A kinetic and molecular study of the purified lipase from *Aspergillus niger*

by

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God, my Creator and the Creator of all life, be the glory.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

$\gamma_{o/w}$	interfacial tension
µm	micrometer
AnL	*Aspergillus niger* lipase
ASP	ammonium sulphate precipitation
BCA	bicinchoninic acid
BSA	bovine serum albumin
CCD	charge coupling device
CDL	colipase dependant lipase
CEL	carboxyl esterase
CHAPS	3-cholamidopropyldimethyl-ammonio-1-1-propane sulfonate
cmc	critical micellar concentration
CTAB	cetyltrimethylammonium bromide
Da	dalton
DEP	diethylpyrocarbonate
DFP	diisopropylfluorophosphate
DIG	digoxigenin
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
FA	fatty acid
g	acceleration due to gravity
HGL	human gastric lipase
HPL	human pancreatic lipase
kbar	kilobar (pressure)
kJ/mol	kilojoules per mole
LD-PCR	long distance polimerase chain reaction
m	metre
MM	molecular mass
mN	millinewton
NaAc	sodium acetate

NMR	nuclear magnetic resonance
OD	optical density
PDA	potato dextrose agar
pfu/µg	plaque forming units per microgram
PLRP	pancreatic lipase related protein
PMSF	phenylmethylsulphonyl fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
S.I.	stereoselectivity index
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sn-x	stereospecific numbering, where x is any position on the glycerol
TE	50 mM Tris and 10 mM EDTA buffer (pH = 7.8)
THF	tetrahydrofuran
Tris	Tris(hydroxymethyl)aminomethane
U/ml	activity expressed in Units per millilitre
V.I.	vicinity index

Felix qui potuit rerum cognoscere causas

Happy is he who gets to know the reasons for things!

Virgil

CHAPTER 1

Literature Review

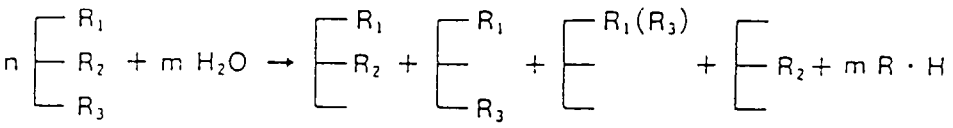
1.1 General introduction

Glycerol ester hydrolases (E.C. 3.1.1.3) or lipases are enzymes that act on the carboxyl ester bonds present in acylglycerols to liberate organic acids and glycerol (Jaeger *et al.*, 1994). Lipases are physiologically important since they catalyse the hydrolysis of oils and fats to free acids and partial acylglycerols, which are essential for metabolic processes such as fatty acid transport, oxidation, and resynthesis of acylglycerols and phospholipids (Shahani, 1975). Although naturally occurring triacylglycerols are normally the preferred substrates, the enzyme can hydrolyse a wide range of insoluble fatty acid esters. It is well demonstrated that the reaction is reversible, and that the enzyme can catalyse ester synthesis from various alcohols and acids, and transesterification, often in nearly anhydrous organic solvents (Figure 1.1) (Kurashige *et al.*, 1989).

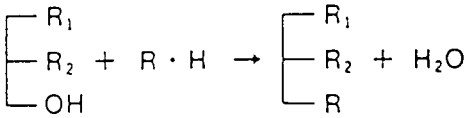
The hydrolysis reaction involves an attack on the ester bond of glycerides in the presence of water molecules to produce both an alcohol functionality and a carboxylic acid (Figure 1.1, Reaction 1). The hydrolysis of fats and oils (triacylglycerols) can be reversed by modifying the reaction conditions. The equilibrium between the forward and reverse reactions is controlled by water content of the reaction mixture, so that in a non-aqueous environment lipases catalyse ester synthesis reactions. Different types of ester synthesis can be distinguished: common ester synthesis from glycerol and fatty acids (Figure 1.1, Reaction 2) and the biotechnologically more important transesterification reactions in which the acyl donor is an ester (Figure 1.1, Reactions 3.1 – 3.3). Transesterification involving fats and oils can further be categorised depending on the type of acyl acceptor. Acidolysis refers to the exchange of acyl radicals between an ester and an acid (Figure 1.1, Reaction 3.1). Alcoholysis and

glycerolysis refer to the transfer of an acyl group from a triacylglycerol to either an alcohol or glycerol (Figure 1.1, Reaction 3.2). In interesterifications, the acyl group is exchanged between acylglycerols (Figure 1.1, Reaction 3.3).

1. Hydrolysis of ester

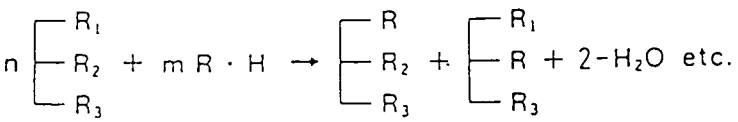


2. Synthesis of ester

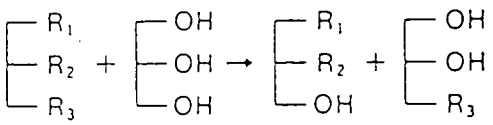


3. Transesterification

3.1. Acidolysis



3.2. Alcoholysis (Glycerolysis)



3.3. Interesterification

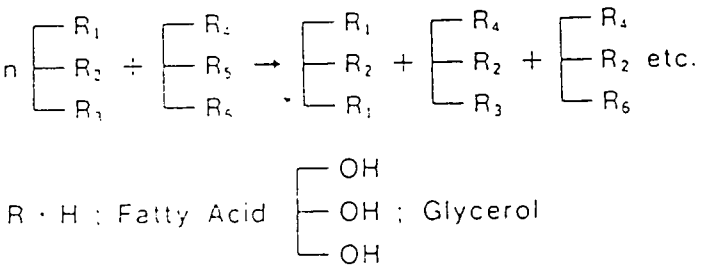


Figure 1.1: Schematic diagrams of lipase-catalysed reactions (Taken from Kurashige *et al.*, 1989).

The lipase enzyme has a wide range of properties, with respect to substrate specificity, pH optimum and thermostability depending on its source. The fact that lipases remain active in organic solvents significantly broadens their biotechnological applications.

The structures of several lipases have been elucidated. The catalytic domains of all lipases whose structures are known have the same α/β hydrolase fold. They are all serine esterases and their catalytic triads are perfectly superimposible. The active site serine is invariably imbedded in a hydrophobic region and mostly buried under a surface loop or "lid". The topology and length of the lid differs depending on the source of the lipase. The exact position and nature of the lid was thought to be an important modulator of lipase activity (Van Tilbeurgh *et al.*, 1993).

1.2 Occurrence and classification of lipases

Lipases are widely distributed in nature, being found in plants, animals and micro-organisms. They have been classified according to their sources, kinetic properties and substrate specificities. More recently, studies on three-dimensional structures of lipases enabled an even better classification of lipases.

1.2.1 Classification according to the source of lipase

1.2.1.1 Plant lipases

It is known that lipases exist in several plant tissues, but few studies have been done so far on the distribution of lipases in whole plants. Most of the studies that have been done on plant lipases have been devoted to seed lipases. During the initial phase of germination, seeds contain a large amount of triacylglycerols, which serve as a compact source of energy for the newly emerging plant, and a small amount of water (Adlercreurtz *et al.*, 1997). Examples of isolated plant lipases are

the lipase from lupin seed (Sanz and Olias, 1990), *Brassica napus* and *Vernonia galamensis* (Adlercreurtz *et al.*, 1997).

1.2.1.2 Animal lipases

Lipases are found in animal tissues such as the muscles, the liver, adipose tissues, the digestive tracts, etc. Animal lipases can be further classified according to their sites of lipolytic action and substrate specificities.

1.2.1.2.1 Preduodenal lipases

The digestion of milk fat is initiated in the stomach by lingual or gastric lipases (Hamosh, 1994). These are a group of closely related enzymes with a sequence homology of 78 %. Rodents, (Hamosh and Scow, 1973) and ruminants (Ramsey *et al.*, 1956; Grosskopf, 1965) depend on enzymes secreted from serous glands on the dorsum of the oropharynx, whereas, many other species such as carnivores depend on gastric lipases (De Nigris *et al.*, 1988; Moreau *et al.*, 1988 a, b). Digestion is then continued in the intestine by pancreatic lipases.

1.2.1.2.2 Pancreatic lipases

The pancreas secretes two lipolytic enzymes, the colipase dependent lipase (CDL) (Sarda and Desnuelle, 1958; Borgström and Erlanson, 1971) and the carboxyl esterase (CEL) (Sarda and Desnuelle, 1958), as well as a group of pancreatic lipase related proteins ($PLRP_1$ and $PLRP_2$) (Giller *et al.*, 1992), whose characteristics differ from those of CDL by exhibiting high phospholipase activity and the absence of the colipase effect in maintaining activity at high salt concentration (Giller *et al.*, 1992). The PLRP proteins in human (Giller *et al.*, 1992) as well as in animal species (Wishart *et al.*, 1993; Hjort *et al*, 1993; Thirstrup *et al.*, 1994) were investigated. There is a high sequence homology between $PLRP_1$ and $PLRP_2$ but somewhat lowers homology with the pancreatic proteins CDL. Because of their high phospholipase activity, and inhibition by bile salts, (which cannot be

overcome by colipase), it has been suggested that they function mainly as phospholipases (Thirstrup *et al.*, 1994).

1.2.1.2.3 Lipoprotein lipase

Lipoprotein lipase belongs to the same protein family as pancreatic lipase, but has evolved to fulfil a different physiological function, namely to hydrolyse triacylglycerols and phospholipids in plasma lipo-proteins. Lipoprotein lipases play an important role in the metabolism of triacylglycerols present in the core of chylomicrons and very-low-density lipoproteins. The enzyme is a glycoprotein and is active in its dimeric form (Bruin *et al.*, 1992).

The lipase shows a number of functional sites, which are distinct from those found in pancreatic lipase. A heparin binding site is located in the cleft between the folding units on the side opposite to the opening of the active site, and serves to anchor the enzyme to heparin sulphate proteoglycans at the luminal side of blood vessels (Olivecrona and Olivecrona, 1993). Apart from the interaction with heparin sulphate, lipoprotein lipase has at least five additional functional domains, including a catalytic active centre, a site for dimerisation, a site for binding to the lipid-water interface and a putative site for binding fatty acids (Bruin *et al.*, 1992).

1.2.1.2.4 Hormone sensitive lipases

Hormone sensitive lipases catalyse the rate-limiting step in the hydrolysis of adipocyte triacylglycerols, and is therefore a key enzyme in lipid metabolism and overall energy homeostasis. The activity of hormone sensitive lipase is under strict hormonal and neuronal control through reversible phosphorylation. Hormone sensitive lipase exhibits a high enzyme activity towards cholesteryl esters, an unusual property of lipases, and has together with the relatively high level of expression in steroidogenic tissues, led to the proposal that the hormone sensitive lipase plays an important role in steroidogenesis (Holm *et al.*, 1994).

1.2.1.3 Microbial lipases

Many lipases from microbial sources have been purified and sequenced. Comparison of amino acid sequences between microbial lipases often revealed no detectable similarities beyond the consensus pentapeptide. The number of amino acid ranges from about 200 in *Pseudomonas fragi* to more than 600 in *Stapylococcus aureus*.

Most microbial lipases are extracellular, being secreted through the external membrane into the culture media. Microbial lipases are subdivided into bacterial and fungal lipases (Taipa *et al.*, 1992). The properties of most microbial lipases are known and are so diverse that with careful screening one can probably find the enzyme or lipase-producing organism with the required properties.

1.2.2 Classification of ester hydrolysis by kinetic properties

1.2.2.1 Esterases

Enzymes that hydrolyse ester bonds in general are esterases (E.C. 3.1.1.1). Esterase enzymes show normal Michaelis-Menten kinetics with respect to substrate concentration. The activity of esterase enzymes does not increase at substrate concentrations exceeding solubility.

1.2.2.2 Cutinases and suberinases

Lipases and esterases have been found to be closely related to cutinases, enzymes that degrade cuticle (the insoluble lipid-polyester matrix covering the surface of plants) and are capable of hydrolysing triacylglycerols. Cutinases differ from classical lipases in that they do not have "lids" covering the active centre of the enzyme and they are active on both soluble and emulsified triacylglycerols (Martinez *et al.*, 1992). Cutinases, therefore establish a bridge between esterases and lipases. Suberinase is an esterase which hydrolyses suberin. Although there

is functional similarity, cutinases and suberinases differ structurally (Derewenda *et al.*, 1994a).

1.2.2.3 Lipases

Figure 1.1 shows reactions that are catalysed by lipases. The very same types of reactions are also catalysed by esterase enzymes. It thus becomes difficult to distinguish between a lipase and an esterase as these two groups of enzymes show considerable overlap in substrate specificities. However, lipases have been found to possess the unique characteristic of being able to be "activated" by interfaces.

Long-chain triacylglycerols, which are the normal substrates of lipase, have hydrophobic properties. In aqueous environments, they form emulsions (lipid-water interfaces) at points of maximum concentration. By contrast, short-chain triacylglycerols posses a distinct solubility due to a higher hydrophilicity. They yield monomers at low concentrations and micelles in more concentrated solutions. It has been shown that whereas the rate of breakdown of a dilute solution of triacylglycerol by a lipase is very slow, the enzymatic activity increases dramatically once the substrate solubility is exceeded (Verger, 1980). This phenomenon was wrongly referred to as "interfacial activation" and was thought to demonstrate a fundamental difference between an esterase and a lipase based upon the presence or absence of "interfacial activation".

1.3 Views on the "interfacial acivation" phenomenon

The four main classes of biological substances are carbohydrates, proteins, nucleic acids and lipids. The first three substances have been clearly defined on the basis of their structural features, whereas the property, which is common to all lipids, is a physicochemical one. Lipids are in fact a group of structurally heterogeneous molecules, which are all insoluble in, water but soluble in apolar and slightly polar solvents such as ether, chloroform and benzene.

Lipids have been classified by Small (1968) depending on how they behave in the presence of water (Figure 1.2). This makes it possible to distinguish between polar and apolar lipids (e.g. hydrocarbon, carotene). The polar lipids can be further subdivided into three classes. Class I consists of those lipids which do not swell in contact with water and form stable monomolecular films (these include triacylglycerols, diacylglycerols, phytols, retinols, vitamin A, K and E, waxes and numerous sterols). The class II lipids (which include phospholipids, monoacylglycerols and fatty acids) spread evenly on the surface of water, but since they become hydrated, they swell up and form well-defined lyotropic (liquid crystalline) phases such as liposomes. The class III lipids (such as lysophospholipids and bile salts) are partly soluble in water and form unstable monomolecular films, and beyond the critical micellar concentration level, micellar solutions.

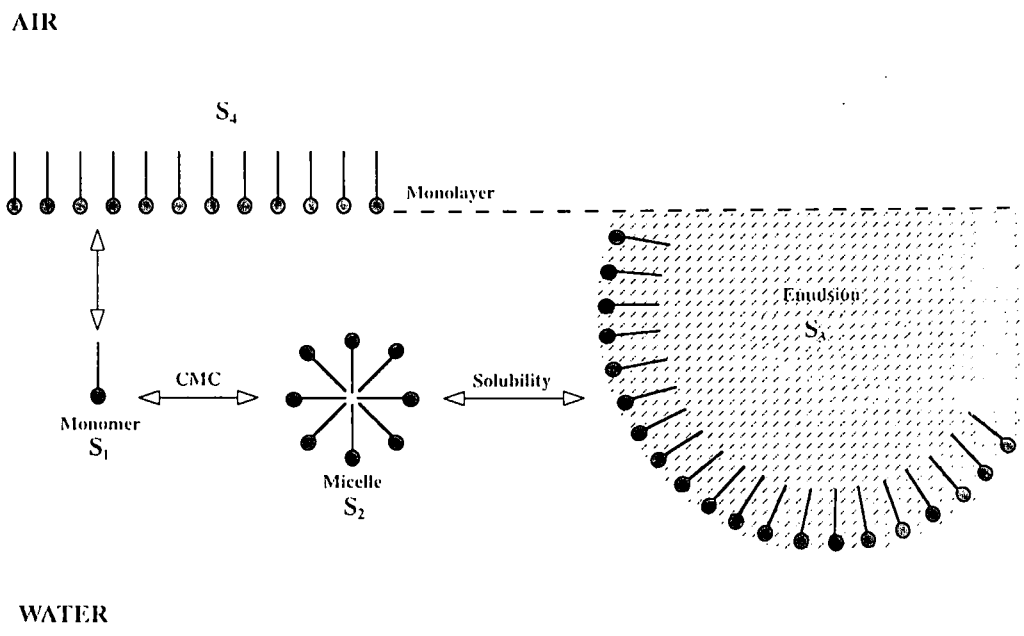


Figure 1.2: A classification of biological lipids based upon their interaction in aqueous systems (Small, 1968). Monomer (S_1), micelle (S_2), emulsion (S_3), and absorbed monolayer (S_4) coexists in equilibrium and represent potential lipase monomeric substrate (S_1) or supersubstrates (S_2, S_3 and S_4) (Taken from Ferrato *et al.*, 1997).

What exactly is a lipase? Is it enough to say that it is a carboxyl esterase which specifically hydrolyses triacylglycerols? In 1958, Sarda and Desnuelle defined lipases in kinetic terms, based on "interfacial activation" phenomenon. This property was not to be found for example among the enzymes which have been classified as esterases, *i.e.* those acting only on carboxylic ester molecules which are soluble in water. The "interfacial activation" phenomenon was in fact first observed as far back as 1936 by Holwerda and co-workers and by Schϕnheyder and Volqvartz, (1945). It amounts to the fact that the activity of lipases is enhanced on insoluble substrates (such as emulsions) rather than on the same substrates in true monomeric solutions. It therefore emerged from the above-mentioned studies that lipases might constitute a special category of esterases which are highly efficient at hydrolysing molecules having a carboxylic ester group and are aggregated in water.

This property was used for a long time to distinguish between lipases and esterases. A conceptual shift has unfortunately occurred however, as the result of which "interfacial activation" has been taken to mean a hypothetical conformational change occurring as the result of interfacial adsorption (Desnuelle *et al.,* 1960).

The preceeding hypothesis gradually drifted and was then progressively transformed to cover an idealised concept, far away from real experimental facts and artifacts. The first three-dimensional structures to be elucidated (Brady *et al.*, 1990; Winkler *et al.*, 1990) suggested that the "interfacial actication" phenomenon might be due to the presence and amphiphilic peptidic loop covering the active site of the enzyme in solution, just like a lid. When contact occurs with a lipid/water interface, this lid must undergo a conformational rearrangement, resulting in the active site becoming accessible. It is worth noting however that the hydrolysis of a substrate having the form of a truly monomeric solution might well also require the lid to be open without any "interfacial activation" being involved. "Interfacial activation" was thought to involve the open and closed forms of lipases.

The results of recent lipase research have nevertheless shown how careful one has to be about extrapolating any kinetics and/or structural characteristics

observed to all lipases in general. The catalytic activity of many lipolytic enzymes has been measured using carboxylic esters which are partly soluble in water, and many differences have been found to exist between the resulting profiles (Figure 1.3).

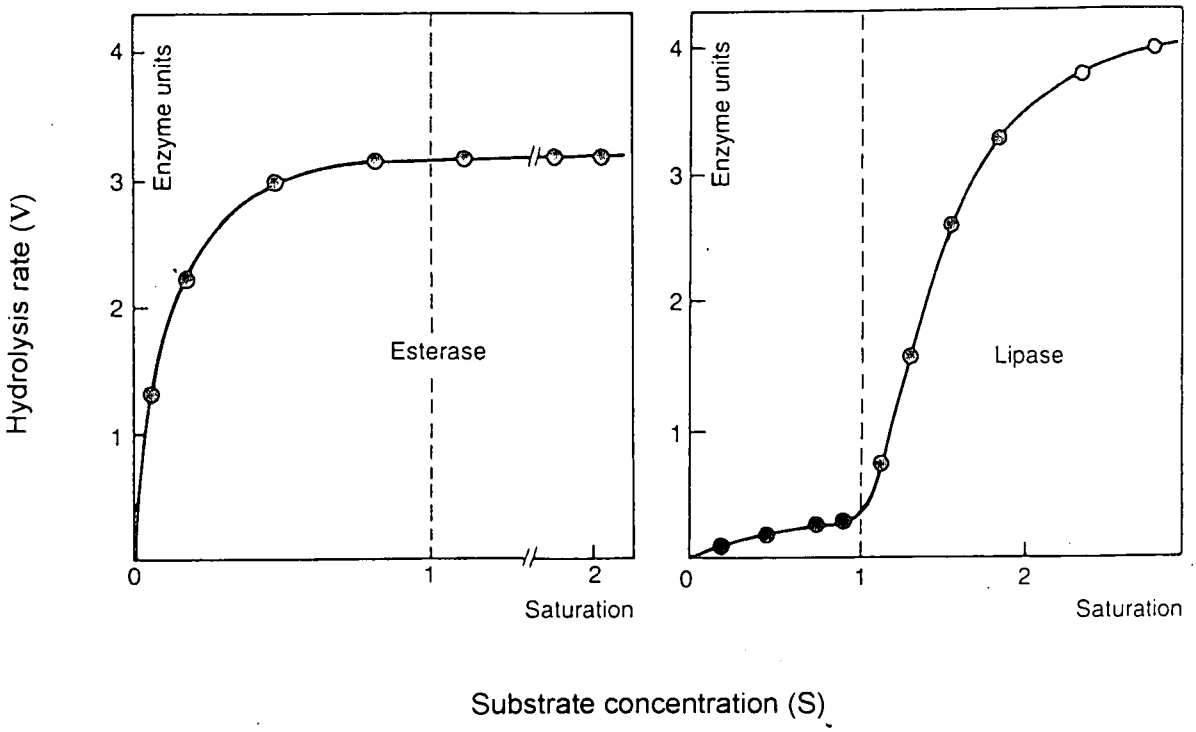


Figure 1.3: Hydrolysis rates (V) as a function of the amount (S) of partly water-soluble ester. Dashed vertical lines represent the limit of solubility or the critical micellar concentration of the ester used. Such kinetic behaviors have been commonly used to discriminate between esterases (left panel) and lipases (right panel) (Taken from Ferrato *et al.*, 1997).

The greatest caution must be exercised both when performing and interpreting kinetic measurements with lipids. Firstly, it is essential to check that the initial lipase velocity is proportional to the amount of enzyme used, both below and above the solubility limit. Substrate depletion, in the monomeric range of substrate concentration, is sometimes a major experimental limitation. Secondly, it is also essential to check that the same lipase active site, and not other unspecified sites, is responsible for the measured catalytic activity on monomeric substrates. Control experiments with nonenzymatic proteins or inhibited lipase should be performed. Thirdly, since the media is heterogeneous, adding any amphiphilic compound to

the system is liable to modify both quantitatively and qualitatively the physicochemical properties of the interface.

In the framework of the European Bridge-T lipase project (1990 to 1994), some new three-dimensional structures and numerous biochemical data provided new insights into lipases. It emerged from these studies that the lipases do not all subscribe to the phenomenon of "interfacial activation". The main exceptions noted was the lipase from *Pseudomonas glumae* (Noble *et al.*, 1993), *Pseudomonas aeruginosa* (Jaeger *et al.*, 1994) and *Candida antarica B* (Uppenberg *et al.*, 1994), all of which have an amphiphilic lid covering the active site. In view of the reinvestigation of the "interfacial activation" one cannot exclude that the optimal experimental conditions have not been found to observe this phenomenon with other lipases.

Some new pancreatic lipases have been identified. Comparisons between their primary amino acid sequences have shown that they have a fairly high degree of homology, but they can nevertheless be divided into three subgroups: (1) the "classical" pancreatic lipases; (2) the pancreatic lipase-related proteins of type 1 ($PLRP_1$), and (3) the pancreatic lipase-related proteins of type 2 ($PLRP_2$). Although the kinetic properties of the classical pancreatic lipases, particularly with regards to "interfacial activation", have been quite fully documented, it was only recently that the $PLRP_2$ lipases of the coypu and the guinea pig were found to show no "interfacial activation". Surprisingly, the coypu lipase has a 23 amino acid lid, which is homologous to that of the classical pancreatic lipases, whereas the guinea pig lipase has a mini-lid consisting of only five amino acid residues (Withers-Martinez *et al.*, 1996).

One can suggest that the molecular explanation for the "interfacial activation" phenomenon had to be investigated not only at the level of the lipase three-dimensional structure, but also in the dynamics of organised multimolecular structures as well as in the interfacial conformations (interfacial quality) of lipids used as lipase substrates. Among other physicochemical parameters, one can imagine that the radius of curvature of the super substrates (S_2, S_3 and S_4 depicted

in Figure 1.2) could play an important role during the interfacial docking of lipases and hence could modulate the catalytic activity, through subtle conformational changes.

"Interfacial activation" as well as the presence of a lid domain are therefore not in the least appropriate criteria on the basis of which to determine whether such an esterase belongs to the lipase subfamily. "Interfacial activation" is thus sometimes wrongly taken as a criterion for predicting the existence of a lid domain in lipases with a unknown three-dimensional structure.

Because naturally occurring triacylglycerols are totally insoluble in water, in contrast to short-chain triacylglycerols, to be little more than an artifact that has stimulated the imaginations of many biochemists, but which has not turned out to be of any very great physiological significance. Lipases might therefore be quite pragmatically redefined as carboxyl esterases that catalyse the hydrolysis of long-chain acylglycerols. In fact, they are simply fat spliting "ferments".

1.4 Specificities of lipase

1.4.1 Positional specificity

Lipases may be unspecific or show specificity for the position of the acyl substituent on the glyceride (Ratledge, 1989). Lipases from *Corynebacterium acnes* (Hassings, 1971), *Chromobacterium viscosum* (Sugiura and Isobe, 1975), *Staphylococcus aureas* (Vadehra and Harmon, 1967), *Staphylococcus hyicus* (Van Oort *et al.,* 1989) and *Aspergillus oryzae* (Ohnishi *et al.*, 1994) are non-specific. They cause complete hydrolysis of triacylglycerols to free fatty acids and glyceride.

Positional specific lipases hydrolyse the ester bonds of triacylglycerides at either the 1,3 or 2 positions. The *sn*-1,3 specific lipases catalyse the hydrolysis of ester bonds of fatty acids located at the *sn*-1 and *sn*-3 positions preferentially to give free fatty acids and the di- and monoglycerides as reaction products. Many microbial

lipases are *sn*-1,3 specific (Omar *et al.*, 1987; Hoshino *et al.*, 1992; Sugihara *et al.*, 1991, 1992 ; Shimada *et al.*, 1993). The *sn*-2 specificity is extremely rare, and has been ascribed to lipases III and IV from *Geotrichum candidum* which have particular abilities to cleave the 2-positioned ester bond nearly twice as fast as 1,3-positioned ester bonds of triacylglycerol molecules (Sugihara *et al.*, 1993).

1.4.2 Fatty acid specificity

Lipases often exhibit a particular ability to release fatty acids whose chain lengths fall within well-defined ranges (Malcata *et al.*, 1992). Microbial lipases have been investigated for chain length specificities and diverse results have been reported. Lipases derived from *Pseudomonas aeruginosa* MB 5001 (Chartrain *et al.*, 1993), *Penicilium caseicolum* (Alhir *et al.*, 1990) and *Candida deformans* (Murderhwa *et al.*, 1985), were found to hydrolyse triacylglycerols containing short-chain fatty acids more readily than those containing long-chain fatty acids. In contrast lipase from *Neurospora crassa* readily hydrolysed triacylglycerols with C-16 and C-18 fatty acids, but hydrolysed short chain fatty acids (C 4 - 10) at a very slow rate (Kundu *et al.*, 1987).

The distribution of activities of some lipases relative to various triacylglycerols changes with temperature, as temperature is increased, the rates of release of long-chain fatty acids increase faster than those of the corresponding short-chain acids. Lipases isolated from *Fusarium heterosporum* and *Bacillus* species showed different preference towards fatty acid chain length depending upon the reaction temperature. At 30 °C the lipase enzyme from *Fusarium heterosporum* hydrolysed triacylglycerols of short-fatty acids with a much higher velocity than the others (Shimada *et al.*, 1993). Elevation of the reaction temperature increased the activity towards the longer fatty acid chain triacylglycerols. The same results were obtained with the studies of the lipase derived from a *Bacillus* specie which showed low activities towards triacylglycerols of long chain length (more than 12 carbons) at 30 °C, but these substrates were readily subjected to enzymatic hydrolysis at 50 °C when this substrate became liquidized at (Sugihara *et al.*, 1991).

For the same chain length of the fatty acid residue, the rate of attack by some lipases seems to increase with the number of double bonds in the hydrocarbon backbone (Malcata *et al.*, 1992). Lipolytic activity of lipase from *Pseudomonas aeruginosa* MB 5001 increased as 18-unsaturated fatty acid content of the oils increased (Chartrain *et al.*, 1993). Low activity was obtained with lard oil (18:0 and *cis*-18:1(9) rich) and olive oil (rich in *cis*-18:1(9)), while higher activity was achieved with sunflower oil (*cis,cis*-18:2(9,12) and *cis,cis,cis*-18:3(9,12,15) rich). Similarly, a higher lipolytic activity was obtained with trilinolein (*cis,cis*-18:2(9,12)) than with triolein (*cis*-18:1(9)) (Chartrain *et al.*, 1993). The rate of triacylglycerol hydrolysis by a lipase from *Pythium ultimum* was also found to increase with an increasing number of double bonds per molecule (Mozaffar and Weete, 1993).

One explanation for the above type of specificity involves the concept of induced fit (Malcata *et al.*, 1992). Although a great many substrates can bind at the active site, only a few can release a proper amount of binding energy required for the change in the conformation of a lipase to a form which is a much more efficient catalyst. Substrates that are too small or possess too few double bonds are not able to release enough binding energy. In such cases the change in conformation of the native lipase to the desired catalytically active conformation does not occur or is, at best, incomplete. Hence, the reaction will proceed slowly. Substrates that are too long or possess too many double bonds are able to release enough binding energy that would in principle be sufficient to effect the desired conformational change. However, some of this energy becomes unavailable for this purpose because it is required to change the conformation of the substrates to make it fit into the active site. Hence only a small fraction of the energy released by the binding process will actually be available to drive the conformational change of the enzyme. Consequently, optimal activity will not be achieved.

The presence of two, and especially three double bonds in the 18-carbon fatty chains reduced the rate of triacylglycerol hydrolysis by some other lipases. Lipase derived from *Candida deformans* hydrolysed triacylglycerols with *cis,cis*-18:2(9,12) and especially with *cis,cis,cis*-18:3(6,9,12) at a slower rate than those with 18:0 and *cis*-18:1(9) (Muderhwa *et al.*, 1985). Similarly, *Humicola lanuginosa* No. 3

lipase catalysed polyethylene sorbitan monooleate (Tween 80) to a higher extent than triolein (*cis*-18:1(9)) and showed low hydrolytic activity towards esters of a higher degree of unsaturation such as methyl linoleate (*cis,cis*-18:2(9,12)) and methyl linolenate (*cis,cis,cis*-18:3(9,12,15)) (Omar *et al.*, 1987).

A special kind of fatty acid specificity has been reported for lipase B from *Geotrichum candidum* which showed high specificity for esters of fatty acids with *cis*-9 double bonds (Jacobsen and Poulsen, 1991; Charton and Macrae, 1991). This feature is resembled by the lipase isolated from *Galactomyces geotrichum* which displayed preference for long chain fatty acids containing a *cis*-9 double bond (Phillips and Pretorius, 1991).

Other lipases can equally hydrolyse saturated and unsaturated triacylglycerols. For example *Neurospora crassa* lipase preferred tripalmitin (16:0), tristearin (18:0), tripalmitolein (16:1), triolein (*cis*-18:1(9)) and trilinolein (*cis,cis*-18:2(9,12)) and hydrolysed them at the same rates (Kundu *et al.*, 1987). A lipase isolated from lupin seed was found to be more active on saturated than on unsaturated fatty acids (Sanz and Olias, 1990). Lipase enzyme from *Fusarium oxysporum f.sp.lini* exhibited a higher affinity to the ester bond of saturated fatty acids than that of unsaturated fatty acids (Hoshino *et al.*, 1992). This preference was exploited in the concentration of poly-unsaturated fatty acid (n-3 PUFA) content of partially hydrolysed glycerides obtained from fish-oil. The lipase gave increases in n-3 PUFA concentration as the hydrolysis progressed.

1.4.3 Glyceride specificity

Some enzymes show unusual specificity towards glyceride i.e. the selectivity among mono-, di- and triacylglycerol as substrates. A lipase from *Penicillium cyclopium* MI has been shown to display its highest activity towards monoglycerides, and much lower activities towards di- and triacyglycerols (Okumura *et al.*, 1980). Yamuguchi and Mase (1991) reported a lipase from *Penicillium camemberti* U-150 with absolute specificity towards mono- and diacylglycerol.

1.4.4 Stereospecifity

Although not much literature is available on the subject of lipase stereoselectivity, a rather large body of literature deals with the preparation of chiral esters and alcohols employing lipase-mediated kinetic resolution of racemic (non-triacylglycerol) substrates. Given the nature of enzymes as chiral catalysts with sophisticated molecular architecture, one might expect selectivity of each type to be the norm, and non-selectivity to be an exception (Sonnet, 1988).

Theil *et al.,* (1992) described double enantioselective transesterification between a meso-diol and a racemic carboxylic ester by lipases. Reaction of the meso-diol with *rac*-2,2,2-trifluoroethyl 2-chloropropanoate in organic solvents in the presence of lipases of different origins gave four stereoisomers (3a-3d) (Figure 1.4).

In general the stereoisomers 3a and 3b are predominant over 3c and 3d (Figure 1.4). Among the lipases tested the highest selectivities were achieved with *Candida* sp. and pancreatin lipases. Lipase B of *Candida antarctica* was found to be responsible for the high selectivity of the acyl transfer from the acyl enzyme to one of the enantiotropic groups of the prochiral diol 1 to give 3a and 3c in high enantiomeric purity (Figure 1.4) (Theil and Bjorkling, 1993).

A lipase from *Candida cylindracea* was shown to possess double enantioselection towards racemic acids and racemic alcohols during esterification reactions in organic (hexane) solvent while the primary alcohols showed lower enantioselectivity than secondary alcohols (Chen and Wang, 1993). However, Hedstrom *et al.*, (1993) found that the enantioselective esterification of racemic ibuprofen, catalysed by a *Candida cylidracea* lipase in a water-oil microemulsion showed high preference for the S(+)-enantiomer of ibuprofen. The R(-)-ibuprofen remained unesterified in the microemulsion. The activity of the enzyme was higher towards short-(propanol and butanol) and long-chained (decanol and dodecanol) alcohols than towards intermediate (pentanol, hexanol and octanol) substrates. However, unlike secondary and tertiary alcohols, all the tested primary alcohols

were substrates for the lipase. Eguchi and Mochida (1993) reported an enantioselective diacylation of 1,3-butanediol carried out using immobilised *Candida* lipase SP382 to produce an optically active (R)-1,3-diacetoxybutane.

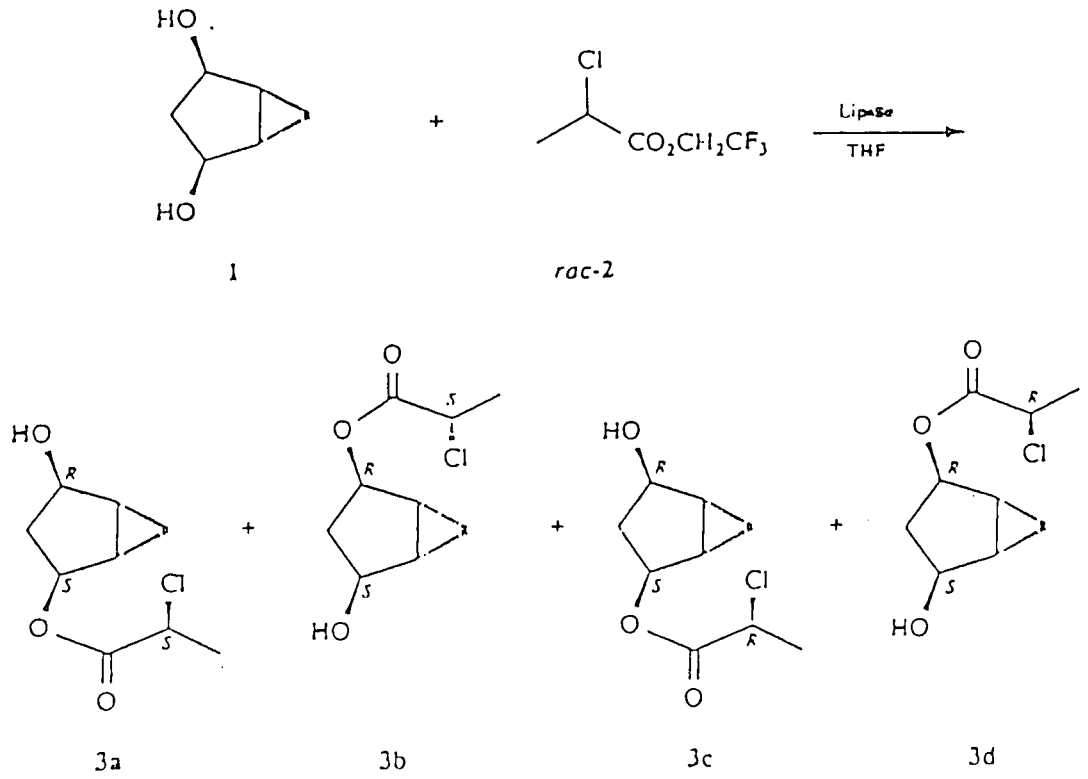


Figure 1.4: Transesterification between a meso-diol and a racemic carboxylic ester (Taken from Theil *et al.*, 1992).

The stereospecificity of the *Pseudomonas fluorescens* lipase YS-catalysed transesterification of primary and secondary alcohols has been reported by Naemura *et al.*, (1994). The enzyme has been used in the separation of stereoisomeric 1,1'-Bis(α-hydroxyethyl) and 1,(1-hydroxy-ethyl)-2-(hydroxymethyl) ferrocenes (Lambusta *et al.*, 1993; Nicolosi *et al.*, 1994) and in the optical resolution of 1-phenylethanol derivatives (Kanerva *et al.*, 1992). Moreover the

enzyme showed best results when used to introduce chirality by enantioselective esterification of 1,2-O protected 2-methylglycerol or enantioselective hydrolysis of its butyryl ester (Wirz *et al.*, 1993).

Lipase enzymes also possess hydrolytic enantioselectivity. Porcine pancreatic lipase enantioselectively catalysed the hydrolysis of n-alkyl sec-alkyl carbonates (Kawashima and Hasegawa, 1993). Stereoselective hydrolysis of 2-acyloxy-3-chloropropyl *p*-toluenesulfonate by lipases derived from *Pseudomonas aeruginosa*, *Aspergillus niger*, *Mucor* species, *Rhizopus delemar* and *Rhizopus japonicus* was reported by Hamaguchi *et al.*, (1986). The *Candida cylindracea* lipase enzyme has been found to carry out enantioselective hydrolytic reaction of racemic ibuprofen ester in the microemulsion (Hedstrom *et al.*, 1993).

1.5 Conclusions

Several applications of lipases have been developed throughout the last two decades, innovative uses of these enzymes for a wide variety of organic syntheses and modification of existing fats and oils have increased exponentially during the last five years. Reasons which may partly account for this trend are the increasing availability of lipases from microbial sources coupled to their special capacity to act as catalysts at hydrophobic-hydrophilic interfaces.

The power tools of molecular biology have been brought to bear, more new lipase amino acid sequences and three-dimensional structures are appearing, and new approaches for handling lipase's complicated interfacial kinetics are being reported. In addition, more ways are being discovered and used to control lipase activity and therefore using their catalytic power for inefficient chemical processes.

Applications for lipases can now be developed in much more rational fashion, however this purpose will not be fully achieved unless careful attempts are made to comprehensively clarify and then integrate all information about the lipase and their properties.

Between the idea
And the reality
Falls the shadow

T. S. Eliot

CHAPTER 2

Introduction into the present study

Microbial lipases are very diverse in their enzymatic properties and substrate specificities, which make them attractive for industrial applications. A large number of lipases have been screened for application as food additives, industrial reagents and as detergent additives, as well as for medical applications.

Most of the microbial lipases are extracellular, being excreted through the external membrane into the culture medium. Optimisation of fermentation conditions for microbial lipases is of great importance, since culture conditions influence the properties of the enzyme produced. The amount of lipase produced is dependent on several environmental factors, such as cultivation temperature, pH, nitrogen composition, carbon and lipid sources, concentration of inorganic salts and availability of oxygen. The fermentation process is usually followed by the removal of cells from the culture broth, either by centrifugation or filtration. The extracellular microbial lipases, after being concentrated and partially isolated are then brought to high purity by a combination of several chromatographic methods.

The high degree of purification obtained for fungal lipases has allowed the successful determination of primary and tertiary structures. Fungal lipases whose three-dimensional structures have been elucidated are; *Rhizomucor miehei* (Brady *et al.*, 1990), *Geotrichum candidum* (Schrag *et al.*, 1991), *Candida rugosa* (Grochulski *et al.*, 1993 and Ghosh *et al.*, 1995), *Candida antartica* (Uppenberg *et al.*, 1994), *Penicillium camemberti* (Derewenda *et al.*, 1994b), *Humicola Langinosa* and *Rhizopus delemar* (Derewenda *et al.*, 1994c) and *Rhizopus niveus* (Kohno *et al.*, 1996).

The aim of this study was, firstly, to screen microbial isolates for their production of lipase enzymes with an objective of finding a lipase with novel properties and

marked stability. Desirable properties would be high lipase activity, high temperature stability, and the ability to function in extremes of pH. Alternatively, the focus could be placed on fungal lipases from less well-characterised groups. In the screening procedure to be reported, an *Aspergillus niger*, which was a prolific enzyme producer, was identified. *Aspergillus niger* lipase is one of the commonly applied enzymes but, surprisingly very little is known about the structure or molecular biology of this enzyme and its relationship with other lipases. A detailed study of the kinetics and molecular properties of the enzyme would therefore be of prime importance.

Kinetic characterisation of the purified enzyme using bulk assays to determine the enzyme's specific physicochemical properties and further characterisation in terms of its surface kinetics would be of prime importance. For this the monolayer and oil droplet method of lipase assay would be employed.

The final aim of the study would be to elucidate the primary structure of the enzyme using molecular techniques and to derive a three-dimensional structure using homology modelling. This would hopefully enable a correlation between the physicochemical and kinetic properties and three-dimensional structure.

Experience is valuable in most human endeavours, but the problem of getting a cat out of a tree is new every time it arises.

Francis Duffy

CHAPTER 3

Purification and Characterisation of *Aspergillus niger* lipase

3.1 Introduction

The pace of lipase research has been accelerating. Many ways are being discovered and used to control lipase activity and for harnessing their catalytic prowess to put greater efficiency into older chemical processes. The potential industrial use of lipases (EC 3.1.1.3) in washing powders, biotransformation, inter- and transesterification reactions has lead to an overwhelming interest in this field. The search continues for lipases with unique properties and features as to gain more insight into this very interesting class of enzymes. In this chapter more insight is provided regarding the production, purification and bulk characterisation of the lipase from *Aspergillus niger*.

3.2 Nutritional factors affecting microbial lipase production

Although lipases may be obtained naturally from different organisms, their production is influenced by culture conditions. A variety of conditions have been described which stimulate or repress the production of lipases by micro-organisms. Lipase production can be induced by the addition of various triacylglycerol substrates to the growth medium. When a *Pseudomonas* strain was cultivated in medium without oil, which consisted of glucose (1 %), peptone (1 %), urea (0,2 %) and minerals, lipase activity was scarcely detected in the culture supernatant in spite of the good growth of the micro-organism (Narasaki *et al.*, 1968). Addition of olive oil resulted in significant lipase activity indicating a stimulation of lipase production by the olive oil. Since then, olive oil has been used to induce lipase

production by various micro-organisms (Yamamoto and Fujiwara, 1988; Suzuki *et al.*, 1988; Phillips and Pretorius, 1991; Christakopoulos *et al.*, 1992). Other oils that have been used to stimulate microbial lipases include soybean oil, corn oil, and sunflower oil (Chistakopoulos *et al.*, 1992). The type of oil used is important for lipase production for a given micro-organism (Espinosa *et al.*, 1990; Hatzinikolaou *et al.*, 1996).

Addition of compounds containing hydrolysable ester groups such as Tweens and Spans in culture medium was found to increase lipase production by other micro-organisms. Of all the carbon sources tested, Tween 80 was by far the best inducer of lipase production by a *Bacillus* specie MC7 (Emanuilova *et al.*,1993). When Tween 80 was replaced by glycerol, glucose or starch, the lipase level was very low and could only be detected by the most sensitive fluorometric assay. The presence of Tween 80 in a culture medium for *Rhizopus delemar* increased lipolytic activity to a level of twice that with olive oil or butyric acid (Espinosa *et al.*, 1990). This effect was postulated to be due to the possible double effect of Tween. It could act as an inducer, as its chemical nature is similar to some substrates of the enzyme, and as a surfactant. When *Trichosporon fermentans* was cultivated in a media containing surfactants such as Tween, Triton and Span, extracellular lipase activities reached levels of 2-3 times as much as that without surfactants (Chen *et al.*, 1994.)

Long chain fatty acids, as end products of lipase activity, have an inhibitory effect on lipase production (Hegedus and Khachatourians, 1988). Contrary to this, oleic acid was found to be better than olive oil in the induction of lipase production by *Candida rugosa* (Del Rio *et al.*, 1990). Studies with different soluble short chain fatty acids have demonstrated that caprylic and capric acids could even be better than oleic acid as inducers of lipase production by *Candida rugosa* (Obradors *et al.*, 1993). Addition of a light divalent cation (Mg^{2+}), in culture media showed enhanced production of lipase activity (Hegedus and Khachatourians, 1988).

Stimulation of lipase production is not only limited to the addition of lipidic substances in the culture media as inducers. Investigations applying one-variable-

at-a-time-optimisation procedures showed that extracellular lipase activity from different micro-organisms achieved maximal values when carbohydrates (Petrovic *et al.*, 1990) were used as carbon sources and certain ammonium salts (Christakopoulos *et al.*, 1992) served as nitrogenous sources. Sztajer and Maliszewska (1988) demonstrated that while starch induced maximal lipolytic activity in *Bacillus circulans*, *Streptomyces* sp., and *Pseudomonas fluorescens*, galactose and sucrose exerted an enhanced activity in *Bacillus* sp. The maximal intracellular lipolytic activity of *Nocardia asteroides* was observed in fructose-supplemented cultures (Nesbit and Gunasekaran, 1993). This was followed by cultures grown in glucose, maltose and sucrose; the least activity was observed in media containing starch and citrate. Although the extracellular lipase activity was much lower than the intracellular activity, in culture grown in monossacharides as the primary carbon source, it was significantly higher than that of cultures with maltose, sucrose and starch.

Lipase production in other micro-organisms is stimulated by the presence of alkanes in the culture media. The ability of *Acinetobacter iwoffi* strain to grow on pure alkanes was associated with the formation of cell-bound lipase (Breuil *et al.*, 1978). Chen *et al.*, (1994), reported that *Trichosporon fermentans* Wu-C12 could produce extracellular lipase with petroleum products as carbon sources.

The studies described above are aimed at defining conditions of optimum lipase production rather than elucidating the mechanisms responsible for the observed effects. Although it is possible to produce substantial amounts of lipase protein from bacteria, no clear general picture is emerging so far from the large amount of experimental data concerning the physiology of lipase biosynthesis and its regulation. Recently, evidence was presented for a complex mechanism of regulation of exoprotein synthesis in *Staphylococcus aureus*. A mutation caused by a chromosomal insertion of transposon Tn551 in *Staphylococcus aureus* resulted in sharply reduced extracellular lipase activity, presumably by inactivating a transcriptional activator (*xpr*) of the lipase structural gene (Smeltzer *et al.*, 1992). The synthesis of different exoproteins including lipase appeared to be regulated by three genetic loci, *agr*, *xpr* and *sar*, interacting at the genetypic level. At least one

of the proteins encoded for by these loci is assumed to be a sensory protein responding to environmental stimuli such as pH and glucose concentration (Hart *et al.*, 1993) suggesting an elegant explanation of a variety of effects which have been observed to influence the level of lipase production.

3.3 Purification of lipases

Taipa *et al.*(1992), analysed 70 articles (between 1970 and 1991) on the isolation and purification of microbial lipases, and made some conclusions about the major chromatographic methods for purification of lipases.

Precipitation is usually used as a fairly crude separation step often in the early stages of a purification procedure, being followed by chromatographic separations. Large quantities of material can be treated and this step is less affected by interfering non-protein materials than chromatographic methods. Almost all the procedures (82 %) used a precipitation step, with 60 % of these using ammonium sulphate precipitation and 35 % using ethanol, acetone or an acid, usually HCl.

Chromatographic methods have been the most popular techniques for lipase purification. Ion-exchange chromatography is the most commonly used chromatographic method, occurring in 67 % of the purification schemes analysed. In 29 % of these purification procedures ion exchange is used more than once. The weak ionogenic groups are the most commonly used ion exchangers; the most frequently employed are the diethylaminoethyl (DEAE) group in anion exchange (58 %) and the carboxymethyl (CM) group in cation exchange (20 %). Strong ion exchangers, especially Mono Q-Sepharose and Q-Sepharose, are becoming more popular in lipase purification.

Although gel exclusion has the lower capacity for loaded protein it can be used at an early stage in the purification scheme (after the precipitation step), instead of ion exchange, or as one of the last steps. Gel exclusion (apart from the desalting

step) is the second most frequently used purification method, used in 60 % of the purification schemes and more than once in 22 % of them.

Affinity methods can be applied at an early stage, but the materials are expensive, and the less costly ion exchange and gel exclusion are preferred after the precipitation step. Affinity chromatography has been applied as a purification step in 27 % of the purification procedures. Hydrophobic interaction chromatography (HIC) was regarded as an affinity chromatography in the analysis and was used as a purification step in 18 % of the purification methods. The most popular used hydrophobic adsorbents were based on agarose with phenyl or octyl functional groups. Adsorption chromatography was applied in 16 % of the purification procedures, adsorbent hydroxylapatite being used in most cases.

The purification protocols did not show adherence to any particular sequence of methods. Methods chosen to purify microbial lipases depended on initial lipase preparation, and even for the same crude lipase preparation, different purification schemes have been used.

The purification achieved per step by ion exchange and gel exclusion chromatography was analysed. A large variation was observed in the relative purification factors attained by these two methods, depending on the stage in the purification procedure. In a purification scheme with 5 steps the purification factor obtained in the last step was lower (50 %) for both these methods compared with factors attained when the same methods were used earlier in the purification scheme. The purification factors for ion exchange chromatography and gel exclusion were approximately 5 when these methods were used as third steps in the purification scheme; these values changed to 7 for ion exchange and 3.5 for gel exclusion, when they were used as fourth steps. The value obtained for precipitation method (used as second step in the purification scheme) are 3-fold. With affinity techniques (including HIC) the purification degree achieved by an affinity step varies from 2 to 10-fold. Precipitation methods had the highest average yield (87 %), while the other techniques led to relatively lower yields (60 - 70 %).

To purify the protein to homogeneity, with an overall yield of 30 % and the purification factors of 320, four or five purification steps are usually required. These values are averages of the overall yields and purification factors obtained for each purification scheme.

3.4 Effect of pH

Changes in pH profoundly affect the degree of ionisation of the amino, carboxyl and other ionisable residues in protein. Since ionisable amino acid residues may be present in the active site of the enzyme, and other ionisable groups may be responsible for maintaining the protein conformation, it is not surprising that the pH of the solution may markedly affect enzyme activity. Moreover, since many substrates are ionic in character, the active site of an enzyme may require particular ionic species of the substrate for optimum activity. These effects are probably the main determinants of the shape of the curve that represents enzyme catalytic activity as a function of pH (Conn *et al.*, 1987). Usually, the catalytic activity of the lipase changes with pH in a bell-shaped fashion, thus yielding a maximum rate at the optimum pH (Zaks and Klibanov, 1985). The plateau of the bell-shaped curve usually is small and the rates decrease rapidly with pH on either side of the maximum. The rate decrease represents changes in the state of ionisation of groups on enzyme or the substrate, or both, that are critical, with regard to the state of ionisation, to the enzyme-catalysed reaction (Conn *et al.*, 1987).

As with other enzymes each lipase has its own optimal pH, ranging from acid to neutral to alkaline (Yamane, 1987). There exists a great diversity in the pH optima of microbial lipases. Development of an alkaline lipase is important, particularly in the use of the enzyme in laundry detergents to enhance cleaning and as a substitute for pancreatic lipase in digestive medicine (Yamane, 1987). Shifts in the pH optimum after immobilisation for various lipases have been observed. After immobilisation, the optimum activity of the lipase from *Candida rugosa* increased to

a more alkaline value (Montero *et al.*, 1993). Shifts in pH optima of immobilised lipases have been reviewed by Malcata *et al.*, (1992). The maxima in the rates of reaction catalysed by immobilised lipases were observed at pH values between 4 and 10. With very few exceptions, the pH optima for the immobilised lipases are equal to or higher than those for their free counterparts. Hence, the immobilisation procedure seems to render catalytically important amino acid residues more basic. An explanation consistent with these results and with the experimental evidence is that upon immobilisation the active site becomes more exposed to the solvent than it was in the globular, folded soluble lipase form. Hence, proton transfer to the amino acid residues at the active site becomes less hindered.

The pH also affects the stability of enzymes. Some lipases are stable over a wide pH range, examples are the lipases from *Pseudomonas cepacia* (which retained 100 % activity after incubation over a pH range of 3 - 11.5 for 24 hours at 30 °C) (Sugihara *et.al.*, 1992) and *Fusarium heterosporum* (stable over a pH range of 4 - 10 at 30 °C for 4 hours) (Shimada *et al.*, 1993). This feature can be exploited in inactivating the enzyme after desired changes have been produced (Kilara, 1985).

3.5 Effect of temperature

The Arrhenius equation relates the specific reaction rate or rate constant, k, to temperature

$$k = Ae^{-E_a/RT} \qquad [3.1]$$

where A is a proportionality constant, E_α is the activation energy, R is the gas constant, and T the absolute temperature. The equation predicts that the rate of the reaction, enzyme-catalysed or not, will increase with increasing temperature. However, since enzymes are proteins and many proteins will be denatured if the temperature is raised sufficiently, enzyme catalysed reactions show an increase in rate with increasing temperature only within relatively small and low temperature

ranges. The optimum temperature of enzyme-catalysed reactions depends on several factors including how long the enzyme is incubated at the test temperature before the substrate is added and the type of organism from which the enzyme was derived (Conn, *et al.*, 1987).

Production of a heat stable lipase is a useful attribute if the lipase is to be used commercially either as a fat splitting enzyme (e.g. as an enzyme additive to the detergents) or in transesterification reactions where little water or solvent is present and the reaction therefore depends upon the substrates being in the liquid phase (Ratledge, 1989). The melting point of fat is very variable and can in some cases be as high as 50 °C, but enzymatic catalysis on solid substrates is limited and therefore becomes difficult for less thermostable enzymes to catalyse the required reactions (Sigurgìsladòttir *et al.*, 1993).

Lipases from plants and animals are in general, not thermostable. Relatively thermostable microbial enzymes have been purified and characterised. Optimum temperatures of 55 °C or above have been reported for bacteria belonging to the genus *Pseudomonas* species (60 °C) (Yamamoto and Fujiwara, 1988), *Pseudomonas. cepacia* (55 - 60 °C) (Sugihara *et al.*, 1992) and *Pseudomonas. aeruginosa* MB 5001 (55 °C) (Chartrain *et al.*, 1993). A fungus identified as *Humicola lanuginosa* S-38 was reported to produce a heat stable lipase (Arima *et al.*, 1972), and the optimal activity of a lipase from *Humicola lanuginosa* No.3 was found to be 45 °C and retained 100 % activity for 20 hours at 60 °C (Omar *et al.*, 1987). A thermophillic *Bacillus* species has been reported to produce a thermostable lipase (Kambourova and Manolov, 1993), and an optimum temperature of 60 °C was reported (Sugihara *et al.*, 1991).

Enzymes, being proteins, are susceptible to heat denaturation. At elevated temperatures the Arrhenius model breaks down due to extensive irreversible denaturation of the lipase (Malcata *et al.*, 1992). Inactivation temperature of lipases is influenced by the composition of the medium in which the inactivation is being determined. For example, it has been shown that in milk higher temperatures and longer times are needed to achieve destruction of lipases than in

buffer systems (Law, 1979). This is probably due to the availability of the substrate of the enzyme which removes excess water from the vicinity of the enzyme and thus restricts its overall conformational mobility (Malcata *et al.*, 1992).

3.6 Effects of metal ions

Numerous studies have been made concerning the effects of various salts on lipase activity and diverse results have been obtained. Most lipases are inhibited by heavy metals (Co^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, Sn^{2+}, Ni^{2+} and Ag^{2+}). However, the lipase isolated from *Penicillium simplicissimum* was found to be resistant to most of the heavy metals tested (Sztajer *et al.*, 1992). It was significantly inhibited by Zn^{2+} and a minor reduction was observed with Ag^{2+}.

In most cases, monovalent cations, Na^{+}, K^{+} and Li^{+}, have been found to have stimulatory or no effect on the rate of lipase -catalysed reactions. A 50 % inhibitory effect by K^{+} was reported on the activity of a lipase isolated from *Pseudomonas* species (Yamamoto and Fujiwara, 1988). Light divalent cations (Mg^{2+} and Ca^{2+}) appear to stimulate the activity of most of the enzymes studied. A significant inhibitory effect by Mg^{2+} was observed on a lipase isolated from *Aspergillus oryzae* (Ohnishi *et al.*, 1994). Often the lost activity can be restored via the addition of metal-chelating agents (Malcata *et al.*, 1992). Porcine pancreatic lipase has been shown to have an absolute requirement for calcium ions in the presence of bile salts (Benzonana and Denuseulle, 1968). The lipase enzyme isolated from castor bean lipid bodies was stimulated 40 fold by 30 mM free Ca^{2+} (Hills and Beevers, 1987).

It is generally known that free fatty acids tend to inhibit lipase-catalysed hydrolysis probably by accumulating at the lipid/water interface, thereby blocking access of the enzyme to the unreacted triacylglycerol molecules (Benzonana and Desnuelle, 1968). The positive effects of metal ions could be due to the formation of complexes with ionised fatty acids which change their solubilities and behaviour at

interfaces, whereas negative effects can be attributed to competitive inhibition at the active site.

3.7 Effects of bile salts and detergents

Most studies on the effect of bile salts on lipases have been made with lipase enzymes derived from animal sources, probably due to the role they play as fat emulsifiers in animal intestinal tracts. In most cases bile salts were found to have stimulatory effects on the activity of animal lipase (Tiruppathi and Balasubramanian, 1982; Gargouri *et al.*, 1986; Carrière *et al.*, 1991). Some animal lipases are characterised by being bile-salt dependent for their activity, particularly lipases purified from milk (Wang, 1991) and from the pancreas of human (Mas *et al.*, 1993) and cod (*Gadus morhua*) (Gjellesvik *et al.*, 1992). It has been shown that *in vitro* pancreatic lipase action on long chain triacylglycerols is inhibited early by the hydrolysed fatty acids and soaps. Bile salts and Ca^{2+} do not increase the initial rate but, rather, counteract the inhibitory effect of the soaps (Shahani, 1975).

Bile salts have also been shown to enhance the activity of lipases purified from *Pseudomonas putida* 3SK (Lee and Rhee, 1993) and *Pseudomonas aeruginosa* MB 5001 (Chartrain *et al.*, 1993). When the activity of a lipase from *Penicillium caseicolum* was tested using tributyrin as a substrate, sodium taurocholate, sodium deoxycholate and $CaCl_2$ inhibited the enzyme, but with butter oil as a substrate, the bile salts enhanced the activity, while $CaCl_2$ weakly inhibited the activity (Alhir *et.al.*, 1990). The activity of *Pseudomonas* sp lipase was enhanced by the addition of sodium cholate and sodium deoxycholate (Yamamoto and Fujiwara, 1988) whereas the activity of *Pseudomonas* sp KW I-56 lipase was inhibited by these bile salts (Iizumi *et al.*, 1990).

The effect of other detergents on lipase activity has been widely studied. Different detergents affect lipases differently. In most studies, anionic detergents inhibited lipase activity while non-ionic detergents (Tween 20 and 80, Triton X-100) enhanced activity (Yamamoto and Fujiwara, 1988; Hoshino *et al.*, 1992; Mozaffar

and Weete,1993; Lin *et al.*, 1996). Lipases from *Pseudomaonas* sp KW I-56 (Iizumi *et al.*, 1990) and *Brassica napus* (Weselake *et al*, 1989) were inhibited by non-ionic detergents. Cetyltrimethyl-ammonium bromide, which is a cationic detergent inhibited *Brassica napus* lipase (Weselake *et al.*, 1989) and *Pseudomonas* sp lipase (Yamamoto and Fujiwara, 1988). Chaps, a zwitterionic detergent, enhanced activity of *Pythium ultimum* lipase (Mozaffar and Weete, 1993) and *Bacillus thermocatenulatus* lipase (Schmidt-Dannert *et al.*, 1994).

The relevant literature provided insight into lipase production, purification and characterisation. With these tools the investigation into properties of lipase from *Aspergillus niger* is discussed.

3.8 Chemicals

Unless otherwise stated, all chemicals were obtained from commercial sources, were of analytical reagent grade or better and were used without further purification.

3.9 Lipase assay conditions

The assays used were done according to standard methods in literature, namely the olive oil assay and a pH stat method with tributyrin as substrate. The assay buffer used contained 1 mM Tris-Glycine (pH 5.5), 0.1 M NaCl and 5 mM $CaCl_2$, unless stated otherwise. Activity assays were always performed in triplicate and the appropriate blanks were also evaluated.

3.9.1 The olive oil assay

The colorimetric method of Duncombe (1963) utilises the formation of a Cu^{2+} soap with the fatty acids liberated by lipase, which is then extracted into an organic phase. The extracted Cu^{2+} is determined by reaction with diethyldithiocarbamate.

The copper reagent was prepared by separately dissolving 16.125 g $Cu(NO_3)_2.3H_2O$ and 32.5 ml triethanolamine in 200 ml volumes of distilled water. After mixing, the pH was adjusted to 7.5 and the volume made up to 500 ml in a volumetric flask. The sodium diethyldithiocarbamate reagent (0.25 %, m/v) was freshly prepared in 2-butanol.

A standard curve was prepared by diluting a stearic acid stock solution to yield a range of 0– 0.4 µmole fatty acid/tube. H_2O (0.5 ml) and copper reagent (2.5 ml) were added to each tube which was vortexed, chloroform (5 ml) was added and again the tubes were vortexed for 30 seconds. It was then centrifuged for 2 min to separate the phases and the aqueous phase was removed by aspiration with a pasteur pipette. An aliquot (2 ml) of the chloroform solution was pipetted into a clean dry tube, care being taken that the pipette did not touch the inner wall of either tube as traces of copper-containing aqueous phase might be transferred to the reagent. Diethyldithiocarbamate reagent (0.5 ml) was added to the chloroform solution and after the solutions had been mixed, the absorbance at 440 nm was measured against a blank solution that had been subjected to the same procedure. The calibration curve obtained is given in Figure 3.1.

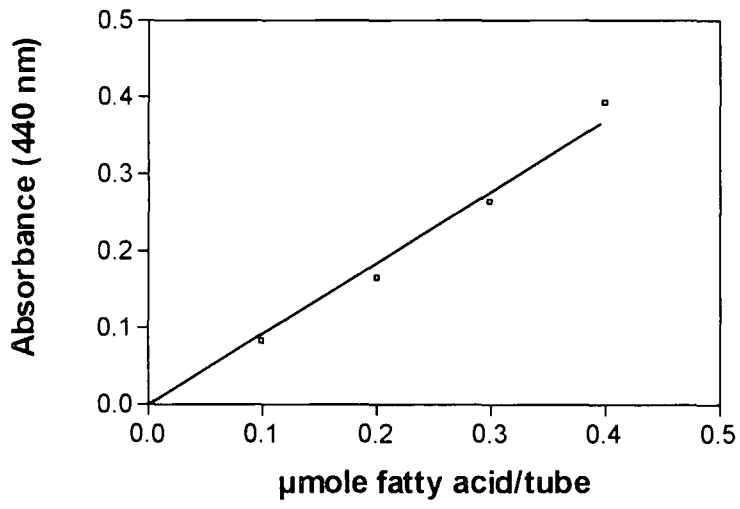


Figure 3.1: Standard curve for assay of fatty acids released with the olive oil assay using stearic acid as standard. Standard deviations for triplicate determinations are smaller than the symbols used for the data points.

The olive oil emulsion used in the assay consisted of 10% (v/v) olive oil and 1% (m/v) gum arabic in distilled water, emulsified by mixing for 2 min with a Branson Sonifier Cell Disrupter B-30. The assay buffer consisted of 1 mM Tris-Glycine, 0.1 M NaCl and 5 mM $CaCl_2$, pH 5.5. The original protocol however suggested a 1 mM Tris-buffer and 5 mM $CaCl_2$, pH 8.5. The pH optimum of the crude enzyme was determined (Section 3.10.2.3) (Figure 3.6) and later confirmed with pH studies on the pure enzyme. This buffer adjustment was made after the screening, initial enzyme assays being done according to the original protocol.

The assay buffer (0.5 ml) was mixed with olive oil emulsion (0.5 ml), where after an appropriately diluted enzyme (0.1 ml) was added and incubated at 30 °C for 60 min in a shaking water bath. The reaction was stopped by addition of copper reagent (2.5 ml) and chloroform (5.0 ml). The rest of the procedure was the same as for the standard curve.

Enzyme activity, U/ml (µmole FA released $min^{-1}ml^{-1}$ enzyme), was calculated using the following formula:

$$U/ml = \frac{V}{v \times E \times d} \times \frac{A_{440nm}}{t} \qquad [3.2]$$

V = final volume of chloroform analysed
v = volume of enzyme assayed
E = gradient of standard curve
t = incubation time of reaction
d = light path of cuvettes (1 cm)

3.9.2 pH stat assay

Although the olive oil assay is a reliable assay of true lipase activity, this procedure is time-consuming and it is prone to high blank readings in some cases. The pH stat assay conditions as described by Sarda and Desneulle (1958) and the

adapted buffer systems as described by Carrière *et al.* (1997) was used. Under these conditions no activity was measured. As these results did not correlate with the olive oil assay, the bile salts (deoxycholate), known to inhibit some lipases (Gargouri *et al.*, 1985) and BSA were omitted from the assay mixture (Carrière *et al.*, 1997). BSA was shown to be a strong inhibitor of lipolysis in spite of its role as a complexing agent for fatty acids released on hydrolysis of the oil (Figure 3.2).

A Metrohm 641 Impulsomat pH stat system was used with a thermostatically controlled titration vessel at 30 °C. The assay buffer consisted of 1 mM Tris-Glycine (pH 5.5), 0.1 M NaCl and 5 mM $CaCl_2$. The liquid triacylglycerol, usually tributyrin, was added directly to the titration vessel and the emulsion formed by vigorous stirring using a propeller agitator. Longer chain triacylglycerols were emulsified before addition as described in Sections 3.9.1 and 3.18.9. Substrate (0.5 ml) and buffer (14.5 ml) were mixed in the titration vessel, the reaction was pretitrated to 5.5 and the appropriate enzyme dilution was added. The hydrolysis rates of the emulsified triglycerides were measured potentiometrically by titrating the liberated fatty acids with standardised NaOH.

A unit of enzyme activity was defined as the amount of enzyme that would release 1 μmole of butyric acid per min under the defined assay conditions.

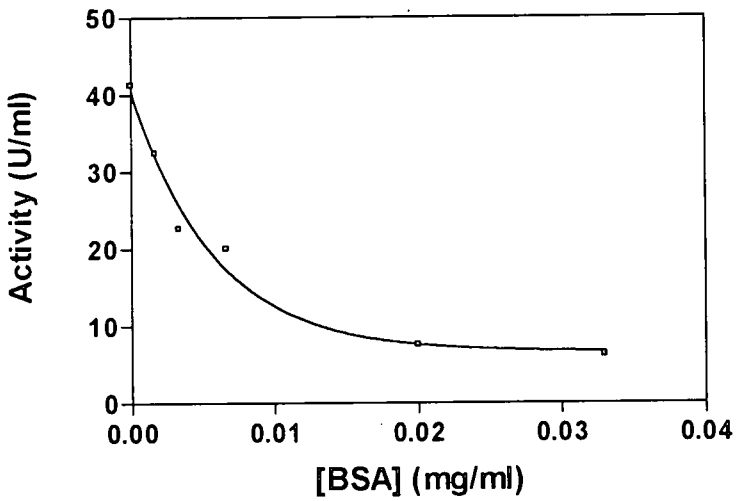


Figure 3.2: Effect of BSA concentration on the rate of hydrolysis by *Aspergillus niger* lipase with tributyrin as substrate. Enzyme (2.5 U/ml) was assayed in the pH stat using 1 mM Tris-glycine (pH 5.5) containing 0.1 M NaCl and 5 mM $CaCl_2$.

Earlier studies done on the inactivation of lipase by proteins (Borgström and Erlanson, 1978; Brockerhoff, 1971), showed that under these conditions, lipases and serum albumin compete for the interface of the substrate. Inhibition is an effect of the physical separation of lipase from the interface (Borgström and Erlanson, 1978). Gargouri *et al.* (1985) also showed that inhibition of the lipase activity neither resulted from the interaction between the enzyme and protein (BSA) in the bulk phase nor was it related to the lowering of interfacial tension. They demonstrated using the monolayer technique, that lipase inactivation is due to the protein associated with the lipid substrate (Gargouri *et al.*, 1984).

The assay conditions were evaluated with regards to the concentration of enzyme used in the assay. A linear relationship between the amount of enzyme used and the activity was apparent (Figure 3.3).

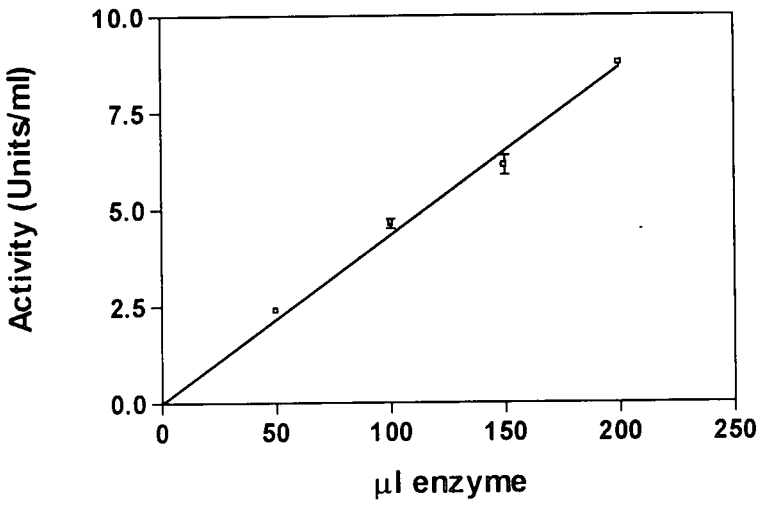


Figure 3.3: Linear relationship between activity and sample volume of culture supernatant assayed using tributyrin as substrate in the pH stat. Standard deviations for triplicate determinations are shown.

3.10 Screening of isolates and identification of fungal isolates

3.10.1 Microbial strains used

A variety of bacterial and fungal isolates were screened for lipase production. Most of the bacterial species were isolated by Microbiology third year students of the

University of the Orange Free State from soil, a variety of water sources, aerobic and anaerobic digesters, milk and cheese samples and were kindly provided to us by Prof. T.J. Britz and Dr. Karl-Heinz Riedel. Five additional bacterial isolates were obtained from SASOL and included in the screening program. SASOL also provided three fungal isolates obtained from bioreactors on site; these three isolates plus an additional thirteen fungal isolates from the Tree Pathology Co-operative Program of the University of the Orange Free State were included in the lipase screening program.

3.10.2 Screening for lipase production

3.10.2.1 Lipase production on agar plates

Screening for lipase production was done by streaking bacterial and plating fungal isolates on agar plates containing four different inducers, namely, (i) an emulsion of olive oil containing Rhodamine B as indicator, pH 7.0 (Kouker and Jaeger, 1987); (ii) Tween 20 and $CaCl_2$, pH 7.0; (iii) Tween 80 and $CaCl_2$, pH 7.0 (Ignjatiovic and Dey, 1993); (iv) Tributyrin (glycerol tributyrate) (Fryer *et al.*, 1966). The plates were incubated for 3-6 days at 30 °C.

The growth medium for olive oil/Rhodamine B plates contained nutrient broth (8 g), NaCl (4 g) and agar (16 g) in distilled H_2O (1 L). Olive oil was emulsified at a concentration of 2.5 % (m/v) by stirring the mixture while emulsifying with a Brason Sonifier Disrupter B-30. The pH was adjusted to 7.0. The medium was autoclaved and cooled to about 70 °C and 10 ml of filter sterilised Rhodamine B solution (0.1 % (m/v) final concentration) was added. The medium was mixed and was allowed to stand for about 10 min to reduce foaming before pouring about 20 ml aliquots into sterile plastic petri dishes.

Tween (20 or 80)/$CaCl_2$ contained nutrient broth (16 g), agar (20 g), Tween 20 or 80 (10 g) and $CaCl_2$ (0.124 g) in distilled H_2O (1 L). After the pH of the medium

was adjusted to 7.0, it was autoclaved and cooled to about 60 °C. 20 ml aliquots were poured into sterile, plastic petri dishes.

The growth medium for tributyrin plates contained: peptone (5 g), yeast extract (3 g), glycerol tributyrate (10 g) and agar (20 g) in distilled H_2O (1 L). The medium was stirred to an emulsion, and the pH was adjusted to 7.0.

Only 1 bacterial and 13 fungal isolates were found to be significantly positive and suitable for the project, i.e. one or more agar plates, with different inducers, gave positive results.

Rhodamine B/olive oil agar plates resulted in pink zones around the colonies of lipase-positive organisms, an indication of extracellular lipase being formed. Under UV light, orange fluorescent halos surround the colonies (Figure 3.4). The Tween 20 or 80/$CaCl_2$ agar plates resulted in white opaque halos around lipase positive colonies caused by crystals formed by precipitation of the Ca^{2+} soap in the media (Figure 3.4). The Tributyrin plates indicated positive lipase production with clear zones around the lipase positive cultures formed where tributyrin was hydrolysed. Unfortunately the clear zones on tributyrin plates are difficult to photograph with fungal isolates and an example of such a plate is not reported.

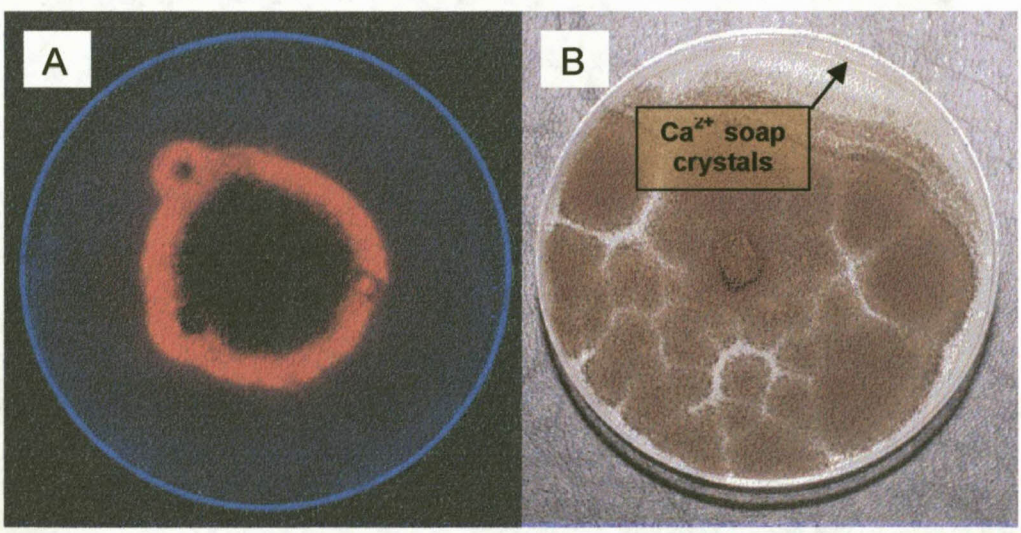


Figure 3.4: Photographs showing positive lipase production by the fungus, *Aspergillus niger*. (A) Rhodamine B and (B) Tween 80 plates.

3.10.2.2 Lipase production in liquid culture media

The eight best lipase producers, as judged by the size and intensity of the zones, on all the plates were allowed to grow in potato dextrose broth. Fresh potatoes (200 g) were autoclaved in water (500 ml) and the filtrate served as the basis of the medium. The culture medium contained the filtrate and olive oil (10 g/L), glucose (5 g/L), gum arabic (3.5 g/L) and $CaCl_2$ (5 g/L). After emulsification with the Branson Sonifer Disrupter B-30, the pH was adjusted to 7.0 and the medium autoclaved.

A pre-inoculum was prepared (5 ml media) and growth was allowed until no oil was visible. The 250 ml conical flasks, containing 80 ml of media, were inoculated and incubated on a rotary shaker (160 oscillation per min) at 30 °C. Aliquots were withdrawn periodically under sterile conditions and centrifuged for 5 min in a microcentrifuge. The supernatants were assayed for activity using the olive oil assay (Table 3.1).

Table 3.1: List of isolates used in secondary screening with lipase activities as determined by the olive oil assay.

Isolate identity	**Identification**	**Activity (U/ml)**
LEN	*Coniothyrium zuluense*	12.3
CMW 118	*Ophiostoma piliferum*	2.9
CMW 997	*Ophiostoma capense*	3.15
CMW 975	*Ophiostoma capense*	8.2
CMW 984	*Ophiostoma capense*	6.8
CMW 1147	*Ophiostoma capense*	2.56
CMW 986	*Ceratocystiopsis protea*	0
WAX 3	Unidentified fungal isolate from SASOL Bioreactor	35

From the results of the screening and initial enzymatic studies the most promising isolate, WAX 3 was chosen for further studies. The fungal-isolate was identified by the Agricultural Research Council's Plant protection Research Institute as *Aspergillus niger* Tiegh.

3.10.2.3 Enzyme production

The production of the enzyme under the growth conditions was monitored. The culture was cultivated as described in the Section 3.10.2.2. An aliquot of the culture was taken aseptically every day and assayed for activity using the olive oil assay. The activity increased to a maximum on days 2 or 3 (Figure 3.5) until the oil-layer was no longer present. The latter was chosen as the moment for harvesting.

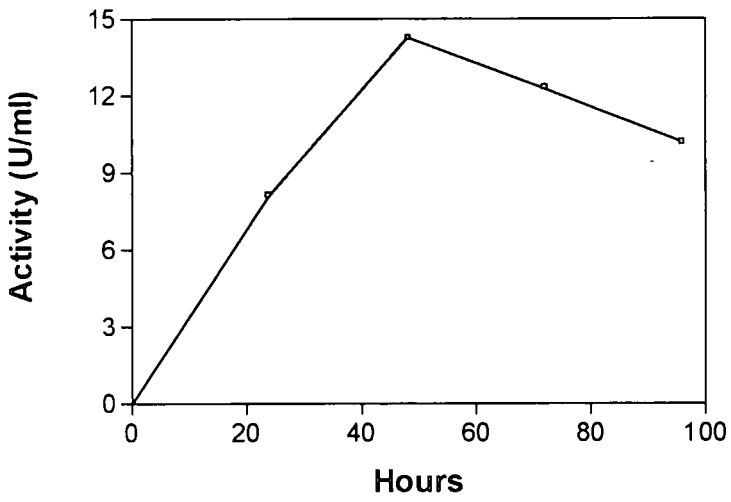


Figure 3.5: A profile of the production of enzyme by *Aspergillus niger* when grown in shake flasks. Enzyme activity was assayed using the olive oil assay.

On the solid media plates *Aspergillus niger* displayed a dark pigmented conidial biomass, but when this organism is cultured in shake flasks the pigmentation is limited. Some of the observed changes in wall structure and pigmentation which take place during maturation are the results of the exposure to the aerial environment (Tokunaga *et al.*, 1973). When conidia are formed in liquid media

they usually lack the surface ornamentations which are produced in subaerial culture (Mangenot and Reisinger, 1976). Conidia of *Aspergillus niger*, when produced in submerged culture do not develop the thick outer cuticle or black pigmentation which is characteristic of the species (Anderson and Smith, 1971).

Another aspect to consider, is that by the time the enzyme was harvested the pH of the media had dropped from 7 to between 3 and 4, possibly due to the production of organic acids by the fungus. The pH activity profile of the crude enzyme was evaluated to make sure that optimal assay conditions were used when evaluating the activity of the lipase (Figure 3.6). The results prompted an adaptation to the assay conditions using a buffer with a pH of 5.5 and not 8.5 as the specified protocol suggested (Duncombe, 1963).

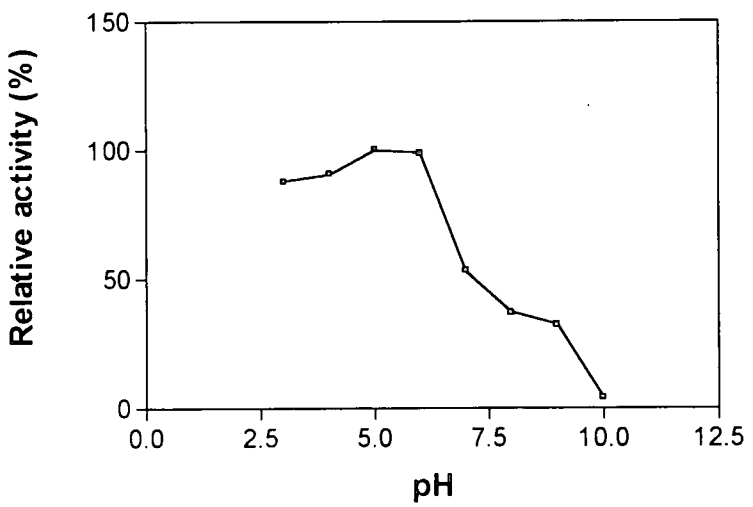


Figure 3.6: Evaluation of the optimum pH for crude lipase from *Aspergillus niger*

3.11 Protein assays

Protein concentrations were estimated either by absorbance at 280 nm (column eluates) or by using the bicinchoninic acid (BCA) method (Smith *et al.*, 1985) which was supplied as a kit by Pierce, (Rockford, IL, USA). Both the standard and micro protein assays were used.

The methods used for assays were those supplied with commercially available kits. A set of protein standards was prepared with bovine serum albumin (BSA), provided by Pierce as part of the BCA protein assay kit in the range of 0 - 800 μg/ml for the protein assay and 0 - 40 μg/ml for the micro protein assay. Standard curves were prepared and used to determine the protein concentration of unknown protein samples (Figures 3.7 and 3.8)

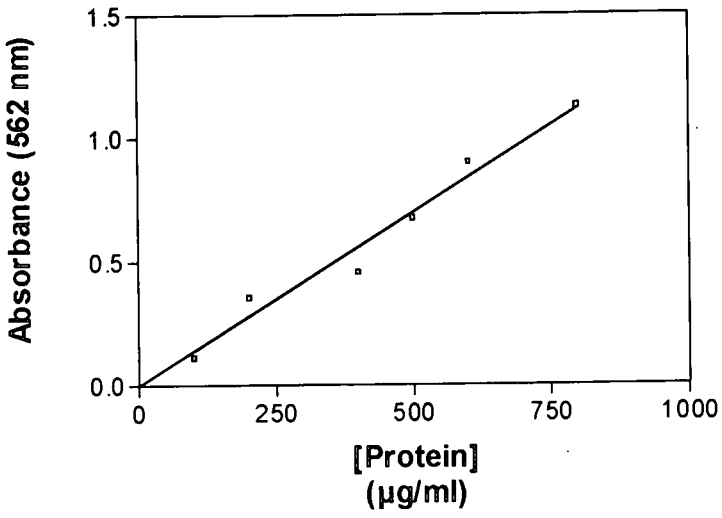


Figure 3.7. Standard curve for the BCA protein assay with BSA as protein standard. Standard deviations for triplicate determinations are smaller than the symbols used for the data points.

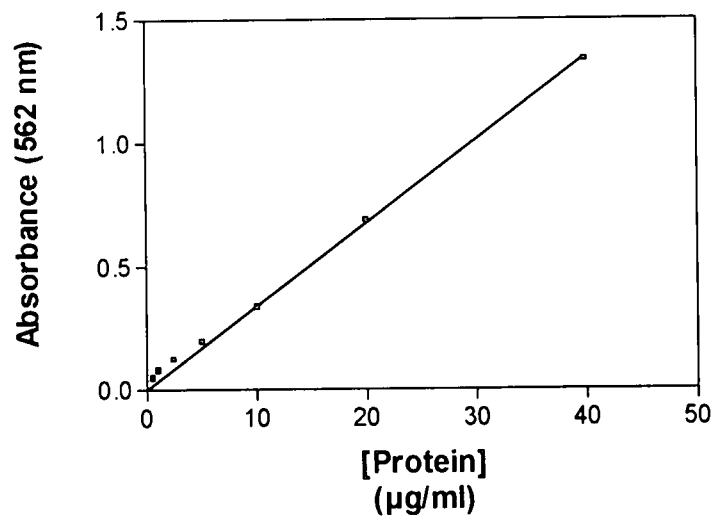


Figure 3.8. Standard curve for the Micro BCA protein assay with BSA as protein standard. Standard deviations for triplicate determinations are smaller than the symbols used for the data points.

3.12 Purification of *Aspergillus niger* lipase

3.12.1 Preparation of the crude enzyme

A pre-inoculum was prepared by inoculating 5 ml media with a plug of the fungal mycelia from PDA-plates in a test tube, as described in Section 3.10.2.2. The pre-inoculum was allowed to grow until no oil layer was visible in the tube, usually more than 48 hours, and used to inoculate 250 ml conical shake flasks containing 80 ml medium. The enzyme activity was monitored on a regular basis, using the olive oil assay, as described in Section 3.9.1. Five conical flasks with the highest activity were selected for further purification. The cultures were centrifuged for 15 min at 4600 rpm to remove the cell mass. The supernant containing the extracellular lipase was decanted and used for further studies.

3.12.2 Ammonium sulphate precipitation

Solid ammonium sulphate was added to the crude extract over 30 min to 40 % saturation (Nishio *et al.*, 1987) at 4 °C. After centrifugation, the clear supernatant was then taken to 100% saturation with solid ammonium sulphate and allowed to stand overnight at 4 °C. After centrifugation for 30 min at 7500 rpm, the precipitate was dissolved in 13 ml Tris-HCl buffer pH 8.0 and assayed for activity.

It was evident that the ammonium sulphate precipitation (ASP) was not suitable as up to 65 % of the initial activity was retained in the supernatant after the final centrifugation step. All attempts to improve the yield in the precipitate were unsuccessful and this isolation attempt was abandoned. This was surprising as many research groups used ASP successfully (Elwan *et al.*, 1987; Sztajer *et al.*, 1992; Wu *et al.*,1996). However, Hatzinikolaou *et al.*, (1996), who also purified a lipase from an *Aspergillus niger* species had a 25 % loss of activity when using ASP.

3.12.3 Ion exchange chromatography

Various authors who reported on lipase purification usually included anion exchange in their protocols (Section 3.3) (Chihara-Siomi *et al.*, 1992; Sztajer *et al.*, 1992; Chen *et al.*, 1995; Labuschagne *et al.*, 1997). Experiments were done with the different anion exchangers available in our laboratory on a small scale (5 ml) to determine to which resin the enzyme would bind most effectively. The Super Q resin from Pharmacia was the most promising, provided that the pH of the enzyme was adjusted to 8.0 after harvesting.

Media filtrate was prepared as before. The pH of the supernatant was too low (approximately 4) for binding to the resin and a dialysis step against 50 mM Tris-HCl (pH 8.0) was employed to raise the pH and remove any unknown components in the media which could act as buffering elements or give a high background in the assays. The dialysis was done overnight and the buffer changed several times until the pH of the retentate was 8. The clear retentate (300 ml) was applied to a Super Q (120 x 25 mm) anion exchange column, previously equilibrated with a 50 mM Tris-HCl (pH 8.0) buffer. The enzyme was eluted with an increasing salt gradient (0 – 0.5 M NaCl (500 ml)) at a flow rate of 3 ml/min. Salt concentration was determined by conductivity measurements and application of a standard curve. Fractions (9 ml) were collected, the absorbance monitored at 280 nm and assayed for activity. Those fractions containing the bulk of activity were pooled.

A fraction of contaminating protein showed no interaction with the resin and eluted before the start of the salt gradient. The lipase activity displayed slight interaction with the column and eluted at low concentrations of NaCl during the early stages of the linear salt gradient (Figure 3.9). The pooled fraction (81 ml) was concentrated using an Amicon ultrafiltration stirred cell unit and a YM-10 ultrafiltration membrane (nominal molecular cut-off of 10 000 Da) from Diaflo. The sample was diluted several times with distilled water and reconcentrated to ensure that the salt and Tris was removed as efficiently as possible as not to interfere with iso-electric focussing to follow. The enzyme experienced no loss of activity and no lipase activity was detected in the filtrate (Table 3.2).

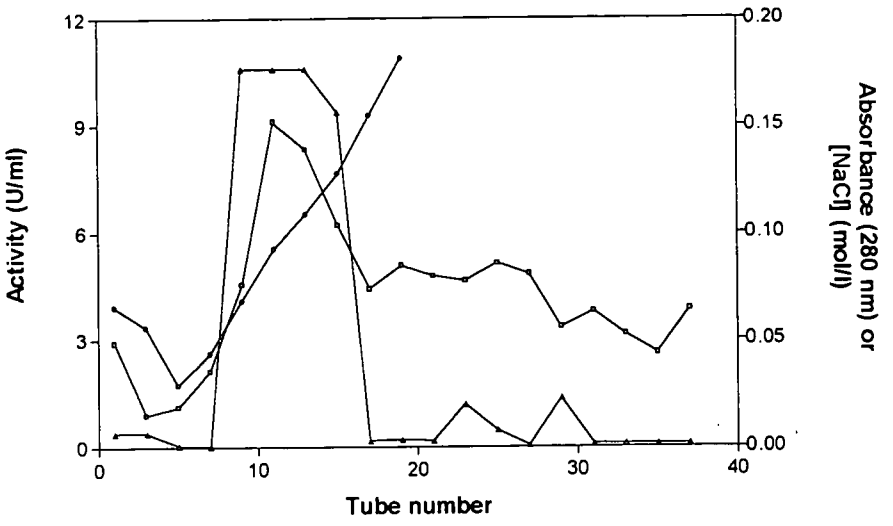


Figure 3.9: A typical profile of a Super Q chromatography column of the crude, dialysed, lipase extract. (A_{280nm} (▣), Activity (△) and [NaCl] (○)).

3.12.4 Iso-electric focussing

Ampholyte (pH 3 - 10) was added to the retentate after the ultrafiltration step, to a final concentration of 2 % (w/v). The protein-ampholyte solution was loaded into a Rotofor Cell (Biorad), which was kept at a constant temperature of 4 °C. The power supply was kept constant at 12 watts and the run was completed in 4 - 6 hours when the voltage had stabilised at 480 V. The fractions were harvested and analysed for activity and protein content (Figure 3.10). The activity focussed between pH 3.3 and 5. The active fractions were pooled and concentrated by ultrafiltration as descibed in the previous section, which removed some of the ampholytes. The next step was the complete removal of ampholytes and further purification by exclusion chromatography.

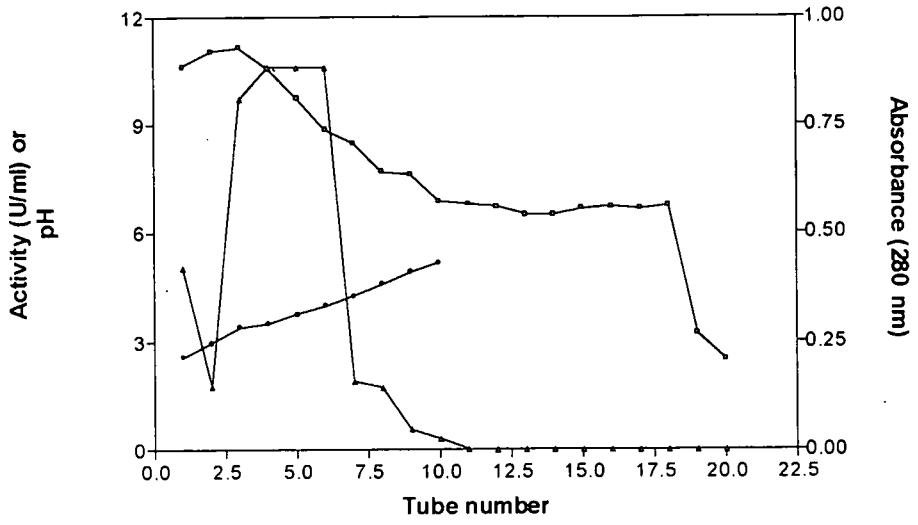


Figure 3.10: A typical profile of iso-electric focussing of the lipase retentate after ultrafiltration, resulting from Super Q chromatography, using the Biorad Rotofor Cell system. (A_{280nm} (■), Activity (▲) and pH (●)).

3.12.5 Sephadex G-75 exclusion chromatography

A Sephadex G-75 column (900 x 17 mm) was pre-equilibrated with a 50 mM Tris-HCl (pH 8.0) buffer and the active enzyme (7.5 ml) from electrofocussing applied to the column at a flow rate of 5 ml/hour. The eluate was analysed for protein content (Figure 3.11). The column yielded two protein fractions. The fractions were pooled on the basis of their A_{230} values and concentrated by ultrafiltration, but only the second peaks displayed lipase activity. The homogeneity of the protein was evaluated by SDS-PAGE as described in Section 3.13. The purified lipase appeared as a single band on SDS-PAGE (Figure 3.14), thus confirming its homogeneity. The purification table for this protocol is given in Table 3.2.

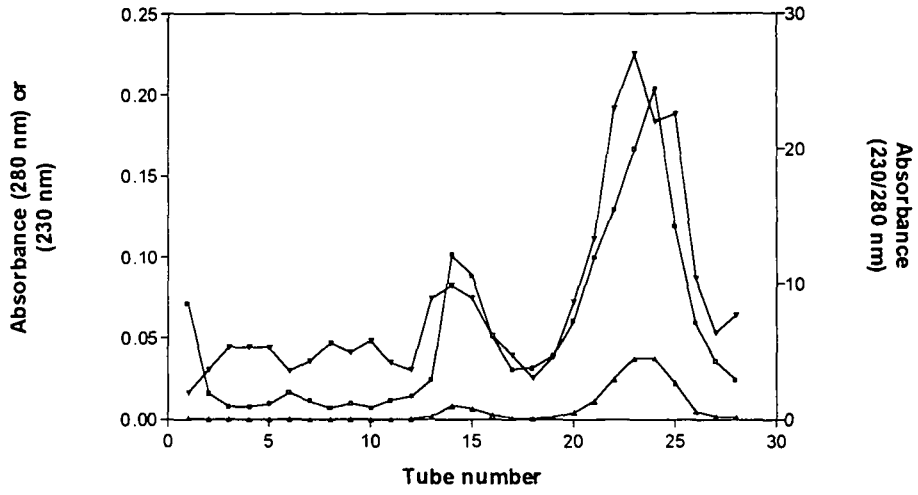


Figure 3.11: A profile of Sephadex G-75 exclusion chromatography of the enzyme fraction after iso-electric focussing (A_{280nm} (▲), $A_{230/280}$ (▽) and A_{230nm}(■)).

Table 3.2 Purification table for the first purification protocol for *Aspergillus lipase*.

Fraction	Volume (ml)	Conc. (µg/ml)	U/ml	Total protein (mg)	Total units	% yield protein	% yield activity	Specific activity (U/mg)	Purification factor
Dialysis	320	2571	0.66	822	211	100	100	0.257	1
Super Q	133	407	3.13	54	416	6.58	197	7.67	29.84
Ultrafiltration	15	1551	44.8	23	672	2.83	318	28.9	112.45
Electrofocussing	17.5	3084	10.2	54	178	6.56	84	3.3	12.84
Ultrafiltration	20	3712	23	74	460	9.02	217	6.18	24.05
Sephadex G-75	5.2	751	12	3.9	62	0.47	30	15.9	61.87

The problems inherent to reliably assay lipase activity are amply illustrated by the yields of enzyme activity obtained during the purification (the same applies for Table 3.3). The presence of other proteins and products of the hydrolysis of the lipid inducer, especially in early stages of the purification, can influence the activity measurements to a large degree. This aspect has been addressed in Section 3.9.2 when the effect of BSA on the lipase activity was assessed. Other contaminating proteins will have a similar effect (Piéroni *et al.*, 1990). For this

reason, specific activities of lipase preparations only have some significance if the preparation is pure and the ability of the enzyme to penetrate the interface is not affected. This will be further addressed in Chapter 4.

3.12.6 Biogel P 60 exclusion chromatography

A second purification procedure was undertaken and the protocol remained as before up to the size exclusion step, excluding the electrofocussing. Biogel P 60 gel filtration chromatography was employed for more effective separation as its functional fractionation range is narrower than that of the Sephadex G-75. This column removed more protein (Figure 3.12) and the purification was just as effective without the iso-electric focussing to yield a single band on SDS-PAGE (Figure 3.14).

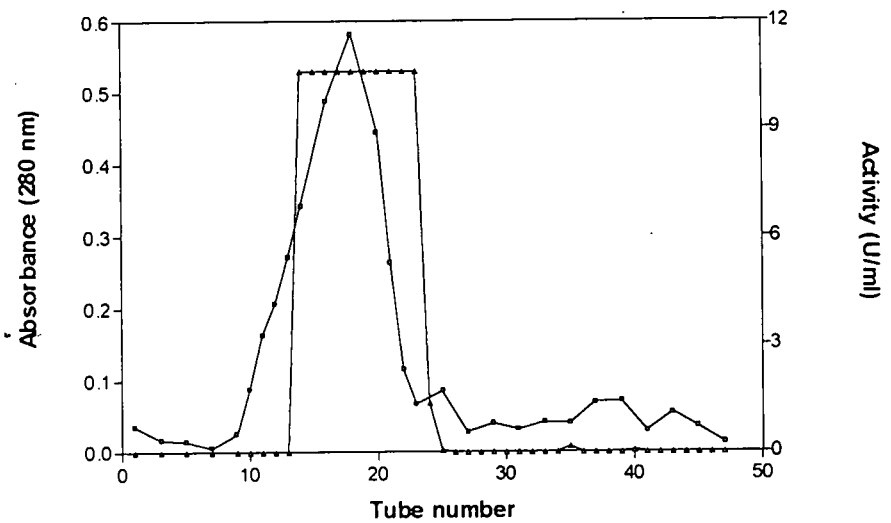


Figure 3.12: A typical profile of exclusion chromatography using Biogel P 60 of the enzyme fraction after Super Q ion exchange chromatography and concentration by ultrafiltration. (A_{280nm} (■) and Activity (▲)).

The purification table is given in Table 3.3. The same restrictions on interpreting enzyme activity data apply as before. It is however clear that the specific activity of the material resulting from Biogel exclusion chromatography is much higher than

that from the Sephadex. The abbreviated protocol was therefore used in future purifications.

Table 3.3 Purification table for the second purification protocol for *Aspergillus lipase.*

Fraction	Volume (ml)	Conc. (µg/ml)	U/ml	Total protein (mg)	Total units	% yield protein	% yield activity	Specific activity (U/mg)	Purification factor
Dialysis	320	2571	0.66	823	211	100	100	0.26	1
Super Q	133	407	3.12	54	415	6.56	196	7.69	30
Ultrafiltration	15	1551	45	23	675	2.79	319	29	112
Biogel P 60	30	703	26	21	780	2.55	369	37	142
Ultrafiltration	10	1404	75	14	750	1.7	355	54	208

3.13 Gel Electrophoresis

The purification process and the relative molecular mass (M_r) of the lipase were assessed using sodium dodecyl sulphate polyacrylamide gel elecrophoresis (SDS-PAGE). The relative molecular mass of the enzyme was estimated by comparing its electrophoretic mobility with those of standard proteins of known molecular masses. A modification of the method of Laemmli (1970) was used as described by Rabillot (1989).

The following methods were used to prepare samples which were too dilute for loading directly onto the gel (Hames, 1990):

i) A volume of the protein sample (± 10 µg of protein) was dried under vacuum for about 60 min and the pellet was dissolved in 20 µl of sample buffer (diluted 1:1 with water).

ii) 1.5 ml of cold (-20 °C) isopropanol was added to a protein sample (± 10 µg of protein) in a 2.0 ml Eppendorf tube. The solution was mixed by inverting the tube and placed on ice for 30 min, centrifuged at

room temperature (10 000 rpm in a microcentrifuge) for 20 min and the supernatant was immediately removed. The protein pellet was dried under vacuum for about 30 min and the precipitate was dissolved in 25 µl of sample buffer (diluted 1:1 with water).

SDS-PAGE was performed using the "Mighty Small" miniature slab gel electrophoresis unit, SE 200, from Hoefer Scientific Instruments. Electrophoresis was performed on approximately 10 µg protein. The protocol used was that described by Sharpiro and Maizel (1969). When the tracking dye almost reached the bottom of the gel, the power supply was turned off and the flow of the coolant was stopped.

The gels were stained with Coomassie blue for 1 hour as described in the Hoefer instruction manual. Thereafter, it was destained with destaining solution 1 (7.5 % ethanol, 5 % acetic acid) and then with destaining solution 2 (40 % ethanol, 10 % acetic acid). To detect bands not visible after Coomassie blue staining, the gel was exposed to silver staining according to Switzer *et al.*, (1979), with a slight modification of the method as described by Rabillot (1989). The gels were stored in 20 % ethanol and then sealed in a small plastic bag.

The protein standards used were: α_2-Macroglobulin (subunit M_r = 170 000), β-galactosidase (M_r = 116 400), fructose-6-phosphate kinase (M_r = 85 200), glutamate dehydrogenase (M_r = 55 600), aldolase (M_r = 39 200), triose phosphate isomerase (M_r = 26 600), trypsin inhibitor (M_r = 20 100) and lysozyme (M_r = 14 300).

SDS-PAGE experiments (10 %, 12 % and 15 %) were performed to separate the respective markers and to construct a plot of the relative distance of migration of each marker versus the logarithm of the molecular mass (Figure 3.13).

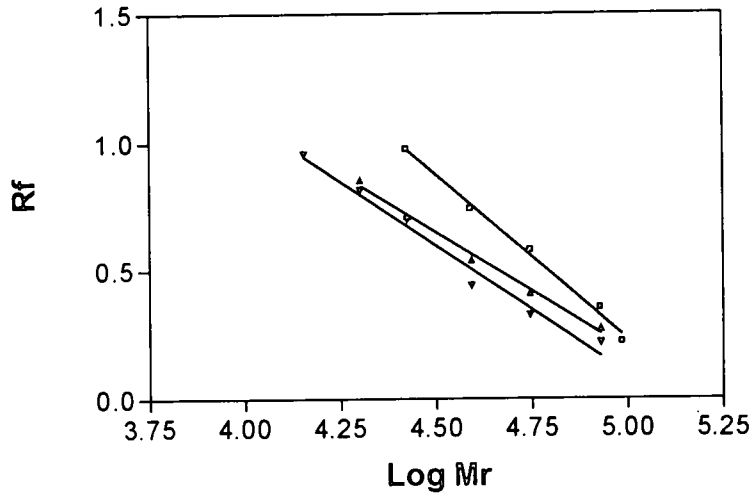


Figure 3.13: A plot of relative mobilities of the marker proteins used versus the logarithm of their molecular masses obtained from reducing SDS-PAGE (10% gel (▫);12 % gel (▵) and 15% gel(▿)).

A single band was observed (Figure 3.14). The unknown protein's molecular mass was estimated by linear regression analysis on the appropriate part of the curve. The migration of the pure lipase protein on the 10 %, 12 % and 15 % gels yielded M_r values 39 800, 38 900 and 43 600, respectively.

Carbohydrate analysis on the purified enzyme (Section 3.15) indicated that the enzyme was glycosylated. Segrest and Jackson (1972) described the estimation of the M_r of glycoproteins in polyacrylamide gels of different acrylamide concentrations (7.5 % to 12.5 %). Although non-glycosylated proteins gave consistent M_r values at all acrylamide concentrations, many glycoproteins behave anomalously during SDS-PAGE electrophoresis. However on increasing the polyacrylamide gel concentration, molecular sieving predominates over charge effect. Therefore, the M_r obtained on the 15 % gel was used as final estimation of the size of the purified protein.

The relative molecular mass compares well with other purified microbial lipases, for example the lipases from *Humicola langinosa* (Omar *et al.*, 1987) and *Pseudomonas aeruginosa* (Shabtai and Daya-mishne, 1992) have a molecular

mass of 39 000 Da. The lipase from *Penicillium simplicissimum* (Sztajer *et al.*, 1992) has a larger M_r value of 56 000 Da. Wu *et al.*, (1996) reported a M_r value of 31 600 Da for the lipase of *Rhizomucor miehei* and the lipase from *Flavobacterium odoratum* has a reported M_r value of 32 500 Da (Labuschagne *et al.*, 1997).

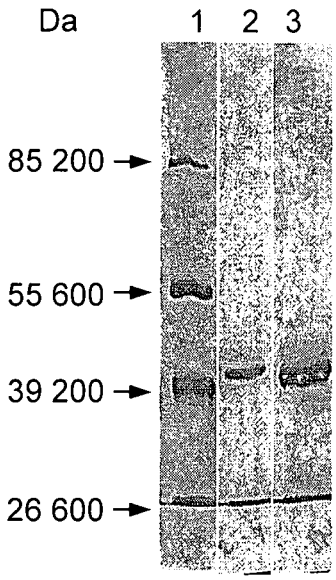


Figure 3.14: SDS-PAGE pattern of the purified AnL (12 % gel). Lane 1 contains some of the molecular weight markers. Lane 2 contains the product after Sephadex chromatography and lane 3 contains the product after Biogel chromatography. The 26 600 marker migrated with the tracking dye.

3.14 Analytical iso-electric focussing

Iso-electric focussing was done using the "Mighty Small II" electrophoresis vertical gel apparatus (Hoefer Scientific Instruments) using the method described by Robertson *et al.* (1987). To detect the bands, the gel was exposed to silver staining according to Switzer *et al.*, (1979), with a slight modification of the method as described by Rabillot *et al.*, (1989). The following Sigma PhastGel pI Markers were used: amyloglucosidase (pI 3.6); trypsin inhibitor (pI 4.6); β-lactoglobulin A (pI 5.1); carbonic anhydrase II (pI 5.9); carbonic anhydrase I (pI 6.6); myoglobin (pI 7.2); lectin (pI 8.6) and trypsinogen (pI 9.3).

The enzyme fraction showed a single band on the gel with an iso-electric point of 6.1, confirming its homogeneity (Figure 3.15).

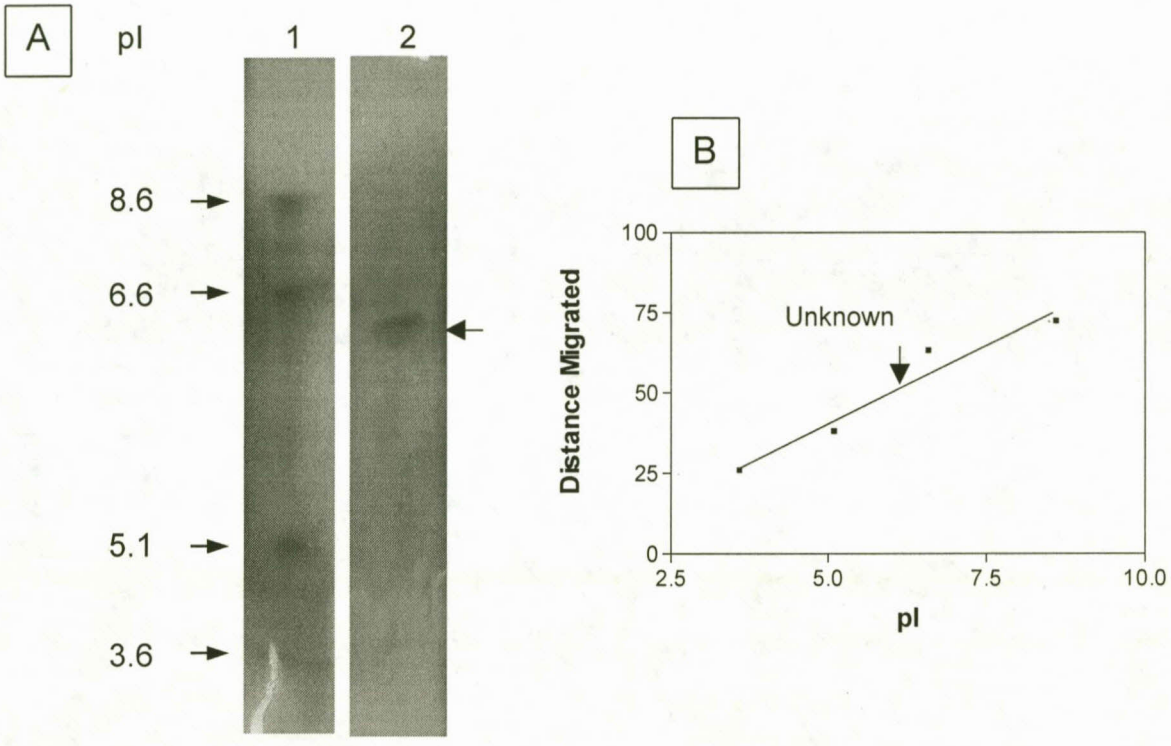


Figure 3.15: Iso-electric focussing gel of the purified AnL (A). Lane 1 contains some of the pI markers. Lane 2 contains the enzyme obtained after Biogel chromatography. The graph was used to calibrate the gel for estimation of the pI of the lipase (B).

The results indicated that the lipase from *Aspergillus niger* has an acidic pI of 6.1. This compares well with other acidic pI values reported in literature for lipases. Sugihara *et al.*, (1993) obtained an iso-electric point of 4.42 for *Geotrichum candidum* lipase. Sztajer *et al.*, 1992 reported an iso-electric point of 4.1 for the lipase of *Penicillium simplissimum*, whereas higher iso-electric points have been reported for *Humicola langinosa* (6.6) (Omar *et al.*, 1987) and *Pseudomonas aeruginosa* (5.8) (Stuer *et al.*, 1986).

3.15 Carbohydrate estimation

The carbohydrate content of an *Aspergillus niger* lipase solution, with a protein concentration of 189 μg/ml, was estimated using a microtitre plate assay as described in the "Glycoprotein carbohydrate content estimation kit" manual as supplied by Pierce. The following Pierce glycoprotein standards were used: lysozyme (0 % carbohydrate content); bovine serum albumin (trace carbohydrate content); ovalbumin (3.2 % carbohydrate content); apo-transferrin (5.8 % carbohydrate content); fetuin (22.9 % carbohydrate content) and α_1-acid glycoprotein (41.4 % carbohydrate content). The calibration curve for the standards is given in Figure 3.16. The high absorbace values were obtained by multiplying the absorbance value of a diluted sample with the appropriate dilution factor. The standard curve is not linear and does not pass through the origin. This is in accordance with the curves given in the user's guide supplied with the kit.

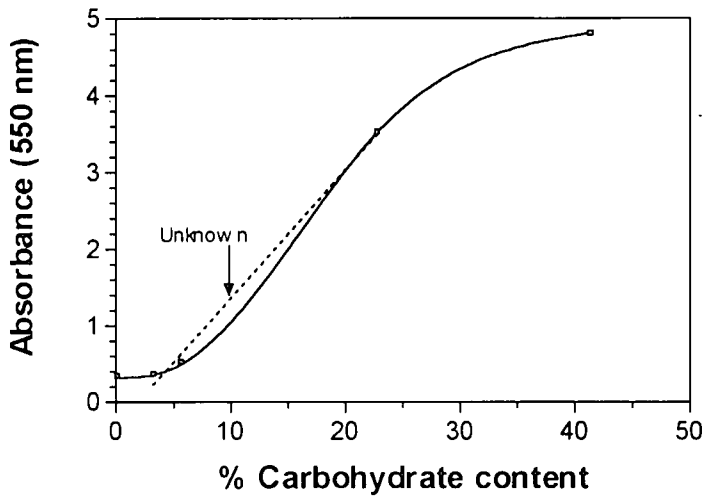


Figure 3.16 Calibration curve for the estimation of carbohydrate by the Pierce Carbohydrate Estimation kit. The arrow indicates the carbohydrate content of the lipase from *Aspergillus niger*.

The absorbance values of diluted samples were used to calculate the percentage carbohydrate of the lipase by doing linear regression on the calibration curve as shown. The results obtained with this kit suggested that the lipase from *Aspergillus niger* has a carbohydrate content of approximately 10 %.

Not many authors reported on the carbohydrate content of lipases, however the literature suggests that fungal lipases have carbohydrate content of between 6 % (Hedrich *et al.*, 1991) and 11,4 % (Baillargeon, 1990) carbohydrate of the total mass. The PhD thesis of Sheba Davidson reported 12.3 % carbohydrate content for the lipase of *Aspergillus carneus* (1998).

3.16 Amino acid analysis

A purified sample of *Aspergillus niger* lipase was sent to the Department of Biochemistry at the University of Pretoria and the CNRS in Marseille for amino acid analysis. Hydrolysis was done in constant boiling HCl at 110 °C for 20 hours in the presence of 1 % phenol (Stein and Moore, 1951, Hirs *et al.*, 1960) and analysed using the Picotag (Pretoria) or a Beckman System 6300 ion exchange system. In general, the two analyses, which were done on different preparations, are very similar (Table 3.4). Glx, Pro, Ala and His show some differences but the significance thereof is questionable. The presence of carbohydrate in the molecule could influence the results from the two laboratories. Tryptophan was not determined. No cysteine was detected in the samples, which coincides with other lipases studied so far.

Table 3.4 Mole percentage amino acid composition of the lipase from *Aspergillus niger*. The analysis data as received from two independent laboratories.

Amino Acid	University of Pretoria	CNRS in Marseille
Asx	6.04	8.04
Thr	12.15	11.88
Ser	22.68	19.37
Glx	6.18	3.67
Pro	3.39	7.59
Cyh	0	0
Gly	9.81	7.45
Ala	9.04	5.25
Val	4.16	3.41
Met	0.51	0.5
Ile	2.50	2.46
Leu	8.97	9.9
Tyr	1.95	1.63
Phe	2.67	2.64
Trp	N.D	N.D
His	5.53	13.43
Lys	3.82	1.73
Arg	0.6	1.07
Total	**100**	**100**

3.17 Amino acid sequencing

The purified protein, after Biogel chromatography, was applied to a 10 % SDS-PAGE gel and electroblotted onto Immobilon-P Transfer membrane using Tris-HCl buffer (25 mM; pH 10.4), containing 10 % methanol. After the electroblotting run at 100 mA was completed, the transferred protein was visualised with Coomassie brilliant blue R stain. The membrane was dried between two folds of Whatman filter paper and submitted for sequencing analysis.

The blotted protein on the Immobilon-P membrane, as well as purified protein (without blotting), were subjected to vapour phase amino acid sequencing by the University of Natal, Molecular Biology Unit, Pietermaritzburg, the protein chemistry Laboratory at the CNRS, Marseille and the Department of Biochemistry, University of Cape Town. In all cases, only traces of amino acids could be seen after each Edman cycle. Sufficient protein was used in each case (~10 - 300 µg). This was probably caused by the high degree of glycosylation or a blocked N-terminus. Similar problems were encountered with a lipase purified from *Aspergillus carneus* by Sheba Davidson at the Delhi University in India (Personal communication, 1998). They were only able to determine the identity of the first 5 amino acid residues as SPTFA.

3.18 Kinetic and Physicochemical Characterisation

The enzyme preparations used for characterisation were stored at 4 °C throughout all the experiments without loss of activity.

3.18.1 Optimum pH

The activity was determined over a range of pH 2.5 - 10 using the olive oil assay described in Section 3.9.1. The pH range was constructed by adjusting the pH of the assay buffers used. The enzyme was added and the reaction was allowed to proceed for 60 min at 30 °C. For each pH, the remaining activity was analysed in triplicate, together with a control. The experiment was repeated twice and the average activity was calculated.

The effect of pH on enzyme activity is depicted in Figure 3.17. The activity at the optimum was taken as 100 %.

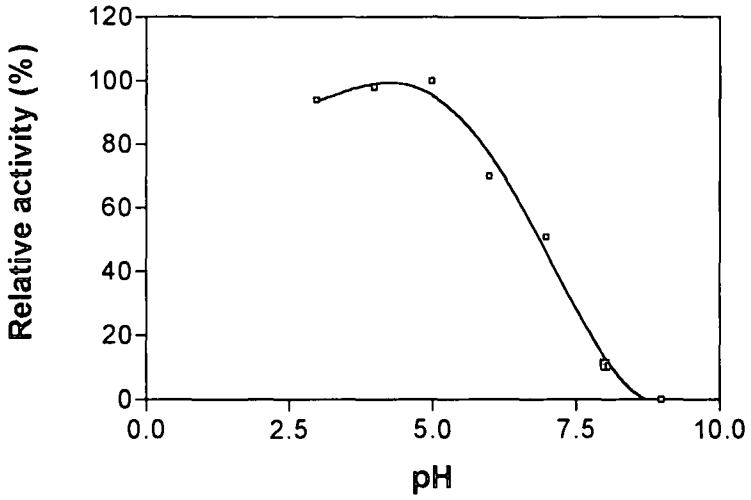


Figure 3.17: Optimum pH of *Aspergillus niger* lipase. Standard deviations for triplicate determinations are smaller than the symbols used for the data points.

The lipase from *Aspergillus niger* showed preference for acidic conditions, with maximal activity at pH 5.0. Sztajer *et al.*, (1992) reported that the lipase from *Penicillium simplissimum* has a pH optimum of 5.0 and the extracellular lipases produced by *Aspergillus niger, Chromobacterium viscosum* and *Rhizopus* spp. are particularly active at acidic pH (Ghosh *et al.*, 1996). Many authors reported on lipases with pH optima in the alkaline regions (Moller *et al.*, 1991; Khyami-Horani, 1996; Labuschagne *et al.*, 1997), mostly of bacterial origin. Other bacterial species showed maximal activity at or around neutral pH (Gowland *et al.*, 1987; Sugihara *et al.*, 1991).

3.18.2 pH stability

The pH of the enzyme solution was adjusted to the required value, between pH 2.5 - 10, using a strong acid or base. The enzyme solution was incubated at 30 °C at the different pH-values and aliquots were periodically withdrawn and immediately placed on ice. The remaining activity was assayed at pH 5.5 in triplicate, using the pH stat with tributyrin as substrate. The experiment was repeated twice on

different days. The experiment was repeated in the presence of 10 mM Ca^{2+} to assess any possible stabilising effect as reported by Pronk *et al.*, (1992).

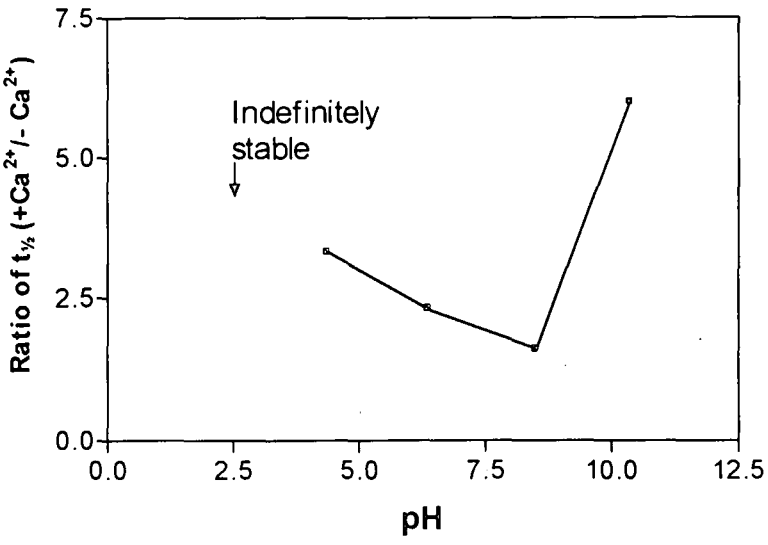


Figure 3.18: pH stability of *Aspergillus niger* lipase, with or without Ca^{2+} in the assay buffer.

Table 3.5 Half lives of *Aspergillus niger* lipase at different pH values in the absence and presence of Ca^{2+}.

pH	$t_{½}$ (hours) no Ca^{2+} added	$t_{½}$ (hours) Ca^{2+} added
4.36	41.5	138
6.38	9.8	22.8
8.51	22.8	36.9
10.36	13.8	82.5

It was evident that in the presence of calcium the enzyme was significantly stabilised, the effect being most dramatic at high pH values (Figure 3.18 and Table 3.5).

The enzyme from our laboratory is much more stable than the other partially purified lipase from *Aspergillus niger* investigated by Hatzinikolaou et al., 1996. Their lipase lost 50% activity after 2 min at pH 9.0.

3.18.3 Optimum temperature

The optimum temperature was determined over a range of 25 - 50 °C. In each case the assay buffer was equilibrated at the required temperature before addition of enzyme. Olive oil assays were done in triplicate together with a blank at each temperature. The experiment was repeated three times and the average activity was calculated.

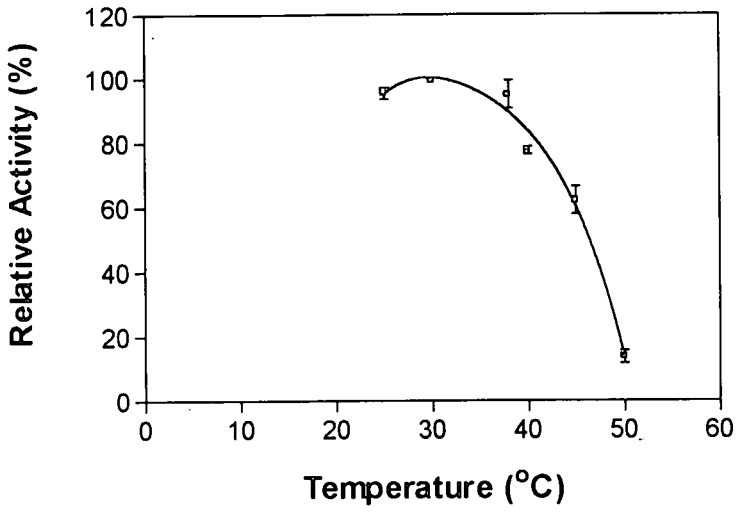


Figure 3.19: Optimum temperature of *Aspergillus niger* lipase. Standard deviations for triplicate determinations are shown.

Although the enzyme is active over a wide range of temperatures the optimum is 30 °C (Figure 3.19). The significance of this data is more apparent in the stability experiments that followed. Mozaffar and Weete (1993) reported an optimum temperature of 30 °C for the lipase of *Pythium ultimum*. In fact several of the microbial lipases reported on displayed an optimum temperature between 30 and 45 °C.

3.18.4 Temperature stability

The temperature stability was determined at temperatures ranging from 30 - 90 °C. The enzyme was incubated at different temperatures and aliquots were periodically withdrawn and immediately placed on ice. The residual activity was assayed in the pH stat with tributyrin as substrate. The experiment was repeated three times and the standard deviations calculated. Typical inactivation curves for the enzyme are shown in Figure 3.20.

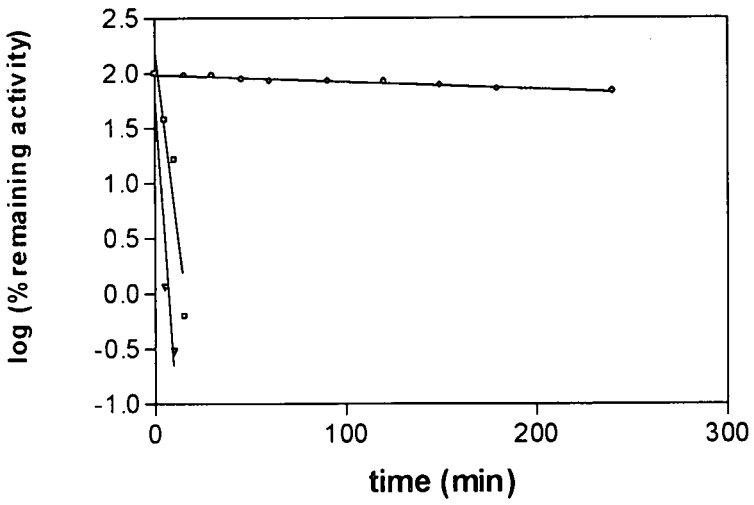


Figure 3.20 Typical decay curves for the inactivation of *Aspergillus* lipase at different temperures. (60 °C (o), 70 °C (□), 80 °C (Δ))

Aspergillus niger lipase lost 20 % activity after 90 min incubation at 60 °C, but after 5 min at 70 °C only 40 % activity remained. Although the enzyme does not compare well with other extremely thermostable enzymes, it is nevertheless encouraging that this enzyme was quite stable over long periods at lower temperatures. Another research group who partially purified a lipase from an *Aspergillus niger* reported that this lipase showed significant loss of activity at temperature above 40 °C; at 50 °C incubation for 20 min 50 % activity was lost (Hatzinikolaou *et al.*, 1996).

The Arrhenius equation (Equation 3.1) (and the corresponding plot) allows calculation of the activation energy and the pre-exponential or frequency factor.

The activation energy is calculated from the gradient and the pre-exponential factor from the intercept of the Arrhenius plots. Rate constants for the inactivation were calculated by linear regression analysis of the plot of log remaining activity (%) vs. time at different temperatures. An Arrhenius plot was used to calculate the activation energy for deactivation of the enzyme without added Ca^{2+} (Figure 3.21).

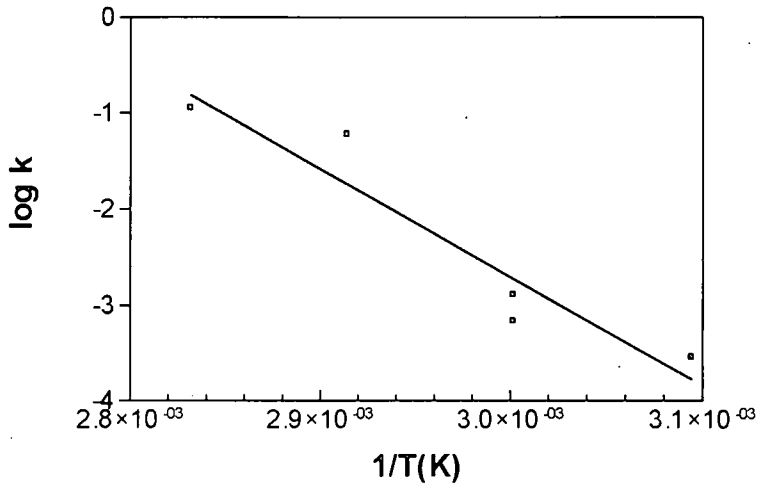


Figure 3.21: Arrhenius plot for the inactivation of *Aspergillus niger* lipase.

The activation energy for inactivation was calculated from the slope of the Arrhenius plot and was found to be 215 kJ/mol. The activation energy for inactivation compares well with that of the lipase from *Candida cylindraceae* (201 kJ/mol) (Pronk *et al.*, 1992).

A stabilising effect of Ca^{2+} ions had been observed under conditions of pH inactivation (Section 3.18.2). This, and the known stabilisation effect of Ca^{2+} on trypsin (Bier and Nord, 1951), prompted a study on the effect of Ca^{2+} on the thermal stability of the *Aspergillus* lipase. The Arrhenius plot for the inactivation of the lipase in the presence and absence of added Ca^{2+} (10 mM) is given in Figure 3.22.

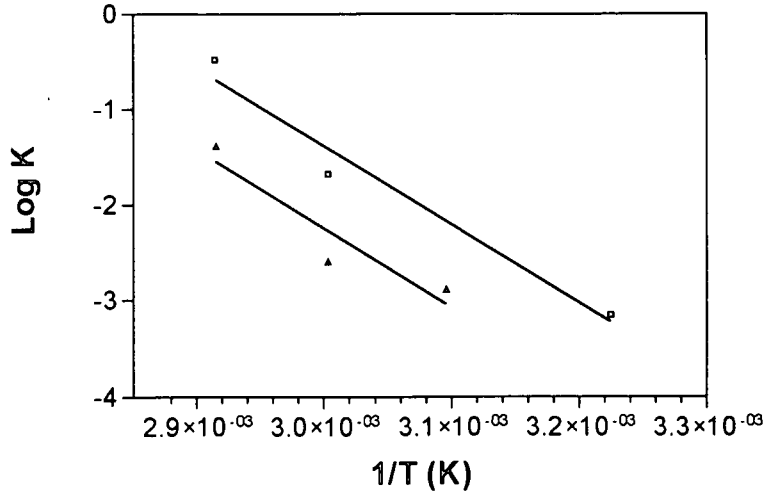


Figure 3.22 Arrhenius plots for the inactivation of *Aspergillus niger* lipase in the absence (△) and presence (▣) of added Ca^{2+}.

The values for the activation energy of inactivation and the frequency factors are given in Table 3.6. The activation energy values of approximately 155 kJ/mol are lower than that obtained previously. This experiment was however done on a different enzyme preparation purified a year later.

Table 3.6 Thermal inactivation parameters obtained for *Aspergillus niger* lipase in the absence and presence of added Ca^{2+}.

Conditions	E_{act} for inactivation (kJ/mol)	Frequency factor (min^{-1})
- Ca^{2+}	156	$1.32X10^{23}$
+Ca^{2+}	158	$3.02X10^{22}$

The Dutch group reported similar data for the lipase of *Candida cylindraceae* (201 kJ/mol) (Pronk *et al.*, 1992); no change in activation energy was observed on immobilisation of their enzyme. A 4.4 fold difference in the value of the frequency factors with and without Ca^{2+} was seen for the *Aspergillus* lipase, the Ca^{2+} therefore leading to a 4.4 fold enhancement in stability. This was similar to the

effect observed by Pronk *et al.* (1992). The frequency factor gives an indication that the event leading to inactivation will take place. Clearly the addition of Ca^{2+} reduces the likelihood of such an event occurring. The exact nature of the binding of the divalent ion is however not known.

3.18.5 Effect of metal ions

The effect of metal ions on lipase activity was assessed using Ag^{+}, Al^{2+}, Ba^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Sn^{2+} and Zn^{2+}. A metal ion concentration range of 0 - 5 mM was investigated. The enzyme was incubated with different concentrations of metals for 30 min at 30 °C and the remaining activity assayed using the pH stat method with tributyrin as substrate. The assay buffer contained the same concentration of metal ion as the incubation mixture.

The effect of metals on enzyme activity is usually not tested at concentrations higher than 1 mM, although some authors have evaluated higher concentrations. (Murderhwa *et al.*, 1985 and 1986; Roussis *et al.*, 1988). The effect of metals on enzyme activity are shown in Figures 3.23 - 3.25. Ba^{2+} and Ca^{2+} are not general enzyme inhibitors, however, it was surprising to experience the slight loss in activity with the incubation of calcium seeing that the presence of this metal increased stability over longer periods of storage. The same effect was reported with trypsin in the presence of these metals (Bier and Nord, 1951). The slight positive effect of low concentrations of Mg^{2+} and Ba^{2+} were however noted. Sn^{2+} (Omar *et al.*, 1987; Iizumi *et al.*, 1990) has been shown to be an inhibitor and also strongly inhibited the action of AnL. Zn^{2+} is known for its strong inhibitory effect (Iizumi *et al.*, 1990; Sugihara *et al.*, 1991; Sztatjer *et al.*, 1992) and this characteristic is emphasised by the *Aspergillus niger* lipase, but to a surprisingly lower effect. Fe^{2+} and Fe^{3+} are also strong inhibitors and had the same effect on AnL (Sugihara *et al.*, 1991 and Iizumi *et al.*, 1990). Although Pb^{2+} is not known for its strong inhibitory effect, the presence of this metal even at very low concentrations inhibited the lipase's action. Hg^{2+}; Mn^{2+}; Al^{3+}; and Ag^{+} are other

metals evaluated in this study, it is quite clear that even very low concentrations of metals have a negative effect of the enzymatic action of AnL.

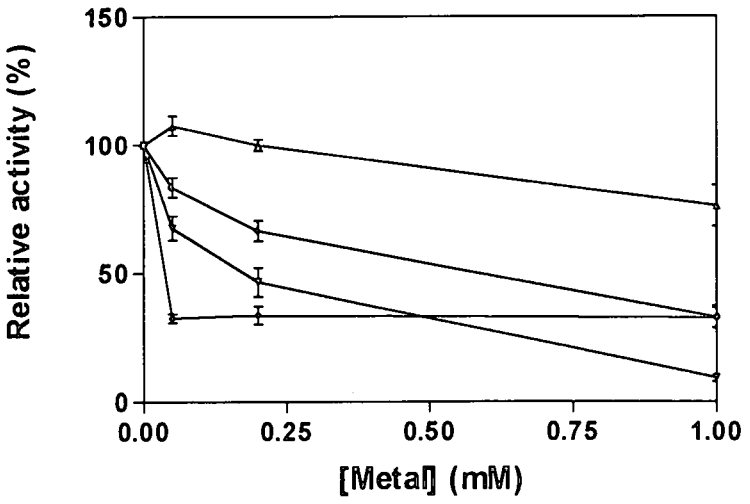


Figure 3.23: Effect of metal ions on *Aspergillus niger* lipase. (Mg^{2+} (▲); Zn^{2+}(▽); Fe^{3+} (◇) and Fe^{2+} (○)). Standard deviations are shown as error bars.

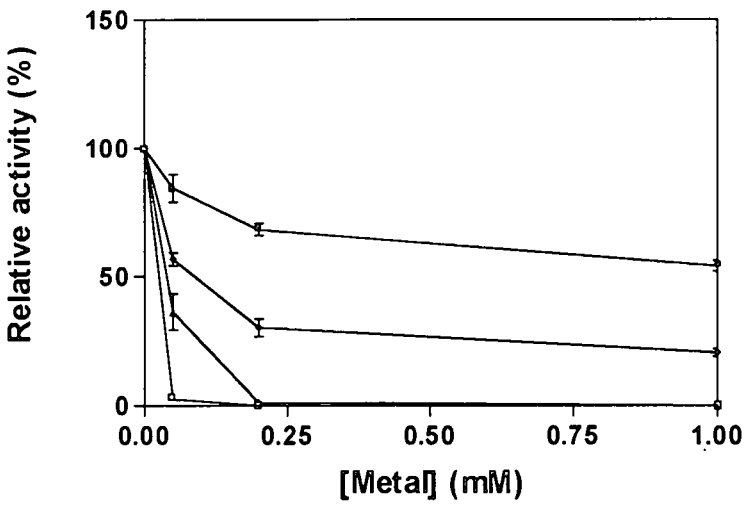


Figure 3.24: Effect of metal ions on *Aspergillus niger* lipase. (Ag^{+} (■); Al^{3+} (□); Mn^{2+}(◇) and Sn^{+} (▲)). Standard deviations are shown as error bars.

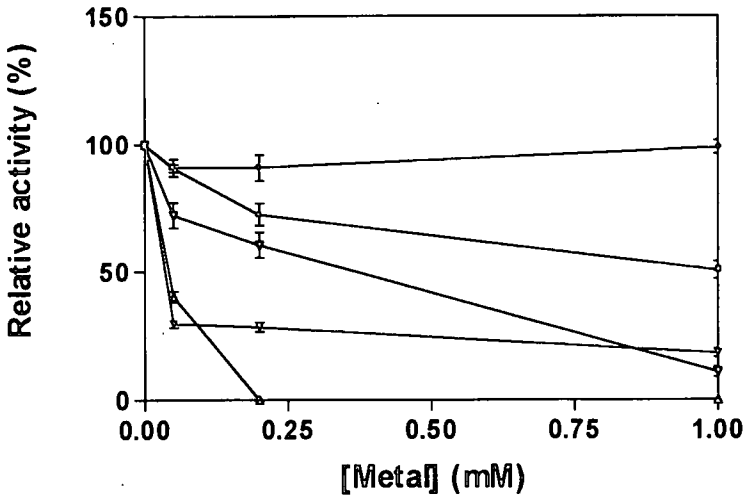


Figure 3.25: Effect of metal ions on *Aspergillus niger* lipase. (Cu^{2+} (▽); Ba^{2+} (•); Pb^{2+} (△); Hg^{2+} (▽) and Ca^{2+} (▣)). Standard deviations are shown as error bars.

3.18.6 Effect of detergents

The effect of detergents on lipase activity was tested at concentrations below and above the critical micelle concentration (cmc = the minimum concentration at which detergents begin to form micelles). Six detergents were tested; the anionic detergents sodium deoxycholate (cmc = 4 mM) and SDS (cmc = 8.2 mM), the cationic detergent cetrimide (hexadecyltrimethylammoniumbromide) (cmc = 0.92 mM), the non-ionic detergents Triton X-100 (cmc = 0.3 mM) and Tween 80 (cmc = 0.01 mM) and the zwitterionic detergent, CHAPS (cmc = 6.5 mM). The enzyme was incubated with different concentrations of different detergents for 30 min at 30 °C and the remaining activity was assayed in triplicate using the pH stat with tributyrin as substrate. The experiment was repeated three times on different days and standard deviations were calculated (Figures 3.26 and 3.27).

The range of detergent concentrations tested is very much higher than the highest concentrations (0.5 %) found in literature (Iizumi *et al.*, 1990). At this concentration only ionic micelle structures showed a significant effect on the lipase. Several researchers (Stuer *et al.*, 1986: Nishio *et al.*, 1987) reported that SDS (0.1 % or 0.4 %) and Cetrimide (0.4 %) have an inhibitory effect on lipase activity. There have

however also been reports on Tween 80 (0.1 %), a non-ionic detergent and sodium deoxycholate (0.5 %), an anionic detergent which had inhibitory effects. Cationic detergents, such as CTAB, also had strong inhibitory effects on lipase catalytic activity (Dellamora-Ortiz *et al.*, 1997). Dellamora-Ortiz *et al.*, (1997) also showed an activation in the presence of Tween 80 (34 %) and a 70 % increase in the presence of Tween 20. This study with the lipase from *Rhizomucor miehei* also addressed an interesting issue, assaying with 7 mM cholate resulted in an increase in activity of 20 %, but when the enzyme was pre-incubated with this detergent it led to considerably higher enzyme activity that reached 250 % of the control. It was suggested by the authors that specific interaction with the monomers of sodium cholate with the enzyme would displace the lid and that binding of the cholate to expose the hydrophobic surface in the open conformation would result in lipase activation (Dellamora-Ortiz *et al.*, 1997). Mozaffar and Weete (1993) reported that in the presence of Triton X-100 and CHAPS the enzyme activity increased 3-fold.

Because of the very low pH optimum of the enzyme the effect of sodium deoxycholate was not evaluated. At pH values less than 7.5, the deoxycholate becomes insoluble and forms a gel, making the assay procedure impossible.

The non-ionic (Tween 80 and Triton X-100) and zwitterionic (CHAPS) detergents had very little or no effect on the lipase of *Aspergillus niger* action even at concentrations high above the cmc (Figure 3.27). However, the enzyme seemed susceptible to ionic micelles (Figure 3.26), in fact even at concentrations below the cmc of the detergents the enzyme lost more than 80 – 90 % of the activity; as can be seen in the discussion above, this is not uncommon for lipases.

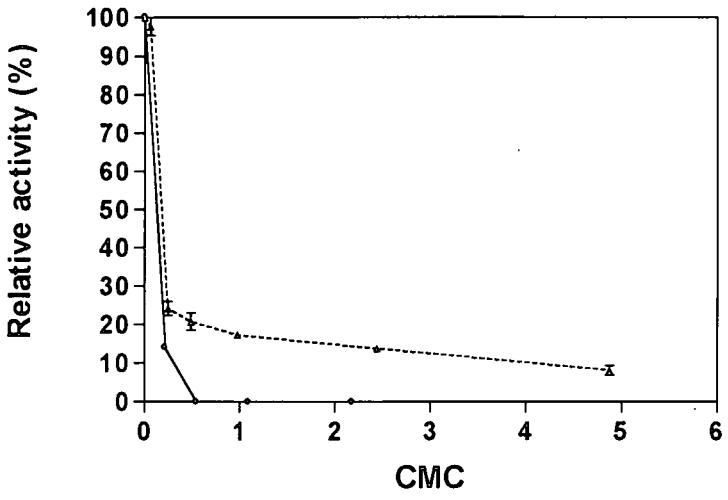


Figure 3.26: Effect of detergents on *Aspergillus niger* lipase. (SDS (▲) and Cetrimide (●)). Standard deviations are shown as error bars.

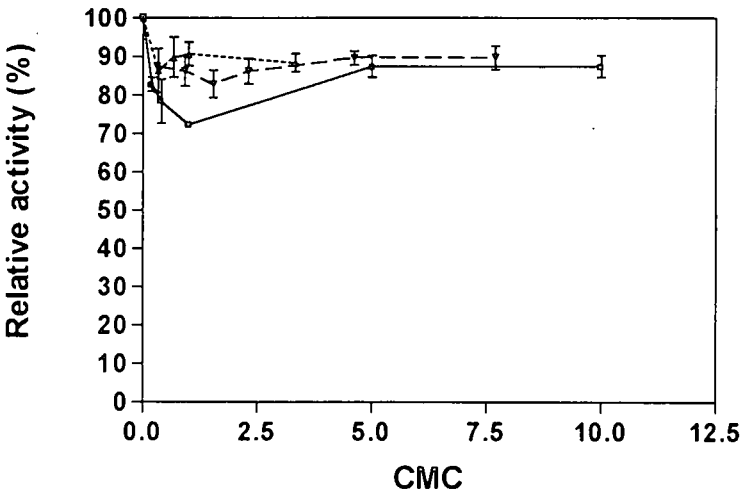


Figure 3.27: Effect of detergents on *Aspergillus niger* lipase. (Tween (■), Triton (▲) and CHAPS (▼)). Standard deviations are shown as error bars.

3.18.7 *Effect of EDTA*

Some enzymes are dependent on metals for their activity, hence they are referred to as metalloenzymes. The dependence of the enzymes on metals is often investigated by the addition of metal chelating agents to the enzyme assay mixture. The metal chelating agent used in most studies are EDTA.

A concentration range of 0.05 - 5 mM EDTA was used. The enzyme was incubated with different concentrations of EDTA for 30 min at 30 °C and the remaining activity assayed using the pH stat method with tributyrin as substrate. The experiment was repeated three times and standard deviations were calculated. The effect of EDTA on lipase activity is shown in Figure 3.28.

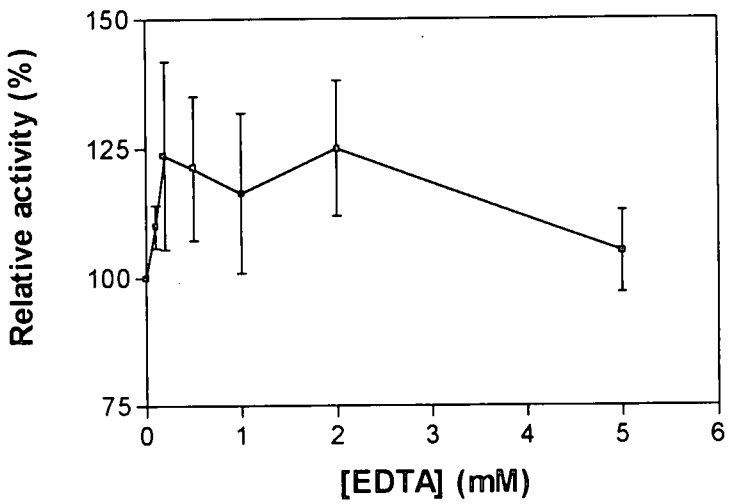


Figure 3.28: Effect of EDTA on *Aspergillus niger* lipase. Standard deviations are shown as error bars.

The activity of the control (enzyme incubated without EDTA) was taken as 100 %. These results are from triplicate values, repeated on different days, explaining the large standard deviations. It is clear that the lipase seems to be activated upon incubation with EDTA, especially at concentrations of EDTA between 0.1 and 2 mM This correlates well with the data shown in Figure 3.25. This clearly is a result of the complexation by EDTA of Ca^{2+} or another inhibitory ion. The addition of

EDTA into the assay mixture had a slight buffering effect in the pH stat, which could account for the high standard deviations. The exact nature of the Ca^{2+} binding is not certain as it was seen to play a stabilising role or possibly stimulate activity (Ghosh *et al.,* 1996). The lipase of *Pseudomonas glumae* contains a bound calcium ion located close to the active site of the enzyme and although no activation was shown in the presence of calcium for this enzyme, the stabilising effect on the *Aspergillus* enzyme might be due to a similar phenomenon (Noble *et al.*,1993). The function of the calcium was postulated to be that of stabilising the local structure adjacent to the active site. Hermoso *et al.* (1996) reported that the Ca^{2+} ion interacts with a highly conserved region forming a distorted square pyramid. In porcine lipase the Ca^{2+} site is located far from the catalytic triad and is not likely to be involved in catalysis, but rather appear to play a purely structural role in the loop (Hermoso *et al.*, 1996). In the case of the lipase from *Rhizopus delemar* this enzyme showed stimulation of activity by calcium, even a three fold increase was observed (Haas *et al.*, 1992).

3.18.8 Identification of catalytic site residues

The three-dimensional structure of a native enzyme is an essential starting point for studies of structure function relationships. The first crystal structures already showed that the catalytic centre is made up of a triad, reminiscent to that found in serine proteases. Serine proteases hydrolyse peptide bonds through nucleophilic attack by a serine residue, which is activated via an Asp-His couple and the accompanying action of the oxyanion hole. This part of the study was to modify the proposed catalytic residues as to receive some insight into the structure of the lipase from *Aspergillus niger.*

3.18.8.1 Modification of serine

The enzyme was incubated with 50 mM PMSF (phenylmethylsulfonyl fluoride) for 24 hours, aliquots were withdrawn periodically and the remaining activity assayed using the pH stat method with tributyrin as substrate. The PMSF was prepared as

a stock solution in either 100 % DMSO or ethanol, the final concentration of the solvent in the reaction being 10 %. The enzyme was also incubated in 10 % solutions of the above to correct for possible inactivation by the organic solvent.

Although one would expect to show modification of serine with PMSF, the lipase from *Aspergillus niger* did not undergo significant inhibition even after long periods of incubation. Sztajer *et al.*, (1992) showed that low concentrations PMSF did not have a big inhibitory effect on their enzyme, which let to the decision to evaluate even higher concentrations. Skein (1994) evaluated this effect in the presence and absence of detergents to see whether the active site was more accessible to the reagent but no significant difference in the absence or presence of detergent was shown.

Inactivation of *Fusarium oxysporum* another fungal lipase, by PMSF could be enhanced when the enzyme was exposed to high concentrations of 2-propanol which was necessary to change the enzyme's conformation thereby making the serine more susceptible to the inhibitor (Hoshino *et al.*, 1992). This approach had no effect on the results obtained. However, the resistance to PMSF has also been observed for other lipases and this has been attributed to the protective burying of the active site within the enzyme by the "lid region" (Brady *et al.*, 1990).

DFP was also assessed as serine modification reagent. An aliquot of the enzyme at pH 5 was treated with 20 mM and 80 mM DFP for 30 min, and the remaining activity assayed as described in Section 3.9.2. Relative to the control, 41 % and 30 % of the activtiy remained respectively. This illustrates the presence of a nucleophilic serine in the catalytic site.

3.18.8.2 Modification of histidine

Diethylpyrocarbonate is the most useful reagent for specific modification of histidine. In a pH range from 5.5 to 7.5, diethylpyrocarbonate is specific for reaction with histidyl residues. The reaction is associated with an increase in

absorbance at 240 nm. The modification is readily reversible at alkaline pH and, in particular, in the presence of nucleophiles such as Tris (Lundland, 1995).

The pH of the enzyme solution was adapted to pH 5.5. The enzyme was incubated with 20 mM DEP (diethylpyrocarbonate) and the reaction followed spectrophotometrically at 240 nm. Aliquots were withdrawn periodically and the remaining activity assayed by pH stat titration, using tributyrin as substrate.

The results (Figure 3.29) show that the addition of DEP inhibited the lipase activity completely and the modification of the residues could be followed at an absorbance of 240 nm. The reaction proceeded very rapidly, leading to a complete loss of activity in less than 10 min. The continued increase in A_{240nm} is probably the result of non-specific modification of other histidine residues by the DEP. The activity was restored the by addition of Tris and by raising the pH which could reverse the action of the DEP. Only about 70 % of the activity was restored but this is sufficient evidence that reversible modification of a histidine involved with catalysis had occurred.

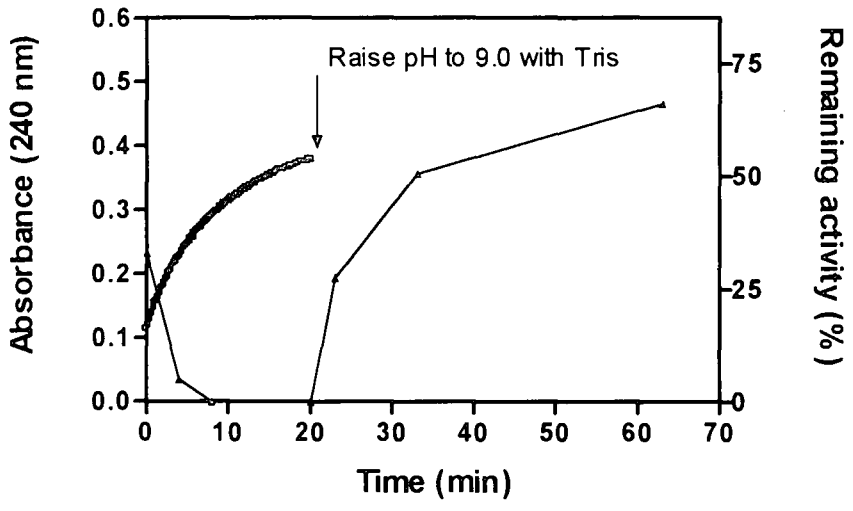


Figure 3.29: Effect of DEP on *Aspergillus niger* lipase activity. The modification of histidine results in inactivation of the lipase, but activity (▲) is restored by reversing the modification.

3.18.8.3 Modification of the carboxylate

The involvement of a carboxylic residue in the lipase catalytic action was evaluated with a water-soluble carbodiimide, (1-ethyl-3 (3-dimethylamino-propyl) carbodiimide), known for modification of acid residues in protein.

The pH of the enzyme solution was adapted to a pH of 4.75. The enzyme was incubated with 0.1 M of the water-soluble carbodiimide and aliquots were withdrawn periodically and the remaining activity assayed. The reagent successfully modified the enzyme until no activity could be detected, this modification is also reversible and the activity could be restored by raising the pH with the addtion of a nucleophile (Lundland, 1995).

The initial activity was taken as 100 %, and after the addition of the carbodiimide the activity was monitored. After 7.5 min only 53 % activity remained and after 17 min no activity could be detected. After the pH was restored, 32 % activity was detected after 10 min and 91 % after 20 min.

From the initial identification of possible catalytic residues and the action of the enzyme the results indicated conclusively that the enzyme was indeed a lipase and displayed a catalytic action similar to the serine proteases.

3.18.9 Specificity/affinity towards liquid triacylglycerols

The assays for hydrolysis of triacylglycerols which are liquid at the temperature of the assay were performed as described in section 3.9.2. The only variable was the triacylglycerol substrate used. The substrates which were evaluated were triacetin (2:0), tributyrin (4:0), tricaproin (6:0), tricaprylin (8:0), triolein (*cis*-18:1(9)), trielaidin (*trans*-18:1(9)) and trilinolein (*cis,cis*-18:2(9,12)). In this experiment the substrates were emulsified using a Branson Cell Disrupter B-30 to ensure an homogeneous emulsion when stirring was insufficient, especially with the longer chain fatty acids. The results are shown in Figure 3.30.

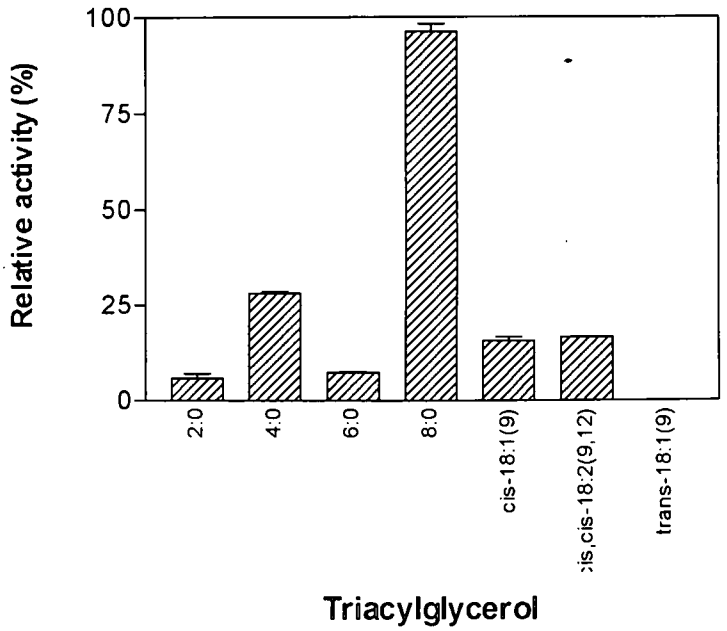


Figure 3.30: Relative reaction rates of the *Aspergillus niger* lipase towards different liquid triacylglycerols. Standard deviations for the triplicate determinations are shown as error bars.

Mozaffar and Weete (1993), evaluated the lipase of *Pythium uitimum*'s preference towards the longer chain triacylglycerols. The authors found an increase in preference with increasing number of double bonds per molecule. The lipase from *Aspergillus niger* did not differentiate between the unsaturated substrates; this was however only true for substrates containing *cis*-double bonds. The lipase from *Pythium uitimum* was introduced to the *trans*-double bond substrate, trieliadin, the rate of hydrolysis (18 %) was very low compared to that of triolein. From the results this preference is also evident as the lipase of *Aspergillus niger* showed no activity towards trieliadin.

Although it is generally difficult to make comparisons between different triacylglycerol substrates due to different properties of the emulsions, the tricaprylin is clearly the preferred substrate. The assays used in this study were usually done using tributyrin or olive oil (*cis*-18:1(9)), which clearly led to underestimation of the activity. This data also highlight the difficulty in interpreting lipase kinetics without well defined interfacial properties. This aspect is addressed in Chapter 4 where

detailed kinetic studies were performed using a monomolecular system where interfacial quality could be defined. Similar complications would make investigation into regio- and stereopreference of the enzyme, in bulk phase, extremely difficult.

3.19 Conclusions

Aspergillus niger is a widespread mould in nature and many strains of the fungus have been isolated from various origins. Moulds are widely recognised as the best lipase source and several references involving different industrial applications depending on the enzyme specificity have been reported (Bjorkling *et al.*, 1991). Studies on fermentation conditions for the production of extracellular lipase from an *Aspergillus niger* showed variations among different strains of the fungus (Pokorny *et al.*, 1994).

In this study, the lipase isolated from *Aspergillus niger,* displayed most characteristics coherent with other fungal lipases isolated for biotechnological applications. The level of enzyme activity obtained with olive oil as substrate was 75 U/ml, this activity is almost double when compared with the lipase from another *Aspergillus niger* which produced what is considered elevated levels of lipase activity (40 U/ml). The enzyme production levels are higher when compared to other hyper-producing fungi.

To date, many properties of lipase from a variety of *Aspergillus* species have been investigated, however, reports on the purification and properties of lipase from *Aspergillus* species are limited. Other attempts to partially purify the lipase included ASP and ion-exchange chromatography. In this study we used this information and developed a successful purification protocol as discussed.

The stability displayed in the presence of Ca^{2+} over a wide range of pH and temperature conditions is especially encouraging. This could possibly indicate a specific effect of calcium on the lipase other than the general removal of inhibitory agents from the lipid-water interface. A calcium binding site has been located on

the three-dimensional structure of the *Pseudomonas glumae* lipase (Noble *et al.*, 1993), unfortunately less is known about the 3-D structures of fungi and such binding has not been reported. It has also been observed that Ca^{2+} stabilises proteins against thermal inactivation. A six fold increase of Ca^{2+} binding affinity was achieved when two uncharged aspartic amino acid residues in the vicinity of the calcium binding site was negatively charged by a change in the pH. This resulted in greater stability against irreversible thermal inactivation of subtilisin (Pantoliano *et al.*, 1988).

In chapter 4 the author will discuss aspects relating to interfacial catalysis of lipases more extensively and more characteristics from the lipase from *Aspergillus niger* will be revealed.

The basic texture of research consists of dreams into which the treads of reasoning, measurement and calculations are woven.

Albert Szent György

CHAPTER 4

Surface Kinetics of *Aspergillus niger* lipase

4.1 The monomolecular film technique

Lipase acts on acylglycerol substrates. Because the plane of symmetry of the glycerol molecule is a prochiral plane, the two primary hydroxyl groups are stereochemically distinct (i.e., they are enantiotropic groups). A Fischer projection of a triacylglycerol molecule is drawn with the secondary alcohol chain branching off to the left of the main hydrocarbon chain (Figure 4.1).

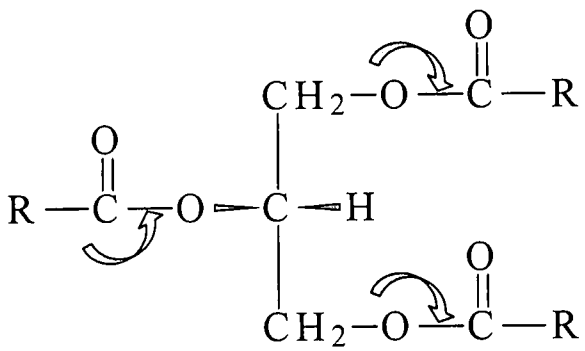


Figure 4.1: Fischer representation of a triacylglycerol molecule. Potentially hydrolysable ester bonds are indicated with arrows.

The carbon atoms are numbered 1, 2 and 3, working downward. With this system of numbering, glycerol becomes *sn*-glycerol (i.e., stereospecifically numbered glycerol). This makes it possible to obtain unambiguous expressions for the stereoisomeric forms of (phospho) glycerides. Natural phospholipids, for example, all belong to the *sn*-glycero-3-phosphate series. In the case of natural

triacylglycerols, the fatty acids that esterify positions *sn*-1, *sn*-2 and *sn*-3 are often different, which results in various chiral substrates.

The lipid monolayer is a suitable model system to study the enzymatic reactions carried out at the interface in a heterogeneous medium. A new field of investigation was opened in 1935 when Hughes used the monolayer technique for the first time for this purpose (Hughes, 1935). He observed that the rate of the phospholipase A-catalyzed hydrolysis of a lecithin film, measured in terms of the decrease in surface potential, decreased considerably when the number of lecithin molecules per square centimeter increased. Since this early study, several laboratories have used the monolayer technique to monitor lipolytic activities, mainly with glycerides and phospholipids as substrates.

There are at least five major reasons for using lipid monolayers as substrates for lipolytic enzymes (Verger and de Haas, 1976; Verger, 1980; Verger and Pattus, 1982; Pièroni *et al.*, 1990; Ransac *et al.*, 1991).

1. It is easy to follow the course of the reaction monitoring one of several physicochemical parameters characteristic of the monolayer film: surface pressure, potential, density, etc.
2. Probably the most important basic reason is that it is possible, with lipid monolayers to vary and control the *interfacial quality*, which depends on the nature of the lipids forming the monolayer, the orientation and conformation of the molecules, the molecular and charge densities, the water structure, the viscosity, etc. One further advantage of the monolayer technique as compared to bulk methods is that with the former, it is possible to transfer the film from one aqueous subphase to another.
3. Using the surface barostat balance, the lipid packing of a monomolecular film of substrate can be kept constant during the course of hydrolysis, and it is therefore possible to obtain accurate pre-steady state kinetic measurements with minimal perturbation caused by increasing amounts of reaction products.
4. The monolayer technique is highly sensitive and very little lipid is needed to obtain kinetic measurements. This advantage can often be decisive in the

case of synthetic or rare lipids. Moreover, a new phospholipase A_2 was discovered using the monolayer technique as an analytical tool (Verger *et al.*, 1982).

5. Inhibition of lipase activity by water-insoluble substrate analogues can be precisely estimated using a zero-order trough and mixed monomolecular films in the absence of any synthetic, nonphysiological detergent.

The monolayer technique is therefore suitable for modeling *in vivo* situations.

4.2 Kinetic models for interfacial enzymatic lipolysis

4.2.1 Kinetics in the presence of substrate only

One of the main assumptions implicitly underlying the classical Michaelis-Menten model is the fact that the enzymatic reaction must take place in an isotropic medium (i.e., both the enzyme and the substrate must be in the same phase). This model therefore cannot be used as it stands to study lipolytic enzymes acting mainly at the interface between a water phase and an insoluble lipid phase. In principle, the mechanism of the chemical reactions carried out at the interfaces in a heterogeneous medium depends strongly on the interfacial organization, steric coordination, and physical interaction between the reacting molecules. The chemical interactions are coupled with processes of adsorption, desorption, convection, and molecular diffusion of the reaction molecules and products of the reaction, etc. One or any combination of these processes may be rate determining.

To adapt the Michaelis-Menten kinetic scheme to interfacial lipolysis, various models were proposed. In the simplest one, described by Verger *et al.*, (1973) an instantaneous solubilisation of the products of the reaction is assumed (Figure 4.2). It is based on the idea that an enzyme-substrate complex might be formed at the interface. The model consists of two successive steps. The first one is a reversible penetration of a water-soluble enzyme (E) into the lipid substrate at the interface. The enzyme is fixed at the interface by an adsorption-desorption

molecular mechanism. One formal consequence of this stage is the dimensional change in the enzyme concentration. The penetration stage (adsorption) sometimes, but not always, involves enzyme activation (e.g. via the opening of the amphiphilic lid covering the active site). This penetration step, leading to a more favorable energy state of the enzyme (E*), is followed by a two-dimensional Michaelis-Menten kinetic scheme. The enzyme in the interface E* binds a substrate molecule S to form the E*S complex followed by its decomposition. The product of reaction P is soluble in the water phase, it desorbs instantaneously, and induces no change with time in the physicochemical properties of the interface. An important conceptual idea should be emphasized here: to be consistent with the fact that the enzyme-catalyzed reaction occurs at the interface, the concentration of E*, E*S and S must be expressed as surface concentration units.

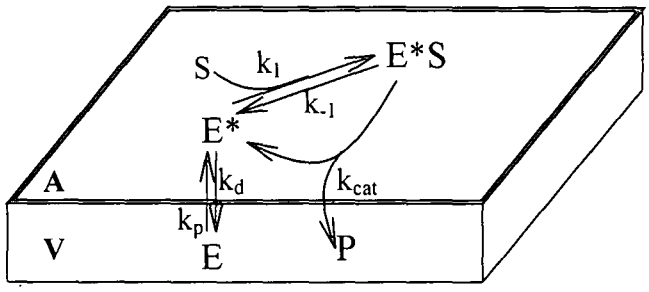


Figure 4.2: Proposed model for lipase kinetics at the interfaces (Adapted from Verger and de Haas, 1976).

The reaction rate dP/dT (product concentration change with time) is given as:

$$v = \frac{dP}{dT} = k_{cat} E_o \frac{S}{K_m^* \left[1 + \frac{k_d}{k_p (A/V)} \right] + S} \qquad [4.1]$$

where:

A = total interfacial area in (surface area units)

E = the bulk enzyme concentration (molecules/volume)

E* = penetrated enzyme concentration (molecule/surface)

E*S = penetrated enzyme-substrate complex concentration (molecules/surface)

E_0 = total enzyme concentration (molecules/volume)

k_1 = rate constant for the complex formation E*S (molecules/surface)$^{-1}$ (time)$^{-1}$

k_{-1} = rate constant for the dissociation of E*S into E* + S (time)$^{-1}$

k_{cat} = catalytic rate constant (time)$^{-1}$

k_d = desorption rate constant (time)$^{-1}$

K_m^* = interfacial 'Michaelis-Menten' constant (molecules/surface)

k_p = penetration rate constant (volume/surface)

P = product concentration (molecules/surface)

S = two-dimensional surface concentration of substrate (molecules/surface)

V = total volume (volume).

4.2.2 Kinetics in the presence of a competitive inhibitor

Kinetic models accounting for the competitive inhibition in the presence and absence of detergent and for irreversible inactivation have also been developed. The rate of product formation, in the stationary state, in the presence of a competitive inhibitor (Figure 4.3) can be written as follows:

$$v = \frac{dP}{dT} = k_{cat} E_o \frac{S}{K_m^* \left[1 + \frac{k_d}{k_p (A/V)} + \frac{I}{K_I^*} \right] + S} \qquad [4.2]$$

where I is a lipase inhibitor.

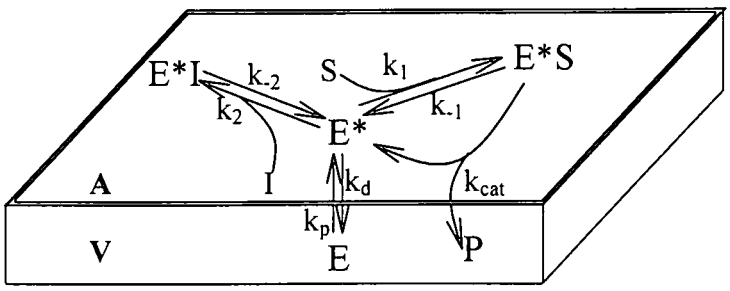


Figure 4.3: Proposed model for competitive inhibition at interfaces (Adapted from Ransac *et al.*, 1991).

The following additional symbols are used in the above model:

E^*I = penetrated enzyme-inhibitor complex concentration (molecules/surface)

I = inhibitor concentration (molecules/surface)

k_2 = rate constant for the complex formation E^*I (molecules/surface)$^{-1}$ (time)$^{-1}$

k_{-2} = rate constant for the dissociation of E^*I into E^* + I (time)$^{-1}$

K_I^* = interfacial dissociation constant for the enzyme-inhibitor complex (molecules/surface).

4.2.3 The interface and enzyme catalysis

To account for the amount of enzyme acting on the interface, two extreme theoretical kinetic situations have been considered by Jain and Berg (1989). In the so-called *Scooting mode* of catalysis (Figure 4.4), the enzyme molecules are irreversibly bound to the surface of vesicles. A bound enzyme molecule E^* remains adsorbed at the interface between the catalytic cycles and hydrolyses the substrate molecules from the outer monolayer of one single vesicle. In pure

hopping mode of catalysis (Figure 4.4), the enzyme molecules are exchanged between vesicles. The binding ($E \rightarrow E^*$) and the desorption ($E^* \rightarrow E$) of a bound enzyme molecule occur after each catalytic cycle. Hopping from one vesicle to the other, ultimately it will hydrolyse available substrate molecules in the outer monolayer of all vesicles. However it has been realised that such a hopping model, if applicable, would be extremely inefficient in terms of catalysis, due to a rate-limiting step in the adsorption-desorption process. (Ransac *et al.*, 1997).

Both models support the general accepted postulation that there are two important steps in lipolysis: binding of the enzyme to the interface ($E \rightarrow E^*$) followed by binding of a single substrate molecule and decomposition into its products.

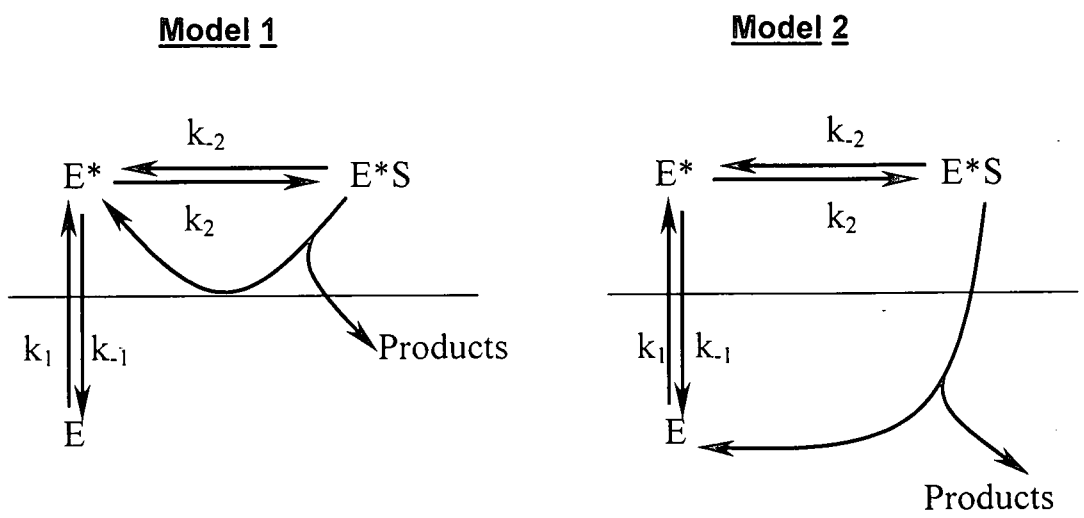


Figure 4.4: Two different models for the action of lipolytic enzymes at interfaces. Model 1 is the scooting mode of catalysis and model 2 describes the pure hopping mode of catalysis.

The experimental hydrolysis rate of both kinetic models under the conditions $K_m^* >> [S]$ can be described by the simplified equation:

$$v = \frac{k_{cat}^{app} \times E_o}{1 + \frac{K_m^{app}}{\frac{1}{V}}}$$

[4.3a]

where

$$k_{cat}^{app} = k_{cat} \times \frac{k_2 S}{k_{-2} + k_{cat}}$$

[4.3b]

From the mathematical treatment it follows that discrimination between the two models is possible on basis of analysis of the K_m^{app} values. In the 'scooting' model the bindings step is not a part of the catalytic cycle and hence K_m^{app} is only dependent on the on-rate (k_1) and the off-rate (k_{-1}):

$$K_m^{app}(scooting) = \frac{k_{-1}}{k_1}$$

[4.4]

An important theoretical aspect of the kinetic model 2 is the prediction that the rate of equilibration of the enzyme over bulk and interface but also catalytic turnover rates influence the value of K_m^{app}

$$K_m^{app}(hopping) = \frac{k_{-1} + k_{cat}^{app}}{k_1}$$

[4.5]

Only when all equilibria are rapid relative to k_{cat}, will the enzyme distribution between bulk and interface not be affected by the rate of hydrolysis. However,

when k_{cat}^{app} is large compared to the off-rate k_{-1}, K_m^{app} will be dependent on k_{cat}^{app} in the hopping mode.

Additional symbols used are defined as:

E = the bulk enzyme concentration (molecules/volume)

E* = penetrated enzyme concentration (molecule/surface)

E*S = penetrated enzyme-substrate complex concentration (molecules/surface)

k_{-1} = desorption rate constant for E* to E $(time)^{-1}$

k_1 = penetration rate constant for E to E* $(surface/volume)^{-1}$ $(time)^{-1}$

k_2 = rate constant for the complex formation E*S $(molecules/surface)^{-1}$ $(time)^{-1}$

k_{-2} = rate constant for the dissociation of E*S into E* + S $(time)^{-1}$

k_{cat} = catalytic rate constant when $K_m^* << S$ $(time)^{-1}$

k_{cat}^{app} = experimentally determined catalytic rate constant $(time)^{-1}$

K_m = dissociation constant for the enzyme-mixed micellar complex (surface/volume)

K_m^* = interfacial 'Michaelis-Menten' constant (molecules/surface)

K_m^{app} = experimentally determined K_m (surface/volume)

4.3 The monolayer technique for studying lipase kinetics

Because lipase acts most efficiently on a lipid-water interface and all interfaces are not identical under conditions of maximal hydrolytic rate, a method of controlling the "quality" of the interface is required. By spreading the lipid phase as a monomolecular film on top of an aqueous phase in a trough, it becomes possible for the surface pressure or the area of the interface to be directly monitored.

4.3.1 The surface barostat

The monolayer technique and instrumentation has been developed to gain insight to the physical and chemical properties of insoluble monolayers at gas-liquid interfaces. The most common and conveniently measured parameter is surface pressure (π) of the monolayer. Surface pressure is defined as the decrease in the interfacial tension produced by the monolayer, i.e. $\pi = \gamma_o - \gamma_m$ where γ_o and γ_m are the interfacial tension in the absence and presence of the monolayer, respectively.

The KSV 2200 Surface Barostat has been designed to measure the surface pressure of a monolayer at gas-liquid interface and control the surface pressure as desired by the user (Figure 4.5). The surface pressure is measured with a KSV 2200 Surface Balance connected to a platinum Wilhelmy plate (Figure 4.6). The information on the surface pressure is received by the Dynamic Film Contol (DFC) system, which commands a step-motor driven mobile Teflon barrier, thereby controlling the surface area occupied by the monolayer and thus subsequently controlling the surface pressure.

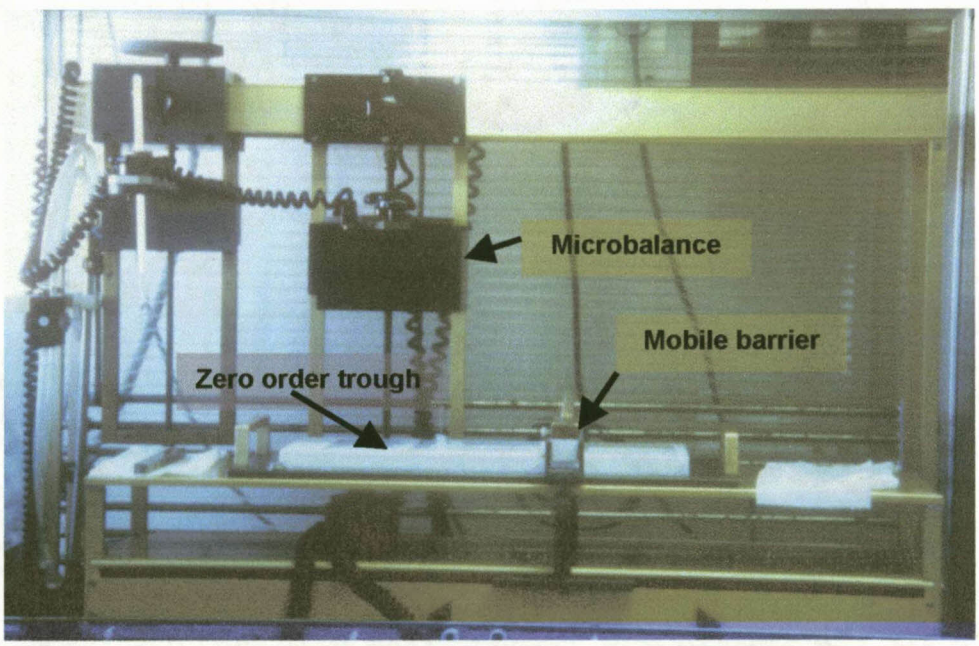


Figure 4.5: KSV 2200 Barostat equipped with "zero-order" Teflon trough for kinetic studies.

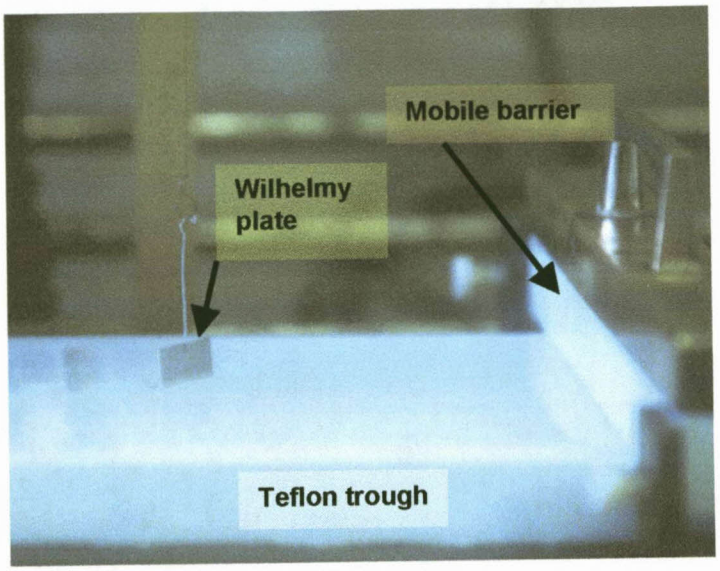


Figure 4.6: Wilhelmy plate suspended by wire leading to an electromicrobalance to control the movement of the mobile barrier, which in turn controls the surface pressure.

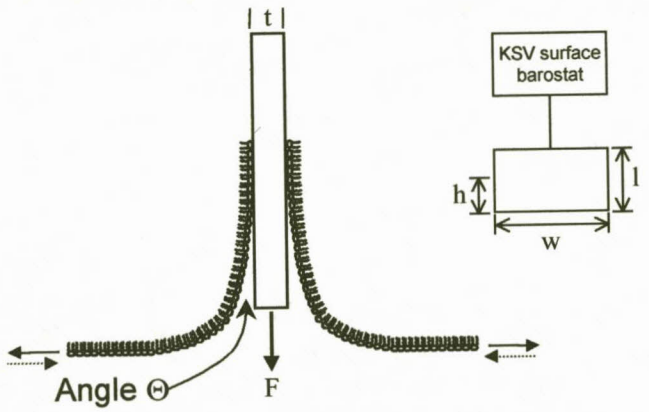


Figure 4.7: Schematical presentation of determination of surface pressure by the Platinum Wilhelmy-plate method. The surface layer pulls the plate in the direction of the solid horizontal arrows resulting in a force F. Surface pressure, applied in the direction of the dotted arrows will "lift" the plate leading to a reduction in the force F.

Figure 4.7 illustrates the principle of measuring the surface pressure by the Wilhelmy plate method. The surface tension of the water exerts a downward force on the plate which is attached to the microbalance. In the presence of a surface layer, the force, F, is given as:

$$F = \rho_p glwt + 2\pi(t + w)\cos\varphi - \rho_l gtwh \qquad [4.6a]$$

where

ρ_p	=	density of the plate
ρ_l	=	density of the liquid
l,w,t	=	dimensions of the plate as shown
h	=	depth of plate in the subphase
g	=	the gravitational constant
φ	=	contact angle of the liquid on the plate
π	=	liquid surface tension

If the width t is negligible compared to w

$$F = 2\pi w\cos\varphi \qquad [4.6b]$$

and if the plate is completely wetted, then cos φ = 1 and

$$F = 2\pi w \qquad [4.6c]$$

4.3.2 Zero-order trough

Several types of troughs have been used to study enzyme kinetics. The simplest of these is made of Teflon that is rectangular in shape (Figure 4.8(A)) but gives non-linear kinetics (Zografi *et al.*, 1971). To obtain rate constants, a semi-logarithmic transformation of data is required. This drawback was overcome by a new through design (Figure 4.8(B)) consisting of a substrate reservoir and a reaction compartment containing the enzyme solution (Verger and de Haas, 1973). A narrow surface canal made of etched glass connects the two compartments. The kinetic recordings obtained with this through are linear, unlike the non-linear plots obtained with the usual one-compartment through. The surface pressure can

be kept constant automatically by the surface barostat method (Verger and de Haas, 1973). Fully automated monolayer systems are commercially available from KSV, Helsinki, Finland.

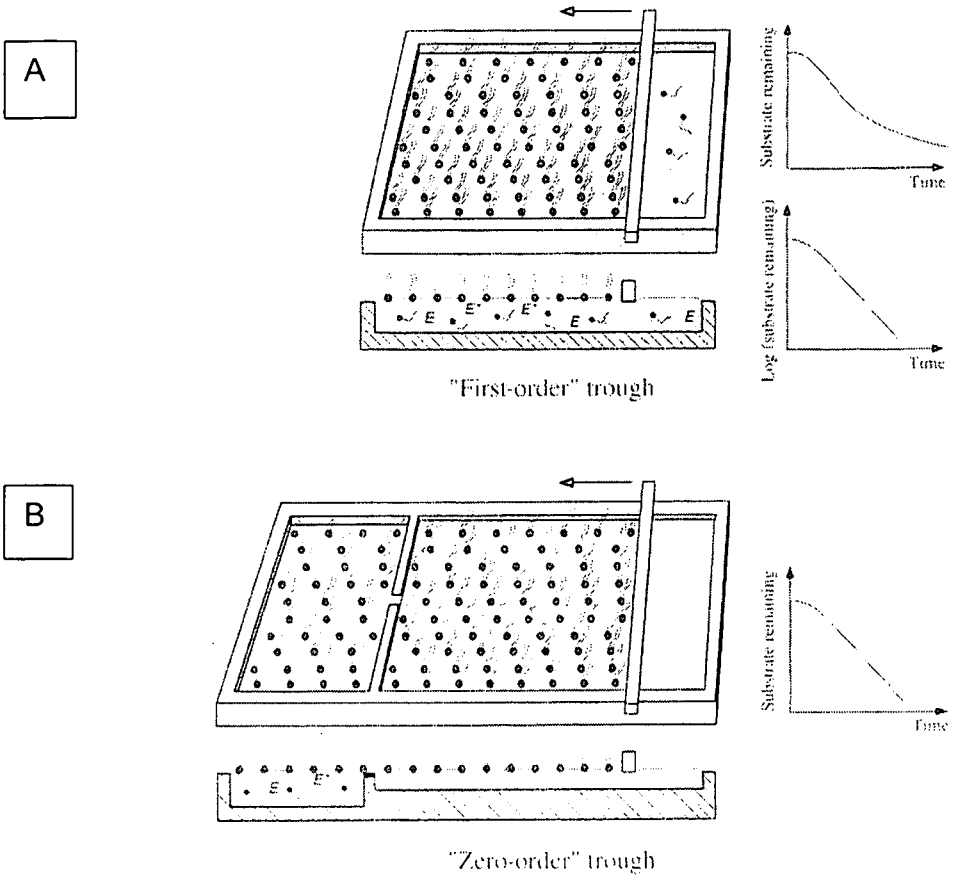


Figure 4.8: Comparison of lipase kinetics obtained with a first-order trough (A) and a zero-order trough (B) (Taken from Ransac *et al.*, 1997)

4.3.3 Pure lipid monolayers as lipase substrates

To use the monolayer technique for lipase kinetics, one necessary condition is that the substrate must be insoluble in the aqueous subphase and must form stable monomolecular films. The enzymatic hydrolysis of short- and mediumchain lipid substrates yielding water-soluble products can be easily interpreted on the basis of Equation 4.1. In the case of short- or medium-chain lipid substrate when the reaction products are water soluble, the enzymatic hydrolysis can be followed at

constant area *A* by measuring the fall of surface pressure (Lagocki *et al.,* 1973). The main objection is related to the fact that the state of the monolayer changes during the reaction, and the kinetic constants (K_d/K_p, K_m and K_{cat}) are not true constants.

4.3.4 Stereoselectivity of lipases is controlled by surface pressure

Lipases, which are lipolytic enzymes acting at the lipid/water interface, display stereoselectivity toward acylglycerols and other esters (Brockerhoff and Jensen, 1974). Biological lipids, which self-organize and orientate at interfaces, are chiral molecules, and their chirality is expected to play an important role in the molecular interaction between proteins and biomembranes. The most unusual aspect of acylglycerol hydrolysis catalysed by pure lipases is its particular stereochemistry (Morley *et al.,* 1974; Rogalska *et al.,* 1990; Cerina *et al.,* 1994).

Lipases can encounter both chiral forms of their substrates, as well as molecules that are prochiral. Physicochemical factors such as temperature (Holmberg and Hunt, 1991), solvent hydrophobicity (Wu *et al.,* 1990; Parida and Dordick, 1991) or the hydrostatic pressure (Kamat *et al.,* 1993), which can affect the stereoselectivity of the reaction, may influence the mechanism whereby an enzyme differentiates between two antipodes of a chiral substrate. To achieve a measurable impact of hydrostatic pressure on a protein in a bulk solution, however, high pressures of the order of 3 kbar need to be used and monitoring the enzyme activity under these conditions is difficult.

However, in the monolayer the surface pressure is easy to manipulate and its effects on the enzyme activity can be readily controlled. Rogalska *et al.,* (1993) investigated the assumption that the stereoselectivity, which is one of the basic factors involved in enzymatic catalysis, may be surface pressure dependent. When working with bulk solutions, the external pressure is not a practical variable because liquids are highly incompressible, whereas the monolayer surface pressure is easy to manipulate.

4.4 Surface kinetics of the purified lipase from *Aspergillus niger*

The experimental work reported on was done at the C.N.R.S, Lipolyse Enzymatique, Marseille, in the laboratory of Dr. Robert Verger (Directeur de Recherches au).

4.4.1 Materials and Methods

The buffers were prepared with double distilled water and were filtered through a 0.45 µm Millipore filter. Any residual surface-active impurities were removed before each assay by sweeping the surface of the buffer with a clean Teflon (polytetrafluoroethylene) bar and suction of the surface with a Pasteur pipette connected to a tap aspirator. Kinetic experiments were performed with the KSV-2200 barostat (KSV, Helsinki) and a "zero-order" Teflon trough (Verger and de Haas, 1973). The width of the reservoir compartment was 147,9 mm, the length 249,2 mm and the surface area 36 815 mm^2. The surface area of the reaction compartment was 10 205 mm^2. The depth of the trough was 8 mm. The reaction compartment was equipped with 2 magnetic stirrer bars operating at 250 rpm. The trough was equipped with a mobile barrier, which was used to compensate for the substrate molecules removed from the film by enzyme hydrolysis, thereby keeping surface pressure constant. The latter was measured using a Wilhelmy plate (Figure 4.6) attached to an electromicrobalance, which was connected in turn to a microprocessor controlling the movement of the mobile barrier (Figure 4.5). The reactions were performed at ambient temperature (25 °C). The enzyme solution was injected through the film with a Hamilton syringe over the magnetic stirrers.

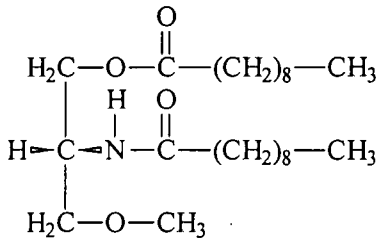
Before each utilisation, the Teflon trough (Figure 4.5) used to for the monolayer film was cleaned with water, then gently brushed in the presence of distilled ethanol, washed again with tap water and finally rinsed with double distilled water. The aqueous sub-phase was composed of glycine (50 mM), 100 mM NaCl and 10 mM $CaCl_2$ (pH 5.5).

4.4.2 Positional preference and stereospecificity of AnL

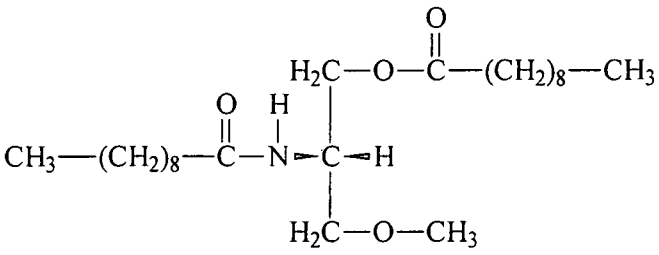
Several research groups have reported on positional selectivity of microbial lipases. Omar *et al.*, (1987) reported that the lipase of *Humicola langinosa* has a *sn*-1,3 positional specificity and Sugihara *et al.*, (1991) reported that the lipase of a *Bacillus* sp. also has a *sn*-1,3 positional specificity. Several other bacterial lipases were depicted as *sn*-1,3 specific (Okeke and Gugnani, 1989; Murderhwa *et al.*, 1986) and untill now it is believed that lipases do not hydrolyse the fatty acid at position *sn*-2 in a triglyceride. Sztajer *et al.*, (1992) however felt that the lipase from the fungus *Penicillium simplissimum* was non-specific, which meant that this lipase hydrolyses any of the three bonds of the triacylglycerol. Sugihara *et al.*, (1991) even suggested in a short contribution that the lipase from *Geotrichum candidum* have some preference for the *sn*-2 position on a triglyceride molecule. These positional specificities were all determined on TLC using a variety of substrates. The problems associated with bulk phase (emulsion) experiments is that the interphase is rather ill defined and that acyl migration in aqueous media can confound the data. Application of pseudolipids containing ether linkages in some positions provides an alternative approach.

In theory, enantiomeric triglycerides containing three different acyl groups should be ideal substrates to investigate the properties of lipases. The six (stereo)-isomeric triglyceride analogs shown in Figure 4.9 were used in the kinetic characterisation of the lipase from *Aspergillus niger*.

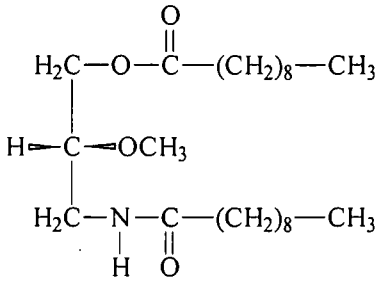
Surface pressures as a function of the mean molecular area isotherms were measured for all compounds. Stock solutions of individual lipids were prepared in distilled $CHCl_3$. Fifty microlitres of a lipid solution with an accurately known concentration was spread on the aqueous buffer. The monolayer was allowed to stabilise for 5 min before it was compressed with a continuous increase in surface pressure per minute (linear compression of 1 mN/m.min). Every 3 seconds data were collected and analysed with proprietary software from KSV Instruments (Helsinki).



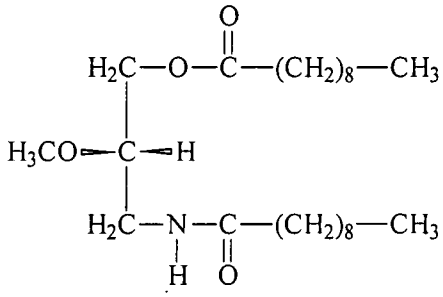
R_1



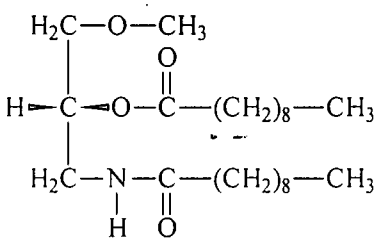
S_1



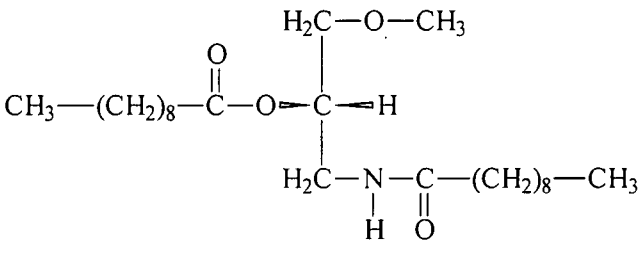
R_2



S_2



R_3



S_3

Figure 4.9: Stereochemical structures of pseudolipid substrates used
(R_1) : (R)-1,2-didecanoyl-2-deoxyamino-3-O-methylglycerol
(R_2) : (R)-1,3-didecanoyl-3-deoxyamino-2-O-methylglycerol
(R_3) : (R)-2,3-didecanoyl-3-deoxyamino-1-O-methylglycerol
S_1, S_2 and S_3 are the optical antipodes of R_1, R_2 and R_3 respectively.

In Figure 4.10 the surface pressure versus mean molecular area isotherms of the three isomeric pseudotriglycerides (R_1, R_2 and R_3 in Figure 4.9) are represented. The three isotherms of the enantiomeric compounds S_1, S_2 and S_3 exactly coincide with those of R_1, R_2 and R_3, respectively, and indicate a high chemical purity of the synthetic triglyceride analogs. These isotherms were used to convert a change in the area of the lipid monolayer on hydrolysis by the enzyme to a molar quantity of substrate hydrolysed. These pseudotriglycerides form stable monomolecular films at the air-water interface up to 32 mN/m.

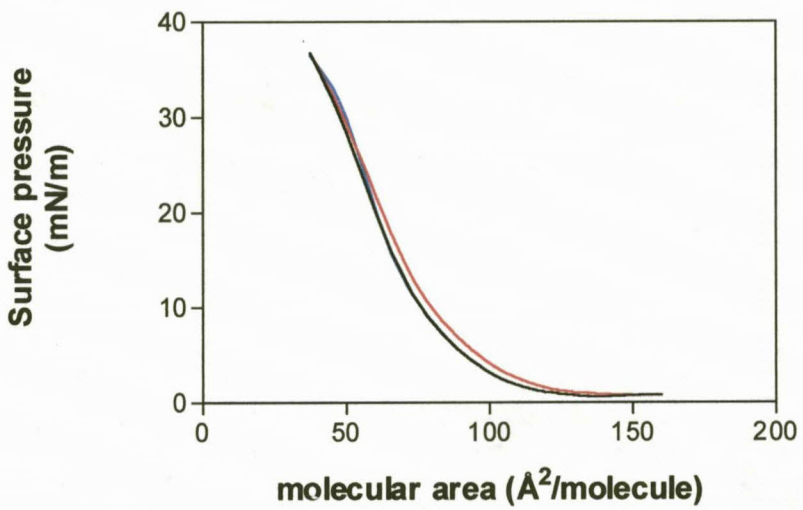


Figure 4.10: Surface pressure versus molecular area in monomolecular films for the pseudotriglycerides in Figure 4.9. (R_1 (--), R_2 (--) and R_3 (--)).

The amount (0.35 µg) of enzyme used for catalysis was also evaluated and it was established that the units being assayed did fall within the linear region of activity for each substrate.

A typical progress curve, as printed by the computerised system, for the reaction is shown in Figure 4.11. The reaction rate is computed from the asymptotic slope of the portion of the progress curve where the barrier speed is constant as indicated by the horizontal arrow. Reaction rates at 15 mN/m are given in Table 4.2.

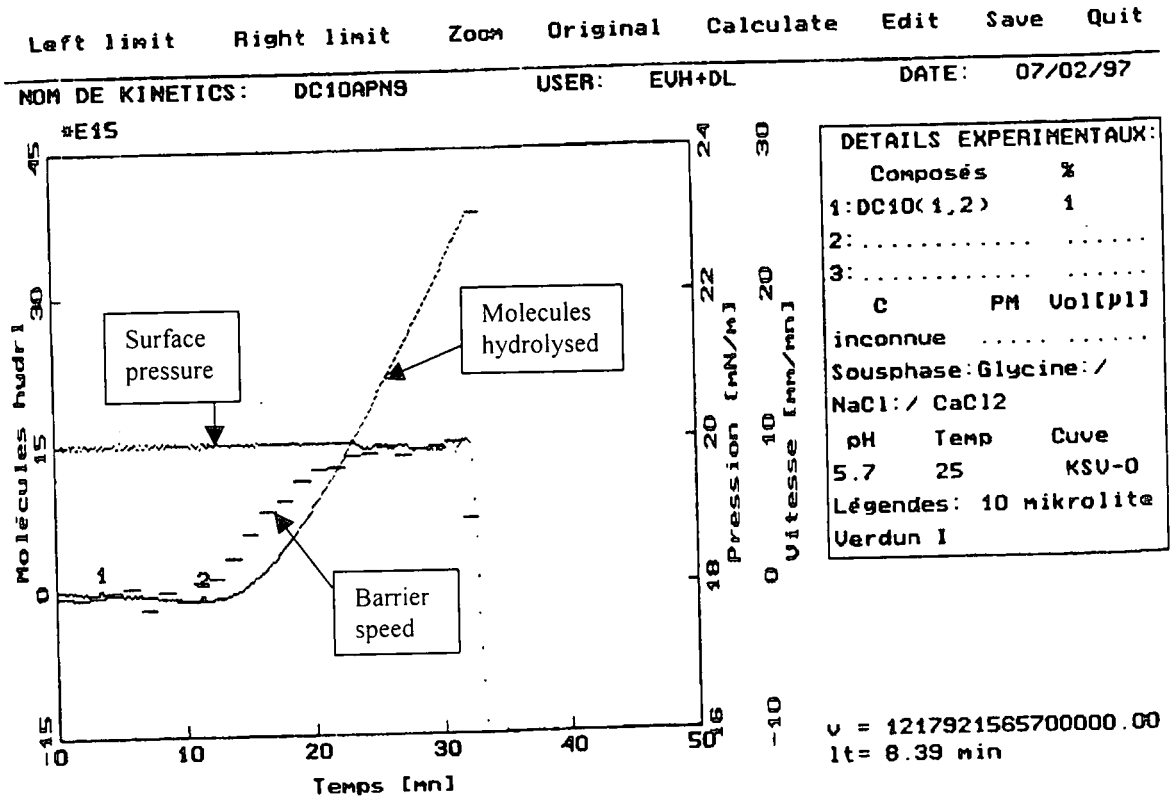


Figure 4.11: A typical progress curve for a hydrolysis reaction from the lipase of *Aspergillus niger* on the computerised KSV 2200 system. The dialect is in French and the appropriate textboxes indicate the parameters measured in such a reaction.

Table 4.2: Enzymatic velocities of *Aspergillus niger* lipase measured with the surface barostat technique for all the six pseudoglycerides. Standard monolayer conditions were used; the full names and stereoconfigurations are given in Figure 4.9.

Pseudolipid substrate	**Rate at 15 mN/m (molec/cm^2/min x 10^{-15})**
R_1	5.9
S_1	2.16
Ratio R_1/S_1	2.73
R_2	2.45
S_2	2.1
Ratio R_2/S_2	1.17
R_3	0.75 x 10^{-3}
S_3	0.299 x 10^{-3}

At low surface pressure it is clear that *Aspergillus niger* lipase displays a stereopreference for the *sn*-1 ester position (R_1/S_1 vs. R_2/S_2) (Table 4.2). Furthermore AnL displays a very low propensity to cleave secondary ester bonds (R_3 and S_3). It is however not very informative to compare substrates at a single surface pressure as the enzyme's reaction to changes in the interfacial quality is not apparent. Changes in surface pressure could dramatically affect the kinetics of the enzyme (Rogalska *et al.*, 1995).

Figures 4.12 to 4.14 show the hydrolysis rates of the isomer-pairs as a function of the surface pressure.

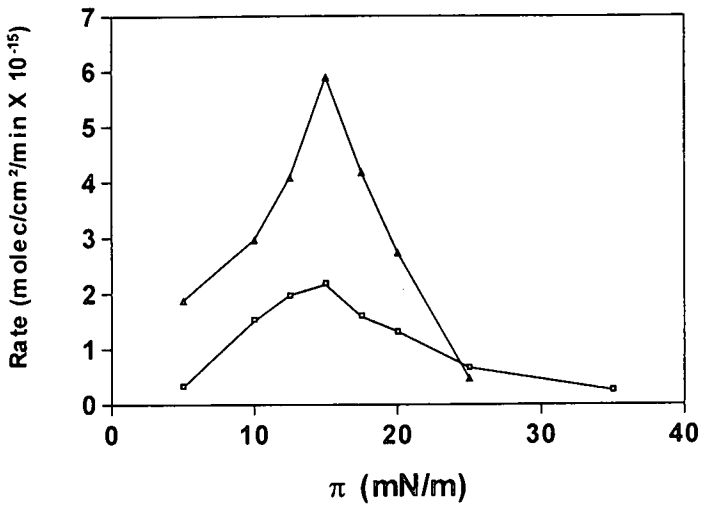


Figure 4.12: Surface pressure profiles of *Aspergillus niger* lipase using triglyceride analogs (R_1 (▲) and S_1. (▣)).

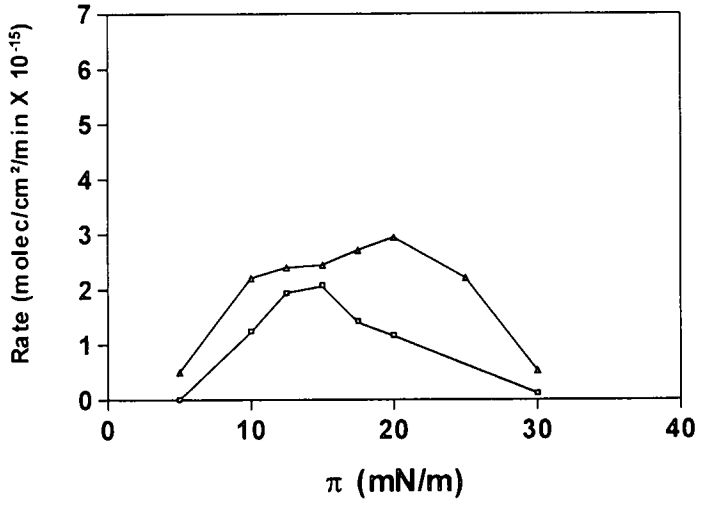


Figure 4.13: Surface pressure profiles of *Aspergillus niger* lipase using triglyceride analogs (R_2 (▲) and S_2 (■)).

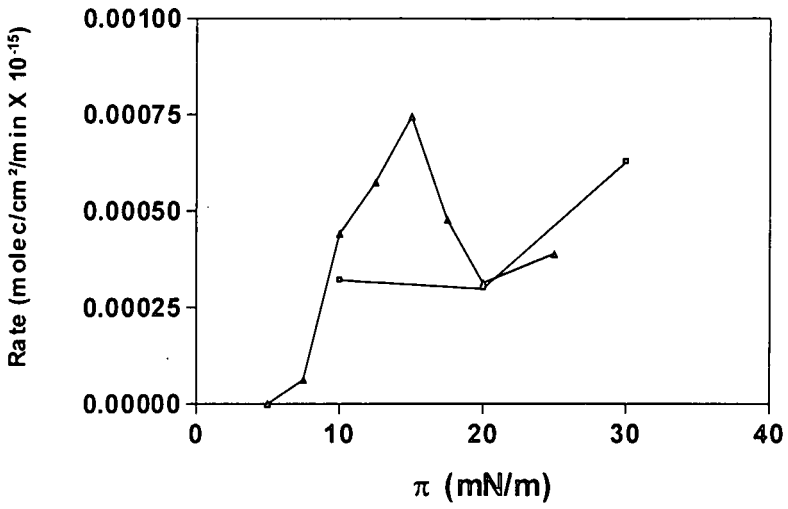


Figure 4.14: Surface pressure profiles of *Aspergillus niger* lipase using triglyceride analogs (R_3 (▲) and S_3 (■)).

The bell-shaped activity profile with an optimum around 15-23 mN/m is well known for enzymes acting on electrically neutral substrate films (Rietsch *et al.*, 1977).

The R-configuration is always preferentially hydrolysed. The hydrolysis rates of the R_1/S_1 and R_2/S_2 substrate couples clearly indicate a stereopreference for the hydrolysis of a primary ester on position 1. A striking stereoselectivity is observed for the R_3/S_3 couple, containing a secondary ester bond (Figure 4.14). Note the 10 000 fold difference in the ordinate scaling of R_3/S_3. The fungal lipase shows a strong stereopreference for the R-isomer. The profound preference for the R_3-isomer, as compared to its optical antipode, appears to be surface dependent. The rates are however very small and the significance of this remains questionable. One can also observe a shift of *sn*-1 (R_1/S_1) to *sn*-3 (R_2/S_2) over increasing surface pressure (Figure 4.15).

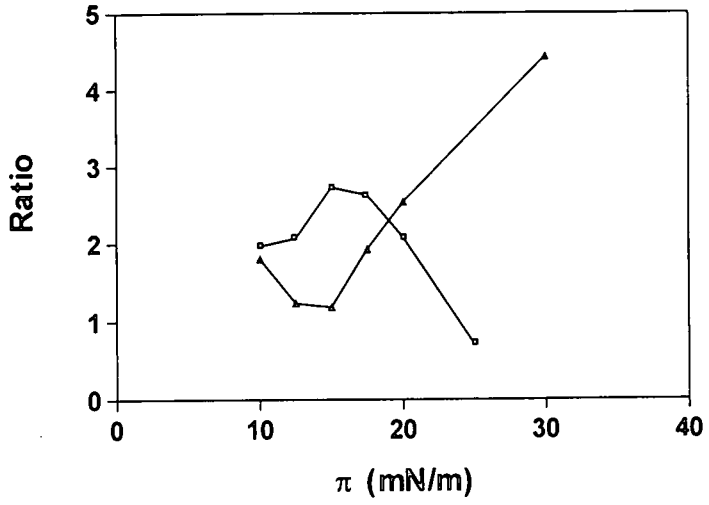


Figure 4.15: The relative ratio of hydrolysis for the *sn*-1 (R_1 and S_1) (□) and *sn*-3 (R_2 and S_2) (▲) isomers.

The determination of the positional- and stereospecific preference of lipase acting on triglycerides is subject to several problems. Enantiomeric triglycerides containing three different acyl groups are in principle ideal substrates to investigate the properties of lipases. However it remains a problem that the hydrolysis products of these compounds are substrates as well. In addition to this, lipases might exert a certain degree of fatty acid specificity. In this study we wanted to investigate whether synthetic triglyceride analogs containing only one single hydrolysable acyl ester bond might be useful to rapidly screen positional specificity of a lipase, without the problem of acyl migration. An additional problem associated with the use of pseudolipid substrates is that an amide or ether bond instead of the usual ester could have a distinct effect on the interaction between enzyme and substrate as the exact stereochemical configuration of these linkages is not identical (Figure 4.16).

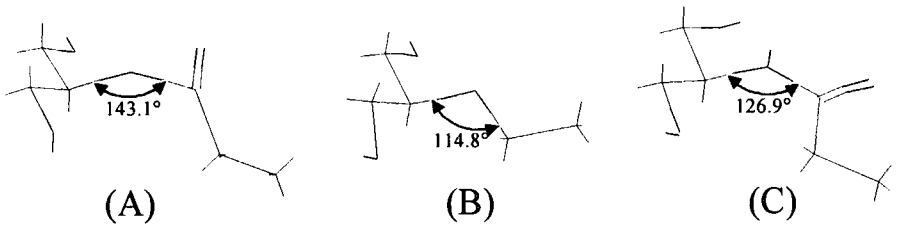


Figure 4.16: Structures of *sn-2* pseudolipids showing the difference in angles between an ester (A), ether (B) and amide (C) linkage. The models were built using Hyperchem software and the geometry optimised by using the Polak-Ribiere conjugate gradient method.

In another study Rogalska *et al.,* (1995) presented the results of an extensive comparative study on the stereoselectivity of lipases. Contrary to previous studies on lipase-acylglycerol chiral recognition where racemic or prochiral substrates were used, the substrates chosen here were three optically pure dicaprin isomers. The monomolecular film technique was chosen as a particularly appropriate method for use in chiral recognition studies (Böhm *et al.,* 1993). To establish the effects of the surface pressure on the stereochemical course of the enzyme action they used optically pure dicaprin enantiomers 1,2-*sn*-dicaprin, 2,3-*sn*-dicaprin, and 1,3-*sn*-dicaprin, spread as monomolecular films at the air/water interface as lipase substrates. One could also evaluate other characteristics of the lipase with dicaprin substrates. In this study, the same substrates were used.

The former two isomers are optically active antipodes (enantiomers), forming stable films up to 40 mN/m, while the latter one is a prochiral compound, which collapses at a surface pressure of 32 mN/m (Figure 4.17).

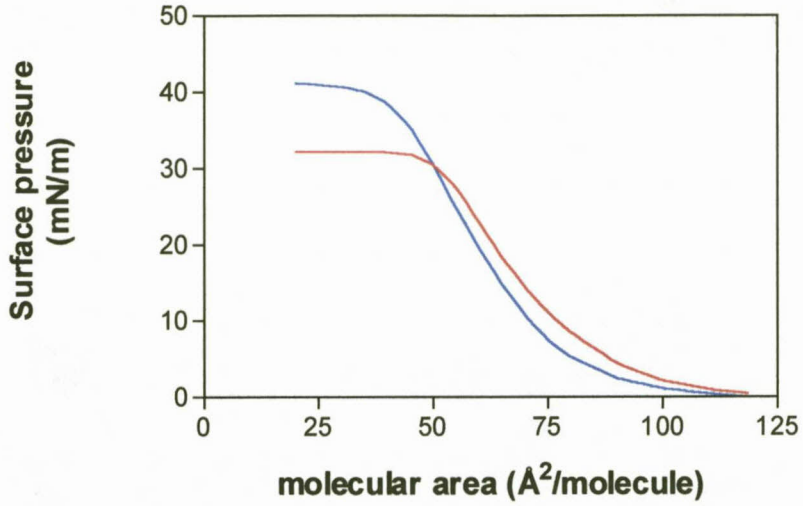


Figure 4.17: Surface pressure versus molecular area in monomolecular films. (*sn*-1,2-dicaprin or *sn*-2,3-dicaprin (---) and *sn*-1,3-dicaprin (---)).

In this study the results show that the regioselectivity, as well as the stereoselectivity, which are the main factors involved in lipolytic catalysis of triacylglycerols, are surface dependent. The lipase from *Aspergillus niger* displayed typical behaviour, which allowed the author classify the lipase into a group (*sn*-1 and R-isomer hydrolytic preference) as defined by Rogalska et al. (1995), on the basis of the enzyme velocity profiles as a function of surface pressure. Their preference for a given diacylglycerol isomer, quantified using parameters coined as the stereoselectivity index (S.I.) and vicinity or regioselectivity index (V.I.) given in Equations 4.7 and 4.8 respectively and the surface pressure threshold, above which the enzymatic activity becomes detectable, were determined.

$$S.I.= {(A_{2,3} - A_{1,2})}\Big/{(A_{2,3} + A_{1,2})} \qquad [4.7]$$

$$V.I.= {[A_{1,3} - 0.5(A_{2,3} + A_{1,2})]}\Big/{[A_{1,3} + 0.5(A_{2,3} + A_{1,2})]} \qquad [4.8]$$

$A_{x,y}$ are the measured rates at identical surface pressures for the diacylglycerol substrates where X and Y indicate the position of the fatty acid.

The general finding was that the three substrates are clearly differentiated, and the differentiation is more pronounced at high interfacial energy (low surface pressures).

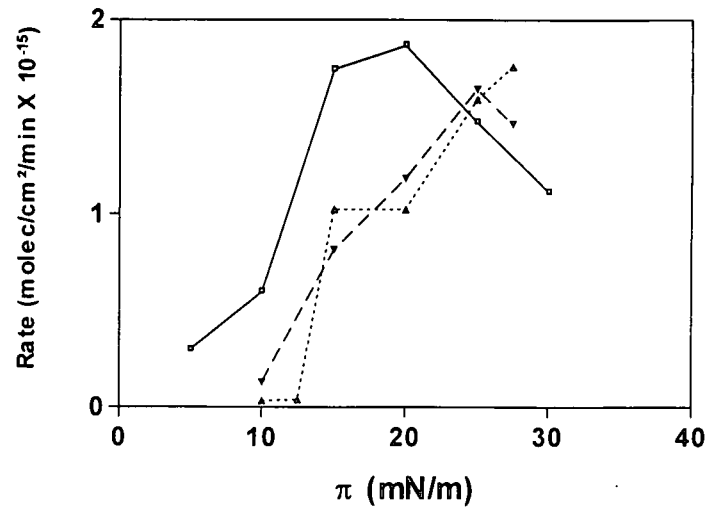


Figure 4.18: Surface pressure profiles of *Aspergillus niger* lipase using dicaprin substrates. (*sn*-1,2-dicaprin (■), *sn*-2,3-dicaprin (▽) and *sn*-1,3-dicaprin (△)).

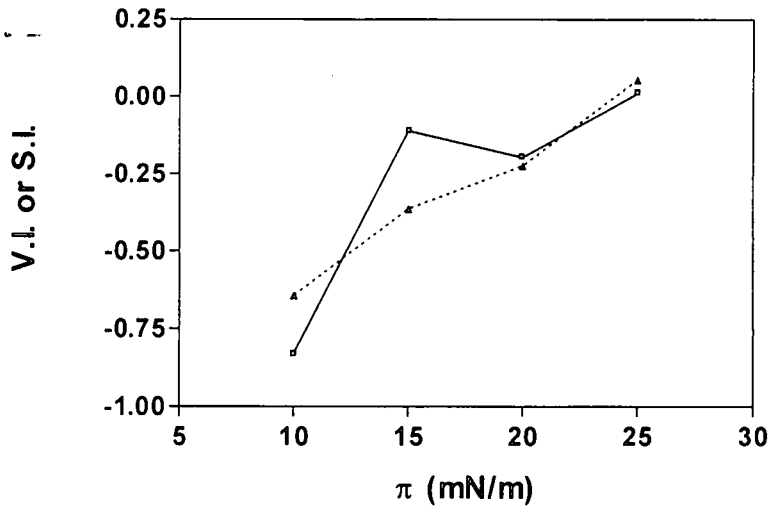


Figure 4.19: The V.I. or S.I. versus surface pressure as calculated from the specific activities measured at different surface pressures respectively.

To describe quantitatively the lipase preference for the distal versus adjacent ester groups of diglyceride isomers, the autor used the V.I. as described previously. These values at different surface pressures were calculated. At low surface pressures the lipase shows an absolute preference for adjacent ester groups, i.e. the 1,3-*sn*-dicaprin is not hydrolysed at all. One can speculate that in the case of 1,3-diglycerides, the "tuning fork" conformation (Grochulski *et al.,* 1994) is responsible for the lack of enzyme recognition. No steric difficulties were encountered in modelling either an *sn*-1,3 or *sn*-2 diglyceride into the *Candida rugosa* binding site by these authors as long as the substrate was in the tuning fork conformation. At high surface pressures when the substrate is less mobile and the fatty acids are forced into the tuning fork conformation, the enzyme is not totally regioselective.

The term S.I., calculated by analogy to enantiomeric excess, was desribed previously (Rogalska *et al.,* 1995). The AnL displayed a preference for the 1,2-*sn*-dicaprin as substrate. However, this stereoselectivity is less pronounced at high surface pressures. At high surface pressures the author observed a slight reverse in stereoselectivity. This is an interesting observation, however unexpected in terms of a simple reaction scheme.

It is striking to note that the stereopreference for either *sn*-1 or *sn*-3 position on glycerides is generally maintained in the case of both di- and triglycerides (Rogalska *et al.,* 1995). Diglycerides are products from triglyceride hydrolysis and are also subsequent substrates giving rise to monoglycerides. One could imagine that the diglyceride resulting from triglyceride hydrolysis could remain preferentially bound in the active site cleft and be reorientated for a subsequent catalytic event. If this were true, one would expect a reversal of stereopreference between di- and triglycerides, which is not the general case. Thus one has to imagine that the intermediate diglycerides are released from the active site between each catalytic step, favouring the transient accumulation of diglycerides.

The first hypothesis, proposed by Hughes (1935) and supported by later workers (Murderhwa and Brockman, 1992) was that a packing-dependent orientation of the substrate may be one of the factors on which the regulation of lipolysis depends.

Another interpretation was put forward by Esposito et al. (1973) who explained the surface pressure optima in terms of changes in lipase conformation upon adsorption at the interface, resulting in an optimal conformation at intermediate values of the interfacial free energy (film pressure). It was suggested that lower and higher values of surface pressure would lead to inactive forms either because of denaturation or because the conformational changes in the enzyme are not sufficiently marked. This view was challenged by Verger and de Haas, (1976) and Pattus *et al.,* (1979). Using radiolabelled enzymes, these authors showed that the observed maxima in the velocity-surface pressure profile disappear when they correlated with the interfacial excess of enzyme. Under bulk conditions the adsorption of nearly all the enzyme occurs at the interface, whereas with a monolayer only one enzyme molecule out of a hundred may be at the interface. Owing to this situation, a small but unknown amount of enzyme, responsible for the observed hydrolysis rate, is adsorbed on the monolayer. Therefore it is necessary to recover and measure the quantity of enzymes adsorbed at the interface before quantifying the activity (Aoubala *et al.,* 1995). This can be achieved by transferring the monolayer to hydrophobic paper and assaying the adsorbed enzyme titrimetrically (Momsen and Brockman, 1981) or by various radiolabelling techniques.

Generally speaking, the stereo preference for either position *sn*-1 or *sn*-3 on acylglycerols is maintained in the case of both tri- and diacylglycerols. This might be due to the fact that the assumed lipase conformational changes involving the active site are occurring on the enzyme/interface interaction. Rogalska et al. (1993) showed that low surface pressures enhance stereoselectivity, while decreasing catalytic activity.

The AnL does not differentiate between the *sn*-2,3- and *sn*-1,3 dicaprin isomers, which are better substrates than the clearly discriminated *sn*-1,2 dicaprin isomer (Figure 4.18). The comparison of the kinetic behaviour of the lipase led to an interesting observation, namely at low surface pressures (<12 mN/m) this enzyme selectively catalyses the hydrolysis of only one of the three isomeric substrates (1,2-*sn*-dicaprin). At higher surface pressures (15 - 25 mN/m), total selectivity was never observed. This supports the author's previous hypothesis that lipase

conformational changes resulting from the enzyme-surface interaction affects the enzyme's specificities. This phenomenon is more pronounced at high interfacial energy i.e. high surface tension (low surface pressure). Surface tension can be defined as the free energy per unit area of surface, or the work that must be done to increase the area of the surface (Adam, 1968).

Results of crystallographic studies (Brzorowski *et al.,* 1991) as well as NMR, UV, and fluorescence spectroscopy data (Deveer *et al.,* 1992; Luthi-Peng and Winkler, 1992), show the ability of lipolytic enzymes to undergo conformational changes upon lipid binding. Assuming that the first step in interfacial catalysis, i.e. lipid binding, is not stereoselective, then the inverse correlation observed between catalytic activity and the S.I. (Figures 4.18 and 4.19), depending on the surface pressure can be explained as follows. The presumed surface pressure-dependent enzyme conformational changes (Verger, 1980) may result in a deterioration of the molecular recognition of both enantiomers due to progressive loss of the residue-specific interactions and the concomitant decrease in catalytic activity at low surface pressures. The deteriorating molecular recognition will, however, have a relatively stronger destabilising effect in the already less well fitted enantiomer, which may then lose the catalytically efficient orientation before its antipode does so, thus enhancing the reaction stereoselectivity.

Rogalska *et al.,* (1995) reported that the surface pressure threshold (S.P.T.), above which the enzymatic activity becomes detectable, differed significantly for different lipases. The AnL was not active at surface pressures below 10mN/m except with 1,2-*sn*-dicaprin, but other lipases were active with all three substrates even below 2 mN/m. The classification of lipases according to S.P.T. can be of practical importance when selecting an enzyme to withstand the presence of tensioactive agents, i.e. lipases for use in detegents. The lipases already selected for these latter types of application (*Humicola langinosa* lipase from Novo-Nordisk) are ranked among the enzymes presenting the lowest S.P.T. The difference in S.P.T. between the three dicaprin isomers might be explained, by assuming that the conformations of the interfacial enzyme/substrate complexes $E^*S_{1,2}$, $E^*S_{2,3}$, and $E^*S_{1,3}$ are different at a given surface pressures. In this case one might refer to a substrate (isomer) stereochemiscal-dependent unfolding of the lipase.

Here the S.P.T. would reflect the stability limits of the three interfacial enzyme/substrate complexes ($E^*S_{1,2}$, $E^*S_{2,3}$, and $E^*S_{1,3}$). One can imagine, however, an alternative and nonmutually exclusive hypothesis: the gradual lipase conformational changes, leading eventually to denaturation with decreasing surface pressure, may proceed in the same way for all three monomolecular films and be solely surface energy dependent. Then, the various S.P.T. values, characteristic for each isomer, may reflect the ability of these isomers to fit in the lipase chiral recognition center at a given conformation corresponding to a given interfacial energy.

4.5 Interaction of lipase with pure egg phosphatidylcholine monolayers

Lipases usually cannot hydrolyse egg lecithin or ester bonds in the *sn*-2 position on triacylglycerols. For this reason surface interactions between the lipase, egg lecithin and pseudolipid S_3 (*sn*-2 ester bond) monolayers can be studied using non-catalytic enzyme concentrations in order to record the variations of surface pressure upon injection of lipase underneath the lecithin monolayer.

The initial surface pressure was measured (5 - 30 mN/m), the enzyme injected under the lipid layer and the increase in surface pressure noted. In these experiments the Gruyere trough, which contains a number of round wells, was used. The volume of the hole was 5ml and the amount of enzyme injected, 200 µl (20.5 µg).

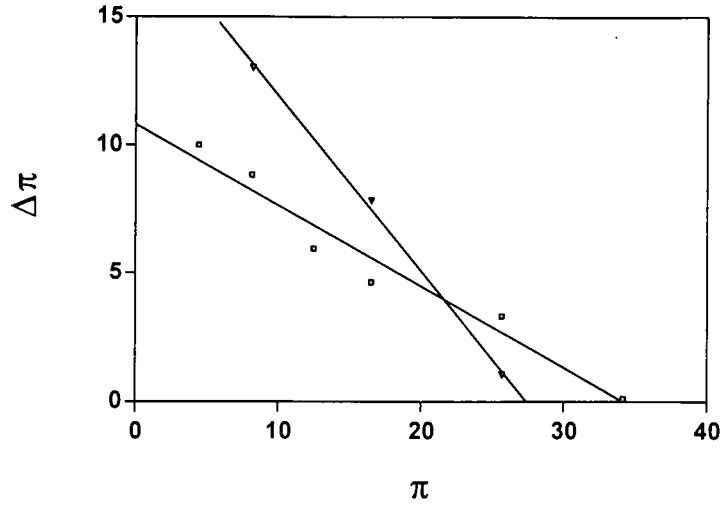


Figure 4.20: Change in surface pressure ($\Delta\pi$) with increasing initial surface pressure upon injection of *Aspergills niger* lipase into unhydrolysable monolayers. (S3 pseudolipid (▽) and egg-phosphatidylcholine (▫)).

With zwitterionic egg phosphatidylcholine monolayers a good correlation is observed between the surface and $\Delta\pi$ (Figure 4.20), in accordance with previous studies (Teissiè, 1981; Mayer *et al.*, 1983). The decrease in surface pressure ($\Delta\pi$) reflects the protein's ability to penetrate or adsorb to the lipid surface. The enzyme does not hydrolyse the phospholipid or the S3 pseudolipid, so noncatalytic concentrations of enzyme can be used to study this phenomenon. The phosphatidylcholine however forms a zwitterionic monolayer which is not necessarily comparable with that formed by a neutral lipid. Although only three data points could be obtained using the pseudolipid, a decrease in $\Delta\pi$ is also observed, indicating that the decrease in reaction rate at high surface pressures is because of the enzyme's decreasing ability to penetrate/adsorb the lipid surface. In the case of S3, the failure of the enzyme to catalyse the hydrolysis of the ester bond is clearly not due to a failure to attach to or penetrate the lipid layer but rather an inability to bind the substrate molecule and to hydrolyse the ester bond.

4.6 The oil-drop tensiometer for studying lipase action

Numerous techniques are available for measuring lipase activity. These can be classified into three groups on the basis of the substrate consumption, the product formation, or the changes with time of one physical property, such as the conductivity, turbidity, or interfacial tension.

Among the interfacial methods, monomolecular film technology at the air/water interface has been extensively developed. Nevertheless, the question remains as to whether the behaviour of the monomolecular lipid film at the air/water interface is actually representative of what is occurring at an oil/water interface. Nury *et al.*, (1987) established that one can gain unique information by measuring the variations in the oil/water interfacial tension ($\gamma_{o/w}$) as a function of time during lipase hydrolysis.

These authors adapted the well-known *oil-drop method* for use in studying the rate of lipase hydrolysis of natural long-chain triacylglycerols. The accumulation of insoluble hydrolysis products at the surface of the drop is responsible for the $\gamma_{o/w}$ decrease, which in turn is correlated with changes with time in the oil-drop profile. The theoretical basis of the calculation of $\gamma_{o/w}$ from a hanging drop profile, using the Laplace equation, has been extensively described in the physics literature since 1938 (Andreas *et al.*, 1938; Girault *et al.*, 1984; Cheng *et al.*, 1990).

Nury *et al.*, (1987) demonstrated that this method constitutes a reliable, sensitive, and convenient means of investigating lipase kinetics by taking oil-drop pictures to determine the interfacial tension from the accurately measured diameters of the drops. The main drawbacks of this technique were the lengthy film processing and profile analysis and the fact that it did not yield real-time measurements. Nury *et al.*, (1991), Grimaldi *et al.*, (1991) and Cagna *et al.*, (1992) developed a new setup whereby the oil-drop profile was automatically digitized and analyzed by image processing; the interfacial tension was calculated in real time using the Laplace equation. The oil-drop methodology can be used to monitor lipase kinetics by following the decrease of interfacial tension due to the lipase action with time. It is

possible to monitor accurately the lipase kinetics by increasing the drop volume to keep $\Upsilon_{o/w}$ constant.

4.7 Oil-drop technology

4.7.1 Automatic oil-drop tensiometer

Figure 4.21 shows a diagram of the experimental setup. An intergrating sphere light source (2), a thermostatted cuvette (3) containing the oil drop within a water phase, and a charge coupling device (CCD) camera attached to a telecentric lens (6) are aligned on an optical bench (1). A drop of liquid A is delivered from a microsyringe filled with liquid A (4), controlled by a dc motor drive (5), into a 25 °C thermostatted optical glass cuvette (1 x 2 x 4.3 cm, Hellma, France) containing liquid B (3). The microsyringe is attached, through a Luer-lock device, to a U-shaped stainless steel laboratory pipetting canula (14 G x 4-inch, Becton-Dickinson, Franklin Lakes) with a flat-cut tip having an external and internal diameter of 2 and 1 mm, respectively.

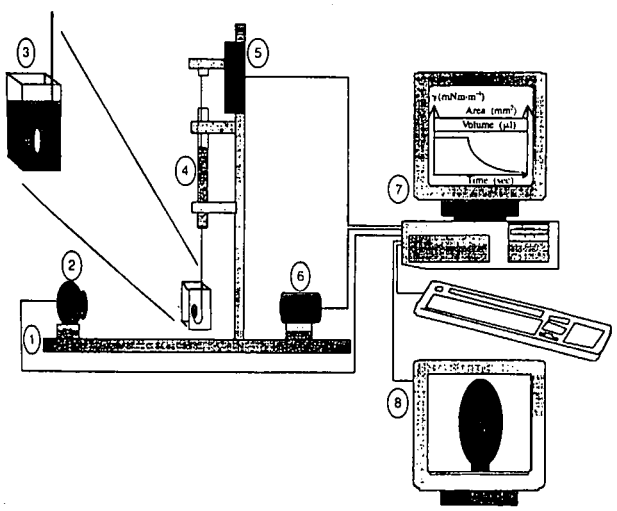


Figure 4.21: Diagram of the experimental set-up. The different components are discussed in the paragraph preceding the figure (Taken from Labourdenne *et al.*, 1997).

Depending on the volumetric masses of the two fluids, the drop is either mounting (e.g. oil drop in water) or hanging (e.g. water drop in oil). The equatorial drop diameters range from 4 to 6 mm. After formation of the drop, its profile is digitized and analyzed through the CCD camera coupled to a video image profile digitizer board (Imaging Technology, model PC Vision Plus) connected to an IBM-PC compatible microcomputer with S-VGA screen (7). To retain permanent visual control, the drop image is continuously visualized on a video monitor (8). The drop profile is processed according to the fundamental Laplace equation applied to the oil-drop profile (Labourdenne *et al.*, 1994). The computer calculates three characteristic parameters of the drop, namely, the area, volume, and interfacial tension ($\gamma_{o/w}$). The average standard accuracy of the interfacial tension measurements is roughly 0.1 mN/m. A typical example of the change in the oil drop profile with time is given in Figure 4.22.

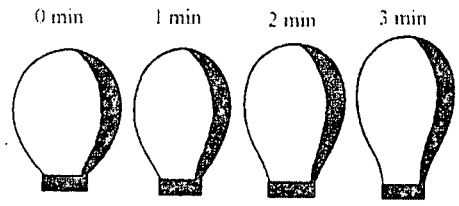


Figure 4.22 Variation in the shape of an oil drop with time resulting from the action of a purified pig pancreatic lipase (Taken from the Labourdenne *et al.*, 1998).

The control unit can work according to one of several modes:

1. Record and plot the changes in $\gamma_{o/w}$ as a function of time (nonregulated mode)
2. Maintain $\gamma_{o/w}$ at a constant endpoint value (barostatic regulation mode) by exerting a feedback regulation on the d.c. motor that automatically increases the drop volume with time. This possibility is of interest for investigating the interfacial tension-dependent phenomena occurring at the interface during kinetic experiments (Labourdenne *et al.*, 1994).

3. An endpoint value of volume or area can be defined by the operator and the control is automatically performed by the control unit. This possibility is of particular interest in following the mass transfer phenomena through the interface.

4.8 Preparation of materials

Cleanliness of materials is a strict requirement for the oil-drop methodology to obtain reliable results. As for the monolayer technique, the water used for washing and buffer preparation was distilled and filtered on a Super Q Millipore filter. Glassware was cleaned with a sulfochromic mixture, then rinsed with specially purified water to wipe out any trace of tensioactive agents that could affect interfacial tension measurements. Before each experiment, the glass cuvette designed to contain the oil drop within a water phase was carefully washed with alcohol then rinsed with tap water and purified water. The syringe and the U-shaped stainless steel needle were sequentially washed with chloroform, methanol, purified water, and once again with methanol and chloroform. Both syringe and needle were dried using filtered compressed air.

4.9 Principle of studying lipase kinetics with the oil-drop tensiometer

Edible soybean oil from Lesieur Company, containing linoleic acid (>50 %), linolenic acid (>9 %), and less than 0.1 % free fatty acids was further purified through a column of silicic acid (Merck) equilibrated in hexane-ethylether. The oil-drop methodology requires the oil to be carefully freed from any natural tensioactive compounds such as free fatty acids, diglycerides, and monoglycerides. As a matter of fact, those contaminants would lower the interfacial tension values because of their amphipathic character.

A drop of 20-100 µl of purified soybean oil is delivered from the microsyringe through the U-shaped stainless steel needle into the thermostatted cuvette containing 5 ml of an aqueous buffer and continuously stirred with a small magnetic bar. The lipase samples are injected into the aqueous phase (using Hamilton syringes) after or before the formation of the oil drop. Soybean oil mainly contains unsaturated long-chain fatty acids, which are released by the enzyme's action at the oil/water interface. The interfacial tension decreases on the accumulation of lipolytic products at the oil/water interface because of their amphipathic character.

A typical profile obtained during the course of an experiment is given in Figure 4.23. The interfacial tension decreases following the lipase injection (first arrow) and the barostatic regulation (second arrow) results in a linear increase of the drop volume and area. One can therefore monitor lipase kinetics by measuring the increase in area (dA) as a function of time to keep $\gamma_{o/w}$ constant. Throughout the kinetic process, the lipase therefore acts on triglyceride substrate molecules present at a preset and constant interfacial composition.

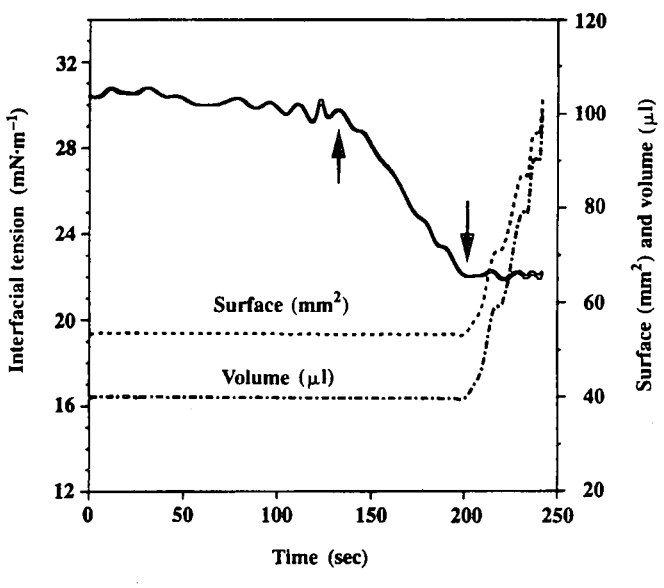


Figure 4.23: A typical kinetic recording of interfacial tension, drop volume, and area during the lipolysis of a mounting soybean drop. The first arrow marks the injection of the lipase solution and the second arrow indicates the beginning of barostatic regulation (Taken from Labourdenne *et al.*, 1997).

The total amount of lipolytic products appearing per time unit (d*n*/d*t*) at the surface of a single oil drop is equal to the oil-drop surface increase (dA) multiplied by the surface density of the lipolytic products (Γ), as shown in Equation 4.9.

$$\frac{dn}{dt} = \frac{dA}{dT} \times \Gamma \qquad [4.9]$$

Γ is, however a difficult parameter to determine and was not determined for this experiment.

Figure 4.24 shows the kinetics of the lipolysis of a mounting soybean oil drop formed in 5 ml of an aqueous phase (10 mM Tris-HCl buffer, pH 5.5) where the AnL (1.5 Units) sample was injected. Interfacial tension ($\gamma_{o/w}$) was recorded as a function of time. The decrease in $\gamma_{o/w}$ is due to formation of hydrolysis products at the interface.

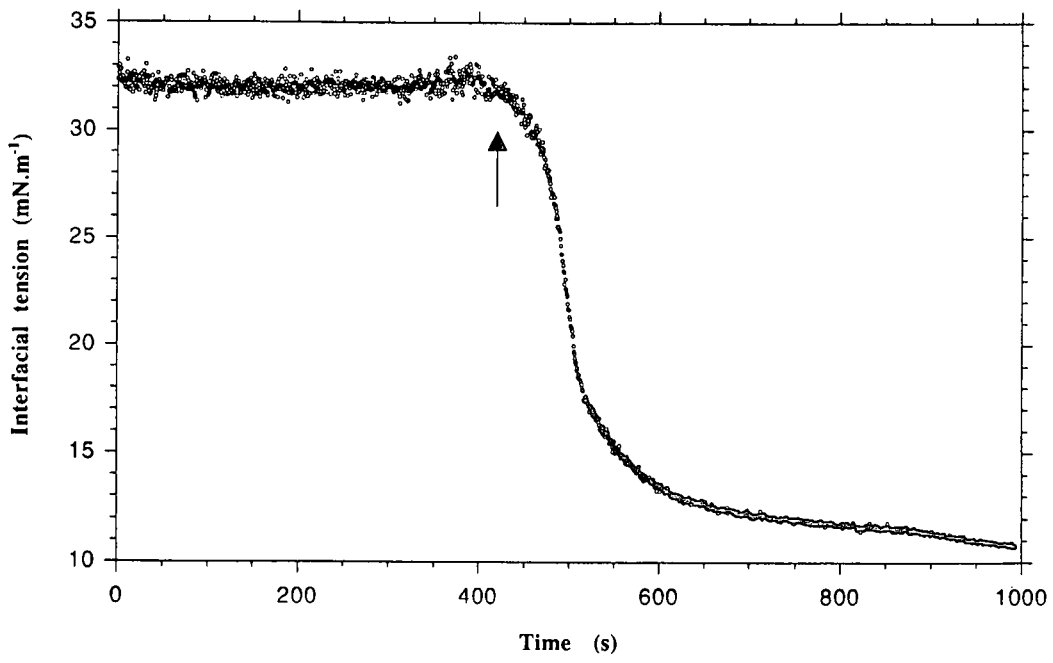


Figure 4.24 A kinetic recording of the reduction of interfacial tension during a lipolysis reaction with the lipase from *Aspergillus niger* (1.5 U/ml). The vertical arrow marks the injection of the enzyme solution.

A series of video photographs of the interfacial changes during hydrolysis was taken (Figure 4.25) over an extended period of time. The variation in shape is a result of the change in oil/water interfacial tension resulting from the accumulation of water insoluble lipolytic products in the surface of the triglyceride oil drop.

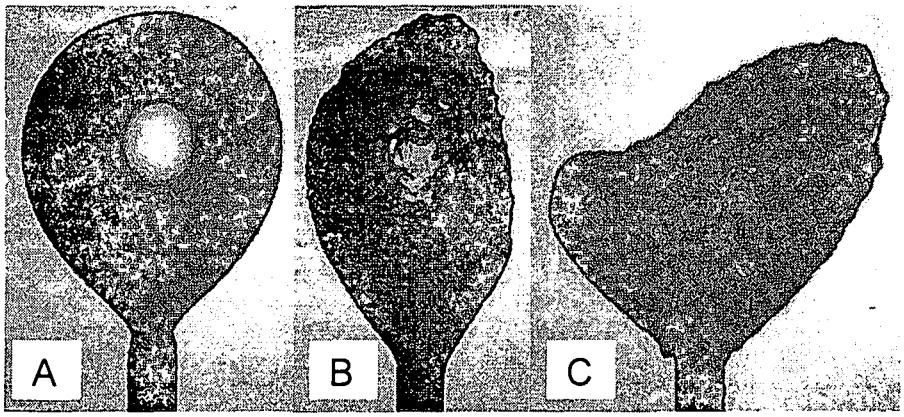


Figure 4.25: A series of photographs to illustrate the change in the interface, during the lipolysis reaction of *Aspergillus niger* lipase. (A) at time zero, (B) after 30 min and (C) after 120 min of hydrolysis.

The activity of AnL was compared to that of the human pancreatic and gastric lipases, which were extensively characterised by the research group of Verger. Subjecting identical amounts of these enzymes to the oil droplet tensiometer one could detemine the activities of the three enzymes. The HPL displayed the lowest ability to hydrolyse the oil at the interface with a slope of –4.2, while the HGL was more active with a comparative value of –9. The lipase from *Aspergillus niger*, however catalysed the hydrolysis of the oil with a resulting slope of –14.5. The data are however only an indication of the relative activities as corrections for the small changes in $\gamma_{o/w}$ due to adsorption of the protein onto the interface could not be made.

4.10 Conclusions

The literature reported various deviations in activity measurements that might be observed as misconceptions such as "interfacial activation" (Sarda and Desnuelle, 1958) and/or conformational changes (Desnuelle *et al.*, 1960). Now it is quite clear that if the substrate presentation is controlled the lipases' activity must correlate (Ferrato *et al.*, 1997).

Recent lipase research has nevertheless shown how careful one has to be about extrapolating any kinetic and/or structural characteristics observed for lipases in general. The catalytic activity of many lipolytic enzymes has been measured using carboxylic esters that are partly soluble in water, and many differences have been found between the resulting profiles.

Therefore extreme caution is necessary when performing and interpreting enzymatic measurements with lipids. It is essential to check that the initial lipase velocity is proportional to the amount of enzyme used, both below and above the solubility limit of the substrate. Because the medium is heterogeneous, adding any amphiphilic compound is liable to modify both quantitatively and qualitatively the physicochemical properties of the interface. Consequently, it is impossible to assess experimentally what interfacial activation may have occurred with substrates of this kind.

In addition, some esters that are partly soluble in water sometimes form monomolecular adsorption films on the surface of the air bubbles produced by stirring of the reaction mixture. This artefact is responsible for the great disparity found among initial velocity measurements when mechanical stirring methods of different efficiency are used.

A typical activity profile which lipase researchers can obtain is seen in Figure 4.26. The experiment was evaluated with the pH stat method, as described in Section 3.9.2, but the triacylglycerol concentration was varied for each assay.

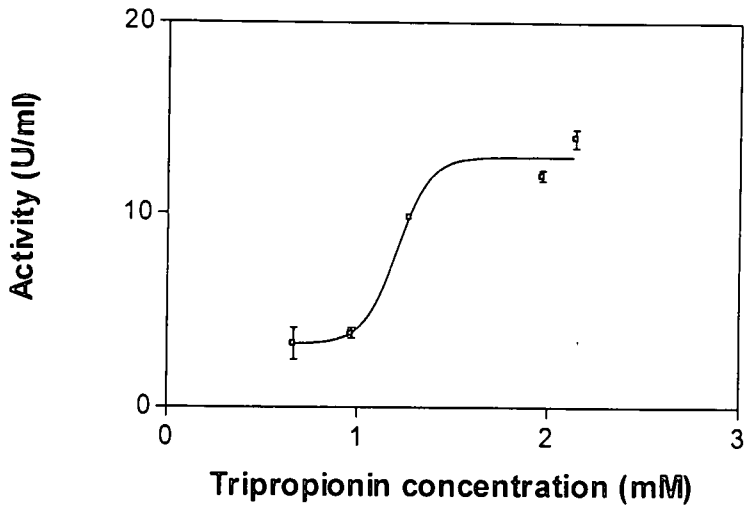


Figure 4.26: A typical curve which would suggest interfacial activation with lipases, but is simply an artefact of experimental procedure.

From the results displayed in Figure 4.26 the initial velocity of the enzyme can not be reported. In addition, when researchers found that the enzyme they were investigating displayed a similar action, there have often been reports that suggested that this phenomenon should be used as a criterium to classify the enzyme as a lipase, and even more daring, that that particular lipase has a lid domain.

One could show a phenomenon such as interfacial activation with tripropionin as substrate if one can avoid the many possible experimental artefacts. One can suggest that the molecular explanations for the "interfacial activation" phenomenon should be investigated not only to the level of the lipase three-dimensional structure, but also explaining the dynamics of organised multimolecular structures as well as in the interfacial conformations (interfacial quality) of lipids used as lipase substrates. Among other physicochemical parameters, one can speculate that the radius of substrates (micelle, emulsion or monolayer) could play an important role during interfacial docking of lipases and hence could modulate the catalytic activity, through subtle conformational changes.

Interfacial activation, as well as the presence of a lid domain. is therefore not in the least appropriate criteria on the basis of which to determine whether such an esterase belongs to the lipase subfamily. Interfacial activation was sometimes wrongly taken as criteria for predicting the existence of a lid domain in lipases with an unknown three-dimensional structure.

For optimal application of lipases it is important, however, to understand their basic properties, e.g. the recognition of lipid-water interfaces. The techniques described in this chapter, monomolecular film and oil drop tensiometer techniques, as well as other titrimetric methods (e.g. pH stat) should all be reliable and comparable techniques when studying lipase kinetics. Lipase activities, which relate to the release of fatty acid per minute per amount of protein from the different assay methods, should compare if the interfacial quality is the same.

Because naturally occurring triacylglycerols are totally insoluble in water, in contrast to short-chain triacylglycerols, interfacial activation can be said to be little more than an artefact that stimulated the imagination of many biochemists, but has not turned out to be of any great physiological significance (Verger 1997). Lipases might therefore be quite pragmatically redefined as carboxyl esterases that catalyse the hydrolysis of long-chain acylglycerols. In fact, they are simply fat splitting "ferments".

Upon my word Watson, you are coming along wonderfully. We have really done very well indeed. It is true that you have missed everything of importance, but you have hit upon the method.

Sir Arthur Conan Doyle

CHAPTER 5

Molecular aspects of *Aspergillus niger* lipase

5.1 General

The great diversity observed in the properties of purified lipases are also reflected in the molecular biology of this class of enzyme. The primary structure of a number of microbial lipases has been determined either by amino acid or nucleic acid sequencing.

Many lipases from microbial sources have been purified and sequenced. Comparison of amino acid sequences between microbial lipases often revealed no detectable similarities beyond the consensus pentapeptide (Section 5.3). The number of amino acid ranges from about 200 too more than 600.

Fungal lipase genes are usually cloned by screening cDNA libraries with either DNA probes deduced from N-terminal sequencing of the purified protein or antibodies raised against the enzyme. Sequence data of cloned genes showed that the microbial extracellular lipases are usually encoded by open reading frames of sizes 930 – 2 900 base pairs, resulting in the enzyme with 135 – 670 amino acids. Signal peptides differ in length from 13 to 40 amino acids.

Fungi sometimes secrete more than one lipase that could be the product of two or more genes. For example, two lipase genes were cloned from *Geotrichum candidum* ATCC 34614 (Shimada *et al.*, 1989 and 1990). Both code for proteins of 544 amino acids and overall identity between them is 84 %. Furthermore, four Cys residues were completely conserved, which may participate in the formation of disulphide bridges. There was some primary structural homology with *Candida*

rugosa lipase. The sequences of several other fungal lipases for example: *Rhizopus niveus, Rhizopus oryzae, Rhizomucor miehei, Fusarium heterosporum, Humicola lanuginosa, Aspergillus oryzae,* and *Penicillium camembertii* etc. are also available.

5.2 Lipases as α/β proteins

Lipases are water-soluble enzymes, but they act on non-soluble substrates. It could be argued that this might impose unique restrictions on the molecular architecture so that these enzymes can be stable in both polar and non-polar environments while undergoing specific structural changes in moving from one to the other. All the lipase enzymes whose three-dimensional structures have been elucidated by X-ray crystallography [*Rhizomucor miehei* (Brady *et al.*, 1990), *Geotrichum candidum* (Schrag *et al.*, 1991), *Candida rugosa* (Grochulski *et al.*, 1993 and Ghosh *et al.*, 1995), *Pseudomonas glumae* (Noble *et al.*, 1993), *Candida antartica* (Uppenberg *et al.*, 1994), *Penicillium camemberti* (Derewenda *et al.*, 1994b), *Humicola Langinosa* and *Rhizopus delemar* (Derewenda *et al.*, 1994c), *Rhizopus niveus* (Kohno *et al.*, 1996) and *Pseudomonas cepacia* (Schrag *et al.*, 1997 and Kim *et al.*, 1997)] are α/β - type proteins with a mixed central β-pleated sheet containing the catalytic residues. The α/β - fold has now been recognised as a general folding pattern for different hydrolases (Ollis *et al.*, 1992) such as acetylcholine esterase (Sussman *et al.*, 1991), serine carboxypeptidase (Liao and Remington, 1990), haloalkane dehalogenase (Franken *et al.*, 1991), and dienelactone hydrolase (Pathak *et al.*, 1988).

5.3 The lipase consensus sequence

The catalytic centre of the lipase contains a serine-protease-like triad consisting of Ser-His-Asp residues, and the active site serine residue is located in a β-εSer-α motif (Derewenda and Derewenda, 1991; Derewenda and Sharp, 1993).

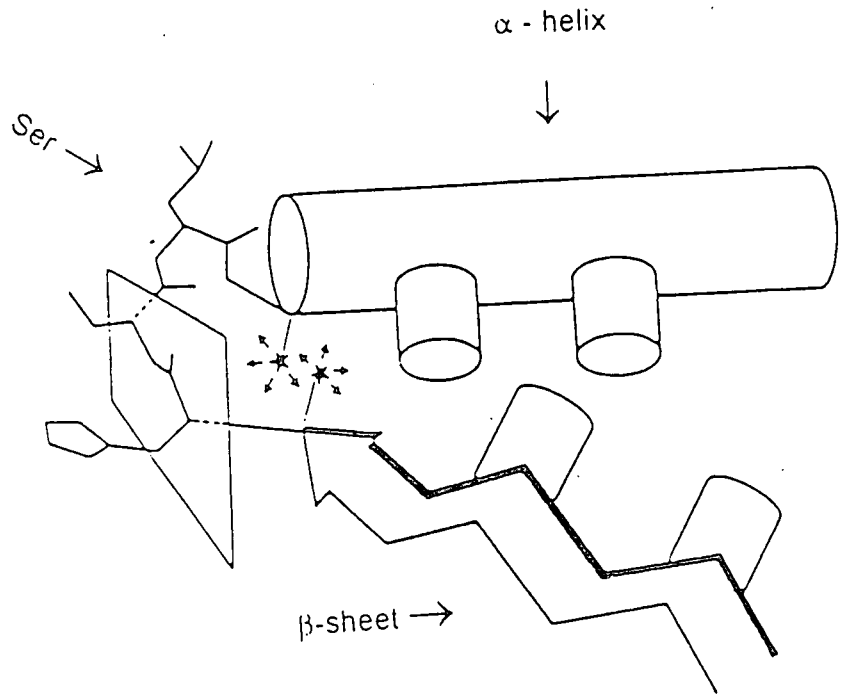


Figure 5.1: A schematic drawing showing the packing within the β-εSer-α motif of some enzymes exhibiting this feature. The helix and strand pack against each other with four amino acids (tinted) forming the interface. The residues nearer the turns are in closer contact and therefore their sidechains are restricted to those of smaller hydrophobic amino acids. The plane of the central peptide of the turn is perpendicular to the axis of the motif, which forces the catalytic Ser to adopt a strained ε conformation. The two stars show the positions that β-carbons of amino acids other than Gly would occupy if the two invariant Gly residues of the GXSXG pentapeptide were mutated (Taken from Derewenda and Sharp, 1993).

This motif consists of a six-residue β-strand and four-residue type II turn with serine in the ε conformation, and a buried α-helix packed parallel against 4 and 5 of the central β-sheet. The first and last glycine residues in the consensus sequence Gly-X-Ser-X-Gly (where X represents any amino acid) of this motif are in extended and helical conformations, respectively, which are conserved because of the steric requirements imposed by the packing stereochemistry of the β-εSer-α motif (Derewenda and Sharp, 1993; Derewenda and Derewenda, 1991). The consensus sequence found in some lipases around the active serine was however not found in *Candida antarctica* lipase (Uppenberg *et al.*, 1994). The sequence around the Ser105 had the highest similarity to the consensus sequence, but the first conserved glycine had been replaced by Threonine to give **Thr**-Trp-Ser-Gln-Gly. The lipases from *Bacillus* strains also appeared to lack the conserved pentapeptide purported to play an essential role in catalysis (Dartois *et al.*, 1992; Möller, *et al.*, 1991). Alignments of several microbial lipase sequences showed significant homology between this conserved peptide and the sequence **Ala**-His-Ser-Met-Gly present in the lipase of *Bacillus subtilis* (Dartois, *et al.*, 1992). The lipase gene of *Bacillus pumilus* described by Möller *et al.*, (1991) has 74 % amino acid sequence homology with that of *Bacillus subtilis*, and the first Gly residue of the conserved sequence is replaced by Ala. It is generally assumed that the central serine is the nucleophilic residue involved in the hydrolytic mechanism (Faustinella *et al.*, 1991) and is part of the catalytic triad Ser-Asp-His equivalent to the active residues seen in the serine proteinases (Brady *et al.*, 1990). The role of the flanking glycines could be less essential in catalysis. These residues may function rather to increase flexibility of this protein region and to reduce steric hindrance in order to optimise accessibility of the catalytic Ser. This hypothesis could explain the relative tolerance of the consensus sequence towards a mutation of the first Gly into Ala. Furthermore, in subtilisin from various micro-organisms, the sequence of the conserved pentapeptide was reported to be Gly-X-Ser-X-Ala (Brenner, 1988).

5.4 The catalytic triad

Determination of the three-dimensional structure of different lipases has confirmed their classification as "serine hydrolases". The active site is composed of three residues; a serine residue hydrogen-bonded to a histidine residue, and a carboxylate residue. In most lipases, the carboxylate residue is an aspartate, but in *Candida cylindracea* (Longhi *et al.*, 1992) and *Geotrichum candidum* (Schrag *et al.*, 1991) and *Galactomyces geotrichum* (Philips and Pretorius, 1991) glutamate replaces aspartate.

The architecture of the catalytic triad of lipases is very similar to the one found in serine proteases (Winkler *et al.*, 1990; Brady *et al.*, 1990). During the reaction a tetrahedral intermediate is formed which decomposes into an acyl-enzyme complex. The free lipase is regenerated by a hydrolytic reaction mediated by a water molecule. Figure 5.2 describes the reaction mechanism of lipases hydrolysing an ester bond. First a nucleophilic attack of the oxygen of the serine side chain on the carbonyl carbon atom of the ester bond leads to the formation of the tetrahedral intermediate (Figure 5.2, Reaction 1). The histidine assists in increasing the nucleophilicity of the serine hydroxyl group. The histidine imidazole ring becomes protonated and positively charged. The positive charge is stabilised by the negative charge of the acid residue (Figure 5.2, Reaction 2). The tetrahedral intermediate is stabilised by two hydrogen bonds formed with amide bonds of residues, which belong to the oxyanion hole. Finally the alcohol is liberated leaving behind the acyl-enzyme complex. (Figure 5.2, Reaction 3). By nucleophilic attack of a hydroxyl ion, the fatty acid is liberated and the enzyme regenerated (Figure 5.2, Reaction 4).

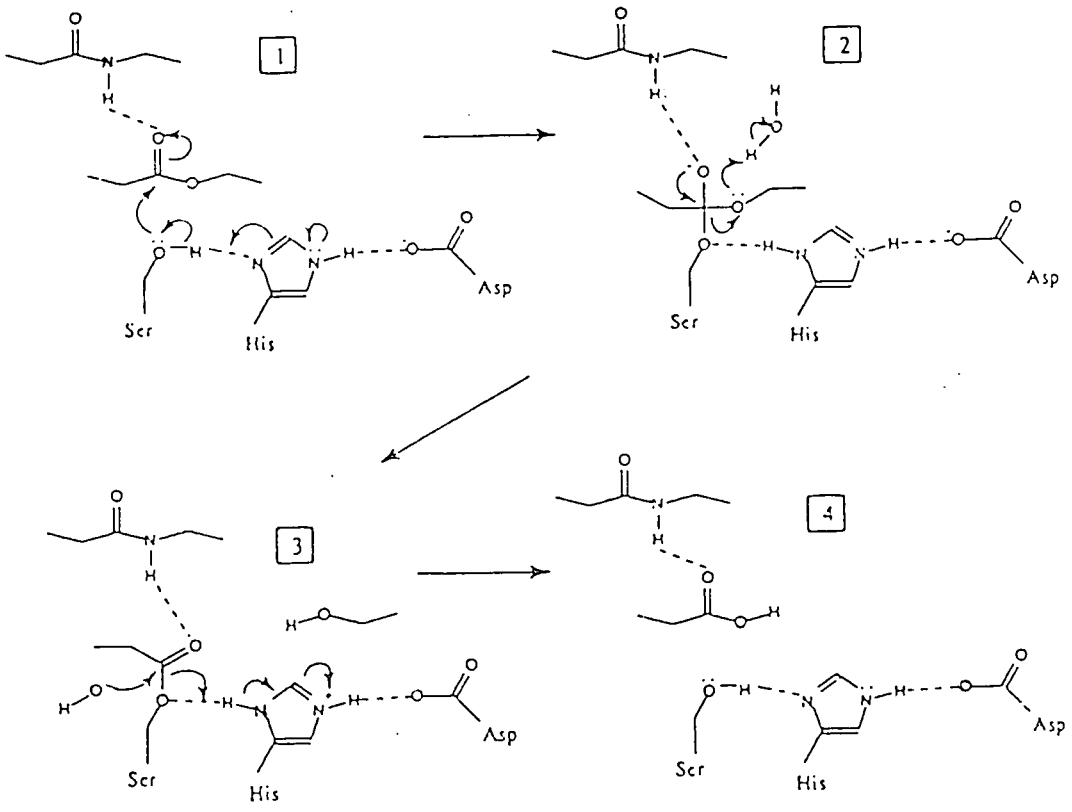


Figure 5.2: Mechanism of hydrolysis of an ester bond by a lipase (Taken from Jaeger *et al.*, 1994).

5.5 Structural relationships between the fungal lipases

When standard alignment routines are applied to lipase sequences, rather inconclusive results are obtained (Svendsen, 1994). The degrees of similarity found within the lipases is very low, the only conserved feature being the "consensus" sequence G-X-S-X-G which is found in many esterases. Even this "consensus' sequence is not conserved. The microbial lipases, and even more surprisingly, the fungal lipases (including yeast) also show very little sequence homology. However, the folding patterns are similar and that the catalytic groups are identical, for many lipases.

An amino acid sequence alignment using Multalin (Corpet, 1988) of the fungal lipases found in the GenBank sequence database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the Swiss-Prot

Databank (http://www.expasy.ch/cgi-bin/sprot-search-ful) is given in Figure 5.3.

The identity of the enzymes is as follows:

CaL = *Candida albicans* lipase LIP1

SpL = *Schizosaccharomyces pombe* chromosome III cosmid lipase sequence

NcL = *Neurospora crassa* lipase cDNA clone W10D7 5'

RnL = *Rhizopus niveus* lipase

TlL = *Thermomyces lanuginosus* lipase

ScL = *Saccharomyces cerevisiae* TGL2

CcL = *Candida cylindracea* lipase I

GcL = *Geotrichum fermentans* lipase I precursor

RnL = *Rhizopus niveus* lipase

PcL = *Penicillium camembertii* mono- and diacylglycerol lipase

AoL = *Aspergillus oryzae* diacylglycerol lipase

FhL = *Fusarium heterosporum* lipase

GcL II = *Geotrichum candidum* lipase II

GgL = *Galactomyces geotrichum* lipase

CcL 5 = *Candida cylindracea* LIP5

CcL 4 = *Candida cylindracea* LIP4

CcL 3 = *Candida cylindracea* LIP3

CcL 2 = *Candida cylindracea* LIP2

CcL 1 = *Candida cylindracea* LIP1

CanL = *Candida antarctica* lipase B

RmL = *Rhizomucor miehei* lipase

YlL = *Yarrowia lipolytica* lipase I

RdL = *Rhizopus delemar* carboxyl ester hydrolase

RdL B = Chain B, Lipase of *Rhizopus delemar*

RdL A = Chain A, Lipase *Rhizopus delemar*

```
                          1                                                50
CaL U34807
CanL Z30645
ScL X98000                                    MG VLHGLTVLDE NGKEKCHRFT GIRYAKPPVG
SpL AL035076                                     GDTITGL NAIIN-EAFL GIPFAEPPVG
CcL X16712
CcL 1 X64703              MELALALLLI ASVAAAPTAT LANGDTITGL NAIIN-EAFL GIPFAEPPVG
CcL 3 X66006 S559397      MKLALALLLI ASVAAAPTAK LANGDTITGL NAIIN-EAFL GIPFAEPPVG
CcL 5 X66008 S55942       MKLALALLLI ASVAAAPTAT LANGDTITGL NAIIN-EAFL GIPFAEPPVG
CcL 4 X66007 S55939       MKLALVLSLI VSVAAAPTAT LANGDTITGL NAIIN-EAFL GIPFAQPPVG
CcL 2 X64704              MKLCL-LALG AAVAAAPTAT LANGDTITGL NAIVN-EKFL GIPFAEPPVG
GfL AB000260
GgL X78032                              MSVTSTSLNG TFNGISEDGI ------EIFK GIKYANR---
YlL Z50020                                    M TLNGNIMKYC LEKGEILISF LLIALESMFR
NcL AI399229                                    MYFPFLGRL SITDYIIV-- VLVYIESIIS
GcL II ATCC34614
RnL AB013496
GRnL D13206
RdL M38352
RdL B 999875
RdL A 999874
RmL A02536
TlL AF054513
PcL D90315
AoL D85895
FhL S77816
Consensus

                          51                                               100
CaL U34807
CanL Z30645
ScL X98000
SpL AL035076              KLRWRRPVTL EDGYDYSGDY NQFKTICPQP FYNNRKNQVR NP--------
CcL X16712                NLRFKDPVPY SGSLD-GQKF TSYGPSCMQQ NPEGTYEENL PKAALDLVMQ
CcL 1 X64703              NLRFKDPVPY SGSLD-GQKF TLYGPLCMQQ NPEGTYEENL PKAALDLVMQ
CcL 3 X66006 S559397      NLRFKDPVPY SGSLN-GQKF TLYGPLCMQQ NPEGTFEENL GKTALDLVMQ
CcL 5 X66008 S55942       NLRFKDPVPY RGSLN-GQSF TAYGPLCMQQ NPEGTYEENL PKVALDLVMQ
CcL 4 X66007 S55939       NLRFKPPVPY SASLN-GQKF TLYGPLCMQM NPLGNWDSSL PKAAINLLMQ
CcL 2 X64704              TLRFKPPVPY SASLN-GQQF TLYGPLCMQM NPMGSFEDTL PKNARHLVLQ
GfL AB000260              FKHPQPF TGSYQ-GLKA NDFSPACMQL DPGNSLTLLD KALGLAKVIP
GgL X78032                FKHPQPF TGTYQ-GLKA NDFSSACMQL NPGNALTILD NALSLASISP
YlL Z50020                RYRWAYAERI DDYDNGVFDC TQEGMACPQV LPFDYNIEKG PK--------
NcL AI399229              ICTVILPSPL RNWFYEQSKK VYSYFLPELL VDDNANKLTD ARDTIDLCAL
GcL II ATCC34614          SVLKLIPQPM INLFEWLIN- -FSTSSDDNT IEE---KLRS APTIHEMCAI
RnL AB013496
GRnL D13206
RdL M38352
RdL B 999875
RdL A 999874
RmL A02536
TlL AF054513
PcL D90315
AoL D85895
FhL S77816
Consensus
```

Figure 5.3: Amino acid sequence alignment of fungal lipases. The accession numbers of the lipases are given in italics. An explanation of the enzyme names is given in the text. Consensus symbols: ! is anyone of IV, $ is anyone of LM, % is anyone of FY, # is anyone of NDQEBZ.

```
                          101                                                 150
                                                                    MQYG APWSTVATQI
CaL U34807                                                          MKLL SLTGVAGVLA
CanL Z30645
ScL X98000
SpL AL035076          ---------- ---DFKYDE- DCLFLNIWVP AGEKPAEGWP VLYFIHGGWL
CcL X16712            SKV------- FEAVSPSSE- DCLTINVVRP PGTKAGANLP VMLWIFGGGF
CcL 1 X64703          SKV------- FEAVLPLSE- DCLTINVVRP PGTKAGANLP VMLWIFGGGF
CcL 3 X66006 S559397  SKV------- FQAVLPQSE- DCLTINVVRP PGTKAGANLP VMLWIFGGGF
CcL 5 X66008 S55942   SKV------- FQAVLPNSE- DCLTINVVRP PGTKAGANLP VMLWIFGGGF
CcL 4 X66007 S55939   SKL------- FQAVLPNGE- DCLTINVVRP LGTKPGANLP VMVWIFGGGF
CcL 2 X64704          SKI------- FQVVLPNDE- DCLTINVIRP PGTRASAGLP VMLWIFGGGF
GfL AB000260          EEFRGPLYDM AKGTVSMNE- DCLYLNVFRP AGTKPDAKLP VMVWIYGGAF
GgL X78032            ENIRGPLYDM AKGSVSMSE- DCLYLNVCRP AGTKPGDKLP VMVWIYGGAF
YlL Z50020            ---------- ---EMPFDEF ECSNLMITRP QG---ATNLP VFVWIHGGGN
NcL AI399229          HGY-----DL EEHFVRTTDG YLLGLHRVYK KKKGKIEELN YLPPVLFIHG
GcL II ATCC34614      FDI-----SV EDHLVRTEDN YILTLHRIPP ISKNRFNN-- --KVVYLHHG
RnL AB013496                                                          M VSFISISQGV
GRnL D13206                                                           M VSFISISQGV
RdL M38352                                                            M VSFISISQGV
RdL B 999875
RdL A 999874                                                                MVLKQRA
RmL A02536
TlL AF054513
PcL D90315
AoL D85895
FhL S77816
Consensus

                                                                              200
                          151
CaL U34807            DMALMVPMLK QGYYVVSPDY EGPKSTFTVG RQSGKATLDS IRAILKSNKF
CanL Z30645           TCVAATPLVK RLPSGSDPAF SQPKSVLDAG LTCQGASPSS VSKPILLVPG
ScL X98000            MKNDN KANDIIIDSV KVPDSYKPPK NPIVFCHGLS GFDKLILIPS
SpL AL035076          QVGNPLHYRQ CDPQDLQ-AD GSPAKFILVS PGHRLNLFGF LAGKELLEED
CcL X16712            EVGGTSTFPP AQMIT-K-SI AMGKPIIHVS VNYRVSSWGF LAGDEIKAEG
CcL 1 X64703          EVGGTSTFPP AQMIT-K-SI AMGKPIIHVS VNYRVSSWGF LAGDEIKAEG
CcL 3 X66006 S559397  EIGSPTIFPP AQMVT-K-SV LMGKPIIHVA VNYRVASWGF LAGDDIKAEG
CcL 5 X66008 S55942   EIGSPTIFPP AQMVS-K-SV LMGKPIIHVA VNYRLASFGF LAGPDIKAEG
CcL 4 X66007 S55939   EVGGSSLFPP AQMIT-A-SV LMGKPIIHVS MNYRVASWGF LAGPDIKAEG
CcL 2 X64704          ELGGSSLFPG DQMVA-K-SV LMGKPVIHVS MNYRVASWGF LAGPDIQNEG
GfL AB000260          VYGSSAAYPG NSYVK-E-SI NMGQPVVFVS INYRTGPFGF LGGDAITAEG
GgL X78032            IFGSSRSYPG NGYVD-E-SV KMGQPVVFVS INYRSGPYGF LGGDGITAEG
YlL Z50020            LAGNGYCSDH NPVPFVKHSI VAGRPVLHVM IEYRLSAFGY LAVPDTNGNW
NcL AI399229          LMMNSESWVC NLKKEDAIPF ALVEQGYDVW LGNLRGNKYS IKNIKFSSQN
GcL II ATCC34614      LLMCSDVWCC NIERHKNLPF VLHDLGYDVW MGNNRGNKYS TAHLNKPPKS
RnL AB013496          SLCLLVSSMM LGSSVVPVSG KSGSSNTAVS ASDNAALPPL ISSRCAPPSN
GRnL D13206           SLCLLVSSMM LGSSAVPVSG KSGSSNTAVS ASDNAALPPL ISSRCAPPSN
RdL M38352            SLCLLVSSMM LGSSAVPVSG KSGSSNTAVS ASDNAALPPL ISSRCAPPSN
RdL B 999875
RdL A 999874
RmL A02536            NYLGFLIVFF TAFLVEAVPI KRQSNSTVDS ------LPPL IPSRTSAPSS
TlL AF054513
PcL D90315
AoL D85895
FhL S77816
Consensus                                  s           v
```

Figure 5.3: Continued

```
                                                                    250
                    201
CaL U34807          TGIKSDAKVA MWGYSGGSLA SGWAAALQPK YAPELKKNLI GAALGGFVTN
CanL Z30645         TGTTGPQSFD SNWIPLSTQL GYTPCWISP- --PPFMLNDT QVNTEYMVNA
ScL X98000          VFHLTNLISN SIVHNMAENF MQDDEDKSDN KYTNLLEIEY WIGVKKFLQS
SpL AL035076        PKSSNFGFWD QRLGLEWTYK HIESFGGNKE NIAVGGISAG SYSALFQLI-
CcL X16712          --SANAGLKD QRLGMQWVAD NIAAFGGDPT KVTIFGESAG SMSVMCHILW
CcL 1 X64703        --SANAGLKD QRLGMQWVAD NIAAFGGDPT KVTIFGELAG SMSVMCHILW
CcL 3 X66006 S559397 --SGNAGLKD QRLGMQWVAD NIAGFGGDPS KVTIFGELAG SMSVLCHLIW
CcL 5 X66008 S55942 --SSNAGLKD QRLGMQWVAD NIAGFGGDPS KVTIFGELAG SMSVLCHLLW
CcL 4 X66007 S55939 --SGNAGLHD QRLGLQWVAD NIAGFGGDPS KVTIFGELAG SMSVMCQLLW
CcL 2 X64704        --SGNAGLHD QRLAMQWVAD NIAGFGGDPS KVTIYGELAG SMSTFVHLVW
GfL AB000260        --NTNAGLHD QRKGLEWVSD NIANFGGDPD KVMIFGESAG AMSVAHQLIA
GgL X78032          --NTNAGLHD QRKGLEWVSD NIANFGGDPC KVMIFGESAG AMSVGHLLIA
YlL Z50020          --VGNWGARD QYTALQWISK HIVEFGGDPS QITIGGESAG SIG-LHALMV
NcL AI399229        PKFWDFSLDS IAIFDIPSIV KYILSVNSFD SISLVGFSQG AILAFAALSI
GcL II ATCC34614    NKFWDFSIDE FAFFDIPNSI EFILDITKVD KVICIGFSQG SAQMFAAFSL
RnL AB013496        KGSKSDLQAE PYNMQKNTEW YESHGGNLTS IGKRDDNLVG GMTLDLPSDA
GRnL D13206         KGSKSDLQAE PYNMQKNTEW YESHGGNLTS IGKRDDNLVG GMTLDLPSDA
RdL M38352          KGSKSDLQAE PYNMQKNTEW YESHGGNLTS IGKRDDNLVG GMTLDLPSDA
RdL B 999875
RdL A 999874
RmL A02536          SPSTTDPEAP AM-------- --SRNGPLPS ----DVETKY GMALNATSYP
TlL AF054513                                                        MRS
PcL D90315                                                        MRLSFFT
AoL D85895                                                      MRFLSGFVS
FhL S77816                                                   MMLVL SLLSIIAFTA
Consensus                 d                 g  p             ges g sm     l a

                                                                    300
                    251
CaL U34807          ITATAEATDG TLFAGLVPNA LSGLANEYPE FKEI-LYQKV SKAATDNLRQ
CanL Z30645         ITALYAGSGN NKLPVLTWSQ GGLVAQWGLT FFPS-IRSKV DRLMAFAPDY
ScL X98000          KGCTVITTKV PGFGSIEERA MALDAQLQKE VKKIESKDKR HSLNLIAHSM
SpL AL035076        YETYHPEANQ IIKRALLLSN G----LSVQP KSVEESQIQF NELAQKFGIP
CcL X16712          NDGDNTYKGK PLFRAGIMQS GAM--VPSDA VDGIYGNEIF DLLASNAGC-
CcL 1 X64703        NDGDNTYKGK PLFRAGIMQL GAM--VPLDA VDGIYGNEIF DLLASNAGC-
CcL 3 X66006 S559397 NDGDNTYKGK PLFRAGIMQL GAM--VPLDP VDGTYGNEIY DLFVSSAGC-
CcL 5 X66008 S55942 NGGDNTYKGK PLFRAGIMQL GAM--VPLDP VDGTYGTQIY DTLVALTGC-
CcL 4 X66007 S55939 NDGDNTYNGK PLFRAAIMQL GAM--VPLDP VDGPYGTQIY DQVVASAGC-
CcL 2 X64704        NDGDNTYNGK PLFRAAIMQL GCM--VPLDP VDGTYGTEIY NQVVASAGC-
GfL AB000260        YGGDNTYNGK KLFHSAILQS GGP--LPYHD SSSVGPDISY NRFAQYAGCD
GgL X78032          YGGDNTYNGK ALFHSAILQS GGP--LPYYN SGWLGPDSAY NRFAKYAGCD
YlL Z50020          HESMKP-KEE CIIHNVILSS GTMDRMGTGT ISEDAFKPIY DGIKTLVGDI
NcL AI399229        DTELRNSVRA FIALAPAIAP KKYSGRTVKS IIHANSQLLY LMFGRNSML-
GcL II ATCC34614    SEKLNRKVSH FIAIAPAMTP KGLHNRIVDT LAKSSPGFMY LFFGRKIVL-
RnL AB013496        PPISLSSSTN SASDGGKVVA ATTAQIQEFT KYAGIAATAY CRSVVPGNKW
GRnL D13206         PPISLSSSTN SASDGGKVVA ATTAQIQEFT KYAGIAATAY CRSVVPGNKW
RdL M38352          PPISLSSSTN SASDGGKVVA ATTAQIQEFT KYAGIAATAY CRSVVPGNKW
RdL B 999875                  SDGGKVVA ATTAQIQEFT KYAGIAATAY CRSVVPGNKW
RdL A 999874                  SDGGKVVA ATTAQIQEFT KYAGIAATAY CRSVVPGNKW
RmL A02536          DSVVQAMSI- ---DGG-IRA ATSQEINELT YYTTLSANSY CRTVIPGATW
TlL AF054513        SLVLFFVSAW TALASPIRRE VSQDLFNQFN LFAQYSAAAY CGKNNDAPAG
PcL D90315          ALSAVASLGY ALPGKLQSRD VSTSELDQFE FWVQYAAASY YEADYTAQVG
AoL D85895          VLSSVALLGY AYPTAIDVRD IPTTQLEDFK FWVQYAAATY CPNNYVAKDG
FhL S77816          AGPVPSVDEN TRVLEHRAVT VTTQDLSNFR FYLQHADAAY C--NFNTAVG
Consensus                 sg   lf gg  ra gtt  l  ft fy   aa aY cr v  a
```

Figure 5.3: Continued

```
                        301                                                 350
CaL U34807              GTEHCIGGAI LYFAEDQYFT GDDRAFPGGY GLLKEEVVNK TISENNLMQM
CanL Z30645             KGTVLAGPLD ALAVSAPSVW QQTTGSALTT ALRNAGGLTQ IVPTTNLYSA
ScL X98000              GGLDCRYLIC NIKNRNYDIL SLTTISTPHR GSEMADYVVD LFENLNALRV
SpL AL035076            LELSSAEKLE KLRAIPFQD- --LADNILNL RLHTFRAVTD GDF-VNPNTF
CcL X16712              --GSASDKLA CLRGVSSDT- --LEDATN-- NTPGFLAYSS LRLSYLPRPD
CcL 1 X64703            --GSASDKLA CLRGVLSDT- --LEDATN-- NTPGFLAYSS LRLLYLPRPD
CcL 3 X66006 S559397    --GSASDKLA CLRSALSDT- --LLDATN-- NTPGFLAYSS LRLLYLPRPD
CcL 5 X66008 S55942     --SSASNKLA CLRGLLTQA- --LLDATN-- DTPGFLSYTS LRLLYLPRPD
CcL 4 X66007 S55939     --GSASDKLA CLRSILNDK- --LFQATS-- DTPGALAYPS LRLLFLPRPD
CcL 2 X64704            --GSASDKLA CLRGLLQDT- --LYQATS-- DTPGVLAYPS LRLLYLPRPD
GfL AB000260            TSASANDTLE CLRSKSSSV- --LHDAQNSY DLKDLFGLLP QFLGFGPRPD
GgL X78032              TSASDVEILQ CLRSKPSST- --LSDAQNSY DLKDLFGLLP QFLGFGPRPD
YlL Z50020              NTCSADELLE AQIKAGLDLG FYLQDDFFPP DWRNVRFKVS RVLLSDVIVD
NcL AI399229            --GSAVFWQA VLYPPVFAKI VDLFLRFF-L SWTGKNISET QKIVAYSHLY
GcL II ATCC34614        --PSAVIWQR TLHPTLFNLC IDIANKIL-F NWKSFNILPR QKIASYAKLY
RnL AB013496            DCVQCQKW-V PDGK------ IITTFTSLLS DTNGYVLRSD KQKTIYLVFR
GRnL D13206             DCVQCQKW-V PDGK------ IITTFTSLLS DTNGYVLRSD KQKTIYLVFR
RdL M38352              DCVQCQKW-V PDGK------ IITTFTSLLS DTNGYVLRSD KQKTIYLVFR
RdL B 999875            DCVQCQKW-V PDGK------ IITTFTSLLS DTNGYVLRSD KQKTIYLVFR
RdL A 999874            DCVQCQKW-V PDGK------ IITTFTSLLS DTNGYVLRSD KQKTIYLVFR
RmL A02536              DCIHCDA--T EDLK------ IIKTWSTLIY DTNAMVARGD SEKTIYIVFR
TlL AF054513            TNITCTGNAC PEVEKADATF LYSFEDSGVG DVTGFLALDN TNKLIVLSFR
PcL D90315              DKLSCSKGNC PEVEATGATV SYDFSDSTIT DTAGYIAVDH TNSAVVLAFR
AoL D85895              EKLNCSVGNC PDVEAAGSTV KLSFSDDTIT DTAGFVAVDN TNKAIVVAFR
FhL S77816              KPVHCSAGNC PDIEK-DAAI VVGSVVGTKT GIGAYVATDN ARKEIVVSVR
Consensus               d  sc  wlc pl k       i tt  sl   #t gyvav d   ktiyl fr


                        351                                                 400
CaL U34807              DKDYLPDIPI FVYHGALDSI VPISNVHVTY KNWCDWGINS FEFSEDLLNG
CanL Z30645             TDEIVQPQ-- -VSNSPLDSS YLFNGKNVQA QAVC--GPLF VIDHAGSLTS
ScL X98000              SQKILP-ICF YQLTTAYMKY FNLVTPNSPK VSYFSYGCSF VPKWYNVFCT
SpL AL035076            KDIYDGTFGK RIRD---SGR ELIIGEVNNE HSIYANTNPP KSKEDLFNQV
CcL X16712              GVNITDDMYA LVREGKYANI PVIIGDQNDE GTFFGTSSLN VTTDAQAREY
CcL 1 X64703            GVNITDDMYA LVREGKYANI PVIIGDQNDE GTFFGTLLLN VTTDAQAREY
CcL 3 X66006 S559397    GKNITDDMYK LVRDGKYASV PVIIGDQNDE GTIFGLLLLN VTTNAQARAY
CcL 5 X66008 S55942     GANITDDMYK LVRDGKYASV PVIIGDQNDE GFLFGLLLLN TTTEADAEAY
CcL 4 X66007 S55939     GTFITDDMFK LVRDGKCANV PVIIGDQNDE GTVFALLLLN VTTDAQARQY
CcL 2 X64704            GTFITDDMYA LVRDGKYAHV PVIIGDQNDE GTLFGLLLLN VTTDAQARAY
GfL AB000260            GNIIPDAAYE LFRSGRYAKV PYISGNQEDE GTAFAPVALN ATTTPHVKKW
GgL X78032              GDIIPDSAYE LYRSGRYAKV PYITGNQEDE GTILAPVAIN ATTTPHVKKW
YlL Z50020              GTNFKNKINP AVR------- --VTPENDFD HKVFKLYNIS -TEDTWEDYH
NcL AI399229            SFTSVKCFVH WAQITRRKVL QMYDDSPGFK PSYYTNLNRI ARYPIE-NIR
GcL II ATCC34614        STTSVKSIVH WFQILRSQKF QMFEESDNML NSL-TRPYQI ANFPTRTNIK
RnL AB013496            GTNSFRSAIT DIVFNFSDYK -PVKGAKVHA GFLSSYEQV- --VNDYFPVV
GRnL D13206             GTNSFRSAIT DIVFNFSDYK -PVKGAKVHA GFLSSYEQV- --VNDYFPVV
RdL M38352              GTNSFRSAIT DIVFNFSDYK -PVKGAKVHA GFLSSYEQV- --VNDYFPVV
RdL B 999875            GTNSFRSAIT DIVFNFSDYK -PVKGAKVHA GFLSSYEQV- --VNDYFPVV
RdL A 999874            GTNSFRSAIT DIVFNFSDYK -PVKGAKVHA GFLSSYEQV- --VNDYFPVV
RmL A02536              GSSSIRNWIA DLTFVPVSYP -PVSGTKVHK GFLDSYGEV- --QNELVATV
TlL AF054513            GSRSIENWIG NLNFDLKEIN DICSGCRGHD GFTSSWRSV- --ADTLRQKV
PcL D90315              GSYSVRNWVA DATFVHTNPG -LCDGCLAEL GFWSSWKLV- --RDDIIKEL
AoL D85895              GSYSIRNWIT DATFPQTDPG -LCDGCKAEL GFWTAWKVV- --RDRIIKTL
FhL S77816              GSINVRNWIT NFNFGQKTCD -LVAGCGVHT GFLDAWEEV- --AANVKAAV
Consensus               Gtnsvrnwit dv fg  d    p! G kvha gflssy  v     dd    v
```

Figure 5.3: Continued

```
                    401                                                 450
CaL U34807          HITETIVGAP AAITWLEARF DGEPVVKGCK KTSRITNFSY PNISDSTSSI
CanL Z30645         QFSYVVGRSA LRSTTGQARS ADYGITDCNP LPANDLTPEQ KVAAAALLAP
ScL X98000          PWKIVYERSK GCPNDGLVTI NSSKWGEYRG TLKDMDHLDV INWKNKLQDD
SpL AL035076        NNYYPEKVT- KALLELYPKV PDMEDEKEYL AAIKALFGSI VSD-MQVYAS
CcL X16712          FKQSFVHAS- DAEIDTLMTA YPXDITQGSP FDTGILNALT PQF-KRISAV
CcL 1 X64703        FKQLFVHAS- DAEIDTLMTA YPGDITQGLP FDTGILNALT PQF-KRILAV
CcL 3 X66006 S559397 FKQLFIHAS- DAEIDTLMAA YPQDITQGLP FDTGIFNAIT PQF-KRILAV
CcL 5 X66008 S55942 LRKLFIHAT- DADITALKAA YPSDVTQGLP FDTGILNALT PQL-KRINAV
CcL 4 X66007 S55939 FKELFIHAS- DAEIDTLMAA YPSDITQGLP FDTGIFNAIT PQF-KRIAAV
CcL 2 X64704        FKQLFIHAS- DAEIDTLMAA YTSDITQGLP FDTGIFNAIT PQF-KRILAL
GfL AB000260        LQYIFYDAS- EASIDRVLSL YPQTLSVGSP FRTGILNALT PQF-KRVAAI
GgL X78032          LKYIFNEAT- DTSLDRVLKL YPETLSEGSP FRTGILNALT PQF-KRVAAI
YlL Z50020          YKMMLFKGD- ETFIRGNQQL ELLFEQENIP VWRQLFDQIH PNDPSRLCHH
NcL AI399229        LPITLVYGSN DNMVDIETLK TQLPPLSQC- IQIPNYEHLD IIMGDTKKDI
GcL II ATCC34614    IPILLIYGGI DSLVDIDVMK KNLPFNSVFD VKVDNYEHLD LIWGKDADTL
RnL AB013496        QEQLTAHPTY KVIVTGHSLG GAQALLAGMD LYQREPRLSP KNL--SIFTV
GRnL D13206         QEQLTAHPTY KVIVTGHSLG GAQALLAGMD LYQREPRLSP KNL--SIFTV
RdL M38352          QEQLTAHPTY KVIVTGHSLG GAQALLAGMD LYQREPRLSP KNL--SIFTV
RdL B 999875        QEQLTAHPTY KVIVTGHSLG GAQALLAGMD LYQREPRLSP KNL--SIFTV
RdL A 999874        QEQLTAHPTY KVIVTGHSLG GAQALLAGMD LYQREPRLSP KNL--SIFTV
RmL A02536          LDQFKQYPSY KVAVTGHSLG GATALLCALG LYQREEGLSS SNL--FLYTQ
TlL AF054513        EDAVREHPDY RVVFTGHSLG GALATVAGAD LRGN----GY -DI--DVFSY
PcL D90315          KEVVAQNPNY ELVVVGHSLG AAVATLAATD LRGK----GY PSA--KLYAY
AoL D85895          DELKPEHSDY KIVVVGHSLG AAIASLAAAD LRTK----NY -DA--ILYAY
FhL S77816          SAAKTANPTF KFVVTGHSLG GAVATIAAAY LRKD----GF -PF--DLYTY
Consensus           qeqlt hpty kvi!tGhslg ga allag d lr r   ls  p#l  slyav


                    451                                                 500
CaL U34807          FEGILNSVTG SELGPGVTSD NITLDGLTGF LGNFIDLK
CanL Z30645         AAAAIVAGPK QNCEPDLMPY ARPFAVGKRT CSGIVTP
ScL X98000          WSKFFHTTTV GEKVDILNFY LKITDDLARK GF
SpL AL035076        TRVLINGLVK GGVPLEKIYR YRI--AFRGK FFDKYEPPES LVPHAGDLGL
CcL X16712          LGDLGFTLAR RYFLNHYTGG TKY--SFLSK QLSGL-PVLG TF-HSNDI-V
CcL 1 X64703        LGDLGFTLAR RYFLNHYTGG TKY--SFLLK QLLGL-PVLG TF-HSNDI-V
CcL 3 X66006 S559397 LGDLAFIHAR RYFLNHFQGG TKY--SFLLK QLLGL-PIMG TF-HANDI-V
CcL 5 X66008 S55942 LGDLTFTLSR RYFLNHYTGG PKY--SFLLK QLLGL-PILG TF-HANDI-V
CcL 4 X66007 S55939 LGDLAFTLPR RYFLNHFQGG TKY--SFLLK QLLGL-PVIG TH-HANDI-V
CcL 2 X64704        LGDLAFTLAR RYFLNYYQGG TKY--SFLLK QLLGL-PVLG TF-HGNDI-I
GfL AB000260        LSDMLFQSPR RVMLSATKDV NRW--TYLST HLHNLVPFLG TF-HGNEL-I
GgL X78032          FTDLLFQSPR DVMLNATKDV NRW--TFLAT QLHNLVPFLG TF-HASDV-L
YlL Z50020          AVDLYYMWDN WEMPEDKHAV ARQYQDTLTK FVYGQDPWPV DKLHYVHDNQ
NcL AI399229        VIQQVVEQLN HVIAGDYFES IKEEFGLDTE LVDGVMNHTI
GcL II ATCC34614    VIAKVLRFIE FFNPGNVSVK TNQLLP-SAS LVEELPSTTW KTTHPTHGLS
RnL AB013496        GGPRVGNPTF AYYV-ESTGI PFQRTVHKRD IVPHVPPQSF GFLHPGVESW
GRnL D13206         GGPRVGNPTF AYYV-ESTGI PFQRTVHKRD IVPHVPPQSF GFLHPGVESW
RdL M38352          GGPRVGNPTF AYYV-ESTGI PFQRTVHKRD IVPHVPPQSF GFLHPGVESW
RdL B 999875        GGPRVGNPTF AYYV-ESTGI PFQRTVHKRD IVPHVPPQSF GFLHPGVESW
RdL A 999874        GGPRVGNPTF AYYV-ESTGI PFQRTVHKRD IVPHVPPQSF GFLHPGVESW
RmL A02536          GQPRVGDPAF ANYV-VSTGI PYRRTVNERD IVPHLPPAAF GFLHAGEEYW
TlL AF054513        GAPRVGNRAF AEFLTVQTGG TLYRITHTND IVPRLPPREF GYSHSSPEYW
PcL D90315          ASPRVGNAAL AKYITAQ--G NNFRFTHTND PVPKLPLLSM GYVHVSPEYW
AoL D85895          AAPRVANKPL AEFITNQ--G NNYRFTHNDD PVPKLPLLTM GYVHISPEYY
FhL S77816          GSPRVGNDFF ANFVTQQT-G AEYRVTHGDD PVPRLPPIVF GYRHTSPEYW
Consensus           ggpr!gn tf aeyv   tgg p qr vhkrd iVp lpP  f gf H g e w
```

Figure 5.3: Continued

```
                    501                                                 550
CaL U34807
CanL Z30645
ScL X98000
SpL AL035076        WFYNVVDGIL PEEIPIYK-A WLKSYGEW-- -----MSTGK TDWGTTKTE-
CcL X16712          FQDYLLGSGS LIYNNAFI-A FATDLDPN-- -----TAGLL VKWPEYTSSS
CcL 1 X64703        FQDYLLGSGS LIYNNAFI-A FATDLDPN-- -----TAGLL VKWPEYTSSL
CcL 3 X66006 S559397 WQDYLLGSGS VIYNNAFI-A FATDLDPN-- -----TAGLL VNWPKYTSSL
CcL 5 X66008 S55942 WQHFLLGSGS VIYNNAFI-A FATDLDPN-- -----TAGLL VQWPKSTSSL
CcL 4 X66007 S55939 WQDFLVSHSS AVYNNAFI-A FANDLDPN-- -----KAGLL VNWPKYTSSL
CcL 2 X64704        WQDYLVGSGS VIYNNAFI-A FANDLDPN-- -----KAGLW TNWPTYTSSL
GfL AB000260        FQFNVNIGPA NSYLRYFI-S FANHHDPN-- -----VGTNL LQWDQYTDE-
GgL X78032          FQYYLNIGPS DSYLRYFI-S FRNHHDPN-- -----VGTGL QNWAKYTDG-
YlL Z50020          FEILDKSQFG DFRNVPAL-K FLLGFSAE-- -----ELGEL TKKYTGEGHY
NcL AI399229
GcL II ATCC34614    YRTHSADRSP LSVQADEA-D EVHNADNSRF LRRVFSTSAI DEDNENEHQD
RnL AB013496        IKSGT-SNVQ ICTSEIETKD CSNSIVP--- -------FTS ILDHLSYFDI
GRnL D13206         IKSGT-SNVQ ICTSEIETKD CSNSIVP--- -------FTS ILDHLSYFDI
RdL M38352          IKSGT-SNVQ ICTSEIETKD CSNSIVP--- -------FTS ILDHLSYFDI
RdL B 999875        IKSGT-SNVQ ICTSEIETKD CSNSIVP--- -------FTS ILDHLSYFDI
RdL A 999874        IKSGT-SNVQ ICTSEIETKD CSNSIVP--- -------FTS ILDHLSYFDI
RmL A02536          ITDNSPETVQ VCTSDLETSD CSNSIVP--- -------FTS VLDHLSYFGI
TlL AF054513        IKS-G-TLVP VTRNDIVKIE GIDATGGNNQ -----PNIPD IPAHLWYFGL
PcL D90315          ITS-P-NNAT VSTSDIKVID GDVSFDGNTG T--GLPLLTD FEAHIWYFVQ
AoL D85895          ITA-P-DNTT VTDNQVTVLD GYVNFKGNTG TSGGLPDLLA FHSHVWYFIH
FhL S77816          LNGGP-LDKD YTVTEIKVCE GIANVMCNGG T-----IGLD ILAHITYFQS
Consensus           i s   snv  v ts ie  d   ns  p           t  ildhlsyf


                    551                                                 592
CaL U34807
CanL Z30645
ScL X98000
SpL AL035076        ----EYRLLD ADGTIKVVDD EKWDWGLKVG RTVAGVFGLN
CcL X16712          QSGNNLMMIN ALGLYTGKDN FRTA-GYDAL FSNPPSFFV
CcL 1 X64703        QLGNNLMMIN ALGLYTGKDN FRTA-GYDAL FSNPPLFFV
CcL 3 X66006 S559397 QLGNNLMMIN ALGLYTGKDN FRTA-GYDAL MTNPLLFFV
CcL 5 X66008 S55942 QAGDNLMQIS ALGLYTGKDN FRTA-GYNAL FADPLHFFV
CcL 4 X66007 S55939 QLGNNLLQIN ALGLYTGKDN FRTA-GYDAL FTNPLLFFV
CcL 2 X64704        QLGNNLMQIN GLGLYTGKDN FRPD-AYSAL FSNPPLFFV
GfL AB000260        --GKEMLEIH MTDNVMRTDD YRIE-GISNF ETDVNLYG
GgL X78032          --GKEMLEIK MLGNSMRTDD FRID-QIANF ESDVTLFG
YlL Z50020          TL
NcL AI399229
GcL II ATCC34614    DTEDQIHKEQ QRRLSAYLES SKDLRQLDAN SSTTALDALN KE
RnL AB013496        NEGSCL
GRnL D13206         NEGSCL
RdL M38352          NEGSCL
RdL B 999875        NEGSCL
RdL A 999874        NEGSCL
RmL A02536          NTGLCT
TlL AF054513        IGTCL
PcL D90315          VDAGKGPGLP FKRV
AoL D85895          ADACKGPGLP LR
FhL S77816          MATCAPIAIP WKRDMSDEEL EKKLTQYSEM DQEFVKQMI
```

Figure 5.3: Continued

Clearly, a high degree of diversity exists between the lipases. This makes application of sequence homology very difficult if, for example, primers are to be designed for PCR. The (lack of) relationship between the lipases is even more apparent when considering the dendrogram illustrating the structural relationship between sequences. Some clustering does occur but there is however no clear relationship between all the fungal lipases. The importance of this will become apparent later when sequence homology is used to attempt to design primers for PCR.

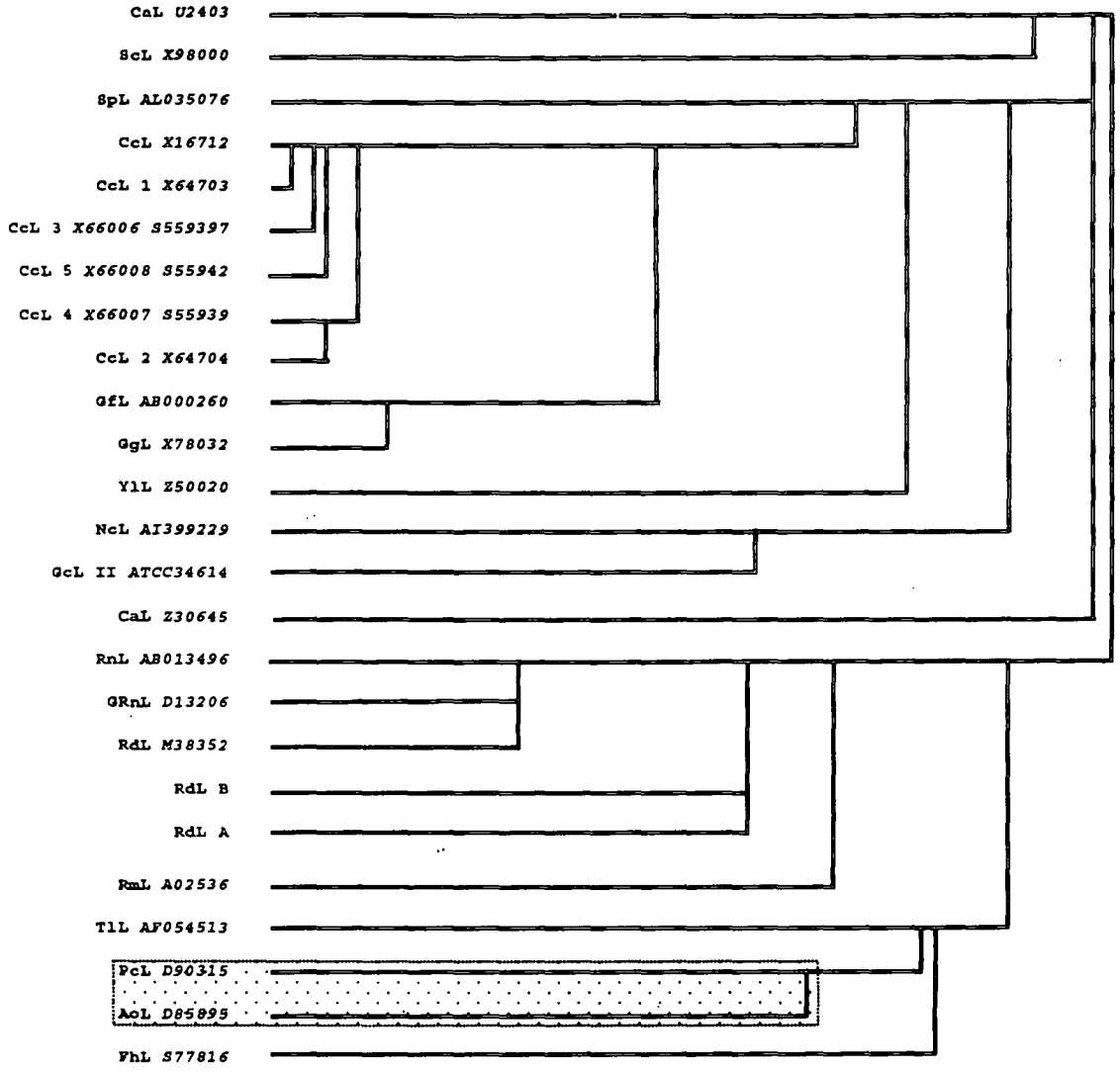


Figure 5.4: Dendrogram showing the relationship between the fungal lipases. Abbreviations used are explained in the text. Accession numbers are given in italics. The shaded area shows the two sequences of the lipase used to design primers for PCR (Section 5.7.2).

With the availability of information on the structural and mechanistic properties of lipases, one could propose a structural model of the fungal lipase of *Aspergillus niger* based on these reported X-ray structures and homology shown in the sequence of the protein. Homology-derived three-dimensional structure prediction is at present the only reliable method to predict protein folding other than crystallographic analysis (and NMR to a lesser degree) of the protein. The motivation for this chapter was to construct a cDNA library, which should contain the sequence of the lipase protein, screen the library for this specific protein, determine the nucleotide sequence and if possible demonstrate any degree of homology in primary structure to other known lipases.

5.6 Construction of a cDNA library

The experimental work reported on was done at the Institut National de la Recherche Agronomique (INRA), Luminy, Marseille,

5.6.1 Extraction of total RNA

The extraction techniques for RNA published thus far have some disadvantages, such as being time consuming, giving low yields of RNA and not being powerful enough for cultures containing high levels of nucleases. The modified method from Vongromoff *et al.*, (1989) made use of fungal mycelia from agar plates and should yield between 200 µg and 3 mg of RNA depending on the growth conditions and strain. From a single colony it was reported that up to 5 µg of total RNA could be isolated. However, the author had very little success with this modified method (Vongromoff *et al.*, 1989).

In the laboratory of Dr. Serge Moukha, (INRA), Luminy, Marseille, techniques were used with which this research group experienced success with another specie of *Aspergillus.* Although this method used for extraction was developed while

working with basidiomycetes, the method is suitable for extraction of RNA for all ascomycetes and basidiomycetes.

The fungal culture was cultivated as described in Section 3.10.2.2. The enzyme activity was monitored as described in Section 3.9.1. The biomass was recovered at optimal enzyme activity by filtration for the extraction of total RNA. The biomass was frozen with liquid nitrogen, and ground in a cold (-20 °C) mortar and pestle, until it was as fine as talc. The total RNA was extracted by adding 10 g biomass powder to 4 ml extraction buffer (0.2 M NaAc, 1% SDS, 10 mM EDTA, pH 5). Hot phenol, equilibrated with a Tris buffer at pH 7.5, pre-warmed to 65 °C was added to the solution, followed by chloroform extraction (Köhrer and Domdey, 1991). The RNA was denatured with a solution of formamide/formaldehyde (100 % / 40 %) for 15 min at 55 °C. The success of the extraction and integrity of the RNA was evaluated on a 1% agarose gel (Figure 5.5). The subunits (28S and 18S) were intact and visible on the gel to confirm the success of the extraction. The concentration of the RNA was calculated spectrophotometrically (1 OD = 40 µg/ml RNA). The isolated RNA had an estimated concentration of 11,5 mg/ml. The total RNA was stored at –20 °C, until further use.

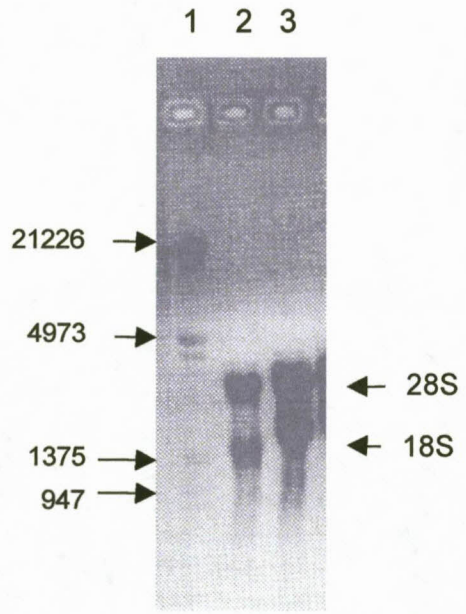


Figure 5.5: A 1 % ethidium bromide stained gel showing the integrity of the isolated RNA. Lane 1 contains DNA molecular weight marker III. Lane 2 and 3 contain the total RNA extracts.

5.6.2 *mRNA extraction from total RNA*

The mRNA was extracted from the total RNA using the Boerhinger Mannheim Magnetic Particle separator and the Anti-digoxigenin Magnetic Particles, with the prescribed protocol. The method relies on base pairing between the poly(A) residues at the 3' ends of the mRNAs and the biotin-labelled oligo $(dT)_{20}$ probe, which itself can be used for immobilisation on avidin or streptavidin solid supports. Non-adenylated RNA species are not bound and washed off. The bound mRNA is eluted from the solid support by lowering the salt concentration.

The isolated mRNA was evaluated on a 1 % agarose gel with non-denaturing conditions (Figure 5.6). The concentration of the mRNA was determined spectrophotometrically. The mRNA was of a good quality and approximately 0.408 µg/µl mRNA was obtained.

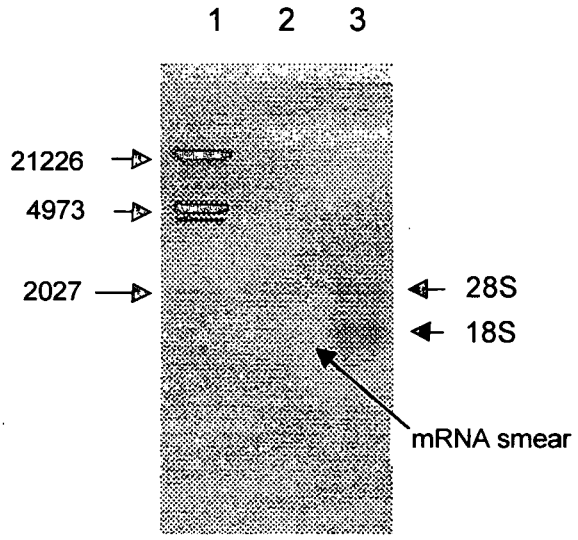


Figure 5.6: A 1 % ethidium bromide stained gel showing the mRNA extraction. Lane 1 contains DNA molecular weight marker III. Lane 2 and Lane 3 contain the smear of mRNA extract and the intact total RNA extract, respectively.

5.6.3 *Purification and packaging of cDNA*

All commonly used cDNA synthesis methods rely on the ability of reverse transcriptase (RT) to transcribe mRNA into ssDNA in the first-strand reaction. However, because the RT cannot always transcribe the entire mRNA sequence, the 5' ends of genes tend to be under-represented in cDNA populations. This is particularly true for long mRNAs, especially if the first-strand synthesis is primed with oligo(dT) primers only, or if the mRNA has persistent secondary structure. Furthermore, the use of T4 DNA polymerase to generate blunt cDNA ends after second-strand synthesis commonly results in heterogeneous 5' ends that are 5-30 nucleotides shorter than the original mRNA (D'Alessio and Gerard, 1988).

The SMART™ PCR cDNA library construction kit provided a novel, PCR-based method for making a high-quality library from very little poly A^+ RNA. The kit utilises the SMART™ oligonucleotide in the first-strand synthesis, followed by long-distance PCR amplification to generate high yields of full-length, double-stranded cDNA (Figure 5.7).

In the SMART cDNA synthesis method, a modified oligo (dT) primer (CDS/3'PCR primer) is set to prime the first-strand reaction, and the SMART oligonucleotide serves as a short, extended template at the 5' end for the RT. When the RT reaches the 5' end of the mRNA, the enzyme switches templates and continues replicating to the end of the SMART oligonucleotide, therefore allowing the synthesis of only full length ss cDNA (Step1). This switching in most cases occurs at the 7-methylguanosine-cap structure, which is present on the 5' end of all eukaryotic mRNAs (Furuichi and Miura, 1975). The resulting full-length ss cDNA contains the complete 5' end of the mRNA as well as the sequence complementary to the SMART oligonucleotide, which serves as a universal PCR priming site in the subsequent amplification.

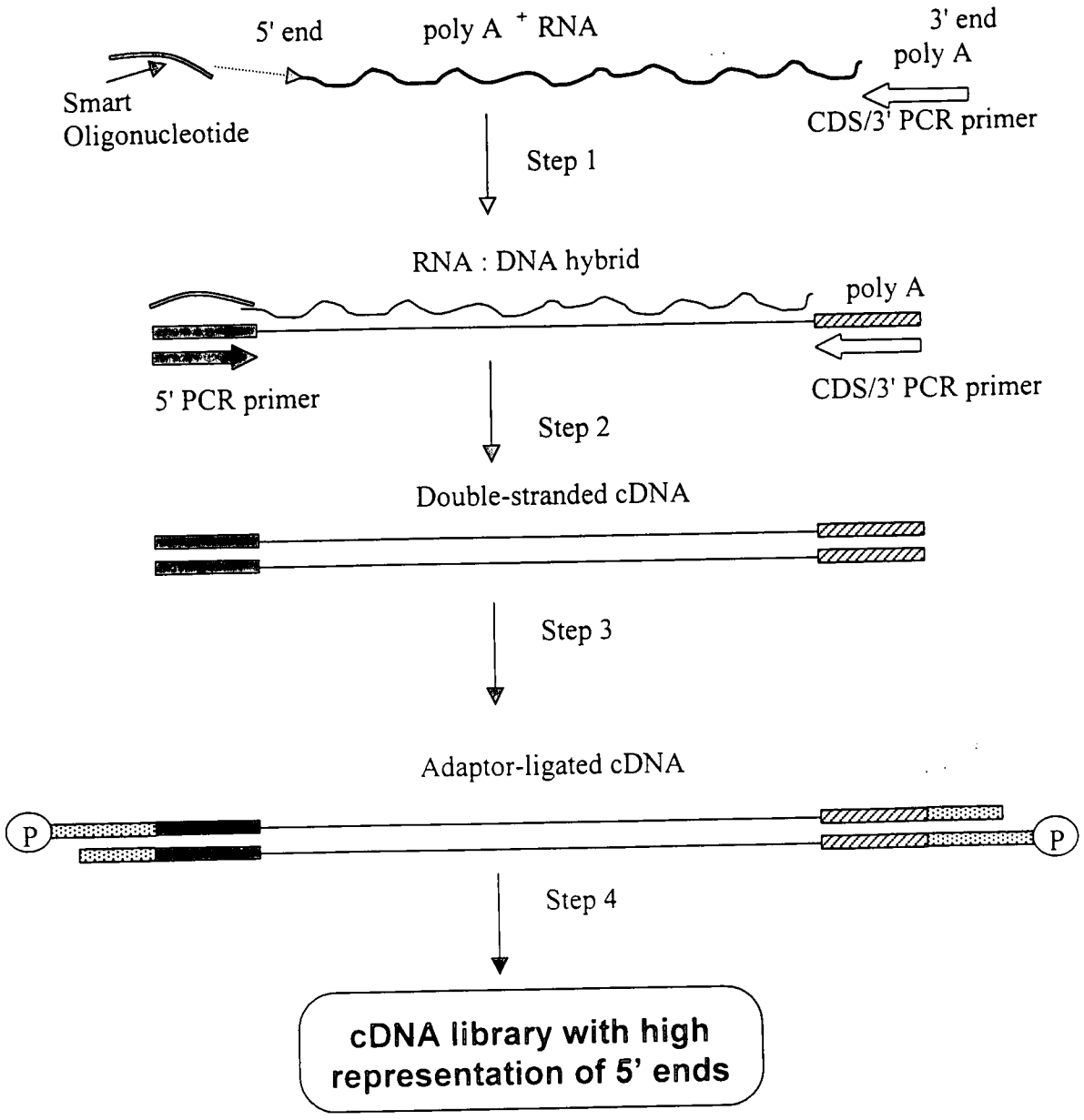


Figure 5.7: Flow chart of the SMART PCR cDNA Library Construction Kit protocol

The first strand cDNA was synthesised as follows: The denaturing reaction was initiated after the addition of a random 3' CDS/PCR primer;

$$Oligo(dT)_{30}N_{-1}N \{N = A,G,C \text{ or } T: N_{-1} = A,G \text{ or } C\}$$

and a SMART oligonucleotide;

5'-TACGGCTGCGAGAAGACGACAGAAGGG-3'

at 72 °C for 2 min. Thereafter elongation was allowed to proceed for 20 min at 68 °C and the cycle ended by decreasing the temperature to 4 °C. This synthesis reaction was performed with two separate reverse transcriptase enzymes, the Expand™ DNA polymerase RNA dependent product (Boerhinger Mannheim) and the CLONETECH™ product. In addition, the synthesis reaction was performed on the purified mRNA as well as the total RNA. The best results were obtained with the purified mRNA and the CLONETECH™ RT.

First strand cDNA amplification was followed by LD-PCR (Step 2). The resulting PCR product was polished and ligated with specially designed adapters that contains a pre-digested EcoR1 restriction site and a phosphorylated blunt end for efficient ligation to the blunt-ended cDNA. Following the adapter ligation, the ds cDNA was phosphorylated at the EcoR1(Step 3) sites and size-fractionated to remove small (<0.5 kb) cDNA fragments and non-cDNA contaminants (such as unincorporated primers and unligated adapters (Step 4). This is important because, the small DNA fragments will be preferentially ligated to the vector, which will reduce the quality of the resulting library.

The resulting cDNA was then ligated into an EcoR1-digested, dephosphorylated λgt11 vector. The ligate was then packed into the Gigapack™ III Gold Packaging Extract Package from SRATAGENE. This packaging extract is used to package recombinant lambda phage with high efficiency. According to the manufacturer Gigapack™ III Gold packaging extract produces a packaging efficiency of 2×10^9 pfu/μg and is specifically designed for cDNA library construction.

Each packaging system was evaluated by titering the resulting library. The control titre was in the order of 10^8 pfu/μg and the various dilutions of the libraries all gave a titre in the order of 10^7 pfu/μg. A library having at least 10^6 independent clones in most cases is representative of the mRNA complexity.

5.7 Screening of the cDNA library

5.7.1 Purified protein N-terminal sequence

After the cDNA library construction, the approach to select for the specific cDNA of the lipase enzyme was to use specific primers designed from the purified protein's N-terminal amino acid sequence. The attempted N-terminal sequencing was discussed in section 3.9. Due to time constraints at INRA, deglycosylation and fragmentation of the protein was not attempted and a molecular approach using degenerate primers was followed.

5.7.2 Degenerate primers

Known lipase amino acid sequences of *Rhizopus niveus, Rhizopus oryzae, Rhizomucor miehei, Fusarium heterosporum, Humicola lanuginosa, Aspergillus oryzae,* and *Penicillium camembertii* deduced from their respective lipase genes, cDNA or known protein sequence, were compared after an alignment using clustal software. *Penicillium camembertii* and *Aspergillus oryzae* monoacylglycerol lipase amino acid sequences were very closely related, showing conserved consensus sequences. Because both are ascomycetes (fungus imperfecti) and taxonomically closely related to *Aspergillus niger,* in comparison to the other fungi, it was chosen to design degenerate oligonucleotides corresponding to conserved regions. The structurally conserved regions have been defined, somewhat arbitrary as those regions, where neither of the structures deviates by more than ~1.5 Å from the mean position of the homologous C_αs (Derewenda *et al.,* 1994b).

Five forward and reverse oligonucleotides were designed (Table 5.1).

Table 5.1 Nucleotide sequences of oligonucleotides designed.

Oligo.	*Specified sequence*	*Base pairs*
F0	CGAGCTCGAC CAGTTCGART TYTGGGT	27
F1	TTCTGGGTYC AATAYGCY	18
F2	TTCTGGGTRC AATAYGCR	18
F3	TTCTGGGTRC AATAYGCY	18
F4	TTCTGGGTYC AATAYGCR	18
R0	GCGTCGACCT GGACGAARTA CCADATRTG	29
R1	RAYGTGMACA TAACCCATAG	20
R2	RAYGTGMACG TAGCCCATAG	20
R3	RAYGTGMACA TAGCCCATAG	20
R4	RAYGTGMACG TAACCCATAG	20

In Figure 5.8 the alignment of the protein sequences analysis of *Penicillium camembertii* and *Aspergillus oryzae* and the degenerate primers are shown.

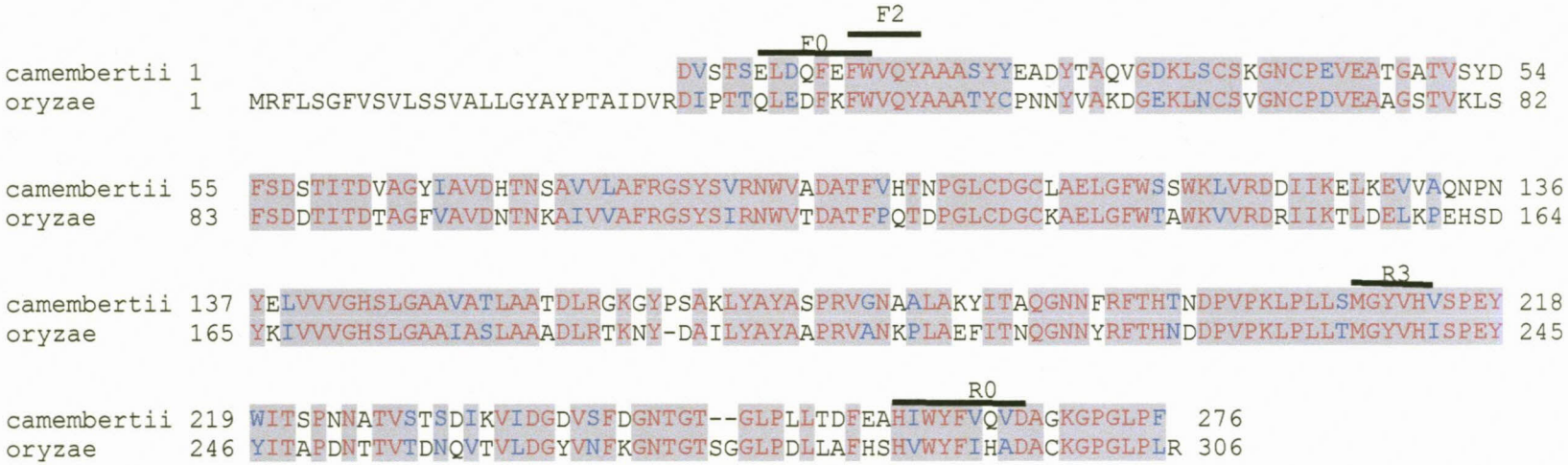


Figure 5.8: The alignment of the protein sequences analysis of *Penicillium camembertii* and *Aspergillus oryzae* and the design of degenerate oligonucleotides corresponding to the conserved regions are shown. The two longer oligonucleotides are indicated as well as the oligonucleotides with which the successful PCR reaction was achieved.

To obtain the DNA needed for the PCR approach, the fungal culture was cultivated as described in Section 3.9.1. The biomass (2.5 g wet weight) was recovered by filtration for the extraction of DNA. The biomass was frozen with liquid nitrogen, and ground in a cold (-20 °C) mortar and pestle, until the biomass was as fine as talc.

The DNA was extracted by shaking the 2.5 g biomass-powder in 12.5 ml extraction buffer (0.2 M Tris-HCl, 0.5% SDS, 25 mM EDTA and 0.25M NaCl, pH 8.5). Hot phenol, equilibrated with a Tris buffer at pH 7.5, pre-warmed to 65 °C was added to the solution, followed by chloroform/isoamyl alcohol (24:1) extraction. The aqueous phase was then treated with an RNase A/T solution and removed by phenol/chloroform extraction. DNA was precipitated by adding 0.54 volume of isopropanol to the aqueous phase. After centrifugation the pellet was washed twice with 70 % ethanol and resuspended in TE buffer. The success of the extraction was evaluated on a 1 % agarose gel (Figure 5.9). The concentration was calculated spectrophotometrically (1 OD = 50 µg/ml DNA), with the isolated DNA having an estimated concentration of 3.54 mg/ml. The total DNA was stored at –20°C, until further use.

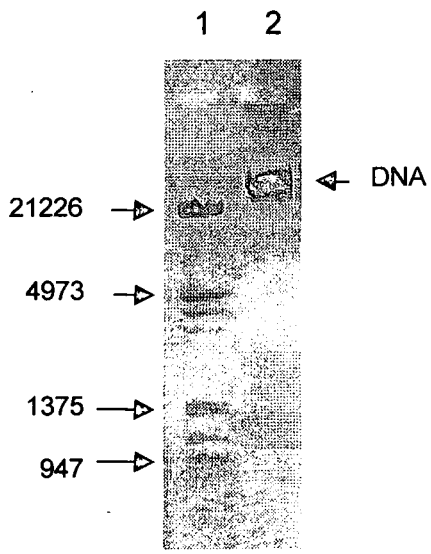


Figure 5.9: A 1 % ethidium bromide stained gel showing the DNA extracted. Lanes 1 and 2 contain DNA molecular weight marker III and the DNA extract, respectively.

The primers were used in all possible combinations to amplify the lipase gene from the genomic DNA. The PCR reactions contained 1.25 mM $MgCl_2$, 1 U Taq polymerase (CLONETECH™), 0.2 mM dNTPs and 0.5 µM of any two primers. The pre-cycle of the reaction had a long denaturing step of 5 min at 95 °C followed by annealing for 30 sec at 55 °C and an elongation for 45 sec at 72 °C (5 cycles). The next 30 cycles had the following three segments: 30 sec at 94 °C, 30 sec at 55 °C and 45 sec at 72 °C. PCR products were analysed by agarose gel electrophoresis.

A PCR product was obtained with two primer combinations (Figure 5.10). The primer pair F2 and R3 yielded a product with an appropriate molecular weight when compared to the other lipase sequence alignments (600 – 700 bp). The only other primer pair (F2 and R2) which yielded a similar PCR product had an additional lower molecular weight product (>564 bp).

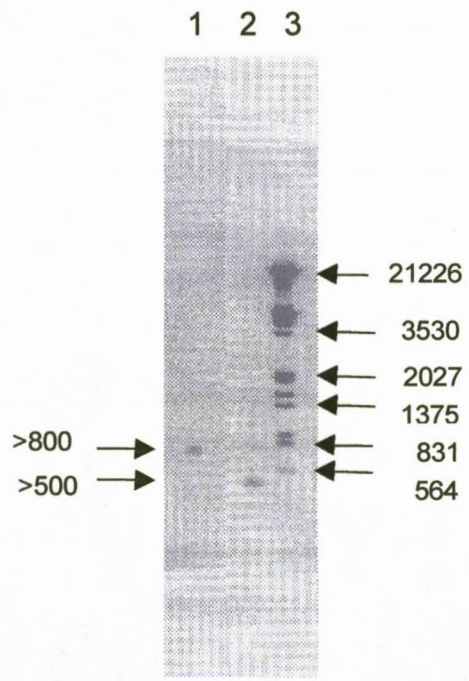


Figure 5.10: A 1 % ethidium bromide stained gel showing the PCR reaction products. Lane 3 contains DNA molecular weight marker III agarose gel. Lane 1 the PCR product obtained with the combination of primers, F2 and R3. Lane 2 the PCR products obtained with the combination of primers, F2 and R2.

The PCR product was recovered from an agarose gel and cleaned using the Geneclean II™ Kit from BIO Inc. The kit contains a silica matrix (GLASSMILK™) that binds single and double stranded DNA without binding DNA contaminants.

The recovered DNA was ligated into pGEM-T™ Easy vector. Competent cells were prepared according to the method of Tang *et al.*, (1994). The ligation mixture was transformed into the competent cells and positive transformants were selected. Plasmid DNA was purified and positive clones were identified by restriction analysis using EcoR1, Sal 1 and Pst 1. The insert appeared to be a product with an appropriate molecular weight when compared to the other lipase sequence alignments (~600 - 700 bp). The PCR product (F2;R3) was labelled using DIG labelling and detection kit. The DIG system uses dioxigenin to label DNA for hybridisation and subsequent luminescence detection.

The plaques from the cDNA library, as discussed in Section 5.7, were transferred to Hybond N^+ membranes. The membrane was peeled from the petri dish containing the plaques, the DNA was fixed on the membranes after being denatured from the plaques according to the method described in Sambrook *et al.*, (1989).

The membranes, containing the blotted cDNA, were allowed to hybridise with the DIG-labelled PCR probe. To enhance the reaction DIG Easy Hyb™ hybridisation solution, which reduces hybridisation time, was used. Positive plaques (30) were identified by superimposing the hybridised membranes on the original petri dishes. A second screening was performed by infecting bacteria using the identified positive plaques.

Positive plaques (24) from the secondary screening were isolated and after infection, allowed to grow overnight. DNA was isolated from the lambda clones using the Promega™ Wizard Lambda Preps DNA Purification Kit. The isolated DNA was subjected to a PCR reaction as described in Section 5.7.2, with the degenerate primers F2 and R3. This product was sequenced manually in both directions.

Two partial sequences obtained a section with the forward primer F2 and another section with the reverse primer R3. The partial sequences were aligned with the nucleotide sequences of *Penicillium camembertii* and *Aspergillus oryzae* lipases to show any degree homology. Figure 5.8 shows the design of these primers, the partial sequences obtained aligned in the regions where the primers were expected to bind, but unfortunately the full sequence of the lipase from *Aspergillus niger* was not obtained to show significant homology with the ascomycetes, used in the initial design of the degenerate primers. The results and alignment of the partial sequences are shown in Figure 5.11.

5.8 Conclusions

A cDNA library for the lipase of *Aspergiluus niger* was successfully obtained with a good titre in the order of 10^7 pfu/µg. When standard alignment routines were applied to lipase sequences, rather inconclusive results were obtained (Svendsen, 1994). The degrees of similarity found within the lipases is very low, the only conserved feature being the "consensus" sequence G-X-S-X-G which is found in many esterases. Even this "consensus' sequence is not always conserved. The microbial lipases and even more surprisingly, the fungal lipases (including yeast) also show very little sequence homology. Therefore the efficacy of degenerate primers with such a diversity among the nucleotide sequences of lipases are very difficult to predict.

However, with the designed primers a partial sequence was obtained for the lipase of *Aspergillus niger*. The sequencing data obtained was aligned with the known lipase sequences of *Aspergillus oryzae* and *Penicilium camembertii*. The homology is apparent for these regions. Further attempts to obtain the whole sequence were unsuccessful. The data suggest that the lipase from *Aspergillus niger* might possibly resemble the sequence patterns of the two known related fungi. However, more extensive research is required.

```
camemberti dna      1    CCGGGAGTAAATTTTCATGTGATCGGGACAACCTTGGAAACCACATGGTGATTACCAATATACGCCAGCCACCA 74
oryzae dna          1                                                                           0
reverse_sequence    1                                                                           0

camemberti_dna     75    TATCATATTCTCACGGCGTACTCCGTACAATAAACTCCGTACTCGGAGTCCACGCGACCTCGGGTGCCGATATT 148
oryzae dna          1                                                                           0
reverse_sequence    1                                                                           0

camemberti dna     149   GGCTAACTTCCAGGGTTCCCGGGCTGACCGAAATGAGACAATAGCCCGGCTCCACCAATGCCCCGCGATTGATA 222
oryzae_dna          1                          AAGCTTTGC-AACCAAGCCTGTCGCC-GTCTG-ATCAACATCCTTCTTGAACAT 51
reverse_sequence    1                                                                           0

camemberti dna     223   GCCAAGGC-GATTG-GAGACTTTTTCGTT---TTTCGGTACCACATCCCCG--GATGTGATCTATACCTTGACG 289
oryzae dna          52   GCCGAGGCTGATTTCGTGGATTTTCCGTCAGCTTAAGCAAGTGGATCCGTGTGGATGGTAGTGTGAACTGAACC 125
reverse_sequence    1                                                                           0

camemberti dna     290   ATGTTTATATGAATCCGGAGTATTCCCCGTGGGCTGAAGTGGGTCGATATCCCTCATCACAAGCAATATCGGAT 363
oryzae dna         126   AA-TTCAGCTCGATCCGGCCAAGAGC--ATCGATGGTAGACTATTGAGATCGCGCATGCCATGTCTCACT--AT 194
reverse_sequence    1                                                                           0

camemberti dna     364   TTTATTCTATGCCGTGGTTCCCCCGACACAAAAATGAGCTGGCGCAATTGTGGAACTAATCCAGGAACCATACT 437
oryzae dna         195   TTTCAGCGGAACCACAAACAACCCGATTGTCGGATCAGGGATATCGATTTTGG--CGAAGTCTGTATACAGAAT 266
reverse_sequence    1                                                                           0

camemberti_dna     438   TCGTACCTGAATGGAACTGTGAGGTG-CAATTGACCATATAAAGCCGGGGGAAACCCCCAGCTTTTGATTTCTC 510
oryzae dna         267   --GTAACT-ATTTAATGGGCGTCGTTTCCACTGTCTGCTGTCTTCATCTGTTCATGCTCA-CAATTGCCTCGTC 336
reverse_sequence    1                                                                           0

camemberti dna     511   CAGTCTCCATCAGCTTCAGCCATTGAC---CTCAAGCCATCATGCGTCTC-TCT-----TTCTTCACAGCTCTA 575
oryzae_dna         337   TC-TATCCATTGAGTAGACCCTGCGAAGCACACTGGCAAAGATGCGCTTCCTCTCCGGCTTCGTTTCTGTTCTG 409
reverse_sequence    1                                                                           0
```

Figure 5.11: Partial DNA sequence of the lipase from *Aspergillus niger* obtained with the degenerate oligonucleotides F2 and R3 in comparison with the sequences of *Penicillium camembertii* and *Aspergillus oryzae*.

```
camemberti dna     576  TCCGCAGTGGCTTCGTTGGGCTATGCCCTCCC--CGGCAAGCTGCAGTCTCGAGGTACATCCTACAACCTTGCC 647
oryzae dna         410  TCCTCAGTGGCCCTGTTGGGTTACGCTTACCCAACGGCAAT-TGATGT-TAGAGGTAC-----TGAATCTGCTT 476
reverse_sequence   1                                                                  GTCGCT 6

camemberti dna     648  CAGTGTAACCTGACCACATTACTGACACATGGTTA-GATGTTTCGACCAGCGAACTGGACCAAT-TCGAATTCT 719
oryzae dna         477  CATGATCTGCCGGACGGAC-ACTGACTCATTGTTAAGACATCCCTACTACCCAGCTCGAAGACT-TCAAGTTCT 548
reverse_sequence   7    CATAGTAT----AACCGAT-ACTAAGTCATAGTTTTGCGTTCGGGTCTA--------GATGACTATCAAAAGAT 67

camemberti_dna     720  GGGTCCAATATGCCGCCGCGTCATACTATGAAGCGGACTACACAGCTCAAGTGGGCGACAAGCTCAGTTGTTCG 793
oryzae dna         549  GGGTGCAATATGCGGCTGCCACCTACTGCCCCAATAACTACGTTGCCAAAGACGGCGAAAAGCTGAATTGCTCT 622
reverse_sequence   68   AGACGCAAGGTTCTTCTT                                                  85

camemberti dna     794  AAGGGCAACTGCCCCGAAGTAGAGGCAACTGGTGCGACTGTATCATATGACTTCTCCGAGTAAGTGAATCCCAC 867
oryzae_dna         623  GTGGGCAACTGCCCTGATGTCGAGGCGGCCGGTTCTACTGTCAAGCTCAGCTTCTCCGAGTAAGTCA--CCTGA 694
reverse_sequence   86                                                                  85

camemberti dna     868  CGTGAAATAACCCAGACGATATACTTACC-CACGCATAGCTCCACTATCACAGATACTGCCGGCTACATTGCAG 940
oryzae dna         695  GTTCCCAGTACCGTGACCTCCAACTAACAACCCTAACAGTGATACCATCACCGACACTGCCGGCTTCGTGGCCG 768
reverse_sequence   86                                                                  85

camemberti dna     941  TCGATCACACCAACTCAGCAGTTGTTCTTGCCTTCCGCGGGTCCTACTCTGTGCGCAACTGGGTCGCTGATGCC 1014
oryzae dna         769  TAGACAACACCAACAAGGCCATCGTCGTCGCTTTCCGTGGCTCCTACTCTATCCGCAACTGGGTCACCGACGCA 842
reverse_sequence   86                                                                  85

camemberti dna     1015 ACATTCGTCCACACAAACCCTGGTCTCTGTGATGGTTGCCTCGCTGAACTCGGCTTCTGGAGCTCCTGGAAGCT 1088
oryzae dna         843  ACCTTCCCCCAAACCGACCCAGGACTGTGCGACGGCTGCAAGGCCGAACTGGGCTTCTGGACCGCCTGGAAGGT 916
reverse_sequence   86                                                                  85

camemberti_dna     1089 CGTCCGTGACGACATCATCAAAGAACTCAAGGAAGTCGTCGCACAGAACCCCAACTACGAGCTGGTCGTAGTGG 1162
oryzae dna         917  CGTCCGCGACCGAATCATCAAGACCCTGGATGAGCTGAAGCCCGAACACAGCGACTACAAAATCGTTGTCGTGG 990
reverse_sequence   86                                                                  85
```

Figure 5.11: Continued

```
camemberti dna     1163 GCCACAGCCTGGGTGCTGCCGTCGCAACCCTTGCTGCCACCGACCTCCGTGGCAAGGGCTACCCATCGGCTAAG 1236
oryzae dna         991  GCCACAGTCTCGGCGCCGCCATCGCCTCGCTCGCAGCTGCGGACCTGCGCACGAAGAATTACGA---CGCGATC 1061
reverse_sequence   86                                                                              85

camemberti dna     1237 CTGTACGCGTACGCCTCTCCTCGCGTGGGTAACGCGGCTTTGGCCAAGTATATCACGGCTCAGGGCAACAACTT 1310
oryzae dna         1062 CTGTACGCCTACGCCGCGCCGCGTGTGGCCAACAAGCCTCTGGCCGAGTTCATCACCAACCAGGGCAACAACTA 1135
reverse_sequence   86                                                                              85

camemberti_dna     1311 CCGCTTCACCCACACCAATGACCCCGTCCCCAAGCTGCCCTTGTTGTCCATGGGTTATGTTCACGTTAGCCCTG 1384
oryzae dna         1136 CCGCTTCACTCACAATGACGACCCCGTGCCCAAGCTGCCGCTCTTGACTATGGGCTACGTGCACATCAGCCCTG 1209
reverse_sequence   86                                                                              85

camemberti dna     1385 AGTACTGGATCACCTCGCCTAACAATGCCACTGTTAGCACTTCTGATATCAAGGTTATTGACGGAGATGTCTCC 1458
oryzae_dna         1210 AATACTATATCACCGCGCCGGACAACACTACCGTCACCGACAACCAAGTCACCGTTCTCGATGGATACGTGAAC 1283
reverse_sequence   86                                                                              85

camemberti dna     1459 TTTGATGGAAACACCGGAACTGGC------CTGCCGTTGCTGACGGACTTTGAGGCGCACATCTGGTACTTTGT 1526
oryzae dna         1284 TTCAAGGGAAACACCGGCACGAGCGGCGGACTGCCTGACCTCCTTGCGTTCCACTCGCATGTCTGGTACTTTAT 1357
reverse_sequence   86                                                                              85

camemberti dna     1527 GCAGGTTGATGCTGGCAAGGGCCCTGGGCTGCCATTCAAGAGGGT--TTAAAATTTAGATCAAGCCCCCTCGAT 1598
oryzae dna         1358 CCACGCCGATGCCTGCAAGGGTCCTGGATTGCCATTGCGCTAATTCGTTACCATTTTTTTGGTGAATTTCTTAC 1431
reverse_sequence   86                                                                              85

camemberti dna     1599 TTTCGTTTGATAGACTCTATAATAGCTGAGCCGACG-TTGATA-TGTATAAAGAATAATGACACATGCAGTTTC 1670
oryzae dna         1432 ATGATTTGGACGGACGATG----AGATGAGTCTATGCTTCGTACTGTATAAGGTTTGATG-----TGGAGTT-- 1494
reverse_sequence   86                                                                              85

camemberti_dna     1671 CGAATATGCAGAGGCAAGATAGAAAGTAGAAATATACCGTAAGTCGATGCAAGGGGT---TGGAAGAAAAAGTA 1741
oryzae dna         1495 -GAA---GCCTACGTAA---ATAATTTA---ATATCCCCTAATCTGAAACAAAATTTAAATCGAACCAAATTTC 1558
reverse_sequence   86                                                                              85
```

Figure 5.11: Continued

```
camemberti dna      1742 --CCAAGATTGCCCATATTATCCTTCAATTCCCAATCTTGCACTAATAAAGTTGGCT-GATAAGCAATT-CGGT 1811
oryzae dna          1559 ATCCCAGTCAAACCTTAATCGCCATAAATTCTCG-TCTC-CACCCACAGAGTAACTTAGGTACACTATTACCTT 1630
reverse_sequence    86                                                                            85

camemberti_dna      1812 CATTTCCGGCTCTCAGGAGACATCCAGTCGGAGATCCGTCGGGGTTAGACCGAAAAGATCAAGTTGGGCGATCT 1885
oryzae dna          1631 CACAACAAAAGATGAGCT-AAAGAAAACTGAAGAAACCCCTCCTCAACCTCCAAAAAATAAAA----GAATTCC 1699
reverse_sequence    86                                                                            85

camemberti dna      1886 TAGTAGTTTTTGGCCTTCTCGTTTGATTCTAGCAATATAAAGTGGATATGGGGAGCCGGGCGGGGTTATCTGGA 1959
oryzae_dna          1700 AAAT-GTCGTCGGCCAAG-CACACTACACCTGCAG                                        1732
reverse_sequence    86                                                                            85

camemberti dna      1960 ATGAACGGTATAAAGATATGACTTGAACCTCGAGTTGAATTGGGGATCTCAATTCAAAATGGTGCTGTATACTA 2033
oryzae dna          1733                                                                          1732
reverse_sequence    86                                                                            85


camemberti_dna      1    CCGGGAGTAAATTTTCATGTGATCGGGACAACCTTGGAAACCACATGGTGATTACCAATATACGCCAGCCACCA 74
oryzae dna          1                                                                             0
forward_sequence    1                                                                             0

camemberti dna      75   TATCATATTCTCACGGCGTACTCCGTACAATAAACTCCGTACTCGGAGTCCACGCGACCTCGGGTGCCGATATT 148
oryzae_dna          1                                                                             0
forward_sequence    1                                                                             0

camemberti dna      149  GGCTAACTTCCAGGGTTCCCGGGCTGACCGAAATGAGACAATAGCCCGGCTCCACCAATGCCCCGCGATTGATA 222
oryzae dna          1                         AAGCTTTGC-AACCAAGCCTGTCGCC-GTCTG-ATCAACATCCTTCTTGAACAT 51
forward_sequence    1                                                                             0

camemberti dna      223  GCCAAGGC-GATTG-GAGACTTTTTCGTT---TTTCGGTACCACATCCCCG--GATGTGATCTATACCTTGACG 289
oryzae dna          52   GCCGAGGCTGATTTCGTGGATTTTCCGTCAGCTTAAGCAAGTGGATCCGTGTGGATGGTAGTGTGAACTGAACC 125
forward_sequence    1                                                                             0

camemberti dna      290  ATGTTTATATGAATCCGGAGTATTCCCCGTGGGCTGAAGTGGGTCGATATCCCTCATCACAAGCAATATCGGAT 363
oryzae dna          126  AA-TTCAGCTCGATCCGGCCAAGAGC--ATCGATGGTAGACTATTGAGATCGCGCATGCCATGTCTCACT--AT 194
forward_sequence    1                                                                             0
```

Figure 5.11: Continued

```
camemberti dna     364  TTTATTCTATGCCGTGGTTCCCCCGACACAAAAATGAGCTGGCGCAATTGTGGAACTAATCCAGGAACCATACT 437
oryzae dna         195  TTTCAGCGGAACCACAAACAACCCGATTGTCGGATCAGGGATATCGATTTTGG--CGAAGTCTGTATACAGAAT 266
forward_sequence   1                                                                              0

camemberti dna     438  TCGTACCTGAATGGAACTGTGAGGTG-CAATTGACCATATAAAGCCGGGGGAAACCCCCAGCTTTTGATTTCTC 510
oryzae dna         267  --GTAACT-ATTTAATGGGCGTCGTTTCCACTGTCTGCTGTCTTCATCTGTTCATGCTCA-CAATTGCCTCGTC 336
forward_sequence   1                                                                              0

camemberti_dna     511  CAGTCTCCATCAGCTTCAGCCATTGAC---CTCAAGCCATCATGCGTCTC-TCT-----TTCTTCACAGCTCTA 575
oryzae dna         337  TC-TATCCATTGAGTAGACCCTGCGAAGCACACTGGCAAAGATGCGCTTCCTCTCCGGCTTCGTTTCTGTTCTG 409
forward_sequence   1                                                                              0

camemberti dna     576  TCCGCAGTGGCTTCGTTGGGCTATGCCCTCCC--CGGCAAGCTGCAGTCTCGAGGTACATCCTACAACCTTGCC 647
oryzae_dna         410  TCCTCAGTGGCCCTGTTGGGTTACGCTTACCCAACGGCAAT-TGATGT-TAGAGGTAC-----TGAATCTGCTT 476
forward_sequence   1                                                                              0

camemberti dna     648  CAGTGTAACCTGACCACATTACTGACACATGGTTA-GATGTTTCGACCAGCGAACTGGACCAATTCGAATTCTG 720
oryzae dna         477  CATGATCTGCCGGACGGAC-ACTGACTCATTGTTAAGACATCCCTACTACCCAGCTCGAAGACTTCAAGTTCTG 549
forward_sequence   1                                                                              0

camemberti dna     721  GGTCCAATATGCCGCCGCGTCATACTATGAAGCGGACTACACAGCTCAAGTGGGCGACAAGCTCAGTTGTTCGA 794
oryzae dna         550  GGTGCAATATGCGGCTGCCACCTACTGCCCCAATAACTACGTTGCCAAAGACGGCGAAAAGCTGAATTGCTCTG 623
forward_sequence   1                                                                              0

camemberti dna     795  AGGGCAACTGCCCCGAAGTAGAGGCAACTGGTGCGACTGTATCATATGACTTCTCCGAGTAAGTGAATCCCACC 868
oryzae dna         624  TGGGCAACTGCCCTGATGTCGAGGCGGCCGGTTCTACTGTCAAGCTCAGCTTCTCCGAGTAAGTCA--CCTGAG 695
forward_sequence   1                                                                              0

camemberti_dna     869  GTGAAATAACCCAGACGATATACTTACC-CACGCATAGCTCCACTATCACAGATACTGCCGGCTACATTGCAGT 941
oryzae dna         696  TTCCCAGTACCGTGACCTCCAACTAACAACCCTAACAGTGATACCATCACCGACACTGCCGGCTTCGTGGCCGT 769
forward_sequence   1                                                                              0
```

Figure 5.11: Continued

```
camemberti dna     942  CGATCACACCAACTCAGCAGTTGTTCTTGCCTTCCGCGGGTCCTACTCTGTGCGCAACTGGGTCGCTGATGCCA 1015
oryzae dna         770  AGACAACACCAACAAGGCCATCGTCGTCGCTTTCCGTGGCTCCTACTCTATCCGCAACTGGGTCACCGACGCAA 843
forward_sequence   1                                                                              0

camemberti_dna    1016 CATTCGTCCACACAAACCCTGGTCTCTGTGATGGTTGCCTCGCTGAACTCGGCTTCTGGAGCTCCTGGAAGCTC 1089
oryzae dna         844  CCTTCCCCCAAACCGACCCAGGACTGTGCGACGGCTGCAAGGCCGAACTGGGCTTCTGGACCGCCTGGAAGGTC 917
forward_sequence   1                                                                              0

camemberti dna    1090 GTCCGTGACGACATCATCAAAGAACTCAAGGAAGTCGTCGCACAGAACCCCAACTACGAGCTGGTCGTAGTGGG 1163
oryzae_dna         918  GTCCGCGACCGAATCATCAAGACCCTGGATGAGCTGAAGCCCGAACACAGCGACTACAAAATCGTTGTCGTGGG 991
forward_sequence   1                                                                              0

camemberti dna    1164 CCACAGCCTGGGTGCTGCCGTCGCAACCCTTGCTGCCACCGACCTCCGTGGCAAGGGCTACCCATCGGCTAAGC 1237
oryzae dna         992  CCACAGTCTCGGCGCCGCCATCGCCTCGCTCGCAGCTGCGGACCTGCGCACGAAGAATTACGA---CGCGATCC 1062
forward_sequence   1                                                                              0

camemberti dna    1238 TGTACGCGTACGCCTCTCCTCGCGTGGGTAACGCGGCTTTGGCCAAGTATATCACGGCTCAGGGCAACAACTTC 1311
oryzae dna        1063 TGTACGCCTACGCCGCGCCGCGTGTGGCCAACAAGCCTCTGGCCGAGTTCATCACCAACCAGGGCAACAACTAC 1136
forward_sequence   1                                                                              0

camemberti dna    1312 CGCTTCACCCACACCAATGACCCCGTCCCCA-AGCTGCCCTTGTTGT----CCATGGGTTATGTTCACGTTAGC 1380
oryzae dna        1137 CGCTTCACTCACAATGACGACCCCGTGCCCA-AGCTGCCGCTCTTGA----CTATGGGCTACGTGCACATCAGC 1205
forward_sequence   1                TTAACGTGCACGTAGCCATAGCCATAGCTTTTGAGCTCCTATGACGGATTTCAAGGCTAGC 61

camemberti_dna    1381 CCTGAGTACTGGATCACCTCGCCTAACAATGCCACTGTTAGCACTTCTGATATCAAGGTTATTGACGGAGATGT 1454
oryzae dna        1206 CCTGAATACTATATCACCGCGCCGGACAACACTACCGTCACCGACAACCAAGTCACCGTTCTCGATGGATACGT 1279
forward_sequence  62   GCAGA--ACTACATGAC--------AGAATTCTTG                                        86

camemberti dna    1455 CTCCTTTGATGGAAACACCGGAACTGGC------CTGCCGTTGCTGACGGACTTTGAGGCGCACATCTGGTACT 1522
oryzae_dna        1280 GAACTTCAAGGGAAACACCGGCACGAGCGGCGGACTGCCTGACCTCCTTGCGTTCCACTCGCATGTCTGGTACT 1353
forward_sequence  87                                                                             86

camemberti dna    1523 TTGTGCAGGTTGATGCTGGCAAGGGCCCTGGGCTGCCATTCAAGAGGGT--TTAAAATTTAGATCAAGCCCCCT 1594
oryzae dna        1354 TTATCCACGCCGATGCCTGCAAGGGTCCTGGATTGCCATTGCGCTAATTCGTTACCATTTTTTTGGTGAATTTC 1427
forward_sequence  87                                                                             86
```

Figure 5.11: Continued

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camemberti dna     1595 CGATTTTCGTTTGATAGACTCTATAATAGCTGAGCCGACG-TTGATA-TGTATAAAGAATAATGACACATGCAG 1666
oryzae dna         1428 TTACATGATTTGGACGGACGATG----AGATGAGTCTATGCTTCGTACTGTATAAGGTTTGATG-----TGGAG 1492
forward_sequence   87                                                                              86

camemberti_dna     1667 TTTCCGAATATGCAGAGGCAAGATAGAAAGTAGAAATATACCGTAAGTCGATGCAAGGGGT---TGGAAGAAAA 1737
oryzae dna         1493 TT---GAA---GCCTACGTAA---ATAATTTA---ATATCCCCTAATCTGAAACAAAATTTAAATCGAACCAAA 1554
forward_sequence   87                                                                              86

camemberti dna     1738 AGTA--CCAAGATTGCCCATATTATCCTTCAATTCCCAATCTTGCACTAATAAAGTTGGCT-GATAAGCAATT- 1807
oryzae_dna         1555 TTTCATCCCAGTCAAACCTTAATCGCCATAAATTCTCG-TCTC-CACCCACAGAGTAACTTAGGTACACTATTA 1626
forward_sequence   87                                                                              86

camemberti dna     1808 CGGTCATTTCCGGCTCTCAGGAGACATCCAGTCGGAGATCCGTCGGGGTTAGACCGAAAAGATCAAGTTGGGCG 1881
oryzae dna         1627 CCTTCACAACAAAAGATGAGCT-AAAGAAAACTGAAGAAACCCCTCCTCAACCTCCAAAAAATAAAA----GAA 1695
forward_sequence   87                                                                              86

camemberti dna     1882 ATCTTAGTAGTTTTTGGCCTTCTCGTTTGATTCTAGCAATATAAAGTGGATATGGGGAGCCGGGCGGGGTTATC 1955
oryzae dna         1696 TTCCAAAT-GTCGTCGGCCAAG-CACACTACACCTGCAG                                     1732
forward_sequence   87                                                                              86

camemberti dna     1956 TGGAATGAACGGTATAAAGATATGACTTGAACCTCGAGTTGAATTGGGGATCTCAATTCAAAATGGTGCTGTAT 2029
oryzae dna         1733                                                                            1732
forward_sequence   87                                                                              86

camemberti_dna     2030 ACTAAGCTT 2038
oryzae dna         1733           1732
forward_sequence   87             86
```

Figure 5.11: Continued

Even though the nucleotide sequences of lipases are diverse the folding patterns of the proteins are similar and the catalytic groups are identical. The partial nucleotide sequence of *Aspergillus niger* lipase, when converted to an amino acid sequence also showed alignment with the known lipases of *Aspergillus oryzae* and *Penicilium camembertii*, but unfortunately the data is not sufficient for structural homology modeling.

Several authors reported on the difficulty when investigating molecular aspects of lipases from fungi, and lipase research in this area should provoke more extensive investigation. Most research attention is focussed on bacterial lipases, and it is only recently that researchers began to realize the potential of fungal lipases.

No good model ever accounted for all the facts since some data was bound to be misleading if not plain wrong.

J. D. Watson

CHAPTER 6

Summary

Lipase (EC 3.1.1.3) catalyses the hydrolysis of triacylglycerols and occur widely in nature. The lipase reaction is reversible and a wide range of trans- and interesterification reactions can be catalysed. These enzymes could be used to manufacture products which could not be obtained by conventional chemical processes, and as the advantages of the use of lipases relative to traditional chemical processes are more and more recognised, lipases may be expected to gain even more importance in the enzyme market. For these purposes, new lipases with a wide range of novel characteristics will be needed.

Several microbial isolates were screened for lipase production on agar plates containing different lipase inducers. The isolates (1 bacterial and 13 fungal) that showed lipase production with at least three inducers, were cultured in shake cultures containing olive oil as inducer of lipase production. The most promising lipase producer was identified as *Aspergillus niger*.

Purification of lipase from *Aspergillus niger* was achieved using ion-exchange chromatography, iso-electric focussing, and size exclusion chromatography. It was shown that the iso-electric focussing was not essential for purification, if a more effective, gel filtration matrix with a narrower functional range was employed. The homogeneity of the lipase was confirmed on SDS-PAGE and iso-electric focussing gels. The purified lipase displayed a relative molecular weight of 43 600 Da in its glycosylated form and a pI of 6.1. The carbohydrate content of the lipase was estimated at 10 %.

The pure lipase showed maximal activity at acidic pH values and a temperature range between 25 - 40 °C. The enzyme was stable over a wide pH range, and the presence of calcium increased the stability with the effect being most dramatic at

high pH values. Even though the *Aspergillus niger* lipase is not considered as thermostable, the enzyme could be stabilised by calcium to such a degree that application even at higher temperatures seems feasible. Some heavy metal ions inhibited the enzyme's activity. The lipase activity was influenced by detergents forming ionic micelles, and the non-ionic and zwitterionic detergents had very little to no effect on the lipase activity. The functional analogy to serine proteases was also confirmed by modification of the "catalytic triad" residues.

The positional and stereospecificity of the *Aspergillus niger* lipase was investigated with the monomolecular film technique. This technique is considered to be the most effective method for studying lipase kinetics. The lipase displayed a stereopreference for the *sn*-1 ester position and as expected, no marked hydrolysis of the ester in *sn*-2 position. As the surface pressure was increased the initial stereoselectivity can be altered to a preference for the *sn*-3 ester position; thus indicating that lipolysis is surface dependant. The regioselectivity of the lipase was also investigated, using this very sensitive technique, and these kinetic studies revealed that the lipase has a preference for adjacent ester groups at low surface pressures, but that the regioselectivity is less marked at higher surface pressures. The kinetic characterisation of *Aspergillus niger* lipase using the oil-drop tensiometer, showed very good lipolysis at the interface of the soybean oil drop. Even when compared to other known and well-characterised lipases, this lipase displayed exceptionally high activity. These studies show interfacial kinetics reported thus far can be misleading and special care must be taken when extracting kinetic parameters from a multiphase (emulsion) system.

The *Aspergillus niger* lipase was also investigated at a molecular level; a successful cDNA library was constructed. Degenerate primers were designed according to amino acid sequence homology displayed between various fungal species, the PCR product obtained with these primers were used to screen the library for the lipase gene. The partial nucleotide sequence of the A*spergillus niger* lipase gene was obtained. The lipase from *Aspergillus niger* shows some unique aspects that should be investigated even more thoroughly to make this hyper producing fungus a prospect for biotechnological application.

Opsomming

Lipase (EC 3.1.1.3), wat algemeen in die natuur voorkom, kataliseer die hidroliese van triasielgliserole. Die lipase-reaksie is omkeerbaar wat 'n wye reeks trans- en interesterifikasie reaksies moontlik maak. Die ensieme kan die vervaardiging van produkte wat andersins nie met konvensionele chemiese metodes gemaak kan word nie, moontlik maak. Na mate die voordele verbonde aan die toepassing van lipases erken word, kan daar verwag word dat lipases 'n groter wordende deel van die markaandeel sal begin inneem. Nuwe en verbeterde lipases sal daarom noodgedwonge gevind moet word.

Verskeie mikrobiese isolate is getoets vir lipase-produksie op agar kwekingsplate met verskillende lipase-induseerders. Die isolate (een bakterie en dertien fungi) wat positief met ten minste drie induseerders getoets het, is in vloeibare skudkulture gekweek met olyfolie as ensiem-induseerder. Die mees belowende isolaat is as *Aspergillus niger* geïdentifiseer.

Die ensiem is gesuiwer deur die toepassing van ioonuitruilingschromatografie, iso-elektriese fokussering en grootte uitsluitingschromatografie. As 'n meer doeltreffende gelfiltrasiemedium met 'n enger fraksioneringswydte gebruik word, kan die iso-elektriese fokusseringstap uitgelaat word. Die homogeniteit van die ensiem is deur middel van "SDS-PAGE" en analitiese iso-elektriese fokussering bevestig. Die gesuiwerde ensiem het 'n relatiewe molekulêre massa van 43 000 Da in die geglikosileerde vorm en 'n pI van 6.1 getoon. Die koolhidraat-inhoud van die lipase was 10 %.

Die gesuiwerde ensiem het maksimale aktiwiteit by lae pH-waardes en 'n by 'n temperatuurgebied van 25 - 45 °C getoon. Die ensiem is oor 'n wye pH-gebied stabiel en Ca^{2+} het 'n dramatiese stabiliseringseffek, veral by hoë pH waardes. Al is die ensiem nie termostabiel nie, kon die ensiem in so 'n mate deur Ca^{2+} gestabiliseer word dat toepassing by hoër temperature moontlik is. Die lipase aktiwiteit is deur sommige swaarmetaalione geïnhibeer. Wasmiddels wat ioniese

miselle vorm het ook die lipase aktiwiteit sterk beïnvloed, terwyl neutrale of zwitterioniese wasmiddels weinig effek gehad het. Die funksionele ooreenstemming met serien proteases is deur modifikasie van die katalitiese triade bevestig.

Die posisionele- en stereoselektiwiteit van die lipase is deur middel van die monomolekulêre filmtegnieke bestudeer. Hierdie tegniek word as die mees betroubare hiervoor beskou. Die lipase het 'n besliste voorkeur vir die *sn*-1 esterbinding getoon en het, volgens verwagting, nie die *sn*-2 posisie teen 'n beduidende tempo gehidroliseer nie. Met 'n toename in oppervlakdruk is die aanvanklike stereovoorkeur verander na dié vir die *sn*-3 posisie. Hidroliese is dus oppervlakdruk afhanklik. Die regioselektiwiteit van die ensiem is ook deur middel van hierdie tegniek ondersoek. Kinetiese studie het daarop gedui dat naasliggende estergroepe by voorkeur by lae oppervlakdruk gehidroliseer word maar dat die regioselektiwiteit by hoë oppervlakdruk begin vervaag. Kinetiese karakterisering van die *Aspergillus niger* lipases deur van die oliedruppel tensiometer gebruik te maak het aangedui dat die ensiem 'n besonder hoë aktiwiteit besit by die interfase van die sojaboon oliedruppel. Hierdie studie dui op die gevaar dat kinetiese data bekom deur die toepassing van multifase (emulsie) sisteme, uiters misleidend kan wees.

Die *Aspergillus niger* lipase is ook op molekulêre vlak ondersoek en 'n cDNS biblioteek is voorberei. Degenererende voorlopers is ontwerp na aanleiding van aminosuurvolgorde homologie met ander fungusspesies. Die PKR-produk wat met hierdie voorlopers verkry is, is gebruik om die cDNS biblioteek te sif vir die lipase-geen. 'n Gedeeltelike nukleotiedvolgorde is vir die *Aspergillus niger* lipase-geen verkry. Die *Aspergillus niger* lipase het sommige unieke eienskappe wat verdere deeglike ondersoek na hierdie hiperproduserende fungus regverdig vir 'n biotegnologiese toepassing.

Absence of evidence is not the evidence of absence.

Anonymous

CHAPTER 7

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