven *Bacilli* found in milk powder production streams could cause changes in WMP during storage. The enzymes were prepared in Section 5.2.3, added to 40% (w/v) reconstituted whole milk and then freeze-dried to powder form. The powder contained 0.01 U/g (*p*NP palmitate units) *Bacillus* lipases and 0.45 U/g (FITC unit) *Bacillus* proteases, respectively (method detailed in Sections 2.6.1).

The freeze-dried WMP was crushed to particles < 500 μm. The moisture content was determined to be 1.8% (est. a_w = 0.12 ± 0.02) using a Karl-Fisher titrator (Anchor Product Morrinsville). The same batch of commercial WMP without enzyme addition was used as the control. Both powders were stored at 37°C for 2 months.

6.2.1 Lipolysis

Total FFA released in the WMPs were monitored at various time points during storage, and quantified in duplicate using the GLC method (details in Section 2.4.2). The results are presented in Table 6.1 and Fig 6.1.

Table 6.1: *Changes in WMPs spiked with the seven* Bacillus *enzymes*

		WMP	WMP plus enzymes[1]
Moisture[2] before storage (%)		2.8	1.8
Total FFA released[3]	Initial	3.3	7.4
(mmole/kg-powder)	14 d	3.1	21.9
	28 d	2.1	24.6
	42 d	3.1	32.7
	59 d	1.9	35.8
FFA released[4](%)	$C_{4:0}$ to $C_{8:0}$	Not detected	1.0
	$C_{10:0}$ to $C_{12:0}$	Not detected	0.2
	$C_{14:0}$ to $C_{18:2}$	Not detected	2.2
	total	Not detected	3.4

[1]Enzymes prepared in Section 5.2.3 were added to 40% (w/v) reconstituted milk and freeze-dried to give a WMP containing 0.01 U/g lipase and 0.45 U/g protease (Section 2.6.1).

[2]Moisture content was tested using a Karl-Fisher titrator at Anchor Product Morrinsville.

[3]FFA were analysed in duplicate using the GLC method (Section 2.4.2).

[4]Percentage of FFA released from total triacylglcyerols in WMP after storage for 59 days at 37°C.

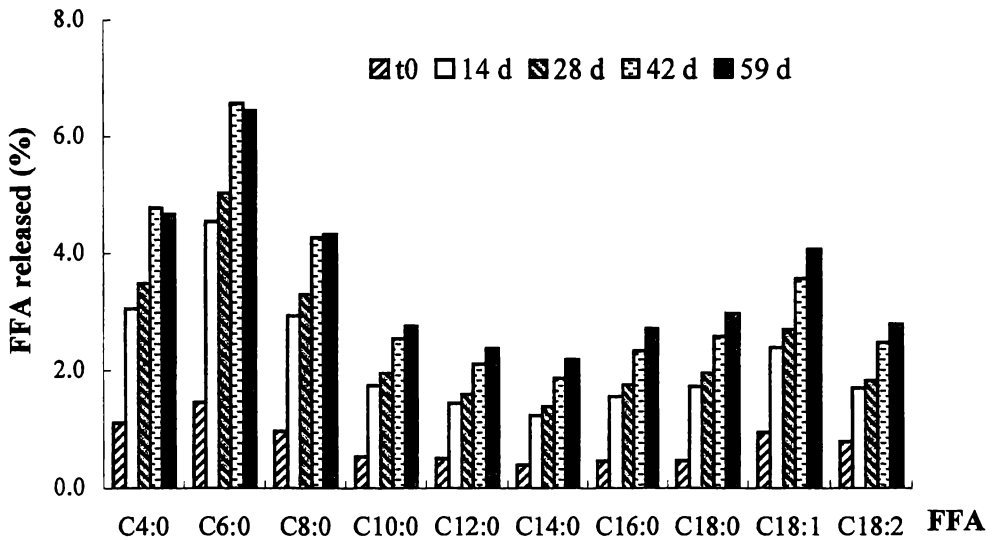


Figure 6.1: FFA released in WMP spiked with *Bacillus* lipases (0.01 *p*NP palmitate U/g-powder) during storage for 59 days at 37°C. Each fatty acid released is shown as a percentage of that FFA available from the triacylglcyerols in the WMP.

The level of total FFA in the WMP spiked with the *Bacillus* enzymes increased rapidly from 7.4 to 21.9 mmole/kg-powder in 14 days of storage at 37°C. A subsequent rise was more gradual over the remaining period of storage. No change in total FFA was observed in the control WMP (Table 6.1). The results clearly demonstrated that the lipases were active in the WMP during storage. This is the first unequivocal evidence that lipolysis is possible in a milk powder at an $a_w < 0.1$. It proves the prediction of Koshonti (1975) that a small amount of lipase and protease might cause defects in products with long storage time, such as milk powder.

Enzyme activity is usually studied in aqueous media *in vitro*. *In vivo*, however, the reactions occur not only in the cytoplasm, but also in the cell membrane and lipid depots. Studies carried out on phospholipase hydrolysis of lecithin (Whitaker, 1996) showed that there was no activity at $a_w < 0.35$ (< 1% water content). As the a_w increased to 0.35, the activity of phosphalipase increased in a non-linear manner. Maximum activity was not reached even at a_w of 0.9 (~ 12% water content) (Whitaker, 1996). In comparison, β-amylase had no activity on starch until a_w was about 0.8 (~ 2% water content), and activity increased 15 times at a_w of 0.95 (Whitaker, 1996). It has been suggested that to prevent enzyme activity, total water content must be less than 1-2% (Whitaker, 1996). These studies with the enzyme-spiked milk powders show that activity still occurs at this water content.

The rate of hydrolysis (lipolysis) rate is very slow in the powder form because of low a_w which limits the substrates and the products diffusion, and there is going to be less water as the hydrolysis processes. However, 3.4% total FFA was released in the *Bacillus* enzyme-spiked WMP during 59 days storage. Palmitic and linoleic acids accounted for 24% each of the total FFA released, followed by butyric acid at 15% and caproic acid at

12%. There was a significant different in odours of enzyme-spiked powder and the control, which could be contributed by the short and medium chain FFA released, as reported by Al-Shabibi (1964). Olivecrona *et al.* (1991) reported that hydrolysis of as little as 1-2% of milk lipids will result a rancid or "lipolyzed' flavour in milk. Although the powder was not tasted, the levels of short chain acids exceeded the threshold values within one week of storage at 37°C (see Table 1.6 for reference).

The pattern of FFA released shown in Fig 6.1 was similar to those given by the semi-purified powder lipase (Fig. 4.4) and the seven *Bacillus* lipases (Table 5.14). Lipases' substrate specificities are the same in a buffer system and in a powder form. Further, the results confirmed that the lipolytic enzymes from the seven *Bacilli* and the WMP are in fact esterases that prefer short chain water-soluble triacylglcyerols. This is also the main reason for persisting on assaying "lipase activity" of the milk powders using short chain *p*NP esters of fatty acids (Section 3.3).

It was noted that the initial level of total FFA in the enzyme-spiked powder was almost twice that in the commercial powder (Table 6.1). This could be lipolysis occurred during preparation of the material and therefore prior to storage. High-speed blending during the spiking process might have damaged milk fat globule membranes and facilitated lipolysis. It is known that the mechanical treatments, such as homogenisation or shaking can enhance lipolysis (Chandan and Shahani, 1964). The freeze-dried powder differed from the commercial powder in terms of the structure of the powder particles (data not shown), the structure effects to lipolysis in WMP during storage was not investigated on this occasion.

6.2.2 Proteolysis

The WMPs obtained in Section 6.2.1 were also dissolved in 10 mL deionised water at 10% (w/v). To 1 mL each sample, 0.5 mL 3% TFA was added and chilled on ice for 10 min. The samples were centrifuged at 10,000 x *g* for 10 min at 4°C, and the supernatants retained for analysis of peptides released using the RP-HPLC method (details see Section 2.3.1). The results are presented in Fig. 6.2.

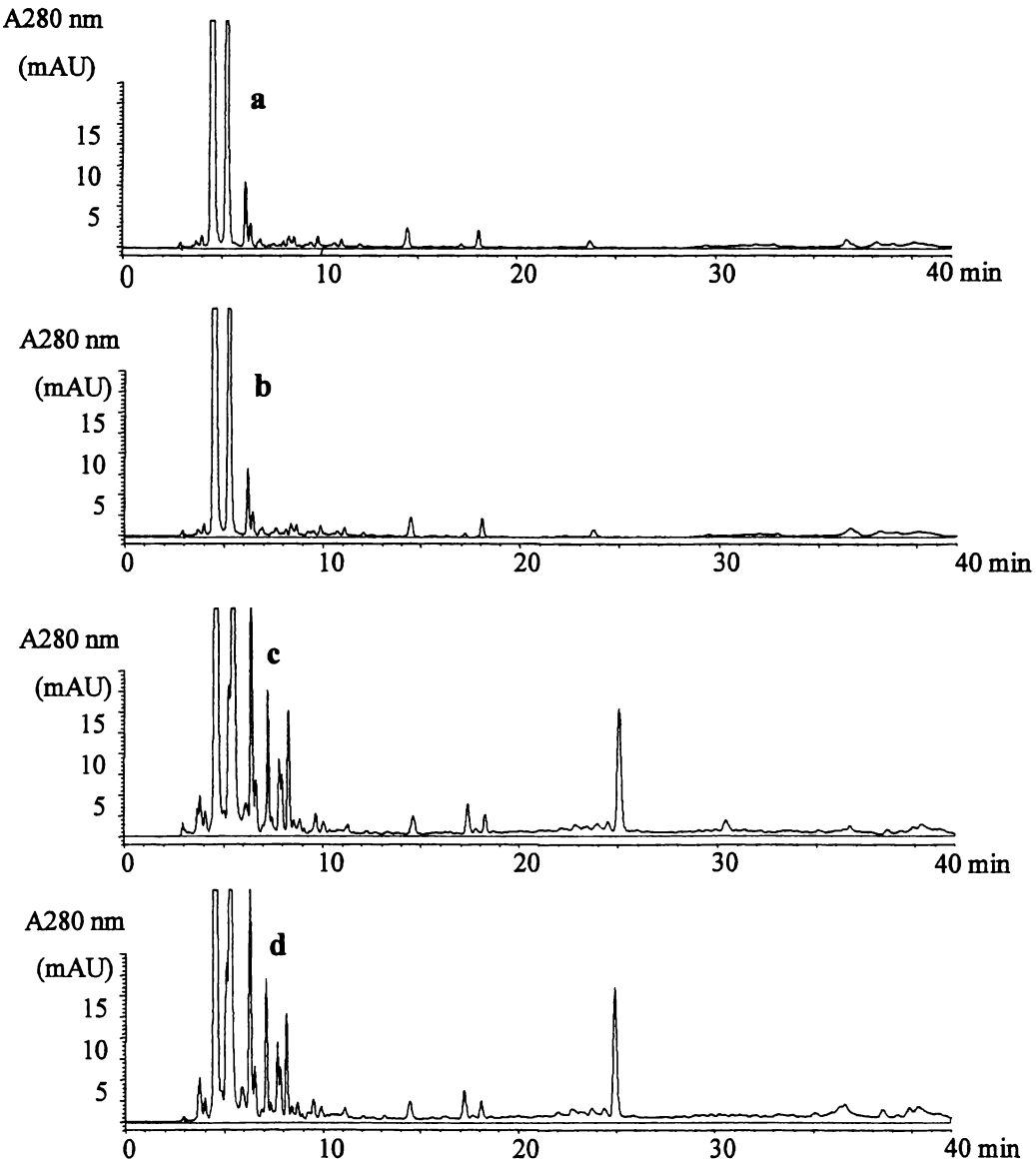


Figure 6.2: RP-HPLC traces of 1% TFA soluble fragments from whole milk powders stored at 37°C. (a) and (b) control powder at zero time and after 59 days storage, respectively. (c) and (d) *Bacillus* enzyme spiked powder at zero time and after 59 days storage, respectively.

Small changes in 1% TFA soluble protein fragments at retention times between 30-40 min were detected in the *Bacillus* enzyme-spiked WMP after storage for 59 days at 37°C, but not in the control powder (Fig. 6.2). The results can be interpreted in two ways: either the changes were the results of proteolysis, or to the physical (structural) changes in the powder particles that resulted in more extractable protein fragments (Walstra *et al.*, 1999). The proteolysis theory is more likely because similar changes were detected in the reconstituted milk made from the same batch of WMP which was incubated at a similar temperature for less time (Section 3.2.3.6). As explained earlier, milk caseins are very stable and not likely to undergo physical changes at temperatures as low as 37°C (Walstra *et al.*, 1999).

The freeze-dried powder had a lower water content of 1.8% (est. a_w of 0.10 ± 0.02) than the commercial powder of 2.8% (est. a_w of 0.20 ± 0.03; Walstra *et al.*, 1999). It would be expected that increasing the water content to 2.8% would possibly result in higher proteolysis in the enzyme-spiked powder because the rate of hydrolysis increases with increasing a_w.

The initial patterns of the 1% TFA soluble protein fragments were different between the control and the *Bacillus* enzyme-spiked powders, so it was possible that proteolysis occurred during sample preparation. However, the majority of the differences are due to the presence of peptides in the enzyme preparation.

6.3 Changes in WMP Spiked with Pure Thermophilic BTL-2 Lipase and AK1 Protease

The experiments in Section 6.2 were carried out with a combination of lipases and proteases from all seven *Bacilli*. In order to define the lipolysis and proteolysis more precisely, and to eliminate possible complication caused by any contaminating esterase activity from the protease, WMP batch IH30 was reconstituted two ways: one with pure thermophilic lipase BTL-2; and the other with pure thermophilic AK1 protease. The samples were prepared following the method detailed in Section 2.6.2, gave 0.14 *p*NP plamitate U/g lipase and 19.2 FITC U/g protease. A third sample was treated the same way without any enzyme added. Because the resulting a_w of the freeze-dried powders was half of that in a commercial powder, the powders were rehydrated over a saturated KCOOH solution for 4 days to reach an equilibrium a_w of ~ 0.30 at room temperature, then put to storage at 37°C for 60days.

6.3.1 Lipolysis with or without BTL-2 Spiked WMP during Storage

The storage powders spiked with BTL-2 lipase, AK1 protease, and control were analysed in duplicate for FFA release using the GLC method (Section 2.4.2). The results are presented in Table 6.2 and Figs. 6.3 and 6.4.

All three powders showed FFA release, and gave a linear relationship between the levels of total FFA released and storage time (Fig. 6.3). The powder treated with BTL-2 lipase gave by far the highest FFA release, it is possibly a combination of the BTL-2 lipase and the initial lipase activity (shown in Sections 4.4 and 4.5) in the powder. The powder spiked with AK1 protease gave less FFA released than the control, which indicated that the protease did not contribute to FFA release. In fact, it may have

lowered the activity of the initial lipase(s) by hydrolysis of the enzymes during preparation. The results confirmed the finding in Section 6.2.1 that lipolysis is possible in milk powders at very low levels.

Table 6.2: *Changes in WMPs spiked with and without BTL-2 lipase and AK1 protease*

ID.		Freeze-dried WMP	WMP+AK1	WMP+BTL-2
a_w[1]	Freeze-dried	0.08	0.11	0.08
	Before storage	0.31	0.34	0.24
Total FFA released[2]	Initial	6.1	4.3	15.4
(mmole/kg-powder)	20 d	9.4	5.2	40.7
	30 d	12.0	5.9	54.3
	48 d	15.9	6.8	69.1
	60 d	18.2	7.2	70.0
FFA released[3] **(%)**	$C_{4:0}$ to $C_{8:0}$	0.4	0.1	1.7
	$C_{10:0}$ to $C_{12:0}$	0.1	0.1	0.5
	$C_{14:0}$ to $C_{18:2}$	1.2	0.5	4.4
	Total	1.7	0.7	6.6

[1]A_w was tested as equilibrium relative humidity at 30°C by Mr. A. dodge (NZDRI).

[2]FFA were analysed in duplicate using the GLC method (details in Section 2.4.2).

[3]Percentage of fatty acids released from the total triacylglcyerols in WMP after 60 day-storage at 37°C.

Total of 6.6% FFA was released in the sample spiked with BTL-2 lipase, palmitic and linoleic acids accounted for 25% and 19%, respectively, followed by butyric acid at 17% and caproic acid at 6% (Table 6.2). The total FFA is higher than the value obtained in Table 6.1 because of the higher dots of lipase. The more lipase added (0.14 and 0.01 U/g-powder), the more FFA released (70 and 35.8 mmole/kg-powder), but is not a linear relationship. This could be due to the differences in specificities between the BTL-2 lipase and lipases from the seven *Bacilli* (see Schmidt-Dannert *et al.*, 1997 and Table 5.14. for reference).

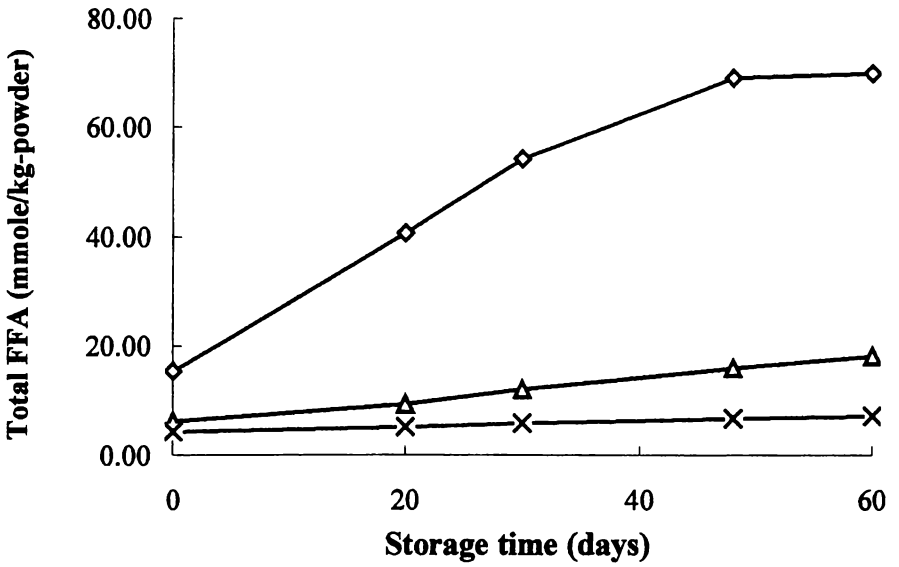


Figure 6.3: Total fatty acid released over time in whole milk powders stored for 2 months at 37°C. Powders were: (Δ) control WMP (y =0.2x + 5.8); (×) WMP + 19.2 FITC U/g-powder thermophilic AK1 protease (y = 0.1x + 4.3); (◊) WMP + 0.14 *p*NP palmitate U/g-powder thermophilic BTL-2 lipase (y = 1.3x + 15.3, up to 30 days). Each data point represents an average of duplicate.

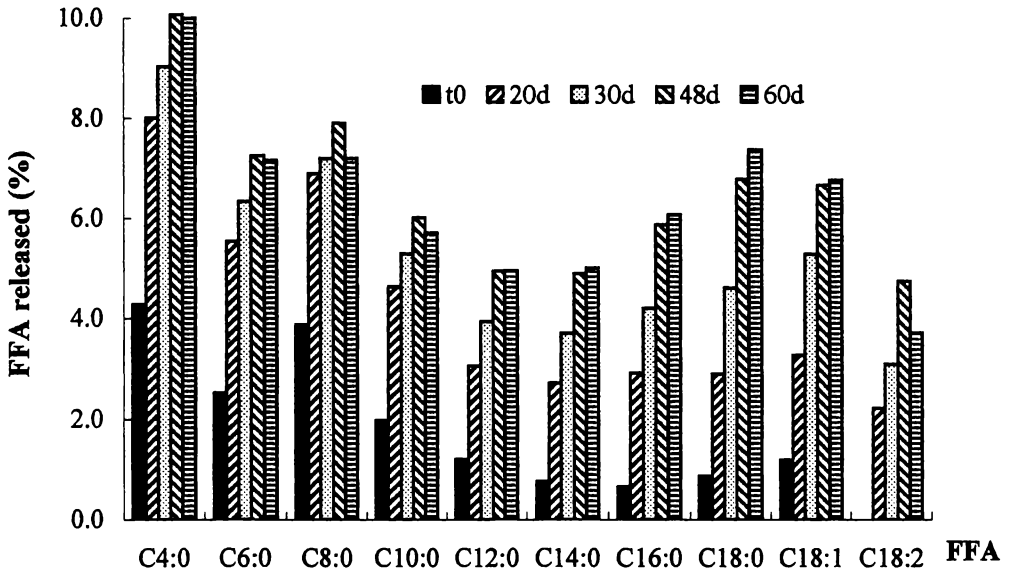


Figure 6.4: Fatty acid released in the whole milk powder spiked with thermophilic BTL-2 lipase (0.14 *p*NP palmitate U/g powder) during storage at 37°C for up to 60 days. Each fatty acid is shown as the percentage of that acid available in the triacylglcyerols in the powder.

The release of each individual fatty acid is shown in Fig. 6.4. The patterns of FFA released were identical to the ones given by the same lipase on the synthetic triacylglycerols in a buffer system (Schmidt-Dannert *et al.*, 1996).

Stead (1986) reported that the rate of lipolysis in olive oil emulsion was initially high, but became very low after 3 months. This phenomenon of a change in rate of hydrolysis (lipolysis) was observed in two storage trials. The amount of FFA released reached maximum after 48 days of storage, and with no further increase up to 60 days (Fig. 6.3). As lipolysis proceeds, more DG and MG are generated. These lipids are sparingly soluble in water which will remain at the interfaces to compete for active sites from the lipase for the triacylglcyerols, therefore cause product inhibition (Olivecrona and Bengtsson, 1984). In a powder, low water content will limit the rate of hydrolysis (lipolysis) by affecting substrate and product diffusion, plus relatively large amount of FFA are produced, so it would be easily reached to product inhibition.

Even though the a_w of the lipase-spiked powders were lower than the rest of freeze-dried powders in two storage trials (Tables 6.1 and 6.2), but this apparently did not prevent lipolysis in the WMPs during storage.

It should also be noted that the initial level of FFA in the powder spiked with the BTL-2 lipase was 2.5-3.6 times higher than that in the control and the AK1 protease-spiked powders (Table 6.2). This reinforces the idea that lipolysis has occurred prior to storage. The lower initial FFA in the protease-spiked powder compared with the control also reinforced the idea that the protease may have destroyed some 'endogenous' lipase activity during powder preparation, and prevented the initial accumulation of FFA.

6.3.2 Proteolysis in WMP during Storage with or without AK1 Protease

The stored WMPs obtained in Section 6.3.1 were also analysed for proteolysis. The powders were first dissolved in deionised water to 10% (w/v) total solids. To determine protein break down, 10 μL each of samples was diluted 20 times, treated with 5 μL 5 x sample buffer, boiled for 5 min, and electrophoresed on 12% SDS-PAGE (method details see Section 2.3.2). The gels were Coomassie stained to identify protein breakdown. The results are presented in Fig. 6.5.

To determine peptide production, 1.0 mL of each sample was treated with 0.5 mL 3% TFA, chilled on ice for 10 min, and then centrifuged at 10,000 x *g* for 10 min at 4°C, and supernatants obtained. The supernatant was separated on a reverse phase C_{18} column (method detailed in Section 2.3.1) and identified by mass spectrometry. The results are presented in Figs. 6.6, 6.7 and 6.8.

No significant changes in protein bands were detected on SDS-PAGE in the three powders spiked with or without thermophilic protease AK1 (Fig 6.5). There were a few bands in the WMP spiked with AK1 protease at R_f between 0.59-0.89 that were not present in the control or BTL-2 lipase-spiked powders. However, these were present immediately after powder preparation, and did not change during storage and are therefore consistent with proteolysis during preparation of the spiked powders.

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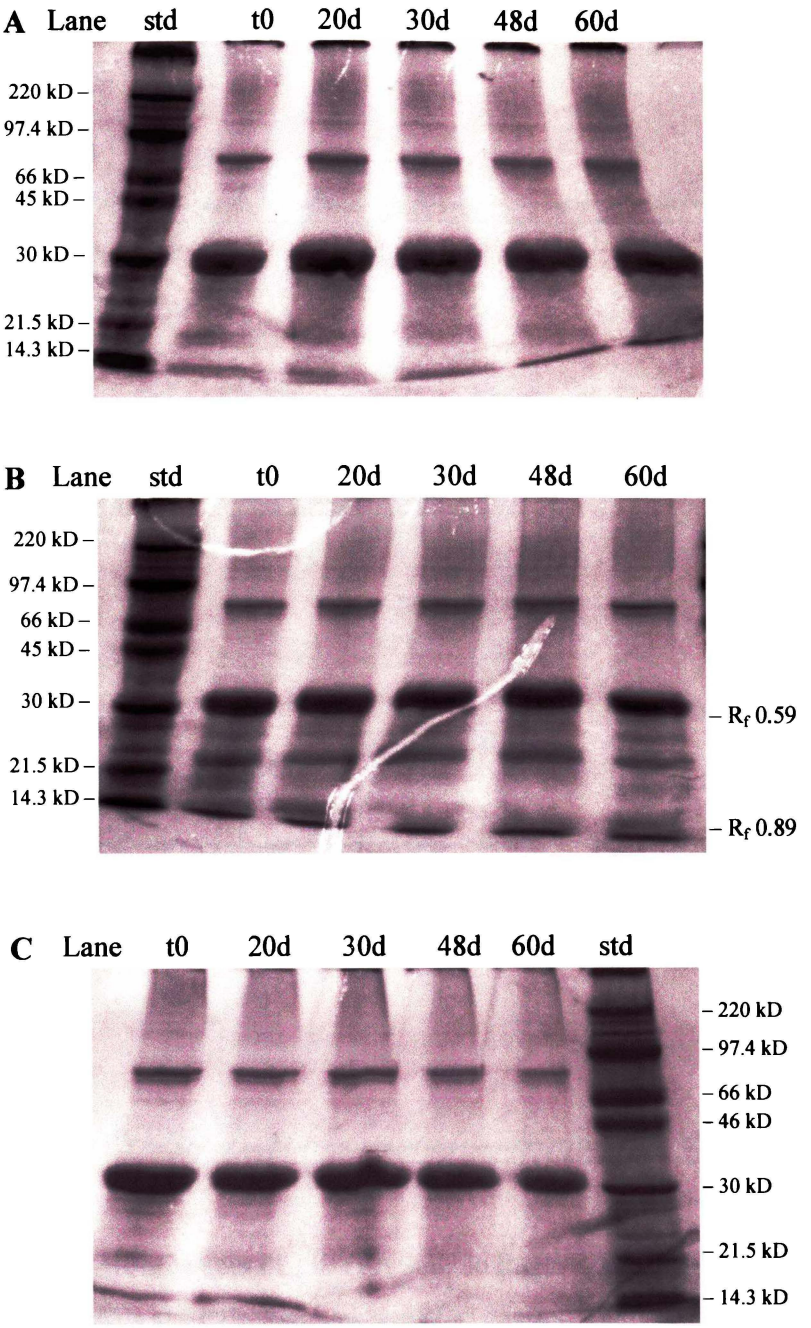

Figure 6.5: SDS-PAGE of WMPs after storage for 60 days at 37°C. Samples were (A) control powder; (B) WMP spiked with AK1 protease; and (C) WMP spiked with BTL-2 lipase. Standards were rainbow markers (Amersham Phamacia Biotech. RPN756).

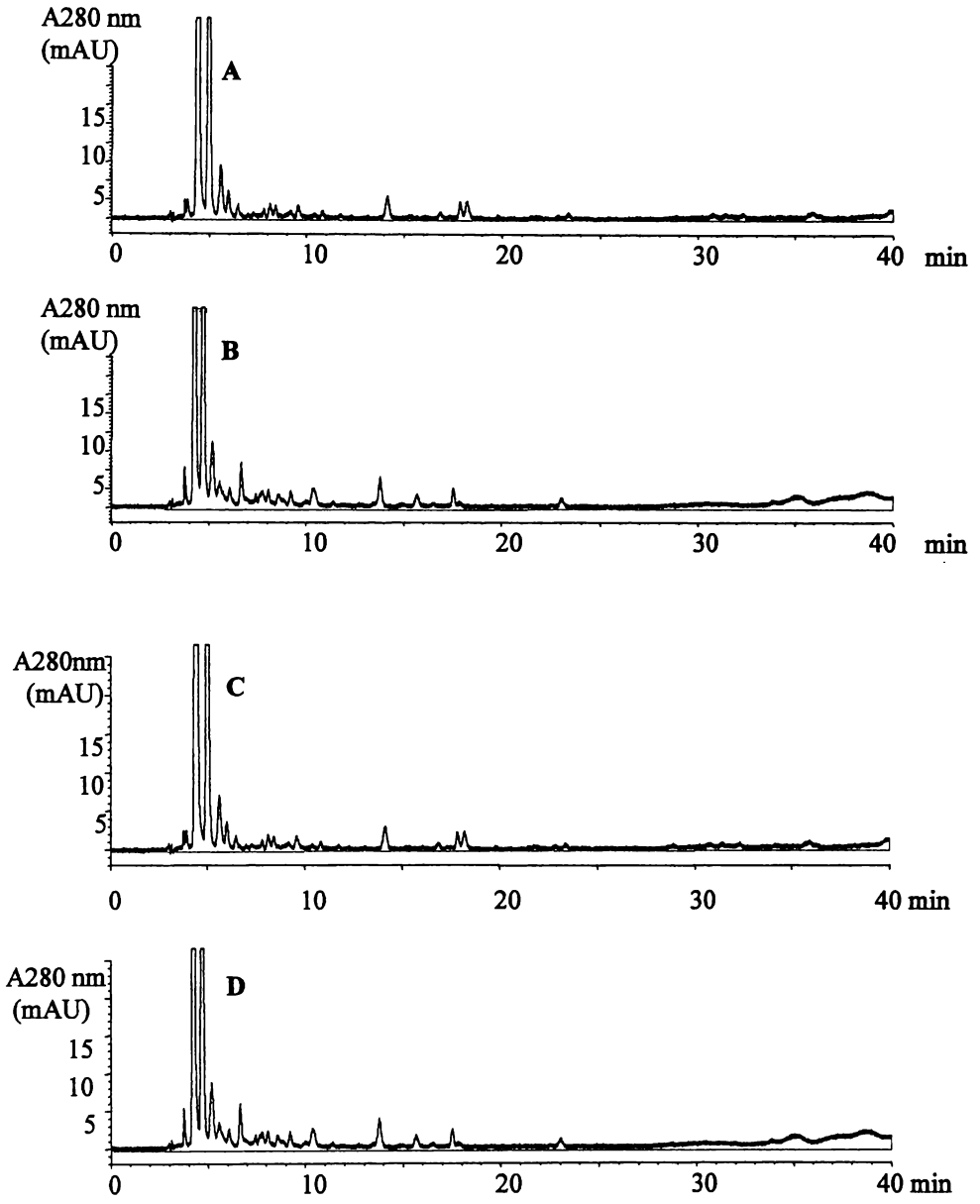

Figure 6.6: RP-HPLC traces of 1% TFA soluble fragments in WMPs after storage for 60 days at 37°C. Samples were (A) control WMP t0; (B) control WMP after 60 days; (C) WMP spiked with BTL-2 lipase t0; and (D) WMP spiked with BTL-2 lipase after 60 days.

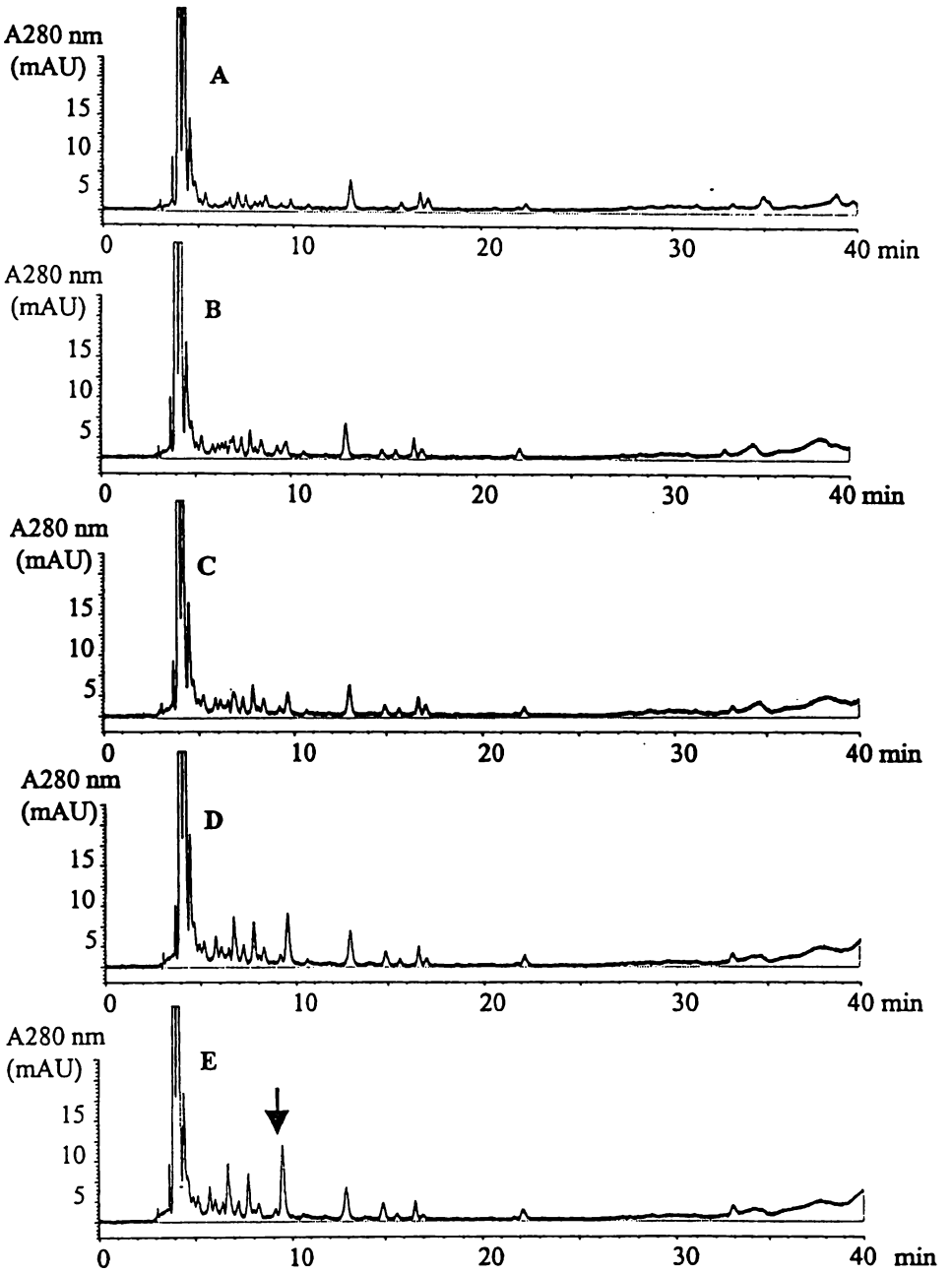

Figure 6.7: RP-HPLC traces of 1% TFA soluble fragments in WMP spiked with AK1 protease after storage at 37°C: (A) t0; (B) 20 day; (C) 30 day; (D) 48 days; and (E) 60 days. The peptide labelled ↓ was Ser-Thr-Ala-Val, derived from the C-terminal of κ-casein.

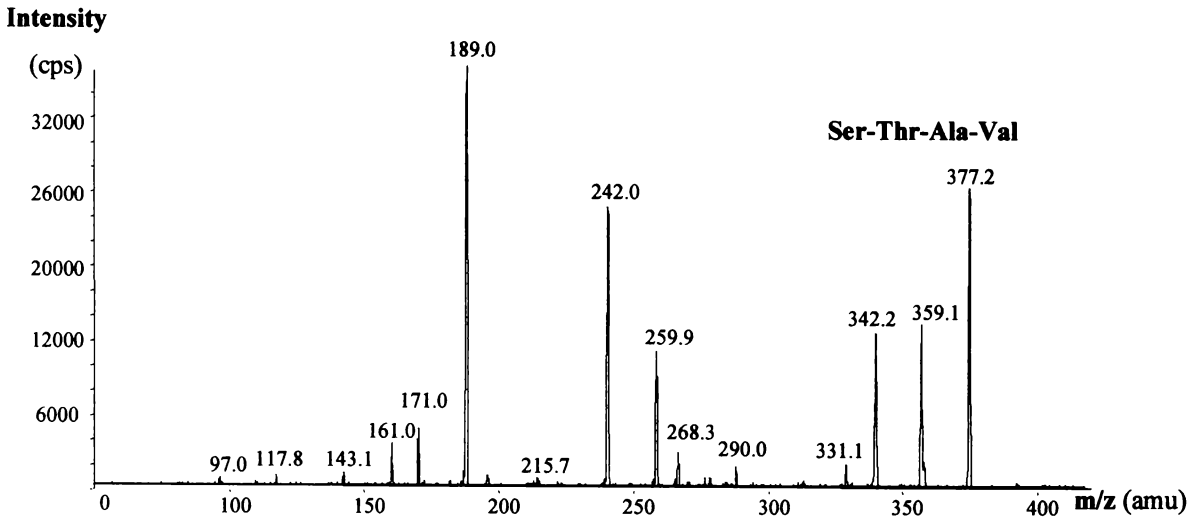

Figure 6.8: Identification of peptide peak by mass spectra. The peptide was separated by RP-HPLC from the WMP spiked with AK1 protease after 60 days of storage at 37°C.

Proteolysis has been reported to cause defects in UHT milk during storage (Adam *et al.*, 1975; Enright *et al.*, 1999). κ-Casein is essential to maintain stability of casein micelles – a stable colloidal structure of milk made of 40-300 μm diameter spherical particles comprise 10^4 casein molecules (Walstra *et al.*, 1999). When κ-casein is hydrolysed by a protease, the result is the precipitation of phosphoproteins of α- and β-casein with Ca^{2+} ions, this is known in cheese making. In the present study, sediments were observed when reconstituting the powders for SDS-PAGE and RP-HPLC work. Such changes in functionality of the freeze-dried powders during storage were not investigated on this occasion, because as mentioned earlier, the freeze-drying process changed the powder particle structure (data not shown). Further investigations should be undertaken to determine the relevance of proteolysis to functionality changes in a powder made under normal industrial conditions.

6.3 Lipolysis in WMP Spiked with *Bacillus* A^m Esterase

The semi-purified A^m esterase (changed name according to the results obtained in Section 5.5.6) from the main contaminating bacterium *B. stearothermophilus* A^m was shown to have greatest activity towards 1,2-DG (Section 5.5.6). In order to determine its specificity in milk powder during storage, three levels of the A^m esterase were added to 40% (w/v) reconstituted whole milk, mixed well and freeze-dried to give powders. Another powder was made without any addition of the A^m esterase (detailed in Section 2.6.3). Powders with low a_w were rehydrated in saturated $MgCl_2$ for 2 days at 37°C to give $a_w \sim 0.3$. All powders were then stored at 37°C for 2 months. FFA were analysed using the GLC method (Section 2.4.2). The results are presented in Table 6.3 and Fig. 6.9.

Table 6.3: *Changes in A^m esterase-spiked WMP during storage*

Sample ID.		**#1**	**#2**	**#3**	**#4**
A^m esterase added[1] (U/g-powder)		0.02	0.06	0.14	none
a_w[2]	Freeze-dried	0.36	0.15	0.11	0.28
	Before storage	0.36	0.30	0.30	0.28
Total FFA[3]	Initial	2.4	1.9	1.5	2.5
(mmole/kg-powder)	15 d	2.5	2.4	2.8	2.0
	30 d	4.1	3.0	3.2	2.1
	45 d	4.5	3.7	3.7	2.2
	60 d	5.7	4.2	3.9	2.8
FFA released[4](%)	$C_{4:0}$ to $C_{8:0}$	0.13	0.14	0.03	0.03
	$C_{10:0}$ to $C_{12:0}$	0.04	0.03	0.01	0.02
	$C_{14:0}$ to $C_{18:2}$	0.37	0.24	0.10	0.19
	total	0.54	0.41	0.14	0.24

[1]Esterase was added to 40% (w/v) reconstituted whole milk and freeze-dried to give three powders containing 0.02, 0.04 and 0.16 U/g (*p*NP palmitate) esterase, respectively (Section 2.6.3).

[2]Aw was tested as equilibrium relative humidity at 30°C by Mr. A. dodge (NZDRI).

[3]FFA were analysed in duplicate using the GLC method (Section 2.4.2).

[4]Percentage of FFA released from the total triacylglcyerols in WMP after 60 days storage at 37°C.

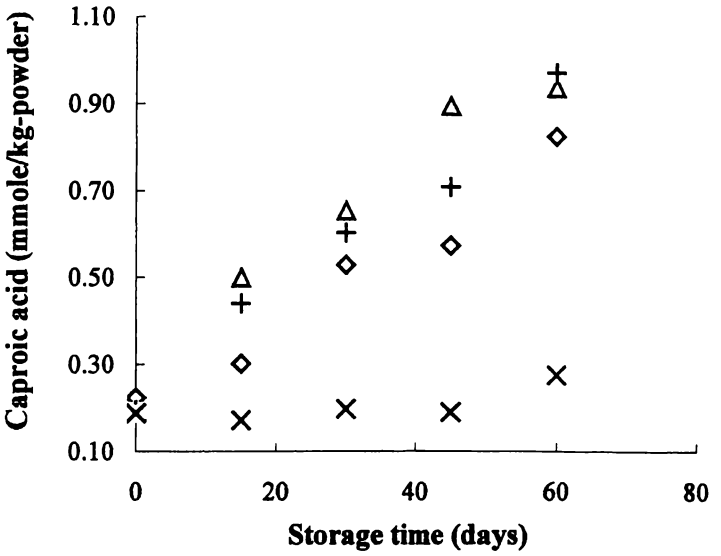

Figure 6.9: Caproic acids released vs time in whole milk powders spiked with different level of A^m esterase at 37°C. Samples were: (×) freeze-dried control powder; (◊) WMP + 0.02 U/g A^m esterase; (+) WMP + 0.06 U/g A^m esterase; and (Δ) WMP + 0.14 U/g A^m esterase.

All powders showed FFA release after storage for 60 days at 37°C (Table 6.3), although the amount of the total FFA released did not give a linear relationship with the amount of A^m esterase added. Short chain butyric and caprioc acids were released predominantly. Three-fold increase in caproic acid level was found after 2 month-storage at 37°C in the powder spiked with 0.14 U/g A^m esterase, but the level will be within the threshold if the powder is to make 12.5% (w/v) reconstituted milk (see Fig. 6.9 and Table 1.6 for reference).

The results agree with the findings in Sections 6.2.1 and 6.3.1 that lipolysis is possible in WMP during storage at low a_w, and the pattern of FFA released correlated well with the specificities obtained in a buffer system (Section 5.5.6). This has been consistent in three lipase-spiked WMPs storage trials. The more A^m esterase added, the more caproic acid released (Fig. 6.9), although clearly the response was not directly proportional.

This may suggest that higher lipase levels may not necessarily lead to higher level of FFA release. It is possibly due to a_w, the predominant factor for lipolysis in a powder form. With the lowest addition of the A^m esterase, the powder had the highest level of FFA released, which had the highest a_w of 0.36. The higher the a_w, the faster the lipolysis (Drapron, 1985).

6.4 Summary

Storage trials of enzyme-spiked WMPs clearly demonstrated that lipolysis is possible in WMPs with an a_w of between 0.1-0.3, and it resulted in the hydrolysis of milk lipids to FFA. The levels of FFA, especially short and medium chain ones were sufficient to cause flavour defects within one week in storage at 37°C. The more lipases (esterases) in the powders, the more FFA released. Specificity data showed that the FFA released in a powder form was identical to the ones given in a buffer system. All lipolytic enzymes from the seven *Bacilli* released more short chain FFA (butyric and caproic acids) than long chain FFA in WMP during storage. These FFA are more potent with respect to flavour.

The data obtained from WMP storage trials indicated that proteolysis is also possible, and changes of solubilities in the WMPs during storage were also noted. Because the main proteolysis product was derived from κ-casein, it could be that the hydrolysis of κ-casein resulted in the destabilised the casein micelle structures and therefore caused increased levels of insoluble casein particles.

Chapter 7 Commercial Whole Milk Powder in storage and Reconstitution

7.1 Introduction

The integrity of milk powders can change during storage and lead to changes in functionality and flavour. The higher the storage temperature and a_w, the higher the rate of deterioration (Pesicky, 1997). WMP normally has a 6-month shelf-life. If to have a 12-month shelf-life, it should be kept under vacuum or gas-packed with nitrogen (Celestino *et al.*, 1997a). Chemical reactions, such as Maillard (browning) reaction has been studied extensively, but little is known about the potential or extent of biochemical reactions (*i.e.* lipolysis and proteolysis) in milk powders during storage.

Experiments detailed in this chapter were designed to examine whether any biochemical reactions would occur in commercial WMPs during storage. Elevated temperatures were used as a tool to shorten the storage time. Attention was paid in particular to flavour (FFA) and functionality changes (peptides) due to lipolysis and proteolysis, respectively.

One of the problems, however, with temperatures above 37°C is that the structure of powder particles are likely to change since milkfat will undergo a phase change from solid to liquid. Milk caseins are known to be heat-stable, only heating at temperatures above 120°C causes the caseins to slowly become insoluble (Walstra *et al.*, 1999). However, there was a question over the stability of milkfat when held at high temperatures for long periods. In order to determine the stability of the milkfat, it was

stored at temperatures up to 85°C and analysed periodically for FFA content and structure changes.

Subsequently, five batches of commercial WMPs, selected solely on the basis of thermophile numbers, were stored at room temperature (22°C), 37°C and 55°C for up to 28 months. During storage, the powders were analysed for enzyme levels, FFA release, peptide profiles and pH changes. Thermophilic bacteria were also counted to check if any correlations existed between thermophile numbers and the keeping quality of the powders.

7.2 Analysis of Milkfat after Storage at Extreme Temperatures

Triacylglycerols is made up 98.3% of milkfat that contains a variety of fatty acids (see abbreviation for reference). A typical composition of New Zealand milkfat (on a w/w basis) is: 27.5% palmitic acid, 18.9% oleic acid, 12.3% myristic acid and 11.2% butyric acid (Creamer and MacGibbon, 1996).

To determine changes in milkfat at elevated temperatures, milkfat samples of 50 g each (FFMR-GG08, Cheese and Milkfat Technology Section, NZDRI) were packed in air-tight foil bags and stored for 4 days at 65°C, 75°C and 85°C. Samples of 0.5 g each were taken in triplicate every day for FFA analysis by the GLC method (Section 2.4.2). Another set of samples of 0.5 g each were first dissolved in 10 mL *tert*-butyl methyl ether/heptane (1:1, v/v) and 10 μL each was separated on TLC (method detailed in Section 2.4.1). The results are presented in Fig. 7.1 and Table 7.1.

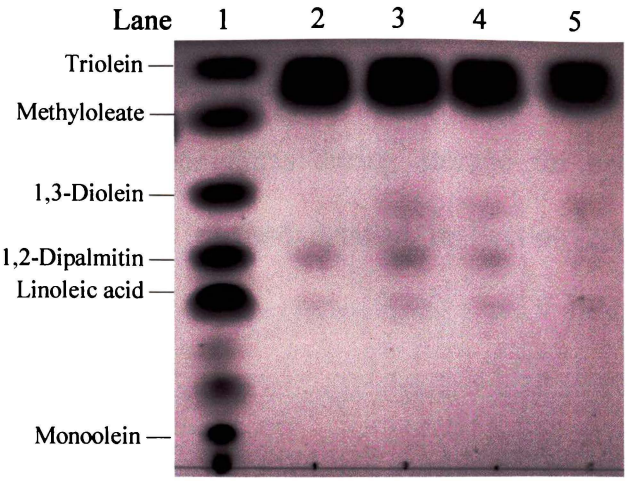

Figure 7.1: TLC analysis of milkfat after storage for 4 days. Lane 1: TLC standards (Nu-Check 18-1A) with addition of 1,2-dipalmitin (Sigma D2135) and linoleic acid (Sigma L1376); lane 2: milkfat before storage; lane 3: after storage at 65°C; lane 4: after storage at 75°C; lane 5: after storage at 85°C.

Table 7.1: *FFA released from milkfat after storage at extreme temperatures*

Storage temperature	FFA (mmole/kg-fat) released after storage for				
	initial	1 day	2 days	3 days	4 days
65°C	3.41	3.49	3.35	3.31	3.38
75°C	3.41	3.44	3.39	3.81	3.58
85°C	3.41	3.39	3.47	3.76	3.71

No significant changes in the levels of total FFA in milkfat were detected by GLC after storage for 4 days up to 85°C (Table 7.1). The results from the TLC-plate did not show any transformational changes in milkfat stored under the same conditions (Fig. 7.1). The results are consistent with those reported by Precht *et al.* (1999) that milkfat undergoes little change in composition even after heating at 200°C for 15 min. This confirmed that the milkfat would be stable when stored at lower temperatures, such as 55°C, for a prolonged time.

7.3 Changes in WMP after Storage at Various Temperatures

To determine temperature effects on WMPs during storage, sample of WMP batch II12 were stored in air-tight foil bags at 4°C, 22°C, 30°C, 37°C and 55°C for up to 2 months. Samples were taken at various times during storage for analysis of thermophile numbers, a_w and FFA levels (method detailed in Section 2.6.4). The results are presented in Table 7.2.

Table 7.2: *Changes in WMP batch II12 during storage at various temperatures*

ID.	Initial status	Status after 60 d at				
		4°C	22°C	30°C	37°C	55°C
Thermophiles[1] (CFU/g-powder)	83,000	82,000	80,000	77,000	61,000	160
a_w[2] after storage	0.16	0.17	0.16	0.17	0.17	0.31
Total FFA[3] (mmole/kg-powder)	0.48	0.42	0.39	0.42	0.45	3.78

[1]Thermophiles (colony forming units per g powder) were tested following the New Zealand Dairy Industry NZTM2 Microbiological Methods Manual Section 60.0.

[2]a_w was measured as equilibrium relative humidity at 30°C by Mr. A. Dodge (NZDRI).

[3]FFA were analysed in duplicate using the GLC method (in Section 2.4.2).

No changes in the levels of total FFA were detected in the WMP after 60 days of storage at up to 37°C (Table 7.2). Although the thermophile numbers dropped from 83,000 to 61,000, it was a change of less than 0.2 log, which is generally considered not significant in the industry, and the results are probably within experimental error. However, at a storage temperature of 55°C, there was almost a 7-fold increase in the total level of FFA after 60 days, and the thermophile numbers fell significantly – more than 2.5 log.

The results may be explained in three possible ways. First, at 55°C, the thermophiles may have lysed and released intracellular lipolytic enzymes into the powder, which led to the significant FFA release. Second, the lipase in the powder was significantly more active at 55°C than at 37°C. Third, milkfat phase changes when the temperature exceeds 37°C (MacGibbon and McLennan, 1987), so the phase transition at 55°C has enhanced the lipolysis. The latter two explanations are independent of thermophile numbers, or cell death and lysis. It is possible that a combination of these explanations is correct. The powder lipase isolated from the same powder showed maximum activity at 60°C (Section 4.5.2). This is consistent with all three scenarios, since (a) the increase in the level of total FFA could be due to greater enzyme activity at higher temperatures; and (b) the greater activity at the higher temperature could partly be because the phase change resulted in the substrates more accessible to lipolysis.

As noted earlier, the thermophile numbers in the powder dropped from 82,000 to 160 CFU/g powder during storage at 55°C. This clearly illustrated that there is no direct correlation between thermophile numbers *per se* and the increase of FFA levels in the WMP during storage.

It should also be noted that the a_w was the highest in the sample stored for 60 days at 55°C. The a_w in milk powders at a moisture content below 12% increases with increasing storage temperature (Pesicky, 1997). This also could have enhanced lipolysis. Enzymatic reaction increases with increasing a_w has been reported (Drapron, 1985; Whitaker, 1996). Enhancement of lipolysis at higher a_w was observed previously on the WMP storage trials described in Section 6.3. However, a correlation

between a_w and the levels of FFA released in WMPs was not investigated further in this study.

7.4 Biochemical Changes in Commercial WMP during Storage

The results in Section 7.3 indicated that commercial WMPs could undergo chemical and biochemical changes that are temperature dependent. To broaden the study, a further five commercial WMPs containing different levels of thermophiles were placed in storage in air-tight foil bags at temperatures of 22°C (room temperature), 37°C and 55°C, for up to 28 months. Samples were taken at various time points and stored at –20°C prior to analysis of thermophile numbers, the levels of FFA, production of volatile compounds, pH, a_w and solubility index (methods detailed in 2.6.4). The results are discussed in Sections 7.4.1 to 7.4.4.

7.4.1 Changes in Thermophile Numbers and Esterase Levels in WMP during Extended Storage

The changes in thermophile numbers in the WMPs during storage at 22°C, 37°C and 55°C were analysed. The lipases activities were separated using Phenyl Sepharose (Section 4.3.4), and then screened for their specificity against *p*NP esters of fatty acids. The results are presented in Table 7.3 and Fig. 7.2.

The results showed that the thermophile numbers remained essentially unchanged in the WMPs stored at 22°C. At higher storage temperatures, the numbers tended to decrease although at 37°C this decrease was not as consistent as at 55°C. No significant differences in the levels of lipase activity were detected either initially or even after storage at 55°C for 2 months in any of the five powders (Table 7.3). Enzymes are

known to be far more stable in dry state than in aqueous medium, so the lipolytic enzymes in the WMP need not be thermophilic *per se*. However, the temperature profile of the semi-purified powder lipase (Section 4.5.2) supports a thermophilic bacterial origin.

Table 7.3: *Changes in thermophile numbers and esterase levels in commercial WMPs*

	Whole milk powder batch number				
	IH30	**II12**	**BI18**	**CI03**	**DI05**
Manufacture date	April 97	April 98	Sept. 98	Oct. 98	Nov. 98
Thermophiles[1](CFU/g)					
Initial	270	41,000	220,000	96,000	320,000
Stored at 22°C[2]	90	42,000	210,000	130,000	630,000
240 d at 37°C	10	21,000	66,000	24,000	440,000
60 d at 55°C	20	4,900	35,000	4,100	67,000
Lipase activity[3](U/g)					
Initial	0.28	0.31	0.31	0.30	0.27
Stored at 22°C	0.25	0.30	0.28	0.29	0.27
240 d at 37°C	0.28	0.26	0.29	0.30	0.30
60 d at 55°C	0.26	0.23	0.28	0.30	0.25

[1]Thermophiles were tested following the New Zealand Dairy Industry NZTM2 Microbiological Methods Manual Section 60.0.

[2]WMPs were stored at 22°C for 28 months (IH30), 16 months (II12), 11 months (BI18), 10 months (CI03) and 9 months (DI05).

[3]Calculated from the data obtained in Table 4.7.

Overall, there was no correlation between the thermophile numbers and the levels of lipase activity detected in the WMPs. This could mean that the level of lipase activity was determined during the manufacture process, rather than caused by thermophile death during storage of the powder. Other researchers have not been able to establish any correlation between the numbers of psychrotrophic bacteria and the levels of FFA in raw milk (Muir *et al.*, 1978). Andersson (1980) reported that there was no correlation

between the total number of *Pseudomonas* cells and the apparent lipase production at different temperatures. These bacteria vary considerably in their ability to produce lipases in milk, so counting bacteria is not a reliable guide to the spoilage potential of milk (Stead, 1986). This reinforces the contention that determination of bacterial numbers will not give a reliable indication of spoilage potential in a product.

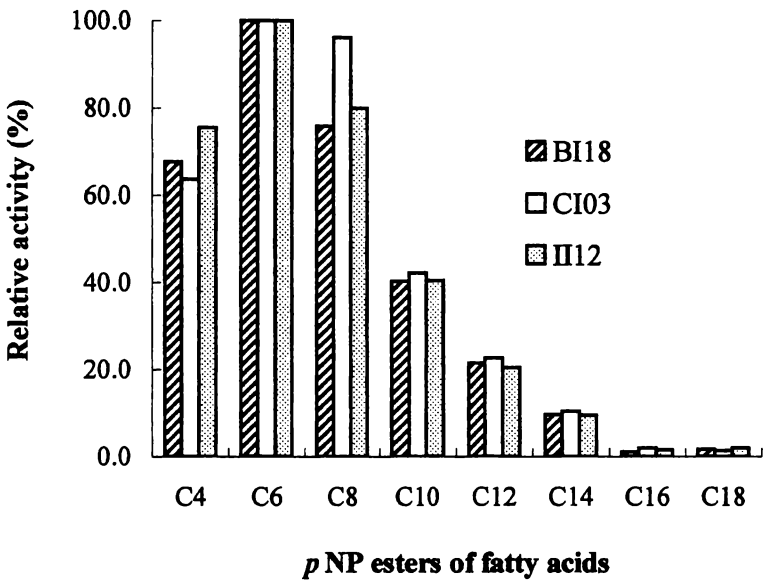

Figure 7.2: Lipase activity from three batches of WMP BI18, CI03 and II12 against *p*-nitrophenyl esters of fatty acids of butyrate, caproate, caprylate, caprate, laurate, myristate, palmitate and stearate. Assay was in 0.1 M Mops buffer, pH 7.2, with 5 mM $CaCl_2$, at 60°C for 30 min. Activity against each substrate is presented as relative to *p*NP-caproate.

The lipolytic activity from three batches of the WMPs showed identical specificities towards *p*NP esters of fatty acids (Fig. 7.2). The patterns were similar to those given by the semi-purified powder lipase (Fig. 4.4) and the seven *Bacillus* esterases (Table 5.14). The results indicated that there was either only one predominant lipase present in the WMP, or several sources enzymes giving identical specificities. The difference is not necessarily academic. From a commercial point perspective, the question is what effect does a certain level of lipase have on WMP quality, and how can they be controlled.

The consistency of the specificity may be good news, because if there is only one type of lipase, it may be easier to control than two widely different types. However, if the pH optima, heat-stabilities, *etc.*, of lipases are different, then the exact identity of the contaminating bacteria becomes very important, regardless of similarities in specificity.

7.4.2 Changes of FFA Levels in WMP during Storage

The WMPs obtained in Section 7.4.1 were also analysed for FFA release in duplicate after storage using the GLC method (Section 2.4.2). The results are presented in Table 7.4.

Table 7.4: *Levels of FFA in WMP after storage at various temperatures*[1]

Storage time and temperature	Total FFA[2] (mmole/kg-powder) in WMP batch number				
	IH30	II12	BI18	CI03	DI05
Fat[3](%)	26.8	26.8	19.5	26.8	29.1
Initial	0.93	0.42	0.92	0.32	1.04
22°C[4]	0.95	0.48	0.95	0.41	1.14
37°C for 14 d	0.86	0.39	0.86	0.37	1.23
42 d	0.76	0.34	0.93	0.35	1.22
72 d	0.81	0.39	0.94	0.35	1.28
105 d	0.90	0.42	0.99	0.35	1.32
240 d	1.40	0.68	1.23	0.35	1.27
55° for 15 d	0.92	0.46	0.80	0.36	0.99
40 d	1.20	0.39	0.91	0.58	1.03
60 d	1.74	1.57	0.99	0.99	1.28

[1]For thermophile numbers and lipase activity see Table 7.3.

[2]FFA were analysed in duplicate using the GLC method (Section 2.4.2).

[3]Information as supplied by manufactures.

[4]WMPs were stored at 22°C for 28 months (IH30), 16 months (II12), 11 months (BI18), 10 months (CI03) and 9 months (DI05).

The results in Table 7.4 showed more than 3-fold difference in the initial levels of FFA between powder CI03 (containing the lowest level of FFA) and powder DI05 (containing the highest level of FFA). This could be related to initial raw milk quality, or lipolysis that occurred during the milk powder manufacture process. No information is available regarding the baseline of FFA level in milk, nor in WMPs, since manufactures do not monitor this.

Storage at 22°C did not lead to changes in the total FFA levels in any of the WMPs, except powder DI05. While the total FFA level in powder CI03 appeared to be higher after 10 months of storage at 22°C, the data obtained at 37°C did not support this as a real change. The FFA levels appeared to remain unchanged over storage for 105 d at 37°C and 15 d at 55°C, again with the exception of powder DI05. However, by the following time of sampling at these two temperatures, increases in FFA levels were observed in almost every sample.

The level of FFA in powder CI03 did not change during 8 months storage at 37°C, but at 55°C, it increased 3-fold after 2 months and was the second fastest amongst five powders. This might suggest that the lipolytic enzyme in powder CI03 required a higher temperature for activity than the enzymes in other powders. This illustrates the points made in the previous section that while all the lipolytic enzymes may have the same specificity, they may not have the same temperature requirements and therefore particular bacteria may need to be targeted for control.

Overall, short chain fatty acids of $C_{4:0}$ to $C_{8:0}$ were released the most at both 37°C and 55°C. The patterns were similar to those given by the seven *Bacillus* lipases. For an

example, caproic acid was the main acid released in powder BI18. The final level of this acid was 8.8 and 1.5 times higher than the initial value after storage at 37°C and at 55°C, respectively. An actual level of FFA in a liquid product made from a WMP will depend on the reconstitution rate. Although the levels of short chain fatty acids increased 2-9 fold, they will still be within the threshold values (Kinsella, 1969) if the stored WMPs were made to 12.5% (w/v) reconstituted milk.

Temperature played a major role in the increase of FFA during WMP storage. The higher the storage temperature, the more FFA released. Increasing storage temperature did not result in the release of FFA from milkfat (Section 7.2), so lipolytic is the only possible cause for FFA release because ester linkages of milk lipids are stable over the pH range of dairy products (Bills *et al.*, 1969).

7.4.3 Release of Volatile Compounds in WMP during Storage

To determine the release of volatile compounds, headspace analysis of WMPs obtained in Section 7.4.1 were undertaken using a combination of SPME and GC/MS (Section 2.4.3). The results from one powder, batch II12, are presented in Fig. 7.3 (similar results were obtained with other powders – data not shown).

GC/MS data showed significant increases in the levels of methyl ketones and volatile free fatty acids ($C_{4:0}$ to $C_{8:0}$) after storage of WMPs at various storage temperatures (Fig. 7.3). The volatile short chain FFA detected correlated well with the results obtained in Section 7.4.2.

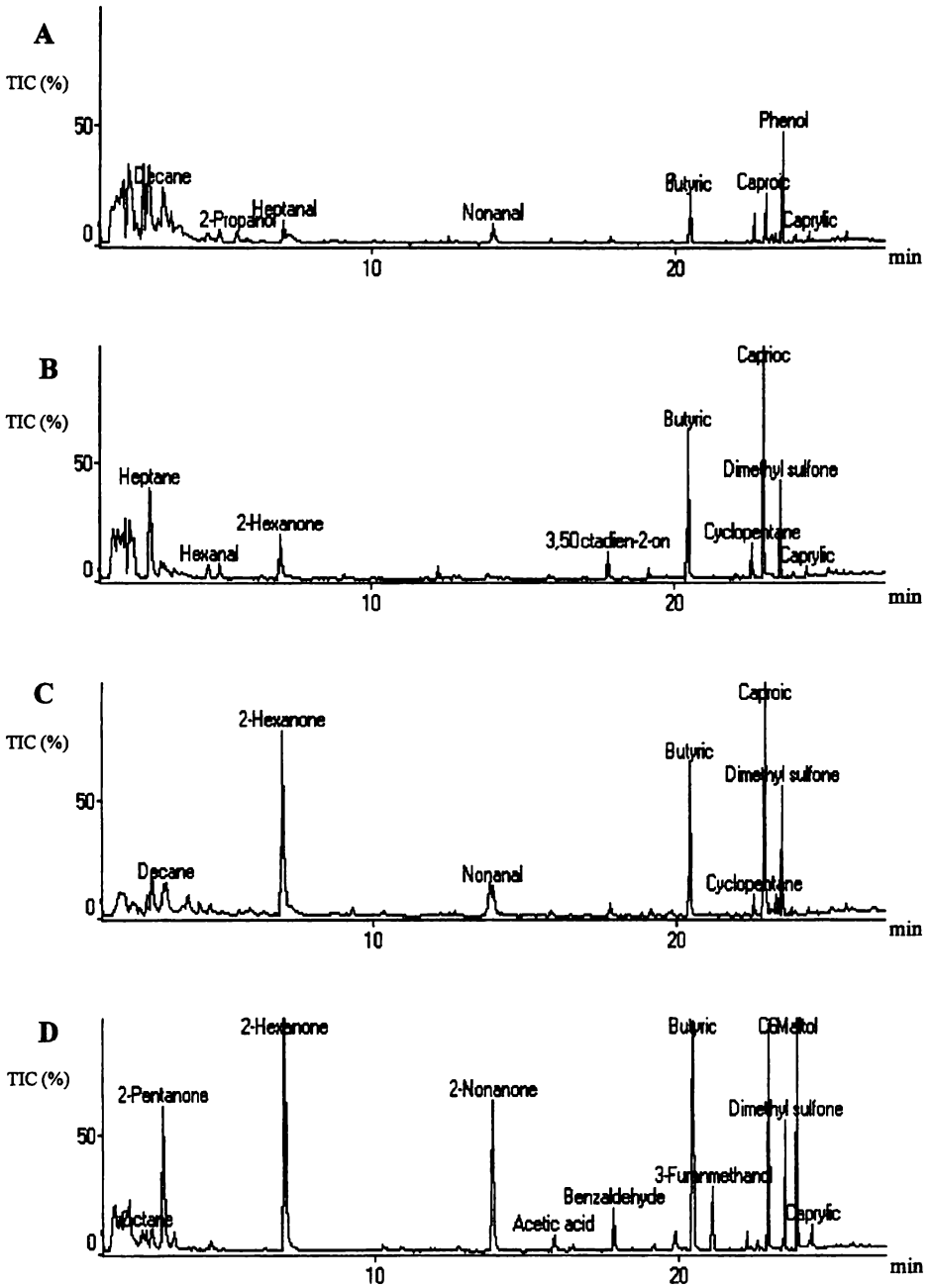

Figure 7.3: Headspace GC/MS analysis of volatiles from WMP. Samples were WMP batch II12: (A) before storage; (B) after 16 months at 22°C; (C) after 8 months at 37°C; and (D) after 2 months at 55°C. Y axis is expressed as percentage of total ion intensity (TIC) at scale 20,000,000.

Methyl ketones are known to cause stale flavours in WMPs and sterile concentrated milks during storage (Parks and Patton, 1961; Parks, 1967). They are formed from β-oxidation of FFA (Day, 1967). So lipolysis resulted in the release of FFA could become the precursor for the formation of the volatile compounds. FFA can also initiate other flavour compounds, such as acetoacetate, β-keto acids, flavour esters and lactones (Vulfson, 1994). If unsaturated fatty acids are released, they are susceptible to oxidisation to aldehydes and ketones which give off-flavours as 'cardboardy, oxidated or metallic' (Shipe *et al.*, 1978).

However, lactose breakdown products, such as furfural, were also detected at the higher storage temperature of 55°C. These compounds are Maillard reaction products (Parks, 1967). Renner (1988) reported that levels of undesirable odours and flavours in milk powder derived from Maillard reaction (such as hydromethylfurfural and aldehydes) increased with storage time and temperature.

7.4.4 Protein and Peptide Profiles in WMP during Storage

The WMPs obtained in Section 7.4.1 were reconstituted at 10% (w/v) in deionised water. To 1 mL sample, 0.5 mL 3% TFA was added, chilled on ice for 10 min and then centrifuged at 10,000 x *g* for 10 min at 4°C. The supernatant was obtained and analysed for protein break down using the RP-HPLC method (Section 2.3.1). The results from one of the WMPs are presented in Fig. 7.4 (similar results were obtained with the other powders – data not shown).

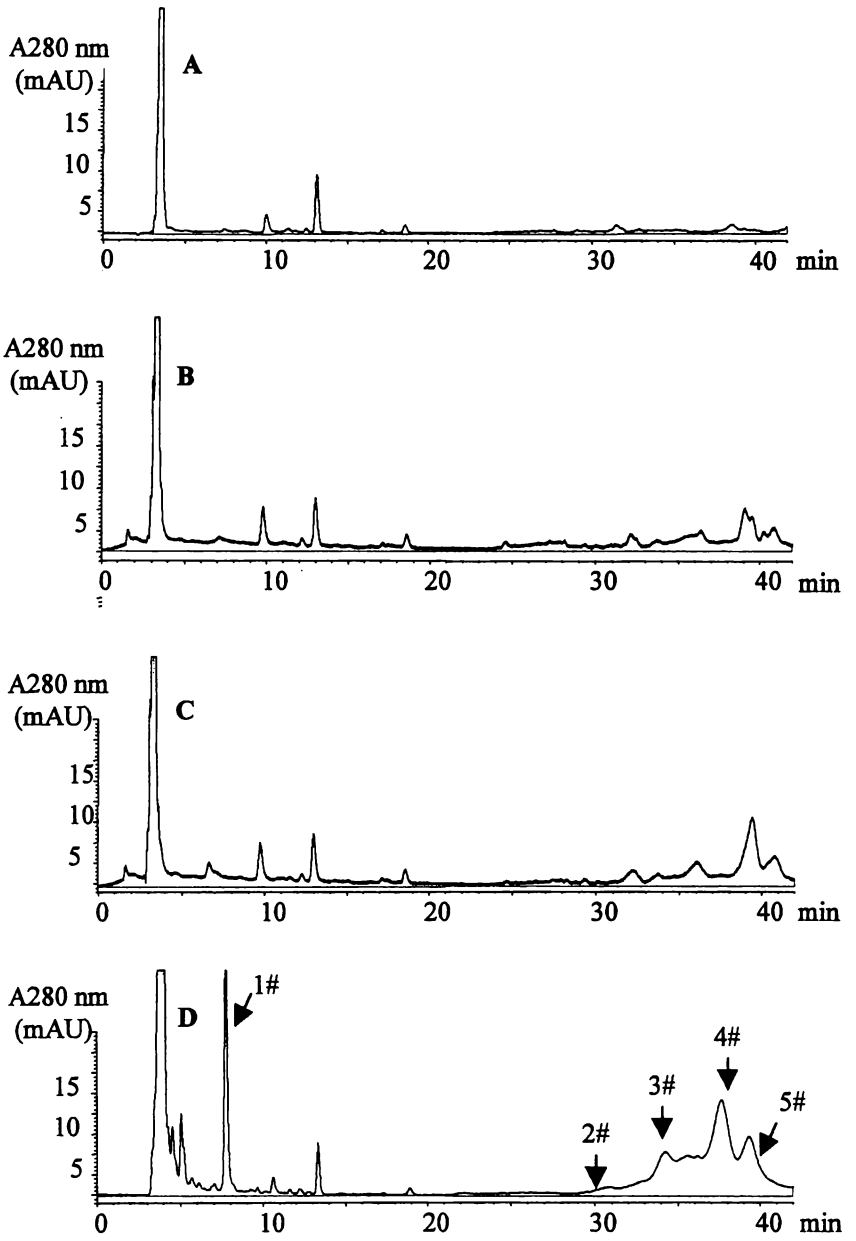

Figure 7.4: RP-HPLC trace of 1% TFA soluble fragments in WMP II12 after storage. (A) zero time; (B) room temperature (22°C) for 16 months; (C) 37°C for 8 months; and (D) 55°C for 2 months. Peaks labelled in (D) were further identified using Automated N-terminal sequence and mass spectra.

Increases in the areas of five major peaks of 1% TFA soluble fragments were found in WMP II12 during storage. The higher temperature for storage, the bigger increase (Fig. 7.4). The rest of four WMPs showed the same trends (data not shown). The patterns were identical with those obtained for the reconstituted milk made from the same powder (Figs. 3.13 and 3.14).

Four peaks shown in Fig 7.4 were identified by automated N-terminal sequencing analysis as: peak 2 – N-terminal of β-casein; peak 3 – N-terminal of α_{s1}-casein; peak 4 – N-terminal of β-casein; and peak 5 – N-terminal of β-lactoglobulin. Peak 1 could not be identified. There was insufficient material to obtain mass spectrometry data, so the size and sequence of the peptides are unknown. As mentioned earlier, milk caseins are heat-stable and do not breakdown at the storage temperature of 55°C, so proteolysis becomes the only possible cause for these peaks.

7.5 Physico-chemical Changes in WMP during Storage

Physico-chemical changes in WMPs obtained in Section 7.4.1, such as pH, a_w, solubility and powder particle structure were analysed (methods detailed in Section 2.6.4). The results of one WMP, batch II12, are presented in Table 7.5, Figs. 7.5 and 7.6.

The pHs of reconstituted milk made from the stored WMPs were slightly lower than of those made from the same powders before storage (Table 7.5). Similar results have been reported by Celestino *et al.* (1997a). The change could be the result of milkfat oxidation as well as lipolysis during storage of powder (Celestino *et al.*, 1997a).

Table 7.5: *Physico-chemical changes in WMP II12 after storage*

WMP II12	pH	a_w[1]	Solubility index[2] (mL) at 24°C	Sediment (mL) at 25°C
Initial	6.68	0.16	0.2	3.8
After 16 m at 22°C	6.75	0.17	0.3	7.0
8 m at 37°C	6.65	0.15	1.5	20.0
2 m at 55°C	6.57	0.24	15.0	17.0

[1]A_w was tested as equilibrium relative humidity at 30°C by Mr. A. Dodge (NZDRI).

[2]Solubility index and sediments were analysed following IDF standard 129 (1985) and NZDRI Method MP-IT-CW0 (Protein, Powder and Environmental Technology Section, NZDRI).

There was also a slight increase in the a_w of the powders at the higher storage temperatures (Table 7.5). This is as expected, when moisture content is below 12%, the a_w rises with increasing storage temperature (Presicky, 1997).

Overall, the higher the storage temperature, the higher the insolubility (shown by the increase of solubility index), and the greater the sediment. The sediment remained insoluble in 12.5% (w/v) reconstituted milk even after vortexing for 10 min at 50°C. Celestino *et al.* (1997a) reported a significant increase in the solubility index of WMPs after storage at 25°C for 8 months. Caseins are heat-stable, and will not become insoluble unless heated at temperatures above 120°C (Walstra *et al.*, 1999), which suggests that the possible cause is proteolysis. This was confirmed by the RP-HPLC results in Section 7.4.4. It should be noted that a single point cleavage on the intact protein by proteolysis can dramatically change its solubility.

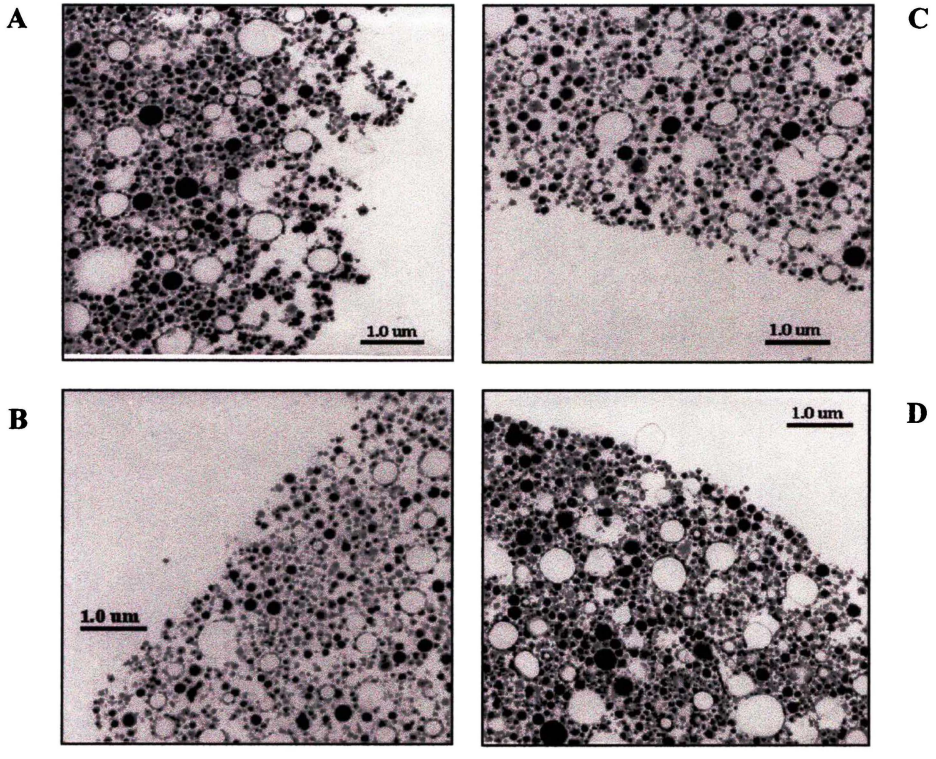

Figure 7.5: Transmission electron microscopy of WMP (batch II12) after storage at different temperatures. Samples were: (A) before storage; (B) after 16 months at room temperature (22°C); (C) after 8 months at 37°C; and (D) after 2 months at 55°C. Dark spots are proteins, while spots are fat globule. Scale bar = 1.0 μm.

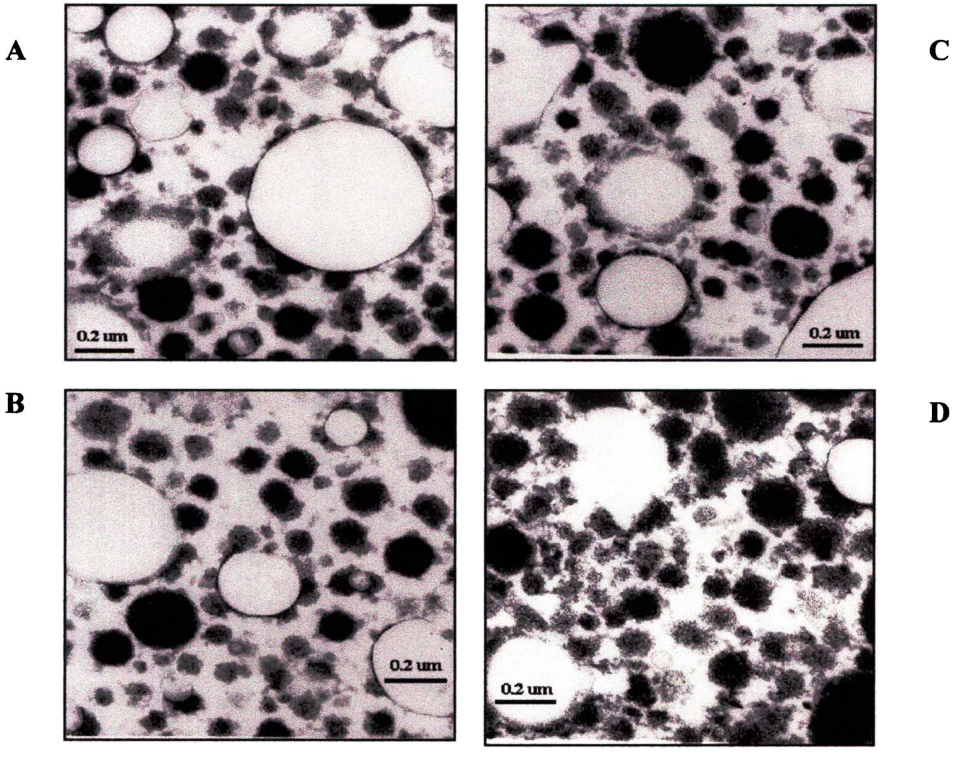

Figure 7.6: High powder transmission electron microscopy of WMP (batch II12) after storage at different temperatures. Samples were: (A) before storage; (B) after 16 months at room temperature (22°C); (C) after 8 months at 37°C; and (D) after 2 months at 55°C. Dark spots are proteins, while spots are fat globules. Scale bar = 0.2 μm.

Structural changes in the powder particles were confirmed by transmission electron microscopy (TEM). The protein particles became denser and less defined with a formation a 'skin' around the edge (Figs. 7.5 and 7.6). These changes became more obvious with increasing storage temperature and time. The formation of the 'skin' was possibly aggregation of whey proteins deposited on the surface of powder particles, which might affect hydration during reconstitution (personal communication, Mr. A. B. McKenna).

7.6 Summary

Both physico-chemical and biochemical changes were detected in the commercial WMPs during storage. However, the causes of the physico-chemical changes were possibly due to proteolysis and lipolysis. Milkfat was heat-stable at extremely high storage temperatures, and milk caseins were reported to be heat-stable as well, so the most probable cause for the changes in WMPs are enzymatic.

The general trend was that the higher the storage temperature, the faster the lipolysis reaction. For example, there was no release of FFA in powder CI03 at 37°C for 8 months, but there was a 3-fold increase after only for 2 months at 55°C. This confirmed that the enzymes were probably thermophilic as increasing storage temperature resulted in greater release of FFA, therefore, it is more like to be an enzymatic reaction rather than chemical reaction.

The more FFA released, the less thermophiles in the WMPs. Although there were differences in the initial levels of FFA amongst different batches of WMPs, there was no correlation with thermophile numbers *per se*. Levels of FFA in the WMP remained

unchanged after storage for 9 months at 22°C, but began to increase after 105 d and 15 d at 37°C and 55°C, respectively. This implies that the lipase in the WMP is likely to be from a thermophilic bacterium.

The pattern of FFA released in the WMP correlated well with the characteristics of the *Bacillus* lipases shown in a buffer system. Short chain fatty acids of $C_{4:0}$ to $C_{8:0}$ were predominantly released. Although the levels of these acids increased between 1-8 fold, they would still be within the threshold values if the powders were used to make 12.5% (w/v) reconstituted milk.

Water activity (a_w) was seen to increase slightly during the storage of WMP at 55°C. This is as expected. However, this would have enhanced the lipolysis in the WMP during storage. The reconstituted milk also showed slight decreases in pH. The solubility index showed significant drop, followed by the observation on changes in powder particle structure.

Overall, the changes in WMP during storage were mainly contributed by lipolysis and proteolysis. The release of FFA not only initiated volatile compound production, but also affected the pH, while the cleavage of protein resulted in the decrease in the solubility index. These changes did not have any correlation with the thermophile numbers in the WMPs, which were originally selected for storage trials based solely on this criterion.

Chapter 8 Overall Discussion and Future Considerations

The data presented in this thesis show that proteases and lipases are present in milk powders. The assays developed for determining the levels of these enzymes gave comparable results amongst powders, and they are useful research tools. However, the assays are not yet simple and straightforward enough to be used in a dairy factory. The main problems are interferences from milk proteins and lipids, especially for lipase assay where it was not possible to obtain a negative control because all 'pure' caseins originated from bovine milk, and have low levels of esterase activity.* Perhaps an antibody assay using specific antibody interaction will overcome the problems.

Separation techniques were developed to obtain semi-purified lipase preparation from powders. The lipase is likely to be from a thermophilic bacterial source as the highest activity was gained at 60°C. Although proteases and lipases from psychrotrophic bacteria may also be heat-stable, they tend to give highest activity at temperatures between 30 to 45°C.

However, the actual source of the lipase in the powder remains to be determined. It did not prove possible during this study to purify the enzyme due to the huge amount of interference from milk proteins, especially caseins. The caseins are hydrophobic in nature and easily form aggregates amongst themselves and between each other. It is likely that the enzymes in the WMP formed strong interactions with the caseins and remained associated with them during the milk powder manufacture process. This strong interaction is not easily overcome, as proven by the difficulties encountered during the attempts to purify the powder lipase.

* See Terminology Section xiv

Preliminary studies on the lipases produced by the seven *Bacilli* found in milk powder production streams showed that, with the exception of the F/G enzyme, they not only hydrolyse short chain *p*-nitrophenyl esters of fatty acids, but also prefer partially hydrolysed milk lipids. The pattern on *p*NP esters was similar to those given by the semi-purified powder lipase. Even though the majority of milkfat is triacyglcyerols, lipolysis is possible, because lipases, such as the F/G enzyme can hydrolyse the milkfat and prime further hydrolysis by any esterases. On the other hand, raw milk quality is also important, if the milkfat is hydrolysed (*e.g.* by LPL) prior to the process, there is a great potential for subsequent lipolysis by the thermophilic esterase to take place.

Three storage trials with WMP spiked with the thermophilic enzymes proved unequivocally that lipolysis and proteolysis are possible during storage in milk powders as low as $a_w < 0.2$ (est. total moisture content of 4%). Although the reaction in WMPs may be very slow, it will become much faster when the powder is reconstituted to liquid milk since increasing a_w results in non-linear increase of enzymatic reaction. Extensive amount of lipase resulted in huge release of FFA, especially short and medium chain acids which exceeded the threshold values only after one week of storage at 37°C.

Studies on the commercial WMP proved that lipolysis and proteolysis is possible with the levels of the enzymes present in the powders after manufacture. Changes measured as the FFA release and solubility index were higher at higher storage temperatures. The selection of powders for storage trials was based solely on thermophile numbers – one of the specifications on milk powder. However, no correlation was found between thermophile numbers, enzyme levels and defects. Therefore, the thermophile specification does not actually give any information about milk powder quality or the potential for spoilage during storage.

The commercial whole milk powders storage trials confirmed that the lipases in WMP were heat-stable, and the levels remained unchanged during storage even at 55°C for 2 months. Further, even at low levels, they could result in more than 3-fold increase in the total FFA at 55°C. These FFA may become precursors for the production of volatile compounds, such as methyl ketones which are known to contribute to "stale" flavour. The solubilities of the WMP decreased over time at higher storage temperatures. This is likely to be due to proteolysis which resulted in cleavage of milk proteins, since even a single point cleavage can lead to a dramatic effect on powder solubility.

The enzymes found in the powder and from the seven *Bacilli* found the milk powder process streams showed considerable heat-stability in a buffer system. Given enhanced stability by milk caseins, they are unlikely to be destroyed by any of the typical heat-treatments applied during manufacture process and will end up in the powder, and remain active for a long time.

It is very important to continue the fundamental studies on these heat-stable enzymes. As the use of milk powders for production of storable liquid products is increasing, these heat-stable enzymes will be a major concern for spoilage in the liquid product. Even the exact amount of enzymes cannot be defined, at least the methods applied in this thesis can give a relative value for spoilage potential. With further modifications, these methods can conceivably provide assays for the industry. Other developments, such as an immunoassay using a specific antibody column could be used to quickly extract the enzymes from most of milk protein and enable rapid assays.

Further work on identification of the source bacterium is also important. This will lead to better understanding of the enzymes released by the thermophiles, and eventually

develop methods to control their presence in milk powders. The enzymes from the seven *Bacilli* needs further characterisation, especially the lipases (esterases) because (1) they are not as clearly classified as proteases, and (2) they can carry out hydrolysis (lipolysis) at extremely low a_w (below 0.1).

The development of an antibody affinity separation technique may lead to identification of the powder enzymes, hence the correlation could be made between the powder enzymes and those from the seven *Bacilli*. Alternatively, enzyme specifications rather than thermophile specifications could be established for milk powders and methods for controlling the levels of enzymes developed.

Further studies on initial levels of FFA in milk and powders will provide information on the quality of the milk for powder manufacture, and alternatively define specifications for FFA which directly correlate with powder quality. Further studies on the relationship between a_w and lipolysis will lead to a possible definition of minimum level of water for preventing enzymatic reactions in milk powder and for keeping quality. Further investigation of functionality changes in relationship to proteolysis will lead to the determination of protease effects on milk powders.

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